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New Detection Method for Uropepsinogen (PGA) Using Isoelectric Focusing and Immunoblotting Techniques*

T. Yasuda, Y. Ikehara, W. Sato, and K. Kishi

Department of Legal Medicine, Fukui Medical School, Matsuoka-cho, Fukui 910-11, Japan

Summary. Uropepsinogen (PGA) was isolated and purified from human urine using a column chromatography series. The purified PGA was injected into a rabbit and a PGA-specific antibody was obtained. PGA isozymogen in human urine could be detected reproducibly by immunoblotting using this antibody after isoelectric focusing electrophoresis (IEF) on polyacrylamide gels. This technique may prove to be useful in the genetic study of PGA polymorphism.

Key words: Urinary isozymogen, uropepsinogen (PGA) – Anti-uropepsinogen (PGA), immunoblotting

Zusammenfassung. Uropepsinogen (PGA) wurde aus Humanharn mit Hilfe einiger Säulenchromatographieverfahren isoliert und gereinigt. Das gereinigte PGA wurde ins Kaninchen eingespritzt und dadurch ein spezifischer Antikörper gegen PGA gewonnen. PGA-Isozymogen im Harn ließ sich durch Einführung der kombinierten Technik des Anti-PGA-benutzten Immunoblotts und isoelektrischer Fokussierung (IEF) auf Polyacrylamidgelen darstellen. Die Anwendung dieser Technik kann sich bei genetischen Untersuchungen des PGA als nützlich erweisen.

Schlüsselwörter: Harnisozymogen, Uropepsinogen (PGA) – Anti-uropepsinogen (PGA), Immunoblottierung

Introduction

Pepsinogen is classified into two immunochemically distinct groups, PGA and PGC. PGA is detected in serum and urine as the uropepsinogen, while PGC is present in serum and seminal fluid. The *PGA* gene is located on chromosome

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Offprint requests to: K. Kishi (adress see above)

11 [1], and its structure is determined at the DNA level [2]. Genetic models of PGA have been proposed by several investigators [3–7], who have used all the proteolytic properties of acid-activated PGA for the detection of the isozymogens on agar or polyacrylamide gels. However, immunologic detection of PGA isozymogen is more important for genetic analysis, since the term, PGA, is immunochemically based on the pepsinogen grouping.

In the present paper, we describe a new detection method for PGA isozymogen utilizing IEF followed by immunoblotting with a specific antibody produced in rabbits by injection of purified PGA.

Materials and Methods

One hundred liters of human urine was collected from a healthy 9-year-old boy and concentrated to 1 l by ultrafiltration for PGA purification.

Antibody to purified PGA was obtained as described previously [8]. A total of 4 mg of the purified PGA was injected into a rabbit.

The protein concentration was determined by the method of Lowry et al. [9], and PGA was assayed after acid activation to pepsin by the spectrophotometric method of Ryle [10], using bovine hemoglobin as the substrate.

IEF was performed as described in our previous paper [7]. Polyacrylamide gels measuring $120 \times 90 \times 0.5$ mm were prepared using the following components: 1.4 ml acrylamide/bisacrylamide (20T, 3C), 2.3 ml sucrose-glycerin (20% w/v, 10% v/v), 1 ml distilled water, 250 µl Pharmalyte 2.5–5 (Pharmacia, Uppsala, Sweden), 5 µl TEMED, and 40 µl of 1.2% ammonium persulfate. Wicks were formed from strips of filter paper no. 526 (Toyo, Tokyo) and soaked in the electrode solution: 0.1 *M* H_2SO_4 at the anode and 0.1 *M* NaOH at the cathode. A 5-µl aliquot of each urine sample was applied with a plastic sample applicator to a point 1.0 cm from the cathode on the gel. An LKB (Uppsala) Multiphor apparatus was employed to run the gel at 3 W for 4 h under cooling. Electrophoresis on sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) was also performed for checking the homogeneity of the purified PGA and for molecular weight determination.

