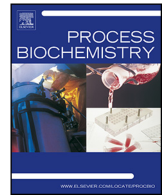
Process Biochemistry 48 (2013) 1303–1310

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio



Constitutive expression of a novel isoamylase from *Bacillus lentus* in *Pichia pastoris* for starch processing

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A R T I C L E I N F O

Article history:
Received 7 April 2013
Received in revised form 27 June 2013
Accepted 1 July 2013
Available online 9 July 2013

Keywords:
Isoamylase
Bacillus lentus
Pichia pastoris
GAP promoter
Starch processing

A B S T R A C T

Isoamylase is essential to saccharifying starch by cleavage of 1,6-glucoside linkages in starch molecules. In this study, a novel isoamylase gene from *Bacillus lentus* JNU3 was cloned. The open reading frame of the gene was 2412 base pairs long and encoded a polypeptide of 804 amino acids with a calculated molecular mass of 90 kDa. The deduced amino acid sequence shared less than 40% homology with that of microbial isoamylase ever reported, which indicated it was a novel isoamylase. A constitutive *GAP* promoter was used to express the recombinant isoamylase in the yeast *Pichia pastoris* by continuous high cell-density fermentation to avoid the use of methanol, which resulted in 318 U/mL extracellular isoamylase activity after 72 h in a 10 L fermenter. The recombinant enzyme was purified and characterized. It had an estimated molecular mass of 90 kDa, with its optimal activity at 70 °C, pH 6.5 and was quite stable between 30 °C and 70 °C. The recombinant isoamylase proves to be superior to pullulanase as an auxiliary enzyme in maltose production from starch. Therefore it will contribute significantly to the starch debranching process.

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1. Introduction

Isoamylase (EC 3.2.1.68, glycogen-6-glucanogydrolase) is one of the starch debranching enzymes that hydrolyzes α-1,6-glucosidic linkages in glycogen, amylopectin and their phosphorylase limit dextrins to yield amylose and oligosaccharides [1]. The native starch used in industry is a mixture of amylose (a linear structure of α-(1,4) linked glucose units) and amylopectin (short α-(1,4) chains linked glucose units highly branched by α-(1,6) bonds) [2], and the ratio of amylopection in the starch of the most botanical origins is more than 70%. Amylopectin can be only partially hydrolyzed by α-amylase because its branch points are resistant to attack from the usual α-amylase. However, debranching enzymes such as isoamylase and pullulanase can exclusively degrade amylopectin, leaving long linear polysaccharides that can be easily hydrolyzed into fermentable sugars. Therefore, application of these enzymes in starch processing industry can significantly improve the efficiency and yield of conversion of starch. Though isoamylase has three advantages over pullulanase, i.e. both endo- and exo-cleavage activity, higher efficient cleavage on α-D-(1,6)-glucoside linkages and less inhibition by its main product maltose, very limited information for isoamylase is available compared with numerous researches concerning pullulanase of various origins [3,4]. This might be mainly due to the serious difficulty in measuring isoamylase activity with interference from activity of other amylolytic enzymes, and also due to the instability of isoamylase itself. Up to date, *Bacillus amyloliquefaciens* [5], *Flavobacterium odoratum* [6], *Escherichia coli* [7–10], and *Pseudomonas amyloderamosa* [11–13] have been isolated with capability of producing this enzyme, but isoamylase of *Bacillus lentus* has, as yet, not been reported.

