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ARTICLE

Characterization of an Exoinulinase Produced by *Aspergillus terreus* CCT 4083 Grown on Sugar Cane Bagasse

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Exoinulinase (β-D-fructan fructohydrolase, EC 3.2.1.80) secreted by *Aspergillus terreus* CCT4083 was obtained using sugar cane bagasse, an agroindustrial residue, as a carbon source. It was further purified from the supernatant culture in a rapid procedure. The enzyme presented 57 kDa on SDS-PAGE and 56 kDa on gel filtration chromatography. Inulin was hydrolyzed by the purified enzyme, yielding D-fructose as the main product. This enzyme showed maximum activity at pH 4.0 and 60 °C and maintained more than 90 and 75% of its original activity at 40 and 50 °C, respectively, after 3.5 h of preincubation. The K_M values for inulin, sucrose, and raffinose were 11, 4.20, and 27.89 mM, respectively, and D-fructose was a competitive inhibitor (K_i = 47.55 mM). The activation energies for sucrose, raffinose, and inulin were 10.4, 5.61, and 4.44 kcal/mol, respectively. The characteristics of *A. terreus* exoinulinase were compared to those of inulinases isolated from other organisms. The exoinulinase traits presented especially good thermostability and the ability to produce pure D-fructose, suggesting its application to the production of high-fructose syrup.

KEYWORDS: Inulinase; *Aspergillus terreus* CCT4083; inulin; agroindustrial residue

INTRODUCTION

Fructans, such as inulin, are reserve carbohydrates present in roots and tubers of plants, including *Helianthus tuberosus* L. (Jerusalem artichoke), *Cichorium intybus* L. (chicory), and *Taraxacum officinale* Weber (dandelion). Inulin is a polydisperse carbohydrate consisting mainly of β-(2,1)-fructosyl-fructose linkages. In general, a D-glucose molecule is linked by sucrose, such as bonds (α-1,2) at the end of each D-fructose chain (*1*, *2*).

The unique aspect of the inulin structure is the presence of β-2,1 bonds, which prevent inulin from being digested as a typical carbohydrate and are responsible for its reduced caloric value and dietary fiber effects (*2*−*4*). This polymer is a recognized source for the production of high D-fructose content syrups. Fructose can be produced from starch by enzymatic methods involving α-amylase, amyloglucosidase, and glucose isomerase, resulting in products consisting of oligosaccharides (8%), fructose (45%), and glucose (50%) (*5*). Fructose can also be obtained by acid hydrolysis of inulin, but this procedure may produce undesirable byproducts (hydroxymethylfurfural, fructose dianhydride) and color-forming compounds that lower the product yield and require more extensive downstream processing and energy expenditure (*6*, *7*). D-Fructose production from inulin catalyzed by exoinulinase is an advantageous process because it consists of a single-step enzymatic reaction and yields up to 95% free fructose, without the inconvenience of undesirable side products. Furthermore, D-fructose syrup from inulin is more soluble than sucrose and provides about 30−50% of the sweetness of table sugar (*3*).

Inulinases are divided into exo- and endoinulinases. Endoinulinases (2,1-β-D-fructan fructanohydrolase, EC 3.2.1.7) are specific for inulin and hydrolyze the internal β-2,1 fructofuranosidic linkages mainly to yield inulotriose, -tetrose, and -pentose as products. Exoinulinases (β-D-fructan fructohydrolase, EC 3.2.1.80) hydrolyze the nonreducing terminal of inulin, raffinose, and sucrose, releasing free D-fructose. The synergistic action of endo- and exoinulinases allows efficient inulin hydrolysis to produce D-fructose (*8*, *9*).

Endo- and exoinulinases belong to the GH32 family, which includes more than 370 members from different species. This family also comprises invertases and other enzymes, such as levanases (EC 3.2.1.65), and transfructosidades, such as sucrose: sucrose 1-fructosyltransferase (EC 2.4.1.99) and fructan-fructan 1-fructosyltransferase (EC 2.4.1.100) (*10*).

Many microorganisms produce inulinases, especially fungi and yeasts such as *Penicillium* (*9*, *11*), *Kluyveromyces* (*12*−*15*), and *Aspergillus*(*16*, *17*). *Aspergillus terreus* has been studied due to the production of large amounts of hydrolytic enzymes, such as α-galactosidase(*18*) and α-arabinofuranosidase (*19*).

