Alkaline Phosphatase Isoenzymes of Liver and Bone Origin Are Incompletely Resolved by Wheat-Germ-Lectin Affinity Chromatography

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We used wheat-germ-lectin affinity chromatography as a tool to investigate the structure of alkaline phosphatase (ALP, EC 3.1.3.1) and to obtain fractions enriched in either bone or liver ALP activity. Liver and bone isoenzymes in serum samples were incompletely resolved except that the activity in the nonretained fraction (fraction 1) always represented pure liver isoenzyme and constituted a larger percentage of total activity in pooled sera with increased liver ALP activity than in pooled sera with increased bone activity. In contrast, a more avidly retained ALP activity, presumably with high glycosylation, was found in human serum with high activity of bone ALP. Using a solid-phase immunoassay, we examined the fractions obtained from the wheat-germ-lectin-Sepharose 4B column to determine whether the isoenzyme preference of the monoclonal antibody was markedly influenced by the degree of glycosylation. Whether samples contained high proportions of liver or of bone isoenzyme activity, the nonretained fraction contained a higher percentage of liver ALP, whereas the more strongly bound fraction contained a higher percentage of bone ALP. Except for eluted fractions that either contained no detectable N-acetylgalactosamine or the highest percentage of it, the avidity of the liver-isoenzyme-specific monoclonal antibody for ALP seemed to be independent of the degree of glycosylation, suggesting that the epitope for monoclonal antibody may be expressed in some structure other than the carbohydrate moieties.

Activities of alkaline phosphatase (ALP, EC 3.1.3.1) in serum are increased in diverse disease states, including hepatoma, cholestatic disease, osteosarcoma, and metabolic bone diseases that increase osteoblastic activity. Consequently, whether a report of increased ALP activity provides clinically useful data often depends on the capacity to quantitatively distinguish between ALP isoenzymes originating in liver and bone. Human liver and bone alkaline phosphatase are believed to be derived from a common gene, such that a post-translational event results in the difference(s) between these two isoenzymes (1–3). The amount of glycosylation of bone and liver ALP isoenzymes has been inferred from the differential mobility on agarose gel impregnated with wheat-germ lectin (4–6) and the quantitative precipitation of about 80% or more of the bone isoenzyme by wheat-germ lectin (4, 7).

Although wheat-germ-lectin affinity chromatography has been used to bind other asparagine-linked oligosaccharide moieties of glycoproteins (8), it has only been briefly compared with other lectins in resolving bone and liver isoenzymes. Because evidence of resolution of ALP species by use of wheat-germ lectin has been more recently obtained (5, 6), we sought to re-evaluate the use of liquid chromatography for resolution of ALP isoenzymes, specifically by using a lectin-Sepharose column. We have observed that both bone and liver ALP have an intermediate avidity for wheat-germ lectin. In addition, wheat-germ-lectin affinity chromatography can be used to obtain ALP from serum that has a high proportion of liver isoenzyme having presumably no glycosylation and ALP from serum that has a high proportion of bone isoenzyme with high glycosylation. Here we demonstrate that for isoenzymes that are considered to be bone or liver ALP on the basis of either inactivation by urea or monoclonal antibody techniques, liquid chromatography does not clearly segregate the major ALP activity into high- or low-glycosylation subtypes, as would have been expected from previous work with lectin–agarose electrophoresis (4–6).

Materials and Methods

ALP isoenzymes

We determined ALP isoenzymes in serum by a kinetic assay, as previously described by Statland et al. (9), finding an interassay coefficient of variation of 2% and a lower detection limit of 3 U/L (mean zero std. + 3 SD). In brief: Total ALP activity was quantified by combining 15 µL of sample with 50 µL of distilled water and 350 µL of a pH 10.15 reagent containing, per liter, 1.0 mol of diethanolamine, 0.5 mmol of MgCl₂, and 16 mmol of p-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO 63178). The reaction rate at 30 °C was monitored by centrifugal analysis (Encore; Baker Instruments Corp., Allentown, PA 18001) that measured the changes in absorbance at 405 nm. One unit (U) of activity was defined as the quantity of enzyme catalyzing the hydrolysis of 1 µmol of substrate per minute under these conditions. The intestinal isoenzyme was estimated by calculating the amount of ALP inactivated by 0.01 mol/L L-phenylalanine (Sigma Chemical Co.) (9). The liver and bone ALP isoenzymes were estimated from the rate of ALP inactivation in 3.2 mol/L urea reagent (Sigma Chemical Co.) as compared with the relative rates of inactivation of ALP in sera from patients with hepatoma (increased liver-isoenzyme activity) and in sera from patients with Paget's disease (increased bone-isoenzyme activity), assayed in the same analytical run.

