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Expression of an exoinulinase gene from *Aspergillus ficuum* in *Escherichia coli* and its characterization

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ABSTRACT

An exoinulinase gene from *Aspergillus ficuum* JNSP5-06 was overexpressed in *Escherichia coli*. Two exons of the exoinulinase gene were amplified separately, joined together by an overlap PCR, and expressed in *E. coli*. The molecular weight of the recombinant exoinulinase was estimated to be 63 kDa. The K_m and V_{max} values for inulin were (7.1 ± 0.2) mM and (1000.0 ± 0.1) μmol/(min mg protein), respectively. The K_m and V_{max} values for sucrose were (347.6 ± 25.9) mM and $(12{,}037.0 \pm 801.9)$ μmol/(min mg protein), respectively. The optimum temperature and pH with inulin as the substrate were 60 °C and 4.0, respectively. The optimum temperature and pH with sucrose as the substrate were 55 °C and 5.0, respectively. Its activity was increased by Mn^{2+}, completely inhibited by Cu^{2+}, and strongly inhibited by Al^{3+}, Ag^{+}, Fe^{3+}, Fe^{2+}, Ni^{2+}, Zn^{2+}, and Mg^{2+}. The product of hydrolysis of inulin by the recombinant exoinulinase was fructose.

1. Introduction

Inulin is a linear polymer of β-2,1-linked D-fructofuranose molecules terminated by a D-glucose residue at the reducing end (Vandamme & Derycke, 1983). It occurs as a reserve carbohydrate in the roots and tubers of plants such as Jerusalem artichoke, chicory, and dahlia. In recent years, inulin has received increasing attention as a raw material for fructose syrup production, ethanol fermentation, and inulo-oligosaccharide production.

Microbial inulinases can be divided into two types: endoinulinase (2,1-β-D-fructan fructanohydrolase; EC 3.2.1.7) and exo-inulinase (β-D-fructan fructohydrolase; EC 3.2.1.80) according to the mode of action on inulin. Endoinulinase are specific for inulin and hydrolyze the internal β-2,1-fructofuranosidic linkages to yield inulotriose, inulotetraose, and inulopentaose as the main products. In contrast, exoinulinase split off terminal fructose units successively from the nonreducing end of the inulin molecule, and also hydrolyze sucrose and raffinose.

In recent years, exoinulinases have been attracting worldwide research interests due to their great potential industrial application in the preparation of high fructose syrups from inulin. However, the yields of exoinulinase from naturally isolated microorganisms are often low and not satisfied with the requirements for the large-scale industrial production. An efficient method for improving the yields of exoinulinase is the usage of recombinant DNA technology. A number of bacterial exoinulinases have cloned and overexpressed using recombinant DNA technology (Kwon et al., 2003; Moriyama, Tanaka, Uwataki, Muguruma, & Ohta, 2003; Tsujimoto et al., 2003; Wang, Huang, Long, Meng, & Liu, 2011; Zhang, Zhao, Zhu, Ohta, & Wang, 2004; Zhang, Yang, Wang, Hua, & Zhao, 2012; Zhang, Gong, Peng, & Chi, 2009).

In our laboratory, we isolated a filamentous fungus, *Aspergillus ficuum* JNSP5-06, from soil samples. It can produce endoinulinase as well as exoinulinase (Chen et al., 2009). Our previous study indicated that endoinulinase-encoding gene from *A. ficuum* JNSP5-06 was expressed in *Escherichia coli* (Chen, Xu, Jin, & Chen, 2012). However, to the best of our knowledge, no study on the expression of an exoinulinase (*exo I*) gene from *A. ficuum* in *E. coli* has been reported. In this study, the expression of this exoinulinase gene from *A. ficuum* JNSP5-06 in *E. coli* and some properties of the recombinant exoinulinase are investigated.

2. Materials and methods

2.1. Strains, plasmids and reagents

A. ficuum JNSP5-06, isolated from soil as reported previously (Chen et al., 2009), was stored in our laboratory. *E. coli* strains JM109

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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 exoinulinase gene, respectively. PCR reagents, T_4 DNA ligase and the restriction exonucleases 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 our previous described method (Chen et al., 2012). 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 at 60 °C for another 20 min. 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 exoinulinase gene by PCR

Genomic DNA were amplified by polymerase chain reaction (PCR) using the upstream primer P1: 5′-GGTGGATCCTTCAACTATGACCAGCCTT-3′ (the underlined sequence is the position of a *BamH* I site) and the downstream primer P2: 5′-ATCGCGGCCGCTTAATTCCACGTCGAAGTAA-3′ (the underlined sequence is the position of a *Not* I site) specifically designed to pick up exoinulinase (purchased from TaKaRa Biotechnology Co., Ltd., Dalian, China). The PCR reaction system consisted of 0.3 μL Taq, 5 μL buffer, 4 μL deoxynucleotides (dNTP mixture, each of 2.5 mmol/L), 4 μL of 25 mmol/L $MgCl_2$, 1 μL primer P1, 1 μL primer P2, 3 μL template DNA (exoinulinase 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.