Pullulanase has been commercially produced by Novozyme and widely used as a processing aid in production of ethanol and sweeteners. By contrast, isoamylase has been mostly used in lab-researches. The application of isoamylase in starch processing industry is restricted by the low-yield ferment strains. *Pichia pastoris* is an industrial methylotrophic yeast. A highly efficient expression system containing the methanol-inducible alcohol oxidase 1 (*AOX1*) promoter and vectors that are integrated into the *Pichia* genome enables it to be easily genetically engineered to express proteins for both basic research and industrial use [14]. High density fermentation and high level expression of proteins are the most prominent advantages of *P. pastoris* system, which make it extremely suitable for foreign protein production. Previous studies indicate that certain proteins that cannot be expressed efficiently in bacteria, *Saccharomyces cerevisiae*, or baculovirus have been successfully produced in functionally active form in *P. pastoris* [15–19]. The powerful genetic techniques available, coupled with its economy of use, make *P. pastoris* a system of choice for heterologous protein expression. So, this study aims to find out if a high level of functional expression of *B. lentus JNU3* isoamylase gene can be achieved in this methylotrophic yeast, and how the recombinant isoamylase reacts to different substrates and catalytic conditions

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http://dx.doi.org/10.1016/j.procbio.2013.07.001

under the "higher eukaryotic" protein modifications such as glycosylation, disulfide-bond formation, and proteolytic processing.

2. Materials and methods

2.1. Strains plasmids and culture media

Supplemental Table 1 shows strains, plasmids and primers used in this study. The isoamylase producing strain *B. lentus* JNU3 was isolated from hot-spring of Tengchong volcano in Yunnan, China. *P. pastoris* GS115 and pGAPZα (Invitrogen) were used as host and vector for heterologous expression of isoamylase. Competent *E. coli* JM109 was used for plasmid construction. pMD18-T (Takara) was used for gene cloning and sequencing. *E. coli* was grown at 37 °C in Luria–Bertani (LB) medium supplemented with 25 μg/mL of zeocin. *P. pastoris* was grown at 30 °C in YPG medium containing 10 g/L yeast extract, 20 g/L peptone, and 20 g/L glycerol or YNB-G plates containing 13.4 g/L yeast nitrogen base without amino acids and 20 g/L glucose supplemented or not with L-histidine (100 g/mL). The medium for fermentation of recombinant *P. pastoris* strain during the batch phase consisted of 10 g/L sucrose, 40 g/L glycerol, 5 g/L yeast extract, 22 g/L $(NH_4)_2SO_4$, 18.2 g/L KH_2PO_4, 7.5 g/L $MgSO_4 \cdot 7H_2O$, and 0.5 g/L $CaCl_2 \cdot 2H_2O$, with vitamins and traces prepared as recommended by Cregg [14].

2.2. Cloning of isoamylase gene

In previous studies, purified isoamylase from *B. lentus* JNU3 was digested by trypsin and subjected to nanoflow liquid chromatography coupled with electrospray ionization quadrupole time-of flight tandem mass spectrometry (nanoLC–ESI-Q-TOF/MS/MS) (submitted but unpublished results). Degenerate primer DNAs of BLID1 and BLID2 (Supplemental Table 1) were designed based on the identified internal peptides sequences (AGFIVRKSMEGND and MSMVITEVSVA) to amplify part of the gene. They were prepared with a DNA synthesizer (Applied Biosystems) and were purified with a DNA Refining System by Sangon Biotech. Genomic DNA from *B. lentus* was prepared by using the DNeasy Kit (Qiagen). PCR was performed in a DNA thermal cycler (TPerformance standard gradient thermalcycler, Biometra), using each pair of primers plus genomic DNA (1.0 μg). The reaction conditions were as follows: 96 °C for 30 s, 52 °C for 30 s, and 72 °C for 2.5 min for 30 cycles. Inverse PCRs were applied to determine the integral sequence flanking the known fragment. First, genomic DNA of *B. lentus* JNU3 was digested with *Xba*I, *Eco*RI, and *Sac*I, respectively, followed by self-ligation under conditions that favored the formation of monomeric circles [20] by T4 DNA ligase. The first round inverse PCRs were conducted with primers IVA1 and IVA2. Then, a new round inverse PCRs were conducted using templates generated by *Sal*I, *Dra*I, and *Bam*HI, respectively, combined with suitably synthesized primers IVB1 and IVB2. Finally, the determined sequences by the two round inverse PCRs, as well as the sequence of the product with primers BLID1 and BLID1 were analyzed with DNAMAN V7 to assemble the whole encoding gene of *B. lentus* isoamylase. Finally, another pair of primers BLIA1 and BLIA2 was designed according to the assembled sequence in order to amplify integral isoamylase gene (*iam*1) from genomic DNA.

