May 10, 1969

To the Graduate Council:

I am submitting herewith a thesis written by Peter Hung Chen entitled "Properties and Partial Purification of Pectin Methyl Esterase in Vegetable-Type 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
Graduate Studies and Research

PROPERTIES AND PARTIAL PURIFICATION OF PECTIN METHYL ESTERASE IN VEGETABLE-TYPE 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

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by

Peter Hung Chen

June 1969

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ABSTRACT

The purpose of this work was to study the effect of sodium chloride, pH, temperature and maturity on soybean pectin methyl esterase activity, to determine enzyme kinetic constants and to describe the inhibition of PME by tannic acid. The enzyme was partially purified.

The source of PME was a slurry of the soybeans which were raised on the University of Tennessee Plant Science Farm at Knoxville. Beckman Autotitrator was used for enzyme assay. The data reflecting effects of salt concentration, pH, temperature and maturity on PME activity were analyzed by the analysis of variance. The Duncan's Multiple Range Test was employed to determine significant differences among means. In the study of enzyme kinetics, the Lineweaver-Burk double reciprocal form of the Michaelis-Menten was utilized. A FORTRAN computer program was written to determine K_m, V_{max}, regression coefficient, b, and the correlation coefficient, r, on the IBM 7040 computer.

Progressive purification steps for this enzyme were made by acetone-dried powders, salt extraction, ammonium sulfate precipitation, dialysis, and DEAE-cellulose ion-exchange column chromatography.

Maximum activity for the PME occurred between 0.25 and 0.40 M concentrations of NaCl, between pH 7.5 and 8.5, and at 50°C. The mean Q_{10} value was 1.23. The more mature the soybeans were, the lower the total PME activity. The K_m value for soybean PME of the first harvest was 0.052 percent pectin and for the third, 0.057 percent pectin. The amount of inhibition of PME by tannic acid was related to inhibitor and substrate concentrations.

An increase in PME specific activity of about 140-fold was obtained by the purification methods. There were at least two isoenzymes of the soybean PME. This hypothesis is based on the elution behavior of the enzyme on the DEAE-cellulose ion-exchange column.

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CHAPTER I

INTRODUCTION

Pectin methyl esterase (PME, 3.1.1.11) occurs in plants, molds, and bacteria, and is capable of catalyzing the demethylation of methylesters of polygalacturonic acid polymers of the pectic substances. In general, alfalfa, tomatoes, citrus fruits, tobacco, potato leaves, and fungi are good sources of PME. This enzyme has been found also in garlic, onions, radishes, grapes, apples, pears, peas, turnips, and other vegetables and fruits.

Since pectic substances play an important role in influencing the character and structure of plant tissue, e.g. cloudiness in orange juice and the texture of tomato products, PME has been of interest to the food scientist. The action of the enzyme results in a decrease of solubility of the pectic substances, particularly in the presence of salts, and an increased firmness and acidity of tissue. Usually, the enzyme is strongly bound to the plant cellular materials and must be treated with salt at an alkaline pH in order to be extracted. The optimum pH, one of important characteristics, is different among the various sources, e.g. for bacteria an alkaline pH is needed (7.5-8.0); for commercially produced fungi, pH 5.0; for citrus fruits and tomatoes, near pH 7.5. For PME of most higher plants temperature optimum is between 45° and 55°C, and the temperature coefficient (Q_{10}) is about 1.4.

Generally, 0.2 M sodium chloride is considered to promote maximum activity of PME. In addition, maturity and other chemical and physical changes are factors to consider when investigating PME activity.

Foods derived from the soybean have been an important part of the diets of Oriental people for thousands of years. Although soybeans, a high protein legume, have been studied extensively, literature was not available concerning PME in soybeans. Therefore, further research is needed.

The purpose of this work was to study the effect of sodium chloride, pH, temperature, and maturity on soybean PME activity, to determine enzyme kinetic constants (K_m and V_{max}) and to describe the inhibition of PME by tannic acid. Partial purification of the enzyme was attempted.

CHAPTER II

REVIEW OF LITERATURE

I. SOURCES OF PECTIN METHYL ESTERASE AND MODE OF ACTION

Pectin methyl esterase hydrolyzes the methyl groups of the methylated carboxyls of the galacturonic residues of pectin (49). The natural substrates are inherent pectic substances in plant tissues.

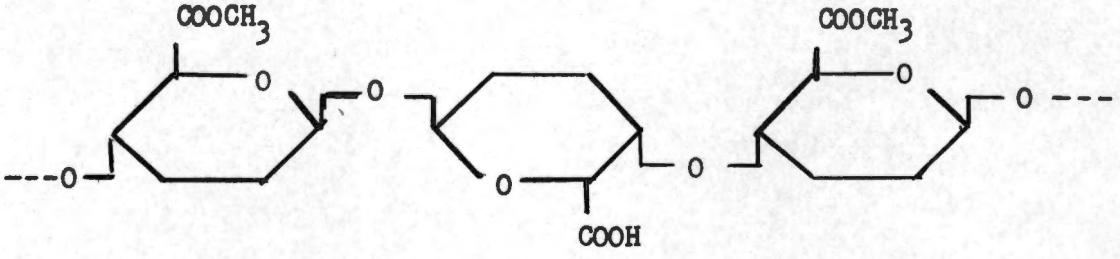
$$\text{Pectin-CH}_3 + \text{HOH} \longrightarrow \text{CH}_3\text{OH} + \text{Pectin}^- + \text{H}^+$$

The PME is highly specific (30). There are two practical methods for measuring PME activity. First, the CH_3OH, one of the end products, can be measured. This method is difficult and time consuming. Secondly, the increase in free carboxyl groups may be followed titrametrically at a constant pH. This procedure is easily conducted, and is used by most investigators.

PME is widely distributed in plants, molds, and bacteria. Sources that contain considerable quantities of PME and that have received attention recently included alfalfa (30), carrots (12, 31), cucumbers (4), oranges (15, 27, 32, 33, 45), pineapples (17), tomatoes (11, 19, 20, 24, 34, 35, 41), beans (47, 51), papaya (1, 7), tobacco leaves (21, 22, 23), bananas (25, 26), Southern peas (8), turnips (9), and fungi (6) and bacteria (36).

II. SUBSTRATE FOR PECTIN METHYL ESTERASE

Pectin, the substrate of PME, is a polymer of D-galacturonic units joined by α-1,4 glycosidic linkages between the pyranose rings. In natural pectins approximately two-thirds of carboxylic acid groups are esterified with methanol (44). The structure of this polymer is shown diagrammatically below (2).



At one time, pectins were thought to contain several carbohydrates, such as arabans and galactans, as impurities. Now it is believed that L-rhamnose, L-arabinose, D-galactose, and traces of other sugars are presented as integral parts of the pectin molecule (44, 53). A commercial pectin made from citrus fruit, Pectin N. F., which has 65-70 percent of its galacturonic acid units esterfied serves as substrate in PME assay studies.

A pretreatment with polygalacturonases (PG), which hydrolyze the α-1,4 glycosidic linkages of pectic substances, decreases the rate of desterification by PME (12).

III. CHARACTERISTICS OF PECTIN METHYL ESTERASE

PME was first discovered in carrots and the name "pectase" was coined by Fremy about a century ago (12, 31). Since Fremy's discovery, several other names have been used, such as pectin demethoxylase, pectin methoxylase, pectolipase, pectinesterase and pectin methyl esterase; the latter two names seem more suitable for the enzyme that deesterifies pectin. Research to determine the properties of this enzyme in several other plant tissues has been conducted.

Lineweaver and Ballou (30) reported on the effect of cations on the activity of alfalfa PME. They illustrated the influence of eleven salts which included monovalent and divalent cations on the activity of a dialyzed aqueous solution of alfalfa PME. In general, divalent cations were much more effective than monovalent cations.

The possibilities of PME playing a role in the softening of cucumber salt-stock for pickles which occurred during the manufacture was studied (4). The PME activity in both the extracted cucumber juice and in the brine was low compared to that observed in the non-brined, green cucumbers. The low activity was attributed chiefly to enzyme inactivation caused by lactic acid produced by fermentation.

MacDonnell et al. (32) studied the properties of orange PME. The observed increase in activity caused by the addition of certain cations was dependent on the pH of the reaction mixture and varied from 5-fold at pH 7.5 to more than 100-fold at pH 5. The pH optimum in 0.15 M NaCl was 7.5 and as the concentration of salt was decreased,

the optimum shifted toward more alkaline pH values. Salt not only affected the activity, but it also affected the extraction, adsorption, elution and solubility of the enzyme. The orange PME was highly specific and it hydrolyzed pectin at least 1000 times faster than any of 50 nongalacturonide other esters tested (33). Edwards and Joslyn (15) described inhibition and activation of orange PME. Silver nitrate decreased PME activity but ascorbic acid and sodium bisulfite increased activity. Potassium cyanide, cysteine hydrochloride, mercuric chloride, sodium sulfide and hydrogen peroxide did not affect activity. Guyer and Miller (17) studied the effect of heat treatment on PME and cloud stability of frozen concentrate made from pineapple and orange. Usually, orange PME activity from peel, membrane, and juice sacs was least when the Brix/acid ratio was relatively low, and higher when the proportion of sugar was higher (43).

