Molecular Characterisation of Insect Amylases and Plant Amylase Inhibitors

Thesis Submitted to AcSIR
For the Award of the Degree of
DOCTOR OF PHILOSOPHY
In
BIOLOGICAL SCIENCES

By
Amey Jayant Bhide
10BB11J26109

Under the guidance of

Dr. Ashok P. Giri (Research Supervisor)
Dr. Vidya S. Gupta (Research Co-supervisor)

BIOCHEMICAL SCIENCES DIVISION
CSIR-NATIONAL CHEMICAL LABORATORY
PUNE-411008, INDIA
August 2016

Dedicated to.....

Aai, Baba

&

Family

सीएसआईआर - राष्ट्रीय रासायनिक प्रयोगशाला
(वैज्ञानिक तथा औद्योगिक अनुसंधान परिषद)
डॉ. होमी भाभा मार्ग, पुणे - 411 008. भारत
CSIR - NATIONAL CHEMICAL LABORATORY
(Council of Scientific & Industrial Research)
Dr. Homi Bhabha Road, Pune - 411 008, India

CERTIFICATE

This is to certify that the work incorporated in this Ph.D. thesis entitled **"Molecular Characterisation of Insect Amylases and Plant Amylase Inhibitors"** submitted by **Mr. Amey Jayant Bhide** to Academy of Scientific and Innovative Research (AcSIR) in fulfillment of the requirement for the award of the Degree of **Doctor of Philosophy**, embodies original research work under my guidance. We further certify that this work has not been submitted to any other University or Institution in part or full for the award of any degree of diploma. Research material obtained from other sources has been duly acknowledged in the thesis. Any text, illustration, table etc., used in the thesis from other sources have been duly cited and acknowledged.

Date: 23rd August 2016
Place: Pune

A.J. Bhide

Amey Jayant Bhide
(Research Student)

Dr. Mrs. Vidya S. Gupta
(Research Co-supervisor)

Dr. Ashok P. Giri
(Research Supervisor)

Dr. Ashok P. Giri
Senior Scientist
Biochemical Sciences Division
National Chemical Laboratory
Pune - 411 008.

Communication Channels
NCL Level DID : 2590
NCL Board No. : +91-20-2590 2000
EPABX : +91-20-2589 3300
: +91-20-2589 3400
FAX
Director's Office : +91-20-2590 2601
COA's Office : +91-20-2590 2660
COS&P's Office : +91-20-2590 2664
WEBSITE
www.ncl-india.org

DECLARATION BY RESEARCH SCHOLAR

I hereby declare that the thesis entitled "**Molecular Characterisation of Insect Amylases and Plant Amylase Inhibitors**"submitted by me for the Degree of Doctor of Philosophy to Academy of Scientific & Innovative Research (AcSIR) is the record of workcarried out by me at Biochemical Sciences Division, CSIR- National Chemical Laboratory, Pune - 411008, India, under the supervision of Dr. Ashok P. Giri (research supervisor) and Dr. Vidya S. Gupta (research co-supervisor). The work is original and has not formed the basis for the award of any degree, diploma, associateship, and fellowship titles in this or any other university or other institute of higher learning. I further declare that the material obtained from other resources has been duly acknowledged in the thesis.

A.J.Bhide

Amey Jayant Bhide

Plant Molecular Biology Group
Biochemical Sciences Division
CSIR-National Chemical Laboratory
Dr.Homi Bhabha Road, Pashan
Pune – 411008
Maharashtra, India

Table of Contents

Acknowledgement

This thesis is the outcome of several people's contribution, without their support this wonderful journey, which is a blend of multitude of things, learning, patience, help, encouragement, aspiration, ecstasy and melancholy would have not been possible. Hence, it becomes my innermost will to acknowledge all those who have extended their support to me scientifically, morally or otherwise to make my way smooth. First and foremost, I owe to my parents and my family members who have walked through myriads of rough patches to pave my way and have been always there for everything in my ups and downs.

I am extremely grateful to Dr. Ashok P. Giri, my research guide, for providing a great support and having faith in me. This work would not have been possible without his guidance, critical inputs and freedom for executing my ideas as well. He has always supported me in every sense throughout these years. He has followed my progress from the first day and helped in a great deal in improving my speaking skill. I also appreciate his meticulous attention to the minute details. I appreciate all his effort, ideas and time to make this happen. I also appreciate his friendly and helpful nature in the lab.

I am also grateful to Dr. Hemlata Kotkar for her inevitable support and encouragement which have accelerated pace of my work and moved to next step. I also acknowledge my Co-guide Dr. (Mrs.) Vidya S. Gupta for kindly replying my queries and giving suggestions out of her busy schedule for my work. She has been associated with my research work from the beginning and has provided critical inputs and suggestions. I am also thankful to Dr. Narendra Y. Kadoo for his critical suggestions and proof reading of my NCL-401 and NCL-402 project works.

I am thankful to my DAC chairman Dr. Mugdha Gadgil and DAC members Dr. Avalokiteshwar Sen, Dr. Narendra Y. Kadoo and Prof. Kisan M. Kodam (SPPU) for their constant support, encouragement and suggestions during DAC meetings. I express my gratitude towards Dr. Sureshkumar Ramasamy for helping me in computational analysis and also for his useful suggestions on protein purification. I am also thankful to Prof. V. L. Maheshwari and Dr. Pankaj K. Pawar for their valuable suggestions on biochemical assays and insect feeding experiments. I am grateful to Dr. R. V. Gadre for allowing me to use HPLC facility and also teaching me basics of fermentation and good laboratory practices during my tenure as a project fellow in his lab. I am thankful to Dr. Mahesh Kulkarni for

giving his valuable advice on proteomics work and also to Dr. Suresh Bhat for allowing me to use confocal microscope. I express special thanks to Dr. Gayathri from IISER-Pune for her help in crystal data collection. I am grateful to Dr. Yogesh Shouche for allowing me to work in his lab as a project trainee during my M. Sc. dissertation. There, I learned basics of cloning and sequencing techniques which I found useful during my Ph.D. work. I also acknowledge Director CSIR-NCL and Dr Archana Pundle, Head, Biochemical Sciences Division and Dr. (Mrs.) Vidya Gupta, former Head, Biochemical Sciences Division for providing the lab facilities and infrastructure.

I express my sincere thanks to Prof. Asaph Aharoni from Weismann Institute of Sciences, Rehovot, Israel for his kind invitation to work in his lab for a period of six months. I learned there basics of plant metabolomics including analysis of targeted and non-targeted metabolites and construction of transgenic plants with over expression or down regulation of particular gene from tomato and potato. I am also grateful to his lab members; Sageet, Iilana, Sergey, Dario, Sam, Uwe, Louis, Pathy, Oren, Maor, Hillary, Guy, Max and Pablo for their time to time help and wonderful time spent during my stay. I thank Luca, Daria, Dana, Nagaraju and Pina from Dormitory for their extraordinary companionship.

I thank Dr. Raghavendra Pai, Director of College of Fisheries, Shirgaon, Ratnagiri and Marine Biological Research Station (MBRS) Zadgaon, Ratnagiri for accepting our request to carry out my CSIR-800 project under his guidance. I also acknowledge Dr. Swapnaja Mohite and Dr. Hukumsingh Dhaker for their valuable time and discussion on aquaculture of marine and fresh water fishes. I am also thankful to staff people of the fisheries college for their useful discussion. My special thanks to Mr. Tejas Jogalekar for his technical support in construction of pH stabiliser.

I am grateful to my lab members Rakesh, Ravi, Vishal, Purushottam, Ram, Meena, Ashwini, Gayatri, Hemangi, Atul, Yojana, Neha M, Neha K, Smrati, Amol J, Amol K, Deepika, Sucheta, Pranjali, Veena, Ramesha, Ranjeet, Gopal and Gaurav for wonderful time and experiences in the lab. I acknowledge project trainees Sucheta, Kabita, Charu, Garima, Haritima and Anmol for their valuable help. I am also grateful to Sonal for her significant help and support. I am thankful to my lab-mates in CSIR-National Chemical Laboratory, Ashish, Rahul, Gouri, Reema, Tejas, Aditi and Priyanka for great time spent with all of them. Special thanks to Sheon, Yogesh, Jagadishprasad, Yashwant and Kedar for

their help in the proteomics studies. I thank Deepak, Manu, Yashpal and Ameya for their help, support and healthy discussion. I also thank Balkrishna (BK) and Shrikant who have always been there for me in every situation. I have always enjoyed their company and spent quality time with them. I express my special gratitude to Dr. Bhushan Dholakia for giving his valuable time for detail discussions and proofreading my book chapter and manuscripts. I also thank all whom I may have forgotten to mention here in this note.

Amey Jayant Bhide

List of Tables

List of Figures

List of Abbreviations

Abbreviation	Full form
aa	Amino acid
AAI	Amaranth Alpha-amylase inhibitor
AgAmy1	*Anthonomus grandis* α-amylase
AhAI	*Amaranthus hypochondriacus* alpha-amylase inhibitor
α-AI	Alpha-amylase inhibitor
ANOVA	Analysis of variance
BASI	Barley Alpha-amylase subtilisin inhibitor
BLAST	Basic Local Alignment Search Tool
BME	Beta-mercaptoethanol
bp	base pair
Bt	*Bacillus thuringiensis*
CcAmy	*Callosobruchus chinensis* α-amylase
CD	Circular Dichroism
CDS	Coding region
CPB	Citrate phosphate buffer
DNSA	Dinitrosalycylic acid
DsAmy	*Diatrea saccharalis* α-amylase
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EF1α	Elongation Factor 1α
FPLC	Fast protein liquid chromatography
GFP	Green Fluorescent Protein
GM	Genetically Modified
HaAmy1	*Helicoverpa armigera* alpha-amylase 1
HaAmy2	*Helicoverpa armigera* alpha-amylase 2
HhAmy	*Hypothenemus hampei* α-amylase
HPLC	High Performance Liquid Chromatography
HsAmyP or HPA	*Homo sapiens* pancreatic alpha-amylase

HsAmyS or HSA	*Homo sapiens* salivary alpha-amylase
HSD	Honestly Significant Difference
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria-Bertani media
LB plot	Lineweaver–Burk plot
MALDI	Matrix-associated Laser Desorption and Ionization
MCPs	Metallo-carboxypeptidase
mJA	Methyl jasmonate
MRE	Mean Residue Ellipticity
NaPI	*Nicotiana alata* proteinase inhibitor
NCBI	National Center for Biotechnology Information
Ni-NTA	Ni2+-nickel-nitrilotriacetic acid
ns	nanoseconds
OD	Optical density
PcAmy	*Phaedon cochleariae* α-amylase
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
RMSD	Root mean square deviation
rpm	Revolutions per minute
RT-PCR	Real-Time Polymerase Chain Reaction
SDS	Sodium dodecyl sulfate
SP	Signal Peptide
TCA	Trichloro acetic acid
TcAmy	*Tribolium castaneum* α-amylase
TMA	*Tenebrio molitor* α-amylase
TmAmy	*Tenebrio molitor* α-amylase
TvD1	*Tephrosia villosa* D1
UTR	Un-translated region
Viz	Videlicet
Vmax	Maximum rate

Abstract

Everlasting molecular arms race exists between insect pests and their host plants. Plants produce variety of defence arsenals as a protection strategy against such insect pests. These include production of secondary metabolites or proteinaceous inhibitors targeted against insect digestive enzymes. On the contrary, insects regulate their digestive enzymes in order to overcome the action of these inhibitors. Among the pool of insect's secreted digestive enzymes, α-amylases play an important role in digestion and assimilation of ingested starchy food materials and ultimately contribute in gaining energy for proper growth and development of the insect. Hence, knowledge acquired through studying biochemical and biophysical properties of these digestive α-amylases will be useful in designing better control strategies against insect pests.

Helicoverpa armigera, a polyphagous insect pest (Order- Lepidoptera) feeds on variety of host plants and imposes major threat to economically important crops. In present thesis, study on two isoforms of digestive α-amylases from *H. armigera* namely, HaAmy1 and HaAmy2 after feeding on different host plants revealed that expression of these isoforms is specific across various tissues and developmental stages. Similarly, both the isoforms showed differential expression pattern across various regions of insect gut. Availability of free sugars in the insect diet was shown to affect the expression of *HaAmy1* and *HaAmy2* along the insect gut. To carry out further studies, full-length nucleotide sequence of *HaAmy1* and HaAmy2 were cloned and proteins heterologously expressed in *Pichia pastoris*. These recombinant enzymes showed distinct biochemical and biophysical properties when compared with each other and with different Lepidopteran α-amylases. A non-proteinaceous α-amylase inhibitor, acarbose, showed similar inhibitory pattern against HaAmy1 and HaAmy2. However, proteinaceous α-amylase inhibitor from wheat showed differential inhibition pattern. Homology modelling and docking study of inhibitor and enzymes revealed an aggregation type interaction between HaAmy1 and inhibitor which was considered to be responsible for higher inhibition shown by Wheat AI against HaAmy1. Overall, these findings will be useful in understanding starch breakdown and sensitivity of *H. armigera* α-amylases towards various inhibitors during digestion.

Along with Lepidopteran herbivores, order Coleoptera consists of several agronomically important pests that mainly feed on stored-grains. α-Amylases from two predominant coleopteran insects namely *Tribolium castaneum* and *Callosobruchus chinensis* were studied as next section in present thesis to understand their biochemical, biophysical and molecular properties as well as their interactions with proteinaceous and non-proteinaceous α-amylase inhibitors. *T. castaneum* α-amylase (TcAmy) and *C. chinensis* α-amylase (CcAmy) differed in their biochemical properties including enzyme kinetics and products generated after amylolytic reaction using starch as a substrate. Acarbose, a non-proteinaceous α-amylase inhibitor was able to completely inhibit the amylolytic activity of TcAmy and CcAmy but not at equimolar concentration. *In silico* analysis revealed that a variation in two amino acids near the active sites of TcAmy and CcAmy would probably have resulted into superficial binding of acarbose near the active site of TcAmy, which was predicted to be responsible for the observed differential inhibition. Outcomes from the present study could be useful in designing inhibitors with higher affinities towards active site of insect α-amylases.

As mentioned earlier, host plants produce different types of proteinaceous α-amylase inhibitors mostly specific towards digestive α-amylases of attacking pests. Therefore, a study of full length native α-amylase inhibitor from *Amaranthus hypochondriacus* (AhAI) highlighting its genomic, biochemical and molecular properties was performed in the next section of the thesis. Identical open reading frame of AhAI was observed in other members of Amaranthaceae family viz. *Amaranthus paniculatus*, *Achyranthes aspera* and *Celosia argentea* although it showed variation at 3' end of the respective transcript. Functional characterization of AhAI signal peptide exhibited its role in extra-cellular secretion of attached protein. Further, it was revealed that transcript abundance of *AhAI* varied across various tissues of related plants from *Amaranthaceae* family as indicated above. Additionally, AhAI was able to completely inhibit amylolytic activity of α-amylases from *T. castaneum* and C. *chinensis* which indicated higher specificity of AhAI towards coleopteran α-amylases. *In silico* analysis, however, reflected its differential molecular interactions with the active sites of these two coleopteran α-amylases.

The present thesis has been organised into six chapters as given below:

Chapter 1: Introduction

Chapter 1 presents the general introduction and provides the review of the literature on insect α-amylases and plant α-amylase inhibitors. This chapter also describes the need to study insect pest's digestive enzymes in order to understand complex interactions during digestion of ingested food material along the insect gut region. Further, it provides insight into strategic exploitation of plant defensive proteinaceous inhibitors against insect pests.

Chapter 2: Study of gene expression patterns of *Helicoverpa armigera* α-Amylases upon feeding on a range of host plants

Chapter 2 presents study of gene expression patterns of *Helicoverpa armigera* α-amylases upon feeding on a range of host plants. The study was focussed on expression patterns of two isoforms of *Helicoverpa armigera* α-amylase namely HaAmy1 and HaAmy2 along the insect's gut region after feeding on number of host plants like Pea, Okra, Marigold and Maize. Further, gene expression pattern across various developmental stages of the insect was analysed. Influence of available free sugars on regulation of expression of digestive α-amylases was also studied.

Chapter 3: Biochemical, structural and functional diversity between two digestive α-amylases from *Helicoverpa armigera*

In continuation with above chapter, the open reading frames of α-amylase isoforms from *Helicoverpa armigera* namely *HaAmy1* and *HaAmy2* were cloned in *Pichia pastoris* and expressed heterologously. Purified recombinant enzymes were characterized for their biochemical and biophysical attributes using established methods. Molecular interactions of these recombinant enzymes with proteinaceous and non-proteinaceous α-amylase inhibitors were also studied.

Chapter 4: Characterization of two coleopteran α-amylases and molecular insight into their differential inhibition by synthetic α-amylase inhibitor, acarbose

The order Coleoptera is the largest order of insects which includes the most common and important stored-grain pests. Digestive α-amylases from two most commonly occurring coleopteran pests namely *Tribolium castaneum* and *Callosobruchus chinensis* were studied with respect to their biochemical and biophysical properties as well as their interactions with proteinaceous (Wheat α-amylase inhibitor) and non-proteinaceous (acarbose) α-amylase inhibitors. Biochemical properties of both the enzymes were compared. Mechanism underlying differential amylolytic reaction inhibition by acarbose was discussed.

Chapter 5: Genomic and functional characterization of coleopteran insect specific α-amylase inhibitor gene from Amaranth species

Chapter 5 highlights genomic, biochemical and molecular properties of α-amylase inhibitor from *Amaranthus hypochondriacus*. A full-length and previously unknown nucleotide sequence of inhibitor was cloned and protein was heterologously expressed in *E. coli*. Further, cellular function of signal peptide of the inhibitor was determined. Transcript abundance of Amaranth α-amylase inhibitor across different tissues of plants from *Amaranthaceae* family was also estimated. Detailed analysis of mechanism underlying differential inhibition of amylolytic activity of above two α-amylases was studied.

Chapter 6: Summary and future prospective

This chapter highlights the important findings from this research topic and their possible future prospects.

Chapter-1

"Introduction"

Contents of chapter-1 have been published in a book chapter……

(Giri AP., **Bhide AJ.** and Gupta VS. (2016) John Wiley & Sons Limited, The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ)

1. Introduction

1.1 Influence of insect pests on agriculture

Insects, a class of invertebrates, represent more than half of the known and classified organisms till now. They have remarkable physiology and adaptability to the various environments and conditions. Insects have unique characteristics which have given them an unusual survival advantage. These characteristics include small size, exoskeleton, ability to fly, reproductive potential, metamorphosis and adaptability. Small size requires minimum resources that are needed for survival and reproduction. Exoskeleton of insects shapes and supports soft tissues of the insect body and also provides protection from injury or physical and chemical attack. Besides, insects are the only invertebrates that can fly. Elasticity of the thorax imparts efficient energy conversion where about 90-95% of the potential energy is converted into the kinetic energy. Moreover, short life cycle and high fecundity enable insects to produce large numbers of offspring. Most insects undergo a unique developmental change known as metamorphosis where alterations in physical, biochemical and behavioral aspects lead to promote overall survival and reproduction of insects. Altogether, these characteristics help insects survive in adverse conditions (https://projects.ncsu.edu).

Insect pests are capable of adapting to toxic materials and new environmental conditions. Similarly, they can escape natural or artificial plant resistance (Roush and McKenzie, 1987). Moreover, insect pests are also responsible for destroying one fifth of the world's total crop production annually (Kerin, 1994). Two major groups of insects are responsible for considerable loss of economically important agricultural crops *viz*. Coleoptera (beetles) and Lepidoptera (moths and butterflies).

The order Coleoptera is the largest order of insects which includes the most common and important stored-grain product pests. A peculiar physical characteristic of coleopteran adults is that they possess forewings which are modified as hard elytra, a protective shield for hindwings. Beetles are able to inhabit a wide variety of habitats and are ubiquitous in nature. Lepidoptera is the second most important order of insect pests of economically important crops. Adults can fly a large distance with two pair of scaly wings ('Lepidon' in Greek means 'scaly'). Mouth parts of adults were adapted to suck nectar or other fluids but are not able to chew plant material while larvae possess well

developed mandible and are able to chew and ingest plant parts. Lepidopteran larvae are well-known for their silk-spinning activities which are responsible for additional loss of agricultural products.

Several species of these two groups are capable of infesting crops both in the field and during storage. As mentioned earlier, Lepidopteran adults do not possess chewing mouth parts, hence, crop damage is only done by the larvae. In the case of Coleoptera, both the developmental stages *viz*. larvae and adults are responsible for crop loss and damage. Some species of Coleoptera are able to infest intact grains but some like *Tribolium* species are able to infest only damaged food grains.

A rapid annual increase in the human population (~2.5-3.0%) in the developing countries like India and China further complicated the problem of competition from insect pests where food resources are only growing by 1% annually (Thind and Singh, 2015). India, with about 1.3 billion people contributes a one fifth of the world's population. According to a study by the Associated Chambers of Commerce and Industry of India, annual crop losses due to insect pests and diseases amount to Rs.50,000 crore ($500 billion), which is significant in a country where every night at least 200 million Indians go to bed hungry. Moreover, agriculture sector contributes 20% of national income and provides employment to 60% of the population. Plant science, therefore, has a prime value in the country (Singh, 2007; Thind and Singh, 2015). The green revolution technologies have relieved the food crisis by increasing food grain production from 82 million tonnes in 1960–61 to 176 million tonnes in 1991–92 and further to 264 million tonnes in 2013–14. This was achieved by steady increase in production of food grains of high yielding varieties of wheat and rice with appropriate agricultural and insect control strategies. According to an estimate, India would require more than 450 million tonnes of food grains to feed 1.65 billion people by 2050 which will be a very challenging task (Thind &Singh, 2015).

In India, where a large number of crops like rice, wheat, pulses, sugarcane and cotton are grown due to the diversified agro-ecosystems, a diverse kinds of insect pests also co-exist which are responsible for severe yield losses all across the country (Figure 1.1). Majority of the insect pests species are from order Lepidoptera, Coleoptera and Diptera. For instance, *Helicoverpa armigera* infests a variety of crops like members from family solanaceae *viz*. tomato and eggplant, crops from family malvaceae *viz*. cotton and okra, crops from family fabaceae *viz*. chickpea and other legumes and plants from family

brassicaceae *viz*. cabbage and cauliflower etc. Some insects, like white flies and aphids also serve as vectors for spreading viral diseases in plants (Thind and Singh, 2015). Evidences indicated that in India, insect pests cause 50% loss in cotton, 25 % in rice, 20% in pulses and sugarcane and 5% in wheat as listed in Table 1.1 (Dhaliwal and Arora, 1996; Singh, 2007).

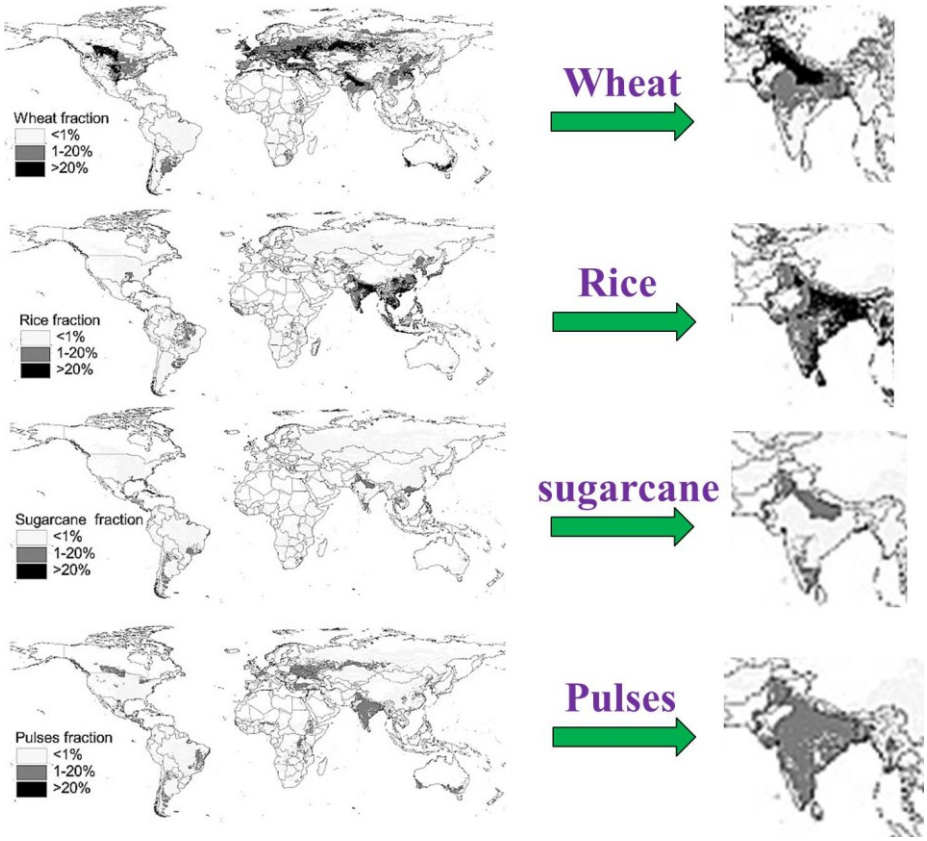


Figure 1.1 Global distribution of major crops cultivated in India. Enlarged images to the right represent distribution of these crops across India (by year 2000) (modified from Leff et al., 2004; Singh, 2007).

Considering the global scenario, with an estimated world population to be 9 billion by 2050, major steps are needed to be taken to meet global food security. Pest control technologies when employed in a satisfactory manner may provide significant economic benefits. For instance, pesticide control provides on an average about $4 benefits for every dollar invested while biological control provides more benefits per

dollar invested than pesticides (Pimentel, 2009). Excessive use of chemical pesticides and fertilizers has further worsened the problem adding environmental pollution, low fertility of soil and emerging insecticide resistance. Since 1987, after the first report of transgenic tobacco plant expressing *Bacillus thuringiensis* toxin (Bt) and providing resistance against insect infestation, genetically modified (GM) crops have reduced use of chemical pesticides by 37%, increased crop yield by 22% and have increased farmers profit by 68% by 2014. Until now 29 GM plants including food crops, fruits and vegetables are commercially available majority of them harboring herbicide and insecticide resistance genes while few have viral resistance gene (ISAAA brief, 49-2014). Recent reports on upcoming insect resistance against economically important GM plants have created a need to generate different GM plants with new targets in insects (Zhu-Salzman et al. 2003; Kluh et al. 2005; Mulligan et al. 2010; Nogueira et al. 2012). Although various reports are available on *in vitro* and *in vivo* interactions and effects of evolutionarily conserved plant defense proteins against insect pests, very few transgenic plants with such defense proteins have reached to field trials while none of them are available commercially (Dunse et al., 2010a). These plant defense proteins include lectins, anti-metabolic peptides, inhibitors of insect's digestive enzymes and other cellular processes. Until now, serine and cysteine proteinases inhibitors have been extensively studied for its bio-potency against various pests where few have reported to be effective through transgenics. To increase inhibitory potential of these defensive proteins, gene fusion, gene stacking and gene pyramiding approaches have been used (Goulet et al. 2008; Zhu et al., 2012). Other than providing insect resistance, few have reported to be beneficial in contributing tolerance against various abiotic stresses. However, detailed understanding of insect's digestive system and its flexibility is necessary to further employ these approaches as a defense against insect pest in fields.

Table 1.1 Estimated losses caused by insect pests to major crops in India*

Crop	**Actual production (million tonnes)**	**Approximate estimated loss in yield due to insect pests**	
		(%)	**Total (million tonnes)**
Rice	93.1	25	31

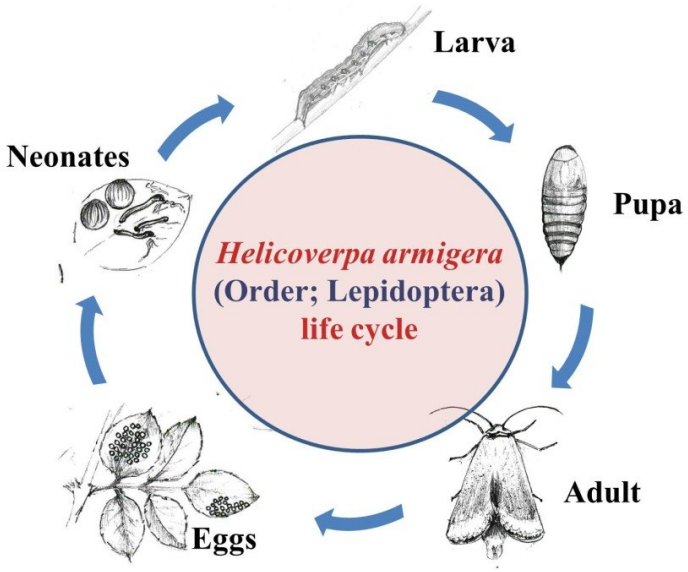
Wheat	71.8	5	3.8
Maize	13.3	25	4.4
Other cereals	20.6	30	8.8
Chickpea	5.3	10	0.6
Other pulses	7.9	20	2
Groundnut	6.9	15	1.2
Rapeseed & mustard	5	30	2.1
Other Oilseeds	8.6	20	2.2
Sugarcane	300.1	20	75
Cotton (lint)	10.1	50	10.1

*Table was adopted from Singh, 2007

1.2 Digestive physiology of insects: Complexity in expression and importance in gaining nutritional requirements

Most of the insects undergo generalized life cycle stages such as egg, larval instars with intermittent molting, pupa and metamorphosis to adults (Figure 1.2A). On the other hand, some part of the life cycle of sucking pest aphids involve parthenogenesis (Figure 1.2B). Diversity in digestive physiology of insects plays a key role in successful completion of life cycle in a particular environment (Jurenka 2015).

A)



B)

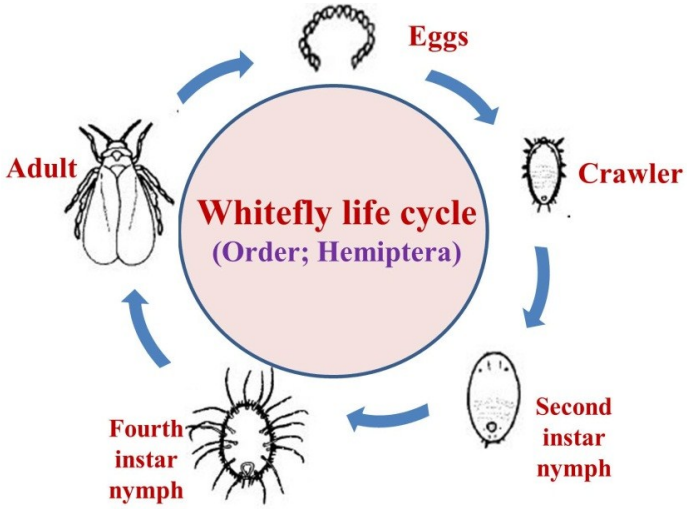


Figure 1.2 Diagrammatic representation of life cycles of insect pests from different orders, A) *H. armigera* from order Lepidoptera and B) Whitefly from order Hemiptera.

Anatomically, alimentary canal or gut of insect is divided into three parts as fore-gut, mid-gut and hind-gut. Food ingested by insect passes through gut with initial churning process in fore-gut while food digestion is carried out in mid-gut region by secretion of various digestive enzymes accompanied by subsequent absorption of nutrients. Finally, water, salts and other beneficial substances are absorbed in hind-gut before excreting remaining frass/ faecal matter. Different classes of digestive enzymes, α-amylases, proteases and lipases primarily carry out digestion of ingested food in insect mid-gut. Depending on nutritive value with protein, carbohydrate and lipid contents of the insect's food, different classes of enzymes play a central role in gaining energy from the ingested food. For example, bruchid weevils, feeding on stored grain pests require to produce considerable amount of α-amylases to digest stored starch or other carbohydrates for energy assimilation. Insects feeding on plant parts like stem, leaves, flowers, seeds or fruits require digestive pool of combination of these enzymes while sucking insects like aphids, fruit flies, jassids etc. produce balanced set of digestive enzyme pool to gain energy from comparatively nutrient deficient plant sap.

Insects regulate production of these enzymes in response to the plant's defense proteins or secondary metabolites or when feeding on various host plants. These

mechanisms vary in specialized to generalist insects (Srinivasan et al. 2005a; Zavala et al. 2008). For example, when fed on different host plant diets, a lepidopteran polyphagous pest *Helicoverpa armigera*, showed variable expression of digestive enzymes in the mid-gut depending on macromolecular composition of the respective diets highlighting diet dependent regulation and adaptability (Figure 1.3); (Patankar et al. 2001; Chougule et al. 2005; Kotkar et al. 2009; Sarate et al. 2012).

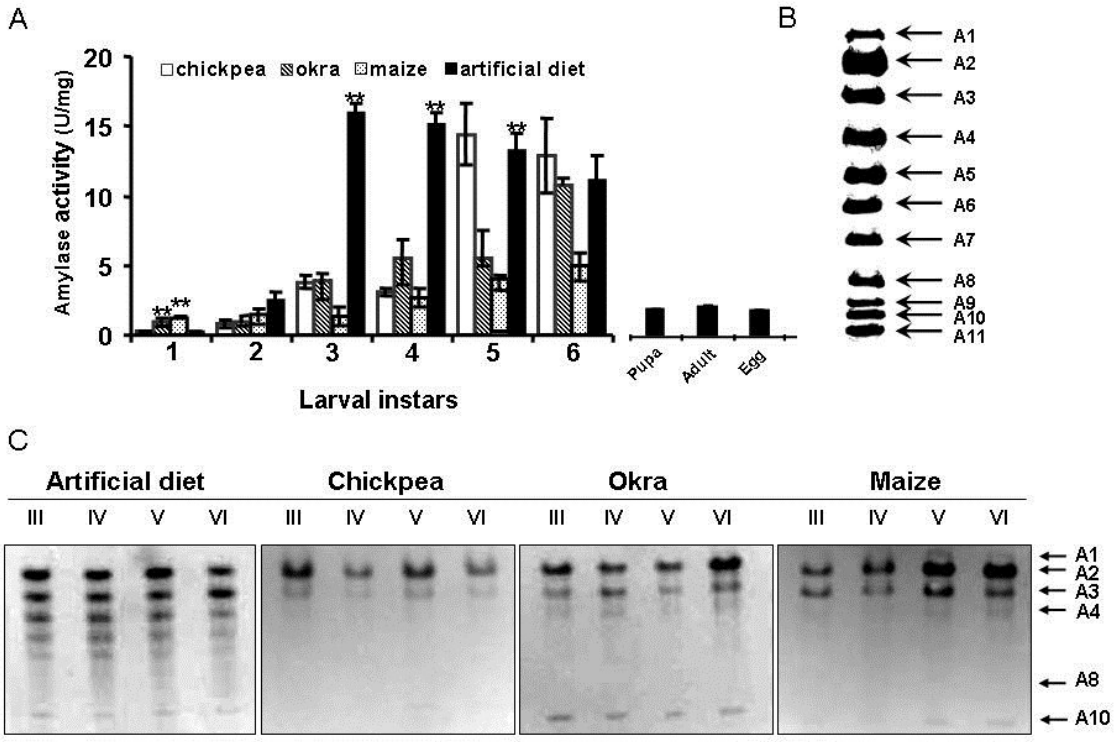


Figure 1.3 Regulation of expression of *Helicoverpa armigera* α-amylase isoforms after feeding on different diets. A. α-amylase activity across different developmental stages of *H. armigera* after feeding on different host plants. **B.** Representation of different α-amylases isoforms. **C.** In-gel α-amylase activity showing regulation of expression of α-amylase isoform after ingestion of various diets. (Figure was adapted from Kotkar et al., 2009)

Similarly, during food break down, regulation of digestive proteases and α-amylases was also evident in cowpea weevil bruchid (Pedra et al. 2003). On the other hand, specialized insect *Manduca sexta* exhibited limited flexibility in digestive proteases upon feeding on native host plant, *Nicotiana attenuata* (Zavala et al. 2008).

Among the three important classes of digestive enzymes, insect proteases have been studied extensively. Besides the proteases, insect α-amylases also play important role in digestion and gaining energy from ingested carbohydrate rich food. Alpha-amylases are found to be more important in insects feeding on storage tissues such as seeds, tubers etc. which are rich source of starch. For example, α-amylases were found to be highly active in feeding stages of stored grain pests coffee berry borer and cowpea weevil (Pedra et al. 2003; Bezerra et al. 2014). Analysis of starch degradation in yellow mealworm gut revealed that the pattern of starch degradation was mainly controlled by type of starch granules from the diet (Meireles et al. 2009). In *H. armigera,* expression of two α-amylase genes was tissue specific and varied across different developmental stages as well as diets (Kotkar et al. 2012). Similarly, transcriptome analysis of salivary gland of western flower thrips and potato leafhopper revealed significant involvement of α-amylases in extra-oral digestion of sugars in plant sap (Stafford-Banks et al. 2014; DeLay et al. 2012). Biochemically, α-amylases of insect pests from different orders were shown to be well adapted to diverse gut environments. For example, α-amylase isoforms from *H. armigera* were found to be stable, optimally active and well-adapted to the alkaline environment of lepidopteran gut lumen (Bhide et al. 2015). However, α-amylases from coleopteran pests showed higher stability towards acidic gut environment (Buonocore et al. 1976). Insect α-amylases possess diversity in their biochemical and biophysical properties. Secondary and tertiary structure variations among these amylases were reflected in their specificities towards various substrates and inhibitors. Very few tertiary structures from insect α-amylases have been reported. Among these available structures, α-amylase from *Tenebrio molitor* was studied extensively to elucidate its catalytic function and molecular interactions with other proteinaceous inhibitors (Figure 1.4).

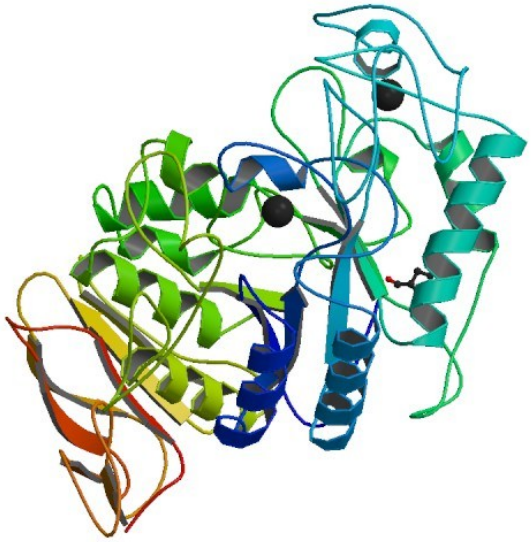


Figure 1.4 Crystal structure of *Tenebrio molitor* α-amylase (PDB ID : 1JAE, Strobl et al., 1998a). Model comprises all 471 amino acid residues of TMA, 261 water molecules, one calcium cation and one chloride anion.

Crystal structure of α-amylase from yellow mealworm, *T. molitor* have revealed that the enzyme consisted of three domains structure named as A, B and C; and all the acidic catalytic residues from the active site of the enzyme were present on "A" domain. Structural alignment and homology modeling of other insect α-amylases indicated that all essential structural and functional residues were conserved across humans to insect α-amylases. A number of 'optional motifs' spanning about three to four amino acids were identified in insect α-amylases while in Drosophila, both the forms of α-amylase with motifs present or absent were reported (Strobl et al. 1998b). Another motif containing nine amino acids stretch in a region called 'variable loop' was present in vertebrates but totally absent in insect α-amylases. In an another comparison, while all of the insect α-amylases had conserved four disulfide bonds, few contained an extra disulfide bridge and this thought to be involved in locking the structure of domain 'C', although exact function of this extra disulfide bridge is still not clear. Comparison of gene structures among insect α-amylases of different orders revealed a considerable difference in the number of introns present (Da Lage et al. 2011).

1.3 Targeting insect pests digestive enzymes with plant proteinaceous inhibitors

Everlasting molecular battle exists between plants and insect pests to overcome each other's defense mechanisms. This leads to the production of myriad of proteinaceous and non-proteinaceous molecules, *viz*. synthesis of toxins, repellents and inhibitors of digestive enzymes in plants. Consequently, insects adapt by producing (i) inhibitor insensitive enzymes, (ii) detoxification machinery and/or (iii) avoidance of toxicity to respective phytochemical(s) that are ingested along with plant part (Figure 1.5).

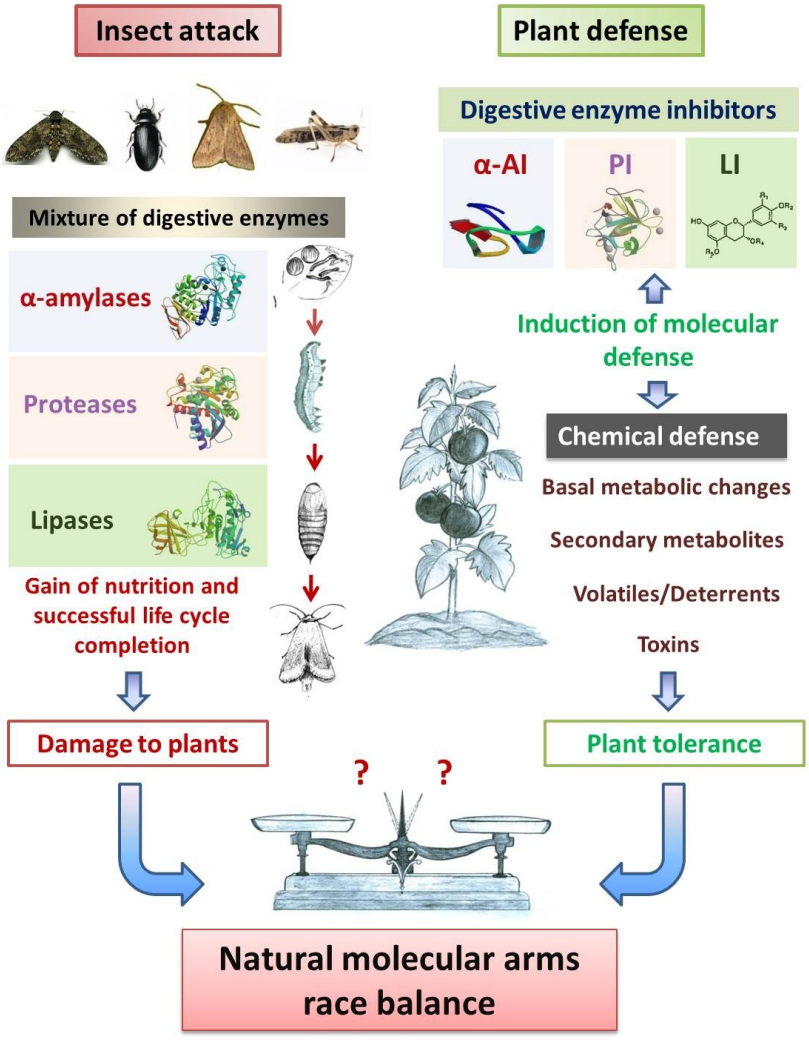


Figure 1.5 Schematics of insect and plant's defense strategies comprising various molecular arsenals and their effects on natural balance between phytophagy and plant tolerance.

Many crop plants severely lack resistance against insect pests, thus, use of chemicals and alternative strategies for protection become prevalent (Simon-Delso et al.

2015). Even though such control measures are being heavily used to cut the losses in crop production, insect infestation remains major challenge in contemporary agriculture (Bass et al. 2015). In recent past, a strategy of transgenic plants expressing proteinaceous toxins from *Bacillus thuringiensis* (Bt toxin) to overcome insect damage on economically important crops has been exploited. Although, effects of these proteins on other organisms and in general to the ecosystem are not fully understood, the use of harmful chemical pesticide is significantly reduced. In addition to the Bt toxin, potentials of other plant derived endogenous proteins have also been evaluated to control the insect infestation (Figure 1.6).

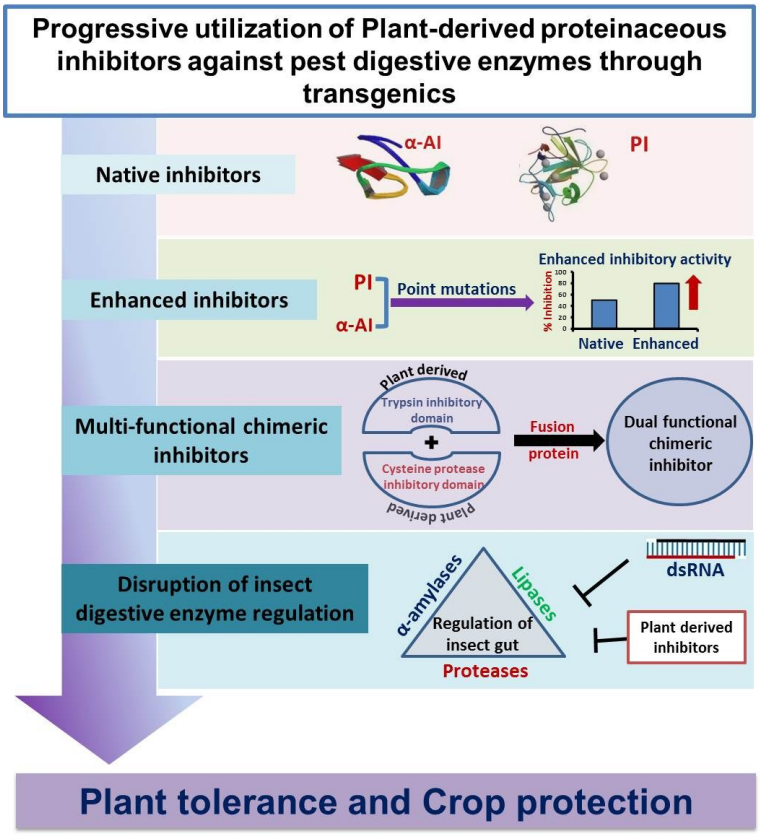


Figure 1.6 Illustration of developments hitherto in utilizing plant-derived proteinaceous inhibitors for effective management of insect pests herbivory.

Plant derived proteinaceous inhibitors against insect's digestive enzymes such as proteases and α-amylases are well studied with the knowledge of their adverse effect on the growth and development of insects (Table 1.2 and Table 1.3). For example, common

bean α-amylase inhibitors (α-AI-1 and α-AI-2) were used to develop transgenic pea and other plants for insect tolerance. Field trials of transgenic pea containing α-AI-1 gene revealed larval mortality in pea weevil while α-AI-2 delayed larval maturation (Pereira et al. 1999). In an another report, cowpea plants expressing α-AI-1 were ineffective against parasitoid of cowpea weevil and hence, predicated to be non-harmful to the surrounding ecosystem (Luthi et al. 2013b). Activation of these inhibitors requires proteolytic processing of its pro-protein form to remove conformational constraint (Pueyo et al. 1993). Bean α-AI-1 was also reported to possess chitinolytic activity against *Zabrotes subfasciatus* (Dayler et al. 2005). Wheat and barley's monomeric, dimeric and tetrameric α-AIs were effective, particularly against storage insect pests (Petrucci et al. 1976; Lazaro et al. 1988; Sanchez-Monge et al. 1989; Gomez et al. 1991). However for these types of inhibitors, contradictory reports exist on the essential role of glycosylation in inhibition (Garcia-Maroto et al. 1991). A biodegradable leaf lectin from timber tree having α-AI activity was reported as a larvicial and was resistant to proteolysis in the gut of *Aedes aegypti* (Napoleao et al. 2012; Lagarda-Diaz et al. 2014). In an another study, lectin-like seed storage protein, Arcelin-1, caused severe damage to the epithelial cells altering gut structure of the insect (Paes et al. 2000). Bi-functional inhibitors of amylases and proteases from rice and castor oil seeds were characterized for their effect on insect digestive enzymes (Yamasaki et al. 2006; Do Nascimento et al. 2011). Antimicrobial peptides from plants (cowpea defensins) were shown to possess inhibitory activity against cowpea weevil α-amylases (Dos Santos et al. 2010; Vieira Bard et al. 2015). Wrightides, a pseudocyclic cysteine-knot α-AIs isolated from water jasmine was effective against bruchid pests (Nguyen et al. 2014). α-AIs from microbial source were investigated for their biochemical properties. For example, *Streptomyces avermitilis* α-AI was reported to inhibit gut α-amylases of *H. armigera* (Zeng et al. 2013). Amylase and α-AI from insect and plants, respectively, were characterized and α-AI activity against *H. armigera* amylases was demonstrated (Giri and Kachole 1998).

Other than Bt toxin, attempts have been made to generate transgenic plants expressing plant protease and α-amylase inhibitors which could be used as an alternative strategy to control insect infestation. Majority of the studies on transgenic plants in laboratories have promised effective employment of plant proteinaceous inhibitors as an

insect control agent although efficacy of these agents in fields and their long-term effects on environment needs to be carefully investigated.

1.4 Molecular insights into mechanism of action of inhibitors against target enzymes

Unraveling fundamental molecular mechanism of inhibitory reactions between plant proteinaceous inhibitors and insect digestive enzymes is elemental to design more potent and effective molecules. Much of the knowledge of inhibitory mechanism came from analysis of effects of various mutations created in inhibitory domain(s) of a particular inhibitor on its inhibitory activity. Additionally, resolution of three-dimensional structures of enzyme-inhibitor complexes imparted special molecular insights for enhancing inhibitory potential. A proteinaceous α-AI from *Tephrosia villosa* (TvD1) was studied extensively by generating peptide variants through *in vitro* mutagenesis. These studies demonstrated that loop β3 was important for the inhibitory activity and involved in binding with active site of the enzyme (Liu et al. 2006; Lin et al. 2007; Vijayan et al. 2012). Similarly, α-AI from Amaranthus spp (AAI) bound to central four sugar binding subsites of the *Tenebrio molitor* α-amylase (TMA) blocked the active site (Figure 1.7).

AAI bound with other insect α-amylases showed relative difference in hydrogen bond formation (Pereira et al. 1999). More complex interactions were observed in case of bi-functional proteinaceous inhibitors. Ragi bi-functional inhibitor (RBI) completely filled the substrate-binding site of TMA (Figure 1.8).

Catalytic residues in the active site of TMA (Asp185, Glu222 and Asp287) were contacted by free N-terminus and first residue (Ser1) of RBI and the complex was further stabilized by extensive interaction between enzyme and α-AI. Usual flexible N-terminal segment of RBI in native form adopted a 3(10) type helical conformation upon binding to TMA whereas structural reorientation in TMA was not observed. Trypsin binding loop was located opposite to the α-amylase binding site and imparted dual α-amylase and trypsin inhibitory property (Strobl et al. 1998b).

