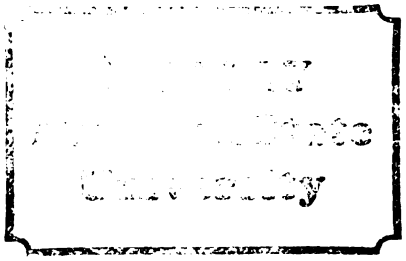

PURIFICATION OF AN ENDOPOLYGALACTURONASE FROM A COMMERCIAL PREPARATION OF *ASPERGILLUS NIGER*

By

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A THESIS

Submitted to

Michigan State University

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

Department of Food Science and Human Nutrition

1981

ABSTRACT

PURIFICATION OF AN ENDOPOLYGALACTURONASE FROM A COMMERCIAL PREPARATION OF *ASPERGILLUS NIGER*

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An endopolygalacturonase (PG) from *Aspergillus niger* was obtained from a commercial source. This enzyme was characterized as a polygalacturonase by its preference for polygalacturonic acid (de-esterified pectin) as a substrate. It was further characterized as having a random mechanism of action (endo-enzyme) by the drop in viscosity of the substrate as related to the increase in reducing groups produced. PG was purified approximately 50-fold using sequential chromatography on gel filtration, DEAE-cellulose, and Phenyl Sepharose. The purity was determined by disc gel electrophoresis. Several bands were present of which one shows polygalacturonase activity. No other pectinolytic enzymes were detected. This preparation has a pH optimum of 4.4, a temperature optimum of 45-50^{o}C, and a Vmax of 1900 micromoles/min/mg protein. The Km is .37 mg/ml. The purification scheme utilized to obtain this preparation was compared to others found in the literature.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to my major professor, Dr. J.R. Brunner, for his valuable discussions and advice during the course of this study and the preparation of this manuscript. I would also like to thank Dr. J.J. Jen, currently with the Campbell Institute for Research and Technology in Camden, N.J., for his help during the initial phases of this research. Appreciation is also extended to Dr. M.A. Uebersax and Dr. H. Momose of the Department of Food Science and Human Nutrition for reviewing this manuscript and for serving on my graduate committee.

My sincere thanks also to Dr. R.N. Costilow of the Department of Microbiology and Public Health who, in addition to reviewing this manuscript and serving on my graduate committee, has put up with me longer than anyone else.

Finally, I would like to thank John Euber for his encouragement and valuable discussion.

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INTRODUCTION

Polygalacturonase (PG, EC .3 .2 .1 .15) belongs to a larger group of enzymes referred to as pectic enzymes. These enzymes are responsible for the degradation of pectic substances and cause plant tissue maceration. They are, therefore, involved in such natural processes as the ripening of fruit, the abscission of leaves and plant organs, the invasion of tissues by plant pathogens, the spoilage of fruits and vegetables, and the rapid decay of plant material. However, the mechanisms and controls by which these enzymes operate in these natural processes are not all clearly understood.

Pectic enzymes are also of significance to the food industry. Their presence can be either beneficial or detrimental depending on the desired properties of the finished product. Generally, when these enzymes are utilized, it is in the form of crude extracts. Thus, they often contain a variety of other enzymes such as cellulases or proteases. These nonpectic enzymes may play a helpful role in many situations but could also contribute to problems with color and taste in more sensitive applications. In these situations, only the presence of a specific pectinolytic enzyme is desired as opposed to the multiple types found in the

crude preparations. This requires a certain amount of purification prior to the use of the enzyme.

Methods employed for the isolation and purification of polygalacturonase have focused on relatively complex protocols involving traditional techniques such as gel filtration and/or ion exchange chromatography. To date, these procedures have yielded enzyme preparations varying in composition and activity. A simplified purification procedure for the enzyme would enhance further studies of the reaction mechanisms as well as its exploitation in applied technology. Thus, the objective of this study was to provide a simple, efficient purification scheme and to characterize the resulting enzyme preparation.

LITERATURE REVIEW

Substrates and Their Degradation by Pectinolytic Enzymes

Pectic substances form a group of heterogenous polysaccharides which occur mainly in the middle lamella and primary cell wall of higher plants (Rombouts and Pilnik, 1978; Fogarty and Ward, 1972; Bateman and Millar, 1966; Whitaker, 1972; Rexova-Benkova and Markovic, 1976; Codner, 1971; Fogarty and Ward, 1974). The principal function of these substances is to maintain the integrity and coherence of the plant tissue. Structurally, they are composed primarily of linear polymeric chains of D-galacturonic acid linked α-1, 4 and esterified to varying degrees with methanol. The nonuronide fraction varies in concentration according to the source of the pectic substances and have been found to consist of L-rhamnose, L-arabinose, D-galactose, D-xylose, and L-fructose (Bateman and Millar, 1966; Rombouts and Pilnik, 1978; Fogarty and Ward, 1974).

The nomenclature of this group of substrates is based upon its varying degrees of methoxylation and neutralization. The water-insoluble parent pectic substance from which the others are derived is referred to as protopectin. Pectic

acid is composed primarily of poly-D-galacturonic acid and is essentially free of methyl ester groups. Pectinic acids are those pectic substances that contain more than a small proportion of methyl ester groups. Pectins are pectinic acids that are capable of forming gels at suitable pH values when sucrose is present (Fogarty and Ward, 1974; Codner, 1971; Pilnik and Rombouts, 1978). The ability to form gels is also affected by the chain length of the polymer (Codner, 1971).

Pectic materials are not uniform and pectins isolated from different plant species are attacked at different rates by a given enzyme system (Bateman and Millar, 1966). The molecular weight of the substrate and degree of esterification as well as the source can significantly alter its degradation by pectic enzymes (Dongowski et al., 1980). Bock et al. (1972) compared several different preparations of pectolytic enzymes and concluded that accurate determination of their specific activities requires the standardization of the substrate with respect to molecular weight and degree of esterification.

One example of the effect of substrate size appeared in the early literature (Demain and Phaff, 1954). A pectic acid specific hydrolase was isolated from Saccharomyces fragilis that exhibited a shift in pH optimum with low molecular weight substrates. Similar shifts in pH optimum have since been reported by a number of authors (Mill and

Tuttobello, 1961; Ayers et al., 1969; Barash and Eyal, 1980). One of these, Pressy and Avants (1971), noted inhibition of tomato PG by higher molecular weight substrates at low pH. Some substrate inhibition was also reported by Koller and Neukom (1969) for PG from Aspergillus niger. Paytner and Jen (1975) pointed out the existence of possible substrate inhibition of PG from Monilinia fructicola.

The rate of substrate degradation is also greatly affected by its size. Nasuno and Starr (1966) isolated an endo-PG from Erwinia carotovora possessing higher rates of activity on longer chain polygalacturonic acids. Barash and Khazzam (1970) observed a similar occurrence with endo-PG from Colletotrichum gloeosporioides. More recently, Liu and Luh (1980) reported that the rate of hydrolysis by endo-PG from Rhizopus arrhizus was a function of substrate size. This endo-PG preferred substrates of intermediate size (degree of polymerization (DP) = 24) rather than larger or smaller ones. Other hydrolytic pectic enzymes prefer oligogalacturonides to polygalacturonides. Hasegawa and Nagel (1967) reported a hydrolase from a Bacillus species that exhibited greatest activity with trigalacturonic acid. Erwinia aroideae produced a similar transeliminase as reported by Hatanaka and Ozawa (1970).

Classification

Polygalacturonase belongs to the group of enzymes collectively referred to as pectic enzymes. Within this group there are two major divisions; the saponifying enzymes called pectin esterases, and the depolymerizing enzymes. Pectin esterase catalyzes the deesterification of pectin whereas the depolymerases catalyze the splitting of the glycosidic (1, 4) bonds of the substrate (MacMillian and Sheiman, 1974; Whitaker, 1972).

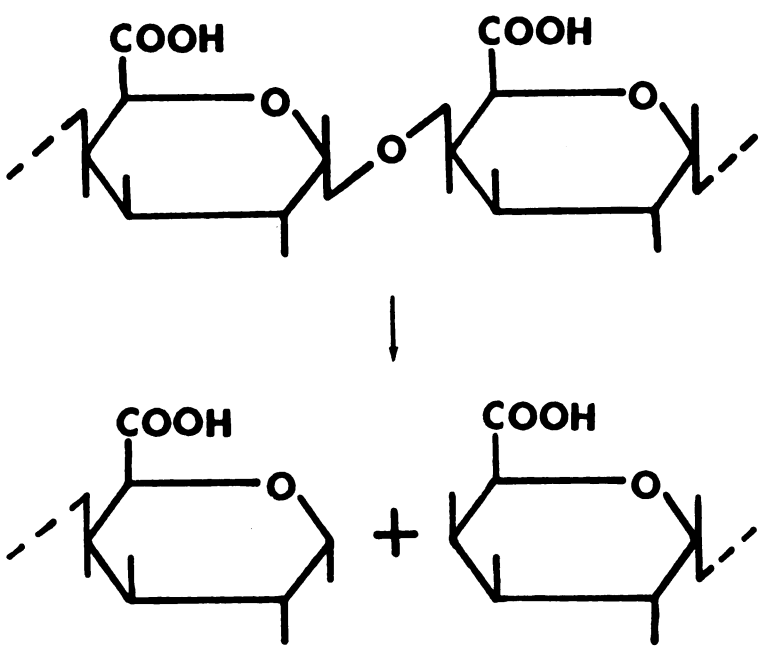
Historically, the depolymerases have been further subdivided into 8 groups on the basis of; a) mechanism of cleavage, b) preference of the enzyme for pectate or pectin, and c) random or terminal splitting of the glycosidic bond, see Table 1, (Kulp, 1975; Voragen and Pilnik, 1970; Rexova-Benkova and Markovic, 1976). The mechanisms of cleavage by the depolymerases are either of the hydrolytic or transeliminative type as illustrated in Figure 1. Enzymes catalyzing trans-eliminative cleavage are referred to as either pectate or pectin lyases, whereas those catalyzing hydrolytic cleavage are polygalacturonases or polymethylgalacturonases (PMG). Some authors now doubt the actual existence of PMG, believing instead that the PMG's cited in the literature were in fact lyases (Rombouts and Pilnik, 1972). Also, enzymes belonging to the exo-PMG and exopectin lyase groups have not been found. In addition, a different

Table 1. Depolymerizing pectic enzymes

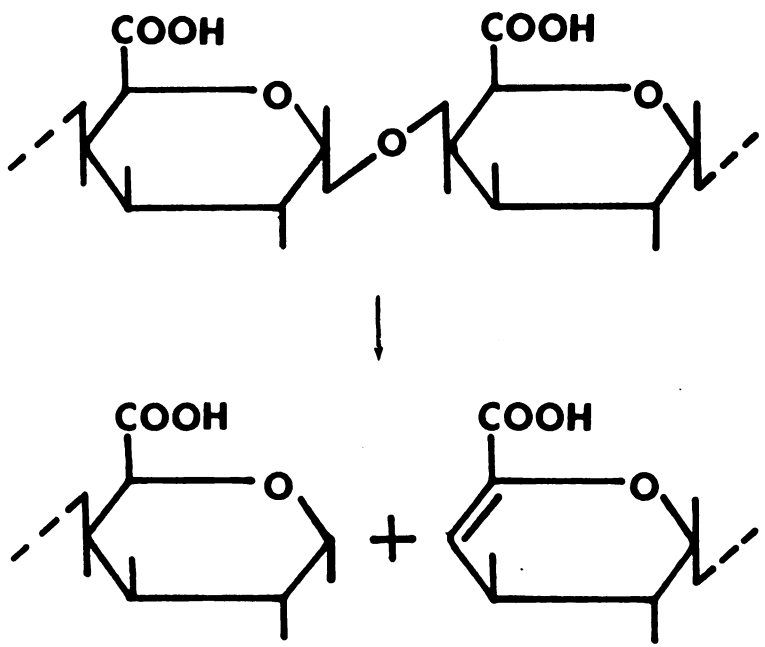
I.	Hydrolytic cleavage
	A. pectin as substrate
	1) endo-polymethygalacturonase
	2) exo-polymethygalacturonase
	B. pectic acid as substrate
	1) endo-polygalacturonase
	2) exo-polygalacturonase
II.	Transeliminative cleavage
	A. pectin as substrate
	1) endo-pectin lyase
	2) exo-pectin lyase
	B. pectic acid as substrate
	1) endo-pectate lyase
	2) exo-pectate lyase

Figure 1. Illustration of hydrolytic and trans-elimi-
native cleavage of polygalacturonic acid by
polygalacturonase and pectate lyase.

Hydrolytic cleavage



Trans-eliminative cleavage



type of hydrolase has been described which prefers oligogalactosideuronates in preference to the polymeric substrates (Hasegawa and Nagel, 1968). It cleaves bonds starting at the nonreducing end of the molecule, releasing digalacturonic acid as the sole reaction product. As a result, Rexova-Benkova and Markovic (1976) proposed the classification scheme shown in Table 2. This table includes suitably modified systematic nomenclature and code numbers.

Occurrences, Uses, and Significance of Pectic Enzymes

Pectinolytic enzymes occur in many plants and microorganisms (Kulp, 1975; Arima et al., 1964; Endo and Miura, 1961; Pilnik and Rombouts, 1978). In plants, their presence has been related to the level of maturity and ripening (Raymond and Phaff, 1965; Ahmed and Labavitch, 1980; Poovaich and Nukaya, 1979; Fogarty and Ward, 1972). They also play a role in the abscission of leaves and plant organs (Huberman and Goren, 1979; Sagee and Goren, 1980; Berger and Reid, 1979). In addition, they play an important role in the degradation of dead plant materials, thus assisting in recycling carbon compounds (MacMillian and Sheiman, 1974). However, the exact mechanism of these natural processes is not understood completely.

