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Overexpression of the endo-inulinase gene from *Arthrobacter* sp. S37 in *Yarrowia lipolytica* and characterization of the recombinant endo-inulinase

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ABSTRACT

The endo-inulinase gene (*EnIA*) from *Arthrobacter* sp. S37 was ligated into the expression vector pINA1317 and over-expressed in *Yarrowia lipolytica* Po1h. It was found that the endo-inulinase activity and specific endo-inulinase activity produced by the transformant 1317-*EnIA* were 16.7 U/mL and 93.4 U/mg, respectively. The recombinant EnIA was purified and characterized. The molecular weight of the purified rEnIA was 78.9 kDa. The optimal pH and temperature of the purified rEnIA were 4 and 50 °C, respectively. The purified rEnIA was stable in the temperature range of 4–40 °C and in the pH range of 2–8. The activity of rEnIA was greatly stimulated in the presence of Li^+. The purified rEnIA could actively convert inulin into disaccharides.

1. Introduction

Inulin consists of linear chains of β-2,1-linked D-fructofuranose molecules terminated by a glucose residue through a sucrose-type linkage at the reducing end. Inulin is present as a reserve carbohydrate in the roots and tubers of plants such as Jerusalem artichoke, chicory, dahlia, and yacon. The dried materials of the tubers contain over 50% inulin [1]. The inulinases are classified among the hydrolases and target on the β-2,1 linkage of inulin and hydrolyze it into fructose and glucose. They can be divided into exo-inulinase and endo-inulinase. The exo-inulinase catalyzes removal of the terminal fructose residues from the non-reducing end of the inulin molecule while the endo-inulinase hydrolyzes the internal linkages in inulin to yield inulotriose, inulotetraose, and inulopentaose as the main products but lack invertase activity [2]. In addition to exo-inulinase, endo-inulinase also has many applications in food, pharmaceutical industries and biotechnology [3]. Furthermore, inulooligo-saccharides produced from inulin using endo-inulinase have very similar structure and functionalities to fructooligosaccharides whose beneficial effects on humans and animals have been well characterized as functional sweeteners [4].

So far, *Penicillium* sp, *Arthrobacter* sp. S37, *Aspergillus ficuum* ATCC, *Aspergillus niger*, *Xanthomonas* sp, *Xanthomonas oryzae*, *Chrysosporium pannorum*, *Bacillus smithii* T7, *Xanthomonas campestris* pv *phaseoli* and *Pseudomonas* sp. have been found to be able to produce endo-inulinase [5–12]. The endo-inulinase from *Arthrobacter* sp. S37 (EnIA) was characterized, the endo-inulinase genes from *Arthrobacter* sp. S37 and *Pseudommonas* sp, and *Pseudomonas mucidolens* have been cloned and expressed in *Escherichia coli* and *Saccharomyces cerevisiae* [6,13–16]. Asp460 was a catalytic residue and the presence of a carboxylate group in this position is a prerequisite for catalysis of *Arthrobacter* sp. S37 endo-inulinase (EnIA) and Asp460 as the residue interacts with the acid/base catalyst Glu519 and elevates its p*K*a [17].

In recent years, it has been found that *Yarrowia lipolytica* appears as one of the most attractive microorganisms for production of heterologous proteins because it has naturally secreting high amount of proteins and a large range of genetic markers and molecular tools [18]. The yeast has been used in several industrial processes and is non-pathogenic (GRAS, generally regarded as safe) [18]. Some recipient strains of *Y. lipolytica* have been optimized for heterologous protein production: (i) extracellular alkaline protease and extracellular acid protease genes have been deleted, and (ii) the production of recombinant invertase allows them to grow on sucrose and molasses [18]. Many molecular tools with the recombinant growth-phase-dependent promoter for heterologous expression in *Y. lipolytica* have been developed [18,19]. Furthermore, different strains of *Y. lipolytica* isolated from natural environments need to be genetically modified using heterologous genes in order to make them more useful in biotechnology [20,21]. Therefore, in the present study, in order to produce

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Table 1
The primers used in this study.

Primer	Sequence
LY1	5′-GGCCGTTCTGGCCGCCACCGGTGACCCCGTCCTG-3′ (the underlined bases encode *Sfi*I site)
LY2	5′-GGATCCCTA*GTGATGGTGATGGTGATG*GAAGGCCGGCGTCGGCCAG-3′ (the underlined bases encode *BamH*I site and both underlined and italic bases encode 6× His)
1317S1	5′-GGAATTGCGGCCGCTGTCGTCGGGAACCGCG-3′
1317A2	5′-CCCCGGGCGGCCGCACTGAGGGCTTTG-3′

the recombinant endo-inulinase using *Y. lipolytica* and engineer different strains of *Y. lipolytica*, the endo-inulinase gene cloned from *Arthrobacter* sp. S37 was over-expressed in *Y. lipolytica* and the recombinant endo-inulinase produced was characterized for hydrolysis of inulin.

