Canine Serum Alkaline Phosphatase Isoenzymes Detected by Polyacrylamide Gel Disk Electrophoresis

Hiroshi ITOH1, Tomoko KAKUTA1, Go GENDA1, Iwao SAKONJU1 and Katsuaki TAKASE1

1Department of Veterinary Surgery, School of Veterinary Medicine and Animal Sciences, Kitasato University, Towada, Aomori 034-8628, Japan

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ABSTRACT. Serum alkaline phosphatase (ALP) isoenzymes were studied in normal dogs using a commercially available polyacrylamide gel disk electrophoresis kit (PAG/disk kit). Serum samples taken from the dogs were incubated with neuraminidase, after which most showed ALP isoenzymes as two characteristic stained bands. To determine the origin of each band, ALP isoenzymes of serum and tissue extracts (liver, intestine and bone) were characterized by heating, wheat germ agglutinin (WGA) and levamisole treatments. The results suggested that the band detected on the anode was liver ALP (LALP) and that the band detected on the cathode represented bone ALP (BALP), and both were corticosteroid-induced ALP (CALP). The percentage of each ALP isoenzyme to total ALP activity was estimated by densitometry. The percentage of BALP was the highest in young dogs (age < 1 year, 64.7%), and this value decreased with age. In contrast, the percentage of LALP in young dogs (22.2%) was much lower than that in middle-aged dogs (ages 1 year to 7 years, 59.3%) and old dogs (ages > 7 years, 50.4%). The present results suggested that a commercially available PAG/disk kit is capable of detecting three serum ALP isoenzymes in dogs, and further that it may have clinical applications in the evaluation of ALP isoenzymes in veterinary medicine.

KEY WORDS: alkaline phosphatase isoenzyme, Alkphor kit, canine serum, polyacrylamide gel disk electrophoresis.
Heat inactivation: Tissue extracts were heated at 56°C for 10 min in a water bath and then cooled on ice to determine remaining ALP activity [10].

Wheat germ agglutinin precipitation (WGA): Sera or tissue extracts (liver, intestine and bone) were mixed with the same volume of WGA (5 mg/ml in deionized water, Honen Co., Ltd., Japan). The mixture was incubated at 37°C for 30 min and centrifuged at 3,000 rpm for 10 min. ALP activity in the supernatant was determined, and the inactivation rate was calculated as follows [12]:

\[
\text{Inactivation rate (\%) = \left\{ \frac{\text{Total ALP} - \text{Supernatant ALP}}{\text{Total ALP}} \right\} \times 100}
\]

Levamisole and L-phenylalanine inhibition: Levamisole (Funakosi Co., Ltd., Japan) and L-phenylalanine (Wako Pure Chemical Industries, Ltd., Japan) were added to the ALP assay buffer at final concentrations of 4.2 mM and 5.0 mM, respectively. The inactivation rate was calculated after each treatment and CALP activity was calculated as follows [7, 9]:

\[
\text{CALP activity value = ALP activity determined by addition of levamisole} \times 100/58
\]

In addition, levamisole was added to the electrophoretic substrate solution at a concentration of 4.2 mM and incubated in staining solution.

PAG/disk electrophoresis: PAG/disk electrophoresis was performed using a commercially available kit (AlkPhor System, Jokoh Co., Ltd., Japan) according to the manufacturer’s instructions. ALP activity was determined by a spectrophotometer (EDC, Helena Laboratories, Co., Ltd., Japan) at 610 nm. Two gel tubes were used for electrophoreses. One tube was stained normally and the other was stained in the presence of levamisole.

Statistical analysis: Results were analyzed statistically for variance (ANOVA) and by Student’s t-test (P < 0.05).

RESULTS

PAG/disk electrophoresis patterns by neuraminidase treatment: According to PAG/disk electrophoresis, serum ALP zymograms in normal dogs had a single band (16/40; 40%) or double bands (24/40; 60%) (Fig. 1a, b, c). An atypical ALP isoenzyme band was observed in the serum of most young dogs (Fig. 1c). After neuraminidase treatment, most serum samples originally showing a single band ALP zymogram had changed to a two band zymogram (14/16; 88%), and the separation of these bands was also improved (Fig. 1a', b', c'). Densitometric analysis of ALP isoenzymes demonstrated that neuraminidase treatment increased the percentage of cathodal bands (+6.3%) and decreased that of the anodal bands (−4.3%).

