

# Molecular Cloning and Biochemical Characterization of Oligo-1,6-Glucosidases from *Bacillus subtilis* and *Bacillus licheniformis*

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## 1 Introduction

Oligo-1,6-glucosidase (O-1,6-G, EC 3.2.1.10), belonging to the subfamily 31 of the glycoside hydrolase family 13 (GH13\_31) [1] catalyzes the exo hydrolysis of  $\alpha$ -1,6-glucoside bonds from the non-reducing ends of  $\alpha$ -limit dextrin, isomaltose and other isomaltooligosaccharides (IMOs), but has no activity towards  $\alpha$ -1,4-glucoside bonds of malto-oligosaccharides [2]. Acting together with maltase, oligo-1,6-glucosidase can completely hydrolyze  $\alpha$ -amylase dextrins, allowing the complete digestion of starch in the gastrointestinal tract of mammals [3, 4]. Moreover, since novel oligosaccharides are finding increasing applications in biotechnological and chemical industries, the debranching enzymes containing oligo-1,6-glucosidases are also valuable [5].

In our study, two genes encoding oligo-1,6-glucosidases from *Bacillus subtilis* and *B. licheniformis* were cloned and overexpressed in *Pichia pastoris*, and their enzymatic properties were comprehensively investigated.

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## 2 Materials and Methods

### 2.1 Bacterial Strains, Plasmids

*Escherichia coli* JM109, *P. pastoris* strain GS115 and vector pPIC9K were obtained from Invitrogen (Carlsbad, CA).

### 2.2 Gene Clone

Gene encoding oligo-1,6-glucosidase from *B. subtilis* (*bsog*) was amplified by PCR using the primer sets Bs-1 5'-GTAAGTGAATGGTGGAAAGAAGCTGTC-3' and Bs-2 5'-TGCTCTAGATCATATACTAATGCCCATCACTGCTT-3'. And primer sets BI-1 5'-GTAAGCCAATGGTGGAAAGAGGC-3' and BI-2 5'-TGCTCTAGATCATGATGTGTAATCCTTTGCCC-3' were used for *blog*.

### 2.3 Enzyme Assays

The described method [6] was used to measure the activities of oligo-1,6-glucosidases from *B. subtilis* and *B. licheniformis*.

### 2.4 pH and Temperature Dependence of Activity

pH optima of BsOG and BIOG were analyzed by incubating them for 15 min at 37 °C as described above.

The optimal reaction temperatures of BsOG and BIOG were determined at temperatures ranging from 15 to 70 °C at pH 6.8.

### 2.5 Determination of Kinetic Parameters

For determination of enzyme kinetics, various amounts of *p*NPG were used as substrates, and the activities of BsOG and BIOG were measured. Isomaltose and isomaltotriose which were dissolved in 0.2 M phosphate buffer (pH 7.0). An Agilent 1200 HPLC system (Waldbronn, Germany) equipped with an evaporative light-scattering detector was used for quantification and identification of various

oligosaccharides in the samples. Separation of oligosaccharides was carried out using a Prevail™ Carbohydrate ES 5 $\mu$  column (GRACE, 4.6 mm  $\times$  250 mm i.d.; particle size, 5  $\mu$ m).

## **2.6 Specificity Towards Natural Substrates**

Isomaltose, isomaltotriose, isomaltulose, panose, maltotriose, maltose, sucrose, amylose, amylopectin and maltodextrin used as substrates to study the specificity towards natural substrates, were dissolved in 0.2 M phosphate buffer (pH 7.0).

## **2.7 Hydrolysis of Isomaltotriose and IMOs by OGs**

Hydrolysis of isomaltotriose and IMOs by BsOG and BIOG were performed and analyzed as described above except that isomaltotriose were dissolved in 0.2 M phosphate buffer (pH 7.0) to a final concentration of 4 mM and that samples were withdrawn at different times for HPLC analysis.

# **3 Results**

## **3.1 Gene Cloning and Expression of Oligo-1,6-Glucosidases**

With genomic DNA as templates, respective genes encoding oligo-1,6-glucosidases from *B. subtilis* and *B. licheniformis* were amplified by PCR, and the fragments obtained were 1700 bp, consistent with their theoretical sizes (*bsog*, 1686 bp; *blog*, 1707 bp). After five days of shake flask fermentation, the activities of oligo-1,6-glucosidases of these GS115-*bsog* and GS115-*blog* were 1085 and 1037 U/mL.

