Serum Alkaline Phosphatase Isoenzymes in Hepatobiliary Disorders Resolved by Use of Immobilized pH Gradients

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This new method for fractionating alkaline phosphatase isoenzymes in hepatobiliary disorders is based on isoelectric focusing on a mixed-type polyacrylamide support containing an immobilized pH gradient with a superimposed carrier ampholyte gradient. The high-M₄ alkaline phosphatase forms typical of hepatobiliary disease (>1 mega-dalton), which cannot migrate into the immobiline gel, are disaggregated in zwitterionic detergents (the most effective being sulfobetaine 3-12)—20 g/L in the sample, 5 g/L in the gel—suggesting that they are still complexed with membrane fragments or that they tend to aggregate spontaneously in solution. These isoenzymes have a pH 5–6 range (while alkaline phosphatases in normal serum focus in the pH 4–5 interval) in immobilized pH gradients, but behave as strongly acidic components by agarose isoelectric focusing in the presence of carrier ampholytes, suggesting that they are strongly complexed with the latter. On treatment with neuraminidase, the low-pI isoenzymes in normal serum focus in the pH 5–6 range typical of the hepatobiliary isoenzymes, suggesting that the latter are poorly glycosylated. By a second-dimension run, in a porosity gradient, followed by activity staining, all alkaline phosphatase forms that have entered the immobiline gel in the first dimension (normal forms and high-M₄ species) exhibit the same M₄ (ca. 140 000 Da), suggesting that no new chains are synthesized in hepatobiliary disorders.

Additional Keyphrases: mixed immobiline carrier-ampholyte gels • high-M₄, alkaline phosphatase • zwitterionic detergents • isoelectric focusing • effect of detergents

We recently described a new fractionation method for use in measuring alkaline phosphatase (AP; EC 3.1.3.1; orthophosphoric monoester phosphohydrolase, alkaline optimum) isoenzymes in sera (1), based on isoelectric focusing (IEF) in immobilized pH gradients (IPG) (2). The latter technique, however, was further modified by adding to the IPG matrix a secondary pH gradient generated by free carrier ampholytes (CA), commonly used in conventional IEF (3). This mixed CA–IPG technique was found to give much sharper AP bands and to allow for a much increased sample load (up to 100 μL of serum). By this method we could show that AP in normal sera consists of an array of ca. 10 isobands isoelectric between pH 3.90 and pH 4.79. In Paget’s disease, two sharp isobands with pIs 4.97 and 5.09 were detected. Placental AP was found to overlap with the higher-pI bands of normal serum; however, upon destruction of the latter by heating, it was found to consist of four sharp isobands with the following pIs: 4.59, 4.62, 4.67, and 4.73, the last one being a minor and the first three being major isofoms, about equally distributed. However, we had no success in mapping APs in hepatobiliary disease: only few bands entered the gel (the ones present in normal sera, focusing in the pH 4–5 region) while, in most cases, a substantial amount of activity was trapped at the edge of the pocket at the application site. It has been reported (4–6) that, in patients with hepatic diseases, APs circulating in serum exhibit a much greater apparent relative molecular mass (M₄ > 1 megadaltons) than the APs in normal sera (M₄, ca. 140 000 Da). Attempts at running IPGs in highly diluted polyacrylamide matrices (ca. 2.8% T) (7) did not alleviate the problem.

We report here the successful fractionation of high-M₄ APs in hepatic disease. This was achieved by disaggregating the high-M₄ complexes in zwitterionic detergent, after which they still are amenable to the focusing process and retain enzyme activity.

Materials and Methods

Apparatus

For the IPG experiments we used a "2217 Ultraphor" chamber (LKB, Bromma, Sweden) with an LKB 2197 constant-power supply and an LKB 2209 "Multitemp" cooling unit. IPG gels were cast with the LKB 2117-901 gradient-gel kit. For the two-dimensional electrophoresis we used a "Protean II" chamber (Bio-Rad Labs., Richmond, CA). All the pH measurements were made with a "pHIM 64" research pH meter (Radiometer, Copenhagen, Denmark).

