Purification and characterization of a 100 kDa DNA polymerase from cauliflower inflorescence

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A DNA polymerase from cauliflower (*Brassica oleracea* var. *botrytis*) inflorescence has been purified to near homogeneity through five successive column chromatographies, and temporally designated cauliflower polymerase 1. Cauliflower polymerase 1 is a monopeptide with a molecular mass of 100 kDa. The enzyme efficiently uses synthetic DNA homopolymers and moderately activated DNA and a synthetic RNA homopolymer as template-primers. The enzyme is strongly sensitive to dideoxythymidine triphosphate and *N*-ethyl-

maleimide, but it is insensitive to aphidicolin. It was stimulated with 250 mM KCl. Its mode of DNA synthesis is high-processive with or without proliferating-cell nuclear antigen. A 3' → 5' exonuclease activity is associated with cauliflower polymerase 1. The enzyme is clearly different from cauliflower mitochondrial polymerase and does not resemble the four different types of wheat DNA polymerase, designated wheat DNA polymerases A, B, CI and CII. In the present paper the role of the enzyme in plant DNA synthesis is discussed.

INTRODUCTION

DNA replication, recombination and repair are key processes to maintain the integrity of the genome. DNA polymerases have important roles in these processes and multiple DNA polymerases have been identified and characterized in mammals, yeast and prokaryotes [1–7]. In this regard, the information on DNA polymerases in plants is much less extensive.

Previously, four different types of plant DNA polymerases have been reported, by using wheat embryos, as wheat DNA polymerases A, B, CI and CII [8,9]. The authors propose that wheat polymerase A could correspond to a DNA polymerase α , wheat polymerase B and CII to the δ -like enzyme, and wheat polymerase CI to DNA polymerase β [9]. A plant γ -like DNA polymerase from *Chenopodium album* L has been reported [10]. A probable alternative form of polymerase A has also been reported from maize germinating tissue, pea, spinach and rice [11–14], and a probable alternative form of polymerase CI has been found in shoot tips of rice [15]. In 1979, two different DNA polymerase classes, DNA polymerase A and B, were reported to be present in cauliflower inflorescence [16]. We have previously reported a DNA polymerase in lily [17]. The lily polymerase shows the greater molecular mass, is insensitive to aphidicolin, a replicative polymerase inhibitor, and is quite sensitive to 2,3-dideoxythymidine-5-triphosphate (ddTTP), a specific inhibitor of DNA polymerase β of mammals [17]. The lily DNA polymerase was different from the wheat polymerases A, B and CII and also from wheat polymerase CI. This observation indicates that higher plants have at least five different species of DNA polymerase.

Our primary concern in this report is to search for a new type of DNA polymerase by using cauliflower inflorescence, which has advantages as a material for studying the enzyme. Cauliflower inflorescence can be collected easily all year round, and exhibits hypertrophic differentiation with continuous growth by mitotic division [18]. Secondly, the apical portion of the inflorescence consists of small cells and contains a lot of nuclei [19]. Finally,

the cauliflower has a close relationship to *Arabidopsis thaliana*, which has become a model for plant molecular genetics [20].

In this study, we found a 100 kDa DNA polymerase in cauliflower inflorescence. The study represents the first extensive purification of this new type of DNA polymerase in the cauliflower inflorescence and therefore in higher plants.

MATERIALS AND METHODS

Materials

Nucleotides, calf thymus DNA and chemically synthetic template-primers such as poly(dA)₄₅₀₀ and oligo(dT)_{12–18} were purchased from Pharmacia LKB. [³H]TTP (80 Ci/mmol) was purchased from New England Nuclear Corp., and [α -³²P]dTTP, [α -³²P]dATP, and [γ -³²P]dATP (3000 Ci/mmol) were from Amersham Life Science. The DNA polymerase inhibitors, ddTTP and aphidicolin were obtained from Pharmacia LKB and Sigma respectively. Phosphocellulose P-11 and DE81 filters were from Whatman Bio System, Ltd. Sephacryl S-300 (HiPrep) and DEAE-Sephacryl were purchased from Pharmacia. Klenow fragment was obtained from TaKaRa Ltd. Bradford reagents and molecular-mass markers for SDS/PAGE were from Bio-Rad. Double-stranded DNA (dsDNA)-Sephacryl was made in our laboratory according to the instruction manual of the HiTrap affinity columns (Pharmacia LKB). The calf thymus DNA, used as a template-primer, was activated with pancreatic DNase I according to the method described by Schlabach et al. [21]. Proliferating cell nuclear antigens (PCNAs) from rice and human were isolated by the methods described previously [22]. All other reagents were of analytical grade and were purchased from Sigma or Wako.

