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Expression of an endoinulinase from *Aspergillus ficuum* JNSP5-06 in *Escherichia coli* and its characterization

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ABSTRACT

In this study, the endoinulinase encoded by the *endo I* gene from *Aspergillus ficuum* JNSP5-06 was over-expressed in *Escherichia coli* and the biochemical characterization of recombinant endoinulinase was investigated. The results showed that the molecular weight of recombinant endoinulinase was estimated to be 60 kDa by SDS-PAGE. The K_m and V_{max} values with inulin as the substrate were found to be (67.4 ± 4.2) mg/mL and (349.2 ± 13.7) mg/mL min, respectively. The optimum pH and temperature of this enzyme were 5.0 and 60 °C, respectively. Its activity was increased by Zn^{2+}, completely inhibited by Ag^{+}, and Cu^{2+}, and strongly inhibited by Al^{3+}, Fe^{2+}, and Fe^{3+}, whereas K^{+}, Ca^{2+}, Mn^{2+}, Mg^{2+}, and Ni^{2+} had no significant influence on this recombinant endoinulinase activity. The major products of hydrolysis of inulin by the recombinant endoinulinase were fructo-oligosaccharides with degree of polymerization (DP) from 3 to 4.

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 molecules terminated by a glucose residue through a sucrose-type linkage at the reducing end (Vandamme & Derycke, 1983). Recently, inulin has attracted more attention as a relatively inexpensive and abundant material for the production of fructo-oligosaccharides, which are extensively used in pharmaceuticals industry and food industry (Vandamme & Derycke, 1983).

Fructo-oligosaccharides can be produced from inulin by using microbial inulinase. 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 hydrolyze the internal β-2,1 fructofuranosidic linkages to yield inulo-oligosaccharides as the main products, such as inulotriose, inulotetraose, and inulopentaose.

Inulinase can be produced by different microorganisms including fungi, yeast, and bacteria. Among the filamentous fungi, *Aspergillus* and *Penicillium* species are common inulinase-producers (Nakamura, Kurokawa, Nakatsu, & Ueda, 1978; Nakamura et al., 1997). Previous studies revealed that several microorganisms produced endoinulinase, such as *Aspergillus niger* (Nakamura, Kurokawa, Nakatsu, & Ueda, 1978), *Aspergillus ficuum* (Ettalibi & Baratti, 1987), *Chrysosporium pannorum* (Xiao, Tanida, & Takao, 1989), and *Penicillium purpurogenum* (Onodera & Shiomi, 1988).

However, the activity of endoinulinase from these microorganisms by submerged fermentation is commonly low, and isolating endoinulinase from exoinulinase-containing culture medium is difficult, it is not suitable for industrial application. In order to make endoinulinase application in the industrial production of fructo-oligosaccharides from inulin, it would be better to express the gene for endoinulinase in a host lacking an extracellular invertase, fructosyltransferase and/or exoinulinase.

Previous studies indicated that the endoinulinase-encoding gene from *A. ficuum* has been expressed in *Saccharomyces cerevisiae* (Kim et al., 1999; Park, Jeong, Kim, Yang, & Chae, 2001). Moreover, recent study also showed that endo-inulinase gene from *Arthrobacter* sp. S37 has been expressed in *Yarrowia lipolytica* (Li, Liu, Wang, Chi, & Madzak, 2012). However, to the best of our knowledge, no study on the expression of an endoinulinase gene from *A. ficuum* in *Escherichia coli* has been reported. In this study, the expression of this endoinulinase gene from *A. ficuum* JNSP5-06 in *E. coli* and some properties of the recombinant endoinulinase are investigated.

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2. Materials and methods

2.1. Strains, plasmids and reagents

A. ficuum JNSP5-06, isolated from soil as reported previously (Wang, Jin, Jiang, & Adamu, 2003), was stored in our laboratory. *E. coli* strains JM109 and BL21 (DE3) were purchased from Novagen Co. Ltd., USA and used as the host strain. The vectors pMD19 and pET-28a(+) were purchased from TaKaRa Biotech Co., Ltd. (Dalian, China) and used for the cloning and expression of the endoinulinase gene, respectively. PCR reagents, T_4 DNA ligase and the restriction endonucleases were purchased from TaKaRa Biotech Co., Ltd. (Dalian, China). All other reagents were purchased from Sigma (St. Louis, MO) and were of analytical grade.

