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Abstract

Further purification and characterization are reported on rat cytosol cornin (RLCC), an antimitotic substance. Fraction I (purified RLCC) was purified more than 10-fold from crude RLCC with Sephadex G-50 column chromatography and showed a remarkable inhibitory effect on division of inseminated sea urchin eggs and mouse fibroblast cells. Fraction I was observed as one spot, and the molecular weight was estimated to be about 25,000 by thin layer gel filtration. Fraction I contained protein (92%) and RNA (8%), but the antimitotic activity was scarcely affected by treatment by pancreatic RNase. The protein of Fraction I was separated into two bands by SDS-polyacrylamide gel electrophoresis, and the molecular weight was estimated as 10,000 and 15,000, respectively. The 50% inhibition dose of Fraction I on the first division of inseminated sea urchin eggs and on proliferation of mouse L cells was about 2.5×10^{-5} g/ml and 5×10^{-4} g/ml, respectively. The yield of fraction I was about 35 mg from 100 g rat liver.

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PURIFICATION AND SOME CHARACTERISTICS OF LIVER CYTOSOL CORNIN, AN ANTIMITOTIC SUBSTANCE FROM RAT LIVER CYTOSOL

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Abstract. Further purification and characterization are reported on rat cytosol cornin (RLCC), an antimitotic substance. Fraction I (purified RLCC) was purified more than 10-fold from crude RLCC with Sephadex G-50 column chromatography and showed a remarkable inhibitory effect on division of inseminated sea urchin eggs and mouse fibroblast cells. Fraction I was observed as one spot, and the molecular weight was estimated to be about 25,000 by thin layer gel filtration. Fraction I contained protein (92%) and RNA (8%), but the antimitotic activity was scarcely affected by treatment by pancreatic RNase. The protein of Fraction I was separated into two bands by SDS-polyacrylamide gel electrophoresis, and the molecular weight was estimated as 10,000 and 15,000, respectively. The 50% inhibition dose of Fraction I on the first division of inseminated sea urchin eggs and on proliferation of mouse L cells was about 2.5×10^{-5} g/ml and 5×10^{-4} g/ml, respectively. The yield of Fraction I was about 35 mg from 100 g rat liver.

Nisida and Murakami (1) reported that a pupillo-contracting substance, cornin (2), extracted from bovine cornea or rabbit muscle showed retarding effects on mitosis of inseminated sea urchin eggs. It has been reported that cornin fraction extracted from bovine liver suppressed the growth of cultured mammalian cells (3) and that injection of cornin fraction extracted from canine intestine prolonged the survival time of mice inoculated with Ehrlich ascites tumor cells (4).

This paper reports on further purification and characterization of the cornin fraction extracted from liver cytosol.

MATERIALS AND METHODS

Extraction of cornin fraction. Livers from adult male rats (Wistar strain) were homogenized with 4 volumes of cold 0.25M sucrose. The homogenate was filtered through 4 layers of gauze, and the filtrate was then centrifuged at $10,000 \times g$ for 10 min. The resulting supernatant was centrifuged at $100,000 \times g$ for 60 min to eliminate the microsomal fraction. The cornin fraction was extracted from this liver cytosol fraction by a modification of the method of Nisida and Mura-

kami (1). The liver cytosol fraction was diluted with 4 volumes of distilled water and boiled for 3 min and cooled immediately in a water bath and then centrifuged at $10,000\times g$ for 10 min. Cold ethanol was added to the resulting supernatant to a final concentration of 70%. The mixture was placed for 2 hr in an ice bath and then centrifuged at $5,000\times g$ for 10 min. Cold ethanol was added to the resulting supernatant to a final concentration of 90%, and the mixture was placed in a cold room (0° to 4°C) overnight and then centrifuged at $5,000\times g$ for 10 min. The resulting precipitate was mixed with 10 volumes of cold acetone, centrifuged and washed with cold ether and then dried in air. This acetone powder was designated as the crude liver cytosol cornin (RLCC) fraction.

Purification of RLCC. Crude RLCC (100 mg) was dissolved in 2 ml of distilled water and charged on top of a Sephadex G-50 column (3×50 cm) and then eluted with 300 ml of distilled water. The eluate at 3 ml fractions was collected, and the ultraviolet (280 nm)-absorbing fractions were lyophilized.

