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Detection of Blotted Antigen Using a Fusion Protein between Protein A and Pepsinogen C

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Human pepsinogen (PG) A and C were fused with protein A and expressed in *Escherichia coli*. Although the fusion proteins (PA-PGA and PA-PGC) were not expressed at high levels and were almost totally recovered from the insoluble fraction, the renaturation and purification procedures were easy and simple. PA-PGA and PA-PGC possessed proteolytic activity equivalent to the gastric mucosal PGA and PGC, respectively. However, the activity of PA-PGC was about 3-fold higher than that of PA-PGA. Therefore, PA-PGC was applied to the subsequent immunoblotting studies. The proteolytic activity of PA-PGC was used for digesting the blocking reagent around the target antigen (*in situ* digestion method) or casein-clotting in the agarose plate containing skimmed milk (caseogram print method). Although the sensitivity of these methods was lower than that of the conventional color detection, the caseogram print method was superior in that the reaction was linear over a wide range. On the other hand, the *in situ* digestion method possessed a unique property on Western blotting, and it was very easy to identify the relative position of the target, which could be recognized as a clear band. For PA-PGC detection, no special chemicals are required, and so the procedure is simple, rapid, and inexpensive.

Key words: immunoblotting, pepsinogen A, pepsinogen C, protein A, recombinant protein.

Pepsinogens (PGs) are aspartic proteases which are synthesized as zymogens in the gastric mucosa. They are activated to pepsins in acidic conditions. On biochemical and immunochemical grounds, human PGs are divided into two groups, PGA and PGC (*1, 2*). PGs are detected not only in the gastric mucosa but also in serum and urine, and the concentration of PGs in serum or urine has been used as a marker for several disease conditions. For example, low serum PGA concentrations are found in patients with atrophic gastritis (*3-5*) or gastric cancer (*6-8*). The serum PGA/PGC index is, therefore, a potentially useful parameter for screening of the high risk group to gastric cancer (*9, 10*). PGs are, of course, of biochemical interest as aspartic proteases. Their structure, function, and molecular evolution have been studied by many researchers.

The proteolytic activity of PGs is usually examined with hemoglobin (*11*) or casein (*12*) as the substrate. We have been analyzing them electrophoretically using a casein-clotting system (*13, 14*) called "caseogram print" (*15*). Here, we describe a unique application of their proteolytic activity for the detection of blotted antigen. Immunoblotting is a basic and important technique in biological and biochemical studies. The blotted antigen is detected using a labeled antibody or antibody-specific ligand such as protein A. For the labeling reagents, enzymes (peroxidase, alkaline phosphatase, β-galactosidase, *etc.*), gold particles, radioisotopes, or fluorochromes are usually used, and many sensitive detection systems have been developed. However, the application of a protease as the labeling enzyme has not been reported. Since the activation of PGs is easily controllable by use of HCl, and the activated PGs (pepsins) possess strong proteolytic activity, they are potentially useful as labeling enzymes. Some aspartic proteases including PGs have been cloned in *Escherichia coli* and expressed as the enzymatically active form (*16-19*). Furthermore, PGs expressed as the fused products also maintain their proteolytic activity (*20, 21*). If the protein A-PG fusion protein (PA-PG) possess this proteolytic ability, it will be a useful tool for immunoblotting.

We previously reported on the construction of a fusion protein between protein A and green fluorescent protein (GFP) and its application to immunoblotting (*22*). The point of that study was the metabolic labeling of protein A and the simple and rapid detection of the blotted protein. In this study, we used PGs instead of GFP and constructed two unique detection systems, the "*in situ* digestion method" and the "caseogram print method."

MATERIALS AND METHODS

Plasmid Construction—The cDNA for human PGA was amplified and modified by standard PCR using two oligonucleotide primers (5'-TTTGAATTCATCATGTACAAGGT-

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Abbreviations: PGA (C), pepsinogen A (C); PGA5, PGA isozymogen 5; PA-PGA (C), protein A-PGA (C) fusion protein; t-PGA (C), PGA (C) purified from human gastric tissue; GFP, green fluorescent protein; IPTG, isopropyl-β-D-thiogalactopyranoside; HRP, horseradish peroxidase; DAB, 3,3'-diaminobenzidine tetrahydrochloride.