Pectic enzymes are also implicated as a feature of many host pathogen interactions (Paynter and Jen, 1974; Bisen and

Table 2. Nomenclature of Pectic Depolymerases

Name	Preferred substrate	Action pattern
Lyases		
endopectate lyase	D-Galacturonan	random
exopectate lyase	D-Galacturonan	penultimate bonds
oligo-galactosiduronate lyase	Oligo-D-galactosiduronate	terminal
pectin lyase	Poly(methyl D-galactosiduronate)	random
Hydrolases		
endo-galacturonase	D-Galacturonan	random
exo-galacturonase	D-Galacturonan	terminal
galacturonandigalacturono hydrolase	D-Galacturonan	penultimate bonds
oligo-galactosiduronate hydrolase	Oligo-D-galactosiduronate	terminal

Modified EC systematic name	EC No.
poly-(1 - 4)-α-D-galactosiduronate lyase	4.2.2.2
poly-(1 - 4)-α-D-galactosiduronate exolyase	4.2.2.9
oligo-D-galactosiduronate lyase	4.2.2.6
poly(methyl D-galactosiduronate) lyase	4.2.2.10
poly-(1 - 4)-α-D-galactosiduronate glycanohydrolase	3.2.1.15
poly-(1 - 4)-α-D-galactosiduronate glycanohydrolase	3.2.1.67
poly-(1 - 4)-α-D-galactosiduronate digalacturonohydrolase	3.2.1.82

Agarwell, 1980; Arinze and Smith, 1979; Weste, 1978; Tseng and Tseng, 1980; Barmore and Brown, 1979; Verhoeff, 1978). They are involved in a variety of diseases of plants such as soft rots, dry rots, wilts, blights, and leafspots. Resistance of some plants to various pathogens may be attributed to the presence of natural inhibitors of the pectinolytic enzymes (Bateman and Millar, 1966; Bell et al., 1965; Albersheim and Anderson, 1971; Fisher et al., 1973).

Industrial applications of pectic enzymes are varied. They are often used in natural fermentations to modify plant tissue, such as the process of retting from which certain textile fibers are produced (MacMillian and Sheiman, 1974; Chesson, 1978). The processing of coffee beans also involves the use of pectic enzymes either in a natural fermentation or through the addition of commerical enzymes to liquify the mucilage which consists of protopectin, pectin, and pectin esters. Addition of commercial enzymes accelerates the digestion, improves quality, and eliminates undesirable fermentations (Sivetz and Foote, 1963).

One of the major uses of pectic enzymes is in fruit and vegetable juice clarification and processing. In apple juice, clarification depends on the ability to hydrolyze the pectin which reduces the viscosity of the juice, making filtration easier (Tressler and Joslyn, 1971; Neubeck, 1975). Treatment of fruit can also increase the yields of juice as in the case of grape juice processing. Here, the

Concord grape, which is often used in this process, has a slimy consistency after crushing, making it very difficult to press without prior enzymatic treatment (Neubeck, 1975).

For juices in which cloud stability is desired, the presence of pectic enzymes can have detrimental effects. Thus juice is often heated to inactivate naturally occurring pectic enzymes (Sherkat and Luh, 1977). In the processing of tomato juice, this procedure is referred to as the "hot break" method (Paul, 1975; Lopez, 1975). A similar problem occurs with citrus juices. In orange juice, only PE is responsible for the "cloud-loss". Cloud-loss occurs after the pectin is demethylated and the resulting insoluble pectinic acids precipitate. Preventing cloud-loss can be accomplished by either heating the orange juice to inactivate the PE or by adding commercial enzymes high in PG activity and low or lacking PE activity (Tressler and Joslyn, 1971). Heating orange juice can cause some undesirable flavor changes. Treatment with PG has no effect on flavor while preventing the precipitation of pectinic acids by cleaving them into soluble pectates (Baker and Bruemmer, 1972). Enzymic treatment can also be used on the citrus pulp in order to increase yields. A minor benefit derived from this treatment would be the improved recovery of citrus seeds (Barmore and Castle, 1979).

Other uses of pectic enzymes include the treatment of wine to improve yields, color and to aid in clarification

in the production of wine. The effect on yield is most significant because improved color is not always obtained (Neubeck, 1975).

Miscellaneous applications for these enzymes include the treatment of commercial softwoods to predispose them to treatment with preservatives, as an aid in the recovery of citrus and olive oils, and in the production of galacturonases of low degrees of polymerization and low methoxyl pectins (Fogarty and Ward, 1972). Low methoxyl pectins are important commercially in the production of low calorie jams and jellies (Lopez, 1975). Structural studies of pectic substances as well as the study of pectin in plant material can also be aided through the use of these enzymes (Neubeck, 1975).

Production of Polygalacturonase

For the commercial production of PG and other pectic enzymes, mainly aspergilli are used in either submerged or surface culture (Pilnik and Rombouts, 1978). In the United States commercial pectinases are derived from Aspergillus niger as required by the FDA. Generally, these preparations are crude extracts containing mixtures of pectolytic enzymes as well as nonpectolytic enzymes. Variations in activity and type of pectolytic enzymes produced can be attributed to culture conditions.

Although the PG of many microorganisms appears to be constitutively produced, culture conditions can have a dramatic effect on the amount and type produced. Mill (1966) found that adding 5% pectin to the medium of Aspergillus niger favored the production of exo-PG at the expense of endo-PG. Yamasaki et al. (1966a) reported that the addition of pectin increased the endo-PG activity of Aspergillus saitoi. Ayers et al. (1969a), Hasija and Agarwal (1978) and Lobanok et al. (1979) also reported constitutive PG whose production could be increased by varying culture conditions. In addition, Brookhouser and Weinhold (1979) reported an increase in endo-PG activity of Rhizoctonia solani in response to cottonseed and hypocotyl exudates. This organism was shown later to produce endo-PG's of varying molecular weights and isoelectric points depending upon culture conditions (Brookhouser, et al., 1980).

In other microorganisms PG production is induceable. Szajer and Bousquet (1975) compared the production of PG by several species of pathogenic fungi and found several that were induceable. Fanelli et al. (1978) reported the isolation of two inducible PG's from culture filtrates of Trichoderma koningii.

Up to this point, only extracellular PG's have been discussed. Intracellular PG's are also known. Cappellini (1966) studied both intra- and extracellular PG from Rhizopus stolonifer. Polygalacturonase within the spores of

Geotrichum candidum was examined by Barash and Klein (1969).

Purification of Endopolygalacturonase

A variety of methods have been utilized in the purification and characterization of various endo-PG's. These methods generally include an initial step to process large quantities such as precipitation from aqueous suspension with acetone, alcohol or ammonium sulfate, followed by ion exchange chromatography and/or gel filtration. Frequently, preparative gel electrophoresis and/or isoelectric focusing is used to achieve final purification (Wang and Keen, 1970; Lim et al., 1980; Cervone et al., 1977; Fanelli et al., 1978).

Three endo-PG's were isolated from Coniothyrium diplodiella by Endo (1964a, b, c), using ammonium sulfate fractionation, DEAE cellulose chromatography and Duolite CS-101 chromatography. Ultracentrifugation studies indicated that each isozyme species was homogeneous. Although this purification scheme was effective in producing three pure isozymes, the yields of each were quite low, ranging from 1.1 to 8.5%. A reasonable explanation may be found in the poor recoveries obtained from the early ammonium sulfate fractionation step. Kaji and Okada (1969) also reported low yields (33%) after ammonium sulfate fractionation during the purification of an endo-PG from Corticium rolfsii.

Fielding and Byrde (1969) cited a poor recovery of enzymic activity following treatments with acetone, ethanol, or ammonium sulfate in the presence of purifying endo-PG from Selerotinia fructigena. Consequently, they began their purification scheme with gel filtration on Sephadex G-75 followed by ion exchange chromatography on Ecteola-cellulose and CM-Sephadex. The final activity yield was 31%. However, criteria for establishing the extent of purity were not given. This enzyme was further resolved by electrophoresis on cellulose acetate strips into two components with molecular weights of 77,000 and 38,500.

Other researchers utilizing alcohol, acetone, or ammonium sulfate treatments as an early purification step include Mill and Tuttobello (1961), Reymond and Phaff (1965), Rexova-Benkova and Slezarik (1966), Nasuno and Starr (1966), Yamasaki et al. (1966), Ayers et al. (1969b), Bateman (1972), Ishii and Yokotsuka (1972), Urbanek and Zalewska-Sobczak (1975), Liu and Luh (1978), Arinze and Smith (1979). Further purification of endo-PG's by the above authors typically include the employment of ion exchangers such as DEAE- or CM-cellulose and gel filtration. Nasuno and Starr (1966) used only repeated chromatography on CM-cellulose after extraction of the acetone precipitate with sodium acetate buffer. Although Mill and Tuttobello (1961) began their isolation with an ammonium sulfate step, they also utilized repeated chromatography on CM-cellulose.

Rexova-Benkova and Slezarik (1966) used DEAE-cellulose instead of CM-cellulose. By contrast, Ayers et al. (1969b) used only gel filtration on Sephadex G-100 after fractionation with ammonium sulfate. The yields of enzymic activity obtained by these procedures were generally around 30%.

A number of authors began purification directly with either ion exchange chromatography or gel filtration. These include: Fielding and Byrde (1969), Fanelli and Cervone (1977), Cervone et al. (1977), Pressy and Avants (1973), Barash and Eyal (1970), Barash and Khazzum (1970), Harman and Corden (1972), Cooke et al. (1976), Lim et al. (1980), Wang and Keen (1970), and Horikoshi (1972). In addition to chromatography on ion exchangers and gel filtration, Wang and Keen (1970) and Harman and Corden (1972) made use of chromatography on hydroxylapatite. Yields for these purification schemes showed greater variations but were still similar to those previously mentioned.

All procedures mentioned thus far are fairly complex and time consuming. A number of workers have applied innovative methods in attempts to simplify purification procedures. Alieva et al. (1978) utilized chromatography on epichlorohydrin cross-linked beet pectin to obtain a 13- to 15-fold purification of an endo-PG from Geotrichum candidum. Rexova-Benkova and Tibensky (1972) made use of pectic acid cross-linked by epichlorohydrin to isolate an endo-PG from Aspergillus niger. This procedure resulted in

approximately a 14-fold purification. Yields were not reported, but the final specific activity was lower than that obtained by a more involved scheme utilizing repeated chromatography on DEAE-cellulose.

Thimbault and Mercier (1978a) attempted to purify an endo-PG also from Aspergillus niger using only agarose gel chromatography. They report a 52% yield of the activity and a 36-fold purification. However, the enzyme preparation was shown by electrophoresis and isoelectric focusing experiments to contain an unknown nonpectolytic protein fraction.

Characteristics of Endopolygalacturonase

Endo-PG generally has a pH optimum in the acid range between pH 4-6 (Cooke et al., 1976; Arinze and Smith, 1979; Fanelli et al., 1978; Lim et al., 1980). One exception to this has been reported by Horikoshi (1972) who studied an alkaline endo-PG obtained from a Bacillus that had a pH optimum of 10-10.5 but was most stable at pH 6.0. It had a molecular weight of 60,000-70,000, which is somewhat larger than most endo-PG's, and exhibited a higher temperature optimum (65^{o}C) than usually associated with PG's. Temperature optimums most frequently reported are generally close to 50^{o}C (Endo, 1964a, b, c; Yamaski et al., 1966b; Lim et al., 1980; Thibault and Mercier, 1978b; Rexova-Benkova,

1967; and Heinrichova and Rexova-Benkova, 1977).

The molecular weights reported for endo-PG's indicate the occurrence of one common size between 30,000 to 40,000 and another less common species in the range of 70,000 to 80,000 (Cooke et al., 1976; Pressy and Avants, 1973; Urbanek and Zalewska-Sobczak, 1975; Ishii and Yokotsuka, 1972; Bateman, 1972; Wang and Keen, 1970). Often, as when isozymes occur, both sizes will be present. Lim et al. (1980) isolated three sizes, 46,000, 50,000 and 30,000, from Saccharomyces fragilis. All three isozymes were glycoproteins whose main sugar components was mannose.

Other endo-PG's also exist as glycoproteins. Fanelli et al. (1978) isolated two isozymes from Trichoderma koningii, distinguishable by differences in their carbohydrate content. Cervone et al. (1977) found similar glycoprotein isozymes in Rhizotonia fragariae.

Many isozymes of endo-PG are distinguishable on the basis of their isoelectric points (Arinze and Smith, 1979). Generally, the isoelectric points of endo-PG's lie in the acid range. Koller and Neukom (1969) reported a pI of 4.5 for an endo-PG from Aspergillus niger, whereas Bateman (1972) reported a pI of 5.2 for an endo-PG from Scleroticum rolfsii. However, there are reports of endo PG isozymes with pI's in the neutral range (Brookhouser et al., 1980). Yuan and Tseng (1980) reported two isozymes in Phytophthora parasitica; one had a neutral pI of 7.3 while the other was

on the basic side with a pI of pH 9.0.

Reports on the isozymic nature of PG have been more frequent in the recent literature. Demain and Phaff (1954) reported only one endo-PG from Saccharomyces fragilis, whereas Lim et al. (1980) reported the presence of three enzymic species. Application of specific staining techniques has aided in the identification of isozymes (Stegeman, 1967; Cruickshank and Wade, 1980).

Further characterization of endo-PG is usually achieved by determining the enzymic parameters, Km and Vmax. Comparison of these reported values becomes difficult due to differences in the substrates used as discussed previously. Thibault and Mercier (1978b) report a Km of 0.44 mg/ml and a Vmax of 239 μmoles/min/mg protein for Aspergillus niger, using a substrate with a degree of polymerization of 44. This rate appears to be in fair agreement with the values given by Heinrichova and Rexova-Benkova (1977) and Mill and Tuttobello (1961) for endo-PG from the same organism.

MATERIALS AND METHODS

Source of Enzyme and Substrates

Spark-L HPG, a liquid preparation high in polygalacturonase activity was obtained in two shipments, A and B, from Miles Laboratories, Inc., and used as the source of endo-PG.

Polygalacturonic acid (98%, grade III) and pectin - grade 1 from citrus fruits with a galacturonic content of 84% and a methoxy content of 7.5% (degree of methylation, DM - 46%) were obtained from Sigma Chemical Company. Pectin of higher methoxy content (11.4-12.0% or DM = 70-73%) was obtained from Atlantic Gelatin, a division of General Foods. Polygalacturonic acids of different molecular weights (110,000 and 3200) as well as di-, tri-, tetra-, and pentagalacturonic acids were obtained from Richard B. Russell Agricultural Research Center, P.O. Box 5677, Athens, GA 30604. The preparation and characteristics of these are described by Pressy and Avants (1971).

