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# A novel silver-activated extracellular β-D-fructofuranosidase from *Aspergillus phoenicis*

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

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

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ABSTRACT

High levels of extracellular β-D-fructofuranosidase from *Aspergillus phoenicis* (=*Aspergillus saitoi*) were obtained when the fungus was grown in Khanna medium supplemented with wheat bran as carbon source, at 40 °C, for 72 h. The extracellular enzyme was purified 12.5-fold to electrophoretic homogeneity, by two chromatographic steps, with a recovery of 7.5%. The purified invertase is a homodimeric glycoprotein with 1.64% carbohydrate content, native apparent molecular mass of 155 kDa and identical subunits of 79 kDa. Optima of temperature and pH were 60 °C and 4.5, respectively. The enzyme was stable for up to 1 h at 60 °C. The β-D-fructofuranosidase activity was stimulated by $Ag^+$ and $K^+$, and inhibited by $Hg^{2+}$, $Mn^{2+}$, $Mg^{2+}$ and $Na^+$. The kinetic parameters ($K_{0.5}$ and $V_{max}$), determined without or with $Ag^+$ and using sucrose as substrate, were 59.9 mM, 954.6 U $mg^{-1}$ protein, and 29.2 mM and 1234.0 U $mg^{-1}$ protein, respectively. Only glucose and fructose were obtained as products of sucrose hydrolysis. *A. phoenicis* extracellular invertase is the first silver-activated β-fructofuranosidase described in the literature.



## 1. Introduction

β-D-Fructofuranosidases (EC 3.2.1.26) are enzymes that can be included in the GH-32 (acid invertases), GH-68 (acid invertases) and GH-100 (neutral and alkaline invertases) families of glycosidases according to CAZY database (http://www.cazy.org) [1]. They hydrolyze β1-4 linkages of carbohydrates such as sucrose, producing an equimolar mixture of D-glucose and D-fructose as products. In plants, sucrose is the major product of photosynthesis and plays important roles in sugar transport, development, carbon storage, signal transduction and stress [2]. This carbohydrate can be irreversibly hydrolyzed by several isoforms of invertases, classified as acid, neutral and alkaline according to optimum pH, when there is a high demand for hexoses [3]. The acidic isoforms are cell-wall or vacuolar enzymes, evolutionary related to yeast and bacterial invertases, whereas alkaline and neutral invertases are accumulated in the cytosol [4]. Many other organisms are able to produce invertases, especially microorganisms, as bacteria [5], yeast [6,7] and filamentous fungi [8,9]. Generally, fungal invertases are included in GH-32 family. *Saccharomyces cerevisae* produces two invertase isoforms, a glycosylated form found in the periplasmic space, responsible for the hydrolysis of extracellular sucrose, and a non-glycosylated cytoplasmic form whose functions have not yet been elucidated [10]. Biotechnologically, the enzymatic cleavage of sucrose is an alternative to acid hydrolysis. In addition, fructose is considered a safer sugar from health standpoint, and may be used by diabetic people. In addition, fructose is non-crystallizable and has higher sweetening capacity compared to sucrose. These characteristics are very interesting for biotechnological applications in candies and soft-centered chocolate food industries. Nowadays, invertases with transfructosylation activity are also being used to produce fructooligosaccharides (FOS) at higher concentrations of sucrose. FOS are prebiotic compounds which are not metabolized by humans, although showing positive influence on *Bifidobacterium* growth in the colon and reducing cholesterol, besides other health benefits [11]. In this work, we describe the properties of a novel silver-activated β-D-fructofuranosidase invertase produced by the filamentous fungus *Aspergillus phoenicis*.

## 2. Materials and methods

### 2.1. Microorganism and culture conditions

*A. phoenicis* (=*Aspergillus saitoi*) was isolated from sugar cane bagasse from São Paulo State (Brazil) [12], identified by the André Tosello Foundation, Campinas and maintained on slants of 4% oatmeal (Quaker). Erlenmeyer flasks (125 mL) containing 25 mL of Khanna medium [13] supplemented with different carbon sources (2% monosaccharides and 1% complex sources; w/v) were inoculated with 1 mL spore solution ($10^5$ spores/mL) and incubated at

* Corresponding author. Tel.: +55 16 3602 4682; fax: +55 16 3602 4886.
*E-mail address:* lhguimaraes@ffclrp.usp.br (L.H.S. Guimarães).

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40 °C under orbital agitation (100 rpm), for convenient time periods.

