PECTINASES FROM MOLDS, THEIR PRODUCTION, PURIFICATION, CHARACTERIZATION AND APPLICATION

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by

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DECLARATION

Certified that the work incorporated in the thesis entitled "Pectinases from molds, their production, purification, characterisation and application" submitted by M. Narsimha Rao was carried out under my supervision. Such material as has been obtained from other sources has been acknowledged in the thesis.

(Dr. Aditi Pant) 18/3

Research Guide

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CONTENTS

SUMMARY

One of the important group of polyaccharidases are the pectin degrading enzymes. Pectinases are a complex group of enzymes, acting on pectins or pectic materials. Over the last 35 years, a number of literature reports and patents have dealt with pectinases from various sources, with respect to their importance in plant physiology, plant-microbe interactions, microbial ecology and the ever-growing scope of their industrial applications. Although some pectinolytic enzymes have been cloned and characterized, more studies are warranted in order to characterize these enzymes, with respect to their catalytic mechanisms and thus be able to obtain a clearer over-view of the significance of this group of enzymes.

The work presented in this thesis is the study of two types of pectin degrading enzymes :

1. Pectate lyase from *Fusarium moniliforme*

2. Endo-polygalacturonase from *Aspergillus ustus*

Chapter 1

General introduction: Pectin degrading enzymes and their characteristics

In this chapter the characteristics of pectin degrading enzymes have been discussed with respect to their catalytic and functional features in comparison with other polysaccharidases in general. It also comprises of a literature survey of pectin degrading enzymes from various sources.

Chapter 2

Induction, purification and characterization of pectate lyase from *Fusarium moniliforme*

During screening for pectinase producers from estuarine and marine environments, a strain of *Fusarium*, later identified morphologically as *Fusarium moniliforme*, was found to produce extracellular pectate lyase (EC 4.2.2.2) and polygalacturonase (EC 3.2.1.15) at alkaline and acidic pH respectively. Both enzymes were strongly induced at their respective pHs in the presence of 1% pectin. This is the first report of a pectate lyase produced by *F.moniliforme*. This organism also showed the production of other polysaccharidases, like endoglucanase

(EC 3.2.1.4), xylanase (EC 3.2.1.8) and amylase (EC 3.2.1.1), when induced by appropriate substrates.

The pectate lyase from *Fusarium moniliforme* was purified to homogeneity by affinity chromatography followed by gel filtration with a yield of 76.5%. Atomic emission spectrometry showed that Ca^{++} was a part of the holo-enzyme held by carboxyl groups of the protein. Loss of Ca^{++} was observed by treatment with EGTA or carboxyl modifying Woodward's reagent K, with subsequent loss of enzyme activity. Kinetic studies and tryptophan fluorescence quenching showed that Ca^{++} was not required in substrate binding.

Chemical modification and substrate-protection studies showed the presence of lysine and tryptophan at or near the active site of the pectate lyase. Chemically modified enzyme showed no major structural changes as determined by circular dichroism.

Amino acid analyses of native, lysine modified and substrate protected enzyme showed that a single lysine residue was present at active-site. Kinetic analysis of partially-inhibited enzyme together with substrate-affinity studies showed that tryptophan was essential for substrate binding, whereas lysine is involved in the catalysis. Fluorescence quenching further confirmed the involvement of tryptophan in substrate binding. The pectate lyase acts on polygalacturonic acid in a typical ß-eliminative fashion. It is suggested that a concerted nucleophilic-electrophilic attack, by the lysine residue in the active-site together with calcium, on the substrate catalyzes the breaking of the α-1,4-glycosidic linkage by the pectate lyase.

Chapter 3

Characterization of endo-polygalacturonase from *Aspergillus ustus*: Elucidation of enzyme mechanism

The enzyme endo-polygalacturonase (poly (1,4-α-D- galacturonide) glycanohydrolase, EC 3.2.1.15) from *Aspergillus ustus* was purified to homogeneity. The endo-polygalacturonase had a molecular weight of 36,000 daltons, a pI of 8.3, specific activity of 785 units/mg., Km of 0.82 mg/ml, and Vmax of 976 micromoles of product min^{-1}, mg^{-1}. Amino-acids involved in the catalysis were identified by

chemical modification. Kinetic constants of partially inhibited enzyme, suggest the involvement of tryptophan in substrate binding and histidine in catalysis. Quenching of tryptophan fluorescence of the enzyme in the presence of polygalacturonic acid, substantiated the conclusion that tryptophan was involved in substrate binding.

The mechanism of action of the endo-polygalacturonase was studied using isotope-effect. The proposed mechanism involves a single proton transfer from the histidine residue of the enzyme to the glycosidic oxygen and subsequent hydrolysis by the addition of a water molecule.

Chapter 4

Production and industrial application of endo-polygalacturonase from *Aspergillus ustus*

In order to develop a process for production, the culture conditions and the media constituents require to be optimized. The optimization of each variable singularly or in conjunction with other variables should lead to an overall process in which maximum yield is obtained using cheap and easily available raw materials.

The present work has been carried out on *Aspergillus ustus* NCIM 1033, a species which has not been previously studied for its production of pectin-degrading enzymes.

In addition to the traditional use of pectinases in food industry, these enzymes are also studied for their use in degumming of natural fibres. For treatment of cellulosic fibres, enzyme preparations free of cellulases would be a major advantage.

A cellulase-free polygalacturonase preparation could be obtained from *Aspergillus ustus* by using cheaply available orange pulp. The optimum conditions for production of the enzyme were obtained by varying the medium constituents and fermentation parameters such as agitation speed, medium pH etc. The enzyme was found to highly stable at 30 °C and in a wide range of pH and hence being advantageous in industrial usage.

A stable mutant strain was obtained by UV-exposure yielding high levels of polyglacturonase activity.

Further process development was done by solid-state fermentation. An high-activity polygalacturonase preparation was obtained by fermentation of *Aspergillus ustus* M26 in medium containing 40 g. wheat bran, 2 g. dried orange pulp and 80 ml. water with 0.5% ammonium nitrate and 0.4% dipotassium hydrogen phosphate.

The enzyme was checked for its juice clarification properties, using apple and citrus juices. It clarifies the juices at a 0.1% concentration of enzyme in one hour.

The endo-polygalacturonase was immobilized on aminated silica gel, in order to increase its applicability. The efficiency of the immobilized enzyme was 28%. Among the other properties studied, the immobilized enzyme was found to have an increased thermostability over the free enzyme.

Chapter 5

General Discussion

The salient features of the thesis have been discussed with respect to the aim and scope of the present work and thereby suggesting future course of investigations.

CHAPTER 1

GENERAL INTRODUCTION: PECTIN DEGRADING ENZYMES AND THEIR CHARACTERISTICS

Polysaccharides are macromolecules occurring in almost all living organisms, and are one of the largest group of all natural compounds. They function as energy sources or as structural units in the morphology of the living material of which they are a part. Polysaccharides are either homoglycans as in the case of amylose, amylopectin, glycogen, cellulose, xylan, mannans, pectin etc. or heteroglycans such as agar, rhamnogalacturonan, heparin, and hyaluronic acid (Aspinall 1983). Alternatively they can be also classified into neutral polysaccharides such as soluble amylose or glycogen and insoluble cellulose, and acidic soluble polysaccharides like pectin, alginic acid, chondroitin sulfate, heparin and hyaluronic acid. Monomeric sugars of the polysaccharides are glycosidically linked between the hydroxyl group at C-1 and an available hydroxyl group of a second monomer. Matheson (1985) suggested that the conformation of the linkage in a polysaccharide such as amylose, which is (1,4)-equatorial-axial, leads to a helical conformation which increases its water solubility and gives a random coil structure in solution. On the other hand a number of homopolymeric polysaccharides that are (1,4)-ß-D-linked have both bonds in the glycosidic linkage, equatorial to the pyranose rings, such as (1,4)-ß-D-mannan, cellulose and (1,4)-ß-D-xylan, and this leads to a characteristic ribbon-like preferred conformation, rendering the polysaccharide insoluble.

An important group of acidic polysaccharides are pectins or pectic substances, commonly found in the intercellular middle lamellae and in the primary cell walls of plant. Pectic polysaccharides or pectins, normally occur in plants in the form of insoluble protopectin, in which the pectin chains are linked with other cell wall components such as cellulose, hemicellulose and lignin. The monomer sugar, D-galacturonic acid, is linked by α-(1,4) glycosidic bonds to form the polymer, Polygalacturonic acid. Polygalacturonic acid, wherein the carboxylate groups are methoxylated is known as pectin. The carboxylic acid groups may be methylated to various degrees, often termed as the degree of esterification. Pectins with degree of esterification greater than 50% are termed as high-methoxyl pectins and those with degree of esterification less than 50% are the low- methoxyl pectins.

Enzymatic degradation of polysaccharides occur either hydrolytically in proton-rich medium or through transeliminative cleavage in basic medium. Hydrolytic cleavage occurs without any major structural changes in the reacting sugar unit. The anomeric configuration at the glycosidic carbon may be retained as

in lysozyme, ß-galactosidase (equatorial-equatorial), α-amylase (axial-axial), or inverted as in the case of ß-xylosidase, cellobiohydrolase (axial-equatorial), ß-amylase and trehalase (equatorial-axial) (Sinnott 1990). The resulting sugars have a free hemiacetal hydroxyl group. Transeliminative cleavage on the other hand, which occurs in a basic medium, causes structural changes in the sugar moiety directly involved in the degradation and leads to the formation of conjugates having a terminally linked 4,5-unsaturated 4-deoxyhexopyranuronate residue, as in the degradation of pectin or chondroitin (Kiss 1974).

In the strictly chemical sense, hydrolytic cleavage is an acid-catalyzed reaction at the glycosidic bond of neutral polysaccharides and is estimated to be a slow reaction which occurs in acid concentrations of 0.01 to 3 N and temperatures between 20° to 100° C (Bochkov 1979). The accepted model for the mechanism, where the acid is 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 the carbonium-oxonium ion with the formation of a reducing end-group and a proton (Smidsrod 1966). The same mechanism has been suggested for biochemical enzymatic hydrolysis of polysaccharides where either carboxyls or histidine of the enzyme functions as a proton-donor. Using kinetic isotope effect studies with ^{18}O and ^{2}H-substituted substrate, enzyme or solvent, the mechanism of action of various enzymes such as exo-ß-(1-3)-D-glucanase (Jeffcoat 1987), ß-glucosidase (Umerzurike 1981), α-L-aribinofuronidase (Selwood 1989), and ß-galactosidase (Rosenburg 1981) have been shown to be a general acid catalysis.

At the molecular level, active-site characterization of the glycosidases, has identified those residues essential for polarization of the glycosidic oxygen in the general acid-hydrolysis mechanism. Matsuura (1984) had identified a glutamic acid and an aspartic acid residue in the structure of Taka-amylase to be catalytically important. Henrissat (1989) also identified aspartic and glutamic acid residues as being actively involved in catalysis, during the amino acid sequence analysis of different 21 ß-glucanases. A glutamic acid residue was identified in the sequence alignments of 28 different cellulase sequences to be essential for enzyme action. Asn-Glu-Pro sequence is highly conserved in the endo-glucanases and it was suggested by the same author that this peptide unit may be involved in pyranose

binding. Lysozyme was also found to contain an aspartate and glutamate in its active site (Anand 1988). Although generally speaking acidic residues have been found to be functionally important in the process of catalysis by various carbohydrases, Jeffcoat (1987) implicated a histidine residue in an exo-ß-glucanase, and a yeast α-glucosidase (Someya 1984) also contained an essential histidine residue in its active-site.

Henrissat (1991) had suggested a classification of glycosyl hydrolases on the basis of their amino acid sequences. He classified 301 hydrolases corresponding to 39 EC entries into 35 families. Enzymes of one family are likely to have similar folding characteristics and active-site morphology. Henrissat (1989), by comparison of amino acid sequences of 21 ß-glucanases, had identified six families on primary structure homology. Svennson (1988) had earlier suggested that amylase, α-glucosidases and transglucanosylases acting on 1,4 and 1,6-α-glucosidic linkages share key structural features in the active-centres. The structure of glycosyl enzymes suggest a common domain motif. The crystal structure of porcine pancreatic α-amylase (Buisson 1987) showed three domains. The larger, N-terminal domain consists of parallel stranded α-ß barrel structure. The C-terminal domain forms a distinct globular unit, where the chain folds into an eight-stranded anti-parallel ß-barrel. The third domain is essentially composed of anti- parallel ß-sheets. MacGregor (1989) had used the predictions of protein secondary structure with amino acid sequence alignments to show that the N-terminal domains of cylcodextrin glucanotransferases and a yeast α-glucosidase, all catalyzing the cleavage of α-1,4-glucosidic bonds, may have the same super- secondary structure as α-amylases, i.e. an $(\alpha/ß)_8$-barrel fold, differing mainly in the loops linking the regular secondary structures.

The eight fold-α/ß barrel conformation is common in 2-keto-3-deoxy-6-phosphogluconate aldolase (Marvidis 1982), chicken muscle triose phosphate isomerase (Banner 1975), cat muscle pyruvate kinase (Stuart 1979) and D-xylose isomerase (Carrel 1984). Lebioda *et al* (1982) used the published data on structure and folding of three carbohydrases; aldolase, pyruvate kinase and triose phosphate isomerase and suggested that protein folding patterns may have significance in molecular evolution. They have presented arguments suggesting both molecular

convergence to a stable protein fold and also evolutionary divergence from an ancestral protein.

The occurrence of enzymes, such as the α-amylases, pyruvate kinase, aldolase and the xylose isomerase, with widely differing function, from widely differing sources but similar super-secondary structure, supports the view that enzymes can converge to a common, limited number of stable protein folds, probably convenient to many catalytical functions (Lebioda 1982). The crystal structure of pectate lyase of *Erwinia chrysanthemi* (Yodder 1993) and *Bacillus subtilis* (Pickersgill 1994) showed that this enzyme folds into a unique motif of ß-sheet barrel. It was suggested by Yodder *et al* (1993) that the stability of pectate lyases was due to this ß-barrel structure. The rationales for both convergent and divergent evolution of carbohydrases are at present equivocal. However, it is interesting to note the similarity in domain structure between pectate lyase (Yodder 1993, Pickersgill 1994) and α-amylase (Buisson 1987) where both proteins have a barrel motif and both cleave a glycosidic bond, although each uses a different mechanism for the actual catalysis. Thus two enzymes, with different functions, both having a highly stable barrel structure can advance the claim for convergent molecular evolution.

Transeliminative cleavage, such as shown by pectate lyase, is involved in the enzymatic degradation of acid polysaccharides such as pectins, algins or heparin, ubiquitously found in plants, animals and micro-organisms. Algin is a linear polymer, comprised of 1,4-linked ß-D-mannuronic acid and ß-D-guluronic acid. Heparin, hyaluronic acid and chondroitin sulfate are all glycosaminoglycans, commonly found in animals. Enzymes such as alginate lyases (EC 4.2.2.3), pectin lyases (EC 4.2.2.10) and pectate lyases (EC 4.2.2.2) which act on polyuronic acids, predominantly found in plants have been reported, as also enzymes such as chondroitin sulphate lyases, hyaluronate lyases and heparin lyases (Linnhardt 1986).

Polysaccharide lyases depolymerize acidic carbohydrates through ß-eliminative cleavage by abstraction of a proton. The abstraction of a proton from the α-carbon atom is assisted by the electron-withdrawing carboxyl group of these polysaccharides. Abstraction of the proton either enzymatically or by a chemical base such as NaOH (Albersheim 1960), results in a direct eliminative cleavage

forming an α-ß- unsaturated uronic acid residue on the non-reducing end and a hemiacetal on the reducing-end of the glycoside linkage.

Polysaccharide lyases have been purified and characterized from various sources (Yamagata 1968, Routela 1973, Elyakova 1974, Michelacci 1975, Linn 1983 and Yang 1985). Alginate lyase of *Littorina* (Elyakova 1974) consisted of six isoforms, one of which was purified and shown to have a molecular mass of 40,000 and a pI of 7.0. The chondroitin lyase from *Flavobacterium heparinum* (Michelacci 1975) had a molecular mass of 80,000 and a pI of 7.0, whereas the hyaluronate lyase from *Staphylococcus aureus* (Rautela 1973) consisted of two isoforms of pI 7.4 and 7.9 and having a molecular mass of 84,000. These enzymes have low to intermediate molecular masses ranging from 20,000 to 110,000 daltons, and are characteristically monomeric. Several of them are glycoproteins, whereas most of them are cationic. These lyases have pH optima for activity above 6.0, and most of them act endo-lytically. Though polysaccharide lyases have been purified and their preliminary characterization reported, there are no studies on the active-site characterization of these enzymes, therefore the residues essential for activity have not been identified.

An understanding of hydrolytic and transeliminative modes of enzyme-catalyzed reactions is fundamental to the study of pectin-degrading enzymes, which form a substantial family by themselves. Pectin-degrading enzymes act on methoxylated polygalacturonic acid (pectin) or non-methoxylated polygalacturonic acid (pectic materials). Polygalacturonic acid is the only substrate known to be degraded by two different groups of enzymes, one acting in a hydrolytic fashion (polygalacturonase) and the other acting in a transeliminative manner (pectate lyase)

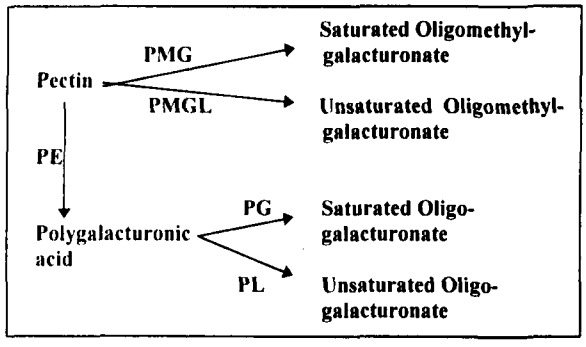
The classification of the pectinases is based on the mechanism of their action. Enzymes acting on pectin or pectic materials are basically of two types: esterases and depolymerases. Pectin esterase catalyses the demethoxylation of pectin to form polygalacturonic acid. The depolymerases are further distinguished by their mode of action and the type of substrate preferred. They fall into two classes, I) Hydrolases and II) Lyases. The classification of the pectic enzymes is illustrated in Table 1.1.

Table 1.1

Classification of Pectinases

Esterases

Pectin esterase or pectin methy hydrolase (PE) Ec 3.1.1.11
Deesterification of methoxy groups of pectin.



Depolymerases

I) Hydrolytic cleavage

Acting on pectin

(a) Endo-polymethylgalacturonase (PMG), EC 3.2.1.4
Random cleavage of α-1,4-glycosidic links of pectin

(b) Exo-polymethylgalacturonase: Causes sequential cleavage of α-1,4-glycosidic links of pectin from the non-reducing end

Acting on polygalacturonic acid

(a) Endo-polygalacturonase (PG) EC 3.2.1.15 : Random cleavage of α-1,4-glycosidic links of pectin from the non- reducing end

(b) Exo-polygalacturonase EC 3.2.1.40 : Hydrolyses in sequential manner α-1,4-glycosidic links

II) Transeliminative cleavage

Acting on pectin

(a) Endo-polymethylgalacturonate lyase (PMGL) EC 4.2.2.10
Causes random cleavage of α-1,4 links of pectin by a β-elimination process resulting in galacturonide esters with unsaturated bonds between C-4 and C-5 at the non-reducing end of the fragment formed

(b) Exo-polymethylgalacturronide lyase : Causes step-wise breakdown of pectin in a β-eliminative fashion

Acting on polygalacturonic acid

(a) Endo-polygalacturonate lyase or pectate lyase (PL), EC 4.2.2.2 : Causes random cleavage of α-1,4-glycosidic links of polygalacturonic acid in a β-eliminative fashion

(b) Exo-polygalacturonate lyase EC 4.2.2.9 : Causes sequential breakdown of polygalacturonic acid in β-eliminative fashion

The mode of action of the various pectinases is illustrated in Fig. 1.1. The hydrolases (PG and PMG) cleave polygalacturonic acid or its methoxylated form in a manner apparently similar to that of other glycosidases acting on neutral polysaccharides. Pectate lyase (PL) and pectin lyase (PMGL) catalyze a typical ß-elimination, common to enzymes acting on acidic polysaccharides (Linhardt 1986).

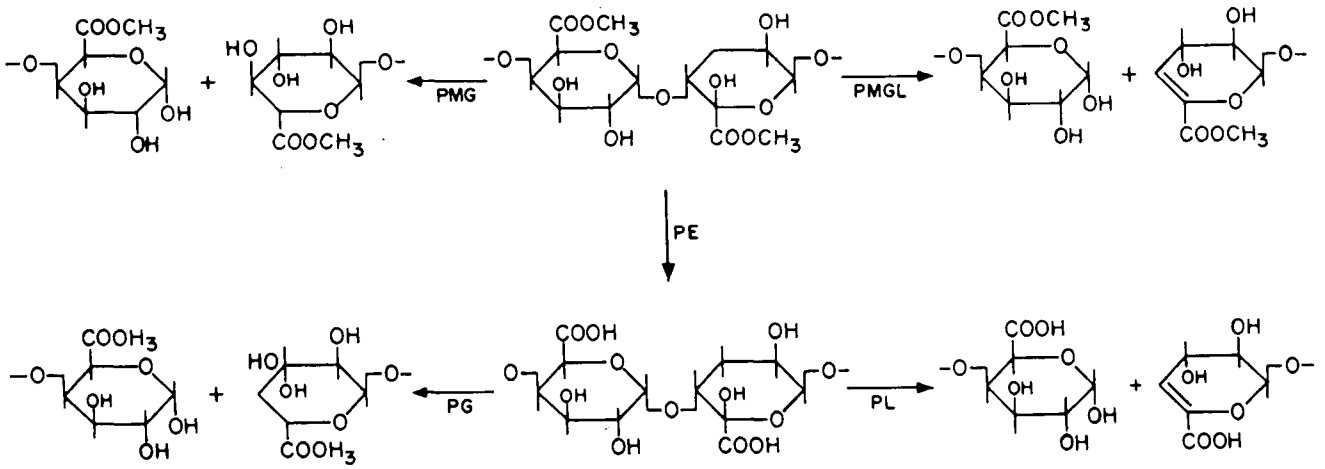
Polygalacturonases have been purified using traditional methods of protein purification. Initial ammonium sulphate or organic solvent concentration followed by ion exchangers such as DEAE-Cellulose, Ecteola-cellulose, CM- Sephadex, or gel filtration have been routinely used. Affinity chromatography has been extensively and profitably used in these purification procedures, where polygalacturonic acid or its derivatives have been linked to various matrices. For example polygalacturonic acid has been linked to alkylamine-controlled pore glass, keratin and polyamide coated silica gel, and acrylonitrile-divinylbenzene copolymer to purify *A.niger* enzymes (Lobarzewski 1985, Ginalska 1988). Cross-linked pectic acid has also been used to purify pectic enzymes although this is less sturdy than ligand-linked matrices. Further, the fact that the volume changes according to salt concentration also makes it less attractive as a purification procedure. Some of the enzymes purified using pectic acid cross-linked with epichlorohydrin are endo-polygalacturonase from *A.niger* (Rexova-Benkova 1972), pectinesterase from orange (Rombouts 1979) and endo-polygalacturonase from *Kluveromyces fragilis* (Inoue 1984).

Among the hydrolytic enzymes polygalacturonases catalyse the hydrolysis of polygalacturonic acid either randomly as in the case of endo-polygalacturonases or sequentially as done by the exo-polygalacturonases. There are two methods to assay polygalacturonase. The first is by measuring the increase in reducing sugars produced by the action of the enzyme on the substrate, where reducing sugars are estimated by the Nelson- Somogyi method as described by Collmer (1988). The second is by assay of the rate of viscosity drop caused by endo-polygalacturonase (Endo 1961). The first method is generally preferred because the viscosity drop measurement sometimes leads to ambiguous results.

Endo-polygalacturonases have been purified from various sources and their preliminary characterization reported (Table 1.2). Most of the endo-polygalacturonases have an optimum pH between 4.0 to 5.0, optimum temperature

Figure 1.1

Mode of action of pectinases



between 30° to 40° C and molecular weight around 30,000 daltons. The isoelectric points of these enzymes are generally between 3.0 to 6.5. Increased methylation of the carboxylic groups results in decrease in rate and extent of cleavage. The rate of hydrolysis also decreases with the shortening of the polymer chain (Waksman 1992).

Table 1.2

Characteristics of endo-polygalacturonases from various sources

Source		Molecular Weight	pH Optimum	pI	Reference
Aspergillus japonicus	I	25000	4.7		Baldwin, 1989
	II	36500	4.0		
Aspergillus nidulans		40000		4.2	Dean, 1989
Aspergillus niger	I	85000	4.9	3.2-3.5	Kester, 1990
	II	38000	4.8	4.6-4.9	
	IIIA	57000	4.3	3.3	
	IIIB	57000	4.5	3.3	
	IV	59000	4.8	3.7	
Crytococcus albidus		45000	5.0-6.0	6.8	Tanabe, 1988
Geotrichum candidum		34500	5.0	6.8	Shastri, 1988
Kluyveromyces fragilis	A	37700		6.2	Inoue, 1984
	B	39800		6.2	
	C	39800		5.8	
Kluyveromyces marxianus	I	49600		6.3	Barnby, 1990
	II	49600		6.0	
	III	46500		6.3	
	IV	49600		5.7	
Neurospora crassa		37000	6.0		Polizeli, 1991
Rhizopus stolonifer		57000	5.0		Manachini, 1987
Saccharomyces fragilis	I	46000	4-5	6.1	Lim, 1980
	II	50000	4-5	6.1	
	III	30000	4-5	5.8	
Trichoderma kaningi	I	32000		6.41	Fanelli, 1978
	II	32000		6.57	

Some reports have described chemical modification studies to identify essential residues in catalysis of this group of enzymes, although a detailed characterization has not been described. Rexova-Benkova (1968) and Cooke (1976) have reported the importance of histidine in the catalysis of endo-polygalacturonase. Histidine has also been implicated in the active-site of other hydrolases, such as exo-glucanase (Ohno 1989), amino-peptidase (Helene 1991) and S1 nuclease (Gite 1992). Waksman *et al* (1992) used substrate fluorescence quenching to investigate reaction kinetics in the enzyme produced by *Colletotrichum lindemuthianum* and suggested the presence of tryptophan in its active-site. Urbanek and Sobczak (1975), through chemical modification had implicated cysteine and tyrosine in the active-site of polygalacturonase from *Botrytis cinera*.

Comparison of the sequences of various glycosidases showed (Henrissat 1991) that polygalacturonases shared no significant homology with other glycoside hydrolases.

Polygalacturonases have been extensively used in the food industry, especially in fruit juice clarification.

Enzymes catalyzing transeliminative cleavage such as pectate lyases have been purified using general chromatographic techniques similar to those used for polygalacturonases. A pectate lyase from *Bacillus stearothermophillus* was purified by covalently linking polygalacturonamide to cynogen bromide activated Sepharose (Karbassi 1980). Ward and DeBoer (1987) had described a rapid method of purification of pectate lyase from *Erwinia caratovora* based on substrate affinity. The enzyme was co-precipitated with polygalacturonic acid in the presence of calcium ions and then eluted out with the addition of salt.

Transeliminative cleavage catalyzed by lyases result in sugar units with a double bond between C-4 and C-3, which absorb at 235 nm. and therefore the lyase activity can be measured by the increase in absorbance at that wavelength (Collmer 1988). The unsaturated products reacts with thiobarbituric acid forming a red-coloured complex, having an absorption maximum at 545 nm. An assay procedure involving reaction with thiobarbituric acid has also been described (Albershiem 1960).

Some of the characteristics of pectate lyases have been listed in Table 1.3. These enzymes act on polygalacturonic acid and have an optimum pH in the alkaline range, from pH 8.0 to 10.0. The molecular masses of the reported enzymes range from 23 kDa to 74 kDa and their isoelectric points from 4.6 to 10.3. All pectate lyases require calcium ion for enzyme activity, although in some cases they are activated to an extent by other metal ions such as Mg^{++}, Ba^{++} and Sr^{++}.

Table 1.3

Characteristics of pectate lyases from various sources

Source		Molecular weight	pH optimum	pI	Reference
Bacillus macerans		35000	9.0	10.3	Miyazaki, 1991
Bacillus stereothermophillus		24000	9.0		Karbassi, 1980
Bacteroides thetaiotaomicron		74000	8.7	7.5	McCarthy, 1985
Cladosporium cucermerinum		27000	9.7		Robertson, 1990
Erwinia caratovora	I II	41000 44000		9.1 9.4	Lei, 1985
Erwinia chrysanthemi	I II III	33000 33000 33000	8.4 8.5 8.0	9.4 9.0 7.8	Bagley, 1979
Fusarium oxysporium	I II	25000 37000			Artes, 1990
Fusarium solani		26000	9.4	8.3	Crawford, 1987
Klebsiella oxytoca	I II	71000 71000	9.0 8.5	5.9 5.3	Pitkanen, 1992
Streptomyces nitrosporeus		41000	10.0	4.6	Sato, 1980
Yersinia enterocolitica		55000	8.8	5.8	Bagley, 1979

Polygalacturonic acid forms gels in presence of calcium. Morris *et al* (1982) had suggested that cooperative binding of calcium to polygalacturonic acid occurs through "egg-box" complexes, and had provided evidence that the primary mechanism of calcium-induced association of polygalacturonate chain sequences is

by dimerization. Crawford *et al* (1987) observed that a ratio of 0.24 of polygalacturonic acid to calcium is required for optimum activity, whereas chain dimerization occurs at a ratio of 0.5 (Morris 1982). Crawford *et al* (1987) suggested that chain dimers are likely to exist at less than the stoichometric ratio, and such dimers of polygalacturonic acid form the actual substrate for the pectate lyase.

The evaluation of the crystal structure of pectate lyase of *Erwinia chrysanthemi* by Yodder *et al* (1993) showed that the enzyme folds into unique motif of parallel ß- strands, folded into a tertiary helix. Because of their sequence and functional similarities, these authors have suggested that all extracellular pectate lyases will have similar structure, differing only in the size and shape of the protruding loops. Although Yodder *et al* (1993) could not use calcium in their crystallization solution, they identified a common site on the protein, where heavy metals ions such as lutetium, lead and uranyl were bound. This site containing three acidic residues, Asp^{131}, Glu^{166} and Asp^{170}, was said to be the putative calcium binding site. Pickersgill *et al* (1984) have crystallized the pectate lyase of *Bacillus subtilis*, complexed with calcium, and have identified a calcium binding site. The calcium was shown to bind to acidic groups of glutamate and aspartate at the bottom of the cleft formed between the ß-helix domain and the loops in the crystal structure.

Pectate lyases have not been exploited for industrial application, though studies have been reported implicating their importance in retting of bast fibres (Tanabe 1986).

Pectate lyases, which act on polygalacturonic acid, are known to require calcium for activity. Whereas pectin lyases, acting on methoxylated olygalacturonic acid, show no such requirement. Pectin lyases and/or polymethylgalacturonate lyases have a pH optimum between 5.0-9.0 (Table 1.4). The molecular masses of the reported pectin lyases are in the range of 25,000 to 40,000 daltons, with their isoelectric points in the range of 5.3 to 9.4.

Table 1.4

Characteristics of pectin lyases from various sources

Source		Molecular weight	pH optimum	pI	Reference
Aspergillus japonicus	A	25000	6.2		Ishi, 1975; Baldwin, 1989
	B	32500	5.9	7.7	
	C	27000	4.9		
Erwinia chrysanthemi		40500	9.4	5.3	Tanabe, 1987
Pseudomonas fluorescence		32000	8.0-8.5	9.4	Sclemmer, 1987

The properties of polymethylgalacturonate lyase from various sources are listed in Table 1.4. These enzymes have not been well characterized yet. Pectin lyases from *Aspergillus* have also been used for fruit juice clarification (Rombouts 1980).

Over the last 35 years, a number of literature reports and patents have dealt with pectinases from various sources, with respect to their importance in plant physiology, plant- microbe interactions, microbial ecology and the ever-growing scope of their industrial applications.

Pectinases are reported to be produced from a variety of bacteria, yeasts, fungi and higher plants (Table 1.5). Micro-organisms producing pectinolytic enzymes are either phytopathogenic or saprophytic.

