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THE PROTEOLYTIC ENZYMES OF A NEW MARINE PSEUDOMONAS

by
Thomas S. Freund

A Dissertation

Presented to the Graduate Committee

of Lehigh University

in candidacy for the Degree of

Doctor of Philosophy

in

Chemistry

Lehigh University

1969

A CERTIFICATE OF APPROVAL

Approved and recommended for acceptance as a dissertation in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

30 April 1969
Date

Joseph R. Merkel
Professor in Charge

Accepted 30 April 1969
Date

Special committee directing the doctoral work of Mr. Thomas S. Freund.

Joseph R. Merkel
Joseph R. Merkel, Chairman

Ned D. Heindel
Ned D. Heindel

Roland W. Lovejoy
Roland W. Lovejoy

Richard J. Malsberger
Richard G. Malsberger

Walter J. Nickerson
Walter J. Nickerson

Thomas E. Young
Thomas E. Young

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ABSTRACT

The characteristics and proteolytic enzymes of a marine bacterium, originally isolated from a 1600 meter Sargasso Sea sample, were studied. Selection of the bacterium was made because of its ability to produce proteolytic enzymes and viscid colonies. It was found to be a gram negative rod, motile by means of a single polar flagellum, its aerobic nature, catalase- and oxidase-production identified this organism as a species of Pseudomonas. After consulting the literature for previously described organisms and finding that none were similar, it is proposed that this Pseudomonas be referred to as a new species. The conditions necessary for maximal growth and enzyme production were determined in shaker flasks and aerated fermentor cultures. Growth and enzyme production were found to depend upon the available nutrients, aeration, stage of growth, temperature, initial pH of the medium and ionic strength of the added salt. The bacterium produced two endopeptidases (I & II) and an aminopeptidase. The endopeptidases were produced extracellularly and the aminopeptidase was found to be an intracellular enzyme. Separation of the proteases in the culture filtrate after ammonium sulfate precipitation was accomplished with ion-exchange chromatography, differential heat treatment and gel-filtration.

Endopeptidase II was purified by gel-filtration and

shown to be homogeneous by electrophoresis and gel-filtration. It was characterized as a metalloenzyme with a neutral pH optimum and a molecular weight of approximately 30,000. Specificity studies with the isolated B-chain of insulin revealed that the amino acid residue contributing the amine portion of the susceptible bond had to contain uncharged groups. The amino acid contributing the carboxyl portion of the bond also had to have a neutral charge. It is suggested that endopeptidase II could be used in short term hydrolytic studies for protein structural determinations.

Endopeptidase I resisted complete separation and purification. An impurity, thought to be an inactive dimer of endopeptidase II, could not be removed. Characterization studies showed endopeptidase I to be similar to endopeptidase II with the exception of a molecular weight of approximately 60,000 and specificity. Specificity studies with the impure endopeptidase I suggested that amino acids with hydroxy residues could serve as the amino contributing amino acid.

The intracellular aminopeptidase was studied in detail. Found to be heat stable, it was separated from the endopeptidases by differential heat treatment and gel-filtration. The enzyme was shown to be homogeneous by electrophoresis, gel-filtration and ultracentrifugation. It requires zinc for activity and has a pH optimum of 8.1. The enzyme is specific for neutral amino acids in the free N-terminal

position, preferring leucine followed by phenylalanine and valine. Rates of hydrolysis of dipeptides revealed specificity for the penultimate position as well. Leucine and isoleucine were preferred in this position. The need for an imidazole and free amine group at the binding site is suggested from the effects of pH on the K_M. Metal complexing agents completely inactivated the enzyme, but the activity could be restored (approximately 70%) by the addition of Zn^{+2}. Dialysis against distilled water also inactivates the aminopeptidase, but reactivation cannot be accomplished by the readdition of Zn^{+2}. The required metal is not lost during dialysis against distilled water but a decrease in ionic strength leads to changes in the enzyme's structure and subsequent loss of heat stability and activity. Changes in the far-ultraviolet, optical rotatory dispersion and the circular dichroism spectra revealed that the native enzyme is in an ordered conformation which becomes disordered in low ionic strength solutions.

It is also suggested that the latter observations on the heat stability and the structure correlation could be expanded to other enzymes and possibly result in the conferring upon heat labile enzymes some measure of heat stability.

INTRODUCTION

Since the recognition of their importance in physiological functions, the proteolytic enzymes have been the subject of numerous investigations (1,2). Their role in the decomposition processes are important to all life. The production of proteases is an essential link in the life-cycle which permits the continued reuse of almost all proteins. Proteolytic enzymes themselves are proteins. It is, therefore, naturally interesting to determine in what manner one protein is able to catalyze the hydrolysis of another. As a result, proteolytic enzymes have been used for many years in studies designed to elucidate the relationship between molecular structure and enzymatic activity.

They have also been used as tools by protein chemists in their investigations into the primary structure of proteins. The search for new proteases which are specific for the catalysis of only certain peptide bonds has been going on for many years (1). The proteolytic enzymes of the extracellular secretions, particularly those of the pancreas, have been studied quite thoroughly, since they are readily available in crystalline form and are well characterized with respect to some chemical and physical properties (3). The narrow specificity of trypsin (for basic amino acid side chains) has resulted in its wide

application for structural studies.

Proteases of microbial origin have not been studied as intensively as the animal proteases. For a number of years it has been contended that most of the microbial enzymes which were studied showed a broad substrate specificity and mechanism of action(1). Many require metals or thiol groups while some do not require any cofactors. However, recent investigations have shown that these proteases are more specific for the type of bond cleaved than was originally suspected (4). Subtilisin hydrolyzes, under certain conditions, only one peptide bond in the protein ribonuclease (5). Recently, separate proteases from Streptomyces fradiae (6) and from Clostridium histolyticum (7) were shown to posses activity similar to trypsin. Studies on the specificity of Bacillus subtilis neutral proteases (8,9) with the insulin B-chain show a similar specificity as thermolysin (10) and a protease from Pseudomonas aeruginosa (11) for uncharged amino acid side chains.

In addition to the endopeptidases, a number of aminopeptidases have recently been examined. Leucine aminopeptidase which is specific for N-terminal leucine residues was first isolated from swine kidney (12). Since that time aminopeptidases specific for acidic amino acid residues (aminopeptidase A) and for basic amino acid residues (aminopeptidase B) have been isolated from mammalian

tissues (13, 14). Westley, et al. (15) demonstrated that many bacteria produced aminopeptidases with varying specificities. The aminopeptidase from Aeromonas proteolytica was purified by Prescott and Wilkes (16) and shown to be a zinc requiring leucine aminopeptidase. The intracellular aminopeptidase from Bacillus subtilis, another leucine aminopeptidase, was found to be similar to the swine kidney enzyme in that manganese was recorded as an activating metal ion (17). The above studies illustrate the growing amount of data in support of the idea that microbial proteases do possess narrow specificities.

Morihara and co-workers (18) noted that those enzymes requiring a metal ion for activity (inhibited by disodium ethylenediaminetetraacetate) and unaffected by diisopropylphosphofluoridate possessed similar pH optima (about 7.0-8.0). Although referred to as neutral proteinases these enzymes could be classed as metalloenzymes (19). Their specificities showed remarkable similarities toward hydrolysis of the B-chain of insulin and synthetic substrates. All were predominently specific for peptide bonds in which L-leucine or L-phenylalanine residues provided the amino group (18). This similarity in hydrolysis furthers the suggestion that some of the proteases of different origins possess similar mechanisms of action (i.e., require similar amino acid side groups and cofactors for activity).

The proteolytic enzymes have been generally divided

into four groups (19): those enzymes requiring a metal ion for activity (metalloenzymes), those enzymes requiring a serine residue for activity (serine-type proteases), those requiring a reduced thiol group (sulfhydryl enzymes) and those whose pH optimum are acidic (acid proteases).

Improved purification techniques have revealed that a single microorganism often produces several proteolytic enzymes (3, 20). The reasons for this multiplicity of protease production are as yet unknown. Possibly, if the specificities of these proteases were narrow, the organism would require more enzymes to provide the necessary nutrients. Two alternate suggestions have raised questions whether the proteolytic enzymes of microorganisms are constitutive or induced. The first suggests that the growing organism changes its microenvironment such that new enzymes would be required with slightly different characteristics (for example, pH optimum). A second suggestion requires the enzymes to be produced at different times in the growth cycle in order to hydrolyze previously synthesized proteins (20).

Many different groups of microorganisms have been found to produce extracellular proteinases (21). Even though Zobell (22) in 1946 reported that "as a group, marine bacteria are actively proteolytic", it was not until recently that an investigation of the proteolytic enzymes of marine bacteria was undertaken (23, 24, 25). A number of

these enzymes have been isolated and partially characterized as to chemical and physical properties (16, 26, 27).

The study of marine bacteria and their proteases could lead to the development of new tools for the protein chemist as well as provide more information concerning the relationship of molecular structure to enzymatic activity. additionally, it is of interest to gain a greater understanding about the production of proteolytic enzymes and the role of the marine bacterium in the sea. It is generally acknowledged (28) that the concentrations of nutrients in the oceans is limited. Were it not for the microorganism's ability to decompose existing material and synthesize other nutrients, life would cease when the nutrients were exhausted. Thus an understanding of the manner in which the proteolytic enzymes are produced by a marine bacterium would be valuable in providing additional information about the marine environment.

In order to help attain the above goals, gain a better understanding of the relationship between a marine bacterium and its proteases and provide additional information about the chemistry and nature of proteolytic enzymes, it was decided to work with the proteolytic enzyme system of a particular marine bacterium. This bacterium was isolated in 1964 by Dr. J.R. Merkel during the time he was conducting a marine bacterial survey in the waters near Bermuda (29). It was isolated because of its predominance in the water

sample, mucoid nature and relatively large proteolytic activity. Upon isolation, it appeared to have different characteristics than the recently described Aeromonas proteolytica (25) and would, therefore,add to our knowledge of marine proteolytic bacteria.

This report contains the results and possible consequences of such studies. The characteristics of the marine bacterium, designated B-207, are described in Part I. In addition, the general characteristics of the proteolytic enzyme system and the composition of the capsular material will also be found there. Part II is concerned with the purification and general characterization of the endopeptidases. The aminopeptidase, its isolation, purification and characterization, is described in Part III.

During the preliminary work with the aminopeptidase it was found that dialysis against distilled water resulted in greatly reduced enzyme activity. It was immediately suspected that during dialysis either a necessary metal ion was removed or that a certain ionic strength was required for optimum activity. Several papers describing aminopeptidases (12, 16, 17) have shown them to be metalloenzymes. However, metalloenzymes do not generally lose their essential metal component by subjecting to dialysis against distilled water (30). On the other hand, it has been reported that neutral salts

in high concentrations (0.3 to 3.0 M) inhibited the activity of various enzymes including trypsin (31). The inactivation appeared to be the result of structural changes in the protein molecule. It was thought that possible loss of salt during dialysis of the aminopeptidase might change the structure, affecting the activity of the aminopeptidase and possibly causing aggregation of the charged protein molecules. These aspects of the aminopeptidase structure-activity relationship were examined and are included in Part III.

EXPERIMENTAL PROCEDURES

Reagents and Media

Reagents: All inorganic compounds and organic solvents used were reagent grade from Baker Chemical Co. (Philipsburg, N. J.) or Fisher Scientific Co. (King of Prussia, Pa.). Enzyme grade ammonium sulfate and tris-(hydroxymethyl)-aminomethane (Tris) were both from Mann Research Laboratories (New York, N. Y.) and Heico Chemical Co. (Stroudsburg, Pa.). Synthetic peptides and substrates as well as dinitrophenyl (DNP)-amino acid standards, molecular weight markers (Kit #8109) and carbohydrate standards were purchased from Mann Research Laboratories, Sigma Chemical Co. (St. Louis, Mo.), Calbiochem Co. (Los Angeles, Calif.), Cyclo Chemical Co. (Los Angeles, Calif.) and Nutritional Biochemical Corp. (Cleveland, Ohio). Ester derivatives of some peptides were prepared from a method described by E. Fischer (32). Trypsin, lysomzyme, ribonuclease and hemoglobin were from Worthington Biochemical Corp. (Freehold, N. J.). Reagents for the gel electrophoresis experiments were made by Eastman Organic Chemical Co. (Rochester, N. Y.) and the cellulose acetate strips were from Gelman Instrument Co. (Sepraphore III, Lot #1155, 1240 and 1694, Ann Arbor, Mich.) and C. Schleicher and Schuell, Co. (Lot #200/5, Keene, N. H.). Diethylaminoethyl (DEAE)-cellulose (Mannex-DEAE, Lot # N2431, Mann Research Laboratories and Whatman Anion Exchanger, DE-50,

W.& R. Balston, Ltd., England) and DEAE-Sephadex (A-25, Lot #8185, Pharmacia Fine Chemicals, Uppsala, Sweden) were purchased for ion-exchange chromatography. The neutral Sephadex resins, used for gel-filtration, came from Pharmacia Fine Chemicals.

Protein substrates: Ribonuclease was isolated from beef pancreas by the procedure of Kunitz (33) or purchased from Worthington Biochemical Corp. Insulin was purchased from Boots Pure Drug Co., Ltd., Nottingham, England and Mann Research Laboraties. Both proteins were oxidized by the performic acid method of Craig (34) and then lyophilized on a commercial freeze-dryer (New Brunswick Scientific Co., Model B66). The A and B chains of insulin were then separated by Prescott's DEAE-Sephadex chromatography method (35). Chromoproteins used for the detection of endopeptidase activity were isolated from Porphyra sp. (36) and separated by calcium phosphate gel chromatography (37). Hemoglobin was denatured with urea by Prescott's modification (26) of Anson's method (38). Five grams of lyophilized salt-free hemoglobin were dissolved in 80 ml of distilled water, followed by the addition of 80 g of urea. After incubation at 37°C for 50 minutes, a solution containing 10 g of urea in 125 ml of 0.134 M phosphate buffer (pH 8.0) was added. The resulting solution could be stored for two weeks at 4°C. The concentration of the protein substrates were determined with the Biuret method (39).

Media: Stock cultures were grown and maintained on peptone sea water agar (PSW) slants prepared with 20 g peptone (Difco Laboratories, Detroit, Mich.), 0.3 g di-sodium phosphate, 40 g Rila Marine Mix (RMM) from Rila Products, Teaneck, N.J., 20 g nutrient agar (Difco Laboratories) per liter of distilled water. The pH of the medium was adjusted to 7.8 and autoclaved at 15 pounds pressure for 15 minutes.

Casein used in the nutritional studies and enzyme experiments came from Mann Research Laboratories. Enzymatically digested casein samples, N-Z Amine type HD (Lot # 30B-20) and pancreatic autolyzate (Lot #20H-27) were gifts from Sheffield Chemical Co., Norwich, N.Y. A cottonseed protein product (Proflo) was a sample from Trader's Protein Division of Trader's Oil Mill Co., Fort Worth, Texas.

Casein and Proflo were digested in the laboratory with Pancreatin (Lot #K1934) from Mann Research Laboratories at pH 8.0, 37°C for 48 hours (7.0 g Pancreatin per 300 g casein or 175 g Proflo). The digest was filtered and stored under toluene at 4°C. For use, it was diluted 1:5 with 3% RMM. This gave approximately a 1% protein concentration as determined by the Lowry method (40) with an ovalbumin (crystallized, Calbiochem Corp.) standard.

Bacteriological Procedures

The marine bacterium used in this study was stored on peptone sea water agar slants at -10°C in a freezer. The stock culture was transferred semi-anually. Subcultures were stored at -10°C and at 22-24°C. They were transferred whenever required for enzyme production.

Bacteriological Tests: The procedures used to identify the bacterium were taken from "The Manual of Microbiological Methods" (41). Pigment production was examined by the method of King, et al. (42). A modification of Kovac's method (43) for the demonstration of oxidase was used in which 1.0 ml of 0.5% p-aminodimethylaniline HCl was added to the bacterial slant. Upon shaking, an immediate change in color to reddish purple was recorded as positive. The sensitivity of the organism to antibiotics was determined with a set of high and low concentration Bacto-Sensitivity Disks (control #481332) from Difco Laboratories. The surfaces of PSW plates were covered with a dilute cell suspension. After 15 minutes the disks were placed on the inoculated plate, leaving 4 cm between disks. The plates were then incubated for 24 and 48 hours at 23°C.

Lytic susceptibility was measured by adding bacterial suspensions to distilled water, 0.05 and 0.5 M concentrations of NaCl, KCl and $MgCl_2$. Four percent RMM was used as a control. One ml of bacterial suspension was diluted

with nine ml of test solution and then incubated at room temperature (22°C) for 15 minutes. The optical densities were read at 660 mu and the residual turbidities were calculated by taking the control after 15 minutes incubation as 100 percent.

Electron microscopy: A drop of the cell suspension was placed on either a Formvar carbon coated copper (400 mesh) or stainless steel (200 mesh) grid. After 30 minutes, excess liquid was removed with filter paper. The grids were dried overnight in a vacuum desicator over P_2O_5. Suspensions were also placed on grids with transfer loops.

Shadowing was accomplished with Pt and Pd-carbon alloy in an Illini Electrodeposition apparatus. Shadow angles of 35-45 degrees were employed. After shadowing, the specimens were examined on an RCA EMU-3G electron microscope.

Cell walls were prepared by combining the osmotic lysis method of Salton and Horne (44) and sonic lysis, followed by the purification procedure of Westphal, et al. (45). Bacterial cells, grown for 18 hours, were washed three times with 3% RMM and then suspended in distilled water. Sonification was accomplished by exposing the ice cooled cell suspension to 20 Kc signals for 10 minutes with a Heat Systems Co. Sonifier Cell Disruptor. Immediately following the cell disruption, the thick suspension was heated in a boiling water bath for 15 minutes. Differential

centrifugation at 4,000 rpm for 10 minutes removed the whole cells and at 9,00 rpm for 15 minutes sedimented the cell walls. The cell wall fraction was then successively treated with 0.5 N NaOH, ribonuclease and trypsin (0.1 mg/ml each). The resultant cell walls were deposited on Formvar-carbon coated grids and shadowed as described above.

Deoxyribonucleic acid (DNA) was isolated from the bacterium by combining the procedures of Marmur (46) and Kay, Simmons and Dounce (47). Lysozyme (0.5 mg/ml) was added to suspensions of bacteria in distilled water and allowed to incubate 6 hours at 20°C. One hundred ml of 5% sodium dodecyl sulfate in 45% ethanol was added per liter of mixture and homogenized for 2 minutes in a Waring blender. Following centrifugation at 25,000 x g for 1 hour, the protein was precipitated by adding 55.0 g sodium chloride per liter of supernatant. Room temperature centrifugation at 15,000 x g for 45 minutes gave a clear supernatant from which the DNA was isolated by the addition of 2/3 volume of cold 95% ethanol. The nucleic acid was wound around a stirring rod as a white thread-like precipitate. The resultant DNA was three times further purified by repetition of the above procedure until the final product had a protein impurity of approximately 0.5% and a negligible amount of ribonucleic acid.

The temperature at which the double stranded DNA unwinds (melting temperature) was determined from Marmur

and Doty's procedure (48). A 0.001% solution of DNA in 0.9% saline-0.1 M citrate was heated in a Haake Circulating Constant Temperature Bath (Series N). The optical density at 260 mu wavelength was determined at several temperatures with a Cary 14R recording spectrophotometer. The percent (G-C) was calculated from the equation Tm = 69.3 + 0.41 (G-C), where Tm = melting temperature and (G-C) = mole percent of guanine plus cytosine.

Capsular polysaccharides were isolated from the bacterium by the procedure of Duguid and Wilkinson (49). The bacterium was grown in a petri dish on a disk of sterilized cellophane dialysis tubing covering the surface of the PSW agar. To increase the polysaccharide production, 2% sucrose was added to the medium. The inoculum was made by suspending an 18 hour PSW slant culture in 3% RMM. A loopful of this suspension was placed in the center of the cellophane disk and spread over the entire surface with a sterile cotton swab. The plates were incubated at 27-28°C. After 48-72 hours the viscous bacterial growth was washed off the cellophane disks with 4% RMM. The resulting suspension was stirred at 23°C for 2 hours. The cells were then removed by centrifugation and the crude polysaccharides in the supernatant fluid were precipitated with three volumes of cold 95% ethanol. Further purification was carried out by subjecting a 1% solution in 0.067 M phosphate buffer (pH 7.0) to ribonuclease (.1 mg/ml) digestion for 2 hours

at 37°C. This was followed by extraction of the protein with hot (60°C) 45% phenol by a procedure described by Westphal and Jann (50). Extraction with phenol was repeated until the ratio of protein to total carbohydrate remained constant. Chromatography on Dowex 1-X8 (Cl^-) and Sephadex G-200 did not remove any further protein.

Quantitative analysis of the isolated polysaccharides was accomplished using the following procedures:

Neutral sugars-	anthrone reagent (51)
	phenol-sulfuric acid method (52)
Amino sugars-	Rondle-Morgan method (53)
Uronic acids-	carbozole reaction (54, 55)
6-deoxy sugars-	cysteine-sulfuric acid method (56)
Pentoses-	orcinol reagent (57)

The polysaccharides were hydrolyzed in 2.0 N HCl for 3, 6 and 16 hours under vacuum at 105°C. The HCl was removed by means of a flash evaporator (Buchler Instruments, Fort Lee, N.J.) and the hydrolyzate was vacuum-dried over NaOH. Ion-exchange chromatography with Dowex 1-X8 (Cl^- form) in a 2.5 x 25 cm column followed by Dowex 50W-X8 (H^+ form) in a similar column yielded neutral, acidic and basic fractions. The identification of the sugars in the hydrolyzate and fractions was accomplished by means of thin layer chromatography on silica gel G and microcrystalline cellulose layers, and by chromatography on Whatman 3MM paper. The following solvent systems were used for one-dimensional chromatographic separation:

A: t-amyl alcohol:isopropanol:water (4:1:2)
B: butanol:acetic acid:water (5:1:2)
C: n-propanol:water (7:1)

D: ethyl acetate:acetic acid:formic acid:water (18:3:1:4)

E: butanol:pyridine:water (6:4:3)

Growth studies: In a typical growth study, 50.0 ml of the medium to be tested was placed in a 250 ml Erlenmeyer flask which contained small baffles or indentations (Triple-baffled shake flasks, Bellco Glass Co., Vineland, N.J.) The pH was adjusted to 7.3-7.4 (unless another pH was required) with 1.0 N NaOH or HCl, a polyfoam stopper was placed in the mouth and the flask was autoclaved at 15 pounds pressure for 15 minutes. Several flasks were prepared at one time and stored at 4°C. These flasks were inoculated by the addition of 0.1 ml of a bacterial suspension. The suspension was prepared from a freshly transferred culture after 18 hours of growth at 23°C on a PSW slant by adding 5 ml of 3% RMM. The shaker flasks were incubated at room temperature (22-24°C) for varying periods of time on a rotating, variable speed shaker (New Brunswick Scientific Co., Model V-5). When more closely controlled temperatures were needed, the flasks were submerged in a constant temperature bath and attached to a wrist action shaker (Burrell, Model BT). Samples were withdrawn, centrifuged at 10,000 rpm for 10 minutes and assayed for endopeptidase, aminopeptidase, pH and growth. Prior to centrifugation, bacterial growth was measured as turbidity at 660 mμ wavelength with a Bausch and Lomb Spectronic 20. The pH was also measured prior to centri-

fugation with a Heath Co. (Benton Harbor, Mich.) pH recording Electrometer equipped with a Sargent (E.H. Sargent & Co., Chicago, Ill.) combination electrode.

After a good medium for growth and enzyme production was found, large batches (5 liters) of enzyme were prepared using a fermentor assembly (New Brunswick Scientific Co., Model FS-307). In a typical fermentor preparation, the N-Z Amine type HD medium (1%) was added to 5 liters of 3% RMM and the pH adjusted to 7.3. The fermentor apparatus was assembled and autoclaved at 15 pounds pressure for 45 minutes. After cooling the fermentor was inoculated with 50 ml of an 18-24 hour shaker flask culture. The temperature of the water bath was maintained at 22-23°C, the stirring speed was 500 rpm and the rate of air flow was maintained between 5 and 6 liters per minute. Foaming was controlled by a pump and electrode system using Antifoam 10 (a silicone emulsion from General Electric Co.). Samples were aseptically withdrawn at varying intervals for analysis of growth and enzyme activity.

Biochemical Procedures

Enzyme assays: Endopeptidase activity (endopep.) was determined by Prescott's modification (26) of Anson's method (38) utilizing urea-denatured hemoglobin as substrate. Two and one-half ml of the hemoglobin solution was incubated with 0.5 ml of enzyme at 37°C for 5 minutes. The reaction

was terminated by the addition of 5.0 ml of 5% trichloroacetic acid (TCA). After 20 minutes, the precipitated, undigested protein was removed by filtration through Whatman No. 2 paper. The absorbance of the soluble material was then determined at 280 mμ. One unit of activity was defined as the amount of enzyme in mg that produced a change in optical density (O.D.) of 1.0 at 280 mμ in five minutes.

Aminopeptidase activity (aminopep.) was determined by a colorimetric procedure (58) in which the hydrolysis product of 0.5 ml of L-leucyl-B-naphthylamide (LNA) was diazotized with 1 ml of 1% sodium nitrate and 1 ml of 0.5% ammonium sulfamate and then coupled with 2.0 ml of 1-naphthylethylenediamine dihydrochloride (0.5 mg/ml in 95% ethanol). The color intensity of the resultant blue complex, as determined at 580 mμ, was a direct measure of the extent of reaction. The reaction required the addition of 0.5 ml of enzyme solution to 0.5 ml of substrate at pH 8.0. Following an incubation period of 10 minutes at 37°C, the reaction was halted by the addition of 0.5 ml of 40% TCA. One aminopeptidase unit is defined as the amount of enzyme in mg that causes the hydrolysis of 0.1 mg of LNA in ten minutes.

Carboxypeptidase activity was tested for with carbobenzoxy glycyl-L-phenylalanine as substrate in the method described by Coleman and Vallee(59).

The presence of collagenase activity in the crude

enzyme mixture was looked for by measuring the release of ninhydrin positive material from samples of collagen (acid extracted calfskin and Achilles tendon). The method described by Rippon (60) was used for this determination.

The presence of elastase activity was tested for by measuring the increase in ninhydrin positive material and in Biuret color of the solubilized elastin. The procedure described by Morihara and Tsuzuki (61) was used with the exception that the Biuret method was substituted for the Lowry procedure.

Esterase activity was determined by measuring the increase in optical density at 254 mμ for the hydrolysis of benzoyl-L-arginine ethyl ester (BAEE) according to Schwert and Tanaka (62) or at 247 mμ for the hydrolysis of tosyl-L-arginine methyl ester (TAME) according to the method of Hummel (63). Percent hydrolysis was determined by comparison with an acid hydrolyzed sample which was assumed to be completely hydrolyzed (105°C, 24 hours).

Enzymatic hydrolysis of proteins: Approximately 40-50 mg of the protein substrate were dissolved in distilled water to make a 10 mg/ml solution. The pH was adjusted to 7.5 by the addition of 0.5 N NH_4OH or HCl. Accurate determination of the protein concentration was then made by means of the Biuret method (39). To 4.0 ml of the substrate solution, 1.0 ml of the enzyme solution (containing 10-100 μg of protein) was added. Hydrolysis was

allowed to proceed at 37°C for varying lengths of time, after which the test tubes containing the reaction mixtures were immersed in a boiling water bath for 10 minutes. The percent hydrolysis was determined by comparing the Biuret color, of 1.0 ml of the reaction mixture, measured at zero time with the Biuret color after the reaction was terminated. The decrease in color was linearly related to percent hydrolysis. In order to eliminate contributions from the enzyme, the optical blank contained an equal quantity of boiled enzyme solution.

Hydrolysis of peptides and amino acid substrates was accomplished in a similar manner except that the reaction was followed by measuring the release of ninhydrin positive material according to the procedure of Stein and Moore (64). In a number of cases 1% methanol was added to aid in dissolution of the peptide substrates. The amino acid-B-naphthylamide substrates were used as described above for the L-leucyl-B-naphthylamide in the aminopeptidase assay. Percent hydrolysis was determined by comparing the ninhydrin color of the test solution to the ninhydrin color of a 100% hydrolyzed sample. The 100% hydrolyzed sample contained equal concentrations of the free amino acids (in the case of peptides) or an acid hydrolyzed sample which was assumed to be completely hydrolyzed (105°C, 24 hours). The ninhydrin color blank contained the substrate and boiled enzyme.

Acid hydrolysis of peptides was carried out with 6 N HCl under vacuum at 105°C for 18 hours. The samples were evaporated to dryness on a flash evaporator, washed several times with distilled water and finally dried under vacuum over NaOH.

Protein concentrations were routinely estimated by reading the optical density (O.D.) at 280 mμ using a 1 cm light path. The approximation that 1.0 O.D. unit corresponded to 1.0 mg of protein was made. The Lowry method (40) using an ovalbumin standard (crystallized, Calbiochem Co.) was used for more accurate determinations of enzyme concentration.

Optical measurements were made on a Bausch and Lomb Spectronic 20 in the visible range and a Bausch and Lomb Spectronic 600 or Beckman DU spectrophotometer in the ultra violet region. A Cary 14R recording spectrophotometer was used in the ultra violet region for DNA and certain protein spectra.

Optical rotatory dispersion and circular dichroism (ORD and CD) were recorded with a Cary 60 ORD spectropolarimeter, equipped with a model 6001 circular dichroism accessory. The spectropolarimeter was continuously purged with nitrogen gas during the recording of a spectrum. Solutions, however, were not degassed. Silica cells with path lengths of 1.0 and 10.0 mm were used. Protein concentrations ranged from 0.05-0.15%. ORD data are expressed as specific activity $[\alpha]$.

Enzyme fractionation: Ammonium sulfate (Baker or Fisher Reagent Grade) was used to study the fractionation of enzyme activity and protein by varying the ionic strength. Increasing amounts of salt were added to tubes containing 5.0 ml of crude enzyme mixture. After sitting for one hour at 4°C, the precipitates were collected by centrifugation at 10,000 rpm for 15 minutes and redissolved in 5.0 ml of 0.02 M Tris-HCl buffer, pH 8.0. The enzyme activity and protein concentration (optical density at 280 mμ) were determined and plotted with respect to percent saturation of ammonium sulfate used.

Acetone (Fisher Reagent Grade) was used in a similar manner to that described above for ammonium sulfate. Varying amounts of cold acetone were added to 5.0 ml of an enzyme solution at 4°C. Results of these tests indicated that acetone fractionation might prove useful. To further test the usefulness, larger samples of enzyme ranging from 100-400 ml were used. Cold acetone was added up to a concentration of 45%. After sitting at 4°C for 1-2 hours the precipitates were removed by centrifugation, and redissolved in 0.02 M Tris-HCl, pH 8.0. Cold acetone was added to the supernatant up to a final concentration of 67%. This solution was left at 4°C overnight. The following morning, the precipitate was collected and redissolved in 0.02 M Tris-HCl buffer, pH 8.0, and assayed.

Column Chromatography: Ion-exchange chromatography was performed with diethylaminoethyl (DEAE)-cellulose and DEAE-Sephadex. The anionic resins were prepared by adding distilled water to the dried material until completely wetted, or, in the case of the DEAE-Sephadex, until completely swollen. The water was removed by filtration on a Buchner funnel and the resins washed alternatively with:

1) 0.5 N HCl
2) distilled water, until approximately pH 7.0
3) 0.5 N NaOH
4) distilled water, until approximately pH 7.0
5) starting buffer, until the pH of the filtrate was the same as that of the buffer

After the above washings, the DEAE-Sephadex was packed by gravity into a 1.2 x 70 cm glass column (bed volume of 50-60 cm). Starting buffer was allowed to run through the column until the bed height had stabilized. One to two ml of a 50 mg/ml solution of enzymes was added with a Pasteur pipette to the top of the column. Elution of the enzymes was then started using the buffer systems described below.

