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Synthetic substrates as amine donors and acceptors in microbial transglutaminase-catalysed reactions

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ABSTRACT

Microbial transglutaminase (EC 2.3.2.13) (mTGase) catalyses a calcium-independent acyl-transfer reaction in which ε-(γ-glutamyl)lysine bonds are formed using the γ-carboxyamide groups of peptide-bound glutamine residues and the amino group of lysine side-chains. Here we present a comparative study on alternative lysine and glutamine substitutes in mTGase catalysis. A homologous series of ω-amino acids, serving as lysine substitutes, was incorporated into carbobenzoxy-L-glutaminylglycine (CBZ-Gln-Gly). The rate constants and particular conversion rates increased with increasing chain length. As for the glutamine substitutes, adipic diamide, glutaric monoamide, and glutaric diamide were converted with monodansylcadaverine (DNS-cadaverine) under mTGase catalysis. For the synthetic glutamine substitutes, the substrates of natural chain length, glutaric mono- and diamide, are better converted than the longer adipic diamide indicating that the window of opportunity seems to be smaller. Synthetic substrates, serving as amine acceptors, offer new opportunities in the field of transglutaminase-catalysed reactions.

1. Introduction

Food chemistry, food technology, and the pharmaceutical industry rely more and more on enzymatic processes as efficient, mild, and environmentally benign source of products [1–3]. Transglutaminases catalyse an acyl-transfer reaction in which ε-(γ-glutamyl)lysine bonds are formed using the γ-carboxyamide groups of peptide-bound glutamine residues and the amino groups of lysine side-chains. In contrast to, e.g. lipase or esterase, which catalyse hydrolytic cleavage in the presence of water and, therefore, require organic solvents, transglutaminase provides an opportunity to operate in aqueous systems without causing hydrolysis. The group of transglutaminases can be classified according to their particular occurrence and scope. The microbial transglutaminase (mTGase), derived from a variant of *Streptomyces mobaraensis*, has an essential advantage in comparison to tissue transglutaminases, such as guinea pig liver transglutaminase, since it catalyses a calcium-independent acyl-transfer reaction. It is, therefore, frequently used in food industry to stabilize products against microbial deterioration by producing a better textural stability and water-binding ability. In addition mTGase has a relatively low-molecular weight of approximately 37 kDa, as compared to guinea pig liver transglutaminase with a molecular weight of 85 kDa, and broader pH and temperature optima [4–6]. Via fermentation it can be isolated easily from the bacteria and, therefore, the access to larger amounts of enzyme for potential industrial application is simplified.

In its natural reaction transglutaminase requires a γ-carboxyamide group of peptide-bound glutamine and the amino group of lysine side-chains. Besides cross-linking of natural proteins, such as casein, collagen, and gluten [7–9], amines, such as putrescine, spermine, *N,N′*-dimethyl-1,4-phenylenediamine, cadaverine, and hydroxylamine [10–18] have only been grafted to glutamine containing natural or synthetic proteins and peptides [7,19–23] without comparable reaction conditions. Nearly all of these reactions were performed with calcium-dependent tissue transglutaminase. Beside these incorporations, a qualitative study of ω-amino acids as the amine donor under mTGase catalysis was reported [24].

However, among the artificial lysine-analogues ω-amino acids stand out, due to their close resemblance of the lysine structure. As AB-reactive compounds, ω-amino acids are valuable substrates in organic and polymer chemistry and, therefore, merit a more detailed investigation.

Here we present a comparative study on the reactivity of ω-amino acids $H_2N–(CH_2)_n–CO_2H$ with $n = 4, 5, 6$ using simplistic pseudo-second-order kinetics. Additionally, we present an initial investigation of possible glutamine substitutes and a fully synthetic mTGase-catalysed reaction.

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2. Experimental

2.1. Materials

The transglutaminase (mTGase) used is ACTIVA WM (Ajinomoto Europe Sales GmbH) and is a fermentation product of *S. mobaraensis*.

Carbobenzoxy-glutaminylglycine (CBZ-Gln-Gly), 7-aminoheptanoic acid, glutarimide, and adipic diamide were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). 5-Aminovaleric acid was obtained from Alfa Aesar GmbH & Co. KG (Karlsruhe, Germany). 6-Aminohexanoic acid, glutaric anhydride, and monodansylcadaverine (DNS-cadaverine) were received from Fluka (Steinheim, Germany).

