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ORIGINAL ARTICLE

Production of transglutaminase by *Streptomyces* isolates in solid-state fermentation

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Keywords
beans, hydroxamate, solid-state fermentation, *Streptomyces,* transglutaminase, Z-Gln-Gly.

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Abstract

Aims: To screen *Streptomyces* isolates for transglutaminase (TGase) production in solid-state fermentation (SSF) on various substrates.
Methods and Results: *Streptomyces mobaraensis* NRRL B-3729, *Streptomyces paucisporogenes* ATCC 12596 and *Streptomyces platensis* NRRL 2364 strains were screened for extracellular TGase production in SSF on different substrates. High-protein-content beans, peas and lentils proved to be the best substrates. Good TGase production was obtained on liver kidney beans and green mung beans in a 4- to 6-day SSF. Temperature optima of the enzymes varied between 45 to 50°C. Molecular weight determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS PAGE) indicated similar size (~37 kDa) for all three enzymes. TGase was the dominating protein band on SDS PAGE for two *Streptomyces* strains in SSF extracts. Other enzymes were present in smaller quantities.
Conclusions: *Streptomyces mobaraensis* NRRL B-3729, *S. paucisporogenes* ATCC 12596 and *S. platensis* NRRL 2364 strains were successfully propagated under SSF conditions on crushed/milled liver kidney bean and green mung bean to obtain good level of TGase.
Significance and Impact of the Study: Owing to much reduced production cost and direct applicability, SSF TGase without downstream processing (cheap *in situ* enzyme, crude enzyme) may be an excellent candidate for some nonfood applications.

Introduction

Transglutaminases (TGase, protein-glutamine γ-glutamyltransferase, EC 2·3·2·13) catalyse the acyl transfer reaction between a γ-carboxyamide group of a lysine residue in a peptide chain and a γ-amino group of a lysine residue, resulting in the formation of an ϵ-(γ-glutamyl) lysine cross-linkage (Folk 1980). TGases are multifunctional enzymes that take part in intra- and extracellular protein stabilization processes (Griffin *et al.* 2002). They are widely distributed in living beings, including humans, plants, animals and micro-organisms (Motoki and Seguro 1998; Griffin *et al.* 2002). TGase are mainly used in food industry as cross-linking agents (Aktas and Kilic 2005; Lantto *et al.* 2006), but their applications are increasingly important in textile and other industries too (Chen *et al.* 2007). TGases can protect wool fabrics from the enzymatic and chemical damage caused by common household detergents; maintain colour and appearance of the wool garment; and reduce shrinking during washing (Cortez *et al.* 2007). TGases can also be used for the preparation of synthetic polymeric films (Di Pierro *et al.* 2007).

For industrial production of the enzyme *Streptomyces mobaraensis*, mutants and/or recombinants are recently used (Ando *et al.* 1989; Nonaka *et al.* 1989; Motoki *et al.* 1994). However, other micro-organisms (mainly *Streptomyces* spp.) are also cited in patents and papers (Cui *et al.* 2006; Langston *et al.* 2007; Bech *et al.* 2000; Lin *et al.* 2003).

Solid-state fermentation (SSF) has a great potential for the production of various extracellular enzymes especially

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in Oriental countries (Pandey *et al.* 1999). The advantage of SSF is operational simplicity and economy in a water-restricted environment, resulting in high volumetric productivity, high product concentration, and possibility for use of the product with little or no downstream processing (Tengerdy and Szakacs 2000). Although SSF best suits for propagation of filamentous fungi, there are a few examples using *Streptomyces* spp. in SSF conditions. Antibiotics, such as tetracycline (Yang and Ling 1988), cephamycin C (Bussari *et al.* 2008) or clavulanic acid (Sircar *et al.* 1998) were produced in SSF using various *Streptomyces* strains. More recently, TGase was successfully produced in SSF with a *Bacillus circulans* isolate (Soares *et al.* 2003).

