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Purification and Characterization of Extracellular Inulinase from a Marine Yeast *Cryptococcus aureus* G7a and Inulin Hydrolysis by the Purified Inulinase

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Abstract The extracellular inulinase in the supernatant of the cell culture of the marine yeast *Cryptococcus aureus* G7a was purified to homogeneity with a 7.2-fold increase in specific inulinase activity compared to that in the supernatant by ultrafiltration, concentration, gel filtration chromatography (Sephadex™ G-75), and anion exchange chromatography (DEAE sepharose fast flow anion exchange). The molecular mass of the purified enzyme was estimated to be 60.0 kDa. The optimal pH and temperature of the purified enzyme were 5.0 and 50 °C, respectively. The enzyme was activated by Ca^{2+}, K^{+}, Na^{+}, Fe^{2+}, and Zn^{2+}. However, Mg^{2+}, Hg^{2+}, and Ag^{+} acted as inhibitors in decreasing the activity of the purified inulinase. The enzyme was strongly inhibited by phenylmethanesulphonyl fluoride (PMSF), iodoacetic acid, EDTA, and 1,10-phenanthroline. The K_m and V_{max} values of the purified enzyme for inulin were 20.06 mg/ml and 0.0085 mg/min, respectively. A large amount of monosaccharides were detected after the hydrolysis of inulin with the purified inulinase, indicating the purified inulinase had a high exoinulinase activity.

Keywords Inulinase · Marine yeasts · Inulin · Characterization · *Cryptococcus aureus* G7a

Introduction

Yeast has been used in food and other industries since ages. They have earned acceptability since long ago and are considered natural. Yeasts are also considered to be easy to handle and grow in comparison to bacteria. Many hydrolytic enzymes such as lipase, protease, and phytase, have been commercially produced by yeasts. Among the enzymes, inulinase has received much attention as it can be widely applied to the production of fuel ethanol and ultra-high fructose syrup from inulin. Inulin is a linear β-(2,1)-linked fructose polymer that occurs as a reserve carbohydrate in Jerusalem artichoke, dahlia tubers, or chicory root [1, 2].

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Fructose is widely used in many foods and beverages instead of sucrose. Inulin can be converted into fructose by chemical approach. However, the chemical approach is currently associated with some drawbacks [1, 3]. Fructose can also be produced from starch by enzymatic methods involving α-amylase, amyloglucosidase, and glucose isomerase [3]. The best procedure involves the use of microbial inulinase, which after one-step enzymatic hydrolysis of inulin, yields 95% pure fructose. Inulinase (EC 3.2.1.7, 2→1-β-D-fructan fructanohydrolase), which catalyzes hydrolysis of inulin, is produced by many microorganisms, such as *Kluyveromyces*, *Aspergillus*, *Staphylococcus*, *Xanthomonas*, and *Pseudomonas*. Yeasts such as *Kluyveromyces fragilis*, *K. marxianus*, *Candida kefyr*, *Debaryomyces cantarelli*, and fungi, *Penicillium* and *Aspergillus* species are the common inulinase producers [1]. Among the yeasts, which can produce inulinases, strains of *Candida* sp., *Sporotrichum* sp., *Pichia* sp., and *Kluyveromyces* sp, two species of *K. fragilis* and *K. marxianus*, have high potential for producing commercially acceptable yields of the enzyme [3]. However, there have been few reports about inulinase from marine yeasts so far [4]. The genes encoding inulinase have been cloned and sequenced in *K. cicerisporus* CBS4857, *K. marxianus* ATCC12424 [5, 6], and inulinases from *K. fragilis* and *K. marxianus* have been purified and characterized [1, 7].

In our previous studies [8], we found that the marine yeast strain *Cryptococcus aureus* G7a isolated from sediment at China South Sea could produce over 85.0 U/ml of inulinase activity within 42 h of fermentation at shake flask level under the optimal conditions. The main purpose of the present study was to purify and characterize extracellular inulinase produced by the marine yeast strain G7a and use the purified inulinase to hydrolyze inulin. To our knowledge, this is the first report about inulinase from *C. aureus* isolated from the marine environment.

Materials and Methods

Yeast Strain

The yeast strain G7a, which was identified to be a strain of *C. aureus* according to the results of routine yeast identification and molecular methods, was isolated from sediment (20 °C, pH 8.1, and 2.89% salinity) at China South Sea [8]. This yeast strain was maintained in YPD medium (prepared with seawater) containing 20.0 g/l glucose, 20.0 g/l yeast extract, and 10.0 g/l polypeptone at 4 °C.

