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Myxobacter AL-1 Protease II: Specific Peptide Bond Cleavage on the Amino Side of Lysine

MARILYN WINGARD, GARY MATSUEDA, AND R. S. WOLFE

Department of Microbiology, University of Illinois, Urbana, Illinois 61801 and the Department of Biochemistry and Biophysics, University of Hawaii, Honolulu, Hawaii 96822

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A second extracellular protease from myxobacter strain AL-1 has been purified to homogeneity and named protease II; the enzyme crystallizes as fine needles. The extracellular, cell wall lytic protease reported previously from the same organism is now designated protease I. Protease II exhibits a *p*H optimum of 8.5 to 9.0 and is stable from *p*H 3.0 to 9.0. The enzyme is heat stable at 50 C for 18 hr. Results of sedimentation equilibrium studies yielded a molecular weight of 17,000, and amino acid analysis revealed 157 residues with a minimal molecular weight of 16,660. Cleavage of peptide bonds in the oxidized B-chain of insulin, cytochrome *c* (horse heart), lysozyme, and vasopressin is restricted to the amino side of lysine. Dilysine and trilysine were not hydrolyzed. Products from digestions of polylysine were lysine and dilysine.

Myxobacter strain AL-1 produces two extracellular proteolytic enzymes. A protease which lyses bacterial cell walls was described initially by Ensign and Wolfe (4, 5) and was further characterized by Jackson and Wolfe under the name, myxobacter AL-1 protease (9). This enzyme was found to cleave the pentaglycine bridge in the cell wall of *Staphylococcus* and to remove peptide moieties from the peptidoglycan (11, 18). We now propose that this enzyme be designated myxobacter AL-1 protease I. In this paper we present the purification procedures, physical properties, amino acid content, and substrate specificity of a second extracellular protease. This enzyme does not possess cell wall lytic activity and has a unique specificity for lysine residues. We propose the trivial name, myxobacter AL-1 protease II, for this enzyme.

MATERIALS AND METHODS

Bacterial cultures. Methods for maintenance of *Arthrobacter crystallopoietes* and myxobacter strain AL-1 as well as procedures for obtaining large batches of growth liquor were the same as described previously by Jackson and Wolfe (9).

Proteolytic and cell lytic assays. The cell wall lytic assays with *A. crystallopoietes* and preparations of cell walls were performed by the methods described by Ensign and Wolfe (4, 5). Proteolytic assays were performed by two procedures. Procedure I: Into a 10-ml Erlenmeyer flask, 10 mg of Azocoll and 3.0 ml of 0.02 M bis [*N*,*N*-bis(-hydroxyethyl) glycine] or Tris [tris(hydroxymethyl)aminomethane], *p*H 9.0, were added. The reaction was initiated by the addition of the enzyme preparation in a 0.1-ml volume, and the flask was placed in a reciprocal shaker bath (108 cycles per min) at 40 C for 15 min. After incubation, the reaction mixture was filtered immediately through Whatman no. 1 filter paper, and the absorbancy of the solution was determined at 520 nm on a Beckman DU spectrophotometer. One unit of activity is defined as an increase of absorbancy of 0.001 at 520 nm in a reaction time of 15 min at 40 C.

Procedure II: This assay was modified from Kunitz (12); the test material was added to 4.0 ml of 1.0% bovine serum albumin (BSA) in 0.02 M Tris, *p*H 9.0, buffer, and the volume was adjusted to 5.0 ml with water. The reaction mixture was incubated at 38 C for 30 min. After incubation a 2.0-ml sample was removed and 3.0 ml of a 10% solution of trichloroacetic acid was added to this sample. This mixture was placed in a boiling water bath for exactly 15 min; it was then filtered through Whatman no. 2 filter paper, and the absorbancy at 280 nm was determined. One unit of activity is defined as that amount of enzyme which yields an increase in absorbancy of 0.001 at 280 nm in 30 min.

Protein assays. Determinations of protein concentrations were performed by the method of Lowry, et al. (13). BSA was used as a standard.

Sedimentation equilibrium. These studies were conducted in a Spinco model E ultracentrifuge with an AN-D rotor at a speed at 39,460 rev/min at 24 C. A six-channel Yphantis cell was used, and enzyme concentrations of 1.0, 0.75, and 0.5 mg per ml were employed (20). After 20 hr the equilibrium state was recorded photographically.

Dansylation. Dansylation of protein digests was performed as described by Gros and Labouesse (7). A

hydrolysis time of 4 hr was followed by chromatography and electrophoresis for 90 min each on Brinkman MN 300 thin-layer chromatography (TLC) plates. One buffer, 0.4% pyridine-0.8% acetic acid, pH 4.4, was used for both procedures as described by Arnott and Ward (1). A Desaga-Brinkman electrophoresis chamber was used at a setting of 400 v.

Aminex A-5 column chromatography. An Aminex A-5 column, 0.9 by 20 cm (designed and prepared by Peter Borgia), was used for separation of peptides from protein digested with protease II (10). An automatic sample injector applied the 1.0-ml sample to the column. A 500-ml gradient from 0.2 M pyridine acetate buffer at pH 3.1 to 1.0 M pyridine acetate at pH 5.0 was used to elute the peptides. For color development an amino acid analyzer coil at 100 C was used for the ninhydrin reaction. The effluent was monitored, and absorbancy was recorded at 570 nm on a Gilford model 300 spectrophotometer. For separation of products from homolysine peptides and polylysine digestions, only the limiting buffer of 1.0 M pyridine acetate, pH 5.0, on a column equilibrated with the same buffer was used.

