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Studies on Alkaline Phosphatase Isoenzyme in the Serum and Organs of the Rat

Won Joon Kim, Hye Seong Cho and Sa Suk Hong

Department of Pharmacology, Yonsei University College of Medicine, Seoul, Korea

Isoenzymes of alkaline phosphatase from purified extracts of liver, intestine, pancreas and bone of rats were determined by their isoelectric points and compared with those from serum.

1) The extracts obtained from homogenized tissues were centrifuged at 65,000xg and filtered through an Ultrogel AcA 34 column. Among the three major peaks obtained by gel filtration, the second peak fractions were further separated by isoelectric focusing. Isoenzymes of alkaline phosphatase were found only in the second peak.
2) Isoenzymes of alkaline phosphatase were distinguishable with pH 3.5-10 ampholytes. When pH 3-6 ampholytes were used, isoenzymes were more clearly separated, e.g., 4 in serum, 5 in intestine and 2 each in the liver, pancreas, and bone.
3) Comparing the bands of the isoenzymes of alkaline phosphatase to those of serum, only the band with 5.04 pI was the same between serum and intestine.

These results indicate that several forms of alkaline phosphatase, even though all are from the rat, may exist; and some of the isoenzymes of alkaline phosphatase found in the serum originated from the intestine.

Key Words: Alkaline phosphatase isoenzyme, Isoelectro focusing, Isoelectric points

Alkaline phosphatase (E.C. 3.1.3.1: orthophosphoric monoester phosphohydrolase) is a glycoprotein enzyme that hydrolyzed organic phosphate esters in alkali media. It is widely distributed in intestinal mucosa, liver, kidney, lung, placenta, and blood. In certain disorders of the liver and in osteoblastic bone diseases, the activity of serum alkaline phosphatase is reported to be increased. Taswell and Jeffers (1963) observed that the serum enzyme activity was also increased in liver cancer and in biliary obstruction. The reason for this difference in the increased level of serum enzyme activity in the

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normal and diseased conditions is probably due to the fact that these enzymes originated from several different organ systems.

Fishman and coworkers (1968) have characterized the enzyme which was isolated and purified from intestine and placenta. Purified enzymes following neuraminidase digestion had different optimum pH and Km values. In support of this, McKenna and coworkers (1979) have demonstrated that while the purified enzymes from bone, kidney and liver shared a common antigenic property, the enzymes isolated from placenta and intestine shared yet another property. Furthermore, the various isoelectric points of the isozymes isolated from liver, bone, and kidney in their native forms could all be converted into a single electrophoretic form by treatment with neuraminidase. However, the isozymes purified from intestine and placenta retained different isoelectric points even after the neuraminidase treatment.

The separation of isoenzymes by electrophoresis on starch gel, agar gel and cellulose acetate were only partially successful. For example, the isoenzymes obtained from bone and liver diseases had migrated to similar distances on these separation media. However, with polyacrylamide gel, Kaplan and Rogers (1969) were able to separate the alkaline phosphatases of human serum into four distinct isoenzymes. One of them was identical with the intestinal enzyme and the others were similar to those present in liver and bone.

In an effort to determine the electrophoretic identity of various isoenzymes of alkaline phosphatase from several organ sources, different tissues of rats were purified and their isoelectric points were determined. Furthermore, the sources of isoenzymes found in the serum were determined and compared to those of the tissues.

MATERIALS AND METHODS

Tissue preparation

Liver, intestine, pancreas, tibia and blood obtained from normal rats were used in this study. Liver and pancrease were rinsed and the remaining blood in the liver was removed by injecting physiological saline through the portal veins. Duodenum was washed out by flushing the luminal side with physiological saline. The washed tissues were homogenized with the Teflon homogenizer (Fisher Scientific) in 1.5 to 5 volumes of an ice-cold 0.25 M sucrose solution. The tibias were pulverized and incubated in 0.05 M Tris-HCl buffer (pH 8.0) for two days at 4°C for the extraction of alkaline phosphatase. The homogenates were centrifuged for 30 minutes at 65,000×g and the supernatants were collected for further purification.

Gel filtration

Ultrogel AcA 34 (LKB, Sweden) was poured into a vertical column (1.6 x 30 cm) and equilibrated with 0.05 M Tris HCl buffer eluted with Tris-HCl buffer at a flow rate of 30 ml/hr. The eluates were scanned continuously for protein at 280 nm using a UVICORD II spectrophotometer (LKB, Sweden) and 30 drops were collected in each tube. The major protein fractions (containing the alkaline phosphatase activity) were dialyzed in distilled water for 10 hours at 4°C and used for isoelectro focusing.