Sugar cane bagasse is an abundant, low-cost lignocellulosic material resulting from juice extraction. This waste basically consists of 50% cellulose, 30% sugars, and 2.4% ashes. Brazil is known as one of the greatest producers of sugar from sugar cane in the world. The Brazilian annual production of sugar cane bagasse is currently estimated at 186 million tons (*20*). The use of sugar cane bagasse in several biotechnological processes has been suggested, and its appropriate destination enhances the value of this material and contributes to the solution for removal of this abundant waste (*21*).

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In this study, we describe the purification and characterization of an inulinase from *A. terreus* CCT 4083 for potential biotechnological applications. The characteristics of this enzyme were compared to those of inulinases isolated from other organisms.

MATERIALS AND METHODS

Microorganism and Growth Conditions. *A. terreus* CCT 4083 was obtained from the André Toselo Tropical Research Foundation, Campinas, SP, Brazil. The stock culture was maintained on potato dextrose agar medium.

For enzyme production, spores from *A. terreus* were transferred to 1.0 L of liquid medium containing 1% (w/v) of sugar cane bagasse as carbon source and (in g/L) 7.0 KH_2PO_4, 2.0 K_2HPO_4, 0.1 $MgSO_4 \cdot 7H_2O$, 1.0 $(NH_4)_2SO_4$, and 0.6 yeast extract. Sugar cane bagasse was obtained from a local market after extraction of the sugar cane juice, drying at 50 °C overnight, and milling.

The culture was incubated under constant agitation for 72 h at 28 °C and 150 rpm. Culture filtrate was collected by filtration through Whatman no. 1 filter paper to remove the cells, which were kept at −20 °C until use.

Enzymatic Assay. Inulinase activity was determined in a reaction system containing 600 μL of a 100 mM sodium acetate buffer, pH 4.0, 150 μL of an enzyme solution, and 250 μL of inulin from chicory [Sigma Chemical Co., St. Louis, MO; 0.5% (w/v)]. The invertase activity was determined according to standard assay described for inulinase, but with 250 μL of 100 mM sucrose as substrate instead of inulin. The reaction was carried out for 20 min at 50 °C and ended with the addition of 1 mL of 3,5-dinitrosalicylate (DNS), followed by boiling (*22*). This procedure was defined as the standard assay. No inulin hydrolysis was detected in control tubes in which the enzyme sample had been replaced by denatured enzyme (enzyme boiled for 10 min). When the effect of D-fructose, melibiose, maltose, lactose, D-galactose, and D-mannose on enzyme activity was tested, product formation was evaluated by the glucose oxidase method (*23*).

Protein Determination. The protein concentration in the enzymatic extracts was determined by the Coomassie Blue binding method (*24*), with bovine serum albumin as standard. The bicinchoninic acid (BCA) method was used to determine the protein concentration during the purification steps (*25*).

Purification. The steps of enzyme purification were carried out at 4 °C. The enzymatic extract was concentrated using an Amicon ultrafiltration cell model 8400 (Bedford, MA) with a 10 kDa molecular cutoff and submitted to an anionic exchange, CM-Sepharose (GE Healthcare, Uppsala, Sweden) column (9.0 × 2.5 cm) pre-equilibrated with 50 mM sodium acetate buffer, pH 4.0. The adsorbed proteins were eluted in a salt gradient (0−2 M NaCl) at a flow rate of 60 mL/h (4 mL per tube). Fractions showing inulinase activity were pooled and concentrated/desalted by ultrafiltration. This sample was applied to a Phenyl-Sepharose (GE Healthcare) column (9.5 × 1.6 cm), pre-equilibrated with 25 mM sodium acetate buffer, pH 4.0, with 1 M ammonium sulfate. The adsorbed proteins were eluted by a decreased ammonium sulfate gradient (1−0 M) at a flow rate of 40 mL/h (3 mL per tube). The purified fractions were concentrated and stored at −20 °C until further analysis.