Electrophoresis and chromatography. All electrophoretic experiments were performed in a Desaga-Brinkmann TLC chamber, at 400 v. Buffers used were: formic acid-acetic acid-water (50:156:1,744), pH 2.1; pyridine-acetic acid-water (100:35:1,796), pH 6.6; and pyridine (0.4%)-acetic acid (0.8%) at pH 4.4. Buffers for chromatography were: pyridine-*n*-butanol-acetic acid-water (40:60:12:48), and pyridine (0.4%)-acetic acid (0.8%) at pH 4.4.

Sedimentation velocity. Experiments were conducted in a Spinco model E centrifuge at a speed of 59,780 rev/min at 20 C. A single-sector capillary type synthetic boundary cell was employed in a standard AN-D rotor. Concentrations of protease II at 8 mg/ml and at 4 mg/ml were used in 0.02 M acetate buffer, pH 4.0. Exposures were taken at 8-min intervals.

Materials. Vasopressin, oxidized B-chain of insulin, polylysine, lysozyme, and soybean trypsin inhibitor were obtained from Mann Research; dansyl amino acids, cytochrome *c*, BSA, and phenylmethane sulfonyl fluoride were from Sigma; all other peptides unless specified from Cyclo; Azocoll from Calbiochem; CM Sephadex C-50 and G-75 from Pharmacia; cellulose TLC plates from Eastman and cellulose MN 300 TLC plates from Brinkmann; *N*-ethylmaleimide from Aldrich; *p*-chloromercuribenzoate from Nutritional Biochemicals.

RESULTS

Purification procedure. In the final purification of certain batches of myxobacter AL-1 culture fluid an unusual event occurred; during the elution of protease I from a CM-cellulose column with glycine buffer (9) protein crystals appeared in a series of tubes in the fraction collector. The crystals had the appearance of fine needles under the microscope. In Fig. 1B their structure is compared to that of protease I (Fig. 1A). When dissolved in water, the crystals exhibited no cell wall lytic activity in contrast to protease I (4, 5, 9), and we were in the unusual position of having first obtained a crystalline protein for which we had no substrate. In screening a series of varied substrates we found that the crystals possessed enzymatic activity toward albumin, azoalbumin, and Azocoll. We have named this enzyme protease II. The following purification procedure was modified from that for protease I (9) so that the purification of both protease I and II could be followed. Results of certain steps in the purification procedure are presented in Table 1. In crude fractions the proteolytic activity detected represents the activities of both proteases. All procedures were performed at 4 C. (Step A) Culture fluid (112 liters in 8 carboys) was precipitated with $ZnCl_2$ as previously described (9). (B) The resulting precipitate was suspended with the aid of a Waring blendor in 4 liters of 0.05 M Tris buffer, pH 9.0, which contained 1 mM ethylenediaminetetraacetic acid (EDTA). The suspension was stirred for 20 min and then centrifuged in a Sharples continuous-flow centrifuge, the effluent being discarded. (C) By repeated use of a Waring Blendor the precipitate was extracted in 4 liters of 0.15 M citrate buffer, pH 5.0. After stirring for 5 min the precipitate was allowed to settle for 5 min. (D) The supernatant solution was then decanted and treated with ammonium sulfate at 70% saturation. (E) The precipitate was removed with a Sharples centrifuge, dissolved in 0.025 M Tris buffer, pH 7.0, and dialyzed against the same buffer for 4 hr prior to assay. (F) Following dialysis the material was stirred with 1 liter of CM-Sephadex C-50 (hydrated but with excess water removed) for 1 hr. (G) The resin was washed on a scintered-glass funnel with 0.025 M Tris, pH 7.0, until it was clear of brown coloration. (H) The enzymes were then eluted from the resin with 0.05 M glycine buffer, pH 9.5, which contained 0.5 M NaCl. (I) To concentrate the material eluted with glycine buffer a precipitation with ammonium sulfate at 90% saturation was performed. (J) After centrifugation the pellet was dissolved in 100 ml of 0.025 M Tris buffer, pH 7.0, and dialyzed against the same buffer for 1 hr. (K) The material was concentrated to approximately 60 ml by dialysis against Carbowax 400. (L) The solution was divided into two portions, and each portion was chromatographed on a separate Sephadex G-75 column (85 by 5 cm) which had been previously equilibrated with 0.025 M Tris at pH 7.0. This modification of the purification procedure (9) allowed separation of protease I and II as soluble fractions. A flow rate of 60 ml per