Isoelectro focusing and measurement of the isoelectric points

The isoelectro focusing was performed on the polyacrylamide thin layer containing Ampholine-carriers in the pH ranges of 3.5 to 10.0 or pH 3.0 to 6.0 (LKB Production,

Sweden) (Kim et al., 1972). The pH values on the surface of the gel were measured with a Contact Electrode (Pye Unicam Ltd., England) every 5 mm from the (+) electrode site. Subsequently, the thin layer gel was incubated for 1 hour with an equilibrium solution containing Trizma-Barbital buffer (pH 8.8), surfactant (pH 7.4) and distilled water in the ratio of 2:1:2.

The alkaline phosphatase bands were developed at 37°C in a buffer solution (pH 10.2) containing amino-2-methyl-1,3-propanediol and 5-bromo-4-chloro-3-indolyl phosphate. The protein bands were stained for 1 hour using Coomassie Brilliant Blue G-250 (Sigma Chemical Co., USA).

RESULTS

When the centrifuged supernatants of rat blood and extracts obtained from various homogenized tissues were directly subjected to isoelectro focusing, the alkaline phosphatase isoenzymes were poorly separated. However, following the gel filtration with Ultrogel AcA 34 which has a resolution limit of molecular weights of 20,000 – 350,000, the alkaline phosphatase activities were found in the second protein peak for every supernatant (Fig. 1,2, 3,4). Fractions of the enzyme activity were pooled and then subjected to isoelectrio focusing.

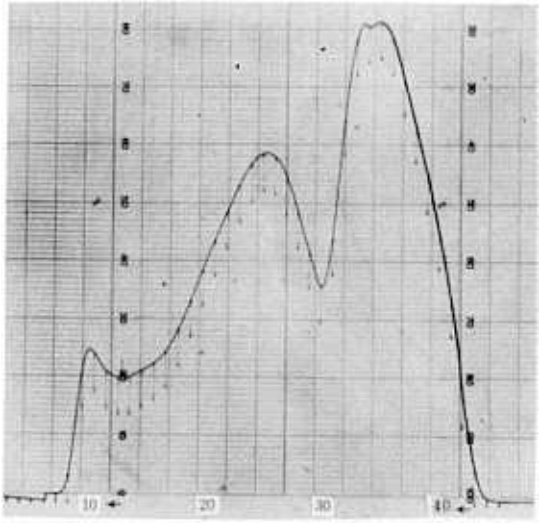

Fig. 2. Gel filtration pattern of intestine extracts.

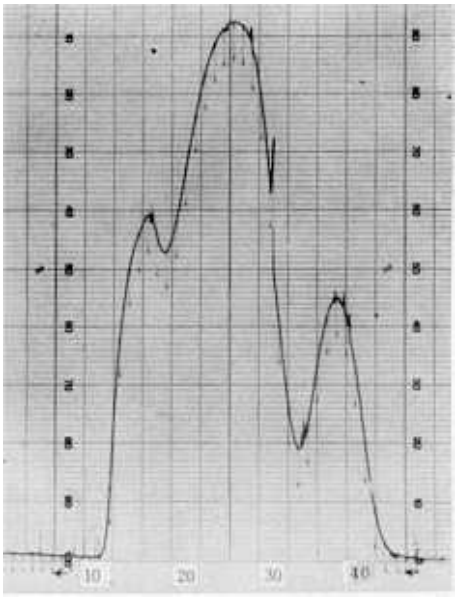

Fig. 1. Gel filtration pattern of serum protein.

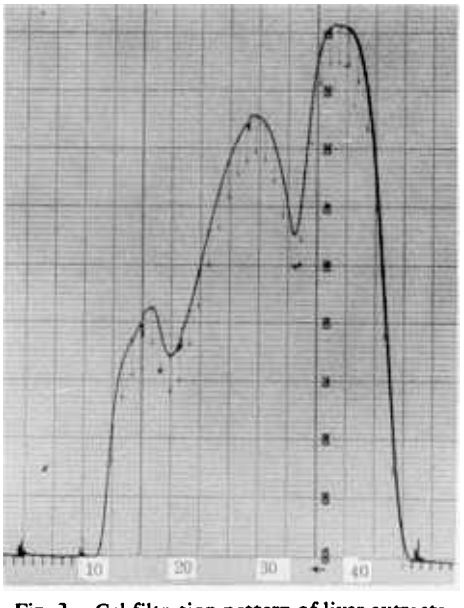

Fig. 3. Gel filtration pattern of liver extracts.