## **3.2 Effects of Temperature on the Activity and Stability of Recombinant Oligo-1,6-Glucosidases**

Effects of temperature on the activity and stability of recombinant oligo-1,6-glucosidases were examined. The temperature optima of BsOG and BIOG were 40 and 45 °C, respectively. BsOG retained 80% of its maximum activity at temperatures ranging from 35 to 50 °C, while BIOG had 80% of its maximum activity at temperatures between 40 and 50 °C. For determination of the thermostability,

BsOG and BIOG were pre-incubated in 0.2 M phosphate buffer (pH 7.0) at 50 °C, and the residual activities were measured at the indicated times. Incubation at 50 °C for 20 min, BsOG and BIOG activity were not detected.

### **3.3 Effects of pH on the Activity and Stability of Recombinant Oligo-1,6-Glucosidases**

The relative activities of BsOG and BIOG at various pHs were measured with two different buffer systems at 37 °C. The effects of pH over a range of 4.0–10.0 on the activities of BsOG and BIOG. The optimal pH of BsOG and BIOG were 7.0 and 6.5. BsOG had a relatively broad pH optimum ranging from 6.0 to 9.5. The optimum pH range of BIOG was 5.5–7.5.

### **3.4 Kinetic Parameters of Recombinant Oligo-1,6-Glucosidases**

Michaelis-Menten and Lineweaver-Burk plots were used to calculate the kinetic parameters. Although both enzymes hydrolyzed *p*NPG, isomaltose and isomaltotriose, their kinetic parameters were different. BsOG catalyzed *p*NPG, isomaltose and isomaltotriose with  $K_{ms}$  of 0.1, 0.59 and 8.60 mM. BIOG showed  $K_m$  values of 0.27, 0.86 and 10.8 mM for *p*NPG, isomaltose and isomaltotriose.

### **3.5 Substrate Specificity of Recombinant Oligo-1,6-Glucosidases**

The ability of BsOG and BIOG to hydrolyze various di- and maltooligosaccharides, as well as  $\alpha$ -glucan polymers, such as amylose and amylopectin, was determined. As shown in Table 1, both BsOG and BIOG hydrolyzed isomaltose, isomaltotriose, isomaltulose, panose, sucrose, amylopectin and maltodextrin, and exhibited weak activity against amylose. However, no activity was observed toward maltose and maltotriose. BsOG and BIOG also exhibited  $\alpha$ -1,2-glucosidase activity on sucrose, in accord with the substrate specificity of isomaltase from *S. cerevisiae* [2]. This restricted substrate specificity indicated that these two enzymes were oligo-1,6-glucosidases [7]. Oligo-1,6-glucosidase prefers isomaltotriose, and hydrolyzes IMOs and dextran [7]. On the other hand, *S. cerevisiae* isomaltase preferentially cleaves

**Table 1** Substrate specificity of recombinant enzymes BsOG and BIOG

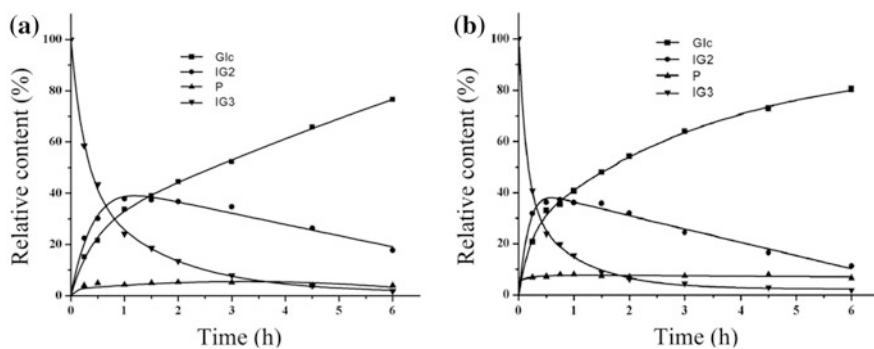
Substrate	BsOG	BIOG
Isomaltose	+	+
Isomaltotriose	+	+
Isomaltulose	+	+
Panose	+	+
Maltose	–	–
Maltotriose	+	+
Sucrose	+	+
Amylose	–/+	–/+
Amylopectin	+	+
Maltodextrin	+	+

