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Purification and Some Properties of Two Boticins

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Two bacteriocins (boticins) were elaborated without induction by strain S5, a nontoxigenic variant of *Clostridium botulinum* type E. After separation of the two active entities by gel filtration on Sephadex G-50, a large particle with boticin activity was isolated by density gradient ultracentrifugation, and a small soluble boticin was purified by continuous curtain electrophoresis and chromatography on sulfoethyl-Sephadex. Large and small boticins were purified 200- and 3,000-fold, respectively, with yields of 50% or more. The small boticin, a basic substance with molecular weight under 30,000, was the predominant species; the large boticin, a negatively charged particle with particle weight greater than 40×10^6, represented less than 20% of the total activity. Both purified boticins were resistant to heat and were attacked by proteolytic enzymes, but the large boticin was less thermostable and less sensitive to proteolytic enzymes than was the smaller variety. The activity of the large boticin was not reduced by treatment with urea or deoxyribonuclease. Both boticins exhibited sporostatic and bactericidal activities for *C. botulinum* type E, strain 070. A suspension of type E strain 070 vegetative cells was rendered nonviable within 9 min by the small boticin. The lethal action of this bacteriocin was not reversed by trypsin.

The production of bacteriocins (boticins) active against toxigenic *Clostridium botulinum* type E strains by several closely related but nontoxigenic organisms was described in an earlier report from this laboratory (25). Since the initial report, we have detected (*unpublished data*) bacteriocin activity against *C. botulinum* type E in filtrates of *C. perfringens* and *C. botulinum* types A, B, and E strains as well. Interference of these bacteriocin-producing organisms with methods for the detection or recovery (or both) of toxin-producing type E has been demonstrated (25).

The status of our knowledge regarding the bacteriocins has been reviewed frequently (3, 9, 10, 17, 21, 30, 34); the recent reviews of Reeves (34) and Bradley (3) are especially pertinent. Bradley's broad definition of bacteriocins as "bactericidal particles which are unable to multiply in a sensitive indicator" is applied in this report.

Many bacteriocinogenic organisms elaborate maximal quantities of bacteriocins only after induction with ultraviolet light, metabolic inhibitors such as mitomycin C, or other agents known to induce lysogenic phages (2, 10, 13, 18, 20, 24, 34, 37). Some organisms release bacteriocin continuously (11, 26); in other cases, an abrupt release of bacteriocin coincides with partial or complete lysis of the culture (11, 14, 17, 20, 24).

As Bradley (3) pointed out, the bacteriocins studied to date fall into two distinct categories. One group consists of bacteriocins of small molecular size, which are usually thermostable and cannot be sedimented by ultracentrifugation; bacteriocins of very large size, which are usually thermolabile and can be sedimented easily, make up the second group. The production of a bacteriocin of each type by one organism is not uncommon among the colicin producers (3, 26).

Although both types of bacteriocin appear to be at least part protein, they differ in sensitivity to proteolytic enzymes such as trypsin (34); as a rule, the large sedimentable bacteriocins are resistant to proteolytic enzymes, but the bacteriocins of small molecular size are not (3).

Purified preparations of some large bacteriocins consist of phagelike particles; several colicins (4, 28, 36), pyocins (3, 13, 16), monocins (4, 12), and a vibriocin (22, 23) are included in this group.

The action of a pesticin and colicins K, E_2, E_3, and F can be reversed by addition of trypsin to cells "killed" by these bacteriocins (6, 7, 29, 31, 35). The fact that trypsin rescue is possible after long periods of treatment with colicins K235-K or E_3 has given rise to speculation that these bacteriocins exert their effects while attached to the cell surface (34), and that their primary action may be bacteriostatic rather than bactericidal (3, 29, 31).

The purpose of this investigation was to provide highly purified boticin preparations for use in elucidating the nature and mode of action of these bacteriocins. This report describes the isolation of two boticins and some of their properties.

MATERIALS AND METHODS

Cultures. Boticin-producing strain S5, a nontoxigenic variant of *C. botulinum* type E, was used throughout these studies. *C. botulinum* type E strain 070 was selected arbitrarily from a number of sensitive organisms. The origin of these strains and methods for their propagation, spore production, and enumeration have been described previously (25).