2. Materials and methods

2.1. Strains and media

E. coli DH5α [*supE44 DlacU169* (*B80lacZDM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] was used to amplify the plasmids carrying the cloned gene. The yeast strain of *Y. lipolytica* for expression of heterologous gene was Po1h (*MatA*, *ura3-302*, *xpr2-322*, *axp1-2*, *ΔAEP*, *ΔAXP*, *Suc+*). *Y. lipolytica* Po1h and the transformant 1317-*EnIA* carrying the endo-inulinase gene (*EnIA*) cloned from *Arthrobacter* sp. S37 were deposited at Marine Microorganisms Culture Collection of China and the collection numbers are 2E01891 and 2E01892, respectively. The yeast strain was grown in YPD medium [21]. The yeast transformants were grown in YNB-N_{5000} medium without uracil [21]. The *E. coli* transformants were grown in LB medium with 100.0 μg/mL of ampicillin or 30.0 μg/mL of kanamycin. To produce heterologous protein by yeast transformants, the PPB medium was used [22].

2.2. Plasmids

pMD19-T simple vector (TaKaRa, Japan) was used to transform *E. coli* DH5α and pINA1317 was used to transform the *Y. lipolytica* yeast strain. The plasmid K1 carrying the endo-inulinase gene (*EnIA*) cloned from *Arthrobacter* sp. S37 was kindly supplied by Dr. Su-Il Kang from Seoul National University, Korea.

2.3. Isolation of DNA, restriction digestions, and transformation

DNA manipulations were performed by using standard methods [23]. Bacterial plasmid DNA was purified by using Perfect-prep plasmid mini kits (Eppendorf). Restriction endonuclease digestions and DNA ligations were performed according to the manufacturer's recommendations. *E. coli* was transformed with plasmid DNA according to Sambrook et al. [23]. Transformants were plated onto LB medium containing 100.0 μg/mL of ampicillin or 30.0 μg/mL of kanamycin. *Y. lipolytica* was transformed according to the methods as described by Xuan et al. [24].

2.4. Expression of the EnIA gene in Y. lipolytica

To express the *EnIA* gene in *Y. lipolytica*, the primers (LY1 and LY2) for amplification of the gene encoding endo-inulinase were designed according to the sequence of the gene (accession number: AJ131562) (Table 1). The plasmid K1 was used as the template for PCR. The reaction system (50.0 μL) was composed of 25.0 μL of 2× GC Buffer I (5.0 mol/L Mg^{2+} Plus), 8.0 μL of dNTP mixture (2.5 mmol/L each), 1.0 μL of the plasmid (plasmid K1), 1.0 μL of the primer LY1, 1.0 μL of the primer LY2, 0.5 μL of TaKaRa LA Taq (5.0 U/μL), and 13.5 μL of distilled water. The conditions for the PCR amplification were as follows: initial denaturation of 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 65 °C for 40 s and 72 °C for 2.5 min, with a final extension at 72 °C for 10 min. The PCR cycle was a GeneAmp PCR System 2400 (PerkinElmer, Waltham, MA, USA). The PCR products were separated by agarose gel electrophoresis and ligated into plasmid pMD19-T simple vector. The recombinant vector was transformed into *E. coli* DH5α. The recombinant vectors carrying the PCR products were extracted from the *E. coli* transformants and purified. The purified recombinant vectors carrying the PCR products were digested with *Sfi*I and *BamH*I, and the digests were ligated into pINA1317 digested with the same enzymes, and transformed into *E. coli* DH5α. The resulting plasmid carrying the *EnIA* gene was designated as pINA1317-*EnIA* (Fig. 1). The DNA fragments carrying the *EnIA* gene were amplified by PCR using the pINA1317-*EnIA* as template and the primers 1317S1 and 1317A2 (Table 1) which were designed according to the sequence of pINA1317 (Fig. 1). The reaction system and the conditions for PCR amplification were the same as described above. The PCR products carrying the *EnIA* gene were separated by agarose gel electrophoresis and recovered using TaKaRa Agarose Gel DNA Purification Kit Ver.2.0. The recovered PCR products were transformed into *Y. lipolytica* Po1h by lithium acetate methods [24]. The transformants were spread on YNB-N_{5000} plates without uracil. The positive transformants carrying the *EnIA* gene were grown in PPB liquid medium for 120 h and endo-inulinase activity in the culture of different positive transformants was determined as described below. *Y. lipolytica* Po1h cells only carrying yeast cassette without the *EnIA* gene and untransformed *Y. lipolytica* Po1h were used as the controls. After determination of endo-inulinase activity in the cultures from over 80 transformants, it was found that the endo-inulinase activity of the transformant 1317-EnIA among them was 16.7 U/mL. Therefore, this transformant was used as the recombinant endo-inulinase producer subsequently.

2.5. Preparation of the crude recombinant endo-inulinase

After the transformant 1317-EnIA was grown in PPB liquid medium for 120 h, the culture was centrifuged at 5000 × *g* and 4 °C for 10 min and the supernatant obtained was used as the crude endo-inulinase preparation.

2.6. Determination of endo-inulinase activity

After 100.0 μL of the crude endoinulinase preparation obtained above was mixed with 900.0 μL of 2.0% (w/v) inulin in 20.0 mmol/L citrate–NaH_2PO_4 buffer (pH 4.0), the mixture was incubated at 50 °C for 15 min. After that, the mixture was immediately inactivated in the boiling water for 10 min. The reducing sugar released from inulin was measured using Nelson–Somogyi method [25]. The same mixture with 100.0 μL of the crude endoinulinase preparation heated at 100 °C for 10 min was used as the control. One unit of endo-inulinase activity was defined as the amount of enzyme causing release of reducing sugars equivalent to 1.0 μmol from inulin in 1 min under the assay conditions. Protein concentration was measured by the method of Bradford, and bovine serum albumin served as standard [26].

