Copyright © 2004 by Humana Press Inc.
All rights of any nature whatsoever reserved.
0273-2289/04/117/0019/$25.00

Purification and Characterization of an Exoinulinase From *Aspergillus fumigatus*

Prabhjot Kaur Gill,[1] Rajesh Kumari Manhas,[2] Jatinder Singh,[3] and Prabhjeet Singh*,[1]

Departments of [1]Biotechnogy, [2]Microbiology, and [3]Biochemistry, Guru Nanak Dev University, Amritsar-143005, Punjab, India, E-mail: Prabhjeet_s@yahoomail.com

Received July 23, 2003; Revised October 22, 2003; Accepted October 30, 2003

Abstract

An extracellular exoinulinase was purified from the crude extract of *Aspergillus fumigatus* by ammonium sulfate precipitation, followed by successive chromatographies on DEAE-Sephacel, Sephacryl S-200, concanavalin A-linked amino-activated silica, and Sepharose 6B columns. The enzyme was purified 25-fold, and the specific activity of the purified enzyme was 171 IU/mg of protein. Gel filtration chromatography revealed a molecular weight of about 200 kDa, and native polyacrylamide gel electrophoresis (PAGE) showed an electrophoretic mobility corresponding to a molecular weight of about 176.5 kDa. Sodium dodecyl sulfate-PAGE analysis revealed three closely moving bands of about 66, 62.7, and 59.4 kDa, thus indicating the heterotrimeric nature of this enzyme. The purified enzyme appeared as a single band on isoelectric focusing, with a p*I* of about 8.8. The enzyme activity was maximum at pH 5.5 and was stable over a pH range of 4.0–9.5, and the optimum temperature for enzyme activity was 60°C. The purified enzyme retained 35.9 and 25.8% activities after 4 h at 50 and 55°C, respectively. The inulin hydrolysis activity was completely abolished with 1 m*M* Hg^{++}, whereas EDTA inhibited about 63% activity. As compared to sucrose, stachyose, and raffinose, the purified enzyme had lower K_m (0.25 m*M*) and higher V_{max} (333.3 IU/mg) values for inulin.

Index Entries: Inulin; exoinulinase; fructose; *Aspergillus fumigatus*; concanavalin A.

Introduction

The importance of fructose and fructooligosaccharides in human nutrition has increased significantly and gained tremendous commercial

*Author to whom all correspondence and reprint requests should be addressed.

importance owing to their favorable functionalities, such as improving the intestinal microflora, relieving constipation, decreasing the total cholesterol and lipid in serum, promoting animal growth, a low-calorie noncariogenic sweetener *(1)*, preventing gastrointestinal infections *(2)*, and enhancing local and systemic immune responses *(3)*. The conventional fructose production from starch needs at least three enzymatic steps, yielding only 45% fructose. However, fructose formation from inulin catalyzed by the enzyme inulinase 2 → 1, β-D fructan-fructanohydrolase (EC 3.2.1.7) is a single-step enzymatic reaction and yields up to 95% free fructose, without the inconvenience of undesirable side products. Inulin is a polysaccharide of β-(2,1)-linked fructose residues attached to a terminal glucose molecule and is accumulated in the underground organs of chicory, dahlia, and Jerusalem artichoke *(4)*.

Industrial inulin hydrolysis is carried out at 60°C in order to prevent microbial contamination and also because it permits the use of a higher soluble inulin substrate concentration owing to increased solubility *(4)*. We previously reported inulinase activity in the extracellular extract of *Aspergillus fumigatus (5)*, which was thermostable. In this article, we report the purification and characterization of an inulinase from the extracellular extract of *A. fumigatus*.

Materials and Methods

Chemicals

Pure inulin from chicory roots, DEAE-Sephacel, and Sepharose 6B were purchased from Sigma (St. Louis, MO), and Sephacryl S-200 was purchased from Pharmacia. All other chemicals were of the highest analytical grade.

Preparation of Crude Enzyme

The strain of *A. fumigatus* (MTCC no. 3009) used was isolated from soil in our laboratory *(6)*. The preparation of inoculum and inoculation procedure of this fungus have been described elsewhere *(5)*. *A. fumigatus* was grown on a medium containing 1% inulin, and after 3 d of growth, the culture was passed through double-layered muslin cloth and the filtrate centrifuged at 10,000*g* for 15 min. The supernatant was used as a crude enzyme extract throughout the purification process.

Estimation of Protein

Protein content was measured by the Bradford method *(7)*, using bovine serum albumin (BSA) as a standard.

Enzyme Assay

Inulinase and invertase activities were assayed and described by Kaur et al. *(5)* and reducing sugars were estimated by Nelson *(8)*. The sucrose/inulin hydrolysis (S/I) ratio was calculated according to Vandamme and Derycke *(4)*.

