Biocatalysis and Agricultural Biotechnology 4 (2015) 362–369

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

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

Original Research Paper

Characterization of a thermo-tolerant mycelial β-fructofuranosidase from *Aspergillus phoenicis* under submerged fermentation using wheat bran as carbon source

Cynthia Barbosa Rustiguel, João Atílio Jorge, Luis Henrique Souza Guimarães *

Departamento de Biologia – Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Avenida Bandeirantes 3900, Monte Alegre, 14040-901 Ribeirão Preto, São Paulo, Brazil

ARTICLE INFO

Article history:
Received 5 February 2015
Received in revised form
9 April 2015
Accepted 22 May 2015
Available online 27 May 2015

Keywords:
Invertase
Fructooligosaccharide
Enzyme purification
Sucrose

ABSTRACT

The filamentous fungus *Aspergillus phoenicis* (*Aspergillus saitoi*) produced high levels of mycelial β-D-fructofuranosidase (invertase) when cultivated under submerged fermentation using Khanna medium with wheat bran as the carbon source for 72 h at 40 °C, under orbital agitation (100 rpm). The mycelial invertase was purified 20-fold with 24% recovery through two chromatographic steps (DEAE-cellulose and Sephacryl S-200). The enzyme was characterized as a monomeric glycoprotein with 2% carbohydrate content and a native molecular mass of 131 kDa comprising two 70-kDa subunits. The optimal temperature and pH for activity were 65 °C and 4.5, respectively. The enzyme was resistant to temperatures of 50 °C and 60 °C and stable at pH 4.0–7.0. Activity increased in the presence of different ions, especially Mn^{2+} (+177 %), and Ag^{+} increased the invertase activity by 91%. The mycelial invertase hydrolyzed sucrose, raffinose, and inulin, with greater specificity for the former. The $K_{1/2}$ and V_{max} values for sucrose were 22.5 mM and 124.9 U mg^{-1}, respectively.

1. Introduction

Invertases (β-D-fructofuranosidase, EC 3.2.1.26) are enzymes that catalyze the breakdown of β-2,1 glycosidic bonds from sucrose molecule to obtain an equimolar mixture of D-glucose and D-fructose known as invert sugar (Álvaro-Benito et al., 2007). These enzymes can be grouped as acid, neutral, and alkaline invertases based on the pH of maximal activity (Winter and Huber, 2000). Neutral and alkaline invertases have been reported in plants such as *Arabidopsis thaliana* (Xiang et al., 2011) and cyanobacteria such as *Anabaena* sp. (Vargas and Salerno, 2010). Comparatively, acid invertases are found in plants and microorganisms such as bacteria and fungi (BRENDA – The Comprehensive Enzyme Information System). Invertase production by filamentous fungi has been described in *Cladosporium cladosporioides* (Almeida et al., 2005), *Aspergillus niger* (Reddy et al., 2010), *Aspergillus caesiellus* (Novaki et al., 2010), *Termitomyces clypeatus* (Chowdhury et al., 2009), and *A. phoenicis* (Rustiguel et al., 2010), among others.

Fungal invertases have attracted interest for various industrial applications, including those in the food and beverage industry. The invert sugar syrup obtained from sucrose hydrolysis by fungal invertases is sweeter than sucrose and does not crystallize at a high concentrations (Bayramoglu et al., 2003). In addition, some microbial fructofuranosidases catalyze the transfructosylating reaction at high sucrose concentrations ($\geq$20%) to obtain fructooligosaccharides (FOS) such as 1-kestose, nystose, and fructosyl nystose (Guimarães, 2012).

The first step to determine the potential applications of a specific enzyme is to investigate its physico-chemical properties. If important and unique properties are observed, further genetic engineering studies are conducted to improve the production and/or enzyme properties. The extracellular fructofuranosidase from *A. phoenicis* was previously characterized and showed interesting and intriguing properties (Rustiguel et al., 2010). However, to our knowledge, the mycelial fructofuranosidase has not been previously investigated. Herein, this manuscript describes the production and characterization of a mycelial fructofuranosidase produced by *A. phoenicis* under submerged fermentation using wheat bran as the carbon source.

2. Material and methods

2.1. Microorganism and culture conditions

The filamentous fungus *A. phoenicis* (*A. saitoi*) was maintained

* Corresponding author. Fax: +55 16 3602 3668.
E-mail address: lhguimaraes@ffclrp.usp.br (L.H.S. Guimarães).

http://dx.doi.org/10.1016/j.bcab.2015.05.004
1878-8181/

at 4 °C in oatmeal slants and subcultured at 20-day intervals. The submerged cultures were obtained by inoculating a spore suspension (10^5 spores/mL) in 25 mL Khanna medium (Khanna et al., 1995) in 125-mL Erlenmeyer flasks with different carbon sources (2% monosaccharides: glucose and raffinose; 1% oligosaccharides: starch and sucrose; or complex sources: sugar cane bagasse, crushed corncob, rice straw, oatmeal, wheat bran, and cassava flour) at initial pH 6.0 (Rustiguel et al., 2010). The media were sterilized at 120 °C and 1.5 atm for 25 min. The inoculated media were kept at 40 °C for 72 h.

2.2. Obtainment of mycelial crude extract

After cultivation, each medium was harvested using a vacuum pump with Whatman No. 1 filter paper. The free cell filtrate was stored for extracellular enzyme quantification. The mycelia obtained were washed three times with distilled water, pressed through the filter paper, macerated with sand sea in a porcelain mortar, and resuspended in 100 mM sodium acetate buffer, pH 5.0. The suspension was centrifuged at 23,000*g* for 15 min, and the free cell extract, termed the mycelial crude extract, was used to determine enzyme activity and for purification.

