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Molecular Homogeneity of Heat-Stable Enterotoxins Produced by Bovine Enterotoxigenic *Escherichia coli*

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Heat-stable enterotoxins (STs) from four strains of bovine enterotoxigenic *Escherichia coli* representing four serogroups were purified to homogeneity by utilizing previously published purification schemata. Biochemical characterization of the purified STs showed that they met the basic criteria for the heat-stable enterotoxins of *E. coli*. Amino acid analysis of the purified STs revealed that they were peptides of identical amino acid composition. This composition consisted of 18 residues of 10 different amino acids, 6 of which were cysteine. The amino acid composition of the four ST peptides was identical to that reported for the STs of human and porcine *E. coli*. In addition, complete sequence analysis of two of the ST peptides and partial sequencing of several others revealed strong homology to the sequences of STs from human and porcine *E. coli* and to the sequence predicted from the last 18 codons of the transposon Tn*1681*. There was also substantial homology to the sequence predicted from the ST-coding genetic element of human *E. coli*, which may indicate the existence of identical bioactive configuration among ST peptides of *E. coli* strains of various host origins. These data support the hypothesis that STs produced by human, bovine, and porcine *E. coli* are coded by a closely related genetic element which may have originated from a single, widely disseminated transposon.

In humans and animals, the pathogenesis of secretory diarrhea caused by infection with enterotoxigenic *Escherichia coli* (ETEC) has been shown to be mediated by two classes of enterotoxins. These include the heat-labile toxin, which has been well characterized and found to be closely related to cholera toxin functionally and structurally (7, 12, 14, 21, 33), and the heat-stable toxin (ST), which is thought to represent a group of subtypes according to biological activity and biochemical characteristics (16, 17, 23, 35, 36). Biological as well as biochemical heterogeneity appears to exist among the STs produced from human and porcine ETEC. However, little information is available on the STs produced by ETEC strains of bovine origins. Recently, we reported on the purification and characterization of the ST produced by some of the prototypes of bovine ETEC (29, 30). In contrast to ETEC strains of other host origins (2, 13, 17, 27 36), which produce both ST and heat-labile toxin, most ETEC strains of bovine origin produce only ST. For this reason, it was of interest to purify and characterize STs from several strains of different serogroups of bovine ETEC to determine their molecular nature and to compare them with STs from ETEC strains of other host origins.

MATERIALS AND METHODS

Bacterial strains. The following bovine ETEC strains were studied: B41, furnished by Ida Orskove from the International Collaborative Escherichia and Klebsiella Center, Copenhagen, Denmark; and B44, M490, and M524, kindly provided by Carlton Gyles, Ontario Veterinary College, Ontario, Canada (Table 1).

Chemicals and reagents. Unless otherwise stated, all chemicals were reagent grades. Pyridine, trifluoroacetic acid (TFA), phenylisothiocyanate, and dimethylaminoazobenzene isothiocyanate were sequanol grade from Pierce Chemical Company, Rockford, Ill.

Growth conditions and toxin production. The selected strains were tested for their growth patterns and ST production in the asparagine-salts medium of Staples et al. (34) as previously described (29).

Toxin assay. The suckling-mouse assay (SMA) of Giannella (11) was used to monitor the production and recovery of ST throughout purification. Only 1-to-2-day-old suckling mice, which were starved for 2 h before inoculation, were used. The protein concentrations of the crude and purified ST preparations were determined by the modified method of Lowry (24).

ST purification. The purification of ST, which included batch adsorption chromatography on Amberlite XAD-2 resin (Rohm & Haas Co., Philadelphia, Pa.) and acetone fractionation, was carried out as previously described (29).

PIFGG. The preparative isoelectric focusing in flatbed granulated gel (PIFGG) was carried out as previously described (29) with the following modifications. The anticonvective surface was made from prewashed and dried Sephadex G-200 superfine gel (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden). Briefly, the Sephadex G-200 gel was washed extensively with double-distilled water purified by the Milli-Q reagent water system (Millipore Corp., Bedford, Mass.) The gel was then placed in a sintered glass funnel and filtered with the aid of an applied vacuum until the gel was dry enough to crack. Absolute ethanol was gradually added to the gel until a milky suspension was formed. The gel was washed further with several volumes of absolute ethanol and then filtered to complete dryness (ca. 35 min). The resulting fine white powder was dried for an additional 24 h under vacuum. A slurry of the gel was made for use in the flatbed isoelectric focusing apparatus by suspending 3.5 g of the gel powder in 130 ml of double-distilled Milli-Q purified water. To this slurry were added Ampholines (2.75 ml [pH 2.5 to 4.0] and 2.25 ml [pH 3.5 to 5.0]; LKB Produkter, Bromma, Sweden). The crude ST (the acetone-fractionated toxin) was then mixed into the gel suspension and refrigerated for an additional 3 h. After refrigeration, the Sephadex-ST slurry was degassed and then poured onto a glass tray (12.5 by 30 cm) to form a flatbed. As previously described, the isoelec-