*Note* Plus sign (+) indicates exhibiting activity toward the corresponding substrate; minus/plus sign (–/+) represents have weak activity; minus sign (–) means no activity is detected

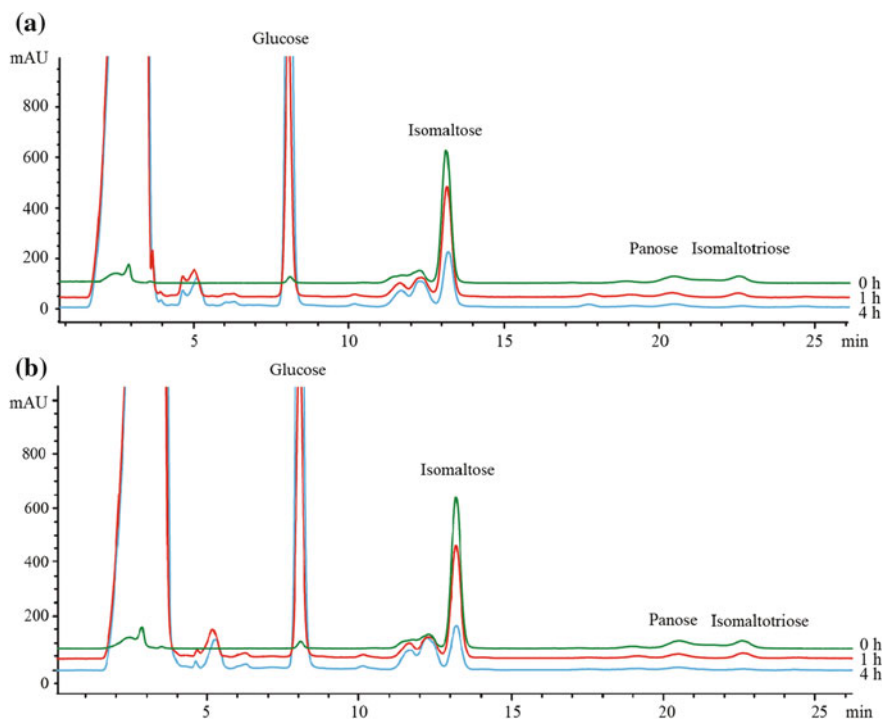
isomaltose and methyl  $\alpha$ -D-glucopyranoside, but does not act on isomaltotriose and isomaltotetraose [8]. These differences in the specificities for substrate chain-length may be partly accounted for by the differences in the shapes of the active sites.

### 3.6 Hydrolysis of Isomaltotriose by Recombinant Oligo-1,6-Glucosidases

As shown in Fig. 1, both BsOG and BIOG hydrolyzed isomaltotriose, their hydrolysis rates were similar. Glucose increased throughout the course of the reactions, whereas isomaltotriose decreased continuously. When isomaltotriose was hydrolyzed by BsOG, isomaltose rapidly accumulated during the first hour and the relative content of isomaltotriose decreased to 6.4%. At the end of this period, the highest content of isomaltose (36%) was achieved, preceding a quick fall in this sugar level. The relative content of panose resulting from the isomerization of isomaltotriose was maintained between 6.5 and 8.0%. In contrast, isomaltotriose hydrolyzed by BIOG was the same as BsOG. The amount of isomaltose rose gradually and reached a maximum (38%) at 1 h of reaction, and slowly decreased since then. The content of panose was between 4 and 5.5% in the process. All these findings indicated that isomaltotriose can be hydrolyzed by BsOG and BIOG to glucose by cleaving single glucosyl groups from the non-reducing end. After hydrolysis by BsOG and BIOG for 18 h, isomaltotriose was completely converted into glucose, and there were no detectable panose and isomaltose (data not shown).



