Alkaline Phosphatase from Human Milk

COMPARISON WITH ISOENZYMES FROM PLACENTA AND LIVER

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Alkaline phosphatase was partially purified from human milk and its antigenic, functional and structural properties were characterized. By immunochemical and enzymic criteria, the enzyme resembled the alkaline phosphatase isoenzyme found in human liver. Two-dimensional electrophoretic analysis showed that the milk enzyme differed from the liver both in subunit molecular weight and in isoelectric point. These differences were shown to result from variation in sialic acid content.

The enzyme alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) is a membrane-bound glycoprotein (Steck & Wallach, 1970) whose exact biological role has not been defined. The enzyme has been found in a variety of tissues, and multiple molecular forms, which are characteristic of selected tissues or cell types, have been documented (for review see Moss, 1970; Fernley, 1971; Fishman, 1974). At least two structurally distinct human alkaline phosphatases have been defined on the basis of peptide 'maps' and *N*-terminal analysis: that found in the full-term placenta and that found in the adult liver (Badger & Sussman, 1976). Although differences in molecular weight, electrophoretic migration, antigenic determinants and response to specific inhibitors have been frequently used to aid analytical detection and demonstrate structural differences (Fishman, 1974), such methods lack specificity owing to immunochemical cross-reactivity and variation in post-translational glycosylation of the peptide moiety (Sussman, 1978). The relationships among isoenzymes of different tissue origin can be more readily defined by probing the structure of the peptide core of the enzyme.

Recent studies have suggested that alkaline phosphatase isoenzymes may be useful in detection and evaluation of cancer of the breast (Coombes *et al.*, 1977). However, no comparative studies of the isoenzyme from normal breast have been done to serve as a control tissue reference. In human breast tissue, alkaline phosphatase is located on the luminal surface of the epithelial cells of the ducts and acinar glands. During lactation, the milk-fat globules are produced by evagination of the plasma membrane from these cells and represent naturally formed vesicles of this cell organelle (Dowben *et al.*, 1967; Saacke & Heald, 1974; Patton & Keenan, 1975). The availability of human breast milk provides a good source for purification and characterization of

alkaline phosphatase from human breast epithelium. Purification and characterization of bovine milk alkaline phosphatase have been previously described (Linden *et al.*, 1974).

In the present study, we report the characterization of alkaline phosphatase from human milk. Functional, antigenic and structural analyses of this enzyme indicate that the breast-type alkaline phosphatase is the same protein species as the enzyme found in adult human liver.

Materials and Methods

Preparation of alkaline phosphatase from human milk

Samples (100–200 ml) of milk were collected from lactating mothers 10–15 weeks *post partum* and stored at -20°C until used. Cream was separated from each sample by centrifugation (6000g, 10 min) and washed twice with buffer A [0.25 M-sucrose/50 mM-Tris/HCl (pH 7.4)/2 mM-MgCl₂]. After the final wash, the cream was again suspended in this buffer and extracted with 20% (v/v) butan-1-ol as described in Morton (1954). After gentle agitation at 37°C for 60 min the aqueous phase was separated by centrifugation (6000g, 10 min). The butanol phase was re-extracted with half the original volume of buffer A. The aqueous phases were pooled, dialysed against buffer B (10 mM-Tris/HCl, pH 7.4) overnight, and loaded on a 10 ml column of DEAE-agarose (Bio-Rad, Richmond, CA, U.S.A.). Enzyme activity was eluted with 10 mM-Tris/HCl (pH 7.4)/30 mM-NaCl. Alkaline phosphatase activity was assayed as described by Bessey *et al.* (1946), and protein was determined by the method of Lowry *et al.* (1951). One unit of enzyme activity hydrolyses $1\ \mu\text{mol}$ of *p*-nitrophenyl phosphate/min at 37°C . Alkaline phosphatases from term placenta or adult liver were

purified as described previously (Badger & Sussman, 1976).

Immunochemical assay

Immunoassays for alkaline phosphatase isoenzymes were done as described by Sussman *et al.* (1968). For [^{32}P]P_i labelling and two-dimensional gel electrophoresis, immunoprecipitation was done as above except that a high-titred rabbit anti-goat γ -globulin (Antibodies, Davis, CA, U.S.A.) was substituted as the second antibody.

Enzyme-inhibition studies

Samples (0.01 unit) of enzyme were assayed in the presence of different concentrations of L-phenylalanine, L-homoarginine, or levamisole {L-(—)-2,3,5,6-tetrahydro-6-phenylimidazo[2,1-*b*]thiazole; Pitman-Moore, Washington Crossing, NJ, U.S.A.}. After 30 min at 37°C, the reaction was stopped with 0.02M-NaOH and the inhibition relative to an untreated control sample calculated.