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TABLE 1. Bovine ETEC strains used[a]

Strain	Serogroup	Source
B41	0101:K$^-$	Orskove
B44	09:K30	Gyles
M490	0101:K30	Myers
M524	08:K85	Myers

[a] All strains were K99 positive of bovine origin and produced ST.

tric focusing was not initiated until approximately one-third of the water had been evaporated from the poured flatbed. The focusing was carried out with a power setting of 8 W and a maximum-voltage setting of 1,400 V for 15 h. Throughout the run, the gel was maintained at 3°C by circulating cold water through the cooling plate with a circulating water bath (Neslab Instruments, Inc., Portsmouth, N.H.).

Collection of the toxin. At the end of the run, the flatbed was viewed under UV illumination (Chromato-vue transilluminator model 0-63, Ultraviolet Products, Inc., San Gabriel, Calif.). To locate the position of the toxin in the flatbed, we fractionated the gel into 30 fractions by using a metal fractionation grid. After measuring the pH of each fraction with a microelectrode, each fraction was suspended in 5 ml of 10% acetic acid and loaded into a 10-ml plastic syringe plugged with glass wool. After elution of the first 5 ml, each fraction was washed with an additional 10 ml of 10% acetic acid which was combined with the first 5 ml. The protein content of each eluate was measured by absorption at 280 nm. The biological activity of the eluates was assessed in the SMA (11) as described above.

RP-HPLC. Fractions from the PIFGG step containing ST protein were further purified by reversed-phase high-pressure liquid chromatography (RP-HPLC) with slight modifications (29). Briefly, RP-HPLC was performed on an Aquapore RP-300 C-18 column (4.6 × 25 cm; Brownlee Laboratories) with a Chromatograph Model 6000 Solvent System (Waters Associates, Inc., Milford, Mass.) equipped with a model 400 variable-wavelength detector. Peak areas were determined with a Waters Associates data module. Semipreparative samples, each consisting of up to 3 mg of ST from the isoelectric focusing step, were chromatographed. Elution was obtained by a two-step linear gradient with methanol as solvent B and 0.1% TFA as solvent A. Fractions (1 ml each) were collected. The UV-absorbing fractions were tested for biological activity in the SMA after the solvents were evaporated down and the residue was dissolved in distilled water.

Biochemical characteristics of ST proteins. Stability to the proteolytic enzymes trypsin, protease V-8, pronase, and subtilisin and to the reducing agents 2-mercaptoethanol and dithiothreitol was tested as previously described (2, 29).

Amino acid analysis. ST samples (20 μg each) from the four ETEC strains, which had been purified to homogeneity by RP-HPLC, were analyzed as previously described (29).

Amino terminal identification and sequence analysis. The partial sequencing of the peptides was performed manually by a modification of the method of Chang (5). Briefly, ca. 100 nmol of each peptide was lyophilized in 0.6-ml reaction vials with V-shaped bottoms. The peptides were dissolved in 50 μl of 50% pyridine. The dimethylaminoazobenzene isothiocyanate (25 μl; 10 nmol/μl of pyridine) was then added to the vials, which were subsequently incubated at 56°C for 1 h. After the excess dye was removed by several washings with heptane-ethylacetate (2:1 [vol/vol], the derivatized peptides were dried. The labeled amino-terminal residue was cleaved by 100 μl of anhyrdrous TFA and incubated at 56°C for 10 min. The cleaved-terminal residue was extracted by vortexing once with 50 μl of distilled water and then twice with 250 μl of butylacetate. After centrifugation to separate the immiscible phases, the organic phase with the cleaved residue was collected and dried. To this was added 100 μl of 50% TFA, and the mixture was then incubated at 56°C for 45 min for conversion of the thiazolinone to the thiohydantoin amino acid. The TFA was evaporated, and the dried residues were dissolved in 150 μl of 80% ethanol. A sample (100 μl) of this solution was chromatographed by RP-HPLC with a C-18 reversed-phase column (Novapack; Waters Associates) adapted in a radial-compression-system "Z Module". The derivatized amino acids were applied to the column in 40% acetonitrile in 35 mM sodium acetate, pH 5.0 (solvent A). They were eluted in three steps with 100% acetonitrile as solvent B. These steps were (i) a 10-min linear gradient ending with 50% solvent B, (ii) 5 min at this percentage of solvents A and B, and (iii) a 15-min linear gradient ending with 65% solvent B. The flow rate was 1 ml/min. Peak areas were monitored at 420 nm. Identification of the released derivatized amino acids was made by comparing the retention times of standard amino acids derivatized and chromatographed under the same conditions.

