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Note

Purification of placental alkaline phosphatase by sulphate-mediated chromatography on Cibacron Blue 3G-A

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The increased interest in placental alkaline phosphatase has arisen from the discovery of a similar type of enzyme in sera and carcinomas of cancer patients[1]. Placental alkaline phosphatase and carcinoplacental antigen are similar with respect to phenylalanine sensitivity, heat stability, cleavage by neuraminidase, optimum pH and immunological specificity[2].

This study was aimed at obtaining an easy method for purification of placental alkaline phosphatase in high yields. The method described is based on sulphate-mediated chromatography on Cibacron Blue–Sepharose and is a quicker process giving higher yields than previously reported procedures[3–5].

EXPERIMENTAL

Materials

Cibacron dyes were gifts from Ciba-Geigy (Manchester, U.K.), Procion dyes were gifts from I.C.I. Organics Division (Manchester, U.K.), Sepharose 4B was obtained from Pharmacia (Uppsala, Sweden), *p*-nitrophenyl phosphate disodium salt was purchased from Sigma (St. Louis, MO, U.S.A.) and ammonium sulphate was obtained from Merck (Darmstadt, F.R.G.). All other chemicals were of the highest grade commercially available.

Enzyme assay

Enzyme assay was carried out at 37°C with 18 m*M* *p*-nitrophenyl phosphate disodium salt and 10 m*M* magnesium chloride in 50 m*M* glycine–sodium hydroxide buffer at pH 10.5[6]. The amount of *p*-nitrophenol liberated in 15 min was calculated from its extinction coefficient at 410 nm, *i.e.*, 18.5 $cm^2/\mu mol$ at 37°C, pH 10.5. The unit of enzyme was defined as that amount which liberated 1 μmol of *p*-nitrophenol per min per ml under the given conditions.

Protein

Protein was assayed by use of the absorbance at 280 nm[7] or by the turbidimetric method of Mejbaum-Katzenellenbogen and Dobryszycka[8].

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Immobilization of triazine dye to agarose

Triazine dyes were coupled to Sepharose 4B according to the method of Baird *et al.*[9]. The dyes used for the screening studies were Cibacron Blue 3G-A and Brilliant Blue BRP, Procion Red HE-7BN, HE-3B, MX-8B and H-8BN, Procion Yellow H-A and MX-4G, Procion Brown MV-5BR and H-5R, Procion Black-BN, Procion Olive H-7G and Procion Blue H-B. The concentration of coupled dye was determined by the method of Leatherbarrow and Dean[10].

Screening of placental alkaline phosphatase on triazine dye–Sepharose 4B adsorbents

Triazine dye–Sepharose adsorbents were prepared and packed into micro columns (column volumes 1 ml). The columns were equilibrated with 50 m*M* Tris–HCl buffer at pH 8.5 with 30 column volumes. The flow-rates were maintained at 4 ml/h. A 0.5-ml volume of the crude enzyme extract (containing six units of enzyme) in the same Tris buffer was applied to each column. The columns were each washed with 10 ml of buffer, followed by a pulse of 1 *M* potassium chloride in equilibration buffer (10 ml). The wash and the salt eluates (2-ml fractions of each) were then assayed for enzyme activity. The experiment was repeated at pH 7.5 in 10 m*M* Tris–HCl buffer.

Purification of placental alkaline phosphatase

Partially purified enzyme was extracted according to the method for placental alkaline phosphatase described by Ghosh and Fishman[3] and was used as the starting material for this process.

Cibacron Blue 3G-A–Sepharose 4B (30 × 2 cm, ligand concentration 3.4 μmol per ml wet weight of gel) was equilibrated with 10 m*M* Tris–HCl buffer, pH 7.5, containing ammonium sulphate to 50% saturation and 1 m*M* zinc sulphate. The flow-rate was adjusted to 40 ml/h.

The enzyme extract (540 ml) in the same equilibration buffer was applied to the column. The column was washed with the equilibration buffer until the absorbance at 280 nm decreased to zero. The enzyme was eluted by decreasing the ammonium sulphate concentration of the equilibration buffer to 30% saturation. Fractions (5 ml) were collected and assayed for enzyme activity and protein. Those exhibiting high specific activity were pooled (450 ml), dialyzed and concentrated.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was carried out according to the method of Davis[11] with 7.5% (w/v) gels (7.5 × 0.5 cm).

Staining of gels for alkaline phosphatase activity

The method of Allen and Hyncik[12] was used to visualize enzyme activity in polyacrylamide gels. An opaque band was obtained after soaking the gel in sodium α-glycerophosphate followed by incubation in lead nitrate solution.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis

This was carried out according to the method of Weber and Osborn[13].

Immunodiffusion experiments

Immunodiffusion tests were carried out in 0.15 *M* phosphate buffer, pH 7.2 in

1.5% (w/v) agar[14]. Diffusion was allowed to proceed for 3 days and the gel was then washed in 0.9% (w/v) sodium chloride before being stained for protein.

