

Purification and Characterization of β -Fructosidase with Inulinase Activity from *Aspergillus niger* - 245

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ABSTRACT

Aspergillus niger - 245, a strain isolated from soil samples showed good β -fructosidase activity when inoculated in medium formulated with dahlia extract tubers. The enzyme was purified by precipitation in ammonium sulphate and percolated in DEAE-Sephadex A-50 and CM-cellulose columns, witch showed a single peack in all the purification steps, maintaining the I/S ratio between 0.32 to 0.39. Optimum pH for inulinase activity (I) was between 4.0 - 4.5 and for invertase activity (S) between 2.5 and 5.0. The optimum temperature was 60^o.C for both activities and no loss in activity was observed when it was maintained at this temperature for 30 min. The K_m value was 1.44 and 5.0, respectively, for I and S and V_m value 10.48 and 30.55, respectively. The I activity was strongly inhibited by Hg^{2+} and Ag^+ and 2×10^{-3} M of glucose, but not by fructose at the same concentration. The enzyme showed an exo-action mechanism, acting on the inulin of different origins. In assay conditions total hydrolysis of all the frutans was obtained, although it has shown larger activity on the chicory inulin than that one from artichoke Jerusalem and dahlia, in the first 30 min. The obtained results suggested that the enzyme presented good potential for industrial application in the preparing the fructose syrups

Key words: inulinase, invertase, β -fructosidase, inulin, fructose syrup, *Aspergillus niger*

INTRODUCTION

D-Fructose is the main component of fructans such as the inulin. In the free form this monosaccharide is found in the pulp fruits and in the bee honey. With sweetening power 1.5 to 2 times more than the sucrose, it presents other advantages over the cane sugar such as larger solubility, smaller tooth decay induction and smaller absorption index, without problems for diabetic individuals. Industrially, fructose is produced by a complex system, the Clinton Corn Process (CCP) for high fructose corn syrup, in which the corn starch is submitted to a bulk of amylolytic enzymes, glucose isomerase and chromatographic separation, reaching a maximum final concentration of 90% (Vandame & Derikre, 1983).

For the past years, several researchers have proposed inulin as fructose alternative source once a fast enzymatic treatment could result in a syrup

with 90% - 95% of fructose and 5% - 10% of glucose in a quite simplified industrial process, compared to the (CCP). This conviction has led European countries to cultivate several inulin rich plants as the Jerusalem artichoke (*Helianthus tuberosus* L.), dahlia (*Dahlia pinnata*) and chicory (*Cichorium intibus*). According to Barta (1995), these are the most suitable inulin sources for utilization at industrial scale, as they have high productivity estimated in 4.5, 2.5 and 0.9 ton/ha, repectively. Roots of some plants of the Compositae and Asteraceae families, native in the Brazilian savannas, contain up to 86% of fructans, representing inulin source and are potentially interesting for industrial use (Isesima *et al.*, 1991).

This work describes the purification and characterization of a β -fructosidase with high inulinase activity produced by a strain of *Aspergillus niger* isolated of soil samples, selected

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as the best enzyme-producing after a wide screening among more than 350 strains.

MATERIAL AND METHODS

Microorganism and culture medium. A strain of *Aspergillus nige* was used in the present studies. It was maintained in Sabraud-dextrose-agar (Difco) medium. For the enzyme production, the spores were inoculated with a platinum loop in 250 ml erlenmeyers flasks with 50 ml dahlia roots extract (Houly *et al.*, 1992) containing 2.0% of total sugars (Dubois *et al.*, 1956), enriched with 2.0% yeasts flour, 0.5% K₂HPO₄, 0.2% NaNO₃, 0.05% KCl, 0.05% MgSO₄·7H₂O, and final pH 5.5. The flasks were agitated at 200 rpm, for 48 h in a rotatory shaker at 28° C. After the fermentation the mycelia were separated through filtration.

