Immunoblot technique to visualise serum pepsinogen A isozymogen patterns

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Abstract

Pepsinogen A (PGA) isozymogen patterns in urine and gastric mucosa can be visualised in non-denatured polyacrylamide gel electrophoresis by showing proteolytic activity after the conversion of pepsinogen into pepsin by acid. This method is not suitable for visualising PGA patterns in serum due to low PGA concentrations. To obtain a more sensitive visualisation method an immunoblotting technique was developed. PGA isozymogen patterns from urine and sonified gastric mucosa specimens obtained by immunoblotting were identical with those obtained by activity staining. The immunostaining method was at least 50 times more sensitive. PGA isozymogen patterns could be visualised in serum. Preliminary results suggest that the PGA patterns in serum and gastric mucosa are identical.

As an association has been found between the genetically determined PGA isozymogen patterns in gastric mucosa and gastric malignancies in man, immunoblotting of PGA isozymogens in serum may provide a screening tool for subjects at risk of malignant gastric disease.

Pepsinogens are low molecular weight proteins synthesised by the gastric mucosa; they are also present in blood and urine. They are the precursors of the gastric pepsins.^{1,2} Using polyacrylamide gel electrophoresis (PAGE) of sonified human gastric mucosa biopsy specimens followed by staining for proteolytic activity, it has been shown that seven pepsinogen isozymogens occur in man. They are numbered Pg1-Pg7 in order of decreasing anodal mobility.³ Biochemically they can be divided into pepsinogen A (PGA), isozymogens Pg1-5, pepsinogen C (PGC) and isozymogens Pg6-7.⁴ PGA is the precursor of pepsin A (EC 3.4.23.1); PGC is the precursor of pepsin C (EC 3.4.23.3).¹

The relative intensities of the PGA isozymogens after electrophoresis are genetically determined.^{5,6} Furthermore, an association between PGA isozymogen patterns with an intense Pg5 band and malignant or premalignant lesions of the gastric mucosa has been reported.⁷⁻¹⁰

PGA concentrations in the urine (200-1000 µg/l) permit visualisation of the PGA isozymogen patterns with activity staining.

The PGA patterns in the urine do not entirely match those in gastric mucosa.^{10,11} The differences have been ascribed to a different reabsorption of the various PGA isozymogens by the renal tubules.¹² Therefore, PGA isozymogen patterns obtained from the blood might reflect more accurately the PGA patterns in the gastric mucosa. Due to low PGA concentrations in the blood and the presence of large amounts of albumin, however, a more sensitive technique is required to visualise serum PGA patterns. In this study we report the development of an immunoblot technique that was used to visualise PGA isozymogen patterns in gastric mucosa biopsy specimens, serum, and urine.

Methods

Serum samples and gastric mucosal biopsy specimens were obtained from patients undergoing routine endoscopy at the Department of Gastroenterology. Samples were frozen at -20°C until analysis. Urine samples were obtained from healthy volunteers. Samples were kept at 4°C and analysed within one week. Supernatants of sonified gastric mucosal biopsy, serum, and urine specimens were treated according to the routine protocol in our laboratory.⁶ Samples were diluted in electrophoresis buffer up to a volume of 50 µl. Subsequently, 25 µl of stacking gel buffer containing 40% (w/v) sucrose and 0.1 g/l bromophenol blue was added. Bromophenol blue was used to monitor both the albumin fraction and the electrophoresis front.

POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

Gels were prepared according to a slight modification of the methods routinely used in our laboratory.⁶ Modifications consisted of an increase in buffer concentrations to reduce the effect of high protein content on the serum: stacking gel buffer: 51 mmol/l TRIS, 49 mmol/l H₃PO₄ (pH 5.5); separating gel buffer: 71 mmol/l TRIS, 60 mmol/l HCl (pH 7.5); and electrode buffer: 8 mmol/l TRIS, 30 mmol/l diethylbarbituric acid (pH 7.0). The gels were 1.5 mm thick. The length of the separating gel was 128 mm and that of the stacking gel 7 mm. Electrophoresis was performed in the Protean vertical slab-gel system (Bio-Rad, Richmond, California, USA), with an LKB 2197 power supply (LKB, Bromma, Sweden) at a 25 mA constant current per gel until the tracking dye was completely separated from the albumin-bound bromophenol blue (about one hour). Subsequently,

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electrophoresis was continued for two hours at a 60 mA constant current per gel. During electrophoresis the system was kept at a temperature of 4°C.

