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I am submitting herewith a thesis written by Kun Kook Park entitled "Partial Purification and Characterization of Pectin Methyl Esterase in Southern Peas (Vigna sinensis)." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Technology and Science.

J. L. Collins, Major Professor

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PARTIAL PURIFICATION AND CHARACTERIZATION OF PECTIN METHYL ESTERASE IN SOUTHERN PEAS (*VIGNA SINENSIS*)

A Thesis

Presented for the

Master of Science

Degree

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Kun Kook Park

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ABSTRACT

The purposes of this study were to partially purify pectin methyl esterase (PME) of Southern peas (Vigna sinensis), to determine optimum pH and NaCl concentration for maximum enzyme activity, to measure the energy of activation and kinetic reactions, and to ascertain the presence of multiple molecular forms of the enzyme and their isoelectric points.

PME was extracted from an acetone powder preparation and purified by a two-step ammonium sulfate fractionation and dialysis. The activity of the partially purified enzyme was significantly ($p < 0.05$) influenced by pH, NaCl concentration, and the interaction between pH and NaCl concentration. The optimum pH and NaCl concentration were 7.9 and 0.25 M, respectively. The energies of activation were calculated to be 4,900, 5,950, and 6,900 calories/mole at pH 6.0, 7.0, and 7.0, respectively. PME had a K_m of 0.781% pectin N.F. and a V_{max} of 24.34 PME units.

Additional purification was achieved by means of column chromatography on DEAE-cellulose and Spehadex G-100. The PME preparation thus obtained was subjected to gel electrofocusing. One PME component and two protein components were detected. The isoelectric point of the PME was 4.5 at 20°C.

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CHAPTER I

INTRODUCTION

Pectin methyl esterase (PME, EC 3.1.1.11) catalyzes the deesterification of the methyl ester groups of polygalacturonic acid units of pectin. It is found in many higher plants and in some microorganisms.

The enzyme is of great interest in plant tissues and has been the subject of a number of studies because of the role of pectic substances in influencing the structure, texture, and character of foods of plant origin. The activity of PME, in the presence of calcium ions, brings about a decrease of solubility of the pectic substances and results in increased firmness of plant tissues. The softening of plant tissues during ripening or storage is also thought to be caused by the PME activity prior to any appreciable degradation by the polygalacturonase.

PME is firmly bound to the plant cell wall materials and extracted with fairly high concentrations of salt at alkaline pH. The activity of PME is influenced considerably by the concentration and type of salt present. Optimum pH of this enzyme is also affected by the presence of salts.

In general, PME of higher plants is somewhat more heat stable and exhibits a broad higher pH optimum as compared with that of microorganisms.

Multiple molecular forms of PME from a variety of higher plants and microorganisms have been isolated and well characterized.

Although the extraction characteristics of PME from different sources and its physicochemical properties have been extensively studied, there is little information concerning the physicochemical properties of PME in Southern peas. The purposes of this study were to partially purify PME of Southern peas (Vigna sinensis), to determine optimum pH and NaCl concentration for maximum enzyme activity, to measure the energy of activation and kinetic reactions, and to ascertain the presence of multiple molecular forms of the enzyme and their isoelectric points.

CHAPTER II

REVIEW OF LITERATURE

I. SOURCES OF PECTIN METHYL ESTERASE

Pectin methyl esterase (PME, EC 3.1.1.11) is widely distributed in the various tissues of many higher plants. It is also produced by numerous microorganisms. Sources of the enzyme which have been studied are alfalfa (32), apple (31), avocado (61), banana (21), carrot (8), cucumber (2), orange (9, 24, 34, 54), papaya (3), pear (45), snap bean (59), Southern pea (5), soybean (4), tobacco leaf (18), tomato (7, 13, 15, 17, 38, 46), fungi (10, 43), and bacteria (42). MacDonnell et al. (34) found that the flavedo in citrus fruits had a higher PME activity than did the albedo. The press juice of the flavedo, as well as the filtered fruit juices, contained little or no enzyme activity. The juice sacs of orange varieties contained the most PME activity, while the greatest amount of pectin was present in the membrane (54, 55). Holden (18) showed that the sap of tobacco leaves contained only a small proportion of the total PME present, most of which was associated with the fiber.

II. SUBSTRATE FOR PECTIN METHYL ESTERASE

Pectin, the substrate of PME, is a polysaccharide composed principally of alpha-D-(1,4)-galacturonic acid units or the methyl ester of the units (36). This substance also possesses varying amounts of L-rhamnose, L-arabinose, D-galactose, and traces of other neutral sugars as its integral parts (41). In natural pectin, approximately two-thirds of the carboxylic acid groups are esterified with methanol. The degree of esterification of a particular pectin depends upon its source and extraction method (36). The activity of PME seems to be little related to the molecular weight of pectin (8).

Pectin has an important structural role in the tissues of higher plants. It is deposited in the cell wall where it acts as "intercellular cement." The enzymatic breakdown of pectin may lead to tissue maceration (36).

It is generally believed, although only partly understood, that the noncellulosic cell wall polysaccharide such as pectin is synthesized in vesicles derived from the Golgi body so that the biosynthesis of the PME may be derived from the Golgi body (44). The newly formed pectin and the PME are kept apart by lipid membranes of the cells as long as the integrity of the structure is maintained (25).

III. MODE OF ACTION OF PECTIN METHYL ESTERASE

PME is a highly specific enzyme. MacDonnell et al. (35) observed that PME from the orange acted on pectin at least 1,000 times as fast as on various nongalacturonide esters; Lineweaver and Ballou (32) found that alfalfa PME deesterified pectin at least several hundred times as fast as it did either alpha-methyl-D-galacturonate or alpha-methyl-D-galacturonate methyl glycoside.

Lee and Macmillan (30) reported that approximately 50% of tomato PME activity was initiated at or near the reducing ends of highly esterified pectin molecules, and suggested that the remaining enzymatic activity would occur at some secondary locus or loci next to free carboxyl groups. However, Deuel and Stutz (8) indicated that PME of the orange hydrolyzed only methyl ester groups adjacent to free carboxyl groups and the hydrolysis would then proceed along the pectin chain by the single chain mechanism.

Pressey and Avants (50) suggested that one of the PME forms that they found in tomato might function to create initiation points for the deesterification by the other enzymes.

A random distribution of the free carboxyl groups is observed during alkaline deesterification of pectin, whereas a blockwise arrangement of the free carboxyl groups is formed during enzymatic deesterification (8, 28).

IV. PROPERTIES OF PECTIN METHYL ESTERASE

Lineweaver and Ballou (33) found that the activity of alfalfa PME at pH 5.7 increased 30-fold in the presence of 0.2 M monovalent or 0.02 M divalent cations, and concluded that a large part of the activation was due to interaction between cations and pectin (32). In general, mono- and divalent cations stimulate the activities of both plant and fungal PME (10, 27, 33, 40), while tri- and tetravalent cations inhibit the enzyme actions (10).

The concentration and type of salt present in the reaction medium not only affected the activity of PME (33), but also affected the extraction, elution, and stability of the enzyme (34). The optimum salt concentration varied considerably depending on the source and degree of purity of PME, assay conditions, and type of salt present (2, 5, 10, 26, 34, 40, 50, 59).

PME activity is markedly affected by the pH of the reaction mixture and optimum pH of this enzyme is also influenced by the amount and kind of salts present (34). Kertesz (27) explained that the effect of added salts is generally to lower the pH optimum and to extend the activity range to lower pH regions. Contrary to the activity of PME of higher plants, one fungal PME was found active in a salt free medium and the addition of salts did not cause a drift of optimum pH ranges (27).

Plant PME shows optimum activity near neutrality. Fungal PME has a pH optimum generally in the acid range, while bacterial PME has a pH optimum in the alkaline range (51).

The firming of canned tomatoes is usually accomplished by the addition of calcium salts. Hsu et al. (20) found that the activity of PME in canned tomatoes was responsible for an increase of low-methoxyl pectin for calcium pectate formation, which in turn, resulted in a higher firmness. Softening of plant tissues during ripening or storage is thought to be associated with deesterification by PME prior to any appreciable degradation by polygalacturonase (8, 17).

PME of higher plants is more heat stable than the fungal preparation. When mold and tomato PME were subjected to various temperatures from 0° to 50°C for one hour at pH 5.8-6.0, the former was rapidly inactivated at 40°C and above, whereas the latter was unaffected even at 50°C (40). In general, PME is more susceptible to heat denaturation at acidic pH than at alkaline pH. Van Buren et al. (59) found that the PME in the pod of snap beans was more heat resistant at pH 6.5 than that in an acetone powder.

Collins (5) observed that Southern peas subjected to frozen storage had a higher apparent activity than the fresh peas, and the increased activity was more pronounced in the more mature peas. Frozen storage did not greatly reduce the

PME activity in snap beans, but the enzyme destroyed by blanching was not regenerated upon frozen storage (59).

The amounts of PME in various higher plants increased with maturity as reported for banana (22), cherry (51), orange (54) and tomato (17), or it decreased as observed for avocado (61), pear (45) and Southern pea (5), or it remained constant as found for cucumber (2) and tobacco leaf (19). The activities of PME also varied with seasonal changes (54, 61), varieties (5), storage conditions (51, 61), fertilizer components (16, 19), and ethylene treatment (11).

