One-Step, High-Yield Purification of Human Prostatic Acid Phosphatase from Seminal Fluid by Gel-Filtration HPLC under Nondenaturing Conditions

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This is a fast, efficient method for purification to homogeneity of human prostatic acid phosphatase [orthophosphoric-monooester phosphohydrolase (acid optimum), EC 3.1.3.2], from seminal fluid. Use of a "high-pressure" liquid-chromatographic gel-filtration column permits high-yield recovery of the purified enzyme with most of its enzymatic and immunological activity retained.

Human prostatic acid phosphatase (PAP, EC 3.1.3.2), a dimeric glycoprotein with a molecular mass of about 102 kDa (reviewed in ref. 1), is secreted into the seminal fluid by prostatic epithelial cells. Although the biological role of this enzyme is not well understood, the amount detected in the serum of patients with prostatic carcinoma appears to be of clinical significance (2–6). Detection and quantification of the prostatic enzyme in serum are impeded by the presence of other acid phosphatases that increase the background readings in assays of enzymatic or immunological activity. The discovery of specific inhibitors of PAP activity—e.g., \( \mu \) (+)-tartrate (7, 8)—and the production of monoclonal antibodies that detect particular antigenic regions of the prostatic enzyme have made it possible to distinguish PAP from other phosphatases of nonprostatic origin (9, 10). Whether for enzymatic or immunological determinations, it is essential to have as a standard of reference a pure enzyme that retains all or most of the enzymatic and immunological properties to be measured.

Many multi-step protocols have been described for purifying human PAP from the prostate gland or from seminal fluid (reviewed in ref. 11), all of which require several purification steps and result in poor analytical recovery of the purified enzyme. We now describe a one-step purification method based on gel-filtration HPLC under nondenaturing conditions, with which we obtain high yields of a native protein that appears to retain most of its enzymatic and immunological characteristics.

Materials and Methods

Preparation of human seminal plasma: Pooled ejaculates from healthy donors were centrifuged at low speed to remove cells, then dialyzed for 24 h vs 200 volumes of 50 mmol/L dibasic sodium phosphate buffered at pH 6.4 with citric acid, with one change of buffer.

After centrifuging the dialysate at 15 000 × g for 30 min, we further centrifuged the resulting supernate at 100 000 × g for 60 min to eliminate most of the particulate material, then stored the supernate in aliquots at −80°C until use.

HPLC chromatography: We used a Waters–Millipore HPLC Model 510 (Waters Associates, Milford, MA) equipped with a Model 441 absorbance detector and a C-83A integrator (Shimadzu, Kyoto, Japan). We loaded 100 µL of the dialyzed sample onto a 7.5 × 600 mm TSK 3000 SW HPLC column (LKB, Bromma, Sweden), and eluted the sample components isocratically at room temperature in the citrate/phosphate buffer at a flow rate of 1.0 mL/min. The column effluent was monitored at 280 nm, and 500-µL fractions were collected every 30 s with an automatic fraction collector (ISCO, Lincoln, NE). The HPLC column had been previously calibrated by using the molecular-mass calibration kit from Pharmacia, Uppsala, Sweden.

Determination of enzymatic activity: Samples, either from unfractionated seminal fluid or from eluted fractions, were diluted, when necessary, in 0.1 mol/L acetate buffer, pH 5.4. Enzymatic activity was tested by mixing 10 µL of sample with 500 µL of the acetate buffer to which had been added p-nitrophenyl phosphate (Calbiochem, San Diego, CA), 5 mmol/L final concentration. After 5 min at 37°C, the reaction was stopped by addition of 2.0 mL of 0.25 mol/L NaOH, and the yellow color was measured at 405 nm with a variable-wavelength spectrophotometer (LKB). Enzyme activity was calculated by using a value for molar absorbivity of 18 600 L cm⁻¹ mol⁻¹ for the p-nitrophenol released. When assayed under these conditions, the PAP obtained from dialyzed seminal fluid was >95% inhibited by the presence of \( \mu \) (+)-tartrate, 6 mmol/L, in the reaction mixture (data not shown).

Determination of immunological activity: The amount of PAP in the sample before and after HPLC purification was determined with an enzymatic immunosassay kit (PAP-EIA; Abbott Labs., Chicago, IL). This assay is based on the recognition by two different monoclonal antibodies of two unique epitopes present on the human prostatic enzyme.

