METHOD

Optimised methods (SDS/PAGE and LC-MS) reveal deamidation in all examined transglutaminase-mediated reactions

Éva Sivadó¹, Meddy El Alaoui¹, Robert Kiraly², László Fesüs², Frédéric Delolme³, Adeline Page³ and Saïd El Alaoui¹

¹ Research Department, Covalab S.A.S, Lyon, France
² Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Debrecen, Hungary
³ Protein Science Facility, SFR BioSciences CNRS UMS 3444, Inserm US 8, ENS, UCBL, Lyon, France

Keywords
deamidation; fluorescence detection; SDS/PAGE; transamidation; transglutaminases

Correspondence
Saïd El Alaoui, Covalab S.A.S, Research Department, 11 Avenue Albert Einstein, 69100 Villeurbanne, Lyon, France
E-mail: elalaoui@covalab.com

(Received 30 July 2018, revised 19 November 2018, accepted 20 November 2018)
doi:10.1002/2211-5463.12575

Transglutaminases (TGs; EC 2.3.2.13) are widely distributed enzymes with pleiotropic functions. Nine members have been described in mammals: keratinocyte (TG1), tissue (TG2), epidermal (TG3), prostate (TG4), type 5 (TG5), neuronal (TG6), type 7 (TG7), blood coagulation factor XIII A-subunit (FXIII-A), and the catalytically inactive erythrocyte band 4.2 protein [1].

The distribution and physiological roles of TGs have been investigated in various cell types and tissues. TG2 is the most studied member of the TGs family. This multifunctional protein has diverse cellular localisation and is implicated in several physiological (regulation of cell survival/death processes, cell adhesion, migration, signal transduction, proliferation) and pathological processes (coeliac disease, neurodegenerative disorders,

Abbreviations
ESI, electrospray ionisation; FAM, fluorescein amidite; FXIII-A, blood coagulation factor XIII A-subunit; HPLC, high-performance liquid chromatography; LC-MS, liquid chromatography–mass spectrometry; PTM, post-translational modification; TG, transglutaminase.
fibrosis, inflammatory diseases, metabolic diseases and cancer) [2]. TG1 mainly exists in the upper spinous and granular layers of the skin; it is involved in the terminal differentiation of keratinocytes by the formation of the crosslinked cell envelope [3]. Thrombin-activated factor FXIII-A plays an essential role in the stabilisation of fibrin clots and in wound healing through the formation of isopeptide bonds [4].

TGs catalyse Ca$^{2+}$-dependent post-translational modifications of proteins by generating a protein–protein crosslink (between a specific γ-carboxamide group of a glutamine and an ε-amino group of a lysine side chain), amine incorporation or deamidation. In the first step active site cysteine reacts with the γ-glutaminy l group of the proteins or peptides leading to the formation of a thiolester intermediate. In the second step the acyl group is transferred to an amine substrate resulting in the formation of an isopeptide bond or the water can act as an alternative nucleophile leading to site-specific deamidation of the glutamine residue [5,6]. Many of these crosslinking reactions occur within 5–10 min, for example in case of FXIII-A between glutamine (Gln398 or -399) and lysine (Lys406) residues of the fibrin γ-chains [7], whereas crosslinking of fibrin α-chains involving Gln221, -237, -328, or -366 and Lys208 or -606 takes place more slower [4,8].

Deamidation of glutamine and asparagine residues is one of the most prevalent post-translational modifications and converts an uncharged amino acid to a negatively charged residue introducing alternations in the protein’s conformation. The protein function can be changed as it is determined by its global structure, and electrostatic protein–protein interaction can be modulated as well [9]. Deamidation can occur in different ways (non-enzymatically, by glutaminases or phosphate-activated glutaminases) regulating several biological processes [10–12]. Moreover, cytotoxic necrotising factors in Escherichia coli and necrotoxin in Bordetella, which are considered to be functional relatives of TGs, can induce the formation of stress fibres by the deamidation of Rho proteins [13].

Deamidation by TGs was believed to be a side reaction, taking place only in the absence of primary amines or at low pH when availability of amines is limited [5], but recently it has been reported that selective deamidation in small heat shock protein [14] and βB2- and βB3-crystallins [15] can occur in a substrate-specific manner. Respectively, the substrate affinity and reaction conditions can influence the propensity for deamidation and transamidation [16]. Many research works are focusing on the examination of TG crosslinking activity, which is connected to some diseases such as fibrosis and neurodegeneration. So far TG-mediated deamidation activity has been related to coeliac disease [17,18], and only a few reports have been published on its role in other physiopathological processes [15,19–21]. These studies support the potential role of TG-dependent deamidation in the regulation of biological processes.