### 2.2. Intra and extracellular enzyme preparations

Cultures were harvested by vacuum filtration and the filtrate, named extracellular crude extract, was saved. The mycelium was disrupted in a porcelain mortar, at 4 °C, with acid-treated sea sand, and the slurry was centrifuged (23,000 × *g*) at 4 °C for 15 min. The supernatant obtained was named intracellular crude extract. Both crude extracts were used to determine the enzymatic activity.

### 2.3. Determination of fructofuranosidase activity

The enzymatic activity was determined using 29 mM sucrose (Mallinckrodt®) as substrate in 100 mM sodium acetate buffer, pH 4.5, at 60 °C, and the reducing sugars released were quantified using dinitrosalicylic acid (DNS) according to Miller (1959) [14]. One activity unit was defined as the enzyme necessary to produce 1 μmol of reducing sugar (glucose) per minute under the experimental conditions. The activity against others substrates, such as inulin from chicory (Sigma®) and raffinose (Difco®) was quantified using the same methodology described above.

### 2.4. Protein and carbohydrate estimation

Protein was quantified according to Lowry et al. [15] using BSA as standard. The protein carbohydrate content was estimated according to Dubois et al. [16] using mannose as standard.

### 2.5. Purification of extracellular fructofuranosidase

The extracellular crude extract was dialyzed overnight against distilled water at 4 °C and loaded into a DEAE-Cellulose column (10.0 cm × 2.0 cm) equilibrated with 10 mM Tris–HCl buffer, pH 7.0. The enzyme was eluted as a single peak using a linear gradient of NaCl (0–1 M). Fractions (3.0 mL) were collected at a flow rate of 1.9 mL/min. Higher activity fractions were pooled, dialyzed overnight against distilled water, lyophilized and loaded into a Sephacryl S-200 column (80.0 cm × 2.0 cm) equilibrated in 20 mM Tris–HCl buffer, pH 7.5, plus 0.5 M NaCl. Fractions (1.0 mL) were collected at a flow rate of 0.38 mL/min. Active fractions were pooled, dialyzed and used to study the enzyme biochemical characteristics.

### 2.6. Optima of temperature and pH, and thermal stability of purified enzyme

Optima of temperature and pH for fructofuranosidase activity were determined, respectively, from 23 to 80 °C and from 3.0 to 8.0. For thermal stability analysis, the enzyme was incubated at different temperatures (50, 60, 65 and 70 °C) for up to 1 h. Residual activity was estimated in enzyme aliquots (100 μL), at 60 °C, after different incubation times. The enzyme was also maintained for 1 h at different pH, using 50 mM sodium acetate buffer (3.5 and 4.5), 50 mM MES (2[N-morpholino]ethane-sulfonic acid) buffer (6.0), 50 mM Tris–HCl (hydroxymethyl-aminomethane) buffer (7.0 and 8.0) and 50 mM CAPS (3-cyclohexylamino-1-propanesulfonic acid) buffer (9.0 and 10.0). Residual activity was estimated at pH 4.5.

### 2.7. Influence of different compounds on enzymatic activity

Different ionic compounds and EDTA were added to the reaction mixture at low (1 mM) and high concentrations (10 mM) and the enzymatic activity was determined according to item 2.3. Activity in the absence of ions or EDTA stands for control.

### 2.8. Molecular mass and homogeneity analyses

The purified enzyme was submitted to non-denaturing (PAGE 7.0%) [17] and denaturing electrophoresis (7.0% SDS-PAGE) [18], and protein bands were stained with silver [19]. Molecular mass makers (Sigma®) were: α-macroglobulin (168 kDa), β-galactosidase (112 kDa), lactoferrin (91 kDa), pyruvate kinase (67 kDa) and dehydrogenase (36 kDa). Fructofuranosidase activity in PAGE was determined by incubating the gel in 100 mM sodium acetate buffer, pH 5.0, containing sucrose 1%, 0.2 mg/mL phenazine methylsulfate, 0.4 mg/mL nitroblue tetrazolium and glucose oxidase (30 U), at 37 °C in the dark. Native molecular mass of purified enzyme was estimated by gel filtration in Sephacryl S-200, as described above. Molecular mass makers were: bovine gamma globulin (158 kDa), alcohol dehydrogenase (150 kDa), BSA (66 kDa), egg albumin (43 kDa) and carbonic anhydrase (29 kDa).

### 2.9. Determination of kinetic parameters

Kinetic parameters as $V_{max}$ (maximum velocity), $K_{0.5}$ (apparent dissociation constant), $V_{max}/K_{0.5}$ (catalytic efficiency) and $n$ (Hill's coefficient) for sucrose hydrolysis in the absence or presence of 1 mM $AgNO_3$ were estimated by non-linear regression using the software Sigraf [20].