An alternative method for adding enzymes was introduced for DEAE-cellulose columns. A measured amount of resin was added to the crude enzyme solution. Following a 20 minute period of equilibration at 4°C, the DEAE-cellulose was washed with the starting buffer until no more protein appeared in the filtrate. The enzyme-laden resin was then packed by gravity into a 25 x 75 cm glass column. The following elution systems were used as linear gradients with both resins:

A: Tris-HCl buffer: 0.02 M, pH 9.5
0.05 M, NaCl

Tris-HCl buffer: 0.02 M, pH 8.4
0.3 M, NaCl

B: Tris-HCl buffer: 0.02 M, pH 8.4
0.05 M, NaCl

Tris-HCl buffer: 0.02 M, pH 8.4
0.3 M, NaCl

The column was finally washed with Tris-HCl buffer: 0.02 M, pH 8.4/0.75 M NaCl in order to remove any remaining protein, and then the resin was regenerated as described above.

Gel-filtration experiments were conducted on polydextran columns with: Sephadex (fine) G-10, G-75, G-100, G-200 and Sephadex (superfine) G-100 at 4°C. The gels were allowed to swell for one week in the elution buffer (0.02 M Tris-HCl buffer at pH 8.0) and were then packed by gravity into glass columns (1.2-2.5 x 75-110 cm). Buffer was allowed to run through the column until the bed height stabilized. Volumes of 45 drops (3 ml) and 75 drops (5 ml) were collected per tube with a Buchler Fractomat or Fractomette fraction collector (Buchler Instruments) at a rate of 6-12 drops per minute. The location of the proteins was accomplished by determining the optical density for each fraction at 280 mu. When peptides were chromatographed on Sephadex G-10 columns with distilled water or acetic acid:pyridine: water (2:5:450) mixture, only 30 drops per tube were collected with a Buchler Sectional fraction collector, at room

temperature (22-23°C).

The several fractions that resulted from column chromatographic separation were concentrated by precipitation with 70% ammonium sulfate, lyophilization or ultrafiltration (Diaflo Apparatus, Amicon Corp.,Lexington, Mass.).

Electrophoresis: Cellulose acetate strip electrophoresis was carried out with a Gelman (Ann Arbor, Mich.) electrophoresis apparatus on cellulose acetate strips. The procedure was that described in the Gelman instruction manual. A Tris-HCl buffer (0.2 M) was used at pH's from 6 to 9. The ionic strength was kept constant by adding the neccessary amount of NaCl. Electrophoretic runs were performed with 150-225 volts at 4°C and at room temperature for periods up to 2 hours. Following electrophoresis, the strips were cut longitudinally. One-half of the strips was stained for protein by immersing it in a Ponceau S dye (National Aniline Division, Allied Chemical Corp., New York, N.Y.) solution (0.4 g per 100 ml of 5% TCA). The other half of the strip was used to determine the location of the proteinases. The endopeptidases were located by laying the strip on a chromoprotein medium and incubating at 28°C (65). The presence of proteinase activity was indicated by the disappearance of the purple color imparted to the medium by the phycocyanin. Endopeptidase activity was verified by cutting separate unstained electrophoretic

strips into 1/8 inch wide pieces parallel to the line of application and eluting the enzymes with 20 ml of distilled water. Each sample was then assayed in the usual manner for both endo- and aminopeptidase activity. The aminopeptidase, which does not hydrolyze the chromoproteins, was detected by cutting sections from an unstained strip and eluting the enzyme as described above. Records of the chromoprotein digestion and protein stained strips were made with a Joyce-Loebl recording densitometer using appropriate filters. In some cases, where it was not possible to obtain a densitometer tracing of the chromoprotein digestion, an accurate drawing was made.

Disc gel electrophoresis in polyacrylamide gels was accomplished with a Buchler Instruments Polyanalyst by the method of Ornstein and Davis (66) with the staining modification of Chrambach, et al. (67): Coomassie Brilliant Blue R (Colab Laboratories Inc., Chicago Heights, Ill.) was used as the protein stain after a fixing period in 12.5% TCA of 1 hour. Proteinases were identified by the same procedure as described above for cellulose acetate strips except that the gels were sliced longitudinally with a Canal Industrial Corp. gel slicer.

High voltage electrophoresis was accomplished with a Savant Instrument, Inc. flat plate electrophoresis apparatus (Model FP-22A, Hicksville, N.Y.) on Whatman #3MM paper. For peptide separations, conditions of 2500

volts for 45 minutes were used with a pyridine:acetic acid:water (1:10:289) buffer (pH 3.7).

Peptide maps were prepared of some of the enzyme-hydrolyzed protein substrates. The procedure followed was similar to the one described by Matsubara, et al. (10). Electrophoresis of a 50 µl sample was done first under the conditions described above at pH 3.7. The paper was then dried, rotated 90 degrees, and descending chromatography performed using a butanol:pyridine:acetic acid:water (15:10:3:12) system. Samples were run in duplicate. In order to locate the peptides, one sheet was sprayed with a 0.2% ninhydrin solution in acetone (Mann Spraytec Reagent). From the second sheet, the located peptides were cut out and eluted with distilled water.

Amino acid analysis: The amino acids from acidic peptide hydrolyzates were identified by means of unidimensional thin layer chromatography on silica gel G plates with a solvent system of butanol:acetic acid:water (3:1:1). For quantitization, the spots were eluted with an ethanol:water (3:1) mixture. Their optical densities were determined at 570 mµ.

Amino acid analysis of the aminopeptidase protein was accomplished with a Beckman Model 120C Automatic Amino Acid Analyzer. The short column consisted of a sulfonated styrene copolymer (Custom Research Resin Type PA-35) while the long column also consisted of a sulfonated

styrene copolymer (Custom Research Resin Type PA-28). Elution of the sample from the short column was with a citrate buffer(pH 5.18),0.35 N in sodium while the long column was eluted step-wise: pH 3.28 citrate buffer (0.2 N in sodium) and pH 4.25 citrate buffer (0.2 N in sodium). Temperature of the run was at 51°C. The protein samples were hydrolyzed for 40 hours with 6 N HCl or 4 N $Ba(OH)_2$ under vacuum at 105°C. Barium was removed by precipitation of barium carbonate.

End-group analyses were performed on the enzyme hydrolyzed proteins and the purified aminopeptidase. The dinitrophenylation (DNP) method of Sanger (68) was used to determine the amino terminal amino acids. Isolation and characterization of the DNP derivatives was done by the Fraenkel-Conrat (69) method of ether extraction and chromatography on silica gel G thin layer plates. The following solvent systems were used for the ether soluble derivatives: the organic phase of a toluene:pyridine: ethylene chlorohydrin:0.8 N ammomium hydroxide (10:3:6:6) mixture and, in the second dimension, chloroform:benzyl alcohol:acetic acid (7:3:0.3). The water soluble, ether insoluble, DNP-amino acids were chromatographed on silica gel G thin layer plates with n-propanol:34% ammonia (7:3). The results were quantitized by scraping out the spots, eluting with 1% $NaHCO_3$ and measuring the absorbance at 360 mμ.

Effects of activators and inhibitors were studied by preincubating several concentrations of the activator or inhibitor under investigation (1.0 ml) with the enzyme (1.0 ml) for 20 minutes. There was always a large excess of activator or inhibitor over the protein concentration. Following incubation, the mixture was added to substrate and enzyme activity determined. Reversibility of inactivation was tested by dialyzing the enzyme plus inhibitor solution against two changes of 5 liter quantities of 0.02 M Tris-buffer (pH 8.0) at 4°C. The effect of diisopropylphosphofluoridate (DFP) was followed by the procedure of Mounter and Shipley (70). Trypsin (1 mg/ml), known to be inhibited by DFP, was used as a positive control.

Heat treatment of enzymes: A glass beaker containing the crude enzyme solution (generally in 0.02 M Tris-HCl buffer at pH 8.0) was heated with occasional stirring in a water bath set at 65°C until the desired temperature was reached. The beaker was then transferred to a water bath at the desired temperature (60°C) and heating was continued for one hour, after which the beaker was removed and cooled. The procedure was repeated until all of the endopeptidase was destroyed.

Heat stability studies were conducted at several temperatures in a similar manner. Enzyme solutions in test tubes were heated and samples were removed at varying in-

tervals for enzyme activity measurements. Enzyme activity measurements were made at 37°C. The resultant activity was compared to a control which was the activity of the enzyme at the time it was immersed in the 60°C water bath.

Temperature optimum measurements were made by pre-incubating enzyme and the required amount of substrate for 5 minutes at the desired temperature. The enzyme was then added to the substrate and assayed at the desired temperature as described earlier. The measurement of released B-naphthylamine after the aminopeptidase assay was conducted at 37°C.

Kinetic measurements were made in several ways. For all kinetic studies the enzymes were diluted with buffer to a point where first order kinetics (K_1) was observed. This was determined by noting the relationship between activity and protein concentration. Where a linear relationship was observed, first order kinetics was assumed to be valid.

The determination of K_M and V_{max} under various conditions was done graphically by the method of Lineweaver and Burk (71) as described by Dixon and Webb (72).

An overall K_1 was calculated from the percent hydrolysis data (73):

$K_1 = [1/t][\log 100/(100-H)]$
t = time in minutes
H = percent hydrolysis

Molecular weight determination was accomplished by

gel-filtration on a Sephadex G-200 column (1.5 x 110 cm) by the method first described by J. R. Whitaker (74). The results were compared to those obtained by sedimentation analysis. The polydextran beads were allowed to swell for one week in the elution buffer (0.02 M Tris-HCl, pH 8.0) at 4°C. The columns were then packed in a similar manner as described above. The void volume was determined by measuring the elution volume of a Blue Dextran 2000 (Pharmacia Fine Chemicals) solution (1 mg/ml). The following standard proteins whose molecular weights are in parenthesis were used: hemoglobin (68,000), ovalbumin (45,000), pepsin (33,000), trypsin (23,800), chymotrypsin (22,500), ribonuclease (13,600) and cytochrome C (13,000).

Sedimentation analyses were performed on a Spinco Model E analytical ultracentrifuge equipped with Schlieren optics at 20°C. Measurements were performed in standard and wedged (1° positive) synthetic boundary cells in an AN-D type rotor at rotational speeds of 60,000 rpm. Boundary positions on the photographic plates were measured on a microcomparator and sedimentation constants were calculated from slopes of a plot of log X vs. time, where X was the radial boundary position (75). An assumed partial specific volume of 0.735 ml/mg and diffusion constant of 7.8×10^{-7} $cm^2 sec^{-1}$ were used in the calculations of molecular weight.

Atomic absorption studies were carried out with one of two Jarrell-Ash atomic absorption units (Model No. 82270

and 82720). The conditions employed for each metal were those described in the Jarrell Ash Methods Manual. Standards were prepared from Fisher Certified Standards (10,000 ppm of each metal) or from the metal chlorides dissolved in 0.1 N HCl.

PART I

A New Marine *Pseudomonas* and the Proteolytic Enzymes it Produces

The marine bacterium, which has been under investigation for the past few years, was isolated from a water sample taken with a Nansen bottle at a depth of 1600 meters at Station S in the Sargasso Sea (29). The purity of the culture was ascertained by repeated isolations of the organism from isolated colonies that developed on the nutrient agar-sea water medium. Upon initial isolation it was designated B-207.

The bacterium was of interest because of its predominance in the water sample, the very mucoid consistency of its growth and its relatively high proteolytic activity.

The results of experiments describing this bacterium, its growth and proteolytic enzyme production, as well as the characteristics of the crude enzyme mixture will be described in this section. All characteristics, that are being described, have remained stable since the isolation and initial investigation in 1964 (29).

EXPERIMENTAL RESULTS

Characteristics of the Organism: The bacterium produced abundant filiform growth with viscid consistancy after 48 hours at 22-24°C on peptone-sea water agar slants. The organism has also been grown on other bacteriological media (nutrient agar-sea water and peptone glucose yeast extract-sea water agar) with similar results. Following initial isolation no growth was observed when distilled water was

substituted for sea water.

This bacterium was found to be a gram negative rod, 0.8 to 1.0 micron in width and 1.2 to 1.5 microns in length, as measured by calibrated electron micrographs (Fig.1). It was motile by means of a single polar flagellum, measuring approximately 150 Å in width and 10 to 15 microns in length. a large capsule was observed (Fig. 2) particularly when glucose or sucrose (2%) was added to the peptone sea water medium. No endospore formation was observed and the

Fig. 1: Electron micrograph of B-207. Pt shadowed.

Fig. 2: B-207 grown in peptone,yeast extract & sea water medium to which 2% sucrose had been added. Stained with nigrosin (0.5%) and with a light shadow of Pt-Pd.

bacterium was not acid fast.

Individual organisms produced colonies which were circular and highly convex. The young colonies (grey-white) became pigmented (yellow-brown) as they grew older (48-72 hours). No diffusible pigments were produced when grown on any of King's media (42).

Some of the biochemical characteristics of the organism are listed in Table I. Those sugars which gave positive results in the media of Hugh and Leifson (76) were all

TABLE I

Biochemical Characteristics of Marine Bacterium B-207

Nutritional and Descriptive Tests

			Reaction*
Utilization of Koser's Citrate			+
Casein Hydrolysis			+
Catalase			+
Gelatin Liquefaction			+
Hydrogen Sulfide Production			-
Indole Production			-
Kovac's Oxidase			+
Lipase			+
Litmus Milk:	Growth		+
	Reaction:	acid	-
		alkaline	+
Methyl Red			-
Nitrates Reduced			+
Nitrites Reduced			-
Pigment Production:	Pyocyanine		-
	Fluoresein		-

*Reaction: + = positive or present
- = negative or absent

Utilization of Carbohydrates

Substrate	Reaction*	Substrate	Reaction*
Arabinose	+	Lactose	-
Xylose	-	Maltose	+
Glucose	±	Sucrose	+
Mannose	+	Dextrin	+
Galactose	-	Cellobiose	-
Fructose	+	Raffinose	-
Sorbitol	-	Starch	-
Inositol	-		

*Reaction: + = Acid Reaction
- = No Reaction or Basic Reaction

utilized aerobically with the production of acid. Arabinose and glucose were sometimes utilized aerobically with the production of acid, but much more slowly and not to the same extent as other sugars.

The results of the antibiotic sensitivity studies (Table II) showed that B-207 was most sensitive to

TABLE II: Antibiotic sensitivity

Antibiotic	Concentration	48 hours growth*
Chloromycetin	5 mcg	++
	30 mcg	+++++
Erythromycin	2 mcg	++
	15 mcg	++++
Kanamycin	5 mcg	-
	30 mcg	-
Neomycin	5 mcg	-
	30 mcg	++
Novobiocin	5 mcg	+
	30 mcg	+
Penicillin	2 units	-
	10 units	-
Streptomycin	2 mcg	-
	10 mcg	-
Aureomycin	5 mcg	-
	30 mcg	-

* each + represents 3 mm of inhibitory zone,
  - indicates no inhibition

chloromycetin and erythromycin. Several colonies were isolated from the chloromycetin and erythromycin inhibitory zones and were shown to be resistant strains. In addition it was noted that early during incubation, enhanced growth was observed around the penicillin disks. Penicillin G (10 units/ml) was added to a 1% N-Z Amine type HD medium in 3% RMM. The bacteria were grown for 18 hours at 23°C. When

compared to a similar medium without added penicillin, approximately 30% of the turbidity, 4% of the endopeptidase activity and 70% of the aminopeptidase activity was observed. In a separate experiment it was found that approximately 15-20% of the endopeptidase activity was inhibited by 10 units/ml of penicillin G. Under the light microscope the penicillin-treated cells appeared elongated as though they were unable to divide.

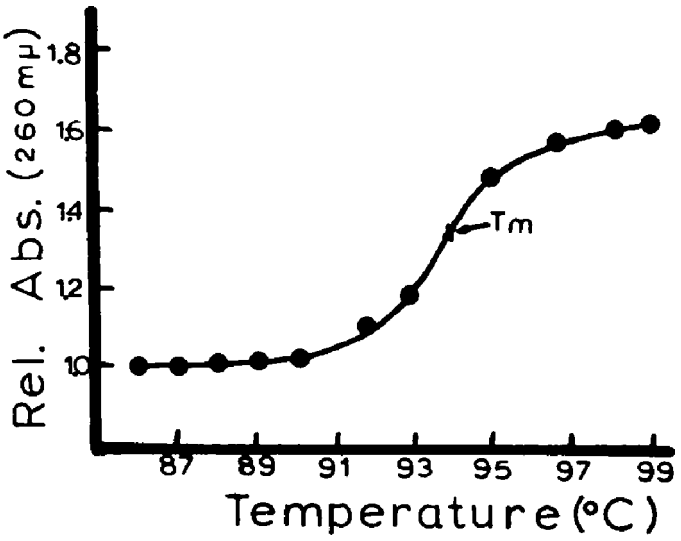


Fig. 3: Determination of melting temperature of DNA from B-207

The melting temperature (Tm) of DNA from B-207 was determined to be 94.2°C (Fig. 3) from which a mole percent (G-C) of 60.6 was calculated.

Lytic susceptibility: The data depicted in Table III revealed that the B-207 organism was osmotically fragile and could be lysed with distilled water or low ionic strength solutions. These results were used to advantage when preparing cytoplasmic materials. The bacteria were suspended

TABLE III

Osmotic Fragility of B-207*

Solution		Residual Turbidity
RMM	4%	100%
$MgCl_2$	0.5 M	92
	0.05M	75
KCl	0.5 M	86
	0.05M	67
NaCl	0.5 M	90
	0.05M	74
Distilled water		65

* 15 minute incubation at 24°C.

in distilled water prior to sonication or lysozyme treatment; thus reducing the time for complete lysis to occur.

Optimal conditions for production of proteolytic enzymes: Merkel and Birchbickler (77) indicated that the production of proteases by bacterium B-207 varied with the cultural conditions. Their carbon and nitrogen sources were hydrolyzates of various proteins with differing concentrations of synthetic sea water (RMM). Some of their results upon which further studies were based were as follows:

(1) Synthetic sea water alone was not enough to support growth and protease production.

(2) A large increase in the pH of the medium from initial conditions was not observed during growth.

(3) Reduction in the concentration of synthetic sea water from 100% (4% RMM) to 75% (3% RMM) did not change the growth and protease production.

(4) Unhydrolyzed protein did not support growth in the absence of some amino acids.

(5) Non-proteinaceous sources of carbon and nitrogen supported growth but only with reduced yields of proteases.

The data in Table IV-A show that the best three hydrolyzed protein substrates for maximum protease production (measured as enzyme activity) were the hydrolyzates of casein, Proflo and beef pancreas. However, the crude material that was precipitated (70% ammonium sulfate) after growth in hydrolyzed casein was white, while that in Proflo and autolyzed panceas was brown to black, indicating large quantitites of unwanted pigment. It was, therefore, decided to use the hydrolyzed casein rather than the Proflo or autolyzed pancreas for further growth studies.

Various commercial casein hydrolyzates were tested for growth and enzyme production (Table IV). Pancreatin hydrolyzed casein and the enzymatic N-Z Amine type HD of Sheffield Chemical Co. gave excellent results. Amino nitrogen and protein content of the Pancreatin hydrolyzed casein varied from batch to batch. On the other hand, the enzymatic hydrolyzate type HD was a commercial preparation of assumed uniform composition. The type HD medium was completely soluble and always gave a clear solution while the Pancreatin hydrolyzed casein was cloudy with much

TABLE IV: Nutritional study of protease production

A) 1% of each nutrient in 3% RMM adjusted to pH 7.3 without buffer in flasks with baffles. (18 hour samples)

medium	pH	turbidity	enzyme activity* endopep.	aminopep.
Casein Hydrolyzates:				
Pancreatin	8.40	----	0.625	0.220
Pancreatic (type A)	8.25	2.68	0.032	0.130
Enzymatic (type YT)	8.05	2.15	0.128	0.140
Enzymatic (type HD)	7.95	2.55	0.660	0.156
Tryptic (peptidase)	7.75	1.28	0.072	0.076
Proflo Hydrolyzate	8.45	----	0.91	-----

B) 0.5% of each nutrient in 3% RMM adjusted to pH 7.3 without buffer in flasks with baffles. (18 hour samples)

medium	pH	turbidity	enzyme activity* endopep.	aminopep.
Casein Hydrolyzates:				
Pancreatin	8.50	----	0.56	0.150
Enzymatic (type HD)	8.15	2.97	0.345	0.150
Edamine (type S)	8.30	----	0.185	0.010
(type T)	8.40	----	0.217	0.050
Soy Peptone	8.30	----	0.093	0.045
Ferm Amine	8.50	----	0.036	0.060
Proflo Hydrolyzate	8.40	----	0.110	0.010
Pancreatic Autolyzate	8.50	----	0.53	0.170

* units per ml, as described in Experimental Procedures

suspended matter. It was therefore decided to use the commercially prepared enzymatic casein hydrolyzate (N-Z Amine type HD) for further studies. Table V and figures 4-10 smmarize the results of those experiments.

TABLE V: Protease production with N-Z Amine type HD as carbon and nitrogen source

18 hour incubation time at a shaker speed of 17

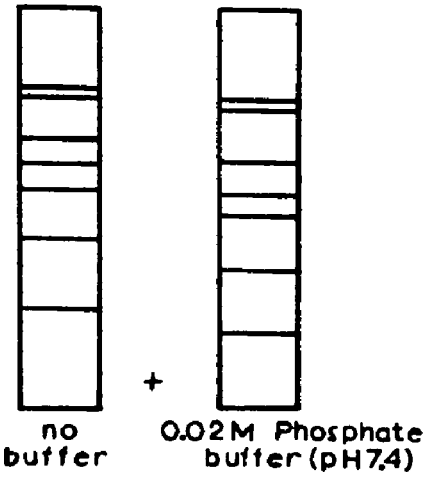
medium	concentration total nutrients (%)	Growth final pH	Growth turbidity	Enzyme Activity** endopep.	Enzyme Activity** aminopep.
A) N-Z Amine type HD	0.25	8.00	1.30	0.93	0.144
	0.50	8.10	2.48	0.88	0.255
	0.75	8,15	3.60	0.87	0.188
	1.00	8.35	4.75	0.94	0.175
	1.50	8.40	5.05	0.90	0.180
	2.00	8.45	5.35	0.90	0.188
B) N-Z Amine type HD no buffer	1.00	8.00	6.35	0.795	0.146
N-Z Amine type HD 0.02 M phosphate buffer, pH 7.4	1.00	7.55	5.50	0.440	0.100
C) N-Z-Amine type HD	1.00	8.25	5.30	1.75	0.260
N-Z Amine type HD with glucose	1.50	7.40	6.05	1.27	0.200
	2.00	7.30	5.95	1.14	0.165
	2.50	7.25	6.10	1.22	0.240
N-Z Amine type HD with glycerol	1.50	7.90	5.30	1.41	0.142
	2.00	7.90	4.85	1.74	0.182
	2.50	7.90	4.75	1.65	0.228

* in 3% RMM; pH adjusted to 7.3-7.4 prior to autoclaving
** units per ml, as described in text in the Experimental Procedure

Comparison of various concentrations of N-Z Amine type HD (Table V-A) showed that a 1% solution in 3% RMM resulted in the greatest endopeptidase activity by B-207, while 0.5% solution, also in 3% RMM, produced the largest aminopeptidase activity in 18 hours. Since it was felt that the overall yield of enzyme would be greatest with a 1% solution, it was decided to continue growth studies with a 1% solution of protein hydrolyzate in 3% RMM.

A buffer was not required as the pH never increased more than 1.0 pH unit above the initial pH of 7.4. The addition of 0.02 M phosphate buffer (pH 7.4), as shown in Table V-B, inhibited growth of B-207 with considerable reduction in proteolytic activity. Electrophoretic studies with polyacrylamide gels (Fig. 4) showed that the same number of proteins were observed regardless of the final pH of the growth medium.

Fig. 4: Tracings of Coomassie Brilliant Blue stained proteins separated by gel electrophoresis when B-207 was grown under different conditions.



The relationships between proteolytic enzyme production and growth of a B-207 culture is shown in Fig. 5. Maximal growth is reached after approximately 15 hours at 23°C. The production of endopeptidase closely followed the growth. The aminopeptidase on the other hand was detected in the medium after the cells began to lyse. In a similar study (Fig. 6) washed log-phase bacteria were sonically lysed, the debris removed by centrifugation at 10,000 rpm for 10 minutes and the intracellular protease activity measured. The results showed that the aminopeptidase production directly followed the growth curve while the endopeptidase production remained low.

The temperature optimum for growth and enzyme production was determined to be 23°C (Fig. 7). There was little growth above 30 and below 10°C. The amount of growth at any particular temperature determined the amount of enzyme production.

By changing the speed of the shaking machine, the amount of aeration received by a growing B-207 culture was varied. In the study depicted in Fig. 8, the amount of aeration was increased from the conditions found in a stationary flask to a strongly aerobic environment. A strong aerobic requirement was indicated by the resultant increase in growth and enzyme production.

When the initial pH of the medium was changed from

Fig. 5: Growth and extracellular protease production

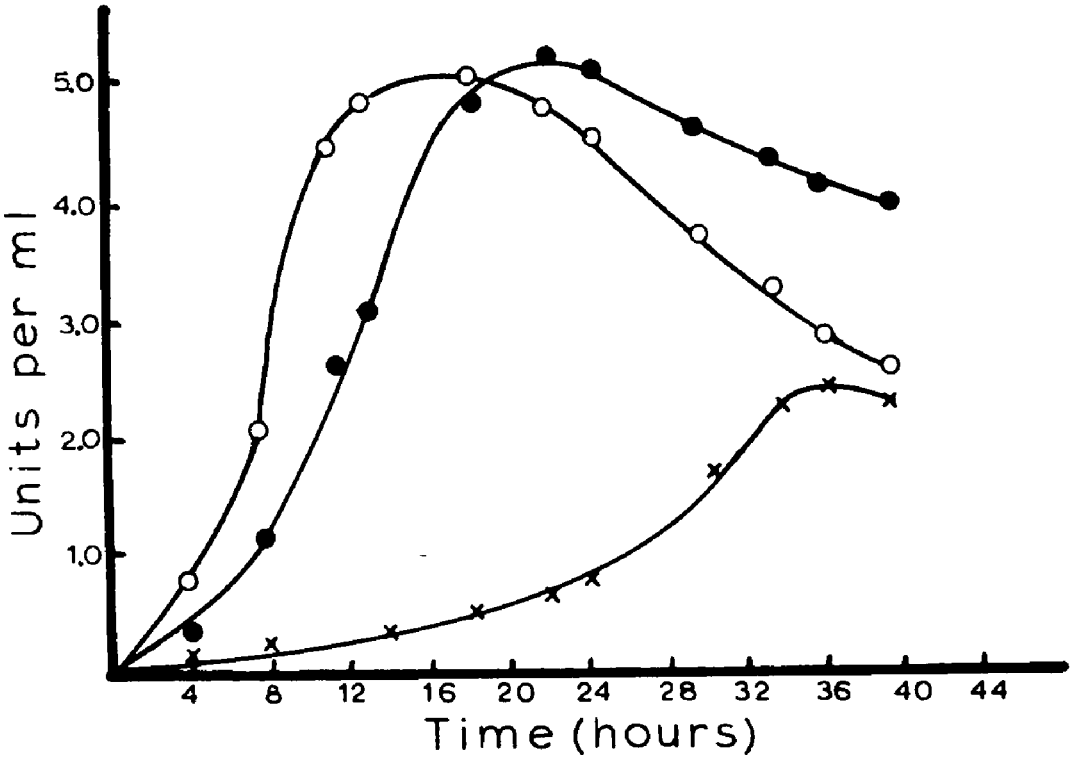
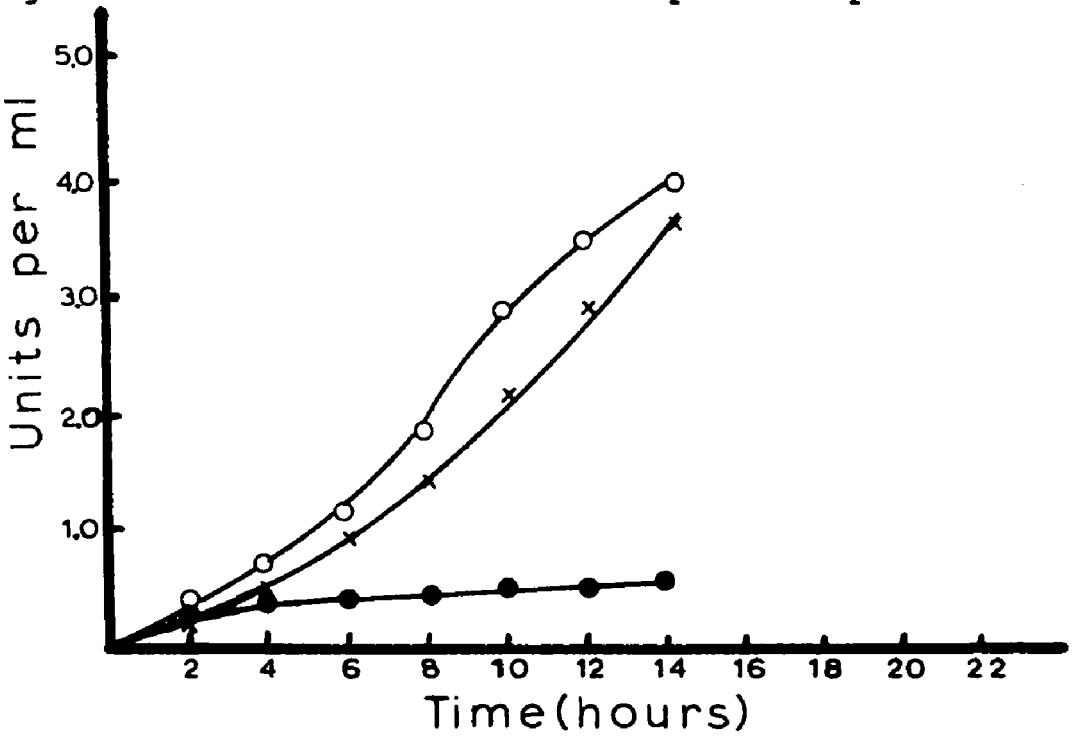


Fig. 6: Growth and intracellular protease production



turbidity endopep. aminopep.

