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Continuous enzyme-coupled assay for microbial transglutaminase activity

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ABSTRACT

Transglutaminases (protein-glutamine:amine γ-glutamyltransferase, EC 2.3.2.13) are a family of calcium-dependent enzymes that catalyze an acyl transfer between glutamine residues and a wide variety of primary amines. When a lysine residue acts as the acyl-acceptor substrate, a γ-glutamyl-ε-lysine isopeptide bond is formed. This isopeptide bond formation represents protein cross-linking, which is critical to several biological processes. Microbial transglutaminase (mTG) is a bacterial variant of the transglutaminase family, distinct by virtue of its calcium-independent catalysis of the isopeptidic bond formation. Furthermore, mTG's promiscuity in acyl-acceptor substrate preference highlights its biocatalytic potential. The acyl-donor substrate, however, is limited in its scope; the amino acid sequences flanking glutamine residues dramatically affect substrate specificity and activity. Here, we have developed and optimized a modified glutamate dehydrogenase assay with the intention of analyzing potential high-affinity peptides. This direct continuous assay presents significant advantages over the commonly used hydroxamate assay, including generality, sensitivity, and ease of manipulation. Furthermore, we identified 7M48 (WALQRPH), a high-affinity peptide that shows greater affinity with mTG (K_M = 3 mM) than the commonly used Cbz-Gln-Gly (K_M = 58 mM), attesting to its potential for application in biocatalysis and bioconjugation.

Transglutaminases (TGs,[1] protein-glutamine:amine γ-glutamyltransferase, EC 2.3.2.13) are a family of calcium-dependent enzymes that catalyze an acyl transfer between glutamine residues and a wide variety of primary amines [1]. When a lysine residue acts as the acyl-acceptor substrate, a γ-glutamyl-ε-lysine isopeptide bond is formed between two proteins [2] (Fig. 1). TG-mediated protein cross-linking is critical to several biological processes and diseases, including extracellular matrix stabilization [3], blood coagulation [4], formation of cataracts [5], type 2 diabetes [6], neurodegenerative diseases [7], and certain cancers [8].

Several TGs have been identified in mammalian tissues; although they play many diverse physiological roles, the requirement of calcium as a cofactor is pervasive with all mammalian members [9]. Microbial transglutaminases (mTGs) are a distinct variant of the transglutaminase family by virtue of their calcium-independent catalysis of the isopeptide bond formation [10,11]. Moreover, the bacterial enzyme is expressed as a zymogen and activated through N-terminal cleavage to the resulting 38-kDa mTG [11–13]. Isolated from *Streptomyces mobaraensis,* mTG has been characterized and shown to have very little sequence similarity to any mammalian TG, whose molecular weights are greater than 70 kDa [14]. Convergent evolution may explain why mTG shares a similar function as its mammalian counterparts but exhibits more robust catalytic properties, including high stability throughout a broad range of pH and temperatures in comparison with other TGs [15–17].

The broad substrate specificity of mTG makes it amenable as a tool for conjugation and opens up its potential as a biocatalyst. For instance, mTG cross-linking has been exploited in the conjugation of biodegradable polymers. Hydroxyethyl starch (HES) is a polymer that has been used as an effect agent for therapeutic drug delivery [18]. The conjugation of the polymer to drugs and proteins is normally executed chemically; however, mTG-mediated cross-linking presents a selective, less expensive alternative [18].

mTG's promiscuity with respect to its acyl-acceptor substrate highlights its biocatalytic potential. The acyl-donor substrate, however, is more limited in its scope; the amino acid sequences flanking glutamine residues dramatically affect their activity. For example, it has been shown that the most likely sequences for mTG specificity have similarities at residues –3, –1, +1, and +2

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[1] *Abbreviations used:* TG, transglutaminase; mTG, microbial transglutaminase; SPPS, solid-phase peptide synthesis; DMF, dimethylformamide; HBTU, *O*-benzotriazolyl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HPLC, high-performance liquid chromatography; FA, formic acid; EDTA, ethylenediaminetetraacetic acid; NADH, nicotinamide adenine dinucleotide (reduced form); GDH, glutamate dehydrogenase; α-KG, α-ketoglutarate.

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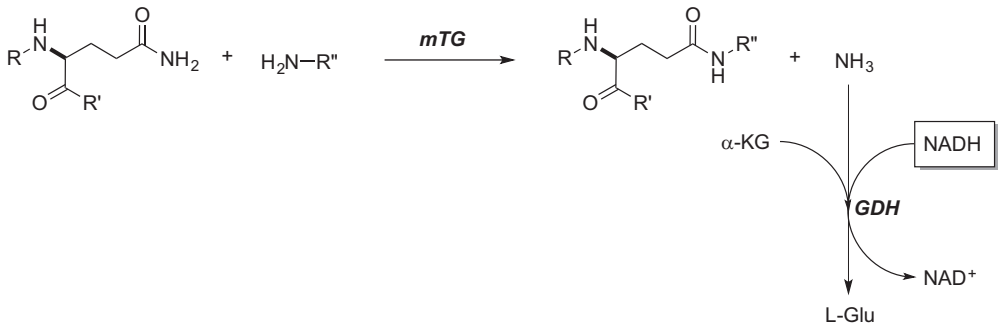

