

Purification and Characterization of Alkaline Phosphatase from Human Seminomas

Tateki YAMAMOTO and Yoji KATSUOKA*

Natural Products Research Department, Teikoku Hormone Mfg. Co., Ltd.

**Department of Urology, Osaka Medical College*

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Despite the apparent link between the presence of alkaline phosphatase (ALP) and various cancers, it has so far been difficult to determine distinct differences between seminoma-derived ALP and placental ALP (PLAP). In order to determine specificity, we purified ALP from a seminoma type of human testicular cancer tissue and compared its biochemical and immunological properties with those of PLAP. The purified ALP had a specific activity of 66 units per mg of protein, and it was possible to obtain 169 μ g of purified preparation from 60 g of tissue. The molecular weight of the purified seminoma enzyme was approximately 500 kDa. We found that a novel type of ALP from human testicular cancer tissue exists, with a high molecular weight and differing in degree, from the seminoma ALP previously reported.

Key words : Seminoma, Alkaline phosphatase (ALP), Placental ALP, Seminoma ALP

INTRODUCTION

Human placental alkaline phosphatase (PLAP) is a heat-stable metalloenzyme present in high levels in the placenta, and at trace levels in the lung and cervix [1, 2]. Fishman *et al.* [3] reported that an alkaline phosphatase (ALP) with properties indistinguishable from the placental enzyme was found in the serum of a patient with bronchial carcinoma and in various cancers, and that this abnormal enzyme, designated as Regan isoenzyme, was produced by the cancer cell itself. Nakayama *et al.* [4] demonstrated the existence of a heat stable ALP in a patient with pleuritis carcinomatosa that was distinct from PLAP or the Regan isoenzyme, and termed it the Nagao isozyme. This latter differs from the Regan isoenzyme in terms of its sensitivity to L-leucine and EDTA. In addition, elevated PLAP activity has been noted in 40% of patients with ovarian carcinoma, 22% of patients with cervical carcinoma, and 41% of patients with endometrial carcinoma [5].

In addition to an association of PLAP activity with the above carcinomas, PLAP

and ALP appear to be associated with seminoma. In 1987, a placental-type ALP was isolated from a seminoma type of human testicular cancer tissue and purified by butanol extraction, ammonium sulfate fractionation and hydrophobic chromatography [6, 7]. It was determined by inhibition studies that this enzyme is a Nagao variant, rather than the Regan type reported in several cancer tissues [8, 9]. It has also been found that 40% of patients with an active seminoma tumor had an elevated serum PLAP [10]. Turker *et al.* [11] demonstrated that of 50 patients with metastatic testicular germ cell tumors, a significant proportion had elevated serum levels of PLAP before treatment, as evaluated by an H17E monoclonal antibody assay. In that study, 88% of 16 patients with seminomas, 54% of 13 patients with mixed seminoma/malignant teratomas, and 33% of 21 patients with malignant teratomas, had an elevated PLAP. With a pure seminoma, the levels of serum PLAP were elevated in 30% of patients with stage I disease and in 56% of patients with stage II-III disease [12]. These results suggest that the measurement of PLAP levels will be useful in the manage-

ment of patients with germ cell tumors, particularly those with a seminoma [13-17].

We purified and ALP from the seminoma type of human testicular cancer tissue, and its biochemical and immunological properties were compared to those of the PLAP and seminoma ALP reported by Qadri and colleagues [6, 7].

MATERIALS AND METHODS

Testicular Cancer Tissue

Human tissue obtained from five Japanese men, 25-35 years old, were diagnosed as a seminoma type of testicular cancer. It was composed of uniform tumor cells, and was classified as stage I. After removal the sample was kept frozen at -80°C until used.

Reagents

Cibacron blue-Sepharose 4B, human PLAP and 4-nitrophenyl phosphate substrate (Sigma 104) were purchased from Sigma Chemical Company, St. Louis, MO, U. S. A. Sephacryl S-300HR and DEAE-Sepharose CL-6B were purchased from Pharmacia Biotech, Uppsala, Sweden and Diaflo membrane YM10 was purchased from Amicon Division, W. R. Grace & Co. Beverly, MO, U. S. A. The standard protein used in the method of Bradford, four-times recrystallized bovine serum albumin (BSA), was from ICN Pharmaceuticals Inc. Cleveland, OH, U. S. A. Marker proteins (thyroglobulin, γ -globulin, ovalbumin, myoglobin, and vitamin B12) for molecular weight determination were purchased from Bio-Rad Laboratories, Richmond, CA, U. S. A. and BSA and ferritin were purchased from Boehringer Mannheim GmbH, Mannheim, Germany. Monoclonal antibody to human PLAP was purchased from Oxoid Limited, Hampshire, England.