2.2. Genomic DNA extraction

Preparation of genomic DNA from *A. ficuum* JNSP5-06 was carried out according to the described method (Garber & Yoder, 1983). The mycelia were washed three times with SE buffer (0.15 mol/L NaCl, 0.1 mol/L EDTA; pH 8.0) and then homogenized in 5 vol. (w/v) of the same buffer. The homogenate was supplemented with SDS and Proteinase K to final concentrations of 0.6% and 100 μg/mL, respectively. The mixture was then incubated at 37 °C for 2 h with shaking at 100 rpm. SDS was again added to the mixture to reach a final concentration of 2.0%, and the mixture was incubated for another 20 min at 60 °C. The mixture was chilled on ice and mixed with an equal volume of phenol/chloroform/isoamylalcohol (25:24:1, v/v/v), followed by shaking at 150 rpm for 30 min. The supernatant was collected after centrifugation at 10,000 rpm for 10 min at 4 °C and extracted with an equal volume of chloroform. DNA was precipitated by adding 2 vol. of ethanol and the precipitate was dissolved in TE buffer (10 mmol/L Tris–HCl, 1 mmol/L EDTA; pH 8.0) supplemented with 50 μg/mL RNase.

2.3. Amplification of the endoinulinase gene by PCR and sequence analysis

Genomic DNA were amplified by polymerase chain reaction (PCR) using the upstream primers P1: 5′-ATCGAATTCCAGTCTAATGATTACCGTCC-3′ and the downstream primers F1: 5′-GACGAATTCGGTACCTGTCTGCACTGC-3′ (the underlined sequence is the position of an *EcoR* I site) specifically designed to pick up endoinulinase (purchased from TaKaRa Biotechnology Co., Ltd, Dalian, China). The PCR reaction system consisted of 0.2 μL Taq, 5 μL buffer, 4 μL deoxynucleotides (dNTP mixture, each of 2.5 mmol/L), 2 μL primer P1, 2 μL primer F1, 3 μL template DNA (endoinulinase genomic DNA), and distilled H_2O made up to a final volume of 50 μL. The PCR program was 95 °C for 5 min followed by 25 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 2 min, followed by an elongation at 72 °C for 10 min. The PCR product was detected by 0.8% agarose gel electrophoresis.

Products of the PCR reaction were cloned into pMD19-T Simple Vector (TaKaRa) and sequenced by Invitrogen Co., Ltd. (Shanghai, China). The sequence was analyzed using the software package DNAMAN 5.0 (Lynnon Biosoft, USA) and the homology was analyzed in the GenBank with the BLAST program.

2.4. DNA manipulations and E. coli transformation

Digestion of DNA with restriction endonucleases, separation of fragments by agarose gel electrophoresis, ligation of DNA fragments, transformation of *E. coli* with plasmidic DNA and extraction of recombinant DNA were all performed according to the standard method (Sambrook et al., 1989). DNA fragments were recovered from agarose gels using the DNA Gel Extraction Kit of TaKaRa.

2.5. Endoinulinase expression

The DNA fragment was ligated into pET-28a(+) with restriction sites of *Eco*R I to generate construct pET-28a-*endo I*. The expression construct was transformed into *E. coli* BL21(DE3) for expression with the empty plasmid pET-28a(+) as the control. The transformants were screened on Luria-Bertani (LB) broth supplemented with 100 μg/mL kanamycin and cultured with shaking at 37 °C overnight. Twenty microliters of seed culture were transferred into fresh medium and cultured until OD_{600} reached 0.6, and IPTG (final concentration 1.0 mmol/L) was then added for induction. The bacterial cells were cultured with shaking at 20 °C for 16 h before collection by centrifugation. SDS-PAGE was applied for confirmation of the expressed product.

2.6. Endoinulinase purification

For endoinulinase purification, all operations were performed at 4 °C unless otherwise mentioned. After growth in liquid medium, cells were harvested by centrifugation, washed with cold saline, and suspended in 20 mL of cold Tris–HCl buffer, pH 7.7. Cells were homogenized by ultrasonic treatment. Supernatant was obtained as crude enzyme solution by centrifugation at 12,000 rpm and 4 °C for 20 min.

