Prostatic Acid Phosphatase in Serum of Patients with Prostatic Cancer Is a Specific Phosphotyrosine Acid Phosphatase

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We developed an assay to measure at acid pH the phosphotyrosine phosphatase activity in sera from patients with prostatic cancer. The method used quantifies the inorganic phosphate liberated from phosphotyrosine after incubation with serum, followed by the deproteinization of the reaction mixture. A high acid phosphatase (EC 3.1.3.2) activity towards phosphotyrosine was observed in all sera from patients with increased activity of prostatic acid phosphatase. This activity represented 96% of prostatic acid phosphatase and 77% of total acid phosphatase activities. Moreover, it was correlated (r = 0.91) with the amount of serum prostatic acid phosphatase determined by radioimmunoasay. When serum acid phosphatase activity was measured on several phosphorylated substrates, preferential hydrolysis was demonstrated for those in which the phosphate group was esterified on an aromatic ring rather than those presenting an aliphatic chain. Among phosphoamino acids, only phosphotyrosine was a good substrate, with little or no activity observed with phosphoserine and phosphothreonine. Human seminal plasma and partially purified prostatic acid phosphatase, tested for their activity on some of these substrates, gave similar results. On the other hand, sera from patients with above-normal alkaline phosphatase activity and no prostatic disease showed little or no activity on phosphotyrosine at both acid and alkaline pH values. Evidence is presented that the prostatic acid phosphatase in serum is a specific phosphotyrosine acid phosphatase.

The presence of prostatic acid phosphatase (PAP; EC 3.1.3.2) in sera of patients with prostatic cancer was first demonstrated five decades ago by Gutman and Gutman (1); since then, the circulating enzyme has been considered a marker for staging and monitoring this disease (2–4). The increase of the PAP concentration in serum is correlated with the progression of prostatic cancer (5), whereas at the same time, in the gland, decreases in both the synthesis of PAP (6) and of its activity (7) have been reported.

Despite numerous reports concerning the biochemical properties of PAP and the clinical relevance of its presence in serum, its physiological role remains to be established. While the search for its natural substrates continues, researchers have found that PAP hydrolyzes the phosphoester bond of phosphorylcholine (8, 9) as well as that of phosphorylated proteins (10, 11). In this respect, glandular and (or) secreted PAP appears to be more specific for phosphoester groups linked to tyrosine than for those linked to serine or to threonine, e.g., in proteins (12) or as phosphoamino acids (13). This specificity for phosphotyrosine (p-tyr) is of great interest because intracellular tyrosine protein kinases (TPKs) and phosphotyrosyl protein phosphatases appear to play important roles in the regulation of cell proliferation, differentiation, and carcinogenesis (reviewed in references 14 and 15). IFPAP turns out to be important locally as an antagonist of prostatic TPK its glandular decrease and leakage in the blood in carcinoma patients might lead to increased prostatic growth owing to the presence of higher concentrations of phosphotyrosylated proteins. Therefore, we decided to investigate whether circulating PAP is a specific p-tyr acid phosphatase. In the present investigation, we developed an assay to measure the p-tyr acid phosphatase activity in sera from patients with prostatic cancer and correlated this activity with those of total acid phosphatase and PAP. We studied the enzyme specificity as follows: by testing the activity of sera from patients with prostatic cancer towards several substrates presenting aromatic or aliphatic structures, by measuring the phosphoamino acid phosphatase activities of serum from patients with above-normal alkaline phosphatase activity but no prostatic disease, and by comparing these activities with the p-tyr acid phosphatase activity of PAP from human seminal plasma.

Materials and Methods

Reagents

Partially purified PAP (from human seminal plasma; specific activity 440 kU/g) and phosphorylated low-molecular-mass substrates—o-naphthyl phosphate, p-nitrophenyl phosphate (p-NPP), β-naphthyl phosphate, o-phospho-L-tyrosine (p-tyr), o-phospho-DL-tyrosine, β-glycero-phosphate, phospholphthalein (mono)phosphate, cholins phosphate, o-phospho-L-serine (p-ser), o-phospho-L-threonine (p-thr), o-phospho-DL-threonine, and L-α-glycero-phosphate—were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium-L-tartarate and ammonium molybdate were obtained from Fisher Scientific Co. (Montreal, Quebec). Sodium orthovanadate (referred to as vanadate), trichloroacetic acid, and L-ascorbic acid were purchased from Aldrich Chemical Co. (Milwaukee, WI), BDH Chemical (Montreal, Quebec), and J. T. Baker Chemical Co. (Phillipsburg, NJ), respectively.

