Enzyme-Linked Immunosorbent Assay for *Escherichia coli* Heat-Stable Enterotoxin Type II

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The gene for *Escherichia coli* heat-stable enterotoxin type II (ST_{II}) was fused to the genes for protein A from *Staphylococcus aureus* and β -galactosidase in two different expression systems. Antibodies raised in rabbits against the protein A-ST_{II} fusion protein recognized the β -galactosidase-ST_{II} fusion protein. The latter fusion protein was used as the immobilized antigen in an enzyme-linked immunosorbent assay (ELISA) for detection of ST_{II}. The correlation between the results of the ELISA and the intestinal loop test in piglets was 95%, suggesting that the ELISA can be used to reliably detect ST_{II}.

Enterotoxigenic *Escherichia coli* (ETEC) can cause severe diarrhea in humans and animals by the production of any of at least three different kinds of enterotoxins; heat-labile enterotoxin (LT) and two heat-stable enterotoxins, types I and II (ST_I and ST_{II}) (3, 6).

The heat-labile enterotoxin has been well characterized. It resembles cholera toxin in both structure and mode of action. LT stimulates adenylate cyclase to raise cyclic AMP levels in gut epithelial cells, leading to fluid secretion and severe diarrhea (4, 5, 10).

Based on methanol solubility and animal species specificity, the two different types of heat-stable enterotoxins can be distinguished (3, 36). ST_I (or ST_a in the nomenclature of Burgess et al. [3]) is methanol soluble and active in the intestine of neonatal piglets and infant mice. This enterotoxin has also been called ST mouse (28). Furthermore, ST_I can be subdivided into two related but genetically distinct forms, ST_{Ia} and ST_{Ib}. ST_{Ia} is composed of 18 amino acids, produced by *E. coli* strains of bovine, porcine, and human origin (24, 32). ST_{Ib} is composed of 19 amino acids and found in ETEC from humans only (1, 23). ST_I has been shown to activate guanylate cyclase (12, 30).

ST_{II} (or ST_b [3]) is methanol insoluble and active in weaned pigs but inactive in infant mice. This enterotoxin is most likely the same as ST pig (Olsson and Söderlind [28]), which is positive in both weaned and neonatal piglets. The enterotoxin ST_{II} has not yet been purified, but the gene for ST_{II} has been cloned (18, 29). The amino acid sequence was deduced from the nucleotide sequence, revealing a 71-amino-acid peptide, including the signal sequence.

Detection of ST_I and ST_{II} has usually been done by the infant mouse assay (28) for ST_I and the weaned pig intestinal loop assay for ST_{II} (33). These tests are laborious, expensive, and not fully accurate.

Other sensitive and simple assays have been developed for the detection of ST_I, such as radioimmunoassay (7, 9) and enzyme-linked immunosorbent assay (ELISA) (32). These

techniques have greatly improved epidemiological and other studies of ST_I.

The pig intestinal loop test as performed by Olsson and Söderlind (28) does not fully discriminate between LT and ST_{II}. The irreproducibility and expense of the intestinal loop test have also limited the number of studies on ST_{II} occurrence. Using colony hybridization, Moon et al. (22) made a survey of the enterotoxin genes among swine ETEC isolates. ST_{II} was found in about three-quarters of the isolates. Among bovine ETEC strains, however, ST_{II} seems to be less common (20).

The aim of the present study was to develop a sensitive ELISA for detection of ST_{II} by using the ST_{II} gene fused to either of two different carrier genes. One fusion product was used as the ELISA coating antigen and the other for production of antibodies. This technique would greatly improve epidemiological and other studies in the future.

