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Cloning and expression of the *Bacillus amyloliquefaciens* transglutaminase gene in *E. coli* using a bicistronic vector construction

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ABSTRACT

Transglutaminases (TGases) are a class of transferases widely used in the food and biotechnology industries. In this work, we describe the production of recombinant *Bacillus amyloliquefaciens* TGase in *Escherichia coli*, obtaining the protein in its soluble and active form. In order to reduce TGase activity inside host cells and consequently its toxicity, we constructed a bicistronic plasmid containing the *B. amyloliquefaciens* TGase gene fused to the inhibitory *Streptomyces caniferus* prodomain. To make the enzyme active and avoid the need of prodomain removal *in vitro*, we also cloned the 3C protease gene into the same plasmid. After a fast single-step purification protocol, we obtained a partially purified recombinant TGase with 37 mU/mg protein activity, that crosslinked bovine serum albumin (BSA). This is the first report on the expression of *B. amyloliquefaciens* TGase in *E. coli* in its mature and active form.

1. Introduction

The use of enzymes in industrial processes has increased steadily in the last years, particularly in the food sector. Enzymes are non-toxic biocatalysts, which are environmentally benign, and their use reduce processing time and energy consumption, increasing industrial efficiency and productivity [1,2]. Recombinant enzymes can be produced in large quantities by genetically-modified microorganisms to attend growing demands, at low costs [3].

Enzyme applications are focused on a variety of markets, including food and beverages. This segment dominates the industrial enzyme market and it is projected to reach US$ 2.3 billion by 2020 [4,5]. Enzyme preparations have been used in food production and processing since the early 20th century [6]. The consumer market demands high-quality products, using less chemical additives and preservatives. Consequently, there is a growing need for new, improved, and more versatile enzymes to develop innovative, sustainable and economically competitive production processes [4].

Transglutaminase (TGase, protein-glutamine γ-glutamyltransferase, EC 2.3.2.13) is an enzyme that catalyzes the formation of inter- and intra-crosslinking reactions among protein molecules. Its mechanism of action is the transamidation of the carboxamide moiety of a glutamine residue (acyl donor) with a primary amine, typically the side chain of lysine residue (acyl acceptor) [7]. This reaction can modify functional characteristics such as viscosity, gelation, solubility, heat stability, and water retention capacity of a given protein system [8,9]. TGase ability to promote reticulation is widely used by the food industry in various processes, as in the manufacture of cheese and other dairy products, in meat processing, in the production of edible films and in the manufacture of bakery products [10–12]. Moreover, TGase has a wide variety of applications in the pharmaceutical industry, such as the production of antibody-drug conjugates (ADCs), in regenerative medicine, as well as in protein PEGylation. It also finds applications in the textile and leather industries, biofilm production and enzyme immobilization [13–20].

TGase has been reported to be found in mammals and other vertebrates, invertebrates, mollusks, plants, and microorganisms [21,22]. It is involved in various physiological functions, from blood coagulation and wound healing to stabilization of photosynthetic complexes and programmed cell death in plants [7,18,23–26]. In 1989, Ando and

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colleagues first reported the detection of a microbial transglutaminase (mTGase), from *Streptomyces* sp. Unlike its mammalian counterparts, *Streptomyces* mTGase was found to be Ca^{+2}-independent, smaller in size and composed of a single domain [27]. mTGase is expressed extracellularly as a zymogen (inactive enzyme) containing an N-terminal prodomain that covers the active site. Along with the zymogen, extracellular proteases are expressed to cleave the prodomain in order to produce the active enzyme [28].

So far, the mTGase produced by fermentation of wild-type *S. mobaraensis*, remains the main industrial source of this enzyme [12]. Thus, there have been many attempts to produce TGase from *Streptomyces* in heterologous microbial hosts. [28–34]. The production of recombinant mTGase has been technically challenging, resulting in insoluble proteins forming inclusion bodies in the host cell, or in preparations with low activity [35–38].

Bacillus transglutaminase (bTGase), discovered in 1996, is involved in the formation of crosslinks of spore-surface coat proteins and seems to be a promising alternative to TGase production [39,40]. bTGase is a single-domain protein, produced in its active form, and the smallest TGase characterized to date (28 kDa) [42]. Moreover, it has little structural homology to its bacterial counterparts [41]. bTGase also catalyzes the formation of inter- and intra-crosslinking reactions forming ε- (γ-glutamyl) -lysine crosslinks that are protease-resistant and stable [40]. Compared to commercial *Streptoverticillium mobaraense* transglutaminase, bTGase was more stable over a wide range of temperatures (30–60 °C) and pH (pH 5.0–9.0), with maximum enzymatic activity at 60 °C and pH 8.0 [42]. Its robust activity may be desirable for various applications as a biocatalyst [43]. Despite these important features, few works have been done to produce or improve current bTGase yields, which are too low and insufficient for large-scale and cost-effective production [44].