CCCCCTC-3′ and 5′-CCCAAGCTTTCATTAAGCCACGG-GGGCCAG-3′, *Eco*RI and *Hin*dIII sites are underlined). One nanogram of human gastric tissue cDNAs (QUICK-Clone, Clontech Laboratories) was used as the template. The amplified PGA cDNA was digested with *Eco*RI and *Hin*dIII, then cloned into the *E. coli* expression vector pKK223-3 (Pharmacia Biotech). The resulting plasmid was designated as pKKPGA. On the other hand, the PGC cDNA was amplified using 5′-TTTGAATTCATCATGGCAGTG-GTCAAAGTG-3′ and 5′-CCCAAGCTTTTACTAGGCGG-CAGTGGCAAAGCC-3′ as primers, and cloned into pKK-223-3. The resulting plasmid was designated as pKKPGC. The DNA fragment encoding protein A was prepared from pPAGFP127 (*22*) by PCR using 5′-GGGGAATTCATGGA-ACAACGCATAACCCTG-3′ and 5′-TTTGAATTCCCCC-GGATCGTCTTTAAGGCT-3′ as primers. The fragment was then cloned into the *Eco*RI site of pKKPGA and pKKPGC, and the resulting plasmids were designated as pPAPGA and pPAPGC, respectively. Enzymes were purchased from Gibco BRL, Life Technologies, or Nippon Gene, and all reactions were performed as specified by the manufacturers. Plasmids were transformed into *E. coli* JM109 with $CaCl_2$, and transformants were selected on LB agar plates containing 50 μg ampicillin/ml. Plasmids were purified with a FlexiPrep kit (Pharmacia Biotech) or by the boiling method as described by Holmes and Quigley (*23*) with slight modifications. The DNA was sequenced using a fluorescence imaging analyzer FMBIO-100 (Takara Shuzo).

Expression and Purification of PA-PGA and PA-PGC—The strain harboring pPAPGA was grown in 100 ml of LB broth. Ten hours after adding 0.3 mM (final concentration) isopropyl-β-D-thiogalactopyranoside (IPTG; Wako Pure Chemical Industries), the cells were harvested by centrifugation. They were then suspended in 10 ml of 10 mM Na-phosphate buffer (pH 7.0), disrupted by sonic oscillation, and centrifuged at 15,000 × *g* for 20 min. The precipitate was dissolved in 0.8 ml of solution A: 50 mM KH_2PO_4, 50 mM NaCl, and 1 mM EDTA (pH 10.7) containing 8 M urea. After centrifugation, the supernatant was slowly diluted to 8.0 ml with solution A without 8 M urea. This solution was adjusted to pH 8.0 with 0.1 M HCl, dialyzed against 10 mM Na-phosphate buffer (pH 7.0), then applied to a HiTrap Q column (1.6 × 2.5 cm, Pharmacia Biotech). The bound materials were eluted with a stepwise gradient of NaCl (0.2, 0.4, and 0.6 M). The fraction containing the PA-PGA fusion protein was dialyzed against 10 mM Na-phosphate buffer (pH 7.0), then applied to a Mono Q HR 5/5 column (Pharmacia Biotech). PA-PGA was eluted with a linear gradient of NaCl from 0.2 to 0.5 M. The purified PA-PGA was diluted with the same volume of glycerine and stored at −20°C. PA-PGC was purified from the strain harboring pPAPGC using the same conditions as described above.

PGA and PGC in human gastric mucosa (designated as t-PGA and t-PGC) were purified from 10 g of tissue as described previously (*21*).