MATERIALS AND METHODS

Bacterial strains. ETEC strains 853/67 (ST_I, ST_{II}, and LT), Bd3505 (ST_I), and Bd2661 (ST_{II}) were porcine clinical isolates obtained from O. Söderlind, National Veterinary Institute. These strains served as reference strains. *E. coli* HB101, JM105, and MZ1 (25) were used as hosts in gene cloning experiments, and strain Bd1427 (no enterotoxin) was used as a negative control. *Staphylococcus aureus* SA113 was used as the host for production of the protein A-ST_{II} (pA-ST_{II}) fusion protein. This strain has no endogenous protein A (14). ETEC strains for evaluation of the ELISA method were obtained from O. Söderlind and tested in the 18-h piglet intestinal loop assay (3, 28, 33). *E. coli* strains were grown in Luria broth (LB) (21), and *S. aureus* strains were grown tryptic soy broth (Difco Laboratories).

Purification of plasmid DNA. The method described by Birnboim and Doly (2) was used to purify plasmid DNA after slight modification of the method for extraction of plasmid DNA.

Transformation of bacteria. *E. coli* strains were transformed with plasmid DNA by the Ca treatment method (21). *S. aureus* SA113 was transformed by protoplasting as described by Götz et al. (11).

Construction of gene fusions. Plasmid pRAS (identical with

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pCHL7), obtained from S. Moseley (18), carries the gene for ST_{II} on a *Hind*III fragment in pBR322. Since the native ST_{II} protein has not been purified, the sequence of the N-terminal end is not known and it is not possible to tell exactly where cleavage of the signal peptide takes place. However, by assuming that cleavage is between Ala-23 and Ser-24 according to the -1, -3 rule of von Heijne (13), the fusion point between protein A and ST_{II} was selected according to this assumption. Therefore, the region between this proposed N-terminal Ser and the *Bgl*II site at Asp-31 was resynthesized as an *Eco*RI-*Bgl*II fragment so that the TC in the *Eco*RI site (GAATTC) constitute the first two nucleotides in the Ser codon. This *Eco*RI site in the *Eco*RI-*Bgl*II 24-mer was joined with plasmid pACE33 cleaved with *Eco*RI and *Pst*I and to the 245-base-pair (bp) *Bgl*II-*Pst*I fragment from pRAS (Fig. 1A). A correct reading frame between protein A and ST_{II} is then obtained.

Plasmid pACE33 is a protein A secretion and expression plasmid based on both pBR322 and pC194 and can therefore replicate in both *E. coli* and *S. aureus*. Plasmid pACE33 has been described (26) under the name pRIT5.

E. coli HB101 was transformed with the ligation mixture, and a clone with plasmid pIG15 (Fig. 1A) was isolated. This plasmid was transformed into *S. aureus* to obtain secretion of the pA-ST_{II} fusion protein into the extracellular medium (27). Identification of pIG15 was done by restriction enzyme analysis.

To obtain a gene fusion between the *lacZ* gene, coding for β -galactosidase, and the ST_{II} gene, the pEX β -galactosidase expression system was used (34). The promoter driving the *lacZ* gene in this plasmid is the *p_R* promoter from phage λ , which becomes temperature inducible if the host bacterium carries a temperature-sensitive repressor. As in the pA-ST_{II} fusion, the fusion point was selected at the proposed native N terminus of ST_{II}. We therefore started with plasmid pIG15 described above and cleaved it with *Eco*RI and *Pst*I to obtain the 270-bp *Eco*RI-*Pst*I fragment. This fragment was inserted in a pEX vector to give a correct reading frame in the 3' end of the *lacZ* gene. *E. coli* MZ1 was transformed, and a transformant with the resulting plasmid pIG30 was isolated (Fig. 1B). Identification of pIG30 was done by restriction enzyme analysis.

Purification of ST_{II} fusion proteins. Even though native ST_{II} has not yet been purified, the fusions to protein A or β -galactosidase could be easily purified. Protein A fusions can be affinity purified on immunoglobulin G (IgG) resins (B. Nilsson, Ph.D. thesis, Royal Institute of Technology, Stockholm, Sweden, 1986), and β -galactosidase can be purified by using substrate analogs (35) or antibodies (8) or as refractory bodies (15).