Human Serum Pools

Two pools were prepared from serum of patients with increased total ALP activity (>1000 U/L). The liver ALP pool was from patients with >90% liver ALP, the bone ALP pool from patients with Paget's disease who had >90% bone ALP, as determined by the urea-inhibition method (9). The total ALP activity for the bone ALP pool was 1345 U/L, for
the liver ALP pool, it was 1145 U/L. Aliquots of each pool were stored at −20 °C. Daily for eight days, one aliquot of the liver ALP pool and one aliquot of the bone ALP pool were thawed and assayed.

**Wheat-Germ-Lectin Affinity Chromatography**

We applied 200 mL of the bone- or liver-isoenzyme-rich pool to a 2-mL wheat-germ-lectin-Sepharose 4B column (Pharmacia, Uppsala, Sweden) and incubated it for 30 min at room temperature. The first and second fractions were eluted with 1 mL of 0.1 mol/L sodium phosphate buffer containing 0.15 mol of NaCl per liter (PBS), pH 7.3. The third fraction was eluted with 1 mL of 0.1 mol/L N-acetylglucosamine in PBS. After an additional 15-min incubation at room temperature, the fourth and fifth fractions were eluted with 1 mL each of the same 0.1 mol/L N-acetylglucosamine buffer. The sixth fraction was eluted with 1 mL of 0.5 mol/L N-acetylglucosamine in PBS, pH 7.3. After an additional 15-min incubation, the seventh and eighth fractions were eluted with 1 mL each of the same 0.5 mol/L N-acetylglucosamine buffer. All fractions were stored at 4 °C until the ALP activity could be determined kinetically by urea inhibition on the same day. Afterwards, the fractions were stored at −20 °C until assayed by immunoassay.

The wheat-germ-lectin-Sepharose 4B column was regenerated with 20 mL of 0.1 mol/L Tris HCl buffer containing 0.5 mol of NaCl per liter, pH 8.5, followed by 20 mL of 0.1 mol/L sodium acetate buffer containing 0.5 mol of NaCl per liter, pH 5.5. The column was then washed with 20 mL of PBS, pH 7.3, and stored overnight filled with PBS at 4 °C. If longer storage was needed, the column was kept at 4 °C in thimerosal solution, 0.1 g/L in PBS, and washed with 20 mL of PBS before use. The N-acetylglucosamine solutions were stored at −20 °C, and aliquots were thawed as needed.

**Wheat-Germ-Lectin-Agarose Electrophoresis**

Electrophoresis of human serum pools was performed according to the procedure of Schreiber and Whitta (5) on agarose gel containing wheat-germ lectin. Agarose Type I and wheat-germ lectin from *Triticum vulgaris* were from Sigma Chemical Co. After pipetting 6 μL of sample into each slot, we electrophoresed the gel in the Corning electrophoresis system (Corning, Palo Alto, CA 94306) at 100 V for 45 min. The bands with ALP activity were stained with Naphthol AS-MX, which fluoresces yellow-green under ultraviolet light (365 nm). The Naphthol AS-MX phosphate substrate was obtained as the Corning Alkaline Phosphatase Isozyme set.

**Immunooassay**

A solid-phase immunoassay to differentiate bone ALP from liver ALP isoenzyme was developed in this laboratory by Lawson et al. (10) and modified by Duda et al. (11). This immunoassay is based on the principle that a murine monoclonal antibody (B450) prepared by Lawson et al. is fivefold more sensitive for liver ALP than for bone ALP, whereas monoclonal antibody B478 binds equally well to liver ALP and bone ALP. For both monoclonal antibodies, the cross-reactivity with human placental and intestinal ALP is <3% relative to liver ALP. A standard curve was prepared by combining various proportions of the human sera with >90% bone ALP and >90% liver ALP (see above). Using the ratio of absorbance at 405 nm after treatment with B450 to that after treatment with B478, one can calculate the percentage of bone ALP or the percentage of liver ALP in the unknown serum by comparison with the standard curve.

**Statistical Analysis**

Statistical analysis of the data obtained from the wheat-germ affinity chromatography of the bone and liver ALP serum pools was performed with the “Statistix” program (IBM version; NH Analytical Software, St. Paul, MN 55117). The data were tested for gaussian distribution by the Wilk–Shapiro test for normality. We compared the nonretained fraction 7 of the bone ALP pool obtained from the wheat-germ lectin affinity column with the nonretained fraction 7 of the liver ALP pool by Student’s paired t-test. To normalize fractions from the two pools, we performed the statistical analysis on the percentage of the total ALP activity recovered from the column for any given day. The total ALP activity applied to the wheat-germ-lectin affinity column was determined by assaying (9) a fivefold dilution of an aliquot of the same serum that was chromatographed. The total ALP activity of a given serum pool recovered from the column was calculated as the sum of the ALP activities of all eight fractions of that pool eluted from the column. The percentage of ALP activity for any given fraction was calculated as the ALP activity for that fraction divided by the total ALP activity of the pool applied to the column.

