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Effect of deglycosylation on the properties of thermophilic invertase purified from the yeast *Candida guilliermondii* MpIIIa

Miguel Plascencia-Espinosa[a,b], Alejandro Santiago-Hernández[a], Patricia Pavón-Orozco[a], Vanessa Vallejo-Becerra[a], Sergio Trejo-Estrada[b], Alejandro Sosa-Peinado[c], Claudia G. Benitez-Cardoza[d], María Eugenia Hidalgo-Lara[a,*]

[a] *Departamento de Biotecnología y Bioingeniería, CINVESTAV-IPN, Av. Instituto Politécnico Nacional No. 2508, D.F. CP 07360, México*
[b] *Centro de Investigación en Biotecnología Aplicada-IPN, Km 1.5 Carretera Estatal Tecuexcomac-Tepetitla, 90700, Tepetitla, Tlaxcala, México*
[c] *Depto. de Bioquímica, Facultad de Medicina, UNAM, Ap. Postal 70-159, Ciudad Universitaria 04510, México, D.F., Mexico*
[d] *Laboratorio de Investigación Bioquímica, ENMH-Instituto Politécnico Nacional, Guillermo Massieu Helguera No. 239 La Escalera Ticoman, México D.F. 07320, México*

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ABSTRACT

Invertase from *Candida guilliermondii* MpIIIa was purified and biochemically characterized. The purified enzyme (INV3a-N) is a glycoprotein with a carbohydrate composition comprising nearly 74% of its total molecular weight (MW) and specific activity of 82,027 U/mg of protein. The enzyme displayed optimal activity at pH 5.0 and 65 °C. The *K*m and V_{max} values for INV3a-N were 0.104 mM and 10.9 μmol/min/mg of protein, respectively, using sucrose as the substrate. The enzyme retained 50% and 20% of its maximal activity after 168 h and 30 days, respectively, at 50 °C. INV3a-N was fully active at sucrose concentrations of 400 mM and the activity of the enzyme dropped slowly at higher substrate concentration. Interestingly, the deglycosylated form of INV3a-N (INV3a-D) displayed 76–92% lower thermostability than that of INV3a-N at all temperatures assayed (50–70 °C), and was inhibited at sucrose concentrations of 200 mM. Findings here indicate glycosylation plays an important role, not only in the thermostability of INV3a-N, but also in the inhibition of the enzyme by sucrose. Since the enzyme is active at high sucrose concentrations, INV3a-N may be considered a suitable candidate for numerous industrial applications involving substrates with high sugar content or for improvement of ethanol production from cane molasses.
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1. Introduction

Invertase (β-fructofuranosidase EC: 3.2.1.26) is an enzyme that catalyzes the hydrolysis of sucrose into glucose and fructose, and it represents one of the most studied enzymes for the purpose of describing and understanding many of the kinetic principles of enzymes [1,2]. The enzyme has been found in some animals, higher plants, filamentous fungi, yeast and bacteria [3–5]. Invertase has a wide range of applications in food and chemical industries and it has been used as an additive for the manufacture of inverted sugar [3–5]. That enzyme is mostly used for the production of jams, candies, soft-centered chocolates, cookies, alcohol and certain organic acids [3–6].

Yeast invertases are among the most important hydrolytic enzymes and have been extensively studied. The majority of research concerning invertase has focused on invertase isolated from *Saccharomyces cerevisiae* [4,7]. The invertase produced by *S. cerevisiae* displays an anticompetitive inhibition by applying a mechanism that involves the reversible binding of substrate on the enzyme molecule at high sucrose concentrations [2,8]. The enzyme became inhibited at a sucrose concentration of 5% [2] (equivalent to 146 mM sucrose), representing a limiting factor in terms of its use in substrates with high sugar concentration such as jams, candies and confectionary products [4,9]. For this reason, it still remains important to search for new biocatalysts, with invertase activity at a sucrose concentration of 150 mM sucrose or higher. Beside this, it would be of great interest to identify new invertases with biochemical properties suitable for industrial applications, e.g., invertases active and stable in a broad range of pH and temperature values.

Invertases from non-conventional yeast, belonging to genera other than *Saccharomyces* or *Schizosaccharomyces* have been described, for example those from the yeasts *Candida utilis* [1,3], *Pichia anomala* [10], *Xanthophyllomyces dendrorhous* [11], *Rhodotorula glutinis* [9] and *Schwanniomyces occidentalis* [12].

* Corresponding author. Tel.: +52 55 57473800x4354; fax: +52 55 57473313.
E-mail address: ehidalgo@cinvestav.mx (M.E. Hidalgo-Lara).

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Candida (*Pichia*) *guilliermondii* has been described as osmotolerant yeast able to produce invertase [13] and inulinase [14] activities. However, there are no biochemical studies describing the catalytical properties of the invertase produced by *C. guilliermondii* strain MpIIIa. In this study, we report the purification and biochemical characterization of INV3-N, the thermostable invertase produced by *C. guilliermondii* MpIIIa. Beside this, we describe the effect of deglycosylation on the biochemical and kinetic properties of the enzyme.

2. Materials and methods

2.1. Chemicals

Culture media were obtained from JT Baker (USA). Sucrose, maltose, raffinose, inulin, lactose, cellobiose, sorbitol and mannitol were purchased from Sigma–Aldrich (USA). Chromatographic media and chemicals used in ion exchange and gel filtration chromatography were purchased from BioRad (USA) and General Electric Company (USA), respectively. The chemicals, including the protein molecular weight markers and the Silver Stain Plus Kit used in the SDS-PAGE analysis were purchased from BioRad. All other chemicals used were analytical grade and purchased from Sigma–Aldrich (USA) and JT Baker (USA), unless otherwise specified.

