A Rapid Transglutaminase Assay for High-Throughput Screening Applications

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A Rapid Transglutaminase Assay for High-Throughput Screening Applications

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Transglutaminases (TGs) are widely distributed enzymes that catalyze posttranslational modification of proteins by Ca^{2+}-dependent cross-linking reactions. The family members of TGs participate in many significant processes of biological functions such as tissue regeneration, cell differentiation, apoptosis, and certain pathologies. A novel technique for TG activity assay was developed in this study. It was based on the rapid capturing, fluorescence quenching, and fast separation of the unreacted fluorescent molecules from the macromolecular product with magnetic dextran-coated charcoal. As few as 3 ng of guinea pig liver transglutaminase (gpTG) could be detected by the method; activities of 96 TG samples could be measured within an hour. The K_m of gpTG determined by this method for monodansylcadaverine (dansyl-CAD) and N,N-dimethylcasein was 14 and 5 μM, respectively. A typical competitive inhibition pattern of cystamine on dansyl-CAD for gpTG activity was also demonstrated. The application of this technique is not limited to the use of dansyl-CAD as the fluorescent substrate of TG; other small fluor-labeled TG substrates may substitute dansyl-CAD. Finally, this method is rapid, highly sensitive, and inexpensive. It is suitable not only for high-throughput screening of enzymes or enzyme inhibitors but also for enzyme kinetic analysis. (*Journal of Biomolecular Screening* 2006:836-843)

Key words: transglutaminase (TG), TG activity, TG kinetics, magnetic dextran-coated charcoal, fluorescence

INTRODUCTION

TRANSGLUTAMINASES (TGs, R-glutaminylpeptide:amine γ-glutamyltransferase, EC 2.3.2.13) are widely distributed enzymes that catalyze Ca^{2+}-dependent acyl-transfer reactions between specific glutamyl residues of peptides/proteins (glutamyl substrate) and primary amines of peptidyl lysine residues or polyamines (lysyl substrate).[1] Although there are 9 TG genes identified in mammals, 8 are found to encode active enzymes, yet only 6 TG enzymes have been isolated and characterized at the protein level. Each enzyme has its specific functions and distributions: 1) factor XIIIa, which is expressed in blood and involved in blood coagulation; 2) the membrane-associated TG1 and soluble TG3, which are intracellular enzymes found in terminally differentiated epithelial cells; 3) TG2, which is also known as G protein, Gαh, exists intracellularly and extracellularly in various tissues to participate in cell adhesion, differentiation, regeneration, apoptosis, and so on; 4) TG4, which is a prostate gland enzyme associated with the mammalian reproductive process; 5) TG5, TG6, and TG7, which are identified at the gene level with unknown functions[2]; and 6) band 4.2, a structural protein that is expressed in erythrocytes without transamidating activity. On the other hand, the family of enzymes is also found to be associated with certain processes of pathologies, such as celiac disease[3] and Huntington's disease.[4] In addition to the Ca^{2+}-dependent TG, Ca^{2+}-independent microbial TGs are also found in certain bacteria. They are valuable for both food and nonfood applications.[5]

Based on the characteristic action of TG, various strategies have been developed to determine the enzyme activity. There are 3 major categories[6]: 1) the fluorometric or radioactive assays that detect the incorporated labeled molecules, such as monodansylcadaverine (dansyl-CAD)[7-11] or [^{3}H]-putrescine,[12,13] respectively, into glutamyl substrates such as casein or synthetic peptides; 2) the enzyme-linked colorimetric or immunochemical assays that detect the biotin-labeled molecules conjugated with TG substrates coated on microplates by enzyme-linked immunosorbent assay (ELISA)–like procedures[14,15]; and 3) the photometric assays that measure photometric change by a spectrophotometer through either a direct TG transamidation reaction[16,17] or an indirect 2nd enzyme-coupled reaction.[18] In recent years, many new TG substrate molecules have been developed to improve enzyme

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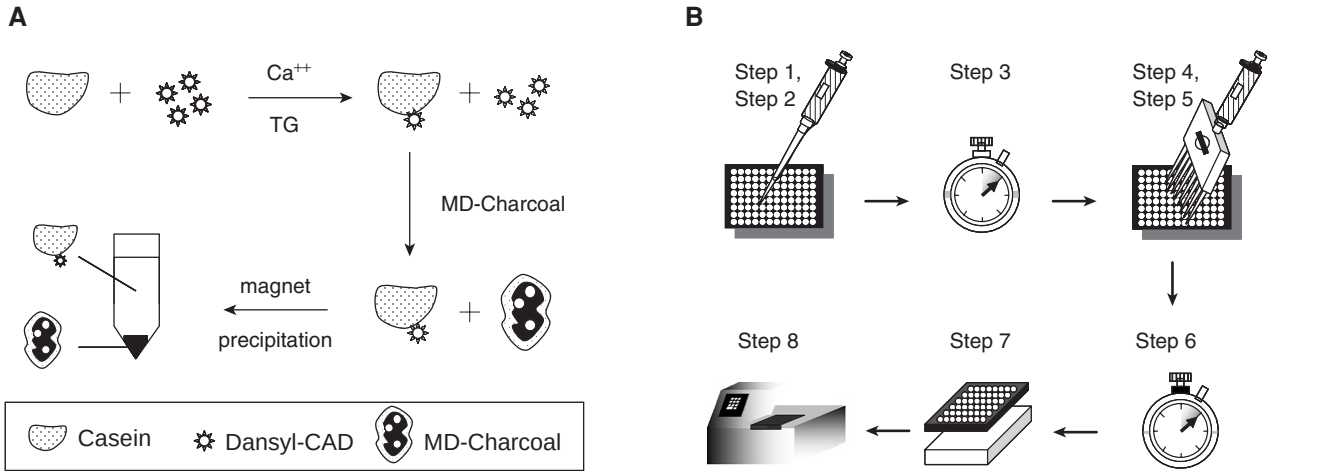

