Characteristics of Enterotoxin H–Producing
Staphylococcus aureus Isolated from Clinical Cases and Properties of the Enterotoxin Productivity

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ABSTRACT

Staphylococcal enterotoxin H (SEH) is predicted to be involved in staphylococcal food poisoning. To characterize SEH-producing Staphylococcus aureus isolates from staphylococcal food poisoning cases in Japan, we investigated the relationship between SEH production and coagulase serotype, which is an epidemiological marker, and compared the properties of SEH production with those of staphylococcal enterotoxins A (SEA) and B (SEB). SEH production was determined by a newly developed sandwich enzyme-linked immunosorbent assay. Eighty-six (59.7%) of 144 isolates from staphylococcal food poisoning cases produced SEH. Seventy-one of the SEH-producing isolates simultaneously produced SEA, SEB, or both. All SEH-producing isolates belonged to coagulase type VII, which was the predominant type, representing 99 (68.8%) of 144 isolates. The amount of SEH produced in brain heart infusion was almost the same as the amount of SEA and approximately 10-fold lower than that of SEB. SEH and SEA were produced mainly during the late exponential phase of growth, whereas SEB was produced mostly during the stationary phase. The production levels of SEH and SEA were greatly affected by decreases in water activity, but the production of SEB was greatly reduced under conditions of low water activity. These findings indicate that SEH-producing S. aureus isolates are of high prevalence in staphylococcal food poisoning cases. Given the unique epidemiological characteristic of these isolates, SEH and SEA probably are responsible for food poisoning.

Staphylococcal enterotoxins (SEs) are exotoxins produced by Staphylococcus aureus. Classical SEs have been classified serologically into five types: SEA, SEB, SEC, SED, and SEE (6). However, new types of SEs have been found in recent years; three new types of SEs (SEG, SEH, and SEI) (15, 26) and 10 staphylococcal enterotoxin–like superantigens (SEls) (10, 11, 19) have been reported. Classical SEs cause staphylococcal food poisoning (SFP), which induces vomiting and diarrhea, and are virulence factors in some cases of toxic shock–like syndrome (6). SEG, SEH, and SEI provoked an emetic response in monkeys (15, 26), whereas some SEls appear to inhibit the emetic ability and others have not yet been tested for emetic potential. It is still unclear whether SEG, SEH, SEI, and SEls are responsible for food poisoning.

S. aureus strains carrying genes for SEG, SEH, SEI, and SEls have been isolated from various foods (2, 4, 12). If SEG, SEH, SEI, and SEls are involved in SFP, strains possessing genes for these toxins must produce large enough amounts of the toxins to cause toxicity. In a previous study, SEH was produced in significant amounts in an appropriate medium by an S. aureus strain carrying seh, but either small amounts of SEG and SEI or no detectable toxin were produced by S. aureus strains carrying seg and sei, respectively (21). In two cases of SFP that occurred in Japan and Norway, SEH was detected from the foods responsible for toxicity (1, 8, 9). These observations suggest that S. aureus strains carrying seh can produce SEH in amounts sufficient to cause toxicity. Therefore, SEH is suspected to be involved in SFP. In the present study, we investigated the epidemiological characteristics of SEH-producing S. aureus isolates associated with SFP and their SEH production in an attempt to establish the relationship between SEH and SFP.

Various epidemiological methods, such as biotyping, pulsed-field gel electrophoresis, phage typing, and coagulase typing (13, 23, 24, 31, 32), have been used in the examination of SFP incidents to discriminate among S. aureus strains. In Japan, coagulase typing has been the most common epidemiological method for characterizing SFP cases (17). This typing method is based on eight different antigenic types (I to VIII) of coagulase proteins. Coagulase type VII has been reported as the predominant type of coagulase responsible for food poisoning incidents throughout Japan, although other S. aureus strains also are capable of producing SEs. There is no association between classical SE production and any particular type of coagulase, but the relationship between SEH and coagulase types has not been investigated.
Production of classical SEs has been relatively well studied under various environmental conditions. The amount of toxin produced varies by toxin type or culture conditions, e.g., type of medium, pH, and water activity \((a_w)\) (3, 22, 29, 30, 33). Although one study was focused on SEH production in specified culture media under anaerobic or low pH conditions (27), the amount of SEH produced has not been compared with the amounts of classical SEs produced under the same conditions. Furthermore, the effect of \(a_w\) on SEH production remains unclear. It is important to examine SEH production under conditions of low \(a_w\) because \(S.\) aureus is an osmotolerant foodborne pathogen.

