To the Graduate Council:

I am submitting herewith a thesis written by Susan Carri Sutherland entitled "Activity of a Streptococcal Proteinase on Soybean Protein." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

J. O. Mundt, Major Professor

We have read this thesis and recommend its acceptance:

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Accepted for the Council:

Vice Chancellor
Graduate Studies and Research

Thesis
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ACTIVITY OF A STREPTOCOCCAL PROTEINASE ON SOYBEAN PROTEIN

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Susan Carri Sutherland

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ABSTRACT

The objective of this investigation was to determine the activities of an extracellular proteinase produced by Streptococcus faecalis var. liquefaciens on Promine-D and to identify and measure the end products of the proteolytic action.

The enzyme was isolated from skim milk by ammonium sulfate fractionation and then lyophilized. The enzyme, lyophilized fraction, concentration was determined by ultraviolet absorption at 260 and 280 nm. Proteolytic activity was demonstrated on the protein substrate Promine-D.

A study of the effect of environmental conditions on the proteinase showed that optimum conditions for activity were at pH 7.5 and 43°C. Tyrosine was measured colorimetrically after enzymatic hydrolysis. Amino acids hydroxyproline, serine, tyrosine and methionine were released by enzymatic action from the protein substrate. The relative amounts of amino acids which increased due to enzymatic hydrolysis were valine, glycine, leucine, proline, threonine, phenylalanine, and glutamic acid.

TABLE OF CONTENTS

CHAPTER		PAGE
I.	SURVEY OF THE LITERATURE	1
II.	MATERIALS AND METHODS	5
	Organism	5
	Culture Medium	5
	Standard Inoculum	5
	Contamination Check	5
	Substrate	6
	Shaker Bath	6
	Measurement of pH	6
	Spectrophotometric Measurements	6
	Freeze-Drier	6
	Centrifugation	6
	Gas-Liquid Chromatography	7
	Procedure for the Isolation and Purification of Streptococcal Proteinase	7
	Determination of Proteolytic Activity	8
	Determination of Tyrosine Content	9
	The Standard Tyrosine Curve	9
	Determination of Protein Concentration in the Enzyme Preparation	10
	The Effect of Temperature and Substrate Concentration on the Activity of the Enzyme	10
	Separation of Free Amino Acids from Unhydrolyzed and Enzyme Hydrolyzed Promine-D	12
	Derivitization Procedure	13
	Statistical Design and Analysis	14
III.	RESULTS AND DISCUSSION	16
	Model for the Equation	16
	Results with Gas-Liquid Chromatography	23
REFERENCES		33
VITA		36

LIST OF FIGURES

FIGURE		PAGE
1.	Standard Tyrosine Curve	11
2.	Response of Tyrosine µg/2 ml Produced as a Function of Substrate Concentration and Temperature with pH Set at 7.5	21
3.	Response of Tyrosine µg/2 ml Produced as a Function of Substrate Concentration and pH with Temperature Set at 43°C	22
4.	Column A Gas-Liquid Chromatographic Separation of N-TFA n-Butyl Esters of Amino Acid Standards	24
5.	Column A Gas-Liquid Chromatographic Separation of N-TFA n-Butyl Esters of Unhydrolyzed Promine-D	25
6.	Column A Gas-Liquid Chromatographic Separation of N-TFA n-Butyl Esters of Hydrolyzed Promine-D	26
7.	Column B Gas-Liquid Chromatographic Separation of N-TFA n-Butyl Esters of Amino Acid Standards	27
8.	Column B Gas-Liquid Chromatographic Separation of N-TFA n-Butyl Esters of Unhydrolyzed Promine-D	28
9.	Column B Gas-Liquid Chromatographic Separation of N-TFA n-Butyl Esters of Hydrolyzed Promine-D	29

CHAPTER I

SURVEY OF THE LITERATURE

It has been known for many years that a number of bacteria produce proteolytic enzymes. Most of the work showed that these enzymes could hydrolyze various protein substrates.

Frobisher and Denny (3) studied Micrococcus zymogenes and came to the conclusion that it should be classified as a streptococcus, possibly Streptococcus liquefaciens. This organism produced a proteolytic enzyme which could digest a variety of proteins.

Sherman (15) condensed the enterococci into four specific species: Streptococcus faecalis, Streptococcus liquefaciens, Streptococcus zymogenes, and Streptococcus durans by means of physiological tests. According to Sherman's criteria, the enterococci share the following characteristics; growth at 10°C and 45°C, growth in the presence of 6.5 percent sodium chloride and in milk containing 0.1 percent methylene blue, the ability to survive 60°C for 30 minutes, the ability to initiate growth in broth at pH 9.6 and the production of ammonia from peptone.

Niven and Sherman (10) examined 19 strains of enterococci of group D Streptococcus. They discovered that the nutritional requirements of group D streptococci were very homogeneous. All the cultures required pantothenic acid, nicotinic acid, pyridoxine and biotin. All strains could synthesize thiamine. Variations in requirements were found for two vitamins, riboflavin and folic acid.

Dudani (2) studied the nutritional aspects of Streptococcus liquefaciens and the effect of pH on the enzyme produced by this organism. He found the enzyme had both proteolytic and coagulating activities when grown on media containing whole proteins. Increased concentrations of milk solids resulted in an increased production of enzyme. A sharp drop in pH of the litmus milk resulted in complete inactivation of the enzyme. The extracellular proteinase had its optimum activity at pH 7.4 for digestion of both casein and lactalbumin. Maximum stability of both the proteolytic and coagulating activities was found to be in the pH range of 6.0 to 8.0. At 62°C both these activities were destroyed in a period of 10 minutes. Vitamin B12 and inorganic cobalt ion did not increase either proteolytic or coagulating activities.

Grutter and Zimmerman (5) used culture filtrates of Streptococcus zymogenes which contained an extracellular proteolytic enzyme to hydrolyze casein, clot milk, and liquefy gelatin. The enzyme was purified 40-fold by ammonium sulfate fractionation. The optimum pH 7.4 of the Streptococcus zymogenes enzyme on casein is the same value Dudani (2) found with the proteinase of Streptococcus liquefaciens. The hydrolysis of casein followed zero order kinetics. It was suggested that only one enzyme was responsible for the different actions of the enzyme.

In 1956 Rabin and Zimmerman (13), using a modified casein medium, studied the nutritional aspects of proteinase production by Streptococcus liquefaciens. They reported that the casein medium could be replaced by a mixture of 12 amino acids and proteinase synthesis could occur. Proteinase biosynthesis depended on a fermentable carbohydrate, arginine and a pH of 6.3.

The rennin-proteolytic producing enterococci were studied by Beishir (1). She found the enterococci followed Sherman's criteria except for one property. They reduced methylene blue more slowly. The cultures curdled litmus milk without rendering it acid. She found the optimum pH for enzyme hydrolysis to be sharply defined at 7.0. A number of protein substrates were hydrolyzed by the enzyme and an increase of soluble nitrogen per milliliter was seen.

Moss (8) studied the extracellular rennin-proteolytic enzyme of Streptococcus liquefaciens. The crude enzyme preparation was derived as a by-product of growth of the organism in sterile skim milk. The activity of the enzyme was estimated by a colorimetric test for tyrosine in protein-free filtrates. A pilot fractionation of the crude enzyme prepared with ammonium sulfate revealed one fraction which contained 146 percent of the original enzyme activity. Heat inactivation occurred between 60°C and 65°C. The peptide bond of the synthetic peptide, L-tyrosylglycine, was hydrolyzed by the enzyme at pH 7.2. Arginine added to the milk substrate affected a 35 percent increase in enzyme activity. The enzyme was able to hydrolyze soy protein with an increase of soluble tyrosine.

Phillips (12) purified the proteolytic enzyme of Streptococcus liquefaciens from skim milk culture medium. A 383-fold purification was achieved by ammonium sulfate fraction, treatment with calcium phosphate gel, filtration with sephadex G-100 and aminoethyl cellulose column chromatography. Synthetic substrates such as n-benzoyl-DL-arginine p-nitroanilide hydrochloride, n-benzoyl-L-arginine ethyl ester

hydrochloride, and polylysine were not attacked. Several metal ions, Mg^{++}, Zn^{++}, Ca^{++}, and KCN at different concentrations were found to stimulate proteinase activity to varying degrees with hemoglobin and casein substrates. The optimum conditions for enzyme activity were shown to be at 35°C and pH 7.5. The proteinase was also noted to have rennin activity.

