Heterogeneity of Hemolytic Efficiency and Isoelectric Point of Streptolysin O

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Using thin-layer agarose gel isoelectric focusing overlaid with thin-layer erythrocyte agar plates, we found that crude streptolysin O (SLO) consisted of a variety of hemolytic components with different isoelectric points (pis) and that the distribution of pis in crude SLO was different even in samples which were produced from a single strain of Streptococcus pyogenes under similar conditions. All of the hemolytic components in crude SLO were shown to have the properties of SLO with respect to their susceptibility to oxygen and anti-SLO serum and their molecular weight. The SLO components showed a single molecular weight of 64,000, but they exhibited various pis ranging from pH 5.4 to 8.3, with major components showing a pi of 6 and/or 7.5. Further examination revealed the slope of the hemolytic titration curve to be dissimilar among the samples of crude SLO. Since the slope of the hemolytic titration curve of a component appears to be based on its hemolytic efficiency, the value of the slope was designated its hemolytic efficiency index. When SLO was purified by isoelectric focusing, the pi of the components was correlated with its hemolytic efficiency index; hemolytic components with lower pis exhibited a lower hemolytic efficiency index. These results indicate that SLO consists of heterogeneous components with different pis and suggest that the differences in hemolytic efficiency indices of SLO components are due to the different electrical charges of SLO molecules, which are related to their polymerization and affect hemolytic efficiency.

Streptolysin O (SLO), a potent membrane-disrupting protein endowed with lethal (2), cardiotoxic (11), cytolytic (7), and hemolytic (1, 12) properties, is produced by Streptococcus pyogenes (group A beta-hemolytic streptococci). Several investigations have described the purification and characterization of SLO, but a general opinion with reference to such basic parameters as molecular weight (1, 3, 4, 12, 15), isoelectric point (pi) (1, 3, 14, 16), and hemolytic capacity of the toxin has not yet been established.

In an attempt to clarify these vague points, we previously determined the pi of SLO from a strain of S. pyogenes by agarose gel isoelectric focusing and found that the culture filtrate contained a variety of SLO molecules with heterogeneous charges. Furthermore, we have determined the pi values of hemolytic components produced by 42 strains of group A type 1, 3, 4, 6, and 12 and group C and G streptococci and found that each strain produced several kinds of SLO with different pis, thus indicating that the distribution of pis of SLO is not related to groups and types of Streptococcus strains (17). Since the distribution of pis of SLO seemed to be strain specific, we assumed that the undefined characteristics of SLO are due to the strains used.

Therefore, we proceeded to purify SLO from one strain of S. pyogenes (group A, type 3). Results show that SLO produced from one strain still exhibited a variety of pi distributions among the samples. Moreover, when each sample was titrated for hemolytic activity, differences in hemolytic efficiency were seen among the samples, suggesting that SLOs with different pi values lyse erythrocytes in slightly different manners. In this paper, we describe the variety of pi distribution among the samples of crude SLO preparations from one strain of S. pyogenes and the differences in lytic efficiencies of SLOs with different pi values.

MATERIALS AND METHODS

**Strain.** S. pyogenes SS265 (group A, type 3), which was obtained from the Centers for Disease Control, Atlanta, Georgia, via the National Institute of Health, Tokyo, Japan, was used in the present study.

**Culture medium.** Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) containing 1% yeast extract (Difco) was filtered through Labo-Module (6,000-molecular-weight cutoff size; Asahi Chemical Industry Co., Ltd., Tokyo, Japan) to eliminate high-molecular-weight substances and was supplemented with 3% glucose before sterilization with a membrane filter (pore size, 0.22 μm).

**Preparation of crude SLO.** Four liters of medium were inoculated with 0.4 ml of bacteria cultured for 7 to 8 h and incubated at 37°C with stirring with the pH held at 7.5 by a pH controller (Tokyo Rikakikai Co., Ltd., Tokyo, Japan). After 16 to 18 h of incubation, culture supernatants were harvested by continuous centrifugation, concentrated to 100 ml by ultrafiltration, treated with 100 U of hyaluronidase (Mochida Pharmaceutical Co., Tokyo, Japan) per ml at 37°C for 5 min, and used as crude SLO. The samples were stored at −40°C and used within 6 months. The samples did not exhibit proteolytic activity against casein when they were assayed by the method of Grushoff et al. (9) with the slight modification of using 2-mercaptoethanol (2-ME) as the reducing agent.

