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Analysis of human bone alkaline phosphatase isoforms: Comparison of isoelectric focusing and ion-exchange high-performance liquid chromatography

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Abstract

Background: Several isoforms of alkaline phosphatase (ALP) can be identified in human tissues and serum after separation by anion-exchange HPLC and isoelectric focusing (IEF).
Methods: We purified four soluble bone ALP (BALP) isoforms (B/I, B1x, B1 and B2) from human SaOS-2 cells, determined their specific pI values by broad range IEF (pH 3.5–9.5), compared these with commercial preparations of bone, intestinal and liver ALPs and established the effects of neuraminidase and wheat germ lectin (WGA) on enzyme activity.
Results: Whilst the isoforms B1x (pI=4.48), B1 (pI=4.32) and B2 (pI=4.12) resolved as well-defined bands, B/I resolved as a complex (pI=4.85–6.84). Neuraminidase altered the migration of all BALP isoforms to pI=6.84 and abolished their binding to the anion-exchange matrix, but increased their enzymatic activities by 11–20%. WGA precipitated the BALP isoforms in IEF gels and the HPLC column and attenuated their enzymatic activities by 54–73%. IEF resolved the commercial BALP into 2 major bands (pI=4.41 and 4.55).
Conclusions: Migration of BALP isoforms is similar in IEF and anion-exchange HPLC and dependent on sialic acid content. HPLC is preferable in smaller scale research applications where samples containing mixtures of BALP isoforms are analysed. Circulating liver ALP (pI=3.85) can be resolved from BALP by either method. IEF represents a simpler approach for routine purposes even though some overlapping of the isoforms may occur.

Keywords: Bone; Alkaline phosphatase; Isoforms; Ion-exchange chromatography; Isoelectric focusing; Sialic acid

1. Introduction

Alkaline phosphatase (ALP, orthophosphoric-monoester phosphohydrolase, alkaline optimum, EC 3.1.3.1) is an ectoenzyme glycoprotein expressed in a wide variety of cells and present in tissues throughout the body. ALP is attached to the outer cell membrane by a glycosylphosphatidylinositol (GPI) anchor (recently reviewed in [1]) and sorted into cell membrane micro-domain rafts [2]. In vivo, ALP circulates as an anchor-less homodimer indicating the conversion of anchor-intact ALP to a soluble, anchor-less form, by the action of endogenous GPI-specific phospholipase D (GPI-PLD) [3,4].

The tissue-nonspecific ALP (TNALP) gene locus 1p36-p34 encodes bone, liver and kidney isoforms of ALP [5]. With a common protein backbone, differences in the TNALP isoforms are due to the type and extent of post-translationally added glycans. All TNALP isoforms are *N*-glycosylated and only the liver ALP isoform (LALP) does not contain O-linked glycans [6]. Removal of glycans, or inhibition of their synthesis, affects both enzyme activity and immunoreactivity [6–9]. In healthy adults, approximately 95% of circulating total ALP activity is derived from bone and liver sources. These, depending on the analytical method, are usually present in a ratio of approximately 1:1 [10].

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The physiological functions of ALP are becoming clearer. Clinical studies of hypophosphatasia, caused by missense mutations in the TNALP gene, and ALP knock-out models in mice have provided compelling evidence for a primary role of bone ALP (BALP), in association with other enzymes including PC-1 (plasma cell membrane glycoprotein-1) and PHOSPHO1 (phosphatase, orphan 1) in skeletal development and mineralization [11–15]. Because of this, and its strong correlation with histomorphometric indices of bone formation, BALP provides a useful and widely used index of osteoblastic activity and bone formation [16].

Separation and quantitation of the ALP isoenzymes and isoforms has traditionally involved electrophoretic methods that rely on differences in their physico-chemical properties [17]. Of these, isoelectric focusing (IEF) has proved useful and capable of separating more than 12 clinically defined ALP bands [18,19], but is strictly dependent upon methodology. Others have applied high-performance liquid chromatography (HPLC)-based methods [20–24] with varying success. To date, only the method based on weak anion-exchange HPLC with post-column reaction detection as described by Magnusson et al. [23,25] has been regularly applied to basic and clinical investigations of bone metabolism. In serum, this technique can separate the four BALP isoforms B/I (bone/intestinal), B1x, B1 and B2, which differ in their sialic acid content [26,7] and three LALP isoforms. The B/I peak comprises, on average, 4% of the serum total ALP activity. B/I is not a pure bone isoform since it co-elutes with circulating intestinal ALP (IALP) isoenzyme and is composed, on average, of 70% bone and 30% IALP. All four bone isoforms have been extracted from bone tissue [10] and cultures of bone-derived cells [27]. Although the functional significances of these isoforms are currently unknown, they can vary independently during the pubertal growth spurt [28] and in metabolic bone diseases [10,23,26,29].