In this study, we cloned and expressed the gene coding for *Bacillus amyloliquefaciens* TGase for the first time in *E. coli*, and the recombinant bTGase protein was produced in its active and mature form. For this purpose, we constructed a bicistronic plasmid containing the *B. amyloliquefaciens* bTGase gene fused to the *Streptomyces caniferus* prodomain. In addition, we also cloned the 3C protease gene in order to make the enzyme active, avoiding the necessity of removing the prodomain *in vitro*. A single-step purification protocol was developed, and the TGase activity of partially purified recombinant bTGase was investigated by both fluorescence and BSA crosslinking assays.

2. Materials and methods

2.1. Materials, bacterial strains, plasmids and cell maintenance

Bacterial strains *E. coli* TOP10 and *E. coli* BL21 (DE3)pLysS were obtained from Invitrogen, San Diego, *ca. E. coli* TOP10 was used as hosts for DNA manipulation, and *E. coli* BL21 (DE3)pLysS was used for recombinant protein production. Bacterial cells were frozen and kept at −20 °C.

PureLink™ Quick Plasmid Miniprep Kit and PureLink™ Quick Gel Extraction and PCR Purification Combo Kit were obtained from Invitrogen, San Diego, *ca.* Plasmid pBAD/HisA (Life Technologies, Gaithersburg, MD) served as a starting point to construct the bicistronic vector used for recombinant protein production.

Lysogeny broth (LB) (yeast extract 5 g/L, tryptone 10 g/L, and NaCl 10 g/L) and Terrific broth (TB) (tryptone 12 g/L, yeast extract 24 g/L, glycerol, 4 mL/L, KH_2PO_4 0.17 M, K_2HPO_4 0.72 M) were used for DNA plasmid cloning and gene expression, respectively [45].

All enzymes used for DNA manipulation were purchased from New England Biolabs (NEB). The chemicals used in this study were of analytical grade or molecular biological grade and purchased from Sigma-Aldrich, unless otherwise stated.

2.2. Bacillus subtilis bTGase sequence identification

The NCBI Protein BLAST web interface was used to search for similar sequences of *Bacillus subtilis* bTGase [46]. The amino acid sequence of B. subtilis protein–glutamine γ-glutamyltransferase (Uniprot accession number: P40746) was entered as a query. We identified a homologue protein from the strain *B. amyloliquefaciens* DSM 7 (GenBank: CBI44050.1). Alignment of *B. subtilis* and *B. amyloliquefaciens* amino acid sequences was performed with Clustal Omega version 1.2.1 [47].

2.3. Plasmid construction

Molecular cloning techniques were performed according to methods described by Sambrook et al. [48]. The genes encoding TGase and 3C protease were chemically synthesized (Biomatik®) and the gene sequence encoding for bTGase from *Bacillus amyloliquefaciens* and 3C protease from *Human rhinovirus* was codon-optimized for *E. coli* expression. The expression plasmid containing bTGase and 3C protease genes was constructed according to Rickert et al. [28] and Liu et al. [44], with some modifications as described below. Genetic sequencing was performed by the company ACTGene Análises Moleculares (actgene@ludwigbiotec.com.br) and confirmed the identity and integrity of the product.

2.3.1. First insert – construction of the expression plasmid pBAD/3C

Expression plasmid pBAD/3C was constructed by cloning the 3C protease gene from Rhinovirus B14 (GenBank accession number NP_740524.1) into pBAD/HisA under the control of the araBAD promoter, introduced in the cloning linker site between restriction enzymes *Sac*I and *Eco*RI at the 5′ and 3′ ends, respectively.

2.3.2. Second insert – construction of the plasmid pBAD/3C/bTGase

Expression plasmid pBAD/3C/bTGase was constructed by subcloning the T7 promoter and the T7 terminator of plasmid pET20b (+) into the plasmid pBAD/3C within the restriction sites *Pci*I and *Bsm*BI at the 5′ and 3′ ends respectively.

The fragment of bTGase gene from *Bacillus amyloliquefaciens* DSM7 (GenBank accession number CBI44050.1) and the *Streptomyces caniferus* prodomain (GenBank accession number AM746294.1) was cloned into pBAD/3C under the control of the T7 promoter. A 3C protease recognition and cleavage site was inserted between the prodomain and bTGase. To optimize bTGase expression, a codon for the amino acid lysine (AAA) was placed shortly after the initial codon of the ATG [49].

2.4. TGase prodomain mutagenesis

To suppress TGase activity and reduce toxicity to host cells, the sequence encoding the TGase prodomain region of *Streptomyces caniferus* was fused upstream to the TGase gene, producing a fused protein containing the prodomain in its N-terminal part [44].

Rickert et al. have suggested that the D20A mutant (*Streptomyces mobaraensis*) achieves the correct balance of prodomain interaction force. At low temperature, during protein expression, it exhibits poor self-crosslinking activity in the *E. coli* cytoplasm. However, this activity can be reestablished after dissociation of the prodomain [28]. To reduce the interaction between the prodomain and TGase, and therefore to facilitate their dissociation and achieve complete enzymatic activity after protein purification, a mutation in the *S. caniferus* prodomain was performed to replace Asp(GAC) by Ala (GCG) at position 22 (D22A), correspondent to the D20A mutation of *Streptomyces* mTGase [28].