### 2.10. Analysis of hydrolysis products

Sucrose hydrolysis products were analyzed by refractive index in a Shimadzu High Performance Liquid Chromatography (HPLC) system, equipped with an EC250/4.6 nucleosil–100.5 $NH_2$ (30 cm × 0.75 cm) column, maintained at 40 °C. The mobile phase was 82% (v/v) acetonitrile in Milli-Q ultra pure water, and the flow rate was 1 mL/min. Sucrose, fructose and glucose solutions (1%, w/v) were used as standards.

### 2.11. Statistical analysis

Experiments were carried out in triplicate, and the results are presented as means ± SD. The results were analyzed by analysis of variance (ANOVA) with α level at 0.05. The *P*-value less than 0.05 was considered significantly different.

## 3. Results and discussion

### 3.1. Invertase production

High levels of extracellular β-D-fructofuranosidase were obtained when the microorganism was cultured in Khanna medium supplemented with 1% wheat bran as carbon source (Table 1), with total activity about 79- and 15-fold higher than that obtained in the absence of carbon source or with sucrose, respectively. A good production was also obtained with oatmeal and sugar cane bagasse as carbon sources. These results are interesting, since inexpensive agroindustrial residues may be used as alternative carbon sources to produce invertases. Production of invertases using agroindustrial residues was reported for *Aspergillus ochraceus* [21] and *Aspergillus niveus* [8]. Otherwise, sucrose, an inductor of invertase production, has been used for *Aureobasidium pullulans* [22] and *Aspergillus niger* IMI303386 [23].

### 3.2. Enzyme purification

After DEAE-cellulose and Sephacryl S-200 chromatographic steps, the *A. phoenicis* extracellular invertase was purified 12.5-fold with a 7.5% recovery (Table 2). The purified enzyme, when run on

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

| Carbon source | Enzymatic activity (total U) | Protein (total mg) | Final pH |
|---|---|---|---|
| No carbon source | 2.5 ± 0.2[a] | 17.3 ± 0.6[a] | 7.1 |
| Starch | 63.4 ± 0.6[b] | 28.8 ± 0.8[b] | 4.3 |
| Oatmeal | 125.4 ± 0.5[c] | 51.8 ± 1.1[c] | 6.9 |
| Sugar cane bagasse | 115.4 ± 1.0[c] | 8.1 ± 0.3[d] | 6.9 |
| Wheat bran | 195.0 ± 1.0[d] | 43.5 ± 0.9[e] | 6.8 |
| Cassava | 17.1 ± 0.5[e,f] | 31.9 ± 0.8[b] | 7.4 |
| Glucose | 2.5 ± 0.1[a] | 31.9 ± 0.7[b] | 4.4 |
| Rice straw | 9.6 ± 2.2[e] | 3.1 ± 0.1[f] | 6.9 |
| Raffinose | 19.6 ± 0.2[f] | 14.1 ± 0.3[g] | 3.0 |
| Crushed corncob | 77.4 ± 2.3[b] | 18.2 ± 0.4[a] | 7.0 |
| Sucrose | 12.9 ± 0.3[e] | 65.1 ± 1.2[c] | 6.6 |

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. Means in the same column with different lower case letters are significantly different ($P < 0.05$).

