[Chem. Pharm. Bull. 36(10)3994—3999(1988)]

The Essential Factor for Streptolysin S Production by *Streptococcus pyogenes*

TERUAKI AKAO, HIRONORI TAMEI and KYOICHI KOBASHI*

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

(Received March 4, 1988)

Neither the supernatant nor the precipitate fraction obtained from sonicated streptococci produced streptolysin S, but the combination of both fractions restored the ability to produce streptolysin S. Streptolysin S production by the precipitate required heat-stable and low-molecular substance(s) in the supernatant in addition to maltose and carrier(s) such as ribonuclease resistant yeast ribonucleic acid. The heat-stable factor could be replaced by brain heart infusion (BHI) broth. Moreover, proteose peptone involved in BHI and protease digests of proteins such as bovine serum albumin (BSA) and casein showed stimulative effects on streptolysin S production, but BSA, casein, casamino acids and an amino acid mixture did not. Some peptides having molecular weights of several thousands were required for streptolysin S production by the precipitate.

These peptides could not be replaced by carriers and maltose for hemolysin production by the precipitate. Thus, three inducer factors, carrier, maltose and peptides, were required for streptolysin S formation. Peptides and maltose were essential for streptolysin S production in the cells, but not for release of the hemolysin from the cells into the medium, which required the carrier.

Keywords——streptolysin S; *Streptococcus pyogenes*; hemolysin production; peptide

Introduction

Streptolysin S is the oxygen-stable hemolysin secreted into the medium by group A streptococci upon exposure to various inducers such as serum albumin, α-lipoprotein, the ribonuclease (RNase)-resistant fraction of yeast ribonucleic acid (RNA) (so-called active fraction, AF), or certain nonionic detergents.[1-3] The entire moiety of streptolysin S appears to be a polypeptide having molecular weight of about 3000, which binds to the inducer agents and transfers from one agent to another.[4,5] Bernheimer,[6] and Bernheimer and Rodbart[7] showed that the formation of streptolysin S occurred at the resting stage in a simple, chemically defined medium, which contained maltose and $MgSO_4$ besides AF in a phosphate buffer.

Ginsburg and Grossowicz[8] reported the presence of a cell-bound streptolysin S which caused lysis of erythrocytes on contact. Schwab,[9] and Taketo and Taketo[10,11] reported the formation of the hemolysin in the cell-free extract of streptococci and showed that the hemolysin was stabilized by the addition of AF. In this system, maltose was required for the intracellular production of hemolysin, and AF was not. These findings suggested that streptolysin S was synthesized in the cell and was released into the medium by the addition of AF. Recently, Calandra and his collaborators[12-15] obtained evidence suggesting the presence of an intracellular hemolysin, mainly localized in the membrane fraction, which could be extracted with a solution containing AF, and named it cellular potential streptolysin S. On the other hand, Taketo and Taketo[16] recently reported that streptolysin S was released into the medium without AF. We obtained results suggesting that proteolysis of the cellular precursor to the active hemolysin proceeds before AF-dependent release of streptolysin S.[17] In this experiment sonicated cells still produced streptolysin S, but neither the supernatant

nor the precipitate fraction alone separated by centrifugation formed hemolysin even in the presence of maltose and AF. The result suggested that some other factor besides maltose and AF was necessary for the formation of streptolysin S.

In the present study, we undertook to find the factor essential for the production of streptolysin S in the supernatant fraction. Consequently, we discovered that brain heart infusion (BHI) involved in the supernatant stimulated the formation of streptolysin S by the precipitate, and proteose peptone in BHI or protease digests of bovine serum albumin (BSA) also stimulated it, suggesting that peptides are the third factor.

Materials and Methods

Streptococcal Strain——Lancefield's group A streptococcus strain C203A (*Streptococcus pyogenes* ATCC 14289) was maintained and cultured as reported previously.[18]

AF and Oligonucleotide Fraction (ONF)——AF was prepared from an RNase A (EC 3.1.27.5) digest of yeast soluble RNA, essentially as described by Bernheimer and Rodbart.[7] AF was further purified by gel filtration on Sephadex G-75. ONF having 5-fold higher inducer activity than AF was obtained as reported previously.[19]

Production of Streptolysin S——Streptolysin S was produced essentially as described by Lai *et al.*,[18] as follows. Streptococci maintained on BHI agar slants were inoculated into BHI medium and grown overnight at 37 °C. This was then added to 9 vol of BHI medium. After cultivation for 4 h the cells were collected by centrifugation and suspended in 0.25 vol of 50 mM potassium phosphate buffer, pH 6.8, containing 5 mM maltose and 5 mM $MgSO_4$ (MPB). The cell suspension was sonicated 4 times for 1 min in ice and centrifuged at $10000 \times g$ for 20 min. The precipitate was resuspended in the same volume of MPB. After the addition of ONF at a final concentration of 2.0 of A_{260} unit/ml, the native cells or the precipitate suspended in MPB were incubated with an equal volume of additives or MPB for 60 min at 37 C and centrifuged to obtain the supernatant containing streptolysin S.