V. CHARACTERIZATION OF PECTIN METHYL ESTERASE

Lee and Macmillan (29) reported that tomato PME stained only faintly with protein stains such as Amido black or aniline black, but more strongly with lipid stains such as sudan black B or oil red O. Lipid components were found by gas chromatography in extracts of alkaline hydrolysates of the purified enzyme, and it was tentatively concluded that tomato PME is a lipoprotein.

Delincee and Radola (7) found that tomato PME stained readily with a carbohydrate stain such as PAS (periodic acid-Schiff), and that its electrophoretic mobility increased in a borax buffer. Consequently, they suggested that tomato PME is a glycoprotein.

Markovic (37) observed that one of the multiple molecular forms of tomato PME, whose quantity was dominant, did not stain with sudan black B. This form was also analyzed for presence of fatty acids by gas chromatography after methanolysis. Not even a trace of fatty acids was found. In view of these results, the assumed lipoprotein character of tomato PME was eliminated. Attempts to confirm the suggested glycoprotein character of tomato PME were unsuccessful because the glycidic, arabinose-containing component could be separated by desalting of the final product.

VI. INHIBITION OF PECTIN METHYL ESTERASE

McColloch and Kertesz (40) found that plant and fungal PME were effectively inactivated by detergents, although fungal enzyme was more resistant than plant enzyme. They theorized that PME activity depended on physical properties of a molecular surface rather than on functional groups, and concluded that the inactivation of this enzyme was due to surface denaturation by detergents.

The inhibitory effect of banana PME by sodium dodecyl sulfate was more evident at pH 3.6 than at pH 7.7 (21). Hall (13) reported that the degree of inhibition of tomato PME by tannic acid was dependent on the concentration of both the inhibitor and the pectin substrate. The decrease in

orange PME activity by silver nitrate was greater at pH 7.0 than at pH 4.0 (9). Bell et al. (2) reported that the lactic acid produced by fermentation was responsible for the low PME activity in both the cucumber extract and the brined green cucumber. Chang et al. (3) observed that the effect of sucrose inhibition on papaya PME was linear up to a concentration of 50% sucrose. Approximately 40% of apple PME inhibition was attained by a 15% sucrose concentration (31).

Tomato PME was found to be competitively inhibited by polygalacturonate with a K_i value of 7×10^{-3} M anhydrogalacturonate residues (29), or by polygalacturonic acid with a K_i value of 0.024% polygalacturonic acid (6).

VII. ACTIVATION ENERGY AND TEMPERATURE COEFFICIENT OF PECTIN METHYL ESTERASE

Energy of activation may be regarded as the amount of energy needed for the substrate molecules to react with the enzyme and be converted to products (31). The reported activation energies were 6,000 calories (6) and 7,280 calories (15) for tomato PME, 5,800 calories for apple PME (31), and 7,600 calories for mold PME (40).

Nakagawa et al. (47) reported a break in the Arrhenius plot at 31°C. NaCl increased the activation energy and lowered the break point to 27°C.

The temperature coefficient (Q_{10}) is conveniently expressed by the increase in rate of an enzyme reaction with increase in temperature (15). Some mean Q_{10} values reported were: 1.40 over the range of 20-50°C for snap bean PME (50); 1.35 over the range of 30-50°C for Southern pea PME (5); 1.47 over the range of 10-50°C for tomato PME (15); 1.60 over the range of 0-20°C for mold PME (40).

VIII. KINETIC REACTIONS FOR PECTIN METHYL ESTERASE

The enzyme kinetics of alfalfa PME (33) showed that the maximum velocity (V_{max}) value at 0.20 M NaCl was about 3.5 times that at 0.025 M NaCl, but the values of the Michaelis-Menten constant (K_m) at the two levels of salt were about the same: 0.04% pectin. Hills and Mottern (15) reported that the values of V_{max} and K_m for tomato PME were 0.0334 and 0.041% pectin, respectively. Other values reported for K_m were 0.08% pectin for orange PME (34), 0.24% pectin (47) and 4×10^{-3} M methyl anhydrogalacturonate residues (29) for tomato PME, 0.09% pectin for tobacco leaf PME (18), and 0.20% pectin for fungal PME (6).

Published studies on PME disagree as to whether the enzymatic deesterification of pectin is a first order or a zero order reaction. Apple PME (31) followed a first order reaction, whereas alfalfa (33) and tomato PME (15) followed a zero order reaction over the initial 40-50% hydrolysis.

However, Speiser et al. (58) failed to prove whether the kinetics of tomato PME were a first or zero order reaction.

IX. PURIFICATION OF PECTIN METHYL ESTERASE

Since PME is firmly bound to the water insoluble cell wall materials, the extraction of the enzyme from plant tissues usually employs a 2-10% NaCl solution adjusted to an alkaline pH (27).

Lee and Macmillan (29) purified tomato PME 19-fold by ammonium sulfate precipitation followed by calcium phosphate gel adsorption and column chromatography on DEAE-cellulose and Sephadex G-75. The purified enzyme showed a single homogeneous band both on ultracentrifugation and disc gel electrophoresis. The sedimentation coefficient was 3.08 S. Nakagawa et al. (46) also purified tomato PME about 60-fold by means of ammonium sulfate fractionation, chromatography on DEAE-cellulose and gel filtration on Sephadex G-100 column. The enzyme thus obtained was homogeneous both on ultracentrifugation and disc gel electrophoresis with a sedimentation coefficient of 3.17 S.

Three fractions of PME have been isolated by differential extraction from banana pulp (21) and further purification of these enzymes was achieved by ammonium sulfate fractionation and DEAE- and CM-cellulose column chromatography (23).

Endo (10) obtained two highly purified forms of fungal PME from culture extract of Coniothyrium diplodiella by utilizing ammonium sulfate fractionation and column chromatographies on Duolite CS-101, Duolite A-2 and DEAE-cellulose. The purified enzymes were homogeneous on free boundary electrophoresis. A fungal PME from the culture broth of Fusarium oxysporum f. sp. vasinfectum was purified 5-fold by a procedure employing column chromatography on DEAE-Sephadex, Sephadex G-75, CM-Sephadex and CM-cellulose (43). The purified enzyme was free of polygalacturonate lyase activity, but a trace of polygalacturonase remained. Disc gel electrophoresis indicated two protein bands at pH 4.3, but none at pH 9.5.

X. MULTIPLE MOLECULAR FORMS OF PECTIN METHYL ESTERASE

Glasziou and Inglis (12) found that tobacco pith contained two PME forms, one of which was firmly bound to the cell wall materials. Artichoke tubers yielded three PME forms, based on whether calcium ions or 2,4-dichlorophenoxy-acetic acid or neither caused binding of the enzyme to the cell walls.

Hultin and Levine (21) observed three molecular forms of banana PME which differed considerably in response to pH, temperature inactivation, and activation or inactivation by

sodium dodecyl sulfate. The further purified enzymes (23) showed different response to cations, inorganic phosphate, nucleotides, sucrose, and initial reaction kinetics.

Markovic et al. (39) recently reported the presence of six molecular forms of banana PME. The thin layer chromatography on Sephadex G-150 Superfine indicated that the molecular weight of each of three forms was about 35,000. The other two forms were 46,000 M.W. each and the last form was 10,000 M.W.

Pressey and Avants (50) separated, by DEAE-Sephadex A-50 chromatography, four forms of tomato PME, with molecular weights of 35,500, 27,000, 23,700, and 24,300. The number and amounts of these components varied with ripeness and variety of tomato, and no single extract contained all four. They were different in heat stability, response to pH and cations.

Endo (10) reported that two molecular forms of a fungal PME were very similar in the specific activity, deesterification of pectin and pectinic acids, optimum pH and heat stability, except that the first form had a broader stable pH-region than that of the second form.

Reid (52) separated two active PME forms, by paper chromatography, from a fungal source and suggested that PME from various fungi differs in its initial rates and total amounts of deesterification of pectin.

CHAPTER III

MATERIALS AND METHODS

I. SOURCES OF SOUTHERN PEAS

Southern peas (Vigna sinensis), Purple Hull Pink Eye variety, were grown on the Plant Science Farm, University of Tennessee, Knoxville. After the peas had developed to the stage when the pods were green (just prior to showing purple coloration), they were harvested and shelled by hand. The peas were placed into polyethylene bags without washing or blanching and placed immediately in an air-blast freezer and frozen at -20°C. The frozen peas were lyophilized and stored in a desiccator at room temperature.

II. PARTIAL PURIFICATION OF ENZYME

Acetone Powder

Acetone powder was prepared by a modification of the method described by Nason (48). The freeze-dried peas were blended with chilled 80% acetone in the ratio of 1:2 (W/V) in a Waring blender for 4 mintues at 4°C. The resultant slurry was filtered on a Buchner funnel with sufficient chilled 80% acetone and washed further with an excess of 100% chilled acetone. The washed residue was spread out on filter paper and allowed to dry overnight at 4°C. The

acetone-dried powder was then pulverized in a water-cooled CRC Micro-Mill (Chemical Rubber Co., Cleveland, Ohio) and stored in a desiccator at 4°C until used.

