

Purification and Properties of Extracellular Endoinulinase from *Aspergillus niger* 20 OSM

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Summary

Extracellular inulinase (E.C. 3.2.1.7) produced by *Aspergillus niger* 20 OSM culture in a 2-litre fermentor was isolated and purified by ion exchange chromatography on DEAE Sepharose, hydrophobic interaction on phenyl Sepharose, chromatofocusing on PBE-94, and size exclusion chromatography (SEC) on Sephadex G-200. The enzyme was homogeneous, as measured by SDS-PAGE with an apparent molecular mass of 69 or 64 kDa, as determined by SEC. Carbohydrate content of the enzyme was estimated at approx. 44.5 %. The optimum temperature and pH for enzyme activity were 55 °C and 5.0, respectively. Some physicochemical properties of the purified inulinase were also determined (isoelectric point, K_m , V_{max} and E_a). Enzyme activity was inhibited by EDTA, pCMB, Hg^{2+} , Mn^{2+} , and some other metal ions. Calcium cations showed a positive effect on the enzyme activity. Thin layer chromatography indicated that the purified enzyme is a typical endoinulinase.

Key words: endoinulinase, enzyme purification, *Aspergillus niger*, extracellular enzyme

Introduction

Inulin is a widespread polyfructan in plants, consisting of a linear chain of β -(2 \rightarrow 1)-linked fructose residues attached to a terminal sucrose molecule (1). This storage polymer is of great interest as it represents a relatively inexpensive and abundant substrate for the production of fructose syrups (2).

While 2,1- β -D-fructan fructohydrolase (E.C. 3.2.1.80), an exoinulinase, hydrolyzes terminal fructose units from inulin, endoinulinase (E.C. 3.2.1.7) splits inulin into inulooligosaccharides, which were suggested to have similar physiological functions to those of fructooligosaccharides (3). Microbial inulinases play an important role in the hydrolysis of inulin for its commercial exploitation. They are used in the production of fructose syrups from plant sources such as chicory, dahlia, Jerusalem artichoke or agave (4,5). Inulinases have also been used for the production of inulooligosaccharides – low caloric sac-

charides acting as a growth factor for beneficial microorganisms in the intestinal flora (2,6,7). Another application of inulinases is the production of ethanol from inulin (5,8).

Fungal inulinases are frequently composed of a mixture of fructanohydrolases with high activity and stability (9). The best known inulinases are those produced by the species of *Penicillium* (10), *Aspergillus* (11) and *Kluyveromyces* (12). For the industrial production of inulooligosaccharides, it is necessary to find a microorganism that produces endoinulinase without exoinulinase.

In a previous study (13), a mutant of 20 OSM with extracellular inulinase activity about 2-fold higher than that of the wild strain was obtained after mutagenic activation of *A. niger*. The aim of the study described in the present paper was to isolate, purify and determine the main characteristics of an extracellular inulinase from this organism.

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Materials and Methods

Strain and growth conditions

A. niger 20 OSM mutant from our collection was used. This strain has been characterized as having high inulinase activity in submerged cultures and as being resistant to some abiotic stresses (13). It was maintained on malt agar slants at 4 °C and grown on a basal medium (BM) which contained (in g/L): sucrose 33.3, yeast extract 19.7, NaNO₃ 3.65, K₂HPO₄ 2.6 and MgSO₄·7H₂O 0.15.

The mycelium of *A. niger* was grown in a 2-litre glass fermentor (Biostat B, B. Braun Biotech International GmbH, Germany) filled with 1.6 L of BM. Aerated (at a rate of 1 L air/min) and stirred (200 rpm) bioreactor free cultures were run at 30 °C for 96 h. Occasionally antifoam A emulsion (Sigma) was added to break the foam. The pH was kept at the value of 6.0 by the addition of 0.1 M NaOH. Dissolved oxygen (DO) concentration in the fermentation broth was measured with an Ingold electrode (Mettler-Toledo GmbH). The values of the readings were expressed as percentage of the initial level of saturation.