Preparation of boticin-containing filtrate. Strain S5 was grown in a liquid medium containing 5.0% Trypticase, 4.0% proteose peptone, 2.0% yeast extract, 1.0% dextrose, and 0.1% sodium thioglycolate, at *p*H 7.0. Tubes containing 40 ml of this medium were heated to 100 C for 15 min, cooled, and inoculated with 0.1 ml of a fresh culture of strain S5. After incubation at 37 C for 18 to 20 hr, the cells were removed by centrifugation for 20 min at 3,000 × *g* in the cold and filtration through a membrane filter with a 0.45-μm pore size. The cell-free filtrate, which served as a crude source of the two boticins, was frozen and held at −20 C until needed.

Test for bacteriophage. Inhibitory preparations were examined for bacteriophage by testing their ability to reproduce in sensitive strains by the procedure described by Paterson (32).

Boticin assay. A spot test was used as a screening procedure for the location of fractions with boticin activity. A sensitive lawn was prepared by spreading 0.1 ml of a suspension containing 10^8 spores of strain 070 per ml on the surface of a Liver Veal Agar (Difco) plate. Spots of undiluted test material, 5 to 7 mm in diameter, were applied to the sensitive lawn, and the plates were incubated anaerobically for 16 to 18 hr at 37 C. The size of clear zones observed was a rough indicator of sample potency.

Quantitative data were obtained by using an assay that involved dilution to extinction of active preparations. Treated and control specimens were diluted serially in Trypticase-peptone-glucose (TPG) broth by using 10-fold and then 2-fold dilution steps. The diluted material was heated at 100 C for 15 min to insure anaerobiosis and to minimize contamination from the nonsterile sample being tested. Each 9.0-ml tube was inoculated with approximately 100 spores of strain 070. After incubation at 37 C for 16 to 18 hr, the tubes were examined and the highest dilution with complete inhibition of growth was noted. The reciprocal of this dilution was recorded as the titer of the sample. The activity of a treated specimen was always compared to that of the untreated control in assessing the effect of the treatment. The assay was reproducible to plus or minus one twofold dilution.

Specific activity. Absorbancy at 280 nm was measured with a Hitachi-Perkin Elmer model 139 spectrophotometer by using appropriate dilutions whenever necessary. Specific activity was defined as boticin titer/absorbancy at 280 nm.

Adsorption of boticins. Adsorption of boticin activity to various solid media was demonstrated after exposure of the test material to active preparations for 10 to 15 min and separation of solid and supernatant fractions. Adsorption was indicated by either a decline in potency of the supernatant fluid or an acquired growth-inhibitory capacity of the solid. Detection of the latter involved immersion of thoroughly washed boticin-treated and control specimens in molten Liver Veal Agar seeded with 10^6 spores of strain 070 per ml and anaerobic incubation for 20 hr at 26 C.

Gel filtration. A column (2.5 by 75 cm) of Sepharose 2B (Pharmacia Fine Chemicals, Inc.), prepared in 0.05 M ammonium acetate buffer at *p*H 5.0 and operated at 4 C with a constant head pressure of 45 cm of buffer, was used to estimate the size of the large boticin.

Gel filtration on Sephadex G-50 (Pharmacia) was used as the initial step for purification of the two boticins. A column (10 by 93 cm), prepared in 0.05 M ammonium acetate buffer at *p*H 5.0, was used for fractionation of up to 1,200 ml of crude filtrate at a time. The column was operated at 4 C with a flow rate between 400 and 500 ml/hr.

Gel filtration on Sephadex G-25 was employed for desalting and buffer exchange with small boticin preparations. A column (5 by 90 cm), equilibrated with an appropriate buffer, was used to process up to 400 ml at a time.

Concentration of active fractions. Fractions containing the small boticin were concentrated in a rotary evaporator at 50 C. The large boticin was sedimented by centrifugation for 2 hr at 35,000 × *g* with an International A-57 rotor.

Density gradient ultracentrifugation. The pellet obtained by sedimentation of the large boticin was suspended in 0.0003 M phosphate buffer at *p*H 7.2, and the preparation was clarified by low-speed centrifugation. Cesium chloride was added at a concentration of 0.4 g/ml, providing an initial density of approximately 1.31 g per cm^3; a gradient was formed by centrifugation for 30 hr at 41,000 rev/min in a swinging bucket rotor (International SB283). Fractions of approximately 0.7 ml were collected through a hole in the bottom of the centrifuge tube. Refractive index was measured at 25 C with a Bausch & Lomb refractometer; density was derived from the table of Anderson and Anderson (1). Cesium chloride was removed by dialysis in the cold against 0.0003 M phosphate buffer, *p*H 7.2.