**Measurement of the slope of the hemolytic curve (hemolytic efficiency).** Hemolytic activity of SLO was spectrophotometrically determined by using 50%-end-point titration (13). SLO was diluted on a 0.1-log-unit dilution scale with 36 mM phosphate buffer (pH 6.5) containing 127 mM NaCl (PBS) which was further supplemented with 0.05 M 2-ME and 0.1% bovine serum albumin. One-half milliliter of 2.5% rabbit erythrocyte (RE) suspension in PBS was added to 1.5 ml of the activated SLO solutions, and the tubes were incubated at 37°C for 60 min. The supernatant in the tubes was read.

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against the blank tube containing the diluent in lieu of SLO. Percent hemolysis calculated for each tube was transformed into probit. When probit was plotted against the log of the SLO dose, a rectilinear curve was obtained. The slope was obtained from a rectilinear curve by using the following formula: Slope = \( \frac{\sum x y - \frac{1}{2} \sum x \sum y}{\sum x^2 - \frac{1}{2} (\sum x)^2} \), where \( x \) is the log of the SLO dose, \( y \) is the percent lysis in probit, and \( N \) is the number of points in the rectilinear curve.

Analytical isoelectric focusing (IEF). Agarose gel (Agarose-IEF; Pharmacia-LKB, Uppsala, Sweden) was used as a medium for IEF (17). Ammonium at pH 3 to 10 and pH 5 to 8 (Pharmacia-LKB) was mixed at a ratio of 2:1 and mixed with 1% agarose solution to a final concentration of 2%. The size of the supporting gel was 1 by 110 by 190 mm. Two microliters of each sample was applied in the middle of the gel, and focusing was performed at 10°C for 2 h by using a power supply (Joko Co., Ltd., Tokyo, Japan) with stepwise increases in voltage (300 V [30 min] at the start, and then 400 V [30 min], 600 V [30 min], and finally 800 V [30 min]). After IEF had been performed, the gel plate was overlaid by an agar plate, which contained 2.5% RE and 0.8% agar in PBS and had been soaked for 30 min in fivefold-concentrated PBS (180 mM phosphate buffer, pH 6.5, containing 635 mM NaCl) supplemented with 0.1 M 2-ME. The hemolytic pattern was observed after incubation for 1 h at 37°C. The pH gradient of the focused plate was confirmed by measuring the pH of the gel extract with a microelectrode.

Preparative IEF. Agar plates containing Ampholine (Pharmacia-LKB) were made as described above, with some modification in plate thickness (2 mm). A 0.5-ml sample was applied in the middle of the gel surface by using a glass fiber filter strip. After IEF had been performed at 100 V for 1 h, 300 V for 1 h, and then 600 V for 2 h, 3-mm gel strips were cut off for extraction. The gel strips were extracted thrice with 1 ml each of PBS supplemented with 0.1% bovine serum albumin, and the extracts were used as purified SLO.

Molecular weight determination by gel filtration. Molecular weight of SLO was determined by thin-layer gel filtration on a TLG-Apparatus (Pharmacia-LKB). Calibration proteins in the range of 18,000 to 300,000 molecular weight (Boehringer GmbH Mannheim, Federal Republic of Germany) were used for determining the molecular weight of SLO. After gel filtration, hemolytic components were detected by overlaying agarose gel containing RE as described above.

Inhibition of hemolytic activity. The inhibition test of SLO was performed by overlaying of cellulose acetate membrane (2 by 100 mm) containing various substances for inhibition on a focused-gel plate. Substances used for the inhibition of SLO activity were anti-SLO serum, which was obtained by immunizing rabbits with crude SLO preparation and confirmed not to inhibit streptolysin S (Sigma Chemical Co., St. Louis, Mo.) activity, and β-lipoprotein (cholesterol, 368 mg/dl; triglycerides, 387 mg/dl; phospholipids, 300 mg/dl), which was prepared by ultracentrifugation of normal human serum. After treatment for 10 min, cellulose acetate membrane was removed and hemolytic components were detected as described above.