Determination of protein

The amount of protein in the preparations was determined by the Bio-Rad coomassie brilliant blue G-250 dye-binding procedure, based on the method of Bradford [18], with BSA used as the standard.

Enzyme assay of ALP

The enzyme assay was performed at 37°C for 30 min, with 18 mM p-nitrophenyl phosphate disodium salt at the substrate, in 50

mM glycine-sodium hydroxide buffer containing 0.5 mM MgCl_2 (pH 10.5), in a final volume of 1 ml [7]. The enzyme reaction was stopped with 10 ml of 0.02 N NaOH and the amount of p-nitrophenol liberated in 30 min was calculated from its absorbance at 410 nm. One unit of enzyme was defined as that amount which liberates 1 μ mole of p-nitrophenol per min per ml.

Estimation of molecular weight

The molecular weight was determined by gel filtration on a HPLC column. To estimate the molecular weight, the column was calibrated with standard proteins [thyroglobulin (670 kDa), ferritin (440 kDa), γ -globulin (158 kDa), BSA (66 kDa), ovalbumin (44 kDa), and myoglobin (17 kDa)] under the same conditions as described above.

Immunoblotting analysis of ALP

Purified enzyme was applied to a nitrocellulose membrane in each lane of the blot apparatus. The membrane was incubated with mAb to human PLAP. Goat anti-mouse horseradish peroxidase (HRP) conjugate and color development reagents were used to visualize the antigen.

Purification of ALP from a Seminoma

All purification procedures were carried out at 4°C .

Step 1. Butanol extraction.

Sixty gm of the testicular tumor tissue were homogenized with 540 ml of 0.05 M Tris-HCl buffer (pH 8.6), at 18,000 rpm for 3 min, in a Polytron homogenizer from Kinematic AG by finely divided mechanical rotation, and then centrifuged at 27,000 g for 20 min. Two hundred forty ml of butanol were added to 600 ml of homogenated supernatant and the mixture stirred for 1 h, then raising and maintaining the temperature at 37°C for 10 min. The bottom aqueous phase was carefully collected and passed through glass wool to remove any suspended particles, then recentrifuged at 35,000 g for 30 min. The supernatant was dialyzed against 5 liters of 0.05 M Tris-HCl buffer (pH 8.6) twice, and 610 ml of butanol supernatant were obtained.

Step 2. Ammonium sulfate fractionation.

Butanol supernatant was precipitated by adding solid ammonium sulfate to 90% saturation, and the mixture centrifuged at

35,000 g for 30 min. The precipitate obtained was then dissolved in 160 ml of 0.05 M Tris-HCl buffer combined with 60% saturated ammonium sulfate (pH 8.5). The inactive supernatant was removed after centrifugation at 35,000 g for 30 min and discarded. The precipitate was dissolved in 160 ml of 0.05 M Tris-HCl buffer combined with 30% saturated ammonium sulfate (pH 8.5), and the supernatant pooled.

Step 3. Heat treatment.

Solid ammonium sulfate was added to the 30-60% saturated ammonium sulfate fraction, and the 70% saturated ammonium sulfate precipitate centrifuged and extracted with 60 ml of 0.01 M Tris-HCl buffer, containing 1 mM Zn (SO₄)₂ combined with 30% saturated ammonium sulfate (pH 7.5). Sixty-two ml of extract were maintained at 55°C for 1 h with occasional agitation. The turbid solution was cooled on ice for 30 min, then centrifuged at 35,000 g for 20 min. The residue was discarded and the heat-treated 30-60% saturated ammonium sulfate recovered.

Step 4. Hydrophobic chromatography on a Cibacron blue-Sepharose 4B column.

The active solution was applied to a column (2.5 × 24.5 cm) of Cibacron blue-Sepharose 4B, equilibrated with 0.01 M Tris-HCl buffer containing 1 mM ZnSO₄ combined with 30% saturated ammonium sulfate (pH 7.5). The column was washed with the same buffer and then eluted with 0.01 M Tris-HCl buffer (pH 7.5). Two hundred ml of the peak fraction containing active ALP were pooled.

