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

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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. Isomaltose 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 from bacteria and yeast, respectively.[1)2)3)4)] Various carbohydrolases acting on maltooligosaccharides or starch have been assigned to GH family 13 (GH13), according to the Carbohydrate-Active enZymes (CAZy) Database.[5)] 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.[6)7)] 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.[8)9)] 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.[10)]

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 oxisporum, 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* NBRC9967 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.[10)] 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*

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Abbreviations: GH, glycoside hydrolase; SDS-PAGE, sodium dodecyl sulfate-denatured polyacrylamide gel electrophoresis; αMG, α-methyl glucoside; pNPαG, *p*-nitrophenyl α-D-glucoside; TLC, thin-layer chromatography.

(FOXG_00152.2), designated *foagl1*, was obtained from the website of Broad Institute (http://www.broadinstitute.org/). PCR primers were designed utilizing the nucleotide sequences of the genes. DNA of *agl1*, *agdC*, and *foagl1* were amplified by PCR using the following sets of primers (forward/reverse): 5′-aaggatccgatggccaaatccgcctcc-cagattc-3′/5′-ttggatccgtaggcctccaccaaaagaacaac-3′, 5′-acgg-gatcccatggccacacc-3′/5′-ctggggatcctaatcaggcctc-3′, and 5′-aaggatccgatgggtagcacggatcaaagtttgc-3′/5′-ttggattactcgag-caggacggcaaagg-3′, respectively. The appropriate cDNAs were used as templates. The isomaltase-encoding genes were inserted into the *Bam*H1 site of plasmid pET-14b (Novagen Inc., Madison, USA) and the plasmid was used to transfect *E. coli* strain AD494 (DE3). Bacterial culture and gene expression was carried out according to the protocol described by Novagen. Gene expression was induced by incubation with 0.1 mM IPTG for 12 h at 20 °C.

Protein purification. Cells were harvested by centrifugation and disrupted by sonication. Proteins soluble in the supernatant were mixed with Ni-chelating resin, COSMOGEL His-Accept (Nacalai Tesque Co., Kyoto, Japan). The suspension was applied to a column that was then washed successively with 20 mM sodium phosphate buffer (pH 7.0) containing 300 mM NaCl, and a buffer containing 800 mM NaCl and 20 mM imidazole. His-tagged protein was eluted with the buffer containing 800 mM NaCl and 250 mM imidazole. The enzymes were dialyzed with 20 mM sodium phosphate buffer (pH 7.0). To isolate Foagl1, further chromatography with DEAE TOYOPEARL (Tosoh Corporation, Tokyo, Japan) was performed in 20 mM sodium phosphate buffer (pH 7.0), and the enzyme was eluted with 0.1 M NaCl. Protein concentration was determined by the method of Bradford using bovine serum albumin as a standard.[11] SDS-PAGE was performed by the Laemmli method.[12] The proteins were then electroblotted onto PVDF membrane and detected using anti-His-tag monoclonal antibody and HRP-conjugated goat anti-mouse IgG (both from Novagen Inc.). The final preparation of AgdC, Agl1, and Foagl1 contained 0.36, 0.28, and 0.33 mg/mL protein, respectively.

Assays. Isomaltase activity was assayed at 30 °C using 0.1 % isomaltose as the substrate in 40 μL 100 mM sodium phosphate buffer (pH 6.0). After stopping the reaction by brief heating, the amount of glucose was determined by the glucose oxidase method using the Glucose CII-Test (Wako Pure Chemicals Industries Ltd., Osaka, Japan).[13] Hydrolysis of pNPαG was examined in a manner similar to that described above, except that the reaction was stopped by adding 200 μL sodium carbonate and the color shift was determined at 405 nm. For TLC, 1 μL reaction mixture was spotted onto a silica gel plate (Merck KGaA, Darmstadt, Germany), which was then developed using a solvent system comprising water:1-butanol:2-propanol (4:3:12). The sugars were visualized using 2 M sulfuric acid containing 0.2 % orcinol.

RESULTS AND DISCUSSION

Characteristics of fungal isomaltases.

The three fungal isomaltase genes, *adgC*, *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 sequences of AgdC and Agl1 were 84.5 % identical, while 50−57 % identity was noted between the sequences of Foagl1 and the other two enzymes (Table 1). The four highly conserved regions (I to IV) characteristic of GH13 enzymes were also found in these fungal enzymes (Fig. 1). The Val residue that is a characteristic of isomaltases

	Region I	Region II	Region III	Region IV
S.c α1,4	106 - DLVINH - 112	209 - DGFRIDTAGL - 218	276 - EVAH - 279	344 - YIENHD - 349
G.s α1,4	98 - DLVINH - 103	194 - DGFRIDAISH - 203	256 - EANG - 259	321 - FLENHD - 326
A.o mal	103 - DVVANH - 138	205 - DGFRVDTVNM - 214	255 - EVLA - 258	340 - FTENHD - 345
S.c α1,6	107 - DLVINH - 112	210 - DGFRIDVGSL - 219	277 - EMQH - 280	347 - YLENHD - 352
G.t α1,6	98 - DLVVNH - 103	194 - DGFRMDVINM - 203	256 - ETPG - 259	325 - YLNNHD - 330
Agl1	105 - DLVVNH - 110	206 - NGFRMDVINF - 215	268 - EMPF - 271	339 - YWENHD - 344
AgdC	105 - DLVVNH - 110	206 - DGFRMDVINF - 215	268 - EMPF - 271	339 - YWENHD - 344
Foagl1	104 - DLVMNH - 109	205 - DGFRMDVINF - 214	262 - EMPC - 265	333 - YLENHD - 338