Enzyme Assay Methods

Pectin-methyl esterase activity was detected by a drop in pH of the reaction mixture resulting from the liberation of carboxyl groups from pectin. The reaction mixture consisted of 0.5% pectin, DM 70-73%, in 0.1% NaCl. One hundred microliters of enzyme preparations at various purification stages were added to 10 ml of reaction mixture at room temperature to initiate the de-esterification of the pectin. The pH was measured at regular intervals for the first two hours and then checked once again after 18 hours.

Pectin and pectate lyase activities were ramified by increased absorbancy (235 nm) in the reaction mixture. Absorbancy was measured on a Gilford model 240 spectrometer. The reaction mixtures contained either 0.5% polygalacturonic acid in 50 mM tris buffer, pH 8.0, or 0.5% pectin in 50 mM Na acetate buffer, pH 5.2. Addition of approximately 50 µl of enzyme at various stages of purity were used to start the reactions at room temperature.

Polygalacturonase activity was determined either by a drop in viscosity of the reaction mixture or by the formation of reducing groups. The reaction mixture consisted of 0.5% polygalacturonic acid in 0.25 M Na acetate at pH 4.4. Viscosity of the mixture was determined in a Cannon-Ubberlohde semi-micro dilution viscometer. Thirty milliliters of reaction mixture was placed in the viscometer and incubated

at 30°C. The reaction was started with the addition of 50 microliters of enzyme solution containing enough endo-PG to produce .1 to .2 micromoles reducing sugar per minute. Viscosity was measured as the flow time in seconds that it takes the solution to pass two reference points within the viscometer bulb. The % drop in viscosity was calculated using the viscosity of the substrate as 100% and the viscosity of H_2O as 0%.

The formation of reducing groups was measured using the arseno-molybdate method of Nelson (1944) as modified by Ashwell (1957), employing galacturonic acid as the standard. Galacturonic acid was obtained from Sigma Chemical Co. The reaction mixture was incubated at 30°C and run with the addition of enzyme. Aliquots of 0.5 ml were taken at 2 to 5 minute intervals, and pipetted into 1 ml of Nelson's copper reagent to stop the enzymic reaction. Rates obtained over different time intervals insured that the activity measured was linear with time. Specific activity was expressed as units/mg protein. Units are defined as μmoles reducing sugar produced per minute.

Molecular Weight Determination of Substrate

The molecular weight of the polygalacturonic acid substrate was determined from viscosity data. Solutions of polygalacturonic acid ranging from 0.5 to .0625% (w/v) were

prepared in 1% Na hexametaphosphate. The viscosity of each solution was determined at 25°C using the Cannon-Ubberlohde viscometer. Intrinsic viscosity (η) was determined as the reduced viscosity where $C \rightarrow 0$ at the point of intersection on a graph of n_{sp}/C and $\frac{\ln \eta_R}{C}$ vs C. (Hewlett-Packard, 1966). Specific viscosity, $\eta_{sp} = \eta_R - 1$, where η_R is the relative viscosity, $\frac{ts}{to}$; ts is the flow time of the sample solution and to is the flow time for 1% Na hexametaphosphate. The concentration, C, is in g per 100 ml of the solute. Molecular weight (m.w.) was obtained from the relationship, m.w. = $\frac{(\eta)}{4.7 \times 10^{-5}}$ according to Christensen (1954).

Protein Determination

Protein determinations were based on the method of Lowry et al. (1951). Bovine serum albumin was used as the standard.

Heat Resistance and Temperature Optimum

Purified endo-PG was heated in 0.5 m Na acetate, pH 4.4 for 5 minutes at 30, 40, 50, 60, 80 and 100°C, then assayed for activity to determine its resistance to heat.

The temperature of optimum activity was determined from assays run at the following temperatures: 25, 30, 35, 40, 45, 50, 55, 60°C. Each assay was sampled at 2, 4, 8 and 16 minutes. Reducing sugars produced were measured and

served as an indication of enzymic activity.

pH Optimum and pH Stability

For pH-optimum studies, assays were run as previously described under enzyme assay methods except that assay mixtures were adjusted to various values of pH, ranging from 3.6 to 5.0.

To determine pH stability, the purified enzyme preparation was diluted 1:10 with either Na acetate or Na phosphate buffers (50 mM) at pH between 3 and 9. Assays were run at pH 4.4 at one week intervals to determine the activity remaining.

Effect of Substrate Molecular Weight on pH Optimum and Rate of Substrate Degradation

Three molecular species of polygalacturonic acids, 110,000, 6000, and 3200 daltons, were used in the assay mixture. The pH for optimum degradation of these substrates was determined as described previously. Substrate degradation rates by endo-PG were evaluated as before using Nelson's (1944) method as modified by Ashwell (1957) at pH 4.4 and 30^{o}C.

Effect of NaCl

NaCl was added to enzyme assay mixtures to give concentrations ranging from 20 to 400 mM. The activity of each assay mixture was assessed by measuring the amount of reducing sugars produced.

Kinetic Studies

The Km and Vmax for endo-PG were determined from Lineweaver-Burk plots of 1/V vs 1/S. Substrate concentrations used ranged from 0.3 to 4 mg/ml. The substrate was polygalacturonic acid (grade III) obtained from Sigma. Enzymic reactions were stopped by pipetting 0.5 ml of the reaction mixture into 1 ml of Nelson's copper reagent.

Dialysis of Enzyme

Dialysis of various enzyme preparations was carried out at $4^{o}C$ using dialyzer-tubing with a $12x10^{3}$ dalton cut-off obtained from Fisher Scientific. The tubing was pretreated by boiling in an aqueous solution of EDTA, rinsed, then boiling in distilled H_2O and rerinsed.

Ion Exchange Chromatography

Separation of proteins based on ion exchange was run with Ecteola-cellulose, DEAE-cellulose, and CM-cellulose. DEAE-cellulose and CM-cellulose were obtained from Bio-Rad; Ecteola-cellulose from Sigma. The effectiveness of Ecteola-cellulose was tested in both batch and column applications. The resins were all pretreated as described by the supplier (Bio-Rad, 1981). Columns were equilibrated with tris-HCl (pH 8.0), Na phosphate (pH 7.0), or Na acetate (pH 4.0-4.2) buffers at 25 mM, 50 mM or 100 mM. Separation of proteins was accomplished through the use of increasing buffer concentrations through a linear salt gradient (0 to 1 M). Eluates were monitored with an ISCO model UA-5 absorbance/florescence monitor.

Thin Layer Chromatography

Thin layer chromatographic studies were run according to Koller and Neukom (1964), using vanillin-H_2SO_4 to visualize resolved residues. The plates used were precoated K4 silica gel G plates obtained from Whatman. Mono-, di-, tri-, tetra-, and penta-galacturonic acids served as standards. Forty microliters of individual reaction mixtures, stopped at various reaction times by boiling, were spotted on the plates to determine the end products of hydrolysis.

Butanol-formic acid-water (2:3:1, v/v) was the solvent used. The extent of substrate hydrolysis was determined at each reaction time by the reducing sugar assay method.

Concentration of Enzyme

Concentration of enzyme preparations were carried out during various stages of purification using an immersible ultrafiltration unit (10,000 dalton cut off) obtained from Millipore.

Gel Filtration

Bio-Gel P-100 obtained from Bio-Rad was employed as a gel filtration matrix to fractionate protein systems and to desalt crude enzymic extracts. The gel was hydrated and poured into a 2.5 cm x 25 cm glass column according to Bio-Rad specifications.

Hydrophobic Chromatography

Separations based on hydrophobic interactions were accomplished on Phenyl-Sepharose CL-4B, obtained from Sigma Chemical Co. Phenyl-Sepharose CL-4B was poured into a glass column (1.5 x 25 cm) and equilibrated as described by Pharmacia Fine Chemicals (1976). Proteins were applied in

high-salt buffer (4 M NaCl 0.4 M Na phosphate pH 7.0 or 100 mM Na acetate pH 4.2) and eluted with a linear salt gradient of decreasing concentration (4M to 0 M) or with a stepwise elution method using decreasing salt concentrations.

SDS and Disc Gel Electrophoresis

Disc gel electrophoresis (pH 9.5) was performed according to the method of Ornstein (1964) and Davis (1964). A Bio-Rad model 155 gel-electrophoresis cell, equipped with either 7.5 or 12.5 cm tubes was employed for these studies. Electrophoresed gels were stained with Coomassie Blue R-250 according to the method by Weber and Osborn (1969). Duplicate gels were sliced and soaked overnight in 0.5 M Na acetate at pH 4.4. The gel extract was tested for enzymic activity to locate the corresponding protein band.

SDS gels were run according to the method of Weber and Osborn (1969), using a 10% gel. Duplicate gels were sliced and soaked overnight in 0.5 M Na acetate at pH 4.4. The gel extract was tested for enzymic activity to lacate the corresponding protein band. A Pharmacia low molecular weight calibration kit was used for a standard curve of molecular weight. The reference proteins included phosphorylase, b, m.w. = 94,000, albumin, m.w. = 67,000, ovalbumin, m.w. = 43,000, carbonic anhydrase, m.w. = 30,000, trypsin inhibitor, m.w. = 20,000, α-lactalbumin, m.w. = 14,400.

The molecular weight of endo-PG was estimated as described in the Pharmacia electrophoresis calibration kits instruction manual. The standard curve is illustrated in Figure 2.

Isoelectric Focusing

Isoelectric focusing was performed on polyacrylamide gel slabs according to the method by Hoyle (1979). Slabs were cut into one centimeter pieces and soaked individually in recently boiled, deionized distilled water. The pH value of each extract was plotted against gel to establish the gradient curve. Each extract was also tested for endo-PG activity to determine the location of the enzyme and its corresponding pI.

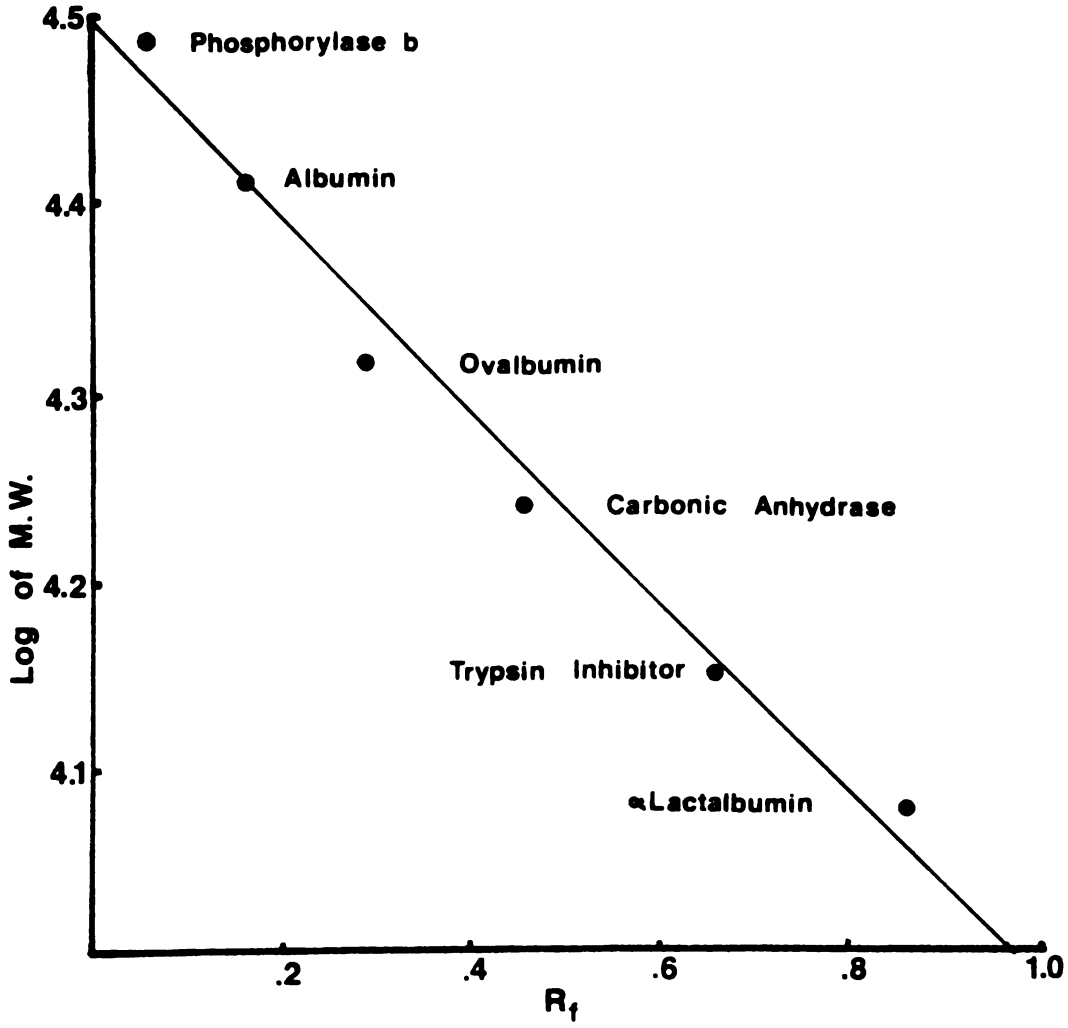


Figure 2. Standard curve for molecular weight determination with SDS gel electrophoresis.

RESULTS AND DISCUSSION

Purification of Endopolygalacturonase

Dialysis

Crude extracts of endo-PG contain sugars and salts of unknown types and quantities. Prior to the use of ion exchangers or Phenyl Sepharose chromatography, these sugars and salts must be removed which is typically accomplished by dialysis or gel filtration. Dialysis of the crude extract resulted in considerable losses in protein and enzymic activity as recorded in Table 3. Losses increased with time of dialysis and were similar in both carrier buffers, viz., 50 mM Na acetate at pH 4.2 and 50 mM Na phosphate at pH 7.0. The observed losses may have been due to the presence of cellulytic enzymes. Similar losses were observed when more purified enzyme preparations were dialyzed, indicating the inadequacy of this procedure in the purification scheme, Table 2. Therefore, dialysis was avoided in the final purification procedure for endo-PG.

Gel Filtration

As an alternative to dialysis, gel filtration on a polyacrylamide gel was utilized. Since it was suspected

Table 3. Dialysis of various enzyme preparations at various pH conditions.

