INFECTION AND IMMUNITY, Nov. 1992, p. 4777–4780
0019-9567/92/114777-04$02.00/0

Vol. 60, No. 11

Purification and Characterization of a Peptide Essential for Formation of Streptolysin S by *Streptococcus pyogenes*

TERUAKI AKAO, TAKAYUKI TAKAHASHI, AND KYOICHI KOBASHI*

Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama-shi, Toyama 930-01, Japan

Received 5 June 1992/Accepted 1 September 1992

Peptides in a pronase digest of bovine serum albumin were required for streptolysin S formation by *Streptococcus pyogenes* besides maltose and a carrier (the oligonucleotide fraction obtained by treatment of *Saccharomyces cerevisiae* RNA with RNase A). A peptide essential for streptolysin S formation was purified to homogeneity from a pronase digest of bovine serum albumin by Sephadex G-25 column chromatography, and anion-exchange, reverse-phase, and gel filtration high-performance liquid chromatography. The purified peptide was divided into more than two peptides by HCOOOH oxidation and was composed of four residues of cysteine, three of leucine, and one each of aspartic acid and glutamic acid. Leucine and cysteine were detected as amino-terminal residues, and leucine and glutamic acid were detected as carboxyl-terminal residues, suggesting that two or three peptides are linked by a disulfide bond(s). A disulfide bond structure in the peptide seemed to be required for streptolysin S formation.

Streptolysin S is an oxygen-stable hemolysin produced by group A streptococci. This toxin is synthesized de novo in the bacteria and released into the medium upon exposure to various substances (so-called carriers), such as serum albumin, α-lipoprotein, the RNase A-resistant fraction (AF) of *Saccharomyces cerevisiae* RNA, and certain nonionic detergents, that form complexes with the toxin (4, 7, 11). Bernheimer (6) and Bernheimer and Rodbart (8) showed that the bacteria at the resting stage produce streptolysin S in a buffer containing maltose and the AF as essential factors.

Recently, we showed the presence of another factor essential for streptolysin S formation in resting cells besides maltose and the AF (1, 2). Thus, streptococcal cells which were sonicated and then washed did not produce the toxin without protease peptone or protease digests of bovine serum albumin (BSA), even in the presence of maltose and the AF, suggesting that the third essential factor for streptolysin S formation is peptide(s).

In the present study, we purified a peptide essential for streptolysin S formation from a pronase digest of BSA and concluded that this peptide is composed of three peptide fragments linked by two disulfide bonds.

MATERIALS AND METHODS

Reagents. Brain heart infusion broth was a product of Difco Laboratories (Detroit, Mich.). BSA (fraction V) was purchased from Sigma Chemical Co. (St. Louis, Mo.). Pronase (bacterial A1 proteinase) was a product of Nagase Biochemicals Ltd. (Osaka, Japan). Amino acid standard solution (type H), hydrazine (anhydrous), hydrochloric acid (20%; super special grade), and phenyl isothiocyanate were purchased from Wako Pure Chemical Co. (Osaka, Japan). Triethylamine was a product of Pierce Chemical Co. (Chicago, Ill.). All other reagents were of the best commercial quality available.

Streptococcal strain. Lancefield's group A *Streptococcus* strain C203A (*Streptococcus pyogenes* ATCC 14289) was maintained and cultured as reported previously (12).

* Corresponding author.

The AF and the oligonucleotide fraction. The AF was prepared from an RNase A (EC 3.1.27.5) digest of *S. cerevisiae* soluble RNA essentially as described by Bernheimer and Rodbart (8, 12). The oligonucleotide fraction, having fivefold higher inducer activity than the AF, was obtained by gel filtration of Sephadex G-25 from the AF as reported previously (3).

Production of streptolysin S and assay of hemolytic activity. Streptolysin S was produced from native and sonicated cells of resting bacteria, and the hemolytic activity of the streptolysin S produced was determined as described in our previous reports (2, 12).