Enzyme concentration

Liquid culture medium was collected after 4 days of fungal growth (when the inulinase activity was maximal), separated from the growing medium by filtration through Miracloth quick filtration material (Chicopee Mills, Inc., USA) and checked immediately for inulinase and invertase activities. Different ways were chosen for the extracellular inulinase concentration from the culture filtrate: (i) precipitation by various agents such as ammonium sulphate or organic solvents (ethanol, propanol, isopropanol, acetone), (ii) vacuum rotary evaporation at 37 °C, and (iii) ultrafiltration using a Filtronmini-Ultra-sette (Filtron, USA). All steps described above were performed at 5 °C.

Purification of inulinase

The culture filtrate containing inulinase activity was preliminarily concentrated on a rotary evaporator, precipitated by isopropanol and used in the following steps of the purification procedure. Next, the inulinase preparation was applied onto a DEAE Sepharose (fast flow) column (2.5×20 cm), equilibrated with 0.05 mM acetate buffer (pH=6.0) and connected to a chromatographic GradiFrac System (Pharmacia Biotech AB, Sweden). The inulinase activity was eluted from the column with a linear NaCl gradient, 0.0–0.25 M, in 0.05 M acetate buffer (pH=6.0) at a flow rate of 1 mL/min. Fractions containing inulinase activity were pooled and applied onto a phenyl Sepharose, PBE-94 or Sephadex G-200 column. Phenyl Sepharose 6 fast flow column (1×20 cm) was equilibrated with 0.75 M (NH₄)₂SO₄ in 0.05 M phosphate buffer (pH=7.0). The column was washed with equilibrating buffer and eluted with a descending gradient of 0.75–0.00 M ammonium sulphate. Chromatofocusing was performed on a Polybuffer Exchanger PBE-94 column (1×20 cm), equilibrated with 0.025 M imidazole-HCl buffer, pH=7.4. The column was eluted with Polybuffer 74 at a flow rate of 0.3 mL/min. Size exclusion chromatography (SEC) was carried out using a Sepha-

dex G-200 (1×100 cm) column eluted with 0.05 M acetate buffer, pH=5.0, at a flow rate of 4 mL/h. Protein elution profile in each chromatography was monitored spectrophotometrically by absorbance at 280 nm.

Molecular mass estimation

Molecular mass was determined by SEC and SDS-PAGE. SEC was performed on Sephadex G-200 column using Bio-Rad Gel Filtration Standards. SDS-PAGE was carried out by the method of Laemmli using 10 % polyacrylamide gel and Pharmacia HMW-SDS Calibration Kit.

Protein identification by mass spectrometry

Protein samples were analyzed by liquid chromatography – electrospray mass spectrometry with collisional fragmentation (LC-ESI-MS-MS/MS). Prior to the analysis the proteins were reduced, alkylated and digested with trypsin. Eluted peptide mixture was applied to RP-18 precolumn (LC Packings) and then transferred to nano-HPLC RP-18 column (LC Packings, 75 mM i.d.) using an acetonitrile gradient (0–50 % AcN in 30 min) in the presence of 0.05 % of formic acid with the flow rate of 200 nL/min. Column outlet was coupled directly to FTICR electrospray mass spectrometer (Thermo Finnigan) working in the regime of data dependent MS to MS/MS. Results were analyzed by a Mascot database search (Matrix Science Ltd.).

Identification of inulin hydrolysis products

For the identification of inulooligosaccharides, thin layer chromatography was carried out on silica gel 60 F₂₅₄ (E. Merck) precoated plates, using the solvent system containing (in volume ratio) nitroethane/acetonitrile/ethanol/water (1:4:3:2). Chromatograms were visualized by spraying with *N*-(1-naphthyl)ethylenediamine dihydrochloride-methanol-sulphuric acid spray.

Determination of K_m , V_{max} and E_a

The kinetic constants (K_m , V_{max}) for inulinase were calculated from standard Lineweaver-Burk plot. The determinations were made at the optimum pH and temperature in the assay reaction mixtures and under the conditions outlined above, except for the fact that substrate concentrations were changed from 0.1 to 2.0 %. The activation energy (E_a) was calculated from the Arrhenius equation.