Inulinase Production

One loop of the cells of the yeast strain was transferred to 50.0 ml of YPD medium in 250 ml flask and aerobically cultivated for 24 h. The cell culture (5.0 ml, $OD_{600\ nm}$=20.0) was transferred to 45.0 ml of the production medium (prepared with the artificial seawater), which contained inulin 40.0 g/l, K_2HPO_4 3.0 g/l, yeast extract 5.0 g/l, KCl 5.0 g/l, $CaCl_2$ 1.2 g/l, NaCl 40.0 g/l, and $MgCl_2 \cdot 6H_2O$ 6.0 g/l, pH 5.0 and grown by shaking at 170 rpm and 28 °C for 42 h.

Determination of Inulinase Activity

The fermented broth was centrifuged at 2,823×*g* and 4 °C for 5 min, and the supernatant obtained was taken as the crude enzyme. The reaction mixture containing 0.1 ml of the

crude enzyme or 0.1 ml of the purified inulinase and 0.9 ml of acetate buffer (0.1 M, pH 5.0) with 20 g/l inulin was incubated at 50 °C for 10 min. The reaction was inactivated immediately by keeping the reaction mixture at 100 °C for 10 min. The same mixture to which the same amount of the inactivated crude enzyme (heated at 100 °C for 10 min) was added before the reaction was used as the control. The amount of reducing sugar in the reaction mixture was assayed by the method of Nelson-Somogyi [9]. One inulinase unit (U) was defined as the amount of enzyme that produces 1 μmol of reducing sugar per minute under the assay conditions used in this study. Protein concentration in the supernatant was measured by the method of Bradford, and bovine serum albumin served as standard [10].

Enzyme Purification

Enzyme purification was carried out at 4 °C. One liter of the culture grown for 42 h was used as the starting material for inulinase purification. After the removal of the cells by centrifugation at 14,000×*g* for 20 min, about 1,000 ml of the supernatant was concentrated to 25.0 ml by ultrafiltration (10 kDa cut-off) with a Labscale™ TFF System (Millipore, USA). Then, the concentrated enzyme solution was applied to the Sephadex™ G-75 column (medium grade; Pharmacia 2.5×100 cm), which had been equilibrated with 20.0 mM phosphate buffer (pH 6.5), and the column eluted at 15 °C with the same phosphate buffer (pH 6.5) by using ÄKTA™ prime with Hitrap™ (Amersham, Biosciences, Sweden). At a flow rate of 0.5 ml/min, 3.0-ml fractions were collected. The inulinase-positive fractions were combined and dialyzed against 20 mM sodium phosphate buffer (pH 6.5) for 24 h at 4 °C. The dialyzed inulinase-positive elute was applied to the DEAE sepharose fast flow anion exchange column (2.5×30 cm), which had been equilibrated with 20 mM sodium phosphate buffer (pH 6.5), and the column was washed with the same buffer for 2 h at a flow rate of 1.0 ml/min. The bound proteins were then eluted with a linear gradient of NaCl solution in the range of 0–0.5 M in the equilibrating buffer. The inulinase-positive fractions were concentrated to 5.0 ml by ultrafiltration (10 kDa cut-off) at room temperature through a Labscale™ TFF System (Millipore, USA). Protein concentration in the elute was measured by the method of Bradford, and bovine serum albumin served as standard [10].

Gel Electrophoresis

The purity and molecular mass of inulinase in the concentrated fractions showing the activity were analyzed in noncontinuous denaturing SDS-PAGE [12] with a two-dimensional electrophoresis system (Amersham, Biosciences, Sweden), stained by Coomassie brilliant blue R-250 (0.01%), and destained with 10% acetic acid [18]. The molecular mass standards for SDS-PAGE comprised β-galactosidase (116 kDa), Bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), and lactate dehydrogenase (35 kDa).

Effects of pH and Temperature on the Inulinase Activity and Stability

The effect of pH on the purified inulinase activity was determined by incubating the purified enzyme between pH 4.0 and 8.0 using the standard assay conditions. The buffers used were 0.1 M acetate buffer (pH 3.6–5.5) and phosphate buffer (pH 5.5–8.0). The pH stability was tested by 120 min preincubation of the purified enzyme in appropriate buffers that had the same ionic concentrations at different pH values ranging from 3.5 to 8.0 at a

temperature of 4 °C. The remaining activities of inulinase were measured immediately after this treatment with the standard method as mentioned above.

The optimal temperature for the activity of the enzyme was determined at temperatures of 35, 40, 45, 50, 55, 60, 65, and 70 °C in the same buffer as described above. Temperature stability of the purified enzyme was tested by preincubating the enzyme at different temperatures ranging from 40 to 80 °C during 120 min; residual activity was measured as described above immediately. The relative inulinase activity of the preincubated sample at 4 °C was regarded as 100%.