Chemicals

"Immobiline" (LKB’s brand of non-amphoteric buffering acrylamide derivatives, with the general structure CH₂=CHCONHR, and having pKa’s of 3.6, 4.6, 6.2, 7.0, 8.5, and 9.3) and "Ampholine" carrier ampholytes in the pH ranges 3.5–5.0 and 5.0–8.0 were all from LKB. "Pharmalyte" carrier ampholytes in the pH 5.0–8.0 and 2.5–5.0 ranges were from Pharmacia Fine Chemicals AB, Upplands, Sweden. Acrylamide, N,N'-methylenebisacrylamide, N,N',N'-tetramethylethylenediamine, and ammonium persulfate were from Bio-Rad Labs. The "Gel Bond PAG" supporting foil was from Marine Colloids, Rockland, ME. L-Glutamic acid, glycerol, ethanol, methylformamide, diethanolamine, acetic acid, zinc sulfate, magnesium chloride, and Nitroblue tetrazolium were from Merck, Darmstadt, F.R.G. 5-Bromo-4-chloro-3-indoxyl phosphate (p-toluidine salt), l-lysine, "Nonidet P-40" (NP-40), and neuraminidase (sialidase, EC 3.2.1.18, from Clostridium perfringens, type VI) were from Sigma Chemical Co., St. Louis, MO. Sulfobetaine ("SB 3-15") and 3-(cholamidopropyl)dimethylammonio)-1-propane-

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6 Nonstandard abbreviations: AP, alkaline phosphatase; CA, carrier ampholytes; IEF, isoelectric focusing; IPG, immobilized pH gradients; WT, (grams of acrylamide + grams of cross linker)/100 mL; %C, grams of cross linker/%; GGT, p-glutamyltransferase.
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sulfonate ("CHAPS") dihydrate were from Serva Feinbiochimica, Heidelberg, F.R.G.

**Gel Preparation**

For IEF in IPG we prepared gels 0.5 mm in thickness containing 20 mmol of Immoblines per liter and an amount of matrix corresponding to 2.8% T, 4% C. An optimized pH gradient in the pH range 3.5 to 6.0 was used (7). On top of the Immobiline gradient we cast a pH 8.0 plateau (2 cm long) containing the sample wells. Gels were polymerized for 1 h at 50 °C. After we removed the gels from the cassette they were washed in distilled water for an hour, dried, and rewollen to their original weights in a mixture of (20 g/L final concentration) Ampholine–Pharmalyte in the pH 3.5–5.0 and 6.0–8.0 intervals for the first and in the pH 2.5–5.0 and 5.0–8.0 ranges for the latter ampholytes. For the two-dimensional electrophoresis the gels were cast according to Görög et al. (8), by preparing a linear porosity-gradient gel of 5–11% T.

**Electrophoretic Conditions**

We used 10 mmol/L glutamic acid and 10 mmol/L lysine at the anode and cathode, respectively. The voltage was kept constant at 300 V (10 mA, 3 W) and the focusing was continued overnight at 10 °C. Then we increased the voltage to 2500 V constant for 3 h. All the two-dimensional electrophoresis was continued at 50 mA constant current at 10 °C until the tracking dye had reached the anode.

**Enzyme Staining**

Staining for alkaline phosphatase was performed as in ref. 1. After IEF the gel slab was incubated for 10 min in a 1 mol/L diethanolamine buffer containing, per liter, 1 mmol of zinc sulfate and 1 mmol of magnesium chloride. Enzyme staining was then performed at room temperature for up to 2 h in the above buffer (fresh) to which was added 1 g of 5-bromo-4-chloro-3-indoxyl phosphate and 0.5 g of Nitroblue tetrazolium salt per liter (these two chemicals should first be dissolved separately in a small volume of dimethylformamide). After staining, the gels were washed extensively in dilute (100 mL) acetic acid, then in water, then dried. Staining for γ-glutamyltransferase (EC 2.3.2.2) was done as in ref. 4.

