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Development of a Solid-Phase Colorimetric Assay for the Screening of Transglutaminase Activities†

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= Abstract =**A solid-phase colorimetric assay method for transglutaminase activities has been developed. The principle of the assay is to monitor cross-linking activities between casein bound to microtiter plates and free biotinylated casein by the sample. Quantitation of immobilized biotin-labeled casein formation through the enzymatic reaction was conducted by avidin or streptavidin conjugated enzymes. For this purpose, the efficiency of four different reporter enzymes (streptavidin or avidin conjugated alkaline phosphatase, streptavidin or avidin conjugated horseradish peroxidase) was compared, especially focusing on the sensitivity and specificity of the respective methods. The newly developed assay method was applied to the procedure of transglutaminase C purification from human erythrocytes and proven to have good correlation with conventional C_{14} putrescine method (r = 0.85, $p<0.05$). Moreover, since this new method can detect enzymatic activity without use of radioisotope and can process a number of samples simultaneously, it is possible to screen a mass population for transglutaminase deficiency, such as factor XIII, in routine clinical laboratories.**

Key Words; *Transglutaminases, Assay, Screening, Development*

INTRODUCTION

Transglutaminases are a group of enzymes that catalyze the calcium-dependent acyl transfer reactions in which γ-carboxamide groups of peptide-bound glutamine residues serve as acyl donors (Folk and Chung 1985; Folk 1980). Many primary amines function as substrates of acyl acceptors in a wide spectrum of cells and body fluids. Several assays to monitor the activity of transglutaminase have been reported (Lorand *et al.* 1972; Stenberg and Stenflo 1979; Velasco *et al.* 1988; Fesus and Arato 1986; Jeon *et al.* 1989; Seiving *et al.* 1991). Among them, filter paper assay using radiolabelled amines (Lorand *et al.* 1972) has been widely used. However, the assay involves exhausting and time-consuming procedures especially when dealing with large numbers of samples. And since the use of radioisotope substrate for the enzymatic analysis requires special facilities and high costs, the application of the method has been limited. Therefore, we tried to set up a colorimetric assay that is fast, simple and can

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process large numbers of samples simultaneously. The advantages of this system enable its application to enzyme purification and detection of factor XIII deficiency. Since assay systems for factor XIII have not been established for multiple samples in Korea until now, this new assay could become a valuable tool for the screening of factor XIII or other transglutaminase-related disorders in clinical hematology laboratories.

MATERIALS AND METHODS

Synthesis of biotinylated casein

For the biotinylation of bovine casein (Merck, Darmstadt, FRG), the NHS-LC-biotinylation kit from Pierce was used. Ten mg of bovine casein was dissolved in 1ml of 0. 1M sodium phosphate buffer pH 7.2. NHS-LC-biotin(sulfosuccinimidyl-6-[biotinamido] hexanoate) was prepared at a concentration of 5mg/0.25ml in distilled water. Immediately, 145ul of the biotin solution was added to the casein solution. The mixture was stirred gently for 30 minutes at room temperature. Ten ml PrestoTM desalting column(cross-linked dextran) from Pierce was equilibrated with 30ml of PBS buffer (0.01M sodium phosphate, 0.15M NaCl pH 7.2). After 30 minutes, the reaction mixture was added to the column. One ml fractions were collected. Then, the protein content of each fraction was monitored at 280nm.

Buffers

Coating buffer was 50mM sodium carbonate buffer (pH 9.8), and washing buffer was 50mM Tris-HCl pH 7.4 containing 0.15M NaCl and 0.1% Tween-20. 0.1M diethanolamine buffer (pH 9.8) with 100mM $MgCl_2$ was used to dissolve the alkaline phosphatase substrate (4-nitrophenyl phosphate disodium salt). Forty mg of ortho-phenylenediamine in 100ml of phosphate-citrate buffer pH 5.0 with 40ul of 35% H_2O_2 was used as the substrate for horseradish peroxidase.

