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STUDIES ON THE NEUTRAL PROTEASE
FROM BACILLUS SUBTILIS.

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THE PURIFICATION AND CHARACTERIZATION STUDIES
ON THE NEUTRAL PROTEASE FROM BACILLUS SUBTILIS

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of the Requirements for the Degree of
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by
James D. McConn

Thesis Committee:

Dr. Robert McKay, Chairman
Dr. Theodore Winnick
Dr. Howard Mower
Dr. John Hall
Dr. Richard Inskeep

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ABBREVIATIONS

CM Cellulose	Carboxymethylcellulose
DEAE	Diethylaminoethylcellulose
EDTA	Ethylenediaminetetraacetate
DFP	Diisopropylphosphorofluoridate
DNP	Dinitrophenyl
mg	milligrams
ug	micrograms
ml	milliliters
gm	grams
M	Molar
°C.	degrees centigrade
mu	millimicrons
$s_{20,w}$	Svedbeurg Units ($\times 10^{-13}$)
SE Sephadex	sulfoethyl Sephadex
cm	centimeters
PU	Protease Units
Vmax	From Michelis and Menten (1913)
Km	
pX	where X is some numerical function (value), pX is the negative logarithm of that function (value).

THE PURIFICATION AND CHARACTERIZATION STUDIES
ON THE NEUTRAL PROTEASE FROM BACILLUS SUBTILIS

ABSTRACT

A neutral protease from Bacillus subtilis was first isolated and partially characterized by Fukumoto, et al. (Nippon Nogei Kazaku Kaishi, 32, 230, 233, and 375 (1958)).

A new purification method starting with a crude commercial enzyme preparation has been developed employing calcium-equilibrated CM-cellulose which gives an enzyme with a higher specific activity than the neutral protease reported by Fukumoto, et al. (Nippon Nogei Kazaku Kaishi, 33, 9 (1959)). The enzyme is shown to be homogeneous by the criteria of ultra-centrifugation, electrophoresis, amino terminal amino acid analysis, and rechromatography.

Physical studies on the neutral protease show that the enzyme is stabilized by calcium ions. Molecular weight determinations by the Archibald procedure yield a value of 44,700. Electrophoretic studies give an isoelectric point of about 9.0. The enzyme is relatively stable in the pH range of 6.0 to 10.0. (0.002 M calcium acetate is present from pH 6.0 to 9.0).

Unlike other known B. subtilis proteases, the neutral protease is a zinc metalloenzyme. The zinc content is about 2.3 ug zinc per mg protein. At the present time, the data indicate that there are 2 moles of zinc per mole of enzyme. This metalloenzyme was found to be inactivated by several chelating agents and heavy metals but not by diisopropylphosphorofluoridate.

The amino acid analysis of the neutral protease shows that the enzyme contains no cysteine. The total sulfur is accounted for in the methionine content.

Preliminary investigations into the substrate specificity of the enzyme show that the enzyme can hydrolyze certain dipeptides, if their amino and carboxyl terminal groups are blocked. The most sensitive synthetic substrate found to date is hippuryl-L-leucinamide. The protease showed no esterase activity against N-acetyl tyrosine ethyl ester or compounds such as methyl butyrate, and amyl acetate.

Detailed kinetic studies with hippuryl-L-leucinamide as substrate show class IV kinetics (Laidler, K. J., Trans. Faraday Soc. 52, 497 (1955) and give a Michaelis constant of 4.7×10^{-2} M at 30°C. Certain thermodynamic parameters and inhibition studies are also reported.

I. Introduction

A. Historical Background

The culture filtrates of Bacillus subtilis and similar bacteria are rich and convenient sources of bacterial proteases. All the extracellular proteases reported and characterized from B. subtilis have had an alkaline pH optimum. There are reports on a neutral protease from another strain of B. subtilis but this enzyme has not been well characterized. (Fukumoto, et al., 1958)

The information known about the neutral protease at the beginning of this work is summarized below. The enzyme was purified using corn starch, $(\text{NH}_4)_2$, an anion and cation exchanger, and acetone. The enzyme could then be crystallized, but this resulted in a loss of specific activity. A stabilization effect of the enzyme with sodium and calcium was found. EDTA would inactivate the enzyme. The inactivated enzyme could be reactivated by zinc, cobalt, manganese, magnesium and calcium. (Fukumoto, et al., 1958)

The most thoroughly studied proteases of B. subtilis are the alkaline proteases: subtilisin (Guntelberg, et al., 1954) and Nagarse (Hagihara, et al., 1958). These proteases have a wide specificity, amidase and esterase activity, are inhibited by DFP, and have been obtained in the crystalline form. Another alkaline protease from B. subtilis has recently been isolated and characterized (Ottesen, et al., 1960).

Subtilisin in particular has been used for work on protein structure. It has been used to isolate the S-S bridges in

ribonuclease (Ryle, et al., 1957), and to release one peptide fragment from ribonuclease (Richards, et al., 1959). Subtilisin was also a tool in sequence studies of oxytocin and glucagon (Tuppy 1953, Sinn, et al., 1957).

A report on alkaline proteases (Wieland, et al., 1960) has compared the properties of Nagarse, subtilisin, and an alkaline protease from B. cereus and has concluded that these enzymes are all the same. The conclusion is drawn from physical data (amino terminal, Sedimentation, mobility, and ultraviolet absorption) and from a comparison of enzymatic activities. There exists disagreement on this point, (Ottesen, et al., 1960) but the problem will not be resolved until the complete structure of these proteins is known.

B. Statement of the Problem

The objective of studying the B. subtilis neutral protease was: 1) to determine its physical and chemical characteristics; 2) to determine how it differed from the alkaline proteases; and 3) to determine if the protease was a metalloenzyme, and if so, the function of the metal.

Since a commercial preparation of the crude enzyme was available (Pacific Enzyme Laboratories, Honolulu, Hawaii), it was used as the starting material in the preparation of the pure enzyme.

At the beginning of this work, the original purification procedure was available only in abstracted form (Chem. Abstracts 1959) and no details were obtainable. Thus it was necessary to devise a new purification procedure.

If the protease were a metalloenzyme, the metal would have to be accurately determined and experiments devised to ascertain its function. The remaining experimental approaches used in this study were well defined: The isolation and purification, demonstration of homogeneity, characterization of the physical and chemical properties, and the study of the enzymatic activity.

II. Materials and Methods

A. Materials

1. Materials and reagents obtained commercially

Carl Schleicher and Schuell Company, New Hampshire

Carboxymethyl Cellulose

Diethylaminoethyl Cellulose

Difco Laboratories

Hemoglobin

Eastman Organic Chemicals, New York

Amyl Acetate

DL-Aspartic Acid

Dimethylglyoxime

S-Diphenylcarbazide

α, α' -Dipyridyl

Methyl Butyrate

Fisher Laboratory Chemicals, New Jersey

Cacodylic Acid

G. Fredrick Smith Chemical Company, Ohio

Bathophenanthroline

2-2 Biquinoline

Neocuproine·HCl

Ortho-phenanthroline

J. T. Baker Chemical Company, New Jersey

Gelatin

8-Hydroxyquinoline

Zincon

Mann Research Laboratories, New York

L-Alanyl-L-leucine

Benzoyl-L-arginine amide

N-Benzoyl-L-tyrosylglycinamide

N-Cbz-d-glutamyl-L-phenylalanine

NCbz-d-glutamyl-L-tyrosine

N-Cbz-glycyl glycine amide

N-Cbz-glycyl-L-phenylalanine

N-Cbz-glycyl-L-phenylalanine amide

N-Cbz-L-leucyl-L-leucineamide

N-Cbz-L-tyrosyl glycinamide

L-cysteine (free base)

Glycyl-DL-phenylalanine

Glycyl-L-phenylalanine amide acetate

Hippuryl-L-leucinamide

L-Histidine

L-Histidine ethyl ester·di HCl

L-Histidyl-L-histidine

L-Leucine

L-Leucyl-L-leucine

L-Phenylalanyl-L-phenylalanine

L-Valyl-L-valine

Matheson, Coleman and Bell, New Jersey

Ethylenediaminetetraacetic Acid (di Na salt)

Nutritional Biochemicals Corporation, Ohio

Casein (Hammersten Quality)

Egg Albumin

Glycyl-glycine

Glycyl-glycyl-glycine

Glycyl-glycyl-glycyl-glycine

Glutathione

L-Leucinamide·HCl

L-Leucyl glycine

Pharmacia Fine Chemicals, Incorporated, New York

S. E. sephadex

Sigma Chemical Company, Missouri

Tris-(hydroxymethyl)-aminomethane

Worthington Biochemical Corporation, New Jersey

Alpha Chymotrypsin 4x Crystallized

Trypsin 3x Crystallized

2. Materials obtained as gifts

Nagarse 1x Crystallized

from Nagase and Company, Osaka, Japan

Papain 3x Crystallized

from Mrs. Joyce Tsunoda, University of Hawaii

B. subtilis Protease Powder

from Pacific Enzyme Laboratories, Honolulu

L-Valyl-L-valine

Matheson, Coleman and Bell, New Jersey

Ethylenediaminetetraacetic Acid (di Na salt)

Nutritional Biochemicals Corporation, Ohio

Casein (Hammersten Quality)

Egg Albumin

Glycyl-glycine

Glycyl-glycyl-glycine

Glycyl-glycyl-glycyl-glycine

Glutathione

L-Leucinamide·HCl

L-Leucyl glycine

Pharmacia Fine Chemicals, Incorporated, New York

S. E. sephadex

Sigma Chemical Company, Missouri

Tris-(hydroxymethyl)-aminomethane

Worthington Biochemical Corporation, New Jersey

Alpha Chymotrypsin 4x Crystallized

Trypsin 3x Crystallized

2. Materials obtained as gifts

Nagarse 1x Crystallized

from Nagase and Company, Osaka, Japan

Papain 3x Crystallized

from Mrs. Joyce Tsunoda, University of Hawaii

B. subtilis Protease Powder

from Pacific Enzyme Laboratories, Honolulu

B. Methods

1. Preparation of resins

- a. Diethylaminoethyl cellulose, obtained as a dry powder was suspended in a large volume of water, decanted and then washed with 1N NaOH. The resin was then washed with water and decanted until the suspension was neutral, thus removing the fines. The resin was next washed with 1N HCl, water, and then titrated to the pH of the starting buffer with NaOH. The resin was then equilibrated with the starting buffer.
- b. Carboxymethyl cellulose, obtained as a dry powder was suspended in a large volume of 0.1N NaOH, decanted, and washed with water until the suspension was neutral. The resin was converted to the H⁺ form with 0.1N HCl. After repeated decantations with water to remove the fines, the resin suspension was titrated to the pH of the starting buffer with NaOH. The resin was then equilibrated with the starting buffer.

2. Ultra-Centrifuge studies

a. Sedimentation Velocity

Sedimentation velocity measurements were made in a Spinco Model E analytical ultra-centrifuge equipped with a RTIC unit for temperature regulation within $\pm 0.1^{\circ}\text{C}$. The conventional 12-mm cell with a 4° centerpiece was used. The employed speed was 59,780 rpm. All runs were carried out at 24°C ., and the sedimentation coefficient (s) was calculated according to equation 1.

$$s = dx/dt/xw^2 \quad 1.$$

where x is distance from center of rotation to peak and w^2 is the angular velocity

The observed sedimentation coefficients were reduced to standard conditions (water as solvent, 20°C.) according to Svedberg, et al. (1940).

Protein concentrations were determined spectrophotometrically with a Beckman D. U. spectrophotometer at 280 mu using an $E_{1\text{cm}}^{1\%}(280)$ of 13.6.

The sedimentation coefficients ($s_{20,w}$) were averaged and then plotted against initial concentration and the line extrapolated to infinite dilution by the method of linear least squares.

b. Archibald "Approach to Equilibrium" Method

Molecular weight determinations by the Archibald "Approach to Equilibrium" method were conducted as outlined by Schachman (1957) and were performed at a speed of 12,590 rpm with the Schlieren phaseplate angles ranging from 60-75°. The molecular weight was calculated only from the meniscus by using equation 3 with $F = w^2 \left(\frac{dc}{dx} \right) / X_m C_m$, where the subscript m denotes the position of the meniscus. The concentration at the meniscus (C_m) was computed by equation 2 of Klainer and Kegeles (1955).

$$C_m = C_o - \int_{x_m}^X \frac{1}{X_m^2} X^2 \left(\frac{dc}{dx} \right) dx \quad 2.$$

where X_m is the distance of the air solution meniscus measured in centimeters from the axis of rotation and X is an arbitrary X coordinated in the plateau region ($dc/dx=0$), and where C_0 , the initial concentration, is determined with a standard 12 mm, 4° sector synthetic boundary cell. All concentrations are expressed in optical units, obtained by trapazodal integration of the areas under the Schlieren curves. In these experiments, measurement of these curves was done with a photographic enlarger.

c. Measurement and Calculation of the Partial Specific Volume

The calculation of the molecular weight by either the sedimentation velocity-diffusion method or the Archibald procedure (Approach to Equilibrium Method) involves the use of the relationship:

$$M = RT / (1 - \bar{v}p) \times F \quad \dots \quad 3.$$

where $R = 8.314 \times 10^7$ erg/degree/mole

T = temperature on the Kelvin Scale

\bar{v} = partial specific volume

p = density of solution

$F = (s/D)$ where D is the diffusion constant standardized to water and 20°C .

(Sedimentation Diffusion Procedure).

$F = w^2(dc/cx)_{m,b} / X_{m,b} C_{m,b}$

(Archibald Procedure).

Thus, the parameter \bar{v} has to be evaluated independently.

The measurement of this parameter was carried out according to the method of densities as outlined by Schachman (1957), in which the densities of the solvent and solution were measured pycnometrically. From these measurements, the apparent partial specific volume could be calculated by means of equation 4.

$$\bar{v}_{app} = 1/d_0 - 1/x(d-d_0)/d_0 \quad \dots \quad 4.$$

where d_0 = density of solvent (buffer)
 d = density of solution
 x = concentration of protein in gm/ml

The protein concentration of the protein was determined spectrophotometrically as discussed above.

3. Electrophoresis

a. Free - Boundary Electrophoresis

Electrophoresis was conducted at 3°C. with a Perkin-Elmer electrophoresis apparatus, Model 38, equipped with a Schlieren optical system. A record of the electrophoretic behavior was obtained by use of a Polaroid Land-Back Camera attached to the instrument.

The buffers were prepared by the method of Miller and Golder (1950).

b. Measurement of the Isoelectric Point

Electrophoresis was carried out as described above.

Buffers of one-tenth ionic strength were prepared according to Miller and Golder, previously cited. At room temperature the pH of the solution was determined using a Beckman pH meter with an expanded scale.

Conductivity measurements of the buffered protein solution were measured in a conductivity cell with platinum electrodes, using a Leeds and Northrup Wheatstone Bridge. The cell constant was determined using 0.10N KCl, and the value of 0.940 was obtained.

Dialysis of the protein solution against the supporting buffer was carried out in the cold room for approximately six hours with continuous stirring.

Prior to the measurement of the conductivity, the protein solution was routinely centrifuged to remove suspended material.

During the electrophoresis run, a minimum of six photographs of the descending boundary patterns were taken at various time intervals.

The mobilities were calculated in the customary manner from the distances of migration of the maximal positions of the individual peaks. The mobilities cited in Table VI represent the average values of the calculated mobilities obtained from six separate determinations of the descending limb. The measurement of the distances of migration were made with the aid of a Gaertner Micro-Comparator.

4. Measurement of Refractive Index Increment (dn/dc)

The refractive index increment of B. subtilis neutral protease was determined at 436 mu and 546 mu at 25°C. using a Thermostated Brice-Phoenix Differential Refractometer (Phoenix Instrument Company, Philadelphia, Pennsylvania). The enzyme was in a solvent of 0.07N NaCl and 0.02M Ca(Ac)₂ to minimize autolysis: A stock solution of this enzyme was used and the measurement of (dn) made on serially diluted solutions. The concentration of the enzyme was determined by taking a suitable aliquot and drying to constant weight in tared bottles.

5. Amino Terminal Amino Acid Analysis

The procedure of Sanger (1945, 1949) was used to

determine the amino terminal acid. In order to identify the dinitrophenyl-amino acids, the solvent system, t-amylalcohol - 3% NH₃ in the first dimension, and 1.5M phosphate buffer pH 6.0 in the second dimension was used to examine the ether phase, and the solvent system, t-amyl alcohol-phthalate buffer pH 6.0, was used to examine the aqueous phase (Fraenkel-Conrat, et al., 1958). No corrections were applied for manipulative losses or destruction during acid hydrolysis.

6. Metal Analyses

a. Zinc Analysis

The zinc content of the enzyme was determined by Zincon Method of Rush and Yoe (1954) using the procedure of Williams et al., 1962, with the following modifications: Zincon was prepared by the original method of Rush and Yoe. Since only zinc was to be determined and since the other metals present did not interfere, the demasking procedure of Williams was not used, so the metal was not converted to its cyanide complex or destroyed with chloral hydrate.

b. Iron Analysis

Iron was determined as the ferrous-bathophenanthroline complex after extraction into isoamyl alcohol under acidic conditions. The procedures used were described in The Iron Reagents by Diehl and Smith published by the G. Fredrick Smith Chemical Company.

c. Manganese, Chromium, and Nickel Analyses

Manganese was determined by oxidation to permanganate, chromium by S-diphenylcarbazide, and nickel by dimethylglyoxime. These methods of analysis are described in detail in Sandell, E. B., Colorimetric Metal Analysis, 3rd Ed., 1959.

7. Methods of Assay and the Measurement of Enzymatic Activity

a. Casein Digestion Method

The proteolytic activity of the B. subtilis neutral protease was determined by the method of Hagihara (1958) using a 1% solution of casein (Hammersten quality) in suitable buffers or dilute NaCl. For most of the assays used in this study, activity measurements were made at 30°C. in 0.01N NaCl, pH 7.30 (chosen as the standard condition). The reaction with casein was carried out in the following manner: to 5 mls of casein solution equilibrated at bath temperature, 1 ml of enzyme solution was added. Digestion was then allowed to proceed for ten minutes, after which the reaction was terminated by the addition of 5 mls of precipitating reagent (0.11M T.C.A. + 0.22M NaAc + 0.33M HAC). The mixture was allowed to stand at room temperature for 30 minutes and then was filtered. The absorbency was then measured at 275 mu in a Beckman DU Spectrophotometer.