Effects of Different Metal Ions on the Inulinase Activity

To examine effects of different metal ions on the inulinase activity, enzyme assay was performed for 1 h in the reaction mixture as described above with various metal ions at a final concentration of 1.0 mM. The activity assayed in the absence of metal ions was defined as the control. The metal ions tested include $ZnSO4{\cdot}7H_2O$, $MgSO_4{\cdot}5H_2O$, $CaCl_2$, KCl, $HgCl_2$, $FeCl_2$, AgCl, and NaCl.

Effects of Protein Inhibitors on the Inulinase Activity

The effects of protein inhibitors (EDTA, PMSF, 1,10-phenanthroline, iodoacetic acid, DDT, and SDS at a final concentration of 10.0 mM) on the inulinase activity were measured in the reaction mixture as described above. The purified enzyme was preincubated with the respective compound for 1 h at 0 °C, followed by the standard enzyme assay as described above. The activity assayed in the absence of the protein inhibitors was defined as the control.

Determination of Kinetics Parameters

To obtain the K_m and V_{max} of the inulinase for inulin, 0.5 ml of inulin solution in 0.1 M acetate buffer (pH 5.0) was mixed with 0.1 ml of the purified inulinase (the final inulinase concentration was 11.1 U/ml and the final inulin concentrations were 0.5, 0.75, 1.0, 1.5, and 2.0 mg/ml, respectively), respectively, and the mixture was incubated at 50 °C for 10 min. The reaction was stopped immediately by keeping the reaction mixture at 100 °C for 10 min. The K_m and V_{max} values were obtained from the Lineweaver–Burk plot and expressed as the mean of the three different experiments.

Inulin Hydrolysis

The effect of inulin concentration on hydrolysis was studied by varying its concentrations from 1.0 to 30.0 g/l in the reaction mixture containing 0.1 ml of 221.9 U/ml of the purified enzyme and 0.4 ml of the acetate buffer (0.1 M, pH 5.0), which was incubated at 50 °C for 16 h. To determine the extent of inulin hydrolysis, reducing sugar estimation was done after 8 or 16 h of incubation. The end products of inulin hydrolysis after 8 or 16 h of incubation at 50 °C were withdrawn and identified to ascertain the extent of hydrolysis by ascending thin-layer chromatography (Silica gel 60, MERCK, Germany) with the solvent system of *n*-butanol–pyridine–water (6:4:3) and a detection reagent comprising 20.0 g/l diphenylamine in acetone–20.0 g/l aniline in acetone–850 g/l phosphoric acid (5:5:1, *v*/*v*/*v*) [11].

The effect of the enzyme concentration was examined by varying its concentrations from 0.04 to 0.4 U/mg inulin in the reaction mixture containing 0.3 ml of 100.0 g/l of inulin at 50 °C.

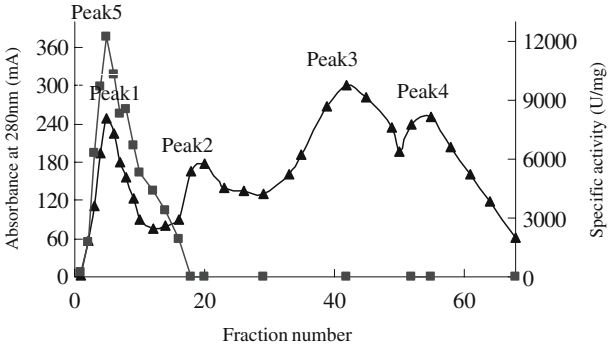

Fig. 1 Elution profile of inulinase from *C. aureus* G7a on Sephadex™G-75 (*filled triangles*, absorbance at 280 nm; *filled squares*, specific inulinase activity)

Results and Discussion

Purification of Inulinase

The extracellular inulinase was purified from the supernatant prepared from the cell culture by ultrafiltration, concentration, gel filtration chromatography (Sephadex™ G-75), and DEAE sepharose fast flow anion exchange chromatography. The elution profile of gel filtration chromatography indicates that peak 1 with the specific inulinase activity from fraction number 1 to 20 showed one single peak (Fig. 1) whereas the elution profile of DEAE sepharose fast flow anion exchange chromatography shows that peak 4 with the specific inulinase activity from fraction number 25 to 38 displayed one single sharp peak (Fig. 2). Therefore, the fractions were collected and concentrated by ultrafiltration. The results in Table 1 show that the enzyme was purified to homogeneity with a 2.44-fold increase in specific inulinase activity with a yield of about 22.4 % compared to that in the supernatant.

Gel Electrophoresis

SDS-PAGE was used to determine the protein purity and estimate the molecular mass of the final concentrated elute as described by Laemmli [12]. The results in Fig. 3 indicate that there was one single protein band from the final concentrated elute, and the relative molecular mass of the purified inulinase was estimated to be 60.0 kDa by SDS-PAGE.

