J. Biochem. **125**, 27-30 (1999)

Unique Synthetic Peptides Stimulating Streptolysin S Production in Streptococci

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Received August 26, 1998; accepted September 14, 1998

A peptide has been isolated from pronase digest of bovine serum albumin as the stimulatory factor of streptolysin S (SLS) production by *Streptococcus pyogenes*, and its primary structure has been deduced [Akao *et al.* (1992) *Infect. Immun.* 60, 4777-4780]. To determine the essential structure for the stimulation, a peptide (P-1) having the deduced structure, in which three peptide fragments are linked by two disulfide bonds, and shorter analogs (P-2 to P-4) of peptide P-1 were chemically synthesized. Another peptide (P-5), in which Ala is inserted between the two Cys residues in the middle peptide chain of P-1, was also synthesized. These synthetic peptides were identified by mass spectrometry and analysis of amino acid compositions. The synthetic P-1 stimulated SLS production in a dose-dependent manner. Other peptide analogs also showed remarkable stimulation of SLS production. Treatment of P-1 with performic acid resulted in loss of its stimulatory activity, indicating that disulfide bridges of the peptides are necessary for their activity on SLS production. These results suggest that the unique primary structure of three peptide chains linked by two disulfide bridges is requisite for the stimulatory effect on SLS production.

Key words: *Streptococcus pyogenes*, streptolysin S, toxin production, unique peptides.

Streptolysin S (SLS) is an oxygen-stable hemolysin produced by group A streptococci. Recently, Stephen *et al.* demonstrated using SLS-deficient Tn*916* insertional mutants that virulence of group A streptococci is closely related to SLS production (*1*). Thus it is important to elucidate the mechanism of SLS production in these bacteria. This toxin is biosynthesized *de novo* 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 yeast RNA, and certain nonionic detergents, which form complexes with the entire moiety, a polypeptide, of the toxin (*2-4*). Bernheimer (*5*) and Bernheimer and Rodbart (*6*) showed that the bacteria at the resting stage produce SLS in a buffer containing maltose and AF as essential factors.

The capacity of resting cells to produce SLS is remarkably diminished by washing or light sonication of the cells, even in the presence of maltose, Mg^{2+}, and AF, but is recovered by the addition of proteose peptone or protease digests of bovine serum albumin (BSA) (*7*). We isolated a peptide from a pronase digest of BSA as another factor besides maltose and AF required for SLS production in the resting cells and predicted its primary structure on the basis of chemical analyses (*8*). This putative peptide (P-1) consists of three peptide fragments linked by two disulfide bonds as shown in Fig. 1.

In the present study, we synthesized peptide P-1 with the putative primary structure mentioned above, and the related peptides (P-2-P-5 in Fig. 1). The synthetic P-1 and the related peptides showed remarkable stimulatory activity on the SLS production, indicating that the peptide structure linked by two disulfide bonds is requisite for the activity.

MATERIALS AND METHODS

Chemicals—All chemicals used for peptide synthesis were of reagent grade. 3-Nitropyridine-2-sulfenyl chloride (Npys-Cl) and Boc-Cys (Npys)-OH were purchased from Kokusan Chemical, Tokyo, and other BOC-amino acid derivatives were obtained from Peptide Institute, Osaka. Brain Heart Infusion (BHI) broth was a product of Difco Laboratories (Detroit, MI, USA). AF and the oligonucleotide fraction having fivefold higher inducer activity than AF were prepared as described previously (*9*). A reversed-phase resin (Cosmosil 5C18) was purchased from Nacalai Tesque, Osaka, and packed into a column (8×250 mm) in our laboratory.

Peptide Synthesis—P-1 with the primary structure shown in Fig. 1 was synthesized according to the scheme shown in Fig. 2. Other peptide analogs were synthesized by the same method. The experimental procedure for P-1 is briefly described below.