Table 1.5

Pectin degrading enzymes from various sources

Organism	PG	PL	PMG	PMGL	PE	References
Bacteria						
Aeromonas liquefaciens	+					Hsu, 1967
Agrobacterium tumefaciens	+					Rodriquez, 1991
Bacillus macerans	+					Miyazaki, 1991
Bacillus polymyxa	+					Nagel, 1961; Nagel,1970
Bacillus pumilus	+	+			+	Dave, 1971
Bacillus subtilis	+	+				Ward, 1974; Jauneau, 1988; Sakamoto, 1994
Bacillus		+				Karbassi, 1980

stereothemophillus						
Bacteriodes theteiotamicron	+	+				McCarthy, 1985
Butyriovibrio fibrisovens		+				Wojciechowicz, 1982
Clostridium mulitfementans		+			+	MacMillan, 1964; Miller, 1970
Cytophaga				+		Kamat, 1967
Erwinia caratovora		+				Zucker, 1970; Reid, 1986
Erwinia chrysanthemi	+	+			+	Bagley, 1979; Collmer, 1982; Reid, 1986
Flavobacterium pectinovorum		+				Ward, 1974
Pseudomonas fluoroscencs		+		+		Zucker, 1970; Schlemmer, 1987
Pseudomonas marginalis				+		Sone, 1988
Selenomonas ruminantium	+					Heinrichkova, 1989
Streptococcus bovis		+				Wojciechowicz, 1984
Streptomyces nitrosporeus		+				Sato, 1977; Sato, 1980
Streptomyces viridochromogenes				+		Agate, 1962
Xanthomonas campestris		+				Nasuno, 1967
Yersinia enterocolitica		+				Bagley, 1979
Yersinia pseudotuberculosis		+				Bagley, 1979
Yeasts						
Aureobasidium pullulans	+					Manachini, 1988
Crytococcus albidus	+					Tanabe, 1988
Kluyveromyces fragilis	+					Inoue, 1984
Saccharomyces fibuliger	+					Fellows, 1989
Saccharomyces fragilis	+					Lim, 1980
Fungi						
Aspergillus awamori	+	+			+	Arima, 1964
Aspergillus flavus	+				+	Arima, 1964
Aspergillus foetidus	+				+	Arima, 1964
Aspergillus fonsaceus					+	Edstrom, 1964
Aspergillus japonicus	+			+	+	Baldwin, 1989
Aspergillus nidulans		+				Dean, 1989
Aspergillus niger	+		+	+	+	Tuttobello, 1961; Maldonado, 1989; Bailey, 1990; Kester, 1990; Leuchtenberger, 1992
Aspergillus satoi	+				+	Arima, 1964; Yamasaki, 1966
Aspergillus sojae	+			+	+	Ishi, 1971
Botrytis cinera	+					Urbanek, 1975; Leone,

						1987; Tobias, 1993
Cladosporium cucermerinum		+				Robertson, 1990
Colletotrichum limdemuthianum	+					English, 1972
Coniothyrium diplodiella	+					Endo, 1964
Fusarium moniliforme	+					Mehta. 1985; DeLorenzo, 1987
Fusarium oxysporium	+	+				Arima, 1964; Strand, 1976; Fernandez, 1993
Fusarium roseum	+		+	+	+	Perley, 1971
Fusarium solani	+	+				Crawford, 1987; Gonzalez, 1992
Geotrichum candidum	+		+			Shastri, 1986
Giberella fujkora	+				+	Arima, 1964
Neurospora crassa	+	+		+		Polizeli, 1991
Penicillium chrysogenum	+				+	Arima, 1964
Penicillium digitatum		+				Bush, 1970
Penicillium islandicum	+					Arima, 1964
Rhizoctonia solani	+	+				Scala, 1980; Ginlaksa, 1988
Rhizopus stolonifer	+					Urbanek, 1975
Sclerotina fructigena	+					Fielding, 1969
Sclerotium sclerotium	+	+			+	Arima, 1964
Trichoderma kaningi	+					Fanelli, 1978
Verticillium alboatrum	+	+			+	Wang, 1970
Plants						
Banana					+	Ward, 1985
Ficus awkeotsung					+	Komae, 1990
Orange					+	Rombouts, 1979
Tomato	+				+	Miyazaki, 1970; Ward, 1985

Phytopathogenic micro-organisms, known to produce one or more of the pectic enzymes, are *Erwinia* (Zucker 1970, Collmer 1982, Reid 1986), *Fusarium* (Perley 1971, Habibullah 1976, Mehta 1985, DeLorenzo 1987, Gonzales 1992) *Aspergillus* (Arima 1964, Ishi 1971), *Verticillium* (Wang 1970), and *Geotrichum* (Shastri 1986). The production of these enzymes and their relation to phytopathogenecity of the organism has been well studied, in fact Leal and Villanueva (1962) have shown that only the phytopathogenic species of *Verticillium* produce pectin-degrading enzymes, whereas the non-phytopathogenic species do not. When supplied with isolated cell walls, pectic enzymes are generally the first polysaccharidases to be induced in a microorganism (Reid 1986) and the degraded

pectin provides further carbon for rapid multiplication of the microbe. Karr and Albersheim (1970) have shown that wall-modifying polygalacturonases assist the attack of other polysaccharidases such as cellulases, xylanases, arabinofuronasidase and galactosidases on isolated plant cell walls.

Higher plants also produce pectic enzymes, and action of such native enzymes result in post-harvest textural changes in fruits and vegetables (Kilara 1985, Zheng 1992). Saprophytic fungi and bacteria have been extensively exploited for their ability to produce industrially useful pectinases.

Fungi have traditionally been regarded as decomposer organisms, whose primary role is the degradation of leaf and wood litter (Newell, 1992). Transformation of complex detrital polymers containing carbon, nitrogen and phosphorus, into easily assimilable substrates is accomplished by release of a battery of extracellular hydrolytic enzymes by the saprophytic fungi (Cromack, 1992). Saprophytic molds probably compete with bacteria in pectin degradation of wood and leaf litter from rich ecosystems like mangal communities. Litter fall rates in mangrove ecosystems recorded in Australia and Thailand vary between 6-20 tons ha^{-1}-y^{-1} and around 60% of the litter may be recycled annually through microbial activity. Microbial biomass in the detritus of mangrove ecosystems comprise 2-5% of the total organic matter and microbial production may reach levels of 0.3-0.5 mg $g^{-1}day^{-1}$ (Subramanian 1988). Raghukumar *et al* (1994) have observed rapid colonization of *Rhizophora* leaves by *Cladosporium herbarum*, *Cirrenalia basiminuta*, *Halophytopthora*, a thaustrochytrid *Scizochytrium mangrovei* and *Fusarium moniliforme*. To achieve mineralization of complex organic resources, fungi posses a wide range of polysaccharidases such as cellulases, hemicellulases, pectinases and ligninases (Chamier 1982, Burns 1983, Subramanian 1988 and Riou 1991) thus playing an important role in the global carbon cycle (Suberkropp 1983 and Zemek 1985).

In the present study, enrichment cultures of pectin-degrading micro-organisms from a marine mangrove environment yielded a strain of *Fusarium*, which produces pectate lyase at alkaline pH and polygalacturonase at acidic pH. The optimum growth conditions for pectic enzyme production by this organism have been studied. The polygalacturonase from *F.moniliforme* has been purified and characterized by DeLorenzo *et al* (1987) and consists of four isoforms of molecular

masses, 38.0, 41.5, 45 and 48.5 kDa, all of which were glycoproteins. This author has not reported the presence of pectate lyase from his species. Our strain, on the other hand, does produce a pectate lyase in addition to polygalacturonase. Pectate lyases have been poorly studied in terms of active-site characterization and their probable mechanism of action. The present thesis deals with the biochemical characterization of the purified enzyme with respect to its active-site and involvement of calcium in catalysis.

The second aspect of this thesis is the characterization of polygalacturonase from a species of *Aspergillus* and its mutant. Polygalacturonase is known to hydrolyze the α-1,4- linkage of polygalacturonic acid. This enzyme has been studied in *Aspergillus niger* and Rexova-Benkova (1968) and Cooke (1976) have reported the importance of histidine in the catalysis of the polygalacturonase. The *Aspergillus* enzyme is widely used in industry. We chose to study the polygalacturonase of *Aspergillus ustus* NCIM 1033, which has not been investigated so far, together with that of its high-producing mutant isolated by us, in order to characterize the active-site of the enzyme and also to determine its industrial potential.

The aim of the present work is to characterize the active- site of pectate lyase, and endo-polygalacturonase, from two different fungi and to suggest the probable mechanism of action of these enzymes. The biotechnology of development of a process to produce polygalacturonase was also undertaken.

CHAPTER 2

INDUCTION, PURIFICATION, AND CHARACTERIZATION OF PECTATE LYASE FROM *FUSARIUM MONILIFORME*

SUMMARY

Fusarium moniliforme, isolated from a tropical mangal community, was found to produce extracellular pectate lyase (EC 4.2.2.2) and polygalacturonase (EC 3.2.1.15) at alkaline and acidic pH respectively. Optimization of growth conditions showed that the best nitrogen source was 0.5% $(NH_4)_2SO_4$ in presence of 0.2% glucose and 1% pectin at pH 8 for pectate lyase and pH 5 for polygalacturonase. Both enzymes were strongly induced at their respective pHs in the presence of 1% pectin. *F.moniliforme* also showed the production of other polysaccharidases, like endoglucanase (EC 3.2.1.4), xylanase (EC 3.2.1.8) and amylase (EC 3.2.1.1), when induced by appropriate substrates.

The organism grew abundantly upto 0.4 M NaCl, whereas growth as well as production of the enzymes decreased at higher salt concentrations showing that this strain of *F.moniliforme* has adapted to the estuarine conditions from which it has been isolated.

The extracellular pectate lyase from *Fusarium moniliforme* was purified to homogeneity by affinity chromatography followed by gel filtration with a yield of 76.5%. The pectate lyase was a glycoprotein with 5% carbohydrate content and had a pI of 9.1. Atomic emission spectrometry showed that Ca^{++} was a part of the holo-enzyme held by carboxyl groups of the protein. Loss of Ca^{++} was observed by treatment with EGTA or carboxyl modifying Woodward's reagent K, with subsequent loss of enzyme activity. Kinetic studies and tryptophan fluorescence quenching showed that Ca^{++} was not required in substrate binding.

Chemical modification and substrate-protection studies showed the presence of lysine and tryptophan at or near the active site of the pectate lyase. There were no major structural changes in the chemically modified enzyme as determined by circular dichroism. Amino acid analyses of native, TNBS-treated and substrate-protected enzyme showed that a single lysine residue is present at or near the active-site.

Inhibition kinetics with trinitrobenzene sulfonate and hydroxynitrobenzyl bromide showed pseudo-first order reaction. Kinetic analysis of partially-inhibited enzyme together with substrate-affinity studies showed that tryptophan was essential for substrate binding, whereas lysine was involved in the catalysis.

Fluorescence quenching further confirmed the involvement of tryptophan in substrate binding.

The reaction mechanism involving ß-elimination, of this enzyme is discussed. A concerted nucleophilic- electrophilic attack by lysine and calcium on the substrate is believed to catalyze the breaking of the 1,4-glycosidic linkage, with formation of a C-4-C-5 double bond in the sugar moiety.

INTRODUCTION

Soil microbial populations possess a remarkable spectrum of metabolic properties, and the success or failure of an individual species will be determined by the availability of suitable substrates and the capacity of the microorganism to respond to those substrates (Burns 1983). A large number of polymeric organic compounds enter the ecosystem as structural components of living tissues. Most are excellent sources of carbon, nitrogen, sulfur and phosphorus, but require extracellular degradation in order to be assimilated.

Many saprophytes as well as pathogenic micro-organisms secrete pectic enzymes, which modify plant cell-walls by degrading pectin, thus increasing accessibility to other enzymes specific to different cell-wall components, resulting in cell-lysis and plant tissue maceration (Martinez 1991). Early reports on pectin degradation (Waksman and Allen 1934) show that soil fungi such as *Aspergillus niger*, and species of *Penicillium* and *Fusarium* produce pectinolytic enzymes, able to degrade pectin and polygalacturonic acid.

Saprophytic molds probably compete with bacteria in pectin degradation of wood and leaf litter from rich ecosystems like mangal communities. Fungi have long been recognized as important members of various natural ecosystems, functioning as decomposers, parasites, pathogens and mycosymbionts with algae (lichens) and higher plants (mycorrhizae) (Cromack 1992). Possessing, as they do a wide range of polysaccharidases (Chamier 1982, Subramanian 1988 and Wainwright 1992), they play an important role in the carbon cycle (Suberkopp 1983 and Zemek 1985).

Mangrove ecosysytems are rich in organic matter (Subramanian 1983). Litter fall rates in mangrove ecosystems recorded in Australia and Thailand vary between 6-20 tons ha^{-1}-y^{-1} and around 60% of the litter may be recycled annually through microbial activity. Microbial biomass in the detritus of mangrove ecosystems comprise 2-5% of the total organic matter and microbial production may reach levels of 0.3-0.5 mg $g^{-1}day^{-1}$.

Fungi of the genus *Phytophthora* (Phycomycetes) were found to be associated with the initial stages of leaf litter decay of *Rhizophora* species (Fell 1975) in estuarine and coastal marine waters. Newell *et al* (1987) had observed that marine zoosporic oomycetes, principally *Phytophthora vesicula* rapidly colonized

fallen leaves of *Rhizophora mangle*. Raghukumar *et al* (1994) have shown that detritus of the mangrove *Rhizophora apiculata* supported the growth of *Cladosporium herbaraum*, *Fusarium moniliforme*, *Cirrenalia basiminuta*, *Halophytophthora vesicula* and Hyphomycete XVII, with consequent production of pectic enzymes, amylases and proteases by these organisms.

The form-genus *Fusarium* is composed of a highly diverse group of saprophytic and plant-pathogenic fungi (Anderson 1992) which cause economically important losses worldwide and synthesize metabolites of commercial importance. Asexual reproduction through microconidia and macroconidia (Subramanian 1971), is thought to predominate in the field, but many *Fusarium* anamorphs have *Gibberella* (*Fusarium moniliforme*) or *Nectria* (*Fusarium solani*) teleomorphs, that are elicited in the laboratory (Anderson 1992).

Most species of *Fusarium* are soil fungi with cosmopolitan distributions and are active in decomposing plant substrates, while some species are plant parasites, causing root and stem rot, vascular wilt, fruit rots or ear diseases. Species of the genus *Fusarium* have been known to produce a range of pectic enzymes. For an enzyme to be beneficial to the producer, it should be stable for long enough to locate the substrate (Burns 1983).

Pectin degradation by species of *Fusarium* has been generally studied with respect to their phytopathogenecity and in host-pathogen relationships. When induced with pectin, *Fusarium solani* has been reported to produce poly-galacturonase at low pH and pectate lyase at high pH (Crawford 1987, Gonzalez 1992). *Fusarium oxysporum* which causes vascular wilts in tomato, produces both pectate lyase and polygalacturonase (Arima 1964, Habibullah 1976, Strand 1976, Fernandez 1993). Perley (1971) had reported the induction of polygalacturonase, pectin lyase and pectinesterase in *Fusarium roseum*. *Fusarium moniliforme* is the only species of the genus *Fusarium* which has been reported to produce only polygalacturonase (Mehta 1985, DeLorenzo 1987) and this species has not yet been reported to produce pectate lyase.

During screening for pectinase-producing micro-organisms in marine and estuarine environments, we isolated a strain of *Fusarium* from decaying wood in a tidal mangrove-dominated creek. The strain which was identified, using

morphological characteristics, as *Fusarium moniliforme*, produced polygalacturonase and pectate lyase. The optimum growth conditions for the production of the pectic enzymes by *F.moniliforme* were studied. The induction studies of other polysaccharidases, like amylase, cellulase and xylanase on different substrates and at different pH, were carried out in order to evaluate the ability of the organism to utilize various carbon sources.

Pectate lyase catalyses a split in the α-1,4-glycosidic bond between two galacturonide units by ß-elimination of a proton from the C-5 of the galacturonide unit at the reducing end (Rexova-Benkova 1976, Gacesa 1987).

Although a lot of work has been done on the purification and characterization of pectate lyase of various micro-organisms (Table 1.3), not much is known about the identity of the functional groups involved in the catalysis.

Chemical modification of reactive amino-acid side-chains help to identify those residues in the active-site essential for catalysis. An active-site can be defined as the sum of the residues which together are involved in the docking of the substrate(s) and consequent make or break of a covalent bond. Any reagent which binds covalently to the amino-acid side chains and which results in detectable changes in the property (or properties) of the enzyme can be used to study the active-site. Of the naturally occurring amino-acids, only those with polar side-chains are the object of chemical modification.

For acidic amino-acids like aspartate or glutamate, isoxasolium salts, such as N-ethyl-5-phenylisoxasolium-3'- sulfonate (Woodward's reagent K) can be used to study their importance in enzyme function (Arana 1981, Sinha 1985). Phenylglyoxal has been first used as an arginine reagent by Takahashi (1968). Phenylglyoxal is a dicarbonyl reagent which reacts with the highly basic guanidino group of arginine. An useful reagent for lysine modification is trinitrobenzene sulfonate (Johnson 1979). Diethylpyrocarbonate, which carboxyethylates on the imidazole nitrogens, is the most commonly used reagent for histidine modification (Miles 1977). N-ethylmaleimide (Ambrose 1976), 5,5'-dithiobis-(2- nitrobenzoic) acid (Ellman's reagent) (Bazaes 1980) and organic mercurials such as p-chloromercuribenzoate (Bai 1979) have been used for the modification of cysteine residues. Horton and Koshland (1965) had shown the specificity of hydroxynitrobenzyl bromide towards

tryptophan. N-actetylimidazole has been shown to be a specific reagent for the modification of functional tyrosine residues (Simpson 1963). Phenylmethyl sufonyl fluoride has been a widely used reagent for modification of serine residues, as in the case of serine proteases (Gold 1967).

Calcium was found to be essential for the activity of all pectate lyases reported, but it was not clear whether it is part of the enzyme or involved in substrate binding. Crawford *et al* (1987) had suggested that a dimer of polygalacturonic acid with calcium ions was the substrate for the pectate lyase whereas Yodder *et al* (1993) had identified a putative calcium site in the crystal structure of pectate lyase from *Erwinia chrysanthemi* and a calcium ion was found to be present in the crystal structure of pectate lyase of *Bacillus* (Pickersgill 1994).

In the present work pectate lyase from *Fusarium moniliforme* was characterized with respect to the active-site nature and the role of calcium in the activity of this enzyme.

MATERIALS AND METHODS

Microorganism

Samples of marine and estuarine waters and soils were put into enrichment media containing pectin as the sole carbon source in artificial sea water. The cultures were then grown on agar medium containing 1% pectin 0.4% K_2HPO_4 in sea water. Resulting colonies were isolated using standard microbiological techniques. The highest pectinase producer was chosen for further studies.

The organism was identified on the basis of morphological studies. It was subcultured on Czapek-Dox agar, modified with the addition of 1.0% pectin. The organism has been deposited in the National Collection of Industrial Micro-organisms as *Fusarium moniliforme* NCIM 1276.

Culture conditions

Fusarium moniliforme was grown in 250 ml. conical flasks with 50 ml. medium. The flasks were incubated at 30° C on a rotary shaker (180 rpm.). After four days of growth, mycelial-free broth was analyzed for pectic enzymes.

Enzyme methods

Polygalacturonase was assayed by measuring reducing sugars released from 0.3% polygalacturonic acid by the method of Nelson (1944) and Somogyi(1952). The reaction mixture was made up in 0.1M sodium acetate buffer, pH 5.0. One unit of activity (PGr) is defined as the amount of enzyme which released 1 μmole of uronic acid per minute at 35° C and pH 5.0. Preliminary results had shown that 35° C was the optimum temperature for the enzyme.

Pectate lyase activity was measured as the increase in absorbance at 232 nm. of 0.24% polygalacturonic acid in 60 mM Tris-HCl Buffer, pH 8.5, with 0.6 mM $CaCl_2.2H_2O$, at its optimum temperature of 40 °C. One unit of lyase activity was defined as the amount of enzyme which produced 1 μmole of unsaturated galacturonide ($E = 4600\ M^{-1}cm^{-1}$) per minute (Collmer 1988).

Effect of salinity of medium on enzyme production

Various concentrations of sodium chloride (0-1.4 M) were used in the growth media containing 1% pectin, 0.2% glucose, 0.2% $(NH_4)_2SO_4$, 0.2% K_2HPO_4, 0.2% KH_2PO_4 and 0.1% $CaCl_2.2H_2O$.

Variation of initial pH of medium

Initial pH of the growth medium was adjusted from 2 to 11 at one unit intervals with 0.1 N HCl or 0.1 N NaOH and inoculated.

Variation of various media constituents

Effect of nitrogen sources on enzyme production were determined by using $(NH_4)_2SO_4$, NH_4NO_3, KNO_3, $NaNO_3$ in 0.075 molar nitrogen concentrations and 0.5% peptone. The best nitrogen source was then supplied at different concentrations in the medium. The effect of the carbon source on enzyme production was studied by varying the percentages of pectin and glucose. Time course of production of pectic enzymes was studied in triplicate with enzyme assays every 24 hours, and concomitant measurement of mycelial dry weight.

Induction of polysaccharidases in F.moniliforme

Induction of polysaccharidases was studied by adding 1.0% (wt./vol.) of different polysaccharides (Table 2.1) to a basal medium containing 0.5% $(NH_4)_2SO_4$, 0.2% K_2HPO_4, 0.2% KH_2PO_4 and 0.1% $CaCL_2.2H_2O$. The activities of the polysaccharidases were determined by estimating the reducing sugars released from respective substrates. One unit of polysaccharidase activity was defined as the amount of enzyme which released 1 μmole of corresponding monosaccharide per minute. The assay mixture contained 0.5% substrate in 0.1M sodium phosphate buffer, pH 7.5.

Purification of pectate lyase

The organism was grown in medium containing 1% pectin, 0.2% glucose, 0.5% $(NH_4)_2SO_4$, 0.2% K_2HPO_4, 0.2% KH_2PO_4, and 0.1% $CaCl_2.2H_2O$. The culture was grown for 4 days and later centrifuged to obtain the cell-free broth.

The pectate lyase was purified by the affinity method of Ward and DeBoer (1987) except that calcium was replaced by barium. All the purification steps were done at 4 °C. 5 gm. of polygalacturonic acid was added to 1 L of cell-free broth with constant stirring. To this mixture, 20 ml. of 1 M barium chloride solution was added drop-wise. The resultant Ba-polygalacturonate gel was washed with 0.1 M Tris-HCl, pH 8.0 buffer with 0.1 M $BaCl_2$. The adsorbed enzyme was eluted with 1.0 M sodium chloride in the wash buffer. The wash was concentrated in an Amicon ultrafiltration unit, using a 5000-cut off cellulose-acetate membrane.

The concentrate was then loaded onto gel filtration column of Biogel P-30, 1.5 m. length and 1.5 cm. I.D., equilibrated with 0.1 M Tris-HCl, pH 8.0 with 0.1 M NaCl and eluted with the same buffer, with a flow-rate of 12 ml/hr. Fractions of 2.5 ml were collected and the fractions containing enzyme activity were pooled.

The homogeneity of the protein was checked by SDS-PAGE, using the Laemmli (1970) method.

Preliminary characterization of pectate lyase

The molecular mass of the pectate lyase was determined by three methods

(1) Gel filtration

(2) Ultracentrifugation

(3) SDS-Polyacrylamide gel electrophoresis

Molecular mass determination by gel filtration was done on a column of Biogel P-30 using standard molecular weight markers such as (a) Trasylol-6500 Da, (b) Cytochrome C-12400 Da, (c) Carbonic anhydrase-29000 Da, (d) Glyceraldehyde dehydrogenase-36000 Da, (e) Ovalbumin-45000 Da and (f) Bovine serum albumin-66000 Da.

The molecular mass was also determined by estimating the sedimentation coefficient on a Beckman Model E Analytical Ultracentrifuge with Schliren optics at a pectate lyase concentration of 6 mg/ml in 20 mM Tris-HCl buffer, pH 7.0 with 50 mM NaCl in an 4°-12 mm cell in an AnHTi rotor at 60000 rpm. An Archibald

sedimentation equilibrium run was done at 22000 rpm. and the synthetic boundary run at 10000 rpm.

Determination of molecular mass by SDS-PAGE was done by using standard molecular weight markers such as (a) Lactalbumin-14.2 kDa, (b) Trypsin inhibitor-20.1 kDa, (c) Trypsinogen -24 kDa, (d) Carbonic anydrase-29 kDa, (e) Glyceraldehyde-3-phosphate dehydrogenase-36 kDa, (f) Ovalbumin-45 kDa and (g) Bovine serum albumin-66 kDa.

The optimum pH of enzyme activity was determined by using substrate solution at different pH. Tris-HCl buffer was used for the pH range 7.0 to 9.0 and glycine-NaOH buffer for the pH range of 9.0 to 10.5. The temperature optimum of the enzyme was determined by incubating the enzyme-substrate mixtures at different temperatures and estimating the increase in absorbance at 235 nm.

The Km and the Vmax of the pectate lyase was determined by assaying the enzyme activity at different substrate concentrations. The constants were obtained by the Lineweaver-Burk double reciprocal plot.

The isoelectric point of the enzyme was determined by the modified straight-tube method (Chinnathambi 1994) using ampholines in the range of pH 3.0 to 10.0.

The glycoprotein nature and the percentage of carbohydrate was determined using the phenol-sulfuric acid method for estimation of total carbohydrate (Dubois 1956). The enzyme was dialyzed in 8 M urea and then used for the assay.

Amino acid analysis was done on a Pharmacia LKB alpha plus amino acid analyser, by hydrolysing the purified enzyme in 6 N HCl at 110 °C for 22 hours. Cysteine, methionine and tyrosine were protected. Tryptophan was estimated by titration with N-bromosuccinimide by the method of Edelhoch (1967).

The metal ion effect on pectate lyase was determined by dialyzing the enzyme in 1 mM EDTA in glycine-NaOH buffer, 0.1 M, pH 9.6, and then in the same buffer without EDTA. Dialysis was done in HNO_3/HCl/deionized water-washed polycarbonate beakers. The dialyzed enzyme was then incubated in various metal ions at 2 mM concentration in the glycine- NaOH buffer, and assayed for the activity in substrate solution containing corresponding metal ion.

Chemical modification

Chemical modification studies were done by using various reagents specific for reactive side-chain of proteins. Inhibition studies were done by incubating 0.73 μM enzyme with various modifiers in appropriate buffers for a period of 30 minutes at 30 C. Control tubes without the inhibitor were incubated under identical conditions. Substrate protection studies were done by addition of polygalacturonic acid to a concentration of 2 mg/ml to the enzyme solution before the addition of the inhibitor.

The pH-effect on enzyme-inhibition was studied by incubating the enzyme and the inhibitor in buffers of various pH. Sodium phosphate buffer was used for pH 5.5, 6.5 and 7.5 and carbonate buffer for pH 8.5, 9.5 and 10.5.

Kinetics of inactivation of pectate lyase by 2,4,6- trinitrobenzene-1-sulfonate (TNBS) and 2-hydroxy-5- nitrophenyl bromide (HNBB) were carried out by incubating the enzyme at various concentrations of the inhibitors. Aliquots were removed at regular intervals and assayed for residual activity. The TNBS incubations were carried out in the dark because of the sensitivity of the TNP-derivatives.

Amino-acid analyses of native enzyme, TNBS-treated enzyme and substrate-protected TNBS-treated enzyme were done. The TNBS-treated pectate lyase was obtained by incubating 20 nmoles of enzyme with 5 mM TNBS for 30 minutes, followed by extensive dialysis in deionized water. For the substrate-protected enzyme, polygalacturonic acid at a concentration of 2 mg/ml was added prior to addition of the inhibitor.

Partially inhibited enzymes were obtained by addition of 20 mM lysine and tryptophan to TNBS and HNBB- treated enzyme.

Circular dichroism

Circular dichroism spectra in the range of 190-350 nm. were obtained for the native pectate lyase and TNBS. HNBB and EGTA treated enzyme using a Jasco J-500 spectropolarimeter.

Tryptophan fluorescence quenching

Fluorescence scans of the pectate lyase and the enzyme- substrate mixtures were obtained using a Kentron SFM-25 spectrofluorimeter. Tryptophan fluorescence was excited at 297 nm. using a 150 W Xenon-High pressure lamp and a monochromator with concave holographic gratings. Emission scans of the range 310 to 390 nm. were obtained using a similar monochromator and a R212 photomultiplier.

Atomic emission spectrometry

Carboxyl groups of pectate lyase were modified with Woodward's reagent K (WRK) by adding aliquots of a 1 M stock of the inhibitor in 1 mM HCl to enzyme solution. The enzyme solution was also dialyzed in 2 mM EGTA to chelate out Ca^{++} ions.

Pectate lyase was modified with Woodward's reagent K (WRK) by adding aliquots of a 1 M stock of the inhibitor in 1 mM HCl. N-ethyl-5-phenylisoxosolium-3'-sulfonate (Woodward's reagent K) has been known to react with acidic side-groups of asparate or glutamate of proteins (Arana 1981, Mehta 1985). Calcium content of 50 μM native pectate lyase, EGTA-treated enzyme and WRK-modified enzyme was determined by inductively coupled plasma-optical emission spectrometry on a Perkin Elmer Plasma 1000 Emission Spectrometer at an emission wavelength of 393.366 nm. Enzyme samples were dialyzed in deionized water in polycarbonate bottles which were previously washed in 1 M HCl, 1 M HNO_3 and deionized water.

RESULTS AND DISCUSSION

Isolation and identification of *Fusarium moniliforme*

Samples from estuarine and marine waters were enriched in pectin containing media. A strain of *Fusarium* was isolated, which produced extracellular pectate lyase and polygalacturonase. The fungus grew on Czapeks agar, producing white, fluffy mycelia. The macroconidia and microconidia produced by the fungus are shown in Fig. 2.1 & 2.2 The macroconidia were fusiform and tapering towards either ends, whereas the microconidia were produced in simple, unbranched chains. The identification of the culture as a strain of *Fusarium moniliforme* was done by Dr. V.G. Rao, MACS-ARI, Pune, India, on the basis of above morphological studies. Microconidial production in chains is characteristic of *Fusarium moniliforme* (Subramanian 1971).

Optimum culture conditions for pectic enzyme production by *F.moniliforme*

Effect of pH of medium

F.moniliforme produced polygalacturonase maximally at a pH of 5.0 and pectate lyase at an alkaline pH of 8.0 (fig. 2.3). In this respect the organism behaved similarly to *Fusarium solani* (Bateman 1966), *Rhizoctonia solani* (Sherwood), *Verticellium albo-atrum* (Durrands 1988) and *Aspergillus nidulans* (Dean 1989). Mehta and Mehta (1985) who isolated *F.moniliforme* from infected potato reported polygalacturonase activity by this organism. DeLorenzo *et al* (1987) studied the same enzyme, produced by their strain in acidic conditions. In the tidal creek from which the present organism was isolated, pH varies between 7.0 to 7.9 depending on the tide and it seems reasonable to suppose that the organism is producing largely pectate lyase in this habitat. The optimum pH of the polygalacturonase and the pectate lyase in the culture filtrate of our isolate was determined to be 5.0 and 9.5 respectively. Thus the induction of the specific pectic enzyme in the acidic and the alkaline range is related to the optimum pH for activity of the enzyme. The present strain of *F.moniliforme* was able to grow in a wide range of pH (Fig. 2.3), though maximal growth was observed between pH 4.0 to 8.0. *F.moniliforme* has been reported to grow in acidic, neutral and alkaline pH (Thind 1979). Thus the ability to grow in a wide range of pH would increase the adaptability of the organism to different environments.

Figure 2.1

Microconidia of *Fusarium* strain

The fungus was grown in slide-cultures on Czapek-Dox agar

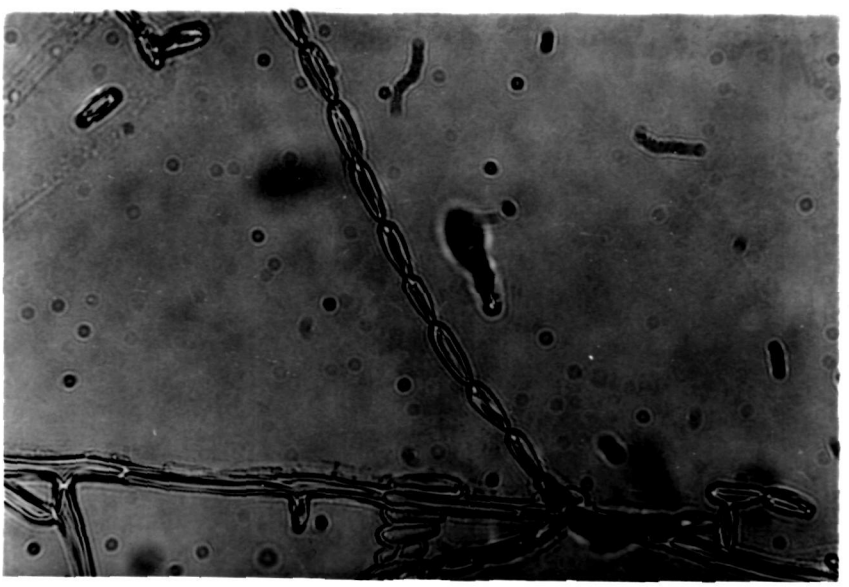


Figure 2.2

Macroconidia of *Fusarium* strain

The fungus was grown in slide-cultures on Czapek-Dox agar

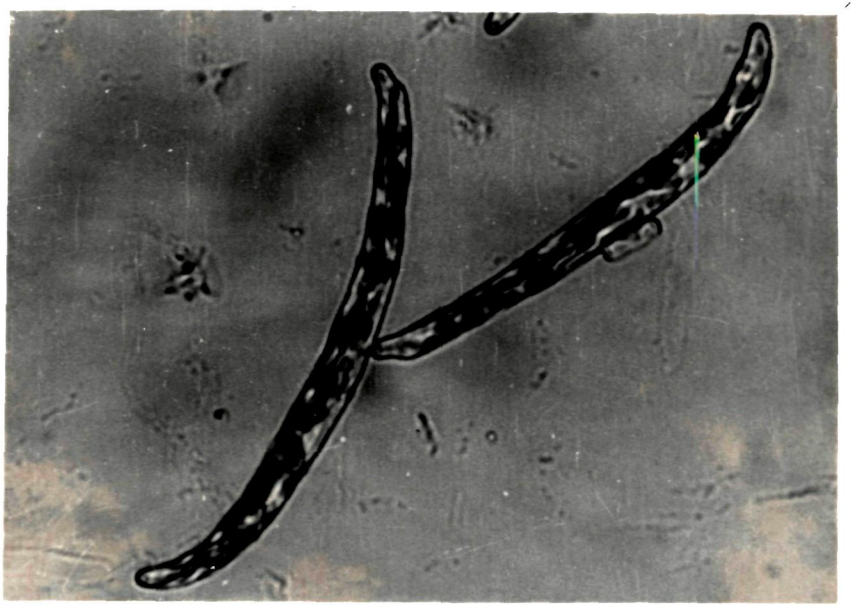
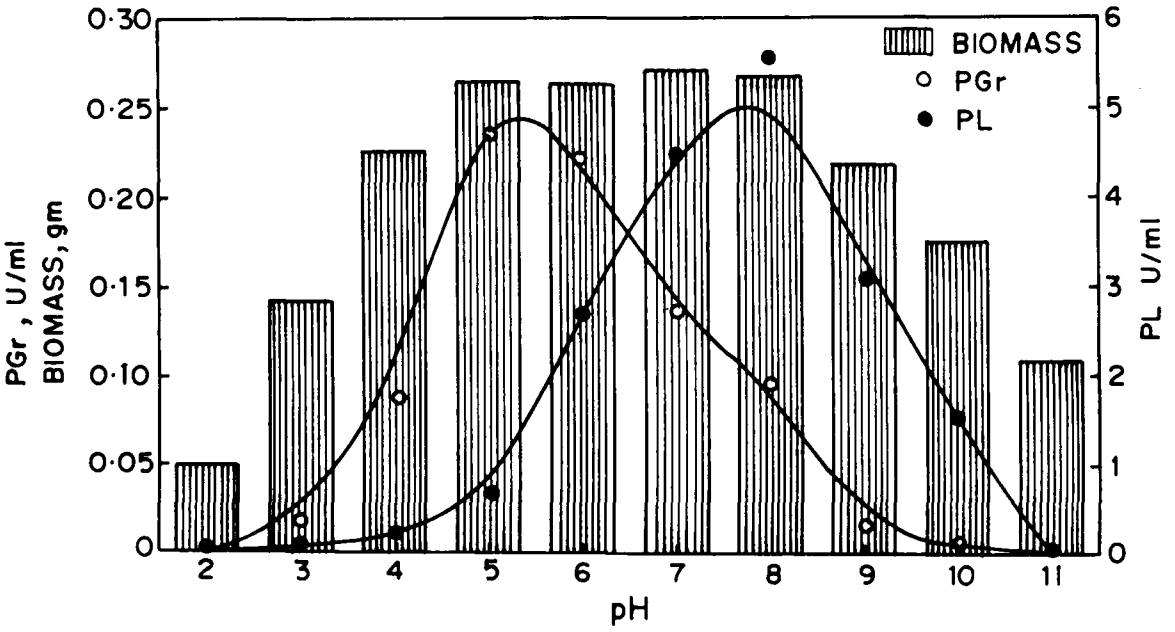


Figure 2.3

Effect of pH variation in the medium on the production of pectic enzymes by *F.moniliforme*.

