Protein Expression and Purification 87 (2013) 41–46

Contents lists available at SciVerse ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Expression, purification and kinetic characterisation of human tissue transglutaminase

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ARTICLE INFO

Article history:
Received 18 August 2012
and in revised form 3 October 2012
Available online 22 October 2012

Keywords:
Enzyme
Tissue transglutaminase
Recombinant expression
GST fusion
PreScission™ Protease

ABSTRACT

The expression of soluble recombinant transglutaminase (TGase) has proven to be a challenge for many research groups. Herein, we report a complementary method for the expression, in BL21(DE3) *Escherichia coli*, of recombinant human tissue transglutaminase (hTG2) whose solubility is enhanced through *N*-terminal fusion to glutathione *S*-transferase (GST). Moreover, we report the cleavage of the GST tag using PreScission™ Protease (PSP) and purification of hTG2 in its untagged form, distinctively suitable for subsequent studies of its remarkable conformational equilibrium. The effects of co-solvents and storage conditions on stability of purified hTG2 are also reported. Furthermore, we demonstrate for the first time the use of a convenient chromogenic assay to measure the activity of the human enzyme. The utility of this assay was demonstrated in the measurement of the kinetic parameters of a wide variety of substrates and inhibitors of both hTG2 and the extensively studied guinea pig liver TGase. Finally, comparison of these results provides further evidence for the functional similarity of the two enzymes.

Transglutaminases are Ca^{2+}-dependent enzymes that catalyse protein cross-linking through isopeptidic bond formation upon acyl transfer between the γ-carboxamide group of a protein-bound glutamine residue and a primary amine, usually the ε-amino group of another protein-bound lysine residue [1–3]. The protein cross-linking activity of TGases is critical to several physiological processes, including the stabilisation of fibrin blood clots [4,5] and deposition of extracellular matrices [6]. However, if their transamidase activity is not carefully regulated, physiological disorders may develop. Tissue TGase (TG2) overexpression and activity have been implicated in pathologies such as the deposition of amyloid plaques associated with Alzheimer's disease, neurodegenerative diseases and Celiac disease [7–11].

Transglutaminases have been crystallized in two dramatically different conformations [12–15], and TG2 is thought to adopt one of these conformations depending on its environment. Further, native gel electrophoresis studies carried out with either guinea pig liver TGase (gplTGase) or rat TGase have shown two bands having different migration times in the presence and absence of GTP (an 'allosteric' inhibitor of the enzyme), calcium, and irreversible inhibitors [13,16–18]. One of the structures solved for human tissue TGase (hTG2) features a bound GDP molecule (PDB code 1KV3) [12], allowing the association of this compact structure with the faster migrating band observed by native gel electrophoresis in the presence of GTP. More recently, Khosla and co-workers solved the structure for hTG2 modified by an irreversible inhibitor that was found to adopt a completely different open conformation (PDB code 2Q3Z) [13], consistent with the slower migrating band on gel electrophoresis.

We have studied the mechanism and inhibition of TGase using recombinant gplTGase. This enzyme shares excellent sequence similarity with the human enzyme (hTG2) and can easily be expressed in a highly soluble form [19]. These qualities led to the use of gplTGase in many mechanistic and inhibition studies, even though gplTGase is not as structurally well characterised as hTG2. In our recent studies of the inhibition of gplTGase, we conducted a photolabelling experiment whose interpretation prompted us to generate 3D homology models for the open and closed conformations [17,20]. However, the reliability of any model of the open-form conformation is questionable, given that it is based on a single X-ray structure. This underlines the importance of working directly with human TG2, for which X-ray structures have been solved. Conversely, the human enzyme suffers from solubility problems that hinder its efficient expression and purification. Therefore, we sought to develop methods for the expression, purification and rapid assay of hTG2, as reported herein.

Materials and methods

Materials

All reagents used were of the highest available purity. Restriction enzymes were from New England Biolabs and MBI Fermentas.

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http://dx.doi.org/10.1016/j.pep.2012.10.002

Synthetic oligonucleotides were obtained from AlphaDNA. All aqueous solutions were prepared using water purified with a Millipore BioCell system.

Construction of expression plasmid

The PreScission™ Protease cleaving site gene was introduced by site-directed mutagenesis by overlap extension (SOE) [21] using plasmid pG-TG [22] as a template and the primers RC-insertPSP-R (CCATGGCCAGTGGACCCTGGAACAGAACTTCCAGCAGATCCGATTTTGGAGGATGGT), RC-insertPSP-F (ACCATCCTCCAAAATCGGATCTGCTGGAAGTTCC-AGGGTCCACTGGCCATGG), hTG-PSP-R (CTCCACAGCATC-TCTTAGTGGA), and hTG-PSP-F (ATCAAACAGGATTTTCGCCTGCT). Initially, two fragments were amplified using primers RC-insertPSP-F and hTG-PSP-R (~300-bp fragment) and RC-insertPSP-R and hTG-PSP-F (~1700-bp fragment). After performing a PCR Cleanup (Qiagen) a second PCR was done using the two initial fragments as overlapping templates and hTG-PSP-F and hTG-PSP-R as primers. The resulting PCR product was purified by agarose gel electrophoresis followed by extraction with the kit from Bio-Rad. This PCR product was then doubly digested by NarI and NcoI and purified by agarose gel electrophoresis, followed by Freeze'N Squeeze extraction and butanol precipitation. The pG-TG vector was digested by NarI, NcoI and SwaI (to prevent re-circularisation) and purified as above. Ligation was effected at 16 °C using a 1:5 molar ratio and yielded the vector pGST-PSP-hTG. This vector which was used to transform BL21(DE3) *Escherichia coli* cells for expression. The entire NarI/NcoI fragment was verified by DNA sequencing at the Institut de Recherche en Immunology et Cancer (IRIC) on an Applied Biosystems 3730 DNA Analyser.