Enzyme Purification

Crude extract was first concentrated by ammonium sulfate precipitation at 40–80% saturation, and the resulting precipitates were removed by centrifuging at 10,000*g* for 20 min. The precipitates were dissolved in a minimum volume of 0.1*M* sodium acetate buffer, pH 5.5, and dialyzed overnight against several changes of the same buffer.

Ion-Exchange Chromatography on DEAE-Sephacel

The dialyzed ammonium sulfate–precipitated fraction was applied on a DEAE-Sephacel column (2.6 × 20 cm) equilibrated with 50 m*M* Tris, pH 8.0. The unadsorbed proteins were eluted from the column with starting buffer (200 mL), and the adsorbed proteins were desorbed with a continuous gradient of NaCl (0 to 1*M*) in the same buffer (500 mL). Fractions of 1 mL each were collected at a flow rate of 40 mL/h.

Gel Filtration on Sephacryl S-200

The active fractions eluted from DEAE-Sephacel were pooled, concentrated, and then loaded onto a Sephacryl S-200 column (1.6 × 95 cm) equilibrated with 0.1*M* sodium acetate buffer, pH 5.5. The proteins were eluted with the same buffer, and 1-mL fractions were collected at a flow rate of 20 mL/h.

Affinity Chromatography on Concanavalin A-Linked Amino-Activated Silica

Concanavalin A (ConA) was immobilized on amino-activated silica as described previously *(9)*. The active fractions eluted from S-200 were concentrated and used for further purification on a column of ConA-linked amino-activated silica (2.6 × 2.0 cm) equilibrated with 10 m*M* phosphate-buffered saline (PBS), pH 7.2. The unadsorbed proteins were eluted from the column with the same buffer, whereas the adsorbed proteins were eluted in a stepwise gradient of methyl-α-D-mannopyranoside (0–0.5 *M*) in 10 m*M* PBS buffer, pH 7.2. The active fractions eluted from ConA were pooled and concentrated and then applied on a Sepharose 6B column (1.6 × 30 cm) equilibrated with 0.1 *M* sodium acetate buffer, pH 5.5. The separated and purified fractions were concentrated and stored at –20°C until further analysis.

Determination of Purity

To monitor the progress of purification of the enzyme, polyacrylamide gel electrophoresis (PAGE) under native conditions was performed according to Laemmli *(10)*. Proteins in the gels were stained by silver staining, and inulinase activity was detected *in situ* by the staining procedure of Gabriel and Wang *(11)*.

Isoelectric Focusing

Nondenaturing isoelectric focusing of the purified fraction was performed in tube gels according to Celis et al. *(12)*. Focusing gels (7-cm length, 0.4-cm diameter) contained 5% acrylamide and 1% (v/v) ampholines, pH 3.6–9.3. Cathodic solution was 20 m*M* NaOH and anodic solution, 10 m*M* H_3PO_4. Running conditions were 20 V/cm gel length for 1 h, then 40 V/cm gel length for 2 h, and after focusing, the samples were loaded at the anodic side and run for 12 h at 400 V. For p*I* determination, the tube gels were loaded with standard protein markers (Sigma). The gels were incubated in 10% trichloroacetic acid for 2 h to remove ampholytes, followed by silver staining.

Determination of Molecular Mass of Purified Enzyme and Subunits

The molecular mass of the purified inulinase was determined by gel filtration on a 1.6 × 90 cm Sephacryl S-200 column, which was calibrated with the following standard proteins: carbonic anhydrase (29 kDa), BSA (66 kDa), β-amylase (200 kDa), and apoferritin (443 kDa).

To determine the molecular weight of the enzyme subunits, sodium dodecyl sulfate (SDS)-PAGE was performed according to Laemmli *(10)*. Proteins in the gel were stained by silver staining, and the apparent M_r was determined using commercially available marker proteins (Sigma).

Determination of Hydrolysis Products

To analyze the product(s) of inulin hydrolysis, purified enzyme was incubated at 60°C for 24 h with 1.1% inulin in 0.1*M* sodium acetate buffer, pH 5.5. The products were analyzed by descending paper chromatography on 3MM Whatman paper using *n*-butanol:acetic acid:water (4:1:5 [v/v/v]) as an irrigating solvent, and the sugars were visualized by staining with $AgNO_3$ in acetone *(13)*.

Kinetic Parameters

The Michaelis-Menten constant (K_m) and maximum reaction velocity (V_{max}) for inulin (0.5–10 m*M*), sucrose (5–250 m*M*), raffinose (2–30 m*M*), and stachyose (2–40 m*M*) were determined from simple Lineweaver-Burk plots using Grafit software.