Synthesis of the Peptide Fragments of P-1—Each peptide fragment (Compounds 1-3 in Fig. 2) was synthesized by the liquid-phase method, by the stepwise elongation of each amino acid using the respective Boc-amino acid derivatives from the corresponding C-terminal Boc-amino acid benzyl esters. The Boc-groups of each segment were

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Abbreviations: SLS, streptolysin S; AF, RNase-resistant fraction; TFA, trifluoroacetic acid.

removed by treatment with 4 M HCl/dioxane, and the peptide bonds were formed by condensation using 1-ethyl-3(3'-dimethylaminopropyl)-carbodiimido (WSCD) in CH_2Cl_2 (*10, 11*). The protected peptide fragments (Compounds 1-3) of P-1 were purified by silica gel column chromatography, crystallized from a mixture of AcOEt and hexane, and identified by mass spectrometric analysis: Boc-Cys(Npys)-Cys(Acm)-OBzl (α_D −95.8°, mp 118-119.5°C), Boc-Cys(MeBzl)-Leu-Leu-OBzl (α_D −30.4°, mp 96-97°C), Boc-Leu-Cys(MeBzl)-Asp(OBzl)-Glu(OBzl)-OBzl (α_D −25.0°, mp 142-143°C).

Selective Formation of the Disulfide Bonds of P-1—The formation of the disulfide bonds of P-1 was carried out in a stepwise manner using three kinds of protecting groups (4-methylbenzyl, MeBzl; acetamidomethyl, Acm; and 3-nitropyridine-2-sulfenyl, Npys) for thiol of the Cys residues by the method of Matsueda *et al.* (*12*) as shown in Fig. 2. Compounds 1 and 2 in Fig. 2 were treated with anhydrous liquid hydrogen fluoride (HF) in the presence of anisole for 1 h on ice to remove all protecting groups except for the Acm and Npys groups. After removal of the HF under reduced pressure, the reaction mixtures were dried up *in vacuo*. The resulting residues of compounds 1 and 2 were mixed in 0.1 M sodium phosphate buffer (pH 6.5) to form the first disulfide linkage, resulting in the production of compound 4 in Fig. 2. After purification by reversed-phase high-performance liquid chromatography (RP-HPLC), compound 4 was treated with Npys-Cl in acetic acid to replace the Acm group with a Npys group, resulting in the production of compound 5 in Fig. 2. Compound 3 in Fig. 2 was treated with HF in the same way as compounds 1 and 2, then mixed with the purified compound 5 to yield P-1. Compounds 4, 5, and P-1 were purified by RP-HPLC and identified by mass spectrometry, and P-1 was analyzed for amino acid composition. The peptide concentration was determined by amino acid analysis.

Reversed-Phase High-Performance Liquid Chromatography—The HPLC apparatus consists of Waters 600 multisolvent delivery system equipped with Hitachi L-3000 photodiode array detector and D-2000 chromatointegrator (Tokyo). The peptides were separated by RP-HPLC on a column of Cosmosil 5C18 (8×250 mm). The peptides were eluted with a linear gradient of acetonitrile (CH_3CN) in 0.05% TFA at a flow rate of 1 ml/min, and the concentration of buffer B was increased at a rate of 1%/min. Gradients were prepared by mixing two buffers: A (0.05% TFA/H_2O) and B (0.05% TFA/CH_3CN).

Analysis of Purified Peptides—The amino acid compositions and molecular masses of purified peptides were determined by amino acid analysis and by fast atom bombardment mass spectrometry, respectively, as described previously (*13, 14*). Molecular weight of each synthetic peptide was measured using glycerol as a matrix by fast atom bombardment ionization double-focusing mass spectrometry (JEOL JMS-HX100).

Treatment of P-1 with Performic Acid—The lyophylized sample of P-1 (100 nmol) was treated for 4 h at 0°C with performic acid, which was prepared by mixing 9 ml of formic acid and 1 ml of hydrogen peroxide and by allowing the mixture to stand for 1 h at room temperature, to oxidize disulfide bonds to cysteic acid residues (*15*).

