PURIFICATION AND SOME PROPERTIES OF AN INDUCIBLE BACTERIOCIN, CLOSTOCIN O, OF *CLOSTRIDIUM SACCHAROPERBUTYLACETONICUM*¹

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A phage tail-like bacteriocin, clostocin O, from *Clostridium saccharoperbutylacetonicum* (ATCC 13564) was purified by two-phase fractionation and sucrose density gradient centrifugation. Clostocin O had two kinds of particle form; one is the intact form (sheathed form) and the other is an inactive form (contracted form). A part of the intact form converted into the contracted form during the purification. The $s_{20, w}$ of clostocin O was 95S for the intact form and 74S for the contracted form. The purified intact clostocin O was homogeneous by several criteria such as sedimentation pattern of sucrose density gradient, sedimentation velocity, and isoelectric focusing. The isoelectric point was pH 4.6. The intact clostocin O mainly consisted of protein, but contained some (2–3%) RNA. Its amino acid composition was also determined with an amino acid analyzer.

Bacteriocins are conveniently divided into two groups, low-molecular weight form and high-molecular weight form. The former, including colicins, is found in various bacterial species (1–5), and its properties have been well studied. Little is known, however, about the latter except pyocins of *Pseudomonas aeruginosa* (6–9).

In the previous paper (10), we reported the induction, isolation, and morphological properties of clostocin O is produced by *Clostridium saccharoperbutylacetonicum* (ATCC 13564) (11). It is an inducible high-molecular weight bacteriocin and phage tail-like particle in shape. Its biological properties were also reported in some other papers (12–15). The present paper describes the purification of

¹ Bacteriocins of Nonpathogenic *Clostridium* species. Part IX.

clostocin O and some of its physical and chemical properties.

MATERIALS AND METHODS

Organisms. *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564) was the strain to produce clostocin O, and No. 8 was the strain sensitive to clostocin O (10).

Media and cultural conditions. The organisms were grown at 30° under a reduced atmospheric pressure (5 to 10 mmHg) in TYA medium (16).

Turbidity measurements. Optical density (OD) of the cultures was measured at 660 nm with a photoelectric colorimeter (Model 7A, Tokyo Koden, Ltd.).

Preparation of clostocin O lysate. Strain N1-4 was anaerobically grown at 30° in TYA broth to 0.3 of OD₆₆₀. To this culture, mitomycin C (Kyowa Hakko Kogyo, Ltd.) was added at 2 µg/ml of final concentration and the culture was incubated for 20 min at 30°. Excess mitomycin C was removed by centrifugation and the harvested organisms were resuspended in the same volume of fresh TYA broth for reincubation. The reincubation was continued until the culture completely lysed (10). The lysed culture was centrifuged twice at $9,000 \times g$ for 10 min, and the supernatant was clostocin O lysate.

Assay of clostocin O. The activity of clostocin O was assayed by the double-layer method (10). Desirable dilutions of clostocin O suspension were prepared, and examined by the spot test on sensitive organisms. An arbitrary unit of activity (units/ml) was defined as the reciprocal of the highest dilution clearly showing an inhibitory zone.

Two-phase fractionation. To 500 ml of clostocin O lysate, 31.6 g of sufficiently crushed poly (ethylene glycol) (PEG) 6000, 0.97 g of dextran sulfate (DS) 500, and 8.7 g of NaCl were added with stirring, as reported by ALBERTSON (17). The mixture was stood overnight at 4° in a separatory funnel. Clostocin O was taken in the DS phase (bottom layer) and the DS phase was centrifuged at $450 \times g$ for 15 min. After this low-speed centrifugation, clostocin O was contained in the interface 'cake' (middle layer). To 2 ml of the middle layer, 5 ml of 1% of DS and 1 ml of 3 M KCl were added to remove the cell debris and DS. The mixture was stood for 2 hr, centrifuged at $4,000 \times g$ for 10 min, and the supernatant was dialysed overnight against M/15 phosphate buffer (pH 5.8) containing 10^{-3} M Mg^{2+} and 5×10^{-2} M NaCl.

