Partial Purification and Some Enzymatic Properties of a Proteinase from *Aeromonas proteolytica** (33884)

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It has been reported previously that culture filtrates of *Aeromonas proteolytica* contain one or more enzymes that catalyze the rapid degradation of protein substrates (1–4). This proteolysis cannot result from the *Aeromonas* aminopeptidase which we recently isolated (3), inasmuch as highly purified preparations of this enzyme are inactive toward protein substrates that are readily cleaved by unfractionated culture filtrate, which therefore must contain one or more proteinases (endopeptidases) in addition to the aminopeptidase. In this paper we report the partial purification and some characteristics of an enzyme that is highly active toward proteins and which hydrolyzes certain synthetic substrates.

Materials and Methods. Enzyme production. A subculture of the marine bacterium *Aeromonas proteolytica* (2), maintained in this laboratory since 1958, was used to produce the proteinase. Large cultures were grown in New Brunswick fermentors by the procedures of Prescott and Wilkes (3), except that growth was ordinarily extended to 18–24 hr to permit appearance of maximum proteolytic activity. The medium consisted of 2% enzymatically hydrolyzed casein in sea water diluted to two thirds of its usual salt concentration. Both natural and artificial sea water (Seven Seas Marine Mix, Utility Chemical Co.) were used. After the autoclaved medium had cooled, a sterile solution of K_2HPO_4 was added to yield a concentration of 50 mg of K_2HPO_4/liter of medium. At the end of the growth period the cells were removed by centrifugation in a Sharples super centrifuge and by successive passage through 0.65 and 0.45 μ Millipore filters. Cells were discarded and the culture filtrate thus prepared was the source of crude enzyme. Ordinarily, the culture filtrates were concentrated to about one fifth or less of their original volume in a rotary evaporator.

Enzyme assays. Proteolytic activity was measured by the procedure described earlier (2), which is a slight modification of the method of Anson (5). One proteinase unit was arbitrarily defined as the amount of enzyme that produced, in a 5-min reaction at 37°, sufficient acid-soluble peptides to give an absorbance of 1.0 at 280 mμ. During the course of this investigation, several synthetic substrates were found to be susceptible to hydrolysis by *Aeromonas* proteinase (see below). In the determination of some catalytic properties of the partially purified enzyme one of these synthetic substrates, benzyloxycarbonylglycyl-L-phenylalanine (CGP) was used; it was made to a concentration of 2 m*M* in 4 m*M* barbital buffer, pH 6.9, and the solution was rendered 0.8 *M* in NaCl. For the assay, 1 ml of enzyme solution was added to 1 ml of substrate, and the mixtures were incubated at 37° in optically matched culture tubes. The reaction was terminated by adding 1 ml of ninhydrin reagent (6) and heating in a boiling water bath for 15 min. Phenylalanine liberated was read at 570 mμ in a Coleman model 6 spectrophotometer and was quantitated from standards. One unit of activity toward CGP was defined as the amount of enzyme that would liberate 1 μmole of phenylalanine in 15 min at 37°. Protein concentrations were determined by the method of Lowry *et al.* (7), using bovine plasma albumin as the standard. Specific ac-

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tivities were expressed as units of enzyme/mg of protein.

Purification procedures. Saturated solutions of ammonium sulfate, neutralized with ammonium hydroxide, were used to precipitate the proteinase. Precipitates were collected in a Servall SS-1 centrifuge at 18,400*g*, then were dissolved in 10 m*M* phosphate buffer (pH 8.0) and dialyzed against this buffer to remove excess ammonium sulfate. For gel filtration experiments, Sephadex G-75 was suspended in 50 m*M* barbital buffer (pH 8.0), the fine particles were removed, and the slurry was poured into a chromatographic column whose size was chosen on the basis of the sample size. The sample was eluted by 50 m*M* barbital buffer, pH 8.0. Ion exchange chromatography was performed on DEAE-cellulose prepared by removing the fines and charging with 1 *N* NaOH, then rinsing successively with distilled water and starting buffer. The charged DEAE-cellulose was poured into a jacketed chromatographic column and packed under an air pressure of about 2 psi; this permitted flow rates of approximately 40–60 ml/hr. Coolant (5°) was circulated through the column jacket during chromatography in which the sample was eluted by increasing concentrations of NaCl from an automatic gradient producing apparatus ("Autograd," Technicon Corp.). Normally a concave gradient from 0 to 0.5 *M* NaCl in 5 m*M* phosphate buffer was used. The culture filtrates and various fractions obtained during purification were concentrated by perevaporation or by freeze–drying.