The urine samples for IEF were concentrated by ultrafiltration, dialyzed against 0.1% glycine, and then lyophilized according to the method described in our previous paper [11].

Hydroxyapatite and celite were purchased from Nakarai (Kyoto, Japan), DEAE-Sepharose CL-6B, Sephadex G-75, and protein standards for estimation of molecular weight from Pharmacia, elastin from Sigma (St. Louis), and DEAE Affi-Gel Blue and peroxidase-labeled goat anti-rabbit immunoglobulin from Bio-Rad (Richmond). Other chemicals used were of reagent grade.

A Durapore (Millipore, Bedford) strip was applied to the gel for transferring migrated PGA isozymogens by pressing with a 1.0-kg weight. The strip was soaked for 15 min in phosphate-buffered saline (PBS) containing 0.1% gelatin and 0.05% Tween 20, followed by washing with PBS containing 0.05% Tween 20 (PBS-T) for 15 min three times. The strip was soaked for 60 min in a solution of rabbit anti-PGA antibody diluted appropriately with PBS-T and then washed for 15 min in PBS-T three times. After reacting for 60 min with diluted peroxidase-labeled goat anti-rabbit immunoglobulin in PBS-T and then washing for 15 min in PBS-T three times, the strip was incubated and developed in a mixture of 3,3′-diaminobenzidine 4HCl and H_2O_2 for 5–30 min.

Results

Purification of PGA

PGA for immunization was purified from concentrated urine principally by the following method described by Minamiura et al. [12]. PGA was fractionally pre-

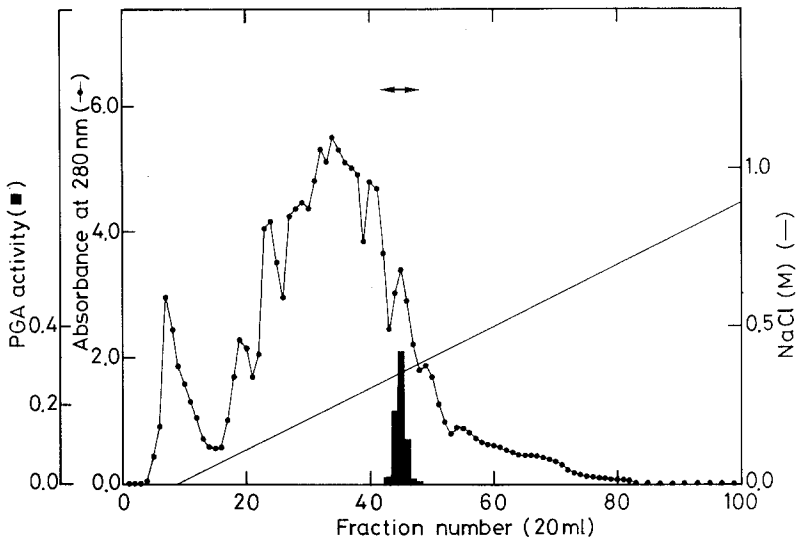

Fig. 1. DEAE-Sepharose CL-6B column (2.6 × 35 cm) chromatography of PGA in the fraction precipitated with ammonium sulfate between 0.25 and 0.8 saturation. The *horizontal bar* indicates those fractions showing PGA activity which were pooled for further purification. See the text for details

