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Amino Acid Composition and Terminal Amino Acids of Staphylococcal Enterotoxin C*

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ABSTRACT: The amino acid compositions of enterotoxin C strain 137 and enterotoxin C strain 361 were determined with a Spinco amino acid analyzer. Glutamic acid was determined as the N-terminal amino acid and glycine as the C-terminal amino acid of both enterotoxins.

This indicates that their structures are single polypeptide chains.

The staphylococcal enterotoxins have been classified according to their reactions with specific antibodies as enterotoxins A, B, etc. (Casman *et al.*, 1963). Identification of a new enterotoxin as enterotoxin C produced by staphylococcal strains 137 and 361 was accomplished (Bergdoll *et al.*, 1965a). Purification procedures and molecular weights calculated from sedimentation, diffusion and viscosity data for enterotoxin C (strain 137) and enterotoxin C (strain 361), respectively, are reported by Borja and Bergdoll (1967) and Avena and Bergdoll (1967). This paper presents a complete amino acid analysis of the enterotoxins C (strains 137 and 361) as well as information about the N- and C-terminal amino acids.

Experimental Section

Materials. The enterotoxins C (strains 137 and 361) used in this investigation were prepared according to the methods of Borja and Bergdoll (1967) and Avena and Bergdoll (1967). The nitrogen content of the enterotoxins C (strains 137 and 361) was determined with an F & M Scientific automatic CHN analyzer to be 16.2 and 16.0%, respectively. All the reagents used in paper chromatography were reagent grade. FDNB[1] and anhydrous hydrazine (95%+) were obtained from Eastman Organic Chemicals. No further purification was made on these two reagents. Benzaldehyde was distilled before use.

Amino Acid Analysis. The amino acid composition of the two enterotoxins C was determined with a Spinco Model 120B amino acid analyzer. In the analyses of calibration mixtures, an average deviation of ±3% was obtained. Trace amounts of ammonia present in the reagents were taken into consideration for calculation of amide nitrogen in enterotoxin C. Hydrolysates were prepared by heating in 6 N HCl at 110° for 24 and 72 hr in evacuated, sealed tubes. The threonine and serine values were calculated by

* From the Food Research Institute and Department of Foods and Nutrition, University of Wisconsin, Madison, Wisconsin. *Received December 7, 1966.* This investigation was supported by Grant AI 04511 from the U. S. Public Health Service and by contributions from various companies and associations of the food industries.

[1] Abbreviations used: FDNB, 1-fluoro-2,4-dinitrobenzene; PTH, phenylthiohydantoin; PTC, phenylthiocarbamyl.

TABLE I: Amino Acid Composition of Staphylococcal Enterotoxin C (strain 137).

Amino Acid	Amino Acid Residues (g/100 g of dry protein)	Nitrogen (g/100 g of protein)	No. of Amino Acid Residues/Molecule	Nearest Integral No. of Amino Acid Residues for 34,100 g of Protein	Integral No. of Residues × Respective Residue Mol Wt
Lys	14.43	3.15	38.4	38	4,864
His	2.91	0.89	7.2	7	959
Arg	1.71	0.61	3.7	4	624
Asp	17.85	2.17	52.9	53	6,095
Thr	5.31	0.74	17.9	18	1,818
Ser	4.58	0.74	18.0	18	1,566
Glu	8.95	0.97	23.7	24	3,096
Pro	2.16	0.31	7.6	8	776
Gly	2.99	0.74	17.9	18	1,026
Ala	1.85	0.36	8.9	9	639
$^1/_2$-Cys	0.72	0.10	2.4	2	204
Val	6.50	0.92	22.4	22	2,178
Met	3.20	0.35	8.3	8	1,048
Ile	4.09	0.51	12.3	12	1,356
Leu	6.54	0.81	19.7	20	2,260
Tyr	9.80	0.84	20.5	21	3,423
Phe	5.35	0.51	12.4	12	1,764
Trp	0.99	0.15	1.8	2	372
Amide NH_2	1.71[a]	1.49	36.4	36[a]	(−36 + 18)
Total	100.00	16.36		296	34,050

[a] Excluded from total.

extrapolation to zero time. The amide nitrogen value was estimated in a similar manner. Cystine was determined with the analyzer after converting cystine to cysteic acid by performic acid oxidation (Hirs, 1956) followed by hydrolysis of the oxidized enterotoxins at 110° for 24 hr. Tryptophan was determined by the method of Beaven and Holiday (1952). Free cysteine content was determined by the titration method of MacDonnell *et al.* (1951) under a variety of conditions.