Electrophoresis and ultracentrifugation. Moving boundary electrophoresis was performed in a Beckman/Spinco model H electrophoresis-diffusion instrument on samples that had been dialyzed overnight in sodium barbital buffer of the selected pH value. Sedimentation was done in a Beckman/Spinco model E ultracentrifuge with an Analytical A rotor and a single sector cell. Samples were dialyzed in phosphate buffer overnight before the experiment.

Results and Discussion. A number of pilot experiments revealed that purification of the enzyme could be achieved by precipitation with ammonium sulfate and with acetone, by gel filtration, and especially by ion exchange chromatography. The exact sequence in which these steps were used was varied from one experiment to another in order to assess their relative effectiveness in combination. The procedures resulted in products which appeared homogenous by moving-boundary electrophoresis or which at worst showed only minor heterogeneity. Some of the preparations were examined by analytical ultracentrifugation and similarly revealed a single peak (Fig. 1). The results of an isolation experiment are shown in Table I, which indicates that a purification of only about fivefold was achieved. The electrophoretic and ultracentrifugal data, however, attest to the

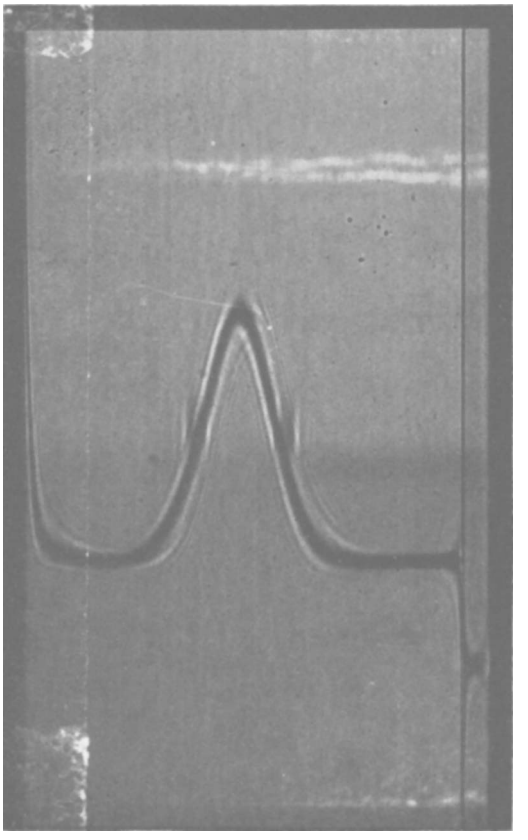

FIG. 1. Ultracentrifugal pattern of a sample of the partially purified *Aeromonas* proteinase in 20 m*M* phosphate buffer, pH 8.7: the exposure was made 144 min after the rotor attained a speed of 59,780 rpm; sample concentration was approximately 1.5%.

TABLE I. Purification of the Proteinase from *A. proteolytica*.

Isolation step	Volume (ml)	Protein (total mg)	Proteinase (total units)	Sp act[a]	Yield (%)
Concentrated culture filtrate	500	12,200	81,500	6.7	100
Ammonium sulfate					
Precipitation (0–50%)	470	4090	94,940	23.2	116
Reprecipitation[b] (35–50%)	180	1100	32,400	29.5	40
Gel filtration (Sephadex G-75)	66	581	19,760	34.0	24
Ion exchange chromatography (DEAE-cellulose)	231	388	14,320	36.9	17

[a] Units (as defined in text) per milligram of protein.

[b] The 0–50% precipitate was dissolved, dialyzed, and reprecipitated; the fraction falling between 35 and 50% contained the principal activity.

success of the procedure and it is likely that the relatively modest increase in specific activity indicates that the proteinase constitutes a large proportion of the total protein present in unfractionated culture filtrates.

The partially purified *Aeromonas* proteinase readily hydrolyzed casein and bovine insulin, in addition to the denatured hemoglobin substrate used in the routine assays. Aminopeptidase activity could be discerned by assays with sufficiently large enzyme concentrations or lengthy incubation periods, even in preparations revealing no heterogeneity by physical assessment. Such residual contaminations are not uncommon in preparations of other proteolytic enzymes; for example, purified samples of trypsin are known to contain chymotrypsin activity (8), and some preparations of leucine aminopeptidase have been shown to possess endopeptidases (9).

