New Exfoliative Toxin Produced by a Plasmid-Carrying Strain of *Staphylococcus hyicus*

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A new serotype of *Staphylococcus hyicus* exfoliative toxin (SHET), serotype B, was isolated from the culture filtrate of a plasmid-carrying strain of *S. hyicus*. The new SHET was purified by precipitation with 70% saturated ammonium sulfate, gel filtration on a Sephadex G-75 column, column chromatography on DEAE-Cellulofine A-500, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The new SHET caused exfoliation of the epidermis as determined by the so-called Nikolsky sign when inoculated into 1-day-old chickens. The new SHET was serologically different from *Staphylococcus aureus* exfoliative toxins (ETs), ETA, ETB, and ETC from the SHET from the plasmidless strain but showed the same molecular weight as the other serotypes of toxins on SDS-PAGE. It was thermostable and lost its toxicity after being heated at 60°C for 30 min. We propose that the new SHET be designated SHETB and that the SHET produced by the plasmidless strain be designated SHETA.

*Staphylococcus hyicus* is the causative agent of exudative epidermitis (EE) in pigs (22). EE is a generalized infection of the skin characterized by greasy exudation, exfoliation, and vesicle formation (5, 11). Sato et al. (19) have isolated an exotoxin from the culture supernatant of *S. hyicus* P-1 and designated it *S. hyicus* exfoliative toxin (SHET). The molecular mass of SHET has been estimated to be 27 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and it differs from *Staphylococcus aureus* exfoliative toxins (ETs) ETA, ETB, and ETC in its antigenicity and in the susceptible animal species (18, 24). Recently, we have confirmed that SHET-producing strains result in clinical signs of EE, whereas non-SHET-producing strains do not cause any clinical signs of EE (25). These findings suggest that SHET, with a molecular mass of 27 kDa, is a causative factor of EE and causes intraepidermal splitting in the granular layer of the epidermis, similar to the case for ETA, ETB, and ETC. However, it differs from ETA, ETB, and ETC in its antigenicity and in the susceptible animal species.

We have recently identified a new serotype of SHET, serotype B (SHETB), from field isolates of *S. hyicus* (20, 25). All of the SHETB-producing strains possess large plasmids as well as the 42-kb plasmid of the ETB-producing strains, and their plasmid-cured substrains have lost their SHET-producing ability (20). In the present paper we describe the antigenicity and some properties of purified SHETB.

MATERIALS AND METHODS

**Bacterial strains.** *S. hyicus* P-23 (20, 25), isolated from a pig affected with EE, was used in this study. The strain was hophilized and stored at 4°C. The hophilized bacteria were suspended in heart infusion broth (Difco Laboratories Inc., Detroit, Mich.) and cultured on heart infusion agar (Difco) at 37°C for 18 h. The bacteria were then suspended in 20% glycerol and stored at −80°C.

**ETs.** SHETB from *S. hyicus* P-1, ETA from *S. aureus* 2M, ETB from *S. aureus* J-sETB-8, and ETC from *S. aureus* Horse-1 were isolated and purified by a method described previously (7, 8, 18, 24). These toxins were used as controls in the suckling-mouse inoculation test, the 1-day-old chicken inoculation test, Western blotting, and the heat stability test.

**Bacterial culture for isolation of SHETB.** For the isolation of SHETB, *S. hyicus* was cultured under the optimum conditions described by Watanabe et al. (27). *S. hyicus* cultured on heart infusion agar was suspended in Dulbecco's phosphate-buffered saline without CaCl2 and MgCl2 (PBS) at a concentration of 108 CFU/ml. A 2-ml portion of this suspension was inoculated in 200 ml of TY broth (6) and cultured at 37°C for 18 h with shaking at 75 oscillations per min. The bacterial culture was centrifuged at 10,000 × g for 20 min at 4°C, and the culture supernatant was passed through a membrane filter (0.45-μm pore size; Toyo Roshi Inc., Tokyo, Japan).

**Determination of optimum concentration of ammonium sulfate.** Ammonium sulfate powder was added to each culture filtrate (100 ml) at various concentrations (10, 20, 30, 40, 50, 60, 70, 80, 90, and 100% saturation). Each of the precipitates was dissolved in a small amount of 10 mM Tris-HCl buffer (pH 7.5) and dialyzed against the same buffer at 4°C for 48 h. Each dialysate was then concentrated to 5 ml with a UP-20 ultrafilter (Toyo Roshi) and twofold diluted serially with the same buffer. Each diluted solution was inoculated subcutaneously in each of two 1-day-old chickens. The exfoliative activity (exfoliation units) of each fraction is shown as the reciprocal of the maximum dilution causing the exfoliation (Nikolsky sign).