RESULTS

Screening studies of the placental alkaline phosphatase on fifteen immobilized triazine dyes in 10 m*M* Tris–HCl buffer, pH 7.5, or 50 m*M* Tris–HCl, pH 8.5, showed that the enzyme had not affinity for the adsorbents under these conditions. However, the enzyme could be sorbed onto Cibacron Blue 3G-A–Sepharose in the presence of a high salt concentration (50% saturation) of ammonium sulphate in 10 m*M* Tris–HCl buffer at pH 7.5. Under these conditions the buffer contained 1 m*M* zinc chloride to prevent inactivation of the alkaline phosphatase at high ammonium sulphate concentration. The enzyme was eluted from the column by lowering the salt concentration of the buffer to 30% saturation with respect to ammonium sulphate.

The method used for purification of placental alkaline phosphatase involved precipitation of partially purified alkaline phosphatase on Cibacron Blue 3G-A–Sepharose (ligand concentration 3.4 μmol per ml wet gel) (Fig. 1). The recovery of the purified enzyme was approximately 95%. The overall yield of the enzyme after the final chromatographic step was 52%. The specific activity was 224 units per mg protein. The results of the purification procedure are summarized in Table I.

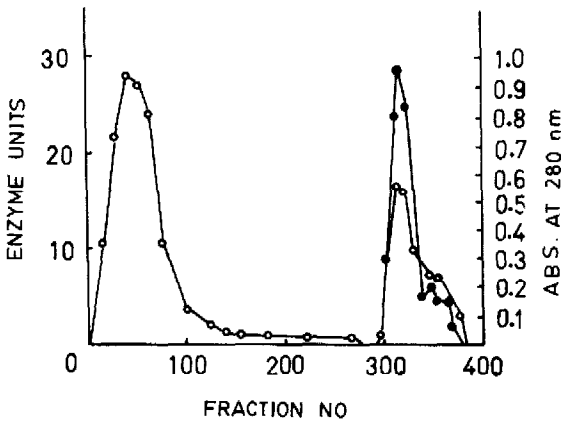

Fig. 1. Elution profile of placental alkaline phosphatase by sulphate-mediated chromatography. Enzyme extract (540 ml) containing 312 mg of protein with a specific activity of 4.9 units/mg was applied to a column (30 × 2 cm) of Cibacron Blue 3G-A–Sepharose 4B in 50% saturated ammonium sulphate (313 g/l), 10 m*M* Tris-HCl buffer, pH 7.5, containing 1m*M* zinc sulphate. The elution performed at room temperature (25°C) with 30% saturated ammonium sulphate (176 g/l), 10 m*M* Tris–HCl buffer, pH 7.5, resulted in a pure enzyme preparation of specific activity 224 units/mg protein. The fraction size was 5 ml. ○—○, Protein; ●—●, activity.

Criteria of purity

Electrophoresis. A single band was obtained after polyacrylamide gel electrophoresis when stained for either protein or enzyme activity. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis also showed the presence of a single protein band.

TABLE I

SUMMARY OF PURIFICATION STEPS USED TO ISOLATE PLACENTAL ALKALINE PHOSPHATASE

Details are provided in Experimental.

Purification step	*Protein (mg)*	*Activity (units)*	*Specific activity (units/mg protein)*	*Purification*	*Yield (%)*
Tris homogenate* supernatant	14,050	2810	0.2	1	100
Dialyzed butanol* supernatant	1110	2444	2.2	11	87
Ammonium sulphate precipitated proteins	565	2135	3.7	18.5	76
Supernatant after heat treatment*	312.7	1545.5	4.9	24.5	55
Chromatography on Cibacron Blue–Sepharose	6.5	1461	224	1120	52

* According to the method of Ghosh and Fishman[3].

Immunological criteria. The immunodiffusion tests with antibody prepared from purified alkaline phosphatase a distinct single line of precipitation formed indicating that the enzyme preparation was homogeneous.

DISCUSSION

Placental alkaline phosphatase was purified successfully by sulphate-mediated chromatography on Cibacron Blue–Sepharose and obtained in high yields (52%). The method is simple and economical, and only a single chromatographic step is needed to purify the placental enzyme. Large quantities of contaminating proteins are removed during the chromatographic phase, the column has a high capacity and ammonium sulphate inhibits placental proteases.

Subforms of placental alkaline phosphatase which have been reported in other purification procedures[3,4] may have arisen due to the activity of such proteases. In the presence of ammonium sulphate the action of such proteolytic enzymes is demonstrably suppressed during the chromatographic process. A purification of placental alkaline phosphatase using sulphate-mediated chromatography on phenylalanine–Sepharose has been reported[5] and gives a yield of 22% in the final step. However, this method involves five consecutive chromatographic steps, two on phenylalanine–Sepharose and three on DEAE-cellulose. In comparison the present method gives a higher yield (52%) and is very simple, involving a single chromatographic step on Cibacron Blue 3G-A–Sepharose. In addition the chromatographic medium is easily made by coupling the dye to Sepharose via the reactive triazine group of the dye. The Cibacron Blue adsorbent can be reused without appreciable loss of capacity.

The enzyme placental alkaline phosphatase has significant clinical importance.

Besides the fact that the enzyme is identical to the carcinoma-associated "Regan isoenzyme," the placental isoenzyme also serves as a reliable marker for clinical diagnosis during pregnancy[15]. It is therefore important to have an easy method for obtaining pure enzyme for use as a standard in clinical laboratories. The purification of placental alkaline phosphatase by sulphate-mediated chromatography on Cibacron Blue may serve for this purpose.

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