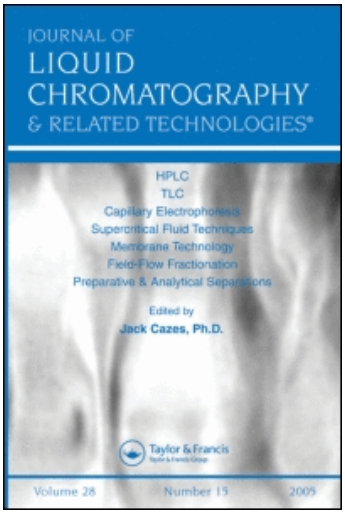

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THE PURIFICATION OF PROSTATIC ACID PHOSPHATASE FROM SEMINAL PLASMA BY REVERSE PHASE HIGH PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

We report here the rapid isolation of PAP directly from dialyzed human seminal plasma by reverse phase high performance liquid chromatography (RP-HPLC). The recovery of PAP of high specific activity was found to be dependent on the treatment of the fractions after separation. The collection of fractions into a stabilizing medium was essential to the preservation of the specific activity of the enzyme. This finding extends the use of RP-HPLC to the purification of active enzymes from complex biological matrixes.

INTRODUCTION

Prostatic acid phosphatase (PAP) is a clinically important marker used for the detection of prostatic cancer. This enzyme is a glycoprotein with a molecular weight of approximately 102,000, and has been purified to near homogeneity from seminal plasma by numerous protein purification techniques. Proteins having high specific enzymatic activity and immunologic re-activity have seldom been

separated using reverse phase high performance liquid chromatography (RPHPLC). The critical role of stabilizing agents in the recovery of enzymatic activity and the chromatographic methodology for the isolation and purification of PAP is hereby described.

MATERIALS

Prostatic acid phosphatase was obtained from human seminal plasma as described by Mahan and Doctor (1). Acetonitrile and trifluoroacetic acid of HPLC grade were obtained from Fisher Scientific, (Silver Spring, MD). The o-carboxyphenyl phosphate was obtained from Sigma Chemical Co., (St. Louis, MO) and the RIA-Quant PAP Test Kit from Mallinckrodt, (St. Louis, MO). All other chemicals and reagents were of the highest purity available.

INSTRUMENTATION

A Waters Associates, (Milford, MA) liquid chromatograph consisting of two M6000A solvent delivery systems, a M660 solvent flow programmer, a U6K universal injector, an M440 absorbance detector set at 280nm, an M450 variable wavelength detector set at 214nm and an M730 data module was utilized. Enzymatic activity was surveyed using a Beckman UV 5230 Spectrophotometer (Fullerton, CA) equipped with an automatic sample changer. An LKB 1270 Rackgamma II gamma counter (Rockville, MD) was used for the RIA analysis. An LKB Multiphor unit, Model 2117 was used for the isoelectric focusing.

HPLC CHROMATOGRAPHIC PROCEDURE

A 30 minute linear gradient was run at a flow rate of 1.5ml/min on a μBondapak C_{18} column (3.9mm x 30cm, Waters Associates). The

mobile phase consisted of solution A, 0.1% aqueous trifluoroacetic acid (TFA) and solution B, 0.1% TFA in acetonitrile, run from 12% to 70% B. Both solvent systems were filtered and degassed prior to use. The column effluent was monitored at 214nm and 280nm. All separations were carried out at ambient temperatures.

PREPARATION OF HUMAN SEMINAL PLASMA

Pooled ejaculates from persons undergoing routine fertility examinations were centrifuged at low speed to remove cells. A 150ml sample of the seminal plasma was dialyzed against 4 liters of 0.1M Tris buffer at pH 7.2 for 48 hours, with one change. The dialyzed sample was centrifuged at 100,000 x g for 30 minutes, and supernatant stored at -70°C.

ENZYMATIC ACTIVITY PROCEDURE

A stock of prostatic acid phosphatase (a gift of Dr. D. Mahan) at a concentration of 1mg/ml was prepared in distilled water. From this stock 200μl was injected into the liquid chromatograph and 30 - 1 minute fractions were collected. Aliquots (0.1ml) of each fraction were added to 2.4ml of a 0.15M sodium acetate, solution pH 5.0, and 0.5ml of 3.65mM o-carboxyphenyl phosphate substrate. The change in absorbance at 300nm over the initial period of linearity was used to calculate the activity. The units of activity were calculated by the following equation:

$$\text{U of Activity/mg} = \frac{A_{300}/\text{min} \times 1000}{3500 \times \text{mg enzyme/ml reaction vol.}} \quad (2)$$

Aliquots of the dialyzed human seminal plasma and fractions from the

resulting chromatographic separation of seminal plasma were assayed similarly. Protein concentrations were estimated by the Lowry method (3) using bovine serum albumin as the standard, and the PAP concentration was determined by RIA.