Cell assay using inseminated sea urchin eggs. Matured eggs of sea urchin (*Temnopleurus toreumaticus*) were collected by an intracoelomic injection of 0.5 M KCl. The shed eggs were then immediately washed with sea water and the fertilization rate was examined. Intact eggs showing high rates (above 95%) were used in this experiment. Test materials were dissolved in sea water and mixed with the egg cell suspension. After treatment for 10 min at 25°C , the egg cells were inseminated. The cleaved cells were counted at 5 min intervals.

Cell assay using mammalian cultured cells. L cells (3×10^4 , mouse fibroblast strain) in stationary state were subcultured at 37°C in tube containing 1 ml of Eagle's minimum essential medium with 10% bovine serum. For assay of cell growth inhibition, the test material dissolved in 1 ml of culture medium was added to the culture 48 hr after subculture. Cells were detached from the tube wall by treatment with 0.05% trypsin-0.025% EDTA in phosphate-buffered saline, pH 7.2, 24 hr and 48 hr after the addition of test materials and counted by a Coulter counter.

Amino acid analysis. Fraction I (2 mg) purified from crude RLCC was hydrolyzed by 4 ml of 6 N HCl at 105°C for 12 hr. The hydrolysate was evaporated to dryness under reduced pressure, and dissolved in 2 ml of 0.01 N HCl and analyzed by an amino acid analyzer (Model JLC-6AH, JEOL Ltd., Japan).

Protein determination. Protein was estimated by the method of Lowry *et al.* (5).

RNA determination. RNA was estimated by the method of Brown (6) after RLCC was treated with cold 5% perchloric acid.

SDS-polyacrylamide gel electrophoresis. To a sample (500 μg /0.3 ml of 10 mM Tris-HCl buffer, pH 8.0), 0.03 ml of 10% SDS (sodium dodecyl sulfate) and 3 drops of glycerol were added, and the mixture was heated in a boiling water bath for one min. The mixture (0.05 ml) was then charged on the top of a 7.5% SDS-polyacrylamide gel column (0.5×8.5 cm). Electrophoresis was carried out at 5 mA/tube for 5 hr and then stained with Coomassie Brilliant Blue. Cytochrome *c* and histone fractions (Sigma Chemical) were used as marker proteins.

RESULTS

The mitosis rate of inseminated sea urchin eggs was retarded by pretreatment of 0.025% crude RLCC for 10 min (Fig. 1).

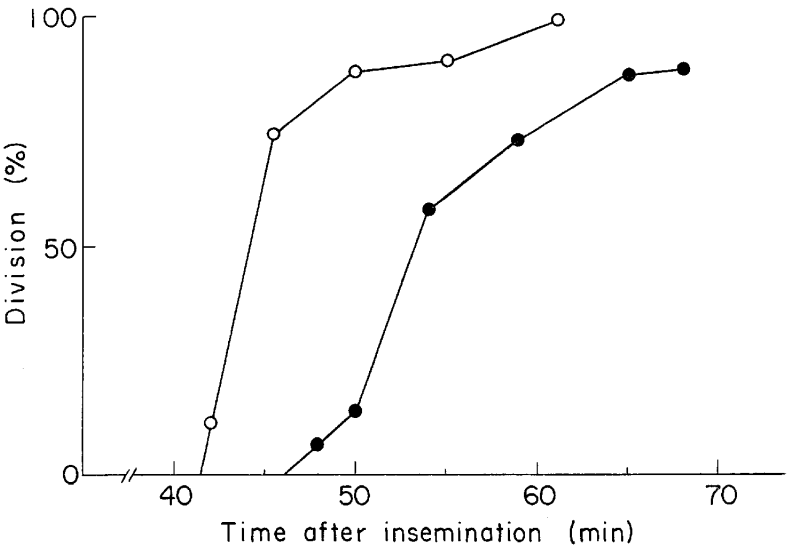


Fig. 1. Effect of crude RLCC on the division of inseminated sea urchin eggs. Ordinate, Percentage of divided eggs to initial eggs. Abscissa, Time after insemination. Open circles, control. Solid circles, crude RLCC (2.5×10^{-4} g/ml).

This crude liver cytosol cornin was further separated into three fractions by Sephadex G-50 column chromatography (Fig. 2).