Initial pH of medium was varied from 2-11 using 0.1 N HCl and 0.1 N NaOH



Effect of salinity of medium

At salt concentrations higher than 0.4 M NaCl, which is the molarity of normal seawater, there is an abrupt decrease in both growth and enzyme production (Fig. 2.4). Upto 0.4 M NaCl however biomass production was similar to that produced in a freshwater medium. It is therefore concluded that this strain of *F.moniliforme* is adapted to the estuarine environment from which it has been isolated, and it is not a true marine form. Raghukumar *et al* (1994) have shown that *Rhizophora* detritus is rapidly colonized by several fungi including *Fusarium moniliforme*.

Effect of medium constituents

For maximum production of pectate lyase as well as polygalacturonase, 0.5 % $(NH_4)_2SO_4$ was found to be the best source of nitrogen (Fig. 2.5). Higher concentrations of $(NH_4)_2SO_4$ decreased the production of the enzymes. Nitrate salts gave poor yields of enzymes. Organic sources also repressed enzyme production.

Of the carbon sources tested (Fig. 2.6), pectin 1% + glucose 0.2% gave maximum yield of pectate lyase and polygalacturonase. Concentrations of glucose above 0.2% repressed enzyme production. Thus the present strain of *F.moniliforme* required pectin for induction of the enzymes. The strain reported by DeLorenzo *et al* (1987) required 1% pectin for induction of polygalacturonase and there was no production of the enzyme when the fungus was grown in minimal medium containing glucose. Mehta's strain was reported to produce polygalacturonase (Mehta 1985) in basal media containing sucrose as sole carbon source, with 70% loss of viscosity of 1.2% sodium polypectate. The present isolate produced 0.018 U/ml polygalacturonase when grown at pH 5 on basal medium and 0.06 U/ml of pectate lyase at pH 8.0 when 0.2% glucose was the only carbon source. Addition of pectin at these pHs resulted in increased activity of 0.27 U/ml of polygalacturonase and 5.4 U/ml of pectate lyase. By comparison Mehta's strain (Mehta 1985) appears to produce the enzyme constitutively.

Figure 2.4

Effect of salt concentration (molarity of NaCl) in medium on production of pectic enzymes by *F.moniliforme*

NaCl concentration was varied from 0-1.4 M in the medium

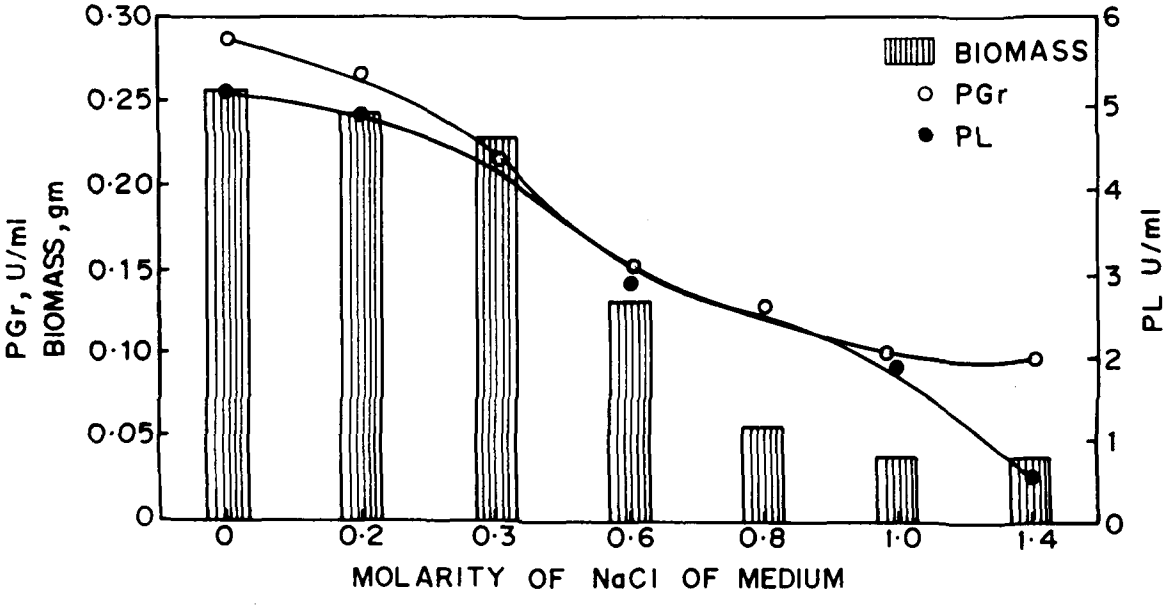


Figure 2.5

Effect of nitrogen source production of pectic enzymes by *F.moniliforme*

Different nitrogen sources at 0.175% molar nitrogen were used. Peptone was used at 0.5%.

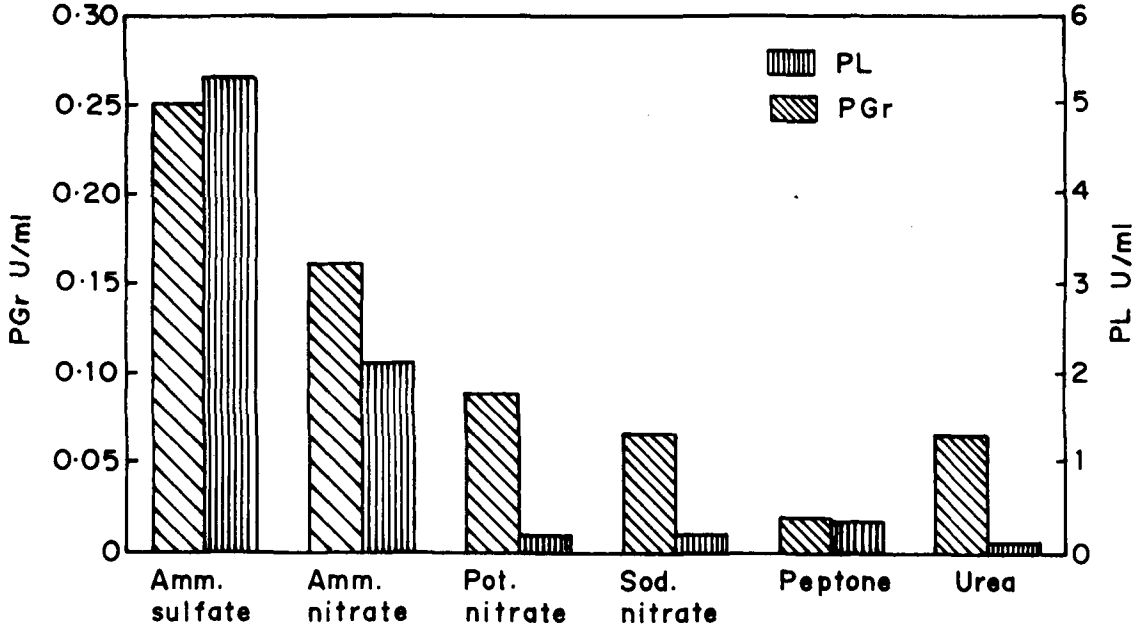
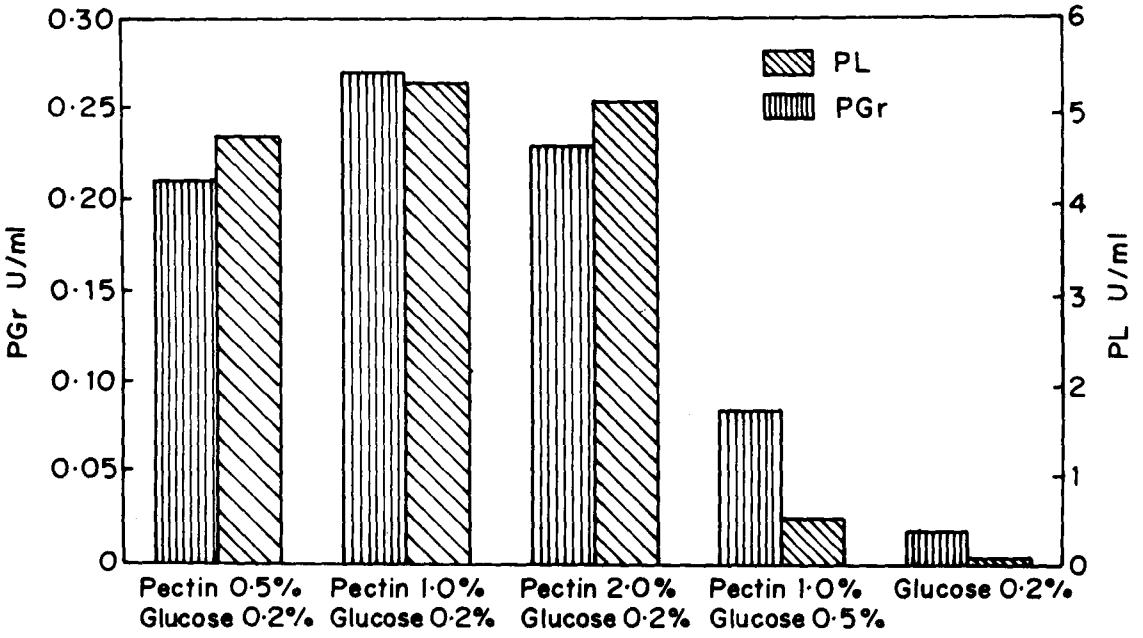


Figure 2.6

Effect of carbon source production of pectic enzymes by *F.moniliforme*

Combinations of different concentration of pectin and glucose were added to basal medium.



Time course of growth and pectic enzyme production

The production of polygalacturonase as well as pectate lyase started on the second day and reached peak values between the third and fourth day (Fig. 2.7). The enzymes were produced in the logarithmic phase and production stopped when the organism reached the stationary phase.

Polysaccharidases of F.moniliforme

As shown in Table 2.1, *F.moniliforme* produced amylase, endoglucanase (CMCase), polygalacturonase, pectate lyase and xylanase in detectable quantities when induced by the corresponding inducer substrate at pH 7.0. When complex polysaccharide sources such as wheat bran were used, most of the enzymes were secreted in quantities comparable to those induced with pure substrates. Pectic enzymes however were poorly induced with wheat bran since the content of pectin in wheat bran is low.

Table 2.1

Polysaccharidases of F.moniliforme at pH 7.0

Substrate	Amylase U/ml	CMCase U/ml	Polygalactu -ronase U/ml	Pectate lyase U/ml	Xylanase
Pectin	0.021	0.011	0.100	3.6	0.009
Cellulose	0.008	0.027	0.07	0.6	0.087
Starch	0.660	0.002	0.032	0.02	0.005
Xylan	0.003	0.014	0.04	0.05	1.15
Wheat bran	0.434	0.043	0.019	0.17	1.5
Glucose	0.006	0.002	0.008	0.05	0.007

At a pH of 4.0 (Table 2.2), the production of amylase, xylanase and pectate lyase decreased, whereas the carboxymethylcellulase production remained almost the same.

Figure 2.8

Gel filtration of pectate lyase on Biogel P-30.

The affinity eluate was concentrated and loaded on P-30 column, equilibrated with 0.1 M glycine-NaOH buffer, pH 9.6. Absorbance at 280 nm. (o), pectate lyase units/ml (Δ).

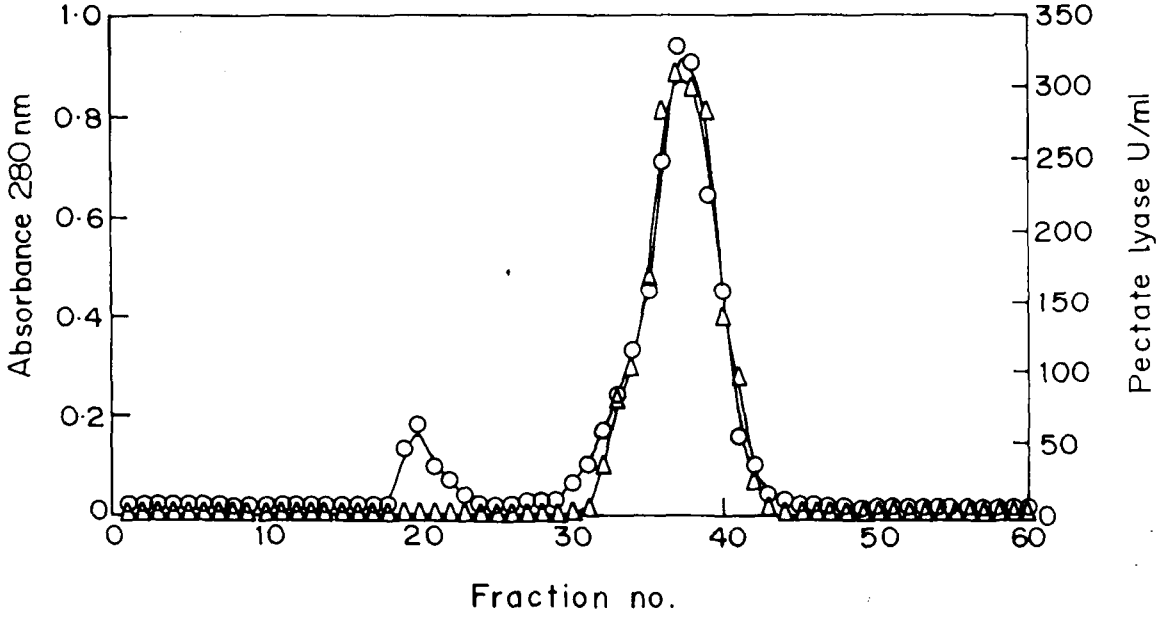


Figure 2.7

Time course of pectic enzyme production by *F.moniliforme*

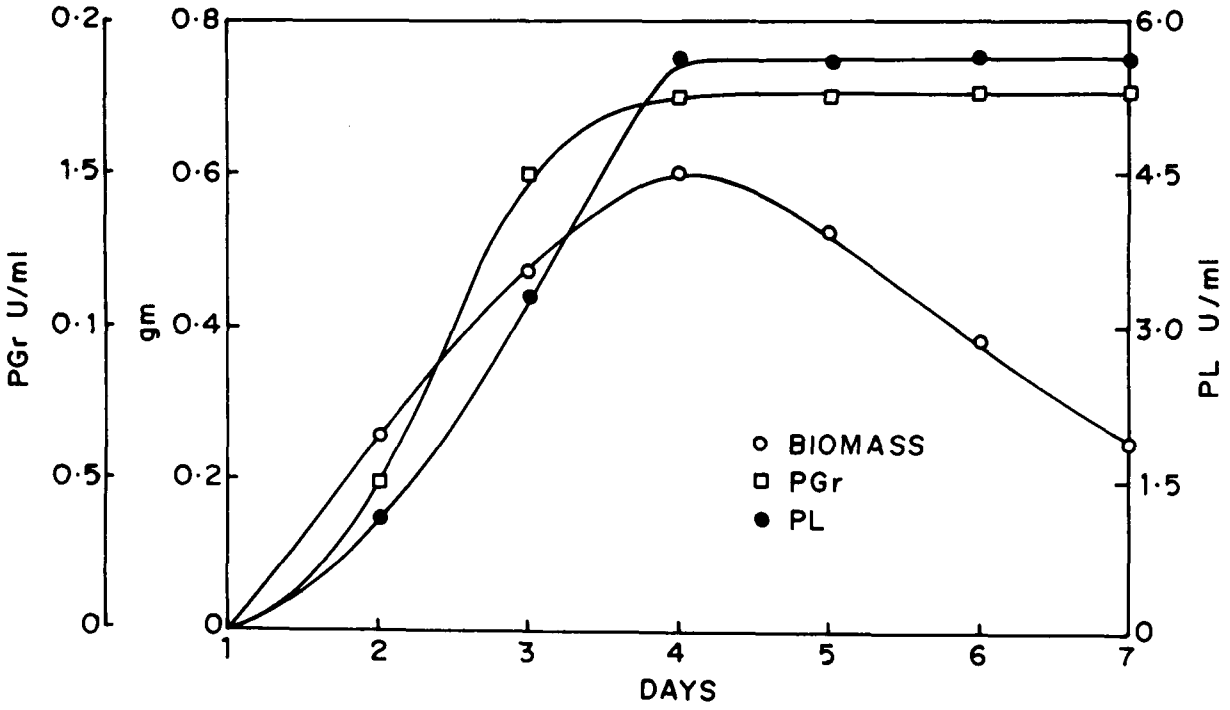


Table 2.2

Polysaccharidases of F.moniliforme at pH 4.0

Substrate	Amylase U/ml	CMCase U/ml	Polygalactu-ronase U/ml	Pectate lyase U/ml	Xylanase
Pectin	0.013	0.009	0.221	0.2	0.005
Cellulose	0.005	0.028	0.011	0	0.053
Starch	0.375	0.002	0.073	0	0.001
Xylan	0.002	0.013	0.062	0	0.441
Wheat bran	0.315	0.044	0.041	0.153	0.559
Glucose	0	0.002	0.015	0.307	0.002

The production of polygalacturonase was higher at this pH. Induction studies at pH 8.0 (Table 2.3) showed an increase in the pectate lyase production but a fall in the activities of the other polysaccharidases as compared to that obtained at pH 7.0.

Table 2.3

Polysaccharidases of F.moniliforme at pH 8.0

Substrate	Amylase U/ml	CMCase U/ml	Polygalactu-ronase U/ml	Pectate lyase U/ml	Xylanase
Pectin	0.018	0.004	0.059	5.2	0.006
Cellulose	0.007	0.012	0.003	0.87	0.042
Starch	0.437	0.001	0.026	0.26	0.002
Xylan	0	0.006	0.017	0.61	0.92
Wheat bran	0.363	0.019	0	1.58	1.1
Glucose	0.002	0.001	0.006	0.46	0.002

Thus the production of various polysaccharidases depended on both the presence of specific substrate inducers and the pH of the medium As discussed by Martinez and Subramanian (1991, 1988), many micro-organisms, pathogens as well as saprophytes secrete enzymes induced by appropriate substrates in their environment, for example, *Sclerotina sclerotium* (Riou 1991) produces polysaccharide depolymerases, necessary to degrade cellulose, pectin and hemicellulose components of higher plant cell-walls. It appears that *F.moniliforme* may be able to secrete "scout" enzymes (Burns 1983) and any increase in the concentration of particular substrates results in higher production of the appropriate enzyme.

Characterization of pectate lyase

The polygalacturonase from *Fusarium moniliforme* has been purified and characterized (DeLorenzo 1987), and has been shown to consisting of 4 isoforms, of molecular masses, 38, 41.5, 45 and 48.5 kDa, all of which were glycoproteins. Strains, obtained from the National Collection of Industrial Micro-organisms, Pune, India; *F.moniliforme* NCIM 1103 and *F.moniliforme* NCIM 1104, were found to produce low levels of pectate lyase in pectin containing media, and therefore it appears that this species does secrete pectate lyase, but which has not been characterized yet.

Enzyme purification

The pectate lyase was purified by affinity chromatography, based on the method of Ward and DeBoer (1987) differing only in the divalent metal ion used for precipitation of polygalacturonic acid. Greater yield was obtained with Ba^{++} than with Ca^{++}. Further purification to obtain a pure enzyme preparation was done by gel filtration on Biogel P-30 (Fig. 2.8). A high yield of 76.5% was obtained with a fold purification of 59.5 (Table 2.4).

Table 2.4

Purification of pectate lyase

	Volume ml	Activity units	Protein mg	Sp. activity units/mg	Yield %	Fold purification
Broth	1000	5200	582.	8.9	-	-
Affinity elution	118	4420	11.2	395	85	44.4
Biogel P-30	15	3978	7.5	530.1	76.5	59.5

Preliminary characterization of pectate lyase

The molecular mass of pectate lyase as determined by gel filtration was 11,000. This was confirmed by equilibrium sedimentation ultracentrifugation, where the molecular mass was determined to be 10,600 by using the Archibald method (Fig. 2.9). SDS-PAGE showed a molecular mass of around 17,500, which could be a result of the glycoprotein nature of this protein. Such proteins are known to

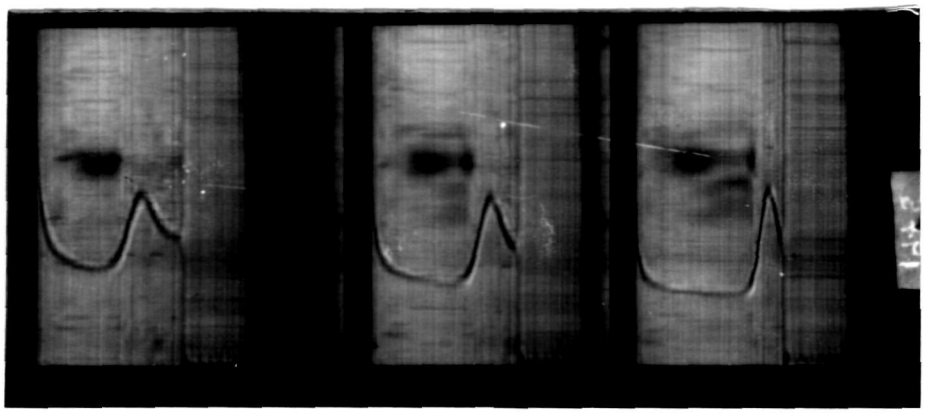
Figure 2.9

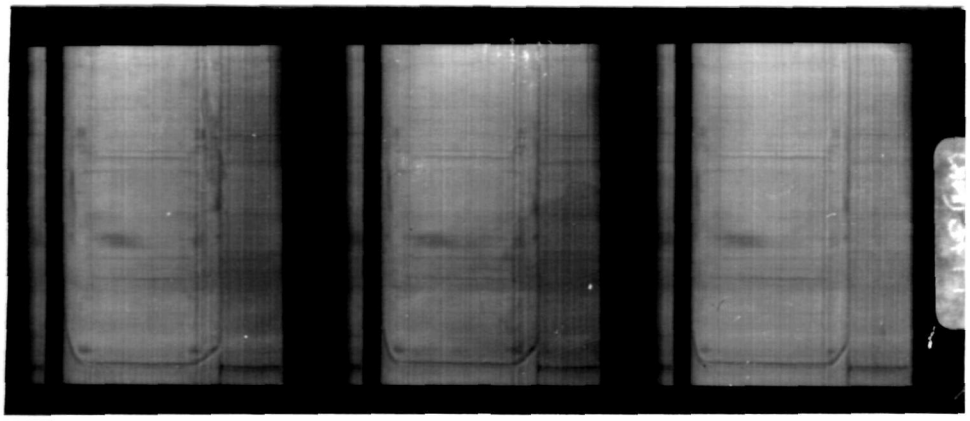
Analytical ultracentrifugation of pectate lyase

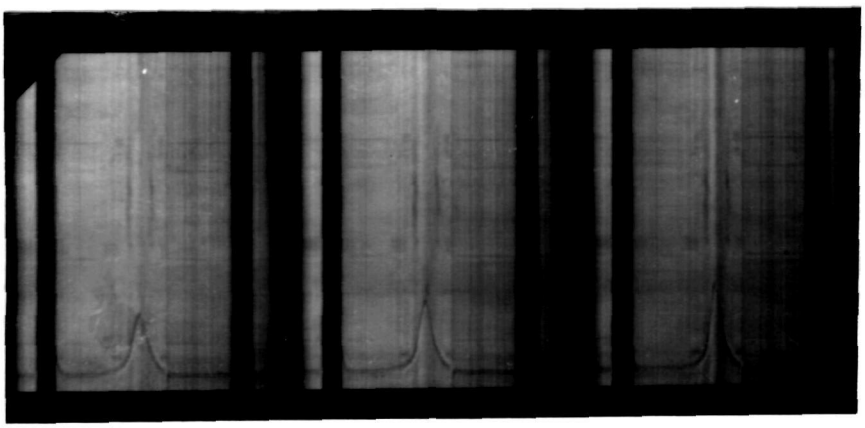
a) Sedimentation coeffecient determination on Beckman Model E Analytical Ultracentrifuge with Scliren optics in a 4 -12 mm cell in an AnHTi rotor at 60000 rpm.

b) Archibald sedimentation equilibrium run at 20000 rpm.

c) Synthetic boundary run at 10000 rpm.







behave differently on SDS-PAGE giving a higher molecular mass. Pectate lyase from *Fusarium solani* has a molecular mass of 26 kDa (Crawford 1987), whereas *F.oxysporum* f.sp. *ciceri* enzyme has a molecular mass of 25 kDa (Artes 1990). The molecular masses of pectate lyases from other species vary from 20 kDa to 74 kDa (Dave 1971, Bagley 1979, Karbassi 1980, Sato 1980, McCarthy 1985, Tamaki 1988 and Robertson 1990). Thus the present pectate lyase is apparently the smallest among the pectate lyases in terms of mass.

The optimum pH for the activity of the purified pectate lyase was 9.6, and the optimum temperature was 40° C. The Km of the purified enzyme was 1.2 mg/ml and the Vmax was 655 units/mg with polygalacturonic acid.

The isoelectric point of the pectate lyase was determined to be 9.1. The enzyme is a glycoprotein with a carbohydrate content of 5%.

The metal ion effect (Table 2.5) showed that the enzyme had an absolute requirement for calcium. No other divalent ion could replace Ca^{++} for activity, though with Ba^{++}, the enzyme showed slight activity.

Table 2.5

Effect of metal ions on pectate lyase

Metal ion	% Activity
Calcium	100
Magnesium	0
Manganese	0
Barium	8
Cobalt	0
Lead	0
Zinc	0

Presence of calcium in the enzyme and its role

The presence of calcium in the molecule of the pectate lyase was determined by atomic emission spectroscopy. Table 2.6 shows that Ca^{++} is bound to the pectate lyase, even after extensive dialysis against deionized water.

Table 2.6

Atomic emission spectrometry

	Ca^{++} ppm.	Molar ratio
Native pectate lyase	1.64	0.82
EGTA - enzyme	0	0
WRK - enzyme	0.1	0.05

Woodward (1961) had found that N-methyl phenyl isoxozolium cation reacts with a carboxylate to form an enol ester under mild reaction conditions. Therefore when allowed to react, Woodward's reagent K would block the free carboxyls of aspartate and glutamate residues of a protein.

When pectate lyase was treated with 10 mM WRK, the enzyme lost total activity along with the loss of metal ion as determined by atomic emission spectroscopy. EGTA, a specific calcium chelator, at 2 mM concentration also inhibited the enzyme through the loss of the Ca^{++} ion.

The molar ratio of metal to protein was 0.82, showing that a single Ca^{++} ion must be bound to the pectate lyase.

Partial blocking by WRK (Fig. 2.10) modified the enzyme at the carboxyl sites and showed loss of activity in proportion to the loss of calcium. Thus the Ca^{++} ion must be held by carboxyl groups, either of aspartate or glutamate residues in the pectate lyase. These results clearly show that the pectate lyase is a metallo-protein.

Circular dichroism spectra of native and EGTA-treated enzyme (Fig. 2.11) were found to be similar, which showed that loss of Ca^{++} did not alter the overall secondary structure of the protein. Thus the metal ion does not appear to contribute to the conformation of the protein.

Yoder *et al* (1993) identified a putative Ca^{++}-binding site in the crystal structure of the pectate lyase C of *E.chrysanthemi* using heavy atoms like lutetium, uranium and lead. These atoms were located at a common site, 2.1 to 2.9 Å from three acidic residues, Asp^{131}, Glu^{166}, and Asp^{170}. They had suggested that these residues that are involved in heavy metal binding may be the putative Ca^{++}

Figure 2.10

Loss of calcium of pectate lyase with WRK inhibition.

The calcium content of 50 μM native pectate lyase, and 28%, 43%, 67%, 88% and 100% WRK-inhibited pectate lyase was determined.

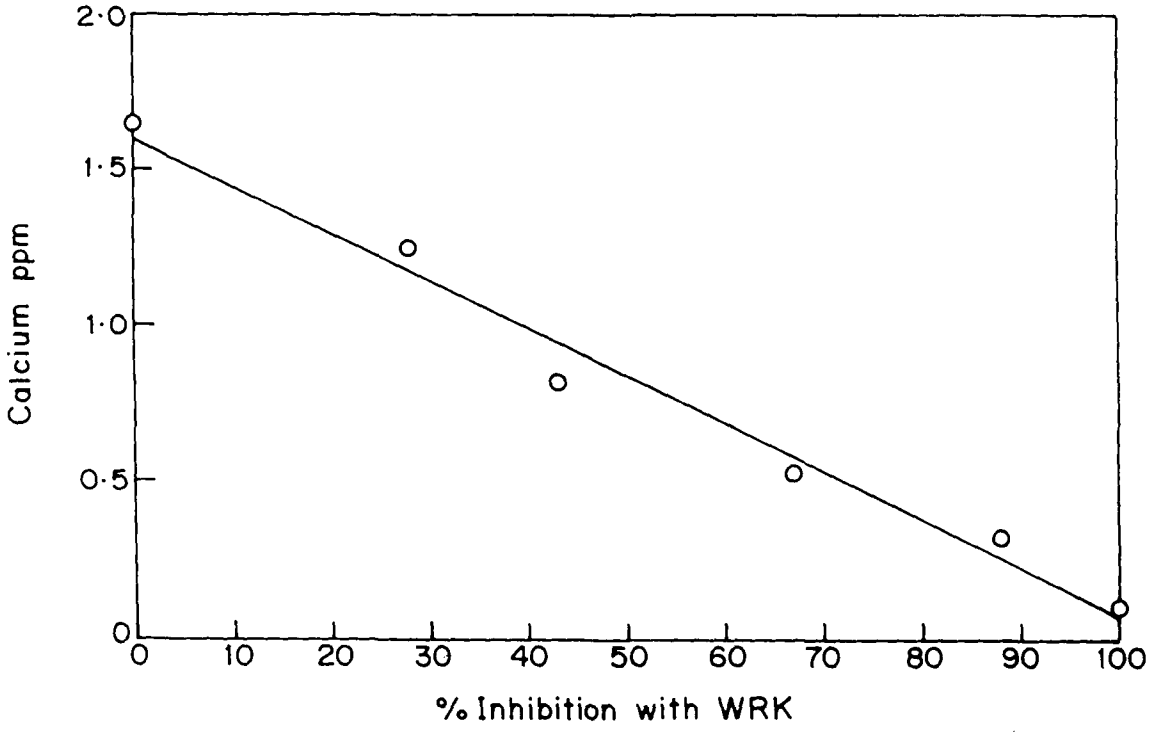
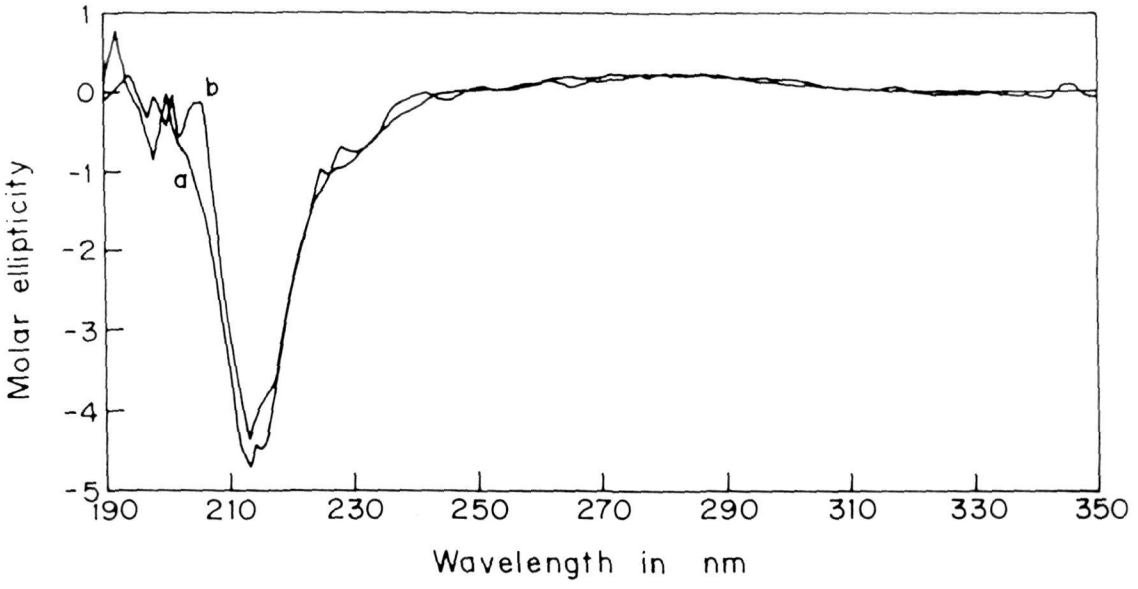


Figure 2.11

CD spectra of native and EGTA-treated pectate lyase.