BLOTTING CONDITIONS

Protein blotting was performed with the Trans-blot system and power supply (model 250/2.5; Bio-Rad, Richmond, California, USA) according to Towbin *et al.*¹³ Protein transfer was performed at a constant voltage of 70 V for one hour. Proteins were blotted on to polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore, Bedford, Massachusetts, USA).¹⁴

IMMUNOSTAINING OF THE BLOTTED PEPSINOGENS

After blotting was complete the membrane was immediately incubated for one hour at 37°C in "blocking" solution (5% (w/v) bovine serum albumin (BSA), 0.9% (w/v) sodium chloride in 20 mmol/l TRIS-HCl, pH 7.4) with gentle agitation. The membrane was washed in 0.1% BSA, 0.9% NaCl, 20 mmol/l TRIS-HCl, pH 7.4, three times for five minutes. The blot was incubated overnight at room temperature with goat antipepsinogen antibodies at 10 µg/ml in 25 ml incubation buffer (1% BSA, 0.05% (v/v) Tween-20, 0.9% (w/v) NaCl, 20 mmol/l TRIS-HCl, pH 7.4). For gastric mucosal biopsy specimens and urine specimens incubation was performed with anti-PGA and anti-PGC antibodies. For serum patterns, only PGA antibodies were used. Affinity purified antibodies against human pepsinogens were obtained from goats immunised with PGA and PGC, antibodies as described elsewhere.¹⁵⁻¹⁷ These antibodies show no cross reactivity between PGA and PGC.¹⁸ To eliminate possible cross reactivity the buffer was supplemented with 10% (v/v) native human plasma (pepsinogens in this plasma were destroyed first by heating the plasma for 45 minutes at 57°C). The blot was then washed as described above and incubated for two hours at room temperature with biotin labelled rabbit anti-goat IgG (1 in 1000 dilution of the stock solution in incubation buffer; Sigma Chemicals, St Louis, Missouri, USA). After another wash cycle the blot was incubated with avidin alkaline phosphatase conjugate (1 in 1000 dilution of the stock solution; Sigma Chemicals) for two hours at room temperature in incubation buffer. After washing as described above the blot was placed in enzyme substrate buffer to develop colour. The enzyme substrate buffer consisted of 100 mmol/l TRIS-HCl (pH 9.55), 100 mmol/l NaCl, 2 mmol/l MgCl₂, 0.03 mmol/l phenazine methosulphate, 0.3 mmol/l 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP), and 0.2 mmol/l nitro-blue tetrazolium (NBT) in high power liquid chromatography grade water. When the incubation was complete (10–15 minutes), the blot was rinsed in water and dried at 37°C. Pepsinogen isozymogen patterns appeared as dark bands on a white background.

VISUALISATION OF THE PEPSINOGEN ISOZYMES IN POLYACRYLAMIDE GELS BY STAINING FOR PEPSIN ACTIVITY

After electrophoresis the isozymogens were converted into pepsin by soaking the gel for 20 minutes at 37°C in a solution of 0.1 mol/l HCl and 2% bovine haemoglobin, followed by incubation in 0.1 mol/l HCl for 40 minutes at 37°C to permit lysis of haemoglobin by pepsins. After incubation the gels were stained for protein for one hour in a solution containing 0.2% Coomassie brilliant blue R250 in 10% (v/v) acetic acid and 25% (v/v) isopropanol. Coomassie brilliant blue—not bound to protein on the gels was removed by washing in 10% acetic acid. Pepsinogen isozymogen patterns appeared as white bands on a blue background.

QUANTITATION OF THE ISOZYMEN PATTERNS

The gels and blots were photographed with a Polaroid MP-4 Land Camera, with Polaroid type 665 Black and White Instant Pack Film (Polaroid Corporation, Cambridge, Massachusetts, USA). For the activity stained gels, a yellow filter was used.