**Incubation with Neuraminidase**

Commercial neuraminidase was dissolved to give a final concentration of 2 kU per liter of pH 5.8 buffer containing, per liter, 50 mmol of sodium acetate, 60 mmol of NaCl, and 4 mmol of CaCl2. Two hundred microliters of serum was added to 20 μL of the neuraminidase solution and incubated for 3 h at 37 °C (final pH ca. 6.3). The reaction was stopped by placing the mixture in an ice bath, and the sample was immediately analyzed by IEF.

**Results**

In preliminary runs of sera in hepatobiliary disorders, a substantial amount of material with AP activity was seen to accumulate on the anodic ridge of the application pocket, thus failing to enter the gel. These could possibly represent the high-Mr AP forms (>1 MDa) often reported in obstructive jaundice (4–6). Even in extremely diluted gels (ca. 2.5% T), these forms failed to migrate to their pl position (the present Mr limit for a protein in an IPG gel appears to be 400 kDa) (9). It occurred to us that these forms could be aggregates, self-associated or formed by association with other protein species. Thus the experiments were repeated with different types of detergents (neutral, such as NP-40, or zwitterionic, such as sulfobetaines and CHAPS) present. As shown in Figure 1, in the control a substantial amount of AP activity was confined to the pocket, but in the detergent-containing gels, higher-pl bands (in the pH 5–6 range) are now seen focused in the polyacrylamide matrix. Because the best-performing detergent appeared to be sulfobetaine, we ran a serial-dilution test, to determine the concentration of it with the best disaggregating power. To the same serum sample we added 0% to 4% sulfobetaine and focused in a gel containing 0.5% sulfobetaine. As shown in Figure 2A, it takes 2% to 3% sulfobetaine (final concentration in the sample) to effectively disaggregate the high-Mr forms and produce a reasonable focusing of these higher-pl components. In Figure 2B, we have run four different sera from patients with cholestasis: it is seen that only the sera with 2% sulfobetaine added (labeled "+") allow entrance of the high-Mr AP components into the gel. Notwithstanding the fact that the focusing gel is impregnated with 0.5% sulfobetaine, failure to add sulfobetaine directly to the serum sample before loading it to the IPG gel (labeled "−") results in precipitation of the high-Mr components in the pocket.

These observations are consistent with the idea that the high-Mr species in cholestasis are indeed aggregates, be-

![Fig. 1. Electrophoretogram of serum of a patient with cholestasis (AP = 572 U/L), run in the presence of different detergents](image-url)
cause they enter the gel after treatment with detergents. It still was not known, however, if the disaggregated AP forms have the same $M_r$, as the normal components in human sera. Thus we ran a two-dimensional map, by using in the second dimension a porosity gradient in the absence of sodium dodecyl sulfate, so as to maintain integrity of the quaternary structure and to be able to prepare a zymogram also for the second-dimension slab. As shown in Figure 3A, all the isoforms that focus in an IPG gel in the presence of sulfobetaine exhibit the same apparent $M_r$ of normal AP components (ca. 140 kDa). In fact, even in an IPG gel run in the absence of detergent, the small amount of AP activity trapped in the pocket that, after solubilization, could enter the second-dimension run also exhibits the same $M_r$ as normal AP species (Figure 3B). Thus the new AP bands released in serum in obstructive jaundice evidently consist of essentially the same polypeptide chains that are typical of normal AP.

There still remains the question of how these aggregates are formed: self-aggregation, aggregation with other serum components, or aggregation with other membrane components? The last is the hypothesis favored by most authors (4–6, 10). To test it, we ran high-$M_r$ APs in the absence and presence of detergents and stained the gels for both AP and GGT activities. As shown in Figure 4 (center and right side), the aggregated form the pocket contains coincident enzymatic activity for both enzymes, neither of which are able to enter the gel. In contrast, on disaggregation the pocket is concurrently cleared of both activities and the two enzymes now exhibit non-coincident isoband distribution. Our data are thus consistent with the model of DeBroe et al. (10), who found that, in cholestasis, the high-$M_r$ AP is part of small vesicles containing leucine aminopeptidase (cytosol aminopeptidase, EC 3.4.11.1) and GGT, typical markers of the plasma membrane (see Discussion).

