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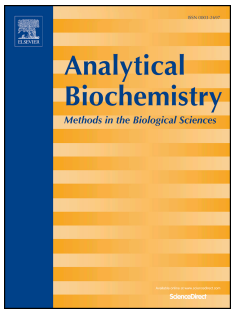

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Real-time kinetic method to monitor isopeptidase activity of transglutaminase 2 on protein substrate

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Short title: Kinetic protein-based isopeptidase assay for TG2

HIGHLIGHTS

Novel protein substrate for isopeptidase activity assay of TG2

Confirmation of specific isopeptidase cleavage of new substrate

Development of a fluorescence polarisation based kinetic assay

Effect of regulatory ligands, mutations and inhibitors

Real-time kinetic method to monitor isopeptidase activity of transglutaminase 2 on protein substrate

ABSTRACT

Transglutaminase 2 (TG2) is a ubiquitously expressed multifunctional protein with Ca^{2+}-dependent transamidase activity forming protease resistant Nε-(γ-glutamyl)lysine crosslinks between proteins. It can also function as an isopeptidase cleaving the previously formed crosslinks. The biological significance of this activity has not been revealed yet mainly because of the lack of protein based method for its characterization. Here we report development of a novel kinetic method for measuring isopeptidase activity of human TG2 by monitoring decrease in the fluorescence polarisation of a protein substrate previously formed by crosslinking fluorescently labelled glutamine donor FLpepT26 to S100A4 at a specific lysine residue. The developed method could be applied to test mutant enzymes and compounds which influence isopeptidase activity of TG2.

Keywords: transglutaminase 2, S100A4, isopeptidase activity, fluorescence anisotropy

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INTRODUCTION

Transglutaminase 2 (TG2, EC 2.3.2.13) is a ubiquitously expressed, multifunctional member of transglutaminases having several catalytic activities and involved in protein-protein interactions both intra- and extracellularly [1]. It has been implicated in a variety of biological processes, including cellular differentiation, apoptosis, angiogenesis, extracellular matrix organisation and linked to immunological, fibrotic, cancer and neurodegenerative disease phenotypes [2,3]. TG2 has various catalytic activities in addition to the well characterized transamidation that is formation of covalent bonds between protein-bound glutamine and lysine residues or primary amines; it can also work as a GTPase, a protein disulphide isomerase and a protein kinase under specific conditions [3]. The Ca^{2+}-dependent transglutaminase activity can also mediate deamidation of glutamine residues and hydrolysis of the previously formed N^{ε}-(γ-glutamyl)lysine as well as γ-glutamylamine derivatives (isopeptidase activity); these reaction mechanisms are reviewed in [4]. The transamidase activity forms a proteinase resistant isopeptide bond which has structural, functional and even industrial implications, for example in clot stabilization by the transglutaminase Factor XIIIa [1], formation of cornified envelopes by transglutaminases in the skin [5], cross-linking of extracellular matrix in kidney fibrosis and food, textile and leather processing to improve flavour, appearance and texture [2,6,7].

Hitherto, only a few reports have been published on the existence and characterization of the isopeptidase activity of TG2. The removal of the previously incorporated monoamines (deaminylation) [8-11] and the isopeptide cleavage between short peptides [12] were demonstrated measuring fluorescence intensity change or using capillary electrophoresis. On a protein level only Factor XIIIa catalysed isopeptidase activity was detected what can reverse the incorporation of α2-plasmin inhibitor into fibrin clots potentially regulating the fibrinolytic processes [13,14]. This raised the possibility that isopeptidase activity of TG2 could also play important roles in regulation of biological processes. However, in the lack of proper and easily accessible assay the full biological and pathological significance of this activity cannot be revealed.

A real-time fluorescence polarization assay has been published [15] to measure transamidase activity of TG2 during crosslinking a fluorescently labelled TG2 specific dodecapeptide (FLpepT26) into bovine serum albumin (BSA) resulting in higher anisotropy of the

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enzymatically crosslinked product. We hypothesized that in case of a proper lysine donor substrate after the forward reaction the cleavage of isopeptide bond by TG2 also leads to anisotropy change which could be monitored using the same biophysical feature utilized in proteinase and deubiquitinating assays [16,17].