In this study we report an optimised SDS/PAGE assay for the rapid and easy detection of both transamidated and deamidated peptides. TG1, TG2, FXIII-A enzymes and a panel of glutamine-containing peptides were examined to determine how the ratio of deamidation to transamidation can be influenced by some reaction parameters, such as affinity for substrates, amine donor concentration, Ca$^{2+}$ concentration and pH. The identity and the relative quantity of the reaction products were confirmed by liquid chromatography–mass spectrometry (LC-MS) analysis. Our results are well correlated with the published data, cited above. Unexpectedly, under our reaction conditions, we found that a particular deamidation always occurs in vitro, even at high excess of the acyl-acceptor substrates.

**Materials and methods**

**Materials**

All materials were obtained from Sigma-Aldrich (Lyon, France) except where otherwise indicated. Recombinant human TG2 (rho-TG2, cat. no. T002), recombinant human keratinocyte TG (TG1, cat. no. T009), and recombinant human blood coagulation factor XIII-A (FXIII-A, cat. no. T027) were purchased from Zedira (Darmstadt, Germany). Peptides were obtained from Covalab (Villeurbanne, France). MilliQ water for mass spectrometry analysis was obtained from an ELGA system (ELGA Labwater, Millipore, Lyon, France).

**Synthetic peptide substrates**

The peptides and their derivatives [fluorescein amidite (FAM)-labelled peptides and deamidated controls] were synthesis by Covalab according to the established method for the production of synthetic peptides using solid-phase peptide synthesis described by Merrifield et al. [22]. Their purity was determined by analytical and preparative reversed-phase HPLC and mass spectrometry. All peptides were dissolved in DMSO at a final concentration of 10 mM. Isoenzyme-specific glutamine-containing peptides have been reported by Sugimura et al. [23,24] and used to develop specific TG activity assays [25,26]. K9 is a natural TG2 sequence based on β-casein [27]. Among the natural reactive glutamine site chains in fibrinogen α-chains a short peptide sequence containing Q238 [8] was chosen for our experiments. The corresponding deamidated control peptides were synthesised by Covalab (Table 1).
**Transglutaminase reaction**

Five micromolar of specific glutamine donor substrates labelled with fluorescent FAM and cadaverine (5–1000 µM) as an acyl acceptor were incubated with their corresponding isoenzymes at 37 °C; 18 mM·L⁻¹ of TG1, TG2, or thrombin (1 U·mL⁻¹)–activated FXIII-A was applied in the assay buffer (20 mM Tris pH 7.2 or 8.0 or MES pH 5.0 or 6.0, 150 mM NaCl, 10 mM DTT) in the presence or absence of 10 mM EDTA. pH was 7.2, unless the effect of pH (5–8) was examined. The enzymatic reaction was initiated by the addition of 5 mM CaCl₂, except in the experiments in which the effect of Ca²⁺ was investigated (0.1–5 mM).

**SDS/PAGE assay**

The assay was previously reported by Kenniston et al. [28] and modified as briefly described below. After the incubation period the enzymatic reaction was stopped by boiling the sample in 6× SDS-loading buffer (9.3 w/w DTT, 12 wt% SDS, 47 v/v% glycerol, 0.06 wt% bromophenol blue in 0.5 m Tris/HCl, pH 6.8). The reaction products were run on SDS/PAGE (15% T, 2.6% C; T represents the total concentration of polyacrylamide monomer expressed in g per 100 mL and C is the percentage of bis-polyacrylamide) and visualised by fluorescence detection (Luminescent Image Analyzer LAS-1000 plus; Fujifilm, Dusseldorf, Germany).

