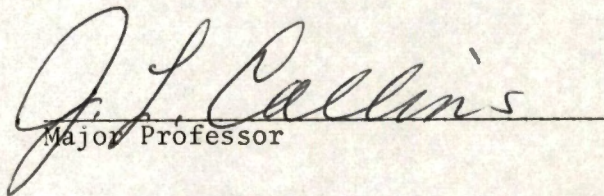


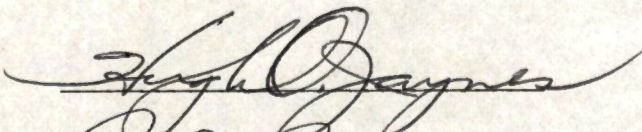
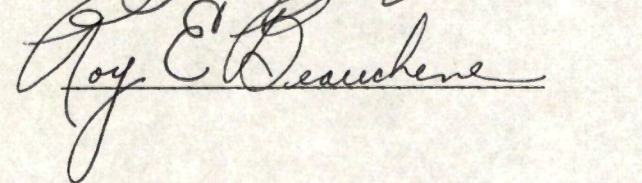
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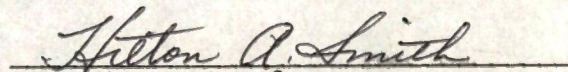
I am submitting herewith a thesis written by James Ronald Lee entitled "Partial Purification and Characterization of Pectin Methyl Esterase in Vegetable Soybeans." I recommend that it be accepted for nine quarter hours of credit in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Technology.


Major Professor

We have read this thesis
and recommend its acceptance:

Accepted for the Council:


Vice Chancellor for
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145

PARTIAL PURIFICATION AND CHARACTERIZATION OF PECTIN
METHYL ESTERASE IN VEGETABLE SOYBEANS

A Thesis
Presented to
the Graduate Council of
The University of Tennessee

In Partial Fulfillment
of the Requirements for the Degree
Master of Science

by
James Ronald Lee
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ABSTRACT

This study was conducted to partially purify pectin methyl esterase (PME) of vegetable soybeans, to determine optimum pH and salt concentration for maximum PME activity, to ascertain heat stability and energy of activation of PME, and to determine the Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) of PME of soybeans.

PME was obtained from soybeans which were raised on the University of Tennessee Plant Science Farm at Knoxville. Progressive purification of PME was accomplished by acetone powder, salt extraction, ammonium sulfate precipitation, dialysis, and pH precipitation in order to obtain partially purified PME which was used for assay of PME activity. The Beckman Model K automatic titrator was used for quantitative determination of PME activity.

The activity values for the effect of salt concentration and pH levels on PME activity were analyzed by analysis of variance using orthogonal comparisons. On the basis of the analysis of variance, a regression equation was chosen which described a three dimensional response surface of PME activity as affected by salt concentration and pH. Interaction between salt concentration and pH was significant at the 0.01 level of probability. No salt concentration or pH level was chosen as the optimum for PME activity.

Partially purified PME in aqueous solution was completely inactivated after a five minute treatment at 80°C. Energy of activation was calculated to be 7,570 calories per mole. By use of the Lineweaver-

Burk form of the Michaelis-Menten equation, K_m was calculated to be 0.111 percent pectin and V_{max} 20.60 PME units ($\times 10^4$).

After soybean PME was partially purified by the progressive purification procedures listed above, the PME containing material was lyophilized and purified further by DEAE- and CM-cellulose column chromatography. Fractions were collected using an automatic fraction collector. There were at least two molecular forms of the soybean PME. Two activity peaks were detected after DEAE-cellulose column chromatography. The PME material from the first activity peak from the DEAE-cellulose column yielded two activity peaks upon CM-cellulose column chromatography. PME material from the second activity peak from the DEAE-cellulose column yielded one activity peak upon CM-cellulose column chromatography. DEAE-cellulose column chromatography was an acceptable means of additional PME purification. CM-cellulose column chromatography was unsatisfactory for additional PME purification due to retention of detectable PME activity by the CM-cellulose column until one-third of the elution volume had been collected.

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CHAPTER I

INTRODUCTION

Pectin methyl esterase (PME, 3.1.1.11) occurs in plants, molds and bacteria and is a true esterase which hydrolyzes the methyl esters of polygalacturonic acid units of pectin. Activity of PME is generally measured by titration of the acid produced by the demethylation of pectin by PME.

The enzyme is of considerable interest in plant tissues and has been studied due to its potential in influencing the structure and character of foods. The activity of PME may result in a decrease of solubility of the pectic substances, and an increase in gel firmness in the presence of polyvalent cations such as calcium. Specifically, initial interest in the enzyme arose because of adverse textural changes of whole tomatoes during canning. Probably the best studied plant PME enzyme is that of the orange and this is because of its important effect on the cloud stability of frozen orange juice concentrates.

PME is believed to be sorbed strongly on the water insoluble cellular plant materials and may be extracted with a salt solution adjusted to an alkaline pH. PME is specific for pectin; glycol and glycerine esters of pectic acids are not attacked. The enzyme is activated by the presence of salts such as sodium chloride and the optimum salt concentration varies with pH of the reaction medium. The pH optimum for maximum PME activity is influenced by the amounts and kinds of

cations present in solution. PME from higher plants is comparatively heat stable; above 55°C the enzyme is gradually inactivated. Activity of PME in plants is closely related to plant maturity.

The soybean is becoming increasingly important world-wide as a source of protein, and may be eaten as a cooked vegetable or snack food in the future (8). Textural considerations will become important and knowledge of the properties of soybean PME will be useful.

This study was conducted to partially purify PME of vegetable soybeans, to determine the optimum pH and salt concentration for maximum PME activity, to ascertain heat stability and energy of activation of PME, and to determine the Michaelis-Menten constant (K_m) and the maximum velocity (V_{max}) of PME of soybeans.

CHAPTER II

REVIEW OF LITERATURE

I. SOURCES AND SUBSTRATE

Pectin methyl esterase is widely distributed in plants, molds and bacteria. Fremy discovered PME in 1840 in carrots (9, 55). Sources of the enzyme which have been studied are tomato (3, 15, 17, 25, 27, 36, 47), orange (11, 23, 33, 50), grapefruit (48), papaya (1, 4), snapbean (56), soybean (5), cucumber (3), southern pea (7), apple (29), banana (18), tobacco leaf (16), alfalfa (31), bacteria (39), and fungi (40). In oranges, the enzyme is bound to the juice sac cell wall, and the attachment may be of an enzyme substrate complex (23). The juice sacs and membrane of the orange contain greater activity than the peel (50).

"Pectin," a substrate for PME, is the term applied to those water soluble polygalacturonic acids of varying methyl ester content and degree of neutralization which are capable of forming gels with sugar and acid under suitable conditions (26). The galacturonic acid units of pectin are linked by α -1,4 glycosidic linkages between the pyranose rings. In natural pectins, approximately two-thirds of the carboxylic acid groups are esterified with methanol. The substrate used in PME assay studies is a commercial pectin, pectin N. F., made from citrus fruits; 65-70 percent of its galacturonic acid units are esterified. It is believed that L-rhamnose, L-arabinose, D-galactose, and traces of other sugar residues are present as integral parts of the pectin molecule (49).

II. MODE OF ACTION AND ASSAY OF ACTIVITY

PME hydrolyzes the methyl ester of the galacturonic acid units of pectin to yield methanol and produce carboxyl groups on the pectin. PME of the orange removes methoxyl groups adjacent to carboxyl groups on pectin at a more rapid rate than those next to the esterified residues (27, 49). Lee and Macmillan (28) showed that 50 percent of tomato PME activity is initiated near the reducing ends of highly esterified pectin molecules. The remainder of the enzymatic activity occurs at other sites.

PME activity has been assayed by several methods. An early method for assay of activity was to determine the time required for formation of a pectic acid gel from pectin in the presence of PME and calcium salts (24). Another method employed was to measure the amount of methanol produced (47). A simplified technique was to record the rate of pH drop from pH 7.5 to pH 7.0 (54). Perhaps the most common method presently used is the continuous titration with a standard alkali while maintaining the pH of the pectin-PME mixture at a constant value (26, 27).

III. PROPERTIES

Since the discovery of PME, names used for this enzyme have been pectase, pectin methoxylase, pectin esterase and pectin methyl esterase (9). Much of the early experimentation with PME was conducted by Kertesz and co-workers (24, 25, 33, 34, 36, 37).

PME has received much attention due to its effect on plant tissue texture. The enzyme is believed to be relatively inactive in most plant tissue, but damage to the raw fruit which destroys permeability barriers without denaturing PME could induce enzymatic activity and accelerate increased firmness due to PME activity on pectic substances (35). In the presence of divalent cations, such as calcium and magnesium, PME brings about decreased solubility of pectic substances which leads to a firmer texture.