Here we report the development of a kinetic fluorescent polarization based assay to follow isopeptidase activity of TG2 on a novel crosslinked protein-peptide substrate. By enzymatic crosslinking of the fluorescently labelled FLpepT26 dodecapeptide and S100A4, a recently characterized specific amine donor of TG2 [18], the purified product was subsequently used as a substrate to demonstrate the cleavage of the isopeptide bond and follow this activity by measuring fluorescence polarization in real time.

MATERIALS AND METHODS

Materials

All materials were purchased from Sigma (St Louis, MO, USA) unless otherwise indicated. The FLpepT26 peptide was obtained as published in [18]. ZDON was sold by Zedira (Darmstadt, Germany).

Expression and purification of proteins

The Val^{224} containing recombinant human TG2 (Uniprot code: P21980) and its mutants were expressed in N-terminally $(His)_6$-tagged form (pET-30 Ek/LIC-TG2; Mw: 82,745 Da) and purified by Ni-NTA affinity chromatography as described previously [11].

N-terminal GST-tagged S100A4 (pETARA-S100A4; Uniprot code: P26447; Mw: 39,559 Da) was expressed in Rosetta 2 (Novagen, Darmstadt, Germany). The overnight culture was inoculated in 1:20 ratio into LB medium containing 50 µg/mL ampicillin and 34 µg/mL chloramphenicol and was grown at 25°C until the optical density reached 0.6–0.8 at 600 nm. The expression was induced with 0.1 mM IPTG at 18°C overnight. Cells were harvested and pellets were dissolved in Buffer A (20 mM Tris-HCl pH 7.2, 150 mM NaCl, 1 mM EDTA, 1 mM DTT) containing 1% Triton X-100, 10% glycerol, 1 mM PMSF and protease inhibitor cocktail. After sonication the supernatant was separated by centrifugation at 20000g, 4°C, 25 min and loaded

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onto Glutathione Sepharose 4B resin (GE Healthcare, UK). After extensive washing with Buffer A the S100A4(GST) protein was eluted using 10 mM reduced glutathione in Tris-HCl pH 8.0 and dialyzed overnight in buffer A. Protein concentration was determined by Bradford method (Bio-Rad Protein Assay, Bio-Rad, München, Germany).

Large scale production of the crosslinked FLpepT26-S100A4(GST)

The mixture of 5 µM FLpepT26, 12.8 µM S100A4(GST) and 5 nM TG2 was incubated for 1 hour in the presence of 5 mM Ca^{2+} in the reaction buffer 20 mM Tris-HCl pH 7.5 containing 150 mM NaCl, 5 mM DTT and 0.01% Tween 20. The reaction was stopped by the addition of 10 mM EDTA (final concentration) to prevent unwanted modification of the crosslinked molecules during their separation. FLpepT26-S100A4(GST) with unmodified S100A4(GST) were purified from the free, unbound FLpepT26 peptide by centrifugal concentrator filter (Amicon ultra, 10 kDa, Millipore, Billerica, MA, USA). Then the buffer was replaced by 20 mM MOPS buffer, pH 6.8 containing 0.5 mM EDTA, 150 mM NaCl, 5 mM DTT, 0.01% Tween 20 because isopeptidase activity prefers slightly acidic pH [19]. Due to co-purification of FLpepT26-S100A4(GST) and S100A4(GST) their ratio was calculated based on the total protein concentration (determined by Bio-Rad Protein Assay) and its fluorescein content (absorption at 493 nm) using 79600 M^{-1} cm^{-1} as molar extinction coefficient for fluorescein. In optimized conditions the FLpepT26-S100A4(GST) content was approximately 15% as an average in the reaction product meaning that 5 µg purified mixture of FLpepT26-S100A4(GST) and S100A4(GST) corresponds to 0.5 µM FLpepT26-S100A4(GST) in 35 µl of the isopeptidase assay.

Preparation of samples for SDS-PAGE analysis

The reaction was stopped by adding 6x denaturation buffer (375 mM Tris-HCl, pH 6.8, 600 mM DTT, 12% (m/v) SDS, 60% (v/v) glycerol, 0.06% (m/v) bromophenol blue) and the samples were boiled for 10 min. SDS-PAGE was performed using 15% Tris-Glycine gel. The fluorescence was detected immediately by an UV gel documentation system (Protein Simple, AlphaImager, HP system).