Bacterial Strain and Culture—Lancefield's group A streptococcal strain C-203A (*Streptococcus pyogenes* ATCC 14289) was maintained and cultured as reported previously (*16*).

Production of Streptolysin S—SLS was produced essentially as reported previously (*7*), as follows. Streptococci were grown to stationary phase, suspended in 50 mM potassium phosphate buffer (pH 6.8) containing 5 mM maltose and 5 mM $MgSO_4$ (MPB), and sonicated to wash out SLS production-stimulating factor(s). After centrifugation, the resulting precipitate was resuspended in MPB and mixed with oligonucleotide fraction at a final concentration of 2.0 of A_{260} unit/ml and a synthetic peptide at a suitable concentration. After incubation for 1 h at 37°C, the reaction mixture was centrifuged to get the supernatant fluid containing the SLS produced. The hemolytic activity of the supernatant was determined as described previously (*16*).

RESULTS

Synthesis of Peptides Composed of Three Peptide Fragments Linked by Two Disulfide Bonds—A peptide stimulating SLS production in streptococci has been purified from pronase digest of BSA (*8*). The putative primary structure of the peptide (P-1) is shown in Fig. 1, which is composed of three peptide fragments linked by two disulfide bonds. To examine the stimulatory activity of P-1 on SLS production, P-1 was chemically synthesized. After deprotection and purification of compounds 1-3 in Fig. 2, each fragment was crosslinked by disulfide bonds in a stepwise manner as shown in Fig. 2. The Cys(Npys) residue specifically reacts with a thiol group of the cysteine residue, resulting in the production of an asymmetric cystinyl peptide (*17*). This reaction was performed in acidic conditions (pH 6.5) in order to avoid the scrambling of disulfide bonds. A single product was observed in the HPLC profile of the reaction for formation of the second disulfide bond, and side reactions were not significant (data not shown), suggesting that two disulfide bonds were correctly formed in P-1. To estimate the effect of amino acid residues in P-1 on the stimulatory activity on SLS production, shorter peptide analogs (P-2, 3, and 4 in Fig. 1) of P-1 were synthesized by the same procedure as P-1. P-5, in which an Ala residue was inserted between the vicinal Cys residues in P-1, was also synthesized, because our preliminary data showed that the pronase digest of RNase A also stimulated SLS production, and RNase A contains a -Cys-Ala-Cys- sequence linked by disulfide bridges. After purifying of the