2.5. Protein expression

E. coli BL21 (DE3) and *E. coli* BL21 (DE3)pLysS were used as hosts for recombinant protein production. Plasmids pBAD/3C/bTGase and

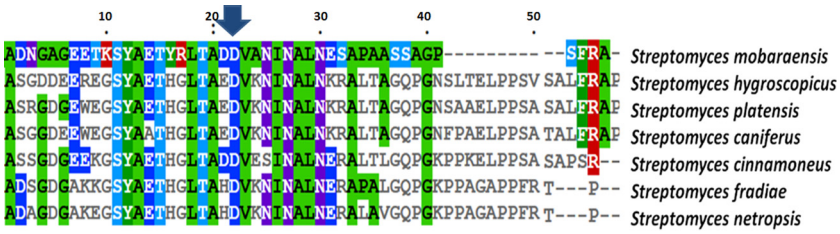

Fig. 1. Alignment the amino acid sequences of transglutaminase prodomain of seven species of *Streptomyces*. In this work, a mutation at D22A was introduced in the sequence of *S. caniferus* in order to reduce the interaction between the prodomain and TGase, facilitating their dissociation and achieving complete enzymatic activity (marked with an arrow).

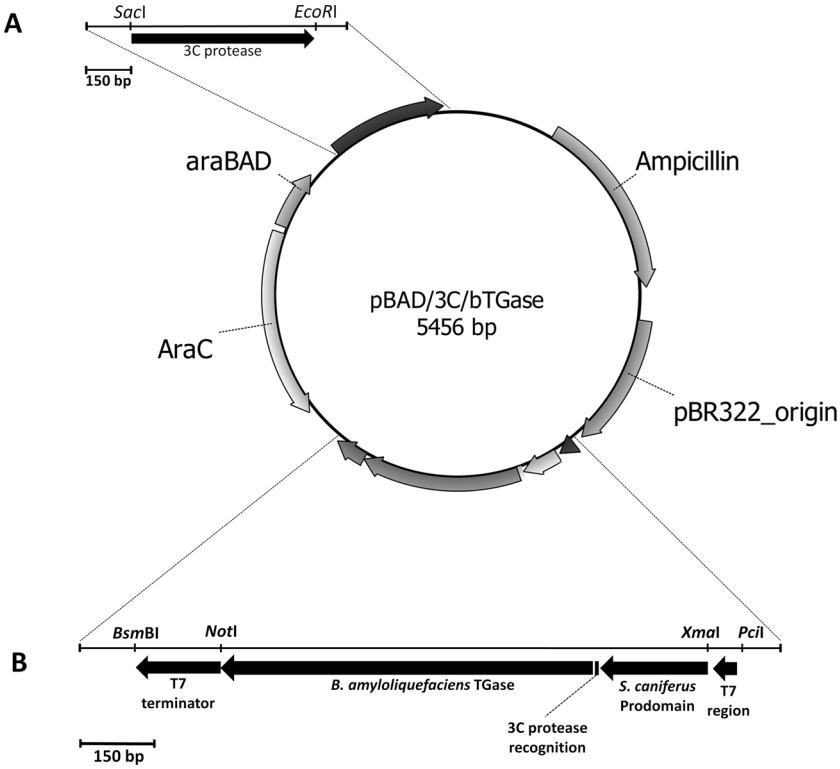

Fig. 2. Structure of the pBAD/3C/bTGase expression plasmid used for transformation into *E. coli* host cells. 2A - Schematic representation of synthetic genes of the first cloning into pBAD/HisA - restriction enzyme sites and 3C protease. 2B - Schematic representation of synthetic genes of the second cloning into pBAD/3C. The construct consists of restriction enzyme sites, the *S. caniferus* prodomain gene, and the *B. amyloliquefaciens* bTGase gene. Between the prodomain and the bTGase gene is the 3C protease recognition site.

pBAD/HisA were transformed into *E. coli* and cultured on LB agar plates containing 100 µg/mL Carbenicillin and 34 µg/mL Chloramphenicol (*E. coli* BL21 (DE3)pLysS). A single colony was grown overnight in 50 mL of LB at 180 rpm at 37 °C with the same antibiotics.

A 1 mL cell culture suspension was added to 100 mL of TB medium, supplemented with 100 µg/mL Carbenicillin and 34 µg/mL Chloramphenicol (*E. coli* BL21 (DE3)pLysS) and incubated at 37 °C on an orbital shaker at 180 rpm until reaching an $O.D._{600}$ of 0.4 – 0.6. The temperature was reduced to 20 °C and cells were equilibrated to the lowered temperature for 30–40 min under continuous shaking before induction.

The pro-bTGase gene was induced with IPTG (0.1 mM, 0.4 mM and 1.0 mM) and the 3C protease gene was induced by L-arabinose (0.002 %, 0.02 % and 0.2 %). Aliquots were taken at 0.5, 1, 2, 3, 4, 6, 9, 12, 24 and 48 h. Attempts were made for co-induction and sequential induction (using first IPTG and L-arabinose).