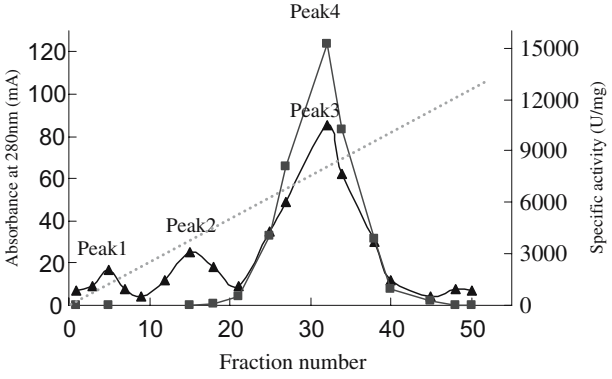

Fig. 2 Elution profile of inulinase from *C. aureus* G7a on DEAE sepharose fast flow anion exchange. (*filled triangles*, absorbance at 280 nm; *filled squares*, specific activity) The *dotted line* represents the concentration of NaCl from 0 to 500 mM

Table 1 Summary of the purification procedures of inulinase from *C. aureus* G7a.

Purification steps	Total protein (mg)	Total activity (U)	Yield (%)	Specific activity (U/mg protein)	Purification factor (fold)
Supernatant concentration	12.5	81,000	100	6,480	1
Sephadex™	7.02	50,680	62.5	7,219	1.11
G-75 gel filtration	2.04	24,841	30.7	12,177	1.88
DEAE sepharose fast flow anion exchange	1.15	18,200	22.4	15,826	2.44

Therefore, it may be concluded that the inulinase from the marine yeast strain was a monomer of 60.0 kDa protein. It has been reported that the extracellular inulinase from the terrestrial yeast, *K fragilis* had 250 kDa of molecular weight whereas the apparent molecular weight of exoinulinase from *K. marxianus* CBS 6556 was 72 kDa (SDS-PAGE) [1, 13]. The purified extracellular inulinase from the yeast *K. marxianus* var. *bulgaricus* was found to be 57 kDa by SDS-PAGE [7]. It has been reported that most of the inulinases from terrestrial fungi have more than 50.0 kDa of molecular weight [1]. This means that the molecular mass of the inulinase from the marine yeast was almost the same as that from terrestrial yeasts.

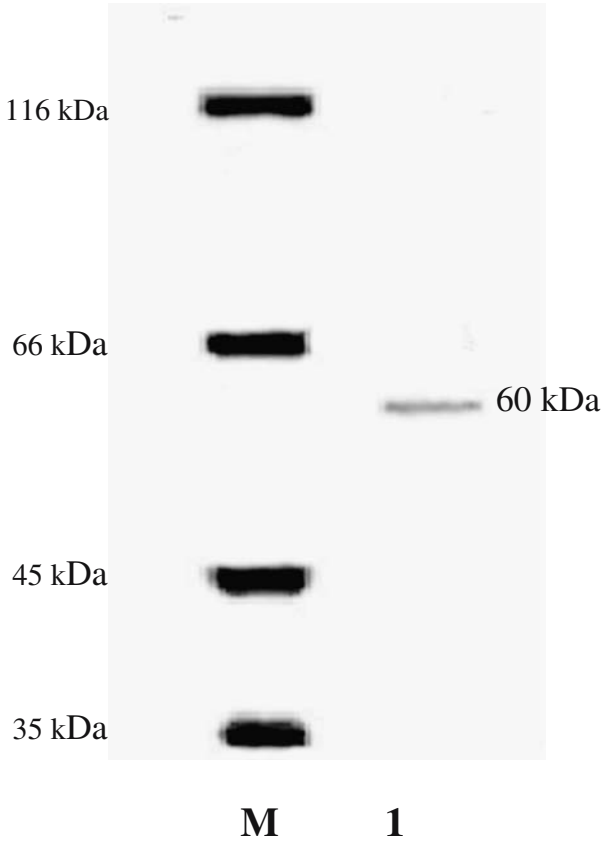

Fig. 3 SDS-PAGE (12%) of the fractions showing inulinase activity obtained during the purification. The lanes are as follows: *M*, marker proteins with relative molecular masses indicated on the *left*; *1*, the elute from DEAE sepharose fast flow anion exchange chromatography. The purified enzyme was a single band, and the molecular mass of the purified enzyme was 60.0 kDa. After SDS-PAGE, the gel was stained by using 0.01% Coomassie brilliant blue R-250 and destained in 10% acetic acid