Streptomyces species may produce high biodiversity of TGase differing from each other in structure (sequence) and mode of cross-linking of most important food, agricultural and textile proteins (Cui *et al.* 2006; Langston *et al.* 2007; Bech *et al.* 2000; Motoki *et al.* 1994; Lin *et al.* 2003). By using SSF process, inexpensive crude (*in situ*) TGase may be produced, without downstream (purification) processes. This crude enzyme may be important in some nonfood applications, where low price is more important factor than the purity of enzyme. In this paper, we present the production of TGase with *S. mobaraensis* NRRL B-3729, *Streptomyces paucisporogenes* ATCC 12596 and *Streptomyces platensis* NRRL 2364 strains in SSF conditions on different agro-industrial substrates.

Materials and methods

Materials

Agro substrates were milled to a 1- to 3-mm average particle size in coffee mill, laboratory hammer mill or manually in mortar in order to obtain good size for SSF.

Z-Gln-Gly (C 6154) was obtained from Sigma (St Louis, MO). All other analytical-grade reagents were purchased either from Sigma or from Reanal Co (Hungary). UV 1601 (Shimadzu, Kyoto, Japan) spectrophotometer was used for the enzyme assays. MiniProtean 3 Electrophoresis Cell (Bio-Rad, Hercules, CA) was applied for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

Micro-organisms and culture conditions

S. mobaraensis NRRL B-3729, *S. paucisporogenes* ATCC 12596 (more recently: *Streptomyces* sp.) and *S. platensis* NRRL 2364 strains were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and from the Northern Regional Research Center (NRRL, USDA, Peoria, IL, USA). The actinomycetes were stored as freeze-dried cultures. Revitalization and sporulation was performed on ATCC-5 medium in Petri plates at 30°C. Composition of ATCC-5 sporulation agar (in g l^{-1}) is the following: yeast extract, 1·0; beef extract, 1·0; tryptose, 2·0; $FeSO_4$, 0·1; glucose, 10·0; bacto agar, 15·0; pH (before sterilization), 7·2.

Screening media and SSF

Various substrates were used at different moisture content in order to screen the strains for TGase production. The composition of the salt solution (in g l^{-1}) used for wetting the substrate was as follows: KH_2PO_4, 5; NH_4NO_3, 5; $MgSO_4.7H_2O$, 1; NaCl, 1; and (in mg l^{-1}) $CoCl_2.6\ H_2O$, 1; $MnSO_4$, 0·8; $ZnSO_4.7\ H_2O$, 1·7; $FeSO_4.7\ H_2O$, 2·5; pH (before sterilization), 6·0.

Solid-state fermentation was carried out in 500-ml cotton-plugged Erlenmeyer flasks containing 10 g of the wetted substrate, autoclaved at 121°C for 20 min. Spores of each strain were aseptically scrapped out from a 5- to 7-day-old plate into 5-ml sterile water, and 1-ml cell suspension was used as inoculum for each flask. The flasks were incubated at 30°C without shaking as still cultures. Extracellular TGase activity was determined from the culture extract of the SSF samples. One flask (initially 10-g dry weight of fermented substrate) was extracted with 100-ml water containing 0·1% Tween-80, by shaking at room temperature (25°C) for 2 h. After extraction, the suspension was centrifuged (9500 ***g***, 15 min) and crude, clear supernatants were stored at 4°C until the enzyme assay was performed. All sets of experiments were done in triplicates and the average value is considered for presentation.