**Partial purification of SHETB.** Ten milliliters of concentrated dialysate was loaded on a Sephadex G-75 (Pharmacia Fine Chemicals Inc., Uppsala, Sweden) column (2.6 by 90 cm) eluted with 10 mM Tris-HCl buffer (pH 7.5) and then eluted with the same buffer. Fractions of the effluent yielding a high concentration of protein were pooled and concentrated to 5 ml by ultrafiltration with a UP-20 ultrafilter. This concentrated solution was placed on a DEAE–Cellulofine A-500 column (2.6 by 90 cm) loaded on a Sephadex G-75 (Pharmacia Fine Chemicals Inc., Uppsala, Sweden) column (2.6 by 90 cm) eluted with 10 mM Tris-HCl buffer (pH 7.5) and then eluted with the same buffer. Fractions of the effluent yielding a high concentration of protein were pooled and concentrated by ultrafiltration to 2 ml with a UP-20 ultrafilter.

**SDS-PAGE.** SDS-PAGE was performed at room temperature by the methods of Laemmli (10) and Sato et al. (19). A mixture of 0.05 ml of 500 mM Tris-HCl (pH 6.8), 0.08 ml of 10% SDS, 0.02 ml of 2-mercaptoethanol (Bio-Rad Laboratories, Richmond, Calif.), and 0.05 ml of 0.02% bromothymol blue in 80% glycerol was added to 0.2 ml of protein solution and then incubated at 37°C for 2 h. The cooled sample solution was layered on SDS-12.5% polyacrylamide gel slabs and run at 30 mA per gel. For purification of the SHETB, the proteins in gel slabs were stained with Coomassie brilliant blue R-250 (E. Merck AG Inc., Dermstadt, Germany) and decolored with 7% acetic acid by the method of Fairbanks et al. (4).

**Extraction of SHETB from SDS-polyacrylamide gels.** After electrophoresis, the gels corresponding to the protein bands were cut out and cut into small pieces. The pieces of these gels were suspended in 10 volumes of 1% SDS in 20 mM Tris-HCl (pH 8.0) and incubated at room temperature overnight with gentle shaking. The suspension was dispensed in an inner tube with a membrane filter (0.45-μm pore size) in a test tube and centrifuged at 400 × g for 15 min. The
filters were concentrated to 2 ml with a UP-20 ultrafilter, and 250 μl of this filtrate was dispensed in an Eppendorf tube. One milliliter of cold acetone was added to the filtrate (250 μl) and stored at −80°C for 1 h. After centrifugation at 10,000 × g for 15 min, the precipitates were dissolved in 50 mM Tris-HCl (pH 7.5).

Protein determination. The protein concentration was determined by the bicinchoninic acid protein assay (21, 23) with bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) as the standard. The bicinchoninic acid protein assay kit was purchased from Pierce Chemical Co., Rockford, Ill.

Animals. Ten inbred specific-pathogen-free (SPF) female BALB/c mice, 1 to 3 days old (Japan Slc Inc., Hamamatsu, Japan), were used for the suckling-mouse inoculation test for each ET. Two mice were used for each toxin sample. Four adult female SPF BALB/c mice (more than 8 weeks old; Japanese SLC) were used for the preparation of antibodies to SHETB. Nine 1-day-old conventional White Leghorn chickens were used for the 1-day-old chicken inoculation test of each protein fraction and each toxin sample.

Inoculation tests. (i) Suckling-mouse inoculation test. Each purified toxin (SHETA, SHETB, ETA, ETB, and ETC; 10 μg each) was injected subcutaneously into 1- to 3-day-old BALB/c mice. The explorative activity was regarded as positive when the Nikolsky sign (peeling off of the skin surface easily caused by slight rubbing with fingertip) (7, 12) was identifiable within 3 h of injection.

(ii) One-day-old chicken inoculation test. Each protein fraction and each purified toxin (SHETA, SHETB, ETA, ETB, and ETC; 10 μg) were injected subcutaneously into 1-day-old chickens. The explorative activity was regarded as positive when the Nikolsky sign was identifiable within 3 h of injection.

