Resolution of Alkaline Phosphatase Isoenzymes in Serum by Isoelectric Focusing in Immobilized pH Gradients

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This new method for fractionation of serum alkaline phosphatase isoenzymes is based on isoelectric focusing on a mixed-type polyacrylamide support containing an immobilized pH gradient with a superimposed carrier-ampholyte gradient. All known forms of alkaline phosphatase are separated in an immobile pH 3.5–6.0 gradient, the sample being applied into pockets cast on a pH 8.0 plateau. Sharp zymogram bands are obtained by substituting alkaline-stable 5-bromo-4-chloro-3-indoxyl phosphate and tetrazolium salts for the standard 1- and 2-naphthyl phosphate–diazonium salt combinations. After hydrolysis of the phosphate group by the alkaline phosphatase the indoxyl moieties reduce tetrazolium salts to nearly insoluble and nondiffusible formazan precipitates. Normal sera show an array of about 10 isobands isoelectric between pH 3.9 and pH 4.79. In Paget’s disease, two sharp isobands with pI of 4.97 and 5.09 are seen. Placental alkaline phosphatase overlaps with the higher pI bands of normal serum; however, upon heat destruction of the latter, it shows four sharp bands with the following pI’s: 4.59, 4.62, 4.67, and 4.73.

Additional Keyphrases: mixed Immobilized-carrier ampholyte gels, Paget’s disease, placental alkaline phosphatase

Determination of alkaline phosphatase in serum, by enzymatic or electrophoretic tests, has been used reliably for several years in the diagnosis of various disorders, especially bone (1) and hepatobiliary (2) diseases. Recently, the recognition of multiple forms of AP from liver, bone, placenta, and other sources and of their diagnostic value in several pathological conditions has been reviewed (3, 4).

Among the methods used to resolve AP isoenzymes, a central role is played by zone electrophoresis in sieving media (e.g., starch and polyacrylamide) (5) or non-sieving media (e.g., agarose and cellulose acetate) (6). These techniques have low resolving power, being barely able to resolve bone and liver AP bands in sera. Often, the presence of an abnormal AP form is inferred more from the broadening of a conventionally migrating band than from any actual resolution of normal vs pathological isofoms present in serum. Due to its high resolving power, isoelectric focusing (IEF) in the presence of carrier ampholytes (CA) would appear to be the technique of choice for resolving and unambiguously identifying closely related isoenzyme forms (7). Few reports dealing with the IEF separation of AP isofoms have appeared (8–11), often demonstrating the presence of multiple components, but in general, contrary to expectations, exhibiting blurred and diffused bands. Some reasons for this might be the metal chelating properties of CA buffers (12), the very low ionic strength environment of conventional IEF (13), and the use of reagents and other reagents (diazonium salts) in the zymography process that are unstable at high pH and partly soluble after the coupling reaction with tetrazolium salts (14).

We report here our use of a novel technique, IEF in immobilized pH gradients (IPG) (15) coupled to the use of a stable reagent, 5-bromo-4-chloro-3-indoxyl phosphate + tetrazolium salts (14), for analysis of serum AP in normal and pathological states.

Materials and Methods

Apparatus

For the IEF experiments we used a 2217 Uльтrophor chamber (LKB, Bromma, Sweden) with an LKB Madrodrive 5 constant power supply and an LKB 2209 Multitemp cooling unit. IEF and IPG gels were cast with the LKB 2117-901 gradient gel kit. All pH measurements were made with a PHM 64 research pH meter (Radiometer, Copenhagen, Denmark).

Chemicals

Immobilines (LKB’s brand of nonamphoteric, buffering acrylamide derivatives with the general structure CH₂ = CH-CO-NH-R) having pKa of 3.6, 4.6, 6.2, 7.0, and 9.3 and Ampholine carrier ampholytes in the pH 4–6.5 range were purchased from LKB. The “Gel Bond PAG” supporting foil was from Marine Colloids, Rockland, ME. Acrylamide, N,N’-methylenebisacrylamide, N,N,N’,N’-tetramethylethylenediamine, and ammonium persulfate were from Bio-Rad Labs., Richmond, CA. L-Lysine (free base) was
from Sigma Chemical Co., St. Louis, MO. Nitroblue tetrazolium salt was from Serva, Heidelberg, F.R.G. l-Glutamic acid, glycerol, ethanol, o-phosphoric acid, acetone, dimethylformamide, diethanolamine, zinc sulfate, magnesium chloride, and acetic acid were from Merck, Darmstadt, F.R.G. 5-Bromo-4-chloro-3-indoxyl phosphate p-toluidine salt was from Bachem, Bubendorf, Switzerland.