Amino- (N-) Terminal Amino Acid Analysis. The N-terminal amino acids of the two enterotoxins C were determined by the FDNB method of Sanger (1945; Fraenkel-Conrat *et al.*, 1955). Dinitrophenol (DNP) protein was prepared by shaking 0.2 μmole of enterotoxins with 10 mg of sodium bicarbonate and excess FDNB in 66% ethanol solution at room temperature for 2 hr. The resultant DNP protein was hydrolyzed in 6 N HCl at 105° for 16 hr and 12 N HCl at 105° for 4 hr in evacuated, sealed tubes. After dilution of the hydrolysates to approximately 1 N acid, ether-soluble DNP-amino acids were extracted with five 5-ml portions of peroxide-free ether. The ether-soluble fraction was dried under reduced pressure and dinitrophenol was removed at 55–60° for 1 hr by Mills' (1952) method. Ether-soluble DNP-amino acids were identified paper chromatographically by using the 1.5 M phosphate (pH 6)–toluene system of Levy (1955) and the *t*-amyl alcohol saturated with pH 5.0 phthalate system of Blackburn and Lowther (1951). DNP-amino acids separated on the chromatograms were cut out and eluted by 1% sodium bicarbonate solution in test tubes at 55° for 15 min. The color intensity was measured at 360 and 390 mμ.

Edman's PTH[1] procedure as modified by Fraenkel-Conrat *et al.* (1955) was employed for qualitative determination of the N-terminal PTH-amino acid of the enterotoxins. Enterotoxin (10 mg) was applied evenly to two paper strips (Whatman No. 1) as a 3% solution. PTC-enterotoxin[1] was prepared at 40° for 3 hr followed by 16 hr at room temperature under 100-mm pressure for cyclization of the PTH-amino acids. The resultant derivative was identified by using solvent A of Sjoquist (1953) on starch-treated paper with 4- and 16-hr descending development. The location of the PTH-amino acid was visualized by spraying with iodine–azide.

Carboxyl- (C-) Terminal Amino Acid Analysis. The C-terminal amino acids of the two enterotoxins C were determined by modified hydrazinolytic procedure (Niu and Fraenkel-Conrat, 1955; Spero *et al.*, 1965). The enterotoxin (0.2 μmole) was introduced into the test tube and dried in an oven at 100° for 3 hr. Anhydrous

TABLE II: Amino Acid Composition of Staphylococcal Enterotoxin C (strain 361).

Amino Acid	Amino Acid Residues (g/100 g of dry protein)	Nitrogen (g/100 g of protein)	No. of Amino Acid Residues/ Molecule	Nearest Integral No. of Amino Acid Residues for 34,000 g of Protein	Integral No. of Residues × Respective Residue Mol Wt
Lys	13.99	3.05	37.2	37	4,736
His	2.87	0.88	7.1	7	959
Arg	1.75	0.63	3.8	4	624
Asp	18.38	2.23	54.3	54	6,210
Thr	5.80	0.80	19.5	20	2,020
Ser	4.81	0.77	18.8	19	1,653
Glu	8.93	0.97	23.5	24	3,096
Pro	2.23	0.32	7.8	8	776
Gly	2.90	0.72	17.3	17	969
Ala	1.61	0.32	7.7	8	568
$^1/_2$-Cys	0.74	0.10	2.4	2	204
Val	5.87	0.83	20.2	20	1,980
Met	3.60	0.39	9.3	9	1,179
Ile	4.02	0.50	12.1	12	1,356
Leu	6.13	0.76	18.4	18	2,034
Tyr	10.27	0.88	21.4	21	3,423
Phe	5.25	0.50	12.1	12	1,764
Trp	0.84	0.13	1.5	2	372
Amide NH_2	1.62[a]	1.41	34.4	34[a]	(−34 + 18)
Total	99.99	16.19		294	33,907

[a] Excluded from total.

hydrazine (0.3 ml) was introduced and sealed under vacuum. Hydrazinolysis was carried out at 100° for 7.5 and 10 hr. The reaction products were dried under reduced pressure over concentrated sulfuric acid for 16 hr. The residues were dissolved in 1 ml of water and transferred to the centrifuge tube. Freshly distilled benzaldehyde (0.25 ml) was added and the mixture was shaken for 2 hr. The tube was centrifuged at 16,000 rpm for 20 min, and the aqueous supernatant was withdrawn. The benzaldehyde treatment was repeated. The aqueous solution was lyophilized and the residues were analyzed with the amino acid analyzer after dissolving in pH 2.2 citrate buffer.

