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# SELECTIVE INHIBITION OF DNA POLYMERASE α BY A POLYSACCHARIDE PURIFIED FROM SLIME OF PHYSARUM POLYCEPHALUM

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SUMMARY : A polysaccharide was purified from the slime of a myxomycete, Physarum polycephalum, and its inhibitory effect on eukaryotic DNA polymerases was examined. Almost all the calf thymus DNA polymerase α activity was inhibited with higher than 0.2 mg/ml of the polysaccharide, when the assay was carried out with activated DNA as a template. The inhibitory effect occurred regardless of the amounts of the enzyme and deoxyribonucleotides, however, kinetic analysis revealed that the inhibition occurs competitively with the template DNA, the $K_i$ value being 4 μg/ml. Inhibition was observed for DNA polymerase α, but not for DNA polymerases β and γ from various eukaryote species. 

The myxomycete, Physarum polycephalum, is regarded as a slime mold because of the secretion of a viscous extracellular slime by its plasmodia ( 1 ). The slime polysaccharide has been purified, and chemical analyses revealed that it was composed of (1→4)-, (1→3)- and (1→6)-linked galactose units, partially substituted by sulfate and phosphate groups ( 2-5 ). The biological functions of the slime polysaccharide have not yet been clarified, but some studies showed that it inhibits cytokinesis and macromolecular biosynthesis in myxoamoebae and bacteria ( 6-8 ).

Three species of DNA polymerases ( α , β and γ ) have been found and characterized in eukaryotes ( 9 ). DNA polymerase α is thought to be essential for nuclear DNA replication, whereas DNA polymerase β is possibly related to DNA repair ( 10 ). DNA polymerase γ participates in mitochondrial DNA replication ( 10 ).

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In the present study, we examined the effects of the slime polysaccharide on DNA polymerases, and found that the polysaccharide inhibits DNA polymerase α specifically, but not other DNA polymerases.

## MATERIALS AND METHODS

Purification of a polysaccharide from slime of Physarum polycephalum: Microplasmodia of Physarum polycephalum were cultured according to Daniel and Rusch ( 11 ) for 4 days. After removal of the organisms by centrifugation at 500 x g for 10 min, the polysaccharide was purified from the supernatant by the method of Farr et al. ( 5 ).

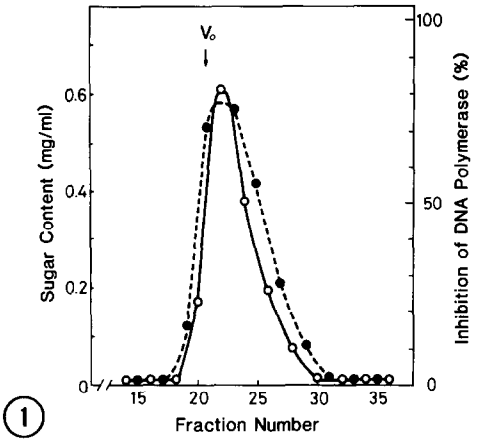
Preparation of DNA polymerases: Nuclei of Physarum polycephalum ( 12 ) were treated with 50 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 1 M NaCl and 1 mg/ml bovine serum albumin, and then centrifuged at 10,000 x g for 30 min. The supernatant was centrifuged in a 15-25 % linear sucrose gradient ( 13 ). Physarum DNA polymerase α (4,000 units/mg; 1 unit corresponds to 1 nmol dNMP incorporated per 1 h at 37 °C) and β (680 units/mg) were, respectively, obtained as the 6-8 S and 3.5 S DNA polymerase activity fractions. Sea urchin DNA polymerases α (12,000 units/mg), β (3,500 units/mg) and γ (1,500 units/mg) were prepared according to the methods of Oguro et al. ( 14 ), Suzuki-Hori et al. ( 15 ) and Habara et al. ( 16 ), respectively. Homogeneous DNA polymerase α (primase-associated form; 75,000 units/mg; Ref.17) from Xenopus laevis ovaries was a generous gift from Dr.R.M.Benbow (Department of Biology, Johns Hopkins University). The 6-8 S DNA polymerase α (1,440 units/mg) and 3.5 S DNA polymerase β (800 units/mg) were prepared from an ovarian extract from immature rats ( 18 ) by the above sucrose gradient centrifugation. Calf thymus DNA polymerase α (10,000 units/mg) and E. coli DNA polymerase I large fragment (100,000 units/mg) were obtained from Pharmacia (Uppsala, Sweden).