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 3.5%/T, 4%/C (16). An optimized pH gradient (17) in the range of pH 3.5 to 6.0 was used (see Table 1). After the polymerization step, we cast a pH 8.0 plateau containing all the sample wells. In the cases where Blue Dextran (18) was included in the plateau, the pH of the sample gel was adjusted to 9.0 by titrating Immobiline pK 9.3 (buffering ion) with the pK 3.6 titrant. After polymerization, the gels were washed, dried, and re-swollen to their original weights in 4% carrier ampholyte in the pH 3.5–8 range (19, 20).

Sample Application

In all cases we used both undiluted and undialyzed serum, because the IPG gels can tolerate heavy salt and especially heavy protein loads (15). In these gels, up to 100 μL of serum can be applied without interference with the separation pattern. For normal sera up to 2 μL and for pathological sera up to 6 μL of alkaline phosphatase activity was applied to the gels.

Electrophoretic Conditions

We performed IEF in IPGs at 2 W constant power for 12 h at 10 °C, using 10 mmol/L of glutamic acid and 10 mmol/L lysine as anolyte and catholyte, respectively.

Enzyme Staining

After IEF, the gel slab was incubated for 10 min in 1 mol/L diethanolamine buffer containing 1 mmol of zinc sulfate and 1 mmol of magnesium chloride per liter. Enzyme staining (14) was then performed at room temperature for up to 2 h in the above buffer (fresh) with 1 g of added 5-bromo-4-chloro-indoxyl phosphate and 0.5 g of nitroblue tetrazolium salt per liter (first dissolve these two chemicals separately in a small volume of dimethyl formamide, about 70 mL/g). After staining, the gels were washed extensively in dilute 100 mL/L acetic acid and water, then dried.

### Table 1. Immobiline Composition for a pH 3.5–6.0 Gradient*

<table>
<thead>
<tr>
<th>Immobiline</th>
<th>Chamber 1</th>
<th>Chamber 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(pH 3.5)*</td>
<td>(pH 6.0)*</td>
</tr>
<tr>
<td>pK 3.57</td>
<td>4.193</td>
<td>3.036</td>
</tr>
<tr>
<td>pK 4.51</td>
<td>3.124</td>
<td>4.393</td>
</tr>
<tr>
<td>pK 6.21</td>
<td>2.192</td>
<td>6.450</td>
</tr>
<tr>
<td>pK 9.59</td>
<td>0</td>
<td>3.069</td>
</tr>
</tbody>
</table>

*This recipe has been calculated to give an average buffering power (β) in the gel of 3.0 meq/(L × pH).

Fig. 1. Physicochemical parameters of the IPG 3.5–6.0 gradient as formulated in Table 1

β: buffering power (meq/(L × pH); range: 2.3–4.5); l: ionic strength (meq/L; range: 2.2–6.0). The pH curve, at any given point, deviates from linearity by not more than 1% of the generated pH interval (i.e., not greater than ±0.025 pH unit)

Results

Because, in preliminary runs with IPGs, most AP bands appeared to focus in the pH 3.5–6.0 range, we first calculated an optimized IPG recipe (Table 1) spanning this pH interval, with the aid of a previously described computer program (17). The physicochemical parameters of this IPG range (buffering power, β, ionic strength, l, and the shape of the pH gradient) are shown in Figure 1: β ranges from 2.3 to 4.5 meq/L × pH, l from 2.2 to 6.0 meq/L, and the pH gradient has a deviation from linearity of only 1% of the stated pH interval. The values in (mmol/L) given in Table 1 represent the customary Immobiline concentration in a conventional IPG gel, which is usually of the order of 12–14 total millimoles of all the species present, including buffers and titrants, per liter (21). However, in the results reported below, we used twice the amounts given in Table 1, so as to obtain double β and l values. In addition, because the AP zones tended to smear and precipitate in an IPG gel alone, we resorted to a mixed-type, primary immobilized pH gradient with an overimposed, secondary, carrier-ampholyte-generated pH gradient (20). The latter ensures, in the transient state, a higher conductivity, which helps protein migration and minimizes precipitation and aggregation at the application point (22).