Hills and Mottern (20) reported that the deesterification of pectin by tomato PME followed a zero order for the initial 40 percent rather than a first order reaction. This fact was utilized to determine rates of reaction and to choose units for expressing activity. Procedures have also been described for preparing highly concentrated solutions of the enzyme from tomatoes (35). These concentrates may be dried *in vacuo* over phosphorous pentoxide at room temperature without significant loss of activity. McColloch *et al.* (34) found that the loss of pectic substances in processed tomato products was the result of the action of PME and pectic acid depolymerase. Consequently, the colloidal properties of the tomato product were lost and poor consistency resulted.

The role of PME, pectic constituents and cations in the firmness of canned tomatoes has been studied (11, 20). Results showed that firmness was related to retention of the original pectic content and controlled demethylation. This induced demethylation and increased the extent of ionic binding which resulted in firmer fruits. Hall (19) described the inhibition of tomato PME by tannic acid. The degree of inhibition was dependent on the concentration of both the inhibitor and the pectic substrate.

Reports of Sistrunk and Cain (47) and Van Buren et al. (51) showed that the firmness of canned green beans was greatly influenced by blanching conditions. Differences in firmness were related to the pectic substance present: the firmer beans had more pectic acid than the softer beans. Conditions under which firming occurred indicated that PME was involved since changes in firmness were stopped when beans were heated to relatively high temperatures. Van Buren et al. (51) described some of the properties of the enzyme, its survival in frozen beans, and the effects of temperature and pH on its activity and denaturation. Freezing and frozen storage did not destroy PME activity significantly in plant tissue. More than 90 percent of PME activity still existed in snap beans after one year of frozen storage.

Heat sensitivity of PME activity in papaya puree has been observed (1). The reduction in the rate of activity in the acidified puree was determined in the range 169-186°F, giving a "z" value of 11°F from the thermal inactivation curve and a D_{180} = 10 minutes. Change et al. (7) described the inhibition of papaya PME by sucrose,

glucose, maltose, and corn syrups. The inhibition effect of sucrose was linear throughout the range investigated (up to the 50 percent sucrose).

The occurrence of multiple molecular forms of PME was discovered by Hultin and Levin (25). Evidence that the banana pulp contained at least three forms of PME was based on differential extraction procedures, pH dependence studies, activation or inactivation by sodium dodecyl sulfate, and differential temperature inactivation. Purification and properties of PME in the banana pulp were studied with these three molecular forms (26).

Holden (21) described the acid-producing mechanisms in minced tobacco leaves. The drift of pH towards the acid side observed when minced tobacco leaves were neutralized with alkali, was due namely to the enzymatic demethylation of pectin when the pH was raised. Holden and Tracey (23) reported that the fertilizer treatment of the tobacco plant affected the quantities of PME of the plant. They indicated that nitrogen supplementation increased the PME content of the plant while phosphorous supplementation decreased it. Holden (22) showed that the sap of tobacco leaves contained only a small proportion of the total PME present, most of PME was associated with the fiber. The liberation of methanol was more rapid in milled than in minced leaf fiber of tobacco plant (21).

Collins (8) investigated the effect of NaCl concentration, pH, temperature, maturity, frozen storage and rinsing of the peas on PME activity <u>in vitro</u> in Southern peas. The more immature the peas were,

the higher the PME activity. Rinsing removed a significant amount of PME from the peas. Peas subjected to frozen storage had a higher apparent activity than the fresh peas with increased activity more pronounced in the more mature peas.

Collins (9) determined the effect of NaCl, pH, temperature and location within the root on PME activity in turnip roots. The peel exhibited the highest PME activity and the lower part of the root had a higher activity than the upper parts. Enzyme kinetic values of PME (K_m and V_{max}) were determined also.

The yield of PME in various plants might vary with maturity as reported for tomatoes (42), apples, pears and cherries (44), Southern peas (8) and turnip roots (9), or it might remain constant as reported for tobacco leaves (23). McCready and McComb (36) studied the pectic substituents in the ripe and unripe fruits such as pears, peaches and avocados. These results suggested that pectic enzymes come in contact with the pectin during ripening and hydrolyze them to smaller molecular materials. Then, the degraded materials were less effective in maintaining firm structures in fruits and less important in contributing to the consistency of processed foods.

Mills (37) described investigations of an extracellular PME secreted by Pseudomonas prunicola, an organism causing a disease in cherry and pear trees. They suggested that destruction of the pectic substances of the middle lamella of the host cells by the bacteria may play a primary role in facilitating the invasion of the tissue. Calesnick et al. (6) reported a commercial preparation of fungal PME was more sensitive

to calcium than to sodium ions, and that deesterification of pectin was apparently of the zero-order for the first 40 percent of hydrolysis.

IV. ENZYME KINETICS OF PECTIN METHYL ESTERASE

Lineweaver and Ballou (30) studied the enzyme kinetics of alfalfa PME. The value of V_{max}, maximum velocity, at the highest salt level (0.20 M) was about 3.5 times greater than at the lowest level (0.025 M) but the value of K_m, Michaelis-Menten constant, which was 0.04 percent of pectin, was about the same, and did not change when the concentration of salt was varied.

MacDonnell *et al.* (32) found that the relative enzyme activity for orange PME increased as the pectin concentration was raised. The corresponding apparent K_m for this enzyme was about 0.08 percent pectin.

Hills and Mottern (20) made a subsequent study which showed that the tomato PME activity was dependent on pectin concentration. The value of K_m was found to be 0.04 percent pectin.

Holden (22) found that the K_m for PME of tobacco leaves was 0.09 percent pectin. This value was found by the definition of K_m, the percentage of pectin concentration which gave half the maximum velocity of hydrolysis.

Collins (9) studied the enzyme kinetics of PME in turnip roots. The value of K_m was 0.155 percent pectin.

Inhibition of PME enzyme by some chemical compounds has been studied. Detergents (25), sucrose (7) and tannic acid (19) were reported to inhibit PME more effectively while cyanide and iodine had

little inhibitory effect. Other phenolics compounds such as gallic acid, chlorogenic acid, caffeic acid and ferulic acid, were tested for PME inhibition, but none was as effective as tannic acid.

V. PURIFICATION OF PECTIN METHYL ESTERASE

PME has been prepared from the juice and from water extracts of such plant tissue as tomatoes (35, 42), oranges (33) and bananas (26).

MacDonnell et al. (33) used a combination of ammonium sulfate fractionation, adsorption and dialysis to prepare orange PME. They found that the precipitation between 0.4 and 0.8 ammonium sulfate saturation had the most PME activity. The enzyme was eluted further with a solution containing 1 M of NaCl and 0.025 M Na_2HPO_4 on a column of diatomaceous earth (Celite) and on an artificial zeolite (Ducil). The PME specific activity was increased more than 100-fold by this method.

McColloch et al. (35) used freeze-drying to concentrate the original extract of tomato PME and repeated dialysis and extraction. Pithawala et al. (42) showed that the PME enzyme was best extracted from tomato pulp with 0.1 M Na_2HPO_4 at pH 8. They also found that ethanol precipitation of the enzyme from phosphate extracts resulted in practically complete loss of activity. However, most of the enzyme was precipitated between 0.3 and 0.5 saturation of ammonium sulfate, a 300-fold increase in concentration of the enzyme was obtained.

The most recent report describing the purification of PME was presented by Hultin et al. (25, 26). The precedures included extraction

by ammonium sulfate precipitation, and ion-exchange chromatography on columns of DEAE- and CM-cellulose for the preparation of PME from banana pulp. They found at least three molecular forms of banana PME, and each had a unique behavior on the ion exchange columns. Also they indicated that the greatest part of the PME activity was retained on the DEAE-cellulose column (anion exchange). A 38-fold increase in specific activity could be obtained under definite procedures.

CHAPTER III

MATERIALS AND METHODS

I. MATERIALS UTILIZED

Source and Handling of Soybeans

The soybeans were raised on the University of Tennessee Plant Science Farm at Knoxville. The variety name of the soybeans could not be ascertained; however, they are commonly referred to as "Soy-lima." The immature beans have an intense green color and resemble baby lima beans in shape.

Fourteen rows (300 feet x 38 inches) were planted on May 21. Prior to planting, 400 pounds of 5-10-10 fertilizer per acre were applied. Weeds were controlled by cultivation.

Pods with the immature, green beans were harvested on three dates: September 23, 26 and 30. A fourth harvest was made on October 15, when the beans had almost matured. They were harvested with the Chisholm-Ryder green bean harvester (16).

The pods were shelled with a Dixie Pea Huller, washed and frozen over-night in an air-blast freezer (-25°C). The frozen beans were packaged in polyethylene bags and moved to and held in a still air freezer (-18°C).

The moisture content of soybeans was determined by freeze-drying samples of the frozen beans from each stage of maturity.

Sample Preparation for Pectin Methyl Esterase Assay

The source of PME was a slurry of the soybeans. The slurry was prepared by blending the thawed soybeans with distilled water (1:2, w/v) for 3 minutes in a Waring Blendor. A mixture of 30 grams of mascerated soybeans, 50 milliliters of 2 percent Pectin N. F. (Sunkist Growers, Inc., Corona, Calif.), 70 milliliters of distilled water and a sufficient amount of NaCl to bring the concentration to the desired level was prepared for PME assay.