The amplified PCR product was purified and cloned into pMD18-T-simple vector to yield pMDBLIA, and for sequencing. With both strands of the insertion sequenced, its sequence was subjected to BLAST analysis. Nucleotide and deduced amino acid sequences were analyzed with the ExPASy Proteomics tool (http://www.expasy.ch/).

2.3. Expression of the iam1 gene in P. pastoris

The construct pMDBLIA was digested with *Not*I to recover the fragment of coding region of the isoamylase gene. The 2.8-kb gel extract fragment (*iam1*) was inserted into the *P. pastoris* expression vector pGAPZα at the *Not*I site, yielding a construct pGAPBLIA. With insertion direction testified, the construct was transformed into *E. coli* JM109 competent cells which were plated on (LB) medium containing 25 μg/mL zeocin. The resulting plasmid pGAPBLIA carries a single copy of the *iam1* gene under the control of the constitutive *GAP* promoter and the *AOX1* terminator. The isolated plasmid was confirmed by checking gel patterns after digestion with various endonucleases and sequencing using the 5′ and 3′ *AOX1* sequencing Primers (Supplemental Table 1).

P. pastoris GS115 (Invitrogen) was prepared for transformation according to the manufacturer's instructions. Prior to the transformation, the pGAPBLIA was linearized by *Avr*II, followed by gel purification using Gel Extraction Kit (Biodev tech). Approximately 25 ng linearized DNA in 1 μL TE buffer and 40 μL competent cells were used during the transformations which were done by electroporation using a Gene Pulser (BIO-RAD), with voltage of 1500 V, capacitance of 25 μF and resistance of 200 Ω, in 2-mm cuvettes. Following electroporation, cells were suspended in 1 mL cold 1 M sorbitol buffer and spread on YNB-G plates containing 0.1 mg/mL zeocin, for screening transformants with *iam1* gene integrated into the host's genomic DNA. After the plates were incubated at 30 °C for 2–4 days, the selection step was carried out based on their resistance to zeocin. The transformants were resuspended into sterile water, and 10^5-diluted cells were plated on YPG plates containing zeocin at a concentration of 0.1, 0.2, 0.5, 1.0, 2.0, and 4.0 mg/mL to select putative multi-copy transformants. Several hundreds of clones that survived higher concentrations (1–2 mg/mL) of zeocin were screened in test tubes as follows. A single colony was inoculated into 5 mL of YPG in a 50 mL tube and incubated for 24 h at 30 °C with shaking at 220 rpm. Cell density was measured by OD600 and a fresh 5 mL of YPG medium was inoculated with 2.5×10^6 cells, and then incubated as described above. This process was repeated until cells from each clone being analyzed were synchronized in growth. Usually, it took two to three days to reach the same growth status. Once synchronized, cells were grown for 72 h, as done earlier. Aliquots of culture (100 μL) were aseptically removed everyday and the supernatant was subjected to isoamylase assay described later.

2.4. High cell density fermentation

For shaking batch experiments, positive *P. pastoris* GS115 transformants screened for *iam1* expression were cultivated in 50 mL of YPG medium [21] at 30 °C for 3 days. The cells were harvested and both extra- and intracellular isoamylase activities were assayed. Supernatant of each sample was performed with SDS (sodium dodecyl sulfate) gel electrophoresis to test isoamylase expression.

Fed-batch fermentation of the isoamylase top-producing transformant was performed in a 10 L biofermenter (Braun) containing 7 L of the medium and inoculated with 0.35 L of a shaking batch culture to an initial OD_{600} around 2. The operation conditions during the batch phase were temperature of 30 °C, pH 6.0, agitation 500 rpm, and aeration 1 vvm. A sharp increase in pH was detected after 20 h of fermentation, and then 500 g/L sucrose and 5 g/L yeast extract were added as feeding medium at a constant flow of 7 mL/h/L for fed-batch fermentation. The operation conditions during the feeding phase were 30 °C, pH 6.0, agitation 1000 rpm, and aeration 2 vvm. The total fermentation was 120 h.