2.7. Confirmation of integration of the target gene into Y. lipolytica genome

Confirmation of integration of the target gene into *Y. lipolytica* genome was performed according to the method described by Zhao net al. [20].

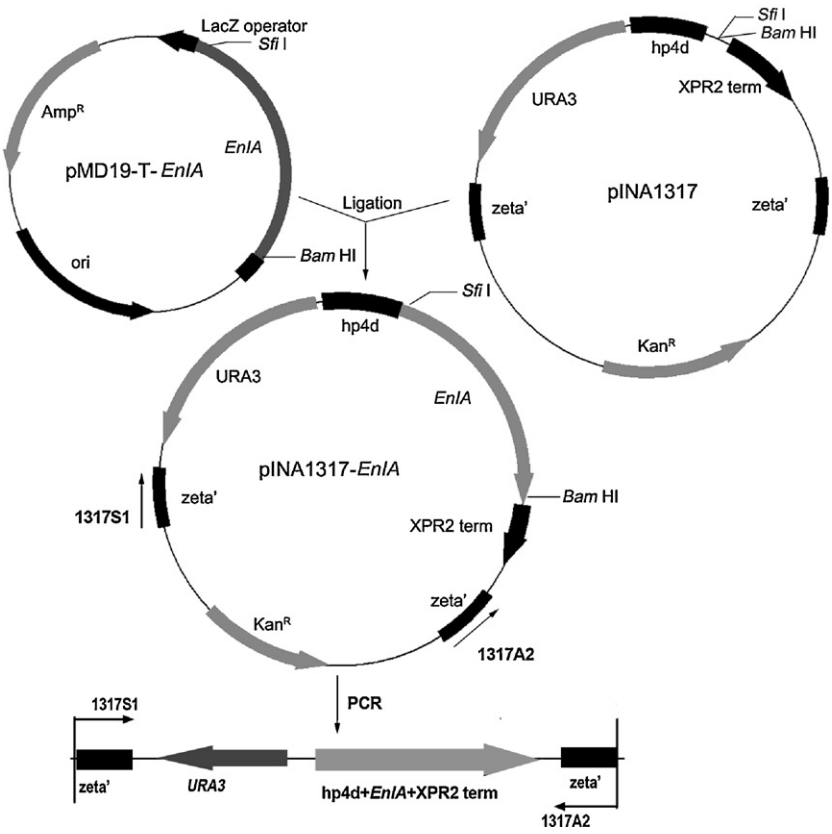

Fig. 1. Construction of the recombinant plasmid carrying the endo-inulinase gene. The detailed procedures were described in Section 2.4.

2.8. Purification of the crude recombinant endoinulinase

Five hundreds milliliters of the crude endo-inulinase preparation were filtrated using 0.2 μm membrane and the filtrate was concentrated to a volume of 50.0 mL by ultrafiltration with a 5-kDa cutoff™ membrane with a Labscale TFF System (Millipore, USA). The concentrate obtained was dialyzed against 50.0 mmol/L Tris–HCl buffer (pH 7.5). The dialysate was applied to DEAE Sepharose Fast Flow anion-exchange column (2.5 cm × 30 cm) that had been equilibrated with 50.0 mmol/L Tris–HCl buffer (pH 7.5). After the unbound proteins in the column were washed with 50.0 mmol/L Tris–HCl buffer (pH 7.5) for 1–2 h, the bound proteins in the column were then eluted with 0–0.5 mol/L NaCl solution in the equilibrating buffer. The endo-inulinase positive fractions were combined and 3.0 mL of the combined fractions was applied to Sephadex™ G-75 column (Pharmacia 2.5 cm × 100 cm), and the column was eluted with 50.0 mmol/L Tris–HCl buffer (pH 7.5). At a flow rate of 0.5 mL/min, 2.0-mL fractions were collected. The endo-inulinase-positive fractions were combined and concentrated by filtration through an AmiconYM3 (MW cutoff 10,000) membrane.

2.9. SDS-PAGE analysis and Western blotting

The recombinant endo-inulinase was confirmed in non-continuous denaturing SDS-PAGE with a two-dimensional electrophoresis system (Amersham Biosciences, Sweden) [27] and stained by Coomassie Brilliant Blue R-250 [28]. The molecular mass standards for SDS-PAGE comprised phosphorylase B (97.2 kDa), bovine serum albumin (66.4 kDa), ovalbumin (44.3 kDa), carbonic anhydrase (29.0 kDa), trypsin inhibitor (20.1 kDa) and lysozyme (14.4 kDa). To confirm the recombinant endo-inulinase expression as a His-tagged fusion protein, Western blotting analysis was carried out according to the method described by Li et al. [29].

