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Human placental alkaline phosphatase in liver and intestine

(immunoassay/peptide/haptene/isozymes)

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ABSTRACT **Three distinct forms of human alkaline phosphatase, presumably isozymes, are known, each apparently associated with a specific tissue. These are placental, intestinal, and liver (kidney and bone). We have used a specific immunoassay and HPLC to show that placental alkaline phosphatase is also present in extracts of liver and intestine in appreciable amounts.**

Based on immunological and chemical (1) findings, human tissues contain three distinct forms of alkaline phosphatase (ALPase). These are placental, intestinal, and liver. Bone and kidney ALPase appear to be variants of the liver enzyme, having the same peptide chain but differing in their carbohydrate moieties (2–5). There are also a number of variants of the placental enzyme (6, 7). All different tissue forms of ALPase may represent isozymes. As with other isozymes it is possible that rather than being limited to a given tissue the ALPase isozymes are widely distributed but present in differing proportions in different tissues, depending on cell type and cell-specific regulatory factors (8).

In an earlier study we reported the purification of human placental ALPase and the sequencing of 42 residues at its amino terminus (9). This information made possible the synthesis of a short peptide sequence that was used to generate an antiserum specific for the amino terminus of placental ALPase. By a combination of immunoassay and HPLC we have been able to demonstrate appreciable amounts of placental ALPase in extracts of human liver and intestine.

MATERIALS AND METHODS

Tissues. Frozen tissues from autopsied subjects were obtained from the Tumor Procurement Center of the Sloan–Kettering Institute (New York). The livers were used intact, whereas intestinal mucosa was used after separation from the tunica muscularis. Subjects 1 (76 yr) and 3 (44 yr), from whom liver was obtained, were diagnosed as having leukemia; subject 2 (77 yr) had no evidence of malignancy; subject 4 (74 yr), from whom liver and intestinal samples were obtained, was shown to have a brain tumor. All subjects were male. None of the tissues used in this study showed evidence of malignancy according to the autopsy reports.

Extraction and Anion-Exchange Chromatography. Total ALPase in fresh term placenta, liver, and intestine was obtained by extraction of homogenates with 1-butanol and subsequent precipitation of the proteins in the aqueous phase with acetone according to standard procedures (10). However, the major source of human placental ALPase used in these studies was a partially purified enzyme prepared from pooled tissues (type XXIV) by Sigma. The latter supposedly represents a mixture of allelic variants of placental ALPase. Material, equivalent to 100 units of ALPase, was dissolved in 20 mM *n*-ethylmorpholine acetate buffer (pH 7.4) containing 1 mM $MgCl_2$ and 20 mM $ZnSO_4$ and loaded at 4°C on a 1 × 10 cm Mono Q HPLC column (Pharmacia). Elution was carried out with a gradient of NaCl (0–400 mM) in the same buffer. The flow rate was 0.6 ml/min and 2-ml fractions were collected.

Assay of ALPase Activity. ALPase activity was determined according to the instructions in the Sigma catalog with *p*-nitrophenyl phosphate as substrate. One unit of enzyme activity corresponds to 1 μmol of substrate hydrolyzed per minute at 37°C.