Assay of DNA polymerase activity: DNA polymerase activity was determined from the incorporation of [$^3$H]dTMP into the acid-insoluble materials with activated DNA as a template (unless otherwise stated). The standard reaction mixture contained 0.01-1.0 μg enzyme protein, 50 mM Tris-HCl (pH 7.5), 20 μM each of dATP, dCTP and dGTP, 10 μM dTTP containing 0.5 μCi [$^3$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 ( 19 ), 10 μg bovine serum albumin and 10 % glycerol in a final volume of 25 μl. To determine the effect of the slime polysaccharide, after mixing all the components, the reaction mixture was preincubated for 10 min at 4 °C in the absence and presence of various concentrations of the polysaccharide, and then the incubation was performed for 30 min at 25 °C, followed by determination of the polymerase activity ( 20 ).

Other methods: The sugar, sulfate, phosphate, nitrogen and protein contents were determined by the methods of Hodge and Hofreiter ( 21 ), Dodgson and Price ( 22 ), Gerlach and Deuticke ( 23 ), Strauch ( 24 ) and Lowry et al. ( 25 ), respectively. The sugar composition was determined by gas-liquid chromatography after acid hydrolysis with 1 N HCl in methanol at 100 °C for 6 h and trimethylsilyl-derivatization.

## RESULTS AND DISCUSSION

The purified slime polysaccharide was composed of carbohydrate (galactose) (86 %), sulfate (6 %), phosphate (5 %) and nitrogen (3 %). This composition is quite similar to that of which purified by Farr et al. ( 5 ), indicating that



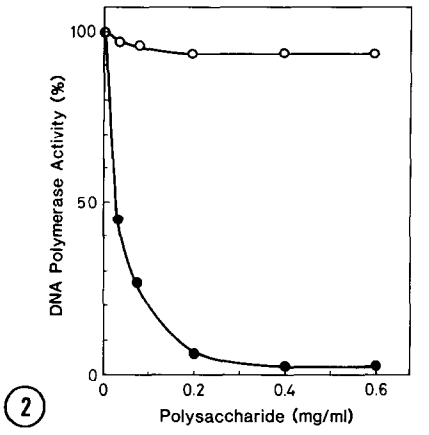




Fig.1. Gel filtration of the slime polysaccharide and its inhibitory effect on DNA polymerase α. The purified slime polysaccharide (2.6 mg) was suspended in 1.3 ml of distilled water and then subjected to Sephadex G-100 column (φ 1.2x30 cm) chromatography. Fractions of 0.5 ml each were collected, and the sugar contents of the fractions were determined. An aliquot (5 μl) of each fraction was added to the reaction mixture containing 0.25 units of calf thymus DNA polymerase α, and then the activity was assayed, as described in the text. DNA polymerase activity is expressed as the ratio of the activity with to without the fraction in %, where 0 % corresponds to 60 pmol dNMP incorporated. $V_o$ represents the void volume of the column determined with blue dextran. (o)Sugar content; (●)DNA polymerase α activity.

Fig.2. Dose dependency of the inhibitory effect of the slime polysaccharide on DNA polymerase α. DNA polymerase α (from calf thymus; 0.5 units) or DNA polymerase β(from rat ovary; 0.01 units) was assayed with various concentrations of the slime polysaccharide. 100 % corresponds to 120 pmol and 10 pmol dNMP incorporated in DNA polymerases α and β, respectively. (●)DNA polymerase α; (o)DNA polymerase β.

the present polysaccharide is the same as the latter. The molecular weight of the polysaccharide was estimated to be about 220 kDa ( the details will be presented elsewhere ).

Figure 1 shows the profile of the purified polysaccharide on Sephadex G-100 column chromatography. When calf thymus DNA polymerase α activity was measured in the presence of an aliquot of each fraction, inhibition of the activity occurred in parallel with the content of the polysaccharide, as shown in Fig.1. This indicates that the polysaccharide inhibits the DNA polymerase, and excludes the possibility that other small substances contaminating the polysaccharide fraction contribute to the inhibition.

The activity of DNA polymerase α was measured with various concentrations of the polysaccharide in the presence of a constant amount of the enzyme ( Fig.2 ). With increasing concentrations of the polysaccharide, the activity

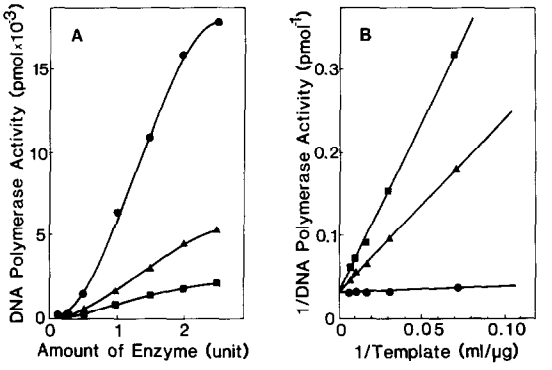



Fig.3. Relationships between the inhibitory effect and the amounts of DNA polymerase α (A) and the template DNA (B). (A)DNA polymerase activity was assayed with increasing amounts of DNA polymerase α from calf thymus with a constant concentration of the slime polysaccharide. (B)DNA polymerase activity was assayed with increasing amounts of activated DNA with a constant amount of DNA polymerase α from calf thymus (0.5 units) and the slime polysaccharide. ●, ▲ and ■ represent the values in the presence of 0, 0.04 and 0.08 mg/ml of the slime polysaccharide, respectively.

