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

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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.

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].

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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.

Table 1
Purification of *C. guilliermondii* MpIIIa invertase.

Step	Protein (mg)	Total activity (units)	Specific-activity (units/mg)	Purification (fold)	Recovery yield (%)
Crude lysate	729	200,585	275	1	100
Ultrafiltration (30 kDa)	516	103,875	2013	7	52
Anion exchange chromatography	0.34	26,537	78,050	283	13
Ultrafiltration (50 kDa)	0.26	21,327	82,027	298	11

2.8. Dynamic light scattering methods

Dynamic light scattering (DLS) analysis was performed at 25 °C using a Zetasizer UV for liquid samples (Malvern Instruments, UK). Samples of INV3a-N and INV3a-D were analyzed at protein concentration of 0.7 mg/mL in Tris–HCl 50 mM, pH 7.5, using a 300 μL quartz cuvette.

2.9. Effect of pH on enzyme activity and stability

The effect of pH on the enzymatic activity of INV3a-N and INV3a-D was determined at different pH values, ranging from 3.5 to 7.0, in 10% (w/v) sucrose solution, prepared in 50 mM citrate phosphate buffer. In order to evaluate the effect of pH on the stability of INV3a-N and INV3a-D, samples of the purified enzymes were incubated in 50 mM citrate–phosphate buffer at different pH values, ranging from 3.0 to 7.0, at 25 °C for 4 h. Subsequently, the remaining invertase activity was measured under standard conditions.

2.10. Effect of temperature on enzyme activity and stability

The effect of temperature on the enzymatic activity of INV3a-N and INV3a-D was estimated by conducting the assay at different temperatures ranging from 35 to 80 °C, in 10% (w/v) sucrose solution prepared in 50 mM sodium acetate buffer, pH 5.0. For determination of thermostability, purified INV3a-N and INV3a-D were incubated at different temperatures (from 50 to 70 °C) in 50 mM acetate buffer, pH 5.0. To determine the half-life of the enzyme preparations, aliquot samples were withdrawn at different time intervals and the residual enzymatic activity was measured under standard conditions.

2.11. Substrate specificity and kinetic parameters

To study the substrate specificity of INV3a-N, the invertase activity was determined under optimal assay conditions using (w/v): 10% sucrose, 10% raffinose, 10% maltose, 10% lactose, 10% mannitol, 10% sorbitol, 0.2% cellobiose or 0.2% inulin as the substrate. For determination of the kinetic parameters K_m and V_{max}, the initial reaction rates for INV3a-N and INV3a-D were studied under the optimal conditions for the activity of each enzyme, using sucrose as substrate, at a concentration ranging from 0.1 to 2 M. The K_m and V_{max} values were determined graphically by applying the linear method devised by Lineweaver-Burk [21].

2.12. Effects of metal ions on enzyme activity

To evaluate the effect of several metal ions on the enzymatic activity of the INV3a-N and INV3a-D invertases, samples of the purified enzymes were diluted to an adequate concentration and the invertase activity was determined in 10% (w/v) sucrose solution prepared in 50 mM sodium acetate buffer, pH 5.0 containing: $NiCl_2$, $MgCl_2$, $CuSO_4$, $ZnSO_4$, $FeSO_4$, $MnCl_2$, $CoCl_2$, LiCl, $HgCl_2$ or $CaCl_2$, at a final concentration of 1 and 10 mM each. Before determining enzyme activities, samples were incubated at 65 °C for 5 min. After adding the metal ion, aliquot samples were withdrawn and used as a blank.