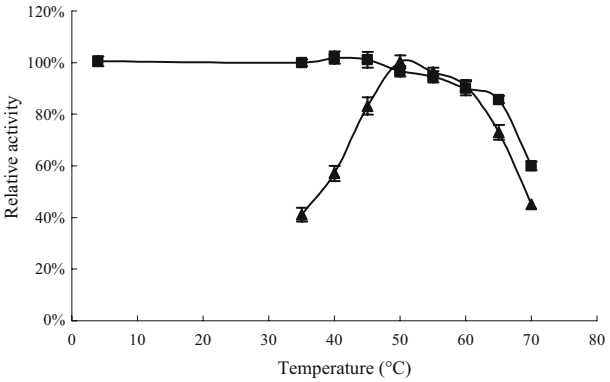

Fig. 4 Effects of different temperature on inulinase activity (*filled triangles*) and stability (*filled squares*). Temperature stability of the purified enzyme was tested by preincubating the enzyme at different temperatures (35, 40, 45, 50, 55, 60, 65, 70 °C) for 2 h; the residual activity was measured as described above immediately. In this figure, the preincubated sample at 4 °C was used as a reference to calculate the residual activity. Data are given as the means±SD, $n=3$. The buffer used was acetate buffer (0.1 mol l^{-1} and pH 5.0) with 18.0 g l^{-1} inulin; incubation time=10 min

Optimum Temperature and Thermal Stability

The inulinase activity measured as a function of temperature from 40 to 80 °C shows that the activity was the highest at 50 °C (Fig. 4). The results in our previous studies indicated that the optimal temperature of the crude inulinase produced by the same yeast strain was also 50 °C [8]. The thermostability was investigated by preincubating the enzyme in the same buffer as described in the "Materials and methods" section for 120 min, and the remaining activity was determined. As shown in Fig. 4, the residual inulinase activity still kept 86.0% of the control after treatment at 65 °C for 2 h, indicating that the enzyme was very stable up to 65 °C. Fig. 4 also reveals that the enzyme was inactivated rapidly at a temperature higher than 65 °C, but still kept 60.0% of the control after treatment at 70 °C for 2 h. From these results, the inulinase seemed to have considerable thermostability. Inulinase from terrestrial microorganisms, in general, shows the highest activity below 50 °C whereas optimum temperature is mostly between 30 and 55 °C [1]. For example, the inulinases produced by *K. marxianus* and *K. fragilis* exhibited optimum activity at 55 °C, respectively [1]. The optimal hydrolysis temperature of the purified extracellular inulinase from the yeast *K. marxianus* var. *bulgaricus* for inulin was 55 °C [7] whereas the optimum temperature of the recombinant exoinulinase from *K. marxianus* expressed in *Pichia pastoris* was 55 °C [14]. The purified extracellular inulinase from the yeast *K. marxianus* var. *bulgaricus* showed good stability at 40 °C over 3.5 h and its half-life at 50 °C was 40 min [7]. This means that the optimal temperature and thermostability of inulinase from the marine yeast were also almost the same as that from the terrestrial *K. marxianus*, *K. marxianus* var. *bulgaricus*, and *K. fragilis*, which have been confirmed to be the common inulinase producers [1] (Fig. 4).

Optimum pH and pH Stability

The inulinase activity was measured at various pHs in buffers with the same ionic concentrations. Our results (Fig. 5) show that the maximum activity was observed at pH 5.0. Our recent studies also showed that the optimal pH of the crude inulinase produced

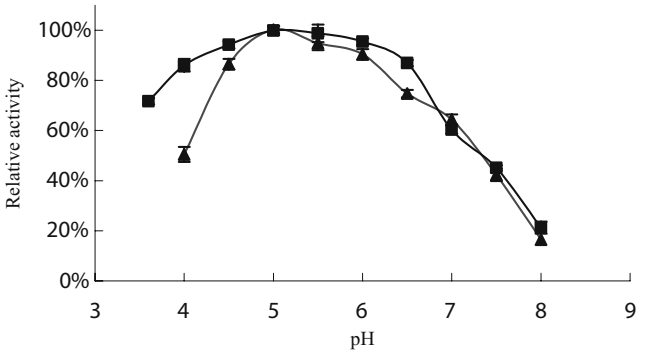

Fig. 5 Effects of different pH on inulinase activity (*filled triangles*) and stability (*filled squares*). The pH stability was tested by 120 min preincubation of the purified enzyme in appropriate buffers that had the same ionic concentrations at different pH values ranging from 3.5 to 8.0 at 4 °C. The remaining activities of inulinase were measured immediately after this treatment with the standard method as mentioned in the text. The inulinase activity of the final concentrated elute without preincubation was regarded as 100%. Data are given as the means±SD, n=3. The buffers with 1.8% (*w*/*v*) inulin; temperature=50 °C; incubation time=10 min