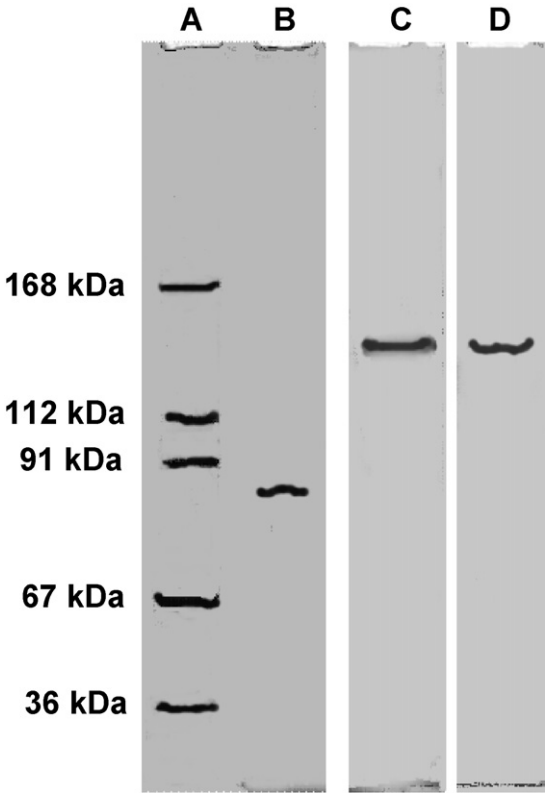


**Fig. 1.** Electrophoretic profile of the extracellular invertase produced by *A. phoenicis* under denaturing (SDS-PAGE) (A and B) and non-denaturing conditions (C and D). Proteins were revealed with silver (A–C) or for enzymatic activity (D). Molecular makers (A): α-macroglobulin (168 kDa), β-galactosidase (112 kDa), lactoferrin (91 kDa), pyruvate kinase (67 kDa) and dehydrogenase (36 kDa).

7% PAGE, produced a single band after silver staining (Fig. 1C), indicating homogeneity of the preparation. Moreover, a duplicate gel stained for invertase activity revealed a single band coincident with the silver stained protein band (Fig. 1D).

### 3.3. Molecular mass and carbohydrate content

The native molecular mass determined by Sephacryl S-200 gel filtration was 155 kDa. SDS–PAGE analysis in 7% gels showed a single band with molecular mass of 79 kDa (Fig. 1B), suggesting a homodimeric structure for the enzyme. The carbohydrate content of the purified invertase was estimated to be 1.64%. The invertases produced by *A. ochraceus* [21], *A. niveus* [8] and *A. niger* IMI303386 [23] are also glycoproteins, with carbohydrate contents higher than that observed for *A. phoenicis* β-D-fructofuranosidase. The invertases produced by *Fusarium solani* and *Rhodotorula glutinis* show molecular masses of 65 kDa and 47 kDa, respectively, estimated by SDS-PAGE [24,25]. A monomeric enzyme produced by *Bifidobacterium infantis* was characterized by Warchol et al. [5].

### 3.4. Optimal temperature and pH of activity

The extracellular invertase from *A. phoenicis* exhibited a temperature optimum of 60 °C (Fig. 2A), higher than those of the enzymes from *A. niger* AS0023 [26] and *Saccharomyces cerevisae* [27], but comparable with that estimated for the invertases from *R. glutinis* [25]. In addition, the purified enzyme was stable for more than 1 h at 50 °C, retaining 80% of control activity at 60 °C (Fig. 2C). At 65–70 °C, the enzyme showed a half-life ($t_{50}$) of 6–9 min. In comparison, the invertase produced by *A. pullulans* DSM 2404 was stable for 1 h at 45 °C [22], while that from *R. glutinis* was stable for just 3 min at 60 °C [25].

The optimum pH of *A. phoenicis* β-D-fructofuranosidase was 4.5 (Fig. 2B), and the enzyme was stable in a range of pH from 4.5 to 8.0 for 1 h (Fig. 2D). The optimum pH value was similar to those reported for *A. ochraceus* [21] and *A. niveus* [8]. Wallis et al. [28] have assayed *A. niger* invertase activity in a pH range from 4.5 to 9.0 and found the optimum value to be 6.0.

### 3.5. Effect of ions and other compounds

The activity of the *A. phoenicis* extracellular β-D-fructofuranosidase was significantly stimulated by $Ag^+$ (Table 3) when the counter ion was nitrate. The activity was not affected by 1 mM $AgNO_3$, but was enhanced about 69% at 10 mM concentration. A lower activation (about 33%) was observed for 10 mM $Ag_2SO_4$. Silver is a non-competitive inhibitor of various enzymes, binding to SH groups of amino acids such as cysteine and promoting conformational changes in the enzyme structure or else altering the global electric charge of the molecule, thus affecting the catalytic activity. This is the first report on a silver-activated invertase, and its activity was also enhanced by $K^+$ (32–40%). Interestingly, the ionic radii of $Ag^+$ and $K^+$ are very similar, 1.26 and 1.38, respectively, suggesting an interaction with a specific region of the enzyme molecule. Indeed, $K^+$ and $Ag^+$ activation is more likely a charge-related effect, since the charge of the counter ion affects the stimulation. Activation by $Ag^+$ was also observed by Mandal et al. [29] for an ATPase produced by *Archaeoglobus fulgidus*. Monovalent salts affect the protein stability by modifying the ionic strength of the solution, which can be slightly stabilizing or destabilizing, depending of the charge distribution within the protein [30].