2.10. Effects of pH and temperature on the recombinant endo-inulinase activity and stability

The effect of pH on the purified recombinant endo-inulinase activity was determined by incubating the purified enzyme in 40.0 mmol/L Na_2HPO_4–citric acid buffer between pH 2.0 and 8.0 using the standard assay conditions. Its pH stability was tested by pre-incubating the purified enzyme for 24 h at 4 °C and pH values from 2.0 to 8.0 in the buffers with the same ionic concentrations. The buffers used were 40.0 mmol/L Na_2HPO_4–citric acid buffer (pH 2.0–8.0). The remaining endo-inulinase activity was measured immediately using the standard method as described above. The optimal temperature for the enzyme activity was determined at temperatures from 35 to 65 °C in 20.0 mmol/L Na_2HPO_4–citric acid buffer (pH 4.0). The temperature stability of the purified enzyme was tested by pre-incubating it at temperatures from 4 to 65 °C for 120 min, and the residual activity was measured immediately as described above. The relative endo-inulinase activity of the pre-incubated sample at 4 °C was considered to be 100%.

2.11. Effects of different metal ion on the recombinant endo-inulinase activity

To examine the effects of metal ion on the recombinant endo-inulinase activity, the assay was performed for 1 h as described above and various metal ions were added to achieve a final concentration of 5.0 mmol/L. The activity in the absence of metal ion was defined as the control. The metal ions tested were Zn^{2+}, Cu^{2+},

Mg^{2+}, Fe^{3+}, Ca^{2+}, K^{+}, Mn^{2+}, Hg^{2+}, Li^{+}, Fe^{2+}, Ag^{+}, Na^{+}, Ba^{2+}, Ni^{2+}, and Co^{2+}.

2.12. The effects of the protein inhibitors

EGTA, EDTA, PMSF, DTT, iodoacetic acid, and SDS (final concentration of 5.0 mmol/L) were tested for their effects on the recombinant endo-inulinase activity. The purified enzyme was pre-incubated with each inhibitor at 4 °C for 60 min, followed by the assay procedure described above. The activity in the absence of the protein inhibitors was defined as the control.

2.13. Determination of kinetics parameters

To obtain K_m and V_{max} of the recombinant endo-inulinase for the inulin substrate, 11.0 mg/mL, 15.0 mg/mL, 20.0 mg/mL, 25.0 mg/mL, 40.0 mg/mL, 70.0 mg/mL and 100.0 mg/mL of inulin were reacted with 25.0 μL of the purified recombinant endo-inulinase dissolved in 20.0 mmol/L citrate–NaH_2PO_4 buffer (pH 4.0) at 50 °C for 15 min, respectively. The reaction was terminated at 100 °C for 10 min. A Lineweaver–Burk plot was generated to obtain the K_m and V_{max}, and the values were expressed as the mean of the triplicate experiments.

2.14. Inulin hydrolysis

One hundred microliters of the diluted endo-inulinase solution (15.5 U/mL) was mixed with 900.0 μL of inulin solution (2.0%, w/v) and the mixture was incubated at 35 °C for different times. The end products of hydrolysis were determined by thin layer chromatography [30]. The reaction mixtures in which the purified recombinant endo-inulinase was inactivated prior to addition by heating at 100 °C for 10 min were used as the controls.

3. Results

3.1. Overexpression of the EnIA gene in Y. lipolytica

As stated in our previous studies [31], the vector pINA1317 and the recipient yeast strain *Y. lipolytica* Po1h have so many advantages. Therefore, the *EnIA* gene cloned from *Arthrobacter* sp. S37 was ligated into pINA1317 and the resulting plasmid pINA1317-*EnIA* was obtained (Fig. 1). After the PCR products carrying the *EnIA* gene were amplified, they were transformed into the yeast strain Po1h. After the different transformants carrying the *EnIA* gene were grown for 120 h, the recombinant EnIA activity of the cultures and in the supernatants was determined. *Y. lipolytica* Po1h cells only carrying yeast cassette without the *EnIA* gene and *Y. lipolytica* Po1h cells not to be transformed were used as the controls. The results in Tables 2 and 3 showed that only the cultures of the different transformants carrying the *EnIA* gene had the endo-inulinase activity, whereas no endo-inulinase activity in the culture of strain Po1h was observed. Among them, the culture of the transformant 1317-EnIA had 16.7 U/mL of endo-inulinase activity and the specific endo-inulinase activity in the supernatant of the transformant 1317-EnIA was 93.4 U/mg (Tables 2 and 3).

It also can be seen in Fig. 1 that the expression vector pINA1317 used in this study carries the zeta elements. It has been confirmed that the zeta elements in this vector allow the "yeast expression cassette" to integrate either by homology in *Y. lipolytica* strains carrying Ylt1, or by non-homologous recombination in strains devoid of this retrotransposon [18]. The multiple copies are dispersed in the genome, increasing the stability of transformants compared to tandem repeated integration [18]. To get the evidence that the linear DNA fragments carrying the cloned endo-inulinase gene have been integrated into the genome of the yeast *Y. lipolytica* Polh, the genomic DNAs from the corresponding transformant 1317-EnIA and its wild-type *Y. lipolytica* Polh were extracted and used as the templates for PCR checking. The PCR products obtained were of the expected size, namely 2317 bp (Fig. 2). This indicated that the DNA fragments encompassing the endo-inulinase gene have indeed been integrated into the genome of the transformant 1317-EnIA. However, no such PCR products were amplified from the genomic DNA of the yeast *Y. lipolytica* Polh without carrying the cloned endo-inulinase gene (Fig. 2).