Proteolytic Activity and Protein Concentration—The proteolytic activity of chromatographic fractions was monitored using 1% agarose gel plates prepared with 0.3 M Na-acetate buffer (pH 5.3) containing 1% skimmed milk (Snow Brand Milk Products). Briefly, 10 μl of each fraction was mixed with same volume of 0.1 M HCl, and following a 20-min incubation at 37°C, 1 μl of the mixture was spotted on the milk-plate. After incubation for 30-60 min at room temperature, a white spot caused by casein-clotting was detected. PGs activity was measured exactly by hemoglobin digestion as described by Kageyama and Takahashi (*11*). The protein concentration was measured with a protein assay kit (Bio-Rad Laboratories) or calculated from the absorbance at 260 and 280 nm.

Antigen and Antibody—Green fluorescent protein (GFP) was used as a model antigen. The cDNA for *Aequorea* GFP (*22*) was cloned into pKK223-3, and to improve the fluorescent activity at high temperatures, Ser147 was changed to Pro (*24*). The resulting plasmid, pGFP147P, was maintained in *E. coli* JM109. Expression and purification of GFP were performed by the standard procedure, and the polyclonal antibody against GFP was raised in young male rabbits.

Electrophoresis and Immunoblotting—SDS-PAGE was performed according to Laemmli (*25*) using a broad range molecular mass standards kit (Bio-Rad Laboratories). The proteins were separated on 0.1% SDS-12% polyacrylamide gels (10 × 8 × 0.15 cm). The *E. coli* strains harboring pGFP147P or pKK223-3 (GFP-negative control) were grown in 10 ml of LB broth until the mid-log phase. After adding 0.3 mM IPTG (final concentration) and a further incubation for 10 h, the cells were harvested. Cells were suspended in 0.5 ml of 10 mM Na-phosphate buffer (pH 7.0) and disrupted by sonic oscillation. Cell extracts (10 μl) were loaded on the gels. The separated proteins were stained with 0.25% Coomassie Brilliant Blue (CBB) R-250 or electroblotted onto polyvinylidene difluoride (PVDF) membrane (Millipore). Electroblotting was carried out at a constant voltage of 20 V for 90 min. On the other hand, dot blotting of purified GFP was performed using the Easy-Titer ELIFA system (Pierce Chemical). The target antigen (GFP) was visualized by the following methods (I, II, and III) and recorded with the gel imaging system "Archiver Eclipse" (Fotodyne). The video images were digitized and the signal intensities were quantified using the densitometry software "FM analysis" (Takara Shuzo).

(I) In situ digestion method: The membrane was blocked in 10 mM Na-phosphate buffer (pH 7.0) containing 2.5% hemoglobin for 30 min before being incubated with the rabbit antiserum against GFP (diluted 1:200-500 with TBS, 50 mM Tris-HCl, 200 mM NaCl, pH 7.4) for 30 min at room temperature, and then with TBS containing 1 μg/ml PA-PGC and 5% bovine serum albumin (BSA) for 30 min. After washing with TBS, the membrane was put on filter paper containing 0.1 M HCl (pH 2.5; adjusted with NaOH). Activation of PA-PGC on the membrane and the digestion of hemoglobin were performed at 37°C for 1 h. The membrane was stained with 0.2% amido black 10B (Wako Pure Chemical Industries) for 2 min and destained with 7% acetic acid containing 10% acetonitrile and 10% Triton X-100.

(II) Caseogram print method: PA-PGC was bound to the antibody under the same conditions described above. It was then activated on the filter paper containing 0.1 M HCl (pH 2.5) for 20 min at 37°C. The membrane was transferred onto filter paper containing 0.3 M Na-acetate buffer (pH 5.3) for 30 s, then onto a 1% agarose gel plate (1.5 mm thick) prepared with the same buffer containing 1% skimmed milk. After incubation for 1 h at room temperature, a white band or spot caused by casein-clotting could be detected.

(III) Conventional color detection method: The membrane was blocked with 5% BSA for 30 min and incubated with antiserum as described above. The membrane was then incubated in TBS containing 2.7 μg/ml second antibody [horseradish peroxidase (HRP)-labeled anti-rabbit IgG] and 5% BSA for 30 min. The visualization of HRP was performed with TBS containing 0.02% 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 0.01% H_2O_2 for 10 min.

