CHARACTERIZATION OF TYPES OF STAPHYLOCOCCAL ENTEROTOXINS[1, 2]

MERLIN S. BERGDOLL AND RUTH N. ROBBINS
Food Research Institute
University of Wisconsin, Madison, Wisconsin 53706

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ABSTRACT

The staphylococcal enterotoxins are single polypeptide chains that contain two half-cystine residues that are cross-linked in the native toxin to form a "cystine" loop. Several amino acid residues involving part of this loop appear to be the same for the different enterotoxins which may represent the toxic site. The other part of the cystine loop may be involved in the antigenicity of the toxin which is the basis for identifying them as enterotoxins A-E. The antigenicty of the enterotoxins varies from the similarity of enterotoxins C_1 and C_2 which have the same major antigenic site to enterotoxins A and B which are apparently unrelated antigenically.

Staphylococcal enterotoxins are a closely related group of simple proteins with a molecular weight of 26,000 to 30,000 *(23)*. The enterotoxins are identified as separate entities with the letters A, B, C, etc. *(10)*, the separation being based on the major antibodies produced by the protein molecules. The specific precipitation reaction given by each enterotoxin with its own antibody is the basis for the tests used to detect the enterotoxins. Enterotoxins A *(9)*, B *(6)*, C *(3)*, D *(11)*, and E *(4)* have been identified, but how many unidentified ones exist is not known. The enterotoxins produced by many strains that are enterotoxigenic by monkey feeding tests have not been associated with specific antibodies.

TOXIC AND ANTIGENIC SITES OF THE ENTEROTOXINS

The enterotoxins are single polypeptide chains composed of relatively large amounts of lysine, aspartic and glutamic acids, and tyrosine (Table 1). They contain only one or two residues of tryptophan and only two residues of half-cystine. The latter are cross-linked to form a cystine residue as evidenced by the fact that there are no free -SH groups in the molecule *(15)*. The half-cystine residues in enterotoxin B are at positions 92 and 112 *(17)* which creates a "cystine" loop of twenty amino acid residues as shown below:

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-Gly-Ala-Asn-Tyr-Tyr-Gln-Cys-Tyr-Phe-Ser-Lys-Lys-Thr-Asn-Asn-Ile-
                                                          ASP
                                                           |
-Thr-Val-Gly-Gly-Tyr-Met-Cys-Thr-Lys-Arg-Lys-Thr-Asn-Glu-His-Ser-
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It is not known yet whether the "cystine" loop in the different enterotoxins is identical, but since only one cystine molecule is present in any of the enterotoxns it is expected that this part of the molecule is involved in some way in the activity of the toxin. Preliminary data from the sequence studies on enterotoxin A show that the six amino acid residues adjacent to the half-cystine residue at position 112 in enterotoxin B are identical. In all probability this is the active site of the molecule since the biological activity of all of the enterotoxns is the same.

The residues near the half-cystine residue at position No. 92 in enterotoxin B may comprise the major antigenic site. There are several tyrosine residues in this part of the molecule but no lysine residues are present either here or in the proposed toxic site. This is consistent with the conclusions of Spero et al. *(24)* that lysine residues are not involved in either the toxic or antigenic sites. They found that substitution of all but one of the E-amino groups of the lysine residues had no effect on the antigen-antibody reaction or the toxic action. The sequence of amino acids in this area is not the same for enterotoxin A and B which would account for these enterotoxins having different major antibodies.

One might conclude that the enterotoxins originated from a single protein and that through the course of evolution by amino acid residue substitutions, several antigenically different proteins with a common toxic site evolved. The difference in antigenicity might be used as the indicator of the degree of evolution having taken place. If a large number of enterotoxins could be purified and their sequence determined, as has been done for the cytochrome C's, one might expect to find a series of proteins that may differ only by a few amino acid residues.

ANTIGENICITY OF THE ENTEROTOXINS

Antigenically homogenous enterotoxins

There does exist within the group of enterotoxins that have been purified a rather wide range of differences in antigenicity. This range varies from those that are antigenically homogenous, but differ otherwise, to those that are distinctly different in this respect. Early work by Bergdoll showed that the enterotoxin produced by staphylococcus strain S-6

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[2]Contribution from the College of Agricultural and Life Sciences, University of Wisconsin, Madison, Wisconsin.

TABLE 1. AMINO ACID COMPOSITION OF THE ENTEROTOXINS IN GRAMS PER 100 GRAMS PROTEIN

Amino acid	Enterotoxin A (23)	B (5)	C_1 (18)	C_2 (18)	E (8)
Lysine	11.26	14.85	14.43	13.99	10.83
Histidine	3.16	2.34	2.91	2.87	3.04
Arginine	4.02	2.69	1.71	1.75	4.50
Aspartic acid	15.53	18.13	17.85	18.38	15.10
Threonine	5.96	4.50	5.31	5.80	6.36
Serine	2.99	4.05	4.58	4.81	4.72
Glutamic acid	12.36	9.45	8.95	8.93	12.15
Proline	1.35	2.11	2.16	2.23	1.93
Glycine	2.96	1.78	2.99	2.90	4.10
Alanine	1.94	1.32	1.85	1.61	2.38
Half-cystine	0.66	0.68	0.79	0.74	0.81
Valine	4.93	5.66	6.50	5.87	4.36
Methionine	0.96	3.52	3.20	3.60	0.45
Isoleucine	4.11	3.53	4.09	4.02	4.30
Leucine	9.78	6.86	6.54	6.13	10.08
Tyrosine	10.63	11.50	9.80	10.27	9.79
Phenylalanine	4.31	6.23	5.35	5.25	4.47
Tryptophan	1.46	0.95	0.99	0.84	1.51
Amide NH_3	1.80	1.66	1.71	1.62	1.66
Total:	98.37	100.15	100.00	99.99	100.88

(later labeled enterotoxin B) could be separated into at least three fractions by careful ion exchange chromatography. All the fractions were toxic and reacted with the same specific antibody. The same type of result was reported in 1971 by Chang et al. *(13)* using hydroxyl apatite chromatography. These investigators reported that all fractions reacted with the same antibody, but that only one gave a toxic reaction in cats. Although no conclusions were drawn concerning the reasons for these differences, the ion-exchange separations would indicate the separations resulted from a difference in charge on the molecules.

