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Purification and Characterization of a Protease from *Clostridium botulinum* Type A That Nicks Single-Chain Type A Botulinum Neurotoxin into the Di-Chain Form

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A protease that nicks the ~150-kilodalton (kDa) single-chain type A botulinum neurotoxin into the ~150-kDa di-chain form in vitro was isolated from *Clostridium botulinum* type A (Hall strain) cultures. The di-chain neurotoxin generated in vitro is composed of an ~50-kDa light chain and an ~100-kDa heavy chain which are disulfide linked and is indistinguishable from the di-chain neurotoxin that forms in vivo and is routinely isolated (M. L. Dekleva and B. R. DasGupta, Biochem. Biophys. Res. Commun. 162:767–772, 1989). This enzyme was purified >1,000-fold by ammonium sulfate precipitation, QAE-Sephadex Q-50, Sephadex G-100, and CM-Sephadex C-50 chromatography steps with the synthetic substrate *N*-benzoyl-DL-arginine-*p*-nitroanilide. The ~62-kDa amidase (protease) is a complex of 15.5- and 48-kDa polypeptides (determined by polyacrylamide gel electrophoresis) that could not be separated without sodium dodecyl sulfate. The enzyme has an isoelectric point of pH 5.73, a pH optimum of 6.2 to 6.4, an absolute requirement for a thiol-reducing agent as well as a divalent metallic cation (probably Ca^{2+}) for activity, and a temperature optimum of 70°C. Tests with several synthetic substrates indicated the high specificity of the enzyme for arginyl amide bonds.

Clostridium botulinum serotype A neurotoxin (NT) is synthesized as an ~150-kilodalton (kDa) single-chain protein. The NT undergoes a posttranslational proteolytic modification(s); a time-dependent cleavage at one-third the distance from the N-terminal end of the protein yields an ~150-kDa di-chain form of the protein (3, 24). Although the type A NT is generally purified in its di-chain form from older (96-h) cultures (4), the single-chain form of this NT has been isolated from young cells (24 h) by interruption of fermentations (16). Trypsinization of this single-chain NT resulted in ~50- and ~100-kDa subunits that were electrophoretically indistinguishable from the subunits derived from the ~150-kDa di-chain NT purified from older cultures (16). We hypothesized that the protease responsible for nicking the type A NT in vivo has a trypsinlike substrate specificity. The enzyme capable of nicking the NT has not previously been isolated.

Another posttranslational change that the botulinum NT undergoes is activation of the relatively low toxicity of the newly synthesized NT to a higher toxicity. Nicking of the single-chain protein at one-third the distance from the N-terminal end has not been found to be directly responsible for this activation (see references in DasGupta [3]). Proteases which are capable of activating but not nicking the serotype B and E botulinum NTs have been isolated from *C. botulinum* strains (2, 5, 21, 22) and other sources (15, 18, 19). In this communication, we describe for the first time the purification and characterization of an amidase (protease) isolated from *C. botulinum* type A cultures that is capable of nicking the single-chain type A NT into its di-chain form.

MATERIALS AND METHODS

Organisms and culture conditions. Stock cultures of *C. botulinum* type A (Hall strain; a kind gift from Lynn Siegel, Fort Detrick, Frederick, Md.) cultured in cooked-meat medium (Difco Laboratories, Detroit, Mich.; 3.2 g in 25 ml of distilled water) and stored at −20°C served as starter cultures. The medium used for the production of the protease and the single-chain NT consisted of (percent [weight/volume]): N-Z Amine type A (Sheffield Farms, Norwich, N.Y.), 2; yeast extract, 0.5; autolyzed yeast paste (Yeastamin; A. E. Staley Manufacturing Co., Decatur, Ill.), 0.6; and glucose, 0.5. The medium (minus glucose) was adjusted to pH 7.3 with NaOH prior to being autoclaved. Glucose solutions were autoclaved separately. All cultures were incubated at 37°C.

Growth curve studies. One liter of production medium was inoculated with 20 ml of a 2-day-old culture of *C. botulinum* type A in cooked-meat medium. Five-milliliter samples were withdrawn at intervals to monitor growth based on the turbidity at 600 nm.

