
Improved Agarose Electrophoretic Method for Separating Alkaline Phosphatase Isoenzymes in Serum

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A modified agarose electrophoretic system for the separation of alkaline phosphatase (ALP, EC 3.1.3.1) isoenzymes is described. Bone, liver, high-molecular-mass, and intestinal ALP are separated with high reproducibility. The sensitivity of the agarose system is superior to cellulose acetate in detecting high-M, ALP. Correlation is good between bone ALP fractions scanned before and after treatment with neuraminidase. Immunoglobulin-bound ALPs, the ALP–lipoprotein-X complex, and the additional ALP fraction observed in transient hyperphosphatasemia in children are detected by their peculiar electrophoretic mobility in the proposed system. Approximately 25% of the samples contained an additional fraction of intestinal-type ALP, as evidenced by neuraminidase treatment and use of polyclonal and monoclonal antibodies. Because the electrophoretic mobilities of this "intestinal variant" and of some immunoglobulin-bound ALP fractions are identical to those of bone and intestinal ALP, respectively, treatment of the samples with a polyclonal antibody that reacts with intestinal ALP is advised.

Additional Keyphrases: agarose and cellulose acetate electrophoresis compared · neuraminidase treatment · polyclonal vs monoclonal antibodies

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5 Received February 22, 1988; accepted May 2, 1988.

Human alkaline phosphatase (ALP, orthophosphoric-monoester phosphohydrolase (alkaline optimum); EC 3.1.3.1) consists of a group of isoenzymes encoded for by at least three different gene loci. They are membrane-bound sequenced ectoenzymes and glycosylated dimeric metalloenzyme with which the exact biological function is still unknown. Their clinical interest stems from the existence of at least six more or less tissue-specific isoenzymes, whose cellular expression and liberation in biological fluids is influenced by physio-pathological factors.

The biochemical determination of ALP activity is technically easy and reliable. For electrophoretic separation of ALP isoenzymes, various supporting media have been proposed: agar (5), agarose (6), starch (7), polyacrylamide (8), and cellulose acetate (9). Some methods are too cumbersome for routine use. In others, a poor resolution of the liver- and bone-derived isoenzymes, an absent or poor visualization of high-molecular-mass ALP, and a lack of sensitivity and reproducibility make interpretation of the isoenzyme pattern difficult.

Here we report our evaluation of the separation of serum ALP isoenzymes with a commercially available agarose electrophoretic system ("Isopal"; Beckman Europe, Analis S.A., Namur, Belgium).

Materials and Methods

Instruments

For absorbance measurements we used an Hitachi 705 apparatus (Boehringer, Mannheim, F.R.G.). Gels were scanned in a computerized densitometer ("Appraise"; Beckman Instruments Inc., Brea, CA).

CLINICAL CHEMISTRY, Vol. 34, No. 9, 1988 1857
Reagents

As earlier described (10), fresh human tissues (liver, bone, small intestine, and placental tissues) were extracted with n-butanol; the aqueous phase obtained after centrifugation was used as a tissue-specific ALP standard material for electrophoresis.

Fresh serum samples associated with a variety of clinical disorders were obtained by venipuncture from hospitalized patients undergoing various medical and surgical departments.

Cellulose acetate gels were from Gelman Sciences Inc., Ann Arbor, MI; agarose gels were Paragon SPE gels from Beckman with buffer components and substrates from the Isopal kit.

Reagents for ALP measurements were commercially available as a kit in which p-nitrophenyl phosphate was the substrate (Baker Chemicals BV, Deventer, Holland).

The ALP substrate bromochloroadipate-phosphate, neuraminidase (from Clostridium perfringens, EC 3.2.1.18), and polyclonal anti-placental ALP antiserum were from Analis.

Lectin (wheat-germ agglutinin), cetrimion bromide (cetyltrimethylammonium bromide, Cetavlon), and phospholipase C (from Bacillus cereus, EC 3.1.4.3) were purchased from Sigma Chemical Co., St. Louis, MO.

The LPX Rapidophor equipment was kindly provided by Immuno Diagnostika, Brussels, Belgium.

Polyclonal antibodies to liver/bone ALP and monoclonal antibodies to placental and intestinal ALP were produced in this laboratory.

Polyclonal antisera to human IgG, IgA, IgM, and kappa and lambda chain were from Behringwerke AG, Marburg, F.R.G.

Procedures

Quantification of ALP and isoenzymes. Total ALP activity of the samples was measured according to the method recommended by the Scandinavian Committee on Enzymes, but at 25 °C (11). The upper normal value for ALP in our laboratory was established as 190 U/L.

To quantify the ALP activity on the scanned surfaces, we visually determined the inflection point between two ALP peaks on the tracing, from which we directed a vertical separation line to be automatically drawn to the base of the scan. The area under the curve was computed by the scanner.