2.4. cDNA construction of exo I gene by overlap-PCR

To construct cDNA of exoinulinase gene, each of the two coding exons, exon 1 and exon 2, was individually re-amplified by PCR and each amplified exon was gel-purified on a 0.8% agarose gel before being joined together with an overlap PCR (Fig. 1).

Exon 1 of *exo I* gene was amplified with PCR primers P1 and P3 (5′-CAACGGGGTAATAGGAAGTATACATGG-3′) in the first round, the reverse primer P3 has 19 bp overlapping region from 5′ end of exon 2. Similarly, exon 2 of *exo I* gene was amplified with primers P4 (5′-ACTTCCTATTACCCCGTTGC-3′) and P2, the forward primer P4 has 19 bp overlapping region from 3′ end of exon 1. Both amplified exon 1 and exon 2 were purified and mixed in 1:1 molar ratio. These fragments were joined and amplified by the second round of PCR with primers P1 and P2 carrying flanking restriction sites (*BamH* I and *Not* I) to facilitate further cloning into pET-28a(+) vector.

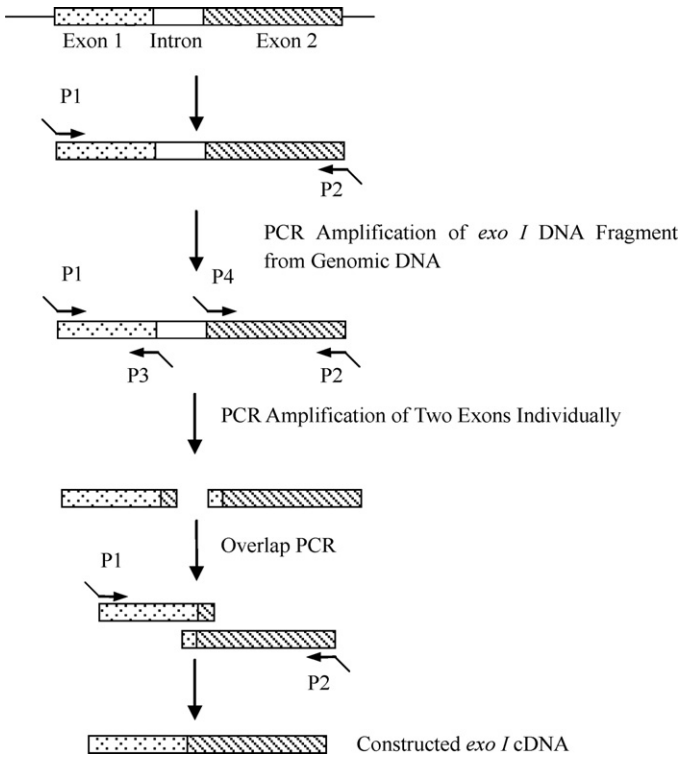

Fig. 1. Amplification of exoinulinase (*exo I*) gene and its cDNA construction. The *exo I* gene was amplified directly from the genomic DNA by PCR using the forward and reverse primers (P1 and P2 respectively). The intron between the two coding exons was removed by amplifying each exon separately, and cDNA of *exo I* gene was constructed by joining the two exons together by a second-round overlap PCR.

2.5. Sequence analysis of the exoinulinase gene

Products of the PCR reaction were cloned into pMD19-T Simple Vector (TaKaRa) and sequenced by Biotech 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.

2.6. DNA manipulations and E. coli transformation

Digestion of DNA with restriction exonucleases, 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, Fritsch, & Maniatis, 1989). DNA fragments were recovered from agarose gels using the DNA Gel Extraction Kit of TaKaRa.

2.7. Expression of exoinulinase in E. coli

The cDNA fragment was ligated into pET-28a(+) with restriction sites of *BamH* I and *Not* I to generate construct pET-28a(+)-*exo 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 0.6–0.8, and IPTG (final concentration 1.0 mmol/L) was then added for induction. The bacterial cells were cultured with shaking at 23 °C for 10 h before collection by centrifugation. SDS-PAGE was applied for confirmation of the expressed product.

2.8. Purification of exoinulinase from E. coli

For exoinulinase 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-tagged purification using a gravity column which could be packed with nickle-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 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 Tris–HCl buffer, pH 7.7) at 0.5 mL/min. The purified His_6-exoinulinase was ultrafiltered by Amicon Ultra-4 (10K) centrifugation devices for removing imidazole.

2.9. SDS-PAGE

To control the expression level via SDS-PAGE, the cells from 1 mL culture medium were re-suspended after centrifugation in 100 μL SDS-PAGE sample buffers. SDS-PAGE was performed on a 12% running gel (Laemmli, 1970) and 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).