Enzymatic characteristics. Experiments were performed with unfractionated enzyme and with various partially purified fractions to find a susceptible synthetic substrate. The enzyme did not attack benzoyl-L-arginine ethyl ester, *p*-toluenesulfonyl-L-arginine methyl ester, acetyl-L-tyrosine ethyl ester or acetyl-L-phenylalanine ethyl ester, all of which are substrates commonly used to assay for trypsin or chymotrypsin, and certain microbial proteinases. No activity was manifest toward the ester bond of benzyloxycarbonylglycyl-L-phenylalanine ethyl ester (CGPEE), but partially purified preparations readily hydrolyzed the peptide bond of this substrate. Moreover, benzyloxycarbonylglycyl-L-phenylalanine amide (CGPN) was cleaved at the peptide bond, but no ammonia was liberated from the amide group. There was no requirement that the carboxyl group of the substrate be blocked, as CGP proved to be susceptible to the proteinase, and in fact was more convenient for routine use than CGPEE or CG PN because of its greater solubility. The enzyme showed maximum activity toward CGP near pH 7.0, and it was essentially insensitive to variations in ionic strength over the range 0–1.0 *M* NaCl. Several other synthetic substrates were tested, with the results shown in Table II. The effects of substrate concentration on the hydrolysis of CGP by the protein-

TABLE II. Action of the *Aeromonas* Proteinase on Some Synthetic Substrates.[a]

Substrate	Extent of hydrolysis[b] (μmoles $\times 10^2$)
Z[c]-glycyl-L-phenylalanine	26.8
Z-glutamyl-L-phenylalanine	17.7
Z-glycyl-L-leucine	7.1
Z-glycyl-L-methionine	1.0
Z-glycyl-L-tryptophan	0.5
chloroacetyl-L-tyrosine	0.5
Z-glutamyl-L-tyrosine	0.4
Z-glycyl-L-proline	0

[a] Substrates (1 mg/ml in 66 m*M* phosphate buffer, pH 8.0) were incubated with 1 ml of enzyme (88 μg/ml) that had been preincubated in 2.5 m*M* Co^{2+} ions for 2 hr.

[b] Amount of substrate hydrolyzed in 30 min at 37°.

[c] *Z* = *N*-benzyloxycarbonyl.

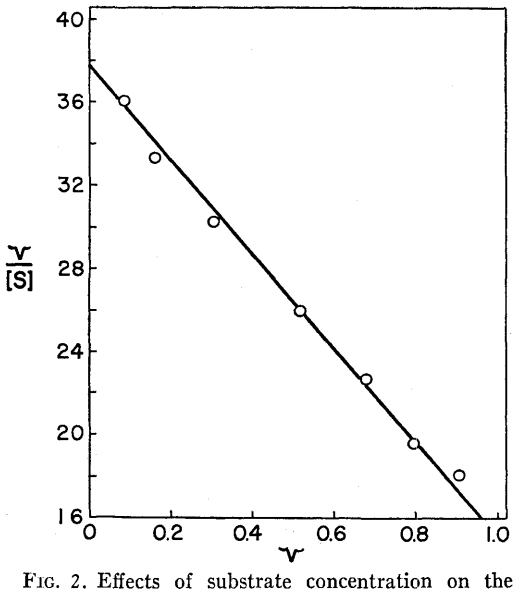

FIG. 2. Effects of substrate concentration on the hydrolysis of CGP by the *Aeromonas* proteinase: the enzyme had been purified through the gel filtration step (Table I) and was used at a concentration of 45 μg/ml of reaction mixture after preincubation for 3 hr in 2.5 m*M* Co^{2+} ions. The data are plotted by the method of Hofstee (10).

ase were determined and the results were plotted by the method of Hofstee (10), as shown in Fig. 2. The K_m calculated from this graph was 4.19×10^{-2} *M*.

In view of the fact that CGP is a widely used substrate for carboxypeptidases, it appeared relevant to investigate whether the hydrolysis of this synthetic substrate resulted from the action of the hemoglobin-hydrolyzing enzyme or from a separate carboxypeptidase. Evidence that a single enzyme is responsible is shown in Fig. 3, which reveals identical losses in the two activities when the enzyme was heated at 70°. It would seem unlikely that two enzymes should possess identical thermal stabilities even at this relatively high temperature, especially since we have shown that the aminopeptidase produced by *A. proteolytica* is stable for 60 min under these conditions (3). Other evidence that a single enzyme is responsible for both CGP and hemoglobin hydrolysis is provided by the virtually identical losses in activities upon dialysis at low pH (see below).

Effects of ions and inhibitors. When the proteinase was incubated in 5 m*M* Co^{2+} ions before the addition of substrate, activity toward both hemoglobin and CGP was substantially enhanced, as is shown in Table III.

TABLE III. Effects of Added Cobalt Ions on Activity of the Proteinase toward Two Substrates.