**Results**

The ALP isoenzyme activity from serum with increased bone or increased liver ALP was assayed by urea inactivation, immunoassay, lectin affinity chromatography, and lectin affinity electrophoresis. The serum with increased bone ALP was found to be 100% bone isoenzyme (n = 2) by urea inactivation and 100% bone by immunoassay (n = 2). The serum with increased liver ALP was 97% liver (n = 2) by urea inactivation and 84% liver (n = 2) by immunoassay. On qualitative analysis by wheat-germ lectin electrophoresis on agarose, the only activity that could be visually detected from pooled sera with increased bone ALP activity did not migrate far from the origin, whereas the pooled sera with increased liver ALP activity had no discernible activity near the origin.

When the fractions obtained from the wheat-germ-lectin affinity column were assayed for ALP isoenzymes by urea inhibition, the isoenzyme type remained either predominantly bone or predominantly liver ALP (Table 1). Applying half the amount of sample to the wheat-germ-lectin column did not alter the chromatographic pattern. Therefore, the incomplete resolution of the glycosylated ALP species was not a result of column overload. As expected, the nonretained fraction from the wheat-germ-lectin–Sepharose 4B column analysis of the liver pool contained the highest proportion of liver ALP by immunoassay (98% ± 8%, n = 8). The strongly bound fraction from the wheat-germ-lectin–Sepharose 4B column analysis of the bone ALP pool contained the highest proportion of bone ALP by immunoassay (94% ± 7%, n = 8) (Figure 1). The column and immunoassay methods appeared to be more sensitive in detecting low concentrations of liver or bone ALP isoenzymes in the presence of very high concentrations of the other isoenzyme.

Most of the ALP activity, whether from a serum containing increased bone ALP activity or one containing increased liver ALP activity, was eluted in the intermediate (0.1 mol/L N-acetylglucosamine) fractions. The most striking difference between the liver- and bone-isoenzyme pools was that
Table 1. Wheat-Germ-Lectin Chromatography of Serum from a Patient with Above-Normal Liver Alkaline Phosphatase Activity (A) and a Patient with Above-Normal Bone Alkaline Phosphatase Activity (B)

<table>
<thead>
<tr>
<th>Eluent</th>
<th>Fraction no.</th>
<th>Total</th>
<th>Liver*</th>
<th>Bone*</th>
<th>Intestine*</th>
<th>Total</th>
<th>Liver</th>
<th>Bone</th>
<th>Intestine</th>
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<td>ND</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>78</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td>23</td>
<td>22</td>
<td>ND</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>183</td>
<td>179</td>
<td></td>
<td>4</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>N-Acetylglucosamine, 0.1 mol/L, in PBS</td>
<td>3</td>
<td>189</td>
<td>183</td>
<td>6</td>
<td>ND</td>
<td>116</td>
<td>ND</td>
<td>116</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>53</td>
<td>53</td>
<td>ND</td>
<td>ND</td>
<td>152</td>
<td>ND</td>
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<td>ND</td>
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<td></td>
<td>5</td>
<td>37</td>
<td>33</td>
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<td>2</td>
<td>58</td>
<td>ND</td>
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<td>ND</td>
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<tr>
<td>N-Acetylglucosamine, 0.5 mol/L, in PBS</td>
<td>6</td>
<td>63</td>
<td>62</td>
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<td>37</td>
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<td>15</td>
<td>ND</td>
<td>3</td>
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<td>17</td>
<td>112</td>
<td>ND</td>
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<td></td>
<td></td>
<td>31</td>
<td>ND</td>
<td>31</td>
<td>ND</td>
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</table>

*Patient's serum was diluted fivefold during the elution procedure as described in Materials and Methods. A 200-μL aliquot of serum was applied to the column and eluted in 1-ml fractions.

The nonretained fraction from the wheat-germ-lectin column contained 25% of the total ALP activity of the liver pool but only 3% of the total ALP activity of the bone pool (Table 2). The difference between the bone ALP isoenzyme-rich pool (31%) and the liver ALP pool (21%) in the strongly bound fraction was not as dramatic, but this finding was consistent with the immunoassay results, which indicated that the liver pool contained 16% ± 7% bone ALP. Nevertheless, the percentage of the total ALP activity in the strongly bound fraction for the liver pool differed significantly from the percentage of total ALP activity for the bone pool (two-tailed t-test, P = 0.001), as calculated by Student’s paired t-test.