The crude enzyme solution was subjected to His-Tag purification using a gravity column which could be packed with nickel-NTA agarose (Qiagen). After the mixture of crude enzyme solution and Ni^{2+}-NTA slurry flowed through it, the column was washed with buffer I (0.5 M NaCl, 0.02 M Tris–HCl buffer, pH 7.7) until the $A_{280\,nm} < 0.01$. The weakly bound protein was washed away from the column using buffer II (20 mM imidazole, 0.5 M NaCl, 0.02 M Tris–HCl buffer, pH 7.7). Then the washing buffer (50 mM imidazole, 0.5 M NaCl, 0.02 M Tris–HCl buffer, pH 7.7) was used to wash the column for the bound protein at flow rate of 1 mL/min. Finally, the recombinant fusion protein was eluted by 250 mM imidazole in buffer III (0.5 M NaCl, 0.02 M NaH_2PO_4, pH 7.7) at 0.5 mL/min. The purified His_6-endoinulinase was ultrafiltered by Amicon Ultra-4 (10 K) centrifugation devices for removing imidazole.

2.7. SDS-PAGE

SDS-PAGE was performed on a 15% running gel (Laemmli, 1970) and the resolved proteins were visualized by staining with Coomassie Brilliant Blue R 250. The standard protein markers used were phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (43 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (20.1 kDa), lysozyme (14.4 kDa).

2.8. Determination of protein concentration

Protein concentration was determined by the Bradford assay (Bradford, 1976) using bovine serum albumin as a standard.

2.9. Assay of endoinulinase activity

Endoinulinase activity was measured using 2.0% (w/v) inulin (Sigma, USA) as a substrate in 50 mmol/L NaAc–HAc buffer, pH 5.0, incubated at 55 °C for 20 min with constant shaking. The liberation of reducing sugars was estimated by dinitrosalicylic acid (DNS) method (Miller, 1959) using fructose as a standard. One unit of enzyme activity was defined as the quantity of enzyme required

to liberate 1 μmol of fructose equivalent per minute at 55 °C, and specific activity was defined as units per mg protein.

2.10. pH optimum and stability

The effect of pH on endoinulinase activity was evaluated at 55 °C over a pH range of 3.0–7.0 for 20 min, using NaAc–HAc buffer (pH 3.0–7.0) under endoinulinase activity assay conditions. The pH stability of endoinulinase was carried out at 55 °C by pre-incubation of the enzyme solutions in the aforementioned buffer system in the absence of substrate at pH 4.5–6.5 for 1–8 h. Residual activities were determined under endoinulinase activity assay conditions.

2.11. Temperature optimum and thermostability

The temperature optimum was measured by performing the endoinulinase activity assay at temperatures ranging from 20 to 80 °C for 20 min under the optimal pH of 5.0. The thermostability of endoinulinase was investigated at pH 5.0 after incubation of the enzyme solutions in absence of substrate at various temperatures (50–70 °C) for 1, 2, 3, 4 h, respectively. Residual activities were determined under endoinulinase activity assay conditions.

2.12. Effect of metal ions on recombinant endoinulinase activity

Metal ions are generally considered as important factors affecting microbial enzyme activity. Each metal ion was added to 5 mL of diluted enzyme solution to a final concentration of 5 mmol/L. The effects of these metal ions were investigated after 20 min of incubation at pH 5.0 and 55 °C.

2.13. Kinetic parameters

The recombinant endoinulinase was used for studying kinetic parameters (K_m and V_{max}). For determining the reaction rate, different substrate (inulin) concentrations were used, ranging from 5.0 to 50.0 mg/mL. The reaction rate versus substrate concentration was plotted to determine whether the enzyme obeys Michaelis–Menten kinetics, and K_m and V_{max} were determined from the Lineweaver–Burk plot.

2.14. Analysis of hydrolysis products of inulin

The hydrolysis was carried out with 5 U/g of purified endoinulinase and 5% inulin. The products of hydrolysis of inulin by purified endoinulinase were analyzed by thin layer chromatography (TLC). Aliquots were removed periodically and 5 μL of each sample was spotted on the TLC plate (Silica gel G). Plates were developed at room temperature with the solvent system of n-propanol/ethyl acetate/water (3:1:1, v/v/v), and inulo-oligosaccharides were located using aniline–diphenylamine–phosphoric acid–acetone reagent. Fructose, sucrose (GF), 1-kestose (GF2), nystose (GF3) were used as standards.