In whole tomatoes, Hsu et al. (17) studied the effect of blanching and holding time prior to peeling on PME activity. They found that a blanching time of 30 seconds at 100°C and a 10 minute holding time gave the highest PME activity and yielded the firmest canned whole tomatoes. Bell and Etchells (3) examined the role which the enzyme played in cucumber salt stock softening. Activity in both the cucumber extract and brine was low compared to activity in non-brined green cucumbers.

Aung and Ross (1) recorded thermal inactivation of papaya PME and found after heat treatment a residual activity which could not be predicted by the resultant thermal inactivation curve. Van Buren et al. (56) indicated that firmness of processed green beans was related to the amount of pectic acid present. Levels of extracted pectic acid could be increased by using heat processing conditions which allowed the enzyme to act on pectin.

The effect of salts on PME activity has been extensively studied and optimum salt concentrations for maximum enzyme activity have been

reported. The optimum salt concentrations have varied greatly depending on the source and degree of purity of PME, the assay conditions, and the type of salt present. Some apparent optimum levels of sodium chloride for maximum activity have been determined to be: 0.05 M for tomato PME (27); 0.15 to 0.20 M for cucumber PME (3); 0.20 M for PME of southern peas (7).

Several workers have purposed that the effect of cations on PME activity is closely linked to pH and that the cationic effect is greater at the lower pH levels (20, 31, 33). The activity of alfalfa PME at pH 5.7 was about 30 times greater in the presence of 0.2 M monovalent cations or 0.02 M divalent cations. At pH 8.5 these concentrations of cations had practically no effect on the activity (31).

PME activity is affected by pH and the pH optimum for maximum activity is influenced by the amount and kind of salt present. Kertesz (26) stated that usually the effect of added salts is to lower the pH at which maximum activity is attained. Generally, plant PME has a broad pH optimum with constant activity in the region of 7.0 to 7.5 (54). Some reported pH optima are: 6.5 to 7.5 for apple PME (29); 7.5 for orange PME (23); 8.5 for PME from the tomato (28). Holden (16) found that PME activity was completely lost at pH 2.0, with some activity being lost between pH 3.0 and 5.0.

The effect of temperature on PME has been studied. Complete inactivation has been reported for green bean PME after holding green bean pods at 90°C for five minutes (56) and for tomato PME after holding aqueous enzyme solutions at 80°C for five minutes (47). Pratt and

Powers (48) calculated the thermal destruction time of PME of grapefruit juice to be 1.06 minutes at 90°C. Van Buren et al. (56) concluded that the enzyme in the pod was more resistant to heat denaturation at pH 6.5 than the enzyme in a suspension of acetone powder. They concluded also that PME was more heat labile at pH 4.5 than at pH 7.5. They found no regeneration of green bean PME activity after frozen storage of blanched green beans.

In temperature related studies, the activation energy (E_a) was calculated to be 5800 calories per mole in apple PME (29). Van Buren et al. (56) found the mean Q_{10} value over the range of 20°C to 50°C to be 1.40 for green bean PME and Collins (7) reported a mean Q_{10} value of 1.35 over the range from 30° to 50°C for PME from Southern peas.

Nagel (42) suggested that the amount of PME activity per gram of pear tissue decreased during maturation of the pear. With immature green southern peas, PME activity was higher than with the more mature peas (7). In the whole orange Rouse et al. (50, 51) found that total PME activity increased as maturity increased. During maturation and post harvest ripening of the banana, enzyme activity increased (19).

A tannic acid concentration of one percent caused a 99 percent inactivation of soybean PME (5). Hall (15) discovered that tannic acid inhibition of tomato PME was reversible. Chang (4) demonstrated that papaya PME was inhibited linearly with increasing sucrose concentration. Sulfur dioxide did not inhibit the enzymatic activity (2).

Recently, tomato PME has been studied extensively. This enzyme is believed to be a lipoprotein, since it can be stained by Sudan Black B

on filter paper. The enzyme exhibited an apparent lack of polarity under electrophoresis at pH 9.5 and 4.3 (27). The apparent molecular weight of tomato PME as determined by ascending chromatography on Sephadex G-75 superfine was 27,500 (40).

Enzyme kinetics of alfalfa PME were studied by Lineweaver and Ballou (31) and the Michaelis-Menten constant (K_m) was 0.04 percent pectin. Other values reported for K_m were 0.08 percent pectin for orange PME (33), 0.05 percent pectin for soybean PME (5), and 0.09 percent pectin for tobacco leaf PME (16).

IV. PURIFICATION

PME from various sources is firmly bound to cell wall materials, thus purification procedures usually employ extraction of the enzyme with a two to ten percent salt solution adjusted to an alkaline pH (26, 49).

Lee and Macmillan (27) purified tomato PME to a high degree of purity in order to study the mode of action. A crude salt extract was obtained from fully ripened tomatoes. The precipitate between 30 and 70 percent saturation of ammonium sulfate was selected for additional purification. The selected precipitated protein was dialyzed against 0.02 M phosphate buffer, pH 8.0. After dialysis, the partially purified PME was not adsorbed on either diethylaminoethyl-- (DEAE) or carboxymethyl-- (CM) cellulose equilibrated and eluted with 0.02 M phosphate buffer at pH values of 6, 7, or 8. The fractions containing PME activity from DEAE-cellulose chromatography were purified on a

column of Sephadex G-75 superfine. PME activity was eluted in a single peak. Dialysis of purified enzyme against deionized water resulted in activity loss of over 95 percent. Fifty percent of the lost activity could be restored by addition of calcium chloride to reaction mixtures.

Hultin et al. (18, 20) extracted three fractions of PME from banana pulp by differential extraction. Two of the fractions could be fractionated by treatment with ammonium sulfate. All three fractions were dialyzed against water and concentrated by dialysis against polyvinyl pyrrolidone (PVP). Aliquots of the concentrated solutions were purified by DEAE- or CM-cellulose column chromatography. The three PME fractions varied in their degree of PME retention on the cellulose columns.

Pithawala et al. (47) extracted tomato PME and Holden (16) extracted tobacco leaf PME with 0.1 M monobasic sodium phosphate, pH 8.0. Somogyi (54) extracted PME from mature cherry fruit with 0.5 M sodium acetate at pH 8.0. Lee and Wiley (29) prepared acetone powder from apples and used acetone powder suspensions for their assays of PME activity.

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CHAPTER III

MATERIALS AND METHODS

I. SOURCE OF SOYBEANS

The cultivar of soybeans used, commonly referred to as "Soylima," was grown on the University of Tennessee Plant Science Farm at Knoxville. In shape and size the immature beans have the appearance of baby lima beans.

The beans were planted on May 30. The seeds were planted at the rate of eight per twelve inches of row. The rows were 40 inches apart. Pods with the mature green beans were harvested by hand on October 9. The pods were shelled with a Taylor Bean Sheller (Taylor Manufacturing Company, Model 675) and the beans were packed in 100 gram lots in polyethylene bags, frozen overnight in an air blast freezer (-28°C) and moved to and held in a still air freezer (-18°C).

II. PARTIAL PURIFICATION OF ENZYME FOR ASSAY

Acetone Powder

Acetone powder was prepared using a combination of the methods of Morton (41) and Nason (43). Eighty percent acetone chilled to 4°C and soybeans which had been allowed to reach the melting point were blended in a 2:1 ratio (v/w) with an Osterizer blender at 4°C . The disintegrated cellular residue was washed on a Buchner funnel, which was attached to a vacuum pump, with sufficient 4°C 80 percent acetone to remove all solubles. The residue was washed further with 100 percent

acetone chilled to 4°C. The washed residue was spread onto filter paper in petri plates and air dried overnight at 4°C allowing all acetone to evaporate. The dried powder was pulverized in a water-cooled CRC Micro-Mill (Chemical Rubber Company) and stored in a desiccator at 4°C.

Enzyme Extraction and Ammonium Sulfate Precipitation

PME was extracted at room temperature (23°C) from the acetone powder with a two percent NaCl solution of pH 8.0. Two percent sodium chloride solution at pH 8.0 was determined in earlier work by Chen (5) as being effective for PME extraction. Ten milliliters of the NaCl solution per gram of acetone powder were used and extraction was continued for one hour. A Beckman Model K automatic titrator was employed to continuously stir the solution and maintain the pH at 8.0 by addition of 0.1 N sodium hydroxide.

After extraction with two percent NaCl, the solution was centrifuged at 1500 x G for 20 minutes and the insoluble residue was discarded. Enzyme grade ammonium sulfate was used to precipitate the suspended substances; this was carried out at 4°C to avoid loss of enzymatic activity. A nomogram giving the amount of ammonium sulfate required to obtain a given degree of saturation was utilized (10). After ammonium sulfate precipitation, the solution was centrifuged at 23,500 x G.

