BBA 66601

ISOLATION AND CHARACTERIZATION OF A PROTEASE FROM *CLOSTRIDIUM BOTULINUM* TYPE B

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(Received January 10th, 1972)

SUMMARY

A protease produced by *Clostridium botulinum* type B (strain Lamanna) was isolated and characterized. Purification steps were precipitation with $(NH_4)_2SO_4$, chromatography on QAE-Sephadex at pH 5.8, gel filtration through Sephadex G-100, and chromatography on SE-Sephadex at pH 5.0. Molecular weight of the enzyme by sedimentation equilibrium is 34 400 and by gel filtration is 40 000; isoelectric pH is 4.62. Optimum pH for amidase activity is 6.2 and for esterase activity is 6.2–7.0. The enzyme acts only on bonds formed by the carboxyl group of arginine and lysine residues. It is not inhibited by trypsin inhibitor from soy bean, lima bean, or ovomucoid. The enzyme is active only when in the reduced state and is more stable when Ca^{2+} is present.

INTRODUCTION

The specific toxicity of *Clostridium botulinum* toxin, a simple protein, increases during incubation of the culture. Such activation of the toxin is particularly evident with proteolytic strains of the bacteria[1,2]. Since change of progenitor toxin* to the more toxic form can be accomplished with trypsin, natural activation occurring in pure cultures should be due to endogenous proteolytic enzyme(s)[3]. The activation probably involves structural alterations similar to the limited proteolysis that activates zymogens[4].

An enzyme of a proteolytic *C. botulinum* type B (strain Lamanna) culture was found to activate progenitor toxin of the same culture[5]. Isolation and characterization of the enzyme were undertaken as a preliminary to a study of the mechanism that is involved in the natural activation of botulinal progenitor toxin.

Abbreviations: BAPNA, *N*-benzoyl-DL-arginine-*p*-nitroanilide; TAME, *p*-toluenesulphonyl-L-arginine methyl ester; SE-Sephadex, sulfoethyl Sephadex; QAE-Sephadex, quaternized aminoethyl Sephadex.

* Progenitor toxin refers to toxin molecule whose specific toxicity can be increased by trypsin and other appropriate proteases.

MATERIALS AND METHODS

The enzyme source was *C. botulinum* type B (strain Lamanna) received from Dr E. J. Schantz. A stock culture, stored at —20 °C, was thawed at room temperature and 2 ml inoculated into 500 ml of the medium (in 500 ml graduated cylinders) used previously[5]. Incubation was at 37 °C for 4–5 days.

Purification procedures were carried out at room temperature (27 ± 2 °C). Sephadex ion exchangers and gels were of particle size 40–120 μm. They were soaked for at least 4 days with appropriate buffer and were washed several times to remove fine particles. Columns were eluted by gravity flow.

Buffers were prepared as described before[6]. Except for phosphate buffers and those indicated, all had 2 mM calcium acetate or $CaCl_2$.

Protein concentrations were determined from absorbances at 260 and 278 nm[7] or compared as absorbance at 278 nm. Very dilute protein solutions, as obtained in gel filtration fractions, were measured as fluorescence intensity (285 nm excitation and 350 nm fluorescence) with an Aminco-Keirs spectrophotofluorimeter.

The enzyme was routinely assayed by amidase activity on *N*-benzoyl-DL-arginine *p*-nitroanilide (BAPNA) (Schwarz/Mann, Orangeburg, N.Y., lot V1121) by a method described for trypsin (EC 3.4.4.4) assay[8] in which liberated *p*-nitroaniline is determined spectrophotometrically ($E_{410\ nm} = 8800$). Hydrolysis of 1 μmole BAPNA per min was taken as 1 unit of enzyme activity and specific activity was units per mg protein (see legends of Fig. 1 and Table I for assay conditions). A 1 mM working solution of BAPNA was prepared by dissolving 43.5 mg in 1 ml of dimethylsulfoxide and diluting the solution to 100 ml with an appropriate buffer. Buffer was usually 0.05 M Tris–HCl, pH 7.5, because Ca^{2+} could be added to it and the enzyme was more stable at this pH than at 6.2 (pH for maximal activity).

L-Lysine-*p*-nitroanilide·2HBr and glycine-*p*-nitroanilide (Nutritional Biochemicals, Cleveland, Ohio, control Nos 1370 and 9704, respectively) and L-leucine-*p*-nitroanilide (Sigma Chemical Co., St. Louis, Mo., lot 90C-2330) were tested as possible substrates of the isolated enzyme. Hydrolysis of these amides was studied at pH 5.8 and 7.8 by recording *p*-nitroaniline formation during incubation at 30°C for up to 14 h. Reaction mixtures were 3 ml reagent (1 mM in 0.1 M acetate buffer, pH 5.8 or 0.1 M Tris–HCl, pH 7.8) added to 0.1 ml enzyme (10 μg) which had been incubated with 0.1 ml of 0.05 M dithiothreitol for 2 h at 30 °C.