Purification of the nicking protease. Twenty milliliters of culture in cooked-meat medium was used to inoculate 2 liters of production medium in a 2-liter Ehrlenmeyer flask. After 20 to 22 h of incubation at 37°C, cells were removed by centrifugation (10,000 × *g*, 20 min). The supernatant was 80% saturated with solid ammonium sulfate (56 g/100 ml). All subsequent steps were performed at 4°C. The material precipitated overnight was harvested by centrifugation (10,000 × *g*, 20 min), suspended in 50 ml of 0.02 M Tris-acetate buffer (pH 6.6) containing 5 mM $CaCl_2$, and dialyzed against a 20× volume of the same buffer for 48 h, with several buffer changes.

The dialyzed extract was loaded onto a QAE-Sephadex Q-50 column (4 by 32 cm) equilibrated with 0.02 M Tris-acetate buffer (pH 6.6) containing 5 mM $CaCl_2$ and washed with 1,200 ml of the same buffer to remove unbound material. The amidase activity (see below) bound to the column was recovered by elution (flow rate, 36 ml/h) with the same buffer containing 0.15 M NaCl. Fractions (7 ml per tube) containing ~10% or more maximum amidase activity were pooled and concentrated by the addition of solid ammonium sulfate to 80% saturation. The protein precipitated overnight

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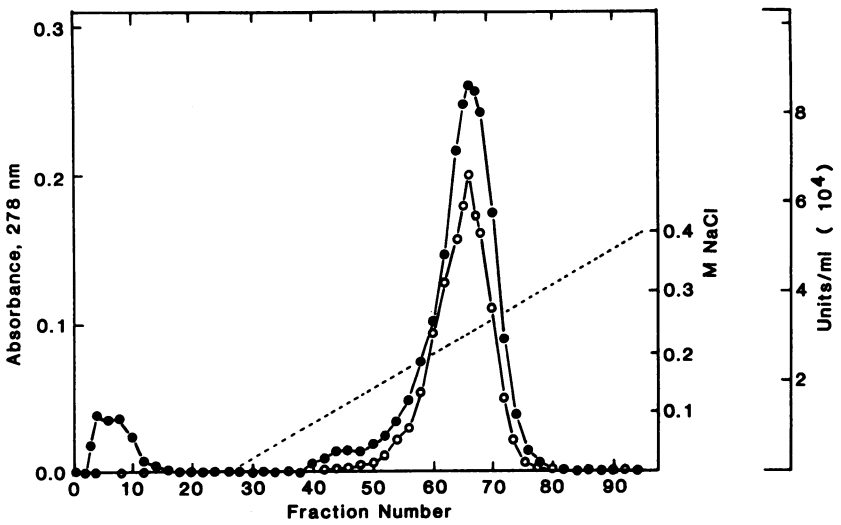

FIG. 1. Chromatography of the amidase on a CM-Sephadex C-50 column (1.5 by 10 cm) equilibrated with 0.02 M acetate buffer (pH 5.4) containing 5 mM $CaCl_2$. Five-milliliter (no. 1 to 24) and 2.5-ml (no. 25 to 90) fractions were monitored for A_{278} (●) and amidase activity with BAPNA (○) as described in Materials and Methods. A linear gradient of increasing NaCl (100 ml of equilibration buffer versus 100 ml of equilibration buffer plus 0.4 M NaCl) was begun at tube number 26 (broken line). The flow rate was 15 to 20 ml/h.

was collected by centrifugation (10,000 × *g*, 20 min) and suspended in a minimum volume (2 to 3 ml) of 0.02 M sodium acetate buffer (pH 5.4) containing 5 mM $CaCl_2$. This sample was applied directly to a Sephadex G-100 column (1.5 by 93 cm); equilibration and elution (flow rate, 30 ml/h) were done with 0.02 M sodium acetate buffer (pH 5.4) containing 5 mM $CaCl_2$. Fractions (3 ml per tube) containing peak enzymatic activity were pooled and loaded on a CM-Sephadex C-50 column equilibrated with 0.02 M sodium acetate buffer (pH 5.4) containing 5 mM $CaCl_2$, and the enzyme was eluted (Fig. 1).