The atypical ALP isoenzyme band that was observed in the serum of most young dogs (Fig. 1c) disappeared after heating at 56°C for 10 min (Fig. 3d'). However, this band was not affected by neuraminidase or PI-PLC treatments (data not shown).

The electrophoretic mobilities of ALP from tissue extracts was observed in order of electrophoretic mobilities from the anode as follows: liver (a), bone (b), kidney (c), placenta (d), and intestine (e).
Analysis of serum ALP isoenzyme: The results of the PAG/disk analysis of each serum ALP isoenzyme were characterized by WGA, levamizole and heat treatments. In WGA treated serum, the cathodal band completely disappeared (a'). In levamisole untreated substrate solution, two bands were observed (b: LALP, BALP). In levamisole treated substrate solution, two bands were completely inhibited (b'). However, the cathodal band observed was not always inhibited by this treatment (c': CALP). In addition, the levamisole resistant cathodal bands did not disappear after heating at 56°C for 10 min (c': CALP). An atypical (d: ←) and cathodal bands (BALP) disappeared after heating at 56°C for 10 min (d'), and after levamizole treatment (d'').

The percentage of BALP was much higher in young dogs than in middle aged and old dogs, and this value decreased gradually with age. In contrast, the percentage of LALP was much lower in young dogs than in middle-aged and old dogs. The percentage of CALP tended to be higher in old dogs.

DISCUSSION

Analysis of serum ALP is an established diagnostic assessment tool in bone and liver diseases [14]. Such disorders are associated with an increase in serum total ALP as a
result of increased entry of bone and liver ALP isoenzymes into serum. In particular, when total ALP activity is within the normal range, quantitative rather than qualitative measurement of bone and liver phosphatase levels increases the sensitivity of isoenzyme analysis by permitting the detection of minor changes in the activities of these enzymes. Quantitative measurements are also needed for serial monitoring of the activity of tissue fractions with bone or liver disease, and the response of these to therapy. However, no simple electrophoresis technique has yet been established for the analysis of ALP in the veterinary clinical setting. Thus, the present study assessed the use of a simple electrophoretic procedure for the analysis of ALP isoenzymes in the serum of dogs using a commercially available PAG/disk electrophoresis kit. In general, separation of isoenzymes in dog serum ALP by cellulose acetate electrophoresis reportedly yields three isoenzymes (LALP, BALP and CALP) [1, 3–5].

In the present study, ALP isoenzymes in all serum samples taken from the dogs showed a single or double band ALP zymogram (Fig. 1). However, since the single band on the ALP zymogram was broad, it may represent an overlap of several ALP isoenzymes. It has been reported that partial neuraminidase digestion affects the separation of ALP isoenzymes by differentially delaying the electrophoretic mobilities of each ALP isoenzyme [11]. We believed that most samples with a single zymogram subsequently separated into two distinct bands on the zymogram after treatment of serum with neuraminidase.

The present study investigated the tissue of origin of each serum ALP isoenzyme band detected by PAG/disk electrophoresis by characterizing the properties of each ALP isoenzyme from liver, bone and intestine. The results of characterization of each dog ALP isoenzyme with heat, WGA and levamizole (Table 1) agreed with the results of previous reports; BALP has been reported to be thermostable [9], readily sedimented with WGA [12], and highly inactivated by levamisole treatment [7]. Another previous study observed that LALP is highly sensitive to levamisole, and slightly resistant to heat and WGA treatment [3]. Furthermore, IALP is reportedly more resistant to heat treatment than BALP and LALP [15, 19], and is also resistant to levamisole treatment [7].