Enzyme Properties

Effect of pH on Enzyme Activity and Its Stability

The effect of pH on inulinase activity was determined in the pH range of 4.0–10.0 using 0.1*M* sodium acetate (pH 4.0–5.5), citrate-phosphate (pH 5.5–6.5), sodium phosphate (pH 6.5–8.0), Tris-HCl (pH 8.0 to 9.0), and glycine-NaOH (pH 9.0 to 10.0) buffers. The enzyme activity was measured at 60°C for 1 h. In addition, the influence of pH on enzyme stability was studied by incubating the enzyme at 40°C for 1 h in 0.1*M* buffer at different

Table 1
Effect of Individual Purification Steps on Inulinase Activity From *A. fumigatus*

Purification step[a]	Total protein (mg)	Total activity (IU)	Specific activity (IU/mg protein)	Activity yield (%)	Purification (fold)	S/I ratio
CE	120.00	810	6.80	100.0	1.00	1.00
ASF	5.64	130	23.00	16.0	3.40	0.83
AECF	1.86	105	56.45	13.0	8.30	0.51
GFCF	0.36	36	100.00	4.4	14.70	0.32
ACF	0.16	22	137.50	2.7	20.20	0.31
Sepharose 6B	0.07	12	171.00	1.5	25.15	0.22

[a]CE, crude extract; ASF, ammonium sulfate fraction, precipitated between 40 and 80%; AECF, anion-exchange chromatography fraction, using DEAE-Sephacel; GFCF, gel filtration chromatography fraction, using Sephacryl S-200; ACF, affinity chromatography fraction, using ConA-linked amino-activated silica.

pH values in the absence of inulin, followed by estimating the residual activity of the enzyme under standard conditions.

Effect of Temperature on Enzyme Activity and Its Stability

The effect of temperature on enzyme activity was measured at pH 5.5 in 0.1*M* sodium acetate buffer over a temperature range of 40–75°C. After incubating at the appropriate temperature for 1 h, the reaction was stopped and reducing sugars were estimated by the Nelson method *(8)*. Thermostability in the presence of inulin was determined by measuring the residual activity of the enzyme at different intervals (up to 5 h) after incubating at various temperatures.

Effect of Metal Ions on Enzyme Activity

The effect of different metal ions and other chemicals on inulinase activity was examined at 60°C (pH 5.5) for 1 h.

Results and Discussion

The results of purification of inulinase from the extracellular extract of *A. fumigatus* are summarized in Table 1. Most of the inulinase activity could be precipitated from the culture filtrate by 40–80% saturation with ammonium sulfate. The precipitates were dissolved in a minimum volume of sodium acetate buffer, pH 5.5, and after dialysis were loaded onto an ion-exchange column of DEAE-Sephacel. A broad peak appeared (fraction no. 16-39) during elution with a linear gradient of 1*M* NaCl (Fig. 1A). Chromatography of active fractions on Sephacryl S-200 improved specific activity considerably and increased the purification by almost 15-fold (Fig. 1B, Table 1). However, after native PAGE there still appeared to be contaminating proteins (data not shown). Further purification was therefore pursued by affinity chromatography on ConA-linked amino-activated silica (Fig. 1C). The retention of inulinase on ConA-affinity gel implied its glycoprotein