All reagents and solvents were of HPLC grade and were used without further purification.

2.2. Methods

For the HPLC measurements (Varian Pro Star) a gradient starting from 10% acetonitrile in water to 100% acetonitrile within 20 min at a flow rate of 1 ml/min was used. Both solvents contained 0.1% trifluoracetic acid (TFA). The injection volume was 20 μl. Compounds were detected by UV at 220 nm.

The MALDI-ToF/MS spectra were recorded on a Bruker Biflex III mass spectrometer α-Cyano-4-hydroxycinnamic acid (20 mg/ml) in water/ acetonitrile 2:1 (v/v) containing 0.1% TFA (TA) was used as matrix. The samples were prepared by the dried droplet method. 5 μl of each sample were mixed with 45 μl of TA. 0.5 μl of this mixture were pipetted onto the target and dried at room temperature.

2.3. Preparations

The mTGase was isolated from ACTIVA WM, which contains 99% maltodextrin. The enzyme was purified by cation exchange chromatography using 20 mM sodium phosphate buffer (NaP) containing 2 mM EDTA at pH 6. Proteins were eluted with a 0–1000 mM gradient of NaCl in 20 mM NaP and 2 mM EDTA at pH 6. The enzyme containing fractions were concentrated, and the total volume finally adjusted to 5 ml with bidistilled water. The purity was confirmed via sodiumdodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The activity of the microbial transglutaminase was determined by using the hydroxamate assay [25].

Glutaric monoamide [26] was synthesized from glutaric anhydride. 10 g of glutaric anhydride were dissolved in 50 ml of aqueous ammonia solution and the reaction mixture was left to stir overnight. The solution was then acidified with concentrated hydrochloric acid, upon which the solid precipitated. The solid was filtered, washed with bidistilled water and then dried overnight. The dried product was extracted with methyl-*tert*-buthyl ether. The ether was then evaporated to give the crystalline product in 35%. Mass spectrometry returned $m/z = 131.06$ ($C_5H_9NO_3$). The melting point was determined at 94 °C. The literature melting point of this compound is 93–94 °C [27]. The product is pure to HPLC.

Glutaric diamide [28] was synthesized from glutarimide. 10 g of glutarimide were dissolved in 100 ml aqueous ammonia solution. The reaction mixture was left to stir overnight after which the product had precipitated (20%). Mass spectrometry returned $m/z = 130.07$ ($C_5H_{10}N_2O_2$). The melting point was determined at 183 °C. The literature melting point of this compound is 182–183 °C [28]. The product is pure to HPLC.

2.4. Enzymatic transformations

The transglutaminase-catalysed reactions were run in an Eppendorf Thermomixer Comfort using a 50 mM ammonium bicarbonate buffer at pH 7.5 as reaction medium. The total volume of each experiment was 1 ml. The reactions were started by adding 400 μl of a 10 U/ml purified mTGase stock-solution and the mixtures were incubated at 37 °C and 450 rpm for up to 24 h. The sample composition and the conversion time profiles were established by HPLC analysis using 100 μl of a 1 mg/ml resorcinol stock-solution as internal standard. The given values could be reproduced in individual experiments within 5%.

For the reaction of the lysine substitutes, a 2-fold molar excess of the amines was added to the buffer containing 1 mg/ml CBZ-Gln-Gly. For the reaction of the glutamine substitutes, 100 μl of a 1.25 mg/ml DNS-cadaverine stock-solution was added to the buffer containing 5 mg of glutaric mono- or diamide.

The assessment of the reactivity of the glutamine substitutes was conducted in analogy to the transglutaminase activity assay from Sigma [25]. The reaction mixture containing 2 mg/ml adipamide, 200 μl of a 200 mM hydroxylamine/glutathione solution in 1 M Tris buffer, and 5 U/mg microbial transglutaminase was incubated at 37 °C for 24 h at 450 rpm. Control samples were set up with pure buffer replacing the transglutaminase. After incubation 100 μl of each sample were diluted with 250 μl trichloric acetic acid (TCA) and mixed by inversion. Then 250 μl $FeCl_3$ and 200 μl bidistilled water were added, mixed, and centrifuged. Then the mixture was transferred into suitable cuvettes and the absorbance was measured at 525 nm in a Varian Cary Win 100 Bio UV–vis spectrophotometer.