Fig. 7: Effect of temperature on growth and protease production

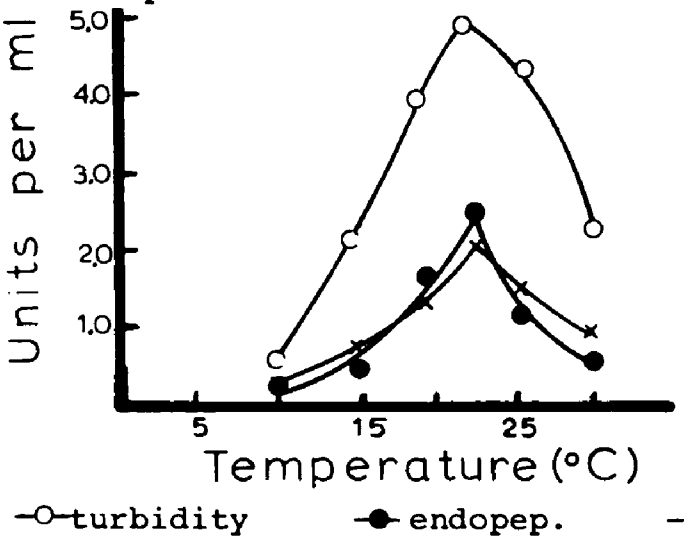


Fig. 8: Effect of aeration on growth and protease production

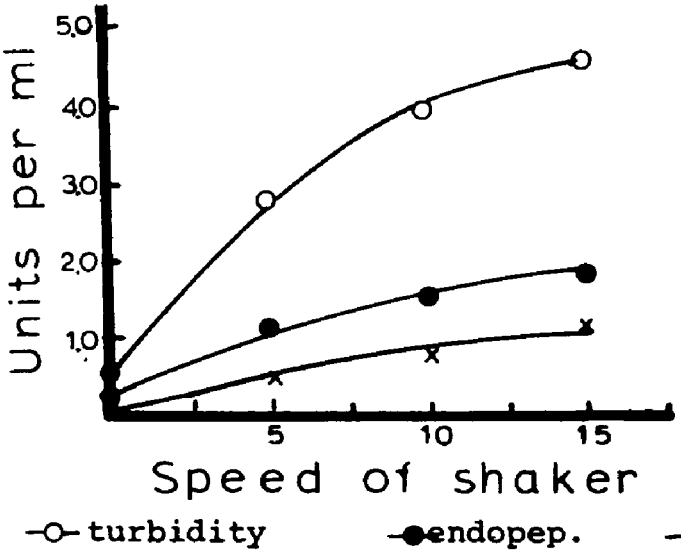


Fig. 9: Effect of initial pH of medium on growth and protease production

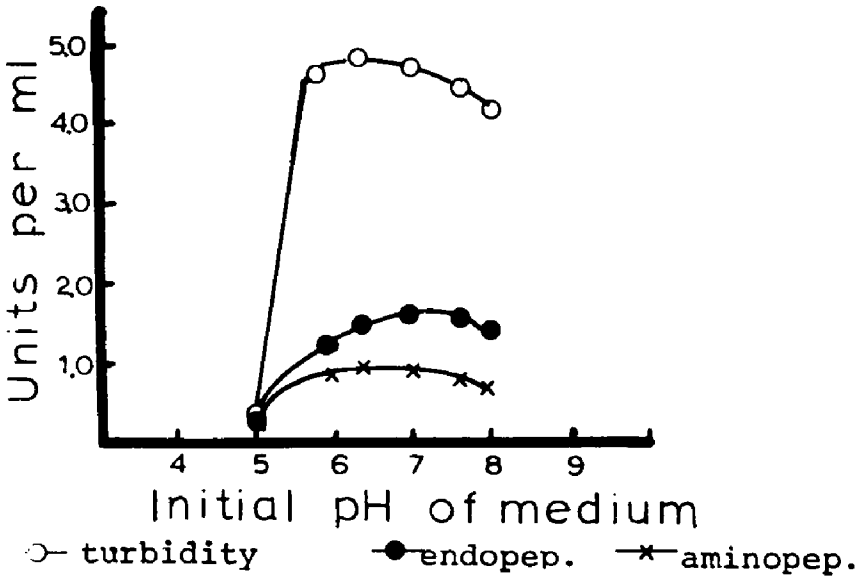
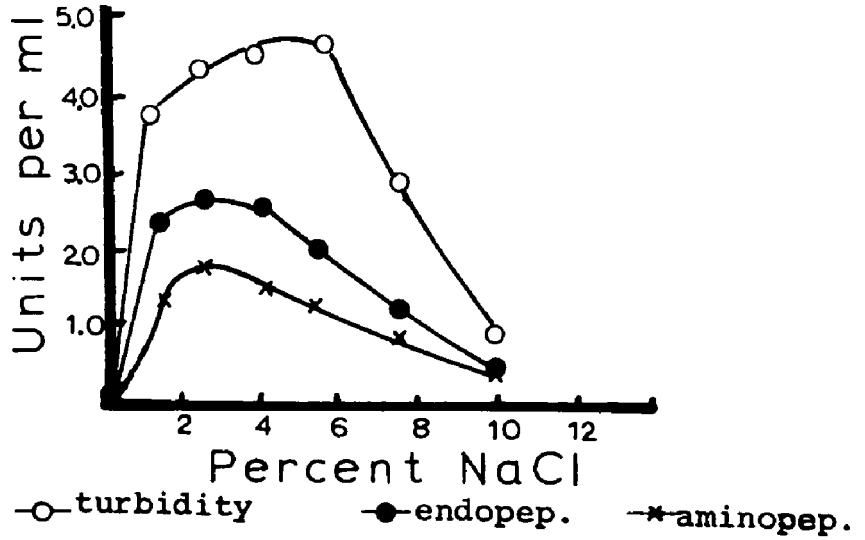


Fig. 10: Effect of sodium chloride on growth and protease production



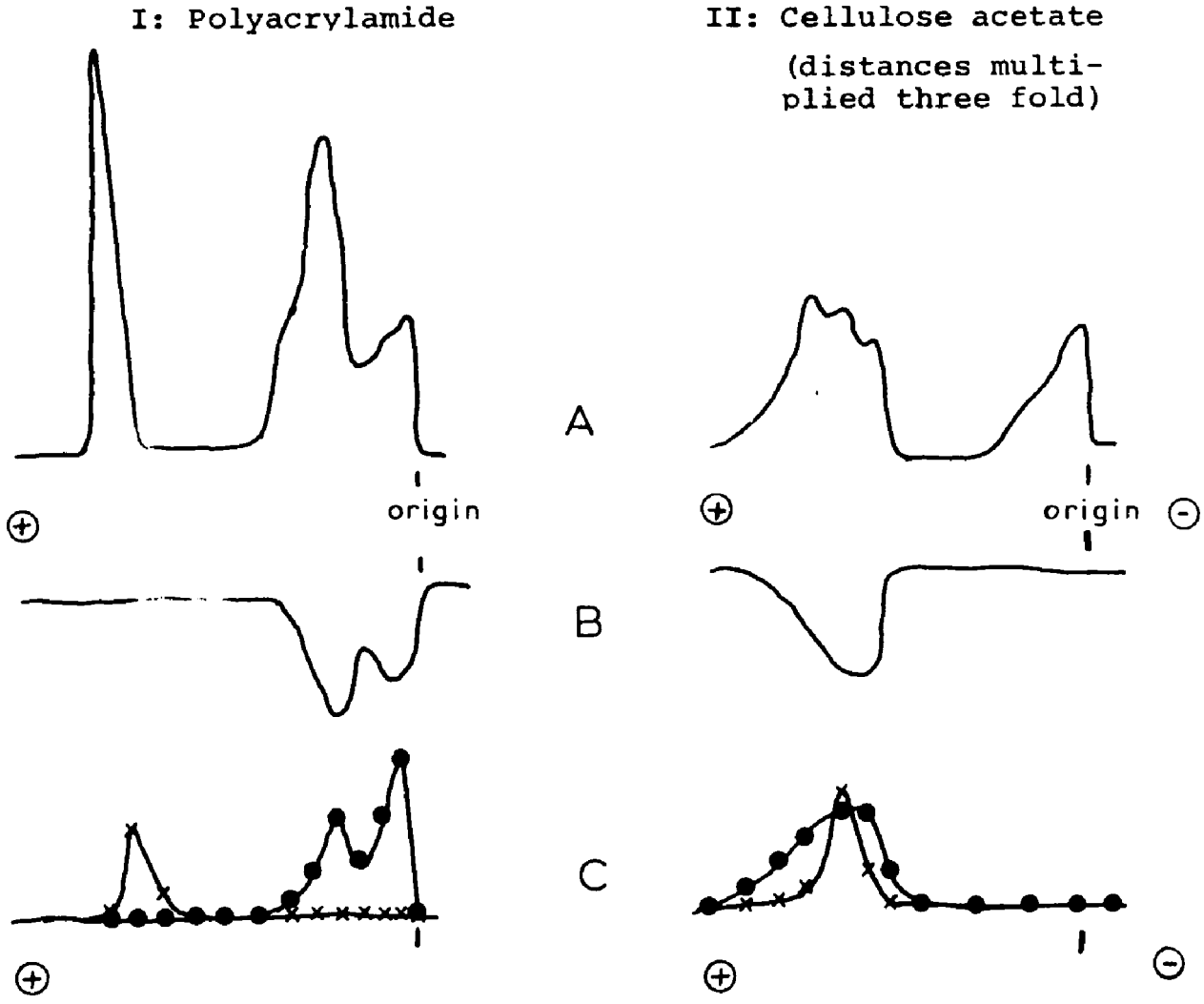
approximately pH 6 through pH 8, no dramatic change was observed in growth or enzyme production (Fig. 9). However, below pH 6, a sharp decline in growth and enzyme production was observed.

Upon initial isolation of bacterium B-207, a requirement for sea water was demonstrated (29). A number of bacteria have been described as halophilic on the basis of growth in sodium chloride (21). The influence of salt on the growth and enzyme production of B-207 was followed by varying the sodium chloride concentration in nutrient broths prepared with distilled water (Fig. 10). The low protease activity at the higher concentrations of salt may have been the result of the small amount of growth or the result of enzyme inhibition.

When B-207 was grown in a medium composed of 3% RMM to which 0.05% of each of the 20 common amino acids was added, a small amount of growth was observed (0.32 turbidity units). Both endopeptidase and aminopeptidase activities were noted. The removal of specific groups of amino acids from the medium (i.e., acidic or basic) did not reveal any requirement for one type of amino acid.

Number and type of proteolytic enzymes produced: Electrophoresis on polyacrylamide gels showed that the crude enzyme mixture contained a large number of proteins (Fig.11). From studies with chromoprotein digestion and elution from gel sections only three proteolytic bands were observed:

Fig. 11: Electrophoresis of crude enzyme mixture from B-207



A) protein stain; B) chromoprotein digestion; C)activity of eluted proteins

—●— endopeptidase —×— aminopeptidase

two endopeptidases and one aminopeptidase. Electrophoresis on cellulose acetate strips yielded three enzymatically active protein bands. On numerous occasions erratic patterns were observed with electrophoesis of crude enzyme on cellulose acetate strips while no problem was encountered with the

polyacrylamide gels. Removal of a high molecular weight fraction consisting primarily of polysaccharide and protein by gel-filtration resulted in more uniform patterns.

Analysis of the isolated capsular material revealed that it contained several polysaccharides and protein (25% by weight). The contents of the polysaccharides and the identities of the carbohydrates are listed: neutral sugar --galactose (47%), uronic acid--glucuronic acid (12%), 6-deoxy sugar--rhamnose (10%) and amino sugar--glucosamine (15%). The totals add up to more than 100% since there is a contribution from other sugars to the neutral sugar color.

The action of the crude enzyme mixture on a number of proteins was examined (Table VI). The results demonstrated

TABLE VI: Enzymatic activity of B-207 crude enzyme mixture

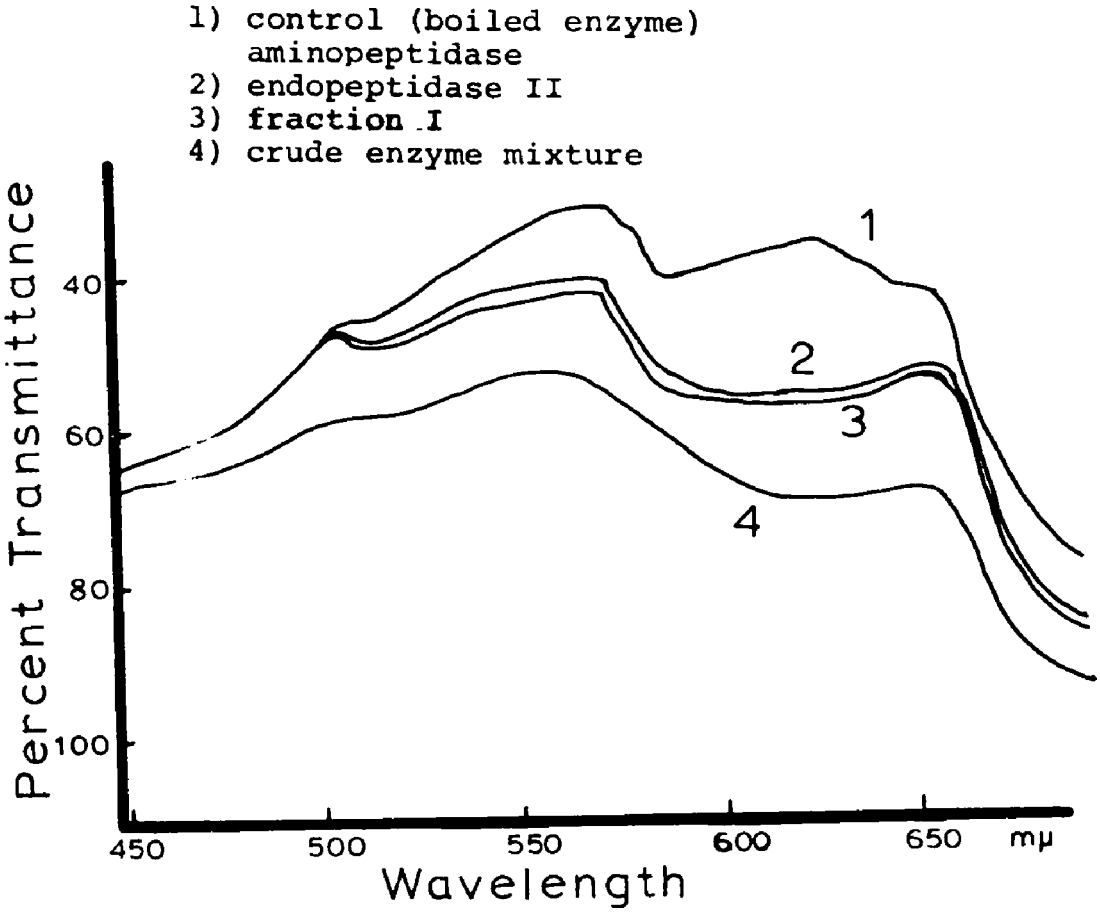
substrate	percent hydrolyzed*	type of activity
RNase	8.0	endopeptidase
Insulin	7.0	endopeptidase
Insulin (oxidized)	12.0	endopeptidase
Casein	7.0	endopeptidase
Hemoglobin	6.0	endopeptidase
Hemoglobin (urea-denatured)	13.0	endopeptidase
Chromoprotein	5.0	endopeptidase
Collagen (calf-skin)	0.0	---
Collagen (tendon)	0.0	---
Elastin	0.0	---
CBZ-glycyl-L-phenylalanine	0.0	---
L-leucyl-B-naphthylamide	25.0	aminopeptidase amidase
BAEE	Trace**	esterase
TAME	Trace	esterase
B-naphthol laurate	Trace	lipase esterase

* 1 hour at 37°C. ** refers to less than 1% hydrol.

that all of the proteins tested were partially hydrolyzed. The denatured proteins were preferred to the native ones. Hydrolysis of L-leucyl-B-naphthylamide revealed the presence of an aminopeptidase while the absence of hydrolysis of CBZ-glycyl-L-phenylalanine indicated that the mixture did not contain a carboxypeptidase. No specific collagenase or elastase activity was observed but trace amounts of esterase activity were noted. Further details of the enzymic attack on the chromoprotein mixture are shown in Fig. 12. The visible spectrum shows the maxima at 497 and 565 mμ for phycoerythrin, 553 and 625 mμ for phycocyanin and 654 mμ for allophycocyanin. The changes in the spectrum are noted for the crude enzyme mixture and several of the enzyme fractions to be discussed below. The aminopeptidase had no activity toward any of the chromoproteins while the endopeptidases and the crude enzyme mixture caused a decrease in the color of all three chromoproteins. However, phycoerythrin was more stable to hydrolysis than the phycocyanin or allophycocyanin.

Characteristics of the mixture of proteolytic enzymes from B-207: In order to learn more about the group of proteolytic enzymes produced by B-207 the chemical and physical characteristics of the enzyme mixture were studied. The results are plotted in Figs. 13-16 and Tables VII and VIII.

Fig. 12: Chromoprotein digestion by B-207 enzymes (1 hour hydrolysis at 37°C)



Incubation of the enzymes at 3-5°C for 24 hours at various pH's in 0.02 M phosphate and Tris-HCl buffers followed by readjustment of the pH to 7.0,resulted in the greatest stability for both types of enzymes being observed in the range 6.4 to 8.4 (Fig. 13). The pH optimum for the endopeptidase-catalyzed hydrolysis of hemoglobin substrate was

Fig. 13: pH stability of protease mixture

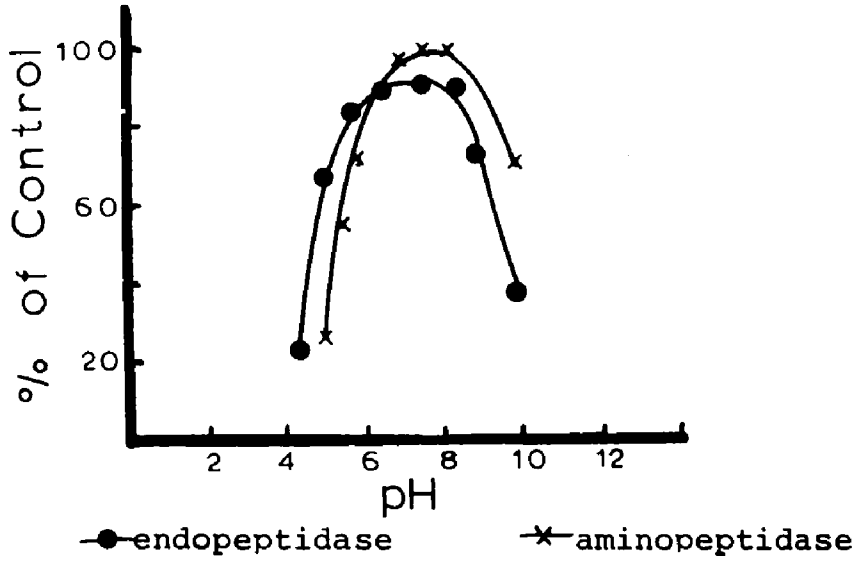


Fig. 14: Effect of pH on protease activity of the crude enzyme mixture

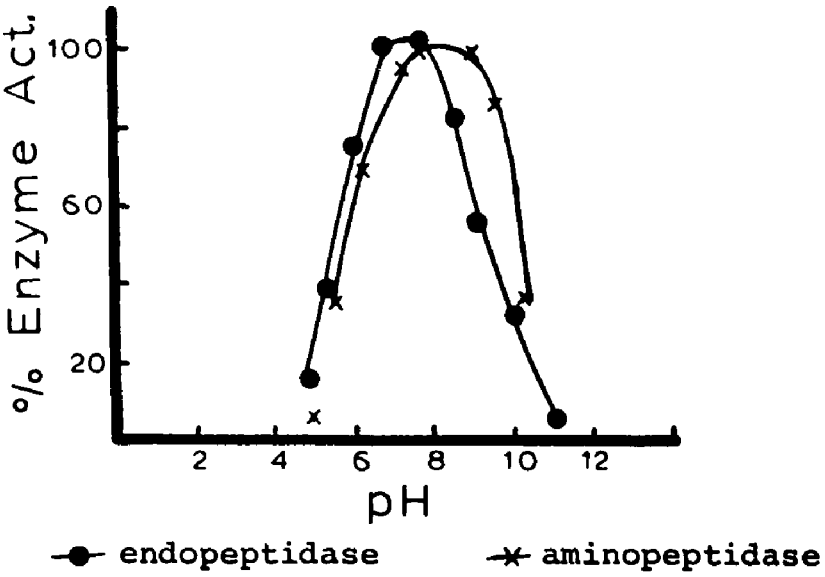


Fig. 15: Heat stability of protease mixture

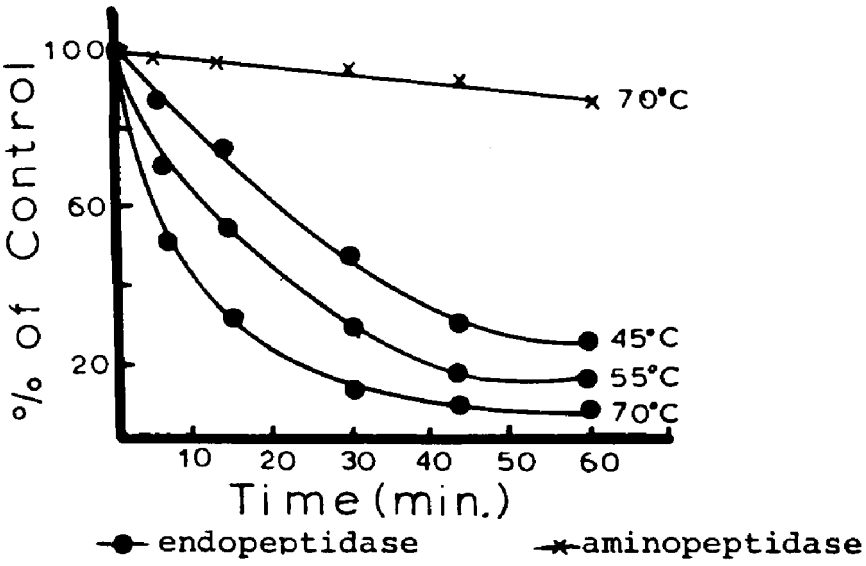
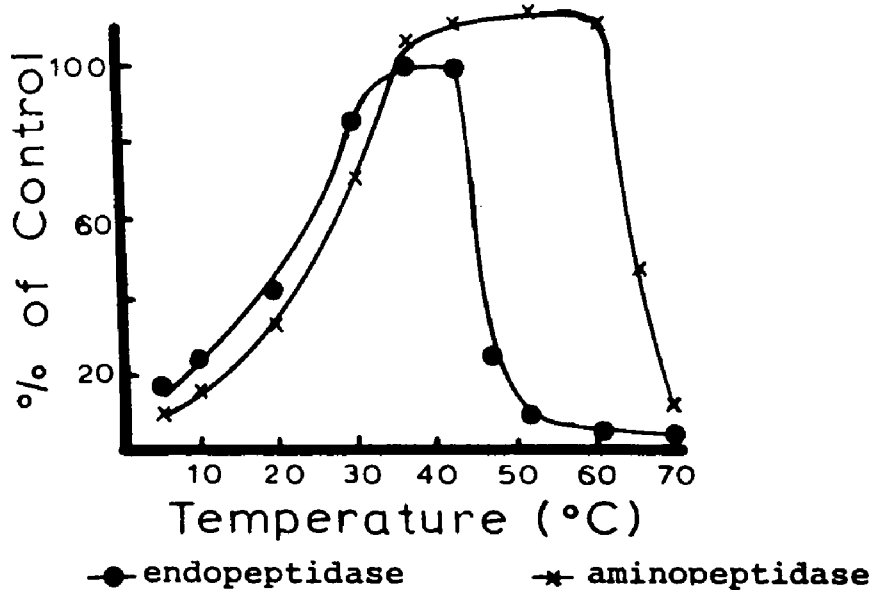


Fig. 16: Effect of temperature on protease activity of the crude enzyme mixture



found to be narrow and near neutrality (pH 6.8 to 8.0). No secondary peaks or shoulders were observed. The pH optimum of the aminopeptidase is broader and more alkaline (pH 6.8 to 8.6). In neither case was any activity observed above pH 10.5 or below pH 4.5.

Heat stability studies (Fig. 15) demonstrated that the aminopeptidase was stable to heating at 65°C for 30 minutes. When exposed to similar conditions, the endopeptidases lost all of their activity. A temperature optimum study (Fig. 16) showed a decrease in activity between 40 and 45°C for the endopeptidase-catalyzed hydrolysis of hemoglobin. On the other hand, a broad optimum with a decrease in enzymatic activity above 65°C was observed for the aminopeptidase.

The results of the activator and inhibitor studies are presented in Table VII. $Copper^{+2}$ and $nickel^{+2}$ inhibited both the endo- and aminopeptidases. For the endopeptidases, Mg^{+2} and Mn^{+2} exhibited inhibitory action, while most of the other metal ions slightly activated or had no effect on the aminopeptidase activity.

The endopeptidases were inhibited by oxidizing agents, while reducing agents and thiol-specific reagents had no effect. By their inhibition with disodium-EDTA, the endopeptidases appear to be metalloenzymes. Reactivation after Na_2EDTA inhibition (Table VIII) was best accomplished with Ca^{+2} and Zn^{+2}.

TABLE VII

Effects of activators and inhibitors on the B-207 enzyme mixture*

Activator or inhibitor	Remaining enzyme act.** endopep.	aminopep.
$CaCl_2$	88	105
$CoCl_2$	103	92
$CuSO_4$	34	39
$FeSO_4$	96	104
$FeCl_3$	100	105
$MgCl_2$	83	105
$MnSO_4$	76	100
$NiCl_2$	80	80
$ZnCl_2$	90	105
p-chloromercuribenzoate	100	102
Iodoacetamide	98	96
Glutathione (oxidized)	56	96
Glutathione (reduced)	96	100
L-cystine	45	100
Potassium permanganate	59	94
Ascorbic acid	100	94
KCN	100	76
EDTA-Na_2	59	60
8-hydroxyquinoline	100	64
L-cysteine	96	80

* 1×10^{-3} M of chemical and 0.5 mg protein/ml in 0.02 M Tris-HCl buffer, pH 8.0; preincubated for 15 minutes at 25°C.

** Percent of original activity after preincubation with distilled water.

TABLE VIII

Reactivation of B-207 proteases after EDTA Inhibition*

Metal ion	Enzyme activity** endopep.	aminopep.
$CaCl_2$	92	5
$CoCl_2$	47	34
$FeSO_4$	45	20
$FeCl_3$	40	18
$MgCl_2$	43	10
$MnSO_4$	45	10
$NiCl_2$	12	8
$ZnCl_2$	80	52
distilled water	6	5

* 1×10^{-3} M concentration of each metal ion and 0.5 mg protein/ml after the enzymes were dialyzed for 6 hours against 10^{-3} M Na_2EDTA, followed by 6 hour dialysis against distilled water.

** Percent of original activity prior to Na_2EDTA inhibition.

The observation that the aminopeptidase was only inhibited by metal chelating agents indicated its metalloenzyme nature. Reactivation after Na_2EDTA treatment was best accomplished with Zn^{+2} and Co^{+2}.

Separation of the proteases into two fractions:

Studies conducted with the various concentrations of ammonium sulfate as precipitating agent led to the conclusion that very little of the inactive protein could be separated from the active material by the precipitation method. Nor could the two types of proteases be separated in that manner. Fractionation with cold acetone separated about 65% of the

inactive protein at 45% concentration while a 67% concentration precipitated the rest of the enzymes. However, large batches required large volumes of acetone. Consequently this method had to be abandoned. It was therefore decided to precipitate all of the proteins with 70% ammonium sulfate and isolate the enzymes by some other means. The precipitated enzymes were redissolved in a small volume of 0.02 M Tris-HCl buffer (pH 8.0) and separated by means of column chromatography.

The results of the column chromatographic separation of the crude enzymes are shown in Figs. 17-19. Many separations were effected by these methods and similar elution patterns were observed in all cases. DEAE-cellulose was chosen as the best method since large samples (approximately 4.00 g per 100 g of dry DEAE-cellulose) could be handled at one time. A study was undertaken to determine at which pH the greatest quantity of enzyme was absorbed. The results from Table IX indicated that the higher the pH, the less aminopeptidase was absorbed relative to the endopeptidase. To insure maximal absorption of active enzyme, it was decided to use pH 8.4 as the initial pH of the buffer. A linear gradient of 0.05to 0.3 M NaCl in 0.02 M Tris-HCl buffer (pH 8.4) was used. After the aminopeptidase was off the column 0.5 M NaCl in 0.02 M NaCl in 0.02 M Tris-HCl buffer (pH 8.4) was used to elute the remaining endopeptidases.

