Journal of Biotechnology 135 (2008) 58–63

Contents lists available at ScienceDirect

Journal of Biotechnology

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

Chromatographic separation and kinetic properties of fructosyltransferase from *Aureobasidium pullulans*

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ARTICLE INFO

Article history:
Received 24 May 2007
Received in revised form 6 February 2008
Accepted 18 February 2008

Keywords:
Fructosyltransferase
Anion-exchange chromatography
Fructooligosaccharides
Enzyme purification
Kinetics
Substrate specificity

ABSTRACT

Efficient chromatographic separation of fructosyltransferase from *Aureobasidium pullulans* was achieved on a preparative scale using a weak anion-exchanger Sepabeads FP-DA. The recovery yield was about 70% and the purification factor reached a value of 28. The molecular weight of the enzyme determined by size-exclusion chromatography was 570,000. The enzyme exhibited both hydrolytic and transferase activity when the latter was higher in the whole concentration range and completely dominating at higher sucrose concentrations. It was found that sucrose was the only donor of fructosyl moiety used in the transfer reaction. The initial rate method was used for the investigation of the kinetics of the action of fructosyltransferase on sucrose in the concentration range 30–2430 mM. The initial transfructosylation rate was well fitted with a linear function of the sucrose activity where the activity coefficient was an exponentially decreasing function of the sucrose concentration.

1. Introduction

Fructosyltransferase (FTase, E.C. 2.1.4.9) is an enzyme transforming sucrose into fructooligosaccharides (FOS). FOS are fructose oligomers with a terminal glucosyl unit and with a general formula GF_n, where typical values of n are 2–4. FOS are classified as prebiotics and have numerous beneficial properties for human health (Yun, 1996). They are widely utilized in food and pharmaceutical industries. Although FTase was found in many higher plants and microorganisms, the most important industrial sources are strains of *Aspergillus niger*, *Aspergillus japonicus* and *Aureobasidium pullulans* (Yun, 1996). The optimal conditions for the production of FOS by FTase action on sucrose are temperatures 50–60 °C, pH values 5–6.5 and sucrose concentrations above 500 g l^{-1}. The yields of produced FOS range from 50 to 65% of the total mass of saccharides (Hayashi et al., 1991; L'Hocine et al., 2000; Sangeetha et al., 2004; Ghazi et al., 2007).

Although *A. pullulans* FTase is used in the industrial production of FOS, there is much less information available about its properties compared with that for FTase from *Aspergillus* sp. Two intracellular and two extracellular FTases from *Aureobasidium* sp. ATCC 20524 were identified and characterized (Hayashi et al., 1991, 1992). Lee et al. (1992) purified intracellular FTase from *A. pullulans* C-23 and described its properties. Yoshikawa et al. (2006) separated five types of FTase differing in the ratio of transfructosylating and hydrolyzing activity from the cell wall of *A. pullulans* DSM 2404. In a subsequent publication, the enzyme with the highest transfructosylating activity was purified and its properties were investigated (Yoshikawa et al., 2007). In spite of the utilization of FTase in the industrial production of FOS and numerous scientific investigations, the only commercially available source of FTase is Pectinex SP-L, a pectinolytic and cellulolytic preparation designated for fruit juice processing.

Our research group selected *A. pullulans* CCY 27-1-94 as a suitable producer of FTase with respect to the yield, specificity and regioselectivity of FOS production (Madlová et al., 1999, 2000). The FTase is stable in a broad range of pH's and temperatures up to 65 °C with an optimum pH 4.4 and temperature of 65 °C (Onderková et al., 2007). The conditions for production of the enzyme in extracellular and intracellular forms were optimized (Antošová et al., 2002; Vandáková et al., 2004). An economic analysis of the industrial production of partially purified FTase from *A. pullulans* was also accomplished (Vaňková et al., 2005). A higher purity and specific activity of the purified FTase preparation could be advantageous not only for laboratory scale experimentation but a higher immobilization efficiency and volumetric activity of immobilized biocatalyst could be achieved as well (Onderková, unpublished results; Ghazi et al., 2005).

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doi:10.1016/j.jbiotec.2008.02.016

An objective of this study was to separate the intracellular FTase from *A. pullulans* by preparative anion-exchange chromatography with respect to a high recovery yield. The main focus of the study was on the characterization of the catalytic properties of the enzyme, in particular the substrate specificity, effect of sucrose concentration on transfructosylation and hydrolytic activities, and kinetics of transfructosylation.

2. Materials and methods

2.1. Materials

Strain *A. pullulans* CCY 27-1-94 was obtained from Culture Collection of Yeasts (Bratislava, Slovakia). Bio-La-Test Glucose GOD was purchased from PLIVA-Lachema Diagnostika (Brno, Czech Republic). Kit for Molecular Weights 29,000–700,000 (MW-GF-1000, Sigma, St. Louis, MO, USA) and Gel Filtration Standard (Bio-Rad Laboratories, Hercules, CA, USA) were used as molecular weight standards for size-exclusion chromatography. The standards for the chromatographic analysis of 1-kestose (GF_2), nystose (GF_3), and 1^F-fructofuranosylnystose (GF_4) were obtained from Wako Pure Chemical Industries (Osaka, Japan). A mixture of FOS, Actilight 950P, was obtained from Beghin Meiji (Neully sur Seine, France). All other chemicals were of analytical grade and were obtained from readily available commercial sources.