Cells were harvested by centrifugation at 8000 × *g* for 30 min at 4 °C and stored at -20 °C. DNA sequencing and mass spectrometry were used to confirm the sequence identity and protein integrity.

2.6. SDS-PAGE

SDS-PAGE was performed using a BioRad Mini-PROTEAN® TGX™ system and a 12 % polyacrylamide gel as described by Laemmli [50]. The lanes of the gel were loaded with 10 µL of the samples plus 2.5 µL of SDS loading dye 5x (Tris-HCl 62.5 mM, pH 6.8; Glycerol 25 %; Sodium Dodecyl Sulfate 2 %; Bromophenol Blue 0.01 %). Then, the gel product was stained using Comassie Brilliant Blue R-250.

2.7. Mass spectrometry

The presence of 3C protease (20 kDa) and bTGase (28 kDa) in protein extracts were investigated by liquid chromatography coupled to mass spectrometry (LC–MS/MS) of sodium dodecyl sulphate 12 % polyacrylamide gels (SDS-PAGE) slices. Proteins were loaded on SDS-PAGE and sections of each protein were excised and submitted to in-gel digestion [51]. Tryptic digest was separated on an in-house made 20 cm reverse-phase column (5 µm ODSAQ C18, Yamamura Chemical Lab, Japan) using a nanoUPLC (nanoLC Ultra 1D plus, Eksigent, USA) and eluted directly to a nanospray ion source connected to a hybrid mass spectrometer (LTQ-XL and LTQ Orbitrap Discovery, Thermo, USA). The flow rate was set to 300 nL/min in a 60 min reverse-phase gradient. The mass spectrometer was operated in a data-dependent mode, with full MS1 scan collected in the Orbitrap, with *m/z* range of 400-1,600 at 30,000 resolution. The eight most abundant ions per scan were selected to CID MS2 in the ion trap. Mass spectra were analyzed using PatternLab platform [52]. MS2 spectra were searched with COMET [53] using a non-redundant database containing forward and reverse *E. coli* BL21 (DE3)pLysS reference proteome and the sequence of both proteins. The validity of the peptide-spectra matches (PSMs) generated by COMET was assessed using Patternlab´s module SEPro [52], with a false discovery rate of 1 % based on the number of decoys.

