"PURIFICATION AND CHARACTERIZATION OF THE N-ACETYL-β-D-GLUCOSAMINIDASES FROM A THERMOTOLERANT *BACILLUS* SP."

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BY

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Dedicated to my parents

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DECLARATION

Certified that the work incorporated in the thesis entitled **"PURIFICATION AND CHARACTERIZATION OF THE N-ACETYL-β-D-GLUCOSAMINIDASES FROM A THERMOTOLERANT *BACILLUS* SP."** *submitted by* ***Ms. B. Amutha*** *was carried out under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.*

Dr. M. I. Khan
Research Guide

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ABSTRACT

Carbohydrates have been projected as the fourth dimension of molecular recognition. The sugar moieties attached to various cellular macro- and micro-molecules (glycoconjugates) play various important roles in cellular recognition processes. They serve as biological recognition markers for the clearance of circulatory serum proteins, specificity for legume-symbiont interaction, allergen recognition, cell-cell recognition and targeting of proteins to different compartments within a cell to name a few. In order to understand the above phenomena, it is essential to determine the exact structure of the carbohydrate moiety of these glycoconjugates. Chemical as well as enzymatic methods have been employed to elucidate the structure of the glycoconjugates. Chemical methods involve permethylation, periodate oxidation and acetolysis. One of the major disadvantages of chemical methods is the destruction / denaturation of the three-dimensional structure of the macromolecules associated with the sugars, especially proteins, leading to loss of functionality of these proteins. Exo- and endo-glycosidases have proved to be specific and non-destructive tools in determining the structure of glycoconjugates owing to their strict substrate specificity.

The exo-N-acetyl-β-D-glucosaminidase (NAGase) and peptide: N-glycanase (PNGase) are two such enzymes that are indispensable in the structure-function elucidation of carbohydrate moieties. The exo-N-acetyl-β-D-glucosaminidase (E.C.3.2.1.30) recognizes and cleaves terminal β(1,4) linked N-acetylglucosamine residues from glycoconjugates. However, the peptide: N-glycanase [peptide-N^4-(N-acetyl-β-glucosaminyl) asparagine amidase] (E.C.3.5. 1.52) recognizes the core glycosylamine linkage (GlcNAc β1-N Asn) unit of N-linked glycoproteins and generates a carbohydrate free peptide and an intact oligosaccharide with the di-N-acetyl chitobiose unit at the reducing end.

The exo-N-acetyl-β-D-glucosaminidase is also one of the key enzymes that constitute the chitinolytic system. Chitin, a linear polymer of N-acetylglucosamine residues occurs widely in nature and is abundant, next only to

cellulose. It is found in the integument of insects and crustaceans and as a cell wall component in fungi. Such chitinous particles are utilized by various fauna with the help of the chitinolytic system comprising of – endo-chitinase, chitobiosidase, β-hexosaminidase and exo-N-acetyl-β-D-glucosaminidase for the complete degradation of chitin and hence reuse of N-acetylglucosamine in the biosphere.

Although selective hydrolysis of glycan structures can be achieved by specific exo- and endo- glycosyl hydrolases, the effective action of these enzymes on glycoprotein substrates often require the presence of denaturing conditions or elevated reaction temperatures. Therefore thermostable glycosyl hydrolases will be of considerable use in the structural elucidation of glycoconjugates. The thermotolerant *Bacillus* sp. NCIM 5120 produced high levels of an exo-N-acetyl-β-D-glucosaminidases (E.C.3.2.1.30) and a peptide-N^4-(N-acetyl-β-glucosylaminyl) asparagine amidase (E. C. 3. 5. 1. 52).

The work presented in this thesis is the study of:

1. Exo-N-acetyl-β-D-glucosaminidase (NAGase) with respect to its purification and extensive characterization regarding its structure-function relationship.

2. Peptide-N^4-(N-acetyl-β-glucosylaminyl) asparagine amidase (PNGase) with respect to its purification, substrate specificity and potential application in the selective deglycosylation of glycoproteins.

CHAPTER I : General Introduction

This part comprises a literature survey of the reported exo-N-acetyl-β-D-glucosaminidases and peptide-N^4-(N-acetyl-β-glucosylaminyl) asparagine amidases with reference to their occurrence, properties, physiological role and applications.

CHAPTER II : Production, purification and characterization of exo-N-acetyl -β-D-glucosaminidase from *Bacillus* sp. NCIM 5120

The thermotolerant *Bacillus* sp. NCIM 5120 produced high levels of an extracellular exo-N-acetyl-β-D-glucosaminidase (25.4 U/L) when grown in a

medium containing peptone (1 %, w/v), yeast extract (1 %, w/v), mannose (0.5 %, w/v) and Speakman's salts solution. The enzyme was purified to homogeneity by chromatography on CM-cellulose, Sephacryl S-300 and phenyl-Sepharose. The M_r of the native enzyme, determined by size exclusion chromatography on Sephacryl S-300 / Sephadex G-200, was 230,000 and it consisted of four identical subunits of M_r 60,000. It is a neutral protein with a pI of 6.8. The optimum pH and temperature for the enzyme activity were 6.0 and 70°C respectively. The enzyme hydrolyzed terminally linked β(1-4) GlcNAc residues specifically from the non-reducing end of neutral oligosaccharides. Comparative studies on the hydrolysis of chitooligosaccharides by exo-N-acetyl-β-D-glucosaminidase indicated chitobiose to be the best substrate with a K_m and k_{cat} of 0.34 mM and 24 μmoles min^{-1} mg^{-1}, respectively. The enzyme also exhibited strict substrate specificity with respect to the glycone substitution as well as anomeric linkage. Analysis of the reaction stereochemistry indicated that the enzyme is a retaining glycosidase, with the (anomer of GlcNAc formed as the first product. Determination of the energy of activation for the exo-N-acetyl-β-D-glucosaminidase revealed that the enzyme exhibits a biphasic / discontinuous Arrhenius plot with two characteristic energy of activation, with an inflection temperature of 50°C. The activation energy at temperatures below the inflection point (50°C) was found to be higher than that at temperatures above the inflection point. A comparison of the energy of activation with different leaving groups (p-nitrophenol and 4-methyl-umbelliferone) showed that the energy of activation for 4-Me-Umb-β-D-GlcNAc was higher, at temperatures below the inflection point, than for pNP-β-D-GlcNAc (60.3 and 43.2 k J / mol respectively).

CHAPTER III : Active site characterization of exo-N-acetyl-β-D-glucosaminidase.

Chemical modification studies on the purified exo-N-acetyl-β-D-glucosaminidase revealed the involvement of a single tryptophan, histidine and carboxylate in the catalytic activity of the enzyme. Spectral analysis and maintenance of total enzyme activities indicated that N-acetylglucosamine

(competitive inhibitor) and p-nitrophenyl-N-acetyl-β-D-glucosaminide (substrate) could prevent the modification of a single essential tryptophan, histidine and carboxylate residue. Analysis of the kinetic parameters of partially inactivated enzyme (by NBS / HNBB) showed the involvement of tryptophan in substrate binding while that of histidine (by photo-oxidation / DEPC) and carboxylate (by EDAC /WRK) in catalysis. The substrate binding properties of chemically modified enzyme samples by fluorescence spectroscopy corroborated the kinetic studies, confirming the participation of histidine and carboxylate in catalysis and the involvement of tryptophan residue in substrate binding. Present studies indicate that the *Bacillus* sp. NCIM 5120 exo-N-acetyl-β-D-glucosaminidase deviates from the reported N-acetyl-β-D-glucosaminidases and β-hexosaminidases that utilize anchimeric assistance in their hydrolytic mechanism.

CHAPTER IV : Fluorimetric studies on exo-N-acetyl-β-D-glucosaminidase

The exo-N-acetyl-β-D-glucosaminidase when excited at 280 nm or 295 nm fluoresces with a λ_{max} of emission at 337 nm. The fluorescence of the enzyme is quenched on titration with substrates / inhibitors without any shift in the λ_{max} of emission. The association constants of various saccharides containing N-acetyl-D-glucosamine were determined by fluorescence titrations. Among the chitooligosaccharides, chitobiose exhibited higher association constant (3.16×10^2 M^{-1}) than chitotriose (1.82×10^2 M^{-1}). However, the relative change in the intrinsic fluorescence of the exo-N-acetyl-β-D-glucosaminidase on binding to chitotetraose was insignificant. These results are consistent with kinetic studies suggesting the enzymes' affinity towards chitobiose > chitotriose > chitotetrose and its exo-mode of action. NBS mediated fluorescence quenching and inactivation process indicated that complete inactivation of the enzyme occurs well before loss in fluorescence. The effect of various solute quenchers, on the native enzyme in presence and absence of N-acetylglucosamine, indicated that acrylamide was more efficient as a quencher (63 %) than succinimide (20 %), KI (28 %) or CsCl (15 %). Treatment of the enzyme with Gdn.HCl resulted in

extensive changes in the fluorescence emission spectral characteristics. There was a shift in the emission maxima from 337 nm in the native protein to 350 nm in the denatured state accompanied by a 36 % decrease in the fluorescence intensity. Gdn.HCl mediated denaturation resulted in increased accessibility of the quenchers to the fluorophors as evidenced by an increase in the $(f_a)_{eff}$ values except for CsCl, which was found to be a poor quencher even under denaturing conditions. Quenching studies also indicate that more than $2/3^{rd}$ of the tryptophan fluorophors in native *Bacillus* sp. exo-N-acetyl-β-D-glucosaminidase are in a fairly rigid hydrophobic environment inaccessible even to the neutral quencher, acrylamide.

Chapter V : Purification and characterization of Peptide-N^4-(N-acetyl-β-glucosylaminyl) asparagine amidase (PNGase) from *Bacillus* sp. NCIM 5120

A glycoamidase, peptide-N^4- (N-acetyl-β-D-glucosaminyl) asparagine amidase (PNGase), was detected in the extracellular culture filtrates of the thermotolerant *Bacillus* sp. NCIM 5120. The enzyme was purified to homogeneity by chromatography on CM-cellulose, CM-Sephadex and Sephacryl S-300. The M_r of the native enzyme, determined by size exclusion chromatography on Sephacryl S-300, was 58,000 and it consisted of two non-identical subunits of M_r 15,000 and 17,000 on SDS-PAGE, indicating the heterotetrameric nature of the enzyme (dimer of a heterodimer). The pI of the enzyme was 6.0. The optimum pH and temperature for the enzyme activity were 5.5 and 50°C respectively. Substrate specificity studies indicated that the PNGase hydrolyzes complex oligosaccharide containing glycans but does not hydrolyze high mannose oligosaccharide containing glycans. It could hydrolyze Dns-Fetuin (-SA) glycopeptide, various complex oligosaccharide containing glycoproteins and the N-linked core disaccharide, Asn-GlcNAc-GlcNAc, suggesting the flexibility of the enzyme towards the polypeptide substitution. Deglycosylation of various glycoprotein substrates indicated that complex, biantennary and complex, triantennary glycoproteins were better substrates than complex, tetraantennary oligosaccharide containing glycoproteins.

CHAPTER VI : General discussion

This chapter compares the salient features of the present investigation with the reported exo-N-acetyl-β-D-glucosaminidases and peptide-N^4- (N-acetyl-β-D-glucosaminyl) asparagine amidases.

List of Abbreviations

β-ME	β-mercaptoethanol
ConA	concanavalin A
CM-cellulose	carboxymethyl cellulose
DEPC	diethylpyrocarbonate
DP	degree of polymerization
DTNB	2,2'-dithiobis nitrobenzoic acid
EDAC	1-ethyl-3-(3-dimethylaminopropyl)azonia carbodiimide
HEPES	(N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid])
HNBB	2-hydroxy-5-nitrobenzylbromide
MES	(2[N-Morpholino]ethanesulphonic acid)
NAI	N-acetylimidazole
NBS	N-bromosuccinimide
NTEE	3-nitro-L-tyrosine ethylester hydrochloride
PAGE	polyacrylamide gel electrophoresis
pNPG	*para*-nitrophenylglyoxal
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
Dns	Dansyl
NAGase	exo-N-acetyl-β-D-glucosaminidase
PNGase	peptide-N^4- (N-acetyl-β-glucosylaminyl) asparagine amidase

Sugar Abbreviations

GlcNAc	N-acetylglucosamine
GalNAc	N-acetylgalactosamine
Man	D-Mannose
Glc	D-Glucose
$GlcNH_2$	D-Glucosamine
Fuc	L-Fucose
SA	Sialic acid
Me-β-D-GlcNAc	Methyl-β-D-N-acetylglucosamine
$(GlcNAc)_2$	Chitobiose
$(GlcNAc)_3$	Chitotriose
$(GlcNAc)_4$	Chitotetraose
pNP-β-D-GlcNAc	*para*-nitrophenyl-β-N-acetylglucosamine
pNP-β-D-GalNAc	*para*-nitrophenyl-β-N-acetylgalactosamine
phenyl-β-D-GlcNAc	phenyl-β-N-acetylglucosamine
pNP-1-thio-β-D-GlcNAc	*para*-nitrophenyl-1-thio-β-N-acetylglucosamine
4-Me-Umb-β-D-GlcNAc	4-methyl-umbelliferyl-β-N-acetylglucosamine
4-Me-Umb-β-D-GalNAc	4-methyl-umbelliferyl-β-N-acetylgalactosamine
4-Me-Umb-β-D-GlcNAc-6-SO_4	4-methyl-umbelliferyl-β-N-acetylglucosamine-6-sulphate
GP	glycopeptide
Neu5Ac	Neuraminic acid

CHAPTER I

GENERAL INTRODUCTION

BACKGROUND

Covalently linked carbohydrate moieties to various cellular macromolecules called "glycoconjugates" are found in bacteria, viruses, fungi, plants and animal cells. They occur usually as glycoproteins, proteoglycans and lipopolysaccharides. The most encountered glycoconjugates are the N- and O-linked glycoproteins [1,2], such as serum proteins, enzymes, hormones, immunoglobulins and animal cell surface proteins. The cell surface glycoproteins have been shown to play important roles in pinocytosis [3,4], differentiation [5,6], tumorogenesis [5,7,8], intercellular recognition and adhesion [7-11], as receptors for hormones and viruses [12] and as mediators of immunological specificities [13]. The secreated glycoproteins function as enzymes, hormones, immunoglobulins and serum transfer factors [13-15]. Although glycoproteins are universal in occurrence and the functional role of the protein entity in many of them has been elucidated, the precise biological role of the constituent oligosaccharide in most of them remains to be studied. Their wide distribution and conserved structures suggest their importance in an undefined but universal physiological role. Incomplete structural analysis as well as diversity of oligosaccharide structures has probably impeded research in this area.

Deglycosylation and structural elucidation of glycans by enzymatic methods

Among the various methods that have been employed to study the structural and functional role of the glycan moiety in glycoproteins (like chemical and enzymatic methods, use of glycosyl deficient mutants or by the use of inhibitors of glycosylation), enzymatic deglycosylation is the most preferred method due to their strict substrate specificity and mild action. Enzymatic degradation of glycoconjugates has been achieved by the use of various exo- and endo-glycosidases. The exo-glycosidases remove monosaccharide units from the non-reducing end of the glycan moiety whereas, the endo-glycosidases remove a part or whole of the glycan entity from the protein *en bloc* [16]. Such endo-

glycosidases have also been called as the "restriction enzymes" of the carbohydrate world. The identification of two important class of endo-glycosidases has enabled a better understanding of the structure and function of the N-linked glycoproteins; the endo-N-acetyl-β-D-glucosaminidase (E. C. 3.2.1.96) and the peptidyl-N^4- (N-acetyl-β-glucosaminyl) asparagine amidase (E. C. 3.5.1.52).

In proteins, basically two different types of carbohydrate linkages have been identified, the N-linked (through the $-NH_2$ group of Asn) and the O-linked (through the –OH group of Ser / Thr or through the -OH group of hydroxylysine and hydroxyproline) [1,2]. The core structure of the N-linked glycoproteins has been well established [1]. Addition of sugars to N-linked glycoproteins involve the transfer of a common precursor, $GlcNAc_2Man_9Glc_3$ (mediated by dolichol phosphate), to the nitrogen in the side chain of an asparagine residue on the protein moiety. Heterogeneity in the N-linked glycoforms arise due to the action of various exo-glycosidases / glycosyltransferases on the precursor glycoprotein giving rise to high mannose, complex and hybrid glycoproteins. Generally, the high mannose glycans possess only Man; the complex glycans, GlcNAc and Gal; while the hybrid glycans possess residues of both glycotypes. Galactose residues are frequently terminated with Neu5Ac, a feature termed "capping". Further modifications include appropriately positioned fucose, sulfate, phosphate and acyl groups giving rise to heterogeneous forms. However, all the N- linked glycoproteins are characterized by the presence of a conserved pentasaccharide inner core region, $GlcNAc_2Man_3$, substituted by a wide variety of oligosaccharide structures at the non-reducing terminal which carry specificity and bear the variable fraction of the glycan [1]. Fig I.1 depicts the involvement of various glycosidases in the ultimate degradation of a typical biantennary N-linked carbohydrate chain.

In contrast, O-linked glycosylation is initiated by the incorporation of a single N-acetylgalactosamine or to a lesser extent mannose, galactose or fucose onto the polypeptide chain (at the –OH group of a Ser / Thr) and elongation of the glycosylation process requires the sequential addition of monosaccharide residues

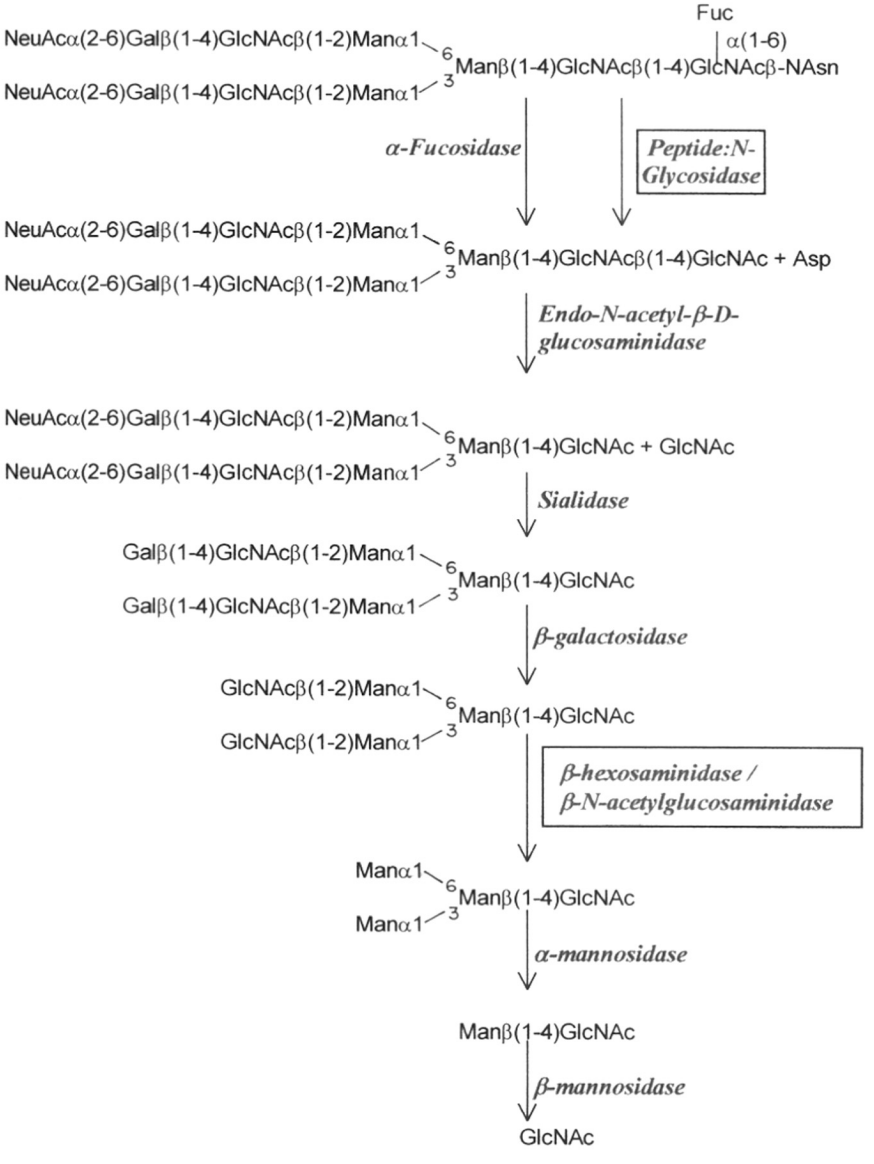

Figure I.1 The hydrolysis of a typical complex, biantennary asparagine linked glycopeptide involving various glycosidases: All the linkages remain same throughout. (Adapted from: *Biology of carbohydrates* (1984), Vol **2, p. 135,** Ginsburg. V. ed., John-Wiley & Sons.)

by glycosyl transferases. O-glycosylated proteins do not exhibit a single, conserved core structure as observed in N-linked glycoproteins and at least eight different core structures have been identified [17].

A complete understanding of the structure and probable functions of glycoconjugates demands a need for exo- and endo-glycosidases with strict glycone and aglycone specificities. The exo-N-acetyl-β-D-glucosaminidase (E. C. 3.2.1.30) and peptidyl-N^4- (N-acetyl-β-glucosaminyl) asparagine amidase (E. C. 3.5.1.52) are two such enzymes that are indispensable in the structure-function elucidation of the glycan moiety of glycoproteins.

EXO-N-ACETYL-β-D-GLUCOSAMINIDASE (NAGase)

Exo-N-acetyl-β-D-glucosaminidase (β-2-acetamido-2-deoxy-D-glucoside acetamido deoxy glucohydrolase, E. C. 3.2.1.30) catalyzes the hydrolysis of terminal, β(1-4) linked N-acetylglucosaminyl residues from the non-reducing end of glycoconjugates. Apart from its specific role in the degradation of glycoconjugates the enzyme plays an important role in the hydrolysis of the structural polysaccharide, chitin.

Chitin is a major component of the biosphere with an annual estimated production of 2.3×10^{12} Kg [18]. It is a linear homopolymer of β(1-4) linked N-acetyl-D-glucosamine, (GlcNAc; 2, acetamido-2-deoxy-D-glucose) residues and has been classified into three major types depending on the structure [19].

α-Chitin: This consists of two linear chains of $(GlcNAc)_n$ polymer, oriented in antiparallel direction. Widely found in arthropods, crustaceae and fungi and exhibit high degree of packing and crystalline structure.

β-Chitin: This is made up of a single linear polymer and many such linear chains come together running in parallel direction and occurs prevalently in marine diatoms and in the chaetae of *Aphrodite aculeata* and squid pens. More readily penetrated by chemical reagents and enzymes than the α-chitin [20].

γ- Chitin: This comprises of three linear chains of $(GlcNAc)_n$ polymer with the central chain running antiparallel between two adjacent chains.

The distribution of the above forms of chitin does not seem to follow any fixed taxonomy and more than one form or all three forms may occur in different parts of the same organism, for example: in Loligo and Lingula [21]. However, except for the β-chitin, which occurs in marine diatoms, chitin has always been found to be cross-linked with various other cellular constituents like proteins, carbohydrates and lipids [22]. They also undergo mineralization in the form of calcification and sclerotinisation on interaction with phenolics and lipid molecules [23].

In view of the above factors, chitin serves as a major carbon and nitrogen nutrient source for bacteria and fungi. To utilize chitin as a nutrient source, there is a need for an efficient degrading system to help such bacteria and fungi utilize chitin in an assimilable form. In an analysis of nearly 500 environmental samples, (soil, fresh water and seawater), among the various chitinolytic bacteria, the best chitin liquefying activity was found in *Pseudomonas aeruginosa* and *Vibrio anguillarum* [24]. *Gooday* [25] has suggested two probable pathways of chitin degradation in conjunction with the presence of chitosan in ocean sediments (Fig I.2). This is mainly done by the chitinolytic system, comprising of the following enzymes, which act synergistically as well as consecutively.

1.) Endo-chitinase: [poly β-(1-4) (2-acetamido-2-deoxy)-D-glucoside glycano-hydrolase, (E.C.3.2.1.14)], hydrolyses GlcNAc polymers randomly with chitooligomers of chain length 3-6 being formed as the product. Chitotetramers and above act as substrates.

2.) Chitobiosidase: Releases chitobiose $(GlcNAc)_2$ units from the chitin polymer, and requires at least the trimer, $(GlcNAc)_3$ for its activity.

3.) Exo-N-acetyl-β-D-glucosaminidase (E.C.3.2.1.30): Exo-hydrolytic in nature and catalyses the hydrolysis of terminal, β(1-4) linked N-acetylglucosamine residues. Chitooligomers of chain length 2-6 serve as substrates.

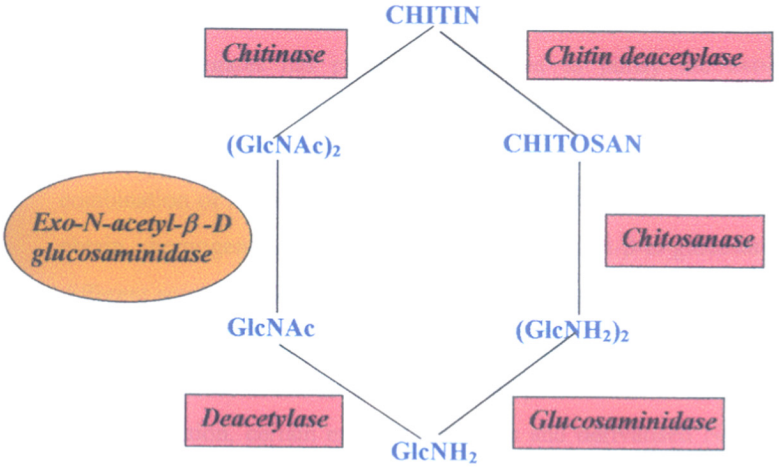

Figure I.2 Alternative routes in the enzymatic degradation of chitin in conjunction with chitosanase. The exo-N-acetyl-β-D-glucosaminidase hydrolyses small length chitooligosaccharides produced by endo-chitinases to the monomer. (Adapted from: *Chitin enzymology*. R. A. A. Muzzarelli ed., Eur. Chitin Soc. Ancona, 1993, **p. 359**)

Purification of exo-N-acetyl-β-D-glucosaminidases

Since the discovery of N-acetyl-β-D-glucosaminidase in mammalian tissues as early as 1939 by Watanabe [26], it has been found to be ubiquitous occurring widely in animal tissues, higher plants and microorganisms. The proposed function of this enzyme in the development of microorganisms, in mobilizing storage food and as a degradative enzyme in the ultimate breakdown of glycoconjugates has prompted the need to purify the exo-N-acetyl-β-D-glucosaminidases to homogeneity. Conventional methods like ion-exchange chromatography, gel filtration, isoelectric focussing, affinity chromatography or a combination of more than one of the above methods have been routinely used to obtain homogeneous preparations of N-acetyl-β-D-glucosaminidase from different sources. Owing to the glycoprotein nature of the β-hexosaminidases / N-acetyl-β-D-glucosaminidases in higher organisms, many investigators have used ConA-Sepharose as one of the steps in the purification of these enzymes [27,28-34]. In addition, certain enzyme inhibitors and derivatised N-acetylglucosamine saccharides have been used as affinity ligands in the purification of these enzymes. N- (ε-Aminohexanoyl)-2-acetamido-2-deoxy-β-D-glucosamine coupled to CNBr activated Sepharose has been used as the affinity matrix in the purification of *Trichomonas foetus* and *Acanthamoeba castellani* β-hexosaminidases [35,36]. The four isoforms of fenugreek seed (*Trigonella foenum greacum*) β-hexosaminidases have been purified using a combination of isoelectric focussing, ConA-Sepharose and affinity chromatography on Sepharose–6B–(6-aminohexanoyl)–(N-acetyl-β-D-glucosaminylamine) [27,37]. N-acetylglucosamine-agarose has been used in the purification of *Prunus serotina* EHRH (black cherry) β-hexosaminidases [38]. In addition to ConA-Sepharose and affinity chromatography on p-Aminophenyl-N-acetyl-β-D-thio-glucosaminide linked to Affi-gel, the purification of *Pisum sativum* L β-hexosaminidases also involves metal chelate chromatography, with Zn^{2+} as the divalent cation [33]. The bovine kidney Hex A and Hex B have been purified

using 2-acetamido-1,2,5-trideoxy-1,5-iminoglucitol (2-acetamido-1,2-dideoxy-nojirimycin) coupled to Sepharose [39].

Enzyme assay

Routine assays of exo-N-acetyl-β-D-glucosaminidases are usually carried out by following the hydrolysis of, chromogenic or fluorescence tag attached substrates like, p-nitrophenyl-N-acetyl-β-D-glucosaminide, phenyl-N-acetyl-β-D-glucosaminide, 3-cresolsulphone-pthaleinyl-N-acetyl-β-D-glucosaminide and 4-methyl-umbelliferyl-N-acetyl-β-D-glucosaminide. The liberated p-nitrophenol is measured spectrophotometrically, at 405 nm as the phenolate anion in alkaline pH (>10). One unit of exo-N-acetyl-β-D-glucosaminidase is defined as the amount of enzyme required to liberate 1 μmole of p-nitrophenol / minute under the assay conditions [40]. Similarly, the hydrolysis of phenyl- substrates or 3-cresolsulphone-pthaleinyl- substrates is followed by measuring the release of the respective aglycone [41,42]. When 4-methyl-umbelliferyl substrates are used, the released 4-methylumbelliferone is determined fluorimetrically using an excitation and emission wavelength of 355 nm and 480 nm respectively [43], and it directly corresponds to the amount of GlcNAc released.

The hydrolysis of natural substrates like chitooligosaccharides or glycoproteins is followed by determining the liberated GlcNAc according to the method of *Ressig et al.* for N-acetyl sugars [44] or by the method of *Nelson-Somogyi* for reducing sugars [45] or by HPLC.

Biochemical properties of the exo-N-acetyl-β-D-glucosaminidases

Table I.1 summarizes the salient features of the reported exo-N-acetyl-β-D-glucosaminidases / β-hexosaminidases. These enzymes show a wide range of variability in their properties and have been characterized from quite a few bacterial sources like *Diplococcus pneumoniae* [47], *Bacillus stearothermophilus* [48], *Bacillus subtilis* [51,52], *Serratia marcesans* [53,54], *Clostridium perfringens* [49] etc. They have also been characterized from many fungi like

Aspergilli [57,58], *Mucor fragilis* [61], *Sclerotina fructigena* [70], *Penicillium oxalicum* [73], *Trichoderma harzianum* [67-69], *Paeliomyces persicinus* [71], *Pycnoporus cinnabarinus* [72], *Physarum polycephalum* [64,65] etc. The optimum pH of the bacterial NAGases / β-hexosaminidases is between 5.0-7.0 [46-55] and they have an acidic pI (3.0-4.5). Fungal NAGases / β-hexosaminidases exhibit their optimum pH between 4.0-5.5 with majority of them on the acidic side and they are stable between pH 3.0-8.0 [61,64,65,67-70]. However, the *Pycnoporus cinnabarinus* β-hexosaminidase shows an unusual pH optimum of 2.2 [72]. pH stability of the reported N-acetylglucosaminidases shows wide variations. The *S. fructigena* β-hexosaminidase [70] is stable over a broad pH range (4-11) while the *M. fragilis* enzyme [61] is stable between 4.5-8.0. The N-acetylglucosaminidases / β-hexosaminidases from *Tremella fuciformis* [63], *Turbatrix aceti* [78,79] and *Alteromonas* sp. Strain O-7 [50] are stable around neutral pH (6.0-8.0). However, the *P. cinnabarinus* β-hexosaminidase is stable only between 2.0-4.0 [72]. NAGases are generally classified as acidic (lysosomal) and neutral (cytosolic) NAGases, depending on their pI. An exception to this is one of the isoforms (Form I) from the fungus *Beauveria bassiana*; which has a pI of 9.5 [62].

The optimum temperature for activity in the bacterial enzymes ranges between 37-50°C [47,52-55] and in the fungal enzymes between 25-50°C [61,64,65,67-70]. Few exceptions like the β-hexosaminidase from *B. stearothermophilus* and that from *B. bassiana* exhibit high temperature optima. The β-hexosaminidase from *B. stearothermophilus* has an optimum temperature of 75°C [48] while that of the *B. bassiana* isoenzyme II (NAGase II) is 57°C [62].

Bacterial enzymes are monomeric proteins with their molecular mass between 60-94 kDa [48-52] and do not exhibit isoenzymic forms except for *C. perfringens*, which has four isoenzymes (Gm 1, Gm 2a, Gm 2b and Gm 3) [49]. With few exceptions, fungal exo-N-acetyl-β-D-glucosaminidases / β-hexosaminidases also do not show multiplicity in the enzymic forms. However, *B. bassiana* showed two isoforms of the enzyme, NAGase I (pI 9.0) and NAGase II

(pI 5.0) [62]. The fungal enzymes have their M_r between 58-150 kDa and many of them are multimeric proteins (homodimers or-tetramers) with a characteristic subunit M_r of 60 kDa [33,62,63,72,73].

The β-hexosaminidases from plants and mammalian tissues are also multimeric proteins, homo- as well as heterodimers or tetramers and the M_r ranges between 110-210 kDa [27,74,77]. The *Pisum sativum* L β-hexosaminidase [33], is a heterotetramer (dimer of a heterodimer with M_r 62 kDa and 64 kDa). They are essentially glycoproteins and this property has been used as a means of purification of these enzymes (by affinity chromatography on Con A Sepharose). The mammalian enzymes are characterized by their αβ nature.

Most of the N-acetylglucosaminidases are neither metalloproteins nor metal requiring enzymes. However, certain plant β-hexosaminidases show enhanced activity in presence of metal ions. For example, one of the four isoforms of β-hexosaminidases from germinating Fenugreek seeds [27,37] showed approximately 1.7 fold enhancement in its activity in presence of Fe^{2+}. However, in case of *Pisum sativum* L β-hexosaminidase II [33], Mg^{2+}, $Mn^{2+}$and Ca^{2+} (10 mM) had only a marginal stimulatory effect. The β-hexosaminidase from *B. stearothermophilus* CH-4 also showed increased activity in presence of Fe^{3+} (2 fold), Ca^{2+} (1.7 fold) and Zn^{2+} (1.4 fold) [48].

Molecular cloning of only three chitobiase genes (E. C. 3. 2. 1. 30) from *Alteromonas* sp. strain O-7 [50], *Serratia marcesans* [53] and *Vibrio harveyi* [87] has been carried out. A comparison of the amino acid sequences of chitobiase genes with other family 20 glycosyl hydrolases reveals considerable regional homologies with the β-hexosaminidases (E. C. 3. 2. 1. 52) cloned from several sources such as bacteria, fungi and animal tissues [66,81,88-93]. Sequence similarities upto 16 to 54 % have been observed among the chitobiases / β-hexosaminidases. The lengths of the encoded NAGase proteins vary from 530 to 600 a.a. with the exception of *V. harveyi* chitobiase, which is considerably longer (883 a.a.) [87].

Table I.1

Properties of the Exo-N-acetyl-β-D-Glucosaminidases / β-hexosaminidases from various sources

Source	Molecular properties: Optimum pH, temperature, pI	Molecular properties: Mol. mass and subunit composition	Properties	Substrate specificity	Ref. No.
Diplococcus pneumoniae	5.3, 37°C	—	Hg^{2+}, Cd^{2+} potent inhibitors	Non-specific* , hydrolyzes 26 % of GlcNAc residues from α-acid glycoprotein [a]	46, 47
Bacillus stearothermophilus CH-4	6.5, 75°C	74 kDa	Fe^{2+}, Ca^{2+}, Zn^{2+} enhance the activity by 204 %, 171 % and 142 %. 15-20 % stable in presence of various organic solvents. $GlcNH_2$ and $GalNH_2$ activate the enzyme.	Non-specific *, hydrolyses chitooligosaccharides upto pentoses	48
Clostridium perfringens				Gm 2a cleaves β 1-3 N-acetylglucosamine linkages six times the rate of Gm 1, however all of them release β1-4 linkages at the same rate. They also hydrolyze α_1- acid glycoprotein [a].	49
Gm 1	6.5	—	Hg^{2+} a potent inhibitor for all the isoforms. Cu^{2+}(2 mM) completely inhibits Gm 1.		
Gm 2a	5.3				
Gm 2b	5.3				
Gm 3	5.7				

Table I.1 Continued...

Alteromonas sp. Strain O-7 cloned into E. coli	7.5, 4.8 (pI), 37°C	60 kDa	—	Specific towards pNP-GlcNAc. Exhibits lower activity towards pNP-GalNAc. Maximum activity against $(GlcNAc)_2$, with the higher oligosaccharides being hydrolyzed at a lesser rate.	50
Bacillus subtilis B	5.9, 3.8 (pI), 37°C	75 kDa	shows anomalous gel filtration behaviour, carried out in presence of 4 M, NaCl	Specific towards non-reducing GlcNAc residues	51, 52
Serratia marcesans cloned into E. coli	6-8, 52°C	94 kDa	Tm of the enzyme, 60°C	Specific towards non-reducing β(1-4) linked GlcNAc residues	53, 54
Vibrio parahaemolyticus cloned into E. coli	7.0, exhibits four isoforms with pI between 4.9-5.1	80 kDa	—	Non-specific *, ratio of hydrolysis of pNP-GlcNAc : pNP-GalNAc, 5:1; acts on β(1-3) and β(1-4) linkages	55
Streptococcus 6646 K		—	—	Non-specific, also acts on PTG[b].	56
I	5.2				
II	5.1				
III	5.5				
	37°C, stable <50°C				

Table I.1 Continued...

Aspergillus niger	4.6, 4.4 (pI), 37°C	149 kDa Glycoprotein	Kinetic studies indicate the probable involvement of acetamido group of substrate in hydrolysis (anchimeric assistance)	Non-specific * . Purified enzyme exhibits β-glucosidase & β-xylosidase activity and hydrolyses 2,4,di-nitrophenyl-β-D-GlcNAc. Also hydrolyses GlcNAc from ovalbumin, fetuin[c] and α_1-acid glycoprotein[a] glycopeptides.	57
Aspergillus nidulans	5.0, 4.3 (pI)	190 kDa, glycoprotein	Hg^{2+}, acetate, benzoate and propionate ions inhibit the activity	Non-specific *, also hydrolyses chito-oligosaccharides upto hexamers	58
Aspergillus oryzae	4.5 / 3.7	140 kDa	Exhibits transglycosylase activity and used in preparation of GlcNAc oligosaccharides like di, tri, and tetrasaccharides	Non-specific *	59, 60
Mucor fragilis			Organism grown in bovine blood as the nutrient source. However maximum activity detected in presence of 0.8 % GalNAc as the inducer.		61
E I	—	—		—	
E II	4.0-5.0 (GlcNAcase) 5.5-6.5 (GalNAcase) stable from 4.5-8.5	125 kDa, glycoprotein		Non-specific * and also acts on $(GlcNAc)_2$, $(GlcNAc)_3$, fetuin[c], and Mycrococcus lysodeikticus cell wall [d].	

Table I.1 Continued...

Beauveria bassiana					
NAGase I	pH, 5.0, 9.0(pI), 57°C	97 kDa	—	Non-specific *, rate of activity of NAGase I on pNP-GlcNAc is 9.5 times more than that of NAGase II	62
NAGase II	pH 5.0, 5.0 (pI), 37°C	dimer of 64 kDa & 66 kDa			
Tremella fuciformis	5.0	125 kDa	Hg^{2+} potent inhibitor, stable around pH 7.0	Non-specific *, also acts on chito-oligosaccharides upto $(GlcNAc)_4$	63
Physarum polycephalum		Glycoproteins	Antibodies prepared against either of them do not cross react.	Intracellular enzyme, non-specific*, catalyses both, terminal GlcNAc & GalNAc. Also hydrolyses terminal GlcNAc from ovalbumin, ovalbumin GPs and various other cell wall fragments. The extracellular enzyme has a similar substrate specificity but with lower hydrolytic rates.	64, 65
Intracellular (Y)	4.2	179 kDa (subunits of 40 kDa)			
Intracellular (Z)	3.5				
Extracellular (X)	4.2	25.3 kDa			
Trichoderma harzianum	4.0 – 5.5, 50°C	150 kDa	Ag^{2+} potent inhibitor	Non-specific*, acts on chitooligosaccharides upto $(GlcNAc)_6$, specific for GlcNAcβ(1-3)Gal, does not hydrolyze GalNAcβ(1-3)Gal or	66, 67-69

Table I.1 Continued...