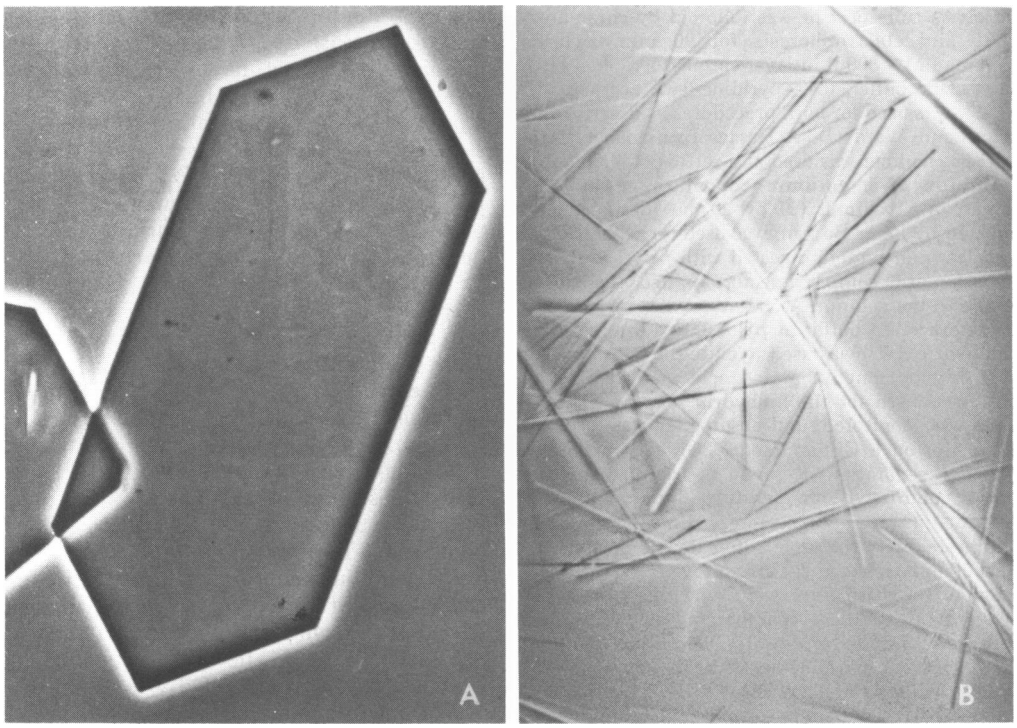

FIG. 1. *Photographs of crystals of protease I and II. Pictures were taken with a phase-contrast microscope; magnification, ×1,360. A, Protease I, the cell wall lytic enzyme; B, protease II. Conditions for crystallization are described in text.*

TABLE 1. *Summary of purification of protease II*

Purification step (see text)	Total activity (10^7 units)	Total protein (mg)	Proteolytic specific activity (units/mg protein)	Percent recovery
Culture fluid	49.8	29,260	19,000	100.0
	24 2	3,562	67,900	48.6
E. $(NH_4)_2SO_4$ precipitate Citrate extraction				
H. Eluate from CM Sephadex C-50	20.4	770	287,100	41.0
K. Applied to Sephadex G-75	18.8	552	343,700	37.8
M. Sephadex G-75				
Protease II	2.0	96.3	207,600	4.0
Protease I	9.6	325.7	300,600	19.3
O. Crystallization				
Protease II	0.47	29.7	158,200	0.94
Protease I	5.7	245.0	232,600	11.4

hr was maintained, and 6-ml fractions were collected. Fractions were monitored by measuring absorbancy at 280 nm. Representative fractions were assayed for cell wall lytic activity and for proteolytic activity with Azocoll; the results are shown in Fig. 2. (M) Fractions from peak *c* which contained proteolytic, but no cell wall lytic activity, were pooled and lyophilyzed to dryness. (N) The dry material was dissolved in 30 ml of water and dialyzed against 0.025 M glycine buffer, *p*H 9.0. After approximately 4 hr crystals began to form. The material was then removed from the dialysis bag and allowed to crystallize for 24 hr. (O) Crystals were removed by centrifugation and washed three times with distilled water which had been adjusted to *p*H 9.0 with NH_4OH. Contaminating protein from peaks *b* and *d* did not crystallize and was removed in the washings. Protease II was dissolved in 0.02 M sodium acetate buffer at *p*H 4.5 and was recrystallized as described above. Preparations which had been recrystallized several times were used in the experiments reported below. The enzyme exhibited the properties of a homogeneous protein, as shown in Fig. 3 during a sedimentation velocity experiment. Similarly the fringe patterns which were photographed after 20 hr in a sedimentation equilibrium experiment yielded perfectly linear plots through all points when the natural loga-

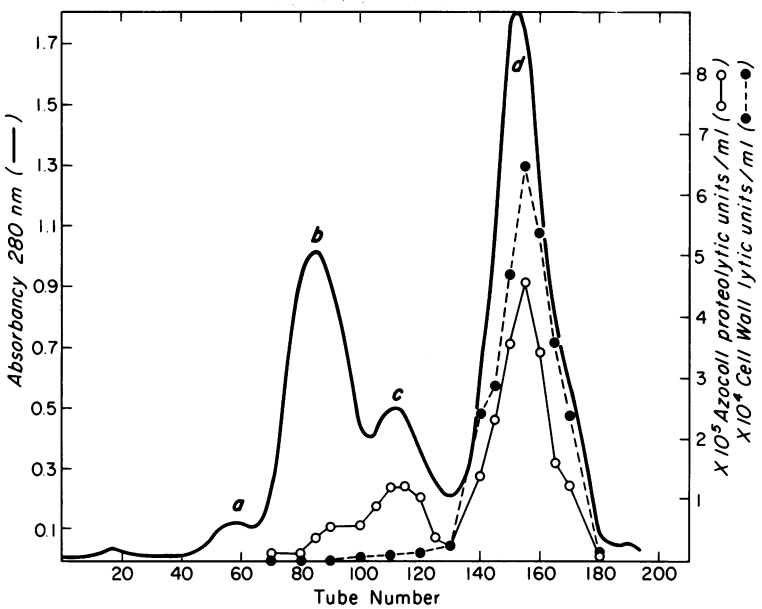

FIG. 2. *Purification of myxobacter AL-1 enzymes on Sephadex G-75 column as described in text. Symbols:* o——o, *proteolytic units/ml;* ●---●, *cell wall lytic units/ml;* ——, *absorbancy at 280 nm.*

