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BRIAN PAUL KLUBEK (Name) for the MASTER OF SCIENCE (Degree)

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Bdellovibrio bacteriovorus 100 and B. stolpii UKi-2 each produce two extracellular proteases which attack Azocoll (collagen) while B. starrii A3.12 produces at least four such proteolytic enzymes. Under standard growth and assay conditions each Bdellovibrio species produces a unique amount of proteolytic activity. The decreasing order of enzyme units was B. starrii > B. stolpii > B. bacteriovorus in a ratio of 10:5:1.

When ammonium sulfate precipitated (ASP) partially purified Bdellovibrio proteases are electrophoresed, bands of proteolytic activity can be developed on casein agar mounts. Each species produced unique and different zymogram patterns at each pH of 7.3, 8.3, and 9.0. The electrophoretic elution profile (pH 8.3) of the proteases from each species is also unique.

There are two types of proteolytic enzymes produced by the Bdellovibrio. One enzyme (metallo protease) is inhibited by 10 mM EDTA, and the other (serine protease) is inhibited by 1 mM phenlymethylsulfonylflouride (PMSF). Both the metallo and the serine proteases are stimulated or inhibited by a variety of charged molecules. These include Tris-glycine (5mM tris, 38 mM glycine), EDTA (10 mM), PMSF (1 mM), Tris (10 mM), Ca^{++} (2mM), Mg^{++} (3mM), and various amino acids (glycine, alanine, cysteine, glutamic acid, and arginine, 20 mM each).

When the purified H-I B. bacteriovorus 100 metallo and serine proteases are dialyzed against Tris-HCl, 49 and 12 percent loss of activity is observed respectively. A complete recovery in proteolytic activity is obtained upon the additon of both Ca^{++} and Mg^{++}. No loss in activity occurs when these same enzymes are dialyzed against pH 7.75 Tris-glycine (5 mM tris, 38 mM glycine). Dialysis against double distilled water results in an 80 and 49 percent loss in activity of the metallo and serine enzymes, and upon the addition of Tris, Ca^{++} and Mg^{++} only a partial recovery in proteolytic activity is observed.

Cysteine and Tris-glycine were found to be stimulatory for both the H-I B. bacteriovorus 100 proteases. Hydrogen peroxide (5 mM) does not cause any inhibition in proteolytic activity.

The serine proteases of both H-I B. bacteriovorus 100 and

H-I B. stolpii UKi-2 have an optimum pH of about 8.0 - 8.1. The metallo enzymes differ in that the H-I B. bacteriovorus 100 has an optimum pH at 7.5, while the H-I B. stolpii UKi-2 has an optimum at 7.75.

The H-I B. bacteriovorus 100 metallo protease has an optimum temperature at 43.5 - 44°C and a Km of 4.8×10^{-5} M using Azocoll as substrate, while the serine protease has a temperature optimum of about 41.5 - 42°C and a Km value of 3.8×10^{-5} M.

The molecular weights of the H-I B. bacteriovorus 100 proteases were also determined. The metallo enzyme has a molecular weight of about 50,000 while the molecular weight of the serine protease is 32,000.

Physical and Chemical Properties of Some Bdellovibrio Extracellular Proteases

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Redacted for privacy

Assistant Professor of Microbiology
in charge of major

Redacted for privacy

Head of Department of Microbiology

Redacted for privacy

Dean of Graduate School

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PHYSICAL AND CHEMICAL PROPERTIES OF SOME *BDELLOVIBRIO* EXTRACELLULAR PROTEASES

INTRODUCTION

In 1962, Stolp and Petzold discovered an obligate ectoparasitic group of vibrios highly specific in their ability to attach to and lyse other gram negative bacteria (24). When propagated on a lawn of host organisms, the vibrios would develop plaques in three to four days. The relative size of the plaques were observed to be larger than those of bacteriophage (25). These parasitic bacteria were subsequently described in more detail and named *Bdellovibrio bacteriovorus* by Stolp and Starr (25).

During the years that followed, it was discovered that *Bdellovibrio* actually penetrate the host wall and lodge within the integument of the host cell (21, 23). Once penetration has occurred, the *Bdellovibrio* begins an intracellular growth phase and gradually consumes the cytoplasmic contents of the host (15, 19, 21, 23). Additional experimental evidence indicates that there exists a major dependence on the host cell as the source of carbon and energy (15, 19, 23, 25). Since the host is nearly two-thirds protein, it seems obvious that these parasites should have evolved biochemical mechanisms by which to assimilate this class of host macromolecules.

To date, two groups have characterized *Bdellovibrio*

extracellular enzymes. Starr and Huang (22) have purified 62-fold a protease, which by column chromatography, exhibited a single peak of activity corresponding to a molecular weight of 11,000. Robinson's group has studied a Bdellovibrio 6-5-S peptidase of 40,000 molecular weight, and has also partially purified a protease having a molecular weight of 100,000 (3, 4). In both cases, Bdellovibrio bacteriovorus 6-5-S was the organism studied.

The present study was initiated in an attempt to clarify these conflicting data (3, 21) and to further characterize the Bdellovibrio proteolytic system with respect to some of its physical and chemical properties.

REVIEW OF LITERATURE

Extracellular proteolytic enzymes are synthesized by a wide variety of non-bacteriolytic microorganisms. The function of these enzymes has been observed to be involved in various activities, such as necrotic virulence factor in Aeromonas (20), to a possible role in the sporulation of Bacillus (13), and in the spoilage of meat products (26).

With respect to the Bdellovibrio, proteases have also been observed to be produced by both the host-dependent (H-D) and host-independent (H-I) strains. The H-D Bdellovibrio are the obligate (wild type) parasites of the host bacterium, while the H-I strains are saphrophytic derivitives (presumably mutants) of these parasites.

In 1963, Stolp and Starr (25) were the first to show the presence of proteolytic activity on heat-killed lawns by cultures of H-I Bdellovibrio A3.12. Later, Shilo and Bruff (19) demonstrated that only damaged host cells were affected by a protease present in the spent cultures of H-I Bdellovibrio A3.12, and not in other Bdellovibrio lysates. These authors postulated that cell lysis (induced by the Bdellovibrio) was a two-stage process, the first being a specific attachment of bdellovibrios (in association with damage) to the cell wall of the host organisms; the second involving a non-specific digestion of cellular components by an extracellular protease produced by

the bdellovibrios. However, Seidler and Starr (16) have shown that protease activity could be detected in the spent growth liquer of all H-I Bdellovibrio isolates. In each instance, the digestion of heat-killed cells was demonstrated, thus suggesting that a proteolytic enzyme(s) was responsible for the digestion of cell protein.

Two groups have recently further characterized the Bdellovibrio extracellular enzymes and have utilized the same test organism, H-D B. bacteriovorus 6-5-S (4, 9). Huang and Starr (9) have purified 62-fold a single protease ("Azocollase") which by column chromatography, exhibited a peak of activity corresponding to a molecular weight of 11,000. Also, evidence was provided for the existence of both an endopeptidase and an exopeptidase which did not have activity on Azocoll.

Fackrell and Robinson (4) have discovered a Bdellovibrio peptidase of 40,000 molecular weight which attacks the DAP-alanine bond of the Spirillum peptidoglycan but does not exhibit proteolytic activity on Azocoll. This group has also partially purified a protease (active on Azocoll) which has been found to have a molecular weight of at least 100,000.

In all of the above cases, only one protease has been observed. In the present study, however, evidence is presented which indicates that there are at least two or more different extracellular proteases produced by the Bdellovibrio. These proteolytic enzymes have been

characterized on the basis of the amount of activity produced, the action of the protease on a natural and a synthetic substrate, the effect of inhibitory substances, and other physical, chemical properties.

MATERIALS AND METHODS

Organisms

The host-independent (H-I) bdellovibrios used in this study were Bdellovibrio starrii A3.12, Bdellovibrio stolpii UKi-2, and Bdellovibrio bacteriovorus 109D, 100, 118, Xty, and 110 (14, 16). The host-dependent (H-D) bdellovibrios used were Bdellovibrio starrii A3.12, Bdellovibrio stolpii UKi-2, and Bdellovibrio bacteriovorus 109D, 6-5-S, 2484 Se-1, and B (25). The Bdellovibrio 6-5-S culture (ICBP 3289) was obtained from Dr. M. P. Starr, curator of the International Collection of Phytopathogenic Bacteria. The host bacterium for the propagation of all the H-D bdellovibrios was Spirillum serpens (ICBP 3227).

Media

The growth medium used for the propagation of all H-I bdellovibrios consisted of 10 gm of Bacto peptone and 3 gm of Difco yeast extract (15), added to 1 liter of 10 mM Tris-HCl buffer. The pH was adjusted to pH 7.5 with 5 N HCl before sterilization, and autoclaved at 15 psi for 20 minutes. Usually 3.25 liters of the peptone yeast extract (PYE) broth was made at a time and one liter volumes of this medium were dispensed into three two-liter Erlenmeyer flasks. The remaining 250 ml of growth medium was equally dispensed into three

125-ml Erlenmeyer flasks.

Plates were prepared by the addition of Difco agar (usually 15 gm/liter) to the PYE broth.

The H-D bdellovibrios were grown in PYE/10 made in 10 mM TCM buffer (3 mM Mg^{++}, 2 mM Ca^{++}, and 10 mM tris, pH 7.5) and dispensed 350 - 400 ml into two-liter Erlenmeyer flasks. PYE/10 was prepared by mixing one volume of PYE broth (15) with nine volumes of TCM buffer.

Growth Conditions

Experiments involving the growth of the H-I strains utilized the following inoculation protocol. From a stock culture, the Bdellovibrio were streaked onto a fresh plate of PYE agar and incubated at 34° C for 24 hours. The newly grown culture was then used to inoculate the 125-ml Erlenmeyer flask containing the PYE broth and incubated at 28° C for 24 hours on a New Brunswick Scientific rotary shaker. Samples were then taken for observation under phase microscopy (Zeiss). After it was determined that no contaminating organisms were present, the broth culture was used as the inoculum for the one-liter volume of PYE broth. The cultures were then incubated at room temperature for 24 - 48 hours on a rotary shaker. After incubation, a 5 ml sample was taken and read at 600 nm on a Bausch and Lomb Spectronic 20 colorimeter. When the optical

density (O.D.) reached a value of 1.0 (the stage approaching maximum protease production for either 24° or 30° C grown cells, Figure 3), samples of the freshly grown culture were again obtained and observed microscopically for contamination and/or the presence of Bdellovibrio spheroplasts (source of intracellular protease). After it was determined that neither of the two were present, the cultures were then centrifuged at 12,000 rpm for ten minutes in a Beckman model J-21 centrifuge. The culture supernatants were retained and stored frozen at -25° C.

The H-D strains were grown according to the following schedule. The 350 - 400 ml of PYE/10 was inoculated with 50 ml of an overnight S. serpens (c.a. 5×10^8 cells/ml) culture grown in PYE broth at 30° C. In addition, 5 to 10 ml of the active lysate from PYE/10 was added. After incubation for 15 hours at 30° C the lysate contained actively motile bdellovibrios (10^9 pfu/ml or greater) and little or no viable S. serpens (less than 10/ml). The cells were removed by centrifugation (12,000 rpm for 20 minutes) and the supernatants were frozen at -25° C.

The PFU/ml were enumerated on double layer plates according to the method of Seidler and Starr (15) except that the PYE/10 in TCM buffer was the nutrient medium. Countable plaques were obtained in two to three days by decreasing the bottom agar to 0.8% concentration, the top to 0.6%, and incubating at 34 - 35° C.

Enzyme Assays

At various times, casein, heat-killed S. serpens, or Azocoll (Calibiochem) were employed to detect protease activity. In the final analysis, Azocoll proved generally most suitable. This substrate is composed of an azo-dye bound to collagen, and upon degradation by a proteolytic enzyme, the pink water-soluble dye is released. Unless otherwise stated, the standard protease assays with Azocoll as substrate (2 mg/ml) were performed in a shaking water bath (63 - 64 strokes/minute, 5.7 cm/stroke) incubated at $40 \pm 1^{\circ}$C. Reactions were carried out in 10 ml volumes in 125-ml Erlenmeyer flasks. The flask contained spent culture medium or partially purified enzyme appropriately diluted with 10 mM TCM buffer. Ten to 15 minutes were allowed for temperature equilibration before the addition of substrate. For some experiments, the influence of inhibitors on protease activity was studied. Reaction mixtures were supplemented with ethylenediaminetetraacetate (EDTA, Baker) at 10 mM final concentration or with 1 mM phenylmethylsulfonylfluoride (PMSF, Calbiochem). The EDTA was prepared as a 100 mM stock solution in glass distilled water, adjusted to pH 7.5. Because of its low solubility in water, PMSF was dissolved in 95% ethanol (100 mM PMSF). Preparation of PMSF were prepared the day of use and reacted with the enzyme mixtures before the assay for 60 minutes

at 34° C. The 1% ethanol controls exhibited less than a 10% reduction in enzyme activity, and reported values consider this influence.

Reactions were terminated by passing samples of the enzyme mixture through scintered glass filters with the amount of reaction determined from colorimetric measurement at 570 nm in a Bausch and Lomb Spectronic 20 colorimeter. Enzyme reactions were linear up to about 0.6 O.D.

Assays of the spent culture medium were generally run for 15 - 30 minutes with B. starrii A3.12 and B. stolpii UKi-2 enzymes while preparations from B. bacteriovorus were incubated for 20 - 60 minutes. Appropriate enzyme controls were run with Azocoll during the extended incubation periods although these readings were generally as low as the blanks. One unit of enzyme activity corresponds to an O.D. change of 0.001/minute of Azocoll degradation. Protein concentration was determined by the method of Lowery, et al. (11).

Digestion of Heat-Killed Host Cells

The host bacterium S. serpens was grown in PYE broth for 24 hours at 30° C on a New Brunswick Scientific rotary shaker. The host cells were then washed and resuspended to their original volume in 10 mM TCM buffer. The cell suspension was then autoclaved at 121° C for seven minutes. The heat-killed cells (4.0 ml) were then added to a 125-ml Erlenmeyer flask containing either the spent

culture medium of the bdellovibrio or the partially purified enzyme appropriately diluted in TCM buffer (Figure 4). The incubation temperature was at 40° C and the final volume of the reaction mixtures in each flask was 25 ml. Controls contained the same enzyme reaction mixture except that the enzyme was heat denatured via sterilization at 121° C for seven minutes. The digestion of the cells was then followed by the decrease in absorption (600 nm) by a Bausch and Lomb Spectronic 20, and phase microscopy (Zeiss).

Partial Purification of the Bdellovibrio Proteases

Figure 1 illustrates a flow-diagram summarizing the initial purification schemes used in this study. The frozen enzyme material was allowed to thaw at 5° C, placed into dialysis tubing (1-1/8 in. diameter, 75 in. length, size 36, V.W.R. Scientific), and concentrated 15 - 20 fold by packing in polyethylene glycol (Baker, 15,000 - 20,000 molecular weight).