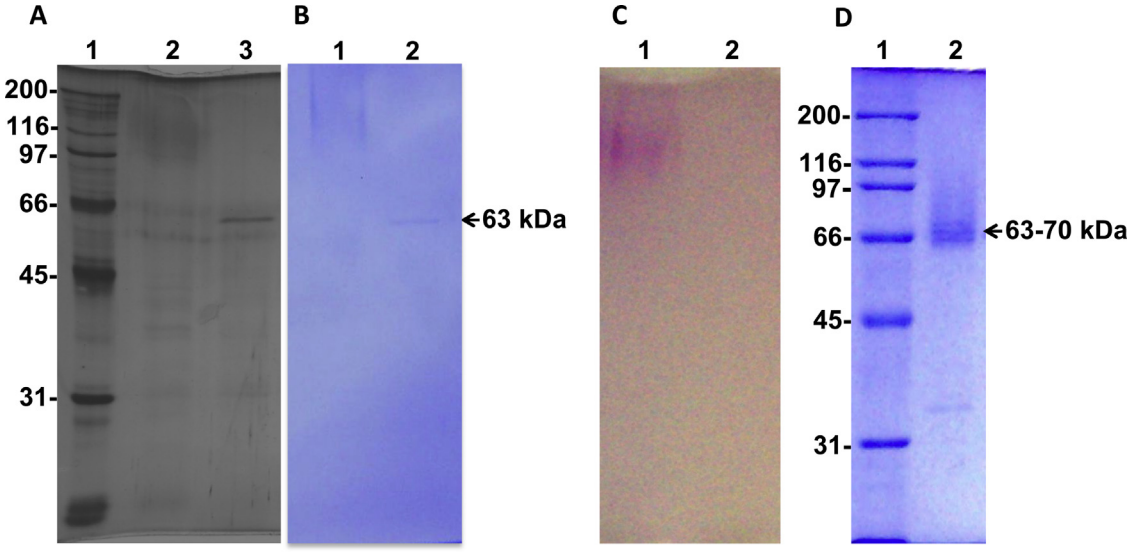

Fig. 1. 10% SDS-PAGE analysis of INV3a-N and INV3a-D. (A) INV3a-N deglycosylated under denaturing conditions. Lane 1: MW markers; lane 2: INV3a-N; lane 3: INV3a-N deglycosylated under denaturing conditions. (B and C) INV3a-N deglycosylated under denaturing conditions. Lane 1: INV3a-N; lane 2: INV3a-N deglycosylated under denaturing conditions. (D) INV3a-D, obtained by the EndoH treatment of INV3a-N under non-denaturing conditions. Lane 1: MW markers; lane 2: INV3a-D. (A) Silver-, (B and D) Coomassie blue and (C) Schiff-stained gels. For deglycosylation under denaturing conditions, INV3a-N was incubated in a boiling water bath for 15 min and then treated with EndoH. For deglycosylation under non-denaturing, INV3a-N was treated with EndoH without incubation in a boiling water bath. An amount of 1.5 μg of protein was loaded by line. All tests were performed in triplicate.

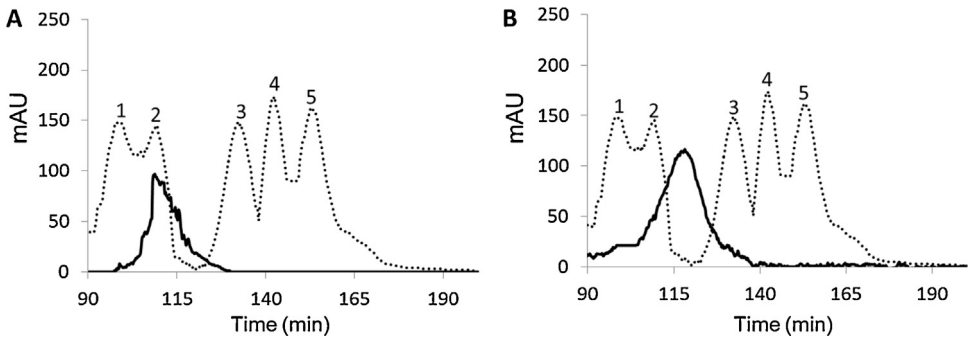

Fig. 2. Gel filtration chromatography of INV3a-N under native (A) and denaturing (6 M Urea) (B) conditions, for MW estimation. MW standards: 1, Thyroglobulin (669 kDa); 2, Ferritin (440 kDa); 3, Aldolase (158 kDa); 4, Conalbumin (75 kDa); 5, Ovalbumin (43 kDa). UV Absorbance at 280 nm is given in milliabsorbance units (mAU). All tests were performed in duplicate.

2.13. Circular dichroism (CD)

Far-UV CD spectra were obtained using a Jasco J-715 Spectropolarimeter (Jasco Inc., Easton, MD). Scans were taken between 200 and 260 nm, using a 0.2 cm path-length cuvette, at 30 °C. Temperature was regulated using a Peltier system. All samples were prepared at a protein concentration of 0.7 mg/mL in 50 mM Tris–HCl buffer, pH 7.5. CD data are reported as mean residue ellipticity $[\theta]_{MRW}$. Each spectrum presented is an average of five measurements.