DNA polymerase assay

A DNA polymerase assay was performed by methods described in a previous report [23]. The assay mixtures (15 μ l) contained

Abbreviations used: ddTTP, 2,3-dideoxythymidine-5-triphosphate; dsDNA, double-stranded DNA; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; PCNA, proliferating cell nuclear antigen.

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the following: (a) 50 mM Tris/HCl (pH 7.5) containing 1 mM MgCl_2 , 1 mM dithiothreitol (DTT), 40 μM of each of dATP, dCTP, dGTP and dTTP, 10 μCi of [^3H]TTP/ml, 200 $\mu\text{g/ml}$ activated calf thymus DNA and 15% (v/v) glycerol; (b) 50 mM Tris/HCl (pH 7.5) containing 1 mM MgCl_2 , 1 mM DTT, 40 μM dTTP, 10 μCi of [^3H]TTP/ml, 20 $\mu\text{g/ml}$ oligo(dT), 40 $\mu\text{g/ml}$ poly(dA) and 15% (v/v) glycerol. Incubation was carried out at 37 °C for 60 min, and then the reactions were spotted on pieces of DE81 filter-paper. The pieces were washed sequentially four times with 5% (w/v) Na_2HPO_4 , twice with distilled water and then twice with 100% ethanol. After drying, the radioactivity of each piece was measured in a toluene-based scintillator using a Beckman liquid-scintillation counter. One unit (1 unit) of DNA polymerase activity was defined as the amount of enzyme catalysing the incorporation of 1 nmol of dTMP into activated DNA in 60 min.

Activity gel methods

The activity gel method was performed as described by Spanos et al. [24] with some modifications. SDS/PAGE was conducted according to the procedures described by Laemmli [25], except that the sample was incubated with sample buffer at 37 °C for 10 min and the 10% running gel contained 144 $\mu\text{g/ml}$ activated DNA. Renaturation of the enzyme in the gel was achieved by washing the gel sequentially in the following solutions: (1) 50 mM Tris/HCl (pH 7.5) containing 1 mM mercaptoethanol for 60 min at room temperature, and (2) 50 mM Tris/HCl (pH 7.5) at 4 °C overnight. After renaturation, the gel was incubated at 37 °C for 8 h in a reaction mixture that contained 50 mM Tris/HCl (pH 7.5), 1 mM DTT, 75 pM of [$\alpha\text{-}^{32}\text{P}$]dTTP (250 μCi), 12.5 $\mu\text{g/ml}$ dNTP (minus dTTP), 1 mM MgCl_2 and 150 mM KCl. After incubation, the gel was washed, to remove the free dNTPs, with 5% trichloroacetic acid containing 1% sodium pyrophosphate for 60 min at room temperature, and then with the same buffer for 16 h at 4 °C. The gel was dried and exposed to an imaging plate and then the radioactivities were quantified with a Bio-Imaging Analyzer BAS 2000 system (Fuji film).

Measurement of processivity

The processivity of the DNA polymerase reaction was measured by gel electrophoresis of the reaction products. The principal methods have been described previously [26]. The template-primer and the nucleotide triphosphate were replaced with poly(dA)/oligo(dT)₁₆ and 50 μM of [$\alpha\text{-}^{32}\text{P}$]dTTP (10 μCi). After a 30 min polymerase reaction at 37 °C, the products were extracted with phenol–chloroform and precipitated with ethanol. The precipitates were dissolved in the dye mixture (90% formamide/5 mM EDTA/0.1% Bromophenol Blue/0.1% Xylene Cyanol) and heat denatured. Electrophoretic analysis was performed in a 6% polyacrylamide/8 M urea gel, which was then dried and exposed to Fuji RX medical X-ray film. The dried gel was also exposed to the imaging plate and the radioactivities were quantified with the Bio-Imaging Analyzer BAS 2000 system (Fuji film).

