**Regular Paper**

**Characterization of Three Fungal Isomaltases Belonging to Glycoside Hydrolase Family 13 That Do not Show Transglycosylation Activity**

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Hiroki Eisawa,¹ Shun Ogawa,² Nobuhiro Yamazaki,¹ Kohki Maekawa,¹ Takahiro Yamaguchi,¹ Shoto Sato,¹ Kazuma Shiota,² and Takashi Yoshida¹,†

¹Department of Biochemistry and Molecular Biology, Faculty of Agriculture and Life Science, Hirosaki University (3 Bunkyo, Hirosaki, Aomori 036–8561, Japan)
²Enzymes and Pharmaceuticals Laboratory, Godo Shusei Co., Ltd. (250 Aza-nakahara, Kamihongo, Chiba 271–0064, Japan)

Abstract: α-1,6-Glucosidase (isomaltase) belongs to glycoside hydrolase (GH) families 13 and 31. Genes encoding 3 isomaltases belonging to GH family 13 were cloned from filamentous fungi, Aspergillus oryzae (agl1), A. niger (agdC), and Fusarium oxysporum (foagl1), and expressed in Escherichia coli. The enzymes hydrolyzed isomaltose and α-glucosides preferentially at a neutral pH, but did not recognize maltose, trehalose, and dextran. The activity of AgdC and Agl1 was inhibited in the presence of 1% glucose, while Foagl1 was more tolerant to glucose than the other two enzymes were. The three fungal isomaltases did not show transglycosylation when isomaltose was used as the substrate and a similar result was observed for AgdC and Agl1 when p-nitrophenyl-α-glucoside was used as the substrate.

Key words: α-glucosidase, isomaltase, Aspergillus, Fusarium, fungi

**INTRODUCTION**

Enzymatic hydrolysis of starch using α-amylase, glucoamylase, and glucose isomerase has been utilized for the production of high-fructose corn syrup. A drawback of the process is the accumulation of isomaltose as a by-product. Isomaltase is a disaccharide consisting of α-1,6-linked glucose molecules. An enzyme capable of degrading isomaltose is expected to improve the yield of glucose. Isomaltase (α-1,6-glucosidase, oligo α-1,6-glucosidase; EC 3.2.1.10) is an α-glucosidase with a high specificity toward α-1,6-glucoside.

Two types of isomaltases, belonging to glycoside hydrolase (GH) families 13 and 31, have been reported in bacteria and yeast, respectively. Isomaltase is a disaccharide consisting of α-1,6-linked glucose molecules. An enzyme capable of degrading isomaltose is expected to improve the yield of glucose. Isomaltase (α-1,6-glucosidase, oligo α-1,6-glucosidase; EC 3.2.1.10) is an α-glucosidase with a high specificity toward α-1,6-glucoside.

The amino acid sequences of the enzymes are rather diverse within the family, but four conserved motifs (Regions I to IV) are reported to be specific to isomaltases.² In yeasts, two types of GH13 α-glucosidases have been reported. Maltase (α-1,4-glucosidase; EC 3.2.1.20) hydrolyzes maltose, amylose, and oligosaccharides, but does not act on methyl-α-glucoside and isomaltose, while isomaltase cleaves isomaltose and methyl-α-glucoside.³ A study on the yeast GH13 α-glucosidase revealed that Val216 in conserved region II is crucial in determining the specificity toward α-1,4- or α-1,6-glucosidic linkage.⁴

During a preliminary screening of isomaltase-producing fungal cultures, we observed hydrolytic activity of several filamentous fungi, including Fusarium and Aspergillus, towards isomaltose. We cloned and expressed three isomaltase-encoding genes from Fusarium oxysporum, Aspergillus oryzae, and A. niger. In this study, we compared the performances of these three fungal isomaltases.