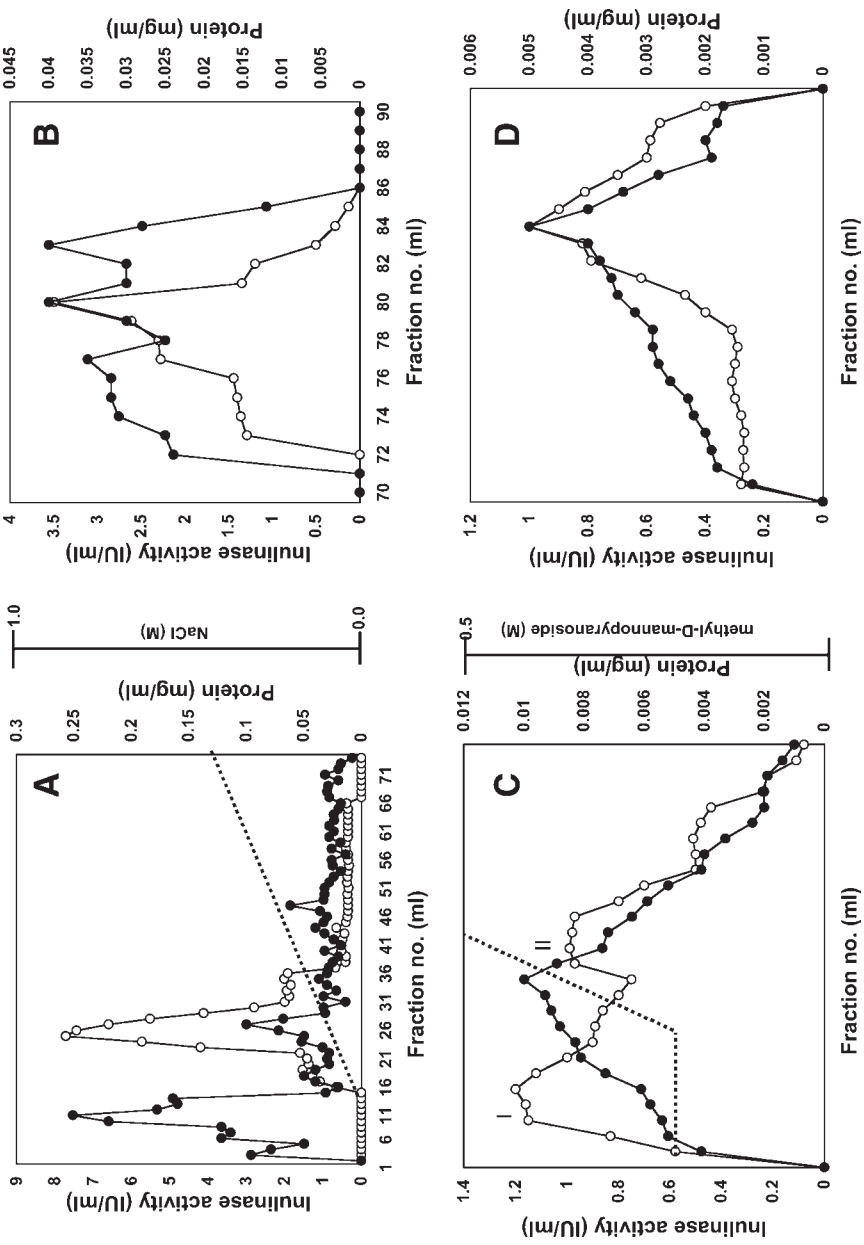

Fig. 1. Elution protein profile (●); inulinase activity profile (○); and eluent gradient profile, NaCl or methyl-α-D-mannopyranoside (– – –), from extracellular extract of *A. fumigatus* by chromatography on **(A)** DEAE-Sephacel, **(B)** Sephacryl S-200, **(C)** ConA-linked amino-activated silica, and **(D)** Sepharose 6B.

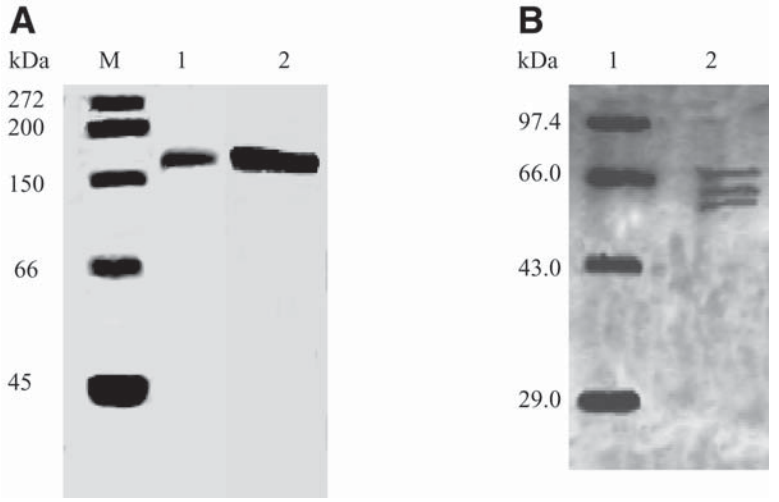

Fig. 2. **(A)** Native PAGE electrophoretogram of purified inulinase fraction. The inulinase band was detected by silver staining *(lane 1)* and by *in situ* detection of activity *(lane 2)*. Lane M, molecular weight standards. **(B)** SDS-PAGE analysis of purified inulinase *(lane 2)* for determining subunit composition.

nature, with α-D-mannopyranosyl residues as its glycosidic moiety. Elution with methyl-α-D-mannopyranoside resulted in two active peaks (I and II). Fractions 2–13 (peak I) were pooled, concentrated, and loaded onto a Sepharose 6B column (Fig. 1D), which resulted in 25-fold purification as compared to the crude extract. The purified inulinase fractions were homogeneous, since native PAGE analysis revealed only a single protein band. The invertase to inulinase activity ratio (S/I) of the purified fraction was about 0.2, compared with 1 for the crude extract. According to Vandamme and Derycke *(4)*, true inulinase has an S/I ratio <10, thus signifying that the purified enzyme from *A. fumigatus* was a true inulinase. The inulinase activity in peak II was contributed by a distinctly different inulinase, which has been partially purified (data not shown).