Generally, invertase activities are enhanced in the presence of $Ca^{2+}$, $Mg^{2+}$ and $Na^+$ (ionic radii of 0.99, 0.72 and 1.02, respectively)

**Table 2**
Purification of extracellular fructofuranosidase produced by the fungus *A. phoenicis*.

| Step | Activity (total U) | Protein (total mg) | Specific activity (U $mg^{-1}$ prot) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|
| Crude extract | 1705.1 | 117.3 | 14.5 | 100.0 | 1.0 |
| DEAE Cellulose | 1271.7 | 31.5 | 40.4 | 74.6 | 2.9 |
| Sephacryl S-200 | 127.3 | 0.7 | 181.8 | 7.5 | 12.5 |

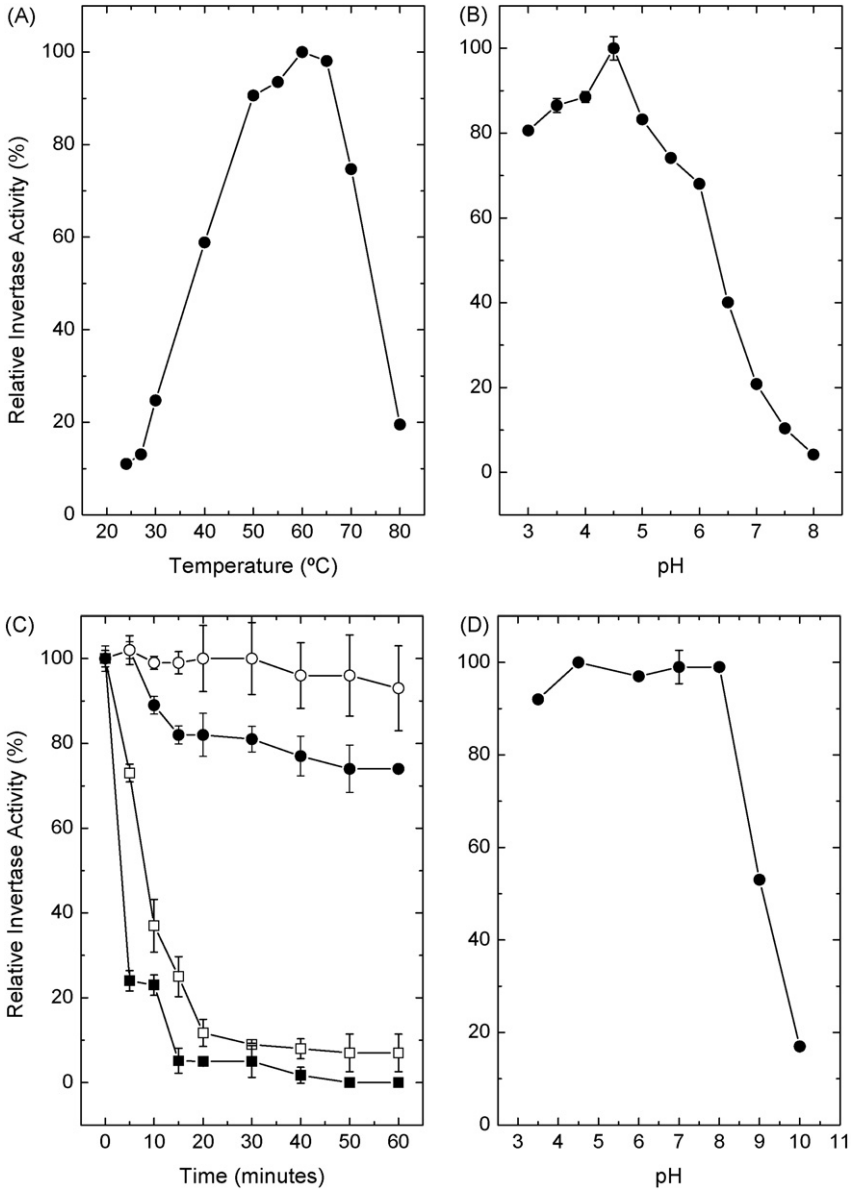


**Fig. 2.** Optimal temperature (A) and pH (B), and thermal (C) and pH stability (D) of the extracellular invertase produced by *A. phoenicis*. Symbols: 50 °C (○), 60 °C (●), 65 °C (□) and 70 °C (■).

