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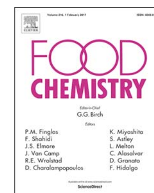

Transglutaminase from newly isolated *Streptomyces* sp. CBMAI 1617: Production optimization, characterization and evaluation in wheat protein and dough systems

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A B S T R A C T

The popularity of transglutaminase (TG) by the food industry and the variation in functionality of this enzyme from different origins, prompted us to isolate and evaluate a high-yielding TG strain. Through the statistical approaches, Plackett-Burman and response surface methodology, a low cost fermentation media was obtained to produce 6.074 ± 0.019 U mL^{-1} of TG from a novel source; *Streptomyces* sp. CBMAI 1617 (SB6). Its potential exploitation was compared to commonly used TG, from *Streptomyces mobaraensis*. Biochemical and FT-IR studies indicated differences between SB6 and commercial TG (Biobond™ TG-M). Additions of TG to wheat protein and flour based doughs revealed that the dough stretching depended on the wheat protein fraction, TG amount and its origin. A higher degree of cross-linking of glutenins and of inclusion of gliadin in the polymers was seen for SB6 as compared to commercial TG. Thus, our results support the potential of SB6 to tailor wheat protein properties within various food applications.

1. Introduction

Microbial transglutaminase (TG; E.C. 2.3.2.13) is today one of the most applied enzymes to cross-link proteins. The enzyme is widely used by the industries in producing meat, dairy and bakery products (Kieliszek & Misiewicz, 2014). TG promotes the formation of intra and intermolecular cross-links through the formation of covalent bonds between lysine and glutamine residues in the proteins (Yokoyama, Nio, & Kikuchi, 2004).

The isolation of a strain of *Streptomyces mobaraense* (Washizu et al., 1994) was the first step towards the extensive commercial exploitation of this enzyme. Thereafter, a number of various microbial strains, such as *Streptomyces lydicus* (Færgemand & Qvist, 1997), *Streptomyces cinnamoneum* CBS 683.68 (Duran, Junqua, Schmitter, Gancet, & Goulas, 1998), *Streptomyces* sp. CBMAI 837 (Macedo, Sette, & Sato, 2007), have been found being able to biosynthesize TG extracellularly. The yield and the properties of the synthesized TG have been found to vary considerably among the strains (Kieliszek & Misiewicz, 2014). Novel TG-producing bacterial strains are still isolated from various environmental sources with an aim to find high-yielding strains (Zhang, Yang, & Chen, 2009). While evaluating novel strains for enzyme production, selection of the most appropriate composition of the media for culturing the specific strains are extremely important (Reddy, Wee, Yun, & Ryu, 2008). The Plackett-Burman design (PB) and response surface methodology (RSM) are powerful statistical tools used to reduce the number of experiments needed through the application of a broad spectrum of interactions among variables to optimize the response of a process (Abd El Aty, Wehaidy, & Mostafa, 2014; Reddy et al., 2008).

Wheat dough extensibility and strength are characters attributed to the main polymeric gluten protein fractions. The amount, type and size distribution of protein present in the wheat flour play a key role in dough development. Genetic and environmental factors are important determinants of the complexity and amount of polymers formed during wheat processing (Johansson et al., 2013). Such polymer differences are

Abbreviations: TG, transglutaminase; RSM, response surface methodology; CCRD, central composite rotatable design; PB, Plackett-Burman design; PP, polymeric proteins

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often measured applying SE-HPLC and determining the percentage of unextractable polymeric protein in total polymeric protein (%UPP) (Hussain, Larsson, Kuktaite, Prieto-Linde, & Johansson, 2012; Malik, Kuktaite, & Johansson, 2013). Beneficial effects of commercially TG from *Streptomyces mobaraensis* on bread making quality have been reported (Larré et al., 2000; Steffolani, Pérez, Ribotta, Puppo, & León, 2008). However, dough development and bread-making quality might be impacted by the use of TGs of various origin and in different concentrations. To better understand effects of functionality of TGs from various strains, protein behavior e.g. solubility and polymerization, can be evaluated in gliadin or glutenin enriched fractions and dough system. The aim of the present study was to detect novel microbial strains producing TG, and to optimize the fermentation media culture for such strains using a low-cost substrate for culturing. An additional aim was to evaluate differences between a novel and a commercial TG enzyme in relation to their effect on protein and polymerization behavior in wheat flour dough and in systems based on the gliadin rich and glutenin rich protein fractions from wheat gluten.

2. Materials and methods

2.1. Materials

Physico-chemical information of wheat flour (W) and gluten powder (GL) was provided by the manufacturers Farina AB (Lilla Harrie, Sweden) and Lantmännen Reppe AB (Lidköping, Sweden), respectively. Wheat flour (Extra bagerivetemjöl) was treated with ascorbic acid (30 mg/kg) by the manufacturer and contained 11.4% protein, 69% starch, 1.4% fat and 2.9% fiber. The gluten powder contained 77.7% protein (N × 5.7) and 5.8% starch. The gluten fractions, gliadin and glutenin, were obtained according to Blomfeldt et al. (2012).

