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Partial Purification and Characterization of Exoinulinase from *Kluyveromyces marxianus* YS-1 for Preparation of High-Fructose Syrup

SINGH, RAM SARUP[1]*, RAJESH DHALIWAL[1], AND MUNISH PURI[2]

[1]*Carbohydrate and Protein Biotechnology Laboratory and*
[2]*Fermentation and Protein Technology Laboratory, Department of Biotechnology, Punjabi University, Patiala - 147 002, Punjab, India*

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Abstract An extracellular exoinulinase (2,1-β-D fructan fructanohydrolase, EC 3.2.1.7), which catalyzes the hydrolysis of inulin into fructose and glucose, was purified 23.5-fold by ethanol precipitation, followed by Sephadex G-100 gel permeation from a cell-free extract of *Kluyveromyces marxianus* YS-1. The partially purified enzyme exhibited considerable activity between pH 5 to 6, with an optimum pH of 5.5, while it remained stable (100%) for 3 h at the optimum temperature of 50°C. Mn^{2+} and Ca^{2+} produced a 2.4-fold and 1.2-fold enhancement in enzyme activity, whereas Hg^{2+} and Ag^{2+} completely inhibited the inulinase. A preparation of the partially purified enzyme effectively hydrolyzed inulin, sucrose, and raffinose, yet no activity was found with starch, lactose, and maltose. The enzyme preparation was then successfully used to hydrolyze pure inulin and raw inulin from *Asparagus racemosus* for the preparation of a high-fructose syrup. In a batch system, the exoinulinase hydrolyzed 84.8% of the pure inulin and 86.7% of the raw *Asparagus racemosus* inulin, where fructose represented 43.6 mg/ml and 41.3 mg/ml, respectively.

Keywords: *Kluyveromyces marxianus*, *Asparagus racemosus*, exoinulinase, partial purification, high-fructose syrup

A wide range of new and more desirable sweeteners has recently emerged on the scene to keep pace with the heightened health awareness among consumers. D-Fructose is an example of such sweeteners, which have also become important owing to their functional properties in addition to than their sweetness. For example, the demand for fructose is increasing because of beneficial role for diabetics, increased iron absorption in children, and increased ethanol removal from the blood of highly intoxicated individuals [11], in addition to its use as a low-calorie sweetener [31]. From an industrial viewpoint, fructose syrup is useful in pastry and confectionary production, as it prevents desiccation and sugar crystallization. Furthermore, it is highly soluble and has a low viscosity [7]. The worldwide commercial production of fructose syrup is based on the multienzymatic conversion of starch to fructose. However, this is now giving way to a more promising technology that includes the enzymatic hydrolysis of inulin, a polyfructan with glucose at the terminating end. Exoinulinase acts on inulin at the fructose terminal by cleaving a fructose residue, while also leaving a glucose unit at the end of the reaction. Various plant materials, such as dahlias, chicory, and Jerusalem artichokes, have already been reported as effective raw materials for high fructose production, yet there has been no previous report on the utilization of *Asparagus racemosus* for high-fructose syrup preparation. *Asparagus racemosus* is a perennial herb, the roots of which contain up to 15% inulin [9], plus it has a high annual world production. Accordingly, the present study investigates the purification and characterization of an exoinulinase from the newly isolated strain *Kluyveromyces marxianus* YS-1 and its application to the preparation of high-fructose syrup from the raw inulin of *Asparagus racemosus*.

*Corresponding author
Phone: 91-175-3046262; Fax: 91-175-2283073;
E-mail: rssingh11@lycos.com

Materials and Methods

Organism and Culture Conditions

The *Kluyveromyces marxianus* YS-1 used in the present study was originally isolated, identified, and maintained by the current authors, as discussed earlier [29]. Raw inulin extracted from dried and powdered roots of *Asparagus racemosus* was used in the fermentation medium for the production of inulinase, as described earlier [30]. The medium was inoculated

with 10% (v/v) of a 12-h-old culture and the fermentation carried out at 30°C under agitation (150 rpm) for 60 h on a shake-flask level. The fermented broth was centrifuged (2,500 ×*g*, 15 min, 4°C), and then the supernatant was assayed for enzyme activity and used for partial purification.

