DNA Polymerase Isolated from Nuclei of Plasmodia of *Physarum polycephalum*

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Summary

DNA polymerase was isolated from nuclei of plasmodia of the true slime mold, *Physarum Polycephalum*. And it was tried to purify the enzyme by DNA cellulose, DEAE-Sephadex A-50 and phosphocellulose chromatography. When the crude enzyme fraction was fractionated on individual chromatography, DNA polymerase activity was separable into two fractions by only phosphocellulose, while by other chromatography it appeared as a single peak. It was also tried to characterize the enzyme fractions from each chromatography.

Introduction

DNA polymerases have been isolated and characterized from various prokaryotic and eukaryotic organisms. Three DNA polymerases isolated from *E. coli* have been studied extensively and it has been concluded that they play a major role in replication or repair. In eukaryotes, most of these studies have been carried out on mammalian cells and four classes of DNA polymerases, DNA polymerases-α, -β, -γ and mitochondrial have been defined.[7-11,21-23] DNA polymerase-α is high molecular weight enzyme (6-8S) found mainly in cytoplasmic extract. DNA polymerase-β is low molecular weight enzyme(3.5S) isolated from nuclear fraction. DNA polymerase-γ is found in both cytoplasmic and nuclear fraction. However the physiological role of these enzymes has not yet been clarified.

On the other hand, in lower eukaryotes such as yeast, green flagellate, several investigators have reported the evidence of only high molecular DNA polymerases of which properties resemble those of DNA polymerase-α. They have suggested that their lower eukaryotes may have different mechanism on DNA synthesis from higher eukaryotes[32-35].

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The slime mold, *Physarum polycephalum* is considered to be suitable for biochemical investigations on DNA synthesis, for it grows as a multinuclear plasmodium and its nuclei divide synchronously in it.

This paper reports the isolation and partial purification of DNA polymerase from nuclei of plasmodia of this organism. It was also attempted to characterize several enzyme fractions obtained during purification procedure.

Materials and Methods

Culture

The plasmodia of *Physarum polycephalum* were cultivated with rolled oats according to the method of Camp.[24]

Template DNA

Calf thymus DNA was purchased from the Sigma Chemical Company. Denatured DNA was obtained by heating native calf thymus DNA at 100°C for 15 min and then cooling immediately.

DNA polymerase assay

DNA polymerase activity was assayed by a disk method.[26),27)] The reaction mixture (150 μl) contained 20 μmoles of Tris-HCl buffer(pH 8.0), 1.5 μmoles of $MgCl_2$, 0.13 μmoles of 2-mercaptoethanol, 3.5 nmoles each of dCTP, dGTP and dATP, 1.75 nmoles of [^{3}H] dTTP (0.25 μCi), 6 nmoles of ATP, 25 μg of native calf thymus DNA, 150 μg of bovine serum albumin and 50 μl of each enzyme fraction. The mixture was incubated at 30°C for 30 min (during which time, the polymerase reaction proceeded linearly with time) and 50 μl aliquots were taken on disk papers. The reaction was stopped by cold 10% trichloroacetic acid containing 1% (w/v) sodium pyrophosphate. The disk paper was washed 3 times with 5% trichloroacetic acid containing 1% (w/v) sodium pyrophosphate, once with 95% ethanol and dried with infrared lamp. The acid insoluble material was measured in 10 ml of scintillator liquid containing 4 g of 2, 5-diphenyloxazole (PPO) and 0.3 g of 1, 4-bis[2-(4-methyl-5-phenyloxazolyl)] benzene (dimethyl POPOP) per liter of toluen. Radioactivity was counted with Packard Tri-carb liquid scintillation spectrometer.

Protein determination

Protein was determined by the method of Lowry et al.[25], with bovine serum albumin as a standard.

Isolation of nuclei

Nuclei were isolated from plasmodia as follows. Frozen plasmodia were thawed and washed with distilled water 3 times to remove slime. Then it was suspended in 5 vols of homogenizing buffer [10 mM sodium

phosphate buffer (pH 6.8), 0.25 M sucrose and 10 mM $MgCl_2$] and homogenized with Tefron homogenizer. The homogenate was filtered through nylon mesh and was layered on an equal vol of 1.0 M sucrose followed by centrifugation at 1000 g for 10 min. The pellet was resuspended in 5 vols of homogenizing buffer and repeated centrifugation on 1.0 M sucrose. After washing, the pellet was suspended in an equal vol of homogenizing buffer and was sonicated till intact nuclei were not observed (crude nuclear fraction).

Phase separation[28),29)]

1.38 m*l* of 20%(w/w) dextran T-500, 3.85 m*l* of 30% (w/w) polyethylen glycole and 4.08 g of solid NaCl were added slowly, with stirring, to the 30 m*l* of the sonicated crude nuclear fraction. The emulsion was viogorously stirred for 2.5 hours and was then centrifuged at 1400 g for 30 min. The upper clear phase containing proteins was separated from the lower phase containing nucleic acids. After removal of the NaCl by dialysis against Sol. A[20%(w/v) glycerol, 2 mM potassium EDTA, 10 mM 2-mercaptoethanol and 20 mM potassium phosphate buffer (pH 7.4)], the sample was centrifuged at 10000 g for 20 min. The supernatant was used as crude enzyme fraction.