SDS-PAGE and Molecular Mass Determination. Enzyme preparations were analyzed by 12% (w/v) SDS-PAGE (*26*), and proteins were silver-stained (*27*). The molecular mass standards (Sigma Chemical, St. Louis, MO) were the following: 66.0 kDa bovine serum albumin, 45.0 kDa ovalbumin, 36.0 kDa glyceraldehyde-3-phosphate dehydrogenase, 29.0 kDa carbonic anhydrase, 24.0 kDa trypsinogen, 20.1 kDa trypsin inhibitor, and 14.2 kDa α-lactolbumin. The enzyme molecular mass was estimated by SDS-PAGE, using a 12% (w/v) polyacrylamide gel.

Molecular Mass Estimation by Gel Filtration Sephadex G-75. The molecular mass of inulinase was also estimated by gel filtration in a Sephadex G-75 column (1.6×70 cm, Amersham Biosciences, Uppsala, Sweden). Ribonuclease A (13.7 kDa), chymotrypsinogen A (25 kDa), ovoalbumin (43 kDa), and bovine serum albumin (67 kDa) were used as standards (GE Healthcare). The standards and the purified inulinase were applied separately in a column pre-equilibrated with 25 mM sodium acetate buffer containing 100 mM NaCl and run with the same buffer. Fractions of 1 mL were collected and assayed for the inulinase activity. The absorbance of standard protein samples was detected at 280 nm.

Thin Layer Chromatography (TLC). TLC on silica gel was used for qualitative analysis of the inulin hydrolysis reaction products. TLC was performed on precoated silica gel plates (Sigma-Aldrich), at room temperature, in a saturated chamber containing the solvent system *n*-propanol/acetic acid/water (1:1:0.1, v/v/v). Sugar was visualized by spraying the plates with 1% α-naphthol (w/v) and 10% orthophosphoric acid (v/v) in absolute ethanol and kept in an oven at 140 °C for 5 min. D-Fructose was used as a sugar standard. The enzymatic reaction was carried out in a solution of sodium acetate buffer, pH 4.0, at 50 °C, for 2.5 h. Aliquots were removed at 0.5, 1, and 2.5 h.

Effect of pH and Temperature on Enzyme Activity. The effect of pH on inulinase activity was determined within the pH range of 3.0−8.0, using McIlvaine buffer (citric acid/sodium phosphate), at 50 °C (*28*), under other standard enzyme assay conditions. The optimum temperature was determined within the temperature range of 25−80 °C, at pH 4.0, using acetate buffer. The highest activity value obtained in each assay was regarded as 100%.

Thermal stability in the absence of substrate was determined by measuring the residual activity of the enzyme after its incubation at 40, 50, 60, and 70 °C for 0−3.5 h.

The effect of pH on inulinase stability was determined by measuring the residual activity of the enzyme after its incubation at pH ranging from 3.0 to 8.0 for 30 min at 50 °C.

Kinetic Parameters. The exoinulinase Michaelis−Menten constant (K_M) for substrate hydrolysis was calculated by nonlinear curve fitting of the data to the Michaelis−Menten plot.

The substrate concentrations were 0.22−41.56 mM for inulin, 2.5−60 mM for sucrose, and 10−200 mM for raffinose. Inulin molecular weight was considered to be 4620 g mol^{-1} (*29*). Kinetic experiments were performed at 60 °C and pH 4.0.

Effect of Ions, Reducing Agents, and Sugars on Enzyme Activity. The effect of different ions, reducing agents, and sugars on enzyme activity was examined by preincubating the enzyme extract with each of the compounds (2 mM) in 100 mM sodium acetate buffer, pH 4.0, for 15 min, at 50 °C. The enzyme activities remaining after incubation were determined according to the standard assay. All assays were performed in triplicate and expressed as the mean ± standard deviation (SD).

Inhibition by D-Fructose. The inhibition constant (K_i) for fructose was calculated by the Dixon plot by using sucrose as substrate at concentrations of 5−100 mM. The fructose concentrations were 30, 60, and 90 mM. All assays were performed in triplicate.

Activation Energy Determination. The activation energies (E_a) for the reaction catalyzed by inulinase, which included sucrose, raffinose, and inulin (30−50 °C), were calculated from the slope of the Arrhenius plot: log of velocity on the ordinate versus $1/T$ in Kelvin on the abscissa. The Arrhenius equation used was slope = $-E_a/2.3R$, where R is the universal gas constant (8.314 J K^{-1} mol^{-1}). All assays were performed in triplicate.