Analytical procedures

The reaction mixture contained 0.05 mL of an appropriately diluted culture filtrate and 0.95 mL of 0.5 % mass per volume ratio of inulin (from Dahlia tubers, Sigma Chemical Co., St Louis, MO, USA) dissolved in 0.1 M acetate buffer (pH=5.0) and was incubated at 50 °C. After 20 min of incubation, the increase in reducing sugars was estimated with the 3,5-dinitrosalicylic acid method (14). Absorbance was measured at 550 nm. One unit (U) of inulinase activity was defined as the amount of the enzyme which produces 1 μmol of reducing sugars per min under the above conditions.

The influence of pH on inulinase was examined in the standard assay mixture except that 0.1 M McIlvaine buffer (pH=3.4–8.5), instead of acetate buffer, was used. The effect of temperature on the enzyme activity was determined under the standard procedure in the range of temperature from 20 to 70 °C at optimal pH value.

Protein concentration was analysed using Lowry or Bradford methods (15,16). Saccharide content in protein molecules was determined according to Dubois *et al.* (17), by the phenol sulphuric assay using glucose as a standard.

The mean standard error for inulinase estimate was ± 0.23 and ranged from 0.003–0.36 U/mL.

Results and Discussion

The main objective of the present study was to purify and determine the dominant physical characteristics of extracellular inulinase of *Aspergillus niger*. Culture filtrate of *A. niger* 20 OSM obtained after 96 h of growth was used as enzyme source for obtaining an active inulinase preparation. The first step of the experiments consisted in finding effective methods of protein isolation and concentration from the culture fluid. Inulinase preparations were obtained by precipitation with ammonium sulphate and organic solvents in various ratios to the culture filtrate volume, ultrafiltration and evaporation. The fraction precipitated with isopropanol in the ratio of 3:1 with culture filtrate indicated a relatively high inulinase activity (about 99 % of initial activity). A little less satisfactory results were obtained after concentrating (10-fold) the culture fluid in a vacuum evaporator. About 95 % of inulinase activity and more than 95 % of initial protein content were observed in this fraction. A similar level of inulinase activity was obtained in the fraction after ultrafiltration or lyophilization (94 and 95 %, respectively).

In the subsequent experiment, the extracellular inulinase of *A. niger*, obtained after the precipitation with isopropanol, was purified on the DEAE Sepharose column. This procedure allowed us to separate the main inulinase activity into one sharp peak fraction, which closely coincided with the protein peak. Inulinase eluted from the column with 0.25 M NaCl was purified over 14-fold with a yield of 67.4 % (Table 1). The main active fraction was applied to a phenyl Sepharose column. Purification level increased to 23-fold with recovery of 40 %. The active fraction after DEAE Sepharose was submitted

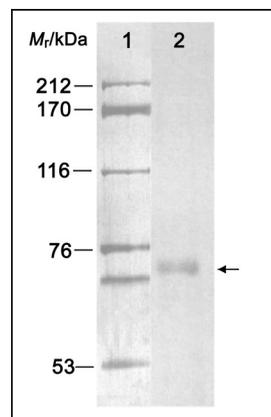


Fig. 1. SDS-PAGE analysis of purified endoinulinase from *A. niger* 20 OSM

Lanes: 1 – standard proteins, 2 – purified endoinulinase

to further fractionation by chromatofocusing on PBE-94. The active inulinase fraction was focused with a single sharp peak which coincided with the protein peak, and its isoelectric point was estimated at 3.85. Another sample of inulinase purified on DEAE Sepharose was also submitted to SEC on Sephadex G-200. Thus purified inulinase showed a similar level of specific activity and a high yield in comparison with the phenyl Sepharose and chromatofocusing purification scheme. The purification factor after the SEC was about 22.5 with the yield of more than 58 % (Table 1). The native molecular mass of the enzyme was determined by SEC as 64 kDa.