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

Triangles indicate catalytic amino acid residues. Enzymes are as follows (the numbers are GenBank IDs): S.cα1,4, *Saccharomyces cerevisiae* maltase (CCB84896.1); G.sα1,4, *Geobacillus stearothermophilus* α-1,4-glucosidase (BAA12704.1); A.omal, *Aspergillus oryzae* maltase (BAB59003.1); S.cα1,6, *S. cerevisiae* oligo-α-1,6-glucosidase (BAD00094.1); G.tα1,6, *G. thermoglucosidasius* oligo-α-1,6-glucosidase (BAA01368.1); Agl1, *A. oryzae* Agl1; AgdC, *A. niger* AgdC; Foagl1, *Fusarium oxysporum lycopersici* Foagl1. The boxed Val residue is conserved among GH13 isomaltases.[10]

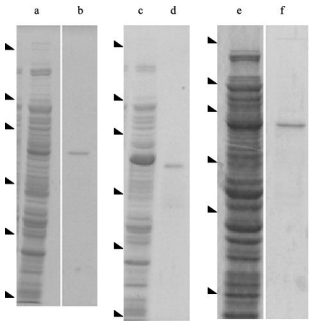

Fig. 2. SDS-PAGE analysis of fungal isomaltases.

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-glucosidases.

(%)	S.cα1,6	G.tα1,6	Agl1	AgdC	Foagl1
S.cα1,6					
G.tα1,6	42.6				
Agl1	40.1	47.8			
AgdC	38.2	46.4	84.5		
Foagl1	37.9	50.4	56.5	57.1	

Identities were calculated by searching in Uniprot (http://www.uniprot.org/). S.cα1,6: *Saccharomyces cerevisiae* oligo-α-1,6-glucosidase; G.tα1,6: *Geobacillus thermoglucosidasius* oligo-α-1,6-glucosidase.

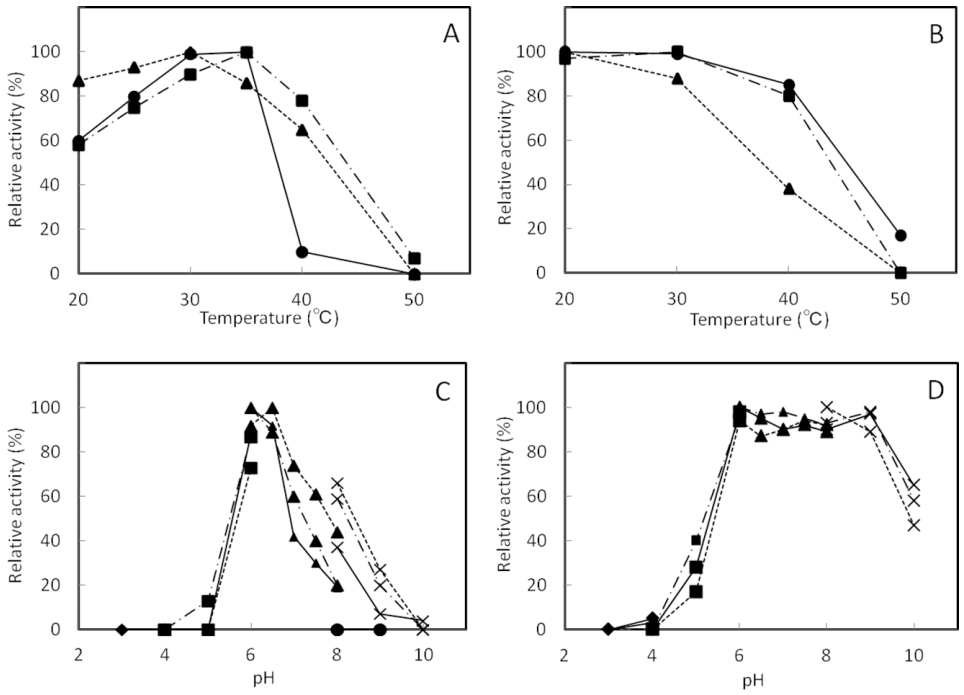

Fig. 3. Optimal conditions for the fungal isomaltases.