Results

DEAE-Sephadex A-50 column chromatography

The crude enzyme fraction (12.3 mg of protein) was applied to DEAE-Sephadex A-50 column (2×10 cm) equilibrated with 0.1 M NaCl in Sol. A. After washing with 0.1 M NaCl in Sol. A, the adsorbed proteins were eluted with a linear gradient of 0.1–0.9 M NaCl in Sol. A. Two m*l* fractions were collected and dialyzed against Sol. A. Fig. 1 shows elution profile from DEAE-Sephadex A-50. A single peak was eluted at 0.33 M NaCl.

Phosphocellulose column chromatography

The crude enzyme fraction (6.4 mg of protein) was applied to phosphocellulose column (1×15 cm) previously equilibrated with Sol. A. With a linear gradient of 0–0.6 M NaCl in Sol. A, the adsorbed proteins were eluted. Three m*l* fractions were collected and dialyzed against Sol. A. Two peaks of enzyme activity were obtained at 0.15 M NaCl and 0.4 M NaCl (Fig. 2) and designated phosphocellulose peak I and peak II, respectively. But peak II was not detected when the fresh crude enzyme fraction which was not stocked at −20°C was applied to the same column.

When the dialyzed DEAE eluate (6.2 mg of protein) described above was further applied to phosphocellulose column (0.8×7.0 cm), two peaks of enzyme activity at 0.20 M and 0.35 M NaCl were obtained (Fig. 3).

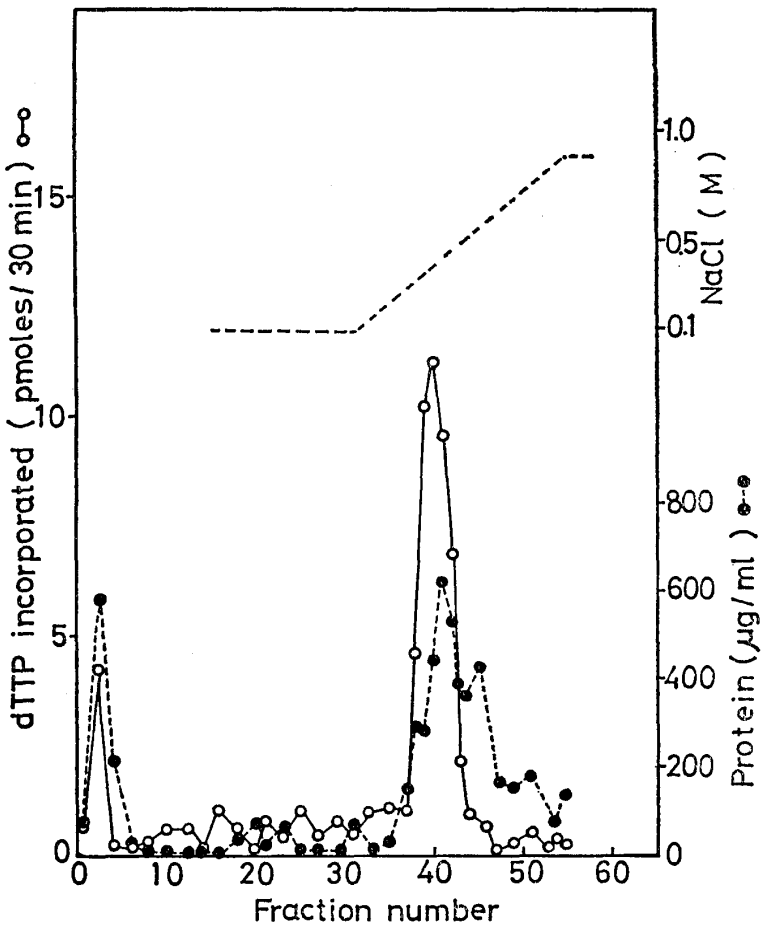

Fig. 1. DEAE-Sephadex A-50 chromatography of crude enzyme fraction. 12.3 mg of protein was applied to DEAE-Sephadex A-50 column (2×10 cm) equilibrated with 0.1 M NaCl in Sol. A. The column was washed with 0.1 M NaCl in Sol. A and eluted with a linear gradient of 0.1-0.9 M NaCl in Sol. A. Fractions (2 ml) were collected and dialyzed against Sol. A. DNA polymerase activity was measured as described in Materials and Methods.

To distinguish them from peak I and peak II, they were designated phosphocellulose peak III and peak IV, respectively.

ss-DNA cellulose column chromatography

DNA cellulose was prepared by the method of Alberts and Herrick.[30] After the column (1×7 cm) was equilibrated with Sol. A, the crude enzyme fraction (59 mg of protein) was applied and eluted stepwise with Sol. A containing increasing NaCl concentrations 0.1, 0.2, 0.4, 0.6, 1.0 and 2.0 M. DNA polymerase activity appeared at 0.4 M NaCl (Fig. 4).

