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ScienceDirect
Enzyme and Microbial Technology 40 (2007) 1373–1380
ENZYME AND MICROBIAL TECHNOLOGY
www.elsevier.com/locate/emt

Molecular cloning and characterization of a novel α-galactosidase gene from *Penicillium* sp. F63 CGMCC 1669 and expression in *Pichia pastoris*

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Received 2 April 2006; received in revised form 19 September 2006; accepted 11 October 2006

Abstract

An extracellular α-galactosidase (EC 3.2.1.22) from *Penicillium* sp. F63 CGMCC 1669, designated Agl1, was purified to homogeneity by means of fast protein liquid chromatography (FPLC) with a molecular mass of 82 kDa on SDS-PAGE while 330 kDa on native gradient PAGE. Based on the partial amino acid sequences of the purified protein, *Agl1* gene encoding the α-galactosidase was cloned and sequenced. The ORF of *Agl1* consisted of 2205 nucleotides encoding a protein of 735 amino acids including 21 residues of a putative signal peptide in its N-terminal. *Agl1* was intron-less, and a GUG codon was considered the start codon of the gene. Agl1 possessed the highest sequence identity of 69.5% with α-galactosidase AglC from *Aspergillus niger* and showed a little homology to those of *Penicillium purpurogenum* and *Penicillium simplicissimum* reported previously, namely, 7.5% and 8.0%, respectively. The mature protein had been expressed extracellularly in *Pichia pastoris* with a yield of 111 U/ml in a 3-l fermenter. The optimum pH and temperature of the recombinant α-galactosidase were 5.0 and 40 °C, respectively. The recombinant α-galactosidase hydrolyzed the release of galactose from natural oligosaccharides, such as melibiose, raffinose, and stachyose. To our knowledge, this is the first report of gene cloning for *Penicillium* α-galactosidase belonging to family 36 of glycosyl hydrolases and expression in *Pichia pastoris*.

Keywords: α-Galactosidase; *Penicillium* sp.; Molecular cloning; Gene expression; *Pichia pastoris*

1. Introduction

α-Galactosidase (EC 3.2.1.22), which is distributed widely in microorganisms, plants and animals, catalyzes the hydrolysis of the terminal non-reducing α-galactosyl residues from various α-galactosides, including oligosaccharides, galacto(gluco)-mannans and galactolipids [1]. The enzyme can be used in various fields, like production of sugar, processing of soymilk, conversion of blood type and treatment of Fabry disease [2–5]. In the past few years, however, α-galactosidase have been considered effective feed additive to remove antinutrient oligosaccharides, which occurred in soybean meal-containing diets [6,7].

Fungal α-galactosidase attracts much attention in term of its suitable pH, fine stability and extracellular secretion. Several fungal α-galactosidases have been isolated, purified and characterized from various sources [8–10]. They have been divided into family 27 and 36 of the glycosyl hydrolases based on amino acid sequence homology [11]. The majority of fungal α-galactosidases are classified into family 27 while a few of them are into family 36. The overwhelming majority of bacterial α-galactosidases belongs to family 36 of glycosyl hydrolases.

Many α-galactosidase genes such as those of *Aspergillus niger*, *Trichoderma reesei* and *Mortierella vinacea* have been cloned [1,12–15]. To date, only two α-galactosidase-encoding genes have been cloned from *Penicillium* spp. One is *AGL1* from *Penicillium simplicissimum* which showed significant similarity with other α-galactosidases of family 27 [16]. The other is the gene from *Penicillium purpurogenum* encoding α-galactosidase of family 27 which was expressed in *Saccharomyces cerevisiae* [17].

Pichia pastoris, a methylotropic yeast strain, has been developed as a host for heterologous protein expression owing to its high expression level, efficient secretion, proper protein folding and the potential to a very high cell density [18,19]. Two kinds of α-galactosidase have been produced using the

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doi:10.1016/j.enzmictec.2006.10.017

Pichia pastoris expression system [20,21]. The cDNA of α-galactosidase isolated from plant, coffee beans has been integrated into the chromosome of *Pichia pastoris* and a biologically active recombinant enzyme has been expressed. The recombinant α-galactosidase was secreted into fermenter culture medium at a level of approximately 12 U/ml with PNPG (*p*-nitrophenyl-α-D-galactopyranoside) as substrate and further induction of methanol did not bring a higher enzyme production [20]. Chen et al. expressed and characterized human α-galactosidase produced in *Pichia pastoris*. The final yield of the α-galactosidase was 1.4×10^5 U/ml fermenter supernatant with MUG (4-methylumbelliferyl-α-galactoside) as substrate [21].

Here we describe gene cloning of α-galactosidase, which belongs to family 36, from *Penicillium* sp. F63 CGMCC 1669 and extracellular expression in *Pichia pastoris*, purification and characterization of the recombinant enzyme and evaluate the potential application of the recombinant enzyme.

2. Materials and methods

2.1. Strains and plasmids

Penicillium sp. F63 CGMCC 1669 was identified by the China General Microbiological Culture Collection (CGMCC, Beijing, China) and preserved under the registered No. CGMCC 1669. *Escherichia coli* JM109 (TaKaRa, Japan) was used as host for the plasmid vector pGEM-T Easy (Promega, USA). The *Pichia pastoris* strain GS115 (Invitrogen, USA) was used for heterologous expression with pPIC9 (Invitrogen, USA) as expression vector.