N-Benzoyl-L-tyrosine-*p*-nitroanilide (Sigma, lot 119B-5390) was tested similarly but only at pH 7.8 because of insolubility at the lower pH. The reagent, 660 μg in 0.1 ml dimethylsulfoxide, was diluted to 7 ml with 0.05 M Tris and adjusted to pH 7.8 with 0.05 M HCl.

Esterase activity of the enzyme was measured with *p*-toluenesulphonyl-L-arginine methyl ester (TAME) (Mann Research Lab., New York, N.Y., lot S3492) using the spectrophotometric method of Hummel[9].

Trypsin inhibitors of soy bean (lot SI 81A), lima bean (lot LBI 1EA) and ovomucoid (lot oI 8DA) were from Worthington Biochemical Corp., Freehold, N.J.).

Action of the bacterial enzyme on egg white lysozyme (3 × crystallized; Sigma, lot L102B-271), oxidized A and B chains of insulin (Schwarz/Mann, lot W-1848 and W-3878, respectively) was followed with a manual ninhydrin assay[10]. Peptide bonds cleaved were deduced by analyzing the enzymatic digest with paper

chromatography, gel filtration and amino acid analysis[11]. Lysozyme was tested at substrate to enzyme ratio of 35:1 (w/w) and incubation for 22 h at 37 °C in pH 5.8 acetate buffer (see legend of Fig. 8 for details).

The A chain of insulin was incubated at substrate: enzyme ratio of 80:1 (w/w) for 12 h at 37 °C in 0.025 M acetate buffer of pH 5.8. The reaction mixture had 4.62 mg substrate per ml, enzyme, and 2.6 mM dithiothreitol. The B chain was not tested at pH 5.8 because of its insolubility. The test was done in 0.02 M phosphate buffer (pH 7.5) with substrate:enzyme ratio of 95:1 (w/w). The reaction mixture contained approx. 6 mg substrate per ml, enzyme, and 3 mM dithiothreitol. Since Ca^{2+} could not be used with phosphate, incubation was longer (22 h) than with the A chain and at a lower temperature (30 °C). Controls of substrate without enzyme and enzyme without substrate were incubated concurrently with the test mixtures.

Molecular weight of the enzyme was determined by the gel filtration method of Andrews[12] and the high speed sedimentation equilibrium (meniscus depletion) method of Yphantis[13]. Calibration of the gel column was with proteins obtained from Mann Research Lab. Ultracentrifuge experiments were performed with the Spinco model E having Raleigh interference optical system. Upon double sector cells with 12-mm light path and Sapphire windows were used.

Isoelectric pH of the enzyme was determined in a 110-ml electrofocusing column (LKB Instruments, Rockville, Md.) using 1% (w/v) ampholyte of pH range 3–10 (Batch No. 30). The 2.0-ml enzyme sample was placed near the middle of the column. During 68–70 h operation at 600 V, water of 12–14 °C circulated through the cooling jacket. The pH of fractions was measured with a Brinkman pH meter, model 101, and protein was monitored as absorbance at 278 nm. Enzyme activity was determined as described in the legend of Table I except for use of 0.2 ml test sample and reaction at 37 °C.

RESULTS

Purification of enzyme

Precipitation of culture filtrate with $(NH_4)_2SO_4$ and collection of precipitate by centrifugation were according to previously reported steps[5]. The precipitate was suspended in 0.05 M Tris–HCl buffer, pH 7.8, equal in volume to 3% of the starting culture filtrate. This suspension, which could be stored at −20 °C for 3–4 months without loss of enzyme activity, was dialyzed first against 20 volumes of 2 mM $CaCl_2$ for 3 h and then against 10 volumes of the pH 7.8 Tris–HCl buffer for 6 h. Solutions used for dialysis were changed hourly. The dialyzed material was centrifuged at 12 000 × *g* for 10 min at 27 °C.

