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Purification and characterisation of exo- and endo-inulinase from *Aspergillus ficuum* JNSP5-06

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ABSTRACT

Three exoinulinases (Exo-I, Exo-II, and Exo-III) and two endoinulinases (Endo-I and Endo-II) were purified from the culture broth of *Aspergillus ficuum* JNSP5-06 by ammonium sulphate precipitation, DEAE-cellulose column chromatography, Sepharose CL-6B column chromatography and preparative electrophoresis. The molecular weights of Exo-I, Exo-II, Exo-III, Endo-I, and Endo-II were determined to be 70 kDa, 40 kDa, 46 kDa, 34 kDa, and 31 kDa, respectively. Using inulin as the substrate, their K_m values were 43.1 mg/ml, 31.5 mg/ml, 25.3 mg/ml, 14.8 mg/ml, and 25.6 mg/ml, respectively. These five inulinases were stable below 50 °C with optimum activity at 45 °C, and were stable at a pH range of 4–8 with an optimum pH at 4.5 for exoinulinase and at 5.0 for endoinulinase. The inulinase activity was completely inhibited by Ag^+ and strongly inhibited by Fe^{2+} and Al^{3+}, whereas K^+, Ca^{2+}, Li^{2+}, EDTA and urea had no significant influence on the inulinase activity.

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1. Introduction

Inulin occurs as a carbohydrate reserve, mainly in the roots and tubers of Jerusalem artichoke, chicory, dandelion, burdock and dahlia. It consists of linear chains of β-2,1-linked D-fructofuranose residues terminated by a glucose residue through a sucrose-type linkage at the reducing end (Vandamme & Derycke, 1983). Recently, inulin has received attention as a relatively inexpensive and abundant material for production of fructose and fructooligosaccharides, which are extensively used in the pharmaceutical industry and the food industry (Vandamme & Derycke, 1983).

Compared with sucrose, fructose is considered to be a safe alternative sweetener because it has higher sweetening capacity, beneficial effects on diabetic patients and increases iron absorption in children. But sucrose will cause a series problems associated with corpulence, cariogenicity, arteriosclerosis and diabetes (Vandamme & Derycke, 1983). Fructooligosaccharides have good functional and nutritional properties, such as for a low calorie diet, *Bifidobacteria* stimulating factor, and a source of dietary fibre in food preparations. Therefore, fructooligosaccharides have been widely used to replace sugars in many food products, such as confectionery, chocolate and dairy products.

Both fructose and fructooligosaccharides can be produced from inulin by using inulinase. Fructose can also be obtained by acid hydrolysis of inulin. Acid hydrolysis of inulin, however, is not an appropriate method for fructose production because it results in formation of difructose anhydrides, which do not have sweetening capacity but cause undesirable colour (Vandamme & Derycke, 1983). Thus, the use of microbial inulinases has been proposed as the most promising approach for obtaining fructose or fructooligosaccharides from inulin.

Microbial inulinases can be divided into exo- and endo-acting enzymes according to their modes of action on inulin. Endoinulinases (2,1-β-D-fructan fructanohydrolase; EC 3.2.1.7) are specific for inulin and hydrolyse the internal β-2,1-fructofuranosidic linkages to yield inulooligosaccharides as the main products, e.g. inulotriose, inulotetraose, and inulopentaose. Exoinulinases (β-D-fructan fructohydrolase; EC 3.2.1.80) successively split off terminal fructose units from the non-reducing end of inulin, and also hydrolyse sucrose and raffinose (Nakamura, Kurokawa, Nakatsu, & Ueda, 1978).

Inulinase can be produced by different microorganisms, including fungi, yeast and bacteria. Among the filamentous fungi, *Aspergillus* and *Penicillium* species are common inulinase-producers (Ettalibi & Baratti, 1987; Nakamura et al., 1978, 1997; Onodera & Shiomi, 1992). Previous studies have revealed that several microorganisms produce endoinulinase as well as exoinulinase,

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e.g. *Aspergillus niger* (Nakamura et al., 1978), *Aspergillus ficuum* (Ettalibi & Baratti, 1987), *Chrysosporium pannorum* (Xiao, Tanida, & Takao, 1989) and *Penicillium purpurogenum* (Onodera & Shiomi, 1988). In our laboratory, we isolated a filamentous fungus, *A. ficuum* JNSP5-06, which also produces endoinulinase, as well as exoinulinase (Wang, Jin, Jiang, & Adamu, 2003). The objective of this study was to isolate and purify three exo-type inulinases and two endo-type inulinases from *A. ficuum* JNSP5-06 and to characterise the properties of those five inulinases.

2. Materials and methods

2.1. Materials

Inulin, DEAE-cellulose-52, Sepharose CL-6B and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). An electrophoresis calibration kit for protein molecular weight determination was purchased from Xibasi (Shanghai, China). All other chemicals used were of analytical grade.

