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The use of Soy hydrolysate for tetanus toxin production

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SUMMARY

The present work was planned to develop a modified system for the growth of *C. tetani* and production of tetanus toxin, the system includes growth media that contain no meat or dairy by-products using non-animal based products. Hydrolyzed soy proteins have been found to replace the meat/dairy product for growth of *C. tetani* and the production of its toxin, these replacements resulted in production of high tetanus toxin titer and of low side effects.

INTRODUCTION

For over 70 years, bacterial strains of the genus *Clostridium* have been used in the manufacture of veterinary and human vaccines to prevent infection and death (Lunney, 2010). The standard medium, known as "MM", was developed in 1954 by Mueller and Miller which contains glucose, beef heart infusion (BHI), an enzymatic digest of casein, some amino acids and vitamins, uracil, and inorganic salts. First problem with the MM medium typically used to culture *C. tetani* for tetanus toxoid preparation is that the medium has proven to be very sensitive to "cooking," in a manner that degrade medium components such as proteins and peptides, thus a less effective growth medium is produced when a particular batch of medium is imperfectly cooked. Finally, the process of *C. tetani* fermentation in MM medium generates H_2S, which is toxic to *C. tetani*, and may also generate some alcohols that can similarly be toxic.

Over the last twenty years, the threat of transmissible protein agent (prion) that causes Bovine Spongiform Encephalopathy (BSE) or antigenic peptides that stimulate undesired immune reactions (e.g., anaphylactic reactions) in immunized subjects. In addition these components cause anaphylactic reactions that range from minor site reactions, such as swelling and tenderness at the injection site, to systemic effects of fever (Jenkins et al., 2007). There is a need for the development of an improved system for

preparing tetanus toxoid that minimizes these risks and problems. Preferably, the system should allow for high levels of toxin production, and should minimize the dangers associated with formation of animal- or dairy-product. So, the present work was undertaken to replace these problematic components by the use of vegetable products instead of the animal one.

MATERIALS AND METHODS

1- Strain

Lyophilized *Clostridium tetani (Harvard strain, 49205)* was originally obtained from the New York State Department of Health.

2- Swiss mice

Two hundred Swiss mice (20-25 gm) were used for determination of the minimal lethal dose of the prepared tetanus toxins, these mice were obtained from the Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo.

3-Toxin fermentation media

Table (1): The formulation of the different types of media that were used in the present study for preparation of tetanus toxin.

Ingredients(g/liter)	Toxin fermentation media			
	M1	M2	M3	M4
	Mueller& Miller (1954)	Modified Mueller&Miller (Abd El-Aziz, 2005)	Demain et. al (2006)	Modified Demain et. al (2006)
Pancreatic digest of casein	22.5 g	22.5 g	-	-
Beef heart infusion	50 ml	50 ml	-	-
Soy hydrolysate enzymatic	-	-	35 g	35 g
Glucose	11 g	11 g	7.5 g	7.5 g
Sodium chloride	2.5 g	2.5 g	5 g	5 g
Potassium chloride	-	0.1 g	-	0.1 g
Disodium hydrogen phosphate	2 g	2 g	0.5 g	0.5 g
Potassium dihydrogen phosphate	0.15 g	0.15 g	175mg	175 mg
Magnesium sulphate	0.15 g	0.15 g	50 mg	50 mg
Cystine	0.25 g	0.25 g	125 mg	-
Tyrosine	0.5 g	-	125 mg	-
Ca pantothenate	1 mg	1 mg	-	-
Uracil	2.5 mg	2.5 mg	-	-
Thiamin	0.25 mg	-	-	-
Riboflavin	0.25 mg	-	-	-
Pyridoxine	0.25 mg	-	-	-
Biotin	2.5 µm	-	-	-
Reduced iron powder	0.5 g	-	0.5g	-
$FeCl_3.6H_2O$	-	32 mg	-	32 mg