Determination of TGase activities

The enzyme assay was adopted from the paper of Grossovitz *et al.* (1950) and used with slight modifications. Briefly, the reaction mixture contained 1·5 ml of TGase substrate (200 mmol l^{-1} Tris-acetate buffer, pH 6·0; containing 5 mmol l^{-1} $CaCl_2$, 10 mmol l^{-1} L-glutathione, 100 mmol l^{-1} hydroxylamine-HCl and 30 mmol l^{-1} Z-Gln-Gly) and 0·5 ml of the properly diluted supernatant. The well-mixed solution was incubated at 37°C for 10 min. The reaction was stopped by the addition of 1·5 ml Stop reagent (100 mmol l^{-1} $FeCl_3$ dissolved in 5% TCA) and the absorbancy of brownish-red (magenta) colour was detected at 525 nm. Assay was carried out using appropriate substrate and enzyme blanks also. One International Unit of enzyme activity was defined as the amount of enzyme that hydrolyzes 1 μmol of Z-Gln-Gly per minute at 37°C and pH 6·0. Standard curve was prepared with L-glutamic acid γ-monohydroxamate. In order

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to eliminate the side effects of potentially present secondary metabolites which may coincide with the hydroxamate assay, crude fermentation extracts were ultrafiltered through a 5-kDa membrane (Amicon Ultra Centrifugal Filter Device; Millipore, Billerica, MA) and resuspended in 200 mmol l^{-1} Tris-acetate buffer (pH 6·0).

Biomass estimation in SSF

Biomass was determined as described by Bussari *et al.* (2008) with slight modifications. We used glucosamine as a standard for calibration and calculation. Briefly, 1-g dried fermented material was incubated with 2 ml conc. H_2SO_4 for 24 h at 24°C, diluted with distilled water and autoclaved for 1 h. The filtrates were neutralized with 1 mol l^{-1} NaOH to pH 7·0. To 1 ml of this solution, 1-ml acetyl acetone reagent (1 ml acetyl acetone in 50 ml 0·25 mol l^{-1} Na_2CO_3) was added and kept in boiling water for 20 min. After cooling, 6 ml ethanol and 1 ml Ehrlich's reagent (2·67 g *p*-dimethyl aminobenzaldehyde in 100 ml 1 : 1 mixture of ethanol and conc. HCl) was added and incubated at 65°C for 10 min. Absorbancies were read against blank, containing the nonfermented medium filtrate, at 530 nm.

Biochemical analysis

The TGase enzymes produced on the best substrate(s) were subjected to further characterizations. The optimum temperature and pH, thermal stability and metal ion dependency of the enzymes were studied.

Effect of temperature and pH
The reaction was carried out at various temperatures ranging from 25 to 80°C at pH 6·0, and the enzyme activity at different temperature points were compared to find out the temperature optimum in each case. Similarly, the enzyme assay was carried out at different pH levels from 5·0 to 9·0 at 37°C.

Thermal stability
Thermal stability of the enzymes was determined, as it was described by Karadzic *et al.* (2002). The crude enzyme extracts were incubated at pH 6·0 at different temperatures (37, 50 and 65°C) for various time intervals (0, 5, 10, 15, 30 and 60 min). After the heat treatment, samples were cooled and the enzyme was subjected to a typical enzyme assay at 37°C for 10 min.

Metal ion influence
Crude SSF extracts were ultrafiltered at 3500 ***g*** for 45 min, using a 5-kDa Amicon Ultra Centrifugal Filter Device (Millipore). Five metal ion salts were applied in three different concentration levels (0·5, 1 and 2 mmol l^{-1}) in the reaction mixture (ultrafiltered SSF extract) to study their effects on enzyme activity. The following salts were used: $CaCl_2.2H_2O$, $CoCl_2.6H_2O$, diamino-ethane-tetra-acetic acid (EDTA), $FeSO_4.7H_2O$ and $ZnSO_4.7H_2O$. Enzyme assay was carried out at 37°C for 10 min in the absence of reducing agent (L-glutathione) as described previously.