Preparation of samples

Transglutaminase C from human erythrocytes was prepared as previously described(Brenner and Wold 1978). The samples were diluted serially in 50mM Tris-HCl, 1mM EDTA, pH 7.4.

Preparation of Reagents

Two ml of 1:100 diluted biotin-casein solution (stock solution 4.3mg/ml) and 1ml of 66mM $CaCl_2$ were mixed. The mixed solutions were used immediately. Streptavidin and avidin conjugated alkaline phosphatase were diluted 1:1000 in washing buffer. Streptavidin and avidin conjugated horseradish peroxidase were diluted to the final concentration of 0.1ug/ml in washing buffer. 4-Nitrophenyl phosphate disodium salt was dissolved in diethanolamine buffer to a concentration of 0.5mg/ml for streptavidin conjugated alkaline phosphatase and 1mg/ml for avidin conjugated alkaline phosphatase. The consecutive phosphatase activity was monitored by measuring the absorbance at 410nm. For horseradish peroxidases, 40ul of 35% H_2O_2 added to 40mg of orthophenylenediamine in 100ml of phosphate-citrate buffer pH 5.0 was used as substrates. This solution was used immediately because of its light sensitivity. The absorbance changes by peroxidase were measured at 490nm.

Assay procedure

Microtitre plates (Falcon probind assay plate) were coated with 150ul sodium carbonate buffer solution of casein (1mg/ml) for one hour at 37°C incubator. Then, each well was washed three times with washing buffer with 1mg/ml dithiothreitol(DTT). One hundred ul of samples diluted in two-fold steps and 100ul biotin-casein solution with $CaCl_2$ were added to each well. The plates were incubated for 30 minutes at 37°C and washed three times with buffer supplemented with DTT. Then 100ul avidin or streptavidin conjugated alkaline phosphatase solution, or avidin or streptavidin conjugated horseradish peroxidase solution was added to each well. The plates were incubated for 1 hour at room temperature followed by washing three times. One hundred ul of substrates for each enzyme were added and

after 30 minutes, the absorbances at each corresponding wavelengths were read with the Titertrek Multiscan ELISA spectrophotometer.

Statistical analysis

The software system used here for data analysis was SAS(SAS Institute 1988). Pearson correlation coefficient was the choice for the statistical calculation.

RESULTS

Assay conditions

The optimal conditions for the screening system were determined by changing each variables(substrate concentration, incubation time, and efficiencies of four different reporter enzymes). In case of casein concentration as a substrate for the assay, the higher concentration showed the better response for enzyme activity screening, which was shown by the absorbance at 410nm (Fig. 1). But, since casein solubility drops significantly at over 1mg/ml concentration, optimal casein concentration for plate coating was set at 1mg/ml. As for the incubation time, thirty minutes' incubation with transglutaminase showed better sensitivity than twenty minutes (Fig. 2). However, incubation longer than 30 minutes caused higher nonspecific background values. Therefore, we chose 30 minutes as the optimal incubation time in this screening system. The optimum concentration of the biotinylated casein for efficient detection by the reporter enzyme was decided by incubating microtiter plates with several dilutions of biotin-labeled casein(stock solution