[^{32}P]P_i labelling and two-dimensional polyacrylamide gel electrophoresis

Enzyme (0.02–0.05 unit of activity) was immunoprecipitated as described above. The precipitates were washed three times with buffer C [20 mM-Tris/HCl (pH 7.4)/150 mM-NaCl/2 mM-MgCl₂] then re-suspended in 200 μl of this buffer. Samples were labelled with 100–200 μCi of [^{32}P]P_i (carrier-free, New England Nuclear, Boston, MA, U.S.A.) as described by Milstein (1964) and modified by Wada *et al.* (1977). The acetone-washed denatured and labelled samples were then subjected to sequential isoelectric focusing and electrophoresis on sodium dodecyl sulphate/polyacrylamide gels as described fully by Wada *et al.* (1977).

Results and Discussion

Table 1 shows results from an immunoprecipitation assay of the post-butanol fraction from seven different milk donors compared with purified placental and liver isoenzymes. The relative percentage of activity corresponding to a specific isoenzyme is determined as the difference in soluble enzyme activity between precipitation with non-immune serum and precipitation with antiserum made against either placental or liver isoenzyme. The antisera used in this assay are specific for the corresponding enzymes and demonstrate no cross-reactivity between them. The amount of milk isoenzyme that precipitates with anti-(liver isoenzyme) serum varies from 87 to 97%. Because the accuracy of the assays is $\pm 10\%$, the small amount of activity that precipitates with anti-(placental isoenzyme) serum can be considered negligible. The consistent lack of >97% precipitation in all seven samples may indicate some differences between liver and milk isoenzymes, but the antigenic similarity between these enzymes and their ready distinction from placental isoenzyme indicate that the liver and milk isoenzymes may be closely related.

Organ-specific alkaline phosphatase isoenzymes have been shown to have differential sensitivity to a variety of inhibitory compounds (Fishman & Sie, 1971). L-Phenylalanine is an effective inhibitor of placental isoenzyme and only mildly affects liver isoenzyme. L-Homoarginine and levamisole (Van Belle, 1976), a veterinary anti-helminthic agent, show opposite specificity towards these two isoenzymes. Inhibitory effects of these three agents on pure or partially pure enzymes from placenta, liver and milk were studied over a range of concentrations for each agent. Milk and liver isoenzymes are similar in their mild response to L-phenylalanine (49 and 32% inhibition respectively, at 10 mM), whereas the placental isoenzyme is more severely inhibited (87%)

Table 1. *Immunochemical assay of milk alkaline phosphatase*

Alkaline phosphatase activity (1×10^{-3} – 2×10^{-3} unit) was added to each tube. The amount precipitated is calculated as the difference between control tubes (non-immune serum) and tubes containing specific antisera. For details of calculation of relative activity see the text.

Donor	$10^{-3} \times$ Soluble enzyme activity (units)			Relative activity (%)	
	Non-immune	Anti-(placental isoenzyme)	Anti-(liver isoenzyme)	Placental isoenzyme	Liver isoenzyme
S. P.	1.1	1.1	0.1	—	91
C. J.	1.3	1.3	0.2	—	87
S. H.	1.9	1.7	0.1	10.5	97.4
S. J.	2.1	2.1	0.2	—	89.5
A. R.	1.9	1.9	0.1	—	95.3
H. S.	2.2	2.2	0.2	—	92
R. S.	1.0	1.0	0.1	—	90

inhibition at 10mM). Inhibitory activity of both L-homoarginine and levamisole supports the apparent close relationship between the milk- and liver-derived isoenzymes (10mM-L-homoarginine: milk isoenzyme, 94% inhibition; liver isoenzyme, 85% inhibition; placental isoenzyme, 33% inhibition; 0.1mM-levamisole: milk isoenzyme, 85% inhibition; liver isoenzyme, 78% inhibition; placental isoenzyme, no inhibition). Enzymes purified from two separate donor samples show identical responses to these compounds. The similar functional properties of liver and milk isoenzymes in response to specific inhibitors support the relationship implied by their immunochemical cross-reactivity. However, as with the antigenic properties, small kinetic differences suggest minor structural differences as well.