Properties of enzyme fractions from different chromatography

The characterization of 5 enzyme fractions obtained above was carried out, namely the peak of DEAE-Sephadex A-50, phosphocellulose

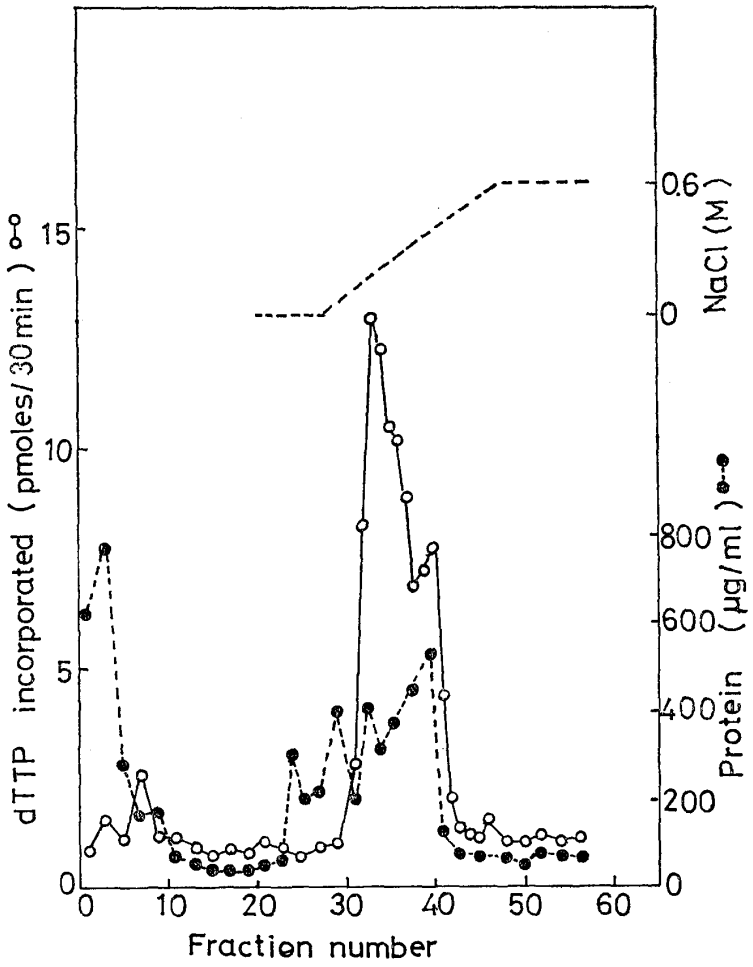

Fig. 2. Phosphocellulose chromatography of crude enzyme fraction. 6.4 mg of protein was applied to phosphocellulose column (1×15 cm) equilibrated with Sol. A. After washing with Sol. A, the adsorbed protein were eluted with a linear gradient of 0-0.6 M NaCl in Sol. A. 3 ml fractions were collected and assayed for activity as described in Materials and Methods.

peak I, II, III, IV and the peak of DNA cellulose. These results are summarized in Table I, II and III.

Fig. 5 shows the effect of a salt concentration (NaCl or KCl) on activity for each enzyme fraction. Optimal NaCl concentration ranged from 4 to 30 mM except phosphocellulose peak IV (150 mM). In contrast, optimal KCl concentration varied for each enzyme fraction.

These enzyme fractions responded similarly to a divalent cation (Mg^{2+} or Mn^{2+}), (Fig. 6). Optimal concentrations of Mg^{2+} and Mn^{2+} were 12 to 20 mM and 1 to 4 mM, respectively.

Fig. 7 shows the effect of pH on DNA polymerase activity. Though optimal pH of most of the enzyme fractions were between pH 8.0 and 9.0, those of more purified fractions, phosphocellulose peak III and IV were pH 9.8.

It was also found that the apparent Km for dTTP was different

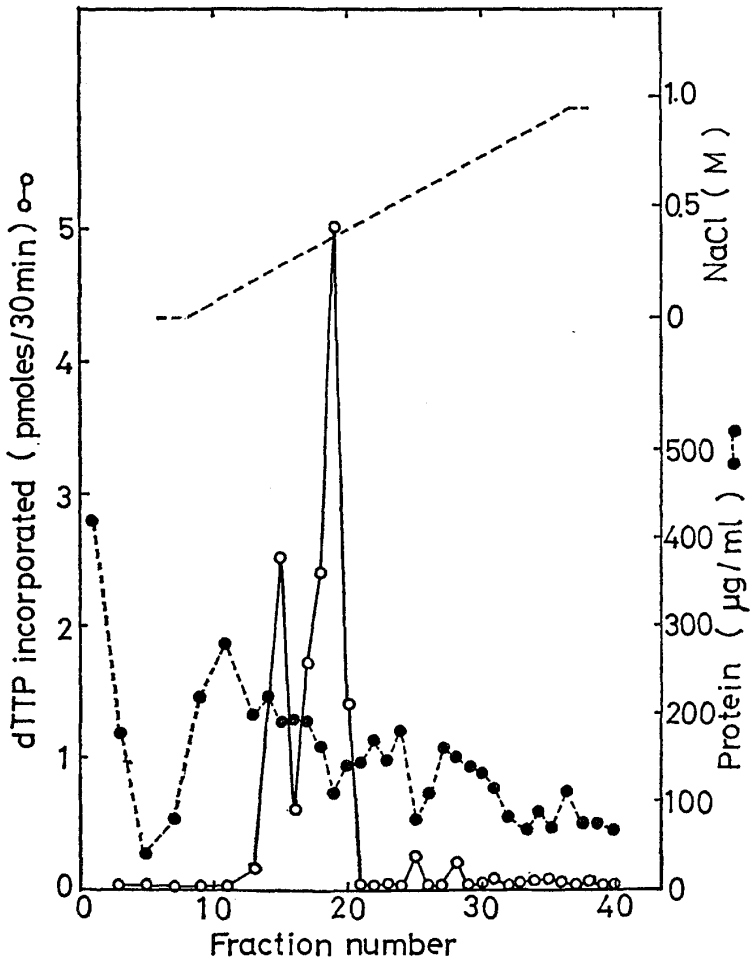

Fig. 3. Phosphocellulose chromatography of the peak of DEAE-Sephadex A-50 chromatography. The peak of activity obtained from DEAE-Sephadex A-50 column (6.2 mg of protein) was applied to phosphocellulose chromatography (0.8×7.0 cm). After washing with Sol. A, DNA polymerase activity was eluted with a linear gradient of 0-0.9 M NaCl in Sol. A. 3 ml fractions were collected and assayed for activity as described in Materials and Methods.