3. Results and discussion

3.1. DNA sequence analysis of the endo I gene

In order to clone *endo I* gene, the degenerated primers P1 and F1 with restriction enzyme *Eco*R I sites were made on NCBI information by using BLAST search of the GenBank Database. Chromosomal DNA of *A. ficuum* JNSP5-06 were used as template genomic for the PCR reaction. The PCR products were cloned into pET-28a(+) vector (Fig. 1) and the nucleotide sequence was subsequently determined. Its open reading frame (ORF) consisted of 1482 bp. And the deduced amino acid sequence was 99.59% and 97.97% identical to that of *A. ficuum* (GenBank Accession No. CAA07345) and *A. niger* CBS513.88 (GenBank Accession No. DQ233221), respectively, however, only 59.17% identical to that of *A. fumigatus* AF293 (GenBank Accession No. EAL86248). The encoding sequence of *endo I* of *A. ficuum* JNSP5-06 was registered in the GenBank and the GeneBank Accession number is FJ984582.

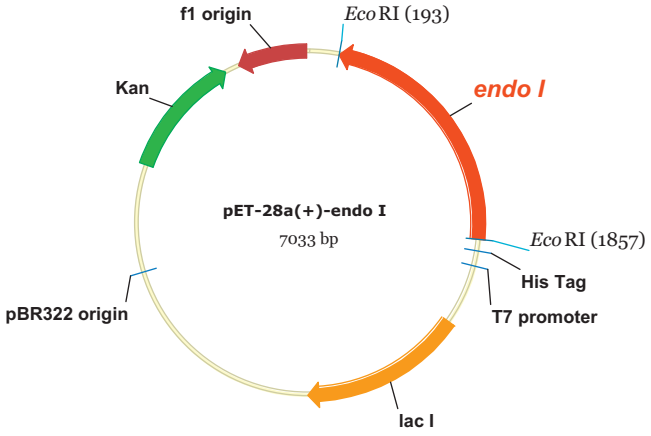

Fig. 1. Schematic representation of the pET-28a (+)-*endo I* vector.

3.2. Expression of endo I in E. coli

To further test whether the cloned sequence truly represents *A. ficuum endo I*, the *endo I* gene with restriction sites of *EcoR* I could be inserted into expression vector pET-28a(+) in the right reading frame. Because of the presence of the His-tag fusion peptide in expression vector pET-28a(+), the recombinant protein had 36 extra amino acids. As shown in Fig. 2(A), a high efficiency of *endo I* protein expression was achieved with pET-28a(+). The recombinant protein showed a molecular weight of about 60 kDa which was close to the theoretical value.

SDS-PAGE showed that the cell extracts from *E. coli* BL21(DE3) harboring pET-28a-*endo I* exhibited a clear band with a molecular weight of about 60 kDa (Fig. 2(A), lanes 7–9), which was a fusion hybrid protein and was the same size as estimated from the deduced amino acid sequence of the fusion region in pET-28a-*endo I*. The expression of the fusion hybrid protein could be induced by IPTG. A maximum activity of 34.6 U/mg was obtained from cellular extract of *E. coli* BL21 (DE3) harboring pET-28a-*endo I*. No band was observed in the extract from the control strain of *E. coli* pET-28a(+) (Fig. 2(A)).

3.3. Purification of endoinulinase

The crude extract was applied to the Ni^{2+}-NTA column. Then the targeted protein was eluted from the column under 250 mM imidazole in pH 7.7 Tris–HCl buffer. As shown in Fig. 2(B), the purified protein showed a single band.

3.4. Characterization of endoinulinase

Purified recombinant endoinulinase was used to evaluate its biochemical properties. From the pH profile, we can see that under assay conditions used, the pH for optimal activity of the endoinulinase was determined to be pH 5.0 (Fig. 3), 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). After the enzyme in the absence of

