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PURIFICATION OF DNA POLYMERASE α-PRIMASE FROM CHERRY SALMON, *ONCORHYNCHUS MASOU* TESTES, USING SPECIFIC AFFINITY COLUMN CHROMATOGRAPHY

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Shunji Izuta and Mineo Saneyoshi (1990) Purification of DNA polymerase α-primase from cherry salmon, *Oncorhynchus masou* testes, using specific affinity column chromatograph. *Bull. Inst. Zool., Academia Sinica* 29 (3, Supplement): 87-94. For the specific purification of DNA-dependent DNA polymerase α-primase, we applied newly prepared affinity ligand bearing 5-(E)-(4-aminostyryl) ara UTP.

Ara UTP resin with spacer (Ara UTP-Affigel 10) was selected to our purpose, because, no DNA polymerase α-primase activity from developing cherry salmon, *Oncorhynchus masou*, testes was bound to affinity ligand with ara UTP without spacer. Selected ara UTP Affigel-10 retains this enzyme activity when poly (dA) or poly (dA)-oligo $(dT)_{12\text{-}18}$ is present. The retained enzyme activity was sharply eluted around 100 mM KCl concentrations as a single peak, and this fraction showed a specific activity of about 170,000 units/mg protein as α-polymerase activity. The highly purified DNA polymerase α-primase isolated using the ara UTP-Affigel 10 contained only three polypeptides, which showed Mr values of 120,000, 62,000 and 58,000, respectively, as judged using sodium dodecyl sulfate-polyacrylamide electrophoresis.

Key words: DNA polymerase α-primase, Cherry salmon, *Oncorhynchus masou*, Affinity chromatography, 5-styryl ara UTP.

Recently, DNA primase activity was found to tightly associate with DNA polymerase α and this enzyme complex has been purified from various sources and characterized (Campbel 1986; Roth, 1987). It is very important to clarify the functions of the polypeptide which corresponds to DNA polymerase α or DNA primase activity. However, there are few reports of success in purifying this enzyme to near homogeneity. One of the reasons causing difficulty in the purification is that DNA polymerase α forms a multiprotein complex (Vishwanatha et al., 1987), and that an appropriate adsorbent which could retain DNA polymerase α strongly and specifically has not been found yet. Immuno-affinity adsorbents which coupled the monoclonal antibody against

DNA polymerase α as a ligand have been developed and used for the specific purification of this enzyme (Hirose et al., 1985; Holmes et al, 1986; Wong et al., 1986), but it is also possible to bind similarly the inactive peptide which would be yielded by degradation of active form.

On the other hand, various affinity adsorbents which contain certain nucleotide analogues have been developed for some enzymes related to nucleic acids biosynthesis or modifications (Ikeda et al., 1984, Rupprecht et al., 1981, Webb et al., 1984).

From this context, we have been attempting to develop a novel affinity adsorbent which contains a strong and selective inhibitor for DNA polymerase α as a ligand. A resin such as this could retain only active polypeptides. Among some candidates of ligand, aphidicolin, which is widely known to strongly and selectively inhibit DNA polymerase α could be considered, however, it is reported that aphidicolin analogs which had modified hydroxyl groups had remarkably reduced affinity for this enzyme (Hiranuma et al., 1987). We have previously found that ara UTP analogues bearing the hydrophobic and steric styryl group at the 5-position showed strong and selective inhibitory effects on DNA polymerase α from cherry salmon, *Oncorhynchus masou*, testes (Izuta and Saneyoshi, 1984, 1987). We had chosen 5-(E)-(4-aminostyryl) are UTP (Izuta and Saneyoshi, 1987) as a ligand and prepared two types of resins. After checking their abilities on affinity of this resin to DNA polymerases from cherry salmon testes, it was reported in preceding paper (Izuta and Saneyoshi, 1988). The utilization of the resin thus obtained for specific purification of DNA polymerase α-primase from cherry salmon are described in this report.

