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Note

Heterogeneity of alkaline phosphatase observed by high-performance liquid chromatography

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Alkaline phosphatases (AP) are reported to be organ specific, and their specificity can be distinguished by thermostability, chemical inhibition, immunochemical cross-reactivity, and electrophoretic mobility. However, their separation into the four common types of isoenzyme (liver, bone, intestine and placenta) or further demonstration of their heterogeneity can only be achieved by electrophoresis[1–8], and the methods are rather cumbersome. Although multiple samples can be run on the same plate simultaneously, their precision and accuracy are less than optimal.

Newer application of high-performance liquid chromatography in protein separation has provided an alternative yet more effective tool in the resolution and isolation of isoenzymes. In this communication, we demonstrate that by using a Mono Q column (50 × 5 mm I.D., Pharmacia) for such chromatography, bovine AP which normally showed as a broad band or peak on electrophoresis or chromatography is highly polydisperse in nature. At least nine consecutive fractions with different retention times are separated by the column chromatography.

EXPERIMENTAL

Materials

Bovine intestinal AP, and AP isotrol (a mixture of human placental, and fast liver AP) are obtained from Sigma (St. Louis, MO, U.S.A.).

High-performance liquid chromatographic conditions

Instrument: fast protein liquid chromatograph with a fraction collector (Pharmacia, Piscataway, NJ, U.S.A.). Column: Mono Q HR 5/5 anion-exchange column (Pharmacia). Sample size: 25 mg total protein or 5 mg AP in 200 μl. Buffers: (A) 20 m*M* Tris–HCl, pH 7.6; (B) 0.5 *M* sodium chloride in solution A. Gradient: formed from buffers A and B using two pumps at room temperature. The shapes of the gradient will be shown in the figures. Washing: 4 min with buffer A, then re-used. Flow-rate: 1.6 ml/min. Pressure: 25 bar (375 p.s.i.). Fractions: 0.5-min fractions (0.8 ml each tube).

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Assay for alkaline phosphatase activity

A sample solution was made up to 0.8 ml with distilled water, and 0.2 ml of 0.5 *M* diethylamine (pH 10.6) containing 50 m*M* *p*-nitrophenylphosphate were added into the solution to initiate the reaction. The mixture was incubated at room temperature until a yellowish color became distinctively visible, and the reaction was terminated by the addition of 1 ml 0.4 *M* sodium hydroxide. The absorbance was rtead at 405 nm using a Coleman Junior spectrophotometer. A blank was prepared by adding the buffer–substrate solution after the enzyme was inactivated by the addition of sodium hydroxide. Standard solution of *p*-nitrophenol was used to calculate the amount of *p*-nitrophenolphosphate hydrolyzed. A unit of enzyme activity is defined as the amount of enzyme which catalyzes the production of 1 μmole of *p*-nitrophenol in 1 min under above conditions.

For the assay of activity in the fractions obtained from fast protein liquid chromatography (FPLC), an aliquot was assayed as the above procedure, or the total volume in each fraction was used to determine the chromatographic profile of AP.

RESULTS AND DISCUSSION

Alkaline phosphatases from different tissues of origin could be separated by the column. Fig. 1 shows that bovine intestinal, human placental and human liver AP are individually eluted out at different retention times using the sodium chloride gradient shown. The intestinal AP gave a peak with extensive tailing. This tailing is not due to the inefficiency of the column but rather due to the heterogeneity of the isoenzyme, as demonstrated by the following two experiments; the tailing fractions actually consist of varied molecular forms, even the consecutive fractions being different from one another.

An amount of 5 mg of the intestinal AP was applied into the column and nine fractions containing the activity were collected and combined at every third tube to yield three combined fractions. When 200 μl each of these combined fractions were analyzed for the elution profile each fraction was separated into three peaks with retention times corresponding to that of the respective fractions before combination. The results shown in Fig. 2 indicate the molecular dispersity of the enzyme.

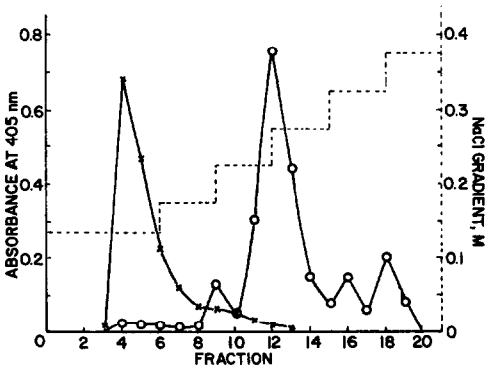


Fig. 1. Separation of alkaline phosphatase of different origins. (×) Bovine intestinal; (O) mixture of placental and liver. The largest peak is identified as placental AP. The broken line indicates the sodium chloride gradient.

