Active Principle of Swine Prostate Extract:  
I. Isolation of Active Principle Activating Prostatic Acid Phosphatase and Its Effect on Testosterone Uptake of the Prostate in Castrated Rats

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ABSTRACT—There have been several reports concerning the therapeutic effect of an extract from animal prostates on benign prostatic hypertrophy. Previously, we reported that the swine prostate extract (PE) had the activity to enhance human prostatic acid phosphatase (PAPase) activity in vitro, and to increase the muscular tonicity of the urinary bladder by directly acting upon vesical muscles, suggesting that PE have an activity to elevate the intravesical voiding pressure in vivo. In the present study, it was attempted to isolate such an active principle of PE as activates human prostatic acid phosphatase (PAPase). The finally purified PE (PPE) was assessed as to some physico-chemical and pharmacological properties. 1) PPE was found to be a peptide with a molecular weight of about 8,800, composed largely of neutral amino acids (approximately 70%) and few of aromatic amino acids. 2) PPE activated PAPase in a dose-dependent fashion, resulting in an increase of the enzyme activity approximately twice in a dose of $2 \times 10^{-5}$ g/ml of PPE. Furthermore, PPE recovered PAPase activity dose-dependently from the 50% inhibition by $2 \times 10^{-3}$ M L-tartaric acid. 3) In castrated rats, the $^3$H-testosterone uptake of the prostate was significantly suppressed by the oral administration of PPE. PPE might be one of active principles of PE for the therapeutic effect on prostatic hypertrophy.

While it is widely accepted that prostatectomy is most effective for the treatment of prostatic hypertrophy, patients suffering from this disease are usually elderly and reluctant to undergo surgical treatment. The majority of the patients are in the early stages of this disease. In such stages of the disease, the operative ectomy is not necessarily required, and conservative treatment is adequate in so far as it eliminates renal impairments and complaints due to the disturbance of urination. There have been several reports (1–4) concerning the effectiveness of an extract from animal prostate on prostatic hypertrophy. Previously, we reported (5–7) that the swine prostate extract (PE), which was prepared by a method devised in our laboratory, enhanced human prostatic acid phosphatase (PAPase) activity in vitro and increased the muscular tonicity of the urinary bladder by directly acting upon vesical muscles, suggesting that the extracts have an activity to elevate the intravesical voiding pressure in vivo.

It was reported that PAPase activity was

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dependent on androgen (8, 9); it showed its peak at around 25 years of age when the physiological function of the prostate became most active, and then the activity decreased with aging (10). It is likely that PAPase plays some role in the physiological function of the prostate, although it is unclear whether PAPase participates in the development of prostatic hypertrophy.

In the present paper, we have tried to isolate and purify the PAPase activating substance from PE to examine the effect of the substance on the prostate. There is a possibility that the purified PE (PPE) obtained here is one of the active principles of PE involved in the therapeutic effect on prostatic hypertrophy. PPE may be useful for examining the physiological role of PAPase in the prostate. In this report, we assessed some of the physicochemical and pharmacological properties of PPE.

MATERIALS AND METHODS

Materials
The prostate extract (PE) subjected to purification was prepared by previously described procedures (5). Briefly, the homogenate of swine prostate (15%) was adjusted to pH 1.5 with 6 N HCl and hydrolyzed by stirring it for 72 hr at 75°C. The hydrolysate was neutralized with 6 N NaOH and then added with m-cresol in a final concentration of 0.3%. The hydrolysate was filtrated by suction through a paper filter (PAD No. 85, Toyo Roshi Co., Ltd.). The yield of PE was 4.5 g from 100 g of the prostate. Other materials used in this study included: Sephadex G-15 and Sephadex G-25 superfine (Pharmacia Fine Chemicals); cellulose powder (Merck); Dowex 50Wx8 (Dow Chemicals); nitrophenyl phosphate, sodium dodecyl sulfate (SDS), dansyl chloride, N,N,N',N'-tetramethylethylenediamine, urea, vitamin B₁₂, testosterone propionate, acetic anhydride and pyridine (Nacalai Tesque); acryl amide (Komune Chemicals); bis-methylene acryl amide (Tokyo Kasei); cytochrome C and insulin (Sigma); 5'-adenosine monophosphate (5'-AMP, Kohjin); and 1α,2α-3H(N)-testosterone (³H-testosterone, specific radioactivity: 57.0 Ci/mmol; New England Nuclear). Human prostatic acid phosphatase (PAPase) was a fraction of peak II obtained by the method of Saito (11); it was dispensed and frozen in aliquots of a few milliliters in vials for storage. Each aliquot was thawed and diluted 250- to 300-fold with water before use.