MATERIALS AND METHODS

Chemicals

Ara UTP-Affigel 10 was prepared as reported previously (Izuta and Saneyoshi, 1988). [^{3}H] dTTP and [^{3}HdGTP were obtained from NEN Research Products, unlabelled nucleotides from Yamasa Shoyu Co. Ltd., poly(dA), poly(dC) and oligo(dT)$_{12\text{-}18}$ from Pharmacia P-L Biochemicals, Sepharose 4B from Pharmacia Fine Chemicals, and other reagents used here were analytical grade.

Partially purified DNA polymerase α-primase (second phosphocellulose fraction): DNA polymerase α-priase of cherry salmon (*O. masou*) testes was partially purified as described previously (Izuta et al., 1985). DNA polymerase α-primase activity extracted from cherry salmon testes was purified by means of sequential column chromatography of DEAE-Sephadex, phosphocellulose, DEAE-cellulose, and second phosphocellulose, and 55% ammonium sulfate fractionation as summarized in Table 2. The second phosphocellulose fraction was used for the experiment of the ara UTP-Affigel 10 chromatography. In the case of conventional purification, the second phosphocellulose fraction was further purified with column chromatographies of hydroxylapatite and single strand DNA cellulose instead of ara UTP-Affigel 10.

Assay of DNA polymerase α and primase activity

DNA polymerase α activity was measured in 25 μl of reaction mixture

containing 50 mM Tris-HCl (pH 8.0), 100μg/ml activated salmon sperm DNA, 4 mM $MgCl_2$, 100 μM each of dATP, dGTP, dCTP and [^{3}H]dTTP (500 cpm/pmole), 1 mM dithiothreitol, 400 μg/ml bovine serum albumin, 15% glycerol and enzyme solution. The mixture was incubated at 37°C for 10-20 min. The reaction was stopped by chilling, and 20 μl of the reaction mixture was transferred to DEAE-cellulose paper disks (Whatman DE-81). The disks were washed six times with 5% Na_2HPO_4, twice with ethanol and with ether, and dried. The remaining radioactivity was measured in Packard liquid scintillation counter with toluene scientillator. One unit of activity was defined to be the amount of enzyme which catalyzes the incorporation of 1 nM of dNMP into DNA at 37°C per hour. DNA primase activity was determined as primer RNA synthesis-dependent DNA synthesis with 25 μl of reaction mixture containing 50 mM Tris-HCl (pH 8.0), 100 μg/ml poly(dC), 4 mM $MgCl_2$, 1 mM GTP, 100 μM [^{3}H]dGTP (500 cpm/pmole), 1 mM dithiothritol, 400 μg/ml bovine serum albumin, 15% glycerol and enzyme. Incubation was performed at 30°C for 10-20 min. The following treatments were similar to the above experiments. One unit of primase activity was defined to be the amount of enzyme which catalyzes the incorporation of dGMP into poly(dC) at 30°C per hour.

SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (1970), with 10% separation gel and 5% condensation gel. The electrophoresis was performed with 20 mA of constant current for 2 h. The gel was fixed and silver stained with the silver staining kit "Daiichi" (Daiichikagaku, Japan).

Preparation of ara UTP-Affigel 10

This affinity ligand was prepared by the method described previously (Izuta and Saneyoshi, 1988).

Binding ability of the enzyme on the ara UTP-Affigel 10

Two hundred units (as DNA polymerase α activity) of DNA polymerase α-primase (second phosphocellulose fraction) in 500 μl of Tris-HCl buffer (pH 8.0) containing 5 mM $MgCl_2$, 1 mM EDTA, 1 mM β-mercaptoethanol, 10% glycerol, 10 mM KCl and several templates or template-primers were added to 0.1 ml settled volume of the ara UTP-Sepharose (Izuta and Saneyoshi, 1988) or the ara UTP-Affigel 10 and gently stirred for 30 min at 4°C. The mixture was settled for 30 min, and the unbound activity in the supernatant was measured. The same resin without a ligand was used as control. Based on these results, the amount of enzyme bound to the resin was determined.

