MOLECULAR SIZE OF *CLOSTRIDIUM BOTULINUM* TYPE E TOXIN IN "IZUSHI"

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Botulogenic "izushi" was prepared from flounders inoculated with spores of *Clostridium botulinum* type E by the method commonly practiced in the local areas. To determine the molecular size and the properties of the toxin in "izushi," the exudate of the "izushi" and the extract of the fish were subjected to gel filtration on Sephadex G-100 and ultracentrifugal separation in sucrose density gradient. Most of the toxin in "izushi" was proved to be in the form of precursor and to possess a large molecular size with a sedimentation constant ($S_{20,w}$) of about 11.5. The tryptic activation of the precursor in the "izushi" caused no appreciable reduction in the molecular size.

INTRODUCTION

We demonstrated that neither the precursor nor the trypsin-activated toxin of *Clostridium botulinum* type E obtained from the bacterial cells penetrated swollen gel of Sephadex G-200. The findings were ascribed to the large molecular weight of 200,000 or larger possessed by both the materials (Sakaguchi, Sakaguchi and Imai, 1964).

Gerwing, Dolman and Arnott (1962) reported a much smaller sedimentation constant ($S_{20,w}$) of 5.6 for a preparation of non-activated type E toxin; more recently, they reported an even smaller $S_{20,w}$ of 1.7 for another preparation (Gerwing, Dolman, Reichmann and Bains, 1964). The discrepancy among their findings and ours must have resulted from the different materials and purification procedures.

Variable molecular sizes have also been reported for type A toxin. The crystalline toxin had a molecular weight of about 900,000 (Putnam, Lamanna and Sharp, 1948), which dissociated into fragments with a sedimentation constant of 7S (Wagman, 1963). By digesting the 7S toxin with pepsin, he obtained a dialyzable unit of 0.64S (Wagman, 1963). The toxin appearing in the lymph of orally poisoned rats had a smaller sedimentation constant than the crystalline toxin; yet its sedimentation constant was about 7.9S (Heckly, Hildebrand and Lamanna, 1960). Gerwing, Dolman and Bains (1965b) claimed that non-aggregated type A toxin had a molecular weight of 12,200. Recently, DasGupta, Boroff and Rothstein (1966) separated crystalline toxin into α and β components with molecular weights of about 150,000 and 500,000, respectively. The α component was

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highly toxic ; while β was non-toxic.

The important question of whether the large molecular form of botulinus toxins is normally produced by the organisms in foods or in cultures or appears only as an artifact of the purification procedures has not been clarified as yet. In the present study, attempts were made to determine the molecular size of type E toxin in "izushi," that is the commonest foodstuff involved in the outbreaks in Japan, by gel filtration on Sephadex G-100 and ultracentrifugal separation in sucrose density gradient.

MATERIALS AND METHODS

Preparation of the "izushi": Fresh flounders were cut into pieces after the heads and visceras were removed. About 250,000 spores of *C. botulinum* type E, strain Iwanai, were introduced by a syringe into each piece of fish. The fish pieces were soaked in water in a plastic container for a period of 3 days at 20 to 23 C, during which time water was changed twice a day. The soaked fish pieces, cooked rice, "koji" and cut-up radish and carrot were placed into a plastic container. The content was compressed tightly and kept standing to allow fermentation at room temperature for 10 days. Afterwards, the container was kept under refrigeration.

Preparation of toxin materials: The turbid fluid with pH 4.28 exudated during fermentation was taken. A portion of the fish meat was ground in a mortar with a pestle with an equal amount of 0.1 M phosphate buffer, pH 6.0. The fish ground and the exudate were centrifuged at $12,000 \times g$ for 20 min and the clear supernatants were used as the toxin materials.

Gel filtration: A column of Sephadex G-100 (2.5×30 cm) buffered with 0.05 M acetate buffer, pH 6.0, was loaded with 5.0 ml of the exudate or the extract. Elution was carried out with the same buffer at room temperature. Protein content in each fraction was determined ; toxicities of the combined fractions of every three tubes were determined before and after tryptic activation. The void volume was measured with a 0.2 % Blue Dextran solution.

Ultracentrifugal separation in sucrose density gradient: Density gradient was prepared in cellulose tubes of 1.3×5.1 cm by mixing two solutions of 0.05 M phosphate buffer, pH 6.0, containing sucrose in 20 % and in 5 %, respectively, so that the final gradient of 20-5 % would be obtained. Two-tenths ml each of the fish extract with and without tryptic activation together with a purified precursor solution containing protein at 1.34 mg/ml and toxicities of 36,400 LD₅₀/ml before activation and 15,500,000 LD₅₀/ml after activation, that had been shown to have an $S_{20,w}$ of approximately 11.5, was placed on top of the gradient. The tubes were centrifuged in a Hitachi swinging bucket rotor model PRS 40 at 40,000 rpm ($105,000 \times g$) for 5 hr.

The fractionation was accomplished by inserting a hypodermic needle into the tube's bottom and controlling the outflow of solution by water pressure. Fractions of 20 drops (18 fractions from the 5-ml sample) were collected. The toxic potency of each fraction was determined after tryptic activation in the case of the nonactivated exudate and the 11.5 S precursor.

The tryptic activation, toxin assay and protein determination were performed as described elsewhere (Sakaguchi *et al.*, 1964).