2.2. Microorganism and culture conditions

The strain isolated from a soil sample was identified as *A. ficuum* JNSP5-06 (Wang et al., 2003). The strain was maintained on solid medium at 4 °C. The medium for enzyme production contained the following (w/v): 2% inulin, 2% yeast extract, 0.5% $NH_4H_2PO_4$, 0.5% NaCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.01% $ZnSO_4 \cdot 7H_2O$, initial pH at 6.5.

2.3. Inulinases production by submerged fermentation

Erlenmeyer flasks (250 ml) containing 30 ml of medium were inoculated with 2.0 ml of spore suspension (10^8 ml^{-1}) of *A. ficuum*. The flasks were incubated at 30 °C for 5 days in a temperature-controlled rotary incubator-shaker operated at 200 rpm. The fermented broth was filtered through double-layered Whatman paper. After centrifugation of the filtrate at 1503 *g* for 20 min, the supernatant was collected as the crude enzyme solution for further purification.

2.4. Inulinase isolation and purification

Crude enzyme solution was first precipitated at an ammonium sulphate concentration of 0–20% of saturation at 4 °C, overnight. After removing contaminating protein by centrifugation, the supernatant was brought to 20–90% of saturation with ammonium sulphate at 4 °C for 6 h. After centrifugation, the precipitate was dissolved in 0.1 M acetate buffer (pH 5.0) and the solution was then dialysed at 4 °C in 0.01 M acetate buffer (pH 5.8) to desalt. After concentration, the resulting enzyme solution was applied to a DEAE-cellulose column (2.6 × 50 cm) pre-equilibrated with the same buffer. The column was eluted with a step gradient of NaCl from 0 to 0.4 M in the same buffer at a flow rate of 0.6 ml/min. Absorbance was monitored at 280 nm. The elution profile revealed four major peaks with inulinase activity on inulin and sucrose, which were designated as A, B, C and D by the order of elution.

The four active fractions of A, B, C and D were pooled, dialysed against distiled water, lyophilised, dissolved in a small volume of acetate buffer and loaded onto a Sepharose CL-6B column (1.0 × 100 cm) equilibrated with the same buffer. The column was eluted with 0.01 M acetate buffer (pH 5.8) at a flow rate of 0.2 ml/min. The effluents were collected, concentrated, and further purified by preparative electrophoresis. After purification by preparative electrophoresis, five purified inulinases were then obtained and they were identified as Exo-I, Exo-II, Exo-III, Endo-I, and Endo-II, respectively.

2.5. Native-polyacrylamide gel electrophoresis (PAGE)

Native-PAGE was performed on a 12% gel at a constant current of 10 mA with Tris–glycine (pH 8.3) as the running buffer. Protein was stained with Coomassie Brilliant Blue G-250.

2.6. Sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis

SDS–PAGE was performed according to a previously described method (Laemmli, 1970) on vertical slab gel with 11% polyacrylamide gel containing 0.1% SDS and Tris–glycine buffer containing 0.1% SDS (pH 8.3 at 18 °C) at a constant current of 10 mA. The inulinase proteins were stained with Coomassie Brilliant Blue G-250.

2.7. Enzyme assays

Enzyme activities were assayed by measuring the concentration of reducing sugars released from inulin and sucrose. The reaction mixture containing 1 ml of diluted crude enzyme and 4 ml of 2% inulin or 2% sucrose (dissolved in 0.1 M acetate buffer, pH 5.0) was incubated at 50 °C for 30 min. Then a 0.5 ml aliquot was withdrawn and the increase in reducing sugar was estimated by the 3,5-dinitrosalicylic acid method (Miller, 1959). Absorbance was read at 575 nm. A high absorbance indicated a high level of reducing sugar produced and consequently a high enzyme activity. One inulinase unit was defined as the amount of enzyme which yields 1 μmol fructose per min. One invertase unit was defined as the amount of enzyme which hydrolyses 1 μmol sucrose per min under the same conditions. The ratio between these two activities was commonly expressed as inulin/sucrose (I/S) (Vandamme & Derycke, 1983).

2.8. Protein determination

Protein content was determined as previously described (Lowry, Rosebrough, Farr, & Randall, 1951), using bovine serum albumin as standard. Protein in the column effluents was monitored by measuring the absorbance at 280 nm.

2.9. Thin-layer chromatography

Thin-layer chromatography on silica gel 60 plates was carried out to analyse the hydrolysis products of the inulinase. Plates were developed at room temperature for 24 h with a solvent system of chloroform–acetic acid–water (30:35:5, v/v/v). Sugar spots were visualised by spraying the plates with 1% α-naphthol (containing 10% phosphoric acid) and heating at 120 °C for 10 min (Azhari, Szlak, Ilan, Sideman, & Lotan, 1989).