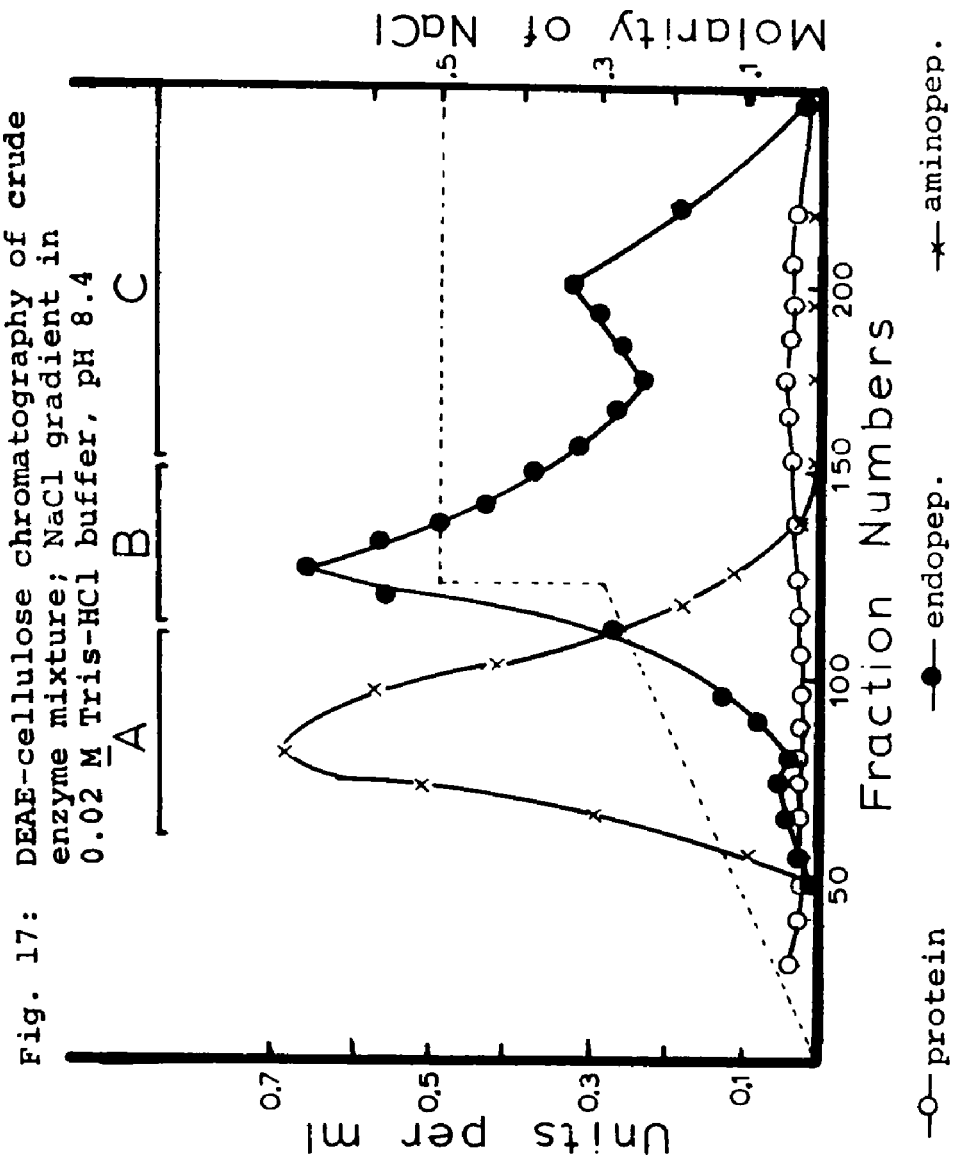


Fig. 17: DEAE-cellulose chromatography of crude enzyme mixture; NaCl gradient in 0.02 M Tris-HCl buffer, pH 8.4

Fig. 18: Chromatography of crude enzyme mixture on Sephadex G-200; elution with 0.02 M Tris-HCl buffer pH 8.4)

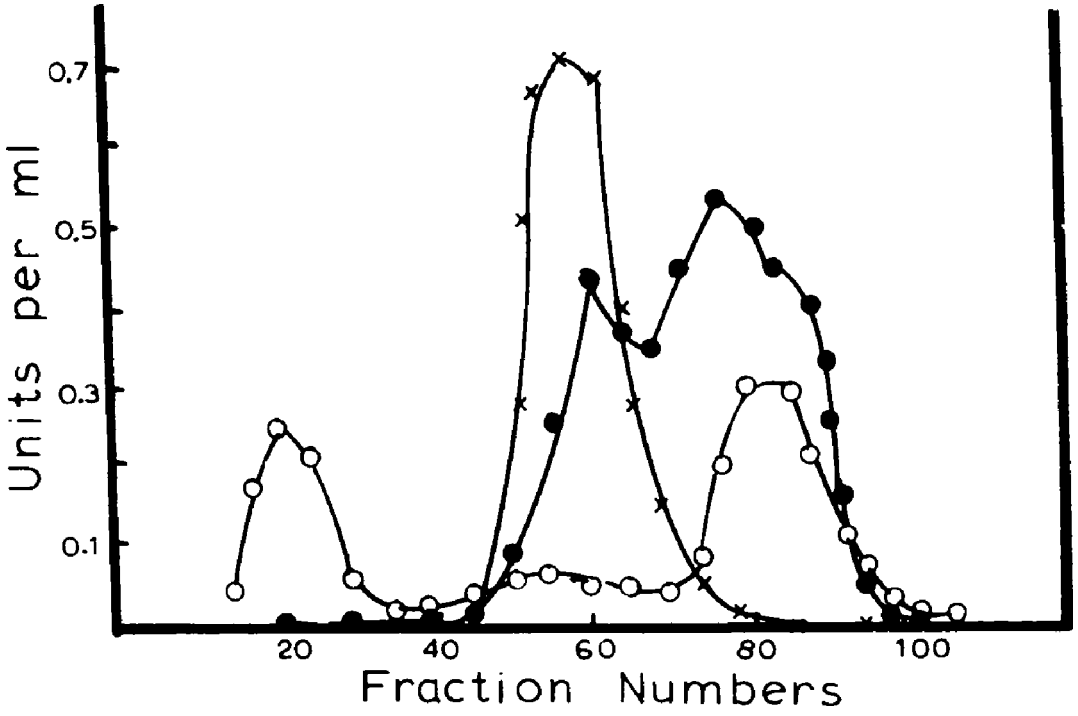


Fig. 19: Chromatography of crude enzyme mixture on DEAE-Sephadex; NaCl gradient in 0.02 M Tris-HCl, (pH 8.4)

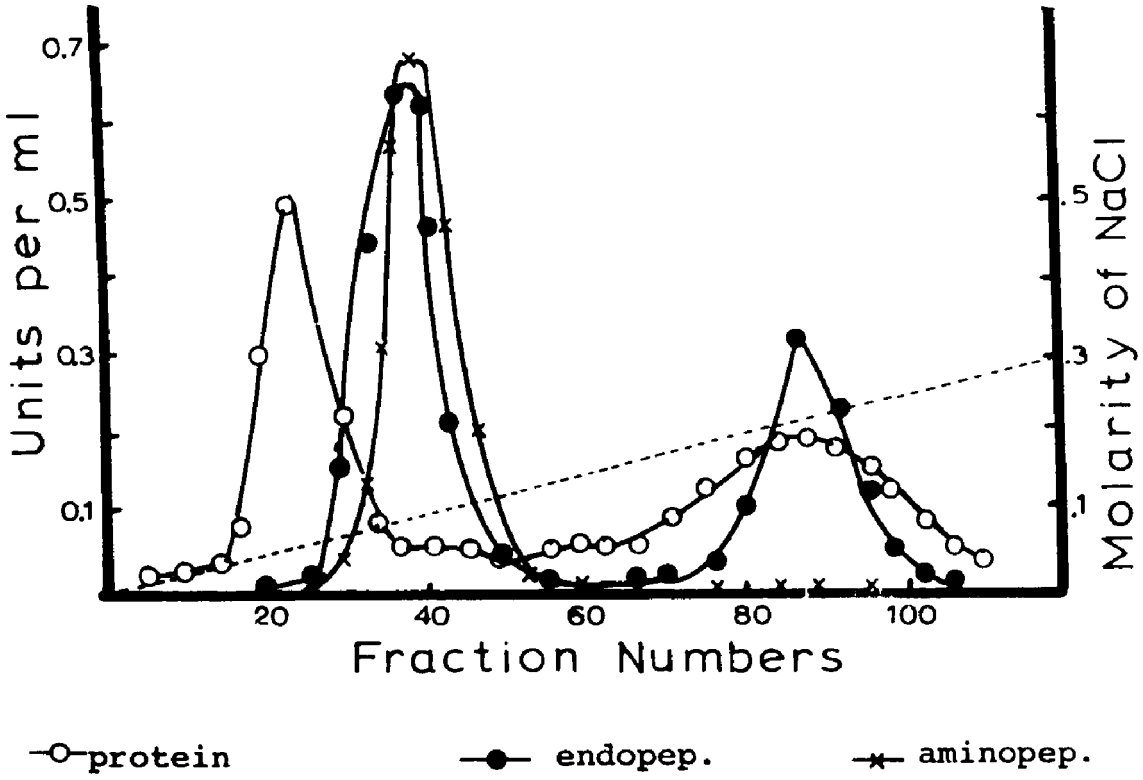


TABLE IX: Absorption of proteases onto DEAE-cellulose*

pH	protein**	endopep.**	aminopep.**
6.4	60	48	15
7.4	65	55	12
8.4	65	65	10
9.5	67	45	6
10.3	70	40	3
11.0	60	38	2

* 100 ml of crude enzyme mixture added to 50 ml suspension of DEAE-cellulose at appropriate pH. After 30 minutes, filtered and assayed for enzyme activity at pH 8.0.

** measured as percent of original enzyme mixture; numbers represent material absorbed onto ion-exchange resin.

The enzyme-containing tubes were pooled into three major fractions (Fig. 17): (A) which contained primarily aminopeptidase with some endopeptidase impurity; (B) contained a little aminopeptidase with a large amount of endopeptidase and (C) contained only endopeptidase. Unabsorbed material, obtained during the packing of the column, was treated in the same manner as fraction B. The purification of the aminopeptidase from fraction A will be discussed in Part III and the isolation of the several endopeptidases from fraction C will be discussed in Part II. Fraction B was lyophilized, pooled with other preparations and stored at -10°C. When enough material became available it was rechromatographed on DEAE-cellulose in a manner similar to that described above and the fractions pooled into A and C.

A summary of a typical purification is given in Table X. The increase in purification of the enzymes was noted by

TABLE X: Isolation and purification of the B-207 proteases*

Procedure	Volume (ml)	Enzyme Activity endopeptidase tot. units	SA	aminopeptidase tot. unit.	SA	Protein tot. mg. Lowry meth.	% yield E	A
1) cell-free medium	10,000	18,000	--	8,000	--	------	100	100
2) after $(NH_4)_2SO_4$ fractionation	1500	12,800	3.0	5,500	1.3	4,300	71	70
3) DEAE-cellulose								
total applied	1500	12,800	3.0	5,500	1.3	4,300	--	--
**A	500	4,000	7.3	4,125	7.5	550	22	52
**B	300	1,000	11.5	400	4.6	87	6	5
**C	600	3,800	15.0	000	---	600	21	0
not absorbed	1000	1,200	0.4	750	0.25	3,000	7	9

* units described in Experimental Procedure, SA represents the specific activity (total units per total mg of protein)

** fractions indicated on Fig. 17.

changes in the specific activity (ratio of total units of enzyme activity to mg of total protein). Because the cell-free medium contained peptides and other Lowry positive reactants which would give misleading results, the specific activity calculations were based on the ammonium sulfate precipitated proteins.

DISCUSSION

The marine bacterium which was designated B-207 is aerobic, catalase- and oxidase-producing, a gram negative rod, motile by means of a single polar flagellum, non spore forming and non acid-fast. This description is in very close agreement with the description given in Bergey's Manual (21) and by several other authors (78, 79, 80, 81) for the genus Pseudomonas. Supporting evidence is given by the similarity of the antibiotic sensitivity (82) and the base ratio of the DNA (48, 83) with other pseudomonads. The greatly increased growth of B-207 on aeration, and the observation of growth only at the top of the test tubes during carbohydrate utilization studies indicated the aerobic nature of this organism, which is typical of the genus Pseudomonas (78).

Consultation with the literature (78, 79, 80, 81, 84, 21) for previously described bacteria with which this organism could be identified, led to the conclusion that this was a novel species. Pseudomonas B-207 could not be placed among any of the existing species described in Bergey's

Manual (21). The characteristics associated with the six groups of marine bacteria described by Quigley and Colowick (78) did not describe the bacterium B-207. However, on the basis of a positive result obtained with the utilization of glucose, B-207 could be identified in Shewan's taxonomic scheme (79) as a Pseudomonas Group II. A negative result on the utilization of glucose would lead to its classification as a representative of the Pseudomonas Group III species.

Growth experiments with shaker flasks showed that proteolytic enzyme production of B-207 was particularly dependent upon the available nutrients, aeration and stage of growth. The temperature, initial pH of the medium and ionic strength of sodium chloride affected the growth of B-207, and consequently the enzyme production. The most favorable conditions for growth and enzyme production were obtained in a 1% solution of enzymatically hydrolyzed casein (N-Z Amine type HD) containing 3% RMM. It is noteworthy to point out that the manufacturer's analysis of the N-Z Amine type HD medium showed a considerably higher ratio of amino nitrogen to total nitrogen than any of his other products (85). Synthesis of the endopeptidases reached a peak after 18-20 hours of growth which coincided with the end of the log phase. The late appearance of the aminopeptidase coincided with the lysis of the cells during the stationary growth phase. Release of the intracellular

materials during the log phase of growth showed the extracellular nature of the endopeptidases and the intracellular nature of the aminopeptidase.

Morphological studies of the penicillin treated cells suggested that penicillin was inhibiting cell wall synthesis (86). Reduction in the activity of the endopeptidases beyond the level of penicillin inhibition indicated that an intact cell wall was required for their production. Further evidence that the aminopeptidase was produced intracellularly was demonstrated by only a slight reduction in its production. The relatively high ratio of aminopeptidase activity to turbidity evident with penicillin treated cells may be due to the bacterium's increased susceptibility to lysis because it lacks a completed cell wall.

Although extensive metabolic studies , employing inhibitors of proteolytic synthesis and radioactively labeled substrates, have not been carried out, one can speculate that marine Pseudomonas B-207 is able to fill its requirement of nitrogen and carbon with amino acids supplied by the following sequence of proteolytic reactions: 1) The two extracellular endopeptidases are produced into the medium; together they are able to hydrolyze both native and denatured proteins (results of Table VI). (2) The peptide products are carried through the membranes of the bacterium, and once inside they are further degraded by the intracellular enzymes, including the aminopeptidase.

The addition of carbohydrates to the hydrolyzed protein medium demonstrates how the organism is able to change its metabolism. From the hydrolyzed protein, the amino acids were obtained which were used as sources of energy and nutrients. The addition of sucrose or fructose (both utilized oxidatively) shifted the metabolism of the organism from the synthesis of proteolytic enzymes to the direct utilization of carbohydrates as sources of energy and nutrients and for the production of polysaccharide. Glucose is apparently converted directly to polysaccharide.

The separation of each polysaccharide from the capsule has not been accomplished, but one can speculate that the endopeptidases might be partially bound to the protein polysaccharide of the capsule. In this way it would be possible to imagine that the bacterium could keep these necessary enzymes in its vicinity, which considering marine currents and the low concentration of nutrients in the environment might cause a problem for the bacterium.

The aminopeptidase shows a heat stability which cannot be correlated with the properties of the organism, since the B-207 cells were observed not to survive much above 30°C. The decrease in activity above 65°C that was observed during the temperature optimum measurement, even though the aminopeptidase was apparently stable for an hour at 70°C, may be explained in two ways. The activity, as determined at various temperatures, is dependent upon a series of

reactions and a number of criteria (87). Amoung them is the formation of the activated enzyme-substrate complex, the enzyme stability and the influence of necessary ionic groups. Gutfreund (88) showed that temperature affected the ionization of histidyl residue and subsequently the activity of the enzyme trypsin. It might be possible for the same sort of interaction to affect the aminopeptidase activity. An alternative explanation might be that the aminopeptidase was reversibly inactivated at 70°C. Since the activity in the temperature optimum study was made at 70°C it would therefore indicate a reduced activity. Upon cooling, the enzyme would be reactivated with the subsequent measurement of activity at 37°C (as was done for the temperature stability) indicating no loss of activity. Both of these explanations, which involve factors affecting the enzyme, may be contributing to the actual situation.

Overall yields in the purification of the proteases after the DEAE-cellulose chromatography were relatively good with a little more then 50% of the activity being accounted for in the three fractions. The enzymes still have to be further purified as will be discussed in the next two sections.

PART II

The Endopeptidases from a New Marine *Pseudomonas*

Studies on the crude protease mixture revealed that two extracellular endopeptidases were produced by B-207. This section of the report describes the procedures used to isolate the individual endopeptidases and the studies conducted in order to characterize them.

EXPERIMENTAL RESULTS

Isolation and purification: The endopeptidases were partially separated from the aminopeptidase by DEAE-cellulose chromatography (Fig. 17). Fraction C, which contained only the endopeptidases, was concentrated by precipitation with the addition of 470 g/ml of solid ammonium sulfate, by means of an Amicon Diaflo ultrafiltration apparatus or by freeze-drying. A solution of crude endopeptidase mixture (25 to 100 mg protein/ml) in 0.02 M Tris-HCl buffer (pH 8.0) was prepared from the concentrates and stored at 4°C. One to two ml aliquots of the solution were then chromatographed by gel-filtration on Sephadex G-100 or G-200 with 0.02 M Tris-HCl buffer (pH 8.4) as elutant (Fig. 20). Two major active fractions were obtained. A third minor peak was observed with Sephadex G-200 which never amounted to more than 1% of the total endopeptidase activity. Its origin is unknown, although interaction between the gel and proteins may have occurred As a result of its minor contribution, it was not included in the studies reported here. A summary of a typical

Fig. 20: GEL-FILTRATION

of

FRACTION C

Sephadex G-200
0.02 M Tris-HCl Buffer, pH 8.0

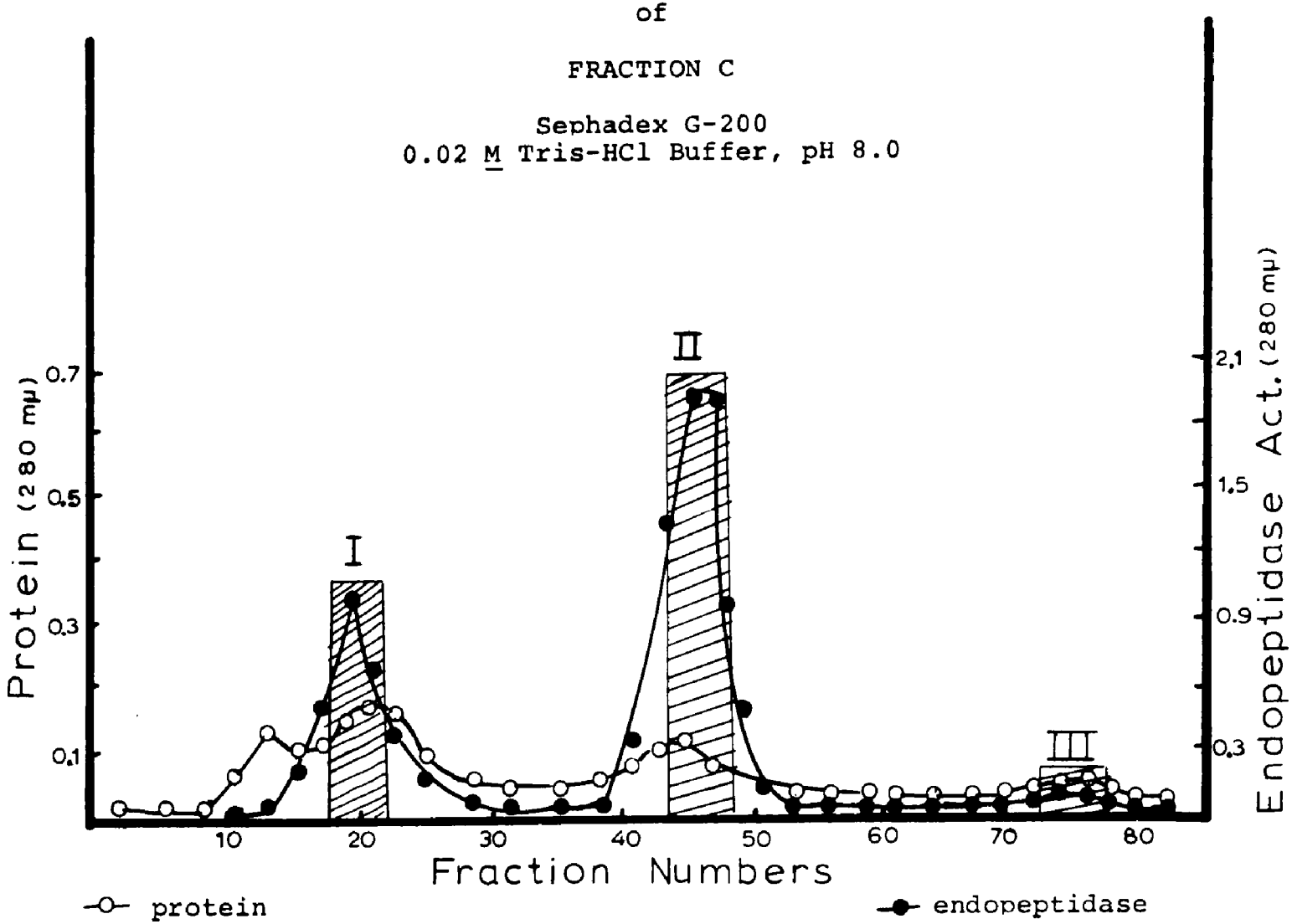


TABLE XI: Isolation and purification of B-207 endopeptidases

Procedure	Volume (ml)	Total Endopeptidase** units	SA	Protein tot. mg Lowry meth.	% Yield Endo.
1) Fraction C (conc.)	10	3600	14.5	250	20
2) Sephadex G-200					
total applied	2.0	720	14.5	50	100
* I	48	100	18.5	5.4	14
* II	75	480	25.2	19.0	67
*III	10	5	3.1	1.6	0.7
				recovered	82%
3) Rechromatography of fraction I on Seph. G-200					
total applied	1.0	25	17.0	1.5	100
* I	15	19	22.0	0.9	76
*II	8	3	10	0.3	12
Rechromatography of fraction II on Seph. G-200					
total applied	1.0	100	23.2	4.3	100
*II	15	83	38.0	2.2	83

* fractions indicated on Fig. 20
** units described in Experimental Procedures; SA represents specific activity; % yield is based on the initial activity of the cell-free medium

purification is shown in Table XI.

Rechromatography of both fraction I and fraction II was accomplished on Sephadex G-200 with the same elutant (Fig. 21). When fraction I was rechromatographed a small active peak of fraction II was obtained. However, the rechromatography of fraction II yielded only one active peak. During the three times that this experiment was repeated, similar results were obtained, regardless of the method of concentration. Molecular weight determinations (by means of a calibrated Sephadex G-200 column) shown in Fig. 33 (Part III) gave a value of approximately 60,000 and 30,000 for the two peaks obtained for fraction I while fraction II was determined to be 30,000.

Cellulose acetate electrophoresis studies (Fig. 22) suggested that fraction I contained three distinct proteins of which two were enzymatically active (by chromoprotein digestion) and that fraction II contained two proteins with only one active band. The inactive protein was observed not to migrate and remained at the origin and was assumed to be denatured fraction II. As a result of the observed single peak on Sephadex G-200 and the presence of one active protein band migrating toward the anode on cellulose acetate and polyacrylamide gel electrophoresis, fraction II was deemed homogeneous. It will be referred to hereafter as endopeptidase II. Fraction I could not be shown to be homogeneous.

Fig. 21: Rechromatography of isolated fractions Sephadex G-200

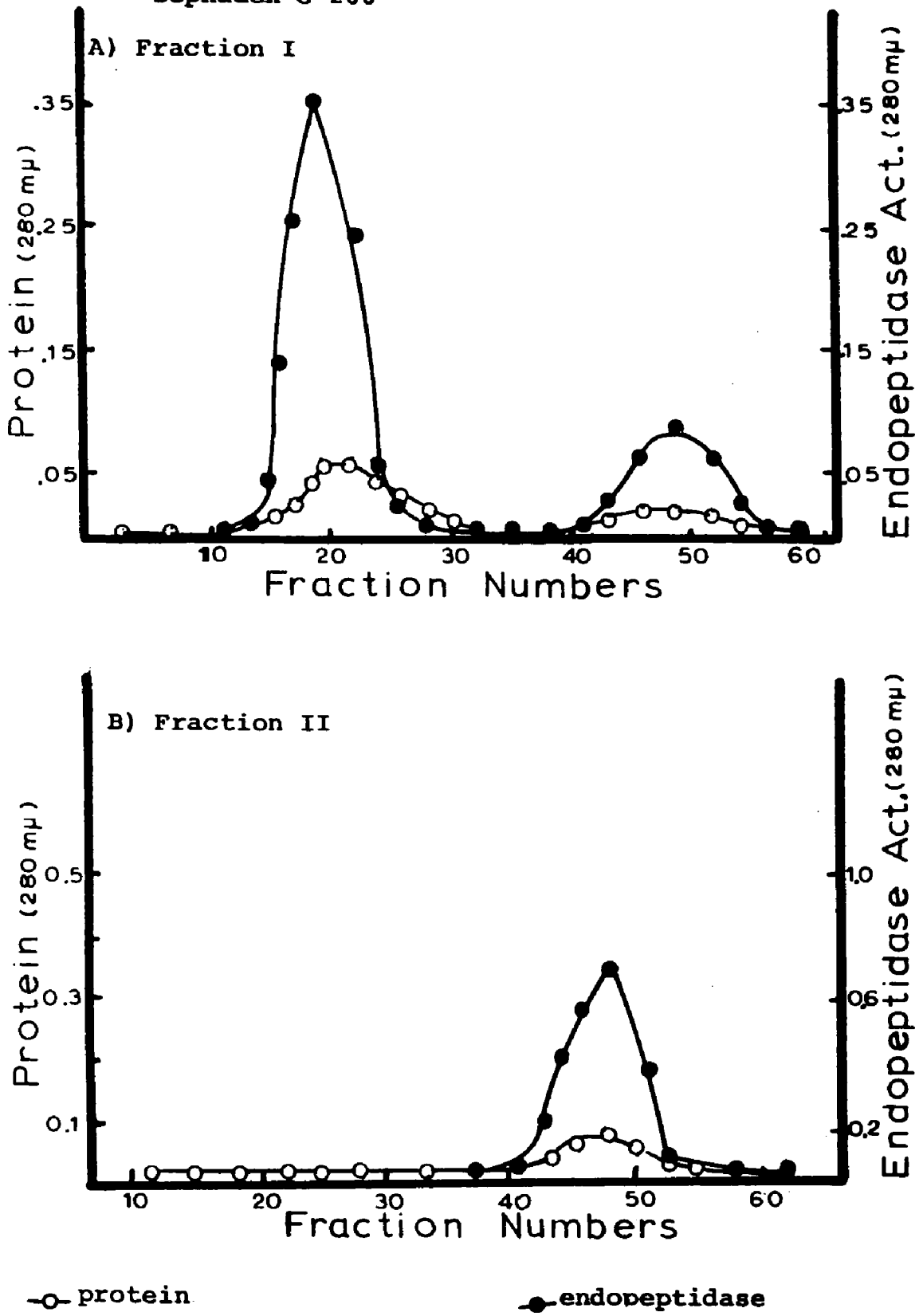
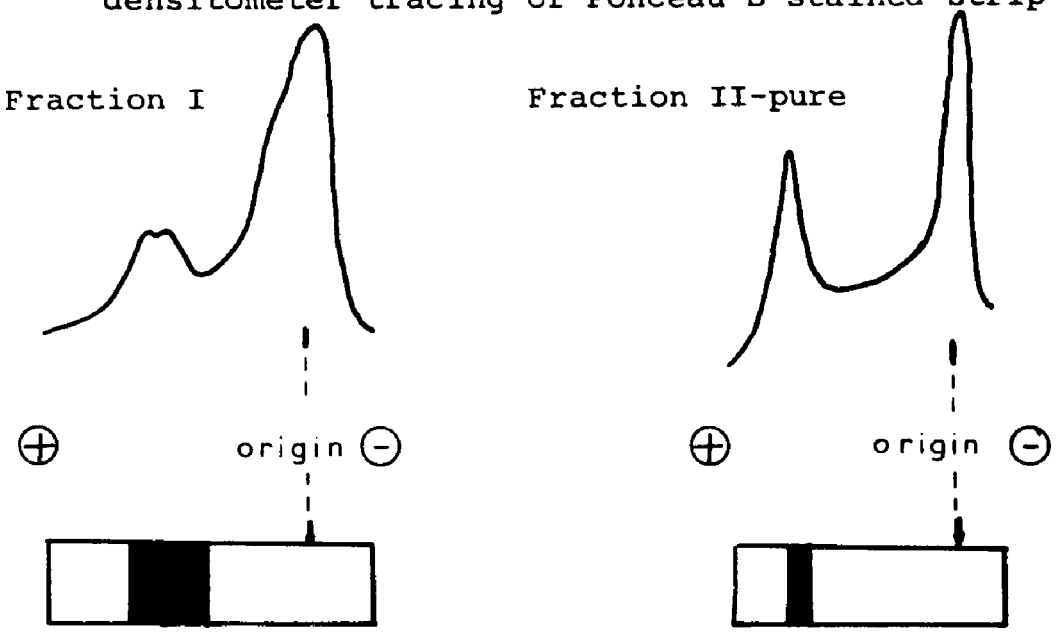


Fig. 22: Electrophoretic studies with the B-207 endopeptidases

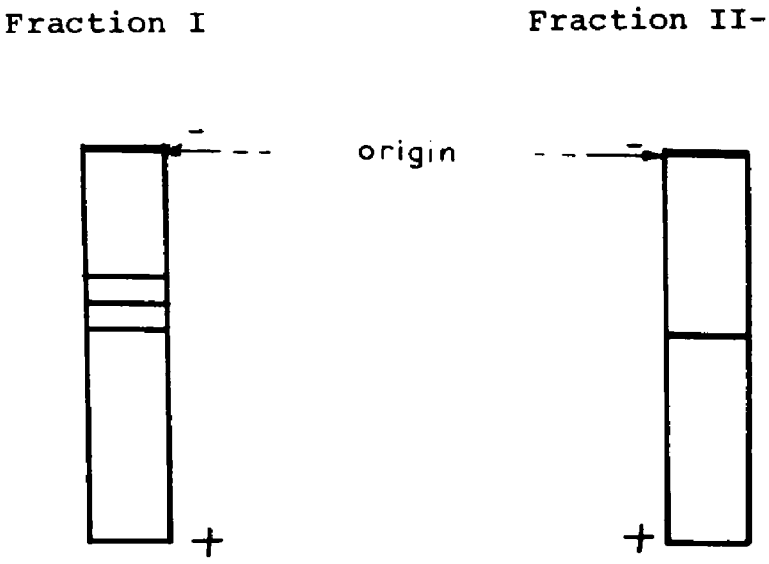
a) Cellulose acetate strip pH 8.0, 1.5 hour, 23°C

densitometer tracing of Ponceau S stained strip



drawing of chromoprotein digestion (shaded areas represent digestion)

b) Gel electrophoresis pH 8.6, 2 hours, 20°C



A number of chromatographic experiments was run in which Sephadex G-100 columns were equilibrated with distilled water or with 0.02 M Tris-HCl buffer (pH 8.4) to which 10^{-3}M disodium EDTA had been added. No appreciable differences in the ratios of enzyme:protein isolated from the two fractions were observed.

Characterization of endopeptidase II and fraction I: The characterization studies described below were carried out on a homogeneous endopeptidase II and a partially purified fraction I which contained a minor endopeptidase II contaminant. In all of the characterization studies, the enzymes were diluted until first-order kinetics was obeyed.

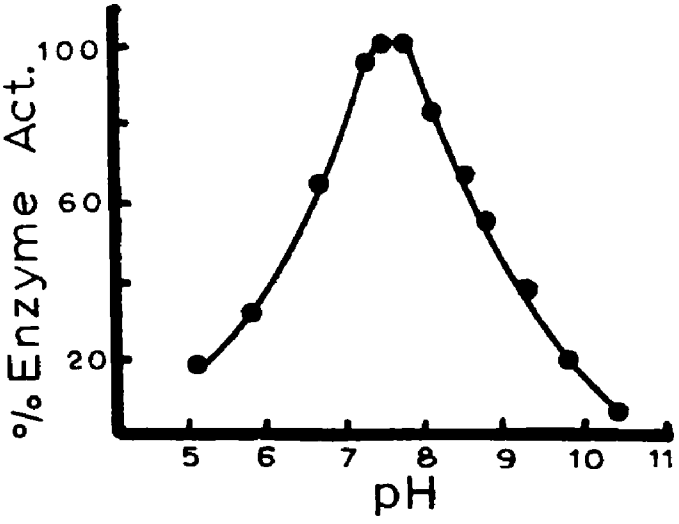
The effect of pH in 0.02 M phosphate-acetate buffer and Tris-HCl buffer on the hemoglobin hydrolyzing activity is shown in Fig. 23. Both enzymes have essentially the same pH optimum (7.3-7.6) with fraction I being slightly broader and more alkaline.

With the purification of endopeptidase II and fraction I from the crude protease mixture, no change in the effect of pH on stability was noted. Both endopeptidase II and fraction I have identical stabilities (greatest pH 6-8).

