EFFECT OF ETHYLENE AND DIAMMONIUM HYDROGEN) PHOSPHATE ON PEROXIDASE AND PROTEIN) DEVELOPMENT IN SWEET POTATO SLICES

by

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Chapter 1

INTRODUCTION

Peroxidase [E.C.1.11.1.7; donor:H_2O_2 oxidoreductase] is found in many species of plants and animals. It is one of the most widely distributed enzymes in nature and one of the most versatile in its action. For many years it has been studied by physical chemists, organic chemists, biochemists, and physiologists; and a very large number of papers has been published on its nature, mode of action, and function (1).

In plants infected by phytopathogens, peroxidase activity increases (2). Peroxidase activity increases in several plant hosts following rust infection (3-10), with greatest increases in resistant-reacting tissues (3-5, 7, 8). Peroxidase occurs widely in healthy plant tissues, but its precise physiological role is uncertain (10). Peroxidase increases have been suggested as being directly responsible for disease resistance.

The disease of sweet potato roots, known as black rot, is caused by certain isolates of the fungus Ceratocystis fimbriata. The surface of root tissue, which is normally susceptible to black rot, can be made resistant to infection by prior inoculation with non-pathogenic isolates of C. fimbriata (11, 12). Root tissue with this induced resistance has increased peroxidase and polyphenol oxidase activity similar to that found in inoculated, naturally-resistant tissue.

Volatile materials from infected root tissue increased the activity of these enzymes. Imaseki et al. (13) and Young et al. (14)

found that the volatile compound is ethylene, and Stahmann et al. (12) were the first to show the increased production of ethylene in sweet potato roots infected by the black rot fungus.

On the other hand, Imaseki et al. (15), using sweet potato roots, demonstrated that when root tissue was injured mechanically, chemically, or by radiation (16-19) ethylene production by the tissue was stimulated and a greater ethylene production occurred when the tissue was more severely injured. The more ethylene is produced, the more activities of the enzymes, including peroxidase, are increased.

Based on these observations, plus our own work using ammonium phosphate as the nitrogen source, the purpose of this thesis is to investigate the role of exogenous ethylene on peroxidase and protein in sweet potato slices. The food aspects of ethylene-treated sweet potatoes are discussed.

Chapter 2

LITERATURE REVIEW

Black Rot Fungus or Injured Tissues and Ethylene Production

Ethylene production by sweet potato roots infected by the black rot fungus Ceratocystis fimbriata increased strikingly after infection (20). Increased ethylene production by plant tissue infected with several pathogens has been reported (21). At least part of the metabolic activation found in diseased tissue had to be stimulated by ethylene, which was produced after infection.

Furthermore, Imaseki et al. (13) reported that the root tissue produced ethylene in response to cut injury. Increasing the cut surface area increased ethylene production, and the amount was proportional to the logarithm of the surface area. When the tissue was treated with chemicals that might destroy the cells, ethylene production also increased remarkably.

The results of the experiments of Stahmann et al. (12) indicate that ethylene can increase resistance of sweet potato tissue to infection by C. fimbriata. They also indicate that such induced resistance is associated with an increase in the peroxidase and polyphenol oxidase activity of ethylene-treated tissues. It is possible that ethylene is one of the stimuli which move from the area of C. fimbriata infection in the sweet potato into adjoining tissue to initiate the metabolic changes which lead to resistance to further penetration by the pathogen.

Since ethylene can increase peroxidase and polyphenol oxidase activity in sweet potato tissue, it appears that these enzymes may be involved in the resistance mechanism. However, as Weber et al. (11) noted, increases in the activity of these enzymes alone cannot be solely responsible for resistance. Other factors are involved, and these may include the rate of synthesis or transport of the substrate to the area of increased polyphenol oxidase activity.

Uritani et al. (22) reported that the fungus had only a little activity of peroxidase; therefore, Kawashima et al. (23) concluded that the increase in the activity of diseased tissue was due to the production of the enzyme by the host cells, but not by the fungus. A drastic increase in the peroxidase activity in diseased tissue was reported by Kawashima et al. (23) in the course of one to three days after inoculation. The activity of the outer layers was much higher than that of the inner layers, but the first layer had smaller activity. The reason may be the injury or death of the host tissue with the fungal infection which may diminish the enzyme formation and inactivate or decompose the enzyme.

Changes in Nitrogen Metabolism in Sweet Potato Infected by Black Rot Fungus

Uritani (24) studied metabolic changes in proteins and amino acids of sweet potato root tissue in response to infection by C. fimbriata by the methods of immunochemistry, electrophoresis in starch gel, and chromatography on modified cellulose columns. Some soluble proteins increased in the tissue adjacent to the infection site; others decreased. Of special interest was the discovery of new antigens in cells adjacent to the area invaded by the fungus which could not be

detected before infection or were remarkably increased in concentration as a result of the adjacent infection. Those new antigens or proteins produced in response to infection were more concentrated in the tissue of resistant varieties than in susceptible ones. One such antigenic component produced near the diseased tissue was identified as a peroxidase. Resistance in such plants may be correlated with the ability of the host tissues to alter their protein metabolism in response to the infecting agent and to form proteins which cannot be detected or can be found at low levels before infection.

Uritani (24) observed a decrease in asparagine in sweet potato roots infected by black rot fungus. Also, Akazawa and Uritani (25), Tomiyama et al. (26), and Suzuki et al. (27) found an apparent increase in protein nitrogen in sweet potato root infection.

Effect of Gamma Radiation on Peroxidase Development in Sweet Potato Tissue

Ogawa et al. (18, 19) reported the effects of gamma radiation on higher plants and demonstrated, using sweet potato roots, that gamma radiation accelerates the typical phenomena of aging such as the increase in polyphenol content and in peroxidase activity (28). Since it is well known that gamma radiation causes an increased production of ethylene in fruits and vegetables (29), some correlation may exist between the effect of gamma radiation and of ethylene on metabolic changes in response to cut injury.

Using 90 K-rads of gamma rays from a ^{60}Co source to irradiate sweet potato slices, Ogawa and Uritani (16) demonstrated the de novo protein synthesis requirement for the peroxidase development by applying cycloheximide to non-irradiated and irradiated disc tissues. They

also demonstrated that exogenous ethylene stimulated peroxidase development in non-irradiated and irradiated slices. Both treatments of gamma radiation and exogenous ethylene showed the additive mode of action with respect to the peroxidase development.

Biochemistry of Ethylene Production

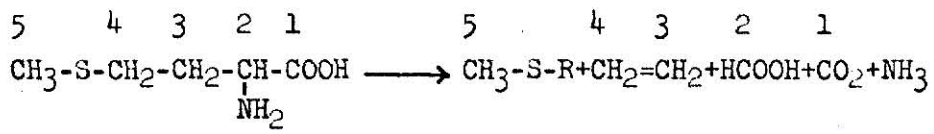
Even though Gane (30, 31) in 1935 established the fact that plants produce ethylene, biochemical studies on the pathway of ethylene production did not start until the late 1950's. Most research has centered on work with higher plants, though ethylene production by fungi has also been studied intensively.

After Miller et al. (32) and Biale (33) demonstrated that penicillium produced ethylene, Fergus (34) found that the best carbon sources were D-mannitol and D-mannose, followed by D-xylose, D-galactose, D-glucose, and other sugars. However, in spite of a considerable amount of effort by a large number of workers, the biochemistry of ethylene formation in fungi remains unknown (35). The pathway with the greatest amount of experimental evidence in its favor is also the simplest, namely the dehydration of ethanol.

$$CH_3\text{-}CH_2OH \longrightarrow CH_2{=}CH_2{+}H_2O$$

In higher plants, a number of substances have been proposed as precursors of ethylene. These include methionine, linolenic acid, β-alanine, propanal, ethanol, organic acids, acrylic acid, thiomalic acid, glycerol, sucrose, glucose, and acetic acid. However, the most probable precursor of ethylene is methionine, according to Liebermann et al. (36, 37). Additional support for the idea that methionine serves as a precursor of ethylene was obtained when they added ^{14}C-methionine

labeled in position 1, 2, 3, 4, or 5 in the methyl group to apple slices. Only methionine labeled in position 3 and 4 produced a significant amount of ethylene.



De Novo Synthesis of Peroxidase Isoenzymes

When stored tissues of sweet potato are cut into slices, many metabolic activities begin to rise (29-45). The rise continues to increase over a period of several days, at the end of which the activity may be many times larger than in fresh tissue. Included among the metabolic changes is the increase in the activities of several enzymes, including peroxidase. Imaseki et al. (46, 47) showed that the peroxidase content of sweet potato slices was significantly enhanced by a low concentration of ethylene exogenously supplied to the slices.

Early work by Kanazawa et al. (48) noted that sweet potato slices treated with inhibitors of protein synthesis contained less peroxidase than that of untreated tissue, thus suggesting that the increase in peroxidase content resulted from synthesis de novo.

Shannon et al. (49) reported that the peroxidase content of sweet potato slices increased nearly one hundredfold following 24 hours of incubation in an air atmosphere containing ethylene. They also showed that the enzymic activity of each peroxidase isoenzyme increased during the incubation period. Each peroxidase isoenzyme appeared to incorporate ^{14}C-leucine. Treatment of fresh slices or slices collected midway in the time course with the inhibitor of protein synthesis Blasticidin S caused an abrupt cessation of peroxidase formation and

simultaneously an abrupt cessation of incorporation of ^{14}C-leucine into peroxidase isoenzymes. They concluded that the rapid increase in peroxidase activity in sweet potato slices resulted from synthesis de novo of the enzyme.

Food Aspect and World Production of Sweet Potato

Ever since its introduction, the sweet potato has been a highly prized food. The sweet potato was introduced in China in 1594 as a result of a search for a crop that would relieve the frequent famines (50). The culture of this plant rapidly increased, and it soon came to be regarded as a boon in the prevention of famine.

Sweet potatoes are grown in almost every garden patch. They constitute an important part of the food of the family of the grower, and they are dug and eaten as needed without entering into trade or commerce of any kind. The consumption is restricted largely to the localities where it is grown. Now, in most countries, storage facilities have been provided, but the highly specialized facilities needed to market this perishable crop have not yet been developed, and extensive shipping is not carried on.

As the high food value of sweet potatoes becomes more generally known and appreciated, it will probably become more sought after as human food and as a stock feed. As may be seen in Table 1, the sweet potato is high in energy value (about 125 calories per 100 g sample) and rich in other important and highly prized food attributes, such as roughage, minerals, and vitamins A and C (51). There are possibilities of greater industrial use, and the manufacture of and commerce in these products will probably be much more extensive in the future than they have been in the past.

Table 1

Nutritive Constituents of the Sweet Potato and the White Potato

Constituent	Sweet Potato	White Potato
Water	68.5%	77.8%
Protein	1.8%	2.0%
Fat	0.7%	0.1%
Carbohydrate	27.9%	19.1%
Fiber	1.0%	0.4%
Sugar	5.4%	0.9%
Starch	20.2%	14.7%
Calories in 100 g sample	125	85
Vitamin A in 100 g sample	3,500 IU	30 IU
Vitamin B_1	31 IU	62 IU
Vitamin B_2	70.0 mg	55.0 mg
Niacin	1.3 mg	2.0 mg
Vitamin C	406 IU	300 IU
Pantothenic acid	1.2 mg	0.65 mg
Calcium	19.0 mg	14.0 mg
Phosphorus	45.0 mg	58.0 mg
Iron	0.92 mg	0.85 mg

Sources: (51, 52).

Chapter 3

EXPERIMENTAL PROCEDURES

Materials

Sweet potato roots (Ipomea batatas) were harvested and stored at 10°C until used. Storage at this temperature caused no apparent cold injury, although there was a slight change during the storage in the degree of biochemical response of the roots to slicing. The roots were washed carefully, trimmed free of any defect, then cut into 3 mm-thick slices.

