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SEPARATION AND HEAT STABILITY OF THE CORTICOSTEROID-INDUCED AND HEPATIC ALKALINE PHOSPHATASE ISOENZYMES IN CANINE PLASMA

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SUMMARY

A convenient method has been developed for the separation of alkaline phosphates (AP) isoenzymes from canine plasma. The various forms of AP activity were extracted by ethanol and separated on an anion exchanger by fast protein liquid chromatography. In this way a complete discrimination was achieved between the increase in plasma AP activity due to liver disease and that due to corticosteroid induction. The corticosteroid-induced form of AP could be separated from the other isoenzymes because of its relative heat stability at 65°C. A quantitation of the contribution of liver and corticosteroid-induced AP isoenzymes to the total plasma AP activity could be made from the respective heat inactivation plots. The separation of the isoenzymes may be valuable in the purification of the different isoenzymes for further characterization.

INTRODUCTION

Alkaline phosphatase (AP) (E.C. 3.1.3.1; orthophosphoric monoester phosphohydrolase) consists of a group of isoenzymes which catalyse the hydrolysis of monophosphate esters at alkaline pH. The heterogeneity of these isoenzymes may be partly due to the expression of different gene loci and partly to post-translational modifications[1]. In man several isoenzymes contribute to the total amount of plasma AP, some of which (liver, intestinal and bone AP) can be found in the plasma of normal healthy individuals, and may have elevated levels in diseased ones. Other isoenzymes are present in plasma only during pregnancy (placental AP) or disease (kidney AP) and sometimes in association with cancer, *e.g.*, Regan isoenzyme[1–5].

In the dog separate AP isoenzymes have also been isolated from the kidney, placenta, intestinal mucosa, liver and bone[6–7]. However, renal, placental and intestinal isoenzymes have never been demonstrated in canine plasma, neither in healthy nor in diseased dogs. This may be due to their very short half-lives (3–6 min in plasma)[8,9]. Besides the normal liver and bone isoenzymes, an additional hepatic isoenzyme has been found in canine plasma following corticosteroid excess[10–12]. This isoenzyme has not yet been found in man or in any other species. There is a marked difference in

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heat stability at 65°C between liver AP and corticosteroid-induced AP isolated from liver tissue[13].

Due to the close similarities between the AP isoenzymes, a quantitative analysis of the contribution of the various forms to the total plasma activity is difficult. Several methods have been employed to distinguish between different AP isoenzymes, including selective heat inactivation, inhibition with L-phenylalanine or L-homoarginine, separation by means of electrophoresis, isoelectric focusing and immunochemical techniques[7,9,12,14–17]. We describe here the results of heat inactivation and of separation by fast protein liquid chromatography (FPLC) of canine plasma AP isoenzymes caused by liver disease and hyperadrenocorticism.

EXPERIMENTAL

Animals

Studies were performed on ten clinically healthy mature Beagles and 26 canine patients (in the Small Animal Clinic of Utrecht State University) which had elevated plasma AP levels. In 14 of the patients, hyperadrenocorticism (Cushing's syndrome) was diagnosed by use of the dexamethasone screening test[18] and in the remaining 12 dogs the presence of liver disease was confirmed by biopsy, and they had no history of corticosteroid treatment or signs of Cushing's syndrome.

Samples

Blood samples were collected by jugular venapuncture in tubes containing lithium–heparine. The plasma was separated by centrifugation at 2800 *g* and stored at −20°C until used for further analysis. Bone AP isoenzyme was isolated from the cystic fluid of a dog with histologically diagnosed osteosarcoma. The canine intestinal AP isoenzyme was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Enzyme measurement

Alkaline phosphatase activity was measured on a Multistat III-FLS centrifugal analyser (Allied, Instrumentation Laboratory, Lexington, MA, U.S.A.) at 30°C, using *p*-nitrophenyl phosphate as substrate (Boehringer Mannheim Diagnostica, Mannheim, F.R.G.). The method used was an "optimized standard method" conforming to the recommendations of the German Society of Clinical Chemistry[19].