Partial Purification of Inulinase

Different concentrations (45–90%, v/v) of ethanol were added to each part of the supernatant and mixed slowly under mild-stirring conditions at 4°C. The samples were then kept undisturbed overnight at 4°C. The precipitates obtained were centrifuged (2,500 ×*g*, 30 min, 4°C) and the pellet dissolved in a sodium acetate buffer (0.1 M, pH 5.5). The dissolved pellet and supernatant were then assayed for their enzyme activity and protein content. The inulinase was further purified by Sephadex G-100 column chromatography, where the column (1×40 cm, Amersham Biosciences, U.S.A.) was first equilibrated with a sodium acetate buffer (0.1 M, pH 5.5), and then the enzyme sample was loaded. The elution was carried out using the same buffer, with the fractions (1 ml) collected at a flow rate of 0.5 ml/min and assayed for their enzyme activity and protein content. The fractions showing the highest enzyme activity were pooled. The partially purified enzyme was subsequently used for characterization and inulin hydrolysis.

Characterization of Inulinase

To identify the optimum temperature and pH for the inulinase, assays were carried out at different temperatures (25–80°C) and pHs (4–8) using a sodium acetate buffer (0.1 M, pH 3–6) and phosphate buffer (0.1 M, pH 7–8). To analyze the effect of metal ions, the inulinase activity was also checked in the presence of various ions (Mg^{2+}, Fe^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Ba^{2+}, Hg^{2+}, Ag^{2+}) and varying concentrations of EDTA (1–10 mM). The apparent K_m and V_{max} values for the exoinulinase were also calculated using a Lineweaver-Burk plot.

Stability Studies

The thermal stability was determined without any substrate or additives. The enzyme solution was kept at 50°C, 60°C, and 70°C in a 0.1 M sodium acetate buffer (pH 5.5) in a temperature-controlled water bath and the residual activity measured at regular time intervals. Similarly, the enzyme solution was incubated at different pHs (4–8) at 50°C to study the effect on the enzyme activity. Samples were withdrawn periodically and the residual activity measured in terms of the relative activity, which was calculated as the percentage ratio of activity at a given pH/temperature to the activity at the optimum pH/temperature.

Hydrolysis of Inulin

To produce a high-fructose syrup using free exoinulinase, the extraction of raw inulin was performed in a sodium acetate buffer (0.1 M, pH 5.5) after boiling dried and powdered *Asparagus racemosus* roots for 10 min. The slurry was then filtered through three layers of cheesecloth. For the hydrolysis, 5% raw *Asparagus racemosus* inulin was incubated with the inulinase (25 IU) under agitation (50–150 rpm) at 50°C for different time intervals (0.5–6 h). For comparison, the hydrolysis of a 5% pure inulin solution was carried out under the same conditions.

Inulinase Assay

A reaction mixture containing 0.1 ml of the enzyme extract and 0.9 ml of a sodium acetate buffer (0.1 M, pH 5.5) containing 2% inulin was incubated at 50°C for 15 min. Thereafter, the enzyme was inactivated by keeping the reaction mixture at 100°C for 10 min. The reaction mixture was then assayed for reducing sugars using the 3,5-dinitrosalicylic acid method. One inulinase unit (IU) was defined as the amount of enzyme that produced one micromole of reducing sugar per minute under standard assay conditions.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The SDS-PAGE was performed using 10% polyacrylamide according to the method of Laemmli [22]. The gels were stained with Coomassie Brillaint Blue R-250 and run on a Mini-Protein III electrophoresis system (Bio-Rad, U.S.A.) at a constant voltage (100 V).