Molecular Mass of Purified Enzyme

The molecular weight of the purified inulinase was determined on a calibrated gel filtration column of Sephacryl S-200 and was estimated as about 200 kDa. However, native PAGE analysis suggested a molecular mass of about 176.5 kDa (Fig. 2A). The SDS-PAGE analysis revealed three closely moving bands of about 66, 62.7, and 59.4 kDa (Fig. 2B), and this pattern remained unaffected even in the presence of iodoacetamide, dithiothreitol, and also increasing concentrations of β-mercaptoethanol, thus indicating the heterotrimeric nature of this enzyme. Considerable variation in molecular weight of inulinases from *Aspergillus* species has been reported, with inulinases from *A. ficuum*, *A. candida*, *A. versicolor*, *A. awamori*, and *A. niger* having a mol wt of 74, 54, 230 ± 20, 69 ± 1, and

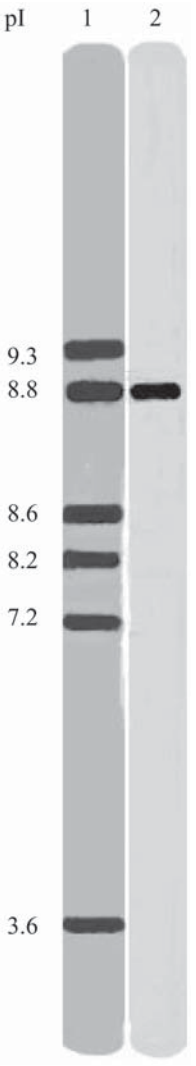

Fig. 3. Isoelectric focusing of purified inulinase: *lane 1*, markers; *lane 2*, purified inulinase.

300 kDa, respectively *(14–18)*. Our results suggest that the inulinase of *A. fumigatus* is likely to be different from other *Aspergillus* inulinases reported so far. Isoelectric focusing of the purified enzyme revealed a single band corresponding to p*I* 8.8 (Fig. 3). Most of the inulinases reported so far have a p*I* ranging from 3.5 to 5.2 (4,17,19), thus supporting the distinct nature of *A. fumigatus* inulinase.

Mode of Action

For determining the exo or endo nature of the purified inulinase, the cleavage products of inulin hydrolysis were analyzed by paper chromatog-

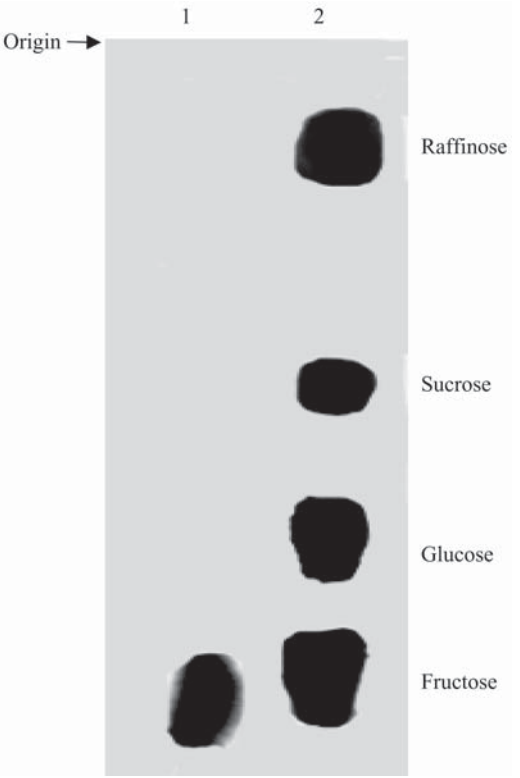

Fig. 4. Paper chromatogram (descending) showing exoinulinolytic activity of purified enzyme *(lane 1)* and standard sugars *(lane 2)*.

Table 2
Substrate Specificity
of Purified Inulinase of *A. fumigatus*

Substrate	K_m (m*M*)	V_{max} (IU/mg)
Inulin	0.25	333
Sucrose	0.85	105
Raffinose	0.71	52
Stachyose	0.66	108

raphy. Fructose was found to be the only product of inulin hydrolysis reaction. The formation of any other fructooligosaccharides was not observed (Fig. 4), which suggests that inulinase of *A. fumigatus* is an exoinulinase. Besides hydrolyzing inulin, the *A. fumigatus* inulinase also hydrolyzed stachyose, raffinose, and sucrose, with a minimum K_m value being observed for inulin (Table 2). Although the purified inulinase showed broad substrate specificity, the comparatively lower K_m (0.25 m*M*) and higher V_{max} (333.3 IU/mg) for inulin demonstrates its greater affinity for inulin. Compared with the K_m values reported for inulinases from other microorgan-