Fig.1. Glutamate dehydrogenase (GDH)-coupled microbial transglutaminase (mTG) activity assay. mTG catalyzes the acyl-transfer reaction from glutamine to a primary amine. (With Cbz-Gln-Gly, R = Cbz and R′ = Gly. With 7M48, R = WAL and R′ = RPH. R″-NH_2 = Gly-OMe). Released ammonia acts as a substrate in the GDH-catalyzed reductive amination of α-ketoglutarate (α-KG). This reaction results in the disappearance of the reduced form of nicotinamide adenine dinucleotide (NADH), monitored by its absorbance at 340 nm.

relative to the position of the glutamine residue, namely aromatic, leucine, arginine, and proline residues [19]. However, very few specific peptide sequences encompassed by these general preferences have been studied in enough detail to provide *quantitative* information regarding their affinity for mTG. For example, although Cbz-Gln-Gly is used as a standard acyl-donor substrate, its affinity for mTG is relatively low [20], requiring that it be used at relatively high concentrations. This illustrates the importance of identifying a high-affinity acyl-donor substrate for mTG if its full potential as a bioconjugation catalyst is to be realized.

In turn, the identification of a high-affinity acyl-donor substrate requires a rapid and convenient activity assay that can be applied to the screening of multiple peptides. However, the assay most commonly used for measuring mTG activity is the hydroxamate assay [21,22], a discontinuous method that requires high quantities of reagents and substrates in order to achieve reliable results. A sensitive, continuous, and rapid assay for mTG activity, therefore, is urgently required.

To that end, we have developed an enzyme-coupled assay in order to measure the activity of mTG with any Gln-containing peptide sequence. Based on a microtiter plate scale, this assay uses little material and is amenable to high-throughput screening applications. We applied it here to quantitatively measure the kinetic parameters for several high-affinity peptide sequences.

Materials and methods

Expression and purification of mTG

The plasmid pDJ1-3 was kindly provided by Joelle Pelletier (Université de Montréal). pDJ1-3 encodes the proenzyme of mTG from *S. mobaraensis* inserted between the *Nde*I and *Xho*I restriction sites of the vector pET20b [23]. mTG was expressed and purified according to a previously published protocol [24] with the following amendments. The plasmid encoding for mTG bearing a C-terminal hexahistidine tag was transformed into *Escherichia coli* BL21-Gold (DE3) in the presence of 100 μg/ml ampicillin. mTG was expressed according to the autoinduction protocol [25]. The culture was centrifuged at 3000*g* for 30 min at 4 °C; the supernatant was discarded and the pellet was resuspended in 50 mM phosphate buffer (pH 8.0) with 300 mM NaCl. Cells were disrupted by sonication over ice (three cycles of 30-s pulse at 20% intensity/1 min pause) using a Branson sonicator. mTG was then activated by cleavage of the proenzyme alpha sequence through incubation in a 1:10 ratio (w/v) of trypsin (1 mg/ml) to unpurified mTG for 75 min at 37 °C. The activated mTG was purified using a 1-ml HisTrap Ni-NTA column (GE Healthcare) equilibrated in 50 mM phosphate buffer (pH 8.0) with 300 mM NaCl and eluted with an imidazole gradient (0–140 mM) on an Åkta FPLC instrument (GE Healthcare). The purified activated mTG was dialyzed against 50 mM phosphate buffer (pH 8.0). The average yield was 75 mg of activated mTG per liter of culture, with greater than 80% purity as estimated through evaluation of 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by staining with Coomassie blue. Aliquots were snap-frozen and stored at −80 °C in 15% glycerol.

Peptide synthesis

Peptides were synthesized using an automated peptide synthesizer (Liberty CEM Microwave Peptide Synthesizer) by conventional Fmoc-based solid-phase peptide synthesis (SPPS). Synthesis was carried out on a scale of 200 mg of Knorr amide resin with a loading capacity of 0.93 mmol/g (0.25 mmol scale). Each round of peptide elongation consists of (i) Fmoc deprotection with 20% piperidine in dimethylformamide (DMF) for 5 min three times; (ii) washing with DMF; (iii) coupling with *O*-benzotriazolyl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), *N*,*N*-diisopropylethylamine (DIEA), *N*-methylpyrrolidinone (NMP), and the appropriate amino acid in DMF for 11 min; and (iv) washing with DMF. The molar ratio of the amino groups on the resin/building block/HBTU was 1:4:3.6. When the peptide was complete, acetylation at the native N terminus (to protect primary amine) was carried out using 20% pyridine in DMF. Peptide was then cleaved from the resin using 90:5:5 trifluoroacetic acid (TFA)/dichloromethane (DCM)/thianisole for 120 min. After cleavage, the resin was filtered and washed three times under vacuum using 1:1 cylcohexane/acetone. The peptide was dissolved in a minimal amount of acetone and precipitated from the solution using cold Et_2O with sonication. The precipitated peptide was then recovered through centrifugation at 1100*g* for 30 min.

HPLC analysis

High-performance liquid chromatography (HPLC) was performed on a Waters 2996 instrument equipped with a photodiode array detector. Ultraviolet (UV) absorbance of peptides was measured at 254 and 274 nm. Analytical HPLC was performed on a Waters C18 column (5 μm, 3.8 × 150 mm) with a run time of 30 min, a flow rate of 0.5 ml/min, and a linear gradient of 50–95% of 0.1% formic acid (FA) in methanol (eluent B) in 0.1% aqueous FA (eluent A).

Hydroxamate assay

The previously reported hydroxamate assay [22,26] was adapted to use with mTG as follows. First, 0.75 ml of 0.2 M acyl-donor substrate was mixed with 0.25 ml of 2 M hydroxylamine,