ELECTROPHOSESIS AND ISOELECTRIC FOCUSING

SDS polyacrylamide tube gels (10% acrylamide, 0.3% bis-acrylamide, and 1.0% sodium dodecyl sulfate) were prepared as a modification of the Maizel method (4). Electrophoresis was carried out in 0.01M Tris-glycine buffer at pH 8.2 containing 1% glacial acetic acid and 10% isopropyl alcohol. The electrophoretic separation of acid phosphatase isoenzymes was based on the buffer system of Reisfeld, et al. (5). Visualization was accomplished by immersing the gels in 50mM acetic acid-sodium acetate buffer at pH 5.0 which contained sodium α-napthyl acid phosphate as a substrate (1mg/ml) and fast garnet salt as a coupler. Isoelectric focusing was performed at 5°C on acrylamide tube gels, pH range 4-6 according to Mahan, et al. (1).

RESULTS AND DISCUSSION

Prostatic acid phosphatase (PAP) is a glycoprotein with a molecular weight of approximately 102,000. It has been purified to near homogeneity from human seminal plasma by a combination of techniques such as gel filtration, affinity chromatography, and preparative gel electrophoresis (1, 6, 7). A stock solution of PAP, was chromatographed on a reverse phase HPLC column under the conditions described in materials and methods. This separation technique revealed only minor impurities, (Figure 1), which corresponded by retention times

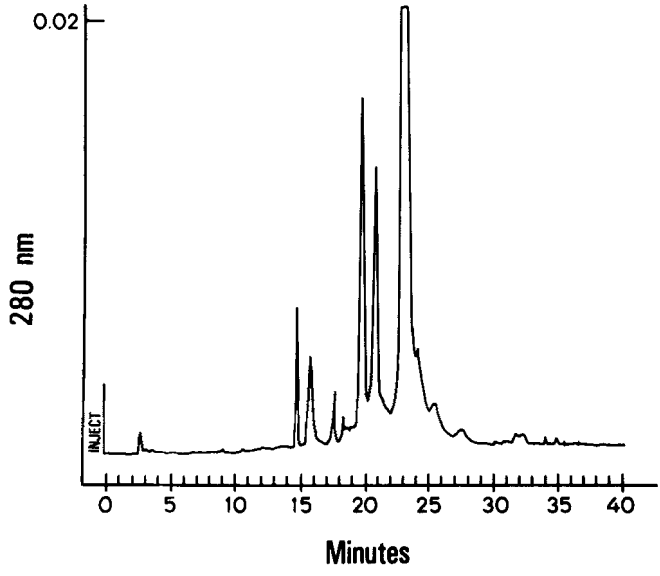

Figure 1: RP-HPLC chromatography of prostatic acid phosphatase purified by gel filtration. Column μBondapak C_{18} (3.9 mm x 30cm), thirty minute linear gradient. Flow rate, 1.5ml/min., mobile phase, 0.1% TFA in water, 12% to 70% 0.1% TFA in acetonitrile, column temperature, ambient. Activity occurs at 22-24 minutes.

to peaks present in the RP-HPLC profile of dialyzed human seminal plasma, (Figure 2). Based on this data, the purification of PAP directly from dialyzed human seminal plasma was attempted.

The chromatographic profile of seminal plasma was reproducible. Injection on column of 100 to 500 microliters of seminal plasma gave a linear response in terms of the area of integration of selected peaks, including the peak at 22-23 minutes which corresponded to PAP. Recovery of active enzyme, though, was negligible. In contrast to our previous experience with trypsin (8) removal of the mobile phase by lyophilization was only marginally effective in restoring enzymatic

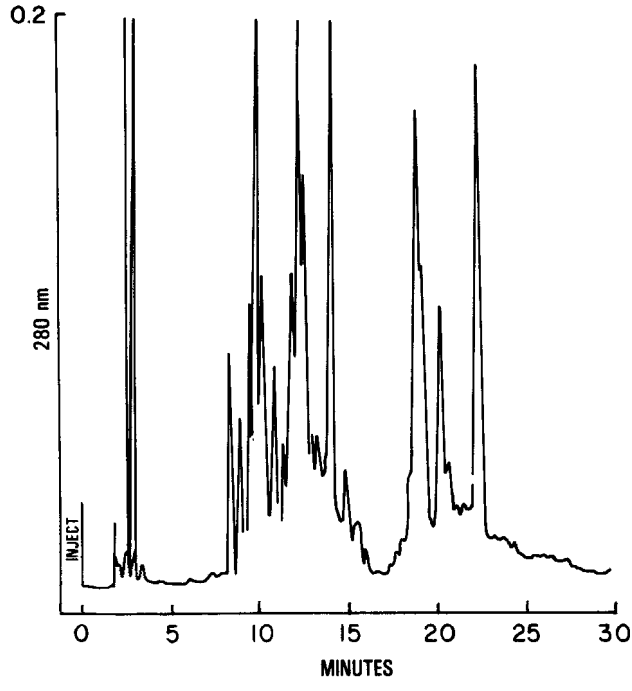

Figure 2: RP-HPLC chromatograph of dialyzed human seminal plasma. 200 µl injected on column. PAP activity at peak elution time 22-24 minutes.