Dialysis and Precipitation of Protein

The protein fraction which precipitated between 0.55 and 0.75 ammonium sulfate saturation was utilized as the source of PME. The precipitate was resuspended in distilled water and dialyzed for 10 hours

against distilled water at 4°C to remove low molecular weight contaminants. Cellulose tubing of 27 millimeters inflated diameter was utilized.

After dialysis, a large portion of the proteins precipitated. The pH of the dialyzed solution was 6.3. The pH was lowered to 6.0 with 0.1 N HCl and additional precipitation occurred. The solution was allowed to stand until the supernatant solution became clear. PME activity was present in the supernatant, but the precipitate contained no activity. Precipitation by pH adjustment to 6.0 after dialysis was the final procedure in purification.

PME partially purified through the above purification procedures of acetone powder, salt extraction, ammonium sulfate precipitation, dialysis and pH precipitation was used for determination of enzyme properties.

III. ENZYME ASSAY METHODS

Bromthymol Blue Indicator Solution

Bromthymol blue is an indicator that changes color in the range from pH 7.6 to 6.0. The indicator solution is prepared by completely dissolving 100 milligrams of bromthymol blue with 3.2 milliliters of 0.05 N sodium hydroxide. Distilled water was added to obtain a total volume of 100 milliliters (5). Bromthymol blue indicator solution was used for a qualitative assay of PME activity. One-half milliliter of sample, two milliliters of one percent pectin (w/v), and two drops of bromthymol blue solution were mixed and the pH adjusted to 7.6 with 0.1 N NaOH. A set of Lamotte Standards, Lamotte Chemical Products Company,

Baltimore, Maryland, was used to make pH and comparative color change judgements. A color change of the indicator from blue at pH 7.6 to greenish yellow at a lower pH indicated the presence of PME.

Assay By Titration

PME activity was determined quantitatively by titration of the acid produced by the demethylation of pectin by PME. A Beckman Model K automatic titrator was utilized to maintain reaction mixtures at constant pH and to titrate the acid with standard 0.0616 N NaOH. The titrator was initially calibrated by use of buffers of pH 4.0 and 10.0. A Beckman 63 millimeter glass electrode and a Beckman 66 millimeter calomel reference electrode were utilized in conjunction with the automatic titrator for assay of PME activity.

A water bath consisting of a plastic vessel insulated with vermiculite was improvised to maintain a constant temperature during enzyme assay. The heating unit, an immersion heater coil, was activated by a Thermistemp Temperature Controller (Model 71) which employed a thermistor immersed in the water bath. A 50 milliliter Pyrex beaker, the reaction mixture container, was suspended in the water bath. The water was constantly stirred with a motor driven stirrer and the enzyme reaction mixture was stirred with a magnetic stirrer.

Assay Conditions

Stock solutions used were: one percent pectin (w/v); one molar NaCl solution; 0.1 N NaOH; and 0.1 N HCl. The pectin and salt solutions were heated to 30°C except where temperature effect was the experimental

variable. Each test mixture was prepared just prior to enzyme assay: total volume was 40 milliliters. Pectin concentration was 0.5 percent except in the enzyme kinetics study. The pH was 7.3, except where pH was an experimental variable. The salt concentration was 0.23 M, except where salt concentration was an experimental variable. Assay of activity was continued for 10 minutes. PME units were calculated by the following equation:

$$\text{PME units} = \frac{\text{milliliters of NaOH} \times \text{Normality of NaOH}}{\text{milliliters of enzyme solution} \times \text{time (minutes)}}$$

From each 100 grams of soybeans approximately 32 grams of acetone powder were obtained. From each 32 grams of acetone powder, 50 milliliters of partially purified PME solution were prepared. Five milliliters of the enzyme containing solution were used for each enzymatic activity measurement.

Protein Determination

Protein determinations were accomplished by measuring the absorbance at 215 and 225 nm according to the method of Waddell (57). A Perkin-Elmer Spectrophotometer (Coleman Model 124) was used to make the absorbance readings. A standard curve was prepared from the difference between the absorbance at 215 and 225 nm with measurements being made at five concentrations of bovine serum albumin. Concentrations were 10, 20, 30, 40, and 50 micrograms. Two replications with two observations at each concentration were made. The standard curve is presented in Figure 1.

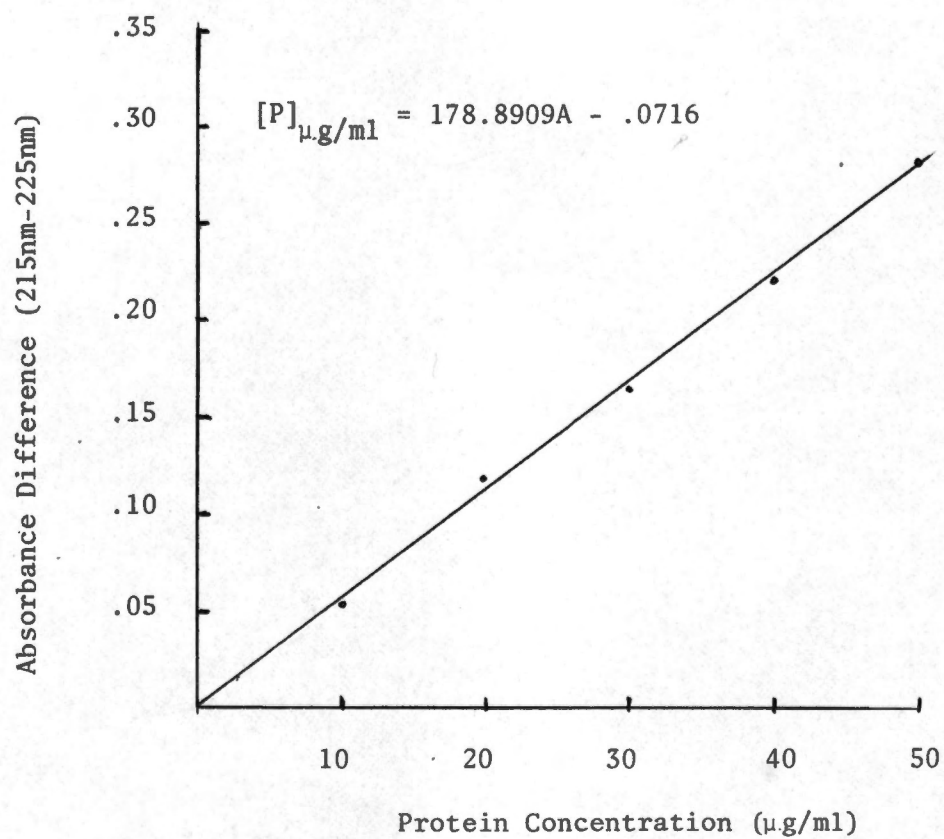


Figure 1. Standard curve prepared by using bovine serum albumin for determining protein concentration.

Sodium Chloride Determination

Sodium chloride determinations of the aliquots resulting from column chromatography were made by the potentiometric method of Cole (6) to measure the linearity of the salt gradient. A silver electrode and a calomel reference electrode were used with a Beckman Zeromatic pH meter. The samples containing salt were titrated with standardized silver nitrate and the end point was taken as the point of maximum rate of change of the potential of the electrode.

IV. DETERMINATION OF OPTIMUM pH AND SALT CONCENTRATION

The experimental design developed to determine the optimum pH and salt concentration necessary for maximum enzymatic activity was a 4 x 4 factorial in two complete blocks. The factors were salt concentrations at levels of 0.16, 0.23, 0.30, and 0.37 M and pH at levels of 6.6, 7.3, 8.0, and 8.7.

PME activity was determined at 30°C and to the test mixture was added sufficient 1 M NaCl solution to give the desired molarity. The pH of the test mixture was adjusted to the desired pH. Five milliliters of enzyme solution were added and the reaction mixture was readjusted to the desired pH. After equilibration, the reaction was continued for 10 minutes. Alkaline deesterification occurred at pH 8.0 and 8.7, thus the test mixtures without enzyme at these pH levels were titrated for 10 minutes in order to make correction for alkaline deesterification.

The PME activity values obtained were subjected to analysis of variance using orthogonal comparisons following the method of Snedecor (53) to determine significance of the effect of salt concentration and

pH on PME activity. On the basis of the analysis of variance the response of PME activity to pH and salt concentration was described by a three dimensional response surface (52). From the response surface a salt concentration level of 0.23 M and a pH level of 7.3 were selected as assay conditions for the remaining PME activity experiments. On the basis of the analysis of variance, a polynomial was chosen according to the method of Sanders (52) to describe the three dimensional response surface.