Sucrose density gradient centrifugation. Three milliliters of sample prepared by the two-phase fractionation was applied on a sucrose gradient (10 to 40%) and centrifuged at 25,000 rpm for 1 to 4 hr at 4° using an SW 25-1 rotor in a Beckman L3 centrifuge (18). The sample solution was divided into 40 fractions by a fractionator (Beckman, Ltd.), and each fraction was measured for its absorbancy at 280 nm and clostocin O activity.

Determination of diffusion coefficient. Sedimentation velocity pattern of clostocin O was determined by ultracentrifugal analysis. Clostocin O was suspended

in 0.01 M tris-HCl buffer (pH 7.2) containing 10^{-3} M Mg^{2+} and 0.1 M NaCl, and centrifuged at 17,980 rpm using Sinco Model E ultracentrifuge at 21.5°.

Chemical components of clostocin O. Lyophilized clostocin O was fractionated by a modified Schmidt-Thannhauser-Schneider (STS) procedure (19), and the amount of protein, DNA, and RNA was determined by Cu-Folin method (20), diphenylamine method (21), and orcinol reaction (22), respectively.

Amino acid analysis. Amino acid was analyzed by a method similar to that described by SPACKMAN *et al.* (23). Lyophilized clostocin O was hydrolyzed with 6 N HCl for 24, 48, or 72 hr at 110°. HCl was removed by evaporation and the dried residue was redissolved in a citrate buffer (pH 2.2). An aliquot was applied to an automatic amino acid analyzer (Model JLC-5, Japan Electron Optics, Ltd.). Tryptophan was estimated from the relationship described by GOODWIN and MORTON (24). The content was determined from the extinction values at 280.0 and 294.4 nm for a solution of clostocin O in 0.1 N NaOH.

Isoelectric focusing. Isoelectric focusing was performed with a 110-ml LKB electrofocusing column containing carrier ampholine with a pH range of 3 to 10. Electrophoresis was carried out by the potential gradient of 400 to 900 V for 48 hr, maintaining column temperature at 4° by circulating chilled water. After electrophoresis, the ampholine was fractionated to 2 ml aliquots and each fraction was measured for its pH, absorbancy at 280 nm and clostocin O activity.

RESULTS

Purification of clostocin O

As shown in Table 1, the salt fractionation and ion-exchange column chromatography with DEAE-Sephadex gave a very low recovery of clostocin O activity. When Sephadex G-100 and CM-cellulose columns were used, 50 to 80% of clostocin O activity was detected at void volume. These procedures may be useful for separating clostocin O from small molecules, but not for a purification step.

Table 1. Specific activity and yield at various steps in the purification of clostocin O.

Fraction	Volume (ml)	Total protein (mg)	Total activity (unit)	Specific activity (units/mg)	Activity recovery (%)
Crude extract (Lysate)	500	750	64,000	85	100
Bottom 'cake'	2	250	51,200	205	80
Supernatant	7	119	44,500	374	70
Sephadex G-100	50	15	19,420	1,302	30
DEAE-Sephadex	50	8	7,450	933	11
Sucrose dens.-grad.	7	7	9,000	1,286	14
Ammonium sulfate	50	400	19,200	48	30

Fig. 1. Purification of clostocin O on sucrose density gradient centrifugation.

The samples of clostocin O partially purified by two phase fractionation were applied on 10 to 40% of sucrose density and the centrifugation was performed at 25,000 rpm for 60 to 240 min. ●, absorbancy at 280 nm; ○, clostocin O activity.

Clostocin O was purified by two-phase fractionation, and then by sucrose density gradient centrifugation. The recovery of clostocin O was 70% by the two-phase fractionation (Table 1). After the two-phase fractionation, clostocin O was concentrated by ultracentrifugation at $105,000 \times g$ for 60 min, and it was applied to sucrose gradients of 10 to 40% and centrifuged for 60, 180, 210, or 240 min at 4° . Figure 1 shows the fractionation pattern. The clostocin O activity was recovered in a sharp peak in parallel with the absorbance peak of 280 nm at 210 and 240 min. There is a small peak of absorbance at 280 nm at the 25th fraction in 240-min

Fig. 2. Sedimentation of clostocin O through a gradient column comparison with Southern bean mosaic virus and Brome mosaic virus.