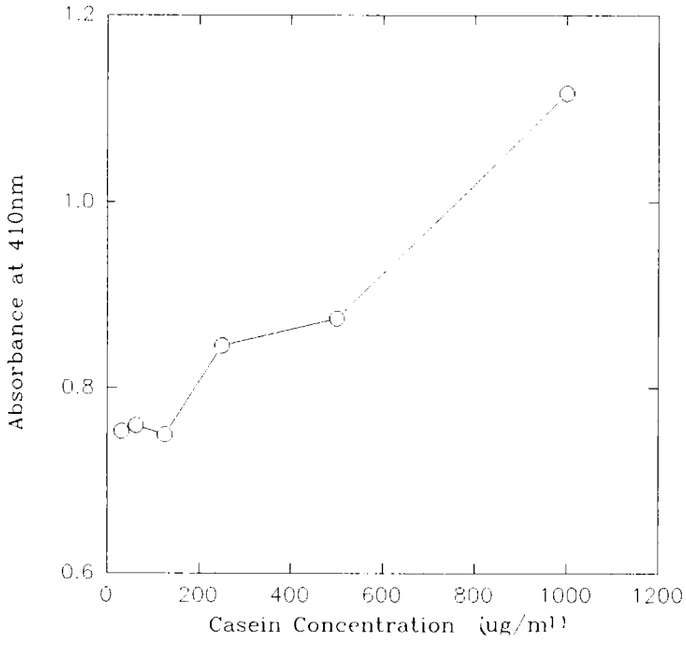

Fig. 1. Effect of casein concentration on color development. One hundred fifty ul of various concentrations of casein were coated on the microtiter plate for 1 hour at 37°C. After washing three times, one hundred ul of transglutaminase C (concnetration 0.43mg/ml) and 100ul of 1:100 diluted biotinylated casein were added to each wells. For the detailed procedure, refer to assay procedure at Methods and Materials.

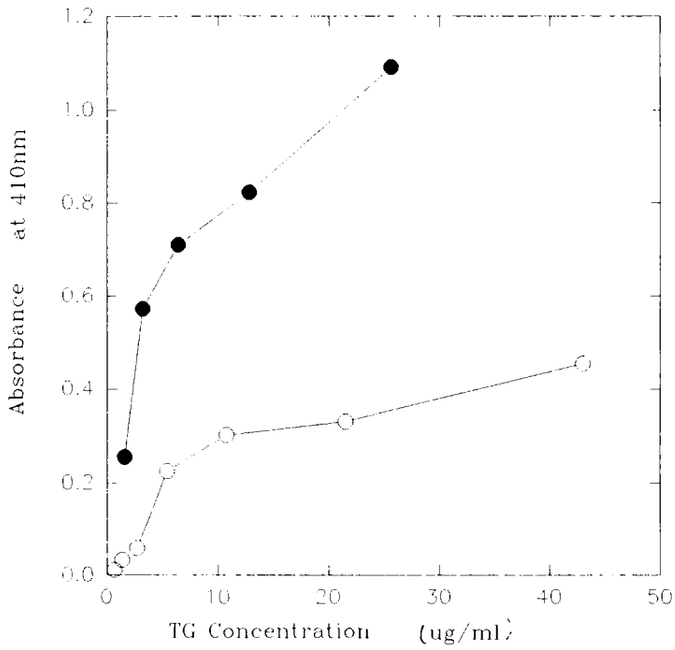

Fig. 2. Effect of incubation time on color development. When 100ul of biotinylated casein and various concentrations of transglutaminase were added to the casein coated plates, the incubation time was varied to see the difference in color development. The detailed procedures were same as the one in Materials and Methods.
Incubation time for transglutaminase with substrates
- ○ 20 minutes
- ● 30 minutes