All experiments were performed with roots cultivated in Manhattan, Kansas.*

Chemicals

Chemicals used in this work were of analytical grade. Acrylamide; N,N'-methylenebisacrylamide (bis); and N,N,N',N'-tetramethylethylenediamine (Temed) were from Eastman. Ampholines were from LKB. Ethylene gas was from the Matheson Company. All reagents were prepared in distilled, deionized water.

Methods

Determination of hydrogen peroxide concentration. According to Fuhrman and Wallace (54), a standard hydrogen peroxide stock solution was

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*We gratefully acknowledge the gift of sweet potatoes from Dr. B. A. Cunningham. We are also indebted to Dr. J. Greig for the storage space for our sample in the Horticulture Department, Kansas State University, Manhattan, Kansas.

prepared by titrating hydrogen peroxide with cerium sulfate. The reaction is as follows:

$$_2Ce(SO_4)_2+H_2O_2 \longrightarrow Ce_2(SO_4)_3+H_2SO_4+O_2$$

A Corning Model 12 pH meter, Pt electrode, and a saturated KCl calomel reference electrode were used to follow the EMF change.

Ten milliliters of approximately 0.03M hydrogen peroxide were added to 25 ml of 3N acetic acid and titrated with 0.02N cerium sulfate. Suppose B ml of 0.02N cerium sulfate was obtained corresponding to the inflection point. From the relation: B x 0.02 = 10 x (hydrogen peroxide concentration), a concentration of hydrogen peroxide is calculated.

On the other hand, the standardization of hydrogen peroxide can be done colorimetrically by using titanium sulfate (55) (Fig. 1). Using the standard curve of Cunningham, if the absorbance at 410 nm is known, the milliliters of 10^{-3}M hydrogen peroxide can be calculated.

A titanium sulfate reagent was prepared by digesting reagent titanium oxide (one gram) in 100 ml concentrated sulfuric acid for 16 hours at 150°C. It was cooled, diluted to 500 ml, and filtered through a sintered glass funnel. An aliquot (125 ml) of that stock solution was diluted to one liter with distilled water to prepare the working reagent. We used the titanium sulfate method in our investigation.

Determination of protein concentration. The Biuret test was used to determine protein concentration (56, 57). Cupric ion, in alkaline tartrate solution, reacts with the poly-α-amino acid structure of protein chains to form a chromophoric complex. This complex has a rather broad absorption peak between 500 and 600 nm. We used a setting of 540 nm to measure it.

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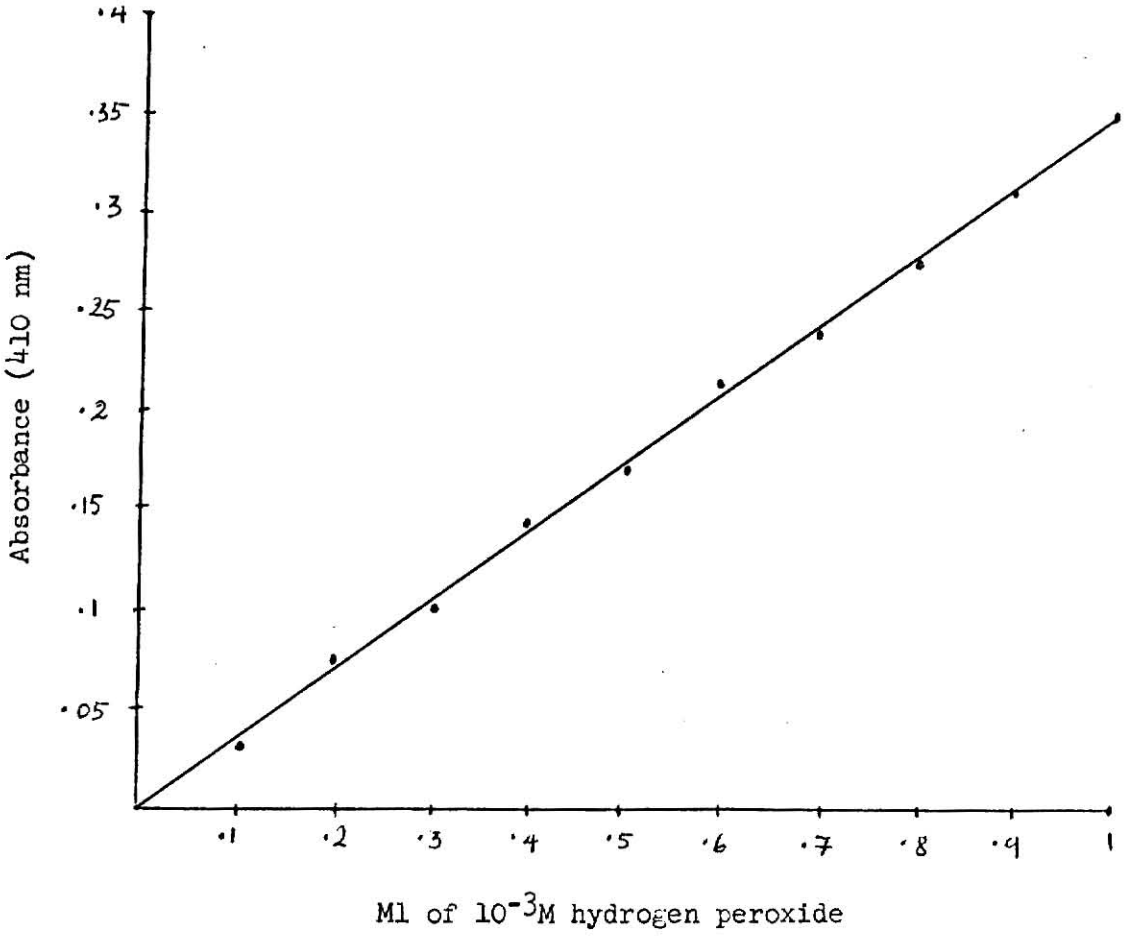
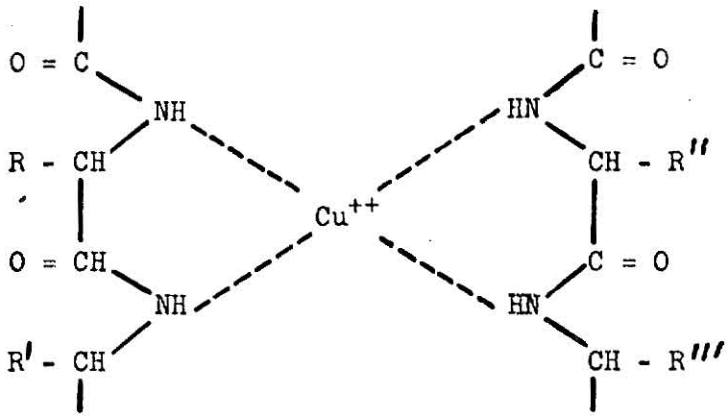


Figure 1. Standard Curve for Determination of Hydrogen Peroxide

Sources:

Cunningham, B. A., "H_2O_2 Standardization," Book 24, page 88, and Book 39, page 92. Unpublished data.



Structure of Chromophoric Complex
of Cupric Ion and Poly-α-Amino Acid

A standard curve was prepared using a solution of bovine serum albumin (8 mg/ml). To each tube containing varied amounts of protein solution, an amount of water was added to make the total volume one milliliter. Biuret reagent (4 ml) was added to each tube and mixed. After 30 minutes, absorbance was measured at 540 nm (Fig. 2). The Biuret reagent consists of 3 g cupric sulfate bonded to five molecules of water, 12 g sodium potassium tartrate, and 30 g sodium hydroxide per liter.

Peroxidase assay. A guaicol method (56) was used with some modification.

1. Reagent:

a. Guaicol (H donor)--0.03 ml 98% guaicol in 100 ml 0.1M potassium phosphate buffer, pH 6. Shake well and wait for complete dissolution.

b. Hydrogen peroxide--The final concentration of hydrogen peroxide should be 10^{-3}M. Dilute the hydrogen peroxide of stock solution with deionized water to desired concentration.

2. Assay:

a. The reaction mixture contains 2 ml of guaicol buffer, 0.1 ml of sweet potato extract, and 0.4 ml water. The reaction is initiated by adding 0.2 ml hydrogen peroxide.

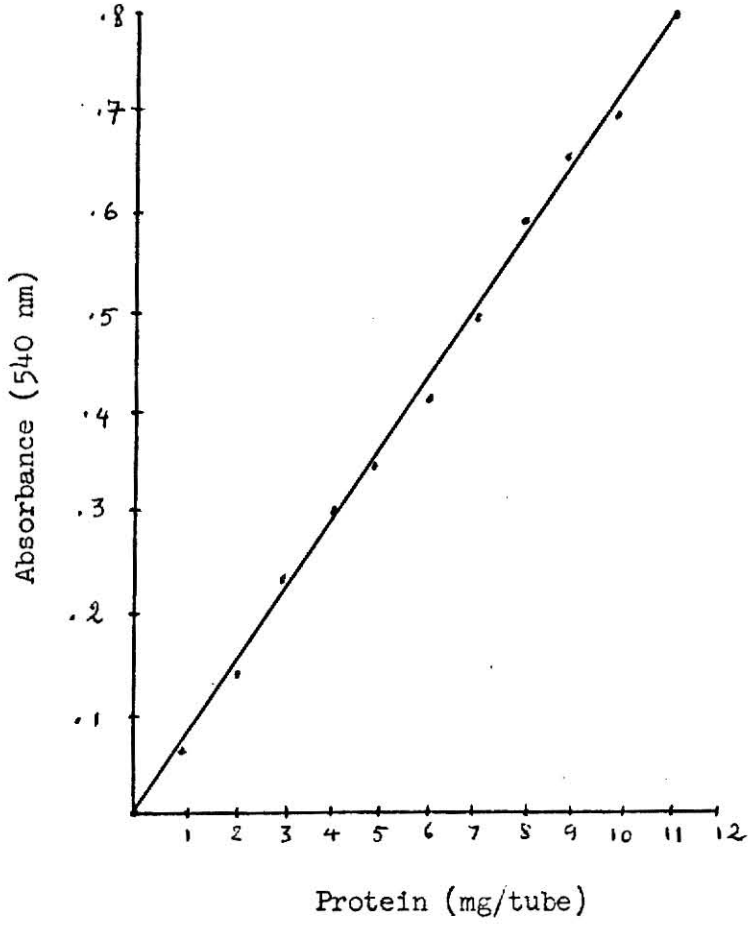


Figure 2. Standard Curve for Determination of Protein by Biuret Procedure

b. The peroxidase activity is recorded by absorbance change at 470 nm on a strip chart. The initial rate is obtained from the linear portion of the curve. The enzyme activity is expressed as absorbance per minute per milliliter of enzyme.

Electrophoresis. Two kinds of electrophoresis were used: thin-layer agarose gel and thin-layer polyacrylamide gel.

1. Thin-layer agarose gel: Electrophoresis was carried out in agarose gels spread on films using the modification described by Cawley (59). The agarose gel was prepared by warming 1% w/v agarose in 0.01M Tris-HCl buffer, pH 8, then spreading 15 ml on a 4 x 5-inch film of DuPont Cronar or 10 ml on a 3 1/4 x 4-inch glass lantern plate.

Samples were applied by streaking 1-2λ of the enzyme on the edge of a sharpened spatula, then piercing straight into the gel and carefully removing the spatula upward with a gentle rocking motion. With that technique it was possible to run four to six channels per gel slab.

Electrophoresis was carried out in an apparatus in the cold room (approximately 4°C ambient). Two pieces of filter paper were used to connect the gel to the reservoirs. Each reservoir contained approximately 400 ml of 0.01M Tris-HCl buffer, pH 8.

After running for two to two and one-half hours at 300 volts, the gel was fixed and stained. For staining in our laboratory we used 0.1M monopotassium hydrogen phosphate buffer, pH 5.5, containing 10 mg of diaminobenzidine tetrachloride and 0.05 ml of 3% hydrogen peroxide per 100 ml buffer. Staining time was about 15 minutes at room temperature. The gel may be dried on a sheet of DuPont Cronar for preservation.