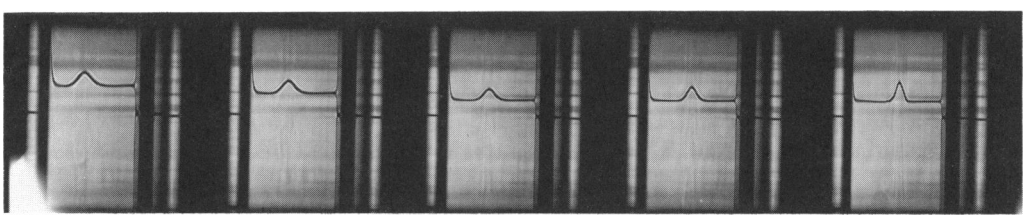

FIG. 3. *Sedimentation velocity experiment with protease II. Conditions were as described in the text. Photographs were taken at 8-min intervals.*

rithm of the initial protein concentration was plotted against the distance from the center of rotation (r^2cm) on the abscissa (20); no evidence for non-homogeneity was obtained. We were unable to devise a disc-gel electrophetic system in which the enzyme remained soluble during migration; the protein precipitated in the gel.

pH optimum and stability. Assays were performed with Azocoll to determine the *p*H optimum of protease II. Buffers were used at a concentration of 0.02 M, and 5 μg of enzyme was used in each assay flask. Figure 4 presents the results of the experiment; the enzyme has a broad *p*H range, peaking in activity at *p*H 8.5 to 9.0. Figure 5 presents the results of an experiment on the stability of protease II at various *p*H values. The enzyme at a concentration of 10 μg per ml was incubated at 37 C in 0.02 M buffers ranging from *p*H 3.0 to 11.0 as used in Fig. 4. At 1 hr and at 18 hr, a 0.1-ml

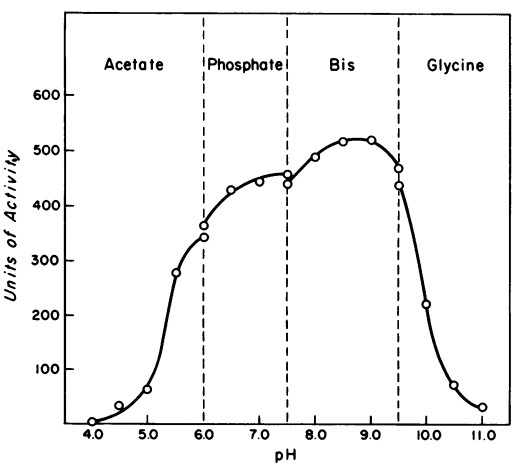

FIG. 4. *Effect of pH on protease II activity. Assays were conducted with 5.0 μg of enzyme in 3.0 ml of each 0.02 M buffer with the Azocoll procedure.*

sample was removed and assayed with the Azocoll procedure. The enzyme was stable for 1 hr at *p*H values from 3 to 11; loss of activity was negligible after incubation for 18 hr at *p*H 4 to 7; thus, the enzyme is resistant to self-digestion.

Temperature optimum and stability. The Azocoll assay was performed at various temperatures as shown in Fig. 6. Although the maximal activity was at 60 C, the temperature of 40 C was selected for standard assays, since high blanks were obtained with Azocoll controls at higher temperatures. In an experiment to test the stability of protease II at various temperatures, 20 μg of enzyme per ml was incubated at 50, 60, and 70 C in 0.02 M Tris at *p*H 7.0. At the time intervals indicated in Fig. 7, 0.1 ml of the enzyme solution was removed and assayed with the Azocoll procedure. The enzyme was stable at 50 C for over 4 hr; at 60 C, 50% of the activity was lost in 4 hr; at 70 C all activity had been destroyed in 2 hr.

Standard assays. When increasing concentrations of protease II were added to standard amounts of Azocoll and of BSA, the rate of reaction was in proportion to concentration over a fourfold concentration of enzyme in both assay systems (Fig. 8). Results of experiments with increasing amounts of substrates are represented in Fig. 9. Enzyme amounts of 1.0 μg in the Azocoll experiment and 4.0 μg in the BSA experiment were used. The nonlinearity of the Azocoll digestion with increasing amounts of Azocoll is probably due to the insolubility of the substrate. An assay with 0.5 μg of enzyme and 10 mg of Azocoll produced a linear reaction rate over a 40-min period.

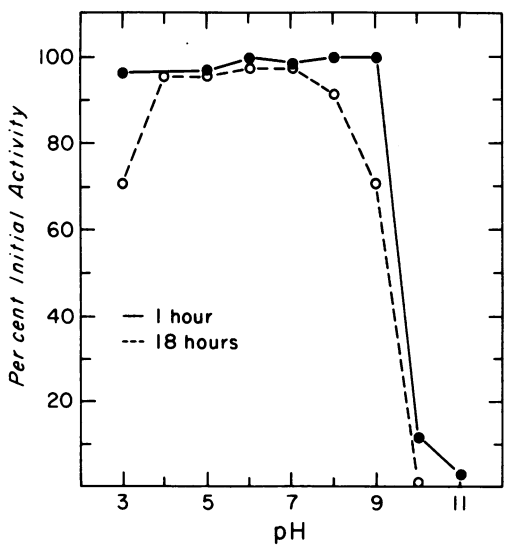

FIG. 5. *Stability of protease II at various pH values. The enzyme was incubated at 37 C in 0.02 M buffers at the indicated pH values. Samples were removed at 1 hr and 18 hr and assayed with the Azocoll procedure.*

