Streptolysin S Formation by Ribosomal RNA of *Escherichia coli**

YORIKO TAKETO and AKIRA FUJIMURA

Department of Pharmacology, School of Medicine, Kanazawa University.
(*Director: Prof. Hajime OKAMOTO*)

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In 1939, Okamoto [1] discovered that yeast ribonucleic acid enhanced the production of streptolysin S in culture of hemolytic streptococci.

Since that time, the specificity of ribonucleic acid for the formation of streptolysin S has been studied with the finding that ribonucleic acids from a variety of sources, *i. e.* liver, kidney, spleen, muscle, Ehrlich carcinoma cells, wheat germ, tobacco leaves, hemolytic streptococci themselves, peptone, were all effective in causing the production of large amounts of streptolysin S.[2] Ribonucleic acid of tobacco mosaic virus was tested, however to be of a feeble activity.[3]

For extending this line of work, ribosomal ribonucleic acid of *Escherichia coli* was subjected to the present study.

MATERIALS AND METHODS

Strain of Streptococcus pyogenes: A strain of *Streptococcus hemolyticus* "S" was used. The culture and cocci suspension were prepared as described in previous paper[4].

Formation of streptolysin S: The appropriate amount of RNA or oligonucleotide was added to 2 ml of the cocci suspension in Bernheimer's basal medium.** After 2-hour incubation at 37°C, the mixture was chilled and centrifuged. The clear supernatant thus obtained was subjected to the estimation of hemolytic activity and optical density at 260 mμ.

Estimation of ribonucleotides: The amount of ribonucleotides was estimated from the optical dencity at 260 mμ.

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** Bernheimer's basal medium [5] was composed of 675 mg maltose, 6 ml 20% KH_2PO_4 (adjusted to pH 7.0 with NaOH), 12 ml 2% $MgSO_4 \cdot 7H_2O$ and 66 ml distilled water.

Estimation of hemolytic activity: The same method as described in previous paper [6] was employed with slight modification, *i.e.* erythrocytes suspension was incubated with the lysin for 2 hours at 37°C.

Isolation of r-RNA from E. coli: RNA was extracted from ribosomes of *E. coli* Kl2Y by phenol method[7], and subsequently purified by repeated precipitation with 2 M NaCl[8]. The 2 M NaCl-precipitable RNA was lyophilized after dialysis and used as the starting material. The ultraviolet absorption spectrum of the sample was shown in Fig. 1.

RESULTS

Streptolysin S forming activity of native r-RNA of E. coli: In the first place, streptolysin S forming activity of *E. coli* r-RNA was compared to that of yeast RNA. As may be seen from Table 1, *E. coli* r-RNA showed only feeble activity in native state.

Table. 1. Streptolysin S forming activity of *E. coli* r-RNA and yeast RNA

Sample	Concentration (mg/ml)	Streptolysin S formed (H.U./ml)
E. coli r-RNA	20	5
E. coli r-RNA	10	5
E. coli r-RNA	5	3
Yeast RNA "Merck" B	10	1075
Yeast RNA "Merck" No. 3	10	512

As mentioned above, it was clearly demonstrated by Bernheimer that treatment of ribonucleic acid with pancreatic ribonuclease markedly increased the streptolysin S forming activity.

Further, it has been repeatedly suggested that ribonucleotides rich in guanylic acid content are responsible for streptolysin S forming activity.[2),9)] On the other hand, guanine content of *E. coli* r-RNA was known to be approximately 32% [10] among four bases. If so, the low streptolysin S forming activity of *E. coli* r-RNA could not be simply explained by its base composition. It was, therefore, of interest to perform RNase I digestion experiment with *E. coli* r-RNA.

Treatment of E. coli r-RNA with RNase I: *E. coli* r-RNA was digested by RNase I for 48 hours at 37°C. The reaction mixture was composed of *E. coli* r-RNA 2.4g, RNase I (Worthington Biochem. corp.) 2.40mg and trace amount of chloroform in 24 ml of distilled water with final pH 7.2. During the incubation, pH was frequently cheked and adjusted to 7.2 if necessary. After incubation, the

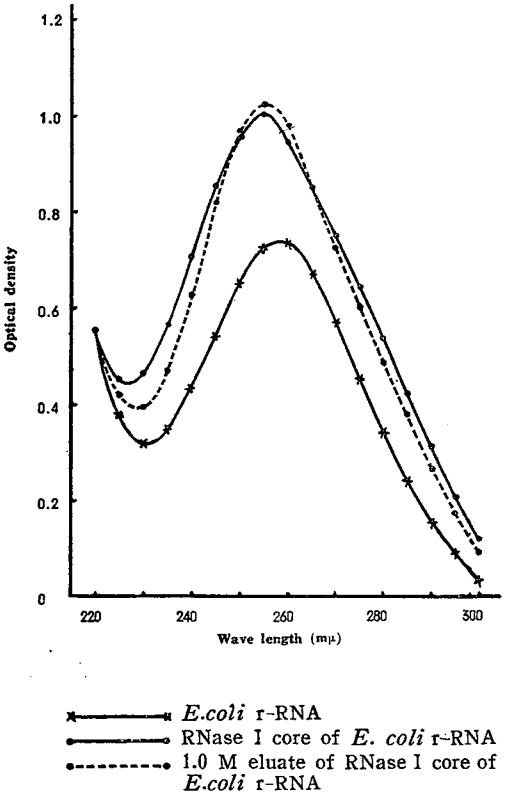
mixtre was shaken with 80% phenol and aqueous phase was removed with centrifugation. The phenol layer was reextracted with distilled water. From the joined extract, RNase I core was collected with ethanol precipitation and dried *in vacuo* after washing with ether. The yield was approximately 5.4%.