Enzyme assay. Amounts of 0.5 ml of enzyme solution were incubated with 1.0 ml of 2.0% inulin solution in 50mM acetate buffer, pH 5.0 for 60 min. The content of fructose liberated was estimated by the Somogyi & Nelson method (1944) and expressed as reducing sugar. The invertase activity was measured by replacing the inulin for a sucrose solution in the same buffer and concentration and the glucose liberated was estimated by the glucose oxidase method (Cruz *et al.*, 1981). One enzyme unit (U) was taken as the enzyme amount necessary to liberate 1 umol of fructose/glucose, per min under the assay condition. The proteins were determined by the procedure of Lowry, modified by Hartree (1972).

Enzyme purification.

Ammonium sulphate insolubilization. A volume of 640 ml of the filtrate containing crude enzyme was saturated with 80% ammonium sulphate and after 48 h, centrifuged at 16,000 rpm for 12 min. The precipitate was redissolved in 34 ml of deionized water, dialized for 48 h against distilled water and for another 48 h against 50 mM acetate buffer, pH 4.7. This fraction was submitted to chromatography in a DEAE-Sephadex A-50 column.

Chromatography in ion exchange column. The (NH₄)₂SO₄ fraction was percolated in a DEAE-

Sephadex A-50 column with 48 x 1.5 cm, previously equilibrated with 50 mM acetate buffer, pH 4.7. The flow was regulated at 5.0 ml/30 minutes and the proteins were eluted with a linear gradient of 0.05 to 0.15 M of NaCl in the same buffer. The fractions were collected in a Pharmacia collector, REDFRAC-100 model and analyzed for proteic content (measure of absorbance at 280 nm), inulinase and invertase activities. The fractions endowed with inulinase activity were collected and precipitated with (NH₄)₂SO₄ (80%), redissolved in 15.6 ml of deionized water and dialized for 48 hours against distilled water and 48 hours against the same buffer. This fraction was then percolated in a 45 cm x 1.5 cm CM-cellulose column equilibrated with the same buffer. The fractions were eluted and analyzed by the procedure as above. All the purification steps were performed at 4° C.

Enzyme characterization. The optimum pH of the inulinase was investigated in range from 2.5 to 7.0 and optimum temperature from 35 to 75° C. For studies on the thermostability, enzyme aliquots with 50 mM acetate buffer, pH 5.0, were maintained at 30 min, at the experimental temperatures and the remaining activity was measured. The effect of the substrate concentration for inulinase and invertase was verified in the range from 0.025 to 20 mM of inulin and from 3.0 to 300 mMol of sucrose (Lineweaver & Burk, 1934). Molecular weight of the inulin was assumed as 5,000. The effect of some metals, monosacharides and organic reagents on the inulinase activity was investigated by its addition to the substrate in the expressed concentrations as shown in Table 2 and the enzymatic activity determined as described in assay

Enzyme action on inulin from different sources.

For verification of the enzyme action on inulin from dahlia, chicory and Jerusalem artichoke as for reaction time, the system was composed by 1.0 ml (14U) of the purified enzyme and 10 ml of the mentioned substrates solution at 5.0% in 50 mM acetate buffer, pH 5.0. The qualitative action of the enzyme was observed by TLC, according to Walkley & Tillman (1977) and measured (quantitatively) by High Performance Liquid Chromatography (HPLC) using a Shimadzu LC-10A, model RID 6-A chromatography, equipped with refraction index detector, and a Supercosyl LC-NH₂ column with 250 x 4.6 mm maintained in acclimatized room at 20° C. The solvent system was

composed of acetonitrile-water (80:20) and flow rate of 2.0 ml/min.

RESULTS AND DISCUSSION

Purification of the β -fructosidase. The filtrate of the growth medium of the *Aspergillus niger*-245

presented inulinase (EC 3.2.1.7) and invertase (EC 3.2.1.26) activities in a I/S ratio from 0.32 to 0.39 in all the purification steps. Table 1 shows the purification details, developed in 3 steps and Fig. 1 and 2 show the chromatograms in DEAE-Sephadex A-50 and in CM-cellulose, respectively.

Table 1: Purification of the β -fructosidase from *Aspergillus niger* -245. Inulinase activity.