II. METHODOLOGY AND INSTRUMENTATION

Beckman Autotitrator Used for Enzyme Assay

PME activity was determined by measuring the amount of standard 0.1000 N NaOH required to maintain the enzyme assay mixture at a given pH for 5 minutes. This procedure was accomplished by the use of a Beckman Autotitrator. Activity of PME was expressed as PME units which is defined as that amount of activity which produced one meq of acid per minute per gram of soybean tissue (dry weight). The following procedures were used for enzyme assay:

1. The test mixture was placed in a 250 milliliters beaker and heated to 30°C in water bath, except where temperature was the study variable.

2. The pH of the mixture was adjusted to pH 8 with 1 N NaOH and 0.1 N HCl, except where pH was the study variable.

3. Titration was carried out for 5 minutes while the slurry was stirred by a magnetic stirrer. Temperature and pH were maintained throughout the titration period.

$$\text{PME units} = \frac{\text{NaOH (milliliters) x normality of NaOH}}{\text{weight of sample (grams) x time (minutes)}}$$

Six experiments were conducted in order to study the properties of soybean PME. Conditions for each experiment were reported. Experiment one: Effect of salt concentration on the activity of soybean PME.

The PME activity was measured at pH 7.5, 30°C and the concentration of NaCl in the test mixture was varied from 0.0 to 0.5 M with increments of 0.05 M. Two observations for each of two replications of 11 treatments were made.

Experiment two: Effect of pH on the activity of soybean PME.

The PME activity was measured at 30°C, 0.35 M (optimum salt concentration as previously determined) and the pH in the test mixture was varied from pH 5.5 to 9.5 with intervals of 0.5 pH units. Two observations for each of two replications were made for each of 9 treatments. At pH values greater than 9, alkali demethylation of the pectin occurred. A correction was made to determine the demethylation effect on the pectin without added enzyme.

Experiment three: Effect of temperature on the activity of soybean PME.

The PME activity was measured at 0.35 M NaCl and pH 8.0 (optimum), starting at 25°C. The temperature was varied with 5°C increments until a sharp inactivation occurred. Two observations for each replication and two replications for each of 8 treatments were made.

Experiment four: Effect of maturity on the activity of soybean PME.

The investigation of the effect of maturity was conducted by utilizing frozen soybeans of the 4 stages of maturity. The PME activity was measured at 0.35 M NaCl, pH 8.0 and 30°C. Two observations for each of two replications were made for each stage of maturity.

Experiment five: Determination of PME enzyme kinetic constants.

The PME activity was measured at 0.35 M NaCl, pH 8.0 and 30°C with various pectin concentrations from 0.03 to 0.20 percent. Soybeans from the first and third stages of maturity were used to ascertain the K_m value. Three observations for each level of substrate were made.

Experiment six: Inhibition of soybeans PME by tannic acid.

The PME activity was measured at the optimum conditions (0.35 M NaCl, and pH 8.0) and 30°C and 0.2 percent of pectin with various tannic acid concentrations ranging from 0.01 to 1 percent. Also, the PME activity was measured with increasing concentrations of substrate from 0.05 to 0.5 percent while the tannic acid was held at 0.05 percent. Inhibition (percent) = $\left(1 - \frac{\text{PME activity with inhibitor}}{\text{PME activity without inhibitor}}\right) \times 100$ percent. Three observations for each level of inhibitor or substrate were made.

Statistical Analyses

The data reflecting effects of salt concentration, pH, temperature and maturity on PME activity were analyzed by the analysis of variance, using the ANOVAR program (5) adapted for the IBM 7040 computer at the University Computing Center. Duncan's Multiple Range Test was used to evaluate differences among mean values (48).

In the study of enzyme kinetics, the Lineweaver-Burk double reciprocal form of the Michaelis-Menten was utilized. A FORTRAN computer program was written to determine K_m, V_{max}, regression coefficient, b, and the correlation coefficient, r, on the IBM 7040 computer (3).

Acetone Dried Powder for Enzyme Extraction

Nason (38) suggested that acetone-dried powder could be used as the starting material for extracting soluble proteins which would contain maximum enzyme activity from plant cells. The powders are very stable and can be stored for long periods of time with little loss in activity (10). Furthermore, acetone powders have the advantage of being chlorophyll-free and tend to have a minimum of gum- and resin-like materials which might interfere in subsequent fractionation.

In higher plants the PME is usually strongly adsorbed on the water-insoluble materials of the tissue. However, the enzyme can be eluted with salt solutions. MacDonnell et al. (32) reported that PME from orange could be extracted in 0.25 M NaCl at pH 7-8 for one hour with alkali addition as required to maintain the pH in this region. Hultin and Levine (25) extracted PME from banana pulp with water, with 0.15 M NaCl and with 0.15 M NaCl at pH 7.7. McColloch et al. (35) also extracted PME from drained and washed tomato pulp with 10 percent NaCl.

The following procedure was utilized to extract PME enzyme from immature soybeans (38):

1. Cover 100 grams of soybeans with at least 5 volumes of chilled acetone and blend vigorously for 3 minutes.

2. Filter the resulting slurry through a Buchner funnel, and wash with an excess of chilled acetone. A vacuum pump may be used to facilitate filtration.

3. Spread the washed residue onto a porcelain dish and allow to dry. The acetone-dried powders were stored in a desiccator at 4°C.

4. Dissolve 10 grams of the powder in 90 milliliters of each of four different solutions, viz. distilled water, 2 percent NaCl, 2 percent NaCl at pH 8.0 and 4 percent NaCl at pH 8.0.

5. Stir with a magnetic stirrer for an hour and adjust the pH with 0.1 N NaOH if needed.

6. Centrifuge at 500×G for 20 minutes and discard the precipitates.

7. Measure the enzyme activity and protein concentration, and select the sample with the highest activity for fractional precipitation by ammonium sulfate.

Ammonium Sulfate Precipitation

Ammonium sulfate precipitation is a very widely used method for preparing purified enzymes due to the large solubility of the salt in water and to the absence of harmful effects on most enzymes. A relatively high percent of total enzyme recovery in all ammonium sulfate fractions is expected, but the separations are not always very sharp. A high quality salt should be used in order to avoid toxic impurities. In addition, time is an important factor in the precipitation of proteins and a period of at least 15 minutes should elapse before centrifugation.

In general, preliminary experiments are needed to determine the salt saturation range in which most of the enzyme of interest is precipitated. In this study the saturation range between 0.55 and 0.70 was used. A nomogram giving the amount of ammonium sulfate required to obtain the degree of saturation was utilized (13). Two hundred milliliters of solution prepared from enzyme extraction procedure mentioned above were considered as the PME containing solution for ammonium sulfate precipitation. The initial level of salt was brought to 0.4 saturation (preliminary experiments) and from this level it was raised to 0.9 saturation in increments of 0.1 units. Each precipitate was dissolved in 40 milliliters distilled water and was assayed for PME activity. It took 30 minutes to precipitate the proteins with the assistance of a magnetic stirrer and 30 minutes for centrifuging. All procedures were performed at 40-45°F.

Dialysis

The principal function of dialysis is to remove salts and other low molecular weight substances from proteins (46). Also, dialysis may contribute to purification by causing the precipitation of undesirable proteins that are insoluble at low salt concentrations.

The protein fraction precipitated between 0.55 and 0.70 saturation was dissolved in distilled water and dialyzed overnight at 40-45°F against distilled water to remove the ammonium sulfate. Cellulose dialysis tubing was used. The dialyzed sample was then placed on a column of diethylaminoethyl (DEAE-) cellulose ion-exchange for fractionation.

Fractional Adsorption with DEAE-Cellulose Column

DEAE-cellulose, purchased from Bio-Rad Laboratories, Richmond, Calif., was prepared as follows (41), 20 grams of DEAE-cellulose was suspended in one liter of 0.1 N NaOH and stirred for 10 minutes. The DEAE-cellulose was then collected on a Buchner funnel and washed with distilled water until all of the excess alkali was removed. Then, the DEAE-cellulose was treated with one liter of 0.1 N HCl with the same process. After the excess acid was washed out of the DEAE-cellulose with distilled water, the cellulose was then held in 0.02 M sodium phosphate buffer at pH 6.8. This buffer was used also as the elution buffer.

The column (2.5 centimeters x 30 centimeters) was packed with treated DEAE-cellulose and eluted with buffer solution overnight for equilibration. Ten milliliters of enzyme preparation from ammonium sulfate fraction were added. A continual increase in the gradient of NaCl with a limiting concentration of 1 M was utilized. The eluant was collected in 6 milliliters fractions by use of an automatic fraction collector (39). All procedures were performed at room temperature (25°C).

A summary of the procedures used for PME enzyme purification is presented in Figure 1.

Assays for PME activity and protein determination for each aliquot collected from DEAE-cellulose ion-exchange column were carried out by use of a bromthymol blue test and a spectrophotometric method, respectively.

