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PREPARATION OF HOMOGENEOUS HUMAN PROSTATIC ACID PHOSPHATASE USING CONCANAVALIN A-SEPHAROSE 4-B

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Summary

A simple, rapid and efficient procedure is presented for the purification of human prostatic acid phosphatase (orthophosphoric-monoester phosphohydrolase (acid optimum), EC 3.1.3.2) to homogeneity. The method employs two steps suitable for use with large quantities of material, followed by chromatography on concanavalin A-Sepharose as its sole column step. The procedure also permits the recovery of purified enzyme in higher yields than earlier methods.

Human prostatic acid phosphatase (orthophosphoric-monoester phosphohydrolase (acid optimum), EC 3.1.3.2) is an enzyme of substantial biochemical and clinical interest, particularly because of its importance as a diagnostic indicator of the development of prostatic cancer [1]. A wide variety of biochemical and immunochemical studies of the enzyme have been described and numerous other studies such as the development of radioimmune assays are underway and it is clear that such studies would be aided by the availability of a simple procedure for the preparation of the homogeneous enzyme. Perhaps the best-described procedure to date, and one which we have frequently employed, is that due to Ostrowski [2]. It employs three separate column procedures including two Sephadex G-100 chromatography steps and an ion exchange chromatography step in which both pH and ionic strength are changed during application of a 'convex' gradient. The column steps are time consuming at best, but in our hands the initial chromatography step did not always provide a good separation of the enzyme from colored impurities including hemoglobin, although this was improved when we employed Sephadex G-150 instead of Sephadex G-100, as might be expected since the prostatic enzyme has a molecular weight of 102 000 [3]. We also found the ion exchange step [2] to be difficult to reproduce, perhaps as a consequence of the rather complex gradient, small variations in salt content or possibly variations in the

autopsy or hypertrophic source material itself. Because we are studying several aspects of the catalytic mechanism of this and related enzymes [4—9] we sought a simple purification procedure which could be readily scaled-up so as to permit us to obtain relatively large quantities of homogeneous enzyme. It has been carefully established that human prostatic acid phosphatase is a glycoprotein having 38—41 carbohydrate residues attached [10] and it therefore seemed possible that this enzyme, like alkaline phosphatase [11] would be bound to concanavalin A-Sepharose and that this could potentially provide a useful purification step. This is indeed the case, and we report here a simple, rapid and efficient procedure for the purification of human prostatic acid phosphatase to homogeneity. The steps include homogenization in a solution containing Tween 80 and EDTA (added to reduce autolysis and degradation during the extraction step), ammonium sulfate fractionation, dialysis against water and finally chromatography on concanavalin A-Sepharose 4-B. The homogeneous enzyme has a specific activity of 240—250 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ when assayed at 25°C with 2.8 mM *p*-nitrophenyl phosphate in 0.1 M sodium acetate buffer (pH 5.0). Experimental procedures and results will now be described.

Enzyme assays were carried out in 0.1 M sodium acetate (pH 5.0) using 2.8 mM *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, Mo.) as a substrate. Following addition of 50 μl of appropriately diluted enzyme solution to 2.0 ml of substrate solution the samples were incubated at 25°C for 5 min and the reaction stopped by the addition of 0.2 ml of 1.25 M NaOH. Product formation was quantitated by measuring the absorption at 400 nm and employing a molar extinction coefficient of $1.8 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ for the *p*-nitrophenoxide ion. Protein determinations were done by the Lowry method [12] using crystalline bovine serum albumin as a standard. The preparation of concanavalin A-Sepharose 4-B was carried out at 4°C by minor modifications of published procedures [13]. Sepharose 4-B (200 ml) was washed twice with equal volumes of water and suspended in 150 ml of water. In a well-ventilated fume hood, an equal volume of 2 M Na_2CO_3 solution was added with stirring, then a solution of cyanogen bromide (15 g) in 8 ml of fresh acetonitrile was rapidly added. After stirring rapidly for 2—3 min the cyanogen bromide-activated Sepharose was poured onto a Buchner funnel and washed successively with 2 l of 0.1 M $NaHCO_3$ (pH 9.5), 2 l of water and finally 2 l of a coupling buffer [14] consisting of 0.13 M $NaHCO_3$ plus 1.0 M NaCl (pH 9.5). Concanavalin A (1 g; Sigma Chem. Co., St. Louis, Mo.) dissolved in 80 ml of coupling buffer was added to the activated Sepharose contained in a beaker. This suspension was allowed to stand 12 h with occasional gentle stirring. In order to remove unbound concanavalin A and to block any remaining reactive sites on the Sepharose it was then washed with 1 l of 1 M glycine, 2 l of 2 M urea, and 2 l of 0.1 M sodium acetate buffer (pH 4.0); all of these solutions were also 1 M in NaCl. The resulting gel suspension was then poured into columns and equilibrated with 5 mM phosphate buffer (pH 6.4) containing also 0.9% NaCl.

