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Production and characterization of β-glucosidase from *Rhizopus oryzae* MIBA348

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A β-glucosidase was purified from the cell extracts of *Rhizopus oryzae* MIBA348 to homogeneity by successive anion exchange and size-exclusion chromatography and characterized. The enzyme has a molecular weight of 105,000. The optimum pH and the optimum temperature was 5.0 and 50℃, respectively. The enzyme was active on *p*-nitrophenyl-β- D-glucopyranoside, cellobiose and salicine. It hydrolyzed gentiobiose and amygdaline, but was inactive on Avicel, carboxymethylcellulose, maltose and *p*-nitrophenyl-α- D- glucopyranoside

Key words: *Rhizopus oryzae*, β-glucosidase, endo-β-D-1,4-glucanases

β-glucan in nature is a biological polymer composed of β-1,3-, β-1,4-, and/or β-1,6-linkages and includes laminarin, pachyman, carboxymethyl cellulose, lichenan and barley beta-glucan (1-6). Cellulose is the most abundant compound that contains only β-1,4-linkages and is considered to be degraded by the synergistic action of three types of enzyme components: endo- glucanases (EC3.2.1.4, endo-β-D-1,4-glucanases), cellobiohydrolases (EC 3.2.1.91), and β-glucosidases (EC3.2.1.21) (7-9). β-Glucosidase is an important enzyme that was able to produce reusable monosaccharide, glucose in the final stage of degradation (10,11).

Zygomycetes group in mesophilic fungi are widely distributed on stored grain, fruit and vegetables, in the air or in compost (12-15). Therefore, they are well known sources of cellulases and have been effectively used for industrial purposes (13-15). For example, members of *Mucor* and *Rhizopus* are important for their use in production of organic acids, while some of them cause rot in ripe and harvested fruits and vegetables (16). Recently, cellulases are found to be composed of different glycosyl hydrolase families (17, 18). To elucidate the enzyme function in the degradation system, the DNA sequences of the respective enzymes should provide important information (19, 20).

In the present study, β-glucosidase was purified from a mesophilic fungus, *Rhizopus oryzae* and characterized.

Materials and methods

Cultivation of fungi

Fungus cultivation was conducted at 30℃ for 2 days, unless otherwise stated. Aliquots of colony of *Rhizopus* species grown on potato dextrose agar plate (21) were inoculated to Erlenmyer flask (200 ml), containing solid medium (50 ml) which was composed of 20% wheat bran and carbon sources (1 %) and incubated. After adding 50 ml of potassium phosphate buffer (pH6.8), the cultures were incubated for 24 hr at 4℃ and filtered through a glass filter (pore size, G1). The filtrate was centrifuged at 10,000 rpm for 10 min and at 4℃ to remove insoluble materials and finally filled up to 55 ml with potassium phosphate buffer (pH 6.8).

In liquid culture, cultivation of *R.oryzae* 348 was performted by shaking 57 Erlenmeyer flasks

(500 ml) containing 50 ml of the medium [0.2% carboxymethylcellulose (CMC), 0.1% KH_2PO_4, 0.3% K_2HPO_4, 0.3% peptone and 0.15% yeast extract (pH 6.8)]. The cells collected by filtration were kept frozen at -30℃ until use.

Enzyme assay

β-Glucosidase activity was determined photometrically at 37℃ in a reaction mixture (1.0 ml) containing 30 mM potassium phosphate buffer (pH 6.8), 2 mM *p*-nitrophenyl-β-D-glucopyranoside and enzyme. One unit (U) of enzyme activity was defined as the amount of enzyme hydrolyzing 1 μmol of the nitrophenyl glucoside/min under the above conditions (22). When various sugars were employed as substrates, either reducing power or glucose formed was assayed by using dinitrosalicylic acid and glucose test kit, respectively (9). One unit (U) of enzyme activity was defined as the amount of 1 μmol sugars hydrolyzed/min. Protein was assayed according to the method of Bradford using bovine serum albumin as the standard (23).