Chromatography of DNA polymerase α-primase on the ara UTP-Affigel 10

To 2900 units (as DNA polymerase α activity) of the enzyme (second phosphocellulose fraction) in 2 ml of Tris-HCl buffer (pH 8.0) containing 5 mM $MgCl_2$, 1 mM EDTA, 1 mM β-mercaptoethanol, 10% glycerol and 10 mM KCl was added poly(dA) (final concentration was 100 μg/ml) and 1 ml settled volume of the ara UTP-Affigel 10, and the mixture was gently stirred for 30 min at 4°C. The resin was packed in a column (0.7 x 3cm),

and washed with 30 ml of the same buffer, followed by elution with a linear gradient of KCl from 10 mM (30 ml) to 400 mM (30 ml) in the same buffer.

RESULTS

Binding abilities of DNA polymerase α to resin

We first examined the binding abilities of DNA polymerase α-primase partially purified from cherry salmon testes on the ara UTP-Sepharose (without spacer) or ara UTP-Affigel 10. Since it has been reported that DNA polymerase α could bind to the substrates after binding with the template or template-primer, several conditions without template or with template-primer were tested. The ara UTP-Sepharose 4B has not retained DNA polymerase α-primase activity in all cases examined. On the other hand, the ara UTP Affigel 10 retained this enzyme when poly(dA-oligodT$_{12\text{-}18}$ (4:1)) or poly(dA) was present. At about 100 μg/ml poly(dA), DNA polymerase α was mostly bound to this resin. Under these conditions without template or with Affigel 10 was calculated to about 1,600 units of DNA polymerase α bound to 1 m ml of settled resin.

Chromatography of DNA polymerase α-primase on the ara UTP-Affigel 10

The bound DNA polymerase α-primase on the ara UTP-Affigel 10 was eluted by the linear gradient of KCl from 10 to 400 mM. The elution profile is very sharp. The enzyme was eluted from this resin around 100 mM KCl concentration as a single peak, and separation of DNA polymerase α activity and primase activity

Table 1. Binding abilities of DNA polymerase α-primase on ara UTP-Sepharose 4B and ara UTP-Affigel 10

	Ara UTP-Sepharose 4B			Ara UTP-Affigel 10		
Condition	Unbound[1] (units)	Bound[2] (units)	Capacity (units/ml resin)	Unbound (units)	Bound (units)	Capacity (units/ml resin)
None	185	15	150	190	10	100
+ 100 μg/ml Activated DNA	100 200	0	0	226	0	0
+ 100 μg/ml poly (dA)	203	0	0	44	156	1560
+ 80 μg/ml poly (dA) 20 μg/ml oligodT$_{12\text{-}18}$	188	12	120	30	170	1700

1. Two hundred units of DNA polymerase α and 0.1 ml of settled resin were used.
2. Determined from the amounts of unbound enzymes.

was not seen. The ultraviolet absorption of this fraction showed a λmax at 260 nm. The results of total purification steps are summarized in Table 2. The final sample showed a specific activity of about 170,000 units/mg protein, as α-polymerase.

Analysis of the polypeptides contained in the final fraction

The polypeptide composition of DNA polymerase α-primase purified by newly developed affinity resin was analyzed with 10% polyacrylamide gel containing 0.1% SDS. The result shows only three distinct bands were detected which correspond with Mr values of 120,000, 62,000, and 58,000, respectively. On the other hand, in the case of conventionally purified sample (single-stranded DNA-cellulose fraction), many protein bands appeared.

DISCUSSION

We compared their ability of two types of ara UTP resin, the ara UTP-Sepharose 4B and the ara UTP-Affigel 10; the latter has a spacer between the matrix and the ligand. As shown in Table 1, the ara UTP-Sepharose 4B (without spacer) did not retain DNA polymerase α-primase in any of the cases examined, however, the ara UTP-Affigel 10 could retain this enzyme, indicating that some distance between the matrix and the ligand is necessary for recognition of the nucleotide inhibitor on the matrix by the enzyme. Among some templates or template-primer, poly(dA)-oligo dT_{12-18} and poly(dA) were effective for this binding, but activated DNA has not been found to be effective so far. Thus, the ability to recognize only dTTP for DNA polymerase α would be needed for the binding. The capacity was changed by the concentration of poly(dA) and the highest capacity was seen when the concentration of poly(dA) was 100 μg/ml. This template concentration would be suitable for DNA polymerase α to recognize the ligand.