3. Results

3.1. Purification of INV3a-N and deglycosilation of the enzyme

The INV3a-N invertase from the yeast *C. guilliermondii* MpIIIa was purified from a microbial culture at the end of the exponential phase of growth, when the highest invertase production was observed (data not shown). The enzyme was purified from the soluble fraction of the cell lysate, by ultrafiltration and ion exchange chromatography. A summary of the results obtained in the purification of INV3a-N is shown in Table 1. A 10% SDS-PAGE analysis of purified INV3a-N revealed a smeared band of high MW (Fig. 1A and B) that gave a positive periodic acid-Schiff reaction (PAS) (Fig. 1C). To assess the glycosylated nature of the enzyme, purified INV3a-N was denatured by boiling and treated with EndoH. SDS-PAGE analysis of the resulting deglycosylated subunit showed a single band with an estimated MW of 63 kDa (Fig. 1A,B), which was negative for PAS staining (Fig. 1C). For the purpose of biochemical studies, the purified INV3a-N was treated with EndoH under non-denaturing conditions, and this active deglycosylated form of the enzyme was named INV3a-D. SDS-PAGE analysis of purified INV3a-D showed several bands with MW ranging from 63 to 70 kDa (Fig. 1D).

3.2. Estimation of molecular weight and oligomeric state

To determine the oligomeric state of the enzyme, the purified INV3a-N was analyzed by gel filtration, under native and denaturing conditions (6 M Urea). The native INV3a-N presented an estimated MW of 463 kDa (Fig. 2A); however, when the enzyme was gel filtered under denaturing conditions (in the presence of 6 M urea) a single peak with an estimated MW of 243 kDa was detected (Fig. 2B).

In order to determine the tendency of native (INV3a-N) and deglycosylated (INV3a-D) enzymes to form a dimeric association, dynamic light scattering was applied to both samples. The analysis of DLS indicated that both samples INV3a-N (98.5%) and INV3a-D (97.3%) were monodisperse, and the apparent molecular weight for INV3a-N was 504.2 ± 109.1 kDa, and for INV3a-D was 137.8 ± 81.4 kDa (supplementary data).

3.3. Estimation of carbohydrate content

Gel filtration chromatography and 10% SDS-PAGE analysis of INV3a-N and INV3a-D allowed us to estimate that INV3a-N presents a carbohydrate content of 74% of its total mass. A glycosylation component of 76 ± 2.3% of the total mass of INV3a-N was confirmed by the Anthrone assay.

3.4. Biochemical characterization

To study the biochemical properties of INV3a-N and INV3a-D purified preparations of both enzymes were used. To know the correct time for assay and protein concentration, enzyme progress curve against varying time, substrate and protein concentration were done (supplementary data).

3.5. Effect of pH on enzyme activity and stability

INV3a-N displayed optimal activity at pH 5.0, whereas in the case of INV3a-D, it occurred at pH 5.5; although, the enzymes were active over a wide range of pH (3.6–6.4) values (Fig. 3A). After 4 h incubation at 25 °C, INV3a-N manifested high stability at a pH range between pH 4 and 6, retaining nearly 60% of its original activity; whereas INV3a-D presented more stability at a pH range between pH 5 and 7, retaining nearly 70% of its original activity (Fig. 3B).

3.6. Effect of temperature on enzyme activity and stability

INV3a-N displayed optimal activity at 65 °C, whereas in the case of INV3a-D it occurred at 60 °C (Fig. 3C). INV3a-N and INV3a-D were active over a wide range of temperature values, (35–80 °C) and (35–70 °C), respectively (Fig. 3C). The thermal stability of INV3a-N and INV3a-D was evaluated at different temperatures (50–70 °C) at their optimal pH and the results obtained are summarized in Table 2. Notably, the half-life for INV3a-D decreases 76–92% when

Table 2
Thermostabilty of INV3a-N and INV3a-D.