The concentrated crude enzymes were then dialyzed against 5 mM TCM buffer (2 mM Ca^{++}, 3 mM Mg^{++}, 5 mM tris, pH 7.5) for six hours at 5° C. After dialysis, ammonium sulfate (Baker) was added to the enzyme concentrate (30% saturation with H-I *B. bacteriovorus* 100; 40% saturation with H-I *B. starrii* A3.12 and H-I *B. stolpii* UKi-2) and allowed to stand overnight at 5° C. The mixture was centrifuged at 10,000 rpm for ten minutes in a Beckman model

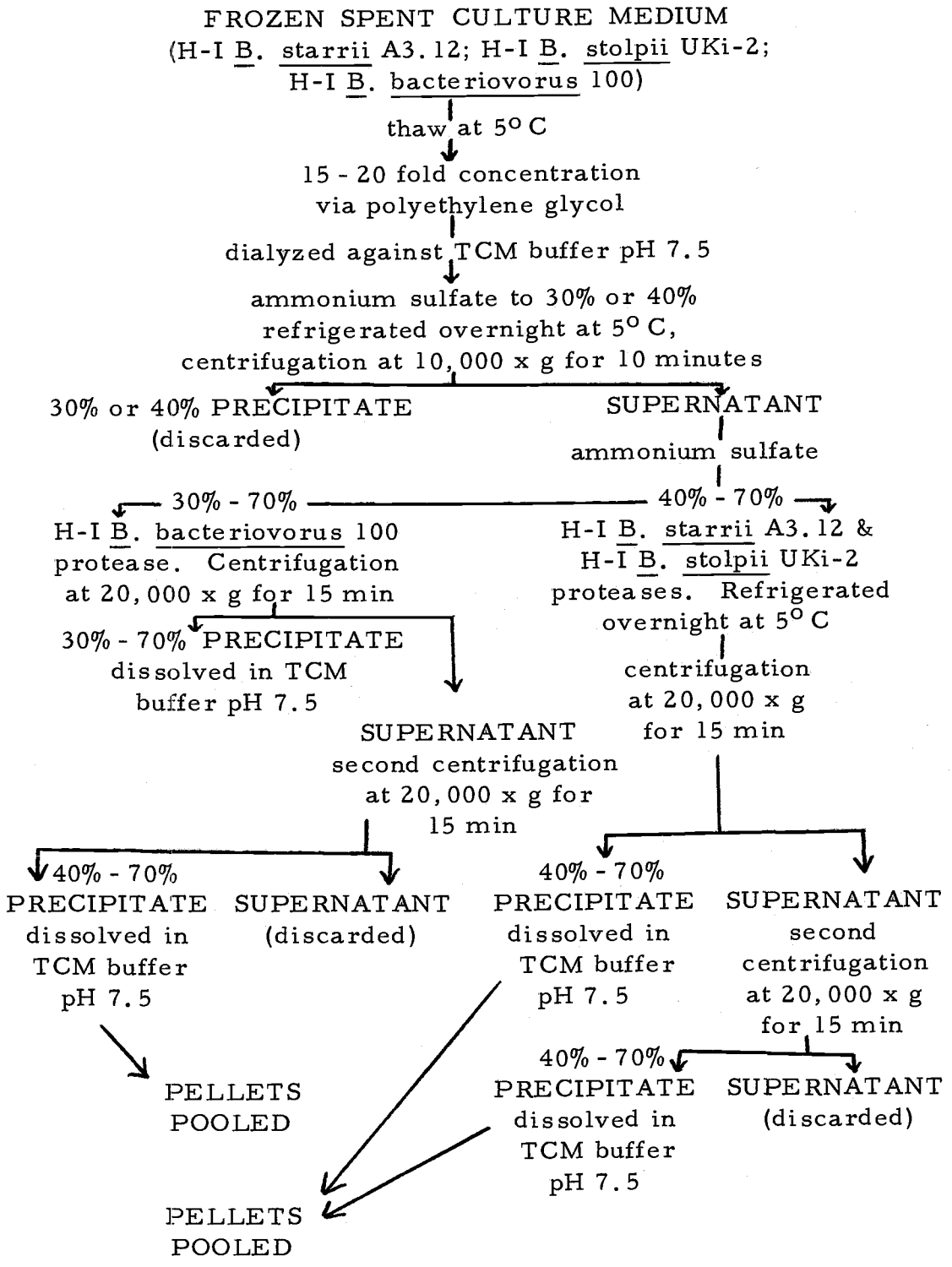

Figure 1. Flow diagram for partial purification of proteases.

J-21 centrifuge and the supernatant was retained. The ammonium sulfate concentration was then increased to 70% of saturation. For the H-I B. starrii A3.12 and H-I B. stolpii UKi-2 proteases, these enzyme mixtures were again allowed to stand overnight at 5° C before centrifuging. The H-I B. bacteriovorus 100 protease, however, was centrifuged at 20,000 rpm for 15 minutes immediately following the addition of ammonium sulfate. The ammonium sulfate precipitates from all species were recovered and dissolved in 1 ml of 5 mM TCM buffer (pH 7.5). Because of some carry-over of the polyethylene glycol, complete precipitation of the proteases was not achieved after the first centrifugation at 20,000 rpm. Therefore, the supernatants were again centrifuged and the pellets were then pooled together (5 ml final volume) and dialyzed against 1 liter of 5 mM TCM buffer at 5° C for 6 - 8 hours. The partially purified and concentrated enzymes were then frozen and stored at -25° C. The H-I B. starrii A3.12 and H-I B. stolpii UKi-2 had undergone the same procedure as outlined above after standing overnight. Enzymes concentrated in this manner and employed in the elution electrophoresis are designated as ammonium sulfate precipitated (ASP) partially purified enzyme. Table 5 summarizes the results of this partial purification.

Storage of Proteolytic Enzymes

Volumes of 5 - 25 ml of crude enzyme (spent culture medium) and 1 ml of the ammonium sulfate precipitated (ASP) partially purified protease were stored at -25° C for up to two months. Samples were removed and thawed at the desired time periods and assayed for activity (Figure 5). For the (ASP) partially purified protease, samples were diluted 1:100 in 10 mM TCM buffer prior to each assay.

Polyacrylamide Gel Electrophoresis

Gels were prepared according to the method of Davis (2). Tables 1 and 2 list the solutions and the procedures for the preparation of polyacrylamide gels. One-tenth strength Tris-glycine buffer (1/10 Tris-glycine, 5 mM tris, 37 mM glycine, pH 8.3) served as the electrophoresis buffer. Two-tenths ml of the ASP partially purified enzyme sample, containing 200 - 550 units of activity, was mixed with 0.2 ml of 40% sucrose and layered over the large-pore gel. Electrophoresis proceeded at 240 volts and 120 amps for about 100 minutes, until the tracking dye (bromphenol blue, 0.001%), reached the bottom of the gel. The flat gel (18.5 cm x 7 cm x 0.35 cm) was removed, sliced into strips (2 cm wide) and assayed for protease activity.

Table 1. Stock Solutions for Polyacrylamide Electrophoresis

(A)		(B)	
1 N HCl	48 ml	1 N HCl	48 ml
Tris (amino-methane)	36.6 gm	Tris	5.98 gm
N,N,N',N'-Tetramethyl-ethylenediamine (TEMED)	0.23 ml	TEMED	0.46 ml
Double distilled water	to 100 ml	H_2O	to 100 ml
Final pH = 8.9		Final pH = 6.7	
(C)		(D)	
Acrylamide	28 gm	Acrylamide	10 gm
N,N -Methyl-enebisacrylamide	0.735 gm	Bis	2.5 gm
H_2O	to 100 ml	H_2O	to 100 ml
(E)		(F)	
Riboflavin	4 mg	Sucrose	10 gm
H_2O	to 100 ml	H_2O	to 100 ml

The composition of the polyacrylamide gels are those recommended by Davis (2). All ingredients were filtered through a Millipore filter (0.45 μm pore size) prior to use.

Table 2. Working Solutions for Polyacrylamide Electrophoresis

Small Pore Solution 1	Small Pore Solution 2	Large Pore Solution	Stock Buffer Solution for Reservoirs	Tracking Dye
1 part A	Ammonium persulfate 0.14 gm	1 part B	Tris 6 gm	Bromphenol blue 1 mg
2 parts C	H_2O to 100 ml	2 parts D	Glycine 28 gm	H_2O to 100 ml
1 part H_2O		1 part E	H_2O to 1 liter	
pH 8.9		4 parts F	Final molarity 0.17 M	
		pH 6.7		

Gels were prepared according to the following schedule: 28 ml of the small pore solution 2 was mixed with 28 ml of small pore solution 1. Five ml of this mixture (small pore solution) was used to wash out the gel chambers. The remaining solution was carefully pipetted into the chamber and a few drops of water were layered on the interface. After 30 minutes, polymerization had occurred and the water at the gel surface was removed. One ml of the large pore solution was then sacrificed to wash out the gel chamber. The remaining 7 ml was applied, and a few drops were again layered on the interface. The electrophoresis unit was placed directly under a light source for a minimum of 30 minutes to permit photopolymerization.

Protease Assays on Polyacrylamide Gels

Bands of proteolytic activity were detected by bringing the polyacrylamide gel strips into contact with casein agar mounted on microscope slides. Casein (Matrix Mother Culture Media, Galloway West Co.) was dissolved at 16% (w/v) in glass distilled water and steamed at 100° C for five minutes. Equal volumes of casein and 3% Bacto agar (cooled to 50° C) were mixed and allowed to solidify. Cleared zones representing hydrolysis of casein were developed by incubating the gel-casein mount in a moist chamber at 40° C. Bands of clearing appeared after one to three hours, depending on the concentration of protease originally applied to the gel (Figure 6).

Elution Electrophoresis

Gel filtration and ion exchange resins were not successful in the separation of proteolytic activities. Only electrophoresis permitted separation of two or more bands of proteases. Therefore, a modification to the electrophoresis unit was made to accommodate larger amounts of protein which could then be eluted off the polyacrylamide gel. Spacers of 8 mm width were inserted between the two halves of the gel unit to increase the gel thickness. A slot was drilled into each spacer at an angle of about 45° which allowed for the fitting of a 22 gauge needle (Figure 2). Gels were prepared as mentioned previ-

Figure 2. Elution Electrophoresis Apparatus.

ously except for the following changes: (1) for the preparation of the small pore solution, 6 ml of solution A was mixed with 12 ml of solution C plus 6 ml of double distilled water and added to 24 ml of freshly prepared and filtered ammonium persulfate solution; (2) the large pore solution was prepared by mixing 2.5 ml of solution B with 5 ml of solution D plus 2.5 ml of solution E. To this mixture, 5 ml of the ASP partially purified protease was added plus 5 ml of an 80% sucrose solution resulting in a final concentration of 20% sucrose. The large pore solution with enzymes was then layered on the small pore gel and allowed to polymerize under a direct light source for 30 minutes. The final gel measured 18.5 cm long, 1.3 cm thick, and 1.8 cm high. Prior to electrophoresis, a dialysis bag (1-1/8 in. diameter and 7.5 - 8.0 in. length) was filled with 100 ml of 1/5 strength Tris-glycine buffer (1/5 Tris-glycine, 10 mM tris, 76 mM glycine, pH 8.3) and placed under the gel to form a base for the elution buffer to be pumped through. The same buffer was used in both top and bottom reservoirs. The molarity of both the tris and the glycine was increased by two fold in order that the mobility of the proteases through the gel might be increased. One and one-half hours after the start of electrophoresis (240 volts, 120 milliamps), the tracking dye reached the bottom of the gel. The same buffer, 1/5 Tris-glycine (pH 8.3) was then pumped through the unit by a Buchler polystaltic pump (Buchler Instruments) set at slow speed and position

1.3. The eluted material (5 ml/fraction) was then collected at a rate of 0.5 ml per minute into a 25 ml graduate cylinder containing 2.5 ml of 10 mM TCM buffer (pH 7.75). A total of 15 - 18 fractions were collected depending upon which Bdellovibrio proteases were being eluted. The fractions were then assayed for proteolytic activity, and the resulting active fractions of each peak were pooled together and stored at -25° C for further experiments.

Dialysis of Eluted Proteases

The serine and metallo proteases were dialysed against 10 mM TCM buffer, the electrophoresis buffer 1/10 Tris-glycine (adjusted to pH 7.75) and glass distilled water (pH 7.5) with one to three changes (1 liter/change) of either the buffer or the distilled water. The dialyzed proteases were then assayed for activity in the presence or absence of EDTA, PMSF, various amino acids, cation(s), Tris (10 mM) and 1/10 Tris-glycine (Tables 7, 8, 9, 10).

pH Optima

The buffers used were as follows: Potassium chloride-HCl (10 mM KCl, 10 mM HCl) pH 2.0; Citrate-phosphate (5 mM citrate, 5 mM phosphate) pH 4.0 - 7.0; Tris-HCl (10 mM) pH 7.0 - 9.0; Tris-glycine (10 mM tris, 10 mM glycine) pH 9.0 - 10.0. The reaction mixtures (5 ml) contained Azocoll (4 mg/ml), the buffer solution,

Ca^{++} and Mg^{++} cations (0.05 ml each, final concentration 2 mM and 3 mM respectively) and the enzyme (1 ml). The final pH of each reaction mixture was adjusted with a single glass electrode pH meter (Corning). These mixtures were then placed in a shaking water bath at $40 \pm 1^{\circ}$ C. Ten minutes were allowed for temperature equilibration before the addition of substrate. The reactions proceeded for 30 minutes, at which time the samples were filtered and read at an O.D. of 570 nm (Figures 9, 11, 12).

Effect of Temperature

Proteolytic activity was measured between 30° and 50° C at 5° C intervals. The reaction mixtures (5 ml) contained TCM buffer (at the optimum pH of the protease tested), Azocoll (4 mg/ml) and enzyme. These test systems were preincubated without substrate 10 - 15 minutes, and after the addition of Azocoll, the reaction proceded for 30 minutes. Samples were then filtered and read at 570 nm (Figure 13).

Km Determination

The reaction mixtures (5 ml) contained Azocoll as the substrate (1 to 19 mg/ml), and the electrophoretically purified enzyme preparation diluted 1:5 with 5 mM TCM buffer. Before dilution, the concentration of the serine protease was 0.11 mg/ml, while the metallo

enzyme was 0.06 mg/ml. The reaction mixtures for both proteases were adjusted to their optimum pH and maintained at their optimum temperature throughout the entire assay. Ten to 15 minutes were allowed for temperature equilibration, and after the addition of Azocoll, the reaction proceeded for 30 minutes. The samples were then filtered and read at 570 nm. The velocities observed for each of the substrate concentrations tested (units/ml) were plotted against the Azocoll concentration (Figures 14, 15). A molecular weight of 30,000 was used for the collagen in calculating the Km (9).

Molecular Weight Determination

For the determination of molecular weights, a Sephadex G-100 column (1) (Pharmacia) measuring 1.5 x 30 cm was prepared and equilibrated with 50 mM Tris-glycine (50 mM tris, 50 mM glycine, pH 7.75). Fractions from the column were collected at a rate of 1 ml (16 drops) per minute. Blue dextran (2 mg/ml final concentration) and 0.5 ml of the following protein standards were used to calibrate the column: cytochrome c (equine heart, 4 mg/ml), ovine albumin (12 mg/ml), yeast alcohol dehydrogenase (4 mg/ml) (Calbiochem), bovine albumin (12 mg/ml) (Miles Research Laboratories, Inc.) (1, 8). The assay for alcohol dehydrogenase used the following protocol: 0.2 ml of each fraction was added to 2 ml of 50 mM Tris-glycine (pH 7.75) and 0.2 ml of 2% ethanol. One-tenth of a ml of

3 mM NAD (Calbiochem) was then added to start the reaction. Activity was measured by a Beckman DU spectrophotometer at 340 nm for 60 seconds. Activity (units/ml) was plotted against the number of fractions collected from the column. The albumins were measured for absorbency at 280 nm while the cytochrome c was read at 412 nm. For the determination of proteolytic activity, each fraction (1 ml) was diluted three fold with 50 mM Tris-glycine (pH 7.75) supplemented with cations (2 mM Ca^{++} and 3 mM Mg^{++} final concentration). The sample was then placed in a shaking water bath at 40 ± 1^{o} C and 10 - 15 minutes were allowed for temperature equilibration. After the addition of Azocoll (4 mg/ml) the reaction proceeded for 60 minutes. The samples were then filtered and measured at 570 nm by a Bausch and Lomb Spectronic 20 colorimeter. The molecular weights of the standard proteins were plotted against the elution volume. The molecular weights of the H-I B. bacteriovorus 100 serine and metallo proteases were then determined from the standard curve (Figures 15, 16).

RESULTS

A. Preliminary Observations of Proteolytic Activity

When all three representatives of the presently recognized Bdellovibrio species were grown in PYE at 30° C, the H-I B. starrii A3.12 and H-I B. stolpii UKi-2 exhibited comparable generation times of three hours, while the H-I B. bacteriovorus 109D had a growth rate of 4.5 hours during the first 12 hours of incubation (Figure 3). Protease production continues throughout all phases, the enzyme activity parallels growth, and the maximum activity corresponds to the stationary phase.

