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Salt-independent thermophilic α-amylase from *Bacillus megaterium* VUMB109: An efficacy testing for preparation of maltooligosaccharides

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ABSTRACT

An amylase (est. M_w 150 kDa) was purified 27.39-folds from the culture broth of *Bacillus megaterium* VUMB109. The purified enzyme was not inhibited by *p*-chloromercuro benzoate and iodoacetamide (10 mM), it rapidly decolorized the blue color of starch–iodine complex and produced α-anomeric products from starch hydrolysis, thus, it is an endo-attacking α-amylase. The enzymatic activity was not affected by any metal ion and EDTA, therefore, it is not in the class of metalloenzyme. The purified α-amylase showed higher affinity (K_m = 1.5 μM; V_{max}/K_m = 0.38 and $K_{cat}/K_m = 2.5 \times 10^6$) to starch than other tested substrates like amylose, amylopectin and glycogen. Maltooligomer mixture with high proportion of maltopentaose (G5) and maltotriose (G3) was produced during hydrolysis of starch, amylopectin and amylose. It exhibited high degree of hydrolysis on raw potato starch than wheat, rice and corn starches. Thus the studied α-amylase could be exploited as a useful catalyst in the bioprocessing of maltooligomer mixture as food supplement for baby and aged people.

1. Introduction

Starch-degrading amylolytic enzymes are now of great significance in biotechnological applications in industries as these replace over 75% acid hydrolysis process of starch. The α-amylase is mostly used in starch saccharification, and finds wide applications in baking, brewing, detergent, textile, paper and distilling industry (Aiyer, 2005; Lin et al., 1997; Pandey et al., 2000). The enzyme can be derived from many sources such as plants, animals and microbes (Jana and Pati, 1997; Maity et al., 2011), but microbial amylases especially those from bacterial origin generally meet industrial demands for their cost-effective production and thermostability (Pandey et al., 2000; Fossi et al., 2009).

Generally most of the amylases produce glucose and maltose as main products from starch hydrolysis, but only a few amylases can produce specific oligosaccharides like maltotriose, maltotetraose and maltopentaose (Fossi et al., 2009; Kobayashi et al., 1990; Ratanakhanokchai et al., 1992; Yang and Liu, 2004; Samanta et al., 2009; Maity et al., 2011). These oligosaccharides have several useful properties like they (i) are low calorigenic; (ii) are less sweet than sucrose (30%, using 3% solution at 20 °C); (iii) inhibit the growth of harmful intestinal microflora; (iv) have low viscosity, high moisture-retaining capacity, and low water activity, convenient for controlling microbial contamination (Dey et al., 2003). Branched oligosaccharides such as isomaltose and panose are also effectively used as anticarcinogenic saccharides (Nakakuki, 2002). These oligosaccharides are highly water soluble and produce clear tasty solutions, which are used as nutrients for infant and aged persons (Fogarty and Kelly, 1990). The Japanese Government legislated for *Foods for Specified Health Use* (FOSHU) and *Foods with Nutrient Function Claims* (FNFC), which enlisted 223 items, more than 50% of which incorporate oligosaccharides as the functional components. The market for oligosaccharides is already substantial and continues to expand gradually. In Japan, several oligosaccharides were produced on an industrial scale by the catalysis of microbial enzymes and annual demands of these saccharides are about 15,000 tons (Nakakuki, 2002). Research and development of novel oligosaccharides with physiologically functional properties is now continuing.

In our laboratory, we have isolated a bacterium *Bacillus megaterium* VUMB109 that can produce thermostable, salt-tolerant and raw starch degrading amylase (Jana et al., 1997). The present paper is concerned with the purification and characterization of malto-oligosaccharide producing α-amylase from *B. megaterium*

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VUMB109. This amylase is quite different with respect to the product specificity from the amylases produced by other strains of *B. megaterium* (Ghollasi et al., 2010; Takasaki, 1989).

2. Materials and methods

2.1. Microorganism

Previously isolated and identified *B. megaterium* VUMB109 (Jana et al., 1997) was used in this study.

2.2. Submerged fermentation

An enriched culture media (pH ~8.2) containing (w/v) 0.2% starch, 0.4% $(NH_4)_2HPO_4$, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.1% KCl was used for submerged fermentation. Enzyme production was carried out in a 250 ml flask containing 50 ml liquid media for 24 h at 40 °C on a rotary shaker (200 rpm). Fermented broth was collected after centrifugation (5000 × *g* for 10 min) and the resultant supernatant was used as the source of enzyme.

2.3. Purification of the amylase

The concentrated culture broth was treated with solid $(NH_4)_2SO_4$ (80% saturation) and kept at 4 °C for 12 h. The precipitate was collected by centrifugation (12,000 × *g* for 30 min, at 4 °C) and dissolved in phosphate buffer (10 mM, pH 7.75). Enzyme solution was dialyzed against the same buffer for 24 h at 4 °C with a periodical change of the buffer solution. The dialyzed enzyme was passed through a DEAE-cellulose column (Merck, Mumbai, India). The active fraction of amylase was eluted with a linear gradient of KCl (0.001–0.2 M). Then the enzyme was passed through Sephadex G-100 column (1.5 cm × 92 cm) that was pre-equilibrated with 10 mM phosphate buffer (pH 7.5) and eluted with the same buffer. The active fractions were collected, concentrated through lyophilization and kept at 4 °C for further use.