2.2. Media and culture conditions

Penicillium sp. F63 CGMCC 1669 was cultivated in an enzyme-producing medium with soybean meal as inducer according to the methods of Adya and Elbein with a few modifications [22]. A loop of spores was inoculated into the medium and cultivation was carried out in 1-l wide-mouth shake flasks at 30 °C and 250 rpm for 7 days. Luria–Bertani (LB) medium was used for cultivation of *E. coli* JM109. The ingredients of RDB (Regeneration Dextrose Base plate), MM (Minimal Methanol Medium), MD (Minimal Dextrose Medium), BMGY (Buffered Glycerol-complex Medium), BMMY (Buffered Methanol-complex Medium), BSM (Fermentation basal salts medium), PTM1 trace salts solution and culture condition of *Pichia pastoris* strain were in reference of the Invitrogen Pichia Expression kit manual (Invitrogen).

2.3. Enzyme purification

Through centrifugation, the mycelia were removed and the supernatant containing α-galactosidase was obtained. The precipitation at an ammonium sulfate saturation of 20–95% (final concentration of the ammonium sulfate, 106–650 g/1) was collected by centrifugation and resuspended in 20 mM Tris/HCl buffer, pH 8.0 (buffer A), and dialyzed against the same buffer. The dialysate was applied to a HiTrap Q Sepharose XL FPLC column (Amersham Pharmacia Biotech, Uppsala, Sweden) previously equilibrated with buffer A. Proteins were eluted with the step gradient of NaCl in the same buffer, α-galactosidase activity was mainly eluted as a single peak at 0.3 M NaCl in buffer A and those with enzyme activity were pooled (Fig. 1A). The active fractions were concentrated by ultrafiltration tube (Pall corporation, USA, MW cut-off, 3 kDa). The concentrated enzyme solution was applied to Sephacryl S-200 HR FPLC column (Amersham Pharmacia Biotech, Uppsala, Sweden) previously equilibrated with buffer A. The column was eluted with the same buffer containing 300 mM NaCl at a flow rate of 0.5 ml/min. Fractions with enzyme activity were collected and concentrated.

2.4. Enzyme activity assays

α-Galactosidase activity was assayed using *p*-nitrophenyl-α-D-galactopyranoside (PNPG, Sigma, USA) as substrate as described previously [15]. One unit of the enzyme was defined as the amount of the enzyme that released 1.0 μmol of PNP from PNPG per minute per milliliter under standard assay conditions. Protein concentration was estimated according to the method of Lowry [23]. The specific activity of the enzyme was expressed in units of enzyme activity per milligram of protein.

2.5. Gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electophoresis (SDS-PAGE) and native gradient PAGE were carried out as described previously ([24]; Pharmacia LKB manual, Uppsala, Sweden). SDS-PAGE was performed in 12% polyacrylamide gel with Tris/Glycine buffer in presence of SDS. Native gradient PAGE was carried out on a continuous gradient polyacrylamide gel (from 4% to 15%) with Tris/Glycine buffer without SDS. Protein was stained with Coomassie Brilliant Blue R-250 (Sigma, USA).

2.6. Determination of N-terminal and inner amino acid sequences of the α-galactosidase (Agl1)

The protein band on SDS-PAGE was transferred onto a polyvinylidence fluoride (PVDF) membrane by eletroblotting method and the protein was subjected to auto Edman degradation to obtain its N-terminal sequence. At the same time, the purified enzyme was treated with trypsin (Roche, Gemany). Briefly, the resulting peptides were isolated by HPLC (HP 1100, Agilent, USA) on a reverse-phase C_{18} column (4.6 mm × 150 mm). The peptides were eluted from

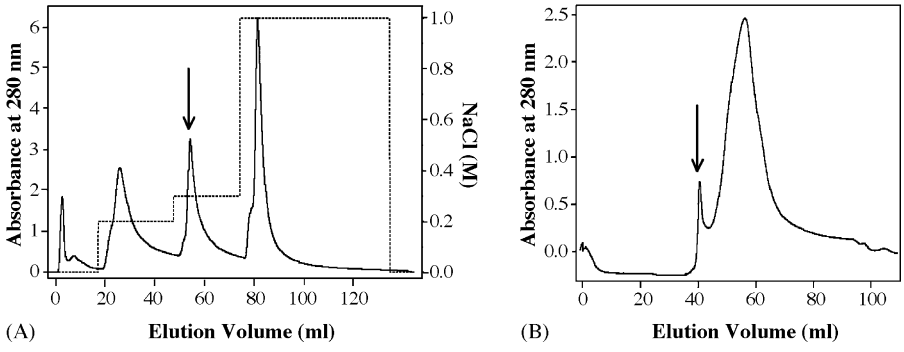

Fig. 1. Purification of an α-galactosidase from *Penicillium* sp. F63. (A) Ion exchange chromatography on FPLC HiTrap Q Sepharose XL column (5 ml). Step elution was used in the isolation of the fraction containing the α-galactosidase. α-Galactosidase activity was mainly in a single peak at 0.3 M NaCl in buffer A. (B) Gel filtration on Sephacryl S-200 HR column (1.0 cm × 120 cm). A homogeneous α-galactosidase was obtained after Sephacryl S-200 HR column. Arrows denote the peaks containing the α-galactosidase activity.