Assay of Hemolytic Activity——The hemolytic activity of streptolysin S was determined as described previously.[18] One hemolytic unit (HU) was defined as the amount of hemolysin which lyses half of the human erythrocytes in a 0.35% suspension in 2.15 ml of 50 mM potassium phosphate buffer, pH 6.8, containing 0.9% NaCl in 1 h at 37 C.

Treatment of BSA and Casein with Proteases——BSA or casein (20 mg) in 10 mM potassium phosphate buffer, pH 7.2, was incubated with 200 μg of trypsin, chymotrypsin, papain, pronase, or pancreatin overnight at room temperature, heated in boiling water for 20 min, and centrifuged at 3000 rpm for 10 min to get a clear supernatant.

Reagents——BHI, proteose peptone, and beef extract were purchased from Difco Laboratories, U.S.A. Casamino acids and general anaerobic medium (GAM) were from Nissui Seiyaku Co., Tokyo, BSA, trypsin, chymotrypsin, and pancreatin were from Sigma Chemical Co., U.S.A., casein was from Merck and Co., Inc., U.S.A., pronase E was from Kaken Seiyaku Co., Tokyo and antistreptolysin-O serum was from Wako Pure Chemical Ind. Ltd., Osaka. Papain was kindly supplied by Dr. N. Nakai, Fukui Medical School, Fukui.

Results

Requirement of Heat-Stable Factor(s) of Low Molecular Weight for Streptolysin S Production

It has been reported that sonicated bacteria produces streptolysin S as well as native bacteria in the presence of maltose and ONF in potassium phosphate buffer, but neither the supernatant nor the precipitate fraction obtained from the sonicated bacteria by centrifugation produces streptolysin S.[1] However, we discovered that a mixture of the supernatant and the precipitate produced the hemolysin as well as native bacteria. The precipitate could not be replaced by that from other bacteria such as *Streptococcus faecalis*, *Eubacterium* sp., *Ruminococcus* sp. and *Clostridium innocuum* (data not shown), but the supernatant could be replaced by that from the other bacteria mentioned above. Moreover, the supernatant filtered through a PM10 membrane (Amicon Co.) and the filtrate heated at 100 °C for 20 min also showed potently stimulative activity toward hemolysin production by the precipitate, but the ashed filtrate showed no activity, as shown in Table I. These findings indicate that heat-stable and low-molecular substance(s) present in the supernatant is (are) effective for streptolysin S production by the precipitate.

TABLE I. Effects of the Supernatant Fraction on Streptolysin S Production by the Precipitate Fraction

Addition	Hemolytic activity (HU/ml)
None	0
Supernatant of *S. pyogenes*	22700
Filtrate of the supernatant	21000
Heated filtrate of the supernatant	16500
Ashed filtrate of the supernatant	0

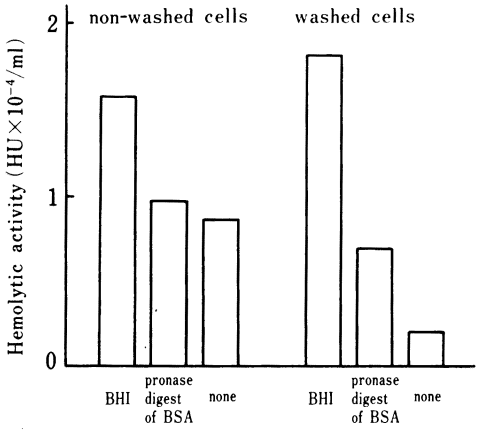

Fig. 1. Effects of BHI and Pronase Digest of BSA on Hemolysin Production by Washed and Non-washed Cells

Streptococci harvested were used immediately or washed once with MPB and suspended in MPB. Production of streptolysin S was carried out according to Materials and Methods. BHI and pronase digest of BSA were added at final concentrations of 18.5 and 5 mg/ml, respectively.