The proteolytic enzyme of Streptococcus liquefaciens was crystallized by Shugart and Beck (17). The enzyme had both proteolytic and rennin activities. Optimal enzyme activity was found to be at pH 7.6 and 35°C. The growth of the organism depended on a medium containing fermentable carbohydrate and arginine. If the medium contained only fermentable carbohydrate with no protein there was a decrease in the amount of proteinase produced. Optimum conditions for synthesis of the enzyme were a pH of 6.3 and 37°C. Proteinase activity was lost as the pH of the medium reached 5.4. Growth of the culture ceased when the carbohydrate or arginine was depleted. During active growth, the extracellular proteinase (18) was found in the whole cells and in the surrounding medium. The usual cell bound enzymes B-galactosidase and the intracellular enzymes LDH and $NADH_2$ peroxidase were not released into the medium. When the whole cells were fractionated, the proteinase was found to be associated with the cell wall and cell membrane.

CHAPTER II

MATERIALS AND METHODS

Organism

The organism R64A used in this investigation was *Streptococcus faecalis* var. *liquefaciens* isolated from a box turtle (9) and maintained in the departmental stock cultures. Stock cultures were kept at -18°C in 16 percent glycerol-trypticase-soy broth. Prior to experimental use, the culture was transferred twice at 24 hour intervals through Mundt and Johnson (MJ) Medium for streptococci. This medium is standard azide dextrose broth (7) with the azide omitted.

Culture Medium

The medium used to produce the enzyme was 11 percent instant nonfat dry milk dissolved in deionized water. The reconstituted milk, having a pH of 6.5, was sterilized in the autoclave under 13 pounds pressure.

Standard Inoculum

The inoculum for 400 ml of milk was 1 ml of cell sediment of strain R64A grown for 18 hours in 5 ml of MJ Medium.

Contamination Check

The method used to detect contamination of R64A was a gram stain of the organism each time the stock culture was used.

Substrate

Promine-D, produced by Central Soya, is an isolated soy protein which contains no less than 90 percent protein on a moisture-free basis. This substrate was dispersed in 0.025 M NaOH.

Shaker Bath

The shaker bath (Blue M Model No. MSB-1122A-1) was used to mix the enzyme and Promine-D dispersion at a constant speed.

Measurement of pH

pH measurements were made at room temperature with a Corning Scientific, Model 12, Research pH Meter.

Spectrophotometric Measurements

Spectrophotometric measurements were made with either a Perkin Elmer Hitachi Model 127 double beam Spectrophotometer or a Bausch and Lomb Colorimeter.

Freeze-Drier

The freeze-drier (American Sterilizing Company) was used to lyophilize the enzyme.

Centrifugation

Centrifugations were performed in a Sorvall RC2-B refrigerated centrifuge (Ivan Sorvall Inc.) at 4°C.

Gas-Liquid Chromatrography

The gas-liquid chromatograph (A Bendix 2500) was used to separate and identify the 20 amino acids from one another by the method of Gehrke et al. (4).

Column A, a 0.32 cm i.d. × 1.8 m long glass column, was packed with 0.65 percent ethylene glycol adipate (EGA) on Chromsorb W, acid washed, mesh size 80-100 (Applied Science, State College, Pennsylvania). The initial temperature was 100°C, and the temperature increased at 4°C per minute to 210°. Carrier gas (nitrogen) flow rate was 40 ml per minute; hydrogen flow rate was 40 ml per minute and air flow rate was 380-400 ml per minute. The column has the ability to remove 17 amino acids from the ester derivative.

Column B, a 0.32 cm i.d. × 1.8 m long glass column, was packed with 2 percent OV-17 and 1 percent OV-210 on Supelcoport, mesh size 100-120. The initial temperature was 110°C and the temperature increased at 8°C per minute to 235°C. Carrier gas, a mixture of hydrogen and air, flow rates were the same as for Column A. This nonpolar column was used to separate the three amino acids arginine, histidine, and cystine and was used to help identify other amino acids which were separated on Column A.

Procedure for the Isolation and Purification of Streptococcal Proteinase

Ten liters of sterile, nonfat dry milk culture medium were inoculated aseptically with cells from a 24 hour starter culture of R64A grown in MJ Medium. After 48 hours of incubation at 37°C, approximately 9-1/2 liters of whey was recovered with the use of a refrigerated

centrifuge at 12,000 × g for 10 minutes at 4°C. The proteolytic enzyme was isolated from the whey employing the procedure of Shugart and Beck (17) and Moss (8). Ammonium sulfate was added to the whey until 55 percent (350 grams per liter) saturation was reached. The mixture was held for eight hours in the refrigerator. The precipitate was recovered by centrifugation at 4°C for 15 minutes, at a speed of 30,000 × g. The precipitate was reconstituted in one-tenth its original volume of whey in deionized water. This solution was dialyzed against 100 volumes of deionized water for 16 hours at 4°C. A freeze-dried preparation was then made from the resulting dialysate.

Determination of Proteolytic Activity

The proteolytic activity of streptococcal proteinase was estimated with casein substate according to the procedure of Moss. One gram of casein (Hammersten) was suspended in 20 ml of 0.1 N NaOH, then titrated to pH 7.0 with 0.1 N HCl, and finally diluted to 100 ml with deionized water and stored at 4°C. Appropriate amounts of enzyme and casein were equilibrated to the desired temperature for five minutes, then mixed thoroughly and samples were withdrawn for analysis after 30 minutes. Each sample was immediately mixed with its own volume of hot (60°C) 12 percent trichloroacetic acid to stop the enzyme reaction. The mixture was stirred rapidly to assure complete precipitation of the unhydrolyzed protein. The solution was centrifuged at 12,000 × g for 10 minutes. Two ml of the supernatant fluid was used for the colorimetric estimation of soluble tyrosine by the method of Ottaway (11).

Determination of Tyrosine Content

A colorimetric estimation of soluble tyrosine present in protein-free filtrate of casein hydrolysates was used as a measure of proteolytic activity (11). This method is the result of the reaction between tyrosine and 1-nitroso-2-naphthol in the presence of nitric acid to produce a red color. Ottaway (11) developed a color-stabilizing reagent which was composed of 0.2 M NaCl, 25 percent acetone and 0.5 M ferric chloride per 300 ml of solution. One-tenth ml of a 0.3 percent solution of 1-nitroso-2-naphthol in acetone was added to the test sample and mixed. The solution was maintained in a 60°C waterbath for 5 minutes, then four drops of concentrated nitric acid were added to initiate the color reaction. The sample was returned to the water bath for 1 minute, then removed and contents mixed. The solution was then air cooled for 5 minutes to allow full color development. The color intensity was measured with a Bausch and Lomb Spectrophotometer at a wavelength of 540 nm.

The Standard Tyrosine Curve

A standard solution of 25 mg of L-tyrosine was dissolved in 5 ml of boiling deionized water and quickly diluted to 100 ml. The standard, stored at 4°C, remains stable for several weeks. Appropriate dilutions of this solution were used in the following system:

	Test Tube No.					
	1	2	3	4	5	6
L-tyrosine (ml)	0.0	0.1	0.2	0.3	0.4	0.6
Water (ml)	2.0	1.9	1.8	1.7	1.6	1.4
µg tyrosine	0	25	50	75	100	150

Each tube was subjected to the Ottaway test for the estimation of tyrosine. The values obtained corresponded to the micrograms of tyrosine per 2 ml of solution. These values were plotted against the optical density readings. Tube one was used as a blank to set the spectrophotometer to 100 percent transmission. The standard tyrosine curve is seen in Figure 1. The equation of the regression line is $y = 0.004891X + 0.0136$. The coefficient of correlation is 0.9987.