RESULTS AND DISCUSSION

Inulinase Production. *A. terreus* was grown using sugar cane bagasse as carbon source and produced extracellular inulinase. Probably the bagasse constituents (sucrose, glucose, fructose, cellulose, and hemicellulose) were used by the fungus to grow, because *A. terreus* CCT 4083 presents cellulolytic system (results not shown). As widely reported, the substrates that contain inulin are good inulinase inducers (*7*, *30*, *31*). Although sugar cane bagasse does not present inulin in its composition, it was able to induce inulinase production. This fact could be due to the presence of sucrose in sugar cane bagasse, because this sugar acts as an inducer of inulinolytic system in *Aspergillus niger* (*32*), *Penicillium janczewskii* (*33*), and *Aspergillus tamarii* (*34*).

Purification of Exoinulinase from *A. terreus*. Results for purification of extracellular inulinase from *A. terreus* are shown in **Table 1**. The sample previously concentrated by ultrafiltration was submitted to ion exchange chromatography. The elution profile obtained from a linear gradient of 2 M NaCl presented a narrow and symmetric activity peak and a distinct peak containing the majority of proteins (**Figure 1**). Fractions with higher activity and lower protein concentrations were pooled and concentrated/desalted by ultrafiltration, followed by submission to

Table 1. Purification of Inulinase from *Aspergillus terreus*

purification step	volume (mL)	total protein (mg)	total activity (U[a])	specific activity (U[a]/mg)	purification fold	yield (%)
crude extract	600	2826	606	0.214	1	100
CM-Sepharose	61	51.1	278.8	5.48	25.5	46
Phenyl-Sepharose	72	5.08	194.4	38.3	179	32.1

[a] 1 U = amount of enzyme that released 1 μmol of reducing sugar (in D-fructose equivalents) per minute under the assay conditions.

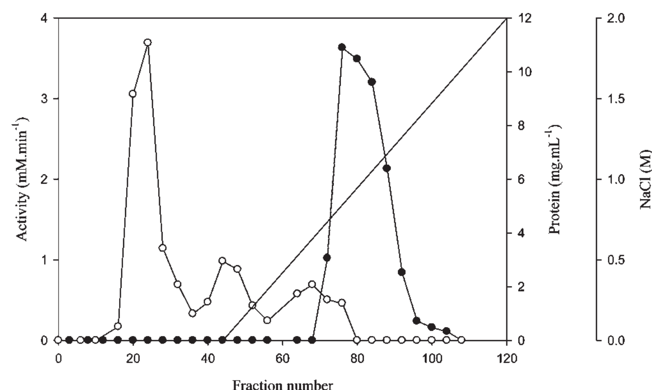

Figure 1. Profile of ion-exchange chromatography with CM-Sepharose for extracellular inulinase from *A. terreus*: (○) protein; (●) activity; (−) NaCl gradient.

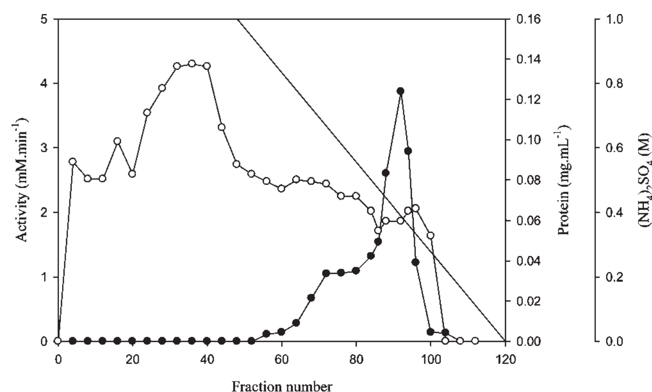

Figure 2. Phenyl-Sepharose elution profile of extracellular inulinase from *A. terreus*: (○) protein; (●) activity; (−) $(NH_4)_2SO_4$ gradient.

hydrophobic interaction chromatography. The elution profile obtained from a decreasing gradient of 1 M ammonium sulfate is shown in **Figure 2**. Two well-separated activity and protein peaks were also observed. This protocol permitted 179-fold purification of the enzyme from the initial extract and a yield of 32.1%. The procedure presented here is reproducible and simple, presents high yield, and can be easily adapted for large-scale purification.