Animals
Eighteen-week-old male rats of the Wistar strain were purchased from Kitayama Labs. The animals were used for the experiment after an acclimatization period of more than 7 days. They were housed in cages in an air-conditioned animal room at 22 ± 2°C with 55 ± 5% relative humidity and supplied with water and solid feed ad libitum.

SDS disc electrophoresis
Dansylated PPE mixed with dansyl chloride as a tracking marker was subjected to SDS disc electrophoresis. The sample specimen was electrophoresed on a 2.5% acrylamide gel added with 0.1% SDS. Electrophoresis was carried out at a constant current of 2.5 mA per polyacrylamide gel in a 5 × 90 mm column, using 0.1 M tris acetate buffer (pH 8.2). The fluorescence of the marker, dansyl-OH, was monitored with a UV monitor (Manaslu lamp) at 360 nm during the 6 hr of electrophoresis.

Estimation of molecular weight
The molecular weight of PPE was estimated by gel filtration chromatography with Sephadex G-25 superfine (18 × 340 mm) and by calculating the ratio of elution volume (Ve) to the total bed volume (Vb). Ve/Vb. Cytochrome C (MW 12,600), insulin (MW 5,733), vitamin B₁₂ (MW 1,355) and 5'-AMP (MW 347) were used as molecular-weight markers. For detection of PPE and the molecular-weight markers, infrared and ultraviolet absorption spectrometries were performed by the usual procedures.
**Amino acid analysis**

One to 3 mg of sample was dissolved in 3.0 ml of 6 N HCl solution, sealed in a hard glass hydrolysis tube under reduced pressure, and heated at 110°C for 48 hr. The hydrolyzed and dried material was then dissolved in 1.0 ml of water and 0.4 to 0.5 ml of the solution was subjected to analysis on a Hitachi KLA-3 Amino Acid Autoanalyzer. Hitachi Spherical Resin No. 3105 was used in a 9 × 550-mm column (resin height: 480 mm) equilibrated with citrate buffers of pH 3.25 and 4.25 for neutral and acid substances, respectively, and in a 9 × 150-mm column (resin height: 95 mm) equilibrated with a citrate buffer of pH 5.28 for basic substances.

**Effect of PPE on PAPase activity**

The effect of PPE on PAPase activity was assessed using the Bessey-Lowry method (12). To 1.0 ml of substrate buffer (mixture of equal volumes of 0.4% disodium p-nitrophenyl phosphate solution and 0.2 M citrate buffer, pH 4.8), 0.1 ml of the test sample solution was added and pre-warmed at 37°C for 5 min. Then, 0.1 ml of PAPase was added to the mixture and incubated at 37°C for 30 min. After termination of the reaction by adding 4.0 ml of 0.1 N NaOH solution, the absorbance at 410 nm was determined against a blank in which PAPase was added after the stop solution. Results were expressed as percent to the OD value of the reaction mixture without PPE.

**Effect of PPE on testosterone uptake in castrated rat prostate**

Two groups of 5 male rats were injected with testosterone propionate, s.c. into their buttocks in a dose of 0.5 mg/kg on days 8, 10, 12 and 14 after bilateral orchiectomy under anesthesia with ether. On day 15, the animals were given 50 μCi of 3H-testosterone, i.p. Blood was drawn, and the ventral lobe of the prostate was removed 2 hr after the injection to measure the radioactivity. PPE was given to one group of rats p.o. in a dose of 3 mg/kg/day, daily, for 7 days beginning on day 8 after the castration. The other group, serving as the control, received the equal volume of water, the vehicle. The ventral lobe of the prostate removed from each rat was placed in a glass liquid scintillation vial, and then it was solubilized by incubation with 0.5 ml of NCS solubilizer (Amersham/Searle) per 100 mg of the tissue for 4 hr at 40°C, followed by 24 hr at room temperature. The determined radioactivity was expressed as DPM/tissue/100 g body weight. The radioactivity in the serum was measured by essentially the same procedure as described above, but the specimen was incubated with NCS solubilizer at 40°C for 20 min for complete solubilization. The result was expressed as DPM/0.1 ml serum.