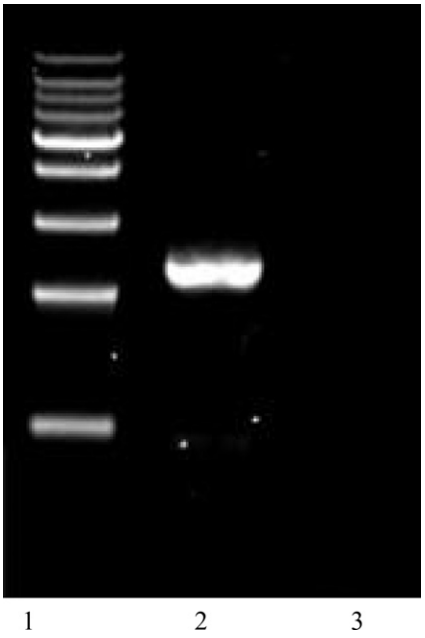

Fig. 2. PCR products (Lane 2) amplified from the genomic DNA of the transformant 1317-EnIA excreting endo-inulinase and no PCR product (Lane 3) amplified from the genomic DNA of *Y. lipolytica* Polh without excreting endo-inulinase by using the primers as described in Table 1. Lane 1 was DNA markers, the marker sizes from top to bottom were 9.0 kb, 8.0 kb, 7.0 kb, 6.0 kb, 5.0 kb, 4.0 kb, 3.0 kb, 2.0 kb and 1.0 kb, respectively.

3.2. Purification of the recombinant endo-inulinase

The recombinant endo-inulinase in the supernatant of the culture of the transformant 1317-EnIA was purified by ultrafiltration, gel filtration chromatography, and DEAE Sepharose Fast Flow anion-exchange chromatography as described in Materials and methods. The elution profile of gel filtration chromatography indicated that there were three peaks, but only peak 2 with the specific endo-inulinase activity in the fractions from 120 to 140 min showed a single sharp peak (data not shown). Therefore, the fractions were collected and concentrated by ultrafiltration. The results in Table 3 showed that the enzyme was purified to homogeneity with a 8.1-fold increase in the specific endo-inulinase activity with a yield of about 25.8% as compared to that in the supernatant and had a specific activity of 753 U/mg protein (Table 3).

3.3. SDS-PAGE analysis and Western blotting

SDS-PAGE showed that the supernatant from the transformant carrying the *EnIA* gene-6His exhibited one unique band with a molecular mass of about 78.9 kDa (Lane 2 in Fig. 3), which was the similar size to that (82.4 kDa) as estimated from the deduced amino acid sequence of the *EnIA* gene. The results of Western blotting (Lanes 3′ and 2′ in Fig. 3) confirmed that this unique band

Table 2
The endo-inulinase activities of different transformants.

Transformant	Endo-inulinase activity (U/mL)	Transformant	Endo-inulinase activity (U/mL)
1317-*EnlA*	16.7 ± 0.6	1317-LY6	6.7 ± 0.4
1317-LY1	13.0 ± 0.2	1317-LY7	4.7 ± 0.1
1317-LY2	13.9 ± 0.3	Untransformed *Y. lipolytica* Polh	0
1317-LY3	6.4 ± 0.1	The supernatant of the transformant 1317-EnLA	7.6 ± 0.2
1317-LY4	5.7 ± 0.4		
1317-LY5	7.6 ± 0.1		
1317-LY6	6.7 ± 0.2		
1317-LY7	4.7 ± 0.3		

The yeast transformant was grown in PBB liquid medium for 120 h and the endo-inulinase activity in the supernatants was determined. Data are given as means ± SD, $n = 3$.

Table 3
Summary of the purification procedures of the endo-inulinase.

Purification step	Total protein (mg)	Total activity (U)	Yield (%)	Specific activity (U/mg)	Purification fold
Crude enzyme	32.5	3040	100	93.4	1.0
DEAE-Sepharose Fast Flow	4.9	1560	51.1	315	3.4
Sephadex G-75	1.0	784	25.8	753	8.1

was indeed the His-tagged fusion protein of the recombinant endo-inulinase.

3.4. Effects of different temperatures and pHs on activity and stability of the recombinant endo-inulinase

The recombinant endo-inulinase activity measured as a function of temperature from 4 °C to 65 °C showed that the activity was the highest at 50 °C (Fig. 4A). The thermo-stability was investigated by pre-incubating the enzyme in the range of 4–65 °C in the same buffer as described in Section 2 for 120 min and the remaining activity was determined. As shown in Fig. 4A, the enzyme was stable up to 40 °C, keeping over 76.7% activity of the control, but inactivated rapidly at temperatures above this. The recombinant endo-inulinase activity was measured in the pH range of 2.0–8.0 in buffers with the same ionic concentrations. The results in Fig. 4B showed that maximum activity was observed at pH 4.0. To investigate pH stability of the purified recombinant endo-inulinase, the enzyme preparation was incubated at 4 °C for 24 h in the solutions with the pH range of 2.0–8.0 that had the same ionic concentrations and residual activity was measured. The activity of the enzyme was stable between pH 2.0 and 8.0 (Fig. 4B). For example, the enzyme still kept 80.1% of the control at pH 8.0 for 24 h. This meant that the enzyme could be active in the wide range of pH.