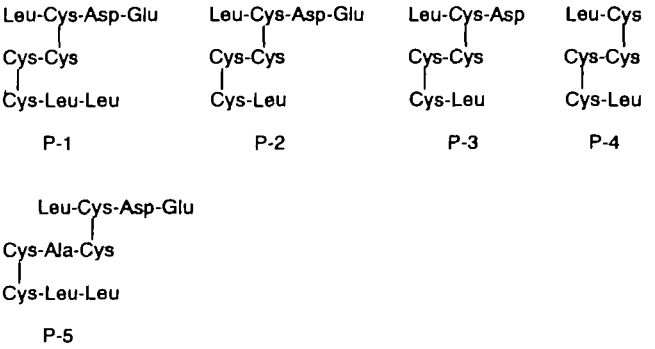

Fig. 1. **Structure of synthetic peptides.**

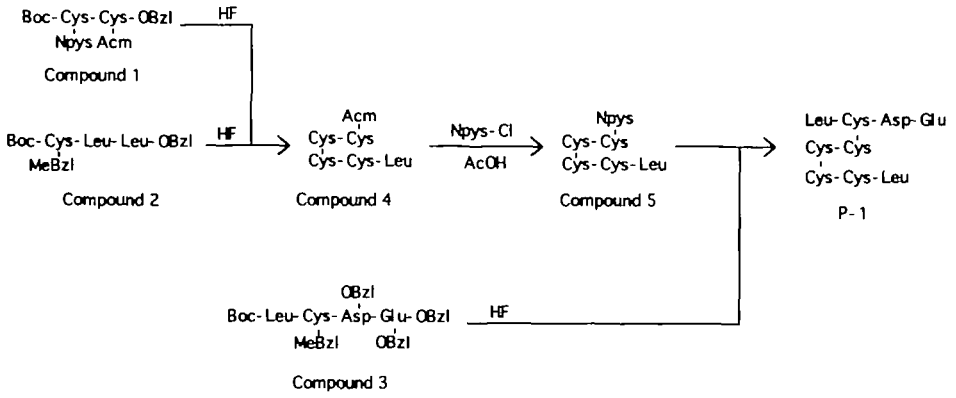

Fig. 2. **Scheme for synthesis of P-1 by stepwise formation of disulfide bonds.** Npys, 3-nitropyridine-2-sulfenyl; Acm, acetoamidomethyl; MeBzl, 4-methylbenzyl.

TABLE I. **Amino acid compositions and molecular weights of synthetic peptides.**

Peptide		P-1	P-2	P-3	P-4	P-5
M.W.	(observed)	1,045.4	932.3	803.5	688.3	1,116.4
	(calculated)	1,045.4	932.3	803.2	688.2	1,116.4
A.A. composition	Leu	3.0	2.0	2.0	2.0	–
	Asp	0.61	0.66	0.63	0	–
	Glu	0.92	0.96	0	0	–

Amino acid composition is indicated relative to Leu.

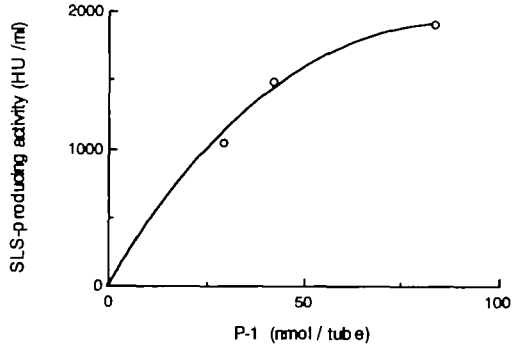

Fig. 3. **Streptolysin S-producing activity of P-1.** The concentration of P-1 was determined by amino acid analysis. SLS-producing activity was not detected when no P-1 was added.

final products by RP-HPLC, the synthetic peptides were identified by mass spectrometry and analysis of amino acid compositions. The observed values agreed well with the values calculated from the primary structures of the peptides, as summarized in Table I.

Stimulation of Streptolysin S Production by Synthetic Peptides—Synthetic peptide P-1, having the presumed structure of the previously purified stimulator of SLS production (*8*), induced SLS production dose-dependently in the resting cells of streptococci as shown in Fig. 3. The related synthetic peptides (P-2-P-4), which were shorter by one, two, and three amino acid residues, respectively, than P-1, also stimulated SLS production (Table II). The synthetic peptide P-5, in which the middle peptide fragment of Cys-Cys in P-1 was replaced by Cys-Ala-Cys, also stimulated SLS production. After treatment of P-1 with performic acid its SLS-producing activity was lost (Fig. 4), as was the purified peptide from pronase-digest of BSA (*8*). These results indicate that the unique structure of these

TABLE II. **Streptolysin S-producing activities of synthetic peptides.**

Peptide	Hemolysin produced (HU/ml)
Exp. 1.	
P-1	3,300
P-2	3,900
P-3	2,700
P-4	3,000
None	N.D.
Exp. 2.	
P-1	3,800
P-5	2,000
None	N.D.

In Exp. 1, 80 nmol of each peptide, which was determined from values obtained by amino acid analyses, was used. In Exp. 2, 100 nmol of each peptide was used.