**MATERIALS AND METHODS**

**Fungal strains.** Aspergillus niger NBRC4066 and Fusarium oxysporum NBRCC9967 were used for cloning the gene encoding isomaltase. mRNA was extracted from lyophilized mycelia using ISOGEN (Nippon Gene Co., Ltd., Tokyo, Japan) and purified by NucleoSpin®RNA Plant (Macherey-Nagel Inc., Bethlehem, USA). cDNA was synthesized using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science, Upper Bavaria, Germany). In case of agl1, DNA of A. oryzae (strain RIB40) EST clone JK2172 was provided from National Research Institute of Brewing (Hiroshima, Japan) and used for gene amplification.

**Cloning and expression of the genes.** We searched for isomaltase genes in the genomes of filamentous fungi by an in silico analysis using the yeast isomaltase gene as the reference.⁵ We identified two genes from Aspergillus spp., one from A. niger (XP_001400455.1), designated agdC, and another from A. oryzae (BAE63400.1), designated agl1. Information about the third gene from Fusarium oxysporum...
sodium phosphate buffer (pH 7.0). To isolate Foagl1, fur-

were used as templates. The isomaltase-encoding genes

pernatant were mixed with Ni-chelating resin, COSMO-
cubation with 0.1 mM IPTG for 12 h at 20 °C.

Protein purification.

transfection and disrupted by sonication. Proteins soluble in the su-

described above, except that the reaction was stopped by add-

 fosagl1

mM imidazole. The enzymes were dialyzed with 20 mM

mM NaCl and 20 mM imidazole. His-tagged protein was

eluted with the buffer containing 800 mM NaCl and 250

ther chromatography with DEAE TOYOPEARL (Tosoh

ly conserved regions (I to IV) characteristic of GH13 en-

The Val residue that is a characteristic of isomaltases

–

Hydroly-

zymes were also found in these fungal enzymes (Fig. 1 ).

Characteristics of fungal isomaltases.

The three fungal isomaltase genes, agdC, agl1, and

foagl1, encoding proteins with 587, 584, and 572 amino

acid residues, have calculated molecular masses of 68,867,

68,621, and 66,102 Da, respectively. The amino acid se-

quences of AgdC and Agl1 were 57 % identity, while

50–57 % identity was noted between the sequences of

Foagl1 and the other two enzymes (Table 1). The four high-

ly conserved regions (I to IV) characteristic of GH13 en-

zymes were also found in these fungal enzymes (Fig. 1).

The Val residue is conserved among GH13 isomaltases.

Table 1. Amino acid sequence identity between GH13 α-1,6-gluco-
sidases.

| (%) | S.cαl1.6 | G.tα1,6 | Agl1 | AgdC | Foagl1 |
|-----|-----------|---------|------|------|--------|
| S.cαl1.6 | 100      | 56.5    | 50.4 | 57.1 | 46.4   |
| G.tα1,6   | 42.6     | 100     | 46.4 | 84.5 | 47.8   |
| Agl1      | 40.1     | 47.8    | 100  | 100  | 84.5   |
| AgdC      | 38.2     | 46.4    | 84.5 | 100  | 100    |
| Foagl1    | 37.9     | 50.4    | 56.5 | 57.1 | 100    |

Fig. 1. Amino acid sequence motifs conserved in GH13 α-gluco-
sidases.

Triangles indicate catalytic amino acid residues. Enzymes are as
follows (the numbers are GenBank IDs): S.cαl1.4, Saccharomyces
cerevisiae maltase (CCB84896.1); G.tα1,4, Geobacillus stea-
thermophilus α-1,4-glucosidase (BAA12704.1); A.omal, Aspergillus ory-
zue maltase (BAB59003.1); S.cαl1.6, S. cerevisiae oligo-α-1,6-glu-
cosidase (BAD00094.1); G.tα1,6, G. thermoglucosidasius oligo-α-1,6-
glucosidase (BA01368.1); Agl1, A. oryzae Agl1; AgdC, A. niger
Agl1; Foagl1, Fusarium oxysporum lycozyme Foagl1. The boxed
Val residue is conserved among GH13 isomaltases. (10)

Fig. 2. SDS-PAGE analysis of fungal isomaltases.