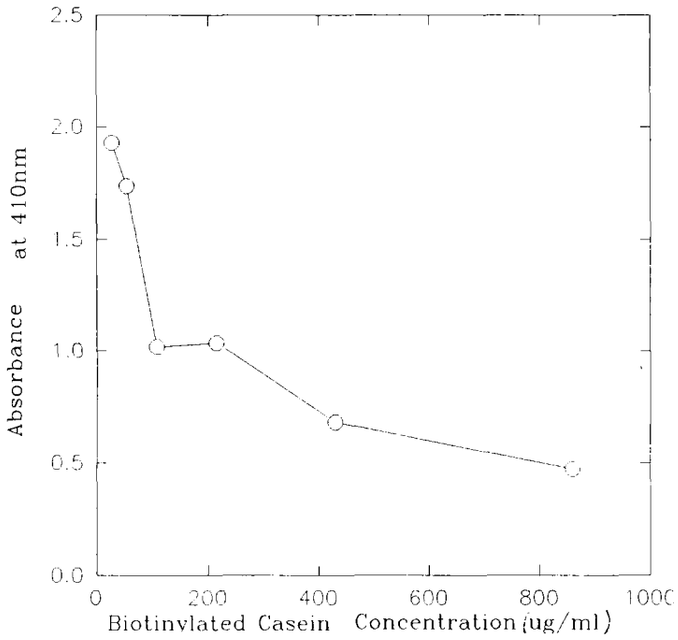

Fig. 3. Effect of concentration of biotinylated casein on color development. One hundred fifty ul of various concentrations of biotinylated casein were directly coated on the microtiter plate for 1 hour at 37°C. 100ul of streptavidin conjugated alkaline phosphatase were added and incubated for 1 hour at room temperature. After 15 minutes' incubation with substrate, color development at 410nm was measured.

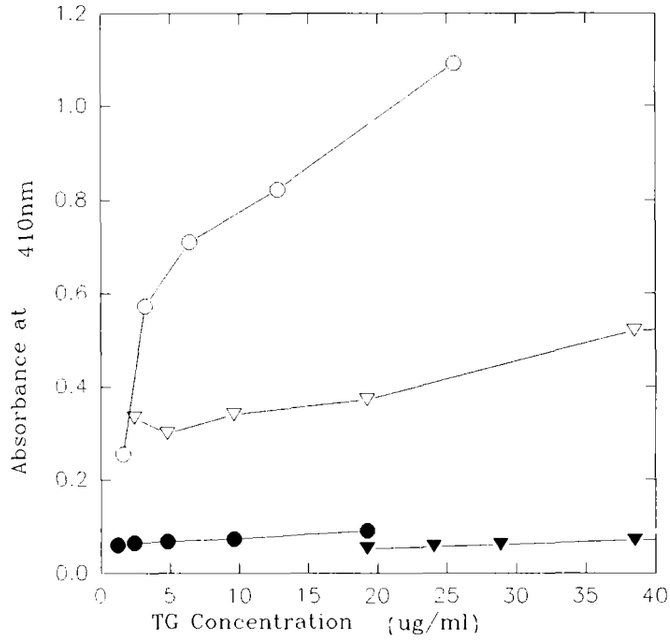

Fig. 4. Effect of enzyme concentration on color development. Reaction conditions were the same as the one in Materials and Methods except four different kinds of reporter enzymes were used
- ○ streptavidin conjugated alkaline phosphatase
- ● avidin conjugated alkaline phosphatase
- ▽ streptavidin conjugated horseradish peroxidase
- ▼ avidin conjugated horseradish peroxidase

3.4mg/ml) and observing color development for the respective reporter enzymes (Fig. 3). The color development seemed to increase in higher dilutions of the biotinylated casein. In 15 minutes' incubation time with the enzyme substrate, dilutions over 1:160 showed values shown by 'over'(too high to detect) in ELISA spectrophotometer. When concentration of biotinylated casein becomes too low, technical errors seem to contribute more significantly than concentration itself. Therefore, the dilution-fold for the biotinylated casein for the assay was set at 1:100(34ug/ml).

Efficiency of the reporter enzymes

Four types of reporter enzymes (streptavidin conjugated alkaline phosphatase, avidin conjugated alkaline phosphatase, streptavidin conjugated horseradish peroxidase, avidin conjugated horseradish peroxidase) were compared in regard to specificity and sensitivity. Here, streptavidin conjugated enzyme systems were better than the avidin conjugated ones in sensitivity. And as a reporter enzyme, alkaline phosphatase (AP) was better than horseradish peroxidase (HRP) in sensitivity (Fig. 4). But when the correlation between color development with the reporter system and transglutaminase concentration was compared (streptavidin conjugated AP: $r = 0.91$, avidin conjugated AP: $r = 0.99$, streptavidine conjugated HRP: $r = 0.89$, avidin conjugated HRP: $r = 0.97$), avidin conjugated alkaline phosphatase($r = 0.99$) seemed to have the highest correlation, though the differences among these systems were insignificant. The correlations for all four reporter enzymes were

