Effect of Streptococcal Extracellular Nuclease on the Carrier Activity of RNA for Streptolysin S

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Upon digestion with a streptococcal extracellular nuclease, yeast RNA yielded acid-insoluble core having increased carrier activity for streptolysin S. The carrier activity was found in minor fractions of the core which were eluted from a DEAE-cellulose column at higher salt concentrations. Upon gel filtration through a Sephadex G-75 column, the effective component (Fr. I) was eluted earlier than bulk oligonucleotides (Fr. II). Nucleotide composition (in mol %) of Fr. I was AMP: 21.8; GMP: 55.1; CMP: 8.2; UMP: 14.9, whereas that of Fr. II was AMP: 38.0; GMP: 33.1; CMP: 8.0; UMP: 20.9. Chromatographic patterns of SLS complex induced by Fr. I were similar to those of the toxin formed in the presence of active fraction prepared from RNase I core. Hemolytic activity of the latter complex was, like the former, unaffected by streptococcal nuclease treatment. The carrier activity of DNA digested with the nuclease was also investigated.

Introduction

Streptolysin S (SLS), an oxygen-stable cytolytic exotoxin of hemolytic streptococci, is produced only in media containing certain carrier (or inducer) substance such as RNA or albumin [1, 2]. Moreover, the active toxin is liberated as a complex of SLS polypeptide and the carrier [3]. Effect of RNA as the SLS carrier (RNA effect) is enhanced by treatment with pancreatic RNase, and the carrier activity is found exclusively in guanylic-acid rich oligonucleotide fraction of the RNase-resistant core [4, 5]. Although pancreatic RNase has extensively been used for preparation of the oligonucleotide carrier, this enzyme is unavailable for streptococci in the affected tissue and its significance for SLS production *in vivo* as well as for the bacterial infection is rather marginal.

RNA added into growing streptococcal culture was substantially degraded during incubation, and the carrier activity of the RNA reisolated from the medium was, when tested in a resting cell system, significantly higher than that of the original material. Similar degradation and enhanced carrier activity of RNA were observed upon treatment with a crude nuclease preparation obtained from streptococcal culture supernatant [6]. These results suggest a potential role of streptococcal nuclease in SLS production *in situ*. In order to elucidate mechanism of SLS production and to clarify biological significance of the extracellular enzyme, streptococcal nuclease was purified and its effect on the carrier activity and on physicochemical properties of RNA were examined.

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Materials and Methods

Chemicals

Yeast RNA was obtained from Merck Co., Darmstadt or Kohjin Co., Tokyo. Calf thymus DNA was purchased from Sigma Chemical Co., St. Louis. AF (guanylic-acid rich oligonucleotide fraction with potent carrier activity for SLS) was prepared from RNase I core of yeast RNA [7, 8]. Preparation and purification of oligonucleotide-SLS complex were performed as described previously [7].

Preparation of streptococcal nuclease

Although various strains of *Streptococcus hemolyticus* (including Sv, C203S and C203U) produced the extracellular nuclease, avirulent mutant Sa [9] was exclusively used in the present experiments. The cells were grown in medium D (made of dialyzable fraction of peptone-meat infusion broth) at 37 °C for 16 h and the culture supernatant was removed by centrifugation at 0 °C. The nuclease was precipitated by addition of 600 g of ammonium sulfate per liter

of the supernatant, collected by centrifugation and dissolved in a small volume of 0.02 M potassium acetate buffer (pH 4.5). After removal of residual ammonium sulfate by filtration through a Sephadex G-25 column, the nuclease sample was applied on a DEAE-cellulose column preequilibrated with the acetate buffer. The pass-through fraction which contained most of the nuclease activity was applied on a CM-cellulose column and elution was carried out with a linear salt gradient from 0.02 M (pH 4.5) to 0.2 M (pH 6.5) potassium acetate buffers. The nuclease activity was eluted in a single symmetrical peak. Both RNase activity and DNase activity co-chromatographed and the ratio of the two activities was constant throughout the peak. The peak fractions were pooled and stored at 0 °C (Fraction CM). Specific activities of Fraction CM were 4.1×10^3 units/OD_{280} unit (RNase activity) and 1.1×10^4 units/OD_{280} unit (DNase activity), respectively. Fraction CM was diluted with 0.02 M potassium acetate (pH 4.5) and applied on a CM-Sephadex C-25 column. The column was developed with 0.2 M glycine-KOH buffer (pH 9.0) and the peak fraction was preserved at 0 °C (Fraction CMS). The purified nuclease preparation was free from acid- and alkaline-phosphatases, 5′-nucleotidase, phosphodiesterase and streptolysin 0.