Co^{2+} ion concentration (m*M*)	Units[a] of activity toward	
	CGP	Hemoglobin
0	1.1	31
0.05	1.7	39
0.15	2.7	44
0.5	3.9	59
1.5	5.3	61
5.0	5.3	39

[a] Units are defined in the text. The enzyme was dialyzed overnight in deionized water; samples were incubated for 1 hr at 37° at the concentration of Co^{2+} ions shown, then were assayed at appropriate dilutions.

In some experiments with CGP and other synthetic substrates, it appeared desirable to achieve the maximal activity possible, and in these tests the enzyme was preincubated in Co^{2+} ions prior to the assays. Such experi-

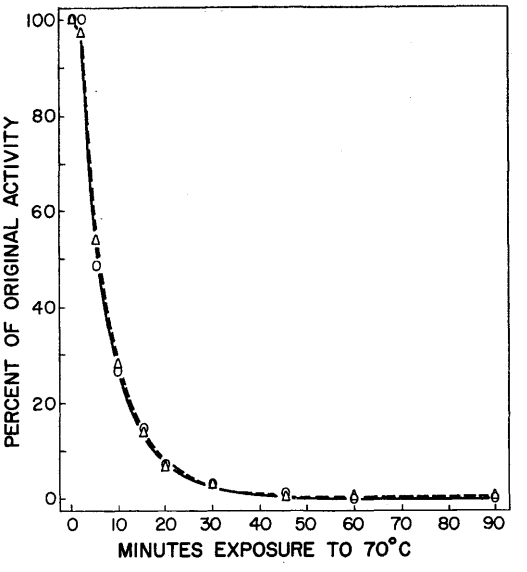

FIG. 3. Effects of heating the *Aeromonas* proteinase at 70° on the activities toward hemoglobin substrate (○) and CGP substrate (△); samples were withdrawn at the time values shown, chilled quickly, then assayed with both substrates.

ments are indicated in the footnotes of the appropriate figures or tables. The involvement of metal ions in the activity of the proteinase was also demonstrated by dialyzing enzyme samples for 16 hr at pH values of 3.6, 4.6, 5.7, 6.7, and 8.0. Dialysis at pH 4.6 reduced the activities toward both hemoglobin and CGP by about 25%, whereas at pH 3.6, these activities were diminished by approximately 75%. Full restoration of activity in the enzyme dialyzed at pH 4.6 was achieved by dialyzing for 16 hr in 2 m*M* $CoCl_2$, then removing the excess $CoCl_2$ by dialysis. Identical treatment failed to restore activity to the sample dialyzed at pH 3.6, however. These results suggest that a reversible loss of metal ions occurred at pH 4.6, and denaturation resulted from the prolonged exposure at pH 3.6. Activity of the purified *Aeromonas* proteinase was markedly reduced by chelating agents; 3.5 m*M* *o*-phenathroline diminished proteolytic activity toward hemoglobin by 75% and completely inhibited activity toward CGP. This difference in the extent of inhibition of activity toward the two substrates may be ascribable to the presence of adventitious ions in the hemoglobin used to prepare the substrate. The inhibition by *o*-phenanthroline was reversed by simple dilution, as it is in the case of carboxypeptidase A (11). Ethylenediaminetetraacetate (EDTA) also affected the proteinase, as dialysis against this reagent at a concentration of 2.5 m*M* completely inhibited activities toward both hemoglobin and CGP. After removal of excess EDTA, attempts were made to reactivate the proteinase by adding various divalent metal ions. One half to two thirds of the original proteolytic activity could be restored to the enzyme by certain ions, whose relative effectiveness was $Co^{2+} > Ca^{2+} = Zn^{2+} > Mg^{2+}$; Sn^{2+} and Mn^{2+} were less effective.

Several reducing agents were tested for their effects on the *Aeromonas* enzyme. At 20 m*M* concentration, Na_2S, cysteine, and reduced glutathione drastically diminished enzymatic activity toward both substrates; KCN and thioglycollate were without effect. The ability of the first three reducing reagents to inhibit enzymatic activity may be ascribable to their ability to complex with metal ions, as shown by Coombs *et al.* (12) to be the case with bovine carboxypeptidase A. Diisopropylphosphorofluoridate was without appreciable effect on the *Aeromonas* proteinase, thus suggesting that it is not a "serine enzyme."

The proteolytic enzyme from culture filtrates of *A. proteolytica* appears to be an endopeptidase capable of rapid hydrolysis of a number of proteins. The most susceptible synthetic substrates found thus far are those to which phenylalanine contributes the amino group of the peptide bond. Divalent metal ions are evidently involved in the activity of the proteinase, although the enzyme as isolated requires no deliberate addition of activating ions. It is likely that the *Aeromonas* proteinase is a metalloenzyme, although the tenacity of ion-binding by the apoenzyme is not presently known. The fact that Co^{2+} ions, when added to the isolated enzyme produced enhanced activity, and the fact that Co^{2+} ions are the most effective of several tried in restoring activity to the apoenzyme do not of themselves prove that Co^{2+} is the metal found in the native enzyme.