Determination of Protein Concentration in the Enzyme Preparation

Protein concentration was estimated by ultraviolet absorption. The optical density of the crude enzyme was measured in a Beckman DB Spectrophotometer at wavelengths of 260 and 280 nm. Layne (6) summarized the procedure of Christian and Warburg by which the milligrams of protein per milliliter of enzyme solution can be calculated according to the formula.

$$\text{Protein Concentration (mg/ml)} = 1.55\ O.D._{280} - 0.76\ O.D._{260}$$

The Effect of Temperature and Substrate Concentration on the Activity of the Enzyme

The activity of the enzyme was monitored by the increase in absorbance at 540 nm after a 30 minute reaction period because there was no increase in tyrosine beyond this time. The statistical design of the experiment was a split plot with two replications. A replication consisted of determination of enzyme activity on all possible combinations of substrate concentration and pH values at 25°, 35°, 45°, 55°,

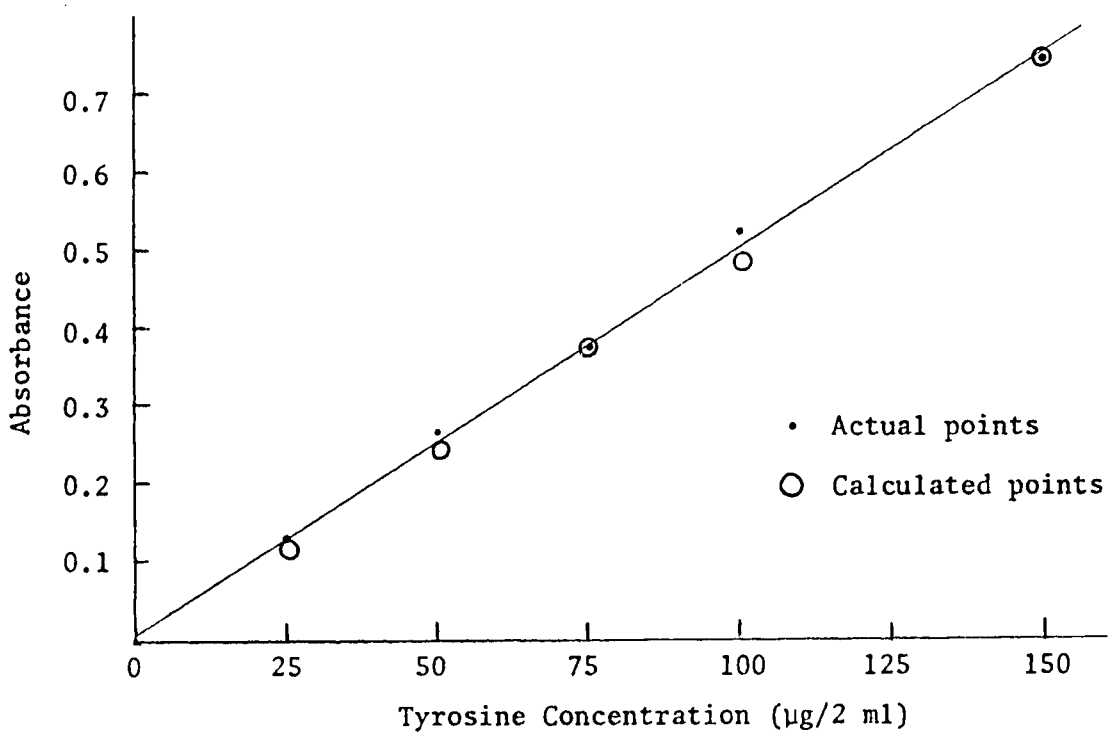


Figure 1. Standard tyrosine curve.

and 65°C. The concentrations of soy protein from Promine-D were 0.167, 0.333, 0.500, 0.677, 0.834, and 1.000 grams per 100 ml.

In a test tube, 10 ml of the following were mixed: 2 ml of substrate dispersion to give the desired concentration; 5 ml of 0.15 M phosphate buffer; 2.5 ml combination 0.05 M HCl and deionized water to achieve the desired pH and 0.5 ml (9 mg crude protein) enzyme suspension.

For each substrate concentration and pH value, the quantity of 0.05 M HCl to achieve the desired pH value was predetermined. For each temperature all ingredients except the enzyme were mixed in a test tube and equilibrated to reach the temperature of the reaction prior to addition of the enzyme. The desired temperature was achieved in a shaker bath and the dispersion was shaken with a stroke of 27 mm at a rate of 84 strokes per minute. Thirty minutes after the addition of enzyme, proteolysis was stopped by the addition of 10 ml of hot (60°C) 12 percent trichloroacetic acid. The resulting mixture was centrifuged at 12,000 × g for 10 minutes and the supernatant fluid was used for tyrosine analysis.

Separation of Free Amino Acids from Unhydrolyzed and Enzyme Hydrolyzed Promine-D

For the unhydrolyzed sample, ten grams of Promine-D were blended in a 100 ml of 80 percent (v/v) ethanol for 5 minutes in a one-half pint osterizer blender jar, and then stirred for 5 minutes on a magnetic stirrer. The sample was centrifuged at 12,000 × g for 15 minutes at 4°C. The suspension was filtered through Whatman No. 1 filter paper. All the filtrate was collected and evaporated to dryness in a 60°C waterbath.

The precipitate was brought back to volume with 10 ml of deionized water. This solution was dialyzed (cellulose dialysis tubing, Sargent-Welch, retain molecular weight 12,000 compounds) against 25 ml of deionized water for 16 hours at 4°C. The dialyzing fluid and 1 ml of tranexamic acid (trans-4-(aminomethyl)cyclohexanecarboxylic acid) was evaporated to dryness in a 125 ml 24/40 ground glass top Erlenmeyer flask in rotary evaporator at 60°C. The dried sample was then taken through the derivitization procedure of Roach and Gehrke (14).

For the hydrolyzed sample 20 ml of soy protein solution (5.6 g protein/100 ml) in 0.025 N NaOH was mixed with 50 ml of 0.15 M phosphate buffer, pH 7.5, 25 ml, of deionized water and 5.0 ml enzyme (90 mg protein/5 ml) and reacted for 30 minutes at 43°C. Four hundred ml of 95 percent ethanol was added at the end of the reaction time to stop enzyme action and precipitate soy protein. The mixture was centrifuged at 12,000 × g, at 4°C for 15 minutes and the supernatant was filtered through Whatman Number 1 filter paper. All of the filtrate was evaporated to dryness, and the residue was dissolved in 10 ml of deionized water. This solution was dialyzed against 25 ml of water in cellulose dialysis tubing. From this point the sample was treated by the same procedure as the unhydrolyzed sample.

Derivitization Procedure

Twelve ml of 3.0 N HCl in n-butanol was added to the precipitate. A teflon coated stirring bar was placed in the flask. The flask was stoppered with a drying tube filled with drierite and placed in a 100°C oil bath for 45 minutes while stirring to esterify. The HCl-butanol

was evaporated from the sample at 60°C under reduced pressure with a rotary evaporator. Next, 5 ml of 25 percent trifluoroacetic anhydride in methylene chloride was added to the residue. The flask was swirled for 30 seconds to dissolve the amino acid-butanol esters. About 2 ml of the solution was transferred to an acylation tube. The tube was sealed tightly with a teflon-lined screw cap. The acylation tube was placed in a 150°C oil bath for 5 minutes. After this, the tube was allowed to cool to room temperature and the sample was ready for injection into the gas chromatograph.

Statistical Design and Analysis

This experiment was run according to a split-plot design with two replications. The whole plot was substrate concentration × pH and the split plot was the temperature. The activity of the enzyme for all possible combinations of substrate concentrations (0.167, 0.333, 0.500, 0.667, 0.834, and 1.000 g protein/100 ml solution) and pH values (4.5, 5.5, 6.5, 7.5, and 8.5) was determined at a randomly selected temperature (25°, 35°, 45°, 55°, or 65°C) at one time.

The order in which the temperature was selected was randomized across replications. One of ten temperatures × replication combinations was randomly selected, then another combination was selected and so forth until the order in which they were run was established. A replication consisted of all possible combinations of substrate concentration and pH values at the five temperatures.

Preliminary analysis of the data indicated heterogeneity of the subgroup variance. It was observed those cells (tyrosine produced at

a given substrate concentration, pH, and temperature) with values less than 5 µg tyrosine/2 ml produced tended to contribute to this heterogeneity. Since there was not any interest in those values below 5 µg and it tended to solve the problem of heterogeneity, all cells with values below 5 µg were deleted from the data. Polynomial regression analysis of the data was run to indicate terms needed for the development of a prediction model.

CHAPTER III

RESULTS AND DISCUSSION

Model for the Equation

The equation for the model which represents 68 percent variation of the data observed is:

$$\hat{c} = 1857.3192 - 665.1474\ X - 47.2883\ Y - 645.5586\ Z + 75.916\ X^2 + 0.5130\ Y^2 + 28.4151\ Z^2 - 2.8077\ X^3 + 13.0657\ XY + 234.4673\ XZ + 0.7554\ YZ - 0.1395\ XY^2 - 5.7118\ XZ^2 - 27.8911\ X^2Z - 0.8527\ X^2Y + 1.1138\ X^3Z - 0.0016\ Y^3Z + 0.00895\ X^2Y^2 \quad (1)$$

where $\hat{c}$ = µg tyrosine produced/2 ml,

X = pH,

Y = temperature, °C, and

Z = substrate concentration, g protein/100 ml.

By selecting the criterion that substrate concentration must be minimal to obtain maximum response of tyrosine produced, a search of means for pH, Table 1, shows that pH 7.5 is a good estimate of an optimum pH. Solving equation (1) for the model with the pH set at 7.5 gave an estimate of optimum temperature range of 42-45°C for maximum response at minimum substrate concentration (Figure 2). The equation for the model with the temperature set at 43°C was then solved and plotted (Figure 3) for maximum response at minimum substrate concentration.