The glutamine substitutes were incubated with a 2-fold molecular excess of mono-dansylcadaverine and 10 U/ml transglutaminase at 37 °C for 24 h. The conversion was determined using MALDI-ToF/MS (glutaric monoamide: $[M+DNS\text{-}Cad]NH_4^+ = 467$ Da; glutaric diamide: $[M+DNS\text{-}Cad]2NH_4^+ = 488$ Da).

3. Results and discussion

3.1. Lysine substitutes

To test for possible lysine substitutes, a homologous series of ω-amino acids consisting of 5-aminovaleric acid, 6-aminohexanoic acid, which contains the amine number of carbon atoms as lysine, and 7-aminoheptanoic acid was chosen. Carbobenzoxy-L-glutaminylglycine (CBZ-Gln-Gly), which is part of the hydroxamate test for TGase activity [25] served as glutamine donor. The conversion was measured by using HPLC applying resorcinol as internal standard. Resorcinol was chosen as internal standard since control experiments revealed that it does not interfere with the catalytic activity. Table 1 shows the results after incubation with mTGase for 24 h.

A typical chromatogram after incubation (Fig. 1A) shows, besides the internal standard, the unreacted CBZ-Gln-Gly, the enzyme, and between those two a new signal, which in all cases

Table 1
Results for the mTGase-catalysed reaction of lysine substitutes with CBZ-Gln-Gly.

Substrate	Retention time (min) after 24 h	Conversion (%) after 24 h	Masses (Da) MALDI-ToF/MS after 24 h
5-Aminovaleric acid	9.2	40	461 $[M+Na]^+$
6-Aminohexanoic acid	9.6	64	467 $[M+NH_4]^+$
7-Aminoheptanoic acid	10.2	70	488 $[M+Na]^+$

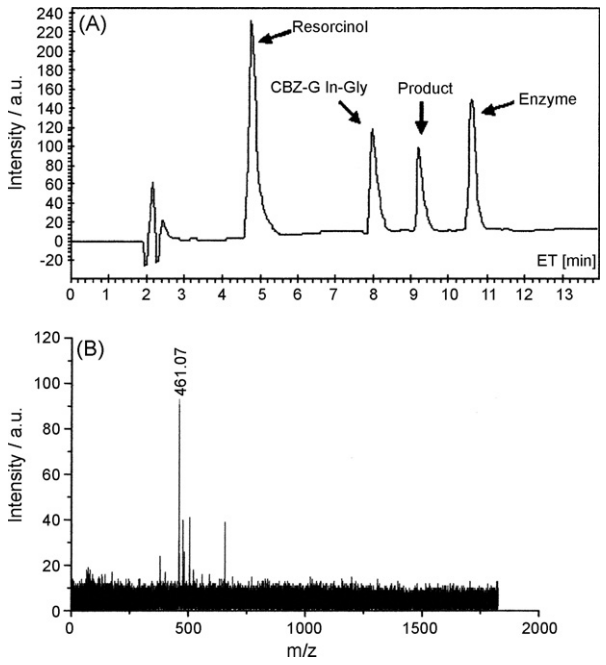


Fig. 1. mTGase-catalysed reaction of CBZ-Gln-Gly with 5-aminovaleric acid after 24 h: (A) HPLC trace; (B) MALDI-ToF/MS.