Determination of peptides. Amounts of peptides were determined by using *o*-phthalaldehyde by the method of Benson and Hare (5) as follows. A sample was treated with 9 nmol of *o*-phthalaldehyde in 3 ml of 0.2 M borate buffer (pH 9.7) containing 12 mM 2-mercaptoethanol. The fluorescence of the reaction mixture was immediately measured at a 455-nm emission wavelength and a 340-nm excitation wavelength with a fluorometer (Shimadzu RF-500). Amounts of peptides were calculated on the basis of a calibration curve made with L-leucine (1 to 30 nmol) as the standard.

Purification of a peptide required for streptolysin S formation. BSA (1 g) in 10 mM potassium phosphate buffer (pH 7.2) was treated with pronase (10 mg) overnight at room temperature, heated in a boiling water bath for 20 min, and centrifuged at 5,000 × *g* for 20 min to obtain a clear supernatant. The pronase digest of BSA was applied to a Sephadex G-25 (Pharmacia LKB Biotechnology, Tokyo, Japan) column (3.4 by 85 cm) equilibrated with 50 mM potassium phosphate buffer (pH 6.8) and eluted with the same buffer. A broad pattern of hemolysin-inducing activity, like that shown in the previous report, was observed (2). One main peak having the lowest molecular weight (1,000 to 2,000) was collected and then applied to a reverse-phase column (TSKgel ODS-80TM; 4.6 by 150 mm; Tosoh Co. Ltd., Tokyo, Japan) equipped with a high-performance liquid chromatography (HPLC) apparatus (Gilson System). The column, equilibrated with water, was eluted with a linear gradient of 0 to 40% acetonitrile at a 0.5-ml/min flow rate, and the A_{230} was monitored (see Fig. 1). Two main peak

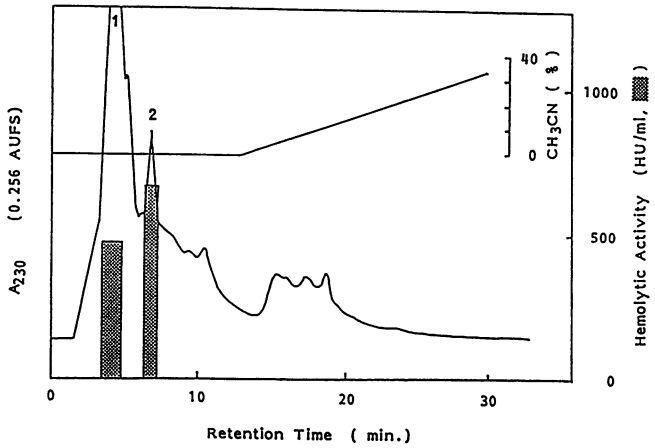


FIG. 1. Separation of streptolysin S-inducing peptides by reverse-phase HPLC. A 200-μl sample of the main Sephadex G-25 peak fraction with streptolysin S-inducing activity was applied to a reverse-phase column (TSKgel ODS-80TM; 4.6 by 150 mm) and eluted as described in Materials and Methods. AUFS, absorbance units, full scale.

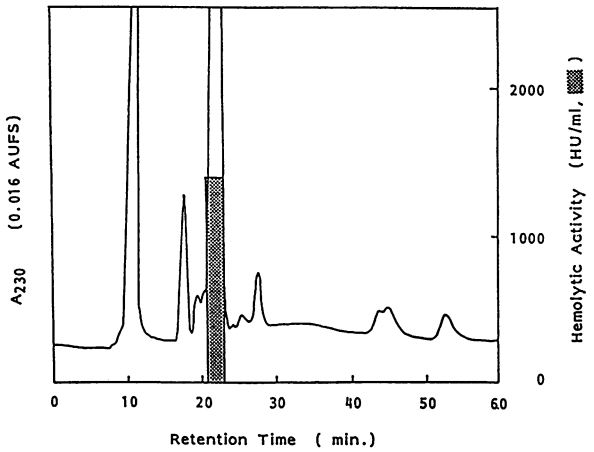


FIG. 2. Separation of a streptolysin S-inducing peptide by gel filtration HPLC. A 200-μl sample of the concentrated product of ion-exchange HPLC was applied to a gel filtration column (TSKgel G-Oligo-PW; 7.8 by 300 mm) and then eluted with water at a 0.5-ml/min flow rate. AUFS, absorbance units, full scale.