2.2. Microorganism and culture conditions

C. guilliermondii MpIIIa was obtained from the CDBB Culture Collection, CINVESTAV, México (accession number CDBB-L-1254). The strain MpIIIa was isolated from sugarcane syrup by Dr. Sergio R. Trejo-Estrada research group and taxonomically identified as *Candida guilliermondii* by Accugenix, Inc. (USA). *C. guilliermondii* MpIIIa was maintained in YEP-agar medium containing (w/v): 1% yeast extract, 2% peptone, 2% agar and 20% sucrose. For conservation, cells were suspended in glycerol (30% v/v) and stored at −70 °C.

2.3. Enzyme assay

Invertase activity was determined by measuring the release of reducing sugars, using 3,5-dinitrosalicylic acid as described by Miller [15]. The standard assay was performed in 10% (w/v) (292.14 mM) sucrose solution prepared in 50 mM sodium acetate buffer, pH 5.0 at 65 °C. An equimolar mixture of fructose and glucose was used as a standard. The results presented are expressed as mean±standard deviation of two independent experiments conducted in triplicate. A single unit of invertase was defined as the amount of enzyme required to hydrolyze 1 μmol of sucrose per minute, under the assay conditions specified above.

2.4. SDS-polyacrylamide gel electrophoresis

Protein analyses were carried out by 10% SDS-PAGE, according to the method of Laemmli [16]. Proteins in the gel were visualized by Coomassie Brilliant blue R-250 or silver staining, following the instructions of the manufacturer. Protein molecular weight (MW) was estimated with reference to broad range molecular weight protein standards. Gels were recorded and analyzed using a gel documentation system (DigiDoc-It Imaging System, UVP). Protein concentration was determined as described by Bradford [17], using bovine serum albumin (Pierce) as standard. Glycoproteins were detected with periodic acid-Schiff staining following the method described by Segrest and Jackson [18].

2.5. Purification of the enzyme

For enzyme production, 2.5 L flasks containing 400 mL of YM broth [19], with (100 g/L) sucrose as carbon source were inoculated to reach a final cell concentration of 1×10^6 colony-forming units/mL and incubated at 30 °C for 72 h and 200 rpm. Cells were harvested by centrifugation (8000 rpm at 4 °C for 5 min). The culture supernatant was tested for invertase activity and the cells were suspended in 100 mL of lysis buffer (10 mM $MgCl_2$, 1 mM EDTA, 0.3 M $(NH_4)_2SO_4$, 5% (v/v) glycerol, 0.2 M Tris–HCl, pH 7.9) supplemented with, 1 mM dithiothreitol, 0.2% (w/v) sodium azide and 1 mM phenylmethylsulfonyl fluoride. Cells were broken using a French Press (Aminco, Maryland, USA) at 500 PSIG pressure, and the cell lysate was centrifuged at 8000 rpm, 4 °C for 15 min. Subsequently, supernatant was ultrafiltered using a regenerated cellulose membrane, with a cut off weight of 30,000 Da (AmiconYM-30, Millipore, USA) and concentrated to a final volume of 10 mL. This enzymatic preparation, named crude extract was used to assess invertase activity.

For enzyme purification, crude extract from *C. guilliermondii* MpIIIa was loaded into a UNOsphere Q column previously equilibrated with buffer A (50 mM KCl, 20 mM Tris–HCl buffer, pH 8.0), using an AKTA system (General Electric Company, USA). Adsorbed proteins were eluted with a linear gradient of KCl (50–250 mM) and a constant flow rate of 0.5 mL min^{-1} and 1.0 mL fractions were collected. Fractions manifesting invertase activity were pooled, dialyzed against 50 mM acetate buffer pH 5.0, and analyzed by 10% SDS-PAGE. Fractions of purified invertase, named INV3a-N were stored at 4 °C for further study.

2.6. Preparation of the deglycosylated form of the enzyme

To obtain an active deglycosylated form of the enzyme, INV3a-N was treated under non-denaturing conditions with Endoglycosydase H (EndoH) (Roche Diagnostics GmbH), following the instructions of the manufacturer. Briefly, the reaction mixture, containing 1.45 mg of purified INV3a-N and 500 mU of EndoH in acetate buffer 50 mM, pH 5.0 was incubated at 37 °C overnight. Then, aliquot samples of EndoH-treated INV3a-N, named INV3a-D, were analyzed by 10% SDS-PAGE and stored at 4 °C for further study.

2.7. Estimation of molecular weight and carbohydrate content

The MW of INV3a-N, under native and denaturing conditions was estimated using gel filtration chromatography in a prepacked HiPrep 16/60 Sephacryl S-300 (General Electric Company, USA) column adapted to an AKTA system, at a flow rate of 0.5 mL min^{-1}. Gel filtration chromatography (GFC) was carried out in 50 mM sodium phosphate buffer, pH 6.8 (native conditions) or in 6 M urea prepared in 50 mM sodium phosphate buffer, pH 6.8 (denaturing conditions). The HiPrep 16/60 S-300 column was calibrated using the HMW Calibration Kit (General Electric Company, USA) and the void volume determined by the elution of Blue Dextran 2000 (supplementary data). To estimate the MW of the deglycosylated subunit, purified INV3a-N was incubated in a boiling water bath for 15 min, treated with EndoH and then analyzed with 10% SDS-PAGE. The carbohydrate content of the enzyme was determined by the difference between the MW of purified INV3a-N, estimated by GFC under denaturing conditions, and the MW of the deglycosylated subunit estimated by 10% SDS-PAGE. The carbohydrate content of INV3a-N was assessed by Anthrone assay, according to the method described by Leyva et al. [20] using mannose as standard. Data obtained were expressed as mean±standard deviation of three independent experiments conducted in triplicate.

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