Characterization of Serum Alkaline Phosphatase of Hepatobiliary and Osteoblastic Origin *

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ABSTRACT

A rapid and reproducible method for the electrophoretic separation and quantitation of serum alkaline phosphatase isoenzymes is described. Sera from patients with biopsy proven parenchymal liver diseases and diseases related to increased osteoblastic activity were subjected to electrophoresis using a continuous tris-borate buffer on a five percent polyacrylamide gel. Following electrophoresis, bands of alkaline phosphatase activity were located on the gels using a substrate of α-napthyl phosphate and a diazonium salt as activator. The stained gels were subsequently photographed, the relative mobility of each isoenzyme fraction measured and the transparencies scanned using an integrating, recording densitometer. This method coupled with heat inactivation of serum alkaline phosphatase provides a reliable indication of the tissue origin of this enzyme in patients with elevations of serum alkaline phosphatase of osteoblastic and/or hepatobiliary origin.

Introduction

Determinations of serum alkaline phosphatase activity are of practical clinical importance in the investigation and management of hepatobiliary and bone diseases. Considerable interest has focused on the nature and properties of the enzyme and on the possible existence of tissue specific variants or isoenzymes.

In 1968, Ivor Smith et al4 showed that acrylamide gel electrophoresis could differentiate alkaline phosphatase isoenzymes which were obtained from extracts of liver, bone, kidney, and intestine. This electrophoretic method has been coupled with the selective difference in the rates of inactivation of human alkaline phosphatases on
heating at $56^\circ C$ in order to define better the nature of the cellular damage in a given disease process.

**Materials and Methods**

**Specimens**

Serum from patients with biopsy proven, parenchymal liver disease and serum from patients with proven osteoblastic disease were used in this study. Pooled control serum was used as a laboratory reference control in daily alkaline phosphatase determinations. Serum alkaline phosphatase activity was determined by a modification of the method of Kind and King$^5$ and expressed as units per dl.

**Procedure**

Two tenths of a ml of serum was diluted in $9.8$ ml of $15$ percent sucrose. This was used as a "stock" sample for each patient and kept frozen until ready for use. The ideal range for measuring isoenzyme activity by gel electrophoresis was between $6$ to $35$ King Armstrong units per dl, and $200 \mu l$ of this stock sample was applied onto the running gel. For serum enzyme activity greater than $35$ King Armstrong units, the "stock" sample was further diluted (usually 1:4) with $15$ percent sucrose, and $100 \mu l$ or $200 \mu l$ used for electrophoresis. Dilution of high activity sera was necessary because the bands of alkaline phosphatase activity which developed on the gel after electrophoresis were too opaque for accurate densitometry.

**Heat Inactivation**

An aliquot of the "stock" sample was incubated at $56^\circ C \pm 0.1^\circ$ for exactly thirty minutes in a constant temperature waterbath. Heated and unheated samples were then subjected to gel electrophoresis and their activities compared.

**Acrylamide Gel Electrophoresis**

The method of Smith$^4$ was followed and Canalco Model 12 electrophoresis apparatus was employed. Siliconized glass tubes $6$ cm in length and $0.6$ mm I.D. were used as gel holders. One ml of running gel was added to each tube. A sample or spacer gel was not used. The sample in $15$ percent sucrose was directly applied onto the running gel. A current of $1.5$ mA per gel was applied over a period of thirty minutes. At the end of this time, a bromphenol blue marker, which complexes with albumin, had run approximately $75$ percent of the way down the gel. The gels were removed from the glass tubes and cut at the albumin-bromphenol blue marker. The section from the origin to the marker was then saved for protein and enzyme location by staining. The length of the stained gel also served as a convenient reference point for relative mobility studies.

**Buffers and Running Gel**

Buffers and five percent acrylamide running gel were made as outlined by Smith.$^4$ A tris-borate buffer at a pH of $9.5$ was used. All solutions were stored at $4^\circ C$ until needed. Electrophoresis was carried out at room temperature.

**Location of Alkaline Phosphatase Activity on the Gel**

The reaction buffer and substrate were made employing the method of Taswell.$^5$ The buffer was a tris-malate at a pH of $9.8$. Sodium a-naphthyl phosphate was used as substrate and 4-amino diphenylamine-diazonium sulfate was used as the coupler. Each gel was placed in a tube containing $7$ ml of the reagent mixture. The reaction was allowed to proceed for one hour at $4^\circ C$. Areas of enzyme activity showed as compact bands of a dark brown color on a yellow background. The yellow background cleared rapidly in a $7$ percent acetic acid
washed. The gels were then photographed and stored at 4°C in small glass tubes containing distilled water.

PHOTOGRAPHY AND SCANNING

The stained gels were photographed in 7.5 cm × 1 cm storage tubes filled with distilled water. A Polaroid MP-3 Land camera with a 127 mm lens and employing No. 146 L film was used. Four gel tubes were placed side by side on a light source and photographed. The resulting transparency of each stained gel was scanned and band density determined using a Beckman Microzone Densitometer Model R-110. This provides a permanent record and allows one to obtain (1) relative density of each alkaline phosphatase band before and after heating, (2) relative mobilities of the band and (3) band character.