Table 1.2 Details of plant derived protease inhibitors against gut proteases from various classes of insect pests

Insect Name	PI from plant	PI name	Tissue	Type of PI	Reference
Lepidoptera					
Alabama argillacea	*Pithecellobium dumosum*	PdKI-4	Seeds	Kunitz	(Rufino et al. 2013)
Anagasta kuehniella	*Entada acaciifolia*	EATI	Seeds	Kunitz	(de Oliveira et al. 2014)
	Acacia polyphylla DC	AcKI	Seeds	Kunitz	(Machado et al. 2013)
	Sapindus saponaria L.	SSTI	Seeds	Trypsin PI	(Macedo et al. 2011)
	Adenanthera pavonina	ApTI	Seeds	Trypsin PI	(Macedo et al. 2010)
	Delonix regia	DrTI	Seeds	Trypsin PI	(Macedo et al. 2009)
	Plathymenia reticulata	PFTI	Seeds	Kunitz	(Ramos et al. 2008)
	Poecilanthe parviflora	PPTI	Seeds	Trypsin PI	(Garcia et al. 2004)
Andoxophynes oana	*Medicago scutellata*	MsTI	Seeds	Bowman-Birk	(Ceciliani et al. 1997)
	Theobroma cacao	TcPIs	Seeds	Serine PI	(Paulillo et al. 2012)
	Sapindus saponaria L.	SSTI	Seeds	Trypsin PI	(Macedo et al. 2011)
	Talisia esculenta	Talisin	Seeds	Trypsin PI	(Macedo et al. 2011)
Chilo suppressalis	*Zea mays* and *Solanum tuberosum*	MPI+PCI fusion protein[1]	--	Serine PI	(Quilis et al. 2014)
Corcyra cephalonica	*Sapindus saponaria L.*	SSTI	Seeds	Trypsin PI	(Macedo et al. 2011)
	Delonix regia	DrTI	Seeds	Trypsin PI	(Macedo et al. 2009)
	Entada scandens	ESTI	Seeds	Kunitz	(Lingaraju & Gowda 2008)
	Poecilanthe parviflora	PPTI	Seeds	Trypsin PI	(Garcia et al. 2004)
Diatraea saccharalis	*Passiflora edulis*	KTIs	Leaves	Kunitz	(Botelho-Junior et al. 2014)
	Adenanthera pavonina	ApTI	Seeds	Trypsin PI	(da Silva et al. 2014)
	Inga laurina	ILTI	Seeds	Trypsin PI	(Ramos et al. 2012)
	Theobroma cacao	TcPIs	Seeds	Serine PI	(Paulillo et al. 2012)
	Sapindus saponaria	SSTI	Seeds	Trypsin PI	(Macedo et al. 2011)
	Poecilanthe parviflora	PPTI	Seeds	Trypsin PI	(Garcia et al. 2004)
Ectomyelois ceratoniae	*Punica granatum*	PgTI	Seeds	Trypsin PI	(Ranjbar et al. 2014)
Epiphyas postvittana	*Nicotiana alata*	AP-TI	Seeds	Kunitz	(Maheswaran et al. 2007)

Galleria mellonella	*Fagopyrum esculentum*	BWI-2c	Seeds	Trypsin PI	(Oparin et al. 2012)
Helicoverpa armigera	*Triticum aestivum*	WCI	Endosperm	Chymotrypsin PI	(Di Maro et al. 2011)
	Glycine max	SKTI	Seeds	Kunitz	(Kuwar et al. 2015)
	Capsicum annum	IRD-7, IRD-9, IRD-12	fruits	Pin-II	(Joshi et al. 2014b)
	Eugenia jambolana	EjTI	Seeds	Trypsin PI	(Singh et al. 2014)
	Nicotiana alata	NaPIs	Flowers	Pin-II	(Stevens et al. 2013)
	Solanum tuberosum	StPin1A		Pin-I	(Dunse et al. 2010)
	Solanum americanum	SaPIN2a	Trichomes	Serine PI	(Luo et al. 2009)/(Luo et al. 2012)
	Acacia nilotica	AnPI	Seeds	Trypsin PI	(Babu et al. 2012)
	Murraya koenigii	MKMLP	Leaves	Kunitz	(Gahloth et al. 2011)
	Capsicum annuum	CanPI-5, -7, -13, -15, -19, 22	Fruits	Pin-II	(Mishra et al. 2010)
	Ipomoea batatas + *Colocasia esculenta*	Sporamin[2]+ CeCPI	--	Trypsin PI	(Senthilkumar et al. 2010)
	Albizzia kalkora	AkTI	Seeds	Kunitz	(Zhou et al. 2008)
	Cicer arietinum	CaKPI	Seeds	Kunitz	(Srinivasan et al. 2005)
	Triticum aestivum	WSCI	Seeds	S/C inhibitor[3]	(Di Gennaro et al. 2005)
	Momordica charantia	BGPI-1 to 4	Seeds	Serine PI	(Telang et al. 2003)
	Psophocarpus tetragonolobus	WBTI-1 to 4	Seeds	Kunitz	(Giri et al. 2003)
	Psophocarpus tetragonolobus	WBTI-5 to 7	Seeds	Bowman-Birk	(Giri et al. 2003)
Helicoverpa punctigera	*Nicotiana alata*	NaTIs and NaCI	Flowers	Pin-II	(Anderson et al. 1997)/ (Dunse et al. 2010)
Helicoverpa zea	*Diplotaxis muralis*	DmTI	Seeds	Trypsin PI	(Volpicella et al. 2009)
	Diplotaxis tenuifolia	DtTI	Seeds	Trypsin PI	(Volpicella et al. 2009)
	Solanum tuberosum	PCI	--	CPI[4]	(Bayes et al. 2006)
Heliothis virescens	*Inga laurina*	ILTI	Seeds	Trypsin PI	(Ramos et al. 2012)
Lacanobia oleracea	*Glycine max*	SKTI	seeds	Kunitz	(Gatehouse et al. 1999)
Manduca sexta	*Beta vulgaris*	BvSTI	Leaves	Serine PI	(Smigocki et al. 2013)
Pieris rapae	*Albizzia kalkora*	AkTI	Seeds	Kunitz	(Zhou et al. 2008)
	Cassia obtusifolia	CoTI	Seeds	Kunitz	(Liao et al. 2007)
Plodia interpuncptella	*Piptadenia moniliformis*	PmTKI	Seeds	Kunitz	(Cruz et al. 2013)
	Pithecellobium dumosum	PdKI-3.1, PdKI-3.2	Seeds	Kunitz	(Oliveira et al. 2009)

	Triticum aestivum	WSCI	Seeds	S/C inhibitor	(Di Gennaro et al. 2005)
plutella xylostella	*Brassica juncea*	MTI2	Seeds	Trypsin PI	(Yang et al. 2009)
Sitotroga cerealella	*Vigna unguiculata*	CpTI	Seeds	Trypsin PI	(Bi et al. 2006)
Spodoptera exigua	*Beta vulgaris*	BvSTI	Leaves	Serine PI	(Smigocki et al. 2013)
	Solidago altissima L.	Crude SPIs	Leaves	Serine PI	(Bode et al. 2013)
	Solanum nigrum	SPI1 and SPI2	Leaves	Serine PI	(Hartl et al. 2010)
	Albizzia kalkora	AkTI	Seeds	Kunitz	(Zhou et al. 2008)
	Helianthus annuus	SMC-T3 SMC-T23	--	Cysteine-serine PI	(Inanaga et al. 2001)
	Hordeum vulgare L.	BTI-CMe	Seeds	Kunitz	(Lara et al. 2000)
Spodoptera frugiperda	*Zea mays*	Oh43 BBI	Leaves	Bowman-Birk	(Johnson et al. 2014)
	Beta vulgaris	BvSTI	Leaves	Serine PI	(Smigocki et al. 2013)
	Entada acaciifolia	EATI	Seeds	Kunitz	(de Oliveira et al. 2014)
	Poecilanthe parviflora	PPTI	Seeds	Trypsin PI	(Garcia et al. 2004)
Spodoptera littoralis	*Vigna unguiculata*	Cowpea PI	Seeds	Serine PI	(Abd El-latif 2015)
	Arabidopsis thaliana	AtSerpin1	Leaves	Serine PI	(Alvarez-Alfageme et al. 2011)
	Soghum bicolor	Sorghum PIs	Seeds	Serine PI	(El-latif 2014)
	Zea mays	Maize PIs	Seeds	Serine PI	(El-latif 2014)
	Solanum nigrum	SPI1	Leaves	Serine PI	(Hartl et al. 2010)
	Brassica juncea	MTI2	Seeds	Trypsin PI	(De Leo & Gallerani 2002)
Spodoptera litura	*Murraya koenigii*	MKMLP	Leaves	Kunitz	(Gahloth et al. 2011)
	Archidendron ellipticum	AeTI	Seeds	Kunitz	(Bhattacharyya et al. 2007)
	Momordica charantia	BGPI-1 to 4	Seeds	Serine PI	(Telang et al. 2003)
	Brassica juncea	BjTI	Seeds	Kunitz	(Mandal et al. 2002)
Telchin licus	*Pithecellobium dumosum*	PdKI-4	Seeds	Kunitz	(Rufino et al. 2013)
Trichoplusia ni	*Arabidopsis thaliana*	UPI	Leaves	Serine PI	(Laluk & Mengiste 2011)
Coleoptera					
Anthonomus grandis	*Cicer arietinum*	CaTI	Seeds	Trypsin PI	(de P G Gomes et al. 2005)
	Piptadenia moniliformis	PmTKI	Seeds	Kunitz	(Cruz et al. 2013)
	Inga umbratica	IUTCI	Seeds	Bowman-Birk	(Calderon et al. 2005)

	Glycine max	SKTI	Seeds	Kunitz	(Franco et al. 2004)
	Vigna unguiculata	BTCI	Seeds	Serine PI	(Franco et al. 2003)
Callosobruchus maculatus	*hybrid Capsicum (Ikeda × UENF 1381)*	HyPep	Seeds	-	(Vieira Bard et al. 2015)
	Pithecellobium dumosum	PdKI-4	Seeds	Kunitz	(Rufino et al. 2013)
	Banksia rufa	BrTI	-	Trypsin PI	(Sumikawa et al. 2010)
	Crotalaria pallida	CpaTI	Seeds	Trypsin PI	(Gomes et al. 2005)
	Tamarindus indica	TTI	Seeds	Trypsin PI	(Araujo et al. 2005)
	Glycine max	SCPI-L1, SCPI-R1, SCPI-N2	Seeds	Cysteine PI	(Lalitha et al. 2005)
	Adenanthera pavonina	ApTI	Seeds	Trypsin PI	(Macedo et al. 2004)
Cosmopolites sordidus	*Oryza sativa*	OsCYS1	-	Cysteine PI	(Kiggundu et al. 2010)
	Carica papaya	CpCYS1	-	Cysteine PI	(Kiggundu et al. 2010)
Diabrotica virgifera virgifera	*Glycine max*	SCPI-L1, SCPI-R1, SCPI-N2	Seeds	Cysteine PI	(Lalitha et al. 2005)
homalinotus coriaceus	*Inga laurina*	IlTI	Seeds	Kunitz	(Macedo et al. 2011)
hypera postica	*Solanum tuberosum*	API	-	Aspartyl PI	(Wilhite et al. 2000)
Hypothenemus hampei	*Lupinus bogotensis*	LbAPI	Seeds	Aspartyl PI	(Molina et al. 2014)
	Phaseolus coccineus	PcBBI1	Seeds	Bowman-Birk	(de Azevedo Pereira et al. 2007)
Leptinotarsa decemlineata	*Solanum lycopersicum and Solanum tuberosum*	CDI-CCII Hybrid inhibitor	-	Aspartyl and Cysteine PI	(Brunelle et al. 2005)
	Glycine max	SCPI-L1, SCPI-R1, SCPI-N2	Seeds	Cysteine PI	(Lalitha et al. 2005)
Prostephanus truncatus	*Hyptis suaveolens L.*	HsTI	Seeds	Trypsin PI	(Aguirre et al. 2004)
Tenebrio molitor	*Triticum aestivum*	WCI	Endosperm	Chymotrypsin PI	(Di Maro et al. 2011)
	Fagopyrum esculentum	BWI-2c	Seeds	Trypsin inhibitor	(Oparin et al. 2012)
	Triticum aestivum	WSCI	Seeds	S/C inhibitor	(Di Gennaro et al. 2005)
	Eleusine coracana	RBI	Seeds	Amylase /trypsin bifunctional inhibitor	(Strobl et al. 1998a)
Tribolium castaneum	*Fagopyrum esculentum*	BWI-2c	Seeds	Trypsin PI	(Oparin et al. 2012)
	Castanea sativa	CsC	Seeds	Cysteine PI	(Pernas et al. 1998)
Zabrotes subfasciatus	*Pithecellobium dumosum*	PdKI-4	Seeds	Kunitz	(Rufino et al. 2013)

	Pithecellobium dumosum	PdKI-3.1, PdKI-3.2	Seeds	Kunitz	(Oliveira et al. 2009)
Diptera					
Aedes aegypti	*Clitoria fairchildiana*	ClfTI	Cotyledon	Trypsin PI	(de Oliveira et al. 2015)
	Myracrodruon urundeuva	MuLL	Leaves	Trypsin PI	(Napoleao et al. 2012)
	Moringa oleifera	MoFTI	Flower	Trypsin PI	(Pontual et al. 2012)
Ceratitis capitata	*Piptadenia moniliformis*	PmTKI	Seeds	Kunitz	(Cruz et al. 2013)
	Pithecellobium dumosum	PdKI-3.1, PdKI-3.2	Seeds	Kunitz	(Oliveira et al. 2009)
	Crotalaria pallida	CpaTI	Seeds	Trypsin PI	(Gomes et al. 2005)
	Tamarindus indica	TTI	Seeds	Trypsin PI	(Araujo et al. 2005)
Liriomyza trifolii	*Solanum tuberosum*	PI-II + PCI hybrid	-	Serine PI	(Abdeen et al. 2005)
Mayetiola destructor	*Vigna umbellata*	RbTI	Seeds	Bowman-Birk	(Katoch et al. 2014)
Hemiptera					
Eurygaster integriceps	*Glycine max*	SBTI	Seeds	Trypsin PI	(Saadati & Bandani 2011)
Ferrisia virgata	*Vigna unguiculata*	CpTI	Seeds	Trypsin PI	(Wu et al. 2014)
Myzus persicae	*Hordeum vulgare*	HvCPI-6	Seeds	Cysteine PI	(Carrillo et al. 2011)
Riptortus clavatus	*Zea mays*	CC1	Seeds	Cysteine PI	(Ishimoto et al. 2012)
Homoptera					
Acyrthosiphon pisum	*Arabidopsis thaliana*	AtSerpin1	Leaves	Serine PI	(Alvarez-Alfageme et al. 2011)
Hymenoptera					
Osmia bicornis	*Oryza sativa*	oryzacystatin-1 (OC-1)	Seeds	Cysteine PI	(Konrad et al. 2008)
Hymenoptera					
Chrysoperla carnea	*Oryza sativa*	oryzacystatin-1 (OC-1)	Seeds	Cysteine PI	(Mulligan et al. 2010)
Orthoptera					
Locusta migratoria	*Amaranthus hypochondriacus*	AmI	Seeds	Serine PI	(Tamir et al. 1996)
Blattodia					
Blattella germanica	*Fagopyrum esculentum*	BWI-2c	Seeds	Trypsin PI	(Oparin et al. 2012)

Table 1.3 Plant derived α-amylase inhibitors against digestive α-amylase from different classes of insect pests

Insect species	Plant species	AI	Tissue	AI type	Reference
Coleoptera					
Acanthoscelides obtectus	*Secale cereale*	BIII	Seeds	-	(Dias et al. 2005)
	Triticum aestivum	Crude wheat α-AI	Kernel	-	(Franco et al. 2005)
	Phaseolus vulgaris	α-AIC3, α-AIA11,α-AIG4	Seeds	Knottin	(da Silva et al. 2013)
Bruchus pisorum	*Phaseolus vulgaris*	αAI-1, αAI-2	Seeds	-	(Suzuki et al. 1994)/ (de Sousa-Majer et al. 2007)
	Pisum sativum	α-AI-1,α-AI-2	Seeds	-	(Morton et al. 2000)
Callosobruchus chinensis	*Phaseolus vulgaris*	αAI-1	Seeds	-	(Luthi et al. 2013a)
Callosobruchus maculatus	*hybrid Capsicum (Ikeda × UENF 1381)*	HyPep	Seeds	-	(Vieira Bard et al. 2015)
	Phaseolus vulgaris	αAI-1	Seeds	-	(Luthi et al. 2013a)
	Ricinus communis	Ric c1, Ric c3	Seeds	-	(Do Nascimento et al. 2011)
	Achyranthes aspera	AI1,AI2	Seeds	-	(Hivrale et al. 2011)
	Vigna radiata	αAI	Seeds	-	(Wisessing et al. 2010)
	Vigna unguiculata	αAI/defensin	Seeds	-	(Dos Santos et al. 2010)
	Dipteryx alata	αAI	Seeds	-	(Bonavides et al. 2007)
	Pterodon pubescens	PpAI	Seeds	-	(Silva et al. 2007)
	Phaseolus acutifolius	α-AI-Pa1,α-AI-Pa2	Seeds	-	(Yamada et al. 2001)
Diabrotica virgifera virgifera	*Triticum aestivum*	WI-1	Seeds	-	(Titarenko & Chrispeels 2000)
	Phaseolus vulgaris	αAI-1	Seeds	-	(Titarenko & Chrispeels 2000)
Hypothenemus hampei	*Phaseolus vulgaris*	αAI-1	Seeds	-	(Barbosa et al. 2010)
	Phaseolus coccineus	αAI	Seeds	-	(Valencia-Jimenez et al. 2008)
	Phaseolus coccineus	α-AI-Pc1	Cotyledon	-	(de Azevedo Pereira et al. 2006)
Mimosestes mimosae	*Gameleira sp.*(ficus)	α-ZSAI	Seeds	-	(Bezerra et al. 2004)
Prostephanus truncatus	*Amaranthus hypochondriacus*	AAI	Seeds	Knottin	(Chagolla-Lopez et al. 1994)
Rhyzopertha dominica	*Triticum aestivum*	α-AI	Kernel	-	(Cinco-Moroyoqui et al. 2006)
Sitophilus oryzae	*Triticum aestivum*	WRP25, WRP26	Seeds	-	(Feng et al. 1996)
Tenebrio molitor	*Wrightia religiosa*	Wrightide/ Wr-AI1	-	Knottin	(Nguyen et al. 2014)
	Ricinus communis	Ric c1, Ric c3	Seeds	-	(Do Nascimento et al. 2011)
	Tephrosia villosa	S32R, D37R (α-TvD1)	-	-	(Vijayan et al. 2012)
	Amaranthus hypochondriacus	AAI	Seeds	Knottin	(Pereira et al. 1999)
	Phaseolus vulgaris	αAI-1	Seeds	-	(Nahoum et al. 1999)
	Eleusine coracana	RBI	Seeds	Amylase /trypsin bifunctional	(Strobl et al. 1998a)

				inhibitor	
	Secale cereale	RDAI-1	Endosperm	-	(Garcia-Casado et al. 1994)
	Triticum aestivum	WMAI-1, WMAI-2, WDAI-3	Endosperm	-	(Sanchez-Monge et al. 1989)/ (Gomez et al. 1991)
	Hordeum vulgare	14.5kDa allergen	Endosperm	-	(Barber et al. 1989)
	Hordeum vulgare	BDAI-1	Endosperm	-	(Lazaro et al. 1988)
	Triticum aestivum	Inhibitor-0.19, Inhibitor-0.28	Kernels	-	(Petrucci et al. 1976)
	Triticum aestivum	WRP25, WRP26	Seeds	-	(Franco et al. 2000)
Tribolium castaneum	*Amaranthus hypochondriacus*	AAI	Seeds	Knottin	(Chagolla-Lopez et al. 1994)
	Oryza sativa	bifunctional subtilisin/ αAI	Seeds	Kunitz	(Ohtsubo & Richardson 1992)
Tribolium confusum	*Achyranthes aspera*	AI1,AI2	Seeds	-	(Hivrale et al. 2011)
Zabrotes subfasciatus	*Olneya tesota*	PF2/lectin	Seeds	-	(Lagarda-Diaz et al. 2014)
	Vigna unguiculata	αAI/defensin	Seeds	-	(Dos Santos et al. 2010)
	Phaseolus vulgaris	α-AI-1, α-AI-2	Seeds	-	(Grossi de Sa et al. 1997)/ (Luthi et al 2013b)
	Ricinus communis	Ric c1, Ric c3	Seeds	-	(Do Nascimento et al. 2011)
	Phaseolus vulgaris	PvCAI	Seeds	-	(Dayler et al. 2005)
	Secale cereale	BIII	Seeds	-	(Dias et al. 2005)
	Gameleira sp.(ficus)	α-PPAI, α-ZSAI	Seeds	-	(Bezerra et al. 2004)
Lepidoptera					
Ephestia kuehniella	*Phaseolus vulgaris*	αAI-1	Seeds	-	(Pytelkova et al. 2009)
	Triticum aestivum	WI-1, WI-3	Seeds	-	(Pytelkova et al. 2009)
Helicoverpa armigera	*Achyranthes aspera*	AI1,AI2	Seeds	-	(Hivrale et al. 2011)
Tecia solanivora	*Phaseolus coccineus*	α-AI	Seeds	-	(Valencia-Jimenez et al. 2008)
	Phaseolus vulgaris	α-AI	Seeds	-	(Valencia-Jimenez et al. 2008)
	Amaranthus blitoides x albus	α-AI	Seeds	-	(Valencia-Jimenez et al. 2008)
Blattodea					
Periplaneta americana	*Sorghum bicolar*	SI alpha-1, SI alpha-2, ST α-3	Seeds	Purothionin like	(Bloch & Richardson 1991)
Hemiptera					
Eurigaster integricep	*Triticale(× Triticosecale)*	T-αAI	Seeds	-	(Mehrabadi et al. 2010)
Orthoptera					
Locusta migratoria	*Sorghum bicolar*	SI alpha-1, SI alpha-2, ST α-3	Seeds	Purothionin like	(Bloch & Richardson 1991)
	Coix lachryma-jobi	αAI	Seeds	-	(Ary et al. 1989)

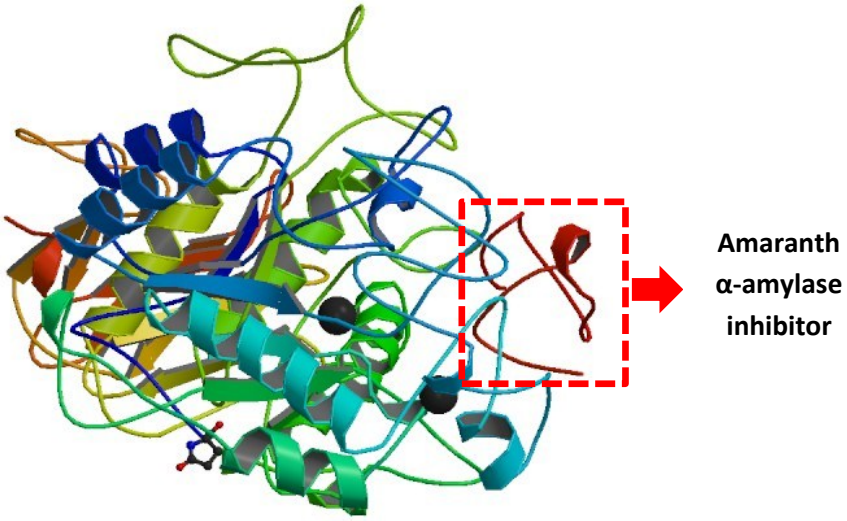


Figure 1.7 Crystal structure of *Tenebrio molitor* alpha-amylase in complex with the amaranth alpha-amylase inhibitor. (PDB ID: 1CLV; Pereira et al., 1999)

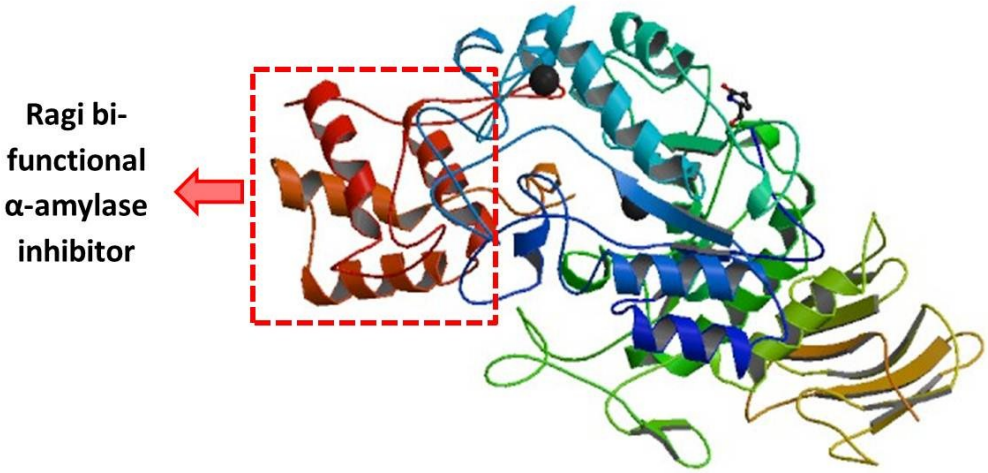


Figure 1.8 Crystal structure of *Tenebrio molitor* alpha-amylase in complex with Ragi bi-functional inhibitor. The Ragi bifunctional alpha-amylase/trypsin inhibitor (RBI) inhibits both trypsin and alpha-amylases and is the prototype of the cereal inhibitor superfamily. (PDB ID:1TMQ, Strobl et al., 1998b)

Another α-AI from barley (BASI) inhibited the endogenous α-amylases by similar steric hindrance to the active site of the α-amylase although in this case an unusual completely solvated calcium ion was located at the protein-protein interface (Vallee et al. 1998). Differential scanning calorimetric analysis of interactions of wheat 0.28 and 0.19 α-AIs with TMA revealed differences in thermal stability which was reflected in their distinct inhibitory activity. However, both the interactions showed significant stabilization relative to free enzyme indicating formation of stable enzyme-inhibitor complexes (Silano and Zahnley 1978). Similarly, FPLC analysis revealed distinct mode of interactions between α-amylase isoforms from *H. armigera* and wheat α-AI. Aggregation reaction of one of the isoform with wheat α-AI reflected into its respective higher inhibition of enzyme activity (Bhide et al. 2015; present thesis).

Although such elucidations of molecular inhibitory mechanisms have been available, the majority of the interactions between insect digestive enzyme and its specific inhibitor remain to be explored. More recent knowledge based on current technologies and research could be useful in developing more effective, potent and specific inhibitors.

1.5 Molecular insights into mechanism of induced resistance of insect against plant proteinaceous inhibitors.

Many studies have reported the development of resistance mechanisms in insect gut digestive enzymes against plant proteinaceous inhibitors. For example, cowpea weevil when encountered with plant AI showed global proteome change. These included expression of cathepsin-B like cysteine proteases, over expression of proteins involved in (i) cell wall degradation, (ii) transcription and translation and (ii) stress (Zhu-Salzman et al. 2003; Mulligan et al. 2010; Nogueira et al. 2012). Another mechanism of resistance reported was induction of insensitive digestive enzymes to the plant proteinaceous inhibitors in the gut region (Bown et al. 1997). Characterization of interactions of common bean α-AI with various insect α-amylases revealed that the insensitivity of insect α-amylases was due to the specific mutations in α-AI binding sites (Kluh et al. 2005). Docking and molecular dynamics simulation analysis of interactions of potato carboxypeptidase inhibitor (PCI) and metallo-carboxypeptidase (MCPs) indicated change in the conformation of β8-α9 loop which narrowed the access to active site and prohibited

entrance of 'C' termini tail of PCI. These interactions also induced disruption of hydrogen bonding in surrounding residues. Thermodynamic analysis of binding of PCI to MCPs further revealed that difference in electrostatic energy was a main contributor for the variation observed in binding affinities between PCI and other insect proteases (Zhang et al. 2012b). Therefore, deeper knowledge of insect resistance mechanisms is very crucial to develop effective insect control strategy based on plant proteinaceous inhibitors.

1.6 Regulation of endogenous and transgenic proteinaceous inhibitors in plants

Successful exploitation and implementation of transgenic technology require complete understanding of regulation of proteinaceous inhibitors in plants and their effect on normal physiological processes. Induction of endogenous proteinaceous inhibitors in plants was reported to occur through metabolites of octadecanoid pathway mainly jasmonic acid (JA) and methyl jasmonate (mJA) in response to external signals or elicitors. One such elicitor, ZmPep3, in maize stimulated production of JA and ethylene which upregulated defense genes including different proteinaceous inhibitors and biosynthetic enzymes for the production of volatile terpenes and benzoxazenoids, all together imparting resistance against *Spodoptera exigua* (Huffaker et al. 2013). After mJA application, leaves of Passion fruit assembled a cocktail of functionally diverse proteinaceous inhibitors and recruited two of them against cysteine proteases (Botelho-Junior et al. 2014). Similarly, monthly application of mJA induced high levels of proteinaceous inhibitors in Datura leaves eventually mitigating loss by herbivory (Kruidhof et al. 2012). Localization of potato cysteine PIs by immunocytochemistry after mJA mediated induction revealed their accumulation in stem vacuoles (Gruden et al. 1997). Comparable study in pigeon pea showed systemic induction of plant PIs after mJA treatment (Lomate and Hivrale 2012). Besides mJA, application of other plant phytohormones like salicylic acid and abscisic acid were also found to be responsible for induction of serine proteases in Arabidopsis (Laluk and Mengiste 2011). Another regulatory peptide from tomato and potato known as systemin was also involved in regulation of protease inhibitors during insect defense (Ryan and Pearce 2003; Bhattacharya et al. 2013). Further, antisense mediated depletion of transcripts of genes involved in wound induced signal transduction pathway were found to be responsible for suppression of gene expression of proteinaceous inhibitors. Insect feeding experiments

with these plants showed reduction of tolerance in plants and weight gain in insects (Royo et al. 1999; Yang et al. 2013). Certain endogenous proteinaceous inhibitors along with their defense function have dual roles in regulating development and physiological processes in plants. They show tissue specific accumulation patterns in response to the herbivory (Hartl et al. 2011; Lepelley et al. 2012).

Gene expression, processing and accumulation of heterologous proteinaceous inhibitors in transgenic plants were influenced by various biotic and abiotic factors. For example, equistatin- a sea anemone PI, when expressed in potato degraded by endogenous arginine or lysine specific and legumain-type Asn-specific cysteine proteases and thus, hampered it's *in planta* accumulation. Transgenic potato lines expressing this PI showed insufficient resistance against Colorado potato beetle (Outchkourov et al. 2003). Biotic external stimuli like extent of oviposition and insect regurgitation induced plant defense response against insect herbivory (Lawrence et al. 2007; Kim et al. 2012; Bandoly et al. 2015). Abiotic factors like high temperature, elevated CO_2 or leaf position also affected accumulation of proteinaceous inhibitors in high levels (Sousa-Majer et al. 2004; Zavala et al. 2009). Till now, studies on induction and regulation of endogenous or heterologous proteinaceous protease inhibitors in plants have highlighted the importance of these regulatory processes in successful implementation of proteinaceous inhibitors as a defense strategy against insect pests. In similar manner, study of *in vivo* regulation of α-amylase inhibitors is necessary to assess the utilization of α-amylase inhibitors as an effective insect control agent.

1.7 Advances in utilization of plant proteinaceous inhibitors against insect digestive enzymes

In recent years, various advance approaches have been used such as structure based protein engineering, synthetic engineering of genes and gene pyramiding to develop more effective and specific proteinaceous inhibitors against digestive enzymes of insect pests. Through the structural analysis and surface charge comparisons of two variants of α-AI plant defensin namely, VrD1 and VrD2, it was revealed that charged residues were responsible for the observed differences in their inhibitory activity. A chimera was constructed by introducing charged residues from VrD1 in functional L3

loop of VrD2 which was found to be more active and potent against α-amylases of insect pest (Lin et al. 2007). Similarly, function of the first inter-cysteine segment AAI was elucidated by inserting this segment into the homologous position of the spider toxin, Huwentoxin-1, where a resulting chimera showed significantly increased inhibitory activity (Lu et al. 1999). Another chimeric inhibitor constructed by the replacement of 2nd and 3rd domain of sunflower multi-cystain with the functional domain of bitter gourd seed inhibitor was effective in simultaneous inhibition of trypsin and papain (Inanaga et al. 2001). In another *in vitro* approach, directed molecular evolution was achieved through shuffling and phage display technique where a combinatorial library was constructed containing 10^8 α-AI variant forms. Further, *in silico* screening was performed to determine the most effective variants against α-amylases of boll weevil (da Silva et al. 2013). In the case of tomato multi-cystatin, 29 single mutants at positively selected amino acid sites of 8th domain were generated which showed improved herbivory potency and specificity towards insect pests cysteine proteases (Goulet et al. 2008). Many studies have reported the construction of fusion Bt protein to enhance toxicity towards insects and delay resistance development. For example, transgenic cotton plant harboring Bt (Cry1Ac) and trypsin inhibitor showed improved resistance and toxicity against insect herbivory (Zhu et al. 2012). Gene pyramiding approach was also used to improve resistance of plants against insect pest. Ingestion of *Nicotiana alata* serine PI (NaPI) by *H. armigera* induced production of inhibitor insensitive chymotrypsins in gut lumen, which conferred resistance towards inhibitor. Tested through field trials, transgenic cotton plants harboring a combination of NaPI (Pin-II type) and Potato Pin-1A (StPin-1A; Pin-1 type) displayed considerable resistance against *H. armigera* infestation (Dunse et al., 2010a).

Along with the introduction of insect-resistant gene in plants, manipulation of plant secondary metabolism and plant mediated RNAi strategy has potential in improving resistance against sap-sucking aphids (Yu et al. 2014). Additionally, the existence of receptor antagonists in plants against regulatory molecules of insect digestive enzymes have been predicted which might be useful in controlling regulation of insect pest digestive enzymes (Jongsma and Beekwilder 2011). Taken together, these advance

approaches would be useful in achieving specificity and improving efficacy of the existing plant proteinaceous inhibitors.

Organization of thesis

Introduction section highlights the necessity of detailed characterization of insect pest's digestive enzymes in order to design effective pest control strategies for management of insect pests in the field. Successful utilization of proteinaceous inhibitors produced against digestive enzymes of insect pests as a defense strategy by plants would be helpful to achieve this goal. In the present thesis, digestive α-amylases from polyphagous pest *Helicoverpa armigera* and two important stored-grain pests *viz. Tribolium castaneum* and *Callosobruchus chinensis* have been studied. Enzymes were heterologously expressed, purified and scrutinized for their biochemical and biophysical properties. Transcript abundance of α-amylases across various developmental stages and body tissues were analyzed to know their tissue specificity and amylolytic activity in different body parts other than gut region. Further, *in silico* analysis of molecular interactions of these α-amylases with various proteinaceous and non-proteinaceous α-amylase inhibitors were carried out to understand underlying mechanism of inhibition and differential inhibitory pattern observed for individual enzyme in biochemical reactions.

Apart from insect digestive α-amylases, genomic, biochemical and molecular properties of the smallest native α-amylase inhibitor from *Amaranthus hypochondriacus* (AhAI) were also investigated. AhAI transcript abundance across various plant parts was analyzed. Fluorescent tag based subcellular localization of AhAI signal peptide was carried out to demonstrate its role in extra-cellular transport. Additionally, inhibitory potential of recombinant AhAI against various α-amylases from number of sources were evaluated. AhAI showed complete inhibition of amylolytic activity of α-amylases from coleopteran insect pests *viz. T. castaneum* and *C. chinensis*. Accordingly, *in silico* analysis was performed to understand differential molecular interactions of AhAI with these α-amylases. Outcomes of overall study are summarized in chapter 6 and its applications are described in future prospective section.

Chapter-2

"Study of gene expression patterns of *Helicoverpa armigera* α-Amylases upon feeding on a range of host plants"

Contents of chapter-2 have been published in a research article……

(Kotkar et al., 2012, Gene, 501, 1-7)

2.1 Introduction

Helicoverpa armigera (Hübner; Lepidoptera: Noctuidae), a generalist herbivore, feeds on a number of plant species across different families (Naseri et al., 2010). Development of insects depends on various biotic and abiotic factors and quality and quantity of food (Bouayad et al., 2008). The digestive tract of *H. armigera* has multiple, diverse and unique combination of enzymes to digest food proteins (proteases), carbohydrates (α-amylases) and lipids (lipases) and might have evolved differently depending on dietary content of a range of host plants. 'Switch on' and 'switch off' mechanisms of digestive enzymes are regulated by feeding response to nutritional quality and availability of diet (Afshar et al., 2010). Absolute amounts and ratios of nutrients which include amino acids, carbohydrates, phospholipids, sterols, vitamins, minerals, trace elements and water etc. required by insect herbivores are highly variable in host plants (Behmer, 2009). Lepidopteran insects mainly feed on tender leaves or bud during their early instars and later instars shift to pods or mature fruits which are rich in proteins. *H. armigera* completes its life cycle on nutritionally diverse plant diets by metabolic adjustments through regulation of digestive enzymes (Sarate et al., 2012).

α-Amylases (α-1, 4 glucan 4 glucanohydrolases; EC 3.2.1.1) is an important class of digestive enzymes that catalyze hydrolysis of α-D-(1, 4)-glucan linkage in starch components, glycogen and various other related carbohydrates to serve as an energy source (Franco et al., 2000). Regulation of α-amylases has been studied in Coleopterans that are dependent on starch as their major dietary source (Bandani and Balvasi, 2006; Franco et al., 2002; Titarenko and Chrispeels, 2000). Although larvae of Lepidoptera rely largely on complex proteases for better performance, α-amylases are also equally important digestive enzymes that encounter varying ratios of starch constituents in the diet to provide energy required for all cellular functions (Terra and Ferriera, 1994; Valencia-Jimenez et al., 2008; Zhu et al., 2003). α-Amylases also play an important role in improving insect's digestive capacity that helps it to survive in diverse conditions and increase its fitness value. This can be corroborated with the fact that α-amylases are active during non-feeding stages like pupa, adult and eggs of *H. armigera* (Kotkar et al., 2009) unlike proteinases, which are expressed when protein is ingested (Zhu et al., 2005). Although, α-amylases have been studied in Lepidopteran insects (Ashwath et al., 2010; Ngernyuang et al., 2011; Parthasarathy and Gopinathan, 2005) their expression and correlation to dietary compositions are relatively unknown.

In our earlier studies, we reported changes in biochemical properties and activities of α-amylases during larval developmental stages and feeding on various host plant tissues. Multiple isoforms of α-amylases specific in response to ingestion of a particular combination of macromolecules in natural diets have been reported (Kotkar et al., 2009). In the present study, we employed semi-quantitative and quantitative Real Time PCR along with biochemical approach to characterize the relative expression of two known *H. armigera* α-amylase gene transcripts (HaAmy1; GenBank ID: EU325552) and HaAmy2; GenBank ID: EF600048). We have studied (i) expression levels of two α-amylase transcripts of *H. armigera* during feeding on different hosts, (ii) tissue and developmental stage specificity of *H. armigera* α-amylase gene expression and (iii) correlations of expression levels with diet quality and availability of sugars.

2.2 Materials and Methods

2.2.1 Insect culture

H. armigera larvae were maintained under laboratory conditions on an artificial diet composed of A) chick pea flour; 50 g, yeast extract; 12 g, wheat germ; 5 g, casein; 3.5 g, sorbic acid; 0.5 g, methyl paraben; 1 g in 150 ml distilled water, B) choline chloride; 0.35 g, streptomycin sulphate; 0.02 g, ascorbic acid; 2 g, cholesterol; 0.15 g, multivitamin multi-mineral capsule (Becadexamin, GlaxoSmithKline Pharmaceuticals Limited, Bangalore, India), Vitamin E; 200 mg, formaldehyde; 1 ml, bavistin; 0.3 g, distilled water; 30 ml and C) agar; 6.5 g in 180 ml distilled water (Nagarkatti and Prakash, 1974; Srinivasan et al., 2005a,b). A and B were mixed together and molten agar (C) was added. Individual insects were fed on 1 cm×1 cm×1 cm cubes of the artificial diet daily and subsequent generations were used for feeding assays.

2.2.2 Feeding assays

Neonates were allowed to feed on fresh legume pods of pea (*Pisum sativum* L.), fresh cut okra (*Abelmoschus esculentus* L.), flowers of marigold (*Tagetus erecta* L.), kernels of maize (*Zea mays* L.) and also on a chickpea (*Cicer arietinum* L.) flour-based artificial diet and reared as detailed in Kotkar et al. (2009). Insect cultures were sampled during mid-second, mid-fourth and mid-sixth instars. For the second instars, whole larvae were snap frozen while all the other larvae were chloroform anaesthetized, dissected ventrally using a Zoom Stereomicroscope (SMZ800, Nikon, Japan) and guts/gut portions

were removed. Foregut included the portion up to the enlarged crop region while the midgut was dissected up to the malphighian tubules. The remaining portion was collected as the hindgut. Care was taken to avoid contamination of gut regions. The foregut was carefully dissected to avoid salivary gland tissue. Whole eggs, adult gut and pupal lumen contents of *H. armigera* fed on artificial diet were also collected. Tissues for head, fat body, haemolymph, oral secretion, integument and whole body were sampled from the mid-fourth instar larvae. All the tissues were snap frozen in liquid nitrogen and stored at −80 °C until further use.

2.2.3 RNA isolation, cDNA synthesis and real time PCR

Total RNA was extracted from foregut, midgut, hindgut, whole larval body, fat body, haemolymph, integument, pupal lumen, adult gut and whole eggs using Trizol reagent (Invitrogen, CA, USA). Crude RNA samples were treated with RQ1 DNase (Promega, USA), followed by phenol/chloroform/isoamyl alcohol (25:24:1) extraction and ethanol precipitation. Two microgram of the DNA-free RNA samples was reverse-transcribed using oligo dT primers and reverse transcriptase (Promega, USA) following the manufacturer's recommendations. Real Time PCR primers pairs were designed in non-homologous regions of *HaAmy1* and *HaAmy2* after alignments using Clustal W software (Table 2.1). cDNA was diluted (1: 10) before use in a PCR reaction. Same primer pairs were used for semi-quantitative RT-PCR performed under the following conditions: initial denaturation at 95 °C for 2 min; 30 cycles at 95 °C for 30 s; 60 °C for 30 s; 72 °C for 30 s and a final extension at 72 °C for 5 min. Quantitative Real Time PCR reactions were performed using AB 7900 Fast Start Real Time PCR System (Applied Biosystems, USA; cycler conditions: 95 °C for 10min; 40 cycles of 3 s at 95 °C and 30 s at 60 °C with an additional dissociation stage of 15 s each at 95 °C, 60 °C and 95 °C) and SYBR Green PCR master mix (Roche Applied Science, Germany). Each plate was run with a standard curve and no template control. Relative quantification was carried out using the standard curve method with β-actin as a reference gene (Table 2.1). Amplification efficiency of each gene was assessed by plotting a standard curve using five serial dilutions of cDNA from a template pool and similar efficiencies were used for comparisons. The target gene expression levels of samples were then normalized using β-actin. For qRT-PCR, typically, data from three biological replicates were used for statistical analysis.

2.2.4 Protein sequence analyses

Amino acid sequences of *H. armigera* α-amylases available from the National Center for Biotechnology Information (NCBI) web site were compared with sequences of other α-amylases deposited in GenBank using the ClustalW and MEGA 5 software. A cladogram was constructed based on the amino acid sequences of known Dipteran, Lepidopteran and Coleopteran α-amylases. A bootstrap analysis was carried out and the robustness of each cluster was verified in 1000 replications. Active sites were predicted using pfam analysis (http://pfam.sanger.ac.uk) detailed by Mistry et al., 2007.

2.2.5 Estimation of starch and sugars

All samples were treated with 80% ethanol to remove reducing sugars and starch content was estimated using anthrone reagent (Thayumanavan and Sadasivam, 1984). Reducing sugars in all the diets and also in the gut extract and haemolymph of *H. armigera* was estimated by the dinitrosalycylic acid (DNSA; Sigma Chemical Co., St. Louis, CA, USA) method (Bernfeld, 1955). Sucrose content in artificial diet, pea, okra, marigold and maize was quantified by High Performance Liquid Chromatography (Agilent, USA) by slightly modifying a method described earlier (Trebbi and McGrath, 2004). Liquid nitrogen-ground samples (10 g each) were extracted in 30 ml distilled water and vortexed for 5 min. Homogenized samples were centrifuged at 12,000 g for 5 min, supernatants (1 ml each) were transferred to 1.5 ml microcentrifuge tubes and re-centrifuged as before. Supernatants (500 µl) were filtered with 0.22 µm filters and transferred to glass vials before analyzing on HPLC. Twenty microliter samples were injected in a Supelcogel Pb HPLC column for carbohydrates (300 mm length×7.8 mm internal diameter; Sigma-Aldrich, USA). The mobile phase was distilled water with a flow rate of 0.5 ml/min and column oven temperature set to 75 °C. Quantifications were based on the peak area measurements of sucrose standard run under identical conditions. Analyses are based on data of two biological replicates of each sample.

2.2.6 α-Amylase activity in oral secretions, haemolymph and faecal matter

Fourth instar larvae fed on artificial diet, pea, okra, marigold and maize were gently prodded on the mouth part with a micropipette tip to cause regurgitation. The crude oral secretions were collected with a micro tip, centrifuged (5 min; 12,000 ×g; 4 °C) and appropriately diluted with sodium phosphate buffer (0.2 M; pH 6.9). Similarly,

the last digit of fourth instar larvae was cut with a small pair of dissecting scissors to allow collection of haemolymph as described above. Faecal matter of actively feeding fourth instar was collected to check residual α-amylase activity. All samples were collected in triplicate, each replicate constituting ten larvae. α-Amylase activity assay was performed as described previously (Kotkar et al., 2009).

2.2.7 Statistical analysis

Single factor ANOVA with replication was followed by comparisons with Tukey's post hoc Honestly Significant Difference (HSD) test. Data was considered to be significantly different within the treatments when the F-value obtained was higher than the critical F-value at a probability level of 0.01. Critical differences (CD) between HSD values were obtained by subtracting subsequent values of the averages and comparing with the calculated CD at pb0.05 and pb0.01 (represented by an asterisk) (Kotkar et al., 2009). Similar lower case letters represent a sub-group within a group with statistically insignificant difference.

2.3 Results

2.3.1 Sequence analyses of HaAmy1 and HaAmy2

A cladogram was generated by Clustal W alignment of α-amylases from three representatives of Insecta using bootstrap values of 1000 replications (Figure 2.1). *Lipomyces kononenkoae* and *Aspergillus shirousami* α-amylase sequences were used as an out-group. Two clades were obtained demonstrating that Coleopteran α-amylases form a distinct group while Lepidopteran and Dipteran have similarities. However, Lepidopteran α-amylases are further forming a separate cluster. HaAmy1 shows higher similarity with *Spodoptera frugiperda* Amy and *Bombyx mori* Amy while HaAmy2 grouped with *Diatraea saccharalis* Amy2 and *Anagasta kuehniella* Amy3. Sequence alignment between HaAmy1 and HaAmy2 aa using Mega 5 shows 75% similarity (Figure 2.2).

2.3.2 Differential expression of *HaAmy1* and *HaAmy2* genes during insect development

To examine the expression profiles of HaAmy1 and HaAmy2 during developmental stages, semi-quantitative RT-PCR was performed with tissues derived

from the second, fourth and sixth instars and also from the pupa, adult and egg. *HaAmy1* gene expression increased progressively from the second instar up to the sixth instar (Figure 2.3A). *HaAmy1* was expressed in the pupal and egg stage, however it was negligible in the adult stage. When compared across the instars, expression of *HaAmy2* was highest in the sixth instar, slightly higher in the pupal stage than the adult stage while very low in the egg stage.

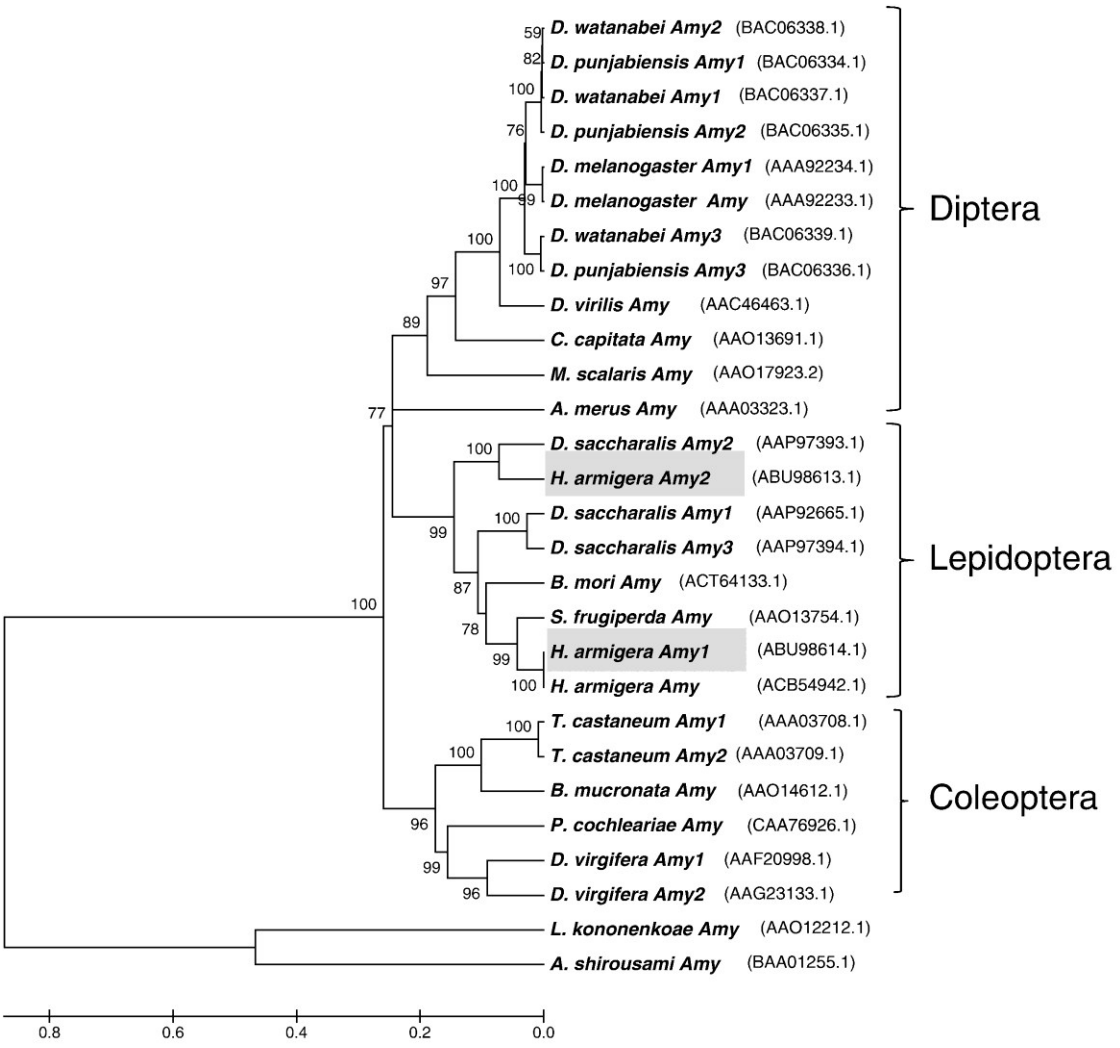


Figure 2.1 Amino acid sequence divergence of insect α-amylases. Cladogram with protein sequences of insect α-amylases from Diptera, Lepidoptera and Coleoptera was constructed using MEGA 5 software and Neighbor Joining method to show genetic variance between HaAmy1 and HaAmy2. Values at branch points indicate bootstrap values of 1000 replications. Fungal α-amylases were used as an out-group. Scale

represents genetic distance between the clades. Accession numbers are given in parentheses.

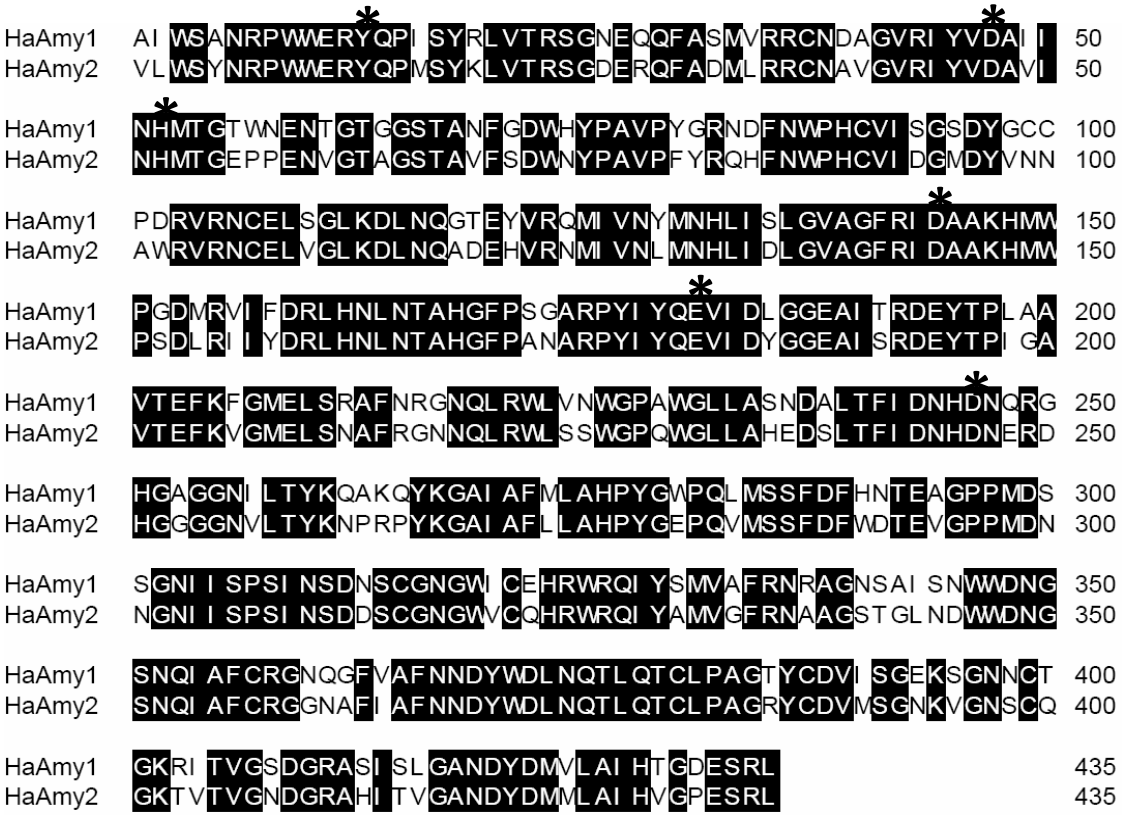


Figure 2.2 ***Helicoverpa armigera*** **α-amylase sequence alignments.** Two amino acid sequences, HaAmy1; GenBank ID: ABU98614 and HaAmy2; GenBank ID: ABU98613 (NCBI database) from coding regions of *Helicoverpa armigera* were aligned using Clustal W. Residues identical to consensus sequences are shaded. Active sites predicted using pfam analyses are indicated by asterisks.

2.3.3 Tissue specific expression of *HaAmy1* and *HaAmy2* genes

Insect head, integument, fat body, haemolymph and whole larvae were used for tissue specific expression analysis by employing semi-quantitative RT-PCR. *HaAmy1* gene was expressed in negligible amounts in the head tissue and integument of the fourth instar larvae fed on artificial diet while it was undetectable in the haemolymph (Figure 2.3). Fat body and whole body showed the highest and almost similar expression of *HaAmy1*. In case of *HaAmy2*, the head tissue showed very low level of expression

remained undetected in the integument while it was highly expressed in the fat body followed by whole body and haemolymph.