RESULTS

pI distribution of hemolytic components in crude SLO produced by S. pyogenes group A type 3. When the pIs of hemolytic components in 50 samples (lots) of crude SLO which were separately produced by one strain of S. pyogenes were determined by analytical agarose gel IEF, many hemolytic components with a wide range of pIs were detected. The distribution patterns of the pIs of hemolytic components varied with each sample, and the major hemolytic components were distributed around pHs 6 and/or 7.5 and were divided into three types: acidic type, in which the pI of the major component is around pH 6; neutral type, in which the pI of the major component is around pH 7.5; and wide type, in which the pIs of the major components are around pHs 6 and 7.5. The proportions of the three types of major components in the 50 samples were 54, 42, and 4%, respectively. The pI distribution patterns of several samples remained unchanged in 15 repeated experiments during a 6-month storage at −40°C. Furthermore, hyaluronidase treatment of crude SLO did not affect the pI distribution patterns. An example of pI distribution patterns of neutral and acidic types of samples is shown in Fig. 1. The number of hemolytic components in a given sample was 1 to 12, and the distribution of the number of hemolytic components of the 50 samples showed a pattern of normal distribution with an average value of 7.5 and a standard deviation of 2.3. These results indicate that hemolytic activity in crude culture supernatant was not solely due to a single molecule and that the pI distribution of hemolysins, including the major component, varied with samples even when they were produced by the same strain under the same conditions.

Properties of hemolytic activity in crude SLO. When IEF of crude SLO samples was performed and hemolytic activity in the absence of 2-ME was assayed, hemolytic activity was not detected, whereas the crude SLO samples were shown to contain several hemolytic components when they were assayed in the presence of 2-ME, indicating susceptibility of the activity of crude samples to oxygen (Fig. 1). The average molecular weight of the hemolytic components in the 50 samples of crude SLO was 64,000, as determined by thin-layer gel chromatography (data not shown). Furthermore, the hemolytic activities of all components were inhibited by rabbit anti-SLO serum but not by β-lipoprotein (data not shown). These results indicate that all components with hemolytic activity are SLO and suggest that the components with various pI values have a molecular weight of 64,000.

Relationship between hemolytic efficiency and pI of SLO. When crude SLO was titrated for hemolytic activity by using

![Graph showing pI distribution patterns of SLO in crude samples produced by S. pyogenes SS265 (group A, type 3) and oxygen susceptibility of hemolytic activity. Two samples of crude SLO were applied to agarose gel containing 2% Ampholine and focused for 2 h; hemolytic activity was detected by overlaying agarose plate containing erythrocytes in the presence or absence of 2-ME. Vertical lines indicate hemolytic bands on the overlaid plate. Samples 4 and 5 show pI distribution patterns of neutral and acidic types, respectively.](http://iai.asm.org/Downloaded from August 14, 2017 by guest)
The relationship from the SUZUKI two with components in formed (0) determined crude SLO. (A) In titers.

6% of the (Fig. 1) varies with their curves reaction and the indicate samples 50 the hemolytic efficiency, samples crude SLO was calculated as described in Materials and Methods. Sample 49 showed a larger HE (9.60) and sample 13 showed a smaller HE (4.83) among 50 samples of crude SLO.

50% end-point titration, the slope of the reaction curve for the 50 samples varied with each sample. Titration curves of hemolytic activities of two samples having larger and smaller values of the slope are shown by percent hemolysis and hemolysis in probit as examples (Fig. 2). The hemolytic titers and the slopes of the curves were shown to be reproducible; in 10 repeated experiments on one sample, standard deviations of the hemolytic titers and slopes of the reaction curves determined by the same preparation of RE were less than 5 and 8% of the mean, respectively. In three experiments on one sample with different preparations of RE, standard deviations of both values were found to be less than 6% of the mean. The variations of the hemolytic titers and slopes of the reaction curves were similar in two other samples tested. As the slope is considered to reflect SLO hemolytic efficiency, we designated the value of the slope the hemolytic efficiency index (HE) in this study. The HE of 50 samples of crude SLO showed an average value of 6.83 with a standard deviation of 1.35; the largest was 11.45, and the smallest was 4.30. These results as well as others which indicate the heterogeneity of pI distribution in crude SLO (Fig. 1) suggest that hemolytic efficiency in SLO components varies with their pI.