FIG. 1. The schemes of the transglutaminase (TG) assay. (**A**) A rapid separation of cross-lined products from free labeled small molecules is illustrated. The conjugation of casein with monodansylcadaverine (dansyl-CAD) is initiated by the calcium-dependent TG reaction. Magnetic dextran-coated charcoal (MD-charcoal) is then added to capture all of the unreacted dansyl-CAD and separated from the resulting conjugated products by magnetic sedimentation. (**B**) The basic protocol for enzyme assay. Enzyme samples are added to (step 1) and mixed with the start reagent (step 2) in the wells of a microplate at 4 °C. The reaction is immediately initiated by incubating the plate at 37 °C for 15 min (step 3) and terminated by adding the stop reagent (step 4). After the addition of MD-charcoal (step 5), the plate is shaken for 10 min to remove all of the free dansyl-CAD (step 6). Finally, MD-charcoal is precipitated by a magnet (step 7), and the fluorescence is measured by a microplate reader at Ex 340/Em 535 (step 8). Transfer of supernatant to another plate for fluorescence measurement is unnecessary because it does not exhibit fluorescent properties. The details for enzyme assay is described in the Materials and Methods section.

detection sensitivity, and all have their own advantages and disadvantages.[10,13,15,17,19-21]

Among the various strategies developed to measure TG activity, the most sensitive and accurate quantification methods are radioactive and fluorometric assays.[6] A problem with the fluorometric assay is the separation, resulting in conjugated products from the free, labeled reactants as a result of using complicated chromatographic fractionation procedures.[7] Although the method of "relative fluorescence enhancement"[8] can be used to detect, monitor, and calculate the resulting products, the interference of free fluorescent molecules cannot be completely avoided. Furthermore, due to the reduced amount of free fluorescent molecules accompanying the increased quantity of resulting conjugated fluorescent molecules, the relative fluorescent value cannot precisely refer to the enzyme reaction. To resolve this difficulty, physically removing the unreactive fluorescent molecules becomes essential.

It has been known that dextran-coated charcoal can work as a molecular sieve to absorb small molecules exclusively and virtually instantaneously.[22] In this study, magnetic dextran-coated charcoal (MD-charcoal) was used for rapid separation of the conjugated products from the free fluorescent molecules, as summarized in **Figure 1A**. Based on this principle, a simple assay protocol was established, as shown in **Figure 1B**. By using this protocol, the TG activities of 96 samples were easily assessed within an hour. In addition, the method was also successfully applied to enzyme kinetic analysis.

MATERIALS AND METHODS

Materials

Guinea pig liver transglutaminase (gpTG), dansyl-CAD hydrochloride, N,N-dimethylcasein (casein), iodoacetamide, and dithiothreitol (DTT) were from Sigma-Aldrich (St. Louis, MO). Dansyl-chloride, 2-mercaptoethanol, and Tris base were from Merck (Darmstadt, Germany). Dansyl-CAD was prepared as 0.1-M stock in 5% acetic acid and stored at 4 °C until use. BioMag MD-charcoal concentrate (40 mg/mL, approximately 1.5 μm of particle size) was from Bangs Laboratories, Inc. (Fishers, IN). MD-charcoal concentrate was diluted 5 times with water and stored at 4 °C for subsequent application. The V96 microwell plate (#249945) was from Nalge Nunc International (Rochester, NY). The flat magnet was from Pierce Biotechnology (Rochford, IL). Water used in this study was prepared by Milli-Q system (Millipore, Bedford, MA). All other chemicals used were analytical grade from either Sigma-Aldrich or Merck.