Antibodies. The purified SHETB was converted to a toxoid by treatment with 0.8% formalin at 37°C for 50 h. The SHETB toxoid was inoculated subcutaneously into adult SPF mice once a week for 4 weeks. The inoculum was a mixture of 50 μg of toxoid and the same volume of Freund’s incomplete adjuvant (Difco). At 4 days after the fourth injection, sarcoma 180 cells (10⁶ cells/0.5 ml) were inoculated intraperitoneally. After 3 days, the toxoid was inoculated into the mice in the same manner. Most of the mice had distended abdomens within 10 to 15 days after the inoculation with sarcoma cells. At that time, the ascitic fluid was withdrawn by paracentesis through an 18-gauge needle into a 10-ml syringe. The ascitic fluids were pooled and centrifuged at 1,500 × g for 20 min. The blood was obtained by cardiac puncture from each of the immunized mice, and the serum was prepared in the same manner as the ascitic fluid. The ascitic fluid and serum were then pooled and designated anti-SHETB antibody. Anti-SHETA, anti-ETA, and anti-ETB antibodies were also prepared from the purified toxins.

ELISA. Enzyme-linked immunosorbent assay (ELISA) was performed by the method of Tanabe et al. (24). A 10-μl portion of each purified toxin (SHETA, SHETB, ETA, ETB, and ETC; 10 μg/ml) in 50 mM carbonate-bicarbonate buffer (pH 9.6) was dispensed into each well of a 96-well microplate (Greiner Labortechnik Inc., Frickenhausen, Germany) and incubated at 4°C for 18 h. The plate was then washed twice with PBS supplemented with 0.05% Tween 20 (T-PBS), and subsequently treated with 25% Block Ace (Yukijirushi Milking Co., Ltd., Tokyo, Japan) at 4°C overnight. After washing with T-PBS, a 100-μl portion of antibody to each toxin was dispensed into the wells, and the plate was then incubated at 4°C for 1 h. After another washing, 100 μl of peroxidase-conjugated anti-mouse immunoglobulin G was mounted onto each strip and incubated at room temperature for 1 h. After washing with T-PBS, the substrate solution (0.05% 3,3′-diaminobenzidine and 0.01% H₂O₂ in 50 mM Tris-HCl, pH 7.7) was mounted on each strip and incubated at room temperature. When the color reaction reached a maximum, each strip was washed with tap water to stop the reaction.

Heat stability of SHETB. Each purified toxin (SHETA, SHETB, ETA, ETB, and ETC) was heated at 100°C for 20 and 40 min and at 60°C for 15 and 30 min. After the heat treatment, 10 μg of each toxin was injected subcutaneously into each of two chickens and two mice, respectively. As a control, the same dose of nontreated toxin was injected subcutaneously into chickens and mice.

RESULTS

Partial purification of SHETB. SHET activity could not be detected in the fraction precipitated with ammonium sulfate at a saturation of 50% or less. The maximum SHET activity was obtained from the fraction precipitated by ammonium sulfate.

![FIG. 1. Sephadex G-75 gel filtration of the 70% saturated ammonium sulfate fraction of culture filtrate of strain P-23.](image-url)
at 70% saturation, and its score was 15 exfoliation units/ml. Figure 1 shows the results of the Sephadex G-75 gel filtration of the toxic fraction precipitated with ammonium sulfate at 70% saturation. Three protein peaks (S-1, S-2, and S-3) were obtained. Fractions of each protein peak were pooled and concentrated to 1 mg/ml. When 10 μg of each preparation was injected subcutaneously into each of two chickens, exfoliative activity was found only with the S-2 preparation (Table 1). In the chickens injected with the S-2 preparation, typical Nikolsky signs appeared at the site of injection within 1 h (Fig. 2). Figure 3 shows the results of the DEAE-Cellulofine column chromatography of the S-2 preparation. Two major peaks (D-1 and D-3) and one minor peak (D-2) were obtained. The fractions for each protein peak were pooled and concentrated to 1 mg/ml. Figure 4 shows the SDS-PAGE pattern for each preparation (D-1, D-2, and D-3). The D-2 preparation gave four major protein bands of 40, 29, 27, and 22 kDa. The 27-kDa protein was detectable in the D-2 preparation but not in the D-1 and D-3 preparations. When 10 μg of each preparation was injected subcutaneously into each of two chickens, exfoliative activity was found only with the D-2 preparation (Table 1).

**Purification of SHETB.** The 40-, 29-, 27-, and 22-kDa proteins were extracted from an SDS-polyacrylamide gel. When 10 μg of each extract was injected subcutaneously into each of two chickens, exfoliative activity was found only with the 27-kDa protein (Table 1). Figure 5 shows the SDS-PAGE pattern for this extract, confirming that the extract consists of the 27-kDa protein alone. Based on these results, we considered the 27-kDa protein to be SHETB.