Baird-Parker and Joseph *(2)* showed that purified enterotoxin B could be separated by starch gel electrophoresis into two fractions which appeared to be identical. Schantz et al. *(22)* obtained a similar separation of enterotoxin B into two fractions using starch gel. Rerunning of the slower moving material gave two bands while rerunning of the faster material gave only the one band. The material from the two bands was identical in toxicity in monkeys, in reaction wth the specific antibody, and in amino acid composition. Joseph and Baird-Parker *(20)* suggested that the difference was possibly in the secondary or tertiary structure. Schantz et al. *(22)* stated that it was uncertain whether the fractionation was due to true heterogeniety of the protein molecules or to some type of molecular interaction.

Attempts to obtain purified enterotoxin in the homogenous state by isoelectric focusing were less than successful *(12, 14)*. Enterotoxins A, B, and C purified by conventional methods gave multiple peaks when subjected to isoelectric focusing. Each peak of a given enterotoxin reacted with the specific antibody for that particular enterotoxin and appeared to be the same in every way except for the charge on the molecule. With one exception *(21)* refocusing of any given peak resulted in multiple peaks with isoelectric points equal to or lower than that of the material refocused *(12, 14)*. The conclusion one might arrive at is that the difference in charge is caused by a difference in the number of amide groups in the molecule. These results indicate that amide groups may be lost during isoelectric focusing while results from ion-exchange chromatography indicate that differences may have arisen during fermentation. These differences do not appear to be sufficiently critical to affect purification of enterotoxins when conventional methods are used.

Enterotoxins C_1 and C_2

Two enterotoxin C's have been purified and labeled C_1 and C_2, primarily on the difference in their isoelectric points, 8.6 for C_1 *(7)* and 7.0 for C_2 *(1)*. Although the major antigenic site of these two enterotoxins is identical, the observation of spur formation on Ouchterlony plates indicated a difference in their minor antigenic sites. It is possible to convert C_1 into a protein with an isoelectric point of 7.0 by removal of amide groups[3], but the antigen-antibody reactions show that the mere removal of amide groups from C_1 does not convert it to a true C_2 enterotoxin. Any differences in the amino acid composition of the two enterotoxins is within the limits of error of the analysis, hence, the difference in structure can be revealed only when the amino acid sequences of the two enterotoxins are worked out. This difference is not of practical concern since either C_1 or C_2 can be detected by the major antibody to either enterotoxin.

Enterotoxins B and C

Gruber and Wright *(16)* observed by ammonium sulfate coprecipitation that enterotoxins B and C contained similar antigenic determinant groups. During identification of enterotoxin C in the Food Research Institute no cross-reaction between the two enterotoxins was noted by antigen-antibody precipitation techniques such as the Ouchterlony gel plate *(3)*, but evidence was available which indicated that enterotoxin B antiserum did neutralize enterotoxin C. In this instance, the enterotoxin C was treated with the B antiserum before intravenous injection into rhesus monkeys. Further experiments in our laboratories indicated that enterotoxin C will no longer give a precipitate with its specific antibody after the enterotoxin is treated with enterotoxin B antiserum and vice versa. Johnson et al. *(19)* noted only a slight

[3]Private communication from Dr. Len Spero.

cross-reaction between enterotoxin B and C_1 in the solid-phase radio-immunoassay test. The amino acid composition of these two enterotoxins are similar in many respects and both have the same N-terminal amino acid. It is possible that the major antigenic sites of these two enterotoxins are sufficiently similar to give a partial cross-reaction.

Enterotoxins A and E

The enterotoxin that was produced by the staphylococcus strain chosen for use for enterotoxin E purification gave no cross-reactions with any of the antisera to the known enterotoxins. When the purification work was nearing an end, a cross-reaction between enterotoxin E and antiserum to A was noted. In this instance, antiserum to enterotoxin A neutralized enterotoxin E when injected intravenously into monkeys. Careful examination by the Ouchterlony plate test revealed that the differences were due to the major antigenic site *(4)*. The amino acid composition of these two enterotoxins are quite similar.

Enterotoxins A and B

The maximal difference in enterotoxins as antigens is between enterotoxins A and B. Although Gruber and Wright *(16)* reported some cross-reaction between these two enterotoxins, this has not been verified. No neutralization of enterotoxin B and the C's with A antiserum or vice versa has been noted, nor has any precipitate cross-reactions been observed. The amino acid composition of the two enterotoxins is different in several respects (Table 1). One of the major differences in the composition is in the methionine content, two residues for A and eight for B.

There are other differences in the enterotoxins, for example, the manner in which they are produced by the organism, but space does not permit a discussion of these differences. In any event, the antigenic differences are of greatest importance since detection methods for the enterotoxins are dependent upon their antigenic reactions.

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