TABLE II. Effects of BHI and Various Nutrients on Streptolysin S Production

Addition	Hemolytic activity (HU/ml)
None	0
BHI	17700
Glucose	0
Beef extract	< 50
Proteose peptone	5000
Synthetic medium	0
Casamino acid	< 50
Mixture of amino acids	0
Casein	0
BSA	0

The precipitate suspended in MPB containing ONF was mixed with additives at the following concentrations. BHI, 18.5 mg/ml; glucose, beef extract, proteose peptone, casamino acids, casein, and BSA, 2.5 mg/ml; a mixture of 19 amino acids, 2.3 mg/ml. An equal volume of synthetic medium prepared as reported by van de Rijn and Kessler[20] was added to the precipitate suspension.

Potent Activity of BHI on Streptolysin S Production

Streptococcal cells harvested from BHI medium produced 5-fold more streptolysin S than the cells washed with the buffer after harvesting, as shown in Fig. 1. Production of streptolysin S by the washed cells was markedly stimulated by the addition of BHI and that by non-washed cells was also stimulated. It was suggested that higher production of streptolysin S by the non-washed cells than that by the washed cells is probably due to the stimulating effect of unknown substance(s) in BHI. The effect of the supernatant fraction on hemolysin production by the sonicated cells (Table I) seems to be also due to BHI contamination in the supernatant fraction. This was confirmed by the remarkably stimulating effect of BHI on streptolysin S production by the precipitate fraction as shown in Table II. BHI heated and filtered by ultrafiltration also showed a potent effect on hemolysin production. The residue after extraction of BHI with chloroform–methanol (2 : 1) had also a potent effect, indicating that lipid is not an activator (data not shown).

Stimulation of Streptolysin S Production by Peptides

Glucose and beef extract involved in BHI had little or no effect on hemolysin production by the sonicated cells, but proteose peptone was potently effective (Table II). A chemically defined medium, which was developed for streptococci by van de Rijn and Kessler,[20] containing amino acids but not proteose peptone, did not affect the production at all.

TABLE III. Effect of Digests of Casein and of BSA on Streptolysin S Production

	Hemolysin production (%)
BHI	100
Digests of casein	
Trypsin digest	4.5
Pronase digest	12
Papain digest	16
Pancreatin digest	3.7
Digests of BSA	
Trypsin digest	3.6
Chymotrypsin digest	6.0
Pronase digest	41
Papain digest	35
Pancreatin digest	4.0

Digests of casein and of BSA were added at the final concentrations of 2.5 mg original protein per ml.

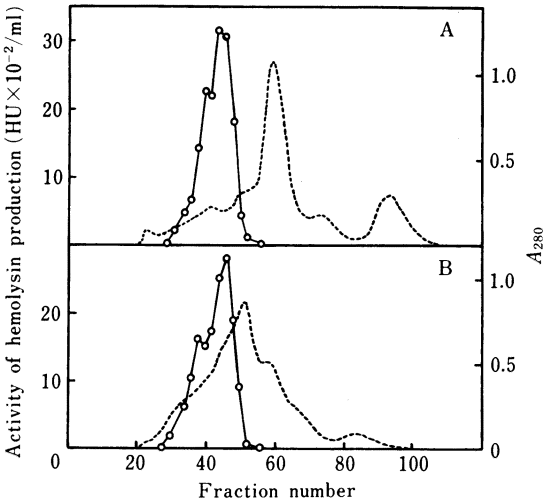

Fig. 2. Sephadex G-25 Column Chromatography of Pronase and Papain Digests of BSA

The column (2×33 cm) was equilibrated with 50 mM potassium phosphate buffer (pH 6.8) and eluted with the same buffer. Digests of BSA (25 mg/ml, 1.5 ml) were applied to the column and fractions of 1.5 ml were collected. Activity of hemolysin production (o—o) and absorbance at 280 nm (----) were measured by using an aliquot from each fraction according to Materials and Methods. A, Pronase digest of BSA; B, papain digest of BSA.

Casamino acids, an amino acid mixture containing 19 amino acids, casein and BSA had no activity, as shown in Table II. Therefore, the effects of digests of casein and of BSA were examined, because proteose peptone is a digest of proteins such as casein. Digests of casein and of BSA with several proteases were active for hemolysin production, as shown in Table III. Digests of BSA were more effective than those of casein, and pronase and papain digests were more effective than trypsin, chymotrypsin and pancreatin digests. Amounts of hemolysin formed were dependent upon the amounts of pronase digest of BSA as in the case of BHI and on the incubation period at 37 °C (data not shown). Furthermore, the pronase digest enhanced hemolysin production in the native washed cells more than 3-fold (Fig. 1). From these results it is suggested that peptides present in proteose peptone, digests of casein and of BSA are required for hemolysin production.