2.5. Purification of recombinant isoamylase

The recombinant isoamylase produced by *P. pastoris* was purified using raw starch adsorption–desorption method described by Saha et al. [22] with certain modifications. Fill in a chromatography column of size 2.0 cm × 20.0 cm with raw starch granules. The column was equilibrated by using 0.04 M, pH 6.0 phosphate buffer containing 20% ammonium sulfate, and then sampled with 50 mL crude enzyme dialyzed by the same buffer at a flow rate of 10 mL/h. Wash the column with equilibration buffer until no protein was detected at 280 nm. Isoamylase adsorbed on to starch granules was washed out by 0.067 M, pH 8.5 phosphate buffer. Then the active fractions were dialyzed and freeze-dried as the purified enzyme.

Protein concentrations were determined by the absorbance at 595 nm due to the binding of Coomassie brilliant blue G-250 (CBBG) to proteins using the original medium as blank, as described above [23].

2.6. Isoamylase activity assays

Isoamylase activity was assayed with glycogen as substrate according to the method described previously [24] with certain modifications. An aliquot (1 mL) of glycogen substrate solution (2% w/v) was pre-incubated at 50 °C for 5 min. An aliquot (1 mL) of pre-incubated isoamylase (0.1 M sodium acetate buffer, pH 6.5) was added, stirred on a vortex mixer and incubated at 50 °C. After exactly 30 min at 50 °C, an aliquot (0.2 mL) of the reaction mixture was removed, added to 5 mL of 0.1% (w/v) iodine solution and stirred vigorously on a vortex mixer. The absorbance at 600 nm was measured immediately (within a minute). Blanks were prepared by mixing 5 mL of 2% glycogen with 5 mL of sodium acetate buffer (0.1 M, pH 6.5), and removing an aliquot (0.2 mL) to mix with iodine solution (5 mL). Isoamylase activity can be determined according to the equation:

$$\text{IAM units/mL} = 10 \times 2 \times \text{Abs} \times \text{dilution factor of the original preparation}$$

where Abs represents the absorbance of the reaction solution against the blank. One unit of isoamylase activity is defined as the amount of enzyme capable of resulting 0.01 increase in absorbance at 600 nm in one hour under the assay conditions.

Pullulanase activity was assayed by measuring the reducing sugars hydrolysed from pullulan as described by Singh et al. [25].

2.7. Biochemical characterization of recombinant isoamylase

The optimal temperature for recombinant isoamylase was determined at pH 6.5. To determine the influence of temperature on the enzymatic stability, standard assay reaction mixtures were incubated at temperatures ranging from 30 to 80 °C for 1 h in 0.05 M sodium phosphate (pH 6.5) buffer. The pH profile of its activity was determined at 50 °C, using three buffer systems containing 0.05 M HAc–NaAc (pH 4.0–5.8), 0.05 M sodium phosphate (pH 5.8–7.8) and 0.05 M Tris–HCl (pH 7.8–9.0), respectively.

Effect of different metal ions and chemicals on activity of recombinant isoamylase was determined at 50 °C, pH 6.5 for 30 min with appropriate metal salts and chemical agents at the final concentrations of 5 mM or 0.5% (w/w). Inhibition by incremental of maltose (1–20%) and cyclodextrin (5–20 mM) was assayed under standard conditions. The extent of inhibition or activation of enzyme activity was described as percentage of the ratio of residual activity to complete enzyme activity in the control sample without addition of metal ions or chemical agents.

Kinetic measurements. All kinetic measurements were performed at 50 °C in 0.05 M sodium acetate buffer (pH 6.5). The kinetic parameters for isoamylase were

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