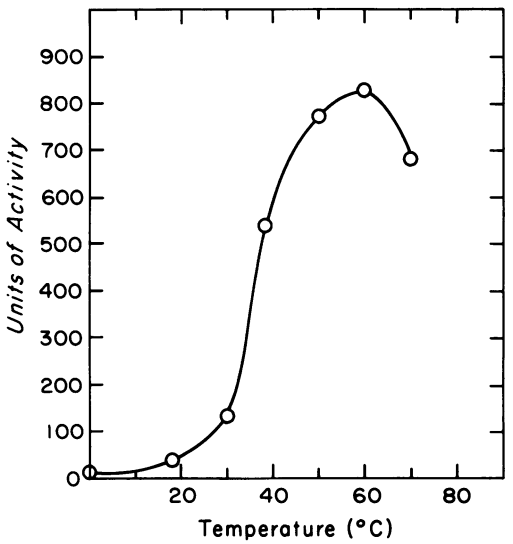

FIG. 6. *Effect of temperature on enzyme activity. Assays were conducted with 10 μg of protease II in 3.0 ml of 0.02 M Tris buffer at pH 9.0 with the Azocoll procedure.*

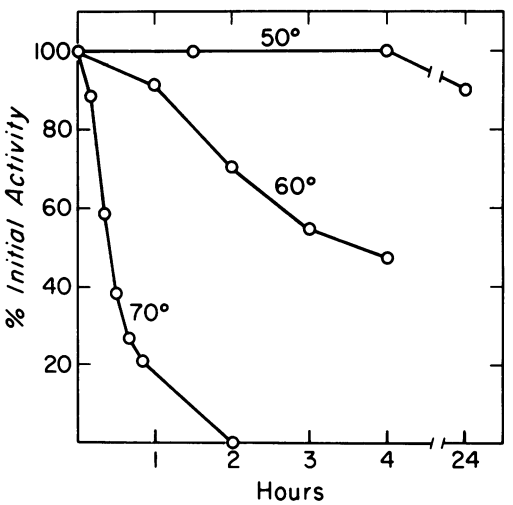

FIG. 7. *Temperature stability of protease II. The enzyme was incubated in 0.02 M Tris buffer at pH 7.0. At times indicated, samples were removed and assayed with the Azocoll procedure.*

Effect of metals, chelating agents, and inhibitors. The effect of various compounds on the activity of protease II was studied. Each compound was added to the Azocoll reaction

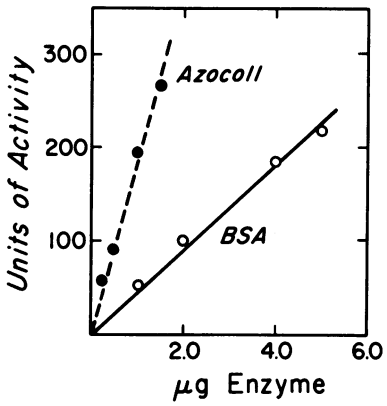

FIG. 8. *Effect of increasing amounts of protease II. Symbols: O---O, 10 mg of Azocoll as substrate in 0.02 M Tris, pH 9.0, at 40 C for 15 min; O——O, 1.0% BSA as substrate in 0.02 M Tris, pH 9.0, at 38 C for 30 min.*

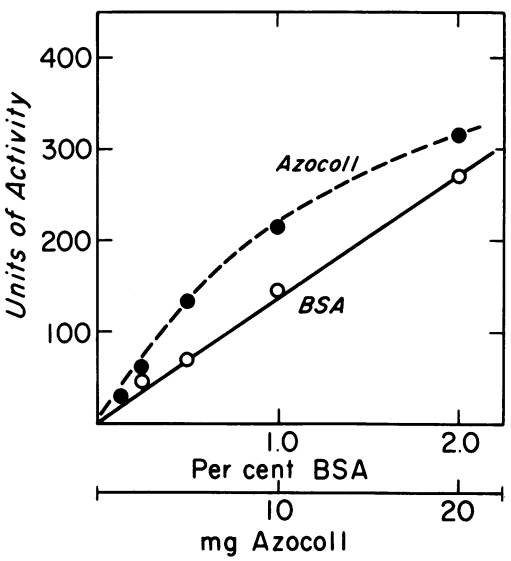

FIG. 9. *Effect of increasing amounts of substrates. Symbols: O---O, Azocoll as substrate, 1.0 μg of protease II per assay conducted in 0.02 M Tris, pH 9.0, at 40 C for 15 min; O——O, BSA as substrate, 4.0 μg of protease II per assay conducted in 0.02 M Tris, pH 9.0, at 38 C for 30 min.*

mixture prior to addition of 2.0 μg of protease II. $MgCl_2$, $MnCl_2$, and $CuSO_4$ had no inhibitory effect at a final concentration of 1×10^{-3} M. $HgCl_2$ exhibited 36% inhibition at 1×10^{-4} M but none at 1×10^{-5} M. Sodium citrate, by the same procedure, inhibited activity 38% at 1×10^{-1} M but showed no effect at 1×10^{-2} M. EDTA inhibited activity completely at 5×10^{-2} M, 39% at 1×10^{-2} M, and 20% at 1×10^{-3} M; no inhibition by phenylmethane sulfonyl fluoride at 1×10^{-4} M and 1×10^{-5} M was demonstrated with the procedure described by Gold and Fahrney (6). Preincubation of 20 μg of protease II in 1×10^{-2} M *N*-ethylmaleimide or in *p*-chloromercuribenzoic acid for 30 min preceding the assays produced no loss of activity. The enzyme proved insensitive to 100 μg of soybean trypsin inhibitor per ml when preincubated for 30 min prior to assay.

