A Milk Clotting Assay for Antibody Using a Fusion Protein between Protein A and Pepsinogen C

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The proteolytic activity of a fusion protein between protein A and human pepsinogen C (PA-PGC) was measured by a modified milk clotting assay on 96-well microtiter plate. The assay ranges for 30 and 120 min-incubation were approximately 0.08–1.25 ng and 0.02–0.16 ng, respectively. Although the absorbance of milk solution decreased by the precipitation of clotted casein, the white precipitate was, if anything, convenient for an easy detection of reaction-positive wells. The borderline between the wells with or without precipitate was very clear and easily detected without the plate reader. This feature was thought to be suitable for the positive-negative judgment of a dilution test in enzyme immunoassay (EIA). Rabbit IgG adsorbed on a microtiter plate was then measured using PA-PGC and this milk assay (designated as PA-PGC assay). Although PA-PGC assay needed slightly longer incubation than the conventional color assay, the sensitivity of both systems was almost identical, and the reaction-positive wells containing white precipitate were easily detected at a very low range by the over night incubation without using the plate reader. PA-PGC assay possesses the unique and useful properties, and it can be used as a convenient and easy EIA technique.

Key words protein A; pepsinogen C; fusion protein; milk clotting assay; enzyme immunoassay

In a previous paper we reported the construction of a fusion protein between protein A (PA) and human pepsinogen C (PGC) and its application to immunoblotting.¹⁾ The objectives of the study were the metabolic labeling of PA and the simple and unique detection of the blotted antigen using the proteolytic activity of PGC. Here, we describe a convenient assay for antibody using PA-PGC fusion protein.

The purification of PA-PGC expressed in *Escherichia coli* is unsophisticated, and the specific activity of PA-PGC is approximately the same as that of human PGC.¹⁾ Since PGC is rapidly activated to pepsin C by the use of HCl, its proteolytic activity is easily controlled. This property is suitable for the labeling enzyme in the immunoassay system. In enzyme immunoassay (EIA), peroxidase, alkaline phosphatase, or β -galactosidase are usually used as the labeling enzyme, and many sensitive detection systems have been developed. However, the application of protease as the labeling enzyme has not been reported. We designed the assay system using PA-PGC with the following concepts; (i) the reaction is performed on a 96-well microtiter plate and measured with a conventionally used microtiter plate reader, (ii) special and expensive reagents or equipment are not required, (iii) the assay procedure is simple, and still the sensitivity is relatively high and sufficient for easy screening.

The most important point is the choice of assay method for pepsin C, which is derived from PA-PGC. The proteolytic activity of pepsin is usually examined either by the hemoglobin digestion method,²⁾ milk clotting assay,³⁾ or by using a fluorescent dye-labeled substrate.^{4–6)} In this study the modified milk clotting assay was used, as it approximated our concept (low assay cost, high sensitivity and simple procedure), and we examined and compared two different assay procedures in which the absorbance change caused by casein clotting was measured using a plate reader or the white precipitate of completely clotted casein was detected through visualization.

MATERIALS AND METHODS

Preparation of PA-PGC PA-PGC was purified from the strain harboring pPAPGC as described previously.¹⁾ The purified PA-PGC was diluted with the same volume of glycerine and stored at -20°C . The protein concentration was calculated by the absorbance at 260 and 280 nm.

Milk Clotting Assay Protease activity of PA-PGC was measured by the milk clotting assay described by McPhie³⁾ with modifications. All reactions proceeded at room temperature (20 – 23°C) on a 96-well microtiter plate (Becton Dickinson Co., Lincoln Park, NJ, U.S.A.). A series of PA-PGC samples (0.01 – $10\text{ ng}/10\text{ }\mu\text{l}$), diluted with TBS; 50 mM Tris-HCl, 200 mM NaCl, pH 7.4, containing 1% bovine serum albumin (BSA), were mixed with $50\text{ }\mu\text{l}$ of 0.1 M HCl-KCl buffer (pH 2.0) and incubated for 30 min at room temperature. During incubation 0.3 M Na-acetate buffer (pH 5.3) containing 1% skimmed milk (Snow Brand Milk Products Co., Tokyo, Japan) was prepared, and $50\text{ }\mu\text{l}$ was added and incubated at room temperature (use freshly prepared milk solution, as old solution causes an acute background). After the incubation for 30, 60, 120 min and 18 h, the absorbance at 492 and 600 nm was measured with a microtiter plate reader MPR-A4 (Tosoh Corp., Tokyo, Japan). In all samples, Abs.492/Abs.600 was approximately 1.65 and Abs.492 was about 2.5-fold higher than the difference of both, [Abs.492–Abs.600]. However, the reaction linearity obtained from [Abs.492–Abs.600] was superior to that obtained from Abs.492. The result was, therefore, indicated with [Abs.492–Abs.600]. When the clotted precipitate was observed without measuring the absorbance change, the clotting reaction proceeded at 37°C . After the incubation for 3, 6 and 18 h, the plate image was recorded with a gel imaging system Archiver Eclipse (Fotodyne Inc., Hartland, WI, U.S.A.).