STEPS	Volume (ml)	Activity (U/ml)	Total activity	Protein (mg/ml)	Spec. activity (U/mg)	Yield (%)	Purif (times)
Crude enzyme	640	5.2	3,328	1.713	3.04	100	1
(NH ₄) ₂ SO ₄ - 80 %	34	27,4	931.6	1.886	14.53	28	4.8
DEAE-Sephad A-50	15.6	19.5	304.2	0.774	26.21	9.1	8.7
CM - Cellulose	16	14.9	238.4	0.442	38.86	7.2	13.0

Successive purification after three stages resulted a fraction with specific activity 13 times that of the crude extract and yield of 7.2%. These values are compatible with the purification and yield obtained for enzymes of another origins (Yokota *et al.*, 1991; Ha & Kin, 1992) by using the same techniques as used in this paper. Korneeva *et al.* (1993) and Campos *et al.* (1992), using more refined techniques such as chromatography by hydrophobic interactions and isoelectric focusing obtained more expressive results with regard to the purification. However, the final yield dropped for just 8%.

with identical values for the I/S ratio, suggesting that both activities were in a same enzymatic form or proteic molecule.

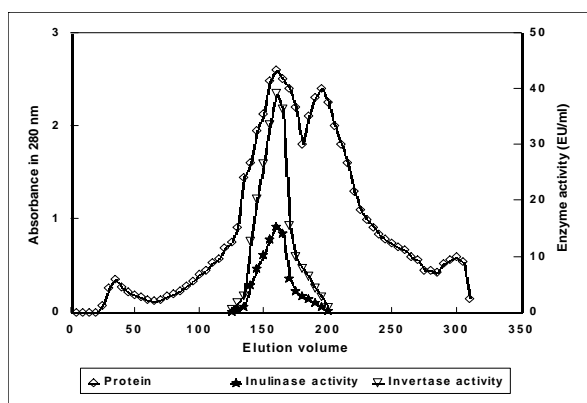


Figure 1: Chromatography in DEAE Sephadex A-50. The (NH₄)₂SO₄ fraction was applied in a column (48 x 1,5 cm) equilibrated with 50 mM acetate buffer, pH 4.7 and the proteins were eluted with 0.05 - 0,15 M NaCl linear gradient

The chromatograms also show that the peaks of inulinase and invertase activity are parallel, so much in DEAE-Sephadex A-50 as in CM-cellulose

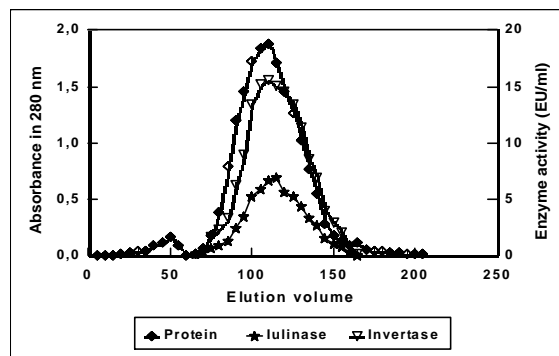


Figure 2: Chromatography in CM-cellulose column. The DEAE-Sephadex A-50 fraction was applied in CM-cellulose column (35 x 1,5 cm) equilibrated with 50 mM acetate buffer, pH 4.7. The fraction were eluted as described for the anionic column.

Several previously published works have already demonstrated that some strains of the *Aspergillus* genus and yeasts produce a β -fructosidase with both activity: inulinase and invertase (Ettalibi & Baratti, 1987; Manzoni & Cavazzoni, 1988; Hayashi *et al.*, 1992).

Characterization of the β -fructosidase

Influence of the pH on the enzymatic activity.