2.10. Determination of molecular weight

The molecular weight of each inulinase was estimated by SDS–PAGE, using an electrophoresis calibration kit, including rabbit phosphorylase b (97 kDa), bovine serum albumin (66 kDa), rabbit actin (43 kDa), bovine carbonic anhydrase (31 kDa), trypsin inhibitor (20 kDa) and hen egg white lysozyme (14 kDa) as standards.

2.11. Effect of pH and temperature on inulinase activity and stability

The effect of pH on inulinase activity was determined in the pH range from 2.0 to 9.0 at 50 °C for 30 min, using 0.1 M acetate buffer (pH 2.0–5.5), or 0.1 M phosphate buffer (pH 5.5–7.0) or 0.1 M Tris-HCl buffer (pH 7.0–9.0). The stability of inulinase at different pH values was determined by estimating the residual activity after

inulinase was pre-incubated at room temperature for 1 h at pH 2.0–10.0.

The optimal temperature for inulinase activity was determined by incubating the enzyme-substrate mixtures for 30 min at various temperatures (20–80 °C) in 0.1 M acetate buffer (pH 5.0) and the liberated reducing sugars were measured. Thermal stability of inulinase was measured in terms of residual activity after incubation of inulinase at different temperatures (40–90 °C) for 1 h.

2.12. Kinetic parameters

The K_m value and maximum reaction velocity (V_{max}) for inulin and sucrose were determined by the method of Lineweaver–Burk plots.

2.13. Effects of metal ions and other reagents on inulinase activity

The effects of metal cations on inulinase activity were investigated by incubating enzyme solution with 2 mM K^+, Fe^{2+}, Mg^{2+}, Ca^{2+}, Mn^{2+}, Al^{3+}, Cu^{2+}, Zn^{2+}, Li^{2+} and Ag^+ in 50 mM sodium phosphate buffer (pH 7.0) at 30 °C for 30 min, then assaying for residual enzyme activity. The effects of 2 mM EDTA, 10 mM urea and 40 mM SDS on inulinase activity were determined under the same conditions as mentioned above.

3. Results and discussion

3.1. Purification of exoinulinase and endoinulinase

To purify the crude enzyme to homogeneity, the enzyme purification procedure included the following three steps: ammonium sulphate precipitation, DEAE-cellulose column chromatography, and Sepharose CL-6B column chromatography. The elution profiles are shown in Figs. 1 and 2.

Three exoinulinases (Exo-I, Exo-II, and Exo-III) and two endoinulinases (Endo-I and Endo-II) were purified from culture broth of *A. ficuum*. The crude enzyme preparation was first fractionated by ammonium sulphate precipitation and then applied to a DEAE-cellulose column. The elution profile (Fig. 1) shows that four major peaks (A–D) exhibited inulinase activity on inulin and sucrose with different I/S ratios. The results from thin-layer chromatography analysis of the hydrolysis products of inulin by four active fractions (data not shown) indicated that the hydrolysis of inulin by A, B, and C yielded fructose as the main product. However, the hydrolysis of inulin by D yielded a mixture of oligosaccharides with degrees of polymerisation of 3, 4, and 5 as the main products. Therefore, it was concluded, from the hydrolysates, that A, B, and C were exo-type inulinases, whereas D was an endo-type inulinase. In addition, it was also found that the I/S ratio of D toward inulin and sucrose was higher than those of A, B, and C (data not shown), which further confirmed the above conclusion. Hence, the four active fractions, A, B, C, and D, were designated as Exo-I, Exo-II, Exo-III, and Endo, respectively.

The active fractions, Exo-I, Exo-II, Exo-III, and Endo, were collected, concentrated, filtered, and loaded onto a Sepharose CL-6B column (Fig. 2), which resulted in 7.62-, 7.30-, 7.96-, and 5.29-fold purifications as compared to the crude extract, respectively (data not shown). However, after native-PAGE, there still appeared to be contaminating proteins present (Fig. 3). Moreover, we also found that the active fraction of Endo contained two bands with endoinulinase activity. These two active fractions were designated as Endo-I and Endo-II.

Fig. 4 shows SDS–PAGE of the five purified inulin-hydrolysing enzymes from *A. ficuum*. Exo-I, Exo-II and Exo-III showed high specific activities not only on inulin, but also on sucrose, but Endo-I and Endo-II were more active on inulin than on sucrose.

3.2. Homogeneity and estimation of molecular weight

As shown in Fig. 4, these five enzymes of Exo-I, Exo-II, Exo-III, Endo-I, and Endo-II give a single band of protein by SDS–PAGE, and their molecular weights were 70 kDa, 40 kDa, 46 kDa, 34 kDa and 31 kDa, respectively.