The heat stability of both endopeptidase II and fraction I is depicted in Fig. 24. Endopeptidase II rapidly loses most of its activity at 60°C while fraction I is more stable. The fact that fraction I had a lower

Fig. 23: Effect of pH on endopeptidase activity

A) Fraction I



B) Endopeptidase II

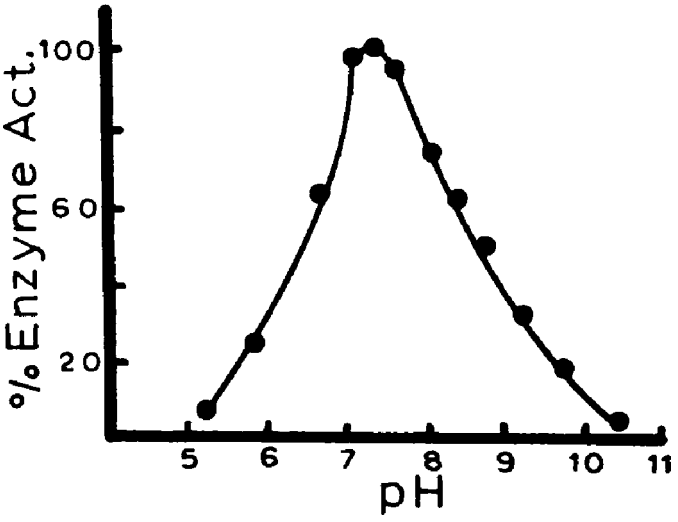
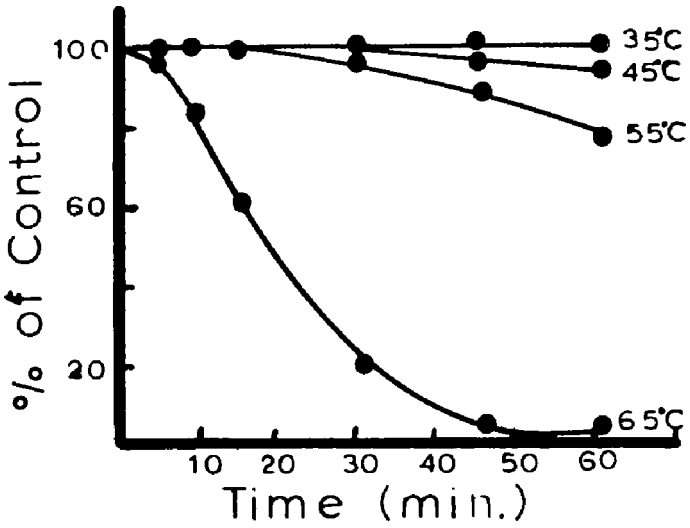
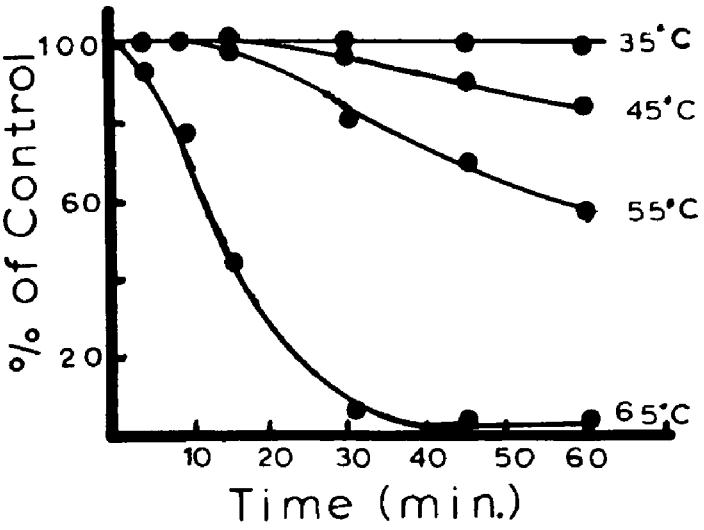


Fig. 24: Temperature stability of B-207 endopeptidases

A) Fraction I



B) Endopeptidase II



specific activity (ratio of active protein to total protein) than endopeptidase II prompted the investigation of the heat stability of the endopeptidases at various specific

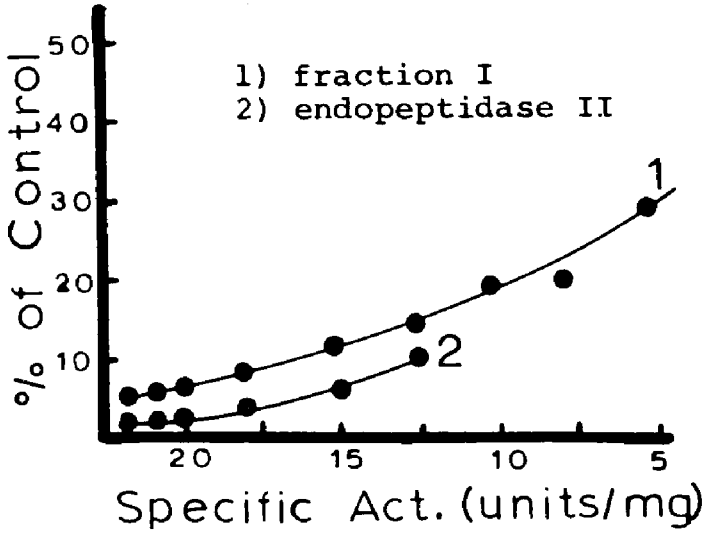


Fig. 26: Influence of protein concentration on heat stability (1 hour at 60°C)

activities. The specific activity was changed by the addition of a calculated amount of non-specific protein (10 mg/ml) in the form of bovine serum albumin. Fig. 26 shows that a lower specific activity results in increased heat stability of both endopeptidases.

The effects of activators and inhibitors on the endopeptidases is shown in Table XII. Both enzymes were inhibited by the metal chelating agents. EDTA was the most effective. Removal of excess disodium-EDTA by dialysis against distilled water at 4°C, followed by treatment with various metals at pH 8.0 resulted in partial reactivation of both endopeptidases (Table XIII). The order of

TABLE XII: Effects of activators and inhibitors on B-207 endopeptidases*

Activator or inhibitor	Enzyme Activity** fraction I	endopep. II
$CaCl_2$	98	100
$CoCl_2$	100	94
$CuSO_4$	34	39
$FeSO_4$	94	96
$FeCl_3$	100	98
$MgCl_2$	86	90
$MnSO_4$	78	82
$NiCl_2$	86	84
$ZnCl_2$	92	94
p-chloromercuribenzoate	95	97
Iodoacetamide	94	96
Glutathione (oxidized)	50	54
Glutathione (reduced)	98	98
L-cystine	40	44
Potassium Permanganate	62	60
Ascorbic Acid	100	98
KCN	70	68
EDTA-Na_2	52	46
8-hydroxyquinoline	72	68
L-cysteine	65	60
DFP	96	98

*$1x10^{-3}$M concentration of chemical and 0.5 mg protein/ml in 0.02 M Tris-HCl buffer, pH 8.0, 15 minute incubation at 25°C before assaying activity.

**Percent of orignal enzyme activity

decreasing reactivating abilitv was determined to be calcium^{+2}, followed by zinc^{+2}, cobalt^{+2}, magnesium^{+2}, manganese^{+2}, iron^{+3} and iron^{+2}.

The reagents possessing some oxidizing ability were able to inhibit both endopeptidases. Oxidized glutathione and L-cystine, both known to be involved with thiol oxidations (89), were the strongest inhibitors. DFP, specific for serine residues (90). had no effect on the proteolvtic activity.

TABLE XIII: Reactivation of B-207 endopeptidases after disodium-EDTA (10^{-3}M) inhibition*

Metal ion	Enzyme Activity** fraction I	endopeptidase II
$CaCl_2$	94	92
$CoCl_2$	38	34
$FeSO_4$	24	20
$FeCl_3$	34	28
$MgCl_2$	36	30
$MnSO_4$	34	28
$NiCl_2$	16	8
$ZnCl_2$	42	38
distilled water	8	6

* $1x10^{-3}$M concentration of metal and 0.5 mg/ml protein after the enzymes were dialyzed for 6 hours against $1x10^{-3}$M Na_2EDTA, followed by 6 hour dialysis against distilled water.

** Percent of the original enzyme activity

Amino acid specificities of endopeptidase II: The specificity of endopeptidase II was examined with native ribonuclease and native bovine insulin, oxidized insulin and the oxidized, isolated B-chain of insulin as substrates. Table XIV shows the results of DNP-amino acid and DNP-peptide end group analysis obtained after 15 and 360 minute hydrolysis at 37°C. The 60 minute hydrolysis experiment gave similar results to those obtained for 360 minute hydrolysis. If we assume that DNP-glutamic and aspartic acids arose from glutamine and asparagine, the results would indicate that endopeptidase II hydrolyzed those bonds in which the amino acid supplying the amino portion was uncharged (not including any hydroxy- or thiol side chain). Di-DNP-lysine was derived from the N-terminal

TABLE XIV: Hydrolysis of protein substrates by endopeptidase II

	Times of hydrolysis (min.)	
	15	360
Ribonuclease:		
DNP-amino acids		
alanine	0.026*(1)**	0.034 (2)
leucine-isoleucine	0.030 (2)	0.034 (2)
valine	0.022 (1)	0.018 (1)
DNP-end groups		
di-lysine	0.016 (1)	0.020 (1)
alanine	0.082 (5)	0.132 (6)
aspartic acid	0.016 (1)	0.030 (2)
glutamic acid	0.048 (3)	0.052 (3)
glycine	0.018 (1)	0.020 (1)
leucine-isoleucine	0.000 (0)	0.020 (1)
phenylalanine	0.024 (1)	0.038 (2)
valine	0.018 (1)	0.020 (1)
Insulin-Native		
DNP-amino acids		
aspartic acid	0.020 (1)	0.046 (3)
glutamic acid	0.018 (1)	0.020 (1)
glycine	0.024 (1)	0.020 (1)
leucine-isoleucine	0.048 (3)	0.082 (5)
phenylalanine	0.042 (3)	0.075 (4)
tyrosine	0.026 (1)	0.030 (2)
valine	0.022 (1)	0.022 (1)
DNP-end groups		
glutamic acid	0.016 (1)	0.022 (1)
tyrosine	0.014 (1)	0.030 (2)
valine	0.020 (1)	0.054 (3)

* Optical density at 360 mμ
** Nearest whole number ratios

TABLE XIV: Hydrolysis of protein substrates by endopeptidase II (con't.)

	Time of hydrolysis (min.)	
	15	360
Insulin-Oxidized		
DNP-amino acids		
aspartic acid	0.016*(1)**	0.024 (1)
glutamic acid	0.018 (1)	0.020 (1)
glycine	0.020 (1)	0.020 (1)
leucine-isoleucine	0.042 (3)	0.084 (5)
phenylalanine	0.042 (3)	0.072 (4)
valine	0.020 (1)	0.024 (1)
DNP-end groups		
aspartic acid	0.016 (1)	0.026 (1)
tyrosine	0.028 (2)	0.042 (3)
valine	0.032 (2)	0.048 (3)
Insulin-B-chain		
DNP-amino acids		
aspartic acid	0.018 (1)	0.028 (2)
leucine	0.020 (1)	0.036 (2)
phenylalanine	0.036 (2)	0.048 (3)
tyrosine	0.014 (1)	0.016 (1)
valine	0.016 (1)	0.016 (1)
DNP-end groups		
aspartic acid	0.006 (0)	0.018 (1)
glutamic acid	0.014 (1)	0.026 (1)
valine	0.018 (1)	0.022 (1)

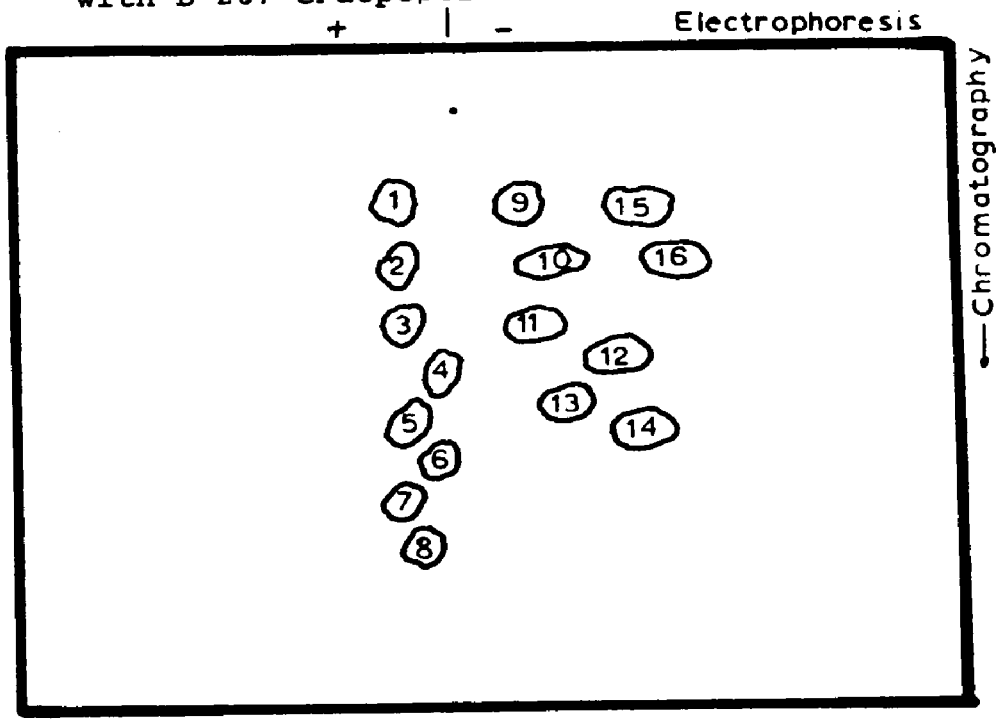
* Optical density at 360 mμ
** Nearest whole number ratios

amino acid of ribonuclease (91).

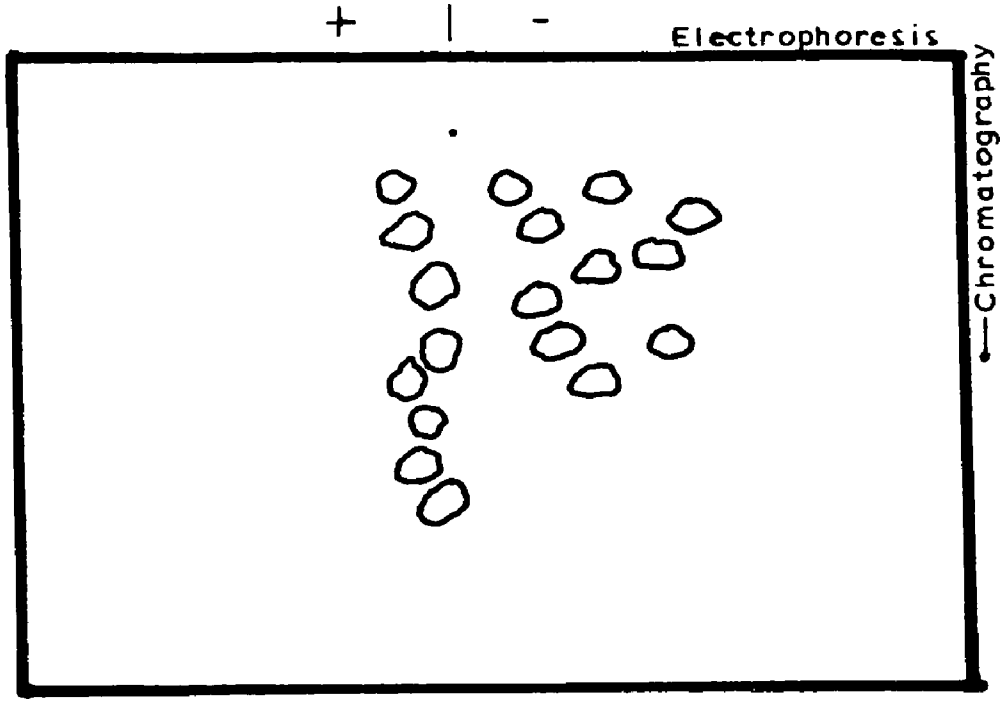
The resulting peptides from a 15 minute hydrolysis of the B-chain of insulin were separated by high voltage electrophoresis at pH 3.7 and chromatography. A peptide map (Fig. 26) was constructed. Sixteen observed spots were isolated and hydrolyzed under vacuum at 105°C for 12 hours with 6 N HCl. The hydrolyzates were evaporated to dryness and chromatographed. Following spraying with ninhydrin reagent, the colors were eluted, optical densities determined and amino acid ratios calculated to nearest whole numbers. Amino acid compositions of the peptides are shown in Table XV. Four individual amino acids are observed as well as a number of larger peptides. The presence of peptides containing residues (see Fig. 27) 1-24, 2-24, 1-16 and 2-16 strongly suggests that the initial points of hydrolysis are centered at the bonds Phe-Phe (24-25), Phe-Val (1-2) and Tyr-Leu (16-17). It appears that subsequent hydrolysis results in all possible uncharged amino acid-uncharged amino acid combinations, with the exception of hydroxy- and thiol side chains, being hydrolyzed. The susceptibility of Leu-Tyr (15-16), Tyr-Leu (16-17) and Phe-Tyr (25-26) may be due to the large amount of unionized tyrosine at pH 7.5.

Specificity studies with endopeptidase II were continued with various di- and tripeptides and some of their derivatives which were available in the laboratory.

Fig. 26: Peptide map of insulin B chain after hydrolysis with B-207 endopeptidases*



A: endopeptidase II



B: fraction I

* 15 minute hydrolysis at 37°C, conditions for electrophoresis and chromatography in Experimental Procedures.

TABLE XV: Amino acid composition of the peptides produced by hydrolysis of the B-chain of insulin with endopeptidase II

	Peptides					
Amino acid	1	2	3	4	5	6
Ala	0.006* (0)**	0.015 (1)	0.018 (1)	0.016 (1)	0.002 (0)	0.000 (0)
Arg	0.000 (0)	0.000 (0)	0.000 (0)	0.000 (0)	0.000 (0)	0.000 (0)
Asp	0.016 (1)	0.018 (1)	0.016 (1)	0.018 (1)	0.004 (0)	0.000 (0)
$CySO_3H$	0.018 (1)	0.020 (1)	0.018 (1)	0.018 (1)	0.000 (0)	0.000 (0)
Glu	0.012 (1)	0.034 (2)	0.038 (2)	0.036 (2)	0.006 (0)	0.000 (0)
Gly	0.015 (1)	0.018 (1)	0.014 (1)	0.015 (1)	0.004 (0)	0.000 (0)
His	0.032 (2)	0.034 (2)	0.040 (2)	0.036 (2)	0.006 (0)	0.000 (0)
Leu	0.036 (2)	0.050 (3)	0.052 (3)	0.046 (3)	0.006 (0)	0.006 (0)
Lys	0.008 (0)	0.006 (0)	0.006 (0)	0.006 (0)	0.000 (0)	0.000 (0)
Phe	0.022 (1)	0.010 (0)	0.018 (1)	0.008 (0)	0.006 (0)	0.006 (0)
Pro	0.006 (0)	0.004 (0)	0.002 (0)	0.004 (0)	0.000 (0)	0.000 (0)
Ser	0.014 (1)	0.012 (1)	0.018 (1)	0.016 (1)	0.004 (0)	0.000 (0)
Thr	0.000 (0)	0.002 (0)	0.004 (0)	0.002 (0)	0.000 (0)	0.000 (0)
Tyr	0.006 (0)	0.008 (0)	0.016 (1)	0.014 (1)	0.020 (1)	0.004 (0)
Val	o.016 (1)	0.038 (2)	0.036 (2)	0.030 (2)	0.006 (0)	0.018 (1)
DNP-end amino acid	Phe	Val	Phe	Val	-	-
Residues in B-chain	1-11	2-15	1-16	2-16		

* the first column represents optical densities at 570 mμ

** the second column represents nearest whole number ratios

TABLE XV: Amino acid composition of the peptides produced by hydrolysis of the B-chain of insulin with endopeptidase II (con't)

	Peptides					
Amino acid	7	8	9	10	11	12
Ala	0.000* (0)**	0.000 (0)	0.004 (0)	0.014 (1)	0.014 (1)	0.006 (0)
Arg	0.000 (0)	0.000 (0)	0.004 (0)	0.016 (1)	0.014 (1)	0.016 (1)
Asp	0.000 (0)	0.000 (0)	0.018 (1)	0.018 (1)	0.016 (1)	0.006 (0)
$CySO_3H$	0.000 (0)	0.000 (0)	0.018 (1)	0.040 (2)	0.044 (2)	0.024 (1)
Glu	0.000 (0)	0.000 (0)	0.016 (1)	0.046 (3)	0.048 (3)	0.022 (1)
Gly	0.000 (0)	0.000 (0)	0.018 (1)	0.056 (3)	0.054 (3)	0.034 (2)
His	0.000 (0)	0.000 (0)	0.032 (2)	0.028 (2)	0.028 (2)	0.010 (0)
Leu	0.002 (0)	0.020 (1)	0.036 (2)	0.086 (4)	0.082 (4)	0.022 (1)
Lys	0.000 (0)	0.000 (0)	0.000 (0)	0.002 (0)	0.000 (0)	0.000 (0)
Phe	0.022 (1)	0.008 (0)	0.006 (0)	0.034 (2)	0.026 (1)	0.024 (1)
Pro	0.000 (0)	0.000 (0)	0.004 (0)	0.002 (0)	0.004 (0)	0.002 (0)
Ser	0.000 (0)	0.000 (0)	0.018 (1)	0.016 (1)	0.018 (1)	0.010 (0)
Thr	0.000 (0)	0.000 (0)	0.002 (0)	0.004 (0)	0.004 (0)	0.006 (0)
Tyr	0.000 (0)	0.000 (0)	0.008 (0)	0.016 (1)	0.016 (1)	0.006 (0)
Val	0.006 (0)	0.004 (0)	0.018 (1)	0.042 (3)	0.044 (3)	0.024 (1)
DNP-end amino acid	-	-	Val	Phe	Val	Leu
Residues in B-chain			2-11	1-24	2-24	17-24

* the first column represents optical densities at 570 mμ

** the second column represents nearest whole number ratios

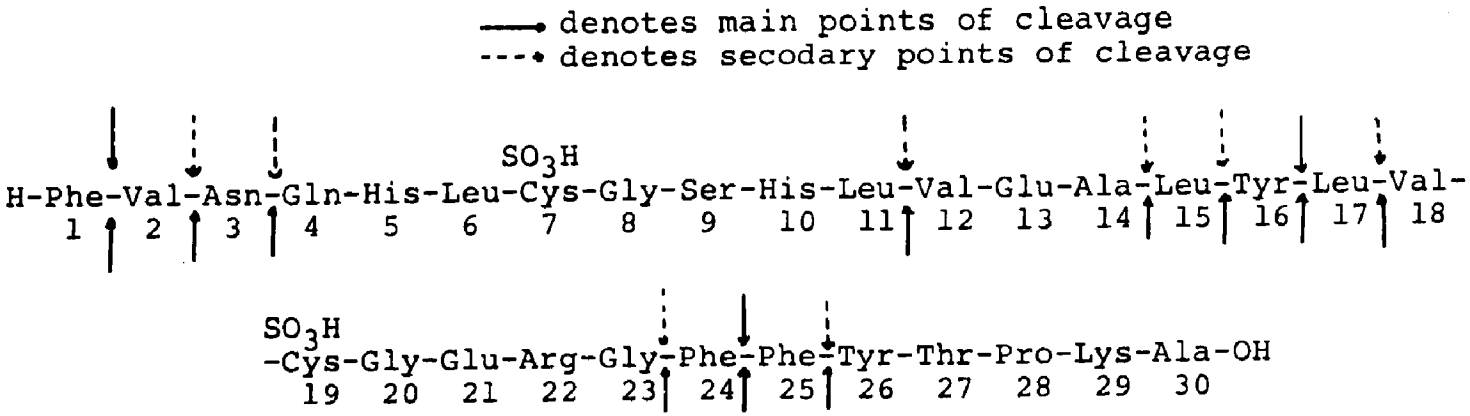
TABLE XV: Amino acid composition of the peptides produced by hydrolysis of the B-chain of insulin with endopeptidase II (con't)

	Peptides			
Amino acid	13	14	15	16
Ala	0.018 (1)	0.020 (1)	0.000 (0)	0.020 (1)
Arg	0.008 (0)	0.004 (0)	0.000 (0)	0.008 (0)
Asp	0.000 (0)	0.000 (0)	0.020 (0)	0.006 (0)
$CySO_3H$	0.008 (0)	0.002 (0)	0.024 (1)	0.004 (0)
Glu	0.016 (1)	0.018 (1)	0.022 (1)	0.004 (0)
Gly	0.006 (0)	0.000 (0)	0.032 (2)	0.006 (0)
His	0.000 (0)	0.004 (0)	0.038 (2)	0.008 (0)
Leu	0.026 (1)	0.024 (1)	0.040 (2)	0.006 (0)
Lys	0.000 (0)	0.000 (0)	0.000 (0)	0.018 (1)
Phe	0.008 (0)	0.008 (0)	0.008 (0)	0.016 (1)
Pro	0.006 (0)	0.006 (0)	0.000 (0)	0.026 (1)
Ser	0.002 (0)	0.002 (0)	0.024 (1)	0.008 (0)
Thr	0.004 (0)	0.004 (0)	0.008 (0)	0.028 (1)
Tyr	0.012 (0)	0.028 (1)	0.008 (0)	0.026 (1)
Val	0.026 (1)	0.026 (1)	0.004 (0)	0.006 (0)
DNP-end amino acid	Val	Val	Asp	Phe
Residues in	12-15	12-16	3-11	25-30

* the first column represents optical densities at 570 mμ

** the second column represents nearest whole number ratios

Fig. 27: Sites of cleavage of oxidized B-chain of insulin by endopeptidase II



The arrows below the B-chain indicate where endopeptidase II attacks this protein, based on the DNP-end groups (Table XIV)

The arrows above the B-chain indicate where endopeptidase II attacks this protein, based on the amino acid composition of the peptides and their DNP-end groups (Table XV)

TABLE XVI: Hydrolysis of synthetic peptides by endopeptidase II

Substrate	Hydrolysis*	
L-leucyl-L-leucine	trace**	
L-leucyl-L-leucine acetate	6.0	
L-leucyl-L-leucine amide	7.5	
L-leucyl-L-leucine methyl ester	6.5	
L-leucyl-D-leucine	0.0	
D-leucyl-L-leucine	0.0	
L-leucyl-L-alanine	trace	
L-leucylglycine	0.0	
L-leucyl-L-isoleucine	trace	
L-leucyl-L-phenylalanine	trace	
L-leucyl-L-proline	0.0	
L-leucyl-L-serine	0.0	
L-leucyl-L-tyrosine	trace	
L-leucyl-L-valine	trace	
L-leucyl-L-valine acetate	6.0	***
L-alanylglycylglycine	trace	ala+glygly
glycylglycylglycine	0.0	
glycyl-L-leucyl-L-tyrosine	trace	gly+leutyr
L-leucylglycylglycine	8.0	leu+glygly
L-leucyl-L-tyrosine-L-leucine	11.0	leu+tyr+ leutyr
CBZ-glycyl-L-tyrosine	0.0	
CBZ-glycyl-L-tyrsone amide	trace	
CBZ-leucyl-L-leucine amide	9.5	
CBZ-leucyl-L-phenylalanine benzyl ester	6.0	
CBZ-leucyl-L-tyrosine	0.0	
CBZ-leucyl-L-tyrosine benzyl ester	trace	
CBZ-leucyl-L-tyrosine hydrazide	trace	

* percent hydrolysis determined after 1 hour at 37°C; 0.02 M of each peptide at pH 7.5.
** trace refers to less than 4% hydrolysis.
***chromatographic results for the hydrolysis of tripeptides

The hydrolysis results are tabulated in Table XVI. The resultant products from tripeptide hydrolyses were examined by thin-layer chromatography. Although the percentage hydrolysis was very low for the examined peptides, it was evident that tripeptides were more susceptible than dipeptides. Those substrates that were blocked at either the C-terminal end with esters or amides, or

the N-terminal end with the carbobenzoxy (CBZ) derivative were hydrolyzed better than the unblocked derivatives. Those dipeptides that were blocked at both ends were still better, but not as susceptible as the tripeptides. Glycylglycylglycine was not hydrolyzed because of the small size of the glycyl reside. Increase to a larger sized N-terminal amino acid (e.g. alanylglycylglycine or leucylglycylglycine) resulted in a larger amount of hydrolysis.

Hydrolysis of proteins with fraction I: The same four protein substrates used in the endopeptidease II specificity studies were subjected to fraction I hydrolysis for various time periods ranging from 15 minutes to six hours. The quantitative data from the DNP-end group analysis of the insulin B-chain is tabulated in Table XVII. A peptide map of a 15 minute hydrolysis experiment was constructed and is drawn in Fig. 26B. The results show that fraction I hydrolyzes those bonds in which uncharged amino acids supply the amino portion.

A comparison of the DNP-end group data between endopeptidase II and fraction I showed that there was considerable overlap. Since this overlap could be due to the endopeptidase II impurity present with fraction I, it was decided that endopeptidase I would first have to be shown to be homogeneous before any definite conclusions as to specificity could be drawn.

TABLE XVII: Hydrolysis of insulin B-chain by fraction I

	Times of hydrolysis (min.)	
	15	360
DNP-amino acids		
aspartic acid	0.016* (1)**	0.022 (1)
leucine	0.018 (1)	0.034 (2)
phenylalanine	0.036 (2)	0.046 (3)
tyrosine	0.030 (2)	0.032 (2)
valine	0.016 (1)	0.018 (1)
DNP-end groups		
aspartic acid	0.018 (1)	0.022 (1)
glutamic acid	0.016 (1)	0.024 (1)
phenylalanine	0.020 (1)	0.028 (2)
serine	0.016 (1)	0.032 (2)
threonine	0.018 (1)	0.030 (2)
valine	0.034 (2)	0.048 (3)

* Optical density at 360 mμ.

** nearest whole number ratios

DISCUSSION

In order to achieve a better understanding of the proteolytic nature of marine bacteria it was decided to isolate the two extracellular endopeptidases produced by B-207 and determine some of their characteristics.

The purification of endopeptidase II was completely successful in that a homogeneous enzyme was obtained with a 13-fold purification. The overall yield could possibly be improved upon in the future. Although realizing a 7-fold purification of endopeptidase I, fraction I is far from being homogeneous. The contamination by endopeptidase II must be taken into account when the results of characterization studies conducted with fraction I are employed.

There are a number of similarities between the two enzymes which deserve mention. The pH stabilities are identical, their pH optima are near neutrality, they are both heat labile and are inhibited by similar reagents. Both are inhibited by disodium EDTA and unaffected by DFP and may be described as neutral proteinases (18). They would fit into the classification scheme as metalloenzymes (19). Based on the inhibitory effect of L-cystine and oxidized glutathione, a definite requirement for a reduced thiol group is evident. The fact that p-chloromercuribenzoate had no inhibitory effect appears contradictory. However it may be that oxidation of a thiol group to the disulfide

results in a change in structure which could be detrimental to the activity of the endopeptidases.

A major difference between the two endopeptidases was observed with the molecular weight determinations. The higher molecular weight peak isolated from fraction I was found to have a molecular weight of approximately twice that found for the lower molecular weight peak and for endopeptidase II (30,000). It was also apparent, as a result of the chromatographic experiments described in the Experimental Results section, that a definite interaction between the two endopeptidases existed and that the low molecular weight peak isolated from fraction I could be identified with endopeptidase II. An explanation of the results may take several forms. The first suggests that endopeptidase II is a monomer of the dimer, endopeptidase I. Fraction I contains both monomer and dimer as active enzymes. This possibility presumably is eliminated by the results obtained on polyacrylamide gel elctrophoresis. A dimer-monomer relationship requires only two proteins. However, the electrophoresis data show the presence of three bands (two active). Two alternative explanations appear more plausible. The first requires fraction I to contain an active endopeptidase I and the inactive dimer of endopeptidase II as well as some active endopeptidase II. The presence of all three forms accounts for the observed three protein bands. The molecular weight relationship and the observed increase in specific

activity upon rechromatography of fraction I suggests that inactive protein (60,000 MW) is being removed and changed to active (30,000 MW) units. Alternatively, endopeptidase II may be present as an inactive zymogen which, after limited proteolytic action, is converted to an active enzyme with an approximate molecular weight of 30,000. Activation might be through the action of endopeptidase II in the autocatalytic manner of trypsin on trypsinogen (92) or by limited proteolysis with endopeptidase I. In either case, the pro-endopeptidase II (inactive) would be converted to endopeptidase II (active). To decide which of the two explanations is more plausible, the following observations are noteworthy: no higher molecular weight peak was observed on the rechromatography of endopeptidase II; nor was the inactive protein observed (molecular weight of 25-30,000) that would result from limited proteolysis. It is felt by the author that the evidence presented above favors the idea of dimer-monomer interaction. In the attempts to convert one form to another, possibly,the proper conditions were not utilized.