Each of the Bdellovibrio species examined produced a different amount of proteolytic activity (Figure 3, Table 3). The H-I B. starrii A3.12 produced over 100 u/ml, the H-I B. stolpii UKi-2 less than 100 u/ml, and the representatives of H-I B. bacteriovorus pro-produced 10 - 25 u/ml.

B. Characterization Studies of the Crude Enzymes

For all three species of the Bdellovibrio tested (H-I B. starrii A3.12, H-I B. stolpii UKi-2, and H-I B. bacteriovorus 100), the digestion of heat-killed host cells (S. serpens) was followed by the change in absorbency (O.D. 600 nm) and by phase microscopy. Figure 4 shows the decrease in turbidity with time of the heat-killed

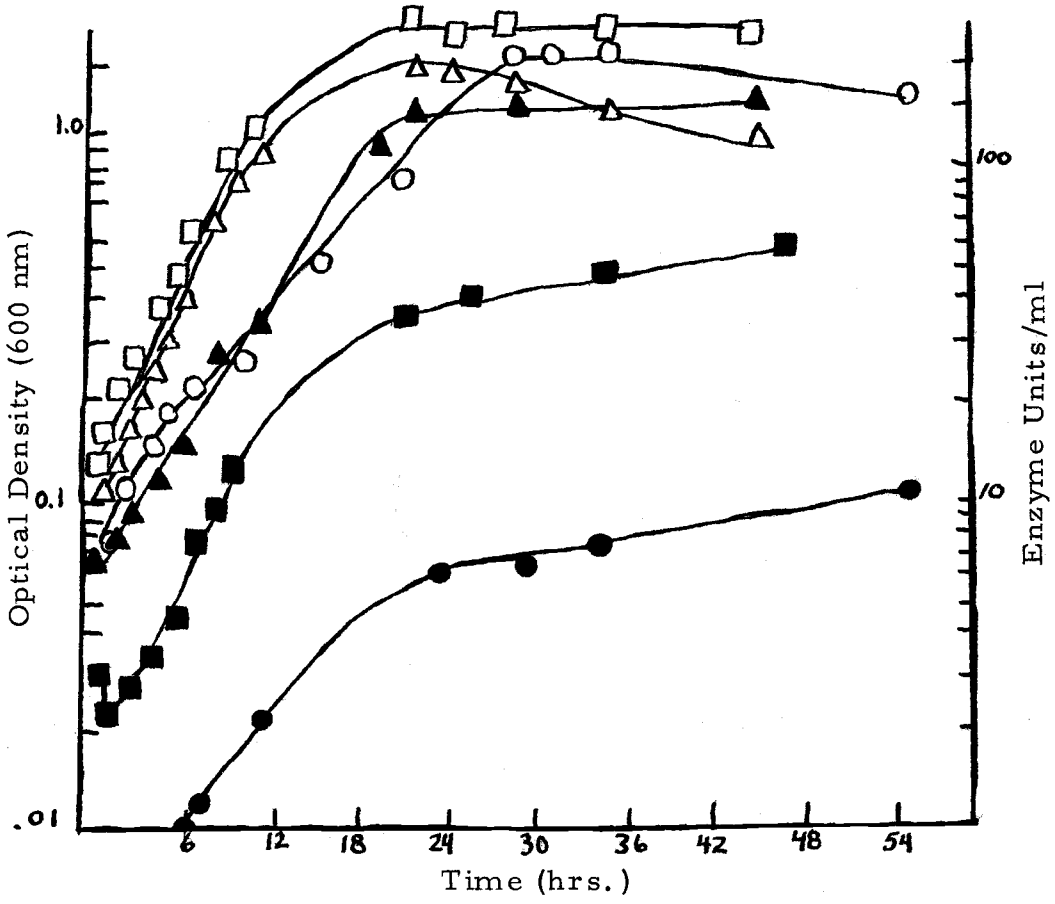

Figure 3. Growth kinetics and protease production by the three species of H-I Bdellovibrio. Growth is in PYE broth at 30° C. Enzyme assays employed Azocoll as substrate (2 mg/ml) and 40° C incubation. The crude enzymes were diluted with 10 mM TCM. Symbols: ▲ and △ H-I B. starrii A3.12 (diluted 1:10); ■ and □ H-I B. stolpii UKi-2 (1:5); ● and ○ H-I B. bacteriovorus 109D (1:1). Solid symbols represent enzyme units per ml while open symbols refer to optical density at 600 nm (growth).

Table 3. Patterns of EDTA and PMSF Inhibition and Total Protease Production for H-I Bdellovibrios

Culture	% Inhibition			
	10 mM EDTA	1 mM PMSF	10 mM EDTA 10 mM PMSF	U/ml
H-I B. starrii A3.12				
OSU #1	81% ± 9 (6)	57% ± 5 (3)	100%	110 - 140
OSU #2	87% (2)	55% (2)		
H-I B. stolpii UKi-2	92% ± (5)	28% ± 8 (4)	100%	40 - 80
H-I B. bacteriovorus				
109D	22% ± 4 (3)	81% ± 3 (3)		
100	41%	70% (2)	100%	
118	20%	79%		10 - 25
Xty	39%	77%		
110	30%	80%		

Assays were performed in spent PYE culture medium diluted with TCM buffer as indicated in the Materials and Methods. Reactions were run at 40° C. Units/ml are defined as a change in O.D. 570 of 0.001/minute. Values for U/ml represent the ranges observed for at least 5 separate cultures of each species. Brackets ( ) indicate the number of separate cultures grown to supply the spent culture medium. The standard deviations are calculated from differences in the average % inhibition observed among the different batches of spent medium. Each individual assay was run in triplicate.

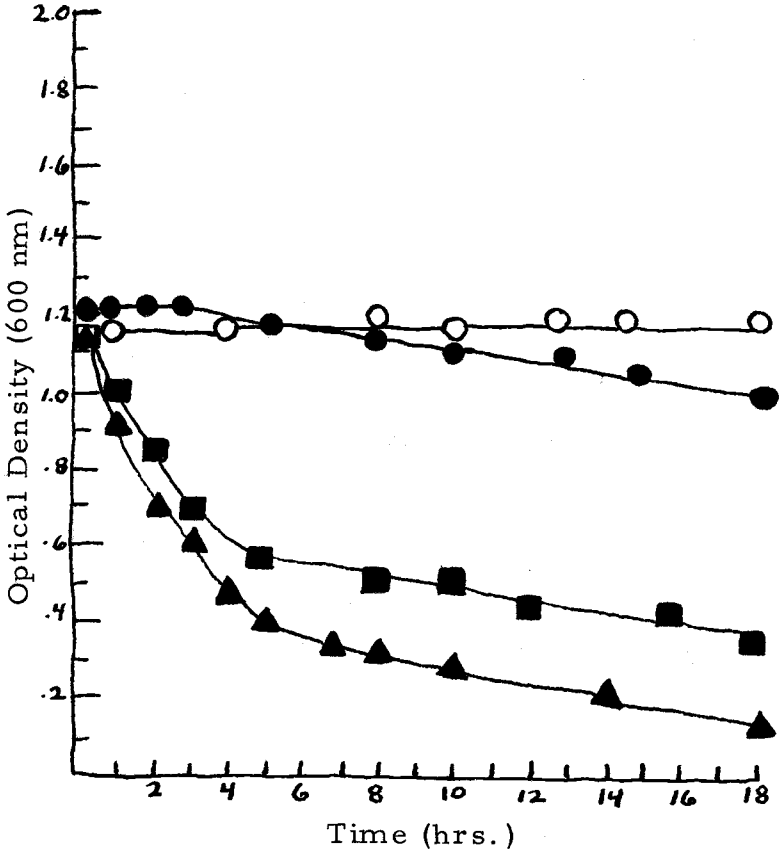

Figure 4. The effect of crude Bdellovibrio enzyme (spent culture medium) on heat-killed cells of S. serpens. The reaction mixtures were incubated in a shaking water bath at 40° C. At specified times, 5 ml of the heat-killed host cell enzyme mixture was removed, the O.D. was measured, and phase observations were made. Symbols: ▲ H-I B. starrii A3.12 (diluted 1:10); ■ H-I B. stolpii UKi-2 (1:5); and ● H-I B. bacteriovorus 100 (1:1); O control.

host cell suspension. It is observed that the H-I B. starrii A3.12 and H-I B. stolpii UKi-2 protease systems digest the host cells at a similar rate, while very little change is observed with the H-I B. bacteriovorus 100 protease. However, when phase observations are made at the same time that absorbency is measured, a different result is obtained.

In the H-I B. starrii A3.12 proteolytic system, most of the internal components of the heat-killed bacteria are dissolved. The empty hulls of the elongated spirals closely resemble the faded appearance of spheroplasts which are found in the H-D systems. With the H-I B. stolpii UKi-2 and H-I B. bacteriovorus 100 protease systems, digestion is not as complete as it is with the H-I B. starrii A3.12 protease, but a clumping or agglutination of the ghosted cells does occur. Furthermore, the degree of clumping is likewise characteristic of each species. In the H-I B. stolpii UKi-2 system, small clusters of cells are found with approximately 10 to 50 cells per cluster. With the H-I B. bacteriovorus 100 protease, large masses of clustered cells are observed and 100 or more cells may be found in each cluster. It is also worthy to note that in the H-I B. starrii A3.12 system, no cluster formations are observed.

Having observed the action of the Bdellovibrio protease systems on heat-killed host cells, other properties of these enzymes were then examined. Since cations are supplemented in the enzyme reaction

mixtures, the effect of EDTA on proteolytic activity was studied. Initially, concentrations of 10 mM, 5 mM, and 1 mM EDTA were tested. The two highest concentrations gave similar results. When EDTA was added to the enzyme reaction mixture, a partial reduction in proteolytic activity was observed in all species tested (Table 3). As can be seen, activity in the H-I B. starrii A3.12 was inhibited 80 - 87% while the H-I B. stolpii UKi-2 was inhibited to a similar but consistently greater degree of 85 - 95%. However, the inhibition patterns in the H-I B. bacteriovorus preparations showed only a 20 - 40% reduction.

The above inhibition data suggests that more than one protease may be present, since a complete inhibition does not occur with EDTA. Therefore, PMSF was employed to detect the possible presence of a different, common class of bacterial proteases, the serine protease (11, 14). In this case, 1 mM and 10 mM concentrations of PMSF were used initially, however, the former concentration proved to be most suitable and was used in the subsequent experiments. When PMSF is added to the enzyme reaction mixtures, the pattern of inhibition is distinct for each species (Table 3). H-I B. starrii A3.12 is inhibited 57% by PMSF, while the inhibition pattern of H-I B. stolpii UKi-2 was 28%, or 1/2 of that observed with the former. In addition, the same inhibition patterns were noted in other independently derived cultures of H-I B. starrii A3.12 and H-I B. stolpii

UKi-2. The H-I B. bacteriovorus proteases, however, demonstrated an inverse pattern of inhibition relative to those of H-I B. starrii A3.12. As a final experiment concerning inhibition of the Bdellovibrio proteases, both EDTA and PMSF (10 mM and 1 mM respectively) were added, and in all cases complete inhibition of activity on Azocoll was observed.

Since the above observations were made, it was relevant to determine whether similar enzyme systems were found when H-D bdellovibrios are grown in the presence of living hosts. Table 4 summarizes the results pertaining to this point. The H-D B. starrii A3.12 and the H-D B. bacteriovorus isolates demonstrated patterns of inhibition quite similar to the corresponding H-I cultures, while the H-D B. stolpii UKi-2 was variable and lower than its H-I counterpart. In addition, the total amount of protease activity (units/ml) of the H-D cultures demonstrated the same progression as the corresponding H-I derivatives, ie., B. starrii > B. stolpii > B. bacteriovorus. The relative ratios of proteolytic activity for both the H-I and H-D cultures is approximately 10:5:1 for the three respective species.

Since more in-depth studies on the Bdellovibrio proteases were to be made, it would be convenient to accumulate and store partially purified enzymes at -25° C for future experiments. Preliminary studies were designed to explore this possibility. Eight samples each of the three species of Bdellovibrio crude enzyme material

Table 4. Patterns of EDTA and PMSF Inhibition and Total Protease Production for H-D Bdellovibrios

Culture	10^{-2} M EDTA	10^{-3} M PMSF	u/10^{9} PFU
H-D *B. starrii* A3.12	84% ± 8 (6)	46% ± 17 (7)	19 - 25
H-D *B. stolpii* UKi-2	43 - 63% (3)	35% (2)	6 - 12
H-D *B. bacteriovorus*			
109D	40% ± 8 (3)	72% ± 15 (3)	1.3 ± 0.1 (3)
6-5-S	38% ± 5 (4)	65% ± 13 (4)	2.2 ± 0.3 (4)
2484 Se-1	44% (2)	N.D.	1.3 (2)
B	43% (2)	N.D.	2.4 (2)

Assays were performed in spect PYE/10 medium which was not usually diluted with TCM buffer. See the legend to Table 2 for further details. N.D. indicates not determined.

(5 - 25 ml) were frozen, and each sample was allowed a single thaw during the desired assay time period. In Figure 5, the change in activity versus time is plotted for all three species. The protease appears to be quite stable with very little, if any loss of activity.

C. Partial Purification and Further Characterization Studies of the Bdellovibrio Proteases

Table 5 summarizes the partial purification scheme used to prepare the proteolytic enzymes for electrophoresis. It may be observed that 14 - 34% of the original enzyme activity was recovered, and activities of 1,000 u/ml were routinely achieved for H-I B. bacteriovorus 100, and 1,000 - 2,000 u/ml for H-I B. starrii A3.12 and H-I B. stolpii UKi-2.

When the ASP partially purified proteases were frozen, stored, and analyzed under identical conditions as previously described for the spent culture medium, some fluctuation in proteolytic activity was observed for all three protease systems. However, essentially 90 - 100% of activity still remained after eight weeks of storage. Thus, a stock supply of partially purified protease for all three species was obtained and stored for future experiments.

Since our major goal was to separate and characterize the Bdellovibrio proteases via elution electrophoresis, the effect of the electrolyte buffer, 1/10 Tris-glycine (pH 8.3) on protease activity

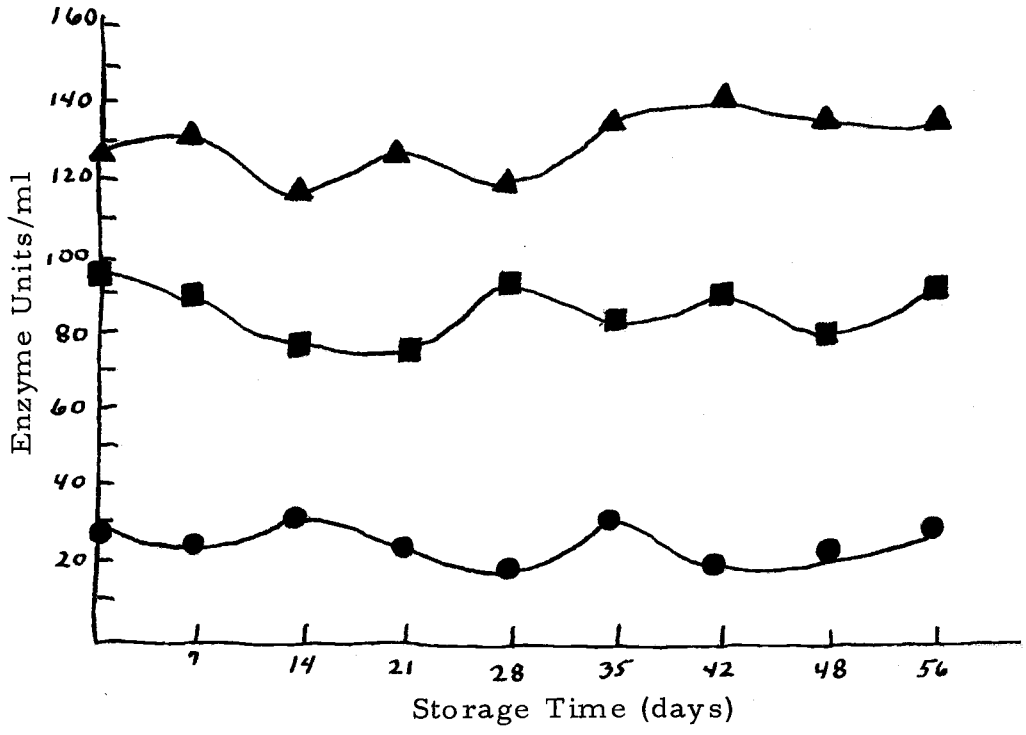

Figure 5. The effect of storage at -25° C on crude enzyme material (spent culture media). Each sample was allowed to thaw at 5° C. Enzyme assays employed Azocoll as substrate and 40° C incubation. Symbols: ● H-I B. bacteriovorus 100 (diluted 1:2 with TCM buffer); ▲ H-I B. starrii A3.12 (diluted 1:10 with TCM buffer); ■ H-I B. stolpii UKi-2 (diluted 1:5 with TCM buffer). Values for units/ml represent activities prior to dilution with TCM.