V. DETERMINATION OF HEAT STABILITY AND ENERGY OF ACTIVATION

Heat Stability

Enzyme solutions consisting of five milliliters at pH 7.3 and 0.23 M NaCl in an 18 x 150 millimeter Kimax culture tube were immersed in a water bath at 40°, 50°, 60°, 70°, and 80°C and held for five minutes. After the holding period, the solutions were removed and immersed for four minutes in an ice water bath. Then the enzyme solution was added to a test mixture of pH 7.0 and the complete reaction mixture was adjusted to pH 7.3, allowed to equilibrate and assayed for 10 minutes at 30°C. One observation was made for each of two replications at each temperature. The enzyme activity at the 40°C treatment was taken to be 100 percent activity.

Energy of Activation

Five milliliters of enzyme solution were added to 35 milliliters of the test mixture having a pH of 7.3. The reaction mixture was readjusted to pH 7.3, allowed to equilibrate, and titrated for 10 minutes.

Assay temperatures were 25°, 30°, 35°, 40°, and 45°C. Energy of activation was determined by calculation but presented graphically by plotting the log of activity against the inverse of temperature according to the method of Neilands (44). One observation was made for each of two replications at each temperature. The line was plotted by calculating the line of best fit.

VI. PECTIN METHYL ESTERASE KINETICS

Pectin concentrations of 0.04, 0.06, 0.08, 0.10, 0.16, and 0.20 percent were used to obtain the K_m and V_{max} . Other assay conditions were 0.23 M NaCl, pH 7.3, and 30°C. Five milliliters of enzyme solution were added to 35 milliliters of the test mixture of pH 7.3 containing the appropriate pectin concentration, and the complete reaction mixture was readjusted to pH 7.3, allowed to equilibrate, and assayed for 10 minutes. One observation was made for each of two replications. The curve for the best fit of the observations, and K_m and V_{max} were determined by calculation.

VII. ADDITIONAL PURIFICATION

After partial purification utilizing acetone powder, salt extraction, ammonium sulfate precipitation, dialysis, and pH precipitation, additional purification by means of DEAE- and CM-cellulose column chromatography was conducted. Changes in the previous purification scheme were made for column chromatography. The PME precipitate of ammonium sulfate fractionation was dissolved in 0.02 M phosphate buffer, pH 7.0.

The PME solution was then dialyzed against 0.02 M phosphate buffer, pH 7.0. Buffer was prepared according to the formula of Gomori (13).

After dialysis for 10 hours, the PME containing solution was decanted off the proteins which had precipitated during dialysis. Concentration of the PME material was accomplished by lyophilization by following the guidelines of Everse (12). The PME solution was frozen rapidly in an air blast freezer (-28°C) prior to lyophilization.

DEAE-cellulose, purchased from Bio-Rad Laboratories, Richmond, California, was prepared by procedures suggested by Whitaker (59). The DEAE-cellulose was sized by suspending 30 grams of material in one liter of 0.25 M HCl. After 90 percent of the DEAE had settled, the supernatant containing the fine particles was siphoned off. Sizing was repeated in one liter quantities of water until the material settled uniformly and the supernatant liquid was clear.

After sizing, the DEAE-cellulose was washed with 500 milliliters of 0.25 M NaCl-0.25 M NaOH and filtered on a Buchner funnel. Following this washing, the DEAE cellulose was washed with two, 500 milliliter quantities of water, with 500 milliliters of 0.25 M HCl, and with five, 500 milliliter quantities of deionized water. The filter cake was suspended in 500 milliliters of 0.1 M phosphate buffer, pH 7.0, and filtered. It was then suspended in 500 milliliters of 0.02 M phosphate buffer, pH 7.0, and filtered. Suspension in 0.02 M buffer was repeated five times and the pH of the final wash was verified.

A column of 2 x 34 centimeters was used for chromatography. The column was pured to a height of 20 centimeters. A two centimeter

buffer layer was maintained above the cellulose. The flow rate of eluant was 96 milliliters per hour. A fraction collector was used to collect 120 fractions of eight milliliters each. A salt gradient apparatus described and depicted by Whitaker (59) was constructed with two identical plastic containers and rubber tubing. Phosphate buffer, 0.02 M and pH 7.0, was used for elution with a linear salt gradient with a limiting concentration of one molar NaCl. The DEAE-cellulose column was equilibrated with 0.02 M buffer for eight hours prior to enzyme chromatography.

Thirty-two grams of acetone powder were used as the source of PME for DEAE-cellulose chromatography. After partial purification and lyophilization, the PME material was dissolved in five milliliters of 0.02 M phosphate buffer and layered beneath the two centimeter buffer zone onto the DEAE-cellulose column. After elution of 120 fractions, the DEAE-cellulose column was washed for eight hours with 0.02 M phosphate buffer, pH 7.0. Another five milliliter aliquot containing PME material was prepared and subjected to chromatography on the same DEAE-cellulose column under the same conditions as the previous aliquot. Two PME activity peaks were obtained from each run. The material from the fractions of the first activity peaks of the two runs was combined and rechromatographed on CM-cellulose. The material from the fractions of the second activity peaks was combined and rechromatographed on CM-cellulose. The fractions were dialyzed against 0.02 M phosphate buffer and lyophilized prior to rechromatography.

The CM-cellulose, also obtained from Bio-Rad Laboratories, was prepared by procedures suggested by Whitaker (59). Thirty grams of

CM-cellulose were sized in one liter portions of distilled water. After sizing, the cellulose was treated with 500 milliliters of 1×10^{-3} M cysteine-HCl and filtered. After filtration, the CM-cellulose was suspended in 0.50 M NaCl-0.50 NaOH and filtered. The CM-cellulose was washed in five, 500 milliliter quantities of deionized water. The material was suspended in 500 milliliters of 0.1 M phosphate buffer, pH 7.0, and filtered. The filter cake was suspended in 500 milliliters 0.02 M phosphate buffer, pH 7.0, and filtered. Suspension in 500 milliliters 0.02 M phosphate buffer, pH 7.0 with subsequent filtration was repeated four more times. The CM-cellulose column was poured and elution of the samples conducted in the same manner as the DEAE-cellulose column.

After chromatography, the fractions collected were qualitatively assayed for PME activity by use of the bromthymol blue indicator solution. Those fractions having activity were quantitatively assayed for PME activity by use of the Beckman automatic titrator. Protein determinations were accomplished by the Waddell method (57) of measuring absorbance at 215 and 225 nm. Determinations of salt concentrations were accomplished by the potentiometric method of Cole (6).

CHAPTER IV

EXPERIMENTAL RESULTS

I. EFFECT OF SALT CONCENTRATION AND pH LEVELS ON PECTIN METHYL ESTERASE ACTIVITY

The summary of the analysis of variance for the effect of treatments on PME activity is presented in Table I. The effect of salt and the interaction between salt and pH were significant at the 0.01 level of probability. The effect of pH was significant at the 0.05 level of probability, however, the cubic pH comparison was significant at the 0.01 level of probability. The linear and quadratic salt comparison was significant at the 0.01 level of probability. Interactions between the linear salt comparison and the linear pH comparison, and between the linear salt comparison and the quadratic pH comparison were significant at the 0.01 level of probability. Interaction between the quadratic salt comparison and the linear pH comparison was significant at the 0.05 level of probability.

The mean values for PME activity resulting from treatments with four salt concentration levels and four pH levels are presented in Table II. The lowest activity value was found for the treatment with highest salt concentration level and highest pH level, the highest activity value with lowest salt concentration level and highest pH level.

Certain characteristics of the response of PME activity to salt concentration and pH are apparent from the three dimensional response

TABLE I

SUMMARY OF ANALYSIS OF VARIANCE FOR EFFECT OF TREATMENTS ON
PECTIN METHYL ESTERASE ACTIVITY ON PECTIN N. F. AT 30°C

Source	Degrees of Freedom	Mean Squares
Total	31	
Salt (S)	(3)	21.25**
Linear (L)	1	48.40**
Quadratic (Q)	1	15.12**
Cubic (C)	1	0.22
pH (P)	(3)	1.25*
Linear (L)	1	0.22
Quadratic (Q)	1	0.50
Cubic (C)	1	3.02**
Salt x pH	(9)	3.85**
S _L x P _L	1	28.12**
S _L x P _Q	1	3.60**
S _L x P _C	1	0.12
S _Q x P _L	1	1.60*
S _Q x P _Q	1	0.12
S _Q x P _C	1	0.40
S _C x P _L	1	0.00
S _C x P _Q	1	0.02
S _C x P _C	1	0.50
Replication	1	0.12
Error	15	0.25

** Significant at the 0.01 level of probability

* Significant at the 0.05 level of probability

TABLE II

PECTIN METHYL ESTERASE ACTIVITY VALUES ON PECTIN N.F. FOR
EFFECT OF TREATMENTS ON ACTIVITY AT 30°C 1,2

Molar Salt Concentrations	pH			
	6.6	7.3	8.0	8.7
0.16	18.0 ³	21.0 ³	21.5 ⁴	22.5 ⁴
0.23	20.0 ³	21.5 ⁴	21.0 ³	21.0 ³
0.30	20.5 ⁴	21.0 ³	19.0 ³	19.5 ⁴
0.37	19.0 ³	17.5 ⁴	16.5 ⁴	16.5 ⁴

¹Means of 2 observations

²Activity expressed as PME units x 10⁴

³Standard deviation = ± 0.0

⁴Standard deviation = ± 0.7

surface depicted in Figure 2. PME activity at the highest pH level increased almost linearly with decreasing salt concentration. At the lowest pH level, a maximum value for PME activity was apparent at 0.27 M NaCl. PME activity at the highest salt concentration level decreased then increased with increasing pH.