Both endopeptidase I and endopeptidase II can be classed as metalloenzymes and neutral proteinases. Morihara and co-workers have examined the specificity of a number of neutral proteinases (18,93). Their findings show that the neutral proteinases studies possess similar specificities toward the bonds cleaved in the B-chain of insulin. With all of the enzymes tested, cleavage occurred at the peptide

bonds of His-Leu (5-6, 10-11), Ala-Leu (14-15), Tyr-Leu (16-17), Gly-Phe (23-24 and Phe-Phe (24-25). Matsubara, et al. (10) showed that thermolysin attacked more bonds than Morihara reported (18). However, the basic conclusion that the enzymes examined were specific for a neutral amino acid supplying the amino portion of the bond is still valid. The sites of cleavage, as deduced from the amino acid analysis and DNP-end groups of the hydrolyzed peptides, of oxidized B-chain of insulin by endopeptidase II is shown in Fig. 27. There were three major points of cleavage: Phe-Val (1-2), Tyr-Leu (16-17) and Phe-Phe (24-25). In all cases the amino supplying portion of the bond was found to be an uncharged amino acid that did not have a hydroxy or thiol side chain. The amino acid residues that contribute the carboxyl groups to the peptide bond seem to vary, but they all have uncharged side chains. In interpreting the data, it should be pointed out that the low optical densities of the amino acids might cover up additional peptides which would show additional points of cleavage. These however would be minor points of cleavage. Specificity studies, conducted by Morihara (18) with synthetic peptides showed a narrow specificity, requiring a blocked amino- and carboxyl end. A minimum substrate of CBZ-glycyl-L-leucyl amide was determined for the neutral proteinases. The studies with endopeptidase II clearly showed the requirement for specific structures of the substrate. A require-

ment for blocked carboxyl- and amino group was evident. Substitution of an ester for the amide was not as effective possibly because the amide resembles the peptide chain to a greater degree than the ester. Tripeptides were more effectively hydrolyzed than dipeptides. This may be due to a requirement, as was recently found for other neutral proteinases (93), for a larger substrate necessary for proper binding. To properly determine this, a series of tri- and tetrpeptides would have to be examined with all possible amino acid combinations.

The points of cleavage, as determined for endopeptidase II action on the insulin B-chain, indicate the possibility that a number of amino acids could be released if all the bonds possible were hydrolyzed. Since a number of di- and tripeptides were hydrolyzed, it can be visualized that the endopeptidase II first attacks the protein substrate in several places and then hydrolyzes some of the remaining peptides. The heavy arrows under the B-chain of insulin in Fig. 27 indicate where endopeptidase II might hydrolyze this protein based on the DNP-end group analyis shown in Table XIV.

The question of broad specificity is still unanswered, partly through a problem in semantics. Endopeptidase II, and the other neutral proteinases (18), will hydrolyze bonds in which the amino contributing group is an uncharged amino acid, preferentially leucine or phenylalanine. This

constitutes a narrow specificity. However, when it is realized that there are a large percentage of uncharged amino acids in an average potential protein substrate it becomes evident that many peptides result and one says that the proteinase has a broad specificity.

Comparison of the peptide maps and the DNP-end group analysis of the hydrolysis products of the B-chain of insulin between endopeptidase II and fraction I shows more peptides and different end groups present from fraction I hydrolysis. Since endopeptidase I has not been purified, the exact specificity cannot at present be determined.

In conclusion it can be said that B-207 produces two extracellular endopeptidases, both classed as metalloenzymes with similar pH optima but different molecular weights and specificities. Endopeptidase II is a neutral proteinase which possesses a specificity toward the B-chain of insulin similar to many of the previously isolated neutral proteinases. Using short term hydrolysis, it would be possible to use endopeptidase II for structural studies.

PART III

The Aminopeptidase from a New Marine *Pseudomonas*

Preliminary studies described in Part I indicated that marine bacterium B-207 produced an intracellular aminopeptidase. This section of the report deals with its further purification and characterization including a possible salt requirement for activity.

EXPERIMENTAL RESULTS

The aminopeptidase was further purified from fraction A (DEAE-cellulose chromatography in Fig. 18) by taking advantage of the differential heat stability between it and the endopeptidases. After the tubes representing fraction A were pooled, the mixture was heated to 60°C and held at that temperature until all of the remaining endopeptidase protein had been denatured. On a number of occasions, when larger quantities of aminopeptidase were desired, the crude unchromatographed enzyme mixture was heated and all of the endopeptidases denatured. Recovery of aminopeptidase varied from 55 to 95%, depending upon the protein concentration and the magnitude of the initial enzyme activities. After cooling at 4°C for 1-2 hours, the coagulated protein was removed by centrifugation at 10,000 rpm for 10 minutes. The supernatant was concentrated with a Diaflo ultrafiltration apparatus or by precipitation with 70% ammonium sulfate.

The impure aminopeptidase concentrate containing denatured protein and active aminopeptidase enzyme was

then brought to an approximate concentration of 50-75 mg protein/ml with 0.02 M Tris-HCl buffer at pH 8.4. A 1-2 ml aliquot was placed on the top of a Sephadex G-75, G-100, or G-200 column and chromatographed with 0.02 M Tris-HCl buffer (pH 8.4) as the elutant. Gel-filtration, depicted

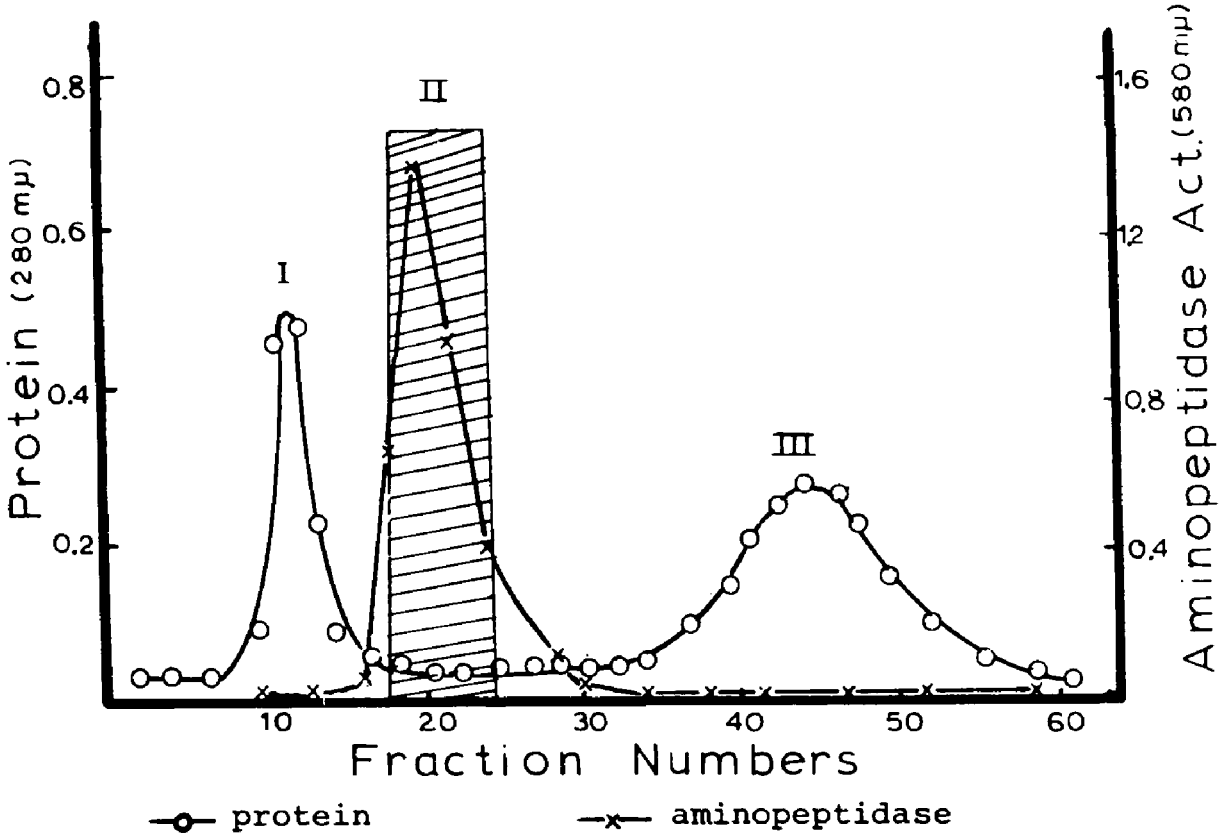
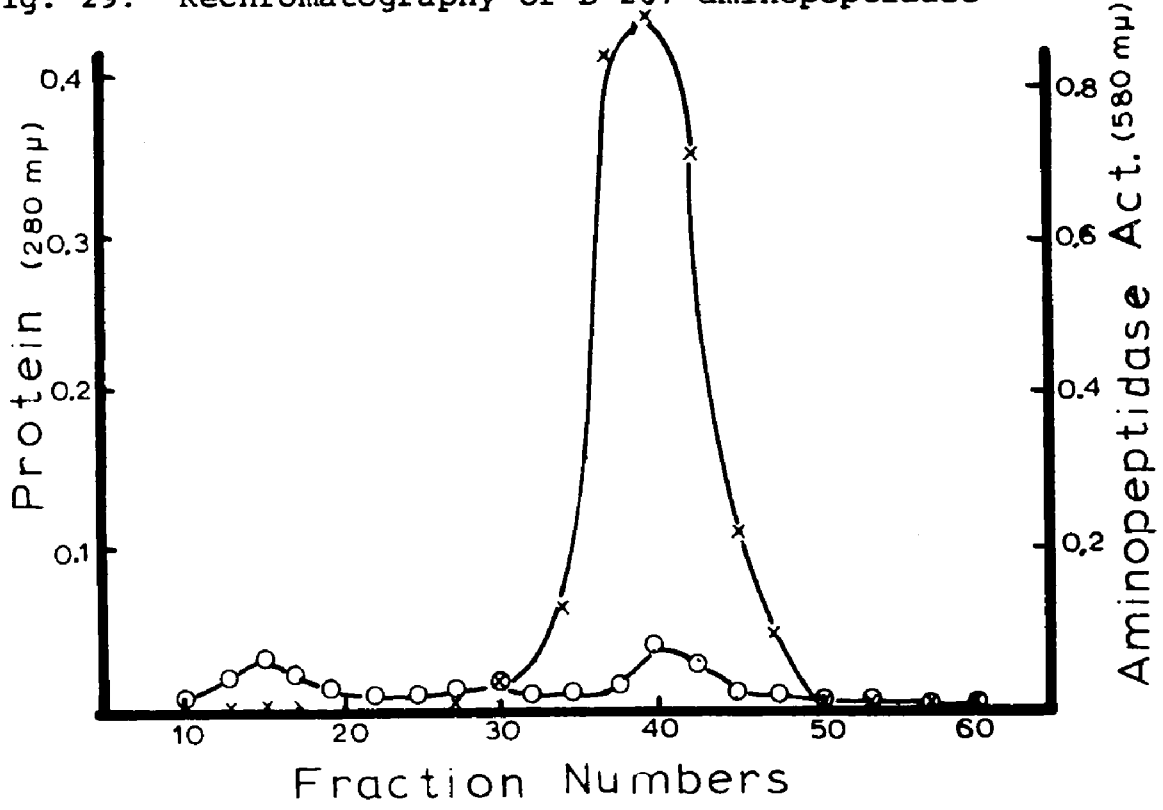


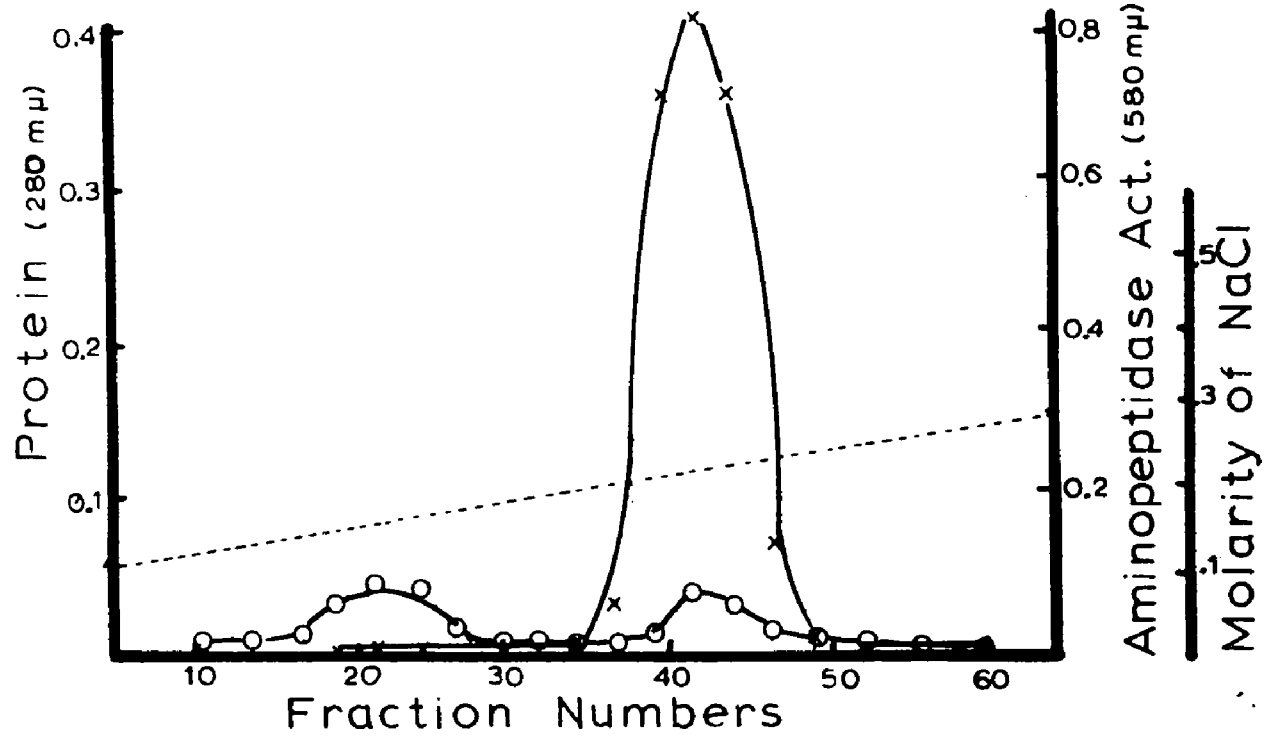
Fig. 28: Gel-filtration on Sephadex G-100 of crude aminopeptidase with 0.02 M Tris-HCl buffer (pH 8.4)

in Fig. 28, separated the inactive protein (I) from the active aminopeptidase (II). A yellowish pigment (III) was also observed as a low molecular weight fraction. The tubes denoted by the hatched lines were pooled and concentrated by precipitation with 70% ammonium sulfate or by ultrafiltration. The aminopeptidase was again brought to

Fig. 29: Rechromatography of B-207 aminopeptidase



A: Sephadex G-200 with 0.02 M Tris-HCl buffer (pH 8.4)



B: DEAE-Sephadex with a linear gradient on 0.02 M Tris-HCl buffer (pH 8.4) from 0.05-0.25 M NaCl

-O- protein -*- aminopeptidase

TABLE XVIII : Isolation and purification of B-207 aminopeptidase

Procedure	Volume (ml)	Total Aminopeptidase* units	SA	Protein tot. mg Lowry method	% Yield* Amino.
1. Fraction A after heating	460	2,900	12.8	225	36.2
2.2. After concentration	25	2,250	10.3	219	28.2
3. Sephadex G-100 total applied	1.0	90	10.3	9.3	---- 100
**II	35	67	24.2	2.8	---- 74
4. Lyophilization *** before	1 mg/ml	24	24	1	---- 100
after	1 mg/ml	15.5	15.5	1	---- 64

* units described in Experimental Procedures; SA represents specific activity; percent yield is based on the initial cell-free medium.

** fraction corresponds to Fig. 28

*** calculated on the basis of the recovery after Sephadex G-100 chromatography assuming an actual concentration to 1 mg/ml.

an approximate concentration of 50 mg protein/ml with 0.02 M Tris-HCl buffer (pH 8.4) and rechromatographed on Sephadex G-200 (Fig. 29A) or DEAE-Sephadex (Fig. 29B). One minor inactive high molecular weight peak was observed with the active aminopeptidase. This high molecular weight peak appeared to be eluted with the void volume(Sephadex G-200 chromatography), indicating possibily an aggregated fraction. The active aminopeptidase was collected, dialyzed for 6 hours against 10^{-3}M Tris-HCl buffer (pH 8.0) at 4°C and used for characterization studies or lyophilized and stored at freezer temperatures. A summary of a typical purification scheme is given in Table XVIII.

The aminopeptidase was shown to be homogeneous on the basis of observing a single, uniform, active peak on Sephadex G-75, G-100, G-200 and DEAE-Sephadex chromatography.

Fig. 30: Tracings of Coomassie Brilliant Blue stained polyacrylamide gel electrophoresis patterns of crude enzyme mixture (A) and pure aminopeptidase (B).

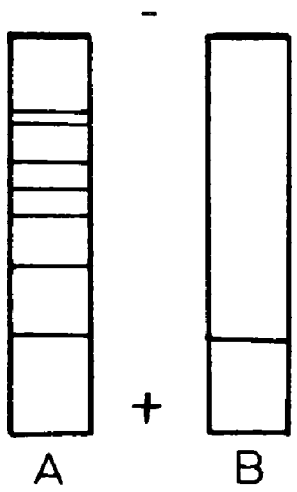
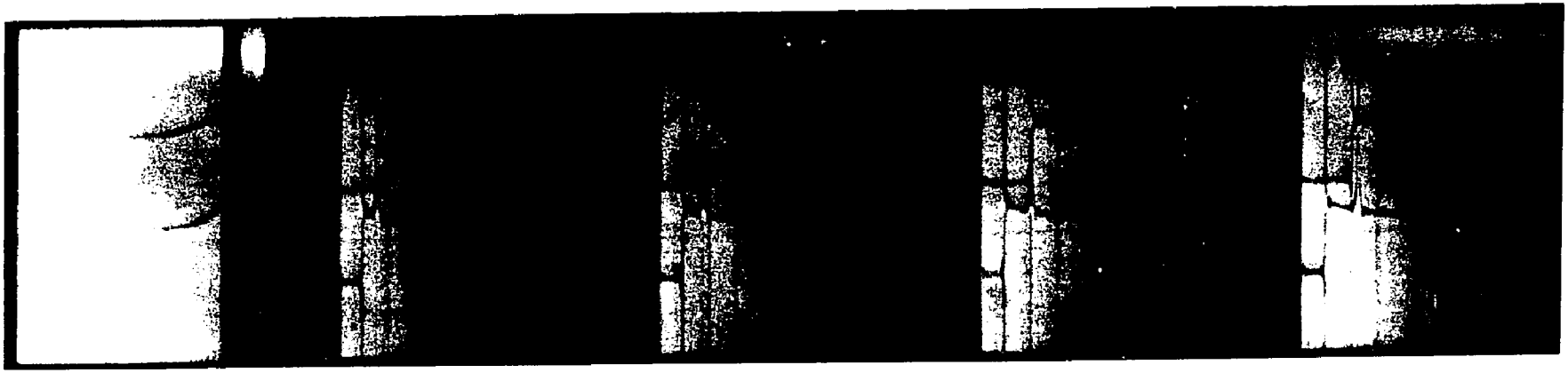
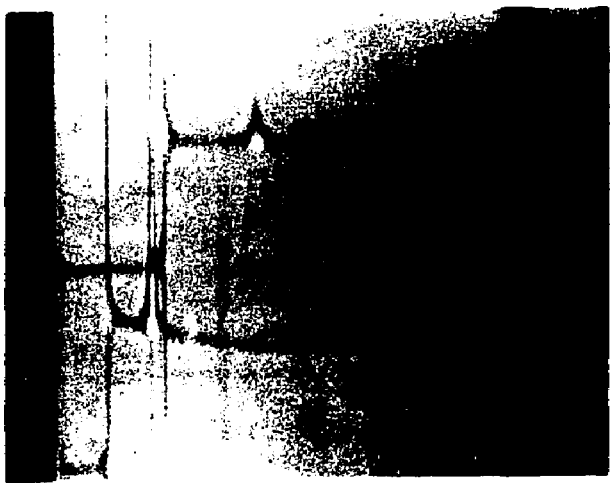


Fig. 31: Ultracentrifugal Schlieren patterns for the B-207 aminopeptidase in 0.1 M NaCl. Analyzer angle of 55°. Photographs taken at the times indicated after attaining full speed of 60,000 rpm. (A) the complete run (B) enlargement of the pattern observed at 52 minutes after speed (lower band is standard cell, upper band is wedged cell)



A 20 min. 36 min. 52 min. 68 min. 84 min.



B

Homogeneity was verified by the demonstration of a single band of active aminopeptidase on polyarcylamide gel electrophoresis (Fig. 30) and a single band in 0.1 M sodium chloride solutions when sedimented in an ultracentrifugal field (Fig. 31).

A sample of the aminopeptidase was crystallized from 50% ammonium sulfate and from 65% acetone. Typical crystals prepared from the acetone crystallization are shown in Fig. 32.

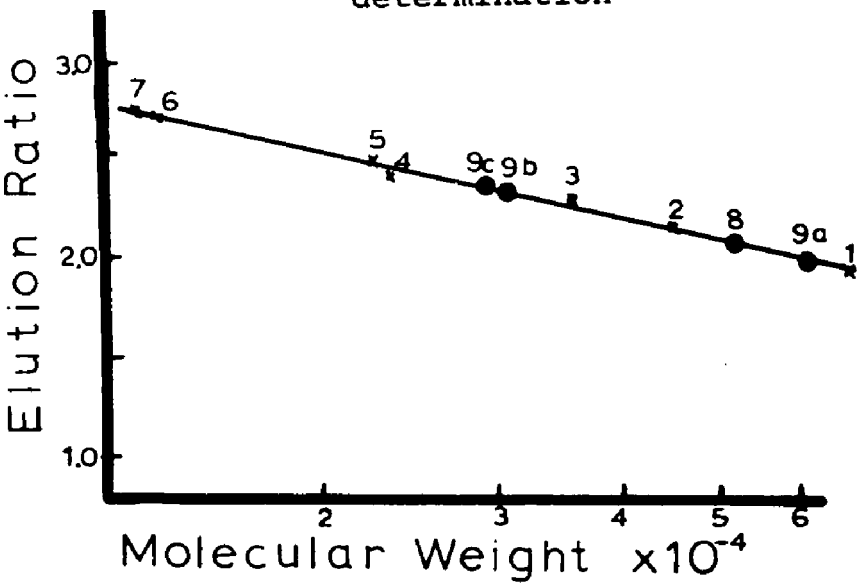
Fig. 32: Crystals of homogeneous B-207 aminopeptidase. Crystallized from 65% acetone. (A) typical field at 320 x mag. (B) 375 x mag.

TABLE XIX: Protein elution ratios

	Protein	Molecular Weight	Elution Ratio*
1.	Hemoglobin	68,000	1.91
2.	Ovalbumin	45,000	2.12
3.	Pepsin	35,500	2.23
4.	Trypsin	23,800	2.38
5.	Chymotrypsin	22,500	2.47
6.	RNase	13,600	2.65
7.	Cytochrome C	13,000	2.71
8.	B-207 Aminopeptidase	------	2.01
9.	B-207 Endopeptidases		
	(a) fraction I--high mw	------	1.97
	(b) low mw	------	2.31
	(c) Endopeptidase II	------	2.38

* Elution ratios were determined from the elution volume of the protein and the elution volume of an excluded high molecular weight polysaccharide.

Fig. 33: Calibration curve for molecular weight determination



The molecular weight of the aminopeptidase was determined by two methods: Gel-filtration on a Sephadex G-200 column as described in the Experimental Procedures section and by sedimentation velocity measurements with calibrated photographs of Schlieren patterns. The elution ratios of the proteins and enzyme fractions are given in Table XIX. Determination of the unknown molecular weights

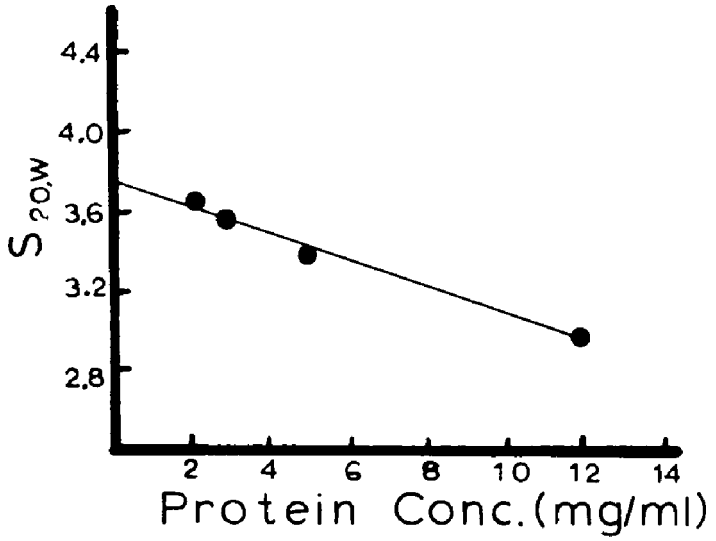


Fig. 34: Concentration dependence of the sedimentation coefficient of the B-207 aminopeptidase

was accomplished by plotting the log of the molecular weight vs. the elution ratio (Fig. 33).

By using four concentrations of the aminopeptidase in 0.1 M sodium chloride, pH 7.5, sedimentation velocity data from several ultracentrifuge runs with Schlieren optics were manipulated (75) to give a plot of corrected sedimentation constant, $S_{20,w}$, vs. protein concentration (Fig. 34). When the line was extrapolated to infinite dilution

TABLE XX: Amino acid composition of the B-207 aminopeptidase

Amino acid	Amino acid residues (g/loo g protein) acid.1	acid.2	alk.1	average**	Residues per 50,000 Mol. Wt.
lysine	6.24	7.37	5.22	6.81	27
histidine	2.83	3.44	3.68	3.18	12
arginine	1.94	2.65	----	2.29	7
aspartic acid	10.24	9.13	8.76	9.68	44
threonine	4.04	4.32	3.86	4.18	23
serine	7.44	6.87	5.12	7.15	41
glutamic acid	8.67	7.94	7.48	8.31	32
proline	6.10	5.88	4.88	5.99	31
glycine	8.80	8.97	8.54	8.89	78
alanine	4.52	4.66	4.42	4.58	32
cysteine	o.00	0.00	0.00	0.00	0
valine	4.96	4.56	4.64	4.86	25
methionine	7.98	7.62	5.88	7.78	30
isoleucine	4.74	4.88	4.76	4.81	21
leucine	5.12	4.98	4.86	5.05	22
tyrosine	4.10	3.86	3.56	3.98	12
phenylalanine	3.66	4.02	3.45	3.84	13
tryptophan	----	----	2.24	2.24	6
ammonia*	----	----	----	----	
glucosamine	0.26	0.41	----	0.34	
				93.60	

* Results from the ammonia peak yielded inconclusive data.
** Average values in g/100g protein calculated from the acid hydrolyzate data. Tryptophan calculated from the alkaline hydrolysis data. Glucosamine not added into total recovery.

of protein, a value of $S_{20,w} = 3.75$ was determined. A molecular weight of 48,600 was calculated and found to be in close agreement with the gel-filtration value of approximately 51,000.

The results of the amino acid analyses are tabulated in Table XX. Two acid hydrolyzed (6 N HCl) and one alkaline hydrolyzed (4 N $Ba(OH)_2$) sample were analyzed. No corrections were made for possible loss through the decomposition of serine, threonine, cystine, tyrosine

and proline or through the incomplete liberation of valine, isoleucine and leucine. Since no peaks were observed, cysteine and its derivatives were assumed to be absent. Tryptophan was determined by quantitatively comparing the area under the peak with that determined for leucine in the alkaline hydrolyzed sample. The value for methionine was found not to be accurate because a buffer induced change in the sensing was reflected in the peak size. Since allo-isoleucine arose from the isoleucine, its integrated value was added on to that of isoleucine. An unknown peak was observed prior to the elution of lysine (possibly hydroxy-lysine). It was not added into the totals.

The presence of glucosamine in the amino acid analysis and neutral hexoses (approximately 1.2% with the phenol-sulfuric acid reagent) indicated that the purified amino-peptidase was a glycoprotein.

The results of two separate N-terminal amino acid analysis on the aminopeptidase resulted in DNP-ε-lysine and DNP-α-his or di-DNP-his (these latter two could not be resolved). These results suggested that either no free amine group was present, suggesting that the enzyme had a blocked N-terminal, or that histidine was the N-terminal amino acid.

Metal analysis for zinc, calcium and magnesium by atomic absorption (Table XXI) was done on the purified aminopeptidase under several conditions. Based on an

TABLE XXI: Atomic absorption analysis of the B-207 aminopeptidase

solvent	protein conc.* (mg/ml)	metal	metal conc** (ppm)
distilled water	1.12	zinc	1.46
		calcium	0.15
distilled water	0.280	zinc	0.31
		calcium	0.09
		magnesium	0.12
dialyzed enzyme (72 hours)	0.276	zinc	0.29
		calcium	0.01
		magnesium	0.00
Tris-HCl, 5×10^{-1} M	0.100	zinc	0.12
5×10^{-3} M	0.100	zinc	0.10
5×10^{-5} M	0.100	zinc	0.09
5×10^{-7} M	0.100	zinc	0.11

* protein concentration was determined by weighing an aliquot after drying at 105°C to constant weight.

** metal conentration was the calculated difference between the sample and the blank in parts per million (ppm).

approximate molecular weight of 50,000, the average content of zinc was approximately one atom per mole of protein. Calcium and magnesium were also found in the pure aminopeptidase but at levels less than one atom per mole of protein. No significant amount of cobalt was found. It should be noted that after dialysis for 72 hours at 4°C, against 4 changes of distilled water, the zinc content remained the same while the calcium and magnesium decreased. When the Tris-HCl buffer (pH 8.0) concentration was lowered while the enzyme concentration remained the same, the zinc concentration was found to remain constant. Only in the 5×10^{-1} M Tris-HCl control was any appreciable zinc found.

The pure aminopeptidase was subjected to similar characterization studies as were previously described for the endopeptidase fractions and the protease mixture, and these are described below.

The effect of pH on the aminopeptidase activity is shown in Fig. 35. A narrow optimum between pH 6.8 and 8.8 was observed with a maximum at pH 8.1. The enzyme was found to exhibit a pH stability similar to that observed with the protease mixture (Fig. 36).

The effects of activators and inhibitors on the pure aminopeptidase are shown in Table XXII. Only the metal

TABLE XXII: Effects of activators and inhibitors on the B-207 aminopeptidase

Activator or inhibitor	Aminopeptidase activity*
$CaCl_2$	101
$CoCl_2$	94
$CuSO_4$	35
$FeSO_4$	100
$FeCl_3$	102
$MgCl_2$	104
$MnSO_4$	98
$NiCl_2$	74
$ZnCl_2$	104
p-chloromercuribenzoate	102
Iodoacetamide	95
Glutathione (oxidized)	97
Glutathione (reduced)	99
L-cystine	101
Potassium permanganate	96
Ascorbic acid	98
KCN	72
EDTA-Na_2	56
8-hydroxyquinoline	62
L-cysteine	74
DFP	96

* 1×10^{-3}M of reagent and 0.5 mg protein/ml in 0.02M Tris-HCl buffer (pH 8.0). Preincubated for 15 min. at 25°C.