**Liquid chromatography–mass spectrometry analysis**

The samples were diluted 10 times in a solution of 0.1% formic acid before analysis. Mass spectrometry analysis was performed on a linear ion trap LTQ Velos (Thermo Scientific, San Jose, CA, USA) with nano-electrospray ionisation (ESI) source coupled in-line to a nanoRSLC system Ultimate 3000 (Thermo Scientific, Germerring, Germany). One microlitre of sample was injected via the autosampler. Samples were first desalted and concentrated on a reverse phase precolumn (C18 PepMap100, 300 µm i.d. × 5 mm, 5 µm, 100 Å; Thermo Scientific) for 3 min at 20 µL·min⁻¹ with H₂O/acetonitrile 98/2–0.1% formic acid. Samples were then separated on a nanocolumn (Acclaim C18, 15 cm × 75 µm i.d., 2 µm; Thermo Scientific). The HPLC gradient was 5–55% solvent B (A = 5% acetonitrile, 0.1% formic acid; B = 80% acetonitrile, 0.1% formic acid) for 30 min followed by 5 min 99% B. The total duration was set to 50 min at a flow rate of 300 nL·min⁻¹. The oven temperature was kept constant at 40 °C.

MS spectra were recorded in the mass range m/z 500–1100 in positive ionisation mode; the enhanced scan rate was used for the full MS spectrum.

**Data analysis**

Relative intensity of fluorescent bands was analysed with IMAGEJ and plotted with PRISM 5 (GraphPad Software Inc., San Diego, CA, USA) software.

**Results and discussion**

**Determination of transamidation and deamidation rates by transglutaminases**

Post-translational modification (PTM) is one of the powerful regulatory elements that confer a specific function to each protein. Dysregulation of PTMs has been the object of a number of studies and was shown to be associated with several diseases [29]. Among the different type of PTMs, deamidation is unique as it has been shown to occur in different ways: spontaneously, chemically and enzymatically [30].

Deamidation is the conversion of selected glutamine and also asparagine residues into glutamate and aspartate/isoaspartate through hydrolytic reaction. Such reaction requires only water, is conditioned by both sequence and structure, and is facilitated by physico-chemical conditions such as high temperatures, extreme pH or high ionic strength. Deamidation of specific proteins such as adrenocorticotropic [31] and lens crystallins [32] has been shown to occur in different ways: spontaneously, chemically and enzymatically [30].

Deamidation of specific proteins such as adrenocorticotropic [31] and lens crystallins [32] has been shown to have biological repercussions due to folding changes and/or modification of life-span. Although some proteins can undergo the deamidation reaction with little or no loss of biological activity, others do lose activity, which can be associated with a growing list of pathological phenomena such as age-related, neurological and autoimmune diseases [33].

While in vivo deamidation of Asn to Asp was clearly established, TG-mediated deamidation of Gln is not fully understood and rather it was believed to be only a side-reaction, occurring when the second substrate is not available or at acidic pH. However, over about a decade many reports focused on the study of the
mechanism of hydrolysis of the specific Gln residues in order to elucidate the role of TGs in the physiopathology of some diseases and coeliac disease in particular.

In order to evaluate the deamidation reaction of TG to convert Gln to Glu, several methods were used depending on whether the Gln substrates were natural (e.g. proteins) or synthetic (e.g. peptides). In the case of proteins, deamidations were analysed through molecular modelling and biological activity [34–36] whereas synthetic peptides were analysed by various techniques such as mass spectrometry (MS and LC-MS), size exclusion chromatography, capillary electrophoresis, HPLC, 2D gels and western blot [14].

In this work we optimised an electrophoresis assay based on SDS/PAGE for the rapid and easy detection of both transamidated and deamidated peptides. All reaction products of FAM fluorescein-labelled peptides could be simultaneously detected with high resolution and specificity. The relative ratio of deamidation and transamidation was evaluated by densitometric analysis (Fig. 1B,E). The identity and the proportion of the reaction products were confirmed by nano-LC–nano-ESI-MS analysis. The relative quantities were calculated from extraction ions chromatogram areas of the doubly charged ions of each species (Fig. 1C,F, Figs S1, S2, Table S1), assuming that all the species have the same response factors. The data obtained by LC-MS are in good correlation with the results of densitometric analysis, indicating that the optimised SDS/PAGE assay is a reliable method for the semi-quantitative examination of both transamidated and deamidated reaction products.

**Catalytic activity of transglutaminases always generates deamidated peptide products**

In this study TG1, TG2, FXIII-A enzymes and five glutamine-containing peptides (K5, T26, F11, K9 and αC(Q328)) (Table 1) were examined to determine how the ratio of deamidation to transamidation can be influenced by some reaction parameters such as substrates affinity, amine donor concentration, Ca$^{2+}$ concentration and pH. K5 [23], T26 and F11 [24] were described as isoenzymes preferring glutamine substrates having high affinity to TG1, TG2 and FXIII-A, respectively, whereas K9 [27] and fibrinogen αC(Q328) peptides [8] are known as natural glutamine donor substrates for TG2 and FXIII-A.