Trichoderma harzianum				GlcNAcβ(1-2)Man. GalNAc does not act as an inhibitor. GlcNAc inhibits GalNAcase activity but not GlcNAcase activity.	
Trichomonas foetus	6.2, 37°C	150 kDa	Purified by affinity matrix on N-(ε-aminohexanoyl)-2-acetamido-2-deoxy-β-D-glucopyranosylamine coupled to Sepharose.	Non-specific *, Ratio of hydrolysis of pNPGlcNAc: pNPGalNAc,. 4:1,however the K_m similar for both the substrates (4-hydroxyl group has no role in substrate binding but determines the rate of hydrolysis.	35
Sclerotina fructigena	Stable at pH 4-11. 37°C	141 kDa	Acetate ions do not inhibit the enzyme activity. Probable involvement of 2 different active sites for both the substrates and shows bisubstrate kinetics with phenyl-β-GlcNAc.	Non-specific*	70
Paecilomyces persicinus	4.6/5.8 35-37°C, 4.8 (pI)	100 kDa	—	Non-specific *, Ratio of hydrolysis of pNPGlcNAc : pNPGalNAc, 1.60.	71
Picnoporus cinnabarinus	2.2/3.7, 5.4 (pI), 37°C	120 kDa, subunits of 65 kDa	pH optimum 2.2, stable at pH 2-4	Non-specific *, hydrolyzes upto tetraoses	72

Table I.1 Continued...

Penicillium oxalicum	3.0-4.5, stable at 7.0-9.0 55°C	143 kDa, 2 subunits of 68 kDa.	—	Non-specific*, hydrolyzes $(GlcNAc)_2$, $(GlcNAc)_3$ and fetuin glycopeptides [c].	73
Pisum sativum L. (four isoforms)				Non-specific* Ratio of hydrolysis of pNPGlcNAc : pNPGalNAc	33
I A	4.5 – 5.0,	210 kDa made	Isozyme II activated (10-20 %) in presence	10.3	
I B	30°C	upof 2 subunits	of 10 mM Mn^{2+}, Mg^{2+}, Ca^{2+}.	9.2	
I C		of 62 & 64 kDa		4.0	
II				12.9	
***Trigonella foenum graecum* (Fenugreek germinating seeds).(four isoforms)**	40 – 45°C			Non-specific* Ratio of hydrolysis of pNPGlcNAc : pNPGalNAc,	27, 37
I	6.0/5.5, 6.78 (pI)	84 kDa	Fe^{2+} (2 mM) enhances activity in Isoform I	1.25	
II	5.0, 6.3 (pI)	72 kDa		2.35	
III	5.0/4.4, 4.9 (pI)	180 kDa		6.50	
IV	4.8/4.6, 4.65(pI)	150 kDa		18.00	
***Gossypium hirsutum L. Im 216* Germinating cotton seeds**	5.6/5.0 37°C	125 kDa	—	Non-specific * also acts on chitin, ovalbumin and wheat germ lectin	32
***Phaseolus vulgaris* seeds**	4.6, 30°C	—	—	Non-specific * also acts on fetuin GPs [c] (–Gal,SA) & bovine pancreatic ribonuclease B.	74, 75

Table I.1 Continued...

***Prunus serotina Ehrh.* Black cherry seeds**	4.5/4.0 30°C	glycoprotein	—	non-specific* also hydrolyses pNP-Glu & oNP-Glu	38
Watermelon fruit	5.0 – 5.5 37°C	150 kDa, glycoprotein	—	hydrolyzes pNP-β-GlcNAc	76
Almond emulsin	5.0-6.0 for (GlcNAcase) 3.5-4.0 for (GalNAcase)		Both the activites are catalyzed by the same active site. Ag^{2+}, Hg^{2+} & Fe^{3+} are potent inhibitors.	Non-specific * , also releases terminal GlcNAc & GalNAc residues from ovalbumin, ovomucoid, $(GlcNAc)_2$, α_1-acid glycoprotein[a], cell wall fragments of S. aureus, human globoside, G_{M2} ganglioside	77
Ficus glabrata	4.0 – 4.5 37°C	glycoprotein	Ag^{2+}, Hg^{2+}, Fe^{3+} potent inhibitors. Evidence for a single active site. Affinity purification on Con A sepharose.	Non-specific *	29
Lupin seeds ***A*** ***B*** ***B 1***	4.8, 25°C glycoprotein	subunit M_r of 66 kDa 40 kDa 40 kDa	Affinity chromatography on Con A Sepharose	Non-specific *	30

Table I.1 Continued...

Turbatrix aceti	4.8/5.4 37°C, stable around neutral pH.	112 kDa (2 subunits of 66 kDa). Glycoprotein (15 %)	Purified on p-aminophenyl 2-acetamido-2-deoxy-1-thio-β-D-glucopyranoside coupled to Sepharose. A single catalytic site is responsible for the hydrolysis of terminal GlcNAc & GalNAc residues.	Non-specific*. Substrate analogs with 'S' substituted at the 'O' of acetamido group are either poor substrates or strong competitive inhibitors. Hydrolyses terminal GlcNAc from ovalbumin, fetuin [c], human salivary glycoprotein oligosaccharide.	78, 79
Acanthamoeba castellani	5.8 (pI)	58 kDa	Affinity purification on 2-acetamido-N-(ε-aminocaproyl)-2-deoxy-β-D-glucopyranosylamine.	—	36
Dictyostellium discoideum Cloned in λgt 11	5.0	168 kDa (2 subunits of 65 kDa & 51` kDa)	—	Non-specific*	80, 81
Manduca sexta (L.) **Tobacco Hornworm**					83, 84
E I	6.0, 5.9 (pI) 25°C	61 kDa, monomer	Detected in larval and pharate pupal moulting fluid, integuments and pupal haemolymph.	Non-specific *, basically a chitinase	
E II	6.0, 5.1 (pI) 25°C,	61 kDa, Dimer of 47 & 14 kDa	Detected in larval and pupal haemolymphs and stabilized by –s-s- bridges.	Non-specific *, essentially a β-hexosaminidase.	

Table I.1 Continued...

Bombyx mori **(haemolymph)**	—	125 kDa , 2 subunits of 61 kDa, glycoprotein	—	catalyses pNP-β-D-GlcNAc, phenyl-β-D-GlcNAc as well as $(GlcNAc)_2$	85, 86
Bovine kidney		glycoprotein	Purification by affinity chromatography on 2-acetamido-1,2-dideoxynojirimycin coupled matrix.	Non-specific*, Hex A also hydrolyses Me-Um-β-D-GlcNAc-6-SO_4 but Hex B does not hydrolyze Me-Um-β-D-GlcNAc-6-SO_4	39
Hex A	5.0, 5.3 (pI), 25°C	120 kDa (subunits of 57 & 52.5 kDa,)			
Hex B	5.0, 7.2 (pI), 25°C	57 kDa, dimer			
Human seminal plasma				Non-specific*	82
I (Hex A)	pH 4.5, 60°C	195 kDa	Thiol reactive agents strongly inhibit the activities. GSH protects from inactivation suggesting presence of thiol groups at the active site.		
II (Hex B)		210 kDa (four subunits of 50 kDa)			

*Non-specific, i. e., hydrolyses both GlcNAc as well as GalNAc containing substrates

Ratio of hydrolysis (RH) indicates the relative rates of hydrolysis observed in presence of pNP-β-D-GlcNAc and pNP-β-D-GalNAc

[a] α_1 – acid glycoprotein (-SA, -Gal) indicates terminal sialic acid and galactose residues removed.

[b] PTG, porcine thyroglobulin

[c] Fetuin (-SA, -Gal) indicates fetuin with the terminal sialic acid and galactose residues removed

[d] Mycrococcus lysodeikticus cell wall fragments obtained from lysozyme treated cell.

Substrate specificity of exo-N-acetyl-β-D-glucosaminidase

The exo-N-acetyl-β-D-glucosaminidase catalyses the hydrolysis of terminal, non-reducing GlcNAc residues from glycoconjugates. However, most of the NAGases reported to date from higher orders (fungi and above) invariably hydrolyze GlcNAc as well as GalNAc containing substrates [27,29-35,37-39,58,74,77,80,83]. Therefore a classification based on the amino acid sequence similarities as well as on the basis of IUBMB nomenclature of enzymes, (based on the substrate specificity) indicates many of the reported exo-N-acetyl-β-D-glucosaminidases to be polyspecific [94-96]. Kinetic and active site characterization studies from many sources have revealed the involvement of a single catalytic site in the hydrolysis of both the substrates [29,78,79]. Certain enzymes like β-N-acetylglucosaminidase from *Aspergillus niger* [57] in addition to its hydrolytic activity on GlcNAc and GalNAc containing substrates also showed activity towards pNP-β-D-Glu and pNP-β-D-Xyl to a lesser extent. The β-hexosaminidase from *Prunus serotina* does not seem to show specificity towards the N-acetyl group and hydrolyses pNP-β-D-Glu as well as oNP-β-D-Glu [38]. Although it has been suggested that NAGases from bacterial sources are strict exo-N-acetyl-β-D-glucosaminidases, this observation is an exception rather than a rule. For example, the NAGases from *B. stearothermophillus* CH-4 [48], *Streptococcus* 6646 K [56], *D. pneumoniae* [46,47], *C. perfringens* [49] and *V. parahaemolyticus* [55] hydrolyze GlcNAc and GalNAc containing substrates to varying extents and have been classified as β-hexosaminidases. However, the corresponding enzymes from *B. subtilis* B [52], *S. marcesans* [53] and *Alteromonas* sp. strain O-7 [50] are strict exo-N-acetyl-β-D-glucosaminidases. The ratio of hydrolysis of GlcNAc and GalNAc containing substrates has been observed to be between 1.25 to 18 [27,33,35,37,71]. Further, the pH optimum for the hydrolysis of both the substrates is also found to vary in a single enzyme. In most of the reported β-hexosaminidases a deviation of ± 0.5 units in the pH optimum has been observed.

The exo-hydrolytic nature of the N-acetyl-β-D-glucosaminidases has been demonstrated by the enzyme's ability to hydrolyze chitooligomers of chain length 2-6 ($GlcNAc_2$ – $GlcNAc_6$). The affinity or catalytic efficiency of the exo-N-acetyl-β-D-glucosaminidases decrease with increase in chain length and exhibit total resistance towards the hydrolysis of chitooligomers with degree of polymerization above 6. In most of the reported enzymes, chitobiose is found to be the best substrate whereas chitotriose as well as chitotetraose are hydrolyzed to a lesser extent, however, chitopentose and chitohexose are resistant to hydrolysis [48,50,58,63,67,72,73]. HPLC analysis of the hydrolysis of chitooligosaccharides in the *Aspergillus nidulans* β-hexosaminidase indicated that the enzyme hydrolyses substrates in an exo-manner [58].

The NAGases exhibit strict specificity towards the anomeric configuration and linkage [97,98]. Few enzymes act on linkages other than β(1,4) GlcNAc / GalNAc residues like the β-hexosaminidases from *Trichoderma harzianum* and *Clostridium perfringens* which are capable of hydrolyzing β(1,3) linked GlcNAc residues as well [67-69,49].

The exo-N-acetyl-β-D-glucosaminidases / β-hexosaminidases also act on various glycoproteins and their corresponding glycopeptide substrates with terminal, non-reducing GlcNAc residues like, fetuin (-Gal, -SA), ovalbumin, pancreatic ribonuclease B, α_1-acid glycoprotein (-Gal, -SA), porcine thyroglobulin, cell wall fragments of *S. aureus*, globoside, G_{M2} ganglioside, *Micrococcus lisodekticus* cell walls etc. [32,56,57,61,64,65,75,77].

Mammalian β-hexosaminidases predominantly occur as two isozymes, the Hex A (αβ) and the Hex B (ββ). Hex A hydrolyses neutral as well as charged substrates like Me-Umb-β-D-GlcNAc-6-SO_4 and glycosphingolipids like G_{M2}, in conjunction with specific activator proteins [99]. However, the Hex B enzyme has restricted substrate specificity and utilizes only neutral GlcNAc / GalNAc containing substrates. Hex A has the broadest substrate specificity in the sense that it can remove non-reducing, terminal GlcNAc and GalNAc residues from all glycosylaminoglycans that occur in human cells. Hex B exhibits similar substrate

specificity with the exception that it is unable to hydrolyze G_{M2} ganglioside. A third form of the enzyme, the S ($\alpha\alpha$) isoenzyme, exists in minor quantities and exhibits limited catalytic activity and is unstable. Inspite of its negligible catalytic activity towards physiological substrates, the S isoenzyme shares with Hex A, the ability to remove GlcNAc-6-SO_4 from glycoconjugates [100].

Although Hex A is capable of hydrolyzing the G_{M2} ganglioside, it requires the presence of an activator protein. The G_{M2} activator protein specifically recognizes the ganglioside, binds to it, and extracts it from the membrane to form a water-soluble complex with a stoichiometry of one ganglioside molecule per activator molecule. However, the exact mode of action of G_{M2} activator is still not known. The activator-lipid complex specifically interacts with the active site of α-subunit of β-hexosaminidase A [101] to bring about hydrolysis. The G_{M2} activator has been mapped to chromosome 5 in the human genome [102]. Physiological significance of the G_{M2} activator protein is highlighted by the occurrence of infantile G_{M2} gangliosidosis (variant AB) due to a deficiency in the activator protein specific for degradation of G_{M2} ganglioside and related gangliolipids by hexosaminidase A [103]. However, this activator protein is not required for the hydrolysis of water-soluble synthetic substrates such as 4-methyl-umbelliferyl-β-D-GlcNAc or p-nitrophenyl-β-D-GlcNAc. Further, presence of the activator protein does not promote hydrolysis of such gangliolipids by Hex B. The exo-N-acetyl-β-D-glucosaminidases / β-hexosaminidases from bacteria, fungi and plant sources also do not hydrolyze the G_{M2} ganglioside.

Active site characterization of the N-acetyl-β-D-glucosaminidases / β-hexosaminidases

Strictly in the chemical sense, hydrolytic cleavage at the glycosidic bond of polysaccharides is a slow, acid-catalyzed reaction that occurs at acid concentrations of 0.01 to 3.0 N and temperatures between 20°C and 100°C [104]. The accepted model of the mechanism, where the acid functions as the proton donor involves three steps, **1)** protonation of the glycosidic oxygen, to give the

conjugate acid, **2)** a unimolecular heterolysis of the conjugate acid, with the formation of a carbonium-oxonium ion and **3)** addition of water to carbonium-oxonium ion with the formation of a reducing end-group and a proton. A similar mechanism has been suggested for the enzymatic hydrolysis of polysaccharides, wherein, certain amino acid side chains function as the proton donor. Several approaches are known for the determination of amino acid residues that participate in enzymatic reactions. Especially, kinetic studies for the estimation of pK values, heat of ionization (ΔH), and chemical modification studies have been done on many enzyme proteins.

Sinott [105] has proposed that glycosyl hydrolases employ an acid-catalytic mechanism involving the participation of two acidic residues (a proton donor and a nucleophile) at the active site as identified in the glycoside cleavage by β-glucosidase [107], α-glucosidase [106,113], β-galactosidase [108], chitinase [109] and lysozyme [106,110]. However, recent evidence suggests the participation of other residues also. Typical examples are the participation of a tyrosine residue in transition state stabilization of a viral neuraminidase [111] and a bacterial sialidase[112]. Some of the other residues that have been implicated alongwith carboxylic acid are cysteine and histidine. Studies on the yeast α-glucosidase II [113], and the exo-β-(1-3)-D-glucanase from *Basidiomycete* sp. QM 806 [114] have indicated the presence of a single histidine at the active site which probably functions as the nucleophile.

The mechanism of action of hen egg white lysozyme (HEWL) widely regarded as the paradigm for enzymatic hydrolysis involves, two acidic residues, Asp 52 and Glu 35 in its catalytic activity [115,116]. The similarity between lysozyme and N-acetyl-β-D-glucosaminidases / β-hexosaminidases is that, all of them hydrolyze C2 N-acetylated β(1-4) linked substrates. In lysozyme, Glu 35 acts as the general acid-base and Asp 52 acts as the nucleophile. Glu 35 initially acts as a general acid, protonating the glycosyl oxygen, leading to bond cleavage and formation of a positively charged oxocarbonium intermediate. Asp 52 proposedly functions as the nucleophile, stabilizing the oxocarbonium intermediate, via electrostatic interactions [116,110] or by the formation of a

covalent intermediate with the C1 atom of –1 sugar residue [109]. Subsequent to the diffusion of the leaving group from the active site, a water molecule is replaced which acts as the nucleophile. This water molecule, assisted by the negatively charged Glu 35, which now functions as the general base, attacks the intermediate to complete the reaction. As the water molecule attacks on the same side of the leaving group, the reaction occurs with net retention in configuration at the anomeric carbon atom.

Few reports exist on the active site characterization of the exo-N-acetyl-β-D-glucosaminidases / β-hexosaminidases. Based on the heat of ionization studies of essential ionisable groups in the planarian, *Turbatrix aceti* β-N-acetylglucosaminidase, *Bedi et al.* [79] suggested the involvement of two carboxyl groups in the reaction mechanism. The active site characterization of *Serratia marcesans* chitobiase and the β-hexosaminidase from human tissues have been studied to some extent. With the help of chemical modifiers specific for carboxyl side chains and *in vitro* mutagenesis of the β cDNA, followed by cellular expression, a single carboxylic acid, βAsp^{196} has been identified as the catalytic acid residue in human β-hexosaminidase A [117,118]. However, *Proia et al.* [119] working on similar lines with the help of specific photo-activable affinity probe, (3-azi-1-[([6-^{3}H] 2-acetamido-2-deoxy-1-β-D-galactopyranosyl) thio]-butane, which modifies active site carboxyl groups and site directed mutagenesis, showed βGlu^{355} as the acid catalyst. Structural determinations in the evolutionarily related enzyme, chitobiase, from *Serratia marcesans* revealed the involvement of Glu^{540}, the counterpart of βGlu^{355} of human β-hexosaminidases, as the catalytic acid [54]. Involvement of an arginine residue in substrate binding has also been implicated in the human HEX A [120].

Unlike other glycosidases, the enzymes from glycosyl hydrolase families 18 and 20 (like chitinase, exo-N-acetyl-β-D-glucosaminidase and β-hexosaminidase) have been identified to possess only a single carboxylate residue at the active site with the absence of a putative nucleophile [54,95,121-126]. Such an observation has led many investigators to propose a substrate assisted catalysis

(anchimeric assistance / neighboring group participation) taking advantage of the natural chemistry of the substrate. This is necessary since oxocarbonium ion stabilization is a fundamental prerequisite in glycosyl hydrolases that undergo hydrolysis via formation of oxocarbonium intermediate due to their extremely short lifetimes in solution [127].

Substantial evidence for a probable substrate assisted catalysis (anchimeric assistance by acetamido group of GlcNAc) in N-acetyl-β-D-glucosaminidases and β-hexosaminidases has been recently found. Kinetic inhibition studies (in the *S. marcesans* chitobiase) using selective de-N-acetylated chito-oligosaccharides at their non-reducing end, resulted in complete resistance towards the hydrolysis of pNP-β-D-GlcNAc, indicating the absolute requirement of the acetamido group [97]. A thiazoline derivative of N-acetylglucosamine, in which the oxazoline ring has been replaced by sulphur, is a potent competitive inhibitor of the jack bean β-hexosaminidase [127]. Jones and Kosman [57], working on the β-N-acetylglucosaminidase from *Aspergillus niger* with the help of various para-substituted phenyl derivatives of GlcNAc (fluroacetamido/ thioacetamido derivatives), suggest a similar anchimeric assistance in glycoside cleavage either by stabilization of the oxocarbonium ion or by facilitating oxozoline formation. In fact even in lysozyme, a p-nitrophenyl-chitobiosyl derivative, in which the C-2 acetamido group of the sugar undergoing hydrolysis has been replaced by a hydroxyl group, was found to be a poor substrate implying that acetamido participation is important for catalysis [129]. The observed decrease in the k_{cat} was of a similar order of magnitude as would be observed with mutations in the supposed oxocarbenium ion stabilizer, Asp-52 [129]. X-ray crystallographic studies on other chitin degrading enzymes like hevamine, (plant chitinase/lysozyme) complexed with the inhibitor allosaminidin [122], the soluble lytic transglycosylase [130] and the *Bacillus* chitinase [131], give proof for a similar substrate assisted catalysis.

																Source	Acc. No.
1.	-	P	D	F	Y	L	H	L	G	G	D	**E**	V	-	324	HEX A-HUMAN	P 06865
2.	-	P	D	F	Y	L	H	L	G	G	D	**E**	V	-	324	HEX A_MOUSE	P 29416
3.	-	P	D	Q	F	I	H	L	G	G	D	**E**	V	-	356	HEX B_HUMAN	M 13519
4.	-	P	D	Q	F	I	H	L	G	G	D	**E**	V	-	335	HEX B_MOUSE	P 20060
5.	-	P	D	H	F	V	H	L	G	G	D	**E**	V	-	298	HEX B_FELCA	S 70340
6.	-	I	D	N	Y	F	H	T	G	G	D	**E**	V	-	309	HEX A_DICDI	P 13723
7.	-	G	T	D	Y	V	H	V	G	G	D	**E**	V	-	302	HEX 1_ENTHI	U 09735
8.	-	I	D	D	V	F	H	V	G	N	D	**E**	L	-	325	HEX 1_CANAL	P 4307
9.	-	S	T	D	M	F	H	M	G	G	D	**E**	V	-	371	HEX C_BOMMO	JC 253
10.	A	P	L	T	T	W	H	F	G	G	D	**E**	A	K	539	CHB_VIBHA	P 13670
11.	Q	P	I	K	T	W	H	F	G	G	D	**E**	A	K	542	CHB_SERMA	-
12.	Q	P	L	T	D	Y	H	I	G	A	D	**E**	T	A	521	HEX_VIBVU	Q 04786
13.	V	P	L	N	T	Y	H	I	G	A	D	**E**	T	A	533	HEX B-ALTSO	D 29665
14.	-	P	G	T	Y	F	H	I	G	G	D	**E**	C	P	337	HEX A-PORGI	X 92379

Fig I.3 Multiple alignment of the homologous region in family 20 glycosyl hydrolases (β-hexosaminidases and exo-N-acetyl-β-D-glucosaminidases) indicating the invariant glutamate residue proposed to be involved in catalysis (the acid catalyst).

The sequences were obtained from **1.** Human α-subunit [90], **2.** mouse α-subunit [133], **3.** human β-subunit [90], **4.** mouse β-subunit [132], **5.** feline β-subunit [134], **6.** β-hexosaminidase from *D. discoideum* [81], **7.** from *E. histolytica* [135] **8.** from *C. albicans* [136], **9.** from *B. mori* [86], **10.** from *V. harveyi* [87], **11.** chitobiase from *S. marcesans* [53], **12.** from *V.fulnificus* [88], **13.** from *Alteromonas* sp. [50] and **14.** β-hexosaminidase from *Porphyromonas gingivalis* [137].

Stereochemistry of the reaction mechanism

Determination of the reaction stereochemistry can give important information regarding the catalytic pathway an enzyme follows. This is usually done by immediate analysis of the reaction products, either by HPLC or H^1 NMR. In glycosidases, catalysis occurs through two major mechanisms, giving rise to an overall retention or inversion of anomeric configuration. Retaining β-glycosyl hydrolases release their saccharide products with retention of configuration at the newly exposed anomeric center [138]. Enzymatic hydrolysis of the glycosidic bond in retaining glycosidases has been proposed to take place via general acid catalysis that requires two critical protein side chain residues, a proton donor and a nucleophile / base. The inverting enzymes hydrolyze the glycosidic bond with net inversion of anomeric configuration via a mechanism involving general base-catalyzed attack of water on the anomeric center of the substrate, coupled with an acid-catalyzed cleavage of the glycosidic bond. The distance between the two catalytic residues is a characteristic of the mode and stereochemistry of the reaction mechanism. A distance of 4.8 – 5.3 Å is typical for hydrolysis with retention of anomeric configuration (via a double displacement mechanism), and about 9.0-9.6 Å for inversion (via single displacement), where an additional water molecule is positioned between the anomeric carbon and the second protein carboxylate (Fig I.3) [139,140]. Most of the β-hexosaminidases and N-acetyl-β-D-glucosaminidases, for which the reaction stereochemistry has been studied, follow the retention mechanism with the β anomer of GlcNAc formed as the first product [97,98]. Retention in stereochemistry has also been observed in other glycosyl hydrolases [141,142].

X-ray crystallography and three dimensional structure

The three dimensional structure of quite a few prokaryotic and eukaryotic chitinases that fall into families 18 and 19 have been reported [124-126,109]. However, except for the *Serratia marcesans* chitobiase there are no reports on the structural elucidation of enzymes from the family 20 glycosyl hydrolases under which the exo-N-β-D-acetylglucosaminidases and β-hexosaminidases have been

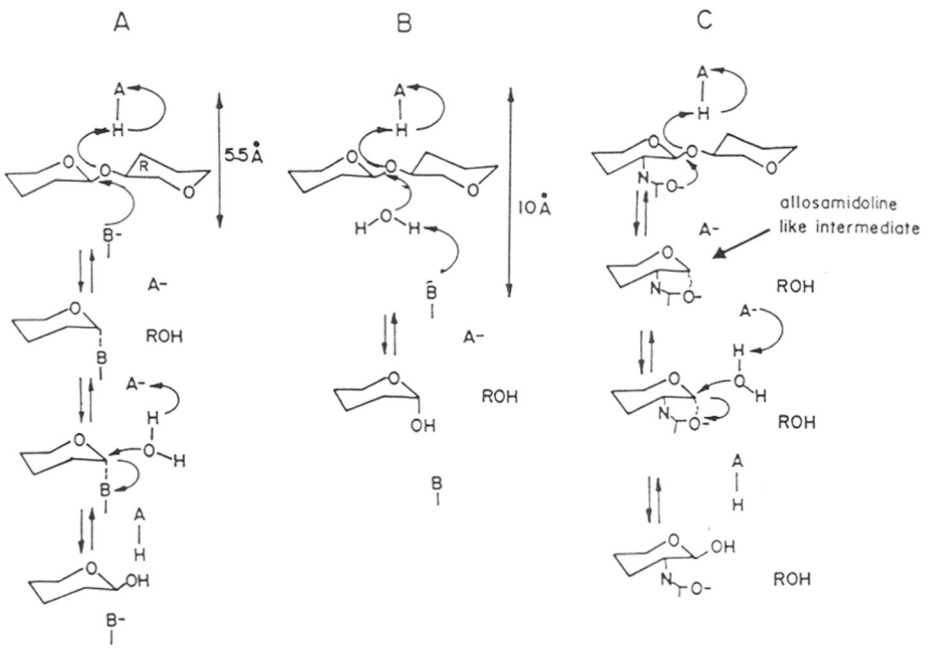

Figure I. 4 The two major mechanisms of enzymatic glycosidic bond hydrolysis as first proposed by *Koshland* [139]. A) retaining mechanism (double displacement), **B)** inverting mechanism (single displacement) and **C)** the proposed substrate assisted catalysis or anchimeric assistance leading to retention in configuration involving the C2 acetamido group [140].

classified. The *Serratia marcesans* chitobiase is a monomeric protein (98.5 kDa) stabilized by three disulphide linkages and it folds into four domains [54]. The active site is situated at the C-terminal end of the central $(\beta\alpha)_8$ – barrel. Based on the structure of the enzyme-substrate complex, in presence of the disaccharide chitobiose, an acid-base reaction mechanism has been proposed with a single protein carboxylate (Glu 540) functioning as the catalytic acid, while the nucleophile is the polar acetamido group of the substrate. At least 4 tryptophan residues have been shown to be involved in substrate stacking; GlcNAc **A** (non-reducing end) is stacked against Trp 737 and is stabilized by Trp 616 and Trp 639 and GlcNAc **B** is stacked against Trp 685.

Physiological role of Exo-N-acetyl-β-D-glucosaminidases / β-hexosaminidases

In bacteria, hydrolysis of peptidoglycan is a part of the pathogenic mechanism of the organisms' invasiveness by providing a means for increased bacterial growth and division, and for damage to the host cell [143]. The enzyme performs a major role in the degradation of the structural polysaccharide, chitin. Utilization of chitin in the marine bacterium *Vibrio furnisii* has been elegantly demonstrated by *Roseman et al.* [144-146]. They propose a three-step process in the degradation of chitin, involving; sensing, attachment and finally degradation. The sensing is mediated by chemotaxis and involves three distinct chemoreceptors; one GlcNAc specific, and two receptors that recognize a limited but overlapping group of small oligosaccharides ($GlcNAc_2$ – $GlcNAc_4$) [144]. The adherence is mediated by a Ca^{2+} dependent, GlcNAc specific lectin [145].

Atleast three different glycosidases have been shown to be involved in the degradation of chitin oligosaccharides to products that can be translocated across the *Vibrio furnisii* cytoplasmic membrane [146]. The colloidal chitin is first solubillized by an extracellular chitinase. The soluble oligosaccharides penetrate the outer membrane and are degraded in the periplasmic space to GlcNAc, $(GlcNAc)_2$ and $(GlcNAc)_3$ by two hydrolases (the chitin dextrinase and the β-N-acetylglucosaminidase) that are probably bound to the cytoplasmic membrane.

GlcNAc is transported and phosphorylated via the PTS, yielding cytoplasmic GlcNAc-6-PO_4. The smaller oligosaccharides found in the periplasmic space are probably mobilized into the cytoplasm with the help of specific permeases wherein they are hydrolyzed by a second cytoplasmic N-acetyl-β-D-glucosaminidase. This observation has been supported by the fact that, two distinct N-acetylglucosaminidases are expressed in *Vibrio furnisii* when grown in a medium containing lactate and 50 % ASW (artificial sea water) under varying concentrations of the inducer, chitobiose, (0.19 μM – 2.4 mM). At lower concentrations of the inducer, the cytosolic N-acetyl-β-D-glucosaminidase was expressed and under high concentrations of the inducer (chitobiose > 24 μM) both the periplasmic as well as the cytosolic N-acetyl-β-D-glucosaminidases were expressed. The cytoplasmic GlcNAc is then phosphorylated by an ATP dependent kinase to yield GlcNAc-6-PO_4. GlcNAc-6-PO_4 generated by either of the pathways is deacetylated and deaminated finally to produce fructose-6-PO_4.

The natural autolysis of filamentous fungi is a degradative process brought about by specific lytic enzymes. A profound increase in the β-hexosaminidase activity in culture filtrates of fungi has been observed during autolysis. Several β-hexosaminidases have been studied from fungi such as *A. nidulans, A. oryzae, T. harzianum, S. fructigena, M. fragilis* etc with respect to their functional role during the developmental stages of the organism [58,59,66,70,61]. Studies on the highly stable *A. nidulans* β-hexosaminidase indicated that the actual function of the enzyme during autolysis is in the hydrolysis of chitooligomers produced by the action of endo-chitinases [58].

β-hexosaminidase has been shown to be developmentally regulated in the cellular slime mould, *Dictyostelium discoideum* [80,147,148]. Low levels of the enzyme activity was observed in the initial stages when the organism was grown on bacterial cells. However enzyme levels increased more than ten-fold in the later stages with maximum levels of the activity found during the aggregation stage of development [147]. Such accumulation in NAGase levels did not occur in mutants lacking the aggregation phenomenon suggesting a role for the enzyme in the developmental program of *Dictyostelium discoideum* [149,150].

Various arthropods and euphausiids produce lytic enzymes capable of hydrolyzing their own chitinous structures favoring resorption of cuticular material and growth. Increased levels of chitinases and N-acetyl-β-D-glucosaminidases were found in the krills, *Euphausia superba* and *Meganytiphanes norvegia* shortly before moulting [151,152]. Chitinolytic enzymes also serve as digestive enzymes in fishes, snails and other animals [153,154]

β-hexosaminidases have been detected in moulting fluids, haemolymph and in the gut tissues of numerous insect species. It has been observed that the level of β-hexosaminidase is considerably higher (more than ten fold) in the pupal haemolymph than in the larval haemolymph suggesting their importance in the moulting process by facilitating degradation of chitinous exo-skeleton found in the cuticle and peritrophic membrane [83]. A pulsed pattern in the enzyme activity has been observed in insects like *Manduca sexta* [83], *Bombyx mori* [85,86] and *Belanus amphitrite* [155] in the larval and pupal stages suggesting the expression of the enzyme to be under hormonal control [156]. The function of β-hexosaminidases in the moulting fluid and integuments is to act as a chitobiase or as an exo-chitinase in the degradation of chitin oligosaccharides to monomers which can subsequently be reabsorbed and used as precursors of cuticular chitin in the next developmental stage. Its physiological role in the pupal haemolymph is not clear and may be involved in tissue processing and restructuring.

In plants, β-hexosaminidases are postulated to act during seed germination by releasing N-acetylglucosaminyl residues from storage glycoproteins which have undergone prior proteolytic degradation [27,74,77]. A marked increase in the β-hexosaminidase levels has indeed been observed during seed germination in plants [74,157].

NAGases have also been proposed to function in the defense mechanisms of vertebrates. External administration of chitosan or chitin to mice showed elevated levels of acid-phosphatase, β-glucuronidase and N-acetylglucosaminidase [158] in the macrophages suggesting their antitumour activity and protection against pathogens. Although, studies on the exact

functional role of N-acetylglucosaminidases in vertebrates are still at a nascent stage, recent findings indicate that NAGases play important roles in the development and growth of vertebrates too. They have been found in the chondro-osseous junction of bovine growth plate suggesting their role in the calcification and dissolution of extracellular matrix [159]. Degradation of glycosylaminoglycans involves the synergistic action of various lysozomal enzymes like β-hexosaminidases, glucuronidases, sulphatases, cathepsins and other proteinases.

A number of inborn errors of glycoprotein catabolism are characterized by the absence of specific lyzosomal hydrolase activity, which results in tissue accumulation of undegraded polysaccharide and / or lipid material (Table I.2). The human β-hexosaminidases are characterized by the presence of two subunits the α- and the β-subunit. A post-translational event of particular importance in the human β-hexosaminidases is the dimerization of its subunits to give catalytically active enzyme. Neither the α-monomer [160] nor the β-monomer [161] is catalytically active by itself. The monomers associate in three different ways to give homo- or heterodimeric isoenzymes: A (αβ), B (ββ) and S (αα). As a characteristic feature of proteins destined for lysosomes, both the subunits of the human β-hexosaminidases undergo numerous post-translational modifications in transit or after reaching the lysosomes. These modifications include removal of the signal peptide, N-glycosylation, formation of disulfide bonds, acquisition of the mannose-6-phosphate recognition marker and limited proteolysis [162,163]. Mutations in the HEX A, HEX B and G_{M2} genes, encoding the α- and β-subunits of β-hexosaminidase A and the G_{M2} activator protein respectively, lead to a group of inherited neurodegenerative diseases, collectively known as the G_{M2} gangliosidosis, that are characterized by lysosomal accumulation of G_{M2} ganglioside mainly in the neuronal tissue. The disorders range in severity from Tay-Sachs disease, a progressive and fatal neurodegenerative disorder of infancy, to clinically milder or later onset forms of G_{M2} gangliosidosis occurring in patients with lower residual activities [164,165]. More than 70 mutations in the human HEX A locus and 14 in the HEX B have been identified [163,166].

Table I.2

Oligosaccharide / Glycopeptide fragments accumulated due to deficiency of lysosomal hydrolases.

Defective lysosomal hydrolase	Major accumulated fragment (in humans)	Disease
Neuraminidase	NeuAc-Gal-GlcNAc-Man Man-GlcNAc NeuAc-Gal-GlcNAc-Man	Sialidosis
β-Galactosidase	Gal-GlcNAc-Man Man-GlcNAc Gal-GlcNAc-Man	G_{M1} Gangliosidosis
N-acetyl-β-D-Glucosaminidase/β-hexosaminidase	GlcNAc-Man Man-GlcNAc GlcNAc-Man and GlcNAc-GlcNAc-Asn	Tay-Sachs, Sandhoff
α-Mannosidase	GlcNAc-Man Man-GlcNAc GlcNAc-Man and GlcNAc-Man Man-GlcNAc	α-Mannosidosis
β-Mannosidase	Man-GlcNAc	β-Mannosidosis
α-L-Fucosidase	Fuc GlcNAc-Asn	Fucosidosis
Glycosylasparaginase	GlcNAc-Asn	Aspartylglycos-aminuria

Clinical significance and applications of the N-acetyl-β-D-glucosaminidases

The antifungal properties of fungi, bacteria and plants which are able to inhibit the growth of phytopathogenic fungi is proposedly due to the synergistic action of various cell wall degrading enzymes, including chitinolytic enzymes of which the exo-N-acetyl-β-D-glucosaminidase is a major component [167]. Certain microorganisms like *Serratia marcesans* and *Kurthia zopfii* and crude chitinolytic preparations from them have been effectively used as biocontrol agents of fungal pathogens (*Fusarium oxysporum*, *Botrytis cenera*, *Trichoderma* spp. and *Saccharomyces* sp.) of crop plants (like tomato, strawberry etc.) [168]. The synergistic action of endo- and exo-chitinases from the culture filtrates of *Serratia marcesans* totally inhibited the growth of the fungal wilt pathogen *Fusarium oxysporum* [169].

The transglycosylase activity of the β-hexosaminidase from *Aspergillus oryzae* has been used to prepare chitooligosaccharides (DP, 2-4) using p-nitrophenyl-N-acetyl-β-D-glucosaminide and N-acetylglucosamine as acceptors [59]. *In vitro* studies on the selective degradation of glycoproteins with terminal GlcNAc residues have been done with many preparations of strict N-acetylglucosaminidases as well as β-hexosaminidases [56,57,61,64,65,75].

Increased levels in exo-N-acetyl-β-D-glucosaminidase activity has been found in thyroiditis [170], diabetes mellitus [171,172], leukemia [173], acromegaly [174] and cancer [175] along with an increase in other lysosomal enzymes. The serum GlcNAcase has been clinically evaluated as a liver-function test [176]. Raised urinary excretion of GlcNAcase is associated with proteinuria and is a clear indication of urinary tract infection [177] and raised levels of the enzyme has been found during renal transplant [178]. The diagnostic value of the urine NAGase activity has been the subject of a recent review [179].

Recent clinical data indicate that modified chitosans with attached amino acid moieties at the nitrogen atom play an active role in wound healing [180]. Such modified chitosans are hydrolyzed *in vivo* by lysozymes and thereby influence the structure of native collagen. The oligomers activate macrophages to

produce GlcNAcases which further hydrolyze chitooligomers to N-acetylglucosamine, glucosamine and substituted glucosamine i.e., the sugar units needed for the biosynthesis of hyaluronic acid and other components of extracellular matrix.

PEPTIDE -N^4- (N-ACETYL-β-GLUCOSAMINYL) ASPARAGINE AMIDASE (PNGase)

The endoglycosidases or oligosaccharide cleaving enzymes of N-linked glycoproteins are basically of two types-

1. The endo-β-N-acetylglucosaminidase, ENGase (E.C.3.2.1.96) and
2. The Peptide-N^4- (N-acetyl-β-glucosaminyl) asparagine amidase, PNGase (E.C.3.5.1.52).

Both the enzymes act at the invariant pentasaccharide core region of asparagine-linked glycans [181]. The endo-β-N-acetylglucosaminidase is a true endo glycosidase, which hydrolyzes the β(1-4) linked di-N-acetylchitobiosyl core region of N-linked glycoproteins. The products formed are an intact oligosaccharide with one of the GlcNAc residues at the reducing end and a peptide / protein moiety with the other GlcNAc residue attached at the asparagine residue. The peptide-N^4-(N-acetyl-β-glucosaminyl) asparagine amidase (PNGase) is essentially an amidase (amidohydrolase) that catalyses the hydrolysis of β-aspartylglucosylamine bond of peptide-bound N-linked glycans generating an intact 1-amino oligosaccharide and a peptide backbone, wherein, the asparagine residue involved in oligosaccharide linkage has been converted to an aspartic acid. The action of PNGases thereby introduces an additional negative charge on the protein molecule. The 1-amino oligosaccharide is further converted non-enzymatically to the corresponding oligosaccharide, following the release of ammonia.

A second amidase, the N^4-(N-acetyl-β-glucosaminyl)-L-asparagine amidase (E.C.3.5.1.26) (Glycosylasparaginase) catalyses a similar reaction. However, it is exactly opposite to PNGase in the peptide specificity [182,183].

The enzyme has an absolute requirement for a free amino- and carboxyl- terminus on the glycosylated asparagine moiety, whereas, the PNGases require a substituted peptide chain on the amino as well as carboxy terminus of the glycosylated asparagine moiety.

Enzyme assay

Detection of PNGase activity is usually done using fluorescent derivatives of the substrates (dansylated / dabsylated / resorufin labelled glycopeptides). Qualitative assays are followed by paper chromatography or TLC [184]. Quantitation of the enzymatic activity is accomplished by chromatographic separation of deglycosylated products from the original substrates (glycopeptides / glycoproteins) by HPLC and / or by using radiolabelled substrates [181]. Deglycosylation of glycoprotein substrates is followed by SDS-PAGE; due to increased mobility of the products (deglycosylated proteins) compared to respective substrate glycoproteins [185].

