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Studies on Human Prostatic Acid Phosphatase. IV.[1] Stabilization of Prostatic Acid Phosphatase against Thermal Inactivation by the Homologous Antibody

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Antibody against purified human prostatic acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) was produced in rabbits. The antiserum stabilized the activity of the enzyme against heat treatment, such as incubation at 56°C for 30 min, whereas normal rabbit serum displayed no stabilizing effect. We isolated IgG by ammonium sulfate fractionation and affinity chromatography on a Protein A-Sepharose CL-4B column. The IgG fraction showed a remarkable heat-stabilizing effect on prostatic acid phosphatase (PAPase) and the heat-stabilizing factor was purified 4.2-fold by the IgG isolation method. Complex of PAPase and PAPase specific IgG was separated from a mixture of PAPase and antiserum by a combination of Protein A-Sepharose CL-4B column and Sephacryl S-300 column chromatographies. The immune complex displayed heat stability. IgG which failed to bind PAPase had no heat-stabilizing effect on PAPase.

Fab fragments obtained by papain digestion showed a low heat-stabilizing effect, whereas $F(ab')_2$ fragments obtained by pepsin digestion displayed a heat-stabilizing effect comparable with that of the IgG fraction.

Keywords——anti-human prostatic acid phosphatase serum; thermo-stabilization; enzyme–antibody complex; Protein A-Sepharose CL-4B; fragments Fab and $F(ab')_2$

The effects of antibodies on enzymes and the nature of these effects have been of considerable interest. Some antibodies inhibit the corresponding enzymatic reactions,[2–7] while others are without effect or may even activate the enzyme reactions.[8–10] Other antibodies protect enzymes against temperature of pH inactivation.[11–13]

Acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2, APase) of human prostate origin is of clinical and biochemical interest as a diagnostic indicator of the development and metastasis of prostatic cancer because of its elevated activity in the serum of individuals with prostatic cancer.[14,15] Sawada *et al.*[16] previously reported a simple, specific assay of serum prostatic APase (PAPase) activity, utilizing the fact that anti-PAPase rabbit serum stabilizes the homologous antigen against heat inactivation.

This report describes the isolation of PAPase stabilizing factor from immune rabbit serum against purified PAPase. We also describe the heat-stabilizing effects of papain-digested IgG molecules and pepsin-digested IgG molecules on PAPase.

Experimental

Materials——Human prostate was obtained from a patient with benign hypertrophy at surgery and stored frozen at −70°C until use. Female rabbits, weighing approximate 2.5 kg were used. *p*-Nitrophenyl phosphate disodium salt (*p*NPP) (Tokyo Kasei Co., Tokyo) was used as a substrate. Bovine serum albumin (BSA) was purchased from Sigma Co. (USA), while papain was from Merck, and pepsin was from Pentex Co. (USA). All other chemicals were of analytical reagent grade or the highest grade available.

Purification of PAPase——Human PAPase was purified by column chromatography on phospho-cellulose, Sephacryl S-300, and DEAE cellulose as reported previously.[17] The PAPase obtained showed only one protein band and a corresponding activity band on polyacrylamide gel electrophoresis. The molecular weight of PAPase was approximately 100000 as determined by exclusion chromatography, and this value is consistent with the previous report by Ostrowski.[18]

Enzyme Assay——Assays were carried out essentially by the method of Smith and Whitby.[19] Enzyme activity was measured by incubating dilute enzyme for 10 min at 37°C with 2 mM *p*NPP in a total volume of

2 ml of 0.1 M citrate buffer, pH 6. The reaction was stopped by addition of 1 ml of 0.4 N NaOH. The absorbance of the liberated *p*-nitrophenol was read at 410 nm. Specific activity was expressed as μmol *p*-nitrophenol per min per mg protein.

Protein Assay——Protein was determined according to the method of Lowry *et al.*[20] with crystalline bovine serum albumin as a standard.

Immunization Procedure——One milliliter of the purified PAPase (1.0 mg/ml) and complete Freund's adjuvant (Iatron) were emulsified and injected into rabbits intramuscularly. This was repeated five times at weekly intervals. Ten days after the last injection, the rabbits were bled through the carotid artery. Serum was separated and deactivated at 56°C for 30 min.

Stability of PAPase——For all the stability measurements, antigen and antigen–antibody mixtures, and antigen in normal rabbit serum, 0.1 M citrate buffer (pH 6.0) was used to prepare solutions.