Amino acid analysis. Six tubes each of which contained 0.5 mg of protease II in 6 M HCl were sealed under vacuum and placed at 110 C; duplicate tubes were removed at 24, 48, and 72 hr. Each hydrolysate was dried down twice under vacuum and dissolved in 1.0 ml of 0.02 M citrate buffer, pH 2.2. The amino acid content of each sample was determined on a Beckman Spinco model 120 amino acid analyzer. The results of the analysis are presented in Table 2. Cysteine was determined as cysteic acid with duplicate 0.5-mg samples of the enzyme being hydrolyzed in 6 M HCl for 20 hr prior to analysis (8).

Results of the analysis showed that there were 2.97 moles of cysteic acid per mole of protein. When tryptophan was determined with the reagent, *N*-bromosuccinimide, as described by Patchornik et al. (15), a content of 2.07 moles per mole of protein was deter-

TABLE 2. *Amino acid composition of protease II*

Amino acid	Protein (g/100 g)	No. of residues[a]	Assumed no. of residues	Amino acid residues (g)
Lysine	1.74	2.30	2	256.4
Histidine	3.29	4.07	4	548.8
Arginine	4.24	4.61	5	781.0
Aspartic acid	15.65	23.12	23	2,647.3
Threonine	9.71	16.12	16	1,617.6
Serine	3.83	7.47	7	609.7
Glutamic acid	9.84	13.05	13	1,666.6
Proline	3.88	6.79	7	679.7
Glycine	4.98	14.83	15	856.5
Alanine	9.16	21.90	22	1,564.2
½ Cystine	1.80	2.97	3	309.6
Valine	6.98	11.98	12	1,189.2
Methionine				
Isoleucine	4.08	6.12	6	679.2
Leucine	3.44	5.16	5	566.0
Tyrosine	8.36	8.70	9	1,468.8
Phenylalanine	4.83	5.57	6	883.2
Tryptophan	2.04	2.07	2	336.0
Total	97.85		157	16,600.0

[a] Calculated on the basis of a molecular weight of 17,000.

mined. When guanidine was used to denature the protein as described by Edelhoch (3), 2.27 moles of tryptophan per mole of protein was detected. An amide content of 15.3 moles per mole of protein was determined by the method of Wilcox (19).

Molecular weight. Two methods were used to estimate the molecular weight of protease II. (i) The minimal molecular weight calculated from amino acid analysis was 16,660 with the assumption that there were eight asparagine and seven glutamine residues per mole of protein. A partial specific volume of 0.711 ml/g was calculated from the standard equation and the values of $\bar{v}$ for each amino acid residue. An $s_{20,w}$ of 2.07S was calculated from results of sedimentation velocity experiments. (ii) The results of sedimentation equilibrium experiments yielded a molecular weight of 17,000. The enzyme exhibited the properties of a homogeneous protein in all ultracentrifugal studies.

Cytochrome c digestion. To determine which peptide bonds were cleaved by protease II a number of proteins and peptides were tested. After digestion of the substrate with protease II the N-terminal amino acids were determined qualitatively by the sensitive dansylation technique (7). A 13-mg sample of horse heart cytochrome *c* was dissolved in 3.0 ml of water. The *p*H was adjusted to 9.0, and 0.1 mg of protease II was added. The *p*H of 9.0 was maintained by the automatic addition of 0.01 M NaOH by a *p*H stat. After 3 hr an additional 0.1 mg of enzyme was added and no change in slope was noted. After 12 hr the resulting material was lyophilized. After lyophilization a fingerprint was made by the following procedure: electrophoresis for 20 min in *p*H 6.6 pyridine acetate buffer on Eastman cellulose TLC plates, followed by chromatography with *n*-butanol-pyridine-acetic acid buffer for 8 hr. Seventeen ninhydrin-positive spots were detected. A sample of the digestion mixture also was chromatographed on an Aminex A-5 column, and sixteen peaks were detected. To determine qualitatively the N-terminal amino acids of the peptides, the digestion mixture was dansylated, and DNS (dansylated) amino acids were separated as described in Materials and Methods. Appropriate dansyl standards were used on control plates. Fluorescent spots were detected corresponding only to di-DNS-lysine and a faint spot of ϵ-DNS-lysine, *O*-DNS-tyrosine always being a product of dansylation when tyrosine is located within a peptide. Figure 10 presents patterns of the TLC plates. We concluded from this experiment that protease II cleaved the peptide bonds on the amino side of lysine. Cytochrome *c* contains 19 lysine reisudes (14), and from the fingerprints it appears that cleavage occurred at the majority of these residues.

Lysozyme digestion. Lysozyme was oxidized with performic acid by the procedure of Sanger (17). A portion which contained 1 μmole was digested with 0.1 mg of protease II. After 4 hr of digestion, a 0.2-μmole amount of the digest was chromatographed on the Aminex A-5 column. The peaks came off the column soon after the void volume, indicating their large size. These results were expected, as lysozyme contains only six lysine residues (2). Dansylation of the digest followed by chromatography and electrophoresis showed only fluorescent spots corresponding to O-DNS-tyrosine and di-DNS-lysine.