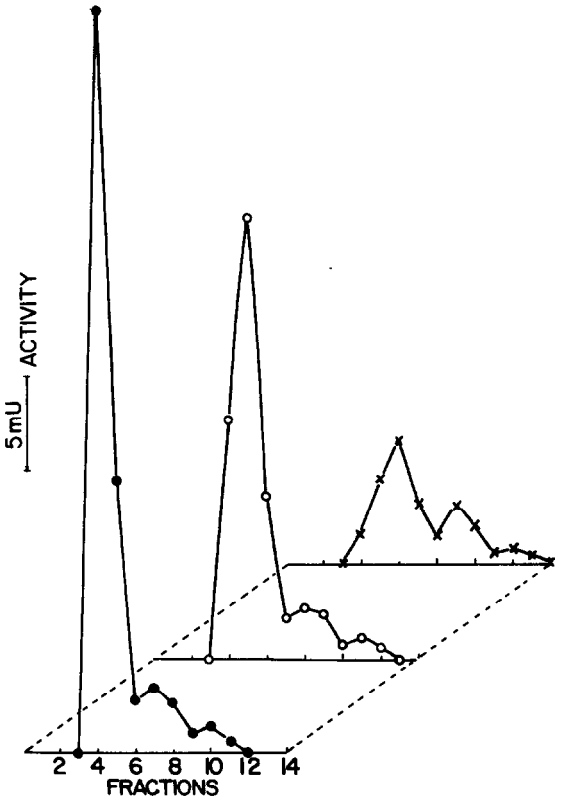
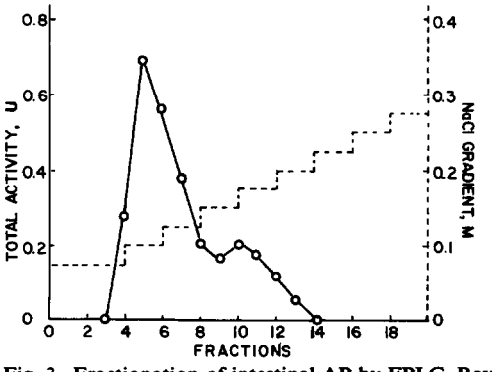


Fig. 2. Re-chromatography of bovine intestinal AP fractions. Bovine intestinal alkaline phosphatase is fractionated into nine fractions according to the chromatography as shown in Fig. 1. Nine fractions (from tune 4 to 12) were combined as follows and re-chromatographed: (●) fractions 4 + 7 + 10; (○) fractions 5 + 8 + 11; (×) fractions 6 + 9 + 12.



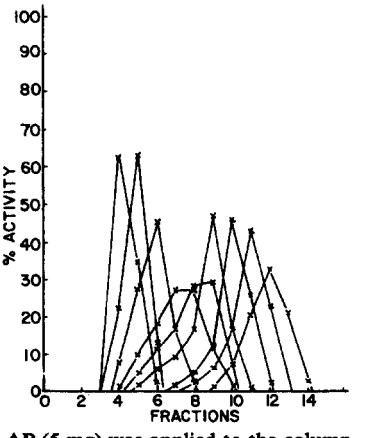


Fig. 3. Fractionation of intestinal AP by FPLC. Bovine intestinal AP (5 mg) was applied to the column and eluted by the sodium chloride gradient shown on the figure as a broken line.

Fig. 4. Distribution of AP in individual fraction. The nine fractions obtained from Fig. 3 were individually re-chromatographed and the percent of activity distribution in different fractions is plotted for each fraction.

By a rather mild sodium chloride gradient as shown in Fig. 3, the intestinal AP shows two broad peaks. When each fraction obtained was re-chromatographed, each showed different compositions as shown in Fig. 4. Although some tailings are evident from the fact that those fractions with longer retention times are more distributed in many tubes, each fraction, however, maintains its characteristic retention time. Thus, intestinal isoenzyme can be separated into at least nine different molecular forms.

Therefore, the FPLC system detects the microheterogeneity of interstinal AP, which has not been previoyusly demonstrated by other analytical methods. Since the neighboring fractions are different from each other, the intestinal AP is highly heterogeneous. The heterogeneity demonstrated by the FPLC is unlikely due to the difference in sialic acid content because the enzyme migrated as a single band on acrylamide gel electrophoresis[9]. It is conceivable that the report on the heterogeneity of mouse duodenal AP[10] caused by complexing with lipids and peptides, describes the factor causing the molecular heterogeneity. However, since the heterogeneity of bovine AP could not be demonstrated by acrylamide gel electrophoresis as that was seen in mouse duodenal AP, the molecular structural change in bovine AP seems to be more subtle. This observation requires further investigation.

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