2. Thin-layer polyacrylamide isoelectric focusing: In protein chemistry, isoelectric focusing is a very powerful method for the

separation of proteins. The determination of the isoelectric points of separated protein components provides a basis for characterization of these components. Svensson (60) and Vesterberg and Svensson (61) developed the technique of isoelectric focusing in sucrose density gradient with ampholine carrier ampholytes. Wrigley (62) used the method of disc isoelectric focusing, which gave good results but presented disadvantages.

The method used in our investigation was that of Bours (63), modified by Vesterberg (64). This method permits an exact comparison of the samples applied and an accurate determination of the isoelectric points of the isoenzymes separated. Our isoelectric focusing technique was carried out in the manner described by Awdeh et al. (65).

Chemicals

--Acrylamide N,N'-methylene-bis acrylamide (bis); acrylamide; and N,N,N',N'-tetramethyl-1,2-diaminoethane were from Eastman Kodak (Rochester, New York).

--Riboflavin was from Merck.

--Ampholine carrier ampholytes were from LKB Produkter (Sweden).

The acrylamide and bis acrylamide were used without recrystallization.

Apparatus. An apparatus was built in our laboratory based on the model of Vesterberg (64). This apparatus consisted of an upper plexiglass plate which was cooled from underneath by water circulating from an ice box by a pump. The electrodes were made of platinum wire.

Preparation of gel. A 5% (w/v) polyacrylamide gel containing 2% (w/v) ampholine was made according to Bours (63). Stock solutions for preparing the gel were as follows:

Solution A--Catalyst Solution: 1 ml N,N,N',N'-tetramethyl-1,2-diaminoethane diluted to 150 ml with distilled water.

Solution B--Acrylamide Solution: 100 g acrylamide, 2.7 g N,N' methylene-bis acrylamide diluted to 400 ml with distilled water.

Solution C--Photoactivation Solution: 2 mg riboflavin diluted to 100 ml with distilled water.

The gel solution was prepared by mixing 7 ml of Solution A, 14 ml of Solution B, 18.5 ml of Solution C, and 3.3 ml of ampholine purchased as 40% solution, pH range 3-10.

A gel mold was prepared (Fig. 3) with two glass plates clamped together in a vertical position and separated by a gasket of flexible, soft polyvinyl chloride about 1.5 mm thick between the plates to make a tight

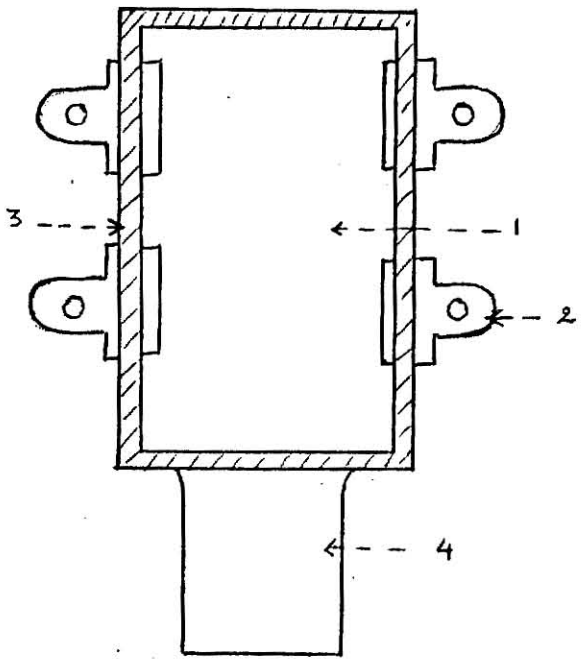


Figure 3. Mold for Preparation of Gel
(1) glass plate; (2) paper clamp; (3) frame of soft polyvinylchloride; and (4) beaker.

seal on three edges of the plates. The plates were kept together by means of paper clamps. One of the plates had been siliconized with dichlorodimethylsilane.

Using a syringe, the gel mixture was carefully poured between the glass plates. Any air bubbles were to be avoided. The solution was polymerized by photoactivation for one to two hours at room temperature using two 20W fluorescent daylight lamps placed vertically about 5-10 cm from the gel. After polymerization, the clamps and the tubing were removed and the siliconized plate was lifted off with a knife. The polymerized gel adhered firmly to the other glass plate.

Application of the samples. In our experiment, 2λ of sample were applied to a filter paper square placed on the gel in the middle.

Isoelectric focusing procedure. Two strips of filter paper, about 1 x 10 cm each, were immersed in the solutions containing 0.5M phosphoric acid and 0.5M sodium hydroxide, respectively. Isoelectric focusing was performed at about 15 v/cm. Focusing was done mostly overnight.

Detection of peroxidase. The staining procedure described for agarose gel was used.

Measurement of pH. Measurement of pH could be done with a microsurfaced electrode 6 mm in diameter (Ingold, Zurich, Switzerland, type lot 403-30-M6) or by cutting the gel into one-centimeter strips, placing each strip in a tube containing 2 ml of distilled water, and using a regular pH meter.

Gel preservation. The method used was originally described in detail by Dangerfield and Faulkner (66) for the preservation of starch gel electrophoresis strips. This could be applied to polyacrylamide gels with a modification, i.e. the gel was covered by a solution of 1% agarose and

5% glycerol. After drying for two days, a hard, smooth, transparent layer was formed on the glass plate.

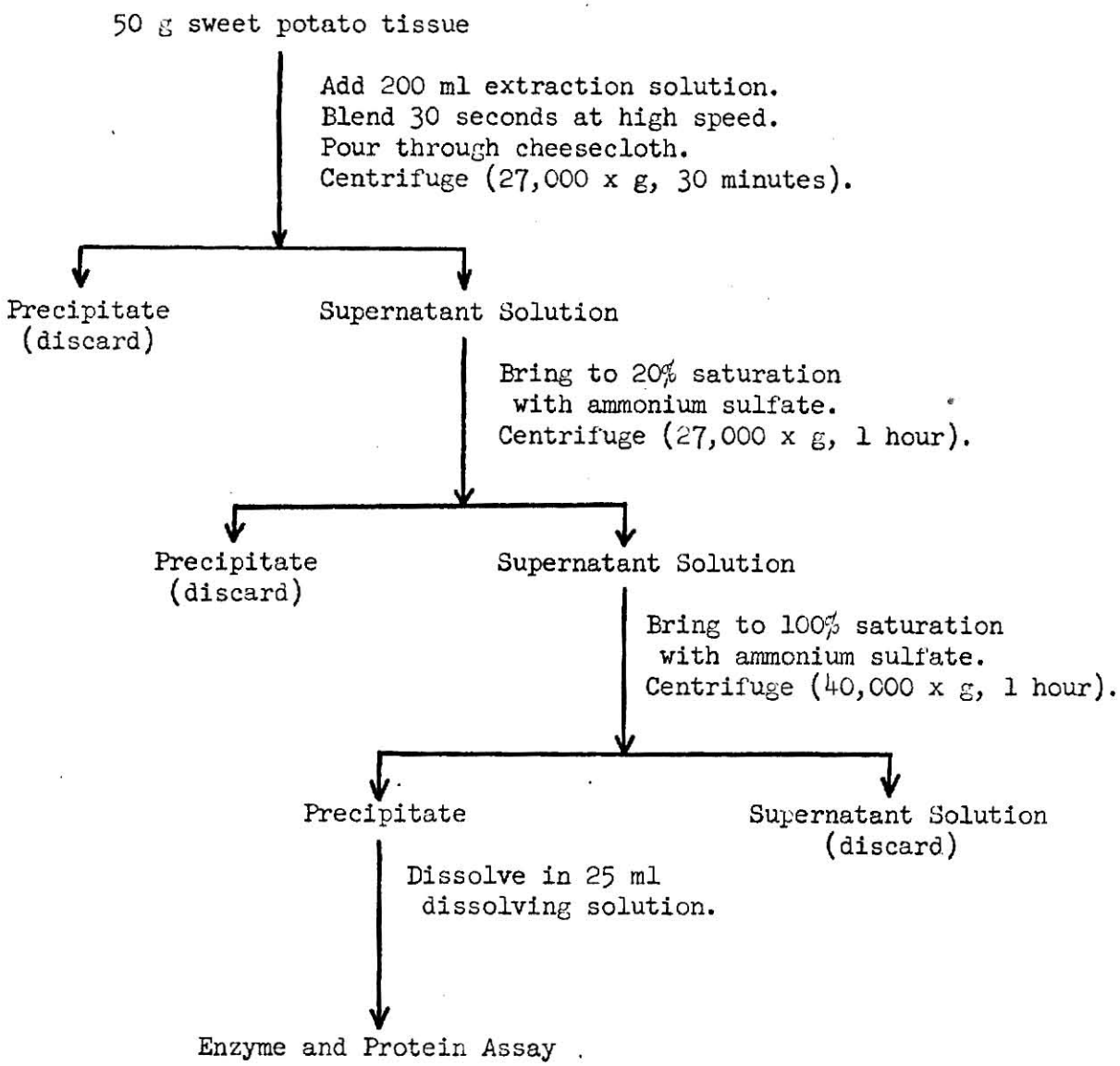


Figure 4. Flow Sheet for Extraction of Sweet Potato Tissue

Chapter 4

OPTIMIZATION STUDIES

Optimum Extraction Solution

A proposed extraction solution contained 0.05M phosphate buffer, pH 7; sodium sulfite; and 1% Polyclar AT. Sodium sulfite was used to prevent the browning effect. It is an antioxidizing agent and also inhibits peroxidase. Finding the correct concentration of sodium sulfite is important. Many workers have used sodium ascorbate, which has a similar role. Polyclar AT was used to absorb polyphenol compounds.

An experiment was set up with different concentrations of sodium sulfite. The results are given in Table 2.

Table 2

Optimum Extraction Solution
(Concentration of Sodium Sulfite)

	Concentration of Sodium Sulfite		
	None	0.005M	0.01M
Enzyme Activity (absorbance/min/ml)	48	56	48
Protein Concentration (mg/ml)	10.13	9.85	8.16

Observations: (1) The extraction solution containing no sodium sulfite became dark in color, indicating the browning effect. This may interfere with the Biuret determination of protein. (2) A concentration

of sodium sulfate above 0.005M decreased the activity of the enzyme. Therefore, the solution 0.05M phosphate buffer, pH 7, containing 0.005M sodium sulfate seemed to be the optimum extraction solution, and we used it throughout the investigation.

Optimum Dissolving Solution

The precipitate of protein and enzyme was dissolved for further measurements. The pH as well as the nature of the solution influences the determination of enzyme activity and protein concentration. Therefore, an experiment was set up to find optimum conditions for dissolving the precipitate.

Two buffer systems were proposed: Tris and phosphate. Tris buffer is known to be a good buffer, but we did not use it because we thought its hydroxyl groups might interfere with guaicol in the determination of peroxidase. We varied the molarity (0.05M and 0.1M) and pH of the phosphate buffer.

The results (Table 3, Figs. 5 and 6) demonstrated clearly that 0.05M phosphate buffer, pH 7, was optimum for the dissolving solution (see Discussion).

Effect of Ethylene Concentration

The sweet potato contains protein (including peroxidase). When the tissue is injured, it will produce ethylene. Ethylene in turn will stimulate the production of peroxidase (10-15) (and protein in general), but the increase is not very important. However, when a certain amount of exogenous ethylene is added to the incubation chamber containing sweet potato slices, at a certain time the increase in enzyme activity is tremendous. Proteins are also increased (49).

