Occurrence of a Complex Form of Staphylokinase in the Course of Cultivation of Staphylococcus aureus

SETSUO FUJIMURA, TOSHIKAZU MAKINO, AND TAKAYOSHI T. A. HAYASHI

Department of Microbiology, Sapporo Medical College, Sapporo, Japan

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Proteins in the culture supernatant of Staphylococcus aureus PS 47 were subjected to Sephadex chromatography. In the early stage of the cultivation, staphylokinase appeared to have a molecular weight of 15,000, and in the later stage it appeared to have a molecular weight of 320,000. The staphylokinase having a lower molecular weight (type A) converted into one having a higher value (type B) during the course of cultivation. It was demonstrated that conversion of type A into type B took place in vitro (monitored by gel filtration after the two types of staphylokinases were mixed), and it was observed that type B reverted to type A when type B was treated with KCl or detergent. Type B seems to be a complex form of type A and some high-molecular-weight substance.

Staphylokinase, known as one of the staphylococal extracellular proteins, converts plasminogen, a precursor of proteolytic enzyme, into an active enzyme, plasmin (6-8, 10). However, the mechanisms of staphylokinase production are largely unknown, except for observations of correlation between production of this protein and lysogenization (2, 4, 5, 14).

We decided, therefore, to further assess the mechanisms of staphylokinase formation by using strain PS 47, which gives a much higher yield of staphylokinase than other strains of our stock cultures (3). We discovered two kinds of staphylokinases having different molecular weights.

This paper deals with the correlation between the two staphylokinases and some of their properties.

MATERIALS AND METHODS

Bacterial strains. Staphylococcus aureus PS 47 (propagating strain for standard typing phase 47) was primarily used. S. aureus 879(756), PS 83A, PS 6, and Terashima were used in some experiments.

Cultural conditions. A medium containing 1% nutrient broth (Difco), 0.3% yeast extract (Difco), and 1% sodium lactate was used throughout the experiments. The pH of the medium was adjusted to 7.2. A 1-ml volume of the 18-h coccus culture was inoculated into 100 ml of the same fresh medium. Cultivations of the cocci were performed in 500-ml Erlenmeyer flasks with a culture volume of 100 ml on a reciprocating shaker at 33 C (100 strokes per min).

Assay of staphylokinase activity. The activity of staphylokinase was measured by the method of Hayashi and Maekawa (7) with some minor modifications. The reaction mixture consisted of the following components: dog plasminogen (0.6 casein unit), 0.1 ml; test sample, 0.2 ml; 0.4% bovine fibrinogen, 0.1 ml; and bovine thrombin (20 U/ml), 0.1 ml. The dog plasminogen was purified by affinity chromatography by the method of Liu and Mertz (11). All reagents were dissolved in 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 8.4). One unit of staphylokinase was defined as the amount of staphylokinase which lysed the fibrin clot in 10 min under these conditions.

Neutralization test. Two kinds of antisera from rabbits immunized separately with types A and B, in conjunction with Freund complete adjuvant, were obtained. Inhibition of the activities of both types of staphylokinase by these sera was measured by the method of Maekawa et al. (12). Types A and B staphylokinase used as antigens were separated by Sephadex gel filtration from culture supernatants of 10- and 24-h cultures of PS 47, respectively. The plots of the log of the remaining staphylokinase activities against dilution of antisera resulted in straight lines (see Fig. 3), and neutralization constants (K) were determined from the plots.

Concentration of samples. Concentration of samples was by dialysis against polyethylene glycol 6000 overnight at 4 C. The concentrated materials were dialyzed against 0.01 M Tris-hydrochloride buffer (pH 8.4) at 4 C for 10 h.

RESULTS

Formation of two kinds of staphylokinases. S. aureus PS 47 was cultured for 10, 15, and 24 h in 100 ml of the medium, and the culture supernatants were concentrated. The
concentrated materials were subjected to gel filtration on Sephadex G-75 so that we could investigate the distribution patterns of staphylokinase activity.

The distribution of staphylokinase activities apparently varied with the incubation period of the cocci (Fig. 1). In the 10-h culture supernatant, only one peak of staphylokinase activity was found at the elution volume of 120 ml (Fig. 1A). The staphylokinase eluted at this position was designated type A. However, a remarkable change of the gel filtration profile was observed in the 15-h culture supernatant (Fig. 1B). This was characterized by appearance of a major peak at 60 ml, which coincided with the void volume of the column; only a slight amount of the activity was found at 120 ml. This minor peak disappeared when the cultivation was continued up to 24 h and all of the activity was eluted in the void volume (Fig. 1C). The fraction of staphylokinase eluted in the void volume was designated type B.

These observations led us to consider that type A, produced in the early stage, converted into type B during the cultivation and that this conversion occurred by alteration of the molecular size of type A.

The gel filtration behavior of staphylokinase was also tested with strains 879(756), PS 83A, PS 6, and Terashima. However, only type A was detected in each of the supernatants even in the case of 24-h cultures.

Comparisons of types A and B. To compare types A and B, we performed the following experiments.

(i) Estimation of molecular weights. The molecular weights of types A and B were determined by the gel filtration method of Andrews (1). They were estimated from the elution volume as 15,000 for type A and 320,000 for type B (Fig. 2A and B).

(ii) Heat stability. The degree of inactivation of activity of each type of staphylokinase was examined by heating at 60 °C for 120 min. Both types were prepared so that they had the same activity (180 U/ml), and protein concentrations were also adjusted to the same level with bovine serum albumin prior to heating. The activities of types A and B after heating decreased to 11 and 42% of those of the original samples, respectively.