In order to isolate the stimulating factor(s), digests of BSA were applied to a Sephadex G-25 column. Both pronase and papain digests of BSA were eluted with similar profiles in terms of hemolysin-producing activity, showing that the molecular weights of active substances were higher than one thousand, though the elution profiles in terms of absorbance at 280 nm were quite different from each other (Fig. 2). A similar elution profile in terms of hemolysin-producing activity was observed on gel filtration of BHI using the same column (data not shown). Moreover, hemolytic activities produced by the addition of BHI, pronase digests of BSA or peptides eluted from a Sephadex G-25 column were inhibited completely by 25 μg/ml of trypan blue or more than 80% by 5 mg/ml of phosphatidylcholine (egg), but were not inhibited by antistreptolysin-O serum (data not shown). These data show that hemolysins produced by the sonicated cells coincided with streptolysin S. These findings indicate that peptides derived from proteose peptone and digests of BSA having molecular weights of several thousands are necessary for streptolysin S production.

TABLE IV. Requirement of Three Factors for Streptolysin S Production

Addition	Hemolytic activity (HU/ml)	Addition	Hemolytic activity (HU/ml)
Exp. 1 (the precipitate in MPB)		Exp. 2 (the precipitate in PB containing ONF)	
BHI	50	BHI	1700
BHI + ONF	16000	BHI + maltose	3500
Pronase digest of BSA	< 50	Pronase digest of BSA	< 50
Pronase digest of BSA + ONF	9300	Pronase digest of BSA + maltose	1400

Exp. 1: Streptococci suspended in MPB were sonicated and centrifuged. BHI (18.5 mg/ml), ONF (2.0 at A_{260}), or pronase digest of BSA (5 mg/ml) was added to the precipitate resuspended in MPB. Exp. 2: Streptococci suspended in 50 mM potassium phosphate buffer (pH 6.8) containing 5 mM $MgSO_4$ were sonicated and centrifuged. BHI (18.5 mg/ml), maltose (2.5 mM), or pronase digest of BSA (5 mg/ml) was added to the precipitate resuspended in the same buffer containing ONF.

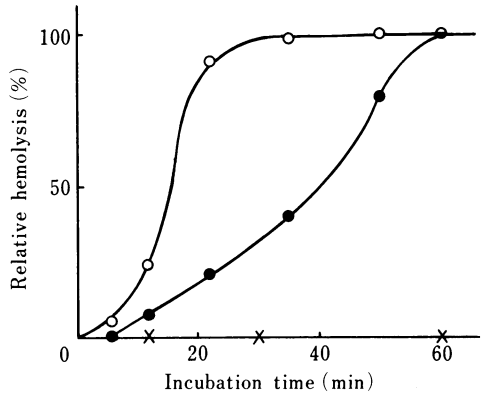

Fig. 3. Effect of BHI and Pronase Digest of BSA on Direct Hemolysis by the Precipitate in the Absence of ONF

The precipitate suspended in MPB without ONF was preincubated with 18.5 mg/ml of BHI or 5 mg/ml of pronase digest of BSA at 37 C for 20 min. Human erythrocytes (0.35%) were incubated with an aliquot (10 μl) of the above suspensions in 2.1 ml of 50 mM potassium phosphate buffer, pH 6.8, containing 0.9% NaCl. After incubation for the indicated period of time, the percentage hemolysis was determined by measuring the absorbance at 540 nm of the supernatant obtained by centrifugation. The same procedure was carried out without BHI and the pronase digest as a control (×). —○—, BHI; —●—, pronase digest of BSA.

Peptides are the Essential Factor for Streptolysin S Production

The streptolysin S production system in the sonicated cells contained maltose and oligonucleotide. Without oligonucleotide, streptolysin S was not formed even in the presence of maltose and BHI or maltose and pronase digest of BSA (Table IV). Moreover, without maltose hemolysin was not produced even in the presence of oligonucleotide and pronase digest of BSA, though streptolysin S was produced only with oligonucleotide and BHI, suggesting that BHI contains maltose or substance(s) acting as a substitute for maltose. These facts indicate that maltose and oligonucleotide can not replace pronase digest of BSA. Thus, peptides such as those in pronase digest of BSA are the essential factor for streptolysin S production in addition to maltose and oligonucleotide.