Measurement of 3' → 5' exonuclease activity

A 3' → 5' exonuclease activity assay was performed by methods described in a previous report [26]. To make a reaction substrate, a synthetic oligonucleotide (15 mer; 5'-CCC AGT CAC GAC GTC-3') was labelled with [$\gamma\text{-}^{32}\text{P}$]ATP by T4 polynucleotide kinase and was hybridized to M13 mp18 DNA to create an incorrect A template–C primer mispair at the 3'-OH end. After

the polymerase reaction, electrophoretic analysis was performed in a 20% polyacrylamide/8 M urea gel, which was then dried and exposed to Fuji RX medical X-ray film.

Buffer

The TEMG buffer contained the following reagents: 50 mM Tris/HCl (pH 7.5), 1 mM EDTA, 5 mM mercaptoethanol, 0.01% (v/v) Nonidet P-40, 15% (v/v) glycerol and protease inhibitors (1 $\mu\text{g/ml}$ each of leupeptin and pepstatin and 1 μM PMSF).

Preparation of mitochondrial fraction

The mitochondrial fraction was prepared from cauliflower inflorescence according to the method described by Fukasawa and Chou [27] with minor changes. The frozen cauliflower inflorescences were ground in 5 vol. of a grinding buffer [50 mM Tris/HCl (pH 7.5)/1 mM DTT/0.5 M sucrose/1 mM EDTA/1 $\mu\text{g/ml}$ each of leupeptin and pepstatin/1 μM PMSF] by a mortar and pestle and then homogenized by a Teflon homogenizer. The homogenized material was filtered with two layers of nylon mesh (30 μm) and the filtrate was centrifuged at 8000 g for 20 min. The sediment was suspended in the grinding buffer and then centrifuged at 500 g for 20 min. The supernatant was centrifuged at 7000 g for 20 min and the sediment obtained was suspended in TEMG buffer and used as a mitochondrial fraction.

Enzyme purification

A DNA polymerase was purified from cauliflower (*Brassica oleracea* var. *botrytis*) inflorescence using the protocol described below. All procedures were carried out at 4 °C.

Step 1. Approx. 100 g of the frozen cauliflower inflorescence tissue was collected and suspended in a small volume of TEMG buffer containing 0.45 M $(\text{NH}_4)_2\text{SO}_4$ and 0.4 M KCl and ground into a paste using a mortar and pestle. The paste was then suspended in 100 ml of TEMG buffer containing 0.45 M $(\text{NH}_4)_2\text{SO}_4$ /0.4 M KCl, ground through a French press and sonicated (20 kHz, 1 min). The supernatant after centrifugation at 12300 g for 10 min was saturated with 70% ammonium sulphate. The ammonium sulphate precipitate was collected by centrifugation. The pellet was resuspended in 70 ml of TEMG buffer, then dialysed against TEMG buffer.

Step 2. The dialysed fraction from step 1 was loaded onto a DEAE-Sepharose column equilibrated with TEMG buffer containing 0.1 M KCl. The column was washed with a sufficient amount of the same buffer and fractions were collected with a 1200 ml linear gradient of KCl from 0.1 to 0.4 M in TEMG buffer. Three DNA polymerase peaks occurred at 0.12 M, 0.2 M and 0.35 M KCl respectively. The major peak was at 0.2 M KCl, and we set out to purify it in this report. The 0.2 M KCl peak was collected and dialysed against the TEMG buffer containing 0.1 M KCl.

Step 3. The fraction from the DEAE-Sepharose column was loaded onto a phosphocellulose column equilibrated with TEMG buffer containing 0.1 M KCl, washed with 1 litre of the same buffer and then eluted with an 800 ml linear gradient of KCl from 0.1 M to 0.8 M in the same buffer. A DNA polymerase activity was eluted at 0.25 M KCl. The active fractions were combined and dialysed against the TEMG buffer.

Step 4. The combined fractions from phosphocellulose column chromatography were loaded onto a heparin-Sepharose column equilibrated with TEMG buffer and then washed with the same buffer. The fraction was collected with a 60 ml linear gradient of NaCl from 0 to 1 M in TEMG buffer. The DNA polymerase

activity was separated into two peaks at 0.2 M and 0.55 M NaCl respectively. The active fractions eluted at 0.55 M NaCl were collected and were processed for the next step.