2.3. Determination of enzyme activity and quantification of proteins and carbohydrates

The enzyme activity was determined using 1% sucrose as the substrate in 100 mM sodium acetate buffer, pH 4.5. The reaction was conducted at different temperatures (30–80 °C) and pH values (3.0–8.0) using McIlvaine buffer. The hydrolysis products were estimated using DNS (3′,5′-dinitrosalicylic acid), as described by Miller (1959). One unit of enzyme activity (*U*) was defined as the amount of enzyme necessary to produce 1 μmol glucose per minute under the assay conditions.

The protein was quantified as described by Lowry et al. (1951) using bovine serum albumin (BSA) as the standard and expressed as mg protein per mL sample. The specific activity was defined as U mg^{-1}. The carbohydrate content was determined according to DuBois et al. (1956) using mannose as the standard.

2.4. Purification

The mycelial crude extract was precipitated with 45% ammonium sulfate, resuspended in 10 mM Tris–HCl, pH 7.5, dialyzed in this same buffer overnight at 4 °C, and loaded onto DEAE-cellulose chromatographic columns (10.0 × 2.0 cm^2) previously equilibrated with 10 mM Tris–HCl, pH 7.5. Fractions containing invertase activity were eluted using a linear gradient of NaCl (0–1 M) in 10 mM Tris–HCl, pH 7.5. Fractions (3.0 mL) were collected at a flow rate of 1.9 mL/min. The invertase activity for each fraction was determined as described above in 100 mM sodium acetate buffer, pH 4.5, at 60 °C. Active fractions were pooled, dialyzed in distilled water overnight at 4 °C, lyophilized, suspended in 50 mM Tris–HCl buffer, pH 7.5, with 50 mM NaCl and loaded onto Sephacryl S-200 chromatographic columns (80.0 × 2.0 cm^2) previously equilibrated in this same buffer. Fractions (1.0 mL) containing invertase activity were eluted in this same buffer at a flow rate of 0.38 mL/min. Fractions with activity were pooled, dialyzed in distilled water overnight at 4 °C, and used for electrophoresis and enzyme characterization.

2.5. Electrophoresis

The purified fraction was submitted to non-denaturing electrophoresis (7% PAGE) as described by Davis (1964) and denaturing electrophoresis (7% SDS-PAGE) as described by Laemmli (1970).

Table 1
Influence of carbon sources on the production of fructofuranosidase by *A. phoenicis*.

Carbon source	Specific Activity (U mg^{-1})
No carbon source	19.81 ± 1.8
Starch	7.15 ± 2.7
Oatmeal	6.83 ± 2.7
Sugar cane bagasse	57.33 ± 7.9
Wheat bran	109.87 ± 10.3
Cassava	8.62 ± 3.1
Glucose	0.41 ± 0.1
Rice straw	53.5 ± 3.3
Raffinose	86.41 ± 2.3
Crushed corncob	6.9 ± 1.6
Sucrose	6.27 ± 4.0

The microorganism was grown in 25 mL Khanna medium at 40 °C under orbital agitation (100 rpm) for 72 h. Total U = U/mL × extracellular extract volume.

Table 2
Purification of mycelial fructofuranosidase produced by *A. phoenicis*.

Steps	Activity (Total U)	Protein (Total mg)	Specific activity (U mg^{-1})	Yield (%)	Purification (X)
Crude extract	806.4	24.6	32.8	100.0	1.0
DEAE-cellulose	872.6	8.6	101.5	108.2	3.1
Sephacryl S-200	196.4	0.3	654.7	24.3	19.9

Proteins were separated at 120 V and 40 mA for 2 h. After running, the gels were stained using silver nitrate using the method described by Blum et al. (2005). To determine invertase activity in 7% PAGE, the gel was washed with 0.5 M sodium acetate buffer, pH 4.5, three times for 30 min each then with 0.1 M sodium acetate buffer, pH 4.5, three times for 20 min each. The washed gel was maintained in a solution containing 0.2 mg/mL phenazine methyl sulfate, 0.4 mg/mL nitroblue tetrazolium, 30 U/gel glucose oxidase, and 1% sucrose in the dark until protein was visible.

2.6. Molecular mass determination

The native molecular mass was determined in a gel-filtration chromatographic column (Sephacryl S-200) as described above. Alcohol dehydrogenase (150 kDa), BSA (66 kDa), egg albumin (43 kDa), and carbonic anhydrase (29 kDa) were used as molecular mass markers.

The molecular mass under denaturing conditions was determined with 7% SDS-PAGE as described above. α-2 macroglobulin (168 kDa), β-galactosidase (112 kda), lactoferrin (91 kDa), pyruvate kinase (67 kDa), and lactic dehydrogenase (36 kDa) were used as molecular mass markers.

2.7. Temperature and pH stabilities

Thermal stability of the purified enzyme was determined in an aqueous solution at different temperatures (50–70 °C) for 1 h. Aliquots were taken at pre-determined times, stored in an ice bath, and used to determine the enzyme activity as described above. The pH stability was determined by incubating enzyme samples at different pH values (100 mM sodium acetate buffer, pH 3.5 and 4.5; 50 mM MES buffer, pH 6.0; 50 mM Tris–HCl buffer, pH 7.0 and 8.0; 50 mM CAPS buffer, pH 9.0 and 10.0) for 1 h before measuring activity. The best temperature for enzyme storage was also investigated by maintaining enzyme samples at −20 °C, 4 °C, and 27 °C for 200 h.