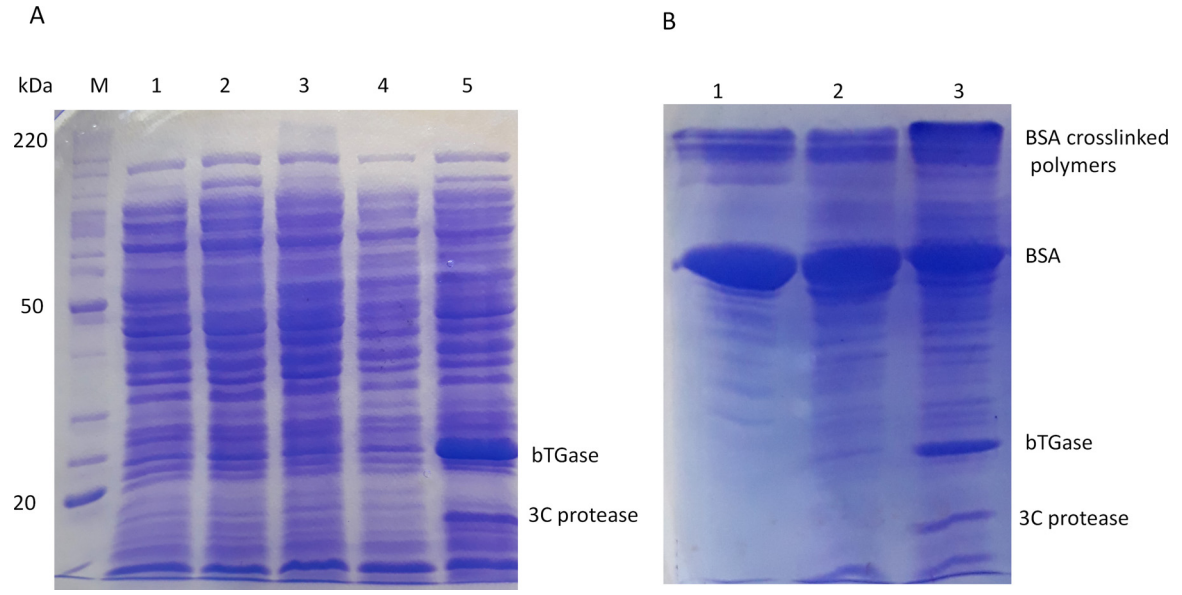

Fig. 3. Gel protein expression and BSA activity of plasmid pBAD/3C/bTGase in *E. coli* BL21 (DE3)pLysS. 3A – SDS-PAGE showing expression of recombinant bTGase and 3C protease in *E. coli* BL 21 (DE3)pLysS. Lane M - Molecular weight marker; Lane 1–3C protease induced with L-arabinose 0.2 % at 24 h. No expression observed; Lane 2 - pBAD/His/*lac*Z induced with IPTG 0.4 mM as positive control of *lac*Z gene. The positive control yielded a 120 kDa protein corresponding to *lac*Z protein; Lane 3 – pBAD/HisA negative control after induction with L-arabinose 0.2 % for 24 h. There is no expression of 3C protease; Lane 4 – pBAD/HisA negative control after induction with IPGT 0.4 mM in 24 h and 4 h of induction with L-arabinose 0.2 %. There is no expression of bTGase and 3C protease. Lane 5 – pBAD/3C/bTGase after induction with IPTG 0.4 mM in 24 h and 4 h of induction with L-arabinose 0.2 %. There is expression of 3C protease and bTGase. 3B – Crosslinking of BSA by the recombinant bTGase. For BSA crosslinking, the crude extract of *E. coli* BL21 (DE3)pLysS transformed with pBAD/HisA as negative control and crude extract of *E. coli* BL21 (DE3)pLysS transformed with plasmid pBAD/3C/bTGase were tested. The reaction was at 50 °C for 12 h. Lane 1 - BSA control; Lane 2 - BSA polymerization by pBAD/HisA transformed into *E. coli* BL21 (DE3)pLysS (negative control). There is no polymerization of proteins; Lane 3 - BSA polymerization by pBAD/3C/bTGase transformed into *E. coli* BL21 (DE3)pLysS. Polymerization was found on the top of the gel, in addition to decreasing the BSA band in the gel medium.

2.8. Purification of TGase

A one-step partial purification protocol was developed. Initially, 8 g of frozen cells were resuspended in 80 mL of 20 mM Bis Tris pH 6 (buffer A) containing 0.2 mg/mL of lysozyme (Ludwig Biotec) and gently stirred for 30 min. Cells were completely disrupted by sonication (10 pulses, 10 s each, 60 % amplitude) and centrifuged at 48,000 × *g* for 30 min. The supernatant was incubated with 1 % (w/v) of streptomycin sulfate for nucleic acid precipitation and stirred for 30 min. The solution was centrifuged at 48,000 × *g* for 30 min and the supernatant was collected. The supernatant was dialyzed twice against 1 L of buffer A using a dialysis tubing with a molecular weight exclusion limit of 12–14 kDa. The sample was centrifuged at 48,000 × *g* for 30 min and the supernatant was loaded on a SP Sepharose Fast Flow cation exchange column (GE Healthcare) pre-equilibrated with buffer A. The column was washed with 5 column volumes (CV) of the buffer A and adsorbed proteins were eluted with a linear gradient (0–75 %) of 15 CV of 20 mM Bis-Tris pH 6 containing 1 M NaCl (buffer B) at 5 mL/min flow rate. Fractions containing the target protein were pooled and protein concentration was determined by the method of Bradford using bovine serum albumin as standard (Quick Start™Bradford BIO-RAD). Recombinant transglutaminase protein fractions were analyzed in 12 % SDS-PAGE stained with Coomassie Brilliant Blue.

2.9. TGase activity assay

2.9.1. Fluorometric assay

The TGase activity was measured by the incorporation of the fluorescent amine, dansylcadaverine [N-(5-aminopentyl)-5-dimethylamino-1-naphthalenesulfonamide, MDC] into N, N-dimethylcasein by the method of fluorescence [44,54,55]. The TGase activity assay system used as substrate 12.5 μM MDC, 0.2 % N, N-dimethylcasein and 4.5 mM dithiothreitol (DTT). The fluorescence intensity was measured in a Shimadzu RF-5301 Spectrofluorometer (Shimadzu, Columbia, MD, USA) using an excitation and emission wavelength of 350 and 500 nm, respectively. One unit of TGase was defined as the amount of enzyme that incorporated 1.0 nM MDC into casein per minute at 37 °C. TGase activity could be calculated using the following formula (Eq. 1):

$$[MDC]_{incorporated} = \frac{I - I_0}{13 \times I_0} \times [MDC]_{total} \quad (1)$$

where I_0 denotes the fluorescence intensity of the reaction mixture without TGase and I denotes the fluorescence intensity of the reaction mixture with TGase.

2.9.2. Crosslinking of BSA with TGase

bTGase activity was also determined as its ability to crosslink to bovine serum albumin (BSA). The reaction system consisting of 5 μL BSA (10 mg/mL in 0.1 M Tris−HCl, pH 8.0) and 35 μL DTT solution (2 mM DTT in 0.1 M Tris-HCl, pH 8,0) was incubated at 50 °C for 12 h with or without 10 μL of bTGase. The *E. coli* BL21 (DE3)pLysS transformed with pBAD/HisA plasmid was used as a negative reaction control. The reaction products were analyzed by 12 % SDS-PAGE and the gel stained with Coomassie R-250 Bright Blue [44,56,57].