Assay of Antibody Adsorbed on Microtiter Plate Rabbit IgG adsorbed on the microtiter plate was assayed or visualized as follows.

(a) Milk Clotting Assay Using PA-PGC (PA-PGC Assay):

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Fig. 1. Milk Clotting Assay for PA-PGC

(A) A series of PA-PGC samples (0.01–1.25 ng/10 μ l) were activated on a 96-well microtiter plate, and the clotting reaction was performed at room temperature. After the incubation for 30 min (○), 60 min (●), 120 min (△) and 18 h (▲), the absorbance of each well was measured (see text for details). To facilitate the arrangement of the data, the results are shown as two graphs: upper panel, 0.01–0.16 ng of PA-PGC; lower panel, 0.08–1.25 ng of PA-PGC. Data are presented as the mean of the two experiments. (B) A series of PA-PGC samples (0.01–10 ng/10 μ l) were activated by the same procedure. However, after adding the milk solution, the plate was incubated at 37 °C. After incubation for 3, 6 and 18 h, the plate image was recorded by a gel imaging system. The reaction-positive wells are indicated by arrows.

A series of rabbit IgG solutions (50 μ l of 0.0125–0.8 μ g/ml, diluted with TBS) was added to the wells and it was adsorbed over night at 4 °C. After washing the wells twice with TBS, they were post-coated with 200 μ l of TBS containing 5% BSA for 2 h at room temperature. The wells were washed again and 50 μ l of the PA-PGC working solution (1 μ g/ml PA-PGC and 1% BSA in TBS) was added and incubated for 1 h at room temperature. The wells were washed four times with TBS containing 0.05% Tween 20, and PA-PGC bound to the wells was activated with 10 μ l of TBS containing 1% BSA and 50 μ l of 0.1 M HCl-KCl buffer (pH 2.0). After incubation for 30 min at room temperature, the milk clotting reaction was performed as described.

(b) Conventional Color Assay: The color assay was performed using horseradish peroxidase (HRP) and *o*-phenylenediamine (*o*-PD). After the adsorption of rabbit IgG and post-coating with BSA, 50 μ l of the second antibody solution (1 μ g/ml HRP-labeled sheep antibody against rabbit IgG and 1% BSA in TBS) was added into the wells and incubated for 1 h at room temperature. The wells were washed with Tween-TBS, and HRP bound to the wells was visualized with 100 μ l of 0.1 M citrate buffer (pH 5.0) containing 0.2% *o*-PD and 0.0035% H₂O₂. After incubation for 1 h at room temperature, the absorbance at 492 and 600 nm was measured, and the result was indicated with [Abs.492–Abs.600].

RESULTS AND DISCUSSION

Optimization of Milk Clotting Assay To perform the milk clotting assay on a 96-well microtiter plate, it was scaled down under optimum conditions. Several parameters (reaction volume, time, temperature, and milk concentration) were examined and the best conditions for our purpose were

described in Materials and Methods. The assay range of PA-PGC was around 0.08–1.25 ng after incubation for 30 min (Fig. 1A), and the longer incubation assay range was 0.08 ng or under. The ranges for 2 h- and 18 h-incubation were approximately 0.02–0.16 ng and 0.01–0.08 ng, respectively (Fig. 1A). Although the data are not shown, it was also possible to assay 2.5 ng or above through a shorter incubation and the use of a lower concentration of milk. When a series of PA-PGC samples diluted with TBS which did not contain BSA were assayed, the sensitivity decreased considerably. This may indicate that BSA in the samples keeps pepsin C from the self-degradation in the activation step. Our assay conditions were suitable for the picogram-level samples, and the sensitivity is clearly higher compared to the standard method.³⁾ The absorbance increased with the clotting reaction, however it decreased by the precipitation of clotted casein (Fig. 1A 0.63 and 1.25 ng of 2 h-incubation). In the milk clotting assay, therefore, the clotting time taken for a certain absorbance change is used as the parameter. This is very complex work when many samples must be dealt with. Although the assay range was relatively limited, our conditions allowed us to assay the picogram-level samples only by measuring the absorbance at two or three appropriate intervals (e.g., 30, 60 or 120 min).