Temperature (°C)	Half-life ($t_{½}$)	
	INV3a-N	INV3a-D
70	5 min	1.2 min
65	7 min	1.5 min
60	60 min	9 min
55	24 h	1.8 h
50	168 h	16 h

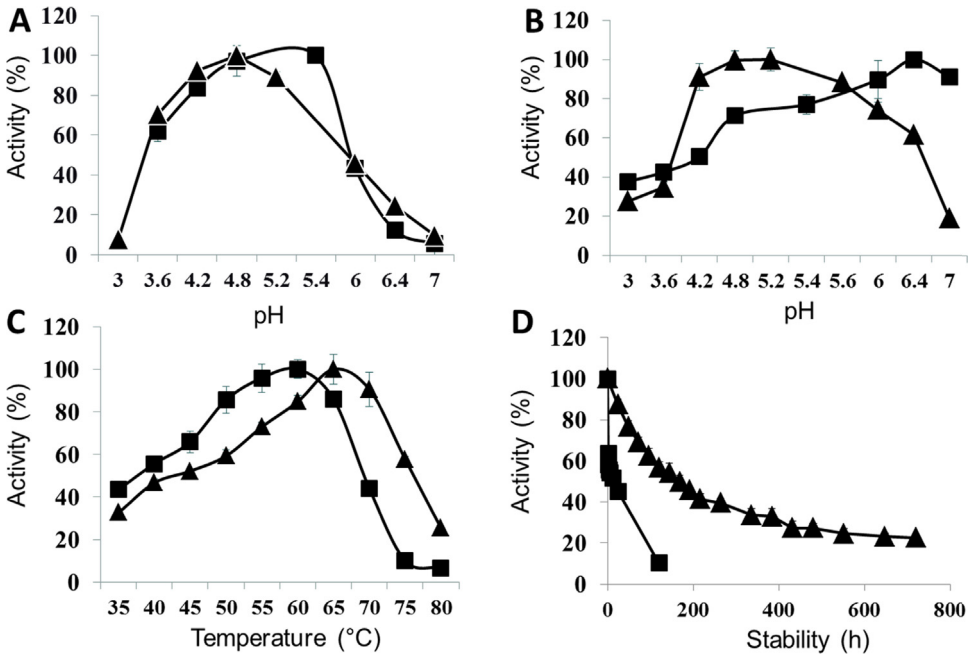

Fig. 3. Effect of pH and temperature on the invertase activity and stability of INV3a-N (▲) and INV3a-D (■). (A) Effect of pH on enzyme activity, (B) effect of pH on enzyme stability, (C) effect of temperature on enzyme activity and (D) effect of temperature (50 °C) on enzyme stability. All tests were performed in triplicate, and error bars indicate standard deviations.

compared to that observed for INV3a-N at all assayed temperatures (Table 2). Furthermore, INV3a-N retained up to 20% of its original activity after 30 days of incubation at 50 °C, whereasINV3a-D was fully inactivated after 7 days (168 h) at the same temperature (Fig. 3D).

3.7. Substrate specificity and kinetic parameters

The purified INV3a-N was fully active on sucrose with a specific activity of 82,027 U/mg of protein and was able to hydrolyze raffinose with a specific activity of 28,708 U/mg of protein; however, the enzyme was not active on maltose, cellobiose, lactose, mannitol, sorbitol and inulin. The K_m and V_{max} values were calculated for both INV3a-N and INV3a-D with sucrose as the substrate. The K_m values for INV3a-N and INV3a-D were 0.121 mM and 0.24 mM, respectively; whereas the V_{max} values for INV3a-N and INV3a-D were 10.9 and 4.2 μmol/min/mg of protein, respectively.

3.8. Inhibition by substrate

The effect of substrate concentration on the enzymatic activity for both INV3a-N and INV3a-D was studied, using sucrose concentrations ranging from 0.1 to 2 M. INV3a-N manifested inhibition at sucrose concentrations of 400 mM and higher; whereas, in the case of INV3a-D inhibition was observed at sucrose concentrations of 200 mM and higher (Fig. 4). INV3a-N and INV3a-D presented nearly 50% and 10% of their original activity respectively, at the highest substrate concentration assayed (2 M sucrose) (Fig. 4).

