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Partial sequencing of human adult, human fetal, and bovine intestinal alkaline phosphatases: Comparison with the human placental and liver isozymes

(enzymes/protein structure/genetics)

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ABSTRACT **Purification, molecular weights, amino acid compositions, and partial sequencing of intestinal alkaline phosphatases (EC 3.1.3.1) from human adult, human fetal, and bovine sources is reported. Additional sequence information is presented for the bovine liver isozyme. Comparisons are made of the partial primary structures of intestinal alkaline phosphatases with those of the isozymes from liver and placenta. Homologies among these isozymes provide structural data to corroborate some concepts of the etiology of these isozymes and refute others.**

Alkaline phosphatases [ALPases; orthophosphoric-monoester phosphohydrolase (alkaline optimum); EC 3.1.3.1] are present on the membranes of almost all animal cells. In higher primates and in man three isozymes are recognized, placental, intestinal, and liver. The liver form is also found in bone, kidney, and other tissues. Each of the isozymic forms is highly glycosylated and, on extraction, mixtures of varying degrees of glycosylated isozyme are found. In some tissues, such as placenta, the ALPase is highly polymorphic (see reviews, refs. 1 and 2). Until recently essentially all of our knowledge of isozymic differences and allelic variations were based on indirect experiments using differential inhibitors, differential heat stability, different peptide patterns produced on partial proteolysis, or different reactions with polyclonal and monoclonal antibodies. Conclusions from such experiments are interesting but are not unequivocal because of the effects of varying degrees of posttranslational modification, mainly glycosylation, on each of the above parameters. Only complete structural data of each of the isozymes and its allelic variants will permit unequivocal conclusions as to their interrelationships and genetic origins.

We have already reported the partial sequencing of human placental ALPase (3) and of the bovine (4) and human liver (5) isozymes. In this paper we report the purification, molecular weights, amino acid compositions, and partial sequencing of the intestinal isozyme from human adult, human fetal, and bovine sources. Additional sequence information is also presented for bovine liver ALPase.

MATERIALS AND METHODS

Specimens of adult human small intestine were obtained from Sloan–Kettering Cancer Research Center, New York. None of the autopsy specimens from adult subjects, who died from various tumors unrelated to intestinal neoplasia, showed evidence of malignancy according to the autopsy reports. The intestine after autopsy was stored at −70°C until use. Meconium, the first stool of newborn infants, was obtained from the Neonatal Division of Mountainside Hospital. Purified bovine intestinal ALPase was purchased from Sigma (type VII-NL). The weak anion exchanger DE Nugel (20-nm pore size) was from Separation Industries (Orange, NJ). Sephadex G-200 was from Pharmacia and the methyl-coated silica-bonded phase support (Synchropak RP-P) was purchased from Synchrom (Linden, IN). The octyl-coated silica column (LC-308, 4.6 × 20 mm) was from Supelco (Bellefonte, PA). Zorbax Bio Series GF-250 (9.4 × 250 mm) was from duPont. Materials for gel electrophoresis were from Bio-Rad. Iodo[^{3}H]acetic acid was from New England Nuclear. All other chemicals were of analytical grade.

ALPase activity was assayed at pH 9.8 in a microtiter plate by using 1.0 M diethanolamine buffer and *p*-nitrophenyl phosphate as substrate according to the instructions in the Sigma catalog. One unit of ALPase activity is defined as 1 μmol of substrate hydrolyzed per min at 37°C. NaDodSO$_4$/PAGE was performed according to Laemmli (6) and silver staining according to Morrissey (7). Molecular weights of native enzyme were determined by high performance gel filtration on a GF-250 column by using 0.1 M Na_2HPO_4 (pH 6.8) containing 0.1 M NaCl as the mobile phase. Reduction and carboxymethylation of ALPase and purification of the carboxymethylated enzyme on an octyl silica column were carried out according to Pan *et al.* (8). Amino acid analyses were performed by the fluorescamine procedure (9). Microsequencing was carried out on an Applied Biosystems Model 470A Sequencer and phenylthiohydantoin-amino acids at each cycle were identified by HPLC by using a Beckman Ultrasphere ODS column and a trifluoroacetic acid/acetonitrile buffer system (10). Deglycosylation was performed with *N*-glycanase (peptide: *N*-glycosidase F from Genzyme, Norwalk, CT) according to the supplier's instructions.

