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Effective levels of tetanus toxin can be made in a production medium totally lacking both animal (e.g., brain heart infusion) and dairy proteins or digests (e.g., casein hydrolysates)

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Abstract

We have developed a fermentation medium for *Clostridium tetani* that results in the formation of tetanus toxin and contains no meat (e.g., beef heart infusion) or dairy (e.g., casein digest) products, thus obviating the problem of possible prion diseases. Particular preparations of hydrolyzed soy proteins, especially Quest Hy-Soy®, have been found to replace both the meat extract and casein digest components of traditional tetanus toxin production media and to yield even higher toxin titers. The comparison of the traditional versus the new medium has been carried out repeatedly by us and the superiority of our medium has been consistently observed. To our knowledge, this is the first time that such a medium has been devised.

Keywords: Tetanus toxin; *Clostridium tetani*; Fermentation medium

1. Introduction

Tetanus is a life-threatening disease caused by infection with *Clostridium tetani* [9]. This ubiquitous (cells and spores are present in feces of domestic animals and humans, soil, house and operating theatre dust, fresh and salt water) anaerobic microbe causes disease by release of the potent, heat labile tetanus neurotoxin (tetanospasmin), a 150-kDa peptide neurotoxin that is hydrolyzed by *C. tetani* peptidases into two peptides (107 and 53 kDa) upon release from the cell. The peptides enter the nerve cells of the host and block release of neurotransmitters for inhibitory synapses. This blockage causes unregulated excitatory synaptic activity of certain host neurons, resulting in spasmic paralysis (uncontrollable muscle contraction and paralysis of facial and back muscles). As of 1989, death resulted in 10–40% of non-immunized hosts. The lethal human dose of tetanus toxin is only 2.5 ng/kg. For over 50 years, vaccination with tetanus toxoid has been practiced to prevent the disease. Toxin is produced and inactivated, usually with formalin, to produce the toxoid. Immunization is completely effective and thus tetanus is rare in the West. However, it is prevalent in the underdeveloped world where vaccination rates can be as low as 20% and/or medical practice is lax [10].

Toxin has been traditionally prepared by growth of *C. tetani* in media containing animal and dairy products (e.g., meat extracts plus casein digests) as sources of proteins, peptides and amino acids needed for good growth. When toxoid is made, it is often contaminated with formalin adducts of animal proteins. Thus, it is possible that some toxoid preparations contain undesirable contaminants such as the prion causing bovine spongiform encephalopathy (BSE; Mad Cow's Disease) or antigenic peptides that stimulate anaphy-

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lactic reactions and other undesirable immune reactions in immunized hosts [11].

The growth medium traditionally used to produce tetanus toxin is MM [2], which contains glucose, beef heart infusion (BHI), a pancreatic digest of casein (e.g., N-Z-Case® or N-Z-Case TT®, Quest Sheffield, Chicago, USA), some amino acids and vitamins, uracil and inorganic salts. BHI has caused problems due to the carryover risk mentioned above [6] and inhibitory materials present [2]. Problems have also been experienced with the casein digests [2,7]. Mueller and Miller [3] isolated three types of acid-labile components from casein digests by resin treatment, all of which were necessary for good toxin production; however, they were not chemically identified. More recently, Porfirio et al. [4] isolated and identified eight peptides, four to eight amino acids in length, which enhance toxin formation. Of course, synthesis of such peptides and their inclusion in the medium would be too expensive for practical use.

As a result of the above problems, there is a need for development of an improved medium lacking both animal and dairy products. The present work addresses this need and describes for the first time a production medium totally lacking brain heart infusion and casein digest which supports production of increased levels of tetanus toxin.

2. Materials and methods

2.1. Culture

The organism used was *C. tetani* from Wyeth-Lederle Vaccine and Pediatrics (Pearl River, NY). Their preparation 3-ABI-13, prepared with cow's milk, was received as a lyophilized culture. After receipt, we stored it before use at 4 °C. When used, it was suspended in 1 ml of step-1 seed medium. All media employed doubly-distilled water.