(iii) Immunological studies. The relationship between dilution of the antisera and inhibition of the staphylokinase activity of each type is illustrated in Fig. 3. $K_p$ (the rate constant for neutralization of type B)/$K_a$ (the corresponding value for neutralization of type A) was calculated as 1.04 and 1.05 when anti-type A and anti-type B sera were used, respectively.

Conversion of type A into type B in vitro. Experiments were carried out to determine
Fig. 2. Determination of molecular weights of types A and B. (A) Type A: A column (90 by 1.5 cm) of Sephadex G-75 was prepared and equilibrated with 0.01 M Tris-hydrochloride buffer (pH 8.4) containing 0.2 M NaCl. The standard proteins (ovalbumin, chymotrypsinogen, and cytochrome c) and type A were applied to this column. (B) Type B: A column (90 by 1.5 cm) of Sephadex G-200 was prepared and equilibrated with 0.01 M Tris-hydrochloride buffer (pH 8.4). Type B was filtered on this column and eluted with the same buffer. The column was equilibrated with the same buffer containing 0.2 M NaCl, and then the standard proteins (bovine serum albumin, human gamma globulin, and horse apoferritin) were separated on and eluted with this buffered saline.

Fig. 3. Neutralization of staphylokinase activities of types A and B by antisera against type A (A) and type B (B). Symbols: O, type A; •, type B.
whether type A is able to convert into type B in vitro. Type B was incubated with type A in an ice bath for 30 min, and then this mixture was subjected to Sephadex chromatography. Most of the activity appeared in the void volume, the position of type B, and only 20% of the recovered activity remained at the position of type A (Fig. 4A–C). This observation indicates that type A does convert into type B in vitro.

If type B was previously treated with Pronase (2 mg/ml) for 30 min at 37 °C, followed by removal of the enzyme by Sephadex gel filtration, added type A never converted into type B.

**Reversion of type B to type A.** An experiment was designed to determine the influence of different eluents on the gel filtration profiles of type B staphylokinase activity.

Eluents containing mercaptoethanol (0.1 M) or urea (6.0 M) and those of low or high pH values (pH 5.0 and 7.0, and pH 9.0, respectively) did not affect the distribution pattern of type B staphylokinase activity! When type B was eluted with KCl (1.0 M), two main peaks appeared (Fig. 5A). The first peak corresponded to the effluent volume of type B and the second peak corresponded to type A. If the staphylokinase which remained to be eluted in the void volume in this run was rechromatographed on Sephadex G-75 under the same conditions, the distribution profile of staphylokinase activity was similar to that of the first gel filtration. When eluent containing Brij-58 (0.1%) was used, a remarkable alteration was observed (Fig. 5B). Almost all activity was found at the position of type A, and only a slight amount of activity was found at the position of type B.

Thus, it seems possible that type B can partially revert to type A in the presence of a high KCl concentration, and this tendency is increased by the action of detergent. Furthermore, these results provide considerable evidence that type B is a complex form of molecule(s) of type A and some other high-molecular-weight substance, tentatively referred to as "complex-forming substance" (CFS), which must be released from the bacterial cells into the surrounding medium in the later stage of the cultivation. The CFS may bind to preexistent type A in the medium and thus type B is formed.

**DISCUSSION**

The existence of two kinds of staphylokinases having different molecular weights has been demonstrated in the culture supernatant of *S. aureus* PS 47 by the gel filtration method. These

![Graph](http://aem.asm.org/Downloaded from http://aem.asm.org on May 7, 2020 by guest)
In the not converted ml/tube. A thousand (molecular weight 47,000) containing buffer (pH 8.4) containing 1.0 M KCl. (B) Eluted with 0.01 M Tris-hydrochloride buffer (pH 8.4) containing 0.1% Brij-58.

Staphylokinases were termed type A and type B. In the early stage of the cultivation, only type A (molecular weight 15,000) was detected, and type B (molecular weight 320,000) was obtained in the later stage. The former value is close to the results of Satoh (13) and Lack and Glanville (9). No staphylokinase having a molecular weight of 320,000 has ever been reported. It is of interest that type A, found in the early stage, converted into type B, in the later stage (Fig. 1). The presence of two kinds of staphylokinases, however, was observed only in strain PS 47. Therefore, this phenomenon may not occur generally over the staphylococcal strains.

The reason why the formation of type B was not observed in strains other than PS 47 is the lack of ability of these strains to synthesize CFS. This assumption is supported strongly by the observation that a staphylokinase produced by strain 879(756), being one of the negative strains for type B, could be converted into type B by mixing with type B produced by PS 47 (S. Fujimura, Ph.D. thesis, Hokkaido University, Sapporo, Japan, 1973). The mechanism of this conversion can be accounted for as follows. The binding sites of CFS are still not saturated, and remaining free binding sites are available for the molecule(s) of type A. This explanation is also quite applicable to the case of the conversion of type A of strain PS 47 into type B in vitro (Fig. 4).

In immunological studies, it was revealed that K value of anti-type A serum against type A was almost equal to that of anti-type A against type B, and the same value was obtained when anti-type B serum was used for the cross-experiment. And reversion of type B to type A occurred by treatment of type B with KCl or detergent (Fig. 5). It seems reasonable, therefore, that the active site of type B is very similar to that of type A.

A significant difference between type A and type B was found in their heat stability, i.e., type B was more stable than type A. This indicates that CFS contributes to the protection of staphylokinase against heat inactivation.

Since the binding activity of CFS was eliminated by treatment with Pronase, CFS itself, or a site which is responsible for the binding activity, is likely to be protein in nature. However, isolation and detailed characterization of CFS are still under investigation.

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