Purification of PNGases

Takahashi and *Nishibe* [186,187] first demonstrated the presence of an amidase in almond emulsin that could release intact oligosaccharides from defined glycopeptides of bromelain and ovalbumin. Since then, PNGases are found to be widely distributed in nature and have been studied from a variety of sources [188-195]. However, these enzymes have been purified from few microbes [181,195], plants [191-194] and animal tissues. In many cases characterization of the PNGases has been done from crude or partially purified preparations [196,197]. Purification procedures usually involve chromatographic techniques like ion exchange, size exclusion, affinity and hydrophobic chromatography. The use of high-resolution hydrophobic interaction chromatography could separate the various endo-N-acetyl-β-D-glucosaminidases (Endo F_1, F_2 and F_3) and the peptide-N^4- (N-acetyl-β-glucosaminyl) asparagine amidase (PNGase F) in *F. meningoscepticum* [181]. Owing to the glycoprotein nature of PNGase R, ConA-Sepharose has been used as one of the steps in the

partial purification of the enzyme from *Raphanus sativus* [194]. In addition to hydrophobic and lectin affinity chromatography, purification of the *Aspergillus tubigensis* PNGase also involves Protein-Pak DEAE chromatography [195]. Protease inhibitors like EDTA, PMSF, DFP, 0-phenanthroline and iodoacetamide have been routinely included in the purification procedures and enzyme assay of the PNGases to eliminate the effect of traces of proteases that may be present in glycoprotein / enzyme preparations [184].

Biochemical properties of PNGases

Although quite a number of endo-β-N-acetylglucosaminidases have been characterized from various sources with respect to their molecular properties, only a few peptide-N^4-(N-acetyl-β-glucosaminyl) asparagine amidases have been characterized with respect to their biochemical properties. In fact only the bacterial glycoamidase, PNGase F from *F. meningoscepticum* [181,198], the fungal glycoamidase PNGase At from *A. tubigensis* [195] and a few plant PNGases [191-194] have been characterized with respect to their physicochemical characteristics (Table I.3). However, they exhibit wide variations in their properties. The pH optimum of the enzymes from jackbean seeds [193], almond emulsin [191-192] and *A. tubigensis* [195] is between 4-6, whereas the PNGase F has a pH optimum of 8.6 [181,198]. The optimum temperature for activity of PNGase F and those from plant sources are between 30-40°C. In this respect the *A. tubigensis* enzyme differs and exhibits a temperature optimum of 62°C and is relatively thermostable [195]. The PNGase F from *F. meningoscepticum* is characterized by its monomeric nature with a molecular mass of 35,000 [181,198]. However, the M_r of the corresponding enzymes from other sources range between 50-80 kDa and also exhibit multimeric nature [191,193,195]. The PNGase At is a heterodimer with subunit M_r of 38 kDa and 28 kDa [195], whereas, the corresponding enzyme from almond emulsin is a single polypeptide of M_r 66.8 kDa [192]. The native M_r of the N-glycanase from jackbean was found to be 69 kDa. However, it exhibited multiple bands on SDS-PAGE ranging from 30-78 kDa [193]. The authors suggest proteolytic processing from the C-terminal

end to be responsible for the multiplicity of the enzyme under denaturing conditions. The PNGases are neither metalloproteins nor metal requiring proteins, however, presence of Mg^{2+}, Zn^{2+}, Co^{2+}, and Cu^{2+} led to increased activities in PNGase A (from almond emulsin) [191].

Substrate specificity of PNGases

The structural determinants of the substrate specificity of PNGases are two fold ; involving primarily, the polypeptide chain and the di-N-acetylchitobiosyl core region of the glycan moiety. The substrate specificity of *F. meningoscepticum* PNGase has been well established [184,200,201]. The peripheral oligosaccharide structure of the glycan moiety is largely unrecognized by the enzyme. Asparagine-linked glycans representing all major oligosaccharide classes like complex, hybrid and high mannose type glycans are hydrolyzed by the enzyme, although, the complex triantennary glycans are the preferred substrates. The only structural feature of the oligosaccharide moiety that seems to confer resistance to PNGase F is the α(1-3) substitution of a fucosyl residue (found in certain plant glycoproteins) at the core chitobiosyl GlcNAc. However, an α(1-6) substitution at the same position does not confer any resistance to hydrolysis. Further, the specificity also seems to depend on the peptide backbone. Both the α-amino and the α-carboxyl groups of the asparagine residue must be substituted by a peptide backbone. While complex glycans could be hydrolyzed in their native state, proteins bearing hybrid or high-mannose glycans could not be hydrolyzed without prior denaturation [202]. In general, prior denaturation of glycoprotein substrates with sodium dodecyl sulphate greatly enhanced the rate of deglycosylation as well as it reduced the amount of enzyme required for complete deglycosylation [203]. The PNGase At from *Aspergillus tubigensis* has a similar substrate specificity as that of PNGase F with a preference for triantennary glycans [195]. However, it hydrolyzed α(1-6) as well as α(1-3) fucosyl substituted glycans equally. In this respect it resembles the plant PNGases which hydrolyze a broad array of oligosaccharide types including very short glycans and oligosaccharides with α(1-6) or α(1-3) core-linked fucose residues [198,204]

Table I.3

Properties of some of the well characterized PNGases from different sources

Source	Optimum pH, temperature and molecular mass	Properties of the enzyme	Substrate specificity	Ref. No.
F. meningo-scepticum PNGase F)	9.3, 37°C, 35 kDa		Hydrolyses all kinds of Asn-linked glycans examined, provided both $-NH_2$ and –COOH of Asn are involved in peptide linkage. Amount of enzyme required for deglycosylation varies with each glycoprotein substrate. Some of them require denaturation.	181
Aspergillus tubegensis (PNGase At)	5.0, 62°C, 80 kDa, subunits of 38 and 28 kDa	No homology with other PNGases and contains a high acidic amino acid content.	Hydrolyses all types of N-glycans with a preference for triantennary glycans. Glycopeptides with α(1-6) fucose substitution also hydrolyzed. Does not hydrolyze glycoproteins and prefers glycopeptides.	195

Table I.3 Continued...

Almond emulsin *(PNGase A)*	5.1, 37°C, 66.8 kDa	Partially purified preparation. Readily inactivated by SDS, Gd-HCl. Mg^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+} increase activity by 50 %.	Hydrolyses both high mannose and complex GPs. Location of the oligosaccharide and the peptide length are the major determinants of enzymatic activity. Glycans at the $-NH_2$ and –COOH terminal are hydrolyzed to a lesser extent. Bulky oligosaccharides are hydrolyzed 15-fold faster than peptide-GlcNAc.	191, 192,
Jack bean meal	5.0, 69 kDa, multiple subunits (78-30 kDa)	—	Liberates high mannose, hybrid and asialo-complex glycans from peptides but the corresponding intact glycoproteins are resistant to hydrolysis. Sialated glycans are not released	193
Raphanus sativus	4.5, 37°C, glycoprotein	suggested to produce 'unconjugated N-glycans' , signalling molecules in plants	Removes complex N-glycans with α(1-3) core fucosyl substitution. Can also deglycosylate RNase B in denatured state	194
Pisum sativum	6.5, 37°C, 80 kDa	partially purified preparation	Hydrolyses all kinds of oligosaccharide types. Complex oligosaccharides preferred (triantennary / biantennary).	199

PNGases from plant sources like *Raphanus sativus* [194], almond emulsin [192,204] and jack bean emulsin [205,206] hydrolyze complex, biantennary and high mannose glycopeptides (with or without α(1-3) core-fucosyl substitution) but not many of the corresponding glycoprotein substrates even after denaturation. The corresponding glycoasparagines are also not hydrolyzed suggesting the absolute requirement of a peptide substitution at the carboxyl and amino terminal of asparagine. Glycopeptides derived from complex glycoproteins are the preferred substrates. However, the almond emulsin PNGase A could hydrolyze denatured ovomucoid and RNase B glycoproteins [192].

Active site characterization and three dimensional structure

Davies and *Henrissat* [140] have suggested that glycosidases fall into three general classes of active-site topologies; the pocket or crater for exoglycosidases, a cleft or groove for endoglycosidases and a tunnel for glycosidases that hydrolyze fibrillar polysaccharides. The endoglycosidases are characterized by the presence of a cleft or groove, an open structure which allows random binding of several sugar units of polysaccharides as has been found in lysozyme, endocellulase, chitinase, α-amylase etc [138].

An analogy of the active site of PNGases with some of the well characterized enzymes that hydrolyze similar substrates like the glycosylasparaginases and L-asparaginases would indicate the involvement of a catalytic triad Asp-Lys-Thr in its activity [207-209]. A unique feature of the glycosylasparaginases is the post translational activation process. Glycosylasparaginases are characterized by the presence of two non-identical α- and β- subunits held by non-covalent forces [208,210]. The protein is encoded by a single gene, synthesized as a single polypeptide precursor and is post-translationally processed. The post-translational processing is a single obligatory proteolytic splicing which results in the formation of a Thr residue at the N-terminal end of the β-subunit [209,211-213]. The N-terminal, β-subunit Thr residue plays an essential role in the activity of the enzyme [214,215]. Aspartylglycosaminuria (AGU) is the most common and severe disorder of

glycoprotein catabolism caused due to the deficiency of glycosylasparaginase. Majority of the cases of AGU are caused due to genetic mutations that result in the failure of the activation process [214,216]. In contrast the PNGases exhibit variations in their molecular mass and subunit composition and no such activation process seems to occur in them. Very few details regarding the mechanism of action and residues involved in the activity of PNGases is known. Active site studies have been confined to the PNGase F from *Flavobacterium meningoscepticum*, which is the only PNGase for which crystallization studies have been done [217,218]. The enzyme is a monomeric protein of 34.8 kDa and folds into 2 domains. Both the domains have eight stranded, anti-parallel, β-sandwich motifs that are similar in geometry. Site directed mutagenesis as well as X-ray crystallographic studies have been done to identify the catalytic and oligosaccharide recognition residues. The active site is located in a cleft at the interface between the two domains of the molecule. Three acidic residues; Asp-60, Glu-118, Glu-206 are essential for the activity. Each residue plays a distinct role in the mechanism of action of the enzyme. Mutagenesis of Glu-118 and Glu-206 to their corresponding amines and co-crystallization experiments with the product N'-N''-diacetylchitobiose revealed their participation in substrate binding [218]. Mutagenesis of Asp-60 (to Asn) resulted in total loss of activity suggesting its role in the catalytic function of the enzyme. The active site cleft is also characterized by the presence of five Trp residues, which are proposed to stabilize the oligosaccharide binding through hydrophobic interactions [218].

Physiological role of PNGases

The predicted functions of endoglycosidases are either by their direct action on various glycoproteins or indirect, via their reaction products which perform several important functions [219]. They degrade glycans to yield free N-glycans also called as oligosaccharins which are suggested to be the precursors of "unconjugated N-glycans", molecules that play important roles in plant physiology [220,221]. PNGases and ENGases are differentially produced during post-germination in raddish (*Raphanus sativus*), the period corresponding to a

critical transition in the sporophytic life cycle of the plant [222]. ENGase was observed only during post-germination, prevalent in the cotyledons suggesting a role in mobilizing storage food, whereas, PNGase was observed throughout the life cycle.

The jack bean lectin, ConA, is synthesized as an inactive precursor and undergoes various post translational processing such as deglycosylation, proteolysis and post translational ligation of peptides to yield the mature protein [193,223]. Removal of the N-glycan from the precursor is a key step in the conversion of the precursor protein to the active lectin, carried out by the jack bean N-glycanase [224].

Plants react to attack by pathogenic microorganisms with a multiplicity of biochemical reactions collectively known as defense or stress responses [225,226]. Elements of these defense responses are the induction of enzymes of secondary metabolism like phenylalanine ammonia lyase [226] and enhanced biosynthesis of the plant growth hormone ethylene [227]. Glycopeptides produced from yeast invertase have been found to act as elicitors of phenylalanine ammonia lyase and ethylene biosynthesis in tomato callus suspension cells [228]. Certain endogenous oligosaccharides are also known to stimulate fruit ripening in tomato [229,230]. It has also been suggested that such elicitors are products of fungal plant pathogens, which play a role in mediating plant-pathogen interactions. Such glycopeptide elicitors have been studied from *Phytophthora megasperma* during infection in soybean [231], from *Cladosporum fulvum*, which causes necrosis in tomato [232] and from *Colleotrichum lagenarium* which induces ethylene biosynthesis in melon plants [233]. The treatment of such glycopeptides with PNGases or ENGases may act as suppressors of the elicitors from which they have been derived [221,230].

Applications of PNGases

The *Flavobacterium meningoscepticum* endoglycosidases have been widely used to determine the exact molecular mass of the peptide backbone of a glycoprotein [234]. Heterogeneity in molecular mass in many cases arises due to

differential glycosylation. This can be ascertained by denaturing the glycoprotein and subjecting to deglycosylation for extended periods (16-24 h), followed by denaturing electrophoresis. Further, by using limiting amounts of the glycosidase, (or by carrying out the reaction under suboptimal pH or temperature), it is possible to generate a "ladder" of partially glycosylated products [235]. The number of bands observed is one greater than the number of glycans present. This can be used to determine whether all the potential sites of glycosylation in a polypeptide has been utilized, based on the nucleotide sequence.

There is increasing awareness of the possibility that antibodies directed against glycoproteins recognize the oligosaccharide moiety rather than the polypeptide. Therefore ENGases and PNGases can be used for deglycosylating glycoproteins and hence study its effect on the antigenecity of the molecule.

The functional role of N-linked carbohydrates on the biological activities of glycoproteins, enzymes and hormones can be assessed by the use of endoglycosidases [236,237].

Objectives of the present investigation

Carbohydrates have been projected as the fourth dimension of molecular recognition. The sugar moieties attached to various cellular macro- and micro-molecules (glycoconjugates) play important roles in cellular recognition processes. Their proposed functions are to act as biological recognition markers for the clearance of circulatory serum proteins, specificity for legume-symbiont interaction, allergen recognition, cell-cell recognition and targeting of proteins to different compartments within a cell. Determination of the carbohydrate composition, type and branching pattern is therefore essential to understand the exact mechanism of these molecules and also in the development of recombinant DNA-derived glycoproteins as pharmaceuticals. Exo- and endo-glycosidases with strict substrate specificity have been proved to be specific and non-destructive tools in determining the structure of glycoconjugates. Thermostable deglycosylating enzymes would be highly effective due to their better stability since denaturing conditions or elevated temperatures are generally required for the effective action of enzymes on glycoprotein substrates. The exo-N-acetyl-β-D-glucosaminidase (NAGase) and peptide-N^4-(N-acetyl-β-glucosaminyl) asparagine amidase (PNGase) are two such enzymes that are indispensable in the structure-function elucidation of carbohydrate moieties.

A thermotolerant *Bacillus* sp. produced high levels of an extracellular exo-N-acetyl-β-D-glucosaminidase (E.C.3.2.1.30) and a peptide-N^4-(N-acetyl-β-glucosylaminyl) asparagine amidase (E. C. 3. 5. 1. 52). The present investigation was undertaken to, **a)** purify and extensively characterize the exo-N-acetyl-β-D-glucosaminidase to understand its structure-function correlation and **b)** to purify the peptide-N^4-(N-acetyl-β-glucosylaminyl) asparagine amidase and evaluate its potential application as an analytical tool in the deglycosylation of glycoproteins.

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CHAPTER II

Production, purification and characterization of exo-N-acetyl-β-D-glucosaminidase from *Bacillus* sp. NCIM 5120

SUMMARY

The thermotolerant *Bacillus* sp. NCIM 5120 produced high levels of an extracellular exo-N-acetyl-β-D-glucosaminidase (25.4 U/L) when grown in a medium containing peptone (1 %, w/v), yeast extract (1 %, w/v), mannose (0.5 %, w/v) and Speakman's salts solution. The enzyme was purified to homogeneity by chromatography on CM-cellulose, Sephacryl S-300 and phenyl-Sepharose. The M_r of the native enzyme, determined by size exclusion chromatography on Sephacryl S-300 / Sephadex G-200, was 230,000 and it consisted of four identical subunits of M_r 60,000. It is a neutral protein with a pI of 6.8. The optimum pH and temperature for the enzyme activity were 6.0 and 70°C respectively. The enzyme hydrolyzed terminally linked β(1-4) GlcNAc residues specifically from the non-reducing end of neutral oligosaccharides. Comparative studies on the hydrolysis of chitooligosaccharides by exo-N-acetyl-β-D-glucosaminidase indicated chitobiose to be the best substrate with a K_m and k_{cat} of 0.34 mM and 24 μmoles min^{-1} mg^{-1}, respectively. The enzyme also exhibited strict substrate specificity with respect to the glycone substitution as well as anomeric linkage. Analysis of the reaction stereochemistry indicated that the enzyme is a retaining glycosidase, with the β anomer of GlcNAc formed as the first product. Determination of the energy of activation for the exo-N-acetyl-β-D-glucosaminidase revealed that the enzyme exhibits a biphasic / discontinuous Arrhenius plot with two characteristic energy of activation, with an inflection temperature of 50°C. The activation energy at temperatures below the inflection point (50°C) was found to be higher than that at temperatures above the inflection point. A comparison of the energy of activation with different leaving groups (p-nitrophenol and 4-methyl-umbelliferone) showed that the energy of activation for 4-Me-Umb-β-D-GlcNAc was higher, at temperatures below the inflection point, than for pNP-β-D-GlcNAc (60.3 and 43.2 k J / mol respectively).

INTRODUCTION

Carbohydrates exhibit wide stereochemical as well as structural variations in their occurrence. Living organisms take advantage of this diversity by using oligosaccharides and polysaccharides for a multitude of biological functions namely, storage, structure and highly specific signaling. Selective hydrolysis of glycosidic bonds is therefore crucial for energy uptake, cell wall expansion and degradation, and turnover of signaling molecules. Exo-N-acetyl-β-D-glucosaminidase (NAGase, β-2-acetamido-2-deoxy-D-glucoside acetamido deoxy glucohydrolase, E.C. 3.2.1.30) is one such glycosidase, which catalyzes the hydrolysis of terminally occurring, β(1-4) linked N-acetylglucosaminyl residues from the non-reducing end of glycoconjugates. The enzyme is widespread in distribution and has been studied from microbes, plants and animals [1-4]. Their functions vary from playing an important role during the cell wall development in bacteria [1], autolyzing activity during fungal cell development [5] to moulting in insect [6]. The NAGases also have an important role in the ultimate breakdown of glycoproteins and mucopolysaccharides. Clinical significance of the human enzyme (also known as the β-hexosaminidase) has been well known for a long time. A defect or deficiency in N-acetylhexosaminidase leads to the lysosomal storage disease, Tay-Sach's / Sandhoff's syndrome, due to the neuronal accumulation of glycoconjugates terminating in GlcNAc/GalNAc residues [7].

Along with its specific function in the degradation of glycoconjugates, the exo-N-acetyl-β-D-glucosaminidase is also a major component of the chitinolytic system. Chitin, a linear polymer of β(1-4) linked GlcNAc residues is the major structural polysaccharide found in the cell wall of fungi and in the exo-skeleton of insects and crustaceans. It is hydrolysed by the chitinolytic system, comprising of endo-chitinase (E. C. 3. 2. 1. 14), chitobiosidase and the exo-N-acetyl-β-D-glucosaminidase (E. C. 3. 2. 1. 30).

Although the exo-N-acetyl-β-D-glucosaminidases have been characterized from various sources, only few reports exist on enzymes from thermophilic organisms. Deglycosylation of glycoproteins often require drastic conditions like

denaturation or elevated temperatures where many of the deglycosylating enzymes are unstable. Further, a classification on the basis of IUBMB nomenclature of enzymes, (based on the substrate specificity) would classify many of the reported exo-N-acetyl-β-D-glucosaminidases to be polyspecific. NAGases from higher organisms exhibit flexibility in their specificity towards the glycone moiety and do not distinguish between β(1-4) linked N-acetylglucosaminyl and β(1-4) linked N-acetylgalactosaminyl residues and are therefore also referred as N-acetylhexosaminidases (E. C. 3.2.1.52). In view of this, thermophilic organisms capable of producing thermostable enzymes, with high linkage specificity, will have considerable application as an analytical tool in the structure-function correlation of glycoconjugates. Hence, a thermotolerant strain of *Bacillus* sp. which exhibited high levels of extracellular exo-N-acetyl-β-D-glucosaminidase activity, when grown at pH 6.0 and 50°C, was selected for further studies. The present chapter describes the production, purification and extensive characterization of the extracellular exo-N-acetyl-β-D-glucosaminidase from the thermotolerant *Bacillus* sp. NCIM 5120.

MATERIALS AND METHODS

MATERIALS

pNP-glycosides, pNP-1-thio-β-D-GlcNAc, N-acetylglucosamine, N-acetylgalactosamine, 3,4,di-NP-β-D-GlcNAc, 4-methyl-umbelliferyl substrates, chitooligosaccharides, methyl-β-D-GlcNAc, N-acetylmannosamine, N-bromosuccinimide and 5,5'-dithiobis 2-nitrobenzoic acid were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Whole yeast cells (Brewer's yeast) obtained from the Indian Yeast Co. Ltd., (Pune, India.) was lyophilized to dryness and used on a w/v basis. CM-cellulose, Sephacryl-S-300 and phenyl-Sepharose were obtained from Pharmacia (Uppsala, Sweden). Dansylated glycopeptides from soybean agglutinin and fetuin were prepared according to Huang *et al.* [8]. All the other reagents used were of the highest purity available from commercial sources.

METHODS

Screening for NAGase activity and fermentation parameters

Soil samples from different sources were suspended in sterile saline and plated on nutrient agar (NA) plates containing 1 % (w/v) whole yeast cells (YC) and incubated at 50°C for 24 h. 26 potential cultures were identified which could grow on NA-YC plates. Single colonies from the plates were transferred to NB liquid medium, (peptone 1.0 % w/v, beef extract 1.0 % w/v and NaCl 0.5 % w/v), incubated at 50°C for 24 h under shaking conditions (200 rpm) and screened for exo-N-acetyl-β-D-glucosaminidase production. Among them, one of the isolates namely, *Bacillus* sp. NCIM 5120 showed maximum extracellular NAGase activity and was selected for further studies.

Microorganism and growth

The thermotolerant *Bacillus* sp. NCIM 5120 was routinely maintained at 50°C on nutrient agar slants (peptone 1.0 % w/v, beef extract 1.0 % w/v, NaCl 0.5 % w/v and agar 2.0 % w/v).

Optimization of fermentation parameters

The basal medium contained peptone (1.0 % w/v) and Speakman's salts solution (NH_4NO_3 0.2 %, KH_2PO_4 0.2 %, $MgSO_4.7H_2O$ 0.1 % and $MnSO_4.2H_2O$ 0.005 % w/v) adjusted to pH 6.0 [9]. Optimization studies were carried out in 250 ml conical flasks, containing 50 ml of the medium, using basal medium as the control medium by transferring 4 % (v/v) inoculum followed by incubation at 50°C for 24 h. The effect of various organic nitrogen sources, namely liver extract, yeast extract, tryptone, casamino acids, malt extract, cornsteep liquor and beef extract, was studied by supplementing the basal medium with the respective nitrogen source at 1.0 % w/v. Similarly the effect of various carbon sources was studied by supplementing the basal medium with various saccharides (glucose, maltose, lactose, fructose, xylose, galactose, sucrose, mannose and arabinose) at 0.5 % w/v. The effect of initial pH on growth and enzyme production was studied

by adjusting the initial pH of the enriched medium (peptone 1.0 % w/v, mannose 0.5 % w/v, yeast extract 1.0 % w/v and Speakman's salts solution) from 5.0 – 9.0. Similarly, the optimum temperature for the growth of the organism and production of enzyme was studied by carrying out the fermentation in enriched medium (pH 6.0) at various temperatures (25°C - 60°C).

Enzyme production

The inoculum was prepared by inoculating 50 ml of enriched medium, containing peptone 1.0 % w/v, yeast extract 1.0 % w/v, mannose 0.5 % w/v and Speakman's salt solution [9], with 1 day old slant. A 4% v/v inoculum was transferred to a fresh medium and incubated at 50°C for 24 h under shaking (200 rpm). Subsequently, the supernatant was collected by centrifugation (7,800 **g**, 20 min, 4°C) and used as the source of enzyme.

Determination of protein

The culture filtrate (0.5 ml) was mixed with 0.5 ml of 12.5 % TCA and incubated in ice for 30 min. The precipitated protein was collected by centrifugation, (7,800 **g**, 5 min) washed with chilled acetone, redissolved in 0.1 N NaOH and the protein concentration was determined by *Lowry's* method [10] using BSA as standard. The protein concentration in column effluents and in the purified samples were determined by *Bradford's* method [11] using BSA as standard.

Enzyme assays

Exo-N-acetyl-β-D-glucosaminidase

The enzyme was assayed, spectrophotometrically, using p-nitrophenyl-N-acetyl-β-D-glucosaminide (pNP-β-D-GlcNAc). Appropriately diluted enzyme was incubated with the substrate (100 µl of 5.86 mM stock) in a final volume of 1 ml in 100 mM citrate-phosphate buffer, pH 6.0. After incubation at 70°C for 15 min, the reaction was terminated by the addition of 2 ml of 1 M Na_2CO_3. The p-nitrophenol liberated was read, as the phenolate anion, at 405 nm. The

concentration of p-nitrophenol liberated was determined using a molar absorption co-efficient of 1.77×10^4 for p-nitrophenol [12]. One unit is defined as the amount of enzyme that liberates 1 μmole of p-nitrophenol min^{-1} under the assay conditions.

When chitooligosaccharides [$(GlcNAc)_2$ - $(GlcNAc)_5$] were used as substrate, the N-acetylglucosamine liberated was quantitated, colorimetrically, according to the method of *Ressig et al.* [13] using N-acetylglucosamine as standard. Hydrolysis of phenyl-β-D-N-acetylglucosaminide was monitored according to *Lowry et al.* [10] using recrystallized phenol as standard. The hydrolysable products of acid swollen chitin, if any, were determined by estimating the reducing sugars liberated [14] using N-acetylglucosamine as standard.

Enzymatic activity on 4-Me-Umb-β-D-GlcNAc, 4-Me-Umb-β-D-GalNAc and 4-Me-Umb-β-D-GlcNAc-6-SO_4 was quantitated by measuring the fluorescence of released 4-methyl-umbelliferon on a Perkin Elmer Spectrofluorimeter, LS 50B. The excitation was done at 355 nm and the emission was measured at 480 nm using a slit width of 5 nm for both the monochromators [15].

Inhibition studies with various monosaccharides (GlcNAc, GalNAc, $GlcNH_2$, $GalNH_2$, 2-deoxy-D-Glc, Glc, methyl-β-D-Glc, methyl-β-D-GlcNAc and ManNAc) was carried out by pre-incubating the enzyme with the respective monosaccharide (5 mM and 10 mM) for 15 min, followed by assaying the enzyme activity under standard assay conditions. The inhibition constant (K_i) for GlcNAc, phenyl-β-D-GlcNAc, 4-Me-Um-β-D-GlcNAc-6-SO_4 and the substrate analogue pNP-1-thio-β-D-GlcNAc was calculated using a minimum of three different concentrations of the respective inhibitor.

Other Glycosidase activities

β-N-acetylgalactosaminidase, β-glucosidase, β-galactosidase, α-N-acetylglucosaminidase, α- & β-mannosidase were assayed using their respective

pNP-substrates. The endo-N-acetylglucosaminidase was detected by its activity on dns-glycopeptides from soybean agglutinin and fetuin. The reaction was followed by chromatography on Whatman 1 paper using butanol: acetic acid: water (3:1:1, by vol.) as the solvent system. The products liberated i.e., dns-a.a-GlcNAc was observed under a U V transilluminator[14].

Effect of temperature

The effect of temperature was studied by monitoring the enzyme activity at various temperatures (25°C – 90°C), in 100 mM citrate-phosphate buffer, pH 6.0, containing 150 mM NaCl. Stability studies were carried out by incubating the enzyme at different temperatures (25°C – 80°C) in absence of substrate and monitoring the activity at definite time intervals (0 min – 120 min) using pNP-β-D-GlcNAc.

To determine the energy of activation of the enzyme, the kinetic rate constants, K_m and V_{max} of the NAGase were determined at various temperatures (30°C - 70°C) with different leaving groups. A fixed concentration of the enzyme (~5 μg) was incubated with varying concentration of the substrates (pNP-β-D-GlcNAc, 25 μg – 150 μg or 4-Me-Um-β-D-GlcNAc, 20 μg – 120 μg). Ln k_{cat} was plotted against the reciprocal of the respective absolute temperature (K) to obtain an Arrhenius plot for both the substrates. Energy of activation was calculated by the equation, slope = – 0.052 [E_{act}] in k J / mol.

Carbohydrate content

This was determined by the phenol-sulfuric acid method [16] using mannose as standard. 200 μg of purified NAGase was denatured by incubation with 6.0 M urea for 24 h at 30°C. The enzyme was then dialyzed extensively against deionised water to remove any non-covalently associated carbohydrate and subjected to carbohydrate analysis. The glycoprotein nature of the enzyme was also assessed by subjecting the purified enzyme to SDS-PAGE followed by staining with thymol-sulphuric acid [17].

Purification of NAGase

All the steps were carried out at 4°C unless otherwise mentioned. Routine enzyme assays during purification steps were based on the hydrolysis of pNP-β-D-GlcNAc. Protein concentrations of column effluents were monitored by the absorbance at 280 nm. Protein concentration of pooled enzyme fractions during purification steps was determined by *Bradford's* method [11].

The crude culture filtrate was concentrated to $1/10^{th}$ of its original volume by ultrafiltration, under reducing atmosphere, using a YM-10 membrane. The concentrated extract was extensively dialyzed against 50 mM Na-acetate buffer, pH 5.5, containing 10 mM EDTA, and loaded onto a CM-cellulose column (2.5 × 50 cm) at a rate of 12 ml / h. The flowthrough fractions, which contained all of the NAGase activity, were pooled, concentrated and dialyzed against 100 mM Tris-HCl buffer, pH 8.0, containing 4 M NaCl. 2 ml of the concentrated enzyme was chromatographed on a Sephacryl-S-300 column (170 × 1.7 cm), pre-equilibrated with 100 mM Tris-HCl buffer, pH 8.0, containing 4 M NaCl. The active fractions (20 ml) were pooled, concentrated, dialyzed against the above buffer and rechromatographed on Sephacryl S-300 column, under similar conditions.

The active fractions, obtained from the above step, was adjusted to 24 % (w/v) ammonium sulphate and loaded onto a phenyl-Sepharose column (1 × 5 cm) pre-equilibrated with 100 mM Tris-buffer, pH 8.0, containing 24 % w/v ammonium sulphate. The column was developed by a step gradient of decreasing concentrations of ammonium sulphate (24,18,12,6,0 %, w/v) in the above buffer. The enzyme, which eluted at 12 % w/v ammonium sulphate, was concentrated, dialyzed against 100 mM Na-citrate-phosphate buffer, pH 6.0, containing 150 mM NaCl and stored at 4°C.

Electrophoresis

Native PAGE was carried out on polyacrylamide slab gels, 7.5 % (w/v), using Tris-glycine buffer pH 8.9 [18] and the bands were visualized by silver staining [19]. Analytical isoelectric focussing was done in 7.5 % (w/v)

polyacrylamide gels over the pH range 3.0-8.0 [20]. The pI of the purified NAGase was also confirmed by subjecting the enzyme to density gradient isoelectric focussing [21].

Determination of M_r

Native molecular mass was determined by gel filtration on Sephacryl S-300 (100 × 0.7 cm) and Sephadex G-200 (100 × 1.0 cm) columns equilibrated with 100 mM Tris-HCl buffer, pH 8.0, containing 4 M NaCl. The columns were calibrated using blue dextran (2000 kDa), thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa) and bovine serum albumin (66 kDa). The subunit molecular mass was determined by SDS-PAGE [22] in 7.5 % w/v polyacrylamide gels using high molecular weight markers (Sigma. SDS 6H) to calibrate the gel. The gels were stained with Coomassie Brilliant Blue R-250.

Amino acid analysis

The amino acid composition of purified NAGase was determined on a liquid phase automated amino acid analyzer, (Hewlett Packard Ti series 1050) equipped with a fluorescence detector. The protein sample was hydrolyzed with 6 N HCl at 110°C for 24 h. Total cysteine content was determined, spectrophotometrically, according to *Habeeb* [23] while total tryptophan content was determined according to *Spande* and *Witkop* [24] and *Eddelhoch* [25].

C D measurements

C D spectra (200-250 nm) were recorded on a JASCO 710 spectropolarimeter, using a thermostatically controlled cell holder with a 1 cm path length cell. For C D measurements at different temperatures, the protein (0.2 mg/ml) in 100 mM Na-citrate-phosphate buffer, pH 6.0, was equilibrated at the chosen temperature for 5 min before recording the spectrum.

Determination of the reaction stereochemistry

pNP-β-D-GlcNAc (200 μg) was incubated with 0.1 U of purified NAGase, in 200 μl of deionised water, for 5 min at 70°C. The reaction was arrested by incubating in a boiling water bath for 3 min, and then subjected to HPLC analyses.

High performance liquid chromatography (LDC Analytical, multisolvent delivery system equipped with an auto-injector, Model 1000 with 20 μl loop) was carried out on a Waters Sugar-PAK 1 column (300 × 6.5 mm). The mobile phase comprising of 0.1 mM Ca-EDTA was used at 25°C and at a flow rate of 0.5 ml/min. 20 μl of the sample or the standard was injected onto the column and the sugar anomers were detected using a RI detector. N-acetylglucosamine, at mutarotation equilibrium, was used as the standard. A C_{18} pre-column guard was used to remove the enzyme before it entered the column.

RESULTS

FERMENTATION PARAMETERS

A number of potential thermophilic isolates from soil samples were screened for the production of extracellular exo-N-acetyl-β-D-glucosaminidase. Among 26 cultures tested the thermotolerant *Bacillus* sp. NCIM 5120 produced maximum levels of the enzyme activity and therefore selected for the present study.

Effect of carbon source **:** All the carbon sources tested, except fructose and xylose, supported growth (Table II. 1a). However, maximum enzyme activity was observed when mannose was used as the sole carbon source. Influence of mannose (0.25 – 2.5 % w/v) on the enzyme production revealed that maximum enzyme levels (25.5 U/L) were obtained in presence of 0.5 % w/v mannose (Table II. 1b). Increase in mannose concentration (> 0.5 % w/v) supported growth but not enzyme production. In case of *Bacillus stearothermophilus* CH-4 [26], chitin compost was found to enhance extracellular NAGase levels. However, in the present case supplementation of chitin (1.0 %, w/v) or whole yeast cells (1.0 %,

w/v) as the sole carbon source did not enhance the enzyme production as compared to mannose (0.5 % w/v).

Effect of nitrogen source : Influence of various organic nitrogen sources tested namely, yeast extract, liver extract, beef extract, malt extract, casamino acids and cornsteep liquor, showed that except malt extract and cornsteep liquor all others supported growth and enzyme production (Table II. 2a). Among them, maximum enzyme levels were observed in presence of yeast extract. Influence of yeast extract concentration (0.5 – 2.5 % w/v), on extracellular NAGase production, revealed that maximum enzyme activity (25.5 U/L) was obtained in presence of 1.0 % (w/v) yeast extract (Table II. 2b).

Effect of initial pH : It was observed that *Bacillus* sp. NCIM 5120 could grow and produce the enzyme over a wide range of pH of 5.0 – 9.0 (Table II. 3). However, maximum enzyme levels (25.5 U/L) were obtained when the initial pH of the medium was adjusted to 6.0. The pH of the medium increased to 8.0 – 8.5 during growth.

Effect of temperature : Influence of temperature, on extracellular exo-N-acetyl-β-D-glucosaminidase production, revealed that *Bacillus* sp. NCIM 5120 could grow and produce the enzyme between 28 - 55°C. However, maximum enzyme activity (25.5 U/L) was obtained when the culture was grown at 50°C. The organism failed to grow above 55°C (Table II. 4).

Profile of growth and enzyme production : The time course of cultivation of *Bacillus* sp. NCIM 5120 in the production medium is shown in Fig II.1. In submerged culture, a gradual increase in the pH (from 6.0 – 8.5) of the broth accompanied growth and maximum enzyme levels were observed after 22 h.

Table II.1a

Effect of different carbon sources on NAGase production by *Bacillus* sp. NCIM 5120*

Carbon Source [a] at 0.5 %, w/v	Final pH	Activity (mU/ml)
Glucose	7.0	-
Maltose	7.5	11.4
Lactose	9.0	4.8
Fructose	no growth	-
Xylose	no growth	-
Galactose	9.0	16.0
Sucrose	7.0	-
Mannose	8.0	25.6
Arabinose	6.5	0.84
Chitin	8.0	23.00
Whole Yeast cells	8.5	15.54
Basal medium	6.0	0.04

* In 24 h.

[a] The effect of the above carbon sources were studied at a concentration of 0.5 % (w/v) in the basal medium (peptone 1.0 % w/v, yeast extract 1.0 % w/v and Speakman's salts solution).

Table II.1b

Effect of varying concentrations of mannose on the production of NAGase by *Bacillus* sp. NCIM 5120*

Mannose conc. (%, w/v)	Final pH	Activity (mU/ml)
0.25	8.31	16.20
0.50	8.04	25.60
1.00	7.26	10.35
1.50	7.45	6.12
2.00	7.00	5.40

* In 24 h.

Table II.2a

Effect of different organic nitrogen sources on the production of NAGase by *Bacillus* sp. NCIM 5120*

Nitrogen Source[a] at 1.0 % w/v	Final pH	Activity (mU/ml)
Liver extract	6.8	4.83
Yeast extract	7.8	25.92
Tryptone	6.5	5.50
Casamino acids	6.6	9.80
Malt extract	6.0	-
Cornsteep liquor	6.0	-
Beef extract	7.0	7.74
Basal madium	6.0	-

* In 24 h.

[a] The effect of the above nitrogen sources were studied at a concentration of 1.0 %, in the medium, peptone 1.0 % w/v, mannose 0.5 % w/v and Speakman's salts solution.

Table II.2b

Effect of varying concentrations of Yeast extract on the production of NAGase by *Bacillus* sp. NCIM 5120*

Yeast extract (%, w/v)	Final pH	Activity (mU/ml)
0.5	8.0	16.30
1.0	8.5	25.92
1.5	8.0	16.02
2.0	8.0	15.10

* In 24 h.

Table II.3

Effect of initial pH on the production of NAGase by *Bacillus* sp. NCIM 5120*

Initial pH	Final pH	Activity (mU/ml)	Relative activity (%)
5.0	6.90	3.33	17.07
6.0	7.27	25.50	100.00
7.0	8.01	15.60	80.00
8.0	8.36	8.10	41.53
9.0	8.23	4.77	24.46

* In 24 h.

Table II.4

Effect of temperature on NAGase production by *Bacillus* sp. NCIM 5120*

Temperature (°C)	Final pH	Activity (mU/ml)	Relative activity (%)
28	6.5	4.62	20.56
37	7.0	16.08	71.56
50	8.5	25.50	100.00
55	8.5	20.20	85.20
60	no growth	-	-

* In 24 h.

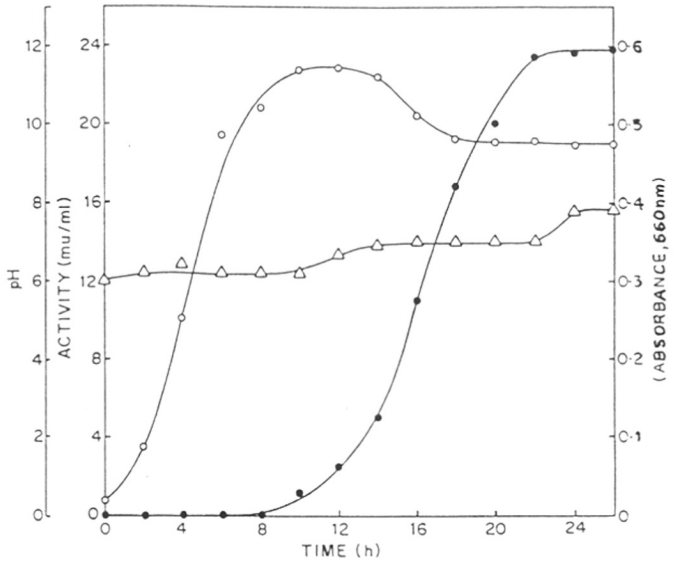

Figure II.1: Growth curve of thermotolerant *Bacillus* sp. NCIM 5120

A typical growth curve experiment carried out in enriched medium containing peptone 1% w/v, yeast extract 1% w/v, mannose 0.5% w/v and Speakman's salts solution. (•) Indicates the amount of NAGase secreted (mU/ml) as a function of time (h). (o) Indicates the cell mass produced (absorbance at 660 nm) as function of time (h). (Δ) Indicates the pH change as the fermentation proceeds from 0 h – 26 h.