2.2. Seed preparation

The seed medium contained 100 ml of BHI, 1.0 g glucose, 1.0 g Bacto-peptone (Difco), 0.5 g NaCl and was adjusted to pH 8.1. Ten milliliters of seed medium were added to a step-1 seed tube (16 × 150 mm), which was autoclaved at 121 °C for 25 min. A step-1 seed tube was inoculated with 0.5 ml of the lyophilized culture and incubated at 34 ± 1 °C for 24 h in a Coy Anaerobic Chamber (CoyLab Prods Inc., Grass Lake, MI). A step-2 seed flask was prepared by adding 40 ml seed medium to a 2510-DeLong Bellco culture flask (125 ml capacity) and autoclaving. One milliliter of step-1 seed culture was inoculated into the step-2 seed flask, which was incubated at 34 ± 1 °C for 24 h in the anaerobic chamber.

2.3. Growth and production

Control fermentation medium contained per liter 7.5 g glucose, 250 ml BHI, 15 g N-Z-Case TT®, 125 mg L-cysteine, 125 mg L-tyrosine, 0.5 g powdered iron, 5.0 g NaCl, 0.5 g Na_2HPO_4, 50 mg $MgSO_4{\cdot}7H_20$, 175 mg KH_2PO_4 and was adjusted to pH 6.8. The N-Z-Case TT® was added last to the medium, solubilized with 3N HCl and the final pH was adjusted to 6.8 with 5N NaOH. The medium was dispensed at 8 ml per each 16 × 100 mm fermentation tube. Tubes were autoclaved, inoculated in triplicate with 40 μl step-2 seed, and incubated in the anaerobic chamber at 34 ± 1 °C. A fourth tube was uninoculated and served to "zero in" the Turner Spectrophotometer (model 330) for growth measurements at 660 nm every 24 h. The experiments were continued until 48 h after cell lysis was observed.

2.4. Toxin assay

The level of tetanus toxin was measured by adding a standard antitoxin, and measuring the elapsed time before flocculation [5]. Both Kf (the time in minutes required for flocculation to occur) and Lf (the limit of flocculation; equivalent to 1 international unit of standard antitoxin, as established by flocculation) were determined as follows. From each of triplicate fermentation tubes 48 h after cell lysis had occurred, 4 ml were removed and combined in a 15 ml centrifuge tube. Tubes were centrifuged at 3400 × *g* for 30 min at 4 °C. One milliliter aliquots of the supernatant fluid were added to tubes containing 0.1–0.6 ml of standard tetanus antiserum and the tubes were carefully shaken to mix their contents. The tubes were placed in a water bath at 45 °C and the time recorded. Tubes were checked frequently and the time at which flocculation began was recorded as Kf. The concentration of toxin in the tube in which flocculation was first initiated was designated LfFF. The concentration of toxin in the tube in which flocculation was initiated second was designated LfF. Calculation was done as follows:

when LfFF > LfF: Lftoxin/ml = LfFF − (LfFF − LfF)/4
when LfFF < LfF: Lftoxin/ml = LfFF + (LfF − LfFF)/4.

3. Results

3.1. Requirement of BHI and N-Z-Case TT® for growth

Medium was prepared without BHI and without N-Z-Case TT® to determine their need for growth of *C. tetani*. Whereas growth in the control medium reached a maximum OD of 0.81 and 0.85 at 3 d, it only reached 0.14 without BHI and 0.06 in the absence of N-Z-Case TT®. Thus, these complex components are required for good growth.

3.2. Ability of soy products to replace BHI for growth

Seven soy products were examined for their ability to replace BHI for growth. All media in this experiment contained N-Z-Case TT®. The control medium reached a maximum OD of 0.92 at 3 d. The maximum values reached with

the soy products were as follows: Quest Hy-Soy® (Quest Sheffield, Chicago, USA) 1.0, Difco Bacto-Soytone (Difco, BD, Franklin Lakes, NJ, USA) 0.91, Gibco Soy Peptone (Gibco, Invitrogen, Carlsbad, CA, USA) 0.90, Quest Hy-Soy T® 0.71, Soybean meal 0.62, ADM Bake NutriSoy® (ADM, Decatur, Il, USA) 0.58, and NutriSoy® flour 0.51. The first three products were soluble and the last four were insoluble. The results thus showed that the growth-supporting property of BHI can be replaced by soy products and that soluble soy preparations are better than the insoluble products. In a subsequent experiment, seven additional soy products were tested and found to replace BHI. These were Quest Hy-Soy®, Quest Amisoy®, Quest NZ-Soy, Quest NZ-Soy BL4®, Quest NZ Soy-BL7®, DMV SE 50 M (DMV International, Veghel, The Netherlands) and DMV SE 50 MK. Media containing these products supported growth to ODs of 0.59–0.97, the control reaching 0.81. The best were DMV SE 50 MK, Quest Hy-Soy® and Quest NZ-Soy.