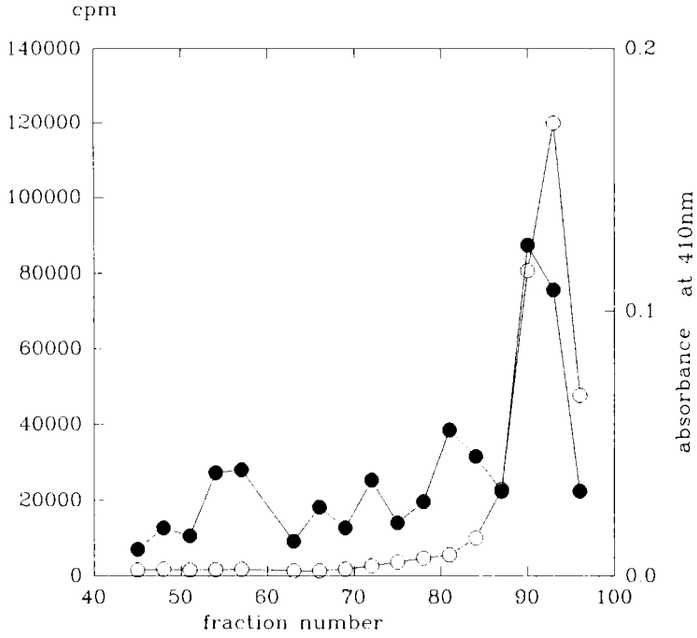

Fig. 5. Correlation of the colorimetric assay (using streptavidin conjugated alkaline phosphatase) with the C_{14} putrescine method in a transglutaminase C purification step. The assay was applied to the fractions collected in a transglutaminase C purification step.
○ The colorimetric assay
● The C_{14} putrescine method

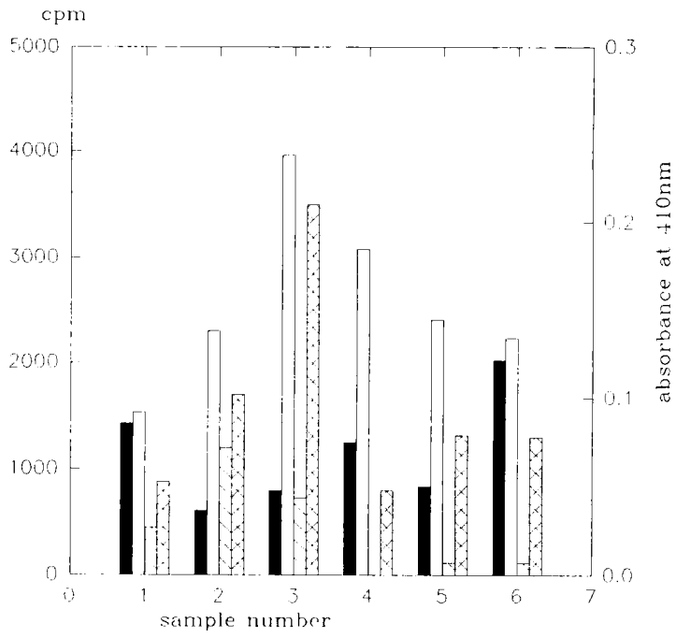

Fig. 6 Comparison of two different assay methods in detecting Factor XIII activity in thrombin activated samples and thrombin nonactivated samples
The colorimetric method(using streptavidine conjugated alkaline phosphatase)
thrombin activated human serum
(20ul of human serum was heated at C for 3 minutes and 5ul of 18 NIH unit of thrombin was added, then the mixture was incubated at 36°C for 20 minutes before assay)
thrombin non activated human serum
(50ul of human serum was heated at C for 3 minutes and incubated at 36°C for 20 minutes before assay)
The C_{14} Putrescine Method
thrombin activated human serum
thrombin non activated human serum

statistically significant($p<0.05$).