RESULTS

Purification and Characterization of PA-PGA and PA-PGC—Two fusion proteins, PA-PGA and PA-PGC, were constructed and expressed in *E. coli* (Fig. 1). From the DNA sequencing, the cloned PGA cDNA was shown to be PGA5 (*26*), and the sequence of PGC cDNA was also confirmed (*27*). Although no proteolytic activity was detected from the cell extracts prepared from the strains harboring pPAPGA or pPAPGC, the expression of these fusion proteins was stable, and they were detected as relatively major bands in a CBB-stained gel (Fig. 1B). They were almost totally expressed as insoluble proteins, but the proteolytic activity was easily regenerated by a simple renaturation procedure. The renatured PA-PGA was purified with HiTrap Q and Mono Q columns (Fig. 2A). PA-PGA was obtained as the major peak, and its purity was confirmed by SDS-PAGE (Fig. 2A). After the two purification steps, 0.2 mg of PA-PGA was obtained from 100 ml of culture. Purification with an affinity column immobilizing rabbit IgG was also performed; however, the Mono Q column was superior in speed and purity. Proteolytic activities of t-PGA (human gastric tissue PGA) and PA-PGA were then compared. The pH dependence of each PGA was almost the same, with their optimal pH around 2.5 (Fig. 2B). The proteolytic activity of different protein concentrations was measured at the optimal pH (Fig. 2C). Their specific activities were as follows: t-PGA, 4.8 units/mg (3.9×10^{-16} units/molecule); and PA-PGA, 3.7 units/mg (4.3×10^{-16} units/molecule), indicating that the activity per molecule was almost the same.

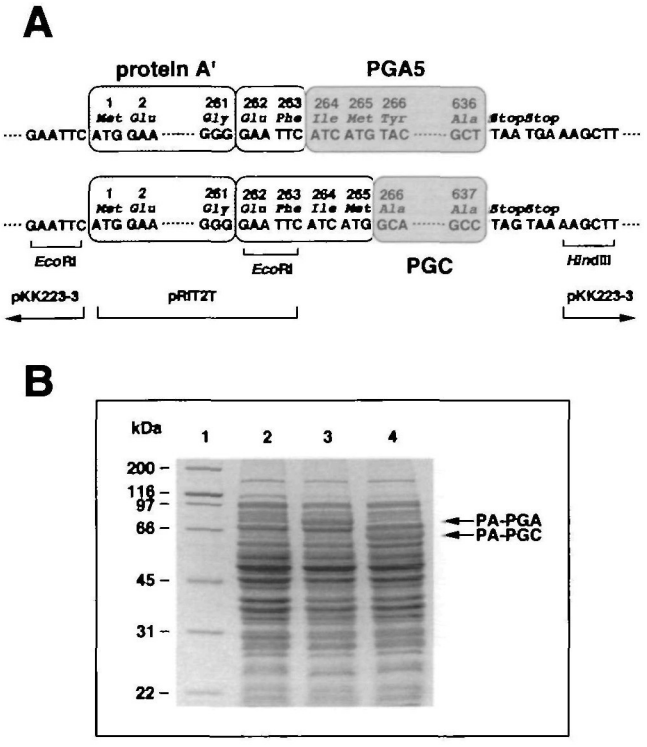

Fig. 1. **Construction of recombinant plasmids (A) and expression of PA-PGs (B).** (A) PGA or PGC cDNA was cloned into pKK223-3, then the DNA fragment encoding protein A was put into the *Eco*RI site. The resulting plasmids were designated as pPAPGA (upper plasmid) and pPAPGC (lower plasmid), respectively. (B) The *E. coli* strains harboring pPAPGA or pPAPGC were grown in 10 ml of LB broth until the mid-log phase. After adding 0.3 mM IPTG (final concentration) and further incubation for 10 h, the cells were harvested. Cells were suspended in 0.5 ml of 10 mM Na-phosphate buffer (pH 7.0) and disrupted by sonic oscillation. Cell extracts (10 μl) were loaded on a 0.1% SDS-12% polyacrylamide gel. The samples loaded are as follows: lane 2, pKK223-3 (control); 3, pPAPGA; 4, pPAPGC. Molecular mass markers were loaded onto lane 1. The products indicated by arrows are PA-PGA (lane 3), PA-PGC (lane 4).