Assay of nuclease

Reaction mixture for assay of the streptococcal nuclease contained, in 0.8 ml, yeast RNA (24 OD_{260} units) or native calf-thymus DNA (20 OD_{260} units), the enzyme, 80 µmol glycine-KOH (pH 9.0), 0.8 µmol $MgSO_4$ and 0.8 µmol $CaCl_2$. The mixture was incubated at 37 °C for 10 min and chilled at 0 °C. After acidification with 1/10 volume of ice-cold 5 N perchloric acid, the mixture was kept at 0 °C for 30 min, centrifuged, and amount of the acid-solubilized fraction was determined by measuring optical density of the supernatant at 260 nm. An increase in optical density at 260 nm of 1.0 under these conditions was defined as one unit of enzyme activity.

Other methods

The carrier activity of poly- or oligo-nucleotides for SLS was determined from their capacity to induce the toxin production in a resting cell system [6, 8]. Titration of SLS and definition of hemolytic unit (HU) were the same as those described previously [7, 8]. Nucleotide analysis was performed after alkaline hydrolysis, as described elsewhere [6].

Results

Properties of streptococcal nuclease

Although enzymatic details of the nuclease will be treated separately, several properties related to the present subject are briefly outlined. The streptococcal extracellular nuclease degraded both RNA and DNA endonucleolytically and the products were mostly oligonucleotides terminated in 5′-phosphate. Extensive digestion of yeast RNA yielded oligoribonucleotides with mean chain length of 4.2 nucleotides and core fraction whose average length was 8.2. Chain length of limit digest prepared from native calf thymus DNA was, on a average, 5.2. The nuclease inactivated infectious MS2 RNA, ΦX174 single-stranded circular DNA and its double-stranded replicative-form DNA. The enzyme required Mg^{2+} (or Mn^{2+}) plus Ca^{2+} and its optimal pH was about 9. The nuclease activity was resistant to 2 mM monoiodoacetic acid but sensitive to 0.5% sodium dodecylsulfate and, unlike RNase I, heat-inactivated at 80–90 °C. Molecular weight of the streptococcal nuclease was somewhat higher than that of RNase I.

Carrier activity for SLS of RNA digested with streptococcal nuclease

Yeast RNA was treated with streptococcal nuclease and change in the carrier activity of cold 0.5 N perchloric-acid insoluble fraction was followed. As shown in Fig. 1, the acid-insoluble fraction exhibited increasing carrier activity for SLS, in parallel with digestion of RNA by the streptococcal enzyme. Thus, SLS-inducing activity of the fraction remaining acid-insoluble after 25 h-treatment was 690 times higher than that of original RNA. On the other hand, its acid- or 70% ethanol-soluble fraction was devoid of the carrier activity (data not shown). When each acid-insoluble sample was passed through a Sephadex G-75 column, the carrier activity was mostly found in fractions eluted earlier, whereas bulk nucleotides gradually appeared in retarded fractions (Fig. 2). Even after exhaustive digestion by streptococcal nuclease, yeast RNA yielded nondialyzable core having considerable carrier activity for SLS. Upon chromatography of the core on a DEAE-cellulose column, the carrier activ-

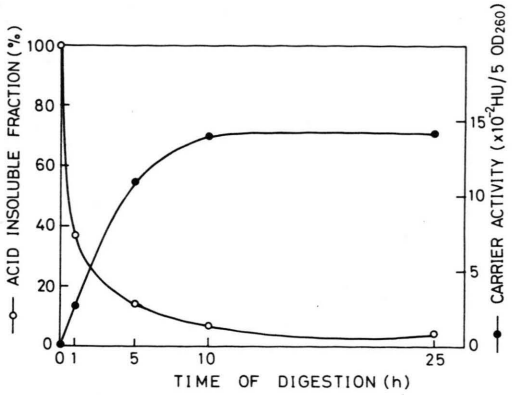

Fig. 1. Development of carrier activity for SLS upon digestion of RNA with streptococcal nuclease. Yeast RNA (1500 OD_{260} units) was incubated at 37 °C, with 680 units of streptococcal nuclease (Fr. CM) in 25 ml of 0.1 M glycine-KOH buffer (pH 9.0) containing 1 mM $MgSO_4$ and 1 mM $CaCl_2$ (buffer G), in the presence of trace amount of chloroform. Acid-insoluble fraction was collected periodically, dissolved in 0.1 M Tris · HCl buffer (pH 9.0), and its carrier activity for SLS was determined.

ity was eluted at KCl concentrations higher than 0.5 M (Table I).