A typical preparation starting with 50 g of frozen hypertrophic human prostates is presented in Table I. All procedures were conducted at 4°C. The barely defrosted material was sliced into small pieces and homogenized for

TABLE I

PREPARATION OF HOMOGENEOUS PROSTATIC ACID PHOSPHATASE USING AFFINITY CHROMATOGRAPHY

Purification step	Volume (ml)	Protein (mg)	Total activity (units)	Specific activity (μmol·min^{-1}·mg^{-1})	Recovery (%)	Relate activity
10 000 x *g* supernatant from initial homogenate*	225	1000	14 750	14.75	100	1
Supernatant from 0—50% $(NH_4)_2SO_4$ pptn.	245	401	9 820	23.0	66.6	1.56
50—70% $(NH_4)_2SO_4$ precipitate	33	271	10 147	37.4	68.8	2.54
Supernatant after dialysis against H_2O	46.5	214	9 586	44.8	65.0	3.03
Sepharose 4-B-concanavalin A chromatography	6.53	6.8	1 639	241.0	11.1	16.3

*Obtained from 50 g of frozen hypertrophic prostates; the yield with postmortem material is somewhat less.

2 min with 200 ml of 0.05 M EDTA/0.01% Tween 80. The homogenate was allowed to stand 12 h with occasional stirring. After centrifugation at 10 000 x *g* for 30 min the supernatant was subjected to an ammonium sulfate fractionation. The 0—50% precipitate obtained after centrifugation at 10 000 x *g* (30 min) was discarded. The 50—70% precipitate obtained after a similar centrifugation was dissolved in 30 ml of a pH 4.0 solution of 0.06 M citric acid/0.08 M sodium phosphate [15]. This solution was dialyzed against several liters of distilled-deionized water for approx. 14 h and centrifuged at 20 000 x *g* (30 min). The supernatant was then dialyzed against 5 mM sodium phosphate/0.9% NaCl (pH 6.4). The dialyzed sample was applied to a 2 x 20 cm column of concanavalin A Sepharose 4-B equilibrated with the same buffer. The flow-rate was 36 ml/h and 9-ml fractions were collected (Fig. 1). When all of the sample had been applied to the column it was allowed to stand for 2 h, then

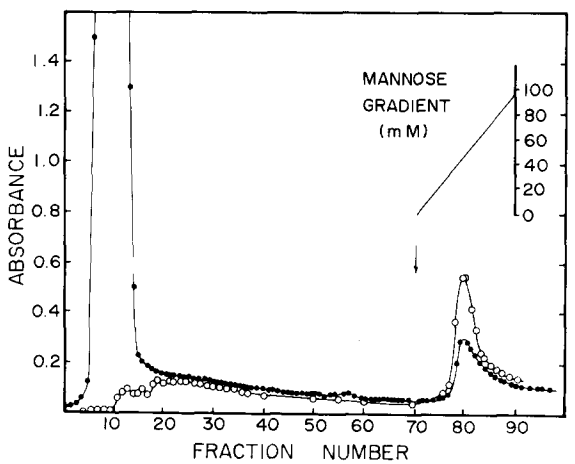

Fig. 1. Purification of human prostatic acid phosphatase by chromatography on concanavalin A-Sepharose 4-B. A 2 x 20 cm column was charged with 214 mg of protein resulting from the water dialysis step (Table I). Elution was conducted initially with 5 mM sodium phosphate/0.9% NaCl (pH 6.4) and at the point indicated by the arrow a linear 0—0.3 M gradient of mannose was applied (made up using 300 ml of the above starting buffer in each reservoir). The flow rate was 36 ml/h and 9-ml fractions were collected. Protein (•—•) was measured by absorbance at 280 nm, and activity (○—○) by measurement of *p*-nitrophenoxide absorbance at 400 nm; inset, initial portion of the mannose gradient (mM).

elution with the starting buffer was continued until a negligible absorbance at 280 nm was obtained (Fig. 1). Protein impurities including hemoglobin and other colored proteins are eluted at this stage. Bound enzyme was then eluted by application of a linear 0—0.3 M mannose gradient (inset, Fig. 1) made up using 300 ml of the pH 6.4 phosphate/salt buffer in each reservoir. The fractions comprising the activity peak were pooled, concentrated on an Amicon Diaflo apparatus (PM-30 membrane) and dialyzed against 12.5 mM Tris maleate/100 mM KCl (pH 6.5).