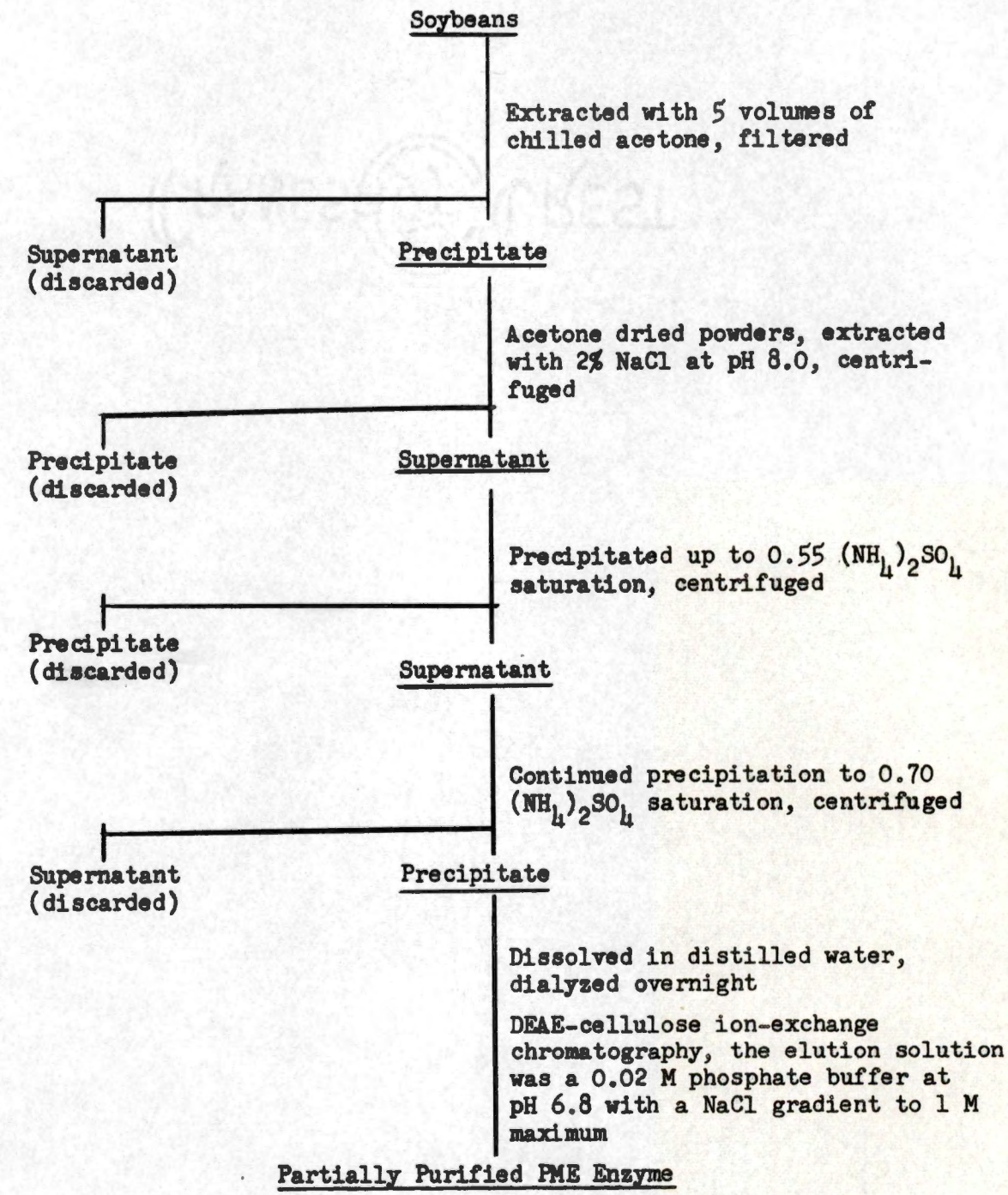


Figure 1. Summary of procedures for PME enzyme partial purification.

Bromthymol Blue Indicator for Enzyme Assay

The Beckman Autotitrator could not be utilized effectively to measure the activity of PME in a relatively small sample. Therefore, another method was adopted which involved a color change of an indicator due to the PME-produced acid. Bromthymol blue is a dye that changes color in the range of pH 6.0-7.6. The blue color disappeared and changed to brown when sufficient acid was produced by PME enzyme action on pectin. A set of Lamotte Standards, Lamotte Chemical Products Co., Baltimore, Maryland, was used to make comparative studies of color change for this enzyme assay.

To prepare the bromthymol blue indicator solution, 100 milligrams of bromthymol blue were mixed with 3.2 milliliters of 1/20 N sodium hydroxide in an agate mortar. After complete dissolution, sufficient distilled water was added to make the volume 100 milliliters (0.1 percent).

This method can be used for a quantitative assay with small amounts of enzyme preparations. The assay was accomplished by adjusting the mixture of one milliliters of sample, 2 milliliters of 1 percent pectin and 2 drops of bromthymol blue to pH 7.6 with 0.1 N NaOH and allowing it to stand for a designed period which was dependent upon the relative enzyme activity.

Spectrophotometric Method for Measuring Proteins

Most proteins exhibit a distinct ultraviolet light absorption maximum at 280 nm, due primarily to the presence of tyrosine and tryptophan (29, 52). Since the tyrosine and tryptophan content of

various enzymes varies only within reasonable narrow limits, the absorption peak at 280 nm has been used as a rapid and a fairly sensitive measure of protein concentration. Unfortunately, nucleic acids, nitrogen-containing compounds, have a strong ultraviolet absorption at 280 nm. However, nucleic acids absorb much more strongly at 260 nm than at 280 nm, and the interference of nucleic acids can be eliminated by calculations involving the following equation suggested by Lowry (29):

$$\text{Protein Concentration (milligram/milliliter)} = 1.45\ D_{280} - 0.74\ D_{260}$$

where D_{280} and D_{260} are absorbance values at 280 nm and 260 nm, respectively. The Perkin-Elmer 202 Spectrophotometer was used to automatically record the absorption spectrum of each protein fraction.

CHAPTER IV

RESULTS AND DISCUSSION

I. EFFECT OF DIFFERENT SALT CONCENTRATIONS ON PECTIN METHYL ESTERASE ACTIVITY

The analysis of variance of the effect of different concentrations of NaCl on PME activity is presented in Table I. The effect of salt was significant at the 0.01 level of probability.

Changes in PME activity with different concentrations of NaCl are shown in Figure 2. Addition of NaCl was not essential for PME activity. However, when salt was added and the concentration was raised to 0.35 M, the activity increased. Enzyme activity was reduced when the concentration was raised above 0.35 M. There was no significant difference among mean PME activity values at 0.25, 0.30, 0.35, 0.40 M concentration of NaCl at the 0.05 level of probability as determined by the Duncan's Multiple Range Test. Since the peak of the activity occurred at 0.35 M NaCl, this value was selected as the working level of NaCl concentration for other experiments. The optimum salt concentration (0.35 M) for soybean PME is somewhat higher than that for maximum PME activity of other plant tissues, viz. 0.20 M for snap beans (51), papaya (7), and alfalfa (30); 0.25 M for orange (32) and Southern peas (8).

Enzyme activity at 0.35 M NaCl was increased 2-fold over the control (0.0 M NaCl). This relative increase was lower than that of

TABLE I

ANALYSES OF VARIANCE FOR EFFECT OF SALT CONCENTRATION, pH, TEMPERATURE AND MATURITY ON THE ACTIVITY OF SOYBEAN PECTIN METHYL ESTERASE

Source	D.F.	S.S.	M.S.	F-ratio
Salt Concentrations	10	402.944	40.294	110.925**
Replications	1	0.309	0.309	0.852
Residual Error	32	11.624	0.363	
Total	43	414.878		
pH	8	787.516	98.440	1172.624**
Replications	1	0.030	0.030	0.360
Residual Error	26	2.188	0.084	
Total	35	789.729		
Temperature	7	1201.788	171.688	441.147**
Replications	1	1.314	1.314	3.38
Residual Error	23	8.951	0.389	
Total	31	1212.051		
Maturity	3	573.196	191.065	2265.849**
Replications	1	0.078	0.078	0.933
Residual Error	11	0.927	0.084	
Total	15	574.202		

**Significant at the 0.01 level of probability.

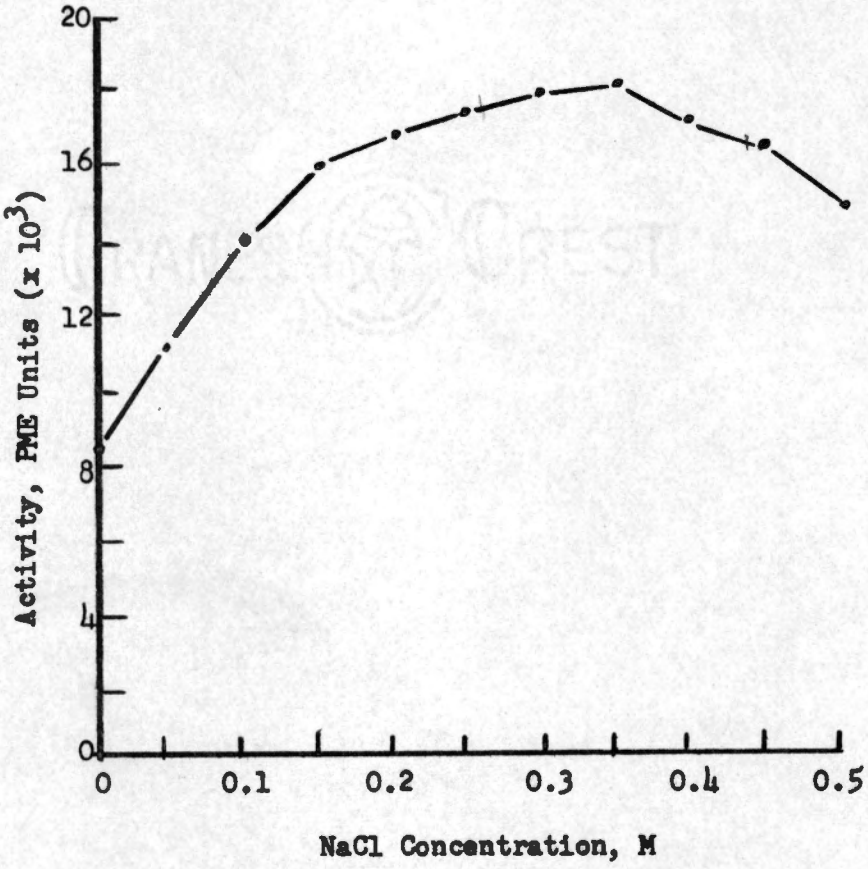


Figure 2. The effect of NaCl concentration on the activity of soybean PME at pH 7.5 and 30°C.

other sources, such as Southern peas, 4-fold (8); orange and snap beans, 5-fold (31, 51); and alfalfa, 30-fold (30).