In terms of the electrophoretic mobilities of ALP isoenzymes, CALP migrates to the anodal side of LALP [1, 3–5] or overlaps with LALP [18] in cellulose acetate electrophoresis. On the other hand, CALP has been shown to be separated on the cathodal side of LALP [15] in starch gel electrophoresis. It is possible that CALP was detected on the cathodal side of LALP at the same position as BALP by the present PAG/disk electrophoresis kit, which has a molecular sieve effect similar to starch gel electrophoresis [15].

The present PAG/disk kit detected an atypical ALP isoenzyme band on the cathodal side in most serum samples taken from young dogs (Fig. 1c). This band was heat labile (Fig. 3d') and was not sensitive to neuraminidase or PI-PLC treatment (data not shown). Variant BALP present in the sera of 99% of normal human children [20] has also been reported to have the above characteristics. In the present study, dog serum BALP was heat labile (Table 1). Therefore, atypical ALP isoenzyme in the serum of young dogs might correspond to the variant BALP of human serum. The densitometric values of the two bands detected in the normally stained gel tube were used to compare the fractionation percentage between LALP and other ALP isoenzymes.

The assessment of ALP bands when using a PAG/disk kit in the clinical setting should proceed as follows. (1) Two gel tubes are concurrently electrophoresed after treatment of the sample with neuraminidase. (2) One tube is stained normally and the other is stained with levamisole. (3) The densitometric values of the two bands detected in the normally stained gel tube are used to compare the fractionation percentage between LALP and other ALP isoenzymes. (4) The band in the gel tube stained with levamisole has been shown to represent CALP. (5) The percentage of CALP and BALP can be estimated by comparison of gel tubes stained normally with levamisole-stained tubes.

The observed age-dependent changes of each ALP isoenzyme in the present serum samples were similar to those reported by Syakalima et al. [17], except that the serum of young dogs showed a much higher percentage of BALP and a much lower percentage of LALP. This disparity could be due to differences in the methods used and the ages of the dogs studied in the present study and those of others. The actual LALP activity in the WGA-treated supernatant fluid was expected to be lower than the calculated value, given that the sedimentation of LALP due to WGA [9] was not considered in the paper of Syakalima et al. [17].

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>TALP (IU/L)</th>
<th>BALP (%)</th>
<th>LALP (%)</th>
<th>CALP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young (age&lt;1)</td>
<td>14</td>
<td>187 ± 67</td>
<td>64.7 ± 9.5</td>
<td>22.2 ± 11.6</td>
<td>1.5 ± 2.5</td>
</tr>
<tr>
<td>Middle (1≤age&lt;7)</td>
<td>13</td>
<td>94 ± 47</td>
<td>31.1 ± 12.7</td>
<td>59.3 ± 15.2</td>
<td>2.5 ± 4.0</td>
</tr>
<tr>
<td>Old (7≥age)</td>
<td>13</td>
<td>97 ± 44</td>
<td>16.0 ± 10.3</td>
<td>50.4 ± 26.2</td>
<td>25.6 ± 26.3</td>
</tr>
</tbody>
</table>

Data are represented by mean ± standard deviations.

n: Number of dogs in the group; TALP: Total alkaline phosphatase activity; BALP: Bone alkaline phosphatase; LALP: Liver alkaline phosphatase; CALP: Corticosteroid-induced alkaline phosphatase.
In the present study, dog serum CALP, which has been reported to be derived from the liver [1] was detectable only in old dogs (Table 2). The CALP percentage was also high in the serum of old dogs in the report of Syakalima et al. [17].

Determination of total serum ALP activity levels is essential for diagnosing bone, liver, and biliary tract diseases. However, the measurement of total ALP activity is not sufficient for the evaluation of the disease condition or for determining the cause of abnormal total ALP levels. The analysis of each ALP isoenzyme is clinically important for these purposes. Using the PAG/disk kit, it was possible, after biochemically processing the serum, to calculate the percentage of each ALP isoenzyme. When disease conditions associated with a high level of ALP activity are encountered, the present method, which uses a commercially available PAG/disk, might be useful in evaluating all three ALP isoenzymes in dog serum.

REFERENCES