Journal of Molecular Catalysis B: Enzymatic 77 (2012) 39–45

Contents lists available at SciVerse ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Immobilization of a recombinant endo-1,5-arabinanase secreted by *Aspergillus nidulans* strain A773

André Ricardo de Lima Damásio[a], Benevides Costa Pessela[b], César Mateo[c], Fernando Segato[d], Rolf Alexander Prade[e], Jose Manuel Guisan[c], Maria de Lourdes Teixeira de Moraes Polizeli[f,*]

[a] *Departamento de Bioquímica e Imunologia, Faculdade de Medicina de Ribeirão Preto Universidade de São Paulo, Ribeirão Preto SP, Brazil*
[b] *Departamento de Biotecnología y Microbiología de los Alimentos, Instituto de Ciencias de la Alimentación, CIAL-CSIC, Calle Nicolás Cabrera 9, Campus UAM, Cantoblanco, 28049 Madrid, Spain*
[c] *Departamento de Biocatálisis, Instituto de Catálisis y Petroleoquímica-CSIC, Campus UAM, Cantoblanco, 28049 Madrid, Spain*
[d] *Laboratório Nacional de Ciência e Tecnologia do Bioetanol (CTBE), Centro Nacional de Pesquisa em Energia e Materiais (CNPEM), Brazil*
[e] *Department of Microbiology and Molecular Biology, Oklahoma State University, Stillwater, OK, USA*
[f] *Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Avenida Bandeirantes 3900, Monte Alegre, 14.040-901 Ribeirão Preto, SP, Brazil*

ARTICLE INFO

Article history:
Received 1 August 2011
Received in revised form
13 December 2011
Accepted 3 January 2012
Available online 10 January 2012

Keywords:
Aspergillus niveus
Aspergillus nidulans
Recombinant endo-1,5-arabinanase
Over-expression
Purification
Immobilization

ABSTRACT

An endo-1,5-arabinanase (*abnA*) encoding gene from *Aspergillus niveus* was identified, cloned and successfully expressed in *Aspergillus nidulans* strain A773. Based on amino acid sequence comparison, the 34-kDa enzyme could be assigned to CAZy GH family 43. Characterization of purified recombinant endo-1,5-arabinanase (AbnA) revealed that it is active at a wide pH range (pH 4.0–7.0) and an optimum temperature at 70 °C. The immobilization of the AbnA was performed via covalent binding onto agarose-modified supports: glyoxyl iminodiacetic acid–Ni^{2+}, glyoxyl amine, glyoxyl (4% and 10%) and cyanogen bromide activated sepharose. The yield of immobilization was similar on glyoxyl amine and glyoxyl (96%), and higher than glyoxyl iminodiacetic acid–Ni^{2+} (43%) support. The thermal inactivation of these immobilized preparations showed that the stability of the AbnA immobilized on glyoxyl 4 and 10% was improved by 4.0 and 10.3-fold factor at 70 °C. The half-life of glyoxyl 4% derivative at 60 °C was >48 h (pH 5), 9 h (pH 7) and 88 min (pH 9). The major hydrolysis product of debranched arabinan or arabinopentaose by glyoxyl agarose-immobilized AbnA was arabinobiose.

1. Introduction

Plant structural polysaccharides are the most abundant source of renewable carbon in the biosphere and represent a valuable industrial substrate in several applications, such as bio-energy production, pulp and paper, food technology, detergent, textile, nutritional or medical research and organic synthesis. Arabinan is a pectic polysaccharide consisting of a backbone of α-1,5-linked L-arabinofuranosyl units, which are further decorated with α-1,2- and α-1,3-linked arabinofuranosides. In the plant cell wall, arabinan is generally linked to rhamnogalacturonan, and often substituted with terminal phenolic esters, particularly feruloyl or coumaroyl, which can dimerize oxidatively to form links between the rhamnogalacturonan polymers [1].