The homogeneity of inulinase obtained from *A. niger* 20 OSM was confirmed by SDS-PAGE. The single protein band visualized as inulinase is shown in Fig. 1. The relative molecular mass of the enzyme determined by this method was 69 kDa. This was very similar to the value estimated by SEC, indicating a monomeric form of the active enzyme. The relative molecular mass of inulinases from *Aspergillus* and *Penicillium*, as determined by SEC and SDS-PAGE, was in the range of 54–78 kDa (9, 11,18). These values are similar to those found for the enzyme from *A. niger* 20 OSM. Protein identity was confirmed by LC-ESI-MS/MS/MS. Results were analyzed by a Mascot database search. The protein was similar to endoinulinase from *Aspergillus niger* 12 (19), with a Mascot score of 1669 and sequence coverage of 45 %. Purified inulinase was identified as a glycoprotein. The carbohydrate content of the enzyme was estimated at 44.5 % by

Table 1. Purification of endoinulinase from *Aspergillus niger* 20 OSM

Method	Total activity	<i>m</i> (total protein)	Specific activity	Purification factor	Yield
	U	mg	U/mg		%
1. Culture filtrate	7708	150.00	51.4	1.00	100.00
2. Concentration in evaporator (5x)	7477	147.50	50.7	0.99	97.00
3. Precipitation by isopropanol (75 %)	7325	57.10	128.3	2.50	95.03
4. DEAE Sepharose	5198	6.92	750.7	14.61	67.44
5a. Phenyl Sepharose	3103	2.58	1201.9	23.39	40.26
5b. PBE-94	3488	3.10	1125.4	21.90	45.25
5c. Sephadex G-200	4483	3.87	1158.3	22.54	58.16

mass. A similar value (40 %) was reported for *K. marxianus* inulinase (20). Next, the basic kinetic characteristics, Michaelis constant (6.7 g/L), maximal speed (0.0476 mg/mL/min), and activating energy (58.33 kJ/mol) were calculated for the purified fraction of inulinase. The optimum temperature and pH for the enzyme activity were 55 °C and 5.0, respectively. The enzyme showed good stability at a pH range from 4.0 to 7.0 and at temperatures up to 55 °C (Fig. 2).

Inulinases from various microorganisms were extensively studied and the kinetic parameters determined for the activities from enzyme preparations of *A. niger* 20 OSM are in agreement with those reported for *A. niger* in the literature (21,22). The behaviour of this enzyme under different temperatures and pH values was also similar to those observed for inulinases from the *Aspergillus* species (22,23).

Inulinase under the influence of *p*-chloromercuribenzoate (*p*-CMB) was strongly inactivated during 30 min of incubation. This indicates that the active center of inulinase contains the SH group of cysteine. *p*-CMB reacts with thiol groups forming mercaptides (a reversible process) and serves as a specific reagent for SH groups (24). Several cations such as Zn²⁺, Cd²⁺, Co²⁺, Ni²⁺, and Fe³⁺ strongly inhibited inulinase activity at 1 mM. Mn²⁺ and Hg²⁺ cations completely inactivated the enzyme. Conversely, some endoinulinases were reported to be activated by Mn²⁺ (25). Divalent metal ion chelator, 1 mM EDTA, also showed higher inhibitory effect (about 17 % of activity left). Several divalent metal ions in a 2 mM concentration were added to EDTA-inactivated inulinase solution to reactivate the enzyme. Calcium ions fully reactivated inulinase activity (data not shown). The positive effect of Ca²⁺ on inulinase activity from *Rhizopus* sp. strain was reported by Ohta *et al.* (25).

Thin layer chromatography of inulin hydrolysis products showed tetra- and pentasaccharides as the main end products. There was no fructose in the hydrolysate (Fig. 3). In general, hydrolysis of fructans by fungal enzymes is catalysed by both endo- and exohydrolases. Most of the fungal inulinases described in the literature are exo-acting enzymes (23,26,27). As inulooligosaccharides were the main product of degradation of fructans by inulinase from *A. niger* 20 OSM, it can be concluded that purified inulinase is an endo-acting enzyme. Dur-

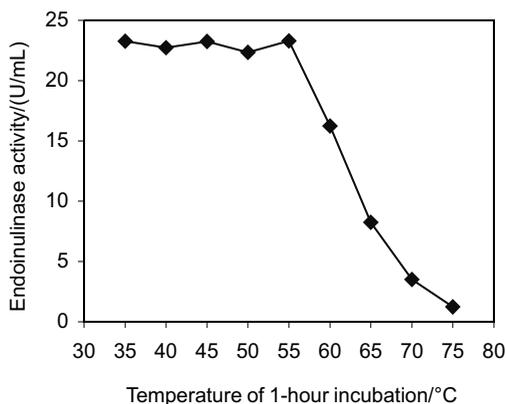


Fig. 2. Stability of endoinulinase from *A. niger* 20 OSM after 1-hour incubation at various temperatures