Specific RIA for Placental ALPase. Placental ALPase was determined with a specific RIA that makes use of a polyclonal antiserum directed against the amino terminus of placental ALPase. The antibody was produced by using the synthetic peptide Ile-Ile-Pro-Val-Glu-Glu-Glu-Asn-Pro-Phe-Gly-Cys (ALPase haptene) conjugated to hemocyanin as an immunogen. The first nine amino acids of the peptide represent the amino terminus of placental ALPase. The Phe-Gly-Cys residues were used to link the haptene to the hemocyanin through the sulfhydryl group of the cysteine (11). Tyrosine was coupled to the ALPase haptene through the sulfhydryl group to provide a site for labeling with ^{125}I. Details of the production of the antiserum in rabbits and the characteristics of the RIA will be published elsewhere. As would be expected, the antiserum crossreacts with the placental ALPase tryptic cleavage product, which corresponds to the 14-residue amino-terminal peptide (placental amino-terminal peptide, PATP) ending with an arginine residue (Ile-Ile-Pro-Val-Glu-Glu-Glu-Asn-Pro-Asp-Phe-Trp-Asn-Arg). All RIAs reported in this paper were carried out after tryptic cleavage of the ALPase. The RIA was carried out in phosphate-buffered saline containing 0.1% bovine serum albumin, 0.1% Triton X-100, 10 mM 2-mercaptoethanol, 10 μl of ^{125}I-labeled ALPase haptene (specific activity, ≈80 Ci/μmol; 1 Ci = 37 GBq), and 10 μl of a 1:1200 dilution of the immune serum. The sample to be assayed (10 μl) was added and, after incubation at 4°C overnight, the immune complex was precipitated with IgGsorb (The Enzyme Center, Malden, MA). The supernatant fluid was removed by aspiration and radioactivity was measured in a γ counter. Calculations of PATP were based on a standard curve prepared by using the peptide as the displacing agent. The linear range of the assay was from 10 to 100 fmol of PATP.

Treatment with Trypsin to Generate the PATP in Partially Purified Extracts. Individual or pooled fractions after Mono Q chromatography were trypsinized at 37°C overnight with an amount of trypsin found to result in maximal release of immunoreactive peptide(s) (50 μg of trypsin per 1000 μg of total protein). Samples were then heated at 100°C for 30 min

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Abbreviations: ALPase, alkaline phosphatase; PATP, placental amino-terminal peptide.

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to inactivate the trypsin and, in the case of placenta, were assayed directly after appropriate dilution in RIA buffer. For liver and intestine, desalting and concentration were required prior to RIA. The trypsinized mixtures were dried in a Savant Speed-vac concentrator, resuspended in equal volumes of 0.5 M pyridine/acetate buffer, pH 4.2, and passed twice through a C_{18} cartridge (Sep-Pak, Waters Associates) to adsorb PATP. Cartridges were then washed with 15 ml of 0.5 M pyridine/acetate, pH 4.2, to remove residual NaCl. PATP was subsequently eluted with 4 ml of the pyridine/acetate buffer containing 30% propanol. The eluted material was dried down and resuspended in 400 μl of RIA buffer, and aliquots were taken for assay.

The overall recovery of generated immunoreactive peptide(s) after desalting and concentration was 40–60% from liver eluates and about 80% from placenta. This was based on both RIA and recovery of ^{125}I-labeled haptene used as an internal standard. Values are presented without correction.

Chromatography of Generated PATP on HPLC Reversed-Phase Columns. One-hundred-microliter aliquots of trypsinized, desalted, and concentrated samples, to which ^{125}I-labeled markers (oxidized [Met]enkephalin, [Met]enkephalin, [Leu]enkephalin, and the haptene) were added, were chromatographed on 4.6 × 250 mm stainless steel HPLC columns of either octyl- or diphenyl-coated silica (Nucleosil 100-5, Macherey & Nagel; 5-μm particle size; 10-nm pore size). Peptides were eluted in 0.5 M pyridine/acetate buffer, pH 4.2, with a superimposed gradient of propanol (0–30%). The columns were run at a flow rate of 0.6 ml/min and 2.1-ml fractions were collected. Recoveries of both the radioactive markers and of generated PATP were excellent on these column (≈85% in each case).

RESULTS

Placental Extracts. When a partially purified, commercial sample of placental ALPase was chromatographed on a Mono Q column at least six bands of enzyme activity were observed (Fig. 1, P). Each of these bands, when treated with trypsin, yielded a peptide(s) that crossreacted with the placental ALPase antiserum. The ratio, units of ALPase activity per pmol of PATP generated, was calculated for each Mono Q band. As shown in Table 1, line 1, the mean value (±SEM) was 0.095 ± 0.021 with most ratios. When an extract from a single fresh term placenta was carried through the same procedure, only one peak of enzyme activity appeared, which coincided with the immunoreactivity. The ratio of ALPase activity per pmol of PATP in this case was 0.07.