Step 5. Gel filtration chromatography on a Sephacryl S-300HR column.

The active Cibacron blue-Sepharose 4B fraction was concentrated to 9 ml by ultrafiltration, using a Diaflow YM10 membrane (10kDa Mr cut-off). The sample was passed through a column (2.5 × 96 cm) of Sephacryl S-300HR equilibrated with 0.01 M Tris-HCl buffer containing 0.15 M NaCl, 2 mM MgCl₂ and 0.025 mM ZnCl₂ (pH 8.0), and eluted with the same buffer. Fifty ml of the active fractions, containing ALP with a molecular weight in the region of 500kDa, were then pooled.

Step 6. Anion exchange chromatography on a DEAE-Sepharose CL-6B column.

The active Sephacryl S-300HR fraction was concentrated by ultrafiltration, using a

Diaflow YM10 membrane, with 0.01 M Tris-HCl buffer containing 2 mM MgCl₂ and 0.025 mM ZnCl₂ (pH 8.0). This sample was applied to a column (1.6 × 5.0 cm) of DEAE-Sepharose CL-6B equilibrated with the same buffer and eluted with a linear gradient of ionic strength from 0 to 0.5 M NaCl. There were two peaks of absorbance and the activity was present in the second peak. The peak of activity, containing ALP of 0.16 M to 0.27 M NaCl, was pooled.

Step 7. Gel filtration chromatography on a Sephacryl S-300HR column.

The active DEAE-Sepharose CL-6B fraction was concentrated to 3 ml by ultrafiltration, using a Diaflow YM10 membrane. The concentrated solution was again fractionated by a 2nd gel filtration on the same Sephacryl S-300HR column (1.5 × 88 cm). There was one peak of absorbance and the activity present in the peak had a molecular weight of 500 kDa. Six ml of the eluted fractions 55-60 were concentrated by ultrafiltration, using a Centricon-10 (10 kDa Mr cut-off), and the final fine preparation was obtained.

HPLC

The purity of purified ALP was analyzed by gel filtration and anion exchange.

HPLC gel filtration chromatography, using a G4000SW column (7.5 mm × 60 cm Toso Co., Ltd.), was carried out at room temperature. The purified active fraction was eluted with 0.05 M sodium phosphate buffer, containing 0.3 M NaCl (pH 7.0), at a flow rate of 1 ml/min. The effluent was monitored for absorbance at 220 nm.

The purified active fraction was loaded onto a strong anion exchange Mono Q HR 5/5 column, (5 mm × 5 cm, Pharmacia Biotech) equilibrated with 10 mM Tris-HCl buffer (pH 8.0), at room temperature. The column was then eluted with a linear gradient of 0 - 1.0 M NaCl at a flow rate of 1 ml/min. The effluent was monitored for absorbance at 280 nm.

RESULTS

Figure 1 shows the final single peak of absorbance of the active fraction after the final fractionation done in Step 7. A total of 0.16 mg of purified seminoma ALP was obtained from 60 g of human testicular cancer tissue. The recovery, as determined by