Preparation of Ammonium Sulfate IgG Fraction[21]——The IgG fraction was isolated from the antiserum by successive precipitations with ammonium sulfate. The first and the second precipitations were carried out at 50% and 20% saturations, while the consective ones were done at 45% and 33% saturations. Saturated ammonium sulfate solution, adjusted to pH 7.8, was prepared at room temperature. The dissolved precipitate was dialyzed against phosphate-buffered saline, pH 7.2 (PBS).

Protein A-Sepharose CL-4B——About 1 g of a freeze-dried preparation of Protein A covalently bound to Sepharose CL-4B (Pharmacia, Uppsala, Sweden) was swollen in PBS and packed in a 0.7×7.5 cm chromatogrophic column. This produced a gel bed of about 3 ml with a rabbit IgG binding capacity of 20 mg IgG/ml. Before each chromatographic run, the gel was thoroughly washed with PBS-0.02% NaN_3: when not in use, the column was kept at 4°C in the same buffer. Preparations of IgG fractions or immune complex were applied directly to the column and the applied material was washed through the column with PBS–0.02% NaN_3. When all the material not bound to the gel matrix had been cleared, elution (20 ml/h) with 0.15 M NaCl–glycine HCl buffer, pH 3.0 was begun to release column-bound material. The column effluent was collected in 3 ml fractions and the absorbance was monitored with an Atto mini UV monitor II, model SJ-1541.

Gel Filtration at pH 7.2——Protein A-Sepharose bound immune complexes released at pH 3.0 with 0.15 M NaCl–glycine HCl buffer described above were applied to a Sephacryl S-300 column (1.9×100 cm) equilibrated with the same buffer. The column was run at 30 ml/h. Fractions (3 ml) corresponding to immune complexes or separated free antibody on the basis of absorbance and/or phosphatase activity were pooled. The pooled fractions were concentrated by ultrafiltration (YM 10-filter).

Gel Filtration at pH 3.0——Complexes obtained by gel filtration at pH 7.2 were applied to a Sephacryl S-300 column (1.9×100 cm) equilibrated with 0.15 M NaCl–glycine HCl buffer, pH 3.0. The column was run at 30 ml/h.

Papain Digestion[22]——IgG fractions of anti-PAPase serum obtained from the Protein A-Sepharose CL-4B column were dialyzed against 0.1 M acetate buffer (pH 5.5). The reaction was conducted for 16 h at 37°C in the presence of cysteine (1 mM) and ethylenediaminetetraacetic acid (2 mM); the papain concentration was 0.13 mg/ml (0.01 mg per 1.0 mg IgG protein). The hydrolysis was stopped by dialyzing the mixture against water for 2 days. After further dialysis against PBS, the reaction mixture was chromatographed on the Protein A-Sepharose CL-4B column.

Pepsin Digestion[22]——IgG fractions of anti-PAPase rabbit serum were dialyzed against several changes of 0.1 M acetate buffer, pH 4.0. The sample (50 mg) was mixed with 1 mg of pepsin in a total volume of 5 ml. The mixture was incubated at 37°C for 18 h. At the end of the digestion period, the reaction mixture was adjusted to pH 8.0 with 1 N NaOH. After dialysis against PBS, the reaction mixture was chromatographed on the Protein A-Sepharose CL-4B column.[23]

Immunodiffusion[24]——Double immunodifusion in 1.2% agarose gel was carried out in plates according to Ouchterlony.

Results

Antibody Stabilization of PAPase against Thermal Inactivation

Rabbit antiserum to PAPase protected the enzyme from thermal inactivation. In this experiment, antigen and antibody were heated together at the desired temperature for 30 min then assayed for activity. The protective effects of antiserum on the heat stability of PAPase are shown in Fig. 1.