Here we describe the separation of human TNALP isoforms by a generally applicable IEF method in polyacrylamide gels with the aim of comparing these separations with that of a validated weak anion-exchange HPLC method. Using IEF we demonstrate that purified BALP isoforms, isolated from cultures of human osteosarcoma SaOS-2 cells, resolve as bands with specific isoelectric points (pI) that correspond to their elution pattern from HPLC. Additionally, we compare the effects of treatment with neuraminidase (a sialidase) and the lectin, wheat germ agglutinin (WGA) on IEF migration and HPLC elution patterns and residual enzyme activities of ALP isoenzymes and isoforms. Finally, we illustrate and discuss the application of IEF to separate TNALP isoforms in serum.

2. Materials and methods

All chemicals and reagents were obtained from Sigma (St. Louis, MO, USA) unless otherwise stated.

2.1. ALP isoenzymes and isoforms

Human bone, liver and intestinal ALP preparations were obtained from Calzyme Laboratories, Inc (San Luis Obispo, CA, USA) and stock dilutions (approximately 4 mg/ml, w/v) made of each.

The BALP isoforms (B/I, B1 and B2) were partially purified from human osteosarcoma SaOS-2 cells and validated as previously described [7]. In brief, cell homogenates were incubated in 30% (v/v) butanol for 24 h at 4 °C. After centrifugation, the aqueous phase was incubated with a crude preparation of GPI-PLD, isolated from human serum, in the presence of 0.01% NP-40 (nonidet P-40, a non-ionic detergent) in order to cleave the GPI anchor. After this, the anchorless BALP was separated from uncleaved forms by phase separation in 2% Triton X-114. Residual detergent was removed by chromatography on a column of Amberlite XAD-2 and the eluate concentrated with Aquacide III (Calbiochem, La Jolla, CA, USA). A final anion-exchange step on Q-Sepharose and elution of ALP over a 0.05–0.20 mol/l gradient of sodium acetate (pH 7.6) resulted in the separation of the BALP isoforms that were identified by HPLC. B1x was not completely separated by this procedure and contained between 10–20% B/I. However, B1x was successfully isolated after a second Q-Sepharose run using a gradient of 0.05–0.10 mol/l sodium acetate.

Sera from two patients with elevated total ALP activities were identified for analysis. One patient had Paget's disease of bone whilst the other had a liver disorder.

2.2. Treatment of ALP with WGA and neuraminidase

WGA (Triticum vulgaris lectin, Sigma L-9640), a lectin that binds *N*-acetyl-D-glucosamine residues and is able to preferentially bind and precipitate BALP, was prepared as a stock solution of 5 mg/ml (w/v) in 0.9% saline. Equal volumes of the WGA solution were added to the ALP containing samples, mixed and incubated in a water bath at 37 °C for 30 min, after which samples were centrifuged at 7500 ×*g* for 10 min. Neuraminidase (Type V, EC 3.2.1.18, Sigma N-2876), a sialidase that removes terminal sialic acid residues from complex glycans, was prepared as a 20 U/ml stock solution in buffer containing 20 mmol/l Tris and 10 μmol/l zinc acetate, pH 7.6. To 50 μl of each ALP sample, 250 μl of the neuraminidase solution was added and incubated in a water bath at 37 °C for 2 h. Aliquots of the treated samples were then assayed for total ALP and analyzed by HPLC and IEF.

2.3. Measurement total ALP activity

Total ALP was measured by a kinetic assay in a 96-well microtitre plate format. In brief, a total volume of 300 μl solution was added per well, containing 1.0 mol/l diethanolamine buffer (pH 9.8), 1.0 mmol/l $MgCl_2$ and 10 mmol/l *p*-nitrophenyl phosphate. The time-dependent increase in absorbance at 405 nm (reflecting *p*-nitrophenol production) was determined on a kinetic microplate reader (Model VMax, Molecular Devices Corp., Sunnyvale, CA, USA). ALP activities are reported as units per liter (U/l), where 1 U reflects the production of 1 micromol/l of *p*-nitrophenol per min at 22±2 °C.

2.4. Separation and quantitation of ALP isoenzymes and isoforms

ALP isoenzymes and isoforms were separated using two independent techniques. A previously validated HPLC method with continuous post-column reaction detection was used [23,25]. A weak anion-exchange column, SynChropak AP300 (250×4.6 mm) (Eichrom Technologies/SynChrom, Darien, IL, USA), was used in place of the referred SynChropak AX 300. The SynChropak AP300 is a slightly modified SynChropak AX300, optimized for BALP isoform analysis [28]. The only difference in the laboratory procedure is that a lower mobile phase pH has to be used, pH 7.6 instead of pH 7.8. Reaction pH in the post-column reactor is not altered by the decreased mobile phase pH because of the low buffer strength, 20 mmol/l Tris acetate, compared with the high buffer strength in the substrate, 0.25 mol/l diethanolamine. A lower mobile phase pH and two pre-columns filled with SynChrosorb AX (Eichrom Technologies/SynChrom) were installed to increase column life and protect the silica-based weak anion-exchanger. These pre-columns were temperature controlled at 30 °C and installed on-line before the gradient mixing module to maintain the same dwell volume as before (i.e., 5.4 ml). Specific details on instrumentation, reagents, peak characterization, precision, analytical recovery, linearity data, and detection limit have been published elsewhere [23,25].