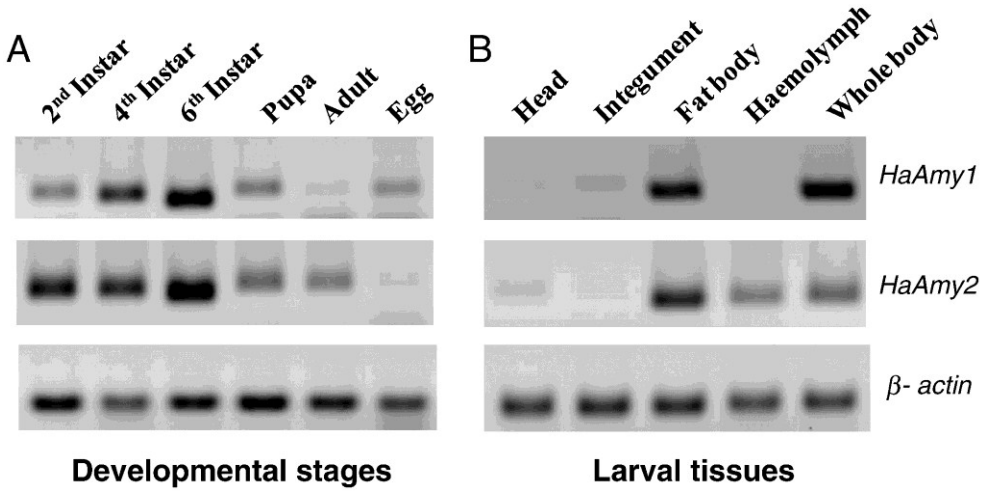


Figure 2.3 Expression of *HaAmy1* and *HaAmy2* during developmental stages and in different tissues. *H. armigera* was reared on artificial diet. Samples were harvested from (A) different developmental stages (whole larvae of second instar, guts of fourth and sixth instar, pupal lumen contents, adult gut and whole eggs) and (B) mid-fourth instar larval tissues (head, integument, fat body, haemolymph and whole body). Semi-quantitative RT-PCR was carried out using *β-actin* as an internal standard.

2.3.4 *HaAmy1* and *HaAmy2* gene expression is influenced by reducing sugar, sucrose and starch availability

To pinpoint dietary factors governing insect α-amylase gene expression, we checked available reducing sugars, sucrose and starch content in host plant tissues used for feeding experiments. Expression level of both the genes, *HaAmy1* and *HaAmy2* was highest in the foregut of larvae fed on marigold while the lowest in maize fed larvae (Figure 2.4). Biochemical analyses depict that marigold flowers and maize kernels had lowest (0.2 mg/g fresh mass; 1.27 mg/g) and highest levels (23.8 mg/g FM; 3.28 mg/g) of sucrose (Figure 2.5A) and starch, respectively. Pea had comparatively moderate levels of sucrose (11.08 mg/g FM) than okra (2.8 mg/g FM), followed by relatively lower levels in artificial diet (1.64 mg/g FM) and marigold (0.2 mg/g FM). On the contrary, reducing sugars in marigold flowers (33.16 mg/g FM) was higher than maize kernels (3.3 mg/g FM). Reducing sugar levels were highest in the gut of fourth instar *H. armigera* larvae

fed on artificial diet (4.28 µg/mg) followed by that of marigold (3.87 µg/mg) while not much variation was observed for the same in haemolymph (2.1 µg/µl) (Figure 2.5B). Larvae fed on all other diets exhibited minor variations in expression levels for *HaAmy1* and *HaAmy2* in the foregut. In the midgut, higher level of transcripts of both the enzymes was detected in artificial diet fed larvae as compared to natural diets while minimum was observed in case of maize (Figure 2.4). Expression of *HaAmy1* and *HaAmy2* was slightly lower in pea (having moderate levels of sucrose) fed larvae than okra (having lower levels of sucrose) fed larvae. Marigold fed larvae exhibited higher expression of *HaAmy2* than pea and okra fed larvae. Expression of *HaAmy1* was lower in the midgut of marigold fed larvae as compared to that in pea and okra fed larvae. Expression of *HaAmy1* and *HaAmy2* in the hindgut of larvae fed on all the diets except okra was almost similar and low. Significantly high but different levels of gene expression of *HaAmy1* and *HaAmy2* were found in the hindgut of okra fed larvae (Figure 2.4).

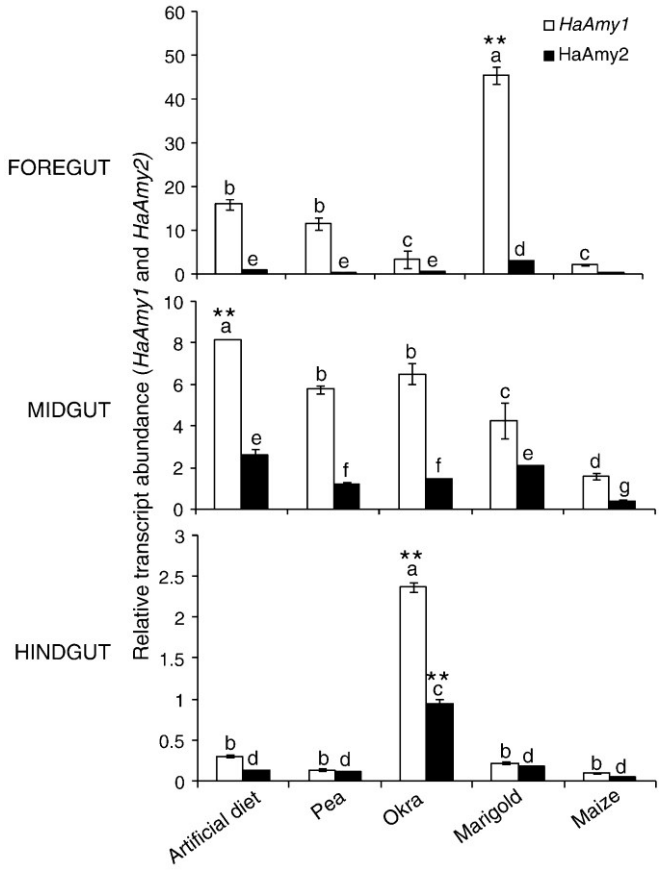


Figure 2.4 Expression of *HaAmy1* and *HaAmy2* in the gut regions of *H. armigera* fourth instar larvae. *H. armigera* larvae were reared on artificial diet, pea, okra, marigold and maize. RNA was isolated from the foregut, midgut and hindgut regions of

fourth instar larvae and cDNA was synthesized. Relative transcript abundance was determined in pooled samples of foregut, midgut and hindgut of fourth instar larvae using quantitative Real Time PCR. *β-actin* was used as an internal standard. *HaAmy1* and *HaAmy2* gene expression data were analyzed by single factor ANOVA followed by Tukey's post hoc HSD suggested significant difference between the data at $p < 0.01$(indicated as '**'). Bars with similar letters represent a group within *HaAmy1* and *HaAmy2*.

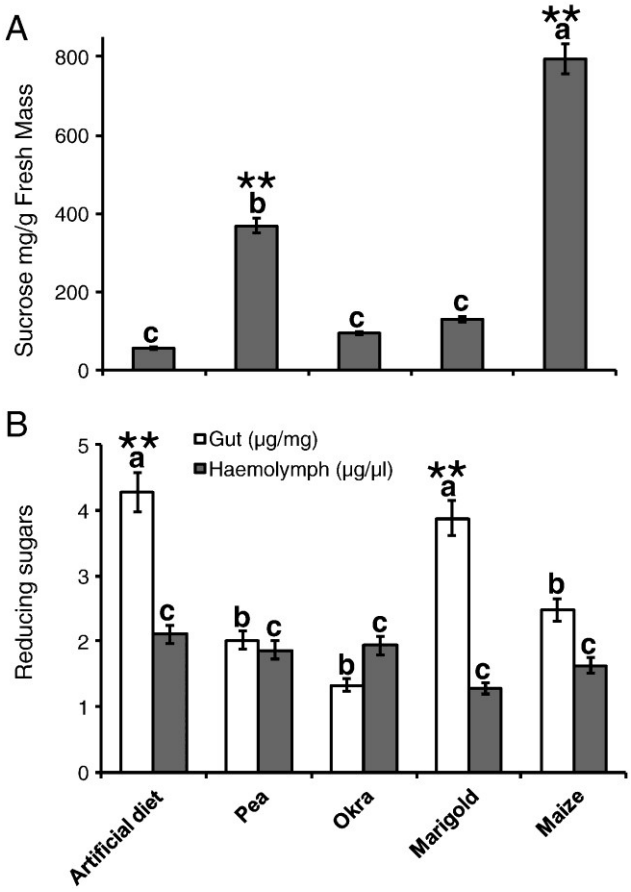


Figure 2.5 Sugars in the host plants, gut and haemolymph of fourth instar *H. armigera* larvae. Neonates of H. armigera were allowed to feed on artificial diet, pea, okra, marigold and maize. (A) Sucrose content in plant parts used for feeding assays was analyzed by HPLC. (B) Gut tissues and haemolymph were collected from the fourth instar larvae and reducing sugar levels from the gut was estimated as mentioned in Methods section. Error bars represent mean ± SE. Single factor ANOVA followed by Tukey's post hoc HSD suggested significant difference between the data at $p < 0.01$

(represented by asterisks) Sub-groups within a group are indicated as by lower case letters.

2.3.5 Expression of *HaAmy1* and *HaAmy2* genes during larval development

Two diets viz. maize and marigold that exhibited differences in expression of α-amylase genes were further selected for profiling patterns during developmental stages of *H. armigera* (Figure 2.6). α-Amylase gene expression was relatively low during the second instar larvae fed on both the diets. Across the diets, relative expression of both the transcripts was much higher in the larvae fed on marigold than on maize while amongst the two transcripts, expression of *HaAmy1* was higher than that of *HaAmy2*. In marigold, fourth instar larvae, while in maize; sixth instar larvae revealed highest expression of both the transcripts.

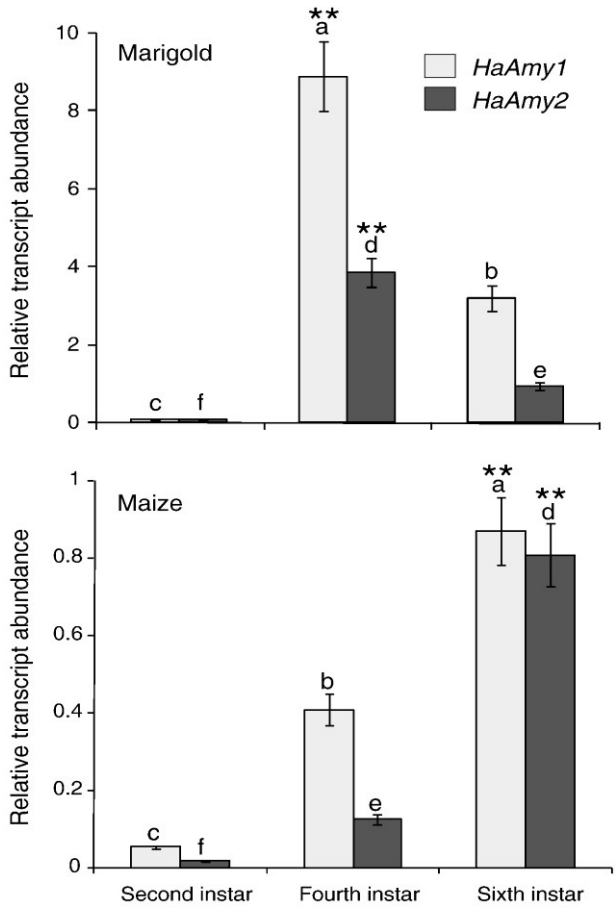


Figure 2.6 *HaAmy1* and *HaAmy2* gene expression during *H. armigera* larval development. Whole second instar larvae and gut tissue of fourth and sixth instar larvae feeding on marigold and maize were collected. RNA was isolated and optimized concentration of cDNA was used. Expression levels of *HaAmy1* and *HaAmy2* of *H.*

armigera were determined by quantitative Real Time PCR with *β-actin* as an internal standard. Single factor ANOVA followed by Tukey's post hoc HSD suggests significantly different expression levels amongst the instars at $p < 0.01$ (represented by **). Sub-groups formed within a group are represented as 'a', 'b' and 'c' for *HaAmy1* and 'd', 'e' and 'f' for *HaAmy2*.

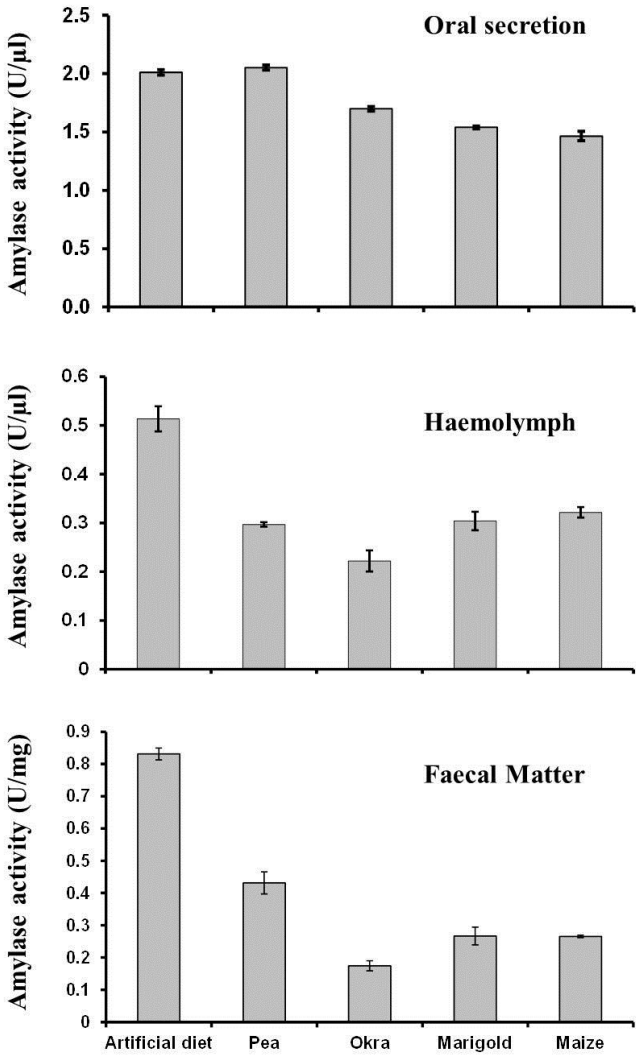


Figure 2.7 α-Amylase activity in oral secretions, haemolymph and faecal matter of *H. armigera* feeding on various diets. Oral secretions, haemolymph and faecal matter of fourth instar larvae of *H. armigera* feeding on artificial diet, pea, okra, marigold and maize were analysed for α-amylase activity. Values are represented as mean ± SE. Asterisks indicate out-groups with significant difference ($p < 0.01$; single factor ANOVA and Tukey's post hoc HSD).

2.3.6 α-Amylase levels in the gut, oral secretion, haemolymph and faecal matter

To investigate presence of α-amylases in insect body fluids, we conducted activity assays with oral secretion and haemolymph of larvae actively feeding on artificial diet, pea, okra, marigold and maize as well as with the faecal matter of *H. armigera* for residual α-amylase activity. Amongst these, α-amylase activity was highest in the oral secretions of *H. armigera*; maximum in the oral secretions of larvae fed on pea and artificial diet followed by okra, marigold and maize (Figure 2.7). In the haemolymph, artificial diet fed larvae had the highest α-amylase activity while maize and marigold fed larvae had similar and okra fed larvae had the lowest α-amylase activity. Significant α-amylase levels were also detected in the faecal matter of larvae fed on all the categories of diets and the trend was similar to that observed in haemolymph (Figure 2.7).

2.4 Discussion

Insect herbivores are efficient nutrient regulators with respect to proteins and carbohydrates wherein absorption and differential utilization of nutrients are significant mechanisms (Behmer, 2009). Polyphagous insects usually prefer macro- and micro-nutrient balance in their food but they can also complete their life cycle on imbalanced diets (Raubenheimer and Simpson, 1999). Thus, insect–plant interactions are regulated by the nutritional and anti-nutritional constituents of the host plant (Cates, 1980). Digestive α-amylases were detected in the larval gut of H. armigera and their sequences have been deposited in the NCBI database (Pauchet et al., 2008) and exhibit similarity with known α-amylases of different insects from Diptera, Lepidoptera and Coleoptera (Figure 2.1). Alpha-amylase catalytic domains for HaAmy1 and HaAmy2 were between 52 to 340 and 12 to 252 amino acids while the C terminal domains were 412 to 419 and 347 to 419, respectively, indicating a common C terminal region but different catalytic domains. Amongst the two transcripts, in the three gut regions, viz. foregut, midgut and hindgut, *HaAmy1* in general was found to be up regulated than *HaAmy2*. *HaAmy1* and *HaAmy2* gene expression also varies across the gut region in response to feeding on nutritionally diverse diets. The present findings corroborate with our earlier data on α-amylase and protease activity of *H. armigera* feeding on nine different diets (Kotkar et al., 2009; Figure 2.8). Such diet-related enzyme up/ down regulation has been observed for

example, function of human salivary α-amylase (Amy1) has been predicted to augment pancreatic α-amylase (Amy2) activity/initiate starch breakdown/speed glucose absorption or release sugars for identifying starch-rich food (Arjamaa and Vuorisalo, 2010; Perry et al., 2007). Similarly, BmAmy from *B. mori* has been found to be expressed only in the foregut (Ngernyuang et al., 2011). Various insect α-amylase isoforms are known to have specificity of expression in different tissues and body fluids such as oral secretions and haemolymph (Da Lage et al., 2003; Parthasarathy and Gopinathan, 2005; Saltzmann et al., 2006). Variations in enzyme levels can also be due to presence of enzyme inhibitors, variations in monomers of complex macromolecules and when the concentrations and ratios of nutrients absorbed from natural diets are unknown (Clissold et al., 2010; Silva et al., 2001). Reducing sugars present in the diet reflected to the levels of reducing sugars occurring in the gut indicating their ready availability for absorption from plants food sources. However, in spite of high reducing sugars in okra as a diet, gut levels remained lower. These levels in the diets were further balanced with sucrose, for example, when reducing sugars in the diet were high, sucrose was low (okra and marigold). Similarly, reducing sugars in the haemolymph correlate with levels of reducing sugars in the gut. Presence of high reducing sugars in the gut of larvae feeding on marigold is probably reflected from the high reducing sugar content in marigold flowers as a diet. Larvae may obtain nutrition from the ingested diet source either by direct absorption of simple molecules or by degradation of macromolecules present in the food with the help of specific digestive enzymes. Pea had a high starch content followed by maize and okra while it was lowest in marigold. Analyses of plant tissues indicate that diets rich in starch have comparatively lower amounts of reducing sugars and vice-versa. As compared to maize fed larvae, *HaAmy1* and *HaAmy2* midgut expression levels of pea and okra fed larvae were higher. Sucrose is a major intermediate product of photosynthesis in plants and is transported from the leaves to the other parts. Upon feeding, in insects, sucrose is hydrolyzed to glucose and fructose which are then metabolized. Sucrose shows an exactly opposite pattern of accumulation in the diets when compared to the reducing sugars. For example, maize is rich in sucrose and starch but low in reducing sugar content while marigold has lower starch and sucrose reserves but moderate levels of reducing sugars. It seems that when sucrose levels are high in the insect diet, a ready supply of 'energy molecules' are instantly available to the larvae upon ingestion of food. This probably reduces the 'expenditure' on utilizing insect α-amylases for degradation of complex molecules such as starch but may involve other enzymes for hydrolysis of already

available sugars in the host plant tissues. In an earlier study, α-chymotrypsin-like and α-amylase like activities were down regulated upon ingestion of excess protein or carbohydrate, respectively by locusts indicating a homeostatic role for enzyme flexibility (Clissold et al., 2010). On the other hand, based on these results, regulation of HaAmy1 and HaAmy2 seems to be suppressed by sucrose suggesting that higher levels of sugars occurring in the natural host plants lower *H. armigera* α-amylase gene expression and this probable 'sucrose sensing' needs further investigation. Considering these correlations, nutrient uptake and regulation of digestive enzymes are important adjustments in polyphagous insects. Levels of α-amylase transcripts in the hindgut of *H. armigera* were negligible during feeding on all the diets and relates to residual α-amylase activity in the faecal matter, except a marginal increase in okra. During feeding assays, we have observed that *H. armigera* prefers to feed on the seeds of okra and later if seeds are unavailable it shifts to other parts of the fruit. Okra has been reported as a carbohydrate-rich diet due to the presence of galacturonic acid, galactose, rhamnose and glucose in its highly mucilaginous fruits (Sarate et al., in press; Woolfe et al., 1977). Our data indicates that it also contains moderate levels of starch as compared to pea, marigold and maize. The uptake, movement through the gut and digestibility of food depends on the nature of food components. Amongst all the diets used in this study, okra was the only highly mucilaginous diet provided to the larvae. Mucilaginous components of okra probably have a longer retention time in the gut regions and affects expression of digestive enzymes. Major hydrolases are distributed in the anterior midgut, middle midgut, posterior midgut and hindgut of *Dermestus maculatus* (Coleoptera) larvae (Caldiera et al., 2007). Furthermore, differences in α-amylase expression levels in oral secretions of the second, fourth and sixth instars feeding on marigold and maize indicate larval-stage specificity. Sixth instar larvae fed on maize had slightly higher levels than that of the fourth instars wherein the larvae seem to utilize available energy reserves for further stages of growth. Sui et al. (2008) have reported expression of lipases during the sixth instar that function to accumulate energy during digestion and as a hydrolytic enzyme for midgut apoptosis during metamorphosis. Our results imply that older larvae might adapt to changes in nutritional and other anti-nutritional components of the diet by controlling the release of enzymes. Although expression of α-amylases was variable, the host plant seems to play a significant role. Changes in the haemolymph in relation to the food intake and larval development have been well documented (Salama et al., 1992; Wyatt, 1961). Even in the haemolymph of larvae feeding on various diets, reducing sugars seem to

dictate the α-amylase levels. Another probable role of haemolymph α-amylases might be degradation of glycogen present in fat bodies (Abraham et al., 1992). HaAmy1 and HaAmy2 are ubiquitous in the larval, pupal, adult and egg stages of *H. armigera*. Cloning and characterization of recombinant proteins will further help to understand the substrate specificities of these enzymes. Transcriptional and post-transcriptional adjustments exhibit flexibility in expression of hydrolases in response to dietary signals *(Karasov et al., 2011)* and regions of the gut (Ngernyuang et al., 2011). The digestive system is costly to run and precise mechanisms exist to detect food components. Therefore, insect feeding usually assures a balance of nutrients via the gustatory or post-ingestive mechanisms wherein the digestive enzymes play a key role. Complexity in α-amylase gene expression in *H. armigera* seems to be strongly correlated with levels of reducing sugars, sucrose and starch in the plant tissues.

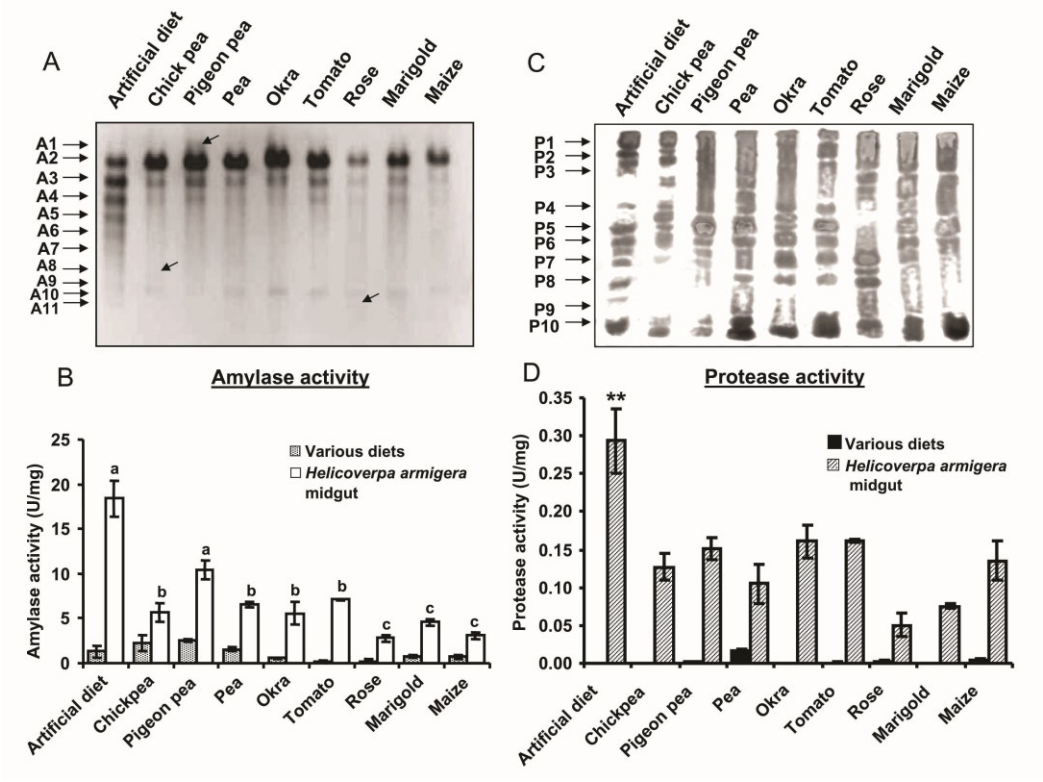


Figure 2.8 *H. armigera* midgut amylase and proteinase activity profiles

Midgut extracts of *H. armigera* larvae fed on artificial and natural diets were separated on native polyacrylamide gels (7%), visualized for amylase (A; arrows indicate isoforms) and protease isoforms (C). The larval gut extracts and diets used for feeding assays of *H. armigera* were further assayed for α-amylase (B) and protease activities (D).

Table 2.1 Primers designed for qRT-PCR analyses of *Helicoverpa armigera* α-amylases

Primer name	Accession no.	Primer sequence	Length (bp)
HaAmy1	EU325552	F5'- GAAGAGCGGTAACAACTGCACC -3' R5'- CACAGCCTCGATTCATCACCAGTG - 3'	22 24
HaAmy2	EF600048	F5'- AACAAGGTGGGCAACTCGTGCCAG -3' F5'- TACAGACGACTCTCAGGGCCGACA -3'	24 24
Actin	AF286059	F5'- GATCGTGCGCGACATCAAG -3'	19
		F5'- GCCATCTCCTGCTCGAAGTC -3'	20

2.5 Summary of the Chapter:

In summary (Figure 2.9), the present study has focussed on (i) Transcript levels of two isoforms of *H. armigera* digestive α-amylases during feeding on different host plants, (ii) gene expression pattern and tissue specificity across different developmental stages of *H. armigera* and (iii) correlations of transcript levels with diet quality and availability of sugars.

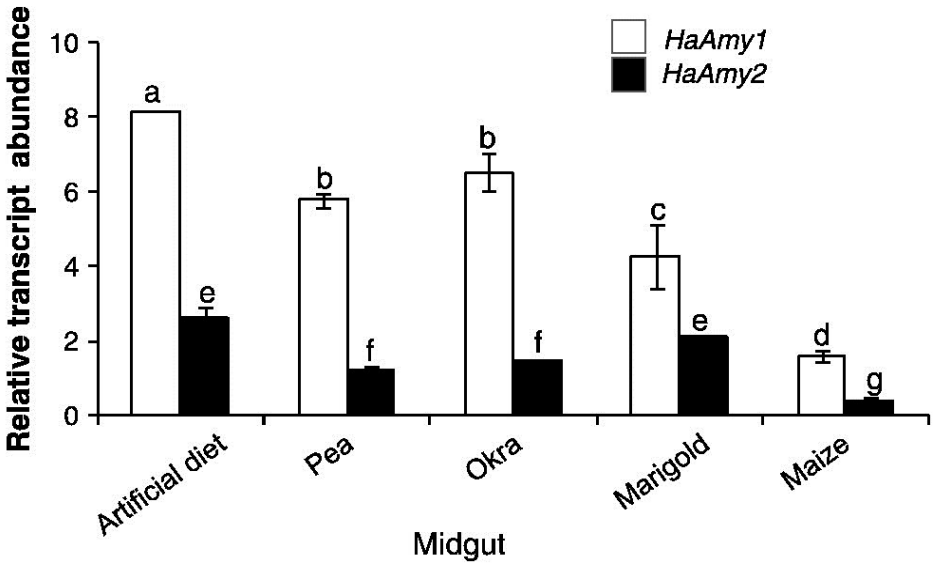


Figure 2.9 Relative transcript abundance of *HaAmy1* and *HaAmy2* in the midgut region of *H. armigera* after feeding upon different host plants.

Together, findings could be highlighted as,

1. Two α-amylase transcripts are differentially expressed in *Helicoverpa armigera*.
2. Expression of *HaAmy1 and HaAmy2 is* not correlated to the host starch content.
3. *HaAmy1 and HaAmy2* tissue specificity can be targeted for insect control.

Biochemical and gene expression data suggests flexibility of α-amylases dependent on the requirement of the insect in relation to nutritional status of the host plant.

Chapter-3

"Biochemical, structural and functional diversity between two digestive α-amylases from *Helicoverpa armigera*"

Contents of chapter-3 have been published in a research article……

(Bhide et al., 2015, BBA General Subjects, 1850, 1719-1728)

3.1 Introduction

Insect pests are responsible for considerable losses and are a major problem for agricultural economy. Digestive enzymes of these insects play a crucial role in adaptation and digestion of complex diets, providing nutrition and energy required for growth, development and reproduction in favorable as well as non-favorable conditions. Particularly, α-amylases significantly contribute to gain energy by hydrolyzing α-D-(1,4) glucan linkages in starch and glycogen releasing glucose, maltose and other oligosaccharides as a nutritional product (Franco et al., 2000; Prigent et al., 2003; Pelegrini et al., 2006; Erban et al., 2009).

Adaptation is a key process through which organism thrives in various environments and is influenced by evolution and diversification of gene families (Chang & Duda, 2012). Reported literature suggests, α-amylase gene family in insects has evolved in rigorous manner and has encountered many duplication, deletion and transposition events which together contribute to the diversity and adaptability of insect α-amylases (Zhang et al., 2003; Schaeffer et al., 2003). Numerous genes encoding α-amylases have been characterized from insect species and for few of them crystal structures are available (Nahoum et al., 1999). However, their characterization, sequence and expression diversity, and influence of dietary components on their regulation at molecular level are poorly understood. In general, regulation of expression of a gene is a cost effective process in terms of fitness of an organism (Langa et al., 2009). Studies on α-amylase gene expression in Opicina (Lepidoptera), Blattella (Blattaria) and Drosophila (Diptera) species indicate that expression of α-amylase genes is regulated by short cardiovascular or brain peptides, and also affected by 5'*cis*-regulating regions or nucleotide polymorphism in coding regions (Harshini et al., 2003; Sakai et al., 2004; Goto et al., 2005; Sakai et al., 2006; Inomata & Nakashima, 2008). Similarly, complex dietary compositions or α-amylase inhibitors are also responsible for inducing α-amylase gene expression in gut region of insects (Lopes et al., 2010; Sarate et al., 2012; Weidlich et al., 2013). Being crucial in obtaining energy from the diet, α-amylases of the insect pests have been targeted by generating transgenic plants expressing different plant α-amylase inhibitors feeding of which showed mitigation effect on insect growth (Oliveira-Neto et al., 2003; EAD Barbosa et al., 2010).

Although detailed study on evolution and functioning of insect α-amylases is available in literature, differential accumulation of α-amylase activity upon (i) ingestion of food components, (ii) in various stages of life cycle and (iii) in different tissues and cellular compartments is not investigated in Lepidopteran insect pests. *Helicoverpa armigera*, a lepidopteran insect is a generalist herbivore and a serious pest on number of plant species across various families (Naseri et al., 2010). In our earlier studies we reported altered biochemical properties and α-amylase activities of *H. armigera* α-amylases after feeding on different various host plants (Kotkar et al., 2009). Further, we showed tissue specificity and correlation of expression levels of two *H. armigera* α-amylases (*HaAmy1* and *HaAmy2*) with diet quality and availability of sugars (Kotkar et al., 2012). These outcomes led us to characterize recombinant HaAmy1 and HaAmy2 for their biochemical, biophysical and differential catalytic properties as well as reactivity with various α-amylase inhibitors.

In the present study, we heterologously expressed two α-amylases *viz., HaAmy1* and *HaAmy2* in yeast (*Pichia pastoris*) and their biochemical and biophysical properties were analyzed. Various substrates were used in enzyme kinetics to distinguish catalytic properties of these two recombinant enzymes. Inhibitory effect of proteinaceous and non-proteinaceous α-amylase inhibitors on these α-amylases was also studied and inhibition profiles with human salivary and pancreatic α-amylases were compared. Further, to understand underlying differential inhibitory mechanisms, homology modeling and molecular docking studies were performed. Reaction products and α-amylase-inhibitor complex was characterized using chromatographic tool in order to understand qualitative and quantitative differences between these two recombinant α-amylases. Together, present study highlights biochemical, biophysical and molecular differences between two isoforms of α-amylases from *H. armigera*. Present knowledge will be useful in understanding starch degradation and enzyme-inhibitor interactions along insect gut.

3.2 Materials and Methods

3.2.1 Insect culture

H. armigera larvae were maintained under laboratory conditions on an artificial diet as described by Nagarkatti and Prakash (Nagarkatti & Prakash, 1974) with some modification as per explained in our earlier publication (Srinivasan et al., 2005a;

Srinivasan et al., 2005b). Individual insects were fed on 1 cm^3 of the artificial diet daily for subsequent generations.

3.2.2 RNA extraction and cDNA synthesis

Total RNA was extracted from gut tissue of 4th instar larvae of *H. armigera* using Trizol reagent (Invitogen, Carlsband, CA,USA) followed by RQ1 DNase (Promega, Madison, WI, USA) treatment. One microgram of total RNA of *HaAmy2* was reverse transcribed using SMARTer™ RACE cDNA Amplification Kit (Clontech, St GermainenLaye, France) following the manufacturer's instructions. Open reading frames of HaAmy1 and HaAmy2 were obtained.

3.2.3 Cloning of HaAmy1 and HaAmy2, expression and purification

α-Amylase genes HaAmy1 and HaAmy2 without the native signal peptide were amplified using P1, R1 & P2, R2 primer pairs, respectively (Table 3.1). Cloning and production of recombinant HaAmy1 and HaAmy2 in *P. pastoris* was carried out as per the protocol mentioned in user's manual (Invitrogen, USA). 5µg of purified protein were loaded on 12% SDS-PAGE gel to check purity.

Table 3.1 Primers for full length cloning of HaAmy1 and HaAmy2

Primer name	Accession numbers	Primer sequence	Length (bp)
HaAmy1 (P1)	EU325552	F5'- (S)TACAAGAACCCACACTATGCG - 3'	51
(R1)		R5'- (N)CAGCCTCGATTCATCACCAGT - 3'	35
HaAmy2 (P2)	EF600048	F5'- (S)TATAAGAACCCGTACTATGCACC – 3'	53
(R2)		R5'- (N)CAGACGACTCTCAGGGCCGACATG – 3'	38
HaAmy2i	EF600048	F5' – TCAACTGGCCGCATTGCGTCAT – 3'	22

(S) = AAAAAACTCGAGAAAAGAGAGGCTGAAGCT ;(N) =AAAAAAGCGGCCGC

3.2.4 Homology modeling of Amy1 and Amy2

To predict the three dimensional structure of HaAmy1 and HaAmy2 proteins, a sequence similarity search was carried out by Blast analysis to find homologous protein

sequences whose structures have been deposited in the Protein Data Bank (PDB). The atomic coordinates of the 1VIW (60%), 1CLV (60%), 1VAH (54%) were obtained from the PDB. The chain A of this X-ray structure was used to build the three-dimensional models of the protein. Modeller-Modweb (https://modbase.compbio.ucsf.edu/scgi/modweb.cgi) was used in building the three-dimensional structure of the Amy1 and Amy2 proteins. The homology model coordinates were then energy minimized with the Schrodinger Macromodel module (Macromodel, version 9.8, Schrodinger, LLC, New York, USA) using *OPLS_2001* force field until the structures reached the final derivative of 0.001 kcal/mol. The stereochemical qualities of the models were checked using the program PROCHECK (Laskowski et al., 1993) and Ramachandran Plots (Ramchandran et al., 1963) were drawn. The visualization of the above studies was done using Pymol (http://www.pymol.org; DeLano Scientific LLC, USA). The conserved residues of Amy1 and Amy2 structures were analysed by ConSurf server (http://consurf.tau.ac.il/). The web-based server NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/) has been used to predict the N-acyl glycosylation sites.

3.2.5 α-Amylase-inhibitor docking and molecular dynamics simulations

Prediction of acarbose binding and wheat inhibitor binding with HaAmy1 and HaAmy2 were carried out using docking studies. Corresponding residues 109-103, 211-214 and 303-207 belonging to the inhibitor binding site, in the *TmAmy* structure (1TMQ) has been assigned. Grid based rigid receptor and flexible ligand docking program Glide (Friesner et al., 2006) and AutoDockVina (Trott & Olson, 2010) were used to accurately predict the binding modes of ligand in the receptor binding site. The topology and parameters for ligands were generated with General Amber Force Field using acpype (Sousa daSilva & Vranken, 2012). The web based protein-protein docking program GRAMM-X with default parameters has been used for docking of proteinaceous wheat α-amylase inhibitor with HaAmy1 and HaAmy2 (Tovchigrechko & Vakser, 2006).

3.2.6 In-gel α-amylase activity and molecular weight determination

Equal quantity (5µg) of each of purified recombinant HaAmy1 and HaAmy2 were loaded on Native-PAGE. After electrophoresis, gel was gently washed with distilled water and incubated in 1% starch solution at 37ºC for 1 hour (Giri & Kachole, 1996). Gel

was rinsed with distilled water and stained by iodine solution (1 mM I_2 in 0.5 M KI). Gel was scanned and image was inverted for better visualization. HaAmy1 and HaAmy2 with 3.6 mg/ml and 3.0 mg/ml, respectively were mixed with sinapinic acid (Sigma-Aldrich, St Louis, MO, USA) (50% CAN, 0.1% TFA) in 1:5 ratio. About 0.5 µl of this mixture was spotted on the MALDI target plate by dry droplet method. The mass spectral analysis was done on AB SCIEX MALDI TOF/TOF^{TM}5800 system. Positive linear mode was used as a mode of operation. MS data were acquired at a laser repetition rate of 400Mz with 1000 laser shots per spectrum over each sample spot in the range of 50-70 kDa for HaAmy1 and HaAmy2.

3.2.7 Determination of α-amylase and α-amylase inhibitory activity

α-Amylase activity was determined by monitoring liberation of reducing sugars from starch. A premix of triplicate reactions was made containing 20mM sodium phosphate buffer (pH 7.0) and purified recombinant enzyme to which 150µl of starch (0.25%) was added. After 15 minutes of incubation at 37°C, reaction was stopped by adding 500µl of Dinitrosalicylic acid (DNSA) reagent and reaction tube was incubated in boiling water bath for five minutes. Absorbance was measured at 540 nm. One Unit (U) is defined as the amount of the enzyme that catalyzes the conversion of 1 micro mole of substrate per minute. α-Amylase inhibitory (AI) activity was performed in a similar way except that enzyme and inhibitor were incubated for 15 minutes at 37ºC before incubating with the substrate. α-Amylase inhibitors *viz* acarbose (non proteinaceous) and wheat AI (proteinaceous) were tested in a concentration range of 10 to 100nM and 2 to 20 nM respectively against HaAmy1, HaAmy2, human salivary and pancreatic α-amylases. α-Amylase inhibitory activity was calculated by measuring reduction in liberated reducing sugars from starch compared to the standard α-amylase activity mentioned above. AI activity was expressed in percentage.

3.2.8 End product analysis of starch hydrolysis

Purified α-amylases HaAmy1 and HaAmy2 were incubated separately with 0.25% potato starch (Sigma-Aldrich, USA) in 20mM phosphate buffer pH 7.0 at 37°C for 6 hours. The reaction was stopped by placing the reaction tube on ice. For quantitative analysis, the end-products of starch hydrolysis by *α*-amylases were analyzed on HPLC system. All samples and standards *viz* Maltose and Glucose (Sigma-Aldrich,USA) were

centrifuged at 11,000 rpm for 20 minutes at 4°C and 10μL of each sample and standard were injected in Supelcogel Pb column (5cm x 4.6mm internal diameter, Sigma-Aldrich, MO, USA). The mobile phase was distilled water at a flow rate of 0.5 ml/min and column oven temperature was set at 70°C. Sugars were detected using refractive index detector (Shodex RI-21, NY, USA) and quantified using standard sugars. Quantifications were based on the peak area measurements of glucose and maltose standard run under identical conditions.

3.2.9 Effect of pH and temperature on the activity of enzyme

To determine the optimum pH value the activity of α-amylase was measured at different pH values ranging from 3 to 12 where equal units of enzyme were used for each reaction. Citrate, sodium phosphate and glycine-NaOH buffers were used for the following specified pH values: 4, 6 to 8, and 10 to 12, respectively. To determine the optimum temperature for starch hydrolysis, the activity of purified α-amylase was measured under pH 7.0 using 20mM sodium phosphate buffer at various temperature ranging from 10 ºC to 80 ºC. An equal amount of enzyme was used for each reaction.

3.2.10 Determination of kinetic parameters

All assays were performed using standard assay conditions at pH7 as mentioned above. Starch and Amylopectin were used as substrates in a concentration range of 0.1 to 3 mg/ml. Kinetic parameters *viz* K_M, V_{max} and K_{cat} of substrate hydrolysis reaction by recombinant HaAmy1 and HaAmy2 were calculated using GraphPad Prizm v.6 software (http://www.graphpad.com/scientific-software/prism/).

3.2.11 Circular Dichroism spectroscopy

Circular Dichroism (CD) assays were carried out using Jasco J-815 spectropolarimeter (Jasco, Tokyo, Japan) under constant nitrogen flow equipped with a Peltier type temperature cuvette holder. Far-UV spectra were recorded using 0.1 cm path length quartz cuvette. For pH study, HaAmy1 and HaAmy2 (0.23 and 0.25 µg/ml respectively) were incubated overnight at 24 ^{o}C in 20mM glycine pH 3.0, 20mM sodium acetate pH 5.0, 20mM phosphate pH 7.0 and 20mM Tris-HCl pH 9.0 and 11.0. Thermal denaturation study was performed by raising the temperature from 30 ºC to 80 ºC at the interval of 10 ºC and 3 minutes incubation time at each temperature. Three consecutive

measurements were accumulated to obtain mean spectra. The observed ellipticities were converted into molar ellipticities (symbol) based on molecular mass per residue of HaAmy1 and HaAmy2.

3.2.12 FPLC analysis of complexes between HaAmy1 and HaAmy2 with wheat α-amylase inhibitor

Individual equimolar (4.4μM) reaction mixture containing HaAmy1 and HaAmy2 with wheat α-amylase inhibitor was incubated at 37 ºC for 60 minutes. The final volume was adjusted to 200μl with 20mM phosphate buffer containing 10mM NaCl and was applied to enrich SEC650 FPLC column (10/300mm) (Bio-Rad, Hercules, CA,USA) followed by elution in same buffer at flow rate of 0.3ml/min and fractions were collected at an interval of 5minute. The fractions were checked on 12% SDS-PAGE.

3.3 Results

3.3.1 Sequence analysis and homology modeling of HaAmy1 and HaAmy2

HaAmy1 and HaAmy2 have predicted secretion signal at N-terminal comprising of 16 and 18 amino acids respectively, which target them to Sec Pathway**.** Sequence analysis and secondary structure predication showed well conserved sequence and a super secondary fold, a parallel $(\beta/\alpha)_8$-barrel for these proteins (Figure 3.1). The catalytic residues Asp209 (Asp210),Glu246 (Glu247) and Asp311 (Asp312) were conserved while the four well conserved sequence regions in α-amylase family enzymes were positioned near β3, β4, β5 and β7 of the catalytic barrel domain. The two His residue at position 215 and 253 that are experimentally established being crucial for the catalysis were well conserved. A potential glycosylation site at Asn270 and Asn292 in HaAmy1 and HaAmy2 protein respectively was predicted by web-based tool. Later one is strictly conserved among all α-amylase family members but Asn270 has been partially conserved. The overall conservation of the catalytic residues in both HaAmy1 and HaAmy2 strongly denote that their enzymatic mechanism might be very similar to the double-displacement catalytic mechanism of other known α-amylase.

The results obtained from homology modeling of HaAmy1 and HaAmy2 proteins

showed that the structures of HaAmy1 and HaAmy2 closely resemble that of other reported insect α-amylase from *Tenebrio molitor* (Figure 3.1). The superimposed structures between *TmAmy* and HaAmy1 and HaAmy2 models are shown in (Figure 3.2) and the root-mean-square deviation of 0.44/0.46 Cα positions. The secondary structure predicted for this protein, indicate the presence of one extra helix in HaAmy2 (Figure 3.3A). All other secondary structure elements in HaAmy1 remained intact in HaAmy2. Sequence alignment and superposition of these α-amylases showed the structure resemblance and high conservation of α-helix and β-strand. The structure consisted of three domains characteristics of the α-amylase family the N-terminal catalytic domain A made up of a $(\beta/\alpha)_8$-barrel, followed by domain B made up of two short α-helix and two short β-strand. This domain showed considerable variation from other α-amylases (Figure 3.1 & 3.2). The C-terminal C-domain was made up of two anti-parallel beta sheets in Greek-key motif. There were few structural differences located especially on the coil and loops regions. In domain B of the template, two β-strands were replaced by coil in the same domain of the target. This unique structural organization of *Ha*Amy1 and *Ha*Amy2 models could highlight the fact that this coil region could be their specific characteristic. Another difference between *Ha*Amy1 and *Ha*Amy2 was the presence of longer helix in *Ha*Amy2 192-199 (256-260 in HaAmy1) and one extra helix in HaAmy2 245-249. Substrate-binding site- three loops around the catalytic site were comparatively shorter than those in *Tm*Amy (Figure 3.1). The disulphide bonds between the residue Cys97 and Cys147 and also between Cys 102 and Cys 149 were conserved in all species, which implies a crucial functional role. Although, most of the α-amylases contain domain C, its specific function remains elusive (Svensson, 1994). The electrostatic surface calculation suggested that the N-terminal region of the HaAmy1 and HaAmy2 protein was predominantly represented with positive charges and in the C-terminal region there were a few patches with negative charges. The Consurf results indicate the presence of many conserved and crucial surface residues in the protein (Figure 3.3B & C). Significant differences that were observed between the HaAmy1 and HaAmy2, might be responsible for the differential interaction with inhibitors. The conserved surface also highlighted the resemblance of HaAmy1 with HSA and HaAmy2 with HPA (Figure 3.4).

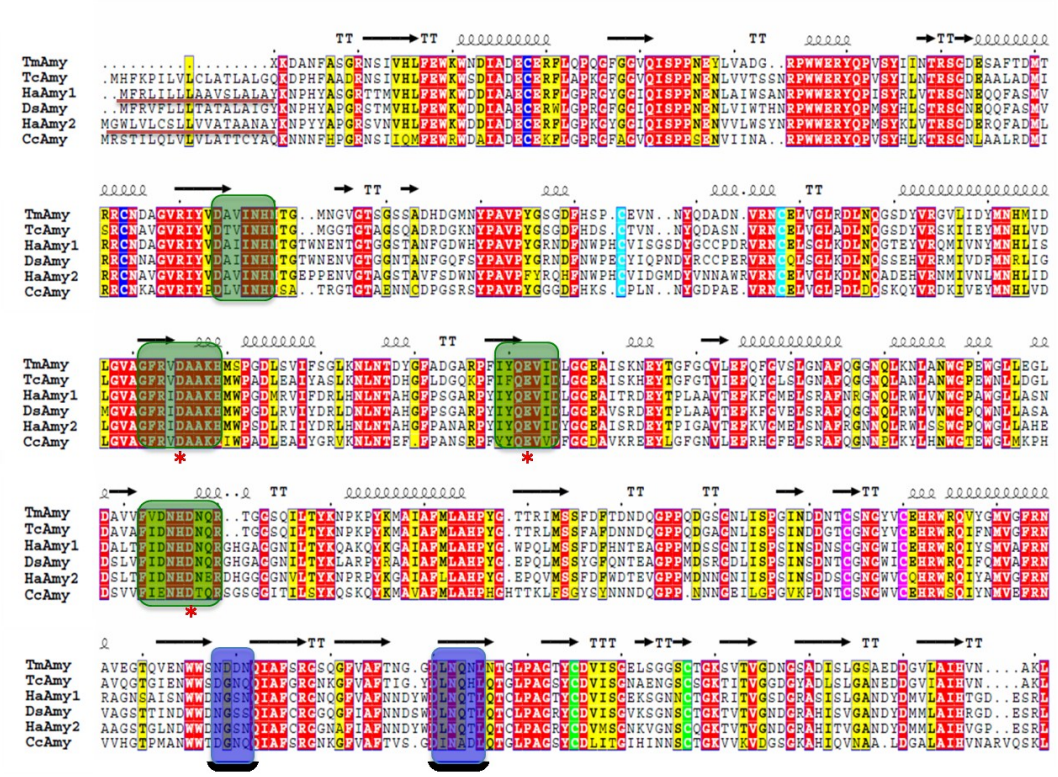


Figure 3.1 Molecular structure prediction of HaAmy1 and HaAmy2 using structure based sequence alignment. **A)** Multiple sequence alignment of HaAmy1 and HaAmy2 (Lepidoptera) with Coleopteran alpha-amylases was performed using ClustalW. The secondary structure elements *above* the sequence blocks correspond to the reported crystal structure of TmAmy. Conserved residues are *boxed in red*. Similar residues are in *black bold* and *boxed in yellow*. *α-helices* are rendered as *squiggles*, *β-turns* as *arrows* and *strict β*-turns as *TT*. The brown lines indicate the signal sequences. Cysteine pairings for disulphide bridges are boxed in *blue, cyan, pink* and *green* color. The box in green represents the conserved regions of α-amylase family with active site residues marked in asterisk (*) and box in purple denotes the predicted N-acyl glycosylation sites. The sequences are as follows, **TmAmy**: *Tenebrio molitor* α-amylase, PDB accession number: 1TMQ_A, **TcAmy**: *Tribolium castaneum* α-amylase, NCBI reference number: NP_001107848.1; **HaAmy1**: *Helicoverpa armigera* α-amylase1, GenBank accession number: ABU98614.1; **DsAmy**: *Diatrea saccharalis* α-amylase, GenBank accession number: AAP97394.1, **HaAmy2**: *Helicoverpa armigera* α-amylase2, GenBank accession number: EF600048.1(missing 5' end was identified using 5'RACE reaction) and **CcAmy**: *Callosobruchus chinensis* α-amylase. The figure was created using ESPript 3.

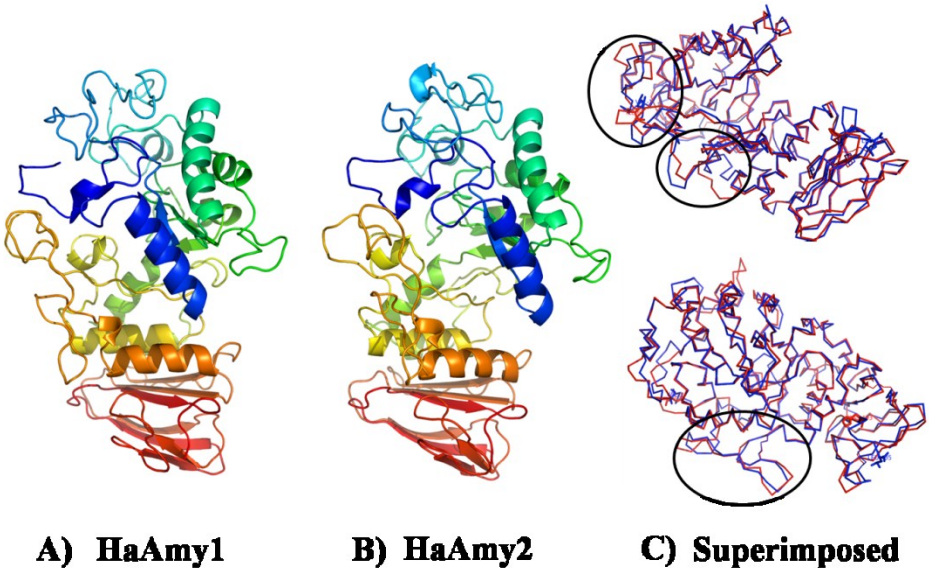


Figure 3.2 Homology modeling of HaAmy1 and HaAmy2. **A)** & **B)** Cartoon diagram of modeled HaAmy1 and HaAmy2 which are color-ramped from N to C terminus (Blue to red).**C)** Superposition of C-alpha of modeled HaAmy1 (Red) and HaAmy2 (Blue), view of 180° rotation. Circles indicate the prominent loop deviations.