3.9. Effect of metal ions on enzyme activity

The effect of several metal ions (Ni^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+}, Co^{2+}, Li^{1+}, Hg^{2+} or Ca^{2+}) on the activity of INV3a-N and INV3a-D was determined at a final concentration of 1 and 10 mM each (Fig. 5A and B). The activity of both INV3a-N and INV3a-D increased 110% and 40% in the presence of Mn^{2+} at 1 mM and 10 mM, respectively (Fig. 5A and B). The ion Co^{2+} increased 60% the activity of INV3a-N at a final concentration of 1 mM; whereas an inhibitory effect on the activity of this enzyme was observed at higher concentration (10 mM) (Fig. 5A and B). The metal ions Ni^{2+}, Li^{+}, Mg^{2+} and Fe^{2+} at 10 mM showed variable effect on the enzymatic activity of INV3a-N and INV3a-D (Fig. 5B); whereas no apparent effect was observed at a final concentration of 1 mM (Fig. 5A). In the presence of Cu^{2+}, Zn^{2+} and Hg^{2+}, the activity of INV3a-N and INV3a-D was partially (1 mM) or completely (10 mM) inhibited; whereas Ca^{2+} had no apparent effect on the enzymatic activity of INV3a-N and INV3a-D at the concentrations tested (Fig. 5A and B).

3.10. Changes on secondary structure

The secondary structure ofINV3a-N and INV3a-D was assessed by CD spectroscopy. Far-UV CD spectra of both samples are shown in Fig. 6. It is observed that INV3a-N presents a negative peak centered around 210 nm; meanwhile INV3a-D displays a CD spectrum

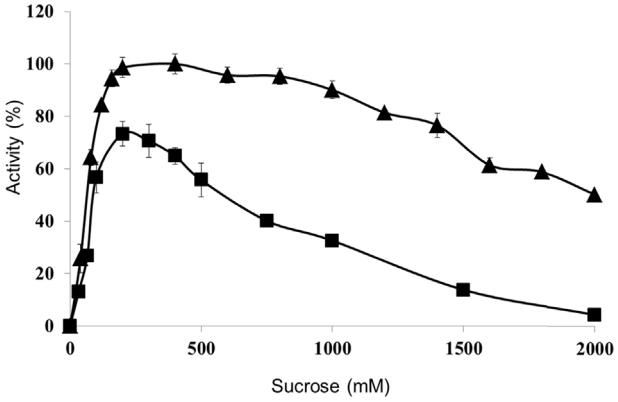

Fig. 4. Effect of sucrose concentration on the invertase activity of INV3a-N (▲) and INV3a-D (■). Purified INV3a-N and INV3a-D were incubated under the optimal conditions (pH and temperature) for the activity of each enzyme, using sucrose as substrate at a concentration ranging from 0.1 to 2 M, prepared in 50 mM sodium acetate buffer. All tests were performed in triplicate, and error bars indicate standard deviations.

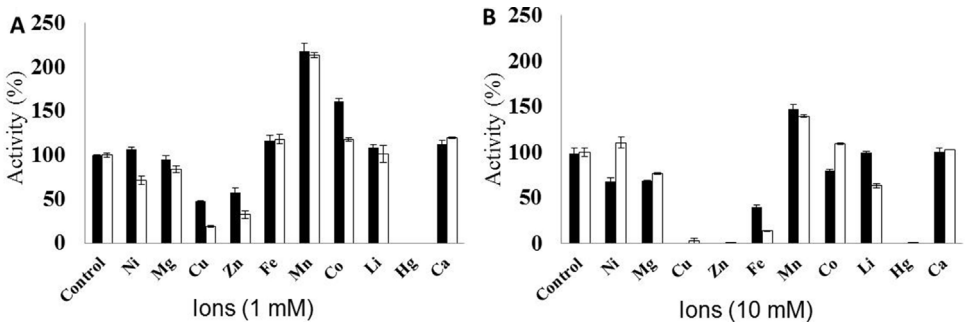

Fig. 5. Effect of metal ions on the invertase activity of INV3a-N (■) and INV3a-D (□) at two different concentrations: 1 mM (A) and 10 mM (B). Purified enzymes were incubated in 10% (w/v) sucrose solution prepared in 50 mM sodium acetate buffer, pH 5.0 containing (1 and10 mM each): Ni^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+}, Co^{2+}, Li^{1+}, Hg^{2+} or Ca^{2+}, at 65 °C for 5 min. All tests were performed in triplicate, and error bars indicate standard deviations.