Enzyme preparations were examined using SDS-PAGE (**Figure 3**). No other protein bands were detected on the SDS-PAGE, indicating that the enzyme was homogeneous.

Polyacrylamide gel was also used to estimate the apparent molecular mass of inulinase. The molecular mass was obtained by linear regression of the standard molecular mass logarithm and relative distance covered by the enzyme. The molecular mass of the purified exoinulinase obtained was 57 kDa. The inulinase molecular mass determined by gel filtration chromatography was 56 kDa, nearly the same value obtained by SDS-PAGE, suggesting that inulinase was secreted by *A. terreus* as a monomer.

A considerable variation in inulinase molecular masses has been observed in other microorganisms. Kushi et al (*12*) studied *Kluyveromyces marxianus* var. *bulgaricus* inulinase and found an identical molecular mass (57 kDa), also using SDS-PAGE.

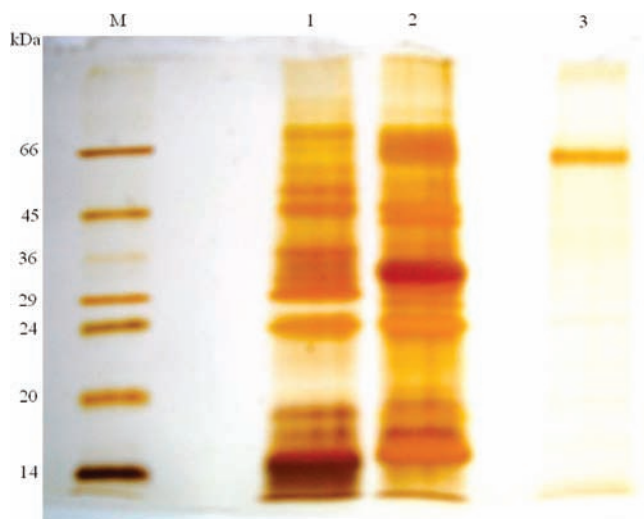

Figure 3. SDS-PAGE of extracellular inulinase from *A. terreus*. SDS-PAGE was carried out in 12% (w/v) polyacrylamide gels stained with silver nitrate. Lane 1, crude enzyme extract; lane 2, fraction from CM-Sepharose column; lane 3, fraction from Phenyl-Sepharose column; lane M, molecular weight markers (bovine albumin, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; trypsin inhibitor, 20 kDa; and α-lactoalbumin, 14 kDa).

In *Rhizopus* sp., the inulinase molecular mass obtained by SDS-PAGE was 84 kDa. *Kluyveromyces fragile* (*35*) and *K. marxianus* CBS 6556 inulinases presented different molecular masses: 250 and 87−102 kDa, respectively (*36*).

Mode of Action. For determining the exo or endo nature of the purified inulinase, the cleavage products of inulin hydrolysis were analyzed by TLC. TLC of the inulin hydrolysis products by purified inulinase indicated that the reaction yielded D-fructose as the main product. The lack of any other fructooligosaccharide suggests that extracellular inulinase from *A. terreus* is an exo-inulinase or β-D-fructan fructohydrolase (**Figure 4**). This enzyme was a β-D-fructan fructohydrolase, rather than a 2,1-β-D-fructan fructanohydrolase, an inulin D-fructosyl-D-fructosyltransferase, or a β-D-fructofuranoside fructohydrolase. Similar results were found for inulinase from *A. fumigates* (*16*) and *Cryptococcus aureus* (*37*). Exoinulinase from *A. terreus* can be used by the food industry to produce high-fructose syrup, because the enzyme released pure fructose as product.

Effect of pH and Temperature on Enzyme Activity. The inulinase activity was measured at different pH values ranging from 2.0 to 8.0 at 50 °C, using sucrose and inulin as substrates (**Figure 5**). The optimum pH of inulinase for inulin and sucrose hydrolysis was the same: near 4.0.

Using sucrose as substrate, at pH values between 3.5 and 5.0, the enzyme maintained about 65% of its activity. Values of pH ranging between 7.0 and 8.0 resulted in total loss of enzyme activity. When inulin was used as substrate, the enzyme maintained high activity between pH values of 3.5 and 5.0.