![Figure 1](image_url)

**Fig. 1.** Relative quantification of TG reaction products using SDS/PAGE analysis and fluorescence detection. TG2 was incubated with FAM-K9 peptide (A–C), thrombin activated FXIII-A with FAM-fibrinogen αC(325–336) peptide (D–F) and with cadaverine in the presence of Ca$^{2+}$ for 20 min (TG2) or 5 h (FXIII-A) at 37 °C. Q, reactive glutamine-containing control peptide; E, deamidated control peptide. (B, E) Densitometry analysis of the SDS/PAGE. (C, F) Relative quantification of the reaction products by nano-LC–nano-ESI-MS.
Applying high excess of the amine donor substrate (cadaverine), the ratio of transamidation to deamidation was increased, whereas deamidation was favoured at low cadaverine concentrations. In the absence of the second substrate, no crosslinked product was detected (Fig. 2). In addition, this effect appeared to be substrate dependent and confirm the results obtained by the group of Sollid [16]. TG2 transamidase activity was rather higher with K9 (Fig. 2B) than with T26 peptide (Fig. 2C) indicating better recognition of natural glutamine substrate. This difference was not observed with FXIII-A as the reactivity is higher with F11 than with fibrinogen αC(Q328) peptides. Indeed, upon the same reaction conditions the relative ratio of deamidated/transamidated fibrinogen αC(Q328) peptide was increased (Fig. 2E) whereas F11 peptide was preferred for transamidation (Fig. 2D). This slight difference in the deamidation/transamidation rate may be explained by the influence of the neighbouring amino acids relative to the targeted Gln and may affect the affinity of the enzymes. Indeed Boros et al. [15] reported that TG2 can process site-specific deamidation, and a proline at position +2 to the specific glutamine residue may positively influence deamidation [35].

Moreover, the results reveal that TG2- and FXIII-A-catalysed substrate deamidation could always occur as an excess of primary amine did not completely inhibit deamidation (Fig. 1B–E). However, deamidation by TG1 is less obvious in all conditions studied and this could explain why K5 may not be good substrate of this enzyme for deamidation (Fig. 2A). Using natural and good substrate for TG1 will be of interest in completing this work.

**Effect of pH on the transglutaminase-catalysed deamidation**

Fleckenstein et al. [35] described that the TG2-catalysed reaction of gliadin substrate is strongly influenced by pH. Here, we extended the investigation for two other isoenzymes and five peptide substrates as detailed in the Materials and methods (Fig. 3). In all cases the transamidation and deamidation reactions were analysed in buffers with pH values ranging from 5 to 8. TG1 (Fig. 3A) and FXIII-A (Fig. 3D,E) seem to not be active at acidic pH, but at pH 6.0 fibrinogen αC(Q328) peptide was deamidated in the majority of cases (Fig. 3E). At pH 8.0 all the peptides were converted to transamidated forms by all the corresponding enzymes. In the condition of neutral pH, TG1 was not active due probably to the low kinetic reaction, whereas TG2 and FXIII-A were able to convert the peptides to transamidated and deamidated products. The proportion of each product depends on the type of the enzyme and also the glutamine substrate. T26 was highly deamidated by TG2 whereas the natural substrate K9 was in the majority of cases transamidated (Fig. 3B,C) and with FXIII-A the deamidation was more important than the transamidation for both substrates (Fig. 3D,E). These results further demonstrate that the rate of the transamidation reactions is significantly increased at alkaline pH indicating the importance of the nucleophilicity of the amine, which must be unprotonated. As the pKa of the cadaverine amine is around 10, it is expected that the transamidation reaction can be favoured over deamidation. Indeed in the report of Fleckenstein et al. [35] using 5-(biotinamido)pentylamine as an amine donor with pKa around 10.5, a general base-catalysed decacylation mechanism was proposed for the transamidation reaction through a nucleophilic attack on the thiol ester.
intermediate. Increasing the pH to narrow the pK$_a$ of the base would decrease its protonation, and consequently the competition by water molecules is blocked explaining the increase of transamidation rate.