To further characterize the peptide generated from placental ALPase as PATP, pooled fractions from the Mono Q column were subjected to HPLC on an octyl-coated silica column. As shown in Fig. 2, P, two immunoreactive peptides were separable. One of them coincided with synthetic PATP; the other has not yet been identified and its possible identity will be discussed below. When the pooled fractions from the Mono Q column were subjected to HPLC on a diphenyl-coated silica column two immunoreactive peptides were again separable, one of them coinciding with synthetic PATP (Fig. 3, P). The proportions of PATP and the second crossreacting peptide in the placental controls (P) shown in Figs. 2 and 3 are not the same because they represent different placental fractions from Fig. 1, P (see *Discussion*).

Placental ALPase in Human Liver. Crude extracts of liver when treated with trypsin yielded material that crossreacted with antiserum to placental ALPase. When the extracts were subjected to chromatography on a Mono Q column several peaks of ALPase activity appeared. Fig. 1, L2–L4, represents data obtained from liver extracts from four different subjects. In all four most of the ALPase activity (70–75% of the total) was the first to be eluted. This major peak did not yield

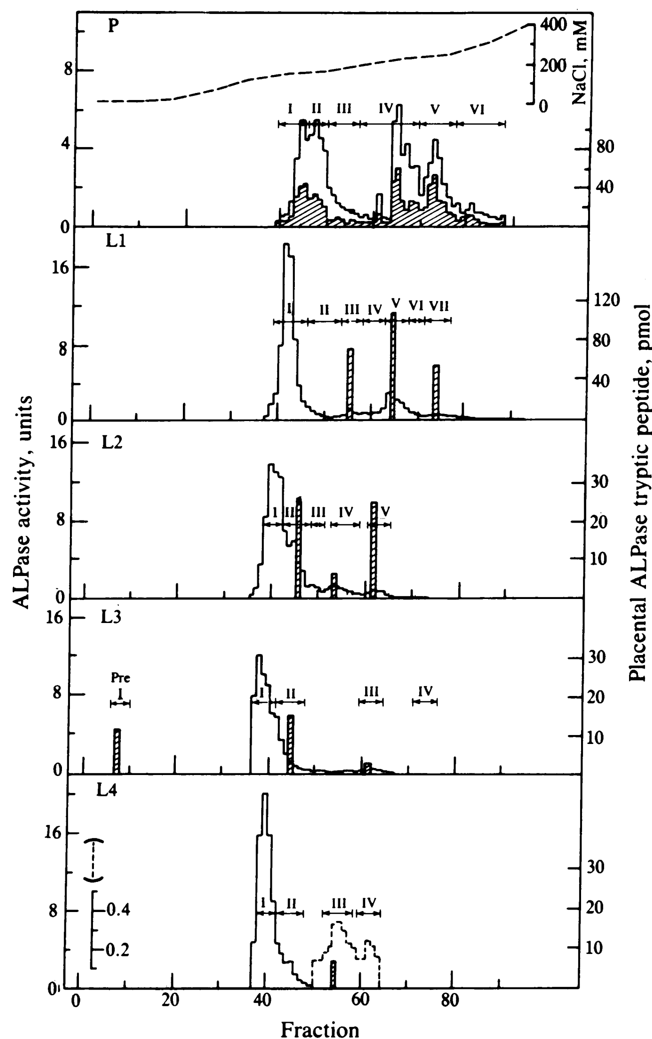

FIG. 1. Chromatography of human placental and liver extracts on Mono Q columns. P is the placental extract and L1–L4 are the extracts of livers from the four subjects. Open bars represent ALPase activity and hatched bars represent PATP. The arrows indicate the fractions that were pooled to assay PATP. The broken lines in panel L4 represent ALPase activity measured at increased sensitivity shown in the inset scale.

PATP-crossreacting material on treatment with trypsin. However, the forms of ALPase that eluted more slowly from the Mono Q columns in almost all cases yielded tryptic material that crossreacted with the PATP antiserum. In many extracts the ratios of ALPase activity per pmol of PATP gave values comparable to those obtained from placental extracts (Table 1). The higher ratios observed in some pooled fractions (livers 2 and 3 and intestine) probably indicate the presence of additional nonplacental forms of ALPase.