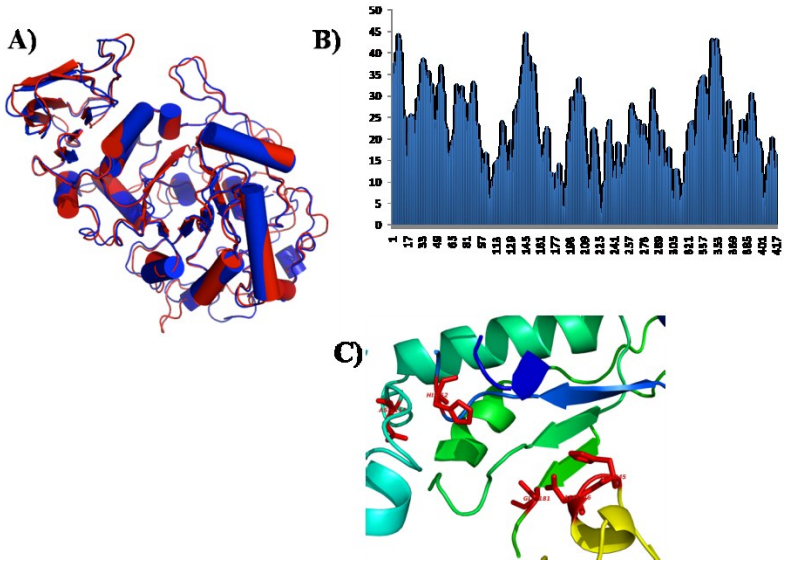


Figure 3.3 Comparisons of HaAmy1 and HaAmy2 tertiary structures. A) The cartoon representation of superposition of modeled HaAmy1 (Red) and HaAmy2 (Blue). The Arrow indicates the presence of extra helices in HaAmy2. **B)** The RMSD plot of C-alpha position of HaAmy1 and HaAmy2 and C) The active site residues are represented in stick in HaAmy1.

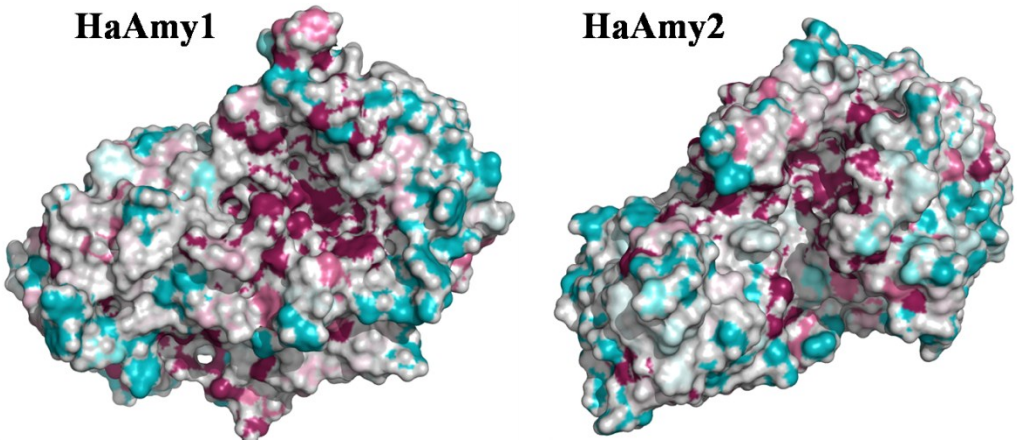


Figure 3.4 The conserved surface of HaAmy1 and HaAmy2 highlighting the resemblance with HSA and HPA respectively. Modeled HaAmy1 and HaAmy2 were surface-coloured based on surface conservation calculated by the ConSurf server as a heatmap with red being the most conserved and blue being the least conserved.

3.3.2 Recombinant protein expression, purification and activity of HaAmy1 and HaAmy2

Predicted molecular weight for HaAmy1 and HaAmy2 was 55.1 and 55.6, kDa respectively. HaAmy1 and HaAmy2 were heterologously expressed and secreted protein was purified and characterized. The average approximate yield was 12.5mg/ L for HaAmy1 and 8.5 mg/L for HaAmy2. Two not well-resolved proteins of molecular weight approximately 60 kDa were detected on SDS-PAGE for HaAmy1 and HaAmy2 (Figure 3.5A). The accurate molecular weight of HaAmy1 was 60.1 and 61.6 kDa while that for HaAmy2 was 55.5 and 57.7 kDa acquired from mass spectrometric measurements (Figure 3.5C). Differences in the molecular weight might be due to N-acyl glycosylation of

predicted sites as described earlier. Both the recombinant α-amylases were active (Figure 3.5B).

Hydrolytic products of starch by recombinant HaAmy1 and HaAmy2 catalysed reactions were glucose, maltose and malto-triose (Figure 3.6A & B). Maltose was a major product that indicated both enzymes lacked exo-amylase activity and thus glucose might be produced as a by-product. HaAmy1 and HaAmy2 can be classified as de-branching enzymes on the basis of major hydrolytic products. No qualitative differences were observed in HaAmy1 and HaAmy2 catalyzed reactions.

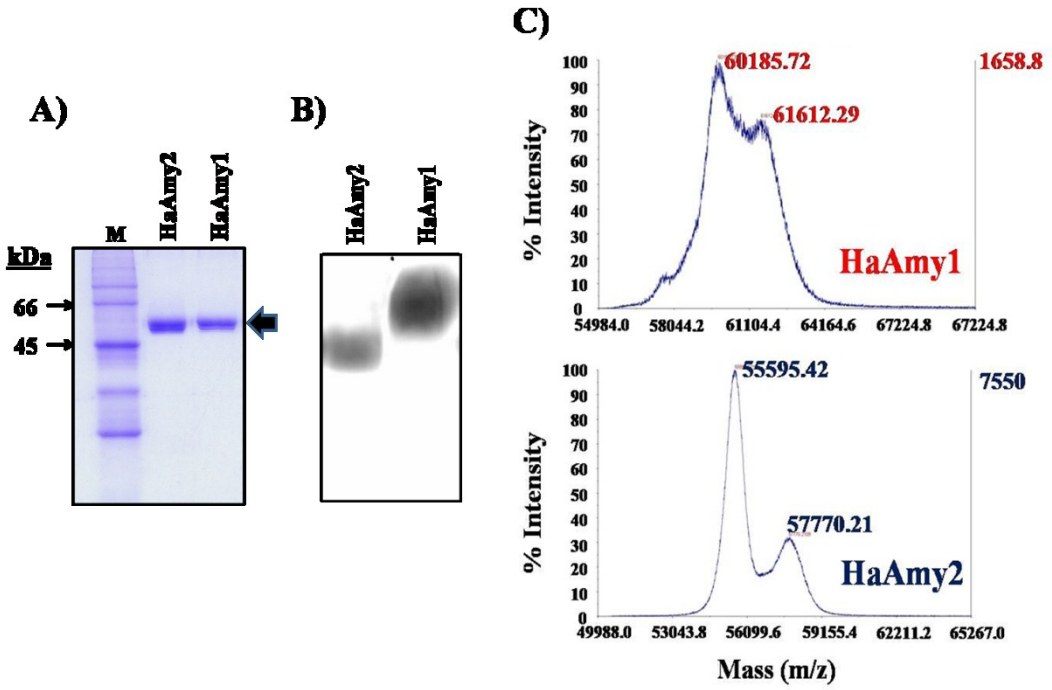


Figure 3.5 SDS-PAGE, In-gel activity and molecular weight identification of HaAmy1 and HaAmy2. *HaAmy1* and *HaAmy2* were cloned in *Pichia pastoris* and expressed protein was purified using Ni-NTA affinity column. 5 µg of purified protein was loaded in each well. **A)** Purified protein was loaded on 12% SDS-PAGE and stained with Coomassie Brilliant Blue R-250 stain. *Lane 1*, Pre-stained protein molecular weight marker; *Lane 2*, HaAmy2; *Lane 3*, HaAmy1. **B)** In-gel α-amylase activity; Purified HaAmy1 and HaAmy2 were loaded on 12% Native PAGE and α-amylase activity was

performed using soluble starch as a substrate .Gel was stained by Iodine solution and image was inverted for better visualization. **C)** Acquisition of accurate molecular weight from mass spectrometric measurements (MALDI).

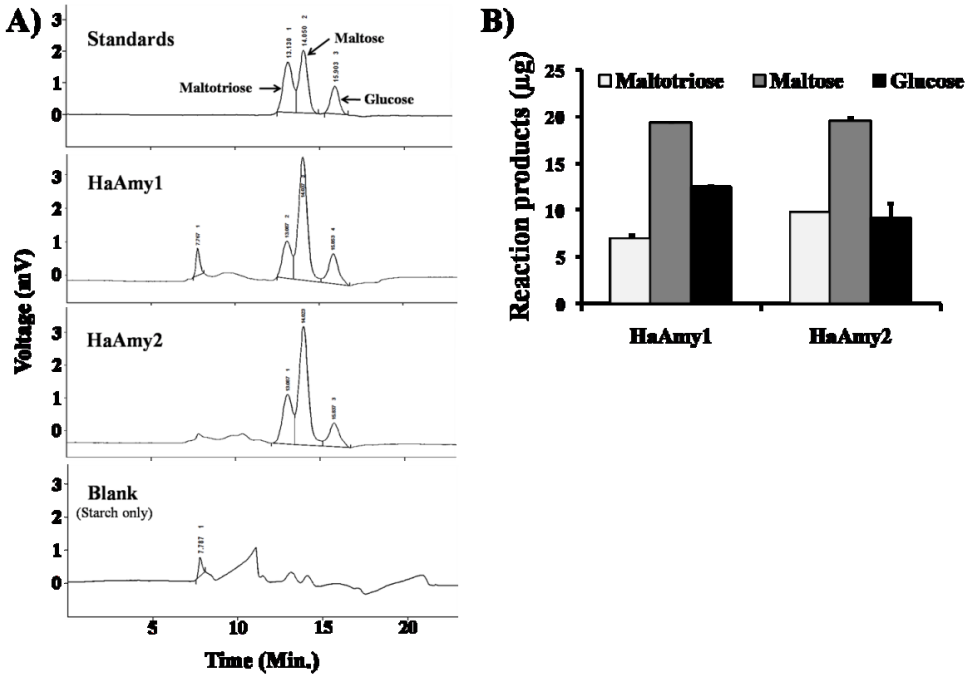


Figure 3.6 HPLC analysis of enzyme catalyzed reaction. A) HPLC chromatograms and **B)** quantitative analysis of HaAmy1 and HaAmy2 enzyme catalyzed reactions. Sugars were detected using refractive index detector.

3.3.3 Biochemical properties of HaAmy1 and HaAmy2 vary with respect to substrates

In order to compare the kinetic parameters of both HaAmy1 and HaAmy2 with various substrates the Michaeli-Menten analysis was performed from substrate saturation assay.K_M, V_{max}, K_{cat} and K_{cat}/ K_M were calculated for HaAmy1 and HaAmy2 using Starch and Amylopectin as substrates (Figure 3.7). Affinity (K_M) of HaAmy1 and HaAmy2 towards Amylopectin was more as compared to that for the starch. Moreover, Amylopectin was converted to the product more efficiently (K_{cat}/ K_M) than Starch when

used as a substrate in case of both the enzymes. Affinity of HaAmy2 for starch was greater than the affinity of HaAmy1 and catalytic efficiency was greater for HaAmy2 than HaAmy1. Although affinity of HaAmy1 for Amylopectin was less in comparison with HaAmy2, enzyme was more efficient in catalyzing the reaction. Comparative analysis between HaAmy1, HaAmy2, and Human salivary and pancreatic α-amylases showed that *H. armigera* α-amylases had higher affinity for the substrate (Starch) but also have reduced rate of the enzyme catalysed reaction than human α-amylases (Table 3.2).Overall, the data showed the prominent difference in substrate preference between the two enzymes.

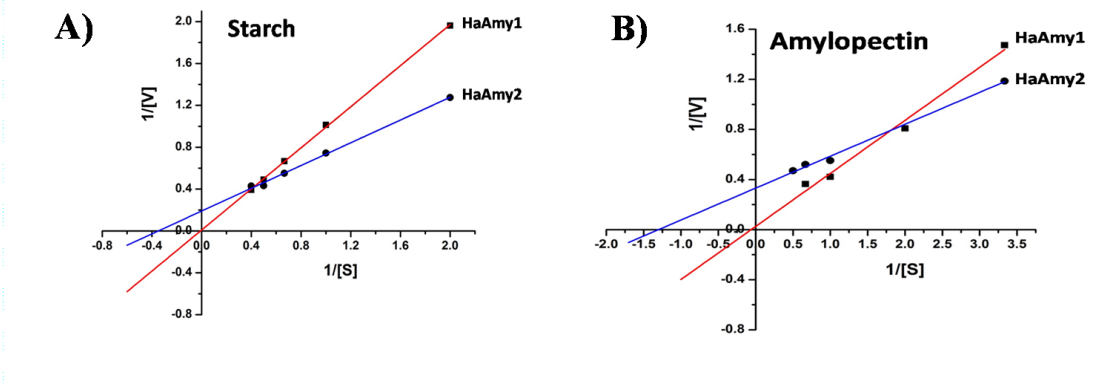


Figure 3.7 Lineweaver–Burk plot of HaAmy1 and HaAmy2 catalyzed reaction using **A)** Strach and **B)** Amylopectin as a substrate for determination of K_m and V_{max}.

3.3.4 Higher thermal conditions have minor effect on biochemistry and conformational stability of HaAmy1 and HaAmy2

Effect of temperature on HaAmy1 and HaAmy2 α-amylase activity was evaluated (Figure 3.8A). Both the enzymes were stable at higher temperatures having optimum activity at 60 ºC. Below 10 ºC and above 80 ºC, activity was found to be negligible. HaAmy2 was more active than HaAmy1 at optimum temperature. Stability of HaAmy1 and HaAmy2 under higher temperatures (*viz.* at 50º C, 60 ºC and 70 ºC) was evaluated

(Figure 3.8B & C). Activity was represented as relative to the activity of non-treated α-amylase preparations. Activity of HaAmy1 when carried out under standard assay conditions was retained at 50 ºC incubation while decreased by 70% when incubated at 70 ºC. Similar trend was observed for HaAmy2 except 15% reduction in relative activity was detected when incubated at 50 ºC. At the same time, comparable results were observed for relative activity carried out at optimum temperature. The Far-UV CD spectrum of HaAmy1 and HaAmy2 at 25 ºC displayed a large negative peak at 210 and 225, respectively and correlated with previously reported studies on α-amylases (Galdino et al., 2011). The estimate of the secondary structural elements calculated from CD data were identical for HaAmy1 and HaAmy2 (Alpha-helix, 18.7%, Beta sheet, 37%; Turns, 19.3% and Unordered structure, 25%) which were very close to the TmAmy structure determined by X-ray crystallography. CD spectral analysis was carried out to investigate structural integrity and thermo-stability of HaAmy1 and HaAmy2 at various temperatures ranging from 20 ºC to 80 ºC (Figure 3.8D & E). Conformation of HaAmy1 and HaAmy2 remained intact up to 60 ºC where shift in elliptical value indicated a distortion in structural integrity with corresponding decrease in enzyme activity observed above 60 ºC (Figure 3.8A). Collectively, data showed the thermal stability as a function of conformational integrity of *H. armigera* α-amylases.

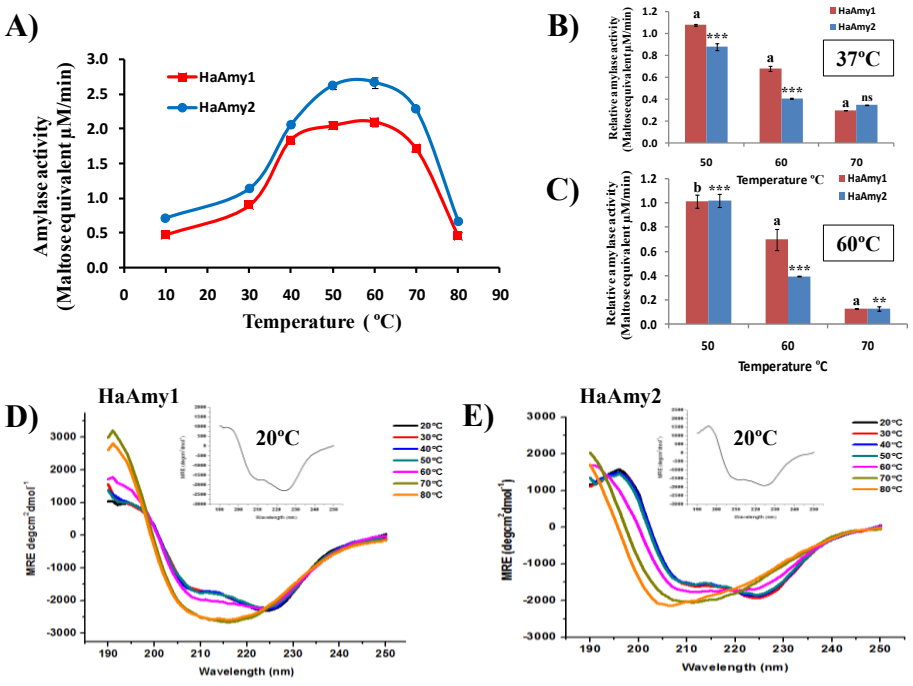


Figure 3.8 Activity and stability of HaAmy1 and HaAmy2 under different thermal conditions. A) α-Amylase activity of HaAmy1 and HaAmy2 at various temperatures (from 10 ºC to 80ºC). **B) & C)** Stability of HaAmy1 and HaAmy2 was checked by incubating enzyme at 50 ºC, 60 ºC and 70 ºC for 30 minutes. Activity was carried out at 37 ºC and 60 ºC using standard assay method. Data was represented as α-amylase activity relative to the activity of non-treated samples. In statistical analysis, One-way ANOVA was performed followed by Tukey's post hoc test. Statistical data is significant at *p*-value (a) or *** < 0.001, (b) or ** < 0.01 and (c) or ns for non-significant. **D) & E)** Far-UV CD spectra of HaAmy1 (62.5 µg/ml) and HaAmy2 (67 µg/ml) at temperatures 20 °C to 80 °C (respectively). Before each reading, protein was held at respective temperature for 5 minutes. A CD spectrum at 20ºC was given as an Insight for better visualization and comparison.

Table 3.2 Comparative properties of HaAmy1, HaAmy2, Human Salivary and Human Pancreatic α-amylases.

α-Amylase	Total amino acids	# % similarity with Tm Amy	MW (kDa)	Active site residues	*No.of di-S bonds	Accession number	PDB ID	Rmsd calpha atoms	p*I*	©K_M	V_{max}	Optimal pH	Optimal temperature
HsAmyP	511	63.40%	57.7	Asp212 Glu248 Asp315	5	P04746	1HNY	0.89(Amy1) 0.81(Amy2)	7.0	12.5	231	7.0	40-50ºC
HsAmyS	511	65.90%	57.76	Asp212 Glu248 Asp315	5	P04745	1SMD_A	0.99 (Amy1) 0.81 (Amy2)	6.4	19.3	428	6.6-6.9	40-50ºC
HaAmy1	500	71.80%	55.13	Asp209 Glu246 Asp311	4	ABU98614	-	1.15 (Amy2)	6.5	9.2	11.6	9.0	50-60 ºC
HaAmy2	502	71.50%	55.61	Asp211 Glu248 Asp313	4	EF600048	-		5.8	2.0	4.3	11.0	50-60 ºC

Part of the data was collected from Takeuchi, 1979; Talvar & Shrivastav, 2004; Ferey-Roux et al., 1998; Yoon & Robyt, 2003 and Ratan, 2004

Percent similarity with *Tenebrio molitor* α-amylase (PDB accession number: 1TMQ_A)

* Number of disufide bonds

© Using starch as a substrate.

3.3.5 HaAmy1 and HaAmy2 were stable under a wide range of pH

Influence of different pH conditions (pH3 to pH12) on HaAmy1 and HaAmy2 α-amylase activity was analyzed (Figure 3.9A & B). HaAmy1 and HaAmy2 showed optimum α-amylase activity at pH9 and pH11, respectively. We have tested stability of both the α-amylases in the range of pH3 to 11. Overall, α-amylase activity of both the enzymes remained unaffected except increase in α-amylase activity was observed by 10 and 5% at pH9 and pH5 for HaAmy1 and HaAmy2, respectively. Structural integrity under different pH conditions were studied using Far-UV CD spectral analysis (Figure 3.9C & D). Except for pH 3, HaAmy1 and HaAmy2 were conformationally stable when incubated with different range of pH. Results indicated that HaAmy1 and HaAmy2 were highly stable and could withstand higher acidic as well as alkaline conditions retaining their activity.

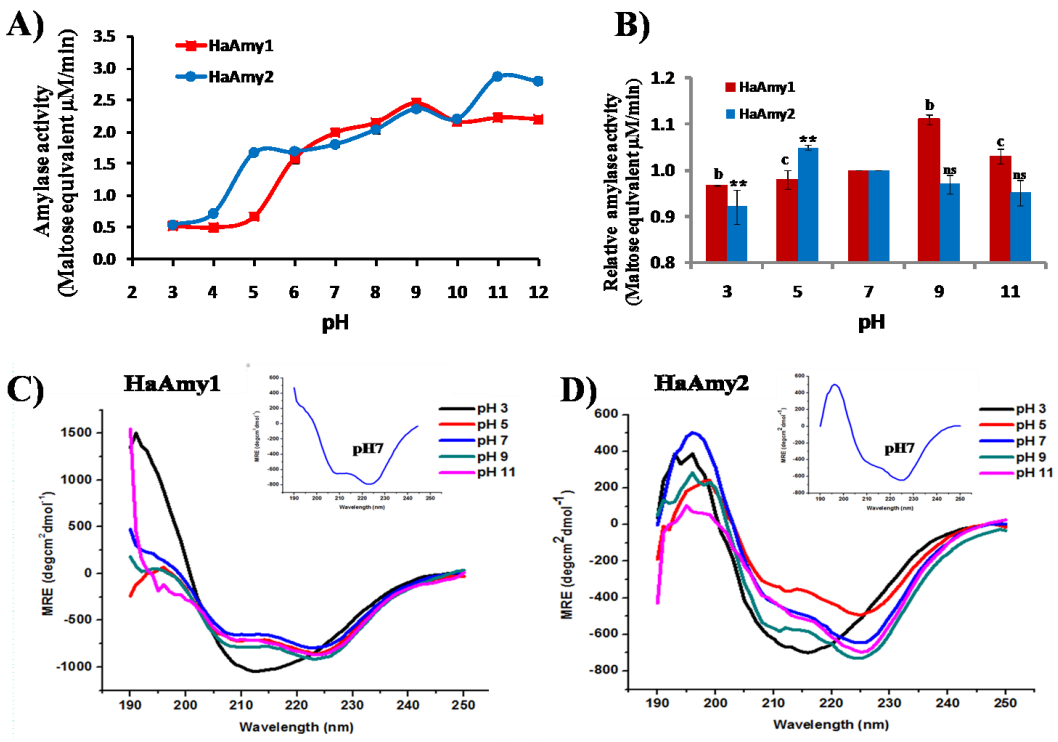


Figure 3.9 Activity and stability of HaAmy1 and HaAmy2 under different pH conditions. A) α-Amylase activity of HaAmy1 and HaAmy2 at various pH. **B)** Stability of HaAmy1 and HaAmy2 was checked by incubating enzyme in the buffer of respective

pH for overnight and activity was carried out under standard assay conditions. Data was represented as α-amylase activity relative to the activity at pH7. In statistical analysis, One-way ANOVA was performed followed by Tukey's post hoc test. Statistical data is significant at *p*-value (a) or *** < 0.001, (b) or ** < 0.01 and (c) or ns as non-significant. **C) & D)** Far-UV CD spectra of HaAmy1 (105 µg/ml) and HaAmy2 (110 µg/ml) at pH 3, 5, 7, 9 and 11. Enzyme was incubated in the buffer of respective pH overnight. A CD spectrum at pH 7 was given as an Insight for better visualization and comparison.

3.3.6 Proteinaceous and non-proteinaceous α-amylase inhibitor had differential activity towards recombinant HaAmy1 and HaAmy2

Acarbose, a chemical and a proteinaceous wheat α-amylase inhibitor was used to compare inhibition of HaAmy1 and HaAmy2 along with human salivary and pancreatic α-amylases. HaAmy2 was inhibited more efficiently (70%) as compared to the HaAmy1 (40%) by acarbose (Figure 3.10B). For equivalent acarbose concentrations, maximum 30% inhibition was attained against human salivary and pancreatic α-amylases. Results indicate that acarbose was more potent towards HaAmy1 and HaAmy2 compared to human α-amylases. Importantly, differential inhibition of HaAmy1 (60%) and HaAmy2 (30%) was apparent using a proteinaceous wheat AI (WAI). Interestingly, WAI failed to inhibit human pancreatic α-amylase (8%) while significant inhibition of salivary α-amylase activity (60%) was recorded (Figure 3.10A). Unlike acarbose, order of percent inhibition of HaAmy1 coincided with that of human salivary α-amylases while HaAmy2 with pancreatic α-amylase.

Size exclusion chromatography studies of these complexes were performed to understand the stoichiometry of these complexes (Figure 3.11A & B). The elution profile clearly indicated the stoichiometry of these complexes was identical and the possible stoichiometry ratio of HaAmy1 and HaAmy2 with WAI was 2:1. These findings suggested that AI's binding site and mode of binding might be similar for both HaAmy1 and HaAmy2 but the affinities could be different. This might account for AI's difference in inhibition of α-amylase activity with the respective isoforms.

3.3.7 Inhibitor docking

Acarbose is known to inhibit α-amylases through competitive binding to the active site. Docking of Acarbose with HaAmy1 and HaAmy2 revealed its similar binding to both as previously reported for α-amylases-acarbose complex (Figure 3.12). The docking of proteinaceous inhibitor WAI with HaAmy1 and HaAmy2 has been shown in Figure 3.13. In both the complexes, the mode and site of binding of WAI were very different. Indeed it was very challenging to predict the complex, which has multimeric complex without definite stoichiometry.

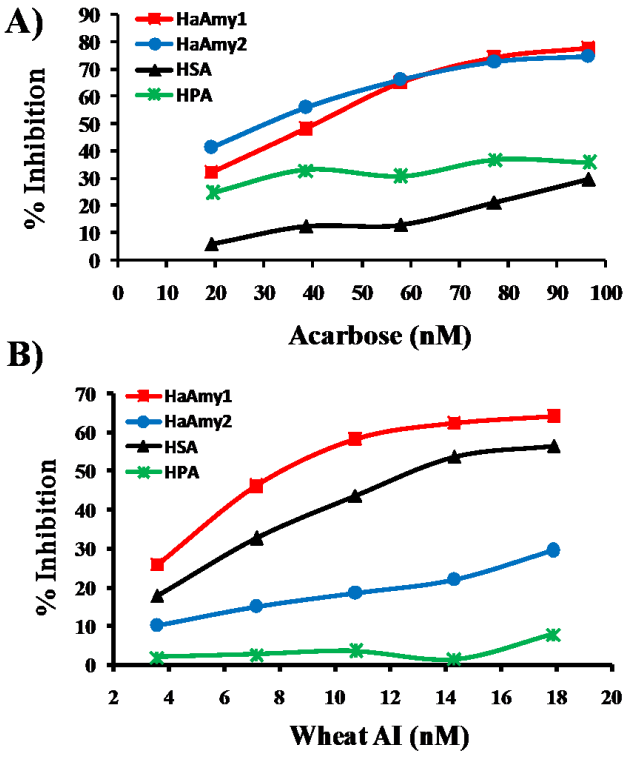


Figure 3.10 Inhibitory activity of acarbose and Wheat α-Amylase inhibitor (AI) against human salivary α-amylase (HSA), human pancreatic α-amylase (HPA), HaAmy1 and HaAmy2.10- 100 nM acarbose, a non proteinaceous α-amylase inhibitor **A)** and 2- 20 nM Wheat AI, a proteinaceous α-amylase inhibitor **B)** was tested for

inhibitory potential against HSA, HPA, HaAmy1 and HaAmy2. Inhibitory activity was represented as percent inhibition.

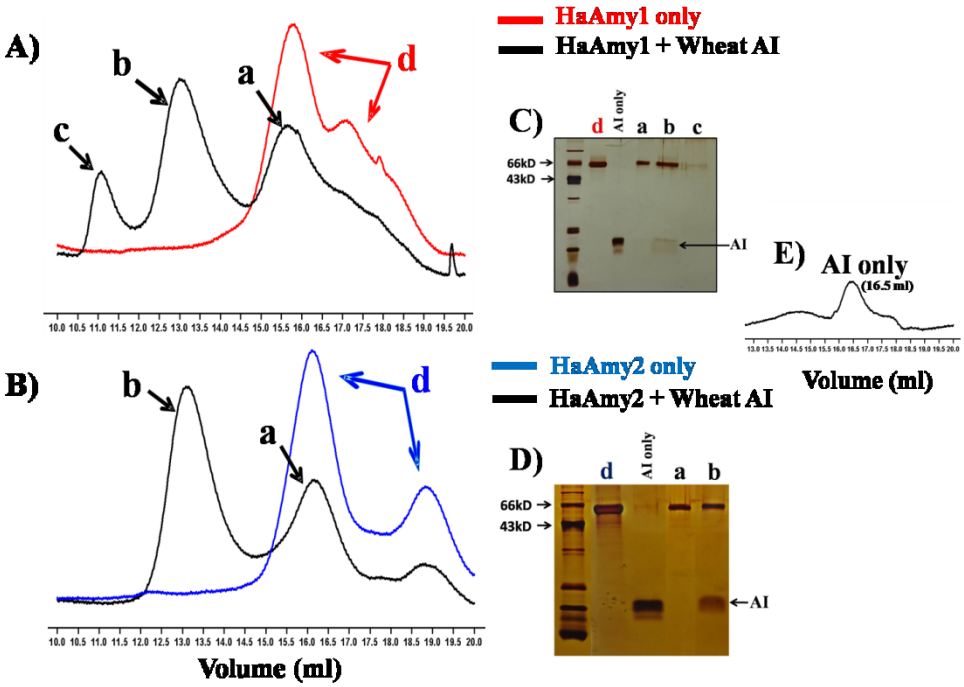


Figure 3.11 FPLC analysis of complexes between HaAmy1 and HaAmy2 with wheat α-amylase inhibitor. A separate equimolar Mixture of HaAmy1 and HaAmy2with Wheat α-amylase inhibitor were incubated at 37°C in phosphate buffer (pH7.0) for 60minutes. 200µl of the entire mixture was applied to Enrich SEC650 column followed by elution in phosphate buffer. In superimposed images, **A) & B)** retention volumes as follows; (d) HaAmy1/HaAmy2 enzyme peaks, (a) free enzyme (HaAmy1/HaAmy2) in reaction mixture, (b) Complex and (c) multimeric complexes between enzyme and wheat inhibitor. **C) & D)** Fractions were checked on SDS-PAGE. **E)** Chromatogram of only AI showing peak at 16.5 ml.

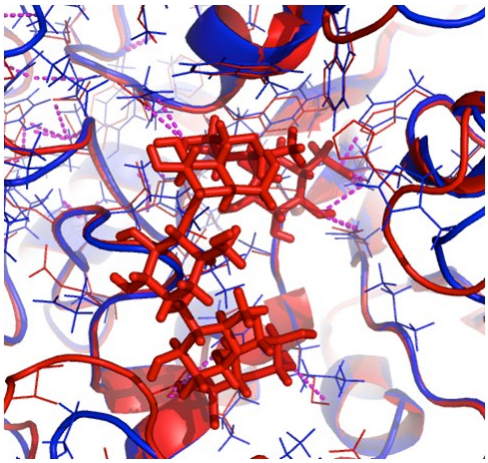


Figure 3.12 The superimposed complex structure of HaAmy1 (Red) and HaAmy2 (blue) complexed with acarbose predicted by molecular docking.

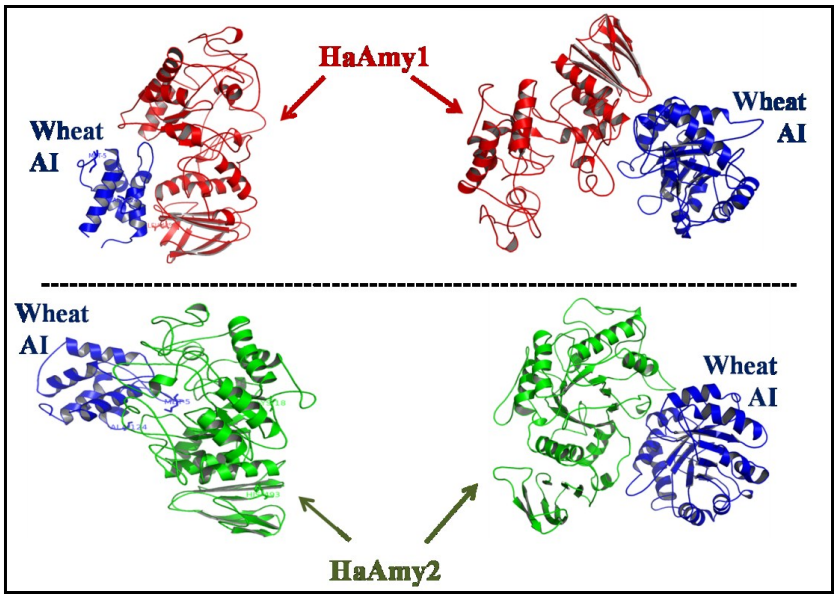


Figure 3.13 Molecular docking studies of HaAmy1 (Red) and HaAmy2 (green) complexed with Wheat AI (blue). The docking modeled resulted by both free docking (left) and forced docking was performed by defining the active site residues from the complex structure of TmAmy-Ragi AI inhibitor (right).

3.4 Discussion

This report intended to throw light on digestive α-amylase variants from *H. armigera*, which is known to feed on complex diets and acquire required nutrition and energy. The kinetic data indicated that HaAmy2 had stronger affinity towards the Starch than HaAmy1. The catalytic efficiency (Kcat/Km) was comparable for both the forms. But in the case of Amylopectin even though HaAmy2 had higher affinity, HaAmy1 had relatively higher catalytic efficiency towards the Amylopectin. The sequence analysis inferred that the critical residues were well conserved among α-amylase from diverged phyla. The result of homology modeling showed that there is no conspicuous difference among these isoforms and also with the other α-amylase from insects. Both, sequence analysis and homology modeling suggested that there wouldn't be diverse mechanism of enzymatic action and diverse function. The mass spectrometric analysis of heterologously expressed and purified proteins revealed that there was different level of glycosylation, which might influence its substrate preference and stability towards different temperature and pH. HaAmy2 showed higher activity under optimum temperature and pH compared to HaAmy1.

The biochemical and binding site dissimilarity were more pronounced between these two isoforms from the result of inhibition kinetic studies. For these investigations both proteinaceous and chemical inhibitors were used to monitor its inhibition effect toward the activity of α-amylase. Only proteinaceous inhibitor, specifically WAI showed the differential inhibition toward its activity. Our docking results showed that the mode of binding between AI with isoforms was different, which might account for the difference in their affinities. To investigate further we attempted to check the complex by size exclusion chromatography analysis. But the result from the size exclusion chromatography exposed that the stoichiometry of complex was the same, perhaps the differential inhibition of inhibitor might be due to the difference in mode of binding in the active sites of isoforms. Altogether, two α-amylases cloned and recombinant protein characterization from *H. armigera* indicated that they had different biochemical properties and specifically their interaction with substrates and plant proteinaceous inhibitors revealed significant variations. Thus diversity and adaptability of *H. armigera* digestive α-amylases in context with its biochemical and biophysical properties as well as

their differential responses towards AI signified additive role of these enzymes for the survival of *H. armigera* on diverse diets.

3.5 Summary of the chapter

In the present study, we heterologously expressed two α-amylases *viz., HaAmy1* and *HaAmy2* in yeast (*Pichia pastoris*) and their biochemical and biophysical properties were analyzed. Distinguishing catalytic properties were investigated by enzyme kinetics using various substrates. We also studied inhibitory effect of proteinaceous and non-proteinaceous α-amylase inhibitors on these α-amylases and compared inhibition profiles with human salivary and pancreatic α-amylases. Further, homology modeling and molecular docking tools were used to understand underlying differential inhibitory mechanisms. To know qualitative and quantitative differences between of these two α-amylases, reaction products and α-amylase-inhibitor complex were characterized using chromatographic tool. Present study will help us to better understand the functioning of α-amylases and hence in context the adaptability of *H. armigera* in various environments.

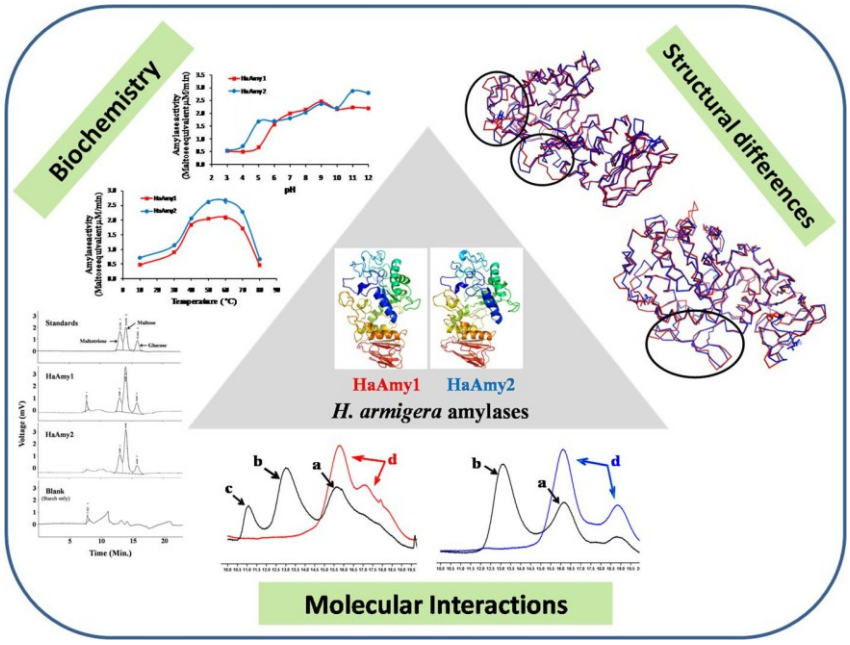


Figure 3.14 Graphical representation of biochemical, biophysical and molecular properties of HaAmy1 and HaAmy2.

Chapter-4

"Characterization of two coleopteran α-amylases and molecular insight into their differential inhibition by synthetic α-amylase inhibitor, acarbose"

Contents of chapter-4 have been published in a research article……

(Channale and Bhide et al., 2016, IBMB, 74, 1-11)

4.1 Introduction

Pulses serve as an important source of income for farmers in developing countries like India. However, cereals and pulses are often infested with insect pests during their storage. Stored grain pests are responsible for about 37% agricultural loss worldwide (Franco et al., 2002). Among Coleopteran pests, species of Chrysomelidae and Tenebrionidae family feed and breed on stored legumes or cereals that eventually make them unfit for human consumption (Southgate, 1979). Stored wheat and mung bean are the preferred host of the red flour beetle (*Tribolium castaneum*) and pulse beetle (*Callosobruchus chinensis*), respectively, for their sustenance and breeding. Infestation of these pests is not only responsible for economic losses but also affect human health. For example, *T. castaneum* is known to secrete benzoquinone in wheat flour, which might show toxic effects on human health, if consumed. *T. castaneum* has the ability to survive dry weather by virtue of a cryptonephridial organ which imparts tolerance towards dry conditions and increases chances of its survival (Ladisch et al., 1967; Lis et al., 2011).

Insect gut is a major interface between insect and its environment (Darvishzadeh et al., 2012). Digestive enzyme pool of the coleopteran insects primarily consists of α-amylases along with proteases to break down starch, other carbohydrate components and proteins that are present in food grains. α-amylases belong to GH13 glycoside hydrolases family of enzymes that hydrolyze α-1,4-glucosidic bond in starch to produce maltose, glucose and other products and these serve as energy sources for insect pests (Svensson, 1994; Janecek et al., 1997; MacGregor et al., 2001; Cantarel et al., 2009). Along with the coleopteran α-amylases, many proteinaceous and non-proteinaceous α-amylase inhibitors (α-AIs) have been studied for their potential α-amylase inhibitory activity towards bruchid digestive α-amylases *in vitro* as well as *in vivo* (Svensson et al., 2004; Nielsen et al., 2004). For example, purified α-AI from *Phaseolus vulgaris* was more effective against α-amylases from larval crude extracts of *T. castaneum* and *C. chinensis* (Gupta et al., 2014). Similarly, purified α-AI from *Carica papaya* was found to be active against *Callosobruchus maculatus* larvae (Farias et al., 2007). In another approach, *Pisum sativum* transgenic plants expressing bean α-AI-1 inhibitor were resistant to the pea weevil infestation in field conditions (Shade et al., 1994; Morton et al., 2000). Several reports are available on biochemical characterization of α-amylases from crude extracts of bruchid larvae but detailed study highlighting their molecular and

structural interactions with α-AIs are lacking (Yamada et al., 2003). Further, crude gut extract represents a mixture of various α-amylase isoforms, which limits systematic interpretation of the molecular basis of α-amylase inhibitions.

In present study, we have cloned and characterized α-amylases from two coleopteran pests viz. *C. chinensis* and *T. castaneum*. Their biochemical and biophysical properties were studied thoroughly in order to understand underlying functional similarities and differences. Interactions of both the enzymes with proteinaceous and non-proteinaceous α-amylase inhibitors highlighted differential mode of interactions and binding of acarbose (non-proteinaceous α-amylase inhibitor) to the active site residues and binding pocket residues at the molecular level. Together, study underlines variable mode of interactions of non-proteinaceous α-amylase inhibitor, acarbose with two coleopteran α-amylases which could be useful in designing improved control agents in order to mitigate insect infestation.

4.2 Materials and methods

4.2.1 Phylogenetic analysis of Coleopteran α-amylases

Full-length amino acid sequences of all the coleopteran α-amylases were extracted from NCBI database. Multiple sequence alignment of these sequences was carried out using ClustalW and the corresponding alignments were used to reconstruct phylogenetic tree. The Neighbor-joining (NJ) tree was generated by MEGA6 (Tamura et al., 2013) using 1000 bootstrap replicates, wherein bacterial (*Bacillus subtilis*) α-amylase (GenBank accession No. ADH93706.1) was used as an out-group.

4.2.2 Insect rearing

C. chinensis and *T. castaneum* cultures were obtained from Entomology department, CSIR-NCL, Pune. Freshly emerged *C. chinensis* adults were transferred to uninfected mung bean seeds and culture was maintained by transferring concurrent generations on fresh seeds. *T. castaneum* adults were sub-cultured on wheat flour. The cultures were maintained under controlled conditions at 25°C, 65% relative humidity with 12h dark/ 12h light cycles in insect growth chamber.

4.2.3 RNA extraction and cDNA synthesis

Total RNA was extracted from 100 mg tissue of *C. chinensis* larvae and *T. castaneum* adults using Trizol reagent (Invitogen, Carlsband, CA, USA) followed by RQ1 DNase (Promega, Madison, WI, USA) treatment. DNA-free RNA (1 µg) isolated from *T. castaneum* adults was reverse transcribed using oligo-dT (Promega, USA) following the manufacturer's instructions. Reverse transcription of 1 µg of total RNA isolated from *C. chinensis* larvae was achieved using SMARTer™ RACE cDNA Amplification Kit (Clontech, St Germainen Laye, France) following the manufacturer's instructions.

4.2.4 Cloning, expression and purification of CcAmy and TcAmy

Open reading frames of CcAmy (GenBank accession: KU641476) and TcAmy (NM_001114376.1) without the native signal peptide of 17 and 18 amino acids, respectively, were amplified using designed primer pairs (Table 4.1) and cloned in pET 28-a expression vector (Novagen, Madison, WI, USA) in frame with N-terminus 6X His-tag. Recombinant plasmids were amplified and transformed into Escherichia coli BL21 Star (DE3) cells. BL21 cells transformed with CcAmy and TcAmy constructs were induced with 0.25 mM and 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG), respectively and incubated at 16°C for 16 h. For CcAmy, bacterial protein homogenate was extracted after cell lysis by sonication (5 min with 47% amplitude, alternating 5 sec on; 5 sec off cycles) on ice in the lysis buffer (25 mM HEPES pH 7.0, 2 mM MgCl2, 20 mM Imidazole, 0.1% Triton X-100, 1 mM β-mercaptoethanol, 150 mM NaCl). Clear lysate obtained by centrifugation at 12000 rpm for 30 min at 4ºC was loaded on pre-equilibrated 300 µl ProBond Ni–NTA resin (Invirogen) in Bio-Rad gravity column (Bio-Rad, Hercules, CA, USA) and washed with 25 mL wash buffer (25 mM HEPES pH 7.0, 2 mM MgCl2, 3mM Imidazole,1 mM β-mercaptoethanol, 150 mM NaCl, 10% glycerol). The bound protein was eluted with 150 mM imidazole solubilized in the same buffer.

For TcAmy, protein was purified from inclusion bodies using on-column refolding method (Oganesyan et al., 2005). In brief, cell lysis was performed in lysis buffer (50mM HEPES, pH 7.0, 100 mM NaCl) by sonication (5 min with 47% amplitude, alternating 5 sec on; 5 sec off cycles) on ice followed by centrifugation at 12000 rpm for 20 min at 4°C. The pellet was washed in same buffer containing 1M Urea, 2% Triton X-100. Further, pellet was solubilized in denaturing buffer (20 mM Tris pH 8.5, 8 M Urea,

100 mM NaCl, 1 mM β-mercaptoethanol) by gently vortexing at room temperature. The solubilized protein from inclusion bodies was bound to Ni-NTA pre-equilibrated resin at room temperature overnight. On-column refolding was achieved with different buffers (10 column volumes for each buffer) under gravity. All the working solutions were prepared in Buffer-A (20 mM Tris, pH 8.5, 100 mM NaCl). First, column was washed with denaturing buffer containing 40 mM imidazole followed by buffer A containing 0.1% Triton X-100 and 500 mM NaCl. The column was further washed with buffer A containing 5 mM cyclodextrin followed by another wash with buffer A. The protein was eluted in buffer A containing 250 mM imidazole and 10% glycerol.

Eluted fractions of CcAmy and TcAmy were pooled separately and were subjected to buffer exchange (25 mM HEPES, pH 7.0 and 20 mM Tris, pH 8.5 respectively) by passing through 10 kDa cutoff column (Millipore, Billerica, MA, USA). Protein was quantified by Bradford assay (Bio-Rad, USA) using BSA as a standard. Purified proteins were loaded on 12% SDS–PAGE to check the purity of samples.

Table 4.1 Primers used for cloning of *Callosobruchus chinensis* α-amylase and *Tribolium castaneum* α-amylase in pET 28a vector.

Primer name	Primer sequence	Length (bp)
CcAmy	F5'GAGCTCCAGAAGAACAACAATTTTCACCCGG 3'	31
	R5'CTCGAGTTACAGTTTGGATTGAACCCTAG 3'	29
TcAmy	F5'GGATCCCAAAAAGACCCACACTTTGCCGC 3'	29
	R5'CTCGAGTTACAATTTGGCATTAACATGAATGGC 3'	33

4.2.5 Homology modeling and docking studies of CcAmy and TcAmy

The three dimensional structures of CcAmy and TcAmy were predicted using MODELLER ver.9.15 (Sali and Blundell, 1993; Webb and Sali, 2014). The crystal structure of *Tenebrio molitor* α-amylase (PDB ID: 1JAE, Strobl et al., 1998a), was used as a template. The models were evaluated on the basis of lowest discrete optimized

protein energy (DOPE) score. The structural assessment of the models was performed using RAMPAGE (Lovell et al., 2003). The visualization of the above models was performed using Pymol (https://www.pymol.org; DeLano Scientific LLC, USA).

Prediction of acarbose binding with CcAmy and TcAmy were carried out using docking studies. Corresponding residues at positions 109-113, 211-214 and 303-307 belonging to inhibitor binding site in *T. molitor* α-amylase (TmAmy) structure was assigned (PDB ID: 1TMQ, Strobl et al., 1998). Docking studies of the acarbose was performed using Glide 6.0 (Schrodinger, LLC, Portland, USA). Grid was generated using porcine pancreatic α-amylase with inhibitor (1OSE, Gilles et al., 1996). Grid based rigid receptor and flexible ligand docking program Glide 6.0 (Friesner et al., 2004) was used to predict binding modes of the inhibitor in the receptor-binding site. Proteins were prepared using protein preparation wizard in Maestro (http://www.schrodinger.com/Maestro/). Hydrogens were added subsequently to carry out restrain using impref utility of Maestro. The root mean square deviation (RMSD) of the atomic displacement for terminating the minimization was set as 0.3 A˚. Similarly, ligands were refined with the help of LigPrep 2.5 (http://www.schrodinger.com/LigPrep/) to define their charged state and enumerate their stereo isomers. The processed receptors and ligands were further used for the docking studies using Glide 6.0 (Friesner et al., 2004).

4.2.6 In-gel α-amylase activity and molecular weight determination

Equal quantity of purified recombinant CcAmy and TcAmy each was loaded on native–PAGE. After electrophoresis, gel was gently washed with 20 mM citrate-phosphate buffer; pH 5.0 and incubated in 2% starch solution, prepared in same buffer, at 37°C for 90 min (Giri and Kachole, 1996). Gel was rinsed with buffer and stained with iodine solution (1 mM Iodine in 0.5 M KI). Gel was scanned and image was inverted for better visualization.

For molecular weight determination, equal quantity (5 µg) of recombinant CcAmy and TcAmy was loaded on SDS-PAGE. After electrophoresis, gel was stained with Coomassie Brilliant Blue R-250 dye. Molecular weight of recombinant α-amylases was determined by calculating relative mobility of the bands (Rf) with known reference run in parallel. Theoretical molecular weights excluding native signal peptide and including 6X His-tag were calculated using ExPASy tool (http://web.expasy.org).

4.2.7 Determination of α-amylase activity

All the assays of α-amylase activity were performed by monitoring liberation of reducing sugars from starch using DNSA (Dinitrosalicylic acid) reagent. A premix of triplicate reactions was prepared containing 20 mM citrate phosphate buffer (pH 5.0) and equal units of purified recombinant enzyme (determined by adjusting the absorbance at 0.5 at 540 nm) to which 150 µL of starch (0.25%) was added. After 15 min of incubation at 37°C, reaction was stopped by adding 500 µL of DNSA reagent and reaction tube was incubated in boiling water bath for 5 min and absorbance was measured at 540 nm. One α-amylase unit was defined as the amount of enzyme required to release 1 µM maltose/min at 37°C from substrate (starch) under the given assay conditions.

4.2.8 Effect of pH and temperature on the activity of enzyme

To determine the optimum pH, the activity of α-amylase was measured at different pH ranging from 3 to 12 where equal units of enzyme were used for each reaction. Citrate-phosphate, sodium phosphate, Tris and glycine-NaOH buffers were used for the following specified pH values: 3 to 6, 7, 8 to 9, and 10 to 12, respectively. For determining the optimum temperature to hydrolyze starch, the activity of purified α-amylase was measured under pH 5.0 using 20 mM citrate sodium phosphate buffer at various temperatures ranging from 10°C to 80°C. An equal amount of enzyme was used for each reaction.

4.2.9 Analysis of end products of starch hydrolysis

Purified α-amylases; CcAmy and TcAmy, were incubated separately with 0.25% potato starch (Sigma-Aldrich, MO, USA) in 20 mM citrate phosphate buffer pH 5.0 at 37°C for 30 min. The reaction was stopped by placing the reaction tube on ice. For quantitative analysis, the end-products of starch hydrolysis by α-amylases were analyzed with HPLC. All the samples and standards viz; glucose, maltose, maltotriose, malto-tetrose and malto-pentose (Sigma-Aldrich, USA) were filtered through 0.22 µm filter and 50 µL of each standard and sample were injected in SupelcogelPb column (5 cm × 4.6mm internal diameter, Sigma-Aldrich). The mobile phase was distilled water at a flow rate of 0.3 mL/min and column oven temperature was set at 70°C. Sugars were detected using refractive index detector (Shodex RI-21, NewYork, USA) and quantified using

standard sugars. Quantifications were based on the peak area measurements of standards run under identical conditions.

4.2.10 Determination of kinetic parameters and α-amylase inhibitory activity

Michaelis-Menten analysis was performed by substrate saturation assay to compare the kinetic parameters of both CcAmy and TcAmy. All the assays were performed at 37°C and pH 5.0 by using starch and amylopectin as substrates. KM, Kcat and Kcat/KM were calculated for CcAmy and TcAmy using GraphPad Prism v6.0 (GraphPad Software, San Diego, CA).

Alpha-amylase inhibitor (α-AI) activity was performed by similar way as indicated in section 2.7 except that enzyme and inhibitor were incubated for 15 min at 37°C before incubating with the substrate. α-AIs viz acarbose (non-proteinaceous) and wheat AI (proteinaceous) were tested in a concentration range of 2 to 193 nM and 0.1 to 21 nM, respectively, against CcAmy and TcAmy. Inhibition of crude α-amylase activity from adult and larval extracts of *T. castaneum* and *C. chinensis* were also performed for comparison. Crude extracts were prepared by incubating 200 mg of crushed larval and adult tissue in 2 mL of 20 mM citrate phosphate buffer; pH 7.0 at 4ºC overnight. Crude extract was centrifuged at 15000 rpm for 20 min and the supernatant was used as crude α-amylase source. α-AI activity was calculated by measuring the reduction in liberated reducing sugars from starch compared to the standard α-amylase activity mentioned above. α-AI activity was expressed as percentage inhibition.