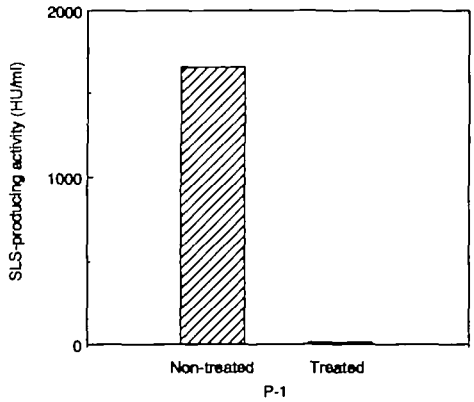

Fig. 4. **Effect of performic acid treatment on streptolysin S-producing activity of P-1.** P-1 (50 nmol) treated or non-treated with performic acid was used for the measurement of SLS production.

peptides linked by two disulfide bonds is requisite for the stimulatory activity of SLS production in streptococci.

DISCUSSION

Production of SLS by streptococci is affected by various factors such as maltose, Mg^{2+}, and carriers [RNase-resistant fraction (AF) of yeast RNA, certain nonionic detergents, serum albumin, and so on]. The entire moiety (polypeptide) of SLS is synthesized *de novo* at the resting stage in the cell suspended in a simple, chemically defined

solution containing maltose and Mg^{2+}, and it then forms a complex with the carrier and is released extracellularly (*2-4*). We have found that another factor, peptides derived from the culture medium, is required for *de novo* synthesis of the toxin besides maltose and Mg^{2+}, and this factor can be replaced by pronase digest of BSA (*7*). SLS production-stimulating peptide has been purified from the pronase digest of BSA, and its unique primary structure of three peptide fragments linked by two disulfide bonds was deduced on the basis of chemical analyses (*8*). In this paper, it was confirmed that a synthetic peptide (P-1) having this unique structure stimulated SLS production. Moreover, the finding that P-1 lost its inducing activity on treatment with performic acid (Fig. 4) indicates that the disulfide bonds in the peptide are requisite for SLS-inducing activity, as reported previously (*8*). In addition to P-1, the related peptides P-2, P-3, and P-4, composed of three peptide fragments linked by two disulfide bonds, also stimulated SLS production. We previously reported that many kinds of SLS-inducing peptides with molecular weights of more than 1,000 were present in pronase digest of BSA, and their activities were destroyed by treatment with reagents that disrupt disulfide bonds such as sulfhydryl compounds and performic acid (*7, 8*). Moreover, pronase digests of other proteins such as casein (*7*), α-chymotrypsinogen, β-lactoglobulin, and RNase A also stimulated SLS production (data not shown), though the digest of BSA, in which structures linked by two disulfide bonds are present at seven loci, was the best. RNase A contains the Cys-Ala-Cys sequence linked by disulfide bonds like P-5. Accordingly, it seems that the unique structure linked by disulfide bonds is requisite for the stimulation of SLS production, regardless of the number and kinds of amino acid residues that constitute the peptides. The minimum structural unit essential for the SLS-inducing activity seems to be a peptide in which two cysteine residues are linked to Cys-Cys by two disulfide bonds, because all synthetic peptides (P-1-P-4), which contain five, four, three and two additional amino acid residues, respectively, in addition to four cysteine residues linked by two disulfide bonds, stimulated SLS production to a similar extent (Table I).

The action mechanism of SLS production-stimulating peptides has not yet been elucidated. However, the peptides seem not to act as carriers, because oligonucleotides were required as carriers even in the presence of the peptides for SLS production (*7*). Moreover, from the requirement of both peptides and maltose for SLS production in streptococci (*7*), it is suggested that these two factors stimulate *de novo* synthesis of SLS in different manners. Finally, it is of interest that a gene, *sag*A, associated with SLS production in group A streptococci is expressed in wild types of the cells, but not in SLS-deficient mutant cells with markedly reduced virulence, as an estimated translational product of 53 amino acids containing several cysteine residues (*1*). Further study on the mechanism of action of the peptide for SLS production is now in progress.