A unit of enzyme activity $\left[(PU)_{275 \text{ mu}}^{\text{casein}} \right]$ is defined as the enzyme activity which gives the extinction at

275 mu equivalent to 1 ug of tyrosine liberated in one minute at 30°C. (Hagihara, et al., 1958). The absorbance was corrected for absorbency contributions of non-enzymatic nature (this blank being prepared by the addition of 5 mls of precipitating reagent to 5 mls of casein before the addition of 1 ml of enzyme solution). Specific activities were expressed as the units of enzymatic activity $\left[\frac{(\text{PU})_{275 \text{ mu}}^{\text{Casein}}}{\text{mg enzyme}} \right]$ per mg enzyme, the value was linear up to a total of 8 ug in the reaction mixture for the pure enzyme.

b. Formol Titration

The proteolytic and esterase activities of the B. subtilis neutral protease were determined with the following substrates: casein (Hammersten quality), gelatin, butyl acetate, amyl acetate, ethyl valerate, ethyl acetate, and methyl-n-butyrate. The protein substrates were dissolved in dilute NaOH and adjusted to pH 7.3 with HCl. The esters were suspended in 0.01M tris and adjusted to pH 7.3 with acetic acid. The reaction with protein substrates was carried out in the following manner: to 5 mls of substrate equilibrated at the bath temperature, 1 ml of enzyme solution was added. Digestion was allowed to proceed for 20 minutes and was then terminated by the addition of 5 mls of 37% formalin solution (neutral pH). This mixture was then titrated potentiometrically with standardized 0.01N NaOH to pH 8.3. After correction for the blank (in the

blank, 5 mls of 37% formalin solution was added before addition of the enzyme) the unit of activity was defined as the micro-equivalents carboxyl liberated per minute per gm substrate. Specific activities were expressed as the units of enzymatic activity per mg enzyme. The maximum value was estimated from the initial slope of a plot of the units of activity versus μg enzyme in the reaction mixture.

The esterase activity was determined by the method of Matsubara, et al., (1958). The procedure was the same as described above except for a two-hour incubation with the enzyme.

c. Hemoglobin Assay

The proteolytic activity of B. subtilis neutral protease was determined by the method of Anson (1938) with the following modification, ammonium phosphate was not used. The pH was adjusted to 7.3 with NaOH. The overall method was adapted to that of Hagihara with the exception of the precipitating reagent. This remained 5% trichloro acetic acid.

d. Egg Albumin Assay

Egg Albumin was treated in the same manner as casein (as described) and the activity was determined accordingly.

e. Peptidase Activity

Studies which required a more accurate measurement of enzymatic activity, particularly in the kinetic

studies, were quantitated by the use of the ninhydrin method of Moore and Stein (1954), since preliminary work had shown that the enzyme hydrolyzed only certain derivatives of a few peptides. Thus, a few well defined substrates were available.

The Ninhydrin Method of Moore and Stein (1954)

Leucinamide liberated during the hydrolysis of hippuryl-L-leucinamide was determined by the method of Moore and Stein (1954). The procedure was calibrated with leucine standards which contained all of the components of the reaction mixture.

For the assay, graduated amounts of a stock solution of 0.03M hippuryl-L-leucinamide in 0.10M cacodylate or tris buffers at the desired pH were added to a series of test tubes. The volume was raised to 2 mls with the corresponding buffer. After incubation for 5 minutes, 0.1 ml of enzyme solution (containing 4.0 to 19 ug enzyme) was added to each tube and a 0 time sample was taken. Every 3 or 5 minutes thereafter, 0.2 mls aliquots were taken and added to 0.4 ml ninhydrin reagent (20 gms ninhydrin, 3 gms hydrindantin, 250 mls 4.0M acetate buffer, pH 5.5 and 750 mls methyl cellosolve), plus 0.2 mls 4.0M acetate buffer, pH 5.5. The resulting mixture was shaken and placed in a boiling water bath. After boiling the mixture for fifteen minutes, it was rapidly cooled to 30°C. and 10 mls of 50% ethanol was added. The absorbency of this

solution was determined at 550 m μ in a Coleman Jr. Spectrophotometer. The blank used in all determinations was the zero time sample. From a plot of the absorbency reading (which can be converted to concentration terms by the standard curve calibrated with leucine) versus time, the rate of the reaction (expressed as absorbency change per unit time, moles per liter leucinamide formed per unit time, or per cent hydrolysis per unit time) was calculated.

8. Kinetic Analysis

Rate measurements were conducted by means of the ninhydrin method of Moore and Stein (1954) discussed previously.

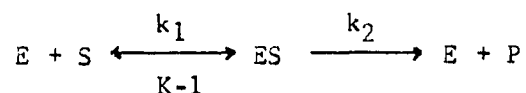
Unless otherwise specified, assays were conducted at 30°C. The reaction mixture consisted of 2 ml of hippuryl-L-leucinamide in a suitable buffer, which was 0.10M and contained 0.004M Ca(Ac)₂, and 0.10 ml of the enzyme solution final concentration 5.3 ug/ml. Immediately after mixing and at suitable intervals thereafter, 0.20 ml aliquots were removed and added to 0.40 ml ninhydrin and 0.20 ml 4.0M acetate buffer, pH 5.5. The tubes were shaken, immediately placed in the boiling water bath, and processed as previously described.

The method was calibrated with leucine solutions containing all of the components of the reaction mixture.

Initial velocities were estimated essentially by the method described by Lumry, et al., (1957) in their studies

with carboxypeptidase A.

Kinetic analyses were made assuming the Michaelis Menten scheme of enzyme catalysis:



Assuming the validity of the steady state approximation, the constants K_m and V_{max} were calculated from plots of the data according to the method of Lineweaver and Burke (1934). K_m and V_{max} were defined as $(k_{-1} + k_2) / k_1$ and $k_2(E)_t$, respectively, where $(E)_t$ was the total enzyme concentration. The values for k_2 were calculated on the assumption that the enzyme contains one catalytic center per molecule.

III. Results

A. Purification and Homogeneity Studies

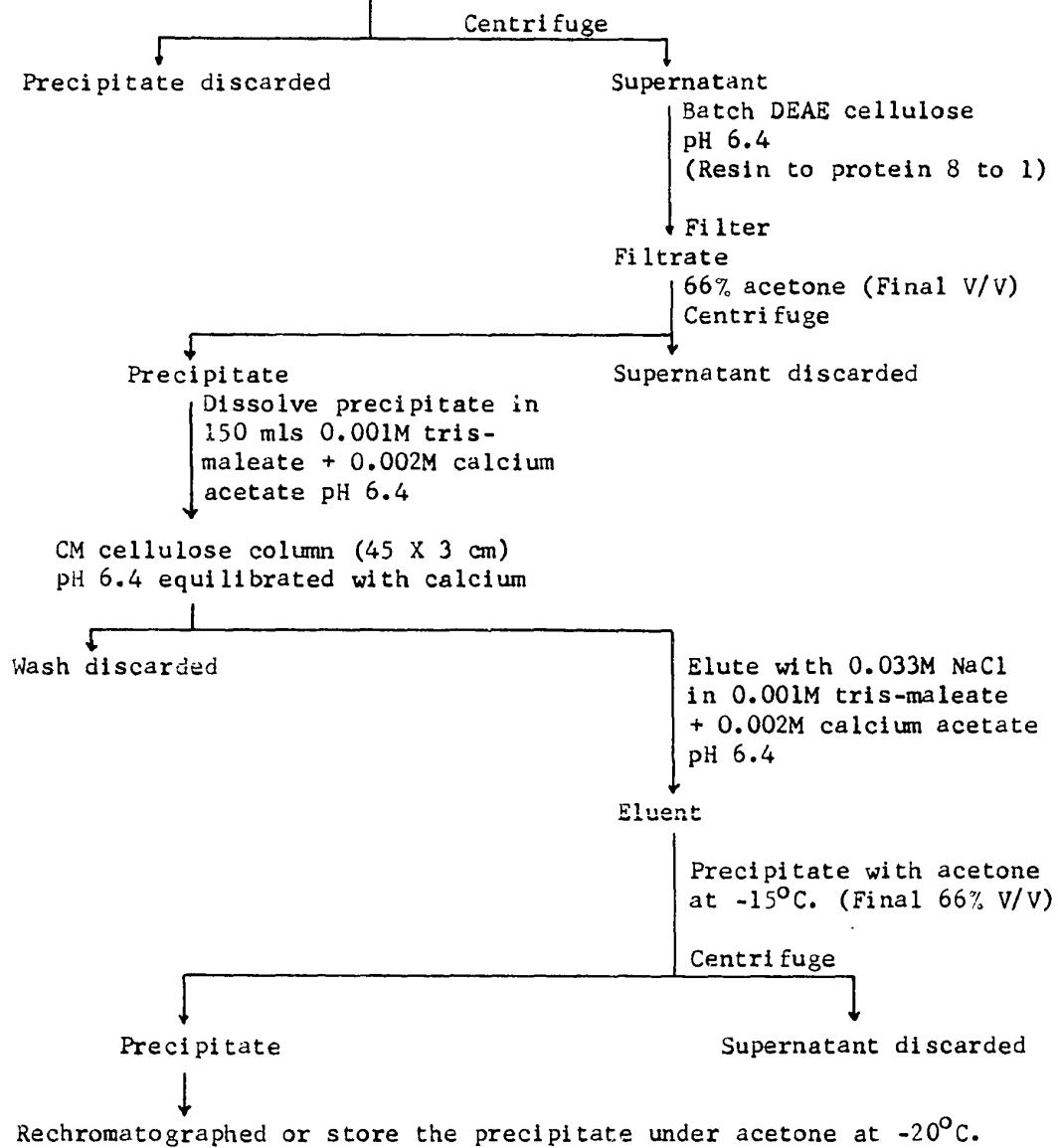
1. Purification

The flow sheet for the purification of the B. subtilis neutral protease is given in Table I while Table II gives the results of one purification. The average time for the complete purification is about four days. This time of purification is kept as short as possible to minimize autolysis and denaturation, and the temperature is kept constant at 4°C. (The enzyme cannot be kept as an acetone precipitate unless the temperature is below -10°C. for periods longer than three days.)

As seen in Table II the purified enzyme makes up about 3% of the starting protein. The enzyme could not be

TABLE I

20 gms starting material dissolved in 400 mls
0.001M tris-maleate + 0.002M calcium
acetate pH 6.4



detected by electrophoresis until after the acetone precipitation. At this point, the enzyme accounted for approximately 30% of the total protein. These approximate results from electrophoresis also agreed with the distribution obtained on carboxymethylcellulose chromatography (Table II and Figure 1).

Figures 1 and 2 show the elution patterns of the enzyme from carboxymethylcellulose. The cross hatched portions indicate the enzyme with the highest specific activity. By using a larger washing volume, the necessity of eluting with 0.01M NaCl is eliminated (Figures 1 and 2). This larger washing volume generally eliminates all the color from the enzyme.

In all subsequent purifications the column was washed with 1.5 to 2 liters of starting buffer before the enzyme was eluted.

Figure 3 illustrates the rechromatography of the enzyme on carboxymethylcellulose. The figure shows that protein, enzyme activity and zinc are eluted from the column at the same place thus indicating that zinc is associated with the enzyme and is not an extraneous metal.

Figure 4 also indicates zinc as a prosthetic group because there is a linear relationship between zinc content and enzyme activity. These studies give support to the proposition that the B. subtilis neutral protease is a zinc metalloenzyme with zinc necessary for enzyme activity. (See Section IIIC1b).

TABLE II

Procedure	Vol. mls	Activity units/ml	Protein mg/ml	Zinc ug/ml	Specific Activity	Total Units	Total Protein	Yield %	ug Zn/mg Protein	Purifi- cation
Original	400	12.9×10^4	118*	23.5	1090	51.4×10^6	47.2 gm	100	0.20	---
DEAE cellulose	880	3.35×10^4	21.3	7.65	1572	29.5×10^6	18.7	57.4	0.36	1.4
Acetone Precipitation	150	2.3×10^5	55	30.1	4347	35.5×10^6	8.25	69.0	0.55	2.8
CM cellulose 0.03M eluant	475	2.61×10^4	2.03	4.67	11346	12.4×10^6	1.09	24.0	2.33	2.6

* Determined by absorbance, the actual weight of the starting material was 30 grams.

FIGURE 1

Chromatography of the acetone precipitate on CM cellulose. A CM cellulose column (40 x 2 cm) was charged with 620 mg of protein containing 9×10^5 units. The flow rate was 20 ml per hour and fractions of 13.3 mls were collected. Stepwise elution was used starting with 0.001M tris-maleate + 0.002M calcium acetate buffer pH 6.4. At the points indicated, portions of the same buffer containing 0.01M, 0.03M, and 0.1M NaCl were used as elutants. The 0.03M NaCl eluant was collected and used for rechromatography on CM cellulose. 0-0-0 and x-x-x represent absorbance at 280 mu and protease activity (units per ml), respectively.

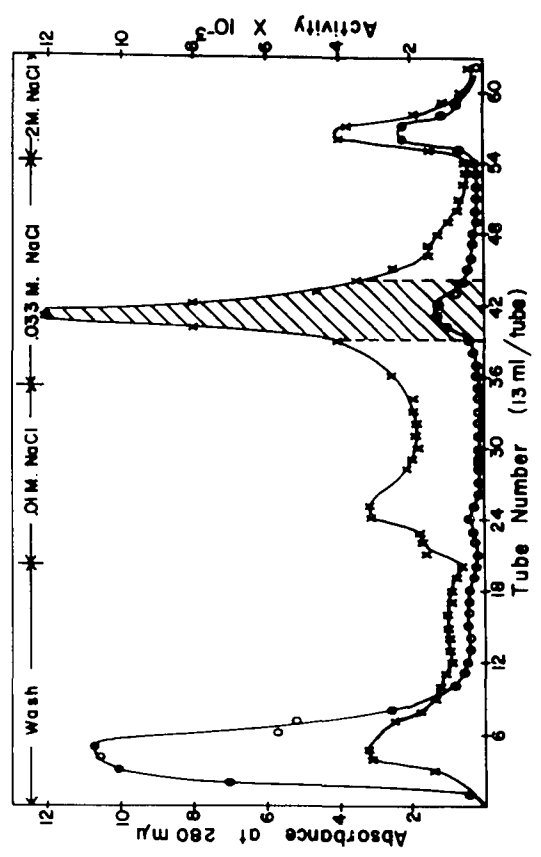


FIGURE 2

Chromatography of the acetone precipitate on CM cellulose. A CM cellulose column (45 X 3 cm) was charged with 15.5 gm of protein containing 2.4×10^7 units. The flow rate was 40 ml per hour and fractions of 17 mls were collected. Stepwise elution was used after the column had been washed with 1.5 liters of starting buffer (0.001M tris-maleate + 0.002M calcium acetate pH 6.4). At the points indicated 0.033M and 0.1M were used as elutants. The 0.033M eluant was collected and used in the physical studies. 0-0-0 and x-x-x represent absorbence at 280 mu and protease activity (units per ml) respectively.

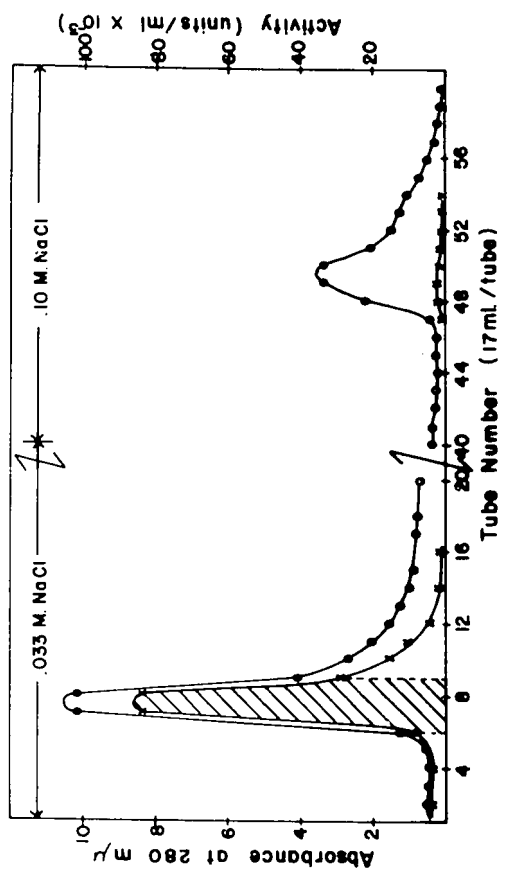


FIGURE 3

Rechromatography on CM cellulose and the relationship of zinc 1.09 gm of the 0.033M NaCl eluant was precipitated with two volumes of acetone, collected by centrifugation, and dissolved in 50 ml of 0.001M tris-maleate + 0.002M calcium acetate buffer, pH 6.4. The enzyme solution was charged on a CM cellulose column (45 X 3 cm) and the column was washed with 1.5 liters of starting buffer. The enzyme was eluted with the same buffer containing 0.033M NaCl. Nine ml fractions were collected at a flow rate of 36 ml per hour. 0-0-0, ▲-▲-▲, and x-x-x represent absorbance at 280 mu, zinc content (ug per ml), and protease activity (units per ml) respectively.

FIGURE 4

The proportionality between zinc content and the specific activity. The points were obtained from the purification and from the dialysis of the enzyme against acidic buffers.