SDS PAGE

Crude enzyme extracts of *S. mobaraensis* NRRL B-3729, *S. paucisporogenes* ATCC 12596 and *S. platensis* NRRL 2364 produced on liver kidney beans were filtered through a 0·22-μm filter (Millipore), concentrated by ultrafiltration using a 5-kDa Amicon Ultra Centrifugal Filter Device (Millipore), and resuspended in 0·5 mol l^{-1} Tris-HCl buffer (pH 6·8). SDS PAGE was performed using the buffer system of Laemmli (1970) at a constant voltage (180 V) with gels containing 10% polyacrylamide in the resolving gel and 5% polyacrylamide in the stacking gel. Prior to electrophoresis, the concentrated and resuspended samples were heated at 100°C for 5 min in dissociating buffer, containing 2% SDS and 5% 2-mercaptoethanol. Precision Plus Protein Standard (Bio-Rad) was used as a molecular mass standard.

Results

Screening in SSF

The screening results showed a great variety in enzyme production, depending on the particular *Streptomyces* strain and on the substrate used for the fermentation. Highest activities were obtained on substrates with high protein content, such as green mung beans, red beans and liver kidney beans, at 60–75% moisture contents. Table 1 shows the best results obtained with *S. mobaraensis* NRRL B-3729, *S. paucisporogenes* ATCC 12596 and *S. platensis* NRRL 2364.

Time course of fermentation and biomass formation (glucosamine content) was studied on liver kidney beans at 67% moisture content with three *Streptomyces* isolates (Fig. 1). *Streptomyces mobaraensis* NRRL B-3729 showed a weak TGase production compared with the other two *Streptomyces* isolates; the peak activity (0·7 IU g^{-1} DM) was achieved in a 3-day SSF. The highest TGase productions were reached in a 4-day SSF with *S. paucisporogenes* ATCC 12596 and in a 7-day SSF with *S. platensis* NRRL 2364, with peak activities 4·2 and 5·1 IU g^{-1} DM, respectively. The growth curves were similar to the production profile of TGase.

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Table 1 Best transglutaminase (TGase) yields obtained with *Streptomyces* isolates on different substrates within solid-state fermentation

Strain	Substrate	Moisture content (%)	Ferm. time (day)	TGase (IU g^{-1} DM)
S. mobaraensis	Wheat bran	70	11	0·9
NRRL B-3729	Wheat bran–soybean meal mixture (9 : 1)	70	7	0·8
	Yellow peas	67	3	0·8
S. paucisporogenes	Lentils	60	4	1·3
ATCC 12596	Yellow peas	67	3	2·4
	Chick peas	67	3	1·1
	Liver kidney bean	67	4	4·2
	Elisabeth beans	67	5	3·1
	Pearl beans	60	7	1·1
	Mung bean	70	4	3·1
	Red kidney beans	67	5	2·8
S. platensis	Wheat bran	75	11	1·1
NRRL 2364	Common wheat	60	5	1·3
	Buckwheat	60	9	1·2
	Lentils	67	6	1·6
	Yellow peas	60	4	2·3
	Green peas	67	6	1·3
	Black beans	67	5	2·6
	Spot kidney beans	70	6	4·2
	Liver kidney bean	67	7	5·1
	Mung bean	70	3	4·9
	Poppy seeds	60	3	2·4

S., Streptomyces.

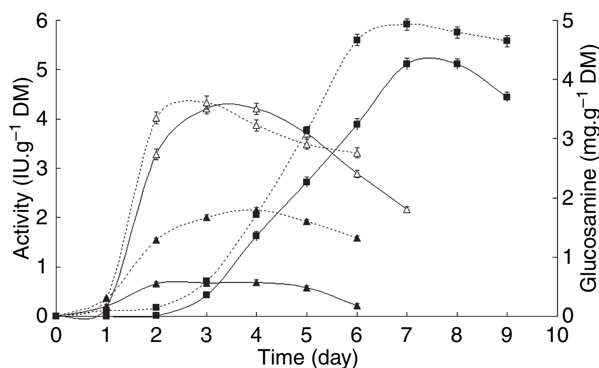

Figure 1 Time course of transglutaminase production (regular/solid line) and biomass formation (dotted line) in solid-state fermentation on liver kidney beans at 67% moisture content with *Streptomyces mobaraensis* NRRL B-3729 (▲), *Streptomyces paucisporogenes* ATCC 12596 (Δ) and *Streptomyces platensis* NRRL 2364 (■) isolates (enzyme assay: hydroxamate, from ultrafiltered supernatants).