Native enzyme (a), EGTA-pectate lyase (b).



binding site on the protein. Pickersgill *et al* (1994), in the crystal structure of pectate lyase of *Bacillus subtilis* had identified aspartic and glutamic residues involved in calcium binding. By using biochemical techniques and atomic emission spectroscopy, the pectate lyase of *Fusarium moniliforme* in solution form was shown to be a calcium containing metallo-protein, and that carboxyl groups are involved in calcium ion binding.

Spectrofluorimeter scans of the present enzyme, with and without substrate, showed that quenching of tryptophan fluorescence occurs in the presence of polygalacturonic acid indicating binding of substrate to the enzyme. This quenching of tryptophan fluorescence was found to occur identically irrespective of the presence or absence of Ca^{++} (Fig. 2.12) supporting the conclusion that Ca^{++} is not directly involved in substrate binding.

Kinetic analysis of the partially inhibited WRK-treated enzyme showed a decrease in Vmax but no change in Km (Fig. 2.13) in comparison to the native enzyme. Since the loss of Ca^{++} ion is proportional to WRK-modification, Ca^{++} is not required for substrate binding, but is necessary for catalysis. These results argue against the suggestions made by Crawford *et al* (1987) who said that Ca^{++} is required to complex the substrate but is not directly involved in catalysis.

Involvement of lysine and tryptophan in the active centre

Pectate lyase was modified with various inhibitors to determine the amino acid side-chains necessary for enzyme activity. There was complete inhibition of pectate lyase by succinic anhydride and TNBS (Table 2.7), indicating that an essential lysine residue was modified by these reagents. The enzyme was inactivated by DEP but there was no reversal of inhibition after addition of hydroxylamine, indicating that histidine was not modified.

Figure 2.12

Fluorescence quenching of pectate lyase in presence of substrate.

Emission scans of pectate lyase when excited at 297 nm. were recorded, (a) 0.25 mg/ml enzyme, (b) 0.25 mg/ml enzyme + 0.08 mg/ml polygalacturonic acid with 1 mM Ca^{++}, (c) 0.25 mg/ml enzyme + 0.08 mg/ml polygalacturonic acid with 1 mM EGTA.

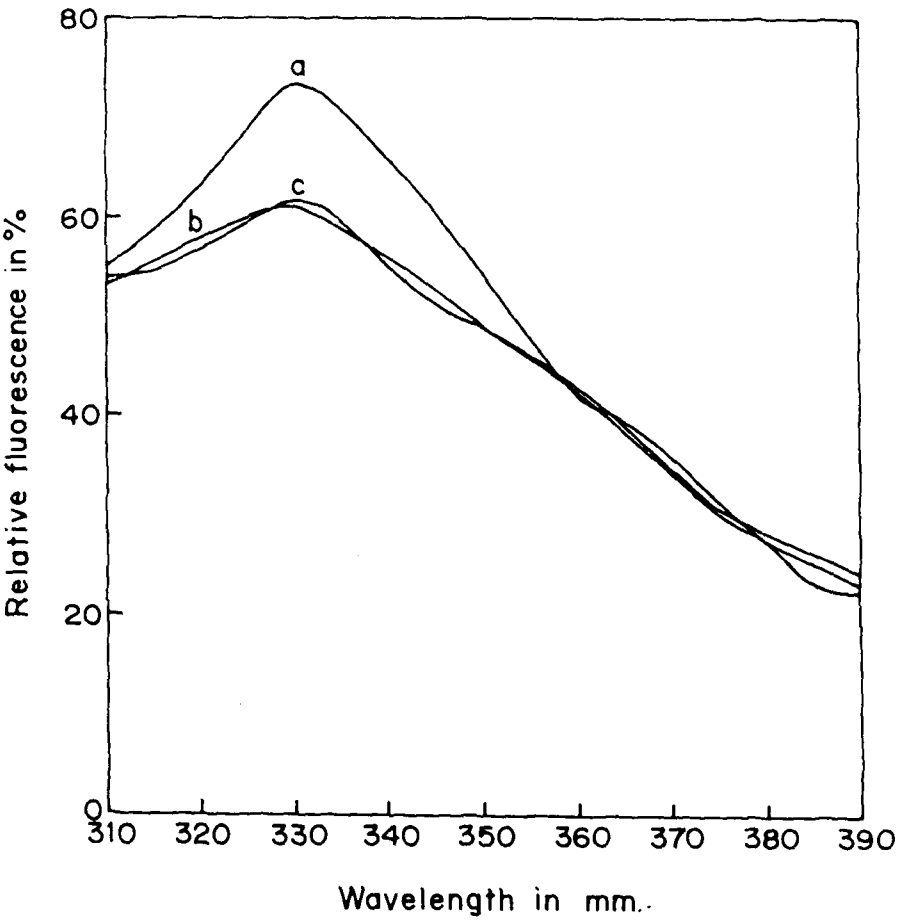


Figure 2.13

Kinetic constants of partially WRK-inhibited enzyme.

The Michaelis-Menten's constants of 67% inhibited (O), 43% inhibited (□), 28% inhibited (Δ) and native pectate lyase (●) were determined.

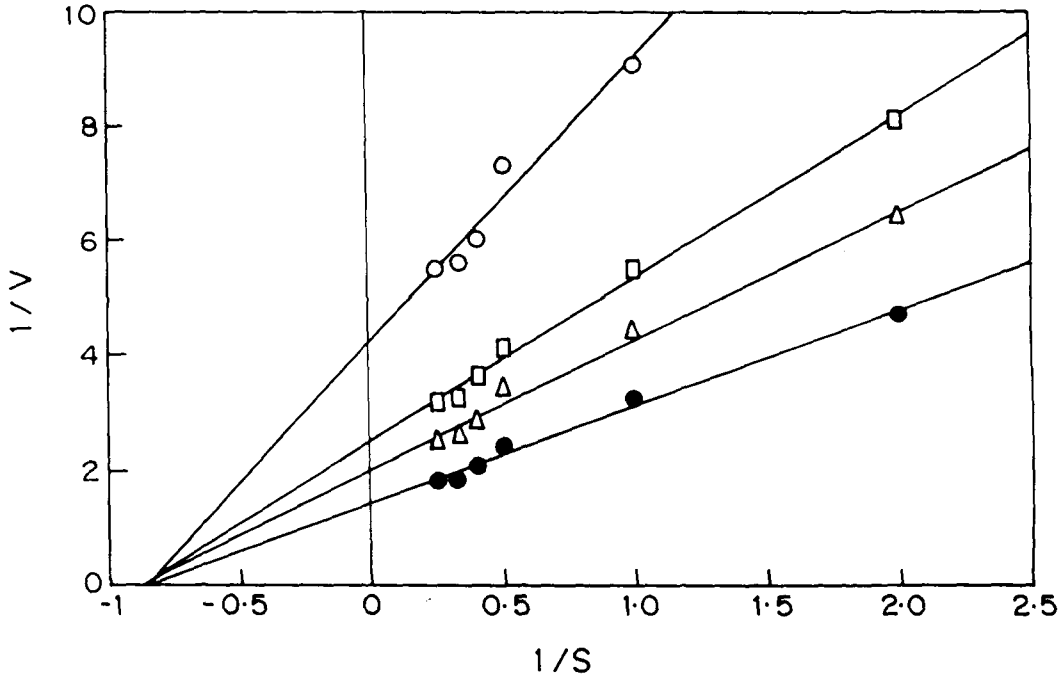


Table 2.7

Chemical modification of pectate lyase

Chemical	Conc. mM	Incubation buffer salt, mM, pH	% Inhibition
N-Acetyl imidazole	10	Phosphate, 50, 7.0	0
PHMB	5	Phosphate, 50, 7.0	0
DTNB	10	Phosphate, 50, 8.0	0
Succinic anhydride	5	Phosphate, 50, 8.0	100
Phenyl glyoxal	10	Carbonate, 50, 8.0	0
DEP	5	Phosphate, 50, 8.0	95
HNBB	10	Phosphate, 50, 7.0	100
TNBS	5	Phosphate, 50, 8.0	100
PMSF	5	Phosphate, 50, 7.0	0

Furthermore pH dependence of inhibition by DEP showed that maximum inactivation was between pH 9.5 to 10.5 (Fig. 2.14), all of which indicate reaction of DEP with lysine residue.

There was no inhibition with DTNB or PHMB therefore the role of cysteine in enzymic activity could be ruled out.

Inhibition by HNBB indicates presence of a tryptophan residue necessary for activity.

2 mg/ml concentration of polygalacturonic acid, added prior to the addition of the inhibitors (succinic anhydride, TNBS, DEP and HNBB) gave complete protection against inhibition. This indicates presence of essential lysine and tryptophan residues at or near the active-site of this pectate lyase.

The inhibition kinetics of pectate lyase by TNBS have been shown in Fig. 2.15. The percentage inhibition was measured at different time intervals and for

Figure 2.14

pH dependent inhibition by DEP

The percent inhibition of 0.73 μM pectate lyase with 5 mM DEP at different pHs was determined.

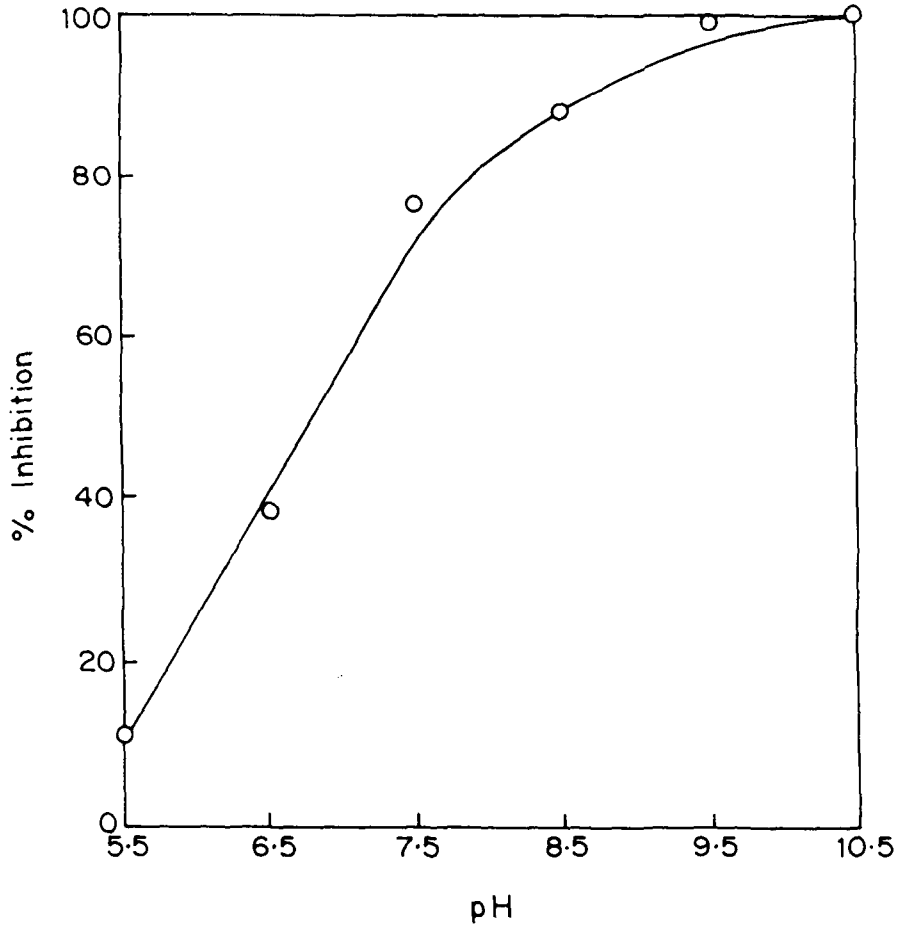
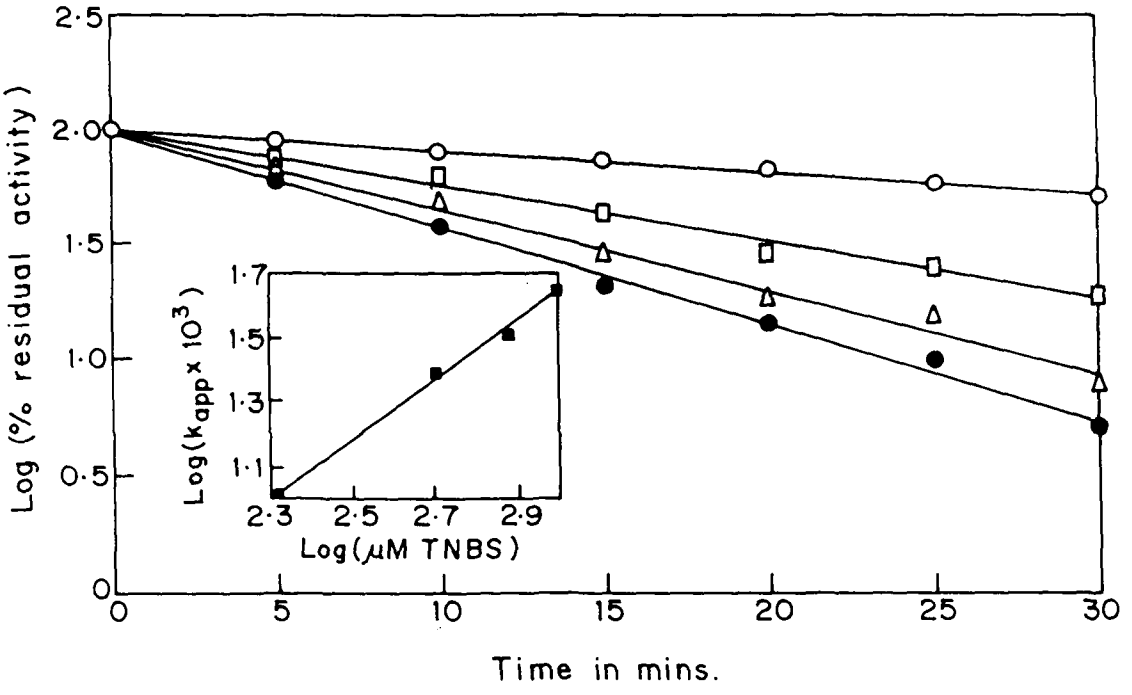


Figure 2.15

Kinetics of inhibition of pectate lyase by TNBS.

The pseudo first-order rate constants (k_{app}) at 0.2 mM (○), 0.5 mM (□), 0.75 mM (Δ) and 1.0 mM (●) TNBS were plotted against the inhibitor concentrations (inset plot) to obtain the order of the reaction.



different inhibitor concentrations. The inhibition followed pseudo- first order kinetics. The logarithm of the percentage inhibtion is plotted against time to obtain the rate constants. The logarithm of the pseudo-first rate constants were then plotted (inset plot) against the logarithm of inhibitor concentration. The slope for TNBS inhibition was determined to be 0.94.

Similar kinetic studies were done for HNBB inhibition of pectate lyase (Fig. 2.16). The pseudo-first order rate constants were determined by plotting the semi-logarithmic plot of inhibition at different concentrations of HNBB. The order of the inhibition reaction (inset plot) was determined to 0.96. Thus the inactivation of the enzyme with both the inhibitors followed a single exponential.

The amino-acid analyses of the native, TNBS-treated and substrate-protected TNBS-treated pectate lyase (Table 2.8) showed that a single residue of lysine is present at or near the active-site, which is protected by substrate against reaction by the inhibitor.

Figure 2.16

Kinetics of inhibition of pectate lyase by HNBB

The pseudo first-order rate constants (k_{app}) at 0.75 mM (O), 1 mM (□), 1.5 mM (Δ) and 2 mM (●) HNBB were plotted against the inhibitor concentrations (inset plot) to obtain the order of the reaction.

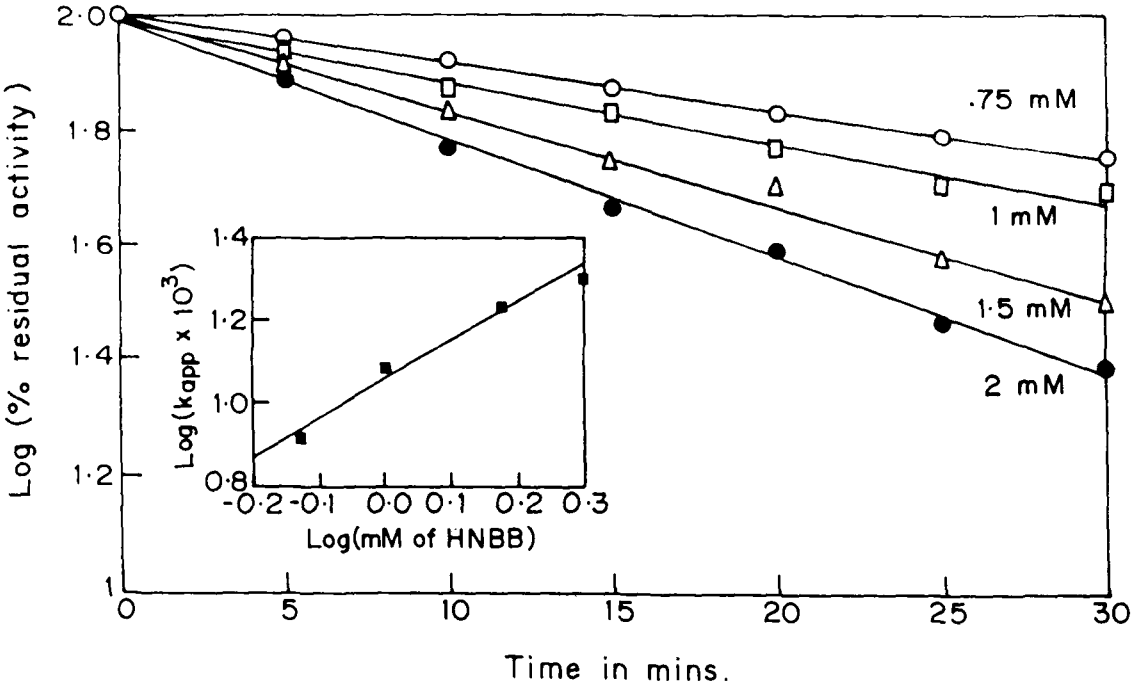


Table 2.8

Amino acid analyses for determination of number of essential lysine residues

Residue	Native PL	TNBS - treated	Substrate protected
Asx	10.8	11.1	10.4
Thr	7.8	7.8	8.3
Ser	6.7	6.6	7.4
Glx	7.7	8.1	8.3
Gly	12.1	12.1	12.5
Ala	6.6	6.9	7.2
Val	6.0	5.8	6.1
Cys	0.8	-	-
Ile	3.2	3.0	3.6
Leu	3.6	3.7	4.1
Met	0.7	-	-
Tyr	4.7	4.9	5.2
Phe	2.7	2.7	3.3
His	1.6	2.0	2.4
Lys	6.6	2.1	3.4
Arg	2.2	2.3	2.1
Trp	3.1	-	-

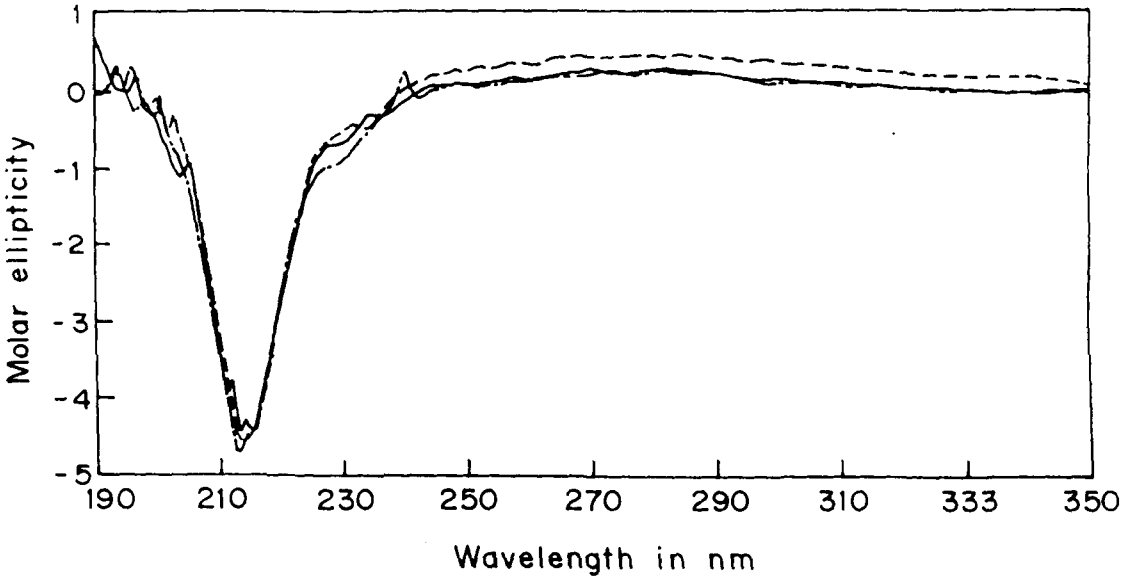
Thus, the inhibition kinetics with TNBS, which follows a single exponential is due to the inactivation of a single essential lysine residue of the enzyme.

The CD spectra of native and chemically modified pectate lyase were found to be similar (Fig. 2.17). This indicates that there were no major structural changes in the protein induced by the chemical reagents, and the loss of activity by chemical modification was a result of a specific blocking of essential amino acid side-chains at

Figure 2.17

CD spectra of native and chemically modified pectate lyase.

CD spectra of native enzyme (—), TNBS-inhibited enzyme (– – –) and HNBB-inhibited enzyme (· - · -) were obtained.



or near the active-site and not due to structural perturbations of the enzyme molecule.

Role of lysine and tryptophan in the active-site of pectate lyase

Kinetic analysis of partially inhibited enzyme was done to evaluate the involvement of lysine and tryptophan in enzyme activity. Kinetic constants of the partial HNBB- inhibited enzyme showed a decrease in Vmax and an increase in Km in proportion to the inactivation (Fig. 2.18), whereas the partial TNBS-inhibited enzyme showed a decrease in the Vmax but the Km remained constant (Fig. 2.19). This suggests that the tryptophan residue could be involved in substrate binding, whereas the lysine could be involved in catalysis. Helene *et al* (1991) had also suggested that increase in the Km of modified enzyme was due to the involvement of arginine, histidine and acidic residues in substrate binding in the active site of aminopeptidase N.

Affinity of the enzyme to polygalacturonic acid and CM- Sephadex was used to evaluate the role of the lysine and tryptophan residues in the enzyme activity. As shown in Table 2.9, lysine-blocked enzyme co-precipitated with polygalacturonic acid on addition of calcium, whereas the tryptophan-blocked enzyme was retained in the supernatent.

Table 2.9

Affinity to polygalacturonic acid

	% Protein in supernatant	% Activity in supernatant
Native enzyme	7	4
HNBB- treated enzyme	95	0
TNBS- treated enzyme	6	0

1ml of polygalacturonic acid soln (0.3 mg/ml) was added to 1ml of enzyme (8µg/ml). 20 µl of 1 M $CaCl_2$ was added and the precipitate was centrifuged. The supernatant was checked for activity and protein.

This would be the case if tryptophan rather than lysine was involved in substrate binding. Secondly although the native protein bound irreversibly to CM-Sephadex, the presence of polygalacturonic acid inhibited such binding probably because the

Figure 2.18

Kinetic constants of partially HNBB-inhibited pectate lyase.

Partially HNBB-inhibited enzyme were obtained by stopping the inhibition reaction of 0.73 μM enzyme with 2 mM HNBB, by addition of 20 mM tryptophan at different intervals. After dialysis, the Michaelis-Menten's constants of 82% inhibited, 60% inhibited, 32% inhibited and native enzyme were determined.

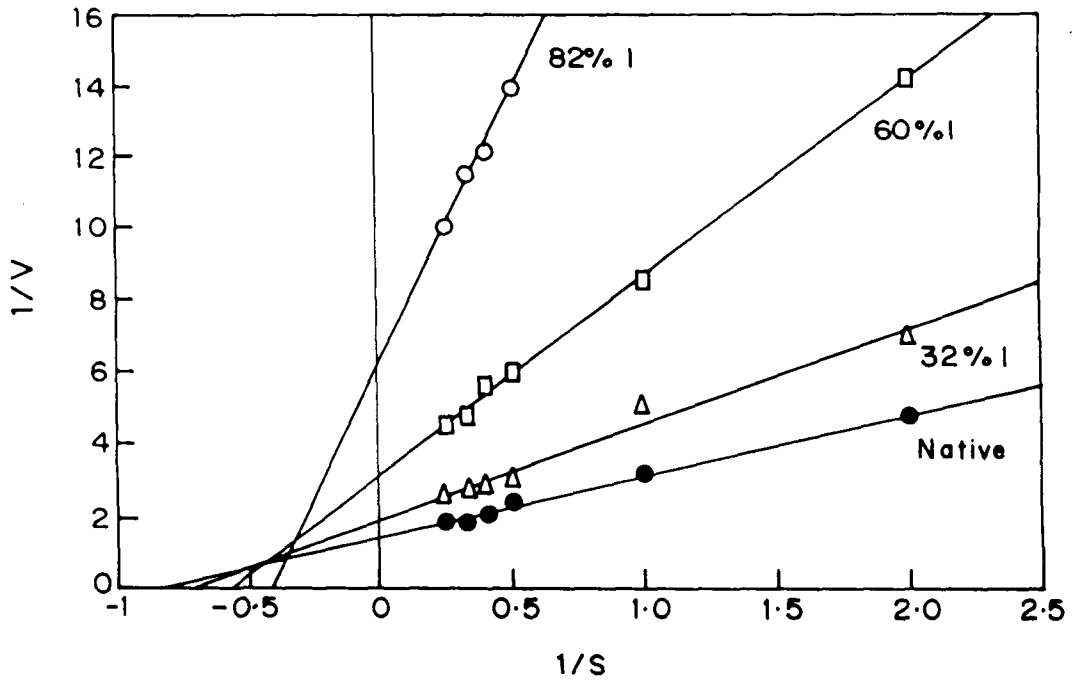
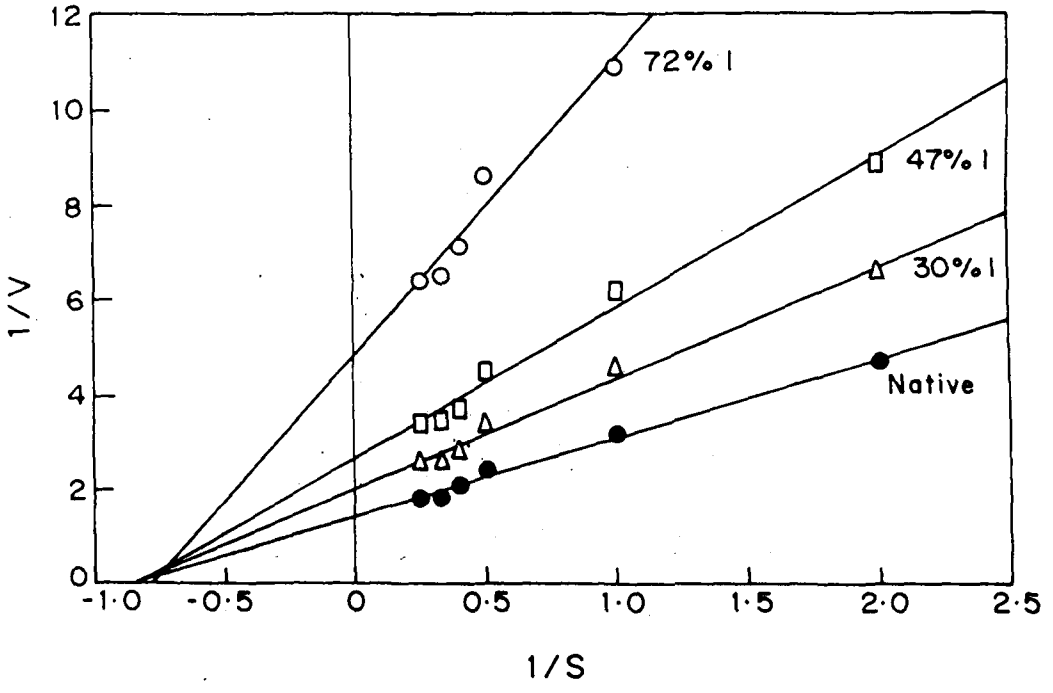


Figure 2.19

Kinetic constants of partially TNBS-inhibited pectate lyase.

Partially TNBS-inhibited enzyme were obtained by stopping the inhibition reaction of 0.73 μM enzyme with 0.75 mM TNBS, by addition of 20 mM lysine at different time intervals. After dialysis, the Michaelis-Menten's constants of 72% inhibited, 47% inhibited, 30% inhibited and native enzyme were determined.



binding took place through the active-site. The presence of a common carboxyl and a pyranose ring in the structures is the most probable reason for CM-Sephadex to act as a substrate analog. Tryptophan-blocked enzyme did not bind to CM-Sephadex, whereas the lysine-blocked enzyme was bound to the matrix (Table 2.10).

Table 2.10

Affinity to CM-Sephadex

	% Protein in supernatant	% Activity in supernatant
Native enzyme	0	0
HNBB- treated enzyme	98	0
TNBS- treated enzyme	0	0

1ml of enzyme (8μg/m) was mixed with 1ml of presoaked CM-Sephadex.
The supernatent was checked for activity and protein.

Tryptophan residues in a protein fluoresce when excited at 297 nm. Fluorescence of pectate lyase when excited at 297 nm. was quenched in the presence of polygalacturonic acid (Fig. 2.20). Increasing concentration of the substrate proportionately effected quenching of fluorescence. These results strongly argue that tryptophan was involved in substrate binding.

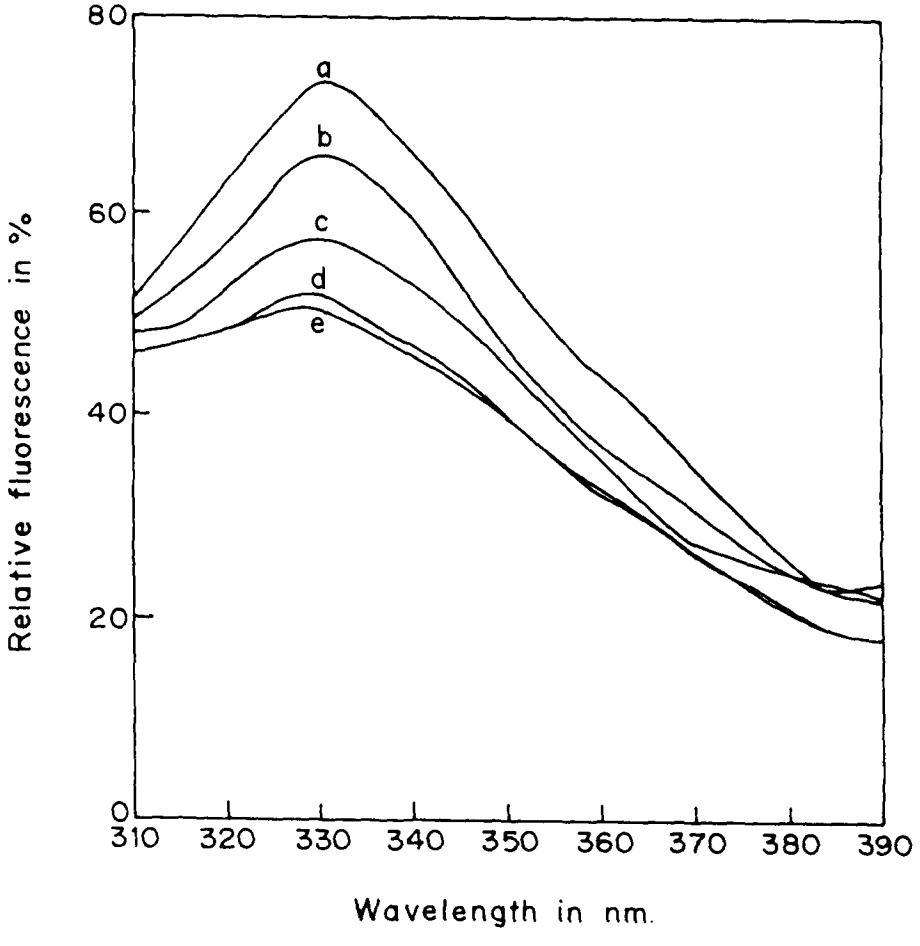
Tryptophan is reported to be present in the active site of various carbohydrases such as endo-1,4- ß-glucanase of *Bacillus* sp.KSM-330 (Ozaki 1991), exo-(1-3)- ß-D-glucanase from *Rhizoctonia solani* (Ohio 1989), the enzyme exhibiting both isomalto-dextranase from *Arthrobacter globiformis* T6 (Okada 1988), and xylanase from *Streptomyces* (Keskar 1989) and also in the active site of Abrin-a, a divalent lectin (Ohba 1991). Tryptophan appears to have affinity to carbohydrates and its involvement in substrate binding of carbohydrate-degrading enzymes therefore is not surprising.