SLS-inducing activity of AF from RNase I core was not significantly altered by additional treatment with streptococcal nuclease. Moreover, hemolytic activity of SLS induced by AF (AF-SLS) was not specifically affected, upon incubation with the streptococcal enzyme (Fig. 3). Similarly, hemolytic activity of streptococcal nuclease core-SLS complex was insensitive to the bacterial enzyme (unpublished observation).

Table I. SLS-inducing activity of DEAE fraction of streptococcal nuclease core. Streptococcal-nuclease resistant core was prepared from yeast-RNA limit digest by extensive dialysis against distilled water followed by ethanol precipitation. The nondialyzable core was fractionated on a DEAE cellulose column by stepwise elution with KCl in 0.05 M Tris-HCl buffer (pH 7.2) and its SLS-inducing activity was compared.

Polynucleotide	OD_{260} unit added	SLS formed [HU/ml]
Original RNA	10	2.1×10^2
Streptococcal nuclease core	4	3.8×10^3
DEAE 0.25 M eluate	1	8.9×10^0
0.50 M eluate	1	6.5×10^2
0.75 M eluate	1	6.3×10^3
1.00 M eluate	1	9.0×10^3
AF	4	1.5×10^4
AF treated with strept. nuclease	4	2.0×10^4

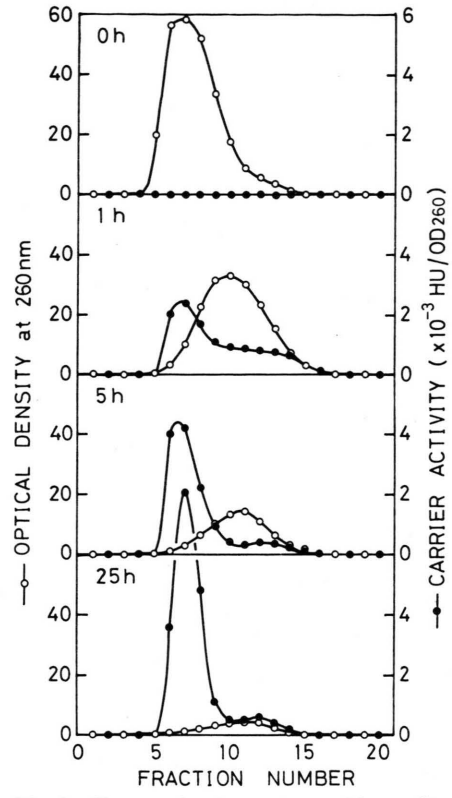

Fig. 2. Changes in chromatographic profile of acid-insoluble fraction during the nuclease treatment. One ml or 0.5 ml (0-time sample only) of the acid-insoluble fraction, collected at the indicated time of the nuclease digestion, was filtrated through a Sephadex G-75 column (1 cm × 21 cm) with 0.05 M potassium phosphate buffer (pH 7.0). Effluent was collected in one-ml fraction and the optical density at 260 nm and carrier activity for SLS were determined.

Nucleotide composition of streptococcal nuclease core

RNA limit digest deproteinized by phenol extraction was dialyzed extensively and remaining core resistant to streptococcal nuclease was precipitated with ethanol. Using a Sephadex G-75 column, the core was further separated into Fr. I with potent

Table II. Nucleotide composition.

Polynucleotide	mol %			
	AMP	GMP	CMP	UMP
Original yeast RNA	26.6	27.7	21.0	25.3
Streptococcal nuclease core	31.9	41.1	6.8	20.2
G-75 Fr. I of the core	21.8	55.1	8.2	14.9
G-75 Fr. II of the core	38.0	33.1	8.0	20.9
AF	20.4	67.3	5.5	6.8
Strept. nuclease-treated AF	20.9	69.4	4.6	3.9