II. EFFECT OF pH ON PECTIN METHYL ESTERASE ACTIVITY

The effect of pH on PME activity was significant at the 0.01 level of probability. The analysis of variance is presented in Table I.

Since enzymes are protein in nature, pH changes will affect primarily the ionic character of the free amino and carboxylic acid groups of the protein molecule (18). Consequently, the catalytic nature of an enzyme will be altered. Therefore, the effect of pH on soybean PME activity was studied by varying the pH from 5.5 to 9.5. PME activity means at different pH levels are shown in Figure 3. There was a steady increase in activity when the pH was raised from 5.5 to 8.0. With further increases in the pH, activity decreased. The activity at the optimum pH (8.0) was slightly less than 3-fold over the activity at pH 5.5.

The optimum pH (8.0) for soybean PME is very near or the same as that of other plant tissues, such as pH 7.5 for papaya (7), tomato (35), orange (32), and turnip (9); pH 8.0 for snap beans (51), Southern peas (8) and tobacco leaves (21). However, in the study of soybean PME, there was no significant difference among mean values at pH 7.5, 8.0, and 8.5 at the 0.05 level of probability as determined by the Duncan's Multiple Range Test.

At pH levels above 9, demethylation of pectin occurred without the presence of PME, and corrections for this were made in the determination of the actual PME activity.

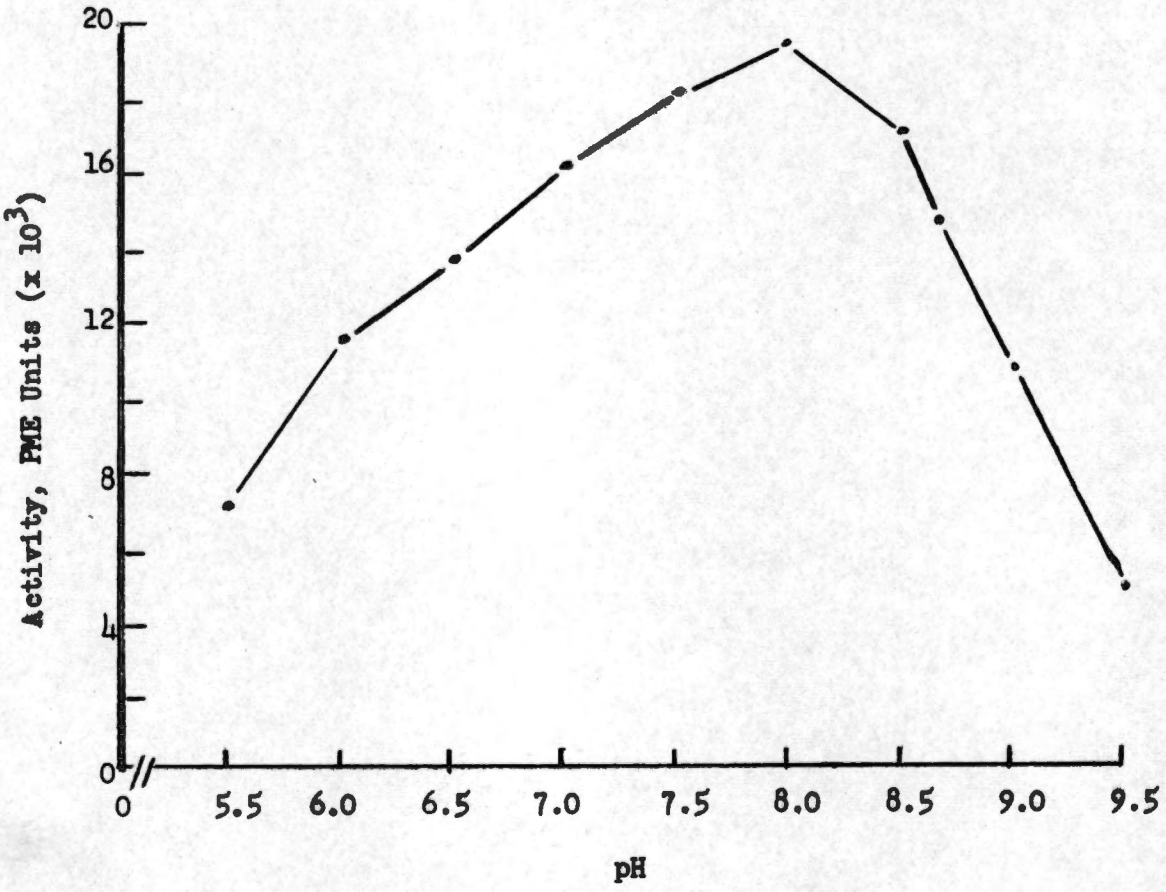


Figure 3. The effect of pH on the activity of soybean PME at 30°C and 0.35 M NaCl.

III. EFFECT OF TEMPERATURE ON PECTIN METHYL ESTERASE ACTIVITY

The analysis of variance showing the effect of temperature on PME activity is presented in Table I, page 25. The effect of temperature was significant at the 0.01 level of probability.

The effect of temperature on PME activity is presented in Figure 4. When the temperature was raised from 25° to 50°C, the rate of increase was almost linear. This increase was almost 1.7-fold. Above 50°C, denaturation of enzyme occurred, and the activity was reduced sharply when the temperature was raised to 60°C. High temperature per se had no significant effect on demethylation of the pectin. Activity at 50°C was significantly higher than at either 45°C or 55°C at the 0.05 level of probability as determined by the Duncan's Multiple Range Test.

The mean Q_{10}, temperature coefficient, was 1.23 over the range of 30° to 50°C. This value is lower than the Q_{10} for PME from other sources: tobacco leaves, 1.45 (21); tomato, 1.52 (35); Southern peas, 1.35 (8); and snap beans, 1.40 (51).

IV. EFFECT OF MATURITY ON PECTIN METHYL ESTERASE ACTIVITY

The maturity level of the soybeans is another factor which influences PME activity. Analysis of variance is shown in Table I. The effect of maturity on PME activity was significant at the 0.01 level of probability.

Table II shows the PME activity and moisture content at four stages of maturity. There were significant differences among PME activity means (the 0.05 level of probability) for the four stages of maturity as