Continuous curtain electrophoresis. The electrophoresis cell was constructed in our machine shop; the design was essentially that of Peeters et al. (33), with the trickle feeding system replaced by a small upper curtain, which dipped into an electrolyte reservoir maintained at a constant level. The electrolyte reservoir and cascade electrodes were fed by three separate veins of a peristaltic pump. Precut curtains (S & S type 470) designed for use in a Beckman model CP cell and a Beckman Constat power supply were used.

Fractionation of small boticin preparations was carried out at 4 C with 0.05 M ammonium acetate buffer, *p*H 3.4, as electrolyte. The cell was carefully leveled before beginning constant voltage operation at a potential of 900 v. A steady state was reached during the 3-hr equilibration period that preceded sample application; the current flow varied only slightly over extended runs from the initial 26 to 30 ma. Sample was fed onto a tab cut from the left side of the curtain at a rate of 8 ml/hr, and fractions were collected from each of 32 points on the bottom of the curtain.

Ion-exchange chromatography. A column (5 by 72 cm) of sulfoethyl-Sephadex C-50 (Pharmacia) was prepared in 0.025 M ammonium acetate buffer at *p*H 5.5. A 100-ml sample was washed into the column with 200 ml of *p*H 5.5 buffer; it was then eluted with a concave gradient of NaCl in *p*H 5.5 buffer. The gradient was produced as described b y Lakshmanan and Lieberman (27) with the exception that rates of flow in and out of the mixing chamber were regulated by pump. The gradient was defined by the following: initial volume of *p*H 5.5 buffer in mixing chamber, $V_0 = 6$ liters; concentration of NaCl added to mixing chamber, $C_0 = 4.0$ M in *p*H 5.5 buffer; inflow rate of NaCl solution into mixing chamber, $R_1 = 0.30$ ml/min; outflow rate from mixing chamber, $R_2 = 2.35$ ml/min.

Some properties of purified boticins. The stability of the small boticin was studied over a *p*H range of 1.1 to 12.4. Hydrochloric acid-potassium chloride buffer was used over the range of *p*H 1.1 to 3.1, universal buffer (5) over the range of *p*H 3.0 to 11.6, and 0.1 to 0.2 M NaOH in the alkaline extreme. Samples of purified boticin were diluted 1:5 with each buffer solution, and the final *p*H was measured. All mixtures were held at room temperature for 4 hr, adjusted to *p*H 7.0, made up to a uniform volume with *p*H 7.0 universal buffer, and assayed for activity.

Purified boticins were heated in 0.05 M phosphate buffer at *p*H 7.0 to assess heat stability.

Crystalline trypsin and chymotrypsin were obtained from Worthington Biochemical Corp.; pepsin and deoxyribonuclease were supplied by Calbiochem. Samples of purified boticins were incubated at 37 C for 20 min with the following: trypsin or chymotrypsin (20 μg/ml) in 0.05 M phosphate buffer, *p*H 7.0; pepsin (20 μg/ml) in 0.05 M ammonium acetate buffer, *p*H 4.0; or deoxyribonuclease (50 μg/ml) in *p*H 7.0 phosphate buffer containing 0.001 M $MgCl_2$. Enzyme action was terminated by boiling for 10 min. Controls received identical treatment except for omission of enzymes.

Crystalline urea was added to purified large boticin to achieve a concentration of 6 M urea, and the preparation was held at 4 C for 18 hr. One portion was assayed for activity; a second was dialyzed in the cold for 16 hr against *p*H 7.0 phosphate buffer, treated with deoxyribonuclease, as described above, and assayed for activity. A third portion was examined for dissociation into active units of smaller size by gel filtration on Sepharose 2B, as described previously.