Mass spectrometric analysis of the peptide after isopeptide cleavage

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Electrospray ionization mass spectrometric measurements were carried out on a Bruker Daltonics Esquire 3000plus (Bremen, Germany) ion trap mass spectrometer using on-line HPLC coupling. HPLC separation was performed on a Jasco PU-2085Plus HPLC system using a Supelco Ascentis C18 column (2.1 x 150 mm, 3 µm). Linear gradient elution (0 min 2% B; 3 min 2% B; 27 min 60% B) with eluent A (0.1% HCOOH in water) and eluent B (0.1% HCOOH in acetonitrile-water (80:20, V/V)) was used at a flow rate of 0.2 ml/min at ambient temperature. The HPLC was directly coupled to the mass spectrometer. Collision induced dissociation (CID) experiments were used for peptide sequencing.

Kinetic isopeptidase activity measurement

In 35 µl reaction volume on 384-well Untreated Polystyrene Black Microplates (Nunc, Thermo Scientific, Denmark; catalog# 262260) 0.5 µM of the FLpepT26-S100A4(GST) crosslinked substrate were tested in 20 mM MOPS pH 6.8 reaction buffer containing, 150 mM NaCl, 6 mM glycine methyl ester, 5 mM DTT, 0.1% Tween 20 and various concentration of TG2. The reaction was started by the addition of 5 mM $CaCl_2$ (5 mM EDTA was used as negative control) and performed at 37°C, measuring the change in fluorescence polarization (FP) value by Synergy H1 microplate reader (GreenFP filter cube, Ex: 485 nm, Em: 528 nm; BioTek, Winooski, VT, USA). The reaction rates were calculated from the initial slopes of the kinetic curves in terms of anisotropy per minutes.

Data analysis

Data analysis, curve fitting, kinetic calculations were performed by GraphPad Prism 5 software (Graphpad Software Inc. La Jolla, CA, USA) using the appropriate incorporated equations and tools mentioned where it is appropriate. In case of Ca^{2+}-dependence experiments the free calcium-ion concentrations were calculated using the online version of MaxChelator (WEBMAXC STANDARD; http://www.stanford.edu/~cpatton/maxc.html) due to the EDTA content of the substrate.

RESULTS AND DISCUSSION

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Design and production of the potential transglutaminase isopeptidase substrate for anisotropy assay

To develop a peptide-protein based isopeptidase assay, the first step was to select proper substrate components for the production of a substance that contains only one N^{ε}-(γ-glutamyl)lysine isopeptide bond and the substrates have significant difference in their size to provide sufficient signal for the detection of anisotropy change. In previous publications fluorescein labelled FLpepT26 peptide was an effective TG2 specific amine acceptor substrate [15] that could be incorporated into the amine donor BSA and recently into S100A4 monitored by fluorescence polarisation assay [18]. Both FLpepT26-BSA and FLpepT26-S100A4 as N^{ε}-(γ-glutamyl)lysine cross-linked products were considered as substrates for isopeptidase activity measurements. However, BSA contains several surface exposed Lys residues compared to S100A4 which has only one TG2 reactive Lys residue (Lys100), and unlike BSA, S100A4 does not contain transglutaminase reactive glutamine residues [18] making S100A4 an ideal candidate to serve as a substrate in an isopeptidase reaction. To compensate the size difference between S100A4 (11.5 kDa) and BSA and to get higher anisotropy change and better fluorescence polarization signal in the designed assay, S100A4 was expressed with GST-tag. GST alone did not show explicit activity neither as an amine donor (in fluorescence anisotropy assay with FLpepT26 peptide, Fig. S1A) nor as an amine acceptor (in dansyl-cadaverine incorporation assay, Fig. S1B). In the transamidase reaction the K_m values for S100A4(GST) and BSA were comparable with earlier observations [15,18], while V_{max} was approximately 10 times higher in case of S100A4(GST) as amine donor (Fig. S1C) suggesting that S100A4(GST) could be a very potent Lys donor to produce an isopeptidase protein substrate with a single isopeptide bond.

The crosslinking reaction was carried out in large scale as described in Materials and Methods to produce the FLpepT26-S100A4(GST) containing the N^{ε}-(γ-glutamyl)lysine bond. The reaction was stopped by the addition of 10 mM EDTA (final concentration) to prevent unwanted further modification of the crosslinked molecules during their separation from free FLpepT26 peptide and the buffer was replaced to MOPS buffer, pH 6.8 for optimal isopeptidase activity [19] (Scheme on Fig. 1A).