Extraction of Crude Enzyme

PME was extracted from the acetone powder with a 2% NaCl solution of pH 8.0 at room temperature according to the method of Chen (4). Thirty grams of acetone powder were slowly added to 300 ml of continuously stirred 2% NaCl solution of pH 8.0 and extraction was continued for 2 hours. An automatic titrator (Model K, Beckman Instruments, Fullerton, Calif.) was employed to continuously stir the mixture and maintain the pH at 8.0 by addition of 0.09937 N NaOH. After the extraction, the mixture was centrifuged at 3,000 × G for 30 minutes and the precipitate was discarded. The supernatant liquid thus obtained was used as the crude enzyme extract.

Ammonium Sulfate Fractionation

Solid ammonium sulfate was slowly added to 250 ml of crude enzyme extract to 0.55 saturation. After stirring for 30 minutes at 4°C, the mixture was centrifuged for 30 minutes at 20,000 × G and the precipitate was discarded. Solid ammonium sulfate was further added to the supernatant to 0.80 saturation and the mixture was recentrifuged. The precipitate was collected and dissolved in 100 ml of deionized water.

This solution was treated again with solid ammonium sulfate to 0.65 and 0.80 saturation and the mixture was recentrifuged. The precipitate was collected and dissolved in 50 ml of deionized water and then dialyzed overnight against 0.02 M sodium phosphate buffer of pH 7.5. The enzyme preparation from the ammonium sulfate fractionation was used for the determination of the following physicochemical properties: (1) optimum pH and NaCl concentration for maximum PME activity, (2) energy of activation, and (3) kinetic reactions.

III. ENZYME ASSAY METHODS

Qualitative Test

Bromthymol blue is an indicator that changes color in the range of pH 6.0-7.6. The indicator solution was prepared by dissolving 100 mg of bromthymol blue with 3.2 ml of 0.05 N NaOH. Deionized water was added to make a volume of 100 ml solution (4).

Bromthymol blue indicator solution was used for a qualitative assay of PME activity. A mixture of 1 ml of sample, 2 ml of 1.0% pectin N.F. (W/V) (Sunkist Growers, Inc., Corona, Calif.) and 2 drops of bromthymol blue indicator was adjusted to pH 7.6 with 0.1 N NaOH. A change in color from blue (at pH 7.6) to greenish yellow (near pH 6.0) indicated the presence of PME.

Quantitative Test

PME activity was quantitatively measured at 30°C by titration of the carboxyl groups of the pectin released by the action of the enzyme. The reaction medium contained 15 ml of 1.0% pectin N.F., 10 ml of 0.75 M NaCl and 5 ml of PME solution. The pH of the reaction was automatically maintained at 7.9 with a titrator (Model K, Beckman Instruments, Fullerton, Calif.) which controlled the amount of 0.09937 N NaOH added to the reaction medium. Preincubation was followed for 2 minutes at 30°C with a water bath during the reaction and then titrated for 10 minutes to the original pH of 7.9.

One unit of PME activity was defined as the amount of enzyme which produces 1×10^{-3} moles of free carboxyl groups per minute under the above conditions.

Protein Determination

The protein concentration of column eluates (described later) was determined by measuring the absorbance at 280 nm with a double-beam grating spectrophotometer (Coleman Model 124, Hitachi Ltd., Tokyo, Japan). A standard curve for determining the protein concentration was developed with various known amounts of bovine serum albumin (Nutritional Biochemicals Corp., Cleveland, Ohio) dissolved in a 1.0% NaCl solution. Two replications with two observations at

each protein concentration were made. The regression equation for the standard curve was: $A = 0.0004[P]_{\mu g/ml} - 0.0001$. The standard curve is presented in Figure 1, and each data point represents the mean of two observations for each of two replications.

Sodium Chloride Determination

The NaCl concentration of eluates from the DEAE-cellulose column was measured with a chloride ion electrode attached to a pH millivolt meter (Model 801, Orion Research Inc., Cambridge, Mass.). A standard curve for determining the chloride ion concentration was prepared with various known amounts of solid NaCl dissolved in 100 ml of 0.02 M sodium phosphate buffer of pH 7.5. Two replications with two observations at each chloride ion concentration were made. The regression equation for the standard curve was: Log [NaCl] = -0.02 mV - 1.748. The standard curve is presented in Figure 2, and each data point represents the mean of two observations for each of two replications.

IV. DETERMINATION OF OPTIMUM pH AND NaCl CONCENTRATION

The experimental design for the determination of the optimum pH and NaCl concentration for maximum PME activity was a 4 × 4 factorial in two complete blocks. The factors were pH at levels of 6.8, 7.2, 7.6 and 8.0 and NaCl concentration at levels of 0.12, 0.18, 0.24 and 0.30 M.

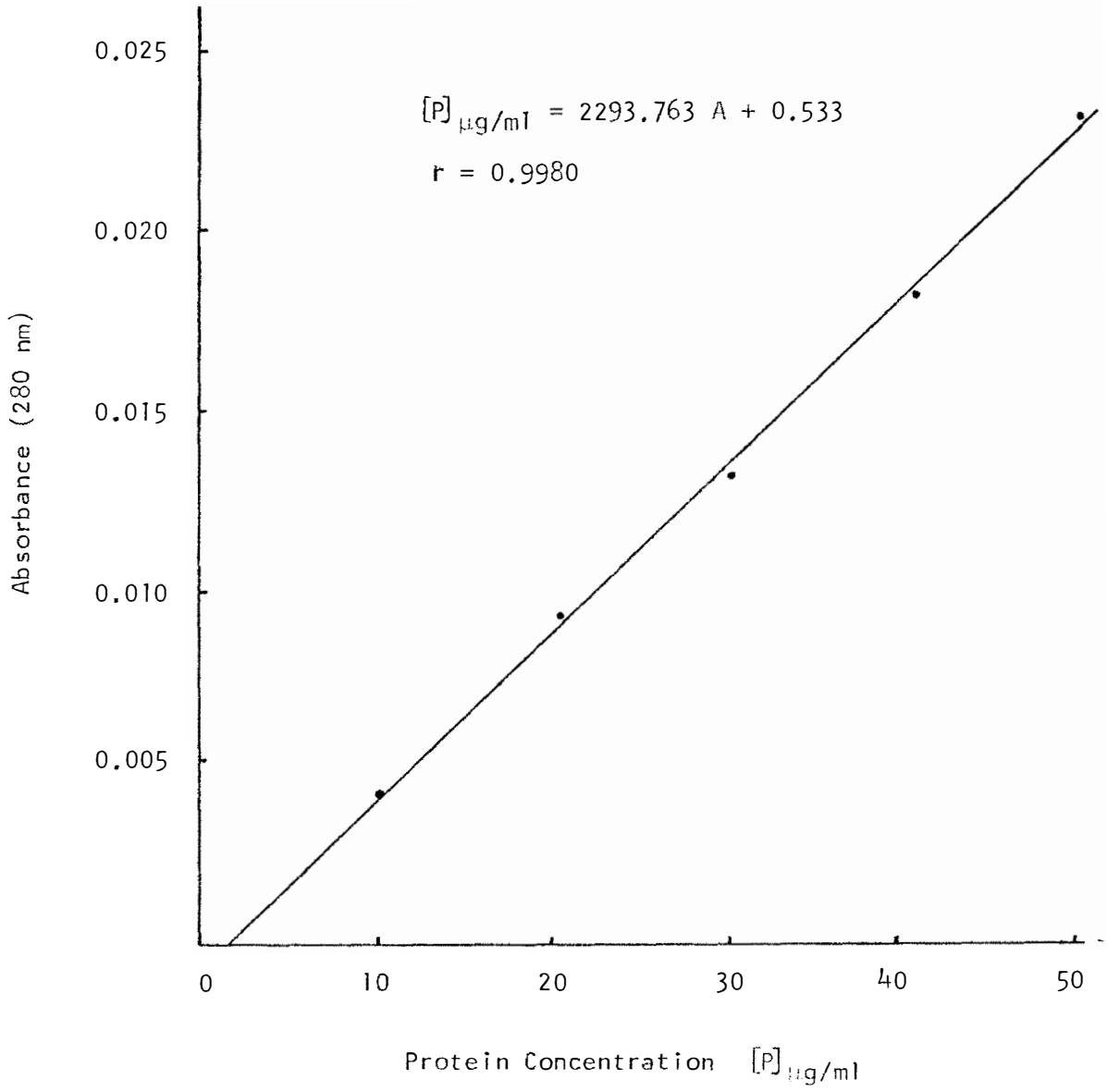


Figure 1. Standard curve for the determination of protein concentration.