1

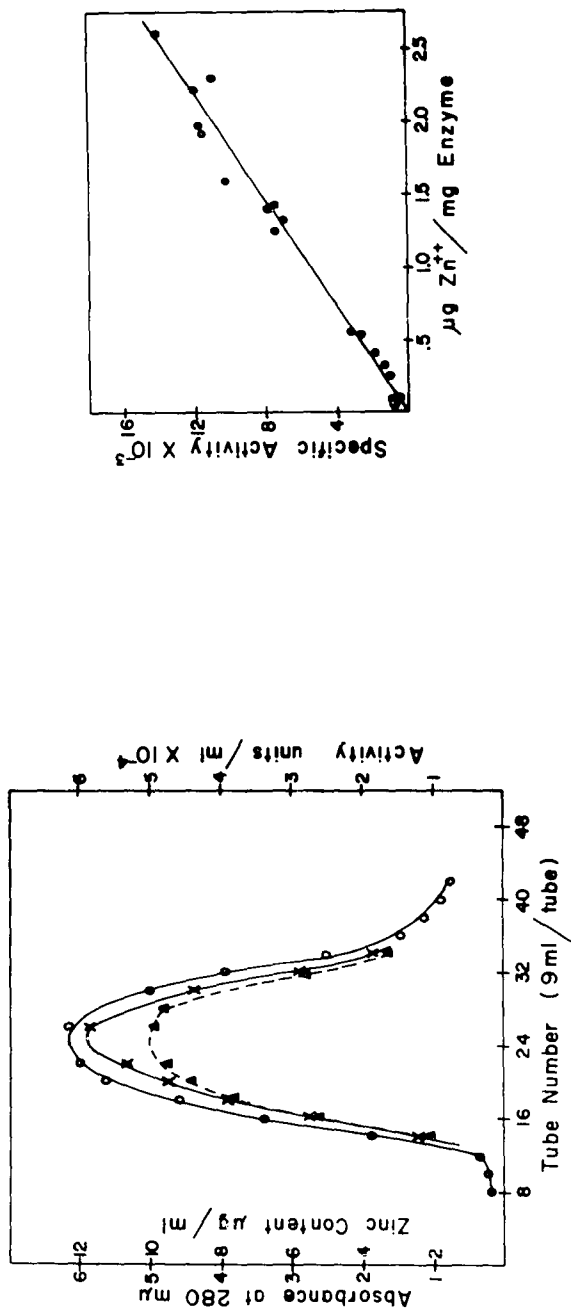
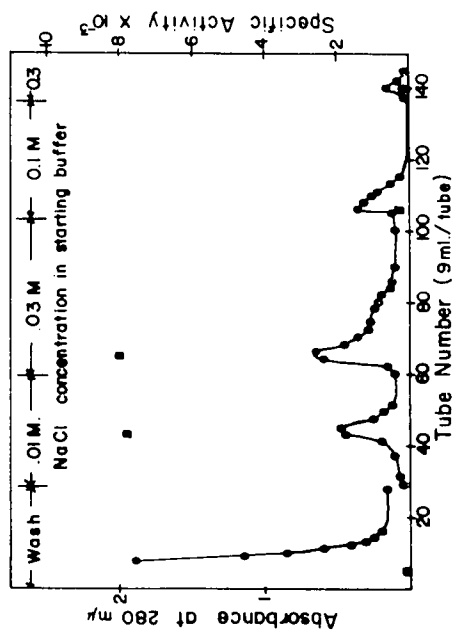


Figure 5 shows the effect of omitting calcium ions during the purification. As can be seen, the 0.03M NaCl elutant contains two species and the 0.01M NaCl elutant is increased. (Compare with Figure 1.) This effect is probably due to autolysis, since this pattern also can be obtained on rechromatography of the calcium prepared enzyme if it has been in solution for about seven days or longer before chromatography. In this case, however, there is no change in activity, but the 0.03M NaCl elutant peak has three shoulders. Chromatography on the stronger resin, SE Sephadex, gives the same results but with a lower specific activity because zinc is removed from the enzyme. Early in this work, before calcium ions were used in the purification, the enzyme was chromatographed on SE Sephadex. The 0.03M NaCl elutant was then rechromatographed on the same resin and a single peak obtained but with a 60% loss in activity. At the time, the reason for the loss in activity was unknown but was thought to be autolysis. Later after the described purification was worked out and the zinc was shown to be a prosthetic group of the enzyme, SE Sephadex was used to remove the excess calcium from the purified enzyme. This experiment was attempted to determine if there were any tightly bound calcium ions in the enzyme and if so, the amount. The experiment was as follows: The enzyme, 28.7 mg, specific activity 13,000, and zinc content of 2.3 ug Zn^{++} per mg enzyme was charged on a 32 x 15 cm column filled with 17 cm of SE Sephadex. The enzyme was

FIGURE 5

Chromatography of the crude enzyme on CM cellulose. (No calcium is present). A CM cellulose column (18 X 1.5 cm) was charged with 0.5 gm of protein containing 6×10^5 units. The flow rate was 20 ml per hour and fractions of 9 mls were collected. Stepwise elution was used starting with 0.001M tris-maleate buffer pH 6.4. At the points indicated 0.01M, 0.03M, 0.1M and 0.3M NaCl were used as elutants. 0-0-0 represents the absorbence at 280 mu while ■ represents the specific activity measured at that point.



eluted with 0.03M NaCl. The eluant contained 70% of the activity and 65% of the zinc. This enzyme was wet ashed with nitric acid and calcium was determined by flame spectrophotometry. The enzyme was shown to contain 1.4 ug Ca^{++} per mg enzyme. A blank containing the same amount of calcium was chromatographed and the calcium content determined. This blank agreed with the zero calcium standard; thus loosely bound calcium was extracted by the resin and firmly bound calcium remained bound to the enzyme. The absolute amount of bound calcium could not be determined because it could not be assumed that zinc and calcium were lost at the same rate. Even though the experiment failed to show the exact moles of calcium per mole of enzyme, it did suggest that the enzyme contained firmly bound calcium.

The loss of zinc in the above experiment altered the hypothesis that autolysis caused the 60% loss of activity during rechromatography on SE Sephadex. The removal of zinc by the resin was the main reason for the loss in activity. The removal of zinc by CM cellulose may also explain why the zinc content of the purified enzyme is 1.6 moles of zinc per mole of enzyme. (Section IIICb).

2. Purity Determinations

It is necessary to determine the purity of a protein preparation when a study of its physical and chemical properties and its biological activity is undertaken. While the determination of purity is often very difficult, physical and chemical methods do exist, which can be used

as criteria for the indication of relative homogeneity or heterogeneity of a protein preparation.

In the present study, the purity of the B. subtilis neutral protease was determined by the criteria of ultracentrifugation, free boundary electrophoresis, amino terminal analysis, and rechromatography.

Discussions of the problems involved in the determination of purity are found in the literature (Li, 1951; Pirie, 1940).

a. Ultra-centrifuge Studies

Examination of the sedimentation patterns obtained indicates no gross heterogeneity at pH 6.5 (Fig. 6). This monodispersity during sedimentation was consistently observed, indicating that the enzyme is relatively homogenous with respect to size and shape. Also, as shown in Section III B 1 a), the calculated sedimentation coefficients show no concentration dependence in the range studied indicative of ideality of solution.

b. Electrophoretic Studies

As previously discussed, electrophoretic studies during purification were of little value.

The electrophoretic studies with the purified enzyme, however, are of importance since over the most stable pH range of the enzyme, they show that the homogeneity of the enzyme is 95% or greater, electrophoretically (Fig. 7). These studies, in addition to others, were used to determine the mobility of the

FIGURE 6

The sedimentation patterns of the neutral protease.

- a. A 0.6% solution of the rechromatographed enzyme (specific activity 13,000) was prepared in 0.1M NaCl + 0.002M calcium acetate pH 6.5 and was centrifuged at 59,780 rpm and 24°C. The photographs were taken at 21.8, 37.8, 53.8, 69.8 and 85.8 minutes after reaching full speed. The bar angle in all photographs was 50°.
- b. A 1.03% solution of the rechromatographed enzyme (specific activity 13,000) was prepared in 0.1M NaCl + 0.002M calcium acetate pH 6.5 and was centrifuged at 59,780 rpm and 24°C. The photographs were taken at 21.9, 37.9, 53.9, 69.9 and 85.9 minutes after reaching full speed. The bar angle for photographs 1, 2, and 3 was 60° and was 50° for photographs 4 and 5.

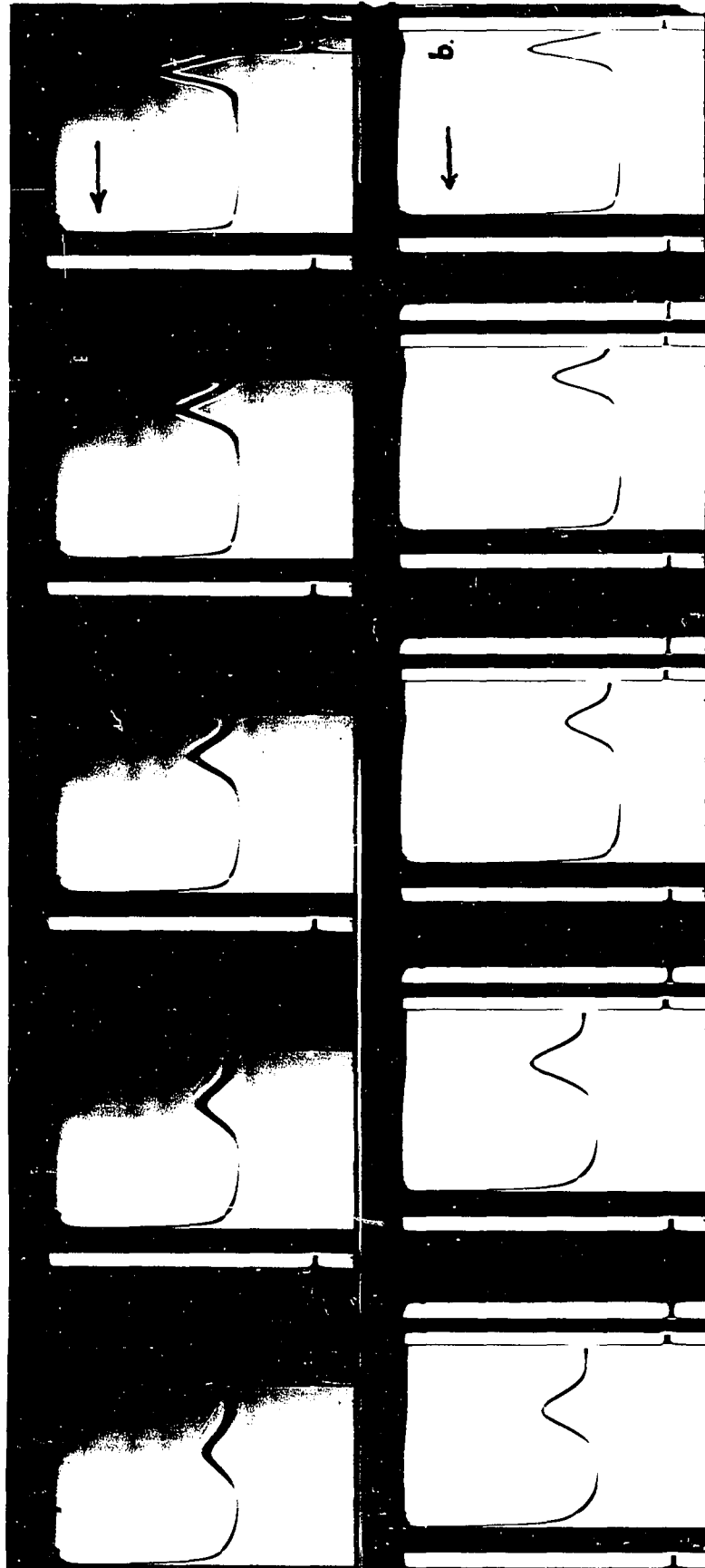


FIGURE 7

Electrophoretic patterns of the neutral protease.

1. Veronal buffer + 0.002M calcium acetate pH 9.0

Descending boundary at 30, 90, and 150 minutes (A, B, and C, respectively).

2. Veronal buffer + 0.002M calcium acetate pH 8.5

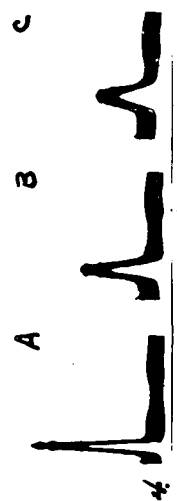
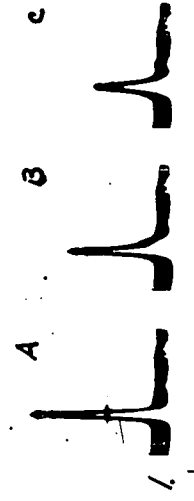
Descending boundary at 30, 90, and 150 minutes (A, B, and C, respectively).

3. Veronal buffer + 0.002M calcium acetate pH 7.9

Descending boundary at 30, 90, and 150 minutes (A, B, and C, respectively).

4. Acetate buffer + 0.002M calcium acetate pH 5.5

Descending boundary at 30, 90, and 150 minutes (A, B, and C, respectively).



purified enzyme as a function of pH.

It was also noted that the length of time between preparation of the enzyme and electrophoresis changed the relative amount of heterogeneity. The explanation of this phenomenon probably lies in the autolysis of the enzyme. (Autolysis was also noted during molecular weight determinations, Section IIIB1b). With these observations, it can be concluded that the B. subtilis neutral protease is, in general, monodisperse in the sense that it contains no grossly contaminating proteins. This monodispersity was also suggested by the fact that after 300 minutes, the peak remained symmetrical.

The absence of complete homogeneity does not appear to reflect any gross contamination of the neutral protease, which is free of other proteins as determined by several criteria, such as amino terminal analysis as studied by the DNP method of Sanger (Section C) sedimentation, just described, and rechromatography (Section D).

c. Amino Terminal Studies

Table III shows the results of the amino terminal analysis on different preparations of the neutral protease. In every preparation, glycine was the only amino terminal amino acid. In one preparation, however, duplicate determinations of the amino terminal did show .02 u Moles of serine in one example. This result in

itself does not imply heterogeneity (Thompson, E. O. P., 1953), since under the more drastic conditions of hydrolysis, DNP glycine was the only amino terminal acid observed.

TABLE III

Amino-Terminal Amino Acid Analyses

DNP Preparation No.	uM Enzyme	Specific Activity	Zinc Content ug/mg	Conditions of Hydrolysis	DNP-amino acid	Yield %
1	0.485	11,073	2.1	12 N HCl for 4 hr	DNP-glycine DNP-serine	58%* 4%
2	0.485	11,073	2.1	12 N HCl for 4 hr	DNP-glycine	62.5%*
3	0.800	12,000	--	6 N HCl for 24 hr	DNP-glycine	--
4	0.448	12,700	--	6 N HCl for 12 hr	DNP-glycine	49%*

* Uncorrected for destruction or handling losses and the molecular weight of the enzyme was assumed to be 44,700.

B. Physical Properties

1. Weight Average Molecular Weight Determinations

A number of physical methods may be used to determine the molecular weight of a protein. Alexander and Block (1961) and Tanford (1962) present methods which are used for the determination of the molecular weight of polymers.

The method used in the present study for the determination of the molecular weight of the neutral protease utilized the analytical ultra-centrifuge. Schachman dis-

cussed the principles of ultra-centrifugation in a monograph (1959) and also outlined the experimental aspects of the molecular weight determination with the ultra-centrifuge (1955).

a. Sedimentation-Velocity

1) Measurement and Calculation of $s_{20,w}$

Table IV summarizes the values obtained for the sedimentation coefficient of the neutral protease as a function of protein concentration at 24°C. Figure 8 illustrates these values. The value of the sedimentation coefficient, corrected to 20°C. in water, is $3.24 \pm .05s$.

TABLE IV

Summary of the Sedimentation Coefficients

Preparation	Temp.	pH	Solvent	Protein Concentration (%)	$s_{obs.} \times 10^{13}$
1	24°C.	6.5	.1N NaCl .001M CaCl	0.46	3.56
1	24°C.	"	"	0.34	3.52
1	24°C.	"	"	0.30	3.43
2	24°C.	"	"	1.03	3.44
2	24°C.	"	"	0.82	3.44
2	24°C.	"	"	0.62	3.55

Under the conditions used and the range of protein concentration studied, the values of sedimentation coefficients did not exhibit concentra-

tion dependence, which indicates, in this region, a thermodynamically ideal solution.

2) Measurement and Calculation of the Partial Specific Volume

The value of the partial specific volume of the neutral protease as determined at 30°C. in the manner outlined under Methods is $.746 \pm .002$ cc/gm.

The value obtained by the method of McMeeken and Marshall, 1952, from the amino acid composition data of D. Tsuru was $.721$ cc/gm.

b. "Approach-to-Equilibrium Method (Archibald Procedure)

Figure 10 shows the patterns obtained from a typical Archibald run. The results of the calculations of the molecular weight of the neutral protease at a series of concentrations and three different preparations are summarized in Figure 11.

The apparent molecular weight showed a negative dependence with time (Figure 9), but after extrapolating the molecular weight to zero time and plotting this value against concentration, the molecular weight did not show dependence on concentration (Figure 11). This type of behavior is consistent with autodigestion of the enzyme, i.e. greater autolysis at higher concentrations. This should give a greater negative slope (molecular weight versus time) at greater concentrations. This is observed in the data in Table V. Any interpretation of the results other than by autolysis

FIGURE 8

The sedimentation coefficients of the neutral protease as a function of protein concentration. The protease was studied in 0.1M NaCl + .002M calcium acetate pH 6.5 at 24°C. The line represents the linear least squares and extrapolation to zero concentration.

FIGURE 9

The plot of $M_{app.}$ as a function of time of centrifugation. The apparent molecular weight as obtained from the photographs in Figure 10 is plotted as a function of time. The line represents the linear least squares.

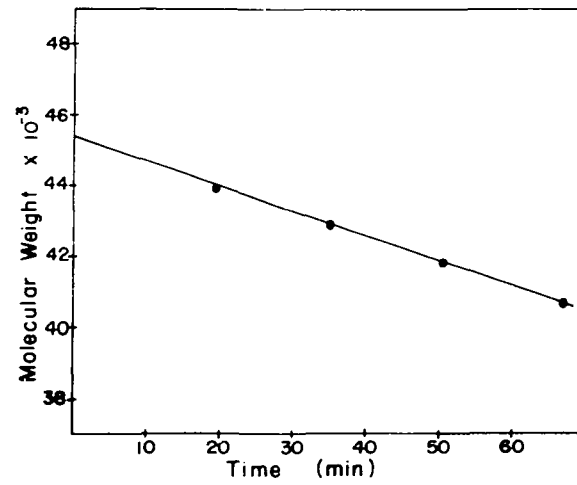
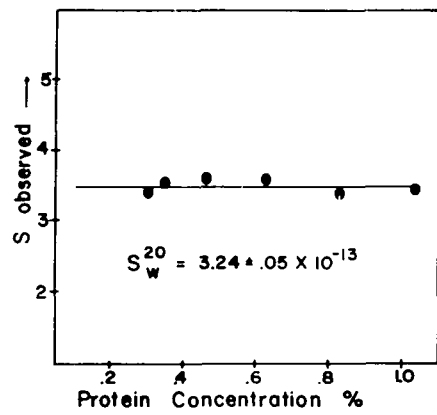


FIGURE 10

Archibald "Approach to Equilibrium" determination on the neutral protease. The protein concentration was 1.2% and the temperature was 24°C.