To clarify the relationship between the HE and pI of SLO, crude SLO was purified by preparative IEF and the HE of each fraction of crude SLO was determined. When a sample of crude SLO (sample 3) which showed a high titer of hemolytic activity was applied to preparative IEF, hemolytic activity was detected in fractions between pHs 5 and 8.5 (Fig. 3). The pI of the major component of the sample observed in fraction 23 was pH 7.4. When the pI of hemolytic components of fractions 20 to 23 of preparative IEF were determined by analytical IEF, each fraction was shown to contain one or two components which had the pI expected from the pI distribution pattern of the original sample (Fig. 4). Thereafter, the HE of the partially purified hemolytic components in each fraction were determined. Purified components from the sample showed different HEs; hemolytic components with lower pIs exhibited lower HEs (Table 1). The relationship between HE and pI of SLO was confirmed by two more experiments with different samples (data not shown). The results suggested that hemolytic actions of SLO components with different pI values are different from each other and can be expressed in the differences of HE.

**DISCUSSION**

The majority of investigators place the molecular weight of SLO in the range of either 60,000 to 70,000 (14, 18) or 53,000 to 58,000 (5) or both (2) and the pI of SLO is placed around pH 6.0 to 6.4 and pH 7.0 to 7.5 (2, 10, 14, 16). The possible existence of two distinct forms of the toxin, which could account for these findings, has been anticipated by Alouf (1), but lines of evidence regarding this point are meager because of difficulties encountered in the isolation of the SLO stemming from the small quantities produced in culture on the one hand and from the proteolytic degradation...
and its hemolytic activity was assayed by 50% endpoint titration. Moreover, we could not obtain such a homogeneous SLO by treatment with 2-ME (data not shown). Furthermore, deamination and decarboxylation of amino acids in SLO molecules by some mechanism is thought to affect the pl and hydrophobicity of the molecules.

During titration of the activity of each sample of crude SLO, we observed differences in HE among the samples. Kusama et al. (13) reported the heterogeneity of the slope of the reaction curve (HE) among the samples of crude SLO which were concentrated by various methods and suggested that different methods of preparation and different time intervals for storage are responsible for the differences of the slopes. However, our results indicate that the heterogeneous HE of SLO is produced under the same conditions of concentration. As the heterogeneity of components in crude SLO was apparent, we assume that the differences in HE are due to the heterogeneity of pls of SLO. As expected, experiments on purified SLO clearly demonstrated a correlation between HE and pl. Furthermore, the relationships between the pl of the major component and the HE of the sample were shown to hold in most crude samples tested, except in crude samples of SLO containing a variety of minor components with a wide range of pls (data not shown).

These results are taken to suggest that the differences in slope are responsible for the differences in HE among molecules with different pls, since the slope showed the relationship between the dose of toxin and the dose of hemoglobin leakage from erythrocytes. In fact, streptolysin S, which is known to destroy erythrocytes by a different mechanism from that of SLO (1, 6, 8), showed an apparently lower HE than that of SLO (unpublished data). It has been reported that SLO binds to cell membrane cholesterol as an acceptor, forms polymers, and generates very large transmembrane channels, after which hemoglobin directly escapes through such channels (4). We postulate that the difference in efficiency may be due to the efficacy of channel formation; SLO with different pls might be polymerized in different degrees to form polymers which intercalate into the membrane, or the polymers of SLO with different pls might possess different affinities to the membrane.

Further studies to elucidate the relationships between the pl value of SLO and its mechanism of action in cell damage and the control of production of homogeneous SLO are in progress in our laboratories.

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LITERATURE CITED

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