With the above information that the high-$M_r$ AP forms can be disaggregated by switwitteronic detergents, that they appear to be part of small plasma membrane vesicles, and that they have the same polypeptide chain as normal APs) there still remains a missing bit of evidence: why should the AP isoforms appearing in cholestasis exhibit such a higher isolectric point (pI) than normal APs (on the average, ca. 1 pH unit higher)? To check for that, we treated both normal and pathological sera with neuraminidase. When analyzed by IPG, the lower-pI (pI range 4–5) normal APs were shifted to the higher pI range (pI 5–6 interval) and were now quite indistinguishable from the pathological AP isoforms (Figure 4, left side). This suggests that the high-$M_r$ AP isoforms are a class of poorly glycosylated APs (or, in any event, containing considerably fewer steric acid residues).

An odd piece of evidence, however, comes from Figure 5: when normal and cholestatic sera are run by conventional IEF in agarose gels, the above data are exactly reversed: the high-$M_r$ AP forms do not focus more alkaline, but instead more acidic as compared with normal AP isobands. This conflict is only apparent, however. In another study on microvillar hydrolases (11) we have demonstrated that these enzymes, formerly membrane-bound, tend to seque-
Fig. 4. Comparison of AP (lanes 3, 4) and GGT (lanes 5, 6) activities in the aggregated and disaggregated states; lanes 1, 2: 1:1 mixture of normal and pathological sera treated with neuraminidase (2nd lane) and untreated (1st lane) 

- AP (3rd from left) and GGT (5th from left) activities in sera in the absence of detergent (note the intense enzyme stains in the pockets). (+): AP (4th lane) and GGT (6th lane) activities in sera after addition of 2% sulfobetaine. The sample in the 2nd track was added also with 2% SB just prior to the run.

ter and complex large amounts of the acidic components of the amphoteric buffers used in conventional IEF (carrier ampholytes), thus giving an artefactual pl distribution. This phenomenon has been described also in a few other cases (12) and in general is ascribed to proteins with an unusual amino acid composition (see also Discussion).

Discussion

On the Nature of the New Isoforms in Cholestasis

In agreement with previous reports (4–6, 10), our findings suggest that, in obstructive jaundice, the new AP isoforms released in serum have the following general properties:

(a) they have a very high apparent Mr (>1 MDa);
(b) upon treatment with zwitterionic detergents (notably sulfobetaine) they are reduced to low-Mr components;
(c) the apparent high Mr seems to be due to the presence of small membrane vesicles, containing clusters of AP and GGT activity (our data) and also leucine aminopeptidase activity (data of DeBroe et al., 10);
(d) in a two-dimensional map, the new AP isoforms present the same native Mr (ca. 140 kDa) as AP circulating in normal sera, suggesting that the polypeptide chains synthesized in cholestasis are no different;
(e) however, unlike APs present in normal sera (ca. 10 isoforms, focusing in the pH 4–5 range), the new APs exhibit substantially higher pl values (four or five isoforms, focusing in the pH 5–6 range);

(f) on treatment with neuraminidase, normal, low-pl APs are converted into higher-pl isoforms, cofocusing with the "pathological" APs, which suggests that the latter are poorly glycosylated forms (i.e., low in sialic acid content);

(g) "pathological" APs, by being less glycosylated, expose to the solvent more hydrophobic domains and thus are able to sequester large amounts of acidic carrier ampholytes, producing spurious, low-pl species in conventional isoelectric focusing.

While points a to c above are common findings now among several laboratories (4–6, 10), our additional findings described in d to g appear to be new and hitherto unreported.

On the Origin of the New AP Isoforms in Cholestasis

This is quite a complex story, and here we can only try to summarize the reported data in the literature. We also suggest a fascinating recent review (13), which should be mandatory reading on this topic.