Enzyme preparation

Lyophilized gpTG was redissolved in water to 1 mg/mL, subdivided into 100 μL per vial, and stored at –80 °C until use. The enzyme was diluted to designated concentrations with Tris buffer (10 mM Tris [pH 7.5], 1 mM EDTA, and 0.025% 2-mercaptoethanol) just before use. Enzyme activity of gpTG

from Sigma was about 3.6 U/mg protein. One unit of enzyme activity was originally defined as the amount of enzyme that catalyzes the formation of 1.0 μmol of hydroxamate per minute from N-α-CBZ-Gln-Gly and hydroxylamine at pH 6.0 at 37 °C.[16]

Enzyme activity assay

The determination of TG activity in this study was based on the incorporation of dansyl-CAD into casein. The assay protocol is summarized in **Figure 1B**. Typically, 30-μL aliquots of enzyme were added into wells at 4 °C. After 30 μL of start reagent (containing 100 mM Tris [pH 9.0], 20 mM $CaCl_2$, 10 mM DTT, 10 mg/mL casein, and 200 μM dansyl-CAD) was added into each well, the reaction was initiated by incubating the plate in a 37 °C humidified incubator for indicated time intervals. The reaction was terminated by adding 60 μL of stop reagent (2 mM iodoacetamide, freshly prepared) and incubated on ice for 2 min, followed by adding 200 μL of suspended MD-charcoal. The plate was shaken by a minishaker (IKA Works, Wilmington, NC) at 300 rpm for another 10 min at room temperature to capture all of the free dansyl-CAD. After MD-charcoal was precipitated by a magnet for 1 min, the fluorescence intensity in each well was read by a microplate reader (Plate Chameleon, Hidex Oy, Finland), with the excitation wavelength setting at 340 nm and the emission wavelength at 535 nm (Ex 340/Em 535), gain 20. The gain value could be adjusted according to the intensity of fluorescence for optimal measurement. Enzyme activity was expressed as net fluorescence per well in this study to reflect the amount of product formed in the reaction.

The capturing properties of MD-charcoal for the 2 transglutaminase substrates

The capturing capacity and rate of MD-charcoal for dansyl-CAD. All reagents used, except gpTG, were replaced with water and are described in the protocol of the enzyme activity assay. To determine the capturing capacity of MD-charcoal for dansyl-CAD, 60 μL of reaction mixture containing 50 mM Tris (pH 9.0), 10 mM $CaCl_2$, 5 mM DTT, 5 mg/mL casein, and 75, 100, 125, 150, or 250 μM of dansyl-CAD was mixed with 60 μL of stop reagent (2 mM iodoacetamide) in each well. After a 200-μL aliquot of MD-charcoal was added per well, the plate was shaken with the aid of a minishaker at 300 rpm at room temperature for 1, 5, 10, or 15 min to determine the capturing rate of MD-charcoal for dansyl-CAD. Then, the plate was placed on the magnet for 1 min to precipitate MD-charcoal. The supernatant in each well was immediately transferred into a respective well of a new plate (within 1 min) to avoid further MD-charcoal capturing of dansyl-CAD. The fluorescence was measured at Ex 340/Em 535, gain 20. The total fluorescence intensities in wells, where MD-charcoal was replaced with water, were measured as the zero time points of MD-charcoal treatment.

Evaluating the loss of casein to MD-charcoal treatment. Dansyl-casein was prepared to access the amount of casein absorbed by MD-charcoal during standard treatment. For dansylation,[23,24] casein was dissolved in 50 mM $NaHCO_3$, pH 9.0, to a concentration of 20 mg/mL (about 0.8 mM) as stock. Dansyl-chloride in acetonitrile at 9.6 mM was prepared freshly just before use. The dansylation reaction was initiated by mixing 200 μL of casein with 50 μL of dansyl-chloride (about 1:3 at molar ratio) in a dark-brown Eppendorf tube and incubated for an hour at room temperature. The reaction was stopped by adding 0.8 to 1.0 mL dimethyl ether and mixed well. The mixture stood for 1 min to allow separation of organic and aqueous layers, and then the organic layer (the upper layer, containing free dansyl-chloride) was removed. After repeated extraction with dimethyl ether to remove the residual free dansyl-chloride, the aqueous phase containing dansyl-casein was air-dried and rehydrated with 10 mM Tris, pH 9.0, and stored at 4 °C until use. The protein concentration was determined by the Bradford method.[25]

The amount of casein lost to MD-charcoal treatment was measured similarly as described above for dansyl-CAD, with some modifications. Briefly, 60 μL of reaction mixture containing 50 mM Tris (pH 9.0), 10 mM $CaCl_2$, 5 mM DTT, and 0.25, 0.5, 1.0, 2.5, or 5.0 mg/mL dansyl-casein was mixed with 60 μL of stop reagent in each well. After 200 μL of MD-charcoal was added per well, the plate was incubated for 10 or 30 min at room temperature with gentle shaking. MD-charcoal was then precipitated by a magnet for 1 min to terminate the incubation, and the fluorescence in each well of the plate was measured at Ex 340/Em 535, gain 20. For control groups (0-min treatment), the total fluorescence intensities in wells were determined by replacing the volume of MD-charcoal with water.