Activity on vegetative cells. The effect of the two boticins on vegetative cells of strain 070 was examined by using TPG cultures in the early stages of exponential growth (22 to 24 hr at 26 C). The clearing of strain 070 cultures was followed at 525 nm with a Coleman model 8 colorimeter; cultures were observed over a period of 6 hr after the addition of either purified boticin (titer approximately 2,000). The killing action of the small boticin was explored further by estimating viable cell numbers at various time intervals after addition of purified boticin (titer 800) and by attempting to rescue "boticin-killed" cells with trypsin. Duplicate portions of a boticin-treated cell suspension were either diluted out immediately or incubated for 2 min at 37 C with trypsin (100 μg/ml). The action of trypsin was terminated by addition of soybean trypsin inhibitor (125 μg/ml); the complete inactivation of boticin was confirmed by analysis of the culture fluid. Tenfold serial dilutions in TPG were prepared from both samples of the cell suspension; the reciprocal of the highest dilution with growth, after 16 hr of incubation at 37 C, was used as an approximation of the number of viable cells/ml. A control cell suspension, which was not treated with boticin, was carried through all other manipulations.

RESULTS

Absence of bacteriophage activity. Filtrates of strain S5, prepared as described, contained no bacteriophage activity, nor was such activity induced by addition of 0.1 to 5.0 μg/ml of mitomycin C.

Boticin production. Unlike many bacteriocin producers, strain S5 elaborates boticin in quantity without induction. The amount of boticin produced is dependent upon the composition of the growth medium. Cultures grown in TPG or TPG supplemented with 1% yeast extract (TPGY) yielded filtrates of relatively low potency (<5,000); the medium described previously for boticin production consistently yielded filtrates with titers between 16,000 and 64,000. Irradiation with ultraviolet light or addition of up to 5.0 μg/ml of mitomycin C to TPG or TPGY cultures in early exponential growth phase did not increase filtrate potency.

Complete lysis of strain S5 cultures was never observed, although sudden fluctuations in culture turbidity suggested that partial lysis was occurring. Boticin was first detected in cultures near the middle of the exponential growth phase. The highest titers were observed in early stationary phase cultures; further incubation resulted in a rapid decline in potency.

Instability in crude filtrate. An enzyme elaborated by the organism was responsible for the decline in potency with further incubation of strain S5 cultures as well as for the instability of crude cell-free filtrate at room temperature. The loss of boticin activity from crude filtrate was *p*H- and temperature-dependent with greatest losses occurring around *p*H 8.0 and 40 C. When heat-

stabilized (10 min at 100 C) and unheated filtrates were combined in various proportions and incubated at 37 C, the rate of boticin inactivation was proportional to the quantity of unheated filtrate in the mixture.

Boticin size and distribution. The presence of two types of boticin activity in crude filtrate was revealed by filtration on Sephadex molecular sieves. Most of the activity was associated with a substance in the 5,000 to 30,000 molecular weight range, since it was excluded by Sephadex G-25 but not by Sephadex G-50. Ten to 20% of the total activity coincided with a particle with apparent particle weight in excess of 40×10^6, since it was excluded by Sepharose 2B.

Adsorption. Both boticins were strongly adsorbed on suitably changed surfaces. The large boticin was adsorbed on anion exchangers; the small boticin was adsorbed on filter paper, glass, plastics, and cation exchangers. In many cases, the adsorption was reversible only under extreme conditions of *p*H.

Purification. The two boticins together represent less than 1% of the ultraviolet-absorbing material in filtrates of strain S5. Isolation of either in quantity necessitates the processing of large volumes of filtrate; the procedures outlined in Fig. 1 were designed with this in mind.

The two boticins were separated from each other and partially purified by gel filtration on Sephadex G-50 (Fig. 2). The large boticin was excluded by the gel and emerged at the column void volume; the small boticin was eluted from the column just ahead of the bulk of peptide material from the growth medium. The two fractions were pooled as indicated in Fig. 2 and subjected to further purification.

The sedimentable portion of the material excluded by Sephadex G-50 was fractionated by isopycnic banding in a CsCl gradient (Fig. 3). The peak of boticin activity coincided with a heavy visible band with a density of 1.32 g/cm³. The pellet and a small band with a density of 1.22 g/cm³ contained no greater activity than adjacent fractions.

