A high-throughput colorimetric assay for the measurement of transglutaminase (type II) ε-(γ-glutamyl) lysine crosslinking activity.

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(Short title: Colorimetric TGase (type II) assay.)

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Abbreviations used: BSA, bovine serum albumin; DMSO, dimethyl sulphoxide; DTT, dithiothreitol; EDC, *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide; EGTA, ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid; HEPES, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid); MES, 2-(N-morpholino)ethanesulphonic acid; TGase, transglutaminase; TMB, 3,3',5,5'-tetramethylbenzidine

Introduction:

Tissue transglutaminase enzyme (EC 2.3.2.13) (Tgase) is involved in the post-translation modification of a wide variety of proteins, either by formation of intra- and inter-molecular protein crosslinks, or by primary amine incorporation (for recent reviews see Griffin, 2002 (1) and Lorand, 2003 (2)). Current Tgase assay methods are based on either the protein crosslinking function of the enzyme, or on its amine incorporating activity.

TGase assays that rely on the amine incorporating activity of the enzyme, for example, the biotin-cadaverine incorporation assay of Slaughter *et al*. (3) and the radiometric [^{14}C]-putrescine incorporation assay of Lorand *et al*. (4), may be compromised by Ca^{2+}-dependent and Ca^{2+}-independent incorporation of polyamines into casein substrates by other enzymes such as diamine oxidases (5) and thioredoxin-family protein disulphide isomerases (6). In contrast, the Ca^{2+}-dependent protein crosslinking reaction appears to be TGase-specific. Further advantages of the protein crosslinking assay over the amine incorporation assay are its relative low cost, its higher-throughput potential, and the absence of safety limitations associated with the use of radioisotopes.

The original protein crosslinking-based assay was developed by Seiving *et al*. (7), and further optimised by Choi *et al*. (8). This procedure employed enzyme-conjugated avidin or streptavidin detection of biotinylated casein that was crosslinked to immobilised casein by TGase activity. The sensitivity of these assay systems was reduced by a high background signal which was, in part, due to the formation of TGase-independent adducts between essential calcium ions in the reaction mixture and acidic residues on the substrate protein. Lilley *et al*. alleviated this problem by using immobilised casein that was EDC-modified at the acidic residues (9).

During routine use of the Lilley protein crosslinking assay in our laboratory, variations in background signal were observed with different batches of biotinylated casein and upon

extended storage. This background signal compromised the sensitivity of the assay quite considerably. To address this problem, free biotinylated casein substrate was replaced in the assay by a biotinylated hexapeptide (Biotin-TVQQEL). This hexapeptide sequence was derived from the N-terminal extension of βA3-crystallin, the major TGase amine-acceptor substrate in lens proteins (10). Biotinylation at the N-terminal residue of the hexapeptide allows detection of crosslinked species with horseradish peroxidase-conjugated extravidin. Substitution of free biotinylated casein with free biotinylated hexapeptide in the cross linking assay reduced the background signal and increased the assay sensitivity. This allowed quantitation of low-level TGase activity in crude plant and mammalian cell extracts. This new version of the protein crosslinking assay was also more sensitive than the [^{14}C]-putrescine incorporation assay when measuring low-level enzyme activity, making it a safe, low-cost, high-throughput alternative to the radiometric polyamine incorporation TGase assay.

Materials and Methods:

Peptide source:

Biotin-TVQQEL peptide (Molecular Weight 942.77) was purchased from the Peptide Synthesis Facility, Department of Biochemistry, University of Bristol, UK. A stock solution was prepared at 10mg.ml^{-1} in DMSO, and stored at -20^{o}C in aliquots.

Preparation of samples for assay:

A stock solution of standard enzyme (guinea-pig liver TGase [Sigma, UK]) was prepared at 1mg.ml^{-1} in 5mM Tris-HCl pH 7.4 and stored in aliquots at –80^{o}C. The stock solution was

thawed on ice, then diluted just prior to use in 100mM Tris-HCl pH 8.5 containing 10mM DTT.

ECV 304 human endothelial cells (ECACC No. 92091712) were grown, harvested, and extracts prepared according to the method described in Lilley *et al*. (9).