Biological Samples

Sera from normal adult males (used as controls for low PAP activity) and semen samples from fertile donors (source of secreted glandular PAP) were provided by the Endocrinology Laboratory of Maisonneuve-Rosemont Hospital. The semen was centrifuged in an IEC clinical centrifuge at 1000 × g for 20 min, and the resulting seminal plasma was frozen in aliquots until use.

Blood from prostatic cancer patients was quickly proc
cessed either by the Clinical Biochemistry Department of the hospital until enzymatic assay of PAP in serum (within two days) or by the Department of Nuclear Medicine, where the amount of PAP activity was measured by radioimmunoassay (RIA). The remaining serum preparations were stored at −80 °C for as long as one month before assay without loss of activity.

Sera from patients without prostatic disease but with above-normal alkaline phosphatase activities (135–1800 U/L) were also collected from the Clinical Biochemistry Department and used for studying phosphatase activities towards phosphoamino acids and p-NPP at both alkaline and acid pH values.

Determination of Phosphatase Activities

Prostatic acid phosphatase. PAP activities in 200 μL of sera were measured routinely by the hydrolysis of α-naphthyl phosphate, 3 mmol/L, in 0.1 mmol/L sodium acetate buffer at pH 5.0 in the presence of diazotized 2-amino-5-chlorotoluene, 1 mmol/L, in a total volume of 3.2 mL. Parallel assays containing 15.5 mmol/L of α-naphthol per liter were also included. Absorbance at 405 nm was determined after 5 and 10 min of incubation at 37 °C, and total acid phosphatase activity was calculated from the difference between these values, which represented the rate of appearance of α-naphthol. The activity was expressed as units (U) per liter of serum, 1 U corresponding to 1 μmol of α-naphthol produced per minute. The tarette-inhibited portion of total acid phosphatase activity, referred to as PAP (16), was also expressed in U/L; values <2 U/L were considered as normal.

The mass concentration of PAP in sera from patients with prostatic cancer and undergoing radiotherapeutic treatments was measured by RIA. The RIA kit (Mallinckrodt Diagnostica Laboratory, Evry-Lisses, Cedex, France) was used according to its instruction manual. In brief, PAP antibody (goat or rabbit) was added to patients’ sera, incubated for 2 h at room temperature, and followed by the addition of 125I-labeled PAP solution. After a second incubation for 24 h at room temperature, the antigen–antibody complexes were immunoprecipitated in the presence of polyethylene glycol by adding the solution of second antibody (anti-rabbit or -goat immunoglobulin). The radioactivity of the pellet, recovered after centrifugation at 1200 × g for 20 min, was then counted, and the amount of PAP in the patients’ sera was determined from a standard curve. Sera containing <2 μg of PAP per liter were considered as normal.

Alkaline phosphatase. Alkaline phosphatase activity in serum was routinely assayed by the Clinical Biochemistry Laboratory, according to the instructions of Boehringer Mannheim (Laval, Quebec) for the Hitachi System 705 automated analyzer. Briefly, 5 μL of serum is added to 420 μL of 1 mol/L diethanolamine buffer at pH 9.8 containing, per liter, 0.5 mmol of MgCl2 and 10 mmol of p-NPP as the substrate. The activity, calculated from the difference of absorbance at 415 nm after 1 and 5 min of incubation at 37 °C, is expressed in units per liter of serum, 1 U corresponding to 1 μmol of p-nitrophenol produced per minute.

Phosphoamino acid phosphatases. The method used to assay the phosphoamino acid phosphatase activities of human seminal plasma and of partially purified PAP (equivalent to 2.5–3.0 mU per assay) was as described elsewhere (13), except that substrates were used at a concentration of 5 mmol/L in sodium acetate buffer (250 mmol/L, pH 5.0), and the reaction was stopped directly by the addition of 700 μL of Ames reagent, consisting of a mixture of ammonium molybdate (4.2 g/L) in 0.5 mol/L of sulfuric acid and ascorbic acid solution (100 g/L) (6:1, by vol) (17). Enzymatic activity was related to the inorganic phosphate (Pi) liberated, which we quantified by measuring the absorbance at 820 nm.