The supernatant solution was made 25 mM with respect to dithiothreitol (solid reagent) and to 2 mM Ca^{2+} with $CaCl_2$. The solution was heated for 15 min after it reached the 50 °C of a water bath and was then rapidly cooled with tap water. The solution was then dialyzed against 10 volumes of 0.025 M acetate buffer (pH 5.8) for 4 h with hourly buffer changes. Centrifugation of the dialyzed sample at 12 000 × *g* for 15 min resulted in a clear supernatant solution which was applied on a 2 cm × 20 cm QAE-Sephadex A-50 column equilibrated with 0.025 M acetate buffer, pH 5.8.

The column was washed with equilibrating buffer until $A_{278\ nm}$ of the eluted fractions was reduced to about 0.08. The enzyme was eluted (Fig. 1) with a linear

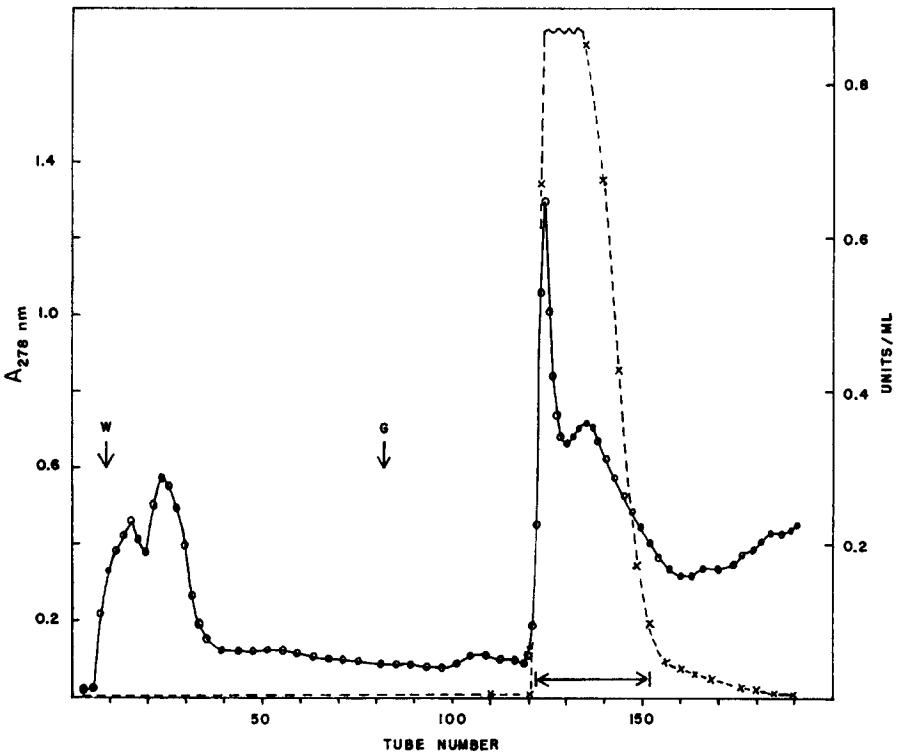

Fig. 1. Chromatography on QAE-Sephadex A-50. A 2 cm × 20 cm column, equilibrated with 0.025 M acetate buffer of pH 5.8, was loaded with 255 mg protein in 36.8 ml. Washing of column with the pH 5.8 buffer and gradient elution (200 ml of the pH 5.8 buffer + 200 ml of the buffer containing 0.4 M NaCl) began at W and G, respectively. Fractions, 4 ml per tube, were collected at 40–44 ml/h. ○—○, absorbance; ×--×, enzyme activity in units per ml. Enzyme assay: 0.1 ml enzyme and 0.1 ml dithiothreitol (0.05 M) were incubated for 2 h at 27 °C before addition of 3.0 ml BAPNA (1 mM in 0.05 M Tris–HCl buffer, pH 7.5). Reaction at 30 °C was followed for 5 min. Fractions 122 to 152 were pooled for concentration with Sephadex G-25.

gradient of increasing NaCl concentration (200 ml equilibrating buffer + 200 ml of the buffer containing 0.4 M NaCl). Fractions with at least 10% of enzyme activity in the peak tube were pooled for concentration.

Dry Sephadex G-25 was added (1 g per 3.5 ml) to the enzyme solution and was allowed to swell for 3 h. Void volume fluid, containing concentrated protein, was collected by centrifugation in ST-930 Separafuge tubes (Occomy Associates, Chicago, Ill.) at 1000 × *g* for 10 min.

The concentrated material was applied in 10 ml portions on 2 cm × 51 cm Sephadex G-100 columns equilibrated and eluted with 0.025 M acetate buffer, pH 5.0. The second of 3 peaks emerging from the column contained the enzyme (Fig. 2) and was immediately transferred to 4 °C for storage. A pool of such fractions from several gel filtration runs was applied on a cation exchange column equilibrated with the pH 5.0 acetate buffer.