According to Fig. 3, the inulinase showed higher activity at pH 4.0 and 4.5, coinciding with the

results described by Vandamme & Derike (1983) working with a strain of *Aspergillus niger*. On the other hand, the optimum pH for the inulinase from another strain of the same species (Oengen *et al.*, 1994) was found at a slightly higher range (5.0 to 6.0), in the same way as the enzymes produced by *Chrysosporium pannorum* (Xiao *et al.*, 1989), *Arthrobacter globiformis* (Haragushi *et al.*, 1990) and *Fusarium oxysporium* (Kaur *et al.*, 1992). For the industrial production of fructose concentrated syrups, enzymes with larger activity in pH range inferior to 5.0 are suitable to render difficult the bacterial contamination of the process. Acting on the sucrose, the enzyme presented two peaks of maximum activity, one in pH 2.5, and another in pH 5.0. However it is possible that the hydrolysis of the sucrose in the first peak has happened, not just for the enzymatic action, but by the high acid concentration in the reaction medium in that pH

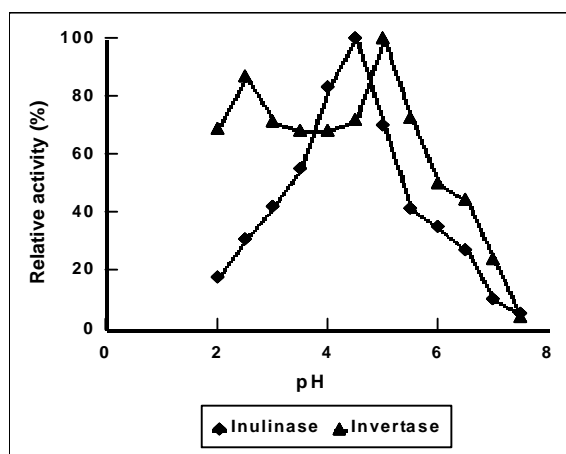


Figure 3: pH effect on the activity of **b**-fructosidase from *Aspergillus niger*-245. Reaction system is described in text

Temperature effect . As can be seen from Fig. 4, inulinase showed higher activity at 60°C and was stable, after treatment for 30 minutes. Gupta *et al.* (1992) concluded that the thermostability of β -fructosidases from several *Aspergillus spp.*, in a general way, presented higher stability when compared to several other inulinases of microbial origin. Such results were also confirmed by Abdel *et al.* (1994) and Korneeva *et al.* (1993), working, respectively with *A. carneus* and *A. awamori*, respectively.

Effect of substrate concentration. Through Fig. 5, the value of the K_m for the inulin was calculated in 0.144 mMol and the value of V_m at 10.48 of fructo-

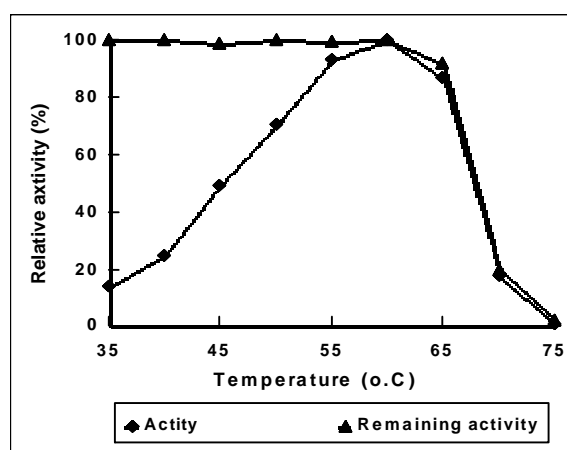


Figure 4: Effect of temperature on the activity and thermostability of **b**-fructosidase from *Aspergillus niger*-245. Reaction system in text.

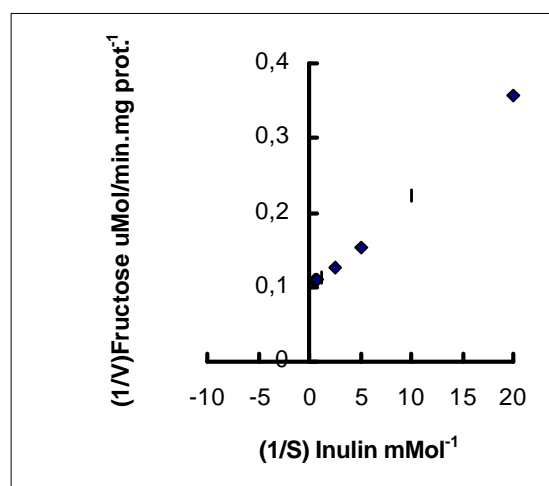


Figure 5: Effect of inulin concentration on the activity of **b**-fructosidase from *Aspergillus niger*-245. Reaction system in text