fractions having hemolysin-inducing activity were obtained, although hemolysin-inducing activity was sometimes detected in fractions other than these two. A slightly retarded fraction (no. 2 in Fig. 1), which was lyophilized and then dissolved in 10 mM Tris-HCl buffer (pH 7.4), was next applied to an ion-exchange column (TSKgel DEAE-3SW; 7.5 by 75 mm; Tosoh Co. Ltd.) equipped with the same HPLC apparatus and eluted with 6 ml of 10 mM Tris-HCl buffer (pH 7.4) and 10 ml of a continuous linear gradient from 0 to 0.2 M NaCl in 10 mM Tris-HCl buffer (pH 7.4) at a 0.5-ml/min flow rate. The two main peak fractions obtained were the nonadsorbed and the adsorbed. The nonadsorbed fraction, lyophilized and dissolved with water, was further purified by using a gel filtration column (TSKgel G-Oligo-PW; 7.8 by 300 mm; Tosoh Co. Ltd.) with the same HPLC apparatus. One peak, showing hemolysin-inducing activity, was eluted with water (see Fig. 2) and rechromatographed by the same column to get a pure peptide essential for streptolysin S formation. The apparent molecular weight of the purified peptide was estimated by using L-cysteic acid and the reduced and oxidized forms of glutathione as standards.

Chemical modification of disulfide bonds. Dried samples of the Sephadex G-25 peak fraction of the pronase digest of BSA and the purified peptide were treated for 4 h at 0°C with performic acid, which was prepared by mixing 9 ml of formic acid (88%) and 1 ml of hydrogen peroxide (30%) and allowing the mixture to stand for 1 h at room temperature to oxidize disulfide bonds to cysteic acid residues (13). The reaction mixture was then evaporated to dryness.

The above-described dried samples were also treated with a 100-fold molar excess of 2-mercaptoethanol for 1 h at 37°C and a 100-fold molar excess of dithiothreitol for 5 min at 100°C to reduce the disulfide bonds.

Analysis of amino acids. Dried samples of the purified peptide (for example, 1 nmol) in small tubes were first hydrolyzed by treatment with 20% HCl vapor for 24 h at 110°C or for 6 h at 130°C under a vacuum. Amino acids of the hydrolysates were next converted to phenylthiocarbamyl derivatives with phenyl isothiocyanate, and each amino acid derivative was separated on a reverse-phase column (Develosil ODS-5; 4.6 by 250 mm; Nomura Chemical Co., Ltd., Aichi, Japan) equipped with an HPLC apparatus as described by Bidlingmeyer et al. (9). The amount of each amino acid was determined by using phenylthiocarbamyl derivatives formed by pretreating a standard amino acid mixture with phenyl isothiocyanate.

Amino-terminal sequence analysis. The amino-terminal sequence was analyzed automatically by four cycles in a gas-phase sequencer (Applied Biosystems).

Determination of the carboxyl-terminal amino acid(s). A dried sample of the purified peptide (1 nmol) was solubilized with 20 μl of anhydrous hydrazine in a small tube, and the tube containing the peptide was next treated with 200 μl of

TABLE 1. Purification of streptolysin S-inducing peptide

Purification step	Total peptides[a] (μmol)	Total hemolysin-inducing activity (HU)	Sp act (HU/μmol)	Purification (fold)
Pronase digestion	733	249,000	339	1
Sephadex G-25 column chromatography	65	50,400	775	2.3
HPLC chromatography				
Reverse phase	13.2	16,900	1,280	3.8
Ion exchange		5,200		
2nd gel filtration	0.071 (0.008)[b]	4,400	61,900 (565,000)[b]	185

[a] Determined by *o*-phthalaldehyde fluorometry.
[b] Calculated from results of amino acid analysis.