The present study was conducted (i) to ascertain the proportion of SEH-producing strains among SFP isolates in Japan and to investigate the relationship between SEH production and coagulase type and (ii) to assess the production of SEH in comparison with that of SEA and SEB in culture medium and under low \(a_w\) conditions. For these purposes, we developed a sandwich enzyme-linked immunosorbent assay (ELISA) by using recombinant SEH and a specific antibody.

**MATERIALS AND METHODS**

**Bacterial strain and growth conditions.** One hundred forty-four \(S.\) aureus isolates from 121 cases of outbreak or sporadic SFP diagnosed by local government laboratories in Japan from 1983 to 1995 were used for the evaluation of enterotoxin production and coagulase typing. The isolates were obtained from the foods involved in the SFP incidents and from patient vomit and feces and were identified as \(S.\) aureus by laboratories according to standard methods for \(S.\) aureus (e.g., the production of coagulase, egg yolk factor, and enterotoxins and other biochemical properties). \(S.\) aureus strains 100 (producing SEA), 243 (SEB), FRI-137 (SEC), 494 (SED), FRI-326 (SEF), and FRI-569 (SEH) were used as reference strains for production of the respective SEs. For the assessment SEs production, \(S.\) aureus was grown in brain heart infusion (BHI) broth (Becton Dickinson, Sparks, Md.) at 35°C for 24 h in a shaking incubator at 200 rpm. To examine the timing of SEH production throughout the growth phase of \(S.\) aureus, SEH-producing isolates (1,000 CFU/ml) were inoculated into 100 ml of BHI and grown at 35°C for 24 h. Growth of \(S.\) aureus isolates was determined by plating serial dilutions of culture fluids onto mannitol salt–containing egg yolk agar plates. To examine the effect of \(a_w\) on SE production, the \(a_w\) of the BHI broth was adjusted with 4.8% sodium chloride to give a final value of 0.97 or with 8.0% sodium chloride to give a final value of 0.95. The \(a_w\) values of the growth media were measured with a Hygrometer AwV C (ROTRONIC AG, Bassersdorf, Switzerland).

**Preparation of recombinant SEH.** The seh gene of \(S.\) aureus strain Osaka 1 from an SFP case was amplified by nested PCR assay. The PCR primers were designed based on DNA sequences in GenBank (accession no. AP 004822): 5′-GTTGGTT-TTTGTTGTGTTTTATATA-3′ (forward) and 5′-AAAGACATGAC-AATTCACACTA-3′ (reverse) for the first PCR and 5′-GGGAT-TCGGAGATTACAGGTTAA-3′ (forward) and 5′-CCCAA-GCTTATACTTTTTCTTAGTATA-3′ (reverse) for the second PCR. EcoRI and HindIII sites were included at the ends of the forward and reverse primers, respectively, for the second PCR. After digestion with EcoRI and HindIII, the PCR products were ligated with T4 DNA ligase (Boehringer Mannheim, Meylan, France) into pET30a (Novagen, Madison, Wis.) or pMAL vector (New England Biolabs, Beverly, Mass.). The insertion was verified by DNA sequencing. Each recombinant plasmid was used to transform Escherichia coli BL21 CodonPlus (DE3)-RIL (Stratagene, La Jolla, Calif.) for pET30a or DH5 for pMAL. \(E.\) coli cells were grown at 37°C in Luria-Bertani broth containing 25 \(\mu\)g/ml kanamycin for pET30a or 50 \(\mu\)g/ml ampicillin for pMAL. After the addition of 50 \(\mu\)M isopropyl-\(\beta\)-D-thiogalactoside, growth was continued at 37°C for an additional 3 h. The cells were harvested and resuspended in BugBuster protein extraction reagent (Novagen) containing a protease inhibitor mixture (Sigma-Aldrich, St. Louis, Mo.). After incubation for 1 h on ice, the cells were sonicated on ice and centrifuged at 27,000 \(\times\) g for 15 min at 4°C. The supernatant was subjected to affinity chromatography with a nickel-nitrilotriacetic Superflow column (Qiagen, Chatsworth, Calif.) for His-tagged SEH (His-SEH) or a amylose-resin column (New England Biolabs) for maltose binding protein fusion SEH (MBP-SEH), according to the respective manufacturer’s instructions.

