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Characterization of a β-glucosidase with transgalactosylation capacity from the zygomycete *Rhizomucor miehei*

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ABSTRACT

An extracellular β-glucosidase from the zygomycete *Rhizomucor miehei* NRRL 5282 cultivated in a wheat bran-based solid state fermentation system was characterized. The purified enzyme exhibited an optimum temperature of 68–70 °C and pH of 5.0. It efficiently hydrolyzed oligosaccharides having β-(1→4) glycosidic linkages and exhibited some β- and α-galactosidase activity. The V_{max} for *p*-nitrophenyl-β-d-glucopyranoside and cellobiose was 468.2 and 115.5 U/mg, respectively, while the K_m was 0.12 mM for both substrates. The enzyme had transglucosylation and transgalactosylation activities resulting in the formation of glycosides from cellobiose, lactose and ethanol. The enzyme increased the amounts of free phenolic antioxidants in sour cherry pomace indicating that its hydrolyzing activity could potentially be applicable to improve the bioavailability of these compounds.

1. Introduction

β-Glucosidases (β-glucoside glucohydrolases; EC 3.2.1.21) are ubiquitous and biologically important enzymes catalyzing the hydrolysis of alkyl- and aryl-β-glycosides as well as di- and oligosaccharides. They have an active role in many biological processes, such as degradation of structural and storage polysaccharides, host–pathogen interactions, cellular signaling and oncogenesis (Bhatia et al., 2002). Their hydrolyzing activity is utilized in various applications, such as fuel ethanol production from cellulosic agro-industrial residues (Harnpicharnchai et al., 2009; Ng et al., 2010), or liberation of aroma compounds from plant-derived products (Su et al., 2010). β-Glucosidases can also be used to liberate phenolic aglycons from their glycosidic bonds, and can therefore be used to increase the amount and nutraceutical activity of phenolic antioxidants (Pham and Shah, 2009). Under certain conditions, β-glucosidases also have synthetic activity, and are able to transfer glycosyl groups to saccharides and alcohols resulting in the formation of oligosaccharides, alkyl-glycosides, and different glycoconjugates, which can be used as therapeutic agents, diagnostic tools and growth promoting agents for probiotic bacteria (Smaali et al., 2007). Enzymatic synthesis of these compounds by transglycosylation or reverse hydrolysis can be achieved in one step instead of several protection/de-protection steps required during chemical synthesis.

In combination with endoglucanases (EC 3.2.1.4) and cellobiohydrolases (EC 3.2.1.91), fungal β-glucosidases have an important role in cellulose degrading enzyme systems, wherein they split short chain oligosaccharides and cellobiose into glucose monomers, preventing inhibition of the other enzymes by cellobiose. The arising glucose, however, is generally a strong inhibitor of β-glucosidases (Eyzaguirre et al., 2005). Due to the fact that high hydrolyzing activity and glucose, alcohol, heat and acid tolerance are important features of enzymes that are potentially applicable in biotechnological and industrial processes, screening for good β-glucosidase producing fungal strains has mainly focused on these parameters. Due to increasing interest in compounds synthesized by glucosidases in the pharmaceutical and food industry, characterization of the transglucosylation activity of fungal β-glucosidases is also an intensively studied area (Christakopoulos et al., 1994; Smaali et al., 2007).

Filamentous fungi are known to be good producers of β-glucosidases and numerous fungal enzymes have been isolated and analyzed (Eyzaguirre et al., 2005); however, only a few β-glucosidases have been purified and characterized from zygomycetes (Yoshioka and Hayashida, 1980, 1981; Borgia and Mehnert, 1982; Petruccioli et al., 1999; Takii et al., 2005). Additionally, synthetic activity of β-glucosidase from zygomycetes has not been described, and only a few reports are available on the liberation of free phenolic antioxidants due to β-glucosidase activity by this fungal group (Vattem and Shetty, 2002; Correia et al., 2004).

The thermophilic fungus *Rhizomucor miehei* is particularly interesting because of its effective extracellular enzyme production,

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e. g. aspartic protease and lipase (Rao et al., 1998; Rodrigues and Fernandez-Lafuente, 2010). In a recent study, β-glucosidase activity of several zygomycetes grown in liquid and solid media was measured and some *R. miehei* strains showed intensive extracellular enzyme activity on wheat bran (Takó et al., 2010a). Based on these results, the β-glucosidase coding gene from the *R. miehei* NRRL 5282 was cloned and characterized and the corresponding enzyme was purified (Takó et al., 2010b).

The present paper presents additional data on the purification of the β-glucosidase from the thermophilic *R. miehei* NRRL 5282 grown on wheat bran; analysis of several biochemical properties and oligosaccharide production are also reported.

2. Methods

2.1. Organism and solid-state fermentation

Spores (10^6 spores/mL) of *R. miehei* NRRL 5282 were inoculated into a 3 L Erlenmeyer flask that contained 130 g of wheat bran and 130 mL distilled water. The culture was incubated at 40 °C for six days. To maintain humidity, 60 mL sterile distilled water was added to the Erlenmeyer flasks every second day.

2.2. Purification of the extracellular β-glucosidase

Details of the *R. miehei* β-glucosidase isolation procedure and the LC–MS analysis were described previously by Takó et al. (2010b).

2.3. Estimation of the protein concentration

Protein content in gel chromatography fractions was monitored by measuring the absorbance at 280 nm. After each purification step, total protein content was determined using a Qubit Fluorometer (Invitrogen) and the Quant-iT Protein Assay Kit (Invitrogen) according to the instructions of the manufacturer.