The bound DNA polymerase α-primase activity was eluted sharply from this resin

Table. 2. Purification of DNA polymerase α-primase from cherry salmon testes

Step	Protein (mg)	Activity: Pol. α (units)	Activity: Primase (units)	Specific Activity: Pol. α (units/mg protein)	Specific Activity: Primase (units/mg protein)
1. 0.5 M KCl extract	200	20,000	9,300	100	47
2. DEAE-Sephadex	180	22,300	10,400	120	58
3. First phosphocellulose	23	14,800	11,200	640	490
4. 55% $(NH_4)_2SO_4$ ppt	6.6	5,300	5,560	800	840
5. DEAE-cellulose	1.4	3,900	1,600	2,790	1,140
6. 2nd phosphocellulose	0.47	2,900	1,060	6,170	2,260
7. Ara UTP-Affigel 10	0.015	2,600	830	173,000	55,300

as a single peak, and the separation of DNA polymerase α activity and DNA primase activity was not determined. The ultraviolet absorption of this fraction showed a λmax at 260 nm, suggesting that poly (dA) was coeluted with this enzyme. It would form an enzyme-poly(dA)-ara UTP analogue complex on the resin.

With this affinity adsorbent, we could obtain highly purified enzyme which showed a specific activity of about 170,000 units/mg protein as α-polymerase. This specific activity was higher than that of the conventionally purified sample, which was about 110,000 units/mg protein (Izuta et al., 1985). And the recovery of enzyme activity before and after the column chromatography was very satisfactory.

The analysis with SDS-polyacrylamide gel electrophoresis revealed that final enzyme preparation contained only three polypeptides which showed Mr values of 120,000, 62,000 and 58,000, respectively, whereas the conventionally purified sample contained a lot of proteins. Thus it appears that this newly developed affinity resin, ara UTP-Affigel 10, can separate DNA polymerase α-primase complex from cherry salmon, *Oncorhynchus masou*, testes, with many impure proteins, which are not removed by several resins such as single-stranded DNA-cellulose.

Since there are no appropriate adsorbents for DNA polymerase α, the resins which contain a strong ibhibitor of this enzyme as ligand, such as reported here, could be very useful tools for the purification of this enzyme from various sources. And we also found that 5-(E)-styryl ara UTP inhibited herpes virus induced DNA polymerases (Suzuki et al., 1987), and this resin should be applicable for purification of this enzymes.

Finally, Fisher and Korn (1981) reported that DNA polymerase α would bind first to a template, second to a primer and finally to the substrates of dNTPs, but our results suggest that a primer is not necessary for α-polymerase to recognize the substrates of the substrate analogues. Like this, the ara UTP-Affigel 10 would be applicable to not only purification but several characterizations of DNA polymerase α from various animals.

REFERENCES

Campbell, J. L. (1986) Eukaryotic DNA replication. *Ann. Rev. Biochem.* 55: 733-77.

Fisher, P.A. and D. Korn (1981) Ordered sequen-mechanism of substrate recognition and binding by KB cell DNA polymerase α. *Biochemistry* 20: 4560-4569.

Holmes, A. M., E. Cheriathundam, E. J. Bollum and L. M. S. Chang (1986) Immunologiccal analysis of the polypeptide structure of calf thymus DNA polymerase-primase complex. *J. Biol. Chem.* 261: 11,924-11,930.

Hiranuma, S., T. Shimizu, H. Yohioka, K. Ono, H. Nakane and T. Takahashi (1987) Chemical modification of aphidicolin and the inhibitory effects of its derivatives on DNA polymerase α *in vitro*. *Chem. Pharm. Bull.* 35: 1641-1644.

Hirose, F., P. Kedar, T. Takahashi and A. Matsukage (1985) Monoclonal antibody specific for chicken DNA polymerase α associated with DNA primase. *Biochem. Biophys. Res. Commun.* 132: 210-216.