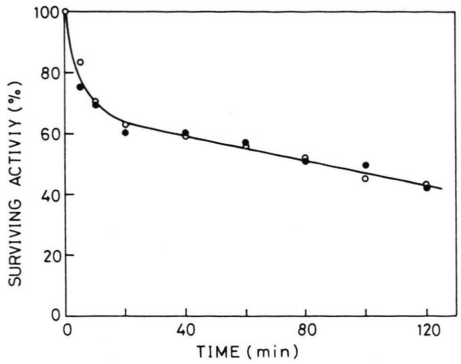

Fig. 3. Effect of streptococcal nuclease on AF-SLS complex. AF-SLS complex (1.6×10^4 HU/ml) was incubated with 106 units/ml of streptococcal nuclease (Fr. CMS) in buffer G, at 37 °C, and change of hemolytic activity was followed. Control without the nuclease (o) was run in parallel.

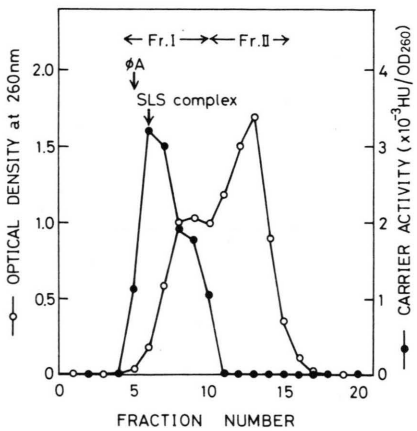

Fig. 4. Fractionation of streptococcal nuclease core through a Sephadex G-75 column. Yeast-RNA limit digest was treated with phenol, dialyzed, and the nuclease-resistant core was collected by ethanol precipitation. One ml of the core sample was passed through a Sephadex G-75 column (1 cm×20.5 cm), using 0.05 M potassium phosphate buffer (pH 7.0). Fraction size was about 1.1 ml. Positions where bacteriophage ΦA and AF-SLS were eluted were marked by arrow.

SLS-inducing activity and Fr. II deficient in the carrier activity (Fig. 4). These fractions were subjected to alkaline hydrolysis and their nucleotide composition was determined (Table II). The streptococcal nuclease core was relatively rich in guanylic acid and adenylic acid residues. Fr. I (minor component) was, like AF, distinctly rich in guanylic acid content, whereas Fr. II (major component) contained moderately higher concentration of adenylic acid and guanylic acid. Treatment of AF with streptococcal nuclease did not particularly alter its nucleotide composition.

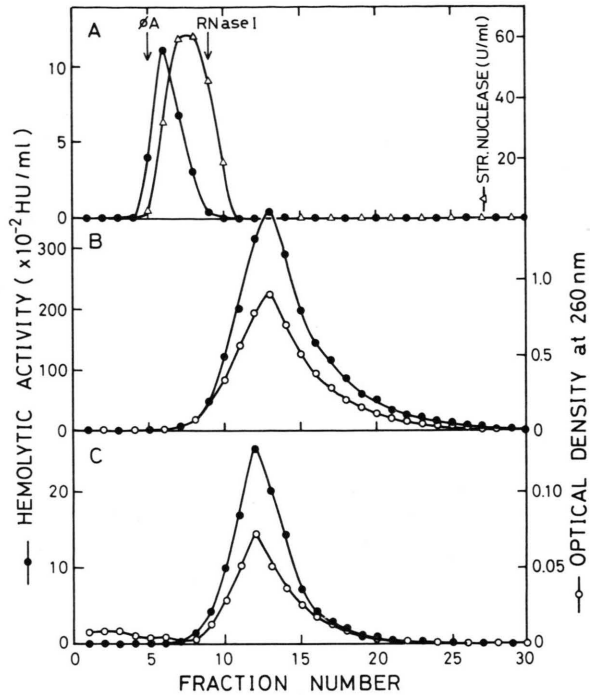

Fig. 5. Chromatographic profiles of streptococcal nuclease core-SLS complex. SLS complex induced by Fr. I of streptococcal nuclease core was partially purified by chromatography on a DEAE-cellulose column [7]. The toxin preparation was further characterized under following conditions. A: gel permeation chromatography through a Sephadex G-75 column (1 cm×21 cm), with 0.05 M potassium phosphate buffer (pH 7.0). Bacteriophage ΦA, streptococcal nuclease (assayed by the DNase activity: $-\triangle-$), and RNase I (assayed in the presence of 0.1 M EDTA) were used as markers. Fraction size was one ml. B: Rechromatography on a DEAE-cellulose column (1 cm × 5 cm). Elution was performed with a linear gradient between 0.3 and 1.0 M KCl in 0.05 M phosphate buffer (pH 7.0). Total volume of the gradient was 90 ml and fraction size was 3 ml. C: Chromatography on a methylated-albumin celite column (1 cm × 5 cm). A linear gradient was formed between 0.3 and 1.0 M KCl in 0.05 M phosphate buffer (pH 6.8). Total volume of the gradient was 90 ml and fraction size was 3 ml.