The pA-ST_{II} fusion protein was purified as follows. *S. aureus* with pIG15 was grown overnight in 1 liter of tryptic soy broth. After centrifugation, the supernatant was passed through a 10-ml IgG-Sepharose Fast Flow column (Pharmacia, Sweden), equilibrated with phosphate-buffered saline (PBS; 10 mM, pH 7.4). The column was washed with 100 ml of PBS and 60 ml of 5 mM ammonium acetate (pH 6.8), and the pA-ST_{II} protein was then eluted with 1 M sodium acetate (pH 2.8). The material was then lyophilized, dissolved in 3 ml of PBS, and dialyzed. The purity of the protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A band corresponding to the expected molecular weight was found (Fig. 2A). The yield was approximately 2 to 3 mg/liter.

The β -galactosidase-ST_{II} fusion protein was purified by means of its insolubility as inclusion bodies. These are

FIG. 1. (A) Construction of plasmid pIG15 with a pA-ST_{II} fusion. (B) Construction of plasmid pIG30 with a β -galactosidase-ST_{II} fusion. Symbols: open bars, ST_{II} gene; solid bars, 24-mer coding for eight NH₂-terminal amino acids in ST_{II}; hatched bars, protein A gene; stippled bars, *lacZ* gene; open circle with arrow, *p_R* promoter; solid circle with arrow, protein A promoter.

formed in the host cells when β -galactosidase-ST_{II} is produced in high amounts. An overnight culture of *E. coli* MZ1(pIG30) in LB at 30°C (Fig. 2A, lane 1) was diluted 50 times into 1 liter of LB, cultivated for 2 h at 30°C, and then shifted to 43°C for an additional 2 h to induce expression (Fig. 2A, lane 2). Cells were then lysed with lysozyme (1 mg/ml) and EDTA (20 mM) in 50 mM Tris (pH 8.0). After lysis,

FIG. 2. (A) SDS-PAGE. Lanes: 1, cell lysate from *E. coli* MZ1(pIG30) grown at 30°C; 2, cell lysate from *E. coli* MZ1(pIG30) grown at 43°C; 3, partially purified (enrichment of insoluble proteins) β -galactoside-ST_{II} fusion protein; 4, partially purified β -galactosidase; 5, markers for molecular weight determination (shown to the left in thousands); 6, purified pA-ST_{II} fusion protein; 7, purified protein A. (B) Lanes 1 to 5 from an SDS-PAGE gel similar to that in panel A were transferred to nitrocellulose paper and probed with sera raised against the pA-ST_{II} fusion protein.

the cells were treated for 30 min with 1% Triton X-100 to solubilize most proteins. The lysate was then centrifuged at $100,000 \times g$ for 15 min. The pellet was suspended in Tris buffer with 1% Triton X-100 to wash away any trapped soluble proteins. After a second similar centrifugation, the pellet was suspended in 5 ml of PBS and kept as a fine suspension. This material was analyzed by SDS-PAGE (Phast System; Pharmacia, Sweden), and two main bands were detected, the β -galactoside-ST_{II} band and a frequently occurring contaminating lysozyme band at the lower part of the gel (Fig. 2A, lane 3). DNase treatment (10 μ g/ml) (Sigma) of the protein prior to Triton X-100 treatment decreased the amount of contaminating lysozyme.

The protein was solubilized in 8 M urea plus 1% 2-mercaptoethanol with agitation at room temperature for 30 min. The solubilized protein was then diluted 5 to 10 times in 6 M urea and dialyzed against PBS. The material was then centrifuged at $100,000 \times g$ for 20 min, and the supernatant was used to coat ELISA plates.