A considerable variation in molecular weight has been earlier reported namely, *Arthrobacter* sp. (75 kDa), *Bacillus stearothermophilus* KP1289 (54 kDa), *Aspergillus candidus* (54 kDa), *Penicillium* sp. TN-88 (68 kDa), *Kluyveromyces marxianus* var. *bulgaricus* (57 kDa), *Streptomyces* sp. (45 kDa) (Kang, Chang, Oh, & Kim, 1998; Kato et al., 1999; Kochhar, Gupta, & Kaur, 1999; Kushi, Monti, & Contiero, 2000; Nakamura et al., 1997; Sharma & Gill, 2007). Ettalibi and Baratti (1987) reported that five exoinulinases showed the same molecular weight of 74 kDa and three endoinulinases had a molecular weight of 64 kDa, which was higher than that of exoinulinases and endoinulinases in the current study.

3.3. Effect of temperature on enzyme activity and stability

To determine the optimum temperature for enzyme activity, enzyme reactions were performed at various temperatures (20–80 °C) at pH 5.0 for 30 min, using inulin and sucrose as substrate. As shown in Fig. 5, the optimum temperature of all exoinulinase and endoinulinase was around 45 °C, which was in accordance with the optimum inulinases temperatures of 45–55 °C from other microorganism (Vandamme & Derycke, 1983).

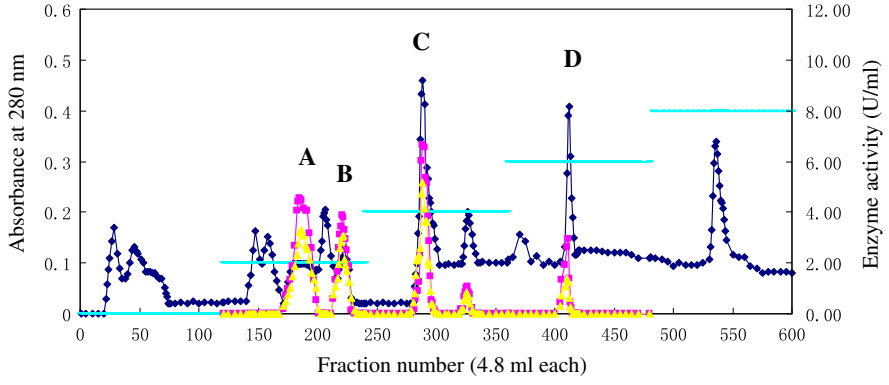

Fig. 1. DEAE-cellulose column chromatography profile of crude preparation after ammonium sulphate fractionation. Crude enzyme protein was loaded onto a DEAE-cellulose 52 column (2.6 × 50 cm) equilibrated with 0.01 M acetate buffer (pH 5.8). The adsorbed enzyme was eluted by a step gradient of NaCl from 0 to 0.4 M in the same buffer, and the effluent was collected in fractions of 4.8 ml at a flow rate of 0.6 ml/min. Absorbance at 280 nm (♦), inulinase activity on inulin (■), inulinase activity on sucrose (▲), and step gradient elution of NaCl (0, 0.1 M, 0.2 M, 0.3 M, 0.4 M) (—) are indicated. A, B, C, and D represent four major peaks with inulinase activity.