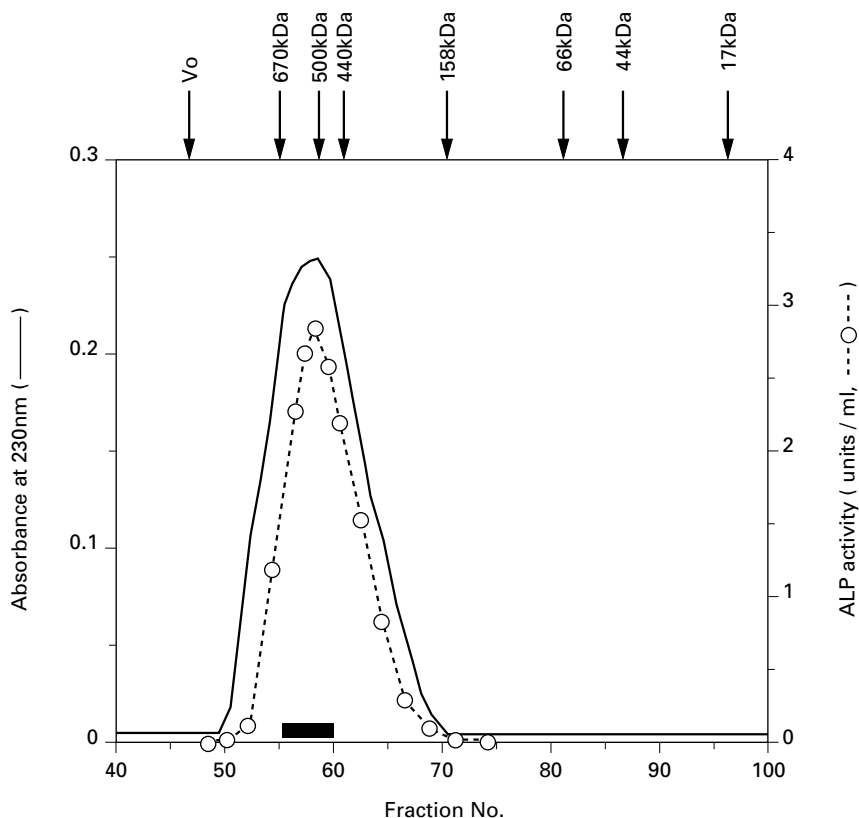


Fig. 1 Gel filtration chromatography on a Sephacryl S-300HR column. Sample : DEAE-Sepharose CL-6B column fraction; Column size : 155 ml (1.5 × 88 cm); Eluent : 0.01 M Tris-HCl containing 0.15 M NaCl, 2 mM MgCl₂, and 0.025 mM ZnCl₂ (pH 8.0); Flow rate : 25 ml/h; Fraction : 1 ml/tube. The active fractions indicated by the solid bar were pooled.

Table 1 Purification of seminoma ALP

Purification Step	Total protein ²⁾ (mg)	Total activity ³⁾ (units)	Specific activity (units/mg)	Recovery (%)	Purification (-fold)
Homogenate ¹⁾	3,710	118	0.032		
Butanol extract	1,750	535	0.306	100	1.0
Ammonium sulfate ppt	456	200	0.439	37.4	1.4
Heat treatment	300	175	0.586	32.7	1.9
Cibacron blue-Sepharose 4B	25.8	94.2	3.65	17.6	11.9
Sephacryl S-300HR (1st)	2.16	71.5	33.0	13.4	108
DEAE-Sepharose CL-6B	0.765	36.9	48.3	6.9	158
Sephacryl S-300HR (2nd)	0.252	16.7	66.2	3.1	216
Centricon-10	0.169	11.4	67.3	2.1	220

1) : As starting material, 60g of tissue was used.

2) : determined by the method of Bradford.

3) : assayed by measuring the release of p-nitrophenol from p-nitrophenyl phosphate at pH 10.5 at 37°C.

One unit of ALP activity was defined as that amount which released 1 μ mole of p-nitrophenol per minute.