2.10. Western blotting

For Western blotting, the proteins were resolved on SDS–PAGE gels and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore), blocked with 5% milk in TBS-0.05% Tween-20 for 1 h and incubated overnight with primary antibodies anti-His (Beyotime Biotechnology). Membranes were washed with TBS–Tween-20, incubated with alkaline phosphatase (AP)-conjugated anti-mouse IgG (Beyotime Biotechnology) for 1 h, and the bound antibodies were visualized using nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Beyotime Biotechnology).

2.11. Determination of protein concentration

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

2.12. Assay of exoinulinase activity

Exoinulinase activity was measured using 2.0% (w/v) inulin or sucrose (Sigma, USA) as a substrate in 50 mmol/L NaAc–HAc buffer, pH 5.0, incubated with constant shaking at 55 °C for 20 min. The liberation of reducing sugars was estimated by the 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.

2.13. pH optimum and stability

The effect of pH on exoinulinase activity was evaluated at 55 °C over a pH range of 2.5–8.0 for 20 min, using sodium phosphate–acid citric buffer (pH 2.5–8.0) under exoinulinase activity assay conditions. The pH stability of exoinulinase 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 2.5–8.0 for 1 h. Residual activities were determined under exoinulinase activity assay conditions.

2.14. Temperature optimum and thermostability

The temperature optimum was measured by performing the exoinulinase activity assay at temperatures ranging from 30 to 80 °C under pH 5.0 for 20 min. The thermostability of exoinulinase was investigated at pH 5.0 after incubation of the enzyme solutions in absence of substrate at various temperatures (30–80 °C) for 1 h. Residual activities were determined under exoinulinase activity assay conditions.

2.15. Effect of metal ions on recombinant exoinulinase 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.

2.16. Kinetic parameters

The recombinant exoinulinase was used for studying kinetic parameters (K_m and V_{max}). For determining the reaction rate, different substrate (inulin or sucrose) 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 obeyed Michaelis–Menten kinetics, and K_m and V_{max} were determined from the Lineweaver–Burk plot.

2.17. Analysis of hydrolysis products of inulin

The hydrolysis was carried out with 5 U/g of purified exoinulinase and 5% inulin. The products of hydrolysis of inulin by purified exoinulinase 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 with the solvent system of n-propanol/ethyl acetate/water (3:1:1, v/v/v) at room temperature, 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. PCR amplification of the exoinulinase gene and sequence analysis

In order to clone *exo I* gene, the degenerated primers P1 and P2 with restriction enzyme *BamH* I and *Not* I sites were made on NCBI information by using BLAST search of the GenBank Database. PCR amplification of the chromosomal DNA of *A. ficuum* JNSP5-06 yielded a product of about 1600 bp when examined on a 0.8% agarose gel (Fig. 2(A)). This DNA fragment was of the expected size for two coding exons and an intron of 60 bp, representing the DNA sequences encoding mature *exo I*. The sequence predicts a 518 amino acid peptide. M_w and pI were 57.2 kDa and 4.89,

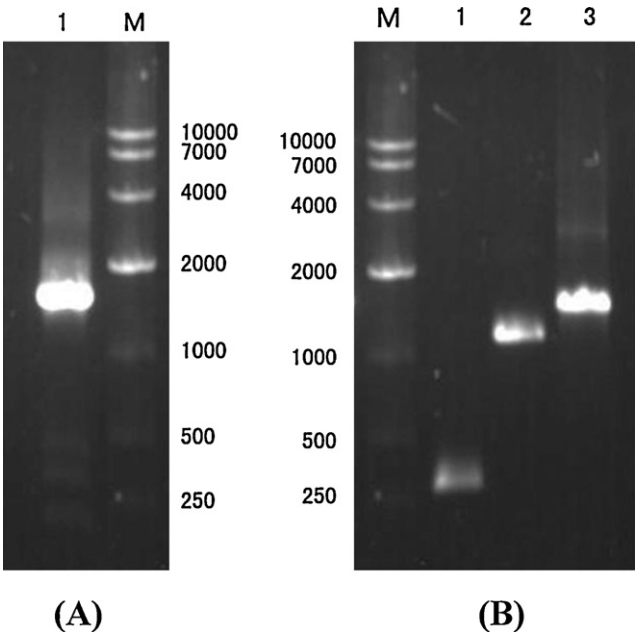

Fig. 2. Agarose gel electrophoresis of PCR products. (A) Amplification of exoinulinase (*exo I*) gene by PCR. Lane M: DNA marker; Lane 1: *exo I* fragment amplified from the genomic DNA of *A. ficuum* JNSP5-06 with primers P1 and P2; (B) cDNA construction of *exo I* gene by overlap PCR. Lane M: DNA marker; Lane 1: exon 1 fragment amplified with primers P1 and P3; Lane 2: exon 2 fragment amplified with primers P4 and P2; Lane 3: cDNA fragment amplified from exon 1 and exon 2 as template by overlap PCR with primers P1 and P2.