Analytical Methods

The total sugars in the root extract were determined as reducing sugars after acid hydrolysis (H_2SO_4, pH 2.0, 45 min, 100°C) using the method of Miller [26]. The glucose was determined using a glucose oxidase-peroxidase kit (Sigma, U.S.A.), whereas the fructose was measured as the difference between the total sugars and the glucose. The protein content was determined according to Lowry [24] with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Partial Purification of Inulinase

The partial purification of the exoinulinase from *Kluyveromyces marxianus* YS-1 is summarized in Table 1. The exoinulinase from the filtrate was neither precipitated by saturation with ammonium sulfate nor acetone. However, the maximum enzyme precipitation was achieved with 85% (v/v) chilled ethanol, producing an 80.2% recovery. Earlier studies also reported that the inulinase from *K. marxianus* could not be precipitated by ammonium sulfate saturation [12, 28]. The precipitates obtained with ethanol were dissolved in a sodium acetate buffer (0.1 M, pH 5.5), and then loaded onto a Sephadex G-100 column. The elution

Table 1. Summary of purification procedures for exoinulinase from culture supernatant of *Kluyveromyces marxianus* YS-1.

Steps	Total units (IU)	Total protein (mg)	Specific activity IU/mg	Purification fold	Yield (%)
1. Crude enzyme	8,940	512	17.5	1.0	100
2. Ethanol precipitation	7,173	153	47	2.7	80.2
3. Sephadex G-100	6,202	15	413	23.5	69.3

pattern is shown in Fig. 1. The fractions with a high inulinase activity (Fractions 12–16) were pooled and used as partially purified inulinase in further experiments. The two-step purification strategy resulted in a 23.5-fold purification of the inulinase with a 69.3% recovery and 413 IU/mg specific activity. The SDS-PAGE of the partially purified inulinase is shown in Fig. 2. Further purification of the enzyme was not carried out, in view of the easy scaling-up and cost effectiveness of the present two-step purification method. The partially purified enzyme was characterized and used for the preparation of a high-fructose syrup.

Characterization of Enzyme

Generally, the characterization of an enzyme is required to develop an efficient process. Therefore, the initial characterization of the partially purified inulinase was then used to develop an efficient process for the preparation of a high-fructose syrup. The partially purified exoinulinase showed a pH optimum of 5.5. Nonetheless, even though the maximum activity appeared at 5.5, the enzyme was also appreciably active between pH 4.5–6.5 (data not shown). Enzymes obtained from different sources normally have variable pH optima, possibly due to different amino acid compositions, which in turn affect their state of ionization in a solution. Thus, an enzyme with a broad pH range is always preferable for application in the food industry. The optimum pH for the inulinase from yeast has been reported to be from 4.4 to 6.5 [2, 6, 13, 21, 27]. The optimum temperature for the partially purified enzyme at pH 5.5 was found to be 50°C. The enzyme exhibited more than 70% of its maximum activity between 35–60°C, which dropped to 60% activity at 65°C and became rapidly inactivated at 70°C and above (data not shown).

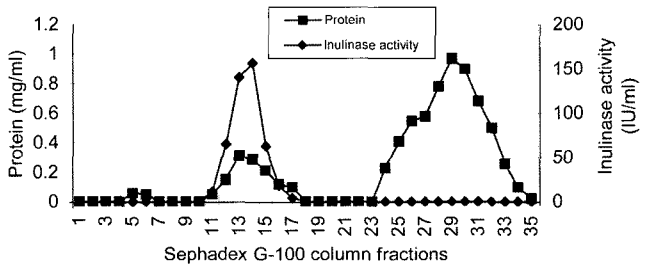

Fig. 1. Elution pattern of inulinase in Sephadex G-100 column (1×40 cm; equilibrated with sodium acetate buffer [0.1 M, pH 5.5]; eluted with same buffer; fraction size 1 ml; flow rate 0.5 ml/min).