Table 5. Partial Purification of Bdellovibrio Proteases

Fraction	Total Enzyme Units	Specific Activity (units/mg protein)	Percent Recovery	Purification
I. Culture supernatant				
H-I B. bacteriovorus 100	23,800	8.5	100	1
H-I B. starrii A3.12	87,000	28.4	100	1
H-I B. stolpii UKi-2	60,800	18.5	100	1
II. PEG Concentrate				
100	13,300	13.8	56	1.6
A3.12	58,300	74.5	70	2.6
UKi-2	48,700	42.1	80	2.3
III. $(NH_4)_2SO_4$				
30 - 70% 100	8,800	109	37	12.8
40 - 70% A3.12	20,100	295	23	10.4
40 - 70% UKi-2	15,100	163	25	8.8
IV. Dialysis (5 mM TCM buffer)				
100	8,025	139	33.8	16.3
A3.12	11,874	340	13.7	12.0
UKi-2	13,134	233	21.6	12.4

Cultures were grown for 24 hours in 1,000 ml volumes of PYE broth and cells were removed by centrifugation. The results represent the average of 2 - 6 experiments. Percent $(NH_4)_2SO_4$ refers to percent saturation of water at 0° C. The final purification and specific activity of H-I B. bacteriovorus 100 proteases were achieved by elution electrophoresis. See Table 8 for these values.

was determined. When the ASP purified enzyme material was assayed in the electrophoresis buffer, essentially no major loss of activity was observed (Table 6). Indeed, a slight stimulatory effect on the protease activity of H-I B. starrii A3.12 by Tris-glycine over the TCM control was noted, although this likely due to the higher pH.

Because of the protocol used in the elution electrophoresis, both enzymes were eluted with 1/5 Tris-glycine buffer. The identification of the eluted metallo proteases, however, is dependent upon the inhibition of proteolytic activity by EDTA. Therefore, the influence of Tris-glycine buffer on the inhibition of proteases by EDTA was determined (Table 6). With the exception of H-I B. stolpii UKi-2, the Tris-glycine buffer effected a type of protective influence over the inhibition by EDTA. Even when the concentration of EDTA in Tris-glycine buffer was increased (60 mM) to sufficiently bind all cations which could possibly contaminate the buffer, the EDTA inhibitions were not as severe as in TCM buffer.

D. Polyacrylamide Gel Electrophoresis

Attempts to routinely separate protease activities by conventional gel filtration or ion exchange chromatography have so far been unsuccessful. With the use of polyacrylamide gel electrophoresis, separation of protease activities has been accomplished, and has demonstrated for the first time that the Bdellovibrio produce at least two

Table 6. Effect of Tris-glycine Buffer on Protease Activity and EDTA Inhibition

Culture	Activity (u/ml) 10 mM TCM (pH 7.5)	Activity (u/ml) 1/10 Tris-glycine (pH 8.3)	10 mM TCM (pH 7.5) 10 mM EDTA	% Inhibition 1/10 Tris-glycine (pH 7.75) 10 mM EDTA	% Inhibition 1/10 Tris-glycine (pH 7.75) 30 mM EDTA	Protease Activity 1/5 Tris-glycine (pH 7.75) 10 mM EDTA	Protease Activity 1/5 Tris-glycine (pH 7.75) 30 mM EDTA	Protease Activity 1/5 Tris-glycine (pH 7.75) 60 mM EDTA
H-I B. starrii A3.12	1190	1320	73%	68%	59%	58%	53%	57%
H-I B. stolpii UKi-2	1210	1255	84%	78%	72%	80%	74%	79%
H-I B. bacteri-ovorus 100	1020	1030	74%	58%	66%	54%	38%	43%

Assays were performed using the following protocol: ASP partially purified protease was diluted 1:100 in buffer [10 mM TCM; 1/5 Tris-glycine, (10 mM tris, 76 mM glycine); 1/10 Tris-glycine, (5 mM tris, 38 mM glycine)]. The final concentration of EDTA are indicated above. Azocoll was the substrate used (2 mg/ml). Reactions were run at 40° C. Units/ml are defined as a change in O.D. 570 of 0.001/minute.

or more proteases.

When the ASP partially purified proteases were electrophoresed through polyacrylamide gels (Figure 6), a unique zymogram pattern was noted for each of the three Bdellovibrio species. The most commonly observed proteolytic activity patterns are indicated by the relative widths of the diagrammed hydrolytic zones. Identical patterns of proteolytic activity were produced by H-I B. bacteriovorus strains 100, 109D, 110, and Xty.

In order to determine which enzyme band corresponds to the serine active protease, the concentrated enzyme material was pre-incubated with PMSF at 34° C for 1 hour before electrophoresis. When this is done, the faster migrating enzyme of both H-I B. stolpii UKi-2 and H-I B. bacteriovorus 100 is eliminated, while with H-I B. starrii A3.12, the slower migrating enzyme is absent. In addition, a partial inhibition of the faster migrating enzyme of H-I B. starrii A3.12 was also noted, thus indicating some influence by the PMSF and/or the ethanol solvent.

E. Elution Electrophoresis

When the ASP Bdellovibrio proteases are eluted, unique elution profiles for each of the three species are obtained (Figure 7). H-I B. bacteriovorus 100 and H-I B. stolpii UKi-2 each exhibit two peaks

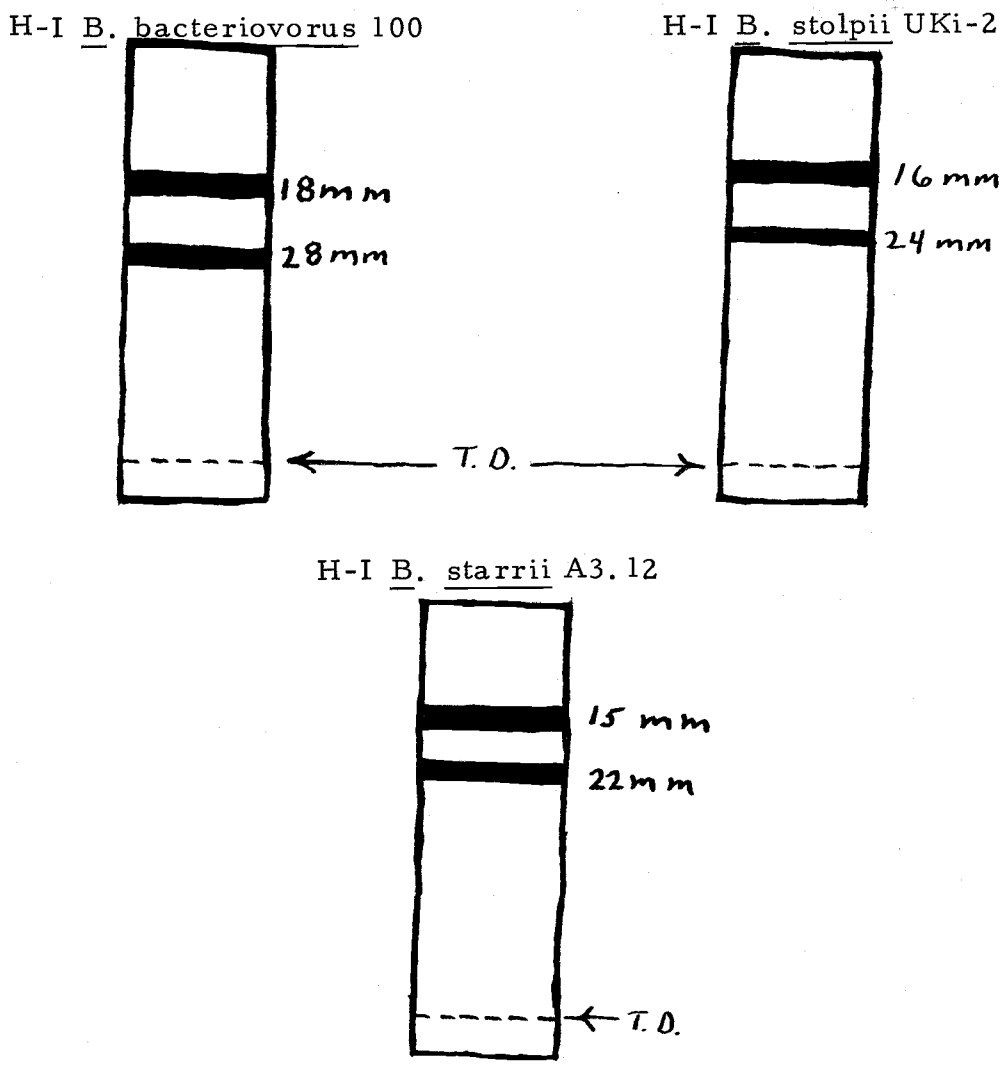

Figure 6. Zymogram patterns for each of the three species of the Bdellovibrio. ASP partially purified protease (0.2 ml of enzyme plus 0.2 ml of 40% sucrose) was layered above the large-pore gel (see Materials and Methods for details). T.D. = TRACKING DYE. Electrophoresis was for 100 minutes, and 1/10 Tris-glycine (pH 8.3) was the buffer used.

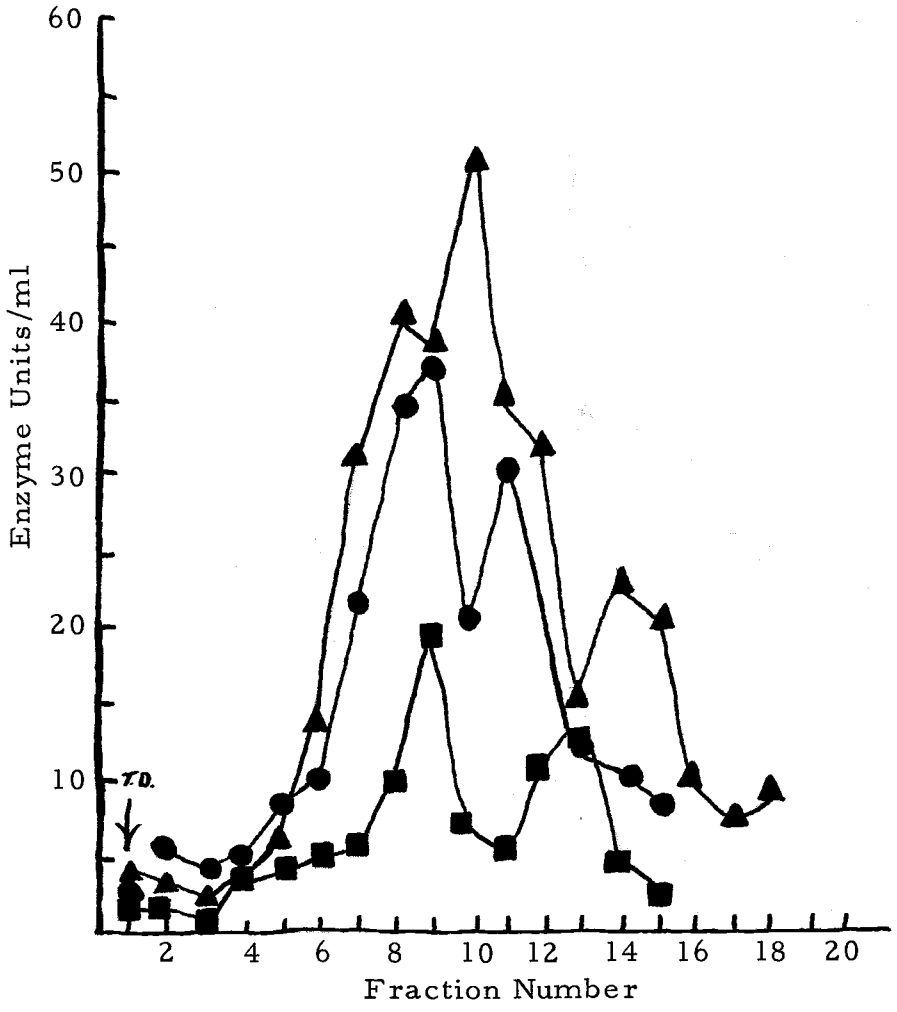

Figure 7. Protease elution profiles of all three species of the Bdellovibrio. The eluted enzymes were collected in a 25 ml graduate cylinder containing 2.5 ml of 5 mM TCM buffer. Five ml per fraction were collected at a rate of 0.5 ml/minute. Enzyme assays employed Azocoll as substrate (4 mg/ml) and 40° C incubation. Symbols: ▲ H-I B. starrii A3.12; ■ H-I B. stolpii UKi-2; ● H-I B. bacteriovorus 100.

while in the H-I B. starrii A3.12 elution three to four peaks are observed.

Since the elution profile of the H-I B. starrii A3.12 proteases was complex, several additional elutions were made to determine if a consistent pattern would develop. Of these elutions, a two peak profile was apparent only once in three experiments. Furthermore, when the pH optimum of the peak fractions of such a "two peak" elution was determined, a shoulder in each curve was observed (Figure 8). This suggests that more than one protease is present in each peak fraction of Figure 8. Finally the EDTA inhibition studies of this two peak profile further suggested that more than two proteases may be present (Table 7). When each peak was dialyzed against three one-liter changes of 5 mM TCM and assayed in the presence of 10 mM EDTA, only a partial inhibition was obtained. This is in contrast to the complete inhibition observed with the eluted H-I B. bacteriovorus 100 metallo enzyme.