Step 5. The combined active fractions eluted from the heparin–Sephacryl S-300 (Hiprep) gel-filtration column equilibrated with TEMG buffer containing 0.2 M NaCl. In the column chromatography, one active peak was found. The active fractions were combined and dialysed against the TEMG buffer.

Step 6. The dialysed fraction in step 5 was loaded onto a dsDNA–Sephacryl (HiTrap) column equilibrated with TEMG buffer. The column was washed with 10 ml of the same buffer and then the proteins were eluted with a 20 ml linear gradient of NaCl from 0 to 0.8 M NaCl in TEMG buffer. The combined active fraction eluted at 0.45 M was dialysed against TEMG buffer. The DNA polymerase fraction from the dsDNA–Sephacryl column was, unless otherwise noted, used as the purified fraction in the following experiments.

Other methods

SDS/PAGE was performed with a 10% gel using the standard Laemmli methods [25]. After electrophoresis, the gel was treated by silver staining as described by Wray et al. [28]. The protein concentration was determined according to the method of Bradford [29] using the protein assay (Bio-Rad) with γ -globulin as the standard.

RESULTS

Purification of the DNA polymerase

A protein with DNA polymerase activity was purified from cauliflower inflorescence as described in the Materials and methods section. In the purification process, we found that the cauliflower inflorescence has at least four different species of DNA polymerase: two peaks at 0.12 M and 0.35 M KCl respectively in the DEAE–Sephacryl chromatography and two peaks at 0.2 M and 0.55 M KCl respectively in heparin–Sephacryl column chromatography (see Materials and methods section). The DNA polymerase activity eluted at 0.55 M KCl in heparin–Sephacryl column chromatography was temporarily designated cauliflower polymerase 1. The remainder of this report is devoted to purification and characterization of the cauliflower polymerase 1. The typical result of purification is summarized in Table 1. The activity value of the cauliflower polymerase 1 before phosphocellulose column chromatography could not be measured precisely because the fractions contained several kinds of DNA polymerase activities. The active fractions from the dsDNA–Sephacryl column chromatography, after

Figure 1 SDS/PAGE analysis of the purified enzyme

The active fractions from dsDNA–Sephacryl column chromatography were combined and analysed by SDS/PAGE. The concentration of the polyacrylamide gel was 10% and the gel was stained with silver nitrate after the electrophoresis.

Figure 2 Activity gel analysis

E. coli Klenow fragment (lane 1) and the purified cauliflower polymerase 1 (lane 2) were subjected to an activity gel system as described in the Materials and methods section.

dialysis, was used in the following experiments because the fraction was sufficiently pure, as described below. Further purification and characterization of the other polymerase activities in the cauliflower inflorescence are in progress.

Table 1 Purification of cauliflower polymerase 1

ND, not determined.

Fraction	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)
Crude extract	175	4908	ND	ND
Ammonium sulphate	70	3391	ND	ND
DEAE–Sephacryl	240	1051	ND	ND
Phosphocellulose	125	24.1	ND	ND
Heparin–Sephacryl	12	6.94	244	35
Sephacryl S-300	12	1.75	239	137
dsDNA–Sephacryl	2	0.05	82	1640

Homogeneity, molecular-mass determination and general enzymic properties of the DNA polymerase

In the Sephacryl S-300 gel filtration, the activity of cauliflower polymerase 1 appeared at a position which was calculated to be 100 kDa (results not shown). The SDS/PAGE of the dsDNA–Sephacryl revealed a large polypeptide band of 100 kDa (Figure 1). The 100 kDa polypeptide was shown to have DNA polymerase activity by activity gel electrophoresis (Figure 2, lane 2). *Escherichia coli* Klenow fragment was used as a protein size marker. These results indicate that cauliflower polymerase 1 is a monopeptide with a molecular mass of 100 kDa, and the monopeptide contains the template-primer-binding domain and is the catalytic subunit of the polymerase. The pH optimum for cauliflower polymerase 1 was 7.0, with about 50% of the

Figure 3 Dependence of cauliflower polymerase on monovalent cations

The polymerase activity was assayed under standard conditions in the presence of the indicated concentrations of NaCl (●) and KCl (□). Activated DNA was used as the template-primer.

Table 2 Template-primer specificity of cauliflower polymerase 1

Cauliflower polymerase 1 activity in the presence of activated DNA and Mg^{2+} is expressed as 100%.