TABLE 1

MEANS FOR µg TYROSINE/2 ML PRODUCED AT DIFFERENT TEMPERATURE, pH AND SUBSTRATE CONCENTRATIONS BY ENZYME HYDROLYSIS OF SOY PROTEIN

		Means	
Temp °C	pH	Protein, g/100 ml	Tyrosine, µg/2 ml*
25	4.5	0.166	0.745
25	4.5	0.332	0.000
25	4.5	0.500	0.000
25	4.5	0.676	0.000
25	4.5	0.833	0.000
25	4.5	1.000	3.800
25	5.5	0.166	8.620
25	5.5	0.332	4.540
25	5.5	0.500	1.760
25	5.5	0.676	5.560
25	5.5	0.833	0.745
25	5.5	1.000	6.580
25	6.5	0.166	0.745
25	6.5	0.332	8.620
25	6.5	0.500	0.745
25	6.5	0.676	10.660
25	6.5	0.833	9.335
25	6.5	1.000	16.780
25	7.5	0.166	0.000
25	7.5	0.332	13.720
25	7.5	0.500	15.760
25	7.5	0.676	25.960
25	7.5	0.833	12.700
25	7.5	1.000	16.780
25	8.5	0.166	8.620
25	8.5	0.332	11.680
25	8.5	0.500	12.700
25	8.5	0.676	17.800
25	8.5	0.833	8.620
25	8.5	1.000	11.375
35	4.5	0.166	0.000
35	4.5	0.332	0.000
35	4.5	0.500	0.000
35	4.5	0.676	3.525
35	4.5	0.833	0.000
35	4.5	1.000	0.000
35	5.5	0.166	3.525
35	5.5	0.332	3.525
35	5.5	0.500	0.000

TABLE 1 (continued)

Temp °C	pH	Means	
		Protein, g/100 ml	Tyrosine, µg/2 ml*
35	5.5	0.676	10.660
35	5.5	0.833	11.710
35	5.5	1.000	9.640
35	6.5	0.166	14.230
35	6.5	0.332	16.780
35	6.5	0.500	7.600
35	6.5	0.676	11.680
35	6.5	0.833	30.035
35	6.5	1.000	31.055
35	7.5	0.166	16.780
35	7.5	0.332	19.840
35	7.5	0.500	25.955
35	7.5	0.676	30.015
35	7.5	0.833	40.230
35	7.5	1.000	45.290
35	8.5	0.166	13.720
35	8.5	0.332	17.800
35	8.5	0.500	19.840
35	8.5	0.676	17.800
35	8.5	0.833	36.145
35	8.5	1.000	24.940
45	4.5	0.166	0.745
45	4.5	0.332	0.000
45	4.5	0.500	3.525
45	4.5	0.676	0.000
45	4.5	0.833	0.000
45	4.5	1.000	3.520
45	5.5	0.166	5.560
45	5.5	0.332	0.745
45	5.5	0.500	5.560
45	5.5	0.676	5.050
45	5.5	0.833	2.000
45	5.5	1.000	5.560
45	6.5	0.166	17.800
45	6.5	0.332	15.760
45	6.5	0.500	24.940
45	6.5	0.676	21.880
45	6.5	0.833	23.920
45	6.5	1.000	17.290
45	7.5	0.166	24.940
45	7.5	0.332	21.880
45	7.5	0.500	32.580

TABLE 1 (continued)

Temp °C	pH	Means	
		Protein, g/100 ml	Tyrosine, µg/2 ml*
45	7.5	0.676	32.070
45	7.5	0.833	29.015
45	7.5	1.000	28.000
45	8.5	0.166	12.700
45	8.5	0.332	15.760
45	8.5	0.500	20.860
45	8.5	0.676	17.290
45	8.5	0.833	24.940
45	8.5	1.000	17.800
55	4.5	0.166	0.000
55	4.5	0.332	0.000
55	4.5	0.500	0.000
55	4.5	0.676	0.000
55	4.5	0.833	0.000
55	4.5	1.000	0.000
55	5.5	0.166	0.000
55	5.5	0.332	10.660
55	5.5	0.500	5.565
55	5.5	0.676	5.050
55	5.5	0.833	9.640
55	5.5	1.000	3.525
55	6.5	0.166	10.660
55	6.5	0.332	26.975
55	6.5	0.500	22.900
55	6.5	0.676	25.960
55	6.5	0.833	30.035
55	6.5	1.000	22.900
55	7.5	0.166	14.740
55	7.5	0.332	29.015
55	7.5	0.500	25.960
55	7.5	0.676	26.980
55	7.5	0.833	33.090
55	7.5	1.000	24.940
55	8.5	0.166	9.130
55	8.5	0.332	12.700
55	8.5	0.500	10.660
55	8.5	0.676	23.920
55	8.5	0.833	12.700
55	8.5	1.000	20.860
65	4.5	0.166	0.000
65	4.5	0.332	0.000
65	4.5	0.500	0.000

TABLE 1 (continued)

Temp °C	pH	Means	
		Protein, g/100 ml	Tyrosine, µg/2 ml*
65	4.5	0.676	0.000
65	4.5	0.833	0.000
65	4.5	1.000	0.000
65	5.5	0.166	0.000
65	5.5	0.332	0.000
65	5.5	0.500	0.745
65	5.5	0.676	0.000
65	5.5	0.833	0.000
65	5.5	1.000	0.000
65	6.5	0.166	0.000
65	6.5	0.332	8.620
65	6.5	0.500	13.720
65	6.5	0.676	9.640
65	6.5	0.833	5.840
65	6.5	1.000	13.720
65	7.5	0.166	0.745
65	7.5	0.332	0.000
65	7.5	0.500	2.780
65	7.5	0.676	10.660
65	7.5	0.833	1.760
65	7.5	1.000	5.560
65	8.5	0.166	0.000
65	8.5	0.332	1.760
65	8.5	0.500	0.000
65	8.5	0.676	0.000
65	8.5	0.833	0.000
65	8.5	1.000	0.000

*Each observation is a mean of two values.

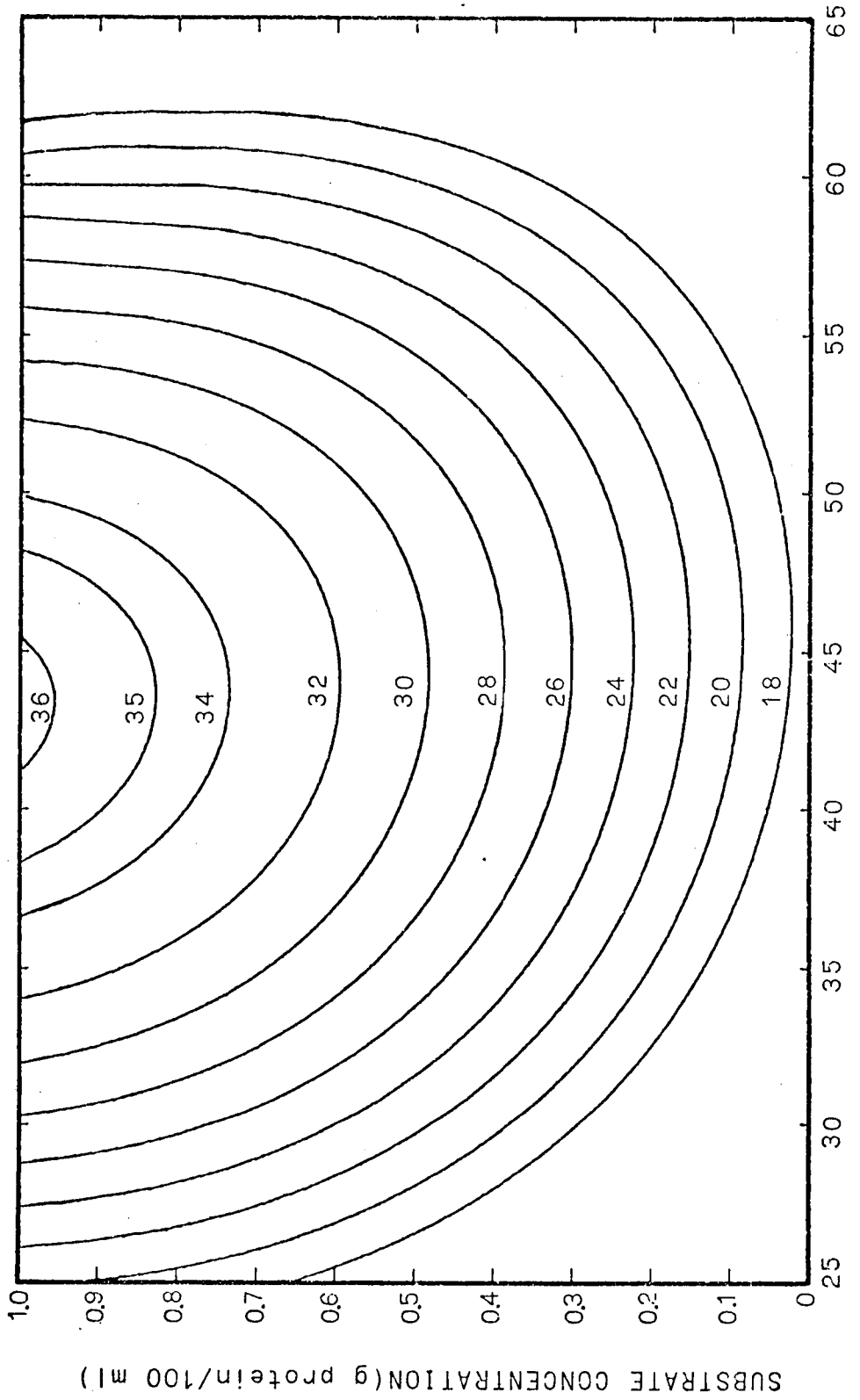


Figure 2. Response of tyrosine µg/2 ml produced as a function of substrate concentration and temperature with pH set at 7.5. Each contour line represents amount of tyrosine produced at those combinations of substrate concentration and temperature.