Lanes a and b are for Agl1, c and d for AgdC, and e and f for
Foagl1. Lanes a, c, and e show cell-free extracts, and lanes b, d, and f
show the purified enzymes. Triangles show the positions of marker
proteins with sizes 25, 37, 50, 75, 100, and 250 kDa (from the bottom
to the top), respectively. Proteins were visualized by staining with
Coomassie Blue.

Table 1. Amino acid sequence identity between GH13 α-1,6-gluco-
sidases.
(Val216 of yeast isomaltase) was also identified in the three fungal enzymes. The molecular masses of fungal isomalta-ses expressed in *E. coli* were 68–70 kDa on SDS-PAGE, which was in agreement with the molecular masses calculated from the DNA (Fig. 2).

**Optimal conditions.**

The maximal activity of AgdC and Agl1 was observed at pH 6.0 and 35 °C and that of Foagl1 was observed at pH 6.5 and 30 °C (Fig. 3). The three enzymes were inhibited by Tris buffer (Fig. 3C). The fungal isomaltases were stable at a pH range of 6.0 to 9.0 at 30 °C for 30 min. Foagl1 was not as heat stable as the other two enzymes above 40 °C.

**Substrate specificity and kinetics.**

The three fungal enzymes showed the highest activity toward isomaltose (Table 2). Isomaltotriose was not as easily hydrolyzed as the biose was. Sucrose and kojibiose were partially hydrolyzed, suggesting that α-1,2-glucoside could also be cleaved by the enzymes. When the hydrolytic activity of Agl1, AgdC, and Foagl1 toward isomaltose, α-MG, and pNPαG were kinetically determined, the highest $k_{cat}/K_m$ for all the three enzymes was obtained with pNPαG (Table 3). This result suggests that these fungal enzymes could be α-glucosidases with a high preference to isomaltose. In contrast, Foagl1 showed lower activity toward α-MG than Agl1 and AgdC did. These findings suggest that a minute difference exists in the structure of catalytic pockets of these fungal isomaltases, although further studies are needed to confirm this notion.

**Glucose tolerance and transglycosylation.**

Many glucosidases are known to be inhibited by a high concentration of glucose. When the fungal isomaltases were incubated with pNPαG as the substrate in the presence of glucose, hydrolysis of pNPαG was inhibited (Fig. 4). AgdC and Agl1 were inhibited when the reaction mixture contained at least 1 % glucose, while Foagl1 was more tolerant to glucose than the other two enzymes were. Isomal-tose was detected following TLC of the products generated in a reaction mixture containing Foagl1, pNPαG, and 1 % glucose (Fig. 5). This indicated that transglycosylation occurred during hydrolysis of pNPαG. In contrast, biose was not detected among the reaction products when AgdC and Agl1 were used under the same conditions. When the three fungal isomaltases were incubated overnight at 30 °C with 1 % isomaltose as the sole substrate, triose was not detected in the TLC (Fig. 6). These results indicated that the two isomaltases from *Aspergillus* did not perform transglycosylation when isomaltose or pNPαG were used as substrates, while Foagl1 catalyzed transglycosylation to some extent when pNPαG was used as the substrate. Notably, none of the three isomaltases catalyzed transglycosylation when isomaltose was used as the substrate. This might be a characteristic of GH13 fungal isomaltases because many GH family 31 α-glucosidases acting on isomaltose are known for their transglycosylation activities. The low transglycosylation activity of the fungal isomaltases with isomal-tose as the substrate seemed to be an advantage for the industrial production of glucose from starch. Improvement of...
some properties, such as heat stability and glucose tolerance, by protein engineering will help produce enzymes with optimal properties, which can then be used for industrial processes.