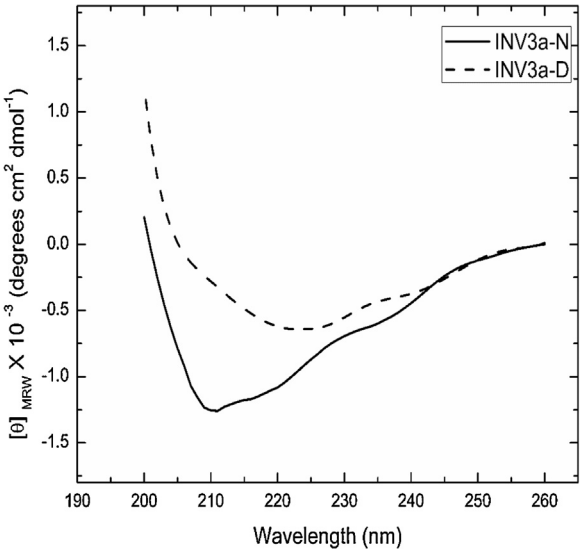

Fig. 6. Far-UV CD spectra between 200 and 260 nm were obtained using a 0.2 cm path-length cuvette. Spectra of INV3a-N and INV3a-D (0.7 mg/ml of protein concentration each) were measured at 30 °C in 50 mM Tris–HCl buffer, pH 7.5, at 30 °C. Each spectrum presented is an average of 5 measurements. The CD data were plotted as mean residue ellipticity $[\theta]_{MRW}$ versus wavelength with smoothing.

225 nm. These observed spectral differences are clearly a reflection of important changes in secondary structure of the protein. According to the analysis by means of the CONTINLL algorithm [22,23], INV3a-N might be characterized by 47% of beta-sheet structure, 23% of turn and 30% of random coil, whereas INV3a-D might gain a considerable percentage of random coil (up to 75%) while keeping about 25% of beta-sheet structure.

4. Discussion

The INV3a-N invertase produced by the yeast *C. guilliermondii* MpIIIa was successfully purified and biochemically characterized. The enzyme was purified from the soluble fraction of the *C. guilliermondii* MpIIIa cell lysate, and it seems to be associated with the whole cell pellet as non invertase activity was detected in the culture supernatant from the yeast. So far, most of yeast invertases have been found associated to the whole cell pellet [10,24–26].

It has been reported that the MW of active yeast invertases is variable, ranging from 112 kDa for *S. cerevisiae* [24] to 1070 kDa for *Schizosaccharomyces pombe* [25]. Beside this, yeast invertases have been found organized in nature as monomers [1,28], dimers [9], tetramers [26], hexamers [25] or octamers [7,27]; besides, the non-glycosylated monomeric form of yeast invertases has an estimated MW ranging from 60 to 65 kDa [28]. To investigate the olimeric state of the invertase, the enzyme was fractionated by GFC under native and denatured conditions (6 M urea). The native INV3a-N presented an estimated MW of 463 kDa (Fig. 2A); however, when the enzyme was gel filtered under denaturing conditions (6 M Urea) a single peak with an estimated MW of 243 kDa was detected (Fig. 2B). Thus, these data suggest the native invertase from the yeast *C. guilliermondii* MpIIIa is organized in nature as a homodimer of two glycosylated subunits with an estimated MW of 243 kDa each. Likewise, data obtained by DLS analysis indicate that the dimeric form is the most preferred state of both the native and deglycosylated forms of the enzyme. It is worth mentioning that the enzyme treated with 6 M Urea did not show enzymatic activity, even after the removal of urea by extensive dialysis; thus suggesting INV3a-N is active as a dimer.

High glycosylation content is a common characteristic of a number of previously studied yeast invertases [1,25,26]. After treatment of the native invertase from the yeast *C. guilliermondii* MpIIIa with EndoH a single band of 63 kDa was observed by 10% SDS-PAGE; thus it is likely that INV3a-N is a highly glycosylated enzyme with a carbohydrate content of 74% of its total mass. The high glycosylation nature of INV3a-N was confirmed by the Anthrone assay, that yield a carbohydrate content of 76% of the total mass of the protein. It is worth pointing out that the carbohydrate content of INV3a-N (~74–76%) is higher than that reported for other yeast invertases, such as *S. pombe* (67%) [24], *Kluyveromyces fragilis* (66%) [29], *S. cerevisiae* (50%) [5,30], and *C. utilis* (47%) [31]. Thus, to investigate the role that glycosylation plays in the biochemical properties of INV3a-N, the catalytical properties of both INV3a-N and its deglycosylated form were compared.