Based on the results of the analysis of variance of the effect of treatments on PME activity, the following polynomial was chosen to describe the response surface (52): $\text{activity} = -663.6 + 231.7 \text{ salt concentration} + 281.1 \text{ salt concentration}^2 + 244.3 \text{ pH} - 29.9 \text{ pH}^2 + 1.3 \text{ pH}^3 + 1.4 (\text{salt concentration} \times \text{pH}) - 3.1 (\text{salt concentration} \times \text{pH}^2) - 113.9 (\text{salt concentration}^2 \times \text{pH}) + 1.0 (\text{salt concentration}^2 \times \text{pH}^3)$. Using the polynomial, the optimum salt concentration for maximum PME activity at pH 7.3 was calculated to be 0.222 M.

II. HEAT STABILITY AND ENERGY OF ACTIVATION

Heat Stability

The effect of heat treatment on the stability of PME activity is shown in Table III. Activity after heating for five minutes at 40°C was considered as 100 percent. Heating at 50°C resulted in a 9.4 percent reduction of PME activity, while heating at 60°C resulted in 80.7 percent reduction. Heating for five minutes at 80°C resulted in complete enzymatic inactivation. The correlation coefficient between the two replications with five observations in each was 0.999.

Energy of Activation

The activation energy was calculated to be 7,570 calories per mole. A graphic representation of the activation energy according to the

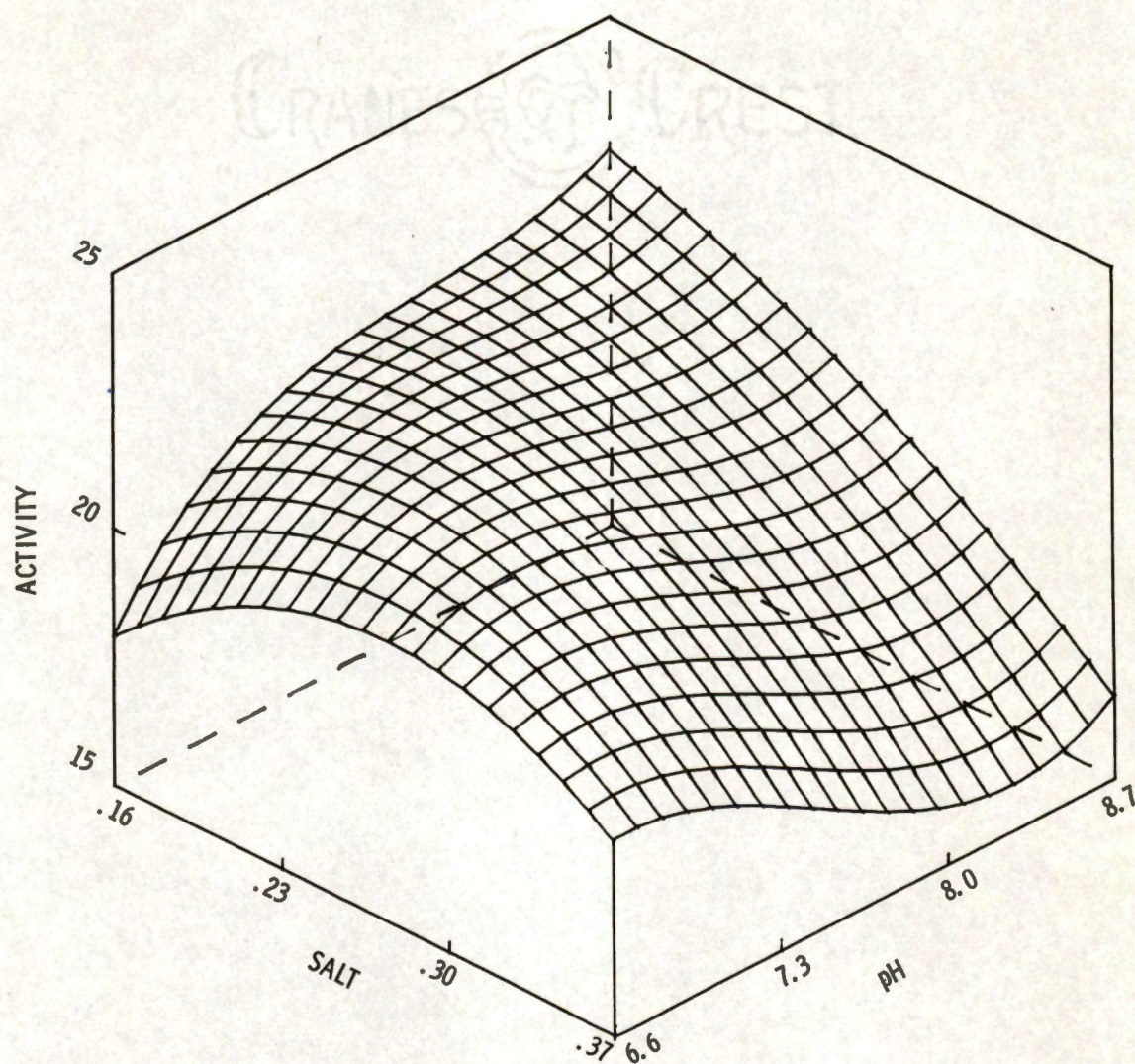


Figure 2. Three dimensional representation of the response surface of pectin methyl esterase activity on pectin N. F. as affected by treatments at 30°C.

TABLE III

REDUCTION OF PECTIN METHYL ESTERASE ACTIVITY ON PECTIN N.F.
AFTER TREATMENT OF AQUEOUS ENZYME¹ SOLUTIONS WITH
DIFFERENT TEMPERATURES FOR FIVE MINUTES

Temperature (°C)	Reduction of Activity ² (percent)
40	0.0
50	9.4 ± 1.8
60	80.7 ± 0.4
70	95.3 ± 0.0
80	100.0 ± 0.0

¹Enzyme solutions were adjusted to pH 7.3 and 0.23M salt concentration prior to temperature treatment.

²Means of two observations with standard deviation.

method of Neilands (44) is shown in Figure 3. The correlation coefficient between reciprocal of temperature ($^{\circ}\text{K}$) and the reciprocal of activity was 0.999.

III. PECTIN METHYL ESTERASE KINETICS

The relationship between PME activity and substrate concentration according to the Lineweaver-Burk plot is presented in Figure 4. The K_m value was calculated by the least squares method to be 0.111 percent pectin N. F. and $V_{\max}$ was 20.60 PME units ($\times 10^4$). The correlation coefficient between the reciprocal of pectin concentration and reciprocal of PME activity was 0.992.

IV. ADDITIONAL PURIFICATION

The behavior of partially purified PME when subjected to DEAE-cellulose chromatography is shown in Figure 5. Two peaks for PME activity were obtained: one from 64 to 96 milliliters of eluant and another from 104 to 160 milliliters. No PME activity was detected after 160 milliliters of eluant. Two protein concentration peaks were detected. One peak was found from 64 to 104 milliliters, the other from 104 to 352 milliliters. No protein was detected after 352 milliliters of eluant.

The linear salt gradient used was similar for all ion exchange chromatography. No sodium chloride was found in the first 64 milliliters, the void volume of the column. The eluant contained 0.10 M NaCl at 152 milliliters, 0.20 M at 240 milliliters, and 0.30 M at 328 milliliters. The gradient continued to 1.0 M at 960 milliliters.