Table 3

Optimum Dissolving Solution (Phosphate Buffer)

	pH 6	pH 7	pH 8
0.05M phosphate buffer			
Enzyme Activity (absorbance/min/ml)	160	200	184
Protein Concentration (mg/ml)	5.2	6.1	5.5
0.1M phosphate buffer			
Enzyme Activity (absorbance/min/ml)	176	196	200
Protein Concentration (mg/ml)	4.87	5.8	5.7

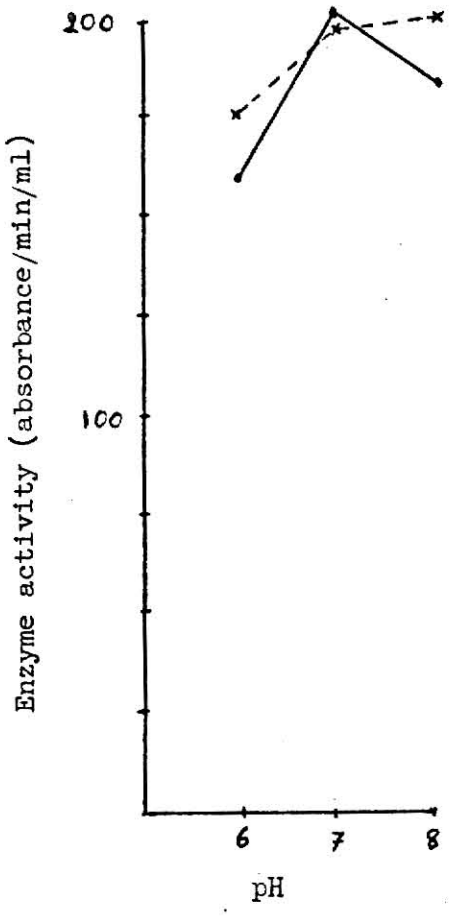


Figure 5. Optimum Dissolving Solution --Peroxidase Activity

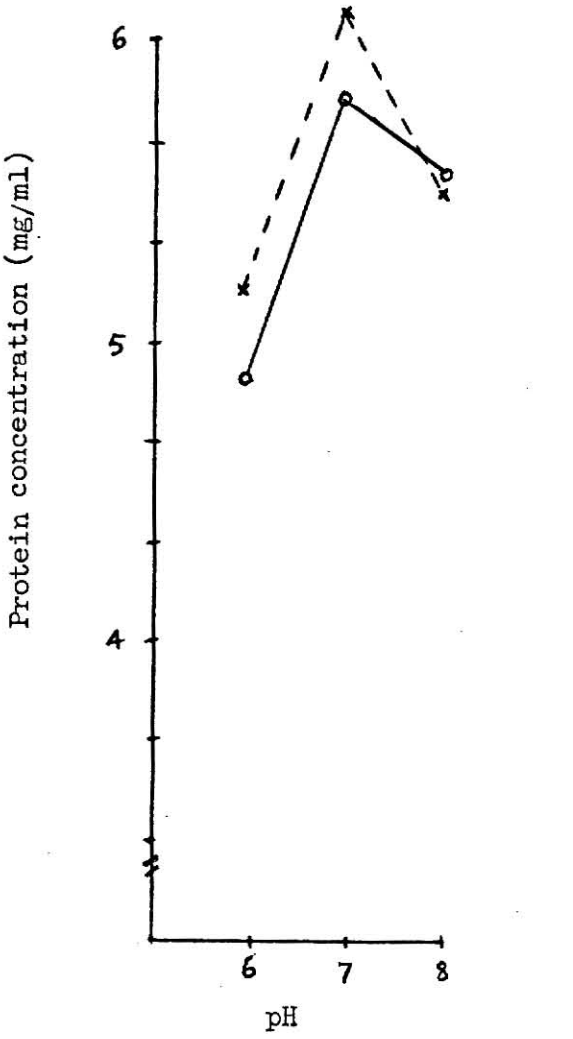


Figure 6. Optimum Dissolving Solution --Protein Determination

In the following experiment, sweet potatoes were cut into slices 3 mm thick. We divided these into three groups, each group consisting of many slices and weighing about 100 g. The first group was used as a control (fresh tissues); the second and third groups were incubated in separate high-moisture chambers with and without ethylene (5 ppm). In order to trap carbon dioxide (because carbon dioxide will inhibit enzyme and protein formation), a beaker containing potassium hydroxide was placed in each chamber. After incubation for 48 hours, these slices were homogenized, and enzyme and protein were analyzed as described previously in the Methods section of Experimental Procedures.

Table 4 shows clearly the effect of exogenous ethylene on peroxidase, as well as on protein in general. The concentration of ethylene and incubation time might play an important role. Sweet potato slices (3 mm thick) were incubated under the same conditions used previously except that the amount of ethylene varied. After a definite time the slices were taken out, homogenized, and analyzed for peroxidase and protein.

Enzyme activity increased from 56 units for the control to 320 units and 1,320 units for untreated and ethylene-treated tissue, respectively (Table 4). This showed clearly the effect of exogenous ethylene on the sweet potato slices. The sweet potato slices themselves also produced ethylene when incubated, and this was responsible for the increase in peroxidase from 56 units to 320 units. However, when exogenous ethylene was added, more enzyme was produced.

In contrast to enzyme activity, protein concentration increased slightly in the presence of exogenous ethylene. There appeared to be an increase after 48 hours' incubation. But when no exogenous ethylene was

Table 4

Comparison Between Untreated and Ethylene-Treated Tissues

	Control (fresh tissue)	Tissue incubated 48 hours	Tissue incubated 48 hours + 5 ppm ethylene
Enzyme Activity (absorbance/min/ml)	56	320	1,320
Relative Increase	No increase	5.7 times	24 times
Protein Concentration (%)	0.475	0.3	0.56
Relative Increase	No increase	No increase	1.2 times

added, after 48 hours' incubation, the amount of protein decreased and enzyme activity increased, to compare with the control. One explanation for that was that protein may be hydrolyzed in order to supply amino acids for enzyme synthesis. Therefore, the amount of total protein dropped when the activity of the enzyme increased.

Moreover, as it was suggested by Abeles and Holm (67) that the cell might use nucleic acids and some non-protein nitrogen as the nitrogen source to synthesize protein, their suggestion could be true in our case; the amount of protein increased when 5 ppm of ethylene were added.

Results shown in Table 5 and Figs. 7 and 8 indicate that there was an increase in enzyme activity when tissue was incubated with 20 ppm of exogenous ethylene. However, there was no increase in total protein. It is suggested that the high level of ethylene may inhibit protein synthesis instead of stimulate it.

Table 5

Effect of 20 ppm Exogenous Ethylene on Enzyme Activity and Protein Concentration

	Incubation time (hours)				
	0	24	48	72	96
Enzyme Activity (absorbance/min/ml)	176	296	1,400	5,120	2,800
Relative Increase (no. of times)	No increase	1.6	8	29	15
Protein Concentration %	1.16	1.07	0.80	0.83	0.95

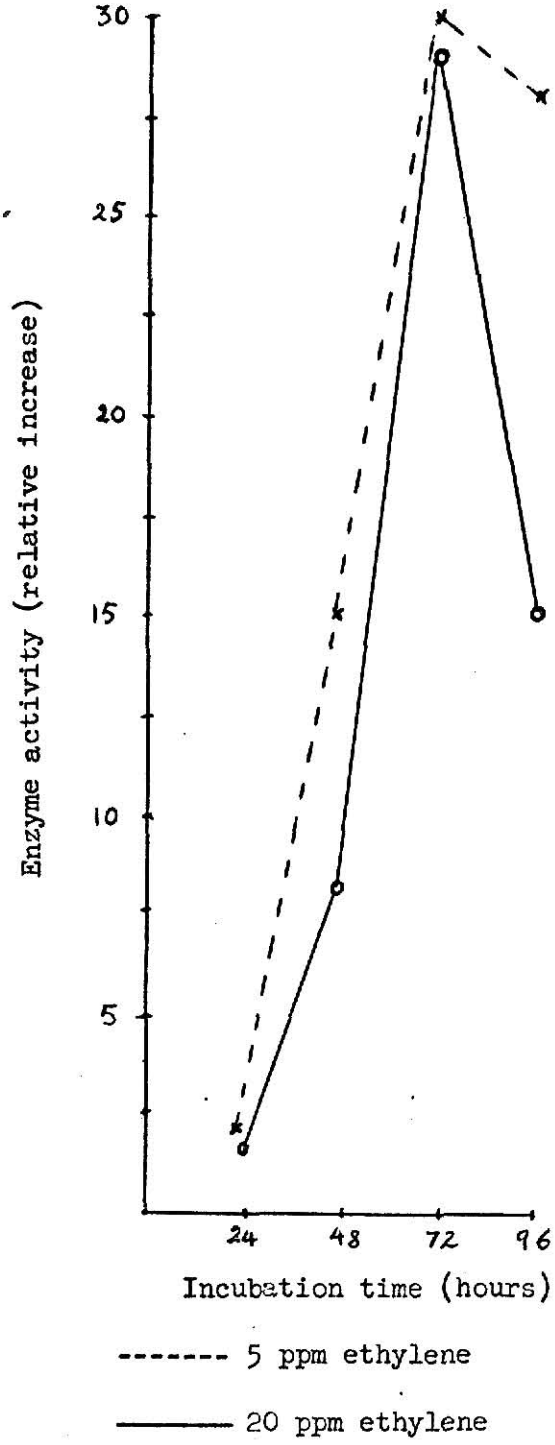


Figure 7. Increase in Peroxidase Activity in Sweet Potato Tissue at Different Concentrations of Exogenous Ethylene

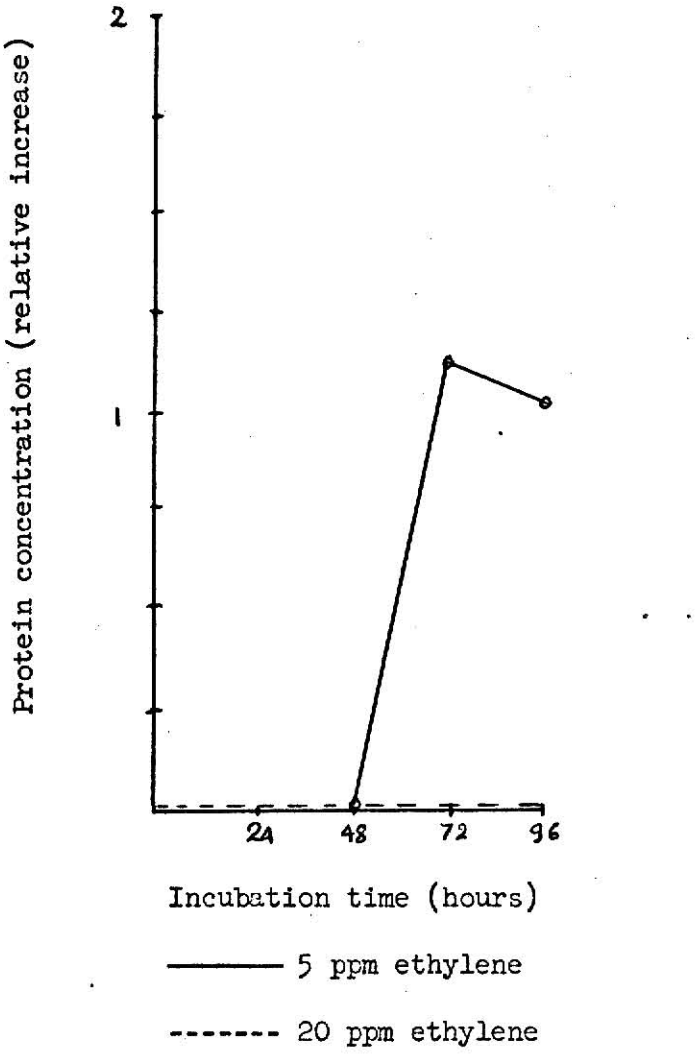


Figure 6. Increase in Protein Concentration in Sweet Potato Tissue at Different Concentrations of Exogenous Ethylene

When 5 ppm of ethylene were used, the results (Table 6 and Figs. 7 and 8) showed an increase in enzyme activity as well as protein concentration, especially after incubation for 72 hours. After 72 hours, both enzyme and protein began to decrease.