Horizontal IEF was carried out on Ampholine™ PAGplate gels (pH 3.5–9.5 and pH 4.0–6.5) (Amersham Biosciences AB, Uppsala, Sweden) using recommended conditions (1 mol/l H_3PO_4 and 1 mol/l NaOH as anode and cathode electrolytes and 1500 V, 50 mA as maximal initial power settings with a run time of 90 min and 0.1 mol/l glutamic acid in 0.5 mol/l H_3PO_4 and 0.1 mol/l β-alanine as anode and cathode electrolytes and 2000 V, 25 mA for approximately 120 min for

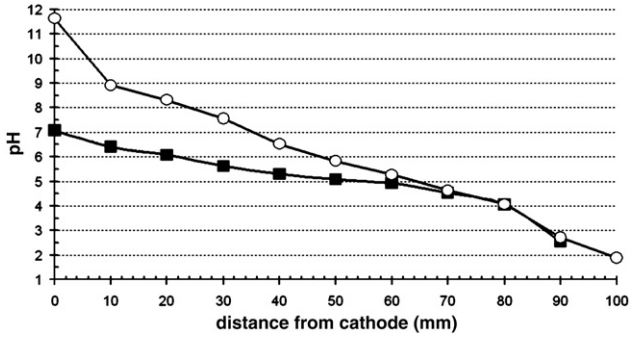

Fig. 1. pH gradients measured across narrow pH range 4–6.5 (filled square) and broad pH range 3.5–9.5 (open circle) Ampholine™ PAGplate gels using a surface pH electrode. Linear regions of the pH gradients (pH 4–6 and 4–8, respectively) were used to calculate the pI values for all ALP preparations.

the broad and narrow pH range gels, respectively). In each case the electrode distance was 90 mm. Samples were applied to the gel 30 mm from the cathode using a sample applicator strip. The volume of sample applied to the gel was kept reasonably constant (approximately 20 μl). Gels were run at 10 °C using a water-cooled LKB 2117 Multiphor II electrophoresis unit and an ECPS 3000/150 high voltage power supply (Pharmacia, Uppsala, Sweden). After completion of the IEF run, the temperature of the cooling plate was increased to 25 °C and the pH gradient of each gel was determined by taking pH measurements at 10 mm intervals between the cathode and anode using a KDCEF11 surface pH electrode (Thermo Electron Corp. Auchtermuchty, Scotland). Isoelectric points of the isoforms were determined using both a standard mix of IEF protein markers of known pIs (IEF Markers 3–10, SERVA liquid mix (Invitrogen AB, Lidingö, Sweden)) and individual protein markers, including amyloglucosidase (pI 3.6), glucose oxidase (pI 4.2), trypsin inhibitor (pI 4.6). β-lactoglobulin A (pI 5.1) and carbonic anhydrase (pI 6.6). After Coomassie staining of the pI markers each gel was calibrated. From initial surface pH measurements the region over which the pH gradient of the gel was linear was determined (Fig. 1). The pI values of the ALP isoforms were estimated from linear regression plots of relative migration versus the pI values of a range of marker proteins.

2.5. Gel staining and detection of protein concentration and ALP activity

In gels, ALP activity was detected using 1.0 mol/l 2-methyl-2-amino-1,3-propanediol (MAP) buffer (pH 10.3), containing 3 mmol/l $MgCl_2$ and 4.4 mmol/l naphthyl phosphate as substrate coupled with 1 g/l Variamine Blue RT salt (4-aminodiphenylamine diazonium sulphate) to precipitate the colored product within the matrix of the gel. Since the diazo-salt is light sensitive, small volumes were freshly made as required by adding 0.059 mg of naphthyl phosphate and 0.050 mg of diazo-coupler to 50 ml of the MAP buffer solution.

Gels were placed directly into staining trays, briefly washed with MAP buffer, decanted and freshly made staining reagent added. Gels were incubated at room temperature for between 20–30 min, or until sufficient staining had occurred. Since the stain is light sensitive and rapidly denatures, bands with very low activity were stained for longer periods with several changes of fresh staining solution. Gels were scanned on a flat-bed scanner to record the image.

For protein staining of the IEF marker standards, appropriate sections of the gel were excised and fixed in an aqueous solution of methanol (50%) and acetic acid (10%) for 15 min. Gels were then rinsed and washed with excess water for 15 min, stained with EZ Blue colloidal Coomassie stain for 30–60 min and then washed with water to minimize background staining before scanning.

3. Results and discussion

3.1. Separation of ALP isoenzymes and isoforms by IEF

Estimates of the pI values for the commercial ALP preparations and purified BALP isoforms made using both broad and narrow pH range PAGplate gels were in general agreement (Table 1). Isoelectric points reported herein are taken from the broad (3.5–9.5 pH) range gels.