	Total protein (mg)	Protein recovery (%)	Specific activity (units/mg)	Total activity (units)	Activity yield (%)
Crude extract A	197	-	9.03	1776	-
dialyzed 8 hrs at pH 7.0	92	46.7	11.6	1067	60
Crude extract B	70.7	-	26.3	1857	-
dialyzed 20 hrs at pH 4.2	20.5	29.0	39.2	803	43
at pH 7.0	20.6	29.1	33.8	696	37
Purified endo-PG	4.05	-	633	2564	
dialyzed 16 hrs at pH 4.0	2.25	56	611	1364	54

that endo-PG would be reasonably large (m.w. $\sim$ 50,000), Bio-gel P-100 was chosen which provides for the removal of smaller molecular weight proteins in addition to salts and sugars. Five milliliters of crude extract were applied to a 2.5 x 25 cm column which separated the components into two peaks as illustrated in Figure 3. Recovery of protein and yields of activity are summarized in Table 4. All of the enzymic activity was located in the first peak eluted. Approximately 42% of the protein was recovered in the second peak which also contained the sugars found in the crude extract. Accordingly, one would expect a corresponding increase in specific activity in the enzyme fraction. To the contrary the specific activity remained the same and activity yields were correspondingly low. A possible explanation for this loss could be the removal of an activating factor of low molecular weight. To test this theory, peaks 1 and 2 were combined in a 1:1 ratio and assayed for enzymic activity. The results of this experiment are shown in Table 5. Specific activity did not return to the level of the crude extract. Whatever the cause of the loss of activity, it appears to be irreversible.

Enzyme preparations were tested before and after gel filtration for the presence of other pecteolytic enzymes. Pectin and pectate lyase activities were not found in the crude extract. As a result, it was not considered necessary to assay for these activities in subsequently purified

Figure 3. Elution pattern from Bio-gel P-100 (2.5 x 25 cm column). Five ml of crude extract containing 118 mg protein was applied and eluted in 25 mM Na phosphate buffer pH 7.0 at a flow rate of 24 mls/mr. Five ml fractions were collected. Key to symbols: ——, absorbance at 280 nm, ●——●, PG activity.

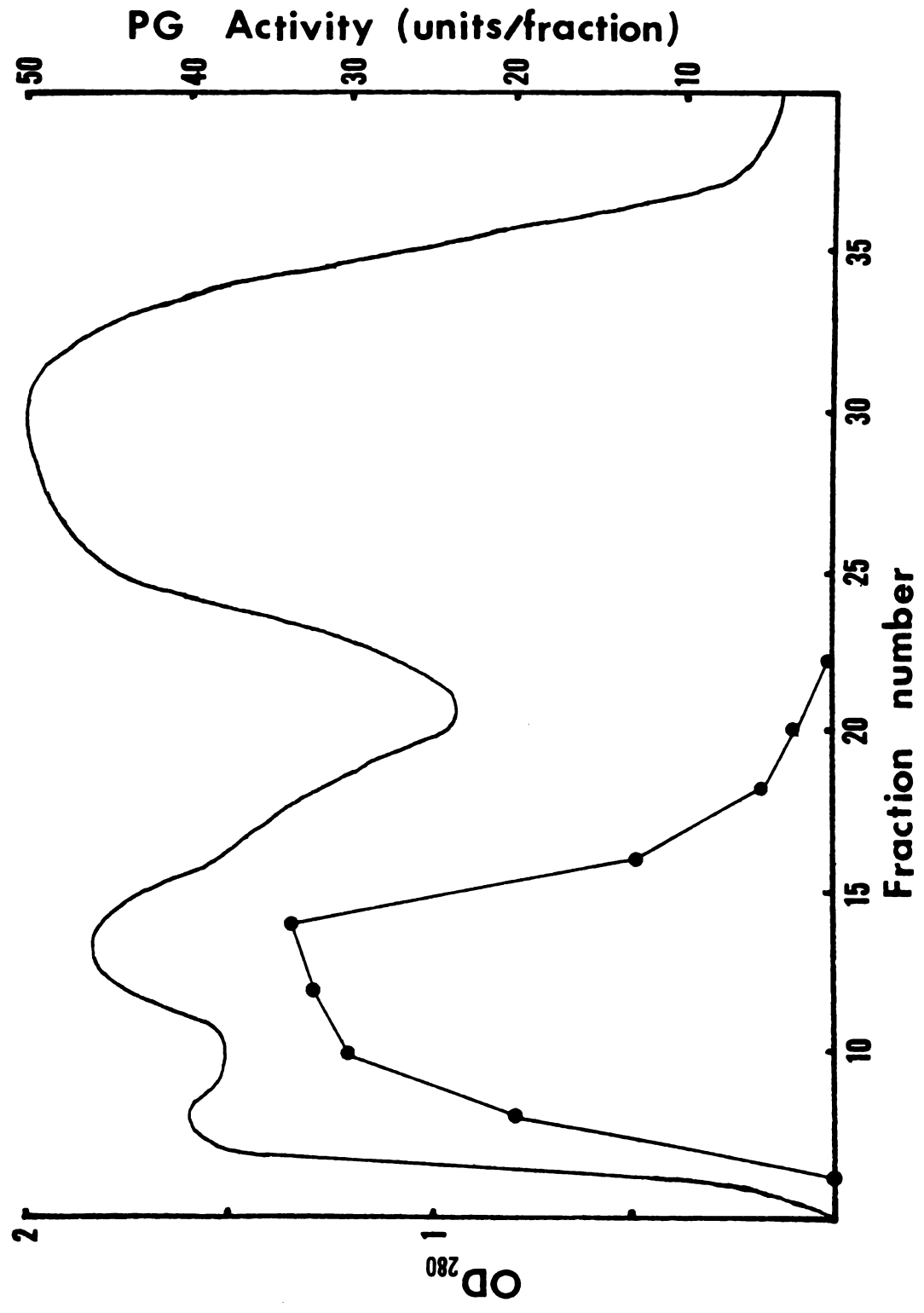

PG Activity (units/fraction)
50
40
30
20
10
35
30
25
20
15
10
Fraction number
2
1
OD280

Table 4. Gel filtration on Biogel P-100 of the crude extract.

	Total protein (mg)	Protein recovery (%)	Specific activity (units/mg)	Total activity (units)	Activity yield (%)
Crude extract B	118	-	26.3	3095	-
Peak 1	59.3	92.5	27.4	1626	52.5
Peak 2	49.8		0	0	-

(Average of 3 experiments)

Table 5. Recombination of the peaks from gel filtration of the crude extract.

	Crude extract B	Peak I	Peak II	Peak I & II	% Yield
Specific activity (units/mg)	26.3	27.4	0	17.5	
Total activity (units)	44.9	23.6	0	21.6	48.1

preparations. However, pectin esterase activity was found in the crude extract and was not removed during gel filtration.

Ecteola-cellulose (Weak anion exchanger)

Although not commonly employed for purification of endo-PG, Ecteola-cellulose was used by several researchers with good results (Paynter, 1975; Fielding and Byrde, 1969). Paynter (1975) used Ecteola-cellulose following gel filtration to obtain a 24-fold purification. Fielding and Byrde (1969) claimed a 61-fold purification over Ecteola-cellulose following cation exchange chromatography on CM-cellulose. Therefore, the possibility of utilizing this anion exchange resin was investigated.

The crude extract was applied to columns of Ecteola-cellulose following dialysis at pH 4.2 and 7.0 and after gel filtration at pH 7.0. In each case most of the endo-PG activity was eluted with the normal flow of the buffer. The small amounts bound to the support was eluted with a 0 to 1 M NaCl gradient. Separation using Ecteola-cellulose in a batch mode was also attempted. Data obtained from three columns and one batch operation are recorded in Table 6.

Results from the Ecteola-cellulose studied indicate that yields of activity were adequate but not exceptional. Overall, this ion exchanger does not appear to be

Table 6. Results of various purification schemes using Ecteola-cellulose.

Purification Procedure	Specific Activity (units/mg)	Total Activity (units)	Protein Concentration (mg/ml)
crude extract A	5.36	1054	5.62
dialyzed pH 4.2	6.12	480	1.96
Ecteola-cellulose (column)	38.7	342	1.16
crude extract A	5.36	1054	5.62
dialyzed pH 7.0	8.37	557	1.90
Ecteola-cellulose (column)	21.5	358	0.26
crude extract B	26.3	3103	23.6
gel filtration pH 7.0	30.2	1666	1.06
Ecteola-cellulose (column)	36.2	1285	0.34
crude extract B	26.3	3103	23.6
gel filtration pH 7.0	24.2	1448	1.00
Ecteola-cellulose (batch method)	24.4	1169	0.42

Total Protein (mg)	Activity Yield of this Procedure (%)	Activity Yield Overall (%)	Fold Purification
196.7	-	100	1.0
78.4	46	46	1.1
8.8	71	32	7.2
196.7	-	100	1.0
66.5	53	53	1.6
16.6	64	34	4.0
118.0	-	100	1.0
55.2	54	54	1.1
35.5	77	41	1.4
118.0	-	100	1.0
59.8	47	47	0.92
48.0	81	38	0.93

particularly adapted to the purification of endo-PG. The greatest degree of purification was achieved when the column was run at pH 4.2 following dialysis of the crude extract. Presumably, further purification following Ecteola-cellulose would require either dialysis or gel filtration; both of which would contribute to further losses. When run in sequence with gel filtration, purification dropped to only 1.4 fold. Disc gel electropherograms of protein preparations obtained from gel filtration and Ecteola-cellulose columns were identical. In addition, pectin esterase activity was co-eluted with endo-PG activity. Thus, it was concluded that Ecteola-cellulose was not applicable in a simplified purification scheme for endo-PG.

Phenyl-Sepharose (Hydrophobic Chromatography)

Hydrophobic interaction chromatography has been used with some success to facilitate the separation of proteins that were difficult to separate by other methods (Widmer and Leuba, 1979; Jen and Flurkey, 1979; Flurkey et al., 1978; Jen et al., 1980). Jen et al. (1980) reported the purification of a homogeneous peroxidase isozyme isolated from tomato juice by including hydrophobic chromatography in the purification scheme. Widmer and Leuba (1979) separated three β-galactosidases of Aspergillus niger by hydrophobic chromatography. Hydrophobic chromatography has not been applied to the purification of polygalacturonase.

Columns of Phenyl-Sepharose, a hydrophobic matrix, were equilibrated with 100 mM Na acetate buffer, pH 4.2, or 400 mM Na phosphate buffer, pH 7.0, both with 4 M NaCl. Enzyme preparations were added in one or the other of these high ionic strength buffers. Other researchers utilized $(NH_4)_2\ SO_4$ instead of, or in addition to, NaCl, to promote hydrophobic interactions (Widmer and Leuba, 1979; Jen et al., 1980). However, ammonium sulfate was found to interfer with color development in the reducing sugar assay used subsequently to estimate endo-PG activity and was therefore not used. Phenyl-Sepharose chromatography at pH 4.2 proved to be of little benefit to the purification scheme because the elution profile, employing decreasing ionic strength, showed endo-PG activity throughout most fractions. Chromatography at pH 7.0 resulted in the elution of most of the endo-PG activity in a large peak between 2.5 and 1.5 M NaCl, see Figure 4.

Phenyl-Sepharose columns were run at various steps in the purification scheme to determine the most effective time for their use. Results of these trials are summarized in Table 7. Large increases in the extent of purification occurred in every application. When used following dialysis of the crude extract, purification was 4.3-fold. When gel filtration was substituted for dialysis, purification increased to 7.9-fold. Greatest increases in the purification occurred when Phenyl-Sepharose was used following

Figure 4. Elution pattern from Phenyl-Sepharose chromatography (1.5 x 20 cm column). Thirty ml containing 19 mg protein was applied and eluted at a buffer flow rate of 34 ml/hr as follows: Na phosphate, pH 7.0 + 4 M NaCl; (fractions 1-30); linear gradient of decreasing ionic strength (fraction 31-100); and water (fraction 101 to 120). Five ml fractions were collected. Key to symbols: ——, OD_{280}, ●——●, PG activity.

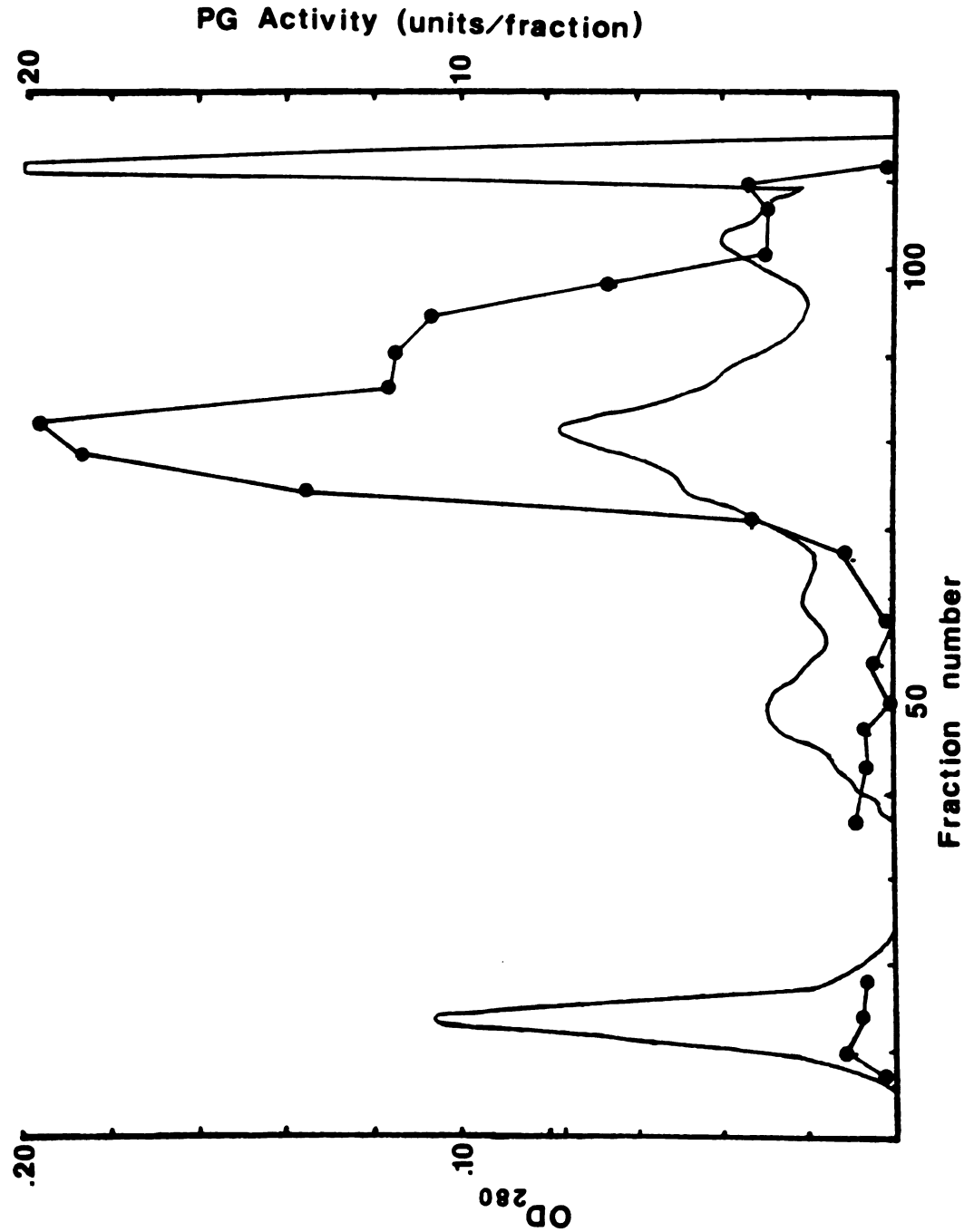

PG Activity (units/fraction)
20
10
100
50
Fraction number
.20
.10
OD280

Table 7. Summary of data from various purification schemes using dialysis, gel filtration, Phenyl Sepharose, and DEAE-cellulose in various combinations and orders.