respectively. And the deduced amino acid sequence was 100%, 99.8% and 91% identical to that of *Aspergillus niger* 12 (GenBank accession no. BAD01476), *A. niger* CBS513.88 (GenBank accession no. ABB59682), and *A. awamorivar* 2250 (GenBank accession no. CAC44220), respectively, however, only 61.3% identical to that of *Penicillium* sp. TN-88 (GenBank accession no. BAC16218). The encoding sequence of *exo I* of *A. ficuum* JNSP5-06 was registered in the GenBank and the GeneBank Accession number is HM587130.

3.2. cDNA construction of exo I gene by overlap-PCR

As the *exo I* gene had only one intron, attempt was made to fuse the exons of *exo I* gene and to clone the fused fragment and express in *E. coli.* Primers were designed to amplify the two exons separately. As shown in Fig. 2(B), exon 1 and exon 2 fragments obtained by PCR amplification were about 400 bp and 1200 bp long, respectively. Two exon fragments purified by agarose gel electrophoresis were used as template in the overlap extension PCR. Finally, the overlap extension PCR resulted in about 1600 bp exon fusion product. The fusion product was sequenced to confirm the coding sequence of *exo I*.

3.3. Expression of exo I in E. coli

To further test whether the cloned sequence truly represents *A. ficuum exo I*, the *exo I* gene with restriction sites of *BamH* I and *Not* I could be inserted into expression vector pET-28a(+), an expression vector with the T7 promoter, a His-tagged, yielding recombinant protein. The sequence and the correct orientation of *exo I* were confirmed by digestion with restriction exonucleases and nucleotide sequencing (data not shown). The construct was used to transform competent *E. coli* BL21 (DE3) cells and a transformant was used for the subsequent induction.

Because of the presence of the His-tagged fusion peptide in expression vector pET-28a(+), the recombinant protein had 34 extra amino acids. As shown in Fig. 3(A), a high efficiency of *exo I* protein expression was achieved with pET-28a(+). The recombinant protein showed a molecular weight of about 63 kDa which was close to the theoretical value.

SDS-PAGE showed that the cell extracts from *E. coli* BL21 (DE3) harboring pET-28a(+)-*exo I* exhibited a clear band with a molecular weight of about 63 kDa (Fig. 3(A), Lanes 4 and 5), 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(+)-*exo I*. The expression of the fusion hybrid protein could be induced by IPTG. No band was observed in the extract from the control strain of *E. coli* pET-28a(+) (Fig. 3(A)). The identity of this band as the recombinant protein was demonstrated by Western blot assay using anti-His antibody to the His-tagged (Fig. 3(B)). A maximum

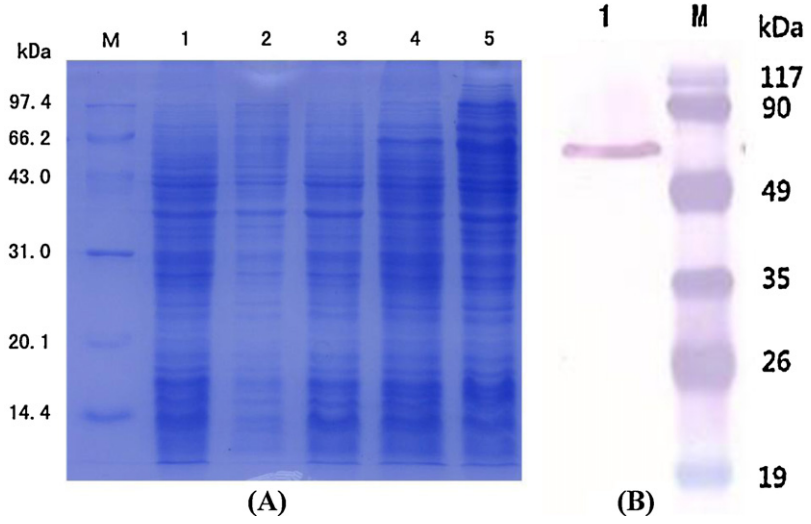

Fig. 3. Expression of *exo I* containing a His-tagged in *E. coli* BL21 (DE3). (A) SDS-PAGE of the whole cell lysate. Lane M: protein markers with the following molecular weight standards: phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (20.1 kDa), lysozyme (14.4 kDa); Lane 1: *E. coli* pET-28a(+) without IPTG induction; Lane 2: *E. coli* pET-28a(+) with IPTG induction for 6 h; Lane 3: *E. coli* pET-28a(+)-*exo I* without IPTG induction; Lanes 4 and 5: *E. coli* pET-28a(+)-*exo I* with IPTG induction for 2 h, 6 h, respectively. (B) Western blot of the whole cell lysate. Lane M: prestained protein molecular weight markers; Lane 1: *E. coli* pET-28a(+)-*exo I* with IPTG induction for 6 h.

