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Dependence of *Clostridium botulinum* Gas and Protease Production on Culture Conditions

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Reports that *Clostridium botulinum* toxin can sometimes be detected in the absence of indicators of overt spoilage led to a systematic study of this phenomenon in a model system. Media with various combinations of pH (5.0 to 7.0) and glucose (0.0 to 1.0%) were inoculated with vegetative cells of *C. botulinum* 62A and incubated anaerobically at 35°C. Although growth and toxin production occurred at all pH and glucose combinations, accumulation of gas was delayed or absent in media with low pH, low glucose levels, or both. Other proteolytic *C. botulinum* strains gave similar results. Trypsin activation was required to detect toxin in some low pH cultures. The trypsinization requirement correlated with low proteolytic activity in the cultures. Proteolytic activity of the strains examined was 5- to 500-fold lower in botulinal assay medium than in cooked meat medium. The results indicate that the absence of gas accumulation does not preclude the presence of botulinal toxin and that proteolytic cultures grown under adverse conditions may require trypsinization for the detection of toxin.

Although *Clostridium botulinum* produces a potent neurotoxin (16), the severity and frequency of botulism outbreaks are often limited by the overt spoilage of the product which discourages people from consuming toxin-containing food. Two manifestations of spoilage are swelling of cans due to gas production and product degradation by proteases. Production of gas from glucose and protease production are key clostridial traits. Proteolytic activity has been used to assign *C. botulinum* strains to different metabolic groups (21). Because proteolytic strains of *C. botulinum* are more heat resistant than nonproteolytic strains (J. P. P. M. Smelt, Ph.D. thesis, Rijkuniversiteit Utrecht, The Netherlands, 1980), proteolytic strains are often used to test the adequacy of heat treatments against *C. botulinum* spores.

The absence of overt spoilage, however, does not assure that a food is free of botulinal toxin (24). Toxin production has been observed in cured meats (9) before they become organoleptically objectionable. In reviewing studies on the minimum pH at which *C. botulinum* will grow, Ito and Chen (11) have suggested that, in addition to determining toxicity of samples at the minimum pH at which growth is obvious, the next lower pH should also be tested for toxin.

The purpose of this study was to systematically investigate conditions under which botulinal toxin could be formed in the absence of biochemical reaction(s) which usually indicate spoilage. The importance of trypsinizing samples from proteolytic strains for the detection of toxin is also presented.

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MATERIALS AND METHODS

Organism and culture conditions. *C. botulinum* strains 62A, CDC 17409, ATCC 25763 (all type A), 53B, B-aphis, and ATCC 7949 (all type B) were maintained as stock cultures in cooked meat medium (CMM) (Difco Laboratories, Detroit, Mich.). Cells of these proteolytic strains were prepared by inoculating botulinum assay medium (12) (BAM) containing 0.2% glucose with 0.1 ml of stock culture. After overnight incubation at 35°C, 0.1 ml was transferred to fresh BAM and incubated for 48 h. The cells were centrifuged at 8,740 × *g* for 90 s in a Microfuge B (Beckman Instruments, Inc., Fullerton, Calif.) and washed with an equal volume of 0.1% peptone water. After five washes, the cells were diluted 100-fold in peptone water. A 10-μl amount of this suspension was used to inoculate tubes containing 10 ml of BAM to a final inoculum of 10^4 vegetative cells per tube. The inoculation sequence was designed to dilute the toxin carryover in the inoculum by a factor of 10^6. A sample (10 μl) of the last wash was diluted with 10 ml of sterile water and tested for toxicity to ensure that no detectable botulinal toxin was carried into the test broth.

The effects of medium pH and glucose concentra-

TABLE 1. Growth, gas production, and toxicity of *C. botulinum* 62A in BAM with different combinations of pH and glucose concentration

pH	Glucose (%)	Growth	Gas[a]	Toxicity	
				Untrypsinized	Trypsinized
7.0	0	+	+	+	+
	0.5	+	+	+	+
	1.0	+	+	+	+
6.0	0	+	+, D	+	+
	1.0	+	+	+	+
5.5	0	+	V, D	+	+
	1.0	+	+	+	+
5.25	0	+	−	−	+
	0.5	+	D	−	+
	1.0	V	+[b]	−	+
5.0	0	+	−	−	+
	0.5	V	−	−	+
	1.0	V	−	−	+

[a] D, Gas production was delayed and was not concurrent with the appearance of turbidity. V, Variable response among triplicate tubes.