PA-PGC was purified from the strain harboring pPAPGC using the same procedures, and 0.3 mg of purified PA-PGC was obtained from 100 ml of culture. The optimal pH of t-PGC and PA-PGC was also around 2.5. Proteolytic activity was measured at the optimal pH, and their specific activities were as follows: t-PGC, 16.2 units/mg (11.3×10^{-16} units/molecule); and PA-PGC, 11.5 units/mg (12.2×10^{-16} units/molecule). Thus, the specific activities for PGCs (t-PGC and PA-PGC) were also almost the same. However, the proteolytic activity of PA-PGC was about 3-fold higher than those of PA-PGA, and so PA-PGC was used for the subsequent immunoblotting experiments.

Application of PA-PGC to Immunoblotting—The target antigen was detected by the *in situ* digestion or caseogram print method using PA-PGC (Fig. 3). The former uses the proteolytic activity of PA-PGC for digesting the blocking reagent (hemoglobin) around the target, while the latter is based on the casein-clotting occurring in an agarose plate containing skimmed milk.

In the dot blotting, the sensitivity and linear reaction of these methods were compared with those of the conventional HRP/DAB color detection (Fig. 4, A and B). Although the HRP/DAB detection was more sensitive, the caseogram print method was superior to others in that the reaction was linear over a wide range of antigen concentrations. In the caseogram print method, the spots of high antigen concentrations diffused considerably, but the reaction response was very good (Fig. 4B). In the *in situ* digestion method, the diffusion of spots and an increase of sensitivity were observed when the digestion and destaining were extended (data not shown), but the quantitative analysis was practicable within a more limited range.

However, the *in situ* digestion method possessed a unique property for Western blotting (Fig. 5B). Since the blotted proteins, including the molecular mass marker proteins, were all stained and clearly visualized, it was very easy to identify the relative position of the target, which was recognized as a clear band. In case of relatively large amounts, the digestion of the target was incomplete. The target itself was, therefore, considerably stained and the area around it was cleared. For the blotting analysis, this property is very useful in many cases. Stained protein bands and the clear band of target were emphasized on the dry and wet membrane, respectively (data not shown). The same sample was then detected by the caseogram print