by the same yeast strain was 5.0 [8]. pH stability was tested by 120 min preincubation of the purified enzyme in appropriate buffers that had the same ionic concentrations at different pH values ranging from 3.5 to 8.0 at 4 °C. The remaining activities of inulinase were measured immediately after this treatment with the standard method as mentioned above. It can be seen from the results in Fig. 5 that the activity profile of the enzyme was stable in the range of pH 4.0 to 6.5. For example, the residual activity still kept over 96.0% after the treatment at pH 6.0 and 4 °C for 2 h. These results suggest that the enzyme was very stable at pH 6.0. However, when the purified inulinase was preincubated at pH values higher than 6.5 or lower than 4.5 for 120 min, the residual activity decreased rapidly, indicating the enzyme was not stable at pH higher than 6.5 or lower than 4.5. Generally, the inulinase from terrestrial fungi and yeasts was stable in the range of pH 4.0–8.0 whereas pH optima of the inulinase were in the range of 4.5–6.0 [1]. For example, the optimum pH of inulinase produced by *K. marxianus* was 4.4 [1]. The optimal hydrolysis pH of the purified extracellular inulinase from the yeast *K. marxianus* var. *bulgaricus* for sucrose was 4.75 [1] whereas the optimum pH of the recombinant exoinulinase from *K. marxianus* expressed in *P. pastoris* was 4.5 [14]. The results indicate that the inulinase from the marine yeast had similar pH optima to that from the terrestrial yeast (Fig. 5).

Effects of Different Cations on the Activity of the Purified Inulinase

It was found that Ca^{2+}, K^{+}, Na^{+}, Fe^{2+}, and Cu^{2+} (at concentrations of 1.0 mM) activated the activity of the purified inulinase. However, Mg^{2+}, Hg^{2+}, and Ag^{+} (at concentrations of 1.0 mM) acted as inhibitors in decreasing the activity of the purified inulinase with Hg^{+} (at a concentration of 1.0 mM) showing the lowest rank (22.0%) (Fig. 6), suggesting that they were able to alter the enzyme conformation [15]. The inhibition by mercuric ions may indicate the importance of thiol-containing amino acid residues in the enzyme function [16]. However, the inulinase from *Penicillium* sp was inhibited by Ag^{2+} and Cu^{2+} [1] whereas inulinases from *Aspergillus* sp. were inhibited by Fe^{3+}, Mn^{2+}, and Mg^{2+} [1]. Fe^{2+} increased the activity of the recombinant exoinulinase from *K. marxianus* expressed in *P. pastoris* by 41.97%, but Mg^{2+}, Cu^{2+}, Zn^{2+}, Ca^{2+}, and Fe^{3+} inhibited the activity of the recombinant

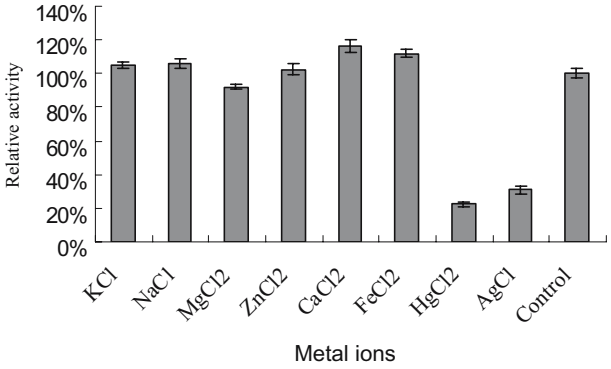

Fig. 6 Effect of different cations on the purified inulinase activity. Data are given as the means±SD, n=3. The inulinase activity in the absence of metal ions was regarded as 100%

exoinulinase from *K. marxianus* expressed in *P. pastoris* dramatically [14]. It was found that Mg^{2+} did not affect the activity of the purified extracellular inulinase from the yeast *K. marxianus* var. *bulgaricus*, but Ca^{2+} inhibited the enzyme by approximately 27%, Ba^{2+}, Zn^{2+}, and Na^{+} inhibited 50% whereas ferric chloride completely inhibited the enzyme [7]. This may imply that some physical and biochemical properties of the inulinase from the marine yeast strain were different from those from terrestrial yeasts.