Semiquantitative analysis of the PGA isozymogens was performed by densitometric scanning of the polaroid negatives with the LKB 2202 Ultrascan laser densitometer (LKB, Bromma, Sweden).¹¹ The area under the curve of each PGA isozymogen was calculated with the LKB 2190-001 gelscan program and expressed as a percentage of the total area under the curve, as described previously.¹¹

The serum PGA concentrations were measured by enzyme linked immunosorbent assay (ELISA), as described previously.¹⁹

Results

To visualise the PGA isozymogens in gastric mucosa with activity staining 5 µl supernatant of a sonified biopsy specimen was required (fig 1, lane 2). The PGA isozymogen pattern of the same biopsy specimen could be visualised in the immunoblot, but required only 0.1 µl supernatant (fig 1, lane 1). To obtain adequate

Figure 1 Pepsinogen isozymogen patterns from gastric mucosa and urine obtained by immunoblot (lanes 1 and 3, respectively) and activity staining (lanes 2 and 4, respectively).

Figure 2 Relative intensities of the pepsinogen isozymogens in gastric mucosa ($n = 5$; open symbols) and urine ($n = 6$; closed symbols), obtained by immunoblot (Y-axis) and activity staining (X-axis).

visualisation of urinary PGA patterns 50 μ l urine was used (fig 1, lane 4). With the immunoblot technique a PGA isozymogen pattern of the same urine sample required only 1 μ l (fig 1, lane 3).

To investigate whether the isozymogens patterns were identical with both methods, the relative intensities of the isozymogens after activity staining and immunoblotting of gastric biopsy specimens and urine samples were compared. An excellent correlation was obtained in gastric and urine samples (fig 2).

PGA isozymogen patterns could be obtained with 25 μ l of serum with a PGA content of 50 μ g/l. Applying larger amounts of serum led to distortion of the patterns. Using smaller aliquots, down to 6 μ l of the same serum sample, visualisation of PGA patterns was still possible, and relative intensities after laser densitometry remained unchanged (table). This dilution series showed linearity of the method when amounts of PGA between 0.3 and 1.25 ng PGA are applied (table). From the relative intensities of the isozymogens in these

Relative intensities (%) of individual isozymogens obtained by densitometric scanning of immunoblots using different amounts of serum sample with PGA concentration of 50 μ g/l

Serum sample (μ l)	6	8	10	15	20	25
PGA applied (ng)	0.3	0.4	0.5	0.75	1.0	1.25
Relative intensities %						
Pg5	32	33	30	31	32	33
Pg4	13	16	13	15	14	12
Pg3	42	39	43	40	42	38
Pg2	13	12	14	13	12	17

patterns and the amount of PGA applied, as little as 0.04 ng of a PGA isozymogen can still be detected.

To assess the coefficient of variation (CV) of this technique, 19 different PGA immunoblots from 18 μ l of this sample were scanned (12 different gels). For the most intense bands (Pg3 and Pg5) the CV was 2.6 and 4.2%, respectively. For bands with a low intensity (Pg2 and Pg4) a CV of 7.6% and 8.0%, respectively, was found.

Serum PGA patterns could be visualised with the immunoblot technique in 18 μ l of native serum of more than 100 subjects, with PGA concentrations ranging from 25 to 110 μ g/l. In four subjects serum PGA patterns were compared with PGA patterns of the gastric mucosa obtained with activity staining. No obvious differences in PGA isozymogen patterns were found (fig 3).

Discussion

Visualisation of PGA isozymogens after PAGE used to be achieved by showing proteolytic activity after conversion of the pepsinogen isozymogens into pepsin by acidification. This technique requires the presence of a considerable amount of PGA, and serum concentrations of PGA are too low to be visualised in native serum by activity staining, and the use of larger volumes of serum is prohibited by the presence of large amounts of albumin because, due to its strong negative charge, albumin migrates along with the pepsinogens. Separation of the pepsinogens from the serum albumin fraction is time consuming and requires large amounts of serum. Moreover, the value of the relative intensities of the PGA isozymogens in the activity staining after such procedures remains questionable.