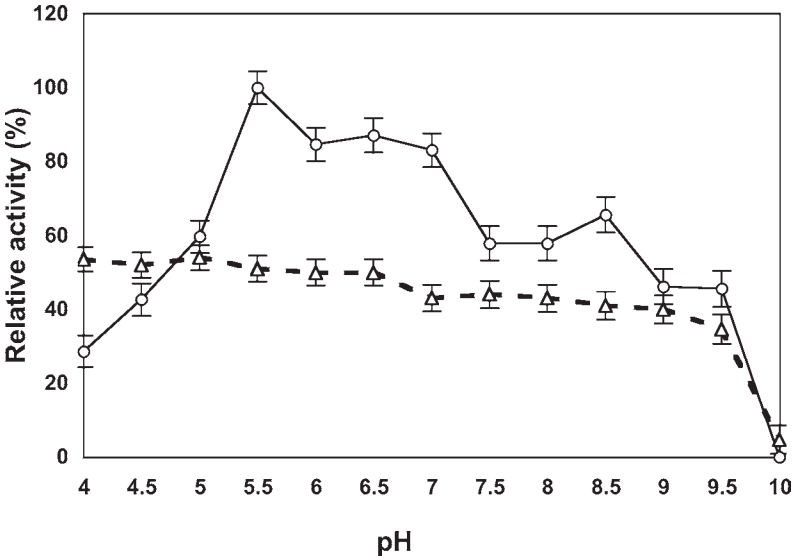

Fig. 5. Effect of pH on activity (○) and stability (△) of purified inulinase from *A. fumigatus*. Data represent the mean of three replicates ±SE.

isms—*A. niger* (1.25–1.87 m*M*) *(4)*, *A. ficuum* (10–15 m*M*) *(14)*, *Debaryomyces cantarelli* (15 m*M*) *(20)*, and *Candida salmenticensis* (17 m*M*) *(21)*—the lower K_m value of the *A. fumigatus* inulinase for inulin makes it a better candidate for inulin hydrolysis.

Effect of pH on Activity and Stability

The activity of purified enzyme was measured at various pH values ranging between 4.0 and 10.0 (Fig. 5). The enzyme was active over a broad pH range, with the optimum pH being 5.5. This pH optimum is comparable with the pH optima of 5.5, 4.5, 4.4, 4.8–5.0, and 4.6 reported for inulinases from *A. versicolor*, *A. awamori*, *A. niger*, *Penicillium janczewskii*, and *Clostridium acetobutylicum*, respectively *(16,17,22–24)*. However, *Fusariun oxysporum* and *Penicillium aculeatum* display optimal activity for various purified inulinases at pH 5.5–7.0 and 6.0, respectively *(25,26)*. The lower pH optima are advantageous for industrial fructose syrup preparations because they prevent undesired color formation *(4)*. The purified inulinase from *A. fumigatus* was stable between pH 4.0 and 9.5, with more than 50% of the maximum activity retained between pH 4.0 and 6.5 and about 40% activity observed even at pH 9.5. The stability of the purified enzyme over a broad range of pH is consistent with the pH stability observed for other inulinases *(14,26)*.

Effect of Temperature on Inulinase Activity and Stability

The optimum temperature of 60°C observed for the purified inulinase (Fig. 6A) is in accordance with that reported for inulinases from other

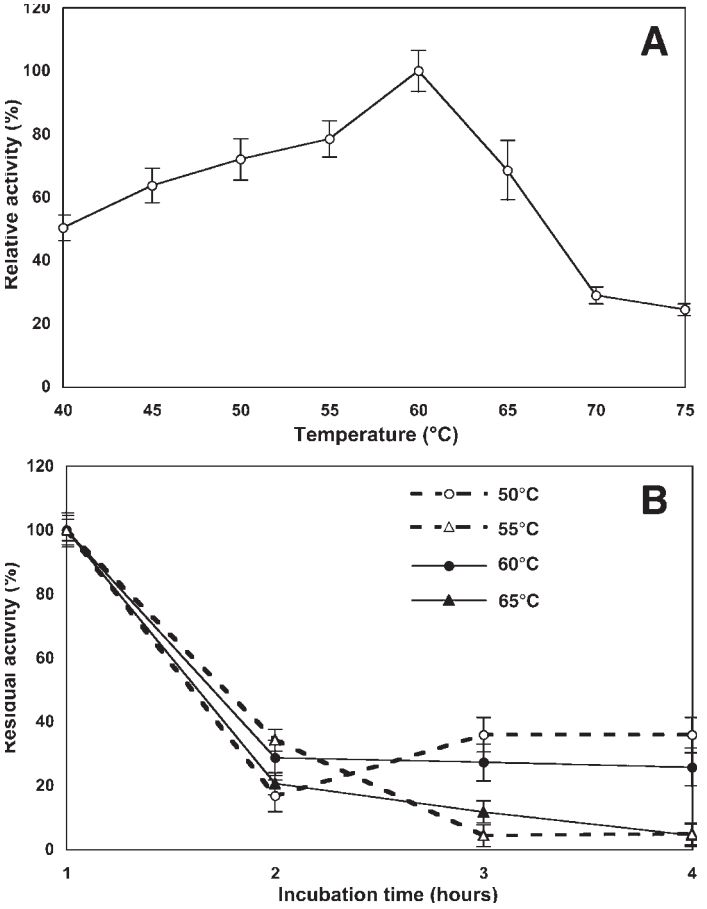

Fig. 6. Effect of different temperatures on **(A)** activity and **(B)** stability of *A. fumigatus* inulinase. Data represent the mean of three replicates ±SE.