The fluorescence intensities of dansyl-CAD after being treated with MD-charcoal. The mixture of dansyl-CAD and MD-charcoal was further fractionated, and the intensity of fluorescence in each fraction was analyzed. In detail, the mixture of dansyl-CAD (100 μM, 50 μL/well) and MD-charcoal (200 μL/well) was incubated at room temperature for 1 min. Then, MD-charcoal was precipitated by a magnet for another minute. The supernatants were collected in respective new wells, and the precipitates were washed twice more with water (250 μL/well). Each washing was performed for 1 min with shaking. The washing fluids after magnetic sedimentation were also transferred separately into other new wells, and the final precipitates were resuspended in aliquots of water (250 μL/well). The fluorescence intensities of the supernatants, washing fluids, and MD-charcoal suspensions were measured at Ex 340/Em 535, gain 20.

Enzyme kinetic assay

The K_m value of gpTG for either dansyl-CAD or casein was determined as previously described[26] with modification. Various concentrations of dansyl-CAD (5, 10, 20, 30, 40, 60, 80, and

Table 1. The Capturing Capacity and Rate of MD-Charcoal for Dansyl-CAD

MD-Charcoal Treatment	*Dansyl-CAD (μM)*				
	75	*100*	*125*	*150*	*250*
	Fluorescence Remained in the Supernatant				
Untreated	4330 ± 261^{1a} (100.0%)	5810 ± 332^{2a} (100.0%)	7116 ± 390^{3a} (100.0%)	8057 ± 398^{4a} (100.0%)	$11{,}446 \pm 590^{5a}$ (100.0%)
1 min	470 ± 16^{1b} (10.9%)	852 ± 7^{2b} (14.7%)	1227 ± 30^{3b} (17.2%)	1738 ± 38^{4b} (21.6%)	3404 ± 86^{5b} (29.7%)
5 min	$307 \pm 9^{1b, 1c}$ (7.1%)	471 ± 15^{2c} (8.1%)	831 ± 26^{3c} (11.7%)	1044 ± 19^{4c} (13.0%)	2341 ± 68^{5c} (20.5%)
10 min	211 ± 10^{1c} (4.9%)	$346 \pm 13^{2c,2d}$ (6.0%)	597 ± 15^{3c} (8.4%)	$809 \pm 15^{4c, 4d}$ (10.0%)	1775 ± 52^{5d} (15.5%)
15 min	188 ± 14^{1c} (4.3%)	$284 \pm 11^{2c,2d}$ (4.9%)	$471 \pm 21^{3c,3d}$ (6.6%)	615 ± 33^{4d} (7.6%)	1460 ± 57^{5e} (12.8%)
Blank	132 ± 6^{1c}	132 ± 6^{2d}	132 ± 6^{3d}	132 ± 6^{4e}	132 ± 6^{5f}

Aliquots of monodansylcadaverine (dansyl-CAD; 60 μL) at various concentrations (75, 100, 125, 150, and 250 μM) were mixed with stop reagent (60 μL) separately and treated with/without magnetic dextran-coated charcoal (MD-charcoal; 200 μL) for 1, 5, 10, or 15 min, as described in the Materials and Methods section. After precipitating the MD-charcoal by magnet, the supernatants were transferred to another plate, and their fluorescence intensities were measured at Ex 340/Em 535, gain 20. Data were analyzed by 1-way ANOVA and Tukey-HSD multiple-range test and are presented as means ± SDs ($n = 3$). Different successive superscripts represent significant difference at $p < 0.05$. The background value from the empty well served as blank.

100 μM) or casein (2, 4, 10, 20, 30, 40, and 60 μM) in reaction mixtures that contained 3 μg of gpTG were reacted at 37 °C for 15 min to obtain the initial velocity (V_0) of the enzyme for lysyl or glutamyl substrate, respectively. The K_m value of the enzyme for each substrate was subsequently assessed by using Lineweaver-Burk double-reciprocal plots, $1/V_0$ versus 1/[S]. For dansyl-CAD, the reaction procedure was as described above in the Enzyme Activity Assay section. For casein, because extreme differences in casein protein concentrations among various assay groups may interfere with their fluorescence detection, the protocol was thus modified by adding 30 μL of 25 mg/mL (about 1 mM) casein per well to all groups at the end of enzyme reaction just prior to the addition of MD-charcoal. The final concentration of casein was about 5 mg/mL for each group before MD-charcoal was added.

For inhibitor kinetic analysis, designated concentrations of cystamine (0, 0.1, and 0.4 mM) were used to compete with various concentrations of dansyl-CAD (5, 10, 20, 30, and 40 μM) in the enzyme reaction mixture. In detail, 10 μL of cystamine (0, 0.6, and 2.4 mM) was premixed with 20 μL of gpTG (0.2 μg) for 10 min on ice prior to the addition of the start reagents (containing 100 mM Tris [pH 9.0], 20 mM $CaCl_2$, 10 mM DTT, 10 mg/mL casein, and 10, 20, 40, 60, or 80 μM of dansyl-CAD, in 30 μL); the reaction was performed at 37 °C for 30 min. The rest of the protocol and the evaluation of K_m were as described above.