Heat inactivation

Plasma samples were incubated at 65°C in a Lauda MT-LCE 004 thermostatic water-bath (Messgeräte-Werk Lauda, Lauda-Königshofen, F.R.G.), which had a guaranteed temperature control of ± 0.1°C. Samples of 3 ml at a temperature of 20°C were placed in thin-walled glass tubes (55 mm × 9 mm, wall thickness 0.5 mm) in the water-bath and at intervals of 0, 0.5, 1, 1.5, 2, 2.5 and 4 min, aliquots of 0.25 ml were transferred to identical glass tubes chilled in ice. The activity remaining after each interval of heat inactivation was expressed as a percentage of the activity in the unheated samples.

Extraction and ion-exchange chromatography

Canine plasma AP was isolated using ethanol extraction according to Dorner

et al.[10]: briefly, 1 ml plasma was diluted in an equal volume of 0.9% sodium chloride and placed in an ice-bath. With continuous stirring, 1 ml of 96% ethanol, cooled to −20°C, was added to give a final ethanol concentration of 32% (v/v). After centrifugation of the mixture for 10 min at 6000 *g*, an equal volume of 96% ethanol was added to the supernatant with continuous stirring. The pellet, collected after centrifugation for 10 min at 6000 *g*, was dissolved in 1 ml of 10 m*M* sodium acetate buffer (pH 5) containing 0.5 m*M* magnesium chloride and dialyzed overnight against the same buffer. After dialysis at 4°C the precipitated protein was removed by centrifugation for 2 min in an Eppendorf microcentrifuge (Eppendorf, Hamburg, F.R.G.).

AP isoenzymes were separated by an FPLC system consisting of a Mono Q anion-exchange column, two P-500 pumps, a GP-250 gradient programmer, an UV-1 single path monitor and an FRAC-100 fraction collector (Pharmacia Fine Chemicals, Uppsala, Sweden). The column was equilibrated with 10 m*M* sodium acetate–acetic acid buffer (pH 5.0) and 0.5 m*M* magnesium chloride. Samples of 0.1–0.5 ml were injected at a solvent flow-rate of 1 ml/min. Proteins were eluted with a linear gradient from 0 to 0.2 *M* sodium chloride in the equilibration buffer, followed by a wash with 1 *M* sodium chloride. The gradient was completed within 20 min. The absorbance was measured at 280 nm and fractions of 0.5 min (0.5 ml) were collected for subsequent estimation of the AP activity.

Statistics

The results were analyzed by the distribution-free method of Wilcoxon–Mann–Whitney[20]. Differences were considered to be significant at $p < 0.05$. The results are presented as the median and range.

RESULTS

Incubation of plasma from the control dogs at 65°C produced a rapid linear decline in the logarithm of AP activity within 2 min, after a short initial plateau (Fig. 1). This linearity indicated the inactivation reaction kinetics to be of pseudo-first order. Inactivation was complete after 2 min (median 0%, range 0–2.1%, $n = 10$). Half-inactivation times, $t_{1/2}$, were derived from the straight lines when plotted on semilog paper. The $t_{1/2}$ for control dogs was 17.3 s (14.0–19.0 s).

Heating for 2 min at a lower temperature was also investigated (Fig. 2). A decrease in temperature of less than 1°C had little effect on the results, but between 64 and 60°C a linear decrease in inactivation was observed.

The results of heat inactivation at 65°C of AP in plasma from dogs with hepatic disease ($n = 12$) were similar to those in plasma from control dogs (Fig. 3). The activities remaining after 2 min (median 2.0%, range 0–8.1%) and $t_{1/2}$ values (median 17.5 s, range 10–25 s) were not significantly different ($p > 0.05$) from those of control dogs. There was only slight heat inactivation of plasma AP from twelve dogs with Cushing's syndrome. Both the remaining activity of 83.1% (62.2–99.9%, $n = 12$) and the $t_{1/2}$ of 350.0 s (138–1080 s) were significantly higher than in the other groups ($p < 0.0001$).

For the plasma of two dogs with hyperadrenocorticism, the plot of the logarithm of the remaining AP activity against time could be resolved into two components (Fig. 3). The $t_{1/2}$ (mean for the two dogs) was 18.5 s for the first component and 262.5 s for the second component.