3. Discussion and results

3.1. Cloning, expression and purification of recombinant bTGase in E. coli

The main objective of the present work was the heterologous production of recombinant TGase from *Bacillus*, an enzyme with different and attractive properties for use in the food industry [42,44,58]. For this, *E. coli* was used as an efficient expression system. In addition to being a versatile bacterium, *E. coli* rapidly grows to a high-cell density in low carbon sources and is the host of choice for the first attempt at recombinant protein production [59].

As TGase from *Bacillus subtilis* was known, we used its amino acid sequence to search for other bacterial homologues. The TGase from *B.*

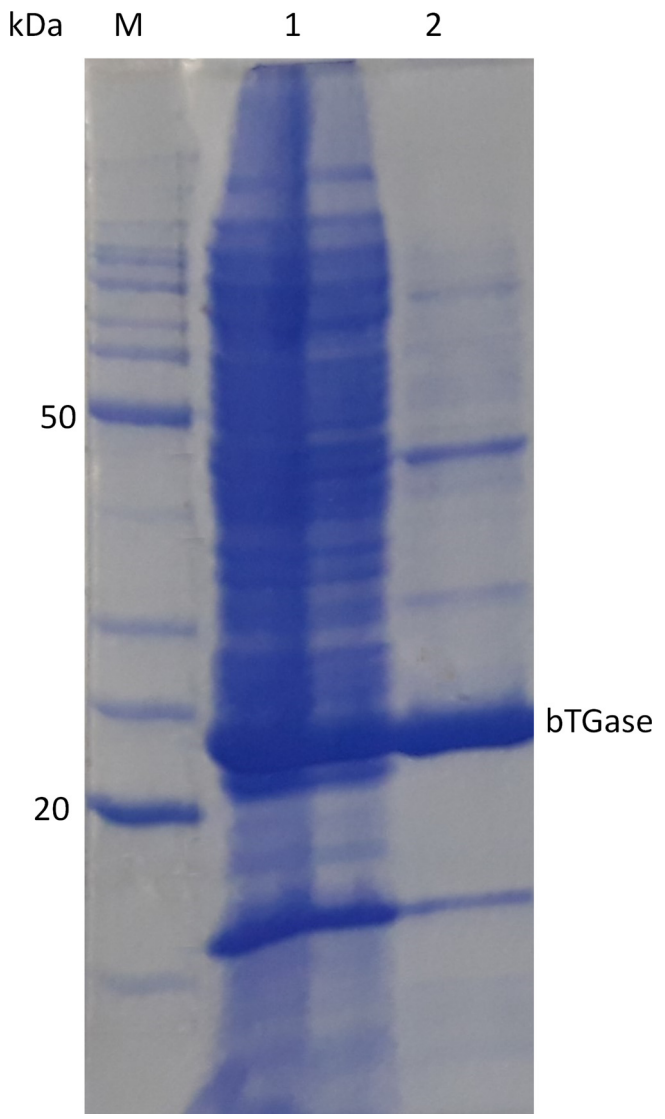

Fig. 4. SDS-PAGE of recombinant bTGase protein purification. Lane M - molecular weight marker. Lane 1 – Crude protein extract loaded in SP Sepharose Fast Flow (cation exchange column); Lane 2 – Elution of bTGase protein from SP Sepharose Fast Flow. The bTGase protein band was indicated in figure.

amyloliquefaciens, a gram-positive spore-forming bacterium that was sequenced in 2011 [60], was among the best matches obtained. Comparison of primary structures of *B. subtilis* and *B. amyloliquefaciens* gene products showed 73 % similarity (Supplementary material of Fig. 1), indicating that the structure and function of these enzymes may be preserved.

However, unlike *S. mobaraensis* TGase, which is synthesized as a proenzyme, the bTGases from Bacillus species are already produced in their mature forms and the expression of bTGase as a heterologous protein in bacteria can be toxic and affect normal cell growth through protein cross-linking within host cells [39,40,43,44]. Therefore, to suppress TGase activity and reduce toxicity in microbial host cells, the sequence encoding the TGase prodomain of *Streptomyces caniferus* was cloned in frame with the bTGase gene to produce a fusion protein.

In a previous work, an approach to suppress bTGase activity and reduce toxicity in microbial hosts was developed, in which TGase prodomains from seven *Streptomyces* species were fused to *Bacillus subtilis* TGase and the yield of recombinant proteins was compared. *Streptomyces caniferus* prodomain was found to have the strongest suppressive effect on bTGase activity [44]. However, to restore the enzymatic activity of bTGase, a downstream step of prodomain removal by proteolytic cleavage after enzyme purification was required [45]. To avoid the need for *in vitro* removal of the prodomain and to directly produce active bTGase, we constructed a bicistronic plasmid containing both the fused prodomain-bTGase encoding gene and the 3C protease gene from Rhinovirus B14. The 3C protease gene is commonly used in molecular biology to remove high specificity protein fusion marks because it recognizes the Leu-Glu-Val-Leu-Phe-Gln-↓-Gly-Pro sequence and cleaves after the glutamine residue [28,61]. A 3C protease recognition site was designed between the *S. caniferus* TGase prodomain and the bTGase sequence to produce bTGase in its mature and active form by proteolytic cleavage. In this construct, two different promoters were used to induce independently 3C protease and bTGase production. The 3C protease gene is controlled by the araBAD promoter and the bTGase gene is controlled by the T7 promoter. Since these promoters are responsive to different inducers (L-arabinose and IPTG for araBAD and T7 promoters, respectively), it is possible to induce production of bTGase and 3C protease simultaneously or sequentially. In this way, the timing of expression can be optimized to improve protein yields.