When ALP activity was ≥100 U/L we performed the electrophoresis on cellulose acetate, using the "Gelman Alk Phos Isoenzyme" system, according to the instructions of the manufacturer. The gels were scanned at 600 nm while still wet. All samples were applied on a second cellulose acetate gel after addition of the lectin wheat-germ agglutinin (final concentration 50 mg/L) to the equilibration buffer (12).

When ALP activity was ≥50 U/L, we used the Isopal agarose electrophoresis system. Agarose gels, precast on a plastic film, were equilibrated for 30 min in the electrophoresis buffer (Tris/H3BO3 buffer, pH 9.45, with a final concentration of 0.38 mol of Tris and 0.06 mol of boric acid per liter) to which a mixture of surfactants was added. Excess buffer was removed and a plastic template was positioned on the gel. We applied 10-µL samples at each application point on the template, let these penetrate into the gel for 10 min, then removed any excess sample and the template. Electrophoresis was started at 150 V for 25 min, after which we incubated the gel for 30 min at 45 °C with 2.5 mL of the substrate 5-bromo-4-chloro-3-indolyl phosphate (final concentration 1.89 mmol/L, pH 10.4). The gel was rinsed in distilled water, dried, and scanned at 600 nm.

For treating samples with neuraminidase (13), before electrophoresis, we added 5 µL of a 2 kU/L neuraminidase solution to 25 µL of serum and incubated for either 30 min or 24 h at 37 °C. Treatment with phospholipase C consisted of adding 5 µL of phospholipase C solution (60 kU/L) to 25 µL of serum, and incubating overnight at 37 °C.

We incubated samples with polyclonal and monoclonal antibodies, specifically directed against one or another isoenzyme, by adding 5 µL of the antibody solution to 25 µL of serum and letting the mixture stand for 5 min, 1 h, or overnight at 37 °C. Final antibody concentrations ranged from 1 to 5 mg/L. In some cases, we combined antisera addition with neuraminidase or phospholipase C treatment. The polyclonal anti-placental isoenzyme cross-reacted with intestinal ALP. The polyclonal antisera to liver/bone ALP consisted of a mixture of three monoclonal antibodies raised against liver ALP. We also used specific mouse monoclonal antibodies against intestinal ALP ("250", "203") and against placental ALP ("17E3"), raised with fresh human intestinal and placental tissue as antigen source.

For heat inactivation we incubated samples either at 65 °C for 15 min (to isolate placental ALP) or at 56 °C for 10 min (to inhibit bone ALP).

Immunoglobulin-bound ALP was identified by an immunofixation technique (14): after electrophoresis we incubated the gel for 50 min at 45 °C with 50 µL of an antisera specific for IgG, IgA, IgM, and kappa and lambda chains, applied by means of a multi-trench applicator strip (Paragon Immunofixation kit, Beckman Instruments Inc.). Non-immunoprecipitated proteins were eliminated by rinsing the gel thoroughly for at least 60 min in isotonic saline (NaCl 150 mmol/L) before incubating the immunoprecipitate with the enzyme substrate.

We also extracted samples with Cetavlon/diethyl ether, adding 5 µL of a 1.4 g/L Cetavlon solution and 50 µL of ether to 25 µL of serum sample (15). After a short centrifugation, the ether extract was discarded and the underlying aqueous phase was used for ALP isoenzyme study.

The lipoprotein-X (LP-X) test was performed by agar electrophoresis ("LP-X Rapidophor") according to the instructions of the manufacturer. ALP activity in the precipitated LP-X band was made visible by incubating the gel with 2.5 mL of 5-bromo-4-chloro-3-indolyl phosphate for 30 min at 37 °C.

Results

Electrophoretic separation of serum samples on cellulose acetate and on agarose gel reveals five common ALP isoenzyme patterns (Figure 1).

In contrast to what is seen on cellulose acetate, liver and bone ALP are already partly separated on agarose. Including lectin in the buffer considerably enhances the electrophoretic bone/liver ALP resolution on cellulose acetate, as does neuraminidase treatment of the sample before agarose electrophoresis (Figure 2). This observation prompted us to compare the estimated percentages of bone ALP after agarose electrophoresis by scanning 165 samples, before and after treatment with neuraminidase. The samples were consecutive routinely processed sera, analyzed and scanned by several technicians, as obtained over a period of four months, but excluding samples with intestinal and high-M₁ ALP. The relative bone ALP activities found before (x) and
after \( y \) neuraminidase treatment correlated well: \( y = 1.31x - 3.95 \) (of total ALP), \( r = 0.86 \). Upon partial desialylation, bone activities were slightly higher (+8.6%). Total ALP activities were unaffected. We also compared the relative bone ALP activity determined after electrophoresis on agarose with that obtained after electrophoresis on cellulose acetate with incorporated lectin. The correlation between both systems was poor \( (r = 0.60) \), the bone ALP activity after lectin inclusion being systematically much higher (20%) than after neuraminidase treatment.