Purification of enzyme

Step 1. The MIBA348 cells were thawed in 5ml PPB and homogenized at 4℃ with pestle, and then centrifuged as described above.

Step 2. The homogenate was dialyzed against several changes of 1000 ml of 10 mM PPB (pH 6.8) and centrifuged. The clear supernatant (350 ml) was applied to DEAE-Sephacel column (1.5 x 40 cm), which was equilibrated with PPB (pH 6.8). After washing thoroughly with PPB, the column was eluted with a linear gradient (500 ml) conducted between PPB in a mixing chamber and PPB/1.0 M NaCl in a reservoir .Fractions of 18 ml were collected and assayed for glucosidase The active enzyme fractions were pooled, dialyzed and centrifuged as described above.

Step 3. The supernatant was applied to DEAE-Sephacel column (1.6 x 8.0 cm), which was equilibrated and exhaustively washed with PPB. The column was eluted with a linear gradient (200 ml) conducted between PPB in a mixing chamber and PPB/1.0 M NaCl in a reservoir. Fractions of 2.0 ml were collectedand assayed for enzyme activity. The active enzyme fractions were pooled, dialyzed and concentrated to 2.5ml on Amicon PM-10 membrane.

Step 4. The concentrate was applied to a Sephacryl S-200 column (1.5 x 100 cm), which was equilibrated with 10 mM PPB/0.3M NaCl and eluted with the same buffer. Fractions of 2.8 ml were collected and assayed for enyme activity. As shown in Fig. 1, the active enzyme fractions eluted from the column were concentrated on Amicon PM-10 membrane filter and subjected to centrifugation and pooled as the final preparation.

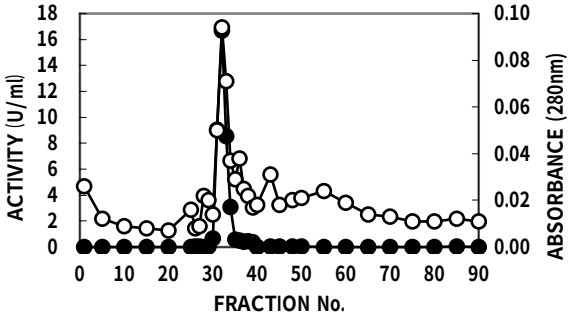

Fig. 1. Elution pattern of *R. oryzae* MIBA348 β-glucosidase from Sephacryl S-200 column.
—○—, absorbance at 280 nm, **——●——:** enzyme activity. The activity is expressed in units (U) per ml of eluate.

Results and Discussion

Twelve *Rhizopus* strains (MIBA341-352) from collections in this laboratory were tested for ability to produce β-glucosidase. *R. oryzae* MIBA348 produced the highest β-glucosidase (0.308 U/mg protein), while other 11 strains gave activities less than 0.05 U/mg protein. The MIBA348 enzyme production was maximum (1.52 U/mg protein) when CMC (0.2%) was used as a carbon source, while the enzyme yield exhibited 80% and 48% of the highest value, respectively when CMC was substituted with 0.2% Avicel or 0.2% cellobiose. The enzyme activity found outside the cells after shaking culture for 2 days was11% of that found inside the cells.

Table 1 shows a summary of the β-glucosidase

purification from strain *R. oryzae* MIBA348. The final preparation (1.0 mg, 103 U/mg protein) was purified 85.7-fold over the cell extracts, the yield being 44.5 %. The protein was homogeneous in terms of its behavior on SDS gel electrophoresis, as shown in Fig. 2. The molecular weight was estimated as105,000 on SDS gel electrophoresis and by gel filtration on Sephacryl S-200.