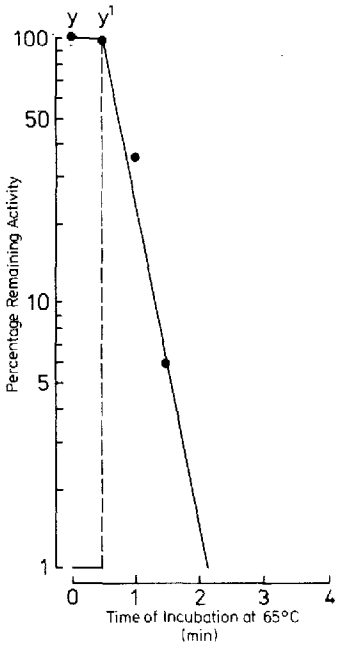

Fig. 1. Heat inactivation at 65°C expressed as the median of the individual activities of alkaline phosphatase in the plasma of ten control dogs. The horizontal part $Y-Y^1$ is due to the warming up of the glass tubes.

Optimum conditions for the separation of AP isoenzymes were determined on an anion-exchange column using plasma from dogs with either hyperadrenocorticism or liver disease. The efficiency of extraction of the total AP activity from the various plasmas with chilled ethanol was 75%, as determined after dialysis against sodium acetate buffer (pH 5) and separation of the precipitated protein by centrifugation. In a buffer of pH 8, normally used for separation by electrophoresis on agarose or

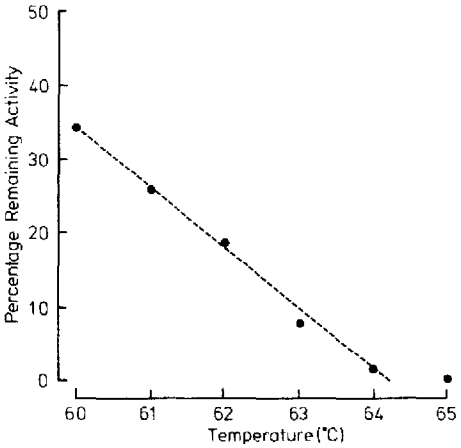

Fig. 2. Effect of different temperatures (2-min exposure) on the mean alkaline phosphatase activity in the plasma of four dogs.

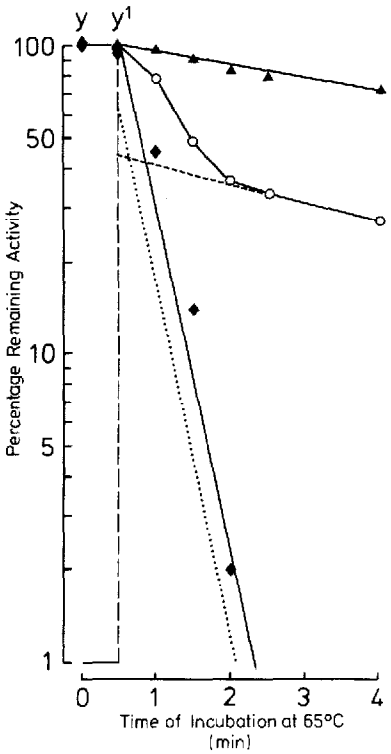

Fig. 3. median percentages of alkaline phosphatase activities in plasma after heating at 65°C for dogs with liver disease (◆—◆; n = 12) and those with hyperadrenocorticism (▲—▲; n = 12). In two other dogs with hyperadrenocorticism (○—○), the biexponential plot could be resolved into two straight lines (..... and – – –).

cellulose acetate, only partial separation was obtained between the liver and corticosteroid-induced AP activity. However, at pH 5 a clear separation was obtained between the two isoenzymes (Fig. 4). A general pattern for the protein absorption at 280 nm was seen in all separations, indicating only small differences in the extraction of plasma proteins with ethanol. The AP activity in the fractions obtained after FPLC was determined before and after heating for 2 min at 65°C. About 60% of the activity added to the Mono Q column was recovered. After heat inactivation the AP of dogs with hyperadrenocorticism appeared to be stable to heat, while the dogs with liver disease only had a heat-labile AP, confirming the identity of the two isoenzymes.