RESULTS

Purification of ALPase from Adult Human Intestine. Frozen human adult small intestine (841 g) was thawed and washed once with cold distilled water. After removing fat, the intestine was chopped into lengths of about 20 cm. The mucosa was scraped off with a glass microscope slide, frozen in liquid nitrogen, and pulverized in a mortar with a pestle. The mucosal powder was homogenized in 450 ml of buffer A (10 mM Tris·HCl, pH 7.4, containing 1 mM $MgCl_2$ and 20 μM $ZnSO_4$) for 2 min with a Polytron unit (Brinkmann), and the homogenate was then stirred at 4°C for 3 hr. Following this, 250 ml of butanol was added, and the mixture was stirred overnight at 4°C. After centrifugation at 8000 × *g* for 30 min, the butanol layer was discarded, the aqueous phase was set aside, and the pellet was reextracted for 2 hr with buffer A and recentrifuged at 8000 × *g*. The two aqueous supernatants were combined, adjusted to pH 5.0 by addition of 2 M acetic

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Abbreviation: ALPase, alkaline phosphatase.

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acid and, after stirring for 30 min, centrifuged at 8000 × *g* for 40 min. The supernatant was adjusted to pH 7 with solid Na_2CO_3, and acetone (−20°C) was added with stirring to bring the concentration of acetone to 30% (vol/vol). After centrifugation at 8000 × *g* for 20 min the precipitate was discarded and acetone (−20°C) was added to the supernatant to a concentration of 50% (vol/vol). The mixture was centrifuged at 8000 × *g* for 20 min, and the precipitated pellet was dissolved in buffer B (20 mM *N*-ethylmorpholine acetate, pH 7.4, containing 1 mM $MgCl_2$ and 20 μM $ZnSO_4$). The solution was applied to a DE Nugel column (2.5 × 10 cm, 20-nm pore size) that had been equilibrated with buffer B and was developed with the same buffer superimposed on a gradient of NaCl. ALPase activity was eluted over a broad area (Fig. 1*A*). The major peak of enzyme activity, which was eluted at 100 mM NaCl was further purified. Those fractions (100–115) with the highest specific activity were pooled and concentrated by ultrafiltration on an Amicon YM 30 membrane. The concentrated solution (1.7 ml) was then subjected to gel filtration on a Sephadex G-200 column (2.5 × 90 cm) developed with buffer B containing 100 mM NaCl. A single peak of ALPase activity was obtained (Fig. 1*B*). The fraction (no. 49) with the highest specific activity was subjected to reverse-phase HPLC on a methyl-coated silica column (4.6 × 250 mm) developed with buffer C (same as buffer B, but adjusted to pH 6.5) and superimposed on a gradient of 1-propanol. A sharp peak with high specific activity was obtained (Fig. 1*C*). Table 1 shows the summary of the purification scheme of the intestinal ALPase. The final yield of adult intestinal ALPase could actually have been much higher than shown because only those fractions with the highest specific activity at each step were used for further purification. The final specific activity of purified ALPase was 3900 units/mg of protein, and the overall purification was about 10,000-fold. Portions of the purified enzyme were carboxymethylated and further purified on an octyl silica column (4).