Stability Studies

The enzyme preparation was incubated at different pHs for different time intervals (0–180 min), and found to remain quite stable over a range of pH 4.5–6.5, where it retained more than 60% of its original activity after 6 h of incubation at 50°C. A rapid decrease in enzyme activity was observed at pH 4 and above 7.5 (Fig. 3A). The thermal activity was then checked at and above the optimum temperatures. The enzyme remained 100% stable at 50°C for 3 h, whereas it lost 50% and 85% of its activity after half an hour at 60°C and 70°C, respectively (Fig. 3B). The thermostability of the inulinase from *K. marxianus* YS-1 was higher than that reported in an earlier study of *K. marxianus,* where a 50% loss of enzyme activity was observed after 40 min at 50°C [21]. Notwithstanding, the inulinase from *Bacillus stearothermophilus* has only been reported to be stable for 10 min at 60°C [16].

Substrate Specificity

The inulinase preparation was found to be active with 2% (w/v) inulin, sucrose, and raffinose, whereas it failed to hydrolyze maltose, lactose, and starch. The enzyme exhibited its maximum activity on sucrose (177.6%), followed by

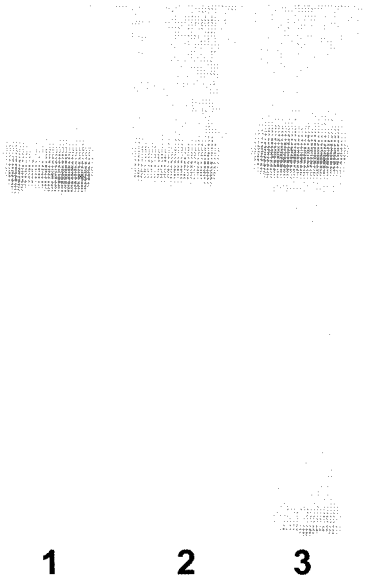

Fig. 2. SDS-PAGE of partially purified inulinase from *Kluyveromyces marxianus* YS-1.
Lane 1, Fraction from Sephadex G-100 column chromatography; lane 2, ethanol precipitates; lane 3, crude enzyme.

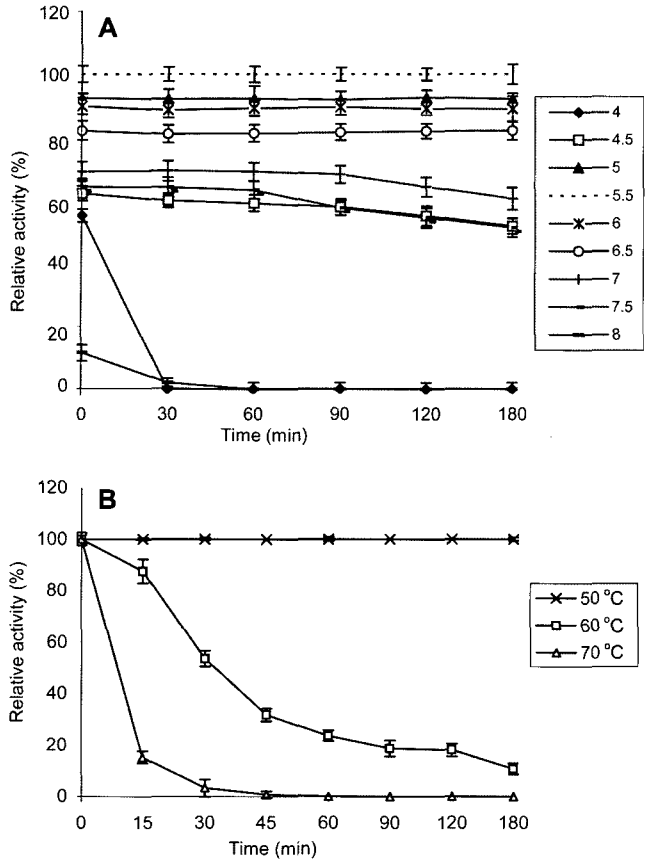

Fig. 3. Stability of inulinase: (**A**) at different pHs, (**B**) at different temperatures.

raffinose (69.0%), in relation to its activity with inulin, which was taken as 100%. The effect of different concentrations of inulin, raffinose, and sucrose on the inulinase activity was investigated and the results plotted using the method of Lineweaver and Burk. The Michaelis constant (K_m) and V_{max} for inulin, raffinose, and sucrose were found to be 3.4 mM and 7.6 μM/min, 27.0 mM and 2 μM/min, and 25.3 mM and 16.6 μM/min, respectively (Table 2). The lower K_m with inulin revealed the greater affinity of the enzyme towards this substrate. The K_m for the hydrolysis

Table 2. Kinetic parameters of exoinulinase.