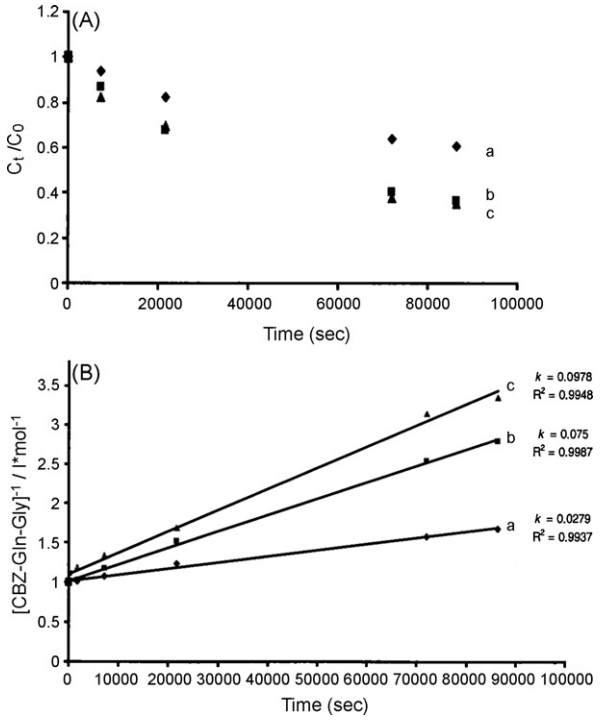


Fig. 2. (A) Decrease of CBZ-Gln-Gly concentration over time during mTGase-catalysed incorporation of (a) 5-aminovaleric acid; (b) 6-aminohexanoic acid; (c) 7-aminoheptanoic acid. (B) Determination of the pseudo-second-order rate constants of Fig. 2A (a–c).

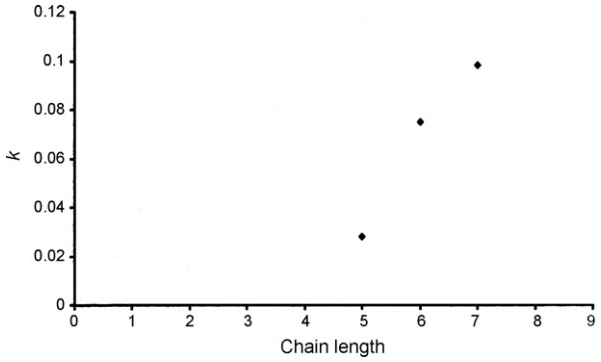


Fig. 3. Rate constant of the mTGase-catalysed reaction of ω-amino acids with CBZ-Gln-Gly in dependence on the chain length: 5: 5-aminovaleric acid; 6: 6-aminohexanoic acid; 7: 7-aminoheptanoic acid.

could be assigned to the particular addition product by MALDI-ToF/MS (Fig. 1B).

The starting ω-amino acids cannot be observed since they do not contain a chromophore.

The retention times of the products increase with the increasing chain length of the ω-amino acids from 9.2 min (5-aminovaleric acid) to 10.2 min (7-aminoheptanoic acid) due to the increased hydrophobicity.

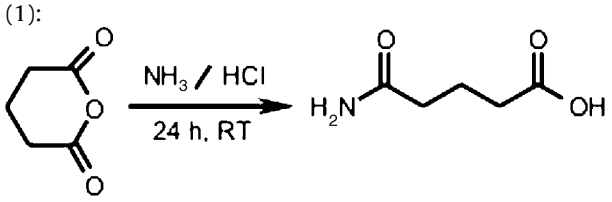
The conversion of the CBZ-Gln-Gly substrate increases from 40% (5-aminovaleric acid) to 70% (7-aminoheptanoic acid). To gain more insight into the reactivity of this homologous series, the conversion-time profiles were recorded (Fig. 2A). Under the premise of a 1:1 reaction stoichiometry the mTGase-catalysed acylation of lysine substitutes with CBZ-Gln-Gly can be treated as pseudo-second-order ($2\ A \rightarrow P$) and thus the rate law becomes $d[A]/dt = -k[A]^2$. For this a plot $1/[A]$ *vs.* t should be linear with the slope being the pseudo-rate constant k. This is indeed the case and the increase of k from 0.028 l mol^{-1} s^{-1} for C_5 to 0.098 l mol^{-1} s^{-1} for C_7 corroborates the increased reactivity of longer carbon chains in mTGase-catalysed reactions (Fig. 2B).

Fig. 3 demonstrates the change of k with the chain length. The run of the curve indicates that substrates with less than five carbon atoms would not react under mTGase catalysis. With increasing chain length, the curve appears to level off. This corresponds well with previous experimental findings [24].