4.3 Results

4.3.1 Phylogenetic analysis of Coleopteran α-amylases

A cladogram was generated by ClustalW through alignment of α-amylases from three families of the insect order Coleoptera (Fig. 4.1). Two distinct clades were generated out of which one clade exhibited similarities between α-amylases from Tenebrionidae and Chrysomelidae families whereas other clade represented α-amylases from Curculionidae family. Alpha-amylases from Tenebrionidae formed separate cluster within single clade. Furthermore, *T. castaneum* α-amylases formed separate sub-cluster within Tenebrionidae. In Chrysomelidae cluster, C. chinensis α-amylases showed higher

similarity with *Zabrotes subfasciatus* α-amylase and formed a distinct sub-cluster. Amino acid sequence alignment showed 74.3% similarity between CcAmy and TcAmy.

4.3.2 Homology modeling and docking analysis of CcAmy and TcAmy

The coding sequences for CcAmy and TcAmy have predicted signal sequence of 17 and 18 amino acids, respectively, which target them to secretory pathway for extracellular secretion. Sequence analysis showed 60% and 78% identity for CcAmy and TcAmy with TmAmy. Secondary structure prediction revealed seven conserved sequences, super secondary fold and (β/α)8 or TIM-barrel. The catalytic residues of CcAmy at the strands beta 4 the nucleophile Asp203 (Asp185), beta 5 the proton donor Glu239 (Glu222) and the beta 7 transition state stabilizer Asp304 (Asp287) and similarly in TcAmy Asp204 (Asp185), Glu241 (Glu222), Asp306 (Asp287) were conserved (Matsuura et al., 1984). The four-disulfide bonds were also conserved among all the six species except in that of *Phaedon cochleariae* α-amylase (PcAmy) (Figure 4.2). The seven conserved regions of α-amylase family were positioned near Aβ2, Aβ3, Bα1, Aβ4, Aβ5, Aβ7 and Aβ8 secondary structure.

The three dimensional structure obtained using homology modeling showed that CcAmy and TcAmy closely resemble to reported crystal structure of TmAmy (Figure 4.3). The RMSD values for the superimposed structure of entire Cα chain of CcAmy and TcAmy with TmAmy were 0.46 and 0.44, respectively. A high structural resemblance on superimposition of α-amylases was observed with respect to conservation of α-helix and β-strand. The architecture consisted of three domains found in α-amylase family, i) domain A, dominating 3-D unit forming (β/α)8 barrel, ii) domain B which inserts into domain A and was the least conserved domain in α-amylase family and iii) domain C - forming Greek key motif made up of exclusively β-strands. The electrostatic surface calculation implied that CcAmy had only a few electropositive residue distribution on the surface compared to TcAmy and TmAmy. Active sites of CcAmy and TcAmy were well conserved and aligned perfectly well in structures.

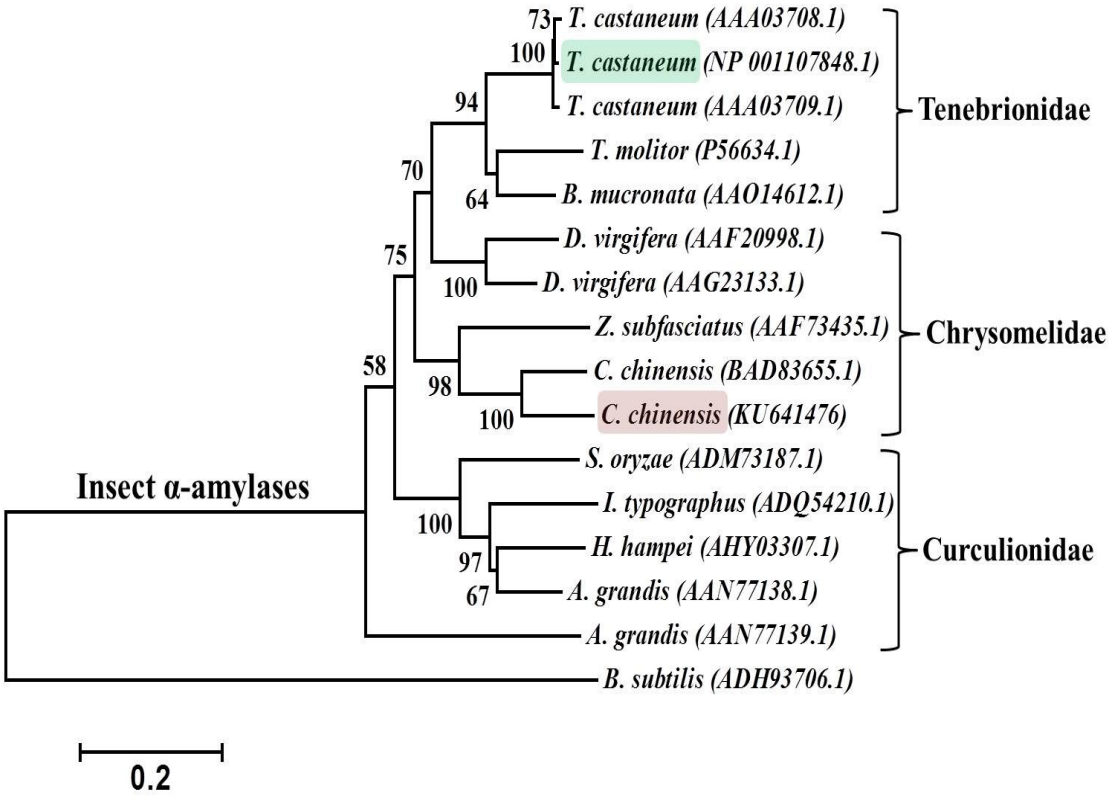


Figure 4.1 Phylogenetic tree of *Coleopteran* α-amylases. A cladogram was constructed using Neighbor-joining method using MEGA6 software with 1000 bootstrap replicates. Bacterial α-amylase *Bacillus subtilis* was used as an out-group. Accession numbers are given in parentheses. For *Tenebrio molitor* α-amylase, the accession number (P56634.1) belongs to UniProt database. All the coleopteran α-amylases belong to subfamily GH13_15 α-amylase whereas *Bacillus subtilis* α-amylase belongs to subfamily GH13_28. The two α-amylases used for the study are highlighted in pink and green color.

4.3.3 Amylolytic products and molecular weight determination of recombinant CcAmy and TcAmy

SDS-PAGE and relative mobility analysis revealed molecular weight of purified recombinant CcAmy to be 55 KDa consistent with the theoretical value and that of TcAmy to be 60 KDa, close to the theoretical molecular weight of 57 KDa (Figure 4.4A). Both recombinant proteins were enzymatically active (Figure 4.4B). Starch hydrolysis products generated as a result of amylolytic reactions catalyzed by i) recombinant

CcAmy were glucose, maltose, malto-triose and ii) recombinant TcAmy included maltose, malto-triose and malto-tetrose (Figure 4.4C and D).

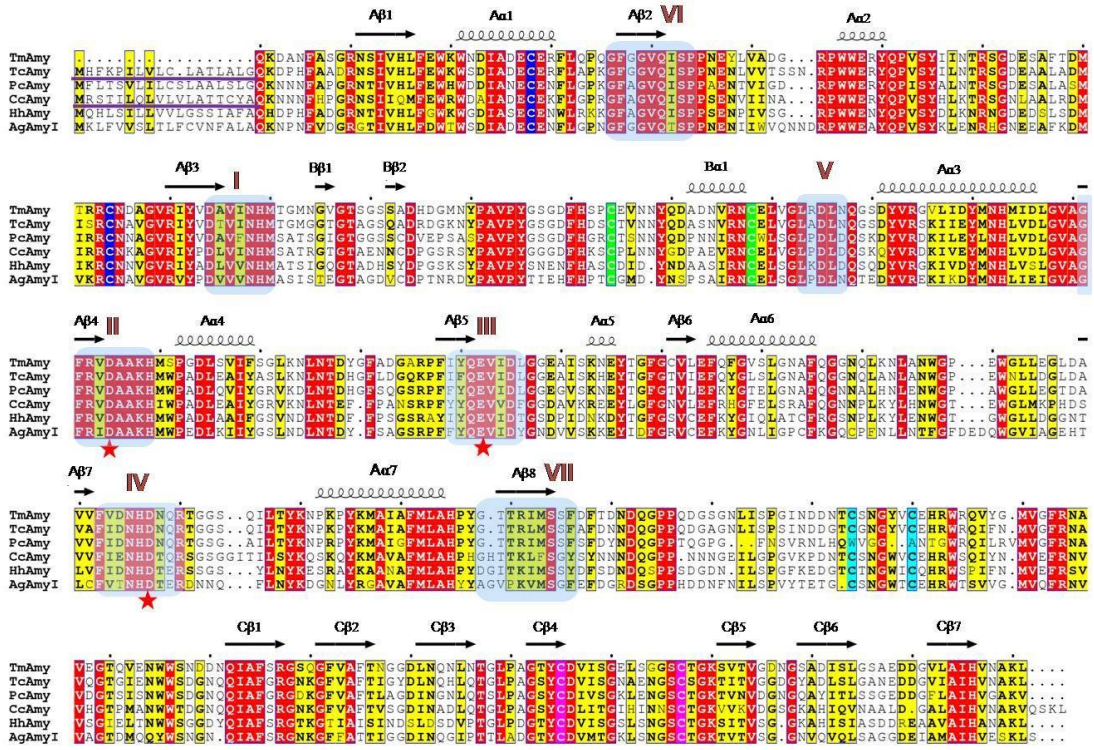


Figure 4.2 Molecular structure prediction of *Coleopteran* α-amylases using structure based sequence alignment. Multiple sequence alignment of Coleopteran α-amylases was performed using ClustalW. The secondary structure elements *above* the sequence blocks correspond to the different domains of α-amylase based on reported crystal structure of TmAmy. Conserved residues are *boxed in red*. Similar residues are in *black bold* and *boxed in yellow*. Cysteine-pairings for disulphide bridges are boxed in *blue, green, cyan* and *pink* color. Signal sequence is indicated with purple line. The seven conserved regions of GH13 a-amylase family are indicated with blue box bearing number on the top and active sites are marked with red asterisk. The sequences are as follows: TmAmy: *Tenebrio molitor α*-amylase, PDB accession number: 1TMQ_A, TcAmy: *Tribolium castaneum α*-amylase, NCBI reference number: NP_001107848.1; AgAmy1: *Anthonomus grandis α*-amylase, AAN77139.1; HhAmy: *Hypothenemus hampei α*-amylase, AHY03307.1;PcAmy: Phaedon *cochleariae α*-amylase, CAA76926.1; CcAmy: *Callosobruchus chinensis* α-amylase, *KU641476*. The figure was created using ESPript 3.

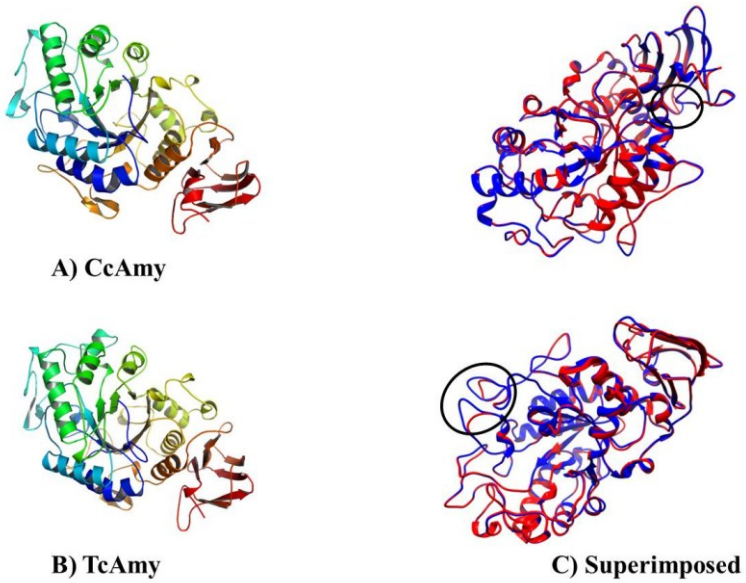


Figure 4.3 Homology modeling of CcAmy and TcAmy. A) and B) Cartoon diagram of modeled CcAmy and TcAmy, which are color-ramped from N (Blue) to C (Red) terminus. C) Superposition of C-alpha of modeled CcAmy (red) and TcAmy (blue), view of 180° rotation. Deviation in loops is indicated by circle.

4.3.4 Kinetic parameters for CcAmy and TcAmy

Kinetic parameters KM, Kcat and Kcat/KM for CcAmy and TcAmy were calculated using starch and amylopectin as substrates. CcAmy showed more substrate affinity towards starch compared to the amylopectin. However, turnover number (Kcat) and catalytic efficiency (Kcat/KM) for CcAmy catalyzed amylolytic reaction using starch and amylopectin as substrates remained equivalent. TcAmy showed higher substrate affinity towards amylopectin as compared to the starch. Similarly in the TcAmy catalyzed reaction, turnover number for amylopectin was higher than that of the starch. Further when compared with CcAmy, TcAmy showed higher substrate affinity and higher catalytic efficiency towards amylopectin as a substrate. On the contrary, CcAmy showed higher substrate affinity towards starch as compared to TcAmy, but catalytic efficiency was similar for both the enzymes (Table 4.2).

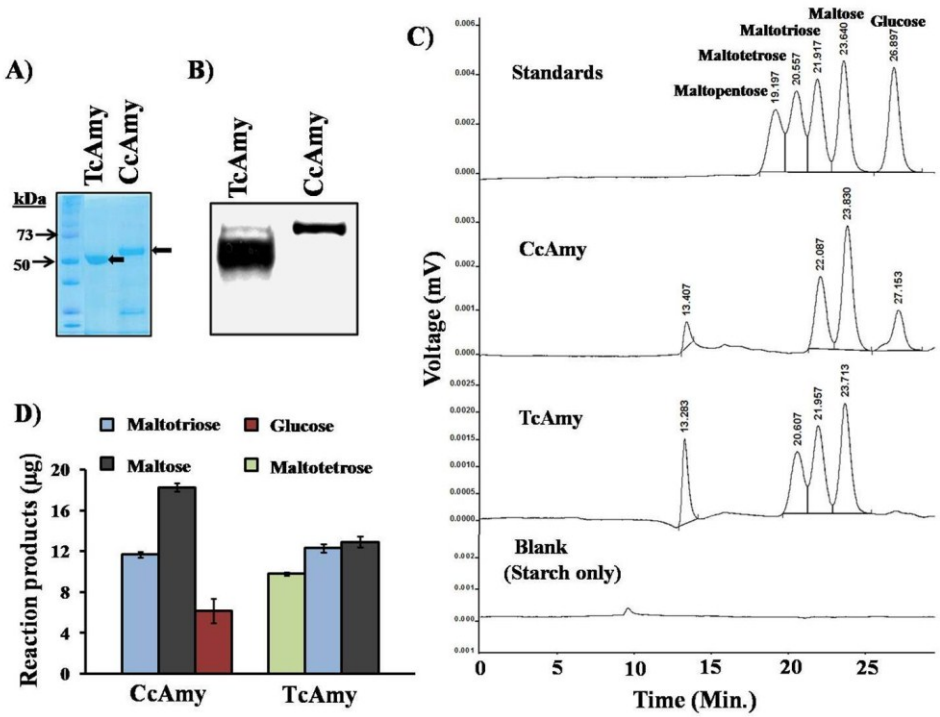


Figure 4.4 SDS–PAGE, in-gel activity and reaction product analysis of CcAmy and TcAmy. CcAmy and TcAmy were cloned in *E. coli* (BL21) and expressed protein was purified using Ni–NTA affinity column. Purified protein was loaded in each well. **A)** Purified protein was loaded on 12% SDS–PAGE and stained with Coomassie Brilliant Blue R-250 stain. Lane 1, Pre-stained protein molecular weight marker; Lane 2, TcAmy; Lane 3, CcAmy. **B)** In-gel α-amylase activity; purified TcAmy and CcAmy were loaded on 8% native PAGE and α-amylase activity was performed using soluble starch as a substrate. Gel was stained by iodine solution and image was inverted for better visualization. **C)** and **D)** HPLC analysis of the products of the reactions of the CcAmy and TcAmy using starch as substrate. The products were identified using standards as described in methodology.

Table 4.2 Enzyme kinetics of CcAmy and TcAmy. Substrates viz starch and amylopectin (concentration range 0.1 to 3 mg/mL) were used for α-amylase catalyzed reaction to compare the kinetic parameters. Kinetic parameters viz K_M, K_{cat} and K_{cat}/K_M for CcAmy and TcAmy were calculated using GraphPad Prizm v.6 software.

Enzyme / substrate	K_M (g/L)×10^{-3}	K_{cat} (s^{-1})	K_{cat}/K_M[(g/L)$^{-1}$ s^{-1}]×10^3
CcAmy			
Starch	3.20 ± 0.41	0.92 ± 0.11	0.29 ± 0.04
Amylopectin	4.11 ± 0.01	1.10 ± 0.008	0.26 ± 0.0002
TcAmy			
Starch	4.20 ± 0.43	1.13 ± 0.08	0.27 ± 0.009
Amylopectin	2.31 ± 0.0007	1.40 ± 0.007	0.59 ± 0.003

4.3.5 Biochemical properties of CcAmy and TcAmy

Coleopteran α-amylases are reported to possess optimal pH activity between slightly acidic to near neutral pH (Vatanparast and Hosseininaveh, 2010; Darvishzadeh et al., 2012). CcAmy and TcAmy showed optimum α-amylase activity at pH 5.0 (Figure 4.5A), which was in agreement with the reported optimum pH values for crude α-amylases from *C. chinensis* and *T. castaneum* (Applebaum and Konijn, 1964; Podoler and Applebaum, 1971). Stability of both the α-amylases was tested in a wide range of pH (3.0 to 11.0) and assay was performed at optimum pH (Figure 4.5B). It was observed that CcAmy and TcAmy displayed increased enzyme stability towards alkaline pH.

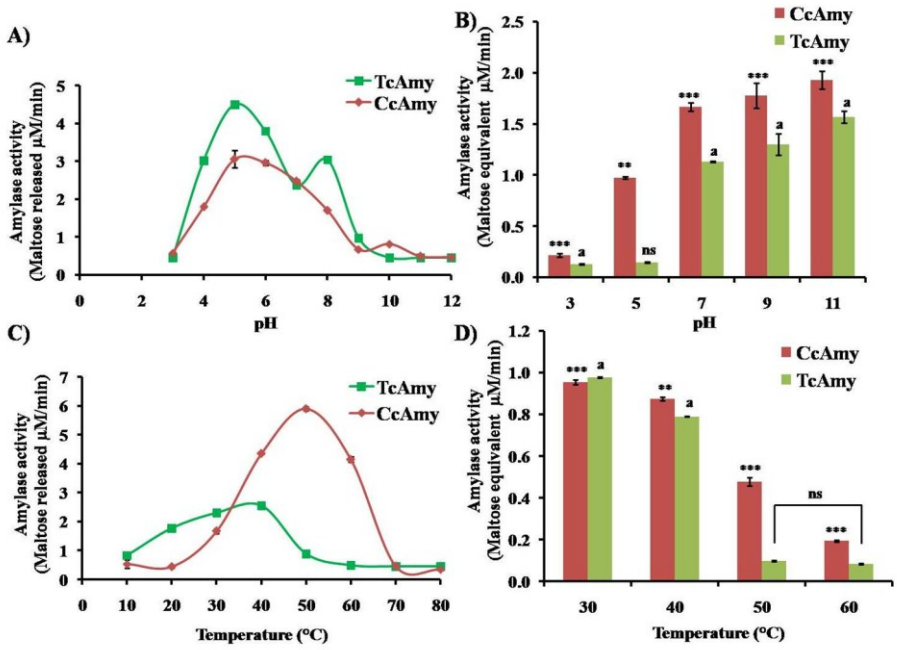


Figure 4.5 Activity and stability of CcAmy and TcAmy under different pH and thermal conditions. A) Alpha-amylase activity of CcAmy and TcAmy at various pH. **B)** Stability of CcAmy and TcAmy was checked by incubating enzyme in the buffer of respective pH overnight and activity was carried out under standard assay conditions. Data was represented as α-amylase activity relative to the activity at pH 5.0. In statistical analysis, One-way ANOVA was performed followed by Tukey's post hoc test. Statistical data is significant at p-value (a) or *** <0.001, (b) or** <0.01 and (c) or ns as non-significant. **C)** Alpha-amylase activity was carried out at various temperatures from 10 to 80°C for CcAmy and TcAmy. **D)** Stability of CcAmy and TcAmy was checked by incubating enzyme at 30°C, 40°C, 50°C and 60°C for 30 min. Activity was carried out using standard assay method. Data was represented as α-amylase activity relative to the activity of non-treated samples. In statistical analysis, One-way ANOVA was performed followed by Tukey's post hoc test. Statistical data is significant at p-value (a) or *** < 0.001, (b) or **< 0.05 and (c) or ns for non-significant.

Effect of various temperatures ranging from 30 to 60°C was studied on amylolytic activity of recombinant CcAmy and TcAmy. Enzymes were incubated at respective temperature and activity was determined at 37°C (pH 5.0). Optimum activity of CcAmy and TcAmy was observed at 50°C and 40°C, respectively (Figure 4.5C). Stability of CcAmy and TcAmy at various temperatures viz. 30°C, 40°C, 50°C and 60°C was evaluated and represented as relative α-amylase activity compared to the equivalent non-incubated standard (Figure 4.5D). CcAmy was stable at 40°C and activity was gradually reduced to 20% at 60°C. Similarly, TcAmy retained 78% amylolytic activity at 40°C which gradually reduced to 10% at 60°C. Though, CcAmy and TcAmy had optimum amylolytic activity between 40 to 50°C, they tend to lose their activity significantly after incubation above 40°C which indicated their sensitivity towards higher temperatures; probably because of rapid denaturation as a function of increased temperatures.

4.3.6 Interactions of proteinaceous and non-proteinaceous inhibitors with CcAmy and TcAmy

Interactions of α-AIs viz. acarbose (non-proteinaceous) and wheat α-AI (proteinaceous) with CcAmy and TcAmy were analyzed using starch as a substrate (Figure 4.6A and B). Amylolytic activities of CcAmy and TcAmy were inhibited from 20 to 100% by acarbose at the concentration of 2 to 40 nM and 2 to 193 nM, respectively, whereas wheat α-AI showed 10 to 100% inhibition at the concentration of 0.1 to 21 nM for both the enzymes. Similar pattern of inhibition was observed for inhibitory reactions of wheat α-AI against CcAmy and TcAmy. However, there was a remarkable difference between inhibitory concentration of acarbose required to completely inhibit the amylolytic activity of CcAmy and TcAmy. Variable pattern of inhibition was observed for inhibitory reactions of wheat α-AI and acarbose against crude extract of CcAmy and TcAmy. Activity of crude CcAmy (adult and larvae) was inhibited up to 80% with 77 nM of acarbose and up to 100% with that of 28 nM of wheat α-AI. In case of crude TcAmy (adult and larvae), 95% of enzyme activity was inhibited using 193 nM of acarbose while 28 nM of wheat α-AI showed 90% inhibition.

Further, to understand the underlying differential inhibitory mechanism at molecular level, docking of CcAmy and TcAmy was performed with acarbose. The ligand interactions were compared based on docking score, which highlights the difference in spatial binding of acarbose in the active sites of CcAmy and TcAmy (Figure 4.7A and B, Table 3). Valienamine unit of acarbose interacted with active site residues of CcAmy viz. Asp185 and Glu221 and binding site residue viz. His189 while α-D glucose and D-glucose moiety interacted with Asp334 residue. In case of TcAmy, acarviosine moiety of acarbose interacted with active site residues viz. Glu224 and Asp289 while α-D glucose and D-glucose moiety interacted with Tyr141 and His191 residues (Figure 4.7, Figure 4.8A and B).

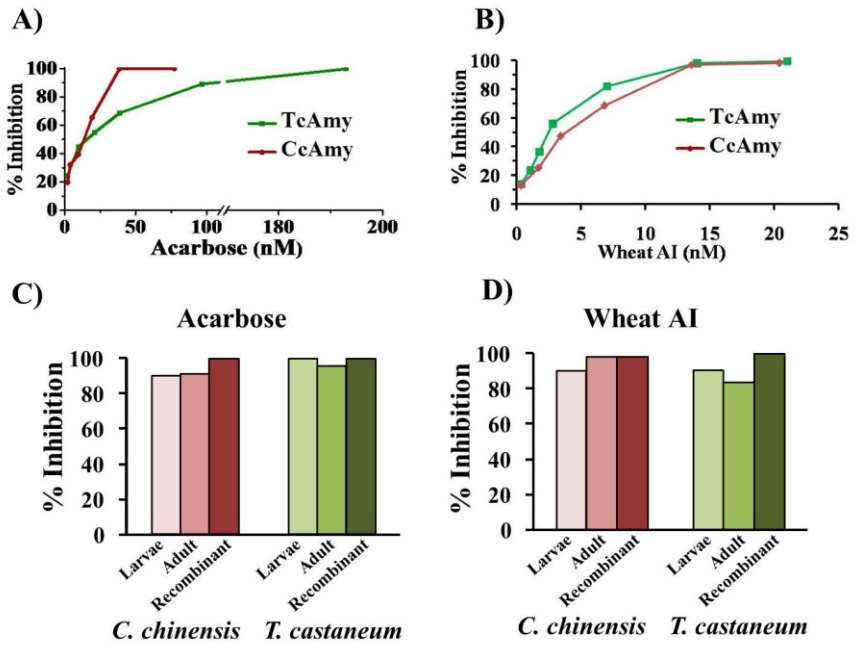


Figure 4.6 Inhibitory activity of acarbose and wheat α-amylase inhibitor (AI) against recombinant and crude CcAmy, TcAmy. A) 2.0 to 193 nM acarbose, a non-proteinaceous α-amylase inhibitor and **B)** 0.1 to 21 nM wheat AI, a proteinaceous α-amylase inhibitor was tested for inhibitory potential against recombinant CcAmy and TcAmy. Inhibitory activity was represented as percent inhibition. **C)** 193 nM acarbose and **D)** 28 nM wheat AI, was tested for inhibitory potential against crude CcAmy and TcAmy. Inhibitory activity was represented as percent inhibition.

Table 4.3 Glide docking showing Standard precision binding scores.

S.No.	Title	Docking score	Glide score	Glide emodel
1	TcAmy_Top Score	-6.95	-6.965	-77.803
2	CcAmy_top Score	-6.368	-6.383	-78.725

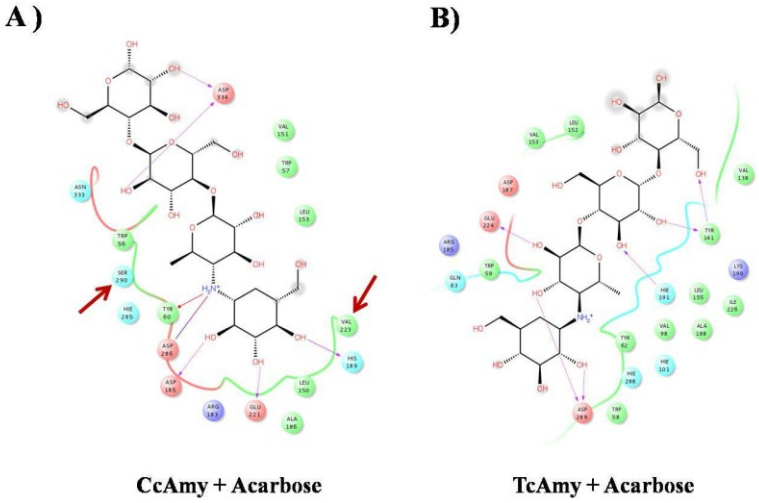


Figure 4.7 Differential mode of interaction of acarbose with CcAmy and TcAmy. 2D-view of ligand-protein interactions. Hydrogen-bonded interactions are shown using purple dotted arrows with the base of the arrow representing the donor and the head representing the acceptor. Cation-π interaction has been shown using a solid red line ending in a filled red circle pointing towards aromatic residue and NH_2^+ represents cation donor. Panel **A)** shows interaction of acarbose with CcAmy. The residues indicated by red solid arrows are not conserved in CcAmy and TcAmy. Hydrogen bond triplet interactions with valienamine unit of acarbose are represented by dotted purple arrows. Panel **B)** shows interaction of acarbose with TcAmy.

Protein sequence alignment between CcAmy and TcAmy revealed that all the catalytic and binding pocket residues were spatially conserved. Except all other identical residues, Ser290 and Val223 in CcAmy were replaced by Thr293 and Ile226 in TcAmy, respectively (Figure 4.9 and Figure 4.10). A spatial replacement of Ser290 toThr293 in TcAmy resulted into introduction of turn in the place of loop as observed in CcAmy (Figure 4.7, Figure 4.8C and D). Moreover, Ser290 in CcAmy formed hydrogen bond with Asp286 while Thr293 in TcAmy formed hydrogen bonds with Asp289 as well as Gly295 (Figure 4.7, Figure 4.8E and F). This dual bonding made binding site more rigid, which hindered the orientation of acarbose and restricted its complete access to TcAmy active site (Figure 4.11).

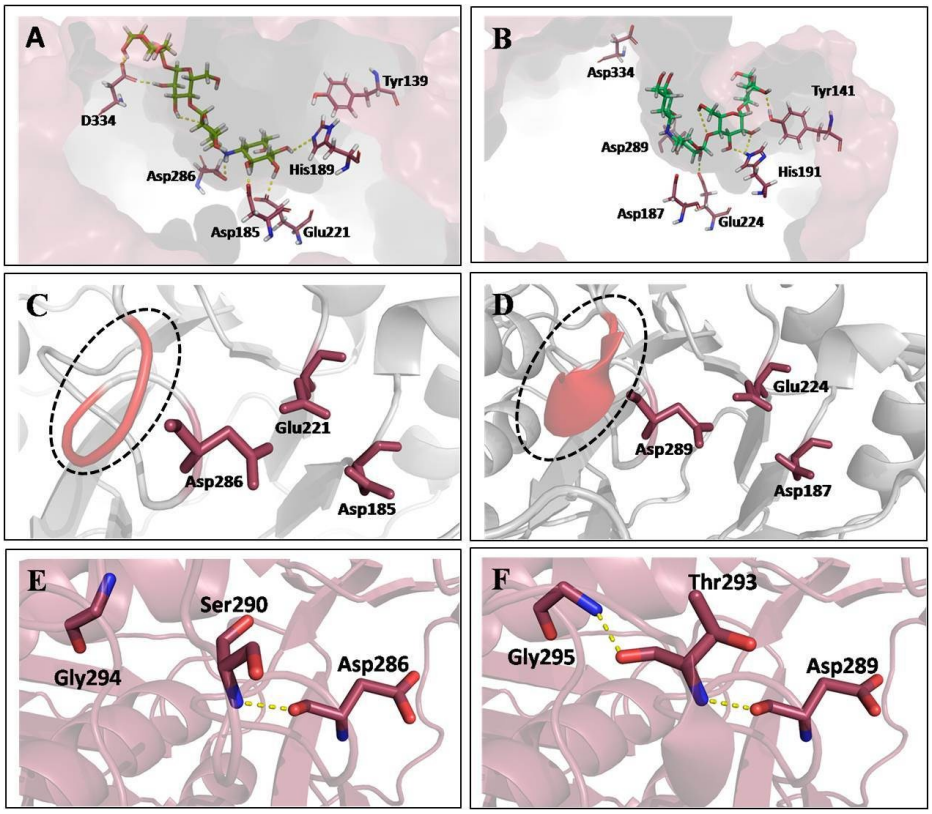


Figure 4.8 Interaction of acarbose with CcAmy and TcAmy. A) and **B)** Surface diagram of CcAmy and TcAmy showing binding of acarbose. Conserved residues in and around active site are shown in stick form. Side view reflect different mode of binding of acarbose with CcAmy and TcAmy. In CcAmy (panel A), active site is accessible to acarbose whereas in TcAmy (panel B) acarbose interacts superficially (refer text). Yellow dotted line shows inter-molecular and intra-molecular hydrogen bonding. **C)** and**D)** Cartoon diagram of CcAmy and TcAmy showing conserved active site residues in stick form. Loop present in CcAmy near active site is replaced by turn in TcAmy (encircled and highlighted in red). **E)** and **F)** Cartoon diagram highlighting the difference between hydrogen bonding of serine and threonine in CcAmy and TcAmy, respectively. Images were prepared in PyMOL, Version 1.7.4.

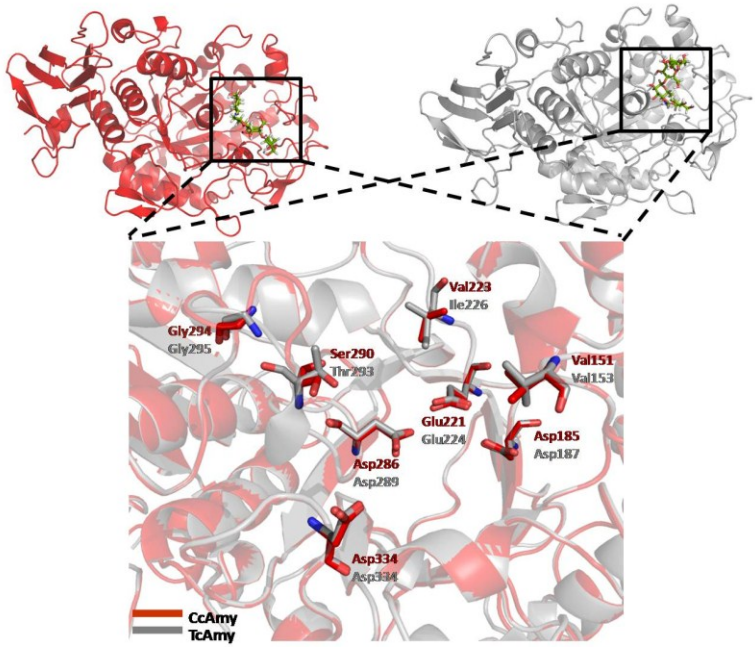


Figure 4.9 Superimposed structures of CcAmy and TcAmy. Stereo-image representing the cartoon diagram of superimposed structure of CcAmy and TcAmy. The position of crucial amino acids is conserved structurally and is shown in stick form. Ser290 and Val223 in CcAmy are replaced by Thr293 and Ile226 in TcAmy. CcAmy and TcAmy are colored red and gray, respectively.

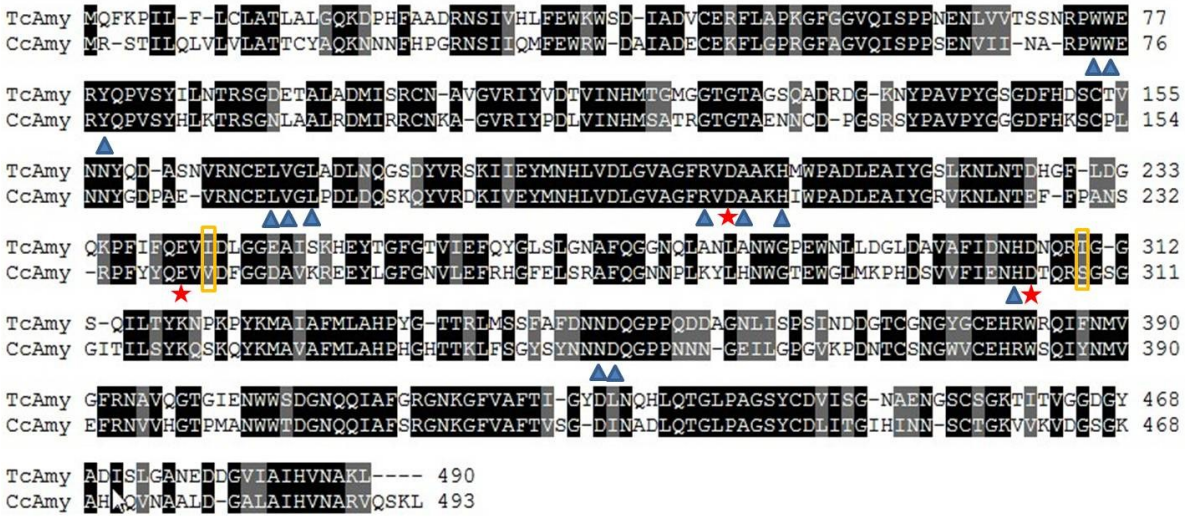


Figure 4.10 *Callosobruchus chinensis* and *Tribolium castaneum* α-amylase sequence alignments. Two amino acid sequences, CcAmy; and TcAmy; Accession number: NP_001107848.1 from coding regions of *C. chinensis* and *T. castaneum* were aligned

using Clustal W and sequences were highlighted using online Sequence Manipulation Suite tool (http://www.bioinformatics.org/sms2/). Residues identical to consensus sequences are shaded. Active sites predicted using pfam analyses are indicated by star whereas residues involved in binding pocket of ligand are marked with triangle. Variable ligand binding residues in binding pocket are boxed in orange.

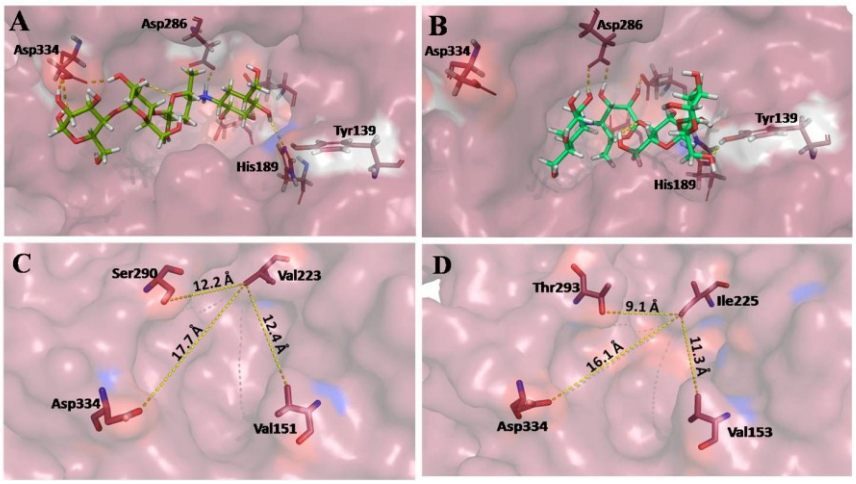


Figure 4.11 Birds eye view of placement of acarbose in active site groove of TcAmy and CcAmy. A) and **B)** Surface View of difference observed between CcAmy and TcAmy, respectively. Residues in and around active site and acarbose are shown in stick form. The active site residues are situated deep inside the groove which is not clearly visible. **Distance mapping of residues near active site of CcAmy and TcAmy** Stereo-image representing surface diagram showing distance between gateway residues. Val223 in CcAmy **C)** is replaced by Ile225 in TcAmy **D)** The interacting residues are shown in stick form. The distance between the residues were represented by dotted yellow line while numbers represent distance in Angstrom unit.

4.4 Discussion

T. castaneum and *C. chinensis* are recognized worldwide as economically important pests which feed on stored grains and are responsible for considerable economic loss. Both of these insects show strong food preference as *T. castaneum* particularly feeds on cereal grains while *C. chinensis* survives on legumes. Thus, unique dietary food preferences led us to investigate the comparative response of insect gut α-amylases with respect to biochemical properties as well as inhibitory potential of proteinaceous and non-proteinaceous inhibitors towards the activity of these enzymes.

Using full-length amino acid sequences of CcAmy and TcAmy with known coleopteran insect α-amylases, NJ tree revealed the phylogenetic relationship of both the α-amylases. Although α-amylases belonging to individual coleopteran families clustered together and were more closely related to each other, it was hard to define a strict evolutionary difference among α-amylases based on the individual enzyme specificities from all the clusters (Janecek, 1995). Our findings were in well accordance with the recently reported phylogenetic analysis of coleopteran insect α-amylases (Bezzera et al., 2014). In order to know the similar or differential biochemical properties, enzymatic characterization viz. enzyme kinetics, pH and temperature optima, enzyme stability under different pH and thermal conditions were performed using recombinant TcAmy and CcAmy. Optimal thermal condition for TcAmy and CcAmy was similar with respect to α-amylases from *Periplanata americana* (order, Blattodea) and *Tecia solanivora* (order, Lepidoptera) whereas comparatively higher thermal optima was reported for *Morimus funerenus* α-amylase (order, Coleoptera) (Agrawal, 1981; Dojnov et al., 2008; Valencia-Jimenez et al., 2008). Further, CcAmy had higher thermal stability than TcAmy. This stability can be explained by considering proline rule proposed by Suzuki and co-workers for the thermo-stability of the proteins. Increased number of proline residues corresponds to increased thermo-stability of the protein which matched well considering higher thermo-stabilities of CcAmy with 25 proline residues compared to TcAmy (19 proline residues) (Suzuki et al., 1987; Suzuki, 1989; Watanabe et al., 1996). Optimal pH condition for TcAmy and CcAmy (pH 5.0) was similar to other reported coleopteran α-amylases from *Hypothenemus hampei* (pH 4.5-5.2), *C. chinensis* (pH 5.2-5.4), *Morimus funerenus* (pH 5.2), *Sitophilus oryzae* (pH 5.0) and *Diabrotica vergifera* (pH 5.7) (Podolar and Applebaum, 1971; Valencia et al., 2000; Titarenko and Chrispeels, 2000; Dojnov et al., 2008; Celinska et al., 2015). According to Svensson and co-workers,

different functional and stability properties of proteins from α-amylase family including stability at various pH conditions were attributed to the domain B which is present as a loop between β3 strand and α3 helix (Rodenburg et al., 1994; Juge et al., 1995). No significant difference was observed in domain B sequences of TcAmy and CcAmy. Both the recombinant enzymes were stable at alkaline pH while the midgut pH of the coleopteran insects was reported to possess acidic environment (Vinokurov et al., 2006; Vatanparast and Hosseininaveh, 2010; Darvishzadeh et al., 2012). However, functional significance of this difference between midgut pH and the pH at which both the enzymes were most stable (i.e alkaline) could not be ascribed. Reaction products were different considering the catalytic reaction by TcAmy and CcAmy to degrade the soluble potato starch. Malto-tetrose was exclusively produced by TcAmy, when compared with the reaction products of *Helicoverpa armigera* (order Lepidoptera) recombinant α-amylases (HaAmy1 and HaAmy2). Maltose was produced as a common major product in both the reactions (Bhide et al., 2015). Experimental evidence suggested that TcAmy and CcAmy lacked the exo-alpha-glucosidic activity; thus, glucose might be formed as a byproduct in hydrolytic reaction by CcAmy. As in the case of TmAmy with respect to the other α-amylases, a similar difference in substrate affinity towards potato starch was observed for TcAmy and CcAmy (Buonocore et al., 1976).

Homology modeled structure of TcAmy and CcAmy displayed overall structural similarity with the existing crystal structures of α-amylases. TcAmy and CcAmy had three domains viz. A, B and C, where domain A found to be similar with TIM barrel except a protruding loop between β3 strand and α3 helix known as domain B and which is a characteristic domain of α-amylases (Banner et al., 1975; Klein and Schulz, 1991; Qian et al., 1993). Initially, α-amylases were known to possess four conserved regions (Toda et al., 1982: Freidberg et al., 1983; Rogers, 1985; Nakajima et al., 1986). Later, the presence of additional conserved sequences was suggested (MacGregor, 1988) which were also found in enzymes of α-amylase family (Svensson, 1988; MacGregor and Svensson, 1989; Jespersen et al., 1991; Jespersen et al., 1993). Further, three additional conserved sequence regions were identified (Janecek, 1992; Janecek, 1994a, b; Janecek, 1995). In the current study of TcAmy and CcAmy, all these seven conserved sites were present (Figure 4.2). Additionally, three His residues were known to be involved in substrate recognition and transition state stabilization near active site (Ishikawa et al., 1992; Sogaard et al., 1993; Robert et al., 2003). These His residues were conserved at the equivalent positions viz. His101/99, His191/189 and His288/285 in TcAmy and CcAmy,

respectively. Moreover, Lys209 residue preceding the very important His residue in Taka-amylase was essential for the substrate binding and hence, crucial for α-amylase activity. Equivalent Lys residue was identified at Lys190/188 position in TcAmy and CcAmy, respectively (Matsuura et al., 1984; Nagashima et al., 1994).

Few reports have highlighted the interactions of coleopteran α-amylases with the proteinaceous α-AIs from various sources although thorough explanation for the mechanism of action of inhibition is largely lacking (Feng et al., 1996; Yamada et al., 2003; Svensson et al., 2004; Farias et al., 2007; Gupta et al., 2014). Similar inhibition pattern was observed for TcAmy and CcAmy with proteinaceous wheat α-AI. On the contrary, acarbose, a non-proteinaceous inhibitor showed differential inhibition and hence, binding interactions of acarbose with TcAmy and CcAmy were explored in-detail to investigate the mode of differential inhibition. Molecular docking indicated that acarbose not only interacted with active site residues of α-amylases as similar to the interactions of inhibitory loop of proteinaceous AIs, but also with the substrate binding residues (Svensson et al., 2004). This interaction differed with respect to the TcAmy and CcAmy binding site residues owing to differential inhibition of α-amylase activity. Moreover, there was a variation in two amino acids near the active sites of TcAmy and CcAmy. Composition of these two residues in TcAmy hampered the entry to the active site which resulted into superficial binding of acarbose with increase in binding energy. This could be one of the potential reasons why TcAmy required more concentration of acarbose for the complete inhibition of enzyme activity. Further mutational studies might be useful to understand molecular events of this enzyme inhibition mechanism.

4.5 Summary of the Chapter:

In present study, we functionally characterized α-amylases from two coleopteran pests viz. *C. chinensis* and *T. castaneum*. Homology modeling of these two recombinant α-amylases, namely, CcAmy and TcAmy revealed the structural differences. Additionally, biochemical properties of CcAmy and TcAmy were determined using starch and amylopectin substrates. Differential biochemical interactions and inhibitory potential of proteinaceous and non-proteinaceous inhibitors against CcAmy and TcAmy were studied. An attempt was made to understand the structural basis of inhibition using molecular docking and dynamics studies. Present study might increase our current

understanding of starch degradation and assimilation in the gut region of coleopteran insect pests. Moreover, insights into underlying mechanism of differential inhibition between coleopteran α-amylases by non-proteinaceous α-amylase inhibitor, acarbose could potentially be useful in designing improved strategies for insect control.

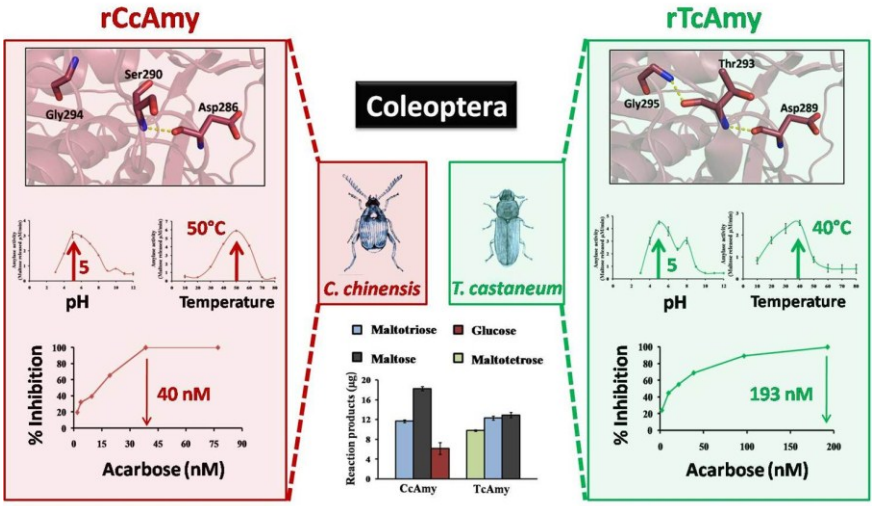


Figure 4.12 Schematics highlighting biochemical and biophysical properties of TcAmy and CcAmy.

Chapter-5

"Genomic and functional characterization of coleopteran insect specific α-amylase inhibitor gene from Amaranth species"

(Bhide et al., 2016, Plant Biotechnology Journal, Under review)

5.1 Introduction

A constant molecular battle exists between host plants and their insect pests. Plants synthesize various defensive proteins including inhibitors against insect digestive enzymes (Ambekar et al., 1996; Giri and Kachole, 1998) while insects overcome the digestive inhibition by producing multiple isoforms of these enzymes having differential sensitivity (Zavala et al., 2008). Insect digestive α-amylases play an important role in digestion and assimilation of starch and carbohydrates ingested by feeding on host plants (Franco et al., 2002). Plants on contrary produce various proteinaceous α-amylase inhibitors in response to insect feeding (Mishra et al., 2012). Wheat and barley's monomeric, dimeric and tertameric α-amylase inhibitors have been studied extensively and particularly against storage insect pests (Petrucci et al. 1976; Lazaro et al. 1988; Sanchez-Monge et al. 1989; Gomez et al. 1991). Similarly, α-amylase inhibitors from rice, castor seeds and water jasmine were also evaluated for effective inhibition of insect digestive enzymes (Yamasaki et al. 2006; Do Nascimento et al. 2011; Nguyen et al. 2014). α-Amylase inhibitory lectins were shown to affect normal growth and physiology of insect pests (Paes et al. 2000; Napoleao et al. 2012; Lagarda-Diaz et al. 2014). Various transgenic plants carrying heterologous α-amylase inhibitors were demonstrated to be effective in mitigating insect infestation (Pereira et al. 1999; Luthi et al. 2013).

Detailed analysis of interactions between plant α-amylase inhibitors and insect digestive α-amylases is essential to design potent and effective inhibitors (Giri et al., 2016). Molecular insights of underlying inhibitory mechanism of α-amylase inhibitors were studied by resolving three-dimensional structures of enzyme-inhibitor complexes. Basic interaction of α-amylase inhibitor with α-amylases involved molecular hydrogen bonding between amino acid residue of inhibitory loop and active sites of the enzyme (Chagolla-Lopez et al., 1994; Pereira et al., 1999). Precise hydrogen bonding of Arginine, Tyrosine and Tryptophan residues present on inhibitory loop of microbial α-amylase inhibitor, Tendamistat and active site residues of porcine pancreatic α-amylase was reported (Wiegand and Huber, 1995). Distinct conformational changes observed in inhibitory loop of *Phaseolous vulgaris* α-amylase inhibitor upon binding to insect and mammalian α-amylases was suggested as an underlying mechanism for differential inhibitory activity shown by inhibitor (Nahoum et al., 1999).Similarly, a distinct mode of interaction between wheat α-AI and α-amylase isoforms from *Helicoverpa armigera* was

detected. Aggregation of one of the isoforms with wheat α-AI was predicted to be responsible for its respective higher inhibition (Bhide et al. 2015).

Much of the knowledge of inhibitory mechanism has come from the analysis of effects of various mutations in inhibitory domain(s) of a particular inhibitor on its inhibitory activity. For example, peptide variants of proteinaceous α-AI from *Tephrosia villosa* (TvD1) were generated through *in vitro* mutagenesis and the effect of mutations on its overall inhibitory activity was studied (Liu et al., 2006; Lin et al., 2007; Vijayan et al., 2012). A mechanism of steric hindrance was observed for bi-functional α-amylase inhibitor from barley (BASI) which inhibits endogenous α-amylases by binding to active site of the enzyme. In this case an unusual completely solvated calcium ion was located at the protein-protein interface (Vallee et al. 1998). Different types of α-amylase inhibitors were also reported to possess proteolyic and chitinolytic activity along with amylolytic activity (Strobl et al. 1998a; Dayler et al., 2005).

α-Amylase inhibitors were reported to have applications in medicine and fermentation industries. For example, barley α-amylase inhibitor was found to be industrially important as it was involved in improving beer foam stability during processing (Limure et al., 2015). Moreover, α-AI also has positive effects on cancer progression. One of the reports indicated that micro-RNA mediated β-catenin signalling was targeted by Barley bi-functional α-amylase inhibitor which eventually impeded glioblastoma progression (Shi et al., 2014). Information regarding induction and regulation of expression of various α-amylase inhibitors under different stress conditions is lacking in the literature. Though, a report suggests synthesis of α-amylase inhibitor could be induced by abscisic acid and dehydration stress (Robertson et al., 1989). In present study, α-amylase inhibitor gene from *Amaranthus hypochondriacus* was thoroughly characterized for its molecular and biochemical function. Interaction of AI with various amylases showed its moderate and no activity against bacterial, fungal, mammalian and lepidoteran α-amylases while strong inhibitory activity against coleopteran insect amylases. Inhibitor's potential for *in vivo* reduction of growth and development of stored grain pest *Callosobruchus chinensis* was also investigated.