Table 1
pI value estimates for the native and neuraminidase treated ALP isoenzymes and isoforms using PAGplate pH 4.0–6.5 and pH 3.5–9.5 gradient IEF gels

ALP	Narrow-range pH 4.0–6.5 gel		Broad-range pH 3.5–9.5 gel	
	pI value native	pI value neuraminidase	pI value native	pI value neuraminidase
Calzyme intestinal	Major band 4.47	Major band 4.47	Major band 4.48 Complex between 5.71–6.52	Major band 4.52 Complex between 5.99–6.70
Calzyme bone	Major bands 4.41 4.52		Major bands 4.41 4.55	
		Complex between 5.99–6.08	Complex between 4.10–4.76	Complex between 5.92–6.70
Calzyme liver	Major band 4.18		Major band 3.85	
		Complex between 6.02–6.08		Complex between 5.92–6.66
BALP B/I	~9 bands 4.69–6.22	6.21	~9 bands 4.85–6.84	6.84
BALP B1x	4.39	6.22	4.48	6.84
BALP B1	4.33	6.22	4.32	6.84
BALP B2	4.23	6.25	4.12	6.84

Of the commercial ALP preparations (Table 2, Fig. 2), the Calzyme IALP resolved as a major band (pI = 4.48) along with a complex of minor bands (pI 5.71–6.52) suggesting the presence of various minor ALP forms. Treatments with neuraminidase, which cleaves terminal sialic acid residues, and WGA had little effect on the migration of the major IALP band consistent with the absence of sialic acid and low degree of IALP precipitation by WGA [30,31]. The Calzyme BALP preparation resolved as a broad complex of bands (pI 4.10–4.76) consisting of two major bands at pI 4.41 and pI 4.55. The Calzyme LALP preparation migrated to the extreme anodal region of the IEF gel suggesting a greater negative charge density than the other ALP preparations. A pI value of 3.85 was estimated for the LALP major band, although this value should not be considered to be definitive since this value was marginally outside the linear

Table 2
Percent remaining ALP activities of purified isoenzyme and isoform preparations after treatment with the lectin WGA and neuraminidase

	ALP activity (U/l)	Remaining ALP activity (%)	
		WGA	Neuraminidase
Calzyme LALP	435	1.5±0.7	97.0±2.0
Calzyme BALP	357	3.9±0.7	109.1±3.9
Calzyme IALP	259	48.3±8.7	87.9±2.5
B/I	377	46.4±2.3	111.7±1.8
B1x	229	27.0±2.7	116.3±1.6
B1	386	31.8±2.3	117.2±2.3
B2	278	31.1±1.3	120.7±1.6

Remaining ALP activity data are presented as the mean±SD of 10 individual assays.

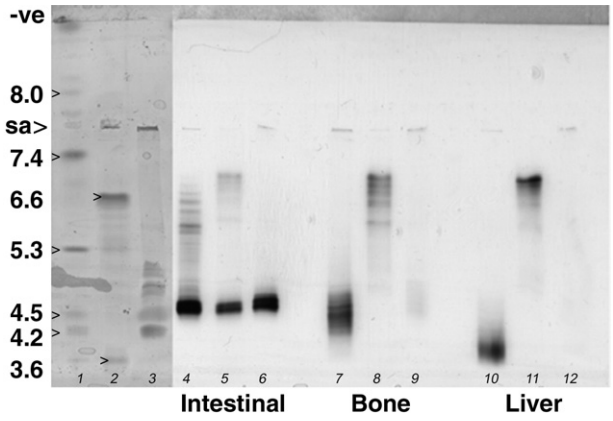

Fig. 2. Commercially prepared human IALP, BALP and LALP before and after treatment with neuraminidase and WGA respectively, separated on a broad range 3.5–9.5 pH PAGplate IEF gel. Lanes 1, 2 and 3 show the Coomassie stained IEF standard protein markers, the cathode is indicated by (-ve) and the sample application point by (sa>). Individual ALP isoforms are shown in groups of 3 lanes (native and after treatment with neuraminidase and WGA lectin respectively).

range of the pH gradient (Fig. 1). As expected, treatment with neuraminidase significantly affected the pIs of both the BALP and LALP preparations, generating similar band complexes of between pI 5.92–6.70 and pI 5.92–6.66, respectively. WGA treatment markedly reduced the gel staining for both the Calzyme BALP and LALP preparations since both are likely to have precipitated out of solution prior to their application to the IEF gel (Fig. 2).

Of the purified BALP isoforms (Fig. 3), B/I focused consistently into 9 discernable bands between pI 4.85–6.84 which represent the most cathodal (least negatively charged) of all of the BALP isoforms. Neuraminidase treatment diminished the in-gel staining of B/I, in contrast to its effects on the activity of the aqueous B/I isoform (Table 2), generating a major band at pI 6.84. Neuraminidase may have altered the biochemical and biophysical properties of the B/I isoform, which in turn could affect the interaction and/or the enzymatic reaction detection within the broad range PAGplate IEF gel. A more detailed molecular characterization of each BALP isoform might add some conclusive evidence for these contradictory results.

The native B1x, B1 and B2 isoforms migrated to pI values of 4.48, 4.32 and 4.12 respectively, indicating differences in the overall surface charge of each BALP isoform. These pI values are comparable with those of the commercial BALP preparation and the observed minor differences in pI may be explained by the different approaches used in the preparation of the isoforms. Treatment with neuraminidase consistently altered the pI values of all of the BALP isoforms to pI 6.84, similar to the value obtained for the most cathodal band of the native B/I component. Pre-treatment with the WGA lectin abolished in-gel ALP staining.