activity. An investigation was therefore undertaken to determine whether the poor recovery was attributable to loss of enzyme on the column or denaturation by the mobile phase after fractionation. The chromatographic fractions were collected into a series of buffers and solvents known to stabilize activity of a variety of enzymes. These substances and their effectiveness in protecting PAP activity are summarized in Table 1. Glycerol was found to be the best protector of PAP activity; Hanks' Balanced Salt Solution and Dulbecco's Phosphate Buffered Saline, though not nearly as good as glycerol,

TABLE 1

Protection of PAP Activity

	STABILIZING AGENT	RELATIVE ACTIVITY
1.	No protective agent [d]	marginal
2.	Hanks' Balanced Salt Solution [a,c]	moderate
3.	Delbecco's Phosphate Buffered Saline [a,c]	moderate
4.	Potassium phosphate, 0.1M, pH 7 [a,c]	none
5.	Sodium Acetate, 0.05M, pH 5 [a,c]	none
6.	Ammonium Bicarbonate, 0.01M pH 8 [a,c]	none
7.	DMSO [b,c]	none
8.	PEG [b,c]	none
9.	Glycerol (45% v/v) [c]	very active

a) Aliquots of 0.5ml of the buffered solutions were added to each 1.5ml fractions.
b) Aliquots of 0.1ml of solvents (7 and 8) were added to each 1.5ml fractions.
c) None of the fractions were lyophilized prior to testing the activity. The acetonitrile, however, was evaporated under N_2.
d) Lyophilization of any of these fractions gave ambigious results, due to the instability of PAP under freezing and thawing conditions. The glycerol was found to be the most effective protector of prostatic acid phosphatase activity.

did significantly increase the recovery of enzymatic activity. The dramatic change in recovery of PAP activity seen in this study suggests that the previous difficulty with reverse phase HPLC may be due to the collection and processing after fractionation and not to denaturation or loss of material in the chromatographic separation.

The homogeneity of the fractions containing PAP was assessed by rechromatography on RP-HPLC, (Figure 3) and sodium dodecyl sulfate

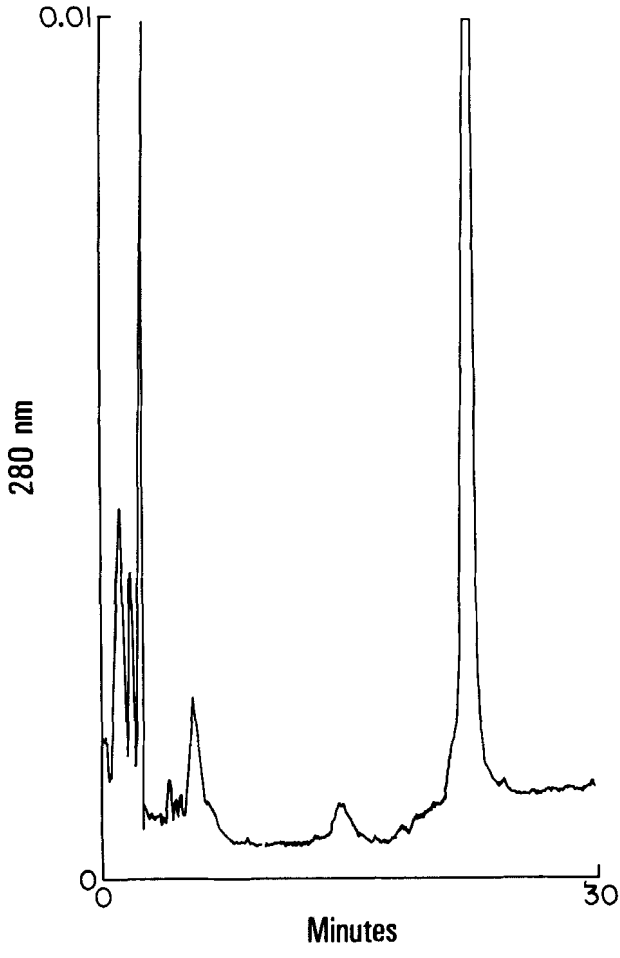

Figure 3: Re-chromatograph of the active fraction obtained from the separation of the human seminal plasma.

gels (Figure 4). The electrophoretic pattern of SDS gels clearly shows one major band corresponding in molecular weight to the purified PAP and to the active enzyme in seminal plasma. Assay of the fractions for isoenzyme activity revealed a single band. This band,