Statistical analysis

Unless otherwise stated, all experiments were performed in triplicate for each datum point, and the data are presented as means ± SDs. One-way analysis of variance (ANOVA) and Tukey-HSD multiple-range comparison were used to evaluate the statistical significance of differences among groups in studying the capturing properties of MD-charcoal for the 2 transglutaminase substrates (**Tables 1-3**).

RESULTS AND DISCUSSION

Capturing properties of MD-charcoal

It has been known that dextran-coated charcoal functions as a molecular sieve to exclusively absorb small molecules immediately.[22] To confirm that these characteristics of dextran-coated charcoal can be applied well in the TG activity assay, the capturing capacities and rates of the suspended MD-charcoal for the 2 substrates of TG were estimated. Dansyl-CAD and dansyl-casein were used as lysine and glutamine donor, respectively. The capturing capacities and rates of the MD-charcoal suspension for various concentrations of dansyl-CAD or dansyl-casein at different time intervals are summarized in **Table 1** and **Table 2**, respectively. The capturing rate and capacity of MD-charcoal for dansyl-CAD depended on the incubation time and the concentration of dansyl-CAD. The bulk (> 70%) of dansyl-CAD was captured by MD-charcoal within 1 min. On the other hand, the large size of dansyl-casein molecules prevents them from being trapped by MD-charcoal. Thus, the fluorescence intensities of MD-charcoal-treated dansyl-casein solutions were not significantly different from those of dansyl-casein solutions without MD-charcoal treatment (**Table 2**). These results are in accordance with the described properties of dextran-coated charcoal.[22] The information obtained from **Table 1** and **Table 2** implies that TG-catalyzed dansyl-CAD-linked caseins would remain in the

Table 2. Evaluating the Loss of Casein to MD-Charcoal Treatment

MD-Charcoal Treatment	*Dansyl-Casein (mg/mL)*				
	0.25	*0.50*	*1.00*	*2.50*	*5.00*
	Fluorescence/Well				
Untreated	3451 ± 304[1a]	6752 ± 569[2a]	13,276 ± 1075[3a]	27,699 ± 1971[4a]	44,474 ± 2680[5a]
10 min	3173 ± 222[1a]	6190 ± 382[2a]	11,667 ± 693[3a]	25,857 ± 1242[4a]	41,973 ± 1915[5a]
30 min	2921 ± 250[1a]	5708 ± 385[2a]	11,764 ± 788[3a]	24,356 ± 1363[4a]	39,673 ± 2078[5a]
p values	0.104	0.068	0.137	0.087	0.088

Aliquots of dansyl-casein (60 µL) at various concentrations (0.25, 0.5, 1.0, 2.5, and 5.0 mg/mL) were mixed with stop reagent (60 µL) and then treated with/without magnetic dextran-coated charcoal (MD-charcoal; 200 µL) for 10 or 30 min, as described in the Materials and Methods section. After precipitating the MD-charcoal by magnet, the fluorescence intensities were measured at Ex 340/Em 535, gain 20. Data were analyzed by 1-way analysis of variance (ANOVA) and Tukey-HSD multiple-range test and are presented as means ± SDs ($n = 3$). The difference among the 3 groups assayed at each dansyl-casein concentration, indicated with different successive superscripts, was not significant ($p > 0.05$).

supernatants after MD-charcoal treatment, and most of the unreacted, free dansyl-CAD was removed by MD-charcoal within a few minutes.

Dansyl-CAD, after being treated with MD-charcoal, was further fractionated and analyzed. The results are as summarized in **Table 3**. The MD-charcoal, after capturing dansyl-CAD, did not exhibit any fluorescent intensity at Ex 340/Em 535, suggesting that dansyl-CAD lost its fluorescent properties after being absorbed by MD-charcoal. This further indicates that free dansyl-CAD molecules in the enzyme reaction mixture, after being captured by MD-charcoal, might be referred to as having "been quenched" by MD-charcoal. Although the fluorescence of free dansyl-CAD was "quenched," the suspended MD-charcoal particles must be precipitated to eliminate their barricade of the excitation light (data not shown). The precipitated pellet did not interfere with the fluorescence detection (**Table 2**). The final detectable fluorescence in each well was generated from the protein-conjugated dansyl-CAD presented in the solution. This phenomenon facilitates the progression of a high-throughput enzyme assay because the transfer of supernatant to a new plate after MD-charcoal precipitation was unnecessary.