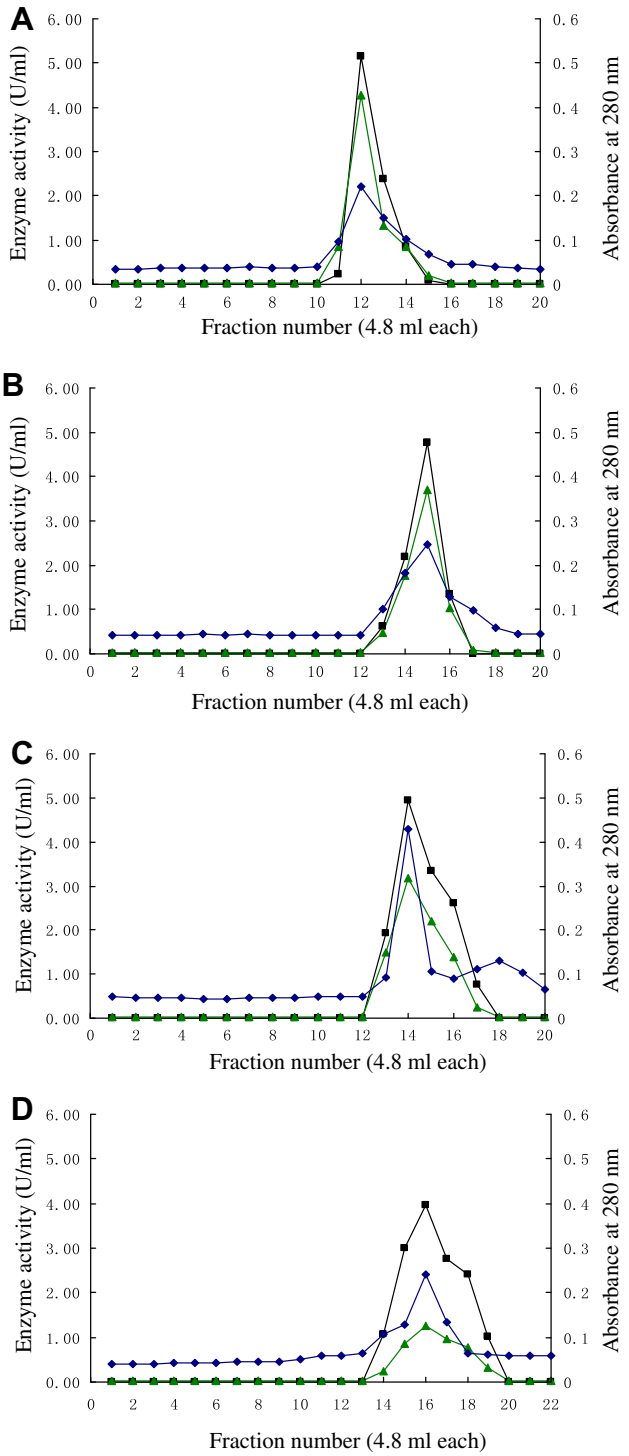

Fig. 2. Sepharose CL-6B column chromatography profile of inulinase. (A) Exo-I, (B) Exo-II, (C) Exo-III, (D) Endo. Four active fractions were loaded onto a Sepharose CL-6B column (1.0 × 100 cm) and were eluted by 0.01 M acetate buffer (pH 5.8), and the effluent was collected in fractions of 4.8 ml at a flow rate of 0.2 ml/min. Absorbance at 280 nm (♦), inulinase activity on inulin (■), and inulinase activity on sucrose (▲) are indicated.

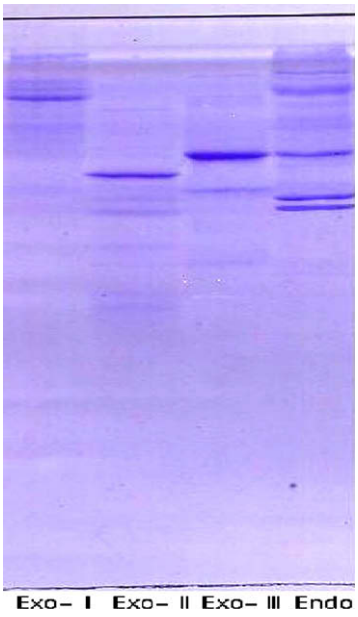

Fig. 3. Native-PAGE of partially purified inulinases from *A. ficuum*.

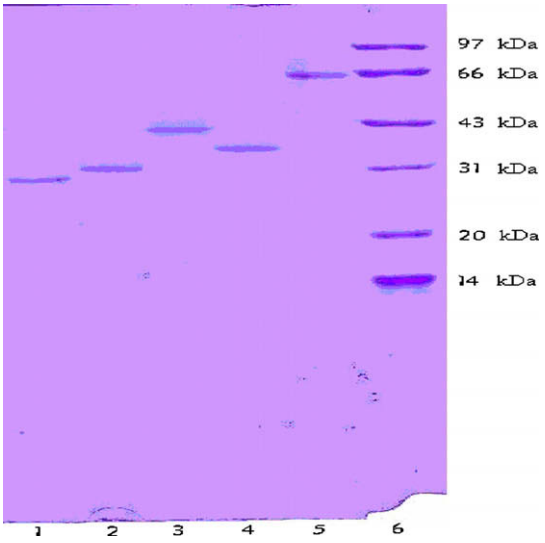

Fig. 4. SDS–PAGE of purified inulinases from *A. ficuum*. Lane 1: Endo-II, Lane 2: Endo-I, Lane 3: Exo-III, Lane 4: Exo-II, Lane 5: Exo-I, Lane 6: molecular weight markers: rabbit phosphorylase b (97 kDa), bovine serum albumin (66 kDa), rabbit actin (43 kDa), bovine carbonic anhydrase (31 kDa), trypsin inhibitor (20 kDa) and hen egg white lysozyme (14 kDa).

For determining the thermostability of purified inulinases, each enzyme solution was heated at various temperatures (40–90 °C) at pH 5.0 for 1 h. After the treatment, the residual activities were measured at various temperatures (data not shown). All five purified inulinase retained 80% relative activity after 1 h (pre-heated at 50 °C), but only 40% relative activity was observed after 1 h (pre-heated at 60 °C). However, complete inactivation was observed when the enzyme was incubated at 80 °C for 1 h. The thermostability of inulinase of *A. ficuum* was lower than that of the inulinase from *Streptomyces* sp. (Sharma & Gill, 2007), which has been reported as a heat-stable inulinase and the inulinase did not show any significant loss of activity after 6 h at 70 °C and it retained about 75% of its activity after 12 h at 70 °C. Moreover, Kim et al. (1994) reported that inulinase from *Scytalidium acidophilum* maintained about 95% of its activity after 6 h incubation at 60 °C.