cipitated with ammonium sulfate between 0.25 and 0.8 saturation after adjusting the pH to 6.0. The resulting precipitate was collected by centrifugation and dialyzed against 0.02 *M* Tris-HCl buffer, pH 7.0. The dialysate was applied to a DEAE-Sepharose CL-6B column equilibrated with 0.02 *M* Tris-HCl buffer, pH 7.0.The adsorbed PGA was eluted with a linear gradient of NaCl from 0 to 1 *M* in the same buffer (Fig. 1). PGA was eluted at around 0.35 *M* NaCl. The PGA-active fractions were combined and precipitated with ammonium sulfate at 0.5 saturation. The precipitate was collected by centrifugation and dialyzed against 0.005 *M* potassium phosphate buffer, pH 6.0. The dialyzed solution was applied to a hydroxyapatite column equilibrated with 0.005 *M* potassium phosphate buffer, pH 6.0. The adsorbed PGA on the column was washed with 0.005 *M* potassium phosphate buffer, pH 6.8, and then eluted with 0.05 *M* potassium phosphate buffer, pH 6.8 (Fig. 2). PGA from the hydroxyapatite column was precipitated with ammonium sulfate at 0.5 saturation. The precipitate was dialyzed against 0.01 *M* potassium phosphate buffer, pH 6.4. After adding solid ammonium sulfate to give 0.35 saturation, the dialysate was applied to a column of elastin-celite (1:5 w/w) [12], which was pre-washed with 0.1 *M* potassium phosphate buffer, pH 6.8, and finally equilibrated with 0.02 *M* potassium phosphate buffer, pH 6.8, containing ammonium sulfate at 0.35 saturation. The column was washed with the same salt solution, and then PGA on the column was eluted with 0.02 *M* sodium acetate buffer, ph 6.0 (Fig. 3). The PGA-active fractions from the previous step were concentrated by ultrafiltration. The concentrate was applied to a Sephadex G-75 column (2.6 × 90 cm) buffered with 0.02 *M* potassium phosphate buffer, pH 6.8, containing 0.2 *M* NaCl. PGA was eluted as a symmetrical and sharp curve separated from a small amount of con-

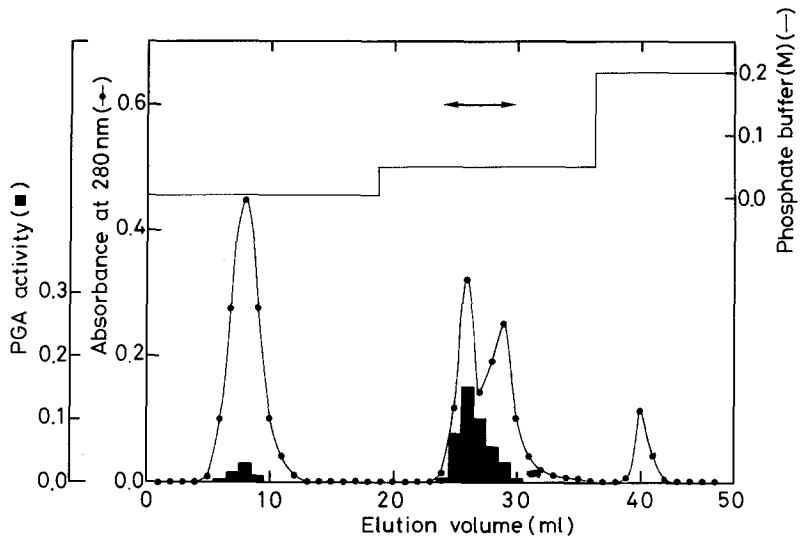

Fig. 2. Hydroxyapatite column (1.6 × 10 cm) chromatography of the PGA active fraction from a DEAE-Sepharose CL-6B column. The *horizontal bar* indicates PGA active fractions which were combined and concentrated for further purification. See the text for details