In an attempt to verify that more than two proteases were produced by H-I B. starrii A3.12, ASP partially purified enzyme was electrophoresed at pH 7.3, 8.0, and 9.0. Figure 9 summarizes the results of these experiments. It is observed that two distinct bands of hydrolytic activity are present at pH 8.0 and 9.0, while at pH 7.3, four distinct bands are found. In addition, when the proteases of H-I B. stolpii UKi-2 and H-I B. bacteriovorus 100 are electrophoresed at

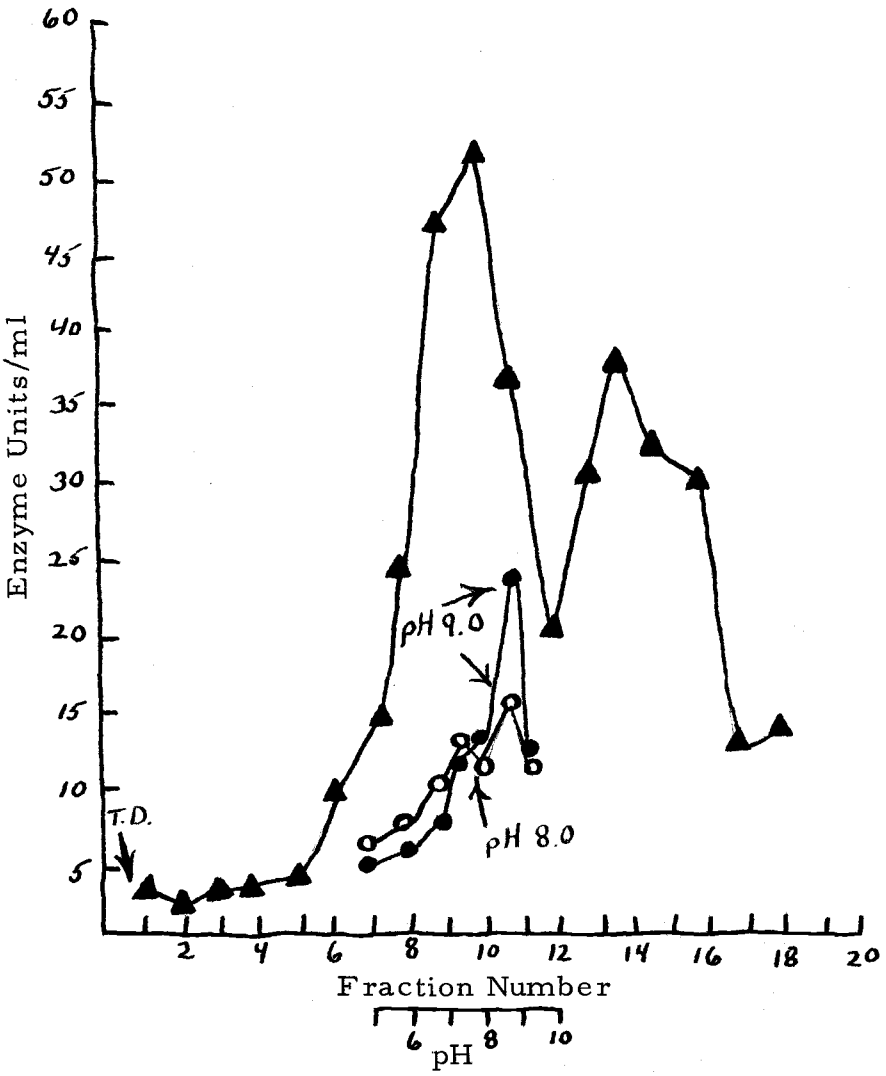

Figure 8. Two peaked protease elution profile and pH profile of H-I B. starrii A3.12. The eluted enzymes were collected as described previously (Figure 7). Fractions 8 - 10 and 13 - 16 were pooled and assayed for pH optima. Enzyme assays for both the eluted fractions and pH profiles used Azocoll as substrate and 40° C incubation. Symbols: ▲ elution profile; ● eluted enzyme form first peak; O eluted enzyme from second peak.

Table 7. Patterns of EDTA and PMSF Inhibition and Total Activity for Eluted Proteases of H-I Bdellovibrios

Culture	10 mM EDTA (non-dialyzed protease)	10 mM EDTA (protease dialyzed against 5 mM TCM)	10 mM PMSF (non-dialyzed protease) % Inhibition	Activity (u/ml)
H-I *B. starrii* A3.12				
Peak 1	29.5%	21%	57%	31
Peak 2	22%	30%	88%	29
H-I *B. stolpii* UKi-2				
Metallo	65%	N.D.	6%	14
Serine	55%	N.D.	70%	18
H-I *B. bacteriovorus* 100				
Metallo	72%	100%	74%	18
Serine	24%	20%	92%	22

Assays were performed using the following protocol: The eluted protease (dialyzed against 3 changes of 5 mM TCM) was diluted 1:5 into 5 mM TCM buffer. EDTA or PMSF was added to the final concentration noted above (see Materials and Methods for details). Azocoll was the substrate used (4 mg/ml) and incubation was at 40° C. N.D. indicates values not determined.

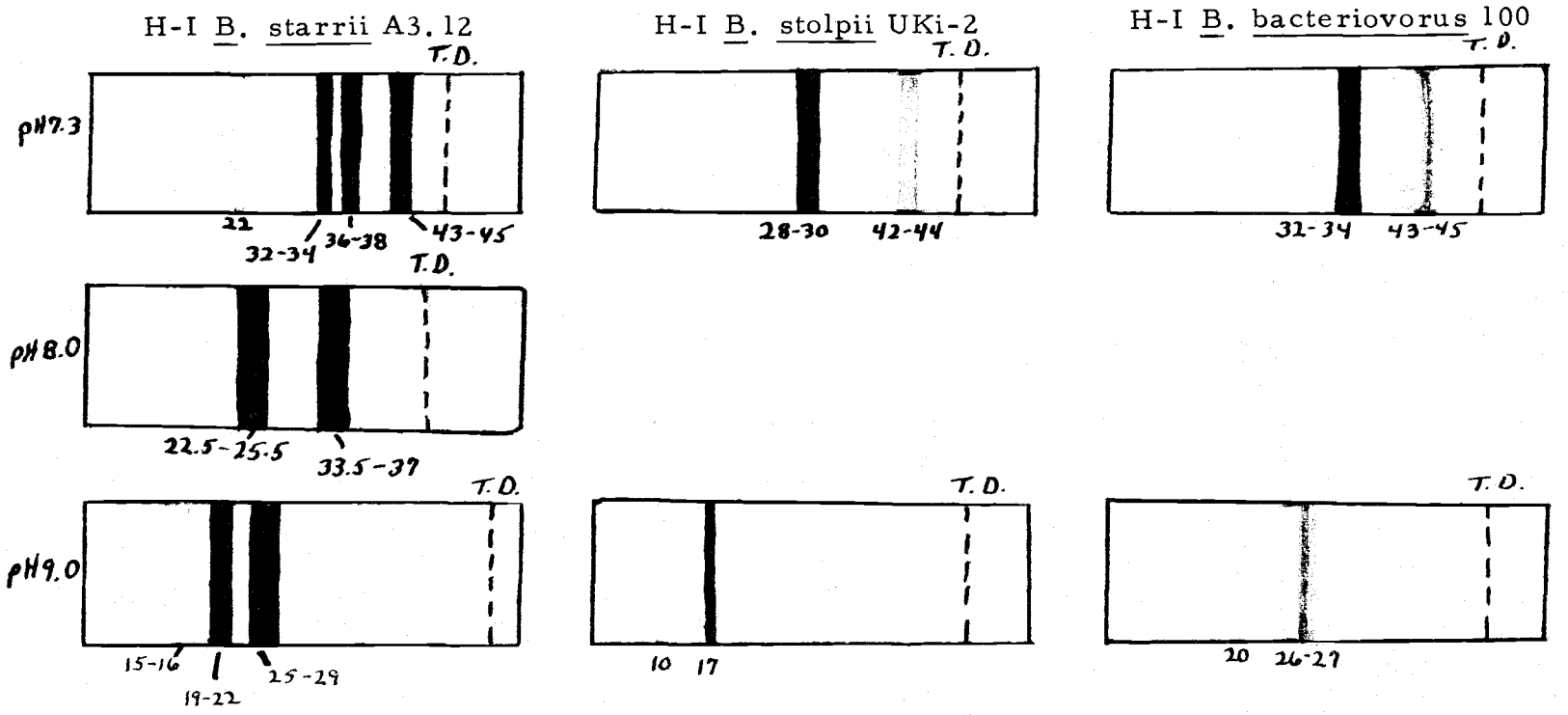

Figure 9. Electrophoresis of partially purified protease of all three species of Bdellovibrio at various pH values. Degree of shading indicates the relative degree of hydrolysis on casein. Time for electrophoretic runs; pH 7.3, 3 hours; pH 8.0, 100 minutes; pH 9.0, 60 minutes. See Figure 6 for pH 8.3 electrophoresis H-I B. stolpii UKi-2 and H-I B. bacteriovorus 100. Numbers indicate mm from origin of proteolytic activity.

pH 7.3, 8.3, and 9.0, only two bands of proteolytic activity are noted (Figures 6, 9). This observation strongly suggests that the latter two species only produce two proteolytic enzymes capable of hydrolyzing casein and Azocoll.

Since separation of four proteolytic activities was successful at pH 7.3, attempts were made to elute the H-I B. starrii A3.12 enzymes at this pH. When an elution of the H-I B. starrii A3.12 system was attempted, the tracking dye changed to a yellow color near the bottom of the gel. This indicated an acidic condition, and therefore no proteases were eluted off the gel. Because of this complex proteolytic system and the difficulty in eluting the enzymes at pH 7.3, further studies with H-I B. starrii A3.12 proteases were not attempted.

When the elution profile of H-I B. stolpii UKi-2 protease is studied (Figure 7), it is noted that the largest amount of protease activity obtained is only 18 units/ml. Several attempts were made to successfully elute these proteolytic enzymes with higher activities as was obtained in H-I B. starrii A3.12 and H-I B. bacteriovorus 100 protease systems. However, only two successful elutions were obtained and low protease activity occurred. For unknown reasons most of this enzyme was lost before or during the elution procedure.

F. Biochemical Properties of the Eluted Enzymes

Table 7 summarizes the results of the PMSF and EDTA inhibition studies on the H-I *B. stolpii* UKi-2 proteases. From these data, the serine protease (the first eluted off the gel) was inhibited 70% by PMSF, while the metallo enzyme showed only a 6% inhibition. EDTA inhibited the serine enzyme 55%, while the metallo enzyme had a 65% loss in activity. Sufficient enzyme was available to also determine the pH optimum of these two enzymes (Figure 10). The serine protease shows an optimum at pH 8.1 while the metallo enzyme is most active at pH 7.75.

Since the H-I *B. bacteriovorus* 100 proteases were consistently eluted off the gels with good activity, more in-depth studies of these enzymes were possible.

Table 8 summarizes the final purification and specific activity of the metallo and serine proteases. As can be seen, a 90-fold purification of the metallo protease, and a 98-fold purification of the serine enzyme was achieved. The final percent recovery of the metallo and serine proteases were 5.3% and 6.5% respectively (12% of initial activity; Table 5).

In Table 7, the effect of PMSF and EDTA on these protease activities is summarized. The serine protease (the first peak) is inhibited 92% by PMSF, while the metallo protease (the second peak)

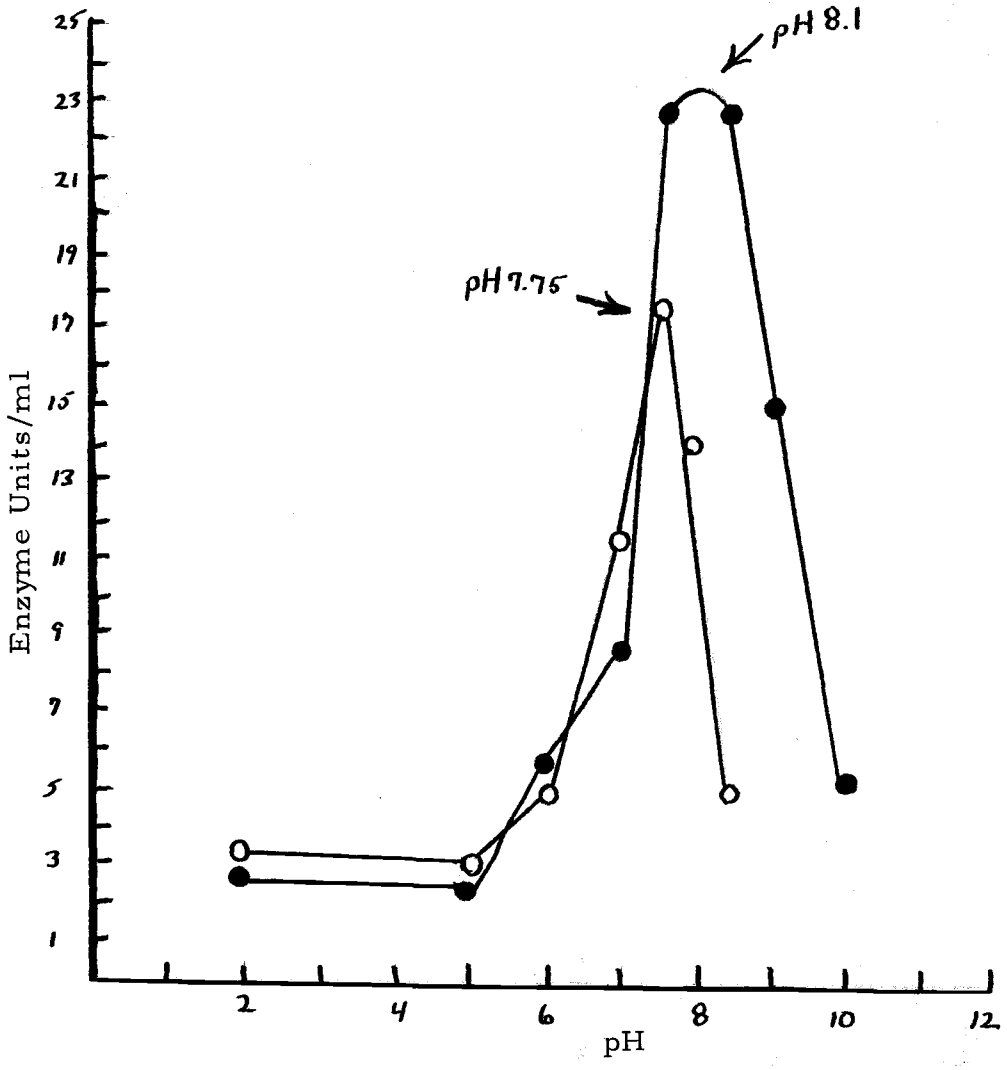

Figure 10. pH profile of the eluted B. stolpii UKi-2 proteases. Enzyme assays employed Azocoll (4 mg/ml) as substrate, 40° C incubation, and the following buffers: KCl - HCl (10 mM, pH 2.0); Citrate - phosphate (5 mM, pH 4 - 7.0); 5 mM TCM (pH 7 - 9.0); Tris-glycine (10 mM, pH 9 - 10.0). Symbols: ● serine protease; O metallo protease.

Table 8. Final Purification and Specific Activity of the H-I B. bacteriovorus 100 Protease

Proteolytic Enzyme	Total Enzyme Units	Specific Activity (units/mg protein)	Percent Recovery	Purification
Metallo protease	506	305	5.3%	90
Serine protease	930	500	6.5%	98

Total enzyme units are the total amount of protease activity in the pooled fractions from the elution electrophoresis. Percent recovery was determined by assuming that 40% of the initial protease activity in step I, Table 5 was due to the metallo protease (Table 3). Purification was likewise determined by assuming that 40% of the specific activity of the crude enzyme (step I, Table 5) was also due to metallo protease. Thus, the initial specific activity of the metallo protease would be 3.4 u/mg and the serine protease would be 5.1 u/mg.

demonstrated a surprising 74% inhibition. In addition, the EDTA completely inhibited the metallo enzyme only when the eluted enzyme was dialyzed against 10 mM TCM buffer, while with the serine protease a 20% inhibition is observed.

Since the metallo enzyme was inhibited 74% by PMSF, it seemed reasonable to check for contamination by the serine protease. Two ml of the eluted metallo protease was preincubated with PMSF for 1 hour at 34° C. After incubation, 1 ml of the enzyme was assayed for activity while the remaining 1 ml was electrophoresed. Also, a control enzyme (treated with 1% ethanol) was likewise incubated in the same manner. When this was done, the PMSF treated protease assayed with Azocoll showed a 67% inhibition, while the ethanol control showed only an 8% loss in activity. However, the PMSF-treated enzyme demonstrated hydrolytic activity comparable to that observed with the control enzyme on the casein mounts. Also, only one proteolytic band was found at 18 mm from the origin, the position corresponding to the metallo enzyme.

Having found that the metallo enzyme was free of any serine protease, the latter enzyme was analyzed to determine if it was likewise in a pure form. When the serine protease was dialyzed against Tris-HCl, and assayed in the presence of EDTA, a 12% reduction in proteolytic activity was observed (Table 9). Since a 20% inhibition by EDTA in 10 mM TCM buffer was noted, the serine protease was

electrophoresed. In this experiment, 1 ml of the serine protease was treated with PMSF, while another 1 ml was treated with 1% ethanol, and both were preincubated at 34° C for 1 hour, followed by electrophoresis. When this is done, the PMSF treated enzyme is completely inhibited, while with the ethanol control, a band 28 mm from the origin is observed on the casein mount. Furthermore, no additional band at 18 mm was observed even upon prolonged incubation.