Enzymes from plant, fungal and bacterial sources that are capable of degrading arabinans and/or arabinose-containing polysaccharides have been described and reviewed [2]. Two major enzymes hydrolyze arabinan: endo-α-1,5-L-arabinanase (EC 3.2.1.99) is specific for α-1,5-L-arabinofuranosidic linkages, and hydrolysis α-1,5-L-arabinan by an endo mechanism. α-L-arabinofuranosidase (3.2.1.55) releases terminal non-reducing α-L-arabinofuranosyl residues present in arabinans, arabinogalactans and arabinoxylans. Arabinanases and arabinofuranosidases are classified by CAZy [5,6] as being members of glycoside hydrolase families GH3, GH43, GH51, GH54 and GH62, which display a wide range of enzymatic activities [3,4].

Multipoint covalent attachment is one of the most interesting approaches to link immobilization and stabilization of enzymes, therefore, stabilization factors ranging between 100 and 1,000,000 have been reported [5–8]. Theoretically, an enzyme molecule attached to a rigid support through many covalent linkages, and via very short spacer arms, should be highly stabilized, that is, the enzyme residues involved in the covalent immobilization should keep their relative positions unaltered during any conformational changes of the protein promoted by any distorting agent (heat, organic solvents, etc.) [8].

* Corresponding author. Tel.: +55 16 36024680; fax: +55 16 36024886.
E-mail address: polizeli@ffclrp.usp.br (M. de Lourdes Teixeira de Moraes Polizeli).

1381-1177/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2012.01.002

This report describes the secretion of an endo-1,5-arabinanase (AbnA) in *Aspergillus nidulans* strain A773 (*pyrG*^{89}). pEXPYR plasmids carrying *abnA* gene was transformed into the host strain and directed protein towards the extracellular medium. It was examined the effect of maltose overexpression followed by studies of temperature and pH, immobilization on modified-agarose supports and analysis of released products from debranched arabinan.

2. Experimental

A. nidulans strain A773 (*pyrG89;wA3;pyroA4*) was purchased from the Fungal Genetics Stock Center (FGSC, St. Louis, MO). The zeocin (pheomycin) was purchased from Invivogen (ant-zn-1) and all other chemicals from the best source possible (Sigma Aldrich, Megazyme and Fisher Scientific). Sepharose 4 or 10BCL and Cyanogen bromide (CNBr)-activated sepharose 4B was purchased from GE Healthcare (Uppsala, Sweden).

2.1. Microbial strains, plasmids and culture conditions

Aspergillus niveus was cultured in defined minimum medium (MM) [9] supplemented with 1% glucose at 37 °C. *A. nidulans* strain A773 (*pyrG*^{89}) was cultured in complete medium (CM) as previously described [10]. One Shot® TOP10 Chemically Competent *Escherichia coli* (Invitrogen, The Netherland) was used to propagate plasmid and to clone purified polymerase chain reaction (PCR) products. The PyrG-containing vector pExPYR was used for expression of AbnA in *A. nidulans*.

2.2. Cloning of endo-1,5-arabinanase-encoding gene

In order to isolate the genomic DNA, *A. niveus* grown in CM-glucose medium, the mycelia was freezed with liquid nitrogen and harvested followed by treatment with 600 μl of genomic extraction solution (10% of 0.5 M EDTA and 1% SDS). The suspension was heated at 68 °C for 10 min, centrifuged at 13,000 × *g* for 5 min. and the supernatant transferred into a fresh tube. A volume of 40 μl of 5 M potassium acetate was added, mixed by inversion and placed on ice for 10 min. The suspension was centrifuged again at 13,000 × *g* for 5 min and the supernatant transferred into a fresh tube. After that, it was added 2.5× vol 95% EtOH and 70% EtOH twice to wash. The pellet was well dried and DNAse-free water added.

The coding sequence for the gene abnA was amplified with the polymerase chain reaction (PCR) using Platinum® Pfx DNA Polymerase (Invitrogen). The primer set used for *abnA* amplification was 5′-NNN **GCG GCC GC**T ATG CCA ACC CGG GGT CGT G-3′ and 5′-NNN **TCT AGA** GGC TAA TTA TCA TAC AAC TGG CCA TCC GCT AGA GAA ATC-3′, bearing restriction sites for directional cloning using *NotI* and *XbaI* sites (indicated in bold). The following touchdown PCR cycle parameters were used: denaturing at 95 °C for 1 min, annealing at 60 °C for 30 s and extension at 68 °C for 3 min, with repetition of previous cycles excepting by lowering the annealing temperature by 2 °C for every cycle until an annealing temperature of 52 °C was reached, then 28 cycles of 95 °C for 30 s, 52 °C for 30 s and 68 °C for 3 min, followed by a final extension at 68 °C for 10 min.