4-Preparation of tetanus toxin: (Mueller and Miller, 1954)

The content of freeze dried ampoule of *C. tetani* strain was reconstituted in few amount of thioglycolate broth (Oxoid) and incubated at 36 °C for 48 hr. Sterility test was performed using nutrient broth, Sabouraud dextrose agar and blood agar media. Two ml of thioglycolate broth was used to inoculate 500 ml of the four (M1, M2, M3 and M4) production media in a cylindrical jar of one liter capacity which has been covered with thin layer of cotton in between two layers of cheese cloth, held firmly in place by a tightened strip of flexible metal. In this cover a long syringe was inserted sterilized with the medium and serve to introduce the inocua. The media was incubated aerobically for 7-10 days at 36 °C. At the fourth day, samples were taken and checked for purity by Gram staining (the Gram-smear shows Gram positive and negative rods of the same thickness but differ in length) centrifuged and checked for toxin production by Flocculation test. This was repeated daily until two similar successive limits of flocculation (LF) values were obtained. Incubation is stopped by exposing the cultures to 4 °C to promote bacterial lysis. Two days later, the completeness of cell disruption was verified by Gram stain. Glycine 5g/liter medium, dissolved in hot water was added to the culture as stabilizer. The culture fluid was filtered through Seitz filter and a presterility test was performed to the filtrate.

3- Evaluation of the prepared tetanus toxin:

A-Determination of Minimal Lethal Dose (MLD).

It was done by determination of death time (according to the British Pharmacopoeia,Veterinary, 1993) as follows:-

- Ten fold serial dilution of produced tetanus toxin in the four media separately using 1% peptone and 0.5% Nacl solution. From each dilution 2 mices were injected s/c at the root of the tail with 0.5 ml and observed for 4 days. MLD is the smallest amount of toxin that killed two mices within 96 hours.

B- Determination of flocculation (LF) values of the prepared toxin.

The flocculation (LF) unites of prepared tetanus toxin in the four media separately were determined according to the procedure of WHO(1997) by using a commercial antitetanic serum obtained from Holding Company for Biological Products and Vaccines (*VACCERA*) , each ampoule contains 1500 IU/ml . The tetanus toxin and tetanus antitoxin aggregate and form visible floccules when mixed in certain proportions in test-tubes. The precipitate is developed more rapidly when equivalent amount of toxin and antitoxin are present than when an excess of either toxin or antitoxin is available.

RESULTS AND DISCUSSION

The most obvious benefit in converting to an animal-free medium base for toxin production is the elimination of any risk of prion-transmissible disease. With no animal source in any of the products, the risk of the potential subsequent introduction into the human food supply is eliminated. (Ellenberg, 2001). The present work provides a method for the growth of *C. tetani* that aimed to maximiz the production of tetanus toxin using media that are substantially free from animal-derived products.

In this study four different fermentation media were tested for growing C. tetani. Two control media contained pancreatic digest of casein and beef heart infusion, M1 (Mueller and Miller, 1954) and M2 (modified Mueller and Miller ,Abd El-Aziz, 2005) were compared with another two experimental medium M3 (Demain et al., 2006) and M4 (modified Demain et al., 2006) that contained soy hydrolysate enzymatic as a replacement for both pancreatic digest of casein and beef heart infusion. The results presented in Table 2 revealed that M1 (MM) and M2 media which contain pancreatic digest of casein and beef heart infusion as a basal source of essential amino acid and peptide support the growth of *C. tetani* and production of tetanus toxin, as also cleared by Porfirio et al. (1997). M2 medium devoid of thiamine, riboflavin, biotin and pyridoxine with the replacement of reduced iron with ferrous chloride in its ingredient and potassium chloride is added. M2 medium induced a significant high yield of tetanus toxin (70 Lf & 600,000- 700,000 MLD) in comparison with M1 medium (40-50 Lf & 300,000 – 400, 000) . These results are supported by findings of Latham et al. (1961) and EL- Helw (2007) who found that a poor yield of tetanus toxin was associated with combined excess of both iron and cystine. Also Zacharias and Bjorklund (1968) found that the addition of potassium chloride doubled the amount of flocculation unites because the potassium ions increased permeability of cell membranes.