In TGases, prodomains also function as chaperones, assisting recombinant proteins to correctly fold within host cells [62]. The D22A mutation was introduced into the *S. caniferus* prodomain sequence in order to maintain the prodomain chaperone function, at the same time converting the interaction between prodomain and bTGase into a temperature-dependent association. In a previous work, an alanine scan mutagenesis on the *S. mobaraensis* prodomain was performed and it was concluded that the Asp20 mutation (D20A) could achieve a relatively high level of active expression of transglutaminase [28]. Thus, bTGase is expected to remain inactive during expression at 20 °C and restore its full activity when the temperature is raised to 37 °C. The alignment of the amino acids sequences of TGase prodomain of seven species of *Streptomyces* mutated at D22A introduced in the sequence of *S. caniferus* is shown in Fig. 1 (marked with arrow).

The construction of the pBAD/3C/bTGase vector consisted of two steps, which are schematically represented in Fig. 2 and described in detail in section 2.3. Briefly, the 3C protease gene was first cloned under control of the araBAD promoter with restriction enzymes *Sac*I and *EcoR*I. (Fig. 2A) and the pro-bTGase insert was introduced under the control of the T7 promoter with the restriction sites *Pci*I and *BsmB*I (Fig. 2B).

After the confirmation of the correct sequence construction of pBAD/3C/bTGase, the *E. coli* host cells were used for recombinant protein production. *E. coli* BL21 (DE3) strains were successfully transformed with plasmids pBAD/3C/bTGase and pBAD/HisA. We tested different expression conditions (using first IPTG and L-arabinose) (Supplementary material of Fig. S2). The best results were obtained under the following conditions: induction with 0.4 mM IPTG for 24 h, followed by induction with 0.2 % L-arabinose for 4 h. In an attempt to obtain better results, we transformed *E. coli* BL21 (DE3)pLysS with plasmid pBAD/3C/bTGase. This host expression strain has been reported to improve the production of toxic proteins that contains the T7

Table 1
Summary of bTGase purification and enzyme activity.

Step	Volume (mL)	Total protein (mg)	Total activity (U)	Specific activity (mU/mg)	Yield (%)	Purification Fold
Crude cell lysate	80	209.60	2.72	13	100	1
Cation exchange chromatography	110	33.02	1.21	37	44.5	2.77

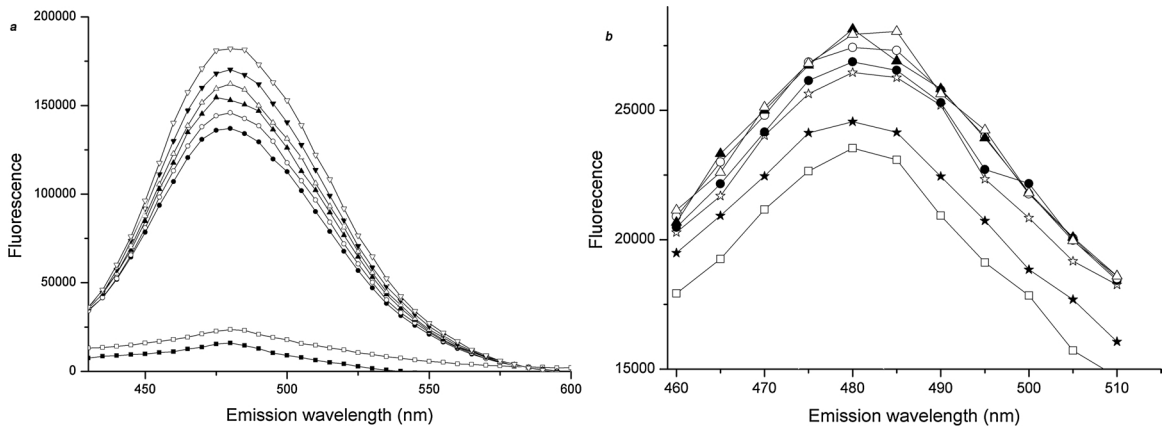

Fig. 5. Determination of incorporation of monodansylcadaverine into dimethylcasein by recombinant bTGase in crude protein extract and purificated bTGase. 5A Crude protein extract of bTGase. Curve ■: only monodansylcadaverine; Curve □: only bTGase without substrate; Curve ●: 20 min of reaction; Curve ○: 30 min of reaction; Curve ▲: 40 min of reaction; Curve △: 50 min of reaction; Curve ▼: 70 min of reaction; Curve▼: 90 min of reaction. 5B Purificated bTGase. Curve □: only bTGase without substrate; Curve ★: 1 min of reaction; Curve ☆: 10 min of reaction; Curve ●: 20 min of reaction; Curve ○: 30 min of reaction; Curve △: 40 min of reaction; Curve ▲: 50 min of reaction.

lysozyme gene, increasing the tolerance of *E. coli* cells towards toxicity and lacking proteases that could degrade expressed proteins [63].