Characterization of the nicking protease. Bicinchoninic acid (Pierce Chemical Co., Rockford, Ill.) was used to determine protein content (23). Two different methods, polyacrylamide gel electrophoresis (PAGE) and gel filtration, were used to estimate the molecular mass. PAGE was performed either with a PhastSystem electrophoresis unit (8 to 25% gradient gels with sodium dodecyl sulfate [SDS]-impregnated buffer strips; Pharmacia, Inc., Piscataway, N.J.) or as described by Dreyfuss et al. (7) in the presence of 0.1% SDS with some modifications. The electrophoresis molecular mass markers (Pharmacia) phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa) were run concurrently with test samples. A TSK-3000 SW size exclusion column (high-pressure liquid chromatography system; 7.5 mm by 30 cm; Beckman Instruments, Inc., Fullerton, Calif.) was used, and elution was done with 0.05 mM Tris hydrochloride buffer (pH 7.0)–5 mM $CaCl_2$–0.1 M NaCl at 0.5 ml/min. Fractions (0.5 ml) were collected and analyzed for amidase activity. Molecular mass markers for high-pressure liquid chromatography (Sigma Chemical Co., St. Louis, Mo.) included bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), trypsin (24 kDa), and cytochrome *c* (12.4 kDa).

Two-dimensional electrophoresis was performed to determine the subunit composition of the purified enzyme. Electrophoresis in the first dimension was performed as described earlier with 7.5% polyacrylamide but without SDS. The single prominent band, migrating between 1.5 and 2.5 cm, was excised from the gel and incubated for 1 to 1.5 h in equilibration buffer containing 10% glycerol (vol/vol), 5% β-mercaptoethanol (vol/vol), 2.3% SDS (wt/vol), and 0.0625 M Tris hydrochloride (pH 6.8) (20). The gel slice was mounted on a second slab gel and run in the presence of 0.1% SDS.

Ferguson plot analysis. PAGE without SDS of the amidase and molecular mass marker protein samples was done at polyacrylamide concentrations of 7.5, 9, and 15% (*%T*; see below). The molecular mass of the amidase was determined by linear regression analysis (10) of the molecular masses of the electrophoresis marker proteins mentioned above, as follows: 100 log (R_f × 100) versus *%T*.

pH optimum. Amidase activity was measured after incubation of the enzyme in 0.1 M phosphate buffers (pH range, 5.8 to 8.0) with *N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) for 30 min at 37°C.

Thermal stability. Two milliliters of BAPNA in 0.1 M sodium acetate buffer (pH 6.2)–5 mM $CaCl_2$–5 mM dithiothreitol (DTT) was heated to different temperatures (25 to 95°C) for 5 min prior to the addition of 0.1 ml of enzyme solution. Each reaction mixture was kept at the desired temperature for 10 min. Reactions stopped by the addition of 0.5 ml of 30% (vol/vol) acetic acid were monitored spectrophotometrically. Control experiments run at selected incubation temperatures were done without the enzyme.

Isoelectric point determination. Isoelectric focusing gels contained 2.42 ml of acrylamide (20 g of acrylamide, 0.6 g of bisacrylamide, and 2 g of Amberlite MB-1 dissolved and diluted to 66.6 ml with distilled water), 6.13 ml of sucrose solution (0.3 g/ml), and 0.95 ml of ampholytes (Isolytes; pH range, 3 to 10; Isolab Inc.) diluted to 14 ml with distilled water. This mixture was degassed for 5 min, and 3 ml was added to 200 μl of protein–50 μl of ammonium persulfate (30 mg/ml)–4.3 μl of N_1,N_1,N',N'-tetramethylethylenediamine. After polymerization for 0.5 to 1 h, the cylindrical gels (0.5 by 8.5 cm) were prefocused for 1.5 h at 0.25 W per gel and run at a constant 300 V for 4 h. The anode and cathode solutions were 1 M phosphoric acid and 1 M NaOH, respectively. Gels were stained with Coomassie brilliant blue or cut

TABLE 1. Purification scheme for the *C. botulinum* type A amidase (protease)

Purification step	Total ml	mg/ml	Total mg	U/ml[a]	Total U (10^6)	% Recovery	U/mg	Purification (fold)
20-h supernatant	2,000	3.76	7,520	2,320	4.6	100	617	
80% $(NH_4)_2SO_4$ precipitate	93	2.20	205	32,150	3.0	65	14,614	24
QAE-Sephadex Q-50 pool	175	0.15	26	12,600	2.2	47	84,000	136
Sephadex G-100 pool	27	0.15	4	31,250	0.8	18	205,592	333
CM-Sephadex C-50 pool	80	0.005	0.4	3,141	0.25	5	668,298	1,083

[a] Units are expressed as an increase of 0.001 A_{410} unit per min during the BAPNA assay as described in Materials and Methods.

into 0.5-cm slices for pH or enzymatic activity determinations.