INV3a-N and INV3a-D manifested optimal activity at pH 5.0 and 5.5, respectively; these optimal pH values are similar to those reported for other yeast invertases, which range from 3.5 to 5.5 [5,11]. INV3a-N and INV3a-D displayed optimal activity at 65 °C y 60 °C, respectively. The optimal temperature for INV3a-N (65 °C) is one of the highest optimal temperature values reported for yeast invertases, and comparable to those reported for invertases from *X. dendrorhous* (65 °C) [11] and *C. utilis* (70 °C) [1]. Concurring with our findings, it has been reported that deglycosylation leads to a slight shift of pH and temperature for optimal enzyme activity, probably due to conformation and/or dynamic properties of a glycosylated protein are likely to differ from its unglycosylated counterpart [32].

Likewise, it has been pointed out the pH stability of an enzyme is an important characteristic for its application in industry [33], and that most of the invertases characterized until now are not stable in neutral or alkaline pH [9]. INV3a-N showed high stability at pH 6.5, retaining 60% of its maximum activity; whereas INV3a-D displayed up to 60% of its maximum activity at pH values ranging from 5 to 7, after 4 h of incubation at the different tested pH values (at the different pH values that were tested). Notably, most of the reported assays for determining the pH stability of yeast invertases

were implemented for short incubation times (15–60 min) [5]. According to Moreno et al. [25], the invertase from *S. cerevisiae* is stable at pH values ranging from 3 to 7 for at least 2 h. The invertase from *R. glutinis* is highly stable at acidic pH values (30 min) ranging from 2 to 5.5, but completely loses its activity at pH 6.5 and higher [9].

Thermostability is one of the most relevant properties of industrial enzymes and is important both for basic research and industrial points of view. Thermostability studies indicated that INV3a-N has low thermal stability at temperatures exceeding 50 °C; however, the enzyme has a half-life of 168 h (7 days) at 50 °C and retains up to 20% of its original activity after 30 days at this temperature. Thus it reveals itself as one of the best examples of thermal stability when compared to any of the yeast invertases reported previously [5,9,10]. Also, it has been reported that glycosylation increases the resistance of yeast invertases to thermal denaturation [17,31]. Concurring with this observation, INV3a-D displayed 76–92% lower thermostability than that observed for INV3a-N, at all temperatures assayed; thus indicating that glycosylation plays a crucial role in the thermostability of INV3a-N. Even though, the thermostability of INV3a-N was drastically affected by deglycosylation, the INV3a-D invertase displayed a half-life of 16 h at 50 °C. These findings showed INV3a-N can be considered as a robust and thermostable enzyme, as even the deglycosylated form of the enzyme had similar or higher thermostability than some native yeast invertases reported previously [5,9,10]. INV3a-N showed the highest specific activity (82,027 U/mg), using sucrose as the substrate, reported so far for a yeast invertase, which is 15-fold higher than the specific activity (5200 U/mg) reported for the yeast invertase from *X. dendrorhous* [11] and 1.6-fold higher than the specific activity (51,833 U/mg) reported for the bacteria *Thermotoga neapolitana* (DSM 4359) [34].

The purified INV3a-N was able to hydrolyze sucrose and, into a lesser extent, raffinose (35% relative to sucrose); however, the enzyme was not active on maltose, cellobiose, lactose, mannitol, sorbitol and inulin. Recently, it was found that invertase SUC2 from *S. cerevisiae* strain JZ1C is the key enzyme responsible for inulin metabolism in the yeast [35]. According to the results obtained in this work, the inulinase activity is not directly associated to the INV3a-N invertase from the yeast *C. guilliermondii* MpIIIa. Most of the yeast invertases reported so far are only active against sucrose and rafinose [1,9–11].

The K_m value for INV3a-N (0.121 mM) is lower than those reported for other yeast invertases [11,12,30], so far; thus indicating INV3a-N represents one of the yeast invertases with the highest affinity to sucrose yet reported. Moreover, the V_{max} value for INV3a-N (10.9 μmol/min/mg of protein) is similar to that reported for an invertase from the fungus *Aspergillus ochreaceus* (10.0 μmol/min/mg of protein) [36] and lower than that reported for an invertase from *R. glutinis* (96 μmol/min/mg of protein) [9].

