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Biochemical Engineering Journal 34 (2007) 44–50

Biochemical Engineering Journal

www.elsevier.com/locate/bej

A β-cyclodextrin glycosyltransferase from a newly isolated *Paenibacillus pabuli* US132 strain: Purification, properties and potential use in bread-making

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Received 1 August 2006; received in revised form 16 November 2006; accepted 16 November 2006

Abstract

A bacterial strain designed US132, isolated from a Tunisian soil was selected for its production of a potent cyclodextrin glycosyltransferase (CGTase) activity. This strain was identified as *Paenibacillus pabuli* by sequencing of the 16S rDNA and the 16S-23S internal transcribed spacer (ITS). The US132 CGTase, purified to homogeneity by hydrophobic interaction chromatography and starch adsorption, is a monomer of approximately 70 kDa. This enzyme exhibited a maximal activity at 65 °C, in presence of 10 mM calcium, and was most active at pH range 5.5–9 with an optimum at 6.5. Using 10% (w/v) of potato starch, this CGTase produced a high level of cyclodextrins reaching 42 g/l with a β-cyclodextrin ratio of 63%. Furthermore, this enzyme can be used in the bread-baking process since its addition in the dough mix improved significantly the loaf volume and decreased the firmness of bread during storage.

Keywords: β-CGTase; *Paenibacillus pabuli*; Purification; Cyclodextrins production; Bread-making

1. Introduction

Cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) is the unique enzyme able to convert starch and related sugars into cyclodextrins (CDs) via a cyclization reaction. CDs are non reducing cyclic structures consisting of 6, 7 or 8 glucose residues, joined by α-(1,4) linkages, for α-, β- and γ-cyclodextrin, respectively. These compounds have an exclusive ability to act as molecular containers by entrapping hydrophobic molecules in their internal cavity. This property has been used for stabilization, solubilization and masking odors and tastes of a wide variety of interesting compounds used in food, pharmaceutical, cosmetic, agricultural and chemical industries [1–3]. Besides the ability of CGTases to catalyze the intramolecular transglycosylation reaction (cyclization), they are also able to perform two intermolecular transglycosylation reactions: coupling, in which a cyclodextrin ring is cleaved and transferred to a linear acceptor substrate and disproportionation, wherein two linear oligosaccharides are converted into linear oligosaccharides of different sizes. In addition, these enzymes possess a weak hydrolyzing activity in which water is the glycosyl acceptor [4,5].

All known CGTases convert starch into a mixture of α-, β- and γ-CD in different ratios. Depending on the main cyclodextrin produced, CGTases are classified as α-, β- or γ-CGTases. Among the three types of cyclodextrins, β-CD is of high interest due to the size of its non-polar cavity which is suitable to accommodate many molecules such as aromatics and drugs; its low solubility in water which facilitate its separation from the reaction mixture. Furthermore, β-CD inclusion complexes are easily prepared and stable [1,6]. The reported β-CGTases generate various CDs yield and proportion, which depends on the microbial source of the enzyme and the bioconversion conditions [4,6–10]. To improve the CDs production yield and selectivity, organic complexing agents can be added [6,10–12]. However, the application of organic solvents has several disadvantages limiting the use of CDs in food and pharmaceutical industries [3,10]. Hence, it is of interest to set out a CGTase activity producing high concentration of β-CD in the absence of organic solvent.

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1369-703X/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.bej.2006.11.016

Additionally, CGTases were reported to improve baked product characteristics such as loaf volume [13,14] and to delay the bread staling during storage [14–16]. Staling phenomenon, caused by the retrogradation of starch, refers to various undesirable changes that occur in the loaf during storage, thereby lowering the consumer's acceptance. The increase of crumb firmness, which means that bread becomes gradually hard and brittle during storage, is the major aspect of staling. Owing to their ability to reduce starch retrogradation, some amylases are described as effective anti-staling agent [14–17]. Among these, the maltogenic (*exo*-hydrolytic) α-amylase (EC 3.2.1.133) from *Bacillus stearothermophilus*, commercially available under the trade name Novamyl (Novozymes A/S) [18], is widely used in industry of bread-baking.