Table 3. The Fluorescence Intensities of Dansyl-CAD after Being Treated with MD-Charcoal

Fractions	*Description*	*Measured Fluorescence*
F1	Total fluorescence of dansyl-CAD	5304.0 ± 164.16[a]
F2	Supernatant, after MD-charcoal treatment	247.7 ± 12.66[b]
F3	Washing fluid 1	188.7 ± 5.69[b]
F4	Washing fluid 2	165.7 ± 15.95[b]
F5	MD-charcoal suspension	173.7 ± 5.13[b]
F6	Background	164.3 ± 4.16[b]

Aliquots of monodansylcadaverine (dansyl-CAD) in triplicate, which were treated with magnetic dextran-coated charcoal (MD-charcoal), were fractionated. Each fraction was measured for fluorescence at Ex 340/Em 535, gain 20. Data represent means ± SDs ($n = 3$) after analyzed by 1-way analysis of variance (ANOVA) and Tukey-HSD multiple-range test. Successive superscripts represent significant difference at $p < 0.05$. The statistic differences among the values of F2 to F6 groups were not significant. F1: Total fluorescence of dansyl-CAD (100 µM, in 50 µL) in which the volume of MD-charcoal was replaced with water (200 µL). F2: Dansyl-CAD (100 µM, in 50 µL) was mixed with MD-charcoal suspension (200 µL) for 1 min. After MD-charcoal was precipitated by magnet, the supernatant was collected and subjected to fluorescence measurement. F3, F4: The precipitated MD-charcoal from F2 was washed twice with water (250 µL) by resuspension. The washing fluids were collected and measured after their separation from MD-charcoal. F5: The precipitated MD-charcoal from F4 was resuspended in 250 µL of water for fluorescence measurement. F6: Fluorescent intensities of empty wells served as background.

Assay sensitivity and linearity

The determination of TG activity in this study was based on the incorporation of dansyl-CAD into casein. The superiority of this improved method to the traditional protocols[7-9] of a similar strategy was the rapid removal of free dansyl-CAD by MD-charcoal and the complete elimination of the interference derived from the unreacted, labeled substrate molecules. Thus, the activities of gpTG could be determined within a short time, without a prolonged incubation interval and separation process of the conjugated products from the free dansyl-CAD molecules. The obtained results also showed that the gpTG activity is dose dependent (**Fig. 2A,B**) and time dependent (**Fig. 3**). Fifteen minutes of reaction time was enough for general TG activity assay, although a longer time of incubation would have resulted in a higher amount of detectable cross-linked products. The minimum dose limit of gpTG was as low as 3 ng per reaction under the described experimental conditions (**Fig. 2B**), equivalent to about 10.8 µU of enzyme activity. This method was highly sensitive as compared to other methods.[11,13,15,21]

Enzyme kinetics for substrates and inhibitor

To prevent the unreacted dansyl-CAD from interference, traditional fluorescence methods for the TG assay must keep dansyl-CAD at a low concentration (about 5 µM) in the reaction mixtures.[8,9] Although the protocol may work well in a regular enzyme activity assay, it has an innate limitation in evaluating the kinetics of TG activity for glutamyl substrate,