Table I. Properties of each enzyme fraction from different chromatography (I). DNA polymerase activity was measured as described in Materials and Methods.

	Mg^{2+} (mM)	Mn^{2+} (mM)	Mg^{2+}/Mn^{2+}	NaCl (mM)	KCl (mM)	pH	dTTP Km (μM)
DEAE Sephadex A-50	12	4	2.0	4	150	8.0	19
DNA cellulose	12	1	2.1	4	8	8.4	5
Phosphocellulose I	20	2	3.2	4	12	8.4	12
II	12	2~4	1.8	25	100	8.6	20
III	12	2	7.3	30	50	9.8	4
IV	20	2~4	1.5	150	150	9.8	26

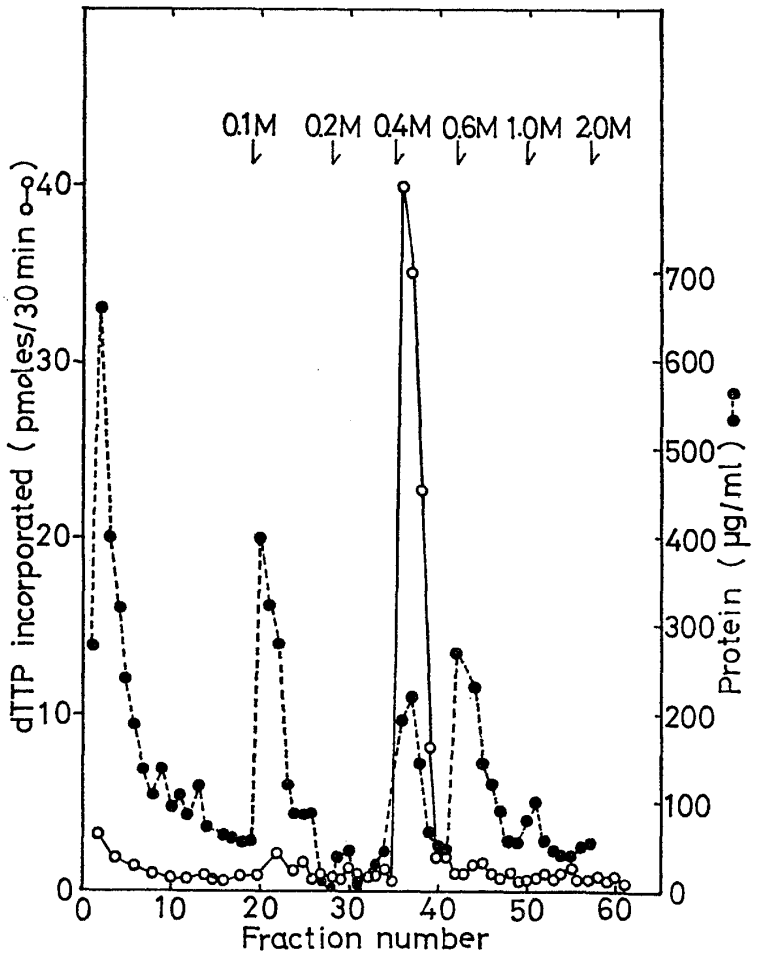

Fig. 4. ss-DNA cellulose chromatography of crude enzyme fraction. 59 mg of protein was applied to ss-DNA cellulose column (1×7 cm). After washing with Sol. A, the adsorbed proteins were eluted with a step wise of 0.1, 0.2, 0.4, 0.6, 1.0 and 2.0 M NaCl in Sol. A. 3 ml fractions were collected and dialyzed against Sol. A. DNA polymerase activity was measured as described in Materials and Methods.

Table II. Properties of each enzyme fraction from different chromatography (II). Incubation conditions were the same as described in Materials and Methods except that the individual component was omitted or added.

	DEAE Sephadex A-50	DNA cellulose	Phospho-cellulose I	Phospho-cellulose II	Phospho-cellulose III	Phospho-cellulose IV
complete system	100	100	100	100	100	100
−dATP	55	24	36	30	46	20
−dCTP	70	14	23	53	53	36
−dGTP	40	26	46	50	72	36
−dATP dCTP dGTP	30	14	18	20	32	25
−ATP	120	90	63	100	100	85
+DNase (3.7μg)	20	5	7	33	48	27
+RNase (3.7μg)	123	104	87	109	148	132
+NEM (12.5 mM)	78	82	128	125	104	95
(25 mM)	76	55	86	108	65	73
+pCBM (0.5 mM)	115	107	107	103	115	120
(3.0 mM)	67	74	55	100	113	100

