Extracellular Polysaccharide from a True Slime Mold, *Physarum Polycephalum*. Polymerization and Auto-Degradation Associated with Cell Growth, and Its Inhibitory Activity on Eukaryotic DNA Polymerase α

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Summary

Plasmodia of a true slime mold, *Physarum polycephalum*, synthesize slime polysaccharide and secrete it outside the organisms. This substance was shown to be synthesized and polymerized with cell growth. And when the cells reached a plateau of growth in a nutrient medium, degradation of the polysaccharide and autolysis of the cells occurred. The composition of polysaccharides from different strains of *Physarum*, F/J or Lu strains, were almost the same, but polymerization rates of them were different. We purified the polysaccharides from these two strains and characterized them. Both of them mainly consisted of galactose (Gal) residue having phosphate ($-PO_4$) and sulfate ($-SO_4$) moieties, and the molar ratios of these constituents were 9 : 1 : 1 for F/J and 9 : 2 : 1 for Lu, respectively.

The biological effect of slime polysacchapide was examined and we found that it had a specific inhibitory activity on eukaryotic DNA polymerase α, and degree of inhibition was not dependent on the length of the polysaccharide.

Introduction

The myxomycete, *Physarum polycephalum* produces a large amount of extracellular slime polysaccharide. Some investigators have studied the nature of the slime (1-3), and Farr *et al.* reported that it was composed of (1-4)-, (1-3)- and (1-6)-linked galactose units with sulfate and phosphate (1). Considering from the nature of this extracellular slime coat, this may act as a protector of the naked, migrating plasmodia.

Henney and Asgari suggested the additional biological function of this substance, and they showed that it inhibited the cytokinesis and macromolecular synthesis in haploid myxoamoebae of *Physarum* and the cell divi-

sion of the bacterium *Bacillus subtilis* (4). They also showed that the respiration of mitochondria isolated from haploid myxoamoebae was inhibited by the slime (5).

On the other hand, in the nuclei of microplasmodia of *Physarum polycephalum*, the presence of a similar galactan has been reported (6, 7), but its roles have also been unclarified.

In order to know the biological significance of this substance, we examined biosynthesis and degradation of it during the cell growth, and studied the effects of the purified substance on DNA polymerase reaction.

Materials and Methods

Organisms. Plasmodia of *Physarum polycephalum*, strains F/J (8) and Lu which was gifted from Dr. S. Kawano of Tokyo University, were cultured by the method ef Daniel and Rush (9).

Chemicals. D-[U-^{14}C]Glc, D-[U-^{14}C]Gal and [^{35}S]H_2SO_4 were obtained from Amersham International Plc (Amersham, UK); calf thymus DNA polymerase α and *E. coli* DNA polymerase I were from Pharmacia (Uppsala, Sweden); DNA polymerase β was prepared from ovarian extract from immature rat (10); [^{3}H]dTTP was from New England Nuclear (Boston, MA, USA); 3% OV-101 on Shimalite W (80-100 mesh) was from Shimadzu (Kyoto, Japan); cetyl piridinium chloride was from Nakarai Tesque Inc. (Kyoto, Japan).

Radioactive labelling of a polysaccharide. To culture media, either 1 μCi/ml of [^{14}C]Glc, [^{14}C]Gal or $^{35}SO_4^{2-}$ was added and incubated at 24°C for various periods. After precipitation of the microplasmodia, 50 μl aliquots of the supernatant were took on filter discs and precipitated the polysaccharide in ethanol and washed several times with ethanol. Then the filters were transferred into scintillation vials and radioactivity was determined.

Purification of a polysaccharide. Purification of the polysaccharide was carried out according to Zaar (11) with slight modifications. The culture media, from 3-day to 7-day culture, were centrifuged at 1,500 rpm for 3 min to remove plasmodia and 2 vols of ethanol were added to the culture media in order to precipitate the polysaccharide. The precipitate was collected by centrifugation at 3,000 rpm for 10 min and dissolved in water at an approximate concentration of 0.5%. Then an equal vol of 10% cetyl piridinium chloride (CPC) was added to the viscous solution and the mixture was kept at 37°C overnight. The resultant precipitate of CPC-

polysaccharide complex was collected by centrifugation at 3,000 rpm for 10 min. This complex was dissolved in 2 M NaCl/10% ethanol, and precipitated again by adding of 3 vols of ethanol. This precipitate was re-dissolved in water and re-precipitated with 3 vols of ethanol, then collected by centrifugation and dried under vacuo.