PURIFICATION OF EXO-N-ACETYL-β-D-GLUCOSAMINIDASE

The results of a typical procedure for the purification of extracellular NAGase, to homogeneity, is given in Table II. 5. The NAGase was purified 184 fold with an overall recovery of approximately 26 %. The $A^{0.1\%,\ 1\ cm}$ of the purified enzyme, at 280 nm, was 1.34. NAGase obtained after CM-cellulose chromatography showed comparatively high specific activity, but contained considerable amount of colored impurities. This could be removed by successive chromatography on Sephacryl S-300. The enzyme after Sephacryl S-300 chromatography, though homogeneous, contained high amounts of polysaccharide. However, this could be removed by chromatography on phenyl-Sepharose. The elution profiles of the NAGase from Sephacryl S-300 and phenyl-Sepharose are shown in Fig II.2a and Fig II.2b respectively. The purified NAGase moved as a single band on native and IEF gels (Fig II. 3a and 3b).

Table II.5

Purification of exo-N-acetyl-β-D-glucosaminidase from *Bacillus* sp NCIM 5120.

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Fold purification	Recovery (%)
Culture Filtrate*	31.44	354.05	0.09	1	100
CM-cellulose unadsorbed	28.33	18.7	1.55	17.6	90.12
S-300 I	16.52	1.38	12.01	136.47	52.56
S-300 II	12.30	0.73	16.78	190.68	39.16
Phenyl-Sepharose	8.0	0.48	16.20	184.09	25.44

* Starting from 1400 ml of culture filtrate

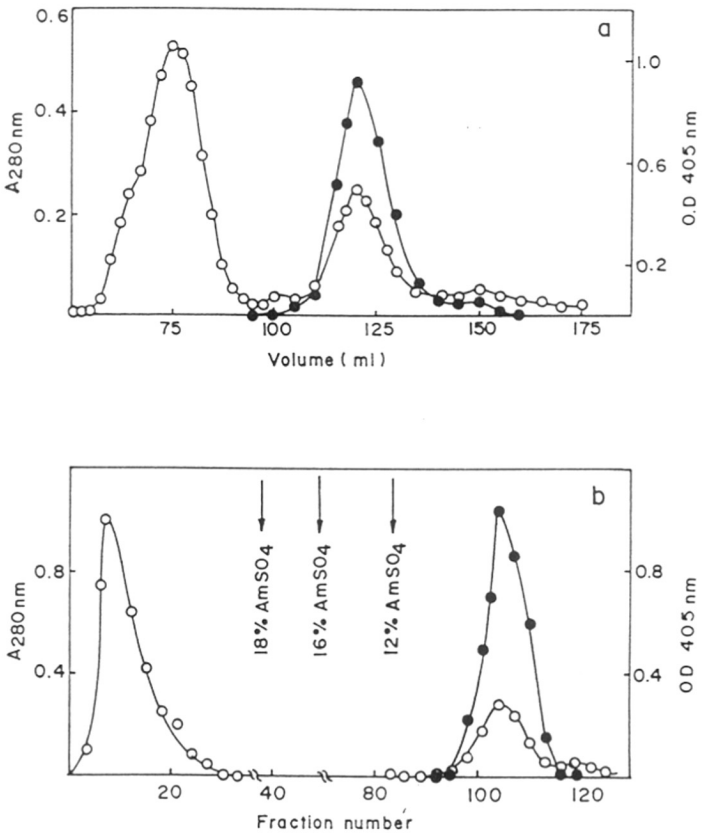

Figure II.2: Elution profiles of *Bacillus* sp. NAGase

a) From Sephacryl S-300 (II): (o) O. D. $_{280\,nm}$ and (•) NAGase activity.

b) From phenyl-Sepharose: (o) O. D. $_{280\,nm}$ and (•) NAGase activity.

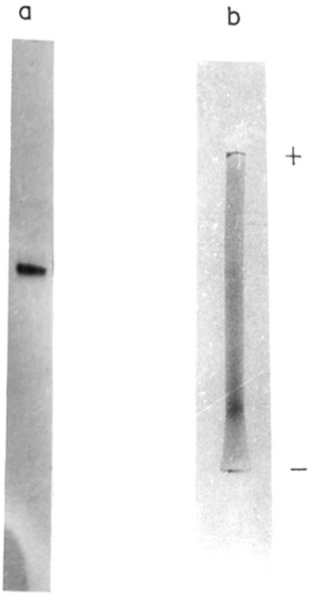

Figure II.3: Electrophoresis of purified NAGase

a) Native gel electrophoresis of purified NAGase (10 μg) in 7.5 % w/v, polyacrylamide slab gel, pH 8.9. The protein was visualized by silver staining.

b) Isoelectric focussing of purified NAGase (50 μg) in 7.5 % w/v, polyacrylamide tube gel using Pharmalytes in the range 3-8. The band was visualized by Coomassie Blue R-250 staining.

Physicochemical properties : The M_r of the purified enzyme, determined by gel filtration, was 230,000 (Fig II. 4). The relative subunit molecular mass of the purified NAGase was 60,000 (Fig II. 5) indicating the homotetrameric nature of the enzyme. Table II. 6 gives the amino acid composition of purified NAGase. The enzyme contained 6 cysteine residues per subunit, but no detectable free cysteine residues indicating the involvement of all the 6 cysteines in intramolecular disulfide linkages. A preponderance of glycine, serine and threonine were also observed.

Optimum pH and pH stability : The enzyme had an optimum pH of 6.0 and the relative activity at pH 5.0 and 7.0 were 39 % and 42 % respectively (Fig II.6). The *Bacillus* sp. NAGase showed comparatively high pH stability and retained a significant amount of its activity (> 70 %) between pH 5.5 and 9.0 (Fig II.6).

Effect of metal ions and EDTA : Among the divalent cations tested for their effect on NAGase activity, Hg^{2+} completely inactivated the enzyme whereas Cu^{2+}, inhibited the enzyme activity by 17 % (Table II.7). None of the other metal ions and EDTA (10 mM) had any significant effect on the enzyme activity.

Optimum temperature and temperature stability : The optimum temperature for enzyme activity was 70°C (Fig II.7a). The enzyme showed high thermostability and retained its full activity at 60°C for 2 h (Fig II.7b). At its optimum temperature (70°C) the enzyme retained its full activity for 30 min.

Substrate specificity, mode of action and kinetic parameters of NAGase : The *Bacillus* sp. NAGase hydrolyzed only GlcNAc containing substrates and no activity could be detected on other glycosidic substrates, suggesting the enzyme to be a true exo-N-acetyl-β-D-glucosaminidase (Table II.8). The K_m and k_{cat} for pNP-β-D-GlcNAc were 69 μM and 20.75 μmoles min^{-1} mg^{-1} respectively. Comparable hydrolysis of 3,4,di-NP-β-D-GlcNAc was observed with a k_{cat} of 18.44 μmoles $min^{-1}mg^{-1}$ but with a higher K_m (800 μM). The enzyme also

hydrolyzed 4-Me-Umb-β-D-GlcNAc with a K_m and k_{cat} of 17 μM and 9.46 μmoles min^{-1} mg^{-1} respectively.

Hydrolysis of chitooligosaccharides indicated chitobiose to be a better substrate, with a K_m and k_{cat} of 0.34 mM and 24 μmoles min^{-1} mg^{-1} respectively (Fig II. 8a). Chitotriose was hydrolyzed to a lesser extent with a K_m and k_{cat} of 1.66 mM and 17.75 μmoles min^{-1} mg^{-1} respectively (Fig II.8b) whereas, chitotetraose was hydrolyzed slowly (only after prolonged incubation, 2 h). Chitooligosaccharides above chitotetraoses were resistant to hydrolysis. Incubation of the enzyme with acid swollen chitin, at 50°C for 24 h, did not show any detectable reducing sugars.

The enzyme neither hydrolyzed pNP-β-D-GalNAc, even after incubation with excess enzyme (ten-fold), nor did it hydrolyze 4-Me-Umb-β-D-GalNAc, indicating its strict specificity towards β(1-4) linked GlcNAc residues. The purified NAGase did not possess any other glycosidase activity when tested against pNP-α-D-GlcNAc, pNP-β-D-Glc, pNP-β-D-Gal, pNP-β-D-Man and pNP-β-D-Xyl.

Phenyl-β-D-GlcNAc, a common β-N-acetylglucosaminidase substrate, was found to be a poor substrate for the *Bacillus* sp. NAGase as only 8.5 % of the original substrate was hydrolyzed after 24 h of incubation at 50°C. Due to the slow rate of hydrolysis, phenyl-β-D-GlcNAc acted as a competitive inhibitor in the hydrolysis of pNP-β-D-GlcNAc with a K_i value of 34.8 μM. Though 4-Me-Umb-β-D-GlcNAc-6-SO_4 could not be hydrolyzed by the enzyme, it exhibited a similar competitive inhibition as observed for phenyl-β-D-GlcNAc with a K_i of 307 μM. The end product, N-acetylglucosamine and the substrate analogue, pNP-1-thio-β-D-glucosamine exhibited competitive inhibition with a K_i value of 0.67 mM and 87.11 μM respectively (Fig II.9a and 9b).

Stereochemistry of reaction mechanism catalyzed by NAGase : HPLC elution profiles of the products of hydrolysis of pNP-β-D-GlcNAc by NAGase is depicted in Fig II. 10. The peak with shorter retention time (9.13 min)

corresponds to the β anomer while the α anomer elutes at 9.83 min (Fig II. 10a). The equilibrium ratio between α and β anomers is approximately 3:2. Analyses of the reaction mixtures after 10 min and 2 h revealed the initial formation of only the β anomer of GlcNAc (Fig II. 10b and 10c). The small amount of α anomer found could be due to the spontaneous mutarotation of the initially formed GlcNAc. Analysis of the reaction mixture, after 24 h, showed the reestablishment of the anomeric equilibrium (Fig II. 10d) indicating that the *Bacillus* sp. NAGase is a retaining glycosidase with the formation of β anomer of GlcNAc as the initial product.

Determination of energy of activation (E_{act}) : The Arrhenius plot of NAGase (Fig II. 11) exhibited a biphasic or discontinuous nature with an inflection temperature of 50°C for both the leaving groups i. e., p-nitrophenol and 4-methyl-umbelliferone. The affinity of the enzyme towards the substrate decreased with decrease in temperature. The energy of activation (E_{act}) was found to be higher at temperatures below the inflection temperature (50°C). The energy of activation with pNP-β-D-GlcNAc was 43.2 k J / mol and 18.5 k J / mol, at temperatures below and above 50°C respectively, while that with 4-Me-Um-β-D-GlcNAc as the substrate it was 60.3 k J / mol and 21.7 k J / mol respectively. Moreover, CD analysis of the NAGase, at different temperatures, showed no change in the ellipticity data indicating the absence of any temperature related changes in the secondary structure (Table II. 9).

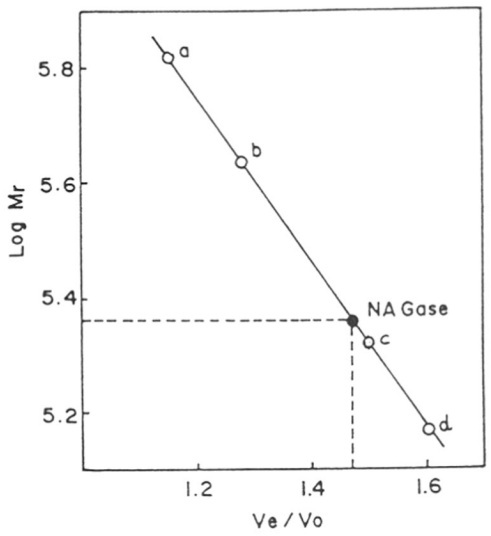

Figure II.4: Molecular weight determination of *Bacillus* sp. NAGase by gel filtration.

A Sephacryl S-300 (100 × 0.7 cm) column was equilibrated with 100 mM Tris-HCl buffer, pH 8.0, containing 4 M NaCl and calibrated with a) Thyroglobulin (669,000), b) Apoferritin (443,000), c) β-Amylase (200,000), d) Alcohol dehydrogenase (150,000). V_0, void volume and V_e, elution volume.

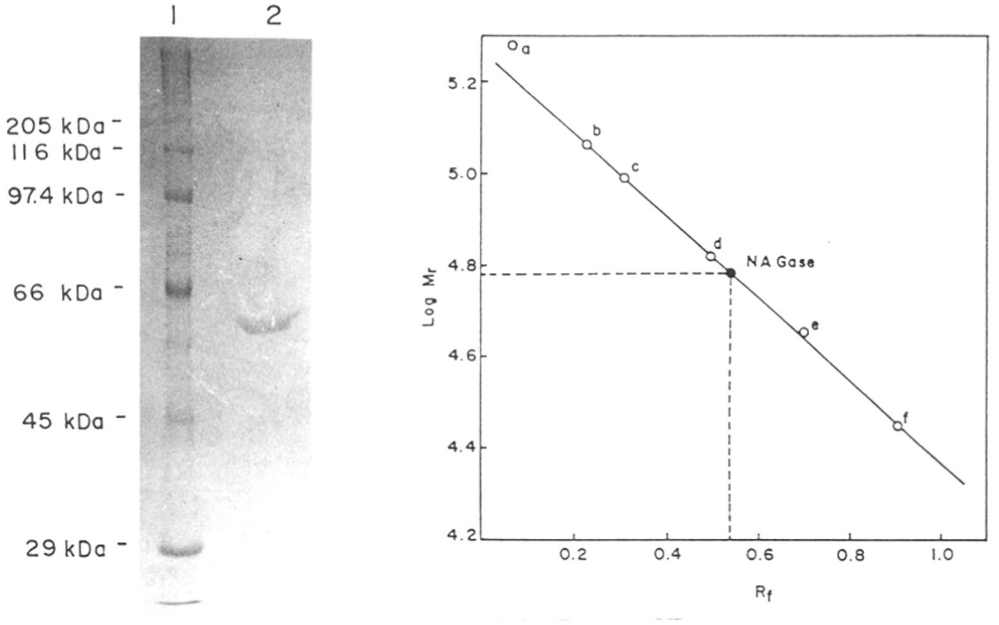

Figure II.5 : SDS-PAGE of NAGase

Purified NAGase (50 μg) subjected to SDS-PAGE in presence of β-ME in 7.5 % w/v acrylamide gels. The molecular masses of marker proteins are indicated alongside the gel. Lane 1, marker proteins (Sigma SDS-6H) a) myosin (205,000), b) phosphorylase b (97,400), c) bovine albumin (66,000), d) egg albumin (45,000), e) carbonic anhydrase (29,000), and; Lane 2, purified NAGase. The gels were stained with Coomassie Brilliant Blue R-250.

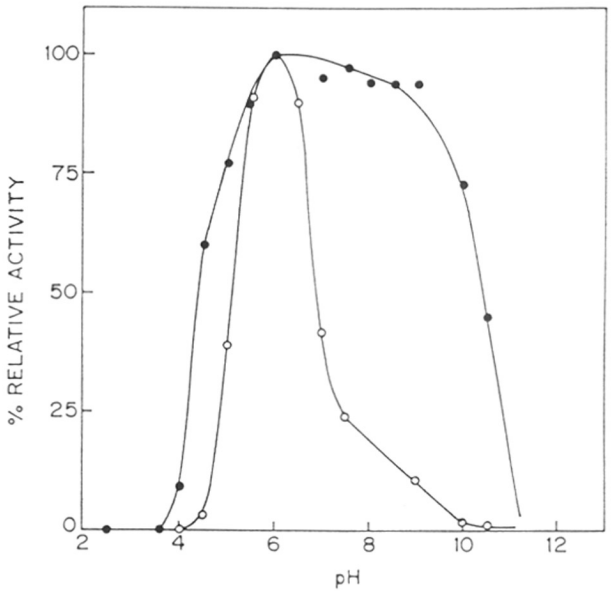

Figure II.6: Effect of pH on the activity and stability of NAGase

Purified NAGase (0.02 U) was assayed at different pH values (2.0 – 11.0), in universal buffers, at 70°C as described in Methods. The relative activities are expressed as a percent of the NAGase activity at pH 6.0.

pH stability studies were carried out by incubating the enzyme (100 μg) at various pH values in universal buffers (2.0 – 11.0) for 24 h and 25°C. The enzyme was brought to pH 6.0 thereafter and assayed for NAGase activity under standard assay conditions. (o) Optimum pH and (•) pH stability.

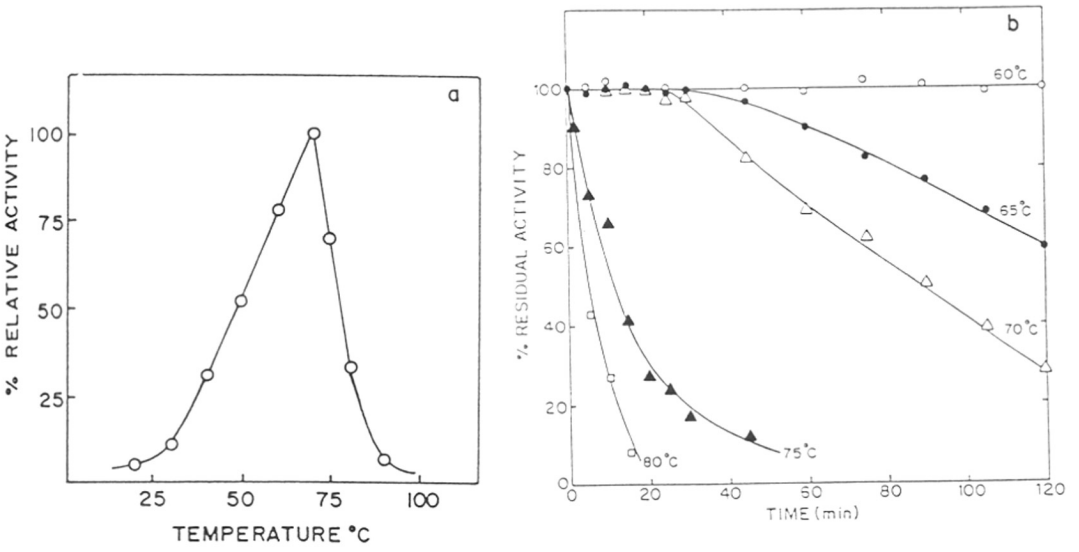

Figure II.7a: Optimum temperature for the *Bacillus* sp. NAGase

Purified NAGase (0.02 U) was assayed at various temperatures (20°C - 80°C) at pH 6.0, as described in Methods. The relative activities are expressed as a percent of the activity at 70°C.

Figure II.7b: Effect of temperature on the stability of NAGase

NAGase (500 μg/ml) after the phenyl-Sepharose step was incubated in Na-citrate-phosphate buffer, pH 6.0, at different temperatures (60°C – 80°C) and aliquots withdrawn at definite time intervals (0 min – 120 min). The aliquots were immediately quenched in an ice bath. Subsequently, the substrate pNP-β-D-GlcNAc was added and the reaction carried out under standard assay conditions.

Table II.6

A comparison of amino acid composition of exo-N-acetyl-β-D-glucosaminidases/ β-hexosaminidases from reported microbial sources.

Amino acid	*P. oxalicum* mol/mol [27]	*A.oryzae* mol/mol [28]	*A.niger* mol/mol [29]	*T.aceti* mol/mol [30]	*Bacillus* sp. NCIM 5120 mol/mol monomer, present investigation
As(x)	95	169.8	172	114	**64**
Thr	62	57.5	122	73.2	**24**
Ser	51	78.6	137.7	85.0	**66**
Gl(x)	74	119.8	105.2	92.0	**81**
Gly	60	74.6	249.1	212.2	**99**
Ala	79	91.6	183.8	120.0	**63**
Val	44	86.7	109.7	54.3	**27**
Met	27	10.0	13.5	6.0	**5**
Ile	44	72.7	58.5	45.6	**28**
Leu	54	82.5	96.6	74.3	**36**
Tyr	42	57.2	45.9	n.d.	**11**
Phe	31	34.8	38.6	35.8	**16**
His	40	32.2	17.3	18.8	**6**
Lys	38	29.9	27.5	33.9	**21**
Arg	35	44.3	27.0	35.0	**21**
Pro	41	68.9	91.5	78.5	**20**
Cys	7	11.5	1.5	31.5	**6**
Trp	27	54.3	10.6	n.d.	**11**
M_r	**141 kDa**	**140 kDa**	**149kDa**	**112 kDa**	**60 kDa**

n.d: not determined

Table II.7

Effect of divalent metal ions and EDTA on the activity of exo-N-acetyl-β-D-glucosaminidase

Metal ion	Relative activity (%)
None	100.00
$CuCl_2$	83.41
$CoCl_2$	109.85
$MnCl_2$	93.06
$MgCl_2$	91.72
$CaCl_2$	98.54
$HgCl_2$	0
$ZnCl_2$	103.04
$NiCl_2$	83.94
EDTA 10 mM	100.00
EDTA 100 mM	90.00

Table II.8

Kinetic constants for various substrates and inhibitors of exo-N-acetyl-β-D-glucosaminidase from *Bacillus* sp. NCIM 5120

Substrate / Inhibitor [a]	K_m	k_{cat} (μmoles min^{-1} mg^{-1})	K_i
pNP-β-D-GlcNAc	69 μM	20.75	
Chitobiose	0.34 mM	24	
Chitotriose	1.66 mM	17.75	
Chitotetrose *	-	-	
3,4,di NP-β-D-GlcNAc	800 μM	18.44	
4-Me-Um-β-D-GlcNAc	17 μM	9.46	
phenyl-β-D-GlcNAc			34.8 μM
4-Me-Um-β-D-GlcNAc-6-SO_4			307 μM
N-acetyl-D-glucosamine			0.67 mM
Methyl-β-D-N-acetylglucosamine			0.24 mM
pNP-1-thio-β-D-GlcNAc			87.11 μM

* After 2 h incubation

[a] pNP-β-D-GalNAc, pNP-α-D-GlcNAc, pNP-β-D-Glc, pNP-β-D-Gal, pNP-β-D-Man and pNP-β-D-Xyl did not show any activity even after 2 h of incubation.

Kinetic constants were calculated from Lineweaver-Burk plots for the respective enzymatic reactions performed in 100 mM Na-Citrate-phosphate buffer pH 6.0 containing 150 mM NaCl at 70°C. ~8 μg of purified NAGase was used / reaction point. k_{cat} values were calculated using the holoenzyme molecular mass value of 240 kDa.

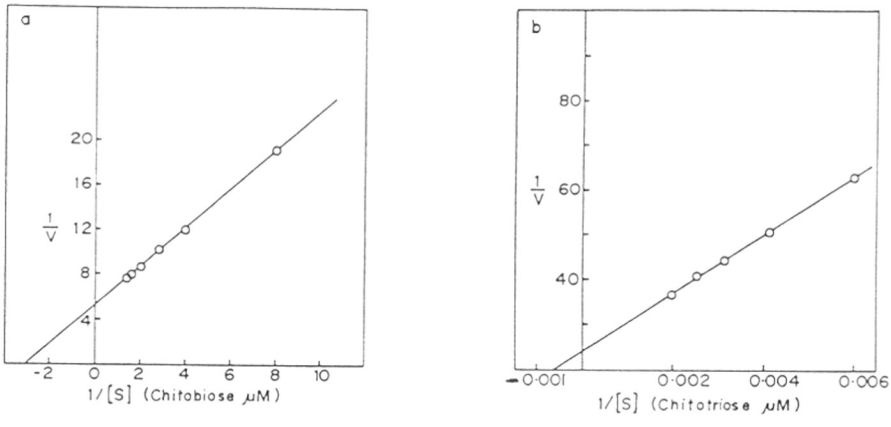

Figure 8: Double reciprocal plot for the hydrolysis of chitobiose and chitotriose by NAGase

Chitobiose (a) and chitotriose (b) were used in the range 25 μg – 150 μg.

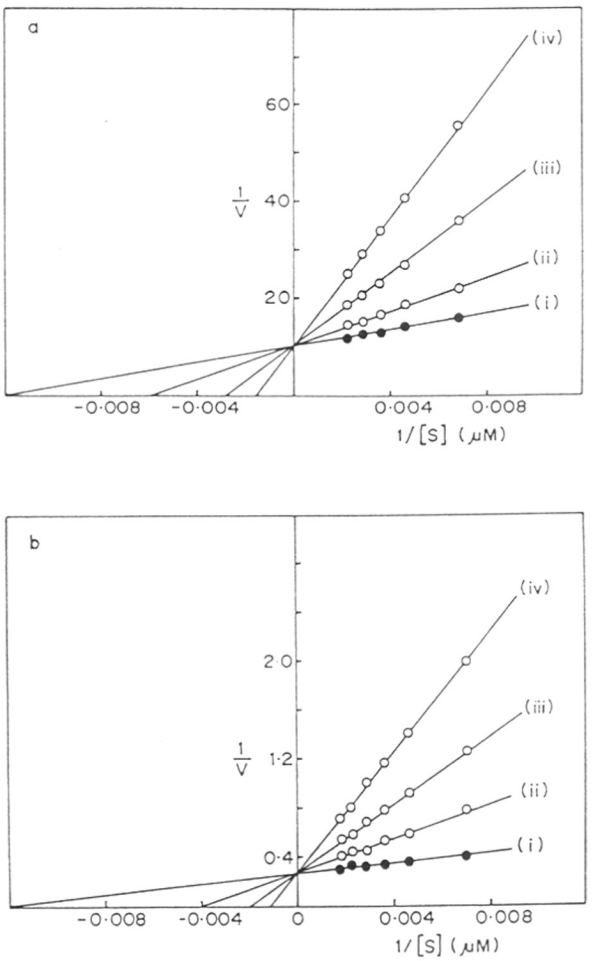

Figure II.9 : Double reciprocal plots of the inhibition by a) GlcNAc and b) pNP-1-thio-β-D-GlcNAc for pNP-β-D-GlcNAc hydrolysis catalyzed by *Bacillus* sp. NAGase.

Reactions were performed as described in Materials and Methods. The concentration of GlcNAc in the reactions were (i) 0, (ii) 2.5, (iii) 5.0, and (iv) 10.0 mM. The concentration of pNP-1-thio-β-D-GlcNAc in the reactions were (i) 0, (ii) 0.5, (iii) 1.0, and (iv) 2.0 mM.

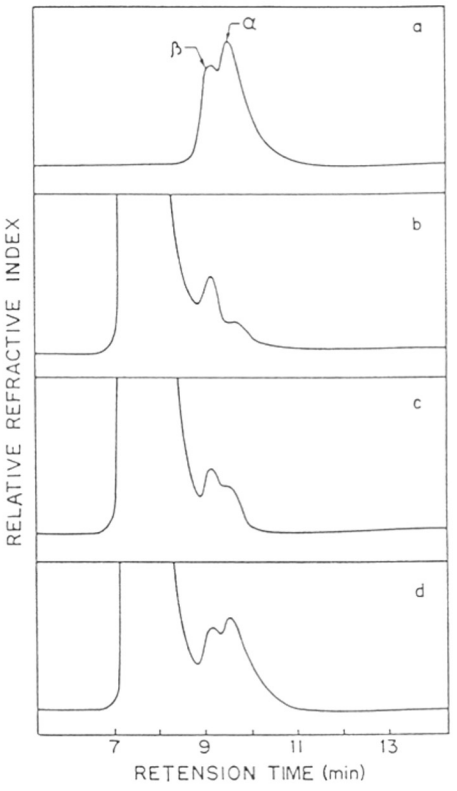

Figure II.10: HPLC determination of the stereochemistry of pNP-β-D-GlcNAc hydrolysis catalyzed by NAGase

200 µg of pNP-β-D-GlcNAc was incubated with 5 µg of purified NAGase in deionised water at 70°C. Samples were subjected to HPLC. **a)** Standard GlcNAc at mutarotation equilibrium (in water), **b)** aliquot of enzymatic reaction after 10 min, **c)** after 2 h and **d)** after 24 h. The X-axes show the retention time (in minutes) and the Y-axes show the signal measured by refractometer.

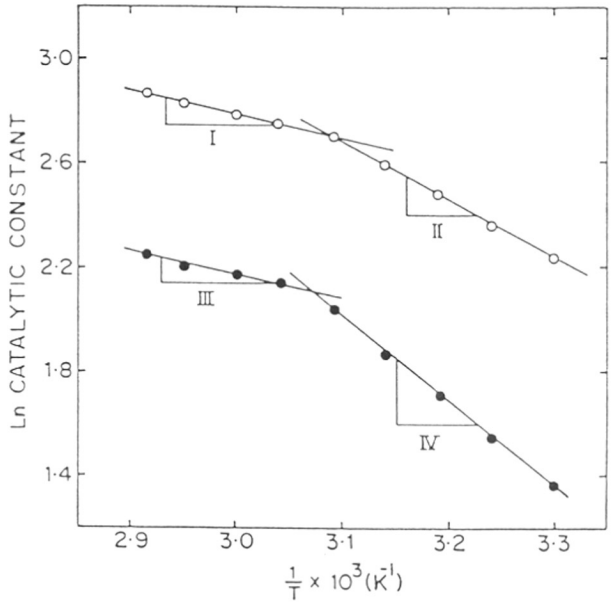

Figure II.11: Arrhenius plot for the NAGase

The reaction rate constant (V_{max}) for NAGase was determined at different temperatures (30°C-70°C) in 100 mM citrate-phosphate buffer, pH 6.0, using saturating concentrations of the substrate pNP-β-D-GlcNAc (25 μg – 150 μg) or 4-Me-Umb-β-D-GlcNAc (20 μg –120 μg). ~5 μg of purified NAGase was used per reaction point and carried out in duplicates. The activation energies were calculated from the slopes of each linear segment. They are 18.5 k J / mol [I] and 43.2 k J / mol [II] for pNP-β-D-GlcNAc (o) and 21.7 k J / mol [III] and 60.3 k J / mol [IV] for 4-Me-Umb-β-D-GlcNAc (•).

Table: II.9

Secondary structure content of *Bacillus* sp. NAGase at pH 6.0 at different temperatures

Secondary structure content (%)				
Temperature °C	**α-helix**	**β-sheet**	**β-turns**	**Unordered / Random coil**
35	4	27	37.5	31.5
65	5	19.5	42	33.5
70	4.5	20	42	33.5

DISCUSSION

Bacillus sp. NCIM 5120 produced high levels of extracellular exo-N-acetyl-β-D-glucosaminidase activity when grown on enriched medium containing 0.5 % (w/v) mannose. Sucrose and glucose, when used as the carbon source supported growth but not enzyme production. Among the various organic nitrogen sources, yeast extract yielded high enzyme levels. Our studies show that the exo-N-acetyl-β-D-glucosaminidase productivity of *Bacillus* sp. NCIM 5120 (25.4 U/L) is comparable to that of the β-hexosaminidase from *Bacillus stearothermophilus* CH-4 (24.83 U/ L) [26] and is much higher than the exo-N-acetyl-β-D-glucosaminidase (6.67 U/L) produced by *Bacillus subtilis* B [1].

Since a large amount of the enzyme is required for structural studies, initial attempts were directed towards the development of a simple and efficient procedure to obtain a homogeneous enzyme preparation in good yield. Although purification of the N-acetylglucosaminidases / β-hexosaminidases from different sources have been achieved by affinity chromatography [3, 34-37], attempts to purify the present enzyme on Bio-gel-GlcNAc were not successful as the enzyme did not show good affinity for the bound sugar. In the present studies, the initial CM-cellulose chromatographic step helped to separate the peptide: N-glycanase from the exo-N-acetyl-β-D-glucosaminidase as only the former was retained on the column. Moreover, gel filtration of the partially purified enzyme was carried out in presence of high salt (4 M NaCl) as, in the absence of high salt, the enzyme exhibited anomalous behaviour (appeared in the void volume). Such anomalous behaviour on gel filtration by N-acetylglucosaminidases / β-hexosaminidases is not uncommon and has been reported in case of the β-hexosaminidase (isoenzyme C) from Bovine brain [4] and the exo-N-acetyl-β-D-glucosaminidase from mesophilic *Bacillus subtilis* B [1]. *Ortiz et al.* [1] attributed this anomalous behaviour of the enzyme to the probable association of cell-membrane material. The above authors also opined that high salt concentration might be disrupting the enzyme: cell-membrane material complex through ionic interactions, leading to the fractionation of the enzyme in the internal volume. Furthermore, it was

observed that the NAGase obtained after the successive Sephacryl S-300 steps was associated with high amounts of extraneous carbohydrate. However, chromatography on phenyl-Sepharose, yielded a homogeneous enzyme preparation devoid of any extraneous carbohydrate. The enzyme after the phenyl-Sepharose step was electrophoretically homogeneous (Fig II.3a)

The M_r of the purified enzyme, determined by gel filtration, was 230,000 (Fig II.4) and it consisted of four identical subunits of M_r 60,000 (Fig II.5). These values are comparable to those obtained from some of the well characterized enzymes from human seminal plasma namely Hex B (M_r 210,000, Chapter I, Table I.1). Moreover, the M_r of *Bacillus* sp. NAGase is higher than other reported enzymes (60,000 – 195,000) (Chapter I, Table I.1). Unlike the *Pisum sativum* L β-hexosaminidase [36], which is a heterotetramer of M_r 62,000 and 64,000, *Bacillus* sp. NAGase consists of 4 identical subunits of M_r 60,000. NAGases are generally classified as acidic (lyzosomal) and neutral (cytosolic) NAGases, depending on their pI. An exception to this is one of the isoforms (Form I) from the fungus *Beauveria bassiana*; which has a pI of 9.5 [38]. The pI of the *Bacillus* sp. NCIM 5120 NAGase was 6.78 suggesting it to be a neutral protein (Fig II.3b). Further, the purified NAGase is a non-glycosylated protein as evidenced by negative carbohydrate staining on SDS-PAGE. The M_r determination based on the amino acid composition (Table II.6) gave a value of 59,722 which is in agreement with the value determined by SDS-PAGE. The amino acid composition showed a preponderance of glycine, serine and threonine compared to other reported β-hexosaminidases and N-acetylglucosaminidases (Table II.6). The *Bacillus* sp. NAGase contained 6 cysteine residues and all of them appear to be involved in disulfide bridge formation because DTNB treatment of the native enzyme did not show the presence of free cysteine. Similar observations have been made on the *Serratia marcescens* chitobiase [39]. The cysteine content of *Bacillus* sp. NAGase is lower than that reported for the β-hexosaminidase from *A. oryzae* (11.5) [28]. Interestingly the β-hexosaminidase from *A. niger* had a low cysteine content (1.5) [29]. *The A. oryzae* β-hexosaminidase also showed a high content of acidic amino acids and tryptophan.

The optimum pH of the purified NAGase was 6.0 (Fig II.6), which is comparable to the N-acetylglucosaminidases from *Trigonella foenum graecum* (pH 6.0) [3], almond emulsin (5.0-6.0) [40], *Acanthamoeba castellani* (5.8) [35], *Mucor fragilis* (5.5-6.5) [41], *Streptococcus* 6646 K (5.2) [33], *Trichomonas foetus* (6.2) [42], *Bacillus stearothermophilus* CH-4 (6.5) [26] and *Clostridium perfringens* (6.5) [32]. However, optimum pH of our enzyme is considerably higher than that of *P. cinnabarinus* β-hexosaminidase [43], which has an optimum pH of 2.2. The *Bacillus* sp. NAGase showed comparatively high pH stability and retained significant amount of its activity between 5.5-9.0 (Fig II.6). pH stability of the reported N-acetylglucosaminidases show wide variations. The *S. fructigena* β-hexosaminidase [44] was stable over a wide pH range (4-11) while the *M. fragilis* enzyme [41] was stable between 4.5-8.0. The N-acetylglucosaminidases / β-hexosaminidases from *Tremella fuciformis* [45], *Turbatrix aceti* [30] and *Alteromonas* sp. Strain O-7 [46] were stable around neutral pH (6.0-8.0). However, the *P. cinnabarinus* β-hexosaminidase was stable only between 2.0-4.0 [43].

The optimum temperature of purified NAGase was 70°C (Fig II.7a), one of the highest reported for similar enzymes from other sources (30-50°C). However, it is less than that of the β-hexosaminidase from *Bacillus stearothermophilus* CH-4, which has an optimum temperature of 75°C [26]. Many of the reported N-acetylglucosaminidases and β-hexosaminidases exhibit lower temperature stabilities (upto 50°C). In this respect the *Bacillus* sp. NAGase show better temperature stability and retained its full activity for 2 h at 60°C. Further, the enzyme was fully stable at 70°C for 30 min. The *Bacillus stearothermophilus* CH-4 β-hexosaminidase was stable at 60°C for 10 min. However, this enzyme is not a true exo-N-acetyl-β-D-glucosaminidase as it also acts on β(1-4) GalNAc containing substrates.

Influence of divalent cations on the activity revealed that Hg^{2+} completely inactivated the enzyme while Cu^{2+} had only a marginal effect. None of the other metal ions and EDTA (upto 10 mM) had any significant effect (Table II.7)

suggesting that the *Bacillus* sp. NAGase is neither a metalloenzyme nor a metal requiring enzyme. Generally, most of the N-acetylglucosaminidases are neither metalloproteins nor metal requiring enzymes. However, certain plant β-hexosaminidases show enhanced activity in presence of metal ions. For example, one of the four isoforms of β-hexosaminidases from germinating Fenugreek seeds [3] showed approximately 1.7 fold enhancement in its activity in presence of Fe^{2+}. However, in case of *Pisum sativum* L β-hexosaminidase II [36], Mg^{2+}, $Mn^{2+}$and Ca^{2+} (10 mM) had only a marginal stimulatory effect. The β-hexosaminidase from *Bacillus stearothermophilus* CH-4 showed increased activity in presence of Fe^{3+} (2 fold), Ca^{2+} (1.7 fold) and Zn^{2+} (1.4 fold) respectively [26].

The purified NAGase did not show any other glycosidase activity when tested against various exo-glycosidase substrates suggesting it to be a true exo-N-acetyl-β-D-glucosaminidase. Substrate specificity studies revealed that unlike most of the N-acetylglucosaminidases the *Bacillus* sp. NAGase could distinguish the linkage between β(1-4) GlcNAc and β(1-4) GalNAc residues (Table II.8). Majority of N-acetylglucosaminidases reported so far do not distinguish between β(1-4) linked GlcNAc and GalNAc residues. One of the fungal N-acetylglucosaminidase from *A. niger* also showed marginal activity towards pNP-β-D-Glc and pNP-β-D-Xyl [29]. Kinetic analyses of NAGase action on various chitooligosaccharide substrates showed that the enzyme could readily hydrolyze upto chitotetraoses with a maximum efficiency for chitobiose (Fig II.8a, Table II.8). Among the chitooligosaccharides the enzyme showed lower affinity for chitotriose (Fig II.8b, Table II.8) while chitotetraose was resistant to hydrolysis as indicated by the prolonged incubation time required for cleavage. Chitooligosaccharides above chitotetraoses were not hydrolyzed at all. The inability of the enzyme to hydrolyze acid swollen chitin and chitooligosaccharides above chitotetraose suggests an exo-mode of action with a preference for low molecular weight substrates. The substrate analogue, pNP-1-thio-β-D-GlcNAc was a potent competitive inhibitor with a K_i value of 87.11 μM (Fig II.9a).

Among the monosaccharides, N-acetylglucosamine was a moderate inhibitor with a K_i value of 0.67 mM (Fig II.9b).

Substrate specificity studies using phenyl-β-D-GlcNAc and pNP-β-D-GlcNAc showed that the former is not a preferred substrate as indicated by the low degree of hydrolysis (8.5 % in 24 h). However, phenyl-β-D-GlcNAc inhibited the hydrolysis of pNP-β-D-GlcNAc competitively (K_i , 34.8 μM) suggesting that it competes for the same substrate-binding site. The competitive inhibition by phenyl-β-D-GlcNAc and its low degree of hydrolysis points towards the aglycone specificity of the *Bacillus* sp. NAGase. Similar competitive inhibition has also been observed in case of *Sclerotina fructigena* β-N-acetylglucosaminidase [44]. 4-Me-Umb-GlcNAc-6-SO_4 could not be hydrolyzed by the enzyme, however, it acted as a competitive inhibitor in the hydrolysis of pNP-β-D-GlcNAc similar to phenyl-β-D-GlcNAc (Table II.8).

β-Hexosaminidase isoenzymes viz., Hex A and Hex B from humans hydrolyze many of the same natural and synthetic substrates containing non-reducing β-linked GlcNAc or GalNAc residues. However, one of the major differences between them is the ability of Hex A to utilize charged substrates, like GlcNAc-6-SO_4 (as in Me-Um-β-D-GlcNAc-6-SO_4) and glycosphingolipids (like G_{M2}, the actual substrate *in vivo*), in conjunction with specific activator protein [47]. The microbial exo-N-acetyl-β-D-glucosaminidases and β-hexosaminidases hydrolyze neutral, uncharged substrates containing terminal β(1-4) linked GlcNAc and GalNAc residues. The above studies indicate that NAGase from *Bacillus* sp. NCIM 5120 is similar to the Hex B family, with a narrower substrate specificity in that, it is specific towards β(1-4) linked GlcNAc residues.