Substrate	K_m (mM)	V_{max} (μM/min)
Inulin	3.4	7.6
Raffinose	2.7	2.0
Sucrose	25.3	16.6

of inulin by inulinase has previously been reported as 2.28 mM [18] and 3.92 mM [20], and in a literature search, the K_m values for inulinase with inulin ranged from 0.003 mM to 60 mM [3, 4].

Effect of Metal Ions

Most metal ions function as enzyme cofactors or prosthetic groups, and can participate with an enzyme to accelerate the rate of reaction through one of several mechanisms: general acid-base catalysis, covalent catalysis, the approximation of the reactants, or the induction of the strain in the enzyme or substrate. Therefore, the effect of metal ions on the inulinase activity was tested using different ions at varying concentrations. The maximum increase in enzyme activity was observed with the addition of $MnSO_4$ (2.4 times) and $CaCl_2$ (1.2 times), when compared with the control with no added metal ions, suggesting that the enzyme required these ions for a better catalytic action. In contrast, $HgCl_2$ and $AgNO_3$ were found to be potent inhibitors of the enzyme, even at lower concentrations than 1 mM, suggesting that certain -SH groups in the protein were essential for the enzyme activity [32]. $Fe_2(SO_4)_3$, $CuSO_4$, and EDTA at a 1 mM concentration inhibited the activity by approximately 38%, 47%, and 50%, respectively. The rest of the metal ions studied had no significant effect at lower concentrations. The stimulatory effect of Mn^{2+}, Ba^{2+}, and Ca^{2+} has already been observed in several previous studies [9, 10, 14, 15, 23], and similarly, inhibition by Fe^{3+}, Cu^{2+}, and Hg^{2+} has also been reported [15].

Hydrolysis Studies

The hydrolysis of the raw inulin from *Asparagus racemosus* and pure inulin was carried out with soluble exoinulinase

Table 3. Hydrolysis of (A) raw *Asparagus racemosus* inulin and (B) pure inulin with time and agitation at 50°C.

Time (min)	Hydrolysis (%) under agitation (rpm)									
	50		75		100		125		150	
	A	B	A	B	A	B	A	B	A	B
30	20.1	18.6	28.8	20.2	29.1	24.1	30.8	25.3	27.5	24.5
60	43.6	40.3	45.7	41.5	54.5	50.3	58.7	52.8	39.1	35.7
120	61.7	57.1	63.3	63.6	68.1	65.8	70.4	67.4	64.8	57.9
180	72.1	69.0	75.9	74.6	81.2	78.6	83.3	79.3	70.7	65.3
240	76.3	73.4	80.8	80.4	87.0	82.9	86.7	84.8	73.1	70.7
300	81.3	79.4	85.8	82.1	88.6	84.3	88.1	85.6	76.1	73.8
360	83.1	81.6	87.3	84.5	89.0	85.0	90.3	87.0	79.8	75.2