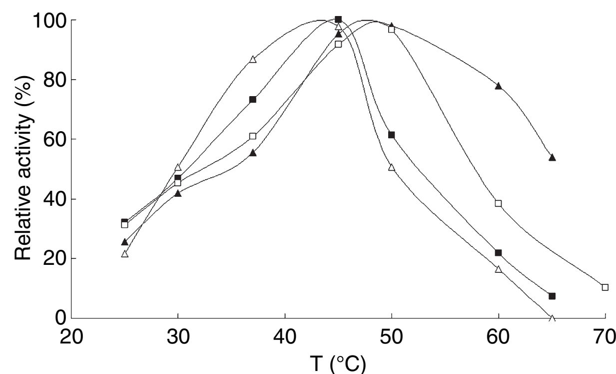

Figure 2 Temperature dependence of activity of transglutaminase produced in solid-state fermentation by *Streptomyces mobaraensis* NRRL B-3729 (▲), *Streptomyces paucisporogenes* ATCC 12596 (Δ) and *Streptomyces platensis* NRRL 2364 (■) isolates. Activa WM (Ajinomoto) (□) was used for comparison (enzyme assay: hydroxamate, from ultrafiltered samples).

Biochemical analysis

Figure 2 shows the effect of temperature (at pH 6·0) on TGase activity. TGase enzymes of *S. platensis* NRRL 2364 and *S. paucisporogenes* ATCC 12596 at pH 6·0 exhibited a optimum temperature at ~45°C, while *S. mobaraensis* NRRL B-3729 and the commercially available *S. mobaraensis* product, Activa WM (Ajinomoto) had somewhat higher temperature optima, ~50°C.

The pH optimum of TGase produced by *S. paucisporogenes* ATCC 12596 and *S. platensis* NRRL 2364 was found to be ~8·0 (data not shown), while in the case of *S. mobaraensis* NRRL B-3729 and Activa WM, the optimum pH was around 7·0, although all three bacterial TGase and Activa WM were also significantly active at the tested pH 5·0-9·0 range.

Activa WM and the TGase produced by *S. mobaraensis* NRRL B-3729 were stable at 37°C. However, Activa WM

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lost its activity after 1-h heat treatment at 60°C, while TGase of *S. mobaraensis* NRRL B-3729 retained 50% of its original activity under these conditions. This might be explained with the presence of other proteins in the crude fermentation extract, which probably stabilize TGase. The TGase enzymes produced by *S. paucisporogenes* ATCC 12596 and *S. platensis* NRRL 2364 retained 20% and 30% of their original activities after 1-h heat treatment at 60°C, respectively.

Streptomyces TGases differ from TGase of eukaryotic origin, as these enzymes are totally independent of Ca^{2+} ions. This property is very useful in the modification of those food proteins which are susceptible to Ca^{2+}, such as milk caseins, soybean globulins or myosins (Motoki and Seguro 1998).

As we did not find any data on *S. paucisporogenes* TGase, we investigated the influence of different metal ions on the TGase activity. From the metal ions tested, only Zn^{2+} inhibited the enzyme activity. Even with the addition of 0·5 mmol l^{-1} $ZnSO_4.7H_2O$ to the reaction mixture, the relative TGase activity was reduced to 30%. Some authors suggest that the cysteine residue might be a part of the active site of microbial TGases (Motoki and Seguro 1998). As Zn^{2+} binds to the thiol group of the single cysteine residue, this may be an explanation of inhibitory effect. The other metal ions did not significantly influence the TGase activity, and the same was observed with the chelating agent EDTA too (data not shown).