**Effect of Ca$^{2+}$ on the transglutaminase-catalysed deamidation**

It is well known that Ca$^{2+}$ is required for the activation of TGs through inducing a large conformational change in the enzyme structure [37,38]. Because of the large difference in the open and closed conformations, we hypothesised that depending on Ca$^{2+}$ binding, more transient conformers with a different hydrodynamic radius can be exhibited. Probably at lower Ca$^{2+}$ concentrations the substrate-binding channel could be particularly covered and the entrance of the second substrate could be inhibited resulting on the deamidation of the glutamine donor substrate via hydrolysis. Transamidation and deamidation were analysed with different Ca$^{2+}$ concentrations ranging from 0.1 to 5 mM as described in Materials and methods (Fig. 4). At lower Ca$^{2+}$ concentration (0.1–0.2 mM) TG2 seems to not be active (Fig. 4B,C) whereas TG1 converted the majority of K5 to transamidated product (Fig. 4A). With FXIII-A we observed high activity with F11 substrate (Fig. 4D) and low activity with natural substrate (Fig. 4E), which can be explained by the high affinity of the enzyme to the synthetic substrate. By increasing Ca$^{2+}$ concentration the enzymes were more active and both transamidation and deamidation occurred at a different rate depending on the Gln substrate: transamidation was observed more with TG1 and TG2 using K5 and T26 substrates, respectively, and more deamidation was obtained with TG2 and FXIII-A using K9 and fibrinogen αC(Q328). Based on these results no significant effect of calcium on the ratio of transamidation/deamidation was obtained.

In conclusion, our assay based on SDS/PAGE provides an easy and rapid method for monitoring the TG reaction with synthetic small peptides. The
fractions of native, deamidated and transamidated peptides can be successfully separated and their rates were confirmed by nano-LC–nano-ESI-MS. Under our experimental conditions we noticed that a particular deamidation always occurs, even at high molar concentration (148–200-fold molar excess) of amine donor substrate and it is preferred at low to neutral pH. These data provide further evidence for the simultaneous reactions of transamidation and deamidation, but further work will be needed to confirm this in vitro and in vivo at the endogenous proteins level. Knowledge of the in vivo regulation of transamidation versus deamidation is therefore crucial for the elucidation of the TG enigma. Most of the TG activity assays are based on detection of the transamidated products. Since the deamidation is no longer believed to be a side reaction, TG activity cannot be accurately evaluated only by detecting the crosslinked products.

Acknowledgements

We would like to thank Agnès Degiuli (University Lyon 1, CNRS, INSA, CPE-Lyon, Institute for Molecular and Supramolecular Chemistry and Biochemistry) for providing free access to the Luminescent Image Analyzer (LAS-1000 plus; Fujifilm). ES was supported by a Marie Curie research grant (‘TRANSPATH’, FP7 No 289964) and RK was supported by Janos Bolyai Research Fellowship of the Hungarian Academy of Science.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

ES performed the majority of the experiments, analysed and interpreted the data and contributed to the writing of the paper; MEA contributed to the experiments and writing of the paper; AP and FD performed LC-MS analysis; RK and LF contributed to the writing of the paper; SEA conceived the project and contributed to the writing of the paper.