The sucrose gradient was 10 to 40% and the centrifugation was performed at 25,000 rpm with SW 25-1 rotor. ○, clostocin O; △, Southern bean mosaic virus (SBMV) 115S; □, Brome mosaic virus (BMV) 86S; —, density.

centrifugation. This peak was confirmed to contain the contracted form (10) of clostocin O by an electron microscope. It will be described in detail later. The sedimentation pattern of clostocin O was compared with those of Brome mosaic virus (BMV) having a sedimentation coefficient of 86S and Southern bean mosaic virus (SBMV) having a sedimentation coefficient of 115S. Figure 2 shows that the sedimentation coefficient of clostocin O is 90S.

Stability and morphological change of clostocin O

The time-course of clostocin O activity was followed by the double-layer method and the shape of clostocin O was observed by an electron microscope. Samples of clostocin O were stored at 2° and in a frozen condition with the addition of 10^{-2} M of mercaptoethanol, 10^{-3} M of glutathione, or 0.5 M of glycerol as a protein-protecting agent. The activities of clostocin O in a frozen condition decreased to one-tenth during 5 days and, even under the best condition, at 2° with 10^{-2} M mercaptoethanol, the activity was reduced to one-tenth during 18 days. This fact shows that clostocin O is a considerably labile bacteriocin. The electron

Fig. 3. Schematic structures of intact and contracted forms of clostocin O.

Fig. 4. Sedimentation velocity pattern of clostocin O.

A solution of clostocin O (2 mg/ml) was prepared in 0.01 M Tris-HCl buffer (pH 7.2), containing 10^{-3} M Mg^{2+} and 0.1 M NaCl, and centrifuged at 17,980 rpm.

microscopic observation revealed two shapes of clostocin O; an intact form with extended sheath of $1,250 \times 220 \text{ \AA}$ and a contracted form with contracted sheath of $800 \times 270 \text{ \AA}$, as shown in Fig. 3. Photographs of both forms have been demonstrated elsewhere (10). In the sample of reduced clostocin O activity with passage of time, contracted form of clostocin O was mainly observed under the electron microscope. The contracted form had no activity to sensitive organisms.

Sedimentation velocity ultracentrifugation

Figure 4 shows the sedimentation velocity pattern of clostocin O. Two peaks are observed. From this result, the sedimentation coefficient ($s_{20, w}$) was calculated to be 95S for the main peak and 74S for the sub-peak. Two peaks of absorbance at 280 nm were also observed in the sucrose density-gradient centrifugation (Fig. 1) and in the DEAE-Sephadex column chromatography (Fig. 5). When each of peak fractions was observed under the electron microscope, each main peak contained the intact clostocin O and another peak contained the contracted form of clostocin O.

Fig. 5. Chromatographic profile of clostocin O on DEAE-Sephadex A-50.

Clostocin O partially purified by two-phase fractionation was applied on a column ($2.5 \times 20 \text{ cm}$), and fraction volume was 5 ml. ●, absorbance at 280 nm; ○, clostocin O activity; —, concentration of NaCl.

Ultraviolet absorption curve of clostocin O

Figure 6 shows the ultraviolet absorption curve of clostocin O. The absorption maximum of clostocin O was at about 280 nm and the curve was absorption spectrum of protein, but the absorbance at 250 nm was higher than that of other bacteriocin which consists of protein only. Therefore, clostocin O may contain a few nucleic acids.

Fig. 6. Ultraviolet absorption curve of clostocin O.

Table 2. Chemical components of clostocin O.

Clostocin O	Protein	RNA	DNA
1	97.4%	2.6%	0%
2	98.6	1.4	0

Data in second column is the value after treatment with 100 $\mu\text{g/ml}$ of RNase for 30 min.