Treatment	Specific Activity (units/mg)	Total Activity (mg)
crude extract	5.36	120.6
dialyzed pH 7.0	8.14	68.4
Phenyl-Sepharose	23.3	41.7
*crude extract	26.3	9310
*gel filtration pH 7.0	30.7	5021
*Phenyl-Sepharose	209	4369
crude extract	26.3	3103
gel filtration pH 8.0	41.2	1890
DEAE-cellulose pH 8.0	284	1489
Phenyl-Sepharose	1044	429
*crude extract	26.3	7054
*gel filtration	32.8	4049
*DEAE-cellulose pH 7.0	255	3503
*Phenyl-Sepharose	1358	2337
crude extract	26.3	2919
gel filtration	35.6	1833
Phenyl Sepharose	269	1291
dialysis pH 8.0	177	850
DEAE-cellulose pH 8.0	274	796
crude extract	26.3	2919
gel filtration	34.6	1833
Phenyl Sepharose	269	1291
dialysis pH 7.0	141	677
DEAE-cellulose pH 7.0	250	488

*Averages of three experiments.

Protein Concentration (mg/ml)	Total Activity (mg)	Activity Yield for this treatment only (%)	Activity Yield (%) overall	Fold purification
5.62	22.5	-	100	1.0
1.95	8.4	57	57	1.5
0.18	1.8	61	35	4.3
23.6	354	-	100	1.0
1.08	165	54	54	1.2
0.28	18.2	87	47	7.9
23.6	118.0	-	100	1.0
10.2	45.9	61	61	1.6
1.50	5.25	79	48	10.8
0.14	0.41	29	14	39.7
23.6	268.2	-	100	1.0
1.92	111.6	57	57	1.2
0.75	14.9	87	50	9.7
0.18	2.26	67	33	51.6
23.6	111.0	-	100	1.0
1.03	51.5	63	63	1.4
0.96	4.80	70	44	10.2
0.96	4.80	66	29	6.7
0.94	2.90	94	27	10.4
23.6	111.0	-	100	1.0
1.03	51.5	63	63	1.4
0.96	4.80	70	44	10.2
0.96	4.80	52	23	5.4
0.48	1.95	72	17	9.5

sequential treatment by gel filtration and DEAE-cellulose chromatography. However, even the most purified preparations when electrophoresed at pH 9.5 showed multiple protein-stained zones, indicating that other components were present. In addition, it was determined that Phenyl-Sepharose chromatography did not remove the pectin esterase activity indigenous in these preparations.

DEAE-cellulose (Strong anion exchange)

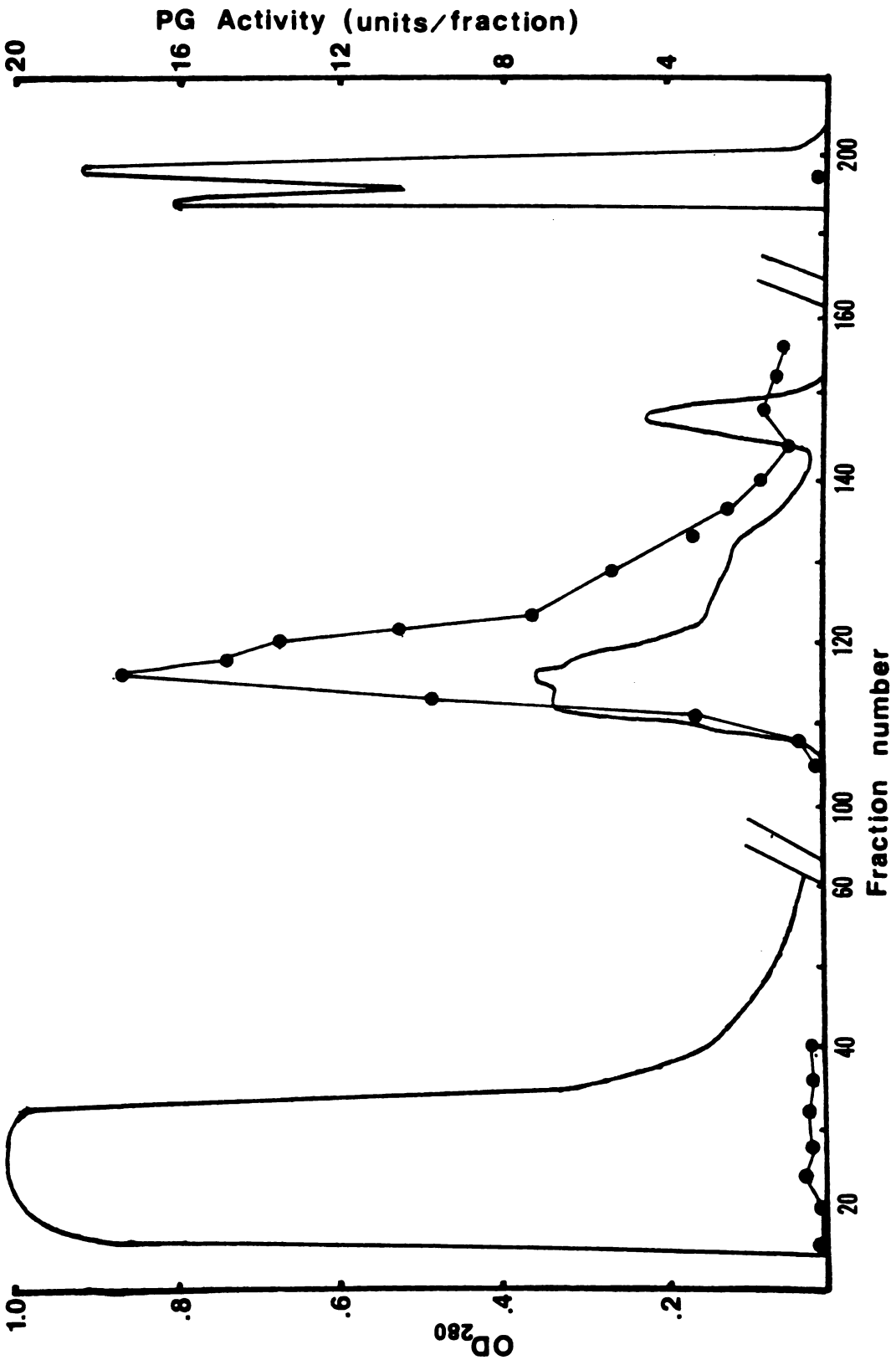
Use of DEAE-cellulose chromatography in purification schemes of PG has been quite common. Uchino et al. (1966) used DEAE-cellulose chromatography to obtain a pure endo-PG from Acroylindrium sp.. Kaji and Okada (1969) obtained a purified endo-PG from Corticium rolfsii also using DEAE-cellulose in their purification scheme. More recently, DEAE-cellulose chromatography was used by Urbanek and Zalewska-Sobczak (1975) to obtain a PG from Botrytis cinera and Liu et al. (1978) to isolate an endo-PG from Rhizopus arrhizus.

DEAE-cellulose has also been used extensively in the purification of endo-PG from Aspergillus niger. Rexova-Benkova and Slezarik (1966) used repeated DEAE cellulose chromatography to purify endo-PG from a surface culture of A. niger. However, values for purification and yields were not given. Cooke et al. (1976) isolated two isozymes of endo-PG from a commercial preparation of Aspergillus niger.

Dialyzed crude extract was submitted to DEAE-cellulose chromatography to achieve a 30-fold purification of the first isozyme and a 6.6-fold purification of the second isozyme. The overall yield of endo-PG activity was 73%. In yet another study with DEAE-cellulose, Heinrichova and Rexova-Benkova (1977) obtained a 7-fold purification and a yield of 87.2% for endo-PG from a commercial extract. By repeating the DEAE-cellulose step, they produced an additional 2-fold purification with a 62% yield in activity. However, the final endo-PG preparation was not completely pure.

In the purification of endo-PG in this study, DEAE-cellulose chromatography was compared with Phenyl-Sepharose in its ability to separate the protein complement. Two buffers differing in pH and ionic composition, i.e., a) 50 mM Tris HCl, pH 8.0 and b) 25 mM Na phosphate pH 7.0 were employed in an effort to maximize purification. Elution was achieved with increasing buffer concentrations, i.e., increasing ionic strength. Results obtained with both buffers were similar, see Table 7. A typical elution pattern for DEAE-cellulose chromatography at pH 7.0 is shown in Figure 5. Both conditions resulted in purifications that were higher than that obtained with Phenyl-Sepharose. At pH 8.0 a 10.8-fold purification with a 79% recovery of activity was obtained. At pH 7.0, the purification was 9.7-fold with an 87% recovery of activity. As

Figure 5. Elution pattern from DEAE-cellulose chromatography (2.5 x 20 cm column). Eight ml containing 120 mg of protein was applied and eluted as follows: 25 mM Na phosphate buffer, pH 7.0 (fractions 1-90); linear gradient of increasing buffer concentration to .4 M Na phosphate buffer, pH 7.0 (fractions 91-180); and 0.4 M Na phosphate buffer, pH 7.0 with 1 M NaCl, (fractions 181-210). Flow rate was 33 ml/hr. Five ml fractions were collected. Key to symbols: ——, absorbance at 280 nm, ●——●, PG activity.


PG Activity (units/fraction)
20
16
12
8
4
200
160
140
120
100
60
40
20
Fraction number
1.0
.8
.6
.4
.2
OD280

noted earlier, a 9.7-fold increase in purity was achieved with Phenyl-Sepharose. In addition to providing a higher degree of purification, DEAE-cellulose chromatography also removes pectin esterase activity.

It would seem, therefore, that DEAE-cellulose is superior to Phenyl-Sepharose as a chromatographic matrix for the purification of endo-PG. Unfortunately, disc gel electropherograms obtained under alkaline conditions for DEAE-cellulose chromatography indicated the presence of other proteins. However, the patterns showed only a few protein zones in common with those in preparations obtained by Phenyl-Sepharose chromatography. Differences in the patterns indicate that complete purification might be achieved in these two protein separation techniques were used in sequence. Further experiments were run with DEAE-cellulose in sequence with Phenyl-Sepharose to test this hypothesis.

When DEAE-cellulose chromatography at pH 7.0 was applied prior to chromatography on a Phenyl-Sepharose column, purification of the endo-PG was increased 51.6-fold with an activity yield of 33%, see Table 7. A similar chromatographic protocol performed at pH 8.0 was slightly less effective as a purification procedure. However, recovery of enzymic activity at pH 8.0 was substantially lower, possibly a consequence of the denaturing effect of the higher pH (Thibault and Mercier, 1978b).

When Phenyl-Sepharose chromatography was used prior to DEAE-cellulose, an additional step was required to remove the high concentration of salt inherent to the technique. As already noted, when either dialysis or gel filtration was employed to achieve this condition, there was a loss of activity in the purified preparation, Table 7. Therefore, the enzymic yield was not as high with DEAE-cellulose chromatography following Phenyl-Sepharose chromatography as when the reverse protocol was employed.

An evaluation of the results achieved by the above procedures indicates that the best purification scheme for endo-PG consists of the following operations in the order listed: gel filtration on Bio-Gel P-100 (pH 7.0), DEAE-cellulose chromatography (pH 7.0), and Phenyl-Sepharose chromatography. Aliquots of the enzyme preparations obtained at each purification step were examined by disc gel electrophoresis, Figure 6. These electropherograms demonstrate an increasing degree of purity with each step in the procedure. Only one enzymically active zone was detected. The final preparation, although lacking other pectolytic enzymes, still contained multiple protein zones. Therefore, to increase the level of purity additional fractionation procedures were necessary.