The white precipitate of clotted casein was, if anything, convenient for our purpose. The wells containing precipitate increased with time, including the 0.01 ng PA-PGC prepared precipitate after incubation for 18 h at 37 °C (Fig. 1B) or for 48 h at room temperature. Although the absorbance of control wells (no PA-PGC) increased slightly, precipitate was not observed at either temperature. When the clotting was performed at 37 °C, the clotted casein rapidly precipitated and it took precedence over the increase of absorbance. It was,

Fig. 2. Conventional Color Assay (A) and PA-PGC Assay (B and C) for Rabbit IgG Adsorbed on a 96-Well Microtiter Plate

(A) Purified rabbit IgG was diluted with TBS (0.0125–0.8 µg/ml) and 50 µl of the solution was added to the wells in duplicate. Rabbit IgG adsorbed on the plate was assayed by the HRP/*o*-PD system as described in the text. Data are presented as the mean of two wells. (B) Rabbit IgG was adsorbed by the same procedure, and the clotting reaction was performed at room temperature. After the incubation for 30 min (○), 60 min (●) and 120 min (△), the absorbance of each wells was measured. Data are presented as the mean of two wells. (C) The clotting reaction continued for 18 h at room temperature. After incubation for 3, 6 and 18 h, the plate image was recorded. The reaction-positive wells are indicated by arrows.

therefore, hard to assay using a microtiter plate reader. However, the borderline between the wells with or without precipitate was very clear and easily detected without the plate reader (Fig. 1B). This feature is suitable for the positive-negative judgment of a dilution test in EIA.

Comparison between Conventional Color Assay and PA-PGC Assay Rabbit IgG adsorbed on a microtiter plate was measured by color assay using the HRP/*o*-PD system and the milk clotting assay using PA-PGC. Although PA-PGC assay needed a slightly longer incubation than the HRP/*o*-PD system, the sensitivity of both assays was almost identical (Fig. 2A and B). Moreover, we assessed the positive wells at a lower range by long incubation at room tempera-

ture (Fig. 2C). Although the data are not shown, 0.025 µg/ml wells also turned positive by the 24 h-incubation. As shown in Fig. 2C, 0.05 µg/ml wells were detected as positive after 18-h incubation. The absorbance change of this point was detectable after 6-h incubation using the plate reader. However, the long incubation reduced assay linearity, and the assay range was limited. The visual detection of precipitate may be suitable for such a weak reaction.

The advantage of PA-PGC assay is the easy visualization of positive wells rather than measuring the exact value with the plate reader. To measure precisely the antigen or antibody in the sample, we have to use the plate reader. However, for a simple assay of antibody titer, the series of diluted samples are examined and the maximum dilution value with a positive signal is determined through visualization. In such cases the exact assay value is not necessary. The clear-cut precipitate produced by PA-PGC assay is suitable for analysis without using the plate reader. Since the baseline in PA-PGC assay does not increase much even after long incubation, it is possible to deal with many samples at the same time and analyze them at any time the following day.

The fluorescent dye-labeled substrate developed recently^{4–6)} may facilitate the speed and sensitivity of the assay, but it is costly to use on a regular basis and requires a fluorescent reader. In conclusion, although the conditions will need to be examined more closely, PA-PGC assay can be used as a convenient and easy EIA technique. The useful properties of PA-PGC assay are that; (i) no special chemicals, including a labeled second antibody, are required, and therefore, the procedure is simple and inexpensive; (ii) the reaction is easily controlled by acidification using HCl; (iii) although PA-PGC assay needs slightly longer incubation than the HRP/*o*-PD system, the sensitivity of both systems was almost identical; and (iv) the reaction-positive wells containing white precipitate are easily detected at a very low range by the over night incubation without using the plate reader.

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REFERENCES

- 1) Aoki T., Satoh M., Watabe H., *J. Biochem. (Tokyo)*, **124**, 572–577 (1998).
- 2) Aoki T., Tomaki E., Morikawa J., Tsubota N., Watabe H., *Biochem. Mol. Biol. Int.*, **39**, 461–469 (1996).
- 3) McPhie P., *Anal. Biochem.*, **73**, 258–261 (1976).
- 4) Bolger R., Checovich W., *Biotechniques*, **17**, 585–589 (1994).
- 5) Levine L. M., Michener M. L., Toth M. V., Holwerda B. C., *Anal. Biochem.*, **247**, 83–88 (1997).
- 6) Jones L. J., Upson R. H., Haugland R. P., Panchuk-Voloshina N., Zhou M., Haugland R. P., *Anal. Biochem.*, **251**, 144–152 (1997).