Owing to the high protein concentration of sera, we slightly modified the assay to measure acid phosphatase activity of serum towards phosphoamino acids and other substrates. Appropriate dilutions of serum (when serum PAP exceeded 200 U/L), and up to 25 μL of undiluted serum, were added to 475 μL of 0.25 mol/L sodium acetate buffer containing 5 mmol of substrate per liter at a final pH of 5.0. In some instances, we also determined the inhibition of acid phosphatase activity by tartrate or vanadate, an inhibitor of phosphotyrosyl protein phosphatase (18–20) and of PAP (13), at a 10 mmol/L concentration of inhibitors. The pH was adjusted to 5.0 once all components were mixed. Parallel assays without substrate, inhibitor, or enzyme preparations were performed and used as blanks. Samples were incubated at 37 °C for 60 min and the reactions were terminated by the addition of 300 μL of trichloroacetic acid solution (final concentration 50 g/L). The mixtures, after standing on ice for 30 min, were centrifuged at 5000 × g for 5 min in a refrigerated Sorvall RC-5B in an HS-4 rotor. We used 300-μL aliquots of the protein-free supernatants to determine the amount of Pi with NaH2PO4 as the standard (17). Phosphoamino acid phosphatase activities were expressed as units per liter of serum, 1 U representing 1 μmol of Pi liberated per minute.

To measure the activity of sera with above-normal alkaline phosphatase activity towards phosphoamino acids and p-NPP, we used the method for measuring p-tyr acid phosphatase activity in serum except that the reaction was performed in 50 mmol/L diethanolamine buffer at pH 9.8 in the presence of 0.5 mmol/L MgCl2 and 10 mmol of substrate per liter.

Results

Validation of serum p-tyr acid phosphatase assay. As described in Materials and Methods, PAP in serum is routinely assayed in 0.1 mol/L sodium acetate buffer at pH 5.0. In attempting to set up an assay for p-tyr acid phosphatase activity in serum, we found that, for accuracy in the determination of Pi, the volume of the reaction mixture needed to be minimal and deproteination was essential. However, in a minimal volume, serum proteins reduced the buffering capacity of sodium acetate. We, therefore, determined that higher concentrations of buffer (0.25 to 1 mol/L) maintained the pH constant at 5.0 without significant effect on the enzymatic activity (data not shown). We then performed subsequent studies of acid phosphatase activity in serum by using sodium acetate buffer at a concentration of 0.25 mol/L and pH 5.0.

Figure 1 shows the results of a typical experiment performed to optimize the assay with serum from a patient with prostatic cancer and above-normal PAP activity. Using p-tyr as the substrate, we found that the liberation of Pi increased linearly as a function of the volume of serum (enzyme concentration) (Figure 1A) and as a function of time up to 60 min at 37 °C (Figure 1B). The concentration of endogenous Pi in serum represented, in the present case, 25% of the measured phosphate and was proportional to the volume of serum (Figure 1A). The remaining 75% of Pi
Intra-Assay activity, endogenous tess In within substrates measured without incubation (0) phosphorylated cancer. For addition, it was determined statistically (Student's t-test, P = 0.05): the mean value of p-tyr acid phosphatase activity (52.2 U/L) was different from that of total acid phosphatase activity (65.8 U/L) but approximately equal to the mean value of PAP activity (53.6 U/L). Figure 2B shows that p-tyr acid phosphatase activity correlated (r = 0.91) with the amount of PAP, measured by RIA, present in serum.

Substrate specificity. To substantiate the hypothesis of a physiological role of PAP through its action on p-tyr, we tested the acid phosphatase activity in serum with above-normal PAP on other phosphaamino acids, as well as on several phosphorylated substrates presenting aromatic or aliphatic structures. The liberated Pi was determined and their rates of hydrolysis were compared with those of p-NPP, arbitrarily referred to as 100%. Figure 3A shows that five of six aromatic substrates, including phosphotyrosine (L and DL conformations), were highly hydrolyzed (>75%), whereas five out of six presenting aliphatic chains, including phosphoserine (p-ser) and phosphothreonine (p-thr, L and DL), were poorly hydrolyzed (<20%). Results were similar for human seminal plasma (Figure 3A). When we tested the activity of partially purified PAP towards phosphaamino acids as substrates (Figure 3B), results were similar to those found with serum. Again, a striking difference was observed: only p-tyr was significantly hydrolyzed, the rates of hydrolysis of p-thr and p-ser being <5%. However, when sera from patients with above-normal

![Image](image-url)