Template/primer	Polymerase activity (%)	
	Mg^{2+}	Mn^{2+}
Activated DNA	100	8
Poly(dA)/oligo(dT) ₁₂₋₁₈	266	118
Poly(rA)/oligo(dT) ₁₂₋₁₈	30	82
Poly(dA-dT)	28	6
Poly(dT)/oligo(dA) ₁₂₋₁₈	270	220
Poly(dC)/oligo(dG) ₁₂₋₁₈	340	130
Poly(rC)/oligo(dG) ₁₂₋₁₈	8	10
Poly(IC)	4	2

optimum activity being expressed at either pH 6.0 or 8.0 (results not shown). The temperature optimum was 37 °C and the activity was completely lost at 50 °C (results not shown). The enzyme required Mg^{2+} ions at a concentration of 1 mM or more, and the Mg^{2+} ions could be replaced with Mn^{2+} ions at 0.5 mM (results not shown). Monovalent cations such as KCl or NaCl stimulated the polymerase activity at the optimum concentration of 250 mM or 200 mM respectively (Figure 3). The structure of the template, activated DNA or poly(dA)/oligo(dT)₁₆, did not influence the ionic-strength effect.

Template-primer specificity and effect of inhibitors of the DNA polymerase

As is well known, each of the five mammalian DNA polymerases has a special template-primer preference. Cauliflower polymerase 1 prefers homopolymer, poly(dC)/oligo(dG)₁₂₋₁₈ and poly(dA)/oligo(dT)₁₆ as a template-primer (Table 2). We could not detect a large difference between 5'-OH-oligo(dT)- or 5'-OH-oligo(dG)- and 5'-PO₄-oligo(dT)- or 5'-PO₄-oligo(dG)-dependent activities. The activated DNA was also used by this polymerase 1 (Table 2). Poly (rA)/oligo(dT)₁₂₋₁₈, a possible substrate of DNA polymerase β -class or DNA polymerase γ -class of mammals could also be used, although not efficiently (Table 2). Poly(dA/dT) and poly(rC)/oligo(dG)₁₂₋₁₈ were poor template-primers for the enzyme (Table 2).

Figure 4 Effect of polymerase inhibitor on cauliflower polymerase 1 activity

Inhibition of cauliflower polymerase 1 (A, C and E) and cauliflower mitochondrial polymerase (B, D and F) by aphidicolin (A and B), NEM (C and E) and ddTTP (E and F). Standard polymerase assays using activated DNA (●) and poly (dA)/oligo(dT) (□) as the template-primer were performed in the presence of the indicated amounts of each inhibitor.

Next, we tested the effects of typical DNA polymerase inhibitors on cauliflower polymerase 1 (Figures 4A, 4C and 4E) in comparison with cauliflower mitochondrial polymerase (probably cauliflower pol γ) (Figures 4B, 4D and 4F). The cauliflower mitochondrial polymerase was prepared as described in the Materials and methods section. ddTTP, an inhibitor of DNA polymerase β of mammals and wheat DNA polymerases A and CI, inhibits cauliflower polymerase 1 activity, even at a ddTTP/TTP ratio of 20:1 (Figure 4E), but not the cauliflower mitochondrial polymerase activity (Figure 4F). Aphidicolin, an inhibitor of DNA polymerases α , δ and ϵ of mammals and wheat DNA polymerases B and CII, did not inhibit this polymerase activity (Figure 4A). Cauliflower mitochondrial polymerase was moderately inhibited by aphidicolin (Figure 4B). On the other hand, *N*-ethylmaleimide (NEM), an inhibitor of polymerases α , γ , δ and ϵ of mammals, the lily meiotic DNA polymerase (β -like) and all of the wheat DNA polymerases, potentially influenced the cauliflower mitochondrial polymerase activity (Figure 4D), and, only when activated DNA was used as the template-primer, also the cauliflower polymerase 1 activity (Figure 4C). NEM has