The ultraviolet absorption spectrum and streptolysin S forming activity of RNase I core of *E. coli* r-RNA were shown in Fig. 1 and Table 2.

Table. 2. Streptolysin S forming activity of RNase I core of *E. coli*. r-RNA in relation to its concentrations

Sample	Concentration (mg or μg/ml)	Streptolysin S formed (H.U./ml)
E. coli r-RNA core	10 mg	1075
	100 μg	1679
	50 μg	1496
	10 μg	614
E. coli r-RNA	10 mg	6

Fig. 1. Ultraviolet absorption spectra (in 0.05 M phosphate buffer pH 7.2)



It is clear that streptolysin S forming activity was markedly enhanced by RNase I digestion. Even at low concentrations, the core showed high lysin forming activity. However, indication was that this sample may contain considerable amount of hemolysis-inhibiting substance* (cf. Fig. 2.). Therefore, RNase I core of *E. coli* r-RNA was further fractionated on a column of DEAE-cellulose (Serva).

Streptolysin S forming activity of various DEAE-cellulose fractions of RNase I core of E. coli r-RNA: 20 mg of the core in 50 ml of 0.05 M phosphate buffer pH 6.8 were applied on a column of DEAE-cellulose (1.5 × 1.5 cm) which had been equilibrated with the same phoshate buffer. Stepwise elution was carried out with 0.25 M, 0.50 M, 0.75M and

* This was similar to the observation by Hayashi et al. [11] that a hemolysis-inhibiting substance was contained in RNase treated yeast RNA preparations.

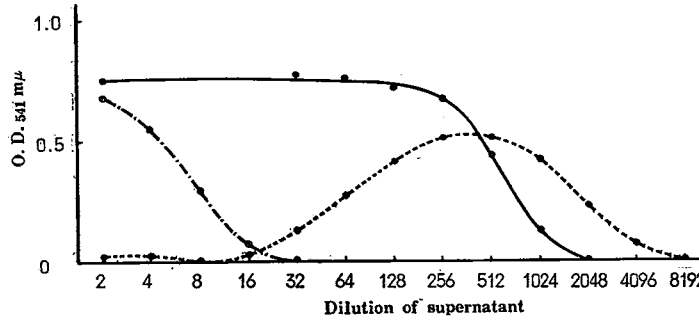
1.0 M NaCl-0.05 M phosphate buffer pH 6.8. The eluate in each step was exhaustively dialysed against deionized water. The non-dialyzable fraction was finally concentrated with a dialysis against Carbowax 4000.

The streptolysin S forming activity of 4 fractions thus obtained was presented in Table 3.

Table 3. Streptolysin S forming activity of DEAE-cellulose fractions of *E. coli* r-RNA core

Fraction	H.U./10 μg sample/ml	H. U. /OD 260mμ of supernatant
0.25 M	1	1
0.50 M	759	867
0.75 M	1009	1184
1.0 M	1155	1388
Original core	535	763

Fig. 2. Hemolysis profile with streptolysin S formed in the presence of *E. coli* r-RNA and its RNase I core



Streptococci were suspended in Bernheimer's basal medium containing a) 10 mg/ml r-RNA (—•—•—), b) 10 mg/ml core (– – •– – •) or C) 10 μg/ml core (—•—•—). After 2-hour incubation at 37°C, the mixture was centrifuged. The clear supernatant was tested for hemolytic activity as described under method, and the hemolysis degree, on a O.D. 541 mμ base, in each dilution tube was protted.

The highest activity was found in 1.0 M eluate, and the eluate was tested to be free from hemolysis-inhibitory substance (cf. Fig. 2).

Using 10 mg/ml of this 1.0 M fraction, supernatants with 1300～1400 H.U./$OD_{260\ m\mu}$, was obtained usually. The ultraviolet absorption spectrum of this fraction was shown in Fig. 1. Nearly the same activity was found in 0.75 M eluate,

However, no activity was found in 0.25 M eluate, the absorption spetrum of which was quite different from that of nucleic acid.* The results presented here strongly suggests the presence of oligonucleotides with high guanylic acid content in *E. coli* r-RNA.

Additional data concerned with the properties of streptolysin S formed in the presence of *E. coli* r-RNA RNase core were that

1) inactivation of the streptolysin S was caused by α-chymotrypsin[5), 12)] and pronase P, (Table 4), and that

Table 4. Effect of α-chymotrypsin and pronase P on the streptolysin S sample

Enzymes (100μg/ml)	Hemolytic activity (H. U.) after 1 hour at 37°C	
	A	B
α-Chymotrypsin (Worthington)	0	17
Pronase P (Kaken)	0	15
Control	407	14172

A : Streptolysin S formed with RNase I core of *E. coli* r-RNA
B : Streptolysin S formed with yeast RNA

2) the lytic action of the streptolysin S upon erythrocytes was antagonized by trypanblue,[13)]

as was in the case of streptolysin S formed in the presence of yeast RNA.

SUMMARY

RNA extracted from ribosomes of *E. coli* by phenol method was almost inactive in streptolysin S formation. After digestion with RNase I, *E. coli* r-RNA showed considerably higher activity of streptolysin S formation. The RNase I core sample, however, was shown to be contaminated with hemolysis-inhibiting substance. From the core, active fractions were obtained by DEAE-cellulose chromatography. The ribonucleotide fractions giving higher lysin formig activity were eluted at 0.75 ~1.0 M NaCl concentration. Using the small amount of the most active fraction, supernatant fluid with 1300~1400 hemolytic units per optical density at 260 mμ was usually obtained.

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* A similar result had been obtained with ribonucleotides isolated from non-dialyzable fraction of commercial peptone [4)].

the course of this work.

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