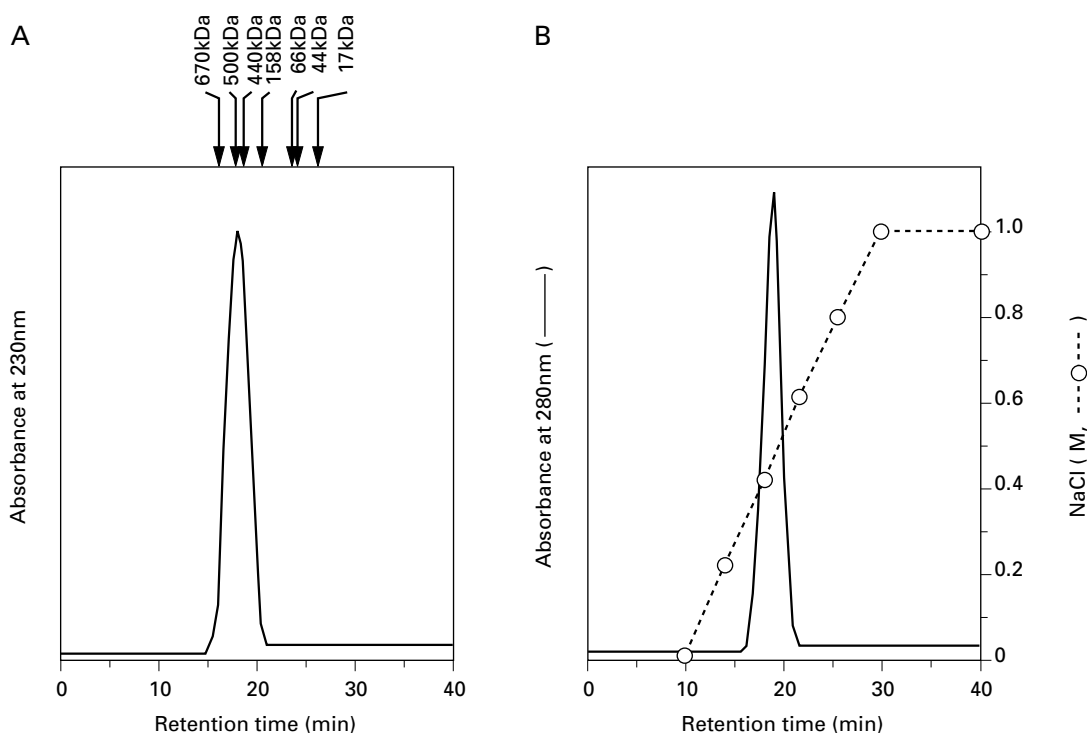


Fig. 2 A) HPLC on a TSK-GEL G4000SW column. Sample : Purified ALP; Column size : 7.5 mm \times 60 cm; Eluent : 0.05 M sodium phosphate buffer containing 0.3 M NaCl (pH 7.0); Flow rate : 1.0 ml/min. B) HPLC on a Mono Q HR 5/5 column. Sample : Purified ALP; Column size : 5.0 mm \times 5.0 cm ; Starting buffer A : 0.02 M Tris-HCl buffer (pH 8.0, 10min); Eluent B : 0.02 M Tris-HCl buffer (pH 8.0) containing 0 - 1.0 M NaCl (20min); Eluent C : 0.02 M Tris-HCl buffer (pH 8.0) containing 1.0 M NaCl (10min); Flow rate : 1.0 ml/min.

the enzyme assay, was 2.1% from the butanol supernatant and 11.4 units of ALP activity were contained in the final preparation. A summary of the purification procedure is shown in Table 1.

The results of preparations from other seminoma patients were in agreement with the data shown here.

Homogeneity of purified ALP

The purified preparation, obtained from an HPLC column using gel filtration and anion exchange, gave a single and symmetrical peak, and appeared to be homogeneous (Fig. 2).

Estimation of molecular weight

The molecular weight of the purified enzyme obtained from the seminomas was estimated to be approximately 500 kDa, by gel filtration on the HPLC.

Chemical inhibition studies

There were clear differences between the testis enzyme and the PLAP in terms of heat stability, inhibition by amino acids [L-phenylalanine (Phe), L-leucine (Leu)] and EDTA (Tables 2A, 2B). The PLAP retained 87.0% of its activity after 30 min at 65°C, while the testis enzyme retained only 4.0% of its activity under the same conditions. The unheated testis enzyme was retained by Phe and Leu, and more sensitive to EDTA than the PLAP. The residual activity of native testis enzyme with 5 mM of Phe, Leu, or 1.5 mM of EDTA, were 89.7, 76.0, and 4.7%, respectively. On the other hand, the activity values of the PLAF were reduced to 36.7 by Phe, 66.0 by Leu, and 84.7% by EDTA (Table 2A). The testis enzyme was also more sensitive to amino acid inhibition and EDTA than was the PLAP, after heating the sample at 65°C

Table 2A Biochemical properties of native ALP from placenta and seminoma

	Residual activity (%) ¹⁾						
	Heat	Phe		Leu		EDTA	
	65°C, 30min	1mM	5mM	1mM	5mM	0.1mM	1.5mM
Placenta ²⁾ (88) ³⁾	87.0 ± 1.0	78.3 ± 1.5	36.7 ± 1.5	89.0 ± 1.0	66.0 ± 2.0	96.0 ± 1.0	84.7 ± 1.5
Seminoma (36)	4.0 ± 2.0	96.7 ± 0.6	89.7 ± 0.6	92.3 ± 1.5	76.0 ± 1.0	78.0 ± 1.0	4.7 ± 1.5

1) : Results are expressed as the mean ± SD (n=3). Data from other seminoma patients are included.

2) : Placental ALP used for stability (Sigma Chemical Co., St. Louis, MO, U. S. A.).

3) : Values in parentheses represent results obtained by Qadri's group [6, 7].

