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Characterization of a protease-resistant α-galactosidase from the thermophilic fungus *Rhizomucor miehei* and its application in removal of raffinose family oligosaccharides

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ABSTRACT

The α-galactosidase gene, *RmGal36*, from *Rhizomucor miehei* was cloned and expressed in *Escherichia coli*. The gene has an open reading frame of 2256 bp encoding 751 amino acid residues. RmGal36 was optimally active at pH 4.5 and 60 °C, but is stable between pH 4.5 and 10.0 and at a temperature of up to 55 °C for 30 min retaining more than 80% of its relative activity. It displayed remarkable resistance to proteases and its activity was not inhibited by galactose concentrations of 100 mM. The relative specificity of RmGal36 towards various substrates is in the order of *p*-nitrophenyl α-galactopyranoside > melibiose > stachyose > raffinose, with a K_m of 0.36, 16.9, 27.6, and 47.9 mM, respectively. The enzyme completely hydrolyzed raffinose and stachyose present in soybeans and kidney beans at 50 °C within 60 min. These features make RmGal36 useful in the food and feed industries and in processing of beet-sugar.

1. Introduction

Soybeans are a rich source of proteins and have many health benefits such as in the prevention of heart-diseases and cancer (Messina, 1999). Soybean meal is used extensively as a protein source in animal feed. However, soybeans and other legumes contain high concentrations of the soluble oligosaccharides, stachyose and raffinose, which cannot be digested by humans and monogastric animals (Girigowda et al., 2007). These sugars pass undigested into the large intestine causing flatulence and gastrointestinal disturbance which reduces the feed efficiency in monogastric animals and general consumer acceptance of soy products (Ghazi et al., 2003).

α-Galactosidases (EC 3.2.1.22) are exo-acting glycoside hydrolases that catalyze the removal of α-linked terminal non-reducing galactose residues from small oligosaccharides as well as from galactopolysaccharides and galactolipids (Ademark et al., 2001). The ability of α-galactosidases to cleave α-1–6-linked-D-galactosyl residues from raffinose family oligosaccharides (RFOs) has rendered them highly useful in many applications, particularly in the food and feed industries (Ghazi et al., 2003; Kapnoor and Mulimani, 2010). These enzymes have also found applications in the sugar industry to increase the sucrose yield by eliminating raffinose, which prevents normal crystallization of beet sugar (Linden, 1982).

α-Galactosidases are distributed widely in microorganisms, plants and animals and can be classified according to their sequence similarity into glycoside hydrolase (GH) families, 4, 27, 36 and 57 (Henrissat and Bairoch 1993). In the recent years, many α-galactosidases have been identified and purified from various microbial sources (Ferreira et al., 2011; Comfort et al., 2007; Goulas et al., 2009). Fungal α-galactosidases have been widely studied since they are particularly suitable for biotechnological applications due to their extracellular localization, acidic pH optima and good stability. Abundant information is available on the isolation and characterization of α-galactosidases from mesophilic fungi (Ademark et al., 2001; Aleksieva et al., 2010; Kapnoor and Mulimani, 2010). Despite their obvious advantages in many bioprocesses requiring high thermostability, relatively few α-galactosidases have been characterized from thermophilic fungi (Kotwal et al., 1999; Puchart et al., 2001; Rezessy-Szabó et al.,

Abbreviations: CAPS, (cyclohexylamino)-1-propanesulphonic acid; CHES, 2-(cyclohexylamino) ethanesulfonic acid; DTT, dithiothreitol; GH, glycoside hydrolase; IPTG, isopropyl β-D-1-thiogalactopyranoside; MES, 2-(*N*-morpholino)ethane sulfonic acid; MOPS, 4-(*N*-morpholino)-propane sulphonic acid; ORF, open reading frame; PCR, polymerase chain reaction; *p*NP, *p*-nitrophenyl; *p*NPG, *p*NP-α-galactopyranoside; RACE, rapid amplification of cDNA ends; RFOs, raffinose family oligosaccharides; RmGal36, GH family 36 α-galactosidase from *Rhizomucor miehei*; TLC, thin-layer chromatography.

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2007). Although genes encoding α-galactosidases have been cloned from several mesophilic fungi (Ademark et al., 2001; Mi et al., 2007; Cao et al., 2009a,b), so far only the α-galactosidase genes from the thermophilic fungi, *Talaromyces emersonii* and *Thermomyces lanuginosus* have been cloned (Janika et al., 2010; Nakai et al., 2010).

Rhizomucor miehei CAU432, is a strain of thermophilic fungus thriving in optimum temperature at 50 °C. In the present study, cDNA cloning, heterologous expression, purification and characterization of a recombinant α-galactosidase (RmGal36) from *R. miehei* CAU432 is described. This is the first report on α-galactosidase from a *Rhizomucor* species. The application of RmGal36 in elimination of RFOs from soybeans and kidney beans was investigated.

2. Methods

2.1. Strains, vectors and reagents

Escherichia coli JM109 and BL21 strains were used for propagation of plasmids and as host for expression of the α-galactosidase gene, respectively. Vector pET-30a (+) was obtained from Novagen (Madison, WI, USA). Restriction endonucleases and pMD18-T vector were purchased from TaKaRa (Tokyo, Japan). *Pfu* DNA polymerase was from Promega (Madison, MI, USA) and T4 DNA ligase from Biolabs (New England biolabs, USA). Multifunctional DNA purification kit was purchased from BioTeke (Beijing, China). The substrates, *p*NP-α-galactopyranoside (*p*NPG), *p*NP-α-glucopyranoside, *p*NP-α-mannopyranoside, *p*NP-β-galactopyranoside, melibiose, stachyose and raffinose were purchased from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals used were of analytical grade unless otherwise stated.