Correlation with C14 putrescine method

The assay method was applied to the transglutaminase C purification steps from human erythrocytes. Color development by our new assay and C_{14} count (Fig. 5) by C_{14} putrescine method for the enzymatic activity of each fraction were highly correlated($r = 0.85$) and the correlation was statistically significant ($p<0.0001$). The assay was also applied to Factor XIII screening and compared with the C14 putrescine method. For the thrombin activated samples, the data of the enzymatic activity in each sample by color development and C_{14} count were highly correlated ($r = 0.75$) and the correlation was of statistical significance ($p < 0.05$). Both assays showed a higher reading in thrombin activated samples (Fig. 6).

In this paper, four different kinds of reporter enzymes were compared in order to establish a method to determine the transglutaminase activity in mass samples without using expensive radioisotope-labelled polyamines. In regard to horseradish peroxidases (HRP), streptavidin conjugated enzyme was more sensitive than avidin conjugated enzyme (Fig. 4). Study with the Pearson equation

DISCUSSION

showed a strong and significant correlation between the concentration of transglutaminase and the enzymatic activities detected by absorbance changes by both streptavidin conjugated HRP ($r = 0.89$, $p<0.05$) and avidin conjugated HRP ($r = 0.97$, $p<0.05$). Compared to avidin conjugated alkaline phosphatase(AP), streptavidin conjugated AP also showed better sensitivity (Fig. 4). The correlation between the concentrations of transglutaminase and the enzymatic activities detected by absorbance changes by streptavidin conjugated AP ($r = 0.91$) or avidin conjugated AP ($r = 0.99$) were similarly strong and statistically significant ($p<0.05$). The present data on the sensitivity of the assay confirm the known fact that streptavidin, when compared to avidin, has the advantage of reduced non-specific binding which eliminates some background noise. The superior properties of streptavidin are attributable to its charge characteristic and low carbohydrate content.

In comparison with horseradish peroxidases, alkaline phosphatase was more sensitive. In 30 minutes' incubation period with substrates, the absorbance reading for alkaline phosphatase was higher than horseradish peroxidase (Fig. 4) In addition, the substrates for horseradish peroxidase started to show unstability in reading toward the end of the 30 minutes incubation period. Therefore, we conclude here that streptavidin conjugated alkaline phosphatase is the best reporter enzyme to use in this assay system.

When compared to other previous assays, our assay has the superior sensitivity of being able to detect transglutaminase activity in as little as 3ug of purified human erythrocyte enzyme. The method developed by Sieving *et al.* (1991) using avidin alkaline phosphatase seems to be less sensitive than ours, but since correlation coefficients for avidin conjugated enzymes were higher than streptavidin conjugated enzymes, in terms of precision, the avidin-conjugated system might have some advantages. However, for the sake of sensitivity, we prefer the streptavidin-conjugated system. Under our assay conditions, the readings with avidin conjugated AP were very low(maximum <0.5) in the 30 minutes incubation period with the substrate, but it could be increased up to around a maximum of 1.3 when the incubation period was prolonged to one hour. The method of Jeon *et al.* with streptavidin-β-galactosidase shows good correlation ($r = 0.9$) between the enzymatic activities illustrated by the reporter enzyme and the concentrations of transglutaminase close to our value ($r = 0.9$). But our assay has the advantage of using easily available substrate (casein) instead of 5-(biotinamido)pentylamine.

One drawback of this assay we found on the process is that when the activity of a transglutaminase overrides the sensitivity range for the assay, readings drop to a meaningless level. But for our purpose, if the dilution of the sample is carried out cautiously enough, this complication could be overcome. From these results, we can say that we have established a very simple, economic and nonisotopic method for the determination of transglultaminase activity in mass samples with high sensitivity and efficiency.

In the application of the new assay, we observed a close relationship between the new assay and the conventional C_{14} putrescine method in a transglutaminase purification step and factor XIII screening. Therefore, the assay has enough potential to replace the exhausting procedures of the C_{14} method in purification procedures. In regard to applying the assay to factor XIII screening, this assay will provide a simple and fast procedure for screening lots of human serum samples simultaneously. We wish that this assay system would find its place in clinical laboratories and become one of useful hematological tests.

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