Immunological Investigation of Intestinal, Liver, Kidney, Bone, Placental and Serum Alkaline Phosphatase in Cattle

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ABSTRACT. The purpose of this study is to investigate the immunological difference between intestinal, liver, kidney, bone, placental and serum alkaline phosphatases (ALPs) in cattle. Kidney, bone and placental ALPs were purified from each tissue by gel filtration and ion-exchange chromatographies, and intestinal, liver, and placental ALPs were obtained from a commercial source. The antibody to each tissue ALP was prepared by immunizing rabbits and tested by double immunodiffusion analysis in cross reactivities. The results indicated that the bovine tissue ALPs are divided into four groups in antigenicity, that is, intestinal, bone, liver and kidney-placental ALPs. The fetus, calf and cow sera contained bone ALP, and calf and cow sera also contained kidney or placental ALP, but all of the sera did not contain intestinal and liver ALP.—**KEY WORDS:** alkaline phosphatase isoenzyme, cattle, immunology, serum, tissue.

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ALP is an enzyme existing in the tissues and body fluids of all vertebrates that hydrolyzes phosphomonoesters [4]. It is well-known that several isoenzymes of ALP are distributed in different tissues with different frequencies [5, 17]. Differences in the properties of serum and some tissue ALP isoenzymes in cattle have been elucidated by inhibition tests [7, 8] and electrophoretic analyses [6]. However, little work has been done to study the immunological properties of ALP isoenzymes of cattle [1] and their tissue distribution.

MATERIALS AND METHODS

Preparation of crude enzyme: All the extraction and purification steps were performed at 4°C. The techniques used in this study are a modification of those described by Engström [3] and Morton [13]. Kidney and bone samples were collected from a slaughterhouse. One kilogram of kidney tissue was homogenized with 3 liters of 0.01

M sodium acetate–0.01 M magnesium acetate and centrifuged at 2800 g for 30 min. A crude ALP fraction was obtained by successive precipitation with 30% N-butanol, 50% acetone, 30% ethanol and 60% ethanol. Ossa antebrachii of cow was brayed with a file and a crude fraction was prepared in the same way.

Purification of the enzyme: 1. Chromatography on TEAE-cellulose: The final ethanol fraction of kidney or bone samples, and one gram of crude placental ALP (Sigma Co., Ltd.), were chromatographed on a 50 ml column of TEAE-cellulose (Serva Feinbiochemica GmbH. & Co.). Elution was performed with a gradient mixture of 800 ml of 0.01 M Tris-acetic acid buffer (pH 8.8) containing 0.1 M magnesium acetate. Fractions of 10 ml were collected every 15 min. The enzyme-active fractions were combined and concentrated to a few milliliters with collodion bag SM13200 (Sartorius GmbH) and dialyzed against 0.01 M Tris-acetic acid buffer (pH

8.8). 2. Re-chromatography on TEAE-cellulose: The enzyme-active fraction was re-chromatographed on TEAE-cellulose, as described above. A gradient mixture was made by mixing 0.01 M Tris-acetic acid buffer (pH 8.8) and 0.05 M Tris-acetic acid buffer (pH 8.8) containing 0.25 M sodium acetate in the same volumes as described above. The enzyme-active fractions were also concentrated and dialyzed against 0.025 M Tris-acetic acid buffer (pH 8.8). 3. Chromatography on Sephadex G-200: The enzyme-active fraction was finally chromatographed on a 50 ml column of Sephadex G-200 (Serva Feinbiochemica GmbH. & Co.). Elution was performed with 0.025 M Tris-acetic acid buffer (pH 8.8) containing 1 M sodium acetate. One or two ml fractions were collected every 30 min.

Protein determinations: The protein concentration of each fraction was determined by absorbance at 280 m μ in a Shimadzu DU spectrophotometer.

Enzyme assays: Enzyme activity was measured by the method of Watanabe [18] (ALP-S Kit, Yatorone).

Preparation of antibody to each tissue isoenzyme: Purified 0.3 mg placental (0.3 ml), 0.29 mg bone (0.25 ml), 0.44 mg kidney (0.5 ml) ALPs, and 200 μ g (210 units) commercial intestinal (0.2 ml) and 3 mg liver (0.3 ml) ALPs, purchased from Sigma Co., Ltd., were diluted to a volume of 1 ml with Tris-acetic acid buffer (0.01 M, pH 8.9). Each antigen preparation (1 ml) was emulsified with an equal volume of Freund's complete adjuvant (Difco) and injected subcutaneously into a rabbit 4 times every week. Sera were collected one month after the final injection.