The efficiency of the separation was checked on SDS-PAGE confirming the presence of the crosslinked, fluorescently labelled FLpepT26-S100A4(GST) molecule at ~42 kDa molecular

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weight in the gel (Fig. 1B) and disappearance of the FLpepT26 peptide which has 1885 Da molecular weight but generally appears in the gel as a 10 kDa diffuse band.

Validation of the potential FLpepT26-S100A4(GST) as isopeptidase substrate for TG2

The prepared product was tested to check whether it can be used as a substrate for TG2 isopeptidase activity measurement. The result of the cleavage of the isopeptide bond could be visualized by SDS-PAGE (Fig. 1B). The acceptance of FLpepT26-S100A4(GST) by TG2 as isopeptidase substrate can be easily followed as the amount of the substrate decreases while increasing amount of the released fluorescein-conjugated peptide appears during the enzymatic reaction.

To further confirm that isopeptidase activity occurred, the product of the isopeptidase reaction was applied to a Jupiter 300 C5 RP-HPLC column (Phenomenex) and the peak corresponding to the deamidated FLpepT26 was further analysed by HPLC-ESI-MS/MS. The results showed that the released peptide was a single compound with a molecular mass of 1884.9 Da with slightly higher retention time compared to FLpepT26 (1886.1 Da) (Fig. S2 and S3). Both peptides were subjected to collision induced dissociation to confirm the location of the mass difference (Fig. S4). MS/MS sequencing of the triply protonated parent ions (m/z 629.3 and 629.7) resulted in a complete set of mainly *b* and *y* type fragment ions (fragmentation pathway is presented in Fig. S4). The identified peptide fragments correlate with the sequence of FLpepT26 confirming the reliability of the analysis. Based on the mass differences of the *N*-terminal peptide fragments, the Gln to Glu transformation in the released peptide was unambiguously confirmed (Table S1).

Testing FLpepT26-S100A4(GST) as a suitable substrate for kinetic measurement of isopeptidase assay

After demonstration of effective hydrolysis of FLpepT26-S100A4 by TG2 we tested its correlation with decreased anisotropy. Since the crosslinking could be followed by the increase of anisotropy [15,18], it was expected that anisotropy should be decreased over time during the isopeptidase reaction. Indeed, anisotropy signal followed an exponential decay; and using linear regression based on the first phase (9-10 minutes) of the decrease, reaction rates were calculated in the range of 10-600 nM TG2 concentration (Fig. 1C). At lower TG2 concentration the decrease

of anisotropy was less significant and the higher noise (deviances of the single measured values from the fitted curve on the values) prevented the determination of isopeptide bond cleavage. The remaining isopeptidase activity of the enzyme used for isopeptide substrate generation was checked by the comparison of the reaction rates in the presence of 5 mM calcium or EDTA without adding any further TG2, but there was no significant difference between the slope of the curves (Fig. 1C). At 5 µg substrate (which corresponds to 0.5 µM concentration) we could detect isopeptidase activity at 10 nM TG2 concentration, while the reaction rate was linearly dependent on TG2 concentration in the 10-300 nM range (Fig. 1D). The commercially available, not protein based kinetic isopeptidase activity assays, which measure deaminylation of small non-specific peptides by TG2 at 50 µM substrate concentration (Zedira assay), has similar sensitivity with a detection limit of approximately 13 nM TG2 [20].

Next, we measured the effect of increasing FLpepT26-S100A4(GST) substrate concentration on the reaction rate at fixed TG2 level (0.3 µM TG2, 5 mM Ca^{2+}) and we have observed saturation kinetics on which curve was fitted based on the Michaelis-Menten equation (Fig. 2A; coefficient of determination: R^2=0.9871). Here, the activity attained a plateau phase and an enzymatic dissociation constant (K_m) was calculated to be 53.9±4.4 nM for FLpepT26-S100A4(GST) and V_{max} to be 57.9±0.9 mr/min/µM TG2 using Graphpad Prism 5. This K_m value is extremely low compared to published values for human TG2. We measured 13.3 µM and 54.3 µM K_m values using Zedira assay with A101 and A102 isopeptidase substrates (deaminylation activity), respectively, in which test the substrates are peptides containing an incorporated unspecific amine (quencher labelled cadaverine) [11]. In other transglutaminase assays when deamidation was detected by kinetic spectrophotometric assay using Cbz-Gln-Gly as substrate, the K_m value was in the mM range [21]. In case of the cleavage of isopeptide bond between cross-linked peptides, the K_m values were also in micromolar range [12]. This demonstrates that our newly developed method based on the novel protein substrate can serve as a highly sensitive and specific reaction to detect and measure TG2 isopeptidase activity. Furthermore, the high reactivity of TG2 with isopeptide bonds shown here suggests that its isopeptidase activity may have physiological relevance not described yet.