se/min.mg of protein. For sucrose, K_m value was 5.0 mM and V_m 30.55 uMol of glucose/min.mg of protein (Fig. 6). These findings, in a general way, confirmed the results previously described (Manzoni & Cavazzoni, 1992; Ku & Hang, 1994) in that the enzyme always exhibited smaller K_m (greater affinity) for the inulin than for sucrose. Onodera & Shiomi (1988) observed that the K_m value of a highly purified inulinase from *Penicillium purpurogenus*, for fructooligosaccharides with 2 to 7 fructose units decreased linearly with the increase of the polymerization degree of these compounds.

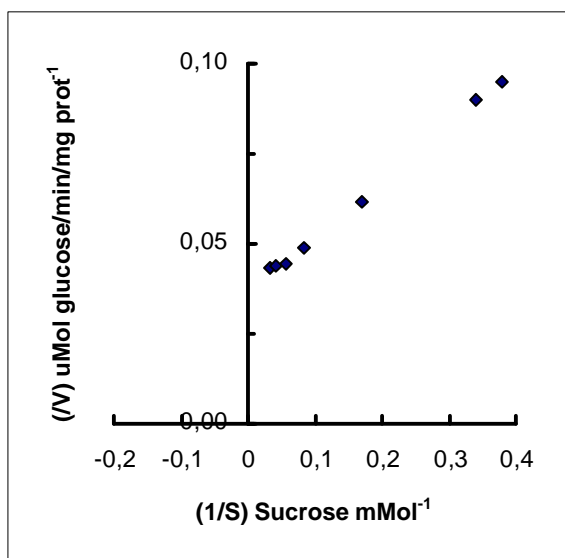


Figure 6: Effect of sucrose concentration on the activity of *b*-fructosidase from *Aspergillus niger* - 245. Reaction system in text.

Inulinases have shown great divergence in K_m values, ranging from 0.042 to 16.51, for the *Aspergillus trzebinkii* (Onodera & Shiomi, 1992) and *Streptomyces sp* (Ha & Kim, 1992). It is possible that the great multiplicity of forms of this enzyme explains these differences.

Effect of inhibitor agents. As shown in Table 2, the inulinase from *Aspergillus niger*-245 was strongly inhibited by the heavy metals Hg^{2+} and Ag^+ in decreasing order, coinciding with the affinity order of these metals for free SH groupings, but not by the iodoacetamide in the tested concentration. Such observation suggested that these groupings were present at the active site of the enzyme, without discarding the presence of other residues as the one of the histidine and other with carboxyl groups, also, affected by heavy metals, but not by *sulphidrilic reagents*. Inulinase activity was affected by glucose (2×10^{-3} M) although not by the same concentration of fructose. The inhibition was observed only when the concentration of the monosaccharide was increased in 10 times, which could represent great advantage of this enzyme for its industrial application. This enzyme exhibited an exo-action model once the fructose was its only hydrolysis product as can be seen in Fig. 7.

Table 2: Metals, monosaccharides and organic reagents on I activity from *Aspergillus niger*-245

Inhibitor	Concentration (M)	Remain. activity (%)
Without	-	100
Iodoacetamide	2.0×10^{-3}	101
EDTA	2.0×10^{-3}	105
Fructose	2.0×10^{-3}	102
	2.0×10^{-2}	88
Glucose	2.0×10^{-2}	49
ZnCl ₂	2.0×10^{-3}	101
CaCl ₂	2.0×10^{-3}	110
MgCl ₂	2.0×10^{-3}	108
CoCl ₂	2.0×10^{-3}	99
BaCl ₂	2.0×10^{-3}	100
FeSO ₄	2.0×10^{-3}	63
MnSO ₄	2.0×10^{-3}	49
HgCl ₂	2.0×10^{-3}	8
AgNO ₃	2.0×10^{-3}	5

Reaction system: amounts of 2.0% inulin solution in 50 mM acetate buffer, pH 5.0 added with each inhibitor in mentioned concentration were incubated with 0.5 ml of purified enzyme and the activity expressed as a percentage from that obtained without inhibitor.