Table 6

Effect of 5 ppm Exogenous Ethylene on Enzyme Activity and Protein Concentration

	Incubation time (hours)				
	0	24	48	72	96
Enzyme Activity (absorbance/min/ml)	92	184	1,360	2,720	2,560
Relative Increase (no. of times)	No increase	2	15	30	28
Protein Concentration (%)	0.8	0.76	0.66	0.9	0.83
Relative Increase (no. of times)	No increase	No increase	No increase	1.125	1.04

Effect of Ethylene and Nitrogen Compounds

From the foregoing observations, it was suggested that the cells of the sweet potato might use some nitrogen compounds in order to synthesize protein. This suggestion led us to the following preliminary experiment. We considered three compounds: ammonium hydroxide, ammonium hydrogen phosphate, and potassium nitrate. Although ammonium hydroxide is a good source of nitrogen, we did not use it because slices of sweet potato became dark in color when they were dipped in ammonium hydroxide solution, even at low concentration. Therefore, 0.05M solutions of

ammonium hydrogen phosphate and potassium nitrate were used in this experiment.

Sweet potatoes, cut into slices 3 mm thick, were divided into two groups, each consisting of many slices and weighing about 200 g. The first group was immersed in 0.05M ammonium hydrogen phosphate and the second group in 0.05M potassium nitrate for about two hours. After that, the slices were taken out, wiped dry with filter paper, and incubated in high moisture chambers containing 5 ppm of ethylene. After a definite time, these slices were taken out, homogenized, and assayed for peroxidase activity and protein concentration as described in the Methods section of Experimental Procedures. The results are shown in Tables 7 and 8 and Figs. 9 and 10.

Table 7

Effect of Ammonium Hydrogen Phosphate on Enzyme Activity and Protein Concentration (5 ppm Ethylene and 0.05M Ammonium Hydrogen Phosphate)

	Incubation time (hours)		
	0 (control, fresh tissue)	72	120
Enzyme Activity (absorbance/min/ml)	24	1,760	4,800
Relative Increase (no. of times)	No increase	70	192
Protein Concentration (%)	0.31	0.16	0.35
Relative Increase (no. of times)	No increase	No increase	1.13

Table 8

Effect of Potassium Nitrate on Enzyme Activity and Protein Concentration (5 ppm Ethylene and 0.05M Potassium Nitrate)

	Incubation time (hours)		
	0 (control, fresh tissue)	72	120
Enzyme Activity (absorbance/min/ml)	24	1,920	2,632
Relative Increase (no. of times)	No increase	77	105
Protein Concentration (%)	0.31	0.18	0.26

These results suggest that the cells can use both ammonium hydrogen phosphate and potassium nitrate for the synthesis of enzyme. However, there was no increase in protein in the case of potassium nitrate and only a small increase after 120 hours' incubation for ammonium hydrogen phosphate. Contrary to the results of the previous experiment, the longer we incubated the more protein and enzyme we obtained.

The concentration of salts may be critical. Therefore, it was necessary to ascertain which concentration was best to obtain the maximum amount of enzyme and protein. We used 0.01M, 0.05M, and 0.2M ammonium hydrogen phosphate and obtained the results found in Tables 9 and 10.

From these results we drew the following conclusions. It seemed that 0.05M ammonium hydrogen phosphate was the best concentration to give maximum enzyme activity (200 to 220 times that of the control) and

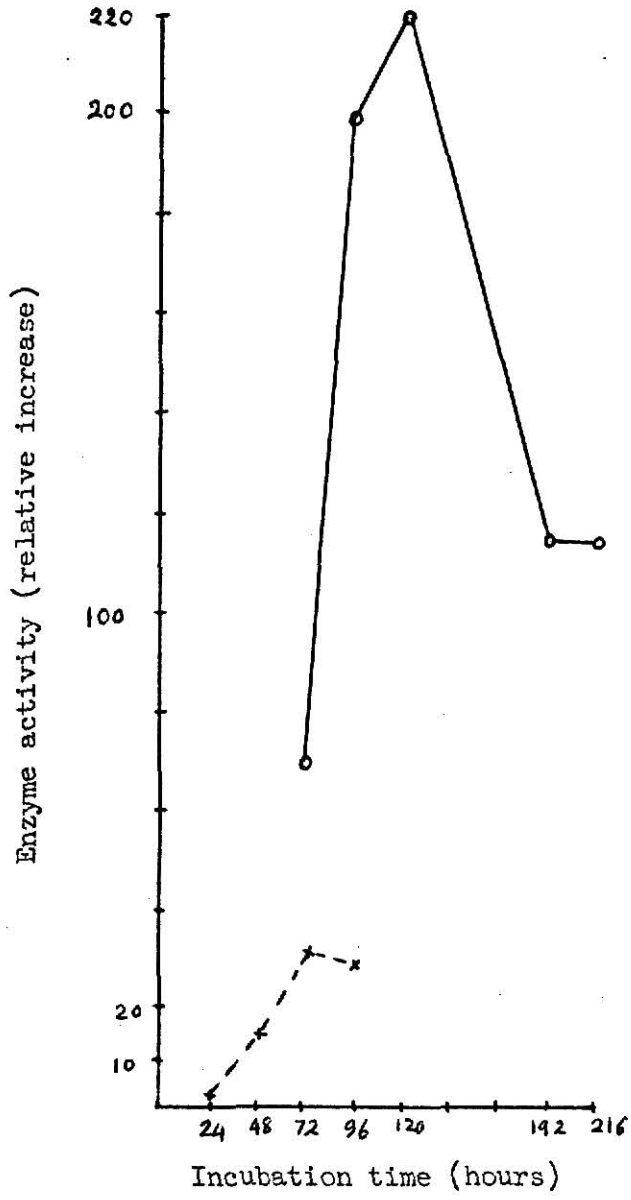
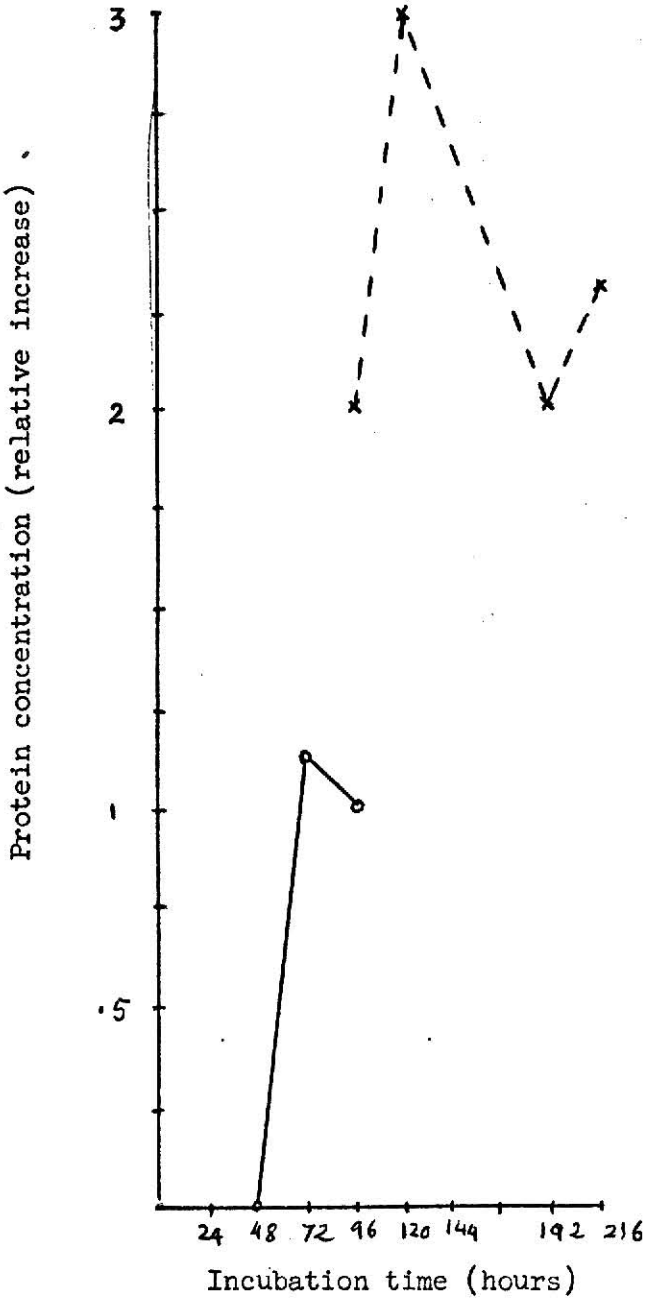


Figure 9. Increase of Enzyme Activity in Tissue Treated with Ethylene and Ethylene Plus 0.05M Ammonium Hydrogen Phosphate



------- 5 ppm ethylene + 0.05M ammonium hydrogen phosphate

——— 5 ppm ethylene

Figure 10. Increase in Protein Concentration in Tissue Treated with Ethylene and Ethylene Plus 0.05M Ammonium Hydrogen Phosphate

Table 9

Effect of Ammonium Hydrogen Phosphate Concentration and Time (96 Hours) on Enzyme Activity and Protein Concentration

Ammonium hydrogen phosphate concentration	Enzyme activity (absorbance/min/ml)	Protein concentration (%)
0.00	24	0.31
0.01M	400 (16 times)	0.54 (1.8 times)
0.05M	5,120 (200 times)	0.62 (2 times)
0.2M	3,520 (140 times)	0.525 (1.75 times)

Table 10

Effect of Ammonium Hydrogen Phosphate Concentration and Time (120 Hours) on Enzyme Activity and Protein Concentration

Ammonium hydrogen phosphate concentration	Enzyme activity (absorbance/min/ml)	Protein concentration (%)
0.00	24	0.31
0.01M	480 (19 times)	0.675 (2 times)
0.05M	5,520 (220 times)	0.875 (3 times)
0.2M	3,280 (130 times)	0.875 (3 times)

protein concentration (two to three times that of the control). Incubation time was another factor that should be considered. Both enzyme activity and protein concentration decreased at incubation times longer than 120 hours, as shown in Table 11. Therefore, the best conditions for incubation were 0.05M ammonium hydrogen phosphate incubated for 120 hours.

Table 11

Effect of Incubation Time on Enzyme Activity and Protein Concentration (5 ppm Ethylene and 0.05M Ammonium Hydrogen Phosphate)

	Incubation time (hours)		
	0	192	246
Enzyme Activity (absorbance/min/ml)	56	6,400	6,600
Relative Increase (no. of times)	No increase	114	114
Protein Concentration (%)	0.4	0.8	0.92
Relative Increase (no. of times)	No increase	2	2.3

One striking factor we noticed was that the rate of ethylene production by root tissue varied significantly with different individual roots. Hence, the enzyme activity and the amount of enzyme varied. It was often observed that the tissue slices prepared from one root produced nearly three times that of the other root. Therefore, the results of the control (fresh tissue) varied from experiment to experiment, which is in agreement with the experience of Imaseki et al. (15).

Ion Exchange Chromatography

Different amino acids are sorted out by the differences in their acid-base behavior. In the process the column is filled with a synthetic resin containing fixed charged groups. There are two major classes of ion exchange resins: cation exchangers and anion exchangers. Amino acids are usually separated on cation exchange columns filled with solid particles of a sulfonated polystyrene resin previously equilibrated with a sodium hydroxide solution so that its sulfonic acid groups are fully charged with sodium ion. This form of the resin is the sodium form. The resin may also be prepared in the protonated form, or hydrogen form, by washing it with acid. To the sodium ion form of the resin is added the crude extraction solution which was previously adjusted to pH acid (pH 5, for example). At this pH, amino acids of the enzyme are largely cations with net positive charge. The cationic amino acids tend to displace some of the bound sodium ions from the resin particles. As the pH and the sodium chloride concentrations of the eluting aqueous medium are gradually increased, the amino acids move down the column at different rates and can be collected in many small fractions.

In this investigation we used a DEAE-cellulose column for absorbing anionic peroxidases and a CM-cellulose column for cationic peroxidases.