A 1.5 cm × 16 cm SE-Sephadex C-50 column was loaded with 200–300 ml of pooled enzyme and was washed with 30–40 ml of equilibrating buffer. The enzyme

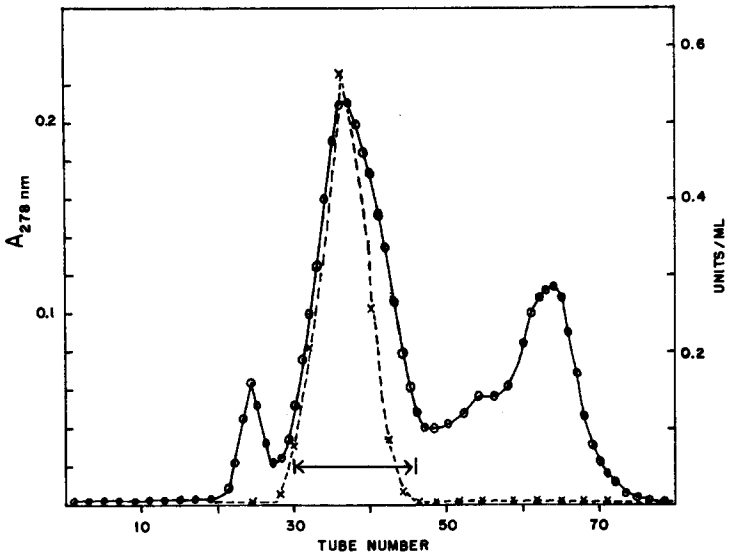

Fig. 2. Gel filtration through Sephadex G-100. A 2 cm × 51 cm column, equilibrated and eluted with 0.025 M acetate buffer, pH 5.0, was loaded with 10.0 ml of sample concentrated with Sephadex G-25. Fractions, 3 ml per tube, were collected at 38–40 ml/h. ○—○, absorbance; ×---×, enzyme activity determined as described in the legend of Fig. 1. Fractions 30 to 46 were pooled for cation-exchange chromatography.

was eluted during a linear gradient of increasing NaCl (100 ml of equilibrating buffer + 100 ml of equilibrating buffer containing 0.2 M NaCl). Small amounts of material were not held during charging of the column (Fig. 3A). An additional small peak eluted ahead of the main peak which contained the enzyme. Fractions of the enzyme peak were pooled and dialyzed against 4 volumes of pH 5.0 buffer for 2 h at 4 °C with buffer change every 0.5 h. The dialyzed material eluted as one peak without inflection points when rechromatographed on a SE-Sephadex C-50 column under conditions identical to the original chromatography (Fig. 3B). A summary of the enzyme purification scheme and recovery data are given in Table I.

The enzyme isolated was analyzed by gel filtration on Sephadex G-200 at pH 8.0 and ultracentrifugation at pH 8.0.

Gel filtration of pooled enzyme on a 1.5 cm × 53 cm Sephadex G-200 column, equilibrated and eluted with 0.05 M Tris–HCl buffer (pH 8.0) containing 0.1 M KCl, also resulted in a sharp peak without inflection points. The sample, 0.42 mg in 0.7 ml, applied on the column were the SE-Sephadex-chromatographed fractions of Fig. 3A concentrated with dry Sephadex G-25. Analysis in the ultracentrifuge is given below.

Properties of enzyme

Molecular weight

A 2.2 cm × 50 cm Sephadex G-200 column, equilibrated and eluted with 0.05 M Tris–HCl buffer (pH 8.0) containing 0.1 M KCl, was calibrated with 4 proteins of known molecular weights[12]. The plot of log molecular weight *vs* elution volume of bovine serum albumin (112.2 ml), ovalbumin (120.9 ml), chymotrypsinogen (141.1