Composition analysis of the polysaccharide. The composition of the carbohydrate was analyzed by gas-liquid chromatography. The polysaccharide was dissolved in 1 N HCl in methanol, and hydrolyzed at 100°C for 24 hrs, then trimethylsilylated. The sample was analyzed by gas-liquid chromatography with 3% OV-101 column at 160-250°C. The carbohydrate content was measured by the phenol-sulfuric acid method (12). Galactose was used as the standard. Phosphate was estimated by the method of Gerlach and Deuticke (13), sulfate by the method of Dodgson and Price (14). For the measurement of reducing group of carbohydrate, Park-Johnson method (15) was used.

Assay of inhibitory activity on DNA polymerases. For the determination of inhibitory activity on DNA polymerases, the reaction mixture consisted of 0.5 unit of DNA polymerase, 50 mM Tris-HCl (pH 7.5), 20 μM each of dATP, dCTP and dGTP, 10 μM dTTP containing 0.5 μCi of [^{3}H]dTTP (1,000 cpm = 1 pmol dTTP), 7 mM $MgCl_2$, 40 mM NaCl, 2 mM 2-mercaptoethanol, 2 μg calf thymus activated DNA prepared by the method of Fansler and Loeb (16), 10 μg bovine serum albumin and 10% glycerol in a final volume of 25 μl. This reaction mixture was incubated at 4°C for 10 min in the presence of different amount and different length of polysaccharide. Then, the reaction mixture was incubated at 25°C for 30 min and the activity was determined from the incorporation of [^{3}H]dTTP into the acid-insoluble fraction.

Results

Polymerization and degradation of a polysaccharide. Microplasmodia of strain F/J were cultured in the media containing labeled Gal or Glc or SO_4^{2-}. Then the polysaccharide was precipitated from the culture media and the incorporation of labeled substances into the polysaccharide was measured (Fig. 1a). This result shows the plasmodia could incorporate both Gal and Glc and polymerized them into a galactan. Incorporated Glc may have been changed into Gal by an epimerase in the cell. Fig. 1b shows the correlation of the length of polysaccharide and cell growth. The rate of polymerization of the polysaccharide was parallel to the rate of cell growth. The polymerization rate reached the maximum

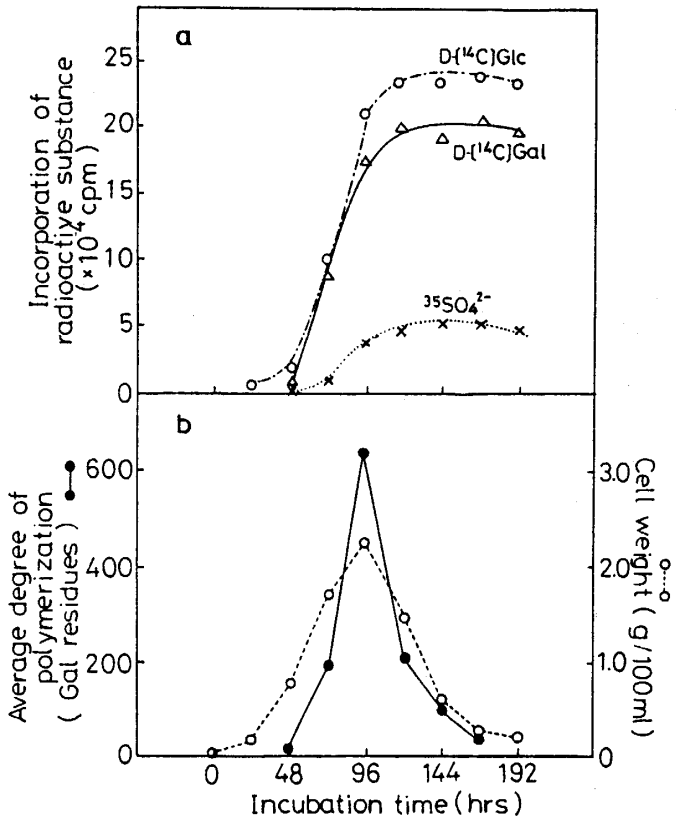

Fig. 1. **(a) Incorporation of radioactive substances into polysaccharide during cell growth.** ○—○, △—△, and ×—× showed the incorporation of D-[^{14}C]Glc, D-[^{14}C]Gal and $^{35}SO_4^{2-}$ into the 50 μl of slime polysaccharide, respectively.
(b) Cell growth and polymerization rate of slime polysaccharide. Polymerization rate was measured by Park-Johnson procedure (15). ●—●, average degree of polymerization (Gal residues); ○—○, cell weight.

at 4 days, and then the polysaccharide was degraded. But the incorporation of labeled substances was kept the same level and did not decrease, indicating that the polymerization and the degradation were performed simultaneously. SO_4^{2-} was also ioncorporated into a sulfated galactan as shown by Zaar (11). When the microplasmodia of strain Lu was used, almost the same results were obtained, but the degree of polymerization in Lu strain was higher than that in strain F/J (Table I).