After induction with 0.4 mM IPTG and 0.2 % L-arabinose (24 h and 4 h, respectively) the whole-cell lysates were analyzed by SDS-PAGE. Total cell lysates from induced samples contained a protein band consistent with the expected size of bTGase (25 kDa), indicating that bTGase gene was successfully expressed in *E. coli* BL21(DE3)pLysS (Fig. 3A, lane 5). We also detected on the same samples a protein band of ~ 20 kDa, consistent with the expected size of 3C protease (Fig. 3A, lane 5).

A BSA crosslinking test was performed to further test the enzymatic activity of recombinant *B. amyloliquefaciens* bTGase, (Fig. 3B). Crosslinking was verified by producing high molecular weight products on top of the separation gel, in addition to decreasing the BSA band in the gel medium (Fig. 3B, lane 3). It has been reported in previous works that crosslinking significantly increases the molecular weight of proteins [42,64]. A negative control with pBAD/HisA in *E. coli* was performed in parallel (Fig. 3B, lane 2). As expected, there was no crosslinking of BSA in the control samples. These results clearly indicated the formation of high-molecular weight polymers resulting from recombinant bTGase catalyzing the BSA cross-linking.

With the confirmation of enzymatic activity of bTGase, cells were disrupted and bTGase was partially purified using a single-step chromatographic (cation exchange) purification procedure (described in detail in Section 2.8). The crude extract and partially purified enzyme were analyzed by SDS-PAGE (Fig. 4). We have decided on a partial purification because, for industrial processes, unnecessary and mandatory cost purifications, especially for the food industry, should be avoided. Additional stages of purification make the process expensive and may lead to loss of enzymatic activity. Only other enzymes and materials that may interfere with the enzymatic catalytic process should be removed [65]. The purification result can be seen in the Table 1.

The 25 kDa SDS-PAGE protein band was excised and subjected to trypsin digestion and the peptides analyzed by LC–MS/MS to confirm the identity of bTGase. Spectra matching from three unique peptides (TYGNTAYWRVTPEGALELK; YRIPASK and NPEFNPAK) from transglutaminase were identified, and around 15 % of coverage of the protein was identified.

3.2. bTGase activity assay

The establishment of reliable assay methods for measuring enzyme activity has been a concern in transglutaminase research. In the last decades, several assay methods have been developed to measure and quantify bTGase activity, but many of them have shown low sensitivity [66].

In this study, we used two methods to identify the enzymatic activity of bTGase, a BSA-crosslinking method and a fluorescence-based. A quantitative fluorescence measurement method involving the TGase-dependent covalent coupling of monodansylcadaverine to N, N-dimethylcasein produces a change in the intensity and wavelength of the dansyl group fluorescence. TGase activity is measured by increasing fluorescence intensity over time [67].

The recombinant bTGase enzyme activity was determined in the crude extract (Fig. 5A) and in the purified fraction (Fig. 5B). Changes in the fluorescence spectrum occurred during the enzymatic reaction in both experiments. An increase in the intensity of emitted fluorescence also can be observed. In the absence of enzyme or the enzyme without the presence of substrate, no time-dependent changes in fluorescence can be seen. The specific activity of TGase in the crude extract was 13 mU/mg protein and of the partially purified recombinant protein was 37 mU/mg protein.

4. Conclusions

This paper describes for the first time the expression and production of *Bacillus amyloliquefaciens* transglutaminase in *E. coli* using a bicistronic vector. The recombinant protein produced was found to be active without the need of the downstream step of prodomain removal by proteolytic cleavage. The protein was purified and identified by mass spectrometry. Further improvements in protein yield can be achieved in large scale production with higher expected biomass concentrations and optimization of cultivation parameters such as temperature, pH, oxygenation, feed rate and cultivation time.

CRediT authorship contribution statement

Lovaine Silva Duarte: Investigation, Conceptualization, Methodology, Writing - original draft. **Laísa Quadros Barsé:** Methodology. **Pedro Ferrari Dalberto:** Methodology. **William Tadeu Santos da Silva:** Methodology. **Rafael Costa Rodrigues:** Methodology. **Pablo Machado:** Resources, Funding acquisition. **Luiz Augusto Basso:** Resources, Funding acquisition. **Cristiano Valim Bizarro:** Resources, Funding acquisition, Conceptualization, Methodology, Writing - review & editing, Supervision. **Marco Antônio Záchia Ayub:** Resources, Conceptualization, Methodology, Writing - review & editing, Funding acquisition, Project administration, Supervision.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.enzmictec.2019.109468.

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