Considering that the added substrate in the assay is a mixture of the isopeptide bond containing FLpepT26-S100A4(GST) and free S100A4(GST), a significant amount of free S100A4(GST) is present raising the possibility that S100A4(GST) may have influence on the isopeptidase

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reaction. To address this concern, the effect of S100A4(GST) on the isopeptidase activity was analysed using the above mentioned Zedira assay which contains small peptide substrate and our protein substrate based assays (Fig. S5A and B, respectively). The increasing S100A4(GST) concentration results in dose dependent increase of isopeptidase activity of TG2 in the Zedira assay; this may be explained by the reported interaction of TG2 and S100A4 [18] leading to the potential stabilization of the active conformation of the enzyme because in previous study amine donor substrates did not influence isopeptidase activity [11]. On the other hand, in the protein-based assay, the increasing concentration of S100A4(GST) did not lead to change of the isopeptidase activity, probably due to the relatively high ratio of S100A4(GST) concentration in the substrate which already could be in saturation without addition of extra S100A4(GST). To further increase the efficiency of the developed assay the peptide incorporation ratio should be further improved to eliminate this potential limitation factor of the method for broad applications. The effect of glycine methyl ester, which was present in the assay solutions, was also checked on the protein-based isopeptidase reaction because it was proposed to facilitate the regeneration of the enzyme from the thiol intermediate state during the isopeptidase reaction (aminolysis) [20]. However, fluorescence polarization was comparable both in the presence or absence of glycine methyl ester (data not shown) in concordance with our earlier observations [11] and the MS analysis has not identified transamidated peptide product in the applied assay conditions suggesting that transamidation does not have significant rate in TG2 catalysed reaction (Fig. S4) [9].

To further characterize the kinetics of the protein-peptide based isopeptidase reaction, Ca^{2+}-dependency was also tested since TG2 undergoes a large conformational change during its activation by Ca^{2+} [22] that could influence substrate accessibility to the active site (Fig. 2B). Generally, the crosslinking and the deaminylation activity of wild type TG2 reaches the maximum reaction rate in the presence of approximately 1.5 mM Ca^{2+} concentration [23]. However, in case of FLpepT26-S100A4(GST) cleavage, 4-5 mM Ca^{2+} concentration was needed to saturate the reaction rate (WT EC_{50} 0.96±0.17 mM, calculated by fitting a dose-response curve hypothesising standard slope) suggesting that isopeptidase activity may have more important role in the extracellular matrix where Ca^{2+} concentration is higher than in the cytosol.

Intracellularly, TG2 activity is regulated reciprocally by Ca^{2+} and nucleotides [3]. The inhibitory effect of GTPγS, a stable GTP compound, was also tested on isopeptidase activity of TG2 (Fig.

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2D). GTPγS had similar inhibitory effect on isopeptidase activity of TG2 in the protein-based as in the Zedira assay (with A102 substrate) [11].

To see the importance of the substrate quality, crosslinked BSA-FLpepT26 was also produced and tested in the isopeptidase assay, but no reaction could be detected by either monitoring polarisation change or by SDS-PAGE analysis (data not shown). This provides further evidence for the importance of the applied protein substrate containing the isopeptide bond to detect specific isopeptide cleavage. The active lysine S100A4 residue, involved in crosslinking, is localized in the C-terminal tail of S100A4 that could be easily accessible for the active site of TG2.