Studies on the reaction stereochemistry indicated that the *Bacillus* sp. NCIM 5120 NAGase is a retaining glycosidase with the initial formation of β anomer of GlcNAc (Fig II.10). Similar mechanism has been observed in β-hexosaminidases [48], the *Serratia marcescens* chitobiase [49] and other glycosidases [50,51]. The retention in mechanism in the jack bean β-hexosaminidase has also been demonstrated by following the stereochemical

course of hydration of glycals (NAglucal) wherein, hydration / protonation of NAglucal occurs at the C-2 carbon from the top face (the correct face) generating an N-acetylglucosamine-enzyme intermediate [48]. All enzymes from a sequence related family have been proposed to hydrolyze substrates with the same stereochemical outcome [52]. For example, family 47 enzymes are inverting enzymes viz., the α (1,2) Mannosidase, class I from *S. cerevesiae* [53]. The exo-N-acetyl-β-D-glucosaminidases and β-hexosaminidases have been classified under the family 20 glycosyl hydrolases and members of this family have been identified to hydrolyze substrates with net retention in anomeric configuration [48,49].

Determination of the energy of activation ($E_{a\alpha}$) with different leaving groups (p-nitrophenol and 4-methylUmbelliferone) revealed that the enzyme exhibits a biphasic Arrhenius plot, with two characteristic energy of activation, with an inflection temperature of 50°C. The activation energy at temperatures below the inflection point was found to be higher than that above the inflection point. The energy of activation for 4-Me-Umb-β-D-GlcNAc was 60. 3and 21.7 k J / mol and for pNP-β-D-GlcNAc, 43.2 k J / mol 18.5 k J / mol below and above the inflection temperature, respectively. Such biphasicity in the energy of activation for the exo-N-acetyl-β-D-glucosaminidases has not been reported so far. The activation energy for the *Phaseolus vulgaris* β-N-acetylglucosaminidase was 41.16 k J / mole [54]. A comparison of the activation energy of the β-hexosaminidases from various crustaceans (euphausiids) range between 47 – 60 k J / mol [55].

Discontinuous Arrhenius plots have been observed in membrane proteins, like the oubain sensitive, (Na^++K^+) ATPase from rabbit kidney [56]. It has also been observed in certain enzymes from thermophiles such as the THF dehydrogenase of *Clostridium thermoaceticum* [57], and fructose-1, 6-diphosphate aldolase from *Bacillus stearothermophilus* NCA 2184 [58]. Various reasons for discontinuous Arrhenius plots have been postulated. In the membrane proteins, the inflection temperature has been related to the melting temperature of the lipid fraction. In addition, Kumamoto *et al.* [59] concluded that a phase

change is responsible for breaks in the slope of Arrhenius plots of biological systems. Another possible hypothesis is the existence of two conformational states of the enzyme above and below the inflection temperature, each with a different catalytic competence [60]. However, far UV CD analysis of the present enzyme at different temperatures (35°C, 65°C, & 70°C) showed no changes in the ellipticity (Table II.9), indicating the absence of any temperature related changes in the secondary structure. As observed earlier, *Bacillus* sp. NAGase is a retaining glycosidase with the β anomer of GlcNAc formed as the initial product of hydrolysis. A comparison of the energy of activation for different leaving groups revealed that at temperatures below the inflection point (50°C), the E_{act} is substantially higher for 4-Me-Umb-β-D-GlcNAc (60.3 k J / mol) than for pNP-β-D-GlcNAc (43.2 k J / mol). However, at temperatures above the inflection point, the energy of activation for both the substrates are comparable (21.7 k J / mol and 18.5 k J / mol).

The general catalytic mechanism proposed for retaining glycosyl hydrolases is a two step process, involving the initial formation of a covalent glycosyl-enzyme intermediate followed by the hydrolysis of the saccharide via formation of oxocarbenium-ion-like intermediates [61]. Further, in β-hexosaminidases and β-N-acetylglucosaminidases the retention in configuration has been attributed to the participation of C 2 acetamido group in formation of the oxazolidine intermediate [62,63]. This suggests that the biphasic Arrhenius plot, in NAGase, is probably due to a change in the rate determining step. At temperatures below the inflection point, the rate determining step would be the formation of the oxazolidine intermediate and at temperatures above, its hydrolysis.

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CHAPTER III

Active site characterization of exo-N-acetyl-β-D-glucosaminidase

SUMMARY

Chemical modification studies on the purified exo-N-acetyl-β-D-glucosaminidase revealed the involvement of a single tryptophan, histidine and carboxylate in the catalytic activity of the enzyme. Spectral analysis and maintenance of total enzyme activities indicated that N-acetylglucosamine (competitive inhibitor) and p-nitrophenyl-N-acetyl-β-D-glucosaminide (substrate) could prevent the modification of a single essential tryptophan, histidine and carboxylate residue. Analysis of the kinetic parameters of partially inactivated enzyme (by NBS / HNBB) showed the involvement of tryptophan in substrate binding while that of histidine (by photo-oxidation / DEPC) and carboxylate (by EDAC /WRK) in catalysis. The substrate binding properties of chemically modified enzyme samples by fluorescence spectroscopy corroborated the kinetic studies, confirming the participation of histidine and carboxylate in catalysis and the involvement of tryptophan residue in substrate binding. Present studies indicate that the *Bacillus* sp. NCIM 5120 exo-N-acetyl-β-D-glucosaminidase deviates from the reported N-acetyl-β-D-glucosaminidases / β-hexosaminidases that utilize anchimeric assistance in their hydrolytic mechanism.

INTRODUCTION

Evaluation of the active site of enzymes can give important information regarding their mechanism of action and structure-function relationship. Several approaches have been used to determine the amino acid residues that participate in enzymatic reactions. Especially, kinetic studies for the estimation of pK_{app} values, heat of ionization (ΔH) and chemical modification using group-specific modifiers coupled with kinetic measurement of the inactivation process have been done on many enzymes. The enzymatic degradation of chitin, a linear polymer of N-acetylglucosamine units (β-1,4 linked 2-acetamido-2-deoxy-glucopyranosyl, NAG) is carried out by endo-chitinase (E. C. 3.2.1.14), chitobiosidase and the exo-N-acetyl-β-D-glucosaminidase (E.C.3.2.1.30) [1]. The exo-N-acetyl-β-D-glucosaminidase catalyses the hydrolysis of terminal, non-reducing GlcNAc residues in chitobiose and higher chitooligosaccharides. The enzyme has been classified under the family 20 glycosyl hydrolases [2]. A unique feature proposed for the family 20 glycosyl hydrolases is the probable anchimeric assistance of the C2 acetamido group in catalysis. The exo-N-acetyl-β-D-glucosaminidases / β-hexosaminidases along with certain other chitinolytic enzymes [3,4] are proposed to follow an acid-base reaction mechanism, with a single protein carboxylate functioning as the catalytic acid, while the nucleophile is the polar acetamido group of the substrate-GlcNAc.

Studies on the substrate specificity of exo-N-acetyl-β-D-glucosaminidase (NAGase) from *Bacillus* sp. NCIM 5120 indicated that it hydrolyses β (1-4) linked GlcNAc residues exclusively from neutral oligosaccharides (Chapter II). The enzyme is a retaining glycosidase and exhibits discontinuous or biphasic Arrhenius plots with two characteristic energy of activation with an inflection temperature of 50°C. The retention in anomerism and a comparison of the activation energy with different leaving groups suggested that the enzymatic hydrolysis probably proceeds via a double-displacement reaction mechanism with the formation of oxazolidine like intermediates. The biphasic Arrhenius plot is the consequence of a change in the rate determining step, i.e., from formation of the

oxazolidine intermediate at temperatures below the inflection point to its hydrolysis above the inflection point (50°C). Although this suggested that the enzymatic reaction proceeds via formation of an enzyme-glycosyl intermediate, probably involving an acid catalyst and a nucleophile / base, it did not give any information regarding the actual amino acid residues that participate in hydrolysis. The present chapter discusses the results of chemical modification studies on the exo-N-acetyl-β-D-glucosaminidase and the identification of amino acid residues involved in the catalytic activity of the enzyme.

MATERIALS AND METHODS

MATERIALS

pNP-β-D-N-acetylglucosaminide (pNP-β-D-GlcNAc), N-acetyl-glucosamine, N-bromosuccinimide (NBS), 2-hydroxy-5-nitrobenzylbromide (HNBBr), diethylpyrocarbonate (DEPC), Woodward's reagent K (WRK), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC), 3-nitro-L-tyrosine ethyl ester (NTEE), phenylglyoxal, 2,3 butanedione (diacetyl), phenylmethylsulfonyl fluoride (PMSF), 5,5'-dithiobis 2-nitrobenzoic acid (DTNB), 2,4,6-trinitrobenzenesulfonic acid (TNBS), N-acetylimidazole, trichloroacetic acid (TCA), dithiothreitol (DTT), HEPES and MES were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Sephadex G-25 was purchased from Pharmacia (Uppsala, Sweden). Methylene Blue, L-ascorbic acid, imidazole and hydroxylamine were purchased from Sisco Research Laboratory, India. p-nitrophenyl glyoxal [5] and tetranitromethane [6] were synthesized in our laboratory and used. All the other reagents used were of the highest purity available from commercial sources.

METHODS

Micro-organism and culture conditions

Growth, maintenance and production of NAGase from *Bacillus* sp. NCIM 5120 was done as described in Chapter II. The purified NAGase after the phenyl-Sepharose step was used in all the chemical modification experiments.

Exo-N-acetyl-β-D-glucosaminidase assay

This was carried out as described in chapter II. The p-nitrophenol liberated following the hydrolysis of p-nitrophenyl-N-acetyl-β-D-glucosaminide at pH 6.0 and 70°C, was measured, spectrophotometrically, at 405 nm. One unit is defined as the amount of enzyme that liberates 1 μmole of p-nitrophenol min^{-1} under the assay conditions [7].

Initial reaction rates were measured at several substrate concentrations in the linear range at the enzyme concentration (~8 μg) used. The kinetic constants K_m and k_{cat} were determined from double reciprocal Lineweaver-Burk plots at any fixed concentration of the modifier. The pH dependent K_m and V_{max} of NAGase was determined similarly in the pH range 4.5-7.0.

Determination of protein concentration

The concentration of purified exo-N-acetyl-β-D-glucosaminidase (NAGase) was determined either by the method of *Bradford* [8] or spectrophotometrically using the relationship, $A^{0.1\%,\,1\,cm}$ at 280 nm = 1.34.

CHEMICAL MODIFICATION

Modification of tryptophan residues

Reaction with N-bromosuccinimide (NBS) : Purified NAGase (8 μM) in 100 mM, Na-acetate buffer, pH 5.5 was titrated with increasing concentrations of NBS (5-100 μM). The reagent was added in 10 installments of 10 μl each. After every addition, an aliquot was removed and the reaction was arrested by the addition of a ten fold excess of L-tryptophan (50 mM stock) and the residual activity was determined under standard assay conditions. The NBS mediated inactivation was monitored, spectrophotometrically, by measuring the decrease in absorbance at 280 nm. The number of tryptophan residues modified were determined using an extinction coefficient of 5500 $M^{-1}cm^{-1}$ [9]. Enzyme samples incubated in absence of NBS served as control.

Reaction with 2-hydroxy-5-nitrobenzylbromide (HNBB) : The reagent was prepared in dry acetone just prior to use and protected from light. Modification of the tryptophan residues of NAGase was carried out by incubating separate aliquots of 4 μM of enzyme with varying concentrations of HNBB (5-50 mM) in 100 mM Na-acetate buffer, pH 5.5 for 30 min at 30°C. The reaction was terminated by the addition of a ten fold excess of L-tryptophan, subjected to gel filtration on Sephadex G-25 (10.0 × 0.5 cm) to remove the excess reagents and the residual activity determined under standard assay conditions. The number of tryptophan residues modified was quantitated using an extinction coefficient of 18,450 M^{-1} cm^{-1} [10]. The acetone concentration in the reaction mixtures did not exceed 5 % (v/v) and had no effect on the activity and stability of the enzyme during the incubation period. Enzyme sample incubated in absence of HNBB was taken as control.

Modification of histidine residues

Photo-oxidation : 500 μl of purified NAGase (100 μg protein) was irradiated in presence of Methylene Blue (0.05 %), at different pH (4.5-9.0), in a glass test tube (15 cm × 1.2 cm), by a 200 W floodlight bulb held at a distance of 10 cm for 30 min at 10 ± 1°C, followed by estimation of the residual activity [11]. Enzyme samples treated under identical conditions, in dark, served as control.

Reaction with diethylpyrocarbonate (DEPC) : Purified NAGase (8 μM) in 100 mM Na-citrate-phosphate buffer, pH 6.0, containing 150 mM NaCl was titrated, at 25°C, with increasing concentrations of DEPC (20 μM - 100 μM) freshly diluted with absolute ethanol. Aliquots were removed at suitable intervals and the reaction was arrested by the addition of an equal volume of 100 mM imidazole buffer, pH 6.8. Subsequently, the residual activity was determined under standard assay conditions. Enzyme samples incubated in absence of DEPC served as control. The ethanol concentration in the reaction mixture did not exceed 5 % (v/v) and had no effect on the activity and stability of the enzyme during the incubation period. The number of histidine residues modified was determined,

spectrophotometrically, by measuring the increase in absorbance at 240 nm as described by *Ovadi et al.* [12]. Further, the DEPC concentration was determined by mixing an aliquot of the diluted DEPC solution with 3 ml of 10 mM imidazole buffer, pH 6.8, followed by monitoring the increase in absorbance at 230 nm. The amount of N-carbethoxyimidazole formed was calculated using a molar absorption coefficient of 3000 M^{-1} cm^{-1} [13]. The concentration of the diluted DEPC solution was 6.3 mM.

Reactivation by hydroxylamine : Decarbethoxylation was carried out according to *Miles* [14]. The DEPC modified enzyme samples were incubated with 200 mM, neutralized hydroxylamine at pH 7.0 and 25°C over a period of 4 h and the enzyme activity determined. Native enzyme incubated in presence of 200 mM hydroxylamine, pH 7.0, served as control.

Modification of carboxylate residues

Reaction with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) : 5 μM of purified NAGase was incubated with varying concentrations of EDAC (5-50 mM) in presence of 30 mM NTEE at pH 6.0, 100 mM MES/HEPES buffer, 75:25 v/v for 45 min at 30°C. Samples (5 μl) were removed and the reaction quenched by the addition of equal amount of 1 M, Na-acetate buffer, pH 5.5 and the residual activity determined under standard assay conditions. To determine the incorporation of number of nitrotyrosyl groups, the EDAC/NTEE treated enzyme samples were precipitated by the rapid addition of an equal volume of 10 % TCA and the mixture left at 4°C for 30 min. The precipitated protein was collected by centrifugation (5000 **g** × 5 min), extensively washed with chilled acetone and reconstituted in 1 ml of 0.1 N, NaOH. The nitrotyrosyl groups incorporated were determined, spectrophotometrically, at 430 nm, assuming a molar extinction coefficient of 4600 M^{-1} cm^{-1} [15]. Enzyme samples incubated in absence EDAC/NTEE served as control.

Reaction with Woodward's reagent K (WRK) : NAGase (4 μM), in 100 mM citrate-phosphate buffer, pH 6.0, was incubated with varying concentrations of WRK (5-50 mM) for 30 min at 25°C. The reaction was arrested by the addition of 1 M Na-acetate buffer pH 5.0. The reaction mixtures were subjected to gel filtration on Sephadex G-25 column (10.0 × 0.5 cm), equilibrated with the above buffer, to remove the excess reagents and the residual activity determined. The number of carboxylate groups modified was quantitated by measuring the increase in absorbance at 340 nm assuming a molar absorption coefficient of 7000 $M^{-1}cm^{-1}$ [16]. Enzyme samples incubated in absence of WRK served as control.

Modification of Arginine residues

Reaction with phenyl-glyoxal : NAGase (5 μM, 1 ml) in 100 mM, Na-borate buffer, pH 8.0 was incubated with phenyl-glyoxal (1.0 - 3.0 mM effective concentration) for 30 min at 30°C [17]. Aliquots were removed at definite time intervals and passed through a Sephadex G-25 column (10 × 0.5 cm) to remove the excess reagent. The residual activity in the reaction mixture was determined under standard assay conditions.

Reaction with 2,3 butanedione (diacetal) : NAGase (5 μM, 1 ml) in 100 mM Na-borate buffer, pH 8.0, was incubated with varying concentrations of 2,3 butanedione (10 mM – 50 mM) at 30°C for 30 min [18]. The reaction mixtures were passed through Sephadex G-25 column (10.0 × 0.5 cm) to remove excess reagents and the residual activity determined under standard assay conditions.

Reaction with p-nitrophenyl glyoxal (pNPG) : 25 μl of pNPG in methanol (to make a final concentration of 0.15 % w/v) was added to NAGase, (5 μM, 1.5 ml) in 100 mM Na-pyrophosphate buffer, pH 9.0, containing 150 mM L-ascorbate. The reaction mixture was incubated at 30°C for 30 min. Subsequently the reaction mixture was cooled in an ice bath and passed through a Sephadex G-25 column (10.0 × 0.5 cm) to remove the excess reagent. The residual activity was

determined and the number of arginyl groups modified was estimated according to the method of *Yamasaki et al.* [19] using L-arginine as the standard.

Modification of cysteine residues

Reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) *:* The NAGase (4 μM, 1 ml) in 100 mM, K-phosphate buffer, pH 8.0, was incubated with 1 mM DTNB at 30°C for 60 min. Aliquots were removed at definite time intervals and the residual activity determined. The number of cysteine residues modified was followed by monitoring the increase in absorbance at 412 nm using an extinction co-efficient of 13,600 M^{-1} cm^{-1} [20].

Modification of tyrosine residues

Reaction with N-acetylimidazole (NAI) *:* The enzyme (5 μM, 1 ml) in 100 mM Na-phosphate buffer, pH 7.0, was incubated with 10 mM NAI for 30 min at room temperature followed by estimation of the residual activity. The enzyme incubated in absence of NAI served as control.

Reaction with tetranitromethane (TNM)**:** TNM was diluted with 95 % ethanol and the concentration of the diluted stock was 1 mM. NAGase (5 μM), in 50 mM Tris-HCl buffer, pH 8.0, was incubated with 100 μM TNM under shaking conditions for 1 h at 25 °C. The reaction mixture was then passed through a Sephadex G-25 column (10.0 x 0.5 cm) and the residual activity determined under standard assay conditions. The number of tyrosine residues modified was quantitated, spectrophotometrically, using a molar absorption coefficient of 4200 M^{-1} cm^{-1} at 428 nm [21]. Enzyme samples incubated in absence of TNM served as control.

Modification of lysine residues

Reaction with 2,4,6 trinitrobenzenesulfonic acid (TNBS) **:** To 0.5 ml of purified NAGase (10 μM) in 50 mM K-phosphate buffer, pH 7.5 was added 0.5 ml of 4 % $NaHCO_3$ (pH 8.5) and 0.5 ml of 0.1 % TNBS in water. The reaction mixture was

incubated in dark at 30°C for 1 h. Subsequently the reaction was terminated by bringing the pH to 4.5 and the residual activity determined [22].

Modification of serine residues

***Reaction with phenylmethylsulfonylfluoride (PMSF)*:** NAGase (5 μM, 1 ml) in 20 mM, K-phosphate buffer, pH 7.5 was incubated with 10 mM of PMSF at 30°C for 1 h. Aliquots were withdrawn at definite time intervals and the percentage residual activity determined [23].

Substrate protection studies

In all the chemical modification reactions, the effect of protective ligands was studied by preincubating the enzyme with excess amounts of GlcNAc (10 mM, in case of tryptophan modification) or pNP-β-D-GlcNAc (0.586 mM, in case of histidine and carboxylate modification) followed by treatment with the respective modifying reagents.

Fluorescence measurements

Fluorescence measurements were performed on a Perkin Elmer Spectrofluorimeter LS 50 B, at 25°C, using a slit width of 5 nm for both excitation as well as emission. The fluorescence spectrum of native and chemically modified enzyme samples (2.5 μM, 2 ml) in 10 mM Na-citrate-phosphate buffer, pH 6.0, containing 150 mM NaCl, was recorded in absence and presence of the substrate, chitobiose (effective concentration 4 mM). In case of histidine and carboxylate modified enzyme samples, the excitation wavelength was fixed at 280 nm and the emission recorded in the range 300-400 nm. Tryptophan modified protein samples were excited at 295 nm and the emission recorded in the range 300-400 nm. Selective modification of tryptophan residues was carried out (either one or two tryptophan residues modified per monomer), by NBS.

C D measurements

C D spectra of native and chemically modified enzyme samples (tryptophan, histidine and carboxylate modified NAGase) in the far U.V range (200-250 nm) were recorded at 25 ± 1°C on a JASCO 710 spectropolarimeter, using a thermostatically controlled cell holder with 1 cm path length cell. A protein concentration of 3 μM in 100 mM, Na-citrate-phosphate buffer, pH 6.0 was used.

RESULTS

Analysis of the pH dependent kinetics indicates that the NAGase follows the classical bell shaped curve with maximum activity at pH 6.0 (Fig III.1). The pH dependence of k_{cat} / k_m can be fitted into a model involving two ionizable groups with apparent pK_a values of 5.3 and 6.2 in the free enzyme. On the basis of observed pK_a values of amino acid side chains, the above pK_a values can be attributed to the probable involvement of carboxylate and imidazole groups in the catalytic activity of NAGase.

The effect of various chemical modifiers on the activity of the enzyme is summarized in Table III.1. Modification of tryptophan, histidine and carboxylate residues led to complete inactivation of the enzyme.

Modification of tryptophan

Purified NAGase when incubated with 100 μM NBS at pH 5.5 and 30°C lost all of its activity. However, no loss of activity was observed in the control samples. The NBS mediated inactivation of the enzyme was accompanied by a decrease in the absorbance of the modified protein at 280 nm. Based on a molar absorption coefficient at 280 nm to be 5500 M^{-1} cm^{-1} [9] and the subunit M_r of NAGase to be 60,000 the total number of tryptophan residues modified was found to be 4. However, a plot of percent residual activity versus the number of tryptophan residues modified showed that the loss of activity resulted from the modification of 2 tryptophan residues per monomer (Fig III.2a). The NBS mediated inactivation of NAGase could be prevented by incubating the enzyme

with excess amounts of the competitive inhibitor, GlcNAc, prior to the modification reaction (Table III. 2a). Moreover, the CD spectra of unmodified and tryptophan modified enzyme were almost identical (Fig III.9) indicating that, modification of tryptophan residues does not result in a gross change in the conformation of the enzyme.

Similarly, modification of the enzyme with HNBB resulted in approximately 90 % loss of its initial activity and the inactivation was concentration dependent. The plot of percent residual activity against the number of tryptophan residues modified revealed that the loss of activity resulted due to the modification of 2 tryptophan residues per monomer of the enzyme (Fig III.2b). GlcNAc (competitive inhibitor) could protect the enzyme against HNBB mediated inactivation (Table III.2a).

The kinetic analysis of partially inactivated enzyme (by NBS / HNBB) samples showed an increase in the K_m but no change in the k_{cat} values (Table III.2b) (Fig III.3).

Modification of histidine

Photo-oxidation of purified NAGase, in presence of 0.05 % (w/v) Methylene Blue, exhibited a pH dependent inactivation and the maximum loss of activity (>90 %) was observed at pH 6.0 (Fig III.4). The inactivation was dependent on the concentration of the reagent. However, no loss of activity was observed in the controls.

Carbethoxylation of NAGase, at pH 6.0, led to complete inactivation of the enzyme. No loss of activity was observed in the control samples. The DEPC mediated inactivation was accompanied by an increase in the absorbance of the modified protein at 240 nm. Based on a molar absorption coefficient of carbethoxyhistidine at 240 nm to be 3200 M^{-1} cm^{-1}[12], the total number of histidine residues modified was found to be 4.0 per monomer. However, the plot of percent residual activity versus the number of histidine residues modified showed that, the loss of activity resulted from the modification of a single histidine residue (Fig III.5). Incubation of the DEPC modified enzyme with 200

mM neutralized hydroxylamine, at pH 7.0 and 30°C for 2 h restored 65-70 % of its initial activity. Methylene Blue and DEPC mediated inactivation of NAGase could be prevented to a significant extent by incubating the enzyme with excess amounts of the substrate, pNP-β-D-GlcNAc, prior to the modification reaction (Table III.3a). The CD spectra of both native and histidine modified NAGase were almost identical (Fig III.9) suggesting that modification does not result in a gross change in the conformation of the enzyme. In addition, determination of the kinetic parameters of partially inactivated enzyme samples showed a decrease in the k_{cat} with no change in the K_m values (Table III.3b) (Fig III.6).

Modification of carboxylate groups

Modification of the enzyme with EDAC in presence of NTEE resulted in 75-80 % loss of its initial activity with concomitant incorporation of 5.5 nitrotyrosyl groups per monomer. A plot of residual enzyme activity versus number of carboxylate groups modified (Fig III.7a) showed the involvement of a single carboxylate in the catalytic activity of the NAGase.

Modification of carboxylate groups of the enzyme with WRK at pH 6.0 resulted in complete loss of its activity with no loss of activity being observed in the control samples. Determination of the number of carboxylate groups modified revealed that 9 carboxylate groups were modified with complete loss in activity. However, a plot of residual enzyme activity against number of carboxylate groups modified (Fig III.7b) showed the involvement of a single carboxylate group in the catalytic activity of the enzyme.

EDAC as well as WRK mediated inactivation could be prevented to a significant extent by preincubating the enzyme with excess amounts of substrate (pNP-β-D-GlcNAc) (Table III.4a). Moreover, the CD spectra of native and carboxylate modified enzyme were almost similar suggesting that the modification does not result in a gross change in the enzyme structure (Fig III.9). Furthermore, the kinetic parameters of the partially inactivated (EDAC / WRK)

enzyme samples showed no change in the K_m but a decrease in the k_{cat} values (Table: III.4b) (Fig III.8).

NAGase when treated with phenyl glyoxal or 2,3 butanedione did not lead to any inactivation at low molar ratios (1:10). However, there was 30 % loss in the activity when the modifying agents were used at higher molar ratios (200-600). Treatment of the enzyme with a more specific reagent like p-nitrophenyl glyoxal revealed a similar observation with no loss in activity at lower reagent concentrations (1:20). However, treatment of the enzyme with excess reagent (molar ratio; 1:200) resulted in the modification of 17 arginine residues (per monomer) with ~30 % loss in activity and was accompanied by protein aggregation and precipitation. Further, incubation of the phenyl glyoxal modified enzyme at pH 8.5, did not bring about any reactivation indicating that arginine residues are not involved in the catalytic activity of NAGase. The low degree of inactivation observed is primarily due to denaturation rather than due to modification of Arg residues. Treatment of the enzyme with TNBS resulted in the modification of 6 lysine residues with no loss in activity. Similarly, modification of Ser by PMSF and Cys by DTNB also did not lead to any inactivation.

Fluorescence studies

The native NAGase exhibited a fluorescence emission maximum (λ_{em}) at 337 nm upon excitation either at 280 nm or 295 nm. There was a decrease in the intrinsic fluorescence (~9 %) on addition of the substrate (chitobiose, effective concentration 4 mM). Histidine and carboxylate modified enzymes on titration with chitobiose to saturation also exhibited quenching of the intrinsic fluorescence to the same extent. Modification of one tryptophan residue (per monomer) did not affect the binding of chitobiose to the enzyme as evident by the appreciable quenching observed (8.5 %). However, addition of chitobiose to NAGase with two tryptophans-oxidized (per monomer) did not bring about any quenching (Table III.5).

Table III.1

Effect of different chemical modifying agents on the activity of exo-N-acetyl-β-D-glucosaminidase from *Bacillus* sp.: Enzyme (6 μM, 1 ml) was incubated with various reagents at room temperature, the reaction terminated and the residual activity determined under standard assay conditions.

Modifying agent	Conc.	Residual activity (%)	Reaction buffer	Residues modified / subunit
None		100		-
NBS	10μM	0	Na-acetate, pH 5.5, 100 mM	4
HNBBr	50 mM	0	Na-acetate, pH 5.0, 100 mM	N.D.
DEPC	50 μM	0	Cit-phosphate, pH 6.0, 100 mM	4
Methylene blue	0.1 %	0	Cit-phosphate, pH 6.0, 100 mM	-
WRK	5 mM	0	Cit-phosphate, pH 6.0, 100 mM	9
EDC	50 mM	0	MES / HEPES, pH 6.0, 50 mM	5.5
Phenylglyoxal	30 mM	70	Na-bicarbonate, pH 8.0, 50 mM	-
2,3 butanedione	50 mM	75	Na-borate, pH 8.0, 50 mM	-
pNPG	0.1 %	72	Na-pyrophosphate, pH 8.5, 50 mM	17
NAI	10 mM	100	Na-phosphate, pH 7.0, 100 mM	N.D.
TNM	50 μM	100	Tris-HCl, pH 8.0, 100 mM	9
PMSF	50 mM	100	K-phosphate, pH 7.2, 100 mM	N.D.
DTNB	1 mM	100	Na-phosphate, pH 8.0, 50 mM	0
TNBS	0.1 %	90	Na-bicarbonate, pH 8.5, 50 mM	6

N.D.: not determined

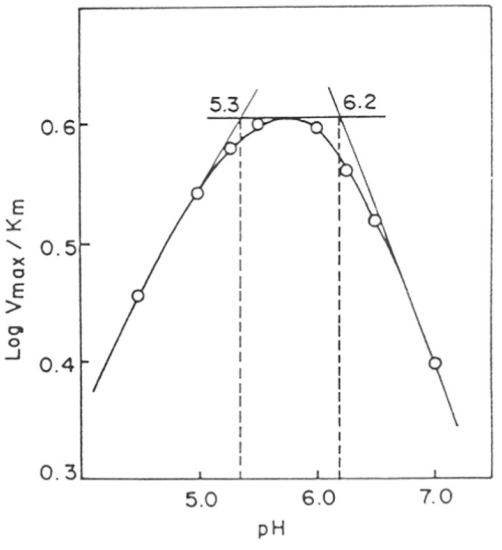

Figure III.1 Effect of pH on the K_m/ k_{cat} of NAGase

A plot of Log K_m /k_{cat} as a function of pH (4.5-7.0). The activity of the NAGase was carried out at different pH values at 70°C for 15 min using pNP-β-D-GlcNAc as the substrate (25-150 μg).

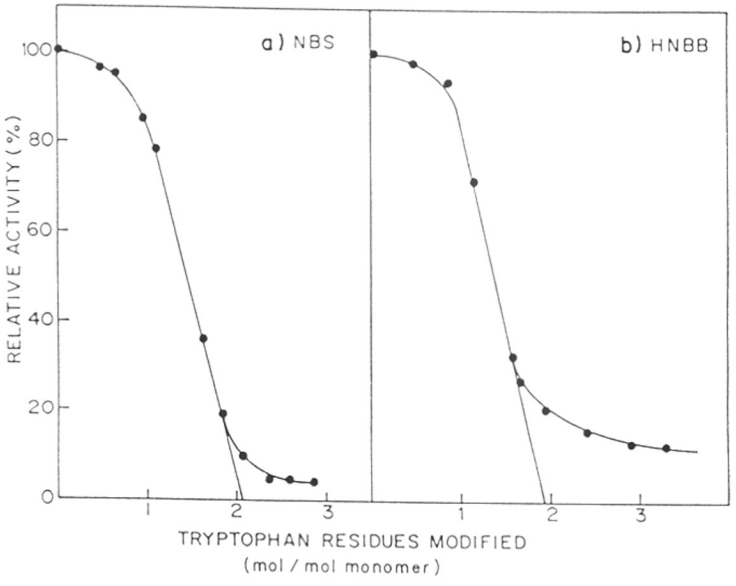

Figure III.2 Plot of percent residual activity versus the number of tryptophan residues modified.

a) By NBS and **b)** by HNBB. The number of tryptophan residues modified were determined as described under Methods.

Table III.2a Influence of tryptophan modification on the activity of NAGase: Substrate protection studies

Modification reaction	No. of residues modified	Residual activity (%)
Control	0	100
Enzyme + NBS (100μM)	4	4
Enzyme + GlcNAc (10 mM) + NBS (100 μM)	3	71.8
Enzyme + HNBB (10 mM)	4	20
Enzyme + GlcNAc (10 mM) + HNBB (10 mM)	3	75.3

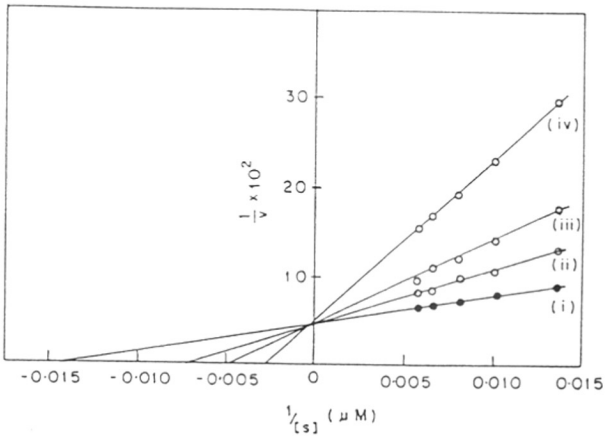

Figure III. 3 Determination of kinetic constants of partially NBS inactivated NAGase. The kinetic constants were calculated by double reciprocal Lineweaver-Burk plots. (i) native, (ii) 26 % inactivated, (iii) 55 % inactivated and (iv) 69 % inactivated.

Table III.2b Kinetic parameters of partially inactivated NAGase (tryptophan modified): The values were determined using double reciprocal Lineweaver-Burk plots under standard assay conditions using the substrate, pNP-β-D-GlcNAc, in the range 25 – 150 μg.

Residue modified	Reagent used	Residual activity (%)	K_m(μM)	k_{cat}(min^{-1})
	None	100	70 ± 0.5	20.75 ± 0.010
Tryptophan	NBS	74	110.52	20.0
		45	210.00	,,
		31	300.00	,,
	HNBB	63	125.00	19.20
		48	205.00	,,
		23	323.00	,,

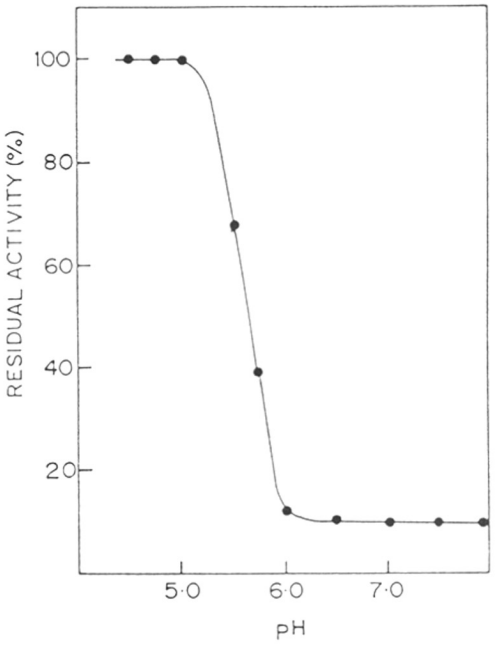

Figure III.4 Effect of pH on photo-oxidation of NAGase.

The enzyme (100 μg in 500 μl) was incubated at different pH values (4.5-9.0) in presence of 0.05 % methylene blue and subjected to photo-oxidation (as described in methods) for 30 min at 10 ± 1°C. Identical samples kept in dark served as controls. The buffers used were Na-acetate, pH 4.5-5.5, MES / NaOH, pH 6.0 and 6.5, and HEPES, pH 7.0 and 7.5 (all at 100 mM conc.). The enzyme activity was measured using pNP-β-D-GlcNAc as the substrate.

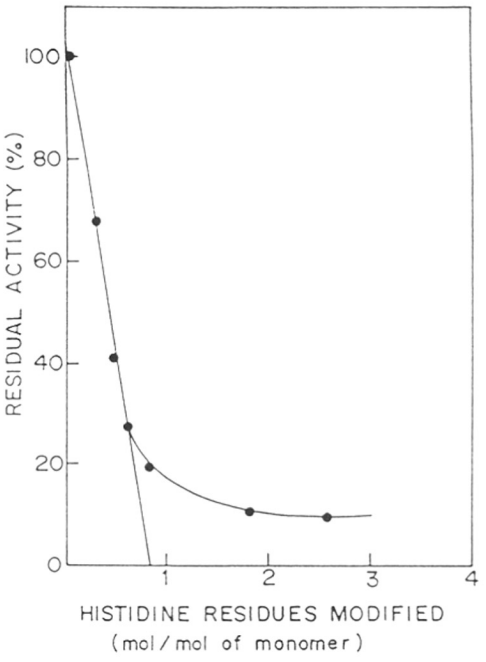

Figure III.5 Plot of percent residual activity versus the number of histidine residues modified (by DEPC).

The number of histidine residues modified was determined as described under Methods.

Table III.3a Influence of histidine modification on the activity of NAGase: Substrate protection studies

Modification reaction	Number of residues modified	Residual activity (%)
Control	0	100
Enzyme + MB (0.05 %)	-	14
Enzyme + pNP-β-D-GlcNAc (0.586 mM) + MB (0.05 %)	-	66
Enzyme + DEPC (90 μM)	4	7.2
Enzyme + pNP-β-D-GlcNAc (0.586 mM) + DEPC (90 μM)	3	73.3

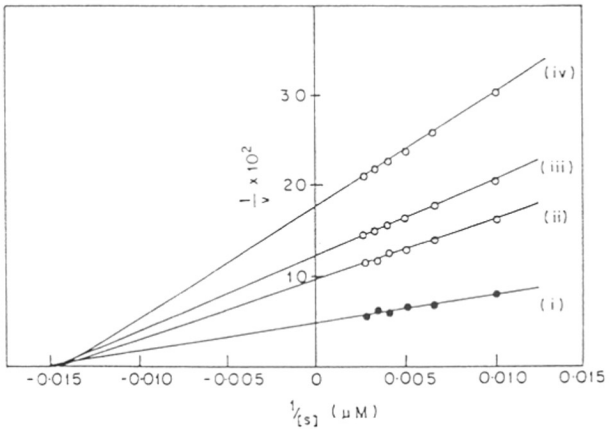

Figure III.6 Determination of kinetic constants of partially DEPC inactivated NAGase. The kinetic constants were calculated by double reciprocal Lineweaver-Burk plots. (i) native, (ii) 44 % inactivated, (iii) 55 % inactivated and (iv) 65 % inactivated.

Table III.3b Kinetic parameters of partially inactivated NAGase (histidine modified): The values were determined using double reciprocal Lineweaver-Burk plots under the standard assay conditions using the substrate, pNP-β-D-GlcNAc, in the range 25 – 150 μg.

Residue modified	Reagent used	Residual activity (%)	K_m(μM)	k_{cat}(min^{-1})
	None	100	70 ± 0.5	20.75 ± 0.010
His	DEPC	66	70	10.35
		45	,,	8.33
		33	,,	5.84
	Photo-oxidation	81	69	14.06
	with MB	62	,,	11.53
		27	,,	7.82

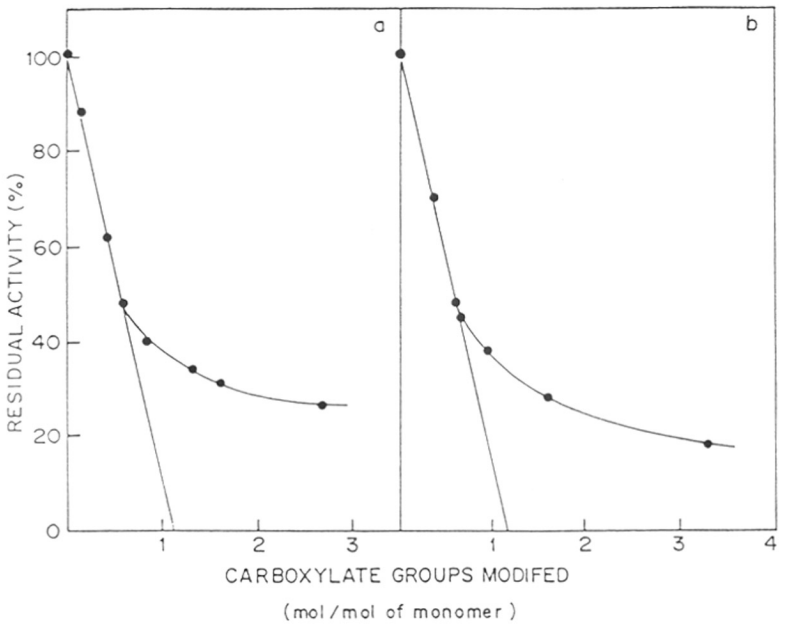

Figure III.7 Plot of percent residual activity versus the number of carboxylate groups modified.

a) by EDAC and **b)** by WRK. The number of carboxylate groups modified were determined as described under Methods.