3.4. Effects of pH on enzyme activity and stability

The effects of pH on the activity and stability of exoinulinase and endoinulinase were also determined. As shown in Fig. 6,

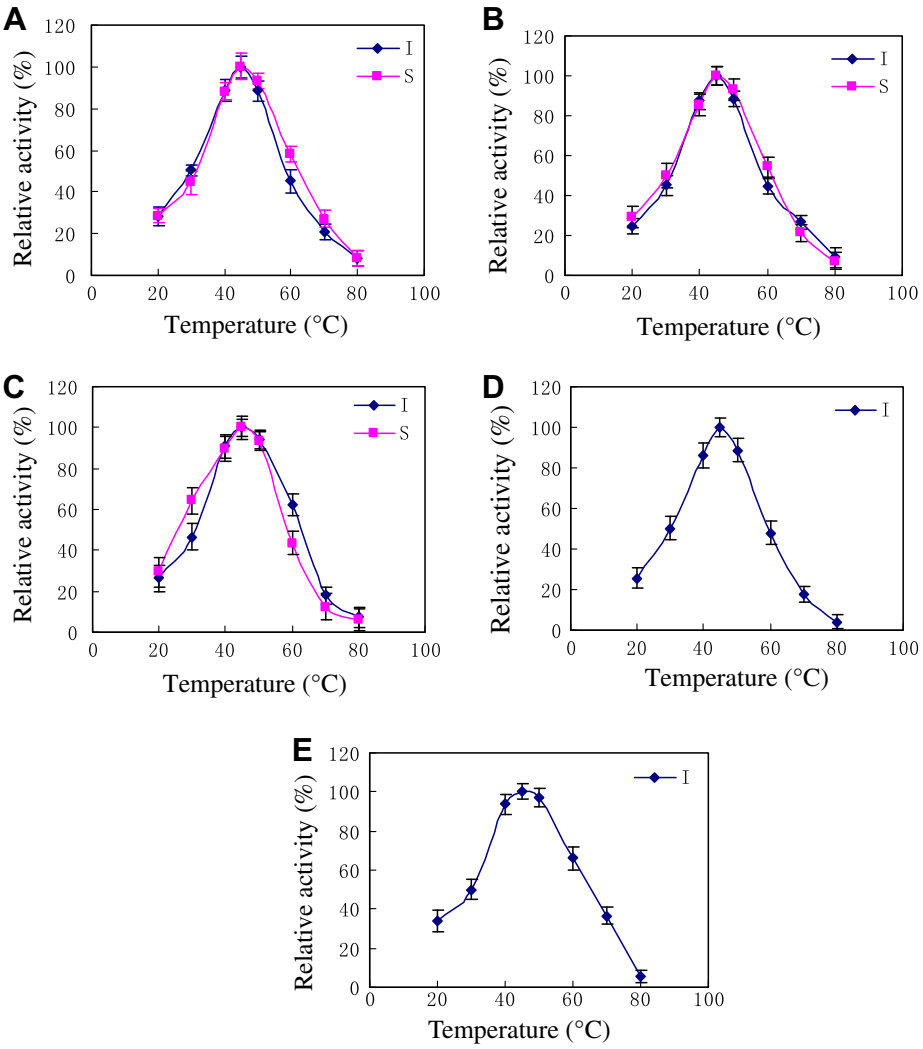

Fig. 5. Effect of temperature on inulinase activity. (A) Exo-I, (B) Exo-II, (C) Exo-III, (D) Endo-I, (E) Endo-II. Data represent the means of three determinations ±SE.

exoinulinase showed maximum activity at pH 4.5, but endoinulinase exhibited the highest activity at pH 5.0, which was in agreement with the general range of many microbial sources reported so far: *A. niger* (pH 4.4) (Derycke & Vandamme, 1984), *A. Awamori* (pH 4.5) (Arand et al., 2002), *Penicillium janczewskii* (pH 4.8–5.0) (Pessoni, Figueiredo, & Braga, 1999), *Penicillium* sp. TN-88 (pH 5.2) (Nakamura et al., 1997).

At 50 °C, both exoinulinase and endoinulinase were stable over a wide pH range (from 4 to 8). However, the enzyme activity decreased dramatically beyond that range, and it almost lost activity below pH 2.0 and above pH 10.0 (data not shown).