2.3. Secretion and purification of AbnA from A. nidulans

After confirmation of cloning into pExPYR plasmid by sequencing (accession number JN222917), the plasmid was transformed into *A. nidulans* [11]. Positive transformants were isolated by their ability to grow in the presence of 100 μg/ml of zeocin. Protein expression was carried out in an Erlenmeyer flask using MM with 100 μg/ml of zeocin with 2% maltose as inducer. The AbnA production was monitored by analyzing the culture supernatant by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and debranched arabinan hydrolysis.

To follow with the AbnA purification, *A. nidulans* was grown in 1 L of MM for 72 h at 37 °C. The mycelium was filtered with Wathman paper n° 1 and the crude extract was dialyzed against water and lyophilized. It was ressuspended in 4 mL of 10 mM ammonium acetate buffer pH 5.0 and applied on a BioGel P100 (Bio-Rad) column (44.9 × 1.2 cm) equilibrated with the same buffer. The protein elution was carried with the same buffer at a flow rate of 0.8 mL h^{-1}. Fractions with activity were concentrated by ultra-centrifugation (10 kDa cut off; Amicon – Millipore, Massachusetts) and analyzed to SDS–PAGE homogeneity.

2.4. Enzyme characterization

Activity against debranched arabinan and others complex carbohydrates were determined for reducing sugar production using dinitrosalicylic acid reagent (DNS) [12]. The reaction mixture, consisting of 50 μl substrate (1%, w/v) in 50 mM ammonium acetate buffer, pH 5.0 and 50 μl enzyme solution, was incubated at 60 °C in a water bath for 5 min. The reaction was stopped by adding 0.1 ml dinitrosalicylic acid and immediately boiled for 5 min. The reducing sugars released as enzyme activity were measured at 540 nm after 1 ml water addition and cooling. One unit of enzymatic activity was defined as the amount of enzyme which produced 1 μmol/min of reducing sugars. Against p-nitrophenyl arabinofuranoside or arabinopyranoside the reaction was assayed using 50 μl of 8-mM substrate and 50 μl of enzyme for 5 min. Reactions were stopped by adding 100 μl saturated sodium tetraborate, and products were measured by absorbance at 405 nm. To determine the optimum pH and temperature profiles, the enzymatic reaction was carried out at different pH in McIlvaine's [13] buffer system (pH 4.0–10.0) and various temperatures (30–80 °C). The protein content was measured by Bradford method [14].

2.5. Activation of agarose with epoxy groups

All the experiments were performed using the same agarose batch and the error was always lower than 10%. For this work, 10 g of agarose 4 or 10 BCL was suspended in 44 mL water, 16 mL acetone, 3.28 g NaOH, 0.2 g $NaBH_4$ and 11 mL epichlorohydrin. The suspension was stirred mildly for 16 h and washed with an excess of water. For quantification of the activated epoxy groups, 1 g of the support was treated with 10 mL 0.5 M H_2SO_4 for 2 h to hydrolyze the epoxy groups. Then, this hydrolyzed support was oxidized with $NaIO_4$, as previously described [15]. The number of epoxy groups was calculated by the difference in periodate consumption between the hydrolyzed support and the initial epoxy support. Periodate consumption was quantified using potassium iodide as previously described [16].

2.6. Modification of agarose supports with different reactive groups

Agarose epoxy-supports were modified with different moieties. In all cases, the ratio of the modifying reactive solution to the support was 1/10 (v/w).

2.6.1. Cationic supports

The epoxy-agarose support was modified with 1 M triethylamine in 50% water/acetone at pH 12 for 24 h at 25 °C.

2.6.2. Anionic supports

The epoxy-agarose support was treated with 0.5 M iminodiacetic acid (IDA) at pH 11 for 24 h at 25 °C.