Aspergillus species, namely, *A. ficuum*, *A. versicolor*, and *A. awamori (14,16,17)*. However, compared with inulinases from *P. janczewskii*, *F. oxysporum*, *P. aculeatum*, and *Penicillium digitatum*, which have temperature optima of 55, 35–45, 40, and 45°C, respectively *(23,25,26)*, the temperature optimum of *A. fumigatus* inulinase is higher. The thermostability of the purified inulinase was determined in the presence of inulin at different temperatures for up to 4 h. It retained 35.9 and 25.8% activities after 4 h at 50 and 55°C, respectively, but lost 89% activity at 60 and 65°C after 3 h (Fig. 6B). The thermal stability of the purified inulinase from *A. fumigatus* is considerably higher (100% for 60 min at 60°C) than that reported for inulinases from some other microbes, namely, *F. oxysporum* (retains 100% activity for 10–15 min at 50°C), *A. niger* (retains 100% activity for 30 min at 55°C), *Penicillium* spp. (100% stable for 30 min at 40°C), and *Kluyveromyces fragilis* (retains 100% activity for 10 min at 60°C) *(25,27–29)*. The higher temperature optimum and greater thermostability of *A. fumigatus*

Table 3
Effect of Metal Ions and Other Chemicals
on Activity of Purified Inulinase of *A. fumigatus*[a]

Compound	Concentration	Relative activity (%)
Control	—	100
$HgCl_2$	1 m*M*	Nil
$NaNO_3$	1 m*M*	83[b]
NaCl	1 m*M*	77[b]
$FeSO_4 \cdot 7H_2O$	1 m*M*	79[b]
KCl	1 m*M*	78[b]
$MgSO_4 \cdot 7H_2O$	1 m*M*	84[b]
$CuSO_4$	1 m*M*	71[b]
$CaCl_2$	1 m*M*	80[b]
$MnCl_2$	1 m*M*	78[b]
$AgNO_3$	1 m*M*	68[b]
$BaCl_2$	1 m*M*	94
$CoCl_2$	1 m*M*	92
EDTA	1 m*M*	37[b]
$(NH_4)_2SO_4$	1 m*M*	81[b]
Urea	1.0 *M*	41[b]
Thiourea	1.0 *M*	06[b]
Guanidine hydrochloride	1.0 *M*	Nil

[a]The data represent the mean of three replicates.
[b]Significantly different at $p \leq 0.05$.

inulinase is a desirable feature for industrial production of fructose from inulin, because higher temperature lowers the chance of microbial contamination of bioreactors and also increases the solubility of inulin.

Tolerance to metal ions is an important aspect in the commercial application of inulinase for fructose production. Of the various metal ions tested, none resulted in enhanced inulinase activity (Table 3). The results imply that *A. fumigatus* inulinase has distinct physicochemical properties from inulinases of *A. ficuum* and *A. niger*, which were reported to be activated by Ca^{2+} and Mn^{2+}, respectively *(14,22)*. Total inhibition of inulin hydrolytic activity was observed in the presence of Hg^{2+}, whereas the inhibitory effect of Ca^{2+}, Fe^{2+}, Cu^{2+}, Mg^{2+}, K^+, Mn^{2+}, Na^+, and Ag^+ was marginal. Inulinase activity was more or less unaffected by the presence of $BaCl_2$ and $CoCl_2$. Similar observations have been previously reported for *A. versicolor* inulinase as well *(16)*. The presence of NH_4^+ ions also resulted in only marginal (20%) inhibition of *A. fumigatus* inulinase.

Enzyme activity was also strongly inhibited (63%) by the metal chelating agent EDTA, thus suggesting the possible involvement of metal for the enzyme. The strong inhibitory effect observed with Hg^{2+} suggested that some –SH– group in the protein was essential for the activity. This has also been previously observed for other microbial inulinases *(14,16)*. The effect of different concentrations of urea, thiourea, and guanidine hydrochloride

was studied on inulin hydrolysis activity of *A. fumigatus* inulinase. Thiourea at a 1*M* concentration resulted in almost total loss of inulinase activity, whereas at the same concentration the inhibition by urea was about 60%. Guanidine hydrochloride at 0.5*M* caused total inactivation of the enzyme. The loss of inulin hydrolytic activity at comparable concentrations of these chaotropic agents has also been reported previously for *Panaeolus papillonaceus* inulinase *(30)*. However, compared with *D. cantarelli* inulinase, which was inhibited in the presence of just 10 µ*M* urea *(20)*, the *A. fumigatus* inulinase appears to be more stable.