**Fig. 1** The hydrolysis process of isomaltotriose by BsOG and BIOG. **a** BsOG; **b** BIOG; *down triangles* isomaltotriose; *up triangles* panose; *closed squares* glucose; *circles* isomaltose. The initial concentration of isomaltotriose was set as 100%. The error bars indicate standard deviations



**Fig. 2** HPLC profiles of IMOs hydrolyzed by BsOG and BIOG for different times. **a** BsOG; **b** BIOG; *red and light blue lines* represent the samples that were hydrolyzed for 1 and 4 h, respectively. *Green line* represents IMOs

### 3.7 Hydrolysis of IMOs by Recombinant Oligo-1,6-Glucosidases

HPLC analysis showed only three products in each digest of IMOs (Fig. 2). One was shown to be glucose, which was the sole product from isomaltose. Another was identified as maltose from panose. The third product was isomaltose from isomaltotriose. The hydrolysis modes of panose and isomaltotriose suggested that the linkage to be split in each isomaltosaccharide was at the non-reducing terminal [7]. We found that isomaltotriose in IMOs was nearly completely converted into isomaltose and glucose after hydrolyzed by BsOG for 4 h, only a small amount of panose left. When IMOs was hydrolyzed by BIOG, isomaltose and isomaltotriose were rapidly converted into glucose, while panose was hydrolyzed into maltose and glucose. At the end of the reaction, isomaltotriose was almost completely hydrolyzed and only a small quantity of panose and isomaltose left.

## 4 Discussion and Conclusion

In our study, the oligo-1,6-glucosidases of *B. subtilis* and *B. licheniformis* were cloned and extracellularly overexpressed with the signal peptide ( $\alpha$ -factor) of pPIC9K in *P. pastoris* GS115. Previously, oligo-1,6-glucosidases from *B. thermoglucosidarius* and *B. cereus* ATCC7064 were expressed in *E. coli*, only 1.7 and 22.8% of recombinant enzymes were secreted into the medium [9]. The high activities of two oligo-1,6-glucosidases show their industrialization prospects.

Some characteristics of the recombinant enzymes BsOG and BIOG were then investigated. Despite BsOG and BIOG, oligo-1,6-glucosidases from *Bacillus mycoides*, *Thermomyces lanuginosus*, *Bifidobacterium*, *Aspergillus niger*, *A. oryzae*, *S. cerevisiae*, were also being expressed and characterized by our group (report elsewhere). Our results have provided a foundation for exploiting oligo-1,6-glucosidases with desired biochemical properties from various microorganisms.

## References

1. Murphy C, Powlowski J, Wu M, Butler G, Tsang A (2011) Curation of characterized glycoside hydrolases of fungal origin. Database bar020
2. Deng X, Petitjean M, Teste M-A, Kooli W, Tranier S, François JM, Parrou J-L (2014) Similarities and differences in the biochemical and enzymological properties of the four isomaltases from *Saccharomyces cerevisiae*. FEBS Open Biol 4:200–212
3. Larner J, McNickle C (1955) Gastrointestinal digestion of starch I. The action of oligo-1,6-glucosidase on branched saccharides. J Biol Chem 215:723–736
4. Hauri H-P, Quaroni A, Isselbacher KJ (1979) Biogenesis of intestinal plasma membrane: posttranslational route and cleavage of sucrase–isomaltase. Proc Natl Acad Sci 76:5183–5186

5. Jespersen HM, MacGregor EA, Henrissat B, Sierks MR, Svensson B (1993) Starch- and glycogen-debranching and branching enzymes: prediction of structural features of the catalytic ( $\beta/\alpha$ ) 8-barrel domain and evolutionary relationship to other amylolytic enzymes. *J Protein Chem* 12:791–805
6. Suzuki Y, Yuki T, Kishigami T, Abe S (1976) Purification and properties of extracellular  $\alpha$ -glucosidase of a thermophile, *Bacillus thermoglucosidius* KP 1006. *Biochim Biophys Acta (BBA)-Enzymol* 445:386–397
7. Suzuki Y, Aoki R, Hayashi H (1982) Assignment of a p-nitrophenyl- $\alpha$ -D-glucopyranoside-hydrolyzing  $\alpha$ -glucosidase of *Bacillus cereus* ATCC 7064 to an exo-oligo-1,6-glucosidase. *Biochim Biophys Acta (BBA)-Protein Struct Mol Enzymol* 704:476–483
8. Yamamoto K, Miyake H, Kusunoki M, Osaki S (2010) Crystal structures of isomaltase from *Saccharomyces cerevisiae* and in complex with its competitive inhibitor maltose. *FEBS J* 277:4205–4214
9. Watanabe K, Kitamura K, Iha H, Suzuki Y (1990) Primary structure of the oligo-1,6-glucosidase of *Bacillus cereus* ATCC7064 deduced from the nucleotide sequence of the cloned gene. *Eur J Biochem* 192:609–620



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