[b] Positive in tubes in which growth occurred.

tion were first examined by using *C. botulinum* 62A inoculated into BAM with 30 combinations of pH (5.00, 5.25, 5.50, 6.00, 6.50, and 7.00) and glucose (0.0, 0.1, 0.2, 0.5, and 1.0%). The initial pH of the media were adjusted with 1 N HCl before autoclaving at 121°C for 15 min. Additional studies with other proteolytic strains used BAM with pH 5.25 and 0.2% glucose. All experiments were done in triplicate in screw-capped test tubes which contained Durham tubes. Incubation was at 35°C for 7 days. All procedures except toxin assays were done in an anaerobic chamber (2).

Growth was determined by the appearance of visible turbidity. The relative amount of gas produced was estimated by measuring the linear displacement (in millimeters) of media by gas in the Durham tubes.

Detection of botulinal toxin. Culture supernatants were obtained by centrifugation at 8,740 × *g* for 90 s in a Microfuge B (Beckman). Some of the supernatants were adjusted to pH 7 and treated with Bacto-Trypsin (Difco) (1:250, at a final concentration of 0.1%) at 30°C for 1 h. Each sample was tested for botulinal toxin by injecting each of two 15- to 20-g Swiss white mice intraperitoneally with 0.4 ml of the putative toxin. The mice were observed for symptomatic botulinic death for 72 h. Samples were scored as toxic if symptomatic death of both mice occurred within 24 h. If symptomatic death was delayed or observed in only one mouse, the sample was scored as weakly toxic. Controls, which included portions of each sample boiled for 10 min, pentavalent antitoxin-neutralized portions of selected samples, appropriate dilutions of the last inoculum wash from each strain, and 0.1% trypsin, were also tested for toxicity to mice.

Determination of protease activity. Because *C. botulinum* cultures at low pH required trypsinization for the detection of toxin, proteolytic activity was also examined in some experiments. Protease activity was detected by hydrolysis of casein and gelatin in a dual-substrate plate diffusion assay (13) with *Bacillus polymyxa* protease (type IX; Sigma Chemical Co., St. Louis, Mo.) as a standard. Because some botulinal proteases require reducing conditions for activity (6, 25) and preliminary experiments demonstrated that cultures assayed anaerobically had significantly more activity than those assayed aerobically (data not shown), all assays were conducted in duplicate under anaerobic conditions. Hydrolysis zone sizes measured after 24 h of incubation at 35°C were proportional to the log of the applied protease activity.

RESULTS

Medium pH and glucose concentrations had marked effects on the growth, gas production, and toxicity of *C. botulinum* 62A. Gas accumulation in Durham tubes was concurrent with the appearance of growth in media containing glucose at pH ≥ 5.50 (Table 1). Gas accumulation in media containing glucose at lower pHs was delayed up to 6 days after the appearance of turbidity and did not occur at pH 5.00. In the absence of exogenous glucose, gas production was delayed in all media with pH < 7. No gas was detected at pH 5.25 and 5.00. At pH 5.50, gas accumulation was variable.

The relative quantity of gas produced was strongly affected by both the pH and glucose concentration of the media (Fig. 1). The addition of only 0.1% glucose to the media resulted in measurable amounts of gas production at all pH ≥ 5.25. Maximum gas production was at pH 6.5. Increasing the glucose concentration at low pH