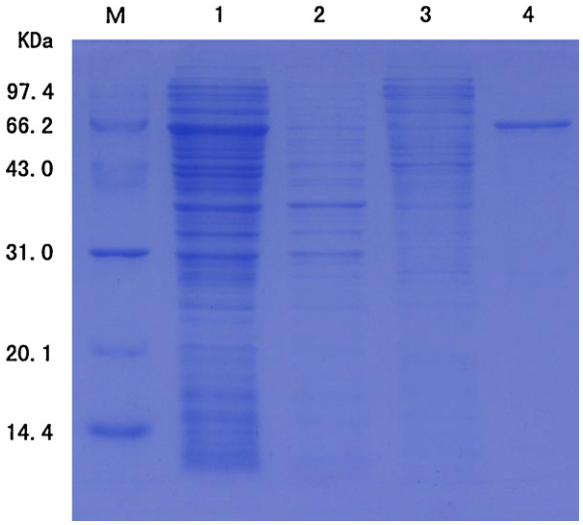

Fig. 4. Analysis of recombinant exoinulinase by SDS-PAGE during the purification process. Lane M: protein molecular weight markers; Lane 1: crude recombinant exoinulinase; Lane 2: flow-through; Lane 3: column washings; Lane 4: recombinant exoinulinase purified by Ni^{2+}-NTA chromatography.

activity of 59.24 U/mg was obtained from cellular extract of *E. coli* BL21 (DE3) harboring pET-28a(+)-*exo I* using inulin as the substrate.

3.4. Purification of exoinulinase

The His_6-tagged allowed a very efficient single-step purification of the fusion protein by Ni^{2+}-NTA column chromatography. 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. 4 (Lane 4), the purified protein showed a single band.

3.5. Characterization of exoinulinase

Purified recombinant exoinulinase 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 exoinulinase with inulin or sucrose as the substrate was determined to be pH 4.0 and 5.0, respectively (Fig. 5(A)), which was in agreement with the general range of many microbial sources reported so far: *Kluyveromyces marxianus* (pH 4.4) (Rouwenhorst, Hensing, Verbakel, Scheffers, & Van Dijken, 1999), *K. marxianus* var. *bulgaricus* (pH 4.4) (Kushi, Monti, & Contiero, 2000), *Kluyveromyces fragilis* (pH 4.75) (Pandey et al., 1999), *Cryptococcus aureus* (pH 5.0) (Sheng, Chi, Gong, & Li, 2008). After the enzyme in the absence of substrate was kept at pH of 3.0–4.5 and 55 °C for 1 h, it retained 80% of maximum activity, but only 70% relative activity was observed after 1-h pre-heated at pH 5.0 or 5.5 under 55 °C (Fig. 5(B)). It was found that the recombinant exoinulinase was sensitive to pH and was stable over a pH range of 3.0–5.0 for sucrose and 3.0–4.5 for inulin. The enzyme activity decreased dramatically beyond that range, and it almost lost activity above pH 8.0.

The effects of temperature on the activity and stability of the recombinant exoinulinase were also determined. As shown in Fig. 6(A), the optimum temperature of the recombinant exoinulinase with inulin or sucrose as the substrate was 60 °C and 55 °C, respectively, which was in agreement with the optimum exoinulinases temperature of 50–60 °C from other microorganisms (Gong, Chi, Sheng, Li, & Wang, 2008; Kushi et al., 2000; Kwon, Kim, & Choi, 2000; Pandey et al., 1999; Rouwenhorst et al., 1999; Sheng et al., 2008; Tsujimoto et al., 2003).