Table 2B Biochemical properties of heat-treated ALP from placenta and seminoma

	Residual activity (%) ¹⁾					
	Phe		Leu		EDTA	
	1mM	5mM	1mM	5mM	0.1mM	1.5mM
Placenta ²⁾ (65) ³⁾	75.3 ± 1.5	37.0 ± 2.0	90.6 ± 0.6	65.7 ± 1.5	98.3 ± 0.6	80.3 ± 1.5
Seminoma (61)	50.6 ± 1.5	16.7 ± 1.5	29.3 ± 1.2	9.0 ± 1.0	89.7 ± 1.5	31.3 ± 1.5
				(86)		(55)
			(14)			(6)

1) : Results are expressed as the mean ± SD (n=3). Data from other seminoma patients are included.

The degree of stability was defined as percentage of initial activity retained after heating samples at 65°C for 30min.

2) : Placental ALP used for stability (Sigma Chemical Co., St. Louis, MO, U. S. A.).

3) : Values in parentheses represent results obtained by Qadri's group [6, 7].

for 30 min. The residual activity of testis enzyme tested with 5 mM of Phe, Leu, or 1.5 mM of EDTA were 16.7, 9.0, and 31.3%, respectively. In contrast, the activity values of the PLAP were reduced to 37.0 by Phe, 65.7 by Leu, and 80.3% by EDTA (Table 2B).

Immunological studies

The testis enzyme crossreacted poorly with the monoclonal antibody to PLAP in the immunoblot assay (Fig. 3).

DISCUSSION

Interest in the study of PLAP has been stimulated by observing the ectopic synthesis of this protein in certain nontrophoblastic tumor cells [19]. Immunohistochemistry has demonstrated the presence of a PLAP-like isoenzyme in germinoma cells. Biochemical analysis has revealed that the heat-stable component of ALP in germinoma cells is consistent with the D-variant of the PLAP-

like isoenzyme [20~22]. The major interest is in the observation that the incidence of the PLAP-like isoenzyme is relatively high in the sera of patients with testicular cancer. When measured by monoclonal immunocatalytic assay, the PLAP-like isoenzyme was elevated in 50% of the stage I seminoma patients, and in all patients with stage II and III disease. Hirano *et al.* [23] have reported that the ALP from seminoma cells is comprised of a heterogeneous population of molecules that demonstrate partial heat sensitivity and microheterogeneity after starch gel electrophoresis. These results contrast with those obtained with pregnancy-related PLAP.

Purified seminoma ALP yields a single peak on a G4000 SW gel filtration HPLC column and exists as a protein with a molecular weight of 500 kDa. Our report is the first to demonstrate that seminoma ALP exists as a 500 kDa protein with a carrier substance. Our results indicate that the 140

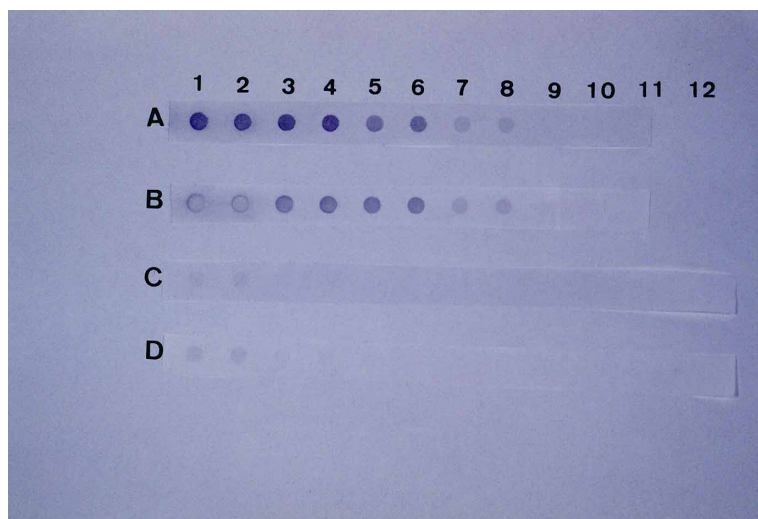


Fig. 3 Immunoblotting analysis of ALP. In each row of the dot apparatus, ALP was applied to nitrocellulose. A, B : crude PLAP (activity : 15 units/mg solid). C, D : seminoma ALP of 1st Sephacryl S-300HR column fraction. Lanes 1, 2 : 100 m units; Lanes 3, 4 : 10 m units; Lanes 5, 6 : 1 m units; Lanes 7, 8 : 100 μ units; Lanes 9, 10 : 10 μ units; Lanes 11, 12 : BSA 1 μ g. Anti-PLAP mAb. A, C : 1 : 100 dilution; B, D : 1 : 1,000 dilution. The purified ALP does not react with anti-bovine serum albumin antibody.