** Percent of the original activity.

Fig. 35: Effect of pH on B-207 aminopeptidase activity

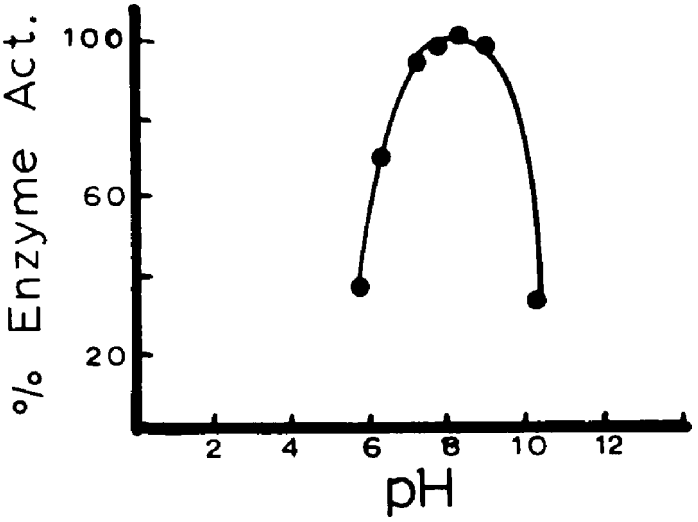


Fig. 36: Effect of pH on the stability of the B-207 aminopeptidase

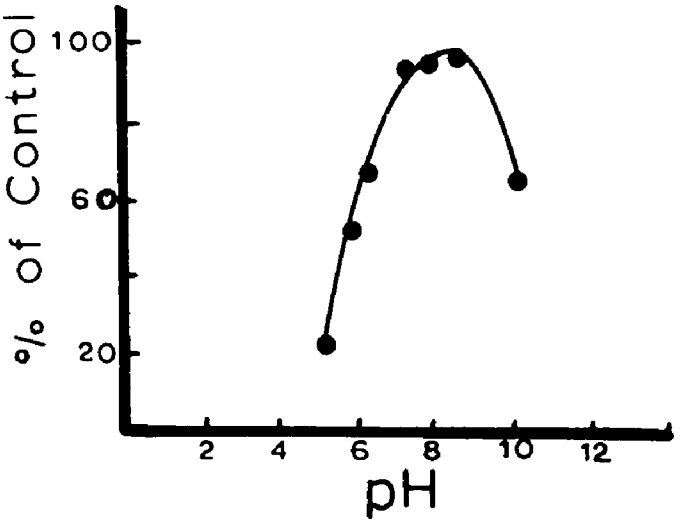


TABLE XXIII: Reactivation of disodium EDTA inhibited B-207 aminopeptidase*

Metal	Aminopeptidase activity**
$CaCl_2$	18
$CoCl_2$	43
$FeSO_4$	22
$FeCl_3$	24
$MgCl_2$	16
$MnSO_4$	14
$NiCl_2$	8
$ZnCl_2$	66
distilled water	6

* 1×10^{-3} M of metal ion and 0.5 mg protein/ml after the enzyme was dialyzed for 6 hours against 10^{-3} M Na_2EDTA, followed by 6 hours dialysis against distilled water. Preincubated for 15 min. at 25°C.

** Percent of original activity

chelating agents had any effect and they were of an inhibitory nature. Reactivation after disodium-EDTA inhibition was best accomplished with zinc and to a lesser extent by cobalt (Table XXIII).

Iodoacetic acid has been found to effect different amino acid residues in enzymes at different pHs (94). The effect of 1×10^{-3} M and 5×10^{-4} M concentrations of the iodoacetic acid on the aminopeptidase was studied at pH 2.8, 5.5 and 8.5. A decrease in activity was observed at pH 5.5 while no effect relative to the control was noted at pH 2.8 and 8.5. The effective K_M for the inhibited aminopeptidase was increased (5.68×10^{-4} M with 10^{-3} M iodoacetic acid when compared to 3.34×10^{-4} M without inhibition) while the maximum velocity decreased by approximately 65%

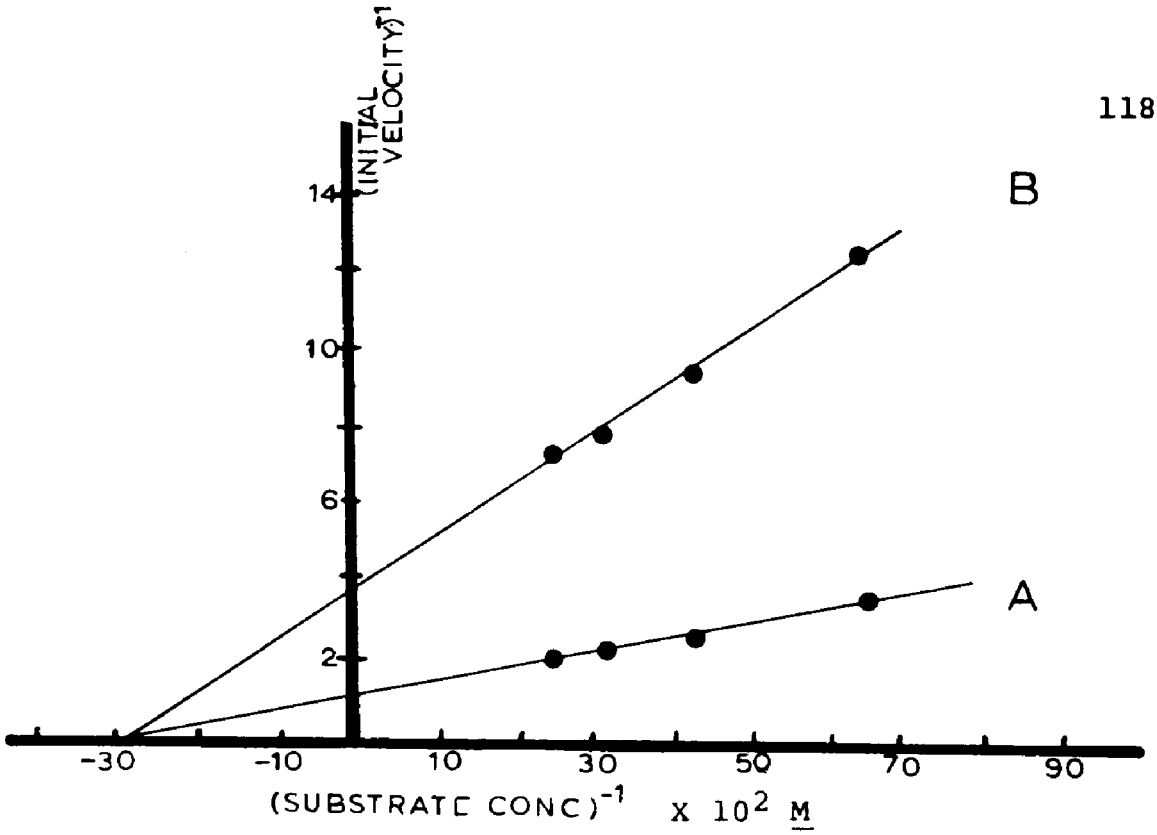


Fig. 37: Determination of the K_M for the B-207 aminopeptidase acting on LNA. (A) before dialysis; (B) after 48 hr. dialysis against distilled water.

The Michaelis constant, K_M, was determined for the aminopeptidase-catalyzed hydrolysis of LNA as substrate in the concentration range 0.1 to 1.0 x 10^{-3} M. A number of other amino acid B-naphthylamides were examined in the same range of concentrations. The calculated K_M for LNA hydrolysis was determined to be 3.3 x 10^{-4} M by a Lineweaver-Burk plot (71) (Fig. 37). Following a 48 hour dialysis against distilled water at 4°C, the K_M was determined and found to be 3.38 x 10^{-4} M, in close agreement with those previously determined. The maximum velocity of the reaction however, decreased sharply to 25% of the previous determinations. The K_M and V_{max} of the several amino acid-B-naphthylamides are shown in Table XXIV.

TABLE XXIV: Hydrolysis of amino acid-B-naphthylamides*

amino acid B-naphthylamide	percent hydrolysis	relative rate of hydrolysis	K_1 ($\times 10^3$)	K_m ($\times 10^4$) M	Vmax (OD/min)	C_1**
alanine	7	11	3.15	45.5	0.05	1.26
aspartic acid	5	8	2.20	----	----	0.88
asparagine	9	13	4.14	----	----	1.65
glutamic acid	6	8	2.71	----	----	1.09
isoleucine	4	6	1.78	33.4	0.019	0.71
leucine	63	100	43.3	3.82	0.10	17.3
ornithine	3	5	1.36	----	----	0.55
phenylalanine	32	48	16.8	5.05	0.067	6.7
serine	2	4	0.94	----	----	0.38
tyrosine	3	4	1.36	----	----	0.55
valine	13	22	6.07	18.5	0.034	2.43

* 1×10^{-3} M of substrate in 0.02 M Tris-HCl buffer, pH 8.0

** C_1 refers to the first order proteolytic coefficient ($C_1 = K_1$/ mg enzyme protein).

The effect of pH on the K_M was determined in the range 5.75 to 8.95. The results, depicted in Fig. 38, revealed two inflection points near pH 7.0-7.3 and 8.25-8.4.

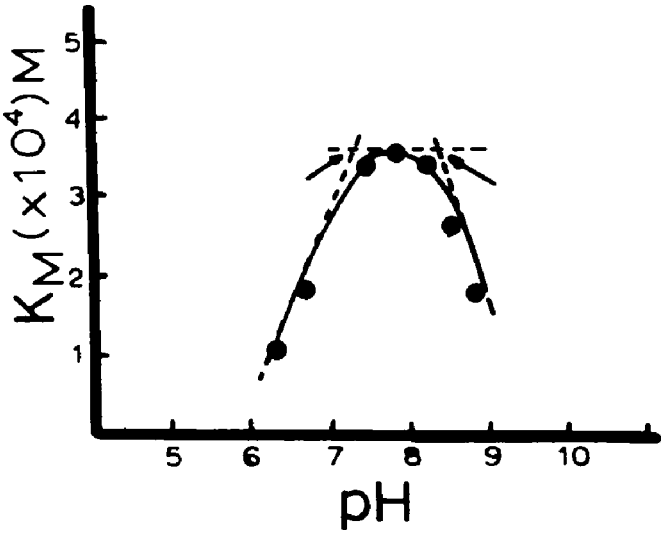


Fig. 38: Effect of pH on the K_M of the aminopeptidase-catalyzed hydrolysis of LNA.

The amino acid specificity of the aminopeptidase was determined with amino acid-B-naphthylamides and di-and tri-peptides. Tables XXIV and XXV list the results in terms of relative rate of hydrolysis (the most susceptible bond being denoted as 100%), the first order rate constant (K_1) and the proteolytic coefficient (C_1) described by Hill, et al. (73). It is readily apparent that L-leucine is the preferred N-terminal amino acid. With the exception of serine and tyrosine, the uncharged amino acid residues were preferred to those ionically charged. This was further emphasized by the comparison, although slight, between L-asparagine and aspartic acid. The influence of the pen-

TABLE XXV: Hydrolysis of synthetic peptides by the B-207 aminopeptidase*

Substrate	K_1 ($x10^3$)**	
L-leucyl-L-leucine	3.9	
L-leucyl-L-leucine acetate	4.4	
L-leucyl-L-leucine methyl ester	4.7	
L-leucyl-L-leucine amide	8.2	
L-leucyl-D-leucine	0.6	
D-leucyl-L-leucine	0.0	
L-leucyl-L-alanine	1.7	
L-leucylglycine	1.2	
L-leucyl-L-isoleucine	3.0	
L-leucyl-L-phenylalanine	2.2	
L-leucyl-L-proline	0.4	
L-leucyl-L-serine	1.4	
L-leucyl-L-tyrosine	1.5	
L-leucyl-L-valine	3.4	
L-leucyl-L-valine acetate	3.9	
L-alanylglycylglycine	0.9	ala+gly+glygly
glycylglycylglycine	0.4	gly+glygly
L-leucylglycylglycine	1.8	leu+gly+glygly
L-leucyl-L-tyrosyl-L-leucine	2.0	leu+tyr+leutyr
CBZ-L-leucyl-L-leucine amide	0.0	
CBZ-L-leucyl-L-tyrosine	0.0	
L-leucinamide***	5.8	

* 1×10^{-3} M of each substrate with 25 ug/ml of enzyme incubated at 37°C for 1 hour (pH 8.0)

** calculated from percent hydrolysis based on ninhydrin color of acid hydrolyzed sample. Chromatographic results are given for the tripeptides.

*** determined by the method of Mitz and Schlueter (111)

ultimate amino acid on the susceptibility of the terminal peptide bond appeared to be the result of a molecular size effect. Small amino acids (glycine, alanine, and serine) and large ones (phenylalanine and tyrosine) were not attacked as readily as were L-leucine, L-isoleucine and L-valine. A requirement for L-amino acids was inferred since neither D-leucyl-L-leucine nor L-leucyl-D-leucine were hydrolyzed

to any extent. The slight hydrolysis observed with L-leucyl-D-leucine might be due to the size rather than spacial requirements of the aminopeptidase for hydrolysis. The lack of hydrolysis of CBZ-L-leucyl-L-leucine amide and CBZ-L-leucyl-L-tyrosine indicated the requirement for an unblocked amino group. The relatively large rate of hydrolysis of L-leucyl-L-leucine amide over the L-leucyl ester derivatives was probably due to the additional hydrolysis of the L-leucinamide. The fact that di-and tripeptides and L-leucinamide were hydrolyzed as well as the amino acid-B-naphthylamides indicates that what has been referred to as an aminopeptidase is indeed an aminopeptidase and not just an arylamidase (95).

To further test the specificity of the aminopeptidase, native bovine insulin and native pancreatic ribonuclease were subjected to hydrolysis by this enzyme. Samples were withdrawn at various times and chromatographed in one dimension with silica gel G layers as described in the Experimental Procedures section. Drawings of the insulin hydrolyzates are depicted in Fig. 39. No amino acids were liberated by the aminopeptidase from native ribonuclease. Hydrolysis of native insulin appeared to progress until the Gln-His (4-5) of the B-chain and the Glu-Gln (3-4) or Gln-Cys-(4-5) of the A-chain were reached. Since glutamic acid was observed on the chromatograms and no distinction between glutamic acid and glutamine could be made, no

conclusions concerning the extent of hydrolysis could be reached. It was however, apparent that hydrolysis was definitely halted at the His (5) of the B-chain and Cys-(6) of the A-chain and that the B-chain was more susceptible than the A-chain. Possible differences between the hydrolysis of of the A- and B-chains might arise from differences in susceptibility between glycine and phenylalanine (the two end groups, respectively) as was evidenced from the previous specificity studies. Since L-lysine is the known end group of native ribonuclease (91), it did not come as a surprise to find that the B-207 aminopeptidase did not attack it.

In view of the decrease in activity observed when the aminopeptidase was subjected to dialysis against distilled

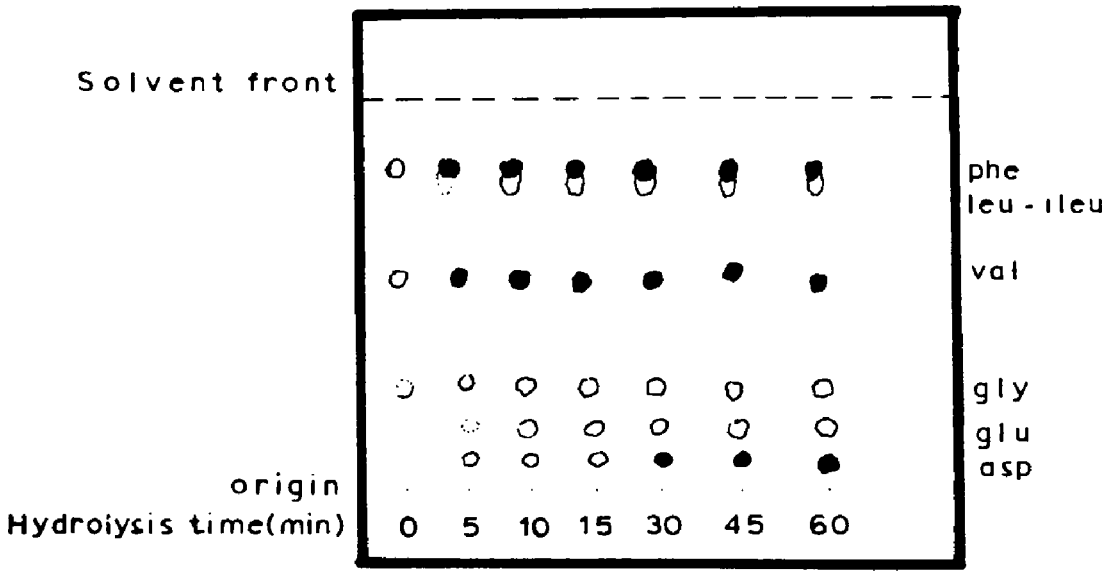


Fig. 39: Hydrolysis of native insulin by B-207 aminopeptidase at 37°C for various times. Sprayed with ninhydrin reagent.

water, a number of experiments were carried out which examined various effects of dialysis on stability and structure. The results of the stability studies are illustrated in Figs. 40-43. The initial activity of the enzyme was reduced as the ionic strength of the solvent was reduced below 0.1 M concentration. It is also apparent, that the activity remaining after 72 hours at 4°C was least at the lowest ionic strength. Although exhibiting the greatest stability, an ionic strength above 0.1 M concentration results in enzyme inhibition. Thus, there appears to be an optimum salt concentration between 0.1 and 0.01 M. The observed effect was not due to the amine of the Tris-HCl buffer because similar effects were noted in both phosphate buffer and NaCl (all at pH 8.0). In addition to affecting the stability of the enzyme at 4°C, the heat stability of the aminopeptidase was also adversly effected by low ionic strength solutions. Figure 43 demonstrates that exposure to 60°C after dialysis against decreased ionic strength solutions resulted in increased denaturation when monitored as decreased enzymatic activity.

Ultraviolet difference spectra in the range 300-190 mμ were recorded for the same solutions that were subjected to stability studies. The sprectra were recorded at various ionic strengths with the aminopeptidase solution in the sample cell and the salt solution with a proper amount of distilled water instead of enzyme in the reference cell.

Fig. 40: Stability of the B-207 aminopeptidase at 4°C in Tris-HCl buffer, pH 8.0

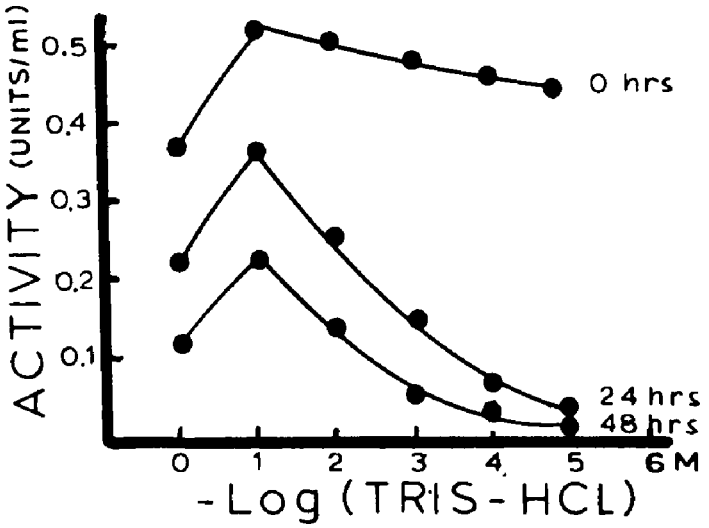


Fig. 41: Stability of the B-207 aminopeptidase at 4°C in sodium chloride, pH 8.0

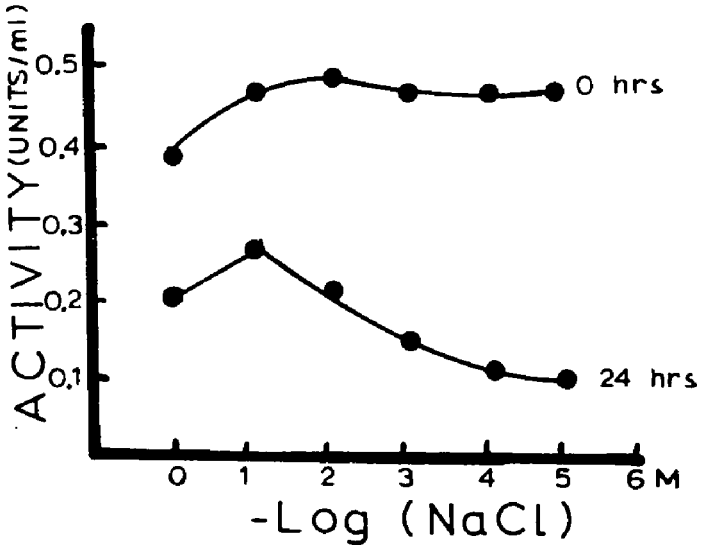


Fig. 42: Stability of the B-207 aminopeptidase at 4°C in phosphate buffer (pH 8.0)

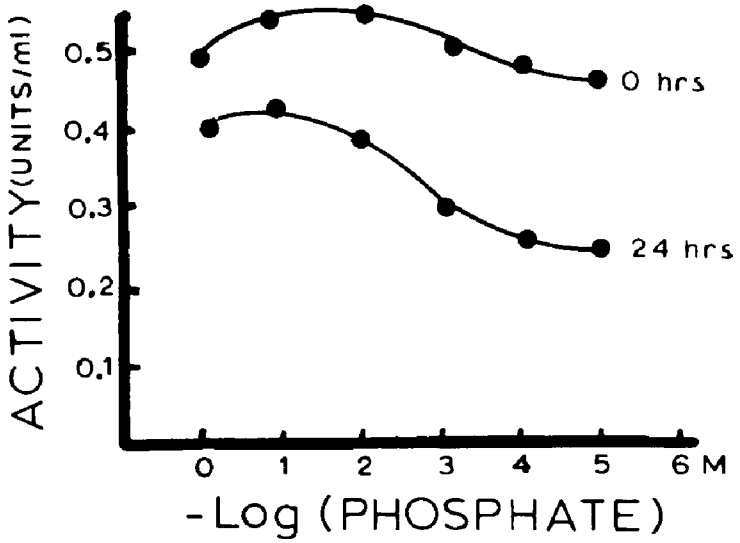
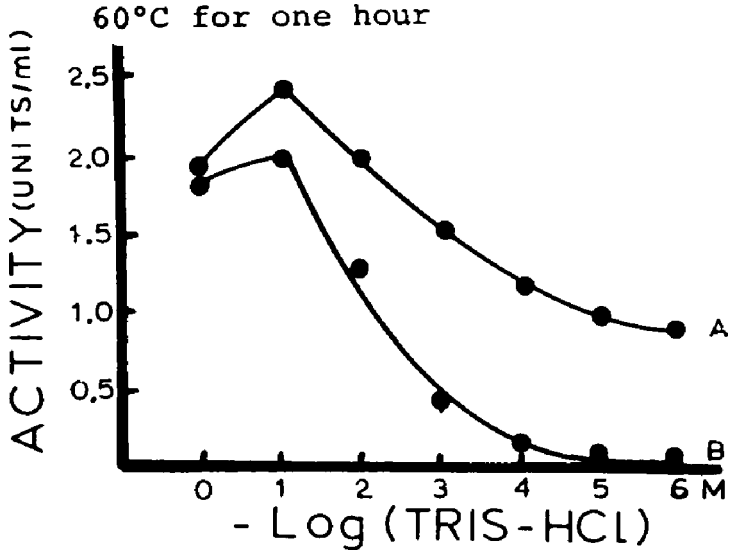


Fig. 43: Stability of the B-207 aminopeptidase after dialysis against Tris-HCl buffer at pH 8.0 for 18 hours at 4°C. (A) unheated; (B) heated at 60°C for one hour



The effects of several concentrations of Tris-HCl buffer (pH 8.0) on the spectrum of the aminopeptidase were primarily noted in the far-ultraviolet region (Fig 44). The absorption peak observed for the aminopeptidase in the dilute buffer was below 200 mμ (using diluted aminopeptidase, it was observed at 185.5-186 mμ) while the peak in the more concentrated buffer was decreased in absorption and shifted toward the red at approximately 212 mμ. A shoulder at 212 mμ is evident for the middle concentration of buffer. Similar results were obtained with concentrations of phosphate buffer (pH 8.0) and sodium chloride solutions at pH 7.0 and 8.0. However, the shift observed with the 0.1 M concentration did not always have the same absorption or maximum wavelength (Fig. 45). The effects of 8M urea and 8M guanidine HCl were also examined but no major differences were noted form the spectra obtained in the 0.1 M salt solutions. The difference spectrum between the aminopeptidase in distilled water and in 0.1 M NaCl (pH 8.0) for two protein concentrations are recorded in Fig. 46. The readings below 210 mu are not valid since the sodium chloride begins to absorb in that vicinity.

Since the effect on the ultraviolet spectrum of the aminopeptidase was observed in the three salt solutions, it was decided to examine the effect of sodium chloride on the optical rotatory dispersion and circular dichroism of

Fig. 44: Ultraviolet spectra of the B-207 aminopeptidase in Tris-HCl buffer (pH 8.0); 20 µg protein/ml.

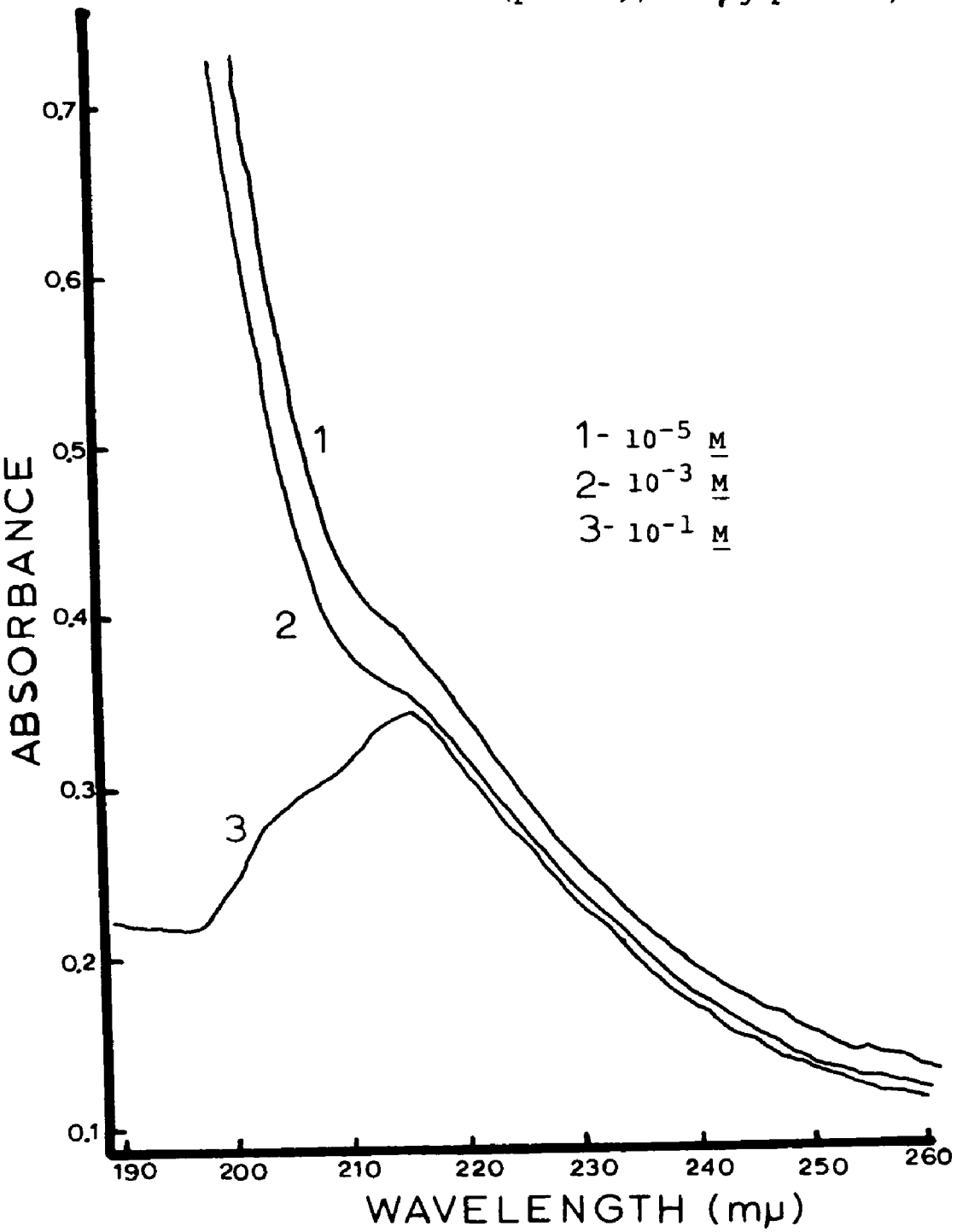
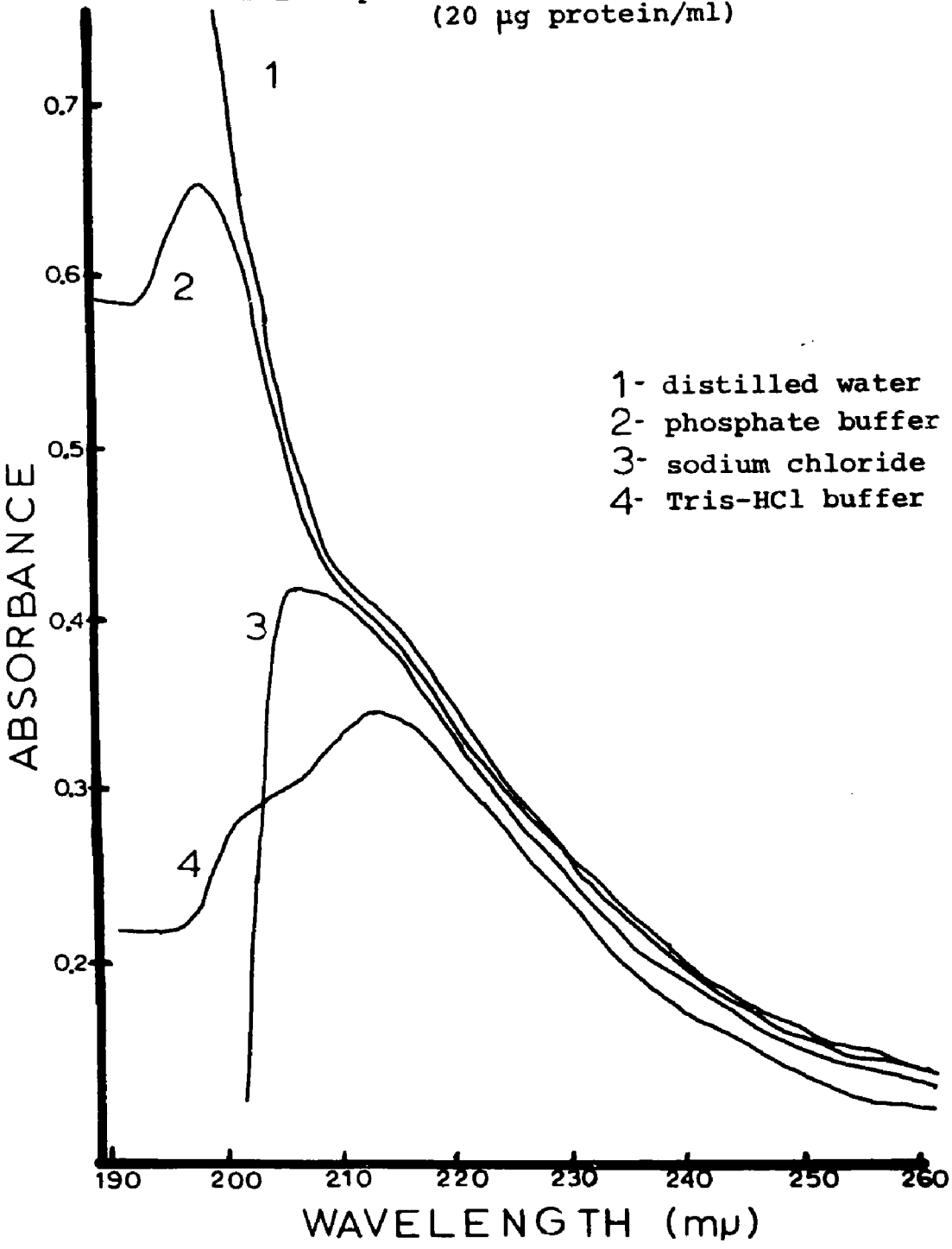


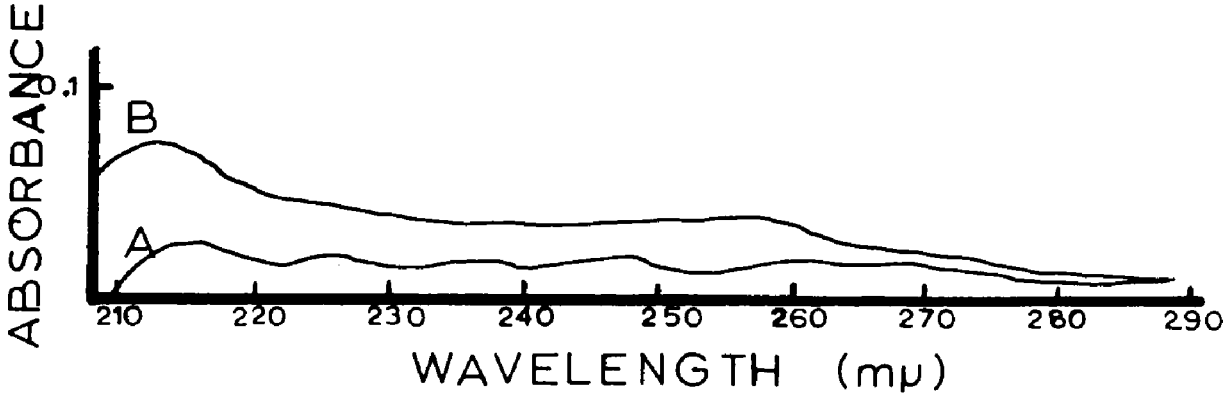
Fig. 45: Effects of salts (10^{-1}M) on the ultraviolet spectrum of the B-207 aminopeptidase. (20 µg protein/ml)



the aminopeptidase in solution. The resultant spectra are recorded in Figs. 47 and 48. The ORD spectra show a broadening of the trough at 220-225 mμ and a disappearance of the peak at about 200 mμ. The spectra of the enzyme in 10^{-5} M NaCl and distilled water were similar to the spectrum of the disorganized form of poly-α-L-glutamate (110). The CD spectra exhibit a decrease in the minimum around 216 mμ and the maximum below 200 mμ as the ionic strength of the solution is lowered. A small minimum appeared in the region of 236-240 mμ in the CD spectrum of the distilled water sample.