Since it was found that charged molecules such as 1/10 Tris-glycine could maintain the full activity of the H-I B. bacteriovorus 100 ASP partially purified protease, and since PMSF and EDTA were inhibitory (in varying degrees) to both of the eluted proteases (Tables 6, 7), the serine and metallo enzymes were dialyzed against Tris-HCl and Tris-glycine buffers, and their response to cations was recorded. Table 9 summarizes the results of these experiments. When the serine and metallo proteases are dialyzed against 10 mM Tris-HCl (pH 7.75), the metallo enzyme loses 50% of its activity, while the serine protease shows very little change. In addition, when either Mg^{++} or Ca^{++} is added back, a 33% increase in activity occurs with the metallo protease whereas the serine protease shows little change in activity. Furthermore, when both Ca^{++} and Mg^{++} cations are added back, full recovery of the metallo enzyme activity is observed, while only a slight increase in activity occurs with the

Table 9. Dialysis of Eluted H-I Bdellovibrio bacteriovorus 100 Proteases: Effect of Buffers, Inhibitors, and Cation(s) on Proteolytic Activity

Proteolytic Enzyme	Control (5mM TCM) Activity (u/ml)	Tris-glycine	Tris-HCl Buffer				% Inhibition by 10 mM EDTA
			Tris	Tris + 2 mM Ca^{++}	Tris + 3 mM Mg^{++}	Tris + 2 mM Ca^{++} 3 mM Mg^{++}	
Metallo protease	20	19.9	10.3	16.4	16.4	19.7	100%
Serine protease	21	21.4	18.4	17	18.4	20.7	12%

Assays were performed using the following protocol: The eluted enzymes were dialyzed against two to three one-liter changes of the following buffer [5 mM TCM (pH 7.75); 1/10 Tris-glycine (pH 7.75); 10 mM Tris-HCl (pH 7.75)]. The dialyzed enzymes were then diluted 1:5 with each of the dialysis buffers. Just prior to the assays, cation(s) or EDTA were added as indicated above. The final volume of the reaction mixture (buffer + enzyme + cation(s) or EDTA) was 5 ml. Azocoll was the substrate used and incubation was at 40° C.

serine protease. Finally, when the serine and the metallo proteases are dialyzed against 1/10 Tris-glycine buffer (pH 7.75), both enzymes show complete activity as compared to the 5 mM TCM control.

Because only 50% of the activity of the metallo protease was lost in Tris-HCl, the two proteases were dialyzed against double distilled water (pH 7.5) (Tables 10, 11). Tris, Tris and Ca^{++} plus Mg^{++}, Tris and an amino acid, and amino acids were added back to the reaction mixtures, and the amount of proteolytic activity determined. It can be seen in Table 10 that 80% of the proteolytic activity of the metallo enzyme is lost when dialyzed against double distilled water. Forty-nine percent of the serine protease activity is also lost. However, when Tris is added back to the reaction mixture, a 188% increase in activity of the metallo protease and a 52% increase in activity of the serine protease is observed.

Also, when Tris plus cations are added to the reaction mixtures, 236% increase by the metallo enzyme and a 43% increase by the serine protease is also noted. Furthermore, when an amino acid was added back, an increase of enzyme activity was also observed (Table 11). With the serine protease, cysteine demonstrated the greatest increase in activity, while glycine, glutamic acid, and arginine showed a slight increase. The metallo enzyme, however, demonstrated increases in activity by all five of the amino acids tested, with cysteine and glutamic acid demonstrating a 214% and

Table 10. Dialysis of Eluted H-I Bdellovibrio bacteriovorus 100 Proteases: Effect of Double Distilled Water, Tris, and TCM on Proteolytic Activity

Proteolytic Enzyme	Control (non-dialyzed enzyme) Activity (u/ml)	Double Distilled Water	Tris		TCM	
		Activity (u/ml)	Activity (u/ml)	% Increase in Activity	Activity (u/ml)	% Increase in Activity
Metallo protease	20.2	4.0	11.5	188	13.4	236
Serine protease	18.4	9.4	14.3	52	13.4	43

Assays were performed using the following protocol: The eluted enzymes were dialyzed against one to two one-liter changes of the double distilled water (pH 7.5). Tris (10 mM) or TCM (10 mM tris + 2 mM Ca^{++} + 3 mM Mg^{++}) was added back as indicated above. The final pH of the reaction mixtures was 7.5. The percent increase in activity was based upon the activity of the metallo and serine proteases in double distilled water. Azocoll was the substrate used (4 mg/ml) and incubation was at 40° C.

Table 11. Dialysis of Eluted H-I Bdellovibrio bacteriovorus 100 Proteases: Effect of Various Amino Acids after Dialysis in Double Distilled Water

Proteolytic Enzyme	I. Activity (u/ml)			II. % Increase in Activity			
	Glycine	Alanine	Cysteine	Glutamic Acid	Arginine	Tris+ glycine	Tris+ cysteine
Metallo protease							
I.	12.2	9.0	12.5	13.4	9.5	15.9	17.0
II.	206	125	214	236	137	298	325
Serine protease							
I.	10.5	9.0	14.2	11.5	10.7	14.7	15.9
II.	12	0%	51	23	14	56	69

Assays were performed using the following protocol: The eluted enzymes were dialyzed against one to two one-liter changes of the double distilled water (pH 7.5). The dialyzed enzymes were then diluted 1:5 with double distilled water (pH 7.5), and an amino acid was added back (final concentration 20 mM), or Tris (10 mM) plus an amino acid (20 mM). The pH of the final reaction mixtures was 7.5 ± 0.3. The percent increase in activity was based upon the activity of the metallo or serine protease in double distilled water (Table 10). Azocoll was the substrate used (4 mg/ml) and incubation was at 40° C.

236% increase of proteolytic activity respectively. In addition, Tris plus cysteine or Tris plus glycine showed a 69% and 56% increase in activity for the serine protease, while with the metallo enzyme a 325% and 298% increase is observed. Finally, since both proteases showed an increase in activity in the presence of cysteine, it suggests that proteolytic activity may be optimal under reduced conditions (7). Therefore, in order to observe what effect oxidized conditions had on protease activity, both enzymes were treated with 5 mM H_2O_2. When this was done, neither the serine nor the metallo proteases showed any sensitivity to the hydrogen peroxide.

In other characterization studies, the pH and temperature optima of the H-I B. bacteriovorus 100 proteases were determined. Figure 11 shows the pH profiles of both the serine and metallo proteases. The serine enzyme has an optimum at pH 8.0 - 8.1, while the metallo protease reaches its maximum activity at pH 7.5. Figure 12 illustrates the temperature optimum for each of the proteases. In this case, the serine protease reaches its maximum amount of activity around 42° C, while the optimum for the metallo enzyme is approximately 44° C.

Since the optimum pH and temperature for each of the H-I B. bacteriovorus 100 proteolytic enzymes were known, the Km of each of these proteases on Azocoll could be determined. Assuming that Azocoll has a molecular weight equal to collagen (30,000; 9), and

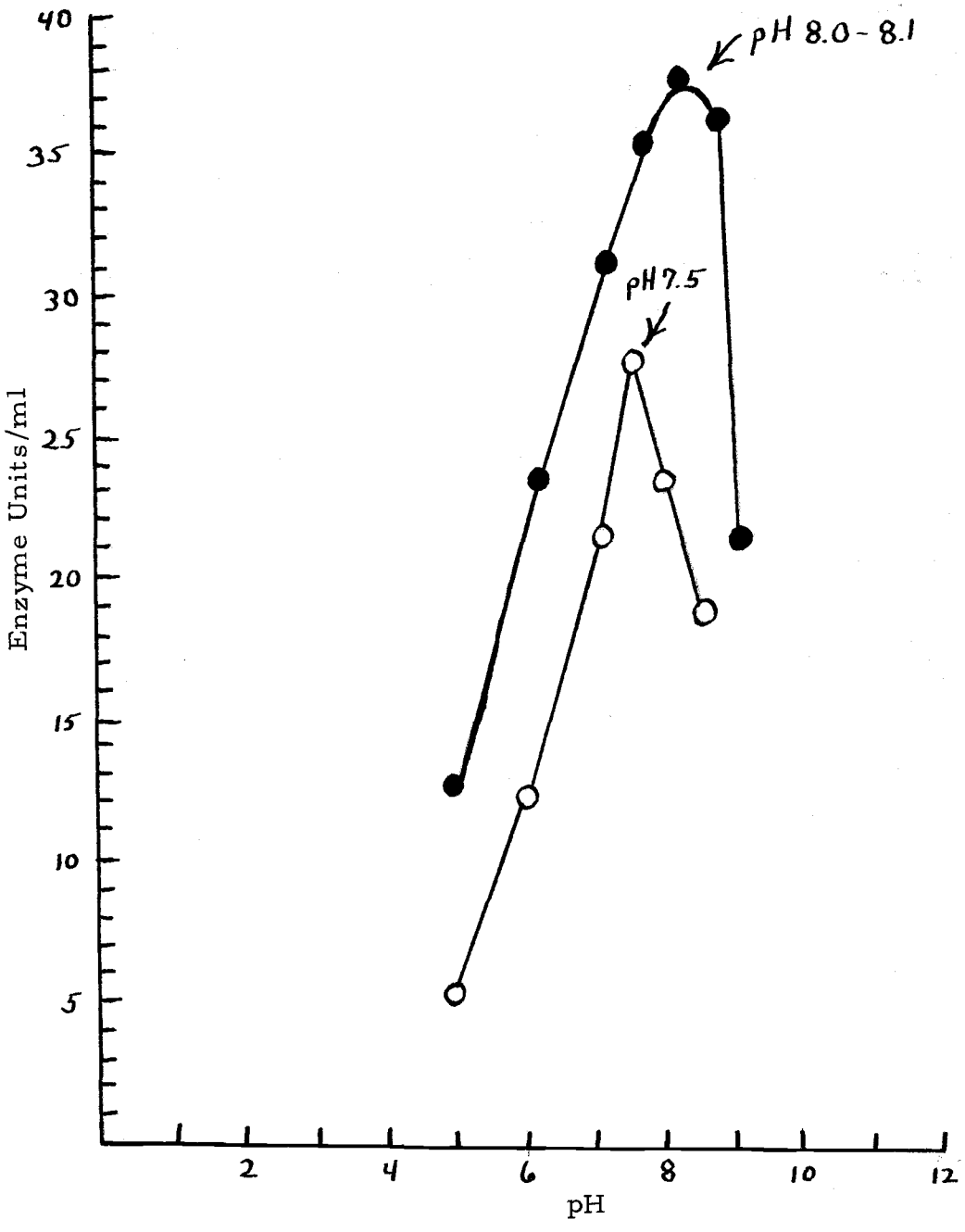

Figure 11. pH profile of H-I B. bacteriovorus 100 proteases. Enzyme assays employed Azocoll as substrate (4 mg/ml) and 40° C incubation. Symbols: ● serine protease; O metallo protease.

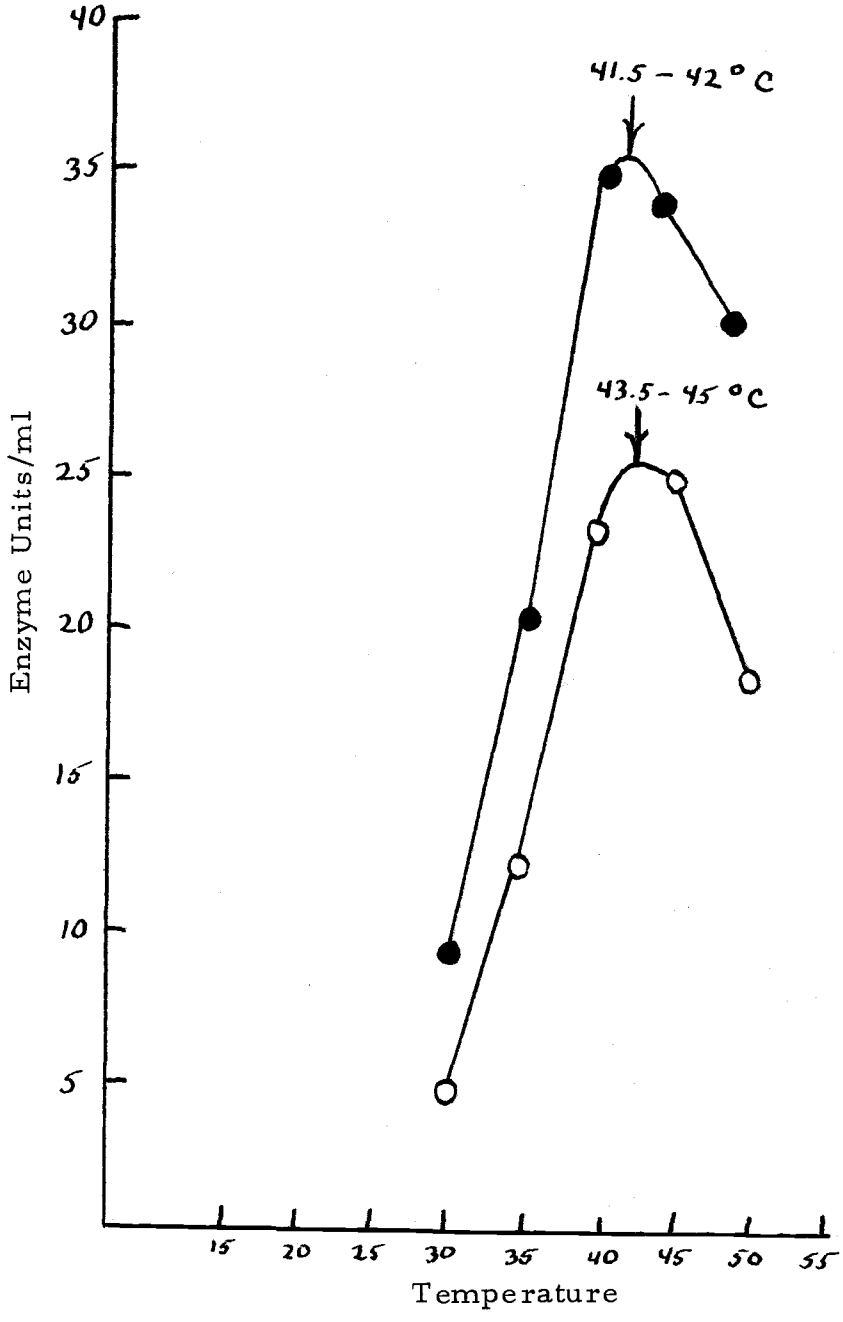

Figure 12. Temperature profile of B. bacteriovorus 100 proteases. Assays employed Azocoll as substrate. Symbols: ● serine protease at pH 8.0; O metallo protease at pH 7.5.

having previously determined that the specific activity of the serine and metallo proteases were 306 units/mg and 500 units/mg respectively, the Km of these enzymes was found. Figures 13 and 14 show typical saturation curves for the serine and metallo proteases. From these data, the serine protease shows half maximum velocity with 4.9 mg/ml of Azocoll (Km value of 3.8×10^{-5} M), while 6.25 mg/ml of Azocoll corresponds with half the maximum velocity of the metallo protease, which is equivalent to a Km of 4.8×10^{-5} M.

For the determination of molecular weight, the protein standards alcohol dehydrogenase (148,000 molecular weight), bovine albumin (67,500 molecular weight), ovine albumin (44,000 molecular weight), and cytochrome c (11,700 molecular weight) were passed through a Sephadex G-100 column. The void volume, as determined by the blue dextran marker, was 11 ml. After the standard curve was determined (by passing the standard proteins through the column three times; Figure 15) the H-I B. bacteriovorus 100 serine and metallo proteases were each passed (once) through the column. The metallo enzyme was found to peak at fraction #17, while the serine protease reached its maximum point of activity at fraction #20 (Figure 16). From these data, it was then determined that the metallo and serine proteases have a molecular weight of about 50,000 and 32,000 respectively.

