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Enzymatic properties of transglutaminase produced by a new strain of *Bacillus circulans* BL32 and its action over food proteins

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ABSTRACT

The aim of this study was to physicochemically characterize transglutaminase (TGase) from *Bacillus circulans* BL32, a strain recently isolated from the Amazon basin region, for its application in food systems. The effects of pH and temperature on the enzyme activity were determined by Central Composite Rotatable Design (CCRD), with maximal TGase activities obtained for pH between 5.7 and 8.7 and temperatures of 25–45 °C. This microbial TGase showed to be remarkably stable: over 90% of its activity was retained after 120 min of incubation at 50 °C. The Ca^{2+} and Mg^{2+} cations enhanced enzyme activity and its thermal stability when in concentrations of up to 2 and 1 mol L^{-1}, respectively. Casein, isolated soy protein, and hydrolysed animal protein were treated with this TGase. The decrease in the amount of free amino groups, especially for casein, showed the cross-linking of protein catalysed by this enzyme, while the emulsifying properties of these proteins were improved with treatment. These results suggest that this microbial TGase has a good potential to be used in food and other industrial applications.

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1. Introduction

Transglutaminase (TGase; protein-glutamine γ-glutamyl-transferase; EC 2.3.2.13) is a family of enzymes that catalyze acyl transfer reactions using peptide-bonded glutamine residues as acyl donors and several primary amines as acceptors. These reactions introduce covalent cross-links between proteins, peptides, and various primary amines (Nonaka et al., 1989). In the absence of amine substrates, TGase catalyses the deamidation of glutamyl residues during which water molecules are used as acyl acceptors. Proteins having TGase activity are abundantly distributed in nature. Calcium-dependent TGases, present mainly in animal and vegetable tissues and in body fluids of vertebrates, are involved in several biological processes (Nielsen, 1995). Although TGases have been isolated from animal tissues and plants (Kuraishi, Yamazaki, & Susa, 2001), only microbial TGases are industrially important due to their properties of calcium independence and relatively low molecular weight. Microbial TGases are extracellular enzymes, implying simpler separation and purification processes (Zhu, Rinzema, Tramper, & Bol, 1995). So far, the TGase obtained from *Streptoverticillium mobaraense* remains the only commercial source of this enzyme (Arrizubieta, 2007).

Interest in TGases has increased recently due to their possible applications in the food industry. Several studies have been carried out on the applications of TGases in the processing of meat, fish, dairy, wheat, and soybean products in order to improve texture, water-holding capacity, elasticity, nutritional value, and appearance (Askin & Kilic, 2009; Gan, Ong, Wong, & Easa, 2009; Gauche, Barreto, & Bordignon-Luiz, 2010; Gauche, Tomazi, Barreto, Ogliari, & Bordignon-Luiz, 2009; Nielsen, 1995; Zhu et al., 1995). Meanwhile, the immunological implications of the use TGase for the modification of gluten for food applications have been studied by Berti et al. (2007), and by Cabrera-Chávez, Rouzaud-Sández, Sotelo-Cruz, and De La Barca (2008), among others. Transglutaminases are also extensively used for biochemical and medical researches, and a range of industries, from pharmaceuticals to chemicals (Arrizubieta, 2007; Hiller & Lorenzen, 2009). A comprehensive review on the applications of TGase in food systems, in particular in dairy products, and the implications of the cross-linking on food proteins with respect to several physicochemical properties, including rheology and mechanical properties, were presented by Jaros, Partschefeldt, Henle, and Rohm (2006). However, most of these applications have been limited due to the high costs of TGase production.

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The purification and preliminary characterization of TGases have been reported for bacteria and fungi such as *Streptoverticillium ladakanum* (Ho, Leu, Hsieh, & Jiang, 2000), *S. mobaraense* (Ando et al., 1989), *Streptomyces hygroscopicus* (Cui, Du, Zhang, Liu, & Chen, 2007), *Bacillus subtilis* (Suzuki et al., 2000), and *Physarum polycephalum* (Klein, Guzman, & Kuehn, 1992). Since different industrial applications may require specific properties of biocatalysts, there is still a need for finding new TGases that could allow novel applications and, more important, be cheaper than the current marketed product. We have recently reported the purification and optimization of the production process of TGase from *Bacillus circulans* BL32, a bacterium isolated from the Amazon basin region (Soares, Assmann, & Ayub, 2003; Souza, Faccin, et al., 2009; Souza, Flôres, & Ayub, 2006; Souza, Rodrigues, & Ayub, 2009). The characterization of this enzyme is essential in order to allow its use in the food industry. Therefore, the purpose of this study was to characterize the TGase from *B. circulans* BL32, investigating its thermal stability, the influence of different salts and their concentrations on both enzymatic activity and thermal stability, the optimal temperature and pH conditions for enzyme activity, and, finally, the enzyme effect on the cross-linking and emulsifying properties of casein, isolated soy protein, and hydrolysed animal protein.