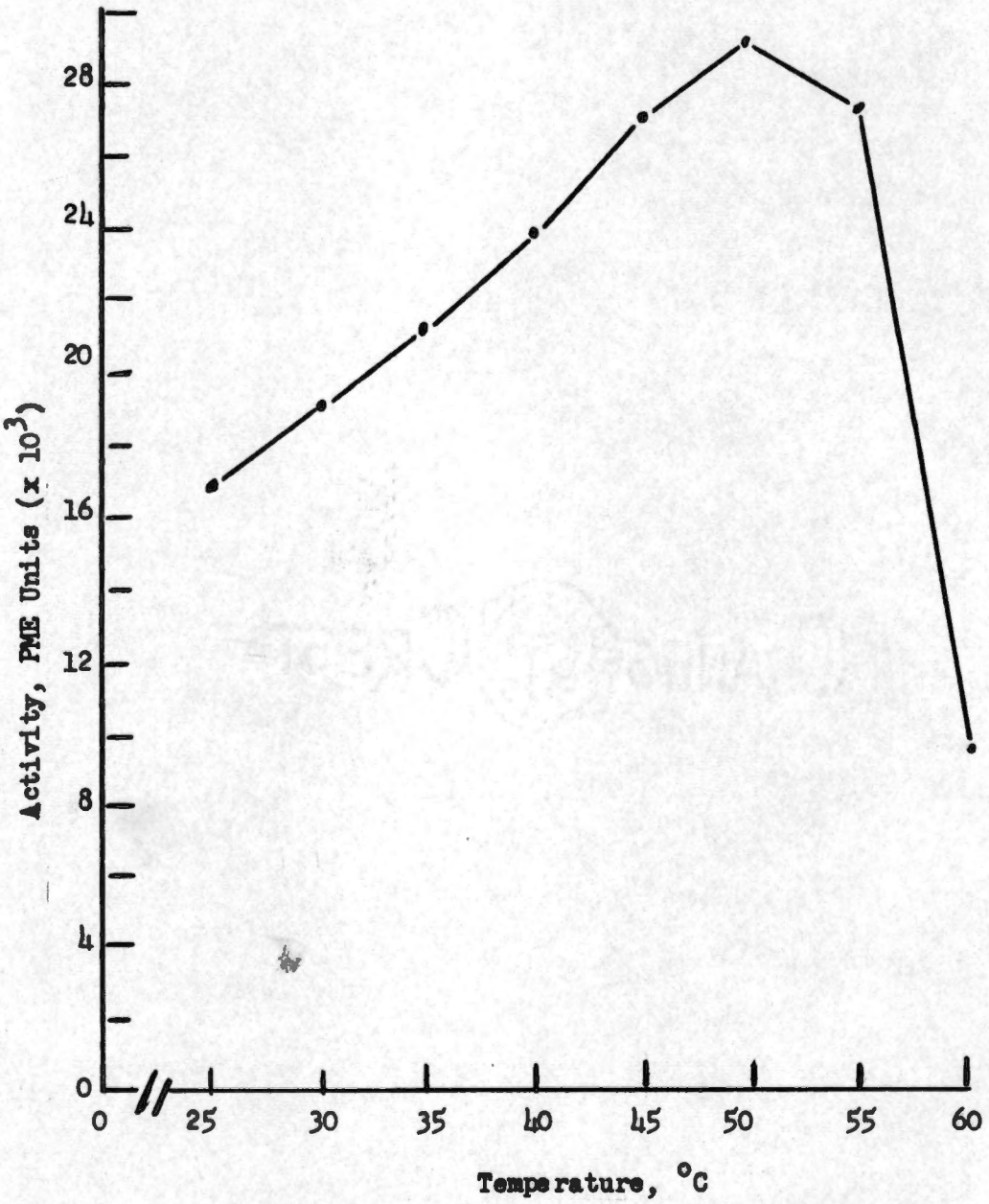


Figure 4. The effect of temperature on the activity of soybean PME at pH 8.0 and 0.35 M NaCl.

TABLE II

THE EFFECT OF MATURITY ON THE ACTIVITY OF SOYBEAN PECTIN METHYL ESTERASE AND MOISTURE CONTENT

Time at Harvest	PME Units[1,2] ($\times 10^3$)	Moisture (percent)
September 23	21.7 a	68.5
September 26	19.6 b	66.5
September 30	17.3 c	63.9
October 15	6.1 d	--

[1] Means of four observations.

[2] Dry weight basis of soybeans.

Means not followed by the same letter are significantly different at the 0.05 level of probability.

determined by the Duncan's Multiple Range Test. The more mature the soybeans were, the lower the PME activity and the lower the moisture content. By observation, the activity and the moisture content were directly related.

The enzyme activity was reduced sharply in the most mature beans. Two possibilities for reduction in PME activity are presented. An actual reduction in PME activity may develop during maturation of the soybeans. Also, the more mature beans were relatively hard and extraction of PME from the tissue by the blending process might have been less effective on these beans than it was on the young, more succulent beans.

V. DETERMINATION OF KINETIC CONSTANTS

The determination of K_m and V_{max} values of PME in soybeans was based on the Lineweaver and Burk equation. The values, computed by use of a FORTRAN computer program, are presented in Figure 5. The K_m for PME of the first harvest is 0.052 percent pectin and for the third harvest, 0.057 percent.

The total enzyme activity was different between the two stages of maturity as indicated by the V_{max} values, but the K_m values did not differ appreciably.

A relatively high correlation was found between the reciprocals of pectin concentration and PME activity. The correlation coefficient, r, was 0.963 for beans of the first maturity and 0.965 for the third. Both were significant at the 0.01 level of probability. The regression coefficient, b, was 0.0031 for the first harvest and 0.0043 for the third.

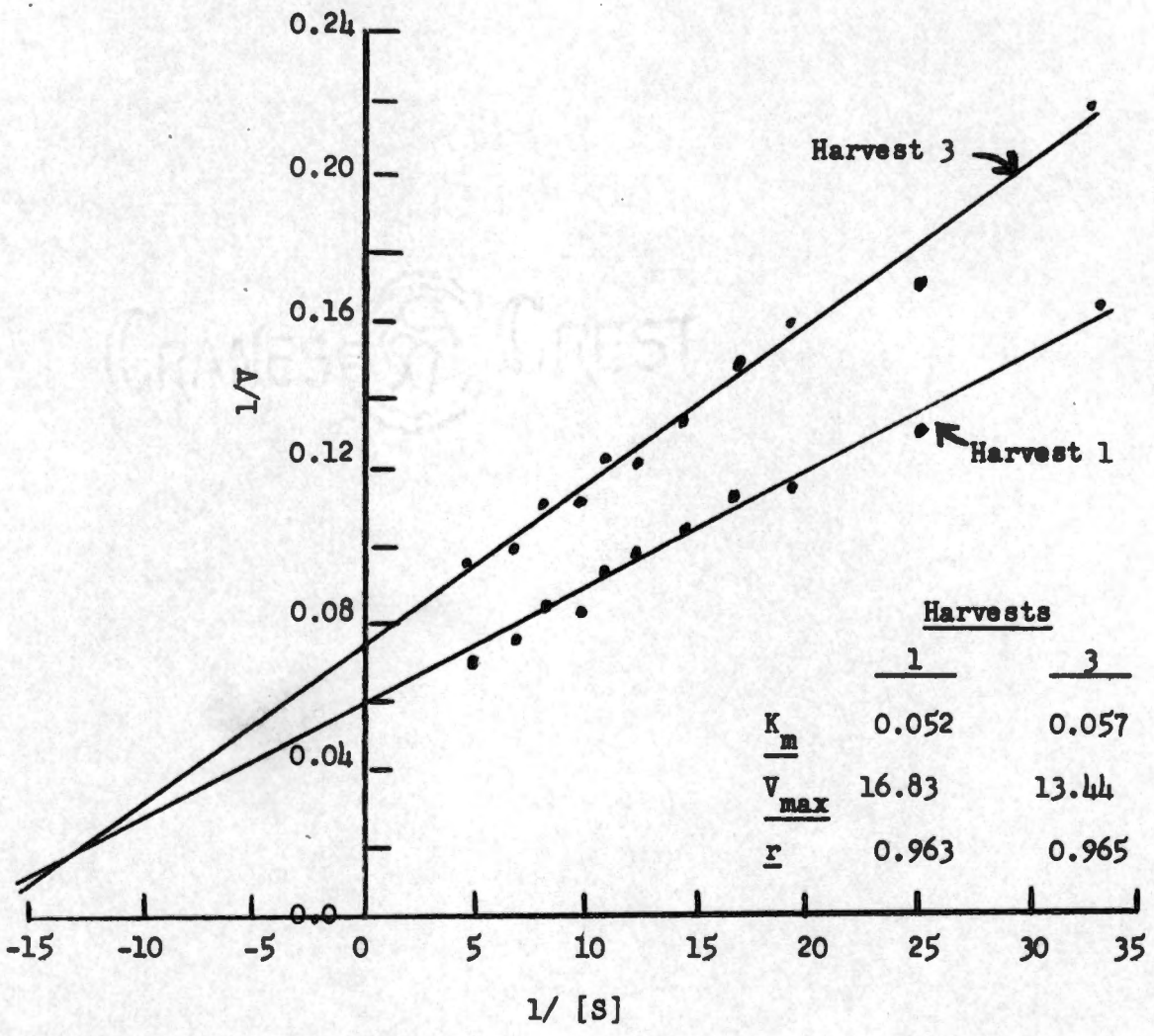


Figure 5. The effect of substrate concentration (percent pectin) on the activity of soybean pectin methyl esterase at pH 8.0, 30°C and 0.35 M NaCl.

VI. INHIBITION OF PECTIN METHYL ESTERASE BY TANNIC ACID

The effect of tannic acid on PME activity is shown in Table III. The substrate concentration was held constant (0.20 percent pectin) and the percentage of inhibitor, tannic acid, was varied from 0.01 percent to 1.0 percent. A tannic acid concentration of 1.0 percent gave almost complete inhibition, while 0.01 percent tannic acid inhibited the PME activity only slightly.

The reversibility of the tannic acid inhibition is indicated in Table IV where the effect of changes of the substrate concentration was varied from 0.05 percent to 0.50 percent pectin while the inhibitor concentration was held constant (0.05 percent tannic acid). With the increase in the pectin concentration, the inhibition decreased in the region observed.

The degree of inhibition of soybean PME by tannic acid was related to the concentration of inhibitor and the substrate. Tannic acid is considered as a competitive inhibitor for soybean PME since the inhibitory effect appears to be reversible. Hall (19) found that inhibition of tomato PME by tannic acid was reversible.

VII. PARTIAL PURIFICATION OF PECTIN METHYL ESTERASE

1. Crude Extraction

Acetone-dried powders were prepared from the thawed soybeans. The yield of powder by this procedure was one-third of the original weight of the beans. Extraction of PME enzyme from acetone powders

TABLE III

DEGREE OF INHIBITION OF PECTIN METHYL ESTERASE ACTIVITY WITH DIFFERENT CONCENTRATIONS OF TANNIC ACID WITH THE SUBSTRATE HELD AT 0.2 PERCENT PECTIN

Tannic Acid Concentration (percent)	Inhibition[1,2] (percent)
0.0	0.0
0.01	5.0
0.05	24.1
0.1	45.7
0.5	86.2
1.0	99.5

[1] Means of three observations.