Purified polysaccharide was mainly composed of galactose, phosphate, and sulfate, and those from different strains, F/J and Lu, showed almost the same composition (Table I).

We examined the change of the composition of the polysaccharide from the culture media from 4 days to 7 days. The constitution did not change as shown in Fig. 2.

Table I. Composition of purified polysaccharide

Strain	Gal	PO_4	SO_4	others	maximal polymerization rate (Gal residues)
	weight % (molar ratio)				
Lu	78 (9)	10 (2)	5 (1)	7 (-)	1,100
F/J	86 (9)	6 (1)	5 (1)	3 (-)	700

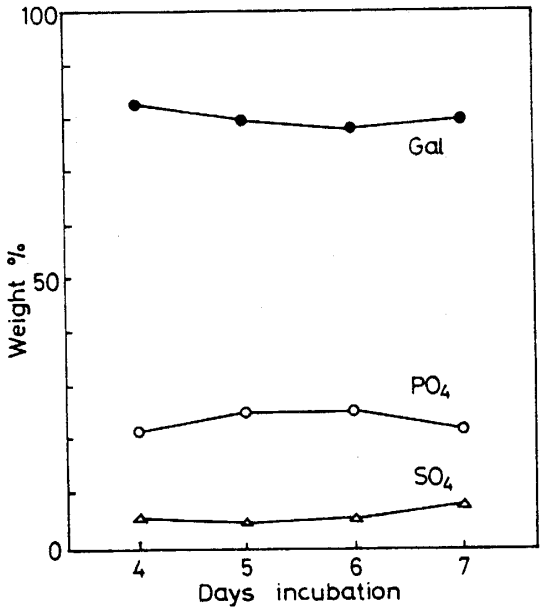

Fig. 2. Composition of polysaccharides from culture media of different incubation periods. The slime polysaccharides from 4-day culture to 7-day calture of Lu strain were purified and analyzed sugar (●), $-PO_4$(○), $-SO_4$(△) contents as described in Materials and Methods. These contents were shown by weight %.

Fig. 3 shows the profiles of purified polysaccharides from 4-day culture to 6-day culture on gel filtration. The peak shifted toward the area of smaller molecular weight as the culture media became older. It was supposed that long polysaccharides were decomposed into shorter ones indicating the presence of a degradation enzyme in older culture media.

Culture media were centrifuged at 100,000×g for 2 hrs and the supernatant was incubated with the polysaccharide. The polysaccharide was degraded with the time and this degradation activity was lost after heat treatment (at 100°C for 2 min) of the supernatant (data not shown).

Effect of the polysaccharide on DNA polymerases. The polysaccharide had an inhibitory activity on DNA polymerase as shown in Fig. 4.