5.2 Materials and Methods

5.2.1 Sequence and phylogenetic analysis of α-amylase inhibitors

Full-length amino acid sequences of all the α-amylase inhibitors were extracted from NCBI database. Multiple sequence alignment of these sequences was carried out using Clustal Omega and the corresponding alignments were used to reconstruct phylogenetic tree. The Neighbor-joining (NJ) tree was generated by MEGA6 (Tamura et al., 2013) using 1000 bootstrap replicates, wherein fungal (*Streptomyces avermitilis*) α-amylase inhibitor (GenBank accession No. ACU45382.1) was used as an out-group. Multiple sequence alignment of AhAI and other α-amylase inhibitors was performed using Clustal Omega and secondary structural features were evaluated and highlighted using online software EsPript3 (Robert and Gouet, 2014).

5.2.2 RNA isolation, cDNA synthesis and real time PCR

Total RNA was extracted from young and mature leaves, young and mature inflorescence and seeds of *Amaranthus hypochondriacus, Amaranthus paniculatus, Celosia argentea and Achyranthes aspera* using Trizol reagent (Invitrogen, CA, USA). Crude RNA samples were treated with RQ1 DNase (Promega, USA). Two microgram of the DNA-free RNA samples were reverse-transcribed using oligo dT primers and reverse transcriptase (Promega, USA) as per the manufacturer's recommendations. Real Time PCR primer pairs were designed in non-homologous regions of α-amylase inhibitor (Table 5.1). cDNA was diluted before use in a PCR reaction and quantitative Real Time PCR reactions were performed using AB 7900 Fast Start Real Time PCR System (Applied Biosystems, USA; cycler conditions: 95 °C for 10min; 40 cycles of 3 s at 95 °C and 30 s at 55 °C with an additional dissociation stage of 15 s each at 95 °C, 55 °C and 95 °C) and SYBR Green PCR master mix (RocheApplied Science, Germany). Each plate was run with a standard curve and no template control. Relative quantification was carried out using the standard curve method with Elongation Factor 1α (EF1α) as a reference gene (Table 5.1). Amplification efficiency of each gene was assessed by plotting a standard curve using five serial dilutions of cDNA from a template pool and similar efficiencies were calculated by LinRegPCR software (http://www.hartfaalcentrum.nl/index.php) used for comparisons. The target gene expression levels of samples were then normalized using EF1α.

5.2.3 Sequence analysis of 3'UTR region

RNA was isolated and cDNA was prepared from young leaves of *Amaranthus hypocondriacus*. Degenerate primers were designed and amplified PCR products (~100bp) were cloned into pGEM-T vector (Promega, USA) and sequenced. Five prime and three prime sequence ends were amplified as per manufacturer's instructions (Clontech, USA) and resulted products were sequenced to obtain full length nucleotide sequence of the inhibitor. Sequenced 3'UTR regions of various full-length AhAI transcripts from *A. hypochondriacus* and its related species vis. *A. paniculatus*, *A. aspera*, *C. argentea* were aligned using clustal Omega.

5.2.4 Cloning, expression and purification of AhAI

Open reading frame of AhAI (GenBank accession: KU641477) without the native signal peptide of 26 amino acids was amplified using designed primer pairs (Table 5.1) and cloned in pET 28-a expression vector (Novagen, Madison, WI, USA) in frame with N-terminus 6X His-tag. Recombinant plasmids were amplified and transformed into *Escherichia coli* BL21 Star (DE3) cells. BL21 cells transformed with AhAI constructs were induced with 0.54 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and incubated at 37°C for 5 h. AhAI protein was purified from inclusion bodies using on-column refolding method (Oganesyan et al., 2005). In brief, cell lysis was performed in lysis buffer (50mM HEPES, pH 7.0, 100 mM NaCl) by sonication (5 min with 45% amplitude, alternating 8 sec on; 12 sec off cycles) on ice followed by centrifugation at 8000 rpm for 20 min. The pellet was washed in same buffer containing 1M Urea, 2% Triton X-100. Further, pellet was solubilized in denaturing buffer (20 mM Tris pH 8.5, 8 M Urea, 100 mM NaCl, 1 mM β-mercaptoethanol) by gently vortexing at room temperature. The solubilized protein from inclusion bodies was bound to Ni-NTA pre-equilibrated resin at room temperature overnight. On-column refolding was achieved with different buffers (10 column volumes for each buffer) under gravity. All the working solutions were prepared in Buffer-A (20 mM Tris, pH 8.5, 100 mM NaCl). First, column was washed with denaturing buffer containing 10 mM imidazole followed by buffer A containing 0.1% Triton X-100 and 500 mM NaCl. The column was further washed with buffer A containing 5 mM cyclodextrin followed by another wash with buffer A. The protein was eluted in buffer A containing 500 mM imidazole and 10% glycerol.

Eluted fractions of AhAI were pooled and subjected to buffer exchange (20 mM Tris, pH 8.5 respectively) by passing through 3 kDa cut off column (Millipore, Billerica, MA, USA). Protein was quantified by Bradford assay (Bio-Rad, USA) using BSA as a standard. Purified proteins were loaded on 12% SDS–PAGE to check the purity of samples (Figure 5a).

5.2.5 Localization of GFP fused with AhAI signal peptide

CDS of GFP was amplified using designed long primer containing complete nucleotide sequence of AhAI signal peptide (Table 5.1). Both Signal peptide with GFP(SP-GFP) and only GFP sequence were cloned in binary plant expression pRI101-AN vector (Takara Bio Inc., Japan). Constructs pRI101-AN:SP:GFP and pRI101-AN:GFP were electroporated into *Agrobacterium tumefaciens* strain GV3101 using a standard electroporation protocol. Empty pRI101-AN vector was used as a negative control. Cultures of *A. tumefaciens* GV3101 containing respective constructs were grown at 28°C in Luria-Bertani medium containing selection markers (25µg/ml Rifampicin and 50µg/ml Kanamycin) at 120 rpm for 24 h. Agrobacterium cells were harvested by centrifugation at 3500 rpm for 5 min and suspended in infiltration buffer (half strength MS medium, pH 5.6, Hi-Media, India and 200 µM acetosyringone). Agrobacterium cells were pelleted and re-suspended in infiltration buffer by adjusting an OD 1.0 at 600 nm. Cultures were incubated at 24°C for 3 h before infiltration. After incubation, agrobacterium cultures were infiltrated into the leaves of two weeks old *Nicotiana benthamiana* seedlings. Plants were maintained at 24°C in a growth chamber. Leaf sections were visualised for subcellular localization of GFP using confocal scanning microscope (Zeiss LSM 710, Germany) at 4 dpi after agro-infiltration.

Table 5.1 Primers used in quantification and expression of rAhAI .

Primer name	Accession No.	Primer sequence	Length (bp)
AI_Forward	P80403	5’TTGCGGACCTAAGATGGATGGAG3’	23
AI_Reverse	P80403	5’AGAGCAATTTCCGTAGTATCAGAAG3’	26
EF1 Forward	BI751166	5'TGGTGTCATCAAGCCTGGTATGGT3'	24
EF1 Reverse	BI751166	5'ACTCATGGTGCATCTCAACGGACT3'	24
AI_GSP1	P80403	5’TCACAACAAGGGACTCCATCCATC3’	24
AI_GSP2	P80403	5’AGAAGTGCAAGTGTATGGTTCAC3’	23
AIBamHI_F	P80403	5’AAAAAAGGATCCGTGCGCGATGACATTGCCATTGC3’	35

AISalI_R	P80403	5'AAAAAAGTCGACAGAGCAATTTCCGTAGTAT CAGAAG3'	37
AhSPGFP_F	P80403	5'AAAAAACATATGATGGATATGGCAAGGAGCA TTCTAGGTCTCATGGCAGCCTTGATGTTGGTAG CCACCATAGCTCCTCCAACCATGGCTCTGTCTA AAGGTGAAGAACTGTTC3'	114
AhSPGFP_R	-	5'AAAAAAGGATCCTTTGTAGAGCTCATCCATGC CG3'	34
GFP_F	-	5'AAAAAACATATGCTGTCTAAAGGTGAAGAAC TGTTC3'	36

5.2.6 Determination of accurate molecular weight and α-amylase inhibitory activity

AhAI (4.0 mg/ml) was mixed with sinapinic acid (Sigma-Aldrich, St Louis, MO, USA) (50% CAN, 0.1% TFA) in 1:5 ratio. About 0.5 µl of this mixture was spotted on the MALDI target plate by dry droplet method. The mass spectral analysis was done on AB SCIEX MALDI TOF/TOF^{TM}5800 system. Positive linear mode was used as a mode of operation. MS data were acquired at a laser repetition rate of 400Mz with 1000 laser shots per spectrum over each sample spot in the range of 5.0 -20 kDa for AhAI.

All the assays of α-amylase activity were performed by monitoring liberation of reducing sugars from starch using DNSA (Dinitrosalicylic acid) reagent. A premix of triplicate reactions was prepared in respective buffers and equal units of purified recombinant enzyme (determined by adjusting the absorbance at 0.5 at 540 nm) to which 150 µL of starch (0.25%) was added. After 15 min of incubation at 37°C, reaction was stopped by adding 500 µL of DNSA reagent and reaction tube was incubated in boiling water bath for 5 min and absorbance was measured at 540 nm. One α-amylase unit was defined as the amount of enzyme required to release 1 µM maltose/min at 37°C from substrate (starch) under the given assay conditions.

5.2.7 Insect rearing and feeding assay

C. chinensis culture was obtained from Entomology department, CSIR-NCL, Pune. Freshly emerged *C. chinensis* adults were transferred to uninfected mung bean seeds and culture was maintained by transferring concurrent generations on fresh seeds. The cultures were maintained under controlled conditions at 25°C, 65% relative humidity with 12h dark/ 12h light cycles in insect growth chamber.

The effect of recombinant α-AI on growth of *C. chinensis* was studied by treating mung seeds with α-AI at 0 (control), 10.0µM, 25.0µM concentration prepared in 20mM Tris pH 8.5, 10% glycerol and 100mM NaCl. For α-AI treatment, 50 mung seeds were soaked in above mentioned concentration of α-AI for 60 mins. The seeds were air-dried for 4 hours. five pairs of freshly emerged insects were allowed to lay eggs on the mung seeds, the insects were then separated from mung seeds after 72hrs. The eggs were counted after seven days of oviposition (Wisessing et al., 2010). The seeds were monitored and adults were counted after their emergence. Total weight of emerged adults was recorded and percent emergence of adults was calculated (Table 5.2).

Table 5.2: Effect of AhAI on growth and development of *Callosobruchus chinenesis.*

AhAI concentration	Number of eggs laid	Number of adults emerged	Total weight of adults (mg)	Average weight / insect (mg)	Adult emergence (%)
Set-1					
Control	143	23	118	5.1	16.0
10 µM	172	11	70	6.3	6.0
25 µM	162	11	66.7	6.0	6.7
Set-2					
Control	117	15	79.6	5.3	12.8
10 µM	139	9	55.4	6.1	6.4
25 µM	143	8	50.6	6.3	5.5

5.2.8 Circular Dichroism spectroscopy and homology modelling of AhAI

Circular Dichroism (CD) assays were carried out using Jasco J-815 spectropolarimeter (Jasco, Tokyo, Japan) under constant nitrogen flow equipped with a Peltier type temperature cuvette holder. Far-UV spectra were recorded using 0.1 cm path length quartz cuvette. Buffer conditions for purified AhAI (at final concentration of 50 µg/ml) were exchanged to 20mM Tris pH 8.5 to remove residual imidazole. Three consecutive measurements were accumulated to obtain mean spectra. The observed ellipticities were converted into molar ellipticities (symbol) based on molecular mass per

residue of AhAI. Percentage of secondary structures was calculated using CD-Pro software.

Homology modelling of 75aa AhAI was performed by submitting protein sequence to I-TASSER server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/). A model was selected out of five predicted models based on CD spectroscopic data (Figure 5.1). A separate motif analysis for 43 aa sequence was performed using Prosite server (http://prosite.expasy.org/).

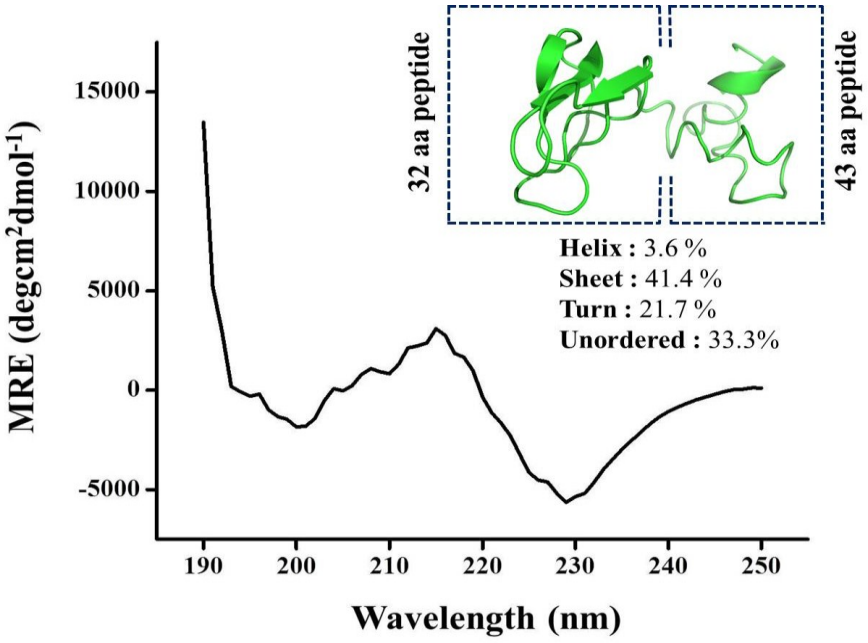


Figure 5.1 Homology modeling and circular dichroism spectroscopy of AhAI. Homology modeling was performed using I-TASSER server. Most suitable model was selected based on CD spectroscopic data. CD spectroscopy was performed at concentration of 50µg/ml AhAI in 20mM Tris (pH 8.5) buffer.

5.2.9 Docking studies of AhAI with CcAmy and TcAmy

The three dimensional structures of CcAmy and TcAmy were predicted using MODELLER ver.9.15 (Sali and Blundell, 1993; Webb and Sali, 2014). The crystal structure of *Tenebrio molitor* α-amylase (PDB ID: 1JAE, Strobl et al., 1998a), was used

as a template. The models were evaluated on the basis of lowest discrete optimised protein energy (DOPE) score. The structural assessment of the models was performed using RAMPAGE (Lovell et al., 2003). The visualization of the above models was performed using Pymol (https://www.pymol.org; DeLano Scientific LLC, USA).

Prediction of AhAI inhibitor binding with CcAmy and TcAmy was carried out using docking studies. Corresponding residues belonging to the inhibitor binding site, in the *TmAmy* structure (1TMQ) were correlated. The web based protein-protein docking program GRAMM-X with default parameters was used for docking of proteinaceous AhAI inhibitor with CcAmy and TcAmy (Tovchigrechko and Vakser, 2006).

5.3 Results

5.3.1 Gene structure and comparison of AhAI with other α-amylase inhibitors

A 32 amino acids long mature peptide showing α-amylase inhibitory activity was reported in the literature (Chagolla-Lopez et al., 1994; Lu et al., 1999). Peptide was purified from Amaranth seeds, amino acid sequence was deduced by Edman degradation and structure was determined by NMR. In present study, complete DNA sequence of AhAI was elucidated by amplifying cDNA ends. AhAI gene possesses 26 amino acids long signal peptide followed by 43 amino acids long region along with 32 amino acids reported mature peptide (Figure5.2a). Biochemical or molecular function could not be assign to internal fragment. Possible mechanism for *in vivo* cleavage of internal fragment from mature peptide also remains unclear. Phylogenetic analysis of AhAI with known α-amylase inhibitors from eudicots and monocots indicated a strict phylogenetic differentiation of AhAI from monocot (Figure5.2b). A similar α-amylase inhibitor showing 53% identity with AhAI was reported from *B. vulgaris* (GenBank accession No. XP_010672947.1). This α-amylase inhibitor showed unique secondary structural identity with AhAI (Figure5.2c). Structural alignment with other α-amylase inhibitors showed conservation of three cysteine residues and single di-sulfide bond across AhAI secondary structure (Figure5.2d). This conserved disulfide bond is crucial in maintaining characteristic disulphide topology of knottin-type α-amylase inhibitor.

5.3.2 AhAI orthologs from Amaranthaceae members exhibit identical open reading frame while variation in 3'UTR sequence

One domestic and two wild relatives of *A. hypochondriacus* viz. *Amaranthus paniculatus*, *Celosia argentea* and *Achyranthes aspera* respectively were found to contain identical nucleotide sequence of AhAI open reading frame. Interestingly genomic regions within this sequence of α-amylase inhibitor were namely indistinguishable. However, various AhAI transcript sequences (clones) from these four species showed variation in their 3'UTR region (Figure 5.3). Polyadenylation recognition site was identified in all of the transcripts. 3'UTR region was known to possess sites for binding of microRNAs which play an important role in regulation of translation of desired protein (Bartel, 2009). *In silico* analysis of AhAI 3'UTR region did not show any possible miRNAs binding sites and no evidence for alternate splicing.

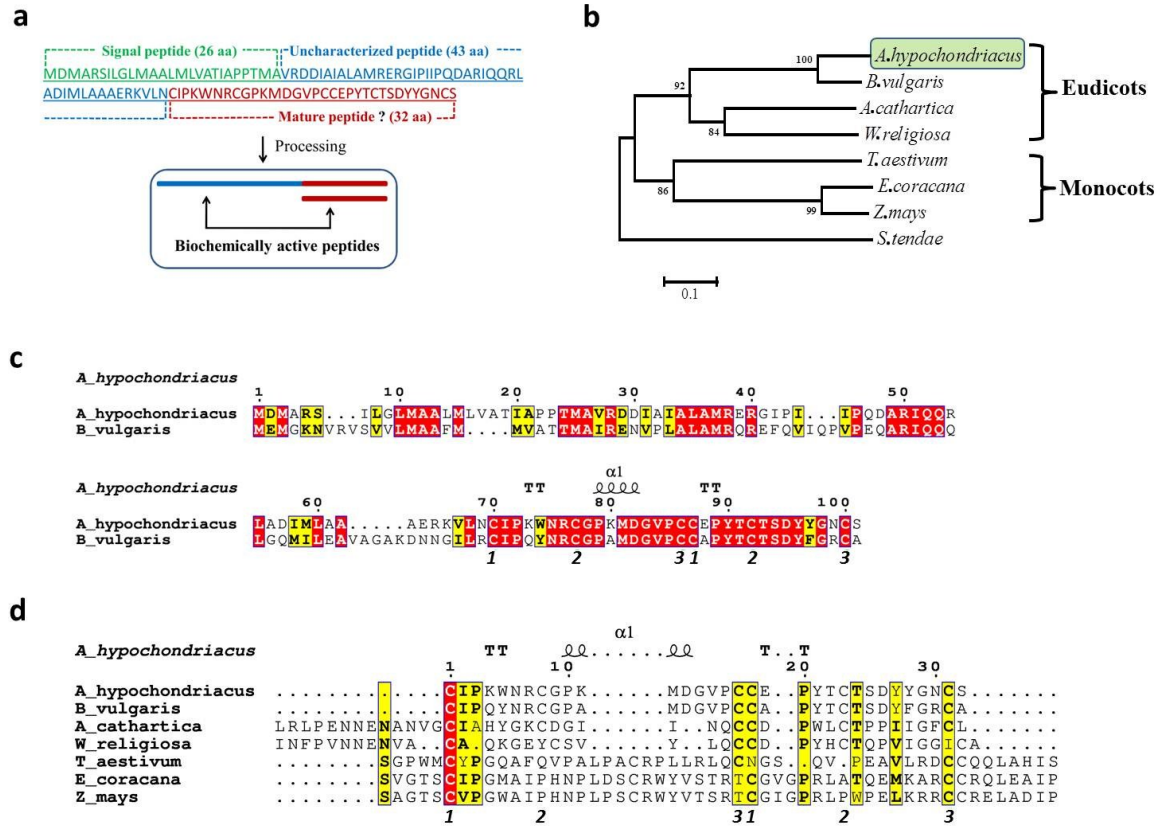


Figure 5.2 Comparison of AhAI protein sequence with known plant α-amylase inhibitors a. Schematic representation of AhAI transcript. **b.** Amino acid sequence

divergence of plant α-amylase inhibitors. Cladogram with protein sequences was constructed using MEGA 6 software and Neighbor-Joining method. Values at branch points indicate bootstrap values of 1000 replications. Fungal (*Streptomyces tendae*) α-amylase inhibitor (GenBank accession No. AAA26686.1) was used as an out-group. Scale represents genetic distance between the clades. **c.** Structure based sequence alignment of AhAI with putative *Beta vulgaris* α-amylase inhibitor (GenBank accession No. XP_010672947.1). Multiple sequence alignment was performed using Clustal Omega. The secondary structure elements above the sequence blocks correspond to the reported crystal structure of Amaranth α-amylase inhibitor (AAI; PDB ID: 1QFD). Conserved residues are boxed in red. Similar residues are in black bold and boxed in yellow. α-helices are rendered as squiggles and strict β-turns as TT. Cysteine pairings for disulfide bridges are numbered as 1, 2 and 3. **d.** Structure based sequence alignment of AhAI with other α-amylase inhibitors. The sequences are as follows, α-amylase inhibitors from A_hypochondriacus: *Amaranthus hypochondriacus* (UniProtKB accession ID: P80403), E_coracana: *Eleusine coracana* (UniProtKB accession ID: P01087), Z_mays; *Zea mays* (UniProtKB accession ID: P01088), W_religiosa: *Wrightia religiosa* (UniProtKB accession ID: V5W9K8), A_cathartica: *Allamanda cathartica* (UniProtKB accession ID: U5JE23), T_aestivum: *Triticum aestivum* (UniProtKB accession ID: P01085) and *Beta vulgaris* α-amylase inhibitor (GenBank accession No. XP_010672947.1). The figure was created using ESPript 3.

5.3.3 AhAI transcript abundance varies across the plant parts of Amaranthaceae members

Relative transcript abundance was analysed in various plant parts of one domestic and two wild relatives of *A.hypochondriacus* viz. *A. paniculatus*, *C. argentea* and *A. aspera,* respectively. *AhAI* transcripts were abundant in *A. hypochondriacus* plant parts specifically in inflorescence as compared to the plant parts from other species. Although abundant α-amylase inhibitor protein was found in *A. hypochondriacus* seeds (Chagolla-Lopez et al., 1994), in our study *AhAI* transcripts remained undetectable in the seeds of all four species. All selected members showed variation in transcript abundance within respective plant tissues *viz.* young leaves, mature leaves, young inflorescence, mature inflorescence and seeds (Figure 5.4).

A. hypochondriacus mature inflorescence showed 40-fold higher expression of *AhAI* transcripts compared to the number of transcripts present in young leaves whereas *A. paniculatus* and *C. argentea* showed only two-fold higher expression in their mature inflorescence. In contrast, *AhAI* transcript abundance in mature inflorescence of *A. aspera* found to be one third of that in young leaves. Similarly, when compared within respective plant parts, number of *AhAI* transcripts was higher in mature inflorescence of all three species except that in *A. aspera* where mature leaves showed the highest AhAI expression. Overall, AhAI protein was reported only from *A. hypochondriacus* seeds, transcripts were found in various plant parts of four relative Amaranthaceae species. Moreover, *AhAI* transcript abundance varies within plant parts and across the four relative species.

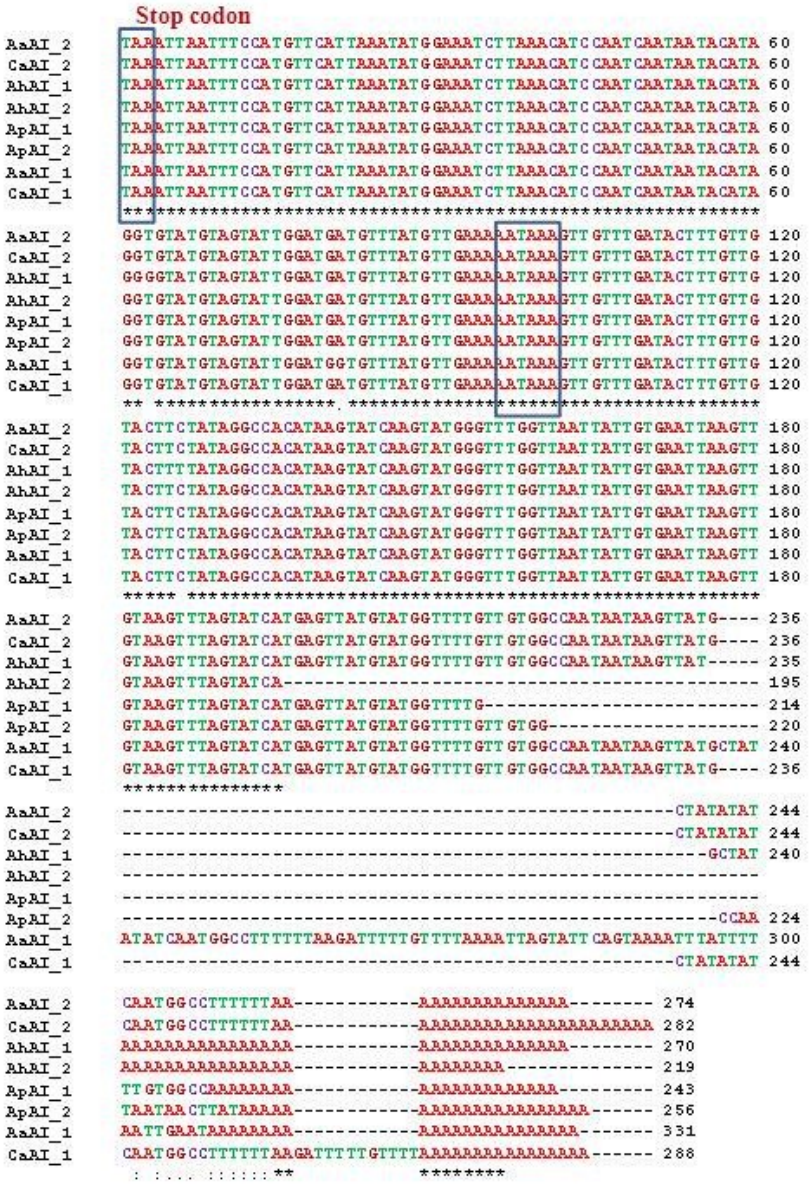


Figure 5.3 Three-prime UTR variation within AhAI transcripts from four species of Amaranthaceae family. Nucleotide sequences of variant AhAI transcripts were aligned using ClustalW. Nucleotides A, T, G and C were colored in red, green, brick red and magenta respectively. Stop codon was boxed in the beginning of the alignment. Poly 'A' tailing signal was highlighted using blue box. Asterisk '*' indicates identical residues, Colon ':' indicates conservation between groups and period '.' indicates conservation between dissimilar groups.

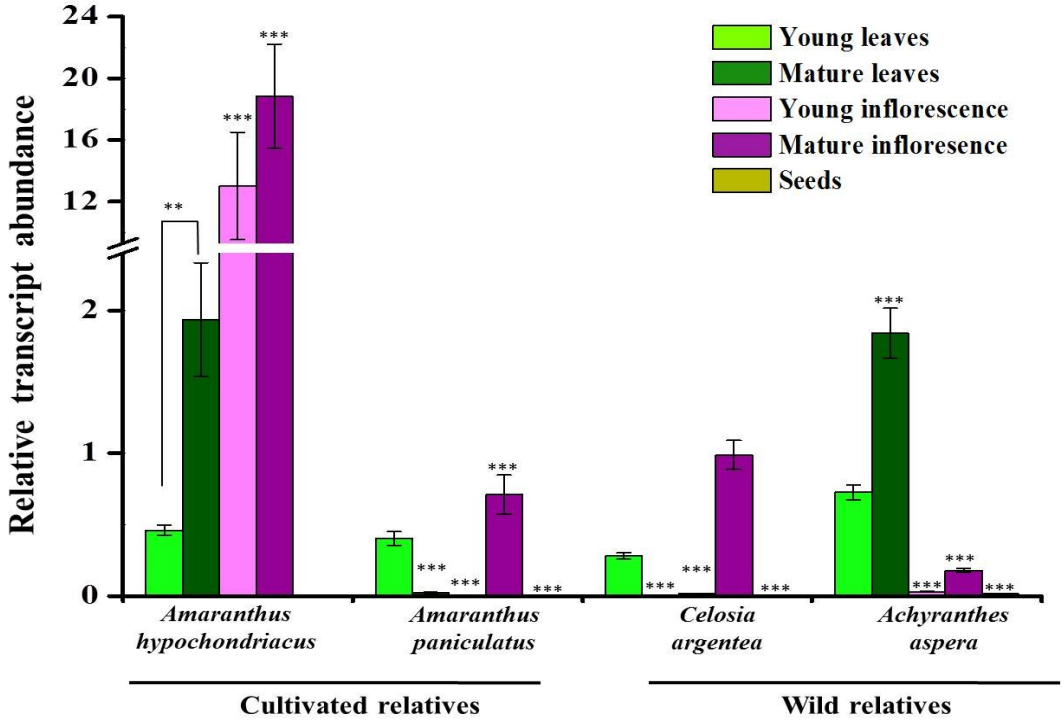


Figure 5.4 *AhAI* transcript abundance across different parts of plants from Amaranthaceae family. AhAI Transcript abundance was analysed in different plant parts *viz.* young leaves (light green), mature leaves (dark green), young inflorescence (light pink) and mature inflorescence (dark pink) using real-time PCR. Transcript abundance was compared between four species of Amaranthaceae family viz. *Amaranthus hypochondriacus*, *Amaranthus paniculatus*, *Celosia argentea* and *Achyranthes aspera*. Absolute quantification was carried out using the standard curve method with Elongation Factor 1-α (*EF1α*) as a reference gene. In statistical analysis,

One-way ANOVA was performed followed by Tukey's post hoc test. Statistical data is significant at P-value *** <0.001 and **< 0.01.

5.3.4 AhAI signal peptide mediates extracellular transport

Although AhAI protein was present in *A. hypochondriacus* seeds in abundant quantity as reported earlier, AhAI transcripts in *A. hypochondriacus* seeds were undetectable (Figure 5.4). Hence it is possible that AhAI synthesized in leaves or inflorescence was being transported to the seeds. Functional analysis of AhAI signal peptide was carried out to understand its possible role in extracellular transport. Signal peptide was fused to GFP and agro-infiltrated into the young tobacco leaves. Transiently expressed GFP protein without signal peptide was prominently observed in nucleus, cytoplasm and periphery, whereas GFP protein fused with AhAI signal peptide was observed only at periphery and in extracellular spaces of the plant cell known as apoplast (Figure 5.5). GFP protein was not visible either in nucleus or in cytoplasm when fused with AhAI signal peptide. Results indicated that signal peptide is involved in transport of attached protein to the extracellular space of the plant cells.

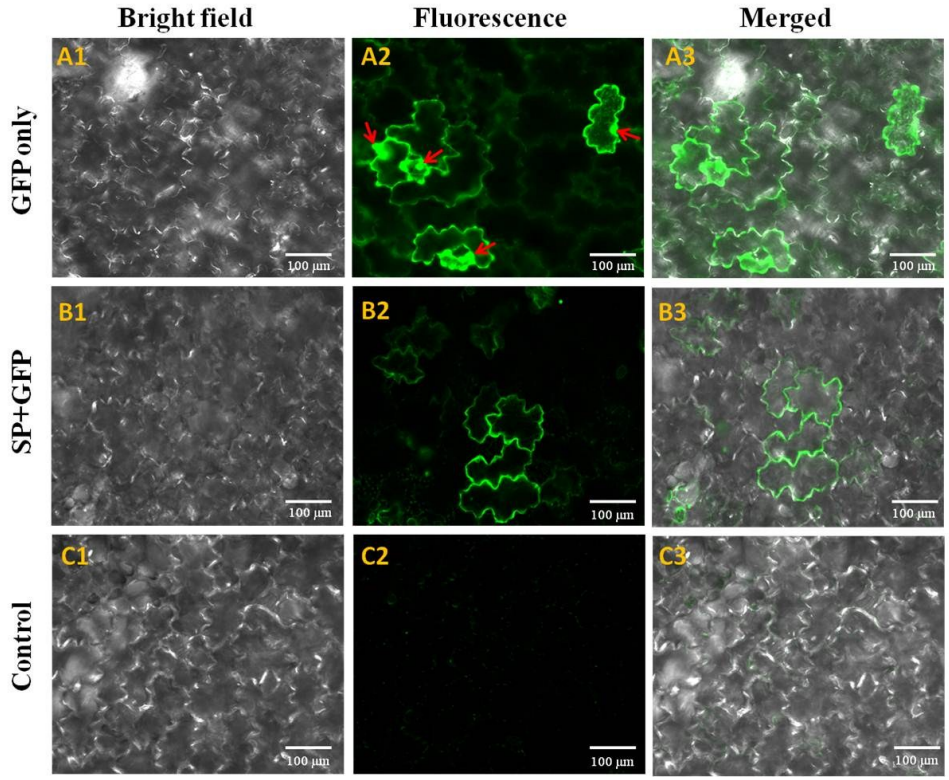


Figure 5.5 Subcellular localization of GFP in absence and presence of AhAI signal peptide visualized in agro-infiltrated tobacco epidermal cells. Images of agro-infiltrated tobacco epidermal cells using constructs pRI101-AN:GFP (A1-A3), pRI101-AN:SP-GFP (B1-B3) and only pRI101-AN (C1-C3) were taken at roughly 4 dpi. All images were taken in a single optical plane. Arrow head point to nucleus. Last panel shows merged images of bright and fluorescence fields. Scale represents 100µm distance. Fluorescence in tobacco epidermal cells infiltrated with only GFP construct was seen mainly in nucleus and cytoplasm whereas for SP-GFP construct, it was mainly seen in apoplasts.

5.3.5 AhAI is a potent inhibitor of coleopteran α-amylases

To evaluate the specific biochemical function, AhAI was heterologously expressed into the *E. coli* cells. Molecular weight of expressed protein was found to be 13.2 kDa as determined by MALDI (Figure 5.6a). Inhibitory potential of recombinant AhAI (rAhAI) was evaluated against a wide range of α-amylases belonging to human, insect, fungi and bacteria (Figure 5.6b). rAhAI was effective in complete inhibition of amylolytic activity of coleopteran α-amylases i.e. crude α-amylases from adult *T. castaneum* and *C. chinensis* larvae. Crude α-amylases from *T. castaneum* larvae and *C. chinensis* adults showed moderate inhibition (48% and 43%. respectively). Interestingly, activity of human salivary α-amylase and crude α-amylases from adults and larvae of *Helicoverpa armigera* were remained unaffected by rAhAI. Moreover, human pancreatic α-amylase and fungal α-amylase diastase showed moderate (38% and 50%, respectively)inhibition whereas purified α-amylase from *Bacillus licheniformis* showed only 15% inhibition by rAHAI. Potential of rAhAI to inhibit amylolytic activity of recombinant lepidopteran and coleopteran α-amylases were also tested (Figure 5.6c). Recombinant α-amylases from *T. castaneum* (TcAmy) and *C. chinensis* (CcAmy) showed complete inhibition while two recombinant α-amylases from *H. armigera* viz. HaAmy1and HaAmy2 showed complete resistance to inhibition even at very high concentration of rAhAI (Figure 5.6c).

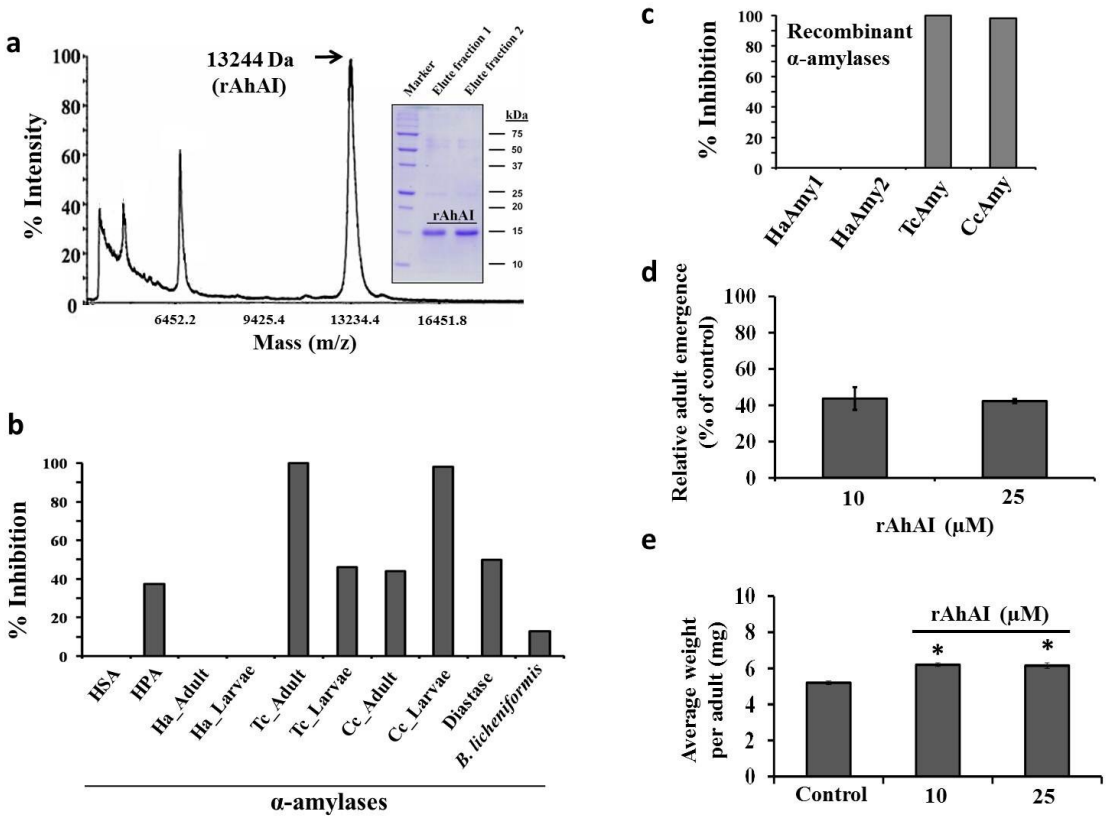


Figure 5.6 Inhibitory activity of rAhAI against various α-amylases.a. Acquisition of accuratemolecular weight of rAhAI from mass spectrometric measurements (MALDI). Insight SDS-PAGE image shows rAhAI fraction purified through Ni–NTA affinity column. **b.** Inhibitory activity rAhAI against human salivary amylase (HSA), human pancreatic amylase (HPA), crude α-amylase from *Helicoverpa armigera* adults (Ha_Adult) and larvae (Ha_Larvae), crude α-amylase from *Tribolium castaneum* adults (Tc_Adult) and larvae (Tc_Larvae), crude α-amylase from *Callosobruchus chinensis* adults (Cc_Adult) and larvae (Cc_Larvae), α-amylase from fungal source i.e. diastase\and α-amylase from bacteria *Bacillus licheniformis*. Insight graph shows Inhibitory activity of rAhAI against recombinant α-amylases viz. *H. armigera* α-amylase 1 and 2 (HaAmy1 and HaAmy2), *T. castaneum* α-amylase (TcAmy) and *C. chinensis* α-amylase (CcAmy). **c.** Effect of different concentrations of rAhAI on *C. chinensis* growth and development. One-way ANOVA was performed followed by Tukey's post hoc test. Statistical data is significant at P-value * <0.005.

A concentration dependant effect of rAhAI on growth and development of *C. chinensis* was evaluated. Effect was measured at a concentration of 10 and 25 µM of rAhAI and indicated as a means of relative adult emergence and average weight per emerged insect (Figure 5.6d). Reduction in adult emergence was noted (40%) in rAhAI exposed insect as compared to the insect fed without inhibitor. Insect fed with 10 and 25 µM rAhAI showed relatively similar adult emergence. Further, average weight for the insects fed with inhibitor was found to be slightly higher than insect fed on control grains (without inhibitor). A similar increase in weight was also evident for *Leptinotarsa decemlineata*, *Psylliodes chrysocephala* and *Spodoptera littoralis* when fed with plant protease inhibitors (De Leo et al., 1998; Girard et al., 1998; Cloutier et al., 2000).

5.3.6 Differential interaction might be responsible for concentration dependent inhibition of coleopteran α-amylases

Inhibitory activity of rAhAI was evaluated against recombinant TcAmy and CcAmy using a concentration range from 0 to 0.4 nM. rTcAmy was completely inhibited at 0.15 nM of rAhAI while 0.4 nM required to inhibit rCcAmy (Figure 5.7). The concentration dependent differential inhibition of rAhAI against rTcAmy and rCcAmy was explained using molecular docking. Inhibitory loop of AhAI showed similar conformational arrangement in the binding pockets of TcAmy and CcAmy except that Arg7 and Tyr28 made differential contacts with active site residues of both the enzymes (Figure 5.7). In TcAmy, Arg7 alone makes contact with two active site residues i.e. Asp187 and glu224 and also with adjacent Tyr28 residue on inhibitory loop. Tyr28 also makes hydrogen bond contact with TcAmy active site residue Asp187. In CcAmy, Arg7 makes single contact with active site residues Asp286 and also with adjacent Tyr28 residue on inhibitory loop. Tyr28 also makes hydrogen bond contact with enzymes active site residue Glu 221. In TcAmy, amino acid residues from inhibitory loops make three hydrogen bond contacts with enzymes active site residues which are energetically more favourable than two hydrogen bond contacts observed in AhAI and CcAmy interaction.

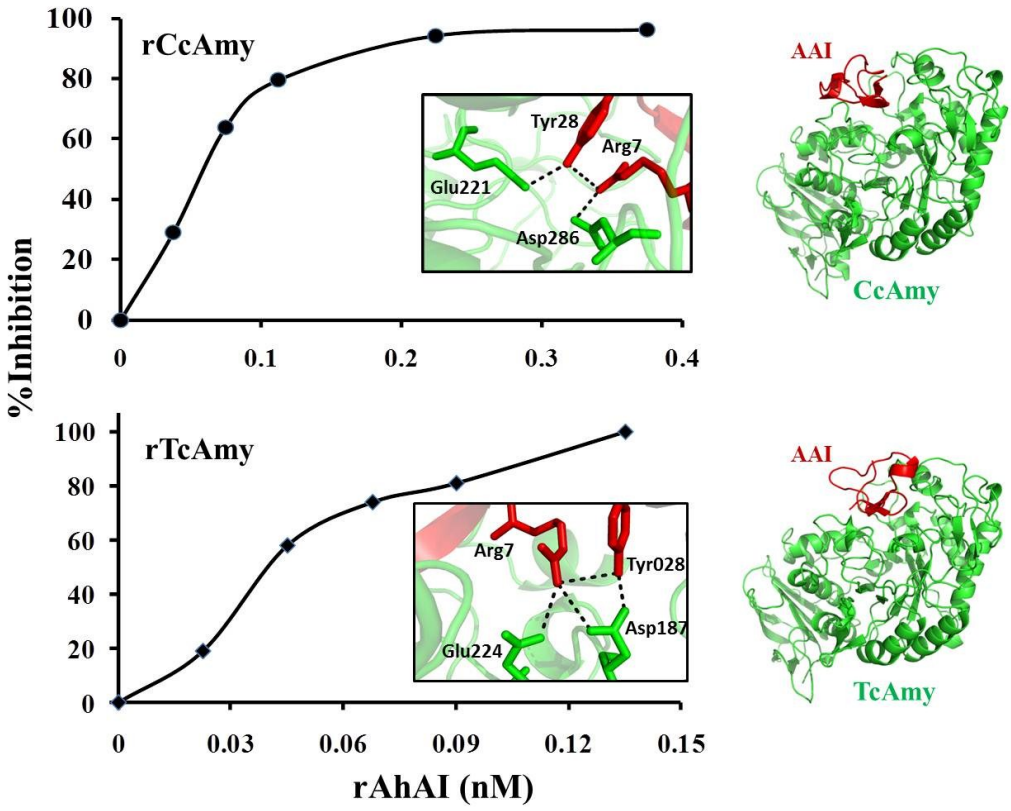


Figure 5.7 Inhibitory potential of rAhAI against recombinant TcAmy and CcAmy. 0.02 to 0.15 nM and 0.04 to 0.4 nM of rAhAI was tested for inhibitory activity against *T. castaneum* α-amylase (TcAmy) and *C. chinensis* α-amylase (CcAmy) respectively. Insight molecular docking figures highlight molecular interactions of rAhAI against both the α-amylases.

5.4 Discussion

Insect pests are major threat to the economically important crops and responsible for severe economic losses. α-Amylases of the insect pests through digestion of starch play a key role in overall energy assimilation and metabolism. Plants on other hand are equipped with various α-amylase inhibitors to impede the growth of insect pests and mitigate the insect infestation. A 32 amino acid long proteinaceous α-amylase inhibitor from *A. hypochondriacus* was reported (Chagolla-Lopez et al., 1994). We cloned the complete DNA sequence of AhAI, which has typical conserved six cysteine residues and three di-sulfide bonds (Chagolla-Lopez et al., 1994; Lu et al., 1999; Franco et al., 2002).

When compared with other α-amylase inhibitors from monocots and dicots, AAI showed conserved α-helix and disulfide bond necessary to retain knottin fold and hence inhibitory activity. Native pre-protein consists of signal peptide and unprocessed protein (AhAI) which is a pro-form of previously reported 32 amino acid long mature peptide. Proteolytic maturation was reported for many types of proteins including peptide hormones, digestive enzymes and coagulation proteins. It was reported that many plant vacuolar proteins before entering to the vacuoles were processed to their carboxyl end of Aspargine amino acid moity by Asn-specific proteases (Hara-Nishimura et al., 1991). Similar activation of mature α-amylase inhibitor peptide from its pro-protein form towards carboxyl end of Asn was reported for *Phaseolus vulgaris*α-amylase inhibitor (Pueyo et al., 1993). Another related *Phaseolus vulgaris* α-amylase inhibitor (α-AI-2) was reported to be processed at particular Asn site to release α and β subunits which assembled after cleavage into its active heterodimer form. It was predicted that processing of pro-protein is necessary to remove conformational constraint and to produce biochemically active form of inhibitor (Pueyo et al., 1993; Nakaguchi et al., 1997). With respect to present AhAI inhibitor, similar Asn69 site is present at N'-terminus of mature peptide. Processing of AhAI to its 32 aa mature form is evident as only mature peptide was reported to be derived in abundant quantity from *A. hypochondriacus* seeds (Chagolla-Lopez et al., 1994; Lu et al., 1999; Martins et al., 2001). Further, we have found recombinant AhAI containing 75 aa is biochemically active in contrast to the earlier prediction of need of processing of pro-protein to become an active form of 32 aa inhibitor. A separate motif search for uncharacterized 43 aa peptide revealed that motifs present on this peptide are identical with the motifs on proteins involved in sensing biological stress. This way an uncharacterized 43aa peptide might be involved in sensing

biological stress. As it is evident that Asn specific pro-protein processing pathway is evolutionary active in *A. hypochondriacus* seeds, presence of active α-amylase inhibitor protein (75 aa AhAI) suggest additional advantage to the inhibitory function.

α-Amylase inhibitors with small variations in their primary structure were reportedly exist in a few plants and their related species. For example, monomeric α-amylase inhibitors with few nucleotide variations were present in wheat and aegilops which were thought to be evolved from common ancestral gene through duplication and mutation (Wang et al., 2008).Similarly, higher diversity of α-amylase inhibitors was present in non-cultivated (wild) accessions of common bean than cultivated accessions (Ishimoto et al., 1995). Hi-lysine barley mutants also showed higher accumulation of mRNA of bi-functional amylase inhibitor in seeds (Leah and Mundy, 1989). Interestingly, in present study, AAI transcripts with identical open reading frame were observed in one cultivated and two wild relatives of *A. hypochondriacus* viz. *A. paniculatus*, *A. aspera* and *C. argentea*, respectively. Variation in expression of AAI transcripts were observed across different plant parts of above mentioned plant species. In *A. hypochondriacus*, highest AhAI expression was observed in mature inflorescence which is an essential plant part in reproduction and requires more protection from insect attack before the seeds mature. A similar variation of expression of α-amylase inhibitors were also observed in wheat and its close relatives (Zoccatelli et al., 2012). Although for most wheat and barley α-amylase inhibitors, genes are expressed only during seed development and remained at an undetectable level in other plant tissues (Sanchez et al., 1994; Altenbach et al., 2011).

Variation in 3'UTR region is known to be responsible for regulation of translation of a particular transcript. We have sequenced various AhAI transcripts from *A. hypochondriacus* and related species mentioned above and noted variation only in 3'UTR region. In the literature, 3'UTR regions of plant viral RNAs and mammalian RNAs have been studied extensively. 3'UTR region in plants however have not been studied thoroughly for their regulatory role under different conditions and very few examples exist in the literature. For example, three prime UTR region of invertase gene in Maize was studied for its involvement in sensing carbon starvation (Cheng et al., 1999). In sweet cherry also, 3'UTR sequences were used as SNP and haplotype markers to improve plant genomic recourses (Koepke et al., 2012). Further, polyadenylation sites in 3'UTR region were experimentally determined in plants by hi-throughput sequencing which has revealed great potential for regulation of gene expression in plants (Ma et al., 2013).In

present study we have identified conserved single polyadenylation site in 3' UTR region of AhAI transcripts from all four Amaranthaceae members. However, no other regulatory sites or secondary structure variations were observed on AhAI 3'UTR region and the molecular role behind 3'UTR polymorphism remains unknown.

Analysis of AhAI transcript abundance in different plant parts of *A. hypochondriacus* and related species indicated negligible expression of *AhAI* transcripts in mature seeds in spite of the fact that abundant mature inhibitor peptide was reported from mature *A. Hypochondriacus* seeds (Chagolla-Lopez et al., 1994). We hypothesize that protein produced in other parts of the plant was being transported to seeds. Appropriate signal sequence is required for effective transport of mature α-amylase inhibitor to seeds. Signal peptide composition in AAI pre-protein showed charged N-terminal, central hydrophobic and polar C-terminal region which is in well accordance with other known eukaryotic signal peptides (Heijne, 1985; Heijne and Abrahmsen, 1989).We have demonstrated the involvement of this signal peptide in *in vivo* extracellular transport of attached protein. To our knowledge, this is the first report of *in vivo* functional characterization of signal peptide from plant α-amylase inhibitors.

To become an effective α-amylase inhibitor, it should be able to inhibit α-amylases from wide range of sources with minimal inhibitory concentration and high specificity. In present study, potential of rAhAI to inhibit α-amylases from different sources showed distinctive inhibition pattern. rAhAI showed more specificity towards coleopteran α-amylases than lepidopteran, fungal, bacterial and mammalian α-amylases. Moderate inhibition was observed in the case of fungal and bacterial α-amylases. A similar differential inhibition with respect to insect and mammalian α-amylases was observed for rye α-amylase inhibitor (Lulek et al., 2000). AhAI showed moderate inhibitory activity against human pancreatic α-amylase and was unable to inhibit human salivary α-amylase as well as α-amylases from *H.armigera*. Similar differential specificities were observed in interactions of wheat α-amylase inhibitor with human salivary and pancreatic α-amylase (O'connor et al., 1981). Moreover, variable inhibitory potential of common bean α-amylases was observed against coleopteran, hymenopteran and dipteran α-amylases (Kluh et al., 2005). Analogous interactions between enzyme-inhibitor complexes of TMA-bean αAI and mammalian α-amylases-bean αAI revealed deviations in interacting inhibitory loop (Nahoum et al., 1999). Further, very low concentrations (nanomolar) of rAhAI were required for complete inhibition of *T.*

castaneum and *C. chinensis* α-amylases. CcAmy required relatively higher quantity of AhAI than TcAmy for complete inhibition of amylolytic activity.

Molecular docking studies highlighted differential hydrogen bonding between active site residues of enzyme and Arg7 and Tyr28 residues of inhibitory loop. AhAI upon binding with α-amylases adopts more compact conformation with increased intramolecular hydrogen bonding (Carugo et al., 2001; Micheelsen et al., 2008). Similar intramolecular hydrogen bonding was observed between Arg7 and Tyr28 of AhAI inhibitory loop after interactions with TcAmy and CcAmy. These interactions stabilise AhAI inhibitory loop over the active sites of the target α-amylases (Martins et al., 2001). Interaction of *Tenebrio molitor* α-amylase (TMA) with AAI revealed that inhibitor binds into the active site groove of the enzyme by blocking substrate entry site and central four sugar-binding subsites (Pereira et al., 1999). Arginine and Tyrosine residues residing in inhibitory loop of other α-amylase inhibitors were demonstrated to be essential for these molecular interactions (Abe et al., 1993; Mirkov et al., 1995; Rodenburg et al., 1995). We have found analogous AhAI residues interacting with active site residues of TcAmy and CcAmy with difference in their hydrogen bonding pattern. This could be the molecular basis behind the concentration dependent inhibition of AhAI to completely deactivate above coleopteran α-amylases.