TABLE 2. Amino acid analysis of purified streptolysin S-inducing peptide

Amino acid	Amt of purified peptide, pmol (no. of residues/mol)	Amt of HCOOOH-oxidized peptide, pmol (no. of residues/mol)
Aspartic acid	131 (1)	112 (1)
Glutamic acid	169 (1)	96.1 (1)
Leucine	410 (3)	294 (3)
Cysteic acid		391 (4)

TABLE 3. Requirement of disulfide bonds in peptides for streptolysin S formation

Treatment	Hemolysin-inducing activity (HU/ml)	
	Sephadex G-25 fraction	Purified peptide
None	4,200	1,400
Dithiothreitol	<40	0
2-Mercaptoethanol	<40	0
HCOOOH oxidation	0	0

anhydrous hydrazine for 20 h at 80°C under a vacuum as reported by Strydom (14). The treated sample was dried and then modified with phenyl isothiocyanate to form phenylthiocarbamyl derivatives. The phenylthiocarbamyl-derivative amino acids obtained were determined as described above.

RESULTS AND DISCUSSION

Purification of a peptide that stimulates streptolysin S formation. Sonicated streptococcal cells required a pronase digest of BSA in addition to maltose and the oligonucleotide fraction (carrier) for streptolysin S formation (2), suggesting that a peptide(s) involved in the pronase digest is essential for streptolysin S formation. Accordingly, we attempted to purify the streptolysin S-inducing peptide(s).

A streptolysin S-inducing peptide was purified from the pronase digest of BSA by sequential Sephadex G-25 chromatography, reverse-phase HPLC (Fig. 1), anion-exchange HPLC, and gel filtration HPLC (Fig. 2). The peptide was purified to homogeneity by repeated gel filtration HPLC. The apparent molecular weight of the purified peptide was estimated to be about 1,000. The purification results are summarized in Table 1. The peptide was purified 185-fold from the pronase digest of BSA, and the streptococcal sonicate was able to produce 61,900 hemolytic units (HU) of streptolysin S after addition of 1 μmol of the peptide in the presence of maltose and the oligonucleotide fraction.

In all of the purification steps, some peaks showing hemolysin-inducing activity were observed, indicating that several streptolysin S-inducing peptides were present in the pronase digest. Moreover, these peptides each have a molecular weight of more than 1,000, as judged from the Sephadex G-25 elution pattern (2).

Structure of the streptolysin S-inducing peptide. Amino acid analysis of the purified peptide showed the existence of aspartic acid, glutamic acid, and leucine (1:1:3 molar ratio) (Table 2). On the other hand, 4 mol of cysteic acid were found, besides 1 mol of aspartic acid, 1 mol of glutamic acid, and 3 mol of leucine in the HCOOOH-oxidized peptide. Moreover, after treatment of the peptide with HCOOOH, the peak with a molecular weight of about 1,000 disappeared and new, smaller peaks appeared on gel filtration HPLC.

Only leucine was found after the first cycle, and nothing was found after the second cycle by amino-terminal sequence analysis of the purified peptide. On the other hand, leucine and cysteic acid were found after the first cycle, cysteic acid was found after the second cycle, and nothing was found after the third cycle in the HCOOOH-oxidized peptide. Moreover, glutamic acid and leucine were found as the carboxyl-terminal amino acids of the purified peptide.

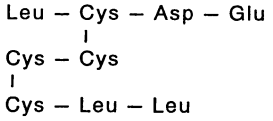
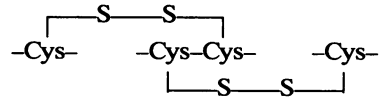


FIG. 3. Possible primary structure of a streptolysin S-inducing peptide.

These results indicate that the purified peptide is composed of three peptides linked with two disulfide bonds. Thus, the primary peptide structure shown in Fig. 3 is predicted on the basis of the above-described results and the primary structure of BSA (10). The molecular weight of the predicted peptide was calculated to be 1,046.42.

The molar amount of the peptide was calculated from the total amounts of amino acids obtained from the HCOOOH-oxidized peptide and the predicted structure. This value was one-ninth of the molar amount measured by *o*-phthalaldehyde fluorometry. The specific streptolysin S-inducing activity calculated from this value was 565,000 HU/μmol of the peptide (Table 1). The difference between the two values seems to be due to the fact that the purified peptide has three amino-terminal residues, because *o*-phthalaldehyde reacts with amino groups to form fluorescent compounds.