**Preparation of polyclonal antibody against recombinant SEH.** His-SEH was emulsified with complete Freund’s adjuvant (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and injected subcutaneously into rabbits. A 10-μg inoculum of His-SEH was injected five times at 2-week intervals and then boosted with 100 μg of His-SEH 40 days after the fifth injection. Rabbit sera were obtained 2 weeks after the final immunization. Antiserum was diluted two- or threefold with 10 mM sodium phosphate buffer (pH 7.0) and separated in a HiTrap-NHS column (GE Healthcare Bio-Sciences Corp., Piscataway, N.J.) with MBP-SEH. The column was washed with 10 mM sodium phosphate buffer (pH 7.0), and the protein bound to the column was eluted with 0.1 M glycine-HCl buffer (pH 2.7). The eluate was immediately neutralized with 1.5 M Tris-HCl buffer (pH 8.8) to prevent inactivation of the antibody. The antibody was then collected by 50% ammonium sulfate precipitation and dialyzed against phosphate-buffered saline (PBS) containing 0.05% sodium azide. For the preparation of biotin-labeled antibody, the purified antibody (1 mg) was incubated with 23 μg/ml EZ-link NHS-Biotin (Pierce Biotechnology, Inc., Rockford, Ill.) in 50 mM sodium bicarbonate buffer (pH 8.5) for 2 h at 0°C. After incubation, excess nonreacted and hydrolyzed biotin reagent was removed with an Amicon Ultra centrifugal filter device (Millipore, Billerica, Mass.). The buffer was changed to PBS by centrifuging three times in the centrifugal filter device.

**Sandwich ELISA.** Culture supernatants from \(S.\) aureus were preincubated with 5% (vol/vol) normal rabbit serum at 4°C overnight to prevent nonspecific reactions caused by Protein A. The antibody solution (2.5 μg/ml) in 0.05 M sodium bicarbonate buffer (pH 8.5) was added to a 96-well plate (100 μl per well; Nalgene Nunc International, Rochester, N.Y.) and incubated overnight at 4°C. The plate was washed three times with a washing buffer of PBS containing 0.1% Tween 20 (pH 7.2) (Sigma-Aldrich). As a blocking treatment, PBS containing 1% bovine serum albumin (BSA; Wako) was added to the plate and incubated for 1 h at room temperature. Native SEH (0.1 mg) in a vial (Toxin Technology, Inc., Sarasota, Fla.) was dissolved in PBS(−) and diluted at various concentrations in a carrier buffer (PBS containing 0.2% BSA) for use as a standard. Samples (100 μl) diluted with carrier buffer were added to triplicate wells and incubated for 1 h at room temperature. After washing three times, the biotin-labeled antibody (0.4 mg/ml) diluted 1,000-fold with carrier buffer was added to each well (100 μl per well), and the plate was incubated for 2 h at room temperature. After washing, an avidin-biotin-peroxidase complex detection system (VECTASTAIN Elite ABC Kit, Vector Laboratories, Inc., Burlingame, Calif.) was used for detection ac-
FIGURE 1. Results of ELISA for SEH. (A) Specificity of sandwich ELISA for the detection of SEH. Culture supernatants of six S. aureus reference strains, which produce the SEs shown in parentheses, were subjected to the sandwich ELISA for the detection of SEH. (B) Linearity of dilution curves. Data are based on serial dilution of culture supernatants of three strains: IFH226 producing both SEA and SEH (♦), IFH55 producing both SEB and SEH (▲), and IFH349 producing SEH alone (▲). Error bars show the standard deviation of triplicate determinations.

TABLE 1. Enterotoxin production by and coagulase types of S. aureus isolates from food poisoning incidents

<table>
<thead>
<tr>
<th>Enterotoxin</th>
<th>No. of isolates</th>
<th>No. of isolates producing coagulase types:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>II</td>
</tr>
<tr>
<td>SEA-SEH</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>SEA-SEB-SEH</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>SEH</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>SEB-SEH</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>SEA</td>
<td>17</td>
<td>3</td>
</tr>
<tr>
<td>SEA-SED</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>SEB</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>SED</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>SEA-SEB</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>SEA-SEC</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>SEB-SED</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Not detected</td>
<td>13</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>144</td>
<td>12</td>
</tr>
</tbody>
</table>

full-strength solution (designated 1:1) and in serial dilutions of this solution (1:2 to 1:16).

Detection of classical SEs. Classical SEs (SEA through SEE) were detected with a reversed passive latex agglutination test kit (Enterotox-F, Denka Seiken Co., Ltd., Tokyo, Japan) or an ELISA kit (Transia Plate, Raisio Diagnostics, Turku, Finland). Purified SEA through SEE (Toxin Technology) were used for the preparation of standard curves.