B-chain of insulin digestion. A 20.0-mg sample of oxidized B-chain of insulin was dissolved in 3.0 ml of water. The *p*H was adjusted to 9.0, and 0.1 mg of protease II was added. The *p*H of 9.0 was maintained by the automatic addition of 0.01 M NaOH by a *p*H stat. After 3 hr an additional 0.1 mg of enzyme was added and no change in the slope was noted. Hydrolysis was allowed to continue for 12 hr and the resulting material was lyophilized to dryness. The digest was dissolved in 0.2 M pyridine acetate buffer at *p*H 2.2, centrifuged, and washed once with the same buffer. Supernatant solutions were pooled and chromatographed on an Aminex A-5 column. Only one peptide was separated. Fractions from the peak were lyophilized and hydrolyzed in 6 N HCl for 24 hr. The hydrolysate was chromatographed on the amino acid analyzer. The peptide was found to contain lysine and alanine.

Thin-layer electrophoresis at *p*H 2.2, 6.6, and 8.0 was attempted with the portion of the digest that was insoluble at *p*H 2.2. No ninhydrin-positive peptides were detectable; one spot was detected close to the origin which corresponded to the undigested B-chain of insulin. A portion of the material was hydrolyzed in 6 N HCl for 24 hr and chromatographed on the amino acid analyzer. Results of this analysis showed that only one lysine and one alanine residue were missing from the complete B-chain of insulin. We concluded from these results that protease II cleaved only the proline-lysine bond in the B-chain of insulin.

Vasopressin digestion. To 2.0 mg of vasopressin (Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly) (16), 10 μg of protease II was added. A *p*H of 9.0 was maintained by the manual addition

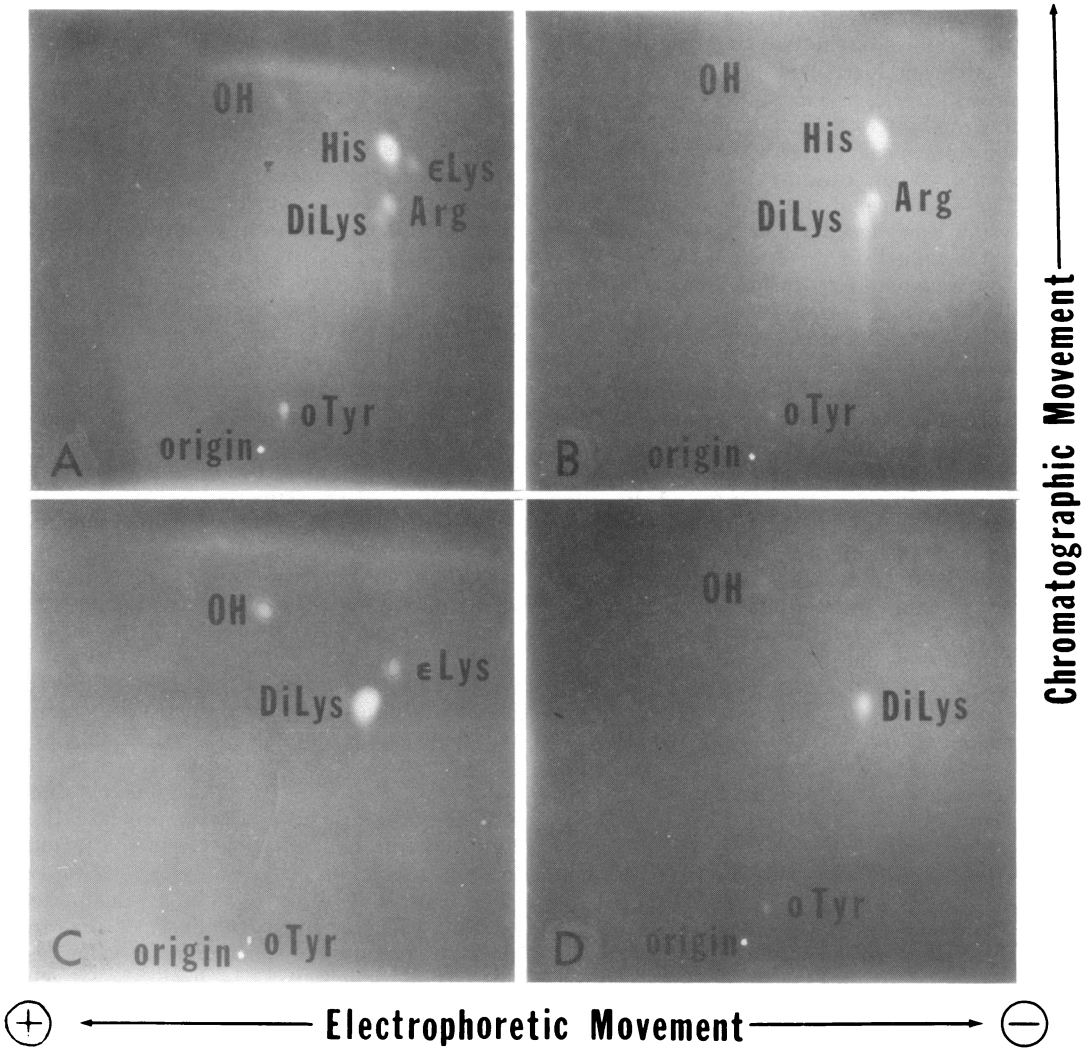

FIG. 10. *Patterns of dansyl (DNS) derivatives after thin-layer chromatography and electrophoresis. A, Dansyl amino acid standards with similar R_F values to dansylated derivatives from cytochrome c digestion. B, Dansylated cytochrome c digestion plus added standards of DNS-Arg and DNS-His. C, Dansylated cytochrome c digestion plus standards of di-DNS-Lys and ε-DNS-Lys. D, Only dansylated cytochrome c digestion. A Wratten N (yellow) filter was used on an MP-3 Polaroid camera with type 52 film to record the UV fluorescent spots in a Chromato-vue cabinet.*

of 0.01 M NaOH. After 2 hr at 37 C, 10 μliters of the digestion mixture was applied to a cellulose TLC plate, and electrophoresis was performed in *p*H 2.2 formic-acetic buffer. After 40 min the plate was dried and stained with ninhydrin. Two ninhydrin-positive spots were detected. The digestion mixture was adjusted to *p*H 2.2 and applied to the Aminex A-5 column. One peak was isolated; the other peptide was not eluted from the resin and was not investigated further. Fractions from this peak were lyophilized and hydrolyzed in 6 N HCl for 20 hr. Amino acid analysis of the hydrolysate showed only lysine and glycine in equimolar amounts. We concluded that the enzyme had cleaved the proline-lysine bond in vasopressin.