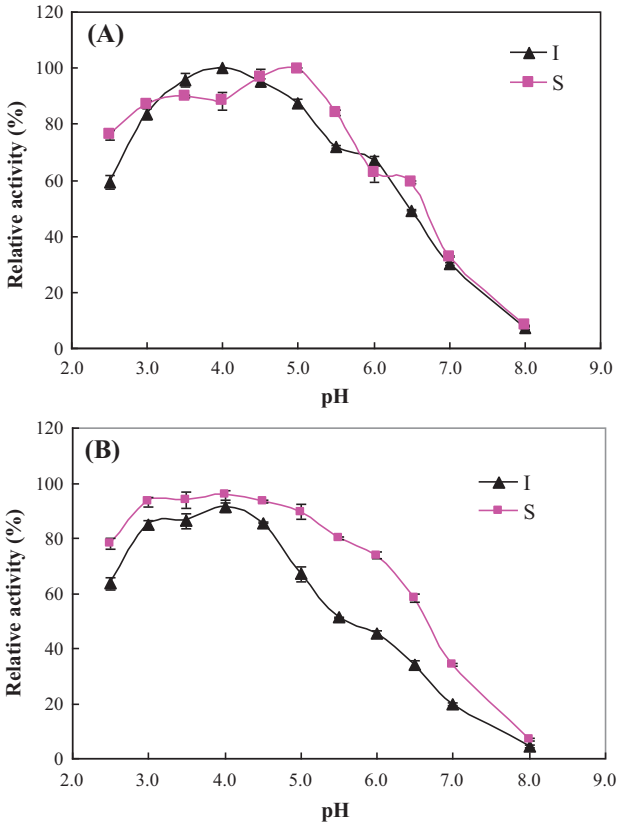

Fig. 5. Effect of pH on the recombinant exoinulinase activity and stability. (A) Effect of pH on the recombinant exoinulinase activity; (B) effect of pH on the recombinant exoinulinase stability. I, inulin; S, sucrose. Data represent the mean of three determinations ± SE.

For determining the thermostability of the recombinant exoinulinase, the enzyme was heated at various temperatures (30–80 °C) and pH 5.0 for 1 h. As shown in Fig. 6(B), the recombinant exoinulinase retained 100% relative activity after 1 h (pre-heated below 55 °C), but only 70% relative activity for inulin was observed after 1 h (pre-heated at 60 °C). However, complete inactivation was observed when the enzyme was incubated at 70 °C for 1 h.

3.6. Kinetic study

The kinetics of the recombinant exoinulinase displayed typical Michaelis–Menten behavior. The affinity of the recombinant exoinulinase for inulin or sucrose were determined at 55 °C and pH 5.0 by a Lineweaver–Burk plot. The kinetic constants K_m and V_{max} values for inulin were (7.1 ± 0.2) mM and (1000.0 ± 0.1) μmol/(min mg protein), respectively. The K_m and V_{max} values for sucrose were (347.6 ± 25.9) mM and (12,037.0 ± 801.9) μmol/(min mg protein), respectively (data not shown). Moriyama et al. (2002) reported that the K_m value of exoinulinase from *Penicillium* sp. TN-88 was 9.0 mM. Sharma and Gill (2007) also reported that the K_m value of exoinulinase from *Streptomyces* sp. was 1.63 mM. Moreover, Kwon et al. (2003) also reported that the K_m and V_{max} values for inulin hydrolysis for exoinulinase from *Bacillus polymyxa* expressed in *E. coli* were 0.7 mM and 2500 μM min^{-1} mg^{-1} protein, respectively, which were different from our results.

3.7. Effects of metal ions on exoinulinase 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

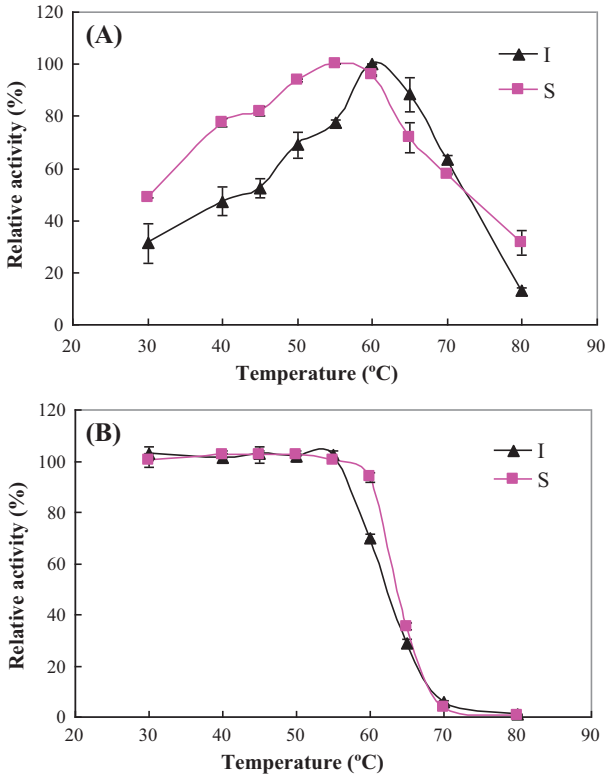

Fig. 6. Effect of temperature on the recombinant exoinulinase activity and stability. (A) Effect of temperature on the recombinant exoinulinase activity; (B) effect of temperature on the recombinant exoinulinase stability. I, inulin; S, sucrose. Data represent the mean of three determinations ± SE.

enzyme activity was determined. As shown in Table 1, the recombinant exoinulinase activity was completely inhibited by Cu^{2+} and strongly inhibited by Al^{3+}, Ag^{+}, Fe^{3+}, Fe^{2+}, Ni^{2+}, Zn^{2+}, and Mg^{2+}, whereas K^{+}, and Ca^{2+} had no significant effect on this recombinant exoinulinase activity. However, Mn^{2+} appeared to slightly stimulate the exoinulinase activity.