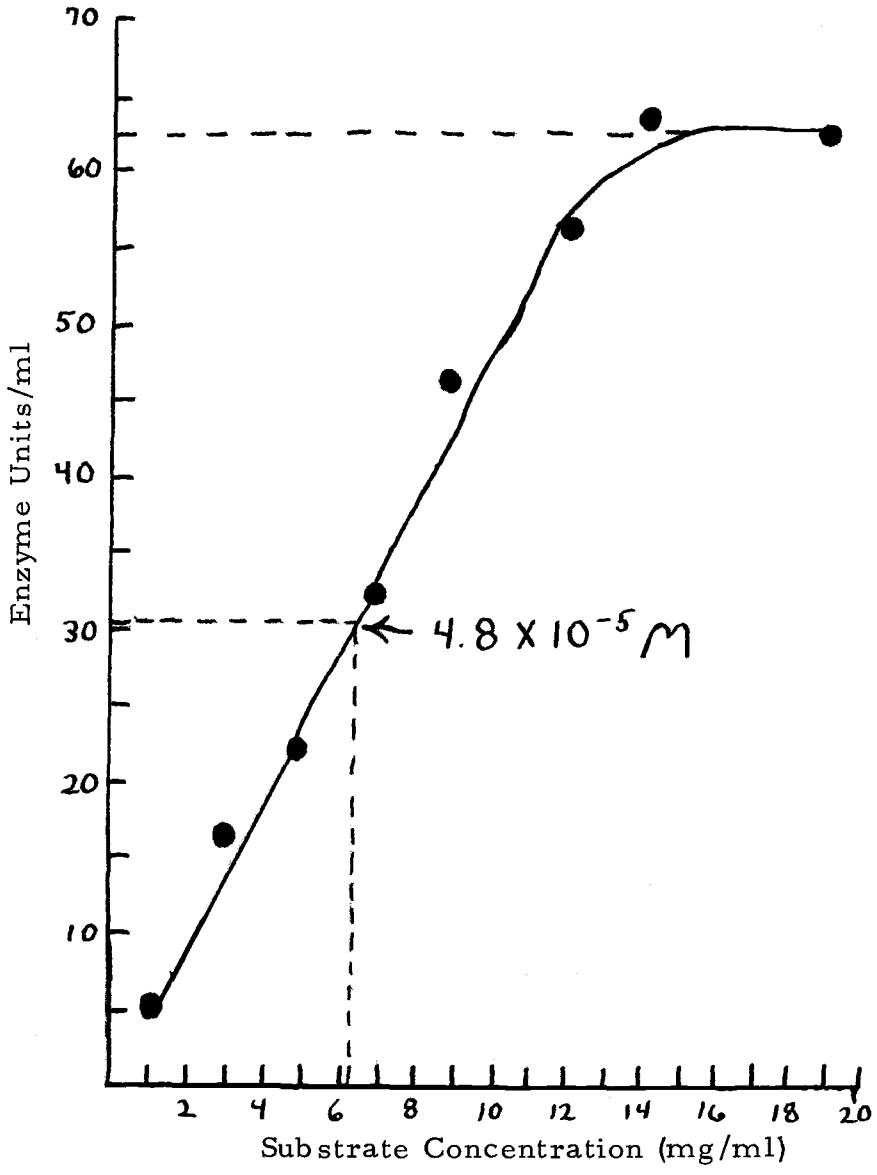

Figure 13. Km determination of H-I B. bacteriovorus 100 metallo protease. Assays employed Azocoll as substrate (1 - 19 mg/ml) and 44° C incubation. The pH of the reaction mixtures was at 7.5.

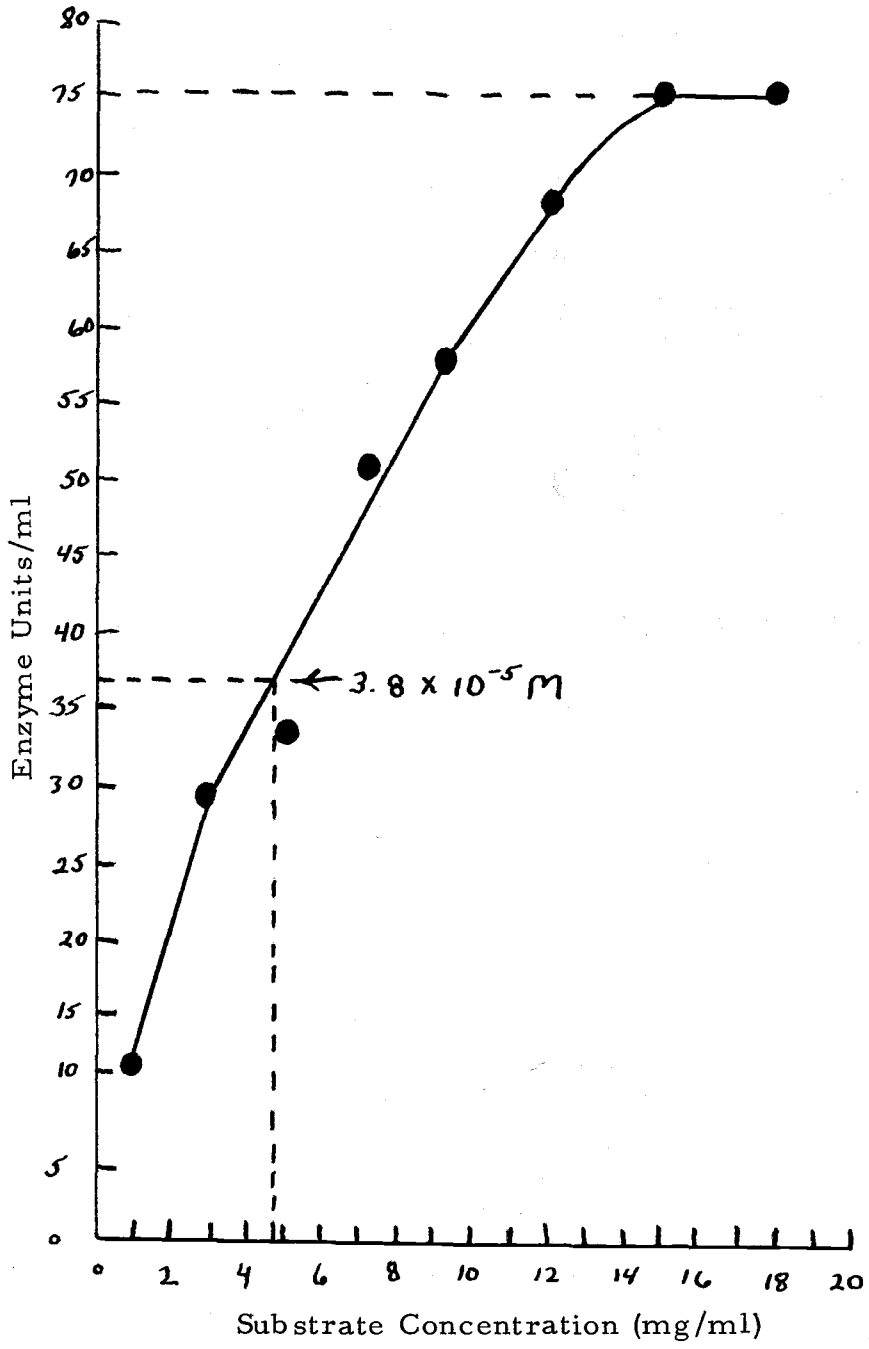

Figure 14. Km determination of H-I B. bacteriovorus 100 serine protease. Assays employed Azocoll as substrate (1 - 18 mg/ml) and 42° C incubation. The pH of the reaction mixtures was at 8.0.

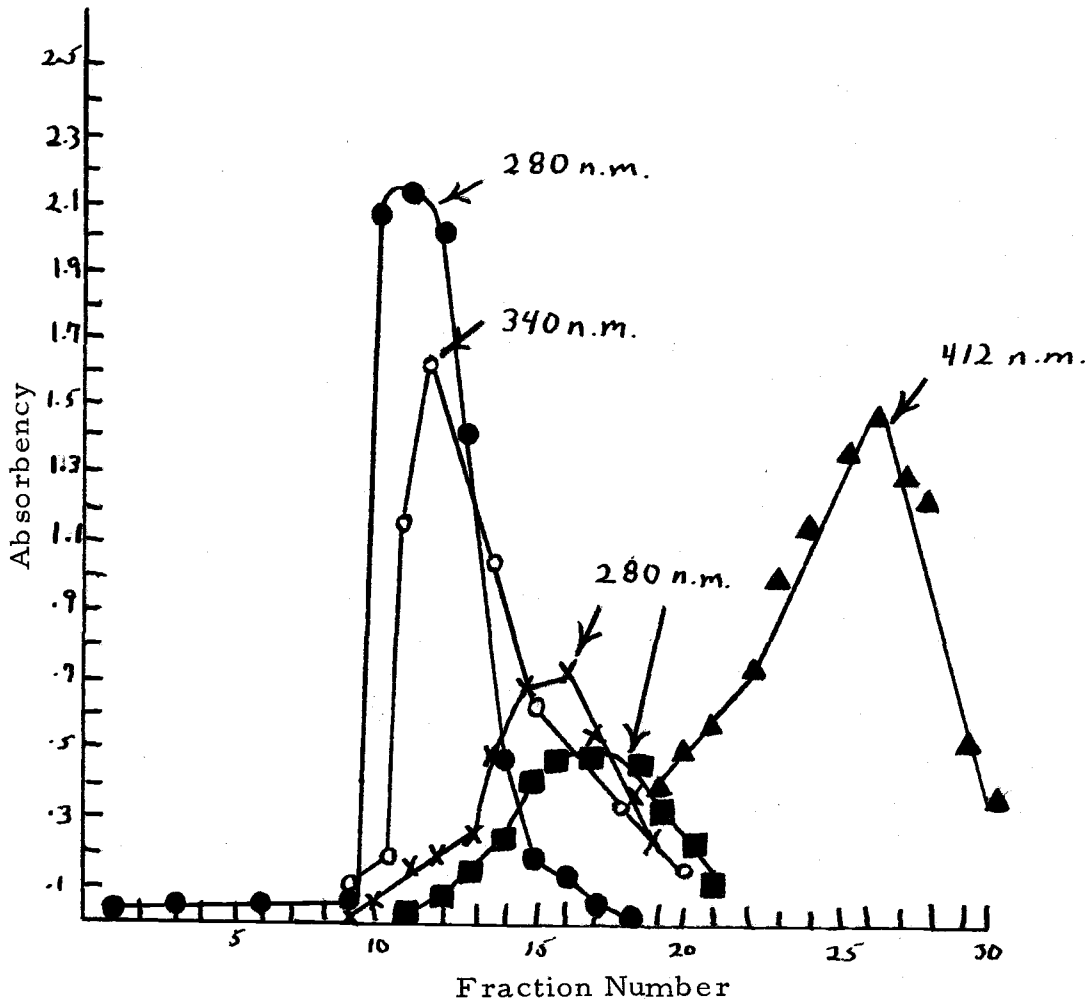

Figure 15. Chromatography of standard proteins on Sephadex G-100 gel in Tris-glycine buffer (50 mM, pH 7.75). The column measured 1.5 x 30 cm. Flow rate was 1 ml/min (16 drops) and 1 ml fractions were collected. Symbols: ● Blue dextran; O Alcohol dehydrogenase; X Bovine albumin; ■ Ovine albumin; ▲ Cytochrome c.

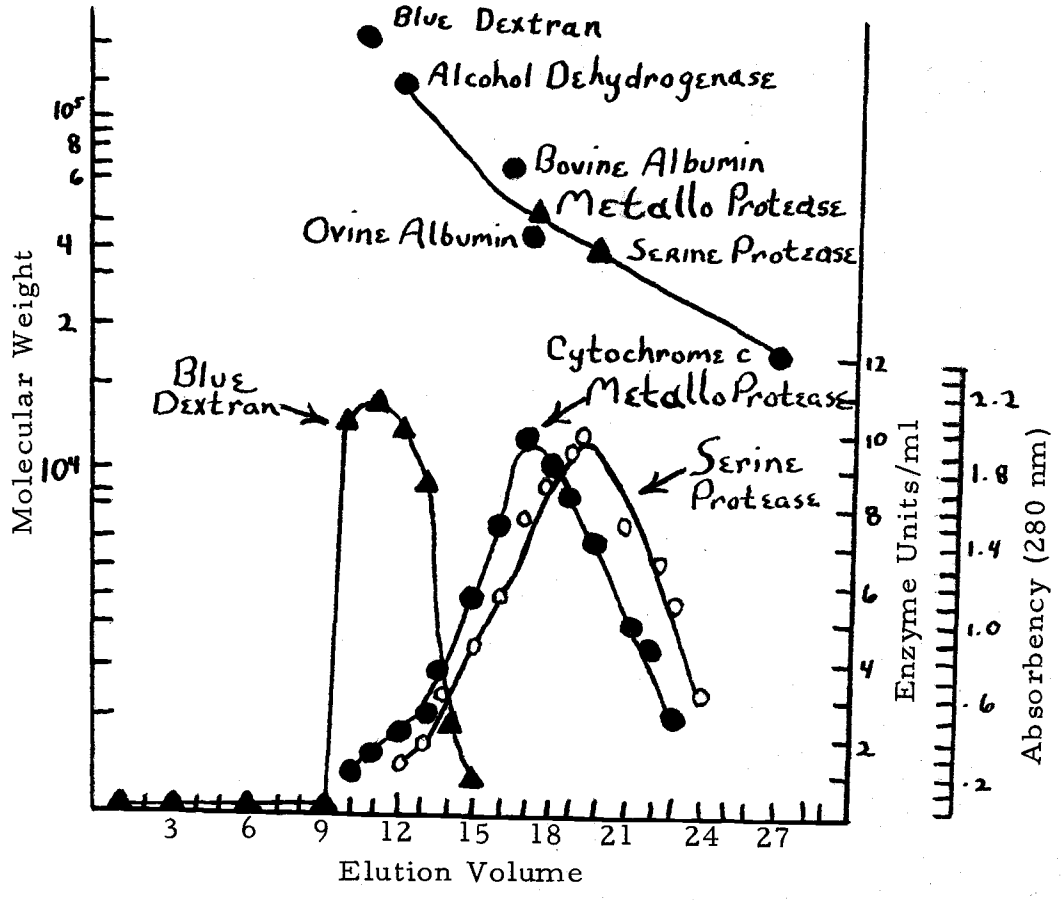

Figure 16. Standard curve for the determination of molecular weight and elution profile of the proteases from the Sephadex G-100 column. The enzyme eluted from the column was diluted 1:3 in 50 mM Tris-glycine (pH 7.75). Azocoll was the substrate used (4 mg/ml), incubation temperature was 40° C. The molecular weights of the H-I B. bacteriovorus 100 serine and metallo proteases are also indicated on the standard curve.

DISCUSSION

The amounts of protease produced by the three species of Bdellovibrio are illustrated in Figure 3 and Tables 3 and 4. The decreasing order of total proteolytic enzyme units was B. starrii A3.12 > B. stolpii UKi-2 > B. bacteriovorus in a ratio of 10:5:1. Thus a convenient means of classifying new isolates of the Bdellovibrio can now be made based on total protease production. Up until this time, no other convenient system was available to distinguish bdellovibrios with the possible exception of host-range specificity.

The action of the proteolytic enzymes does not appear to be limited to specific substrates. Gloor and Seidler (6) have demonstrated that host cell protein, collagen, albumin, casein, and gelatin are degraded by these proteases. Shilo and Bruff (19) have reported that heat-killed cells of both the gram negative and the gram positive bacteria are degraded by an exocellular enzyme of H-I B. starrii A3.12, but did not examine other H-I Bdellovibrio strains. In the present study, it was observed that the degree of digestion of heat-killed cells is dependent upon a species specific protease. The digestion of the heat-killed cells of S. serpens by the H-I B. starrii A3.12 proteases differs from that observed with the H-I B. stolpii UKi-2 proteases or the H-I B. bacteriovorus 100 proteases, which are also different from each other.