Table III.4a Influence of carboxylate modification on the activity of NAGase: Substrate protection studies

Modification reaction	Number of residues modified	Residual activity (%)
Control	0	100
Enzyme + EDAC (3.5 mM)	5.5	19
Enzyme + pNP-β-D-GlcNAc (0.586 mM) + EDAC (3.5 mM)	4.3	86.24
Enzyme + WRK (5 mM)	9	0
Enzyme + pNP-β-D-GlcNAc (0.586 mM) + WRK (5 mM)	8.1	78.64

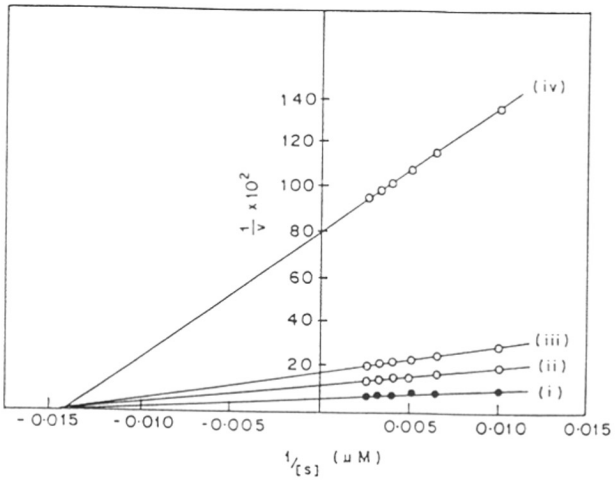

Figure III.8 Determination of kinetic constants of partially EDAC inactivated NAGase. The kinetic constants were calculated by double reciprocal Lineweaver-Burk plots. (i) native, (ii) 44 % inactivated, (iii) 70 % inactivated and (iv) 92 % inactivated.

Table III.4b Kinetic parameters of partially inactivated NAGase (carboxyl groups modified) : The values were determined using double reciprocal Lineweaver-Burk plots under the standard assay conditions using the substrate, pNP-β-D-GlcNAc, in the range 25 – 150 μg.

Residue modified	Reagent used	Residual activity (%)	K_m(μM)	k_{cat}(min^{-1})
	None	100	70 ± 0.5	20.75 ± 0.010
Carboxylate group	EDAC	56	68.5	9.4
		30	,,	6.48
		8	,,	1.23
	WRK	85	70	13.5
		66	,,	10.45
		16	,,	3.12

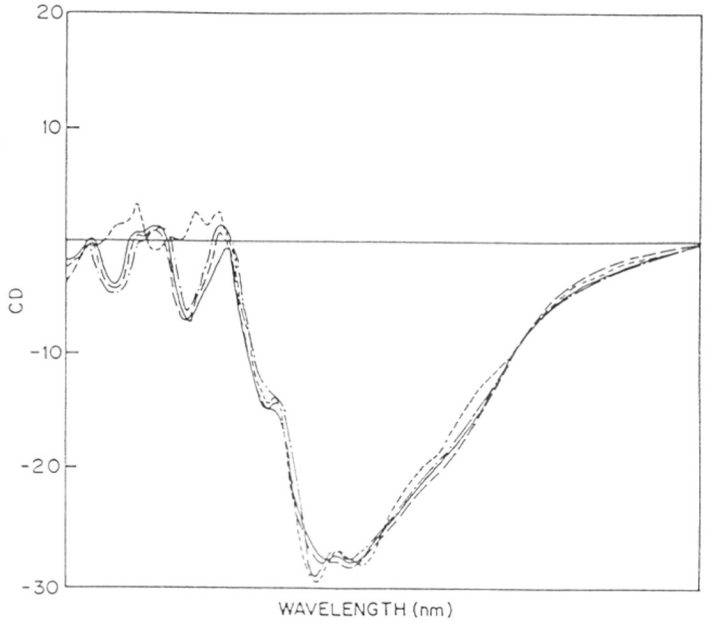

Figure III.9 C D spectra of native, NBS, DEPC and EDAC treated NAGase samples.

The spectra were recorded in the far UV range (200-250 nm) using a 1 cm path length at 25°C in 50 mM citrate phosphate buffer pH 6.0. ~ 3 μM NAGase (2 ml) was used in all the experiments. (—) Native NAGase, (–·–) NBS treated, (—) DEPC treated and (---) EDAC treated.

Table III.5 Fluorescence quenching of native and chemically modified NAGase

2 ml of NAGase samples (2.5 μM) in 10 mM citrate-phosphate buffer, pH 6.0, was subjected to chitobiose mediated quenching (final concentration 4 mM) at 25°C and the values corrected for dilution effect.

Sample	% Residual activity	$\lambda_{max, em}$	ΔF_{max} (%) *
Native	100	337	9.0
Tryptophan modified (one)	80	336	8.5
Tryptophan modified (two)	0	332	0
Histidine modified	0	337	9.0
Carboxylate modified	0	337	9.0

* The fluorescence intensities are expressed as a percentage of the fluorescence intensity difference, $\Delta F_{max}(\%)$, relative to that of the free enzyme i. e., $\Delta F_{max}(\%) = (F_i - F_f / F_i) \times 100$, where F_i and F_f are the initial and final fluorescence respectively.

DISCUSSION

Except for the human hexosaminidases and the chitobiase from *Serratia marcescens*, little information is available on the active site nature of other N-acetylglucosaminidases [24-27]. Depending on the pK_{app} values of 4.7 and 5.2 and heat of ionization studies, *Bedi et al* [28] have suggested the probable involvement of two carboxyl groups in the activity of *Turbatrix aceti* β-N-acetylglucosaminidase. Site directed mutagenesis in the human hexosaminidase indicate the involvement of βArg^{211} and βGlu^{355} in the catalytic function [24-26]. However, the human hexosaminidase hydrolyses both terminally linked GlcNAc as well as GalNAc residues from the non-reducing end of glycoconjugates. Based on the X-ray structure of *S. marcescens* chitobiase complexed with the substrate (chitobiose), *Vorgais et al* [27] gave evidence for the involvement of a single carboxylic acid in the catalytic mechanism.

Oxidation of the *Bacillus* sp. NAGase by N-bromosuccinimide resulted in complete loss of its activity, suggesting a role for tryptophan residues in the catalytic activity of the enzyme. Spectral analysis of NBS mediated inactivation revealed that, the loss of activity occurred due to the modification of 2 tryptophan residues (Fig III.2a). It was noted that the enzyme retained significant amount of its initial activity (>85 %) upon modification of the first tryptophan residue, however, subsequent modification of the second tryptophan residue was accompanied by a rapid loss of activity (>95 %) indicating the involvement of one tryptophan residue in the catalytic activity of the enzyme. The above data also suggests that the modification of the first tryptophan residue either facilitates the modification of the catalytically active, second tryptophan residue or the catalytically active tryptophan is a slow reacting residue. Similar results were obtained when the modification reaction was performed with a tryptophan specific reagent viz., HNBB (Fig III.2b).

NBS as well as HNBB mediated inactivation of NAGase could be prevented to a significant extent by incubating the enzyme with excess amounts of GlcNAc prior to the modification reaction (Table III.2a). Determination of the

number of tryptophan residues modified by NBS / HNBB in absence and presence of the competitive inhibitor, GlcNAc, gave a value of 4 and 3 respectively (calculated on the basis of the subunit M_r of 60,000), suggesting the involvement of one tryptophan residues / monomer, in the activity of the enzyme. Additionally, tryptophan modification did not bring about any gross change in the enzyme structure indicating that the loss of enzyme activity is due to tryptophan modification rather than structural changes (Fig III.9). Furthermore, the kinetic parameters of partially inactivated enzyme samples showed an increase in the K_m and no change in the k_{cat} pointing towards the involvement of tryptophan in substrate binding (Table III.2b) (Fig III.3).

Photo-oxidation of the purified NAGase in presence of Methylene Blue, at pH 6.0 and 10 ± 1°C for 30 min, resulted in approximately 90 % loss of its initial activity and the inactivation was concentration dependent. The inactivation of the enzyme could be prevented by shielding the enzyme-Methylene Blue mixture from irradiation. The pH dependent, Methylene Blue mediated inactivation of the enzyme (Fig III.4) was similar to that observed in case of free histidine [29] and also in the photo-inactivation of several other enzymes with histidine at their active site [30-32], suggesting the presence of histidine at or near the active site of *Bacillus* sp. NAGase.

The involvement of histidine in the catalytic activity of NAGase was also determined by modifying the enzyme with a histidine specific reagent namely DEPC. DEPC mediated inactivation was accompanied by an increase in the absorbance of the modified enzyme at 240 nm, characteristic of carbethoxylation of histidine residues and it resulted in total loss of enzyme activity. Determination of the number of essential histidine residues following DEPC modification indicated that a single histidine residue is responsible for the loss of enzyme activity (Fig III.5). DEPC treatment of the enzyme in absence and presence of the substrate (pNP-β-D-GlcNAc) gave a value of 4 and 3 residues respectively (based on the monomer M_r of 60,000) suggesting the involvement of a single histidine residue in the catalytic activity of the enzyme. Hydroxylamine treatment of the

DEPC inactivated enzyme restored significant amount of the initial activity (66 %) substantiating the role of histidine in the catalytic activity of NAGase.

Though, DEPC is specific for histidine at and around neutral pH, it also reacts to a lesser extent, with tyrosine, cysteine and lysine residues [14]. However, modification of tyrosine residues by tetranitromethane, though resulting in the modification of 9 out of 11 residues, did not have any effect on the activity suggesting that tyrosine residues do not have a role in the catalytic activity of NAGase (Table III.1). Moreover, modification of tyrosine as a result of DEPC treatment was ruled out by the observation that there was no significant decrease in the absorbance of the modified protein at 278 nm. Though the above observations support the presence of histidine at or near the active site, they still do not rule out the possible involvement of cysteine residues. However, in the present case, DEPC mediated inactivation of the enzyme cannot be attributed to modification of cysteine residues as DTNB treatment of the enzyme did not reveal the presence of free cysteine (Chapter II). Furthermore, the loss of activity of *Bacillus* sp. NAGase, as a result of DEPC treatment, cannot be correlated to lysine modification since the DEPC modified enzyme could regain substantial amount of its initial activity in presence of hydroxylamine. Had the inactivation of the enzyme been due to the modification of lysine residues, then hydroxylamine treatment would not have restored its activity. This conclusion is supported by the observation that modification of the enzyme with a lysine specific reagent like trinitrobenzenesulfonic acid did not result in any loss of enzyme activity (Table III.1).

Studies on substrate protection revealed that Methylene Blue or DEPC mediated inactivation could be prevented by incubating the enzyme in presence of excess amounts of the substrate (pNP-β-D-GlcNAc) prior to the modification reaction (Table III.3a). Additionally, DEPC modification did not bring about any gross change in the structure indicating that the loss of enzyme activity is due to histidine modification rather than structural changes (Fig III.9). Moreover, no change in the K_m values of the partially inactivated enzyme with concomitant

decrease in the k_{cat} (Table III.3b) (Fig III.6) suggest the involvement of histidine in catalysis rather than substrate binding.

Modification of the carboxylate groups by EDAC resulted in a concentration dependent inactivation of the enzyme indicating that carboxyl groups may have a role in the catalytic activity of NAGase. However, no loss of activity was observed in the controls. Determination of the number of nitrotyrosyl groups incorporated showed that, the loss of activity occurred due to the modification of a single carboxyl residue per monomer of the enzyme (Fig III.7a). Similar results were obtained when the modification was carried out with WRK (Fig III.7b). Both EDAC and WRK mediated inactivation could be prevented to a significant extent by preincubating the enzyme with excess amounts of pNP-β-D-GlcNAc. Moreover, determination of the number of carboxylate residues modified by EDAC, in absence and presence of the substrate, gave a value of 5.5 and 4.3 respectively, with significant retention of initial activity indicating the involvement of one carboxylate group in the catalytic activity of the enzyme. Similar results were observed when the modification reaction was carried out in presence of WRK (Table III.4a). The CD spectra of native and the EDAC inactivated enzyme were identical indicating that the loss in activity is due to carboxylate group modification rather than structural changes (Fig III.9). The K_m and k_{cat} of partially inactivated enzyme samples showed a decrease in the k_{cat} with no change in the K_m (Table III.4b) (Fig III.8), suggesting the involvement of carboxylate in catalysis rather than substrate binding.

The substrate binding properties of various NAGase derivatives (tryptophan, histidine and carboxylate modified) was studied by fluorescence spectroscopy. Native NAGase when titrated with chitobiose (0.5 – 4 mM) exhibited approximately 9 % decrease in the intrinsic fluorescence with no change in the emission maxima (337 nm). Histidine and carboxylate modified enzyme samples when saturated with chitobiose revealed a similar observation suggesting that modification of histidine or carboxylate residues does not impede substrate binding (Table III.5). The above observations along with kinetic data confirm the participation of histidine and carboxylate residues in catalysis.

The functional role of essential tryptophan residues in the active site of *Bacillus* sp. NAGase was probed by selective oxidation of tryptophan residues by NBS. The intrinsic fluorescence of both, native enzyme and NAGase with one tryptophan oxidized was quenched to a similar extent (ΔF_{max}, ~9 %). However, addition of chitobiose to the modified enzyme with two tryptophans oxidized did not lead to any quenching in the fluorescence. This indicates that modification of the first tryptophan residue in NAGase does not affect substrate binding, but oxidation of the second tryptophan residue completely abolishes this property. This substantiates the observation made by kinetic analysis that the essential tryptophan residue is involved in substrate binding rather than in the catalytic activity of NAGase. Involvement of tryptophan residues in substrate binding has been observed in other glycosidases as well [33-37]. At least 4 Trp residues have been implicated in the stacking of the substrate (chitobiose) against the active site of the *Serratia marcescens* chitobiase [27].

The classical mechanism proposed for general glycoside hydrolysis with retention of configuration is a double displacement reaction involving, two carboxyl groups (one protonated and the other unprotonated), with one of the carboxylate groups donating a proton to the β(1-4) glycosidic oxygen whereas the second unprotonated carboxylate residue facilitates bond cleavage either by forming a covalent glycosyl intermediate or by providing an ion-pair stabilization to the oxocarbonium ion intermediate [3,4,43]. Such a mechanism would be well justified for glycosidases catalyzing simple, unsubstituted sugars. But for N-acetyl substituted sugars, (as in GlcNAc) a possible mode of intramolecular stabilization by the substrate through the N-acetyl group (anchimeric assistance or neighboring group participation) has been proposed. *Jones* and *Kosman* [38], working on the β-N-acetylglucosaminidase from *Aspergillus niger*, using various para-substituted phenyl derivatives of GlcNAc (fluroacetamido/ thioacetamido derivatives), suggested such an anchimeric assistance in glycoside cleavage either through stabilization of the oxocarbonium ion or by facilitating oxozoline formation. X-ray crystallographic studies on the closely related enzyme,

hevamine, (plant chitinase/lysozyme) complexed with the inhibitor allosaminidin, give proof for a similar substrate assisted catalysis [39].

Investigations on the active site characterization of the human β-hexosaminidase [24-26,40] and the *Serratia marcescens* chitobiase [27] have shown the involvement of a single carboxylate residue in the catalytic activity of these enzymes. Although, the involvement of an arginine residue has also been reported in the catalytic activity of human β-hexosaminidase [41], its exact role is still under investigation. Given the high pK_a of arginine (12.0) *Fernandes et al.* [40] and *Pennybacker et al.* [26] have suggested that the role of active-site arginine may be in maintaining the pK_a of the acid catalyst (carboxyl group) in its protonated state at a pH which is significantly higher than the pK_a of dicarboxilic acids or it may have a role in substrate binding. In fact the crystal structure of the *Serratia marcescens* chitobiase indicates that Arg^{349}, the counterpart of βArg^{211} of the human enzyme, is involved in substrate binding and not in catalysis. Modification studies on the *Bacillus* sp. NAGase indicated that arginine residues do not have a role in the catalytic activity of the enzyme.

Enzymatic hydrolysis of a saccharide substrate with overall retention of configuration implies the participation of nucleophile / negatively charged residue resulting the formation of enzyme-glycosyl intermediate which is subsequently hydrolyzed by a water molecule [42]. The absence of a putative nucleophile in the family 20 glycosyl hydrolases has suggested a probable anchimeric assistance of the substrate (the C2 acetamido group) in catalysis [3,4]. However, chemical modification studies on the *Bacillus* sp. NAGase revealed the involvement of carboxylate as well as histidine residues in the catalytic activity of the enzyme. Studies on the reaction stereochemistry of the *Bacillus* sp. NAGase revealed that the enzyme is a retaining glycosidase, with the formation of β anomer of GlcNAc as the first product [Chapter II]. The above observations i.e., the involvement of histidine and carboxylate residues in the catalytic activity of the NAGase and the retention in product configuration suggests that, the unprotonated carboxyl group found in other glycosidases has probably been replaced by an active histidine residue. The imidazole side chain of histidine residue, given its pK_a, (with the

observed pK_{app} values for the free enzyme, 5.3 and 6.2, Fig III.1) would act by removing a proton from the acetamido group of the bound substrate, producing an internal oxazoline structure as in a Transition State or a reactive intermediate. The involvement of active site histidine as a general base in the initial abstraction of a hydrogen ion from substrates has been demonstrated in the Group B Streptococcal hyaluronate lyase [44] and other hydrolases [45-47]. The essential carboxylate residue might function as the acid catalyst although a probable anchimeric assistance in the catalytic mechanism of NAGase cannot be ruled out. The role of the essential histidine residue would then be in stabilizing the oxocarbonium intermediate. The present studies show that the *Bacillus* sp. NAGase is different from other reported β-hexosaminidases and exo-N-acetyl-β-D-glucosaminidases in that, apart from a carboxylate residue, a histidine residue is also involved in the catalytic mechanism and a tryptophan residue is involved in substrate binding.

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CHAPTER IV

Fluorimetric studies on exo-N-acetyl-β-D-glucosaminidase

SUMMARY

The exo-N-acetyl-β-D-glucosaminidase when excited at 280 nm or 295 nm fluoresces with a λ_{max} of emission at 337 nm. The fluorescence of the enzyme was quenched on titration with substrates / inhibitors without any shift in the λ_{max} of emission. The association constants of various saccharides containing N-acetyl-D-glucosamine were determined by fluorescence titrations. Among the chitooligosaccharides, chitobiose exhibited higher association constant (3.16×10^2 M^{-1}) than chitotriose (1.82×10^2 M^{-1}). However, the relative change in the intrinsic fluorescence of the exo-N-acetyl-β-D-glucosaminidase on binding to chitotetraose was insignificant. These results are consistent with kinetic studies suggesting the enzymes' affinity towards chitobiose > chitotriose > chitotetrose and its exo-mode of action. NBS mediated fluorescence quenching and inactivation process indicated that complete inactivation of the enzyme occurs well before loss in fluorescence. The effect of various solute quenchers, on the native enzyme in presence and absence of N-acetylglucosamine, indicated that acrylamide was more efficient as a quencher (63 %) than succinimide (20 %), KI (28 %) or CsCl (15 %). Treatment of the enzyme with Gdn.HCl resulted in extensive changes in the fluorescence emission spectral characteristics. There was a shift in the emission maxima from 337 nm in the native protein to 350 nm in the denatured state accompanied by a 36 % decrease in the fluorescence intensity. Gdn.HCl mediated denaturation resulted in increased accessibility of the quenchers to the fluorophors as evidenced by an increase in the $f_{a(eff)}$ values except for CsCl, which was found to be a poor quencher even under denaturing conditions. Quenching studies also indicate that more than 2/3rd of the tryptophan fluorophors in native *Bacillus* sp. exo-N-acetyl-β-D-glucosaminidase are in a fairly rigid hydrophobic environment inaccessible even to the neutral quencher, acrylamide.

INTRODUCTION

Determination of the association constants and evaluation of the thermodynamic parameters associated with the binding process can give important information regarding the forces involved in stabilization of macromolecule-ligand interactions. Various approaches like UV difference spectroscopy, equilibrium dialysis, fluorescence spectroscopy and X-ray crystallography have been used to study such interactions [1-4]. The sensitivity of the indole chromophore to its environment has been used as an effective tool in the study of protein and protein-ligand complexes by fluorescence spectroscopy [2]. Remarkable differences in the fluorescence behaviour of lysozyme and its complexes with substrates / inhibitors has provided valuable information regarding their interactions at the enzyme binding site in solution, and has complemented X-ray studies of the binding site in crystal [5]. However, little information is available on the nature of substrate binding to exo-N-acetyl-β-D-glucosaminidases / β-hexosaminidases.

Chemical modification studies on the *Bacillus* sp. exo-N-acetyl-β-D-glucosaminidase indicated the involvement of a single tryptophan residue in substrate binding (Chapter III). Kinetics of the inactivation process and substrate protection studies revealed that modification of the active site tryptophan was preceded by the modification of more than one non-essential tryptophan residues. This suggested that the active site tryptophan residue is either a slow reacting residue or the modification of the first tryptophan residue facilitates the modification of the active site tryptophan. The present studies were undertaken to evaluate the binding of various N-acetylglucosamine containing saccharides to the enzyme. The sensitivity of tryptophan fluorophors to the local environment manifests in changes in the wavelength and / or intensity of its fluorescence. Therefore, the microenvironment of tryptophan residues and its accessibility in the exo-N-acetyl-β-D-glucosaminidase were assessed by comparing the quenching profiles using different solute quenchers in the absence and presence of inhibitor and denaturant.

MATERIALS AND METHODS

MATERIALS

Chitooligosaccharides ($GlcNAc_2$ – $GlcNAc_4$), p-nitrophenyl-β-D-N-acetylglucosaminide (pNP-β-D-GlcNAc), p-nitrophenyl-1-thio-β-D-N-acetyl-glucosaminide (pNP-1-thio-β-D-GlcNAc), N-acetyl-D-glucosamine (GlcNAc), methyl-β-D-N-acetylglucosamine (Me-β-D-GlcNAc), N-bromosuccinimide (NBS), acrylamide, succinimide, and ultrapure guanidine hydrochloride (Gdn.HCl) were obtained from Sigma Chemical Co. (St. Louis, MO, U. S. A.). Analytical grade cesium chloride (CsCl) and potassium iodide (KI) were purchased from Sisco Research Laboratories (India). All the other chemicals were of the highest purity available from commercial sources.

METHODS

Microorganism and culture conditions

Growth, maintenance of *Bacillus* sp. NCIM 5120, production and the purification of exo-N-acetyl-β-D-glucosaminidase (NAGase) were carried out as described in Chapter II.

Enzyme assay

The exo-N-acetyl-β-D-glucosaminidase activity was assayed, spectrophotometrically, using p-nitrophenyl-β-D-N-acetylglucosaminide at pH 6.0 and 70°C. The p-nitrophenol liberated was read as the phenolate anion, at 405 nm (Chapter II) [6].

Protein determination

Protein determination of the purified enzyme was routinely performed by *Bradford's* method [7] using BSA as the standard.

Preparation of enzyme solution

For all fluorescence studies, the enzyme solution was dialyzed against 20 mM Na-acetate buffer, pH 5.5 or 20 mM Na-citrate-phosphate buffer, pH 6.0,

containing 150 mM NaCl, for 24 h, filtered through 0.45 μm filter and the protein concentration determined.

Preparation of saccharide solutions

The sugar solutions were prepared (GlcNAc, Me-β-D-GlcNAc, chitobiose, chitotriose, chitotetraose, pNP-β-D-GlcNAc and pNP-1-thio-β-D-GlcNAc) by weighing out the dry powder and dissolving in 20 mM Na-citrate-phosphate buffer, pH 6.0, containing 150 mM NaCl. The solutions were filtered through 0.45 μM filter before use. The saccharide concentration was determined by total carbohydrate estimation [8] as well as by reducing sugar estimation [9].

Fluorescence measurements

Fluorescence measurements were performed on a Perkin-Elmer Spectrofluorimeter LS 50B, using an excitation and emission slit width of 5 nm. The excitation wavelength was fixed either at 280 nm or 295 nm and the emission recorded in the range 300 – 400 nm. Fluorescence quenching experiments were carried out by titrating 2 ml of the enzyme solution (2 μM in 20 mM Na-citrate-phosphate buffer, pH 6.0, containing 150 mM NaCl) at 25 ± 1°C with the saccharide solutions (5-50 μl aliquots) followed by monitoring the change at 337 nm ($\lambda_{max,\ em}$ of the enzyme). The temperature of the enzyme and the saccharide solutions were maintained using a circulating cryobath. Fluorescence of the buffer and the saccharide solutions (if any) were measured at identical wavelengths and corrected for, in the observed fluorescence. Corrections were also done for dilution effect and the final volume change on addition of the saccharide solution was less than 5 % of the enzyme solution. Care was taken to ensure that the enzymatic activity of NAGase did not interfere in the determination of association constants. The relative fluorescence intensity of NAGase, saturated with saccharide, F_∞, was extrapolated from the experimental data by plotting $1/F_0$-F against 1/[C] where F is the measured fluorescence of a solution containing the enzyme with a given substrate concentration [C] and F_0 is the fluorescence of a solution of enzyme alone. Log

(F_0-F/F-F_∞) was plotted against Log [C] and the association constants determined from the intercept on the X-axis [1].

Influence of N-bromosuccinimide (NBS) on the intrinsic fluorescence and activity of NAGase was studied by titrating native NAGase (2 μM, 2 ml) with increasing concentrations of NBS (1 μM - 50 μM) in 20 mM Na-acetate buffer, pH 5.5, containing 150 mM NaCl at 25 ± 1°C. The decrease in fluorescence and residual activity were plotted as a function of molar ratio of NBS : NAGase. In a parallel experiment, the number of tryptophan residues oxidized per subunit mol of the enzyme was determined spectrophotometrically according to *Spande* and *Witkop* [10].

Solute quenching

The exo-N-acetyl-β-D-glucosaminidase (2 μM, 2 ml in 20 mM Na-citrate buffer, pH 5.5) was titrated with acrylamide, succinimide, KI and CsCl in absence and presence of 0.025 M GlcNAc. A stock solution of acrylamide (8.0 M), succinimide (2.0 M), KI (5.0 M) and CsCl (5.0 M) was prepared in 20 mM Na-citrate buffer, pH 6.0 and 20 aliquots of 5-10 μl were added to the protein solution. The stock solution of KI was routinely made to 0.1 mM $Na_2S_2O_3$ to eliminate the small amount of I_3^- present and to prevent further formation of I_3^-. An excitation wavelength of 295 nm was used to selectively follow the tryptophan fluorescence of the protein and the relative fluorescence intensity of the protein was recorded in the range 300-400 nm. The quenching data were analyzed by the Stern-Volmer and modified Stern-Volmer equations [11,12] using MicroCal Origin 4.0.

$$F_0/F = 1 + K_{sv}[Q] \quad (1)$$

$$F_0/\Delta F = \{1/(f_a . K_Q)\ 1/[Q] + 1/f_a\} \quad (2)$$

where F_0 and F are the fluorescence intensities of the protein in absence and presence of the quencher at a concentration [Q]. f_a is the fractional degree of fluorescence at concentration [Q], K_{sv} is the effective quenching or Stern-Volmer

constant and ΔF is the difference in fluorescence intensity in the absence and presence of the quencher.

A stock solution of 8.0 M guanidine hydrochloride (Gdn.HCl) was prepared in deionised water and the pH of the solution was adjusted to 6.0 by the addition of dilute NaOH. The effect of Gdn.HCl was studied by incubating the enzyme solution (2 μM, 2 ml in 20 mM Na-citrate-phosphate, pH 6.0) in presence of varying concentrations of the denaturant (1.5 M-6.0 M) at 4°C for 24 h. Fluorescence quenching in presence of varying concentrations of Gdn.HCl was performed with solute quenchers and the differences in quenching parameters compared.

RESULTS

Determination of binding constants : The intrinsic fluorescence of native NAGase exhibited an emission maximum of 337 nm on excitation either at 280 nm or 295 nm. Titration of exo-N-acetyl-β-D-glucosaminidase with chitooligosaccharides resulted in quenching of the intrinsic fluorescence with no change in the emission maximum. Maximum quenching was observed with chitobiose and chitotriose (9 %). There was no appreciable quenching on addition of chitotetraose. The association constants for the binding of chitobiose and chitotriose at 25 ± 1°C were 3.16×10^2 M^{-1} (Fig IV.1A) and 1.82×10^2 M^{-1} (Fig IV.1B) (Table IV.1) respectively. The association constants for pNP-β-D-GlcNAc and pNP-1-thio-β-D-GlcNAc at 25 ± 1°C were substantially higher (5.012×10^4 M^{-1} and 1.18×10^4 M^{-1} respectively) and the fluorescence quenching was accompanied by a small red shift ($\lambda_{max\ em}$, 340 nm). Me-β-D-GlcNAc was found to be a better inhibitor than GlcNAc (with K_a values 1.02×10^2 M^{-1} and 31.62 M^{-1} respectively) (Table IV.1).

Influence of NBS on the fluorescence and activity of NAGase : Fig IV.2 shows the effect of N-bromosuccinimide (NBS) on the activity and intrinsic fluorescence of NAGase. Oxidation of tryptophan residues by NBS resulted in a

decrease in the intrinsic fluorescence and there was a shift in the λ_{max} to 332 nm. About 74 % of the original fluorescence could be abolished on extensive titration with NBS (upto a molar ratio of NBS : enzyme 20 : 1). Further addition of NBS led to spectral blue shift as well as an increase in A_{280}. It was observed that the enzyme lost its total activity when only 20 % of the intrinsic fluorescence was quenched by NBS (at a molar ratio of NBS : enzyme, 5:1), suggesting that only a few tryptophan residues in NAGase are responsible for enzyme activity.

Solute Quenching : Titration of native NAGase with various non-ionic and ionic solute quenchers indicated that acrylamide was a better quencher (63 %) as compared to succinimide (20 %), KI (28 %) or CsCl (15 %) (Table IV.2). The direct Stern-Volmer plots were found to be linear in case of acrylamide and succinimide where as it was found to be downward curving in case of CsCl and KI (Fig IV.3iii and 3iv). The modified Stern-Volmer plots were linear for acrylamide, succinimide and CsCl, however, it was found to be bimodal in case of KI giving rise to two K_Q and $f_{a(eff)}$ values at lower and higher concentrations of the quencher (Fig IV.3) (Table IV.2). There was a marginal increase in the fraction of accessible fluorescence when the titration was carried out in presence of 0.025 M GlcNAc with KI. However, in case of acrylamide, succinimide and CsCl the fluorescence spectral characteristics as well as quenching parameters were similar to that of the native protein.

Denaturation of the protein with 3.0 M Gdn.HCl led to increased percentage quenching as well an increase in the fraction of the accessible fluorescence and exhibited almost linear Stern-Volmer plots for all the solute quenchers except CsCl which was found to be downward curving (Fig IV.5iv). Treatment of the protein with 6.0 M Gdn.HCL led to an upward curving Stern-Volmer plot for acrylamide and the efficiency of quenching with KI reduced from 71.8 % at 3.0 M to 39 % at 6.0 M Gdn.HCl (Table IV.3).

Spectral changes : There was a small blue shift from 337 nm in the native protein to 335 nm after titration with various quenchers in presence or absence of

GlcNAc. Gdn.HCl mediated denaturation of the exo-N-acetyl-β-D-glucosaminidase was concentration dependent and maximal effects were observed at 3.0 M Gdn.HCl (Fig IV.4). The denaturation was accompanied by a large red shift (from 337 nm in the native protein to 350 nm in the denatured enzyme) and a 36 % decrease in the fluorescence intensity relative to the native enzyme. Titration of Gdn.HCl denatured protein with various solute quenchers also revealed a blue shift in $\lambda_{em,\ max}$ ($\Delta\lambda_{max\ shift}$, ~5 nm) suggesting that the fluorescence associated with the enzyme after titration with various solute quenchers are due to buried tryptophan residues.

Table IV.1

Association constants for the binding of various GlcNAc containing saccharides with *Bacillus* sp. NAGase

Saccharide	K_a (M^{-1})
Chitobiose	3.16×10^2
Chitotriose	1.82×10^2
Chitotetrose	-
pNP-β-D-GlcNAc	5.01×10^4
pNP-1-thio-β-D-GlcNAc	1.18×10^4
GlcNAc	31.62
Me-β-D-GlcNAc	1.02×10^2

The binding constants were determined at 25 ± 1 °C

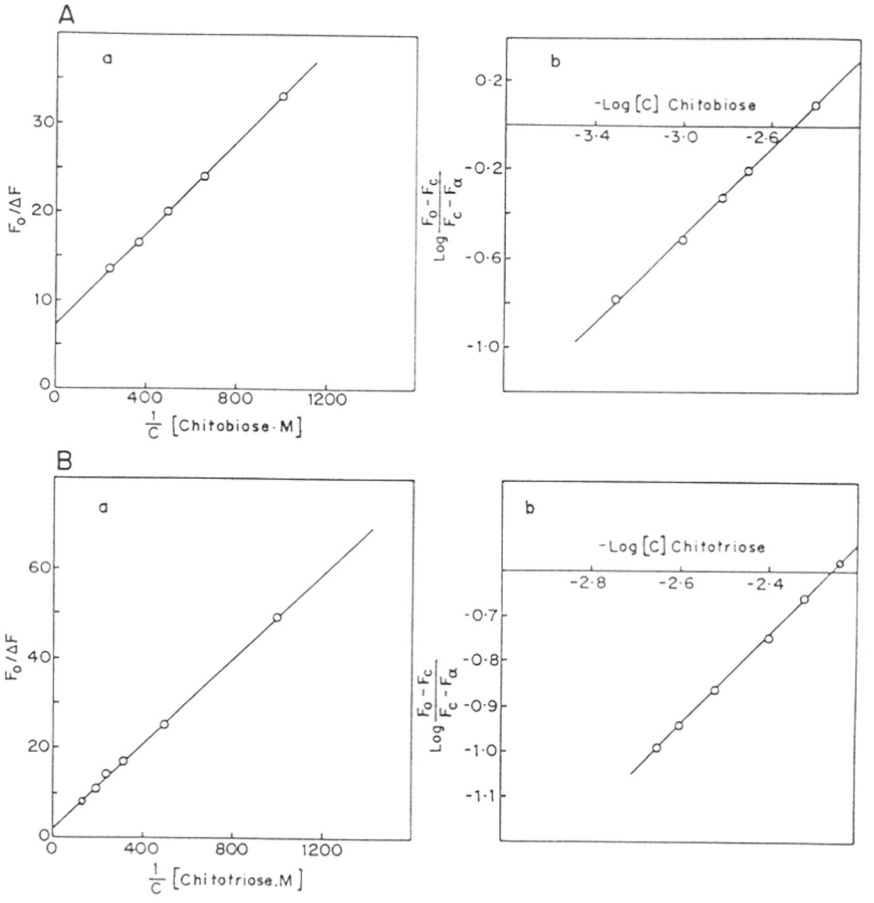

Figure IV.1 Determination of the association constants for the binding of chitobiose and chitotriose to *Bacillus* sp. exo-N-acetyl-β-D-glucosaminidase.
A) Chitobiose and **B)** Chitotriose.

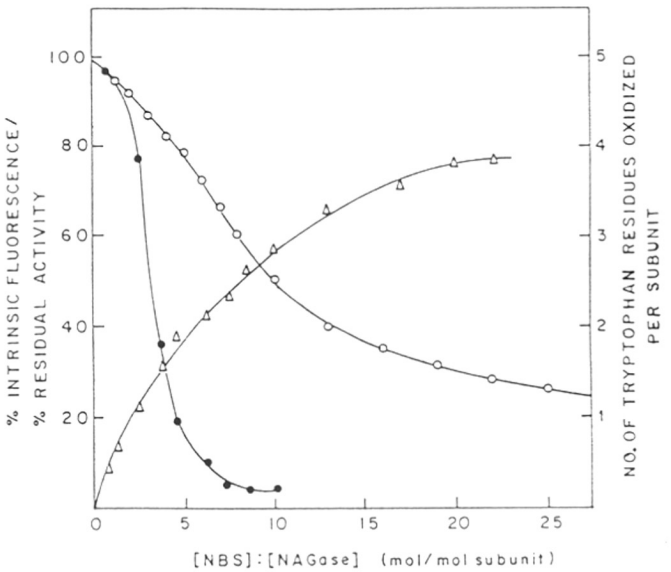

Figure IV.2 Effect of NBS on the activity and intrinsic fluorescence of *Bacillus sp.* exo-N-acetyl-β-D-glucosaminidase. (•) indicates residual activity and (o) fluorescence of exo-N-acetyl-β-D-glucosaminidase. (Δ) indicates the number of tryptophan residues oxidized per subunit.

Table IV.2

Quenching parameters of neutral and ionic quenchers on the fluorescence of native exo-N-acetyl-β-D-glucosaminidase from *Bacillus*. sp. NCIM 5120: The protein (2 μM, 2 ml in 20 mM Na-citrate-phosphate buffer pH 6.0, containing 150 mM NaCl) was titrated with various solute quenchers in absence and presence of N-acetylglucosamine (0.025 M) using an excitation wavelength of 295 nm and the emission recorded in the range 300-400 nm.

Quencher	GlcNAc (0.025 M)	$f_{a(eff)}$	K_Q	% Quenching	$\lambda_{max, em}$
None					337
Acrylamide (0.8 M)	-	0.66	5.7	63	335
,,	+	0.66	5.7	63	
Succinimide (0.2 M)	-	0.24	12.59	21	334
,,	+	0.23	12.61	22.5	
KI (0-0.1 M)	-	0.21	15.85	27	335
KI (0.1-0.4 M)	-	0.44	4.54		
KI (0-0.16 M)	+	0.25	11.28	26	
KI (0.16-0.4 M)	+	0.57	2.34		
CsCl (0.4 M)	-	0.33	2.27	15	335
,,	+	0.33	1.62	16	

The fraction of accessible fluorescence $f_{a(eff)}$ in case of acrylamide, succinimide, KI and CsCl were obtained by extrapolating the modified Stern-Volmer plots onto the Y-axis.

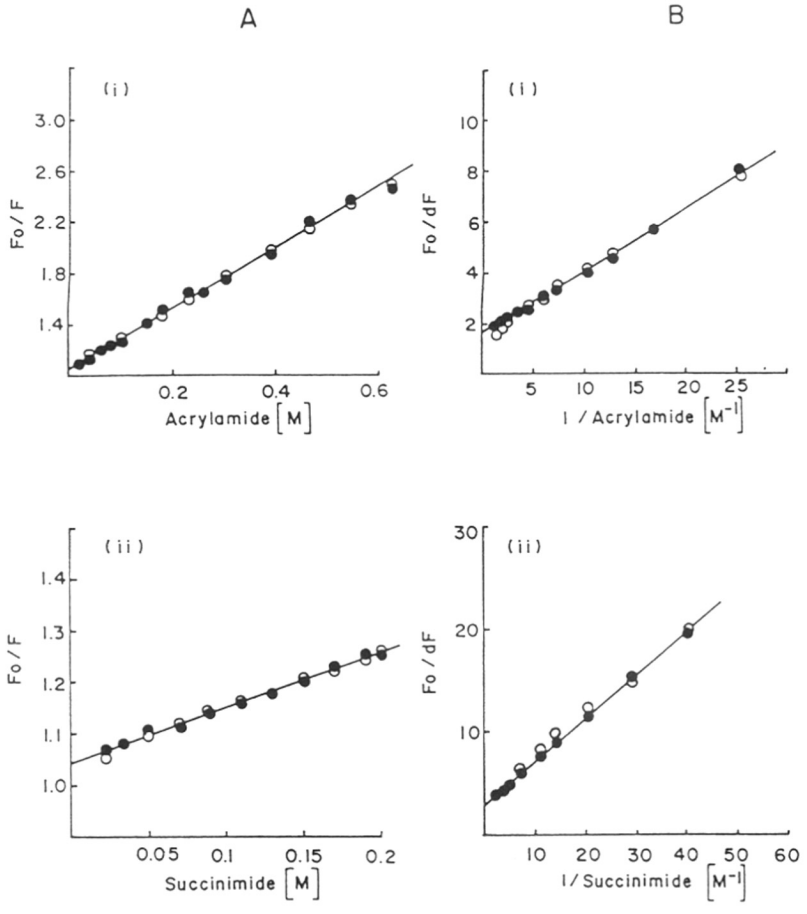

Figure IV.3 Stern-Volmer and modified Stern-Volmer plots for the quenching of fluorescence of native exo-N-acetyl-β-D-glucosaminidase.

A) Stern-Volmer plots; i) Acrylamide, ii) Succinimide.

B) Modified Stern-Volmer plots; i) Acrylamide, ii) Succinimide. Quenching in (o) absence and (•) presence of GlcNAc (0.025 M).