Extraction and Purification of ALPase from Fetal Intestine. Meconium, a rich source of fetal intestinal ALPase (15), was used as the source of the enzyme. Frozen meconium (182 g) was thawed and homogenized in 182 ml of buffer A (10 mM Tris·HCl, pH 7.4, containing 1 mM $MgCl_2$ and 20 μM $ZnSO_4$) for 1 min in a Waring Blendor. Cold butanol was added four times (25 ml each), and the suspension was homogenized for

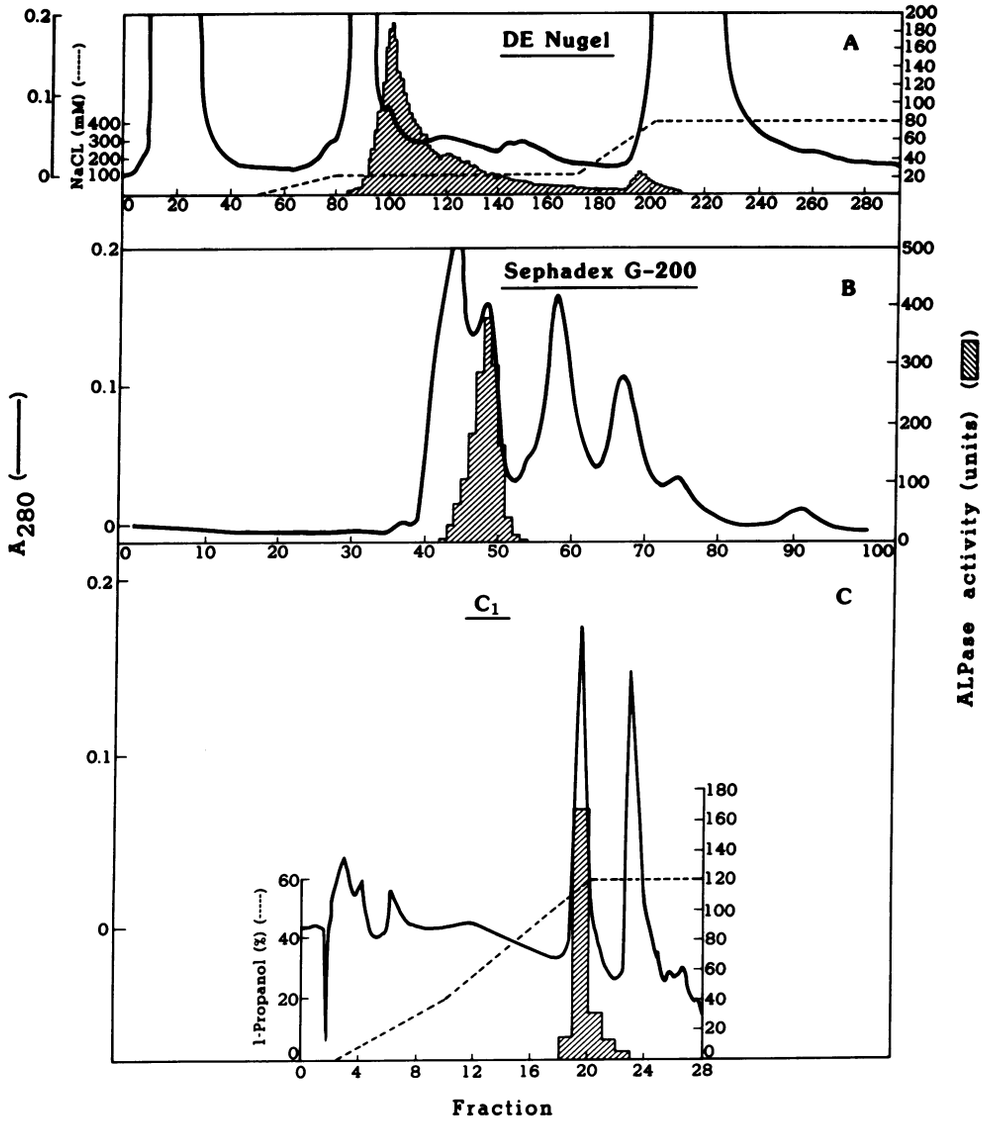

FIG. 1. Steps in the purification of human intestinal ALPase. (*A*) Chromatography on a DE Nugel column. (*B*) Chromatography of fractions 110–115 from the DE Nugel column on a Sephadex G-200 column. (*C*) HPLC of fraction 49 from the Sephadex G-200 column on a methyl-bonded (C_1) column.