**Table 1. Precision of Assays of Phosphotyrosine Acid Phosphatase in Serum**

<table>
<thead>
<tr>
<th>Serum*</th>
<th>PAP</th>
<th>Phosphotyrosine acid phosphatase, mean (SD)</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-Assay</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.5</td>
<td>2.3 (0.1)</td>
<td>2.7</td>
</tr>
<tr>
<td>2</td>
<td>18.6</td>
<td>23.5 (1.4)</td>
<td>5.8</td>
</tr>
<tr>
<td>3</td>
<td>30.9</td>
<td>33.8 (2.7)</td>
<td>7.3</td>
</tr>
<tr>
<td>4</td>
<td>46.0</td>
<td>48.3 (3.8)</td>
<td>7.9</td>
</tr>
<tr>
<td>5</td>
<td>110.3</td>
<td>105.8 (8.1)</td>
<td>7.7</td>
</tr>
<tr>
<td>Interassay</td>
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<tr>
<td>1</td>
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<td>42.6 (1.0)</td>
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</tr>
<tr>
<td>2</td>
<td>69.8</td>
<td>66.0 (2.1)</td>
<td>2.2</td>
</tr>
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</table>

*n = 8 for each assay

![Image](image-url)
Table 2. Comparison of Kinetic Parameters of Phosphotyrosine Acid Phosphatases from Serum and Seminal Plasma

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Serum</th>
<th>Seminal Plasma</th>
</tr>
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<tbody>
<tr>
<td>K_m, mmol/L, phosphotyrosine</td>
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<td>1.95</td>
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<tr>
<td>K', μmol/L, vanadate</td>
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<tr>
<td>tartrate</td>
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<td>69</td>
</tr>
<tr>
<td>D_{50}, μmol/L, vanadate</td>
<td>53</td>
<td>35</td>
</tr>
<tr>
<td>tartrate</td>
<td>355</td>
<td>365</td>
</tr>
</tbody>
</table>

Fig. 3. Activity of acid and alkaline phosphatases towards phospho-ylated substrates presenting aromatic or aliphatic structures

The phosphatase activity of different preparations was determined as described in Materials and Methods. A: Acid phosphatase activity of sera from patients with prostate cancer and with above-normal PAP activity (70–100 U/L) was measured towards 12 substrates (dark bars) and compared with that of seminal plasma from semen donors (open bars). B: Acid phosphatase activity of commercial PAP (open bars) and alkaline phosphatase activity of sera with above-normal alkaline phosphatase activity (145–1800 U/L) from patients without prostatic disease (dark bars) were measured with substrates of phosphoamino acids. The phosphate liberated from all substrates was measured by the Ames reaction (17), according to the procedure described for determining phosphoamino acid phosphatase activity. Results, expressed relative to the hydrolysis of p-NPP (100%), are the mean of three preparations (serum and seminal plasma).

Alkaline phosphatase activity (diseases other than prostatic) were tested for activity on the three phosphoamino acids at pH 9.8 and compared with p-NPP taken as 100%, the rates of hydrolysis of p-tyr and p-thr were similar (25% and 22%, respectively), whereas that of p-ser was 6.5% (Figure 3B). When these sera were tested at pH 5.0, we did not detect any phosphatase activity towards the three phosphoamino acids as substrates (not shown).

Kinetic parameters. The Michaelis–Menten plot presented in Figure 4A shows that the p-tyr acid phosphatase in serum was saturated at a substrate concentration of 6 mmol/L. The enzymatic activity was inhibited by both tartrate and vanadate with 50% inhibition dose (ID_{50}) values of 355 and 53 μmol/L, respectively (Table 2); maximal inhibition was reached at a concentration of 10 mmol/L (Figure 4B). When calculated from the Lineweaver–Burk plot (Figure 4C), a K_m value of 1.92 ± 0.10 mmol/L was obtained for p-tyr. Figure 4C also shows that the inhibition of p-tyr acid phosphatase activity in serum by tartrate and vanadate was competitive, with K_i values of 69 ± 5 and 5.0 ± 0.5 μmol/L, respectively. When similar studies were performed with human seminal plasma (Table 2), the K_m for p-tyr was 1.95 ± 0.54 mmol/L with ID_{50} concentrations for tartrate and vanadate of 365 ± 28 and 35 ± 4 μmol/L, respectively. K_i values were 67 ± 7 μmol/L for tartrate and 2.45 ± 0.1 μmol/L for vanadate. We calculated from K_i values that vanadate was 14- to 28-fold more potent than tartrate in inhibiting p-tyr acid phosphatase activities in serum and seminal plasma.