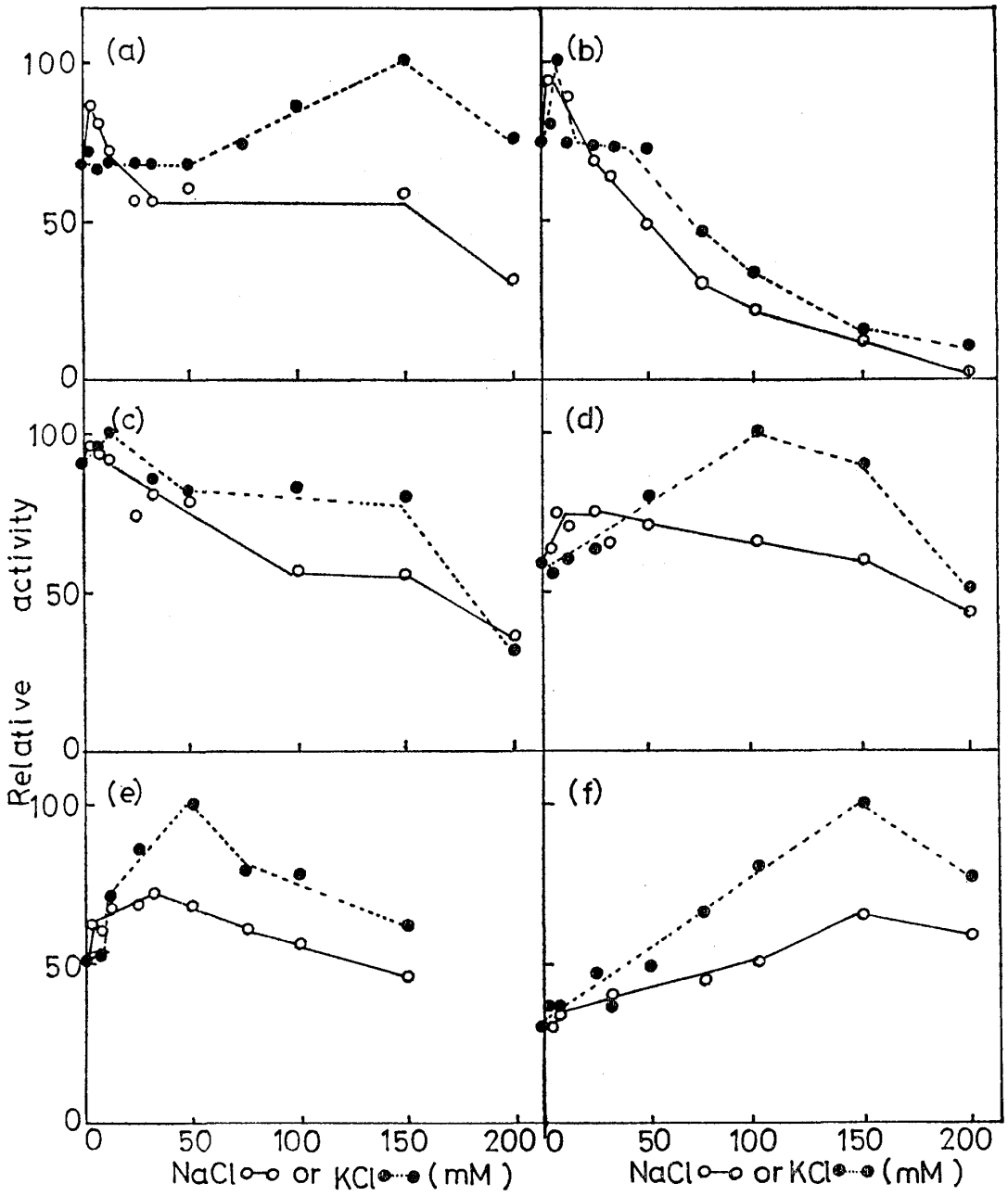

Fig. 5. Effect of a salt concentration (NaCl or KCl) for each enzyme fraction from different chromatography. The reaction mixture is as described in Materials and Methods except for the concentration of NaCl or KCl. The value with each optimal KCl concentration, was set at 100%. (a) the peak of DEAE-Sephadex A-50, (b) the peak of ss-DNA cellulose, (c) phosphocellulose peak I, (d) phosphocellulose peak II, (e) phosphocellulose peak III, (f) phosphocellulose peak IV.

for each fraction (Table I).

These enzyme fractions required all four deoxyribonucleoside triphosphates for their maximal activity (Table II). Omission of one deoxyribonucleoside triphosphate in the reaction mixture reduced their activities. And omission of three deoxyribonucleoside triphosphates reduced activities even further. These results suggest that the reaction is not