Table 1. Purification of human intestinal alkaline phosphatase

Purification step	Activity, units	Protein, mg	Specific activity, units/mg	Yield, %	Purification factor
Butanol extract	4640	11,450	0.4	100	1
Acetone precipitate	3660	1,960	1.9	79	4.7
DE Nugel	1640 (2920)	17.5	94	35.3 (63)	235
Sephadex G-200	1060 (1430)	1.56	729	22.8 (30.8)	1820
C_1 HPLC	163 (763)	0.04	3920	3.5 (16.4)	9800

The scheme used to purify material for sequencing utilized at each step only the fraction or fractions with the highest specific activity. Values in parentheses represent the activity and yield at each step if all the fractions of high specific activity had been pooled.

30 sec after each addition. The homogenate was incubated at 37°C for 30 min in a shaking water bath and then centrifuged at 10,000 × *g* for 1 hr at 4°C. The butanol layer was discarded, the aqueous layer was saved, and the meconium pellet was reextracted with buffer A. The two extracts were combined and filtered through four layers of cheesecloth to remove solid debris. All subsequent procedures were carried out essentially as described for the adult enzyme. The specific activity of the purified fetal enzyme was 1590 units/mg of protein representing an overall purification of about 100-fold. Portions of this material were carboxymethylated and purified as above.

Purification of Bovine Intestinal ALPase. The purification of commercial bovine intestinal ALPase was accomplished in one step by dissolving 2 mg of the powder in 1 ml of buffer C and subjecting it to reverse-phase HPLC on a C_1 column (4.6 × 250 mm). The chromatography conditions were the same as those used for purification of human intestinal ALPase. One major and two minor peaks of enzyme activity were obtained. NaDodSO$_4$/PAGE showed that all three peaks of enzyme activity were homogeneous and had similar molecular weights (data not shown). Only the major peak, with a specific activity of 3110 units/mg was used for further analysis.

Molecular Weights of the Native ALPase and Their Subunits. Purified human and bovine intestinal ALPases were shown to be homogeneous by NaDodSO$_4$/PAGE (Fig. 2). The apparent molecular weights of the subunits of the human fetal, human adult, and bovine ALPases observed on the gels were 72,000, 72,000, and 62,000, respectively. The apparent molecular weights of native adult and fetal human ALPase as determined by HPLC (gel filtration) were approximately 143,000; that of the bovine enzyme was 127,000. These values are in good agreement with homodimeric structures consisting of two monomers each of M_r 72,000 in the case of human enzymes and M_r 62,000 for the bovine enzyme. Since ALPases are known to be highly glycosylated, the purified enzymes were treated with glycanase, an enzyme that removes asparagine-linked oligosaccharides from glycoproteins (8). Fig. 3 also shows that deglycosylation shifts the M_r values of the subunits from 72,000 to 62,000 for the human adult enzyme and from 62,000 to 57,000 for bovine ALPase. The adult form of human intestinal ALPase apparently contains twice as much N-linked carbohydrate (16.7%) as does the bovine enzyme (8%).

Amino Acid Compositions and Amino Terminal Sequences. Table 2 shows the amino acid compositions of human adult, human fetal, and bovine intestinal ALPase. The human placental isozyme is presented for comparison. The amino acid compositions of the human adult and fetal ALPases are the same, within experimental error. Intestinal and placental ALPase show remarkable similarity.

Amino terminal sequencing up to about 40 residues was obtained with all the intestinal ALPases (Fig. 4). Data for the human fetal enzyme are not shown separately because they were identical to those for the adult isozyme through residue 39. The data for bovine liver represent additional sequencing over what was reported (4).