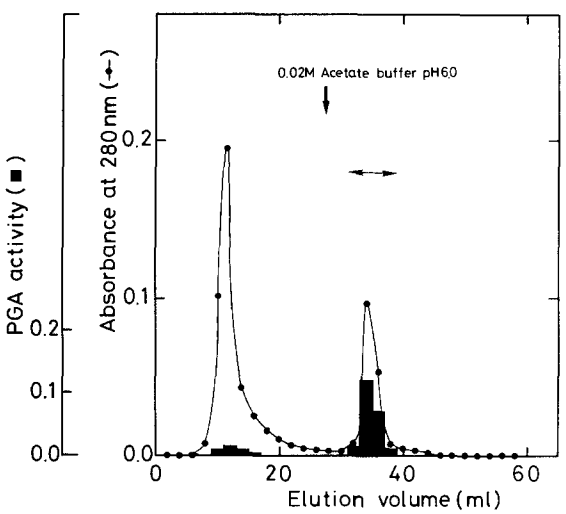

Fig. 3. Elastin-celite column (1.6 × 10 cm) chromatography of the fraction containing PGA from the hydroxyapatite column. The *horizontal bar* indicates the PGA active fractions which were pooled for further analysis. Other chromatographic conditions are described in the text

taminating protein. PGA-active fractions from the Sephadex G-75 column were pooled and concentrated to a small volume for further analyses and immunization.

Homogeneity of the purified PGA

The elution profile of PGA showed an almost symmetrical peak in its absorbances at 280 nm for protein and enzymatic activity, and the molecular weight

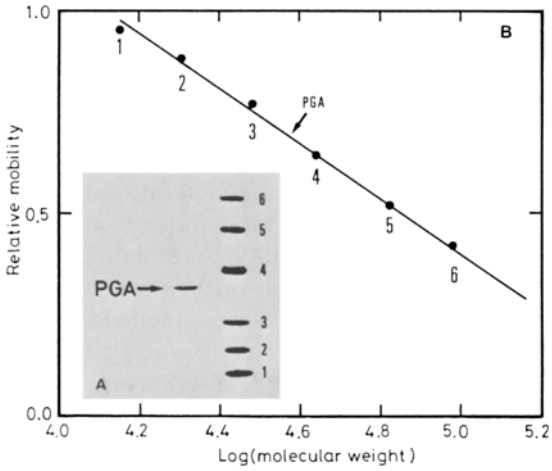

Fig. 4. Molecular weight determination of the purified PGA by SDS-PAGE (4%–10%). *A* SDS-PAGE patterns of PGA and standard proteins (1, α-lactoalbumin 14,400; 2, trypsin inhibitor 20,000; 3, carbonic anhydrase 30,000; 4, ovalbumin 45,000; 5, bovine serum albumin 67,000; 6, phosphorylase 94,000) under reducing conditions. *B* Calibration curve for molecular weight determination of PGA

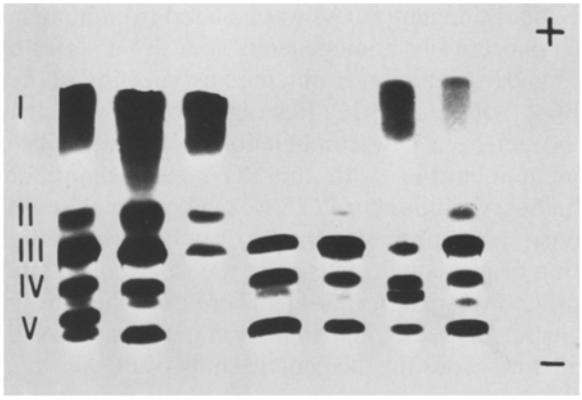

Fig. 5. Electrophoretic patterns of PGA isoenzymogens in human urine samples from several different subjects detected using the method presented in this paper

of PGA was estimated to be around 39,000. The purified PGA was also homogenous on SDS-PAGE after reduction and also showed a molecular weight of around 39,000 (Fig. 4).

Specificity of Anti-PGA Antibody

IgG fraction purified by DEAE Affi-Gel Blue chromatography was used as a PGA antibody. The antibody inhibited the enzymatic activity of the purified PGA and urine samples from several subjects of different ages. A double diffusion test in agar gel, showed only one precipitin line formed by the antibody with either purified PGA or concentrated urine samples from young and adult individuals, and these lines fused with one another. The antibody showed neither cross-reaction with human seminal fluid containing pepsinogen C group isozymogen (PGC) nor with sera at the original concentration upon agar diffusion.