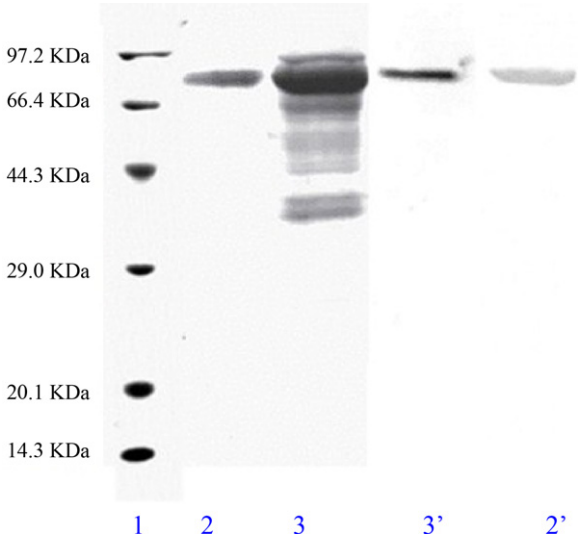

Fig. 3. SDS-PAGE (12%) of the fractions showing endo-inulinase activity obtained during the purification and Western blotting analysis of the expressed fusion protein rEnlA-6His. The mouse anti-His antibody (GE Healthcare, American) was used at a dilution of 1:1500 as the primary antibody and goat anti-mouse IgG antibody conjugated with horseradish peroxidase (HRP; TianGen, Beijing, China) was used at a dilution of 1:2000 as the secondary antibody. Lane 1 marker proteins with relative molecular masses indicated on the right; Lane 2 the purified rEnlA; Lane 3 supernatant; Lane 3′ and Lane 2′ were the results of Western blot of the supernatant and the purified rEnlA.

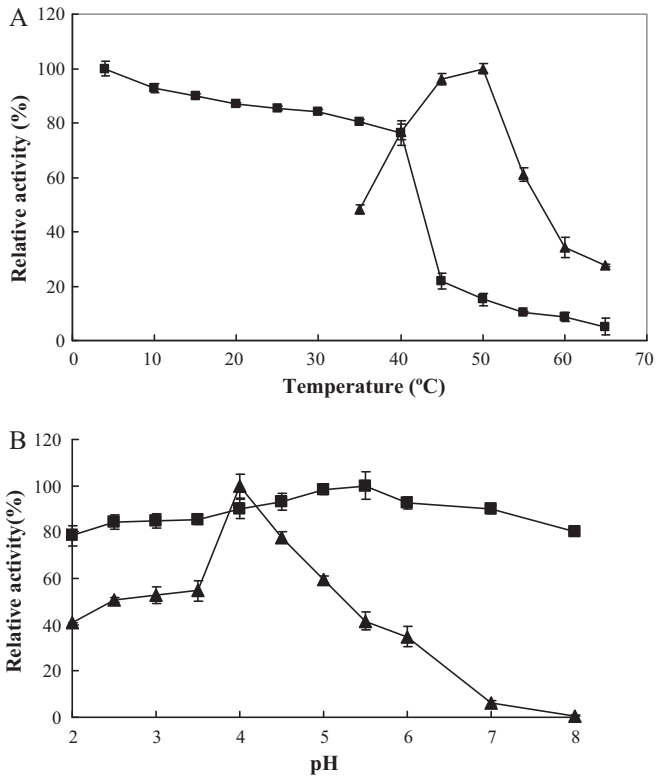

Fig. 4. Effects of different temperatures (A) and pHs (B) on rEnlA activity (filled upright triangle) and stability (filled square). The optimal temperature for the enzyme activity was determined at temperatures from 35 to 65 °C and pH 4.0. The temperature stability of the purified enzyme was tested by pre-incubating it at temperatures from 4 to 65 °C for 120 min. The optimal pH of the purified recombinant endo-inulinase was determined by incubating the purified enzyme in the pH range from 2.0 to 8.0 and its pH stability was tested by pre-incubating the purified enzyme for 24 h at 4 °C and pH values from 2.0 to 8.0. Data are given as means ± SD, $n = 3$.

Table 4
Effect of different cations and protein inhibitors on the purified recombinant endo-inulinase activity.

Cations	Concentration (mM)	Relative activity (%)	Inhibitors	Concentration (mM)	Relative activity (%)
Control	0.0	100 ± 4.5	Control	0	100 ± 4.1
Zn^{2+}	5.0	25.8 ± 3.3	DTT	5.0	88.8 ± 2.9
Mg^{2+}	5.0	96.4 ± 5.3	EGTA	5.0	88.4 ± 4.5
Ca^{2+}	5.0	96.9 ± 4.5	PMSF	5.0	86.6 ± 3.3
Hg^{2+}	5.0	2.2 ± 1.3	EDTA	5.0	89.4 ± 2.5
Cu^{2+}	5.0	2.8 ± 1.6	SDS	5.0	17.3 ± 1.8
Mn^{2+}	5.0	5.00 ± 1.0	Iodoacetic acid	5.0	67.8 ± 5.9
Fe^{3+}	5.0	20.7 ± 4.6			
Fe^{2+}	5.0	58.8 ± 1.5			
Ba^{2+}	5.0	103.6 ± 4.2			
K^{+}	5.0	105.1 ± 4.9			
Co^{2+}	5.0	69.8 ± 2.9			
Ag^{+}	5.0	24.4 ± 0.8			
Ni^{2+}	5.0	44.2 ± 2.9			
Li^{+}	5.0	113.6 ± 2.6			

The recombinant endo-inulinase was mixed with different ions or different protein inhibitors and the mixtures were incubated at 4 °C for 1 h. Then, endo-inulinase activity was determined. Data are given as means ± SD, $n = 3$.