The elution positions of intestinal and bone AP were also established (Fig. 5). Both isoenzymes appeared to be heat labile. Hence all four AP isoenzymes could be separated by anion-exchange chromatography, intestinal AP being eluted with the column volume, bone AP at 0.06 M sodium chloride, liver AP at 0.11 M sodium chloride, and corticosteroid-induced AP at 0.16 M sodium chloride. In the plasma samples investigated, no intestinal or bone AP isoenzyme was found. Occasionally the liver AP and corticosteroid-induced AP isoenzymes coexisted in the same plasma of dogs with Cushing's syndrome (Fig. 5), which was confirmed by differences in heat

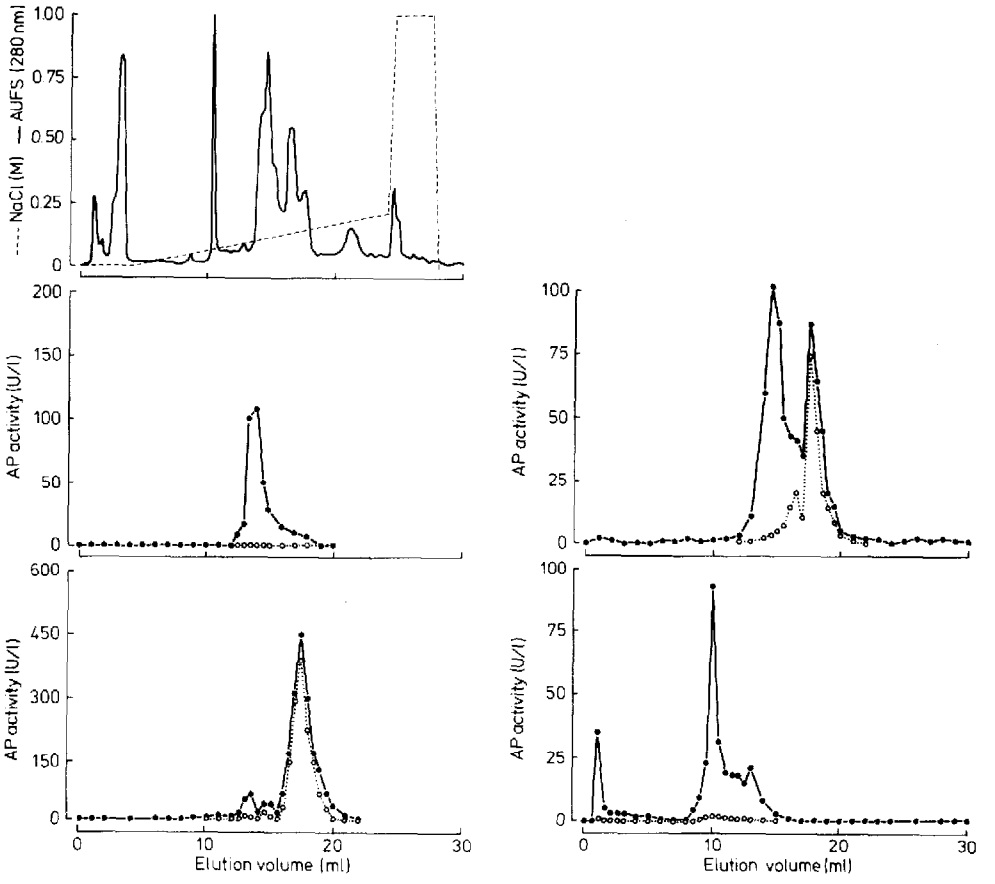

Fig. 4. Anion-exchange chromatography of canine plasma alkaline phosphatases extracted with ethanol on a Mono Q column. The column was equilibrated with 10 m*M* sodium acetate–acetic acid buffer (pH 5) and 0.5 *M* magnesium chloride. The proteins were then eluted with a linear gradient of 0–0.2 *M* sodium chloride in the same buffer at a flow-rate of 1 ml/min. The upper figure shows the general absorption pattern at 280 nm. The middle and bottom figures show the AP enzyme activity in the collected fractions before (●—●) and after (○--○) heating for 2 min at 65°C of plasma from animals with liver disease and animals with Cushing's disease, respectively. The AP from the plasma of a dog with liver disease was eluted at 0.11 *M* sodium chloride, while that corticosteroid-induced AP was eluted at 0.16 *M* sodium chloride.