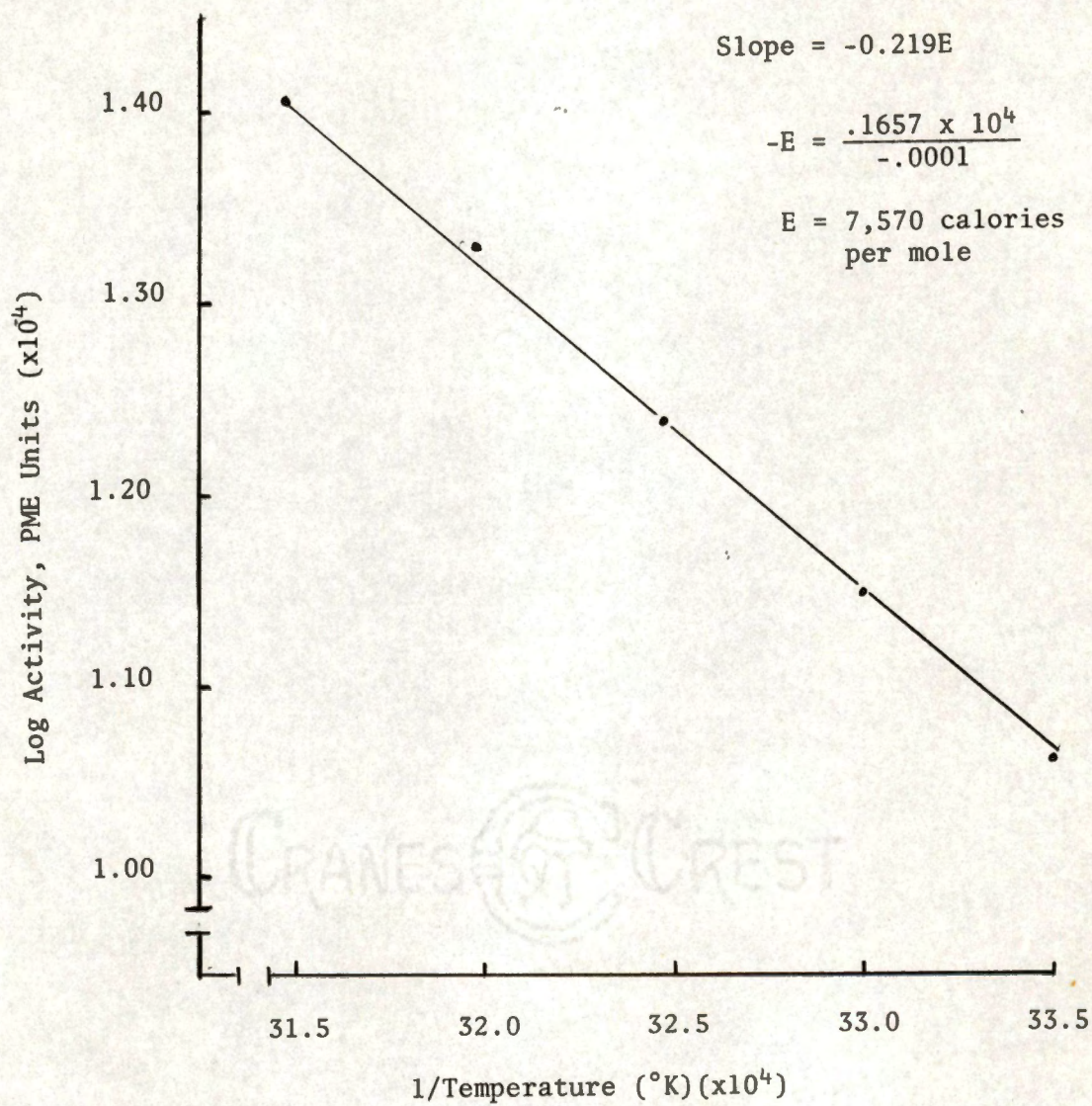


Figure 3. Activation energy of Pectin Methyl Esterase on pectin N. F. over a temperature range of 25° to $45^{\circ}C$, at 5° intervals.

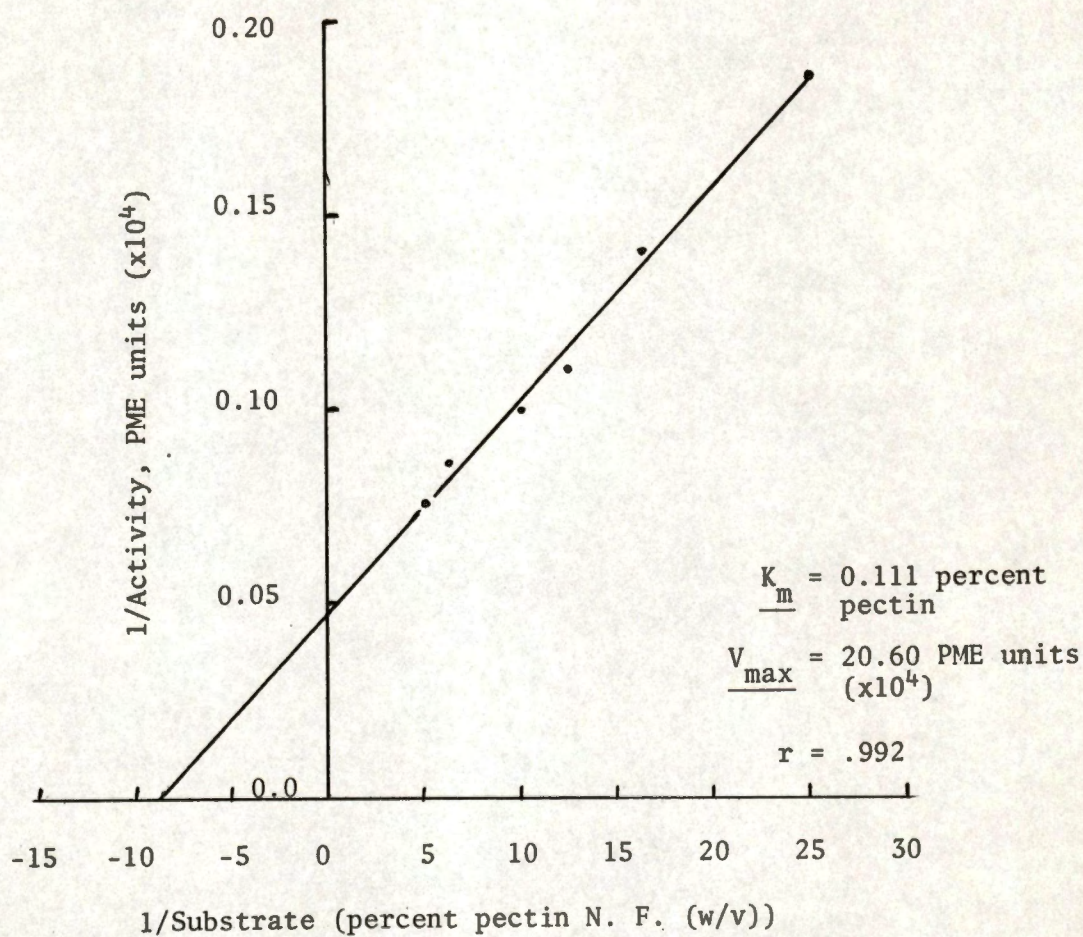


Figure 4. Effect of substrate concentration (percent pectin N. F.) on the activity of pectin methyl esterase. Conditions: pH 7.3; 0.23 M NaCl; 30°C.

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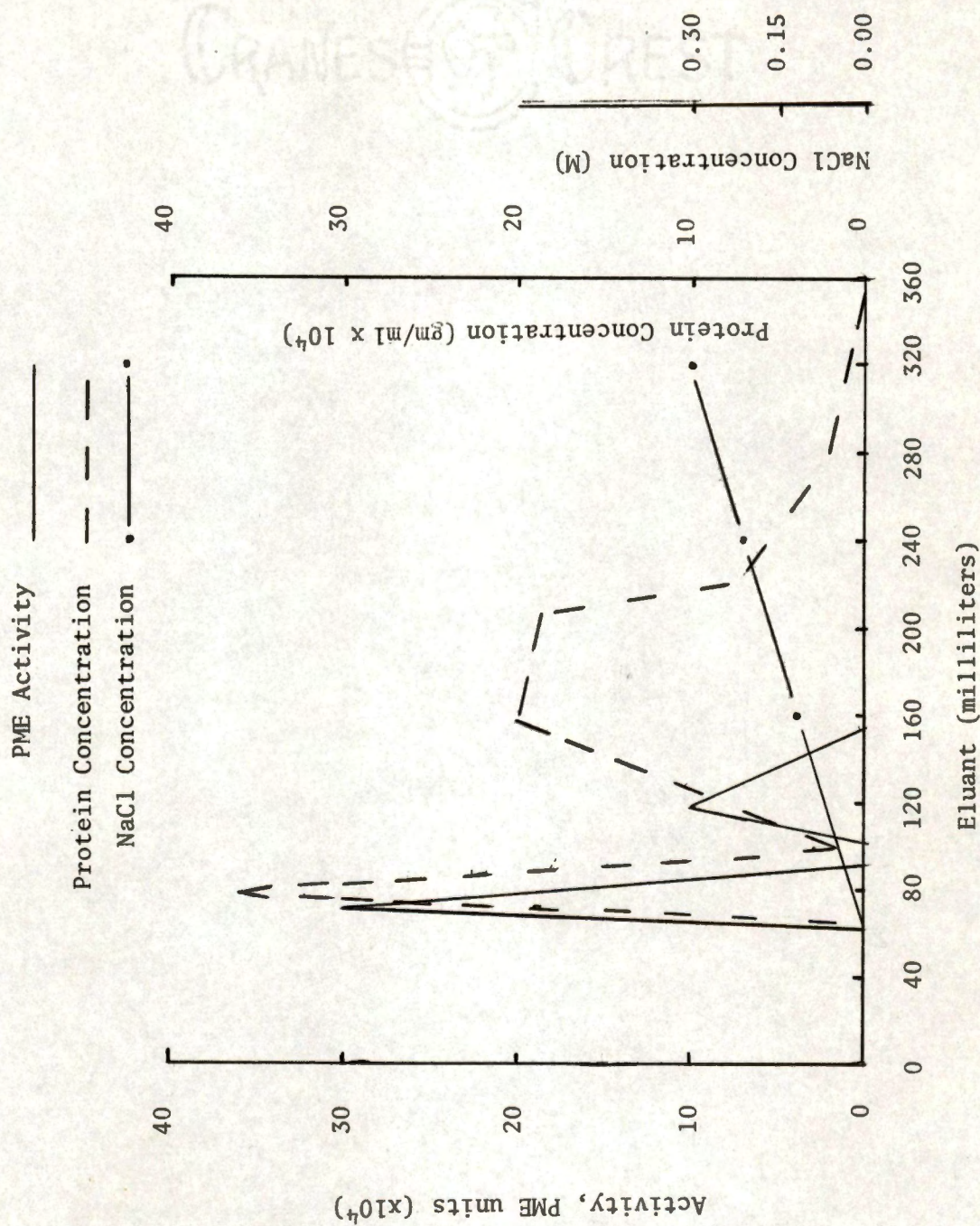