3.2. Separation of ALP isoenzymes and isoforms by HPLC

Chromatographic profiles of the Calzyme IALP, BALP and LALP preparations are shown in Fig. 4A to I. Native and neuraminidase treated IALP preparations eluted at 4.50 and 4.30 min respectively indicating minimal interaction with the anion-exchange media. After WGA treatment, residual ALP activity was detected suggesting the presence of non-sialated forms within the IALP preparation. However, the major IALP peak was greatly reduced, which was in contrast to the modest affect of WGA on IALP detected by IEF analysis. The biochemical and biophysical basis for the separation by IEF and HPLC are to some extent similar, however, not identical. The WGA treatment could, theoretically, have altered the molecular properties for IALP without causing precipitation and without affecting the active site of ALP, that is, IALP still presented as a dark stain due to ALP activity. With respect to the HPLC analysis, altered molecular properties could affect the interaction with the matrix of the analytical column and possibly even with the guard column, which could result in a strongly retained IALP to either, or both, of these columns. Both the analytical and guard columns are weak anion-exchange resins that have an average pore size of 300 Å, which allows analysis of proteins of up to 200 kDa. The BALP preparation comprised the same set of BALP isoforms (i.e., B/I, B1x, B1 and B2) as previously reported in human bone tissue extracts and serum [27]. Treatment with neuraminidase converted all BALP isoforms to a single narrow peak eluting at 4.28 min indicating minimal interaction of these desialated components with the column matrix. The LALP preparation eluted as a broad peak at 13.52 min with a peak baseline between approximately 9 and 19 min, which was again converted after neuraminidase treatment to a narrow peak eluting at 4.27 min that showed little interaction with the column matrix. In blood, three LALP isoforms (L1, L2 and L3) are generally separated by HPLC with approximate retention times between 14 and 18 min [10,29]. The endogenous release of LALP into the circulation is dependent on solubilization by GPI-PLD catalyzed by bile salts [32]. Although the majority (>95%) of this LALP preparation was soluble, other as yet uncharacterized, modifications

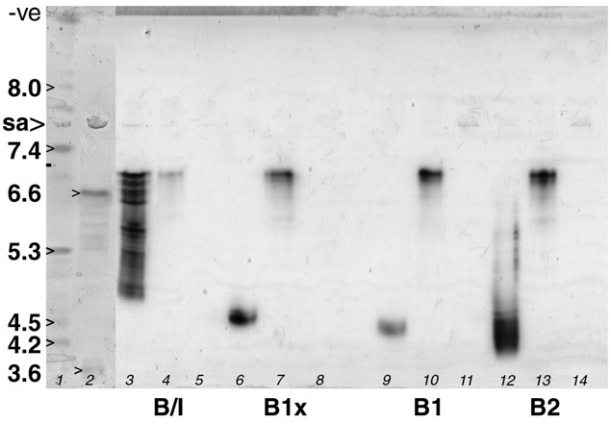

Fig. 3. Purified human BALP isoforms B/I, B1x, B1 and B2 before and after treatment with neuraminidase and WGA separated on a broad range 3.5–9.5 pH PAGplate IEF gel. Lanes 1 and 2 show the Coomassie stained IEF standard protein markers, the cathode is indicated by (-ve) and the sample application point by (sa>). Individual BALP isoforms are shown in groups of 3 lanes (native and after treatment with neuraminidase and WGA, respectively).