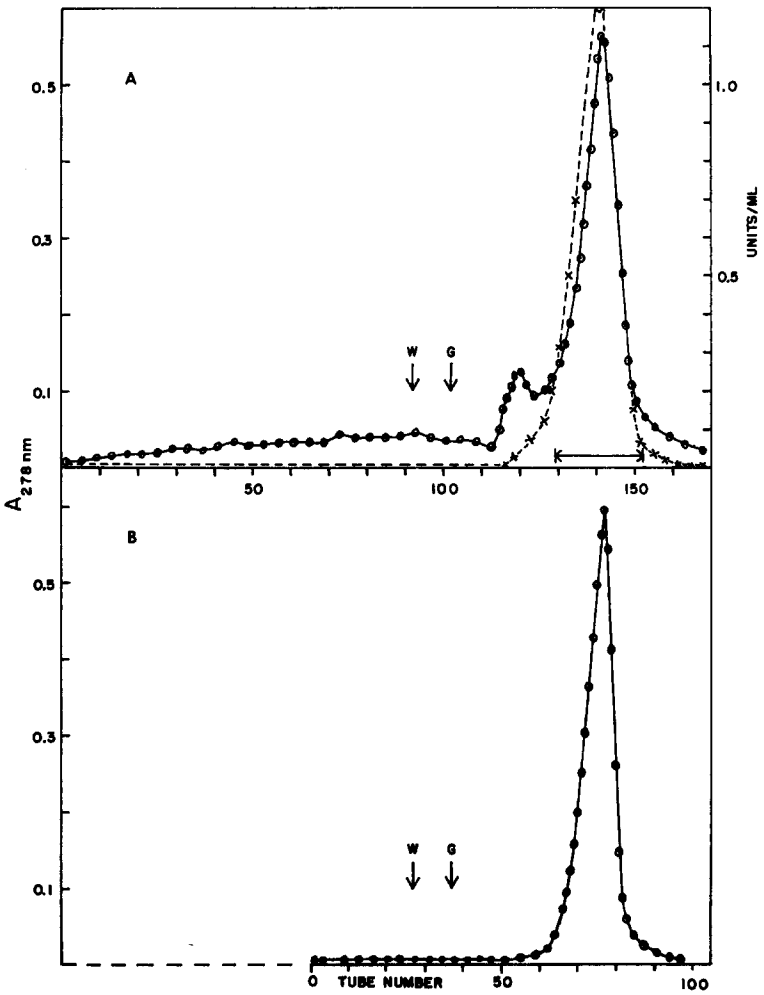

Fig. 3. (A) Chromatography on SE-Sephadex C-50. A 1.5 cm × 16 cm column, equilibrated with 0.025 M acetate buffer, pH 5.0, was loaded with 36.4 mg protein in 280 ml and eluted at 36 ml/h. Washing of column with the pH 5.0 buffer and gradient elution (100 ml of the pH 5.0 buffer + 100 ml of the pH 5.0 buffer containing 0.2 M NaCl), at flow rate of 55 ml/h, began at W and G, respectively. Fractions were 3 ml per tube. ○—○, absorbance; ×---×, enzyme activity determined as in the legend of Fig. 1. Fractions 129 to 152 were pooled for further tests. (B) Elution profile when enzyme pool obtained from step shown in (A) was rechromatographed on SE-Sephadex C-50. Applied sample was 21 mg in 72 ml; conditions of chromatography was same as in (A).

ml) and myoglobin (150.4 ml), showed the molecular weight of the enzyme with elution volume 124.0 ml to be 40 000.

High speed sedimentation equilibrium experiments were carried out with initial protein concentration 0.06% in 0.05 M Tris–HCl buffer (pH 8.0) containing 0.1 M KCl and 2 mM dithiothreitol at rotor speed 23 500 rev./min and 20 °C. Pictures were taken 21.5 h after reaching operational speed. A plot of log fringe displacement *vs* square of distance from center of rotation showed a straight line with a slope 0.505.

TABLE I

SUMMARY OF ENZYME PURIFICATION STEPS AND RECOVERIES

The specific activity was determined from amidase assays: 0.1 ml sample was incubated with 0.2 ml dithiothreitol (0.05 M) and 1.0 ml of 0.1 M Tris–HCl buffer (pH 7.5) for 2 h at 30 °C. Reaction was followed for 5 min at 30 °C following addition of 2.0 ml BAPNA (65 mg/100 ml) in 0.05 M Tris–HCl buffer (pH 7.5). High 0.1 M buffer concentration was used to assure all samples (initial pH range of 5 to 7) would have same pH for assay.

Step	*Vol. (ml)*	*Protein (mg/ml)*	*Specific activity*	*Total activity*	*Purification (-fold)*	*Yield (%)*
1. Culture filtrate	975	20.80	0.02	401	—	100
2. Sample applied on QAE-Sephadex*	36.8	6.93	0.58	144	30	36
3. Pool from QAE-Sephadex	124	0.54	1.74	117	90	29
4. Concentrated with Sephadex G-25	56.5	1.05	1.69	100	87	25
5. Pool from Sephadex G-100	280	0.13	2.0	73.3	103	18
6. Pool from SE-Sephadex	72	0.29	2.1	44.3	109	11

* Clarified solution obtained form culture filtrate by precipitation with $(NH_4)_2SO_4$, several dialyses, heating, and centrifugation.