Amidase assay and the synthetic substrates tested. The enzyme was routinely assayed on the basis of amidase activity with BAPNA as the substrate; *p*-nitroaniline liberated after hydrolysis of the amide bond was determined spectrophotometrically at 410 nm (8). A 1 mM working solution of BAPNA was prepared by dissolving 43.5 mg of BAPNA in1 ml of dimethyl sulfoxide and then diluting the solution to 100 ml with an appropriate buffer. Aliquots of 1.5 ml were added to 100 μl of sample and incubated at 35°C. The buffer was usually 0.1 M acetate (pH 6.2) (pH 6.2 to 6.4 was maximal for activity) containing 5 mM $CaCl_2$ and 5 mM DTT. We defined 1 U of activity as being equivalent to an increase of 0.001 A_{410} unit at 35°C/min. Specific activity was expressed as units per milligram. L-Lysine-*p*-nitroanilide-2HBr, glycine-*p*-nitroanilide, and L-leucine-*p*-nitroanilide (Sigma) were tested as possible substrates for the enzyme at pHs 5.5, 6.2, and 7.5 by recording *p*-nitroaniline formation. Reaction mixtures containing 2.0 ml of reagent (1 mM in 0.1 M acetate buffer–5 mM $CaCl_2$–5 mM DTT [pH 5.5 or 6.2] or 0.1 M Tris hydrochloride buffer–5 mM $CaCl_2$–5 mM DTT [pH 7.5]) and 0.1 ml of enzyme were incubated at 35°C for up to 60 min.

The esterase activity of the enzyme was tested with Nα-*p*-tosyl-L-arginine methyl ester (TAME), Nα-*p*-tosyl-L-lysine methyl ester (TLME), and *N*-acetyl-L-tyrosine ethyl ester (ATEE) (all from Sigma) as substrates. Reaction mixtures contained 2.0 ml of reagent (10 mM in 0.1 M acetate buffer–5 mM $CaCl_2$–5 mM DTT [pH 5.5 or 6.2] or 0.1 M Tris hydrochloride–5 mM $CaCl_2$–5 mM DTT [pH 7.0]) and 0.1 ml of enzyme. The increase in the A_{247} after incubation at 35°C was monitored to measure esterase activity (12, 25).

Effects of inhibitors and activators on the enzyme. The enzyme preparations were pretreated with the following well-known protease inhibitors before BAPNA assays were performed: 1 and 5 mM phenylmethylsulfonyl fluoride (PMSF) in 5% aqueous isopropanol for 1 h; 2.5 mM tosylamide-2-phenylethylchloromethyl ketone (TPCK) in 5% aqueous isopropanol for 30 min; 5 mM 1,10-phenanthroline (PHEN) in 5% aqueous isopropanol for 30 min; 10 μg of soybean trypsin inhibitor (SBTI; aqueous) per ml for 30 min; 0.1, 0.5, 1, and 5 mM aqueous EDTA for 30 min; and DTT at concentrations of 0, 1, 5, 10, and 50 mM. All experiments were performed in either 0.1 M phosphate or Tris-acetate buffer (pH 6.2), and all but the DTT dependency experiments contained 5 mM DTT. The enzyme concentration in test solutions was ~5,000 U/ml.

Reversal of EDTA inhibition with metals. Enzyme solutions (~100 μg/ml) were diluted 1:10 with distilled water. Because the buffers in which the enzyme were stored typically contained 5 mM $CaCl_2$, the level of Ca^{2+} was reduced by dilution to 0.5 mM. EDTA was added (final concentration, 5 mM) to the diluted enzyme solutions. After 15 min at room temperature, individual metals (Zn, Mg, Mn, Ni, Cu, Cr, Fe, and Ca) were added (final concentration, 20 mM) to test solutions. After another 15 min of incubation at room temperature, the samples were assayed for amidase activity but without $CaCl_2$. Control samples were those in which no EDTA or additional metals were added to enzyme solutions (positive control) or those in which EDTA alone was added to enzyme solutions (negative control).