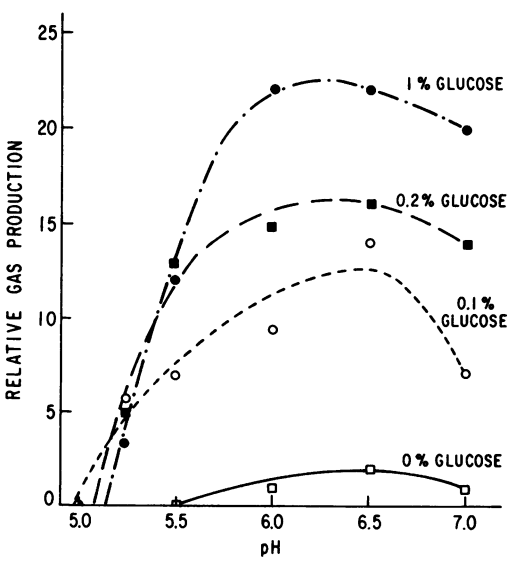

FIG. 1. Relative amount of gas produced by *C. botulinum* 62A when cultured for 7 days at 35°C in BAM with different combinations of pH and glucose concentration.

TABLE 2. Growth, gas production, toxicity, and proteolytic activity of *C. botulinum* strains in BAM with 0.2% glucose and pH 5.25 after 7 days at 35°C

Strain	Sample no.	Growth	Gas	Proteolytic activity (IU/ml)	Toxicity	
					Untrypsinized	Trypsinized
62A	1	+	−	0.01	−	+
	2	+	−	0.01	−	+
	3	+	−	<.01	+	+
B-aphis	4	−	−	<.01	−	−
	5	+	−	<.01	−	+
	6	+	−	<.01	−	+
CDC 17409	7	+	−	<.01	−	−
	8	+	−	<.01	−	−
	9	+	−	<.01	−	−
53B	10	+	−	.02	W[a]	+
	11	+	−	.01	W	+
	12	+	−	<.01	W	+
ATCC 25763	13	+	+	<.01	+	+
	14	+	+	0.05	+	+
	15	+	+	0.04	+	+
ATCC 7949	16	−	−	<.01	−	−
	17	+	+	0.26	+	+
	18	+	+	0.31	+	+

[a] W, Weak toxicity.

often caused variable growth, probably due to further lowering of the pH by the acids produced from the glucose. In most cases, toxin from *C. botulinum* cultured at pH 5.0 and 5.25 could be detected only if the samples had been trypsinized.

Other proteolytic *C. botulinum* strains gave similar results in BAM at pH 5.25 and 0.2% glucose (Table 2). Strains 62A, B-aphis, CDC 17409, and 53B all exhibited dense growth without gas production. Trypsinization was required to detect the toxin in two of the strains. Trypsinization increased toxicity in one strain and had no effect on the toxicity of three strains. Examination of the proteolytic activities of the cultures showed a correlation between low-protease activities and the lack of toxicity in untrypsinized samples. Usually, samples with proteolytic activities ≤0.01 required trypsinization for the detection of toxin; those with higher proteolytic activities did not.

None of the controls elicited a toxic response in the mouse bioassay. The absence of toxicity in samples which did not exhibit growth (Table 2, samples 4 and 16) also confirms that the toxicity of positive samples was not due to toxin carried over with the inocula.

Since both gas production and proteolytic activity were attenuated in BAM at low pH, these parameters were also examined in BAM at pH 6.80 and in CMM. Growth and gas production were copious in both media (Table 3). In agreement with the observation in Table 2, samples with proteolytic activities >0.01 did not require trypsinization for the detection of toxin, although in some weakly proteolytic cultures, increased toxicity was observed. Cultures in CMM had protease activities that were 5 to 500 times greater than those of strains cultured in BAM at a similar pH. Under the assay condition used, 0.1% trypsin has an activity of 3.1 IU/ml, roughly comparable to the protease levels in CMM.

DISCUSSION

C. botulinum ferments glucose to ethanol and carbon dioxide (5) via the Embden-Meyerhof-Parnas pathway (19). The relationship between gas formation and glucose concentration (Fig. 1) was indicative of carbon dioxide production due to glucose catabolism. Gas accumulation observed in the absence of added glucose may have been low levels of ammonia and carbon dioxide produced by the Strickland degradation of amino acids or carbon dioxide produced from trace amounts of fermentable carbohydrates in the basal medium.