Because of overlap in catalytic properties between isoenzymes and cross-reactivity among various antisera, verification of their identity demands more stringent criteria. To satisfy the uncertainty inherent in these previous methods, we characterized milk

alkaline phosphatase by isoelectric focusing and electrophoresis in sodium dodecyl sulphate/polyacrylamide-gel slabs after affinity labelling of the enzyme active centre with [^{32}P]P_i. Fig. 1 shows two-dimensional radioautograms of immunoprecipitates of purified placental and liver isoenzymes and the post-butanol fraction of milk isoenzyme. Fig. 1(a) shows a gel of liver isoenzyme precipitated with anti-(liver isoenzyme) serum. This isoenzyme focuses at an isoelectric point (pI) of 5.4–6.0 and has an apparent molecular weight ($\bar{M}_r$) of 81 000. Fig. 1(b) shows placental isoenzyme precipitated with anti-(placental isoenzyme) serum and this isoenzyme has a pI of 5.9–6.0 and a $\bar{M}_r$ of 64 000. Surprisingly, milk isoenzyme (Fig. 1c) runs in this gel system in a unique position, with a more basic pI (6.3–6.7) and a $\bar{M}_r$ (72 000) intermediate between those of the placental and liver isoenzymes. Fig. 2(d) shows co-electrophoresis of all three labelled immunoprecipitates and confirms their non-overlapping distinct positions in the gels. In this last gel the apparent relationship between

Fig. 1. Two-dimensional electrophoretic radioautograms of [^{32}P]P_i-labelled alkaline phosphatase from liver, placenta and milk. Placental isoenzyme (0.02 unit) and liver and milk isoenzymes (0.05 unit) were immunoprecipitated with 2 or 5 μl of anti-(placental isoenzyme) or anti-(liver isoenzyme) serum respectively. The washed immunoprecipitates were labelled with [^{32}P]P_i and subjected to two-dimensional electrophoresis in polyacrylamide gels as described in the Materials and Methods section. (a) Placental isoenzyme, anti-(placental isoenzyme); (b) liver isoenzyme, anti-(liver isoenzyme); (c) milk isoenzyme, anti-(liver isoenzyme); (d) placental isoenzyme, anti-(placental isoenzyme); milk and liver isoenzymes, anti-(liver isoenzyme).

Fig. 2. Two-dimensional electrophoretic radioautograms of neuraminidase-digested milk and liver isoenzymes

Liver or milk isoenzymes (0.05 unit) were immunoprecipitated with or without previous digestion by 0.1 mg of neuraminidase/ml (*Clostridium perfringens*; Worthington Biochemical Corp., Freehold, NJ, U.S.A.) for 10 min at 37°C. The reaction was stopped by cooling on ice. Electrophoretic analysis was as described in Fig. 1. (a) Liver isoenzyme and neuraminidase-digested liver isoenzyme; (b) neuraminidase-digested liver and milk isoenzymes; (c) liver and milk isoenzymes, and neuraminidase-digested milk isoenzyme.

the isoenzymes from milk and liver is noteworthy. Although these components are distinct, their positions in the gel define a diagonal axis in the two-dimensional matrix. Because some alkaline phosphatases are known to be sialoglycoproteins, the

differences between liver and milk isoenzymes might be due to sialic acid residues, whose removal would result in proportional changes in charge and $\bar{M}_r$.

Fig. 2 shows the results of three gels that test this hypothesis. Fig. 2(a) shows a mixture of native liver isoenzyme and liver isoenzyme that has been digested with 100 μ g of neuraminidase/ml. The component with higher $\bar{M}_r$ and more acidic pI is the undigested form and the more basic, smaller component has been stripped of sialic acid residues. This gel shows the electrophoretic relationship between native and asialo-(liver isoenzyme) and confirms the assumption that such structural differences between enzymes of identical peptide structure produce components that run along a diagonal axis in this type of two-dimensional gel. Fig. 2(b) shows a mixture of neuraminidase-digested liver and milk isoenzymes and shows that after cleavage of the sialic acid moieties, these isoenzymes run as a single component in both dimensions and cannot be separated. Fig. 2(c) shows a gel run with a mixture of liver isoenzyme, native milk phosphatase and neuraminidase-digested milk phosphatase. Components seen in this gel span the entire range from native liver isoenzyme to the asialo form of this enzyme family. This result indicates that milk alkaline phosphatase is more heterogeneous in sialic acid content than liver isoenzyme and may contain both asialo and sialylated forms of the enzyme. Heterogeneity of alkaline phosphatase from several sources including pig kidney and human kidney has been previously described and can be largely, if not entirely, explained on the basis of post-translational glycosylation (Wachsmuth & Hiwada, 1974; Butterworth & Moss, 1966). The variation seen in milk isoenzyme sialic acid content might arise after the secretion of the enzyme containing milk-fat globules.

The results described in the present report are strong evidence indicating that liver and milk isoenzymes have similar or perhaps identical peptide structures and may therefore be coded by the same gene. At the very least they are closely related members of the same enzyme class and are readily distinguished from a second class of alkaline phosphatase represented by the placental isoenzyme. The differences between the milk and liver isoenzymes as investigated here point out that similar or very closely related gene products may be differentially modified post-translationally in different tissues and cell types. The enzymes responsible for these changes appear to exhibit well-regulated specificity.

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