Curcubita pepo (zucchini) extract was prepared by Waring Blender homogenisation of plant material (10ml per 100g fresh weight) in 10mM Tris-HCl pH 7.4 containing 140mM NaCl, 1mM EDTA, 0.2mM phenylmethanesulphonyl fluoride, 2μM E-64, 20μM leupeptin. The homogenate was filtered through four layers of muslin into a chilled tube prior to centrifugation at 1050*g* for 10 minutes. The resulting supernatant was centrifuged at 48000*g* for a further 2 hours at 4^{o}C. The extract was stored frozen in aliquots at –80^{o}C.

Extracts were thawed on ice, then diluted just prior to use in 100mM Tris pH 8.5 containing 10mM DTT.

Crosslinking Assay:

Microtitre plate wells (Nunc Maxisorp) were coated by incubation overnight at 20^{o}C with EDC-modified casein at 1mg.ml^{-1} in 50mM carbonate buffer pH 9.8 (250μl per well). During each incubation, the plate was covered with a lid and sealed with laboratory film.

Wells were washed three times with distilled water before the addition of 250μl of blocking solution (0.1% (w/v) BSA [Fraction V] in 50mM carbonate buffer pH 9.8) to each well. The plate incubated at 37^{o}C for 1 hour. Wells were washed, before the addition of 150μl of reaction buffer (100mM Tris-HCl pH 8.5 containing 13.3mM dithiothreitol, 5μM biotinylated hexapeptide and either 6.7mM $CaCl_2$ or 5mM EGTA) to each well. The reaction was started by the addition of 50μl of extract in triplicate, in the presence and absence of Ca^{2+} for hour at 37^{o}C. The wells were washed as before and the crosslinked biotinylated peptide was detected by the addition of 200μl probing solution (extravidin peroxidase [Sigma, UK] diluted 1:10

000 in 1% (w/v) BSA / 100mM Tris-HCl pH 8.5) to each well for 1 hr at 37^{o}C. After washing the wells extravidin peroxidase binding was revealed by the addition of 200µl of developing solution (100mM sodium acetate pH 6.0 containing 75µg ml^{-1} TMB [Sigma, UK] and 3% (v/v) H_2O_2) to each well. Colour development was stopped by the addition of 50µl 10N H_2SO_4. Extract-containing wells were developed for either 5 or 15 minutes, depending on the level of the enzyme activity in the extract. Standard wells were developed for the same time to allow quantitation of the TGase activity within the extracts. Absorbance was measured at 450nm in a microtitre plate-compatible spectrophotometer.

EDC-modified casein preparation:

Casein modification by *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide (EDC) was based on the method outlined by Lilley *et al*. (9). Casein, sodium salt [Sigma, UK], was dissolved in 25mM MES pH 6.0 to give a final concentration of 10mg.ml^{-1}. Solid urea and glycine methyl ester [ICN Pharmaceuticals, Ltd, UK] were added to give final concentrations of 7.5M and 1M, respectively. N-hydroxysuccinimide, at a final concentration of 2.5mM, was included to increase the yield of EDC-modified product (11). Following the drop-wise addition of freshly prepared EDC solution (in 25mM MES pH 6.0) to give a 2.5mM final concentration, the reaction was allowed to proceed at room temperature for 2 hours, keeping the pH at 6.0 throughout. The modified casein solution was dialysed extensively against 10mM HEPES pH 7.2.

Radiometric polyamine incorporation assay:

This procedure was carried out according to the method in Lilley *et al*. (9).

Results:

Preliminary assays using standard TGase indicated a Km value of 1μM for the biotinylated hexapeptide (data not shown). In subsequent experiments, the hexapeptide was used at a final concentration of 5μM.

The data in Figure 1a illustrate the linear relationship between absorbance signal and standard TGase activity in the range of 10-120ng per well after 5 minutes development time. From this graph it was not possible to approximate the level of TGase activity in the cell extract from 1500 ECV304 cells or in 250μg of soluble plant protein per well, as the signal was at the lower extreme of the graph.