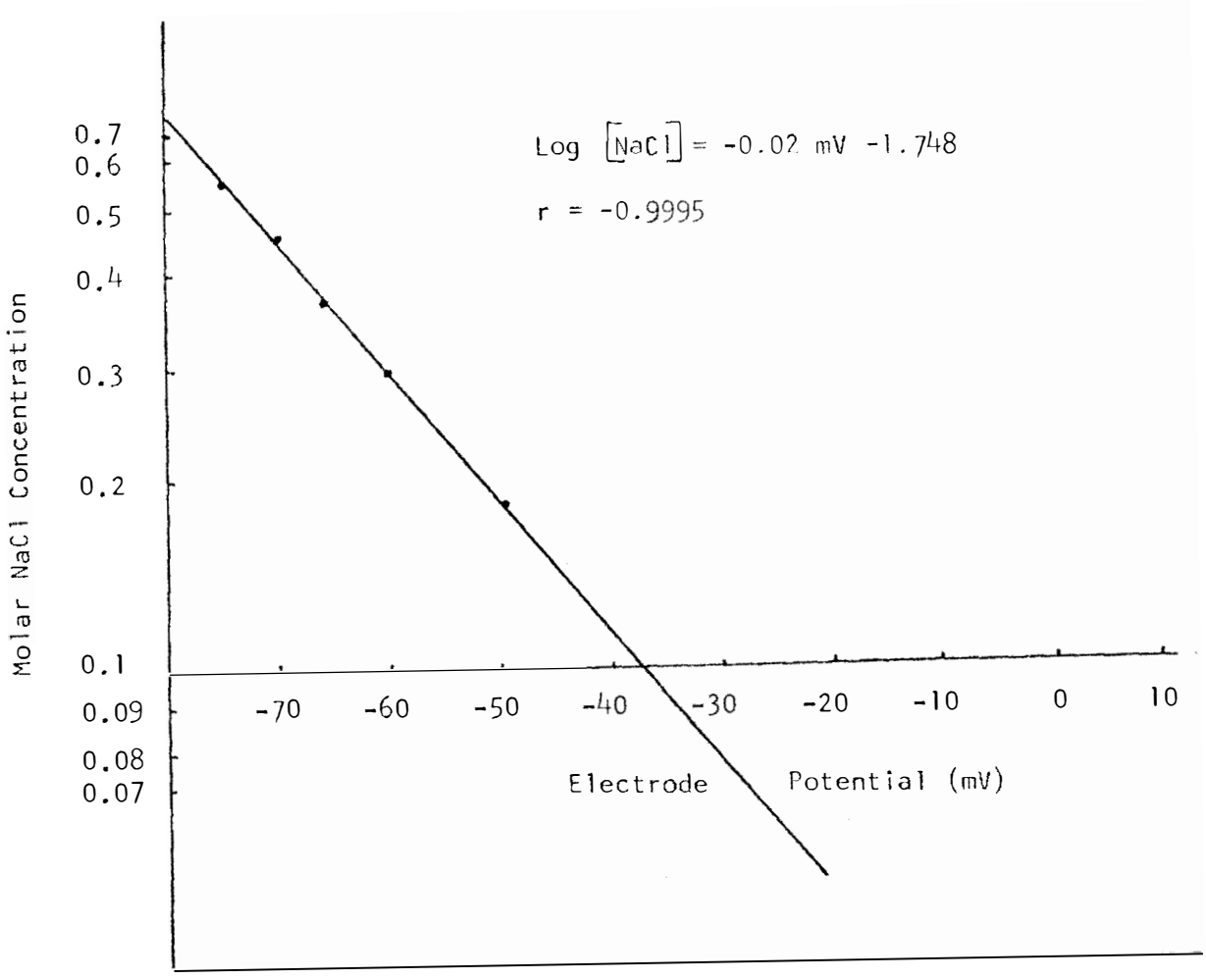


Figure 2. Standard curve for the determination of NaCl concentration.

For the reaction mixture in which PME activity was measured, 5 ml of enzyme solution, 15 ml of 1.0% pectin N.F., 0.75 M NaCl and a sufficient amount of deionized water were mixed to bring the NaCl concentration to the desired level with a total volume of 30 ml. The temperature of the mixture was 30°C, and was maintained by holding the beaker containing the mixture in a constant temperature water bath. The pH of the mixture was then adjusted to the desired level with 0.09937 N NaOH. The pH was maintained by an automatic titrator during the reaction period of 10 minutes. Since alkaline deesterification occurred at pH 7.6 and 8.0, rates for alkaline deesterification were determined at these pH levels in the absence of PME and corrections were applied to the rates obtained enzymatically. One observation was made for each of two replications. The values of PME activity thus obtained were analyzed by the analysis of variance, using the Statistical Analysis System (57) adapted for computation by the IBM 360-65 computer at the University Computing Center (56). The response of PME activity to different levels of pH and NaCl concentration was expressed as a three dimensional response surface map (56).

V. DETERMINATION OF ACTIVATION ENERGY

The reaction mixtures containing PME solution were incubated at 6 different temperatures, 20, 25, 30, 35, 40 and

45°C. The initial reaction velocities were determined at these temperatures, and the determinations were conducted for 0, 1, 2, 3, 4 and 5 minutes at each temperature according to the method of Nakagawa et al. (47). The pH values of the reaction mixtures employed for this experiment were 6.0, 7.0 and 7.9, respectively. The energy of activation was calculated by the graphic procedure from the Arrhenius plot (49). One observation was made for each of two replications at each temperature.

VI. DETERMINATION OF KINETIC REACTIONS

The initial rates for PME activity were measured at various concentrations of pectin N.F. The assay conditions of the reaction mixture containing PME solution were 0.25 M NaCl, pH 7.9 and 30°C. The reaction was carried out for 10 minutes. One observation was made for each of two replications at each concentration of pectin N.F. The values of K_m and V_{max} were determined by calculation, and the data and regression curve were plotted on the Lineweaver-Burk plot.

VII. ADDITIONAL PURIFICATION

After partial purification of the PME by a two-step ammonium sulfate fractionation and dialysis, additional purification was performed in order to ascertain the

presence of possible multiple molecular forms of PME in Southern peas and to determine their isoelectric points.

DEAE-Cellulose Column Chromatography

According to procedures suggested by Whitaker (60), DEAE-cellulose (J. T. Baker Chemical Co., Phillipsburg, N.J.) was sized by suspending 30 grams of material in one liter of 0.25 M HCl. The suspended material was occasionally stirred to prevent clumping. After 70-90% of the DEAE-cellulose had settled, the supernatant liquid was siphoned off to remove the fine particles. This washing procedure was continued with sufficient quantities of deionized water until the material settled uniformly and the supernatant liquid was clear. After sizing, the DEAE-cellulose was washed first with 500 ml of 0.25 M NaCl-0.25 M NaOH and filtered on a Buchner funnel. The resin was then washed with two 500 ml of deionized water and filtered again. The filter cake was resuspended in 500 ml of 0.25 M HCl and filtered again. The DEAE-cellulose was washed finally with five, 500 ml aliquots of deionized water and filtered again. The filter cake was suspended in 500 ml of 0.1 M sodium phosphate buffer at pH 7.5 and filtered. It was then suspended in 500 ml of 0.02 M sodium phosphate buffer of pH 7.5 and filtered. This step was repeated five times and the pH of the suspension was finally adjusted to 7.5.

The DEAE-cellulose slurry was poured carefully into the column of 1.6 × 40 cm to a height of 30 cm. Cold water (0-1°C) was continuously circulated through a thermostated jacket of the column. Sufficient volumes of 0.02 M sodium phosphate buffer of pH 7.5 were allowed to pass through the column in order to stabilize and equilibrate the DEAE-cellulose bed prior to sample application. The flow rate of eluate was adjusted to 0.27 ml per minute. A salt gradient mixer (GM-1, Pharmacia Fine Chemicals, Inc., Piscataway, N.J.), consisting of two identical reservoirs, was utilized to achieve a linear salt gradient. The mixing reservoir was filled with 0.02 M sodium phosphate buffer of pH 7.5 and the other reservoir was filled with an equal amount of 0.02 M sodium phosphate buffer, pH 7.5, containing 1.0 M NaCl.

Five ml of partially purified PME solution, which had been previously purified by a two-step ammonium sulfate fractionation and dialysis against 0.02 M sodium phosphate buffer of pH 7.5, was applied to the DEAE-cellulose column. An automatic fraction collector (MRA Corp., Boston, Mass.) was employed to collect 120 fractions of 4.28 ml each. A qualitative and a quantitative test were used to detect the PME activity from the eluates. After elution of 120 fractions, the DEAE-cellulose column was washed with 0.02 M sodium phosphate buffer of pH 7.5 for 10 hours. Another 5 ml of PME solution was applied to the DEAE-cellulose column under

the same conditions as previously described. The eluates containing the PME activity fractions from the first and second DEAE-cellulose column chromatography were combined, and concentrated by precipitation with 0.90 saturated solid ammonium sulfate. The precipitate was dissolved in 10 ml of 0.02 M sodium phosphate buffer of pH 7.5 and used for the gel filtration chromatography.

Sephadex G-100 Gel Filtration

Ten grams of Sephadex G-100 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) were allowed to swell in 600 ml of deionized water for 3 days at room temperature. The gel slurry was then packed into the column of 1.6 × 40 cm to a height of 30 cm with the aid of a gel and eluant reservoir. Cold water (0-1°C) was continuously circulated through a thermostated jacket of the column. Sufficient volumes of 0.02 M sodium phosphate buffer of pH 7.5 were allowed to pass through the column in order to stabilize and equilibrate the gel bed before sample application. The quality of packing was checked by observing the bed in transmitted light. The operating pressure on the column was maintained at 66.3 cm of water which gave a flow rate of 0.30 ml per minute.