Chapter 5

RESULTS AND DISCUSSION

A great number of methods are available for the extraction of plant protein. However, data on the relative advantages of these methods are rare. Proteins vary greatly in their ionic properties; therefore, the yield of different proteins will depend on the method of extraction. Usually the extraction is carried out in a medium of low ionic strength and a neutral pH as these two factors seem to favor the preservation of proteins in their native state (68). The results shown in Tables 2 and 3 indicated the influence of pH and molarity on enzyme activity and protein concentration.

A number of workers have shown that peroxidase activity of incubating sweet potato root discs increases with time (69, 46). We duplicated these results (Tables 4 and 5). However, the increase was not as great as that reported by Shannon et al. (49). The increase was maximum after 72 hours' incubation, then dropped afterwards. This fact can be explained by many reasons such as damaged tissue or lack of nitrogen source to synthesize the new enzymes and protein. The first reason seems improbable because we did not observe any damage of the tissue after 72 hours' incubation. Therefore, the most reasonable explanation is lack of a nitrogen source. Fortunately, the results found in Tables 7, 8, and 9 supported our hypothesis when we used ammonium hydrogen phosphate as the nitrogen source to supply the cells. Of course, after a long period of time (about eight or nine days), the tissue

became damaged. This explained why the activity of the enzyme decreased.

In order to examine the effect of different levels of exogenous ethylene on sweet potato tissue in relation to the injury caused by cutting, 3 mm-thick slices of roots were incubated for zero, 24, 48, 72, and 96 hours with two levels of ethylene, 5 ppm and 20 ppm. The results (Tables 5 and 6) showed that at certain levels ethylene, instead of acting as a stimulant, became an inhibitor of protein synthesis, as Imaseki (46) had observed.

A question may be asked: Is the increase in enzyme activity and protein concentration due to activation of a precursor of the enzyme or due to de novo synthesis? The question will be answered clearly when we look at the results given by electrophoresis.

For ethylene-treated tissue (Fig. 11), seven bands, numbered 1 to 7, were obtained on agarose electrophoresis. For the control, five bands appeared (bands 2, 3, 5, 6, and 7). After incubating, new bands appeared (bands 1 and 4) and all old bands were strongly enhanced.

For ethylene- and ammonium hydrogen phosphate-treated tissue (Fig. 12), four bands (bands 2, 3, 5, and 6) appeared for the control. After incubating, new bands were seen (bands 1, 4, and 7), and all old bands were strongly stimulated.

These results showed that the increase in peroxidase activity was due both to de novo synthesis of new isoenzymes and to activation of the precursors of the enzyme already existing.

Our hypothesis was strengthened when we examined thin-layer polyacrylamide gel isoelectric focusing. For ethylene-treated tissue, the control (Fig. 13) showed four weak bands (E1, E5, E9, and E11). After

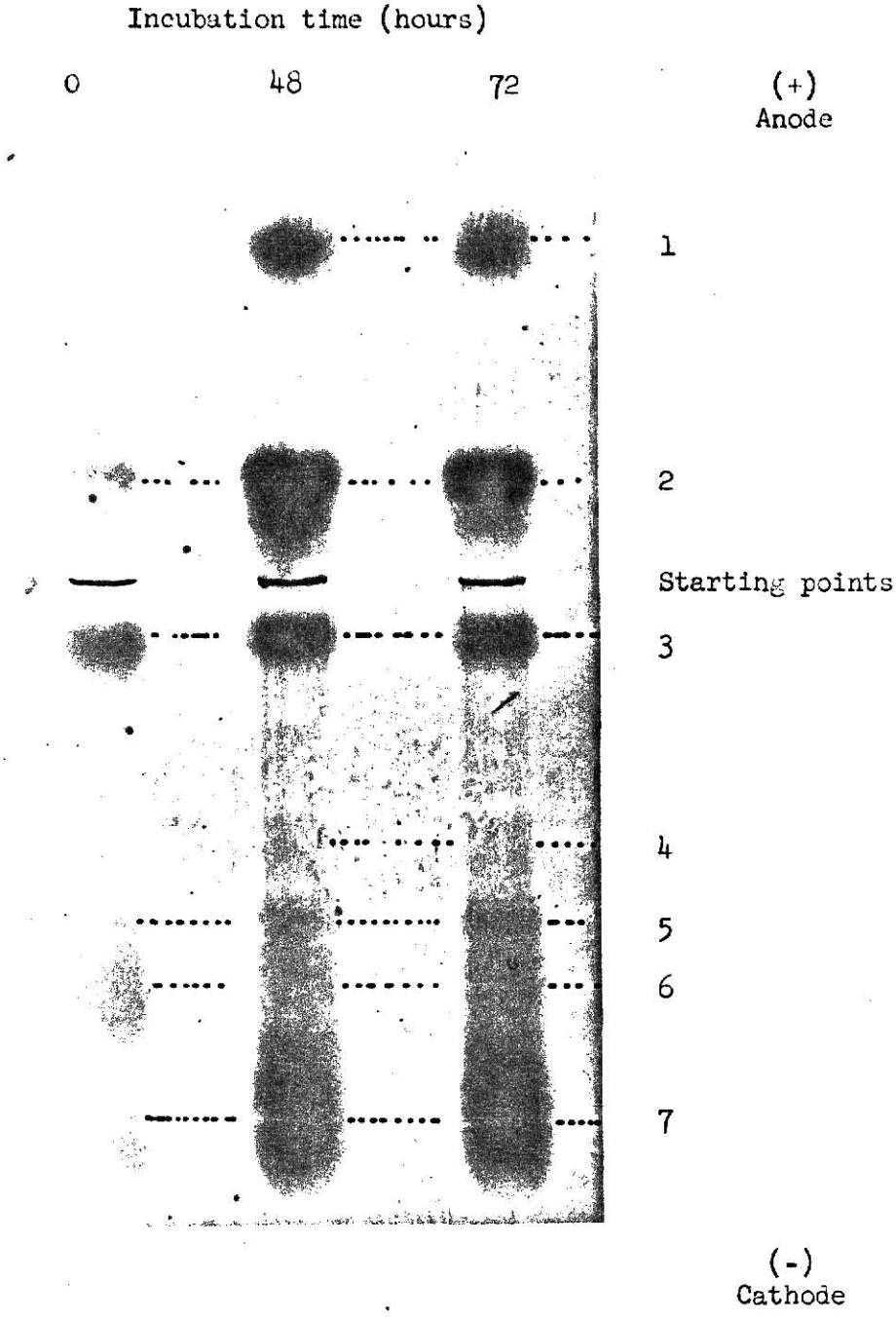


Figure 11. Agarose Electrophoresis (Ethylene Effect).

Description: Electrophoretogram of the activity of the enzyme of sweet potato slices which had been incubated in a high moisture chamber containing 5 ppm ethylene. Maximum activity at 72 hours, then decreased (see Fig. 9). Amount of sample applied: 25λ. Running time: 5 hours.

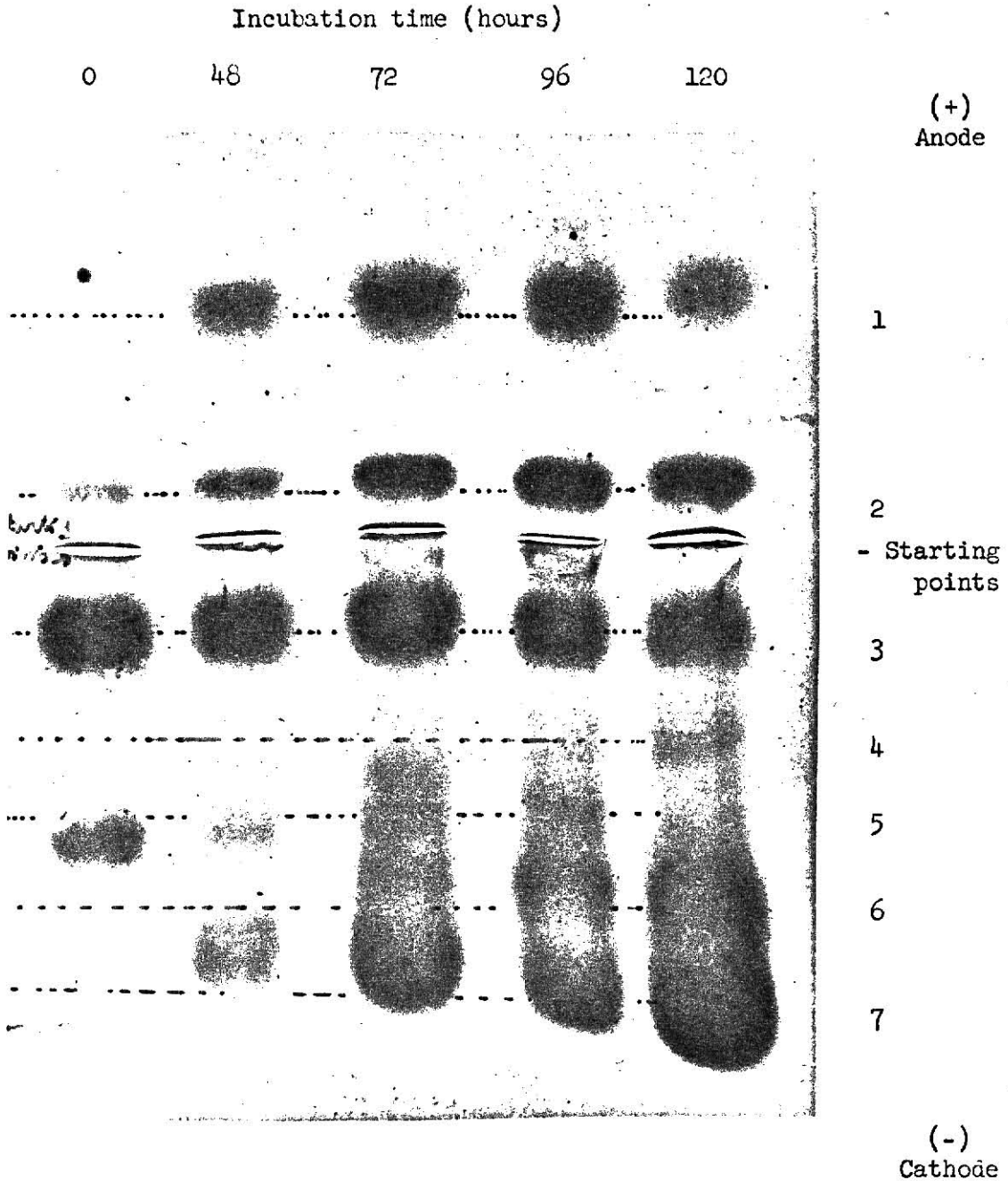


Figure 12. Agarose Electrophoresis (Ethylene and Ammonium Hydrogen Phosphate Effect)

Description: Electrophoretogram of the activity of the enzyme of sweet potato slices which had been immersed in a 0.05M solution of ammonium hydrogen phosphate for three hours, then incubated in chambers containing 5 ppm of ethylene. Maximum activity at 120 hours (see Fig. 9). Amount of sample applied: 25λ. Running time: 3 hours.