Effects of Ammonium Sulfate Fractions on PAPase Stability

The stabilizing effects of 33% and 45% ammonium sulfate (AS) precipitates, 50% AS supernatant, and 20% AS precipitate fraction to PAPase were measured. When 1.2 mg of each fraction was added to 0.06 unit/ml PAPase, the 33% AS precipitate gave 92.6% residual

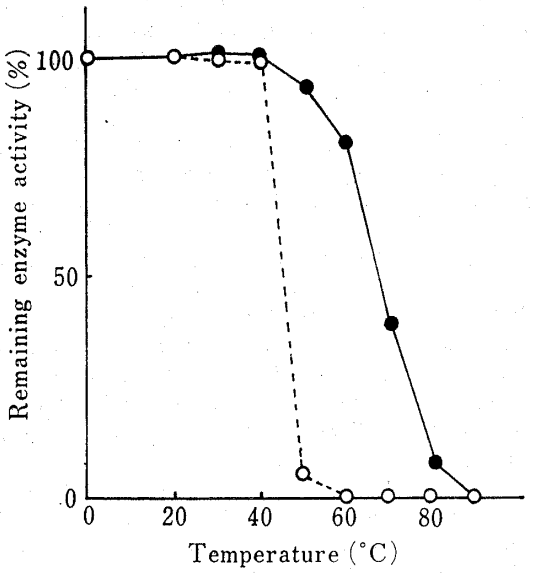

Fig. 1. Heat Inactivation of PAPase

One milligram of normal rabbit serum or anti-PAPase serum was added to 0.06 Unit of PAPase and the residual activity after heat treatment at each temperature was measured. ○——○; normal rabbit serum, ●——●; anti-PAPase serum.

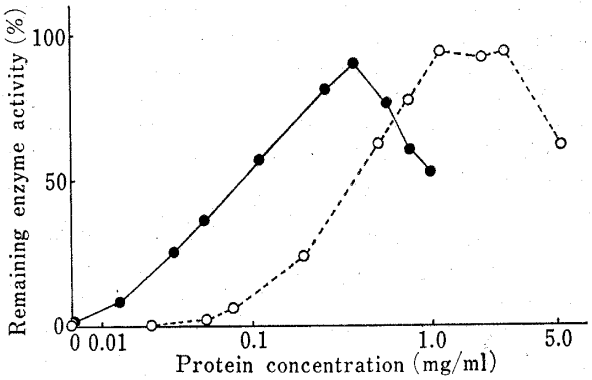

Fig. 2. Stabilization of PAPase against Heat by IgG Fraction and by Anti-PAPase Serum

Various amounts of anti-PAPase serum or anti-PAPase IgG fraction were added to 0.06 Unit of PAPase and the residual activity after heat treatment was measured. ○——○; anti-PAPase serum, ●——●; anti-PAPase IgG fraction.

activity, and the 45% AS precipitate gave 95.9% residual activity after heat treatment, while with 50% AS supernatant and 20% AS precipitate, PAPase lost its activity completely.

Further assayes were performed with increasing amounts of either 33% or 45% AS precipitate fraction (with a constant amount of enzyme) and PAPase (with a constant amount of antibody fraction). In both cases, the 33% and 45% AS precipitate fractions showed higher stabilization effects than antiserum assayed as a control (figure not shown).

Effect of Anti-PAPase IgG Fraction on the Heat Stability of PAPase

Thirty-three percent and 45% AS precipitates were chromatographed on the Protein A-Sepharose CL-4B column, separately. After the unbound material had been eluted with PBS, 0.15 M NaCl–glycine HCl buffer was used to elute the column-bound fraction. The pass-through peak consisted of immune serum constituents. The second peak was identified as IgG. It showed a single precipitin line with either anti-rabbit IgG goat serum or anti-rabbit whole serum goat serum by the Ouchterlony method.[23] The effects of various amounts of IgG and antiserum on the residual activity of antibody mixture are shown in Fig. 2. Although the protein concentration of antiserum at which 50% residual activity is obtained is 0.36 mg/ml, that of IgG is 0.086 mg/ml. The heat-stabilizing factor was therefore purified 4.2-fold by IgG isolation.

Characterization of PAPase Anti-PAPase Complex

Initially PAPase anti-PAPase complexes were chromatographed on the Protein A-Sepharose CL-4B column at 4°C (Fig. 3). The first peak eluted with PBS was unbound antigen (28.7% of the applied PAPase). The coincidence of the second peak of absorbance at 280 nm of protein and PAPase activity (71.2% of the applied PAPase) indicated the release of IgG-containing complexes from the column when the pH was lowered to 3.0. The glycine–HCl-eluted fractions were pooled and dialyzed against PBS. A 9 ml portion of the dialyzate was then chromatographed on Sephacryl S-300 in the same buffer. Complexed material was separated from non-complexed serum IgG (Fig. 4). The complexes contained 0.0587 mg of protein and 0.2267 unit/ml of phosphatase activity. On the assumption that the specific

activity of PAPase, approximately 300, was not decreased, the molar ratio of antigen to antibody of the complex was calculated as 1 to 48. The complex fraction, however, showed about 40% residual activity after heat treatment. IgG fraction which failed to bind PAPase showed no heat-stabilizing effect on PAPase.