RESULTS AND DISCUSSION

The ~150-kDa single-chain type A botulinum NT was nicked into ~50-kDa light-chain and ~100-kDa heavy-chain subunits by trypsin (16). These trypsin-derived subunits were electrophoretically identical to the light- and heavy-chain subunits obtained from type A NT purified from 96-h cultures in its di-chain form. Because the single-chain type A NT could be nicked by trypsin, we hypothesized that an endogenous protease present in the culture supernatant may, like trypsin, have a preference for arginyl and/or lysyl bonds and also be capable of nicking the type A NT. We therefore tested BAPNA, a synthetic substrate for trypsin (8), as a substrate to search for the NT-nicking protease in cultures and during purification steps and characterization studies. Casein and azocasein were also tested as possible proteolytic substrates but proved far less sensitive than BAPNA. The amidase at various purification steps nicked the single-chain type A NT (data not shown) and thus exhibited its proteolytic activity. The results of nicking the single-chain type A NT with the amidase following its >1,000-fold purification were reported previously (6).

The pattern observed for the growth of *C. botulinum* type A was similar to that described by others (1, 14): rapid growth in production medium from 4 to 12 h after a short initial lag period. Maximum cell density (measured as turbidity) was attained at about 22 h, followed by rapid cell lysis. An increase in amidase activity paralleled the growth of the organisms, reached near maximum levels by 12 h, and then remained constant for the remainder of the 96-h culture incubation. We chose to isolate the amidase from 18-h cultures to minimize contamination of the extracellular amidase with intracellular proteins and nucleic acids which would be released into the medium as a result of cell lysis.

The scheme for isolating the enzyme is outlined in Table 1. The dark brown material obtained by ammonium sulfate precipitation of 20- to 22-h culture supernatants contained about 65% of the total amidase activity originally present. Because of the large load of the crude material in this column, ~4 ml of anion-exchange gel per ml of extract was required to completely bind the enzyme present in the sample. Nearly all of the dark brown pigment was retained on the column, and the eluted amidase peak fractions, light amber in color, were pooled.

Two well-resolved peak fractions were eluted from the

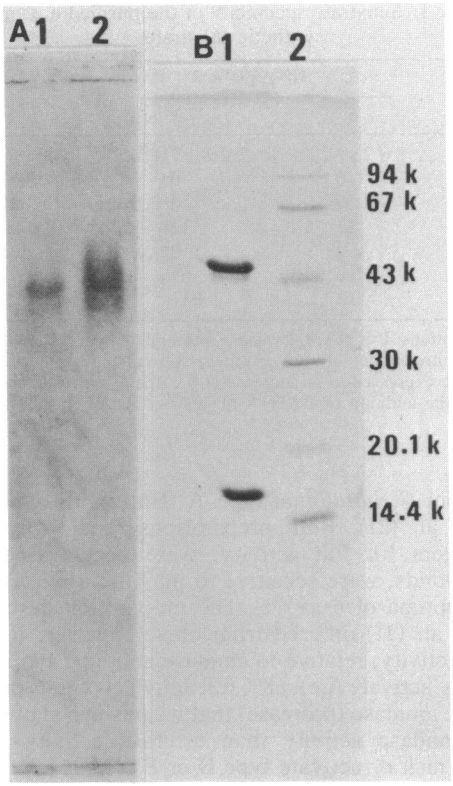

FIG. 2. Electrophoresis of the ~1,000-fold-purified amidase under native (A) or SDS-denatured (B) conditions. Lanes 1 and 2 of panel A contained 2 and 4 μg of enzyme, respectively. Lane 1 of panel B was loaded with 8 μg of enzyme, and lane 2 of panel B was loaded with the molecular mass markers indicated in Materials and Methods. k, Thousands.

Sephadex G-100 column. A small peak, not resolved from the ascending slope of the highest peak, contained predominantly a 55-kDa protein (analyzed by SDS-PAGE; data not shown). The highest protein peak contained the amidase activity. The trailing small peak (the apex was ~60 ml away from the main peak apex) contained some brown-pigmented material.

A pool of the fractions from the highest protein peak containing predominantly the amidase activity was resolved by the CM-Sephadex C-50 column (Fig. 1). A small amount of protein did not bind and was eluted by washing. A major protein peak emerged during elution with an increasing salt gradient. This protein peak alone contained the amidase activity. The amidase at this stage of purification (Table 1) was of 1,000-fold-greater purity than that in culture supernatants.