Immunization of rabbits and analysis of sera. Rabbits were

immunized with 50 μ g of pA-ST_{II} fusion protein in Freund complete adjuvant (Sigma Chemical Co.) at a 1:1 ratio. Injections were given at six to eight places subcutaneously. A booster was given after 4 weeks with incomplete adjuvant. Sera were collected after a further 4 weeks and stored at -20°C until assayed.

Sera from five rabbits immunized with the pA-ST_{II} fusion protein were tested in Western blotting (immunoblotting). To test for ST_{II}-specific antibodies by this technique, the protein A fusion protein causes two problems. First, protein A will bind to the rabbit antibodies by the Fc interaction (17). This interaction can be blocked by preincubation with non-specific antibodies (19). Second, specific antibodies are likely to be raised against protein A as well as ST_{II}, and this interaction is not blocked by unspecific antibodies (17). Figure 2B shows that rabbit sera raised against pA-ST_{II} cross-reacted with β -galactosidase-ST_{II} but not with native β -galactosidase. Similar results were obtained with five different rabbit antisera (data not shown). Preimmune sera from the rabbits gave no signals in Western blots.

Pig intestinal loop assay. Piglets (3 to 8 weeks old) were used for the detection of ST_{II} activity by the ligated intestinal loop assay (28). Bacteria-free culture supernatants were injected into 5-cm loops in volumes of 2 or 4 ml.

Fluid accumulation, expressed as the volume-to-length ratio, was determined after 6 h (28). Values of ≥ 1 ml of thin fluid per cm of intestine were regarded as positive. Positive and negative controls were always included both anterior and posterior to samples.

Enzymes and chemicals. Restriction enzymes were from Boehringer Mannheim and used according to the recommendations of the manufacturer. Methods for gene cloning were from Maniatis et al. (21).

RESULTS

Development of an ST_{II} ELISA. An ELISA based on binding of antibodies against pA-ST_{II} to immobilized β -galactosidase-ST_{II} was used for development of an inhibition ELISA for quantification of ST_{II} in supernatants from ETEC strains (16, 31).

Flat-bottomed polystyrene microtiter plates (Immuno-plates; Nunc, Copenhagen, Denmark) were coated with antigen (0.1 to 10^4 μ g) by incubating 100 μ l of various dilutions of β -galactosidase-ST_{II} and β -galactosidase in coating buffer (50 mM carbonate buffer, pH 9.6) per well at room temperature for 18 h. After addition of 100 μ l of 5% milk powder in coating buffer for 1 h, to block remaining binding sites, the plates were washed three times with PBST (10 mM PBS [pH 7.4] containing 0.05% [vol/vol] Tween-20). The wells were then filled with 100 μ l of antiserum against pA-ST_{II} diluted in PBST and incubated for 3 h at room temperature. After three washes, a secondary goat anti-rabbit antibody labeled with alkaline phosphatase (Sigma Chemical Co.) was diluted in PBST, and 100- μ l fractions were added to each well.

Following overnight incubation at room temperature, the plates were washed four times, and 100 μ l of substrate (4-nitrophenylphosphate tablets [Sigma] dissolved in 1 M diethanolamine and 0.5 mM MgCl_2 [pH 9.8] as indicated by the manufacturer) was added. After 30 to 40 min of enzyme-substrate reaction at room temperature, the A_{405} was recorded. All samples were measured in duplicate. Figure 3 shows binding of anti-pA-ST_{II} as a function of antigen concentration. A dilution of antigen was then chosen that gave a significant difference in binding between antiserum

FIG. 3. ELISA of β -galactosidase-ST₁₁. The antigens were used at the indicated dilutions to coat microtiter plates, and serum raised against pA-ST₁₁ at a 400-fold dilution was added. Preimmune serum was used as a control. Symbols: $\circ$, β -galactosidase-ST₁₁ as antigen with anti-pA-ST₁₁ serum; $\bullet$, β -galactosidase as antigen with anti-pA-ST₁₁ serum; $\triangle$, β -galactosidase-ST₁₁ as antigen with preimmune serum; $\blacktriangle$, β -galactosidase as antigen with preimmune serum. Duplicate measurements were made.

and preimmune serum. With the batch of antigen used in the experiments shown in Fig. 3, a concentration of 100 ng/100 μ l was used.