3.5. Effects of different cations and protein inhibitors on activity of the recombinant endo-inulinase

As indicated in Table 4, only Li^{+} (5.0 mmol/L) had an activating effect on the recombinant endo-inulinase activity, while Mg^{2+}, Ca^{2+}, Ba^{2+} and K^{+} had no significant effect on the recombinant endo-inulinase activity. However, Co^{2+}, Zn^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Hg^{2+}, Cu^{2+}, Ag^{+}, and Ni^{2+} (5.0 mmol/L) tested had negative influence on the recombinant endo-inulinase activity and Cu^{2+}, Hg^{2+}, and Mn^{2+} demonstrated the greatest inhibitory effect (less than 5.0%; Table 4).

Table 4 also depicted the effects observed in the presence of protein inhibitors on the enzyme. It could be clearly observed from the results in Table 4 that iodoacetic acid, EDTA, EGTA, PMSF, DTT, and SDS (5.0 mmol/L) had negative effect on the enzyme activity.

3.6. Kinetics parameters

The Lineweaver–Burk plots showed that the apparent K_m and V_{max} values of the recombinant endo-inulinase for inulin were 37.1 mg/mL and 3.9 mg/min, respectively (data not shown).

3.7. Inulin hydrolysis

The results in Fig. 5 showed that after the hydrolysis of inulin by the recombinant endo-inulinase obtained in this study, only disaccharides were produced, indicating that the recombinant enzyme produced by the transformant carrying the *EnIA* gene was indeed endo-inulinase.

4. Discussion

The transformant 1317-EnIA in which the *EnIA* gene had been integrated into its chromosomal DNA (Fig. 2) had 16.7 U/mL of endo-inulinase activity and 93.4 U/mg of the specific endo-inulinase activity (Tables 2 and 3). It has been reported that the specific activity of the native endo-inulinase produced by *Arthrobacter* sp. S37 was 0.8 U/mg protein [6]. This meant that the *EnIA* gene was over-expressed in *Y. lipolytica* Po1h. It has been reported that the endo-inulinase gene (*inul*) of *Pseudomonas* sp. was expressed in *E. coli* HBlOI and the endo-inulinase (1.5 U/mL within 20 h) from *E. coli* HBlOI/pKMG50 was constitutively expressed, producing a high yield of IOS (78%) while the endo-inulinase activity in a culture broth of *Pseudomonas* sp was 1.8 U/mL [14]. However, after the *INU2* gene encoding an endo-inulinase of *A. ficuum* was expressed in a *SUC2*-deleted *S. cerevisiae*, the recombinant *S. cerevisiae* produced 4.0 U/mL of the endo-inulinase free of an exo-inulinase on YPS medium [32]. This indicated that after the bacterial endo-inulinase gene used in this study was expressed in *Y. lipolytica*, the endo-inulinase activity produced by the recombinant *Y. lipolytica* was very high.

The molecular mass of the recombinant endo-inulinase was about 78.9 kDa because of the added 6His tag (Lane 2 in Fig. 3). However, the molecular mass of the native endo-inulinase produced by *Arthrobacter* sp. S37 was estimated as 75.0 kDa by SDS-PAGE [6]. The molecular mass of the secreted endo-inulinase by the recombinant *S. cerevisiae* was 67.0 kDa as measured by SDS-PAGE and Western blotting analysis, which was larger by 1–3 kDa than those of the purified enzymes [33]. The purified endo-inulinase from *X. oryzae* No. 5 has molecular mass of 139.0 kDa [34].

The optimal pH and temperature of the purified rEnIA were 4 and 50 °C, respectively. The purified rEnIA was stable in the temperature range of 4–40 °C and in the pH range of 2–8 (Fig. 4). However, the native endo-inulinase produced by *Arthrobacter* sp. S37 hydrolyzed inulin optimally at pH 7.5 and 50 °C [6] and the enzyme activity at the range of pH 5.0–10.5 and 30–40 °C was stable more than 80% of the maximum activity [6]. This meant that the optimal pH of the recombinant endo-inulinase produced *Y. lipolytica* was much lower than that of the native endo-inulinase produced