The complete sequence analysis of the purified ST of strain M490 was also performed by Lowell Ericssen (Triple A Laboratory, Mercer Island, Wash.) by using the method of Crestfield et al. (6). Briefly, about 500 nmol of the high-pressure liquid chromatography (HPLC)-purified ST was alkylated with [^{14}C]iodoacetic acid by using 5 M excess over the cysteine content of the peptide. The alkylation was carried out in 7 M guanidine hydrochloride–5% EDTA–0.5 M Tris (pH 8.6) under argon in the dark for 4 h. Dithiothreitol (300 M excess over iodoacetic acid) was then added, and the reduction was allowed to proceed at room temperature for 9 h. A twofold molar excess of cold iodoacetic acid over

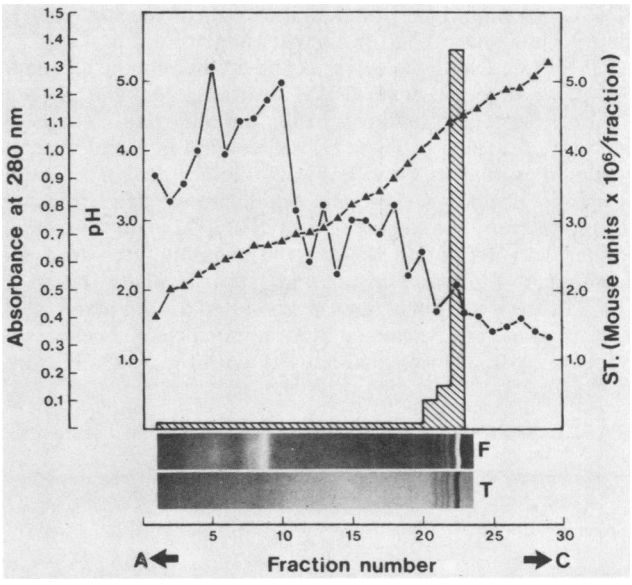

FIG. 1. PIFGG of acetone-fractioned ST. Anticonvective stabilizing gel, Sephadex G-200 superfine; ▲, pH gradient (1.8 to 5.2); load, 570 mg of crude ST; voltage, 1,400 V for 15 h; power, 8 W; A, anode; C, cathode; ●, absorbence at 280 nm; ▨, ST concentration in mouse units; F, gel viewed under UV illumination; T, gel viewed under tungsten light.

dithiothreitol was added, and a second alkylation was allowed to proceed for 4 h. The radioactive peptide was recovered from the mixture by chromatography on a Sephadex G-25 fine column with 0.1 M ammonium bicarbonate for elution. The peptide peak was dissolved in 1 ml of 9% acetic acid. Samples (300 μl) were used for sequence analysis. The sequence analysis was performed on a sequencer (model 890B; Beckman Instruments, Inc., Fullerton, Calif.) in the presence of 5 mg of polybrene (Pierce Chemical Company) by the method of Edman and Begg (9) with the 0.1 M Quadrol program of Brauer et al. (3). Phenylthiohydantoin derivatives of the amino acids were also identified by RP-HPLC by the procedure of Hunkapiller and Hood (15).

RESULTS

Bacterial growth and ST production. The four bovine ETEC strains grew in the chemically defined medium to comparable density after 24 h of culturing at 39°C. The relative efficacy of ST production was determined by the quantitation of ST in the filtered supernatant of each 10-liter batch by the SMA.

Purification. Quantitative recovery of ST from the XAD-2 adsorption chromatography, preparative isoelectric focusing, and RP-HPLC was comparable with that reported earlier (29).