An unexpected finding was that immunoreactive PATP appeared in areas of the chromatogram that contained no enzyme activity (Fig. 1, L3, Pre I). This was inadvertantly discovered when these regions were used as negative controls. The material generated from the Pre I peak was identified as PATP by HPLC on two systems (see below). It apparently represents denatured or fragmented placental ALPase.

To further identify the putative PATP generated from the Mono Q eluates of the liver extracts (Fig. 1), each of the numbered pools was subjected to sequential reversed-phase HPLC on octyl- and diphenyl-coated silica columns. The results obtained on chromatography of the tryptic peptides of peaks III, V, and VII from extracts of subject 1 (Fig. 1, L2)

Table 1. Placental ALPase in liver and intestine

Tissue	Sample*	Enzyme activity, units	PATP,† pmol	Unit/pmol	Placental ALPase as fraction of total enzyme,‡ %
Placenta	Total§	135.8	1436	0.095 ± 0.021¶	100
Liver 1	I	50.4	—	—	26
	II	7.3	—	—	
	III	2.7	75	0.04	
	IV	3.4	—	—	
	V	11.7	110	0.11	
	VI	6.1	—	—	
	VII	6.0	57	0.11	
	Total	87.6	242		
Liver 2	I	48.4	—	—	6.3
	II	25.2	26	0.10	
	III	3.6	—	—	
	IV	6.4	7	0.90	
	V	4.5	25	0.18	
	Total	88.1	58		
Liver 3	Pre I	—	13	—	4.9
	I	39.2	—	—	
	II	18.9	15	1.30	
	III	1.9	3	0.60	
	Total	60.0	31		
Liver 4	I	60.0	—	—	0.9
	II	13.5	—	—	
	III	1.6	7	0.23	
	Total	76.0	7		
Intestine	I	25.4	—	—	3.0
	II	6.8	—	—	
	III	4.1	3	1.37	
	IV	0.4	9	0.05	
	V	1.9	—	—	
	Total	38.6	12		

*Represents the fractions that were pooled for assay in Figs. 1 and 4.
†Assuming that the more hydrophobic immunoreactive tryptic peptide is equivalent to PATP in the immunoassay.
‡[(Total PATP × 0.095)/total enzyme activity] × 100, where 0.095 is the average unit of the pooled fractions per pmol shown in Fig. 1 P. The value for placenta is arbitrarily taken as 100%.
§The sum of the ALPase activities and measured PATP in each pooled fraction shown in Fig. 1 P.
¶Mean ± SEM.

on an octyl-coated silica column are shown as an example (Fig. 2) along with material obtained from placenta. Note that the same two crossreacting peaks appeared in both liver and placental extracts. However, peak V yielded only PATP. The significance of this will be discussed later. Examples of the behavior of the putative PATP on diphenyl-coated silica HPLC are shown in Fig. 3. Peaks II and V from the Mono Q chromatography of extracts of subject 2 (Fig. 2, L2) each yielded essentially one crossreactive tryptic peptide that coeluted with PATP.

Placental ALPase in Intestine. Extracts of intestine, on treatment with trypsin, crossreacted with the antiserum. When the extracts were chromatographed on a Mono Q column most of the enzyme activity appeared in peaks I and II (Fig. 4), which, on trypsinization, did not crossreact with the antiserum. The material in peak III, which represented about 11% of the total ALPase activity, did crossreact with the antiserum after trypsinization. When the trypsin digests were subjected to HPLC on octyl- and diphenyl-coated silica columns one crossreacting peptide was observed that behaved exactly as PATP (data not shown). Although peak IV had essentially no ALPase activity, it yielded a significant amount of immunoreactivity after trypsinization that migrated with synthetic PATP on octyl- and diphenyl-coated silica HPLC.

