NOTE

EFFECT OF SOME SYNTHETIC PEPTIDES ON THE ALPHA-TOXIN PRODUCTION BY CLOSTRIDIUM PERFRINGENS PB6K

In a previous paper, it was shown that a certain fraction derived from a peptone was effective in promoting the growth and alpha-toxin production by *Cl. perfringens* PB6K (Murata *et al.*, 1958 b). However, isolation and identification of the active principle(s) have not yet been successful, although it was suggested that substances of peptide nature might be responsible for this activity. In 1959, Jayko *et al.* showed by using 29 synthetic peptides that some combinations of three or four peptides remarkably stimulated the lecithinase production when added to the synthetic medium. Among these peptides, glycyl-L-asparagine appeared to be most effective in lecithinase synthesis. The present authors tried to follow their experiments but were not able to confirm the toxin promoting effect of the synthetic peptides used by them. However, it was evident that fractions obtained from a protein digest were effective in the toxin production.

Materials and Methods.

Medium: Two types of synthetic medium were used as the basal medium. They were in principle similar to the chemically defined medium described by Boyd *et al.* (1948).^{*} One of them was the modification by Jayko *et al.* (1959) and the other was that by the present authors. In the former medium (called SMJ), amounts of all amino acids were doubled, glucose was replaced by sucrose and glutamine was added in concentrations of 0.01-0.05 percent. In the latter (called SMT), fructose was used as carbohydrate, ascorbic acid was replaced by thioglycolic acid (0.01 %), Na_2HPO_4 and KH_2PO_4 were used as the phosphate and glutamine was added in a concentration of 0.025 percent. Besides, a slight modification was given as regards vitamins and minerals (ref. Murata *et al.*, 1958 a).

A peptone medium was also used as the control in which a potent toxin is produced. It contains the following materials per 100 cc: 5 g of Proteose Peptone No. 3 (Difco), 1 g of fructose, 0.92 g of K_2HPO_4 , 0.14 g of KH_2PO_4 , 20 mg of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 mg of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.6 mg of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.7 mg of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and thioglycolic acid in a concentration of 0.01 percent. pH was adjusted to 7.6.

Synthetic peptides were obtained from Nutritional Biochemicals Corporation.

Preparation of fractions from proteose peptone: Dialysate of Proteose Peptone No. 3 (Difco) was concentrated in vacuo and fractionated with various ion exchange resins by batch method. A glass column of 250 by 35 mm filled with resin was employed except in the case of Amberlite CG 400 where a column of 600 by 33 mm was used. A procedure of preparing active fractions is described below.

One hundred and ten cc of the dialysate (106 mg/cc) was first passed through a column of Amberlite CG 120 (H-type). The column was washed with 2 l of water until ninhydrin reaction became negative. The column was then eluted by passing through 2N- NH_4OH . The eluate and washings were combined and it was distilled in vacuo to dryness (Fraction 2). The fraction was dissolved in 140 cc of distilled water and 120 cc of this solution was passed through Amberlite CG 400 (OH-type), and the column was eluted by 2N- CH_3COOH . The mixture of eluate and washings was neutralized with N- NaOH and then passed consecutively through three columns of

^{*} Boyd's medium consists of: aminoacids, glucose, ascorbic acid, phosphate buffer (K_2HPO_4 & KH_2PO_4), several accessory growth factors and minerals.

Amberlite CG 45 (OH-type), Amberlite CG 50 (H-type) and Amberlite CG 120 (H-type). The first column (Amberlite CG 45) was eluted by 2N-CH₃COOH and the pool of eluate and washings was passed through CG 120 to remove acetic acid, and the latter resin was, in turn, eluted by 0.2N-NH₄OH. The pool of the eluate and washings was distilled in vacuo to dryness (Fraction 2-A, yield 1.668 g). The second one (Amberlite CG 50) was eluted by 0.1 N-HCl and the pool of the eluate and washings was passed through Amberlite CG 45 to remove HCl, and the pool of the filtrate and washings was concentrated in vacuo (Fraction 2-B, yield 1.618 g). The last one (Amberlite CG 120) was eluted by 0.2N-NH₄OH and the pool of the eluate and washings was distilled in vacuo to dryness (Fraction 2-N, yield 3.6 g).

Inoculum: A strain of *Clostridium perfringens* PB 6 K-N5 was grown in a seed medium consisting of the papain digest of beef supplemented with fructose (Murata *et al.* 1958 a). Cells from actively growing culture were washed twice with saline and resuspended in saline of a half volume of the seed culture and 0.1 cc was used to inoculate into 4 cc of the medium.