3.8. Analysis of hydrolysis products of inulin

TLC analysis (Fig. 7) showed that after the hydrolysis of inulin by the recombinant exoinulinase obtained in this study, fructose was produced as the only hydrolysis product of inulin, which was consistent with previous studies (Kwon et al., 2003; Zhang et al., 2009), indicating that the recombinant enzyme was indeed exoinulinase.

Table 1
Effect of metal ions on the recombinant exoinulinase activity.

Metal ions	Concentration (mmol/L)	Relative activity (%)	
		Inulin	Sucrose
Control		100.0 ± 4.3	100.0 ± 1.8
K^{+}	5	101.6 ± 6.7[b]	88.7 ± 4.3[a]
Ca^{2+}	5	92.8 ± 5.1[b]	86.3 ± 5.6[a]
Al^{3+}	5	9.6 ± 0.6[a]	45.7 ± 2.2[a]
Cu^{2+}	5	0[a]	10.9 ± 0.7[a]
Mg^{2+}	5	75.0 ± 2.0[a]	55.2 ± 3.0[a]
Ag^{+}	5	54.4 ± 5.2[a]	17.1 ± 0.9[a]
Mn^{2+}	5	103.2 ± 1.7[b]	117.2 ± 0.6[a]
Zn^{2+}	5	57.4 ± 3.5[a]	89.8 ± 3.9[a]
Fe^{2+}	5	36.6 ± 2.9[a]	49.2 ± 3.4[a]
Fe^{3+}	5	27.2 ± 3.9[a]	52.8 ± 3.0[a]
Ni^{2+}	5	38.3 ± 5.5[a]	49.6 ± 1.0[a]

After pre-incubation of enzyme with metal ions at a concentrations 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 versus control at $P < 0.05$.
[b] Not significant.

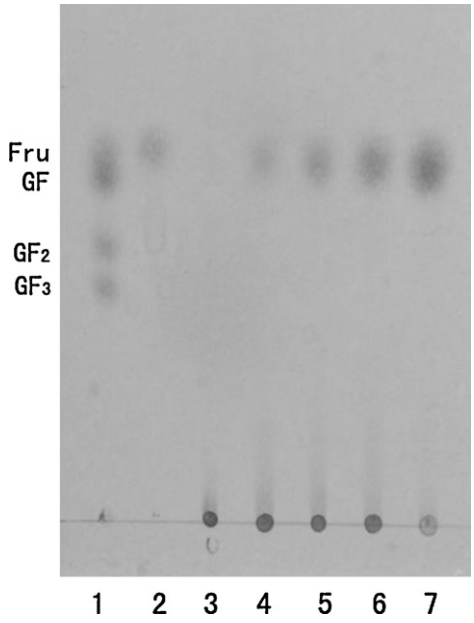

Fig. 7. TLC profile of hydrolysis product of inulin by the recombinant exoinulinase. Lane 1: Fructose (Fru), Sucrose (GF), 1-Kestose (GF2), Nystose (GF3); Lane 2: fructose standard; Lanes 3–7: reaction mixture at 0, 2, 4, 16, 24 h, respectively.

4. Conclusions

In this study, the exoinulinase encoded by the *exo I* gene from *A. ficuum* JNSP5-06 was overexpressed in *E. coli* and the biochemical characterization of the recombinant exoinulinase was investigated.

The molecular weight of the recombinant exoinulinase was estimated to be approximately 63 kDa by SDS-PAGE. The K_m and V_{max} values of the recombinant exoinulinase for inulin were (7.1 ± 0.2) mM and (1000.0 ± 0.1) μmol/(min mg protein), respectively. The K_m and V_{max} values of this enzyme for sucrose were (347.6 ± 25.9) mM and (12,037.0 ± 801.9) μmol/(min mg protein), respectively. The optimum temperature and pH of the recombinant exoinulinase with inulin as the substrate were 60 °C and 4.0, respectively. The optimum temperature and pH of this enzyme with sucrose as the substrate were 55 °C and 5.0, respectively. Its activity was increased by Mn^{2+}, completely inhibited by Cu^{2+}, and strongly inhibited by Al^{3+}, Ag^{+}, Fe^{3+}, Fe^{2+}, Ni^{2+}, Zn^{2+}, and Mg^{2+}, whereas K^{+}, and Ca^{2+} had no significant influence on this recombinant exoinulinase activity. The product of hydrolysis of inulin by the recombinant exoinulinase was fructose.

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References

Bradford, M. M. (1976). A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry*, *72*, 248–254.

Chen, H.-Q., Chen, X.-M., Li, Y., Wang, J., Jin, Z.-Y., Xu, X.-M., et al. (2009). Purification and characterization of exo- and endo-inulinase from *Aspergillus ficuum* JNSP 5-06. *Food Chemistry*, *115*, 1206–1212.