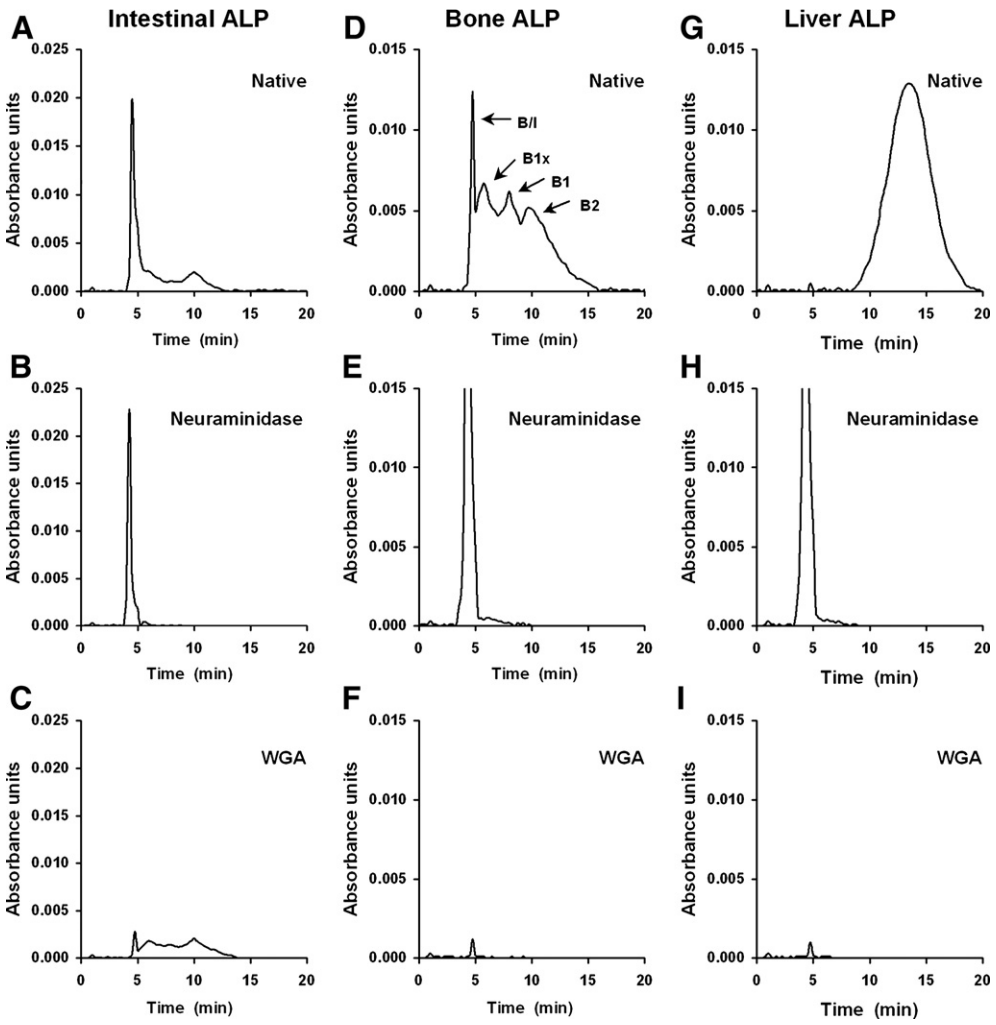

Fig. 4. HPLC profiles of the Calzyme human IALP, BALP and LALP preparations. IALP: (A) native, retention time 4.50 min; (B) after neuraminidase treatment, retention time 4.30 min; and (C) after WGA treatment. BALP: (D) native, retention times 4.85 min, 5.67 min, 7.90 min, 9.63 min for the B/I, B1x, B1 and B2 isoforms, respectively; (E) after neuraminidase treatment, retention time 4.28 min; and (F) after WGA treatment. LALP: (G) native, retention time 13.52 min; (H) after neuraminidase treatment, retention time 4.27 min; and (I) after WGA treatment. Note the differences in the scale of the *y*-axes in A, B and C.

may explain the differences in resolution between the commercial preparation and those forms identified in human serum that may conceivably be the products of various specialized hepatic tissues [18,19]. Treatment with WGA abolished separation of both the BALP and LALP preparations due possibly to either precipitation or retention of WGA/ALP aggregates by the column matrix (Fig. 4).

HPLC profiles for the purified BALP isoforms are shown in Fig. 5. All BALP isoforms eluted at characteristic elution times (see legend to Fig. 5). In contrast to the complex banding pattern on IEF (Fig. 3), the B/I isoform eluted as a single narrow peak, as previously reported for B/I in serum. From this, B/I may represent a mixture of species that interact poorly with the column matrix since they contain less sialic acid relative to the other BALP isoforms. Since the B/I isoform comprises, on average, only 4% of the total ALP activity in human serum, its clinical utility is limited. All BALP isoforms were similarly affected by neuraminidase and WGA treatments indicting the presence of terminal sialic acid residues, the amounts of which are likely to both define and dictate the elution of the individual BALP isoform (Fig. 5).

3.3. Effects of neuraminidase and WGA treatment on ALP enzymatic activity

Total ALP activity was assessed on all ALP preparations before and after treatment with WGA and neuraminidase (Table 2). Of the commercial preparations, the majority of both LALP and BALP were inhibited by WGA, which contrasts with the original report of the WGA precipitation of serum LALP [31]. The reason for this contradictory result could be due to the fact that the Calzyme preparations are purified from tissue sources and not from serum. The endogenous release of LALP into the circulation is not fully characterized and some aspects modifying the molecular properties could be overlooked (as discussed above). The Calzyme BALP preparation might be suitable for standardization and/or as a control for BALP assays,