**Statistical analysis**

Data were statistically evaluated using Student's t-test.

**RESULTS**

**Isolation of active principle from PE**

The isolation procedure for the active principle from PE is shown in Fig. 1. The PE sample was extracted with absolute methanol, and the soluble portion (Fr-A) was evaporated to dryness. Then, Fr-A was extracted with absolute ethanol. The ethanol-soluble portion and insoluble portion were defined as Fr-B and Fr-C, respectively. Since PAPase activating activity was recovered in Fr-C, this fraction was subjected to Sephadex G-15 gel filtration (Fig. 2). The PAPase activating activity was recovered in the eluate corresponding to 0.34-0.60 Ve/Vb (Fr-D). Fr-D was then subjected to ion-exchange chromatography on Dowex-50Wx8 by stepwise elution using five different solvents: 0.01 M ammonium formate buffer (pH 2.49, Fr-D1), 0.1 M ammonium acetate buffer (pH 5.50, Fr-D2), 0.2 M ammonium acetate buffer (pH 6.88, Fr-D3), 0.1 N ammonium solution (Fr-D4) and 1.0 N ammonium solution (Fr-D5), in this order. PAPase activating activity was detected in three fractions, Fr-D2, Fr-D3 and Fr-D4. Fr-D3 that had the highest activity among them was re-chroma-
Fig. 1. Schematic diagram for preparation of purified prostate extract (PPE) from swine prostate extract (PE).

Fig. 2. Gel filtration of Fr-C on Sephadex G-15. The column (70 X 700 mm) was operated using water as a solvent with a flow rate of 50 ml/hr, and 15-ml fractions were collected. The fractions were monitored for absorbance at 276 nm and activation of human prostatic acid phosphatase (PAPase) activity.
tographed on Sephadex G-15 gel using a solvent of 0.1 N acetic acid. Three peaks of absorbance at 276 nm were found in the elution pattern, and PAPase activating activity was found in the first peak (Fr-D3G). A 150-mg sample of Fr-D3G and 1 g of cellulose powder were added to a 20-ml mixture of n-butanol, acetic acid and water (4:1:5), and the suspension was evaporated to dryness. The dried material was packed in a chromatographic column and washed with the mixture mentioned above and then eluted with water. The water eluate showing the highest PAPase activating activity was designated as fraction E4 (Fr-E4). Gel filtration of Fr-E4 using a column of Sephadex G-25 superfine and 0.2 N acetic acid solution as an eluent yielded two distinctive peaks with absorbance at 276 nm (Fig. 3). The PAPase activating activity was in the region of 0.4 Ve/Vb. Acetic acid in the fraction was removed by several repeated cycles of dissolving it in water and evaporating. This final fraction was used as purified PE (PPE) in the following experiments. The yield of PPE was 0.6 g from 100 g of PE.

**SDS-disc electrophoresis**

SDS-disc electrophoretic analysis of dansylated PPE is shown in Fig. 4. The fluorescent band (yellowish green) at the front was dansyl-OH, and the band (orange) located close to the starting point was dansyl-tris. PPE showed a yellowish-green fluorescent between the two bands. The ratio of the distance traveled by PPE to that traveled by dansyl-OH was approximately 0.3.

**Estimation of molecular weight**

The molecular weight of PPE was estimated by gel filtration on Sephadex G-25 superfine (Fig. 5). The Ve/Vb of PPE was approximately 0.4, and the values of the marker compounds, cytochrome C, insulin, vitamin B₁₂ and 5'-AMP, were 0.34, 0.41, 0.58 and 0.70, respectively. From these data, the molecular weight of PPE was estimated to be 8,000–9,000.