RESULTS

Development of Toxin in "izushi"

A piece of the fish after soaking in water contained toxic potencies of 320 LD₅₀/g and 64,000 LD₅₀/g before and after tryptic activation, respectively. On the 10th day after pickling, the exudate of the "izushi" contained 810 LD₅₀/ml and 29,000 LD₅₀/ml and the fish pieces 4,100 LD₅₀/g and 250,000 LD₅₀/g before and after activation, respectively. On the 77th day after the start of pickling, the extract from other pieces of fish still contained 6,400 LD₅₀/g and 210,000 LD₅₀/g before and after activation.

Gel Filtration on Sephadex G-100

The elution patterns of the exudate and the fish extract are shown in Fig. 1. The majority of the toxic activities of both the samples were eluted in the void volume or in Fraction A being accompanied by a very small amount of protein. No significant

Fig. 1. Elution patterns of type E botulinus toxin in the exudate of "izushi" and in the extract of the fish from a column of Sephadex G-100 (2.5×30 cm).

amount of toxic activity was detected in the retarded fractions or in Fractions B, C, D and E. The majority of the proteinous substances was eluted in the retarded fractions.

Ultracentrifugal Separation in Sucrose Density Gradient

The patterns of ultracentrifugal separation in sucrose density gradient of the fish extract before and after tryptic activation in comparison with the 11.5S precursor are shown in Fig. 2. The toxic activities of the non-treated and the activated extract were sedimented to the same relative position; that of the purified precursor sedimented to a lesser extent. The position of the peak of toxic activity and that of protein were the same with the purified precursor; while most proteinous materials were separated from the toxic activity with the fish extract.

Fig. 2. Sedimentation patterns of type E botulinus toxin in the extract of the fish before and after tryptic activation and of the 11.5S precursor in sucrose density gradient.

Since the toxic potency of the extract was much lower than that of the 11.5 S precursor solution, the extract was concentrated by precipitation with ammonium sulfate at 50% saturation. The precipitate was dissolved in a small amount of 0.1 M phosphate buffer, pH 6.0, and dialyzed against the same buffer. The potential toxicity of the dialyzate was 6 times higher than that of the original extract.

The same experiments were run with the concentrated fish extract before and after tryptic activation and also with the 11.5 S precursor as the control. As can be seen in Fig. 3, the toxic activities of the three materials were sedimented to the same relative position.

Fig. 3. Sedimentation patterns of the concentrated extract of the fish before and after tryptic activation and of the 11.5 S precursor in sucrose density gradient.

DISCUSSION

Gerwing et al. (1962, 1964) reported inconsistent molecular sizes of non-activated type E toxin, $S_{20,w}$ = 5.6 and 1.7. They ascribed the discrepancy in their own work to molecular degradation and aggregation due to the use of ethanol and lyophilization in the

former work. We reported that both the precursor and the trypsin-activated toxin possessed molecular weight larger than 200,000 (Sakaguchi *et al.*, 1964). Gerwing, Dolman and Ko (1965a) claimed that our purification procedures for the precursor, especially the extraction of the toxin from bacterial cells with 1 M acetate buffer and its repeated precipitation with solid ammonium sulfate, are conducive to the formation of molecular aggregates. However, they did not demonstrate aggregation of their small molecular-sized toxin by exposure to ethanol, ammonium sulfate or 1 M acetate buffer. They also claimed that the true molecular weight of toxin should approximate the smallest figure.

Before attempting to obtain the smallest possible figure of the molecular size, one must answer a rather simple but fundamental question of what is the true molecular size of type E toxin normally produced by the organisms in foods or in cultures.

The results presented here clearly indicate that type E toxin appearing in "izushi" is for the most part in the form of the precursor and of a large molecular size. Since the toxin materials precipitated with ammonium sulfate had the same sedimentation pattern as the purified precursor with $S_{20.w}$ of approximately 11.5, the type E toxin in "izushi" may approximate this size.

Wagman and Bateman (1951, 1953) and Wagman (1954) stated that the molecular size of crystalline type A toxin is largely dependent upon the pH and ionic strength of the solvent. The pH of "izushi" usually lies between 4 and 5 (Kanzawa and Iida, 1957) and the present "izushi" had a pH of 4.28, which was on the same level as the final pH of type E cultures of our routine use (Sakaguchi *et al.*, 1964). On the other hand, Gerwing and her associates utilized dialyzing culture with daily changes of culture medium outside the casing (Gerwing, Dolman and Arnott, 1961). The method may have provided a fairly constant pH at or above 7.0, thus providing more chances for molecular dissociation or enzymatic degradation with the proteolytic enzyme produced by the toxigenic phase (Skulberg, 1964) or by the proteolytic mutant phase (Dolman, 1957).

At any rate, it may be true that the victims in this country ate principally the large molecular sized precursor in "izushi." The present investigation also demonstrated that the precursor in "izushi" was not appreciably reduced in size when acted upon by trypsin at pH 6.0. If tryptic activation takes place at the upper part of the duodenum where the pH may be still acidic, the activated toxin should be of a large molecular size. At the lower part of the intestine where the pH is higher, the toxin may undergo dissociation and detoxification rather slowly by trypsin, chymotrypsin (Sakaguchi and Sakaguchi, to be published) and possibly other proteolytic enzymes.

The present data seem to support the use of the precursor of a large molecular size in the studies on the mechanism of tryptic activation of type E toxin.

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