3.5. Kinetic parameters of inulinases

The affinity of the inulinases for inulin and sucrose were determined at 50 °C and pH 5.0 by a Lineweaver-Burk plot. The K_m values of Exo-I, Exo-II, Exo-III, Endo-I, and Endo-II were 43.1 mg/ml, 31.5 mg/ml, 25.3 mg/ml, 14.8 mg/ml, and 25.6 mg/ml, respectively, with inulin as the substrate. The V_{max} values were 32.7 mg/ml × min, 217 mg/ml × min, 46.3 mg/ml × min, 40.8 mg/ml × min, and 53.8 mg/ml × min, respectively (data not shown). The values of K_m and V_{max} of these five inulinases were different. Kushi et al. (2000) reported that the apparent K_m values of inulinase for sucrose and inulin were 4.58 mg/ml and 86.9 mg/ml, respectively, different from our result.

3.6. Effect of metal ions and other reagents on inulinase activity

The various metal ions and chemical reagents were added to the enzyme solution at different concentrations. After pre-incubation at 30 °C for 30 min, the remaining enzyme activity was measured. As shown in Table 1, K^+, Ca^{2+} and Li^{2+} had no obvious effect on the activities of exoinulinase and endoinulinase. However, both inulinases were completely inhibited by Ag^+, which was in agreement with previous studies (Gaye, Sukan, & Vassilew, 1994; Nakamura et al., 1978). It was previously reported that some –SH groups were essential for the activity of inulinase produced by molds (Ettalibi & Baratti, 1987, 1990), some yeasts (Parekl & Margaritis, 1986; Rouwenhorst, Ritmeester, Scheffers, & van Dijken, 1990) and bacteria (Elyachioui, Horeiz, & Taillez, 1992; Vullo, Coto, & Sinerez, 1991), and that Ag^+ formed a complex with SH.

In addition, exo- and endo-inulinase activities were strongly inhibited by Fe^{2+} and Al^{3+} and slightly inhibited by Mn^{2+} and Zn^{2+}. Cu^{2+} caused decrease in exoinulinase activity against inulin and sucrose by 28.4% and 15.4%, respectively, and decreased endoinulinase activity by 16.9%. Mg^{2+} appeared to slightly stimulate the inulinase activity. Ettalibi and Baratti (1990) and Azhari et al. (1989) also demonstrated that the activity of purified inulinase from *A. ficuum* was inhibited by Mn^{2+}. However, Nakamura et al. (1978) reported an increase in inulinase activity of *A. niger* in the presence of Mn^{2+}.