*K*m values of the enzyme in the complex and of the native enzyme were very similar, 7.7×10^{-4} M and 3.0×10^{-4} M, respectively. The enzyme in the antibody complex had an optimal pH of 5.5, while that of the purified enzyme was at 5.9.

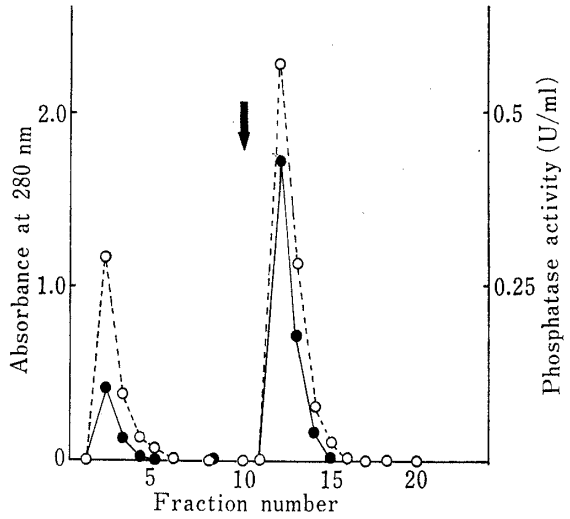

Fig. 3. Column Chromatography of PAPase Anti-PAPase Complexes on Protein A-Sepharose CL-4B

A 52.1 mg sample of 33% ammonium sulfate precipitate and PAPase mixture was applied to a Protein A-Sepharose CL-4B column (0.7×7.5 cm), equilibrated with PBS. ○----○; protein, ●——●; phosphatase activity. The arrow indicates elution with 0.15 M NaCl-glycine HCl buffer, pH 3.0.

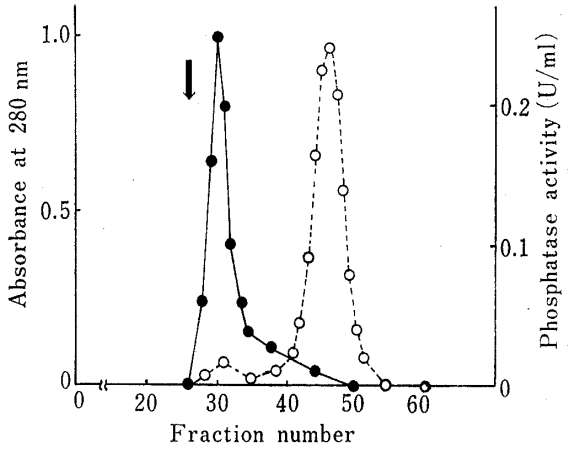

Fig. 4. Column Chromatography of PAPase Anti-PAPase Complexes on Sephacryl S-300

A 25.7 mg sample of the Protein A-Sepharose CL-4B bound fraction of 33% ammonium sulfate precipitate and PAPase mixture was applied to a Sephacryl S-300 column (1.9×100 cm), equilibrated with PBS. ○----○; protein, ●——●; phosphatase activity. The arrow indicates the column void volume.

Gel Filtration at pH 3.0

The complex was applied to a column of Sephacryl S-300 preequilibrated with 0.15 M NaCl–glycine HCl buffer (pH 3.0). The antigen and antibody were incompletely separated by the gel filtration. One symmetrical protein peak and one phosphatase peak were obtained at positions corresponding to molecular weights of 160000 and 100000. The dissociation of the immune complex was apparently complete because no protein or phosphatase activity peak was seen at around the void volume. The difference of molecular weights of antigen and antibody was relatively small, so that their elution peaks overlapped. Fractions which contained various ratios of antigen to antibody were obtained. The fractions were heated at 56°C for 30 min and the residual activity of each fraction was measured,