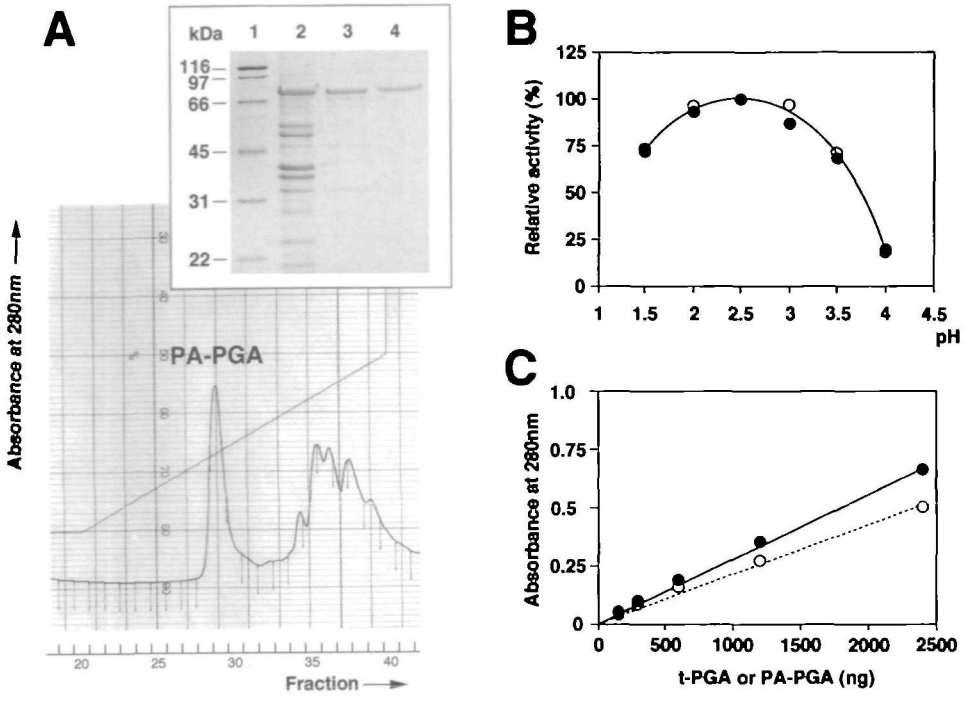

Fig. 2. **Purification of PA-PGA (A) and its proteolytic activity (B and C).** (A) The insoluble fraction obtained from the cell extract of the pPAPGA-harboring strain was solubilized with 8 M urea. After renaturation, the solution was applied to a HiTrap Q column. The fraction containing PA-PGA was then applied to a Mono Q column. SDS-PAGE lanes are as follows: 1, markers; 2, the insoluble fraction after the renaturation; 3, PA-PGA after HiTrap Q; 4, PA-PGA after Mono Q. (B) PGAs (1.5 μg of PA-PGA, ○; t-PGA, ●) were mixed with 1 ml of 0.1 M HCl (pH 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0; adjusted with NaOH) containing 2% hemoglobin and incubated for 60 min at 37°C, then 2 ml of 5% trichloroacetic acid was added. After 30 min at room temperature, the mixture was centrifuged and the absorbance of the supernatant at 280 nm was measured. The highest activity obtained from each PGA sample was taken as 100%. (C) The specific activity of PGAs was determined at pH 2.5. PGAs (150, 300, 600, 1,200, and 2,400 ng of PA-PGA, ○ or t-PGA, ●) were examined as described above. One unit of the enzyme was defined as the amount which caused an absorbance change of 1.0 per minute.

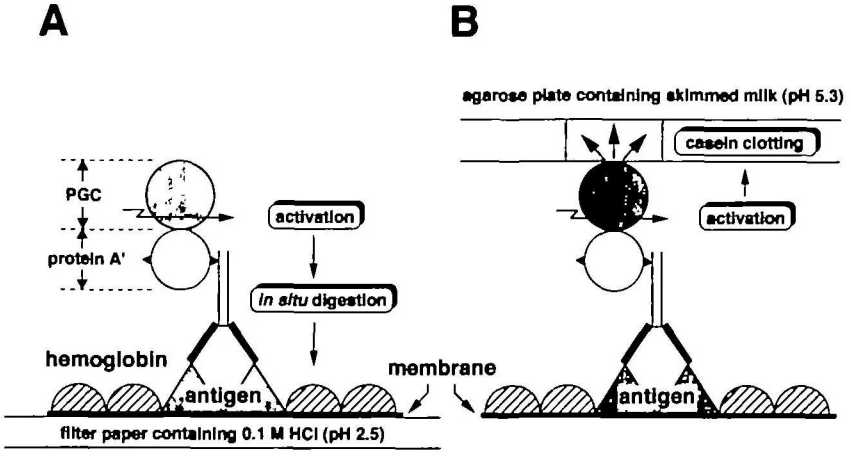

Fig. 3. **Principle of *in situ* digestion method (A) and caseogram print method (B).** PA-PGC bound to the antibody is activated by 0.1 M HCl, and the resulting pepsin C digests the blocking reagent around the target antigen (A) or causes casein-clotting in an agarose plate containing skimmed milk (B).