Conclusion

We have reported the purification of a novel inulinase from an extracellular extract of *A. fumigatus*. The purified *A. fumigatus* inulinase is an exoacting enzyme that hydrolyzes inulin to produce fructose monomer. The purified inulinase is a trimeric protein of dissimilar subunit structure with a p*I* of 8.8. The temperature and pH optima of 60°C and 5.5, respectively, coupled with its thermostable nature, make this inulinase a potential candidate for production of fructose from inulin. Further studies are in progress to exploit its fructose-producing potential at the industrial level in various biotechnological applications. Immobilization and full-length cloning of this inulinase is also under way.

Acknowledgment

We are grateful to the Department of Biotechnology, Government of India, New Delhi, for supporting this research.

References

1 Chen, W. C. and Liu, C. H. (1996), *Enzyme Microb. Technol.* **1296,** 153–160.
2 Elmer, G. W. (1986), *JAMA* **275,** 870–876.
3 Hatcher, G. E. and Lambrecht, R. S. (1993), *J. Dairy Sci.* **76,** 2485–2492.
4 Vandamme, E. J. and Derycke, D. G. (1983), *Adv. Appl. Microbiol.* **29,** 139–176.
5 Kaur, A., Sharma, D., Harchand, R. K., Singh, P., Bhullar, S. S., and Kaur, A. (1999), *Indian J. Microbiol.* **39,** 99–103.
6 Sharma, D., Kaur, A., Harchand, R. K., Singh, P., Bhullar, S. S., and Kaur, A. (1998), *Indian J. Microbiol*. **38,** 235, 236.
7 Bradford, M. M. (1976), *Anal. Biochem*. **72,** 248–254.
8 Nelson, N. (1944), *J. Biol. Chem.* **153,** 375–380.
9 Singh, J., Kamboj, S. S., Sandhu, R. S., Shangary, S., and Kamboj, K. K. (1993), *Phytochemistry* **33,** 979–983.
10 Laemmli, U. (1970), *Nature* **227,** 680–685.
11 Gabriel, O. and Wang, S. F. (1969), *Anal. Biochem*. **27,** 545–554.
12. Celis, J. E., Lauridsen, J. B., and Basse, B., eds. (1994), in *Cell Biology: A Laboratory Handbook*, 2nd ed., Academic, San Diego, pp. 305–313.
13 Trevelyan, W. E., Procter D. P., and Harrison, J. S. (1950), *Nature* **166,** 444, 445.
14 Ettalibi, M. and Baratti, J. C. (1987), *Agric. Biol. Chem.* **54,** 61–68.
15 Kochhar, A., Gupta, A. K., and Kaur, N. (1999), *J. Sci. Food Agric*. **79,** 549–554.
16 Kochhar, A., Kaur, N., and Gupta, A. K. (1997), *J. Sci. Ind. Res*. **57,** 184–187.
17 Arand, M., Golubev, A. M., Neto, J. R. B., et al. (2002), *Biochem. J.* **362,** 131–135.

18. Nakamura, T., Kurokawa, T., Nakatsu, S., and Ueda, S. (1978), *Nippon. Nogeikagaku. Kaishi*. **52,** 581–587.
19 Uhm, T. A. and Byun, S. M. (1987), *Biotechnol. Lett*. **9,** 287–290.
20 Beluche, I., Guiraud, J. P., and Galzy, P. (1980), *Folio Microbiol*. **25,** 32–39.
21 Guirad, J. P., Viard-Gaudin, C., and Galzy, P. (1980), *Agric. Biol. Chem*. **44,** 245–252.
22 Derycke, D. G. and Vandamme, E. J. (1984), *J. Chem. Tech. Biotechnol*. **34,** 45–51.
23 Pessoni, R. A. B., Figueiredo, R., and Braga, M. R. (1999), *J. Appl. Microbiol*. **87,** 141–147.
24 Efstathiou, I., Reysset, G., and Truffaut, N. (1986), *Appl. Microbiol. Biotechnol*. **25,** 143–149.
25 Kaur, N., Kaur, M., Gupta, A. K., and Singh, R. (1992), *J. Chem. Tech. Biotechnol*. **53,** 279–284.
26. Hazaa, M. M. (1999), *J. Union Arab. Biol*. **8,** 467–482.
27 Nakamura, T., Ogata, Y., Shitara, A., Nakamura, A., and Ohta, K. (1995), *J. Ferment. Bioeng*. **80,** 164–169.
28 Nakamura, T., Shitara, A., Matsuda, S., Matsuo, T., Suiko, M., and Ohta, K. (1997), *J. Ferment. Bioeng*. **84,** 313–318.
29 Nahm, B. H. and Byun, M. (1977), *Korean Biochem. J.* **10,** 95–108.
30. Mukherjee, K. and Sengupta, S. (1987), *Can. J. Microbiol*. **33,** 520–524.