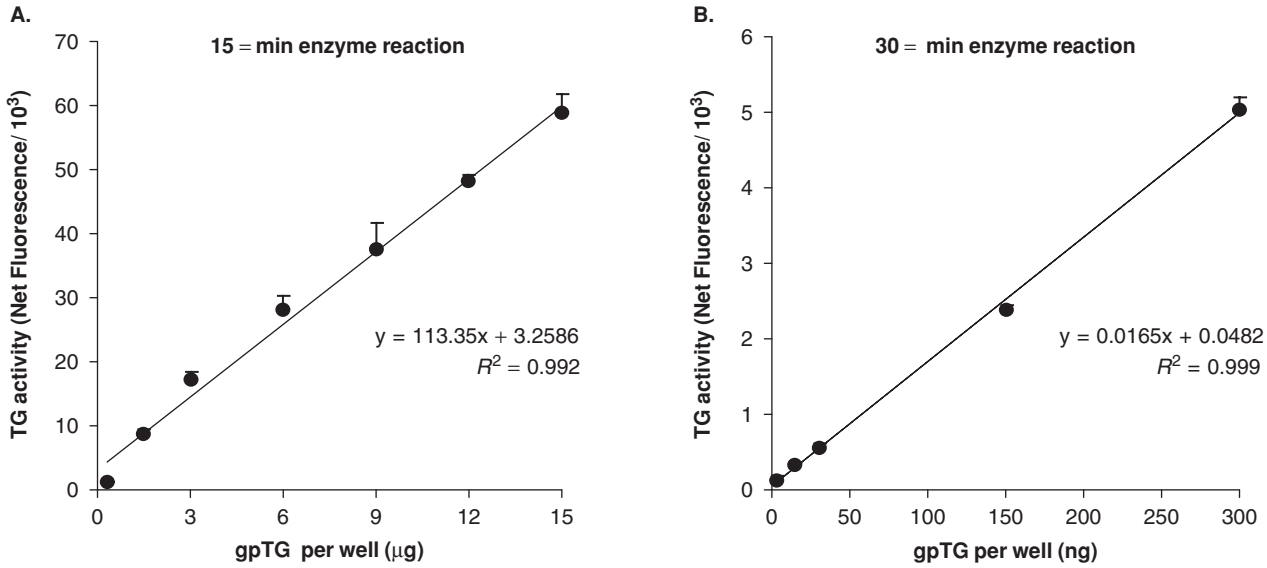

FIG. 2. The linear relationship between the net fluorescence intensities of resulting products and the doses of guinea pig liver transglutaminase (gpTG) enzyme. The enzyme reaction mixtures (50 mM Tris [pH 9.0], 10 mM $CaCl_2$, 5 mM dithiothreitol [DTT], 5 mg/mL casein, 100 μM monodansylcadaverine [dansyl-CAD], and various doses of gpTG) were incubated at 37 °C for (**A**) 15 or (**B**) 30 min. The net fluorescent activities were in linear correlation with the increased amounts of gpTG, which were in the range of (**A**) 0.3 to 15 μg and (**B**) 3 to 300 ng for the 15- and 30-min reactions, respectively. Fluorescence intensities were measured at Ex 340/Em 535, gain 25. Data presented are means ± SDs ($n = 3$).

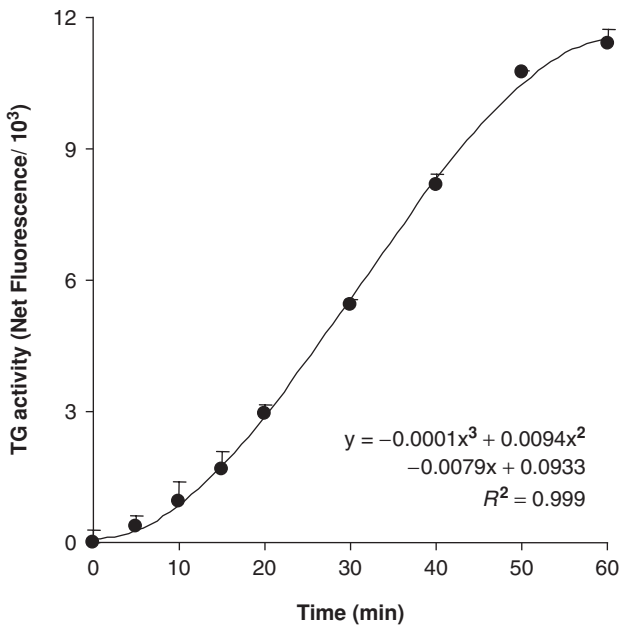

FIG. 3. Time course of enzyme activities. Enzyme reaction mixture (containing 0.3 μg of guinea pig liver transglutaminase [gpTG], 50 mM Tris [pH 9.0], 10 mM $CaCl_2$, 5 mM dithiothreitol [DTT], 5 mg/mL casein, and 100 μM monodansylcadaverine [dansyl-CAD]) was incubated at 37 °C for 0, 5, 10, 15, 20, 30, 40, 50, or 60 min. The fluorescence intensities were measured at Ex 340/Em 535, gain 25. Data presented are means ± SDs ($n = 3$).

such as casein, because dansyl-CAD should be at its saturated concentration in a kinetic analysis. However, in our study, such difficulty is completely eliminated because the saturated, free dansyl-CAD can almost be completely removed by MD-charcoal at the end of assay under the designed experimental condition. The enzymological kinetics of gpTG for both substrates was estimated by Lineweaver-Burk plots (**Fig. 4A,B**); the K_m values estimated for dansyl-CAD and casein in this study were 14 μM and 5 μM, respectively. The K_m value for dansyl-CAD was similar to that obtained by Lorand et al.[8] (14 μM, whereas saturated α-casein was used as glutamyl substrate). On the other hand, the inhibitory kinetics of the competitive inhibitor on TG activity could also be assessed by including inhibitors, such as cystamine, in the reaction mixture of this protocol. A typical competitive inhibition pattern similar to that in another publication[27] was observed (**Fig. 5**).

Perspectives

Based on the rapid separation of unreacted, labeled small molecules, the enzyme substrates applied in the current method may not be limited to dansyl-CAD and casein. Nowadays, many fluorescent lysyl substrates (e.g., KXD)[20] and glutamyl substrates (e.g., CGG-DNS)[10,11] have been developed for TG assays. These small molecules may also be applied to this protocol. For example, to identify a protein as a glutamyl or a lysyl substrate of TG, KXD or CGG-DNS may be used as a fluor-labeled lysyl or