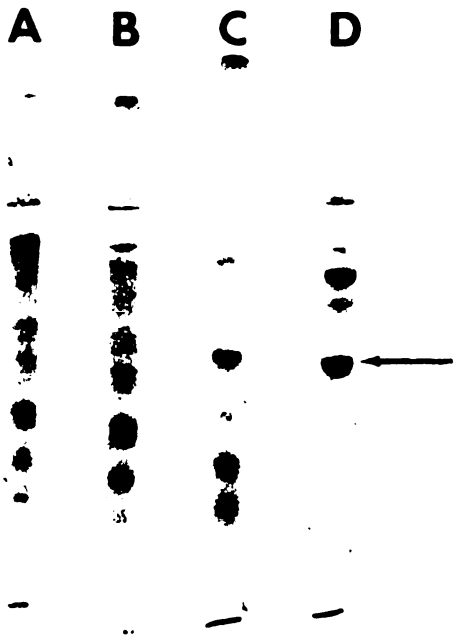


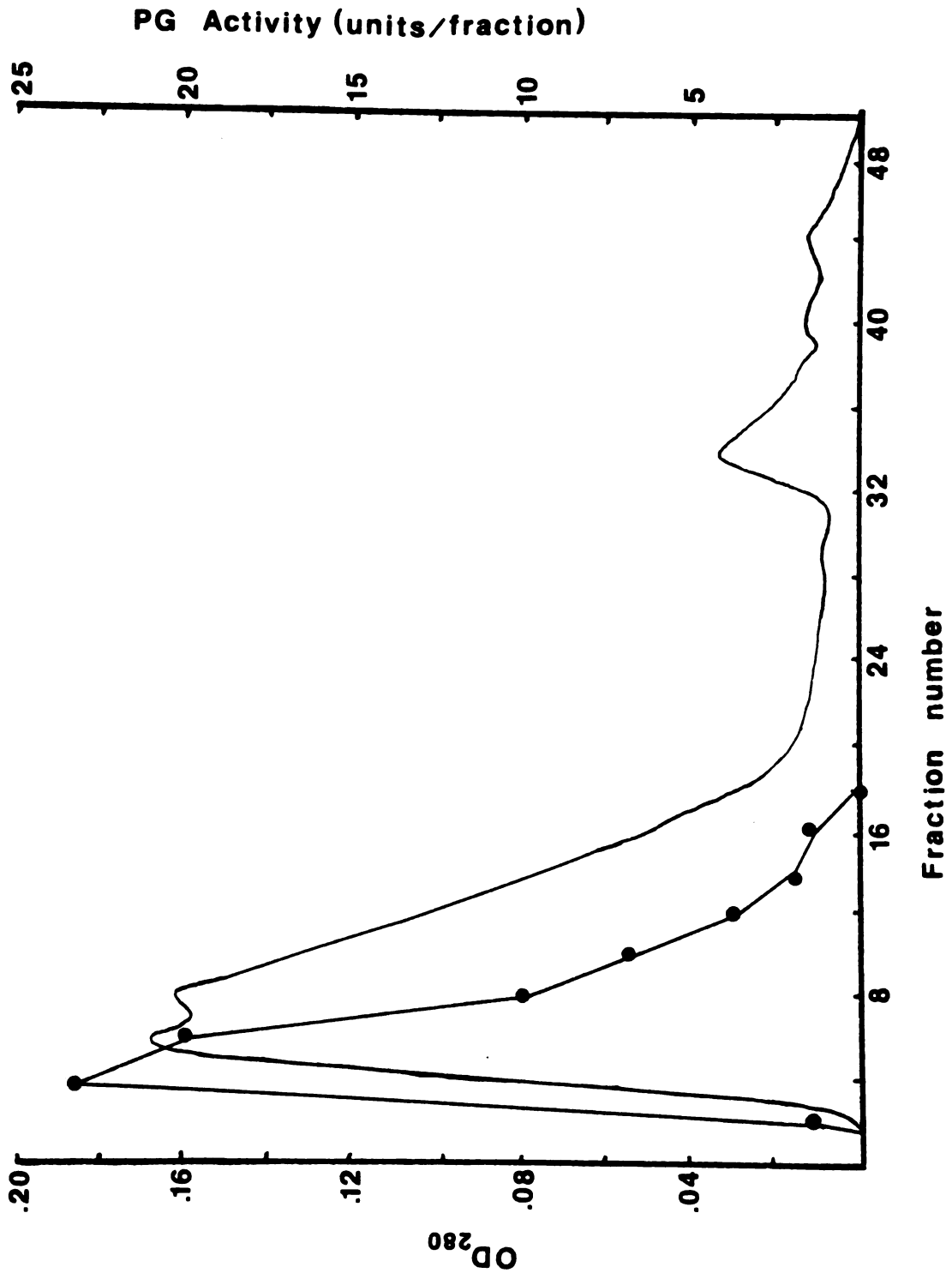
Figure 6. Electropherograms of preparations from various purification steps. Key: A, crude extract, B, preparation after gel filtration, C, preparation after DEAE-cellulose chromatography, D, preparation after Phenyl-Sepharose chromatography. Arrow indicates an enzymically active zone.

CM-cellulose (cation exchange)

CM-cellulose chromatography is another commonly used method for the purification of PG. Mill and Tuttobello (1961) used it to purify an endo-PG from Aspergillus niger. Nasuno and Starr (1966) used repeated chromatography on columns of CM-cellulose to purify a PG from Erwinia carotovora. More recently Fanelli et al. (1978) isolated two inducible PG isozymes from Trichoduma koningii, using CM-cellulose as the critical procedure in their purification scheme. Several researchers have used the combination of CM-cellulose and DEAE-cellulose chromatography to achieve purification of the enzyme (Reymond and Phaff, 1965; Harman and Corden, 1972).

Since the endo-PG in Spark-L HPG contained extraneous proteins after chromatography on Bio-Gel P-100, DEAE-cellulose and Phenyl-Sepharose, an attempt was made to add CM-cellulose chromatography to the purification scheme. A partially purified enzyme preparation was applied to the column after dialysis in 25 mM Na acetate, pH 4.0, buffer. The initial specific activity of the enzyme preparation prior to chromatography on CM cellulose was 611 micromoles/min/mg protein. After application of the enzyme to the column it was eluted by an increasing gradient of buffer concentration. The endo-PG was not adsorbed to the CM-cellulose under these conditions. The elution pattern from this column is shown in Figure 7. Early fractions

Figure 7. Elution profile from CM-cellulose chromatography (1.5 x 20 cm column). A partially purified enzyme preparation, containing approximately 2 mg protein, was applied and eluted at a flow rate of 30 mls/hr with 25 mM Na Acetate buffer, pH 4.0 (fractions 1 to 30), followed by .5 M Na Acetate buffer, pH 4.0 (fractions 31 to 50). Five ml fractions were collected. Key: ——, OD_{280}, ●——●, PG activity.


PG Activity (units/fraction)
25
20
15
10
5
48
40
32
24
16
8
Fraction number
.20
.16
.12
.08
.04
OD280

eluted from this column were combined and concentrated. Only 10.1% of the initial activity applied to the column remained in the concentrated solution. The loss may be a consequence of decreased stability as the enzyme approaches a pure state.

Characterization

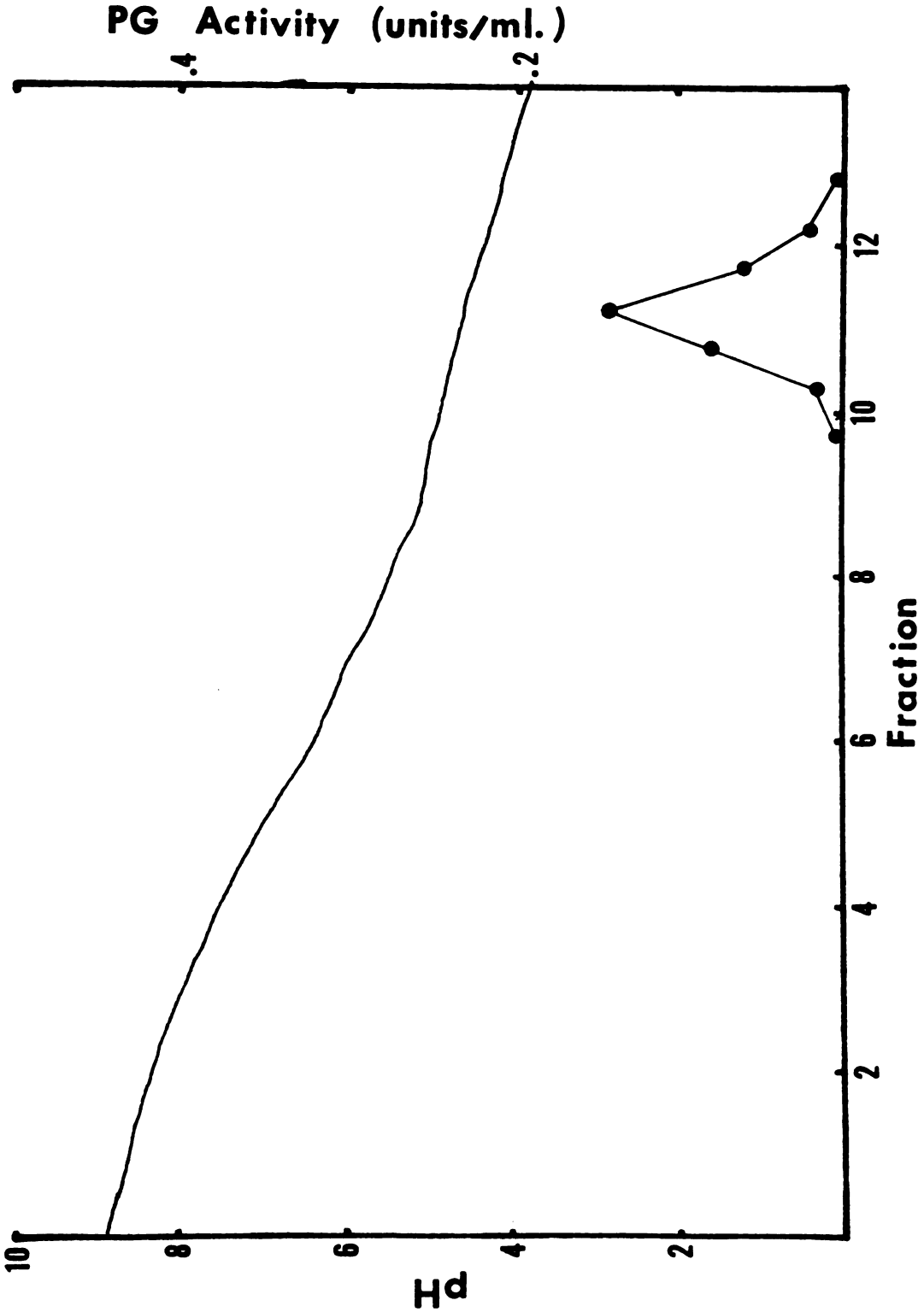
Molecular Weight

Molecular weight of the purified endo-PG was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The molecular weight of endo-PG determined in this manner was 46,000 to 48,000. This value is similar to that found by Heinrichova and Rexova-Benkova (1977). They used thin-layer chromatography on Sephadex G-1500 Superfine to estimate a molecular weight of 46,000 for an endo-PG purified from a commercial preparation.

Isoelectric Point

The plot of pH vs length of the gel is shown in Figure 8. Enzymic activity was found to peak at pH 4.65 ± 0.1. This corresponds to the value of 4.5 published by Koller and Neukom (1969) for endo-PG from Aspergillus niger.

Figure 8. Data from isoelectric focusing experiment on a slab gel. Gels were cut into 1 cm fraction. The pH of each fraction was determined and plotted against fraction number. Key: ——, pH, ●——●, PG activity.


PG Activity (units/ml.)
.4
.2
12
10
8
6
4
2
Fraction
10
8
6
4
2
pH

Substrate Preference

Rates of degradation were run as described under Materials and Methods using both polygalacturonic acid, free of methyl ester groups, and pectin with a methoxy content of 7.5% as substrates. The specific activity of the partially purified enzyme preparation on polygalacturonic acid was 1845 micromoles/min/mg. On pectin, the specific activity was 286 micromoles/min/mg. Thus, the enzyme showed a definite preference for polygalacturonic acid, defining it as a polygalacturonase as opposed to a polymethygalacturonase.

Mode of Degradation

To determine whether this PG works in a random or ordered cleavage, the rate of reducing sugars produced was compared to the % loss in viscosity of the substrate. This relationship is illustrated in Figure 9. The substrate used was polygalacturonic acid with an average molecular weight of 8000 to 8500 as determined from viscosity measurements. A 50% drop in the viscosity of the substrate was obtained when only 8% hydrolysis had occurred. Hydrolysis continued to 55.7% after 18 hours which compared well to the value of 56.7% obtained by Thibault and Mercier (1978b) for complete hydrolysis of this substrate.

In addition, the end products of the hydrolysis were examined using the T.L.C. method described under Materials

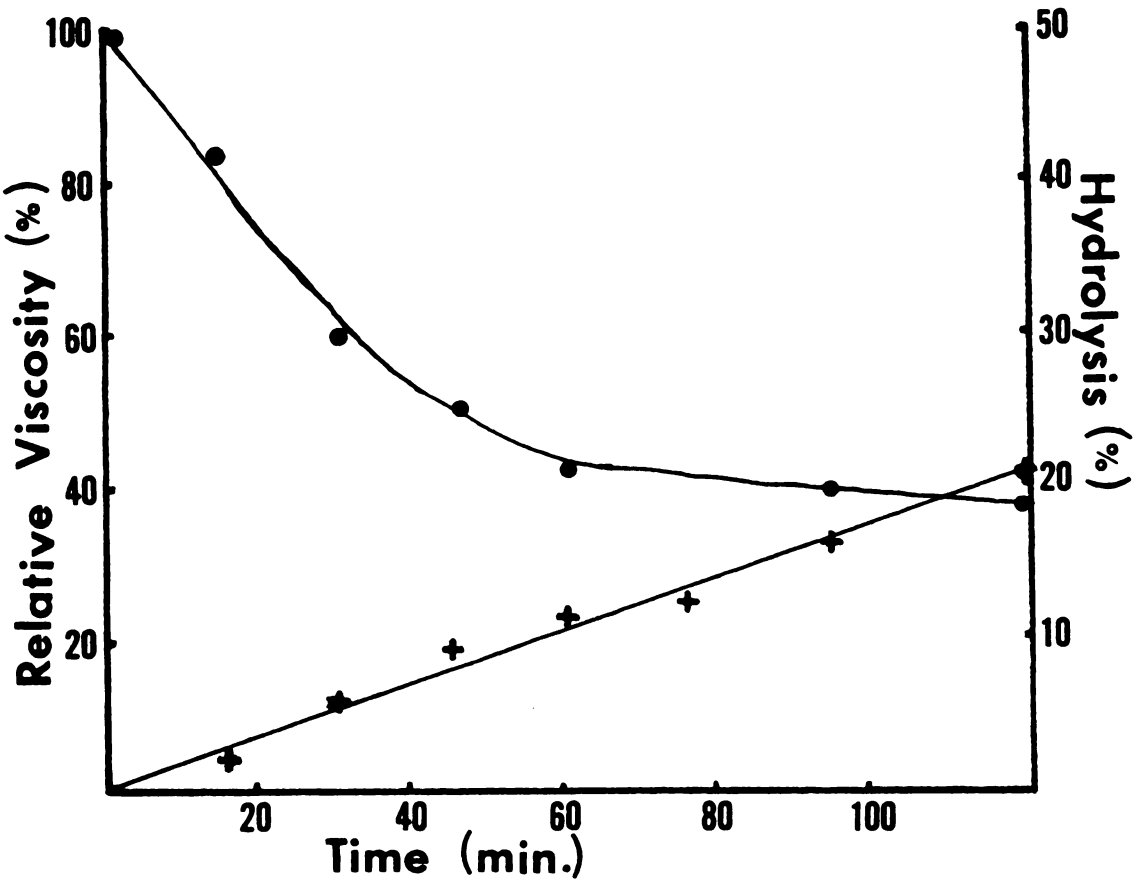


Figure 9. Viscosity reduction vs hydrolysis of polygalacturonic acid by PG. Key to symbols: ●——●, viscosity, +——+, hydrolysis.

and Methods. A chromatogram showing standards and reaction products vs time of hydrolysis are shown in Figure 10. Early reaction products contained no oligogalacturides smaller than pentagalacturonides. After 32 minutes (approximately 17.6% hydrolysis), smaller galacturonides were produced. At 64 minutes, an obvious disappearance of the longer chain galacturonides is apparent. This information, in addition to the viscosity data discussed above, indicates that the enzyme operates by a random cleavage mechanism and can be characterized as an endo-PG.