- A. Run in a 12 mm cell with a 4° sector aluminum centerpiece.
The times after full speed are 3, 19, 35, 51, and 67 minutes.
- B. Synthetic boundary run in 0.07M NaCl + 0.02M calcium acetate at 12,590 rpm. The bar angle was 70° in all photographs.

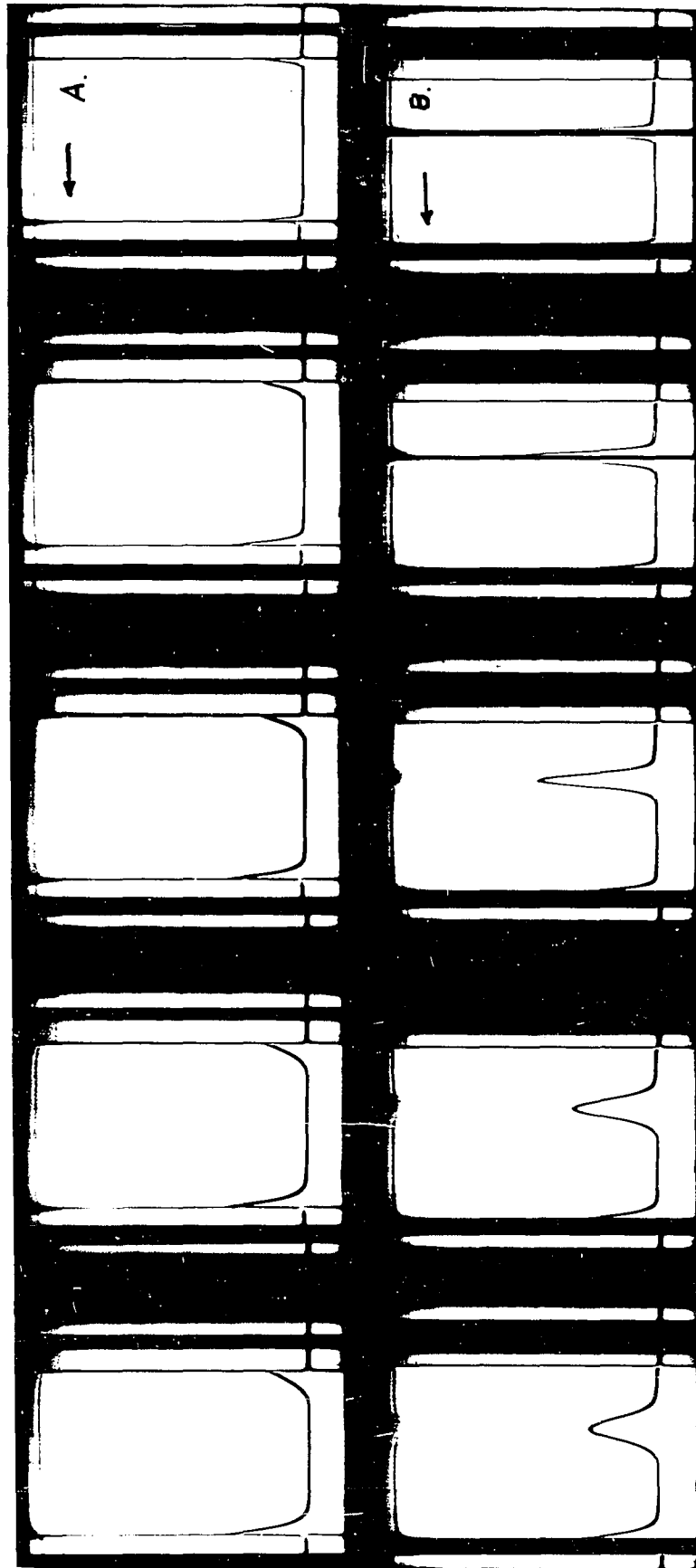


FIGURE 11

The plot of M_{app} . (extrapolated to zero time) as a function of protein concentration. The line represents the average molecular weight. Different symbols represent different preparations.

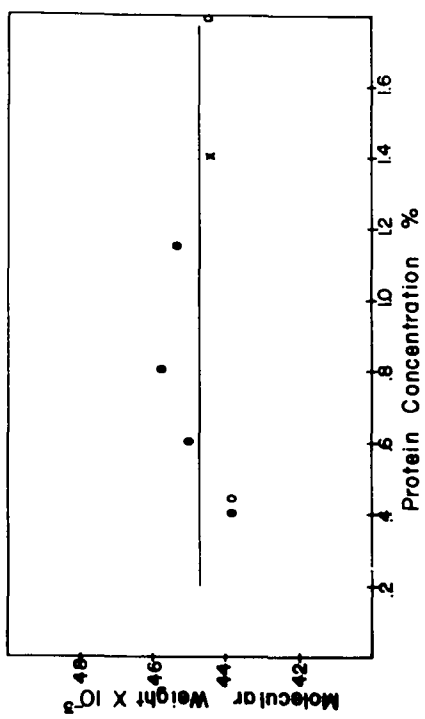


TABLE V

Molecular Weight Variation with Time

<u>Sample</u>	<u>Protein Concentration</u>	<u>Temperature of Run</u>	<u>Slope M.W./time</u>
1. d.	.40%	24°C.	- .032
2. b.	.44%	21°C.	- .003
1. c.	.60%	24°C.	- .052
1. b.	.80%	24°C.	- .070
1. a.	1.20%	24°C.	- .073
3	1.40%	24°C.	- .056
2. a.	1.70%	24°C.	- .107

<u>Sample</u>	<u>Solvent</u>
1a, 1b, 1c, 1d, 2a	0.07M NaCl + 0.02M Ca(Ac) ₂
3	0.10M NaCl + 0.002M Ca(Ac) ₂
2b	0.002M Ca(Ac) ₂

should give a decreasing molecular weight with decreasing protein concentration (positive concentration dependence). The differences in slope of the molecular weight versus time are probably due to temperature and ionic strength effects (see Section IID6c and Figure 25A).

Treatment of the data obtained with the neutral protease yields a weight average molecular weight of $44,700 \pm 800$. The standard deviation does not include the uncertainty in the partial specific volume.

For a discussion of the ideality of the sedimentation coefficient and the non-ideality of the molecular weight in the ultra-centrifuge, see Appendix I.

2. Electrophoretic Studies

The variation of Electrophoretic Mobility with pH: The isoelectric point.

Figure 12 plots the calculated mobility as a function of pH. Interpolation of the mobility to zero mobility yields an isoelectric point of $8.95 \pm .06$ pH units.

It is seen in the figure and Table VI that the mobility does not vary greatly over the pH range 5.0 - 8.00. This is generally indicative of ion binding or of an ionic strength effect (ion-atmosphere, Moore, 1958). Data from the amino acid composition gives a calculated isoionic point of 4.8. The difference of 4.0 pH units can be reconciled when ion binding is considered, thus the charges of aspartic and glutamic acid residues are neutra-

TABLE VI

Summary of Electrophoretic Mobility Measurements

Run No.	Buffer	pH*	Ionic Strength	F**	$u \times 10^5$ ***
1	Acetate +0.002M Ca(Ac) ₂	5.5	.10	8.8045	+1.33 ± .14
2	Veronal +0.002M Ca(Ac) ₂	7.9	.10	8.2476	+0.632 ± .017
3	Veronal +0.002M Ca(Ac) ₂	8.5	.10	7.9276	+0.30 ± .15
4	Veronal +0.002M Ca(Ac) ₂	9.0	.10	8.5768	0 ± .26
5	Glycine	9.4	.10	8.2454	-0.96 ± .08
6	Glycine	10.0	.10	7.9687	-1.97 ± .32

* pH measured at room temperature with Beckman expanded scale pH meter.

** Potential Gradient = i/Ak (volts/cm)
 where i = current (m amps)
 A = cross-sectional area of cell
 k = specific conductance of solution

*** Electrophoretic mobility in units of $\text{cm}^2/\text{volt-sec}$

lized with cations.

3. The $E_{1\text{cm}}^{1\%}$ (280 mu) of the *B. subtilis* neutral protease

The $E_{1\text{cm}}^{1\%}$ (280 mu) was determined by relating the absorbency to a known weight of protein. The value obtained was $13.6 \pm .2$. The value calculated from the amino acid composition was 13.4.

4. Optical Properties

a. Measurement of Refractive Index Increment (dn/dc)

The measurement of the increment (dn) was determined as a function of concentration. The average values obtained at 25°C. were $0.182 \pm .003$ at 436 mu and $0.172 \pm .002$ at 546 mu. These values are lower than those reported for other enzymes. (Wilcox, et al., 1957 and Stacey 1956).

5. Stability Studies

Most of these studies were conducted with the help of Dr. D. Tsuru of our laboratory or by him alone. They are included here for reasons of clarity and continuity.

a. Stability as a Function of pH

As seen in Figure 13 the *B. subtilis* neutral protease is stable over the pH range 6.0 to 10.0. After 24 hours, however, the stability in the alkaline range decreases. This is probably due to loss of residual calcium so actually the effect may be caused by both denaturation and autolysis or by only a single factor.

The instability of the enzyme over the acid pH

range can be explained by the loss of zinc (Section IIIA1) which is necessary for activity. It is well known that metalloenzymes are not stable in acid solution. Metalloenzymes generally begin to lose their metal below pH 6.0, thus the B. subtilis neutral protease is no different in this respect. (Vallee,et al., 1960 and 1958, and Yamada,et al., 1962)

b. Stability as a Function of Calcium Ion Concentration

Figure 14 shows the relative activity of the B. subtilis neutral protease as a function of calcium ion concentration. The increase in ninhydrin color at low concentrations of calcium also indicates that the instability of the enzyme is due to autolysis and not to denaturation or other possible effects. From the conditions used for the experiment, it is also demonstrated that the activity of the enzyme in dilute solutions is stable for about two days. This study also points out the necessity of using at least $2 \times 10^{-3}M$ calcium ions throughout the entire purification.

Calcium, however, is not the only cation that tends to stabilize the enzyme (McConn et al. 1964). Other alkaline earth cations such as strontium, magnesium, and barium also show this effect. The latter cations do not have as great an effect as calcium.

c. Stability as a Function of Temperature

Figure 15 illustrates the stability of the B. subtilis neutral protease at various temperatures in

FIGURE 12

The electrophoretic mobilities of the neutral protease as a function of pH. Approximate 1% solutions of the enzyme were prepared in Miller and Golder buffers of 0.1 ionic strength. All buffers except pH 9.5 and pH 10.0 contained 0.002M calcium acetate.

FIGURE 13

The effect of pH on the stability of the neutral protease. The enzyme solution contained 17.2 ug of enzyme per ml (specific activity 12,000) and was prepared with and without calcium acetate. After incubation at the indicated pH at 25°C. for one hour and 24 hours, 0.5 ml aliquots were removed and protease activity determined at standard conditions. 0-0-0 represents one hour incubation (+0.002M calcium acetate). x-x-x represents one hour incubation and ●-●-● represents a 24-hour incubation, both lacking calcium acetate.

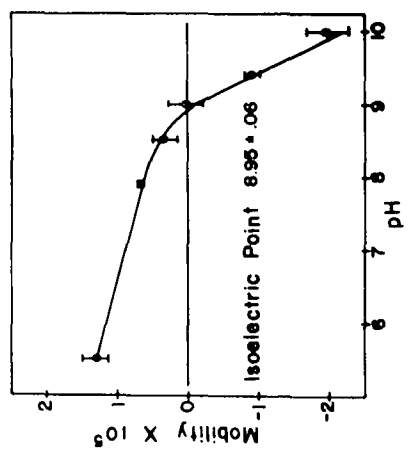
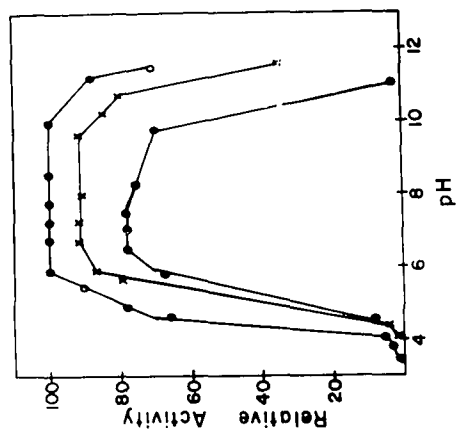


FIGURE 14

The effect of calcium ions on the stability of the neutral protease. The incubation mixture consisted of 240 ug enzyme (specific activity 12,000), 0.001M tris-maleate buffer, pH 6.4, and varying concentrations of calcium acetate in a total volume of 3 mls. The mixture was incubated at 32°C. for 40 hours. ✕-✕-✕ represents the remaining protease activity and ●-●-● represents the ninhydrin color value (uM leucine equivalents per ml).

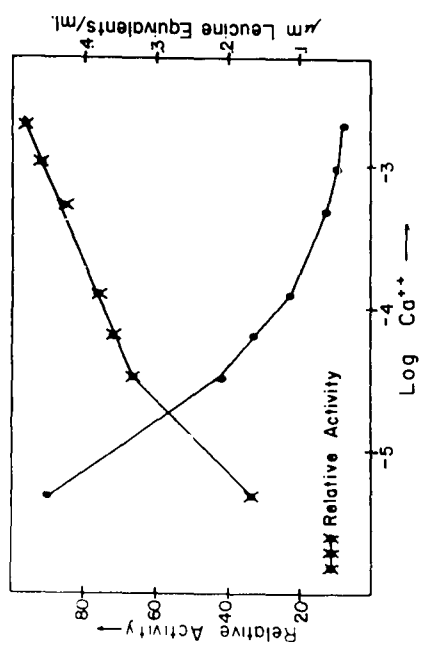
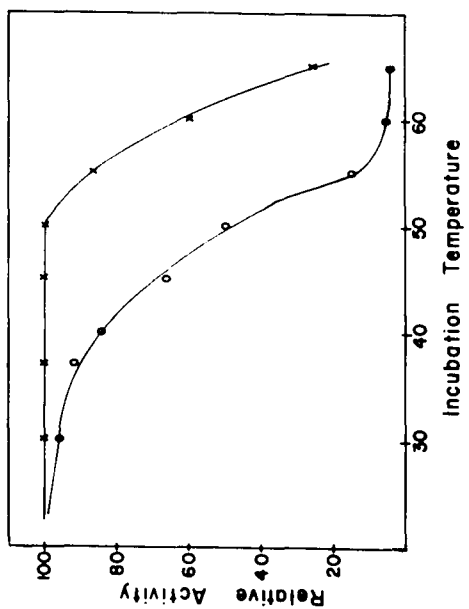


FIGURE 15

The effect of temperature on the stability of the neutral protease. The enzyme solution contained 176 ug of protein per ml (specific activity 12,000) in 0.02M tris-maleate buffer, pH 7.4 was incubated for 15 minutes at the indicated temperatures. At the end of this time, a one ml aliquot was diluted ten fold with cold tris-maleate buffer and the activity remaining was determined. x-x-x represents 0.004M calcium acetate present. 0-0-0 represents no calcium acetate present.



the presence and absence of calcium. The large difference between the two curves at high temperatures is due to the processes that can occur, autolysis and denaturation. As previously discussed, autolysis occurs in the absence of Calcium, so this, as well as denaturation, is the cause for the difference in the curves. Since the absence of calcium may also promote denaturation at high temperatures, the two processes cannot be differentiated.

C. Chemical Properties

1. Elementary Composition

a. Nitrogen and Sulfur Analysis

The nitrogen and sulfur analyses were performed in duplicate by the Bernhardt Laboratories, Max Planck Institute, Mulheim, Germany. The results obtained were 15.7% nitrogen and 0.36% sulfur. As can be seen below in the amino acid composition, methionine accounts for all the sulfur.

b. Metal Analysis

1) Spectrographic Analysis

Qualitative spectrographic analysis on the pure enzyme showed the presence of the following metals: calcium, copper, iron, nickel, chromium, sodium, and zinc.

2) Microchemical Metal Analysis

Table VII gives the results of the microchemical analysis on the enzyme for the metals

TABLE VII

Microchemical Determination of Metal Content of the Purified Enzyme

Metal Tested	mg enzyme used	Metal content ug/mg enzyme	Method of analysis	Reference
1. Zn ⁺⁺	5	2.1	Dithizone	*
2. Zn ⁺⁺	5	2.3 ± 0.1	Zincon	**
3. Fe ⁺⁺	2.2	0.03	Bathophenanthroline	***
4. Cu ⁺⁺	10	0.0	Cuprizone	****
5. Mn ⁺⁺	50	0.0	Oxidation to permanganate	*
6. Ni ⁺⁺	38.6	0.12	Dimethylglyoxime	*
7. Cr ⁺⁺	38.6	0.0	S-Diphenylcarbazide	*

ug Zn added to 1.7 mg enzyme	Recovery of Standard (Zincon Method) Zn Recovered ug	%
1. 2.60	2.5 ± .05	97
2. 3.25	3.1 ± .05	95
3. 1.30	1.25 ± .05	95
4. 1.30	1.3 ± .05	100

References:

- * Sandell, E. B. (1959) Colorimetric Metal Analysis. 3rd ed. Interscience Publishers Inc., New York.
- ** Rush, R. M. and J. H. Yoe (1954) Anal. Chem. 26, 1345.
- *** Diehl, H. and G. G. Smith (1960) The Iron Reagents. G. Fredrick Smith Chemical Co., Columbus, Ohio.
- **** Peterson, R. E. and M. E. Bollier (1955). Anal. Chem. 27, 1195.

present in the spectrographic analysis with the exception of calcium and sodium. These two metals were expected to be present since they were used in the purification of the enzyme. This does not rule out, however, that each is not the prosthetic group. Sodium is unlikely to be the prosthetic group because the enzyme is routinely dialyzed against calcium salts with no loss in activity. Thus zinc or calcium or both are the possible prosthetic groups. As described in Section IIIA1, the zinc content was determined during purification and rechromatography (Figures 3 and 4). It was directly proportional to specific activity and it followed the activity and protein content during rechromatography. Zinc was also the only cation present in the spectrographic analysis, which would reactivate the apoenzyme (Tables VIII and XII). These data indicate that zinc is necessary for activity but does not affect the indication that nondialyzable calcium is present in the enzyme (Section IIIA1). Since there is no cysteine, calcium may function as a means of tertiary structure stability (Stein, et al., 1964).