Coagulase typing. Coagulase typing was performed with a coagulase typing kit (Denka Seiken) according to the procedure of Ushioda et al. (31), with rabbit-specific antisera against coagulase types I through VIII.

RESULTS

Sandwich ELISA for the detection of SEH. A sandwich ELISA was developed for the detection of SEH with the purified polyclonal antibody against recombinant SEH. The reactivities of the sandwich ELISA with the culture supernatants of six different type strains of S. aureus are shown in Figure 1A. Only the culture supernatant of strain FR1569 exhibited high reactivity, indicating that this ELISA possessed no cross-reactivity with the other type culture supernatants. The minimum concentration of SEH detected by the ELISA was 1 ng/ml, and SEH was detectable quantitatively between 1 to 25 ng/ml (data not shown). When the culture supernatants of three strains were diluted and assayed, each sample gave results close to linearity (r = 0.997 to 1.000), confirming parallelism between calibrators and culture samples (Fig. 1B).

SE production and coagulase types in S. aureus isolates from cases of SFP. The relationship between the SE and coagulase types in 144 isolates is shown in Table 1. The enterotoxin-producing isolates were classified into 12 types, 7 of which included isolates that produced two or more SEs. Eighty-six (59.7%) of the isolates produced SEH. SEA-producing isolates were the most common (62.5%). SEB and SEC were detected in 31.9 and 11.1%
Comparison of the amount of toxin produced by S. aureus isolates that produce SEA-SEH and SEB-SEH. A reversed passive latex agglutination test was used for the detection of SEA and SEB, and SEH levels were determined by ELISA. The data represent the mean ± standard deviation for isolates producing SEA-SEH (n = 35) and for isolates producing SEB-SEH (n = 13).

Comparison of SEA, SEB, and SEH levels. In the 35 isolates that produced both SEA and SEH and 13 isolates that produced both SEB and SEH, the individual levels of SEA, SEB, and SEH in BHI were determined (Fig. 2). The level of SEH produced by the SEA-SEH isolates was almost the same as that of SEA (SEA, 0.41 ± 0.30 μg/ml; SEH, 0.51 ± 0.24 μg/ml). There was no difference in the levels of SEH produced by the SEA-SEH isolates and the SEB-SEH isolates. The SEB-SEH isolates produced higher amounts of SEB (10.5 ± 9.71 μg/ml) than of SEH (0.91 ± 0.55 μg/ml).

Relationship between growth phase and SE production. The timing of SEH production was compared with those of SEA and SEB to examine the production of SEH throughout the growth cycle (Fig. 3). S. aureus IFH226, producing both SEA and SEH, and S. aureus IFH55, producing both SEB and SEH, were used. The cultures of these two strains had a lag phase of 2 h, a phase of exponential growth, and then entered a stationary phase at 12 h of incubation for IFH226 and 10 h of incubation for IFH55. In strain IFH226, SEA and SEH were detected after 8 h of incubation, corresponding to the late exponential phase. In strain IFH55, most SEB was produced during the stationary phase (after 10 h of incubation), whereas SEH appeared mainly during the late exponential phase (8 to 10 h of incubation). Although the SEB level of IFH55 continued to increase during the stationary phase, SEH was maintained at the same level during the stationary phase.

Effect of aw on SE production. S. aureus strains IFH226, IFH55, and IFH349 were cultivated in BHI adjusted to aw of 0.97 and 0.95 by the addition of NaCl. BHI (without added NaCl) with an aw of 1.00 served as the control. All strains grew well under every aw condition. The
TABLE 2. Effects of a_w adjusted by NaCl on enterotoxin production by S. aureus isolates grown in BHI

| Isolate | Enterotoxin | Enterotoxin production (µg/ml) at a_w:\n| | | 1.00 | 0.97 | 0.95 |
|---------|-------------|-----------------|-----------------|-----------------|
| IFH226  | SEH         | 1.3             | 2               | 0.7             |
|         | SEA         | 1.2             | 1.1             | 0.8             |
| IFH55   | SEH         | 2               | 3.3             | 1.2             |
|         | SEB         | 13.7            | 4               | 0.3             |
| IFH349  | SEH         | 0.6             | 1.4             | 0.8             |

* The a_w value was adjusted to 0.97 or 0.95 by the addition of NaCl to BHI. The a_w of BHI without NaCl was 1.00.