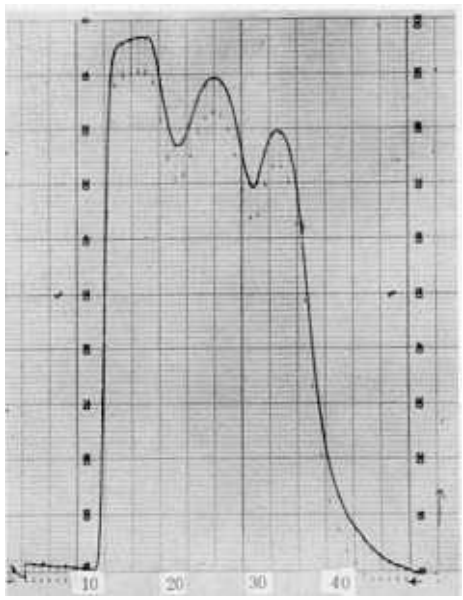

Fig. 4. Gel filtration pattern of bone extracts.

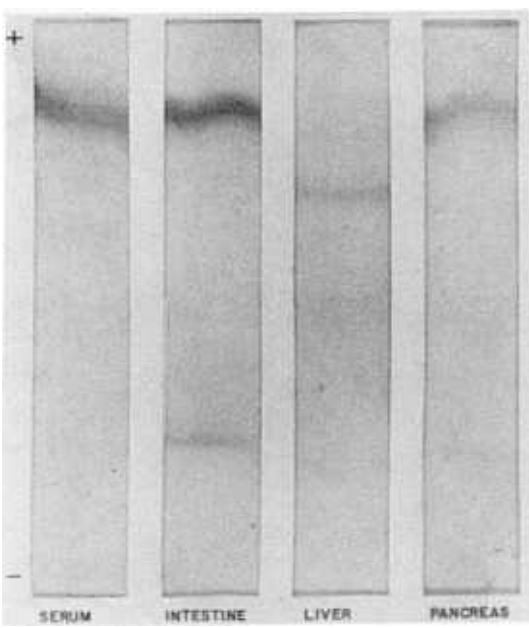

Fig. 5. Isoelectro focusing patterns with pH 3.5-10 ampholytes.

After such preliminary purification, all of the isoenzymes of alkaline phosphatase appeared within the fraction numbers of 20 and 30.

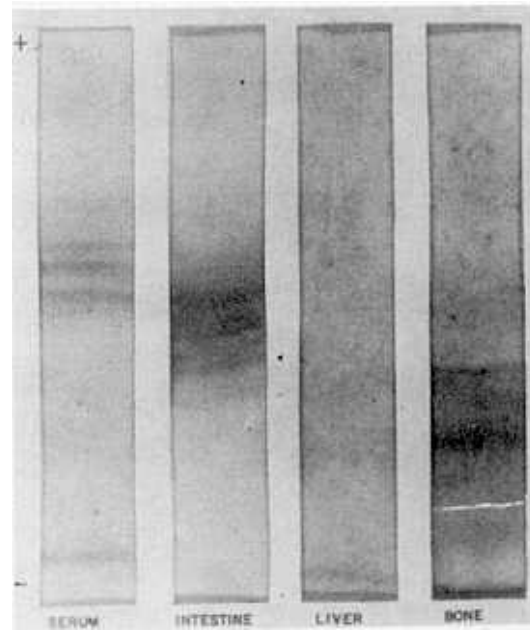

Fig 6. Isoelectro focusing patterns with pH 3-6 ampholytes.

Isoelectric points of the isoenzymes of serum alkaline phosphatases

Upon isoelectro focusing with the wide range ampholytes (pH 3.5-10.0), the poorly separated isoenzyme bands appeared in the range of pI of 4.80 to 5.04 (Fig. 5). When these were subjected to another isoelectro focusing with the short range ampholytes (pH 3.0-6.0), the isoenzymes were separated into four with the short range ampholytes (pH 3.0-6.0), the isoenzymes were separated into four distinct bands with the pI of 4.89, 4.94, 5.04 and 5.89 (Fig. 6.).

Isoelectric points of the isozymes of tissue specific alkaline phosphatase

Initial isoelectro focusing with the wide range ampholyte buffer for the peak fraction obtained from gel filtration of the supernatants of intestinal extracts yielded isoenzyme bands with a pI of 4.80 − 5.00 and a pI of

6.80 (Fig. 5). Secondary isoelectro focusing of the first diffuse band with the short range ampholyte buffer yielded five distinct band with the pI values of 5.04, 5.10, 5.14, 5.22 and 5.32 (Fig. 6). The band with a pI value of 5.04 obtained from intestinal extracts was identical with that obtained from serum.

The pooled protein fractions obtained from the gel filtration of the pancreatic extract supernatants were subjected to the same isoelectro focusing steps as the intestinal extracts. Results obtained were the same as those of the intestinal extracts, but the protein bands were more or less faint (Fig. 5).