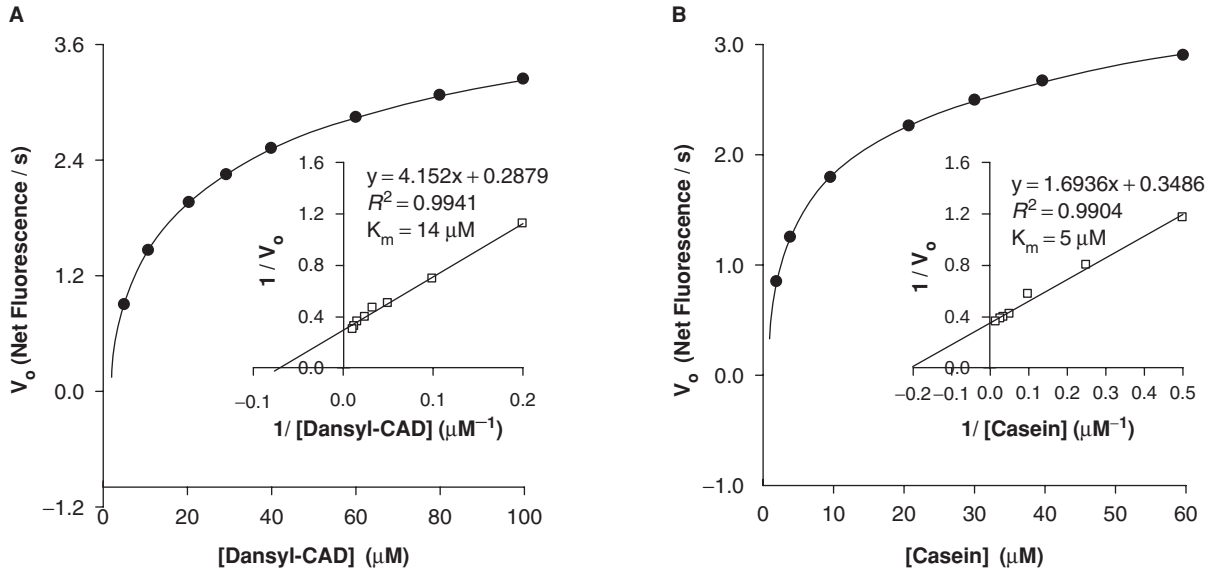

FIG. 4. Steady-state kinetic analysis of guinea pig liver transglutaminase (gpTG)–catalyzed incorporation of monodansylcadaverine (dansyl-CAD) into casein. The gpTG transamidation activity was dependent on the concentration of lysyl substrate, (**A**) dansyl-CAD, and glutamyl substrate, (**B**) casein. The inset shows the double-reciprocal Lineweaver-Burk plot for each result. The calculated K_m values for dansyl-CAD and casein were 14 and 5 μM, respectively. The fluorescence intensity was measured at Ex 340/Em 535, gain 20.

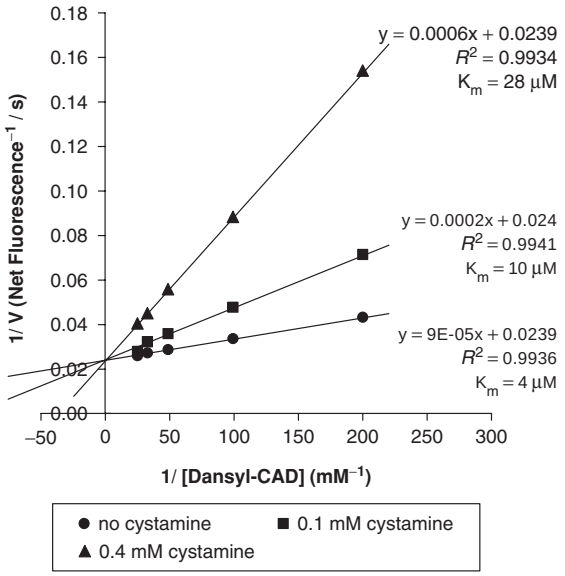

FIG. 5. Competitive inhibition of the guinea pig liver transglutaminase (gpTG)–catalyzed incorporation of monodansylcadaverine (dansyl-CAD) into casein by cystamine. Designated concentrations of cystamine (0, 0.1, and 0.4 mM) were used to compete with dansyl-CAD (5, 10, 20, 30, and 40 μM) for its incorporation into casein in the presence of 0.2 μg of gpTG in reaction. The enzyme activities obtained from the 15-min reaction at 37 °C were expressed as net fluorescence measured at Ex 340/Em 535, gain 40. The enzyme kinetics in the presence of cystamine was analyzed by a double-reciprocal Lineweaver-Burk plot that demonstrated a typical competitive inhibition pattern.

a glutamyl substrate, respectively, in the reaction. The limitation of this application was on the molecular size of the 2 substrates: either a small fluorescent lysyl substrate coupled to a large glutamyl substrate or a large lysyl substrate coupled to a small fluorescent glutamyl substrate. Based on the similar rule, other molecular-binding reactions, such as ligand-receptor binding, may also be assessed accordingly. After the submission of this article, we have subsequently found that fluorescein isothiocyanate (FITC)–labeled cadaverine greatly improves the detection limit. This implies that other brighter fluorescent molecules may also be applied to enhance the sensitivity of this assay.

CONCLUSIONS

In conclusion, the method developed in this study is an easy, rapid, highly sensitive, accurate, safe, inexpensive, and high-throughput method for TG activity assay. It is also suitable for enzyme kinetic analysis. Furthermore, it has the potential to perform a fast and high-throughput screening for identifying protein fractions with TG activity during chromatographic purification, clinical diagnosis, and screening for TG inhibitors. In addition, this method can also be applied to other studies on the binding of fluorescent small molecules to large molecules.

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