Izuta, S., and M. Saneyoshi (1984) Inhibitory effects of 1-β-D-arabinofuranosyl-(E)-5-styryluracil 5′-triphosphates on DNA-d-pendent DNA polymerase α and β from developing cherry salmon, *Oncorhynchus masou*, testes.: On the approach to the selective affinity chromatography for DNA polymerase α. *Nucleic Acids Res. Sym. Ser.* 15:

73-77.

Izuta, S., M. Kohsaka and M. Saneyoshi (1985) Inhibitory effects of various 3′-deoxyribonucleotides on DNA polymerase α_2-primase from developing cherry salmon, *Oncorhynchus masou*, testes. *Nucleic Acids Res. Sym. Ser.* 16: 241-244.

Izuta, S., and M. Saneyoshi (1987) Selective inhibition of DNA polymerase α by 1-β-D-arabiinofuranosyl-5-styryluracil 5′-triphosphates and related nucleotides: Influence of hydrophobic and steric factors on inhibitory action. *Chem. Pharm. Bull.* 36: 1889-1901.

Izuta, S., and *M. Saneyoshi* (1988) Ara UTP-Affigel 10: A novel affinity adsorbent for the specific purification of DNA polymerase α-primase. *Anal. Biochem.* 174: 318-324.

Ikeda, S., I. Park, P. Gardner and D. H. Ives (1984) New affinity adsorbent containing deoxcytidine, deoxyadenosine, or deoxyguanosine and their interaction with deoxynucleoside metabolizing enzymes. *Biochemistry* 23: 1914-1921.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (London)* 227: 680-685.

Roth, Y. F. (1987) Eucaryotic primase, *Eur. J. Biochem.* 165: 473-481.

Rupprecht, K. M., N. Sonenberg, A. J. Shatkin and S. M. Hecht (1981) Design and preparation of affinity columns for the purification of eukaryotic messenger ribonucleic acid cap protein. *Biochemistry* 20: 6570-6576.

Suzuki, S., S. Izuta, C. Nakayama, and M. Saneyoshi (1987) Inhibitory effects of 5-alkyl- and 5-alkenyl-1-β-D-arabinofuranosyluracil 5′-triphosphates on herpesvirus-induced DNA polymerase. *J. Biochem.* (Tokyo) 102: 853-857.

Vishwanatha, J. K., S. A. Coughlin, M. Wesolowski-Owen and E. F. Baril (1986) A multiprotein form of DNA polymerase α from calf thymus. *J. Biol. Chem.* 261: 6619-6628.

Webb, N. R., R. V. J. Chari, G. DePhilis, J. W. Kozarich and R. E. Roads (1984) Purification of the messenger RNA cap-binding protein using a new affinity medium. *Biochemistry* 23: 177-181.

Wong, S. W., L. R. Paborsky, P. A. Fisher, T. S. F. Wong and D. Korn (1986) Structural and enzymological characterization of immunoaffinity purified DNA polymerase α-*primase complex* from KB cells. *J. Biol. Chem.* 261: 7958-7968.

利用特定親和柱狀色層分析法純化櫻鮭(*Oncorhynchus masou*)精巢之DNA聚合酶α-引導合成酶

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依全新製備之 5-(E)-(4-aminostyryl) ara UTP 爲親和配體，進行 DNA 聚合酶-引導合成酶之特異純化，以 ara UTP 樹脂加上間隔者 (Ara UTP-Affigel 10) 爲本研究之原因，乃是在 ara UTP 親和配體缺乏間隔者時，成長中櫻鮭 (*Oncorhynchus masou*) 精巢之 DNA 聚合酶 α-引導合成酶無法結合。在選定之 ara UTP Affigel-10 含有 poly(dA) 或 poly(dA)-oligo$(dT)_{12-18}$ 時，酵素活性可保留。被保留之酵素活性可在 100 mM KCl 冲洗出來成一單峰，這部份 α-聚合酶活性之特異活性爲 170,000 單位/毫克蛋白質，高度純化之 DNA 聚合酶 α-引導合成酶由 ara UTP-Affigel 10 層析法分離後，仍然含有三種蛋白質，依硫酸十二酯鈉一聚丙烯醯胺膠體電泳法 (SDS-PAGE) 分析，其分子量分別是 120,000，62,000 及 58,000。