Liver extracts produced a single protein band with a pI value of 5.22 upon initial wide range isoelectro focusing. Secondary isoelectro focusing of this band with the short range ampholyte buffer again yielded a single band with the pI value of 6.02.

Isoelectro focusing with the short range ampholyte buffer for the supernatants obtained from pulverized tibias yielded two distinct bands with the pI values of 5.22 and 5.45 and a third band which was at the cathode electrode site.

DISCUSSION

The elevation of serum alkaline phosphatase activity has been used as a diagnostic index for hepatobiliary and skeletal muscle disorders. However, the mechanism for such elevations in these disorders is not clear.

Many separation methods have been developed for the isoenzymes of alkaline phosphatase. These include electrophoresis on various gels (i.e., starch, agar, cellulose acetate and polyacrylamide), gel filtration, ultracentrifugation, and immunological (antigen-antibody reaction), biochemical (neuraminidase cleavage, L-phenylalanine inhibition) and physical (heat inactivation) methods (Smith et al., 1969; Fishman et al., 1968). Combined methods have also been employed (Rosenberg, 1959; Taswell and Jeffers, 1963; Warnes et al., 1976; Smith et al., 1968; Sörensen et al., 1981). For example, the gel filtration and ultracentrifugation methods were used in combination to learn that the normal serum, liver and bone enzymes were obtained in the 7S-peak fractions. However, in patients with metastatic liver and bone diseases, the serum alkaline phosphatase activities were found both in the 19S and 7S fractions (Dunne et al., 1967; Price and Sammons, 1974). The appearance of 19S fractions may have resulted from the formation of IgG-alkaline phosphatase complex with the liver and bone, but not with the intestinal enzymes. Once the IgG complex was formed, the enzymes moved very slowly upon electrophoresis (Crofton and Smith, 1978; Cha et al., 1975).

Inactivation of the electrophoretically purified enzyme activity with heat (56°C, 10 min) has also been employed to differentiate the tissue specificity of alkaline phosphatases. Heat treatment produced sharp decreases of bone phosphatase activity, while there was less change in the liver enzyme and no change in the placental enzyme activities (Sundblad et al., 1973). In addition to these combinations, enzymatic digestion by sialic acid with neuraminidase has been combined with electrophoretic methods. The isoenzymes obtained from liver, bone and kidney, each with a unique isoelectric point in their native form, could be converted into a single form. However, the intestinal isoenzyme was not affected by the neuraminidase treatment. This result suggests that the purified intestinal enzyme did not contain sialic acid (Kamoda and Sakagishi, 1978).

In this study, we have learned that simple

homogenization combined with ultracentrifugation to obtain tissue extracts for separation by isoelectric focusing on polyacrylamide gel was not sufficient. Both the serum and tissue alkaline phosphatase isoenzymes were poorly separated. However, upon using the combination methodology (i.e., gel filtration and isoelectro focusing), we have obtained clearly separable isoenzymes from various tissue extracts. Thus, by using the Ultrogel AcA 34, initial selection for the 7S fractions has been achieved. With the addition of such a simple procedure as specific gel filtration, isoelectric focusing becomes a powerful tool in separating the isoenzymes of alkaline phosphatase, not only in serum but also in various tissue extracts.

The results of this study indicated that even in normal serum, four distinct isoenzymes were present. Furthermore, there were at least two separate isoenzymes in each of the normal tissues such as liver, pancreas, and bone. While 5 distinct isoenzymes were found in the intestinal extracts, only the band with the pI value of 5.04 was the same between serum and intestine. This result suggests that among the isoenzyme bands found in serum, one may be identical with that found in the intestine and may also originate from the intestine.

Using this combined methodology, we were able to detect the presence of 2 – 5 isoenzsymes of alkaline phosphatase even within one organ. Conventionally, a simple electrophoresis of serum on polyacrylamide has been employed to detect the elevation of serum enzyme activity in various disorders. While the major band of the enzyme obtained in bone diseases moved slightly behind the one commonly seen in liver diseases, they were not clearly separated. However, based on such observations, Moss (1962, 1963) stated that every organ probably contains a single alkaline phophatase; and in disease states, it may undergo structural changes. Alternatively, the alkaline phosphatase had been purified from the intestinal mucosa and the presence of three isozymes were detected by gel filtration. According to Nakasaki et al. (1979), this molecular heterogeneity resulted from aggregation of the monomer molecules of the enzyme. Furthermore, the liver enzyme has also been separated into two components which differed in electrophoretic mobility and in some biochemical characteristics (Adeniyi and Heaton, 1982). These reports support our finding that under normal conditions and even within one organ, several different forms of alkaline phosphatase may exist. This conclusion concurs with those made by Mulivor et al. (1978), Etzler and Moog (1968), and Hodson (1983).

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