Inhibition of *Clostridium botulinum* Okra B by N-Acyl Amino Acid Ester (Nα-Cocoil Arginine Ethylester-DL-Pyrrolidone Carbonate)

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**ABSTRACT**

An amino acid ester, Nα-cocoil-L-arginine ethylester-DL-pyrrolidone carbonate (CAE), was inhibitory to growth and toxin production of *Clostridium botulinum* okra in peptone-yeast extract-glucose (PYG) medium, pH 7.0, at 30°C. Addition of 10 mg of CAE/L to PYG medium delayed toxin production and 25 mg of CAE/L inhibited growth and toxin production, whereas 5 mg of CAE/L had no effect on both growth and toxin production.

Some amino acid derivatives have surface active effects and antibacterial activity (1-3). In Japan, they are used in shampoo, toothpaste, and cosmetics because their toxicities are rather low and their skin-irritating activities are weak compared to common surface active agents (1). Among them, Nα-cocoil-L-arginine ethylester-DL-pyrrolidone carbonate (CAE) is highly inhibitory to bacteria. Hirose (1) found that CAE is also greatly inhibitory to fungi. The objective of this study was to determine the effect of CAE on growth and toxin production of *Clostridium botulinum* in a laboratory medium.

**MATERIALS AND METHODS**

**Test organism**

The spore suspension (approx. 4 × 10^8 spores/ml) of *C. botulinum* okra (type B) was kindly supplied by Prof. G. Sakaguchi of Osaka Prefectural University. It was held at -20°C until used.

**Test material**

CAE was supplied by Ajinomoto Company. It was dissolved in distilled water to a final concentration of 3 mg/ml and sterilized by filtering through a Millipore filter (0.45 μm).

**Growth medium**

PYG medium containing 2% peptone (Mikuni), 0.5% yeast extract (Oriental yeast), 0.5% glucose and 0.1% cysteine-HCl, pH 7.0, was used as growth medium. Culture tubes containing 3 ml of PYG medium were autoclaved at 121°C for 15 min. CAE was aseptically added to the desired concentration.

**Growth studies**

Each culture tube containing 3 ml of PYG medium and an appropriate amount of CAE was inoculated with a *C. botulinum* spore suspension prepared by diluting the frozen stock of a known concentration in 0.8% saline to approx. 3 × 10^7 spores/ml. The spores were not heat-shocked. Spore suspension (0.1 ml) was inoculated into each tube and tubes were incubated at 30°C. Growth in culture tubes was monitored by determining the absorbance at 550 nm in a Shimadzu Spectronic 20A spectrophotometer. More than twenty replicate tubes were prepared for each experimental condition and a fresh tube was used daily for the determination of cell concentration because growth of the organism was affected by agitation during sample collection.

**Detection of toxin**

Toxin was detected according to the CDC method (4). The 6-d cultures were centrifuged at 10,000 × g for 20 min. Resulting supernatant fluid (0.1 ml) was incubated with 0.3 ml of trypsin solution (0.27 mg of trypsin in 1 ml of 0.2% gelatin-0.05 M Na_2HPO_4, pH 6.2) at 37°C for 30 min to activate toxin. The resulting toxin solution was tested for its toxicity by injecting i.v. 0.1 ml into each of two mice [strain dd (Shizuoka Laboratory Animal Center, Shizuoka), male, 17 to 20 g]. The mice were observed for 3 d.

**RESULTS AND DISCUSSION**

**Growth inhibition**

Yoshida et al. (5) reported the MIC of CAE to *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* in nutrient broth, pH 7.0, at 30°C for 48 h were 10, 50 and 250 mg/L, respectively. The effect of CAE on growth of spores of *C. botulinum* okra in PYG medium, pH 7.0, is presented in Figure 1. The small increase in absorption in the presence of 25 and 100 mg/L of CAE was not caused by growth of the organism because no turbidity was observed, but was likely due to browning of PYG medium. The addition of 5 mg of CAE/L did not cause inhibition, but addition of 25 mg of CAE/L caused complete inhibition throughout an incubation period of 8 d. We compared the inhibitory effect of CAE with that of nitrite. The addition of 25 mg/L of filter-sterilized nitrite was also completely inhibitory to growth of *C. botulinum* okra in PYG medium at 30°C for 8 d (data not shown).

**Inhibitory effect on toxin production**

The effect of CAE on toxin production by *C. botulinum* okra in PYG medium incubated at 30°C for 6 d is shown in Table 1. The addition of 25 mg of CAE/L did not cause inhibition, but addition of 25 mg of CAE/L caused complete inhibition throughout an incubation period of 8 d. We compared the inhibitory effect of CAE with that of nitrite. The addition of 25 mg/L of filter-sterilized nitrite was also completely inhibitory to growth of *C. botulinum* okra in PYG medium at 30°C for 8 d (data not shown).
Figure 1. Effect of CAE on the growth of C. botulinum okra in PYG medium incubated at 30°C for 8 d. CAE concentration: ●—●, 0 mg/L; ●—●, 5 mg/L; ●—●, 25 mg/L; and ●—●, 100 mg/L.

TABLE 1. Effect of CAE on the toxin production of C. botulinum okra in PYG medium incubated at 30°C for 6 d.

| CAE (mg/L) | Toxin*
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*aSurvival time of mice after intravenous injection: less than 3 h (+ +); 3 to 20 h (+); more than 20 h (−).

CAE may be a useful preservative for some cured meats; however, the inhibitory concentration of antibacterial compounds in foods are generally much higher than that in culture medium. We will next study the effect of CAE on C. botulinum in cured meat.

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REFERENCES