2.4. Assay of β-glucosidase activity

The β-glucosidase activity was determined by using *p*-nitrophenyl-β-d-glucopyranoside (*p*NPG; Sigma) as substrate in a reaction mixture containing 180 μL diluted enzyme solution and 20 μL of 7 mM *p*NPG. The reaction was carried out at 50 °C for 30 min and was stopped by adding 50 μL of 0.1 M Na_2CO_3. *Para*-nitrophenol release was monitored at 405 nm in 96-well microtiter plates using an ASYS Jupiter HD microplate reader (ASYS Hitech). One unit of β-glucosidase activity corresponded to the release of 1 μM *p*-nitrophenol per minute under the conditions of the assay. Enzyme activities were determined in three independent experiments.

2.5. Isoelectric focusing

Isoelectric focusing (IEF) was performed by using Novex IEF gels (Invitrogen) containing 5% polyacrylamide and 2% ampholytes (pH range 3.0–10.0). Running conditions were set up according to the manufacturer's instructions. The gel was fixed in 12% (w/v) trichloroacetic acid containing 3.5% (w/v) sulfosalicylic acid for 30 min, and was stained with 0.0025% (w/v) Coomassie Brilliant Blue R-250. The pI of the purified β-glucosidase was determined by using an IEF standard marker mix (Sigma) containing proteins with pI values from pH 3.6 to 9.3.

2.6. Characterization of the β-glucosidase

2.6.1. Effect of pH and temperature

The pH optimum of the purified β-glucosidase activity was determined at 50 °C for 30 min in the range from pH 2.2 to 8.0 by using 50 mM McIlvaine buffer supplemented with 0.7 mM *p*NPG. For the pH stability studies, the enzyme was pre-incubated in the same buffer for 24 h at 4 °C, and then the residual activity was evaluated by incubation for 30 min at 50 °C using 0.7 mM *p*NPG as a substrate.

Optimum temperature for the activity was determined by incubating the purified enzyme in the range from 20 to 80 °C for 30 min in 0.1 M acetate buffer (pH 5.0) containing 0.7 mM *p*NPG. Thermal stability was established by incubating the enzyme for 4 h at the desired temperature, and then the residual activity was estimated at 50 °C for 30 min using 0.7 mM *p*NPG. Thermal inactivation was followed at 70, 75 and 80 °C by measuring the residual activity at different time intervals in the presence and absence of cellobiose. Half-life ($t_{(1/2)}$) was determined for each temperature.

2.6.2. Substrate specificity assays

Substrate specificity of the β-glucosidase was estimated by incubating the purified enzyme (0.1 U/mL) in 0.1 M acetate buffer (pH 5.0) containing 0.7 mM aryl-glycosides (*p*NP-β-d-glucopyranoside, *p*NP-α-d-glucopyranoside, *p*NP-N-acetyl-β-d-glucosaminide, *p*NP-α-d-maltohexaoside, *p*NP-β-d-cellobioside, *p*NP-α-d-galactopyranoside, *p*NP-β-d-galactopyranoside, *p*NP-α-d-mannopyranoside, *o*NP-β-d-glucopyranoside and *p*NP-β-d-xylopyranoside; Sigma) or 0.2% (w/v) saccharides (cellobiose, sucrose, salicin, trehalose, amygdalin, lactose, cellulose, sophorose, laminaribiose, maltose and laminarin; Sigma) at 50 °C for 30 min. The activities were monitored by measuring the liberated *p*-nitrophenol and reducing sugars (Miller, 1959), or quantifying the released glucose using glucose oxidase/peroxidase reagent (Sigma) according to the instructions of the manufacturer. The relative rate of hydrolysis on aryl-glycosides and saccharides was determined as percentages of the initial rate of hydrolysis obtained with *p*NPG and cellobiose, respectively.

2.6.3. Determination of kinetic parameters

The apparent Michaelis–Menten constants (K_m) and maximum velocities (V_{max}) for the purified β-glucosidase were assessed from Lineweaver–Burk plots by using *p*NPG or cellobiose as substrates. The enzyme was incubated in acetate buffer (0.1 M, pH 5.0) with the substrates in concentrations ranging from 0.2 to 1.2 mM of *p*NPG or 0.07–1.02 mM of cellobiose at 50 °C for 30 min.

The inhibition constant (K_i) for glucose inhibition was determined by the intersections of the lines on Dixon plots. The β-glucosidase was incubated with 1.2 and 1.6 mM *p*NPG by adding glucose at concentrations up to 55 mM.

2.6.4. Determination of the effect of metal ions and chemical reagents

Enzyme activity was measured under standard assay conditions in the presence of 5 mM $CoCl_2$, $HgCl_2$, $CuSO_4$, $ZnCl_2$, $MnCl_2$, $CaCl_2$, $MgSO_4$, NaCl and KCl, and 10 mM N-bromosuccinimide (NBS), dimethyl sulfoxide (DMSO), diethylpyrocarbonate (DEPC), ethylenediaminetetraacetic acid (EDTA) and sodium dodecyl sulfate (SDS).

2.6.5. Effect of various sugars and ethanol

The effect of mono- and disaccharides on the enzyme activity was determined by using standard enzyme assay conditions in the presence of fructose, xylose, galactose, arabinose, lactose and sucrose at concentrations ranging from 5% to 20% (w/v). The effect of ethanol on the *p*NPG hydrolyzing activity was studied in the range from 5% to 25% (v/v).