kDa ALP binds to a carrier protein, and the molecular weight of the seminoma ALP increases to 500 kDa as the testicular cancer proceeds. In fact, it has been reported that the molecular weight of liver cancer ALP change to more than 1,000 kDa. Furthermore, the purified ALP gave a single peak on the Mono Q anion exchange HPLC column. However, when examined for purity, the purified seminoma ALP yielded a few bands after SDS-polyacrylamide gel electrophoresis. It is of interest that the first twenty N-terminal amino acid sequence of the purified seminoma ALP are perfectly homologous to those of apolipoprotein A-1 (data not shown). It seems likely that the seminoma-derived purified enzyme exists as a complex with apolipoprotein A-1. Although we attempted to isolate substances corresponding to a homodimer with a 70 kDa subunit, no active ALP with a molecular weight of 140 kDa was detected at any step of the purification procedure. All structures of the seminoma high molecular weight ALP are not required for ALP activity. It seems most probable that the active site with ALP activity is the 70 kDa subunit.

The seminoma enzyme was more sensitive to temperature than the PLAP. However, the placental enzyme under the same conditions retained more than 87.0% of its activity. Without heating the enzyme, the activity of the seminoma ALP was stable to Phe, and was more sensitive to EDTA. Purified ALP is found to be similar in properties to the ALP reported by Qadri's group (Tables 2A, 2B). However, the purified seminoma ALP was more susceptible to heat-stability and resistant to inhibition by EDTA than was the ALP reported by Shameen and Qadri [6] after heat treatment at 65°C for 30 min (Table 2B). The results suggest that the cause for the difference is due to the molecular form. Purified ALP binds to the carrier protein and its properties reflect the living reaction. The most interesting characteristic of the heat-stable testicular enzyme is its greater sensitivity to Phe and Leu inhibition. These inhibition studies showed that the enzyme is similar to the Kasahara isoenzyme for heat stability and to the Nagao isoenzyme for Leu inhibition, rather than the Regan and the Kasahara isoenzymes.

The seminoma enzyme crossreacted poor-

ly with the monoclonal antibody to PLAP in the immunoblot assay. The sensitivity for seminoma ALP displayed 1,000-fold decreases of magnitude when compared with PLAP. Opaque staining is observed in dot blot analysis on B-1, 2 (Fig. 3). As the antigen and antibody interact in high concentration, it seems likely that the reaction produces inhibition and opaque staining results. The differences in reactivity between seminoma ALP and PLAP may depend on molecular size or the epitope sequence of both ALPs.

The recent cloning of DNAs coding for PLAP, tissue-unspecific or liver/bone/kidney-type ALP, and intestinal ALP confirms the existence of at least three distinct genes in this family. Millan *et al.* [24-26] demonstrated the existence and revealed the structure of a germ-cell ALP gene, and provided evidence that this gene encodes the Nagao isozyme which is overproduced by seminoma of the testis. However, the question of identity between the PLAP and Nagao isozymes has remained unsolved.

The cDNA variants of one of the isozymes of PLAP have now been cloned and sequenced in several laboratories [27~34]. These studies have conclusively demonstrated that the isozymes constitute separate genetic entities and also demonstrated the complex intron/exon structure of their genes. In addition, genomic DNA clones representing a minor form of human ALP have been isolated. An enzyme encoded at one particular locus of these clones has been designated as "PLAP-like", or "germ cell" ALP. The cloning of other isozymes is necessary to understand the relationships among the isozymes. In addition, such cloning will provide tools with which to elucidate the function and regulation of ALPs, and to determine their mode of attachment to plasma membranes.

We are trying to isolate two kind of cDNA clones encoding germ-cell ALP and liver-type ALP genes from a seminoma cDNA library. Nucleotide sequence analysis suggested that one of these clones encoded an allelic variant of the germ-cell ALP gene. The molecular cloning of the cDNAs encoding seminoma ALP and the clarification of all structures of seminoma ALP with a high molecular weight are goals for future studies [35].

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