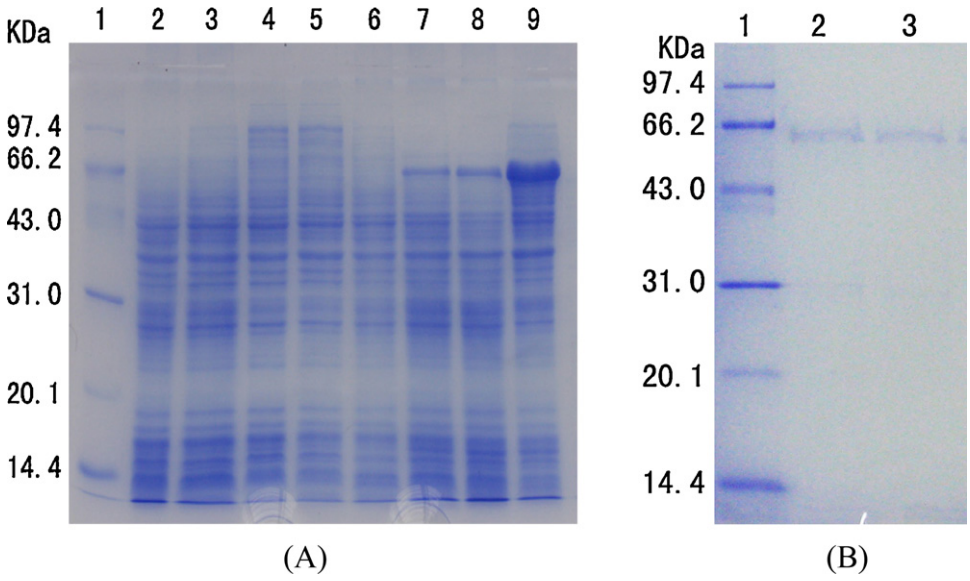

Fig. 2. SDS-PAGE analysis of different protein samples. (A) Lane 1: protein markers with the following molecular weight standards: phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (20.1 kDa), lysozyme (14.4 kDa); lane 2: *E. coli* pET-28a without IPTG induction; lanes 3–5: *E. coli* pET-28a with IPTG induction for 2 h, 6 h, 16 h, respectively; lane 6: *E. coli* pET-28a-*endo I* without IPTG induction; lanes 7–9: *E. coli* pET-28a-*endo I* with IPTG induction for 2 h, 6 h, 16 h, respectively. (B) Lane 1: protein molecular weight markers; lanes 2 and 3: endoinulinase purified by Ni^{2+}-chelating chromatography.

substrate was kept at pH 5.0 and 55 °C for 36 h (data not shown), it retained 75% of maximum activity, but only 70% relative activity was observed after 1-h pre-heated at pH 4.5 or pH 6.0 under 55 °C (Fig. 4). It was found that recombinant endoinulinase was sensitive to pH and was stable over a pH range of 4.5–6.0. The enzyme activity decreased dramatically beyond that range, and it almost lost activity above pH 6.5.

The effects of temperature on the activity and stability of the recombinant endoinulinase were also determined. As shown in Fig. 5, the optimum temperature of the recombinant endoinulinase was around 60 °C, which was higher than the optimum inulinases temperature of 45–55 °C from other microorganism (Vandamme & Derycke, 1983).

For determining the thermostability of recombinant endoinulinase, the enzyme was heated at various temperature (50–70 °C) at pH 5.0 for 1–4 h. As shown in Fig. 6, recombinant endoinulinase retained 100% relative activity after 4 h (pre-heated below 55 °C), but only 60% relative activity was observed after 4 h (pre-heated at 60 °C). However, complete inactivation was observed when the enzyme was incubated at 70 °C for 1 h.

3.5. Kinetic study

The kinetics of the recombinant endoinulinase displayed typical Michaelis–Menten behavior. The affinity of recombinant

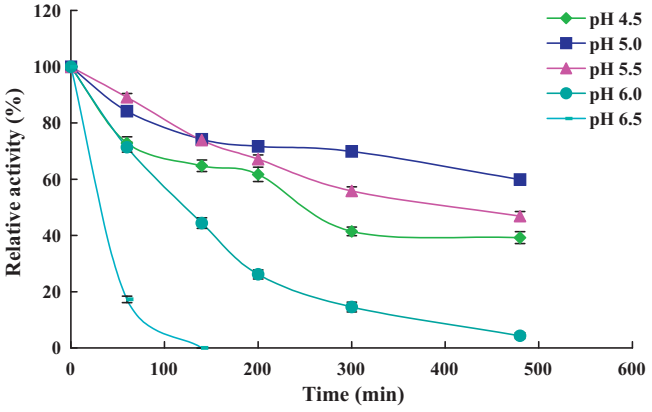

Fig. 4. Effect of pH on recombinant endoinulinase stability. Data represent the mean of three determinations ± SE.