Results

In figure 1 is shown a characteristic serum alkaline phosphatase pattern from two patients, one with biopsy proven hepatitis (figures 1a and 1b). The other had metastatic breast carcinoma to bone with a negative liver scan and biopsy (figures 1c and 1d). Polaroid transparencies and densitometer tracings demonstrate that the characteristic features of liver alkaline phosphatase are (1) a narrow sharply defined band...
of enzyme activity, (2) relative heat stability and (3) a mobility of between 54 to 58 mm.

Characteristic features of bone alkaline phosphatase (figures 1c and 1d) are (1) a diffuse broad band, (2) marked inactivation by heat and (3) a mobility of between 50 to 54 mm.

**TABLE I**

VALUES OF RESIDUAL SERUM ALKALINE PHOSPHATASE ACTIVITY PERCENTAGES OBTAINED BY HEAT INACTIVATION TEST

<table>
<thead>
<tr>
<th>Number of Patients</th>
<th>Diagnosis</th>
<th>Residual Serum Alkaline Phosphatase* Activity (Mean ± S.D.) %</th>
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<tbody>
<tr>
<td>51</td>
<td>Hepatic disease</td>
<td>34.5 ± 6.8</td>
</tr>
<tr>
<td>31</td>
<td>Skeletal disease</td>
<td>10.8 ± 8.4</td>
</tr>
<tr>
<td>12</td>
<td>Mixed hepatic and skeletal disease</td>
<td>26.1 ± 8.7</td>
</tr>
<tr>
<td>11</td>
<td>Intestinal disease</td>
<td>52.6 ± 11.9</td>
</tr>
</tbody>
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* Serum alkaline phosphatase remaining after heating at 56°C for 15 minutes.

Results of the heat inactivation of bone and hepatic alkaline phosphatase are shown in figure 2. It can be seen that alkaline phosphatase from skeletal origin is rapidly inactivated (56°C) at an exponential rate; half the enzyme disappearing in 5.6 ± 1.5 minutes. In the case of hepatic alkaline phosphatase, heat inactivation occurs at a much slower rate, half the enzyme disappearing in 12 to 15 minutes. Intestinal alkaline phosphatase is quite stable and placental alkaline phosphatase almost completely heat stable. Mixtures of hepatic and bone phosphatases usually gave a "normal" heat inactivation pattern. Sera in the so-called osteoblastic group showed alkaline phosphatase heat inactivation (56°C, 15 min) to less than 20 percent of original activity and hepatic sera to greater than 34 percent of original activity. Intestinal alkaline phosphatase showed greater than 52 percent of its activity remaining in serum after heat inactivation.

The heat inactivation pattern of subjects with both osteoblastic and hepatic diseases
remained in an intermediate position between that of the rapidly inactivated serum of subjects with increased osteoblastic activity and the slowly inactivated serum of patients with hepatic disease. The heat inactivation pattern of alkaline phosphatase in normal serum also lies in an intermediate position between the serum of subjects with increased osteoblastic activity and the serum of patients with hepatic disease. Normal serum seemed to behave like a mixture containing 50 to 60 percent skeletal isoenzyme. The determination of alkaline phosphatase isoenzymes in normal subjects by electrophoretic and thermostability techniques should be studied further before their significance is reported. Only serums with significantly elevated serum alkaline phosphatase levels should be characterized by our procedure.

Serum with elevated bone alkaline phosphatase was also mixed with serum containing liver alkaline phosphatase. This combination was subjected to electrophoresis and stained and scanned in the usual manner. This study was carried out in order to determine if one could characterize serum containing increased bone and liver isoenzymes. The results are shown in Figures 3c and 3d. These patterns demonstrate (1) a band with characteristics between those of liver and bone alkaline phosphatase and (2) bone alkaline phosphatase activity greatly reduced on heating, yielding a narrow band characteristic of liver alkaline phosphatase.

Thus, this method is capable of separating liver from bone alkaline phosphatase on the basis of their relative mobilities after heat inactivation. Rf values were calculated by the ratio of the distance from the origin of the run to the band center (or peak heights on densitometer tracings) in centimeters to gel length in centimeters. In table II is shown the relative mobilities of the alkaline phosphatase bands shown in figures 1 and 3.

**Discussion**

The identification of alkaline phosphatase isoenzymes of different tissue origin in hu-
man serum is of important diagnostic significance. The results presented in this study seem to imply that heat inactivation and gel electrophoresis can provide a reliable parameter for measuring the presence of serum alkaline phosphatase activity of skeletal or hepatic origin. Using the method described it has been possible to identify liver or bone alkaline phosphatase in pathological serum. Qualitative identification of the tissue of origin can be accomplished by a visual comparison of the stained isoenzyme in gels of both heated and unheated specimens. When serum contains alkaline phosphatases of both skeletal and hepatic origin, heat inactivation clearly removes the broad “bone” band leaving a sharp residual “liver” band. This combined procedure can provide rapid and reliable information concerning the tissue origin of an elevated serum alkaline phosphatase.

References

“Nothing is more simple than greatness; indeed, to be simple is to be great.”

Miscellanies: Literary Ethics

Emerson