3.3. Ability of soy products to replace BHI for toxin production

Eight of the soy products examined above and found to support excellent growth were compared to BHI for ability to replace it for toxin production. All media in this experiment contained N-Z-Case TT®. The results with three active peptones (Quest Hy-Soy®, Quest NZ-Soy, DMV SE 50 MK) are shown in Table 1. The Quest Hy-Soy® performed best and yielded higher toxin levels than the control medium. Five other peptones (Quest NZ-Soy BL4®, Quest NZ-Soy BL7, DMV SE 50 M, Gibco Soy Peptone and Difco Bac-Soytone) supported excellent growth but toxin formation was not observed.

3.4. Removal of N-Z-Case TT® from the medium

We had already seen that removal of N-Z-Case TT® from the control BHI-containing medium totally inhibited growth. However, when soy products were present as BHI replacements, growth did take place. Growth was sub-optimal however and, except in one case (DMV SE 50 MK), toxin production was not measurable. Thus, we searched for other replacements for N-Z-Case TT®. At 15 g/l, neither Traders Protein (Traders Protein, Memphis, TN, USA) Proflo (a yellow flour made from cottonseed) nor corn steep liquor could support growth without N-Z-Case TT®, even in the presence of BHI. Various yeast extracts were tested at 7.5, 15, 30 and 45 g/l in the presence of Quest Hy-Soy®. These included Difco Yeast Extract, Gibco Yeast Extract, and Quest products HyYest 412®, HyYest 441®, HyYest 444® and HyYest 455®. In general, they stimulated growth but interfered in toxin formation. Difco Malt Extract slightly inhibited growth and totally inhibited toxin formation.

We next compared different concentrations of Quest Hy-Soy® versus the traditional combination of BHI and N-Z-Case TT® for growth and toxin formation (Table 2). As can be seen, growth was equivalent but toxin production was higher in the soy-based medium. The optimum concentration of Quest Hy-Soy® was 34 g/l.

Table 1
Ability of soy peptones to replace BHI for growth and toxin production

Additive (g/l)	Maximum	
	Growth (OD)	Toxin (Lf/ml)
BHI (22.8) (control)	0.71	27.5
(34.1)	0.97	32.5
Quest Hy-Soy® (22.8)	0.94	52.5
(34.1)	1.02	37.5
Quest NZ-Soy (22.8)	0.75	17.5
(34.1)	0.87	27.5
DMV SE 50 MK (22.8)	0.93	37.5
(34.1)	1.10	Inactive

All flasks contained 15 g N-Z-Case TT® per liter.

Table 2
Comparison of the traditional BHI and N-Z-Case TT medium with that containing Quest Hy-Soy®[a]

BHI	N-Z-Case TT	Soy product	Maximum	
			Growth (OD)	Toxin(Lf/ml)
+	+ (control)	–	0.80	27.5
–	+	Quest Hy-Soy® (22.8 g/l)	0.83	37.5
–	–	Quest Hy-Soy® (34.1 g/l)	0.75	42.5
–	–	Quest Hy-Soy® (45.5 g/l)	0.85	27.5
–	–	Quest Hy-Soy® (56.9 g/l)	1.03	27.5

[a] When added, BHI was added at 22.8 g/l and N-Z-Case TT at 15 g/l.

4. Discussion

For many years, the media used to grow *C. tetani* and to produce tetanus toxin contained complex additives such as BHI and casein digests. Attempts to remove animal protein were made by Stone [8] by adapting the culture to lower and lower concentrations of veal infusion, but toxin titers were low. Furthermore, when beef heart or veal infusion was omitted, performance became dependent on the quality of the casein digests. However, many problems were experienced with the quality of casein digests [7]. For example, work on an improved medium lacking animal protein [1] revealed that only three out of six batches of N-Z-Case® gave satisfactory production, whereas two were low and one was very poor.

With the aim of removal of potential dangerous animal and dairy products from vaccines, we examined the nutrition of the bacterium. We found that in a traditional medium, removal of either the beef preparation or the casein preparation eliminated growth. It was indeed surprising to find that both of these undesirable additives could be replaced by a single soy product. Indeed, a suitable concentration

of Quest Hy-Soy® not only supported growth equivalent to that of the traditional type of medium but also provided higher toxin titers. We have repeated these experiments many times and have observed improved production repeatedly. Other useful soy products were DMV SE 50 MK and Quest NZ-Soy.

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