Further purification of the small boticin by continuous curtain electrophoresis at *p*H 3.4 is illustrated in Fig. 4. The boticin was adsorbed to the filter paper initially, and no activity emerged from the bottom of the curtain until about 8 hr after the separation was begun. Under the condi-

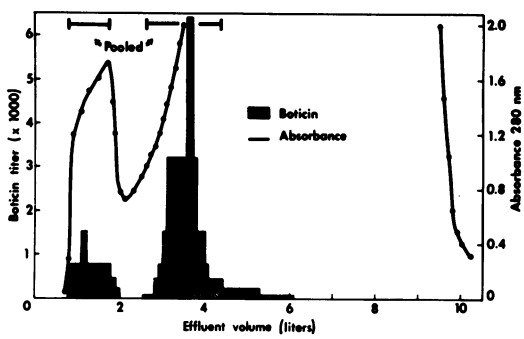

FIG. 2. *Separation and partial purification of two boticins by gel filtration on Sephadex G-50.*

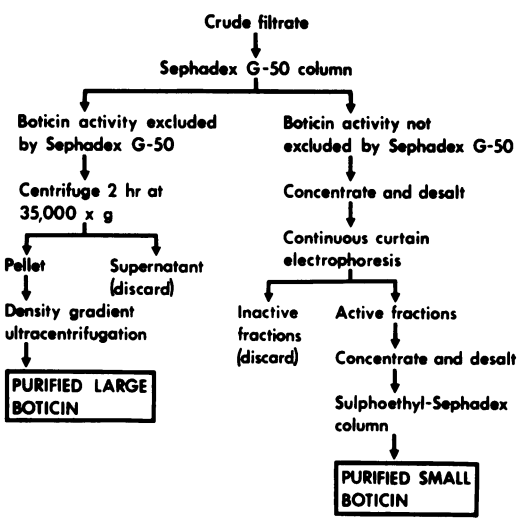

FIG. 1. *Schematic summary of procedures used in the isolation of two boticins from filtrate of strain S5.*

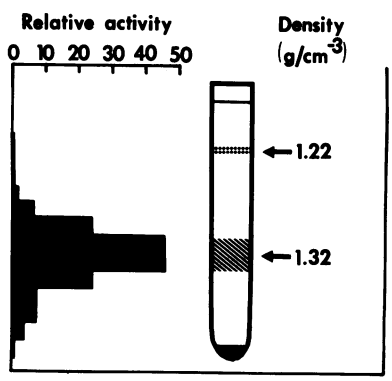

FIG. 3. *Purification of large boticin in a cesium chloride density gradient. Cesium chloride (0.4 g/ml) was added, and a density gradient was formed by centrifugation for 30 hr at 41,000 rev/min in an International SB283 rotor. The centrifuge tube, as it appeared after centrifugation, is represented schematically in the center. Boticin activity at various levels in the centrifuge tube is represented by the profile on the left; densities of bands observed are shown on the right.*

tions employed, the small boticin moved more strongly toward the cathode than did most of the ultraviolet-absorbing material.

The final step in the isolation of the small boticin, chromatography on sulfoethyl-Sephadex, is represented in Fig. 5. A small percentage of the total activity emerged with the two absorbancy peaks. Most of the small boticin remained on the column to be eluted as the NaCl concentration of the eluant reached approximately 0.3 M.

Table 1 summarizes the results obtained at each step in the purification of the two boticins.

pH stability. Purified small boticin was stable over a wide *p*H range. No loss was incurred over the range of *p*H 1.1 to 9.5, but the activity was diminished at *p*H 12.4 and above. The *p*H stability of the large boticin was not studied.

Effect of heat, enzyme, and urea treatments. The effect of these treatments on purified boticin preparations is shown in Table 2. In analyzing these data, it is important to keep in mind that the titration assay is reproducible to plus or minus one dilution. A difference of one dilution, which would appear to be a 50% loss of activity, is not significant.

The activity of purified small boticin was not significantly reduced by boiling for 10 min in *p*H 7 buffer; purified large boticin was slightly less stable under these conditions. Crude filtrate could be boiled 1 hr or autoclaved 30 min without detectable loss of activity. Although tolerance for heat was reduced by purification, significant quantities of both types of boticin activity survived autoclaving for 30 min.

The small boticin was inactivated almost completely by trypsin or chymotrypsin. Large boticin activity was reduced by treatment with trypsin or chymotrypsin, but it was less susceptible to these enzymes than was the small boticin. Pepsin was less effective than either trypsin or chymotrypsin in inactivating the small boticin; the large boticin