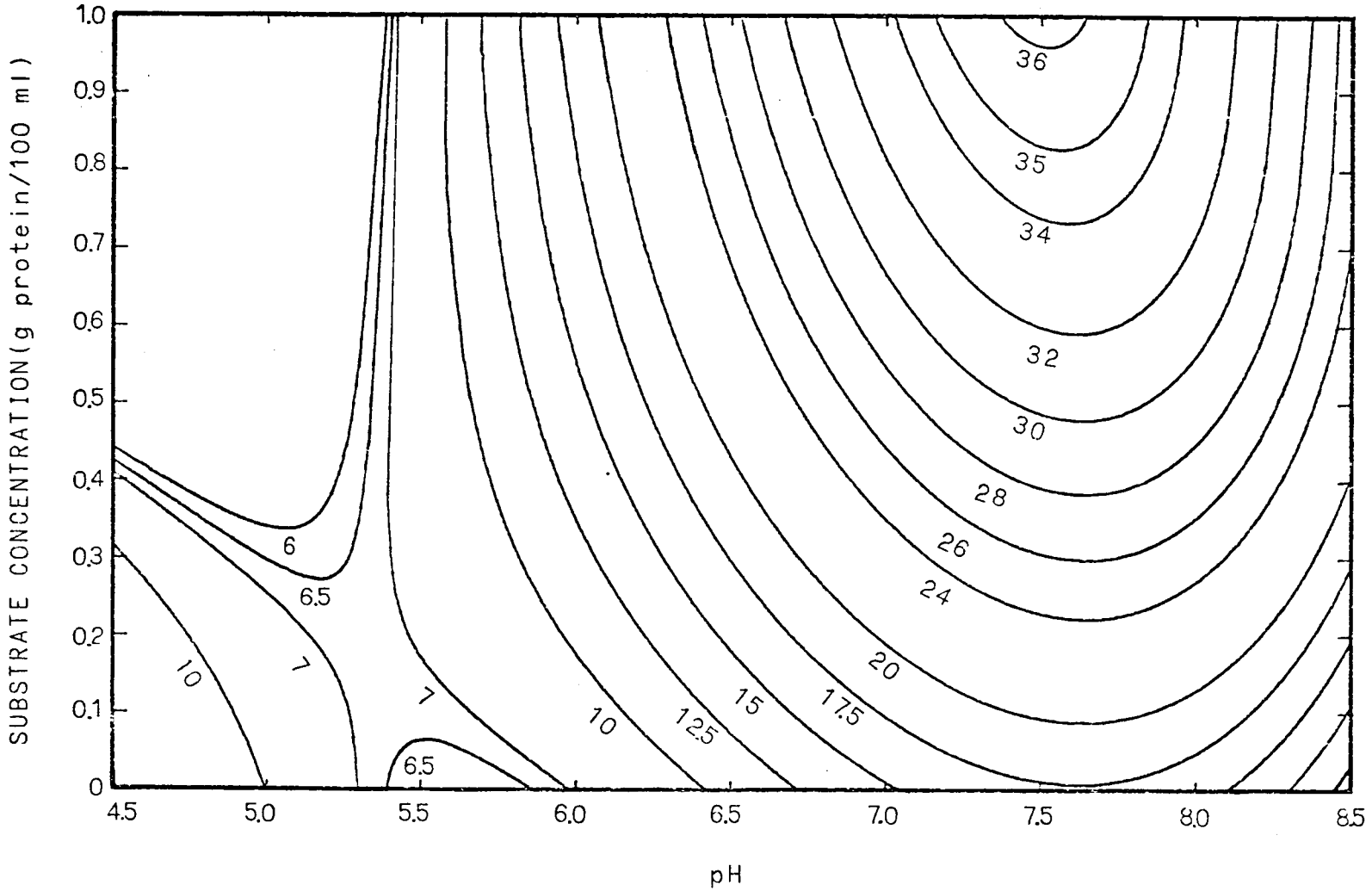


Figure 3. Response of tyrosine µg/2 ml produced as a function of substrate concentration and pH with temperature set at 43°C. Each contour line represents the amount of tyrosine produced at those combinations of substrate concentration and pH.

Figure 3 shows that the optimum pH for maximum response at 43°C is in the pH range of 7.5 to 7.7.

It can be observed from Figure 2 with the pH set at 7.5 that as the concentration of the substrate changes, the optimum temperature for maximum enzyme activity (maximum tyrosine produced) also changes. The same type of observation may also be made from Figure 3 with the temperature set at 43°C that as the substrate concentration changes the pH for maximum enzyme activity also changes slightly.

In this study, it was observed that at 65°C for 30 minutes the enzyme activity was not completely destroyed. These results can be seen in Table 1. This disagrees with the results of Dudani (2) and Moss (8). Dudani states that all proteolytic activity was completely destroyed at 62°C in 10 minutes. Moss found that the enzyme was inactivated between 60°C and 65°C in 30 minutes.

Optimal conditions were found to be at 43°C and pH 7.5, Figures 2 and 3, respectively. Similar pH optima were reported by Phillips (12) and Shugart and Beck (17). The optimum temperature of 43°C was different from that found by all other investigators of the enzyme. This may be due to the fact that Promine-D did not form a homogeneous dispersion as does casein.

Results with Gas-Liquid Chromatography

The results with the gas-liquid chromatography are presented in Figures 4, 5, 6, 7, 8, and 9. Figures 4 and 7 are prepared from known amino acid standards. Figures 5 and 6, column A represent unhydrolyzed and hydrolyzed Promine-D, respectively. Figures 8 and 9, column B represent unhydrolyzed and hydrolyzed Promine-D, respectively. The