The effect of pH on inulinase stability was tested by incubating the enzyme at different pH values, at 50 °C for 30 min. The pH

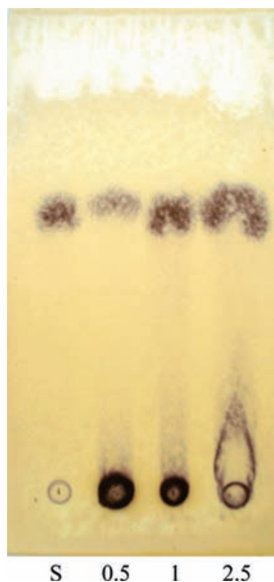

Figure 4. Thin layer chromatography analysis of the products of inulin hydrolysis by *A. terreus* purified inulinase. Standard (S), D-fructose; reaction time, 0.5, 1, and 2.5 h.

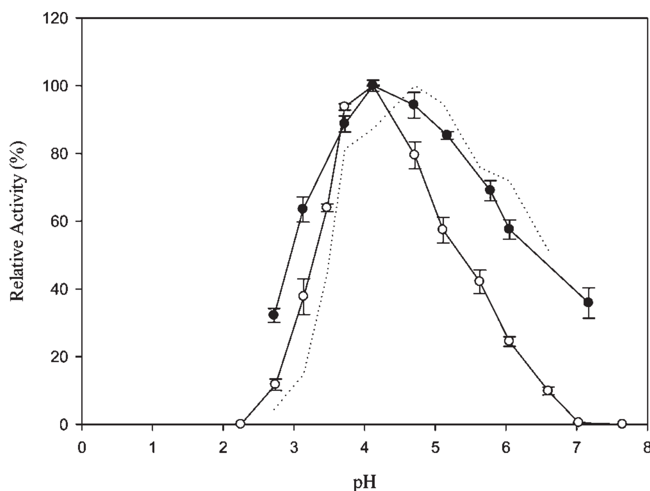

Figure 5. Effect of pH on inulinase from *A. terreus* in sucrose (○) and inulin (●) hydrolysis and (···) inulinase pH stability. Relative activities were calculated in relation to activities determined in pH 4.0, which were considered as 100%.

stability of inulinase is shown in **Figure 5**. Exoinulinase presented high pH stability in pH ranging from 4.0 to 6.0. However, when it was incubated at pH values lower than 4.0, most of its activity was lost.

In general, inulinases present acidic optimal pH: *K. marxianus* var. *bulgaricus*, 4.75; *Rhizopus* sp., 5.5; *A. niger*, 4.3; and *K. marxianus* CBS 6556, 5.0 (*7*, *12*, *36*, *38*). One advantage of the enzyme from *A. terreus* is its stability in acidic pH values (4.0−6.0).

The enzyme activity was assayed at different temperatures between 25 and 80 °C at pH 4.0, using sucrose and inulin as substrates. The optimum temperature for both substrates was 60 °C (**Figure 6**).

When sucrose was the substrate, the enzyme activity was >85% within the temperature range of 55−65 °C. Between 45 and 70 °C, the activity remained >50% for both substrates. At temperatures >70 °C, the activity fell dramatically, whereas at temperatures <40 °C, the activity was higher when inulin was the substrate.

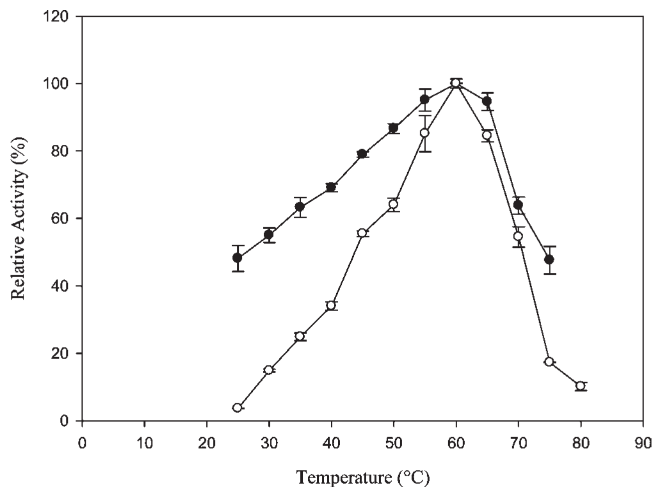

Figure 6. Effect of temperature on inulinase from *A. terreus* in sucrose (○) and inulin (●) hydrolysis.