In the agarose electrophoretic system the scanned area under the curve obtained for a liver ALP fraction of about 60 U/L showed a within-gel CV of 2% and a between-gel CV of 3%. Low ALP activity bands (intestinal and high-Mg fractions) also demonstrated fair reproducibility (Table 1).

When the same sample was applied on the 10 application points of five different gels, we saw no significant difference (\( \alpha > 0.05 \), Friedman test) between the scanned surfaces in the 10 positions of the gels, or between the mobilities of the bone/liver/high-Mg ALP fractions relative to the intestinal isoenzyme. Within-gel differences in relative mobility were 1% to 2% for liver/bone and high-Mg ALP; between gels, the difference in relative mobility was 5% for liver/bone and 6% for high-Mg ALP.

The sensitivity of the agarose system for high-Mg and intestinal fractions was tested in 100 consecutive samples. The detection limit for both fractions was 2 U/L if the total ALP activity exceeded 50 U/L.

Cellulose acetate and agarose were compared with respect to their accuracy for detecting high-Mg and intestinal fractions in 53 patients’ samples. Samples with an ALP activity >100 U/L were selected, so that ALP would be visible on cellulose acetate. We found that only half (19) of the 38 samples revealing a high-Mg ALP band on agarose also showed this fraction on cellulose acetate. When they were detectable in both systems, the high-Mg ALP fractions averaged 6% higher in the agarose system. Of the four samples determined to contain an intestinal-type ALP on agarose, one was not detected by cellulose acetate electrophoresis. None of the samples was positive for either of these isoenzymes on cellulose acetate and negative by agarose electrophoresis.

Migration of placental ALP in the agarose system coincided with that of bone ALP. The major band was often accompanied by a second band with a more anodal electrophoretic mobility and of placental origin, as demonstrated by heat denaturation and reaction with specific monoclonal and polyclonal anti-placental ALP antisera (Figure 3).

In approximately 25% of the samples sent to the laboratory for ALP isoenzyme evaluation we encountered an unusual fraction that could be detected only on agarose gel. It coincided with bone ALP, but was less diffuse. As shown in Figure 4, neuraminidase treatment had no effect on its position. The change in electrophoretic mobility of this fraction after incubation with a monoclonal anti-intestinal antiserum prompted us to call it an "intestinal variant." In most clinical conditions where an intestinal ALP was present, this "intestinal variant" was also detected. The coincidental appearance of intestinal ALP and "intestinal variant" was apparent in a selected population of carriers of the hepatitis B virus (chronically dialyzed and renal-transplant

### Table 1. Reproducibility of the Agarose System

<table>
<thead>
<tr>
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<th>Within gel</th>
<th>Between gel</th>
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<tr>
<td></td>
<td>( \bar{x} \pm SD, U/L )</td>
<td>CV, %</td>
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<tr>
<td>Intestinal</td>
<td>3 ± 0.7</td>
<td>24</td>
</tr>
<tr>
<td>Bone</td>
<td>37 ± 0.6</td>
<td>2</td>
</tr>
<tr>
<td>Liver</td>
<td>90 ± 1.8</td>
<td>2</td>
</tr>
<tr>
<td>High-Mg</td>
<td>9 ± 1.7</td>
<td>18</td>
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*The same sample was applied 10 times on one gel. *The same sample was applied on 10 different gels.
nants, e.g., LP-X, in cholestasis. This complex migrated well anodal to the high-Mr fraction in agarose: “ultra-fast” high-Mr ALP (16). The LP-X immunoprecipitate showed a clear ALP activity. After selective extraction of the serum with Cetavlon/ether (15), both LP-X and the “ultra-fast” high-Mr fraction(s) disappeared from the electrophoretic profile (Figure 6A), without affecting the ALP activity of the samples. ALP can also form complexes with immunoglobulins. So far, we have found six patients’ sera containing immunoglobulin-bound ALP, as follows: four IgG lambda types, two of which were complexed to liver ALP, one to liver/bone ALP, and one to intestinal ALP; and two IgG kappa types, one of which was complexed to liver/bone, the other to intestinal ALP (Figures 6B and 7).

Discussion

The clinical value of the measurement of total ALP activity is substantially increased by qualitative and quantitative analyses of the ALP isoenzyme pattern. One of the purposes of this paper was to compare a modified agarose electrophoretic system with that of the widely used cellulose acetate system.