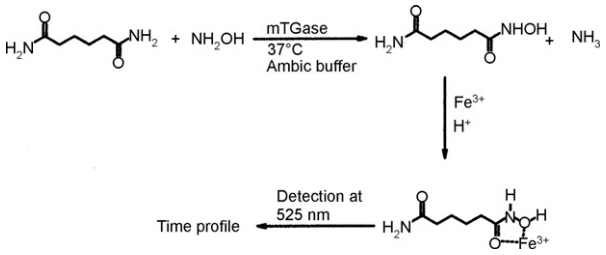
None of the examined reactions showed complete conversion. After 48 h of incubation, the increase in conversion as compared to 24 h was negligible. An mTGase activity test revealed a decrease of the enzyme activity after 24 h to approximately one-third, while after 48 h the enzyme activity had totally ceased. The enzyme has a high tendency for self-cross-linking, not detectable with SDS-PAGE due to the upper size limit of the gel (66,000 Da). However, a spot of considerable size was present at the starting point after the reaction, which indicates the formation of higher molecular-weight fractions. Cross-linking could potentially circumvented by immobilisation of the enzyme or higher dilution.

3.2. Glutamine substitutes

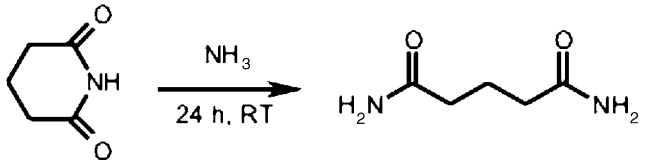
For this study adipic diamide, glutaric monoamide, and glutaric diamide were used as glutamine substitutes. Adipic diamide is commercially available. Glutaric monoamide [26] was prepared in 35% yield from glutaric anhydride according to Eq. (1):



Glutaric diamide [27] was prepared from glutaric imide according to Eq. (2) in 20% yield:



Scheme 1. Formation of the Fe^{3+}/hydroxamate complex instancing adipic diamide.



To assess the overall reactivity of these compounds, a preliminary test in analogy to the hydroxamate activity assay [25] was performed in which adipic diamide, glutaric monoamide, and glutaric diamide served as CBZ-Gln-Gly substitute. The progress of the reaction was followed spectrometrically at $\lambda = 525$ nm, which is the absorption maximum of the Fe^{3+} complex formed with hydroxamate (Scheme 1).

The complex is formed with all three glutamine substitutes (Fig. 4). Following the reaction with time, an increase in colour intensity due to the cumulative formation of the Fe^{3+}/hydroxamate complex is noticeable. Control experiments did not show the presence of compounds that form coloured complexes with Fe^{3+} in the starting materials, but their formation cannot be ruled out completely. The intensities are, therefore, only compared qualitatively under the premise that the local environments around the metal centres are similar for all three complexes. Two observations are evident: when comparing glutaric diamide (C_5) and adipic diamide (C_6) the substrate of natural chain length reacts

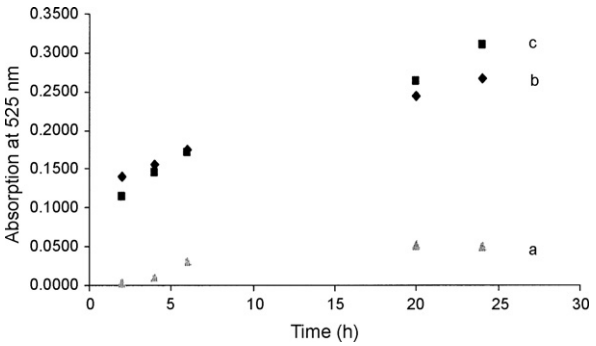


Fig. 4. Progress of the mTGase-catalysed reaction of glutamine substitutes with hydroxylamine determined colouremetrically after addition of Fe^{3+}: (a) adipic diamide; (b) glutaric monoamide; (c) glutaric diamide.

significantly faster and reaches a higher conversion than the substrate with one carbon atom too many. This is in contrast to the lysine substitutes, where 7-aminoheptanoic acid shows the highest rate and conversion. Secondly, considering the above restrictions, the rate of conversion is similar for glutaric mono- and diamide.