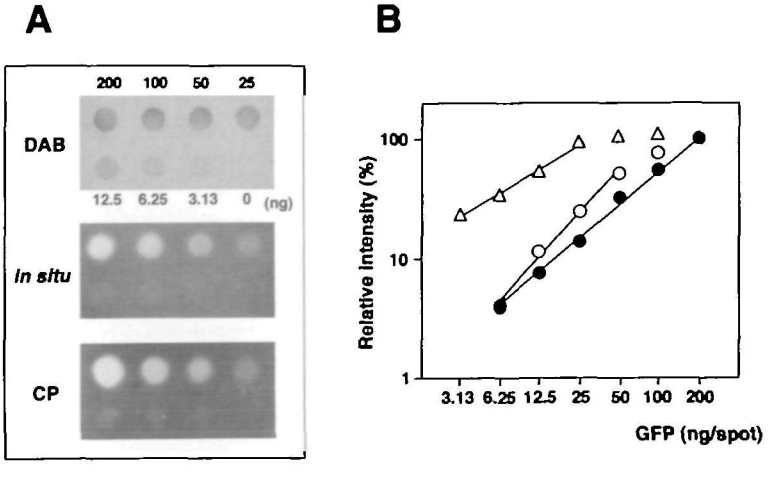

Fig. 4. **Application of PA-PGC detection to dot blotting.** (A) The antigen (GFP) was blotted on a PVDF membrane as a 4-mm spot. The spots were detected by HRP/DAB detection (indicated as DAB), *in situ* digestion (*in situ*), or caseogram print (CP). (B) Spot intensity was digitized and quantified (see text for details). The intensity of 200 ng was taken as 100% in each membrane (△, DAB; ○, *in situ*; ●, CP). Data are presented as the mean of three experiments.

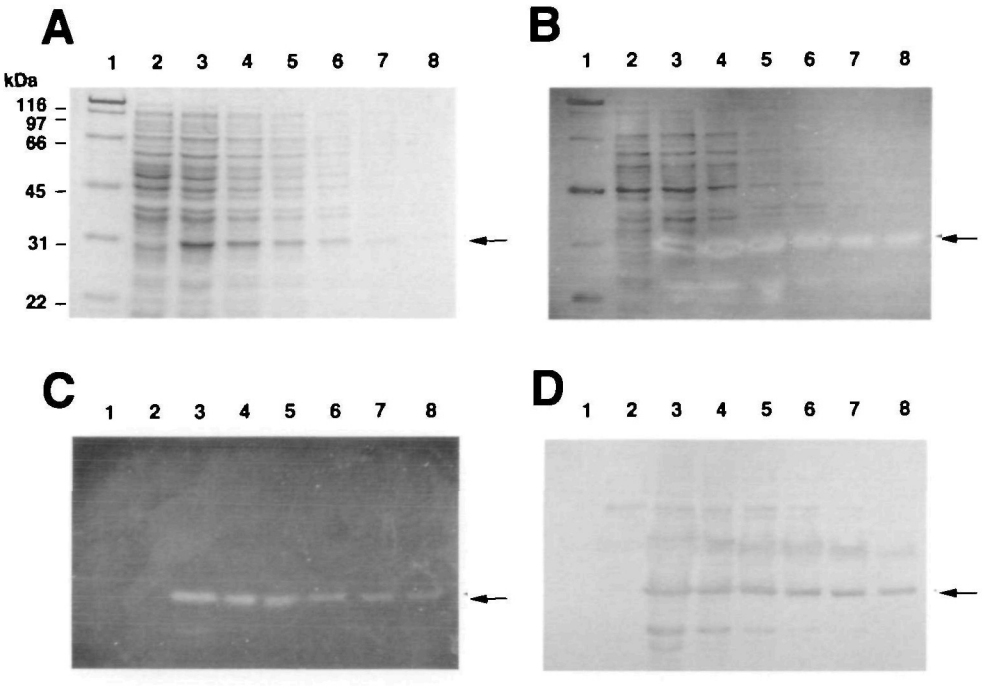

Fig. 5. **Application of PA-PGC detection to Western blotting.** The cell extracts were prepared from the *E. coli* strains harboring pGFP147P or pKK223-3 by the procedure described in the text. Ten microliters of cell extract was loaded on four polyacrylamide gels (lane 3). The sample was diluted ×2, 4, 8, 16, and 32 and loaded onto lanes 4, 5, 6, 7, and 8, respectively. Molecular mass markers and the GFP-negative control (cell extract prepared from the strain harboring pKK223-3) were loaded onto lanes 1 and 2, respectively. The separated proteins were stained with CBB (A) or electroblotted onto PVDF membrane (B-D), and the GFP bands (indicated by arrows) were detected by *in situ* digestion (B), caseogram print (C), or HRP/DAB (D).