as observed for the enzymes of *R. glutinis* [25] and *A. ochraceus* [21], but these ions inhibited the invertase from *A. phoenicis*. The inhibition by $Ca^{2+}$ might be explained by favoring the formation of low activity aggregates, as aggregation by $Ca^{2+}$ has been reported for different fungal enzymes [31,32]. Mercuric ions (ionic radius of 1.02) also severely inhibited the enzyme, signalizing the presence of thiol groups that are important for the catalytic activity [24]. Taken together, data suggest that ions with radii around 1.0 or lower are inhibitors of enzyme activity, while those with higher radii are enhancers. Apparently, the $SO^{4-}$ groups have negative influence on the enzymatic activity, which can justify the differences between the sulfate and chlorate compounds. Both the activation by $Ag^{+}$ and inhibition by $Ca^{2+}$, $Mg^{2+}$ and $Na^{+}$ suggest a new kind of β-D-fructofuranosidase produced by filamentous fungi. However, the molecular mechanism of β-D-fructofuranosidase silver stimulation has not been investigated yet, and deserves more studies in the future.

Enzyme immobilization may be conducted at different supports by site-specific attachment of proteins to surfaces, reducing the denaturing tendency and increasing the orientational order. Uniform films of silver have been used with this aim [33]. Since silver ions have positive influence on the activity of the β-D-fructofuranosidase produced by *A. phoenicis*, it is possible to carry out its immobilization on silver-covered surfaces for biotechnological applications. On the other hand, nanorods using $Ag^{+}$ have been produced, as for instance BSA–$Ag^{+}$ nanorods [34]. The biological modification of semiconductor and metal nanoparticles surface with proteins may provide bioactive functionalities for the nanocrystal surface, resulting in additional biological interactions or couplings. In addition, they may be useful in life sciences for luminescence tagging, drug delivery, and many other purposes [34].

### 3.6. Substrate specificity and kinetic parameters

The extracellular β-D-fructofuranosidase produced by *A. phoenicis* was able to hydrolyze all the substrates tested, especially sucrose (Table 4). The values of hydrolysis obtained for the

**Table 3**
Influence of ions and other compounds on the extracellular invertase activity from *A. phoenicis*.

| Compound | Relative invertase activity (%) | |
|---|---|---|
| | 1 mM | 10 mM |
| No addition | 100[a,A] | 100[a,A] |
| $AgNO_3$ | 118.7 ± 1.1[a,A] | 169.3 ± 1.0[b,B] |
| $Ag_2SO_4$ | 102.4 ± 2.8[a,A] | 132.6 ± 8.4[c,A] |
| $AgC_2H_3O_2$ | 103.1 ± 1.5[a,A] | 140.2 ± 3.2[c,B] |
| $BaCl_2$ | 48.9 ± 1.1[a,A] | 41.0 ± 18.1[d,A] |
| $CaCl_2$ | 37.6 ± 2.8[b,A] | 30.3 ± 4.8[e,B] |
| $CoCl_2$ | 104.1 ± 10.8[a,A] | 94.8 ± 0.7[f,A] |
| $CuCl_2$ | 26.2 ± 0.6[b,c,A] | 13.9 ± 13.5[g,A] |
| $CuSO_4$ | 16.8 ± 0.6[c,A] | 15.2 ± 6.2[g,B] |
| EDTA | 78.0 ± 1.1[d,f,A] | 79.0 ± 1.8[h,A] |
| $HgCl_2$ | 57.9 ± 5.8[d,A] | 0[i,B] |
| KCl | 140.6 ± 4.2[e,A] | 132.2 ± 1.5[c,B] |
| $MgCl_2$ | 87.2 ± 2.2[a,A] | 64.8 ± 10.2[j,B] |
| $MgSO_4$ | 34.4 ± 0.9[b,A] | 28.9 ± 9.3[e,g,A] |
| $MnCl_2$ | 82.3 ± 1.4[a,f,A] | 41.3 ± 1.5[d,B] |
| NaCl | 84.7 ± 2.2[a,A] | 43.8 ± 5.7[d,B] |
| $NaNO_3$ | 102.0 ± 0.2[a,A] | 100.0 ± 0.1[a,A] |
| $NH_4Cl$ | 30.1 ± 1.2[b,A] | 23.6 ± 0.5[g,B] |
| $ZnCl_2$ | 99.6 ± 12.0[a,A] | 99.3 ± 0.2[a,f,h,A] |

Means in the same column with different lower case letters are significantly different ($P < 0.05$). Means in the same line with different higher case letters are significantly different ($P < 0.05$).