![Fig. 3. Gel filtration of Fr-E4 on Sephadex G-25. The column (18 x 340 mm) was eluted with 0.2 N acetic acid, and 5-ml fractions were monitored for absorbance at 276 nm and activation of the PAPase activity.](image)

![Fig. 4. The SDS-polyacrylamide gel electrophoretic pattern of purified prostate extract (PPE). Band a: dansyl-tris, Band b: dansyl-PPE, Band c: dansyl-OH.](image)
**UV absorption spectrum**

The UV absorption spectrum of PPE dissolved in water at a concentration of $6 \times 10^{-4}$ mg/ml revealed a marked absorption at 190–210 nm. PPE in this concentration showed no detectable absorption within a range from 260 to 280 nm.

**Infrared absorption spectrum**

The IR absorption spectrum of PPE was determined with a KBr tablet containing 600 µg of PPE on a Hitachi EPI-G3 Infrared Spectrom-

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**Fig. 5.** Estimation of molecular weight of purified prostate extract (PPE) by Sephadex G-25 superfine. The column (18 × 340 mm) was eluted with a 0.2 N acetic acid and 5-ml fractions were monitored for absorbance at 276 and 260 nm. Ve: the elution volume, Vb: the total bed volume of column.

**Fig. 6.** The IR spectrum of purified prostate extract (PPE). The IR absorption spectrum of PPE was determined using KBr tablets containing about 600 µg of PPE.
eter (Fig. 6). Absorption bands at 1,650, 1,550 and 1,260 cm⁻¹ were considered to be due to amido I (C = O stretching), amido II (N-H deformation and C-N stretching) and amido III (C-N stretching) vibrations, respectively. There was no band for an aromatic amino acid.

**Amino acid analysis**

Table 1 shows the results of amino acid analysis performed on the hydrolysates of three independently purified PPE specimens. The analysis revealed the presence of aspartic acid, glutamic acid, serine, glycine, alanine, valine, proline, isoleucine, leucine, lysine, arginine and no aromatic amino acids except for phenylalanine. The PPE molecule was estimated to be comprised of a total of 82 amino acids, from which its molecular weight was calculated to be 8,835, which was approximately the value determined by gel filtration on Sephadex G-25 superfine.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>No. 1 (μmol)</th>
<th>No. 2 (μmol)</th>
<th>No. 3 (μmol)</th>
<th>Tentative composition</th>
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<td>0.128</td>
<td>0.099</td>
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<tr>
<td>His.</td>
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<td></td>
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<td>NH₃</td>
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<td>Ile.</td>
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<td>0.047</td>
<td>0.038</td>
<td>1</td>
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<td>Leu.</td>
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<td>0.103</td>
<td>0.096</td>
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<td>Tyr.</td>
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<tr>
<td>Phe.</td>
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<td>0.040</td>
<td>0.039</td>
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<td>Try.</td>
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<tr>
<td>Total</td>
<td>3.734</td>
<td>2.903</td>
<td>4.340</td>
<td>82</td>
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</table>

N = 1, MW = 8,835

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*: Not detected

**Effect of PPE on PAPase activity**

Figure 7 shows the effect of PPE (10⁻⁶ to 10⁻⁴ g/ml) on the activity of PAPase. PPE increased the PAPase activity dose-dependently; PPE at a concentration of 2 × 10⁻⁵ g/ml caused an approximately two-fold increase in the PAPase activity. To investigate the mechanism of action, the Lineweaver-Burk reciprocal plot of data was utilized (Fig. 8). The values estimated from the graphic analysis were as follows: Vmax = 2.6 μmole/μg protein/min and Km = 286 μM in the absence of PPE and Vmax = 5.0 μmole/μg protein/min and Km = 248 μM in the presence of PPE. Thus, the Km value did not differ virtually between the presence and absence of PPE, and the Vmax value showed an approximately two-fold increase in the presence of PPE.

L-Tartaric acid is known to inhibit PAPase specifically (12). The 50% inhibitory concentration of L-tartaric acid on the enzyme activity was determined as 2 × 10⁻³ M. The kine-
tic parameters obtained from Lineweaver-Burk reciprocal plots of the data (Fig. 9) were as follows: $V_{\text{max}} = 2.4 \mu \text{mole/\mu g protein/min}$ and $K_m = 206 \mu \text{M}$ in the absence of L-tartaric acid and $V_{\text{max}} = 2.7 \mu \text{mole/\mu g protein/min}$ and $K_m = 8,252 \mu \text{M}$ in the presence of L-tartaric acid. It is evident, therefore, that L-tartaric acid inhibits PAPase competitively. Then the effect of PPE was examined on the PAPase activity inhibited by $2 \times 10^{-3} \text{ M}$ L-tartaric acid. As shown in Fig. 10, PPE produced a dose-dependent recovery of the inhibited PAPase activity.