SDS-PAGE results revealed that the enzymatic peak from the final CM-Sephadex chromatography step consisted of 48- and 15.5-kDa proteins (Fig. 2B). The same two electrophoretic bands were obtained whether the purified enzyme was reduced with β-mercaptoethanol or not, indicating that the two proteins were not disulfide linked. The nature of the interaction of the 15.5- and 48-kDa proteins was further examined by PAGE in the absence of SDS. A single prominent band that had amidase activity when sliced from the gel and assayed with BAPNA appeared (Fig. 2A). Ferguson plot analysis (10) of the band (migration as a function of cross-linkage of the gel) yielded a molecular mass of 62 kDa. Furthermore, when this band was sliced from the native gel and run in a second dimension under denaturing conditions in the presence of SDS, two bands corresponding to 48 and 15.5 kDa were seen. In three separate gel filtration high-pressure liquid chromatography runs in the absence of SDS, a single amidase peak appeared at elution times of 24, 25.5, and 27 min. These elution times corresponded to molecular masses of 37, 38, and 41 kDa, respectively, determined from the elution times of molecular mass marker proteins. When the NaCl concentration in the elution buffer (0.05 M Tris hydrochloride [pH 7.0] plus 5 mM $CaCl_2$) was increased from 0.1 to 0.3 M, the amidase was retained on the column for a longer time, thus appearing as a 31.5-kDa protein. Apparently the enzyme was exhibiting a hydrophobic interaction with the column (9). This result also suggests that the estimated molecular mass (37 to 41 kDa) found in the presence of 0.1 M NaCl could be higher at a lower salt concentration of the buffer and be closer to the value of 62 kDa estimated by Ferguson plot analysis. We surmise from these studies that the enzyme, a 62-kDA protein, is composed of 15.5- and 48-kDa polypeptides.

Although it is probable that the two polypeptides are the structural components of the enzymatically active 62-kDa protein, the possibility exists that one of the two polypeptides is a contaminant bound to the other polypeptide, which is actually the enzyme. This possibility cannot be ruled out until the polypeptides can be separated in the absence of denaturants (e.g., SDS) and their enzymatic activities are tested individually. Trypsin and α-chymotrypsin are two very familiar examples of proteases that are composed of more than a single polypeptide chain (11, 13). Transformation of streptococcal proteinase from its inactive (zymogen) to its active enzyme form by an autocatalytic reaction following reduction (contact with —SH groups in the bacterial cell walls) involves proteolytic processing (17). Future research can also establish whether the ~62-kDa active amidase (protease) isolated from *C. botulinum* type A is the end product of a proteolytically processed larger precursor protein or a ~62-kDa precursor protein which undergoes cleavage of one peptide bond located outside a disulfide loop.

Isoelectric focusing of the purified enzyme in one experiment yielded the major band at pH 5.6 (Coomassie stained). In a second experiment, amidase activity focused in replicate gels at a pH of 5.85 and corresponded to a strongly staining (Coomassie) protein band. A value of 5.73 was therefore assigned as the isoelectric point for the enzyme. The peak activity of the enzyme (hydrolysis of BAPNA) was observed in the pH range of 6.2 to 6.4. The enzyme exhibited an increase in activity up to 70°C (Fig. 3); above that there was a rapid loss of activity. The amidase required 5 mM DTT for full activity. In the absence of a reducing agent, only 1 to 5% of full enzymatic activity was observed. Although no diminution of amidase activity was observed when the enzyme was pretreated with PMSF, PHEN, or SBTI, a 35% decrease in activity was observed upon exposure to 2.5 mM TPCK. EDTA acted as a potent inhibitor of this enzyme; 1 mM EDTA completely inhibited enzymatic activity (Table 2). The effect of 5 mM EDTA inhibition could be completely reversed by the addition of 20 mM $CaCl_2$; 60 and 30% of enzymatic activities were restored by $MnCl_2$ and $FeCl_2$, respectively; and Zn, Mg, Ni, and Cr were 10% or less effective. Cu had no reversal activity.