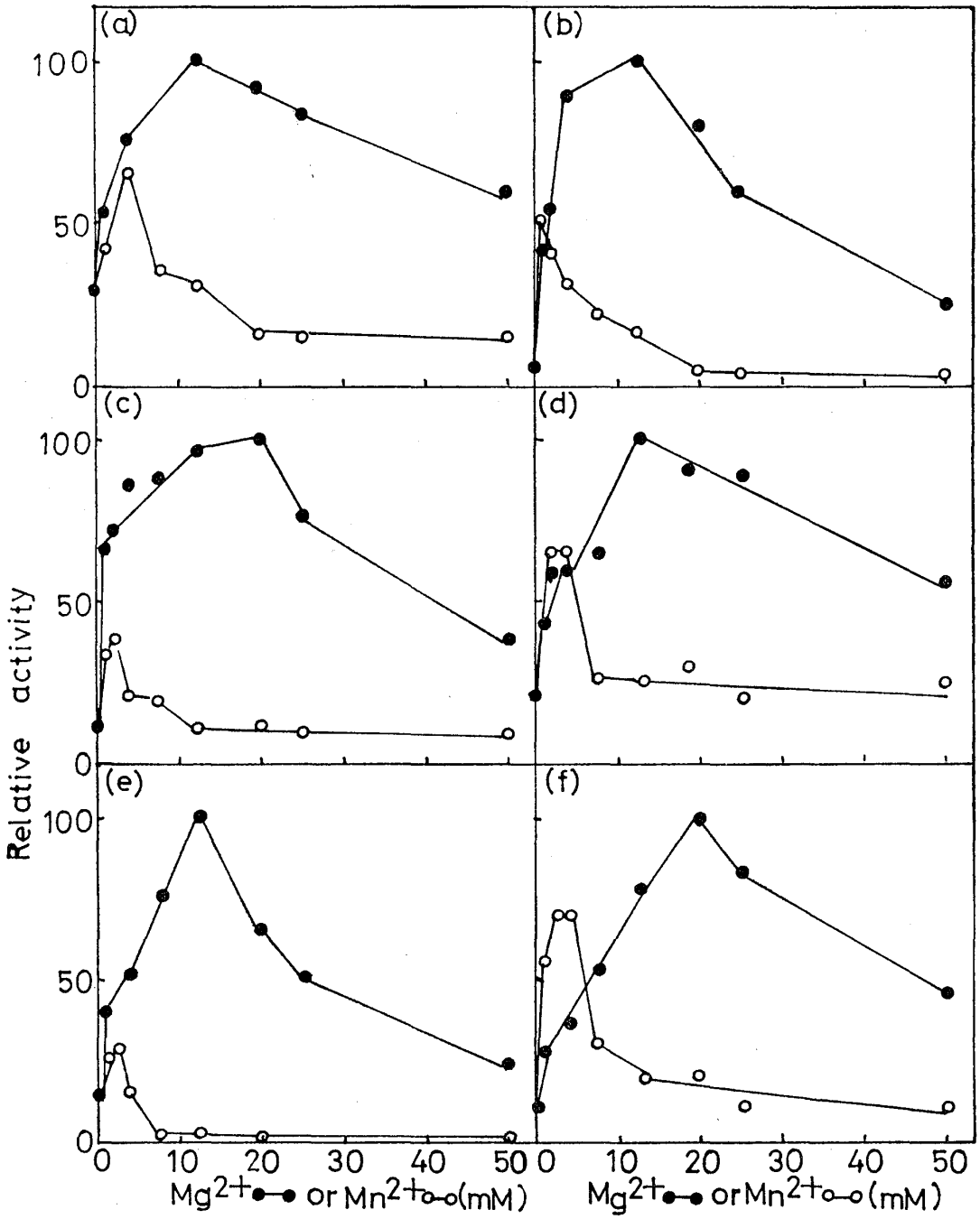

Fig. 6. Effect of a divalent cation (Mg^{2+} or Mn^{2+}) for each enzyme fraction from different chromatography. The reaction mixture is as described in Materials and Methods except for the concentration of $MgCl_2$ or $MnCl_2$. The value with each optimal concentration of Mg^{2+}, was set at 100%. (a) the peak of DEAE-Sephadex A-50, (b) the peak of ss-DNA cellulose, (c) phosphocellulose peak I, (d) phosphocellulose peak II, (e) phosphocellulose peak III and (f) phosphocellulose peak IV.