Figure 2 shows the focusing, in a mixed IPG pH 3.5–6.0 + 4% CA (pH 3.5–8) gel, of AP from nine normal human sera and three patients with Paget's disease. The IPG gradient spans an 8.5 cm gel length. On top of it, a pH 8.0 plateau, 3.5 cm long, is cast, containing the pockets for sample seeding. This high-pH shelf ensures a high net-negative charge on most of the proteins in the sample, facilitating migration out of the pocket and preventing its precipitation and (or) aggregation at the pocket/gel interface (essentially no residual AP activity is seen at the gel edge of the application zone). The values of AP enzymatic activity (in U/L) are given in each pocket. By our assay (Technicon AutoAnalyzer), the normal values range between 30 and 95 U/L. In normal sera, at least 10 AP isoforms are resolved by our IPG technique, with pIs in the pH 3.90–4.79 range. In Paget's disease, with its bone pathology, the total activity in serum is much higher, 250–300 U/L. On IEF, most of the isobands are seen in the same pH 3.90–4.79 interval as normal samples, with the exception of two sharp, higher-pI forms.
In sample 3 from the left, the heavy, higher pl bands have been associated with placental AP, because they originated from the serum of a pregnant woman. To prove it, we ran a heat-stability test (incubation of serum for 15 min at 65 °C). As shown in Figure 3, in the heated serum (Pr) all the acidic bands of the control serum (Pc) have disappeared, leaving four sharp isobands with the following pl:s: 4.59, 4.62, 4.67, and 4.73, the former three representing major, almost equal isoforms.

**Discussion**

The above data, although preliminary in nature, involved some novel features worth discussing.

**The Immobilin system.** With the new IPG technique, especially when combined with a secondary, CA-generated pH gradient, it is possible to couple a high resolving power with a high reproducibility in band position. In previous reports on focusing of AP isoforms (8–11), poor patterns (smear, unreproducible in spot position, number, and intensity) were generally obtained. We believe this is ascribable to several drawbacks of the conventional CA-based technique, notably: (a) the extremely low ionic-strength environment (13), (b) the cathodic drift (23), and (c) potential artefactual bands generated by complexes among AP and different CA species (24). In the first instance, the low-I milieu could produce near-isoelectric precipitation and band smearing (salting-out effect). In the second case, it would generate positional unreproducibility of the AP zones. In the last case it would produce new, artefactual AP "pseudo-

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**Fig. 2.** Focusing of serum alkaline phosphatases

Top: Gel: 3.5% T, 4% C, containing Immobilines in twice the amounts shown in Table 1, in the pH 3.5–8.0 interval, plus 4% Ampholine pH 3.5–8.0. A 3.5-cm-long pH 8.0 plateau is cast on top of it, containing the pockets for sample application. The first three samples from the left are from three different patients with Paget’s disease, the additional nine are normal sera (25 μL, applied in all cases). In the normals, the activities (U/L) are given in the pockets. The two arrowheads at the left indicate two isoforms that are specific for Paget’s disease. Focusing: 12 h at 2 W, 10 °C, 1500 V at equilibrium. Staining as in ref. 14.

Bottom: All conditions as described above. Samples 1 to 10, from left, contain abnormally high AP activities (marked in the pockets in U/L); tracks 11 and 12 are from normal individuals. For identification of the various suspected pathologies see the Results section.

banding at pl’s 4.97 and 5.09 (indicated in Figure 2 by the horizontal arrowheads in the left three samples).

Figure 2 (bottom panel) compares a series of sera with abnormal AP values ranging from 145 to 405 U/L. Essentially all of them contain the group of 10 isoforms in the pH 3.90–4.79 range typical of normal sera, although with quite differing relative abundance of the various isobands. In addition, quite a few of them contain substantial amounts of higher-pl isoenzymes, banding in the pH 5.03–5.40 range (notably samples 146, 201, 151, and 195 U/L, from left). Several of them have been associated with hepatobiliary disorders, not only because of the extremely high values for aspartate and alanine aminotransferases and abnormal bilirubin concentrations, but also because of the presence of the high-M₉ aggregates of AP, typical of liver disease (see also Discussion). Strong AP activity is in fact seen (in Figure 2, bottom) precipitated at the anodic edge of the pocket in the following samples (from left): 228, 201, 245, 405, 349 and 195 U/L. That they represent high-M₉ aggregates (possibly >1 000 000 Da) has been demonstrated by running them in highly diluted polyacrylamide matrices (2.5% T): all the AP activity entered the gel, but moved down from the pocket as a smear and did not focus during the time allotted to the experiment.