Heat Resistance

The resistance of endo-PG to various temperatures is illustrated in Figure 11. The percentage of residual activity drops off rapidly after 40°C, falling from 93% at 40°C to 41% at 50°C. This observed sensitivity to heat was greater than reported by Mill and Tuttobello (1961).

Temperature Optimum

Rates of degradation vs temperature are illustrated in Figure 12. Rates of degradation continue to increase to about 55°C then abruptly drop off. This temperature optimum is higher than that reported by other researchers (Thibault and Mercier, 1978b; Heinrichova and Rexova-Benkova, 1977).

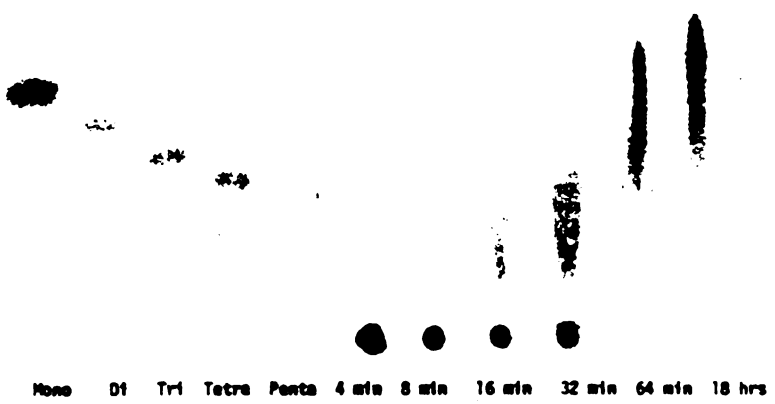


Figure 10. Thin layer chromatogram of end products of the endo-PG reaction. The reaction mixtures consisted of 1 ml of .5% polygalacturonic acid in .25 M Na Acetate pH 4.4 with 10 microliters of purified enzyme preparation. Reactions were stopped at the various times indicated by boiling for 5 min. The first five spots represent standards: mono-, di-, tri-, tetra-, and penta-galacturonic acid as indicated.

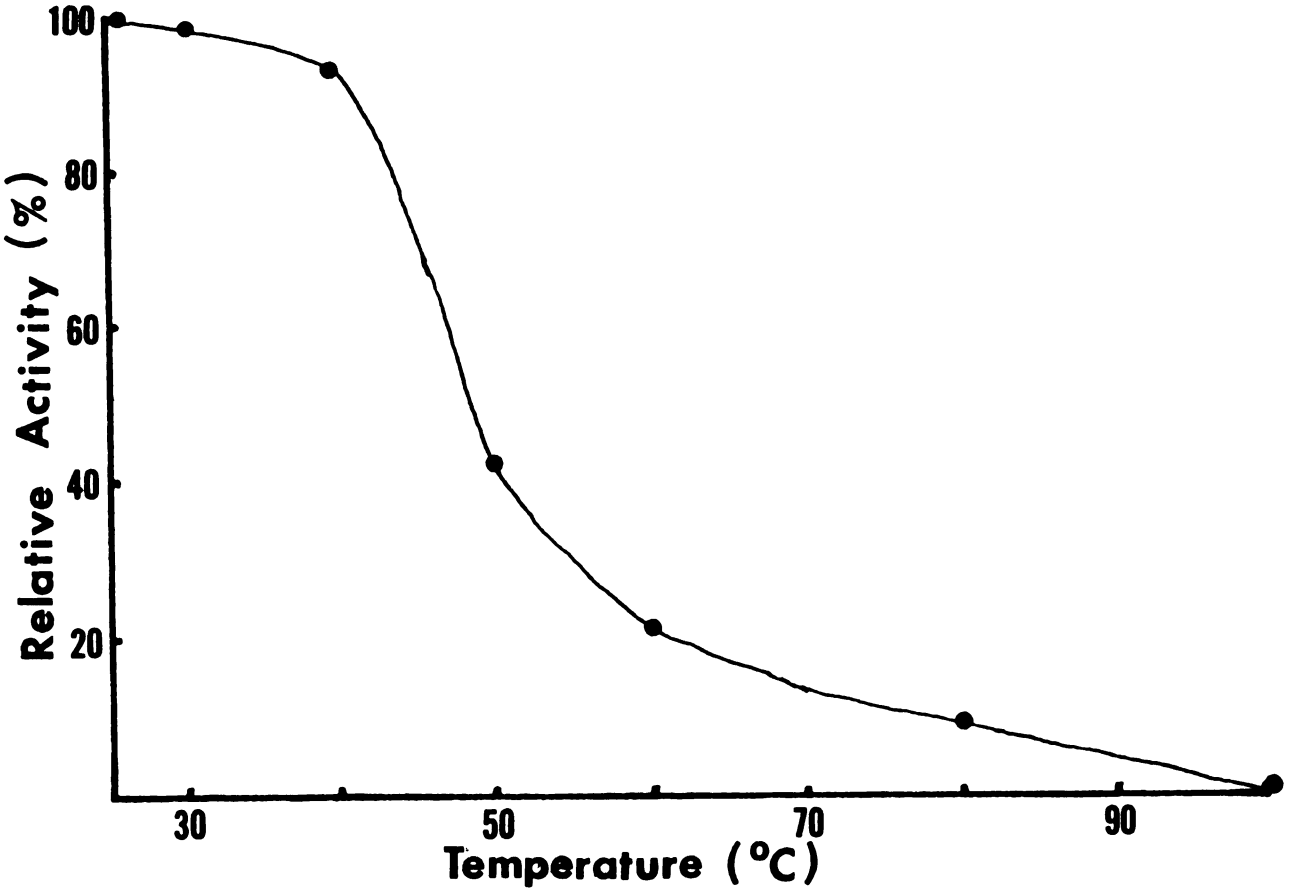


Figure 11. Stability of PG to temperature. Enzyme was incubated at various temperatures for 5 min then assayed at 30°C for activity.

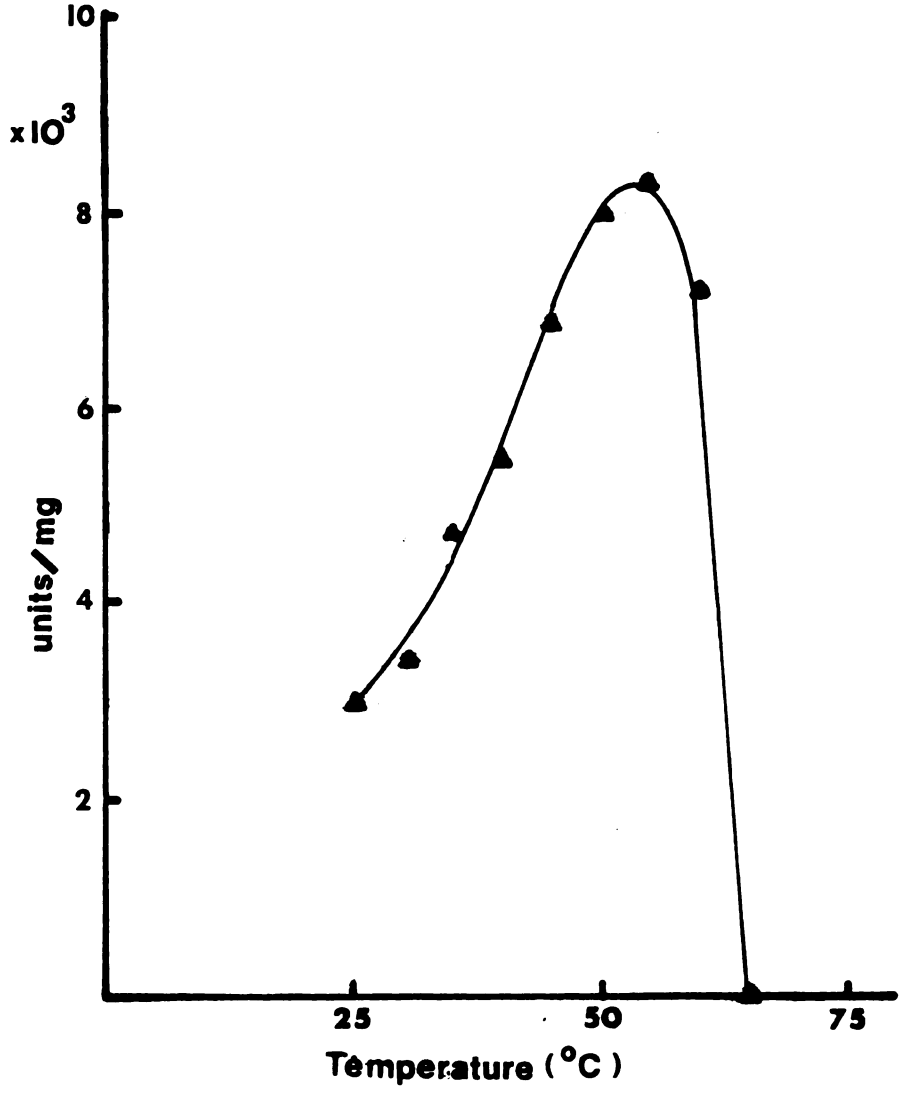


Figure 12. Temperature/activity profile for purified endo-PG, from Aspergillus niger.

pH Stability

The pH stability of the purified enzyme was tested across the range of pH 3-9 over a period of one month while stored at 4°C. Activity at all pH values showed an average of 35% loss in activity after the first week. No additional loss occurred after four weeks. Stability over a wide range of pH has been reported previously (Kaji and Okada, 1969; Ishii and Yokotsuka, 1972).

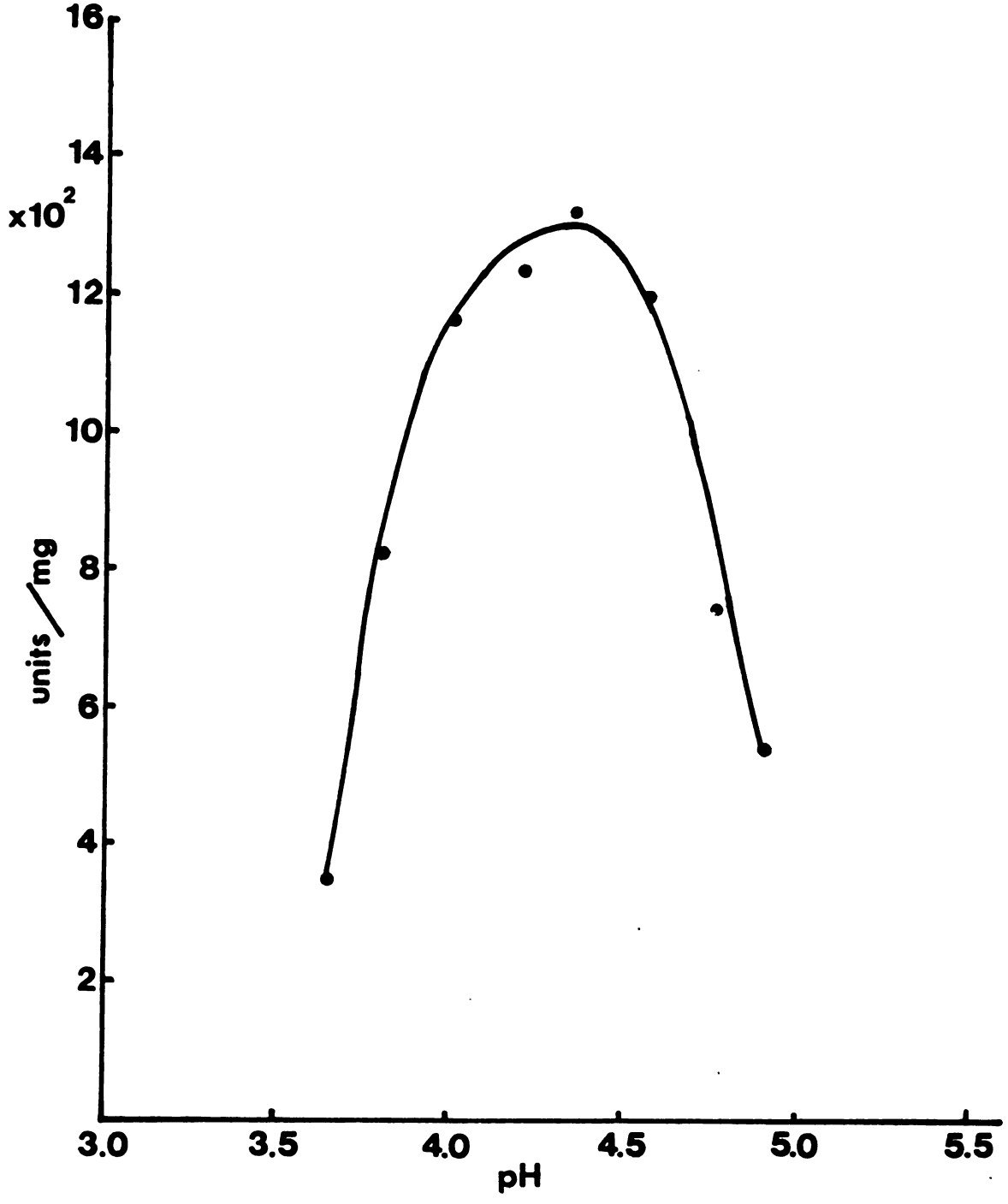
pH Optimum

The activity of endo-PG at various pH values is illustrated in Figure 13. The pH optimum was found to be at 4.4. This was slightly higher than that reported by Thibault and Mercier (1978), Rexova-Benkova (1967) and Mill and Tutto-bello (1961), but lower than that reported by Koller and Neukom (1969) and Heinrichova and Rexova-Benkova (1977) for an endo-PG from Aspergillus niger.