The possibility of aggregation occurring during the equilibration of the aminopeptidase in distilled water was examined by gel-filtration studies on Sephadex G-100 superfine columns (1.3 x 73 and 1.8 x 127 cm). The columns were first equilibrated with distilled water and samples

Fig. 46: Difference spectra between aminopeptidase in distilled water vs. 0.1 M NaCl (pH 8.0). (A) 80 μg protein/ml; (B) 20 μg protein/ml



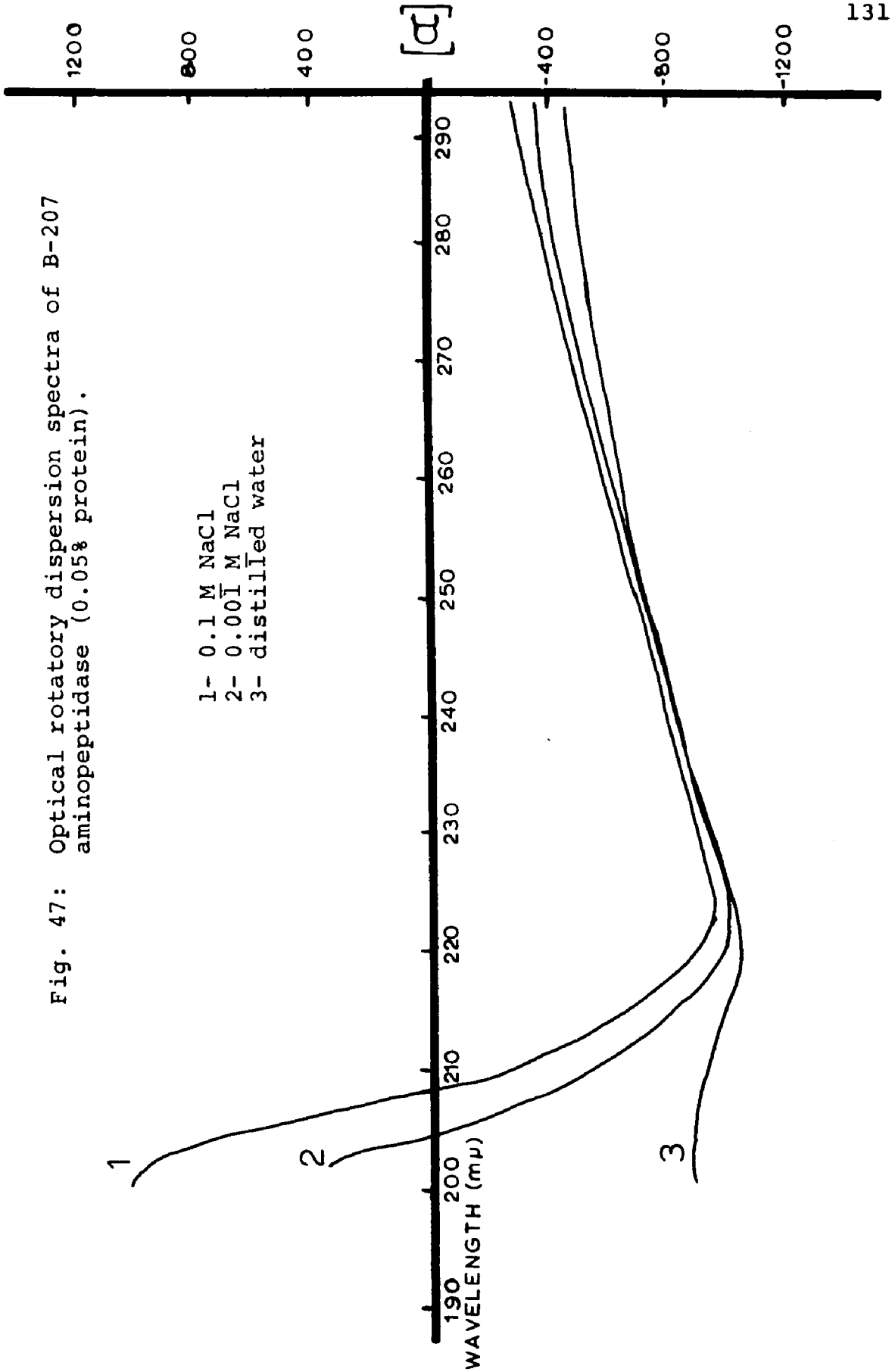
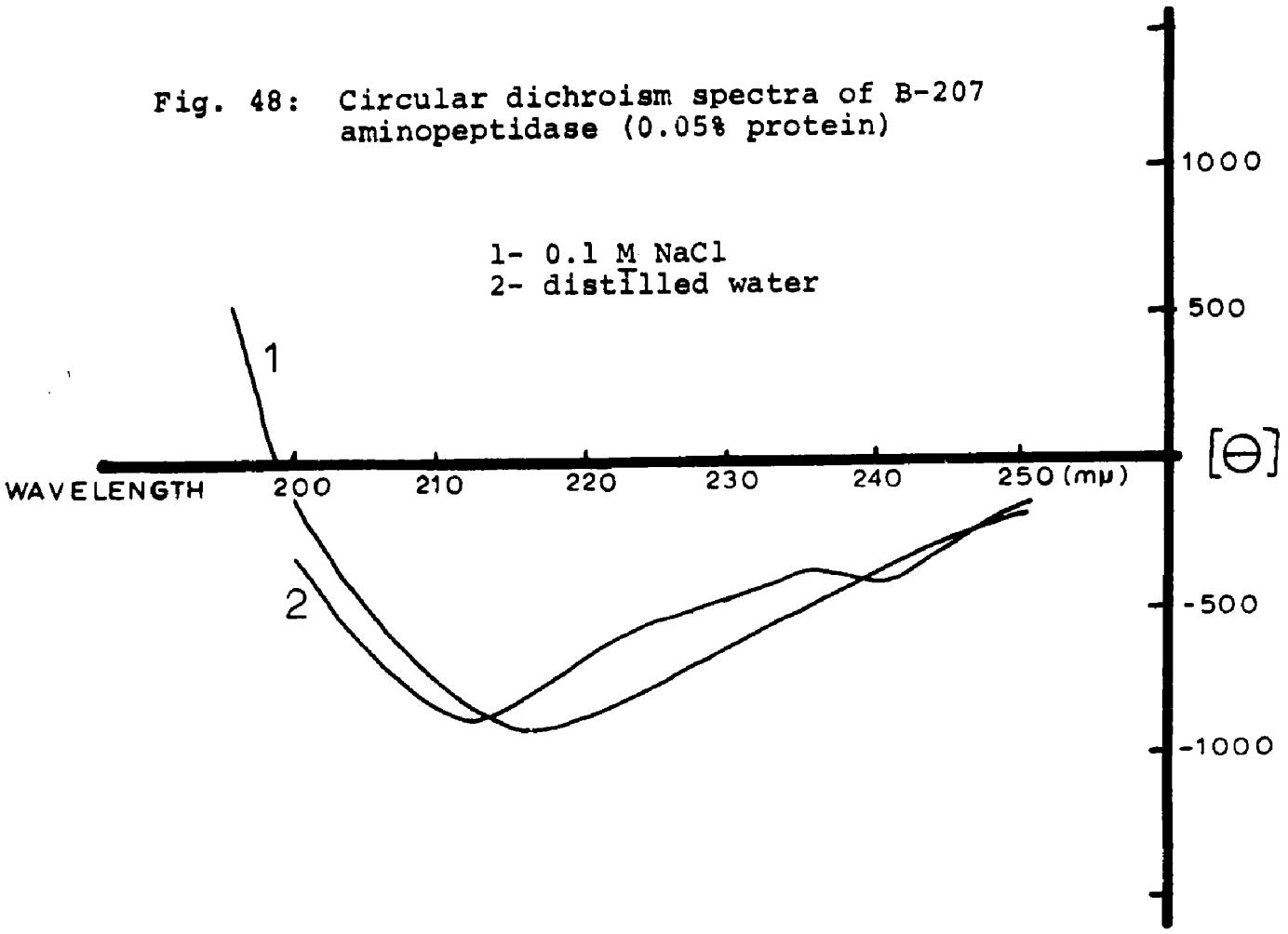


Fig. 47: Optical rotatory dispersion spectra of B-207 aminopeptidase (0.05% protein).

1- 0.1 M NaCl
2- 0.001 M NaCl
3- distilled water

Fig. 48: Circular dichroism spectra of B-207 aminopeptidase (0.05% protein)

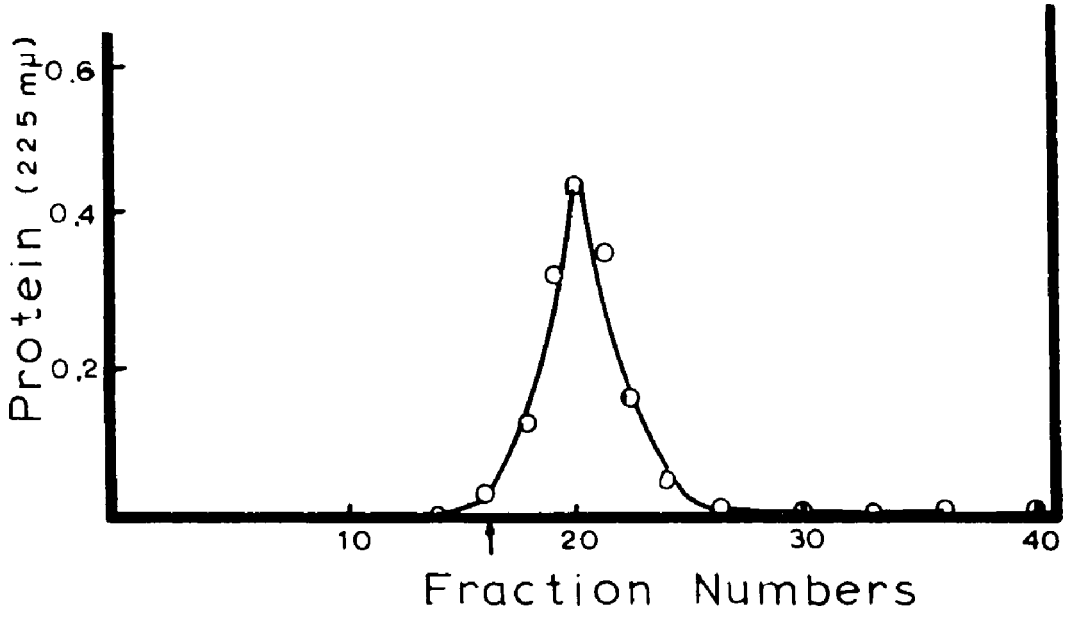
1- 0.1 M NaCl
2- distilled water



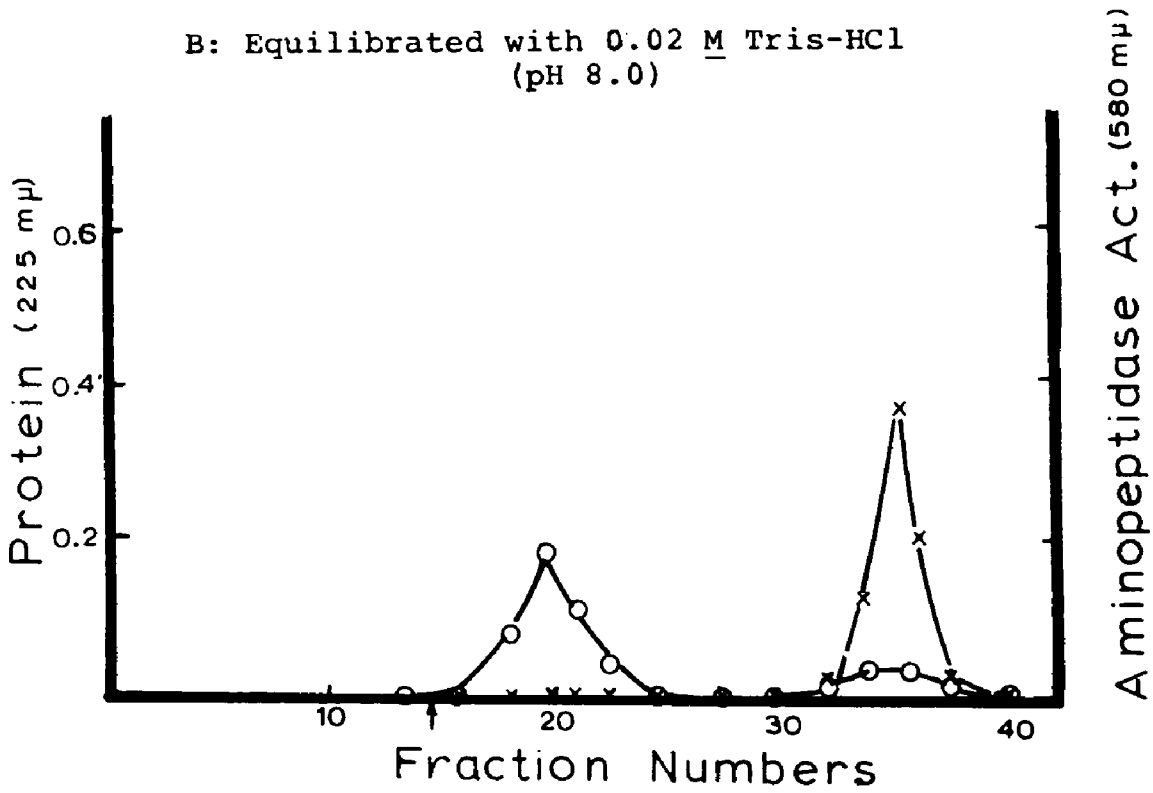
(1-2 ml of a 50 mg protein/ml solution) of crude aminopeptidase were chromatographed at 4°C. The results (Fig. 49A showed only one major protein peak (determined by measuring the optical density at 225 mμ) which appeared just after the blue dextran (void volume marker). Similar results were obtained when the longer column was used with a larger sample (2-3 ml). The isolated peak did not possess any aminopeptidase activity. However, upon dialysis against 0.02 M Tris-HCl buffer (pH 8.0) approximately 66% of the original activity was recovered. When similar experiments were conducted with columns equilibrated with 0.02 M Tris-HCl buffer, pH 8.0, a pattern similar to the one depicted in Fig. 49B was obtained. This pattern is similar to the one originally observed during the isolation and purification procedures (Fig. 28). After the isolated peak from the distilled water equilibrated column was concentrated, it was rechromatographed on the buffer-equilibrated column. A pattern similar to the one observed in Fig. 49B was obtained.

Fig. 49: Chromatography of B-207 aminopeptidase on Sephadex G-100 superfine

A: Equilibrated with distilled water



B: Equilibrated with 0.02 M Tris-HCl (pH 8.0)



Arrows indicate blue dextran marker

—O— protein —×— aminopeptidase

DISCUSSION

The intracellular aminopeptidase produced by B-207 was easily purified by taking advantage of its heat stability. Studies with the homogeneous enzyme showed it to be a metalloenzyme with an alkaline pH optimum at 8.1 and a molecular weight of approximately 50,000.

As a result of the widespread use of the amino acid B-naphthylamides in the aminopeptidase assay, a study of the human liver enzymes was conducted by Behal, et al. (95) and it was suggested, from this study, that some of the enzymes hydrolyzing the B-naphthylamides were not aminopeptidases but arylamidases. The fact that the B-207 aminopeptidase hydrolyzed di- and tripeptides and leucinamide in addition to various B-naphthylamides and did not hydrolyze any of the CBZ-derivatives should be sufficient evidence that this enzyme is an aminopeptidase and not an arylamidase or naphthylamidase.

The relatively low recovery (93.6%) of the amino acids from acid and base hydrolysis of the aminopeptidase may have been in part due to the several uncertainities expressed in the results (i.e. the unknown peak, which may be hydroxylysine, eluted prior to the lysine peak or the carbohydrate content). A more complete amino acid analysis would include larger samples hydrolyzed for 24, 48, 72 and 96 hours. Since the light absorption of the

aminopeptidase in solution did not show any maximum in the region of 280 mμ, the primary purpose of the amino acid analysis was to determine the relative amounts of the aromatic amino acids which absorb in that region. Based on the enzymes listed in Table XIII of Tristram and Smith's review of amino acid composition (96) an average value was calculated in grams per 100 grams of protein for phenylalanine (4.88), tyrosine (5.75) and tryptophan (3.75). The values for the B-207 aminopeptidase are lower for tyrosine and tryptophan but close to average for phenylalanine. Since phenylalanine does not contribute very much to the absorption at 280 mμ(its maximum is between 250-260 mμ) (97), the low tyrosine and tryptophan should result in low 280 mμ absorption. In addition, possible folding of the peptide chain might hide any of the aromatic amino acids that were present. However, spectra taken in 8 M urea and 8 M guanidine HCl did not demonstrate any increased absorption as a result of unfolding. Another aspect of the results from the amino acid analysis was the slightly higher proline content (5.99 vs. 4.11 for the averaged enzymes). This might result in increased folding, thus providing the enzyme with a more compact structure and adding to its heat stability.

Although K_M values by themselves do not have a great deal of meaning, when several are compared, information concerning the binding of the substrate to the enzyme

can be inferred. The similarity of the K_M values before and after dialysis (3.30×10^{-4} M and 3.38×10^{-4}M) indicated that the rate of binding and subsequently the binding site of the enzyme was not altered by the dialysis against distilled water. As a result of the dialysis, the enzyme was apparently inhibited in a non-competitive fashion (100) (i.e. an area of the enzyme other than the binding site was affected). The K_M was found to vary with the pH. The two inflection points indicate that groups with pKa values of 7.0-7.3 and 8.25-8.40 are important for the binding of LNA to the aminopeptidase. The lower pKa is close to that reported for the imidazole of histidine and the higher pKa can be assigned to the N-terminal amino acid of the enzyme or substrate (98).

Smith suggested that the aminopeptidase acts upon the substrate by chelation of the metal ion by two groups on the enzyme and by the N-terminal amino nitrogen of the substrate and the imide nitrogen of the susceptible bond (99). The possibility of a third group on the enzyme interacting with the side chain was also considered. Through the work with the B-207 aminopeptidase we have evidence that one of the groups on the enzyme described by Smith is a histidyl residue.

The inhibition by iodoacetic acid at pH 5.5, by reacting with a histidyl residue (94), apparently is an example of a mixed type of inhibitor (100) in which the

overall reaction is inhibited by the failure of the inhibited enzyme-substrate complex to break down.

Since no free N-terminal amino acid was apparently detected on the enzyme, the N-terminal amino acid of the substrate is therefore required for proper binding to take place. This would satisfy the requirement of aminopeptidases for free N-terminal amino groups. The results thus far strongly suggest that the aminopeptidase is a zinc requiring enzyme whose binding site contains at least one histidyl residue.

The specificity of the aminopeptidase has been defined by the experiments described under the Results. The N-terminal residue of the substrate exerted a considerable influence on the rate of hydrolysis and the binding of the substrate. Phenylalanine, which followed leucine in preference had a larger K_M and approximately a similar V_{max} as was noted for LNA hydrolysis. The rest of the neutral amino acids that were examined, all had much larger K_Ms and approximately similar maximum velocities. Thus, the binding of the substrate to the enzyme is a major factor in determining the specificity. It may be envisioned that the ionic charge of the N-terminal amino acid of the substrate binds it through the zinc to a histidyl residue. Only those peptides whose N-terminal amino acids are free with neutral side chains are hydrolyzed. Those amino acids which are susceptible

would then be required to possess the correct spacial arrangement to fit into the binding site. L-leucine followed by L-phenylalanine and L-valine possess the proper characteristics. The requirement for the penultimate amino acid was revealed through the rates of hydrolysis of the di- and tripeptides. Evidently a secondary binding site is present which apparently is smaller in size or differently shaped than the primary site of attachment. Under the restrictions set forth above, L-leucyl-L-leucine amide would be optimally hydrolyzed. As a result of its specificity, the aminopeptidase could be used for protein structural determinations. The additional advantage over the commercially available leucine aminopeptidase from hog kidney is that it does not contain any endopeptidases (101). The hog kidney leucine aminopeptidase also requires activation with metal ions, not necessary for the B-207 aminopeptidase. From the first order proteolytic coefficients of hog kidney leucine aminopeptidase (0.4), Aeromonas aminopeptidase (23.0) and the B-207 aminopeptidase (17.3), it is evident that the two microbial aminopeptidases hydrolyze LNA at the fastest rate. The Aeromonas aminopeptidase is apparently quite similar to the B-207 aminopeptidase in specificity and metal requirement (16).

The loss of activity on dialysis against distilled water suggested that the aminopeptidase was affected by

one or two parameters which resulted in inhibition and loss of heat stability. One explanation was that the required zinc was being removed during dialysis. Atomic absorption studies revealed that only one atom of zinc per mole of enzyme was present and that it was not removed during dialysis. Furthermore, if we assume that Smith's model (99) for binding is correct, then the loss of a metal during dialysis would result in an alteration of the binding site with a subsequent change in K_M. Since the K_M did not change, we can assume that the binding site was not altered. The effect of the small amount of calcium and magnesium found with the zinc during atomic absorption analysis is unclear. Since the amounts were never constant relative to zinc or protein concentration and were reduced during dialysis it was assumed to be an impurity. The possibility that a second metal is loosely bound to another part of the molecule and that it is removed by dialysis has been ruled out by the data.

Alternatively it is possible to suggest that the aminopeptidase has a salt requirement. During dialysis, the ionic strength would be lowered below the necessary level for the enzyme's structural integrity and catalytic function. Von Hippel and Wong (102) reported that increased ionic strength of neutral salts affected the native form of a number of proteins and DNA. Their results, along with those of Warren, et al. (31) suggested

that the higher salt concentrations disrupted the structure of the protein. Although many studies have been conducted on the effects of cations and anions on enzyme activity, no all inclusive results are available. At salt concentrations from 0.3 to 3.0 M the ions have been ordered by von Hippel and Wong (102) on the basis of capacity to inhibit enzymes. Gordon (103) has recently looked at the structural changes occurring when the protein concentration was increased in various salt solutions. His conclusion was that ORD spectra of high concentrations of chymotrypsin were affected by changes in ionic strength and that those effects could be correlated to aggregation of the molecule. The heat stability of α-amylase is known to require calcium which confers the required molecular rigidity (104). Several proteinases have been reported where calcium acts to increase the heat stability (105). Sipos and Merkel demonstrated (106) that trypsin forms a more ordered conformation after the addition of calcium.

The results of studies with the B-207 aminopeptidase have shown that the enzyme lost heat stability and enzyme activity when the salt solution was diluted. The far-ultraviolet spectra have shown a decrease in absorption and a red shift in the absorption maximum between the dialyzed enzyme and the same enzyme in 0.1 M salt solution. Tinoco, et al. (107) reported that both poly-L-lysine

at pH 10.8 and poly-L-glutamic acid at pH 4.9 exhibited helical structures with decreased absorption and shifts in the absorption maximum when compared to the same polyamino acids in the random coil conformation. Since we are not dealing with synthetic polyamino acids, we can only approximate the results and suggest that in distilled water the aminopeptidase is inactive, heat labile and exists in an unordered structure, while the increased ionic strength solutions yield an enzyme that is active, heat stable and is in a changed conformation. The results indicated that this was a reversible change.

To further test this hypothesis, the ORD and CD spectra were recorded (Figs. 47 and 48). Like the ultraviolet spectra, the interpretations are based on synthetic polyamino acids whose structures themselves have been determined only through X-ray analysis. The ORD spectrum of the aminopeptidase in 0.1 M NaCl (#1 in Fig. 47) shows a minimum in the range of 220-225 mμ and a maximum near 203 mμ. Both of these parameters are similar to the ORD curves reported for the B-form of silk fibroin (108) and a number of other enzymes (109). The CD spectrum also corresponds to that for the B-structure (108). The ORD spectrum and CD spectrum of the enzyme in distilled water (#3 in Fig. 49) resembles the spectra and parameters described by Timash ff, et al. (110) for the unordered conformations (random coiled).

It is interesting to note that the native, heat stable B-207 aminopeptidase is apparently in a conformation akin to the B-structure (ordered). Trypsin was shown (106) to increase its heat stability upon the addition of calcium and shift its conformation toward the B-structure. Possibily it may be found, that possession of some form of B-structure is required for heat stability.

Aggregation is usually maximal at very low ionic strength (103). It is, therefore, possible to imagine the no longer active, unordered aminopeptidase molecules aggregating in distilled water. The results of the gel-filtration experiments (Fig. 50) indicated that this was what was happening. The high molecular weight peak isolated from the distilled water column was later shown to be active aminopeptidase in 0.02 M Tris-HCl buffer (pH 8.0).

The above described results are apparently valid for dilute protein solutions. Fig. 46 showed that when the protein concentration was increased the effective change in the ultraviolet spectrum was decreased. In addition it had been found that increased protein concentration increased the heat stability.

In conclusion it can be said that an active, crystalline, heat-stable, zinc-requiring aminopeptidase was isolated from Pseudomonas B-207. Its specificity

is that of a leucine aminopeptidase, but it is considerably more active toward LNA than the hog kidney leucine aminopeptidase. The aminopeptidase requires an optimal ionic strength for activity and heat stability which are also dependent upon a specific conformation.

SUMMATION

The marine bacterium which was desiqnated B-207 is aerobic, catalase- and oxidase- producing, a gram negative rod, motile by means of a single polar flagellum, non-spore forming and non acid fast. These characteristics were found to be similar for those organisms described in the genus Pseudomonas. Although no organisms described in the literature could be identified with this particular bacterium, a number of characteristics were similar to those described for the taxonomic groupings of various authors for marine bacteria and pseudomonads (78, 79, 80, 84). At present, however, there is no unifomly accepted system other than Bergey's Manual (21) for classifying the bacterial isolates and consequently those characteristics deemed important by one author may be neglected by another.

Marine Pseudomonas B-207 produced relatively large quantities of proteolytic enzymes and capsular material. The growth and enzyme production was found to be dependent upon a large number of variables, primarily the available nutrients, aeration and stage of growth. The temperature, initial pH of the medium and ionic strength of the added salt all affected the growth and consequently the enzyme production. Two endopeptidases were produced into the medium while the single aminopeptidase was found to be an intracellular enzyme which was present in the medium

after the older cells began to lyse during the stationary growth phase.

The observed multiplicity of protease production by a single organism was again observed with Pseudomonas B-207. The presence of two different endopeptidases and one aminopeptidase was examined. The fact that both endopeptidase and aminopeptidase activity was noted when only amino acids were used as the medium for growth suggests that at least one of the endopeptidases and the aminopeptidase are constitutive enzymes. The apparent differences in specificities of the two endopeptidases (endopeptidase II for uncharged amino acid residues and endopeptidase I for hydroxylated amino acid residues) suggests that their narrow specificities require the bacterium to produce more than one enzyme.

After examining the specificities of the endopeptidases (by DNP-end group analysis of protein hydrolyzates and di- and tripeptide hydrolysis) and the aminopeptidase (with B-naphthylamides and di- and tripeptide hydrolysis) it appears that what was suggested in Part I concerning the mode by which the bacterium was able to utilize protein may someday be shown to be correct. If we assume the endopeptidases to primarily hydrolyze proteins at the amino side of uncharged amino acid residues, then the resulting peptides would have N-terminal uncharged amino acids. If we further assume that these peptides were able to enter

the cell, then it could be imagined how the aminopeptidase, specific for the free neutral amino acid residue of the peptides, would be able, with the aid of some other peptidases, to supply individual amino acids toward the metabolism of the bacterium.

The exact role of the protein-polysaccharide capsule is unclear, but its production may represent part of the marine bacterium's contribution to the life-cycle in the oceans. The production of proteolytic enzymes which digest the available proteins and provide peptide and amino acid nutrients may result in the amino acids being converted during metabolism to carbohydrates and ultimately to capsule. This capsule could be used as food by other marine organisms. It may also be visualized that many capsulated organisms could be able to adhere through the mucoid nature of the capsule and move about as large groups in the oceans.

The requirement for a specific conformation was demonstrated by the aminopeptidase in order to maintain heat stability and activity. This heretofore unknown relationship between the enzymatic function, characteristics and protein structure has led to several possibilities for future work. It could be envisioned that the particular requirement of the aminopeptidase for a specific structure, necessary for heat stability, and maintained by a proper ionic strength, would be widespread and found in other enzymes. The previously mentioned studies by other investi-

gators point out the interest in this area of endeavor and that isolated effects of ionic strength (generally increased concentration of salt) upon enzyme activity have been noted. More important is the possible application of this work in providing increased heat stability to the generally labile enzymes by changing the structure of the enzymes through manipulation of the ionic strength.

Although a number of questions were raised and others left unanswered during the just completed investigation, several results were obtained which provide new information about marine bacteria and their production of proteolytic enzymes. Furthermore, two new tools for protein chemists have been isolated, purified and characterized. Both endopeptidase II and the aminopeptidase should prove useful in protein sequence determinations. Finally, the work on the aminopeptidase has shown for the first time that a specific structure is required and provided for by the proper ionic strength which confers activity and heat stability upon an enzyme.

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VITA

Thomas S. Freund, the son of William and Marianne Freund was born in New York City on January 11, 1944. Along with a younger sister, he attended the public schools of Kew Gardens and Forest Hills, New York and graduated from Forest Hills High School in June, 1961.

Mr. Freund attended Lehigh University and received a Bachelor of Science degree with Departmental Honors in Chemistry when he graduated in June,1965. He remained at Lehigh after graduation in order to pursue graduate study in biochemistry.

He has had industrial experience as a laboratory technician with the Organic Chemistry Section in the Division of Research and Development of Lever Brothers,Co.

Following graduation, Mr. Freund will assume a position as Postdoctoral Research Fellow with the Department of Ophthalmology, College of Physicians and Surgeons, Columbia University, New York.

Mr. Freund is married to the former Mary Braunberg of Mineola, New York. They have a daughter, Catherine Louise, born December 1, 1967.