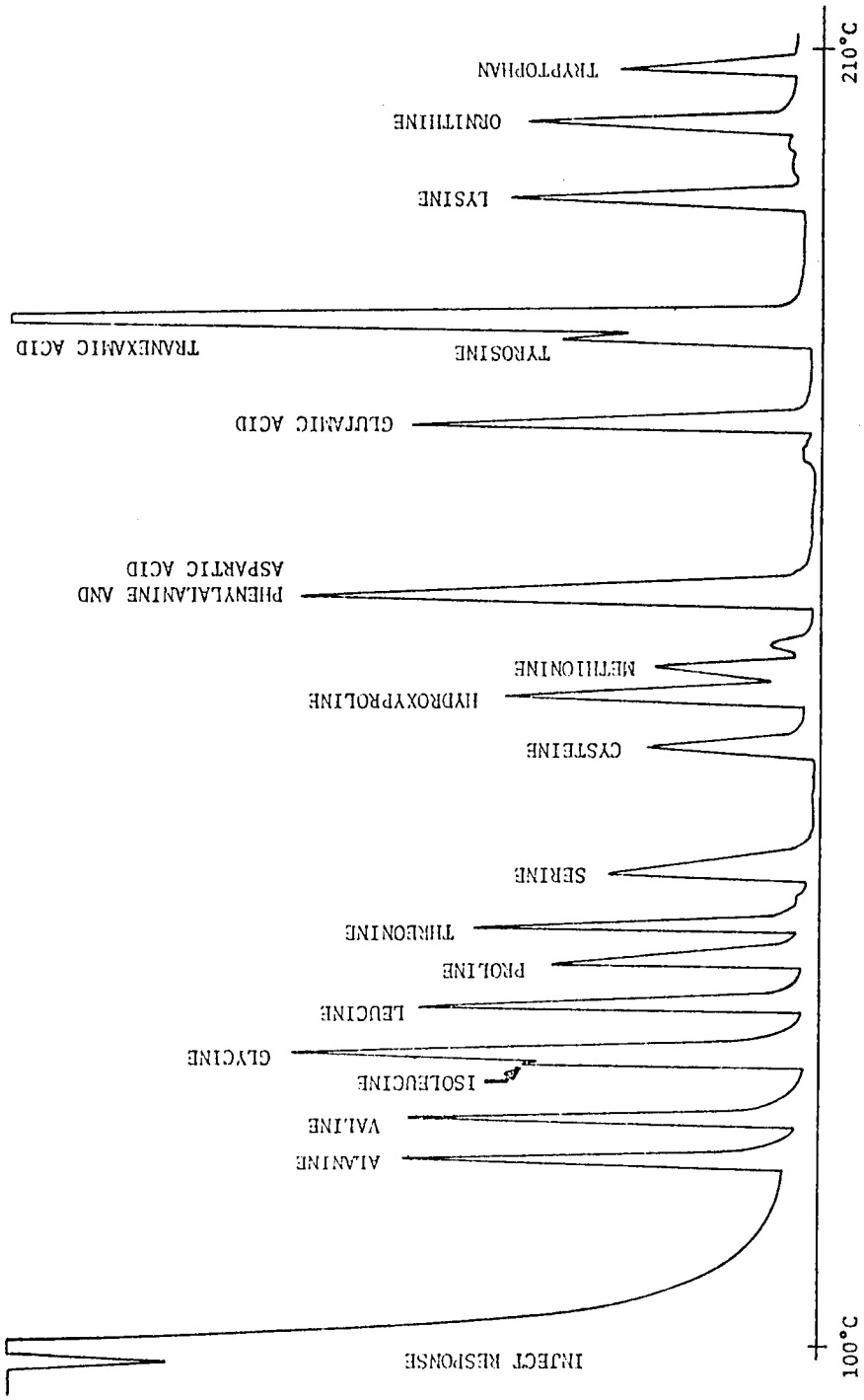


Figure 4. Column A gas-liquid chromatographic separation of N-TFA n-butyl esters of amino acid standards. Column A: 0.65% EGA on 80/100 mesh AW Chromosorb W, 1.5 m × 4 mm I.D. glass. Injected: ca. 6 µl. Conditions: initial temperature, 100°C; program rate 4°C/min; final temperature, 210°C. Internal standard, tranexamic acid. Unlabeled peaks are unknown. Attenuation was set at 1K.

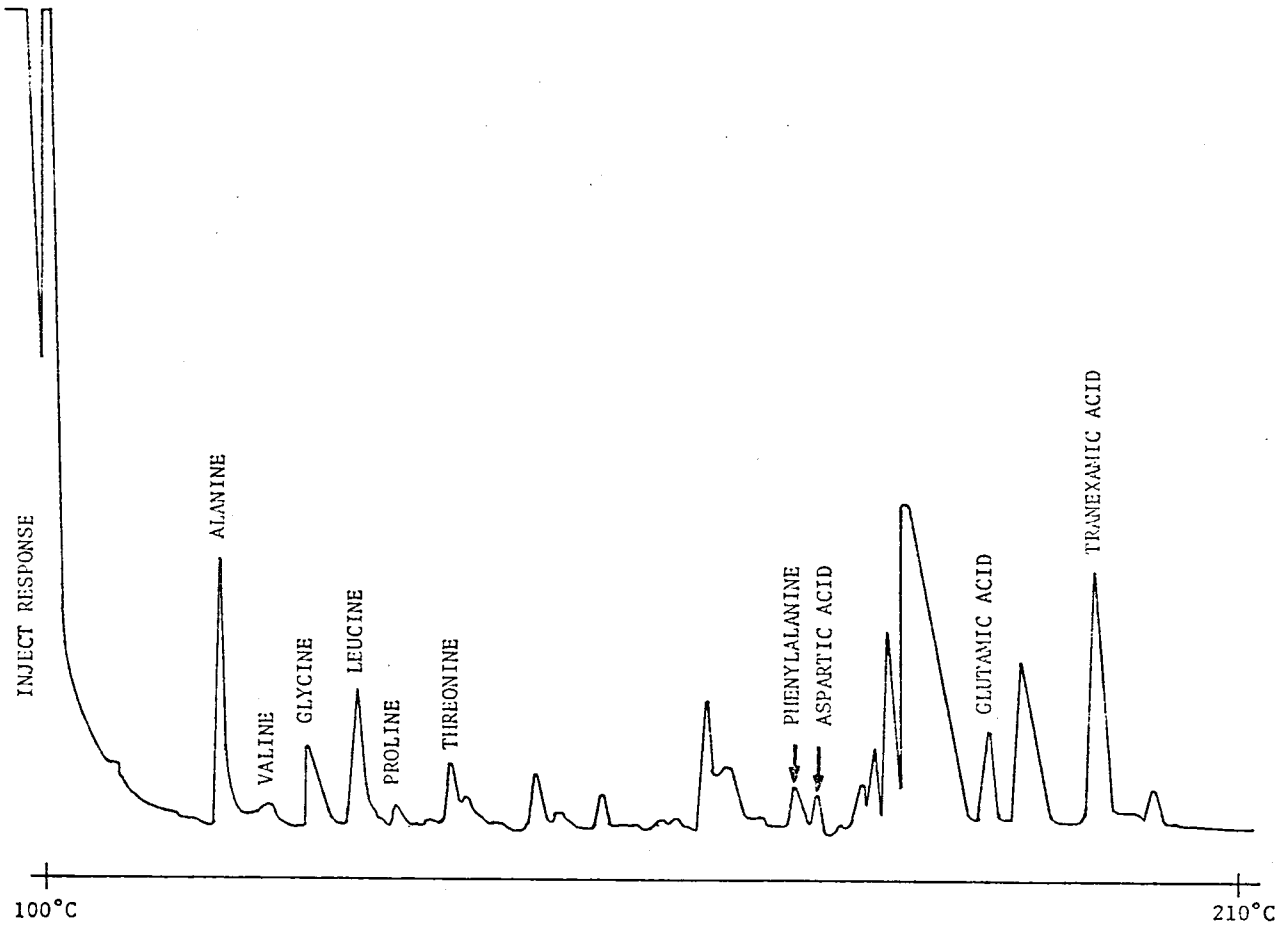


Figure 5. Column A gas-liquid chromatographic separation of N-TFA n-butyl esters of unhydrolyzed Promine-D. Column A: 0.65% EGA on 80/100 mesh AW Chromosorb W, 1.5 m × 4 mm I.D. glass. Injected: ca. 6 µl. Conditions: initial temperature, 100°C; program rate 4°C/min; final temperature, 210°C. Internal standard, tranexamic acid. Unlabeled peaks are unknown. Attenuation was set at 2K.