Until now, effectiveness of 32 aa AAI in controlling insect pests growth and development has been studied by *in vitro* interactions of AhAI with purified insect digestive α-amylases. AAI was found to be effective in inhibiting amylolytic activity of α-amylases from a lepidopteran pest*, Tecia solanivora* (Valencia-Jimenez et al., 2008). Another report highlighted the potential of AAI in inhibiting amylolytic activity of α-amylases from coleopteran pests viz. *T. castaneum* and *Prostephanus truncates* (Chagolla-Lopez et al., 1994). This is the first report showing effectiveness of 75 aa AhAI in mitigating growth and development of *C. chinensis*, a coleopteran pest by incorporating the inhibitor into the insect diet. Besides AhAI, various other α-amylase inhibitors were expressed in host plants of insect pests and the efficacy was tested by performing inset feeding assays (Morton et al., 2000; De Sousa-Majer et al., 2007; Barbosa et al., 2010; Luthi et al., 2013). In the present study, detailed genomic and functional analysis of AhAI suggested its potential in mitigating insect infestation. Generating transgenic host plants expressing AhAI would be the next strategy to evaluate the efficacy of the AhAI inhibitor.

5.5 Summary of the Chapter:

In present study, we cloned the complete DNA sequence of AhAI, which has typical three cysteine residues and one di-sulfide bond conserved within known α-amylase inhibitors from eudicots and monocots. Identical genomic and open reading frames from four members Amaranthaceae family having 26 aa signal peptide and 75 aa pro- peptide were characterized. Although 32 aa peptide alone reported to possess inhibitory activity. AI transcripts were differentially expressed across various plant parts with the highest abundance in flowers. Fluorescent tag based subcellular localization revealed that AhAI signal peptide was involved in extracellular secretion. Recombinant full-length 75 aa α-amylase inhibitor (rAhAI) showed wide range of inhibition against α-amylases. I*n vitro* and *in vivo* studies revealed that rAhAI was efficient in inhibiting amylolytic activity of two coleopteran insects. Further, insect feeding assays on mung bean seeds showed potential of rAhAI in limiting overall development and adult emergence in case of *C. chinensis*. On other hand rAhAI failed to inhibit *H. armigera* α-amylases as well as human salivary α-amylase. Overall study emphasizes potential of native amaranth α-amylase inhibitor towards lessening insect infestation and damage.

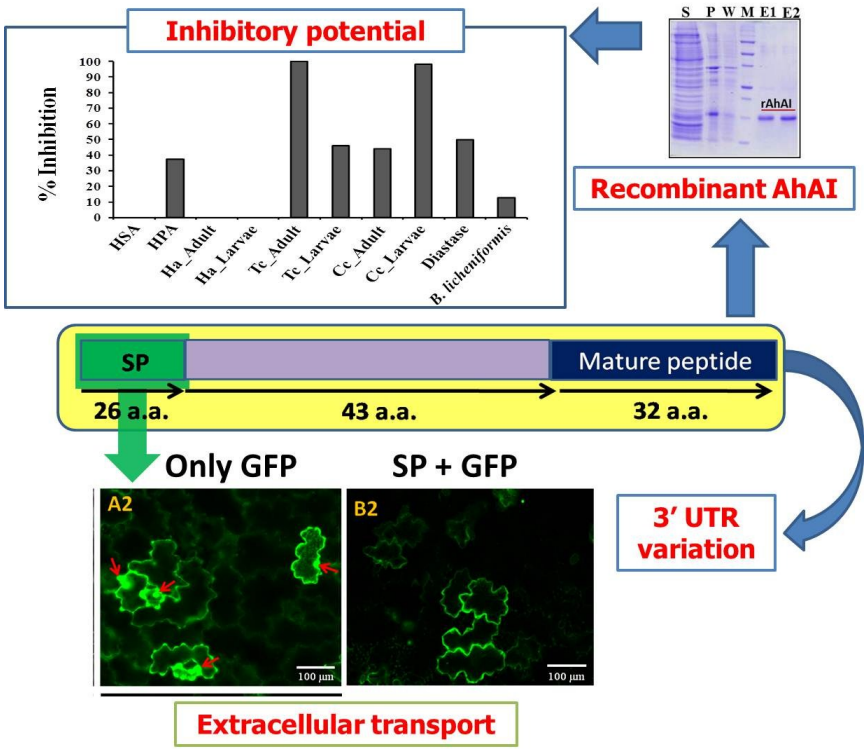


Figure 5.8 Schematics representing biochemical and molecular features of α-amylase inhibitor from *Amaranthus hypochondriacus*.

Chapter-6

"Summary

&

Future prospective"

6.1 Summary

Alpha-amylases from Lepidopteran and Coleopteran insect pests as well as α-amylase inhibitor from Amaranth species have been studied thoroughly in this thesis. Main objective of the study was to understand biochemical and biophysical properties of α-amylases from insect pests and inhibitory potential of plant α-amylase inhibitors against these classes of enzymes. Lepidopteran (*Helicoverpa armigera*, Hubner) and Coleopteran (*Tribolium castaneum* and *Callosobruchus chinensis*) α-amylases belong to Glycoside hydrolase enzyme family (GH-13) and display diversity in their biochemical, biophysical and molecular properties. Classified within the knottin fold super family, full length native α-amylase inhibitor from *Amaranthus hypochondriacus* exhibited the highest specificity towards coleopteran α-amylases. This inhibitor also showed inhibitory potential against human pancreatic as well as fungal and bacterial α-amylases. Further, *in silico* analysis revealed that inhibitor interacted differentially with coleopteran α-amylases at molecular level, highlighting its distinct specificities towards these α-amylases. Overall, the work provides insight into basic enzymatic functionality of insect pest's digestive α-amylases and potential of amaranth α-amylase inhibitor to affect growth and development of coleopteran insect by inhibiting starch degradation along the insect gut.

H. armigera (Hübner; Lepidoptera: Noctuidae), a generalist herbivore, feeds on a number of plant species across various families and imposes threat to the economy of important crops. Expression of two α-amylase genes (*HaAmy1* and *HaAmy2*) was studied in *H. armigera* feeding on various host plants and during larval development. Alignment of *HaAmy1* and *HaAmy2* with other insect α-amylases showed similarities with known Lepidopteran α-amylase transcripts. *H. armigera* α-amylase gene expression was influenced by the availability of reducing sugars, sucrose and starch content of host plants and further correlated to the pool of reducing sugars in the gut and haemolymph of larvae. *HaAmy1* and *HaAmy2* during larval development on two host plants *viz*., maize (cereal) and marigold (ornamental) showed differences. Results supported the view that when host plants differed in their macronutrients, relationships of enzymes and substrates were flexible. The present work highlights the distribution of *HaAmy1* and *HaAmy2* (i) during various stages of insect development (second, fourth and sixth instar, pupa, adult and

egg), (ii) in various tissues *viz*., head, haemolymph, fat body, integument and whole larval body of *H. armigera* feeding on artificial diet and (iii) in three gut regions of larvae fed on various diets. Further, to evaluate biochemical and biophysical properties of these two α-amylases, the open reading frames of *HaAmy1* and *HaAmy2* were cloned and heterologously expressed in *Pichia pastoris*. Purified recombinant enzymes were characterized for their biochemical and biophysical attributes using established methods. Sequence alignment and homology modeling showed that HaAmy1 and HaAmy2 are sequentially and structurally conserved. Both the isozymes showed optimum activity at 60ºC. However, the isozymes differed in their pH optimum (HaAmy1, pH 9 and HaAmy2, pH 11). Further, HaAmy2 showed higher affinity for starch and amylopectin whereas HaAmy1 possessed higher catalytic efficiency. HaAmy1 and HaAmy2 were inhibited to the same magnitude by acarbose (a synthetic α-amylase inhibitor) while wheat proteinaceous α-amylase inhibitor showed 3-fold more inhibition of HaAmy1 than HaAmy2. Interactions of HaAmy1 and HaAmy2 with wheat AI revealed 2:1 stoichiometric ratio with more complex interaction with HaAmy1. Altogether, study revealed importance of these α-amylase isoforms in starch digestion and their differential interactions with proteinaceous and non-proteinaceous α-amylase inhibitors.

Insect pests not only infest standing crops in the fields but also stored-grains. The order Coleoptera is the largest order of insects which includes the most common and important stored-grain product pests. Post-harvest insect infestation of stored grains makes them unfit for human consumption and leads to severe economic loss. Digestive α-amylases play important role in digestion of ingested starchy material along the bruchid's gut. In present thesis, Functional and structural characterization of two coleopteran α-amylases *viz*. *C. chinensis* α-amylase (CcAmy) and *T. castaneum* α-amylase (TcAmy) along with their interactions with proteinaceous and non-proteinaceous α-amylase inhibitors have been reported. Secondary structure alignment and molecular docking studies of CcAmy and TcAmy with other coleopteran α-amylases revealed conserved motifs, active sites, di-sulfide bonds and two amino acid variations at spatially conserved substrate or inhibitor-binding sites. Homology modeling and molecular docking showed structural differences between these two enzymes. Based on biochemical characterization, both the enzymes had similar optimum pH values but had different optimum temperatures. Overall, patterns of enzyme stabilities were similar under various temperature and pH conditions. Further, CcAmy and TcAmy differed in their substrate

affinity and catalytic efficiency towards starch and amylopectin. HPLC analysis detected common amylolytic products like maltose and maltotriose while glucose and maltotetrose were unique in CcAmy and TcAmy catalysed reactions, respectively. At very low concentration, wheat α-amylase inhibitor was found to be superior over the acarbose as far as complete inhibition of amylolytic activities of CcAmy and TcAmy was concerned. Although, a variation in two amino acids near the active sites of TcAmy and CcAmy resulted into superficial binding of acarbose near the active site of TcAmy and may forms the basis for the observed differential inhibition of amylolytic reactions.

Naturally plants are known to produce various proteinaceous α-amylase inhibitors as a defence strategy against insect pests digestive α-amylases. Various types of α-amylase inhibitors have been described until now and their usefulness in pest management has been studied extensively. The smallest 32 amino acid (aa) α-amylase inhibitor from *Amaranthus hypochondriacus* (AAI) had been reported in the literature. The complete gene of pre-protein (*AhAI)* encoding 26 aa signal peptide followed by 43 aa region and previously identified 32 aa peptide was successfully cloned. Three cysteine residues and one di-sulfide bond conserved within known α-amylase inhibitors were present in AhAI. Identical genomic and open reading frame were found to be present in close relatives of *A. hypochondriacus* namely *Amaranthus paniculatus*, *Achyranthes aspera* and *Celosia argentea*. Interestingly, 3'UTR of *AhAI* varied in these species. The highest expression of *AhAI* was observed in *A. hypochondriacus* inflorescence; however, it was not detected in seeds. We hypothesized that inhibitor expressed in leaves and inflorescence might be transported to the seeds. Subcellular localization studies indeed indicated involvement of AhAI signal peptide in extracellular secretion. Recombinant full-length 75 aa AhAI showed differential inhibition against α-amylases from human, insects, fungi and bacteria. Particularly, α-amylases from *H. armigera* (Lepidoptera) were not inhibited while *T. castaneum* and *C. chinensis* (Coleoptera) α-amylases were completely inhibited by AhAI. Molecular docking studies with AhAI revealed tighter interactions with active site residues of *T. castaneum* α-amylase compared to *C. chinensis* α-amylase, which could be the rationale behind disparity in IC_{50}. Normal growth, development and adult emergence of *C. chinensis* was found to be hampered after feeding on rAhAI. Altogether, the ability of Amaranth α-amylase inhibitor to affect the growth of *C. chinensis* demonstrates its potential to be used as an efficient biocontrol agent especially for stored-grain pests.

6.2 Future prospective

- Complexity in expression of *H. armigera* α-amylase genes suggests existence of mechanisms involved in detecting nutrient balance required to avoid fitness costs and focus their importance in insect nutrition. The diversity and adaptability of *H. armigera* digestive α-amylases in context with their biochemical and biophysical properties as well as their differential responses towards AI signifies additive role of these enzymes for survival of *H. armigera* on diverse diets. Further, characterization of digestive enzymes of *H. armigera* provides the basis for the insect's polyphagous nature and ways to design strategies for their control.

- Characterization of α-amylases from *T. castaneum* and *C. chinensis*, the most important pests of stored-grains revealed their differential biochemical properties and distinct spatial molecular interactions with non-proteinaceous inhibitor, acarbose. Biochemical and molecular information generated through above study will be useful in designing potent inhibitor(s) against coleopteran α-amylases.

- *In vivo* functional characterization of signal peptide from Amaranth α-amylase inhibitor has provided a tool to lead any attached protein to extra-cellular spaces of plant. In future, this signal peptide can be used to direct insecticidal proteins into the phloem which has potential application against sap sucking aphids. Analysis of molecular interactions of amaranth α-amylase inhibitor with coleopteran α-amylases has provided insight into underlying mechanism of inhibition. This information can be used to generate mutants or to construct chimeric peptides which will have higher affinity towards coleopteran α-amylases.

Bibliography

- Abd El-latif, A.O., 2015. Biopotency of serine protease inhibitors from cowpea (Vigna unguiculata) seeds on digestive proteases and the development of Spodoptera littoralis (Boisduval). Archives of Insect Biochemistry and Physiology, 89(1), pp.1–17.
- Abdeen, A. et al., 2005. Multiple insect resistance in transgenic tomato plants over-expressing two families of plant proteinase inhibitors. Plant Molecular Biology, 57(2), pp.189–202.
- Abe, J., Sidenius, U. & Svensson, B. 1993. Arginine is essential for the alpha-amylase inhibitory activity of the alpha-amylase/subtilisin inhibitor (BASI) from barley seeds. Biochemical Journal, 293, pp.151-155.
- Abraham, E.G., Nagaraju, J. & Datta, R.K., 1992. Biochemical studies of amylases in the silkworm, Bombyx mori: competitive analysis in diapausing and non diapausing strains. Insect Biochemistry and Molecular Biology, 22, pp.867–873.
- Afshar, K. et al., 2010. Diet-specific salivary gene expression and glucose oxidase activity in Spodoptera exigua (Lepidoptera: Noctuidae) larvae. Journal of Insect Physiology, 56, pp.1798–1806.
- Agrawal, O.P., 1980. Amylase activity in the alimentary tract and salivary glands of Periplaneta americana L. Acta physiologica Polonica, 32, pp.29-36.
- Aguirre, C. et al., 2004. A novel 8.7 kDa protease inhibitor from chan seeds (Hyptis suaveolens L.) inhibits proteases from the larger grain borer Prostephanus truncatus (Coleoptera: Bostrichidae). Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology, 138(1), pp.81–89.
- Altenbach, S.B., Vensel, W.H. & Dupont, F.M. 2011. The spectrum of low molecular weight alpha-amylase/protease inhibitor genes expressed in the US bread wheat cultivar Butte 86. BMC Research Notes, 20, pp.242.
- Alvarez-Alfageme, F. et al., 2011. Potential use of a serpin from Arabidopsis for pest control. PloS One, 6(5), p.e20278.
- Alvarez-Alfageme, F., Luthi, C. & Romeis, J., 2012. Characterization of digestive enzymes of bruchid parasitoids-initial steps for environmental risk assessment of genetically modified legumes. PloS one, 7(5), p.e36862.
- Ambekar, S.S., Patil, S.C., Giri, A.P. & Kachole, M.S. 1996. Proteinaceous Inhibitors of Trypsin and of Amylases in Developing and Germinating Seeds of

Pigeon Pea (Cajanus cajan(L) Millsp). Journal of the Science of Food and Agriculture, 72, pp.57-62.

- Anderson, M.A. et al., 1997. Proteinase inhibitors from Nicotiana alata enhance plant resistance to insect pests. Journal of insect physiology, 43(9), pp.833–842.
- Applebaum, S.W. & Konijn, A.M., 1964. The Utilization of Starch by Larvae of the Flour Beetle, Tribolium castaneum. Journal of Nutrition, 85, pp.275-282.
- Araujo, C.L. et al., 2005. In vivo bioinsecticidal activity toward Ceratitis capitata (fruit fly) and Callosobruchus maculatus (cowpea weevil) and in vitro bioinsecticidal activity toward different orders of insect pests of a trypsin inhibitor purified from tamarind tree (Tamarindu). Journal of Agricultural and Food Chemistry, 53(11), pp.4381–4387.
- Arjamaa, O. & Vuorisalo, T., 2010. Gene-culture coevolution and human diet. American Scientist, 98, pp.140–147.
- Ary, M.B., Richardson, M. & Shewry, P.R., 1989. Purification and characterization of an insect alpha-amylase inhibitor/endochitinase from seeds of Job's Tears (Coix lachryma-jobi). Biochimica et Biophysica Acta, 999(3), pp.260–266.
- Ashwath, S.K. et al., 2010. Identification of RAPD markers linked to digestive amylase genes using near isogenic lines of the silkworm, Bombyx mori. Journal of Insect Science, 10, pp.1–10.
- Azzouz, H. et al., 2005. Potential effects of plant protease inhibitors, oryzacystatin I and soybean Bowman-Birk inhibitor, on the aphid parasitoid Aphidius ervi Haliday (Hymenoptera, Braconidae). Journal of Insect Physiology, 51(8), pp.941–951.
- Babu, S.R. et al., 2012. In vivo and in vitro effect of Acacia nilotica seed proteinase inhibitors on Helicoverpa armigera (Hubner) larvae. Journal of Biosciences, 37(2), pp.269–276.
- Bandani, A.R. & Balvasi, A., 2006. Comparison of alpha-amylase activity in larval stages of flour beetles, Tribolium confusum (Coleoptera: Tenebrionidae). Communications in agricultural and applied biological sciences, 71, pp.537–541.
- Bandoly, M., Hilker, M. & Steppuhn, A., 2015. Oviposition by Spodoptera exigua on Nicotiana attenuata primes induced plant defence against larval herbivory. The Plant Journal?: for Cell and Molecular Biology, 83(4), pp.661–672.

- Banner, D.W. et al., 1975. Structure of chicken muscle triose phosphate isomerase determined crystallographically at 2.5 Å resolution: using amino acid sequence data. Nature 255, pp.609-614.
- Barber, D. et al., 1989. A barley flour inhibitor of insect alpha-amylase is a major allergen associated with baker's asthma disease. FEBS Letters, 248(1-2), pp.119–122.
- Barbosa, A.E. et al., 2010. Alpha-amylase inhibitor-1 gene from Phaseolus vulgaris expressed in Coffea arabica plants inhibits alpha-amylases from the coffee berry borer pest. BMC Biotechnology, 10: 44.
- Bartel, D.P. 2009. MicroRNAs: target recognition and regulatory functions. Cell, 136, pp.215-233.
- Bass, C. et al., 2015. The global status of insect resistance to neonicotinoid insecticides. Pesticide Biochemistry and Physiology, 121, pp.78–87.
- Bayes, A. et al., 2006. Response of the digestive system of Helicoverpa zea to ingestion of potato carboxypeptidase inhibitor and characterization of an uninhibited carboxypeptidase B. Insect Biochemistry and Molecular Biology, 36(8), pp.654–664.
- Behmer, S.T., 2009. Insect herbivore nutrient regulation. Annual Review of Entomology, 54, pp.165–187.
- Bernfeld, P., 1955. a-Amylases. Methods in Enzymology, 1, pp.149–151.
- Bezerra, C.A. et al., 2014. Molecular cloning and characterization of an a-amylase cDNA highly expressed in major feeding stages of the coffee berry borer, Hypothenemus hampei. Gene 553, pp.7-16.
- Bezerra, C.A. et al., 2014. Molecular cloning and characterization of an alpha-amylase cDNA highly expressed in major feeding stages of the coffee berry borer, Hypothenemus hampei. Gene, 553(1), pp.7–16.
- Bezerra, I.W.L. et al., 2004. Alpha-amylase inhibitors from Ficus sp. seeds and their activities towards coleoptera insect pests. Protein and Peptide Letters, 11(2), pp.181–187.
- Bhattacharya, R. et al., 2013. Hydroxyproline-rich glycopeptide signals in potato elicit signalling associated with defense against insects and pathogens. Plant science?: an international Journal of Experimental Plant Biology, 207, pp.88–97.
- Bhattacharyya, A., Mazumdar Leighton, S. & Babu, C.R., 2007. Bioinsecticidal activity of Archidendron ellipticum trypsin inhibitor on growth and serine

digestive enzymes during larval development of Spodoptera litura. Comparative Biochemistry and Physiology. Toxicology & Pharmacology?: CBP, 145(4), pp.669–677.

- Bhide, A.J. et al., 2015. Biochemical, structural and functional diversity between two digestive a-amylases from Helicoverpa armigera. Biochimica et Biophysica Acta General Subjects, 1850, pp.1719–1728.
- Bi, R.-M. et al., 2006. [Transgenic wheat (Triticum aestivum L.) with increased resistance to the storage pest obtained by Agrobacterium tumefaciens--mediated]. Sheng Wu Gong Cheng Xue Bao = Chinese Journal of Biotechnology, 22(3), pp.431–437.
- Bloch, C.J. & Richardson, M., 1991. A new family of small (5 kDa) protein inhibitors of insect alpha-amylases from seeds or sorghum (Sorghum bicolar (L) Moench) have sequence homologies with wheat gamma-purothionins. FEBS Letters, 279(1), pp.101–104.
- Bode, R.F., Halitschke, R. & Kessler, A., 2013. Herbivore damage-induced production and specific anti-digestive function of serine and cysteine protease inhibitors in tall goldenrod, Solidago altissima L. (Asteraceae). Planta, 237(5), pp.1287–1296.
- Bonavides, K.B. et al., 2007. Molecular identification of four different alpha-amylase inhibitors from baru (Dipteryx alata) seeds with activity toward insect enzymes. Journal of Biochemistry and Molecular Biology, 40(4), pp.494–500.
- Botelho-Junior, S. et al., 2014. Defense response in non-genomic model species: methyl jasmonate exposure reveals the passion fruit leaves' ability to assemble a cocktail of functionally diversified Kunitz-type trypsin inhibitors and recruit two of them against papain. Planta, 240(2), pp.345–356.
- Bouayad, N. et al., 2008. Effects of different food commodities on larval development and a-amylase activity of Plodia interpunctella (Hubner) (Lepidoptera: Pyralidae). Journal of Stored Products Research, 44, pp.373–378.
- Bown, D.P., Wilkinson, H.S. & Gatehouse, J.A., 1997. Differentially regulated inhibitor-sensitive and insensitive protease genes from the phytophagous insect pest, Helicoverpa armigera, are members of complex multigene families. Insect Biochemistry and Molecular Biology, 27(7), pp.625–638.

- Brunelle, F. et al., 2005. A hybrid, broad-spectrum inhibitor of Colorado potato beetle aspartate and cysteine digestive proteinases. Archives of Insect Biochemistry and Physiology, 60(1), pp.20–31.
- Buonocore, V. et al., 1976. Physical and catalytic properties of a-amylase from Tenebrio molitor L. larvae. Biochemical Journal, 153, pp.621-625.
- Calderon, L.A. et al., 2005. Purification of a 6.5 kDa protease inhibitor from Amazon Inga umbratica seeds effective against serine proteases of the boll weevil Anthonomus grandis. Protein and Peptide Letters, 12(6), pp.583–587.
- Caldiera, W. et al., 2007. Digestives enzyme compartmentalization and recycling and sites of absorption and secretion along the midgut of Dermestus maculatus (Coleoptera) larvae. Archives of Insect Biochemistry and Physiology, 64, pp.1–18.
- Cantarel, B.L. et al., 2009. The Carbohydrate-Active Enzymes database (CAZy): an expert resource for glycogenomics. Nucleic Acids Research, 37, D233-D238.
- Carrillo, L. et al., 2011. A barley cysteine-proteinase inhibitor reduces the performance of two aphid species in artificial diets and transgenic Arabidopsis plants. Transgenic Research, 20(2), pp.305–319.
- Carugo, O. et al., 2001. Structural analysis of free and enzyme-bound amaranth alpha-amylase inhibitor: classification within the knottin fold superfamily and analysis of its functional flexibility. Protein Engineering, Design & Selection, 14, pp.639-646.
- Cates, R.G., 1980. Feeding patterns of monophagous, oligophagous and polyphagous insect herbivores: the effect of resource abundance and plant chemistry. Oecologia 46, pp.22–31.
- Ceciliani, F. et al., 1997. A trypsin inhibitor from snail medic seeds active against pest proteases. Phytochemistry, 44(3), pp.393–398.
- Celinska, E. et al., 2015. Cloning, expression, and purification of insect (Sitophilus oryzae) alpha-amylase, able to digest granular starch, in Yarrowia lipolytica host. Applied Microbiology and Biotechnology, 99, pp.2727-2739.
- Chagolla-Lopez, A. et al., 1994. A novel alpha-amylase inhibitor from amaranth (Amaranthus hypocondriacus) seeds. The Journal of Biological Chemistry, 269(38), pp.23675–23680.

- Chang, D. & Duda Jr, T. F. 2012. Extensive and continuous duplication facilitates rapid evolution and diversification of gene families. Molecular Biology and Evolution, pp 1-11.
- Cheng, W.H., Taliercio, E.W. & Chourey, P.S. 1999. Sugars modulate an unusual mode of control of the cell-wall invertase gene (Incw1) through its 3' untranslated region in a cell suspension culture of maize. Proceedings of the National Academy of Sciences U S A., 96, pp.10512-10517.
- Chougule, N.P. et al., 2005. Gene expression patterns of Helicoverpa armigera gut proteases. Insect Biochemistry and Molecular Biology, 35(4), pp.355–367.
- Christy, L.A. et al., 2009. Engineering sugarcane cultivars with bovine pancreatic trypsin inhibitor (aprotinin) gene for protection against top borer (Scirpophaga excerptalis Walker). Plant Cell Reports, 28(2), pp.175–184.
- Cinco-Moroyoqui, F.J. et al., 2006. Alpha-amylase activity of Rhyzopertha dominica (Coleoptera: Bostrichidae) reared on several wheat varieties and its inhibition with kernel extracts. Journal of Economic Entomology, 99(6), pp.2146–2150.
- Cipolla, L. et al., 2014. N-Bridged 1-deoxynojirimycin dimers as selective insect trehalase inhibitors. Carbohydrate Research, 389, pp.46–49.
- Clissold, F.J. et al., 2010. The gastrointestinal tract as a nutrient-balancing organ. Archive of Proceedings of the Royal Society B: Biological Sciences, 1688, pp.1751–1759.
- Cloutier, C. et al., 2000. Adult Colorado potato beetles, Leptinotarsa decemlineata compensate for nutritional stress on oryzacystatin I-transgenic potato plants by hypertrophic behavior and over-production of insensitive proteases. Archives of Insect Biochemistry and Physiology, 44, pp.69–81.
- Craik, D.J. et al., 2004. Discovery, structure and biological activities of the cyclotides. Current Protein & Peptide Science, 5(5), pp.297–315.
- Cruz, A.C.B. et al., 2013. Bioinsecticidal activity of a novel Kunitz trypsin inhibitor from Catanduva (Piptadenia moniliformis) seeds. Plant Physiology and Biochemistry?: PPB / Societe francaise de physiologie vegetale, 70, pp.61–68.
- D'Adamio, G. et al., 2015. New synthesis and biological evaluation of uniflorine A derivatives: towards specific insect trehalase inhibitors. Organic & Biomolecular Chemistry, 13(3), pp.886–892.

- Da Lage, J.L. et al., 2003. A nested alpha-amylase gene in Drosophila ananassae. Journal of Molecular evolution, 57, pp.355–362.
- Da Lage, J.-L., Maczkowiak, F. & Cariou, M.-L., 2011. Phylogenetic distribution of intron positions in alpha-amylase genes of bilateria suggests numerous gains and losses. PloS One, 6(5), p.e19673.
- da Silva, D.S. et al., 2014. Short and long-term antinutritional effect of the trypsin inhibitor ApTI for biological control of sugarcane borer. Journal of Insect Physiology, 61, pp.1–7.
- da Silva, M.C.M. et al., 2013. Employing in vitro directed molecular evolution for the selection of alpha-amylase variant inhibitors with activity toward cotton boll weevil enzyme. Journal of Biotechnology, 167(4), pp.377–385.
- Darvishzadeh, A. et al., 2012. Biochemical characterisation of digestive a-amylase of Red Palm Weevil, Rhynchophorus ferrugineus (Olivier, 1790) (Coleoptera: Curculionidae). Archives of Phytopathology and Plant Protection, 45, pp.2132–2142.
- Dayler, C.S. 2005. Identification of a novel bean alpha-amylase inhibitor with chitinolytic activity. FEBS Letters, 579, pp.5616-5620.
- de Azevedo Pereira, R. et al., 2006. An alpha-amylase inhibitor gene from Phaseolus coccineus encodes a protein with potential for control of coffee berry borer (Hypothenemus hampei). Phytochemistry, 67(18), pp.2009–2016.
- de Azevedo Pereira, R. et al., 2007. Effect of a Bowman-Birk proteinase inhibitor from Phaseolus coccineus on Hypothenemus hampei gut proteinases in vitro. Journal of Agricultural and Food Chemistry, 55(26), pp.10714–10719.
- De Leo, F. & Gallerani, R., 2002. The mustard trypsin inhibitor 2 affects the fertility of Spodoptera littoralis larvae fed on transgenic plants. Insect Biochemistry and Molecular Biology, 32(5), pp.489–496.
- De Leo, F. et al., 1998. Opposite effects on Spodoptera littoralis larvae of high expression level of a trypsin proteinase inhibitor in transgenic plants. Plant Physiology, 118, pp.997–1004.
- de Oliveira, C.F.R., Marangoni, S. & Macedo, M.L.R., 2014. The trypsin inhibitor from Entada acaciifolia seeds affects negatively the development of Mediterranean flour moth, Anagasta kuehniella. Pesticide biochemistry and physiology, 108, pp.74–79.

- de Oliveira, L.O. et al., 2015. A Trypsin Inhibitor from *Clitoria fairchildiana* Cotyledons is Active Against Digestive Enzymes of Aedes aegypti Larvae. Protein and Peptide letters, 22(10), pp.893–902.
- de P G Gomes, A. et al., 2005. Toxicity to cotton boll weevil Anthonomus grandis of a trypsin inhibitor from chickpea seeds. Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology, 140(2), pp.313–319.
- de Sousa-Majer, M.J. et al., 2007. Bean alpha-amylase inhibitors in transgenic peas inhibit development of pea weevil larvae. Journal of Economic Entomology, 100(4), pp.1416–1422.
- DeLay, B. et al., 2012. Transcriptome analysis of the salivary glands of potato leafhopper, *Empoasca fabae*. Journal of Insect Physiology, 58(12), pp.1626–1634.
- Dhaliwal, G.S. and R. Arora. 1996. Principles of insect management. Commonwealth Publishers, New Delhi.
- Di Gennaro, S. et al., 2005. cDNA cloning and heterologous expression of a wheat proteinase inhibitor of subtilisin and chymotrypsin (WSCI) that interferes with digestive enzymes of insect pests. Biological Chemistry, 386(4), pp.383–389.
- Di Maro, A. et al., 2011. WCI, a novel wheat chymotrypsin inhibitor: purification, primary structure, inhibitory properties and heterologous expression. Planta, 234(4), pp.723–735.
- Dias, S.C. et al., 2005. Molecular cloning and expression of an alpha-amylase inhibitor from rye with potential for controlling insect pests. The Protein Journal, 24(2), pp.113–123.
- Do Nascimento, V.V. et al., 2011. In silico structural characteristics and a-amylase inhibitory properties of Ric c 1 and Ric c 3, allergenic 2S albumins from Ricinus communis seeds. Journal of Agricultural and Food Chemistry, 59, pp.4814-4821.
- Dojnov, B. et al., 2008. Purification and properties of midgut a-amylase isolated from Morimus funereus (Coleoptera: Cerambycidae) larvae. Comparative Biochemistry and Physiology, 149B, pp.153-160.
- Dos Santos, I.S. et al., 2010. Purification of a defensin isolated from Vigna unguiculata seeds, its functional expression in Escherichia coli, and assessment of its insect alpha-amylase inhibitory activity. Protein Expression and Purification, 71(1), pp.8–15.

- Dunse, K.M., Kaas, Q., et al., 2010b. Molecular basis for the resistance of an insect chymotrypsin to a potato type II proteinase inhibitor. Proceedings of the National Academy of Sciences of the United States of America, 107(34), pp.15016–15021.
- Dunse, K.M., Stevens, J.A., et al., 2010a. Coexpression of potato type I and II proteinase inhibitors gives cotton plants protection against insect damage in the field. Proceedings of the National Academy of Sciences of the United States of America, 107(34), pp.15011–15015.
- EAD Barbosa, A. et al., 2010. a-amylase inhibitor-1 gene from Phaseolus vulgaris expressed in Coffea Arabica plants inhibits a-amylases from the coffee berry borer pest. BMC Biotechnology, 10, pp.2-8.
- El-latif, A.O.A., 2014. In vivo and in vitro inhibition of Spodoptera littoralis gut-serine protease by protease inhibitors isolated from maize and sorghum seeds. Pesticide Biochemistry and Physiology, 116, pp.40–48.
- Erban, T., et al., 2009. The importance of starch and sucrose digestion in nutritive biology of synanthropic acaridid mites: alpha-amylases and alpha-glucosidases are suitable targets for inhibitor-based strategies of mite control. Archives of Insect Biochemistry and Physiology 71, pp.139-158.
- Farias, L.R. et al., 2007. Isolation of a novel Carica papaya a-amylase inhibitor with deleterious activity toward Callosobruchus maculatus. Pesticide Biochemistry and Physiology, 87, pp.255–260.
- Feng, G.H. et al., 1996. a-Amylase inhibitors from wheat: amino acid sequences and patterns of inhibition of insect and human a-amylases. Insect Biochemistry and Molecular Biology, 26, pp.419-26.
- Feng, G.H. et al., 1996. alpha-Amylase inhibitors from wheat: amino acid sequences and patterns of inhibition of insect and human alpha-amylases. Insect Biochemistry and Molecular Biology, 26(5), pp.419–426.
- Ferey-Roux, G. et al., 1998. The human pancreatic alpha-amylase isoforms: isolation, structural studies and kinetics of inhibition by acarbose. Biochimica et Biophysica Acta, 1388, pp.10-20.
- Franco, A.L. et al., 2002. Plant a-amylase inhibitors and their interaction with insect a-amylases. European Journal of Biochemistry, 269, pp.397-412.

- Franco, O.L. et al., 2000. Activity of wheat a-amylase inhibitors towards bruchid a-amylases and structural explanation of observed specificities. European Journal of Biochemistry, 267, pp.2166–2173.
- Franco, O.L. et al., 2002. Overlapping binding sites for trypsin and papain on a Kunitz-type proteinase inhibitor from Prosopis juliflora. Proteins, 49(3), pp.335–341.
- Franco, O.L. et al., 2003. Effects of black-eyed pea trypsin/chymotrypsin inhibitor on proteolytic activity and on development of Anthonomus grandis. Phytochemistry, 63(3), pp.343–349.
- Franco, O.L. et al., 2004. Effects of soybean Kunitz trypsin inhibitor on the cotton boll weevil (Anthonomus grandis). Phytochemistry, 65(1), pp.81–89.
- Franco, O.L. et al., 2005. Characterization of two Acanthoscelides obtectus alpha-amylases and their inactivation by wheat inhibitors. Journal of Agricultural and Food Chemistry, 53(5), pp.1585–1590.
- Friedberg, F., 1983. On the primary structure of amylases. Federation of European Biochemical Societies Letters, 152, pp.139-140.
- Friesner, R. A. et al., 2006. Extra Precision Glide: Docking and Scoring Incorporating a Model of Hydrophobic Enclosure for Protein-Ligand Complexes. Journal of Medicinal Chemistry, 49, pp.6177-6196.
- Friesner, R.A. et al., 2004. Glide : A New Approach for Rapid , Accurate Docking and Scoring .1. Method and Assessment of Docking Accuracy. Journal of Medicinal Chemistry, 47, pp.1739-1749.
- Gahloth, D. et al., 2011. Bioinsecticidal activity of Murraya koenigii miraculin-like protein against Helicoverpa armigera and Spodoptera litura. Archives of Insect Biochemistry and Physiology, 78(3), pp.132–144.
- Galdino, A. S. et al., 2011. Biochemical and structural characterization of Amy1: An Alpha-amylase from Cryptococcus flavus expressed in Saccharomyces cerevisiae. Enzyme Research, Article ID 157294, 7 pages.
- Garcia, V.A. et al., 2004. Trypsin inhibitor from Poecilanthe parviflora seeds: purification, characterization, and activity against pest proteases. The Protein Journal, 23(5), pp.343–350.
- Garcia-Casado, G. et al., 1994. Rye chromosome arm 3RS encodes a homodimeric inhibitor of insect alpha-amylase. TAG. Theoretical and Applied Genetics. Theoretische und Angewandte Genetik, 89(1), pp.60–63.

- Garcia-Maroto, F., Carbonero, P. & Garcia-Olmedo, F., 1991. Site-directed mutagenesis and expression in Escherichia coli of WMAI-1, a wheat monomeric inhibitor of insect alpha-amylase. Plant Molecular Biology, 17(5), pp.1005–1011.
- Gatehouse, A.M.R. et al., 1999. Digestive proteolytic activity in larvae of tomato moth, Lacanobia oleracea; effects of plant protease inhibitors in vitro and in vivo. Journal of Insect Physiology, 45(6), pp.545–558.
- Gilles, C. et al., 1996. Crystal structure of pig pancreatic a-amylase isoenzyme II, in complex with the carbohydrate inhibitor acarbose. European Journal of Biochemistry, 238, pp.561-569.
- Girard, C. et al., 1998. Growth stimulation of beetle larvae reared on a transgenic oilseed rape expressing a cysteine proteinase inhibitor. Journal of Insect Physiology, 44, pp.263–270.
- Giri A. P. & Kachole M. S., 1996. Detection of electrophoretically separated amylase inhibitors in starch-polyacrylamide gels. Journal of Chromatography A, 752, pp.261-264.
- Giri, A.P. & Kachole, M.S. 1998. Amylase inhibitors of pigeonpea (Cajanus cajan) seeds. Phytochemistry, 47, pp.197-202.
- Giri, A.P. et al., 2003. Identification of potent inhibitors of Helicoverpa armigera gut proteinases from winged bean seeds. Phytochemistry, 63(5), pp.523–532.
- Giri, A.P., Bhide, A.J. & Gupta, V.S. 2016. Targeting digestive physiology: Trends in strategic exploitation of plant defensive proteinaceous inhibitors against insect pests. Genetic Engineering of Plants – Enhancing Productivity and Product Value, John Wiley & Sons Limited.
- Gomes, C.E.M. et al., 2005. Effect of trypsin inhibitor from Crotalaria pallida seeds on Callosobruchus maculatus (cowpea weevil) and Ceratitis capitata (fruit fly). Plant Physiology and Biochemistry?: PPB / Societe Francaise de Physiologie Vegetale, 43(12), pp.1095–1102.
- Gomez, L. et al., 1989. Wheat tetrameric inhibitors of insect alpha-amylases: Alloploid heterosis at the molecular level. Proceedings of the National Academy of Sciences U S A., 86, pp.3242-3246.
- Gomez, L. et al., 1991. Wheat Inhibitors of Heterologous alpha-Amylases?: Characterization of Major Components from the Monomeric Class. Plant Physiology, 96(3), pp.768–774.

- Goto, H. et al., 2005. Effect of nucleotide polymorphism in cis regulatory and coding regions on amylase activity and fitness in Drosophila melanogaster. Heredity, 95, pp.369–376.
- Goulet, M.-C. et al., 2008. Tailoring the specificity of a plant cystatin toward herbivorous insect digestive cysteine proteases by single mutations at positively selected amino acid sites. Plant Physiology, 146(3), pp.1010–1019.
- Grossi de Sa, M.F. et al., 1997. Molecular characterization of a bean alpha-amylase inhibitor that inhibits the alpha-amylase of the mexican bean weevil Zabrotes subfasciatus. Planta, 203(3), pp.295–303.
- Gruden, K. et al., 1997. Potato cysteine proteinase inhibitor gene family: molecular cloning, characterisation and immunocytochemical localisation studies. Plant Molecular Biology, 34(2), pp.317–323.
- Gruden, K. et al., 1998. The cysteine protease activity of Colorado potato beetle (Leptinotarsa decemlineata Say) guts, which is insensitive to potato protease inhibitors, is inhibited by thyroglobulin type-1 domain inhibitors. Insect Biochemistry and Molecular Biology, 28(8), pp.549–560.
- Gujar, T. et al., 2004. Potentiation of insecticidal activity of Bacillus thuringiensis subsp. kurstaki HD-1 by proteinase inhibitors in the American bollworm, Helicoverpa armigera (Hubner). Indian Journal of Experimental Biology, 42(2), pp.157–163.
- Gunnar von Heijne & Abrahmsen, L. 1989. Species-specific variation in signal peptide design Implications for protein secretion in foreign hosts. FEBS letters, 244, pp.439-446.
- Gunnar von Heijne 1985. Signal sequences: The limits of variation. Journal of Molecular Biology, 184, pp.99-105.
- Gupta, M., Sharma, P. & Nath, A.K., 2014. Purification of a novel a -amylase inhibitor from local Himalayan bean (Phaseolus vulgaris) seeds with activity towards bruchid pests and human salivary amylase. Journal of Food Science and Technology, 51, pp.1286–1293.
- Hara-Nishimura, I., Inoue, K. & Nishimura, M. 1991. A unique vacuolar processing enzyme responsible for conversion of several proprotein precursors into the mature forms. FEBS letters, 294, pp.89-93.

- Harshini, S., Reshmi, V. & Sreekumar, S., 2003. A brain peptide stimulates release of amylase from the midgut tissue of larvae of Opisina arenosella Walk. (Lepidoptera: Cryptophasidae). Neuropeptides, 37, pp.133-139.
- Hartl, M. et al., 2010. Serine protease inhibitors specifically defend Solanum nigrum against generalist herbivores but do not influence plant growth and development. The Plant Cell, 22(12), pp.4158–4175.
- Hartl, M. et al., 2011. The multiple functions of plant serine protease inhibitors: defense against herbivores and beyond. Plant Signaling & Behavior, 6(7), pp.1009–1011.
- Hivrale, V.K. et al., 2011. Biochemical characterisation of alpha-amylase inhibitors from Achyranthes aspera and their interactions with digestive amylases of coleopteran and lepidopteran insects. Journal of the Science of Food and Agriculture, 91(10), pp.1773–1780.
- Huffaker, A. et al., 2013. Plant elicitor peptides are conserved signals regulating direct and indirect antiherbivore defense. Proceedings of the National Academy of Sciences of the United States of America, 110(14), pp.5707–5712.
- Iimure, T. et al., 2015. Purification of barley dimeric a-amylase inhibitor-1 (BDAI-1) and avenin-like protein-a (ALP) from beer and their impact on beer foam stability. Food Chemistry, 172, pp.257-264.
- Inanaga, H. et al., 2001. Protein engineering of novel proteinase inhibitors and their effects on the growth of Spodoptera exigua larvae. Bioscience, Biotechnology, and Biochemistry, 65(10), pp.2259–2264.
- Inomata, N. & Nakashima, S. 2008. Short 5'-flanking regions of the Amy gene of Drosophila kikkawai affect amylase gene expression and respond to food environments. Gene 412, pp.102-109.
- Ishikawa, K. et al., 1992. Multi-functional roles of a histidine residue in human pancreatic a-amylase. Biochemical and Biophysical Research Communications, 183, pp.286–291.
- Ishimoto, M. et al., 1995. Variation of seed a-amylase inhibitors in the common bean. Theoretical and Applied Genetics, 90, pp.425-429.
- Ishimoto, M. et al., 2012. Heterologous expression of corn cystatin in soybean and effect on growth of the stink bug. Bioscience, Biotechnology, and Biochemistry, 76(11), pp.2142–2145.

- Iulek, J. et al., 2000. Purification, biochemical characterisation and partial primary structure of a new alpha-amylase inhibitor from Secale cereale (rye). The International Journal of Biochemistry & Cell Biology, 32, pp.1195-204.
- Janecek, S., 1992. New conserved amino acid region of alpha-amylases in the third loop of their (beta/alpha) 8-barrel domains. Biochemical Journal, 288, pp.1069-1070.
- Janecek, S., 1994a. Parallel ß/a-barrels of a-amylase, cyclodextrin glycosyltransferase and oligo-1, 6-glucosidase versus the barrel of ß-amylase: Evolutionary distance is a reflection of unrelated sequences. Federation of European Biochemical Societies Letters, 353, pp.119-123.
- Janecek, S., 1994b. Sequence Similarities and Evolutionary Relationships of Microbial, Plant and Animal a-amylases. European Journal of Biochemistry, 224(2), pp.519-24.
- Janecek, S., 1995. Close evolutionary relatedness among functionally distantly related members of the (a/ß) 8-barrel glycosyl hydrolases suggested by the similarity of their fifth conserved sequence region. Federation of European Biochemical Societies Letters, 377, pp.6-8.
- Janecek, S., 2002. How many conserved sequence regions are there in the a-amylase family? Biologia, 57, pp.29-41.
- Janecek, S., Svensson, B. & Henrissat, B., 1997. Domain evolution in the a-amylase family. Journal of Molecular Evolution, 45, pp.322-31.
- Jespersen, H.M. et al., 1991. Comparison of the domain-level organization of starch hydrolases and related enzymes. Biochemical Journal, 280, pp.51-55.
- Jespersen, H.M. et al., 1993. Starch-and glycogen-debranching and branching enzymes: prediction of structural features of the catalytic (ß/a) 8-barrel domain and evolutionary relationship to other amylolytic enzymes. Journal of Protein Chemistry, 12, pp.791-805.
- Jin, D. et al., 2014. Effects of a protease inhibitor protein from Xenorhabdus bovienii on physiology of pea aphid (Acyrthosiphon pisum). Pesticide Biochemistry and Physiology, 108, pp.86–91.
- Johnson, E.T., Skory, C. & Dowd, P.F., 2014. Identification of a bioactive Bowman-Birk inhibitor from an insect-resistant early maize inbred. Journal of Agricultural and Food Chemistry, 62(24), pp.5458–5465.

- Jongsma, M.A. & Beekwilder, J., 2011. Co-evolution of insect proteases and plant protease inhibitors. Current Protein & Peptide Science, 12(5), pp.437–447.
- Josephrajkumar, A., Chakrabarty, R. & Thomas, G., 2006. Midgut proteases of the cardamom shoot and capsule borer Conogethes punctiferalis (Lepidoptera: Pyralidae) and their interaction with aprotinin. Bulletin of Entomological Research, 96(1), pp.91–98.
- Joshi, R.S., Gupta, V.S. & Giri, A.P., 2014b. Differential antibiosis against Helicoverpa armigera exerted by distinct inhibitory repeat domains of Capsicum annuum proteinase inhibitors. Phytochemistry, 101, pp.16–22.
- Joshi, R.S., Mishra, M., et al., 2014a. The remarkable efficiency of a Pin-II proteinase inhibitor sans two conserved disulfide bonds is due to enhanced flexibility and hydrogen bond density in the reactive site loop. Journal of biomolecular structure & dynamics, 32(1), pp.13–26.
- Juge, N. et al., 1995. Isozyme hybrids within the protruding third loop domain of the barley a-amylase (ß/a) 8-barrel implication for BASI sensitivity and substrate affinity. Federation of European Biochemical Societies Letters, 363, pp.299-303.
- Jurenka, R.A., 2015. Insect physiology. Available at: http://accessscience.com/content/insect-physiology/346000 OP - AccessScience.
- Karasov, W.H., Martinez del Rio, C. & Caviedes-Vidal, E., 2011. Ecological physiology of diet and digestive systems. Annual Review of Physiology, 73, pp.69–93.
- Katoch, R. et al., 2014. Cloning, characterization, expression analysis and inhibition studies of a novel gene encoding Bowman-Birk type protease inhibitor from rice bean. Gene, 546(2), pp.342–351.
- Kaur, R., Gupta, A.K. & Taggar, G.K., 2014. Nitrate reductase and nitrite as additional components of defense system in pigeonpea (Cajanus cajan L.) against Helicoverpa armigera herbivory. Pesticide Biochemistry and Physiology, 115, pp.39–47.
- Kfir, R. (1997). Natural control of the cereal stemborers Busseola fusca and Chilo partellus in South Africa. Insect Science and its application. 17, 1, 61-67.
- Kiggundu, A. et al., 2010. Deleterious effects of plant cystatins against the banana weevil Cosmopolites sordidus. Archives of Insect Biochemistry and Physiology, 73(2), pp.87–105.

- Kim, J. et al., 2012. Insect eggs can enhance wound response in plants: a study system of tomato Solanum lycopersicum L. and Helicoverpa zea Boddie. PloS One, 7(5), p.e37420.
- Klein, C. & Schulz, G.E., 1991. Structure of cyclodextrin glycosyltransferase refined at 2.0 Å resolution. Journal of Molecular Biology, 217, pp.737-50.
- Kluh, I. et al., 2005. Inhibitory specificity and insecticidal selectivity of alpha-amylase inhibitor from Phaseolus vulgaris. Phytochemistry, 66(1), pp.31–39.
- Koepke, T. et al., 2012. Rapid gene-based SNP and haplotype marker development in non-model eukaryotes using 3'UTR sequencing. BMC Genomics, 1186, pp.1471-2164.
- Kondrak, M. et al., 2005. Inhibition of Colorado potato beetle larvae by a locust proteinase inhibitor peptide expressed in potato. Biotechnology Letters, 27(12), pp.829–834.
- Konrad, R. et al., 2008. Potential effects of oilseed rape expressing oryzacystatin-1 (OC-1) and of purified insecticidal proteins on larvae of the solitary bee Osmia bicornis. PloS One, 3(7), p.e2664.
- Kotkar, H.M. et al., 2009. Responses of midgut amylases of Helicoverpa armigera to feeding on various host plants, Journal of Insect Physiology, 55, pp.663–670.
- Kotkar, H.M. et al., 2012. Amylase gene expression patterns in Helicoverpa armigera upon feeding on a range of host plants. Gene, 501(1), pp.1–7.
- Kruidhof, H.M., Allison, J.D. & Hare, J.D., 2012. Abiotic induction affects the costs and benefits of inducible herbivore defenses in Datura wrightii. Journal of Chemical Ecology, 38(10), pp.1215–1224.
- Kuwar, S.S. et al., 2015. Adaptive regulation of digestive serine proteases in the larval midgut of Helicoverpa armigera in response to a plant protease inhibitor. Insect Biochemistry and Molecular Biology, 59, pp.18–29.
- Ladisch, R., Ladisch, S. & Howe, P., 1967. Quinoid Secretions in Grain and Flour Beetles. Nature 214, pp.572–573.
- Lagarda-Diaz, I. et al., 2014. Recognition and binding of the PF2 lectin to a-amylase from Zabrotes subfasciatus (Coleoptera:Bruchidae) larval midgut. J Insect Science, 14,pp. 204.
- Lalitha, S. et al., 2005. Comparison of chemical characteristics of three soybean cysteine proteinase inhibitors. Journal of Agricultural and Food Chemistry, 53(5), pp.1591–1597.

- Laluk, K. & Mengiste, T., 2011. The Arabidopsis extracellular unusual serine protease inhibitor functions in resistance to necrotrophic fungi and insect herbivory. The Plant Journal for Cell and Molecular Biology, 68(3), pp.480–494.
- Langa, G. I., Murrayb, A. W. & Botsteina, D. 2009. The cost of gene expression underlies a fitness trade-off in yeast. Proceedings of the National Academy of SciencesU. S. A, 106, pp.5755–5760.
- Lara, P. et al., 2000. Adaptation of Spodoptera exigua (Lepidoptera: Noctuidae) to barley trypsin inhibitor BTI-CMe expressed in transgenic tobacco. Transgenic Research, 9(3), pp.169–178.
- Laskowski, R. A. et al., 1993. A program to check the stereochemicalquality of protein structures. Journal of Applied Crystallography, 26, pp.283–291.
- Lawrence, S.D., Novak, N.G. & Blackburn, M.B., 2007. Inhibition of proteinase inhibitor transcripts by Leptinotarsa decemlineata regurgitant in Solanum lycopersicum. Journal of Chemical Ecology, 33(5), pp.1041–1048.
- Lazaro, A. et al., 1988. A dimeric inhibitor or insect alpha-amylase from barley. Cloning of the cDNA and identification of the protein. European Journal of Biochemistry / FEBS, 172(1), pp.129–134.
- Lázaro, A. et al., 1998. Signal peptide homology between the sweet protein thaumatin II and unrelated cereal alpha-amylase/trypsin inhibitors. FEBS Letters, 239, pp.147-150.
- Leah R. & Mundy, J. 1989. The bifunctional a-amylase/subtilisin inhibitor of barley: nucleotide sequence and patterns of seed-specific expression. Plant Molecular Biology, 12(6), pp.673-682.
- Leff B., Ramankutty N. & Foley J. A. 2004. Geographic distribution of major crops across the world. Global Biochemical Cycles, 18, GB1009
- Lepelley, M. et al., 2012. Coffee cysteine proteinases and related inhibitors with high expression during grain maturation and germination. BMC Plant Biology, 12, p.31.
- Liao, H. et al., 2007. A trypsin inhibitor from Cassia obtusifolia seeds: isolation, characterization and activity against Pieris rapae. Biotechnology Letters, 29(4), pp.653–658.
- Lin, K.-F. et al., 2007. Structure-based protein engineering for alpha-amylase inhibitory activity of plant defensin. Proteins, 68(2), pp.530–540.