When we determined p-tyr acid phosphatase activities in serum and seminal plasma at pH values between 4 and 6, both showed identical variations in activity, which was maximal at pH 5.0 (Figure 5). We obtained similar results when p-NPP was used as substrate (not shown).

Discussion

To verify whether PAP in sera from patients with prostatic cancer was a specific p-tyr acid phosphatase, we developed an enzymatic assay, using p-tyr as the substrate, and quantified the liberated phosphate by spectrophotometry, according to the method of Ames (17). Deproteinization of the assay mixture was required before the Ames
reaction to prevent serum protein interference with the reagent and precipitation of the reduced complex of phosphomolybdate, which would produce inaccurate absorbance measurements. After deproteinization with trichloroacetic acid, no significant interference with the reaction was noticed and all the Pi remained soluble.

With this method, excellent correlations were observed between p-tyr acid phosphatase activity and total acid phosphatase \((r = 0.98)\), as well as with PAP activity (tartrate-inhibited portion of total acid phosphatase; \(r = 0.99)\) in serum. More importantly, the p-tyr acid phosphatase activity in serum was equal to PAP (96%), yet represented only 77% of total acid phosphatase activity. A linear correlation \((r = 0.91)\) was also observed with the mass concentration of PAP (determined by RIA) in serum. Though time-consuming, this method provides the advantage of measuring PAP activity directly in serum. The precision of the assay is also excellent and eliminates the need to measure total acid phosphatase activity. However, endogenous Pi in serum must be determined. That sera from patients with no prostatic disease and with above-normal alkaline phosphatase activity were devoid of any phosphatase activity at acid pH ensures the specificity of this assay. Furthermore, the kinetic properties of p-tyr acid phosphatase in serum, \(K_m\) for p-tyr, \(K_i\) for tartrate and vanadate, and optimal pH were, in all instances, similar to those of p-tyr acid phosphatase in seminal plasma, which were equivalent to PAP and were inhibited by vanadate and tartrate \((13)\). The \(K_m\) values obtained in this study for p-tyr with acid phosphatases in serum also correspond to those reported for PAP from human seminal plasma \((10)\). The data obtained with partially purified PAP towards phosphoamino acids, which show that it behaves the same as the acid phosphatases in sera from patients with prostatic cancer, also reinforce our conclusion that p-tyr acid phosphatase in serum is equivalent to PAP.

Over the years, PAP activity in serum has been assayed under different experimental conditions (types and concentrations of substrates and buffers used, optimal pH) and, even though some variations might have been observed in turnover rates of substrates, researchers have concluded that the best substrates for PAP possess an aromatic structure. In general, their hydrolysis has been monitored directly on the basis of the absorbance of their aromatic ring or indirectly by means of the formation of a chromophore-complex \((21-24)\). In the present study, in which we have measured the liberated Pi, several substrates could be compared under identical conditions; we found that PAP preferentially hydrolyzed substrates with a phosphate linked to an aromatic ring, including p-tyr, whereas substrates presenting a phosphate on an aliphatic chain such as p-ser and p-thr, were quite unreactive to enzymatic hydrolysis. These results suggest that those amino acid involved in the catalytic site of PAP—histidine, arginine, glutamate, and aspartate \((25)\)—recognize the aromatic structure of phosphorylated substrates. The physiologic importance of the present data lies principally in the difference in phosphoamino acid hydrolysis by PAP i serum. Interestingly, glandular and secreted PAP (seminal plasma) are also specific for the hydrolysis of phosphotyrosine \((13)\) and phosphoantibodies \((10, 12, 26)\). In addition, the PAP activity of prostatic cells appears to be inversely related to their TPK activity \((20, 27)\). Because we have not detected any TPK activity in normal serum nor in sera from patients with benign prostatic hyperplasia or prostatic cancer (with or above-normal PAP) \((28)\), it is tempting to propose a local role for PAP, whether it leakage into circulation is a cause or a consequence of tumor progression. Indeed, a decrease of the intracellular PAP concentration and (or) of its activity might lead to the presence of higher concentrations of phosphoantibodies to prostatic proteins. Therefore, if PAP is acting as an antagonist of TPK, it could be a key element in the overall regulation of prostatic functions and the identification of its intracellular physiological substrates, a subject of great interest.

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