Results

Amino Acid Composition. Tables I and II show the results of the amino acid analyses of enterotoxins C, strains 137 and 361, respectively. The figures presented are the average of four determinations with an average deviation of ±3%. As mentioned above, serine, threonine, and amide values were estimated by extrapolation to zero time. The other amino acids were essentially constant during the time of hydrolysis, except for valine which shows a higher value after 72 hr than after 24 hr of hydrolysis. As shown in Tables I and II, the total nitrogen calculated for enterotoxins C, strains 137 and 361, is 16.36 and 16.19%, respectively. These values are in good agreement with 16.2 and 16.0% for the two enterotoxins determined by the automatic analyzer.

The molecular weights of 34,100 and 34,000 for enterotoxins C, strains 137 and 361, respectively, calculated from the sedimentation, diffusion, and viscosity data (Borja and Bergdoll (1967); Avena and Bergdoll, 1967) were used to calculate the number of amino acid residues in the two enterotoxins. The number of residues for enterotoxins C, strains 137 and 361, was found to be 296 and 294, respectively.

No free cysteine was found to be present in enterotoxin C molecule by the method mentioned previously. The numbers of half-cystine for the two enterotoxins (Table I and II) are slightly higher than two residues per mole of the enterotoxins. This may be caused by difficulty in determining relatively small amounts of half-cystine in the enterotoxins accurately by the performic acid oxidation procedure.

N-Terminal Amino Acid. The results obtained from Sanger's (1945) FDNB method showed only the presence of DNP-glutamic acid in both enterotoxins except for usual by-products of dinitroaniline and trace amounts of dinitrophenol. Dinitrophenol was almost completely eliminated by Mills' (1952) method.

The recoveries of DNP-glutamic acid were calculated to be 0.78 and 0.94 mole/mole of enterotoxins C, strains 137 and 361, respectively, after correcting for destruction during the hydrolysis and for loss in paper chromatography. The ratio of optical density at 390:360 mμ in 1% bicarbonate solution was found to be 0.64 for DNP-glutamic acid obtained from both DNP-enterotoxins. DNP-arginine was not detected paper chromatographically in the water-soluble DNP-amino acid fractions of either of the hydrolysates of the DNP-enterotoxins. The large amount of ε-DNP-lysine, the trace amounts of Im-DNP-histidine, and the tailing spot of *O*-DNP tyrosine were the major products of water-soluble DNP-amino acid fractions of both DNP-enterotoxin hydrolysates. A short hydrolysis (4 hr) of the DNP-enterotoxins in 12 N HCl was performed as mentioned previously. No DNP-proline or DNP-glycine was detected paper chromatographically. In order to distinguish between terminal glutamic acid and glutamine, the PTH procedure as mentioned above was employed. Under the conditions described above, glutamine is not converted to glutamic acid. The results showed that the PTH-terminal amino acids of the enterotoxins C when compared chromatographically with PTH-glutamic acid and PTH-glutamine corresponded to PTH-glutamic acid. Therefore, it is apparent that glutamic acid is the N-terminal amino acid of both enterotoxins C.

C-Terminal Amino Acid. The method Spero *et al.* (1965) used in their investigation of the C-terminal amino acid of staphylococcal enterotoxin B was found to be excellent for identification and determination of the C-terminal amino acid of the two enterotoxins C. Almost 1 mole of glycine/mole of protein was obtained for both enterotoxins after correcting for destruction during the hydrazinolytic process. It is interesting to note that higher recovery was obtained in 7.5 hr than in 10 hr of hydrazinolysis.

From the long column (150 cm), in addition to glycine, trace amounts of several other amino acids were obtained. In order to confirm these results a 60-cm column was also employed. From the short column (15 cm) a small peak (one-tenth of glycine peak) was always obtained at the histidine position. However, this peak shifted to the lysine position if a 5-cm column was employed. Therefore, this basic ninhydrin-positive substance may be a by-product of the hydrazinolytic process.

The carboxypeptidase method, which gave satisfactory results in our investigation of C-terminal amino acid sequence of enterotoxin B (Bergdoll *et al.*, 1965b) was not effective in the study of the enterotoxin C. Even when high concentrations of carboxypeptidases A and B and an enterotoxin sample denatured by 6 M urea (Halsey and Neurath, 1955) were employed, no clear results were obtained. The failure of carboxypeptidases to release glycine from the C-terminal end of the enterotoxin C molecule may indicate the presence of a proline residue adjacent to the C-terminal glycine. From the data presented, it is concluded that glycine is the C-terminal amino acid of both enterotoxins.