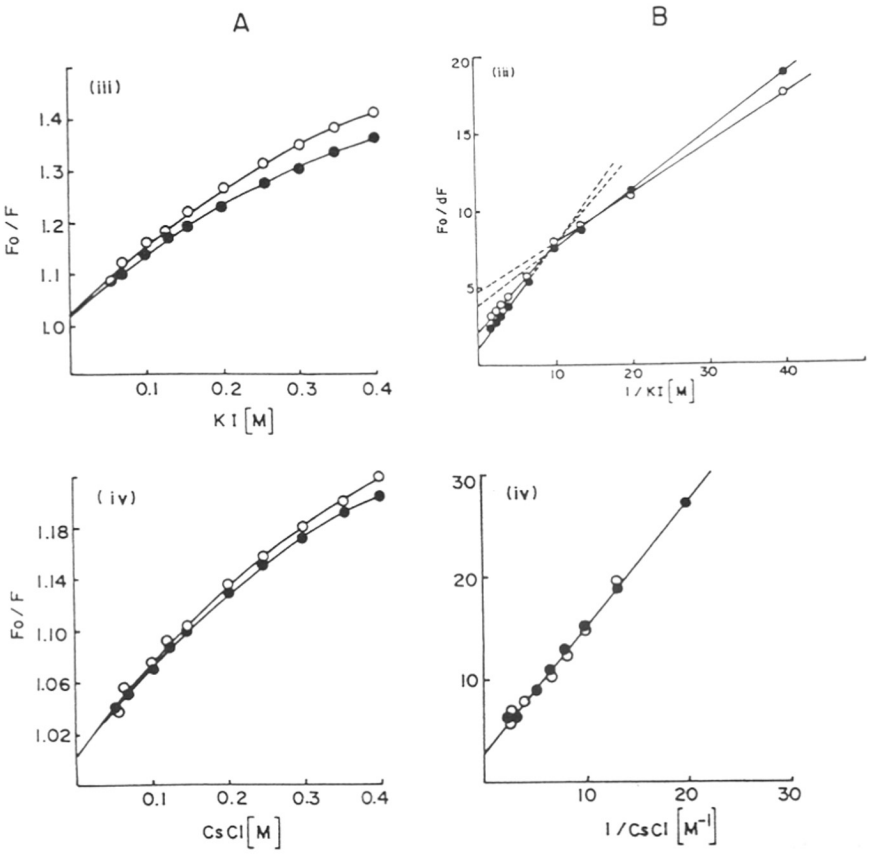

Figure IV.3 Stern-Volmer and modified Stern-Volmer plots for the quenching of fluorescence of native exo-N-acetyl-β-D-glucosaminidase.

A) Stern-Volmer plots; iii) KI and iv) CsCl.

B) Modified Stern-Volmer plots; iii) KI and iv) CsCl. Quenching in (o) absence and (•) presence of GlcNAc (0.025 M).

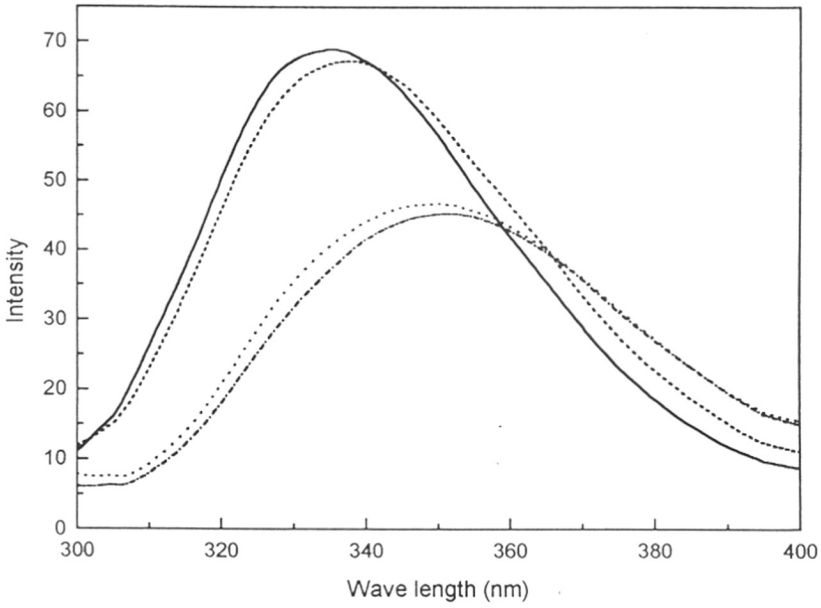

Figure IV.4 Effect of guanidine hydrochloride (Gdn.HCl) on the fluorescence emission spectrum of exo-N-acetyl-β-D-glucosaminidase from *Bacillus* sp. NCIM 5120.

NAGase (2 μM in 2 ml 20 mM sodium-citrate-phosphate buffer) was treated with varying concentrations of Gdn.HCl at 4°C for 24 h. The treated proteins were exicited at 295 nm and the emission recorded in the range 300 - 400 nm at 25±1 C. (—) native enzyme, (- - -) 1.5 M, (·····) 3.0 M and (·-·-·-) 6.0 M Gdn.HCl treated enzyme.

Table IV.3

Quenching parameters of neutral and ionic quenchers on the fluorescence of exo-N-acetyl-β-D-glucosaminidase from *Bacillus* sp. NCIM 5120 on denaturation with Gdn.HCl : The protein (2 μM, 2 ml in 20 mM Na-citrate-phosphate-buffer pH 6.0) was equilibrated with different concentrations of Gdn.HCl (1.5 M, 3.0 M and 6.0 M) and titrated with various solute quenchers using an excitation wavelength of 295 nm and the emission recorded in the range 300-400 nm.

Gdn.HCl	Quencher	$f_{a(eff)}$	K_Q	% Quenching	$\lambda_{max, em}$	$\lambda_{max, em}$ shift
Native NAGase					337	
1.5 M	Acrylamide	0.71	7.18	71.03	340	335
	Succinimide	0.25	11.80	22.40		
	KI	0.26	10.60	30.00		
	CsCl	0.33	8.90	20.00		
3.0 M	Acrylamide	0.89	10.75	77.26	347	342
	Succinimide	0.72	8.79	45.09		
	KI	0.83	8.21	71.85		
	CsCl	0.27	7.60	23.82		
6.0 M	Acrylamide	1.0	7.80	93.16	350	345
	Succinimide	1.0	13.10	90.10		
	KI	0.45	13.00	39.10		
	CsCl	0.23	6.10	23.60		

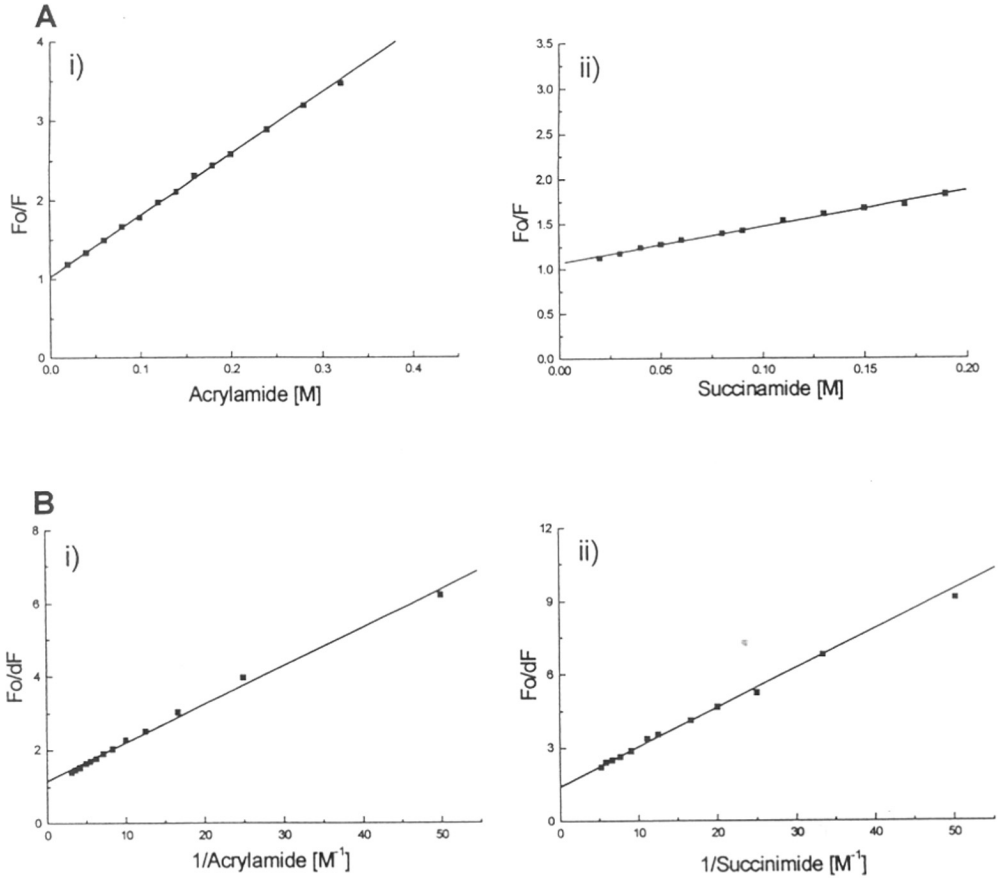

Figure IV.5 Stern-Volmer and modified Stern-Volmer plots for the quenching of fluorescence of 3.0 M guanidine hydrochloride treated exo-N-acetyl-β-D-glucosaminidase

A) **Stern-Volmer plots**; i) Acrylamide and ii) Succinimide
B) **Modified Stern-Volmer plots**; i) Acrylamide and ii) Succinimide

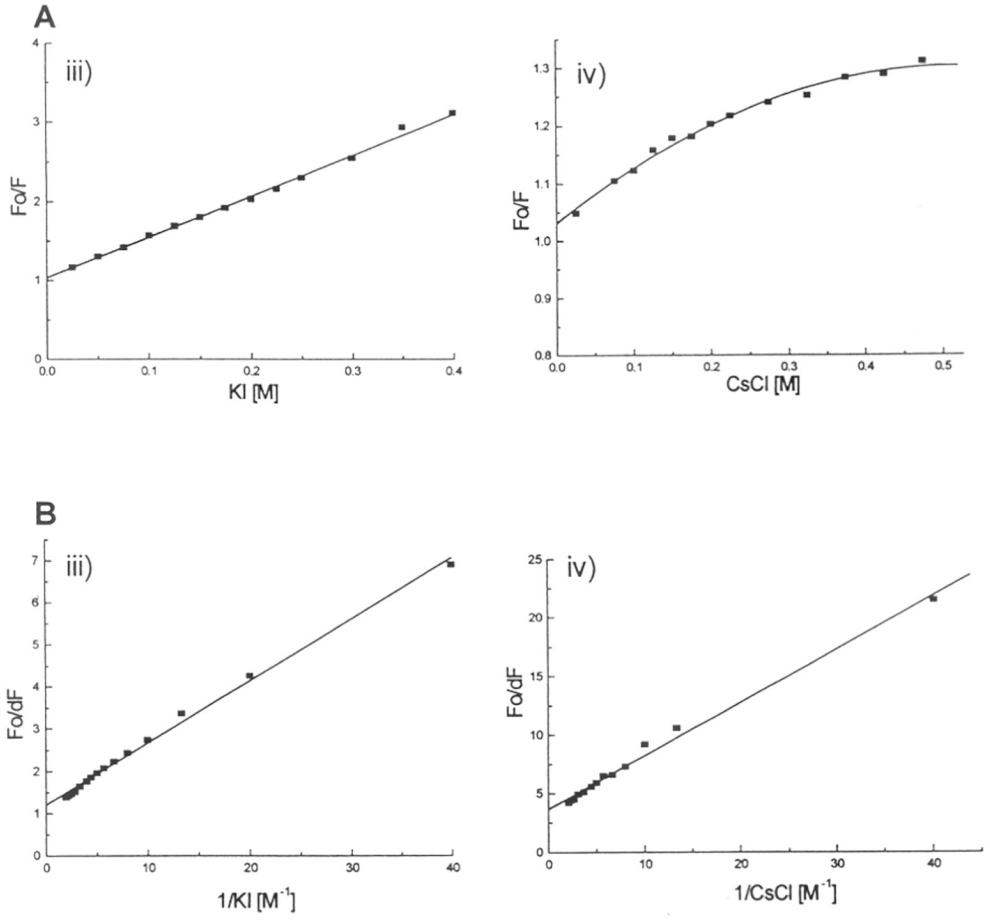

Figure IV.5 Stern-Volmer and modified Stern-Volmer plots for the quenching of fluorescence of 3.0 M guanidine hydrochloride treated exo-N-acetyl-β-D-glucosaminidase

A) **Stern-Volmer plots**;iii) KI and iv) CsCl
B) **Modified Stern-Volmer plots**; iii) KI and iv) CsCl

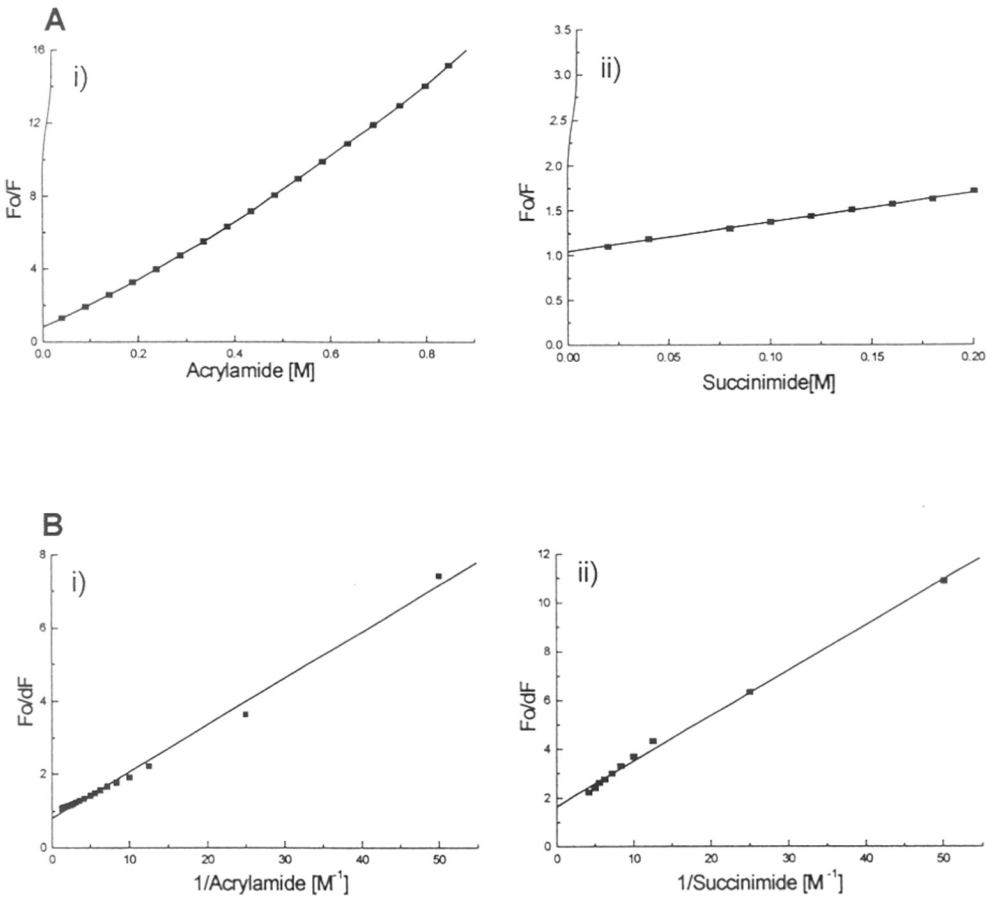

Figure IV.6 Stern-Volmer and modified Stern-Volmer plots for the quenching of fluorescence of 6.0 M guanidine hydrochloride treated exo-N-acetyl-β-D-glucosaminidase

A) **Stern-Volmer plots**; i) Acrylamide and ii) Succinimide
B) **Modified Stern-Volmer plots**; i) Acrylamide and ii) Succinimide

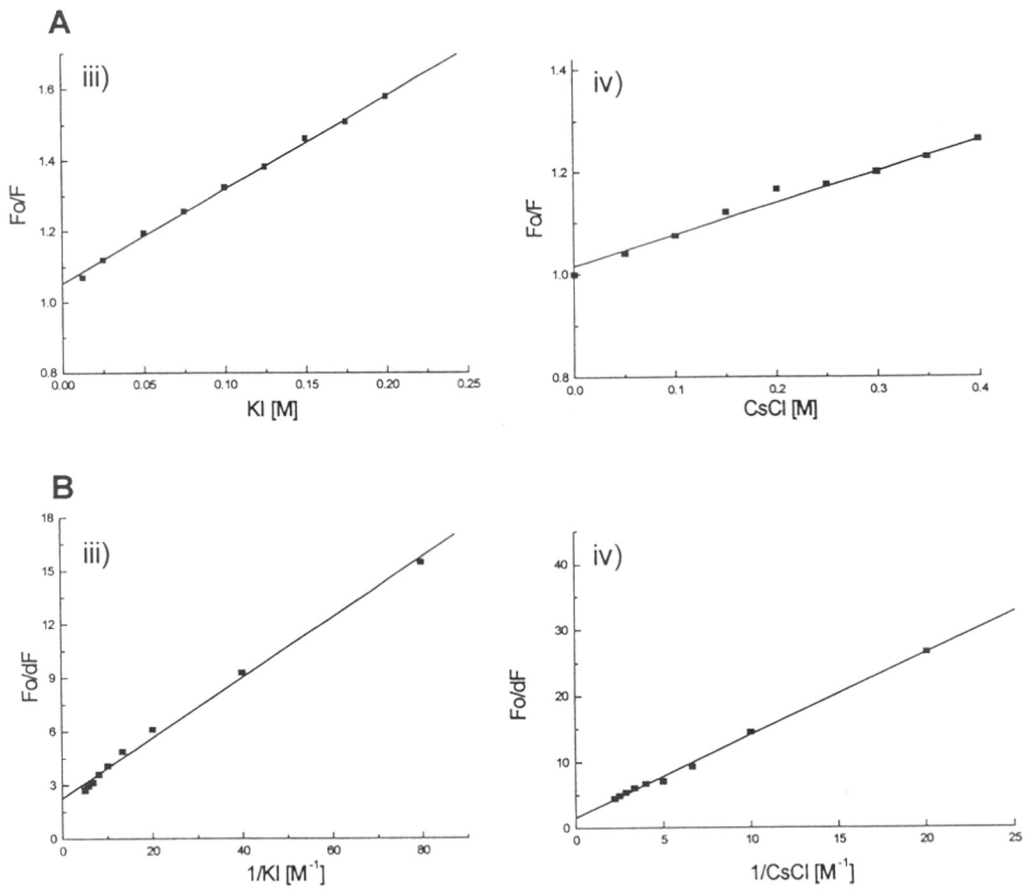

Figure IV.6 Stern-Volmer and modified Stern-Volmer plots for the quenching of fluorescence of 6.0 M guanidine hydrochloride treated exo-N-acetyl-β-D-glucosaminidase

A) **Stern-Volmer plots**;iii) KI and iv) CsCl
B) **Modified Stern-Volmer plots**; iii) KI and iv) CsCl

DISCUSSION

The exo-N-acetyl-β-D-glucosaminidase exhibits a fluorescence emission maximum at 337 nm on excitation either at 280nm or 295 nm. There was a decrease in the intrinsic fluorescence of the enzyme on binding to various substrates or inhibitors although to varying degrees. The maximum decrease in fluorescence was observed with the natural substrates, chitobiose and chitotriose (9 %). GlcNAc quenched the fluorescence by 7 % whereas quenching observed with other substrates / inhibitors were ~6 %. The was no change in the emission maxima on addition of the various saccharides except for pNP-β-D-GlcNAc and pNP-1-thio-β-D-GlcNAc, which exhibited a small red shift to 340 nm. The slope of the plot of Log F_0-F/F-F∞ versus Log [C] was found to be unity with each of the saccharides tested, indicating the formation of a one-to-one complex (Fig IV.1A and 1B). Although the calculated association constants from fluorescence studies for all the saccharides were lower than those obtained during kinetic studies (Chapter II, Table II.8), a similar trend in the values were observed. Such differences in the values of association constant determined by different methods are not uncommon [13].

The association constants for chitobiose and chitotriose was calculated to be 3.16×10^2 M^{-1} and 1.82×10^2 M^{-1} respectively (Table IV.1) (Fig IV.1A and 1B). Addition of chitotetraose to NAGase did not bring about any significant quenching in the intrinsic fluorescence under similar concentrations. These results are in accordance with kinetic studies, wherein, chitobiose was found to be a better substrate than chitotriose (k_{cat}, 24 μmoles min^{-1} mg^{-1} and 17.75 μmoles min^{-1} mg^{-1} respectively), and chitotetraose was found to be a weak substrate (hydrolysis observed after prolonged incubation, 2 h). Further, the affinity towards chitobiose was greater than chitotriose (K_m, 0.34 mM and 1.66 mM respectively) (Chapter II, Table II.8).

A comparison of the association constants of various substrates / inhibitors indicate that, substitution of a methyl group at the reducing end of N-acetylglucosamine is comparable to the substitution of another GlcNAc residue at the same position. The increase in association constant from 31.62 M^{-1} for

GlcNAc to 1.02×10^2 M^{-1} for Me-β-D-GlcNAc is similar in the order of magnitude as observed in case of $GlcNAc_2$ or $GlcNAc_3$. However, Me-β-D-GlcNAc was not an effective substrate for the enzyme and exhibited competitive inhibition towards the hydrolysis of pNP-β-D-GlcNAc (K_i , 0.24 mM). The β-hexosaminidase from *Streptococcus* 6646 K also could not hydrolyze Me-β-D-GlcNAc [14]. Substitution of a p-nitrophenyl group at the same position enhanced the association constant more than 100 fold (Table IV.1) and the $\lambda_{max,em}$ was shifted to 340 nm. As observed from kinetic studies the K_m for pNP-β-D-GlcNAc and the K_i for the substrate analogue, pNP-1-thio-β-D-GlcNAc were 69 μM and 87.11 μM respectively, suggesting a strong affinity for these two saccharides to the enzyme. However, the rate of hydrolysis for pNP-β-D-GlcNAc was lower than that of the most preferred substrate, chitobiose (20.75 μmoles min^{-1} mg^{-1} and 24 μmoles min^{-1} mg^{-1} respectively). The small red shift observed in the emission maximum of the enzyme on titration with the pNP substituted saccharides is indicative of hydrophobic interactions between the pNP group and the tryptophan residues of the protein. The increased affinity for these saccharides could be due to interaction of the pNP group with a hydrophobic patch at the active site.

Amino acid analysis of the NAGase indicated the presence of 11 tryptophan residues per subunit of the enzyme (Chapter II, Table II.7). Fig IV.2 shows the effect of NBS on the intrinsic fluorescence and activity of NAGase. There was a rapid decrease in the fluorescence intensity of the enzyme on addition of NBS upto a molar ratio of 10. Further addition of NBS resulted in a marginal decrease of the intrinsic fluorescence, suggesting variations in the reactivity of tryptophan residues of NAGase towards NBS. Also, only 74 % of the intrinsic fluorescence could be quenched on extensive titration with NBS (molar ratio 1:20) suggesting that not all the tryptophan residues are accessible to NBS mediated oxidation under native conditions. A spectral blue shift from 337 nm to 332 nm after NBS mediated fluorescence quenching indicates that the residual fluorescence is due to buried tryptophan residues [15,16]. Treatment of the enzyme with NBS led to a loss in fluorescence as well as activity but

complete loss in activity was observed when < 20 % of the tryptophan fluorescence had been quenched indicating that not all the tryptophan residues are involved in the activity of the enzyme. Chemical modification studies indicated the involvement of a single tryptophan residue / subunit in the activity of the enzyme although more than one non-essential tryptophan residues do get modified prior to inactivation (Chapter III). Further, spectrophotometric titration of NAGase with NBS under similar conditions revealed that only four tryptophan residues could be modified under native conditions on extensive titration with NBS (at NBS : enzyme ratio 20:1). This would suggest that the major contribution towards the protein fluorescence (74 % of the fluorescence) in native state is from four tryptophan residues. However, energy transfer between the non-fluorescent and fluorescent tryptophan residues cannot be ruled out [17].

The accessibility of tryptophan fluorophors and its local environment in native NAGase was investigated by studying the quenching parameters with two neutral quenchers (acrylamide and succinimide), a cationic quencher (Cs^+) and an anionic quencher (I^-) in absence and presence of a bound ligand (GlcNAc, 0.025 M). The percentage quenching for acrylamide, succinimide, KI and CsCl were 63 %, 20 %, 28 % and 15 % respectively (Table IV.2). The solute quenchers by themselves had no effect on the activity of the enzyme suggesting the absence of conformational changes on titration with various quenchers. Iodide (I^-) and Cesium (Cs^+) are hydrated ionic quenchers and are capable of quenching fluorophors at or near the protein surface. The low quenching observed with KI and CsCl indicates that not all the tryptophan residues are *accessible to ionic quenchers and may also be solvent masked in the native state.*

The Stern-Volmer plots in case of acrylamide, succinimide almost linear (Fig 3A.i, ii), however, it was found to be downward curving in case of KI (Fig IV.3A.iii). This suggests that the surface exposed tryptophan residues, accessible to quenching with KI, are in an electropositive environment and the deviation of the Stern-Volmer plot towards the concentration axis indicates the non-permeability of the quencher to the interior of the protein and variations in the accessible fluorophors [18]. At higher concentrations of the quencher, essentially

all the accessible residues are quenched. The remaining fluorescence is from inaccessible residues, whose fluorescence is independent of the quencher concentration.

The modified Stern-Volmer plots were linear for acrylamide, succinimide and CsCl (Fig IV.3B.i, ii and iv), whereas, it was found to be bimodal in case of KI, giving rise to two different K_Q and $f_{a(eff)}$ values for low and high concentrations of the quencher (Fig IV.3B.iii). The observation of a bimodal modified Stern-Volmer plot is indicative of the heterogeneity in the class of accessible flourophors affected at the two concentration ranges (Table IV.2). There was no noticeable difference in the extent of quenching with acrylamide, succinimide and CsCl in the presence or absence of GlcNAc, whereas, there was a marginal increase in the $f_{a(eff)}$ with KI (0.20 and 0.25 at lower and 0.44 and 0.57 at higher concentrations of the solute quencher in absence and presence of GlcNAc respectively). This suggests that binding of GlcNAc to the protein exposes a fraction of tryptophan residues onto the surface rendering them accessible to quenching with KI. Although an emission maximum of 337 nm indicates that the tryptophan residues are present in a moderately hydrophobic environment, the above quenching studies along with NBS mediated oxidation of tryptophan residues indicate that, more than $2/3^{rd}$ of the tryptophan residues in the native NAGase are in a hydrophobic, rigid environment.

As the percentage quenching observed in case of a neutral quencher like acrylamide was only 63 % in the native enzyme, the protein was treated to varying concentrations of the denaturant, guanidine hydrochloride (1.5 M, 3.0 M and 6.0 M), and its effect on the protein conformation and quenching parameters were evaluated. The extent of denaturation of NAGase with Gdn.HCl depended upon the concentration of the denaturant and maximal effects were observed at 3.0 M Gdn.HCl (Fig IV.4). Denaturation in presence of Gdn.HCl caused extensive changes in the fluorescence emission, evidenced by a large red shift in the tryptophan emission peak and a marked decrease in the fluorescence intensity. The emission maxima shifted from 337 nm in the native protein to 347 nm in the denatured state (3.0 M Gdn.HCl) suggesting that the fluorescent

tryptophan residues in the native enzyme are in a hydrophobic environment. A 36 % decrease in the intensity was observed in the denatured protein compared to the native NAGase (Fig IV.4). At 6.0 M Gdn. HCl there was a marginal decrease in the intensity and a small red shift of 3 nm (i. e., λ_{max} 350 nm) in the emission maxima as compared to 3.0 M Gdn.HCl. The extensive changes observed on treatment with Gdn.HCl suggests dissociation of the subunits as well as randomization of the conformation of each chain. The localization of the emission maxima at 350 nm on denaturation indicates exposure of tryptophan side chains to the aqueous environment [19]. Increased fluorescence intensities and quantum yields have been observed in many multi-tryptophan proteins on treatment with denaturing agents [20]. However, in the present case there was a decrease in the fluorescence intensity on treatment with Gdn.HCl. It has been observed that presence of certain amino acid residues close to tryptophan fluorophors can react reversibly with Gdn.HCl and hence shield the fluorophors [21]. A small blue shift of 2-3 nm in the native enzyme and ~5 nm in the denatured protein was observed on titration with the solute quenchers. The most probable reason for this shift is the heterogeneity in the environment of tryptophan residues in a multi-tryptophan protein like NAGase. Similar blue shifts upto 5 nm has been observed in lysozyme [3] and pepsin [22]. This also indicates that the protein is not totally denatured even with 6.0 M Gdn.HCL treatment.

In presence of 3.0 M Gdn.HCl, almost linear direct Stern-Volmer plots were observed in all the quenchers except for CsCl (Fig IV.5) with increased quenching efficiencies (Table IV.3). Treatment of the NAGase with 6.0 M Gdn.HCl exhibited linear direct Stern-Volmer plots for succinimide, KI and CsCl, whereas it was found to be upward curving for acrylamide (Fig IV.6.i) indicating the occurrence of both collisional and static quenching. The lower K_Q values with increasing concentrations of Gdn.HCl can be explained due to the effect of increased viscosity associated with higher concentrations of the denaturant [12]. Also, there was a decrease in the percentage quenching and fraction of accessible fluorescence in case of KI (72 % and 39 % in presence of

3.0 and 6.0 M Gdn.HCl respectively) indicating a role for charge effects to be responsible for the decrease in quenching. The ionic quenchers KI as well as CsCl have similar hydrated ionic radii, however, the differences in their quenching is as a result of the difference in charge distribution around tryptophan fluorophors [23]. At 3.0 M Gdn.HCl most of the exposed tryptophan residues are in a electropositive environment thereby accessible to quenching with KI (% quenching 71.8 %, $(f_a)_{eff}$, 0.83) but become inaccessible at higher concentrations of the denaturant (probably due to shielding effects; % quenching, 39 % and $f_{a(eff)}$, 0.45). Complete accessibility for the cationic quencher, Cs^+ could not be achieved even under denaturing conditions suggesting that the tryptophan residues of the exo-N-acetyl-β-D-glucosaminidase are in a electropositive environment.

The present fluorescence studies on the *Bacillus* sp. exo-N-acetyl-β-D-glucosaminidase with various chitooligosaccharides confirmed its exo-hydrolytic nature with maximum efficiency towards chitobiose. NBS mediated fluorescence quenching indicates that only 1/3rd of the tryptophan residues are responsible for 74 % of the enzyme fluorescence. Analysis of quenching characteristics with various solute quenchers indicates variations in the class of accessible tryptophan fluorophors and that the tryptophan fluorophors in the native enzyme are in a electropositive environment. At least 2/3rd of the tryptophan residues in the native enzyme are in a relatively rigid hydrophobic environment and do not contribute significantly towards the intrinsic fluorescence of the protein.

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CHAPTER V

Purification and characterization of Peptide-N^4- (N-acetyl-β-glucosylaminyl) asparagine amidase (PNGase) from *Bacillus* sp. NCIM 5120

SUMMARY

A glycoamidase, peptide-N^4-(N-acetyl-β-D-glucosaminyl) asparagine amidase (PNGase), was detected in the extracellular culture filtrates of the thermotolerant *Bacillus* sp. NCIM 5120. The enzyme was purified to homogeneity by chromatography on CM-cellulose, CM-Sephadex and Sephacryl S-300. The M_r of the native enzyme, determined by size exclusion chromatography on Sephacryl S-300, was 58,000 and it was made up of two non-identical subunits of M_r 15,000 and 17,000 indicating the heterotetrameric nature of the enzyme (dimer of a heterodimer). The pI of the enzyme was 6.0. The optimum pH and temperature for the enzyme activity were 5.5 and 50°C respectively. Substrate specificity studies indicate that the PNGase hydrolyzes complex oligosaccharide containing glycans but does not hydrolyze high mannose oligosaccharide containing glycans. It could hydrolyze Dns-Fetuin (-SA) glycopeptide, various complex oligosaccharide containing glycoproteins and the N-linked core disaccharide, Asn-GlcNAc-GlcNAc, suggesting the flexibility of the enzyme towards the polypeptide substitution. Deglycosylation of various glycoprotein substrates indicated that complex biantennary and complex triantennary glycoproteins are better substrates than complex tetraantennary oligosaccharide containing glycoproteins.

INTRODUCTION

The oligosaccharide cleaving enzymes of N-linked glycoproteins are basically of two types, the endo-N-acetyl-β-D-glucosaminidase (ENGase, E. C. 3.2.1.96) and the peptide-N^4- (N-acetyl-β-D-glucosaminyl) asparagine amidase (PNGase; E. C. 3.5.1.52) [1-3]. Both the enzymes have been used as effective tools in investigating oligosaccharide structure [4] glycoprotein biosynthesis [5-7] and structure-function relationships of N-glycosylated proteins [8,9]. The peptide-N^4- (N-acetyl-β-D-glucosaminyl) asparagine amidase catalyses the hydrolysis of peptide-bound asparagine-linked glycans, converting the asparagine residue to an aspartic acid and releases an intact 1-amino oligosaccharide, thereby introducing an extra negative charge on the peptide backbone [10]. The 1-amino oligosaccharide further hydrolyses spontaneously to the corresponding saccharide by the release of an ammonia molecule. The functional role of peptide-N^4- (N-acetyl-β-D-glucosaminyl) asparagine amidase is in the degradation of glycoproteins [4] and has also been postulated to have a regulatory role in the maturation of mammalian glycoproteins [11-14] and in plant developmental processes [15-18]. The enzyme has been studied from microbes [1,19], plants [15-17,20-22] and animal tissue [14]. Studies on the substrate specificity indicate that these enzymes readily hydrolyze all kinds of N-linked oligosaccharides from defined glycopeptides, but, deglycosylation of glycoprotein substrates often require the presence of chaotropic agents or denaturants or elevated reaction temperatures [23].

During the course of purification of the exo-N-acetyl-β-D-glucosaminidase from the thermotolerant *Bacillus* sp. NCIM 5120, an endo-glycosidase, capable of hydrolyzing dansyl labelled fetuin glycopeptides was detected. The present chapter discusses the purification of this endo-glycosidase, its substrate specificity and applicability in the deglycosylation of glycoproteins.

MATERIALS AND METHODS

MATERIALS

Fetuin (Type III from Fetal Calf Serum), holotransferrin, ovalbumin, RNase B, α_1-acid glycoprotein, fibrinogen, pronase, carboxypeptidase, leucine aminopeptidase, neuraminidase, hemoglobin, dansyl chloride, pNP-glycosides, Asn-GlcNAc-GlcNAc, CM-cellulose and CM-Sephadex were purchased from Sigma Chemical Co. (St. Louis. MO, U.S.A.). Sephacryl S-300 was obtained from Pharmacia (Uppasala, Sweden).

METHODS

Soybean and *Ricinus communis* (castor bean) agglutinin were prepared from the respective seeds [24,25]. Desialated fetuin and desialated fibrinogen were prepared by treating the glycoproteins with 0.1 N H_2SO_4, for 2 h, at 50°C followed by extensive dialysis to remove the released N-acetyl neuraminic acid residues. The acid treated glycoproteins were then subjected to neuraminidase digestion to ensure complete desialation.

Preparation of glycopeptide substrates

A) Preparation of glycopeptides from soybean agglutinin

Purification of soybean agglutinin : The agglutinin from soybean seeds was purified according to the method of *Gordon et al.* [24] with slight modifications. Soybean seeds (100 g), untoasted and undefatted, were macerated dry in a blender and stirred with 10 fold v/v, 10 mM K-phosphate buffer, pH 7.2, containing 150 mM NaCl (PBS) for 24 h at 4°C. The suspension was filtered through cheese cloth to remove the insoluble material and the pH of the supernatant was adjusted to 4.5 with 1 M acetic acid. The precipitated proteins were removed by centrifugation (5000 **g**, 20 min, 4°C) and the supernatant readjusted to pH 7.2 with 1 N NaOH and subjected to 70 % ammonium sulphate saturation. The precipitated protein was collected by centrifugation (5000 **g**, 20 min, 4°C) and dialyzed against PBS extensively. The dialysate was then loaded onto a cross-

linked guargum affinity matrix (60 × 5 cm) pre-equilibrated with PBS. The column was washed with PBS to remove the unadsorbed proteins (O. D. $_{280\ nm}$ < 0.05) and the bound agglutinin was eluted with 0.1 M lactose in PBS. The active fractions were pooled and subjected to 90 % ammonium sulphate saturation. The precipitated protein was collected by centrifugation (7,800 g, 20 min, 4°C), dissolved in minimum amount of deionised water, dialyzed extensively against deionised water, freeze dried and used for further processing.

Digestion of soybean agglutinin : The freeze dried agglutinin (~2 g) was denatured by incubation with 50 ml of 0.1 N HCl, for 2 h, at 60°C. The suspension thus formed was brought to pH 8.0 by the addition of 0.1 N NaOH and mixed with Tris-HCl buffer, (100 mM, effective concentration) pH 8.0. 30 mg of pronase was added to the suspension and incubated at 37°C, for 24 h, under mild stirring (50 rpm). Two more additions of pronase (10 mg each) were done at an interval of 24 h. Sodium azide (0.005 %) was added to the reaction mixture to prevent microbial contamination. The digest was freeze dried, extracted with minimum volume of 10 mM acetic acid and subjected to Sephadex G-25 column chromatography (125 × 1 cm, pre-equilibrated with 10 mM acetic acid). The carbohydrate positive fractions were pooled and freeze dried. Glycopeptides obtained from the Sephadex G-25 column were further digested with leucine aminopeptidase and carboxypeptidase and chromatographed on a Bio-gel P-4 column (100 × 1 cm) equilibrated with 10 mM acetic acid. The carbohydrate positive fractions were pooled, freeze dried and finally passed through a Dowex 1X4-50 anion exchanger (20 × 1 cm, acetate form), to separate the glycopeptides from the oligosaccharides, generated during proteolytic digestion.

B) Preparation of fetuin glycopeptides

The procedure used for the preparation of fetuin glycopeptides was essentially the same as described for soybean agglutinin glycopeptides, with few modifications.

Digestion of fetuin : Fetuin (~2 g) was dissolved in 50 ml of 0.2 N H_2SO_4 and incubated at 50°C for 2 h. The resulting suspension was dialyzed extensively against deionised water to remove the N-acetyl neuraminic acid residues and then digested with pronase (24 h, 37°C). The pronase treated samples were desalted on Sephadex G-25 column (125 × 1 cm, preequilibrated with 10 mM acetic acid) and subjected to leucine amino peptidase and carboxy peptidase digestion. Subsequently, the digested samples were again incubated with neuraminidase to ensure complete removal of terminal N-acetyl neuraminic acid residues. Neuraminidase treated glycopeptides were desalted on Bio-gel P-4 column (100 × 1 cm) and used for dansylation.

Dansylation of glycopeptides

Dansylation of the glycopeptides was done according to the method of *Gray* [27]. A glass test tube containing 20-50 mg of glycopeptides was dissolved in minimum amount of 10 mM $NaHCO_3$ and vacuum dried. The glycopeptides were redissolved in deionised water and the pH of the solution adjusted between 8.5 – 9.0. Dansyl chloride (dissolved in dry acetone) was added to the glycopeptides to make a final concentration of 10 mM. The reaction mixtures were thoroughly mixed and incubated in dark, at 37°C, under shaking conditions (200 rpm) for 24 h. The dansylated glycopeptides were dried under vacuum and desalted by chromatography on Bio-gel P-4 (100 × 1 cm).

Organism, culture conditions and enzyme production

The thermotolerant *Bacillus* sp. NCIM 5120 was routinely maintained at 50°C on nutrient agar slants (peptone 1.0 % w/v, beef extract 1.0 % w/v, NaCl 0.5 % w/v and agar 2.0 % w/v). The inoculum was prepared by inoculating 50 ml of enriched medium, containing peptone 1.0 % w/v, yeast extract 1.0 % w/v, mannose 0.5 % w/v and Speakman's salt solution [28], with 1 day old slant. A 4% v/v inoculum was transferred to a fresh medium and incubated at 50°C for 24 h under shaking (200 rpm). Subsequently, the supernatant was collected by

centrifugation (7,800 **g**, 20 min, 4°C) and used as the source of enzyme (Chapter II).

Enzyme assay

The enzyme (~1 μg) was incubated with either soybean agglutinin or fetuin dansylated glycopeptides (~20 μg neutral sugar) in 10 mM Na-acetate buffer, pH 5.5, containing 0.1 % bovine serum albumin and 10 mM EDTA at 50°C for 18 h. Subsequently, the reaction was arrested on ice and the products were analyzed by ascending paper chromatography on Whatman 1 paper (butanol: acetic acid: water system, 3:1:1 by vol.). The chromatograms were dried and the liberated products visualized under an UV transilluminator [23]. Time course of the reaction was followed by removing aliquots of the reaction mixture at definite time intervals (2 h - 20 h), followed by analyzing the products by paper chromatography.

Hydrolysis of the N-linked core disaccharide, Asn-GlcNAc-GlcNAc and fetuin glycopeptide (-SA) was also followed, fluorimetrically, by measuring the release of ammonia [29]. o-pthalaldehyde-mercaptoethanol reagent was prepared by mixing 4.5 ml of 750 mM o-pthalaldehyde (10 mg / ml in absolute ethanol) and 4.5 ml of 7.2 mM β-mercaptoethanol (5 μl / ml in absolute ethanol) with 81 ml of 0.2 M Na-phosphate buffer, pH 7.4. 100 μg (by neutral sugar) of either, Asn-GlcNAc-GlcNAc or fetuin (-SA) glycopeptide, in 100 μl of 100 mM Na-acetate buffer, pH 5.5 was incubated with 5 μg of the purified PNGase at 50°C, for 18 h. Freshly prepared o-pthalaldehyde-mercaptoethanol reagent (2.9 ml) was added to the reaction mixture and incubated at 25°C for 45 min. The liberated ammonia was measured, fluorimetrically, on a Perkin-Elmer Spectrofluorimeter LS 50B with an excitation and emission wavelength of 410 nm and 470 nm respectively using ammonium sulphate (5-500 n moles) as the standard. A slit width of 5 nm was used for both the monochromators. The fluorescence of the

buffer and the substrates (if any) were measured at identical wavelengths and corrected for, in the observed fluorescence.