Lysine has been reported to be involved in the catalytic site of arginosuccinase (Lusty 1987), γ-cystathionase (Fearon 1982) and aspartate aminotransferase (Morino 1978) probably as a proton acceptor. In the present case

Figure 2.20

Fluorescence quenching of pectate lyase at different substrate concentrations.

Emission scans of pectate lyase when excited at 297 nm. were recorded, (a) 0.25 mg/ml enzyme,(b) 0.25 mg/ml enzyme + 0.5 mg/mg/ml polygalacturouronic acid, (c) 0.25 mg/ml enzyme + 0.1 mg/ml polygalacturonic acid, (d) 0.25 mg/ml enzyme + 0.15 mg/ml polygalacturonic acid and (e) 0.25 mg/ml enzyme + 0.2 mg/ml polygalacturonic acid.



we suggest that lysine is a good candidate for proton abstraction in the ß-elimination cleavage of polygalacturonic acid.

Secondary structure of pectate lyase

Circular dichroism has been an alternate biophysical technique in structural analysis of proteins. The CD spectrum of the native pectate lyase (Fig. 2.11) was investigated to correlate the shape of the spectrum with the tertiary class of the protein as described by Venyaminov (1994). Cluster analysis using pattern recognition algorithms showed that our pectate lyase falls in the all-ß tertiary structure class. Yodder *et al* (1993) have shown from its crystal structure that the *Erwinia* pectate lyase folds into a unique motif of parallel ß strands coiled into a large helix. The authors had also suggested that because of the sequence similarities, all extracellular pectate lyases may have a common ß helix motif, differing only in the size and confirmation of the protruding loops. The crystal structure of pectate lyase of *B. subtilis* also has a similar topology of a parallel ß-helix. The dominance of ß-folds in the stucture of the present pectate lyase suggests that this protein would have a similar tertiary structure as other reported bacterial pectate lyases.

Reaction mechanism of pectate lyase

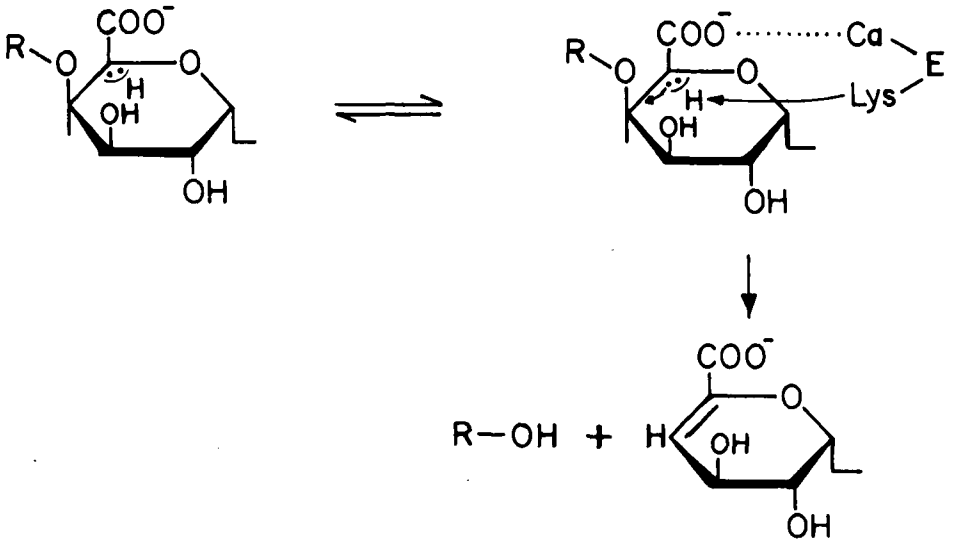
The catalytic mechanism of the degradation of polygalacturonic acid by pectate lyase is a typical ß- elimination process (Rexova-Benkova 1967). The basic mechanism is illustrated in Scheme 2.1, in which a proton is abstracted from C-5 of one galacturonide moiety by the enzyme. The abstraction of a proton, possibly by the ε- amino group of lysine in the active-site of the enzyme, results in the breaking of the C-O bond and formation of a double bond between C-4 and C-5.

The rapid rates of enzyme-catalyzed abstraction of proton from a carbon adjacent to a carbonyl or carboxyl group would not be explained by a simple base-catalyzed heterolytic cleavage of C-H bonds. Although the α-proton of a carbon acid is more acidic than an aliphatic proton, the inductive effect of the carboxyl group is insufficient to decrease the pK_a difference of the α-proton and the catalytic base. According to Gerlt and Gassman (1992), the pK_a of the carbon acid would be sufficiently decreased by an additional acid catalyst acting on the carbonyl or carboxyl group. These authors have suggested that concurrently occuring general-

Scheme 2.1

Catalytic mechanism proposed for ß-elimination by pectate lyase

A concerted nuclephilic-electrophilic attack by lysine and calcium of the enzyme is proposed to catalyze the cleavage of the α-1,4-glycosidic linkage, with formation of a double bond between C-4 and C-5



acid:general base-catalysis would explain the high rates of ß-elimination reactions. They later (Gerlt and Gassman 1993) proposed that pre-positioning of the electrophilic catalyst adjacent to the carboxyl group would decrease the intrinsic kinetic component of the activation energy barrier of the reaction, thus creating a climate favourable to base catalyzed proton abstraction from the α-carbon.

In extending the above theory of a concerted acid-base catalysis to the ß-elimination reaction of pectate lyase, the abstraction of the C-5 proton by the base, presumed to be the ε-amino group of lysine, would require a general acid catalyst acting on the carboxyl group. We propose that the Ca^{++} of the pectate lyase would satisfy such a requirement by acting as a Lewis acid. The polarization of the carboxyl group by Ca^{++} would acidify the α-proton at C-5 sufficiently for abstraction by the catalytic base of the enzyme. Although calcium is generally known to have structural role in most calcium containing metallo-enzymes, it has also been reported to be catalytically important in some enzymes. For example, Cotton *et al* (1979) had proposed that calcium facilitates the nucleophilic attack of staphylococcal nuclease on thymidine 3',5'-biphosphate, by polarizing the 5'-phosphate group. Similarly Suck and Oefner (1986) have proposed that Ca^{++} of bovine pancreatic deoxyribonuclease I facilitates nucleophilic attack on the substrate through polarization of a phosphate group.

Though Pearson (1968) in his theory of hard and soft acids and bases (HSAB), had grouped calcium in the category of hard Lewis acids, with low polarizability, it would not be too difficult to visualize an entirely different environment for the calcium coordinately bound in a protein molecule. Williams (1971) had proposed that such a micro-environment as exists in a metallo-protein would lead to an energised ground state of the metal ion, termed as the "entactic state", capable of decreasing the activation energy for a reaction. It is therefore proposed that pectate lyase catalyzes a concerted general-acid:general-base ß-elimination, using Ca^{++} as a Lewis acid and the ε-amino group of lysine as a base.

Conclusion

The strain of *Fusarium moniliforme* isolated from an estuarine environment produced an interesting pectate lyase. The above study has shown the requirement of a lysine, tryptophan and calcium ion in the activity of the pectate lyase from *F.moniliforme*. Tryptophan was found to be involved in substrate binding whereas lysine is suggested to play the role of proton-acceptor in the reaction mechanism of the enzyme. The ß-elimination cleavage would involve proton-abstraction from α-carbon of the galacturonide monomer of the substrate by the enzyme with a concerted electrophilic attack by calcium.

CHAPTER 3

CHARACTERIZATION OF ENDO-POLYGALACTURONASE FROM *ASPERGILLUS USTUS*: ELUCIDATION OF ENZYME MECHANISM

SUMMARY

The mechanism of action for the hydrolysis of polygalacturonic acid by the enzyme endo-polygalacturonase (poly (1,4-α-D-galacturonide) glycanohydrolase, EC 3.2.1.15) was investigated. The enzyme from *Aspergillus ustus* was purified to homogeneity and used for the study. The endo-polygalacturonase had a molecular weight of 36,000 daltons, a pI of 8.3, specific activity of 785 units/mg., Km of 0.82 mg/ml, and Vmax of 976 micromoles of product min^{-1}, mg^{-1}. Amino-acids involved in the catalysis were identified by chemical modification and the active-site characterized. Inhibition by hydroxynitrobenzyl bromide and diethylpyrocarbonate, followed by substrate protection studies showed that tryptophan and histidine were involved at or near the active-site. Analysis of the inhibition showed that it follows pseudo-first order kinetics. Kinetic constants of partially inhibited enzyme, suggest the involvement of tryptophan in substrate binding and histidine in catalysis. Quenching of tryptophan fluorescence of the enzyme in the presence of polygalacturonic acid, substantiated the conclusion that tryptophan was involved in substrate binding. An isotope effect of 1.8 was observed with deuterated water on the Vmax of the endo-polygalacturonase, with the proton inventory giving a linear relationship. The proposed mechanism involves a single proton transfer from the histidine residue of the enzyme to the glycosidic oxygen and subsequent hydrolysis by the addition of a water molecule.

INTRODUCTION

Endo-polygalacturonases catalyze the hydrolysis of polygalacturonic acid in a random fashion. Preliminary characterization of purified enzymes from various sources have been reported (Table 1.2). Most of these proteins have an optimum pH between 4.0 to 5.0, optimum temperature between 30° to 40° C and a molecular mass around 30,000 to 40,000 daltons.

Chemical modification studies, used to characterize the active-site of this enzyme, have been described. Rexova- Benkova (1968) and Cooke (1976) have reported the importance of histidine in the catalysis of endo-polygalacturonase of *A.niger*, whereas Urbanek and Sobczak (1975) had implicated cysteine and tyrosine in the active-site of the enzyme from *Botrytis cinera*. A detailed active-site characterization of polygalacturonases has not yet been reported.

Isotope effect on the kinetics of enzymes has yielded a lot of information on their mechanism of action (Northrop 1974, Rosenburg 1981, Umerzurike 1981, Schowwen 1982, Cleland 1987, Jeffcoat 1987, Julin 1989, LaReau 1989, Selwood 1990 and Pryor 1992). Isotope effect is caused by substitution of a heavy atom for a light one (^{2}H, ^{3}H for H, ^{13}C or ^{14}C for ^{12}C, ^{15}N for ^{14}N and ^{18}O for ^{16}O) in the substrate, solvent or the enzyme.

Proton inventories, where the rate constants are measured in different fractions of $^{2}H_2O$, are useful in determining the number of hydrogenic sites contributing to the overall isotopic effect (Schowwen 1982, Cleland 1987, Selwood 1990).

The mechanism of hydrolysis of the substrate by polygalacturonase has not been investigated yet.

The present study involves a detailed active-site characterization of the endo-polygalacturonase of *Aspergillus ustus* NCIM 1033, by chemical modification studies. The reaction mechanism of the enzyme was studied using deuterated water.

MATERIALS AND METHODS

Microorganism

Aspergillus ustus NCIM 1033 obtained from the National Collection of Industrial Microorganism, was subcultured on Czapek Dox agar, modified with the addition of 0.5 % pectin.

Enzyme assay

Polygalacturonase activity was assayed by measuring the reducing sugars released from 0.3% polygalacturonic acid in 0.1 M sodium acetate buffer, pH 5.0 at 40°C (Collmer 1988). One unit of enzyme activity was defined as the amount of enzyme which released 1 μmol of uronic acid per minute.

Purification of endo-polygalacturonase

Endo-polygalacturonase was purified from the culture broth of *Aspergillus ustus* NCIM 1033. The fungus was grown in medium containing 1% pectin, 0.2% glucose, 0.5% NH_4NO_3, 0.4% K_2HPO_4 and 0.1% $MgSO_4$, at pH of 5.0, at 30 °C on a rotatory shaker.

After an incubation period of 96 hours, the culture was filtered. All further steps of purification were done at 4- 6 °C. The culture filtrate was concentrated by ultrafiltration in an Amicon unit using a 10,000 daltons cut-out cellulase-acetate membrane.

The concentrate was then passed through a column of CM- Sephadex, 20 cm. length and 3.0 cm. I.D., equilibrated with 50 mM acetate buffer, pH 4.8. After washing off the unadsorbed protein, a linear gradient of 0.01-0.5 M NaCl in 0.1 M acetate buffer of pH 4.8 was applied to the column. The fractions of 2.5 ml each, were assayed for protein and polygalacturonase activity. The enzyme fractions were pooled and concentrated.

The concentrate, 2ml, was loaded on a column of Sephadex G-75, 1.2 m length and 1.5 cm. I.D., equilibrated with 50 mM acetate buffer, pH 4.8 with 0.1 M NaCl. Fraction of 2.5 ml were collected. The endo-polygalacturonase fractions were pooled and rechromatographed on the same gel- filtration column.

Homogeneity was determined by SDS- polyacrylamide gel electrophoresis using 10% polyacrylamide gel by the Laemmli (1970) method.

Characterization of enzyme

The molecular weight was determined by SDS-PAGE, using molecular weight markers of the range 14,000 to 66,000 daltons (Sigma Chemicals). and also by determining elution volume of the polygalacturonase on Sephadex G-75, along with standard proteins of the molecular weight range of 12,400 to 66,000 daltons (Sigma Chemicals).

The pH and temperature optima of the enzyme was determined. The Km and Vmax of the endo-polygalacturonase were obtained by the double-reciprocal plot of the rate of reaction at different substrate concentration.

Amino acid analysis was done on the Pharmacia LKB alpha plus amino acid analyser, by hydrolyzing the purified enzyme using 6 N HCl at 110 °C for 22 hours. Cysteine, methionine and tyrosine were protected. Tryptophan was determined by titration with N-bromosuccinimide (Edelhoch 1964).

Isoelectrofocussing was performed in the modified straight- tube method (Chinnathambi 1994) to determine the pI of the enzyme. Analytical isoelectrofocussing was performed in polyacrylamide gels using ampholines in the pH range of 3.5 to 10 (Righetti 1983).

Chemical modification studies

Chemical modification studies were carried out by incubating 0.14 nmoles of the purified enzyme in presence of various reagents at appropriate concentrations as listed in Table 3.2, specific for certain reactive side chains available in proteins, in 1 ml reaction mixture. After incubation for an hour, the enzyme-reagent mixtures were extensively dialyzed in respective incubation buffers (Table 3.2), and residual activity of the enzyme determined. The stock of HNBB was in prepared in 1,4-dioxane, the stock of WRK was in 1 mM HCl, whereas the stock of DEP was made in ethanol. The stocks of the other reagents were in corresponding incubation buffers as shown in Table 3.2. The incubation of TNBS with the enzyme was carried out in the dark.

Substrate protection studies were carried out by incubating the enzyme with the respective reagent in presence of 2 mg/ml polygalacturonic acid. Reversal of diethylpyrocarbonate inhibition by hydroxylamine was carried out by incubating 1 ml of 50 mM phosphate buffer pH 7.0 with 0.14 nmoles of enzyme with 5 mM DEP for 30 minutes. 1 ml of 2 M hydroxylamine in the same buffer, along with 2 mM EDTA was added to the DEP-inhibited enzyme. After an incubation for 60 minutes, the mixture was dialyzed in 0.1 M acetate buffer, pH 4.8. The activity of the dialyzed enzyme was determined, along with a control in which DEP was omitted in the above steps.

Kinetics of inhibition with DEP and HNBB were carried out by incubating the enzyme with different concentrations of the inhibitors, and determining the residual activity at each time interval.

Partially inhibited enzymes were obtained by stopping the inhibition reaction by the addition of specific amino-acid. Partial-DEP modified enzyme was obtained by the addition of 5 mM histidine to the reaction mixture of DEP and enzyme at intervals. Partial-HNBB modified enzyme was obtained similarly by the addition of 20 mM tryptophan to HNBB-enzyme reaction mixture. The Km and Vmax of partially inhibited polygalacturonase, with DEP and HNBB, were determined by the double-reciprocal plots and compared with native enzyme.

Fluorescence scans of the endo-polygalacturonase and the enzyme-substrate mixtures were obtained using a Kentron SFM-25 spectrofluorimeter. Tryptophan fluorescence was excited at 297 nm. using a 150 W Xenon-High pressure lamp and a monochromator with concave holographic gratings. Emission scans from 310 to 360 nm. were obtained using a similar monochromator and a R212 photomultiplier. The emission scan of the endo-polygalacturonase was obtained with 1.5 ml with 0.37 mg of enzyme mixed with 1.5 ml of acetate buffer, 0.1 M, pH 4.6. Scans of enzyme-substrate mixtures were taken by mixing 1.5 ml of enzyme (0.37 mg) and 1.5 ml of buffer with 0.05, 0.1, 0.15 and 0.2 mg/ml concentrations of polygalacturonic acid.

Kinetic isotope studies

Isotope effect on the kinetics of the endo- polygalacturonase was studied by incubating the substrate and the enzyme in $^{2}H_2O$ for twenty-four hours and then

used for determining the kinetic parameters of the enzyme in H_2O and ^2H_2O. The pH of the reaction mixture remained constant at 4.6, without addition of any buffer ions.

RESULTS AND DISCUSSIONS

Purification of endo-polygalacturonase

The endo-polygalacturonase was purified 41 fold, from the culture filtrate of *Aspergillus ustus* NCIM 1033 (Table 3.1).

Table 3.1

Purification of endo-polygalacturonase

	Total activity units	Total protein mg	Sp. activity units/mg	Yield %	Fold purification
Broth	15250	800	19.06	-	-
CM-Sephadex	14025	33.7	416.17	21.8	91.9
Spehadex G-75					
PG-I	4849	21.75	222.9	11.7	31.8
PG-II	8352	10.98	759.9	39.9	54.8
Spehadex G-75					
PG-II	7850	10	785	41.2	51.5

The enzyme fraction from the salt eluate from CM-Sephadex, when loaded on Sephadex G-75, yielded two peaks of polygalacturonase activity. The first peak, PG-I was determined to be an exo- polygalacturonase, while the second major peak, PG-II was an endo-polygalacturonase as determined by the ratios of the viscosity drop to percent reducing sugars released. The endo- polygalacturonase (PG-II) gave 50% reduction in viscosity, with release of only 5% of the total reducing sugars, whereas the exo-polygalacturonase (PG-I) showed only 8% reduction in viscosity with the release of 5% of the total reducing sugars. The PG-II peak was reloaded on Sephadex G-75 to obtain a homogenous peak (Fig. 3.1) of the endo-polygalacturonase.

The molecular weight of the endo-polygalacturonase, as determined on SDS-PAGE (Fig. 3.2) and molecular sieve chromatography, using molecular weight standards, was 36,000 daltons. The molecular weight of the endo-polygalacturonases of *A.niger* (Kester 1990) varies from 38,000 to 85,000 daltons, the enzyme from *A.nidulans* (Dean 1989) has a molecular weight of 40,000, the

Figure 3.1

Gel filtration of endo-polygalacturonase on Sephadex G- 75

The endo-polygalacturonase fractions from the first Sephadex G-75 chromatography was concentrated and reloaded on the same column.

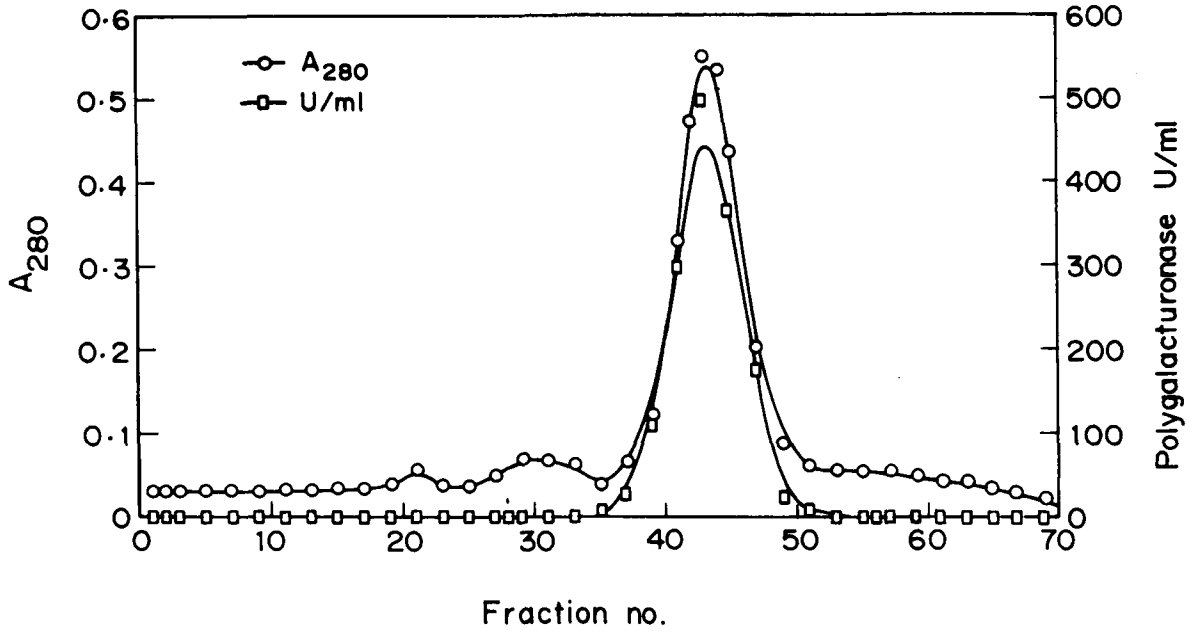


Figure 3.2

SDS-PAGE of endo-polygalacturonase with markers

10% acrylamide gel was used with Sigma (70L) molecular weight markers (from top to bottom) (a) Bovine serum albumin, 66 kDa; (b) Egg albumin, 45 kDa; (c) Glyceraldehyde-3- phosphate dehydrogenase, 36 kDa; (d) Carbonic anhydrase, 29 kDa; (e) Trypsinogen, 24 kDa; (f) Trypsin inhibitor, 20.1 kDa; and (g) α-Lactalbumin, 14.2 kDa.

enzyme from *A.japonicus* (Baldwin 1989) is 36,500 daltons, *Trichoderma kaningi* releases an endo-polygalacturonase of a molecular weight of 32,000 and the enzyme from *Neurospora crassa* is of 37,000 daltons. Thus it appears that most fungal endo-polygalacturonases have a molecular weight of around 30,000 to 40,000 daltons.

The isoelectric point of the enzyme was determined to be 8.3. Only a single molecular species of endo-polygalacturonase was found to be present without any isoforms (Fig. 3.3). In contrast, *Botrytis cinera* (Tobais 1993) releases 5 isoforms of polygalacturonase, four with acidic pI and one with an alkaline pI, *Sclerotina sclerotium* (Riou 1992) was reported to have numerous isoforms, only one of which has an alkaline pI of 8.3, and 5 isoforms of endo-polygalacturonase has been characterized from *Aspergillus niger* (Kester 1990) all of which have acidic pI.

The Km and the Vmax of the enzyme were determined to be 0.82 mg/ml and 976 units/mg. The optimum pH for activity was 4.6, while the optimum temperature was 50 °C. The amino acid composition of the endo-polygalacturonase was as shown in Table 3.2.

Table 3.2

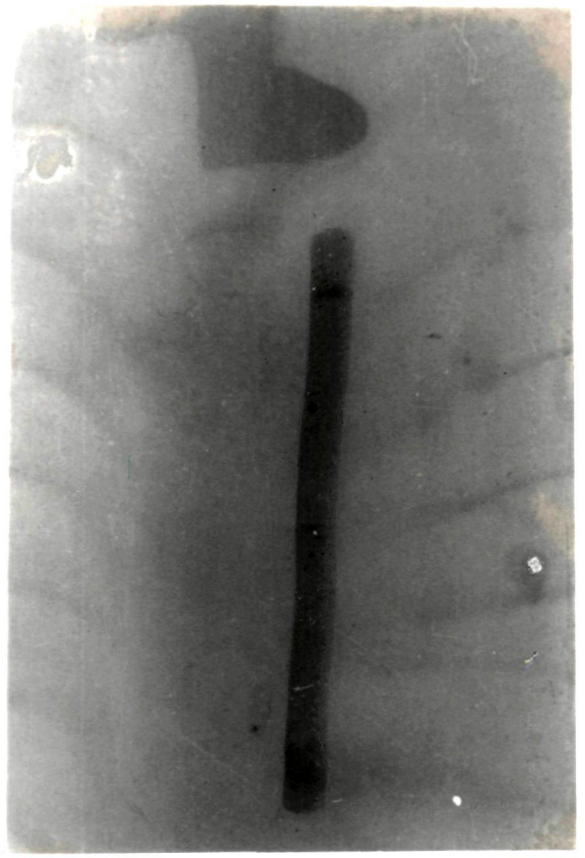
Amino acid composition of endo-polygalacturonase

Residue	Molar ratio
Asx	38
Thr	35
Ser	36
Glx	22
Gly	48
Ala	20
Cys	2
Val	23
Met	1
Ile	24
Leu	17
Tyr	13
Phe	13
His	7

Figure 3.3

Analytical isoelectrophoresis of endo-polygalacturonase

Isolectrofocussing was performed in 5% acrylamide gels with ampholines of the range 3.0 to 10.0



Active-site characterization

The chemical modification of the endo-polygalacturonase showed complete enzyme inhibition with DEP and HNBB (Table 3.3), whereas there was no substantial inhibition with the other modifiers. According to Horton *et al* (1965), HNBB reacts very specifically with tryptophan residues (Horton 1965) in acidic and neutral pH, but in alkaline pH, it is reactive to cysteine and tyrosine also. However, NEM and PHMB, which are specific for cysteine residues and N-acetylimidazole, specific for tyrosine, do not inhibit activity of this enzyme. Therefore loss of activity with HNBB is suggested to be due to tryptophan modification. Furthermore treatment of DEP-inhibited enzyme with hydroxylamine showed 92% reversal of activity, confirming that histidine residue was being modified by DEP which resulted in loss of activity of the endo-polygalacturonase. Thus both histidine and tryptophan is implicated in the activity of the enzyme.

Table 3.3

Chemical modification of endo-polygalacturonase

Chemical	Conc. mM	Incubation buffer salt, mM, pH	% Inhibition
N-Acetyl imidazole	10	Phosphate, 50, 6.0	0
PHMB	10	Phosphate, 50, 6.0	0
N-ethyl maleimide	5	Phosphate, 50, 6.0	0
Phenyl glyoxal	10	Carbonate, 50, 7.0	0
DEP	5	Phosphate, 50, 6.0	100
HNBB	20	Phosphate, 50, 6.0	96
TNBS	10	Phosphate, 50, 7.0	0
WRK	10	Phosphate, 50, 6.0	0
PMSF	5	Phosphate, 50, 6.0	0

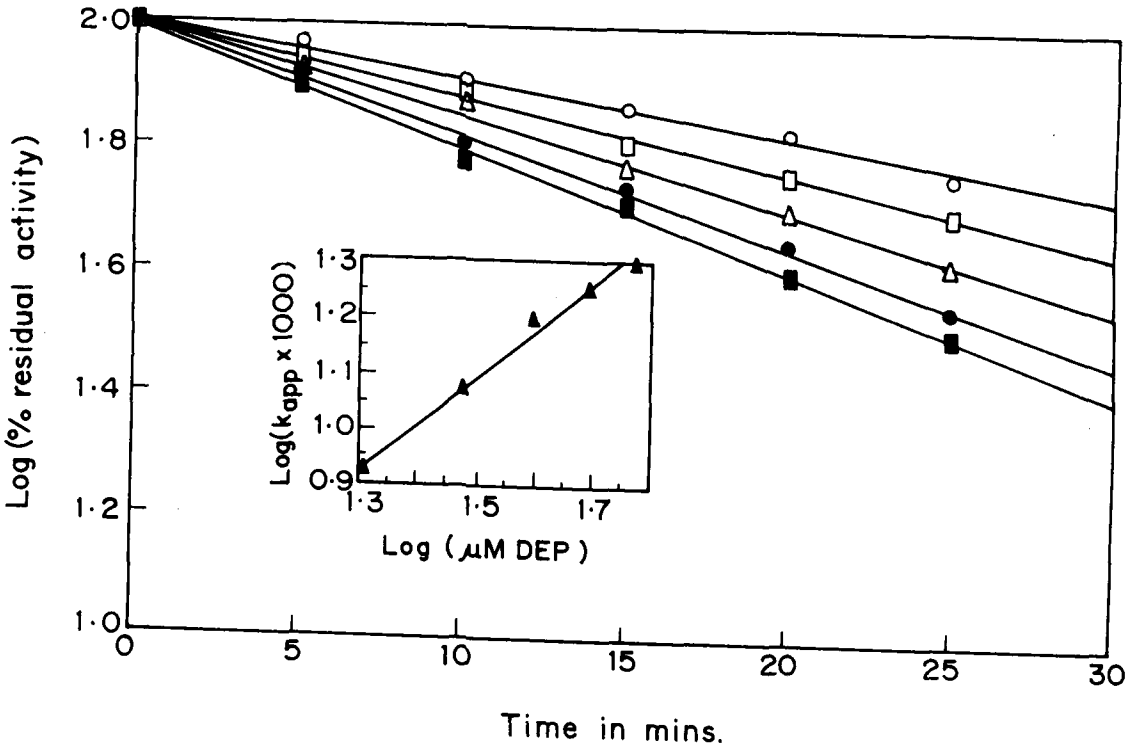
In the presence of substrate, the enzyme modified with DEP retained 90% activity, whereas the enzyme modified with HNBB showed 85% retention of activity. Substrate protection shows that the residues modified with specific inhibitors, are situated at or near the active-site of the enzyme.

Kinetic analysis of the inactivation of the enzyme by DEP, was done by plotting the log of residual activity against time at different concentrations of the inhibitor (Fig. 3.4). The plots indicate pseudo first-order rate kinetics, and applying the analysis described by Levy *et al* (1963), the rate constants (k_{app}) were plotted

Figure 3.4

Kinetics of inhibition of endo-polygalacturonase by DEP

The pseudo-first order rate constants (k_{app}) at 20 μM (O), 30 μM (□), 40 μM (Δ), 50 μM (●), 60 μM (■) were plotted against the inhibitor concentrations (inset plot).



against the logarithm of inhibitor concentration to obtain the order of the reaction. The plot gave a slope of 0.85, indicating that the inhibition kinetics follow a single exponential. Histidine has been earlier reported to be essential for the activity of polygalacturonase of *A.niger* (Rexova-Benkova 1968, Cooke 1976).

HNBB was found to inhibit endo-polygalacturonase completely, whereas in the presence of substrate, there was no inhibition. Inhibition kinetics of the enzyme with HNBB (Fig. 3.5), analyzed as earlier, gave the order of the reaction as 1.18, showing single exponential kinetics.

The emission scans between 310 to 360 nm. of the protein, when excited at 297 nm., showed tryptophan fluorescence. In presence of increasing concentrations of substrate, the fluorescence of the enzyme was found to be quenched in proportion to the concentration of polygalacturonic acid (Fig. 3.6). Thus binding of substrate to enzyme quenched the fluorescence implicating the presence of tryptophan at or near the active site. Waksman *et al* (1992) have earlier studied the reaction kinetics of the polygalacturonase from *Colletotrichum lindemuthianum*, by fluorescence quenching in the presence of the substrate polygalacturonic acid, indicating presence of tryptophan near the binding site. Urbanek and Sobczak (1975) had reported the inhibition of polygalacturonase from *Botrytis cinera*, by p-chloromercury benzoate and tetranitromethane, indicating the importance of cysteine and tyrosine for activity. We found no inhibition of the endo-polygalacturonase of *A.ustus* by PHMB or N-ethyl maleimide, specific for cysteine, nor by N-acetyl imidazole, a reagent specific for tyrosine, suggesting these residues are not important in the catalysis of this enzyme.

Comparison of the kinetic constants of the native endo- polygalacturonase and the partially inhibited enzyme, showed a close dependency on the residues modified. Partially active DEP-modified enzyme exhibited lower catalytic rates, while the Km remained the same (Fig. 3.7). On the other hand HNBB-modified partially active enzyme showed lowering of the catalytic rate as well as increase in Km (Fig. 3.8). Thus the HNBB-modified enzyme has a lower affinity to substrate, whereas the DEP-modified enzyme showed no alteration in the affinity to substrate. This suggests that the tryptophan could be involved in the binding of the substrate to the enzyme. It has been earlier reported that tryptophan is present in the active-site of various other saccharidases like endo-1,4-ß-glucanase (Horton 1965), exo-(1-3)-ß-D-

Figure 3.5

Kinetics of inhibition of endo-polygalacturonase by HNBB

The pseudo-first order rate constants (k_{app}) at 5 mM (O), 7.5 mM (□), 10 mM (Δ), 12.5 mM (●), 15 mM (■) were plotted against the inhibitor concentrations (inset plot).