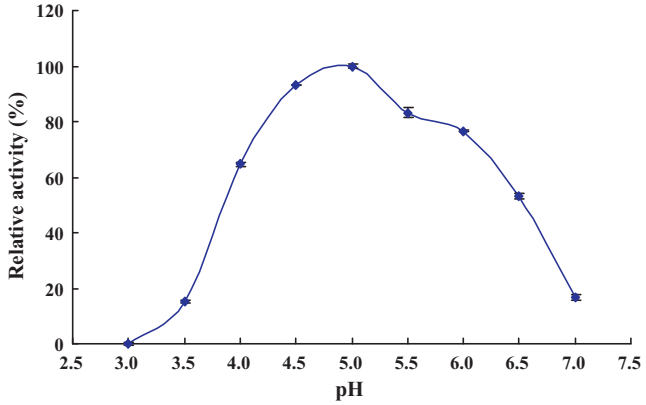

Fig. 3. Effect of pH on recombinant endoinulinase activity. Data represent the mean of three determinations ± SE.

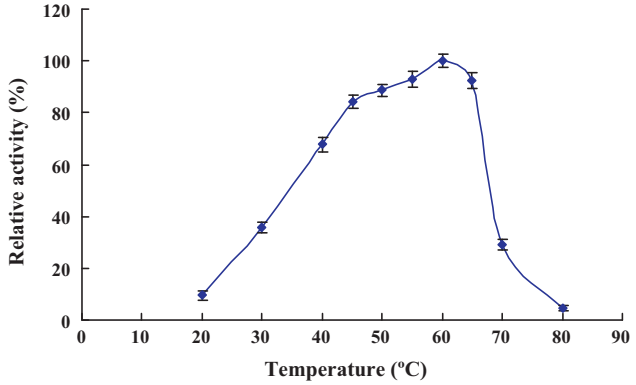

Fig. 5. Effect of temperature on recombinant endoinulinase activity. Data represent the mean of three determinations ± SE.

Table 1
Effect of metal ions on recombinant endoinulinase activity.

Metal ions	Concentration (mmol/L)	Relative activity (%)	Metal ions	Concentration (mmol/L)	Relative activity (%)
Control	–	100.0 ± 2.7	Ag^{+}	5	0[a]
K^{+}	5	113.0 ± 3.8[b]	Mn^{2+}	5	113.9 ± 3.9[b]
Ca^{2+}	5	107.9 ± 4.1[b]	Zn^{2+}	5	123.8 ± 3.8[a]
Al^{3+}	5	17.2 ± 3.1[a]	Fe^{3+}	5	54.3 ± 4.9[a]
Cu^{2+}	5	0[a]	Fe^{2+}	5	29.5 ± 4.4[a]
Mg^{2+}	5	99.9 ± 4.4[b]	Ni^{2+}	5	100.3 ± 3.1[b]

After pre-incubation of enzyme with metal ions at a concentration of 5 mmol/L at 55 °C and pH 5.0 for 20 min, the remaining enzyme activity was measured. Data represent the mean of three determinations ± SD.
[a] Significantly different vs. control at $P < 0.05$.
[b] Not significant.

endoinulinase for inulin were determined at 55 °C and pH 5.0 by a Lineweaver–Burk plot. The kinetic constants K_m and V_{max} values were (67.4 ± 4.2) mg/mL and (349.2 ± 13.7) mg/mL min, respectively (data not shown). Nakamura et al. (1978) reported that the K_m value of endoinulinase from *A. niger*-12 was 1.25 mmol/L. Nakamura et al. (1997) also reported that the K_m value of endoinulinase from *Penicillium* sp. TN-88 was 0.2 mmol/L. Moreover, recent study showed that the K_m value of recombinant endoinulinase expressed in *Yarrowia lipolytica* was 37.1 mg/mL (Li et al., 2012), which was different from our result.

3.6. Effects of metal ions on endoinulinase activity

The various metal ions were added to the enzyme solution, after pre-incubation at pH 5.0 and 55 °C for 20 min, the remaining enzyme activity was measured. As shown in Table 1, the recombinant endoinulinase activity was completely inhibited by Ag^{+} and Cu^{2+}, which was in agreement with previous studies (Gaye, Sukan, & Vassilew, 1994; Nakamura et al., 1978). In addition, the recombinant endoinulinase activity was strongly inhibited by Fe^{3+}, Fe^{2+} and Al^{3+}, whereas K^{+}, Ca^{2+}, Mn^{2+}, Mg^{2+}, and Ni^{2+} had no significant influence on this recombinant endoinulinase activity. However, Zn^{2+} appeared to slightly stimulate the endoinulinase activity.