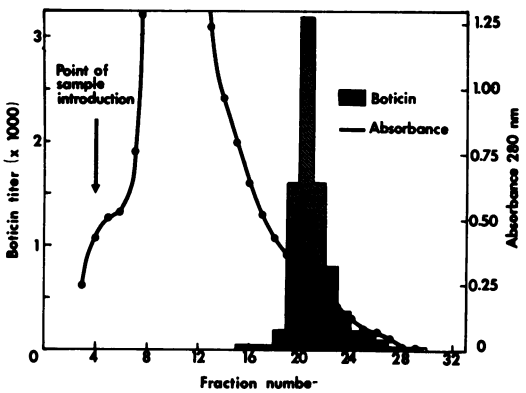

FIG. 4. *Fractionation of a preparation containing small boticin by continuous curtain electrophoresis.*

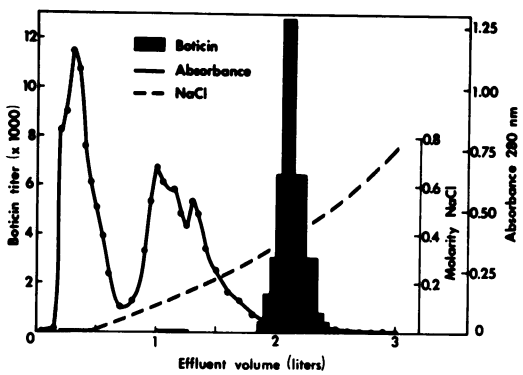

FIG. 5. *Further purification of small boticin by chromatography on sulfoethyl-Sephadex.*

TABLE 1. *Purification and recovery at various steps in the isolation of two boticins from filtrate of strain S5*

Step in purification	Specific activity[a]	Purification factor	Estimated recovery[b]
			%
Large boticin			
Crude filtrate	12[b]		
Gel filtration Sephadex G-50	587	49	88
Ultracentrifugation	2,352	196	62
Small boticin			
Crude filtrate	62[b]		
Gel filtration Sephadex G-50	903	15	91
Continuous curtain electrophoresis	9,243	149	70
Chromatography sulfoethyl-Sephadex	177,320	2,860	53

[a] Specific activity expressed as boticin titer per absorbancy at 280 nm.

[b] Large and small boticins represented approximately 15 and 85%, respectively, of the activity in this batch of filtrate. Specific activity of crude filtrate and recovery data were computed on the basis of these proportions.

TABLE 2. *Effect of heat, enzyme, and urea treatments on the activity of purified boticin preparations*

Treatment	Small boticin		Large boticin	
	Control	Treated	Control	Treated
	titer	*titer*	*titer*	*titer*
Boiled 10 min	32,000	16,000	12,800	3,200
Autoclaved 30 min	32,000	8,000	12,800	1,600
Trypsin	16,000	10	6,400	1,600
Chymotrypsin	16,000	10	6,400	1,600
Pepsin	3,200	200	6,400	6,400
Deoxyribonuclease			3,200	3,200
Urea (6 M)			6,400	3,200
Deoxyribonuclease following 6 M urea			1,600	1,600

was not attacked by this enzyme. Neither deoxyribonuclease, 6 M urea treatment, nor the combination of urea treatment followed by deoxyribonuclease caused a significant reduction in the activity of the large boticin preparation. Treatment with 6 M urea did not cause dissociation of the large boticin into active units of smaller size.

Activity on vegetative cells. In addition to their ability to prevent the outgrowth of type E spores, both boticins have bactericidal activity for vegetative cells of strain 070. Addition of either purified boticin (titer approximately 2,000) to exponentially growing cultures resulted in an abrupt cessation of growth and a gradual decrease in culture turbidity. Cultures were cleared completely within 6 hr after boticin addition.

Further study of the killing action of purified small boticin revealed that strain 070 cells were rendered nonviable long before the culture was cleared. Table 3 shows the effect of the small boticin (titer 800) on a suspension of exponential-phase cells. Within 9 min, a population of 10^7 cells/ml was reduced to fewer than 100 viable cells/ml. The effect of the small boticin was not reversed by trypsin even though the enzyme completely inactivated all boticin in the surrounding medium and, presumably, on the cell surface.

DISCUSSION

The elaboration of bacteriocins by strain S5 is apparently not inducible since filtrate potency is influenced by composition of the growth medium but not by the use of inducing agents. Although inadvertent induction cannot be ruled out, it seems likely that the enhanced titers obtained with the more complex growth medium are a consequence of the greater cell densities supported by this medium.

Cultures of strain S5 do not undergo mass lysis such as many bacteriocin producers undergo. But sudden fluctuations in culture turbidity suggest that the boticins are liberated by lysis of cells producing them.