Cultures: Experiments were carried out with tubes containing 4 cc of the medium. All media were inoculated immediately after autoclaving. Anaerobic culture was carried out in an atmosphere of hydrogen at 37°C.

Assay of alpha toxin: Toxin was titrated by the lecithovitellin method (Murata *et al.*, 1956). When cell free supernatant was used, it was possible to titrate alpha toxin to 2 EU/cc (=6 LD₅₀ by mice intravenous method).

Other methods: Nitrogen content was determined by micro-Kjeldahl method and phosphate by Allen's method. Tests for carbohydrate were carried out by Molish- and orcinol-reaction (Kabat & Mayer, 1961).

Results and Discussion.

Table 1 shows the effect of several peptides on the toxin production. Contrary to our expectation, little amount of toxin was detected by the prescription recommended by Jayko *et al.* (1959), although the organism was able to grow abundantly in the medium. In the synthetic medium modified by the present authors, growth was rather poorer than in the Jayko *et al.* medium. However, alpha toxin was detected, though in a low titer. No favorable effect was observed by the addition of peptides. The experiment was repeated several times under anaerobic and aerobic conditions. Similar

Table 1. Effect of some peptides on the growth and toxin production

Medium	Growth O. D. §	Toxin EU/cc
SMJ	0.57	0
SMJ+GA+GT+LGG	0.61	0
SMJ+GA+LGG+AA	0.61	0
SMT	0.25	8
SMT+GA+GT+LGG	0.23	8
SMT+GA+LGG+AA	0.22	6
Peptone medium	0.95	142

Incubated for 6 hours anaerobically.

Abbreviations

SMJ: Synthetic medium (Boyd) modified by Jayko *et al.* (1959).

SMT: Synthetic medium (Boyd) modified by the present authors.

AA: DL-alanyl-DL-alanine.

GA: glycyl-L-asparagine.

GT: glycyl-L-tryptophan.

LGG: L-leucyl-glycyl-glycine.

§. O. D.: Optical density at 660 mμ with 1 cm light path by Coleman Junior Spectrophotometer.

results were obtained by aerobic culture.

Then, the effect on the toxin production of Proteose Peptone No. 3 (Difco) and its derivatives was investigated by using the synthetic medium modified by us. As shown in Table 2, growth and toxin production were enhanced by three fractions of the peptone dialysate, 2B, 2N and 2A, the first two of these three fractions being very active, although the titer of toxin was inferior to that in the complex medium. However, it may be possible to state that such fraction(s) are necessary to promote the toxin production in the synthetic medium. The chemical nature of the active principles in these fractions have not yet been elucidated, but results of some chemical analyses are attached in Table 2. The three active fractions were negative by orcinol test and weakly positive (or doubtful) by Molish test. These results show that peptides and amino acids are main components of the active fractions. Since various amino acids and/or sugars (including glucosamine) were not able to stimulate the toxin production when added singly or in various combinations to the basal medium, it is most probable that some peptides may be responsible for the activity.

Table 2. Effect of Proteose Peptone and its derivatives on growth and toxin production

Medium	Growth O. D.	Toxin EU/cc
SMT	0.15	3
SMT+Proteose Peptone 1 mg/cc	0.50	50
2.5 mg/cc	0.77	100
SMT+Dialysate of Proteose Peptone 1 mg/cc	0.55	38
2.5 mg/cc	0.82	77
SMT+Fraction 2, 1 mg/cc	0.22	18
SMT+Fraction 2 B, 1 mg/cc	0.34	30
SMT+Fraction 2 N, 1 mg/cc	0.53	39
SMT+Fraction 2 A, 1 mg/cc	0.21	16
Peptone medium	0.95	200

Incubated for 6 hours aerobically at 35°C in a water bath.

Chemical nature of the fractions of the peptone :

	N%	P%
Dialysate	15.2	0.60
Fr. 2	13.6	0.55
2 B	13.4	0
2 N	12.0	0.51
2 A	12.7	0.10

The reason why we obtained results different from those of Jayko *et al.* is not understandable. We followed their experiment exactly according to their description except that the strain was different. Therefore, it is plausible that the discrepancy between their and our results may be due to the difference in the strain used, although both strains are the substrains of *Cl. perfringens* PB6K.

Summary.

Some synthetic peptides, reportedly active in toxin synthesis, failed to promote the

alpha toxin production by a strain of *Cl. perfringens* PB6K. However, it is evident that certain fractions derived from a protein digest supported the production of a potent toxin.

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