Homolysine peptides. Five micromoles of trilysine was treated with 5 μg of protease II at 37 C for 3 hr. Treated material was spotted on cellulose TLC plates, and electrophoresis was performed for 60 min in *p*H 2.2 formic-acetic buffer. No ninhydrin-positive spot other than

trilysine was observed. After a 12-hr reaction time, 0.2 μmoles of the trilysine reaction mixture was chromatographed on the Aminex A-5 column which was previously equilibrated with the limiting buffer of 2.0 M pyridine-acetic, pH 5.0. Only one peak corresponding to trilysine was found. When dilysine was treated similarly, no evidence of digestion was obtained. Polylysine was digested and 0.005 μmole was chromatographed on the Aminex A-5 column. Dilysine was found to be the major product with lysine as a minor product.

Peptides not digested by protease II. To a solution which contained 10 mg of each peptide in 1 ml of 0.02 M Tris buffer at pH 9.0, 50 μg of protease II was added. Each sample was incubated at 38 C for 3 hr. Controls which contained the peptide alone and the enzyme alone were treated in the same manner. A 10-μliter sample of each reaction mixture was subjected to electrophoresis at pH 2.2 in formic-acetic buffer for 90 min. None of the following treated peptides showed any ninhydrin-positive spots which did not correspond to those on their corresponding controls: Gly-DL-Leu; Gly-DL-Val; Gly-DL-Ser; Gly-DL-Phe; Gly-DL-ethionine; Gly-DL-Nor; Gly-L-Try; L-Leu-L-Tyr; D-Leu-Gly; L-Leu-L-Leu; DL-Leu-DL-Ala; DL-Leu-DL-Ile; DL-Leu-Gly; D-Leu-L-Tyr; D-Leu-Gly-Gly; *N*-CBZ-L-Leu-Gly-Gly; Gly-Gly-Gly; *N*-CBZ-Gly-L-Phe; *N*-CBZ-L-Ala-L-Leu amide; *N*-CBZ-Gly-Gly-*p*-nitrophenylester; *N*-CBZ-L-Pro-Gly-Gly-Met; *N*-CBZ-L-Ala-Gly methylester; Pro-Phe-Pro-Gly; polyproline; polyglycine; poly-D-lysine; Ala-Pro-Lys; Ala-Pro-Ala-Asp-Gly-Leu-Lys.

DISCUSSION

It is of interest that the two extracellular proteases of myxobacter strain AL-1 which have been studied are relatively small proteases. Both proteases have pH optima at alkaline pH and are very stable enzymes; protease II is much more heat stable, exhibiting optimal activity at 60 C. The enzymes differ markedly in specificity, with protease II showing extreme specificity. In routine fractionation the crystalline yield of protease II is about 10% that of protease I from the same batch of culture fluid.

Although extensive studies on the active site of protease II have not been performed, it probably does not involve a serine residue, since 10^{-4} M phenylmethane sulfonyl fluoride does not cause inhibition, and the failure of 10^{-2} M *N*-ethylmaleimide or *p*-chloromercuribenzoate to inhibit the enzyme indicates that SH groups are not involved in catalysis.

In addition to the work reported here, protease II is being used in two other laboratories; these studies confirm the specificity of the enzyme, and we mention the findings briefly. In the laboratory of Henry S. Kingdon, protease II was tested on glutamine synthetase (*personal communication*); 100 nmoles of glutamine synthetase (which had been reduced and carboxymethylated) was digested with protease II at a ratio of 1 mg of protease to 100 mg of glutamine synthetase. Digestion in a titrimeter was followed by uptake of base and was terminated when base uptake ceased. Amino terminal residues were determined. From an expected 2,600 nmoles of amino terminal lysine, 3,100 nmoles of lysine was observed on the first cycle of Edman degradation in an automated peptide sequencer (Spinco). Other amino acids were at a level 100-fold less. On the second cycle of the automated degradation a large number of other amino acids were seen, and no lysine was detected, indicating cleavage only on the amino side of lysine residues. Protease II has been used in the laboratory of J. M. Clark, Jr. (R. E. Lundquist and J. M. Clark, Jr., Fed. Proc., p. 1138, 1971). The in vitro synthesized peptide had the N-terminal sequence F-Met-Ala-Lys. When this peptide was digested with protease II, a dipeptide with the sequence F-Met-Ala was obtained. An assay was developed with protease II to follow synthesis of the peptide in the in vitro system.

Protease II could prove to be a valuable tool in amino acid sequence analysis of proteins, since it has a unique specificity for lysine residues. This specificity has been shown with proteins as well as with peptides; however, the enzyme does not cleave terminal lysine residues, trylysine or dilysine. In the digestion of proteins or large peptides, 1 mg of enzyme readily digests 100 to 200 mg of protein, and there is no self-digestion of the enzyme.

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