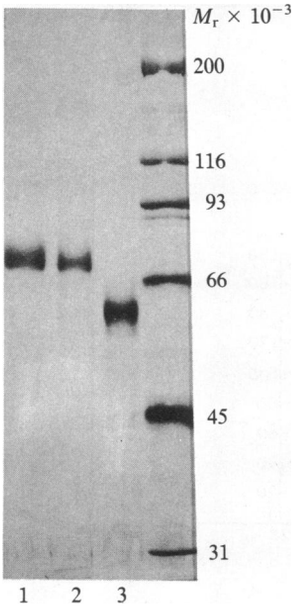

FIG. 2. NaDodSO$_4$/PAGE of purified human adult (lane 1), human fetal (lane 2), and bovine (lane 3) intestinal ALPase.

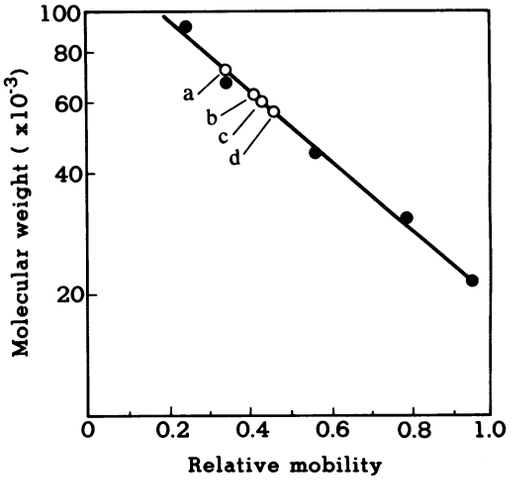

FIG. 3. Molecular weight estimation of subunits of human (a) and bovine (b) intestinal ALPase and their deglycosylated (c, human; d, bovine) derivatives (on a 9% gel). Molecular weight markers (●) are phosphorylase *b* (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), and soybean trypsin inhibitor (21,500).

Table 2. Amino acid compositions of alkaline phosphatases

Amino acid	Intestinal				
	Human adult	Human fetal	Bovine	Bovine*	Human placental†
Asx	59	64	54	61	55
Thr	40	44	33	37	40
Ser	32	38	21	24	32
Glx	64	75	45	53	60
Gly	56	58	43	45	54
Ala	70	70	58	60	66
Val	40	42	40	42	35
Met	11	10	13	13	14
Ile	20	21	14	12	18
Leu	50	52	37	38	48
Tyr	22	22	20	20	22
Phe	23	22	15	15	22
His	19	18	14	14	16
Lys	22	24	23	22	24
Arg	33	29	25	21	40
Pro	30	—	24	30	33
Trp	8	—	6	—	4
Cys	6	—	6	6	5

Values are presented as residues per mole of subunit. Each value represents the average of several analyses. Cysteine was assayed both as cysteic acid and as carboxymethylated cysteine.
*The values from Besman and Coleman (11) were recalculated.
†Values from Ezra *et al.* (3).
—, Not determined.

DISCUSSION

ALPases from human placenta (3) and from human (5) and bovine liver (4) have already been purified and appreciable sequencing of their amino termini has been obtained. With the sequencing of human and bovine intestinal ALPases sufficient structural information becomes available to begin to deduce the relationships of the human isozymes and their possible evolutionary development.

Based on a number of indirect criteria the consensus has been that fetal human intestinal ALPase differs from the adult isozyme. However, as we noted above, the fetal enzyme is apparently identical to the adult form through the first 39 residues. The claims of Komoda *et al.* (12) based on a difference at the amino terminus, therefore, are not valid. The suggestion that the fetal enzyme is a heterodimer (13) is unlikely because not only were the subunits not resolved on gels but only one residue was observed at each cycle during sequencing of the fetal enzyme. Differences between fetal and adult enzyme based on CNBr peptides reported by Volkley *et al.* (14), if substantiated by structural studies, must represent differences closer to their carboxyl termini.