On the other hand, when the precipitate was incubated directly with erythrocytes in the maltose solution, hemolysis was observed without oligonucleotide by the addition of pronase digest of BSA or BHI, though hemolysis did not occur without pronase digest of BSA and BHI, as shown in Fig. 3. From the results, it is suggested that peptides do not act on the release of streptolysin S from the cell to the medium (like a carrier such as oligonucleotide), but on the production of streptolysin S in the cell.

Discussion

Maltose and a carrier such as RNase-resistant fraction of yeast RNA were required for production of streptolysin S by streptococci suspended in a simple, chemically defined solution.[6,7] The bacteria harvested at the stationary phase showed high capacity to produce

streptolysin S.[18] However, this capacity of streptococci depended on the culture conditions and was decreased by washing the cells. In the present study, it was confirmed that constituents in BHI broth, which were washed out partially and removed completely by sonication, were necessary for streptolysin S production. The precipitate fraction obtained from the sonicated bacteria did not produce streptolysin S, but it produced streptolysin S on the addition of BHI, proteose peptone or digests of BSA (Tables II and III). However, since glucose and amino acids used as nutrients were not effective on streptolysin S production, proteose peptone and the digests of BSA seemed to affect specifically streptolysin S production by unknown mechanisms, but not by stimulation of protein synthesis. From the results that BSA and amino acids had no effect in spite of the potent activity of the digests of BSA, it is verified that peptides involved in the digests of BSA are effectors, and this was confirmed by the elution profile of the digests of BSA on a Sephadex G-25 column (Fig. 2). Proteose peptone and the digests of BSA were about one-half less active than BHI (Tables II and III). Other constituents in BHI may potentiate streptolysin S production, for example, by stimulation of protein synthesis.

Although streptolysin S production was enhanced by the pretreatment or treatment of native streptococci with trypsin,[16,21] it is not clear whether this production is stimulated by tryptic digests of streptococcal proteins.

The attempts to purify a single active peptide from the digests of BSA were unsuccessful and instead we found several kinds of peptides to be effective. Elucidation of the common sequence of these peptides for the hemolysin production is in progress.

Acknowledgments We thank Miss S. Takayanagi for her secretarial assistance.

References

1) I. Ginsburg, "Microbial Toxins," ed. by T. C. Montie, S. Kadis and S. J. Ajl, Academic Press Inc., New York, 1970, pp. 100—171.
2) A. W. Bernheimer, "Streptococci and Streptococcal Diseases," ed. by L. E. Wannamer and J. M. Matson, Academic Press Inc., New York, 1972, pp. 19—31.
3) J. H. Alouf, *Pharmacol. Ther.*, **11**, 661 (1980).
4) J. Koyama, *J. Biochem.* (Tokyo), **54**, 146 (1963).
5) A. W. Bernheimer, *J. Bacteriol.*, **93**, 2024 (1967).
6) A. W. Bernheimer, *J. Exp. Med.*, **90**, 373 (1949).
7) A. W. Bernheimer and M. J. Rodbart, *J. Exp. Med.*, **80**, 149 (1948).
8) I. Ginsburg and N. Grossowicz, *Bull. Res. Counc. Isr.*, **713**, 237 (1958).
9) J. H. Schwab, *J. Bacteriol.*, **71**, 100 (1956).
10) A. Taketo and Y. Taketo, *J. Biochem.* (Tokyo), **56**, 552 (1964).
11) A. Taketo and Y. Taketo, *J. Biochem.* (Tokyo), **57**, 787 (1965).
12) G. B. Calandra and E. L. Ogindky, *Infect. Immun.*, **12**, 13 (1975).
13) G. B. Calandra, R. S. Whitt and R. M. Cole, *Infect. Immun.*, **13**, 813 (1976).
14) G. B. Calandra, *Infect. Immun.*, **29**, 306 (1980).
15) G. B. Calandra and M. C. Roger, *Infect. Immun.*, **31**, 386 (1981).
16) A. Taketo and Y. Taketo, *Z. Naturforsch.*, **41c**, 258 (1986).
17) T. Akao, T. Akao, K. Kobashi and C. Y. Lai, *Arch. Biochem. Biophys.*, **233**, 556 (1983).
18) C. Y. Lai, M.-T. Wang, J. B. de Faria and T. Akao, *Arch. Biochem. Biophys.*, **191**, 804 (1978).
19) T. Akao, M.-T. Wang and C. Y. Lai, *Arch. Biochem. Biophys.*, **201**, 56 (1980).
20) I. van de Rijn and R. E. Kessler, *Infect. Immun.*, **27**, 444 (1980).
21) A. Taketo and Y. Taketo, *Z. Naturforsch.*, **40c**, 166 (1985).