Deglycosylation of glycoproteins

Glycoprotein stocks (10 mg / ml) were prepared in 20 mM Na-acetate buffer, pH 5.5, containing 10 mM EDTA. Deglycosylation reactions were performed with 500 µg of the respective glycoprotein and 5-10 µg of purified PNGase in a total volume of 200 µl, at 50°C, for 18 h. When denatured glycoproteins (boiled in presence of 0.1 % SDS) were used as the substrates, the reaction mixtures were made to 0.1 % Nonidet P-40 prior to addition of the enzyme. The reaction was terminated by the addition of 1:1 volume of 20 % TCA and the precipitated protein collected by centrifugation at 7800 **g** (5 min, 4°C). Carbohydrate content in the deglycosylated protein samples was determined by the phenol-sulfuric acid method [30]. The O. D. $_{280\ nm}$ of the supernatant was also measured to detect any protease contamination. Glycoprotein samples incubated under identical conditions, in absence of the enzyme, served as control.

Protease assay

Protease activity in the crude culture filtrate and during the course of the purification of the enzyme was assayed by measuring the acid soluble, A_{280} positive material following the hydrolysis of hemoglobin at pH 5.5 and 50°C for 18 h [31].

Exo-glycosidase activity in the purified enzyme preparation was measured by incubating the enzyme with the respective pNP-glycoside and the released p-nitrophenol was measured at 405 nm [32].

Carbohydrate estimation

Neutral sugar content of the glycoproteins and glycopeptides was determined by the phenol-sulphuric acid method [30]. The TCA precipitated protein samples were redissolved in 1ml of 0.1 N NaOH. 400 µl of the protein solution was mixed with 400 µl of 5 % phenol and incubated at room temperature

for 10 min. 2 ml concentrated sulphuric acid was then added to the mixture, allowed to cool for 20 min and the absorbance measured at 490 nm. Mannose was used as the standard for high mannose containing glycoproteins and a 1:1 mixture of galactose : mannose was used as the standard for complex glycoprotein substrates.

Determination of protein concentration

Protein concentration of the purified enzyme was determined by the method of *Bradford* [33] using BSA as the standard.

Electrophoresis

Native PAGE was performed on polyacrylamide slab gels, 7.5 % (w/v) at pH 4.3, using Methylene Blue as marker and the bands were visualized by silver staining [34]. SDS-PAGE was carried out according to Laemmli [35] and the protein bands visualized by silver staining [34]. Bovine serum albumin (M_r, 66,000), Ovalbumin (M_r, 45,000), Glyceraldehyde 3-phosphate dehydrogenase (M_r, 36,000), Carbonic anhydrase (M_r, 29,000), Trypsinogen (M_r, 24,000), Trypsin inhibitor (M_r, 20,100) and α-lactalbumin (M_r, 14,200) were used as the reference proteins. The pI of the purified enzyme was determined by density gradient isoelectric focussing using Pharmalytes in the range 3-10 [36].

Determination of M_r

The molecular mass of the purified enzyme was determined by gel filtration on a Sephacryl S-300 column (100 × 0.7 cm) equilibrated in 100 mM Na-acetate buffer, pH 5.5, containing 150 mM NaCl and 10 mM EDTA. The column was calibrated using bovine serum albumin (M_r, 66,000), carbonic anhydrase (M_r, 29,000) and cytochrome C (M_r, 12,400).

Purification of PNGase

All the purification steps were carried out at 4°C unless otherwise mentioned. Routine enzyme assays, during purification steps, were based on the

visual observation of the hydrolysis of Dns-Fetuin (-SA) glycopeptides. Protein concentration of the column effluents were monitored by measuring the absorbance at 280 nm. Protein concentration of pooled enzyme fractions during purification steps was determined by *Bradford's* method [34].

The crude culture filtrate was concentrated to $1/10^{th}$ of its original volume by ultrafiltration, under reducing atmosphere, using a YM-10 membrane. The concentrated extract was extensively dialyzed against 50 mM Na-acetate buffer, pH 5.5, containing 10 mM EDTA (Buffer A), and loaded onto a CM-cellulose column (50 × 2.5 cm), pre- equilibrated with Buffer A, at a flow rate of 12 ml / h. The column was washed with the same buffer untill the absorbance at 280 nm was < 0.020 and then the adsorbed protein was eluted with 0.5 M NaCl in Buffer A. The active fractions were pooled, dialyzed extensively against Buffer A and loaded on a CM-Sephadex column (20 × 2.5 cm) pre-equilibrated with the same buffer. The column was developed by a linear gradient of 0-0.5 M NaCl, and fractions of 2 ml were collected. The PNGase positive fractions were pooled (20 ml), concentrated to 2 ml and dialyzed extensively against 100 mM, Na-acetate buffer, pH 5.5 containing 10 mM EDTA.

The partially purified protein, obtained from the above step, was chromatographed on a Sephacryl S-300 column (170 × 1.7 cm) equilibrated with 100 mM, Na-acetate buffer, pH 5.5, containing 150 mM NaCl and 10 mM EDTA. Fractions of 2 ml were collected at a flow rate of 12 ml / h and the active fractions were pooled, dialyzed against Buffer A, lyophilized and stored at -20°C.

RESULTS

Purification of PNGase : The enzyme was purified to homogeneity by chromatography on CM-cellulose, CM-Sephadex and Sephacryl S-300. The elution profile of the enzyme from CM-Sephadex and Sephacryl S-300 are shown in Fig V.1. The PNGase obtained from the Sephacryl S-300 step was found to be homogeneous on native gels (Fig V.2).

Physicochemical properties : The M_r of the purified enzyme was 58,000 as determined by gel filtration (Fig V.3). The enzyme showed two bands of M_r 15,000 and 17,000 on SDS-PAGE, in presence or absence of β-mercaptoethanol, indicating it to be a dimer of a heterodimer (Fig V.4). The pI of the enzyme was 6.0. The PNGase had an optimum pH of 5.5 (Fig V.6) and was stable in the pH range, 5.0-8.0, for 24 h, at 4°C. The optimum temperature for activity was found to be 50°C, and significant amount of the activity was observed upto 70°C (Fig V.7). The enzyme was stable at 50°C for 24 h.

Type of glycan linkage hydrolyzed and substrate specificity : The purified enzyme hydrolyzed Dns-Fetuin (-SA) glycopeptides but could not hydrolyze Dns-Soybean agglutinin glycopeptides (Fig V.5). A time course analysis of the reaction indicated that maximum hydrolysis occurred after 18 h of incubation at 50°C. The type of linkage catalyzed by the enzyme was assessed by its action on Dns-Fetuin (-SA) glycopeptide and the core chitobiosyl disaccharide, Asn-GlcNAc-GlcNAc by fluorescence spectroscopy. It was observed that the enzymatic hydrolysis of the above substrates was accompanied by release of ammonia suggesting that the enzyme is a peptidyl: N-Glycanase rather than an endo-N-acetyl-β-D-glucosaminidase. The purified peptide:N-Glycanase hydrolyzed Dns-Fetuin (-SA) glycopeptides and was free of any protease activity. It did not exhibit any other exo-glycosidase activity (as tested against pNP-β-D-GalNAc, pNP-β-D-$GlcNH_2$, pNP-β-D-$GalNH_2$, pNP-β-D-Glu, pNP-β-D-Gal, and pNP-β-D-Man) except for a negligible pNP-β-D-GlcNAcase activity (0.423 pmoles of p-nitrophenol liberated / h / mg of protein, at 50°C, Table V.1).

The ability of the PNGase to deglycosylate various glycoprotein substrates is tabulated in Table V.1. The percentage deglycosylation varied with different glycoproteins and maximum deglycosylation was observed with fetuin (38.2 %), desialated fetuin (31.3 %), fibrinogen (38.5 %) and desialated fibrinogen (35.0 %). The percent deglycosylation observed with α_1-acid glycoprotein (19.20 %), holotransferrin (20.3 %) and ovalbumin (18.0 %) were lower. However, RNase B,

Ricinus communis agglutinin and Soybean agglutinin were resistant to hydrolysis. No appreciable change in percent deglycosylation was observed when the hydrolytic efficiency was tested on denatured glycoproteins (0.1 % SDS, 100°C, 3 min) (Table V.1).

DISCUSSION

A simple procedure involving successive chromatography on CM-cellulose, CM-Sephadex and Sephacryl S-300 yielded homogeneous preparations of a peptide: N-Glycosidase from the thermotolerant *Bacillus sp.* NCIM 5120. The initial CM-cellulose chromatographic step helped to remove the coloring matter present in the crude culture filtrate and also eliminated the bulk of the proteins (> 90 %) including the exo-N-acetyl-β-D-glucosaminidase which came unbound from the column. The requirement of high molarity of NaCl (0.3-0.4 M NaCl) to elute the enzyme from the second CM-Sephadex column (Fig V.1a) point towards a strong interaction of the enzyme with the matrix. The final gel filtration step on Sephacryl S-300 (Fig V.1b) resulted in homogenous preparation of the PNGase (Fig V.2).

The M_r of the enzyme determined by gel filtration was 58,000 (Fig V.3), but on SDS-PAGE it split into two bands of M_r 15,000 and 17,000 (Fig V.4) suggesting it to be a heterotetramer (dimer of heterodimer). PNGases studied from different sources show wide variations in the molecular mass and subunit composition. PNGase F (from *F. meningoscepticum*) and PNGase A (from almond emulsin) are monomeric proteins with a M_r of 35,000 and 69,000 respectively [10,21]. However, the PNGase At from A. *tubigensis* is a heterodimer with subunit M_r of 38,000 and 28,000 [19] and the jack bean N-Glycanase exhibits multiple bands from 78,000-30,000 on SDS-PAGE [17]. However, the L-asparagine amidase from *F. meningoscepticum*, which catalyses a similar type of reaction as PNGases, has a subunit M_r of 15,000 and 17,000 [37]. In this respect our enzyme is comparable to the *F. meningoscepticum* L-asparaginase in αβ nature and subunit size.

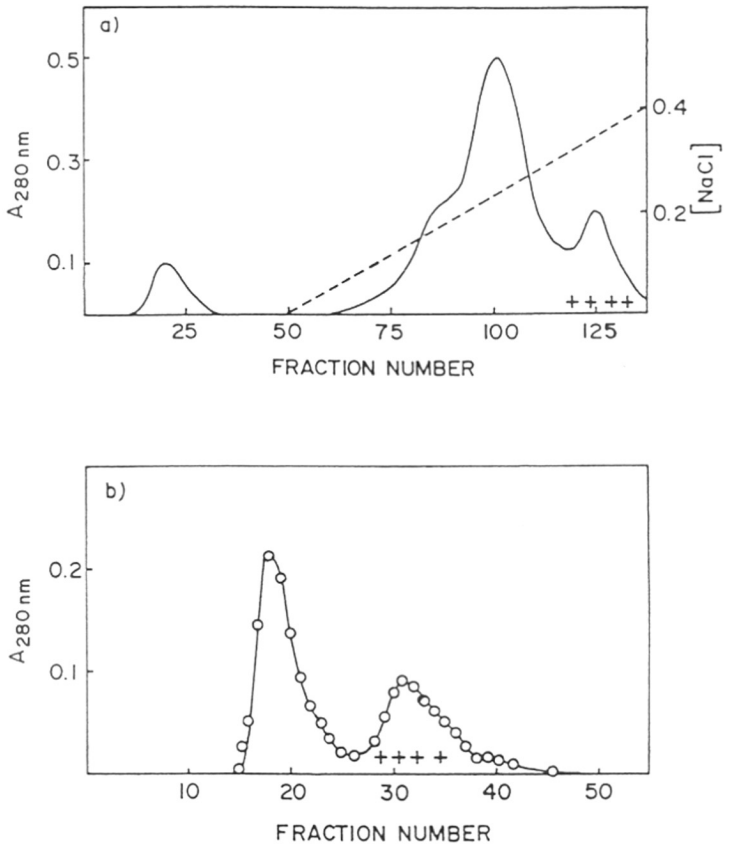

Fig V.1 Elution profile of *Bacillus* sp. PNGase

a) From CM-Sephadex: (o) O. D. $_{280\ nm}$ and (+) indicates PNGase positive fractions.

b) From Sephacryl S-300: (o) O. D. $_{280\ nm}$ and (+) indicates PNGase positive fractions.

Fig V.2 Native gel electrophoresis of purified PNGase from *Bacillus* sp. NCIM 5120 in 7.5 % w/v polyacrylamide gels, pH 4.3.

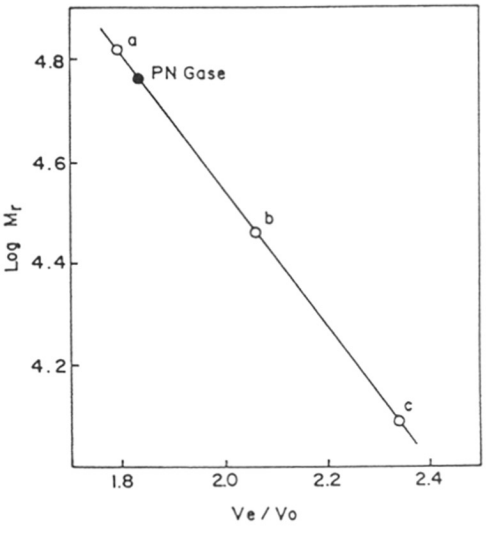

Fig V.3: Molecular weight determination of *Bacillus* sp. PNGase by gel filtration

A Sephacryl S-300 (100 × 0.7 cm) column was equilibrated with 100 mM Na-acetate buffer, pH 5.5, containing 150 mM NaCl and 10 mM EDTA and calibrated with **a)** Bovine Serum Albumin (66,000), **b)** Carbonic anhydrase (29,000) and **c)** Cytochrome C (12,400). V_0, void volume and V_e, elution volume.

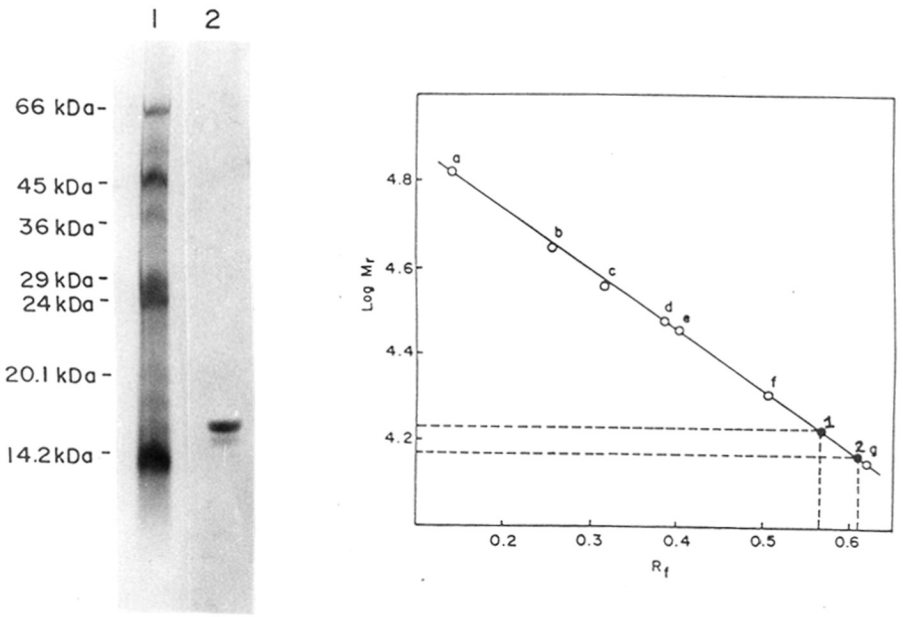

Fig V.4 Determination of molecular weight of purified PNGase by SDS-PAGE.

SDS-Polyacrylamide gel electrophoresis was carried out in 12 % w/v gels. The molecular mass of marker proteins are indicated alongside the gel.

Lane 1, marker proteins (Sigma 70 L) **a)** Bovine serum albumin (M_r 66,000), **b)** ovalbumin (M_r 45,000), **c)** glyceraldehyde 3-phosphate dehydrogenase (M_r 36,000), **d)** carbonic anhydrase (M_r 29,000), **e)** trypsinogen (M_r 24,000), **f)** trypsin inhibitor (M_r 20,100) and **g)** α-lactalbumin (M_r 14,200).

Lane 2, Purified PNGase, α-subunit (1) and β-subunit (2).

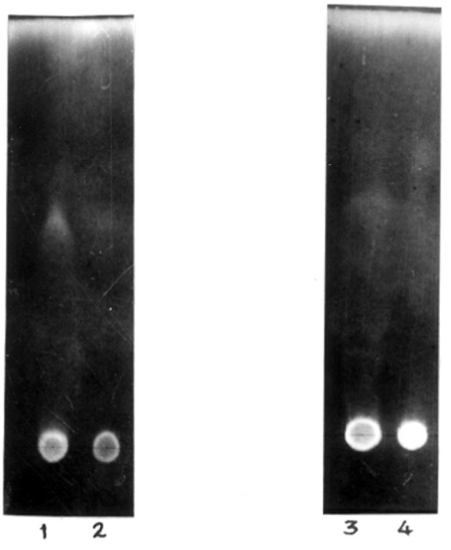

Fig V. 5 Paper chromatogram of the products released by *Bacillus* sp. PNGase on incubation with Dns-fetuin and Dns-soybean agglutinin glycopeptides.

The glycopeptides (20 μg reducing sugar) were incubated with ~1 μg of purified PNGase as described in Methods. Reaction mixtures containing **1)** Dns-fetuin glycopeptide and PNGase, **2)** Dns-fetuin glycopeptide and inactivated PNGase, **3)** Dns-soybean agglutinin and PNGase and **4)** Dns-soybean agglutinin and inactivated PNGase.

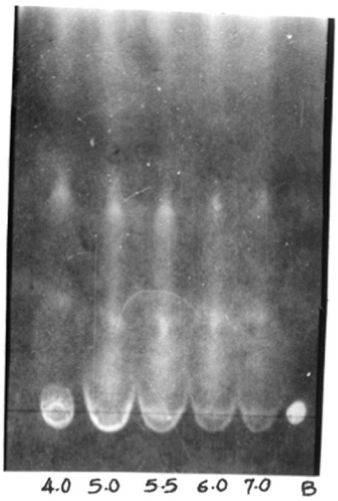

Fig V. 6 Effect of pH on the hydrolysis of Dns-fetuin glycopeptides by *Bacillus* sp. PNGase

Dns-fetuin glycopeptide (20 μg neutral sugar) was incubated with ~1 μg of purified PNGase in a total volume of 20 μl, at 50°C, for 18 h at different pH values as described in Methods.

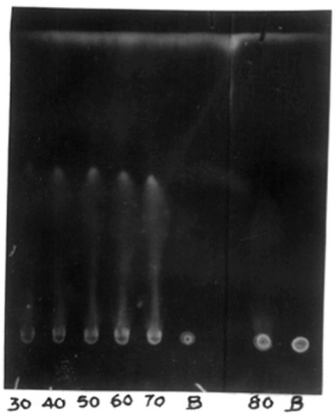

Fig V. 7 Effect of temperature on the hydrolysis of Dns-fetuin glycopeptides by *Bacillus* sp. PNGase

The glycopeptide (20 μg neutral sugar) was incubated with ~1 μg of purified PNGase in 10 mM Na-acetate buffer, pH 5.5, in a total volume of 20 μl, for 18 h at different temperatures.

Table V.1

Deglycosylation of glycoproteins, glycopeptides and oligosaccharides by the peptide: N-Glycanase from *Bacillus* sp. NCIM 5120

Substrate	Type of glycan structure hydrolyzed	% neutral sugar released, under		O.D 280 nm
		native conditions	on denaturation with 0.1 % SDS	
Fetuin	complex, triantennary	38.2	40.6	0.036
Desialated fetuin	complex, triantennary(-SA)*	31.3	35.0	0.023
Fibrinogen	complex, biantennary	38.5	40.5	0.025
Desialated fibrinogen	complex, biantennary(-SA)*	35.0	36.0	0.047
Holotransferrin	complex, biantennary	20.3	20.0	0.015
α_1-acid glycoprotein	complex, tetraantennary	19.2	20.0	0.020
Ovalbumin	heterogeneous	18.0	15.0	0.013
Ricinus communis agglutinin	high mannose	0	0	0.020
Soybean agglutinin	high mannose	0	0	0.018
RNase B	high mannose	0	0	0.034
		Relative ammonia released		
Fetuin glycopeptide (-SA)*		1.0		
Asn-GlcNAc-GlcNAc		0.7		
		p-nitrophenol released		
pNP-β-D-GlcNAc		0.423 pmoles / h / mg		

* (-SA) indicates desialated glycoproteins / glycopeptides

The optimum pH of the enzyme was 5.5 (Fig V.6) and it was stable in the pH range 5.0-8.0. This is comparable to the PNGases studied from various plant sources like jack bean seeds [17], almond emulsin [21], *Raphanus sativus* [15] and *Selina alba* [16] and the fungal glycoamidase from *A. tubigensis* [19] (optimum pH, 4.0-6.0). However, it is different from the *F. meningoscepticum* PNGase F, which has an optimum pH of 8.6 [10]. The pI of the *Bacillus* sp. peptide: N-Glycanase was 6.0. The optimum temperature was found to be 50°C (Fig V.7) and the enzyme exhibited considerable activity upto 70°C. The purified enzyme showed high temperature stability and retained its activity at 50°C for 24 h. In this respect it is comparable to the *A. tubigensis* PNGase At [19] which has an optimum temperature of 62°C and is relatively thermostable. Temperature optimum for the PNGases from other sources are between 30-40°C [10,17,21]. The relatively high optimum temperature and thermal stability of the *Bacillus* sp. peptide: N-Glycanase suggests its applicability in deglycosylation reactions at elevated temperatures.

The purified enzyme was free of proteolytic activity and did not exhibit any other exo-hydrolytic activity except for a negligible activity towards pNP-β-D-GlcNAc (Table V.1). The purified enzyme hydrolyzed Dns-Fetuin (-SA) glycopeptides, but not Dns-Soybean agglutinin glycopeptides (Fig V.5) indicating its specificity for complex oligosaccharide containing substrates. However, this does not reveal the linkage specificity of the enzyme since, both endo-N-acetyl-β-D-glucosaminidase as well as peptide-N^4-(N-acetyl-β-D-glucosaminyl) asparagine amidase yield products with increased mobility on paper chromatography. However, fluorimetric studies of the reaction products obtained with fetuin glycopeptides and the N-linked core disaccharide, Asn-GlcNAc-GlcNAc showed the presence of ammonia suggesting that the enzyme is a glycoamidase and not an endo-N-acetyl-β-D-glucosaminidase (Table V.1).

The substrate specificity of both endo-N-acetyl-β-D-glucosaminidase and the peptide-N^4-(N-acetyl-β-D-glucosaminyl) asparagine amidase are directed towards the invariant core region of the N-linked glycoproteins. However, the

endo-N-acetyl-β-D-glucosaminidase cleaves the β(1-4) linked di-N-acetyl-chitobiosyl bond generating a peptide backbone with one of the N-acetylglucosamine residue attached to asparagine and an oligosaccharide with the other N-acetylglucosamine residue at the reducing end [10]. The peptide-N^4-(N-acetyl-β-D-glucosaminyl) asparagine amidase is essentially an amidase and recognizes the glycosylamine linkage of the glycoprotein generating an aspartic acid on the peptide backbone and a 1-amino oligosaccharide. The 1-amino oligosaccharide spontaneously hydrolyzes non-enzymatically to the corresponding oligosaccharide with the liberation of ammonia. Therefore, the observed release of ammonia indicates that the *Bacillus* sp. enzyme is a peptidyl: N-Glycanase. Interestingly, the enzyme hydrolyzes both the Dns-Fetuin (-SA) glycopeptide and the core disaccharide, Asn-GlcNAc-GlcNAc, although at a lower rate (Table V.1) suggesting that it could be a L-asparagine amidase rather than a peptide-N^4- (N-acetyl-β-D-glucosaminyl) asparagine amidase. The peptide-N^4- (N-acetyl-β-D-glucosaminyl) asparagine amidases studied from various sources exhibit strict substrate specificity with respect to the polypeptide chain and require that the glycosyl asparagine residue be substituted on its amino and carboxyl terminus [38]. The L-asparagine amidase (Glycoamidase) is exactly opposite to PNGase in its peptide specificity and has an absolute requirement for a free amino and carboxyl terminus on the glycosylated asparagine moiety [38]. However, the present enzyme could hydrolyze Dns-Fetuin (-SA) glycopeptide and various glycoprotein substrates (Table V.1) suggesting it to be a peptide-N^4- (N-acetyl-β-D-glucosaminyl) asparagine amidase probably with a flexible polypeptide specificity.

The hydrolytic efficiency of the enzyme on various glycoproteins is given in Table V.1. The enzyme could liberate glycans from complex glycoproteins but not from high mannose glycoproteins. Further, biantennary and triantennary complex oligosaccharides were found to be better substrates than the tetraantennary complex oligosaccharides. Desialation of the terminal sialic acid residues did not have a significant effect on the catalytic efficiency. Further, denaturation of the substrates in presence of 0.1 % SDS did not enhance the

percent hydrolysis under similar conditions as for non-denatured substrates. The PNGases from bacteria, fungi, plants, and animal tissues (PNGase F [10], PNGase At [19], PNGase A [21], PNGase R [15], PNGase Se [16], N-Glycanase from jack bean seeds [17] etc.) are insensitive towards the oligosaccharide composition and can hydrolyze high mannose, hybrid and complex glycans with a preference towards complex triantennary type of glycoproteins. However, the *Bacillus* sp. peptide: N-Glycosidase is specific towards complex oligosaccharide containing glycoproteins and does not hydrolyze high mannose oligosaccharide containing glycoproteins.

In conclusion, the *Bacillus* sp. NCIM 5120 enzyme is a typical glycoamidase which shows preference for complex oligosaccharide containing glycoproteins. The enzyme appears to be flexible towards the polypeptide substitution. Furthermore, the relatively high optimum temperature and temperature stability of the enzyme will be useful in the deglycosylation of glycoproteins at elevated temperatures.

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CHAPTER VI

GENERAL DISCUSSION

Glycoproteins constitute a major protein profile in living systems. Covalently attached carbohydrate moieties to such glycoproteins have been proposed to perform central roles in a diverse array of biological processes such as, structure, food storage and utilization, cell-cell recognition and highly selective cellular signaling [1,2]. The enormous number of possible combinations of carbohydrate structures compared to peptides or nucleotides envisages their role in such specific functions [3]. A complete understanding of the precise roles of these cellular macromolecules would involve a detailed study of the glycan structure and branching pattern. The cellular degradation of such glycan structures is brought about by specific enzymes, the glycosyl hydrolases. Exo- and endo-glycosidases with strict glycone and aglycone specificities therefore serve as indispensable tools in studying the anomeric configuration, sequential arrangement of saccharides units in heterosaccharide chains and their possible functions. Heritable deficiencies in glycosyl hydrolases are the cause of a number of lysosomal storage diseases [4,5], the most frequently observed genetic disorder in man. The objective of the present investigation was directed at studying two important glycosyl hydrolases; an exo-N-acetyl-β-D-glucosaminidase and a peptide-N^4-(N-acetyl-β-D-glucosaminyl) asparagine amidase produced by the thermotolerant *Bacillus* sp. NCIM 5120. This discussion attempts at evaluating the position of exo-N-acetyl-β-D-glucosaminidase (NAGase) and the peptide-N^4-(N-acetyl-β-D-glucosaminyl) asparagine amidase (PNGase) in comparison with such enzymes studied from other sources.

The thermotolerant *Bacillus* sp. NCIM 5120 produced maximum levels of an extracellular exo-N-acetyl-β-D-glucosaminidase (25.4 U/L) when grown in a medium containing peptone (1 %, w/v), yeast extract (1 %, w/v), mannose (0.5 %, w/v) and Speakmans' salt solution. The productivity of *Bacillus* sp. NAGase is comparable to the β-hexosaminidase produced by *Bacillus stearothermophilus* CH-4 (24.83 U/L) [9] and is much higher than the exo-N-acetyl-β-D-glucosaminidase produced by *Bacillus subtilis* B (6.67 U/L) [6]. The NAGase has been purified to homogeneity using CM-cellulose, Sephacryl S-300 and phenyl-

Sepharose chromatography. The enzyme is a homotetramer with a subunit M_r 60,000. Substrate specificity studies indicate that the enzyme is a strict exo-N-acetyl-β-D-glucosaminidase and hydrolyses terminal, non-reducing β(1-4) linked GlcNAc residues exclusively. It did not hydrolyze β(1-4) linked GalNAc residues. With few exceptions most of the reported N-acetylglucosaminidases do not distinguish between β(1-4) linked GlcNAc and GalNAc residues and have therefore been classified as β-hexosaminidases. In this respect the *Bacillus* sp. NAGase is similar to the N-acetylglucosaminidases from *Bacillus subtilis B* [6], *Alteromonas* sp. Strain O-7 [7] and the *Serratia marcescens* chitobiase [8] which are also strict exo-N-acetyl-β-D-glucosaminidases.

The optimum temperature for activity was 70°C, one of the highest among the reported exo-N-acetyl-β-D-glucosaminidases. High temperature optimum has been observed in *Bacillus stearothermophilus* β-hexosaminidase [9] and in one of β-hexosaminidase isoforms (NAGase II) from *Beauveria bassiana* [10] (75°C and 57°C respectively). However, both the enzymes hydrolyze GlcNAc as well as GalNAc containing substrates from the non-reducing end.

The Arrhenius plot for the NAGase exhibits a biphasic or discontinuous plot with an inflection temperature of 50°C. As observed in other enzymes that exhibit discontinuous Arrhenius plots, the energy of activation (E_{act}) at temperatures below the inflection temperature (50°C) was found to be higher than that above the inflection temperature. Such biphasicity in the energy of activation for the exo-N-acetyl-β-D-glucosaminidases has not been reported so far.

Biphasic Arrhenius plot has been observed in membrane proteins [11] and in certain enzymes from thermophiles [12-15]. Various reasons for discontinuous Arrhenius plots have been postulated. In the membrane proteins the inflection temperature has been related to the melting temperature of the lipid fraction. Involvement of a phase change or the existence of two conformational states of the enzyme above and below the inflection temperature, each with a different catalytic competence has also been proposed for breaks in the slope of Arrhenius plots of biological systems [16,17]. However, far UV CD analysis of the present

enzyme at different temperatures (35°C - 70°C) showed no changes in the ellipticity, indicating the absence of any temperature related changes in the secondary structure of the enzyme. Further, a comparison of the energy of activation with different leaving groups (p-nitrophenol and 4-methyl-umbelliferone) reveal that at temperatures below the inflection point (50°C), the E_{act} is substantially higher for 4-Me-Umb-β-D-GlcNAc (60.3 k J / mol) than that for pNP-β-D-GlcNAc (43.2 k J / mol). However, at temperatures above the inflection point the energy of activation for both the substrates are comparable (21.7 k J / mol and 18.5 k J / mol). Determination of the reaction stereochemistry indicates that the NAGase is a retaining glycosidase with the β anomer of GlcNAc formed as the initial product. The proposed general catalytic mechanism for retaining glycosyl hydrolases involves the formation of an initial covalent glycosyl-enzyme intermediate and the hydrolysis of the saccharide takes place via formation of oxocarbonium ion-like intermediates [3,18,19]. This suggests that the observation of biphasic Arrhenius plot in NAGase is probably due to a change in the rate determining step. At temperatures below the inflection point the rate-determining step is the formation of the oxazolidine intermediate and at temperatures above the inflection point, its hydrolysis.

Henrissat has classified glycosyl hydrolases into 63 families based on the amino acid sequence similarities and at least one member from 24 of these families have been characterized with respect to their three dimensional structure [20-22]. The exo-N-acetyl-β-D-glucosaminidase / β-hexosaminidase have been classified under family 20 glycosyl hydrolases. Glycosyl hydrolases have been proposed to perform catalysis with two critical amino acid side chains: a proton donor and a nucleophile / base [3,18,23]. Few reports are available regarding the active site architecture of the exo-N-acetyl-β-D-glucosaminidase / β-hexosaminidase [24-27] and unlike other glycosidases the β-hexosaminidases and the exo-N-acetyl-β-D-glucosaminidases have been identified to possess only a single catalytic residue (carboxylate) with the absence of a putative nucleophile [24,25]. Our studies on the *Bacillus* sp. exo-N-acetyl-β-D-glucosaminidase

revealed the involvement of a single carboxylate residue as well as a single histidine residue in the catalytic activity of the enzyme. The retention in anomerism and the biphasic Arrhenius plot are suggestive of the reaction proceeding via a double displacement reaction mechanism involving an oxocarbonium intermediate. Although retention in anomerism has been observed in the *Serratia marcescens* chitobiase [28] and the jack bean β-hexosaminidase [29], the absence of a putative nucleophile has suggested an alternative mechanism in the transition state stabilization of these enzymes. A substrate assisted catalysis involving the C1 N-acetyl group of N-acetylglucosamine has been proposed and evidence for a similar phenomenon has been recently found [28-30]. The exo-N-acetyl-β-D-glucosaminidase from *Bacillus* sp. NCIM 5120 seems to differ from the reported β-hexosaminidases and N-acetylglucosaminidases in that it involves a second catalytic residue (histidine) along with a carboxylate. The function of the carboxylate residue would be to act as the acid catalyst and histidine probably functions as the nucleophile, although, a substrate assisted catalysis cannot be ruled out. The role of the histidine residue would then be in stabilizing the oxcarbonium intermediate. Further studies are required to assign such a role. The involvement of active site histidine as a general base has been demonstrated in the Group B *Streptococcal* hyaluronate lyase [31] and other hydrolases [32-34]. Chemical modification studies also show the involvement of a single tryptophan residue in substrate binding. Our studies also reveal that the active site Trp residue is a slow reacting residue. Involvement of tryptophan residues in the stacking of substrates in the active site of *Serratia marcescens* chitobiase [24] has also been observed. The involvement of active site tryptophan residues in the stacking of substrates in the active site of glycosyl hydrolases is a commonly observed phenomenon [35,36].

The binding constants observed for chitooligosaccharides (chitobiose > chitotriose > chitotetraose) are in concurrence with kinetic studies confirming the exo-hydrolytic nature of the enzyme and indicating its preference for low molecular weight substrates. *Bacillus* sp. NAGase contains 11 tryptophan residues

per subunit. From the present studies it appears that the major contribution towards the intrinsic fluorescence of the protein (74 %) is from four tryptophan residues. Solute quenching studies indicate that at least 2/3rd of the tryptophan residues are inaccessible to quenching with the neutral quencher, acrylamide and that the environment around tryptophan fluorophors is electropositive. Guanidine hydrochloride (Gdn.HCl) mediated denaturation indicates that the denaturation process is concentration dependent and maximum denaturation is observed at 3.0 M Gdn.HCl. The denaturation of the enzyme led to a decrease in the intrinsic fluorescence and an increase in the quenching of intrinsic fluorescence. The spectral characteristics of denatured NAGase suggest a change in the local environment of tryptophan fluorophors on denaturation accompanied by dissociation of the subunits and randomization of the protein backbone. Present studies also indicate that at least 2/3rd of the tryptophan residues in native NAGase are in the hydrophobic interior of the protein and do not contribute significantly to the intrinsic protein fluorescence.

Enzymes that hydrolyze the glycosylamine linkage of asparagine-linked glycans have been described previously. However, only a few PNGases from microbial sources have been characterized like the *Flavobacterium meningoscepticum* PNGase F [37] and the fungal glycoamidase PNGase At from *Aspergillus tubigensis* [38]. The *Bacillus* sp. peptide-N^4-(N-acetyl-β-glucosylaminyl) asparagine amidase has been purified by sequential chromatography on ion-exchange chromatography and gel filtration. It has a native M_r of 58,000 and exhibited 2 non-identical bands of M_r 15,000 and 17,000 on SDS-PAGE indicating the heterotetrameric nature of the enzyme. The reported PNGases exhibit wide variations in their molecular mass and subunit composition and the *Bacillus* sp. PNGase does not seem to resemble any of the reported enzymes. Instead it resembles the L-asparaginases in the αβ nature and subunit size [39]. The enzyme could hydrolyze Dns-Fetuin (-SA) glycopeptides, various complex oligosaccharide containing glycoproteins and the N-linked core disaccharide, Asn-GlcNAc-GlcNAc, indicating the flexibility of the enzyme

towards the polypeptide substitution. The percentage hydrolysis observed for various complex oligosaccharide containing glycoproteins varied (fetuin 38.2 %, desialated fetuin 31.3 %, fibrinogen 38.5 %, desialated fibrinogen 35.0 %) but high mannose containing glycoproteins were resistant to hydrolysis indicating the preference of the *Bacillus* sp. PNGase towards complex oligosaccharide containing glycoprotein substrates. Desialation or denaturation of the glycoproteins did not have a profound effect on the percent hydrolysis. The PNGases from *Flavobacterium meningoscepticum* [37,40], *Aspergillus tubigensis* [38], jack bean seeds [41,42], almond emulsin [43,44] and *Raphanus sativus* [45] hydrolyze all types of glycan structures like high mannose, hybrid and complex oligosaccharides although the complex triantennary glycoproteins are the preferred substrates. However, they exhibit restricted specificity towards the polypeptide substitution and prefer oligosaccharides substituted with bulky peptide chains. The restricted oligosaccharide specificity and the flexibility towards the polypeptide substitution of the *Bacillus* sp. PNGase coupled with its relatively high optimum temperature and temperature stability can be of use in the selective deglycosylation of complex oligosaccharide containing glycoproteins.

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CONCLUSIONS

1. *The thermotolerant Bacillus sp. NCIM 5120 produces an extracellular exo-N-acetyl-β-D-glucosaminidase (E. C. 3.2.1.30) and a peptide-N^4-(N-acetyl-β-glucosylaminyl) asparagine amidase (E. C. 3.5.1.52).*

2. *The exo-N-acetyl-β-D-glucosaminidase is a retaining β-glycosidase and exhibits strict substrate specificity in hydrolyzing terminal β(1-4) GlcNAc containing substrates.*

3. *The optimum temperature for the activity of NAGase is 70℃, one of the highest among the exo-N-acetyl-β-D-glucosaminidases reported so far.*

4. *The enzyme exhibits a biphasic Arrhenius plot with two characteristic energy of activation with an inflection temperature of 50℃. The energy of activation at temperatures below the inflection point (50℃) is higher than that above the inflexion temperature.*

5. *A comparison of the energy of activation with different leaving groups (p-nitrophenol and 4-Methyl-Umbelliferone) suggests that the enzymatic hydrolysis probably proceeds by a double displacement reaction via formation of an oxazolidine intermediate.*

6. *Active site characterization indicates the participation of a single tryptophan in substrate binding and that the substrate binding tryptophan is a slow reacting residue.*

7. *A single histidine and a carboxylate residue are involved in the catalytic activity of the enzyme.*

8. *Binding constants observed for chito-oligosaccharides are in accordance with kinetic data suggesting that chitobiose is a better substrate than chitotriose and chitotriose is a better substrate than chitotetrose indicating the enzymes' preference for low molecular weight substrates and its exo-hydrolytic nature.*

9. *Fluorescence studies indicate that at least 2/3rd of the Trp residues in NAGase are present in a rigid, highly hydrophobic environment and do not contribute significantly to the intrinsic fluorescence of the enzyme.*

10. *Solute quenching studies indicate that the accessible fluorophors are in a electropositive environment.*

11. *The peptide: N-Glycanase is a specific N-glycosidase, which hydrolyzes complex oligosaccharide containing glycoproteins but not high mannose oligosaccharide containing glycoproteins and exhibits flexibility towards the polypeptide substitution.*

List of Publications

1. Characterization of a novel exo-N-acetyl-β-D-glucosaminidase from the thermotolerant *Bacillus* sp. NCIM 5120.

   B. Amutha, J. M. Khire and M. I. Khan, (1998) **Biochim. Biophys. Acta,** 1425 (2), 300-310.

2. Active site characterization of the exo-N-acetyl-β-D-glucosaminidase from thermotolerant *Bacillus* sp. NCIM 5120: involvement of tryptophan, histidine and carboxylate residues in catalytic activity. (Communicated)

3. Quenching of intrinsic fluorescence of exo-N-acetyl-β-D-glucosaminidase: Substrate binding and tryptophan microenvironment. (Manuscript under preparation)

4. Thermostable peptide-N^4-(N-acetyl-β-glucosylaminyl) asparagine amidase from *Bacillus* sp. NCIM 5120: Purification and Characterization (Manuscript under preparation)