Experiments utilizing differential enzyme inhibitors indicate that the Bdellovibrio produce at least two major types of proteolytic enzymes. One enzyme (metallo protease; 5, 18) is inhibited by EDTA, while the other (serine protease) contains the amino acid serine in the active site and is thus inactivated by PMSF (10, 13). In addition, complete inhibition in the presence of EDTA and PMSF indicates that no other class of protease is present which degrades Azocoll (Table 3).

The effect of the electrophoresis buffer (1/10 Tris-glycine, pH 8.3) as a substitute for Ca^{++} and Mg^{++} in supporting proteolytic activity of the ASP partially purified protease was the first indication that other charged molecules could function in this manner (Table 6). This observation was further established when it was shown that the buffer itself could lower the percent inhibition of 60 mM EDTA by 16 - 31%. Thus, in the presence of Tris-glycine, the percent inhibition by EDTA was always less than that observed in the TCM control buffer.

The initial attempts to separate the two proteases by conventional gel filtration and ion exchange resins proved unsatisfactory. Therefore, conformation on the existence of two major proteases for H-I B. stolpii UKi-2 and H-I B. bacteriovorus 100 was attempted and achieved through separation on polyacrylamide gels (Figures 6, 9). The use of this same technique has also provided evidence for the

existence of four proteases for H-I B. starrii A3.12. This latter observation, however, did not become apparent until electrophoresis at several pH values was studied (Figure 9).

Identification of the electrophoretically separated proteases was achieved with use of 1 mM PMSF. With H-I B. stolpii UKi-2 and H-I B. bacteriovorus 100, the serine protease had the faster migrating enzyme. With the H-I B. starrii A3.12, however, serine proteases were believed to have been detected in both hydrolytic bands. In this case, after the PMSF treated ASP partially purified protease was electrophoresed at pH 8.3, the slower migrating enzyme was completely inhibited, while the faster migrating enzyme showed only partial inhibition. Therefore, either the ethanol solvent caused a partial inhibition or more than one serine protease is produced by the H-I B. starrii A3.12. If the latter is true, there is at least one serine protease in each of the two hydrolytic bands after electrophoresis at pH 8.3. Further evidence that the H-I B. starrii A3.12 produces more than two proteases will be discussed later.

The elution patterns of both the H-I B. stolpii UKi-2 proteases and the H-I B. bacteriovorus 100 proteases agreed with the electrophoresis data that two proteases were produced by each of these species. However, with the H-I B. starrii A3.12 proteases, no correlation was found between the elution profile and the zymogram pattern from an electrophoretic run at pH 8.3. For this reason, a

more in-depth study of this protease system was carried out.

When the H-I B. starrii A3.12 ASP partially purified protease was electrophoresed at pH 7.3, 8.0, and 9.0, only at pH 7.3 were four major bands of proteolytic activity detected (Figure 9). Furthermore, the elution profile (Figure 7) of these enzymes is complimentary to the electrophoretic run at pH 7.3, where four peaks can be seen. Also, when the pH optima of the only two-peaked elution profile were determined, a shoulder in each fraction was observed (Figure 8). Hence, these data strongly suggested that the H-I B. starrii A3.12 produces more than two proteolytic enzymes. It was unfortunate, however, that further studies on this protease system were prevented since elutions at pH 7.3 could not be successfully achieved.

When the H-I B. stolpii UKi-2 ASP partially purified protease was electrophoresed at pH 7.3 and 9.0, only two hydrolytic bands were observed. This indicated that there were only two proteases (Figure 9).

In other characterization studies involving the H-I B. stolpii UKi-2 protease system, both the eluted serine and metallo enzymes showed sensitivity (in varying degrees) to both PMSF and EDTA (Table 7). The serine protease was inhibited 70% by PMSF and 55% by EDTA, while the metallo enzyme was inhibited 65% by EDTA and 6% by PMSF. Thus, these data suggest cross contamination by the two proteolytic enzymes. However, since only two successful

elutions of these proteases were obtained, verification of this possibility was not achieved. Nevertheless, other data seemed to indicate that the two eluted proteases were in a pure form.

Since neither of the eluted H-I B. stolpii UKi-2 eluted enzymes (serine and metallo) were dialyzed against 10 mM TCM buffer, Tris-glycine was still present, thus preventing a complete inhibition of the metallo enzyme by EDTA. This would explain the observed 65% EDTA inhibition of the eluted metallo enzyme; but not, however, the 55% inhibition of the eluted serine protease. Therefore, either the EDTA molecule acted in some inhibitory manner on the serine protease, or the eluted enzyme was indeed a mixture of both metallo and serine proteases. Other data (which will be discussed below) indicates that the EDTA molecule does exert some inhibitory influence on the Bdellovibrio serine protease.

In other studies involving the H-I B. stolpii UKi-2 eluted proteases, the pH optimum for each enzyme was found. The metallo protease had an optimum at pH 7.75, while the serine protease has an optimum at pH 8.0 - 8.1 (Figure 10). It should also be noted that the pH profile of both the metallo and the serine protease demonstrates only one peak, thus further indicating the probable purity of each protease.

As mentioned previously, further studies on the H-I B. stolpii UKi-2 proteases were not carried out since the elution of these

enzymes could not be routinely achieved. For reasons unknown, upon completion of the elution electrophoresis usually no trace of proteolytic activity could be found in either the eluted fractions (by assaying with Azocoll as substrate) or in the gel itself (mounted on casein). The two successful elutions of these enzymes were obtained only when several stops were made during the electrophoretic run. These stops were necessary, as plugging occurred and the outflow slot of the gel unit had to be cleared of polyacrylamide debris.

The H-I B. bacteriovorus 100 proteases were the only proteolytic enzymes that have undergone an extensive characterization. After the elution electrophoresis, the serine and the metallo proteases were purified 98-fold and 90-fold respectively (Table 8). These values were determined by assuming that 40% of the initial specific activity of the crude enzyme (Table 5, step I) was due to the metallo protease (Table 3).

In other studies, when these eluted enzymes were treated with PMSF the metallo protease unexpectedly lost about 2/3 of its activity. Because the PMSF molecule is highly negatively charged (10), and since it is possible that the H-I B. bacteriovorus 100 metallo protease may belong to that group of metallo proteases that contains the Zn^{++} cation in its active site and requires Ca^{++} to permit protein conformation around the zinc atom (18), it suggests that an electrostatic attraction may occur between the two molecules, thus the

PMSF could block or alter the active site of the enzyme as a competitive inhibitor. Under these conditions it would require energy to break this attraction, and when under the influence of the electric field during electrophoresis such energy is provided. Another possibility is that the charge on these molecules may be altered during electrophoresis and the molecules would dissociate. Hence, the metallo protease is allowed to express its full normal activity on the casein mount after PMSF treatment, even though it is partially inhibited in the presence of PMSF when assayed with Azocoll on a shaking bath (Table 7).

The charged EDTA molecule may also exert some influence on the serine protease. Supporting evidence for this is found in the fact that when the H-I B. bacteriovorus 100 serine protease was dialyzed against 10 mM Tris-HCl and assayed for proteolytic activity in the same buffer plus 10 mM EDTA, a 12% loss in activity was observed (Table 9). In addition, since all members of the serine protease group may be classified as endopeptidases, it has been generally observed that the cleavage of the terminal peptide bonds are inhibited by the charge on the amino or carboxyl group of a terminal residue (27). Thus it seems likely that the observed inhibition by EDTA on both the H-I B. stolpii UKi-2 and H-I B. bacteriovorus 100 eluted serine proteases is not due to contamination by the metallo protease. This is further supported by the fact that when the H-I B.

bacteriovorus 100 eluted serine protease was electrophoresed, only one hydrolytic band (28 mm from the origin) was observed.

From the above observations it becomes apparent that charged molecules such as EDTA and PMSF exert inhibitions of varying degrees on both of the H-I B. stolpii UKi-2 and H-I B. bacteriovorus 100 metallo and serine proteases. Other charged molecules such as Tris, Ca^{++}, Mg^{++}, and amino acids also exert their influence.

Protease activity was increased by 1/3 when either Ca^{++} or Mg^{++} was added back to the H-I B. bacteriovorus 100 eluted metallo enzyme dialyzed against Tris-HCl. It was also determined that full recovery is obtained when both cations are added back to the eluted metallo protease. The eluted serine protease (from the same species) showed no loss in activity after dialysis (Table 9). Robinson et al. had observed similar results of increased activity with the Bdellovibrio 6-5-S protease (3), however they made no distinction concerning which class of protease their enzyme belonged. (Further comments on the data of this group will be discussed below.)

In other studies, when the H-I B. bacteriovorus 100 serine and metallo proteases were each dialyzed against double distilled water at pH 7.5, a 52% increase in activity of the serine protease and a 188% increase in activity of the metallo enzyme occurred upon the addition of 10 mM Tris to the distilled water (Table 10). Since the pH of the double distilled water alone, and the double distilled water plus Tris

were the same, it suggests that the increase in proteolytic activity may be due to the positive charge on the Tris molecule rather than pH.

When the H-I B. bacteriovorus 100 eluted serine and metallo proteases are dialyzed against double distilled water at pH 7.5, and Tris plus cations are added back, a 43% increase in activity of the serine protease and a 236% increase in activity of the metallo enzyme is observed (Table 10). However, both the serine and the metallo proteases (dialyzed and having Tris plus cations added back) have less activity (5 - 7 units/ml) than the non-dialyzed control. Thus, it is apparent that some irreversible denaturation of both proteases occurs in a system devoid of cations. It is also apparent, however (Table 10), that a complete loss of activity by the eluted metallo protease does not occur even after two one-liter changes of double distilled water (pH 7.5). Therefore, contaminating traces of cations (from the water itself) may prevent complete denaturation of the enzyme during dialysis. Thus the stimulatory effect of Tris or Tris plus cations may be dependent upon the presence of trace amounts of Ca^{++} and/or Mg^{++}.

When an amino acid is added back after dialysis in double distilled water (pH 7.5), the observed increase in protease activity varies (Table 11). Only cysteine caused a noticeable increase (51%) in proteolytic activity for the H-I B. bacteriovorus 100 eluted serine

protease, while with the eluted metallo enzyme (from the same species), all five of the amino acids tested enhanced enzyme activity. Thus, with respect to the metallo protease, glycine, cysteine, alanine, glutamic acid, and arginine may substitute to a degree for Ca^{++} and Mg^{++} (5). The question may be raised concerning the possibility that the amino acids may have been contaminated with trace amounts of cations. Since the concentration of the amino acids tested were 20 mM, and since 1/5 Tris-glycine reduced the inhibition by 60 mM EDTA (which is sufficient to chelate all cations present; Table 6), it suggests that an amino acid will support Bdellovibrio proteolytic activity even in the absence of cations. Furthermore, the stimulatory effect by amino acids is not due to a buffering action or a change in pH. When both Tris and an amino acid (cysteine or glycine) are added back (pH 7.5) the recovery in activity is greater than with Tris alone (Table 11). Finally, since cysteine had a noticeable effect on both proteases, it suggests that the enzymes might express more activity under reduced conditions (7). In the presence of 5 mM H_2O_2, no decrease in activity was observed.

In other characterization studies of the H-I B. bacteriovorus 100 proteolytic system, the pH optimum and the molecular weight of each protease was in general representative of their own group. The metallo enzyme exhibits maximum activity at pH 7.5 and has a molecular weight of 50,000, while the serine protease is most active at pH

8.0 and has a molecular weight of about 32,000.

In other studies involving the above proteases the temperature optimum and Km (using Azocoll as substrate) of each enzyme were found (Figures 12, 13, 14). The metallo enzyme has a temperature optimum of 44° C, and a Km value of 4.8×10^{-5} M. The serine protease was very similar in that its temperature optimum was at 42° C, and the Km value was 3.8×10^{-5} M.

Recently, there have been three studies on the extracellular enzymes of H-D Bdellovibrio 6-5-S (3, 4, 9, 22). Fackrell and Robinson have purified a peptidase enzyme (3,000-fold) which digests the DAP-alanine bond in the peptidoglycan of S. serpens (3, 4), but does not degrade Azocoll. This group has also described a protease which degrades Azocoll and has a molecular weight of 100,000 (4). This proteolytic enzyme, as mentioned previously, has not been classified as a metallo or serine protease. It was obtained from a 0 - 50% ammonium sulfate fraction and purified 7.1-fold by passage through a Sephadex G-100 column. When the molecular weights of the two proteases in this study are summed, a value of 82,000 is obtained. Hence, Fackrell and Robinson may have characterized a mixture of proteolytic activity containing both the metallo and serine proteases.

It is worthy to note that the only other reported experiment with this protease enzyme involves the effect of water, buffer (25 mM TCM, pH 7.5), and buffer plus Ca^{++} and/or Mg^{++} (2 mM) on protease

activity (4). Although there is no indication that the protease was dialyzed against water, buffer, or buffer plus cation(s) before the experiment, there is a detectable progression in the amount of activity regained with various charged molecules: activity in water < activity in buffer < activity in buffer plus Ca^{++} or Mg^{++} < activity in buffer plus Ca^{++} and Mg^{++}. These results are similar to what was obtained in the present study when the eluted metallo protease was first dialyzed against double distilled water or Tris (Tables 9, 10). No further comparisons between these two studies can be made since it is assumed that Fackrell and Robinson's protease was not dialyzed against water or buffer, in which case the effect of initial contamination by cations is very likely. The optimum temperature and pH of this protease(s) was not reported (4).

Another group, Huang and Starr (9, 22) have partially purified a proteolytic enzyme ("Azocollase") from H-D Bdellovibrio 6-5-S lysates. This enzyme was purified 62-fold and demonstrated a single peak of activity after it was obtained from a DEAE cellulose column. However, it is our experience that this single peak usually contains both the metallo and serine proteases. Again, no mention was made by these authors as to which class of protease their Azocollase belonged. Furthermore, since the yield of purified enzyme in that study was less than 2%, and since a molecular weight of 11,000 was determined for that protease, it is very probable that only one

proteolytic enzyme had remained. However, if this group characterized only one protease, then differences in molecular weight determination are apparent compared to the values obtained in both the present study and those of Robinson (4). In the present study the serine protease was found to have the lower molecular weight of 32,000. This is three times the estimation of the Huang and Starr Azocollase. Either gross differences in the molecular weight of the proteases occur within the Bdellovibrio species, or the molecular weight determination of the Azocollase of Huang and Starr is in error.

In the present study the serine protease was purified 98-fold and the metallo enzyme 90-fold (Table 8). This compares to a 62-fold purification by Huang and Starr. The greater degree of purification of enzymes in this study probably is due to the use of elution electrophoresis, which has also permitted a final total recovery of 12% (Table 8).

In other studies by Huang and Starr, Ca^{++} but not Mg^{++} was found to be required for stabilizing the protease during dialysis. When the Azocollase was dialyzed against Tris-HCl, and Ca^{++} plus Mg^{++} were added back, a 30% stimulation of proteolytic activity was observed (9). Similar experiments in the present study demonstrated that either Ca^{++} or Mg^{++} stimulated protease activity by 33% (Table 9), while Ca^{++} plus Mg^{++} stimulated activity by 50%.

The pH optimum and the Km of Azocollase was also determined

(9). The pH optimum was 8.5 and a Km value of 2.5×10^{-5} M on Azocoll and 5.1×10^{-5} M or N,N-dimethyl-casein was obtained. These data are more similar to the serine protease in the present study, which has a pH optimum of 8.0 and a Km of 3.8×10^{-5} M on Azocoll. If their molecular weight determinations are in error, it may be an indication that Huang and Starr have characterized the serine protease.

Finally, Huang and Starr have also detected endopeptidase and exopeptidase activity from H-D Bdellovibrio 6-5-S (9, 10). However, this group did not demonstrate the possibility that these peptidases degrade Azocoll. Thus, the question arose as to whether either or both peptidases corresponded to the metallo or serine protease in the present study. From Huang and Starr's data, however, five Azocollase negative mutants exhibited partial to full endopeptidase and/or exopeptidase activity. Therefore, these data make it clear that the serine and metallo proteases are distinct from Huang and Starr's exopeptidases and endopeptidases.

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