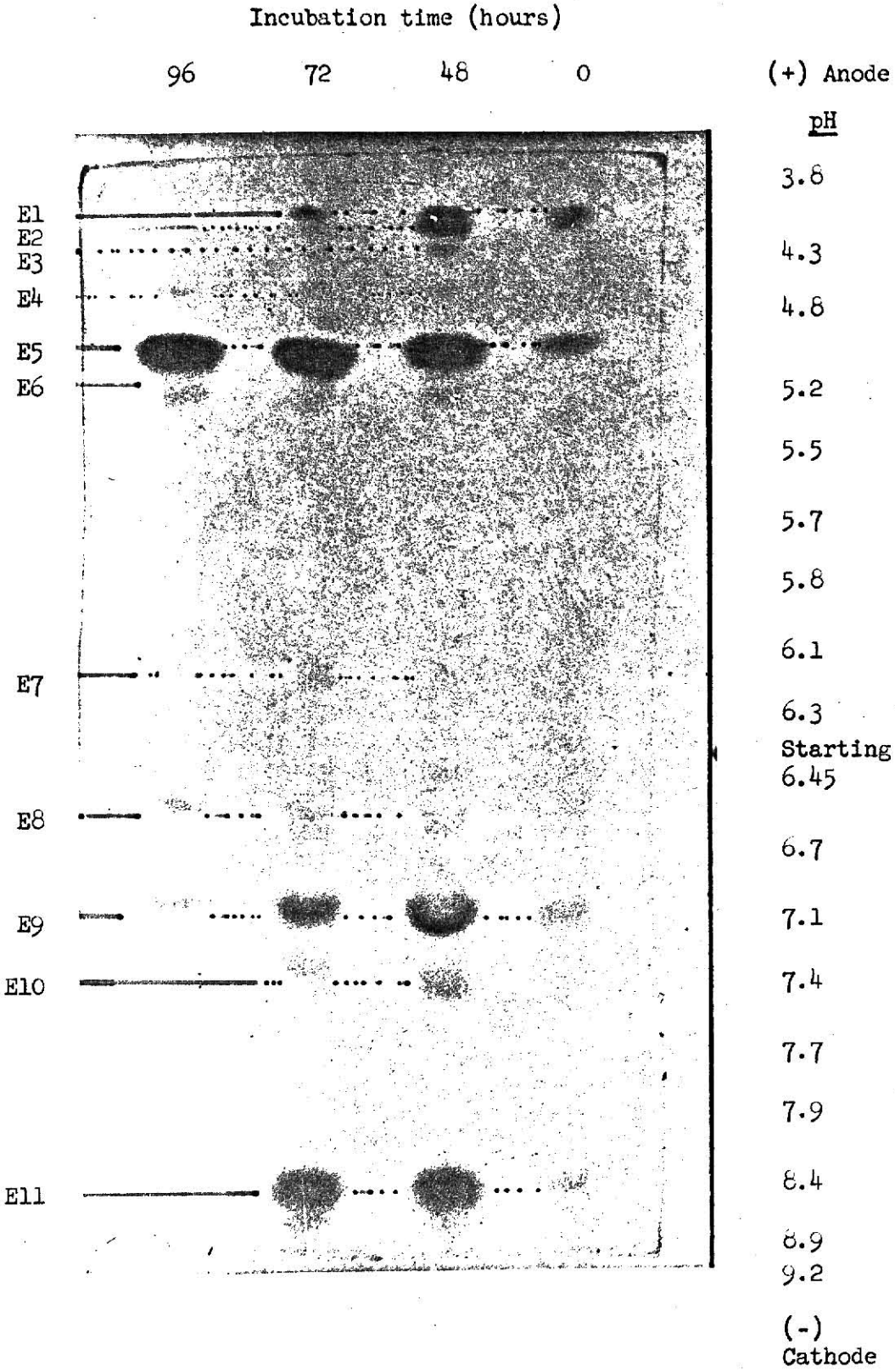


Figure 13. Thin-Layer Polyacrylamide Gel Isoelectric Focusing (Ethylene Effect)

Description: Sweet potato tissues were incubated for zero, 48, 72, and 96 hours in a chamber containing 5 ppm ethylene. Maximum activity at 72 hours. Amount of sample applied: 2λ. Focusing time: 24 hours.

incubating, new bands appeared (E2, E3, E4, E6, E7, E8, and E10), and after 72 hours' incubation some bands disappeared (E3 and E6).

When we looked at two focusing electrophoretograms, we saw that the pattern shown in Fig. 13 was somewhat different from that in Fig. 14. In Fig. 13, from pH 6 to 7.7, only four weak bands were seen (E7, E8, E9, and E10); in Fig. 14, in the same pH range, we saw a long strip with three strong bands (EA8, EA9, and EA10). This showed clearly the involvement of ammonium hydrogen phosphate in the synthesis of protein and enzymes.

There was no doubt that de novo synthesis of enzyme and protein was responsible for the increase in the concentration after incubation for a period of time. However, it seemed to us that this was not adequate. Not only de novo synthesis, but stimulation of the precursors of the enzymes which already existed are the reasons for the increase in the activity of the enzyme. Gahagan et al. (69) reported the same as we had observed, using protein biosynthesis inhibitors.

On the other hand, Kanazawa et al. (48) reported that peroxidase formation in sweet potato slices was prevented not only by inhibitors of protein synthesis, but also by those of the respiratory system. Peroxidases in sweet potato are homoprotein containing polysaccharide (70, 71). Inhibition may be caused by suppression of enzymes involved in formation of either protoheme or polysaccharide.

Multiple forms of peroxidases also were detected by using DEAE- and CM-cellulose column chromatography. The tissue extract was chromatographed on a column (2 x 15 cm) of DEAE-cellulose, which previously had been calibrated with 0.005M Tris-hydrochloric acid buffer, pH 8.5. That fraction of peroxidase which was absorbed on the

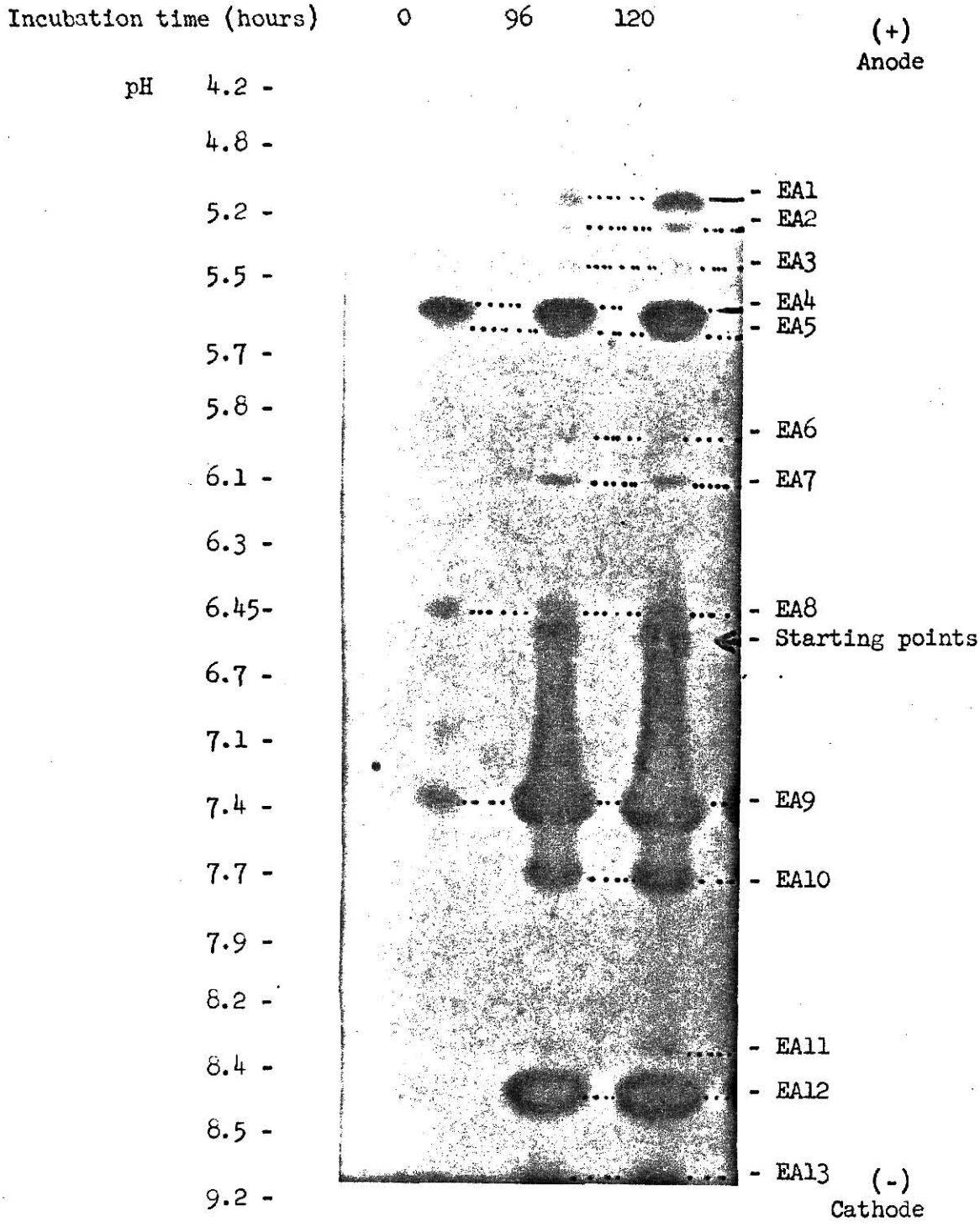


Figure 14. Thin-Layer Polyacrylamide Gel Isoelectric Focusing (Ethylene Plus Ammonium Hydrogen Phosphate Effect)

Description: Sweet potato tissues immersed in 0.05M ammonium hydrogen phosphate for three hours were incubated in 5 ppm ethylene. Maximum activity at 120 hours. Amount of sample applied: 2λ. Focusing time: 24 hours.

DEAE-cellulose column was eluted with a linear gradient in salt concentration (0.025M Tris-hydrochloric acid buffer, pH 8.5, plus 0.25M sodium chloride). The eluted fraction was designated as anionic peroxidases.

The fraction of peroxidase which was not absorbed to DEAE-cellulose was dialyzed for 24 hours against 0.005M sodium acetate, pH 5, and passed through a column (2 x 15 cm) of CM-cellulose previously equilibrated with 0.005M sodium acetate, pH 5. That fraction of peroxidase which was absorbed to the CM-cellulose column was eluted batchwise with 0.05M sodium acetate, pH 5, plus 0.5M sodium chloride. The eluted fraction was designated cationic peroxidases. The fraction of peroxidase which was not absorbed to CM-cellulose was collected and designated neutral peroxidase.

Fig. 15 presents the elution profile of peroxidase activity obtained from the DEAE-cellulose column (anionic fraction). Three major peaks and several minor ones were obtained. Only one peak of peroxidase activity (cationic peroxidase) was obtained from the column of CM-cellulose (Fig. 16). No peroxidase activity was found in the neutral fraction. We obtained a total of 28% recovery of all three fractions from a crude extract, which had 10,000 absorbances per minute.

Fig. 17 gives the electrophoresis patterns of these peaks on agarose gel, which indicate the purification of the enzyme after passing the DEAE- and CM-cellulose columns. Peak 1, corresponding to the tube number 7 of the DEAE-cellulose column, was not pure, but peaks 2 and 3 were pure cationic and anionic peroxidase.