In the zinc analysis by the Zincon method the precision was three to four per cent and the absolute accuracy, as calculated from the extinction coefficient (Rush and Yoe, 1954), was within five

TABLE VIII
Preparation of Metal-Free Enzyme and
the Reactivation by Various Divalent Metals

Experiment No.	I*		II*	
	Specific Activity, units/mg protein	Zn Content, ug/mg protein	Specific Activity, units/mg protein	Zn Content, ug/mg protein
1. None	10400	1.70	12400	1.92
2. After EDTA and ion exchange resin treatment				
a. Control	1500	0.30	334	0.06
b. a + CaCl ₂	1720	-	1230	-
c. a + MnCl ₂	4760	-	1435	-
d. a + CoCl ₂	6140	-	4240	-
e. a + ZnSO ₄	8140	-	6900	-
3. Exp't. 2e was dialyzed against ZnSO ₄ and then Zn free buffer	8000	1.25	7100	1.09

* EDTA concentration: $1.33 \times 10^{-4}M$ for Exp't. I. $2.50 \times 10^{-4}M$ for Exp't. II. Metal concentration in the casein assay was $1 \times 10^{-4}M$. The experiment was performed using 100 mg of enzyme, the concentration of enzyme in the casein assay was approximately 10 ug.

per cent.

The recovery of zinc standard from the enzyme was, in all determinations, within five per cent.

2. Amino Acid Composition

This data is taken from Dr. D. Tsuru (unpublished) to illustrate the sulfur analysis and to calculate the molecular weight and partial specific volume.

Table IX summarizes the results of the amino acid analysis. As was stated above, methionine contains the only sulfur in the enzyme. The recovery of 101.8% of the protein nitrogen does not indicate a gross contamination, such as carbohydrates.

The abundance of aspartic and glutamic acid residues (amide nitrogen subtracted) are evident, indicating an acidic protein. The electrophoretic results do not agree (see Section IIIB2 for a discussion of isoelectric and isoionic points).

TABLE IX

Summary of the Amino Acid Composition

Amino Acid or Compound	Hydrolysis Time (hr)				Average gm/100 gm Protein	Amino Acid		Minimum Molecular Wt.	No. Residue per Mol. Wt. 44,700	Integral No. A.A. Residues per Mole
	<u>gm Amino Acid</u> 100 gm Protein					Average Residue/ 100 gm Protein	N%			
	18	24	48	72						
Aspartic Acid	17.95	17.87	18.40	18.20	18.10	15.65	11.94	735	60.8	61
Threonine	9.62	9.50	9.29	9.15	9.73 ^a	8.27	7.15	1,224	36.5	37
Serine	9.57	9.43	9.00	8.50	9.80 ^a	8.04	8.19	1,072	41.6	42
Proline	3.41	3.55	3.86	3.56	3.59	3.07	2.73	3,207	13.9	14
Glutamic Acid	10.54	10.70	10.75	10.90	10.72	9.41	6.41	1,372	32.5	33
Glycine	6.33	6.06	6.17	6.32	6.22	4.72	7.28	1,207	37.0	37
Alanine	7.05	6.93	7.01	7.07	7.02	5.61	6.92	1,269	35.2	35
Valine	5.67	5.85	6.13	6.24	6.24 ^b	5.28	4.71	1,880	23.7	24
Methionine	1.65	1.46	1.45	1.43	1.55 ^b	1.45	.97	9,043	4.9	5
Isoleucine	4.56	4.85	5.09	5.37	5.37 ^b	4.64	3.62	2,443	18.2	18
Leucine	7.54	7.51	7.39	7.58	7.50	6.67	5.06	1,749	25.5	26
Tyrosine	11.77	11.71	11.46	12.30	11.81	10.68	5.71	1,534	29.1	29
Phenylalanine	5.28	4.85	4.93	5.06	5.03	4.48	2.69	3,284	13.6	14
Ammonia	1.68	1.74	1.82	1.92					39.1	39

Alanine	7.05	6.93	7.01	7.07	7.02	5.61	6.92	1,269	35.2	35
Valine	5.67	5.85	6.13	6.24	6.24 ^b	5.28	4.71	1,880	23.7	24
Methionine	1.65	1.46	1.45	1.43	1.55 ^b	1.45	.97	9,043	4.9	5
Isoleucine	4.56	4.85	5.09	5.37	5.37 ^b	4.64	3.62	2,443	18.2	18
Leucine	7.54	7.51	7.39	7.58	7.50	6.67	5.06	1,749	25.5	26
Tyrosine	11.77	11.71	11.46	12.30	11.81	10.68	5.71	1,534	29.1	29
Phenylalanine	5.28	4.85	4.93	5.06	5.03	4.48	2.69	3,284	13.6	14
Ammonia	1.68	1.74	1.82	1.92					39.1	39
Lysine	6.68	6.44	6.48	6.80	6.60	5.79	7.92	2,215	20.1	20
Histidine	2.29	2.28	2.30	2.34	2.30	2.03	3.90	6,746	6.6	7
Tryptophan*					1.95	1.78	1.68	10,473	4.2	4
Arginine	3.97	4.00	3.98	4.10	4.01	3.61	8.10	4,344	10.3	10
TOTALS						101.18	101.9			416

Nitrogen 15.9%

a = extrapolated to zero time

b = maximum value used

Experimental \bar{v} = .746

Calculated \bar{v} = .721

* Tryptophan analysis:

1. Goodwin, T. W. and R. A. Morton *Biochem. J.* 40, 628 (1946).
2. Spies, J. R. and D. C. Chambers *Anal. Chem.* 21, 1249 (1949).

D. Enzymatic Properties

1. Enzymatic Activitya. Casein Digestion

The digestion of casein by various proteases is illustrated in Figure 16: The specific activities of these enzymes were calculated as described in Methods and are tabulated in Table X.

TABLE X

Comparative Specific Activities of Proteases

Enzyme	Assay Conditions	Specific* Activity
<u>B. subtilis</u> neutral protease	0.01M NaCl, pH 7.30	13,600
Crystalline papain	0.005M EDTA in 0.01M NaCl, pH 7.30 + 0.05M Cysteine	2,550
Crystalline Chymotrypsin	0.01M NaCl, pH 7.30	1,813
Crystalline Trypsin	0.01M NaCl, pH 7.30	1,653

* Enzyme concentrations determined by using $E_{1\text{cm}}^{1\%}$ (280)
 B.S.N. protease = 13.6
 Papain = 24
 Chymotrypsin = 20
 Trypsin = 15.2

It should be noted that the neutral protease is 4 to 5 times more active than papain, which makes it one of the most active proteases known. The other very active protease "pronase" (Nomoto, et al., 1960) has recently been shown to consist of more than two enzymes by a separation of peptidase and protease activities

FIGURE 16

Casein digestion by various proteases.

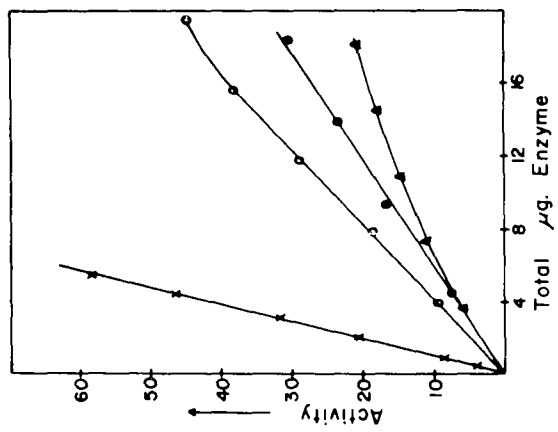
All digestions were carried out under standard conditions.

x-x-x B. subtilis neutral protease

0-0-0 Papain (cysteine activated + 0.005M EDTA)

●-●-● Chymotrypsin

▲-▲-▲ Trypsin



(Hiramatsu, et al., 1963). It is difficult to compare proteases on the basis of only one substrate since a change in the protein substrate may change the order of relative activities.

b. Digestion of other proteins

Figure 17 and Figure 18 illustrate the digestion of various proteins by the B. subtilis neutral protease. Table XI summarizes the specific activities of the neutral protease on these proteins.

TABLE XI
The Digestion of Proteins with the Neutral Protease

Protein	Specific Activity*	Carboxyl * Liberated	Relative Initial Rate of Hydrolysis
Casein	11,390	1,700	100
Hemoglobin	9,180		81
Egg Albumin	2,550		22
Gelatin		720	42

* Assay methods under Methods (Section IIB7).

As shown, the protease is very active on casein and hemoglobin but not very active on egg albumin or gelatin. There seems to be a limited number of bonds in gelatin which can be hydrolyzed. These bonds are hydrolyzed rapidly, then the reaction almost ceases.

FIGURE 17

Protein digestion by the neutral protease.
The digestion of various proteins by the
neutral protease using the formol titra-
tion method and standard conditions.

0-0-0 Casein

x-x-x Gelatin

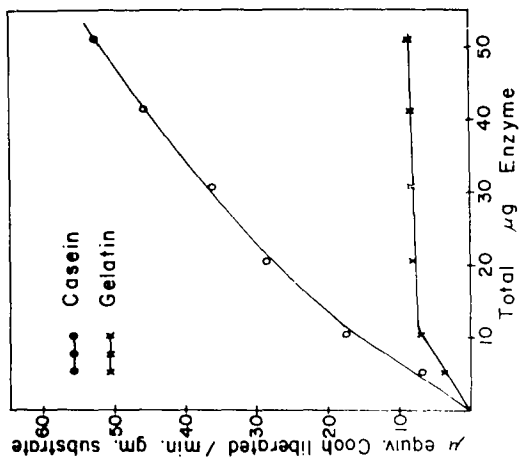
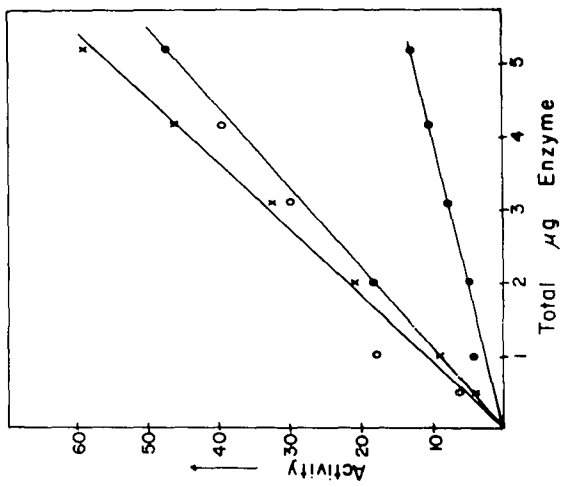
FIGURE 18

Protein digestion by the neutral protease.
The digestion of various proteins by the
neutral protease using the Hagihara assay
and standard conditions.

x-x-x Casein

0-0-0 Hemoglobin

●-●-● Egg Albumin



2. Inhibition

a. Inhibition with chelating agents

Figure 19 illustrates the inhibition of the B. subtilis neutral protease with some chelating agents. As shown in Sections IIIA1 and IIIC1b2), the enzyme contains zinc so chelating agents in general which react most strongly with zinc would be expected to maximally inhibit the enzyme. This type of inhibition also suggests that zinc is necessary for activity, either for substrate binding or for the hydrolysis step. It has been shown (Coleman, et al., 1962) in the case of carboxypeptidase A that zinc is necessary for the hydrolysis step of peptides, but is needed for the binding of esters as well as their hydrolysis. Similar experiments with the neutral protease have not been carried out, due to the instability of the apoenzyme.

Other chelating agents which inhibit the enzyme are: dithizone, NaCN, and sodium diethyldithiocarbamate.

One difficulty in all the experiments with chelating agents was the interference of calcium ions. Calcium at concentrations used in the purification (Section IIIA1) generally increased the concentration of chelating agent needed for inhibition by several orders of magnitude. This effect is not surprising since most chelating agents combine with calcium and thus decrease the effective inhibitor concentration.

FIGURE 19

The inhibition of the neutral protease by chelating agents. To nine ml of .001M tris buffer pH 7.3 containing various concentrations of chelating agents, one ml of enzyme solution was added, mixed, and a one ml aliquot containing 5 ug of enzyme (free of extraneous calcium) was used in the casein assay. (The enzyme was prepared by SE sephadex chromatography.)

- EDTA
- 0-0-0 Aspartic acid
- Δ-Δ-Δ o-phenanthroline
- x-x-x Cysteine
- ▲-▲-▲ Histidine
- 8 Hydroxy quinoline

FIGURE 20

The inhibition of the neutral protease by metal ions. To nine ml of .001M tris + .002M calcium acetate pH 7.3 containing various concentrations of metal ions, one ml of enzyme solution was added, mixed, and a one ml aliquot containing 8.8 ug of enzyme (specific activity 12,000) was used in the casein assay.

- Mercury
- 0-0-0 Lead
- Cadmium
- Δ-Δ-Δ Nickel
- x-x-x Copper

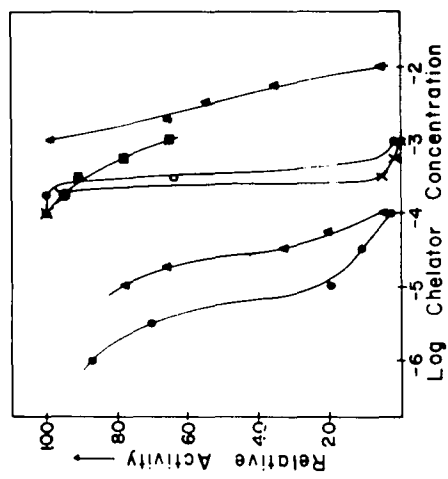
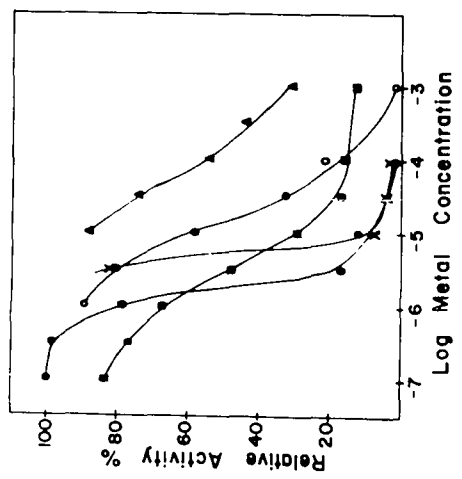


TABLE XII

Effect of Various Metal Ions on the B. Subtilis Protease*

Metal added	Activity	% Inhibition
1. No addition	100	0
2. ZnSO ₄	112	0
3. CaCl ₂	100	0
4. CuSO ₄	19	81
5. MnCl ₂	100	0
6. NiSO ₄	50	50
7. CoCl ₂	100	0
8. HgCl ₂	1	99
9. PbNO ₃	14	86
10. CdCl ₂	14	86
11. FeCl ₃	7	93
12. FeCl ₂	60	40

* For each experiment 34.3 ug of enzyme (specific activity 12,000) was added to the various metal solutions in a total volume of 10 ml. One ml aliquots containing 3.43 ug of enzyme ($7.7 \times 10^{-8}M$) and $10^{-4}M$ metallic salt solution was used for the casein assay.

This problem, however, necessitated that very low levels of calcium be used during the experiments. These conditions increased the uncertainty of the results, but did not negate the conclusions.

More detailed experiments are described in Section IIID6g2) on the kinetics of EDTA inhibition.

b. Inhibition by Heavy Metals

Table XII shows the effect of various metals on the neutral protease, while Figure 20 gives in more detail the inhibitory effect of some of these metals. These metals in general are inhibitors of sulfhydryl enzymes, but in this case, the enzyme contains no sulfhydryl groups. There are two possible explanations of the inhibition by metals: 1) The zinc is replaced by the inhibitory metal; (Carboxypeptidase A, Vallee, 1961), and 2) The inhibitory metal is bound by another site in the active center.

Table XIII shows the results of dialysis of the inhibited enzyme against calcium acetate.

TABLE XIII

Dialysis of Metal Inhibited Enzyme

Metal	Metal Concentration	Inhibited Activity % of Control	Activity after Dialysis against 0.002M Ca(Ac) ₂ * % of Control
Control	-----	100	100
Hg ⁺⁺	3 x 10 ⁻² M	7.2	7.2
Pb ⁺⁺	5 x 10 ⁻² M	65	115
Ni ⁺⁺	5 x 10 ⁻² M	73	100
Co ⁺⁺	5 x 10 ⁻² M	81	115
Cd ⁺⁺	5 x 10 ⁻² M	63	96

* 3 mls of enzyme solution, containing the metal ions and 3.2 mg enzyme per ml (5.6×10^{-5} M) were dialyzed 24 hours against 4 changes of 0.002M Ca(Ac)₂ (pH 6.5). The metal salts were adjusted to pH 6.5 with NaOH or HCl.

In the case where the inhibitory metal replaces zinc two possibilities can exist: (1) the inhibitory metal replaces zinc giving an active enzyme, and (2) the inhibitory metal is in equilibrium with zinc. In either case the inhibition would be from an excess concentration of metal ions. If the metal is bound to another site in the "active center", the results of cadmium, lead, and nickel in Table XIII can be explained in terms of equilibrium. The actual effect of metals on the neutral protease can be determined only with additional experimentation.

More detailed experiments are described in Section IIID6 on the kinetics of metal inhibition.