In this paper, we report the purification and the characterization of a CGTase, from the newly isolated *Paenibacillus pabuli* US132 strain, producing a high concentration of cyclodextrins, mainly formed by β-CD. The effect of this enzyme on the loaf volume and the crumb firmness, compared with that of Novamyl, is also reported and discussed.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture media

The strain US132 was isolated from Tunisian soil as a potent β-CGTase producer. *Escherichia coli* DH5α (F^- *sup*E44 φ80 δ*lac*ZΔ M15 Δ(*lac*ZYA-*arg*F) U169 *end*A1 *rec*A1 *hsd*R17 (r_k^-, m_k^-) *deo*R thi-1 λ^- gyrA96 *rel*A1) [19] was used as host strain. The plasmids pJS3 and pJS7, derivatives of pGEM-T easy vector (Promega), carried, respectively, the 16S rRNA gene and the 16S-23S internal transcribed spacer (ITS) of the US132 strain. *E. coli* recombinant strains were grown on Luria Bertani (LB) plates [20] containing ampicillin (100 μg/ml). For US132 strain a medium composed of 1% potato starch, 1% peptone, 0.5% yeast extract, 0.5% beef extract and 0.1% NaCl was used. The initial pH of culture media was adjusted to 8.

2.2. Isolation and identification of the strain US132

The soil samples for screening were taken from different regions in Tunisia. The microorganisms were isolated on agar plates of LB medium containing 1% starch. After incubation at appropriate temperature for 24 h, the plates were stained with iodine vapor. All colonies exhibiting a clear zone, indicating starch degradation, were isolated for further investigation. The bacterium designated US132 displayed a large halo of starch degradation versus a very low level of reducing sugars. Hence, it was retained for its production of a CGTase activity and was used as source of chromosomal DNA. PCR amplifications of the 16S rRNA gene and the 16S-23S ITS were performed using primers designed from conserved regions within the rRNA operon of *E. coli* [21]. The PCR products were purified and cloned into pGEM-T easy vector. Then, the corresponding nucleotide sequences were determined by the BigDye Terminator v3.1 Cycle Sequencing Kit and the automated ABI Prism® 3100-Avant Genetic Analyser (Applied Biosystems). Homology search was carried out using BLAST search algorithm. The nucleotide sequences of the 16S rRNA gene and the 16S-23S ITS have been submitted to the EMBL data bank under accession numbers AM087615 and AM087616, respectively.

2.3. Purification of the US132 CGTase

The bacterial cells of the fermentation broth were removed by centrifugation at 6000 × *g* for 15 min at 4 °C and the cell-free supernatant was used as source of the enzyme. After the addition of $(NH_4)_2SO_4$ at 35% saturation, the resultant mixture was applied to Phenyl Sepharose™ 6 Fast Flow column (Amersham Biosciences) previously equilibrated with 50 mM sodium acetate buffer (pH 6.5) saturated by 35% $(NH_4)_2SO_4$. The enzyme was eluted with a decreasing linear gradient, 35–0% of $(NH_4)_2SO_4$ in sodium acetate buffer, at a flow rate of 10 ml/min. The fractions containing CGTase activity were pooled, concentrated and dialyzed against sodium acetate buffer at 4 °C. The dialyzed solution was adsorbed to 7% insoluble potato starch in the presence of 1 M $(NH_4)_2SO_4$ at 4 °C as described by Martins and Hatti-Kaul [7] with some modifications. The mixture was centrifuged at 5000 × *g* for 10 min and the pellet was washed with cold water until the absorbance at 280 nm reached zero. To allow enzyme elution, the pellet was incubated with 50 mM sodium acetate buffer (pH 6.5) at 40 °C for 30 min followed by centrifugation at 5000 × *g* for 10 min. The elution was repeated twice and the fractions were concentrated and stored at 4 °C. The purity of the US132 CGTase was checked by using SDS-PAGE and HPLC size exclusion column (Shodex KW-802.5, Bio-Rad).

2.4. Enzyme activity assays

The amount of the reducing sugars released by the action of amylases on starch was determined according to the DNS method of Miller [22].

The CGTase activity was determined as starch-degrading activity and monitored by measuring the decrease in absorbance (blue value) of iodine–amylose complex [23]. The reaction mixture contains 50 μl of appropriately diluted enzyme, 10 mM calcium and 500 μl of 1% potato starch (Sigma) dissolved in 100 mM sodium acetate buffer (pH 6.5), in a total volume of 1 ml. In standard conditions, assays were incubated for 10 min at 65 °C and the reaction was stopped by addition of 1 ml of 1.5 M acetic acid; then 1 ml of iodine reagent (0.02% I_2, 0.2% KI) was supplemented to the mixture and the absorbance was measured at 700 nm. One activity unit was defined as the amount of enzyme able to decrease 10% of amylose–iodine complex optical density per min under the assay conditions.

The *exo*-hydrolytic activity introduced in dough mix was assayed using the Betamyl Kit commercialized by Megazyme (Wicklow, Ireland). The procedure employs as substrate the *p*-nitrophenyl maltopentaoside (PNPG5) and an excess level of high purity α-glucosidase in the presence of stabilizers which significantly reduce the rate of cleavage of PNPG5 by α-glucosidase. The assay was performed by incubating 100 μl of appropriately diluted enzyme with 100 μl of substrate mix-