Other methods: Protein was quantified according to the method of Lowry et al. (12), with bovine serum albumin (Calbiochem) as standard. We performed polyacrylamide gel electrophoresis under nondenaturing, nonreducing conditions on 100 g/L slab gels at pH 9.5 (13), using a Bio-Rad (Richmond, CA) minigel apparatus run at 4°C and 10 mA for 30 min. Protein bands were made visible with Coomassie Blue R-250; the enzymatic activity of acid phosphatase was detected by staining the gel with a buffered solution containing α-naphthyl phosphate and Fast Red B as chromogens (kindly provided by our Diagnostic Department at Sclavo; patent pending). Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed in 100 g/L slab gels according to Laemmli (14). Protein bands were stained with a silver-stain kit (Bio-Rad) according to the manufacturer’s instructions.

Results and Discussion

Figure 1 shows the elution profile obtained after injection of 100 µL of dialyzed seminal fluid onto the HPLC gel filtration column. The peak of PAP activity coincides with the protein peak at 16 min, and it is only partly overlapped by the peak at 18 min, which represents serum albumin, as demonstrated by an immunoblot experiment with specific anti-human serum albumin antibodies (data not shown). The material eluted in the fractions collected from 15 to 18 min was then analyzed separately on SDS–polyacrylamide
gel electrophoresis under reducing conditions (Materials and Methods) to assess its purity (data not shown). The fractions eluted between 15.5 and 17 min were devoid of any contamination detectable by the silver-stain method, and they were pooled for further analysis.

Figure 2 shows the results of the polyacrylamide gel electrophoresis of the pooled material under reducing and nonreducing conditions. Silver staining of the reducing gel reveals only one band at a molecular mass of about 50 kDa, which corresponds to that of the subunit of the prostatic enzyme (lane A), while in the nonreducing, nondenaturing gel two bands were evident, both by protein and enzymatic stain (lanes B and D). Two bands were also seen in the crude sample of seminal plasma electrophoresed under the same conditions (lane C). The presence of these two species can be explained by the fact that the alkaline pH conditions used to run the nonreducing, nondenaturing gel are known to cause some supramolecular aggregation of the enzyme (15). Furthermore, the observation that rechromatography of the pooled fractions on the same HPLC column resulted in all the proteins eluting as a single peak (see inset in Figure 1), confirms the interpretation that aggregation only occurs under the conditions used to run the nonreducing gel. Therefore, both analyses—i.e., gel filtration and gel electrophoresis—conducted on the material isolated from the peak at 16 min confirmed its purity. The yields obtained by processing 100 μL of dialyzed seminal plasma are presented in Table 1. The analytical recovery of enzymatic activity also always exceeded 60% in other purifications done subsequently (not shown), which represents one of the best recoveries reported in the literature. A similarly high recovery (72%) was also found when PAP was assayed immunologically (Table 1). Because the assay used to quantify PAP is based on two different monoclonal antibodies directed to two different epitopes of human prostatic protein, any alteration of the protein structure that affects at least one of these two sites will disturb the recognition of the antigen. Therefore, the high analytical recovery of both enzymatic and immunological activity strongly suggests that the integral structure of the enzyme is well preserved during our purification procedure. The difference between the PAP values obtained with the Lowry determination method and the immunoenzymatic test could be due to the fact that different reference proteins are used in the two methods.

Many attempts have been made to purify the human prostatic enzyme from seminal fluid or from the prostate gland (16-20). Most of the protocols described are time-consuming because they involve several purification steps.
resulting in a considerable loss of protein. In 1978, Van Etten and Saini (21) described a method that allowed a good recovery (around 70%) of the human prostatic enzyme from prostate glands, but required a rather complicated preparation of an affinity matrix to be coupled with Sepharose 4B.

More recently, a one-step purification method based on reversed-phase HPLC (22) was described. However, in this system PAP was eluted at an acetonitrile concentration of 70%, so these conditions resulted in a strong inactivation of the enzyme with regard to both enzymatic and immunological activity, even when fractions were collected in 45% glycerol (as suggested by the authors) and the purified material was further dialyzed (after evaporation of the acetonitrile) against citrate/phosphate buffer, pH 6.4, containing 45% glycerol (data not shown). When the thus-purified enzyme was analyzed by polyacrylamide gel electrophoresis under reducing conditions, only one band could be detected. However, when this material was analyzed by gel-filtration HPLC, it was eluted as two peaks: a larger one (representing roughly 53% of the total material), which eluted at the void volume, and a smaller one (28% of the total), which eluted in the position corresponding to native PAP at 16 min (data not shown), indicating that enzyme aggregation had occurred during reversed-phase purification. The residual 19% represented contamination with serum albumin.

In conclusion, the method we present in this paper allows the recovery of at least 60% of the PAP originally present in the seminal-fluid sample in a single purification step and, more important, the purified enzyme retains most of its biological properties, such as enzymatic activity and immunological characteristics. This method can be easily scaled up for the purification of milligram quantities of PAP by use of a preparative HPLC column, and it should therefore permit a better standardization of diagnostic kits and facilitate the structural analysis of this protein.

References