![Fig. 7. Effect of purified prostate extract (PPE) on the activity of human prostatic acid phosphatase. Each value represents the mean ± S.E. of 10 experiments. PAPase activity was measured by the Bessey-Lowry method.](image)

![Fig. 8. Lineweaver-Burk’s plot of human prostatic acid phosphatase activity with or without $2 \times 10^{-5} \text{ g/ml purified prostate extract (PPE).}](image)
Fig. 9. Lineweaver-Burk's plot of human prostatic acid phosphatase activity inhibited in the presence of 2 × 10⁻³ M L(+)-tartaric acid (LTA).

**Effect of PPE on testosterone uptake in castrated rat prostate**

The serum level of ³H-testosterone of animals given PPE p.o. was almost the same as that of the control, whereas the amount of ³H-testosterone in the ventral lobe of the prostate of the PPE-treated rats was significantly lower than that of the control group, thus indicating PPE suppressed the testosterone uptake of prostates in the castrated rats (Table 2).

**Table 2. Effect of purified prostate extract (PPE) on testosterone uptake of prostates in castrated rats**

<table>
<thead>
<tr>
<th>Radioactivity (³H-testosterone)</th>
<th>DPM/0.1 ml serum (× 10⁴)</th>
<th>DPM/tissue/100 g body weight (× 10²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.70 ± 0.19</td>
<td>9.27 ± 0.31</td>
</tr>
<tr>
<td>PPE</td>
<td>3.65 ± 0.31</td>
<td>6.88 ± 0.65*</td>
</tr>
</tbody>
</table>

Testosterone propionate (0.5 mg/kg/day, s.c.) was given every other day and PPE (3 mg/kg/day, p.o.) was administered daily for 7 days starting 8 days after the castration. On day 15, the rats were injected with 50 μCi of ³H-testosterone, i.p.; and 2-hr later, blood and prostates were isolated for measuring the radioactivity. Each value indicates the mean ± S.E. of 5 animals. *: Statistical significance of difference from the control at P < 0.05 (t-test).
DISCUSSION

The PAPase activating substance was isolated from PE. After the final purification step, the activity migrated as a single band in SDS-disc gel electrophoresis. The molecular weight of PPE was between 8,000 and 9,000 in the analysis by column chromatography, which was approximately the same as the calculated value, 8,835, from amino acid analysis. UV and IR spectral analyses revealed that the PPE molecule contained few aromatic amino acids, and this finding was consistent with the results of amino acid analysis of the substance. These findings indicated that PPE was a highly pure substance, being a peptide composed largely of neutral amino acids, including about 60% glycine, and it contained no cysteine.

PPE activated human PAPase activity dose-dependently; PPE increased the $V_{\text{max}}$ value of the enzyme without affecting its $K_m$ value. PAPase was inhibited competitively in the presence of L-tartaric acid. PPE also dose-dependently activated the PAPase activity in the presence of L-tartaric acid. These findings suggest that PPE did not affect the affinity of the enzyme to the substrate, but accelerated the release of the product from the enzyme-substrate complex. However, further studies must be performed to determine the details of the activation mechanism.

There have been several reports (1–4) concerning the effectiveness of extracts from animal prostates on prostatic hypertrophy. In these cases, the extracts were administered via a parenteral route. Prostatic hypertrophy is a chronic disease, requiring long-term medication. Therefore, it is desirable to medicate such drugs through the oral route rather than parenteral routes. As shown in the present study, testosterone uptake into the prostate was reduced by the oral administration of PPE. Currently, excessive accumulation of testosterone or dihydrotestosterone in the prostate is widely thought to be one of the major pathogenetic causes of benign prostatic hypertrophy. Shida et al. (13) have reported successful treatment with a synthetic gestagen for patients with prostatic hypertrophy. The present study suggests that PPE is one of the active principles in the therapeutic effect of on PE prostatic hypertrophy, which can be taken via the oral route. It, however, remains unclear whether the effect of PPE to suppress the testosterone uptake into the prostate is through the PAPase activating activity of PPE or not.

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