PIFGG. When the flatbed from the PIFGG was viewed under UV illumination at the end of the run, bright fluorescent bands on the anodic end of the flatbed were evident. This area (fractions 5 thorugh 8) corresponded to the highest UV-absorbing fractions at 280 nm (Fig. 1). At the cathodic end of the flatbed, a sharply focused band was observed at pH 4.3 with minor bands between pH 4 and 4.3. Under tungsten illumination, however, only the focused ST band was evident. All fractions of the flatbed were screened for biological activity in the SMA. The majority of the activity was found in fraction 23 which had a pH of 4.3. Only a minor amount of ST activity was found in the rest of the flatbed. Table 2 shows the loads and recovery of the four STs in the PIFGG. It should be noted that >60% of the loaded STs focused in a sharp band in the pH range of 4.2 to 4.6.

RP-HPLC. Using the solvents and gradient in the legend to Fig. 2, we detected several UV-absorbing peaks at 214 and 280 nm. Screening of these peaks for biological activity in the SMA revealed that the ST was eluted in a major peak, indicated as peak 4, as well as in two minor peaks, indicated as peaks 3 and 5 (Fig. 2). As can be seen from the chromatogram, the absorption of ST at 214 nm was much greater than absorption at 280 nm. The ampholytes were also detected at 214 nm and are indicated as peaks 1 and 2. The STs from all four strains were eluted as one major peak with, in some cases, one or more minor peaks. These were detected by their biological activity with the SMA. However, the elution profiles of the four major ST peaks were identical to that of strain B41 (Fig. 3).

TABLE 2. Recovery of ST purified from four strains of bovine ETEC by PIFGG

Strain	ST load[a] (MU[b] × 10^6)	Recovery from the major peak		No. of exp
		MU × 10^6 (%)	pH	
B41	32	22 (68)	4.3–4.6	4
B44	18	11 (61)	4.2–4.4	3
M490	50	30 (60)	4.2	2
M524	16	10 (60)	4.3	2

[a] Loads represent the biologically active STs from 10-liter batch cultures.
[b] MU, Mouse units.

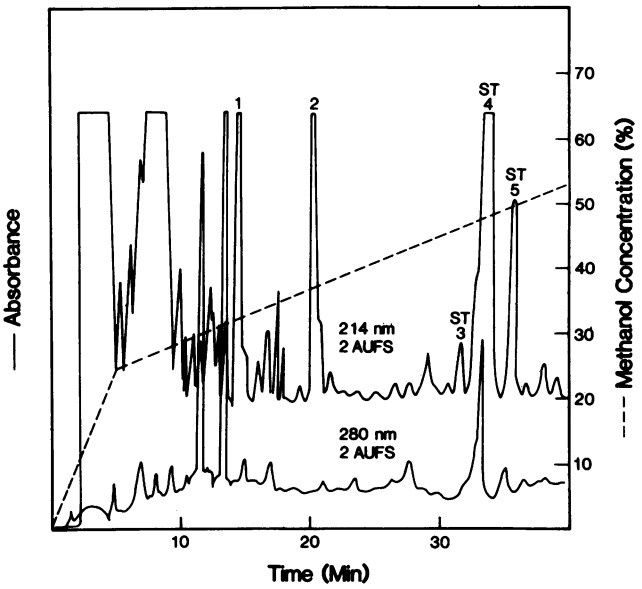

FIG. 2. Semipreparative RP-HPLC separation of PIFGG-prepared ST protein. Partially purified ST (ca. 3 mg) was separated on an Aquapore RP-300 C-18 column. Solvent A, 0.1% TFA; solvent B, methanol; flow rate, 1 ml/min; gradient, 0 to 50% B in 5 min and 50 to 100% B in 45 min.

Amino acid analysis. The amino acid composition of STs obtained from the major RP-HPLC peaks for all four strains as well as from the minor RP-HPLC peaks for strains B41 and M490 is presented in Table 3. Values for the number of amino acid residues have been rounded to nearest integer values for comparison. Complete sequence analysis of the ST peptides from strains M490 and B44 is shown along with the previously determined sequences of ST peptides from human and porcine ETEC in Fig. 4.