[2] Dry weight basis of soybeans.

TABLE IV

DEGREE OF INHIBITION OF PECTIN METHYL ESTERASE ACTIVITY WITH DIFFERENT CONCENTRATIONS OF PECTIN WITH THE INHIBITOR HELD AT 0.05 PERCENT TANNIC ACID

Pectin Concentration (percent)	Inhibition[1,2] (percent)
0.05	39.5
0.10	30.0
0.20	24.1
0.30	19.8
0.50	16.3

1/ Means of three observations.

2/ Dry weight basis of soybeans.

with four solvent systems was performed. The results of a quantitative comparison of the PME activities and protein concentrations of four extracts are shown in Table V. The greatest PME activity was obtained by extraction with 2 percent NaCl at pH 8.0. The salt concentration and pH of the extractive medium are important factors in preparing PME extracts.

Determination of protein concentration was made by a spectrophotometric method. This rough estimate of protein content was made quickly and easily on a small quantity of material and in the presence of salts, sodium chloride or ammonium sulfate. The adsorption spectra of fraction extracts from acetone dried powders with different solvent systems are presented in Figure 6.

2. Ammonium Sulfate

Fractional ammonium sulfate precipitation was performed as the second step. The amount of protein precipitated was dependent on the degree of saturation of the solution by ammonium sulfate. To the supernatant obtained from the extractive procedure, ammonium sulfate was added to obtain saturation levels of 0.4 to 0.9. The PME activity was determined on each fraction obtained. The results are shown in Figure 7. More PME was precipitated between 0.6 and 0.7 saturation of ammonium sulfate than at other levels. However, since there was a comparatively high activity in the precipitate obtained between 0.5 and 0.6 saturation, a wider saturation range (between 0.55 and 0.70) of ammonium sulfate was adapted in order to obtain more PME in the precipitate.

TABLE V

RESULTS OF COMPARATIVE STUDIES FOR EXTRACTING PECTIN METHYL ESTERASE FROM ACETONE DRIED POWDERS

Extracting Solvents	PME Units/ml[1] (x 10^2)	Absorbance[2]		Proteins (mg/ml)
		280nm	260 nm	
Distilled H_2O	5.3	0.98	1.12	15.5
2% NaCl	8.5	1.10	1.25	16.8
2% NaCl at pH 8.0	10.0	1.24	1.41	18.9
4% NaCl at pH 8.0	8.7	1.22	1.38	18.7

[1] Means of two observations.

[2] Diluted with distilled water to the ratio of 1:25 before spectrophotometric reading.

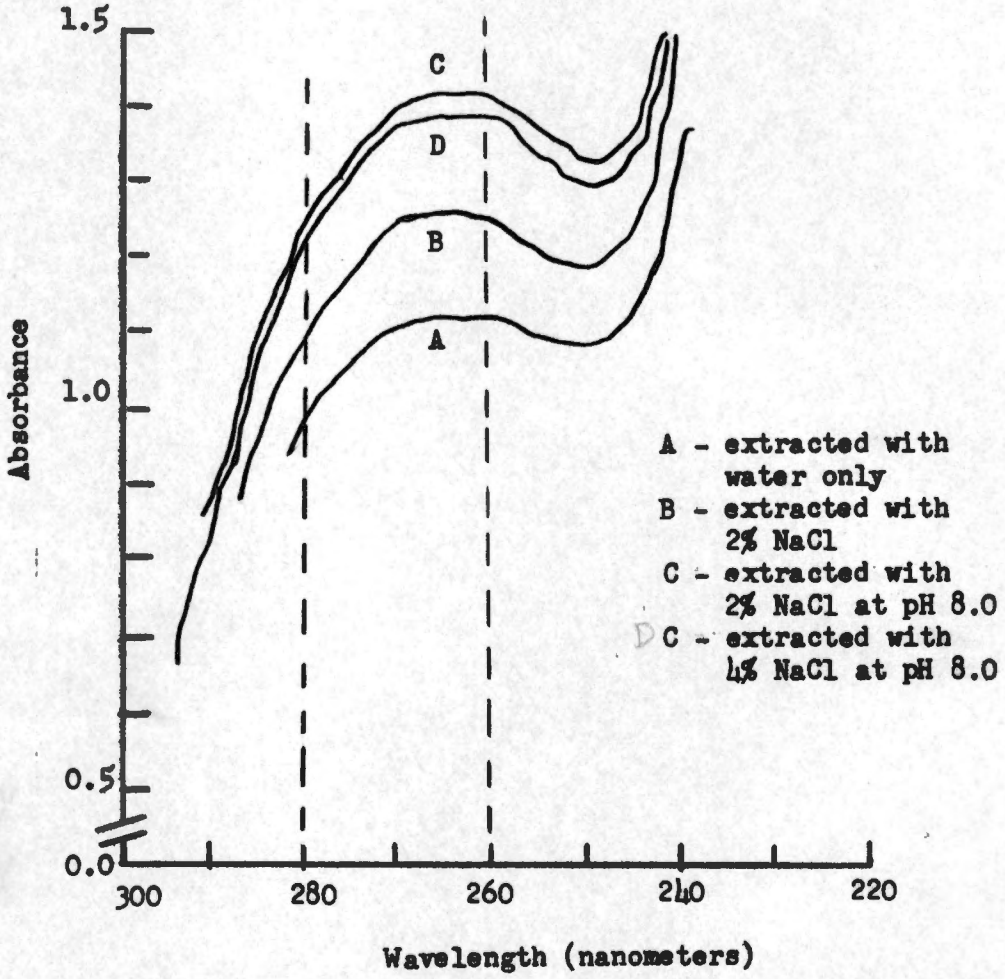


Figure 6. The absorption spectra of fraction extracted from acetone-dried powder with different solvent systems.

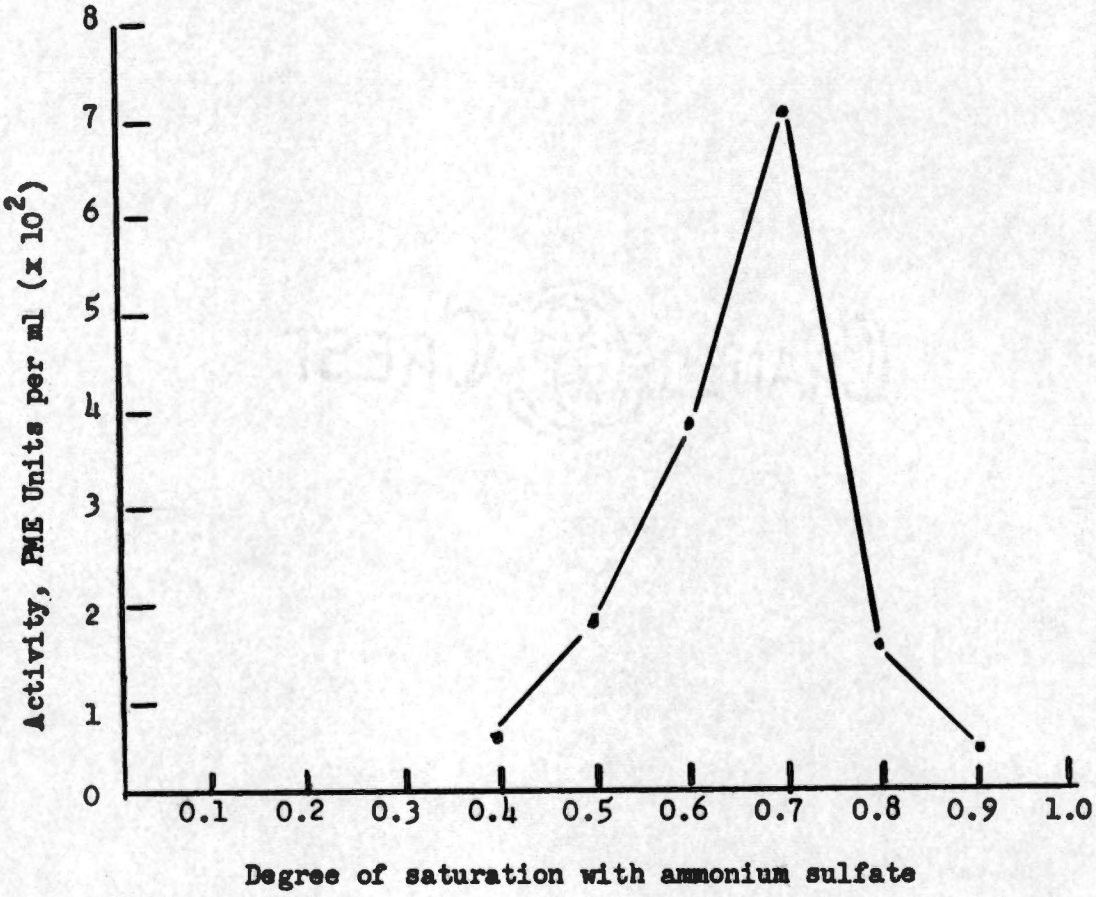


Figure 7. Comparative studies for fractional precipitation of soybean pectin methyl esterase from solution with ammonium sulfate.