In the agarose system the separation of liver and bone ALP in untreated samples corresponds well with the liver/bone activities found in neuraminidase-treated samples. Only when bone ALP activity exceeds 50% of total ALP activity (e.g., in children) is neuraminidase treatment sometimes required. The higher bone fraction obtained when lectin is included in the cellulose acetate system (12, 13), as compared with the bone fraction obtained after neuraminidase treatment in the agarose system, might be attributable to the lack of specific binding of lectins to ALP sugar side-chains (17).

The ALP fraction typically found in the serum of patients with cholestasis or hepatic metastasis of solid tumors (16, 18–20) migrates more anodally than the liver fraction in this agarose system and is called high-Mr ALP (7). It has also been referred to as “bile ALP” (21), “fast liver ALP”

patients). The “intestinal variant” was present in 13 of the 20 patients’ samples tested (65%) and was accompanied by an intestinal fraction in 12 of these 13 patients (92%).

The ALP isoenzyme pattern in serum of children with transient hyperphosphatasemia (Figure 5) showed a pronounced bone fraction accompanied by an additional ALP band with an electrophoretic mobility intermediate between that of liver and high-Mr ALP. After heat denaturation and incubation with a monospecific anti-liver/bone ALP antiserum it was shown that this fraction was more heat resistant than bone ALP, and that it was liver/bone in nature. Addition of phospholipase C did not alter its migration.

Although ALP most frequently circulates as free isoenzymes, these can be complexed with other serum compo-
The ALP isoenzyme of placental origin, encountered in pregnant women, was electrophoretically identified as a single band or a doublet. Occasionally, serum from patients with malignant disease revealed the presence of placental ALP, ectopically expressed into the circulation (26). The proposed agarose system is not sensitive enough to detect most of these fractions. Rather, one should use the highly specific and sensitive immunological methods developed to detect these placental ALP isoenzymes (27).

A striking observation during this study was the appearance of an additional fraction frequently associated with intestinal ALP. We provisionally called it "intestinal variant" in view of its biochemical properties and its reaction with monoclonal anti-intestinal antibody. Although the clinical significance of this variant in serum is not yet known, we found high activities of it in patients with liver cirrhosis, neoplastic disease, or undergoing chronic dialysis. Because this "intestinal variant" migrates similarly to bone ALP, its presence can lead to an overestimation of the latter. Currently we treat all samples with a polyclonal anti-placental antiserum that cross-reacts with intestinal ALP. This alters the electrophoretic mobility of the "intestinal variant" without affecting the mobility of the bone fraction (Figure 4).

The ALP isoenzyme predominating in childhood is of the bone type. Secondary to viral infection, young children (ages ≤5 years) can develop a transient hyperphosphataseemia, with ALP activities ranging from 700 to >1000 U/L, without evidence of liver or bone disease (28). The additional fraction present in these children reacts with an anti-liver/bone ALP antiserum. The apparently greater resistance to heat treatment of this fraction, vs that of a normal bone fraction, suggests liver as its source, as suggested by Rosalki and Foo (29).

The presence of one or more "ultra-fast" high- Mr bands in serum samples with a high direct bilirubin content has generated controversy on the origin of these fractions. Peretz et al. (30) proposed that these bands reflect an artifactual binding of bilirubin with the enzyme substrate; i.e., they have no ALP activity. Koett et al. (16) suggested that they represent a complex of ALP with lipoprotein-X, which we have confirmed here.

ALP, mostly of liver or bone origin, and sometimes of intestinal origin, can also be complexed to circulating immunoglobulins, most frequently of the IgG kappa type (10, 14). These fractions are usually easily recognized on agarose gels because of their peculiar migration patterns. However, when present only in small amounts, the complex may have the same electrophoretic mobility as intestinal ALP. Treatment with neuraminidase, polyclonal anti-placental antiserum, or more specifically with monoclonal antibodies directed against the complexed ALP identifies the nature of the ALP type involved, and immunofixation procedures can be used to establish the immunoglobulin type of the complex.

The proposed agarose system is easy to handle, sensitive, and reproducible for the ready separation and quantification of most of the actually clinically relevant ALP isoenzymes in serum. It is particularly useful for detecting the high- Mr fraction. When dried, the transparent gels can easily be stored. The clinical usefulness of additional fractions such as the "intestinal variant" needs further study.
We thank all the technicians—especially Marina Van Mullem of the Department of Clinical Chemistry of the Antwerp University Hospital, and Hilde Geryl of the Department of Nephrology and Hypertension of the University of Antwerp—for their excellent technical assistance. We also thank the Pediatric Department of the Antwerp University Hospital and the pathologists from peripheral hospitals and laboratories for providing clinical data on the samples they sent to our department.

References