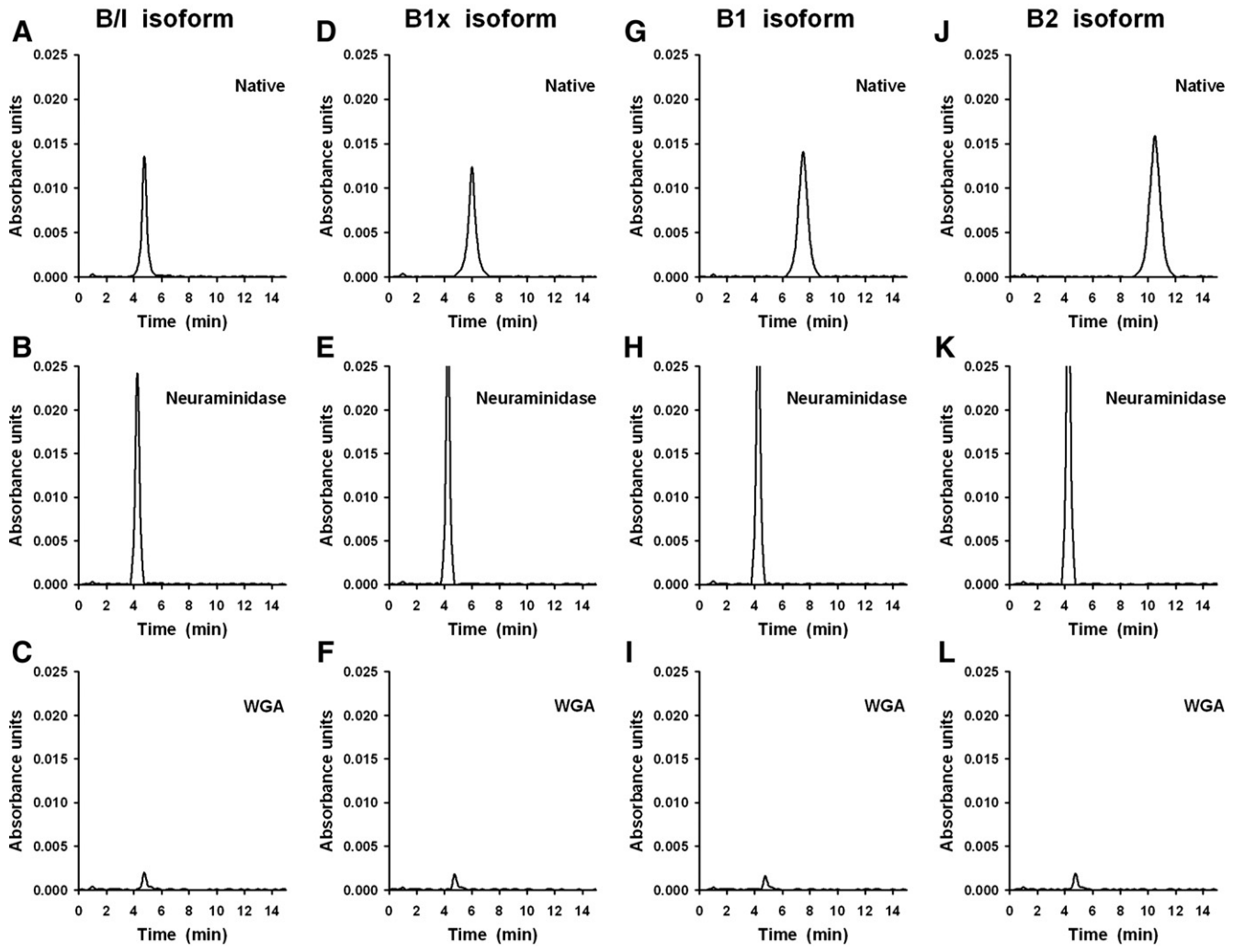

Fig. 5. HPLC profiles of the BALP isoforms B/I, B1x, B1 and B2 preparations. BALP isoform B/I: (A) native, retention time 4.75 min; (B) after neuraminidase treatment, retention time 4.25 min; and (C) after WGA treatment. BALP isoform B1x: (D) native, retention time 5.97 min; (E) after neuraminidase treatment, retention time 4.25 min; and (F) after WGA treatment. BALP isoform B1: (G) native, retention time 7.48 min; (H) after neuraminidase treatment, retention time 4.26 min; and (I) after WGA treatment. BALP isoform B2: (J) native, retention time 10.48 min; (K) after neuraminidase treatment, retention time 4.27 min; and (L) after WGA treatment.

but the LALP preparation is more questionable. However, additional aspects including both the type of bone and its anatomical location also need to be considered since the relative amounts of the B1 and B2 isoforms may differ between trabecular and cortical bone [10]. ALP isoenzyme/isoform standardization is complex and its further evaluation would be ideally suited to a Working Group format. Neuraminidase had little effect on the Calzyme LALP preparation but increased the activity of the BALP preparation. WGA inhibited about half of the IALP activity and neuraminidase reduced the activity by about 12%. In contrast to the commercial BALP preparation in which activity was reduced by 96%, individual BALP isoforms were inhibited by between 54–73% by WGA and increased by 10–20% by neuraminidase, which had a marginally greater effect on the B1x, B1 and B2 isoforms than either B/I or the commercial BALP preparation. After WGA treatment the differences observed between the total activity measured in solution (Table 2) and HPLC (Fig. 5, panels C, F, I, and L) for the BALP isoforms are likely to be due to altered molecular properties. Such changes could affect the retention and/or interaction with the column matrix with a pore size of 300 Å as discussed above for HPLC analysis of IALP after WGA treatment.

The observed differences, with respect to precipitation by WGA, between the Calzyme tissue preparations versus previously reported values for serum ALPs and purified BALP isoforms in this study are possibly due to different methods employed in their preparation. BALP purification was developed to mimic the endogenous removal of the GPI-anchor by GPI-PLD. This releases the BALP isoforms from membrane fragments so that they resemble the naturally occurring circulating isoforms [3,4]. Although the commercial ALP preparations are 100% soluble, that is they bear no cell membrane fragments, these tissue preparations may still contain residual structures, or have been inadvertently modified in someway by the proprietary procedure used by Calzyme. These are all

plausible explanations that could effect the interaction between WGA lectin and ALP isoenzymes and isoforms.