Potential applications of the newly developed kinetic isopeptidase assay

There is an increasing interest from the pharmacological industry to regulate transglutaminase activity by inhibitors, particularly in neurodegenerative diseases, kidney or pulmonary fibrosis and to prevent thrombosis [3]. However, the effects of newly developed inhibitors were always tested on the crosslinking activity of TG2 and there has been no comparison of their effect on isopeptidase activity of TG2, particularly in a kinetic, protein substrate assay that has not existed so far. A commercially available, cell permeable, irreversible active site directed inhibitor of TG2, the ZDON (Zedira, [24]) was tested in our newly developed method (Fig. 2C). Approximately 0.3 μM ZDON was enough for complete inhibition of the isopeptidase activity which is in a good correlation with the expected value in case of an irreversible inhibitor in the presence of 0.3 μM TG2 although the apparent IC50 value was 30.7 nM (calculated by fitting a dose-response curve hypothesising standard slope) which is lower than a reported one in an *in vitro* transamidation assay [24].

Enzyme assays are useful to characterise the effect of mutations on activity. In our recent study two special mutants have been produced [11]. One which is deficient in transamidase activity but has higher isopeptidase activity compared to the wild type (isopeptidase mutant W332F), and another with opposite characteristics (transamidase mutant W278F). Interestingly, in the protein-based assay both mutants demonstrated lower activity than wild type TG2 (Fig. 2E; 46.6% and 78.6%, respectively). Moreover, the W332F mutant showed lower while the W278F mutant higher activity compared to the Zedira deaminylation assay [11]. The larger size of the substrate

may mask the earlier observed differential effect of these mutations [11] suggesting that steric features have a strong control on the TG2 isopeptidase activity.

For more detailed study of isopeptidase activity and its biological significance, the replacement of the substrate components with other TG2 specific substrates could provide further mechanistic details about the isopeptidase reaction. Results of such studies can lead to pharmacological approaches to accelerate the removal of pathologically accumulated, N^{ε}-(γ-glutamyl)lysine cross-linked proteins from fibrotic tissues or in case of neurodegenerative disorders. These bonds otherwise are catabolized slowly requiring degradation by proteases to yield γ-glutamyl-ε-lysine isodipeptide that is released into the circulation to be cleaved by γ-glutamilamine cyclotransferase in the intestine and kidney [25,26].

CONCLUSIONS

We have developed a kinetic method to follow isopeptidase activity of TG2 in real time using a cross-linked protein substrate in the presence and absence of different effectors/inhibitors. Reversible covalent modifications of proteins have crucial regulatory functions in cells and tissues like those mediated by ubiquitin ligases and deubiquitinases or kinases and phosphatases. Unlike phosphorylation and ubiquitination there are no enzymes in nature to reverse transglutaminase mediated protein cross-linking. Instead, transglutaminases themselves, such as TG2 possess hydrolytic activity to cleave the N^{ε}-(γ-glutamyl)lysine bonds which they produce. The biological and regulatory significance of this reaction as well as the potential use of the isopeptidase activity of transglutaminases in pathologic conditions require further investigations. We believe that the presented assay can serve as a useful tool for those kind of studies.

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FIGURES:

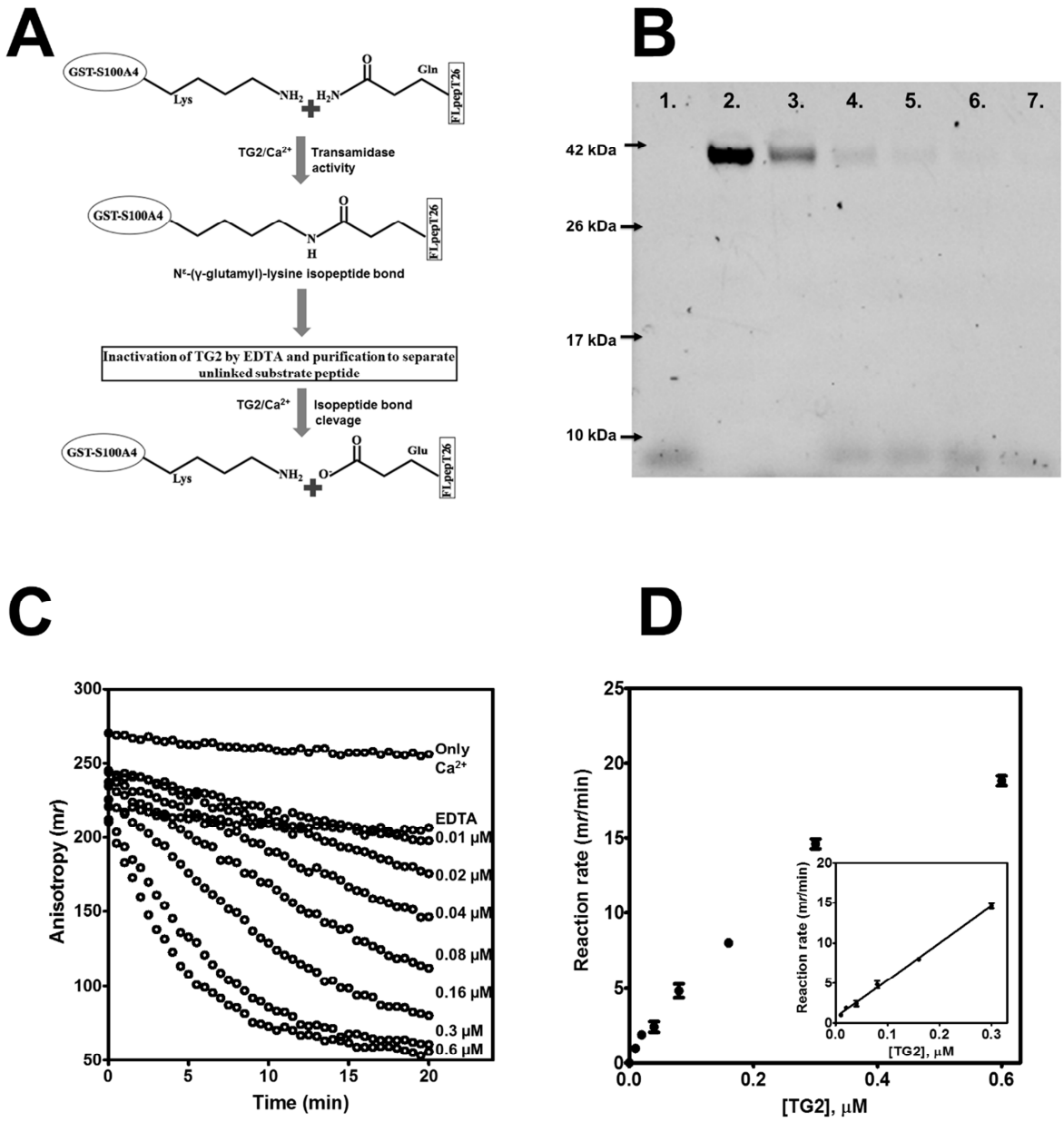

Fig 1. Design and implementation of the fluorescence anisotropy assay that monitors isopeptidase activity of TG2. (A) Work scheme: FLpepT26 peptide and S100A4(GST) are crosslinked by TG2 and after separation of the free peptide the isopeptide containing FLpepT26-S100A4(GST) serves as isopeptidase assay substrate. (B) Visualization of the isopeptide cleavage on FLpepT26-S100A4(GST); the monitored kinetic reactions were performed for 90 mins at 37°C then products were separated by 15% SDS-PAGE and detected in an UV gel documentation system.

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Representative gel image of FLpepT26 peptide (11.3 ng peptide, lane 1), separated FLpepT26-S100A4(GST) isopeptidase substrate (8.6 µg protein, lane 2), product of isopeptidase reactions on the substrate in the presence of 300 nM TG2 and 5 mM EDTA (lane 3) or in the presence of 5 mM Ca^{2+} and different TG2 concentrations (0.06, 0.3, 0.6, 1.2 µM, lane 4-7, respectively; 2.8 µg substrate protein). (C) Real-time monitoring of the isopeptide cleavage by measuring the anisotropy change using 5 µg substrate protein mixture in 35 µl reaction mix (0.5 µM FLpepT26-S100A4(GST)), 5 mM Ca^{2+} or EDTA and various concentrations of TG2. (D) The rate of anisotropy change shows linear correlation between 10-300 nM TG2 concentration. The inset shows the linear range analysed by GraphPad Prism 5 using Pearson correlation analysis ($P<0.0001$, $r=0.990$). Data are presented as means with ±SD from three separate experiments done in triplicate.