Properties of SLS induced by streptococcal nuclease core

Chromatographic properties of SLS complex formed in the presence of Fr. I of streptococcal nuclease core were investigated, in comparison with those of AF-SLS complex. As shown in Fig. 5, Fr. I-induced SLS complex (Fr. I-SLS) was eluted from a Sephadex G-75 column immediately after bacteriophage ΦA (a marker nonpermeable into the matrix), followed by streptococcal nuclease and then by RNase I. Upon the gel permeation chromatography,

specific hemolytic activity of Fr. I-SLS complex amounted to more than 4.5×10^4 HU/OD_{260}. Indicating its complex structure, UV-absorbing carrier nucleotide was detected in the Fr. I-induced hemolysin, even after chromatography on DEAE-cellulose column or methylated-albumin celite column. These elution profiles closely resembled those of AF-SLS complex (not illustrated). Like AF-SLS, Fr. I-SLS was sensitive to α-chymotrypsin and pronase but insensitive to trypsin or thermolysin. In addition, trypan blue and methylated albumin inhibited hemolytic activity of both toxins.

Feeble carrier activity of DNA treated with streptococcal nuclease

DNA preparations from *E. coli* or calf thymus showed very low carrier activity for SLS: about 2×10^2 HU of SLS were formed per mg DNA. The carrier activity was, however, retained even after treatment with 0.3 N KOH at 37 °C for 20 h, excluding possible contribution of contaminated RNA. Because streptococcal nuclease possessed potent DNase activity, effect of the enzyme digestion on the carrier activity was tested, using alkali-treated calf thymus DNA. Core fraction of DNA remaining after extensive digestion was not particularly effective in SLS induction. Upon DEAE-cellulose chromatography of the DNA core, marginal carrier activity was detected in fractions eluted at low salt concentration, whereas fractions eluted with 0.5–0.75 M NaCl were totally inactive. When SLS formed in the presence of the DNA core was subjected to DEAE cellulose chromatography, hemolytic activity was found in fractions eluted with 0.5 M KCl. Owing to its low specific activity, properties of this SLS complex were not investigated further.

Discussion

Digestion of yeast RNA with a streptococcal nuclease yields guanylic-acid rich core fraction with enhanced carrier activity for SLS. This result coincides well with previous observations that AF from RNase I core is rich in guanylyl residue [5] and that, among four homoribopolymers tested, only polyguanylic acid is effective as SLS carrier [6]. Chromatographic properties of SLS complexes are very similar whether the carrier oligonucleotide is prepared by streptococcal nuclease treatment or by RNase I digestion. In these respects, the streptococcal nuclease apparently resembles pancreatic RNase, though the former is entirely different from the latter in other properties such as molecular weight, requirement for Mg^{2+} and Ca^{2+}, optimal pH, and associated DNase activity. The streptococcal nuclease is constitutively produced in medium D or in peptone-meat infusion broth, without supplementation of RNA or AF. (AF preparations usually contain significant amount of RNase I used for digestion of RNA. In present experiments, precautions have been taken to avoid contamination with this robust RNase.)

Although mean chain length of the streptococcal nuclease core is about 8, Fr. I of the core is eluted from Sephadex G-75 column at nearly void volume. In addition, the oligonucleotide(s) is eluted from DEAE cellulose column at higher salt concentrations. These results suggest that the oligonucleotides are in an aggregated conformation which may be essential for the carrier activity. Unlike RNA, DNA was inactive even after digestion with the streptococcal enzyme, indicating crucial role of ribose portion in the carrier oligonucleotide.

Present data demonstrate that an extracellular streptococcal nuclease is involved in SLS production, through "processing" of carrier RNA. This conclusion is further strengthened by feeble carrier activity of RNA in streptococcal mutants deficient in the extracellular nuclease (manuscript in preparation). It is thus quite probable that the nuclease, together with host RNases liberated from injured tissue, enhances the RNA effect for SLS production *in vivo*.

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