References

1 Griffin M, Casadio R and Bergamini CM (2002) Transglutaminases: nature’s biological glues. Biochem J 368, 377–396.
2 Fesus L and Piacentini M (2002) Transglutaminase 2: an enigmatic enzyme with diverse functions. Trends Biochem Sci 27, 534–539.
3 Kim SY, Chung SI, Yoneda K and Steinert PM (1995) Expression of transglutaminase 1 in human epidermis. J Invest Dermatol 104, 211–217.
4 Arienès RAS, Lai T, Weisel JW, Greenberg CS, Grant PJ and Ariës RAS (2002) Role of factor XIII in fibrin clot formation and effects of genetic polymorphisms. Blood J 100, 743–754.
5 Folk JE (1983) Mechanism and basis for specificity of transglutaminase-catalyzed ε-(γ-glutamyl) lysine bond formation. Adv Enzymol Relat Areas Mol Biol 54, 1–56.
6 Lorand L and Graham RM (2003) Transglutaminases: crosslinking enzymes with pleiotropic functions. Nat Rev Mol Cell Biol 4, 140–156.
7 Chen R and Doolittle RF (1971) Gamma-gamma crosslinking sites in human and bovine fibrin. Biochemistry 10, 4486–4491.
8 Matsuka Y, Medved LV, Migliorini MM and Ingham KC (1996) Factor XIIIA-catalyzed cross-linking of recombinant alpha C fragments of human fibrinogen. Biochemistry 35, 5810–5816.
9 Berg J, Tymoczko J and Stryer L (2002) Biochemistry, 5th edn. W H Free, New York. ISBN 0-7167-3051-0, pp. 319–344.
10 Robinson NE and Robinson AB (2001) Molecular clocks. Proc Natl Acad Sci U S A 98, 944–949.
11 Hensley CT, Wasti AT and Deberardinis RJ (2013) Glutamine and cancer: cell biology, physiology, and clinical opportunities. J Clin Invest 123, 3678–3684.
12 Schousboe A, Scafidi S, Bak LK and Waagepetersen HSM (2014) Glutamate metabolism in the brain focusing on astrocytes. Adv Neurobiol 11, 11–30.
13 Schmidt G, Selzer J, Lerm M and Aktories K (1998) The Rho-deamidating cytotoxic necrotizing factor 1 from Escherichia coli possesses transglutaminase activity: cysteine 866 and histidine 881 are essential for enzyme activity. J Biol Chem 273, 13669–13674.
14 Boros S, Åhrman E, Wunderink L, Kamps B, De Jong WW, Boelens WC and Emmanuelsen CS (2006) Site-specific transamidation and deamidation of the small heat-shock protein Hsp20 by tissue transglutaminase. Proteins Struct Funct Genet 62, 1044–1052.
15 Boros S, Wilmarth PA, Kamps B, de Jong WW, Bloemendal H, Lampi K and Boelens WC (2008) Tissue transglutaminase catalyzes the deamidation of glutamines in lens βB2- and βB3-crystallins. Exp Eye Res 86, 383–393.
16 Stamnaes J, Fleckenstein B and Solld LM (2008) The propensity for deamidation and transamidation of peptides by transglutaminase 2 is dependent on substrate affinity and reaction conditions. Biochim Biophys Acta 1784, 1804–1811.
17 Van De Wal Y, Kooy Y, Van Veelen P, Peña S, Mearin L, Papadopoulos G, Van De Wal Y, Kooy Y, Van Veelen P and Pen S (1998) Cutting edge: selective deamidation by tissue transglutaminase strongly enhances gliadin-specific T cell reactivity. J Immunol 161, 1585–1588.

18 Molberg Ø, McAdam SN, Körner R, Quaasen H, Kristiansen C, Madsen L, Fugger L, Scott H, Norén O, Roepstorff P et al. (1998) Tissue transglutaminase selectively modifies gliadin peptides that are recognized by gut-derived T cells in celiac disease. Nat Med 4, 713–717.

19 Nemes Z, Demény M, Marekov LN, Fésüs L and Steinert PM (2000) Cholesterol 3-sulfate interferes with cornified envelope assembly by diverting transglutaminase I activity from the formation of cross-links and esters to the hydrolysis of glutamine. J Biol Chem 275, 2636–2646.

20 Schmid AW, Condepi E, Tuchserer G, Chiappe D, Mutter M, Vogel H, Moniatté M and Tsymbin YO (2011) Tissue transglutaminase-mediated glutamine deamidation of β-amyloid peptide increases peptide solubility, whereas enzymatic cross-linking and peptide fragmentation may serve as molecular triggers for rapid peptide aggregation. J Biol Chem 286, 12172–12188.

21 Iwai K, Shibukawa Y, Yamazaki N and Wada Y (2014) Transglutaminase 2-dependent deamidation of glyceraldehyde-3-phosphate dehydrogenase promotes trophoblastic cell fusion. J Biol Chem 289, 4989–4999.

22 Merrifield RB (1963) Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. J Am Chem Soc 85, 2149–2154.

23 Sugimura Y, Hosono M, Kitamura M, Tsuda T, Yamamishi K, Maki M and Hitomi K (2008) Identification of preferred substrate sequences for transglutaminase 1 – development of a novel peptide that can efficiently detect cross-linking enzyme activity in the skin. FEBS J 275, 5667–5677.

24 Sugimura Y, Hosono M, Wada F, Yoshimura T, Maki M and Hitomi K (2006) Screening for the preferred substrate sequence of transglutaminase using a phage-displayed peptide library. J Biol Chem 281, 17699–17706.

25 Thomas V, El Alaoui S, Massignon D, Clement S, Simonet F and Quash G (2006) Development and evaluation of a modified colorimetric solid-phase microassay for measuring the activity of cellular and plasma (Factor XIII) transglutaminases. Biotechnol Appl Biochem 43, 171–179.