In view of the chromatographic (Fig. 3B) and gel filtration results, the slight upward curvature at the bottom of the cell is considered as non-ideal behavior of the protein or association at high concentration. A molecular weight of 34 400 was calculated from the slope of this plot and an assumed partial specific volume of 0.72.

pH optima

Amidase and esterase activities of the isolated enzyme were studied with BAPNA and TAME, respectively, using phosphate buffers of pH range 5.8–8.0. Amidase activity was maximal at pH 6.2 while esterase activity was maximal over a range of pH 6.2–7.0 (Fig. 4).

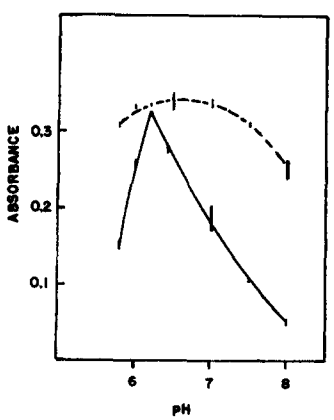

Fig. 4. Effect of pH on enzyme activity. ———, amidase activity expressed as increase in absorbance at 410 nm; — — —, esterase activity expressed as increase in absorbance at 247 nm. Reaction mixtures consisted of 0.1 ml enzyme incubated with 0.1 ml of 0.05 M dithiothreitol for 2 h at 30 °C before addition of 3.0 ml BAPNA (1 mM) in 0.05 M phosphate buffer or 2.8 ml TAME (1 mM) in 0.05 M phosphate buffer. Amidase and esterase activities were followed for 5 and 4 min, respectively, at 30 °C. Buffers were pH 5.8, 6.0, 6.2, 6.4, 7.5 and 8.0.

Requirement for reducing agent

Enzyme was incubated in 10 mM dithiothreitol for 2 hr at 30 °C. The test mixture, made 0.6 mM dithiothreitol by addition of substrate in 0.05 M Tris–HCl, buffer, pH 7.5, hydrolyzed 0.036 μmole of BAPNA per min. The same amount of enzyme had no enzymatic activity when treated identically in the absence of dithiothreitol.

Ca^{2+} and enzyme stability

Enzyme was held 16 h at 3 temperatures as solutions of different pH that differed in the presence or absence of 2.4 mM Ca^{2+}. Under conditions where loss of enzymatic activity occurred, less inactivation resulted if Ca^{2+} was present (Table II).

TABLE II

ENZYME STABILITY DURING STORAGE IN DIFFERENT CONDITIONS

Isolated enzyme was dialyzed at 4 °C against 40 vol. of water for 4 h with hourly change of water. Enzyme, 0.2 ml, diluted with 1.0 ml buffer made with or without 2.4 mM $CaCl_2$, was stored at indicated temperatures for 16 h. Buffers were 0.2 M acetate, pH 5.0 and 5.8 and 0.2 M Tris–HCl, pH 8.0. Stored samples, after equilibration to 30 °C, were incubated with 0.2 ml of 0.05 M dithiothreitol for 1 h at 30 °C. Enzyme assays followed for 5 min at 30 °C, started by adding 2.0 ml BAPNA (65 mg per ml water) to these incubated solutions, were at pH of storage conditions. Final 2 mM Ca^{2+} in assay systems was obtained by using solutions of substrate made with appropriate concentration of $CaCl_2$. Enzyme activity after storage expressed as percentage of that found at start of holding period.

Temp. (°C)	*pH 8.0*		*pH 5.8*		*pH 5.0*	
	Ca^{2+}	*No Ca^{2+}*	*Ca^{2+}*	*No Ca^{2+}*	*Ca^{2+}*	*No Ca^{2+}*
−20	100	104	100	68	88	21
4	97	98	89	73	91	15
37	82	71	72	48	83	4

Effect of trypsin inhibitors

Enzyme was treated separately with trypsin inhibitor from soy bean, lima bean, and ovomucoid at an enzyme:inhibitor ratio of 1:15 (w/w). Mixtures of enzyme and inhibitor in 0.05 M Tris–HCl buffer of pH 7.5 were incubated for 15 min at 30 °C. In one procedure, 0.1 ml of enzyme was first incubated with 0.1 ml inhibitor; further incubation of 1 h followed the addition of 0.1 ml of 0.05 M dithiothreitol. In the second method, enzyme was first incubated with dithiothreitol and then with inhibitor. BAPNA (3.0 ml, in 0.05 M Tris–HCl buffer, pH 7.5) was added to the incubated mixtures and the reaction was followed for 5 min at 30 °C. Whether inhibitors were added before or after reduction of enzyme with dithiothreitol, about 0.031 units of activity were found. Controls of enzyme without inhibitors showed the same level of activity.