Summary. A proteinase possessing the characteristics of an endopeptidase was partially purified from culture filtrates of *Aeromonas proteolytica*. The enzyme readily hydrolyzed hemoglobin, casein, insulin, CGPN, CGPEE, and CGP; with the latter substrate K_m was 4.19×10^{-2} *M*. Chelating agents or dialysis at low pH inhibited the proteinase, which could be reactivated under favorable conditions by the addition of certain divalent metal ions.

1. Prescott, J. M. and Willms, C. R., Proc. Soc. Exptl. Biol. Med. **103**, 410 (1960).

2. Merkel, J. R., Traganza, E. D., Murkherjee, B. B., Griffin, T. B., and Prescott, J. M., J. Bacteriol. **87**, 1227 (1964).

3. Prescott, J. M. and Wilkes, S. H., Arch. Biochem. Biophys. **117**, 328 (1966).

4. Merkel, J. R., Anal. Biochem. **17**, 84 (1966).

5. Anson, M. L., J. Gen. Physiol. **22**, 79 (1938).

6. Moore, S. and Stein, W. H., J. Biol. Chem. **211**, 907 (1954).

7. Lowry, O. H., Rosebrough, N. J., Farr, A. L.,

and Randall, R. J., J. Biol. Chem. **193**, 265 (1951).
8. Kostra, V. and Carpenter, F. H., J. Biol. Chem. **239**, 1799 (1963).
9. Frater, R., Light, A., and Smith, E. L., J. Biol. Chem. **240**, 253 (1965).
10. Hofstee, B. H. J., Enzymologia **17**, 273 (1956).
11. Felber, J-P., Coombs, T. L., and Vallee, B. L., Biochemistry **1**, 231 (1962).
12. Coombs, T. L., Felber, J-P., and Vallee, B. L., Biochemistry **1**, 899 (1962).

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Accumulation of Endogenous Carbohydrate-Containing Compounds in the Cecum of the Germfree Rat* (33885)

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Considerable amounts of protein- and carbohydrate-containing material are found in the enlarged ceca of germfree rats and mice (1). Much of the protein is endogenous in origin accumulating in the cecum apparently due to a failure in its degradation and reabsorption in the terminal small intestine (2).

The cecal carbohydrates could be derived from either unabsorbed dietary carbohydrates or undergraded intestinal mucins. The present investigation attempted to determine the contribution of dietary carbohydrate to the cecal contents. For this purpose germfree rats were starved or given a diet containing glucose as the sole carbohydrate. Under conditions of starvation any carbohydrate containing material found in the cecum would be endogenous in origin and most likely mucinous in nature. On the high glucose diet, any unabsorbed dietary carbohydrate could be readily estimated by measuring the free glucose level in the cecum.

Materials and Methods. Young (30–35 day old) gnotobiotic CDF female rats were obtained from the Charles River Breeding Laboratories (North Wilmington, Mass.) and were housed in plastic germfree isolators. The animals were maintained on irradiated diet 585 (3) modified to contain 66% glucose as the sole dietary carbohydrate and 1% chromic oxide to serve as a dietary marker. After 2.5 weeks ingestion of this diet the animals were distributed so as to give comparable weight animals in both the fed and starved groups. All animals were fed for another 3 days and then the control animals were sacrificed; and food but not water was removed from the animals scheduled to be starved. Starved animals were sacrificed at 1 and 4 days and other starved animals died overnight between days 5 and 6.

All animals were killed by neck fracture and the cecum was removed, weighed and the cecal contents were expressed. The contents of each cecum were analyzed individually for total soluble protein and carbohydrate; high molecular weight proteins and glycoproteins (10% trichloroacetic acid precipitate); mucoproteins and mucopolysaccharides (80% ethanol precipitate) and low molecular weight carbohydrate and peptides (TCA and ethanol soluble material) by the fractionation procedure used previously (1). The dry weight of the insoluble residue was determined as well as its carbohydrate and chromic oxide content (1, 4). Galactose was used as the standard in the anthrone procedure because glucose was found to be a minor component of the cecal carbohydrates. Free glucose was measured enzymatically with glucose oxidase (Glucostat, Worthington Biochemical Corp., Freehold, N.J.) with suitable controls to correct for the contribution of the cecal pigment to color development.

In other experiments the soluble fractions

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