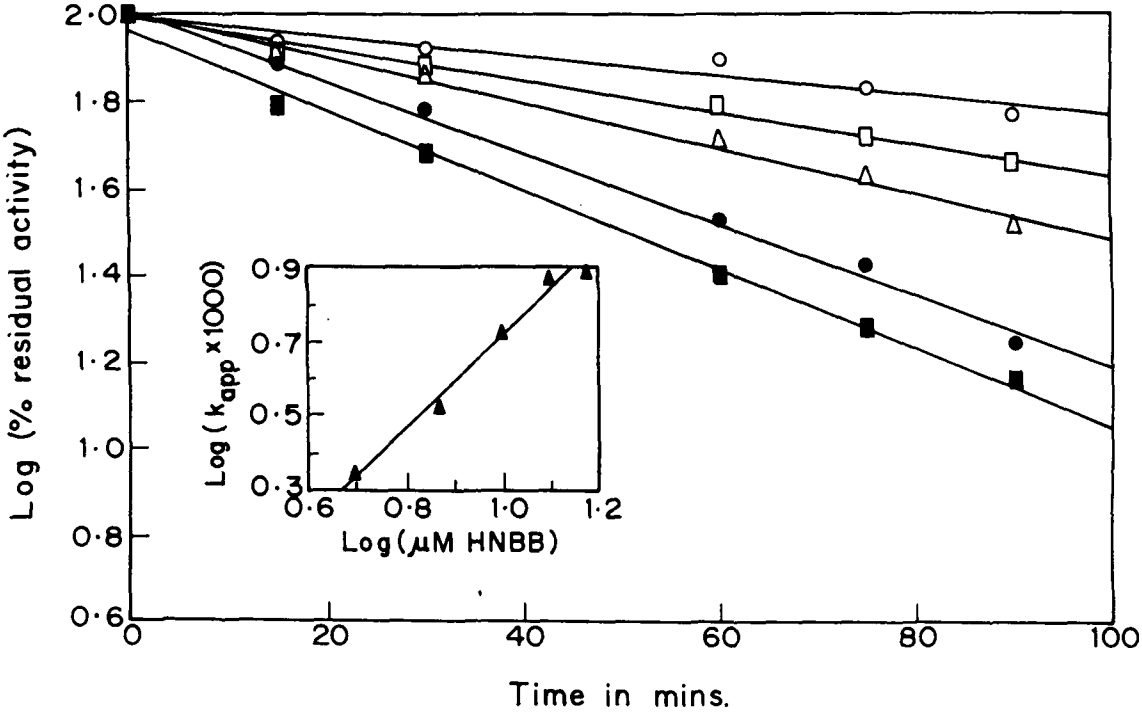


Figure 3.6

Fluorescence quenching of enzyme in presence of substrate.

Emission scans of endo-polygalacturonase when excited at 297 nm. (a) 7.5 uM enzyme, (b) 7.5 uM enzyme + 0.05 mg/ml polygalacturonic acid, (c) 7.5 uM enzyme + 0.1 mg/ml polygalacturonic acid, (d) 7.5 uM enzyme + 0.15 mg/ml polygalacturonic acid, (e) 7.5 uM enzyme + 0.2 mg/ml polygalacturonic acid.

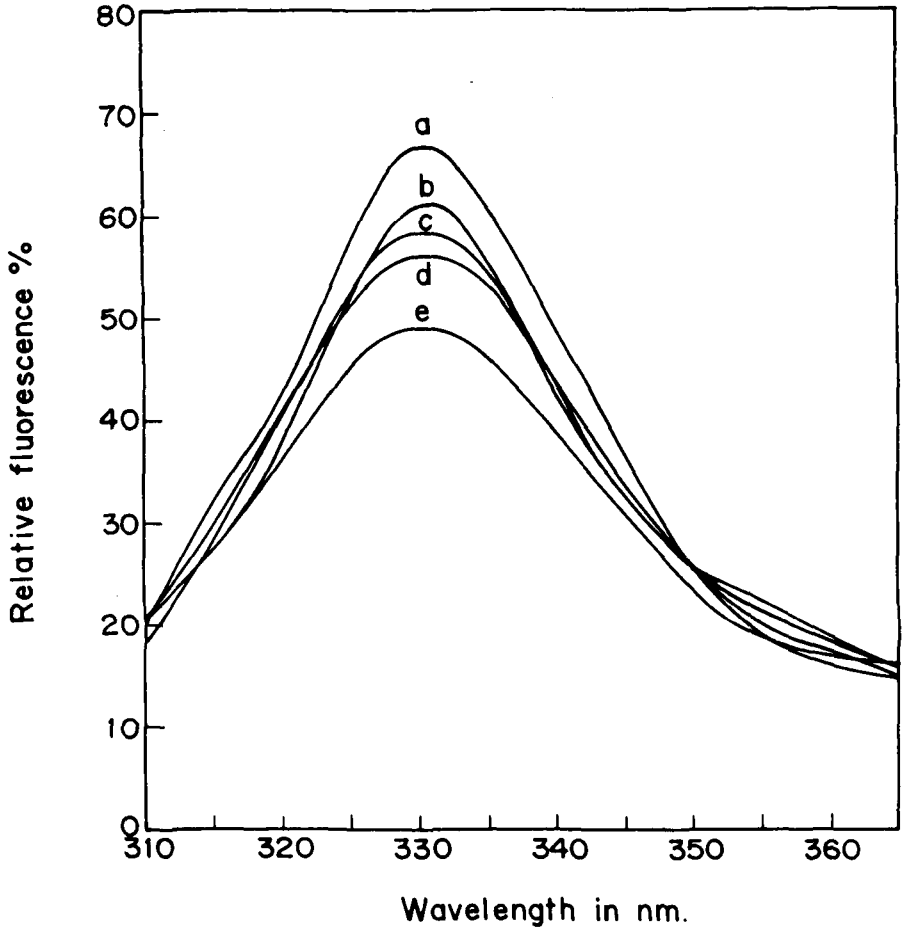


Figure 3.7

Determination of kinetic constants of partially DEP-inhibited enzyme

Partially DEP-inhibited enzyme was obtained by stopping the inhibition reaction of 0.14 nmoles of enzyme with 40 uM DEP, by addition of 10 mM histidine at different time intervals. After dialysis the Michaelis-Menten's constants of 65% inhibited (○), 46% inhibited (□), 30% inhibited (Δ) and native enzyme (●) were determined.

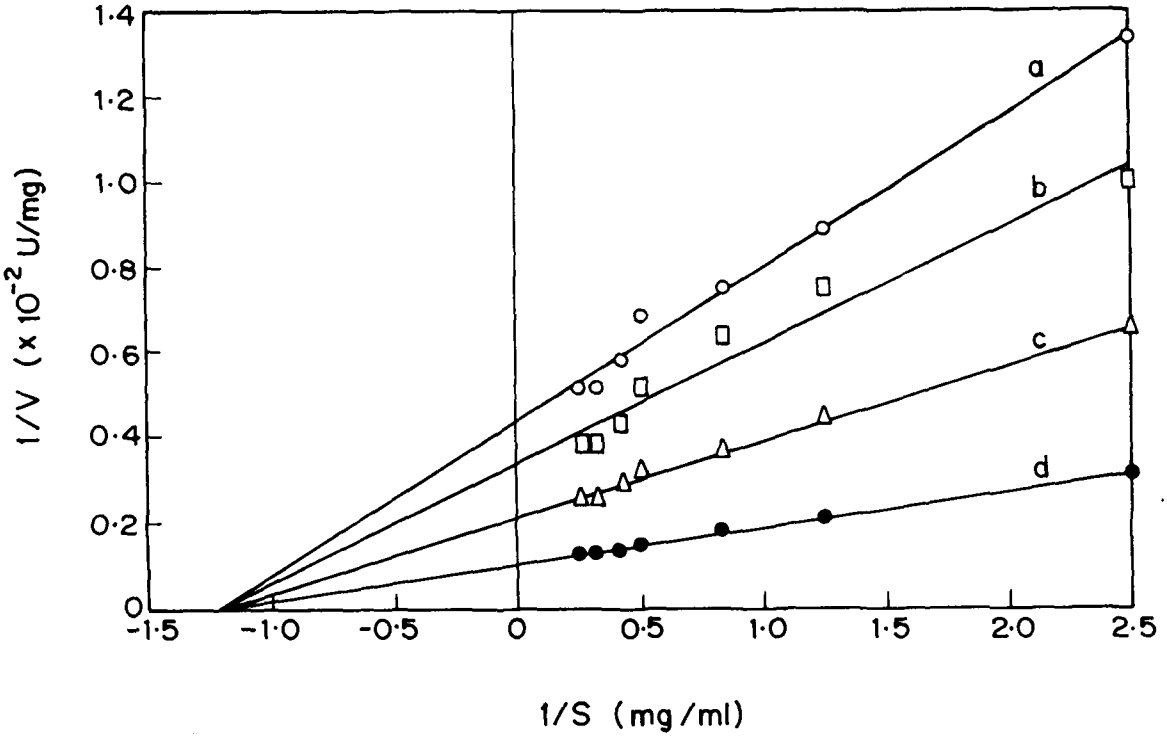
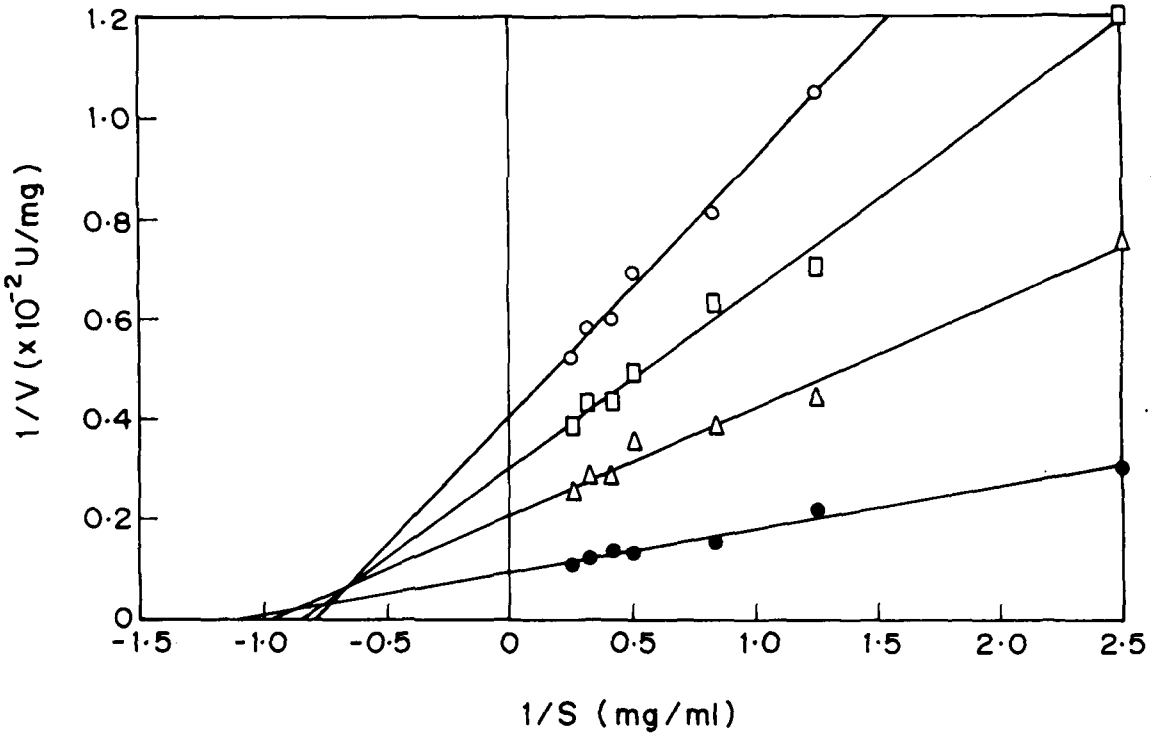


Figure 3.8

Determination of kinetic constants of partially HNBB-inhibited enzyme

Partially HNBB-inhibited enzyme was obtained by stopping the inhibition reaction of 0.14 nmoles of enzyme with 10 mM HNBB, by addition of 50 mM tryptophan at different time intervals. After dialysis, the Michaelis-Menten's constants of 69% inhibited (O), 48% inhibited (□), 22% inhibited (Δ) and native enzyme (●) were determined.



glucanase (Ohio 1989), isomalto-dextranase (Okada 1988) and xylanase (Keskar 1989). Tryptophan thus appears to have affinity towards carbohydrates, and could be involved in substrate binding in carbohydrate-degrading enzymes. It is likely that the histidine is involved in the catalytic process. Histidine has been implicated in catalysis of other hydrolases like exo-(1-3)-ß-D-glucanase (Ohno 1989), amino-peptidase (Helene 1991) and S1 nuclease (Gite 1992).

Elucidation of mechanism of enzyme action

In the presence of ^2H_2O, there was a distinct isotope effect on Vmax (Fig. 3.9), whereas the Km remained unchanged. An isotope effect of 1.8 was observed when the exchangeable protons of the enzyme were replaced by deuterium and H_2O was replaced by ^2H_2O as the solvent. The proton inventory, plotting Vmax at different fractions of ^2H_2O and H_2O against corresponding fraction, is linear (Fig. 3.10), with a correlation coefficient of 0.98, suggesting that a single proton transfer is the rate-limiting step in the catalysis by the endo- polygalacturonase. From the above findings, the mechanism of action of the enzyme seems to be a general acid-hydrolysis, involving the proton transfer from the imidazole of the histidine moiety in the active-site of this enzyme to the glycosidic oxygen of the substrate (Scheme 3.1). The mechanism of acid- hydrolysis appears to be similar to that reported for other carbohydrases such as α-L-arabinofuranosidase of *Monilinia fructigena* (Selwood 1990) and exo-ß-(1-3)-D-glucanase of a *Basidiomycete* sp. QM 806 (Jeffcoat 1987), and involves the protonation of the glycosidic oxygen, followed by attack of a water molecule on C-1. As suggested by Jeffcoat *et al* (1987), due to the electronegativity of oxygen, the rate-limiting step would be the protonation of the glycosidic oxygen atom linking two galacturonic acid residues, rather than the attack of water molecule on C-1 of one of the monomer. This protonation involves the breaking of the N-H bond and the formation of the O-H bond, and would favour the attack on the electrophillic carbon, thus produced by H_2O, resulting in hydrolysis. Due to its position between two oxygen atoms, C-1 would be more electron-deficient than C-4 of the second galacturonic residue, and hence H_2O would attack preferably on C-1.

Figure 3.9

Kinetic isotope effect on endo-polygalacturonase

Kinetic constants were determined of (□) deuterated enzyme in various concentrations of substrate in ^2H_2O, and (O) native enzyme in various concentrations of substrate in H_2O.

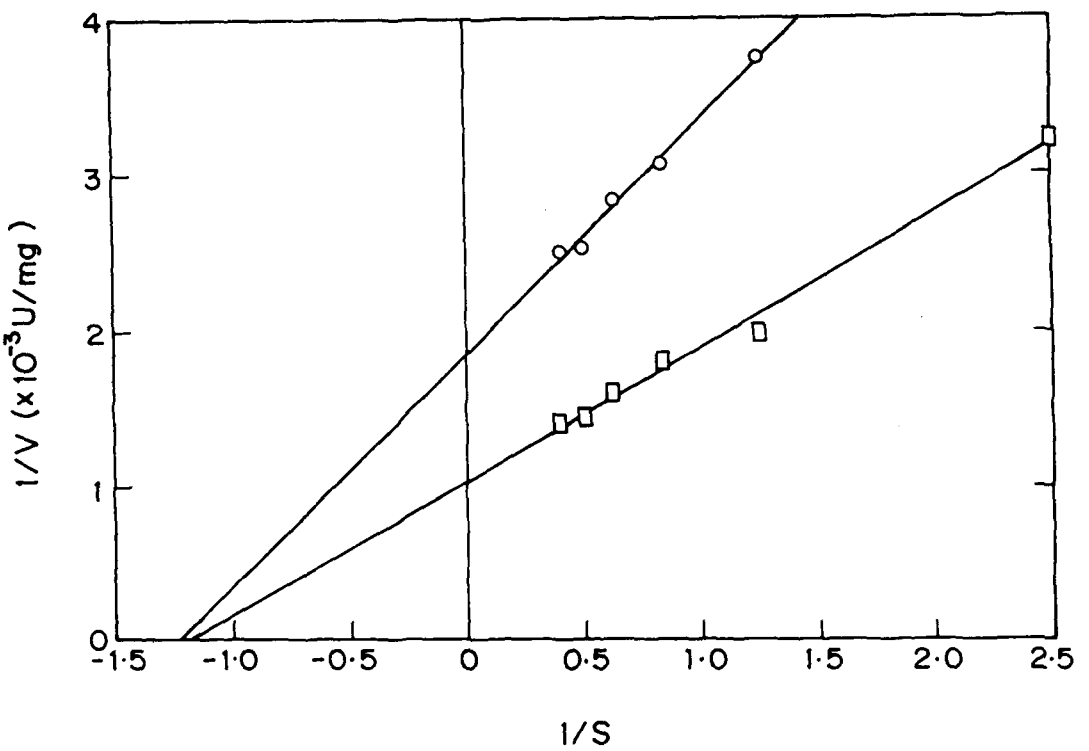
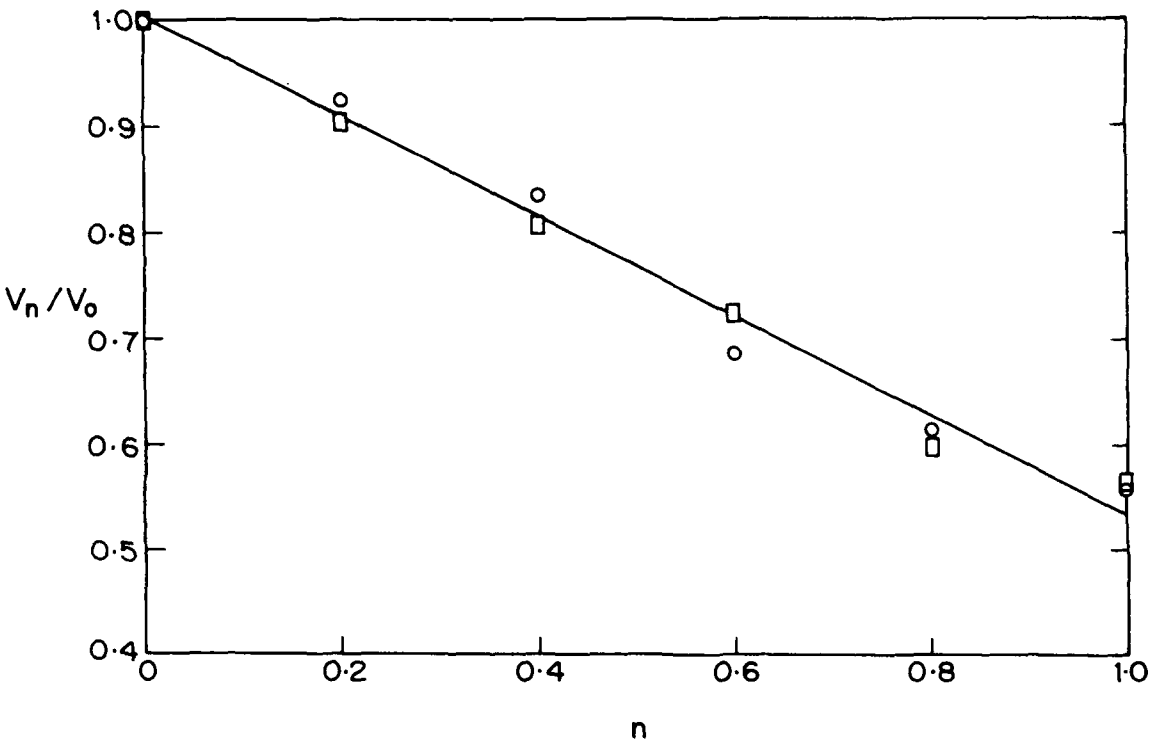


Figure 3.10

Proton inventory of endo-polygalacturonase

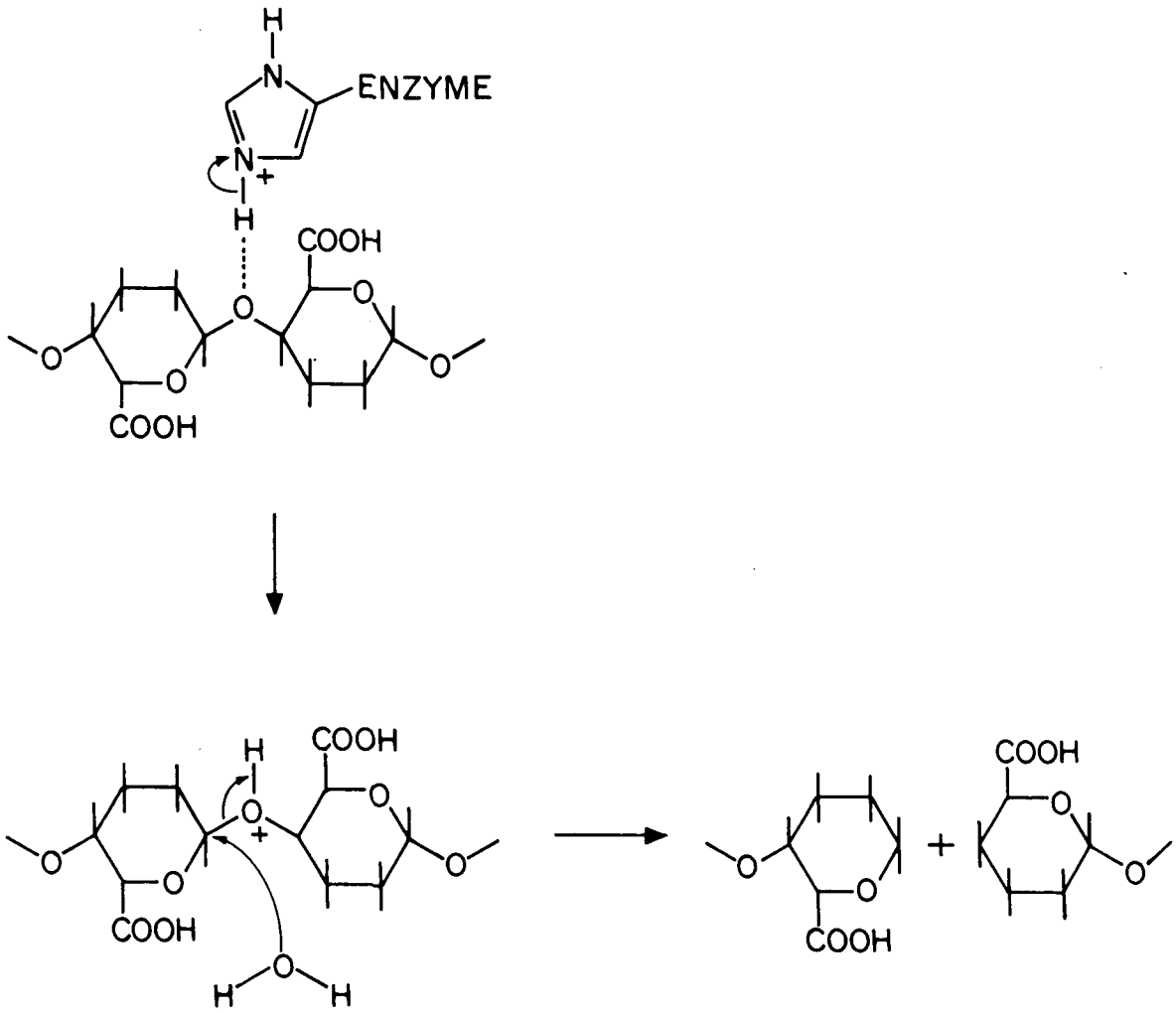
Values of Vmax were obtained at different fractions of ^2H_2O- H_2O mixtures. Ratio of maximal rate at 100% ^2H_2O and the maximal rate at 100% H_2O were plotted against the fractions (n). Two experimental values (O) and (□) were obtained for each fraction.



Scheme 3.1

Proposed acid-hydrolysis of polygalacturonic acid by endo-polygalactruonase

A catalytic mechanism is suggested where a proton is transferred from histidine of the enzyme to the glycosidic oxygen, with subsequent addition of a water molecule resulting in hydrolysis.



CONCLUSION

This is the first report of the purification and characterization of the active-site nature, of an endo- polygalacturonase from *Aspergillus ustus*. The active-site characterization of the enzyme revealed the presence of tryptophan, which is essential in substrate binding and histidine residue, involved in the catalysis. The action of the endo- polygalacturonase involving the general acid-hydrolysis by histidine residue is similar to the mechanism of hydrolysis of other carbohydrases.

CHAPTER 4

PRODUCTION AND INDUSTRIAL APPLICATIONS OF ENDO-POLYGALACTURONASE FROM *ASPERGILLUS USTUS*

SUMMARY

A cellulase-free polygalacturonase preparation was obtained by culturing a strain of *Aspergillus ustus* NCIM 1033. The media optimization for the production of polygalacturonase in submerged, shake-flask cultures was done. The enzyme was found to be inducible and maximum yield was obtained in presence of 1 % pectin, 0.2 % glucose and 0.5 % NH_4NO_3. Orange pulp, a cheaper substitute, gave more yield than pure pectin. *A.ustus* NCIM 1033 did not produce any cellulolytic enzymes, even in induction media.

The strain of *A. ustus* NCIM 1033 was subjected to UV radiation and a mutant M26 was obtained, which yielded 300% of the activity of the wild strain.

The polygalacturonase thus produced was stable for upto 3 months at 30 °C. The enzyme was also stable in a wide range of pH, from 3.0 to 8.0.

Solid-state fermentation of the *A.ustus* M26 mutant strain yielded a high activity enzyme preparation. In a medium containing 40 g. of wheat bran, 2 g. of dried orange pulp, 0.5 g. of ammonium sulphate, 0.4 g. of dipotassium hydrogen phosphate and 80 ml of water, fermentation by the mutant strain yielded around 960 polygalacturonase units per gram of dry bran medium. Wheat bran yielded more enzyme than defatted rice bran. A bran to water ratio of 1:2 was optimal for enzyme production.

Purified endo-polygalacturonase from *A.ustus* was immobilized onto modified silica gel. 60 units of enzyme when used for binding to 1 g. of grafted silica gel, gave maximum efficiency of 28%. The binding of enzyme molecules through the ε-amino groups of lysine, to glutaraldehyde-linked silica gel yielded a stable immobilized endo-polygalacturonase preparation, which retained activity even after 10 cycles. The immobilized enzyme was found to have increased thermostability over its soluble form.

INTRODUCTION

The secretion of pectic enzymes by saprophytic micro-organisms has been exploited in order to meet the industrial demand for pectinases. Pectinases have been generally used in the food processing industry (Rombouts 1978, Uhlig 1990) and are recently being studied for application in retting of natural fibers.

Industrial applications of pectinases

Fruit juice manufacture

The objective of clarification of fruit juices is to remove fragments of plant cells and insoluble particles of skin and seeds. The concentration of pectin in ripe fruits varies not only with the type of fruit but also with the variety, degree of ripeness, agricultural process and storage of harvested products. For most fruits, the pectin concentration varies from 0.5 to 1.0 % (Frost 1987). In apple juice, pectin acts as a stabilizing colloid for insoluble cell debris. Hydrolysis of pectin lowers the viscosity of juices and eliminates the stabilizing effect of pectin. The insoluble particles form aggregates and these flocs settle rapidly, permitting efficient filtration of the juices to a stable clear form (Whitaker 1984). After enzymic treatment, flocculation of the particles is accelerated by the addition of gelatin, silica or bentonite (Uhlig 1990)

Preparation of vegetable and fruit nectars

Pectinases are an important aid in the maceration and liquefaction of vegetables and fruits in the manufacture of nectars and concentrates (Rombouts 1978). Fruit juice concentrates are commercially important because of their longer shelf life, as well as lower storage and transportation costs than juices (Uhlig 1990).

Production of wine

Pectinases have been used in the making of red wine and white wine. Use of pectinases facilitates preliminary mash preparation, by improving the ability of the grapes to be crushed (Uhlig 1990) and increasing the rate of filtration. The addition of pectinases prior to crushing results in considerable increase in juice yield, often with more intense colour (Whitaker 1984), which is particularly advantageous in the manufacture of red wines.

Treatment of cellulosic materials

In addition to the traditional use of pectinases in the food industry, these enzymes are also being studied for their use in degumming of natural fibres (Baracat, 1991) and in biochemical pulping of bast fibres (Tanabe, 1986), a process wherein the cellulose fibres are extracted by degrading pectic middle lamellae of the plant cell walls. Cao *et al* (1992) obtained four strains of alkalophilic bacteria, which secreted polygalacturonase and xylanase, with a potential application in degumming of ramie fibres. For treatment of cellulosic fibres, enzyme preparations free of cellulases would be a major advantage.

Miscellaneous applications

Pectinases have been found to be useful in wood preservation. Commercial softwoods like Sitka and Norway spruce, when treated with enzyme preparation of pectinolytic bacteria absorbed the preservatives more rapidly and effectively (Ward 1972).

The scope for application of pectinases is ever widening. Newer uses have been recently described, such as extraction of active ingredients from medicinal plants (Nissen-Food 1986, JP 61233626), production of vanilla flavour from pods (Mane- Fils 1992) and in the recovery of oils and fats from plant materials (Petruccioli 1988).

Production of Pectinases

Most of the industrially significant work in the field of pectinase production has been carried out with species of *Aspergillus*, especially with *A.niger*. Other than their usefulness, their acceptability in the food industry has greatly dictated the choice of Aspergillus enzymes. Suitable organisms include *A.niger*, *A.oryzae*, *A.wentii* and *A.flavus* (Fogarty 1974). Several processes have been described in patents and scientific literature. Three general methods of production have been studied; I) Submerged fermentation II) Solid-state fermentation and III) Fungal immobilization.

Submerged fermentation

Submerged fermentation requires critical control of media components, pH of the medium, temperature of fermentation, aeration and agitation. These parameters need to be optimized for each organism to suit its specific physiology.

Most of the pectolytic enzymes have been reported to be induced in presence of pectin (Manachini 1988, Dean 1989, Larios 1989, Bailey 1990 and McKay 1990) although reports do exist on the constitutive production of polygalacturonase (Chopra 1985, Leuchtenberger 1989). Commercial sugar has been used to produce pectolytic enzymes from *Aspergillus niger* (Friedrich 1992). In the production of pectolytic enzymes, crude sources of pectin like dried sugar beet extraction waste (Bailey 1990), lemon peel (Larios 1989), citrus pulp (Siessere 1989, Baracat 1991), and apple pomace (Uhlig 1990) have often been used.

Corn steep liquor, yeast extract, and nitrogenous inorganic salts have been used as nitrogen source in pectinase production (Fogarty 1974, Mehta 1985).

Solid-state fermentation

Solid-state fermentation (SSF) processes have existed for many years and have wide application in production of food, enzymes, organic acids, antibiotics and other microbial products (Aidoo 1982).

Solid-state fermentation involves growth of micro-organisms on moist solid substrates and in the absence of free-flowing water. The necessary moisture in SSF exists in an absorbed form, possibly resulting in efficient oxygen transfer (Murthy 1993). Some of the advantages of SSF include cheap media components, usually agricultural wastes, simpler fermentor specifications unlike in submerged fermentations, less risk of contamination due to low moisture and increased product yield (Hesseltine 1972).

Production of pectinases by solid-state fermentation has been done by growing the pectinolytic mold in wheat bran, rice bran, sugar-cane bagasse, with or without pectin additives. Pectin in the form of beet pulp, citrus peel or apple pomace is usually added. Fermentation is carried out in trays or rotating drums in chambers with controlled temperature and humidity. Pereira *et al* (1993) have

described a process wherein sugarcane bagasse is filled in glass columns and aerated at a rate of 0.4 l air/ g dry matter. The mouldy bran is then extracted with water, filtered and concentrated in vacuo or through salt or solvent precipitation.

Generally solid-state fermentation gives higher productivity as compared to submerged fermentation (Pereira 1993), using cheaper medium constituents and without involving the costs of high levels of controls required for submerged fermentation. Addition of glucose or D-galacturonic acid enhanced production of pectolytic enzymes by *A.niger* in solid-state culture, whereas they repressed production in submerged fermentation (Pereira 1993). This is possibly related to the inherent differences in mixing and nutrient diffusion between solid and liquid fermentation.

Immobilized mycelium

Recent patents have described the use of immobilized mycelium in the production of pectinases. *A.niger* has been immobilized on various matrices, such as textile fibres, and used for the production of the enzyme (Leuchtenberger 1987, Morrek, 1987). Although this method of production is still in the preliminary stages of process development, it has a promising future in the continuous production of pectinases.

Mutation

Mutation of micro-organisms in order to get higher yielding strains has been extensively used in biotechnological process development. Hyper-producing mutants of pectinase producers have been obtained using mutagenic agents such as UV-radiation, nitrosoguanidine (NTG) and ethyl methyl sulfonate (EMS). Antier *et al* (1993) had isolated high yielding strains of *A. niger* using UV-radiation. Mutants of *Penicillium occitanis* with hyper- production of pectinase and cellulase were obtained using NTG, EMS and UV-radiation (Jain 1990). Leuchtenberger and Mayer (1992) had isolated various mutants of *A. niger* producing different ratios of polygalacturonase and pectin esterase after exposure to UV-rays.

Enzyme-Immobilization

Immobilized enzymes are enzymes which are physically confined or localized in a defined region, with retention of catalytic activity. Enzymes are usually bound to inert matrices by chemical or physical methods. Enzymes covalently bound to matrices through the reactive groups of amino-acids such as lysine, arginine, serine, threonine, cysteine, glutamic acid and aspartic acid, have longer shelf-life and increased stability.