Chemical components of clostocin O

Two milligrams of lyophilized clostocin O was fractionated by the modified STS procedure and the amount of protein, DNA, and RNA was determined by the Cu-Folin method, diphenylamine method, and orcinol reaction, respectively. As shown in Table 2, clostocin O consisted of 97.4% of protein and 2.6% of RNA, but DNA was not detected at all. After the treatment with 100 $\mu\text{g/ml}$ of RNase in $\text{m}/15$ phosphate buffer (pH 5.0) at 30° for 30 min, clostocin O still contained 1.4% of RNA. It seems that this RNA is involved somewhere in clostocin O particle, such as rapidosome (25) and cannot be removed completely by RNase treatment. RNase-treated clostocin O had the same killing activity as native clostocin O. Therefore, RNA in clostocin O may be independent of killing action of clostocin O.

Isoelectric focusing of clostocin O

The isoelectric fractionation profile of clostocin O in a pH 3 to 10 linear gradient is shown in Fig. 7. The peak of clostocin O activity was at pH 4.6. It was also indicated that clostocin O was an acidic protein having an isoelectric point at pH 4.6.

Amino acid analysis of clostocin O

Table 3 shows average values of amino acids for the samples of clostocin O after several times of hydrolysis, compared with the amino acid composition of

Fig. 7. Isoelectric focusing of clostocin O with carrier ampholytes.

The experimental procedure is described in MATERIALS AND METHODS.

●, absorbancy at 280 nm; ○, clostocin O activity; —, pH.

Table 3. Amino acid compositions of clostocin O and pyocin R.

	Clostocin O ^a	Pyocin R ^b		Clostocin O ^a	Ryocin R ^b
Lys	8.25%	4.75%	Ala	6.12%	9.30%
His	0.69	0.65	Cys	0.41	0.75
Arg	2.14	7.35	Val	6.33	8.15
Asp	11.26	11.95	Met	1.46	2.05
Thr	6.89	4.70	Leu	6.75	8.75
Ser	5.59	4.60	Ile	6.63	4.15
Glu	8.25	10.25	Tyr	3.29	3.00
Pro	1.35	4.10	Phe	2.71	5.05
Gly	6.61	4.95	Try	3.20 ^c	5.60

^a Average values for 24-, 48-, and 72-hr hydrolysates of purified clostocin O.

^b Data taken from Kageyama (7).

^c Determined by spectrophotometric analysis.

pyocin (7). Clostocin O has a higher content of lysine and isoleucine, and lower content of alanine than those of pyocin R. The content of acidic amino acids in clostocin O is greater than that of basic amino acid, so that clostocin O shows the feature of acidic protein.

DISCUSSION

Clostocin O was purified by a two-phase fractionation and sucrose density-gradient centrifugation. It is more suitable for the purification of clostocin O to apply the method of virus purification than that of general protein purification.

The salt fractionation and ion-exchange column chromatography gave low recovery of clostocin O activity. Low recovery of the activity caused the increase of contracted form of clostocin O. It seems that clostocin O loses the activity when it takes the contracted form. The contraction seems to occur easily even during the process of purification of clostocin O. Therefore, the recovery of clostocin O activity is considerably low, compared to that of pyocin (6). This difference may depend on the facile occurrence of the contracted form.

Another remarkable feature of clostocin O is the presence of about 2% of RNA in its particle. Though this RNA is not essential component of clostocin O, there is no report about a large molecular bacteriocin containing RNA in its particle. However, rapidosome was reported to be non-bactericidal particle containing RNA which was produced by *Escherichia coli* (25). The RNA of clostocin O was not a contaminant, but it could not be removed completely even by efficient RNase treatment. The killing activity of clostocin O was not affected by RNase treatment. It seemed that RNA in clostocin O is not concerned with the killing activity. From these results, it is considered that RNA is involved in clostocin O particle at the production of clostocin O in its producing organism. The origin of RNA is still unknown, but it will be of interest to know why RNA is involved in clostocin O particles. This RNA may be needed for assembling of clostocin O subunits.

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