Discussion

Based on the data presented in this paper, both enterotoxins have one N-terminal glutamic acid and one C-terminal glycine. If there are no other N-terminal amino acids such as a cystinyl or an acylated residue as detected in tobacco mosaic virus (Tsugita *et al.*, 1960; Anderer *et al.*, 1960), and no other C-terminal amino acids such as an asparaginyl, glutaminyl, or cystinyl residue, then the enterotoxins C should be single polypeptide chains. These exceptional residues could not be detected by the methods used herein.

Both enterotoxins C have an extremely high content of lysine and aspartic acid which comprise about one-third of the total amino acid residues in the enterotoxin molecules. An unusually high content of lysine and aspartic acid is also observed in enterotoxin B (Spero *et al.*, 1965; Bergdoll *et al.*, 1965b). It is interesting to note that enterotoxins B and C have the same N-terminal amino acid but different C-terminal amino acids.

As reported in accompanying papers (Borja and Bergdoll, 1967; Avena and Bergdoll, 1967), sedimentation, diffusion, viscosity, and molecular weight values of both enterotoxins C are nearly the same. The only significant difference in the two enterotoxins was found to be in their movement in an electric field. In this study, the same end groups and almost the same number of residues of each of the amino acids (within the limit of error) for both enterotoxins C were observed. However, from calculation of the acid–base balance, there is an excess of eight basic groups in the enterotoxin C (strain 137) molecule while only an excess of four basic groups in the enterotoxin C (strain 361) molecule. This may be the reason for the significant difference in the electrical mobilities of the two enterotoxins C.

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Ascaris Cuticle Collagen: on the Disulfide Cross-Linkages and the Molecular Properties of the Subunits*

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ABSTRACT: The subunit polypeptide chains of neutral salt-soluble *Ascaris* cuticle collagen (mol wt 900,000) are covalently linked by disulfide cross bridges. Reducing agents produce a rapid transition in the molecular properties of this collagen in the presence or absence of denaturing conditions. (1) The molecular weight decreases by a factor of 10–15. (2) The reduced viscosity (η_{sp}/c) falls to values in the range 0.5–1 dl/g. (3) The sedimentation coefficient ($s^0_{20,w}$) decreases from 7.0 to about 2.2 S. (4) The alkylated product (RCM *Ascaris*) shows a quantitative conversion of cystine to *S*-carboxymethylcysteine. Nevertheless, in the absence of denaturing agents >75% of the collagen-fold conformation is retained by the subunit polypeptide chains when the SS bridges are cleaved as judged by optical rotation studies. Gel filtration and high- and low-speed sedimentation equilibrium studies indicate that RCM *Ascaris* is relatively homogeneous with respect to size with a molecular weight of 62,000. At low temperature the collagen-fold conformation is regenerated in RCM *Ascaris* and the helix–coil transition in this material occurs without any change in molecular weight. Reduction and alkylation of the insoluble collagen matrix of *Ascaris* cuticle disperse virtually all of the collagen into solution, and the reduced alkylated product is indistinguishable in physical and chemical properties from that prepared from the neutral salt-soluble material.

Reoxidation of reduced *Ascaris* collagen gives a heterogeneous mixture of particle sizes, but the optical rotatory properties of the native protein are regenerated. The structure of the native collagen is considered in terms of the physical properties of the subunits.

Neutral salt-soluble collagen isolated from the cuticle of the invertebrate *Ascaris lumbricoides* exhibits a number of unusual physical and chemical properties. The molecular weight of this material, estimated at about 900,000 (Josse and Harrington, 1964), is significantly higher than that of the vertebrate tropocollagens. Moreover, the individual polypeptide chains of the structure appear to be covalently cross-linked since the molecular weight remains unchanged on denaturation, and since all of the physical properties of the native structure are completely regenerated at low temperature following thermal denaturation. Aside from the unusual pyrrolidine composition (29% proline and 2% hydroxyproline), *Ascaris* cuticle collagen exhibits an anomalously low glycine content (28%) and a small, but significant amount of half-cystine. The consistent presence of half-cystine residues in amino acid analyses of purified *Ascaris* collagen has prompted us to investigate the effect of reductive cleavage agents on the properties of this material. As we will demonstrate below, the polypeptide chains of *Ascaris* cuticle collagen are cross-linked through disulfide bridges. This report will focus on the properties of the subunit polypeptide chains derived from *Ascaris* on reductive cleavage of the disulfide bridges and will consider the significance of these findings in terms of the structure of the native collagen. A preliminary

* Publication No. 497 of the McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland. *Received November 28, 1966.* This work was supported by a U. S. Public Health Service research grant (AM 04349) from the National Institute of Arthritis and Metabolic Diseases.

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