3. Apparent pH Optimum

The hydrolysis of a 1% casein solution as a function of pH by the B. subtilis neutral protease is illustrated in Figure 21. The protease exhibits a single optimum with maximal activity from pH 6.5 to pH 7.5.

The hydrolysis of a synthetic substrate (hippuryl-L-leucinamide) as a function of pH is described in Section IIID6a.

4. Apparent Temperature Optimum

The hydrolysis of casein as a function of temperature by the neutral protease is illustrated in Figure 22. As the figure shows, calcium ions reduce the rate of autolysis and affect the heat stability of the enzyme as shown by the increased temperature optimum. The hydrolysis of hippuryl-L-leucinamide as a function of temperature is described in Section IIID6d.

5. Specificity

a. Action on some Peptide, Amide, and Ester Bonds

Since there was no data on the specificity of the B. subtilis neutral protease, qualitative paper chromatography was employed as a means of detecting hydrolysis. The results are shown in Table XIV.

Two facts are evident from the Table: 1) the substrate cannot be charged; (as is seen from the variations of glycyl phenylalanine, only when both the carboxyl and amino groups are blocked is the peptide bond hydrolyzed), and 2) one of the amino acids at the

FIGURE 21

The digestion of casein by the neutral protease as a function of pH. A 0.5% casein solution was prepared in 0.1M cacodylate or 0.1M tris buffer at the indicated pHs. The assays were conducted under standard conditions using 4.7 ug of enzyme (specific activity 11,000). Cacodylate buffer was used in the pH range 5.5 to 7.0 and tris buffer was used in the pH range 7.0 to 9.0.

FIGURE 22

The digestion of casein by the neutral protease as a function of temperature. Solutions of 0.5% casein were equilibrated at the temperatures indicated, as were the enzyme solutions. One ml of enzyme solution (8.6 ug enzyme, specific activity 12,000) was then added to five ml casein substrate. After ten minutes the reaction was terminated by the addition of protein precipitant. x-x-x represents the results with 0.002M calcium acetate and 0-0-0 represents the results without calcium acetate.

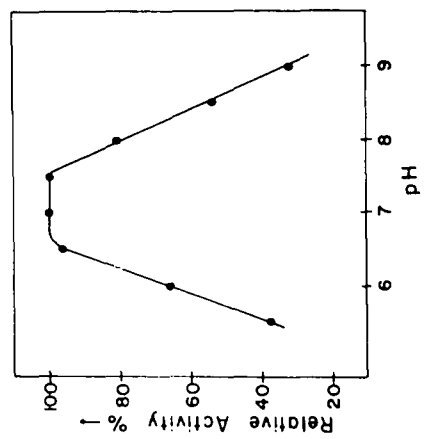
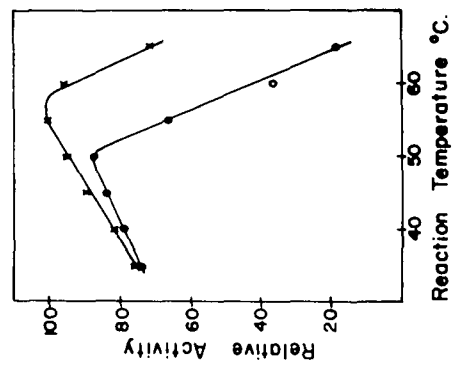


TABLE XIV

Substrate Specificity of the B. subtilis Neutral Proteinase

Peptide	Assay Method*	Hydrolysis	Peptide	Assay Method*	Hydrolysis
1. Gly-L-Pro	P.C.	Negative	23. Benzoyl-Arginine Amide	Nin.	Negative
2. L-Pro-Gly	P.C.	Negative	24. N-Acetyl-Tyrosine ethyl ester	S., 10 min.	Negative
3. L-Lys-methyl ester	P.C.	Negative			
4. L-His-ethyl ester	P.C.	Negative	25. Dl-Ala-Dl-Met	P.C.	Negative
5. L-Leu-L-Tyr	P.C.	Negative	26. Dl-Ala-Dl-Aspamide	P.C.	Negative
6. L-Leu-Amide	P.C.	Negative	27. Dl-Ala-Dl-Al	P.C.	Negative
7. L-His-L-His	P.C.	Negative	28. Dl-Ala-Dl-Ala	P.C.	Negative
8. Gly-Gly	P.C.	Negative	29. Dl-Ala-Gly-Gly	P.C.	Negative
9. Gly-L-Tyr	P.C.	Negative	30. N-Acetyl-Diiodo-DL-Tyr-Gly	P.C.	Negative
10. Gly-Gly-Gly	P.C.	Negative	31. Gly-DL-Phe	P.C.	Negative
11. Glutathione	P.C.	Negative	32. Gly-L-Phe-Amide-Acetate	P.C.	Negative
12. Cbz-L-Glu-L-Phe	P.C.	Negative	33. Cbz-Gly-L-Phe	P.C.	Negative
13. Cbz-Gly-Gly-Amide	P.C.	Negative	34. Cbz-Gly-L-Phe-Amide	P.C.,Nin.	Positive
14. L-Tyr-L-Leu	P.C.	Negative	35. Cbz-L-Tyr-Gly-Amide	P.C.,Nin.	Positive
15. L-Phe-L-Phe	P.C.	Negative	36. Cbz-L-Leu-L-Leuamide	P.C.	Positive
16. L-Val-L-Val	P.C.	Negative	37. Benzoyl-L-Tyrosyl-Gly-Amide	P.C.	Positive
17. L-Ala-L-Leu	P.C.	Negative	38. Hippuryl-L-Leucinamide	P.C.,Nin.	Positive
18. L-Leu-L-Leu	P.C.	Negative	39. Amyl Acetate	F.	Negative
19. L-Leu-Gly-Gly	P.C.	Negative	40. Butyl-Acetate	F.	Negative

3.	L-Lys-methyl ester	P.C.	Negative			
4.	L-His-ethyl ester	P.C.	Negative	25.	Di-Ala-Di-Met	P.C. Negative
5.	L-Leu-L-Tyr	P.C.	Negative	26.	Di-Ala-Di-Aspamide	P.C. Negative
6.	L-Leu-Amide	P.C.	Negative	27.	Di-Ala-Di-Al	P.C. Negative
7.	L-His-L-His	P.C.	Negative	28.	Di-Ala-Di-Ala	P.C. Negative
8.	Gly-Gly	P.C.	Negative	29.	Di-Ala-Gly-Gly	P.C. Negative
9.	Gly-L-Tyr	P.C.	Negative	30.	N-Acetyl-Diiodo-DL-Tyr-Gly	P.C. Negative
10.	Gly-Gly-Gly	P.C.	Negative	31.	Gly-DL-Phe	P.C. Negative
11.	Glutathione	P.C.	Negative	32.	Gly-L-Phe-Amide-Acetate	P.C. Negative
12.	Cbz-L-Glu-L-Phe	P.C.	Negative	33.	Cbz-Gly-L-Phe	P.C. Negative
13.	Cbz-Gly-Gly-Amide	P.C.	Negative	34.	Cbz-Gly-L-Phe-Amide	P.C., Nin. Positive
14.	L-Tyr-L-Leu	P.C.	Negative	35.	Cbz-L-Tyr-Gly-Amide	P.C., Nin. Positive
15.	L-Phe-L-Phe	P.C.	Negative	36.	Cbz-L-Leu-L-Leuamide	P.C. Positive
16.	L-Val-L-Val	P.C.	Negative	37.	Benzoyl-L-Tyrosyl-Gly-Amide	P.C. Positive
17.	L-Ala-L-Leu	P.C.	Negative	38.	Hippuryl-L-Leucinamide	P.C., Nin. Positive
18.	L-Leu-L-Leu	P.C.	Negative	39.	Amyl Acetate	F. Negative
19.	L-Leu-Gly-Gly	P.C.	Negative	40.	Butyl-Acetate	F. Negative
20.	Cbz-L-Glu-L-Tyr	P.C.	Negative	41.	Ethyl Acetate	F. Negative
21.	N-Acetyl-L-Phe-L-Tyr	P.C.	Negative	42.	Ethyl Valerate	F. Negative
22.	Gly-Gly-Gly-Gly	P.C.	Negative	43.	Methyl Buterate	F. Negative

* The following abbreviations were adopted: P.C., Nin., S., and F., which represent paper chromatography, ninhydrin, spectrophotometric determination, and the formol titration method, respectively.

bond to be hydrolyzed must have a side chain. The first condition is met in the case of cbzglyglycinamide but not the second so this peptide is not hydrolyzed. Thus van der Waal's forces may be the means of substrate binding. This is not the only possibility, however, because chelation may bind the substrate. In Section IIID6 the kinetic analysis gives similar results, but the actual means of substrate binding requires a stable apoenzyme (Coleman, et al., 1962).

As seen from Table XIV, the enzyme does not exhibit esterase activity toward the fatty acid esters or amino acid esters tested.

The actual specificity of the B. subtilis neutral protease cannot be defined from the above data and table. It is unknown how many amino acids have the type of side chain that will function in hydrolysis. At present the amino acids at which hydrolysis is known to occur are leucine, tyrosine, and phenylalanine.

D Amino Acid substrates have not yet been investigated.

6. Kinetics of Hydrolysis of Hippuryl-L-leucinamide

a. Effect of pH

Figure 23 shows a detailed study of the effect of pH on the rate of hydrolysis of 0.03M hippuryl-L-leucinamide by the B. subtilis neutral protease in the presence of 0.004M $\text{Ca}(\text{Ac})_2$. The velocity is maximum at slightly less than pH 7. The buffers in all experiments (tris and cacodylate) were chosen because they showed

little or no ion effects with the enzyme. The enzyme was stable at all pH's used for periods of time longer than the longest kinetic determination.

b. Effect of temperature

Figure 24 shows the effect of temperature on the rate of hydrolysis of hippuryl-L-leucinamide. As the figure illustrates, the temperature optimum is 45°C. or slightly less than that observed for casein (Figure 22). The enzyme was shown to be stable up to 50°C. for periods longer than the longest kinetic determination.

c. Effect of Ionic Strength

Figure 25A shows the effects of ionic strength on the rate of hydrolysis of hippuryl-L-leucinamide. All subsequent studies were performed at 0.1 ionic strength.

d. Effect of Ca⁺⁺ Concentration

Figure 25B shows the effect of calcium concentration on the hydrolysis of hippuryl-L-leucinamide. As the figure shows, high concentrations of calcium tend to inhibit the reaction.

e. Effect of Enzyme Concentration

The influence of enzyme on the rate of hydrolysis of hippuryl-L-leucinamide under pseudo-first order kinetic conditions is shown in Figure 26. The rate is proportional to enzyme concentrations up to 10 ug.

f. Effect of Substrate Concentration

Figure 27 shows a typical plot of the leucinamide produced as a function of time of incubation at various

FIGURE 23

The velocity of hydrolysis of hippuryl-L-leucinamide as a function of pH. The assay method is described under Methods. The enzyme concentration was 5.3 ug per ml (specific activity 10,000). The substrate concentration was 0.03M. The velocities were calculated from first order rate constants. x-x-x and 0-0-0 represent cacodylate and tris buffer, respectively.

FIGURE 24

The velocity of hydrolysis of hippuryl-L-leucinamide as a function of temperature. The assays were carried out under standard conditions with the reaction mixture incubated at the indicated temperatures. The reaction mixture consisted of 5.3 ug of enzyme per ml, 0.017M substrate and 0.004M calcium acetate in 0.1M cacodylate buffer, pH 7.00. 0-0-0 represents the temperature optimum of the assay. x-x-x represents the temperature stability of the enzyme after incubation for 30 minutes at the indicated temperatures. The assays were carried out under standard conditions (30°C.). The activity remaining was determined by comparison of the first order rate constants at no time of incubation and after 30 minutes incubation.

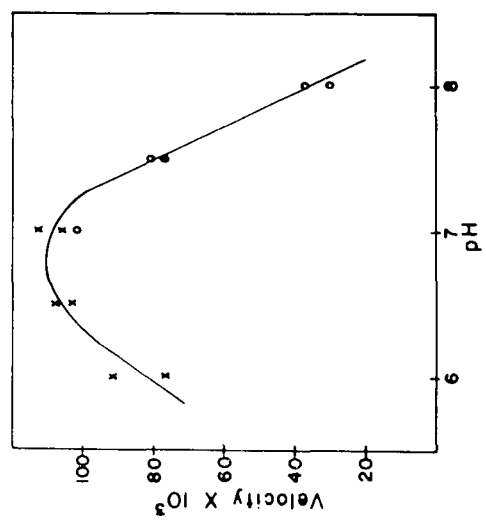
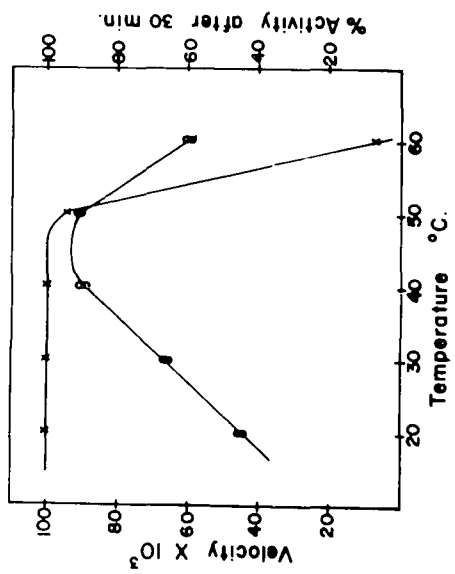


FIGURE 25

- A. The effect of ionic strength on the velocity of hydrolysis of hippuryl-L-leucinamide. The assay was carried out in 0.01M cacodylate buffer pH 7.00 and adjusting the ionic strength with NaCl. The enzyme concentration was 5.3 ug per ml (specific activity 10,000) and the substrate concentration was 0.025M. The calcium acetate concentration was 0.004M and the velocities were calculated from the first order rate constants.
- B. The effect of calcium concentration on the velocity of hydrolysis of hippuryl-L-leucinamide. The assay was carried out using 0.1M cacodylate buffer pH 7.00 and adjusting the calcium concentration with calcium acetate. The enzyme concentration was 5.3 ug per ml (specific activity 10,000) and the substrate concentration was 0.017M. Velocities were calculated from first order rate constants.

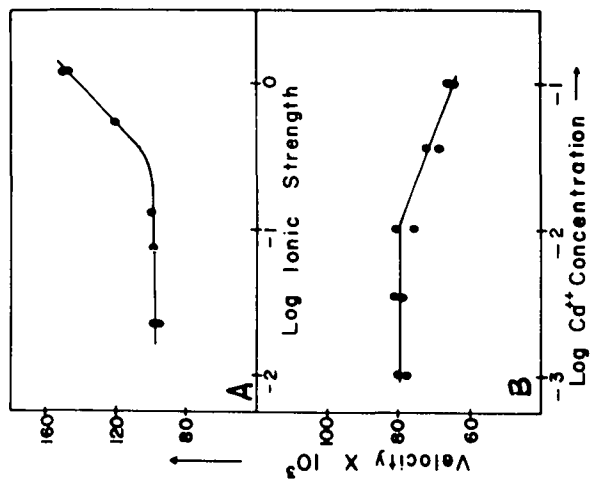


FIGURE 26

The velocity of hydrolysis of hippuryl-L-leucinamide as a function of enzyme concentration. The assay was carried out under standard conditions. The reaction mixture consisted of 0.017M substrate, 0.004M calcium acetate, and varying amounts of enzyme in 0.10M cacodylate buffer, pH 7.00. The velocity is calculated from the first order rate constants.

FIGURE 27

The velocity of hydrolysis of hippuryl-L-leucinamide as a function of substrate concentration. The assays were carried out under standard conditions in cacodylate buffer, pH 7.00. The reaction mixture consisted of 5.3 ug of enzyme (specific activity 10,000) per ml and 0.004M calcium acetate with the following substrate concentrations: 0.03M, 0.024M, 0.020M, 0.012M, and 0.009M hippuryl-L-leucinamide represented by 0-0-0, x-x-x, 0-0-0, ■-■-■, and Δ - Δ - Δ , respectively.

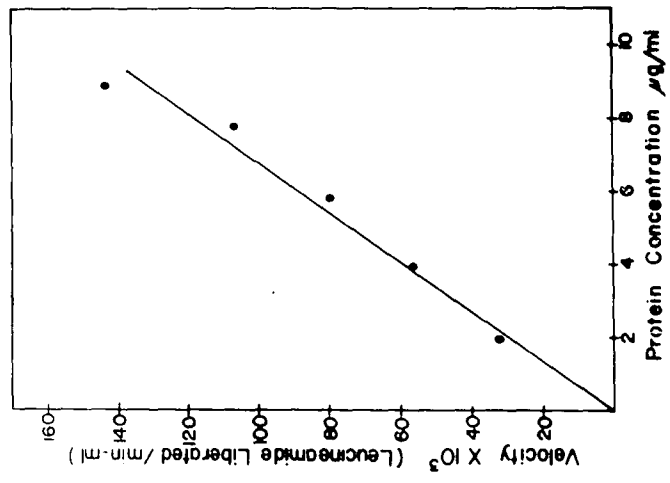
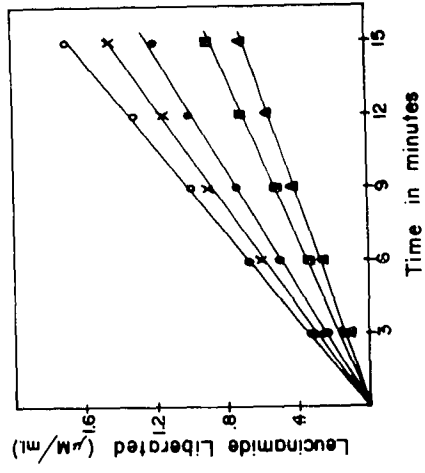
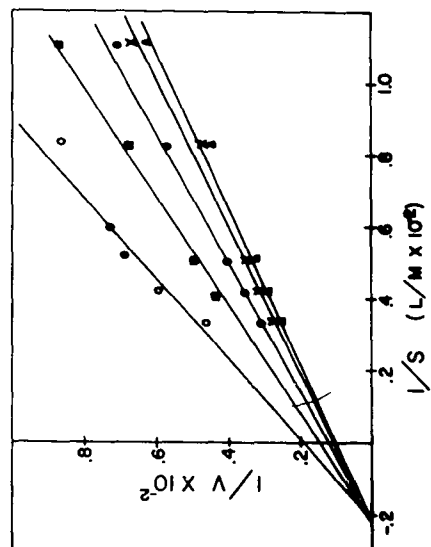


FIGURE 28

Double reciprocal plot (Lineweaver Burke) of the velocity-concentration data. The assays were carried out in 0.1M cacodylate buffer, pH 7.00. The reaction mixture consisted of 5.3 ug of enzyme per ml (specific activity 10,000) and 0.004M calcium acetate and various substrate concentrations. O-O-O, ■-■-■, ●-●-●, x-x-x, and Δ-Δ-Δ represent temperatures of reaction of 20°, 25°, 30°, 35° and 40°C., respectively.



substrate concentrations. It can be seen that under experimental conditions employed, the initial reaction velocity can be either pseudo-zero or pseudo-first order because both plots are linear.