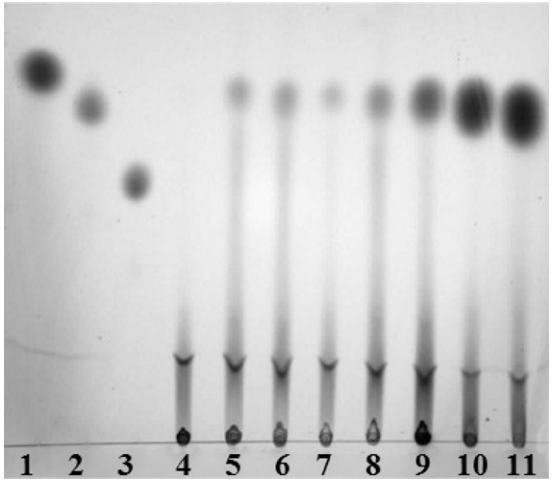

Fig. 5. TLC analysis of the hydrolysate of inulin by rEnIA. Lane 1: Glucose; Lane 2: Sucrose; Lane 3: Raffinose; Lane 4: Inulin+ inactivated the purified recombinant endo-inulinase at 100 °C for 10 min; Lanes 5–11: The hydrolysis products after inulin was hydrolyzed by the purified recombinant endo-inulinase for 10, 20, 30, 60, 300, 1200 and 1440 min.

by *Arthrobacter* sp. S37, suggesting that the characteristics of the recombinant endo-inulinase were greatly different from those of the native one. The optimal temperature of the endo-inulinase produced by *B. smithii* T7 was 70 °C [11]. The endo-inulinase produced by *Xanthomonas* sp. was optimally active at 45 °C and pH 6.0 [9] while the activity of endo-inulinase from *X. oryzae* No. 5 was the highest at pH 7.5 and 50 °C, and stable over a pH range of 6.0–9.0 and up to 45 °C [34].

The activity of rEnIA was greatly stimulated in the presence of Li^+ while Mg^{2+}, Ca^{2+}, Ba^{2+} and K^+ had no significant effect on the recombinant endo-inulinase activity and other ions tested had negative influence on the recombinant endo-inulinase activity (Table 4). It has been reported that Hg^{2+} and EDTA also decreased the native endo-inulinase activity produced by *Arthrobacter* sp. S37 to 67% and 59% of the control, respectively while Ca^{2+} and Mg^{2+} have no effects on the endo-inulinase activity produced by *Arthrobacter* sp. S37 [6]. This again demonstrated that the properties of the recombinant endo-inulinase produced by *Y. lipolytica* were greatly different from those of the native endo-inulinase produced by *Arthrobacter* sp. S37. The activity of endo-inulinase I, and endo-inulinase II produced by *A. ficuum* JNSP5-06 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 [35]. The activity of the endo-inulinase of *Penicillium* sp. TN-88 was completely inactivated by Ag^+ and Hg^{2+} [5].

The apparent K_m and V_{max} values of the recombinant endo-inulinase for inulin were 37.1 mg/mL and 3.9 mg/min, respectively (data not shown). However, the K_m of the purified endo-inulinase produced by *Arthrobacter* sp. S37 was estimated to be 1.7 mmol/L by Lineweaver–Burk plot [6]. The K_m of the purified endo-inulinase produced by *B. smithii* T7 was 4.17 mmol/L [11]. The Michaelis constant (K_m) and maximum reaction velocity (V_{max}) of the endo-inulinase from *X. oryzae* No. 5 were 16.7 g/L and 12.1 g/L h, respectively [34].

After the hydrolysis of inulin by the recombinant endo-inulinase obtained in this study, only disaccharides were produced (Fig. 5). However, the native endo-inulinase produced by *Arthrobacter* sp. S37 hydrolyzed inulin mainly into inulo-triose (F3), inulo-tetraose (F4) and inulo-pentaose (F5) optimally at pH 7.5 and 50 °C [6]. We think that the higher recombinant endo-inulinase activity was probably responsible for the appearance of inulobiose (Tables 1 and 2). Meanwhile, inulobiose was also the major product with immobilized endo-inulinase produced by *Pseudomonas* sp. No. 65 or immobilized recombinant *E. coli*, possessing endo-inulinase gene [2]. In soluble form, the endo-inulinase produced by *Pseudomonas* sp. No. 65 produced two major components, inulobiose and DP3 oligosaccharides [2]. When inulo-oligosaccharide from chicory juice was hydrolyzed by an endo-inulinase from *Pseudomonas* sp, DP2, DP3 and DP4 were the major components [9]. After the endo-inulinase gene (*inul*) of *Pseudomonas* sp. was expressed in *E. coli* HblOl, the intact cells of the recombinant *E. coli* and the native enzyme from *Pseudomonas* sp. were used to produce IOS. It was found that higher levels of inulobiose (the smallest molecule in the product) were observed when intact cells were employed. They thought that the difference in microenvironments within the cells is probably responsible for the appearance of high levels of inulobiose [14].

5. Conclusions

In summary, the endo-inulinase gene (*EnIA*) from *Arthrobacter* sp. S37 was over-expressed in *Y. lipolytica* Po1h. The molecular weight of the purified rEnIA was 78.9 kDa. The optimal pH and temperature of the purified rEnIA were 4 and 50 °C, respectively. The purified rEnIA was stable in the temperature range of 4–40 °C and in the pH range of 2–8. The activity of rEnIA was greatly stimulated in the presence of Li^+. The purified rEnIA could actively convert inulin into disaccharides. In order to improve properties of the endo-inulinase, the chemical modification of it may be carried out [36].

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