Five ml of PME solution, which had been previously purified by DEAE-cellulose column chromatography, was loaded

onto the Sephadex G-100 column and eluted with 0.02 M sodium phosphate buffer of pH 7.5. Forty fractions of 4.05 ml each were collected by an automatic fraction collector. A qualitative and a quantitative test were used to detect the PME activity from the eluates. After elution of 40 fractions, the Sephadex G-100 column was washed with 0.02 M sodium phosphate buffer of pH 7.5 for 10 hours. Another 5 ml of PME solution was applied to the Sephadex G-100 column under the same conditions as previously described. The eluates containing the PME activity fractions from the first and second Sephadex G-100 column chromatography were combined, and concentrated by precipitation with 0.90 saturated solid ammonium sulfate. The precipitate was dissolved in 5 ml of deionized water and dialyzed against deionized water overnight at 4°C. This solution was used for the polyacrylamide gel electrofocusing.

VIII. POLYACRYLAMIDE GEL ELECTROFOCUSING

For the preparation of gel electrofocusing, ampholyte solutions (40% W/V) with buffering capacities in the pH range of 3-10 were obtained from LKB Produkter, Bromma I, Sweden. N,N,N',N'-tetramethylethylenediamine (TEMED), acrylamide and N, N'-methylene bisacrylamide were obtained from Eastman Kodak Co., Rochester, N.Y. Ammonium persulphate was obtained from Fisher Scientific Co., Fair Lawn, N.J.

A stock solution was prepared by dissolving 25 grams of acrylamide and 0.8 grams of N, N'-methylene bisacrylamide in 100 ml of deionized water. A 4% (W/V) ammonium persulphate solution was prepared weekly.

The gel solution was a mixture of 8.6 ml of deionized water, 2.4 ml of stock gel solution, 0.05 ml of TEMED and 0.6 ml of LKB Ampholines. According to methods of Righetti and Drysdale (53), the gel solution was degassed under vacuum for 20 seconds. Four drops of 4% ammonium persulphate were added, mixed and pipetted immediately into tubes (10 × 0.3 cm) which were sealed at one end with Parafilm. Air bubbles were removed by tapping the sides of the tubes. Finally, water was layered on top of the gel solution to give a flat gel surface and polymerization of the gel was then completed. When the gels had formed, the tubes were inserted into the gel electrofocusing apparatus (Model M 137-A, Metaloglass Inc., Boston, Mass.) and cooled to 0-1°C. The electrode vessels were then filled with solutions of 0.01 M H_3PO_4 and 0.02 M NaOH as anolyte (bottom) and catholyte (top), respectively. After 30 minutes of electrolysis (1 mA per tube) to discharge ammonium persulphate, the sample was applied to the top of the gel. The sample was a mixture of 1 ml of PME solution, 20% sucrose, 2% ampholytes (pH 3-10) and a trace of bromophenol blue. After sample application, the current was restored and maintained at a

level of 1 mA per tube until the voltage had risen to 300 V. Thereafter, the voltage was maintained at this level for 6 hours. When the focusing was complete, the gels were removed from the tubes by rimming with a Number 26 needle attached to a 50 ml syringe filled with cold water and injecting the water between the gel and the glass (14).

IX. ASCERTAINMENT OF THE PRESENCE OF MULTIPLE MOLECULAR FORMS AND THEIR ISOELECTRIC POINTS

Detection of Proteins

Following the method of Amorim and Josephson (1), gels were dialyzed against several changes of 12.5% trichloroacetic acid for 2 days to remove ampholytes, stained with 0.52% Amido black 10 B solution for 6 hours and destained in 7% acetic acid.

Detection of Pectin Methyl Esterase

To detect PME activity, the gels were transferred to a medium containing 1.0% pectin N.F., 0.1 N NaOH and bromthymol blue indicator solution. The reaction was allowed to proceed at room temperature. The yellowish color development on the gels indicated the presence of PME. The incubation time required to give adequate color development usually varied from 20 to 30 minutes, depending on the amount of enzyme present.

Determination of pH Gradient and Isoelectric Point

The pH gradient developed in the gels was measured from eluates of gel sections. The gels were sectioned with a razor blade into 0.5 cm segments which were dispersed into 0.5 ml of distilled water and then shaken for one hour with an automatic shaker (Model 75, Burrell Corp., Pittsburgh, Penn.) according to the method of Righetti and Drysdale (53). pH of the gel eluates was measured at 20°C with a combination microelectrode (Fisher Scientific Co., Fair Lawn, N.J.) in conjunction with a pH meter.

The isoelectric point of the PME was approximated by correlating the position of the histochemically stained band with the pH gradient in a replicate gel.

CHAPTER IV

RESULTS AND DISCUSSION

I. EFFECT OF pH LEVELS AND NaCl CONCENTRATIONS ON PECTIN METHYL ESTERASE ACTIVITY

The summary of the analysis of variance for the effect of pH levels and NaCl concentrations on the activity of PME prepared by ammonium sulfate fractionation is presented in Table I. The effect of pH and NaCl concentration were significant at the 0.05 level of probability with the linear effect of both factors being dominant over the quadratic effect. The interaction between pH and NaCl concentration was significant at the 0.05 level of probability, being due to the interaction between the linear pH comparison and the linear NaCl comparison. In view of these results, it can be stated that the linear pH comparison and the linear NaCl comparison are the major factors for the influence on PME activity.

Figure 3 shows the three dimensional response surface map, in which the response of PME activity to pH and NaCl concentration was described. The values for PME activity from treatments with four pH levels and four NaCl concentrations are presented as triangular areas on the map. The treatment with lowest pH (6.8) and lowest NaCl concentration (0.12 M) gave the lowest PME activity. As the pH or NaCl

TABLE I

SUMMARY OF ANALYSIS OF VARIANCE FOR EFFECT OF pH AND NaCl CONCENTRATION ON THE ACTIVITY OF PECTIN METHYL ESTERASE OF SOUTHERN PEAS

Source		Degrees of Freedom	Mean Squares
Total		31	
pH (P)		3	4.01*
Linear	(L)	1	10.99*
Quadratic	(Q)	1	0.96*
Cubic	(C)	1	0.09
NaCl (S)		3	2.52*
Linear	(L)	1	6.79*
Quadratic	(Q)	1	0.76*
Cubic	(C)	1	0.01
pH × NaCl		9	0.18*
$P_L \times S_L$		1	1.42*
$P_L \times S_Q$		1	0.01
$P_L \times S_C$		1	0.00
$P_Q \times S_L$		1	0.04
$P_Q \times S_Q$		1	0.00
$P_Q \times S_C$		1	0.07
$P_C \times S_L$		1	0.08
$P_C \times S_Q$		1	0.01
$P_C \times S_C$		1	0.00
Replication		1	0.18
Residual Error		15	0.04

*Significant at the 0.05 level of probability.

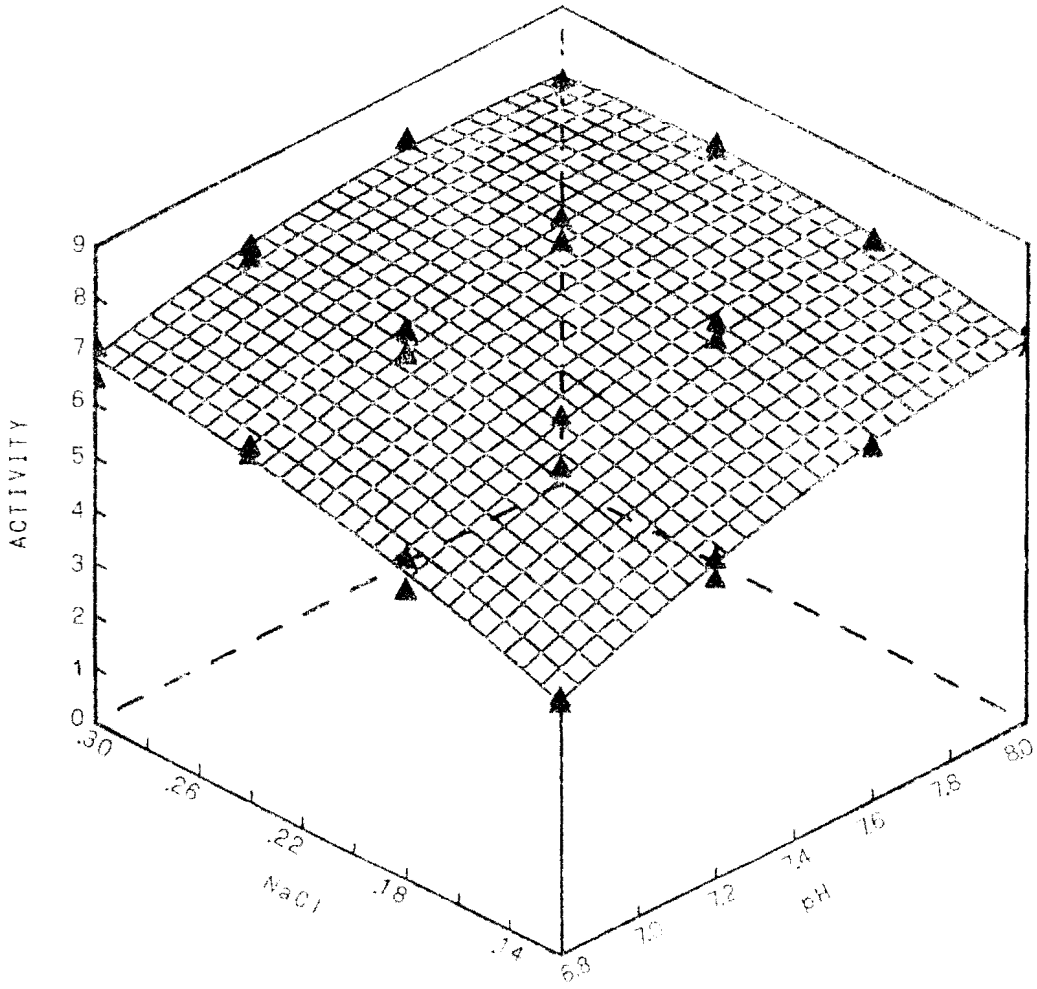


Figure 3. Three dimensional representation of the response surface of pectin methyl esterase activity of Southern peas on 1.0% pectin N.F.

concentration was increased, PME activity increased almost linearly until the conditions for maximum PME activity were reached. This observation is substantiated by the significant factors presented in the analysis of variance (Table I, page 32).