Effect of Molecular Weight of the Substrate on the Rate of Degradation and pH Optimum

Several groups of researchers have reported on the effect of the molecular weight of the substrate on the reaction rate and pH optimum of endo-PG (Pressy and Avants, 1971; Barash and Eyal, 1970, Liu and Luh, 1980). Molecular weight differences in substrates used for activity of endo-PG could account for the variation in values reported.

Figure 13. pH/activity profile for purified endo-PG from Aspergillus niger.


16
14
x10²
12
10
units/mg
8
6
4
2
3.0
3.5
4.0
4.5
5.0
5.5
pH

Therefore, the present studies were undertaken to assess the possible effects substrate size would have in the reaction with the partially purified endo-PG. Substrates with average molecular weights of 110,000 and 3,200 were obtained as stated in Materials and Methods. The molecular weight of the intermediate range substrate was determined to be 6000-6500 using the method described under Materials and Methods. The pH optimum for the reaction was the same for each of three substrates used. However, the rate of degradation of the substrate increased with increasing molecular weight. For three species of polygalacturonic acid with average molecular weights of 110,000, 6,000-6,500, and 3,200, the rates of degradation by the enzyme preparation were 625, 504, and 460 micromoles/min/mg, respectively. These results suggest that reported variations in apparent Km and Vmax values may be attributed to substrate size, but that variations in pH optimum must be due to other parameters. Also, rates of activity can only be described as apparent rates because the actual rate appears to decline with the decline in substrate size.

Effect of NaCl

Thibault and Mercier (1978b) reported an increase in activity in response to NaCl up to 0.125 M. Since high concentration of NaCl were used during Phenyl-Sepharose chromatography, its effect on endo-PG is noteworthy. A

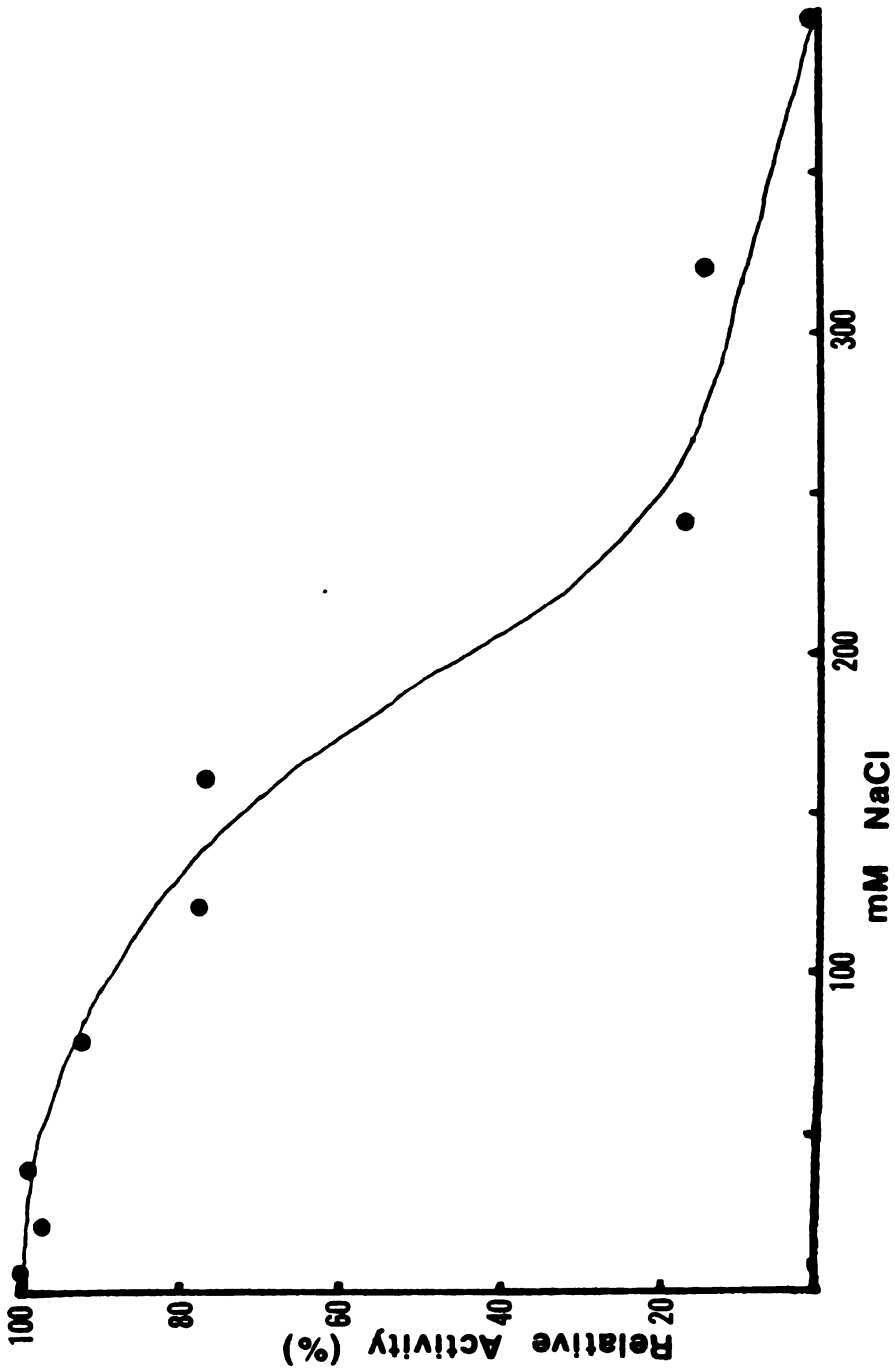


Figure 14. Activity of PG as affected by NaCl concentration.

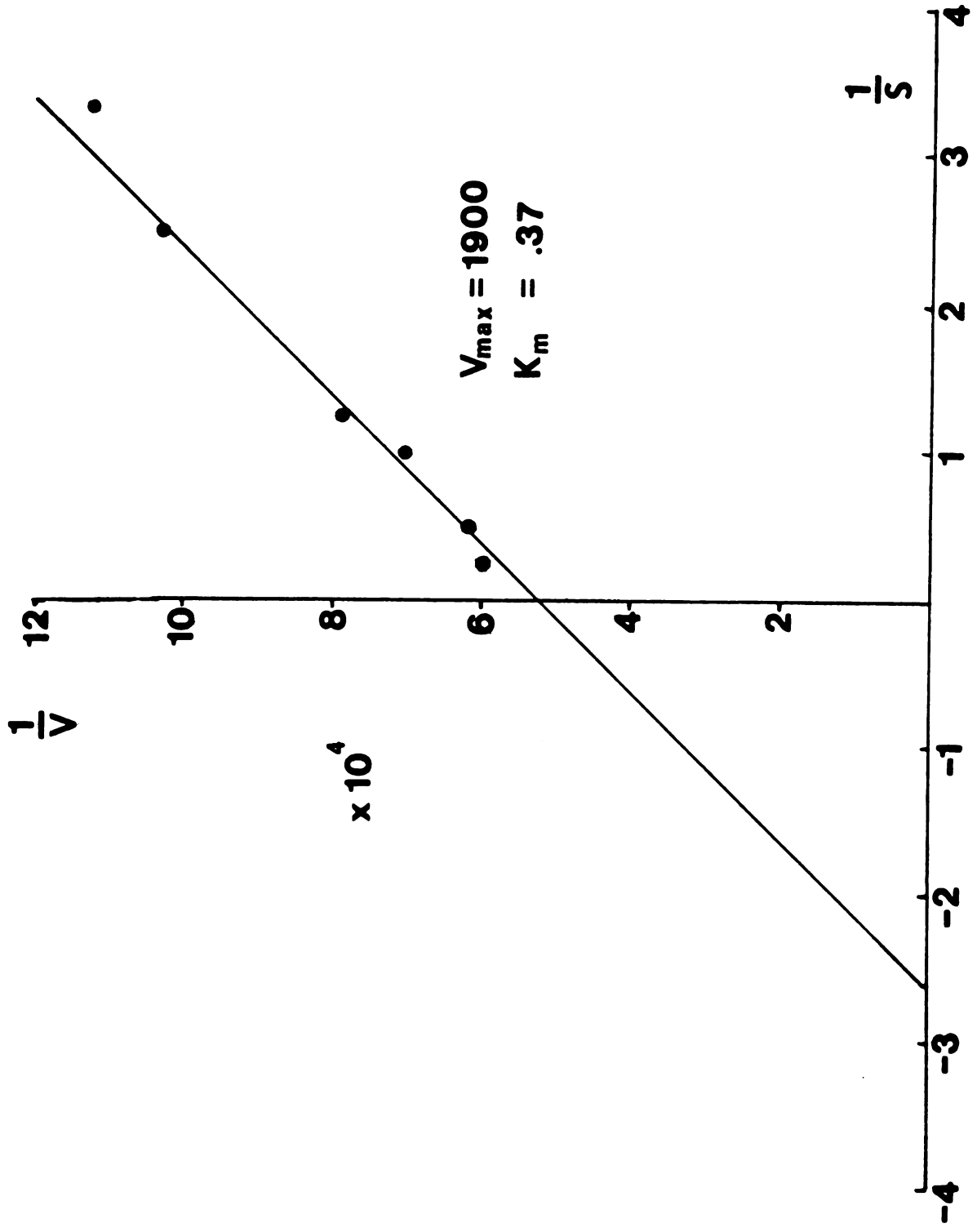
plot of percentage relative activity of endo-PG vs salt concentration is represented in Figure 14. Below 50 mM NaCl no significant influence was detected. At higher concentrations activity began declining. It is important to note that beyond 50 mM NaCl the assay substrate also began to precipitate. Therefore, the inhibition at higher salt concentrations may be due more to the availability of the substrate than to the actual effect of NaCl on the enzyme. A similar drop in activity associated with high salt concentrations was noted by Thibault and Mercier (1978b) after initial stimulation of the enzyme by NaCl at lower concentrations. They also cite precipitation of the substrate as a possible explanation for this inhibition.

Kinetic Studies

The results of kinetic studies using polygalacturonic acid (8000 to 8500 daltons, DP = 41-44) as the substrate are shown on the Lineweaver-Burk plot in Figure 15. The partially purified enzyme exhibited Michaelis-Menten kinetic parameters of apparent Vmax = 1900 moles/min/mg and an apparent Km = .37 mg/ml (4.3 to 4.6 x 10^{-5}M assuming substrate mass of 8000 to 8500 daltons).

Among the various Km values reported previously, two are within the same order of magnitude as the apparent Km of this study. Heinrichova and Rexova-Benkova (1977) reported a Km of 1.96 x 10^{-5}M and Thibault and Mercier

Figure 15. Lineweaver-Burk plot for purified endo-PG with polygalacturonic acid as the substrate. $\underline{V}$ represents rate of the reaction in micromoles of reducing groups formed/min/mg protein. S represents the substrate concentration in mg/ml.


$\frac{1}{V}$
x 10^4
12
10
8
6
4
2
V_{max} = 1900
K_m = .37
$\frac{1}{S}$
4
3
2
1
-1
-2
-3
-4

(1978b) reported a Km of 5.18×10^{-5} M. However, both groups reported Vmax values considerably smaller (224 and 239 μmoles/min/mg) than found in this study. Neither of these groups reported purifying their enzyme preparations to homogeneity. Possibly, the enzyme preparation prepared in this study was more homogeneous than preparations previously studied.

SUMMARY AND CONCLUSIONS

Several methods and procedures were utilized in an effort to develop an effective and rapid method for the purification of endo-PG from a commercially available crude extract of Aspergillus niger. Gel filtration was utilized as a first step to remove salts and sugars as well as smaller molecular weight proteins. Dialysis was avoided due to losses of enzymic activity and protein content. DEAE-cellulose was employed as the second step because it provided a 10-fold purification of the enzyme. The final step in the scheme utilized hydrophobic chromatography on Phenyl-Sepharose which provided additional purification. In tandem the three chromatographic procedures provided a rapid purification method yielding a 51.6-fold purification of the endo-PG.

The purified enzyme preparation produced in this manner was characterized as a PG on the basis of its preference for polygalacturonic acid over pectin as a substrate. It was further characterized as an endo-enzyme on the basis of the rapid reduction in viscosity of its substrate as compared to a slow accumulation of reducing sugars and by the absence of oligogalacturonides early in the reaction as shown by TLC analysis of reaction end products.

Kinetic studies showed that the enzyme preferred larger molecular weight substrates, following Michaelis-Menten kinetics within a specified substrate molecular weight range. Apparent Km and Vmax values were 4.3-4.6 x 10^{-5} M and 1900 micromoles/min/mg, respectively. The Km value is essentially similar to Km values reported previously. Corresponding Vmax values reported in the literature are significantly lower than the value determined here. The enzyme preparation produced in this study appears to be more highly purified than previously reported preparations.

The purification scheme developed and evaluated in this study differs from previously reported schemes primarily in the use of hydrophobic chromatography as a final step. Hydrophobic chromatography has not been previously utilized for the purification of PG from any source. It proved to be a valuable addition to the purification of this enzyme.

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