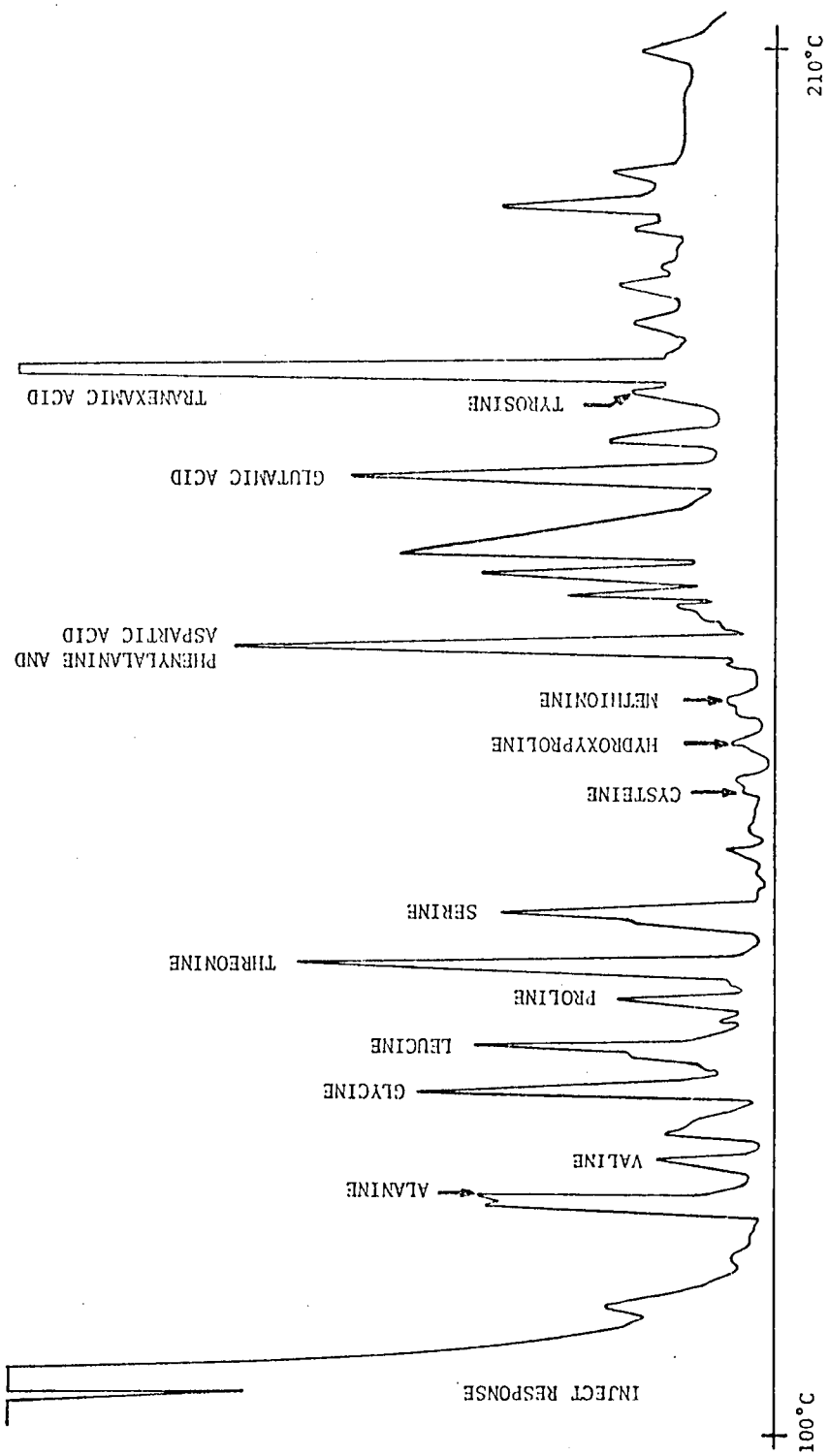


Figure 6. Column A gas-liquid chromatographic separation of N-TFA n-butyl esters of hydrolyzed Promine-D. Column A: 0.65% EGA on 80/100 mesh AW Chromosorb W, 1.5 m × 4 mm I.D. glass. Injected: ca. 6 µl. Conditions: initial temperature, 100°C; program rate 4°C/min; final temperature, 210°C. Internal standard, tranexamic acid. Unlabeled peaks are unknown. Attenuation was set at 1K.

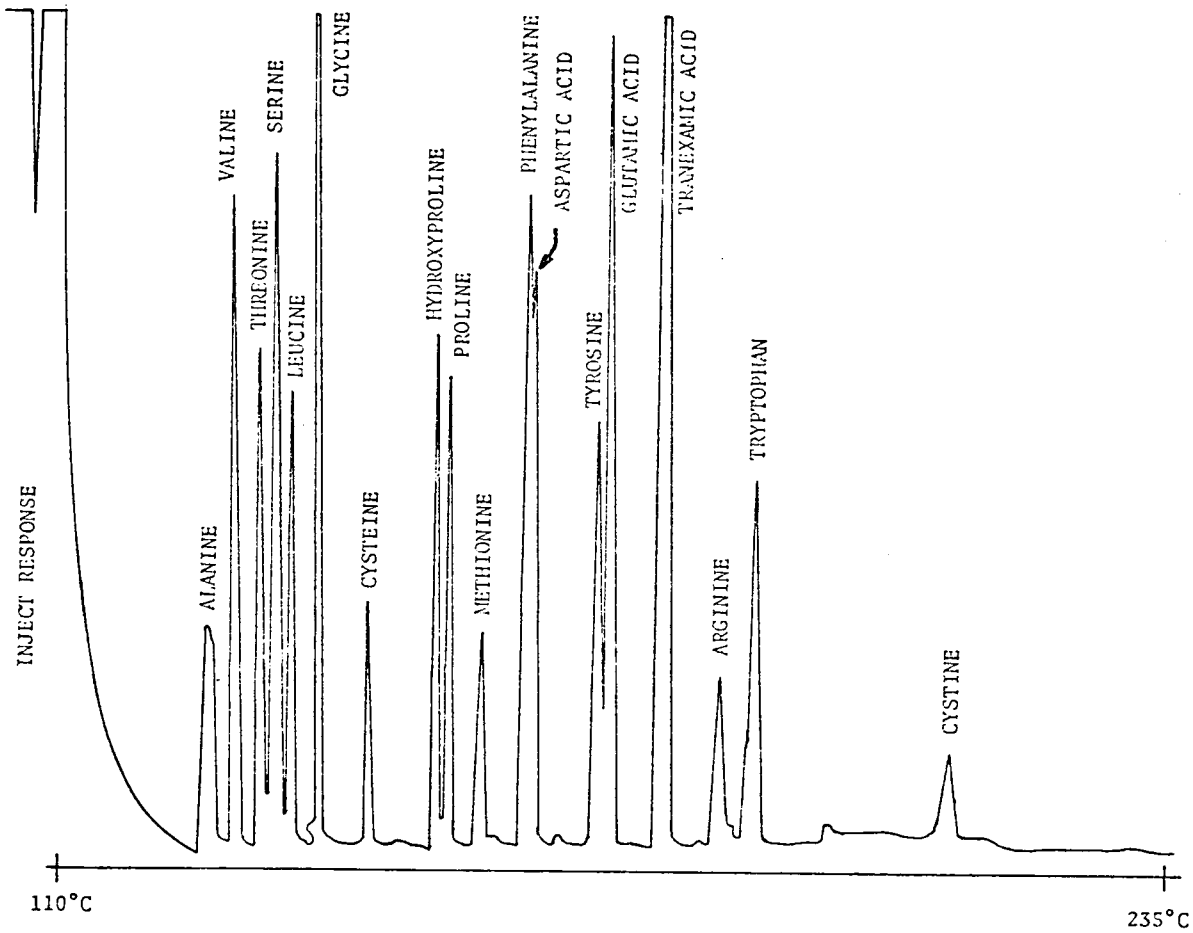


Figure 7. Column B gas-liquid chromatographic separation of N-TFA n-butyl esters of amino acid standards. Column B: 2.0% OV-17, 1.0% OV-210 on 100/120 mesh Supelcoport, 1.5 m × 4 mm I.D. glass. Injected: ca. 6 µl. Conditions: initial temperature 110°C; program rate 8°C/min; final temperature, 235°C. Internal standard, tranexanic acid. Unlabeled peaks are unknown. Attenuation set at 1K.