Extending the development time to 15 minutes, as shown in Figure 1b, allows a greater sensitivity for detection of low-level enzyme activity. From this plot of standard enzyme concentrations of 0.5-8ng per well, it was possible to estimate the level of active TGase as 8ng per 1500 ECV304 cells, and 7ng per 250μg of soluble plant protein (Table 1). The extended development time allowed quantitation of as little as 3ng active enzyme in 400 ECV304 cells (Table 1).

In a standard enzyme assay comparing freshly prepared and stored biotinylated hexapeptide (1 month at -20^{o}C), there was no reduction in signal after storage of the peptide under the recommended conditions (data not shown).

TGase activity may also be determined by radioactive ([^{14}C] or [^{3}H]) polyamine incorporation (4). Any alternative assay for TGase activity must give results that correlate well to those obtained in this radiometric assay. The data in Table 2 shows good correlation between the two assays at high-level enzyme activity, and a definite higher sensitivity of the peptide assay over the radiometric [^{14}C]-putrescine incorporation assay at lower enzyme levels.

Summary:

Direct substitution of free biotinylated casein with free biotinylated hexapeptide in the TGase (type II) ε-(γ-glutamyl) lysine crosslinking assay results in a reproducibly low background signal. This allows extended development times in the assay procedure to enable detection of levels in the order of 3ng of Tgase per sample.

TGase activity was detected in crude mammalian cell extract at 3ng per 400 cells, and in a crude plant extract at 7ng per 250μg of soluble protein.

Good correlation was shown between the peptide assay and the Lorand radiometric assay, with the peptide assay giving greater sensitivity when assaying samples containing low-level TGase activity.

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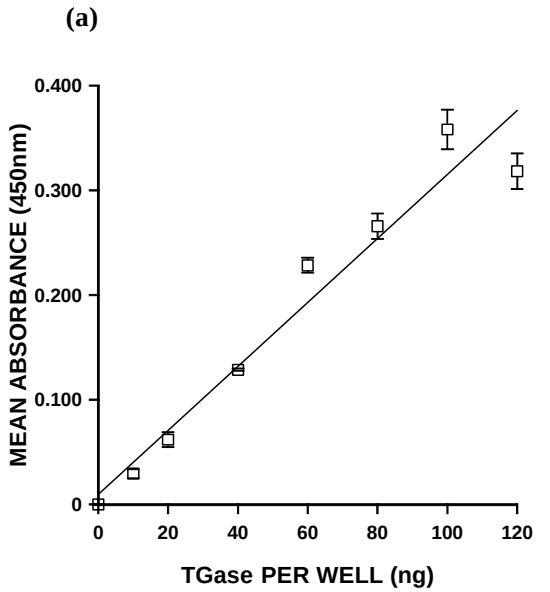


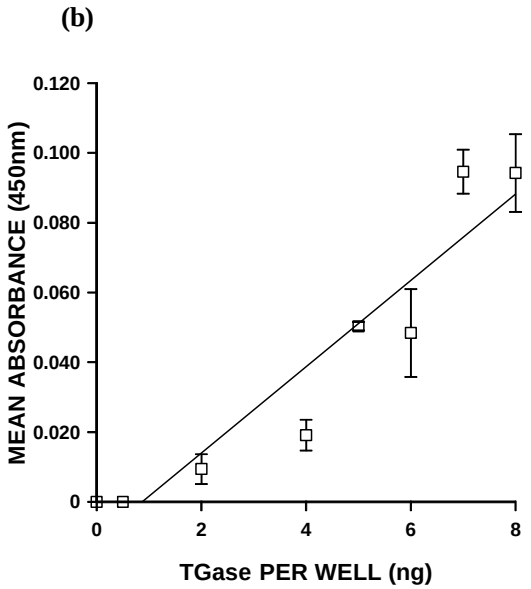


Figure 1: Mean absorbance signal at 450nm resulting from the crosslinking activity of 10-120ng per well **(a)** and 0.5-8ng per well **(b)** standard TGase enzyme, after 5 and 15 minutes

assay development time, respectively. Signal values plotted are those obtained in the presence of calcium, minus the signal obtained from the EGTA-containing parallel control sample.

Table 1: Correlation between development time and detectable amount of TGase in the peptide crosslinking assay.