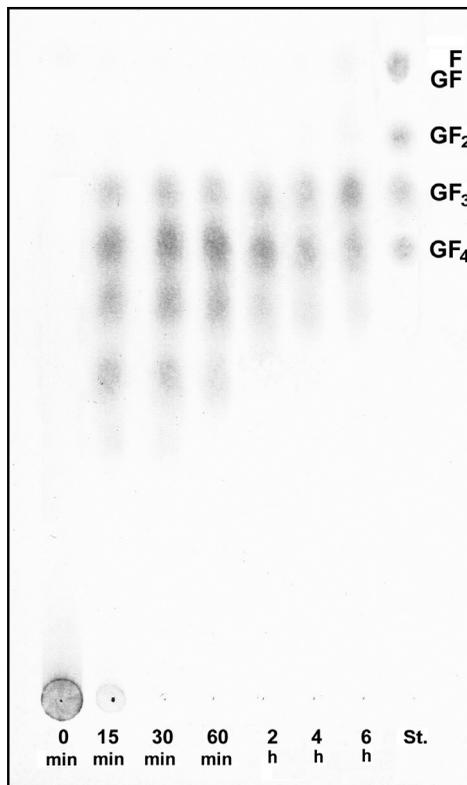


Fig. 3. Thin layer chromatography of inulin hydrolysate by the purified enzyme in the course of reaction Standards (St.): fructose (F), sucrose (GF), 1-kestose (GF₂), nystose (GF₃), 1'-β-fructofuranosylnystose (GF₄)

ing the hydrolysis process, at first longer saccharide chains appeared, which were subsequently cut into shorter fragments. Purified inulinase showed no detectable activity toward sucrose and little activity toward raffinose (0.8 % of relative enzyme activity on inulin). This confirms the claim that the enzyme is a typical endoinulinase. Using culture supernatant instead of purified inulinase led to producing fructose from inulin. This suggests that apart from endoinulinase, an exo-acting enzyme also occurs in culture filtrate.

Conclusion

For the industrial production of inulooligosaccharides, it is necessary to find an enzyme without traces of exoinulinase activity. Purified endoinulinase obtained from *A. niger* 20 OSM meets these requirements. Our results indicate that *A. niger* 20 OSM is a microorganism which can be successfully used for efficient industrial production of extracellular endoinulinase, as high amounts of oligofructans can be released from β-(2,1)-fructans after a very short period of incubation. Therefore, further studies are required to examine the use of the immobilized enzyme for inulin hydrolysis on a bioreactor scale.

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Pročišćavanje i svojstva ekstracelularne endoinulinaze iz soja *Aspergillus niger* 20 OSM

Sažetak

Ekstracelularna inulinaza (E.C. 3.2.1.7), proizvedena pri uzgoju *Aspergillus niger* 20 OSM u dvolitrenom fermentoru, izolirana je i pročišćena ionskom kromatografijom na DEAE sefarozi hidrofobnom interakcijom na fenil sefarozi, kromatofokusiranjem na koloni PBE-94 (Polybuffer Exchanger) te SEC kromatografijom (Size Exclusion Chromatography) na Sephadexu G-200. Enzim je homogen, sudeći prema SDS-PAGE elektroforezi, prividne

relativne molekularne mase od 69 ili 64 kDa, određene SEC kromatografijom. Enzim sadržava približno 44,5 % ugljikohidrata. Za enzimsku aktivnost optimalna je temperatura 55 °C uz pH=5,0. Određena su i neka fizikalno-kemijska svojstva (izoelektrična točka, K_m , V_{max} i E_a). Enzimska je aktivnost inhibirana s EDTA, pCMB, Hg^{2+} , Mn^{2+} i nekim drugim ionima metala. Kationi kalcija pozitivno su utjecali na enzimsku aktivnost. Kromatografijom na tankom sloju ustanovljeno je da je pročišćeni enzim tipična endoinulinaza.