Based on the results of the analysis of variance, the following polynomial was developed to describe the response surface (56): PME activity = $-75.60 + 18.78\ [pH] - 1.08\ [pH]^2 + 76.13\ [NaCl] - 42.71\ [NaCl]^2 - 6.94\ [pH \times NaCl]$. According to the polynomial calculation, the optimum pH was 7.9 and the optimum NaCl concentration was 0.25 M.

II. DETERMINATION OF ACTIVATION ENERGY

The activation energies were calculated by the graphic procedure from the Arrhenius plot and are presented in Figure 4. The activity of PME yields a linear relationship when the pH levels were 6.0, 7.0, and 7.9. The continuation of the linear rate of the PME activity at the elevated temperature indicates the absence of thermal denaturation of the enzyme, which obviously would have interferred with the resultant PME activity.

The data in Table II are the calculated values for activation energies at pH 6.0, 7.0, and 7.9 (Figure 4). The value for activation energy at pH 7.9 is about 1.5 times greater than that at pH 6.0. This indicates that the activiation energy is influenced by pH of the reaction medium.

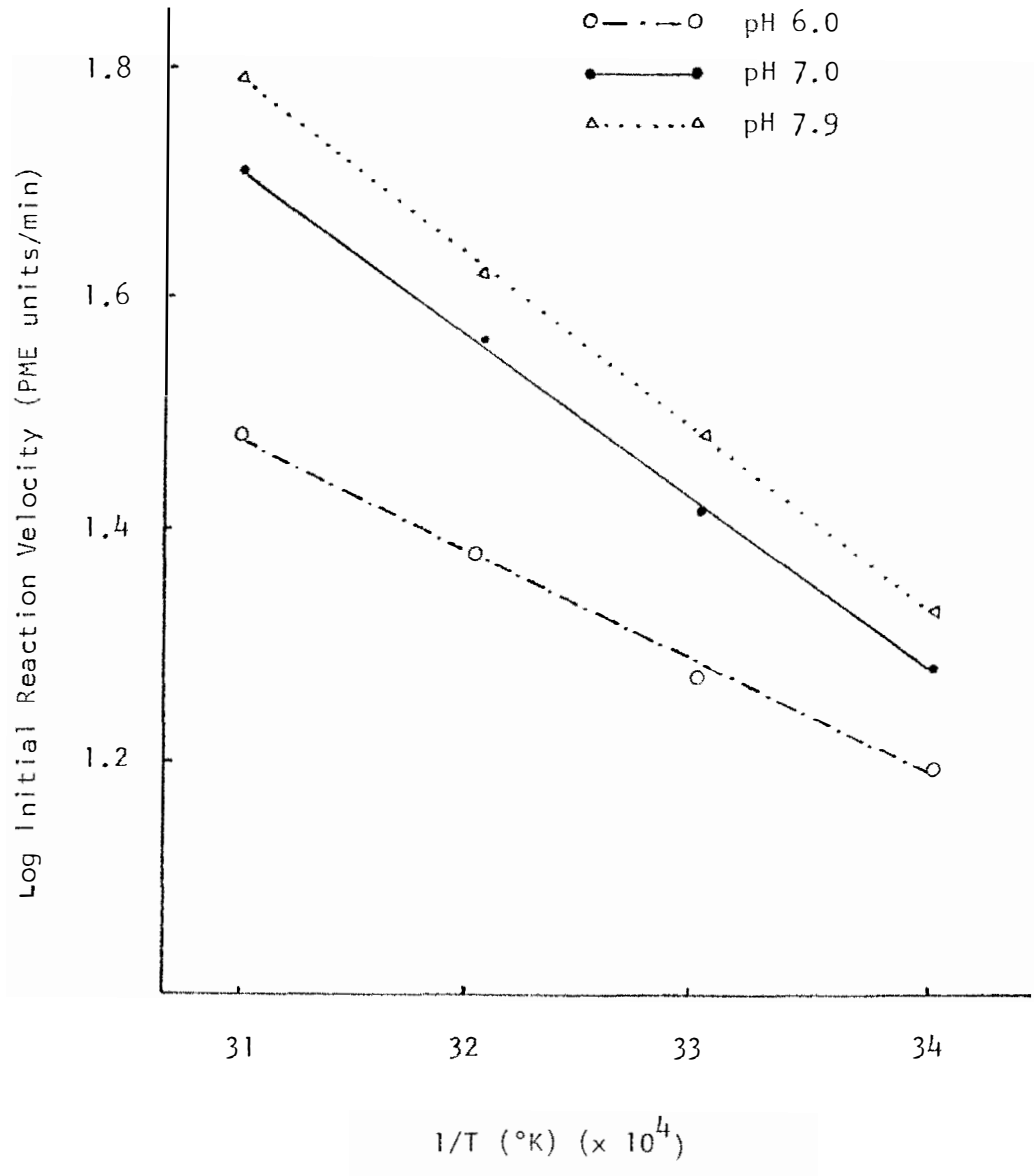


Figure 4. Arrhenius plot for pectin methyl esterase of Southern peas.

TABLE II

ACTIVATION ENERGIES OF PECTIN METHYL ESTERASE FROM SOUTHERN PEAS

pH	Activation[1] Energy (calories/mole)	Correlation Coefficient (r)
6.0	4,900	0.9948
7.0	5,950	0.9942
7.9	6,950	0.9935

[1]Means of one observation for each of two replications.

III. DETERMINATION OF KINETIC REACTIONS

The effect of different substrate (pectin N.F.) concentrations on the activity of PME is presented on the Lineweaver-Burk plot (Figure 5). The calculated apparent K_m is 0.781% pectin N.F. and the V_{max} is 24.34 PME units. The K_m is higher than that reported from PME of other sources (6, 33, 47).

IV. ADDITIONAL PURIFICATION

The PME solution, which had been partially purified by a two-step ammonium sulfate fractionation and dialysis, was subjected to DEAE-cellulose and Sephadex G-100 column chromatography for additional purification.

DEAE-Cellulose Column Chromatography

The chromatographic pattern of PME on DEAE-cellulose column is shown in Figure 6. Two active PME fractions were detected by a qualitative test. The enzyme activity from the first-appearing fraction was strong enough for a quantitative test, but that of the second-appearing fraction (fraction number, 41-47) was too weak for the quantitative test and consequently it is not presented in Figure 6. Therefore, the first-appearing PME fraction was collected, purified and used for the remaining experiments. Four distinct protein peaks were detected.