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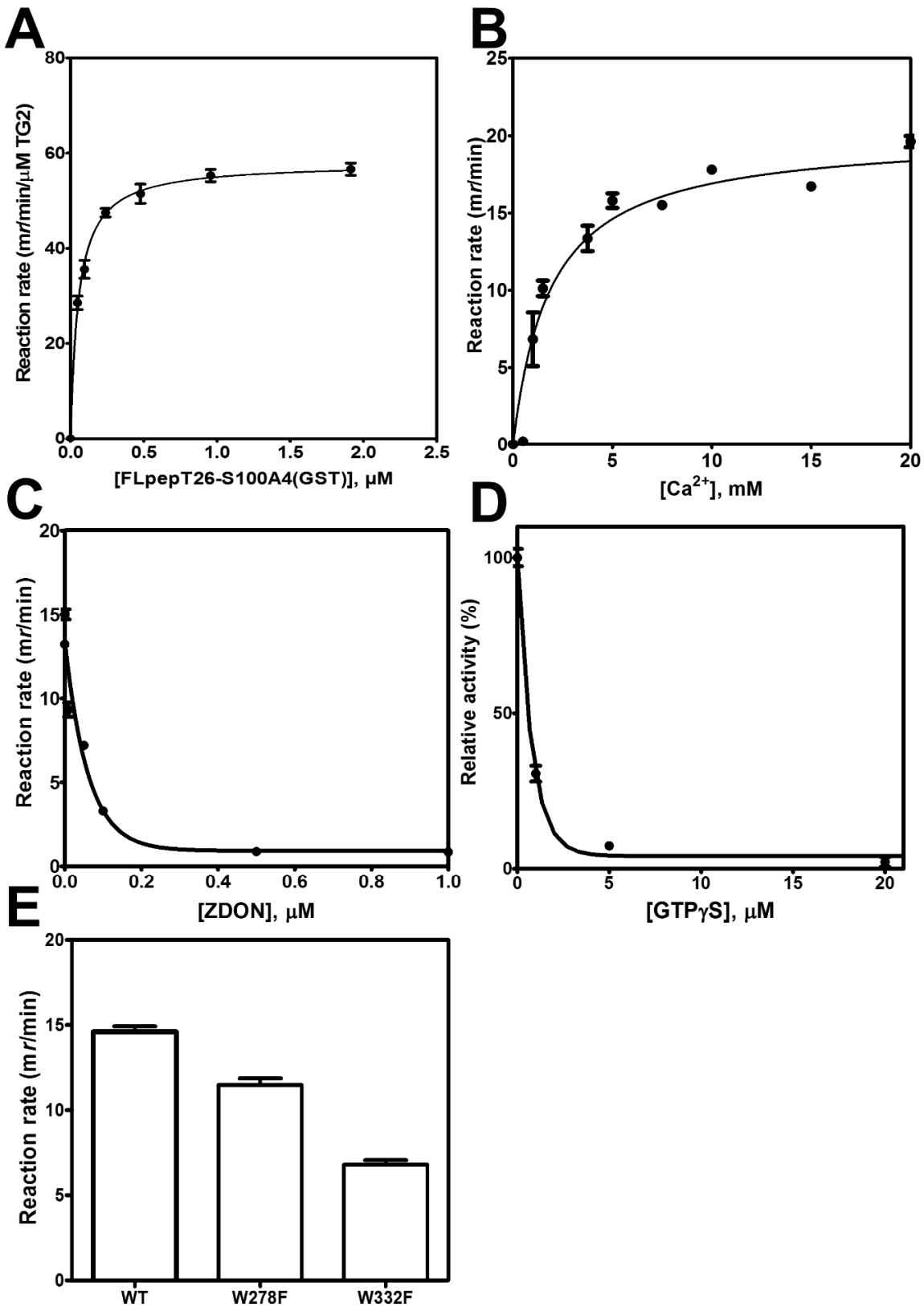

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Fig 2 Characterisation of the newly developed protein based isopeptidase assay. Determination of the Michaelis constant (A), the Ca^{2+}-dependence (B), the inhibitory effect of ZDON (C) and GTPγS (D). ZDON inhibitor was tested after 5 minutes preincubation in the presence of 5 mM Ca^{2+}, 0.3 μM TG2 and 5 μg of substrate while in the case of GTPγS-dependence the Ca^{2+} concentration was 2 mM. Mutants (E) were also tested in the presence of 5 mM Ca^{2+} and at 300 nM TG2 concentration. In all negative control experiments 5 mM EDTA was present instead of Ca^{2+}. Data are presented as means with ±SD from two separate experiments done in triplicate.