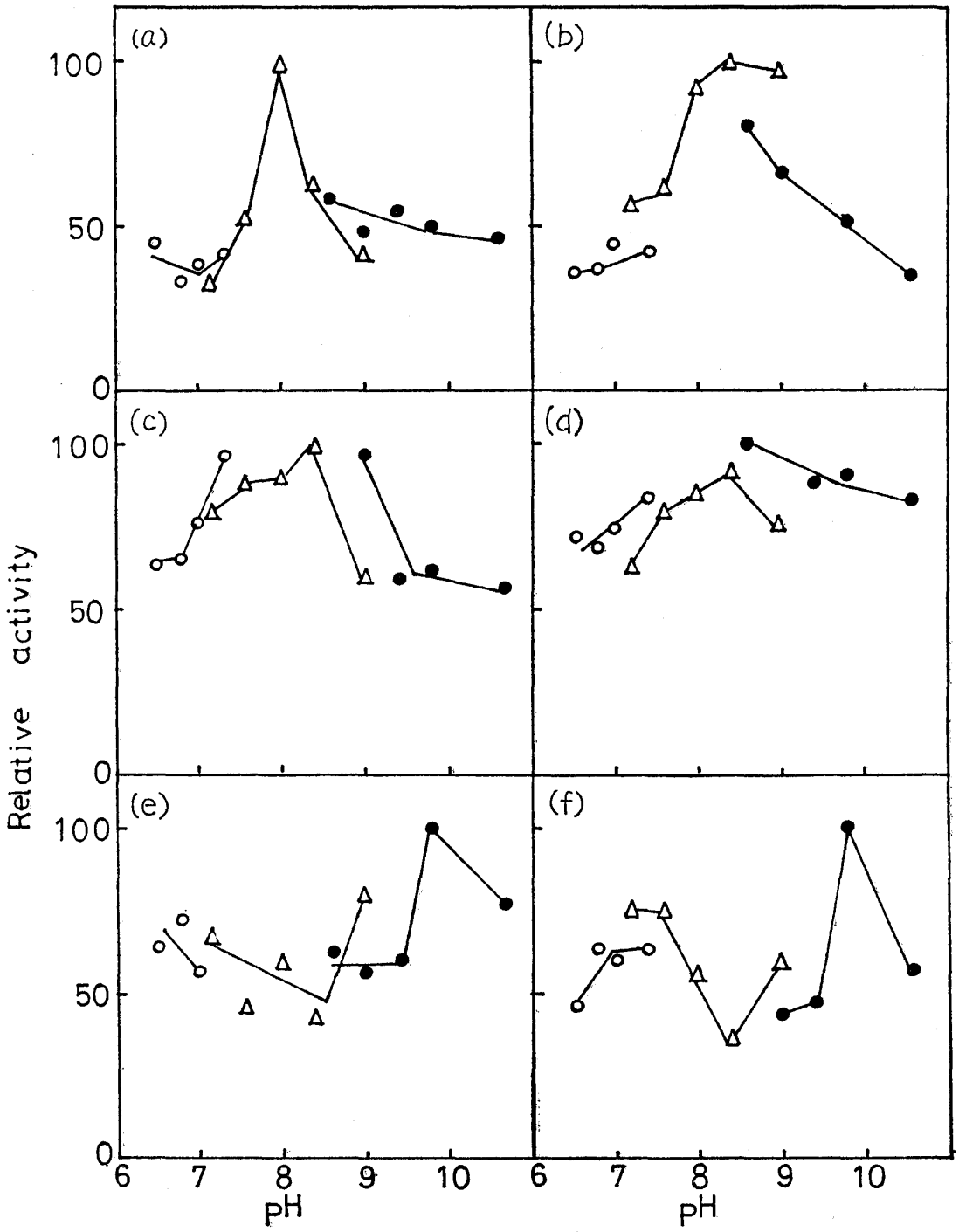

Fig. 7. Effect of pH for each enzyme fraction from different chromatography. Each enzyme fraction was assayed as described in Materials and Methods except that the indicated buffer was used. The value with each optimal pH, was set at 100%. (a) the peak of DEAE-Sephadex A-50, (b) the peak of ss-DNA cellulose (c), phosphocellulose peak I, (d) phosphocellulose peak II, (e) phosphocellulose peak III, (f) phosphocellulose peak IV, ○—○ potassium phosphate buffer, △—△ Tris-HCl buffer, ●—● glycin-NaOH buffer.

due to a terminal transferase activity.

In the absence of ATP, the activity of phosphocellulose peak I was reduced to 63% of the control. However, it was observed that ATP was not necessary in activities of other enzyme fractions.

In order to check whether or not the reaction product was DNA, and not RNA, it was treated with DNase and RNase. Radioactivity was no longer found in acid insoluble fraction after treatment with DNase, while RNase had no such effect. It was concluded that [^{3}H] dTTP was indeed incorporated into DNA.

These enzyme fractions were also insensitive to PCMB and NEM which were SH-inhibitor.

Table III shows template requirement for each DNA polymerase activity. Denatured DNA and native DNA were almost equally active as template for the peak of DEAE-Sephadex A-50 and phosphocellulose

Table III. Template requirement for each enzyme fraction from different chromatography. The reaction was incubated at 30°C for 30 min and DNA polymerase activity was measured as described in Materials and Methods except for template DNA. The value with each native DNA was, set at 100%.

	native	denatured
DEAE Sephadex A-50	100	92
DNA cellulose	100	22
Phosphocellulose I	100	42
II	100	104
III	100	28
IV	100	33

peak II. On the other hand, for other enzyme fractions, native was more active than denatured. Especially, this tendency was remarkable for the peak of DNA cellulose, phosphocellulose peak III and IV.

Fig. 8 is isoelectrofocusing of crude enzyme fraction. Isoelectric point was at pH 5.4.

Purification of DNA polymerase

The purification of DNA polymerase is summarized in Table IV. Crude enzyme fraction prepared by phase separation as described in Materials and Methods was applied to DEAE-Sephadex A-50 column chromatography (Fig. 1), followed by phosphocellulose column chromatography (Fig. 3). Two peaks obtained from phosphocellulose (peak III and IV) were further applied to ss-DNA cellulose, respectively. But both peaks had no affinity to ss-DNA cellulose and were eluted at void volume. At this step these DNA polymerase activities were purified about 120 and 150 folds, respectively.