OD_{600} values of the cultures exceeded 1.0 after 24 h, although the growth in the low a_w media was slower than that in the control medium (data not shown). Levels of SEs in culture supernatant after 24 h of incubation for the three isolates are shown in Table 2. SEH production by the three isolates increased by approximately 150 to 230% when the a_w of the medium was reduced from 1.00 to 0.97 and decreased to approximately 35.0 to 57.1% when the a_w of the medium was reduced from 0.97 to 0.95. SEA production by IFH226 reduced gradually as the a_w decreased whereas SEB production by IFH55 in a_w 0.95 medium was rapidly reduced to 2.2% of the control production.

**DISCUSSION**

Recent studies revealed that the genes encoding SEG, SEH, and SEI are distributed widely among *S. aureus* isolates from a variety of sources (16, 18, 20, 25). However, few attempts have been made to examine the production of SEG, SEH, and SEI because no commercial kits to detect these toxins are available. In this study, we developed a sandwich ELISA to detect SEH using an antibody against recombinant SEH expressed in *E. coli*. The anti-SEH antibody was purified by affinity chromatography using a column coupled with recombinant SEH; consequently, the ELISA with this antibody specifically detected SEH, even though SEH shares significant sequence homology with other SEs (6). Dilution curves from three strains producing SEH indicated no significant interference by culture constituents. The ELISA was sufficiently sensitive and specific to detect SEH in *S. aureus* culture.

We evaluated 144 *S. aureus* isolates from 121 cases of sporadic SFP in Japan that occurred from 1983 to 1995 and were associated with different food vehicles. The findings that 62.5% of isolates examined produced SEA and 68.8% of isolates belonged to coagulase type VII are consistent with results of an earlier epidemiological study of SFP in Japan (17). SEA is produced in a variety of food environments (14), which may be one of the reasons why SEA is the most common toxin associated with SFP. SEA-producing isolates produced not only coagulase type VII but also types II, III, IV, and VI. The reason for the dominance of coagulase type VII in SFP isolates remains to be clarified.

We found that 59.7% of the isolates produced SEH, indicating that SEH-producing *S. aureus*, in addition to SEA-producing *S. aureus*, was of high prevalence in SFP cases in Japan. All of the SEH-producing isolates were classified as coagulase type VII, although none of the classical SEs has been linked to a particular type of coagulase. The linkage between SEH production and coagulase type VII may be the reason for the predominance of coagulase type VII in strains from SFP cases. Some attempts have been made to characterize SEs in relation to other epidemiological markers such as phage type or pulse-field gel electrophoresis type (24, 32). However, no adequate marker relating to SE production has been found. The correlation between SEH production and coagulase type VII is therefore considered unique and informative.

The production of classical SEs under different environmental conditions has been relatively well studied. In particular, the production of SEA differs from that of SEB. When *S. aureus* is cultivated, the toxin yield is much lower for strains producing SEA than for those strains producing SEB (3). SEA is produced mainly during the late exponential phase of the growth cycle, whereas most SEB emerges during the stationary phase of the growth cycle (5). The production of SEs also is affected by decreases in a_w, although SEA production is less sensitive to a_w than in production of SEA (29, 30, 33). In the present study, the yield of SEH was compared with that of SEA and SEB. SEH production closely resembled that of SEA, the amount of which was only about one tenth that of SEB in BHI. SEH and SEA production was observed during the late exponential phase, and SEB was produced during the stationary phase. Under conditions of low a_w, SEH and SEA production was affected gradually by decreases in a_w, whereas SEB production decreased rapidly, as described previously. These results indicate that the SEH production is similar to that of SEA, providing important information for understanding the conditions needed for SEH production. The production of SEA and SEB is regulated by different mechanisms (7, 28). SEH may be produced under conditions where SEB production is suppressed. This hypothesis is supported by the fact that SEH and SEA was detected in food associated with an outbreak of SFP in Japan in 2000 (1, 8).

Slight differences in production of SEH and SEA were observed for strains grown in BHI with an a_w of 0.97. SEH production increased when the a_w of the medium was reduced from 1.00 to 0.97, whereas SEA production slightly decreased. It is unclear whether the increase of SEH production in the medium with an a_w of 0.97 is associated with high prevalence of SEH-producing *S. aureus* in SFP cases. It remains to be determined in what food matrices SEH would be produced in amounts sufficient to cause SFP. SEH production in various foods should be studied further.

SEH-producing *S. aureus* strains were highly prevalent in SFP cases and belonged to coagulase type VII, and SEH production was similar to that of SEA. This finding suggests that SEH, in addition to SEA, is responsible for food poisoning. We propose further careful monitoring and surveillance of SEH in SFP incidents to confirm the food poisoning risk associated with SEH.
REFERENCES