mixtures (sucrose + inulin; sucrose + raffinose) were intermediary if compared with the hypothetical sum of the individual values, suggesting that sucrose, raffinose and inulin are hydrolyzed by the same enzyme. Moreover, the results indicate either the existence of three catalytic sites with common specificity or a unique catalytic site, the last hypothesis seeming more reasonable [21]. The distinction between invertase and inulinase activities is controversial [35]. The S/I ratio (sucrose hydrolysis/inulin hydrolysis) is commonly used to distinguish both activities, and in the case of *A. phoenicis* invertase this ratio was 5.08. However, only the S/I ratio is not satisfactory to distinguish invertase and innulinase activities, since inulin source, substrate concentration and buffer composition may affect both activities [35]. The extracellular invertase from *A. phoenicis* was able to act on β(2-1) and β(2-1) with α(1-6) linkages. Only glucose and fructose were obtained as hydrolysis products of 20% sucrose. Some invertases are able to produce fructooligosaccharides in the presence of high concentrations of sucrose, as those from *Schwanniomyces occidentalis* [36], *Aspergillus japonicus* [37] and *A. niger* IMI 303386 [23], characterizing a transfructosilating activity which was not observed for the enzyme from *A. phoenicis*.

The enzyme showed alosteric behavior (data not show) and the kinetic parameters $K_{0.5}$ and $V_{max}$ were 59.89 mM and 954.6 U mg$^{-1}$, or 29.21 mM and 1234.0 U mg$^{-1}$ in the absence or presence of 1 mM of silver nitrate, respectively (Table 5). $K_{0.5}$ and $V_{max}$ values for the enzyme from *A. ochraceus* were 13.4 mM and 42.13 U mg$^{-1}$ [21], while for *A. niveus* enzyme they corresponded to 5.78 mM and 30.46 U mg$^{-1}$ [8]. Addition of silver nitrate enhanced $V_{max}$ approximately 1.3-fold, confirming the results shown in Table 3, and improved the affinity for the substrate sucrose about 2-fold.

**Table 4**
Hydrolysis of different substrates by the fructofuranosidase produced by *A. phoenicis*.

| Substrate (1%, w/v) | Extracellular invertase activity (U/mL) |
|---|---|
| Sucrose | 24.3 ± 0.7 |
| Inulin | 4.8 ± 0.3 |
| Raffinose | 7.9 ± 0.2 |
| Sucrose + inulin | 17.0 ± 0.5 |
| Sucrose + raffinose | 5.6 ± 0.1 |
| Inulin + raffinose | 7.8 ± 0.2 |
| Sucrose + inulin + raffinose | 15.7 ± 0.6 |

**Table 5**
Kinetic parameters for sucrose hydrolysis by the extracellular invertase produced by *A. phoenicis*.

| Parameter | Without $AgNO_3$ | With 1 mM $AgNO_3$ |
|---|---|---|
| $K_{0.5}$ (mM) | 59.9 ± 1.8 | 29.2 ± 1.0 |
| $V_{max}$ (U mg$^{-1}$) | 954.6 ± 7.5 | 1234.0 ± 2.1 |
| $V_{max}/K_{0.5}$ (U mg$^{-1}$ mM$^{-1}$) | 16.0 ± 0.51 | 42.0 ± 0.6 |
| $n$ (Hill's coefficient) | 0.9 ± 0.1 | 1.1 ± 0.1 |

## 4. Conclusion

In conclusion, we suggest that the extracellular invertase produced by *A. phoenicis* is a new kind of thermostable fructofuranosidase activated by silver, devoid of transfructosilating activity, and may represent an enzyme with biotechnological potential.