Isoelectric point

Isoelectric focusing was done with enzyme preparations that had been rechromatographed on SE-Sephadex. All runs showed a narrow band of protein precipitate; precipitation could not be prevented with higher ampholyte concentrations (up to 5%, w/v) or less protein sample. When the anode was at the bottom, precipitate adhering to the column walls gradually dissolved as the more alkaline gradient moved down during collection of the fractions. Thus, a sharp protein peak was not obtained but protein concentration and enzymatic activity were both maximal in a fraction

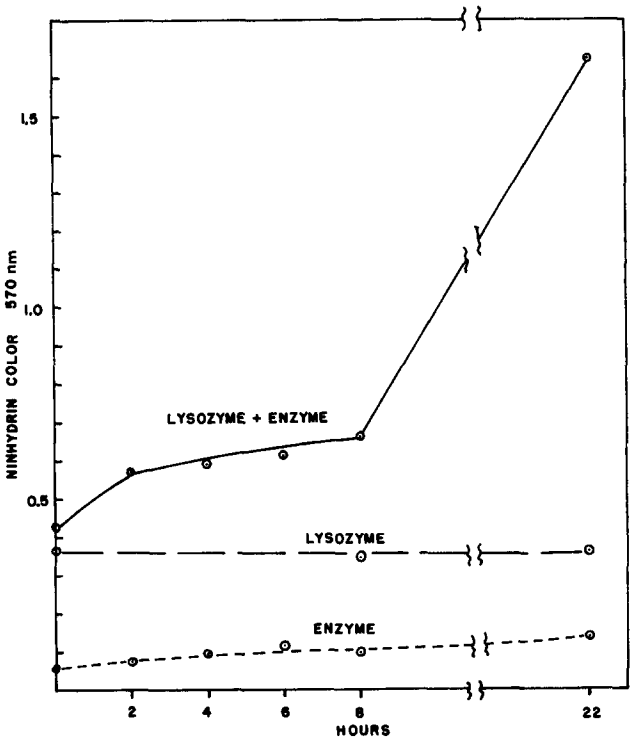

Fig. 5. Hydrolysis of lysozyme by the enzyme. Lysozyme, 3.0 mg per ml of 0.05 M dithiothreitol, was incubated at 37 °C for 5.5 h and centrifuged to remove insoluble materials. Working substrate was supernatant solution diluted with an equal volume of 0.2 M acetate buffer, pH 5.8. To 0.2 ml enzyme (40 μg), incubated with 0.1 ml dithiothreitol (0.05 M) for 2 h at 27 °C, was added 1.0 ml of substrate solution. The final mixture of 1.3 ml was incubated at 37 °C. Controls were test mixtures *minus* lysozyme or enzyme. Aliquots of 0.2 ml were withdrawn at indicated times, boiled 5 min, and stored at −20 °C until assayed with ninhydrin.

of pH 4.75 in one test and in a fraction of pH 4.48 in a second. When polarity was reversed, a much sharper peak of enzyme activity with its apex in a fraction of pH 4.65 was found. The mean of the 3 determinations or pH 4.62 was taken as the isoelectric point of the enzyme.

Substrate specificity

An amount of enzyme hydrolyzed 0.113 and 0.013 μmoles of BAPNA per min at pH 5.8 and 7.8, respectively. During incubation up to 14 h, the same amount of enzyme did not hydrolyze L-lysine-*p*-nitroanilide, L-leucine-*p*-nitroanilide, glycine-*p*-nitroanilide and *N*-benzoyl-L-tyrosine-*p*-nitroanilide (tested at pH 7.8 only).

Ninhydrin tests showed lysozyme was hydrolyzed by the isolated enzyme (Fig. 5) but the A chain of insulin was not. Descending paper chromatography (Whatman paper No. 1; pyridine–methanol–water) (2:4:1, by vol., 29 °C) of the A chain incubated with enzyme gave a pattern similar to that obtained with the control of A chain incubated without enzyme.

Hydrolysis of the B chain of insulin by the enzyme was indicated by (i) ninhydrin color of the test mixture that was 1.8 times greater than that produced by a control of substrate incubated without enzyme and (ii) a paper chromatographic

pattern of the test mixture that differed from the control in indicating the presence of alanine (R_F 0.45–0.46).