Figure 5. DEAE-cellulose column chromatography of partially purified pectin methyl esterase.

The protein fraction eluted in the first PME activity peak on the DEAE-cellulose column was rechromatographed on a CM-cellulose column and the results are shown in Figure 6. Two PME activity peaks were obtained. One sharp activity peak occurred from 64 to 112 milliliters of eluant and a broad peak of lower activity from 112 to 280 milliliters. No PME activity was detected after 280 milliliters of eluant. A distinct protein concentration peak was eluted from 64 to 96 milliliters of eluant, and another from 96 to 152 milliliters. No protein was detected after 480 milliliters of eluant.

Protein eluted in the second PME activity peak on DEAE-cellulose was rechromatographed on a CM-cellulose column, and the results are shown in Figure 7. One PME activity peak was detected from 96 to 136 milliliters of eluant. Two protein concentration peaks were detected; one from 64 to 88 milliliters of eluant and the other from 96 to 136 milliliters. No PME activity was detected after 136 milliliters of eluant, and no protein was detected past 200 milliliters of eluant.

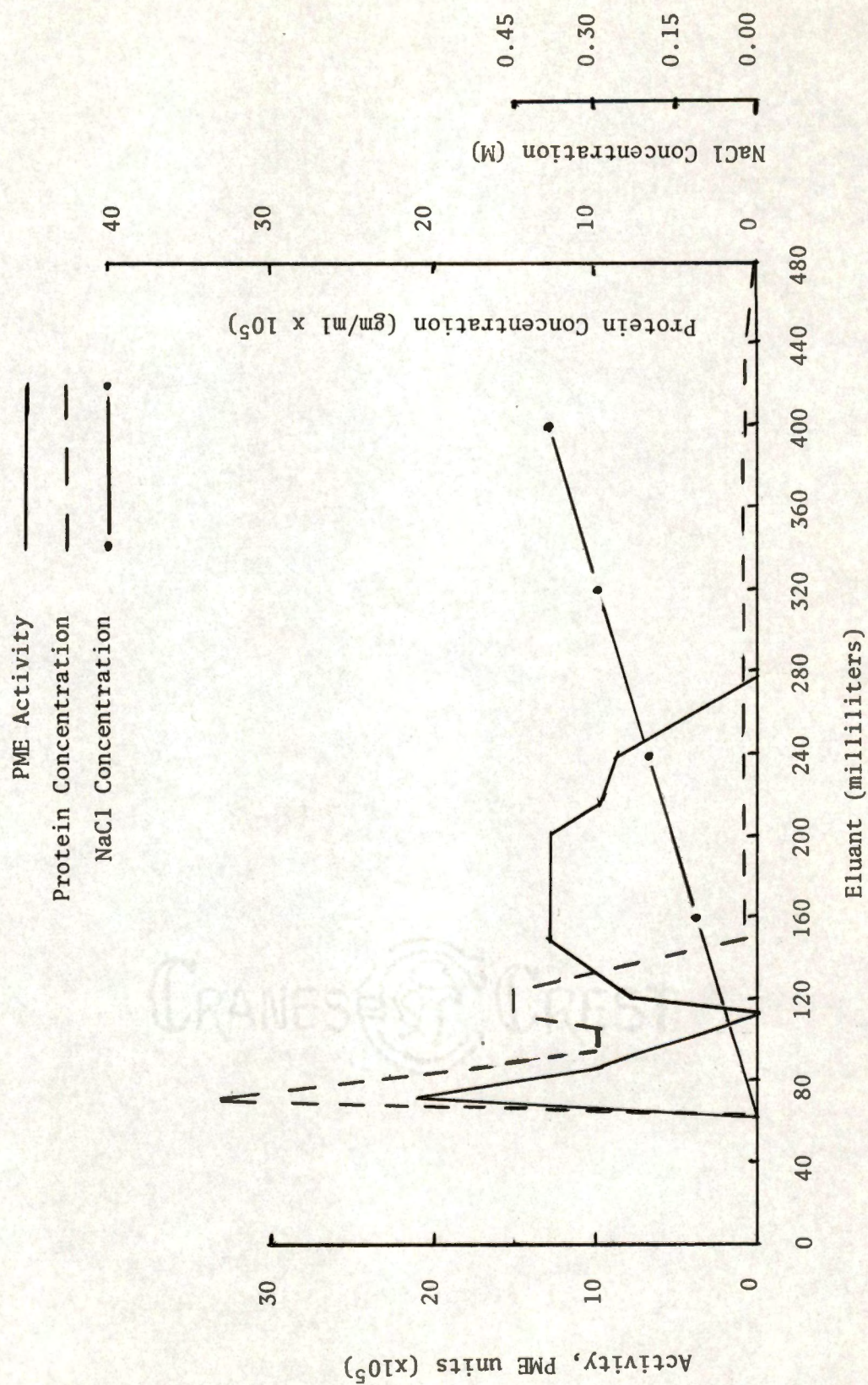


Figure 6. CM-cellulose column chromatography of the first pectin methyl esterase activity peak from the DEAE-cellulose column.