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## References

[1] B.L. Cantarel, P.M. Coutinho, C. Rancurel, T. Bernard, V. Lombard, B. Henrissat, Nucleic Acids Res. 37 (2008) 233 (PMID: 18838391).
[2] W. Vargas, A. Cumino, G.L. Salerno, Planta 216 (2003) 951.
[3] H. Winter, S.C. Huber, Crit. Rev. Plant Sci. 19 (2000) 31.
[4] A. Sturm, M.J. Chrispeels, Plant Cell 2 (1990) 1107.
[5] M. Warchol, S. Perrin, J.P. Grill, F. Scheneider, Lett. Appl. Microbiol. 35 (2002) 462.
[6] N. Tanaka, N. Ohuchi, Y. Mukai, Y. Osaka, Y. Ohyani, M. Tabuchi, M.S.A. Bhuiyan, H. Fukui, S. Harashima, K. Takegawa, Res. Commun. 245 (1998) 246.
[7] A. Belcarz, G. Ginalska, J. Lobarzewski, C. Penel, Biochim. Biophys. Acta 1594 (2002) 40.
[8] L.H.S. Guimarães, A.F. Somera, H.F. Terenzi, M.L.T.M. Polizeli, J.A. Jorge, Process Biochem. 44 (2009) 237.
[9] B. Wolska-Mitaszko, J. Jaroszuk-Scisel, K. Pszeniczna, Mycol. Res. 111 (2007) 456.
[10] C. Herwig, C. Doerries, I. Marison, U. von-Stockar, Biotechnol. Bioeng. 76 (2001) 247.
[11] R.C. Fernandez, C.A. Ottoni, E.S. Silva, R.M.S. Matsubara, J.M. Carter, L.R. Magossi, M.A.A. Wada, M.F.A. Rodrigues, B.G. Maresma, A.E. Maiorano, Appl. Microbiol. Biotechnol. 75 (2007) 87.
[12] L.H.S. Guimarães, S.C. Peixoto-Nogueira, M. Michelin, A.C.S. Rizzatti, V.C. Sandrim, F.F. Zanoelo, A.C.M.M. Aquino, A. Barbosa-Junior, M.L.T.M. Polizeli, Braz. J. Microbiol. 37 (2006) 474.
[13] P. Khanna, S.S. Sundari, N.J. Kumar, World J. Microbiol. Biotechnol. 11 (1995) 242.
[14] G.L. Miller, Anal. Chem. 31 (1959) 427.
[15] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[16] M. Dubois, K. Gilles, J. Hamilton, P. Rebers, F. Smith, Anal. Chem. 28 (1956) 350.
[17] B.J. Davis, Ann. NY Acad. Sci. 11 (1964) 407.
[18] U.K. Laemmli, Nature 222 (1970) 680.
[19] H. Blum, H. Beier, H.J. Gross, Electrophoresis 81 (1987) 93.
[20] F.A. Leone, L. Degreve, J.A. Baranauskas, Biochem. Educ. 20 (1992) 94.
[21] L.H.S. Guimarães, H.F. Terenzi, M.L.T.M. Polizeli, J.A. Jorge, Enzyme Microb. Technol. 42 (2007) 52.
[22] J. Yoshikawa, S. Amachi, H. Shinoyama, T. Fujii, J. Biosci. Bioeng. 103 (2007) 491.
[23] Q.D. Nguyen, J.M. Rezessy-Szabó, M.K. Bhat, A. Hoschke, Process Biochem. 40 (2005) 2461.
[24] H.N. Bhatti, M. Asgher, A. Abbas, R. Nawaz, M. Sheiki, J. Agric. Food Chem. 54 (2006) 4617.
[25] M.C. Rubio, R. Runco, A.R. Navarro, Phytochemistry 61 (2002) 605.
[26] L. L'Hocine, Z. Wang, B. Jiang, S. Xu, J. Biotechnol. 81 (2000) 73.
[27] K. Shafiq, S. Ali, Ikram-ul-Haq, Biotechnology 1 (2002) 40.
[28] G.L.F. Wallis, F.W. Hemmig, J.F. Peberdy, Arch. Biochem. Biophys. 345 (1997) 214.
[29] A.K. Mandal, W.D. Cheung, J.M. Argüello, J. Biol. Chem. 277 (2002) 7201.
[30] B.N. Dominy, D. Perl, F.X. Schimid, C.L. Brooks, J. Mol. Biol. 319 (2002) 541.
[31] L. Cardello, H.F. Terenzi, J.A. Jorge, Microbiology 140 (1994) 1671.
[32] L.H.S. Guimarães, H.F. Terenzi, J.A. Jorge, F.A. Leone, M.L.T.M. Polizeli, Folia Microbiol. 48 (2003) 627.
[33] X. Liu, C.-H. Jang, F. Zheng, A. Jürgensen, J.D. Denlinger, K.A. Dickson, R.T. Raines, N.L. Abbott, F.J. Himpsel, Langmuir 22 (2006) 7719.

[34] L. Yang, R. Xing, Q. Shen, K. Jiang, F. Ye, J. Wang, Q. Ren, J. Phys. Chem. B 110 (2006) 10534.
[35] R.J. Rouwenhorst, L.E. Visser, A.A. Van der Ban, W.A. Scheffers, J.P. Van, Dijken, Appl. Environ. Microbiol. 19 (1988) 293.
[36] M. Álvaro-Benito, M. de-Abreu, F. Fernández-Arrojo, F.J. Plou, J. Jiménez-Barbero, A. Ballesteros, J. Poliana, M.J. Fernández-Lobato, Biotechnology 132 (2005) 75.
[37] Y. Yang, J. Wang, D. Teng, F. Zhang, J. Agric. Food Chem. 56 (2008) 2805.