Requirement of a disulfide bond(s) for streptolysin S formation. As the purified peptide had two disulfide bonds, we examined whether disulfide bonds in the peptide are necessary for streptolysin S formation and whether disulfide bonds are involved in other streptolysin S-inducing peptides which have not been purified. The Sephadex G-25 peak fraction and the purified peptide were treated with HCOO OH, dithiothreitol, or 2-mercaptoethanol. All treated preparations showed no streptolysin S-inducing activity, despite the high activity of the original samples (Table 3). These results indicate that disulfide bonds in the purified peptide are required for streptolysin S formation and suggest that other peptides contained in the Sephadex G-25 fraction also have disulfide bonds essential for streptolysin S formation. As the primary structure, which is as follows:



is present on seven loci of BSA (10), many peptides with this primary structure seem to be obtained by pronase digests of BSA. Thus, the unique structure in which three peptide chains are linked by two disulfide bonds may be important for streptolysin S formation, and the mechanism of action of these peptides for streptolysin S formation requires further study.

ACKNOWLEDGMENTS

We thank I. Takemura for technical assistance and S. Takayanagi for secretarial assistance.

REFERENCES

1. **Akao, T., T. Akao, K. Kobashi, and C. Y. Lai.** 1983. The role of protease in streptolysin S formation. Arch. Biochem. Biophys. **233**:556–561.
2. **Akao, T., H. Tamei, and K. Kobashi.** 1988. The essential factor for streptolysin S production by *Streptococcus pyogenes*. Chem. Pharm. Bull. **36**:3994–3999.
3. **Akao, T., M. T. Wang, and C. Y. Lai.** 1980. Purification of oligonucleotide with streptolysin S inducer activity and *de novo* synthesis of the hemolysin. Arch. Biochem. Biophys. **201**:56–63.
4. **Alouf, J. E.** 1980. Streptococcal toxins (streptolysin O, streptolysin S, erythrogenic toxin). Pharmacol. Ther. **11**:661–717.
5. **Benson, J. R., and P. E. Hare.** 1975. *o*-Phthalaldhyde: fluorogenic detection of primary amines in the picomole range. Comparison with fluorescamine and ninhydrin. Proc. Natl. Acad. Sci. USA **72**:619–622.
6. **Bernheimer, A. W.** 1949. Formation of a bacterial toxin (streptolysin S) by resting cells. J. Exp. Med. **90**:373–392.
7. **Bernheimer, A. W.** 1972. Hemolysins of streptococci: characterization and effects on biological membranes, p. 19–31. *In* L. E. Wannamer and J. M. Matsen (ed.), Streptococci and streptococcal diseases. Academic Press, Inc., New York.
8. **Bernheimer, A. W., and M. J. Rodbart.** 1948. The effect of nucleic acids and of carbohydrates on the formation of streptolysin S. J. Exp. Med. **80**:149–168.
9. **Bidlingmeyer, B. A., S. A. Cohen, and T. L. Tarvin.** 1984. Rapid analysis of amino acids using pre-column derivatization. J. Chromatogr. **336**:93–104.
10. **Brown, J. R.** 1976. Structural origins of mammalian albumin. Fed. Proc. **35**:2141–2144.
11. **Ginsburg, I.** 1970. Streptolysin S, p. 100–171. *In* T. C. Montie, S. Kadis, and S. J. Ajl (ed.), Microbial toxins. Academic Press, Inc., New York.
12. **Lai, C. Y., M.-T. Wang, J. B. de Faria, and T. Akao.** 1978. Streptolysin S: improved purification and characterization. Arch. Biochem. Biophys. **191**:804–812.
13. **Schram, E., S. Moore, and E. J. Bigwood.** 1954. Chromatographic determination of cystine as cysteic acid. Biochem. J. **57**:33–37.
14. **Strydom, D. T.** 1988. A micromethod for the determination of carboxyl-terminal amino acids of peptides and proteins: phenylisothiocyanate reaction with hydrazinolysates. Anal. Biochem. **174**:679–686.