The peak of enzymatic activity obtained following application of the mannose gradient (Fig. 1) in the concanavalin A-Sepharose chromatography step of the purification procedure (Table I) is homogeneous acid phosphatase. The specific activity of 240—250 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ (when assayed with 2.8 mM *p*-nitrophenyl phosphate in 0.1 M sodium acetate buffer, pH 5.0) is that characteristic of the homogeneous enzyme as obtained in our laboratory and by others (cf. footnote 2 in ref. 16)*. This has additionally been confirmed by gel electrophoresis studies of the enzyme obtained in the final step. Single coincident protein and activity bands [17] were obtained at a variety of protein concentrations (Fig. 2) and confirm that this procedure results in the isolation of homogeneous enzyme.

The preparative procedure is remarkable for its simplicity since it employs but a single column chromatography step, circumventing entirely the ion exchange steps and repeated gel chromatography columns necessary to obtain homogeneous enzyme by previous methods. The capacity of the concanavalin A-Sepharose 4-B column is easily exceeded and as a result a portion of the enzyme may be eluted as a broad flat activity peak during the initial washing stages (Fig. 1). The recovery obtained under the conditions described in Table I is also comparable with previously reported literature procedures but it can in fact be significantly improved by the simple expedient of using a smaller sample size or larger column size in the concanavalin A chromatography step. Thus, in another preparation starting with 52 g of tissue, a combined yield of 10 mg of homogeneous enzyme corresponding to a 30% recovery of the enzyme units present in the initial supernatant was obtained when the concanavalin A chromatography step was carried out in three batches instead of the one shown in Table I. This is significantly improved recovery compared to literature procedures. Because the initial steps are easily amenable the large-scale manipulations and because the column, which is charged with a relatively small amount of protein, can be readily scaled-up, this procedure is very convenient for the large-scale purification to homogeneity of human prostatic acid phosphatase.

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*The exact numerical value can be somewhat increased if higher substrate concentrations are employed, and we also find values 8—10% higher when citrate buffer is employed instead of acetate.

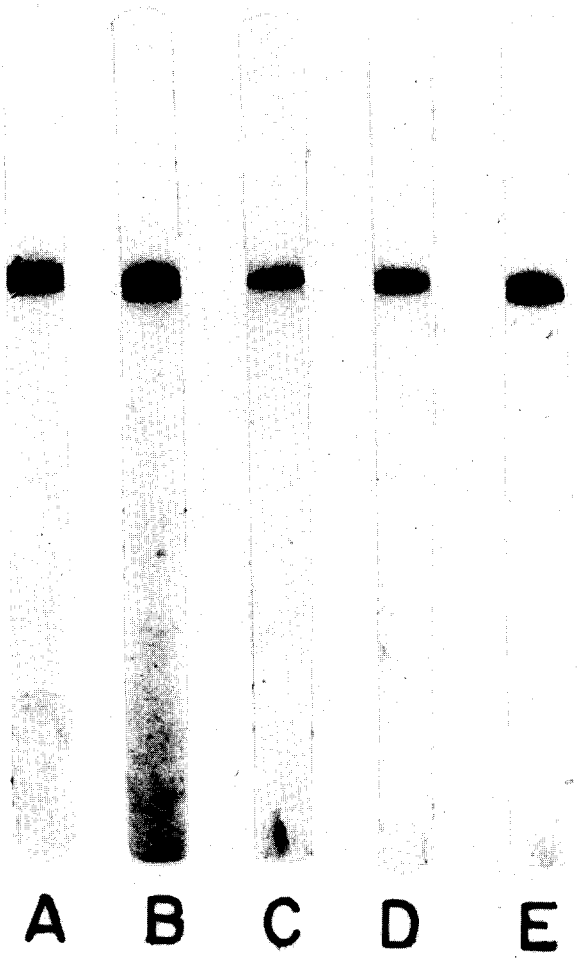

Fig. 2. Polyacrylamide gel electrophoresis of human prostatic acid phosphatase obtained following concanavalin A-Sepharose 4-B chromatography (Table I). The 9% gels were stacked using 0.37 M Tris/HCl (pH 8.9) and run (using 1 : 20-diluted buffer) with a current of 4 mA per gel for 6 h at 4°C. The protein and activity band migrated just behind the tracking dye. From left to right are illustrated gels stained for protein with Coomassie Blue (6 and 12 μg protein/gel, A and B) and stained for activity [16] with α-naphthyl phosphate-Fast Garnet GBC salt (6, 9 and 12 μg of protein/gel, C—E, respectively).

Acknowledgements

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