Expression of GST-hTGase

BL21(DE3) *E. coli* cells harbouring the expression plasmid pGST-PSP-hTG were taken from frozen stock and grown overnight in 5 mL Terrific Broth (TB) medium containing 100 μg/mL ampicillin. This bacterial suspension was then used to inoculated 1 L of fresh TB medium and the culture was incubated at 37 °C with shaking (240 rpm). When the optical density at 600 nm reached approximately 0.6, IPTG was added and the culture was incubated for an additional 20 h at 28 °C with shaking at 240 rpm. Cells were then harvested by centrifugation (30 min, 2000*g*, 4 °C) and the resulting 8-g pellet was resuspended in 30–40 mL of lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EGTA, 1 mM TCEP, and 15% glycerol at pH 8.0). The cells were lysed with a cell disrupter (Constant Systems) and the crude lysate was clarified by centrifugation (60 min, 44000*g*, 4 °C) and filtration (0.22-μm pore size).

Purification of hTG2

All purification steps were carried out at 0–4 °C. To the lysate was added 1 mL glutathione Sepharose 4B resin that had been previously equilibrated with lysis buffer. The slurry was mixed gently (100 rpm on a rotary shaker) for 2 h and then loaded into a gravity filtration column (Bio-Rad Econo-Column chromatography column 1 × 10 cm). This column was then washed with 10 mL of lysis buffer containing 0.5% Triton X-100, 10 mL of lysis buffer and 10 mL of storage buffer (25 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM TCEP, pH 7.2). These combined fractions were retained as flow-through, while the hTG2 remains bound on the column. One millilitre of a 0.375 mg/mL solution of PreScission™ Protease (GE Healthcare) was then added to the column, which was allowed to stand for 18 h. hTG2 was then eluted from the column with an additional 3 mL of storage buffer; the PreScission™ Protease remains bound to the column. hTG2 activity and concentration were determined as described below. Glycerol was added to a final concentration of 10% and the solution was then aliquoted into micro-centrifuge tubes (~0.1 U each), flash-frozen on dry ice and stored at −80 °C.

Protein concentrations

Total protein concentration was generally determined using the Bio-Rad protein assay, a method based on the Bradford assay, using BSA as a standard. Colour development took place over 20 min and absorbance values were measured using a Varian Cary 100 Bio and compared to standard curves performed daily.

Determination of specific activity

TGase activity was measured by the colorimetric assay procedure [23] using 925 μL of assay buffer (0.05 mM EDTA, 3.3 mM $CaCl_2$, in 0.11 M MOPS at pH 7.0), 25 μL of 17.4 mM *N*-Cbz-Glu(γ-*p*-nitrophenylester)Gly in DMF and 50 μL of hTGase solution at 25 °C. The activity assay solution was prepared fresh daily. One unit of enzyme activity (U) is defined as the amount of TGase that catalyses the formation of 1 μmol of *p*-nitrophenolate per minute. Specific activity is reported as units per milligram of protein.

Electrophoresis

Separation of proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was conducted using the Bio-Rad Mini-PROTEAN® Tetra Cell electrophoresis system according to procedures of Laemmli using minigels (86 × 68 mm) prepared with 10% acrylamide resolving gel at pH 8.8, 4% acrylamide stacking gel at pH 6.8, and 2.7% crosslinker concentrations. The protein sample (15 μL) was mixed with 5 μL of loading buffer (50 mM Tris–HCl (pH 6.8), 100 mM DTT, 2% w/v SDS, 0.1% w/v bromophenol blue, and 10% v/v glycerol) and boiled for 5 min prior to loading. Protein bands were revealed using 250R Coomassie blue staining followed by drying. The SDS–PAGE broad-range molecular weight markers (Bio-Rad) were used as standards.

Kinetic assays

During the optimisation of hTG2 expression and purification, enzyme activity was determined with the chromogenic substrate Cbz-Glu(OpNP)-Gly (AL5), according to conditions reported previously for gplTGase [23]. Namely, activity assays were performed by observing an increase in absorbance at 405 nm at 25 °C using 55 μM 'AL5' in buffer composed of 50 mM $CaCl_2$, 50 μM EDTA and 0.1 M MOPS at pH 7. Blank runs were performed under the same conditions in the absence of enzyme.

For the determination of the K_M value of AL5, 2.5 mU of purified hTG2 was used in 1 mL of assay buffer and the concentration of AL5 was varied from 13.5–432 μM. The K_a value of calcium was determined at 55 μM AL5 by varying the concentration of $CaCl_2$ from 0.1 to 3 mM. The apparent K_M value of Ac-PNPQLPF-OH was determined through a competition experiment as described previously [24,25] where the concentration of AL5 was varied from 30 to 400 μM in the presence of 9.8 to 75 μM Ac-PNPQLPF-OH.

Inhibition constants (K_i values) were measured for the reversible inhibitors CP4d and CP30a as described previously [26,27] using concentration ranges from 2.5 to 10 μM for CP4d and from 25 to 100 μM for CP30a, in the presence of 30–400 μM AL5. For the irreversible inhibitor NC-I052, K_I and k_{inact} values were determined as described previously [28] through analysis of the time-dependent loss of activity [29,30] observed through the AL5 assay described above [23], using 60 μM AL5 and 9–101 μM NC-I052.