Figure 5 Processivity of cauliflower polymerase 1

(A) Time-course of reaction. The reactions were assayed under standard conditions and incubated for 2 min, 5 min and 10 min (lanes 1–3 respectively). (B) Effect of a monovalent cation on the processivity. The reactions were performed in the presence of 0, 100, 200 and 300 mM KCl (lanes 1–4 respectively). (C) Effect of a bivalent cation on the processivity. The reactions were performed in the presence of 0, 1, 5 and 10 mM $MgCl_2$ (lanes 1–4 respectively). (D) Effect in rice of human PCNA on processivity. Lane 1, control (no PCNA); lane 2, 0.08 $\mu g/\mu l$ rice PCNA–maltose-binding protein fusion protein; and lane 3, 0.018 $\mu g/\mu l$ human PCNA. In these reactions, the mixture contained poly (dA)₄₅₀₀/oligo(dT)₁₆ as a template-primer.

almost no inhibitory effect on cauliflower polymerase 1 if the template-primer is poly(dA)/oligo(dT)_{12–18}. Since the assay conditions used were the same as those in the original experiments, the reason why NEM potentially influenced the activity only with activated DNA is unclear. These characteristics indicate that the cauliflower polymerase 1 is not the cauliflower mitochondrial polymerase.

Processivity of the DNA polymerase

To determine whether cauliflower polymerase 1 replicates DNA processively or distributively, the reaction products were analysed using poly(dA)₄₅₀₀/oligo(dT)₁₆ as a template and denaturing PAGE. Cauliflower polymerase 1 appears to have a high processive ability (Figure 5A). We also investigated the replication mode in relation to the KCl effect. Figure 3 and Figure 5(B) show the dose-dependent curve of the KCl effect on poly(dA)/oligo(dT)₁₆-dependent DNA polymerase activity and on the replication mode respectively. Increasing ionic strength, although the polymerase activity increased at 250 mM KCl or below (Figure 3), decreased the processivity (Figure 5B), suggesting that KCl ions enhance the accessibility to the template-primer, but lower the elongation activity. Cauliflower polymerase 1 was most highly processive at 100 mM KCl where the enzyme is less active, and is moderately processive or distributive at

Figure 6 The 3' → 5' exonuclease activity of cauliflower polymerase 1

(A) Elution profile of heparin–Sepharose column chromatography. Activated DNA was used as a template-primer. (B) 3' → 5' Exonuclease activity of cauliflower polymerase 1. The numbers indicate the fraction numbers on the heparin–Sepharose column chromatography shown in (A).

200–300 mM KCl where it is most active (Figure 3 and Figure 5B). The processivities of plant DNA polymerases were studied only in the enzymes from wheat [9]. DNA polymerase A was a highly processive enzyme [9], and DNA polymerases B and CII could processively replicate DNA in addition to PCNA [9]. The processivity of cauliflower polymerase 1 was not changed by adding PCNA from rice or human (Figure 5D), indicating that it is not of the wheat DNA polymerase B or CII class. In the absence of KCl, the processivity of cauliflower polymerase 1 was also not changed by adding the PCNA (results not shown). In mammals or yeast, both the activity and processivity of DNA polymerase δ are PCNA dependent, and DNA polymerase ϵ is PCNA independent but requires PCNA when monovalent cations are present in the reaction mixture *in vitro* [30]. The presence of rice or human PCNA did not affect the processivity of the cauliflower polymerase 1 with or without KCl, suggesting that cauliflower polymerase 1 is also not of the mammalian DNA polymerase δ or ϵ class. The processivity of cauliflower polymerase 1 was also slightly affected with bivalent cations, Mg^{2+} ions (Figure 5C). Increasing the bivalent cation concentration led to a decrease both in the activity and the processivity to the same extent (Figure 5C). The results in this section suggest that as far as the processivity is concerned, cauliflower polymerase 1 seems to resemble wheat DNA polymerase A.

3' → 5' Exonuclease activity of the DNA polymerase

DNA polymerases γ , δ and ϵ , and wheat DNA polymerase B, corresponding to DNA polymerase δ , have a 3' → 5' exonuclease

activity for proofreading DNA, but wheat DNA polymerase A, corresponding to DNA polymerase α , does not have this activity and neither do wheat DNA polymerases CI and CII [31]. Such DNA polymerases require the DNA proofreading activity to repair DNA mismatch in the DNA polymerization. To determine whether cauliflower polymerase 1 has a 3' $\rightarrow$ 5' exonuclease activity, we performed a 3' $\rightarrow$ 5' exonuclease assay with mismatched base-paired termini on the fractions of heparin-Sepharose column chromatography. As described in the Materials and methods section, for the 3' $\rightarrow$ 5' exonuclease activity assay, a synthetic oligonucleotide (15 mer; 5'-CCC AGT CAC GAC GTC-3') was labelled with [γ - 32 P]ATP by T4 polynucleotide kinase and was hybridized to M13 mp18 DNA to create a deliberately incorrect A template-C primer mispair at the 3'-OH end, and the products were analysed by PAGE using a 20% polyacrylamide gel and 8 M urea.