1) Measurement of the Michaelis-Menten Constants and of the Maximal Velocity

The data which were obtained were in accord with normal Michaelis-Menten kinetics. When $1/v$ was plotted against $1/S$, a linear relationship existed (Figure 28).

Similar measurements were made at various pH's ranging from 6.0 to 8.0. The values of K_m and V_{max} obtained by graphical treatment of the data were summarized in Table XV.

TABLE XV
Variation of K_m and V_{max} with Temperature

$K_m \times 10^2$	V_{max} at pH 7.00	°C.
4.53 ± .08M	.161 μ M leucinamide liberated/min-ml	20
4.58 ± .27M	.215 μ M " " "	25
4.71 ± .23M	.268 μ M " " "	30
4.73 ± .28M	.291 μ M " " "	35
4.64 ± .20M	.323 μ M " " "	40

Similarly, the data obtained at the different pH's were in accord with the Michaelis-Menten kinetics.

2) Variation of $\log V_{\max}$ and pK_m with pH

Figure 29 illustrates the relationship existing between $\log V_{\max}$ and pK_m as a function of pH. This data was treated as described by Dixon (1953) and provides a means whereby information about the pK values for the groupings at the "active center" of the enzyme could be deduced. The plot of pK_m versus pH gives information about the ionization of the enzyme and enzyme-substrate complex while the $\log V_{\max}$ versus pH plot gives information about the ionization of the enzyme substrate complex. This information is based on the assumption that the substrate does not ionize. For more information see Laidler, 1958 and Dixon and Webb, 1958.

The data presented here do not unequivocally determine the pK's of the essential groupings present in the "active center" of the neutral protease, but they do suggest the possibility of at least two residues: (1) a grouping with a slightly acidic pK, and (2) a grouping with a slightly basic pK value. These values are consistent with those of histidine and an alpha amino group, respectively.

The assignment of pK's to a particular grouping must be considered provisional, but the next section does indicate that histidine could be involved.

3) Variation of V_{max} , K_m and the pK 's with Temperature

Figure 30 shows the variation of pK_a and $-\log \bar{K}$ ($\log K_m$) versus $1/T$. The thermodynamics are discussed in the next section and are given in Table XVI. The pK_b could not be determined directly because pK_a was the only value which fell within the stability range of the enzyme.

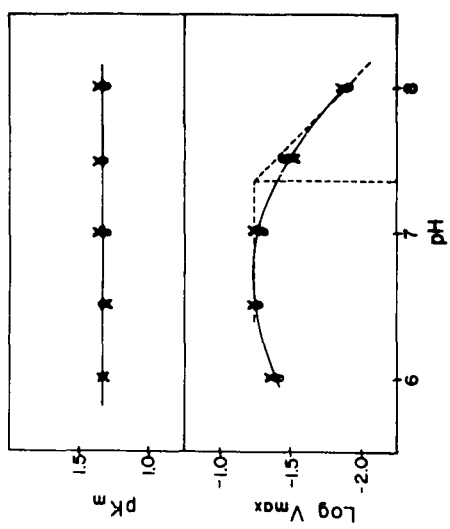
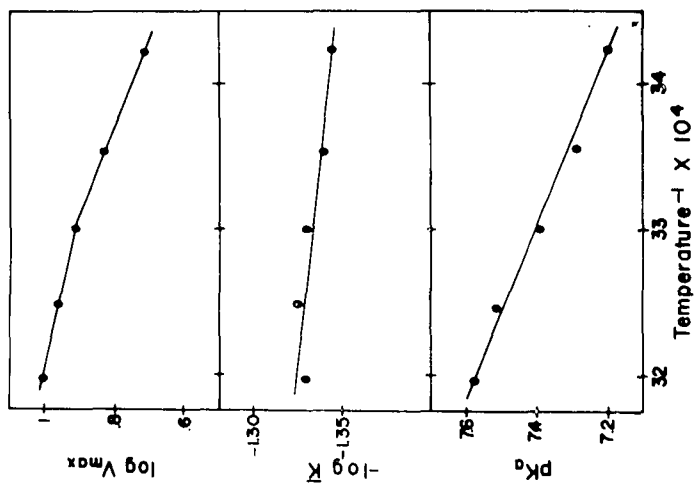
The pK_b could be calculated from the relationship $[H]_{opt} = (K_a K_b)^{\frac{1}{2}} = \text{constant}$, since as Figure 31 shows, the pH optimum does not change with temperature. Thus, since the ΔH_i for K_a is known, (from Figure 30) the ΔH_i for K_b must be exactly equal to the heat of ionization of K_a but opposite in sign. This value (-7,500 cal/mole for ΔH_i of K_a) is then +7,500 cal/mole for K_b which corresponds to the value for histidine (Dixon and Webb, 1958). If it were assumed that the evidence for histidine, though indirect, were correct, then the heat of ionization of K_a is not consistent with an alpha amino group. It might be postulated that some zinc chelate is responsible for this K_a and its heat of ionization. Dixon and Webb state that the effect of the protein molecule on heats of ionization are unknown, thus the two possibilities of what K_a may represent are unchanged. Chemical experiments must be used to confirm and to distinguish between the possible residues at the "active center" of the

FIGURE 29

The $\log_{10} V_{\max}$ and pK_m as functions of pH. The reactions were carried out at 30°C. in 0.1M cacodylate buffer in the pH range 7.0 to 8.0. pK_a is located at the intersection of the dashed line with the pH axis. Different symbols represent different determinations.

FIGURE 30

The $\log_{10} V_{\max}$, $-\log_{10} \bar{K}$, and pK_a as functions of reciprocal temperature. These are secondary plots obtained from velocity, concentration, and temperature data.



B. subtilis neutral protease.

The variation of the $\log V_{\max}$ versus $1/T$ in Figure 30 has a break at 30°C . The thermodynamics were calculated on the portion of the curve 30°C . and above because from the data obtained, there seems to be an interaction between the acidic and basic groups at the "active center" of the enzyme at low temperatures. Thus, the V_{\max} is lowered. Other examples of this type of behavior are in the literature (Massey, 1953-54).

4) Thermodynamic Functions from the Hydrolysis of Hippuryl-L-leucinamide

The following table represents the thermodynamic results obtained from the kinetics of the B. subtilis neutral protease on hippuryl-L-leucinamide.

TABLE XVI
Heat and Entropy Values for
the Formation of the Enzyme-Substrate Complex

pH	T°C.	ΔH	ΔS eu.	ΔF
7.00	30°	- 296.8	+ 5.10	- 1,841

Kinetic Values Relating to
the Breakdown of the Enzyme-Substrate Complex

T°C.	pH	k_2^* (sec ⁻¹)	ΔE	ΔS eu.	ΔF	ΔH
30°	7.00	40	3,460	- 54.9	13,785	2,858

Kinetic Values Relating to
the Formation of the Enzyme-Substrate Complex

T°C.	pH	$k_2^{**} \bar{K}$ (M ⁻¹ sec ⁻¹)	ΔE	ΔS eu.	ΔF	ΔH
30°	7.00	8.49×10^2	6,398	- 50.5	9,506	5,796

* $k_2 = v_{\max}/[E]$ total

** $\bar{K} = 1/K_m$

ΔF , ΔH , and ΔE are expressed in units of cal/mole.

The first values in the table were calculated using $K_m = k_{-1} / k_1$ from the Michaelis-Menten Equation. Evidence that $K_2 \ll k_{-1}$ if from non-competitive pH dependence (Laidler, et al., 1956) and non-competitive inhibition (discussed in the next section) from Webb, 1963. The non-competitive pH dependence is based on the evidence that the

shape of the pH optimum curve does not change with increasing substrate concentration (Figure 32). This would demonstrate non-competitive behavior with respect to hydrogen or hydroxyl ions. The values for the breakdown of the enzyme-substrate complex were calculated from V_{max} . The kinetic values for the formation of the enzyme substrate complex were calculated from first order rate constants. These thermodynamic values are in agreement as can be seen from the entropy.

g. Kinetics of Inhibition

1) Inhibition by metals

Figure 33 shows the inhibition of the neutral protease with Hg^{++} . Inhibition with Cu^{++} is similar. This type of inhibition is unknown. It is similar to the kinetics of substrate reacting with inhibitor but this seems very unlikely since the substrate concentration is three orders of magnitude greater than inhibitor concentration. The inhibition is too large to be explained by the inhibitor reacting with substrate, however the following explanations are possible: (1) The effective inhibitor concentration is reduced by the products. (At high substrate there are more products thus as the substrate concentration is increased the inhibitor concentration is decreased.) (2) The inhibitor denatures the enzyme, but there

is also some protection of the enzyme with substrate. (3) The inhibitor replaces zinc.

Figure 34 shows the inhibition of the B. subtilis neutral protease with cadmium. As the kinetics illustrate, the inhibition is non-competitive with a K_i of 7.2 to $7.9 \times 10^{-6}M$.

2) Inhibition with Chelators

Figure 35 shows the inhibition of the B. subtilis neutral protease with EDTA. As shown the inhibition is competitive, with a K_i ranging from 6×10^{-6} to 6×10^{-5} . This range in K_i is probably due to the fact that preincubation of the chelator with the enzyme was not used. With EDTA, a finite amount of time is required for equilibrium conditions (McConn, et al., 1964). Thus, this aspect can account for the spread in K_i . The type of inhibition should not change with preincubation, but preincubation should define a more consistent K_i .

FIGURE 31

The velocity as a function of pH and temperature. pH optimum curves which were determined in 0.004M calcium acetate and 0.1M cacodylate or 0.1M tris buffer. The enzyme concentration was 5.3 ug per ml (specific activity 10,000) and the substrate concentration was 0.030M. x-x-x, 0-0-0, and ●-●-● represent the temperatures of 35°, 30°, and 25°C., respectively.

FIGURE 32

The velocity as a function of pH and substrate concentration. pH optimum curves which were determined in 0.004M calcium acetate and 0.1M cacodylate or 0.1M tris buffer. The enzyme concentration was 5.3 ug per ml (specific activity 10,000) and the temperature was 30°C. 0-0-0, x-x-x, and ●-●-● represent the substrate concentrations of 0.03M, 0.02M, and 0.009M, respectively.

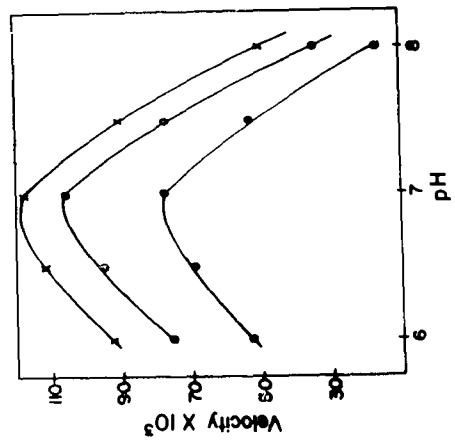
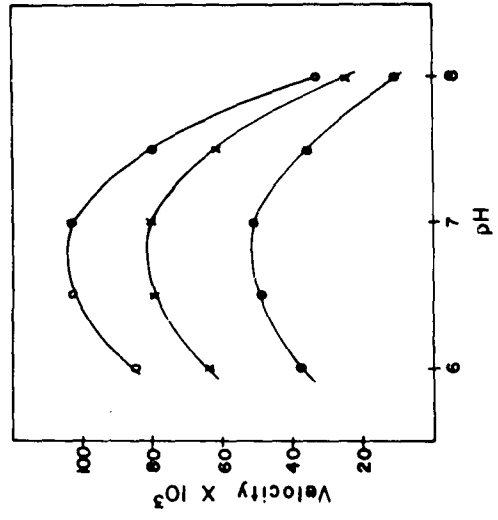


FIGURE 33

Reciprocal and double reciprocal plots of the inhibition by mercury. The assays were carried out at standard conditions. The reaction mixture contained 5.3 ug of enzyme per ml (specific activity 10,000), 0.004M calcium acetate, 0.1M cacodylate buffer pH 7.00, and from $1 \times 10^{-6}M$ to $7 \times 10^{-6}M$ mercuric chloride. The mercury concentration used for the double reciprocal plot was $5 \times 10^{-6}M$. The substrate concentrations used for the reciprocal plot were 0.023M and 0.013M. In the reciprocal plot x-x-x represents a 30 minute preincubation of mercury with the enzyme while 0-0-0 represents no preincubation period with mercury.

FIGURE 34

Reciprocal and double reciprocal plots of the inhibition by cadmium. The assays were carried out at standard conditions. The reaction mixture contained 5.3 ug of enzyme per ml (specific activity 10,000), 0.004M calcium acetate, 0.1M cacodylate buffer, pH 7.00, and from $1 \times 10^{-6}M$ to $7 \times 10^{-6}M$ cadmium chloride. The cadmium concentration used for the double reciprocal plot was $5 \times 10^{-6}M$. The substrate concentrations used for the reciprocal plot were 0.023M and 0.013M. No preincubation of the enzyme with the inhibitor was employed.

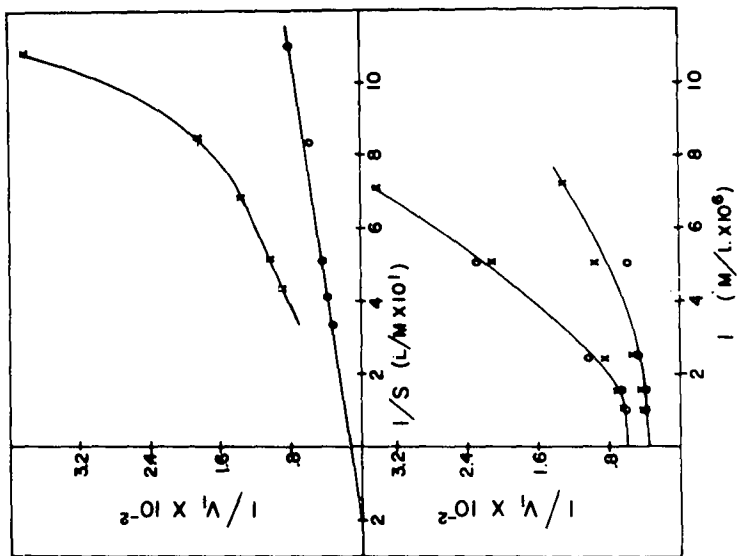
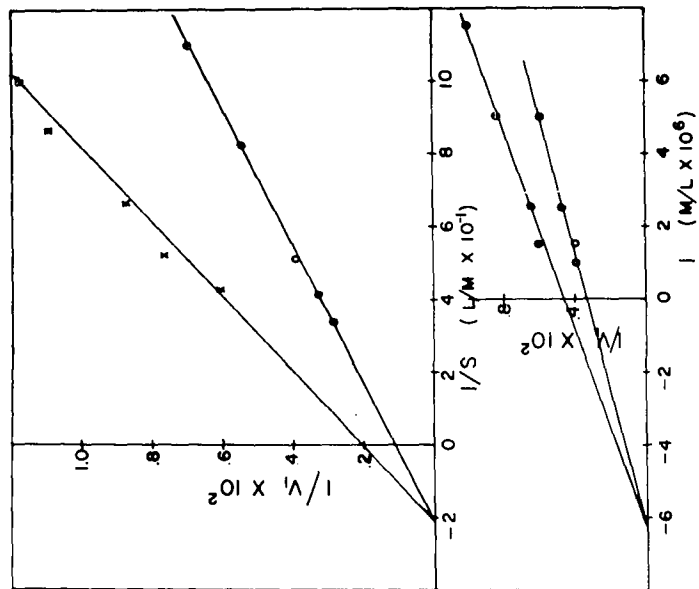
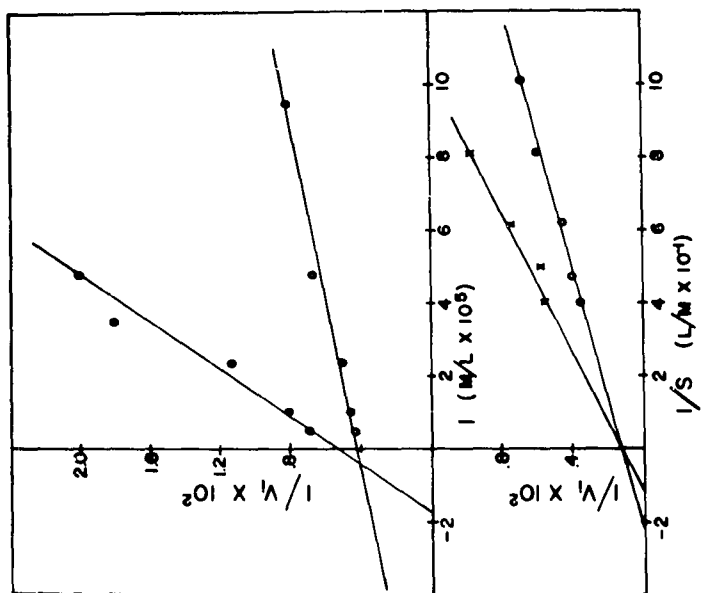


FIGURE 35

Reciprocal and double reciprocal plots of the inhibition by EDTA. The reaction mixture consisted of 5.3 ug of enzyme per ml (specific activity 10,000), 0.1M cacodylate buffer, pH 7.00 and from 5×10^6 M to 5×10^5 M EDTA (no calcium acetate was used in the experiments). The EDTA concentration used for the double reciprocal plot was 5×10^5 M. The substrate concentrations used for the reciprocal plot were 0.022M and 0.012M. No preincubation of the enzyme and inhibitor was employed.