Pectinases have also been immobilized on various matrices. The endo-polygalacturonase from *A.niger* has been immobilized on porous polyethylene terephthalate by adsorption (Rexova-Benkova 1982). Omelkova et al (1986) immobilized endo-polygalacturonase onto porous poly(6-caprolactam) activated by glutaraldehyde with a relative activity of 24%. Other matrices which have been used for the immobilization of polygalacturonases are poly(2,6-dimethyl-p-phenylene oxide) (Rexova-Benkova 1983), poultry bone (Findlay 1986), porous glass (Romero 1987) and nylon (Lozano 1987). In order to develop a process for production, the culture conditions and the media constituents require to be optimized. The optimization of each variable singularly or in conjunction with other variables should lead to an overall process in which maximum yield is obtained using cheap and easily available raw materials.

The present work has been carried out on *Aspergillus ustus* NCIM 1033, a species which has not been previously investigated for its production of pectin-degrading enzymes. *A.ustus* has been reported to produce cellulases and xylanases (Marcis 1986, Shamala 1986).

The present study involves production of cellulase-free polygalacturonase by *Aspergillus ustus* NCIM 1033, in shake- flask cultures, mutation of the strain, and solid-state fermentation with an high-yielding strain. Application of the enzyme in fruit-juice clarification and enzyme immobilization was studied.

MATERIALS AND METHODS

Microorganism:

Aspergillus ustus NCIM 1033 obtained from the National Collection of Industrial Micro-organisms, was subcultured on Czapek Dox agar, modified with the addition of 0.5 % pectin.

Enzyme assay:

Polygalacturonase activity was assayed by measuring the reducing sugars released from 0.3% polygalacturonic acid in 0.1 M sodium acetate buffer, pH 5.0 at 40 °C (Collmer 1988). One unit of enzyme activity, expressed as PGr, was defined as the amount of enzyme which released 1 μmole of uronic acid per minute.

Optimization of growth conditions in shake-flask cultures

Combinations of various concentrations of pectin and glucose in addition to basal medium containing 0.5% NH_4NO_3, 0.2% K_2HPO_4, 0.2% KH_2PO_4 and 0.1% $MgSO_4$ were used to determine the optimum carbon source for enzyme yield. Orange pulp, orange peel, apple pulp, lemon peel were dried in an oven at 50°C, powdered and then used at a concentration of 2.0% (w/v) in the medium for determining the best source of pectin for enzyme production by the fungus. $(NH_4)_2So_4$, NH_4NO_3, KNO_3, $NaNO_3$, urea at molar nitrogen concentrations of 0.075 and peptone at 0.5% concentration were used to determine the best N-source for polygalacturonase production.

Three different concentrations of NH_4NO_3 at 0.2%, 0.5% and 1.0% and dried orange pulp at 1.0%, 2.0%, and 4% were varied in the medium, to determine the optimum concentration of both for polygalacturonase production by *A.ustus* NCIM 1033.

The variation of the initial pH of the medium containing 2% orange pulp, 0.5% NH_4NO_3, 0.2% glucose, 0.2% K_2HPO_4, 0.2% KH_2PO_4 and 0.1% $MgSO_4$, was done by adjusting from pH 2.5 to 7.5 with 0.1 N NaOH or 0.1 N HCl. After 4 days of incubation, the mycelium-free broth was analyzed for polygalacturonase activity.

Effect of agitation rates on enzyme production

Flask cultures were incubated at varying agitation rates (50 rpm. to 200 rpm.) on a variable-speed rotatory shaker and the time course of the enzyme production was studied. Effect of a static culture on the polygalacturonase production was also determined.

Kinetics of Polygalacturonase Production and Growth of *Aspergillus ustus* NCIM 1033

50 ml media in 250 ml conical flasks were inoculated. The flasks were incubated at 30°C on a rotary shaker (200 rpm.). The experiment was run in triplicates. The mycelium was centrifuged out and dried to a constant weight. The broth was analyzed for polygalacturonase activity, and reducing sugars.

Cellulase production

The fungus was grown in media containing 1% cellulose powder in basal medium containing 0.5 % NH_4NO_3, 0.2 % glucose, 0.2% K_2HPO_4, 0.2 % KH_2PO_4 and 0.1 % $MgSO_4.7H_2O$ and 1% carboxymethylcellulose in above medium. The cellulase activity in the culture supernatant was determined by carboxymethylcellulase (CMCase) activity and filter paper activity (Mandels 1974) assays. Endoglucanase (CMCase) activity was assayed using 0.5 ml of enzyme broth and 0.5 ml of 1 % carboxymethyl cellulose in 0.1 M phosphate buffer, pH 6.5, with incubation at 40 °C for a period of 60 minutes. In the filter-paper assay, 50 mg. of Whatman no. 1 filter paper in 1 ml of buffer was incubated with 1 ml of enzyme broth for 12 hours, at 40 °C. After incubation the reducing sugars were estimated with DNSA reagent, and the activity was defined in terms of glucose equivalents.

Mutation of *A.ustus* NCIM 1033

UV-mutation was carried out with the strain of *A.ustus* NCIM 1033 in order to isolate a high yielding strain for the enzyme polygalacturonase. A spore suspension was made in 0.1 % Tween- 80, 0.9% saline, from a slant of the organism. A 10^6 spores/ml suspension was exposed to UV-radiation for different time intervals from one to ten minutes. The exposed spore suspension was diluted

from 1:10 to $1:10^5$, and streaked on pectin-agar medium, containing 1% pectin, 0.5% NH_4NO_3, 0.2% K_2HPO_4, 0.2% KH_2PO_4, 0.1% MgSO and 2.5% agar. Colonies of viable spores were picked after growth at 30 °C for 76 hours and inoculated in media containing 2.0% orange pulp, 0.5 % NH_4NO_3, 0.2% glucose, 0.2 % KH_2PO_4, 0.2% K_2HPO_4, and 0.1% $MgSO_4$. After incubation for 4 days, the culture broth was assayed for polygalacturonase activity.

Properties of the crude enzyme preparation

The temperature stability of the crude polygalacturonase was studied by incubating it at different temperatures (4°, 30°, 40°, 50° and 60°C), and the residual activity was determined at different time intervals.

The pH stability was determined by dialyzing the crude enzyme in buffers of different pH for 48 hours and then determining the residual activity. From pH 2.2 to 8.0, citrate- phosphate buffers were used and for pH 9.0 and 10.0, tris-HCl and carbonate buffers were used respectively.

The presence of alflatoxin in the culture broth was analyzed by HPLC, on a Nucleosil C-18 column, using standard aflatoxin.

Solid-state fermentation

A spore suspension was prepared from a 4 day-old slant of *A.ustus* M26 in 0.1% Tween-80-saline. The spore suspension was used to inoculate the bran medium in a 1 L conical flask.

The media constituents for solid-state fermentation were varied by using wheat bran and rice bran, in combination with dried orange peel, orange pulp and lemon peel. Defatted rice bran was obtained by treating rice bran with hexane.

For variation of orange pulp content in the medium, basal medium used consisted of 40 g. wheat bran, mixed with 80 ml water. 0.5 g. of NH_4NO_3 and 0.4 g. of K_2HPO_4 was added into the water. Orange pulp was varied from 0 to 8 g. in the basal medium. A preinoculum of *A.ustus* M26 was made in medium containing 2% orange pulp, 0.2% glucose, 0.5% NH_4NO_3, 0.2% K_2HPO_4, 0.2% KH_2PO_4. 1 ml of the 96 hours old inoculum was added to the bran medium and incubated at 30 °C. After four days of incubation, the fermented bran was

extracted with 200 ml distilled water. Extraction was done by agitation of the mouldy bran and water on a rotatory shaker for 2 hours. The extract so obtained was filtered, and the filtrate measured for polygalacturonase activity as described earlier. The activity was expressed as units of polygalacturonase per gram of wheat bran.

The moisture content of the bran medium was varied from a ratio of 1:1 to 1:2.5 of the wheat bran. 40 g. of wheat bran with 2 g. of orange pulp, 0.5 g. NH_4NO_3 and 0.4 g. K_2HPO_4 was mixed with 40, 60, 80, and 100 ml water.

The time-course of enzyme production by solid-state fermentation of *A.ustus* M26, was studied by extraction of one batch of 40 g. wheat bran fermentation each day, and assaying for polygalacturonase.

Immobilization of endo-polygalacturonase

Aminated silica gel was used as support for the covalent immobilization of endo-polygalacturonase. Aminated silica gel was prepared by refluxing 50 g. of silica gel 60-120 mesh in 300 ml of ethanolamine for 3 hours. The silica gel was washed thrice in 600 ml of acetone, dried and activated with 250 ml of 4% glutaraldehyde in 0.1 M phosphate buffer, pH 8.0, with slow shaking at 4 °C for 2 hours. The activated silica gel was washed with 0.1 M acetate buffer, pH 4.6 to remove excess glutaraldehyde. The grafted silica gel was used for the immobilization of the purified endo-polygalacturonase. The enzyme in 0.1 M acetate buffer, pH 4.6, was mixed with 1 g. of the activated silica gel and incubated at 4 °C, with slow shaking for a period of 16 hours.

The activity of the immobilized enzyme was assayed by incubating 0.1 g. of silica gel in 5 ml of 0.3% polygalacturonic acid in 0.1 M acetate buffer, pH 4.6. The increase in reducing sugars, as expressed as equivalents of D-galacturonic acid, was measured using the Nelson-Somogyi method (Collmer 1988). The units are expressed as μmoles of reducing sugars released per minute per g. of silica gel.

The efficiency of binding of an immobilized enzyme is the ratio of the activity expressed by the enzyme bound, to the total activity, in terms of soluble enzyme, actually linked to the matrix. In case of a pure enzyme being immobilized, the

efficiency would simply be the ratio of the activity expressed per mg. protein bound, to the specific activity of the enzyme in soluble form.

The effect of enzyme loading was studied by varying the enzyme concentration from 10 units to 100 units per gram of silica gel.

The time required for binding of the enzyme to the grafted silica gel was estimated by incubating 1 g. of the silica gel with 40 units of endo-polygalacturonase, and determining the soluble enzyme at different time intervals.

The immobilized enzyme was analyzed for the number of cycles over which it could be used. After each assay for the immobilized enzyme, the silica gel was washed with acetate buffer, 0.1 M, pH 4.6.

The Km and Vmax of the immobilized enzyme were determined using the double reciprocal plot of the activity per gram of silica gel versus substrate concentration.

The temperature stability of the immobilized endo- polygalacturonase was studied by incubating 0.1 g. aliquots of matrix at different temperatures (4, 30, 40, 50, 60 °C). At every time interval, residual activity was determined.

Clarification of apple and citrus juice by polygalacturonase

Apple juice clarification was studied using the enzyme obtained by solid-state fermentation. Freshly obtained apples were cut, crushed in a juicer and filtered. The juice obtained was used for clarification. 0.2 ml of enzyme extract, with 30 units of polygalacturonase and 0.36 mg. of protein, was mixed with 200 ml of fresh apple juice and incubated at 40 °C, with slow shaking. At different time periods, an aliquot was taken, centrifuged, and assayed for viscosity drop and absorbance at 660 nm. A control of untreated apple juice was used for comparison. Orange juice clarification by the polygalacturonase was performed in a similar way. Fresh orange juice was obtained by crushing oranges, and filtering the juice.

RESULTS AND DISCUSSION

Medium optimization for production of polygalacturonase by *A.ustus*:

A 1 % (w/v) concentration of pectin in the medium was found to be optimum (Fig. 4.1) for production of the enzyme by *A.ustus* NCIM 1033. When no pectin is added in the medium, there is only a trace level of the enzyme, suggesting that the polygalacturonase is an inducible enzyme. Pectolytic enzymes have been reported to be both constitutively produced (Chopra 1985, Leuchtenberger 1989 and Freidrich 1992) in media containing simple sugars, and also induced (Manachini 1988, Dean 1989, Larios 1989, Bailey 1990 and McKay 1990) in presence of pectin in various native forms. A concentration of 0.2% of glucose was found necessary for maximum yield of the enzyme in a medium containing 1% pectin. A higher concentration of glucose represses enzyme production. The role of glucose in enzyme induction has not been investigated in the present thesis. Aguilar and Huitron (1987) had observed in *Aspergillus* sp. that 0.1% glucose, when added to pectin-containing medium, had no effect on production of pectinolytic enzymes, whereas 0.5 % glucose repressed enzyme production.

As shown in Table 4.1, of the nitrogen sources tested, organic sources such as peptone and casein hydrolysate, repressed polygalacturonase production. Of the mineral salts containing nitrogen, NH_4NO_3 gave maximum yield of the enzyme. Chopra and Mehta (1985) have reported that *Aspergillus niger* too required NH_4NO_3 or KNO_3 for maximum production of polygalacturonase.

Table 4.1

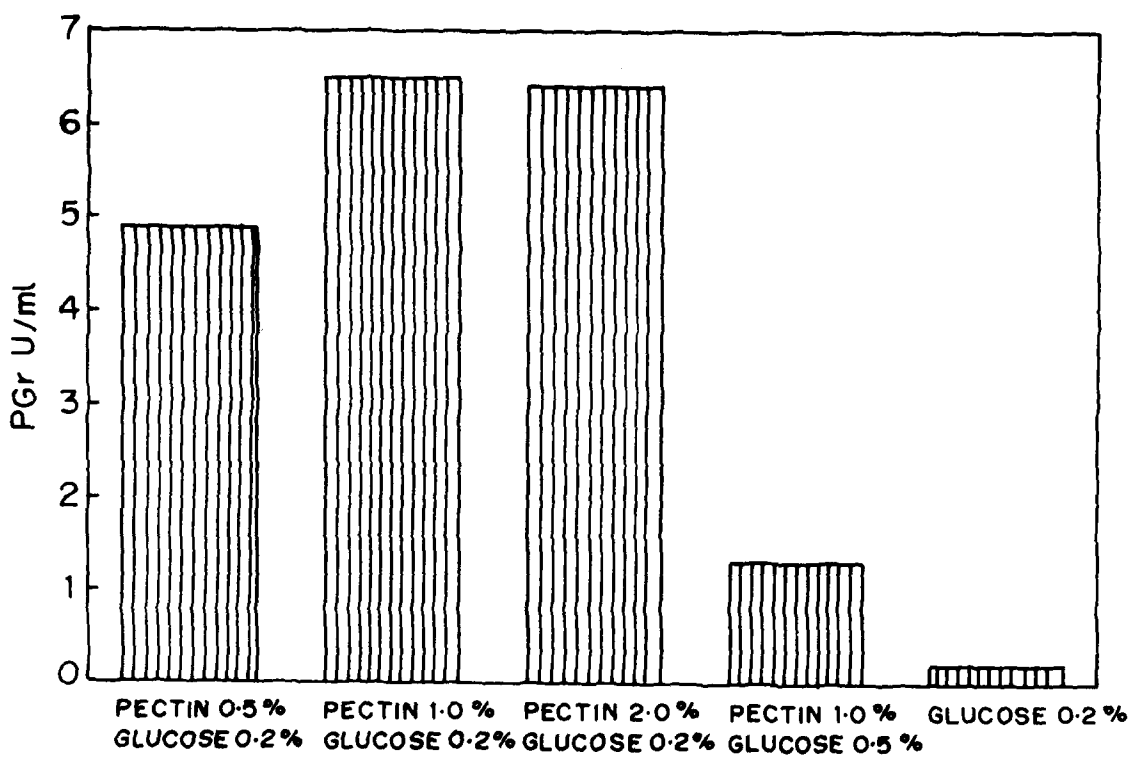
Effect of nitrogen source on enzyme production by *A.ustus*

N-Source	*Activity* U/ml
Ammonium sulfate	5.96
Ammonium nitrate	6.74
Potassium nitrate	3.72
Soya peptone	0
Casein hydrolysate	0

Figure 4.1

***Induction and repression of* polygalacturonase production by *A.ustus*.**

The effect of carbon source on enzyme production was studied using combinations of pectin and glucose at different concentrations.



In the production of pectolytic enzymes, crude sources of pectin like dried sugar beet extraction waste (Bailey 1990), lemon peel (Larios 1989), citrus pulp (Siessere 1989, Baracat 1991), apple pomace and also rice and wheat bran (Uhlig 1990) have been often used. Of the various sources of pectin, dried orange pulp was best for production of the enzyme by *A.ustus* NCIM 1033 (Table 4.2). Orange pulp has a pectin concentration of around 25-30% dry weight.

Table 4.2

Effect of source of pectin on enzyme production by *A.ustus*

Source	Activity U/ml
Orange pulp	10.4
Orange peel	5.03
Apple pulp	3.8
Lemon peel	1.2
Pectin	6.8

Determination of the minimum concentration of the nitrogen and carbon source for maximum yield, would help in estimating the cost effectiveness of the production of the enzyme. Zetelaki (1976) had determined the optimum concentration of sugar beet extract at unit carbon and unit cost for production of pectolytic enzymes from *Aspergillus awamori* and *Aspergillus foetidus* to be 2%. A combination of 0.5 % NH_4NO_3 and 2.0% dried orange pulp resulted in optimum production by *A.ustus* NCIM 1033 (Table 4.3).

Table 4.3

Optimum concentration of C-source and N-source required for enzyme production

Orange pulp %	Ammonium sulfate % 0.2	0.5	1.0
1.0	3.6	3.28	3.98
2.0	5.76	10.49	8.11
4.0	4.56	10.52	5.4

Variation of pH in the medium has been reported to be highly influential in the production of pectinases by fungi, with polygalacturonase production usually obtained in the acidic range (Perley 1971, Lisker 1975, Dean 1989, Aguillar 1991).

The production of the polygalacturonase by the present strain is maximum when the initial pH of the medium is between 4.5 and 5.5 (Fig. 4.2). There is no appreciable change in the final pH of the medium after 96 hours fermentation.

An interesting observation made during the optimization of agitation rate, was that there was no effect on polygalacturonase production, either on the yield nor on the rate of production with increased agitation (Fig. 4.3) and 50 rpm. was enough for maximum production of the enzyme. In static culture, the yield of the enzyme was lower. Bailey (1990) had also reported that low agitation rates in a laboratory fermentor were sufficient to maintain necessary dissolved oxygen levels for maximum yield of polygalacturonase from *A.niger*, whereas Freidrich et al (1989) had observed in their strain of *A.niger* that increased agitation and aeration rates had not only increased production of pectolytic enzymes but also reduced the fermentation time. There was no distinct morphological variation in *A.ustus* with increased agitation rates. This observation is in contrast to that seen in *A.niger* (Leuchtenberger 1984), which grew in pellet or diffuse mycelial form depending on the agitation rate. Metz et al (1977) have also stated that agitation rates have a distinct effect on fungal morphology.

Time course of Polygalacturonase production by *A.ustus* NCIM 1033

Production of polygalacturonase started after 24 hours and activity was maximum after 96 hours (Fig. 4.4). The production of the enzyme is concurrent to the logarithmic phase of the growth of *A.ustus* NCIM 1033.

Cellulase-free polygalacturonase

The present strain of *A.ustus* did not produce any cellulolytic activity, in terms of CMCase and filter-paper assays, neither in medium containing cellulose, nor in medium containing orange pulp. 200 ml of broth (0.36 mg/ml protein) was concentrated to 10 ml by ultrafiltration and used for determination of cellulase activity as described in the methods. Even on concentration, the culture broth showed no cellulolytic activity. Thus, we conclude that the polygalacturonase preparation from *A.ustus* NCIM 1033 is cellulase free. Cellulase-free polygalacturonase would be particularly useful in treatment of raw cellulosic fibres, such as jute, hemp, flax and bast.

Figure 4.2

Effect of initial pH of medium on enzyme production by *A.ustus*

(O)-Polygalactuornase U/ml, (Δ)-Biomass

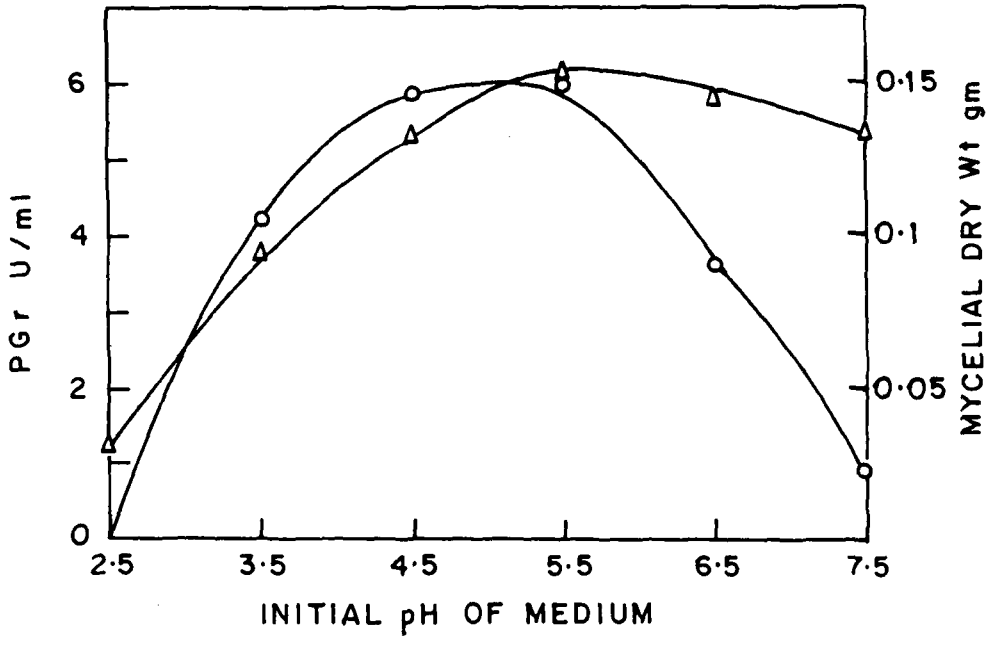


Figure 4.3

Effect of agitation rate on polygalacturonase production by *A.ustus*

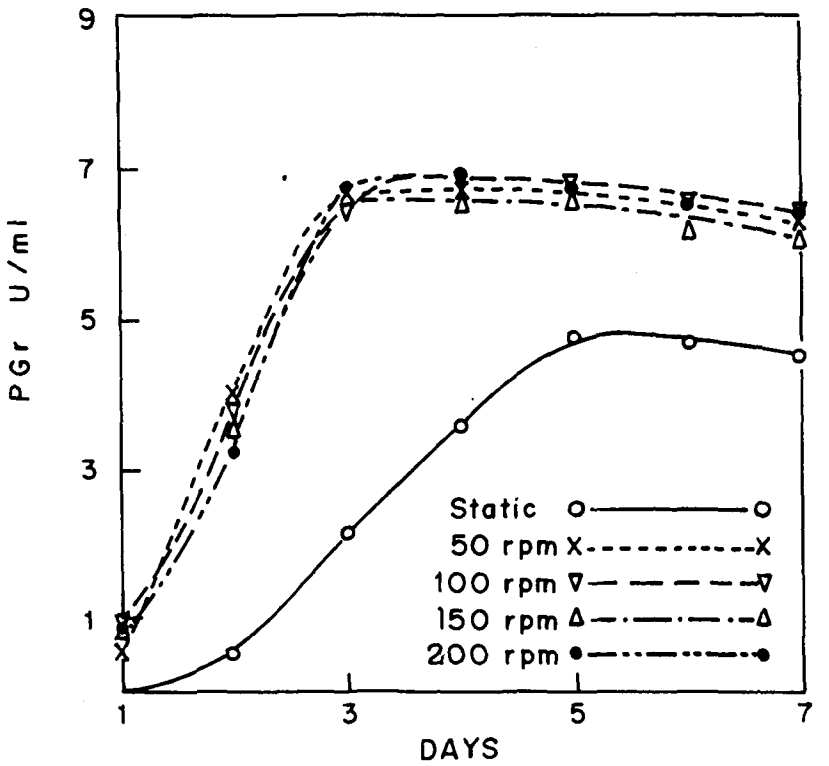
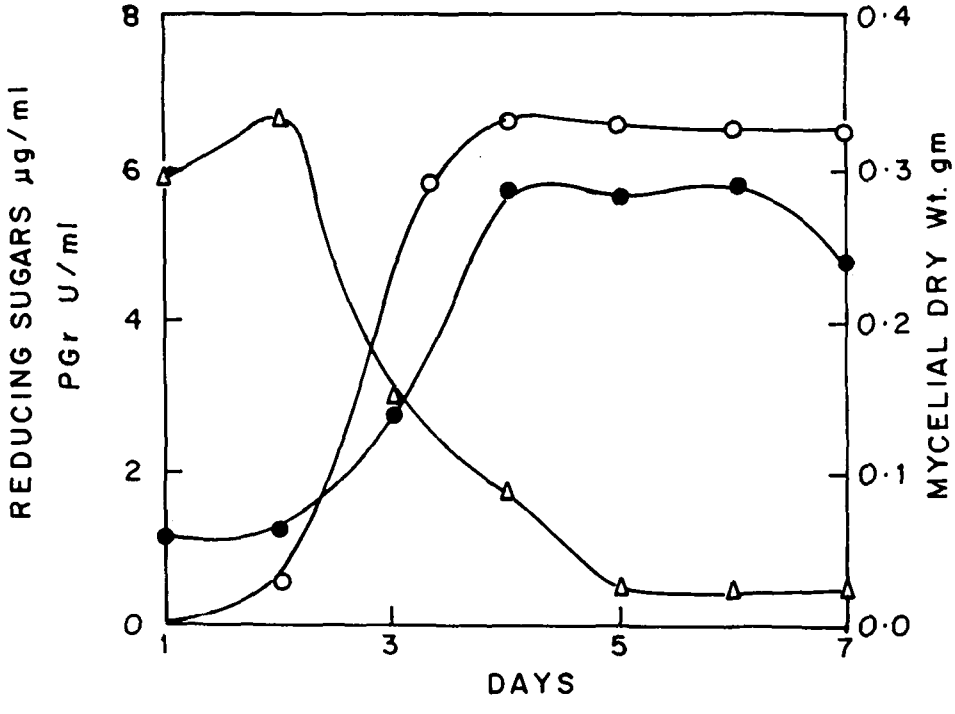


Figure 4.4

Kinetics of growth and enzyme production by *A.ustus*.

(O)-Polygalacturonase U/ml, (●)-Biomass, (Δ)- Reducing sugars in broth



Mutation of *A.ustus* NCIM 1033

Screening of the colonies from the viable spores obtained after the UV-exposure yielded some strains having higher activity than the wild. The strain, M26, gave maximum yield of 31.6 units/ml, three times that of the parent strain. The M26 mutant, grown on Czapek-Dox agar with pectin, gave constant yield of polygalacturonase in liquid culture over 20 subcultures, indicating that the mutant is stable. This is an important factor to be considered during isolation of high-yielding mutants, as reversion of mutants is a major problem associated with industrial usage of such organisms for primary or secondary metabolite production.

A major difference of the mutant and the wild strain was in the morphology obtained in liquid culture. M26 grew in culture as diffuse mycelial form, while the wild strain grew in pellet form (Fig. 4.5) under the same growth conditions. The increase in production of polygalacturonase by diffuse mycelia of the mutant over the pellet form of the wild strain is in contrast to the production of the enzyme by the strain of *A.niger* reported by Leuchtenberger (1984), which gave higher yield of polygalacturonase in pellet form rather than in the diffuse mycelial form.

Properties of the crude polygalacturonase

The enzyme is highly stable at 30 °C and 4 °C (Fig. 4.6). It can withstand storage at 30 °C for over 90 days, retaining over 90% of its activity. The enzyme rapidly loses activity at temperatures above 50 °C, with a half-life of just 30 minutes. This property of the polygalacturonase can be effectively put in use when the enzyme has to be inactivated after a specific usage, by pasteurizing the treated material. The polygalacturonase of *A.niger* (Rexova-Benkova 1967) was reported to be stable at 30 °C for only 24 hours, and at 50 °C, total activity was lost within 60 minutes, and Mill and Tuttobello (1961) reported that the polygalacturonase of their strain of *A.niger* loses 15% activity when stored at 5 °C for 5 days. In contrast Sreekantiah *et al* (1969) had reported that the polygalacturonase of *A.niger* retains 88% of its activity after 3 months at 28-30 °C.

The polygalacturonase of *A.ustus* was also stable over a wide range of pH. It retains over 90% activity on storage for 48 hours between pH 3.0 to 8.0 (Fig. 4.7). The polygalacturonase of *A.niger* reported by Mill and Tuttobello (1961) had a

Figure 4.5

Culture morphology of wild-type and mutant (M26) in liquid medium.

a- wild type *A.ustus* NCIM 1033, b- mutant *A.ustus* M26

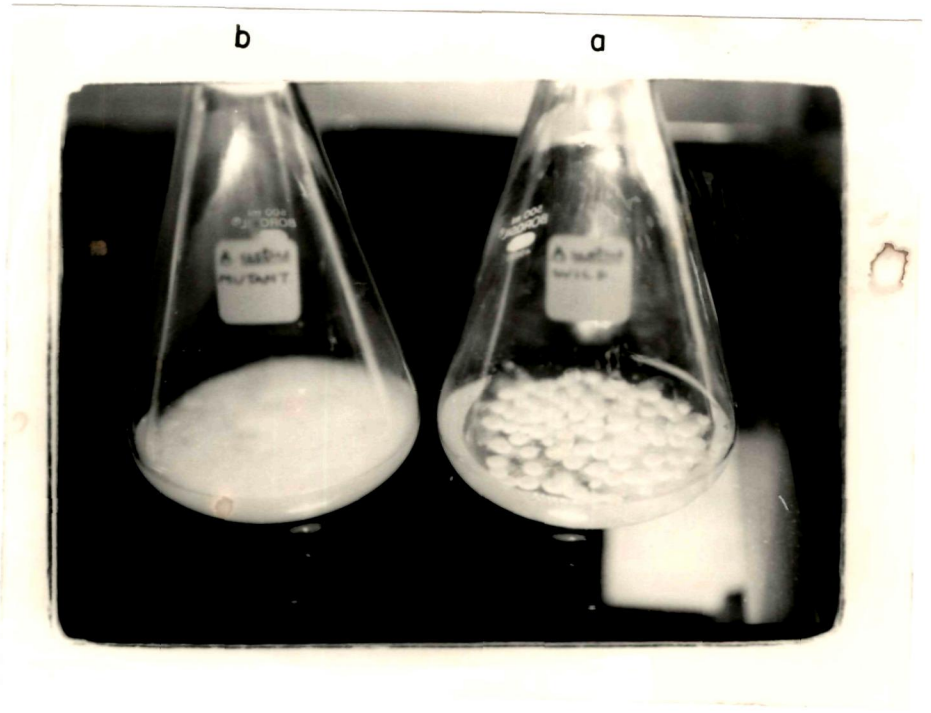


Figure 4.6

Temperature stability of polygalacturonase of *A.ustus*.

(O)- 4 °C, (●)- 30 °C, (Δ)- 40 °C, (▲)- 50 °C, (□)- 60 °C

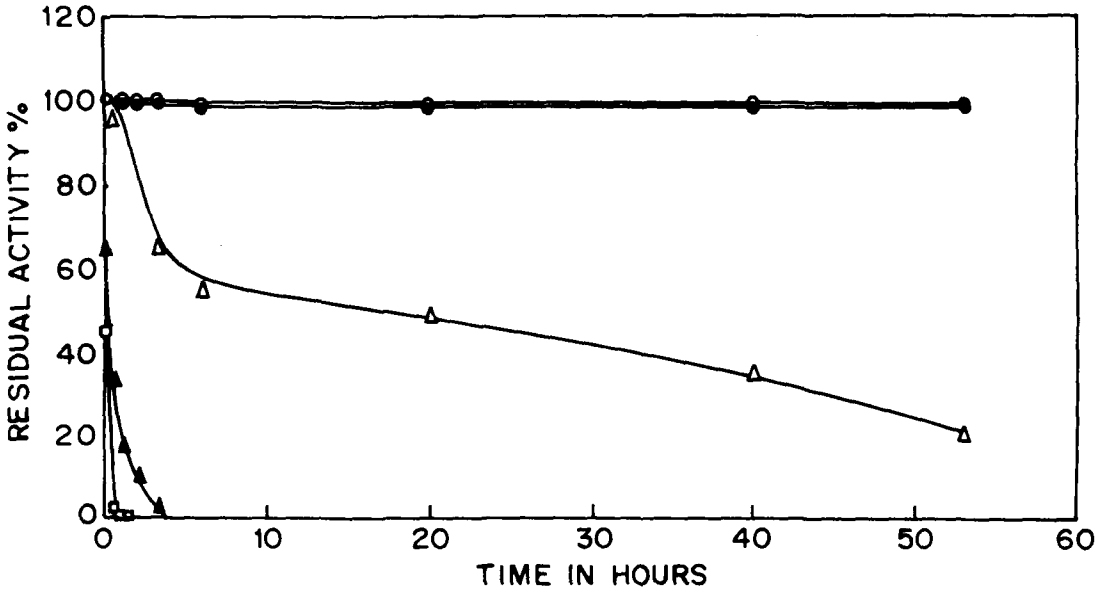
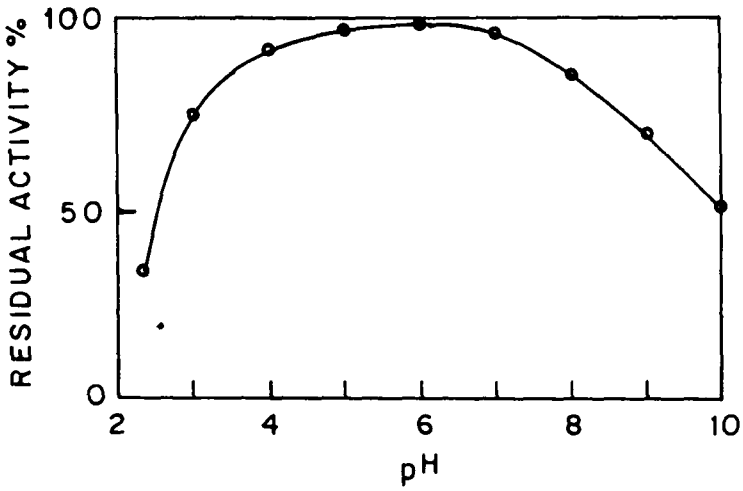


Figure 4.7

pH stability of polygalacturonase of *A.ustus*.

pH 2.2-8.0: citrate-phosphate buffer, 0.1 M; pH 9.0: Tris- HCl buffer, 0.1 M; pH 10.0: carbonate buffer, 0.1 M



narrow stability range around pH 6.0, whereas the enzyme from *A.carbonarius* was found to be stable for one month in the pH range 3.0-4.5 (Sreekantiah 1975).