We thank Professor Y. Shimonishi (Protein Research Institute, Osaka University) for his helpful advice.

REFERENCES

1. Betschel, S.D., Borgia, S.M., Barg, N.L., Low, D.E., and de Azavedo, J.C. (1998) Reduced virulence of group A streptococcal Tn916 mutants that do not produce streptolysin S. *Infect. Immun.* **66**, 1671-1679
2. Ginsburg, I. (1970) Streptolysin S in *Microbial Toxins* (Montie, T.C., Kadis, S., and Ajl, S.J., eds.) pp. 100-171, Academic Press, New York
3. Bernheimer, A.W. (1972) Hemolysins of streptococci: Characterization and effects on biological membranes in *Streptococci and Streptococcal Diseases* (Wannamer, L.E. and Matsen, J.M., eds.) pp. 19-31, Academic Press, New York
4. Alouf, J.E. (1980) Streptococcal toxins (streptolysin O, streptolysin S, erythrogenic toxin). *Pharmacol. Ther.* **11**, 661-717
5. Bernheimer, A.W. (1949) Formation of a bacterial toxin (streptolysin S) by resting cells. *J. Exp. Med.* **90**, 373-392
6. Bernheimer, A.W. and Rodbart, M.J. (1948) The effect of nucleic acids and of carbohydrates on the formation of streptolysin S. *J. Exp. Med.* **80**, 149-168
7. Akao, T., Tamei, H., and Kobashi, K. (1988) The essential factor for streptolysin S production by *Streptococcus pyogenes*. *Chem. Pharm. Bull.* **36**, 3994-3999
8. Akao, T., Takahashi, T., and Kobashi, K. (1992) Purification and characterization of a peptide essential for formation of streptolysin S by *Streptococcus pyogenes*. *Infect. Immun.* **60**, 4777-4780
9. Akao, T., Wang, M.-T., and Lai, C.Y. (1980) Purification of oligonucleotide with streptolysin S inducer activity and *de novo* synthesis of the hemolysin. *Arch. Biochem. Biophys.* **201**, 56-63
10. Sheeham, J.C. and Hess, G.P. (1955) A new method of forming peptide bonds. *J. Am. Chem. Soc.* **77**, 1067-1068
11. Sheeham, J.C., Cruickshank, P.A., and Boshart, G.L. (1961) A convenient synthesis of water-soluble carbodiimide. *J. Org. Chem.* **26**, 2525-2528
12. Matsueda, R., Higashida, S., Ridge, R.J., and Matsueda, G.R. (1982) Activation of conventional S-protecting groups of cysteine by conversion into the 3-nitro-2-pyridinesulfenyl (Npys) group. *Chem. Lett.* 921-924
13. Takao, T., Tominaga, N., Yoshimura, S., Shimonishi, Y., Hara, S., Inoue, T., and Miyano, A. (1985) Isolation, primary structure and synthesis of heat-stable enterotoxin produced by *Yersinia enterocolitis*. *Eur. J. Biochem.* **152**, 199-206
14. Bidlingmeyer, B.A., Cohen, S.A., and Tarvin, T.L. (1984) Rapid analysis of amino acids using pre-column derivatization. *J. Chromatogr.* **336**, 93-104
15. Schram, E., Moore, S., and Bigwood, E.J. (1954) Chromatographic determination of cystine as cysteic acid. *Biochem. J.* **57**, 33-37
16. Lai, C.Y., Wang, M.-T., deFaria, J.B., and Akao, T. (1978) Streptolysin S: improved purification and characterization. *Arch. Biochem. Biophys.* **191**, 804-812
17. Chino, N., Kubo, S., Nishiuchi, Y., Kumagaye, S., Kumagaye, K.Y., Takai, M., Kimura, T., and Sakakibara, S. (1988) Synthesis of porcine C5a anaphylatoxin by the solution procedure and confirmation of the reported structure. *Biochem. Biophys. Res. Commun.* **151**, 1285-1292