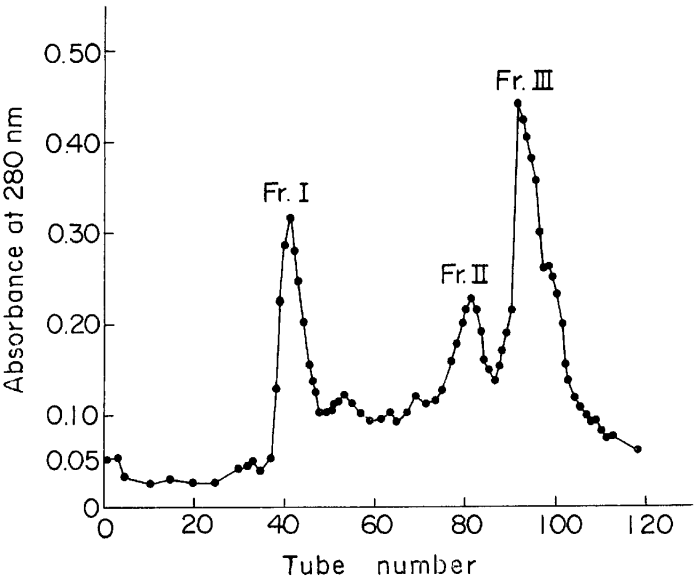


Fig. 2. Sephadex G-50 column chromatography of crude RLCC. Ordinate, Absorption of UV (280 nm). Abscissa, Tube number.

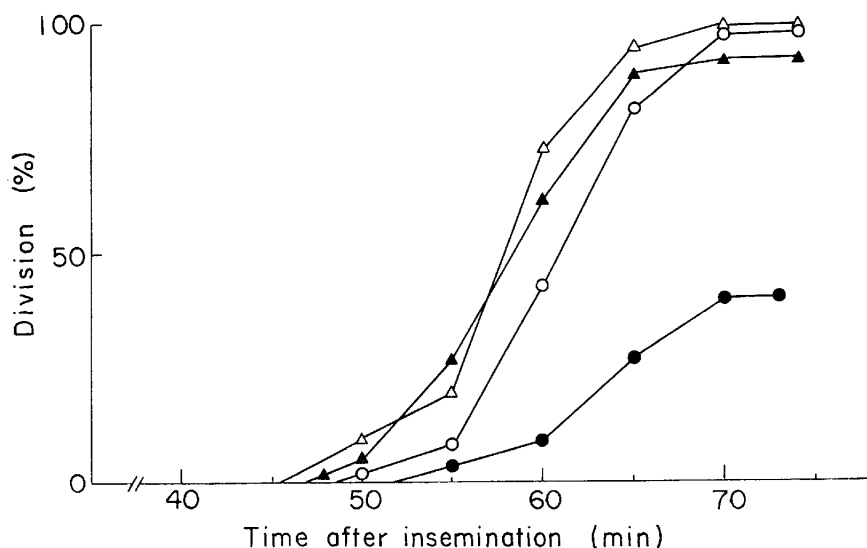


Fig. 3. Effect of fractions separated from crude RLCC on division of inseminated sea urchin eggs. Ordinate, Percentage of divided eggs to initial eggs. Abscissa, Time after insemination. Open circles, control. Solid circles, Fraction I (2.5×10^{-5} g/ml). Solid triangles, Fraction II (2.5×10^{-4} g/ml). Open triangles, Fraction III (2.5×10^{-4} g/ml).

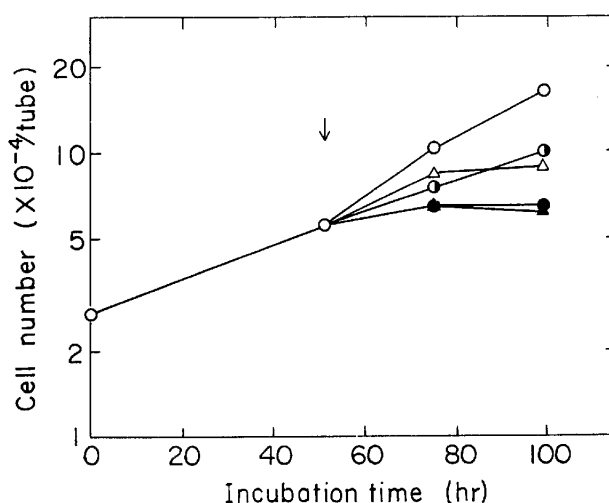


Fig. 4. Effect of RNase on the antimittotic activity of Fraction I isolated from crude RLCC. Ordinate, Cell numbers per tube. Abscissa, Incubation time at 37°C. Open circles, control. Solid circles, Fraction I (1×10^{-3} g/ml). Half solid circles, Fraction I (5×10^{-4} g/ml). Open triangles, Fraction I (RNA free, 5×10^{-4} g/ml). Solid triangles, Fraction I (RNA free, 1×10^{-3} g/ml). Fraction I free of RNA was prepared from crude RLCC which was previously treated with pancreatic RNase ($25 \mu\text{g}/50\text{mg}$ crude RLCC/ml) for 60 min at 37°C. Arrow shows the time when an equal volume of the culture medium containing test material was added to the culture.