**Partial characterization of SHETB.** Table 2 shows the animal susceptibilities of five ETs. In the suckling-mouse inoculation test, the Nikolsky sign was observed in each of two mice inoculated with ETA, ETB, and ETC, whereas no sign was observed in the mice inoculated with SHETA and SHETB. In the 1-day-old chicken inoculation test, the Nikolsky sign was observed in each of two chickens inoculated with SHETA, SHETB, and ETC, whereas no sign was observed in the chickens inoculated with ETA and ETB. Table 3 shows the heat-stabilities of five ETs. The toxic activities of SHETB, ETB, and ETC were eliminated after treatment at 60°C for 30 min. The activity of SHETA was eliminated after treatment at 60°C for 15 min. ETA was stable after being heated at 100°C for 20 min. Figure 6 shows the results of the Western blot analysis of the ETs. The anti-SHETB antibody reacted with the 27-kDa protein band of only SHETB. Similarly, the anti-ETA, anti-ETB, and anti-SHETA antibodies reacted with the 27-kDa protein bands of their homologous toxins.

**DISCUSSION**

*S. hyicus* is a causative agent of EE in pigs (22). Amtsberg (1) has suggested that SHET is one of the virulence factors of *S. hyicus*; at the time of Amtsberg’s report, however, the isolation and purification of SHET had not yet been achieved. In 1991, we reported the isolation of SHET from culture filtrates of *S. hyicus* and the production of exfoliation in piglets injected with SHET (19). We also stated that the molecular mass of SHET seemed to be 27 kDa (24) and that the susceptible animal species were piglets and chickens (17). We have recently clar-

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**TABLE 2. Animal species susceptible to SHETB**

<table>
<thead>
<tr>
<th>Animal</th>
<th>ETA</th>
<th>ETB</th>
<th>ETC</th>
<th>SHETA</th>
<th>SHETB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suckling mouse</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>One-day-old chicken</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>
ified that SHET can be divided into at least two serotypes (SHETA and SHETB) and that SHET-producing strains of *S. hyicus* cause EE in piglets, whereas non-SHET-producing strains do not (20, 25). Similar findings have been reported by Andresen (2), Andresen et al. (3), and Wegener et al. (28). Moreover, we have reported that the production of SHETA and SHETB is genetically controlled by chromosomal DNA and a 42-kb plasmid (pKUH-1) (20).

Several investigators have isolated two serotypes of ETs (ETA and ETB) from *S. aureus* strains derived from patients with staphylococcal scalded-skin syndrome (7–9, 29). ETA is a heat-stable toxin and its production is genetically controlled by chromosomal DNA (7, 14, 16), while ETB is a heat-labile toxin and its production is controlled by a 42-kb plasmid (8, 13, 15). However, both toxins have the same molecular mass (27 kDa), the same susceptible animal species (humans and suckling mice), and the same toxic activities (formation of an intraepidermal cleavage plane). The SHETA produced by the plasmid-carrying strain of *S. hyicus* is a new serotype of *S. hyicus* is not fully understood. We therefore attempted to purify SHETB in this study.

In the purification of SHETA (24, 27), SHETB activity was highest in the fraction precipitated with a 90% saturation of ammonium sulfate, and the SHETB activity could be detected in the second peak on Sephadex G-75 gel filtration and in the first peak (at 0 to 0.05 M NaCl) on ion-exchange chromatography. The SHETA activity was highest in the fraction precipitated with a 70% saturation of ammonium sulfate, and the SHETB activity could be detected in the second peak on gel filtration and the second peak (at 0.12 to 0.15 M NaCl) on ion-exchange chromatography. Such differences in the purification steps for the SHETs seem to reflect differences in composition between the SHETA and SHETB molecules. Differences in the optimal concentrations of ammonium sulfate and different elution profiles on ion-exchange chromatography were also seen in the purification steps for ETA and ETB (7, 8).

SHETA is a heat-labile toxin, since its toxicity is lost after heating at 60°C for 15 min, and its molecular weight is approximately 27,000 (19, 24). SHETB is also a heat-labile toxin with a molecular weight of 27,000, but it retains its toxicity longer, finally losing its toxic activity after heating at 60°C for 30 min. The differences in heat stability between SHETA and SHETB also seem to reflect the composition differences between SHETA and SHETB molecules.

In the Western blotting analysis, antibody to SHETB reacted only with SHETB and not with ETA, ETB, and SHETA. Similarly, each toxin reacted with antibody to the same toxin. These results suggest that the SHETB obtained from the plasmid-carrying strain (P-23) of *S. hyicus* is a new serotype of SHET.

**ACKNOWLEDGMENTS**

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**REFERENCES**

17. Sato, H., M. Kuramoto, T. Tanabe, and H. Saito. 1991. Susceptibility of various animals and cultured cells to exfoliative toxin produced by *Staphy-

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