Effects of Protein Inhibitors on the Activity of the Purified Inulinase

Figure 7 depicts the effects observed in the presence of protein inhibitors of the purified inulinase. The presence of the chelating agents, EDTA and 1,10-phenanthroline, inhibited the enzyme activity, demonstrating that the purified enzyme was metalloenzyme [17]. The enzyme activity was strongly inhibited by PMSF, indicating that Ser residues were essential for the enzyme active sites [18] because PMSF sulfonates the essential serine residue in the active site and results in the loss of activity. The results in Fig. 7 also show that iodoacetic acid had a negative effect on the enzyme activity, suggesting that Cys residues were important for the active sites of the enzyme. It was also found that DTT could protect the purified inulinase, but SDS could damage it. However, little is still known about the effects of protein inhibitors on inulinase activity produced by terrestrial yeasts [1].

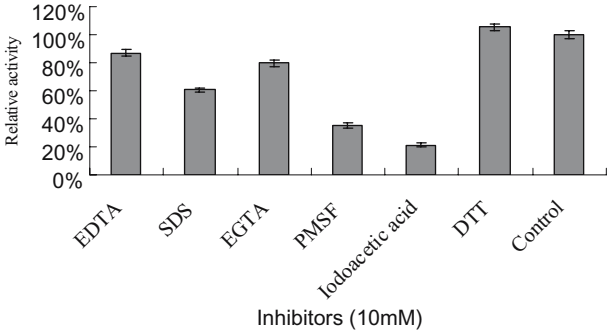

Fig. 7 Effect of protein inhibitors on the purified inulinase activity. Data are given as the means±SD, n=3. The inulinase activity in the absence of the protein inhibitors was regarded as 100%

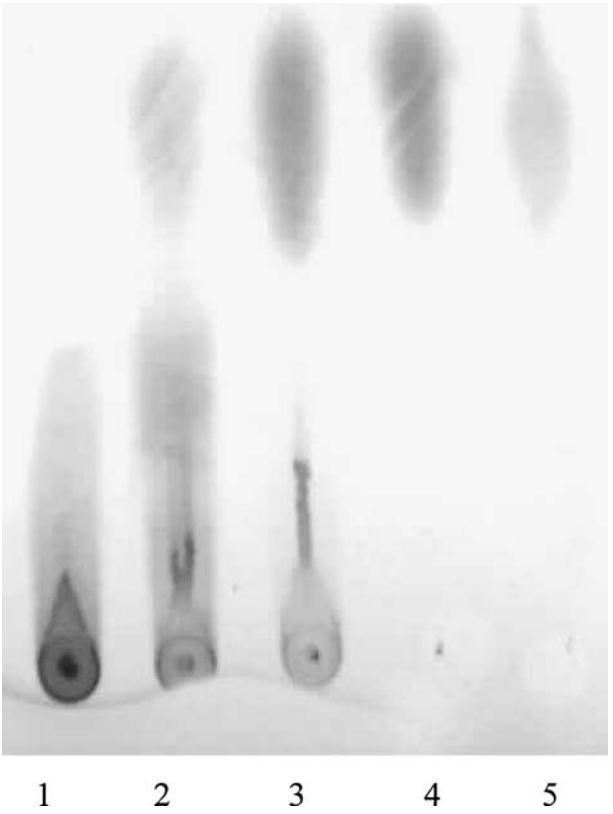

Fig. 8 Thin-layer chromatogram of the hydrolysis products of inulin with the purified inulinase. *Lane 1*, control (inulin+inactivated inulinase by heating at 100 °C for 10 min); *lane2*, the hydrolysis products for 8 h; *lane 3*, the hydrolysis products for 16 h; *lane 4*, fructose; *lane 5*, glucose. The end products of inulin hydrolysis was analyzed using the TLC plate (Silica gel 60, MERCK, Germany) with the solvent system *n*-butanol–pyridine–water (6:4:3) and a detection reagent comprising 2.0% (*w*/*v*) diphenylamine in acetone–2.0% (*w*/*v*) aniline in acetone–85% (*w*/*v*) phosphoric acid (5:5:1 by volume)

Kinetics Parameters

Lineweaver–Burk plots show that apparent K_m and V_{max} values of the enzyme for inulin were 20.06 mg/ml and 0.0085 mg/min, respectively (data not shown). However, the inulinase from terrestrial yeast *K. marxianus* had a K_m value of 11.9 and 3.92 mM for sucrose and inulin, respectively [1], whereas the apparent K_m value of the purified extracellular inulinase from the yeast *K. marxianus* var. *bulgaricus* for inulin was 86.9 mg/ml [7]. These results reveal that the inulinase from the marine yeast displayed very high affinity for inulin.