Purification of small quantities of the boticins involves the processing of large volumes of crude filtrate. In addition to the problem of extracting very small quantities of the boticins from a complex filtrate, the job is complicated by the presence in crude filtrate of an enzyme that inactivates the boticins, and by adsorption of the small boticin to laboratory utensils. Large and small boticins can be purified 200- and 3,000-fold, respectively, with yields of 50% or more if care is exercised to minimize enzymatic and adsorption losses. Enzyme inactivation is minimized by working at 4 C, and *p*H 5.0 or below. Adsorption of the small boticin is minimized by limiting exposure to glass or plastic utensils and by avoiding equipment with sintered glass or glass wool supports.

The two types of boticin isolated from strain S5 filtrates fit the two categories of bacteriocins described by Bradley (3). The small boticin has a molecular weight under 30,000 and is nonsedimentable. It is sensitive to trypsin and chymotrypsin and, to a lesser extent, pepsin. The large boticin, with an apparent particle weight in excess of 40×10^6, is sedimented easily. Although the large boticin is partially inactivated by trypsin and chymotrypsin, it is less sensitive to

TABLE 3. *Effect of purified small boticin on vegetative cells of Clostridium botulinum type E strain 070*[a]

Length of treatment	No. of viable cells/ml		
	Boticin-treated[b]	Boticin-treated, trypsin added at end of boticin treatment[c]	Control (no boticin treatment), trypsin added[c]
min			
0	10^6	10^6	10^7
3	10^5	10^4	10^7
6	10^2	10^3	10^8
9	$<10^2$	$<10^2$	10^7
12	$<10^2$	$<10^2$	10^8

[a] Boticin titer 800.

[b] At the end of the time specified, the cell suspension was diluted out immediately for estimation of viable cell numbers.

[c] At the end of the time specified, the cell suspension was treated for 2 min with trypsin (100 μg/ml at 37 C, *p*H 7.0) to inactivate the boticin. Trypsin action was terminated by addition of soybean trypsin inhibitor (125 μg/ml), and the cells were diluted serially for estimation of viable cell numbers.

proteolytic enzymes than is the smaller variety. If 10 min at 100 C is used as the criterion, the small boticin is thermostable and the large boticin is slightly less stable.

The possibility that the small boticin represents a breakdown product of the large particle cannot be ruled out. However, the large boticin cannot be dissociated into smaller active units by urea treatment, and the two active entities carry strong but opposite charges.

Whether the large boticin is similar in structure to the many phagelike bacteriocins remains to be resolved. Treatment with urea does not diminish large boticin activity, whereas urea causes complete inactivation of pyocin C9 and structural alteration of pyocins C9 and R (13, 16). Urea causes partial inactivation of vibriocin, a deoxyribonucleic acid- and ribonucleic acid-containing bacteriocin produced by *Vibrio comma*, and urea pretreatment renders the nucleic acids of vibriocin susceptible to attack by nucleases, causing further reduction of this activity (8). The activity of the large boticin is not affected by deoxyribonuclease alone or deoxyribonuclease following urea pretreatment, indicating that deoxyribonucleic acid is not essential to its activity.

To our knowledge, the ability of the boticins to prevent the germination and outgrowth of spores of sensitive strains without affecting spore viability (25) is a characteristic that has not been reported for any other bacteriocin. Both boticins also have bactericidal activity for vegetative cells of strain 070. Addition of either purified boticin to exponential-phase cultures results in an immediate cessation of growth and gradual loss of turbidity. The lengthy time requirement for clearing sensitive cultures suggests that a gradual leakage of cell contents may be involved, rather than lysis. The boticins may be similar in action to megacin 216, which appears to disrupt the permeability barrier of sensitive cells, causing a gradual loss of cell contents until all cells are converted to empty ghosts (15, 19).

A concentration of purified small boticin equivalent to an 80-fold dilution of crude filtrate from strain S5 kills the cells in exponential-phase cultures of strain 070 in minutes. This indicates that the clearing of sensitive cultures is not the primary effect of this boticin, but rather a consequence of it. The killing action of the small boticin is not reversed by trypsin, even though trypsin completely inactivates this bacteriocin. The irreversible nature of the killing action suggests two possibilities. (i) The small boticin rapidly penetrates into the cell where it is protected from trypsin inactivation; or (ii) the small boticin acts very rapidly to produce some lesion, which is irreparable even after boticin is removed from the environment.

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