IV. Discussion

A. Comparison of the B. subtilis Neutral Protease with Other B. subtilis Proteases

A comparison of the B. subtilis neutral protease and other proteases from B. subtilis seems warranted because it would show the differences and similarities between these proteases.

Table XVII lists three B. subtilis enzymes and gives some of their physical and chemical data. On the basis of the data in the table and as discussed in this thesis, the neutral protease is different from the other proteases in activity, amino terminal, molecular weight, sedimentation coefficient, amino acid composition, and specificity. The neutral protease is not inhibited by diisopropylfluorophosphate but is inhibited by chelators and some heavy metals.

It is very unlikely that all B. subtilis proteases utilize the same mechanism for enzymatic activity. The differences between the alkaline and neutral proteases are great enough to justify this conclusion. Kinetic support for this conclusion is not available because of the lack of kinetic data on the B. subtilis alkaline proteases.

The greatest similarity between the B. subtilis proteases is the observation that none of the enzymes contain any cysteine. This was shown for the neutral protease, Nagarse, and subtilisin by the amino acid composition. (Fuks, et al., 1959 and Johansen, et al., 1964)

TABLE XVII

Comparison of Proteolytic Enzymes of Bacillus subtilis

	Subtilisin	Nagarse	Neutral Protease
Crystalline form	Needle	Needle	-----
Analysis	N = 16.6%	N = 15.1 S = 1.0	N = 15.9 S = 0.36
Amino Terminal	Alanine	Alanine	Glycine
Carboxyl Terminal	-----	-----	Leucine
Extinction at 280 mu (1%)	8.6	8.8	13.6 - 13.8
Molecular Weight			
From Sedimentation-Diffusion	25,000 - 30,000	30,500	
From Archibald Method	-----	-----	44,700
Isoelectric Point	pH 9.4	About pH 7.0	pH 8.95 (in 0.002M Ca ⁺⁺)
pH Optimum (Casein digestion)	pH 10 - 11	pH 10 - 11	pH 6.5 - 7.5
Stable pH	pH 6.0 - 11.0	pH 6.0 - 10.0	pH 6.0 - 10.0 (in 0.002M Ca ⁺⁺)
Protecting factors	-----	Ca ⁺⁺ , Na ⁺ , Co ⁺⁺ NH ₄ cell extract	Ca ⁺⁺ , Sr ⁺⁺ , Mg ⁺⁺ , Mn ⁺ , Ba ⁺ , Co ⁺⁺
s _{20,w}	2.85	2.92 at 18°C. in M/50 phosphate [*]	3.24
\bar{v}	.749	-----	.746
f/f ₀ (frictional Ratio)	1.1	-----	1.3

* From Wieland, T. Archiv. fur Mikrobiologie 35 415 (1960).

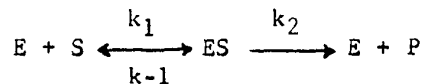
B. Kinetics and Mechanism of Enzyme Action

The neutral protease hydrolyzes hippuryl-L-leucinamide with the kinetics of the reaction following steady state kinetics of the type described by Laidler (1958). According to the data this reaction gives Type IV kinetics, indicating that the substrate is bound to neither an acidic nor basic group of the enzyme, but at a neighboring site. Since a pH optimum exists for this enzyme, the acidic and basic groups, determined by the study of V_{\max} , must enter into the subsequent breakdown of the enzyme-substrate complex.

This type of kinetic analysis then gives zinc a possible role in substrate binding. The specificity studies (Section III D5) indicate that van der Waal's forces would be expected to function in substrate binding, from the type of substrates hydrolyzed. The thermodynamics for the binding of substrate are of the magnitude required for van der Waal's forces or hydrogen bonding. Thus van der Waal's forces and zinc may function in substrate binding.

Van der Waal's forces may be responsible for substrate binding if zinc functions only in the hydrolysis of substrate. (The peptidase activity of carboxypeptidase A Coleman, et al., 1962) The only evidence for this assumption is the inconsistency of the heat of ionization with an alpha amino group. The pK_a would then represent the pK of some zinc-enzyme complex with the observed heat of ionization.

The kinetics are also indicative of the original Michaelis-Menten assumptions, i.e., K_m represents an equilibrium constant.



$$K_{eq} = [ES] / [E][S] = k_1/k_{-1} = 1/K_m = \bar{K}$$

Thus $k_2 \ll k_{-1}$. The evidence for these conclusions are:

(1) The shape of the pH optimum curve does not change with substrate concentration, which demonstrates non-competitive pH dependence. (2) Non-competitive inhibition is observed with cadmium ions. It has been shown (Dixon and Webb, 1958) that non-competitive inhibition is impossible in any case where $k_2 \gg k_{-1}$. When the constants are purely kinetic and do not represent equilibrium, the inhibition would be mixed and would not be non-competitive.

The complete thermodynamics of the kinetics cannot be interpreted without more data. For the entropies of activation, structural and solvent effects must be determined. However, the signs are significant in the entropy as in the energy of activation, and they indicate an unfolding or loosening of the enzyme structure as the enzyme-substrate complex is formed. The free energy of formation of the enzyme-substrate complex is negative, so the formation of the complex is spontaneous.

The determination of structural and solvent effects (electrostatic and non-electrostatic effects) have yet to be studied as a function of the dielectric constant of the reaction mixture. Using chymotrypsin as a model, since the type of kinetics (on methyl-hydrocinnamate) are the same as the neutral protease (on hippuryl-L-leucinamide), and since chymotrypsin

has no metal prosthetic group, it can be postulated that substrate binding is due to van der Waal's forces. (There is some evidence for this assumption as previously discussed.) With this analogy and using the mechanism postulated for chymotrypsin (Laidler 1956), it is necessary only to replace the acidic alpha amino group in chymotrypsin with the acidic zinc complex in the neutral protease. This gives a mechanism very similar to that proposed by Laidler for chymotrypsin.

This type of mechanism must be considered tentative since the exact role of zinc at the present time is unknown. When the exact function of zinc is determined, a more concrete mechanism may be postulated.

C. Summary and Discussion of the Properties and Kinetics of the B. subtilis Neutral Protease

1. This protease represents the first neutral protease from B. subtilis that has been isolated and characterized.

The enzyme is homogenous by several criteria. It is monodisperse during ultra-centrifugation and free boundary electrophoresis. Amino-terminal studies reveal a single polypeptide chain with glycine occupying the amino terminal position.

Electrophoresis indicates an isoelectric point of 8.95. Most electrophoretic patterns over the range of enzyme stability indicate the presence of a 5% contaminate which is believed to be autolysis products.

The neutral protease exhibits ideal sedimentation behavior at pH 6.5 and yields a sedimentation coefficient

($s_{20,w}$) of $3.25 \pm .05$ Svedberg units. These values of the sedimentation coefficients show no concentration dependence. The molecular weight, as determined by the Archibald "approach-to-equilibrium" method is $44,700 \pm 800$. Individual determinations of molecular weight by the above method exhibit a negative time dependence, which suggests autolysis (see Appendix I).

The enzyme is relatively stable from pH 6.0 to 9.0 and up to 40°C . in the presence of 0.002M calcium acetate. The instability of the enzyme at low pH is due to the loss of zinc and at other pH's, due to autolysis.

The amino acid composition of the neutral protease indicates an abundance of aspartic and glutamic acids. It also establishes the absence of cysteine since all the sulfur present in the enzyme is accounted for by methionine. Calculation of the isoionic point gave a value of 4.8. The large difference between the isoionic and isoelectric points is thought to be caused by the binding of cations.

The neutral protease is a zinc metalloenzyme with the zinc necessary for enzymatic activity. Thus the enzyme is inhibited by chelating agents such as EDTA, dithizone, and ortho-phenanthroline.

Besides being inhibited by chelating agents the enzyme is also inhibited by heavy metal ions. With mercury and copper the type of inhibition is unknown. Cadmium ions exhibit non-competitive inhibition.

The pH optimum of proteolytic activity is at pH 7.0

when assayed with casein or hippuryl-L-leucinamide and a temperature optimum (under the conditions of assay) is in the region of 45 to 55°C.

The limited studies on the specificity of the enzyme indicate the need for blocked groups, both carboxyl and alpha-amino groups. The enzyme seems also to require a side chain on one or both of the amino acids. The types of amino acids with different side chains which can be hydrolyzed are unknown except for the substrates given in the text. The substrate hippuryl-L-leucinamide is the most sensitive to hydrolysis by the neutral protease. The enzyme exhibits no esterase activity on all the esters tested.

Kinetic studies with hippuryl-L-leucinamide indicate that the enzyme hydrolyzes this substrate with "Type IV" steady state kinetics (Laidler, 1955). Examination of the V_{\max} versus pH curve indicates the groups of histidine and an alpha amino acid may be involved in the catalytic process. The plot of the pK_a versus temperature also indirectly indicates histidine. The kinetic data are amenable to treatment by the classical Michaelis-Menten equation, and the observed values of K_m are constant over the entire pH range studied. The overall kinetics and inhibition with EDTA indicate that zinc is essential for enzymatic activity.

APPENDIX I

The independence of the sedimentation coefficient of the neutral protease with concentration is unusual when the time dependence of molecular weight is considered. With this data it is obvious that autolysis must also occur during the sedimentation determinations.

Because no effect of autolysis on the sedimentation coefficient could be detected, the protein concentration during sedimentation (area under the sedimenting peak at time t) was investigated. An analysis of the autolysis was also made using the molecular weight data in conjunction with the equation for the weight average molecular weight. Assumptions were then made on the possible breakdown of the enzyme, and the mole fraction of undigested enzyme was calculated. The results are as follows:

Only one example of the calculated mole fraction will be presented since the assumptions used cannot cover the actual situation.

$$\text{Wt. average M.W.} = \frac{\sum_{i=1}^n m_i (M_i)^2}{\sum_{i=1}^n m_i (M_i)}$$

where m = moles of particles

M = particle weight

If we assume 1 mole of enzyme, and that each molecule which is digested is broken into eight fragments of molecular weight 5,600, the resulting equation is:

$$40,300^* = \left[\frac{(1-\chi)(44,700)^2 + 8\chi(5,600)^2}{(1-\chi)(44,700) + 8\chi(5,600)} \right]$$

where χ is the moles of enzyme digested.

$$18.01 \times 10^8 = 19.98 \times 10^8 - 19.98 \times 10^8 \chi + 2.51 \times 10^8 \chi$$

$$17.47 \chi = 1.97$$

$$\chi = .113$$

Thus using these assumptions, 88.7% of the enzyme is intact.

These assumptions are reasonable because this is approximately the same result as is given by the boundary analysis. The fractions of area lost in 65 minutes at protein concentrations of 0.6, 0.8, and 0.85 were 11%, 17%, and 22%, respectively.**

The assumed fragment weight is low enough to diffuse into the solution having only the intact protein sedimenting. Thus although the system is no longer homogenous with respect to particle size, the contribution if any made by the smaller particles must be negligible.

* This is the value for the molecular weight at 65 minutes as is given in Figure 10.

**

Protein Concentration					
0.60		0.80		0.85	
Time	wt area in mg	Time	wt area in mg	Time	wt area in mg
36	17	22	40.8	23	93.2
38	15.5	38	41	39	90.8
54	13.8	54	37.4	55	88.8
70	13.3	70	40	71	80.3
86	14.5	86	35	87	73.5
				103	67.8
				119	60.5

The slopes were determined with linear least squares.

Another possibility exists for the assumptions about autolysis. This is that each autodigested molecule is broken into a large fragment and a small fragment. Either possibility occurring exclusively is considered unlikely, although most of the data favors the assumption that when a molecule is broken down it is completely broken down into small fragments (i.e., no loss in specific activity after dialysis for 48 hours but a loss in total activity and total protein).

APPENDIX II

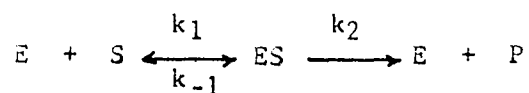
In enzyme reactions which at the steady state follow the Michaelis-Menten rate law, there are fifteen experimental thermodynamic changes, not all independent: $\Delta F_{\alpha}^{\ddagger}, \Delta H_{\alpha}^{\ddagger}, \Delta E_{\alpha}^{\ddagger}, \Delta S_{\alpha}^{\ddagger}, \Delta V_{\alpha}^{\ddagger}, \Delta F_{\beta}^{\ddagger}$

$\Delta H_{\beta}^{\ddagger}, \Delta E_{\beta}^{\ddagger}, \Delta S_{\beta}^{\ddagger}, \Delta V_{\beta}^{\ddagger}, \Delta F_{\alpha/\beta}, \Delta H_{\alpha/\beta}, \Delta E_{\alpha/\beta}, \Delta S_{\alpha/\beta},$ and $\Delta V_{\alpha/\beta}$.

When the reverse process (products to reactants) can also be studied an additional set of fifteen thermodynamic quantities can be measured.

The measured quantities α, β , and α/β (first order rate constant at low substrate concentration, maximum velocity constant, and the equilibrium constant for the binding of substrate) are all that can be determined from steady state velocity studies. They are no more than parameters which are related in some way to the actual chemical events of the catalyzed reaction.

As has been shown in the text, (Section IIID6f4) the neutral protease follows Michaelis-Menten kinetics, K_m being an equilibrium constant. i.e.:



$$K_m = [E][S] / [ES] = k_{-1}/k_1$$

Thus the equilibrium constant for the formation of the enzyme-substrate complex is $\bar{K} = k_1/k_{-1}$. The free energy for the formation of the enzyme-substrate complex can be calculated by using the following equation:

$$\Delta F_{\alpha/\beta} = -RT \ln \bar{K} \quad \dots \dots \dots 1.$$

Using the van't Hoff equation

$$\frac{d(\ln \bar{K})}{dT} = \frac{\Delta H_{A/B}}{RT^2} \dots\dots\dots 2.$$

with the variation of \bar{K} with temperature, the enthalpy for the formation of the enzyme-substrate complex can be calculated. Utilizing the values ΔF from equation 1 and ΔH from equation 2, the entropy for the formation of the enzyme-substrate complex can be determined with the following equation

$$\Delta S_{A/B} = \frac{\Delta H_{A/B} - \Delta F_{A/B}}{T} \dots\dots\dots 3.$$

The above three equations give the values $\Delta F_{A/B}$, $\Delta H_{A/B}$, and $\Delta S_{A/B}$. $\Delta E_{A/B}$ and $\Delta V_{A/B}$ were not determined.

By using the theory of absolute reaction rates (Eyring, 1935), the energies and entropies of activation can be calculated. The theory states that the rate of any reaction at a given temperature depends only on the concentration of an energy rich activated complex which is in equilibrium with unactivated reactants. The rate is then given by $k_B T/h$; i.e., the rate of breakdown is independent of the nature of the complex.

The reaction velocity constant k is given by

$$k = \frac{k_B T}{h} \bar{K} \dots\dots\dots 4.$$

Since

$$\Delta F^* = \Delta H^* - T\Delta S^* = -RT \ln \bar{K} \dots\dots\dots 5.$$

and from the definition $\bar{K} = K^*$, then the substitution of equation 5 in equation 4 gives

$$k = \frac{k_B T}{h} e^{-\frac{\Delta F^*}{RT}} = \frac{k_B T}{h} e^{-\frac{\Delta H}{RT}} e^{\frac{\Delta S}{R}} \dots\dots\dots 6$$

If it is assumed that ΔS^* does not vary with temperature, the following equation may be obtained by taking logarithms and differentiating with respect to temperature

$$\frac{d(\ln k)}{dT} = \frac{1}{T} + \frac{\Delta H^*}{RT^2} = \frac{\Delta H + RT}{RT^2} \dots\dots\dots 7.$$

By comparing equation 7 with the Arrhenius equation

$$\frac{d(\ln k)}{dT} = \frac{E^*}{RT^2} \dots\dots\dots 8.$$

it is noted that

$$E^* = \Delta H^* + RT \dots\dots\dots 9.$$

The variation of V_{\max} and the first order rate constant at low substrate concentration with temperature will give ΔE_{β}^* and ΔE_{α}^* , respectively. Using these values of ΔE^* and equation 9 the corresponding values of enthalpy can be calculated at a defined temperature.

The entropy can be calculated from equation 6 by using the calculated enthalpy values and k_2 (the maximum velocity constant) or the corresponding first order rate constant at low substrate concentration. With the information obtained (i.e., ΔE_{α}^* , ΔE_{β}^* , ΔH_{α}^* , ΔH_{β}^* , $\Delta S_{\alpha}^{\ddagger}$, and $\Delta S_{\beta}^{\ddagger}$) the free energy, $\Delta F_{\alpha}^{\ddagger}$ and $\Delta F_{\beta}^{\ddagger}$ can be calculated from equation 3 or equation 6.

The maximum velocity constant k_2 is calculated from the equation

$$V_{\max} = k_2 [E] \text{ total} \dots\dots\dots 10.$$

A final indication that k_m is an equilibrium constant is the fact that when $k_2 \ll k_1$ the value for $k_2 \bar{K} M^{-1} \text{sec}^{-1}$ is the first order rate constant at low substrate concentration (Laidler, 1958). The value of 8.49×10^2 for $k_2 \bar{K}$ (Table XVI) is approximately equal to the value

obtained from first order rate constants ($5.5 \times 10^2 \text{ M}^{-1}\text{sec}^{-1}$).

Thus all assumptions used in the text in interpreting the kinetics of the neutral protease on hippuryl-L-leucinamide are consistent. The only possible exception could be in the number of active centers per molecule because the zinc data may indicate two. Even in that case, they must be equivalent and independent.

Definition of Symbols

ΔF	free energy change
ΔH	enthalpy change
ΔE	energy change
ΔS	entropy change
ΔV	volume change
k_B	Boltzman constant
h	Planck's constant
T	absolute temperature
R	gas constant
K_m	Michaelis constant
k	rate constants
V_{\max}	maximum velocity
$[E]_{\text{total}}$	total enzyme concentration
$\bar{K} = 1/K_m$	or the equilibrium constant for the formation of the enzyme-substrate complex

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