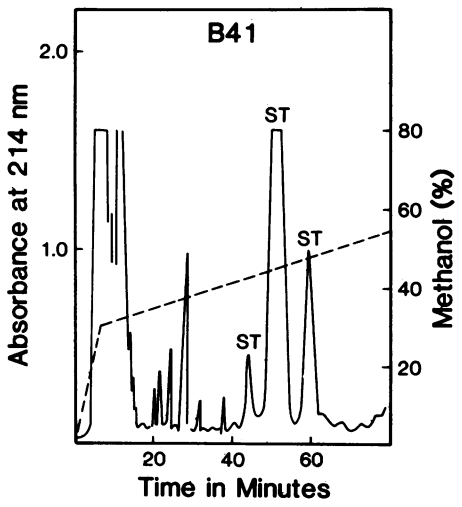

FIG. 3. Purification of ST peptides from the bovine ETEC strain B41 by RP-HPLC. Column, Aquapore 300, RP-C18 (Brownlee Laboratories). Chromatographic conditions were the same as those described in the legend to Fig. 2. Peaks marked with ST contained biological activity detected by the SMA.

TABLE 3. Amino acid analysis of ST purified from four strains of bovine ETEC

Amino acid residue	HPLC peaks for the following strains[a]:					
	B41		M490		M524[b]	B44[b]
	Major	Minor	Major	Minor	Major	Minor
Alanine	1.96 (2)	1.93 (2)	1.97 (2)	1.97 (2)	1.96 (2)	1.96 (2)
Asparagine[c]	2.0 (2)	2.04 (2)	1.99 (2)	1.99 (2)	1.99 (2)	1.99 (2)
Cysteine	6.02 (6)	4.89 (6)	5.63 (6)	4.97 (6)	4.75 (6)	5.10 (6)
Glutamic acid	1.07 (1)	1.04 (1)	1.08 (1)	1.04 (1)	1.01 (1)	1.01 (1)
Glycine	1.13 (1)	1.11 (1)	1.28 (1)	1.14 (1)	1.03 (1)	1.03 (1)
Leucine	1.01 (1)	1.00 (1)	1.08 (1)	1.04 (1)	0.98 (1)	1.02 (1)
Phenylalanine	0.97 (1)	0.95 (1)	0.95 (1)	0.99 (1)	0.99 (1)	0.98 (1)
Proline	1.00 (1)	0.93 (1)	1.05 (1)	1.00 (1)	1.02 (1)	0.98 (1)
Threonine	0.98 (1)	0.99 (1)	0.95 (1)	0.97 (1)	0.96 (1)	0.99 (1)
Tyrosine[d]	1.66 (2)	1.65 (2)	1.64 (2)	1.64 (2)	1.97 (2)	1.97 (2)

[a] Values in parenthesis are rounded to the nearest integer.
[b] Minor peaks in the HPLC chromatograms were evident, but insufficient materials were obtained for amino acid analysis.
[c] Identified as aspartic acid from the peptide lysate. However, sequence analysis revealed that it is asparagine and the amino-terminal residue in the sequenced ST peptides of the four strains.
[d] Carboxy-terminal residue of the ST peptide.

Biochemical characteristics of ST proteins. Comparable results were obtained for STs from all four strains, as well as from other field isolates of bovine *E. coli* that we have tested in our laboratory (Saeed et al., unpublished data). These STs retained their biological activity after heat and enzymatic treatments, but they were completely inactivated by treatment with the reducing agents 2-mercaptoethanol and dithiothreitol at concentrations of 0.1 M and 4×10^{-5} M, respectively.

DISCUSSION

The STs from four strains of ETEC pathogenic for cattle were purified to homogeneity, and their molecular properties were compared. Data obtained from this study indicate that the STs produced by the four bovine ETEC strains share a great number of similar molecular properties. This was indicated by their identical isoelectric points, similar elution times in analytical RP-HPLC, and identical amino acid compositions.

When the electrofocused toxin was purified by RP-HPLC, the majority (over 80%) of the biological activity of the loaded preparation was eluted in the form of one major peak at 42% methanol (solvent B) in the described gradient. ST obtained from this peak was found to be homogeneous based on its high biological activity, its uniform composition upon repeated amino acid analyses of the toxin from several batches, and its sequence analysis, in which the toxin peptide had single amino-terminal and carboxy-terminal residues. Identical profiles were obtained from several RP-HPLC runs. However, additional minor peaks with detectable biological activity were also evident. Such peaks were eluted immediately before and next to the major peak. When adequate, samples from these peaks were subjected to amino acid analysis, and the composition turned out to be identical to ST preparations from the major peaks (Table 2). It is possible that these minor peaks were created by partial reduction or denaturation of the native ST protein and had a retention time in the HPLC gradient different from that of the major peak. Such partial denaturation could also have happened during purification before HPLC. However, the percentage of the biologically active toxin in these peaks was so minimal (less than 1% of the total ST activity from the major peak) that no further investigation into the significance of the minor peaks was undertaken.