decreased exponentially. Half of the activity was inhibited on the addition of 0.025 mg/ml polysaccharide. Almost all of the activity was inhibited with higher than 0.2 mg/ml polysaccharide. On the contrary, almost all rat ovary DNA polymerase β activity remained resistant even in the presence of 0.6 mg/ml polysaccharide. Thus, the polysaccharide was shown to inhibit DNA polymerase α in a dose-dependent manner.

To elucidate the relationship between the amount of the enzyme and the inhibitory effect, DNA polymerase α activity was measured with increasing amounts of the enzyme (0.12-2.3 units) in the presence of a constant concentration of the polysaccharide ( 0, 0.04 or 0.12 mg/ml) ( Fig.3A ). Figure 3A shows that the inhibition occurs to a constant extent with a constant concentration of the polysaccharide, regardless of the amount of the enzyme. This indicates that the inhibition is independent of the amount of the enzyme.

The extent of the inhibition did not change when the assay was carried out with higher concentrations (50 μM or 100 μM each) of each of dATP, dCTP, dGTP and dTTP ( data not shown ), indicating that the inhibition is also independent of the concentration of deoxyribonucleotides.

On the contrary, the extent of the inhibition decreased with increasing concentrations of the template DNA; 0.04 mg/ml polysaccharide inhibited

Table 1. Inhibitory effect of the slime polysaccharide on various kinds of DNA polymerases

| Source of enzymes | DNA polymerase activity ( % )[a] | | | |
|---|---|---|---|---|
| | α | β | γ[b] | pol I[c] |
| Physarum polycephalum | 36 | 75 | - | - |
| sea urchin embryos | 1 | 85 | 88 | - |
| Xenopus ovary | 3 | - | - | - |
| rat ovary | 3 | 95 | - | - |
| calf thymus | 1 | - | - | - |
| E. coli | - | - | - | 84 |

[a]Each DNA polymerase activity (0.1-0.5 units) was assayed in the absence or presence of the slime polysaccharide (0.6 mg/ml), and the ratio of the activity with to without the polysaccaride is presented in %.
[b]The assay was carried out in 50 mM Tris-maleate buffer (pH 8.0), 10 μM each of dATP, dCTP and dGTP, 5 μM dTTP containing 2 μCi [$^3$H]dTTP, 5 mM $MgCl_2$, 100 mM NaCl, 20 mM potassium phosphate (pH 7.9), 0.5 μg poly(rA), 0.025 μg oligo$(dT)_{10}$, 400 μg/ml bovine serum albumin and 10 % glycerol in a final volume of 25 μl. Under the conditions, 75 % of calf thymus DNA polymerase α activity was inhibited by the polysaccharide (0.6 mg/ml) in the presence of 80 μg/ml activated DNA without poly(rA)·oligo$(dT)_{10}$.
[c]DNA polymerase I large fragment from E. coli.

the activity by 80 % in the presence of 15 μg/ml activated DNA, while the inhibition was reduced to 55 % and 20 % in the presence of 80 μg/ml and 150 μg/ml activated DNA, respectively. A similar decrease was observed with each concentration of the polysaccharide. Figure 3B reveals that the polysaccharide increases the $K_m$ value of template DNA for DNA polymerase α reaction without changing $V_{max}$. This clearly shows that the polysaccharide inhibits DNA polymerase α by competing with the template DNA. The $K_i$ value was estimated to be 4 μg/ml.

The polysaccharide inhibited DNA polymerase α almost completely, but not DNA polymerases β and γ, prepared from a wide range of eukaryote species, from invertebrates to vertebrates ( Table 1 ). In the case of Physarum DNA polymerases, the inhibitory effect was not as great as in the cases of others. However, the results may be reasonable, since both DNA polymerases α and β from Physarum have been reported to differ little from those of other higher

eukaryotes ( 26,27 ). DNA polymerase I from E. coli was resistant to the polysaccharide.

Previously, we also found a DNA polymerase inhibitor in Physarum polycephalum ( 28 ), but the present inhibitor seems to differ from the previous one, judging from the mode of action and the chemical composition. Aphidicolin is known as a specific inhibitor of DNA polymerase α ( 10 ). It inhibits the enzyme by competing with only dCTP in vitro ( 14 ). The present polysaccharide is the first reported inhibitor which inhibits DNA polymerase α, but not β or γ, competitively with the template DNA. Thus, the polysaccharide is not only useful for assaying DNA polymerases selectively, but also it may be available for analyzing the template-enzyme reaction.

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