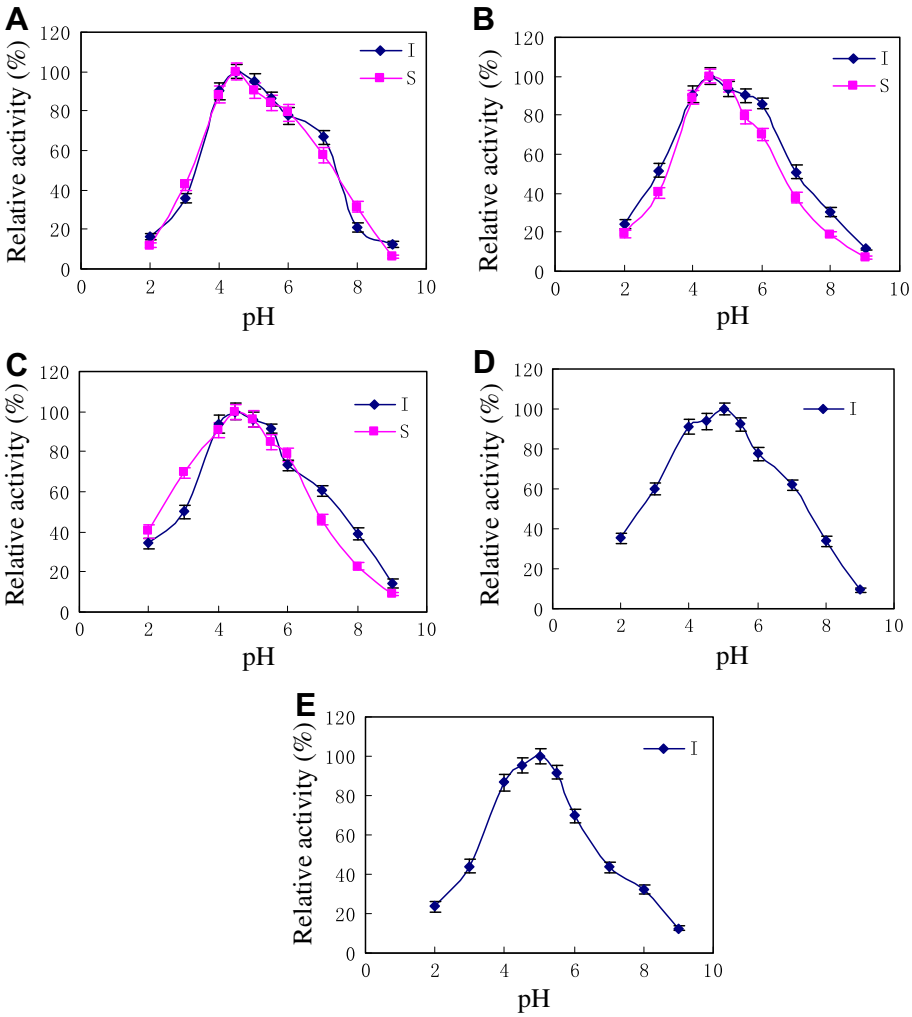

Fig. 6. Effect of pH on inulinase activity. (A): Exo-I, (B): Exo-II, (C): Exo-III, (D): Endo-I, (E): Endo-II. Data represent the means of three determinations ± SE.

Table 1
Effect of metal ions and other chemicals on activities of exoinulinase and endoinulinase from *A. ficuum*.

Compound	Concentration (mmol/L)	Relative activity (%)		
		Exoinulinase		Endoinulinase
		Inulin	Sucrose	Inulin
Control		100 ± 4.4	100 ± 3.7	100 ± 5.2
K^+	2	98.4 ± 3.2[b]	99.4 ± 3.3[b]	101 ± 3.6[b]
Fe^{2+}	2	38.7 ± 0.7[a]	44.3 ± 1.3[a]	65.6 ± 1.6[a]
Mg^{2+}	2	107 ± 4.1[b]	105 ± 3.5[b]	112 ± 5.2[b]
Ca^{2+}	2	101 ± 3.6[b]	101 ± 2.9[b]	98.9 ± 3.4[b]
Mn^{2+}	2	91.4 ± 1.8[b]	91.9 ± 1.9[b]	92.6 ± 2.0[b]
Al^{3+}	2	29.6 ± 0.6[a]	44.5 ± 0.9[a]	65.6 ± 1.4[a]
Cu^{2+}	2	71.6 ± 1.5[a]	84.6 ± 1.7[a]	83.1 ± 1.7[a]
Zn^{2+}	2	92.1 ± 1.9[b]	94.0 ± 2.0[b]	91.0 ± 1.8[b]
Li^{2+}	2	103 ± 3.7[b]	105 ± 2.8[b]	97.4 ± 2.4[b]
Ag^+	2	0	0	0
EDTA	2	102 ± 3.8[b]	101 ± 4.4[b]	101 ± 3.3[b]
Urea	10	104 ± 2.8[b]	101 ± 2.7[b]	102 ± 3.4[b]
SDS	40	93.1 ± 1.9[b]	98.6 ± 2.2[b]	91.5 ± 1.8[b]

After pre-incubation of enzyme with metal ions and chemical reagents at different concentrations at 30 °C for 30 min, the remaining enzyme activity was measured. Data represent the means of three determinations ±SE.

[a] Significantly different vs. control at $P < 0.05$.

[b] Not significant.

From Table 1, it also appears that neither the metal chelator EDTA nor urea affected the activity of inulinases. However, SDS, at the concentration of 40 mM, slightly inhibited inulinase activity.

4. Conclusion

By ammonium sulphate precipitation, DEAE-cellulose column chromatography, Sepharose CL-6B column chromatography and preparative electrophoresis, three exo-inulinases (Exo-I, Exo-II, and Exo-III) and two endo-inulinases (Endo-I and Endo-II) were purified from the culture broth of *A. ficuum* JNSP5-06. The molecular weights of Exo-I, Exo-II, Exo-III, Endo-I and Endo-II were determined to be 70 kDa, 40 kDa, 46 kDa, 34 kDa, and 31 kDa, respectively. Using inulin as substrate, the K_m values of these five inulinases were 43.1 mg/ml, 31.5 mg/ml, 25.3 mg/ml, 14.8 mg/ml, and 25.6 mg/ml, respectively. Their V_{max} values were 32.7 mg/ml × min, 217 mg/ml × min, 46.3 mg/ml × min, 40.8 mg/ml × min, and 53.8 mg/ml × min, respectively. These five inulinases were stable below 50 °C with an optimum activity at 45 °C, and were stable in the pH range of 4–8, with an optimum pH at 4.5 for exoinulinase and at 5.0 for endoinulinase. The inulinase activity was completely inhibited by Ag^+, strongly inhibited by Fe^{2+} and Al^{3+}, moderately inhibited by Cu^{2+} and slightly inhibited by Mn^{2+}, Zn^{2+} and SDS.

K^+, Ca^{2+}, Li^{2+}, EDTA and urea had no significant influence on the inulinase activity, while Mg^{2+} enhanced the inulinase activity.

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