- Lingaraju, M.H. & Gowda, L.R., 2008. A Kunitz trypsin inhibitor of Entada scandens seeds: another member with single disulfide bridge. Biochimica et Biophysica Acta, 1784(5), pp.850–855.
- Lis, L.B. et al., 2011. The carcinogenic effects of benzoquinones produced by the flour beetle. Polish Journal of Veterinary Sciences, 14, pp.159–164.
- Liu, Y.-J. et al., 2006. Solution structure of the plant defensin VrD1 from mung bean and its possible role in insecticidal activity against bruchids. Proteins, 63(4), pp.777–786.
- Lomate, P.R. & Hivrale, V.K., 2012. Wound and methyl jasmonate induced pigeon pea defensive proteinase inhibitor has potency to inhibit insect digestive proteinases. Plant Physiology and Biochemistry?: PPB / Societe Francaise de Physiologie Vegetale, 57, pp.193–199.
- Lopes, K.V. et al., 2010. Guedes, Modified alpha-amylase activity among insecticide-resistant and -susceptible strains of the maize weevil, Sitophilus zeamais. Journal of Insect Physiology, 56, pp.1050-1057.
- Lovell, S.C. et al., 2003. Structure validation by Ca geometry: ?, ? and Cß deviation. Proteins 50, pp.437-450.
- Lu, S. et al., 1999. Solution structure of the major alpha-amylase inhibitor of the crop plant amaranth. The Journal of Biological Chemistry, 274(29), pp.20473–20478.
- Luo, M. et al., 2009. Overexpression of a weed (Solanum americanum) proteinase inhibitor in transgenic tobacco results in increased glandular trichome density and enhanced resistance to Helicoverpa armigera and Spodoptera litura. International Journal of Molecular Sciences, 10(4), pp.1896–1910.
- Luo, M. et al., 2012. The characterization of SaPIN2b, a plant trichome-localized proteinase inhibitor from Solanum americanum. International Journal of Molecular Sciences, 13(11), pp.15162–15176.
- Lüthi, C. et al., 2013. Resistance of aAI-1 transgenic chickpea (Cicer arietinum) and cowpea (Vigna unguiculata) dry grains to bruchid beetles (Coleoptera: Chrysomelidae). Bulletin of Entomological Research, 103, pp.373-381.
- Luthi, C., Alvarez-Alfageme, F. & Romeis, J., 2013b. Impact of alphaAI-1 expressed in genetically modified cowpea on Zabrotes subfasciatus (Coleoptera: Chrysomelidae) and its parasitoid, Dinarmus basalis (Hymenoptera: Pteromalidae). PloS One, 8(6), p.e67785.

- Luthi, C., Alvarez-Alfageme, F., Ehlers, J.D., et al., 2013a. Resistance of alphaAI-1 transgenic chickpea (Cicer arietinum) and cowpea (Vigna unguiculata) dry grains to bruchid beetles (Coleoptera: Chrysomelidae). Bulletin of Entomological Research, 103(4), pp.373–381.
- Ma, L. et al., 2014.High throughput characterizations of poly(A) site choice in plants. Methods, 67, pp.74-83.
- Macedo, M.L.R. et al., 2004. A Kunitz-type inhibitor of coleopteran proteases, isolated from Adenanthera pavonina L. seeds and its effect on Callosobruchus maculatus. Journal of Agricultural and Food Chemistry, 52(9), pp.2533–2540.
- Macedo, M.L.R. et al., 2009. Properties of a Kunitz-type trypsin inhibitor from Delonix regia seeds against digestive proteinases of Anagasta kuehniella (Z.) and Corcyra cephalonica (S.) (Lepidoptera: Pyralidae). Protein and Peptide Letters, 16(12), pp.1459–1465.
- Macedo, M.L.R. et al., 2010. Adenanthera pavonina trypsin inhibitor retard growth of Anagasta kuehniella (Lepidoptera: Pyralidae). Archives of Insect Biochemistry and Physiology, 73(4), pp.213–231.
- Macedo, M.L.R. et al., 2011. Practical and theoretical characterization of Inga laurina Kunitz inhibitor on the control of Homalinotus coriaceus. Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology, 158(2), pp.164–172.
- Macedo, M.L.R., Diz Filho, E.B.S., et al., 2011a. A trypsin inhibitor from Sapindus saponaria L. seeds: purification, characterization, and activity towards pest insect digestive enzyme. The Protein Journal, 30(1), pp.9–19.
- Macedo, M.L.R., Freire, M. das G.M., et al., 2011b. Bioinsecticidal activity of Talisia esculenta reserve protein on growth and serine digestive enzymes during larval development of Anticarsia gemmatalis. Comparative Biochemistry and Physiology. Toxicology & Pharmacology?: CBP, 153(1), pp.24–33.
- MacGregor, E.A. & Svensson, B., 1989. A super-secondary structure predicted to be common to several a-1, 4-D-glucan-cleaving enzymes. Biochemical Journal, 259, pp.145-152.
- MacGregor, E.A., 1988. a-Amylase structure and activity. Journal of Protein Chemistry, 7, pp.399-415.

- MacGregor, E.A., Janecek, S. & Svensson B., 2001. Relationship of sequence and structure to specificity in the a-amylase family of enzymes. Biochimica et Biophysica Acta, 1546, pp.1-20.
- Machado, S.W. et al., 2013. Purification of a Kunitz-type inhibitor from Acacia polyphylla DC seeds: characterization and insecticidal properties against Anagasta kuehniella Zeller (Lepidoptera: Pyralidae). Journal of Agricultural and Food Chemistry, 61(10), pp.2469–2478.
- Mahajan, N.S. et al., 2013. Plasticity of protease gene expression in Helicoverpa armigera upon exposure to multi-domain Capsicum annuum protease inhibitor. Biochimica et Biophysica Acta, 1830(6), pp.3414–3420.
- Maheswaran, G. et al., 2007. A proteinase inhibitor from Nicotiana alata inhibits the normal development of light-brown apple moth, Epiphyas postvittana in transgenic apple plants. Plant Cell Reports, 26(6), pp.773–782.
- Mandal, S. et al., 2002. Precursor of the inactive 2S seed storage protein from the Indian mustard Brassica juncea is a novel trypsin inhibitor. Charaterization, post-translational processing studies, and transgenic expression to develop insect-resistant plants. The Journal of Biological Chemistry, 277(40), pp.37161–37168.
- Marinho-Prado, J.S. et al., 2012. Enzymatic response of the eucalypt defoliator Thyrinteina arnobia (Stoll) (Lepidoptera: Geometridae) to a bis-benzamidine proteinase Inhibitor. i. Neotropical Entomology, 41(5), pp.420–425.
- Martins, J.C. et al., 2001. Solution structure of the main alpha-amylase inhibitor from amaranth seeds. Eur Journal of Biochemistry, 268, pp. 2379-2389.
- Matsuura, Y. et al., 1984. Structure and possible catalytic residues of Taka-amylase A. The Journal of Biochemistry, 95, pp.697-702.
- Mehrabadi, M., Bandani, A.R. & Saadati, F., 2010. Inhibition of Sunn pest, Eurygaster integriceps, alpha-amylases by alpha-amylase inhibitors (T-alphaAI) from Triticale. Journal of Insect Science (Online), 10, p.179.
- Meireles, E.A. et al., 2009. Digestion of starch granules from maize, potato and wheat by larvae of the the yellow mealworm, Tenebrio molitor and the Mexican bean weevil, Zabrotes subfasciatus. Journal of Insect Science (Online), 9, p.43.
- Micheelsen, P.O. et al., 2008. Structural and mutational analyses of the interaction between the barley alpha-amylase/subtilisin inhibitor and the subtilisin savinase reveal a novel mode of inhibition. Journal of Molecular Biology, 380, pp.681-690.

- Mirkov, T.E. et al., 1995. Location of the active site of the bean alpha-amylase inhibitor and involvement of a Trp, Arg, Tyr triad. Glycobiology, 5(1), pp.45-50.
- Mishra, M. et al., 2010. Interaction of recombinant CanPIs with Helicoverpa armigera gut proteases reveals their processing patterns, stability and efficiency. Proteomics, 10(15), pp.2845–2857.
- Mishra, M. et al., 2012. Stress inducible proteinase inhibitor diversity in Capsicum annuum. BMC Plant Biology, 12, pp.217.
- Mishra, M. et al., 2015. Ecological turmoil in evolutionary dynamics of plant-insect interactions: defense to offence. Planta, 242(4), pp.761–771.
- Mistry, J., Bateman, A. & Finn, R.B., 2007. Predicting active site residue annotations in the Pfam database. BMC Bioinformatics 8, p.298.
- Molina, D. et al., 2014. Effects of the aspartic protease inhibitor from Lupinus bogotensis seeds on the growth and development of Hypothenemus hampei: an inhibitor showing high homology with storage proteins. Phytochemistry, 98, pp.69–77.
- Morton, R.L. et al., 2000. Bean alpha amylase inhibitor 1 in transgenic peas (Pisum sativum) provides complete protection from pea weevil (Bruchus pisorum) under field conditions. Proceedings of the National Academy of Sciences USA, 97, pp.3820-3825.
- Morton, R.L. et al., 2000. Bean alpha-amylase inhibitor 1 in transgenic peas (Pisum sativum) provides complete protection from pea weevil (Bruchus pisorum) under field conditions. Proceedings of the National Academy of Sciences USA., 97, pp.3820-3825.
- Mulligan, E.A. et al., 2010. Characterisation of adult green lacewing (Chrysoperla carnea) digestive physiology: impact of a cysteine protease inhibitor and a synthetic pyrethroid. Pest Management Science, 66(3), pp.325–336.
- Nagarkatti, S. & Prakash, A. 1974. Rearing Heliothis armigera (Hubn.) on artificial diet, Technical Bulletin, 17. Commonwealth Institute of Biological Control, Bangalore .
- Nagashima, T. et al., 1994. Site-directed mutagenesis of catalytic active-site residues of Taka-amylase A. Bioscience, Biotechnology, and Biochemistry, 56, pp.207-210.

- Nahoum, V. et al., 1999. A plant-seed inhibitor of two classes of alpha-amylases: X-ray analysis of Tenebrio molitor larvae alpha-amylase in complex with the bean Phaseolus vulgaris inhibitor. Acta Crystallographica Section D., 55, pp.360-372.
- Nahoum, V., et al., 1999. Plant-seed inhibitor of two classes of alpha-amylases: X-ray analysis of Tenebrio molitor larvae alpha-amylase in complex with the bean Phaseolus vulgaris inhibitor. Acta Crystallographica Section-D, 55, pp.360-362.
- Nakaguchi, T. et al., 1997. Structural characterization of an alpha-amylase inhibitor from a wild common bean (Phaseolus vulgaris): insight into the common structural features of leguminous alpha-amylase inhibitors. The Journal of Biochemistry, 121, pp.350-354.
- Nakajima, R., Imanaka, T. & Aiba, S., 1986. Comparison of amino acid sequences of eleven different a-amylases. Applied Microbiology and Biotechnology, 23, pp.355-60.
- Napoleao, T.H. et al., 2012. Effect of Myracrodruon urundeuva leaf lectin on survival and digestive enzymes of Aedes aegypti larvae. Parasitology Research, 110(2), pp.609–616.
- Naseri, B. et al., 2010. Digestive proteolytic and amylolytic activities of Helicoverpa armigera in response to feeding on different soybean cultivars. Pest Management Science, 66, pp.1316–1323.
- Ngernyuang, N. et al., 2011. Cloning and expression analysis of the Bombyx mori a-amylase gene (Amy) from the indigenous Thai silkworm strain, Nanglai. Journal of Insect Science, 11, 38 (available online: insectscience.org/11.38).
- Nguyen, P.Q. et al., 2014. Discovery and characterization of pseudocyclic cystine-knot a-amylase inhibitors with high resistance to heat and proteolytic degradation. FEBS Journal, 281, pp.4351-4366.
- Nielsen, P.K. et al., 2004. Barley a-amylase/subtilisin inhibitor: structure, biophysics and protein engineering. Biochimica et Biophysica Acta,1696, pp.157-164.
- Nogueira, F.C.S. et al., 2012. Global proteome changes in larvae of Callosobruchus maculatus Coleoptera:Chrysomelidae:Bruchinae) following ingestion of a cysteine proteinase inhibitor. Proteomics, 12(17), pp.2704–2715.
- O'Connor C.M. & McGeeney, K.F. 1981. Isolation and characterization of four inhibitors from wheat flour which display differential inhibition specificities for

human salivary and human pancreatic alpha-amylases. Biochimica et Biophysica Acta, 658, pp.387-396.

- Oganesyan, N., Kim, S. & Kim, R. 2005. On-column protein refolding for crystallization. Journal of Structural and Functional Genomics, 6, pp.177-182.
- Ohtsubo, K. & Richardson, M., 1992. The amino acid sequence of a 20 kDa bifunctional subtilisin/alpha-amylase inhibitor from bran of rice (Oryza sativa L.) seeds. FEBS Letters, 309(1), pp.68–72.
- Oliveira, A.S. et al., 2009. Two Kunitz-type inhibitors with activity against trypsin and papain from Pithecellobium dumosum seeds: purification, characterization, and activity towards pest insect digestive enzyme. Protein and Peptide Letters, 16(12), pp.1526–1532.
- Oliveira-Neto, et al., 2003. Molecular cloning of alpha-amylases from cotton boll weevil, Anthonomus grandis and structural relations to plant inhibitors: an approach to insect resistance. Journal of Protein Chemistry, 1, pp.77-87.
- Oparin, P.B. et al., 2012. Buckwheat trypsin inhibitor with helical hairpin structure belongs to a new family of plant defence peptides. The Biochemical Journal, 446(1), pp.69–77.
- Outchkourov, N.S. et al., 2003. Expression of sea anemone equistatin in potato. Effects of plant proteases on heterologous protein production. Plant Physiology, 133(1), pp.379–390.
- Paes, N.S. et al., 2000. The effect of arcelin-1 on the structure of the midgut of bruchid larvae and immunolocalization of the arcelin protein. Journal of Insect Physiology, 46, pp.393-402.
- Paixao, G.P. et al., 2013. Biochemical responses of Anticarsia gemmatalis (Lepidoptera: Noctuidae) in soybean cultivars sprayed with the protease inhibitor berenil. Journal of Agricultural and Food Chemistry, 61(34), pp.8034–8038.
- Parthasarathy, R. & Gopinathan, K.P., 2005. Comparative analysis of the development of the mandibular salivary glands and the labial silk glands in the mulberry silkworm, Bombyx mori. Gene Exprression Patterns, 5, pp.323–339.
- Patankar, A.G. et al., 2001. Complexity in specificities and expression of Helicoverpa armigera gut proteinases explains polyphagous nature of the insect pest. Insect Biochemistry and Molecular Biology, 31(4-5), pp.453–464.

- Pauchet, Y. et al., 2008. Mapping the larval midgut lumen proteome of Helicoverpa armigera, a generalist herbivorous insect. Journal of Proteome Research, 7, pp.1629–1639.
- Paulillo, L.C.M.S. et al., 2012. Evaluation of in vitro and in vivo effects of semipurified proteinase inhibitors from Theobroma seeds on midgut protease activity of Lepidopteran pest insects. Archives of Insect Biochemistry and Physiology, 81(1), pp.34–52.
- Pedra, J.H.F. et al., 2003. Transcriptome analysis of the cowpea weevil bruchid: identification of putative proteinases and alpha-amylases associated with food breakdown. Insect Molecular Biology, 12(4), pp.405–412.
- Pelegrini, P. B., et al., 2006. Structure and enzyme properties of Zabrotes subfasciatus a-Amylase. Archives of Insect Biochemistry and Physiology, 61, pp.77-86.
- Pereira, P.J. et al., 1999. Specific inhibition of insect alpha-amylases: yellow meal worm alpha-amylase in complex with the amaranth alpha-amylase inhibitor at 2.0 A resolution. Structure (London, England?: 1993), 7(9), pp.1079–1088.
- Pernas, M. et al., 1998. A chestnut seed cystatin differentially effective against cysteine proteinases from closely related pests. Plant Molecular Biology, 38(6), pp.1235–1242.
- Perry, G.H., et al., 2007. Diet and the evolution of human amylase gene copy number variation. Nature Genetics, 39, pp.1256–1260.
- Petrucci, T. et al., 1976. Further characterization studies of the alpha-amylase protein inhibitor of gel electrophoretic mobility 0.19 from the wheat kernel. Biochimica et Biophysica Acta, 420(2), pp.288–297.
- Pimentel D, 2009. Pest control in world agriculture. Agricultural Sciences, Volume II, EOLSS publishers Co. Ltd., Oxford, United Kingdom.
- Podoler, H. & Applebaum S.W., 1971. The a-amylase of beetle, Callosobruchus chinensis. Biochemical Journal, 121, pp.321-325.
- Pontual, E.V. et al., 2012. Effect of Moringa oleifera flower extract on larval trypsin and acetylcholinesterase activities in Aedes aegypti. Archives of Insect Biochemistry and Physiology, 79(3), pp.135–152.
- Prigent, S., Renard, E. & Cariou, M.L. 2003. Electrophoretic mobility of amylase in Drosophilids indicates adaptation to ecological diversity. Genetica, 119, pp.133-145.

- Pueyo, J.J., Hunt, D.C. & Chrispeels, M.J. 1993. Activation of bean (Phaseolus vulgaris) alpha-amylase inhibitor requires proteolytic processing of the proprotein. Plant Physiology, 101, pp.1341-1348.
- Pytelkova, J. et al., 2009. Digestive alpha-amylases of the flour moth Ephestia kuehniella-adaptation to alkaline environment and plant inhibitors. The FEBS Journal, 276(13), pp.3531–3546.
- Qian, M., Haser, R. & Payan, F., 1993. Structure and molecular model refinement of pig pancreatic a-amylase at 2.1 Å resolution Journal of Molecular Biology, 231, pp.785-799.
- Quilis, J. et al., 2014. Inducible expression of a fusion gene encoding two proteinase inhibitors leads to insect and pathogen resistance in transgenic rice. Plant Biotechnology Journal, 12(3), pp.367–377.
- Rahbe, Y. et al., 2003. Toxicity to the pea aphid Acyrthosiphon pisum of anti-chymotrypsin isoforms and fragments of Bowman-Birk protease inhibitors from pea seeds. Insect Biochemistry and Molecular Biology, 33(3), pp.299–306.
- Ramachandran, G. N., Ramakrishnan, C. & Sasisekharan, V. 1963. Stereochemistry of polypeptide chain configurations. Journal of Molecular Biology, 7, pp.795–99.
- Ramos, V. da S. et al., 2008. Purification and characterization of a trypsin inhibitor from Plathymenia foliolosa seeds. Journal of Agricultural and Food Chemistry, 56(23), pp.11348–11355.
- Ramos, V. da S. et al., 2012. Molecular cloning and insecticidal effect of Inga laurina trypsin inhibitor on Diatraea saccharalis and Heliothis virescens. Comparative Biochemistry and Physiology. Toxicology & Pharmacology?: CBP, 156(3-4), pp.148–158.
- Ranjbar, M., Zibaee, A. & Sendi, J.J., 2014. A trypsin-like proteinase in the midgut of Ectomyelois ceratoniae Zeller (Lepidoptera: Pyralidae): purification, characterization, and host plant inhibitors. Archives of Insect Biochemistry and Physiology, 85(1), pp.1–12.
- Ratan, V. 2004.Handbook of Human physiology, Jaypee Brothers Medical Pub (p) Ltd.
- Raubenheimer, D. & Simpson, S.J., 1999. Integrating nutrition a geometrical approach. Entomologia Experimentalis et Applicata, 91, pp.67–82.

- Robert, X. & Gouet, P. 2014. Deciphering key features in protein structures with the new ENDscript server. Nucl. Acids Research, 42, pp.320-324.
- Robert, X. et al., 2003. The structure of barley a-amylase isozyme 1 reveals a novel role of domain C in substrate recognition and binding: a pair of sugar tongs. Structure, 11, pp.973-984.
- Robertson, M. et al., 1989. Induction of alpha-amylase inhibitor synthesis in barley embryos and young seedlings by abscisic Acid and dehydration stress. Plant Physiology, 91, pp.415-420.
- Rodenburg, K.W. et al., 1994. Domain B protruding at the third strand of the a/ß barrel in barley a-amylase confers distinct isozyme-specific properties. European Journal of Biochemistry, 221, pp.277–284.
- Rodenburg, K.W. et al., 1995. Arg-27, Arg-127 and Arg-155 in the beta-trefoil protein barley alpha-amylase/subtilisin inhibitor are interface residues in the complex with barley alpha-amylase 2. The Biochem Journal, 309, pp.969-976.
- Rogers, J.C., 1985. Conserved amino acid sequence domains in alpha-amylases from plants, mammals, and bacteria. Biochemical and Biophysical Research Communications, 128, pp.470-476.
- Roush, D.K. and McKenzie, J.A. (1987). Ecological genetics of insecticide and acaricide resistance. Annual Review of Entomology. 32: 361-380.
- Royo, J. et al., 1999. Antisense-mediated depletion of a potato lipoxygenase reduces wound induction of proteinase inhibitors and increases weight gain of insect pests. Proceedings of the National Academy of Sciences of the United States of America, 96(3), pp.1146–1151.
- Rufino, F.P.S. et al., 2013. Inhibitory effects of a Kunitz-type inhibitor from Pithecellobium dumosum (Benth) seeds against insect-pests' digestive proteinases. Plant Physiology and Biochemistry?: PPB / Societe Francaise de Physiologie Vegetale, 63, pp.70–76.
- Ryan, C.A. & Pearce, G., 2003. Systemins: a functionally defined family of peptide signals that regulate defensive genes in Solanaceae species. Proceedings of the National Academy of Sciences of the United States of America, 100 Suppl , pp.14577–14580.
- Saadati, F. & Bandani, A.R., 2011. Effects of serine protease inhibitors on growth and development and digestive serine proteinases of the Sunn pest, Eurygaster integriceps. Journal of Insect Science (Online), 11, p.72.

- Sakai, T. et al., 2004. Characterization of crustacean cardio active peptide as a novel insect midgut factor: isolation, localization, and stimulation of alpha-amylase activity and gut contraction. Endocrinology, 145, pp.5671-5678.
- Sakai, T., Satake, H. & Takeda M. 2006. Nutrient-induced a-amylase and protease activity is regulated by crustacean cardioactive peptide (CCAP) in the cockroach midgut. Peptides, 27, pp.2157-2164.
- Salama, M.S., Schouest, L.P. & Miller, T.A., 1992. Effect of diet on the esterase patterns in the hemolymph of the corn earworm and the tobacco budworm (Lepidoptera: Noctuidae). Journal of Economic Entomology, 85, pp.1079–1087.
- Sali, A. & Blundell, T.L. 1993. Comparative protein modelling by satisfaction of spatial restraints. Journal of Molecular Biology, 234, pp.779-815.
- Saltzmann, K.D. et al., 2006. Characterization of BGTG-1, a tergal gland-secreted alpha-amylase, from the German cockroach, Blattella germanica (L.). Insect Molecular Biology, 15, pp425–433.
- Sanchez de la Hoz, P., Castagnaro, A. & Carbonero, P. 1994. Sharp divergence between wheat and barley at loci encoding novel members of the trypsin/alpha-amylase inhibitors family. Plant Molecular Biology, 26, pp.1231-1236.
- Sanchez-Monge, R. et al., 1989. New dimeric inhibitor of heterologous alpha-amylases encoded by a duplicated gene in the short arm of chromosome 3B of wheat (Triticum aestivum L.). European Journal of Biochemistry / FEBS, 183(1), pp.37–40.
- Sarate, P.J. et al., 2012 Developmental and digestive flexibilities in the midgut of a polyphagous insect, the cotton bollworm, Helicoverpa armigera. Journal of Insect Science, 12, pp.42.
- Schaeffer, S. W., Bernhardt, M. J. & Anderson, W. W. 2003. Evolutionary rearrangement of the amylase genomic regions between Drosophila melanogaster and Drosophila pseudoobscura. Journal of Heredity, 94, pp.464–471.
- Senthilkumar, R., Cheng, C.-P. & Yeh, K.-W., 2010. Genetically pyramiding protease-inhibitor genes for dual broad-spectrum resistance against insect and phytopathogens in transgenic tobacco. Plant Biotechnology Journal, 8(1), pp.65–75.
- Shade, R.E. et al., 1994. Transgenic Pea Seeds Expressing the a-Amylase Inhibitor of the Common Bean are Resistant to Bruchid Beetles. Nature Biotechnology, 12, pp.793–796.

- Shi, Z.D. et al., 2014. BASI, a potent small molecular inhibitor, inhibits glioblastoma progression by targeting microRNA-mediated ß-catenin signaling. CNS Neuroscience & Therapeutics, 9, pp.830-839.
- Silano, V. & Zahnley, J.C., 1978. Association of Tenebrio molitor L. alpha-amylase with two protein inhibitors--one monomeric, one dimeric--from wheat flour. Differential scanning calorimetric comparison of heat stabilities. Biochimica et Biophysica Acta, 533(1), pp.181–185.
- Silva, C.P., et al., 2001. Digestion of legume starch granules by larvae of Zabrotes subfasciatus (Coleoptera: bruchidae) and the induction of alpha-amylases in response to different diets. Insect Biochemistry and Molecular Biology, 31, pp.41–50.
- Silva, D.P. et al., 2007. Identification of an alpha-amylase inhibitor from Pterodon pubescens with ability to inhibit cowpea weevil digestive enzymes. Journal of Agricultural and Food Chemistry, 55(11), pp.4382–4387.
- Simon-Delso, N. et al., 2015. Systemic insecticides (neonicotinoids and fipronil): trends, uses, mode of action and metabolites. Environmental Science and Pollution Research International, 22(1), pp.5–34.
- Singh D. K., 2007. Insecticidal methods of pest control. Applied Entomology, New Delhi
- Singh, D., Jamal, F. & Pandey, P.K., 2014. Kinetic assessment and effect on developmental physiology of a trypsin inhibitor from Eugenia jambolana (Jambul) seeds on Helicoverpa armigera (Hubner). Archives of Insect Biochemistry and Physiology, 85(2), pp.94–113.
- Smid, I. et al., 2013. Inhibition of the growth of colorado potato beetle larvae by macrocypins, protease inhibitors from the parasol mushroom. Journal of Agricultural and Food Chemistry, 61(51), pp.12499–12509.
- Smigocki, A.C. et al., 2013. Pest protection conferred by a Beta vulgaris serine proteinase inhibitor gene. PloS One, 8(2), p.e57303.
- Sogaard, M. et al., 1993. Site-directed mutagenesis of histidine 93, aspartic acid 180, glutamic acid 205, histidine 290, and aspartic acid 291 at the active site and tryptophan 279 at the raw starch binding site in barley a-amylase. The Journal of Biological Chemistry, 268, pp.22480-22484.
- Sousa da Silva, A. W. & Vranken, W. F. 2012. ACPYPE - AnteChamberPYthon Parser interface. BMC Research Notes, 5, pp.367.

- Sousa-Majer, M.J. de et al., 2004. Response to water deficit and high temperature of transgenic peas (Pisum sativum L.) containing a seed-specific alpha-amylase inhibitor and the subsequent effects on pea weevil (Bruchus pisorum L.) survival. Journal of Experimental Botany, 55(396), pp.497–505.
- Southgate, B.J., 1979. Biology of the Bruchidae. Annual Review of Entomology, 24, pp.449–473.
- Srinivasan, A. et al., 2005a. trypsin inhibitor from chickpea (Cicer arietinum L.) that exerts anti-metabolic effect on podborer (Helicoverpa armigera) larvae. Plant Molecular Biology, 57, pp.359–374.
- Srinivasan, A. et al., 2005a. Podborer (Helicoverpa armigera Hubn.) does not show specific adaptations in gut proteinases to dietary Cicer arietinum kunitz proteinase inhibitor. Journal of Insect Physiology, 51, pp.268–1276.
- Srinivasan, A. et al., 2005b. A Kunitz trypsin inhibitor from chickpea (Cicer arietinum L.) that exerts anti-metabolic effect on podborer (Helicoverpa armigera) larvae. Plant Molecular Biology, 57, pp.359–374.
- Srinivasan, T., Kumar, K.R.R. & Kirti, P.B., 2009. Constitutive expression of a trypsin protease inhibitor confers multiple stress tolerance in transgenic tobacco. Plant & Cell Physiology, 50(3), pp.541–553.
- Stafford-Banks, C.A. et al., 2014. Analysis of the salivary gland transcriptome of Frankliniella occidentalis. PloS One, 9(4), p.e94447.
- Stevens, J.A. et al., 2013. The impact of ingested potato type II inhibitors on the production of the major serine proteases in the gut of Helicoverpa armigera. Insect Biochemistry and Molecular Biology, 43(2), pp.197–208.
- Strobl, S. et al., 1998. A novel strategy for inhibition of alpha-amylases: yellow meal worm alpha-amylase in complex with the Ragi bifunctional inhibitor at 2.5 A resolution. Structure, 6(7), pp.911-921.
- Strobl, S. et al., 1998a. Crystal structure of yellow meal worm a-amylase at 1.64 Å resolution. Journal of Molecular Biology, 278, pp.617-628.
- Sui, Y., Wang, J. & Zhao, X., 2008. Effects of classical insect hormones on the expression profiles of a lipase gene from the cotton bollworm (Helicoverpa armigera). Insect Molecular Biology, 17, pp.523–529.
- Sumikawa, J.T. et al., 2010. The defensive functions of plant inhibitors are not restricted to insect enzyme inhibition. Phytochemistry, 71(2-3), pp.214–220.

- Suzuki, K., Ishimoto, M. & Kitamura, K., 1994. cDNA sequence and deduced primary structure of an alpha-amylase inhibitor from a bruchid-resistant wild common bean. Biochimica et Biophysica Acta, 1206(2), pp.289–291.
- Suzuki, Y. et al., 1987. A strong correlation between the increase in number of proline residues and the rise in thermostability of five Bacillus oligo-1, 6-glucosidases. Applied Microbiology and Biotechnology, 26, pp.546-551.
- Suzuki, Y., 1989. A general principle of increasing protein thermostability. Proceedings of the Japan Academy Ser B Physical and Biological Sciences, 65, pp.146-148.
- Svensson, B. et al., 2004. Proteinaceous a-amylase inhibitors. Biochimica et Biophysica Acta, 1696, pp.145-156.
- Svensson, B., 1988. Regional distant sequence homology between amylases, a-glucosidases and transglucanosylases. Federation of European Biochemical Societies Letters, 230, pp.72-76.
- Svensson, B., 1994. Protein engineering in the a-amylase family: catalytic mechanism, substrate specificity, and stability. Plant Molecular Biology, 25, pp.141-57.
- Takeuchi, T. 1979. Human amylase isoenzymes separated on concanavalin A—Sepharose. Clinical Chemistry, 8, pp.1406-1410.
- Talwar, G.P. & Shrivastav, L.M. 2004. Textbook of Biochemistry and Human biology, Third ed., Prentice-Hall of India Pvt. Ltd.
- Tamhane, V.A. et al., 2007. Diverse forms of Pin-II family proteinase inhibitors from Capsicum annuum adversely affect the growth and development of Helicoverpa armigera. Gene, 403(1-2), pp.29–38.
- Tamir, S. et al., 1996. Isolation, characterization, and properties of a trypsin-chymotrypsin inhibitor from amaranth seeds. Journal of Protein Chemistry, 15(2), pp.219–229.
- Tamura, K. et al., 2013. MEGA 6: Molecular Evolutionary genetics analysis version6.0. Molecular Biology and Evolution 30, pp.2725-2729.
- Taylor, M.A. & Lee, M.J., 1997. Trypsin isolated from the midgut of the tobacco hornworm, Manduca sexta, is inhibited by synthetic pro-peptides in vitro. Biochemical and Biophysical Research Communications, 235(3), pp.606–609.

- Tedeschi, F. et al., 2012. Wheat Subtilisin/Chymotrypsin Inhibitor (WSCI) as a scaffold for novel serine protease inhibitors with a given specificity. Molecular BioSystems, 8(12), pp.3335–3343.
- Telang, M. et al., 2003. Bitter gourd proteinase inhibitors: potential growth inhibitors of Helicoverpa armigera and Spodoptera litura. Phytochemistry, 63(6), pp.643–652.
- Terra, W.R. & Ferriera, C., 1994. Insect digestive enzymes: properties, compartmentalization and function. Comparative Biochemistry and Physiology, 109, pp.1–62.
- Thayumanavan, B. & Sadasivam, S., 1984. Physicochemical basis for the preferential uses of certain rice varieties. Plant Foods Human Nutrition, 253.
- Titarenko, E. & Chrispeels, M.J., 2000. cDNA cloning, biochemical characterization and inhibition by plant inhibitors of the alpha-amylases of the Western corn rootworm, Diabrotica virgifera virgifera. Insect Biochemistry and Molecular Biology, 30(10), pp.979–990.
- Titarenko, E. & Chrispeels, M.J., 2000. cDNA cloning, biochemical characterization and inhibition by plant inhibitors of the a-amylases of the Western corn rootworm, Diabrotica virgifera virgifera. Insect Biochemistry and Molecular Biology, 30, pp.979–990.
- Toda, H., Kondo, K. & Narita K., 1982. The Complete Amino Acid Sequence of Taka-Amylase A. Proceedings of the Japan Academy, 58(B), pp.8-12.
- Tovchigrechko A & Vakser, I.A. 2006. GRAMM-X public web server for protein-protein docking. Nucleic Acids Research, 34, pp.310-324.
- Trebbi, D. & McGrath, M., 2004. Fluorometric sucrose evaluation for sugar beet. Journal of Agricultural and Food Chemistry, 52, pp.6862–6867.
- Trott, O. & Olson, A. J. 2010. AutoDockVina: improving the speed and accuracy of docking with a new scoring function, efficient optimization and multithreading. Journal of Computational Chemistry, 31, pp.455-461.
- Turra, D. & Lorito, M., 2011. Potato type I and II proteinase inhibitors: modulating plant physiology and host resistance. Current Protein & Peptide Science, 12(5), pp.374–385.
- Valencia, A. et al., 2000. a-Amylases of the coffee berry borer (Hypothenemus hampei) and their inhibition by two plant amylase inhibitors. Insect Biochemistry and Molecular Biology, 30, pp.207-213.

- Valencia-Jimenez, A. et al., 2008. Digestive a-amylases from Tecia solanivora larvae (Lepidoptera: Gelechiidae): response to pH, temperature and plant amylase inhibitors. Bulletin of entomological research, 98, pp.575–579.
- Valencia-Jimenez, A. et al., 2008a. Digestive alpha-amylases from Tecia solanivora larvae (Lepidoptera: Gelechiidae): response to pH, temperature and plant amylase inhibitors. Bulletin of Entomological Research, 98(6), pp.575–579.
- Valencia-Jimenez, A., Arboleda Valencia, J.W. & Grossi-De-Sa, M.F., 2008b. Activity of alpha-amylase inhibitors from Phaseolus coccineus on digestive alpha-amylases of the coffee berry borer. Journal of Agricultural and Food Chemistry, 56(7), pp.2315–2320.
- Vallee, F. et al., 1998. Barley alpha-amylase bound to its endogenous protein inhibitor BASI: crystal structure of the complex at 1.9 A resolution. Structure (London, England?: 1993), 6(5), pp.649–659.
- Vatanparast, M. & Hosseininaveh, V., 2010. Digestive amylase and pectinase activity in the larvae of alfalfa weevil Hypera postica (Coleoptera: Curculionidae). Journal of the Entomological Research, 40, pp.328–335.
- Vieira Bard, G.C. et al., 2015. Characterization of Peptides from Capsicum annuum Hybrid Seeds with Inhibitory Activity Against alpha-Amylase, Serine Proteinases and Fungi. The Protein Journal, 34(2), pp.122–129.
- Vijayan, S. et al., 2012. Enhanced antifungal and insect a-amylase inhibitory activities of Alpha-TvD1, a peptide variant of Tephrosia villosa defensin (TvD1) generated through in vitro mutagenesis. Peptides, 33, pp.220-229.
- Vinokurov, K.S. et al., 2006. Diversity of digestive proteinases in Tenebrio molitor (Coleoptera: Tenebrionidae) larvae. Comparative Biochemistry and Physiology - Part B, 145, pp.126-137.
- Volpicella, M. et al., 2009. Identification and characterization of protease inhibitors in Diplotaxis species. Plant Physiology and Biochemistry?: PPB / Societe Francaise de Physiologie Vegetale, 47(3), pp.175–180.
- Volpicella, M. et al., 2010. Analysis by phage display selection and site-directed retromutagenesis of the Mustard Trypsin Inhibitor 2 reactive site. Journal of Plant Physiology, 167(17), pp.1507–1511.
- Wang, J.R. et al., 2008. SNP and haplotype identification of the wheat monomeric alpha-amylase inhibitor genes. Genetica, 134, pp.277-285.

- Wang, S., Magoulas, C. & Hickey, D., 1999. Concerted evolution within a trypsin gene cluster in Drosophila. Molecular Biology and Evolution, 16(9), pp.1117–1124.
- Watanabe, K., Kitamura, K. & Suzuki, Y., 1996. Analysis of the critical sites for protein thermostabilization by proline substitution in oligo-1, 6-glucosidase from Bacillus coagulans ATCC 7050 and the evolutionary consideration of proline residues. Applied and Environmental Microbiology, 62, pp.2066-2073.
- Webb, B. & Sali, A. 2014. Comparative Protein Structure Modeling Using MODELLER. Curr. Protoc. Bioinformatics, 47, 5.6., pp.1–32.
- Weidlich, S. et al., 2013. Woodring, Regulation of amylase, cellulase and chitinase secretion in the digestive tract of the two-spotted field cricket, Gryllus bimaculatus. Archives of Insect Biochemistry and Physiology, 83, pp.69–85.
- Weidlich, S., Hoffmann, K.H. & Woodring, J., 2015. Secretion of lipases in the digestive tract of cricket Gryllus bimaculatus. Archives of Insect Biochemistry and Physiology.
- Wiegand, G., Epp, O. & Huber, R. 1995. The crystal structure of porcine pancreatic alpha-amylase in complex with the microbial inhibitor Tendamistat. Journal of Molecular Biology, 247, pp.99-110.
- Wilhite, S.E. et al., 2000. Inhibition of cysteine and aspartyl proteinases in the alfalfa weevil midgut with biochemical and plant-derived proteinase inhibitors. Insect Biochemistry and Molecular Biology, 30(12), pp.1181–1188.
- Wisessing, A. et al., 2010. Biochemical characterization of the alpha-amylase inhibitor in mungbeans and its application in inhibiting the growth of Callosobruchus maculatus. Journal of Agricultural and Food Chemistry, 58(4), pp.2131–2137.
- Woolfe, M.L., Chaplin, M.F. & Otchere, G., 1977. Studies on the mucilages extracted from okra fruits (Hibiscus esculentus L.) and baobab leaves (Adansonia digitata L.). Journal of the Science of Food and Agriculture, 28, pp.519–529.
- Wu, H. et al., 2014. Effects of transgenic Cry1Ac + CpTI cotton on non-target mealybug pest Ferrisia virgata and its predator Cryptolaemus montrouzieri. PloS One, 9(4), p.e95537.
- Wyatt, G.R., 1961. The biochemistry of insect haemolymph. Annual Review of Entomology, 6, pp.75–102.

- Yamada, T. et al., 2003. Cloning and Expression of cDNA Encoding Larval alpha Amylase of Azuki Bean Weevil, Callosobruchus chinensis. Journal of Insect Biotechnology and Sericology, 72, pp.139-148.
- Yamada, T., Hattori, K. & Ishimoto, M., 2001. Purification and characterization of two alpha-amylase inhibitors from seeds of tepary bean (Phaseolus acutifolius A. Gray). Phytochemistry, 58(1), pp.59–66.
- Yamasaki, T. et al., 2006. Rice bifunctional alpha-amylase/subtilisin inhibitor: cloning and characterization of the recombinant inhibitor expressed in Escherichia coli. Bioscience, Biotechnology, and Biochemistry, 70(5), pp.1200–1209.
- Yang, D.-H., Baldwin, I.T. & Wu, J., 2013. Silencing brassinosteroid receptor BRI1 impairs herbivory-elicited accumulation of jasmonic acid-isoleucine and diterpene glycosides, but not jasmonic acid and trypsin proteinase inhibitors in Nicotiana attenuata. Journal of Integrative Plant Biology, 55(6), pp.514–526.
- Yang, L. et al., 2009. The diamondback moth, Plutella xylostella, specifically inactivates Mustard Trypsin Inhibitor 2 (MTI2) to overcome host plant defence. Insect Biochemistry and Molecular Biology, 39(1), pp.55–61.
- Yoon, S. H. & Robyt, J. F. 2003. Study of the inhibition of four alpha amylases by acarbose and its 4IV-a-maltohexaosyl and 4IV-a-maltododecaosyl analogues. Carbohydrate Research, 338, pp.1969-1980.
- Yu, X. et al., 2014. Engineering plants for aphid resistance: current status and future perspectives. TAG. Theoretical and Applied Genetics. Theoretische und Angewandte Genetik, 127(10), pp.2065–2083.
- Zavala, J.A. et al., 2009. Role of cysteine proteinase inhibitors in preference of Japanese beetles (Popillia japonica) for soybean (Glycine max) leaves of different ages and grown under elevated CO2. Oecologia, 161(1), pp.35–41.
- Zavala, J.A., Giri, A.P., Jongsma, M.A. & Baldwin, I.T. 2008. Digestive duet: midgut digestive proteinases of Manduca sexta ingesting Nicotiana attenuata with manipulated trypsin proteinase inhibitor expression. Plos One, 3(4):e2008.
- Zeng, F. et al., 2012. A new gene from Xenorhabdus bovienii and its encoded protease inhibitor protein against Acyrthosiphon pisum. Pest Management Science, 68(10), pp.1345–1351.
- Zeng, F. et al., 2013. Effects of a new microbial alpha-amylase inhibitor protein on Helicoverpa armigera larvae. Journal of Agricultural and Food Chemistry, 61(9), pp.2028–2032.

- Zhang, H. et al., 2012a. Expression of a nematode symbiotic bacterium-derived protease inhibitor protein in tobacco enhanced tolerance against *Myzus persicae*. Plant Cell Reports, 31(11), pp.1981–1989.
- Zhang, H. et al., 2012b. Molecular dynamics and free energy studies on the carboxypeptidases complexed with peptide/small molecular inhibitor: mechanism for drug resistance. Insect Biochemistry and Molecular Biology, 42(8), pp.583–595.
- Zhang, Z., et al., 2003. Evolutionary history and mode of the amylase multigene family in Drosophila. Journal of Molecular Evolution, 57, pp.702–709.
- Zhou, J.-Y. et al., 2008. Identification of a Kunitz inhibitor from Albizzia kalkora and its inhibitory effect against pest midgut proteases. Biotechnology Letters, 30(8), pp.1495–1499.
- Zhu, Y.C. et al., 2005. Cloning and characterization of chymotrypsin- and trypsin-like cDNAs from the gut of the Hessian fly [Mayetiola destructor (say)]. Insect Biochemistry and Molecular Biology, 35, pp.23–32.
- Zhu, Y.C. et al., 2012. Interaction of proteinase inhibitors with Cry1Ac toxicity and the presence of 15 chymotrypsin cDNAs in the midgut of the tobacco budworm, Heliothis virescens (F.) (Lepidoptera: Noctuidae). Pest Management Science, 68(5), pp.692–701.
- Zhu, Y.C., Zeng, F. & Oppert, B., 2003. Molecular cloning of trypsin-like cDNAs and comparison of proteinase activities in the salivary glands and gut of the tarnished plant bug Lygus lineolaris (Heteroptera: Miridae). Insect Biochemistry and Molecular Biology, 33, pp.889–899.
- Zhu-Salzman, K. et al., 2003. Cowpea bruchid Callosobruchus maculatus uses a three-component strategy to overcome a plant defensive cysteine protease inhibitor. Insect Molecular Biology, 12(2), pp.135–145.
- Zoccatelli, G. et al., 2012. Expression of a-amylase inhibitors in diploid Triticum species. Food Chemistry, 135, pp.2643-2649.

List of publications

Research paper(s):

- Khan A., **Bhide AJ.**, Gadre RV. (2009) Mannitol production from glycerol by resting cells of *Candida magnoliae*. **Bioresource Technology, 100,** 4911-4913

- Kotkar HM., **Bhide AJ.**, Gupta VS., Giri AP. (2012) Amylase gene expression patterns in *Helicoverpa armigera* upon feeding on a range of host plants. **Gene 501,** 1-7

- Itkin M., Heinig U., Tzfadia O., **Bhide AJ.**, Shinde B., Cardenas P., Bocobza SE., Unger T., Malitsky S., Finkers R., Tikunov Y., bovy A., Chikate Y., Singh P., Rogachev I., Beekwilder J., Giri AP., Aharoni A. (2013) Biosynthesis of antinutritional alkaloids in solanaceous crops is mediated by clustered genes. **Science 341,** 175-179

- **Bhide AJ.**, Channale SM., Patil SS., Gupta VS., Ramasamy S., Giri AP. (2015) Biochemical, structural and functional diversity among *Helicoverpa armigera* amylases. **Biochemica et Biophysica Acta – General Subjects 1850,** 1719-1728

- Channale SM.*, **Bhide AJ.***, Yadav Y., Kashyap G., Pawar PK., Maheshwari VL., Ramasamy S., Giri AP. (2016) Characterization of two Coleopteran a-amylases and molecular insights into their differential inhibition by synthetic α-amylase inhibitor, acarbose. **Insect Biochemistry and Molecular Biology 74,** 1-11

- **Bhide AJ.**, Channale SM., Yadav Y., Bhattacharjee K., Pawar PK., Maheshwari VL., Gupta VS., Ramasamy S., Giri AP. (2016) Genomic and functional characterization of coleopteran insect specific a-amylase inhibitor gene from *Amaranthus* species. **Plant Biotechnology Journal** (Under review)

- Kasar SS., Marathe K., **Bhide AJ.**, Herwade A., Giri AP., Maheshwari VL., Pawar PK. (2016) Thermostable α-amylase inhibitor glycoprotein from *Withania somnifera* differentially inhibits various α-amylases and affects growth and

development of *Teniflorum castaneum*. **Biochemica et Biophysica Acta – General Subjects** (Under review)

Book chapter(s):

- Giri AP., **Bhide AJ.**, Gupta VS. (2016) Targeting digestive physiology: Trends in strategic exploitation of plant defensive proteinaceous inhibitors against insect pests. **Genetic Engineering of Plants – Enhancing Productivity and Product Value**, John Wiley & Sons Limited, The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ (In Press)

Patent(s)

- Compositions and method for effective management of storage and sucking insect-pests. Patent file numbers - India (3706/DEL/2014, 15.12.2014) and World (PCT/IN2015/050200, 15.12.2014)

Personal Details

Name: **Mr. Amey Jayant Bhide**

Address: H-59, NCL colony,
Pashan Road,
Pune 411008

Email address: ameybhidey123@gmail.com

Telephone No.: +91-20-2590-2245/2207
Mobile No.: +91-9423856303

Marital Status: Married

Birth Date: April 30th 1985

Educational Qualification:

Secondary School Certificate Examination	March 2001	First class with Distinction **82.66%**	Maharashtra State Board of Secondary and Higher Education, Pune
Higher Secondary Certificate Examination	Feb 2003	First class with Distinction **81**%	Maharashtra State Board of Secondary and Higher Education, Pune
B.Sc. (Microbiology)	(I-IIIrd) Years 2004-2006	First class **76**%	Shivaji University, Kolhapur
M.Sc. (Microbiology)	(I-IVth) Semester 2007-2008	First class **65**%	Abasaheb Garware College, Pune affiliated to Savitribai Phule-Pune University

Research experience

- **From July 2010 to August 2016**

  Pursuing Ph.D. at CSIR-National Chemical Laboratory, Pune, Maharashtra on the topic '*Molecular Characterization of Insect α-amylases and Plant α-amylase Inhibitors*'.

- **From October 2011 to April 2012**

  Visited **Weismann Institute of Science**, Rehovot, Israel as a research student in the lab of Prof. Asaph Aharoni and worked on the topic '*Biosynthesis of antinutritional alkaloids in solanaceous crops*'.

- **From August 1st 2008 to August 7th 2009.**

  Worked as a project assistant on 'Microbial Production of Erythritol' at the Biochemical Engineering Unit, Chemical Engineering and Process Development division, CSIR-National Chemical Laboratory, Pune, Maharashtra.

- **From April 2007- March 2008.**

  Worked on M. Sc.'s dissertation project entitled 'Identification of Microbial Flora from Human Skin' at the Molecular Biology Laboratory, National Centre for Cell Science (NCCS), Pune, Maharashtra

Published work

Research paper(s):

- Khan A., **Bhide AJ.**, Gadre RV. (2009) Mannitol production from glycerol by resting cells of *Candida magnoliae*. ***Bioresource Technology***, 100, 4911-4913

- Kotkar HM.**, Bhide AJ.**, Gupta VS., Giri AP. (2012) Amylase gene expression patterns in *Helicoverpa armigera* upon feeding on a range of host plants. ***Gene*** 501, 1-7

- Itkin M., Heinig U., Tzfadia O., **Bhide AJ.**, Shinde B., Cardenas P., Bocobza SE., Unger T., Malitsky S., Finkers R., Tikunov Y., bovy A., Chikate Y., Singh P., Rogachev I., Beekwilder J., Giri AP., Aharoni A. (2013) Biosynthesis of antinutritional alkaloids in solanaceous crops is mediated by clustered genes. ***Science*** 341, 175-179

- **Bhide AJ**, Channale SM, Patil SS, Gupta VS, Ramasamy S and Giri AP (2015) Biochemical, structural and functional diversity among *Helicoverpa armigera* amylases. ***Biochemica et Biophysica Acta*** 1850, 1719-1728

- Channale SM.*, **Bhide AJ.***, Yadav Y., Kashyap G., Pawar PK., Maheshwari VL., Ramasamy S., Giri AP. (2016) Characterization of two coleopteran a-amylases and molecular insights into their differential inhibition by synthetic α-amylase inhibitor, acarbose. ***Insect Biochemistry and Molecular Biology*** 74, 1-11

- **Bhide AJ.**, Channale SM., Yadav Y., Bhattacharjee K., Pawar PK., Maheshwari VL., Gupta VS., Ramasamy S., Giri AP. (2016) Genomic and functional characterization of coleopteran insect specific α-amylase inhibitor gene from *Amaranthus* species. ***Plant Biotechnology Journal*** (Under review)

- Kasar SS., Marathe K., **Bhide AJ**., Herwade A., Giri AP., Maheshwari VL., Pawar PK. (2016) Thermostable α-amylase inhibitor glycoprotein from *Withania somnifera* differentially inhibits various α-amylases and affects growth and development of *T. castaneum*. ***Biochemica et Biophysica Acta*** (Under review)

Book chapter(s):

- Giri AP., **Bhide AJ**., Gupta VS. (2016) Targeting digestive physiology: Trends in strategic exploitation of plant defensive proteinaceous inhibitors against insect pests. **Genetic Engineering of Plants – Enhancing Productivity and Product Value**, John Wiley & Sons Limited, The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ (In Press)

Patent

- Compositions and method for effective management of storage and sucking insect-pests. Patent file numbers - India (3706/DEL/2014, 15.12.2014) and World (PCT/IN2015/050200, 15.12.2014)

Professional activities:

Short term training

- Certificate for Medical Laboratory Techniques (CMLT) conducted by Shivaji University, held at Yashwanrao Chavan College of Science, Karad, Maharashtra in 2005

- Industrial Quality Control Management (IQCM) conducted by Shivaji University, held at Yashwantrao Chavan College of Science, Karad, Maharashtra in 2006

- **'C' Programming** course from SEED INFOTECH, Pune (from June 2007 to December 2007)

Achievements

- Qualified for CSIR-JRF (Rank 159) in joint CSIR-UGC National Eligibility Test conducted by Council of Scientific and Industrial Research in December 2009

- Qualified GATE 2010 in subject Life Sciences having score 453 with rank 422

Skills

- Basic Biochemistry and Microbiology techniques
- Handling fermenter, soxhlet apparatus, Mass Spectrometer, HPLC, FPLC, CD spectrometer, PCR, DGGE, Real-Time PCR etc.
- Expertise in gene cloning methods, protein purification, secondary metabolite purification and analysis
- Usage of LINUX, DOS and WINDOWS based systems, Coding in 'C'

Other details

Language known: English, Marathi, Hindi and Sanskrit

Hobbies

Listening to music, riding bicycle and reading

References

1. **Dr. Ashok P. Giri**
   Sr. Scientist
   Plant Molecular Biology Unit,
   Division of Biochemical Sciences,
   CSIR-National Chemical Laboratory,
   Pune 411 008 (M. S.), India
   Tel: +91-20-25902710
   Fax: +91 20 25902648
   Email ID: ap.giri@ncl.res.in

2. **Dr. Vidya Gupta**, FNAAS
   Former Chair and Chief Scientist
   Biochemical Sciences Division
   CSIR-National Chemical Laboratory
   Dr. Homi Bhabha Road, Pashan,
   Pune 411008 (M. S.) India
   Tel: +91-20-2590 2237/2647
   Fax: +91-20-2590 2648
   Email ID: vs.gupta@ncl.res.in