3.7. Analysis of hydrolysis products of inulin

TLC analysis (Fig. 7) showed that after the hydrolysis of inulin by the recombinant endoinulinase obtained in this study, inulotriose (F3) and inulotetraose (F4) were produced as the main hydrolysis products of inulin, indicating that the recombinant enzyme was indeed endoinulinase.

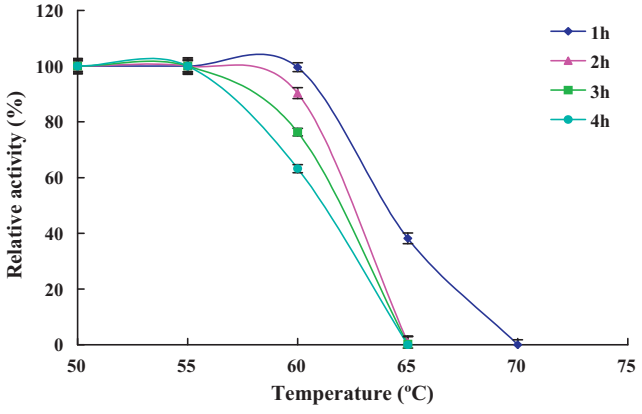

Fig. 6. Effect of temperature on recombinant endoinulinase stability. Data represent the mean of three determinations ± SE.

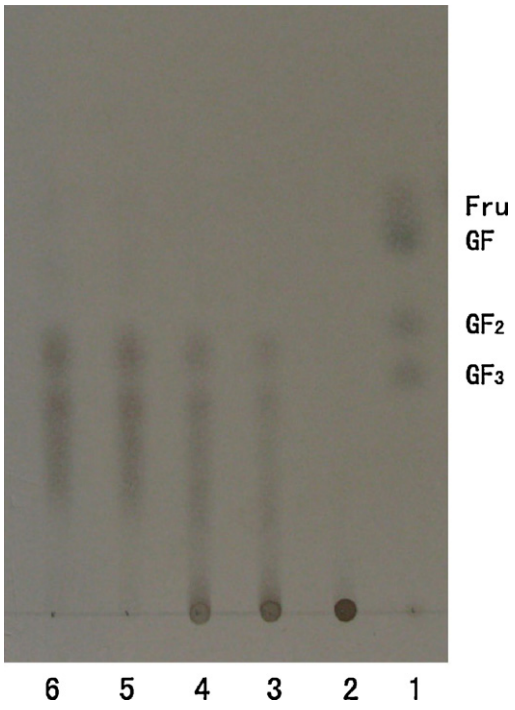

Fig. 7. TLC profile of hydrolysis product of inulin by recombinant endoinulinase. Lane 1: fructose, sucrose (GF), 1-kestose (GF2), nystose (GF3); lane 2–6: reaction mixture at 0, 2, 4, 16, 24 h, respectively.

4. Conclusions

Previous studies indicated that the endoinulinase-encoding gene from *A. ficuum* has been expressed in *S. cerevisiae*. However, to our knowledge, the expression of an endoinulinase gene from *A. ficuum* in *E. coli* has not been reported. In this study, the expression of the endoinulinase gene from *A. ficuum* JNSP5-06 in *E. coli* and some properties of the recombinant endoinulinase were investigated.

The endoinulinase encoded by the *endo I* gene from *A. ficuum* JNSP5-06 was overexpressed in *E. coli*. The molecular weight of the recombinant endoinulinase was about 60 kDa. The K_m and V_{max} values with inulin as the substrate were (67.4 ± 4.2) mg/mL and (349.2 ± 13.7) mg/mL min, respectively. The optimum pH and temperature of this enzyme were 5.0 and 60 °C, respectively. Its activity was increased by Zn^{2+}, and K^{+}, completely inhibited by Ag^{+}, and Cu^{2+}, and strongly inhibited by Al^{3+}, Fe^{2+}, and Fe^{3+}, whereas Ca^{2+}, Mn^{2+}, Mg^{2+}, and Ni^{2+} had no significant influence on this recombinant endoinulinase activity. The major products of hydrolysis of inulin by the recombinant endoinulinase were fructo-oligosaccharides with degree of polymerization (DP) from 3 to 4.

This recombinant endoinulinase may be potential application in the industrial production of fructo-oligosaccharides from inulin.

Acknowledgements

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