26 Perez Alea M, Kitamura M, Martin G, Thomas V, Hitomi K and El Alaoui S (2009) Development of an isoenzyme-specific colorimetric assay for tissue transglutaminase 2 cross-linking activity. Anal Biochem 389, 150–156.

27 Cleary DB and Maurer MC (2006) Characterizing the specificity of activated Factor XIII for glutamine-containing substrate peptides. Biochim Biophys Acta 1764, 1207–1217.

28 Kenniston JA, Conley GP, Sexton DJ and Nixon AE (2013) A homogeneous fluorescence anisotropy assay for measuring transglutaminase 2 activity. Anal Biochem 436, 13–15.

29 Huang Q, Chang J, Cheung MK, Nong W, Li L, Lee MT and Kwan HS (2014) Human proteins with target sites of multiple post-translational modification types are more prone to be involved in disease. J Proteome Res 13, 2735–2748.

30 Riggs DL, Gomez SV and Julian RR (2017) Sequence and solution effects on the prevalence of α-isomers produced by deamidation. ACS Chem Biol 12, 2875–2882.

31 Dixon H and Stack-Dunne M (1955) Chromatographic studies on corticotropin. Biochem J 3, 483–495.

32 Kramps JA, de Jong WW, Wollensak J and Hoenders HJ (1978) The polypeptide chains of α-crystallin from old human eye lenses. Biochim Biophys Acta 533, 487–495.

33 Lindner H and Hellinger W (2001) Age-dependent deamidation of asparagine residues in proteins. Exp Gerontol 36, 1551–1563.

34 Sjöström H, Lundin KEA, Molberg Körner R, Mcdam SN, Anthonsen D, Quaasen H, Norén O, Roepstorff P, Thorsey E and Sollid LM (1998) Identification of a gliadin T-cell epitope in coeliac disease: general importance of gliadin deamidation for intestinal T-cell recognition. Scand J Immunol 48, 111–115.

35 Fleckenstein B, Molberg Ø, Qiao SW, Schmid DG, Von Mülle F, Der Elgstoen K, Jung G and Sollid LM (2002) Gliadin T cell epitope selection by tissue transglutaminase in celiac disease. Role of enzyme specificity and pH influence on the transamidation versus deamidation reactions. J Biol Chem 277, 34109–34116.

36 Fleckenstein B, Molberg Ø, Qiao S, Schmid DG, von der Mülle F, Elgstoen K and Sollid LM (2002) Gliadin T cell epitope selection by tissue transglutaminase in Celiac Disease. J Biol Chem 277, 34109–34116.

37 Di Venere A, Rossi A, De Matteis F, Rosato N, Agró AF and Mei G (2000) Opposite effects of Ca2+ and GTP binding on tissue transglutaminase tertiary structure. J Biol Chem 275, 3915–3921.

38 Pinkas DM, Strop P, Brunger AT and Khosla C (2007) Transglutaminase 2 undergoes a large conformational change upon activation. PLoS Biol 5, 2788–2796.
Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** Nano-LC–nano-ESI-MS analysis transglutaminase reaction products eXtracted Ions Chromatograms (XIC). TG2 was incubated with FAM-K9 peptide in the absence (a) or with 150 μM of cadaverine (b) in the presence of Ca²⁺ for 20 min at 37 °C. From top to bottom, XICs of m/z 649.84 from FAM-K9 peptide native form, m/z 692.40 from FAM-K9 peptide transamidated form and m/z 650.32 from FAM-K9 peptide deamidated form (MA = peak area).

**Fig. S2.** Nano-LC–nano-ESI-MS analysis of transglutaminase reaction products eXtracted Ions Chromatograms (XIC). Thrombin-activated FXIII-A was incubated with FAM-fibrinogen αC(325–336) peptide in the absence (a) or with 1000 μM of cadaverine (b) in the presence of Ca²⁺ for 5 h at 37 °C. From top to bottom, XICs of m/z 770.36 from FAM-fibrinogen αC(325–336) peptide native form, m/z 770.88 from FAM-fibrinogen αC(325–336) peptide deamidated form and m/z 812.88 from FAM-fibrinogen αC(325–336) peptide transamidated form (MA = peak area).

**Table S1.** Molecular mass and m/z (mass-to-charge ratio) of K9 and fibrinogen αC(325–336) glutamine donor peptides.