As a control, native β -galactosidase was also used as the immobilized antigen. Significantly less binding of anti-pA-ST₁₁ was then observed (Fig. 3).

The antiserum was diluted 400 times in the studies shown in Fig. 3, but to titrate the antiserum, several dilutions were tested. A dilution of the antiserum that gave the most significant difference between binding to β -galactosidase-ST₁₁ and to native β -galactosidase was then chosen for the following experiments (data not shown).

ST₁₁ inhibition ELISA. To quantify ST₁₁ in bacteria-free culture supernatants of ETEC, 50 μ l of a fivefold dilution series of ST₁₁ samples was added to the wells prior to addition of 50 μ l of anti-pA-ST₁₁ antiserum at a final concen-

FIG. 4. Inhibition ELISA. Inhibition of binding of anti-pA-ST₁₁ to β -galactosidase-ST₁₁ was measured in the presence of various amounts of supernatants from ST₁₁-producing strains. A fivefold dilution series of ST₁₁ with an initial dilution of 1/10 was used. (A) Supernatants were from *E. coli*(pRAS) (ST₁₁ producer) ($\triangle$) and *E. coli*(pBR322) (nonproducer) ($\blacktriangle$). (B) Supernatants were from *E. coli* 853/67 (ST₁, ST₁₁, LT) ($\circ$), *E. coli* Bd1427 (no enterotoxin) ($\bullet$), *E. coli* Bd2661 (ST₁₁) ($\square$), and *E. coli* Bd3505 (ST₁) ($\blacksquare$). Duplicate measurements were done with insignificant variation.

TABLE 1. Correlation between intestinal loop test and ELISA results

Loop test result	No. of ELISA results	
	Positive	Negative
Positive	78	9
Negative	1	103

tration of 1/400. Some wells were filled with 100 μ l of PBST and others with 50 μ l of PBST plus 50 μ l of antiserum (for determination of minimum and maximum binding, respectively). Values were routinely recorded when the A_{405} of the maximum binding was approximately equal to 1. Percent inhibition was calculated as follows: % inhibition = $[(A - B)/A] \times 100$, where A is the absorbance in the absence of any added ST_{II} (maximum absorbance) and B is the absorbance in the presence of added ST_{II} from the sample.

Competition between immobilized β -galactosidase-ST_{II} and ST_{II} is shown in Fig. 4. Inhibition values exceeding 20% were considered positive, and values below 10% were considered negative.

Screening of ST_{II} strains. A collection of 191 porcine ETEC strains from the Swedish National Veterinary Institute were tested in the inhibition ELISA of ST_{II} described in Materials and Methods. Table 1 shows the correlation between the ST_{II} inhibition ELISA and the pig intestinal loop test results. The correlation was 95%. Of the nine samples that were positive in the loop test but negative in the ELISA, five were only weakly positive in the intestinal loop test, i.e., volume-to-length ratio, ca. 0.8, whereas in the same pig, other positive samples had a ratio of about 2. Six of the nine isolates were also found to carry ST_I, which is active in newborn piglets. A variation in the intestinal loop test is not uncommon and depends on variations in sensitivity from one pig to another.

Some samples were tested several times in the ELISA and showed no or only a slight variation in response.

We therefore conclude that the ELISA method for ST_{II} detection is reliable enough to be used in routine diagnosis and for extensive epidemiological studies.