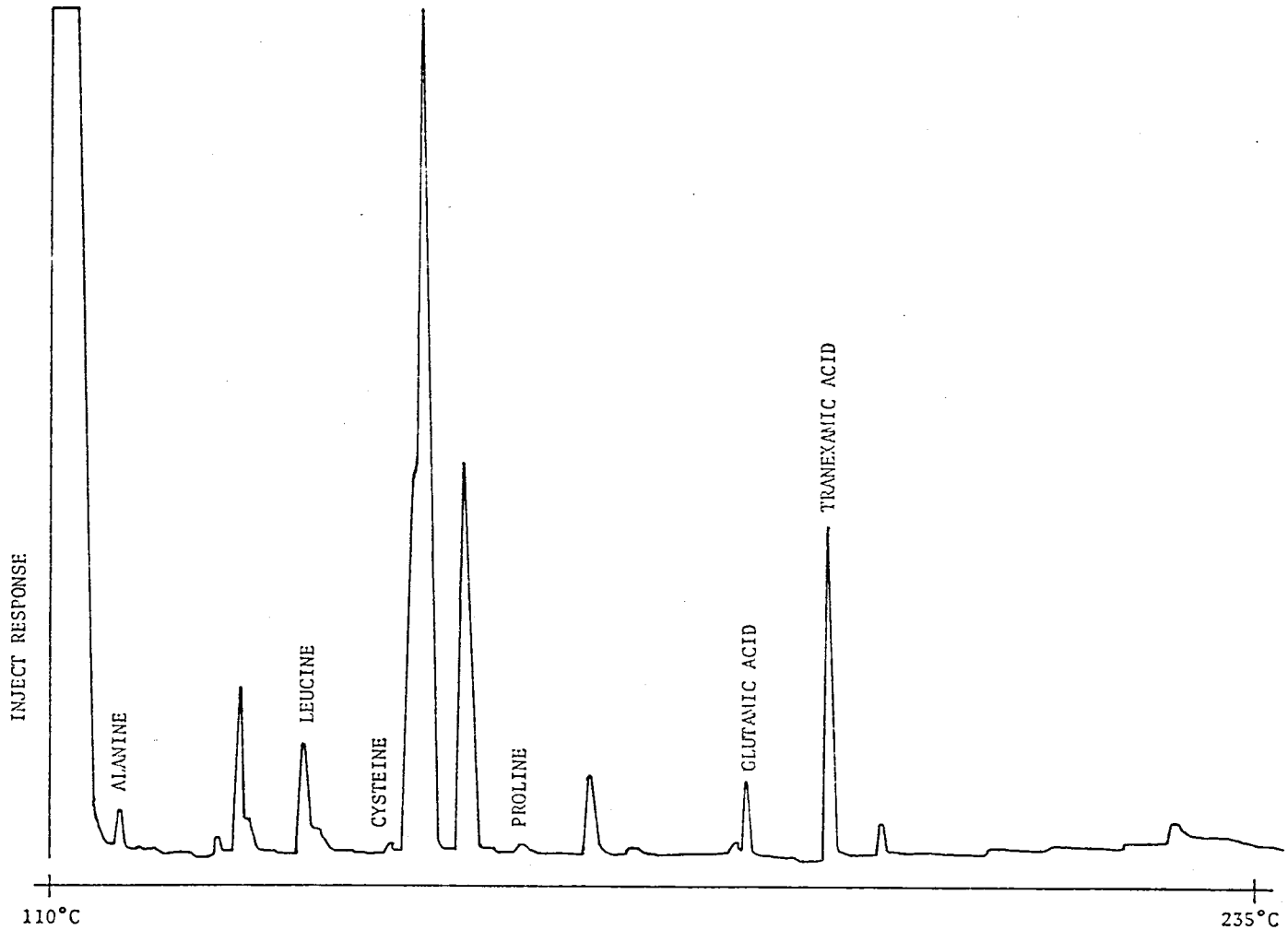


Figure 8. Column B gas-liquid chromatographic separation of N-TFA n-butyl esters of unhydrolyzed Promine-D. Column B: 2.0% OV-17, OV-210 on 100/120 mesh Supelcoport, 1.5 m × 4 mm I.D. glass. Injected: ca. 6 µl. Conditions: initial temperature 110°C; program rate 8°C/min; final temperature, 235°C. Internal standard, tranexamic acid. Unlabeled peaks are unknown. Attenuation set at 2K.

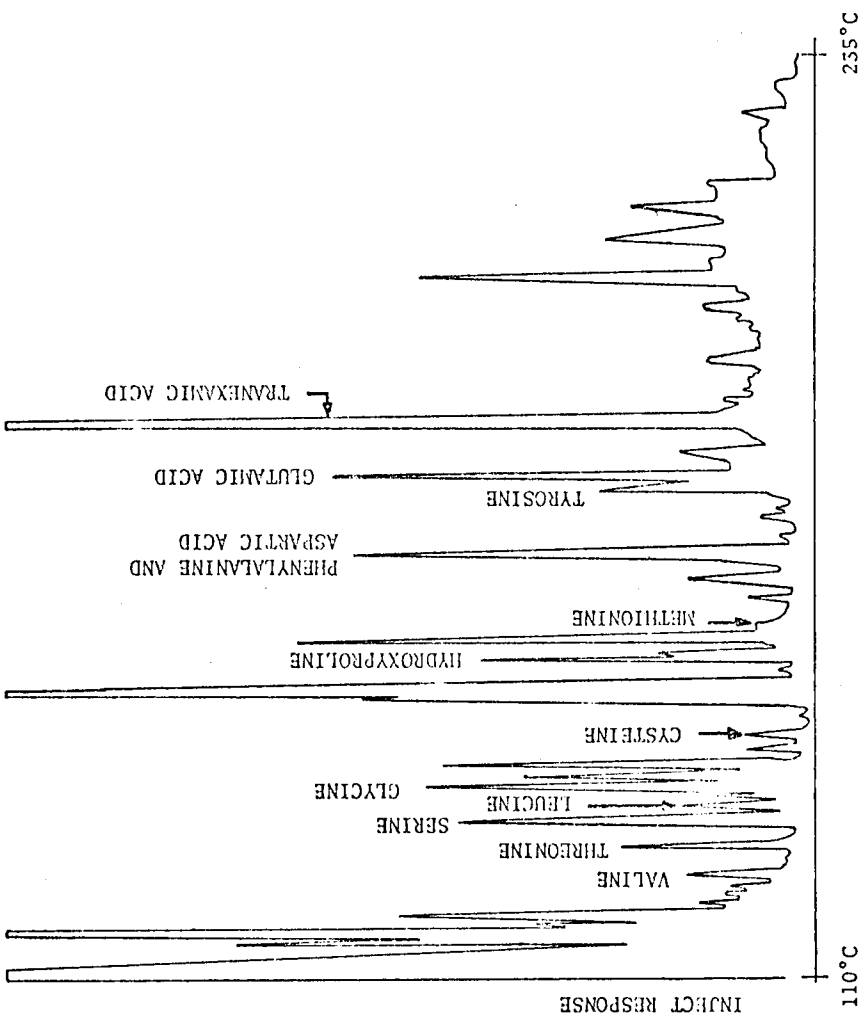


Figure 9. Column B gas-liquid chromatographic separation of N-TFA n-butyl esters of hydrolyzed Promine-D. Column B: 2.0% OV-17, 1.0% OV-210 on 100/120 mesh Supelcoport, 1.5 m × 4 mm I.D. glass. Injected: ca. 6 µl. Conditions: initial temperature 110°C; program rate 8°C/min; final temperature, 235°C. Internal standard, tranexamic acid. Unlabeled peaks are unknown. Attenuation set at 1K.

amino acids released by enzymatic hydrolysis can be detected clearly by use of the gas chromatography.

The gas-liquid chromatographic analysis was performed as a qualitative procedure so the amounts of amino acids freed were not calculated. In Figures 5 and 6 the attenuation was set at 2K for the unhydrolyzed protein and set at 1K for the hydrolyzed protein. Since the extract from the unhydrolyzed protein was from 10 g of Promine-D and the extract from the enzyme hydrolyzed Promine-D was 5.6 g the peak area for a given concentration of a compound should be approximately the same in both figures unless the concentration of that compound has changed.

Unhydrolyzed Promine-D, Figures 5 and 8, had the free amino acids alanine, leucine, cysteine, proline, glutamic acid, valine, glycine, aspartic acid, threonine, and phenylalanine. The relative amounts of amino acids which increased due to enzymatic hydrolysis were valine, glycine, leucine, proline, threonine, phenylalanine, and glutamic acid. When the soy protein was hydrolyzed enzymatically, Figures 6 and 9, additional free amino acids which appeared were serine, methionine, hydroxyproline, and tyrosine.

The enzyme in this study was not a pure preparation and no attempt was made to separate the rennin and proteolytic activities. Therefore, no conclusion can be made concerning the number(s) of enzymes involved in rennin and proteolytic activity.

The contour graph (Figure 2, page 21) shows that for maximum production of tyrosine at any substrate concentration the temperature should be 43°C. The pH for maximum response at 43°C is in the range of 7.5 and 7.7 and this is seen in Figure 3, page 22. A saddle effect

is seen in the region of pH 6.0 to 6.5. A hypothesis which may explain the saddle effect is that two enzymes are responsible for the proteolytic activities of the enzyme under study, or it may indicate one enzyme with two optimum pH values. The work of Sekine et al. (15) may support the hypothesis that more than one enzyme is present. They concluded that Aspergillus sojae produces proteinase which is able to digest soybean protein due to a number of different enzymes. After putting the crude enzyme extract through a DEAE-sephadex A-50 column three peaks with proteinase activity were obtained. Dudani (2) felt that the proteolytic enzyme of Streptococcus faecalis var. liquefaciens was a manifestation of one enzyme. Moss (8), using a crude ammonium sulfate enzyme preparation, described the proteolytic enzyme of Streptococcus faecalis as one enzyme which was responsible for both rennin and proteolytic activity. There may be two reasons why Moss (8) and Dudani (2) were not able to separate the two activities of the enzyme: (1) they had a different strain of bacteria than the one used in this study; and (2) their method for separating the enzyme activities, salting out technique, was too crude. Grutter and Zimmerman (5) reported a 40-fold increase in the purified proteinase of Streptococcus zymogenes which coagulated milk and hydrolyzed casein and hemoglobin. They felt this was all due to one enzyme with multiple activities. Phillips (12) used a highly purified enzyme which possessed both rennin and proteolytic activity. He concluded this was due to one enzyme having double activity. Phillips purified the enzyme using very sophisticated techniques. By use of sephadex G-100 column, he was able to obtain an enzyme with one

activity peak. This fraction was able to coagulate milk and hydrolyze casein.

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VITA

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