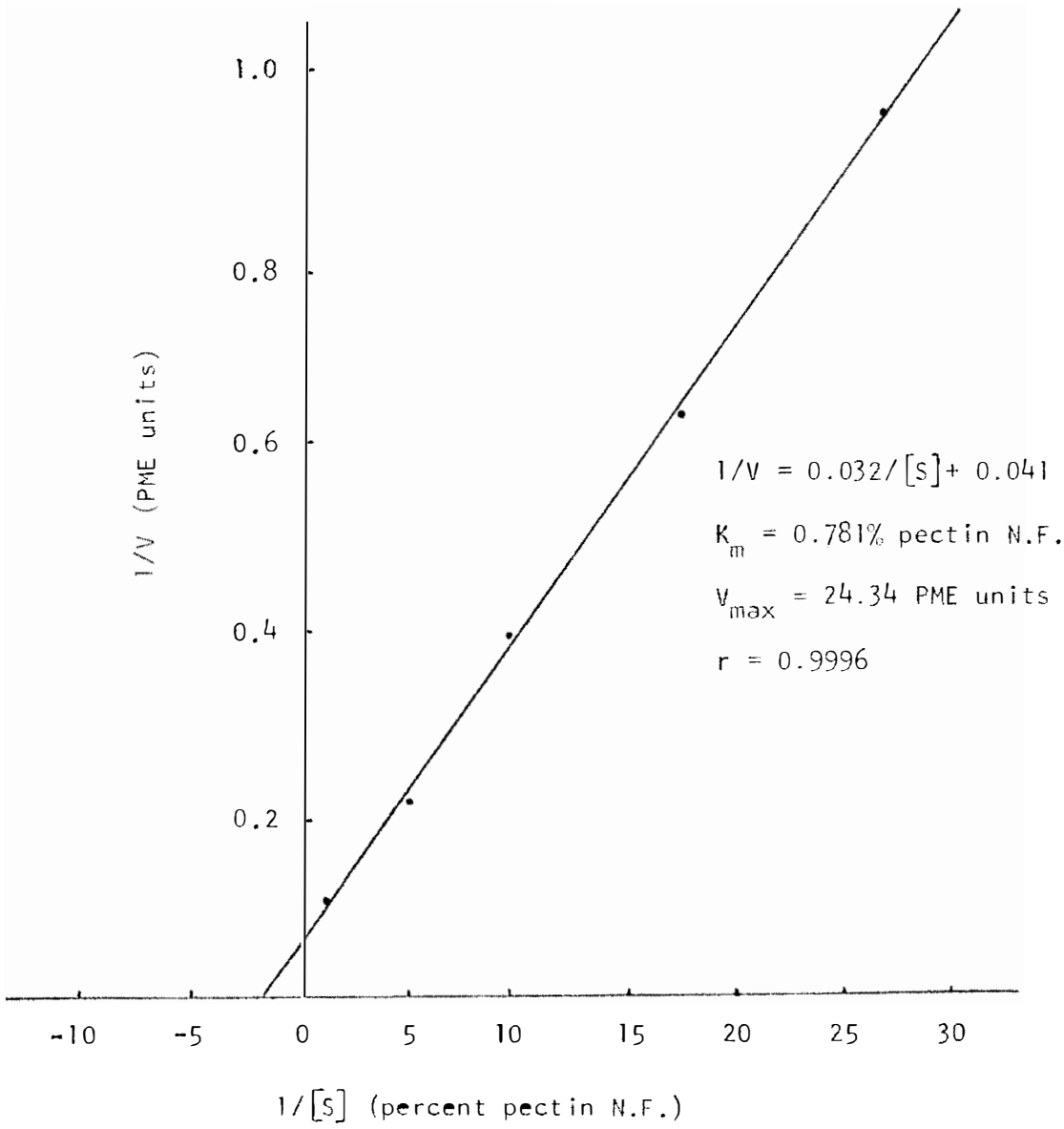


Figure 5. Lineweaver-Burk plot for effect of substrate concentration (percent pectin N.F.) on the activity of pectin methyl esterase from Southern peas.

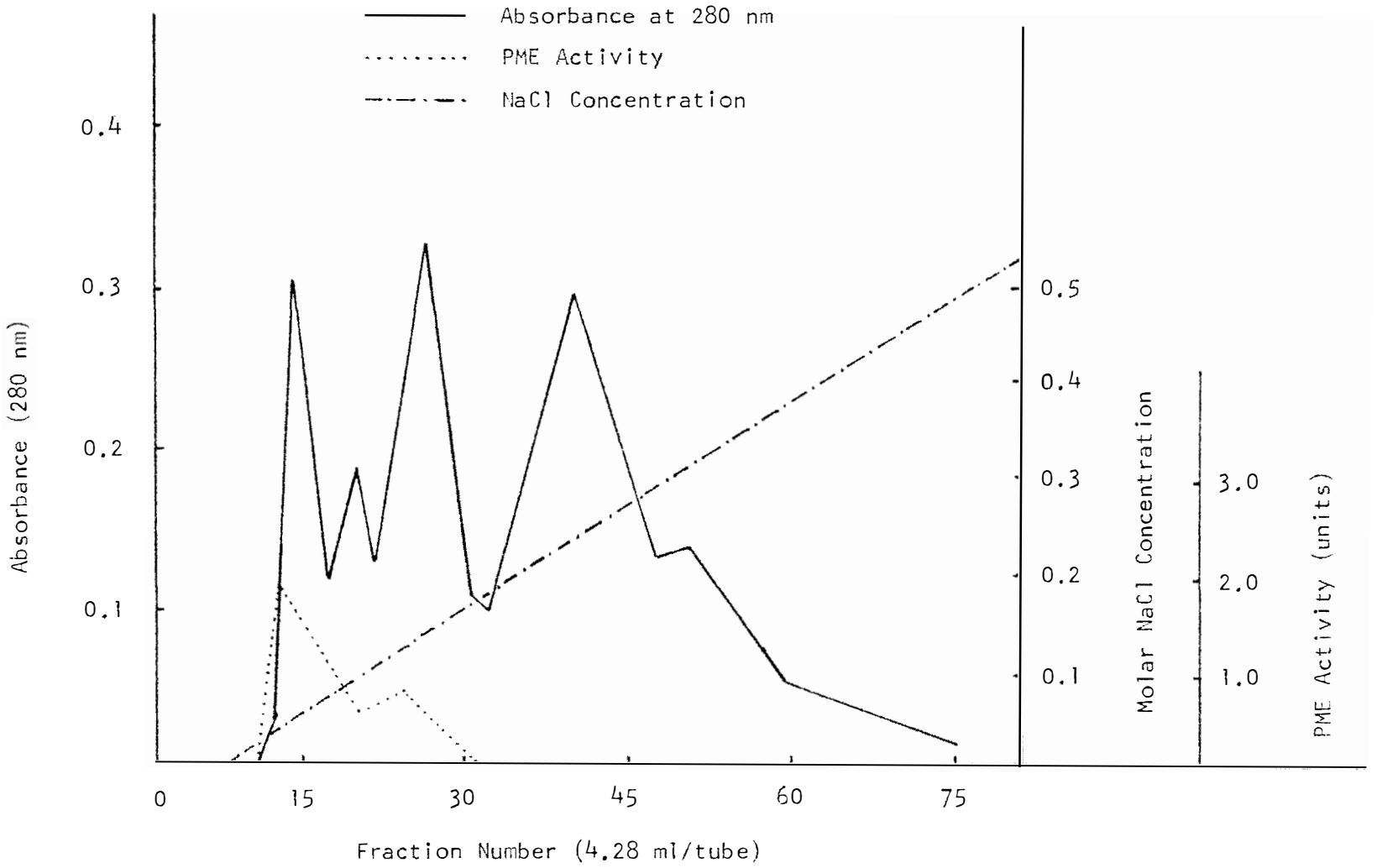


Figure 6. Chromatography of pectin methyl esterase from Southern peas of DEAE-cellulose column.

A linear salt gradient with a limiting concentration of 1 M NaCl was used to desorb the PME from its attachment to the DEAE-cellulose material. The fractions containing PME activity came off the DEAE-cellulose column early in the course of elution. This phenomenon can be explained by the fact that the first-appearing PME fraction did not adsorb on DEAE-cellulose, however, passage of the material through the column removed significant amounts of extraneous protein (29). The PME from the DEAE-cellulose column represents an increase of 198-fold (Table III) in the specific activity of the enzyme over that of the crude enzyme extract.

In general, the purification of PME based on different charge properties is relatively difficult because of difficulty in finding suitable conditions where the enzyme is most stable.

Sephadex G-100 Gel Filtration

The chromatographic pattern of PME on Sephadex G-100 column is given in Figure 7. A qualitative test showed one active PME fraction, which was confirmed by a quantitative test. A single protein peak was observed. This observation indicates that the PME was purified to a greater fold than that of DEAE-cellulose column chromatography. The PME activity represents an increase of 354-fold (Table III) in the specific activity of the enzyme over that of the crude

TABLE III

PURIFICATION OF PECTIN METHYL ESTERASE FROM SOUTHERN PEAS

Fraction	Volume (ml)	PME Activity (units)	Protein (mg)	Specific Activity (units/mg)	Yield (%)	Purification (X)
Crude enzyme extract	250	1,980	12,270	0.16	100	1
1st $(NH_4)_2SO_4$ fractionation (0.55-0.80 saturation)	80	625	1,470	0.43	32	3
2nd $(NH_4)_2SO_4$ fractionation (0.65-0.80 saturation)	40	330	250	1.32	17	8
DEAE-cellulose eluate	85.6	114	3.6	31.7	5.8	198
Sephadex G-100 eluate	37.5	102	1.8	56.7	5.2	354