Growth and toxin production were consistently observed in the absence of gas production when strains 62A, B-aphis, CDC 17409, and 53B were cultured at low pH in the presence of exogenous glucose. A variety of conditions ap-

TABLE 3. Growth, gas production, toxicity, and proteolytic activity of *C. botulinum* strains in BAM (0.2% glucose and pH 6.8) and CMM after 7 days at 35°C

Strain	Medium	Growth	Gas	Proteolytic activity (IU/ml)	Toxicity	
					Untrypsinized	Trypsinized
62A	BAM	+	+	0.04	+	+
	CMM	+	+	1.03	+	NT[a]
B-aphis	BAM	+	+	0.09	+	+
	CMM	+	+	1.53	+	NT
CDC 17409	BAM	+	+	0.03	W[b]	–
	CMM	+	+	2.27	+	NT
53B	BAM	+	+	0.09	+	+
	CMM	+	+	0.47	+	NT
ATCC 25763	BAM	+	+	0.03	W	+
	CMM	+	+	16.33	+	NT
ATCC 7949	BAM	+	+	0.04	W	+
	CMM	+	+	1.03	+	NT

[a] NT, Not tested.
[b] W, Weak toxicity.

pear to support toxin production in the absence of gas accumulation. Toxin in the absence of gas has been observed in cured meat (22), vacuum-packed potatoes incubated at suboptimal temperatures (14), acidified foods (31), and in processes that utilize radappertization and high brine concentrations to inhibit botulinal growth (1, 24).

This report appears to contradict the belief that exogenous carbohydrates are required for the production of botulinal toxin. Examination of the literature (3, 4, 17, 18) indicates that detectable levels of toxin were produced, but carbohydrate addition caused at least a 10-fold (4) and as much as a 1,000-fold (17, 18) increase in the level of toxin.

Types A and B botulinal toxin are synthesized as prototoxins which are subsequently activated by their own proteases (4). Although both trypsin and clostridial proteases cleave botulinal toxin at an arginine site (6, 7), Tjaberg (28) reported that trypsin was a better activator of type A and B toxins than endogenous proteases. Toxin activation is important for the detection of toxin administered intraperitoneally in the mouse bioassay. Unactivated toxin not detected by the bioassay would be toxic to humans due to activation by gut proteases. Activation of a proteolytic type A strain by trypsin has been observed only during the early stages of growth (10). Presumably, the endogenous proteases had not had time to act or were not yet synthesized. This is the first report which demonstrates that mature cultures of both proteolytic type A and B strains may require trypsinization for the detection of toxin. Because proteases from different *C. botulinum* strains behave similarly when exposed to inhibitors (29) and are serologically related (30), the results obtained in this report with six strains may extend to other proteolytic strains.

The reduced level of endogenous proteolytic activity observed at low pH (Table 2) is consistent with current knowledge of clostridial proteases. Botulinal protease activity declines dramatically with decreasing pH (6, 8, 27). Furthermore, the protease produced by type B *C. botulinum* is irreversibly inactivated at pH 4.5 (26). Because the samples in this study were assayed at pH 7, the low activity observed must be attributed to the inhibition of protease synthesis or protease inactivation at low pH or both.

The marked effect of growth media on protease activity (Table 3) was unexpected. Although different media have been reported to influence protease levels (26), the dramatic differences observed between BAM and CMM suggest that the proteases are under some mode of negative metabolic control. The high concentrations of amino acids and peptides in BAM may cause enzyme repression or inhibition. However, the cultures showed little activity when assayed in the absence of high free amino acid levels. This suggests that only low levels of protease were synthesized in BAM.

These results may shed light on the mechanisms by which *C. botulinum* can grow at pH < 4.6. Smelt and co-workers have theorized that metabiosis with other organisms plays an impor-

tant role in this process (15, 20), whereas Tanaka (23) has postulated the existence of microenvironments with permissive pH. Neither group reported a trypsinization requirement to detect the toxin produced at low pH. Since, in a homogeneous system, clostridial proteases would be inactive at these low pHs, a microenvironment must exist for them to be active.

In summary, this paper demonstrates that gas production is an unreliable indicator of growth and toxin production by *C. botulinum* and that endogenous proteases are not always sufficient to activate botulinal toxin. Samples being tested for botulinal toxin should be assayed for proteolytic activity and trypsinized if only low levels are found.

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