2. Materials and methods

2.1. Materials

All chemicals used were of analytical grade and purchased from Merck (Darmstadt, Germany) unless otherwise mentioned. *N*-carboxybenzoyl-L-glutaminyl-glycine (*N*-CBZ-Gln-Gly), bovine serum albumin (BSA), and trinitrobenzenesulphonic acid (TNBS) were obtained from Sigma–Aldrich Co. Ltd. (MO, USA). Q-Sepharose fast flow (FF) was from Pharmacia (Uppsala, Sweden). Casein was Spraynol produced by Coatec Industrial Ltd. (RS, Brazil). Isolated soy protein (ISP) was Supro 780, produced by The Solae Company S.A. (RS, Brazil). Hydrolysed animal protein (HAP) was obtained in our laboratory as mechanically deboned poultry meat treated with Flavourzyme protease from Novo Nordisk Bioindustrial do Brazil Ltd. (PR, Brazil) according to Soares, Marques, Albuquerque, and Ayub (2000).

2.2. Microorganism and cultivation procedure

A strain of *B. circulans*, isolated from the aquatic environment of the Amazon rain forest, characterized in our group and coded as BL32, was used in this study and described elsewhere (Souza et al., 2006). This strain is kept as lyophilized reference stocks in the Culture Collection of the Microbiology Department of The Federal University of Rio Grande do Sul State, Brazil. Working stocks of *B. circulans* BL32 were kept in glycerol (50 g/100 g) vials at −20 °C. Prior to culture, cells were recovered from frozen stocks and were platted on to Mueller-Hinton agar from where isolated colonies were picked to initiate cultures.

Pre-inocula were prepared by seeding a single colony from plates into Erlenmeyer flasks (250 mL) filled with 50 mL of medium M1, which composition was optimized and described in previous works (Souza et al., 2006; Souza, Rodrigues, et al., 2009). Inoculations were carried out at 30 °C in a rotatory shaker at 100 rpm and cells were grown to optical density (OD) of 1.0 (600 nm). Final cultures were carried out in Erlenmeyer flasks (1 L) filled with 200 mL of M1, pH 8.5, inoculated with 10 mL of pre-inoculum and incubated at 30 °C in shaker at 100 rpm. After 8 days of incubation, cultures were collected and centrifuged at 17,000*g*, 4 °C, 20 min; the supernatant was used for enzyme purification.

2.3. TGase purification

The enzyme was produced and purified according to previous works of our group in which these processes were optimized (Soares et al., 2003; Souza, Faccin, et al., 2009). Basically, TGase from *B. circulans* BL32 was precipitated with 60 g/100 mL ammonium sulphate, resuspended in a 20 mmol L^{-1} Tris–HCl buffer pH 8.0, and dialyzed against the same buffer. After dialysis, the enzymatic preparation was applied to a Q-Sepharose fast flow (FF) ion-exchange column pre-equilibrated with 20 mmol L^{-1} Tris–HCl buffer, pH 8.0, followed by a linear gradient (0–1 mol L^{-1}) of NaCl. Fractions containing the highest enzymatic activity were pooled, dialyzed against the 20 mmol L^{-1} Tris–HCl buffer, pH 8.0, and lyophilized. This cleaned fraction of enzyme was then used for further analysis.

2.4. Determination of enzyme activity and protein concentration of samples

TGase activity was determined by hydroxamate formation using the specific substrate, *N*-CBZ-Gln-Gly, described by Grossowicz, Wainfan, Borek, and Waelsch (1950). A calibration curve was prepared with L-glutamic acid γ-monohydroxamate. One TGase unit (U) was defined as the amount of enzyme that causes the formation of 1 μmol L^{-1} glutamic acid γ-monohydroxamate per minute. Protein concentration was determined by the Lowry assay (Lowry, Rosebrough, Farr, & Randall, 1951), using a standard curve of bovine serum albumin, fraction V.

2.5. Thermal stability of the TGase

The thermal stability of the enzyme was determined by incubating 1 mL of 1 U mL^{-1} TGase solutions (20 mmol L^{-1} Tris–HCl buffer, pH 8.0) at 30, 40, 50, 60, and 70 °C. Sample aliquots were withdrawn at intervals of 0, 1, 2, 5, 10, 20, 30, 60, and 120 min, immediately iced and the residual enzyme activities were measured using the method described above. Three replicates of each measurement were made. The enzymatic activity was expressed as percentage of relative activity. Activity without incubation was considered to be 100%. The relative activity was calculated as:

$$\text{Relative TGase activity, } \% = 100(C_t/C_0) \quad (1)$$

where C_t is the activity at time t (min), and C_0 is the activity at time $t = 0$ min.

2.6. Effects of salts on TGase stability

The salts NaCl, KCl, $CaCl_2$, and $MgCl_2$ were added to 1 mL of an enzyme preparation with 1 U mL^{-1} of TGase activity in 20 mmol L^{-1} Tris–HCl buffer, pH 8.0. Tested salt concentrations were 0, 0.25, 0.5, 1, 2, and 3 mol L^{-1}. After incubations at 35, 40, 45, 50, and 55 °C, for 30 min, samples were immediately placed in ice and TGase activity was determined as described above. Measurements were done in triplicates. The enzymatic activity was expressed as percentage of relative activity, where incubation without salts was considered to be 100%.

2.7. Effects of pH and temperature on TGase activity

The effects of pH and temperature on the enzyme activity were determined by Central Composite Rotatable Design (CCRD), with $k = 2$, which generates 11 treatment combinations. Five levels of each variable were chosen, the upper and lower limits of them set to

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