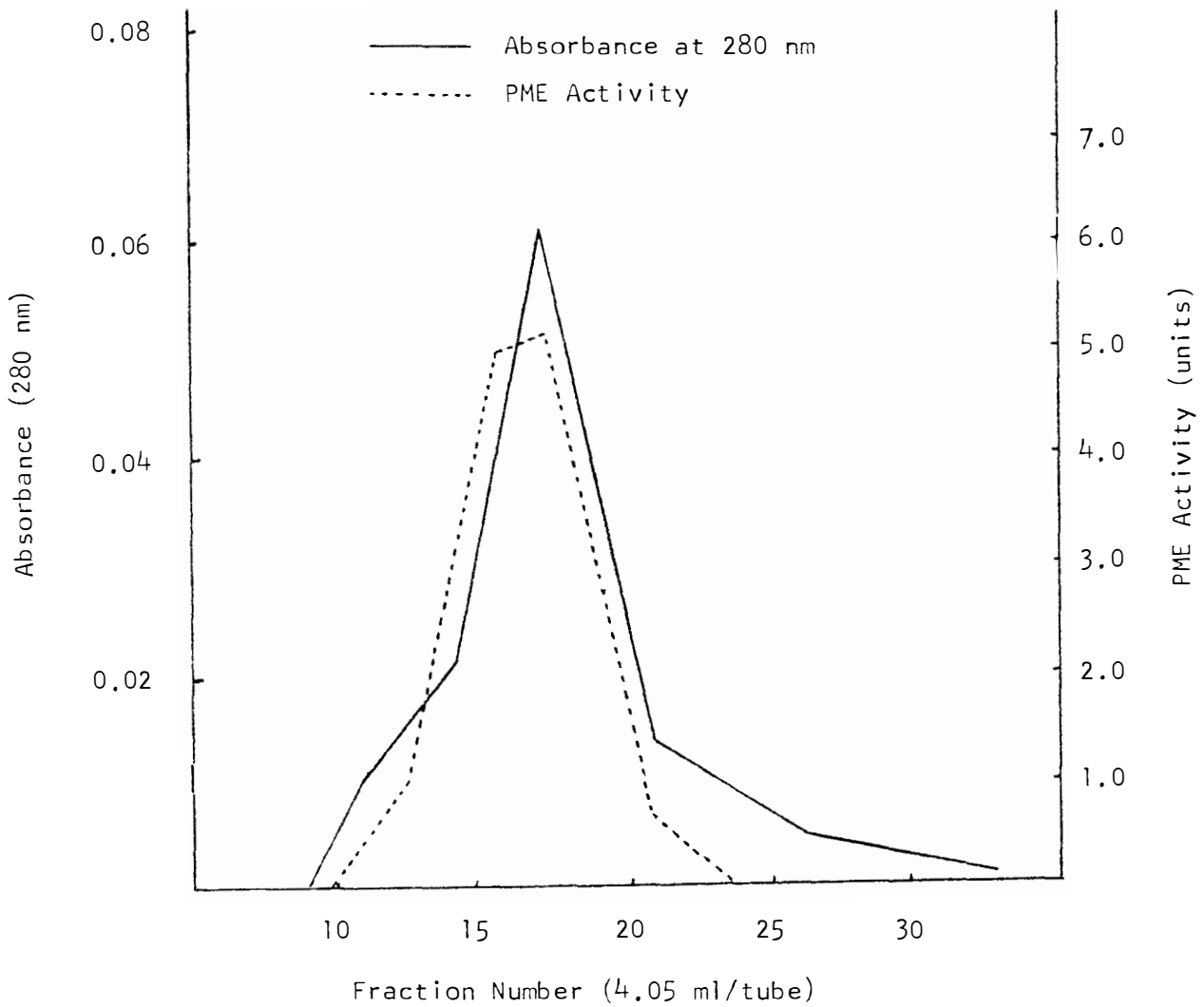


Figure 7. Chromatography of pectin methyl esterase from Southern peas on Sephadex G-100 column.

enzyme extract. These results clearly demonstrate that the method used for characterization of the size properties of PME can be well employed for purification of the enzyme. In general, gel filtration on Sephadex provides an effective means for removing much of the inactive accompanying proteins (7).

Table III, page 41, shows that the PME extracted from Southern peas was purified progressively by: first and second ammonium sulfate fractionation, DEAE-cellulose column chromatography, and Sephadex G-100 column chromatography with 3, 8, 198, and 354-fold purification, respectively.

V. ASCERTAINMENT OF THE PRESENCE OF MULTIPLE MOLECULAR FORMS AND THEIR ISOELECTRIC POINTS

The gel electrofocusing of the partially purified PME, in a pH 3-10 ampholyte system, indicated that at least two protein components were present. One component was stained strongly with Amido black 10 B, but the other one was stained faintly.

The qualitative PME activity test showed at least one enzymatically active component. The isoelectric point of the PME was approximated to be 4.5 at 20°C and is shown in Figure 8.

A stable pH gradient was developed across the column of gel. However, it was relatively difficult to develop a pH gradient that was sufficiently stable to discount molecular sieving effect in the gel.

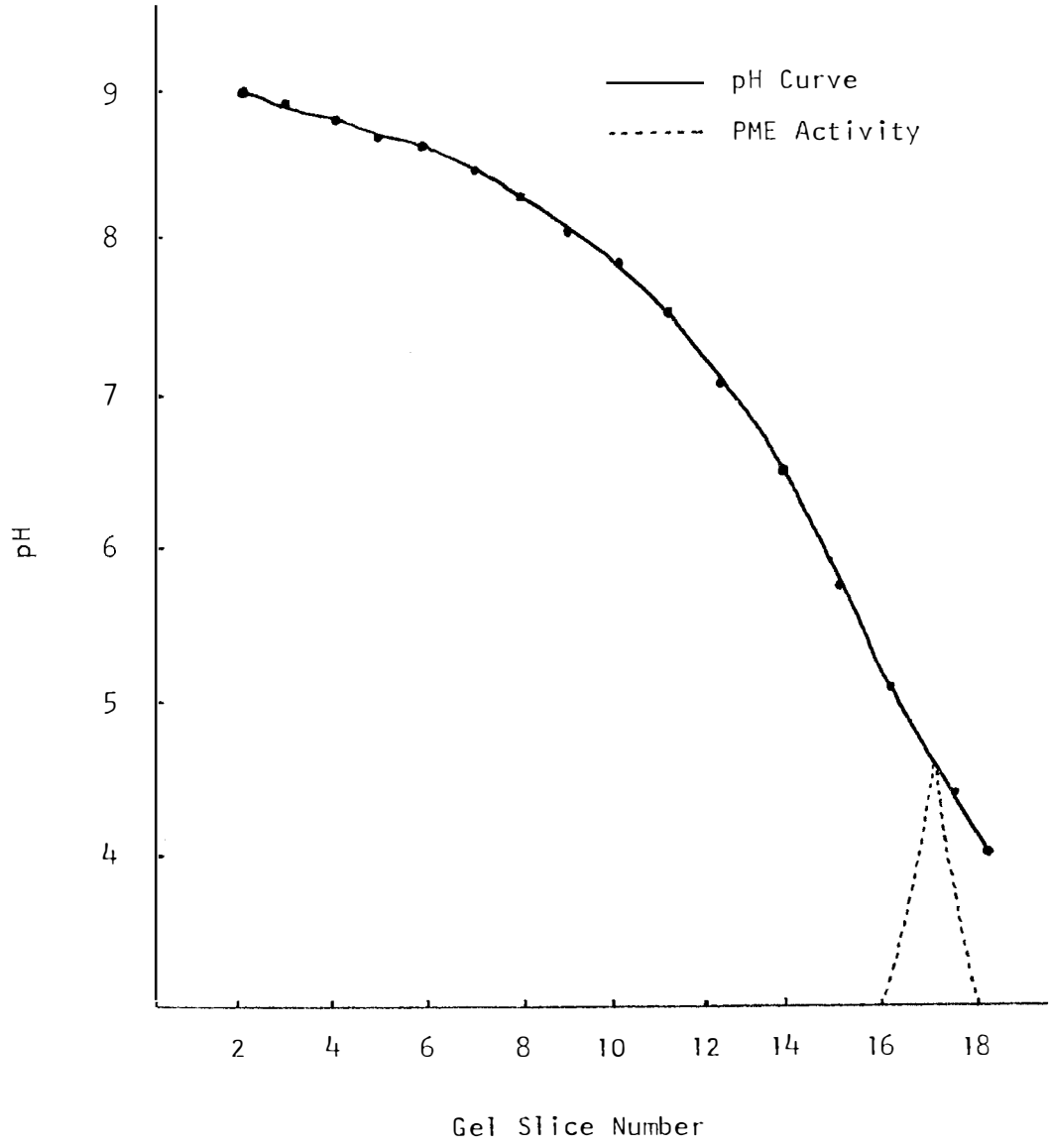


Figure 8. Development of a pH gradient in the pH 3-10 ampholyte system.

Although the experimental results with a gel electrofocusing showed a single enzymatically active component, it should not be assumed that Southern peas do not contain isoenzymes. As was described previously, DEAE-cellulose column chromatography separated at least two PME fractions. Since only one PME fraction was used in this experiment, it is quite possible that there is more than one PME in Southern peas.

The pH gradient is affected considerably by various factors such as choice of electrolytes, temperature, or concentration of ampholytes (53) so that the isoelectric point of PME should be read under those conditions described in the Materials and Methods.

CHAPTER V

SUMMARY

In this study, experiments were conducted to determine some of physicochemical properties of pectin methyl esterase (PME) from Southern peas (Vigna sinensis).

Based on the results of this study, the following conclusions were made:

1. PME activity was significantly ($p < 0.05$) influenced by pH, NaCl concentration, and the interaction between pH and NaCl concentration. Both, the linear and quadratic effects had a significant influence ($p < 0.05$), but the linear effect was predominant.

2. The optimum pH and NaCl concentration were calculated as 7.9 and 0.25 M, respectively.

3. The calculated energies of activation were 4,900, 5,950, and 6,900 calories/mole at pH 6.0, 7.0, and 7.9, respectively.

4. K_m was 0.781% pectin N.F. and V_{max} was 24.34 PME units.

5. PME was purified progressively by: first $(NH_4)_2SO_4$ fractionation, second $(NH_4)_2SO_4$ fractionation, DEAE-cellulose column chromatography, and Sephadex G-100 column chromatography with 3, 8, 198, and 354-fold purification, respectively.

6. Gel electrofocusing, resulting from the first-appearing PME fraction of the DEAE-cellulose column chromatography, indicated that there were at least one PME component and two protein components.

7. The isoelectric point of one PME component was approximated to be 4.5 at 20°C.

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LITERATURE CITED

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