Fractional precipitation with ammonium sulfate rather than with organic solvents has been recommended for purification of PME by several workers (26, 33, 42). The level of ammonium sulfate saturation used to precipitate PME was dependent on the enzyme sources. Most of the PME was precipitated from tomatoes at ammonium sulfate saturation between 0.3 and 0.5 (42); from orange, at 0.4 and 0.8 (33). In the present study the preponderance of soybean PME was precipitated between 0.55 and 0.70 saturation.

The definite separation between supernatant and precipitate by centrifugation was not easily distinguishable and the supernatant was always brown. The insufficient centrifugal force, 6,000 rpm applied, might have been the reason. In general, more than 10,000 rpm are required to produce a distinct separation between precipitate and supernatant.

3. Dialysis

For the third step, the enzyme preparation obtained by ammonium sulfate fractionation was dialyzed against distilled water overnight to remove the salts. After dialysis, the supernatant became clearer and some proteins were precipitated. Loss of PME activity in the solution was about 20 percent.

McColloch et al. (35) adopted a procedure of purification by dialysing tomato PME after extraction with NaCl solution. They observed that dialysis resulted in nearly 40 percent loss of the PME activity. MacDonnell et al. (33) indicated that the orange PME could not be adsorbed on an artificial zeolite (Ducil) without dialysis. Almost 30

percent of the PME activity was loss in the purification of orange PME with dialysis. Although the loss of PME activity took place during dialysis, this procedure was recommended in order to remove salts and other low molecular weight substances.

4. Ion-exchange Chromatography

As the final step, the solution containing the enzyme after dialysis was fractionated on a column of diethylaminoethyl (DEAE)-cellulose. The results, relative PME activity value and absorbance value at 280 nm for each aliquot, are given in Figure 8. There are two peaks for PME activity; one was around aliquot 19 (114 milliliters) and another around aliquot 38 (228 milliliters). The two peaks indicate the possible presence of two molecular forms of the soybean PME.

Ninety aliquots were collected, but only the first sixty are indicated in Figure 8, since the latter thirty had neither PME activity nor absorbance at 280 nm.

Several attempts were made to use Sephadex, a three-dimensional polysaccharide used to separate a mixture of proteins according to their molecular size (40, 43). Certain enzymes of the preparation reacted with the polysacchride and caused the eluates to become cloudy. This problem occurred at room temperature but it could possibly have been prevented at lower temperatures. Refrigerated working areas were not available.

Generally, procedures for purification of enzyme should be performed at low temperature, 4°C (14). Since adequate equipment was not

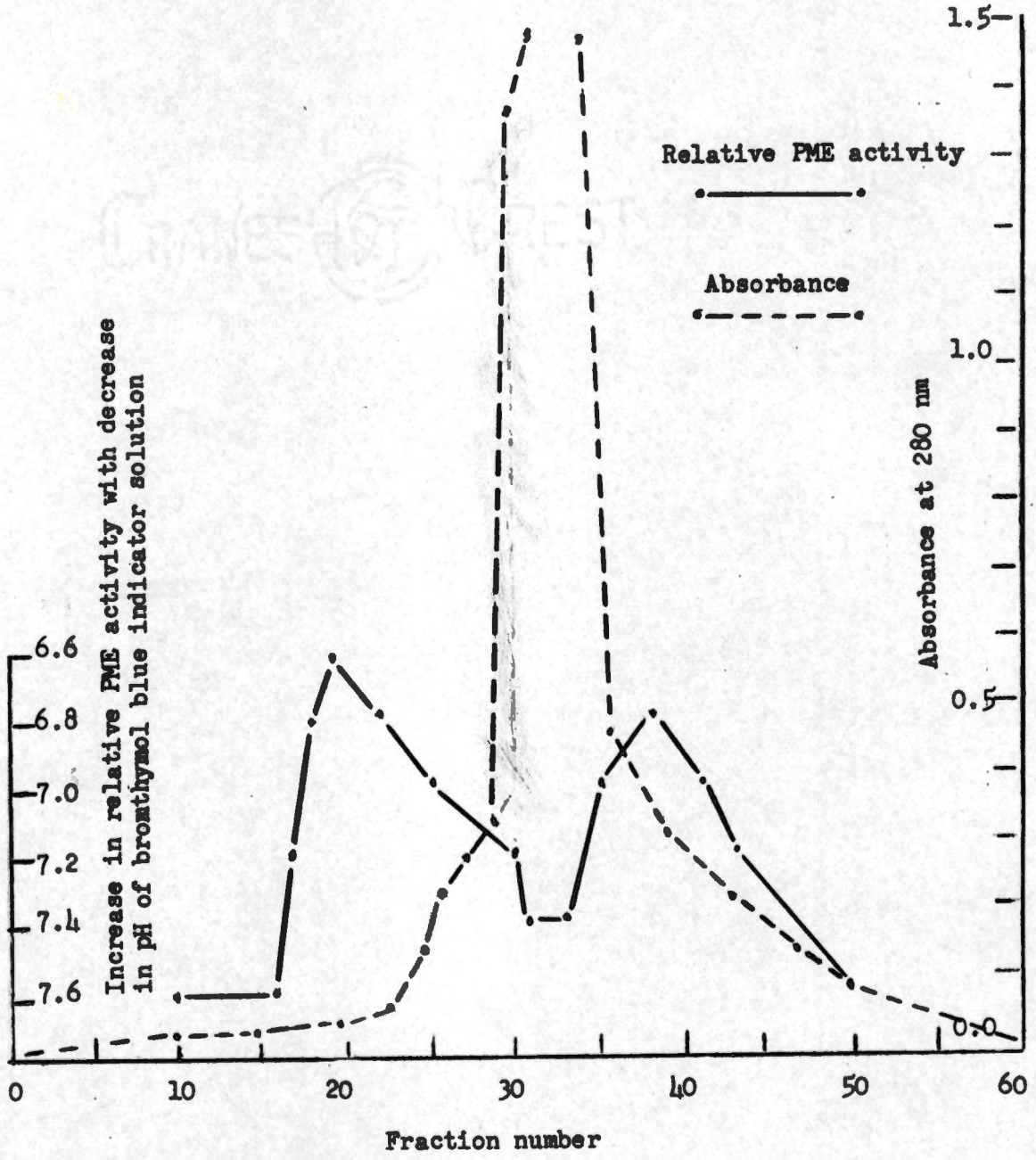


Figure 8. Chromatography of PME precipitation on DEAE-cellulose.

available, soybean PME was fractionated on a DEAE-cellulose column for one day at room temperature.

5. Increase in Specific Activities

Results of partial purification of soybean PME are presented in Table VI. Specific activities were increased with each procedure. Since there were two peaks for PME activity (Figure 8), an increase of about 140-fold in specific activity was obtained in aliquot 19 and 18-fold in aliquot 38 compared to that in the crude extract.

VIII. SUGGESTIONS FOR FURTHER STUDY

It was concluded that there are at least two molecular forms of soybean PME. This hypothesis is based on the elution behavior (two peaks) of the enzyme on the ion-exchange column. Further study is necessary to ascertain the physical and chemical properties of the two forms of soybean PME.

Determination of molecular weight is one method for describing an enzyme. Literature was not available concerning the determination of molecular weight of PME. Therefore, further study is needed to determine this property.

TABLE VI

RESULTS OF PROGRESSIVE PURIFICATION OF SOYBEAN PECTIN METHYL ESTERASE

Progressive Purification Steps	PME Units/ml[1] ($\times 10^2$)	Proteins (mg/ml)	Specific Activities (units/mg)
1. Crude extract (with 2% NaCl at pH 8.0)	10.6	27	0.40
2. Ammonium sulfate (between 0.55 and 0.70)	24.0	30	0.80
3. Dialysis	8.3	13	0.64
4. DEAE-cellulose (aliquot 19)	1.7	0.03	56.66[2]
5. DEAE-cellulose (aliquot 38)	1.7	0.23	7.40[3]

[1] Means of two observations.

[2] $\frac{56.66}{0.40}$ = 141.6 fold increase in PME specific activities over crude extract.

[3] $\frac{7.40}{0.40}$ = 18.5 fold increase in PME specific activities over crude extract.

CHAPTER V

SUMMARY

In this study, experiments were conducted to determine the effect of chemical and physical changes on pectin methyl esterase activity in vegetable-type soybeans. The following conclusions may be made from the studies conducted.

1. The NaCl concentration, pH, temperature and maturity had a significant effect on the soybean PME activity at the 0.01 level of probability.

2. Maximum activity for the PME occurred between 0.25 and 0.40 M concentrations of NaCl, between pH 7.5 and 8.5, and at 50°C. The mean Q_{10} value was 1.23.

3. The more mature the soybeans were, the lower the PME activity. The enzyme activity was reduced sharply in the most mature beans.

4. The K_m value for soybean PME of the first harvest was 0.052 percent pectin and for the third harvest, 0.057 percent.

5. The amount of inhibition of soybean PME by tannic acid was related to inhibitor concentration and substrate concentration. The inhibitory effect appeared to be reversible.

6. There were at least two isoenzymes of soybean PME. This hypothesis is based on the elution behavior of the enzyme on the ion-exchange column.

7. An increase in PME specific activity of about 140-fold was obtained by the purification methods.

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