In normal individuals, bone and intestine are known to be sources of serum alkaline phosphatase (14), while, in obstructive jaundice, the elevated APs are of liver origin (13). Striking increases in alkaline phosphatase activity are usually confined to patients with cholestatic syndromes; in viral hepatitis, for instance, or in massive hepatic necrosis, serum AP is not appreciably increased (15). What is the mechanism of this large increase of AP activity in cholestasis? Recent studies suggest that this increase is ascribable to increased synthesis of alkaline phosphatase (16), owing to enhanced translation of mRNA rather than to increased transcription (17). At least two mechanisms have been
proposed for the subsequent appearance of hepatic AP in serum. In one, it is suggested that alkaline phosphatase bound to bile canaliculus membranes may be solubilized by the bile acids that accumulate in cholestasis. AP then enters the bile canaliculus and regurgitates into serum through the intercellular junctional complex (the paracellular shunt pathway). According to this hypothesis, AP gains access to blood through leaky tight junctions. The interpretation is that cholate and deoxycholate, accumulated in the bile duct upon obstruction, lower the bile secretory pressure by disrupting the (formerly tight) junctions, thus rendering them permeable to such high-M₉ aggregates.

An alternative explanation is that hepatic AP enters serum directly from the plasma membrane. Normally, AP is found primarily on the exterior surface of the bile canaliculus membrane (18); however, it rapidly changes distribution after bile-duct obstruction (19). It is then found intracellularly and on all parts of hepatocyte plasma membrane. DeBroe et al. (19) have in fact found that in cholestasis there is a high-molecular-mass form of AP in blood that is derived from the hepatic plasma membrane and is part of small vesicles containing, in addition to AP, leucine aminopeptidase and GGT activities. Thus, it would appear that some hepatic alkaline phosphatase can enter the blood stream directly.

It should be appreciated that the two suggested routes are not necessarily mutually exclusive; they might be complementary to each other. In both pathways, high intrahepatic concentrations of bile acids appear to play an essential role. Further studies will be needed to elucidate these aspects.

Enzyme Staining

As both reviewers of this paper have criticized our enzyme staining protocol, on the grounds that 1 mmol/L Zn is highly inhibitory and that the Zn/Mg ratio has not been optimized, we should like to add the following comments. Rej and Bretaudiere (20) have in fact stated that 15 μmol of Zn⁺ per liter is stimulatory while 150 μmol/L is inhibitory of AP activity in humans and other mammals. However, their experiments involved direct measurements of enzyme activity in tissue extracts, rather than after an electrophoretic migration step. In our case, the enzyme staining has been performed after isoelectric focusing in immobilized pH gradients, with 2% added carrier ampholytes: the latter buffers are strong metal chelators (21), so that, if substantial amounts of metals are not added during the post-electrophoretic incubation, no enzyme activity appears at all (22). In separate experiments on rat kidney and ileum (not reported here, for brevity) we could show that incubation with substrate alone gave almost no staining, addition of 1 mmol of Zn²⁺ per liter gave the most intense AP bands, 5 mmol/L inhibited the staining by ca. 50% (as compared with 1 mmol/L), and 10 mmol/L was extremely inhibitory (stain intensity reduced almost to the level of the control, without zinc). In addition, especially in the case of rat ileum, admixing 1 mmol of Mg²⁺ per liter to the 1 mmol/L Zn²⁺ solution had the effect of intensifying staining some minor AP bands, which appeared very faint in the sample track incubated with only zinc. Thus it appears that, at least after an isoelectric focusing analysis of AP isozymes, 1 mmol each of Zn²⁺ and Mg²⁺ per liter is the best solution for producing the most intense staining. There could be two reasons for this: (a) as just stated, this amount overcomes the chelating power of carrier ampholytes (which are still present in the gel during the enzyme staining) and (b) diffusion of Zn and Mg ions in the gel might proceed at substantially lower rates than in free solution, so that the concentration of these two ions in the gel phases is less than 1 mmol/L and thus not inhibitory.

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References


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