After having established that TGase tolerates both lysine and glutamine substitutes, an entirely artificial mTGase-catalysed reaction was attempted. For this glutaric diamide was incubated with DNS-cadaverine at 37 °C for 24 h. After this time, a total conversion of 87% and two new signals were detected (Fig. 5) in the HPLC trace. The product ration, i.e. monoadduct:diadduct, was formed to be 2:98. A simple Monte Carlo simulation of the reaction sequence outlined in Fig. 5 reveals that for these values the ratio of the pseudo-second-order rate constant k_1/k_2 of 0.13. The transformation of the second amide group is approximately 10 times faster than the first one indicating that the presence of a long substituent at one end of the molecule is beneficial for the introduction of the other substituent.

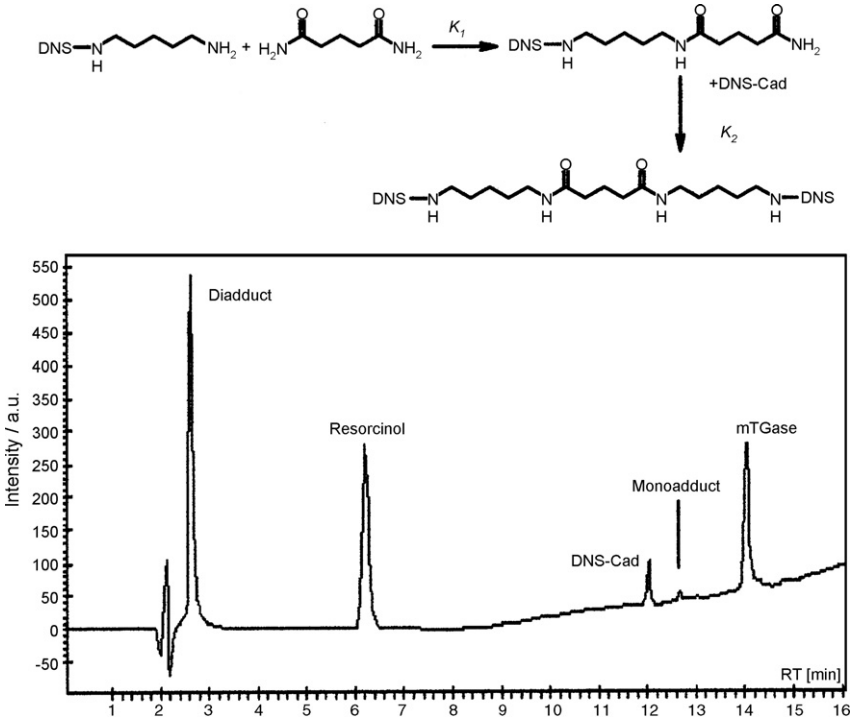


Fig. 5. Reaction sequence and HPLC trace of an mTGase-catalysed reaction of gluraric acid diamide with DNS-cadaverine after 24 h.

4. Conclusions

It was shown that the microbial transglutaminase, derived from a variant of *S. mobaraensis*, tolerates both synthetic lysine and glutamine substitutes. None of the tested reactions go to complete conversion as the enzyme likely cross-links itself during the reaction leading to its deactivation, but high conversions are possible with appropriate substrates. For the lysine substitutes, pseudo-second-order rate constants were calculated, which reveal that 7-aminoheptanoic acid is the best substrate in terms of reactivity and conversion. However, for ω-amino acids the set of possible substrates appears to be fairly small as 4-aminobutyric acid is not converted at all [24] and the rate constants for acids containing more than seven carbon atoms are extrapolated to be very low. For the synthetic glutamine substitutes, the substrates of natural chain length, glutaric mono- and diamide, are better converted than the longer adipic diamide indicating that the window of opportunity seems to be smaller. In an entirely artificial reaction, DNS-cadaverine is reacted with glutaric diamide to the mono- and diadduct. Having properly established the reactivity and potential of both synthetic lysine and glutamine substitutes, new opportunities in the field of transglutaminase-catalysed reactions such as protein and polymer functionalisation as well as modification of synthetic surfaces are at hand. Grafting of antimicrobial substances onto wool and polymer surfaces as well as a surface modification which enables specific reactions at distinct sites of polymers can be an environmental friendly and a future-oriented opportunity for the industries.

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