Immunological technique: Ouchterlony's double immunodiffusion tests were performed in 1% agar gel containing 0.025 M veronal buffer (pH 8.6), allowing the gels to stand for 1 or 2 days at 4°C. Precipitin lines were stained with amidoblack after soaking

the gel in 0.85% NaCl solution for 4 days. Positive or negative results were indicated by the respective presence or absence of precipitation lines. Anti-bovine tissue ALP sera and tissue ALP preparations were serially diluted, and each dilution of antiserum was diffused against each dilution of antigen preparation to find their suitable concentrations to give a fine precipitin line. For absorption tests, each antiserum and tissue ALP preparation were mixed in each proper concentration and incubated at 37°C for 24 hours. Then the precipitate was removed by centrifugation at 12000 g and the supernatant was used for the immunodiffusion test.

RESULTS

Rechromatography using TEAE-cellulose of bone ALP and final gel chromatographs of placental and kidney ALPs are shown in Figs. 1, 2, and 3, respectively. The enzyme activities of the finally purified placenta, bone and kidney were 3.7, 40.0 and 5.3 KAU/mg.N, respectively. These ALP preparations were used for immunization. The purities of these preparations were demonstrated by a single precipitin line produced against each antiserum in the double diffusion test and by immunoelectrophoretic analyses (data not shown). The precipitin tests are shown in Fig. 4 and the results summarized in Table 1.

Anti-intestinal ALP gave only one clear precipitin line against the homologous ALP (Fig. 4a), and the precipitin was not absorbed with any other tissue ALP preparations (Table 1).

Anti-liver ALP gave a precipitin line against liver, kidney and placental ALPs, and the precipitation line against the homologous ALP was partially fused with those against kidney or placental ALP (Fig. 4b). It indicates that liver ALP contains another determinant than those possessed by kidney

Fig. 1. Re-chromatography of bone alkaline phosphatase on TEAE-cellulose. The enzyme-active fraction (158.3 mg) which was obtained by TEAE-cellulose chromatography was applied to a 1.6×25cm column. Gradient elution was performed as described in Materials and Methods. Fractions 14–17 were pooled and used for immunization. Open circles: Protein concentration (absorbance at 280mμ). Closed circles: Enzyme activity.

and placental ALPs.

Anti-placental and anti-kidney ALPs gave a fused precipitin line against liver, placental and kidney ALPs (Fig. 4c, 4e), and they also reacted with calf and cow sera but not with fetal sera (Fig. 4f, 4g).

Anti-bone ALP gave a precipitin line against the homologous ALP and the precipitin was not absorbed with any other tissue ALP (Fig. 4d). A fused precipitin line was observed in its reaction with fetal, calf and cow sera (Fig. 4h).

DISCUSSION

Our results indicating that bovine intestinal ALP has unique immunological properties perfectly coincide with other reports described for intestinal ALP of other species [14, 15, 16]. Healy [7] also reported that only intestinal ALP but not other tissue ALPs from cattle was inactivated by urea and L-phenylalanine. Gahne [6] divided bovine ALP into two groups, intestinal ALP and other organ ALP, by electrophoretical analyses of neuraminidase-treated ALPs. However intestinal and placental ALPs were mutually cross-reactive [9, 10], and liver, bone and spleen ALPs were also

Fig. 2. Re-chromatography of placental alkaline phosphatase on Sephadex G-200. The enzyme-active fraction (3.0 mg) which was obtained by Sephadex G-200 chromatography was applied to a 1.6×25cm column. Elution was performed as described in Materials and Methods. Fractions 8–11 were pooled and used for immunization. Symbols are the same as those shown in Fig. 1.

Fig. 3. Re-chromatography of kidney alkaline phosphatase on Sephadex-G200. The enzyme-active fraction (36.4 mg) which was obtained by Sephadex G-200 chromatography was applied to a 1.6×25cm column. Elution was performed as described in Materials and Methods. Fractions 10–14 were pooled and used for immunization. Symbols are the same as those shown in Fig. 1.

mutually cross-reactive [2]. Saini and Saini [15] reported that bone and liver ALPs from dog were cross-reacting but other tissue ALPs were not cross-reactive. Our finding that cattle bone ALP also possessed a unique antigenicity was not consistent with these reports. The reasons for those contra-

dictory findings are unknown. The differences in distribution of ALP isoenzymes in different species may depend on differences in the physiological adaptation of each animal.