Fig. 5. Elution profiles of ethanol-extracted canine plasma AP on a Mono Q anion exchanger (●—●). The chromatographic conditions were as in Fig. 4. The upper figure shows the existence of two AP activity peaks in the plasma of an animal with Cushing's disease, at 0.11 and 0.16 *M* sodium chloride, respectively. After heating at 65°C (○—○) the second peak remained, which proved it to be due to corticosteroid-induced AP. In the bottom figure the elution position of bone AP is shown. Bone AP was eluted mainly at 0.06 *M* sodium chloride. Intestinal AP was eluted completely within the column volume.

stability. A small unidentified peak of AP activity was sometimes present between the liver AP and corticosteroid-induced AP peaks obtained by FPLC.

DISCUSSION

Heat inactivation as a means of separation of different AP isoenzymes is a simple method in comparison with electrophoresis, isoelectric focusing or immunochemical techniques. Because of its simplicity, it can easily be adapted to routine laboratory use. The results of this study demonstrate that AP in plasma from dogs with hyperadrenocorticism is more heat resistant than is that from normal dogs or those with hepatic disease. Heat inactivation at 65°C for 2 min allows the two isoenzymes to be distinguished. Because of individual variations in the half-inactivation times of corticosteroid-induced AP, a quantitative measurement of the different isoenzymes can be obtained only by construction of a multi-point inactivation plot. The possible presence of bone AP in canine plasma does not influence the determination of corticosteroid-induced AP, because the former has an even lower heat stability than liver AP[7,9,12].

Two dogs with hyperadrenocorticism had different denaturation plots than the other twelve dogs of this group. The plots could be resolved into two components with mean half-inactivation times of 18.5 and 262.5 s, indicating the existence of both liver AP and corticosteroid AP in the plasma. The non-linear part is due to the simultaneous inactivation of the two isoenzymes, the heat-labile liver AP being the cause of the rapid decline in the first part of the plot and the heat-stable corticosteroid AP representing the linear part. The contribution of both isoenzymes to the plasma AP could be calculated by extrapolation of each part of the plot.

The rate of heat inactivation of plasma AP isoenzymes appears to be higher than that of AP in liver extracts as reported by Wellman *et al.*[13]. This difference may be due to the effects of precipitation of plasma proteins that would enhance the denaturation of plasma AP[21].

The separation of hepatic and corticosteroid-induced AP isoenzymes by electrophoresis on cellulose acetate may result in overlapping bands, making this technique less useful for quantitative measurement of these AP isoenzymes. In the present study a new technique is described for separation of AP isoenzymes by FPLC. One isoenzyme is predominant in plasma of healthy dogs and dogs with liver disease. Corticosteroid induction leads to the presence of an isoenzyme more stable to heat which is eluted from the anion-exchange column at higher sodium chloride concentrations. Both the heat stability and the difference in elution of the corticosteroid-induced enzyme from the anion-exchange column may be caused by an increase in sialic acid residues as reported by Wellman *et al.* The isoelectric point of the corticosteroid-induced enzyme, p*I* 3.5, indicates an acidic isoenzyme in contrast with p*I* 4.5 for the liver isoenzyme[14]. This lower isoelectric point may be one of the reasons for the stronger retention of this isoenzyme by the Mono Q column at pH 5. The close similarity of liver and corticosteroid-induced isoenzymes after treatment with neuraminidase suggests that both isoenzymes are derived from a single gene and that differences are due to post-translational modification resulting in the addition of sialic acid residues. The small heat-resistant peak between the main liver and corticosteroid-induced peaks in FPLC may be a transient form between different stages of incorporation of sialic acid residues.

The separation of isoenzymes by FPLC, giving isolated isoenzyme fractions, may be valuable for further characterization of AP isoenzymes as well as for the

generation of isoenzyme-specific antiserum, which in turn may have diagnostic value in some diseases and in oncology.

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