Biological activity of pA-ST_{II} fusion protein. The pA-ST_{II} fusion protein was tested for its biological effect in the intestinal loop assay together with positive controls (Table 2). pA-ST_{II}, when purified on IgG-Sepharose, had very low biological activity compared with native ST_{II} from an 853/67 supernatant. However, nonpurified pA-ST_{II} from an *S.*

aureus SA113(pIG15) supernatant had biological activity. This is probably because the fusion protein as such is inactive, but in a supernatant from *S. aureus* a portion of pA-ST_{II} is proteolytically degraded to smaller molecular forms. Some of these molecules may have only a short remaining part of protein A left at the NH₂-terminal end of ST_{II}, increasing the biological activity of ST_{II}. Such proteolytically degraded products would not be purified on IgG-Sepharose.

This hypothesis was strengthened by the finding that dialysis of nonpurified pA-ST_{II} in dialysis bags with a molecular weight cutoff of 14,000 resulted in loss of more than 50% of the activity. Dialysis bags with a molecular weight of cutoff of 1,000 gave no detectable loss of activity.

The other fusion protein, β -galactosidase-ST_{II}, was not tested in the intestinal loop assay. This protein is not suitable for testing due to its insolubility.

DISCUSSION

Gene fusion technology has been used to facilitate the development of an ELISA for enterotoxin type ST_{II} (STb). This enterotoxin has not previously been purified in its native form. By making a gene fusion, the corresponding fusion protein could be purified by methods developed for the carrier protein. In the case of protein A, this was done on IgG-Sepharose, and in the case of β -galactosidase, purification was based on its insolubility when produced intracellularly in huge amounts.

We examined two features of the fusion proteins: relative immunogenicity and cross-reactivity with the native protein, and biological activity. One can assume that both aspects are altered for fusion proteins compared with the native protein.

Small proteins are usually not good immunogens unless conjugated to a carrier protein. This could be simplified by making a gene fusion, thereby obtaining an increased-molecular-weight ST_{II}. The molecular weight of ST_{II} is ca. 5,000, that of pA-ST_{II} is 35,000, and that of β -galactosidase is 120,000.

Cross-reactivity between native ST_{II} and the β -galactosidase-ST_{II} fusion was demonstrated here by using antisera raised against another fusion protein, pA-ST_{II}.

Thus, two important advantages were achieved by making fusion proteins involving ST_{II}: simplified purification and proposed improved immunogenicity, although it has not yet been compared with that of native low-molecular-weight ST_{II}.

The other aspect of fusion proteins, biological activity, is questionable in this case. The purified pA-ST_{II} fusion protein had very low biological activity in the intestinal loop test, whereas the nonpurified fusion protein had better activity. Partial proteolysis, leaving NH-terminal extensions to the ST_{II} protein of various lengths, may be the reason for this observation. These results are in agreement with those of Nilsson et al. (27) for protein A-insulinlike growth factor I. The insulin-like growth factor I activity, as determined by binding to placenta membranes, was demonstrated only after specific cleavage of the fusion protein at the junction between the two proteins.

We have shown here that the technique of making fusion proteins may be a powerful tool for protein purification and development of a quantitative assay for ST_{II}. Purification of native ST_{II} from a strain producing high quantities of ST_{II} due to introduction of a strong inducible promoter is now being carried out. Detection of ST_{II}-containing fractions is facilitated by the ELISA.

TABLE 2. Biological activity of ST_{II}

ST _{II} source	Estimated toxin concn (μ g/5 ml)	Loop ratio range (ml/cm) ^a
<i>E. coli</i> 853/67 supernatant	1-4	1.3-3.1 (10)
<i>S. aureus</i> (pIG15) supernatant (pA-ST _{II})	2-10	1.0-1.6 (11)
<i>S. aureus</i> pACE33 supernatant (protein A)	2-10	0.1-0.7 (4)
Purified pA-ST _{II}	1	0.0-0.4
	10	0.0-0.6
	100	1.0-2.0
Purified protein A	100	0.0
	1,000	0.0

^a Values are milliliters of fluid accumulated per centimeter of intestinal loop. The ranges given for supernatants are the 95% statistical confidence intervals, and the number of measurements is shown in parentheses.

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