The result obtained from our investigation may be applied to produce high protein food. As shown in Table 10, the amount of protein

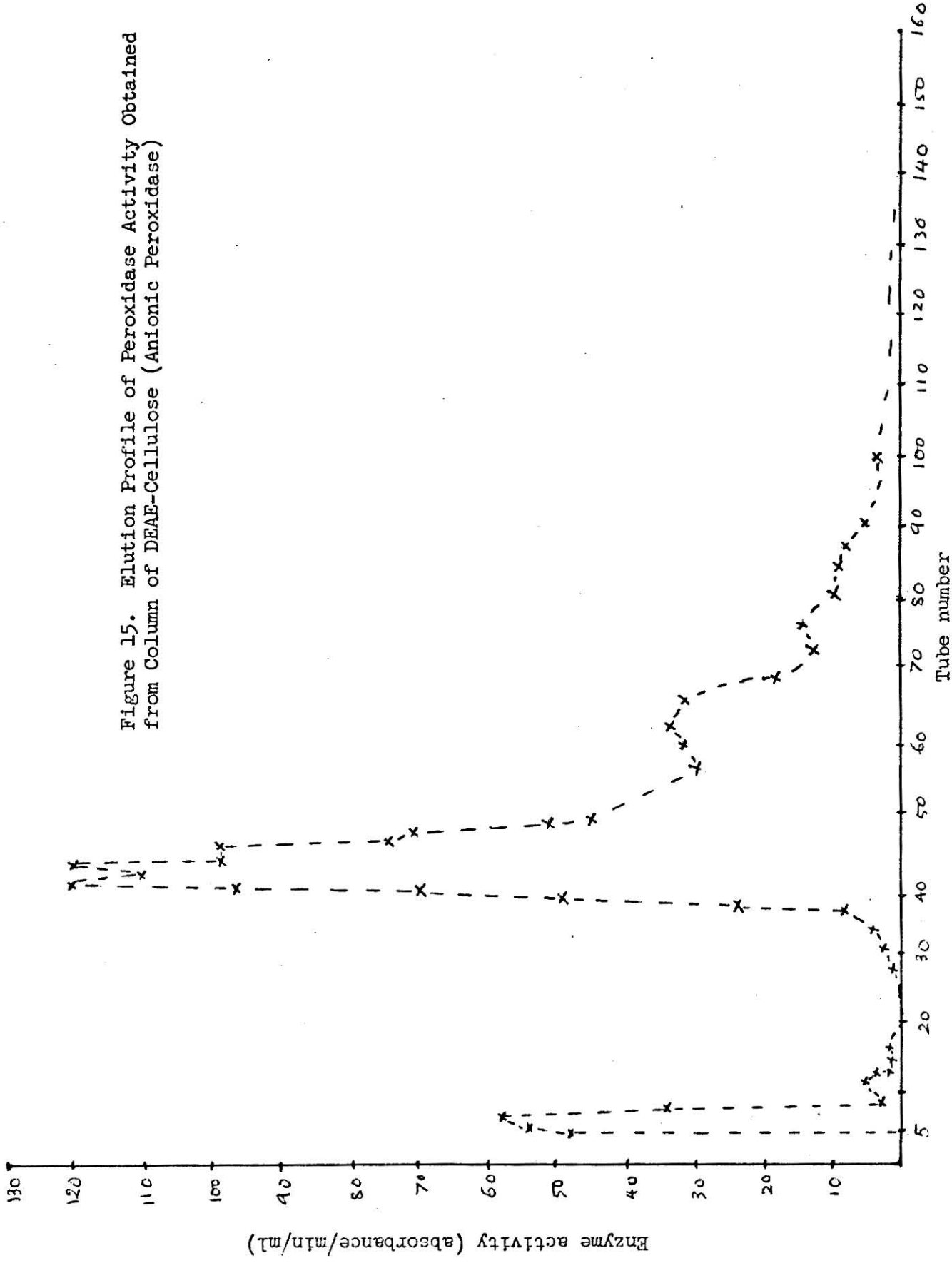
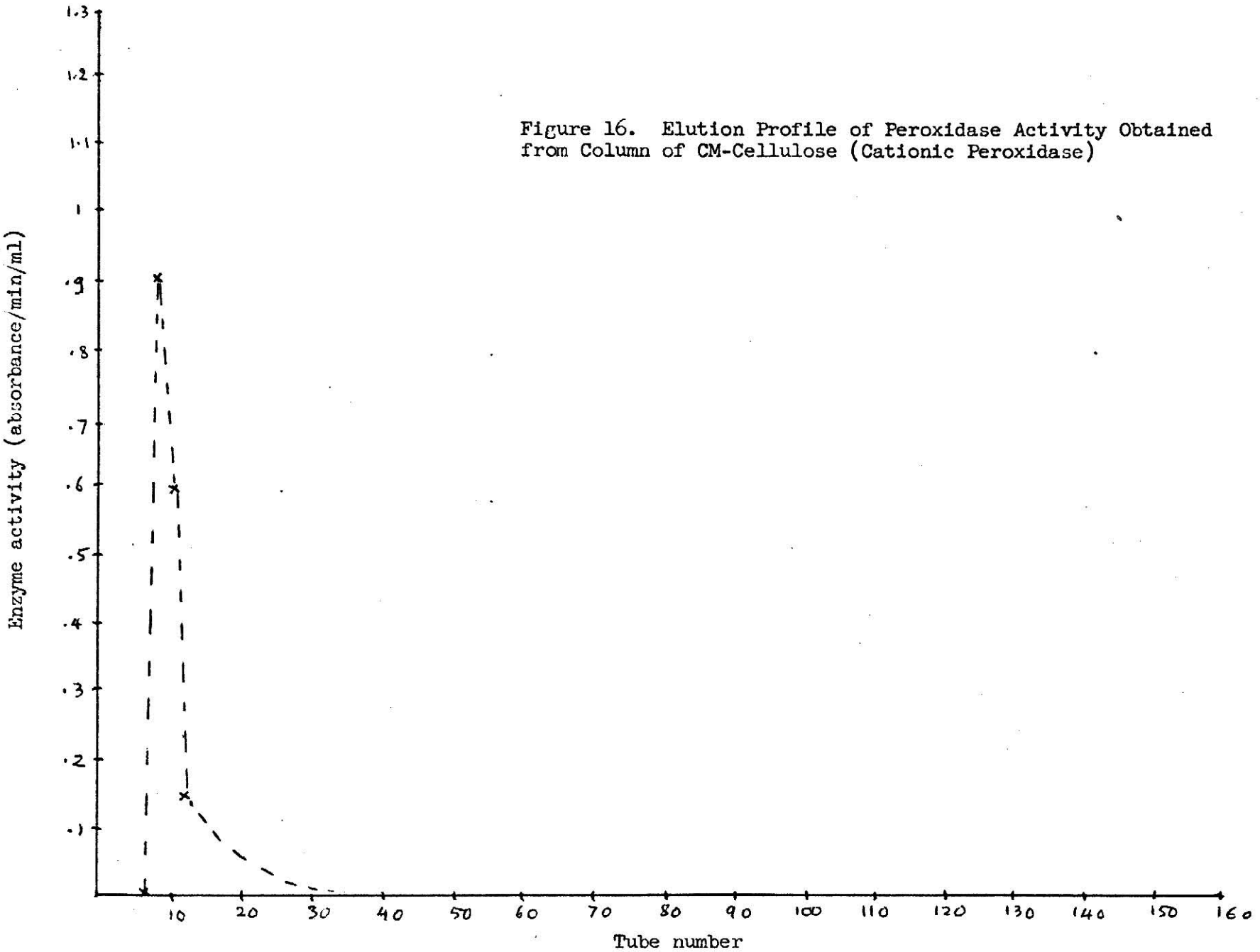


Figure 15. Elution Profile of Peroxidase Activity Obtained from Column of DEAE-Cellulose (Anionic Peroxidase)

Figure 16. Elution Profile of Peroxidase Activity Obtained from Column of CM-Cellulose (Cationic Peroxidase)



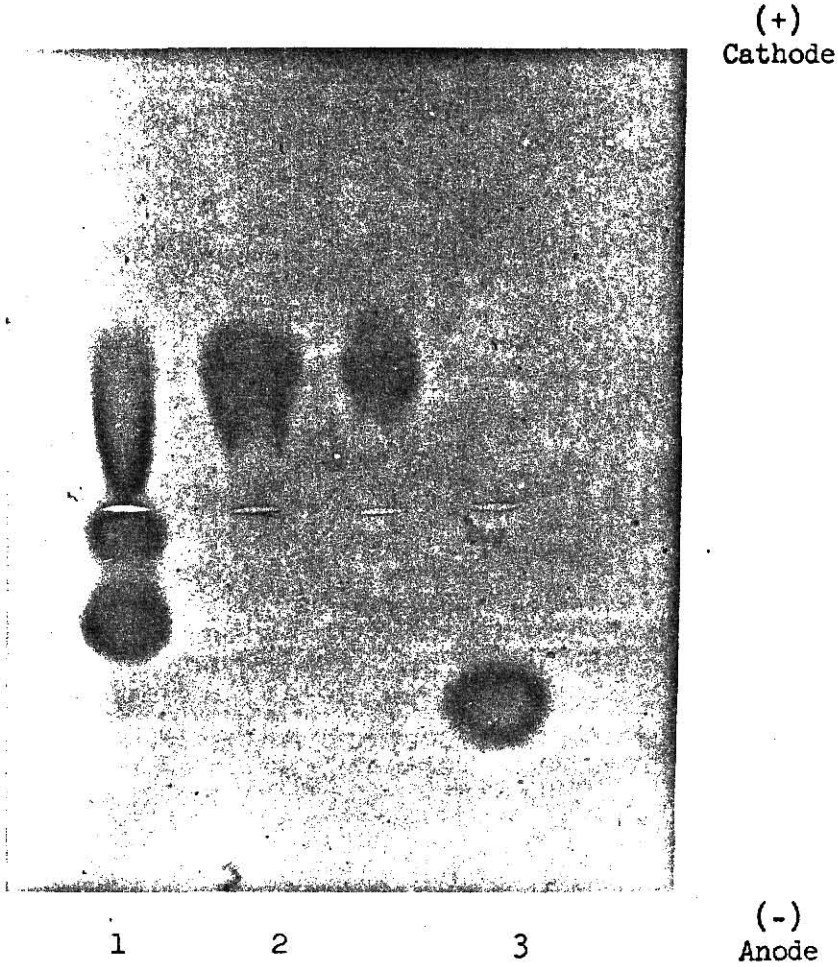


Figure 17. Pattern of Peaks from Agarose Electrophoresis

Description: Peak 1 and 2 of DEAE-cellulose column.
Peak 3 of CM-cellulose column.

increased threefold after treatment with ammonium hydrogen phosphate. This is very important because we know that large segments of the population of developing countries suffer from the effects of protein deficiency in their diet.

Food legumes are major sources of protein and other nutrients in the diets of many developing countries. However, they have been seriously neglected in terms of research necessary to increase their yield and to correct certain defects in the nutritional and food use quality.

It is our hope that this investigation will stimulate further research in the production of plant food with high protein content. High-protein plant food would hold considerable promise for solving world protein shortages.

Chapter 6

CONCLUSIONS

From our investigation, we reached the following conclusions:

(1) The best solution for extracting the proteins (and enzymes) from sweet potato would be 0.05M phosphate buffer, pH 7, containing 0.005M sodium sulfite.

(2) The optimum dissolving solution would be 0.05M phosphate buffer, pH 7, too.

(3) Exogenous ethylene optimally increases the formation of proteins (including peroxidase) at concentration 5 ppm.

(4) Diammonium hydrogen phosphate appears to be a nitrogen source for synthesizing proteins (including enzymes) as it significantly extends the time period over which increases occur in the presence of 5 ppm ethylene.

(5) Time of incubation is an important factor.

(6) The increase in peroxidase concentration is probably due to de novo synthesis, based on the appearance of new electrophoretic bands, as well as enhanced activity in bands from the control. However, activation of peroxidase precursors cannot be ruled out. Time did not allow inhibitor or tritium labeling studies.

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EFFECT OF ETHYLENE AND DIAMMONIUM HYDROGEN PHOSPHATE ON PEROXIDASE AND PROTEIN DEVELOPMENT IN SWEET POTATO SLICES

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ABSTRACT

Conditions for the extraction and dissolution of the peroxidase and protein content of the sweet potato were studied. Both pH and molarity of both extraction and dissolution solution played an important role in the recovery of the protein and enzyme. When the tissue of the sweet potato was injured, it produced ethylene. Ethylene in turn acted as a stimulus for the formation of the enzyme and protein. When exogenous ethylene was added, more protein and enzyme were produced. However, after 72 hours of incubation, both enzyme activity and protein content dropped sharply. When the tissue was dipped in a solution of diammonium hydrogen phosphate, protein concentration and enzyme activity were increased--about 200 to 220 times more for peroxidase and two to three times more for protein--compared with the control (fresh tissue). Electrophoresis indicated that the increased enzyme activity might be due to de novo synthesis of the enzyme. The potential application of this investigation to produce a high-protein food was discussed briefly.