### Table 2. Substrate specificity of fungal isomaltases.

| Substrate | Linkage | Hydrolytic Ratio (%) |
|-----------|---------|----------------------|
| Isomaltose | Glcα1-6Glc | AgdC 100 |
|           |         | AgI 100 |
|           |         | Foagl1 100 |
| Isomaltotriose | Glcα1-6Glcα1-6Glc | AgdC 19 |
|              |         | AgI weak |
|              |         | Foagl1 40 |
| Kojibiose | Glcα1-2Glc | AgdC 30 |
|           |         | AgI 14 |
|           |         | Foagl1 22 |
| Maltose   | Glcα1-4Glc | AgdC n.d. |
|           |         | AgI n.d. |
|           |         | Foagl1 n.d. |
| Nigerose  | Glcα1-3Glc | AgdC n.d. |
|           |         | AgI n.d. |
|           |         | Foagl1 n.d. |
| Panose    | Glcα1-6Glcα1-4Glc | AgdC n.d. |
|           |         | AgI n.d. |
|           |         | Foagl1 n.d. |
| Sucrose   | Glcα1-2βFru | AgdC 14 |
|           |         | AgI weak |
|           |         | Foagl1 18 |
| Trehalose | Glcα1-1otGlc | AgdC 31.3 |
|           |         | AgI 12.9 |
|           |         | Foagl1 24.3 |
| Dextran   | α1,6-glucan | AgdC n.d. |
|           |         | AgI n.d. |
|           |         | Foagl1 n.d. |
| Starch    | α1,4, α1,6-glucan | AgdC n.d. |
|           |         | AgI n.d. |
|           |         | Foagl1 n.d. |

The sugars were used at 0.1 % (w/v) concentration.

### Table 3. Kinetic parameters of isomaltases.

| Substrate | Enzyme | $K_m$ (mM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$・M$^{-1}$) |
|-----------|--------|------------|----------------------|----------------------------------|
| Isomaltose | AgI    | 15         | 5.7                  | 381                              |
|           | AgdC   | 41         | 3.7                  | 91                               |
|           | Foagl1 | 5.8        | 4.8                  | 828                              |
| α-MG      | AgI    | 31.3       | 12.9                 | 411                              |
|           | AgdC   | 32.8       | 5.0                  | 151                              |
|           | Foagl1 | 24.3       | 2.2                  | 90                               |
| pNPαG     | AgI    | 8.3        | 17.5                 | 2125                             |
|           | AgdC   | 7.8        | 19.8                 | 2557                             |
|           | Foagl1 | 1.0        | 8.7                  | 8700                             |

### Fig. 4. Hydrolysis of pNPαG by fungal isomaltases in the presence of glucose.

Enzymatic activity of AgI (●), AgdC (■), and Foagl1 (▲) toward 0.1 % pNPαG was determined in 20 mM sodium phosphate buffer (pH 6.0) at 30 °C for 10 min in the presence of various concentrations of glucose.

### Fig. 5. Hydrolysis of pNPαG by fungal isomaltases in the presence of glucose.

Agl1 (lane 3), AgdC (lane 4), or Foagl1 (lane 5) was incubated overnight with 0.25 % pNPαG in the presence of 1 % glucose at 30 °C. The reaction products were analyzed by TLC. Lanes 1 and 2 show the positions of isomaltose (IM), glucose, and pNPαG.

### Fig. 6. Hydrolysis of isomaltose by fungal isomaltases.

AgdC (lanes 2, 3, and 4), Agl1 (lane 5), or Foagl1 (lane 6) was incubated with 1 % isomaltose in 50 mM sodium phosphate buffer (pH 6.0) at 30 °C for 3 h (lane 2), 6 h (lane 3), or 18 h (lanes 4, 5, and 6). The reaction products were analyzed by TLC. Lane 1 shows the position of isomaltose (IM).
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