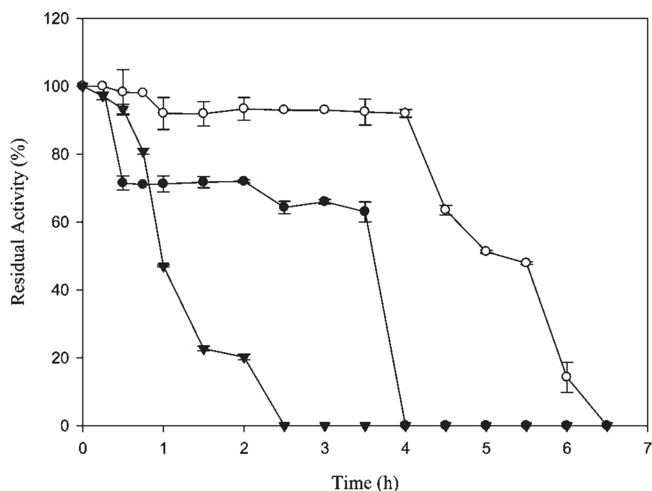

Figure 7. Effect of temperature on stability of inulinase from *A. terreus*: (○) 40 °C; (●) 50 °C; (▼) 60 °C. The enzyme sample was preincubated for several periods at different temperatures and assayed as described for the standard assay. The activity at 0 min of preincubation was considered to be 100%.

For the determination of thermal stability, the enzyme was preincubated at different temperatures for up to 3 h, at pH 4.0. Residual activities were measured at 60 °C. A significant activity loss was observed after preincubation for 30 min at 60 °C. However, at 40 and 50 °C, inulinase retained 90 and 75% of activity, respectively, for >3.5 h (**Figure 7**).

The optimum temperature observed for the purified *A. terreus* inulinase was in accordance with many reported inulinases, including those from *A. fumigatus* (*16*), *Penicillium* sp. strain TN-88 (*4*), and *P. janczeiskii* (*39*).

Kinetic Parameters. The kinetic constant (K_M), maximum velocity (V_{max}), and catalytic constant (K_{cat}) obtained for sucrose, raffinose, and inulin as substrates are shown in **Table 2**. Extracellular inulinase from *A. terreus* showed lower K_M for sucrose, compared with inulin and raffinose. However, K_M for inulin was lower when compared to those for inulinases from other microorganisms. The K_M of inulinase for inulin was 11 mM (48.09 mg/mL), which is lower than the value reported for Fructozyme L, a commercial inulinase from *A. niger* (60 mg mL^{-1}) (*40*). The apparent K_M of inulinase from *K. marxianus* for inulin was 61.83 mM (*41*), also higher than our results. It is suggested that

Table 2. Kinetics Parameters of Inulinase from *Aspergillus terreus*

substrate	K_M (mM)	V_{max} (mM min^{-1})	K_{cat} (min^{-1})	K_{cat}/K_M (min^{-1} mM^{-1})
sucrose	4.20	0.47	2032.15	483.84
raffinose	27.89	2.54	1098.45	39.38
inulin	11.00	18.49	2672.9	242.99

extracellular inulinase from *A. terreus* has a great potential for inulin hydrolysis, due to its high relative affinity to this substrate. The catalytic constant for inulin was 2672.9 min^{-1}, which is higher than K_{cat} for sucrose and raffinose, which were 2032.15 and 1098.45 min^{-1}, respectively. This indicates that inulinase presents higher potential to hydrolyze inulin compared to sucrose and raffinose hydrolysis.

The catalytic efficiency was 483.84, 242.99, and 39.38 min^{-1} mM^{-1} for sucrose, inulin, and raffinose, respectively. These values indicated that exoinulinase from *A. terreus* efficiently hydrolyzes inulin and sucrose. The catalytic efficiency to inulin was >47.92 min^{-1} mM^{-1}, observed for inulinase from *Bacillus smithii* T7 (*31*).