SDS PAGE

Activa WM, the commercially available TGase product of Ajinomoto Co. with a molecular mass of 37 kDa was used as a comparison. The main bands of *S. paucisporogenes* and *S. platensis* TGases were at the same position, at ~37 kDa, indicating that these two isolates have a TGase with similar molecular mass as the commercially available TGase (Fig. 3). As *S. mobaraensis* showed a weak TGase production in SSF, clear TGase band was not detected. Surprisingly, crude fermentation extracts from *S. paucisporogenes* and *S. platensis* SSF products contained mainly the TGase enzyme and only a lesser amount of other proteins were present. In contrary, *S. mobaraensis* secreted relatively many proteins (enzymes) during the fermentation on liver kidney beans.

Discussion

Three *Streptomyces* isolates were propagated in SSF for transglutaminase production on different agro-food materials. Altogether, 26 different agro-substrates were tested, including various cereals (rice, barley, wheat, rye, oat, buckwheat, broomcorn millet), beans, peas, lentils, poppy seeds, wheat bran, etc. While *S. mobaraensis* (Ando *et al.* 1989) and *S. platensis* (Bech *et al.* 2000) strains are known producers of TGase enzymes, no such data for *S. paucisporogens* ATCC 12596 may be found in previous papers. Good TGase production was observed on substrates with high protein content, such as different beans, peas or lentils. Maximal production was obtained on liver kidney beans with *S. paucisporogenes* ATCC 12596 isolate in a 4-day SSF (4·2 IU g^{-1} DM) and with *S. platensis* NRRL 2364 in a 7-day SSF (5·1 IU g^{-1} DM). Although *S. mobaraensis* NRRL B-3729 showed a fine growth under solid-state conditions, it did not produce significant amount of TGase. However, in shake flask fermentation *S. mobaraensis* proved to be an excellent producer of TGase enzyme (data not shown). All three enzymes exhibited temperature optima around 50°C, pH optima around pH 7–8, and proved to be not thermostable.

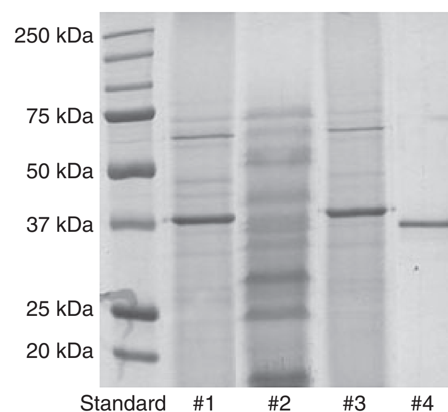

Figure 3 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 20-μl concentrated crude fermentation extracts of *Streptomyces platensis* NRRL 2364 (#1), *Streptomyces mobaraensis* NRRL B-3729 (#2), *Streptomyces paucisporogenes* ATCC 12596 (#3) and Activa WM (#4). Precision Plus Protein Standard (Bio-Rad) was used as a molecular mass standard.

The TGase produced by these three *Streptomyces* isolates in SSF were Ca^{2+} ion independent and their activity could not be inhibited by the addition of EDTA.

The SDS PAGE analysis revealed that both *S. paucisporogenes* ATCC 12596 and *S. platensis* NRRL 2364 secrete a TGase enzyme with a molecular mass of ~37 kDa. SDS PAGE results also showed that extracts of enzyme-enriched beans produced by *S. paucisporogenes* ATCC 12596 and *S. platensis* NRRL 2364 contain TGase in higher amount, and other proteins (enzymes) are present in smaller quantities. It was shown in a previous study (Szakacs *et al.* 2001) that inexpensive *in situ* crude cellulase enzyme may be produced in SSF. Therefore, the authors are convinced that owing to much reduced production cost and direct applicability, SSF TGase without

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downstream processing (cheap *in situ* enzyme, crude enzyme) may be an excellent candidate for some nonfood areas, such as textile and leather industry.

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