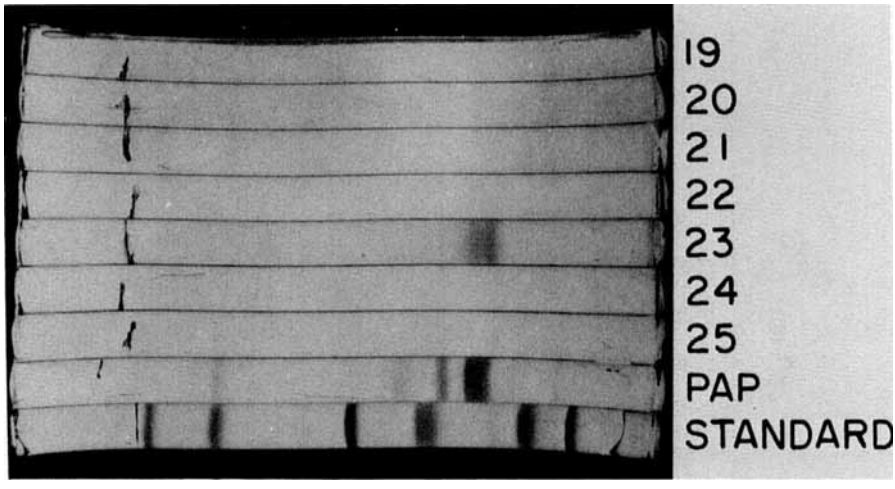

Figure 4: SDS polyacrylamide gel electrophoresis analysis of RP-HPLC fractions. PAP, purified by gel filtration, was included for identification of the HPLC fraction containing the enzyme; protein standards were obtained from Pharmacia (HMW).

compared with the PAP purified by affinity chromatography, showed a similar electrophoretic mobility. To test the validity of the previous assay, aliquots resulting from the fractionation were subjected to isoelectric focusing as per the methods section. The profile showed that all samples contained the same number of bands focused at the same area.

The enzymatic activity of the fractionated PAP was surveyed by the reaction to the substrate o-carboxyphenyl phosphate and with a RIA. The latter also confirms the immunological integrity of the purified PAP. The recovery of immunologically re-active protein from 100µl of human seminal plasma, injected on column, was 57.5%. The PAP activity and protein concentration of each fraction from the chromatographic separation was determined and is graphically shown in Figure 5.

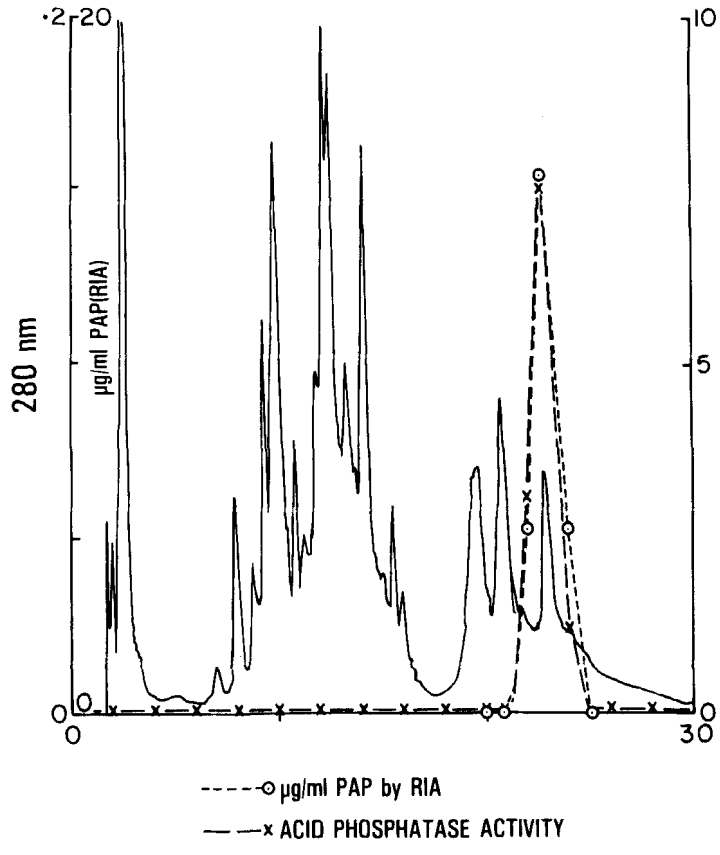

Figure 5: PAP activity and protein concentrations of each fraction from the chromatographic separation of human seminal plasma.

Purification of PAP by RP-HPLC yields highly active, immunologically and chromatographically homogenous enzyme, directly from dialyzed human seminal plasma in thirty minutes. With the proper selection of post-fractionation conditions, RP-HPLC can be demonstrated to be a highly effecient and simple technique for the isolation of enzymes.

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