Culture broth which was concentratred 100 times contained no ,aflatoxins, as analyzed by HPLC. Absence of aflatoxins would permit use of the enzyme in food processing.

Solid-state fermentation

Medium constituents for solid-state fermentation by *Aspergillus ustus* M26 for production of polygalacturonase

As seen in Table 4.4, wheat bran with the supplementation of orange pulp gave the maximum yield of polygalacturonase.

Table 4.4

Solid-state fermentation on different media

Media	Yield U/g
Wheat bran-40 g	284
Wheat bran-40 g + lemon peel-2 g	336
Wheat bran-40 g + orange peel-2 g	452
Wheat bran-40 g + orange pulp-2 g	961
Rice bran-40 g	236
Rice bran-40 g + lemon peel-2 g	284
Rice bran-40 g + orange peel-2 g	367
Rice bran-40 g + orange pulp-2 g	621

An addition of 2 g. of orange pulp was optimal for maximum yield of the enzyme (Table 4.5). Very little enzyme was produced with only wheat bran. Wheat bran contains low quantities of pectin, and hence an additional pectin source is required for induction of the polygalacturonase. Periera *et al* (1993) had added pectin additionally to sugarcane bagasse in their solid-state medium for *A.niger*, whereas Siessere and Said (1989) had used citrus peel pellets in the solid-state fermentation of *Talaromyces flavus*, *Tubercularia vulgaris* and *Penicillium charlessi* for production of pectic enzymes. On the contrary, Endo (1961) had reported that addition of beet pulp as an additional source of pectin to rice bran gave lower yield of pectolytic enzymes as compared to that in rice bran medium by *Coniothyrium diplodiella*. The present organism preferred orange pulp to lemon or orange peel, which was similar

to the results obtained with submerged shake-flask cultures. Defatted rice bran medium gave lower yields in comparison to wheat bran on fermentation by *A.ustus* M26, in contrast to the observation of Endo (1961) that *Coniothyrium diplodiella* gave better activity of pectolytic enzyme on defatted rice bran than wheat bran.

Table 4.5

Effect of orange pulp content in bran medium on yield of polygalacturonase

Orange pulp g	Yield U/g
0	288
2	960
4	955
6	925
8	882

Effect of moisture content of bran medium on production of polygalacturonase by *A.ustus* M26

Moisture content is an important parameter in the optimization of solid-state fermentation processes. In the present study, of the various ratios of bran to water, maximum yield of the enzyme was obtained when 40 g. of wheat bran was mixed with 80 ml of water (Table 4.6). Periera *et al* (1993) had also used a 1:2 ratio of sugarcane bagasse and water for production of polygalacturonase using *A.niger*, whereas Nishio *et al* (1979) had found an optimum moisture content of 52% for production of macerating enzymes by *A.niger*.

Table 4.6

Effect of moisture content of bran medium on yield of polygalacturonase

Wheat bran g	Water ml	Yield U/g
40	40	689
40	60	755
40	80	952
40	100	532

Kinetics of enzyme production by solid-state fermentation

As seen in Fig. 4.8, polygalacturonase production started after 48 hours of fermentation, and was maximum after 96 hours. The yield per gram substrate remained constant thereafter, which was similar to the observation in shake-flask cultures, wherein the activity per unit volume remained constant after 4 days, though the mycelial dry weight decreased slightly after the 6^{th} day. This could be due to the stability of the enzyme at 30 °C.

As seen above, the maximum yield of polygalacturonase from solid-state fermentation of *A.ustus* M26 on wheat bran and orange pulp medium, was 952 units per gram of dry medium. The yield from submerged fermentation, in shake-flask cultures, by the mutant was 30 U/ml. Thus, notwithstanding the difference in the yield expression between the two, much higher production of polygalacturonase was obtained in solid-state fermentation in comparison to submerged fermentation. Periera *et al* (1993) had also observed that higher yields of polygalacturonase were obtained in solid-state than in submerged fermentation. The reasons have not been investigated in the present study.

In the simple downstream processing for obtaining the enzyme from the fermented bran, 200 ml of water was added to the bran and kept on a shaker with mild agitation. 90% of the enzyme was found to be extracted by the first aliquot. Extraction with further volumes of water did not give any appreciable yield of the enzyme.

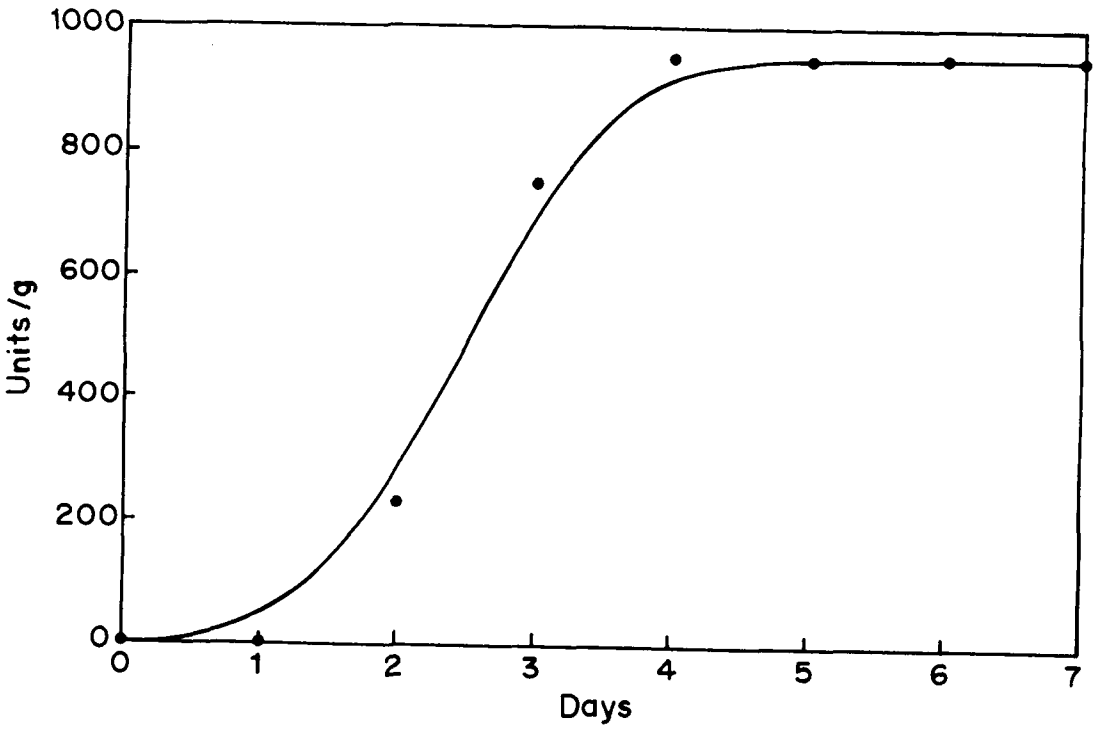
Immobilization of the endo-polygalacturonase

The 51-fold purified endo-polygalacturonase, with a specific activity of 785 units/mg., obtained by ion-exchange chromatography on CM-Sephadex and gel filtration on Sephadex G-75 (Chapter 3), was immobilized onto silica gel. The free aldehyde obtained by grafting glutaraldehyde to ethanolamine treated silica gel was linked to the side-chain amino groups of the enzyme, through Schiff's base formation. When the enzyme was treated with trinitrobenzene sulfonate, a reagent which binds to lysine residues in proteins, the enzyme failed to bind to the matrix, indicating that binding takes place through the ε-amino groups of lysines on the surface of the enzyme. Previous studies on chemical modification of the active-site

Figure 4.8

Enzyme production in solid-state fermentation by *A.ustus* M26

Solid-state fermentation in the optimized medium, containing 40 g. wheat bran, 2 g. orange pulp, 0.5 g. NH_4SO_4, 0.4 g. K_2HPO_4 and 80 ml water, was carried out and the yield of polygalacturonase per gram medium was estimated on each day



of the endo-polygalacturonase had shown that lysine is not essential for catalytic activity (Chapter 3).

The optimum loading for an enzyme onto a matrix, refers to the amount of enzyme that can be immobilized, with maximum efficiency.

Table 4.7

Effect of enzyme load on effeciency of binding

Enzyme loaded U	Enzyme bound U/g	% bound	Activity U/g	Effeciency %
10	10	100	2.7	27
20	20	100	5.2	26
40	38	95	10.5	27.5
60	53	88	14.9	28
80	71	88	16.3	23
100	85	85	17.0	20

When the amount of polygalacturonase loading onto silica gel was varied (Table 4.7), it was found that when 60 units of enzyme per g. of matrix was used for binding, a maximum efficiency of 28% was observed. Higher binding lead to lower efficiency, probably due to crowding of the enzyme molecules on the support. Fig. 4.9 shows the time required for binding of the enzyme to the grafted silica gel. 60 units of polygalacturonase were mixed with 1 g. of the treated silica gel. Within 2 hours, 88% of the enzyme was bound, and further incubation did not increase binding appreciably.

Properties of the immobilized endo-polygalacturonase

The number of catalytic cycles, which the immobilized enzyme could pass through with maximum retention of its activity, would determine the efficiency of the binding. The present immobilized endo-polygalacturonase could pass through 10 cycles, with 90% retention of its original activity (Fig. 4.10). Thus tight binding to the support allowed very little leaching of the enzyme.

The Lineweaver-Burk's plot yielded a Km value of 1.32 mg/ml (Fig. 4.11) for the bound enzyme, as compared to 0.82 mg/ml for the soluble enzyme. The higher

Figure 4.9

Binding kinetics of endo-polygalacturonase immobilization

1 gram of grafted silica gel was incubated with 40 units of enzyme, and soluble activity was determined at regular time intervals

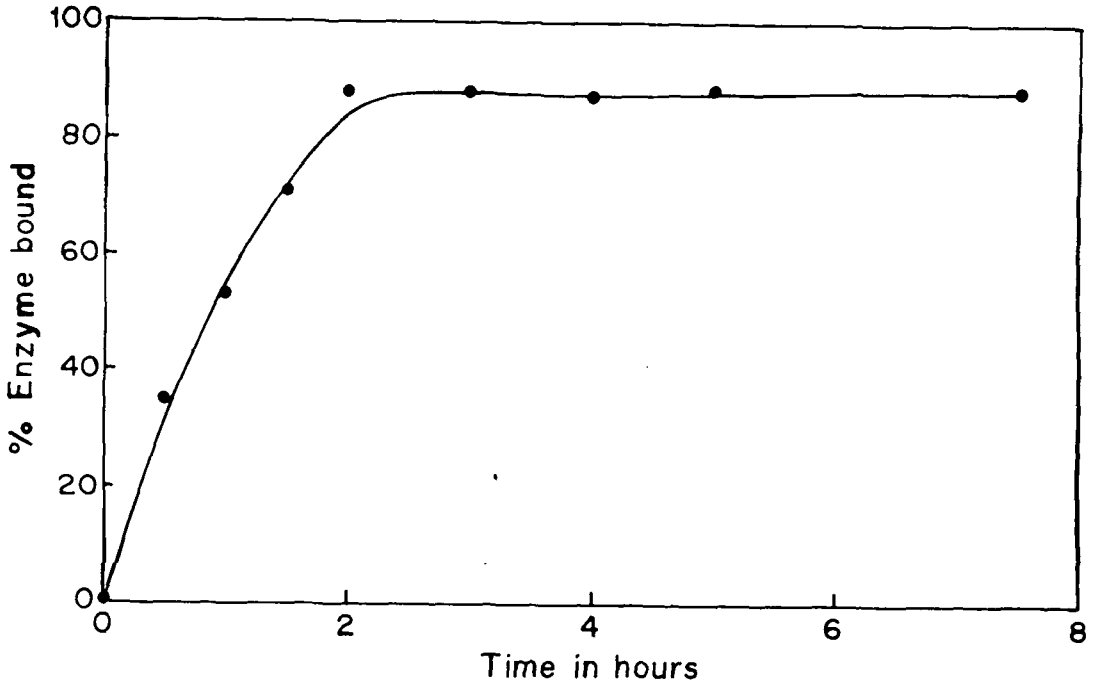


Figure 4.10

Reusability of immobilized endo-polygalacturonase

The reusability was determined by passing the immobilized enzyme through cycles of incubation with substrate

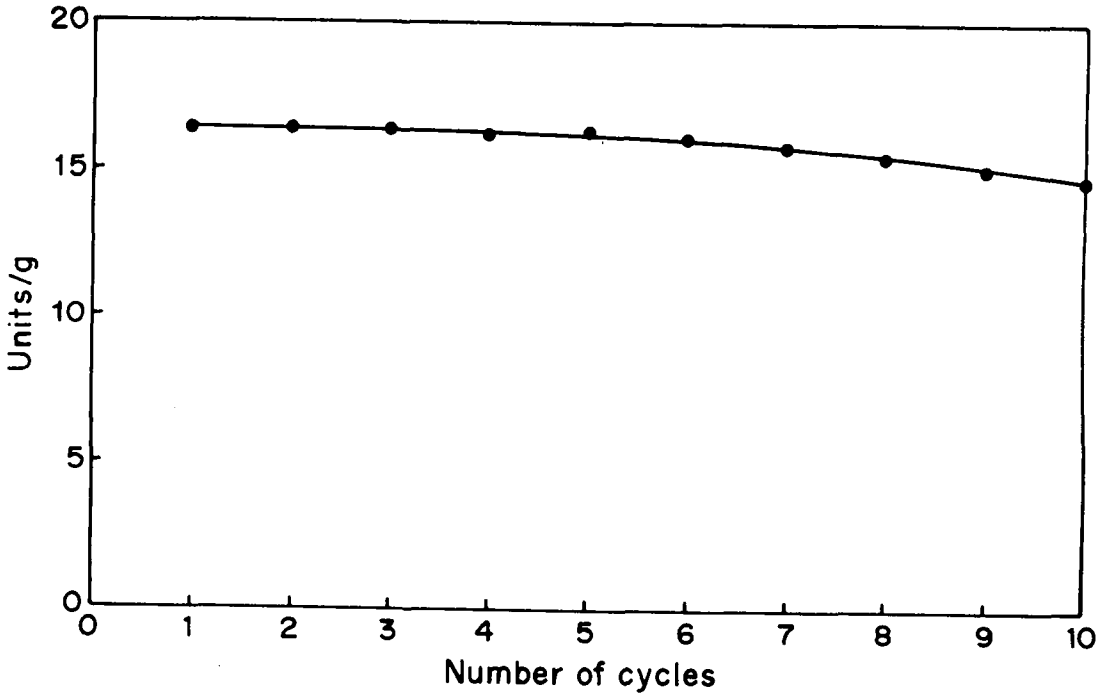
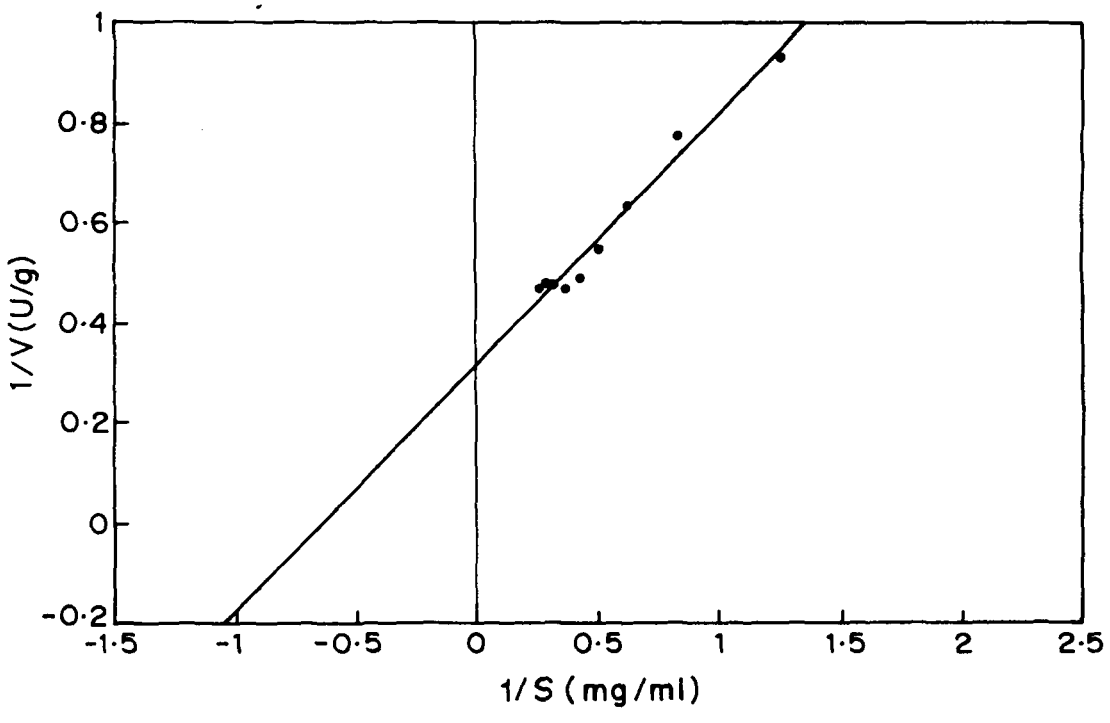


Figure 4.11

Km of immobilized endo-polygalacturonase

Activity of the immobilized enzyme was determined at different substrate concentrations



apparent Km of the immobilized enzyme could be due to diffusional limitations of the polymeric nature of the substrate.

It is generally found that immobilized enzymes have increased thermal stability over the soluble enzymes, though this is not true for all enzymes (Woodward 1985). As seen from the comparison of Fig. 4.6 and Fig. 4.12, the immobilized endo-polygalacturonase too was found to have increased thermal stability. At 40 °C, 50% loss of activity was obtained within 10 hours for the soluble enzyme, whereas the immobilized enzyme retains 82% of its activity in 10 hours at 40 °C. Similarly the half-life (50% retention of activity) of the soluble enzyme at 50 °C was 30 minutes, whereas the half life of the immobilized enzyme at 50 °C was 5 hours. This increased stability would be advantageous in the industrial usage of the immobilized polygalacturonase. The thermal resistance could be due to the sturdiness of the silica gel matrix, onto which the enzyme was immobilized, which would impede diffusional denaturation of the enzyme.

Application of polygalacturonase in fruit juice clarification

The traditional and major use of pectic enzymes has been in food processing (Uhlig 1990). Polygalacturonase obtained from solid-state fermentation was analyzed for its ability to clarify apple and orange juices.

When apple juice was treated with 0.1% v/v of the crude extract obtained from the mouldy bran, flocs started appearing within 30 minutes of reaction. According to Whitaker (1984), when pectin of the apple juice is degraded, the stabilizing effect is lost, and the insoluble particles present in the juice start coagulating and settle down. The clarification process results in a decrease in viscosity and simultaneous decrease in the turbidity of the juice. As shown in Fig. 4.13, the drop in viscosity and the turbidity, as measured by the absorbance at 660 nm., of the treated apple juice reach saturation after 60 minutes, and a clear fluid was obtained. Similarly, orange juice was also clarified when treated with 0.1% v/v of enzyme extract (Fig. 4.13). No further decrease in viscosity and turbidity, was observed after 90 minutes of treatment and clear orange juice was obtained. The time period required for clarifying orange juice was longer as compared to that for apple juice, due to difference in pH of the two juices. Orange juice was found to

Figure 4.12

Temperature stability of immobilized endo-polygalacturonase

0.1 gram of matrix with immobilized enzyme was incubated at different temperatures and the residual activity determined

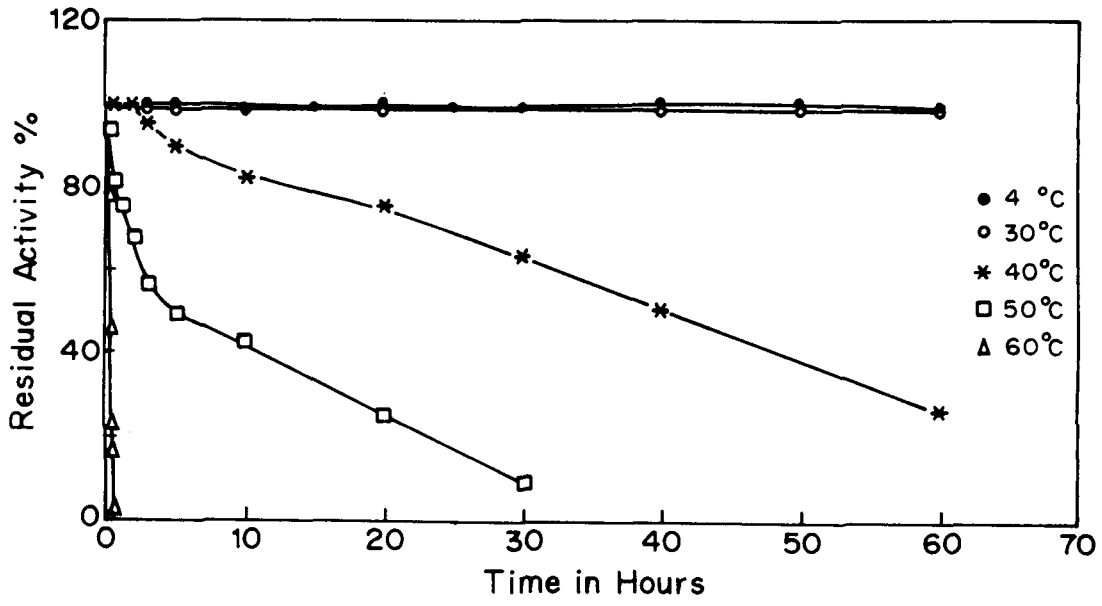
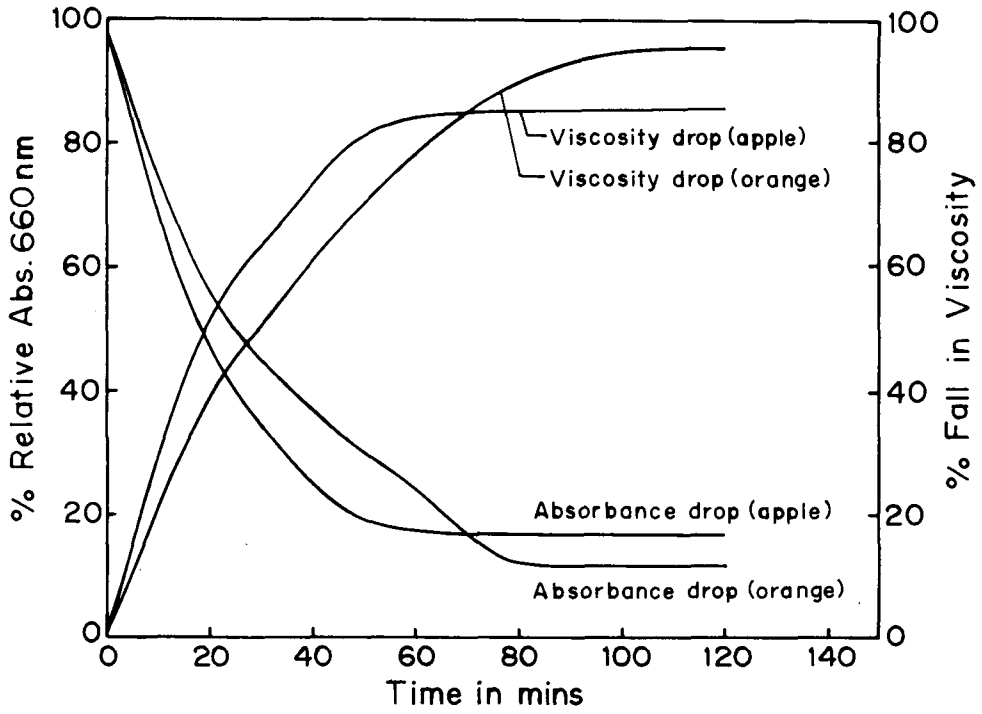


Figure 4.13

Fruit juice clarification by polygalacturonase

200 ml of fresh apple and orange juice were incubated with 0.2 ml (30 units) of enzyme preparation. Aliquots were heated at 60 °C for 5 minutes, and their viscosity and absorbance at 660 nm. determined.



have a low pH of 3.3, whereas the apple juice had a pH of 5.2. The optimum pH for activity of the polygalacturonase was 4.6. as shown earlier.

Conclusions

Cellulase-free polygalacturonase was produced by a mutant strain of *Aspergillus ustus*, in submerged shake-flasks cultures and solid-state fermentation. Media optimization showed that orange pulp addition increased yield in both types of fermentation. The enzyme was stable over a wide range of pH and at room temperatures. The enzyme preparation was successfully utilized to clarify apple and orange juices. The purified endo-polygalacturonase was immobilized on modified silica gel. The immobilized enzyme would be advantageous with respect to its reusability and its increased thermostability.

CHAPTER 5:

GENERAL DISCUSSION

Two types of depolymerases acting on polygalacturonic acid have been characterized in this thesis. Pectate lyase from *Fusarium moniliforme*, isolated from a marine mangrove ecosystem and an endo-polygalacturonase from *Aspergillus ustus* NCIM 1033 have been purified and their active-site characterized. The results of this study have been used to discuss the two enzymes, the first of which catalyzes ß-eliminative cleavage of the glycosidic bond and the second which acts hydrolytically on the same substrate, in the light of current understanding of electrophilic/nucleophilic catalysis.

The present strain was identified as *Fusarium moniliforme* on the basis of morphological characteristics. The form-genus *Fusarium* is composed of a highly diverse group of saprophytic and plant-pathogenic fungi (Anderson 1992). Asexual reproduction through microconidia and macroconidia (Subramanian 1971), is thought to predominate in the field, but many *Fusarium* anamorphs have *Gibberella* (*Fusarium moniliforme*) or *Nectria* (*Fusarium solani*) teleomorphs, that are elicited in the laboratory (Anderson 1992). Our strain has been presently grouped as a fungi imperfecti.

The induction of pectate lyase and polygalacturonase from *F.moniliforme* in different culture conditions was studied and it was found that the fungus releases pectate lyase (EC 4.2.2.2) at pH 8.0 and polygalacturonase (EC 3.2.1.15) at pH 5.0. The present strain, isolated from a mangrove environment, where pH is between 7.0 to 7.9, would probably secrete pectate lyase in order to assimilate pectic materials of leaf detritus in such an alkaline environment. At pH 7.0, the organism produces about 15% of the polygalacturonase activity produced at pH 5.0. This enzyme may either complement the pectate lyase in degrading pectin or it may regulate pectate lyase activity. Collmer *et al* (1982), in their studies on *Erwinia chrysanthemi*, implicated a polygalacturonase in the release of pectate lyase by the same organism, suggesting that the oligomeric products of the degraded pectin material may trigger the induction of pectate lyase. It is possible that a similar regulatory mechanism occurs in this species. Low levels of both polygalacturonase and pectate lyase, "scout enzymes" (Burns 1993), are produced by the present organism which may also help in production of oligomeric enhancers.

Opportunistic organisms, like the present strain of *F.moniliforme*, produce various other polysaccharidases like endoglucanase (EC 3.2.1.4), xylanase (EC

3.2.1.8) and amylase (EC 3.2.1.1), in addition to pectin degrading enzymes, when induced by appropriate substrates at different pHs. The secretion of polysaccharidases presumably helps the organism in the saprophytic colonization of mangrove leaf detritus. A study by Raghukumar *et al* (1994) has shown colonization by various fungi, including *F.moniliforme* on mangrove leaf litter. The triggers that result in the induction of different enzymes would form an interesting eclogical study.

As the polygalacturonase of *F.moniliforme* has been already studied (DeLorenzo 1987), we have concentrated on the extracellular pectate lyase from the strain and this enzyme was purified and characterized. Circular dichroism spectroscopy has shown that the pectate lyase falls in the all-ß group of structures. This adds support to the earlier studies on the crystal structures of pectate lyase of *E.chrysanthemi* (Yodder 1993) and that from *B.subtilis* (Pickersgill 1994) which were shown to consist of a unique motif of parallel ß-sheets folded into a helix. The barrel structure may be the reason for the stability of these enzymes, including the present protein. The similarity of these structures with the α/ß barrel structures of α-amylase (Buisson 1987), aldolase (Marvidis 1982), pyruvate kinase (Stuart 1979), triose phosphate kinase (Banner 1975) and xylose isomerase (Carrel 1984), which have widely differing functions, supports the hypothesis of a convergent evolution of carbohydrases towards a stable structure.

Our studies, which show that Ca^{++} is a part of the holo- enzyme held by carboxyl groups of the protein adds, for the first time, biochemical evidence to X-ray crystallographic data of Yodder *et al* (1993) and Pickersgill *et al* (1994) that this metal is indeed essential for the activity of the enzyme and is a part of the protein. Pickersgill *et al* (1994) had shown that calcium is present in the cleft formed between the ß-barrel domain and the loops, and suggested that the cleft is the probable active-site on the pectate lyase produced by *B.subtilis*. We have shown that calcium is involved in the catalysis, not in substrate binding, which contradicts an earlier theory of Crawford *et al* (1987) that calcium only forms dimers of polygalacturonate chains and plays no further role in catalysis. Lewis acids such as Zn^{++} are known to assist catalytic conversion of substrates by enzymes such as carboxypeptidase and carbonic anhydrase (Williams 1971). Although, calcium was grouped in the strong Lewis acids having low polarizibility by

Pearson (1970), Williams (1971) proposed that a micro- environment such as exists in a metallo-protein would lead to an energised ground state of the metal ion, termed as the "entactic state", capable of decreasing the activation energy for a reaction. Thus such an "entactic state" (Williams 1971) of the calcium may increase its polarizibility necessary for electrophilic catalysis.

In order to explain the high rates of reaction in ß-elimination, Gerlt and Gassman (1992) suggested that a general acid attack on the carboxyl group assists the abstraction of an α- proton. We therefore suggest that a concerted nucleophilic-electrophillic attack by the lysine moiety in the active-site of the enzyme, together with calcium on the substrate catalyzes the breaking of the α-1,4-glycosidic linkage by pectate lyase.

The present studies on .the endo-polygalacturonase (poly (1,4-α-D-galacturonide) glycanohydrolase, EC 3.2.1.15) produced by *Aspergillus ustus* suggests the involvement of histidine in catalysis and tryptophan in substrate binding. Rexova-Benkova (1968) and Cooke (1976) had shown earlier that histidine is essential for activity of endo-polygalacturonase from *A.niger*.

Tryptophan has been implicated in substrate binding in other carbohydrases, for example endo-1,4- ß-glucanase of *Bacillus* sp.KSM-330 (Ozaki 1991), exo-(1-3)-ß-D-glucanase from *Rhizoctonia solani* (Ohio 1989), and xylanase from *Streptomyces* (Keskar 1989). Tryptophan has also been shown in the active-site of an interesting enzyme from *Arthrobacter globiformis* T6 which exhibits both isomaltase and dextranase activity (Okada 1988). Thus tryptophan appears to have an affinity to pyranose rings. Not much is known about the thermodynamics of such interactions.

Isotope effect studies with deuterated water were used to investigate the catalytic mechanism of the present polygalacturonase. The mechanism of action proposed is that a single proton is transferred from the histidine residue of the enzyme to the glycosidic oxygen. Subsequent hydrolysis is effected by the addition of a water molecule. Thus the enzyme acts as a general acid catalyst, as is the case of other hydrolases, such as exo-ß-(1,3)-D-glucanase (Jeffcoat 1987), α-L-aribi-nofuronidase (Selwood 1989), ß-glucosidase (Umerzurike 1981) and ß-galactosidase

(Rosenburg 1981). Crystallization of this endo- polygalacturonase is currently being undertaken in our laboratory.

Pectinases are an important group of industrial enzymes, with applications ranging from food processing to treatment of raw-cellulosic fibres. The industrial aspects of pectinases were studied, using a mutant strain of *Aspergillus ustus* NCIM 1033 and a cellulase-free polygalacturonase preparation was obtained. Media optimization in solid-state fermentation gave a yield of 950 units of the enzyme per gram medium as compared to 30 units/ml obtained in submerged cultures. This has been known in the case of other metabolites too (Hesseltine 1972), though the reasons are not yet clear. The fact that the present organism lacks cellulase may increase its value in retting of natural fibres. The present organism does not produce any aflatoxins, as determined by HPLC analysis of a broth concentrated 100 times broth. The absence of aflatoxins would permit its use in the food industry.

In conclusion, a pectate lyase from a strain of *F.moniliforme* and an endo-polygalacturonase from *A.ustus* NCIM 1033, have been characterized and their active-site residues were identified. A bench-scale process for producing a cellulase-free polygalacturonase has also been developed.

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Abbreviations

Da	Dalton
DEP	Diethyl pyrocarbonate
DTNB	Dithiobisnitrobenzoic acid
EGTA	Ethylene glycol-bis-(β-aminoethyl ether) N,N,N,N-tetra acetic acid
HNBB	Hydroxy nitro benzyl bromide
NCIM	National Collection of Industrial Microorganisms
NEM	N-ethyl maleimide
PHMB	p-hydroxymercuric benzoate
PMSF	Phenylmethyl sulphonyl flouride
TNBS	Trinitrobenzene sulphonate
TNP	Trinitrophenyl
WRK	Woodward's reagent K