method (Fig. 5C) and the HRP/DAB system (Fig. 5D). Although the sensitivity of the two new methods was somewhat lower than that of the HRP/DAB detection, a considerably small amount of antigen was detectable by these methods.

DISCUSSION

In our previous paper, we reported on the construction of fusion proteins between human PGs and maltose-binding protein in *E. coli* (*21*). These fusion proteins were activated as the intact pepsins by acidification and exhibited proteolytic activity. Their specific activities were almost the same as those of native PGs purified from gastric tissues. Tanaka and Yada reported on the expression of a thioredoxin-porcine PGA fusion protein in *E. coli* (*20*) and its activation mechanism (*28*). PA-PGA and PA-PGC constructed in this study were also activated by acidification and possessed almost equivalent specific activity to those of native PGs. These findings suggesting that it is possible to fuse PGs with many other useful and functional proteins without compromising their proteolytic activity.

We applied PA-PGC to immunoblotting as a labeled ligand. This was based on the following facts: (i) since PG is rapidly activated to pepsin at acidic condition (< pH 4), its proteolytic activity is easily controlled. This property is suitable for the labeling enzyme in the immunoblotting system. (ii) The proteolytic activity of PG is strong, and its detection is relatively easy and simple. (iii) We have already reported on the construction of a PA-GFP fusion protein and its application to immunoblotting. Therefore, we thought that the application of PA-PG to blotting was possible.

The detection of PA-PGC bound to antibody was performed by the two different methods, *in situ* digestion and caseogram print. In electrophoretic analysis of PG, its proteolytic activity in the gel has been detected by the hemoglobin digestion (*1*) or casein-clotting (*13-15*). We used these techniques with some modifications for the detection of PA-PGC on the membranes. The *in situ* digestion method based on hemoglobin digestion possessed a unique property. The point of this method is the visualization of all blotted proteins as well as the detection of the target antigen. This property results in the easy analysis of complex patterns obtained from one- or two-dimensional gels. The blotted proteins are clearly visualized in spite of the blocking protein (hemoglobin), which is also stained. We speculate that the blocking protein may bind weakly to the membrane and much of it (but not all) comes off with the destaining reagent. However, the electroblotted proteins are tightly bound to the membrane, and so they are held on the membrane. On the other hand, the caseogram print method based on casein-clotting was useful for the quantitative analysis. The point of this method is that the proteolytic reaction progresses on the milk-containing agarose plate (not on the membrane). Although the bands or spots were considerably diffuse, the reaction progresses slowly and the reaction response may increase.

The proteolytic activity of PA-PGC is clearly higher than PA-PGA in solution. Therefore, in this study we used PA-PGC for immunoblotting. However, the PA-PGA and PA-PGC detections performed under the conditions described here were almost equal in sensitivity (data not shown). In conclusion, although the detection procedure will need to be examined more closely, it is clear that PA-PGC (and PA-PGA) can be used in blotting studies. The useful properties of PA-PG detection are that (i) no special chemicals for detection, including a labeled second antibody, are required, and therefore the procedure is simple, rapid, and inexpensive; (ii) the reaction is controlled easily by acidification using HCl; and (iii) the *in situ* digestion and caseogram print methods are suitable for the recognition and quantification of a target, respectively. When a sample possesses high endogenous activity of the standard labeling

enzymes (peroxidase, alkaline phosphatase, *etc.*), the PA-PGC detection may be a good option.

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