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**Fig. 3.** Identification of placental alkaline phosphatases in serum

All conditions as in Fig. 2. The two samples at the left are normals, the two at the right contain placental isoenzymes before (P₀) and after (P₀) heat inactivation (15 min at 60 °C). In P₀, note the complete disappearance of the acidic, serum-type isoforms.
forms” not existing in vivo. The high I milieu typical of IPCs (in the present case, at least 20 times higher than in conventional CA-IEF) would ensure full protein solubility and would disrupt any potential binding with CA species. Needless to say, cathodic drift does not exist in IPCs, so that band position reproducibility is fully guaranteed, no matter how prolonged the IEF run. It is typical of the IPG technique to generate protein pI values significant to the second decimal place (as given here), while in conventional IEF not even the first decimal place is often accurate (25). This will allow much more reproducible and comparable AP patterns among different laboratories, while permitting a close scrutiny of the fine spectrum of isofoms present in a specific tissue. This is not possible with conventional electrophoretic techniques. In fact, although by treatment with neuraminidase and lectin affinity electrophoresis (26, 27) the resolution of APs in samples from different tissues has recently been increased, the zone-electrophoretic method used did not allow resolution of each band into its constituent spectrum of isofoms. It is quite clear from our data that each band obtained by zone electrophoresis in cellulose acetate is an envelope of sub-bands, which can be fully resolved by the present IPG technique.

The staining technique. Instead of the standard azo-coupling technique in which 1- and 2-naphthyl phosphates and diazonium salts are commonly used, we have adopted the indoxyl phosphate–tetrazolium salt combination, first described by McCadey (28) and recently reported by Sinha (14). The azo-coupling technique has the disadvantage that diazonium salts are relatively unstable at the high pH values (>pH 9.5) used during the enzyme staining procedure. This could lead to partial destruction of these salts, required for the coupling reaction, during the digestion process. Diazonium salts can also inhibit enzymes and might change their kinetic properties. In addition, 1- and 2-naphthols, owing to their higher solubility in aqueous buffers, do not precipitate completely at the sites of AP activities in the gel, so the resulting xymogram is often diffused and the bands blurred. With the present staining technique, after hydrolysis of the indoxyl-phosphate, the released indoxyl molecules reduce the nitroblue tetrazolium salt to insoluble formazan at the sites of enzyme activity, thus producing sharp bands, contrasting clearly with the background and allowing resolution of zones that are less than 0.5 mm apart.

Sample load. A major problem with detecting AP isofoms after conventional IEF of normal sera is the low amount of sample that must be used with the IEF procedure. At the tolerated volumes (3–5 μL of serum), as the activity is distributed over at least 10 zones, the bands become almost undetectable. At least 25 to 30 μL of serum is needed to intensify the zones, but at these very high protein loads (ca. 2 mg of protein/track) the pH gradient in conventional IEF breaks down, and wavy and blurred zones are produced. In IPCs, we routinely load 25 to 30 μL of whole, undialyzed serum without any negative side effects. In the present technique, in fact, where Immobilines in double the usual concentration and as much as 4% CA are used, we have loaded as much as 100 μL of serum before overloading became apparent. Not even albumin, which in general greatly disturbs the IEF process, is affecting the distribution and the quality of the AP isobands. In fact, at the beginning we had tried to eliminate it by preparing a sample application plateau containing an affinity ligand (Blue Dextran), but we later abandoned this procedure because albumin was not interfering in the AP pattern and, in addition, some AP activity appeared to be lost in the affinity plateau, possibly bound to Blue Dextran.

Conclusions. While still preliminary in nature, our data show the great potential of the present IPG-CA technique as a fine tool for a close scrutiny at the spectrum of AP isofoms in serum. Work is in progress to isolate AP from different tissues for a clear mapping of the numerous AP bands present in serum. At the moment, it appears that the two isoenzymes of Paget’s disease and the four placental isofoms are clearly recognizable (their relative positions in the pI spectrum being in full agreement with their migration in disc electrophoresis, cf. our figures with Figure 3 of ref. 3). The hepatobiliary disorders, by producing different patterns consisting of additional, higher-pI components and greatly altered ratios among the 10 isofoms present in normal serum, will need closer scrutiny to see if the different spectra of isofoms can be correlated to specific liver diseases.

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