As shown in Figure 6(B), the 3' $\rightarrow$ 5' exonuclease activity on the elution profile of a heparin-Sepharose column (Figure 6A), coincided well with the cauliflower polymerase 1 activity peak, suggesting that cauliflower polymerase 1 may have the mismatched DNA proofreading function. The fraction numbers in Figure 6(B) indicate those of the heparin-Sepharose column in Figure 6(A). Since the enzyme in the final dsDNA column was very unstable, we used the active fractions from the heparin-Sepharose column for the exonuclease assay. The enzyme in the dsDNA column also showed the exonuclease activity, although the band signal was weak (results not shown). Thus, cauliflower polymerase 1 appears to be neither a cauliflower cognate of wheat DNA polymerase A nor of any of the other wheat polymerases reported.

DISCUSSION

This report represents the first extensive purification of the DNA polymerase from cauliflower inflorescence. As described in the Introduction, several polymerase classes have been reported from higher plant species as follows: the DNA polymerase from lily [17], the Yamaguchi cauliflower DNA polymerases A and B [16] and four different types of DNA polymerases in wheat embryos, i.e. wheat DNA polymerases A, B, CI and CII [9]. Lily DNA polymerase and Yamaguchi cauliflower DNA polymerase B are thought to correspond to a β -like enzyme. The Yamaguchi cauliflower DNA polymerase A, although partially degraded (H. Seto, M. Hatanaka, S. Kimura and K. Sakaguchi, unpublished work) might be an alternative form of wheat DNA polymerase A. The wheat DNA polymerases A, B, CI and CII may be DNA polymerase α , the δ -like enzyme, the β -like enzyme and another δ -like enzyme respectively. Probable alternative forms of wheat polymerase A have also been reported from maize germinating tissue, pea, spinach and rice [11–14], and a probable alternative form of the polymerase CI has been obtained from shoot tips of rice [15]. Cauliflower polymerase 1 as reported here is probably an enzyme corresponding to the Yamaguchi cauliflower DNA polymerase B (75 kDa enzyme), although it is thought that the Yamaguchi polymerase was partially degraded.

Cauliflower polymerase 1 was purified as a 100 kDa enzyme which is composed of a monopolyptide. This enzyme requires strong monovalent ionic strength at more than 200 mM. It prefers poly(dC)/oligo(dG)₁₆ and poly(dA)/oligo(dT)₁₆ as a template-primer rather than activated DNA, and can moderately utilize synthetic RNA homopolymers such as poly(rA)/oligo(dT)₁₆. It is sensitive to ddTTP and NEM, but insensitive to aphidicolin. Its mode of DNA synthesis is processive, irrespective

of the presence of PCNA. Interestingly, the enzyme has a 3' $\rightarrow$ 5' exonuclease activity.

Cauliflower polymerase 1 appeared to be relatively similar to wheat DNA polymerase A (an α -like replicative enzyme) or lily DNA polymerase (a kind of β -class repair enzyme), but wheat DNA polymerase A lacked the 3' $\rightarrow$ 5' exonuclease activity of cauliflower polymerase 1. These characteristics suggest that cauliflower polymerase 1 does not belong to any of the DNA polymerases of wheat, the study of which, among higher plants, has progressed furthest to date, and does not resemble any of the mammalian, *Drosophila* or yeast DNA polymerase species taxonomically [32]. Since cauliflower polymerase 1 is a monopolyptide polymerase which requires extremely high-salt conditions for the assay, it may be much closer to the lily DNA polymerase than to the wheat replicative polymerase.

Our isolation and characterization of cauliflower polymerase 1 places us in a position to apply molecular tools to an analysis of that enzyme's function, and moreover, the DNA polymerase system in higher plants. Mass isolation makes cloning of the gene easily possible. The possibility of producing structural gene mutations following gene cloning holds the potential for more precise definition of the DNA polymerase function.

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