An amount of enzyme corresponding to ~6,000 U/ml at pH 6.2 was monitored for its ability to hydrolyze L-lysine-*p*-nitroanilide, L-leucine-*p*-nitroanilide, glycine-*p*-nitroanilide, and *N*-benzoyl-L-tyrosine nitroanilide at pHs 5.5, 6.2,

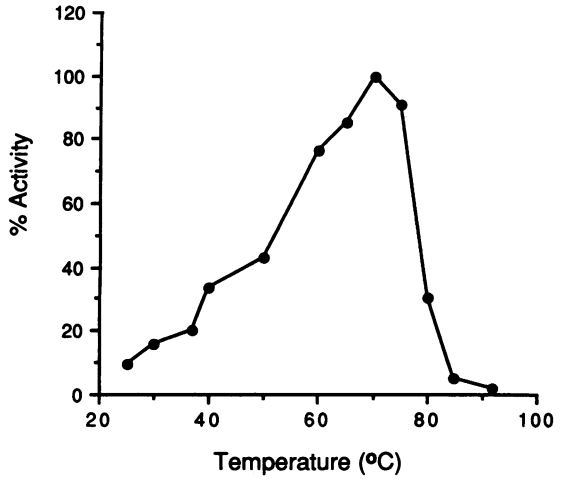

FIG. 3. Thermal stability of the amidase. The enzyme was assayed with BAPNA at temperatures ranging from 25 to 95°C in 0.1 M acetate buffer (pH 6.2) containing 5 mM $CaCl_2$ and 5 mM DTT as described in Materials and Methods.

and 7.5 (Table 3). After 1 h of incubation at 37°C, only the lysine and leucine amides exhibited slight hydrolysis, with pH optima near 7.5, and at levels less than 0.1% the BAPNA activity. The same amount of enzyme was also tested for esterase activity with TAME and TLME as substrates. Again, both substrates were hydrolyzed, predominantly at a pH near 7.5, but at levels less than 0.5% the BAPNA activity.

The 1,000-fold-purified amidase had some properties similar to those found in the crude protease preparation obtained from *C. botulinum* type A (Hall strain) cultures by Ohishi et al. (21). Both preparations required a thiol-reducing agent for full activity, were very active against arginine bonds, were sensitive to inhibition by EDTA, and had pH optima of 6 to 6.5. The preparation described by Ohishi et al. (21) differed from ours, however, in its high esterase activity, relative to amidase activity, and its ability to partially activate (i.e., 68% full activity) type E progenitor toxin. The amidase (protease) that we purified showed much greater amidase activity than esterase activity and was unable to nick or activate type B or E NTs (6).

Knowledge of the characteristics of this protease has important applications in efforts to purify type A botulinum NT. We observed that exposure of the NT to this protease for 1 h at pH 6.0 to 6.4 considerably degraded the NT into various fragments. This result indicates that prolonged exposure of the NT during purification to the active protease must be avoided to minimize degradation of the NT. Measures to be taken would be to include metal chelators (such as EDTA) in buffers and/or to use divalent metal cation-sequestering buffers (e.g., citrate) during the purification process.

TABLE 2. Effect of inhibitors on the activity of the purified enzyme[a]

Treatment	Concn	Activity (% of control)
No enzyme		0
Boiled enzyme		0
DTT	0 mM	0
	1 mM	3
	5 mM	100
	20 mM	98
Isopropyl alcohol	5%	110
PMSF	1 mM	123
	5 mM	124
TPCK	2.5 mM	65
PHEN	5 mM	99
SBTI	10 μg/ml	121
EDTA	0.1 mM	88
	0.5 mM	10
	1 mM	0
	5 mM	0

[a] BAPNA assays were performed at pH 6.2 in the presence of protease inhibitors as described in Materials and Methods. Results are presented as percentages of the results for the control containing 5 mM DTT but no inhibitor and are averages of duplicate values.

TABLE 3. Substrate specificity of the purified enzyme with synthetic substrates

Substrate[a]	U[b] at pH:		%
	6.2	7.3	
BAPNA	6.2×10^6	2.8×10^6	100
Lys NA	6	6	<0.001
Leu NA	6	6	<0.001
Gly NA	12	12	<0.001
TAME	0	0	<0.001
TLME	12	65	0.002 (pH 7.3)
ATEE	12	241	0.009 (pH 7.3)

[a] Abbreviations: Lys NA, L-Lysine-*p*-nitroanilide hydrobromide; Leu NA, L-leucine-*p*-nitroanilide; Gly-NA, glycine-*p*-nitroanilide.

[b] Units are expressed as an increase of 0.001 A_{410} unit per ml of protease per h after the addition of BAPNA reagent as described in Materials and Methods.

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