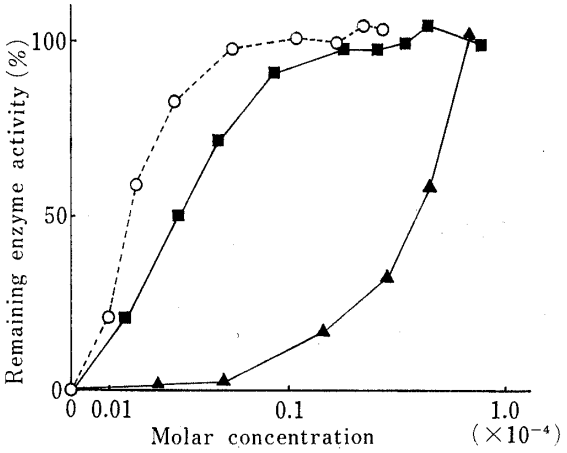

Fig. 5. Stabilization of PAPase against Heat by Fragments Fab and $F(ab')_2$

Various amounts of IgG, Fab fragments, or $F(ab')_2$ fragments were added to 0.06 Unit of PAPase and the residual activity after heat treatment was measured. ○----○; IgG, ▲——▲; Fab fragments, ■——■; $F(ab')_2$ fragments.

while the molar ratio of antigen to antibody was also calculated. The highest residual activity, 96.6%, was obtained when the molar ratio of antigen to antibody was 1 to 60. Molar ratios above or below this point gave lower heat stabilities (figure not shown).

Effect of Fab Fragments and $F(ab')_2$ Fragments of Anti-PAPase IgG on the Heat Stability of the Homologous Antigen

To determine whether any fragment of IgG can exhibit this PAPase heat-stabilizing effect or not, the IgG anti-PAPase antibodies were papain-digested or pepsin-digested and passed through the Protein A-Sepharose CL-4B column. The residual activity of PAPase after heat treatment in the presence of thus-obtained Fab fragments and of $F(ab')_2$ fragments is illustrated in Fig. 5. The data obtained show differences between the enzyme-digested fragments of IgG molecules. $F(ab')_2$ fragments exerted a strong protective effect against thermal inactivation of PAPase, whereas the effect of Fab fragment was low. The molar concentration of $F(ab')_2$ fragments at which 50% residual activity is obtained was 1.9 times that of undigested IgG, whereas that of Fab fragments was 17.0 times that of intact IgG, when the molar concentration of Fab fragments was doubled for direct comparison with IgG.

Discussion

Several enzymes have been reported to be stabilized against heat or pH inactivation in the presence of the homologous antibody. Most studies, however, were done with antiserum or ammonium sulfate fraction as the antibody. Thus, it is not certain whether the stabilizing effect of anti-PAPase serum is caused by its homologous IgG or by some other substance.

From the data obtained, it is evident that the Protein A-Sepharose CL-4B column-bound IgG fraction is responsible for stabilizing PAPase against heat. This IgG fraction, containing PAPase non-specific IgG as well as PAPase specific IgG, was purified 4.2-fold compared with antiserum. This stabilization effect occurred even when the amount of antigen and antibody was too small to give a precipitate.

The enzyme-digested IgG molecule fractions that have been separated in this study differ from one another in PAPase stabilizing behavior. The stabilizing effect of univalent Fab fragments was 17.0 times lower than that of intact IgG. (The molar concentrations of Fab fragment and IgG which give 50% residual activities of PAPase after heat treatment are compared in Fig. 5). Divalent $F(ab')_2$ fragments possess a stabilizing effect comparable with that of IgG. These findings led us to speculate that the heat-stabilizing effect might be caused by a lattice-forming event in which divalent $F(ab')_2$ fragments could participate but univalent Fab fragment could not.

The immune complex which eluted in the void volume fraction, (large molecular weight) showed 40% residual activity when heat-treated. However, in a fraction which contains antigen and antibody at the ratio of 1 to 60 the enzyme activity was completely heat-resistant. This suggests that heat stabilization requires rather more antibody than the amount that gives large immune complexes.

Since PAPase is only slightly inhibited by the antibody as measured in terms of activity towards *p*NPP as a substrate, the antigenic determinants seem to be located some distance apart from the substrate binding site, *i. e.*, the substrate binding site is accessible to the substrate in the antibody–enzyme complex.

Arnon[25] suggested in his review article that conformational changes caused by either partial or complete denaturation of the enzyme influence both the enzymatic and the immunological properties. In the case of PAPase, the interaction with specific anti-enzyme antibodies might change the conformation of the enzyme to a more heat-stable form which is still catalytically active. The nature of the change that occurs during denaturation in the absence of antibody also remains to be elucidated.

References and Notes

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