Amounts of Placental ALPase in Other Tissues. From the data shown in Table 1 it was possible to estimate what fraction of total ALPase activity in extracts of liver and intestine represents placental enzyme. The average of assays on all of the pooled fractions from commercial and fresh placenta yielded a ratio of 0.095 unit of enzyme per pmol of PATP. This value was used to estimate the fraction of enzyme activity due to placental ALPase in the two other tissues by the calculations shown in Table 1. The values for liver and intestinal extracts ranged from about 1% to 26%. These may be an overestimation because placental enzyme is apparently extracted in higher yield than the liver or intestinal enzyme. In the case of the liver from subject 1 the amount of enzyme activity extracted was unusually low. This may have contributed to the high proportion of placental enzyme. On a weight basis liver contains 0.9–2.6 pmol of placental ALPase per g and intestine contains 6.4 pmol/g. This compares to 200 pmol of placental ALPase per g of placenta.

DISCUSSION

Since expression of most isozymes is generally not limited to a single tissue one might expect that each of the isozymes of ALPase should appear even in tissues that largely contain another form. The present findings show that placental ALPase is present in both liver and intestine. It should be noted that all of these subjects were males. It is conceivable that at least some of the previous reports of immunocross-

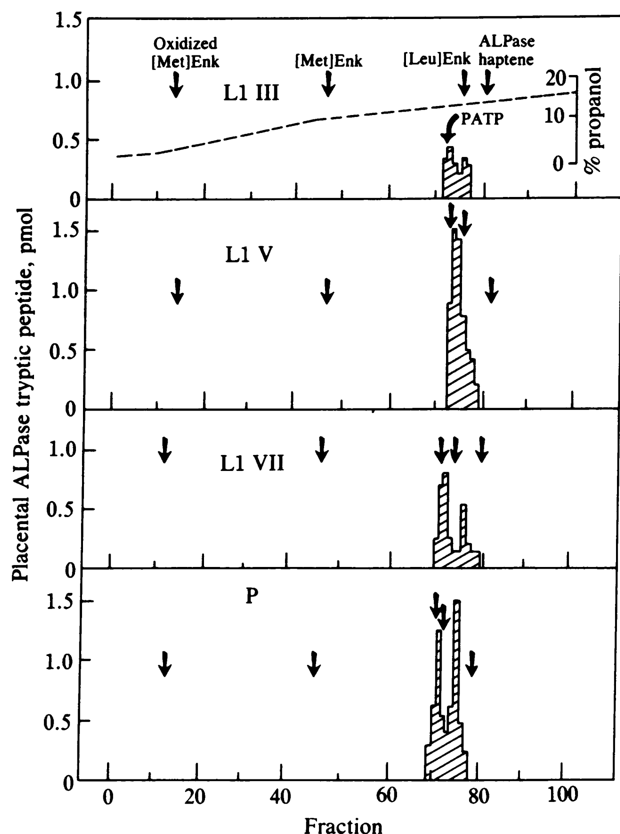

FIG. 2. HPLC of immunoreactive tryptic products from liver and placenta on an octyl-coated silica column: L1 III, L1 V, and L1 VII represent the pooled fractions in Fig. 1, L1. P represents pooled fraction II in Fig. 1, P. Enk, enkephalin.