In this study we have shown that PGA isozymogen patterns can also be obtained with an immunoblot technique from sonified gastric mucosal biopsy and urine specimens. Usually, proteins are blotted on to nitrocellulose sheets, but we favour the use of PVDF membranes for this purpose as in earlier experiments only weak PGA patterns from gastric mucosal biopsy specimens were obtained with nitrocellulose membranes. This was most probably due to a lack of binding of the pepsinogens to these membranes, as the presence of PGA could also be shown on two other nitrocellulose membranes placed at the anodal side of the first one. In similar experiments with PVDF membranes, pepsinogens could only be shown on the first membrane, even after prolonged blotting.

Both PGA and PGC isozymogens can be shown in the gels with activity staining. Therefore, to compare activity staining and

Figure 3 Serum PGA patterns (immunoblot) in four subjects (lanes 1 to 4) and their corresponding gastric mucosal PGA patterns obtained with activation staining (lanes 5 to 8) (A). Densitometric scans of the PGA patterns are shown (B) as well as the calculated relative intensities of the PGA isozymogens (C).

immunoblotting of gastric mucosal biopsy and urine specimens, incubation with antibodies against PGA and antibodies against PGC was carried out. Both PGA and PGC isozymogens were shown in gastric mucosa. In urine PGC isozymogens are almost absent and were therefore neither visualised by activity staining nor by immunoblotting (fig 1).

Peptic enzyme activity does not necessarily reflect the amount of protein present in the gel. Relative intensities of the isozymogen bands in the blot were similar to those obtained by the proteolytic activity staining, suggesting that the differences in intensity in the activity stained gels did, indeed, reflect a difference in the amount of isozymogen and not merely a difference in proteolytic activity between the PGA isozymogens.

Laser densitometric scanning of pepsinogen isozymogens after activity staining of gels has been described previously, and the linearity of the method was shown in a dilution series.¹¹ In our study densitometric scanning was applied to immunoblotting. Dilution of a serum sample did not affect the relative intensities of the PGA isozymogens in the immunoblot (table). This illustrates the reliability of densitometric scanning in immunoblotting when used within a range corresponding to serum PGA concentrations from about 12 µg/l to 210 µg/l. We were able to visualise PGA isozymogen patterns over the entire physiological range (45.8 SD 17.3 µg/l),²⁰ using 18 µl of native serum. Furthermore, our data show that serum samples can be diluted without affecting the PGA isozymogen pattern, even if higher serum concentrations are present.

Phenotyping of the PGA patterns relies on differences in intensity of the bands. The high correlation of the relative intensities obtained with both methods means that the results of immunoblotting can be compared with those previously reported with activity staining.

PGA isozymogen patterns have attracted the attention of clinicians for several reasons. The familial occurrence of peptic ulcers in which pepsin is a factor, led to the investigation of the genetics of the PGA isozymogens.^{21,22} For obvious reasons, gastric mucosal biopsy specimens cannot be obtained on a large scale for genetic studies. Urinary patterns are not adequate for this purpose as they do not correspond very well with the gastric patterns. Our results suggest that PGA patterns in serum are identical with those in gastric mucosa. Therefore, the immunoblot technique for visualisation of the serum PGA patterns might be important, as it may provide a new tool with which to investigate the genetics of pepsinogens in man. Moreover, this technique is readily applicable for large population studies and might, therefore, contribute to the detection of subjects at risk of malignant gastric disease.

A preferential tubular reabsorption of Pg5 isozymogen from the glomerular filtrate has been shown.^{12,23} Due to the absence of immunological heterogeneity, no specific antibody for each PGA isozymogen is currently available to develop a specific immunoassay for each individual isozymogen.²⁴ Moreover, the close similarity in amino acid sequence of the PGA

isozymogens makes it unlikely that a specific monoclonal antibody for each individual isozymogen could be obtained.²⁵ The relative intensities of the individual isozymogens in the serum PGA patterns obtained by densitometric scanning, however, will enable us to calculate the serum concentrations of the individual isozymogens. Thus, in addition to genetic and gastroenterologic research, determination of PGA isozymogen patterns in serum by immunoblotting might be of major importance for the investigation of differences in renal tubular reabsorption of the PGA isozymogens in man.

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