2.2. Microorganism and cultivation

R. miehei CAU432 has been deposited in the China General Microbiological Culture Collection Center (web-site: http://www.cgmcc.net/) under CGMCC No.4967. For isolation of genomic DNA, *R. miehei* CAU432 was cultivated at 50 °C for 2 days in medium (gL^{-1}): glucose, 10; tryptone, 10; yeast extract, 10; $MgSO_4 \cdot 7H_2O$, 0.3; $FeSO_4$, 0.3; $CaCl_2$, 0.3. For isolation of RNA, the organism was cultured as above except that barley β-glucan was used instead of glucose as carbon source. The mycelia were collected and ground to a fine powder under liquid nitrogen.

2.3. Cloning of an α-galactosidase gene and sequence analysis

DNA manipulations were performed as described by Sambrook and Russell (2001). Genomic DNA was isolated from *R. miehei* CAU432 using the CTAB method (Lodhi et al., 1994). For isolation of RNA for RT-PCR, cells were grown, collected and ground as described above. The total RNA was isolated using the Trizol kit (Invitrogen, Carlsbad, USA). The quality and integrity of RNA was determined by gel electrophoresis in 1.0% agarose containing 3.5% formaldehyde. mRNAs were purified using the Oligotex mRNA Midi Kit (Qiagen, Germany). The degenerate primers, GalDF and GalDR (Table 1) were designed based on the conserved blocks of amino acid residues IKWDMN and DNTRPD of known fungal α-galactosidase belonging to GH family 36 using the CODEHOP (Consensus Degenerate Hybrid Oligonucleotide Primers) programme (http://bioinfo.weizmann.ac.il/blocks/codehop.html). A putative homologous consensus region of the α-galactosidase gene was amplified using the degenerate primers and analyzed by sequencing the PCR products. The PCR conditions were as follows: a hot start at 95 °C for 5 min followed by first five cycles of 95 °C for 30 s, 60 °C for 45 s and 72 °C for 1 min, with 1 °C decrease in annealing temperature per cycle, then 30 cycles of 95 °C for 30 s with constant annealing temperature of 55 °C for 45 s and 72 °C for 1 min followed by a final extension for 8 min at 72 °C. The PCR amplified product was cloned into vector pMD18-T, transformed into *E. coli* DH5α competent cells and sequenced.

The full length cDNA sequence of the α-galactosidase was obtained by 5′ and 3′ RACE (Rapid Amplification of cDNA Ends) using a BD SMART™ RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA). To amplify the 5′ end of the cDNA, the RACE product was amplified with primer Gal5′GSP and an adapter primer (UPM) and subjected to nested PCR using nested gene-specific primer Gal5′NGSP and adapter primer NUP (Table 1). The PCR condition for RACE was: one cycle of 1 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 68 °C, and 1 min at 72 °C, and finally 8 min at 72 °C. For the 3′ RACE, the primary PCR was performed with primers Gal3′GSP and UPM which was followed by a nested PCR using nested gene-specific primer (Gal3′NGSP) and NUP. The PCR product thus obtained was purified, cloned and sequenced. The 5′ and 3′ flanking sequences obtained by 5′ and 3′ RACE were assembled with that of the consensus region to form the full-length cDNA sequence containing the ORF of the α-galactosidase gene. The sequence was subjected to BLAST analysis. The α-galactosidase cDNA sequence from *R. miehei* CAU432 was deposited in the GenBank nucleotide sequence database under the Accession No. JF340459.

Sequence assembly was done using DNAMAN software (LynnonBiosoft, USA). BLAST analysis was performed at the NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple alignment analysis was performed by Clustal W2.0 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Structural analysis of deduced protein was carried out on the website of ExPASy Proteomics Server (http://www.expasy.ch/tools/). Transcription start site was predicted using Neural Network Promoter Prediction software (http://www.fruitfly.org/seq_tools/promoter.html). Signal peptide was analyzed by SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/). Putative catalytic residues were predicted using Motif Scan (http://myhits.isb-sib.ch/cgi-bin/motif_scan) and CAZY (http://www.cazy.org/).

2.4. Heterologous expression of RmGal36 in E. coli

The coding region of the gene without the signal peptide sequence was amplified by PCR from the cDNA of *R. miehei* CAU432 with primers RmGal36F and RmGal36R. *Nde* I and *Not* I sites (underlined) were added to the forward and reverse primers, respectively (Table 1) so that the expressed protein carries a C-terminal His-Tag encoded by the vector. The PCR product was cloned into the *Nde* I/*Not* I site of pET30a (+) vector (Novagen), and transformed into *E. coli* BL21 for protein expression. A single colony of *E. coli* BL21 harboring *RmGal36* in pET30a was inoculated into LB medium containing kanamycin (50 $\mu g\ mL^{-1}$) and incubated on a rotary shaker (200 rpm, 37 °C) until the optical density OD_{600} reached about 0.8–1.0. Lactose was added to a final concentration of 2% to induce expression and the culture was grown further at 37 °C for 22 h.

2.5. Purification of the recombinant α-galactosidase

One liter of *E. coli* culture was harvested by centrifugation, suspended in lysis buffer (50 mM pH 8.0 sodium phosphate, 150 mM NaCl) and disrupted by sonication. The lysate was clarified by centrifugation at 10,000*g* and the clear supernatant was applied to a Ni-IDA column (1 cm × 5 cm) (GE Life Sciences, USA) pre-equilibrated with buffer A (50 mM pH 8.0 sodium phosphate, 500 mM NaCl, 30 mM imidazole). The column was washed with 15 column volume (CV) of buffer A followed by 5 CV of buffer B (50 mM pH 8.0