The amino acid sequence of the TNALP gene suggests five putative *N*-glycosylation sites. Nosjean et al. [6] have confirmed that both BALP and LALP are *N*-glycosylated, however, the specific number of *N*-glycosylated sites and their internal structures have yet to be determined. Moreover, their data suggest that the major difference between BALP and LALP is the presence of O-linked glycans in BALP that are absent in LALP. Both the HPLC elution pattern and the migration of the TNALP isoforms in a pH gradient are dependent on the surface charge characteristics of the individual isoform which is largely determined by the number and type of glycans added post-translationally to the TNALP protein core. Differences in terminal sialic acid residues among the TNALP isoforms are, in particular, the basis for the charge-dependent separation in methodologies such as anion-exchange HPLC and IEF. Available evidence (based on theoretical estimates) indicates that as circulating homodimers the BALP isoforms B1 and B2 contain 29 and 45 terminal sialic acid residues respectively [7], whereas the B/I component is more akin to the intestinal isoenzyme that does not contain sialic acid [30]. Removal of sialic acid residues by neuraminidase, in particular a glycan located near to the active site of each monomer [8,5] may reduce stearic hindrance allowing the substrate better access to the active site thus potentiating the activity of the enzyme. Our study only included one type of neuraminidase, however, other sialidases with specificities for different sialic acid linkages are also available and these could be used in future investigations of the BALP isoforms [33]. Although speculative, from the elution and migration characteristics of the BALP isoforms, the B1x isoform may be sialylated to an extent intermediate of that of the B/I (few residues) and the 29 residues estimated in B1. Furthermore, the degree of sialylation may itself govern the enzyme activities of the individual BALP isoforms [7,8].

3.4. Analysis of human serum TNALP isoforms

IEF of two serum samples with pathologically high total ALP activities on broad range gels are shown in Fig. 6. The ALP activity in the patient with Paget's disease of bone (lane 1) was concentrated in the region of the gel (approximately pI 4.1–4.5) corresponding to the pI of the characterized BALP isoform mixture shown in lane 3. It could be inferred from this that BALP isoforms were responsible for the elevated ALP activity, which concurs with the pathology of Paget's disease [34]. Conversely, the ALP activity in the patient with liver disease (lane 2) was concentrated at the anodal margin of the gel (approximately pI 3.8), beyond the range for assignment of an accurate pI value. This corresponds to the location of the LALP isoforms presented in Fig. 2 and is consistent with liver pathology [17]. IEF provides, in comparison with HPLC, a less complicated, less expensive (with respect to instrumentation) and less time consuming approach for the separation of human serum samples with increased total ALP activities. We were not able to achieve similar separations as previously demonstrated by the Isopal system (which is no longer commercially available) [18] since we had some overlapping between the BALP and LALP isoforms, and also among the individual BALP isoforms. However, it was possible to clarify the reason for the increased serum total ALP result. We believe the described IEF method to have the potential to achieve similar isoform separations as reported for the Isopal system but requires further optimization. We can not recommend the use of HPLC for routine purposes due the cumbersome and time-consuming methodology. However, HPLC is, in our hands, the methodology of choice for separation and quantitation of BALP isoforms for research objectives.

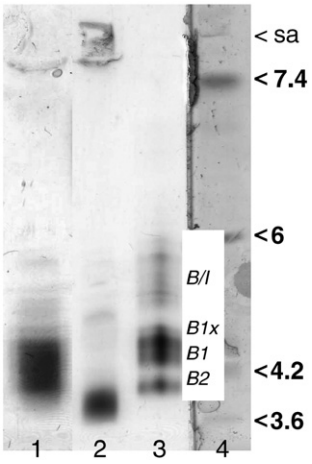

Fig. 6. Composite image of bands cut from two broad range 3.5–9.5 pH PAGplate IEF gels illustrating the migration patterns of TNALPs in: (1) serum from a patient with elevated total ALP with Paget's disease of bone (10 μl, 612 U/l); (2) serum from a patient with elevated total ALP with liver disease (5 μl, 960 U/l); (3) a mixture of the purified BALP isoforms (10 μl, 317 U/l) and; (4) Coomassie stained IEF standard protein markers.

In conclusion, our study illustrates characteristic molecular differences between the isoforms of BALP as demonstrated by the two charge-dependent methodologies IEF and anion-exchange HPLC. We have further characterized the BALP isoforms with respect to their isoelectric points and compared them with commercially available preparations of human ALP. Additionally, we have shown that the BALP and LALP isoforms may be separated by IEF using broad pH range polyacrylamide gels and may represent a simpler and more applicable approach to routine ALP isoform analysis than HPLC-based methods. Whether BALP isoforms represent specific bio-markers for the differentiation of mesenchymal cells, in particular the development of the osteoblastic phenotype, or merely reflect intermediate products of BALP biosynthesis remains to be determined. In order to fully understand the molecular differences that define the individual TNALP isoforms and their clinical significance, future work should include a detailed characterization of the N-linked and O-linked oligosaccharides.

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