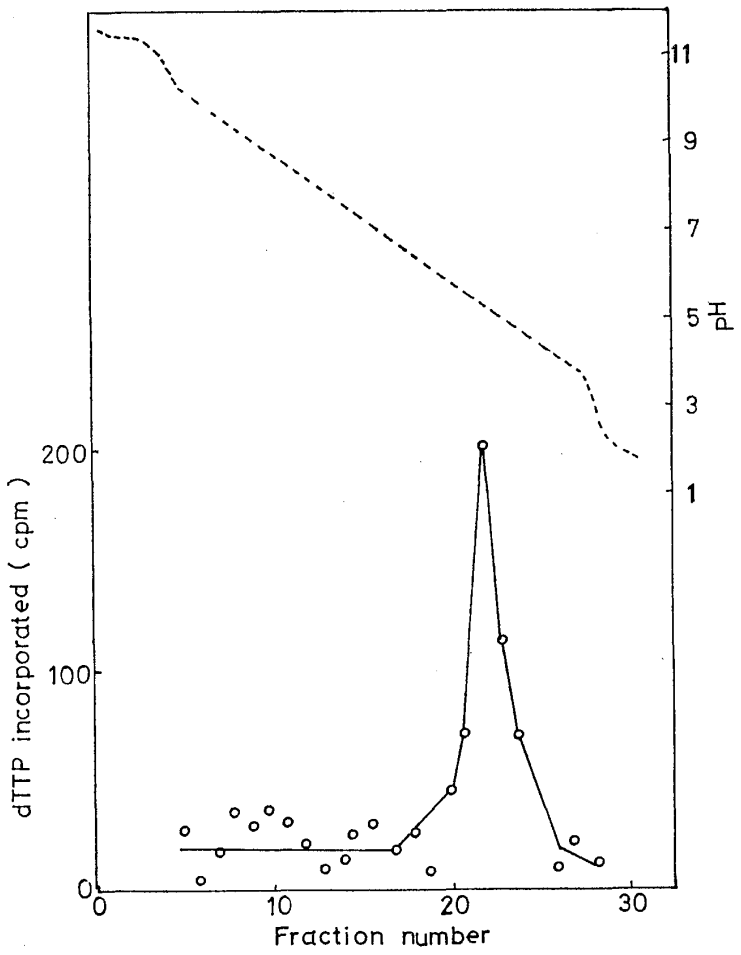

Fig. 8. Isoelectrofocusing of crude enzyme fraction. DNA polymerase activity was assayed as described in Materials and Methods.

Table IV. Purification of DNA polymerase. DNA polymerase activity was assayed as described in Materials and Methods. Phosphocellulose (1) and (2) correspond to phosphocellulose peak III and peak IV, respectively. DNA cellulose (1) and (2) are obtained from phosphocellulose peak III and IV.

	Total activity (pmoles)	Protein (mg)	Specific activity (pmoles/mg protein)	Yield (%)
Nuclei	11200	200	56	100
Phase separation	9840		464	88
DEAE Sephadex A-50	9680	3.2	3025	86
Phosphocellulose (1)	1276	0.24	5316	37
(2)	2872	0.39	7364	
DNA cellulose (1)	421	0.05	6014	7
(2)	390	0.045	8666	

Discussion

The study of physiological role of DNA polymerases in eukaryotes is less advanced because of their complicated system and the difficulties in finding mutants for DNA synthesis.

Recently four DNA polymerases namely DNA polymerases-α, -β, -γ and mitochondrial have been classified by the properties such as molecular size, template requirement and sensitivity to various inhibitors.[7~11,21~23]

On the other hand, multiple species of DNA polymerase besides those listed above have been reported. And the functions and properties of these species also still remain to be solved.[1~6,10,12~20]

Hecht showed that DNA polymerase-α, high molecular weight enzyme (6-8S), was convertible by salt [0.125 M $(NH_4)_2SO_4$, 0.25 M NH_4Cl, 0.25 M NaCl etc.] to a form of DNA polymerase-β, low molecular weight enzyme (3.5S).[19,20] And Yoshida et al. reported that the high molecular weight enzymes (6.7S-8.5S) are dissociated into active smaller molecules (5.4S and 3.8S) upon freezing and thawing or 2 M urea treatment or gel-filtration.[14] They suggested these enzymes were capable to convert in form from one molecular to another.

In contrast, Spadari et al. demonstrated DNA polymerase-β was immunologically distinct from DNA polymerase-α.[31]

In this paper, it was tried to purify DNA polymerase from nuclei of *Physarum polycephalum* by DEAE-Sephadex A-50, phosphocellulose and ss-DNA cellulose. As the result, it was found that the chromatographic behavior by each column was different. Two peaks of enzyme activity were separated only by phosphocellulose using sample frozen once at $-20°C$ though it was not observed under condition using fresh extract (Fig. 2, 3).

By DNA cellulose, one peak appeared at 0.4 M NaCl (Fig. 4), while by DNA cellulose following DEAE-Sephadex A-50 and phosphocellulose, DNA polymerase lost its affinity to it.

It was also shown that enzyme activity from each chromatography has different properties for template requirement, effect of a monovalent cation, optimal pH, the apparent Km for dTTP etc. Particularly, it is interesting to note that phosphocellulose peak I and II (from crude enzyme fraction) showed difference in properties from phosphocellulose peak III and IV (from DEAE eluate).

One possible explanation of these results may be that DNA polymerase is modified during the experimental procedure.

Another possible explanation is that this DNA polymerase consists of subunits and is dissociated into them by phosphocellulose chromatography.

On the other hand, some investigators reported that only DNA polymerases with high molecular weight exist in primitive eukaryotes such as yeast *Saccharomyces cerevisiae*, green flagellate *Euglena gracilis*.[32~35]

It was reported here that isoelectric point of crude enzyme fraction was pH 5.4. Generally, it is revealed that low molecular weight polymerases have high isoelectric point (9.0–9.4), while high molecular weight enzymes have lower isoelectric points (4.5–5.8).[10,36,37] It follows that DNA polymerase in nuclei from *Physarum polycephalum* may well be a high molecular weight enzyme.

In order to clarify these problems, further studies such as determination of molecular weight for each enzyme fraction are required.

Acknowledgments

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