The homogeneity of the purified amylase was tested through SDS-polyacrylamide (12%) gel electrophoresis according to the method of Laemmli (1970). After electrophoresis, the gel was stained with coomassie brilliant blue (R250) and the molecular weight of the amylase was determined against marker proteins (Genei, Bangalore, India).

2.4. Assay of amylase

Saccharification activity of amylase was measured following the method of Achi and Nijoku-Obi (1992). Briefly, reaction mixture contained 0.5 ml of 1% (w/v) soluble starch, 0.4 ml of 10 mM phosphate buffer (pH 7.75) and 0.1 ml of enzyme solution. Then the mixture was incubated at 93 °C for 5 min. The liberated reducing sugar in the reaction mixture was quantified using 3,5-dinitrosalicylic acid (DNS) as coloring reagent and absorbance was measured at 540 nm. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol reducing sugars (measured as glucose) ml^{-1} min^{-1} under the specified assay conditions.

2.5. Estimation of protein

Soluble protein was estimated following the procedure of Lowry et al. (1951) using bovine serum albumin (fraction V) as the standard.

2.6. Determination of blue loss percentage of starch

The reduction in starch–iodine color intensity upon enzyme hydrolysis was determined following the method of Teodoro and Martins (2000). The substrate solution of 1.0 ml [1% (w/v) soluble starch (Himedia, Mumbai, India) in phoshphate buffer, 0.02 M, pH 7.2] was incubated with 0.1 ml of the enzyme at 90 °C for 30 min. Enzymatic reaction was periodically stopped by adding 10 ml of 0.1 N HCl. Then this solution was diluted 10 times with iodine reagent (0.05% iodine and 0.5% potassium iodide) and the absorbance was measured at 660 nm.

2.7. Measurement of optical rotation of hydrolyzed products

The purified amylase was employed for complete hydrolysis of potato starch. A reaction mixture (1 ml) consisting of 1% (w/v) starch solution in 10 mM phosphate buffer (pH 7.75) and 100 μl of purified amylase was added to 1 cm cell (cuvette). The optical rotation of the mixture was periodically measured in polarimeter (Perkin Elmer) using sodium light. The mutarotation of the hydrolysate was determined by adding 5.0 mg of solid sodium carbonate per milliliter of mixture after the optical rotation became almost constant (Konsula and Liakopoulou-Kyriakides, 2004).

2.8. Degree of hydrolysis of starchy foods

Different starchy foods (1%, w/v in phosphate buffer, pH 7.75) like wheat, potato, rice and corn were separately mixed with enzyme solution (1.0 U/ml) and incubated at 90 °C. Acid hydrolysis was carried out by treating the food stuffs with 6.0 M HCl at 100 °C for 2 h. Total reducing sugar as maltose equivalents was estimated by DNS method (Dubois et al., 1956).

$$\text{Degree of hydrolysis (\%)} = \frac{\text{reducing sugar produced by enzyme hydrolysis}}{\text{reducing sugar produced by acid hydrolysis}}$$

2.9. Chromatographic studies for product identification

The enzymatic hydrolyzed products of different polysaccharides were detected on 1 mm Whatman no. 1 chromatographic paper. Starch, amylase and amylopectin [1 g% (w/v) in 10 mM phosphate buffer, pH 7.5] were digested with the purified amylase at 93 °C and the periodic hydrolytic products (100 μl) were spotted on the papers. For identification of hydrolyzed products, the maltooligosaccharide marker (Sigma, USA) was employed. A descending mode of solvent system of *n*-butanol–acetic acid–water (4:1:5, v/v) was used for paper chromatography. Chromatogram was developed by dipping the papers in alkaline silver nitrate/sodium hydroxide reagent (1.2% $AgNO_3$ + 0.1% KOH + 5% $Na_2S_2O_3$).

The quantity of maltooligosaccharides in the enzymatic hydrolyzate was determined in thin layer chromatography following the principle of HPTLC. For thin layer chromatography, Merck classical silica TLC plate (silica gel 60 on aluminum backed, 10 cm × 20 cm) was used. 10 μl of sample was spotted on activated plate and dried with hair drier. The plates were then dipped in the twin trough chamber with solvent system of *n*-butanol, methanol and 16% (v/v) aqueous ammonia (5:4:3). After 10 min of run, plates were dried and the oligosaccharides were stained by spraying a mixture of 2.4% (w/v) phosphomolybdic acid, 5% (v/v) H_2SO_4 and 1.5% (v/v) H_3PO_4. In similar manner known quantities of different oligosaccharides (Sigma, USA) were also run simultaneously. The quantity of each oligosaccharide was estimated through scanning densitometry study using Immage Lab 2.0 software (Bio Rad) and expressed as relative percentage.

2.10. Estimation of kinetic parameters

The kinetic parameters of purified amylase like K_m and V_{max} were estimated from Eadie–Hofstee plot (v_0 vs. v_0/[s]) using