Scanning Density Gradient Isoelectric Focusing of *Staphylococcus aureus* Enterotoxins B and C₁

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Purified *Staphylococcus aureus* enterotoxins B and C₁, the causative agents of staphylococcal food poisoning, were resolved into several components by scanning isoelectric focusing in sucrose density gradients in the absence and presence of 6 M urea and 5 mM dithiothreitol. The observed heterogeneity persisted in the denaturing medium, which suggests that the primary structure of protein enterotoxins B and C₁ may be partly responsible for the isoelectric point differences of the various forms. To our knowledge, the scanning isoelectric focusing patterns of staphylococcal enterotoxins B and C₁ are reported for the first time.

Staphylococcal enterotoxins are responsible for the acute symptoms of food poisoning. Susceptibility to enterotoxins appears to be restricted to humans and a few animals, such as certain monkeys. In man, a gastrointestinal upset follows ingestion of the enterotoxin and is characterized by vomiting and diarrhea.

Scanning isoelectric focusing (SCIF) in microdensity gradients (1, 2, 5) is a high-resolution technique for obtaining isoelectric spectra of proteins available in microgram amounts. The main advantage of this method over the standard isoelectric focusing procedure is that the quartz microcolumn is continuously scanned in situ with ultraviolet absorption optics; thus, it is possible to know exactly when a protein has been focused. The scanning procedure allows the final isoelectric spectrum to be recorded at the "steady state" (4). In the present report, we have applied the SCIF method to highly purified *Staphylococcus aureus* enterotoxins B and C₁ in normal and denaturant media.

*Staphylococcus aureus* enterotoxins B and C₁ were prepared as described previously (7, 8). The SCIF assembly and the "on-line" digital data acquisition and processing system have been described in detail elsewhere (2, 4). The microdensity gradient, in the absence or presence of 6 M urea and 5 mM dithiothreitol (5), was formed with a modified apparatus reported previously (2). Isoelectric focusing was performed in a 2% (wt/vol) mixture of pH 3 to 10 (9 parts) and pH 7 to 10 (1 part) Ampholine (LKB, Bromma, Sweden) at 20 V/cm until the steady state was reached (usually 9 to 11 h) by the criteria described (4). Approximately 100 μg of protein was used for each scanning experiment. The sample was uniformly distributed in the density gradient.

Because evaluation of the isoelectric points of a multicomponent protein cannot be assayed very accurately by SCIF, three colored proteins provided a convenient control during focusing: horse ferritin in the acidic, horse myoglobin in the neutral, and horse cytochrome c in the alkaline pH range.

SCIF patterns of enterotoxin B in the absence and presence of 6 M urea and 5 mM dithiothreitol are shown in Fig. 1 and 2, respectively. The isoelectric pattern in the absence of urea and in the basic portion of the separation path resembles, in general, those reported by Metzger et al. (8) and by Chang and Dickie (6) by preparative isoelectric focusing. However, these authors (6, 8) restricted their experiments to the pH range above 7.0, so that at least two bands that appear in the acidic region of the spectrum were missed. Thus, the present results extend the above findings. The possibility that the different isoelectric forms of enterotoxin B are due only to conformational changes of the protein was investigated by carrying out the isoelectric focusing experiments in a urea-dithiothreitol medium. Under these conditions, enterotoxin B produced five major bands in the basic region with a few minor components in the
major basic components of enterotoxin C₁ are apparently more "acidic" than those of enterotoxin B.

Visual inspection of the patterns shown in Fig. 1 through 4 offers additional information to ponder, such as the apparent ratio of the areas of the observed bands and their position before and after treatment with urea-dithiothreitol. Attempts to explain the observed isoelectric heterogeneity can be considered entirely premature at the present stage of this work. Preparative fractionation of these components and study of their biological, chemical, and physical differences may further elucidate our present findings. The aspects of micro-heterogeneity as revealed by isoelectric focusing have been reviewed by Williamson et al. (10). They are either synthetic or postsynthetic. Synthetic heterogeneity arises during gene duplication from acidic region. These results indicate that the observed heterogeneity persists in the unfolded form of the protein, and it is probably due to differences in the primary structure of the protein chain.

Enterotoxin C₁ in its native form produced three major (and one minor) components in the near basic region and two major components in the acidic region (Fig. 3). Upon denaturation in urea-dithiothreitol, the protein exhibited again three major (and one minor) components in the near basic region and at least three other components in the acidic region (Fig. 4). The

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Fig. 1. SCIF spectrum of purified S. aureus enterotoxin B. Carrier ampholytes in a 2% mixture of pH 3 to 10 (9 parts) and pH 7 to 10 (1 part) were used. SCIF was performed at 280 nm at 20 V/cm for 9 h. Approximately 100 µg of enterotoxin B was employed. Samples without enterotoxin were employed as controls and did not produce any definite peaks at 280 nm. Full scale, 1.0 optical density unit.

Fig. 2. SCIF spectrum of S. aureus enterotoxin B in 6 M urea and 5 mM dithiothreitol. Carrier ampholytes in a 2% mixture of pH 3 to 10 (9 parts) and pH 7 to 10 (1 part) were used. The enterotoxin was incubated with 6 M urea and 5 mM dithiothreitol for 1 h prior to scanning at 20 V/cm for 11 h. Approximately 100 µg of enterotoxin B was employed. Samples without enterotoxin B served as controls. Full scale, 0.5 optical density unit.

Fig. 3. SCIF spectrum of purified S. aureus enterotoxin C₁. Experimental conditions were the same as in Fig. 1; enterotoxin C₁ was employed. Full scale, 0.5 optical density unit.

Fig. 4. SCIF spectrum of S. aureus enterotoxin C₁ in 6 M urea and 5 mM dithiothreitol. Experimental conditions were the same as in Fig. 2; enterotoxin C₁ was employed. Full scale, 1.0 optical density unit.
random assortment of different subunits, errors made during transcription or translation of protein synthesis, incomplete carbohydrate addition, and substitution of side chains. Postsynthetic heterogeneity arises from cleavage of N-terminal amino acid residues, cleavage of carbohydrate chains, deamination, binding of ligands, and formation of conformational isomers.

With the exception of the exfoliative toxin, the enterotoxins of *S. aureus* are the only other staphylococcal products which have been shown to have a specific clinically recognizable effect. Characterizations of enterotoxins by recent fine resolution techniques are incomplete, and such delineation of the toxin molecule is a prerequisite for the understanding of the mode of action of staphylococcal enterotoxins. The application of SCIF to *S. aureus* enterotoxins may be considered for comparative studies on possible distinct forms of enterotoxins within a given staphylococcal strain and varying genetic forms of these toxins among different strains. Thus, SCIF offers some new and unique advantages for the characterization of microbial toxins. It should be stated that at its present stage of development, the method renders the collection and analysis of the resolved components of a toxin very difficult. Thus, nothing can be said regarding the toxicity and immunological reactivity of the various subunits of the enterotoxins B and C₁ as they were revealed by SCIF. However, it was stated in the report on the isoelectric focusing of enterotoxin B by Metzger et al. (8) that all four components of the toxin found in the basic pH region showed lines of identity by Ouchterlony gel diffusion and that materials from the main and secondary subunits were toxic for monkeys. Furthermore, when the main component of enterotoxin was re-electrofocused, it yielded only a single peak.

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