Table 1. Purification of β–glucosidase from *R. oryzae* MIBA348

Step	Total Protein (mg)	Total Activity (U)	Yield (%)	Specific Activity (U/mg)	Purification
Crude extract	200	231	100	1.2	1.0
1st DEAE-Sephacel	8.5	226	97.8	27	22
2nd DEAE-Sephacel	3.0	197	85.3	65	54
Sephacryl S-200	1.0	103	44.5	103	86

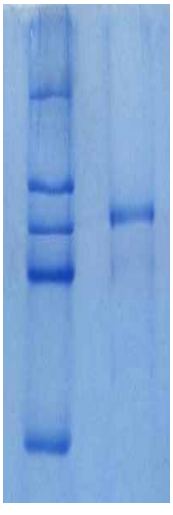

Fig. 2. SDS polyacryamide gel electrophoresis of *R. oryzae* MIBA 348 β-glucosidase.

The SDS gel contained 7.5% acryl acryl-amide. Electrophoresis was carried out for 90 min at 25 mA/gel and at 25°C, using 10 μg protein. After electrophoresis, the gel was stained with Coomassie brilliant blue. **Lane1**: standard molecular weight markers (200,200, 116,250, 97,400, 66,200, 45,000), **lane 2**: purified MIBA348 β-glucosidase

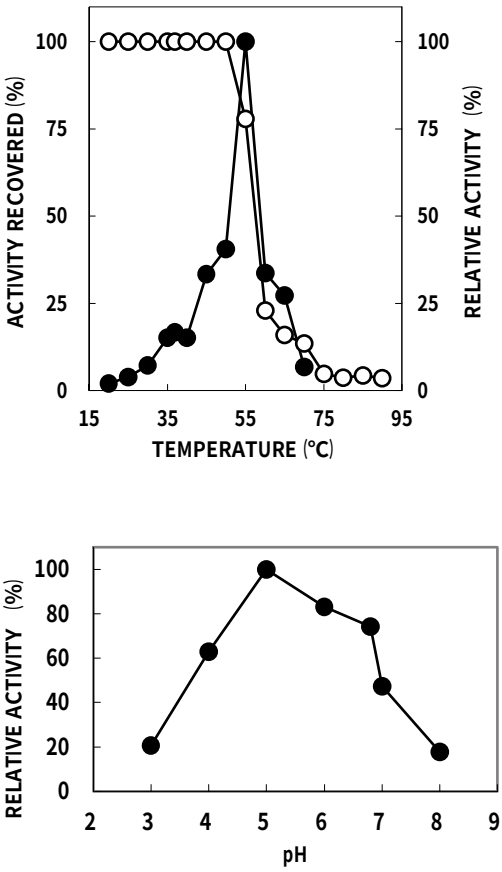

Fig. 3. Effect of temperature on the activity and stability and pH profile of activity of *R. oryzae* MIBA348 β-glucosidase.

(*upper panel*): The activity (●) was determined as given in the text, except that the incubation temperature was changed. The enzyme activity observed at 50°C was expressed as 100%. Thermostability (○) was examined after treating the enzyme for 15 min at different temperatures and at pH 6.8, as shown in the figure, and then the mixture were assayed for the remaining activities. The activity observed at 4°C was defined as 100%.

(*lower panel*): The activity was determined as given in the text, except that the enzyme was incubated in 10-fold volume each of 100 mM sodium acetate buffer (pH 3.0 – 5.5), 100 mM McIlvaine buffer (pH5.0 – 6.5) and 100 mM potassium phosphate buffer (6.0-8.0), respectively prepared with different pHs, as shown in the figure. The activity observed at pH 5.0 was taken as 100%.