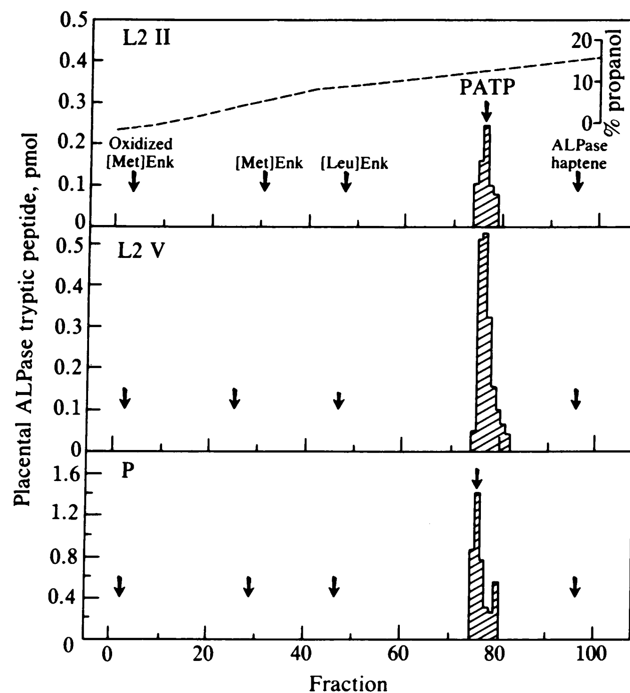

FIG. 3. HPLC of immunoreactive tryptic products from liver and placenta on a diphenyl-coated silica column. L2 II and L2 V represent the corresponding pooled fractions in Fig. 1, L2. Enk, enkephalin.

reactivity among the ALPases (12, 13) may be due to the presence of the placental enzyme in purified preparations of intestine and liver ALPase. It will be interesting to see whether the other isozymes are also widely expressed.

Other explanations for the presence of placental ALPase in liver and intestine must be considered. One possibility is contamination with blood. However, blood normally contains little placental ALPase, particularly in males. Some of the subjects (1, 2, and 4) had had malignancies and it is conceivable that the placental ALPase could be of tumor origin. However, subject 2 had no diagnosis of malignancy, yet his liver contained fairly large amounts of placental ALPase.

It may be worth repeating that the experimentally determined proportions of placental ALPase in extracts of liver and intestine could be somewhat higher than those actually present in the intact tissue since the procedures used for extraction of the enzyme give higher yields with placenta than with liver or intestine (unpublished observations).

The immunoassay detected placental ALPase in regions of Mono Q chromatograms that contained no enzyme activity, such as the Pre I pooled fractions Fig. 1, L3, and Fig. 4, IV. In both cases the PATP generated by trypsin was identified by its behavior on two different HPLC systems. The proteins from which the PATP was generated probably represent denatured or degraded forms of placental ALPase that would not have been detected by any other method. It should be noted that only a few of the fractions shown in Figs. 1 and 4 were tested for denatured placental enzyme so that much more could have been present in the tissues.

The two separable forms of placental ALPase obtained by Ezra et al. (9) yielded the same amino-terminal sequence through 42 residues. In a subsequent study by Abu-Hasan and Sutcliffe (14) three forms of placental ALPase were separated. Sequencing of all three forms through residue 13 was identical to that reported by Ezra et al. (9). One would therefore have expected that PATP would be the only immunoreactive peptide cleaved from placental ALPase by trypsin. The second, and slightly more hydrophobic, tryptic peptide may represent the action of a peptidase on PATP during isolation. However, it may very well be that the more hydrophobic tryptic peptide is derived from an, as yet, uncharacterized variant that differs only slightly at the amino terminus. It is of interest that the six forms of placental ALPase separated from extracts of mixed placenta on a Mono Q column (Fig. 1 P) yielded varying ratios of the two tryptic peptides. As shown in Figs. 2 and 3 some of the pooled fractions separated on Mono Q columns yielded exclusively PATP. The enzyme from a single fresh term placenta, which showed a single peak on a Mono Q column, yielded mainly PATP on trypsinization but it did contain a small but significant amount of the more hydrophobic peptide. Cloning and sequencing of placental ALPase cDNA will probably be the best way to determine the nature of the additional tryptic peptide.

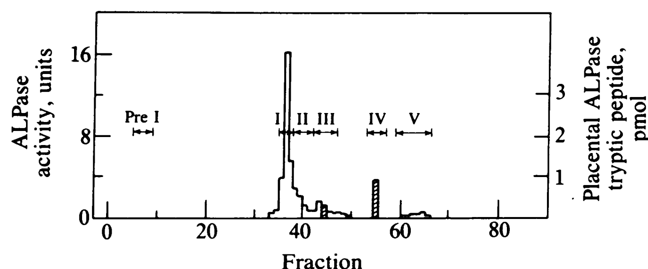

FIG. 4. Chromatography of human intestinal extracts on a Mono Q column. Open bars represent ALPase activity and hatched bars represent PATP. The arrows represent fractions that were pooled to assay PATP.

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