Besman and Coleman (11) presented over 30 residues at the amino terminus of the bovine intestinal isozyme. These are identical to those found by us (Fig. 4). They also reported sequence differences between calf and adult bovine intestine. One of the calf sequences they reported differed from the adult form by only three residues; isoleucine for valine at residue 2, alanine for valine at residue 4, and asparagine for aspartic acid at residue 8. These appeared as minor constituents (about 15%) at the same cycles in our sequencing. Their significance became apparent to us when the findings of Besman and Coleman (11) appeared. Since our material had been obtained from a commercial source, we cannot be certain of the significance of the presence of some calf enzyme in adult bovine alkaline phosphatase.

Comparison of the amino termini of intestinal bovine ALPase with the human enzyme yields 80% homology indicating their close genetic origins. The evolution of the human placental enzyme from human intestinal ALPase has been fairly well accepted based on immunological studies, selective enzyme inhibitors and stability studies. The structural data in Fig. 4 clearly show their common origins (80% homology). Even bovine intestinal ALPase exhibits over 70% homology with the human placental enzyme.

In a report (5) we have compared human liver and placental ALPases. Although the homologies were not high (*ca.* 25%) we noted many structural similarities. In both proteins the hydrophobic dipeptide at the amino terminus is followed by a proline. From the third residue on both are helical with a probable β turn at proline-9. The intestinal enzymes follow the same pattern. What is of great interest is that there is even greater homology between human liver ALPase and the bovine intestinal enzyme than between human liver ALPase and any of the other human isozymes. The amino termini of human and bovine liver ALPase are identical through the first 21 residues, and they exhibit about 50% homology with the bovine intestinal enzyme. Good homology between bovine liver and human intestinal ALPase is also maintained as far as sequence information is available. This is of interest and provides structural data supporting current concepts that the liver and intestinal enzymes arose from a common ancestral form (1, 2). ALPase from mouse placenta, liver, and kidney appear to be identical and also exhibit great homology with the bovine and human liver enzymes (M. Terao, personal communication). The limited protein sequence data now available correct a number of mistaken conclusions drawn from indirect methods but support widely held views that the liver and intestinal ALPases arose from a common ancestral molecule. Apparently the liver isozyme has been fairly well preserved from the murine to the bovine to the human enzyme. Their homology with the liver ALPases confirms speculations that mutations to the intestinal forms occurred prior to the appearance of the higher primates. The mutation of intestinal ALPase to the human placental form, which is generally believed to have occurred after the appearance of the higher primates, was apparently a rather conservative one.

The sequence data that has been obtained provide the information necessary to clone the various ALPase cDNAs and thereby elucidate their entire structures. Only through such experimentation will it be possible to determine the number of ALPase genes in man and elucidate the structures of the many reported allelic forms (2) of the human placental enzyme. Such information will also be needed to determine

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                    5         10        15        20        25        30        35        40
PLACENTA (H)    I I P V E E E N P D F W N R E A A E A L G A A K K L Q P A Q T A A K N L I I F L G D
INTESTINE (H)   V I P A E E E N P A F W N R Q A A E A L D A A K K L Q P I Q T V A K N L I L F
INTESTINE (B)   L V P V E E E D P A F W N R Q A A Q A L D V A K K L Q P I Q T A A K N V I L F L G D ? M
LIVER (B)       L V P E K E K D P K Y W R D Q A Q Q T L K N A L R L Q T I N T N V
LIVER (H)       L V P E K E K D P K Y ? R D Q A Q Q T L K Y
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FIG. 4. Amino terminal sequences of human (H) and bovine (B) ALPases. Data for human placenta are from Ezra *et al.* (3) and for human liver from Garattini *et al.* (5). Each protein was subjected to three independent sequence analyses.

the mechanism of biosynthesis of ALPases and how they are inserted into the cell membrane.

Note Added in Proof. After this paper was submitted, Culp *et al.* (16) reported sequencing of 16 residues at the amino terminus of bovine intestinal ALP. Their sequence was identical to the one reported in this paper.

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