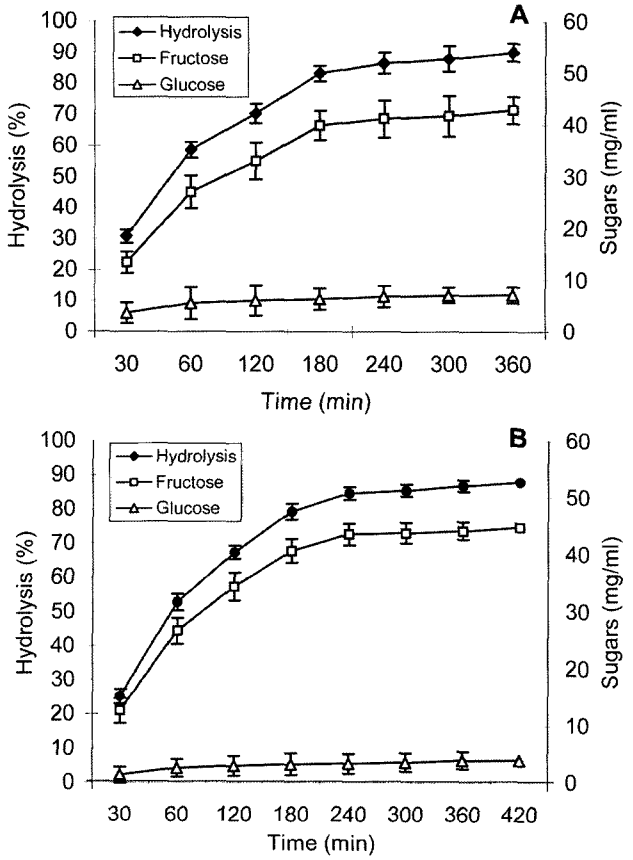

Fig. 4. Time course of sugars released from (**A**) *Asparagus racemosus* raw inulin and (**B**) pure inulin under agitation (125 rpm) at 50°C.

(25 IU) under agitation (50–150 rpm) at 50°C. For both substrates, shaking at 125 rpm was found to be optimal for the hydrolysis (Table 3). Therefore, the hydrolysis was studied as a function of time using this mode of agitation. The results showed that the hydrolysis of the raw inulin was initially very fast, yet after 4 h, a distinct slowdown was observed. After 4 h, 86.7% of the added raw inulin was hydrolyzed, whereas after 6 h, only 90% was hydrolyzed (Fig. 4A). The total reducing sugars released from the *A. racemosus* inulin were 48.1 mg/ml after 4 h, where fructose represented 41.3 mg/ml. A similar hydrolysis pattern was observed with the pure inulin (Table 3), where 84.8% and 87.0% were hydrolyzed after 4 and 6 h, respectively (Fig. 4B), and the total reducing sugars liberated after 4 h were 47.2 mg/ml, where fructose represented 43.6 mg/ml. The production of glucose and fructose was faster in the raw inulin when compared with the pure inulin during the initial stages of hydrolysis, indicating that the polymer with the lower degree of polymerization was hydrolyzed more rapidly than the one with the higher degree of polymerization. Similar results have also been reported in earlier studies [5], which were mostly carried out using Jerusalem artichokes. In a batch system, a 90% conversion of a Jerusalem artichoke extract yielded 34 mg/ml [17] and 77.5 mg/ml [19] of D-fructose, whereas the hydrolysis of a dried topinamber extract yielded 85.7% fructose in the total reducing sugars [25]. In a continuous system, the hydrolysis of Jerusalem artichokes yielded a mixture of 85% D-fructose and 15% D-glucose [34], whereas the hydrolysis of a chicory extract produced a mixture of fructose and glucose with a ratio of 3:1 [8], and kuth roots yielded 70% fructose after hydrolysis with inulinase [33]. As such, the amount of fructose obtained from the hydrolysis of the raw inulin from *Asparagus racemosus* was comparable to the fructose produced from Jerusalem artichokes, chicory, and kuth roots in previous studies.

The two-step purified exoinulinase displayed a good thermal and pH stability, plus significant kinetic properties. The hydrolysis of more than 85% of the raw inulin from *Asparagus racemosus* without prior treatment was encouraging. Furthermore, the preparation of a high-fructose syrup containing 85.7% fructose and only 14.3% glucose from *Asparagus racemosus* roots was also very promising. Consequently, with comparable results to chicory and Jerusalem artichokes, and yet a much higher world production [1], *Asparagus* plants would seem to be a good candidate for high-fructose syrup production.

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