The animal source component has proven to be particularly problematic, both because of the risk that it may contain undesirable products that may be carried over into the final toxoid preparation. So the present work provides a method for the growth of *C. tetani* that maximizes the production of tetanus toxin using media that are substantially free of animal-derived products.

The result in Table (2) showed that both Demain (M3) and modified Demain media that contained 35 g/L of Hy-Soy supported the growth of *C. tetani* and production of tetanus toxin at high levels of toxin reaching (60 Lf &500,000-600,000 MLD) and (80Lf & 700,000-800,000 MLD) respectively in comparison to the other two media. Such results indicate that the Hy-Soy

could be a satisfactory replacement for pancreatic digest of casein and beef heart infusion for growth of *C. tetani*, and it is preferred for production of tetanus toxin. It was also observed that elimination of Ca pantothenate, Cystine, Tyrosine, Thiamin, Riboflavin, Pyridoxine, and Biotin from the fermentation media, did not affect on toxin production. On other hand, addition of potassium chloride in modified Demain media (M4) induced a significant high yield of tetanus toxin in comparison to Demain media (M3). These results were conducted with the findings of Latham et al. (1961) and EL-Helw (2007) and Zacharias and Bjorklund (1968). So it could be concluded that replacement of animal source media with plant source is more beneficial and economic in production of efficient tetanus toxin that used in preparation of antitetanic serum and tetanus vaccine.

Table (2): The mean Flocculation unites and Minimal Lethal Dose (MLD) of tetanus toxin batches prepared in different media.

Types of media	Flocculation unites/ml	MLD/ml
1- Mueller& Miller (1954)	40-50	300.000-400.000
2- Modified Muller&Miller (Abd El-Aziz,2005)	70	600.000-700.000
3- Demain et al., (2006)	60	500.000-600.000
4- Modified Demain et al., (2006)	80	700.000-800.000

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استخدام الصويا هيدروليزات لإنتاج توكسين التيتانوس

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فى هذه الدراسة تم مقارنة بعض المستنبتات البكتيرية ذات الأصل الحيواني الخاصة بنمو بكتيريا التيتانوس وذلك مع مستنبتات ذات أصل نباتي و ذلك بغرض استبدال المستنبتات ذات الأصل الحيواني بمستنبتات ذات اصل نباتى . اثبتت التجارب ان ميديا مولر و ميلر ١٩٥٤ و تعديلها ٢٠٠٥ ذات الاصل الحيوانى قد اعطى سمية على التوالي كالاتي (٤٠-٥٠ وحدة تندفية /ملى، ٣٠٠,٠٠٠- ٤٠٠,٠٠٠اقل جرعة مميته) و (٧٠ وحدة تندفية، /ملى ٦٠٠,٠٠٠- ٧٠٠,٠٠٠اقل جرعة مميته) .

اما المستنبتات ذات الأصل النباتي قد أعطت سمية عالية فى رقم ٣ و رقم ٤ على التوالى كالاتى (٦٠ وحدة تندفية /ملى، ٥٠٠,٠٠٠- ٦٠٠,٠٠٠ اقل جرعة مميته) و (٨٠ وحدة تندفية، /ملى ٧٠٠,٠٠٠- ٨٠٠,٠٠٠ أقل جرعة مميته) .

كما أوضحت الدراسة أنة يمكن استبدال المستنبتات ذات الأصل الحيوانى بمثيلتها ذات الاصل النباتى في انتاج توكسين عالي العياريه نادراً ما يحدث منه اثار جانبيه مثل ما يحدث من التوكسين المحضر على المستنبتات ذات الأصل الحيواني