(A) Optimal temperatures and (B) effects of temperature on the stability were determined for Agl1 (circles), AgdC (rectangles), and for Foagl1 (triangles). For the determination of stability, the enzymes were pre-heated for 30 min at each given temperature. (C) For the determination of optimal pH and (D) the effects of pH on the stability of Agl1 (straight line), AgdC (dashed line), and Foagl1 (dotted line), various buffers, including CH_3COONa-HCl buffer (◆, pH 3.0 to 4.0), CH_3COONa-CH_3COOH buffer (■, pH 4.0 to 6.0), NaH_2PO_4-Na_2HPO_4 buffer (▲, pH 6.0 to 8.0), Tris-HCl buffer (●, pH 8.0 to 9.0), and glycine-NaOH buffer (×, pH 8.0 to 10.0) were used. For the determination of stability, the enzymes were preheated for 30 min at 30 °C in 50 mM each buffer. Assays were carried out in 50 mM NaH_2PO_4-Na_2HPO_4 buffer (pH 6.0) with isomaltose as the substrate at 30 °C for 1 h.

(Val216 of yeast isomaltase) was also identified in the three fungal enzymes. The molecular masses of fungal isomaltases 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.[14)15)] 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. Isomaltose 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.[16)17)] The low transglycosylation activity of the fungal isomaltases with isomaltose as the substrate seemed to be an advantage for the industrial production of glucose from starch. Improvement of

Table 2. Substrate specificity of fungal isomaltases.

Substrate	Linkage	Hydrolytic Ratio (%)	
Isomaltose	Glcα1-6Glc	AgdC	100
		Agl1	100
		Foagl1	100
Isomaltotriose	Glcα1-6Glcα1-6Glc	AgdC	19
		Agl1	weak
		Foagl1	40
Kojibiose	Glcα1-2Glc	AgdC	30
		Agl1	14
		Foagl1	22
Maltose	Glcα1-4Glc	AgdC	n.d.
		Agl1	n.d.
		Foagl1	n.d.
Nigerose	Glcα1-3Glc	AgdC	n.d.
		Agl1	n.d.
		Foagl1	n.d.
Panose	Glcα1-6Glcα1-4Glc	AgdC	n.d.
		Agl1	n.d.
		Foagl1	18
Sucrose	Glcα1-2βFru	AgdC	14
		Agl1	weak
		Foagl1	18
Trehalose	Glcα1-1αGlc	AgdC	n.d.
		Agl1	n.d.
		Foagl1	n.d.
Dextran	α1,6-glucan	AgdC	n.d.
		Agl1	n.d.
		Foagl1	n.d.
Starch	α1,4, α1,6-glucan	AgdC	n.d.
		Agl1	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 ($s^{-1} \cdot M^{-1}$)
Isomaltose	Agl1	15	5.7	381
	AgdC	41	3.7	91
	Foagl1	5.8	4.8	828
α-MG	Agl1	31.3	12.9	411
	AgdC	32.8	5.0	151
	Foagl1	24.3	2.2	90
pNPαG	Agl1	8.3	17.5	2125
	AgdC	7.8	19.8	2557
	Foagl1	1.0	8.7	8700

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.

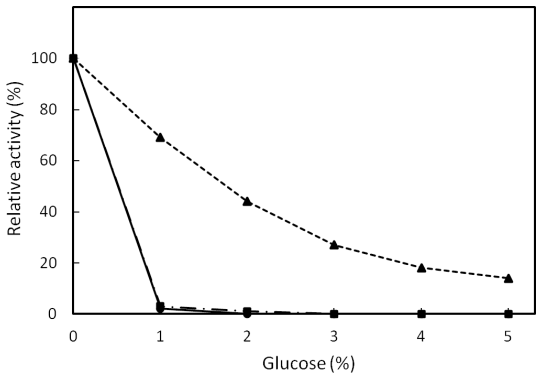

Fig. 4. Hydrolysis of pNPαG by fungal isomaltases in the presence of glucose.

Enzymatic activity of Agl1 (●), 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.

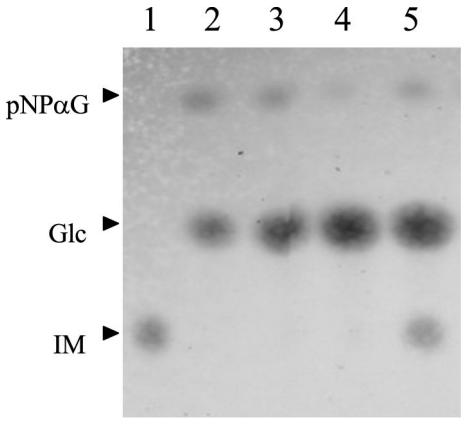

Fig. 5. Hydrolysis of pNPαG by fungal isomaltases in the presence of glucose.

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.

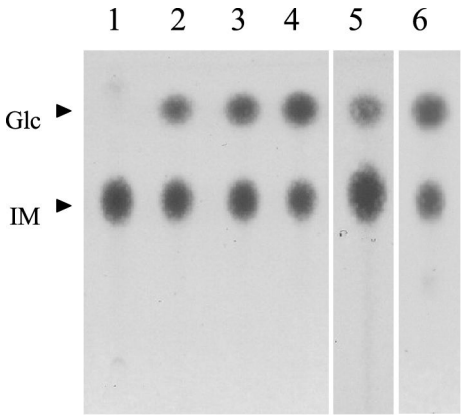

Fig. 6. Hydrolysis of isomaltose by fungal isomaltases.

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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