Notably, the kinetic parameters K_m and V_{max} value for INV3a-N were negatively affected by deglycosylation treatment. The K_m value for INV3a-D (0.24 mM) was twice that determined for INV3a-N (0.121 mM), and comparable to that reported for a fructooligosacharide-producing β-fructofuranosidase (0.29 mM) from the fungus *Aspergillus niger* ATCC 20611 [37]. Likewise, the V_{max} value for INV3a-D (4.2 μmol/min/mg of protein) was 60% lower, when compared to that observed for INV3a-N (10.9 μmol/min/mg of protein). Findings here suggest glycosylation plays an important role concerning both the substrate affinity of INV3a-N when sucrose is the substrate, and in the catalytic activity of INV3a-N. Concurring with these results, Belcarz et al. [1] found the K_m value obtained for the glycosylated form of invertase from *C. utilis* was 33% higher when compared to that observed for the non-glycosylated form of the enzyme, attributing these differences to the carbohydrate content [1].

It has been reported that the invertase from *S. cerevisiae* is inhibited by 5% sucrose (~146 mM sucrose) and displayed only 30% of its highest activity at 2 M of sucrose [2]. In this study, it was observed that INV3a-N and INV3a-D are inhibited at sucrose concentrations of 400 mM and 200 mM or higher respectively, retaining about 50% and 10% of their highest activity at 2 M of sucrose, respectively; thus suggesting that glycosylation also plays an important role in the inhibition of INV3a-N by sucrose as the substrate. Furthermore, the identification of an invertase that is active at 2 M of sucrose is relevant, taking into account that most of the industrial applications for invertases involve substrates with a high sucrose content; even as high as 70% (w/v) sucrose [38], which corresponds to approximately 2 M sucrose.

It has been reported that invertases can be inhibited or activated by the presence of certain metal ions [9]. The invertase activity of INV3a-N and INV3a-D was increased in nearly 110 and 50% in the presence of Mn^{2+} at 1 mM and 10 mM, respectively; while this effect was not observed with Mg^{2+} and Ca^{2+} at any of the concentrations tested. Also, there was an increase of 60% in the enzymatic activity of INV3a-N in the presence of Co^{2+} 1 mM; while an inhibitory effect was observed for this enzyme with Co^{2+} 10 mM. Likewise, an increase of 47% and 91% in the invertase activity of *A. ochraceus* [36] and *A. terreus* [39], respectively, was observed in the presence of Mn^{2+} (1 mM). Additionally, an increase in the invertase activity has been reported in the presence of Mn^{2+}, Fe^{3+} and Al^{3+} [40]. Inhibition by Hg^{2+}, Cu^{2+} and Zn^{2+} was also described for invertases from other sources [37,39]. The ion Hg^{2+} is widely recognized for its reactivity against sulfhydryl groups in proteins, leading to conformational modifications that may promote aggregation and/or precipitation. Alterations in total electric charge of the protein may also occur, inhibiting enzymatic activity [39]. It has been reported that although ion additions clearly affect invertase activity, the results obtained from many previous reports indicate that no clear pattern exists to predict which ion will lead to a dramatic improvement or reduction in invertase activity [41].

Structural evidences obtained by CD suggest that there were some changes in the secondary structure of the enzyme after deglycosylation involving mainly the loss of about the half of the beta-sheet content and all the structure described as turn to gain random coil. Although these differences in secondary structure are not sufficient to cause inactivation of invertase from *C. guilliermondii* MpIIIa; it is plausible that they are responsible of modifying the biochemical properties and thermostability of INV3a-N after deglycosylation, as described above.

5. Conclusion

We report the purification and biochemical characterization of the thermophilic INV3a-N invertase from the yeast *C. guilliermondii* MpIIIa. The purified enzyme, active at a wide range of pH and temperature values, displayed good thermal stability, high affinity to sucrose as the substrate, and was active at high sucrose concentrations. These properties distinguish INV3a-N from other native yeast invertases, qualifying it for application in industrial processes. This study offers an understanding of the biochemical properties of a remarkable invertase, and a basis for further biochemical, molecular and structural studies, as well as for the optimization of industrial processes involving the invertase activity of this enzyme.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.procbio.2014.05.022.

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