Calf and cow sera reacted with anti-placental, anti-kidney and anti-bone ALP. As calves and cows were not pregnant, ALP isoenzymes of the sera should be derived from the kidney and the bone tissue. Miyazawa *et al.* [12] reported that ALP activity of bovine fetal kidney was quite low, but it increased immediately after birth in the epithelial cells of renal tubules. It increases in parallel with development of kidney function [11]. This indicates that

elevation of ALP levels in calf and cow sera is related to the functional development of kidney rather than growth of bone tissue.

In the present studies, immunological properties of each ALP isoenzyme from different tissue and serum were examined. In further studies, ALP stain of precipitation lines and comparison with these biochemical properties will be useful.

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Table 1. Ouchterlony's double immunodiffusion analyses of bovine tissue and serum ALPs

Anti-tissue ALP	Bovine tissue ALP used for absorption	Tissue ALP					Serum		
		I	L	K	B	P	Fetus	Calf	Cow
Anti-I ALP		+	-	-	-	-	-	-	-
	L ALP	+	-	-	-	-	-	-	-
	K ALP	+	-	-	-	-	-	-	-
	B ALP	+	-	-	-	-	-	-	-
Anti-L ALP	P ALP	+	-	-	-	-	-	-	-
		-	+	+	-	+	-	-	-
	I ALP	-	+	+	-	+	-	-	-
	K ALP	-	+	-	-	-	-	-	-
Anti-K ALP	B ALP	-	+	+	-	-	-	-	-
	P ALP	-	+	-	-	-	-	-	-
		-	+	+	-	+	-	+	+
	I ALP	-	+	+	-	+	-	+	+
Anti-B ALP	L ALP	-	-	+	-	+	-	+	+
	K ALP	-	-	-	+	-	+	+	+
	B ALP	-	-	-	+	-	+	+	+
	P ALP	-	-	-	-	-	+	+	+
Anti-P ALP		-	-	-	+	-	+	+	+
	I ALP	-	+	+	-	+	-	+	+
	L ALP	-	-	+	-	+	-	+	+
	K ALP	-	-	-	-	-	-	-	-
	B ALP	-	+	+	-	+	-	+	+

+: precipitation line observed. -: precipitation line not observed.

ALP: alkaline phosphatase. I: intestine. L: liver. K: kidney. B: bone. P: placenta.

Fig. 4. Double immunodiffusion analysis in agar gel. AI, Anti-intestinal ALP, serum diluted 160 fold ($\times 160$); AL, Antiliver ALP ($\times 160$); AP, Anti-placental ALP ($\times 40$); AB, Anti-bone ALP ($\times 5$); AK, Anti-kidney ALP ($\times 10$); I, Intestinal ALP ($0.6\mu\text{g}$); L, Liver ALP ($12\mu\text{g}$); P, Placental ALP ($1.2\mu\text{g}$); K, Kidney ALP ($1.1\mu\text{g}$); B, Bone ALP ($1.4\mu\text{g}$); F, Bovine fetal serum at the original concentration ($\times 1$); Cf, Calf serum ($\times 1$); C, Cow serum ($\times 1$); Quantity of each antigen and antibody applied was 0.006 ml .

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要 約

ウシ腸，肝，腎，骨，胎盤ならびに血清アルカリホスファターゼの免疫学的性質：宮澤清志・友田 勇¹⁾（帯広畜産大学・¹⁾日本獣医畜産大学）——腎，骨および胎盤アルカリホスファターゼ（ALP）は精製したものを，腸および肝 ALP は市販の精製標品を免疫して各臓器 ALP 抗体を作成した．二重免疫拡散法により，各臓器 ALP は免疫学的に腸，骨，肝ならびに腎-胎盤の 4 グループに分類された．骨 ALP は胎児，仔ウシおよび成牛の血清中に，腎-胎盤 ALP は仔ウシおよび成牛の血清中に認められたが腸および肝 ALP は，いずれの血清中にも認められなかった．