Effect of Metal Ions and Other Reagents. The metal ions Mn^{2+} and Mg^{2+} enhanced invertase activity by approximately 20 and 40%, respectively. Mn^{2+} enhanced inulinase activity by about 30%, but Mg^{2+} did not affect inulinase activity. Inulinase from *A. niger* AF10 (*17*) also has its activity enhanced by Mn^{2+}, but Mg^{2+} was an inhibitor. Galactose, melibiose, maltose, and lactose had no significant effect on the invertase activity, whereas raffinose and stachyose, which are also substrates of this enzyme, enhanced the activity by almost 40%. The addition of β-mercaptoethanol decreased the invertase activity by about 45%, suggesting that a disulfide bond contributes to the enzyme structure stability. This reducing agent promoted a slight decrease in the inulinase activity (86.7%). In the presence of $CuSO_4$ and $AgNO_3$, the invertase activity was reduced to about 60 and 23%, respectively, whereas the inulinase activity was reduced to 69 and 61%, respectively (**Table 3**). Inhibition by $AgNO_3$ was also observed in inulinase from *Penicillium* sp. (*11*) and in invertases from plants (*42*). This inhibition suggests the presence of a sulfhydryl group in the enzyme structure (*42*). The denaturant agent SDS completely inhibited the invertase activity and reduced the inulinase activity to about 60%. β-Mercaptoethanol, $AgNO_3$, and SDS promoted more drastic effects on the invertase activity in comparison to the inulinase activity.

Effect of D-Fructose Concentration on the Inulinase Reaction Rate. The effect of D-fructose concentration on the inulinase initial velocity of sucrose hydrolysis was evaluated. The plot showed an increase in K_M value and a tendency for constant V_{max} with the increase in the initial D-fructose concentration, in the presence or absence of the inhibitor, suggesting that D-fructose acts as a competitive inhibitor for the exoinulinase. The K_i value was 47.55 mM, as determined by the Dixon plot. Similarly, exoinulinase from *Aspergillus ficuum* and invertase from *Pycnoporus sanguineus* were competitively inhibited by D-fructose (*43*, *44*).

Determination of Activation Energy (E_a). Arrhenius plots for sucrose, raffinose, and inulin hydrolysis were linear, and the activation energies of these substrates were 10.48, 5.61, and 4.41 kcal/mol, respectively. The lower E_a value indicated that inulin hydrolysis is faster than raffinose and sucrose hydrolysis because the reaction transition state is more quickly reached for this substrate.

Kushi et al. (*12*) found similar values for inulinase from *K. marxianus* using sucrose as a substrate (8.21 kcal/mol), but higher values were observed for raffinose and inulin, 13.57 and 17.9 kcal/mol, respectively. Ettalibi et al. (*45*) encountered lower values for sucrose when working with two fractions presenting activity of inulinase from *A. ficuum*: 5.43 and 6.81 kcal/mol. Catana et al. (*46*) estimated the activation energy for inulin as 7.18 kcal/mol, for a commercial *A. niger* inulinase, which is higher than our result. This information demonstrates the variability among different sources of inulinase.

Table 3. Effects of Various Metal Ions and Sugars on Invertase and Inulinase Activities

effector (2 mM)	relative activity[a] (%) ± SD	
	invertase	inulinase
none	100.0 ± 2.0	100 ± 2.5
EDTA	88.5 ± 1.1	95.3 ± 0.4
KCl	96.3 ± 0.6	93.3 ± 6.9
SDS	0.0	31.5 ± 1.7
NaCl	80.8 ± 0.2	93.8 ± 8.8
$CuSO_4$	59.9 ± 5.4	69.4 ± 0.8
$CaCl_2$	105.0 ± 5.6	115.8 ± 5.6
$MnCl_2$	113.5 ± 6.3	129.3 ± 4.0
β-mercaptoethanol	67.2 ± 1.4	86.7 ± 5.7
$MgCl_2$	141.9 ± 7.7	96.0 ± 2.9
$AgNO_3$	23.1 ± 0	61.4 ± 0.9
raffinose	135.7 ± 5.4	nd[b]
stachyose	130.0 ± 6.8	nd
mannose	101.7 ± 2.1	nd
lactose	109.7 ± 1.5	nd
galactose	111.3 ± 0.5	nd
melibiose	109.2 ± 3.4	nd
maltose	112.6 ± 2.7	nd

[a] Relative activities were calculated in relation to the invertase and inulinase activities without effectors, which were considered to be 100%. [b] Not determined.

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