Detection of PGA Isozymogen on IEF Gels by Immunoblotting Using the Anti-PGA Antibody

A 0.5%–1.0% solution of freeze-dried urinary samples, corresponding to about 100 times the concentration of the original urine, was used for IEF analysis. The electrophoretic patterns of PGA isozymogen from several individuals are shown in Fig. 5. PGA migrated into five fundamental fractions on the IEF gel between pI 3 and 4 and these fractions were tentatively named I to V in order of decreasing anodal mobility. Several different phenotypes were characterized by the lack of certain bands or the presence of extra bands. These PGA patterns were very reproducible for each individual. PGA samples were very stable in a lyophilized state, since they showed no change in the electrophoretic patterns even after storage for several years at −20° or 4°C.

Discussion

Uropepsinogen (PGA) was isolated from human urine and purified to an electrophoretically homogeneous state by a series of several column chromatographies. An antibody obtained by injection of the purified PGA was specific to PGA but not to PGC. PGA isozymogen was clearly separated and detected on the gel by a new combination technique utilizing both IEF and subsequent immunoblotting with the PGA-specific antibody. This technique provided higher resolution for PGA isozymogen analyses than those based on either ordinary electrophoresis on agar or polyacrylamide gels and those following detection of proteolytic activity of the isozymogen after acid activation, reported by other investigators [3–6]. Therefore, the present method facilitates a much clearer degree of discrimination between PGA phenotypes, and thus is greatly advantageous for the genetic study of PGA.

References

1. Taggart RT, Mohandas TK, Shows TB, Bell GI (1985) Variable numbers of pepsinogen genes are located in the centromeric region of human chromosome 11 and determine the high-frequency electrophoretic polymorphism. Proc Natl Acad Sci USA 82:6240–6244
2. Sogawa K, Fujii-Kuriyama Y, Mizukami Y, Ichikawa Y, Takahashi K (1983) Primary structure of human pepsinogen gene. J Biol Chem 258:5306–5311
3. Samloff IM, Townes PL (1970) Pepsinogen: genetic polymorphism in man. Science 168:144–145
4. Taggart RT, Karn RC, Merrit AD, Yu PL, Conneally PM (1979) Urinary pepsinogen isozymogens: a highly polymorphic locus in man. Hum Genet 52:227–238
5. Korsnes L, Gedde-Dahl T Jr (1980) Genetics of pepsinogen I. Ann Hum Genet 43:199–212
6. Frants RR, Pronk JC, Pals G, Defize J, Westerveld BD, Meuwissen SGM, Kreuning J, Eriksson AW (1984) Genetics of urinary pepsinogen: a new hypothesis. Hum Genet 65:385–390
7. Kishi K, Yasuda T (1987) Newly characterized genetic polymorphism of uropepsinogen group A (PGA) using both isoelectric focusing and immunoblotting. Hum Genet 75:209–212

8. Kishi K, Iseki S (1983) Specific antibody to the young age-related glycoproteins (Ugl-Y) in normal human urine. Proc Jpn Acad 59B : 240–242
9. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193 : 265–275
10. Ryle AP (1970) The porcine pepsins and pepsinogens. In: Perlman GE, Lorand L (eds) Methods in enzymology vol. 19: Proteolytic enzymes. Academic Press, New York San Francisco London, pp 316–336
11. Kishi K, Yasuda T, Iida R (1985) Glutamate-pyruvate transaminase (GPT) polymorphism detected in human urine by isoelectric focusing. Proc Jpn Acad 61B : 490–493
12. Minamiura N, Ito K, Kobayashi O, Yamamoto T (1984) Uropepsinogen in human urine: its protein nature, activation and enzymatic properties of activated enzyme. J Biochem 96 : 1061–1069

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