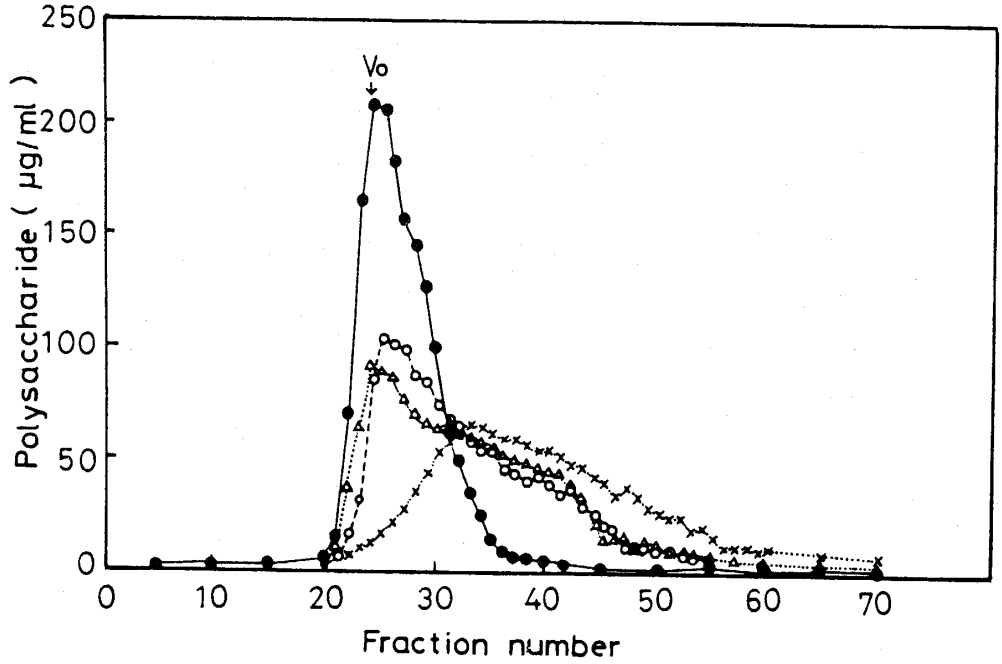

Fig. 3. Sephadex G-100 gel filtration profile of purified polysaccharide. 1.5 mg of polysaccharide purified from the culture media of the plasmodia of strain F/J were dissolved in 1.5 ml of distilled water and were applied on the column (1.2×60 cm) of Sephadex G-100 and eluted with distilled water, 1 ml each fraction was collected and polysaccharide was determined by the phenol/sulfuric acid method of Hodge and Hofreiter (12) using Gal as a standard. △—△, 3-day culture; ●—●, 4-day culture; ○—○, 5-day culture; ×—×, 6-day culture.

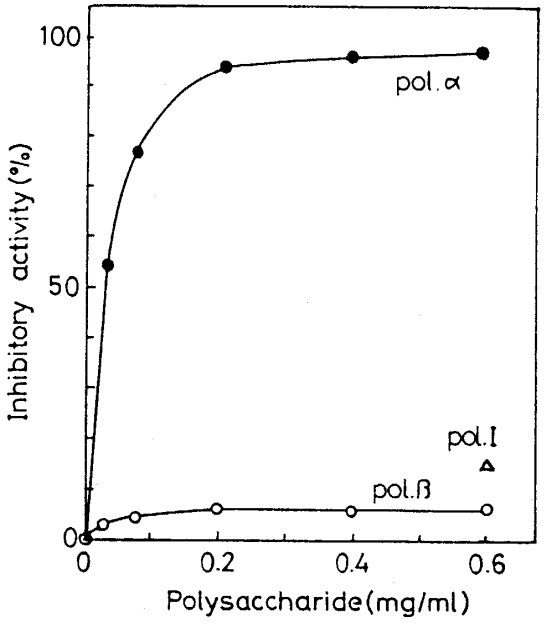

Fig. 4. Inhibitory effect of the polysaccharide on DNA polymerases. The activities of DNA polymerases were measured with various concentrations of the slime polysaccharide purified from the culture media of Lu-strain plasmodia. The degree of polymerization of used polysaccharide was about 700 Gal residues/molecule. ●—●, DNA polymerase α from calf thymus; ○—○, DNA polymerase β from immature rats; △, DNA polymerase I from *E. coli*.

Eukaryotic DNA polymerases were assayed under the various concentrations of the polysaccharide which had a high molecular weight (>200 kD). The activity of DNA polymerase α was inhibited exponentially with increasing concentration of polysaccharide. And at a concentration of 0.2