The commercial enzyme Biobond™ TG-M (designated as commercial TG from here and onwards) was supplied by Shanghai Kinry Food Ingredients Co. (China) and presented an activity of 109.9 U g^{-1}. *N*-Carbobenzoxy-L-glutaminyl glycine (*N*-CBZ-Gln-Gly), L-glutamic acid γ-monohydroxamate were purchased from Sigma-Aldrich Co. (Germany). Urea and sodium dodecyl sulfate (SDS) were purchased from Duchefa Biochemie (Netherlands). NaH_2PO_4 and trifluoroacetic acid (TFA) were purchased from Merck (Germany), and HPLC grade acetonitrile from VWR BDH Prolabo (VWR Chemicals, Sweden). Isopropanol was supplied by Honeywell, Germany. All chemicals were of analytical grade. Water was purified by a Milli-Q system (Millipore Corporation, Billerica, USA).

2.2. Methods

2.2.1. Isolation and screening of soil actinomycetes for TG production

Actinomycetes were isolated from soil samples from different locations in Brazil according to El-Nakeeb and Lechevalier (1963) with modifications. Growth of actinomycetes was favored over other bacteria, by mixing air-dried soil with calcium carbonate (1:1). Mixtures were serially diluted and inoculated in inorganic salts-starch agar (ISP medium 4; Shirling & Gottlieb, 1966), then incubated at 30 °C and 40 °C for 7 days. The strains were maintained as mycelial fragments and spores in 20% (v/v) glycerol at -80 °C or in yeast malt extract agar (ISP medium 2; Shirling & Gottlieb, 1966).

Inocula was prepared by incubating the culture on petri dish containing ISP medium 2 for 4 days at 30 °C. Six disks of 0.6 cm diameter and uniformly covered by bacteria were cut, inoculated in fermentation flasks of 250 mL containing 50 mL of culture broth and incubated at 30 °C, constant agitation at 150 rpm. The medium used for the screening of TG producing actinomycetes under submerged fermentation was composed of 2.5% soybean meal, 2.0% potato starch, 0.1% glucose, 1.0% bacteriological peptone, 0.4% $KH_2PO4{\cdot}7H_2O$ and 0.2% $MgSO_4{\cdot}7H_2O$, pH 7.0 (Macedo et al., 2007). Transglutaminase activity was determined using the hydroxamate assay (Folk & Cole, 1966) with slight modifications (Macedo et al., 2007). The calibration curve was prepared with L-glutamic acid γ-monohydroxamate. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol of hydroxamic acid per minute at 37 °C. The B6 strain was selected for further analysis due to its high TG activity.

2.2.2. The 16S rRNA gene sequence and the phylogenetic analysis

The comparative 16S rRNA gene sequence is considered a reference method for bacteria identification, supporting the elucidation of complex taxonomic relations within *Streptomyces* genus (Zucchi et al., 2012). The isolate B6 was deposited to the Brazilian Collection of Microorganisms from the Environment and Industry and was given strain number CBMAI 1617 (the identification method is detailed in the supplementary section, Appendix A).

2.2.3. Screening for optimizing fermentation media using the Plackett-Burman design

Seven independent variables (supplementary section; listed in Table B1; Appendix B) were screened for their effect on the B6 fermentation and TG production. The real values of this screening were transferred into coded values as presented in Table B1.

2.2.4. Optimization of fermentation media using central composite rotatable design (CCRD)

Three variables (supplementary section; Table B2; Appendix B) were evaluated at two levels and at their axial points applying the CCRD. Similarly as for PB, the real values of this optimization were transferred into coded values as presented in Table B2. CCRD was performed with three replicates at the central point and six axial points, summing 17 runs. A second-order polynomial equation was used to predict the optimal concentrations of nutrients for TG production (1):

$$Y = b_0 + \sum_{i} b_i X_i + \sum_{ii} b_{ii} X_i^2 + \sum_{ij} b_{ij} X_i X_j \quad (1)$$

where Y is the estimated response (TG activity), b_0, b_i, b_{ii}, b_{ij} are offset term, linear coefficient, quadratic coefficient, and interaction coefficient. The coded independent variables are x_i and x_j. The results were validated by performing experiments using the predicted optimized concentrations. The quality of fit of the statistical model equation obtained was demonstrated by the coefficient of determination (R^2). The statistical significance was determined by an *F* test (ANOVA).

2.2.5. Enzyme preparation

To prepare TG extract from B6 applying the optimized media, Erlenmeyer flasks of 500 mL with 100 mL working volume were inoculated following the method described on item 2.2.1., incubated at 30 °C, 150 rpm during 96 h.

The cell free supernatant was fractionated with 40% (saturation) ammonium sulfate. After centrifugation (16,576*g*; 10 min, Hitachi Himac CR 21 GII, Japan) ammonium sulfate was added to bring the supernatant to 80% saturation. The precipitate was dialyzed against distilled water and lyophilized. The finally prepared enzyme was designated as SB6 from here and throughout the manuscript.

2.2.6. Effects of pH and temperature on enzyme activity of SB6 and commercial TG

The effect of pH on the enzyme activity was evaluated at 37 °C using the following 50 mM buffers: sodium citrate (pH 4.0–6.5), Tris-HCl (pH 7.0–9.0), Glycine-NaOH (pH 10.0–11.0) and NaOH solution (pH 12.5). To evaluate the temperature effect on enzyme activity, incubation

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