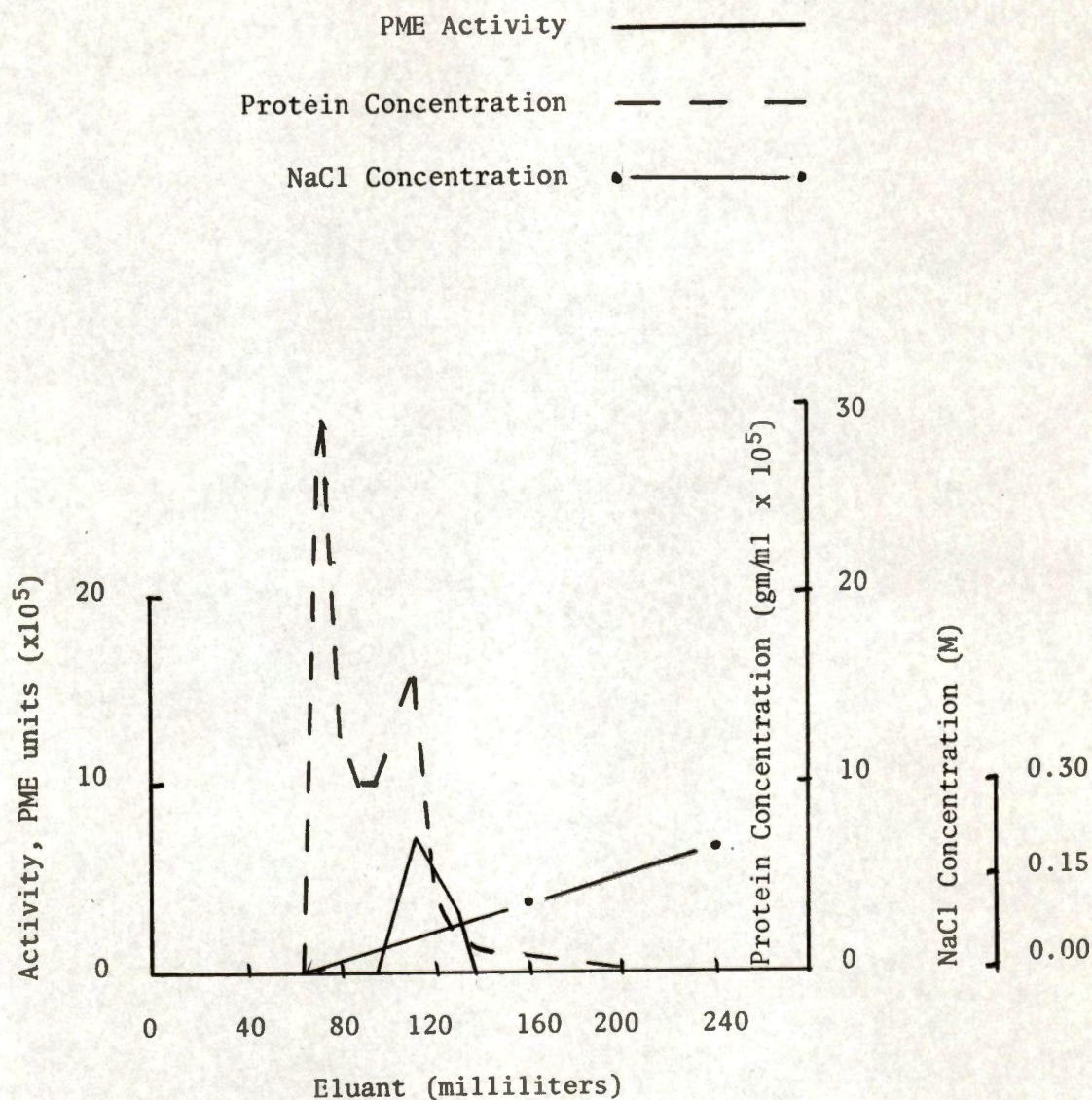


Figure 7. CM-cellulose column chromatography of the second pectin methyl esterase activity peak from the DEAE-cellulose column.

CHAPTER V

DISCUSSION

In the attempt to determine optimum salt concentration and pH for maximum PME activity, a 4 x 4 factorial experimental design was used. The two factors were salt concentration and pH and the levels of each were chosen in an attempt to include the levels for maximum PME activity. The factorial experimental design was developed to detect interaction, which diminishes the additivity of the main factors (53). PME activity was influenced by salt concentrations at the lowest pH level differently than by salt concentrations at the highest pH level.

Reporting the optimum salt concentration or the optimum pH for maximum soybean PME activity would not be appropriate, because one would report that maximum PME activity occurred at 0.16 M NaCl and pH 8.7. However, by observation of the three dimensional response surface of PME activity at pH 8.7, PME activity showed an increase as salt concentration was decreased, and consequently no activity peak was apparent. The optimum value obtained for a given salt concentration level and pH level could conceivably be obtained from a different set of levels due to the effect of interaction.

By using the polynomial chosen to describe the response surface of PME activity, the optimum salt concentration at pH 8.7 for maximum PME activity was calculated to be 0.06 M. An optimum salt concentration for maximum PME activity at a pH level included in the range

of this experiment can be calculated; or an optimum pH level for a salt concentration.

The choice of 0.23 M NaCl and pH 7.3 as conditions for the remaining PME studies was based on the observation that at those values, PME activity reached a peak that was relatively broad. Thus, a slight inadvertent variation of either salt concentration or pH would not effect experimental results appreciably.

Lee and Macmillan (28) proposed an explanation for the apparent optimum value of pH 8.7 for maximum PME activity. The apparent optimum may have been due to experimental technique and to the mode of action of PME. The pH of the reaction mixture was adjusted to the test pH before addition of enzyme. Above pH 7, random alkaline deesterification of pectin begins to occur and at even higher pH levels the effect becomes greater. The alkaline condition demethylates the pectin and produces free carboxyl groups on the pectin chain before and during enzymatic hydrolysis. It has been suggested that tomato PME may attack at loci next to free carboxyl groups and at the reducing ends of pectin chains. The newly created carboxyl groups could consequently act as initiation points for PME hydrolysis. The number of sites for PME hydrolysis would be increased, and higher PME activities would conceivably result.

Usually the effect of added salts is to lower the pH at which maximum PME activity is attained (26). This generalization applies to the response of soybean PME. When referring to the optimum salt concentration for PME activity, the fact that pectin N. F. contains

a certain amount of NaCl should be remembered. The acid (HCl) and alkali (NaOH) used for pH adjustment also contribute a certain amount of ions.

The reduction of PME activity after being heated in aqueous solutions at different temperatures for five minutes were as expected. Complete inactivation of activity after the enzyme was held at 80°C for five minutes has been reported for tobacco leaf PME (16).

The activation energy of soybean PME was calculated to be 7,570 calories per mole. Lee and Wiley (29) calculated the activation energy of apple PME to be 5,800 calories per mole.

The calculated K_m value, 0.111 percent pectin N. F., agrees closely with the value of 0.09 percent pectin N. F. obtained for tobacco leaf PME (16). The K_m value is useful in estimating the substrate concentration necessary to give maximum velocity. The K_m value was determined under one set of conditions. A different K_m value might be obtained at a pH other than 7.3 and NaCl concentration other than 0.25 M.

The ammonium sulfate saturation range between 0.55 and 0.75 was chosen in order to obtain sufficient PME for assay after partial purification. After dialysis against distilled water of the protein fraction which precipitated between 0.55 and 0.75 ammonium sulfate saturation, a large portion of the proteins precipitated. The precipitate was a thick, ropy, whitish material which contained no detectable PME activity. This material was soluble in a 0.20 M NaCl solution.

The results obtained from chromatography of the ammonium sulfate precipitate on DEAE- and CM-cellulose columns were predictable since similar studies had been conducted by Hultin et al. (18, 20) on bananas

and Lee and Macmillan (27) on tomatoes. The presence of two ⁵isoenzymes of soybean PME was definitely indicated and the possible presence of a third was indicated.

The second PME activity peak from the DEAE-cellulose column was of lower activity than the first PME activity peak from the DEAE-cellulose column, and the PME containing material lost much of the PME activity after being subjected to dialysis, lyophilization, and chromatography on the CM-cellulose column. Hultin et al. (20) reported that one PME fraction from banana pulp was susceptible to inactivation by freezing.

PME activity was present through the first 280 milliliters of eluant from the CM-cellulose column. Hultin et al. (20) found activity through the first 200 milliliters of eluant when working with banana pulp PME.

Use of DEAE-cellulose column chromatography was an acceptable means of improving purity of soybean PME. Both PME activity peaks were eluted before the majority of the extraneous protein was eluted. A column packed to a height greater than 20 centimeters may cause greater resolution between the two activity peaks.

CM-cellulose column chromatography was not as desirable for purification of soybean PME as DEAE-cellulose column chromatography. However, CM-cellulose column chromatography may be used satisfactorily by changing the buffer system or the pH of the buffer.

The Waddell method (57) of protein determination was selected because at 215 and 225 nm absorbance is due to the peptide bonds, thus absorbance is almost independent of the protein amino acid

composition. By utilizing the wavelengths of 215 and 225 nm sensitivity is improved over that at 280 nm. However extra care must be used when working at such low wavelengths.

Relatively low values of PME activity were consistently obtained throughout the experiment. Although the soybeans were green when harvested, PME activity may have begun to decline. Chen (5) found that soybean PME activity decreased as maturity increased.

This work could be used as the basis for further purification and study of soybean PME with a minimum of preliminary work. By using the progressive purification procedures previously outlined, partially purified PME could be obtained and further purified on a DEAE-cellulose column. The fractions containing PME activity could be subjected again to chromatography on the DEAE-cellulose column. Those fractions containing PME activity could be dialyzed and lyophilized. Crystallization would be of value as a final stage of purification to remove minor impurities (22). PME at this stage of purity could be subjected to molecular weight and isoelectric point determinations. The enzyme could be immobilized to study local environment effects on PME activity.

CHAPTER VI

SUMMARY

This study was conducted to partially purify PME of vegetable soybeans, to determine optimum salt concentration and pH for maximum PME activity, to ascertain heat stability and energy of activation of PME, and to determine the Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) of PME of soybeans.

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

1. PME activity was influenced by interaction between salt concentration and pH and no optimum levels were chosen.
2. Partially purified PME in aqueous solution was completely inactivated after a five minute treatment at 80°C.
3. Energy of activation was 7,570 calories per mole.
4. K_m was 0.111 percent pectin and V_{max} was 20.60 PME units.
5. Based on the behavior of partially purified PME on DEAE-cellulose and CM-cellulose column chromatography at least two isoenzymes of soybean PME were observed.
6. DEAE-cellulose column chromatography was an acceptable purification procedure for soybean PME. CM-cellulose was unsatisfactory.



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VITA

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