Sample	Amount detectable after 5 mins development time (ng) [absorbance signal (n=3)]	Amount detectable after 15 mins development time (ng) [absorbance signal (n=3)]
ECV304 cell extract (1500 cells)	n.d.	8 [0.084±0.0004]
ECV304 cell extract (400 cells)	n.d.	3 [0.017±0.001]
Zucchini extract (250µg protein)	n.d.	7 [0.073±0.001]

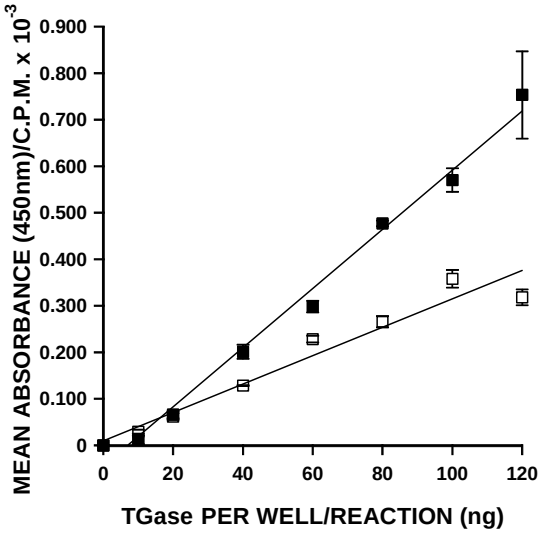
n.d.: not detectable.

Table 2: Comparison of peptide crosslinking and radiometric polyamine incorporation TGase assays using standard enzyme.

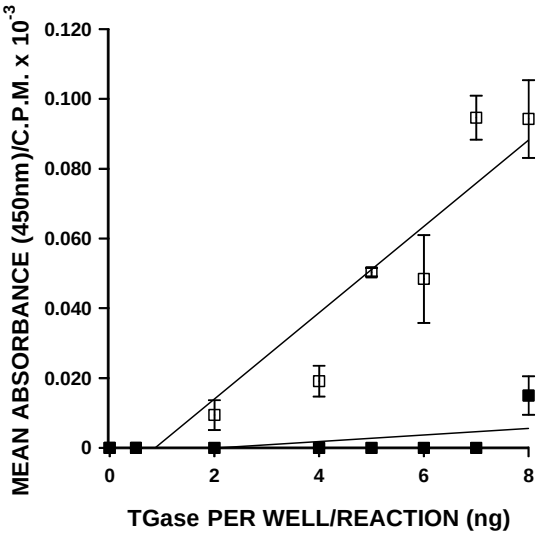
TGase per sample (ng)	Counts per minute in radiometric TGase assay (n=3)	Absorbance signal in peptide assay (n=3) [minutes development time]
0.5	n.d	n.d [15]
2	n.d	0.009±0.005 [15]
4	n.d	0.019±0.005 [15]
5	n.d	0.050±0.001 [15]
7	n.d	0.094±0.008 [15]
8	15±7	0.094±0.014 [15]
20	66±9	0.062±0.009 [5]
40	201±19	0.129±0.001 [5]
80	477±8	0.266±0.015 [5]
120	753±115	0.318±0.021 [5]

n.d.: not detectable.

(a)



(b)



ALTERNATIVE TO TABLE 2 Figure 2: Comparison of the peptide assay (open symbols) with the radiometric assay (solid symbols) at 10-120ng enzyme per

well/reaction (a), and 0.5-8ng enzyme per well/reaction (b). Mean absorbance signal at 450nm resulting from crosslinking activity is plotted in the case of the peptide assay; counts per minute (c.p.m.) value (x 10^{-3}) as a result of [^{14}C]-putrescine incorporation by the enzyme is plotted in the case of the radiometric assay. Signal values plotted in each case are those obtained in the presence of calcium, minus the signal obtained from the EGTA-containing parallel control sample.

Notes:

1. **Figure Legends should be separate in actual manuscript.**
2. **Choose either Table 2 or Figure 2 for Lorand assay comparison.**
3. **Size of the graphs here are approximately the size the will appear in the journal, hence the (recommended) use of uppercase in the axis titles. Axes have been changed to make a 'square' graph. This is how they appear in a manuscript I have got from the journal.**
4. **r2 values??**

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