Peptide bonds of the B chain of insulin cleaved by the enzyme were determined by identifying hydrolytic products that were isolated by gel filtration. Elution volumes of blue dextran (18 ml), B chain of insulin (19 ml) and alanine (32 ml) were determined for a 0.6 cm × 70 cm Sephadex G-25 column eluted (1.0 ml per tube per 3 min) with 0.2 M pyridine. The column resolved a mixture of B chain of insulin and alanine by yielding ninhydrin positive peaks at 19 and 32 ml.

When 0.6 ml of the B chain digested with enzyme was gel filtered through the same column, a sharp peak at 19 ml and a broad peak with its apex at 31 ml were obtained. Fractions around the broad peak, beginning 3 tubes after the first peak, were pooled. After evaporation, the sample was divided into 2 portions; one was acid hydrolyzed (6.6 M HCl, 121 °C for 24 h *in vacuo*) and the other was not. Amino acid analyses[11] showed the latter had alanine as the only free amino acid and a very slowly eluting ninhydrin positive peak that was suggestive of peptide. The acid hydrolyzed sample had glycine, phenylalanine, tyrosine, threonine, proline, lysine and alanine.

DISCUSSION

The protease isolated from the culture filtrate of *C. botulinum* type B (strain Lamanna) hydrolyzed polypeptides (lysozyme and B chain of insulin), an amide (BAPNA) and an ester (TAME). All these substrates have at least one arginine residue. Substrate specificity of the protease was indicated by its failure to hydrolyze the A chain of insulin, lysine-*p*-nitroanilide, leucine-*p*-nitroanilide, glycine-*p*-nitroanilide, *N*-benzoyl-tyrosine-*p*-nitroanilide, and *N*-acetyl-tyrosine-ethyl ester[5]. All of these lack arginine.

The bonds of the B chain of insulin cleaved by the enzyme are deduced by relating the identified hydrolytic products to the substrate's structure[14]. Amino acids 21 to 30 of the C-terminal end of the substrate are –Glu–Arg–Gly–Phe–Phe–Tyr–Thr–Pro–Lys–Ala. Only amino acids that correspond to residues 23 through 30 of the substrate were found in the acid hydrolyzed portion of the pool made from fractions obtained by gel filtration of the incubated enzyme–substrate mixture. This showed cleavage of the peptide bond formed by the carboxyl group of arginine; the particular amino acids identified could not arise by cleavage at any other bond. The free alanine, found in the enzymatic digest by paper chromatography and in the pool (not acid hydrolyzed) of gel filtration fractions, results from cleavage of the peptide bond formed by the lysine residue. It is unlikely that the other alanine in the B chain of insulin was freed since this would give rise to 2 other peptides. These peptides, if present, should have been in the gel filtered fractions analysed and contributed amino acids other than those actually found.

Activation of botulinal progenitor toxins by trypsin had suggested that the endogenous enzyme responsible for activation during growth of pure cultures may have trypsin-like specificity. Such a specificity (cleavage of the peptide bond formed by the carboxyl group of arginine and lysine residues) is now shown for the bacterial enzyme previously reported to activate progenitor toxin[5].

This enzyme differs from trypsin in several respects. The 3 natural inhibitors of trypsin do not inhibit the bacterial protease at concentrations approx. 15-fold

greater than that needed for complete inhibition of trypsin. The optimum pH of trypsin acting on BAPNA is near 8.1[8] compared to pH 6.2 for the bacterial enzyme. Trypsin hydrolyzes both BAPNA and L-lysine-*p*-nitroanilide[8] but the bacterial enzyme does not act on the latter compound. Although lysine-*p*-nitroanilide is susceptible to tryptic hydrolysis, presence of the free α-amino group may make it refractory to the bacterial enzyme. This proposition was not tested because α-benzoyl-lysine-*p*-nitroanilide is not commercially available.

Substrate specificity of this enzyme is similar to that of Clostridiopeptidase B (EC 3.4.4.20) produced by *Clostridium histolyticum*[15]. Both enzymes require a reducing agent for activity and are more stable in the presence of Ca^{2+}. Clostridiopeptidase B differs from the *C. botulinum* enzyme in molecular weight (50 000 *vs* 34 400 or 40 000), isoelectric point (pH 4.8–4.9 *vs* 4.62) and pH optimum for esterase activity (7.4–7.8 *vs* 6.2–7.0).

ACKNOWLEDGEMENTS

Research supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison, by grant S-R01-FD-0090 of the U.S. Public Health Service and contributions from various companies and associations of the food industries.

Authors thank Dr J. E. Blair, Institute of Enzyme Research, University of Wisconsin, for ultracentrifugation experiments.

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