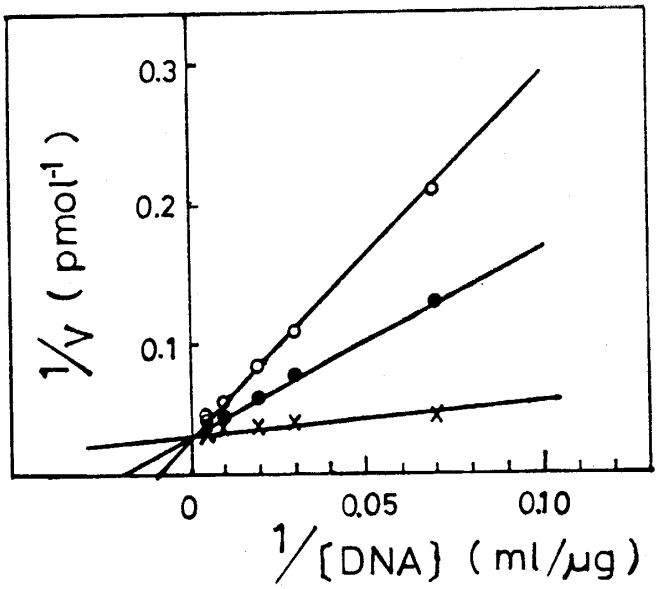

Fig. 5. DNA polymerase α was assayed with increasing amounts of template, activated DNA with a constant amounts of the enzyme (0.5 unit) and the polysaccharide. ×, ● and ○ represent the values in the presence of 0, 40 and 80 μg/ml of the polysaccharide, respectively.

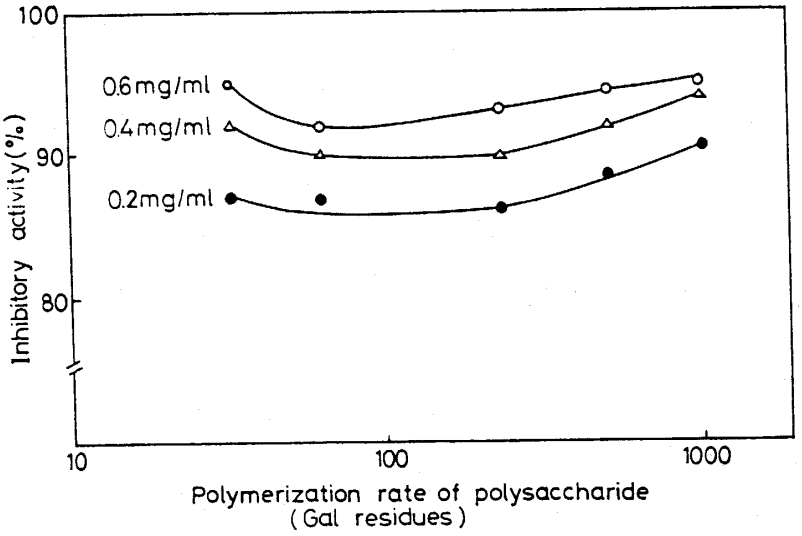

Fig. 6. Relationship between the inhibitory activity on DNA polymerase α and the polymerization rate of the polysaccharide. Constant amount of DNA polymerase α was assayed in the presence of polysaccharide with different length. Polysaccharides were purified from culture media of strain-Lu plasmodia and polymerization rate was measured as described in the text.

mg/ml, the enzyme was completely inhibited. But the activity of DNA polymerase β was not inhibited even under a high concentration of this compound. As *E. coli* pol I was not inhibited either, the inhibitory activity was specific for eukaryotic DNA polymerase α. This inhibition was dose dependent (Fig. 4), and occurs competitively with the template DNA as shown in Fig. 5. The polysaccharide increased the K_m value of template DNA for DNA polymerase α reaction.

We showed that different length of polysaccharides were synthesized during cell growth (Fig. 1b) and examined whether the inhibitory effect on DNA polymerase α differ by different degree of polymerization of polysaccharide (Fig. 6). A little increase of the inhibitory effect was observed associating with the elongation of polysaccharide, but the difference of the effect was not significant. The results suppose that the length of the galactan was not important for the inhibitory effect.

Discussion

We showed that the polysaccharides were polymerized and sequentially degraded during the exponential growth of plasmodia of *Physarum polycephalum*. The chemical analysis on the purified polysaccharides of *Physarum* was performed and the result showed a good agreement with that reported by Farr *et al.* (1). Sequential degradation was supposed to be carried out by the extracellular degradation enzyme, β-galactosidase. We obtained partially purified β-galactosidase from ultracentrifugal supernatant of culture media by gel filtration, and now the trial of the purification and characterization of this enzyme is under way.

The function of this extracellular polysaccharide is still unclear, but our finding of its inhibitory effect on DNA polymerase α may postulate a valuable clue to resolve this problem. Farr and Horisberger have found an existence of a similar polysaccharide in the nuclei of *Physarum* (6, 7). This polysaccharide was constituted of galactose (89%), phosphate (2.5%) and sulfate (9.6%) and had an average degree of polymerization of 560. If such an intranuclear polysaccharide normally exists and plays important roles in nuclear function, our finding suggests the possibility that this polysaccharide works on a regulation of DNA synthesis *in vivo*.

Acknowledgement

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