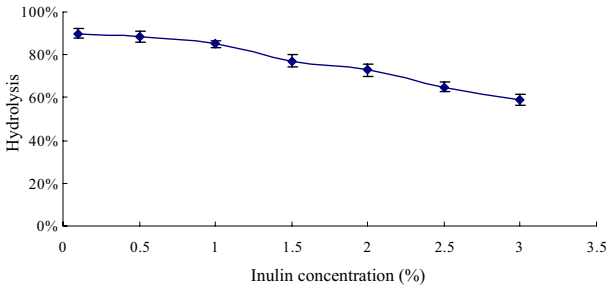

Fig. 9 Effects of different concentrations of inulin on hydrolysis extent by the purified inulinase. Data are given as the means±SD, n=3

Inulin Hydrolysis

It is very interesting to note that only monosaccharides (glucose and fructose) were released from inulin by the action of the purified inulinase (Fig. 8). These results in Fig. 8 strongly suggest that the inulinase produced by the marine yeast had high exoinulinase activity. However, analysis of the hydrolysis products of inulin by the crude inulinase produced by the same yeast strain showed that a large amount of monosaccharides and oligosaccharides with different molecular sizes were released from inulin after inulin hydrolysis by the crude inulinase [8]. The monosaccharides and oligosaccharides were also detected after inulin hydrolysis for more than 2 h by the purified exoinulinase produced by *K. marxianus* var. *bulgaricus* [7]. Therefore, the inulinase may have great potential use in the direct digestion of inulin in food and fermentation industries.

The potential application of inulinase from marine yeast was evaluated by studying the extent of hydrolysis of inulin at a temperature of 50 °C for 16 h. It can be observed in Fig. 9 that the hydrolysis extent of inulin by the purified enzyme decreased from 90.0% to 59.0% when the concentration of inulin was increased from 10.0 to 30.0 g/l within 16 h. When the enzyme dose varied from 0.04 to 0.4 U/mg inulin and incubated with 30.0 g/l of inulin, the highest extent of hydrolysis of inulin was observed when the enzyme dose was 0.2 U/mg inulin (data not shown). This strongly suggests that the concentration of enzyme dose (0.2 U/mg inulin) is sufficient to bring about an appreciable hydrolysis of inulin at the concentration of 30.0 g/l.

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References

1. Pandey, A., Soccol, C. R., Selvakumar, P., Soccol, V. T., Kriege, J. D., & Fontana, J. D. (1999). *Applied Biochemistry and Biotechnology, 81*, 35–52.
2. Rocha, J. R., Catana, R., Ferreira, B. S., Cabral, J. M. S., & Fernandes, P. (2006). *Food Chemistry, 95*, 77–82.
3. Gill, P. K., Manhas, R. K., & Singh, P. (2006). *Jounal of Food Engineering, 76*, 369–375.
4. Chi, Z., Liu, Z., Gao, L., Gong, F., Ma, C., Wang, X., et al. (2006). *Journal of Ocean University of China, 5*, 251–256.
5. Laloux, O., Cassart, J. P., Delcour, J., Van Beeumen, J., & Vandenhaute, J. (1991). *FEBS Letters, 289*, 64–68.
6. Wen, T., Liu, F., Huo, K., & Li, Y. Y. (2003). *World Journal of Microbiology & Biotechnology, 19*, 423–426.
7. Kushi, R. T., Monti, R., & Contiero, J. (2000). *Journal of Industrial Microbiology & Biotechnology, 25*, 63–69.
8. Sheng, J., Chi, Z., Li, J., Gao, L. M., & Gong, F. (2007). *Process Biochemistry, 42*, 805–811.
9. Spiro, R. G. (1966). *Methods in Enzymology, 8*, 3–26.
10. Bradford, M. M. (1976). *Analytical Biochemistry, 72*, 248–253.
11. Gong, F., Sheng, J., Chi, Z., & Li, J. (2007). *Journal of Industrial Microbiology and Biotechnology, 34*, 179–185.
12. Laemmli, U. K. (1970). *Nature, 227*, 680–685.
13. Rouwenhorst, R. J., Hensing, M., Verbakel, J., Scheffers, W. A., & Van Dijken, J. P. (1990). *Applied and Environmental Microbiology, 11*, 3337–3345.
14. Zhang, L., Zhao, C., Wang, J., Ohta, Y., & Wang, Y. (2005). *Process Biochemistry, 40*, 1541–1545.
15. Sharon, C., Furugoh, S., Yamakido, T., Ogawa, H., & Kato, Y. (1998). *Journal of Industrial Microbiology and Biotechnology, 20*, 304–307.
16. Barth, G., & Gaillardin, C. (1997). *FEMS Microbiology Reviews, 19*, 219–237.
17. Ramirez-Zavala, B., Mercado-Flores, Y., Hernadez-Rodriguez, C., & Villa-Tanaca, L. (2004). *FEMS Microbiology Letters, 235*, 369–375.
18. George, V., & Diwan, A. M. (1983). *Analytical Biochemistry, 132*, 481–483.