These chromatographic results indicate that there are at least two behaviorally different fractions from both the bone ALP and the liver ALP pools. Although both ALP pools contain a common fraction that binds to the wheat-germ-lectin column, the liver ALP pool contains some ALP species that fail to bind to wheat-germ lectin. The bone ALP pool contains some ALP species that are more difficult to displace from the wheat-germ lectin with N-acetylglucosamine. On the basis of the defined properties of wheat-germ lectin binding, the wheat-germ-lectin chromatograms suggest that the predominant ALP species of both the bone and the liver pools have an intermediate degree of glycosylation. This finding was surprising, because of the affinity electrophoresis results from our and other (4–6) laboratories, which have demonstrated that the bone isoenzyme remains near the origin and the liver ALP isoenzyme is the one that migrates most rapidly in patients’ sera.

Discussion

Our observation that the major fraction of sera containing both increased liver and increased bone ALP activities has similar and intermediate affinity for wheat-germ lectin

Table 2. Wheat-Germ-Lectin–Sepharose 4B Column Chromatography of Serum with Increased Bone or Liver Alkaline Phosphatase

<table>
<thead>
<tr>
<th>Eluent*</th>
<th>Fraction no.</th>
<th>Bone</th>
<th>Liver</th>
<th>Bone</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS, pH 7.3</td>
<td>1</td>
<td>5±1</td>
<td>50±4</td>
<td>2</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3±1</td>
<td>7±2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>N-Acetylglucosamine, 0.1 mol/L, in PBS</td>
<td>3</td>
<td>50±2</td>
<td>25±2</td>
<td>19</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>90±3</td>
<td>78±4</td>
<td>34</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>30±1</td>
<td>19±1</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>N-Acetylglucosamine, 0.5 mol/L, in PBS</td>
<td>6</td>
<td>17±2</td>
<td>10±1</td>
<td>6</td>
<td>5</td>
</tr>
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<td></td>
<td>7</td>
<td>50±2</td>
<td>29±2</td>
<td>19</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>15±1</td>
<td>7±1</td>
<td>6</td>
<td>3</td>
</tr>
</tbody>
</table>

Total     | 260         | 225   | 99    | 100  |
| Serum pool | 263±9       | 225±5 | 100   | 100  |

*Two pools were prepared from patients’ serum with increased total ALP (>1000 U/L): one with >90% bone ALP and one with >90% liver ALP isoenzyme (by urea inhibition). The 200 mL of pool was applied to the column and eluted with 1 mL of elution buffer, as outlined in Materials and Methods.

Table 1. Wheat-Germ-Lectin Chromatography of Serum from a Patient with Above-Normal Liver Alkaline Phosphatase Activity (A) and a Patient with Above-Normal Bone Alkaline Phosphatase Activity (B)

**Fig. 1.** Immunoassay of alkaline phosphatase fractions obtained from the wheat-germ-lectin affinity column

Pooled serum samples with increased liver ALP or increased bone ALP were applied to the wheat-germ-lectin affinity column. The nonretained fraction was eluted with PBS, pH 7.3. The weak bound fraction was eluted with N-acetylglucosamine, 0.1 mol/L, in PBS. The strongly bound fraction was eluted with N-acetylglucosamine, 0.5 mol/L, in PBS. The fractions obtained from the wheat-germ-lectin column were then assayed for liver ALP by monoclonal antibody immunoassay. Bars indicate the mean (and SE) percentage of liver ALP in the high bone-ALP pool (lighter bars) and the percentage of liver ALP in the high liver-ALP pool (darker bars) (n = 8)
suggests that the carbohydrate content of these isoenzymes and their sensitivity toward urea inactivation and monoclonal antibody avidity are independent variables in ALP structures. The liver ALP pool contains a fraction of ALP species that completely lacks the essential carbohydrate structure required for wheat-germ-lectin binding. The bone ALP pool contains a fraction of ALP species with additional carbohydrate groups attached to the polypeptide chain.

It is believed that human liver and bone ALP are derived from a common gene and that a post-translational event results in the difference between liver and bone ALP (1). Although the results of wheat-germ-lectin affinity chromatography do not directly substantiate this theory, the wheat-germ-lectin affinity chromatograms are certainly consistent with the hypothesis that there is a post-translational modification of a common gene product. In addition, the cDNA encoding bone ALP, which was isolated by Weiss et al. (3), has five possible glycosylation sites. Not only must the Aan-X-Ser(Thr)-tripeptide sites be sterically available for glycosylation, the unknown glycosyltransferases also must differ in their substrate specificity for different oligosaccharide structures (12). Tissue-specific post-translational modification of the liver or bone ALP isoenzyme could involve modifications affecting the steric availability of the glycosylation sites as well as the presence or absence of the various glycosyltransferases. Alternatively, the isoenzymes could have identical structures immediately after processing and differ by subsequent degradative events. The binding of cellular constituents unique to bone cells or biliary epithelium that are unrelated to the carbohydrate moieties may be the primary determinant in urea and antibody reactivity.

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References