Complete sequencing of two of the ST peptides (M490 and B44) revealed that they have identical sequences. Partial sequencing of the other two peptides (B41 and M524) strongly suggested that they have the same sequence as the STs from strains M490 and B44. This is indicated by similar amino acid compositions and homology of the sequences of the first six amino acid residues. The results for the molecular properties of STs produced by human and porcine ETEC (1, 4, 18), along with the sequences specified by the last 18 codons of the ST-encoding genetic elements reported by So and McCarthy (32) and Moseley et al. (22), suggest that a great deal of homology exists among these STs (Fig 4). In particular, it is noteworthy that the six cysteine residues are located at the same positions in the amino acid sequences. This conservation in the position of the cysteines is possibly related to the bioactive configuration and may explain, in part, the similarity in the biological properties among STs produced by ETEC strains of various host origins.

The structure and sequence of the ST purified from strain B41 and the other strains is strikingly homologous to the sequence of the last 18 codons of Tn*1681*, the transposon isolated and characterized from bovine ETEC strain B41. Furthermore, the substantial homology to the sequence predicted from the ST-encoding genetic probe (22) suggests that this type of enterotoxin of ETEC may be encoded as a larger molecule which is processed and then transported through the membrane of the *E. coli* cells (19). It appears that the genetic element which codes for ST in the various ETEC strains is highly conserved. The observations of So et al. (31) also suggest that this genetic element is widely disseminated among ETEC strains of various host origins. This is supported by the findings of Dreyfus et al. (8), who demonstrated the similarities among the purified STs from human, bovine, and porcine origins. These included similar amino acid compositions having the same amino-terminal and carboxy-terminal amino acid residues. Our data are in agreement with those findings and suggest further that STs from ETEC strains may have one common genetic origin.

In our attempt to improve the separation of ST by isoelectric focusing and RP-HPLC, we found the modification reported in this paper to have several advantages. The gel used in isoelectric focusing allowed greater resolution and was of higher load capacity than the one previously reported (25, 26, 29). We concluded that the ST has a good pH slope

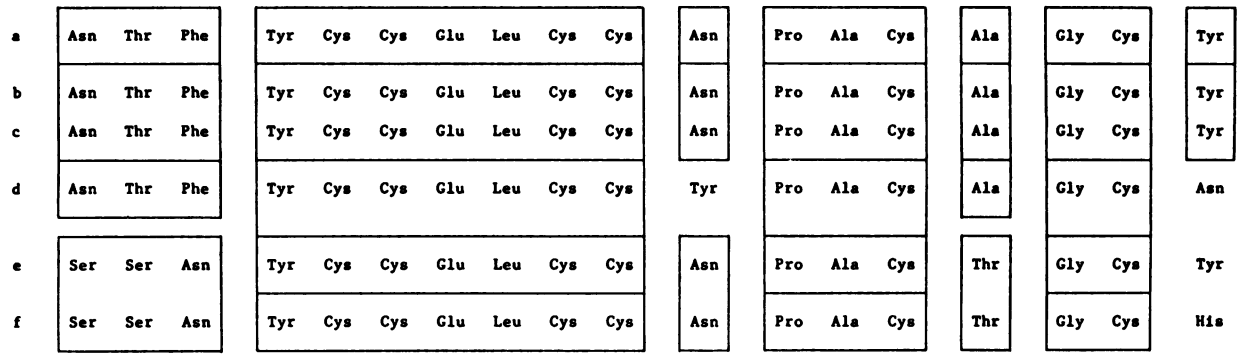

FIG. 4. Comparison of amino acid sequences from STs of bovine, human, and porcine ETEC. Rows: a, amino acid sequence predicted from the last 18 codons of the transposon Tn*1681* of bovine ETEC strain B41 (32); b, amino acid sequence of purified ST peptides from bovine ETEC strains B44 and M490 as determined by our laboratory; c, amino acid sequence of purified ST from porcine ETEC (18); d, amino acid sequence of purified ST from human ETEC (11); e, predicted amino acid sequence from the last 18 codons of an ST-encoding genetic probe from human ETEC (22); f, amino acid sequence of an ST from human ETEC (1). Boxed regions indicate amino acid sequence homology.

mobility and could be effectively purified by isoelectric focusing (28).

In RP-HPLC, the modified gradient and solvents yielded more consistent and reproducible results than the previously used conditions (29). This procedure allowed accurate determination of the amino acid composition and other biochemical features of the STs as the contaminating ampholines were effectively separated (10).

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