The effect of each fraction separated from crude RLCC was examined on mitosis of inseminated sea urchin eggs (Fig. 3). The mitosis was suppressed by 60% and 96% with pretreatment of 0.0025% and 0.025%, respectively, of Fraction I. Fractions II and III had no effect on mitosis of inseminated sea urchin eggs. The yield of Fraction I was about 35 mg from 100 g of rat liver.

As Fraction I contains protein (92%) and RNA (8%), the effect of RNase on antimitotic activity of Fraction I was examined by the cultured cell assay system (Fig. 4). The cell growth inhibitory activity of Fraction I was not affected significantly by RNase treatment. RNA isolated from Fraction I by the SDS-phenol method had no effect on mitosis. The antimitotic activity of Fraction I was eliminated with digestion using bacterial protease (Nagarse, Katayama Chemical Co., Osaka) at 200 μ g or 160 PUN/10 mg of Fraction I, at 37°C for 1 hr.

Fraction I was shown to be a single spot and its molecular weight was calculated as about 25,000 by thin layer gel filtration on Sephadex G-100. Fraction I was separated into two bands by SDS-polyacrylamide gel electrophoresis, and

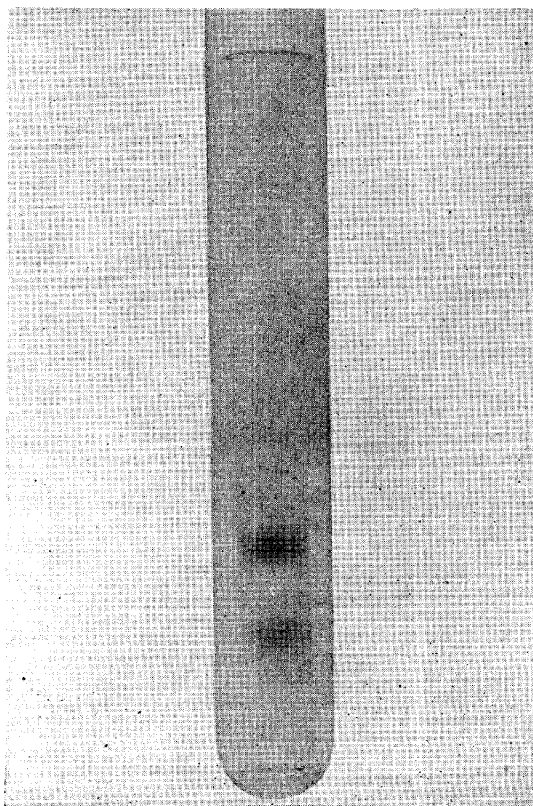


Fig. 5. SDS-polyacrylamide gel electrophoresis of Fraction I isolated from crude RLCC.

their molecular weights were calculated as about 10,000 and 15,000, respectively (Fig. 5).

The amino acid composition of the hydrolysate of Fraction I is shown in Table 1. The content of the acidic amino acids was relatively high compared with that of basic amino acids. The content of sulfur-containing amino acids in Fraction I was very low.

TABLE 1. AMINO ACID COMPOSITION OF FRACTION I OF RLCC

Amino acid	Percent in moles
L-Arginine	2.2
L-Histidine	1.6
L-Lysine	5.8
L-Phenylalanine	2.5
L-Tyrosine	1.1
L-Leucine	6.5
L-Isoleucine	3.1
L-Methionine	0.7
L-Cysteine	0.6
L-Valine	6.0
L-Alanine	10.7
Glycine	9.8
L-Proline	7.4
L-Glutamic acid	14.0
L-Serine	8.8
L-Threonine	8.1
L-Aspartic acid	11.3

DISCUSSION

Since the antimetabolic activity of Fraction I purified from crude RLCC was eliminated by protease treatment and was little affected by RNase treatment, the activity of Fraction I is probably associated with the protein in Fraction I, and the RNA in Fraction I may be considered a contaminant.

Numerous investigators (7-11) have reported that cell growth inhibiting substances were extracted from mammalian liver. It was found that some of them are closely associated with arginase (10, 11). These substances were generally heat labile in contrast with the cornin substance. The antimetabolic activity of RLCC does not seem to be tissue-specific in contrast with liver chalone (12), though neither is species-specific.

Further purification is necessary for critical characterization of RLCC, and

the purification of Fraction I with protamine sulfate treatment is under investigation.

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