2.2. FTase isolation

FTase was prepared by the aerobic cultivation of *A. pullulans* CCY 27-1-94 (Vandáková et al., 2004). The cells were separated by centrifugation and re-suspended in a 0.1 M phosphate–citrate buffer with the pH 5.5 to the concentration of 150 g l^{-1}. The suspension was disintegrated in a French press (Thermo Electron Corp., Waltham, MA, USA). The disintegration was performed at the pressure of 240 MPa using four repeated passes of the suspension through the disintegration cell when more than 80% of FTase was released from the cells into the solution. The cell debris was removed by centrifugation for 20 min at 16,400 × *g* and microfiltration through a 0.65 μm membrane. The supernatant was used as a crude enzyme preparation. It was then dialyzed through a membrane SpectraPor, MWCO 50000 (Spectrum Medical Industries, Houston, TX, USA) at 6 °C against 0.02 M phosphate–citrate buffer with pH 6.0 containing 0.02% NaN_3 (buffer A).

2.3. Separation of FTase by ion-exchange chromatography

A glass, semi-preparative column Pharmacia XK 16/40, 400 mm × 16 mm i.d. (Amersham Biosciences, Uppsala, Sweden) with the bed volume of 60 ml was used. The column was filled with a weak anion-exchange resin Sepabeads FP-DA with particle size of 250–300 μm (Mitsubishi Chemical Co., Tokyo, Japan) and was equilibrated with buffer A. 30 ml of a feed was then applied to the column using a peristaltic pump Alitea-VS (FIAlab Instruments, Stockholm, Sweden) and non-retained compounds were washed with 225 ml of the equilibration buffer. FTase and other adsorbed proteins were eluted with buffer A containing 0.15 M NaCl (75 ml) and subsequently by a linear gradient of NaCl in buffer A ranging from 0.25 to 0.75 M (150 ml). The constant flow rate of 2.5 ml min^{-1} was provided by a pump MaxiStar K-1000 (Knauer, Berlin, Germany) during the whole process. Five millilitre-fractions of the eluate were collected with a fraction collector FRAC-100 (Amersham Biosciences, Uppsala, Sweden). The FTase activity was determined in each fraction. The signal from a UV detector (Knauer, Berlin, Germany) operated at the wavelength of 280 nm was recorded by the CSW DataStation (DataApex, Praha, Czech Republic). After each separation run, the column was regenerated with a 0.5 M NaOH solution, washed with water, activated with the 1 M NaCl in buffer A and again equilibrated with the equilibration buffer.

2.4. Size-exclusion chromatography of FTase

For size-exclusion chromatography, an Agilent 1100 system (Agilent, Vernon Hills, CA, USA) equipped with a BioSep-SEC-S3000 column (300 mm × 4.6 mm i.d., Phenomenex, Torrance, CA, USA) was used. The mobile phase was a 0.1 M phosphate buffer with the pH 6.8 at the flow rate 0.35 ml min^{-1}. Detection was performed by a diode-array detector at the wavelength of 280 nm. The molecular weight of FTase was determined using calibration curve constructed by means of the following molecular weight standards: blue dextran, 2,000,000; thyroglobulin, 669,000; apoferritin, 443,000; β-amylase, 200,000; bovine γ-globulin, 158,000; alcohol dehydrogenase, 150,000; bovine serum albumin, 66,000; chicken ovalbumin, 44,000; carbonic anhydrase, 29,000; equine myoglobin, 17,000.

2.5. FTase activity assay

In order to determine the FTase activity, 40 μl of enzyme solution was added into 800 μl of 100 g l^{-1} sucrose solution prepared in a 0.1 M phosphate–citrate buffer with pH 5.5. The enzyme reaction was carried out at 45 °C. The reaction was stopped in a predefined time by the addition of 75 μl of 1 M NaOH. The amount of glucose produced in the reaction was determined by a glucose assay with the Bio-La-Test GOD. One unit of transfructosylation activity was defined as the amount of enzyme producing 1 μmol of glucose during 1 min. It was verified that, at the sucrose conversion below 5%, the amounts of glucose and 1-kestose produced were equal.

2.6. Protein assay

The protein content was assayed by Lowry's method (Lowry et al., 1951) with bovine serum albumin as a standard.

2.7. HPLC analysis of FOS

The analyses of FOS were performed by HPLC (Knauer, Berlin, Germany) as described previously (Antošová et al., 2002). The column Watrex Polymer IEC Pb form, 250 mm × 8 mm i.d. (Watrex, Praha, Czech Republic) was maintained at 80 °C and double-distilled water was used as the mobile phase at a flow rate of 0.8 ml min^{-1}. A 10 μl sample was injected by the autosampler Gilson 234 (Gilson, Villiers le Bel, France) and the detection was performed by a differential flow refractometer (Knauer, Germany) at 25 °C.

2.8. Substrate specificity of FTase

The substrate was here Actilight 950P in a form of water solution with the concentration of 277.8 g l^{-1}. Actilight 950P is a mixture of saccharides containing 35 ± 3% of 1-kestose, 50 ± 3% of nystose, 10 ± 3% of 1^F-fructofuranosylnystose and in total 5% of sucrose, fructose and glucose. One millilitre of enzyme solution was added into 9 ml of the substrate solution so the initial substrate concentration was 250 g l^{-1}. The reaction was carried out in a stirred reactor for 22 h at 28 °C and 560 min^{-1}. Samples of the reaction mixture were withdrawn from the reactor in predetermined time intervals. The reaction in the samples was stopped by heating in boiling water and the concentrations of saccharides were determined by HPLC.