The enzyme activity was maximal at 55°C with half-optima at 48°C and 62°C (Fig. 3). The enzyme was most active at pH 5.0 with half-optima at pH3.8 and 7.1 (Fig. 3). The β-1,4-linkages of cellobiose and salicin were cleaved by the enzyme, 20% and 8.4%, respectively, as compared with that of the nitrophenyl glucosaide. The following compounds were not utilized as the substrates: *p*-nitrophenyl-α-D-glucopyranoside, arbutin, maltose, and β-lactose (Table2). Neither Avicel nor CMC was effective

Table2 Hidrolysis of various sugars by *R.oryzae* MIBA348 β-glucosidase

Sugar	Final concentration	Relative activity (%)	Linkage
β-pNPG	20mM	100.	β-1,4-
D-Cellobiose	20mM	20.3	β-1,4-
Salicin	20mM	8.38	β-1,4-
Arbutin	20mM	0.0	β-1,4-
β-Lactose	20mM	0.0	β-1,4-
Avicel	0.1%	0.0	β-1,4-
CMC	0.1%	0.0	β-1,4-
p-nitrophenyl-D-glucopyranoside	20mM	0.0	α-1,4-
Maltose	20mM	0.0	α-1,4-
Amygdalin	20mM	9.01	β-1,6-
Gentiobiose	20mM	22.9	β-1,6-

substrates for the enzyme. The enzyme lost no activity at 4-50°C for 15min at pH 6.8, and activity recovered was 100% at 50°C, 50% at 62°C, and 20% at 70°C, showing the enzyme was quite resistant to heat treatment. This stability of enzyme will be applicable to hydrolyze β-glucan found in nature.

So far, fungal cellulases have been isolated mainly from the members of *Deuteromycotina* (12, 15). *Trichoderma reesei* produces eight cellulase components belonging to seven different glycosyl hydrolase families (CBHI [family 7], CBHII [family 6], EGI [family 7], EGII [family 5], EGIII [family 12], EGV [family 45], BGLI [family 3], and BGLII [family 1] (17, 19). These cellulase components have been shown to degrade crystalline cellulose synergistically (1, 2). The variety of cellulase components might arise mainly through multiple gene transfer events rather than gene duplication events, since it is thought that genes encoding cellulase components belonging to the different glycosyl hydrolase families evolved from different ancestral genes (12).

Although many fungal cellulase genes have been isolated and characterized, the isolation of glucosidase genes from food borne origins has not been reported (21, 22, 24, 26). To determine the cellulase system of *Rhizopus* species, we selected β-glucosidase-producing fungi. As a result, β-glucosidase of *Rhizopus oryzae* MIBA348 was purified to electrophoretically homogeneous state. The final preparation was found to be active towards gentiobiose and amygdaline which contained β- 1, 6-linkages, as shown in Table 2. This glucosidase was able to hydrolyze β- 1, 6- linkages as well as β-1,4- linkages, since it exhibited the similar activity on gentiobiose as that on D-cellobiose.

Aspergillus oryzae α-amylase production is stimulated by soluble starch, glycogen and malto-oligosaccharides (26). The molecular mechanism study of the α-amylase induction in this fungus indicated a gene, designated *amyR* was a transcriptional activator gene involved in starch induced efficient expression of the amylolytic genes (30). On the other hand, cellulose and its oligosaccharides were reported to be inducers in *Tricoderma* species (27, 28). Recently, genes encoding FI-carboxymethylcellulase (CMCase) and β-glucosidase from the fungus *Aspergillus aculeatus* were individually fused with the gene encoding the C-terminal half (320 amino acid residues from the C terminus) of yeast alpha-agglutinin and introduced into *S. cerevisiae*. (29). The genes were expressed by the glyceraldehyde-3-phosphate dehydrogenase promoter from *S. cerevisiae*. The cells displaying these cellulases could grow on cellobiose or water-soluble cellooligosaccharides as the sole carbon source. The degradation and assimilation of cellooligosaccharides were confirmed by thin-layer chromatography. This result showed that the cell surface-engineered yeast with these enzymes could be endowed with the ability to assimilate cellooligosaccharides. This is the first step in the

assimilation of cellulosic materials by *S. cerevisiae* expressing heterologous cellulase genes.

To investigate the substrate specificity observed towards compounds such as amygdaline and gentiobiose which were composed of β-1,6-linkages, detailed molecular analysis of the enzyme is required. This approach is underway to clone genes coding for MIBA348 enzyme and expression in our strong expression system using *Aspergillus oryzae* as a host.

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