Hydrolysis of the inulin from different sources.

Table 3 shows that the inulinase from *Aspergillus niger*-245 acted more quickly on the chicory inulin than on the fructans from dahlia and Jerusalem artichoke. In 30 min of reaction 72% of the chicory inulin was already transformed into free fructose against only 27% of the dahlia and Jerusalem artichoke inulins. After 60 min of reaction, those hydrolysis values rose to 88% and 80%, respectively. In the end of 3 hours they equaled around 90%, which can be considered as very close to the total inulin hydrolysis (Vandame & Derikre, 1983). The largest activity of the enzyme on the inulin from chicory leads to the supposition that some fructans present a structure more susceptible to the enzymatic attack (perhaps less condensed or with different polymerization degrees) than the other ones. Unfortunately, similar studies were not found in the literature for comparison purposes.

Table 3: Fructose liberation from inulin of different sources by inulinase from *Aspergillus niger*-245.

Inulin sources			
Reaction time (min)	Chicory (%)	Dahlia (%)	Artichoke (%)
30	72.0	27.5	26.9
60	88.1	80.9	79.8
120	87.1	84.7	85.9
180	85.4	85.5	88.0
240	90.0	88.0	91.2

Reaction system: 1.0 ml (14U) of the purified enzyme and 1.0 ml of the mentioned substrates solution at 5.0% in 50 mM acetate buffer, pH 5.0. Samples were collected and analysed by HPLC. When the reaction time reached 120 min no more inulin was found in the medium. The differences of the obtained fructose to 100% was identified as glucose.

CONCLUSION

The β -fructosidase activity produced by *Aspergillus niger*-245 in medium formulated with dahlia tubers extract showed good pH and temperature properties, low inhibition by the final product and fast action on inulins from different origins. It suggested that the microorganism currently studied

presented good potentiality for industrial application

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RESUMO

Aspergillus niger - 245, isolado do solo mostrou boa atividade de β -frutosidase meio formulado com extrato de tubérculos de dahlia. A enzima foi purificada por precipitação em sulfato de amônia e percolada em colunas de DEAE-Sephadex A-50 e CM-celulose, produzindo um único pico em todas as fases de purificação e mantendo a relação I/S entre 0,32 a 0,39. O pH ótimo para a atividade de inulinase (I) foi encontrado entre 4,0 - 4,5 e para a atividade de invertase (S) em 2,5 e 5,0. A temperatura ótima foi de 60°C para ambas as atividades e nenhuma perda foi observada quando

Foto (Fig. 7) de posse da revista

Figure 7: Effect of reaction time on hydrolysis of inulin by inulinase from *Aspergillus niger*-245. 1- Fructose; 2- Glucose; 3- Sucrose; 4- Inulin, 0 min; 5- Inulin, 30 min; 6- Inulin, 60 min; 7- Inulin, 120 min; 8- Inulin, 180 min; 9- Inulin, 240 min.

mantida nesta temperatura por 30 min. Os valores de K_m foram de 1,44 e 5,0, respectivamente, para I e S e os valores de V_m de 10,48 e 30,55, respectivamente. A atividade I foi fortemente inibida por Hg^{2+} , Ag^+ e 2×10^{-3} M de glicose, mas não por frutose na mesma concentração. A enzima mostrou um mecanismo de exo-ação, atuando sobre a inulina de diferentes origens. Em condições de ensaio foi obtida hidrólise total de frutanas, apesar de ter mostrado maior atividade sobre a inulina de chicória que sobre as de alcachofra de Jerusalém e dahlia, nos primeiros 30 minutos de reação. Os resultados obtidos sugerem que a enzima apresenta bom potencial para aplicações industriais na preparação de xaropes de frutose.

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