Chen, X.-M., Xu, X.-M., Jin, Z.-Y., & Chen, H.-Q. (2012). Expression of an endoinulinase from *Aspergillus ficuum* JNSP 5-06 in *Escherichia coli* and its characterization. *Carbohydrate Polymers*, *88*, 748–753.

Gong, F., Chi, Z. M., Sheng, J., Li, J., & Wang, X. H. (2008). Purification and characterization of extracellular inulinase from a marine yeast *Pichia guilliermondii* and inulin hydrolysis by the purified inulinase. *Biotechnology and Bioprocess Engineering*, *13*, 533–539.

Kushi, R. T., Monti, R., & Contiero, J. (2000). Production, purification and characterization of an extracellular inulinase came from *Kluyveromyces marxianus* var. *bulgaricus*. *Journal of Industrial Microbiology and Biotechnology*, *25*, 63–69.

Kwon, H.-J., Jeon, S.-J., You, D.-J., Kim, K.-H., Jeong, Y.-K., Kim, Y.-H., et al. (2003). Cloning and characterization of an exoinulinase from *Bacillus polymyxa*. *Biotechnology Letters*, *25*, 155–159.

Kwon, Y. M., Kim, H. Y., & Choi, Y. J. (2000). Cloning and characterization of *Pseudomonas mucidolens* exoinulinase. *Journal of Microbiology and Biotechnology*, *10*, 238–243.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature*, *227*, 680–685.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, *31*, 426–428.

Moriyama, S., Akimoto, H., Suetsugu, N., Kawasaki, S., Nakamura, T., & Ohta, K. (2002). Purification and properties of an extracellular exoinulinase from *Penicillium* sp strain TN-88 and sequence analysis of the encoding gene. *Bioscience Biotechnology and Biochemistry*, *66*, 1887–1896.

Moriyama, S., Tanaka, H., Uwataki, M., Muguruma, M., & Ohta, K. (2003). Molecular cloning and characterization of an exoinulinase gene from *Aspergillus niger* strain 12 and its expression in *Pichia pastoris*. *Journal of Bioscience and Bioengineering*, *96*, 324–331.

Pandey, A., Soccol, C. R., Selvakumar, P., Soccol, V. T., Krieger, N., & Jose, D. (1999). Recent development in microbial inulinases, its production, properties and industrial application. *Applied Biochemistry and Biotechnology*, *81*, 35–52.

Rouwenhorst, R. J., Hensing, M., Verbakel, J., Scheffers, W. A., & Van Dijken, J. P. (1999). Structure and properties of the extracellular inulinase of *Kluyveromyces marxianus* CBS 6556. *Applied and Environmental Microbiology*, *11*, 3337–3345.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning: A laboratory manual* (second ed.). Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Sharma, A. D., & Gill, P. K. (2007). Purification and characterization of heat-stable exo-inulinase from *Streptomyces* sp. *Journal of Food Engineering*, *79*, 1172–1178.

Sheng, J., Chi, Z. M., Gong, F., & Li, J. (2008). Purification and characterization of extracellular inulinase from a marine yeast *Cryptococcus aureus* G7a and inulin hydrolysis by the purified inulinase. *Applied Biochemistry and Biotechnology*, *144*, 111–121.

Tsujimoto, Y., Watanabe, A., Nakano, K., Watanabe, K., Matsui, H., Tsuji, K., et al. (2003). Gene cloning, expression, and crystallization of a thermostable exo-inulinase from *Geobacillus stearothermophilus* KP1289. *Applied Microbiology and Biotechnology*, *62*, 180–185.

Vandamme, E. J., & Derycke, D. G. (1983). Microbial inulinases: Fermentation process, properties, and applications. *Advances in Applied Microbiology*, *29*, 139–176.

Wang, L., Huang, Y., Long, X., Meng, X., & Liu, Z. (2011). Cloning of exoinulinase gene from *Penicillium janthinellum* strain B01 and its high-level expression in *Pichia pastoris*. *Journal of Applied Microbiology*, *111*, 1371–1380.

Zhang, L., Zhao, C., Zhu, D., Ohta, Y., & Wang, Y. (2004). Purification and characterization of inulinase from *Aspergillus niger* AF10 expressed in *Pichia pastoris*. *Protein Expression and Purification*, *35*, 272–275.

Zhang, S., Yang, F., Wang, Q., Hua, Y., & Zhao, Z. (2012). High-level secretory expression and characterization of the recombinant *Kluyveromyces marxianus* inulinase. *Process Biochemistry*, *47*, 151–155.

Zhang, T., Gong, F., Peng, Y., & Chi, Z. (2009). Optimization for high-level expression of the *Pichia guilliermondii* recombinant inulinase in *Pichia pastoris* and characterization of the recombinant inulinase. *Process Biochemistry*, *44*, 1335–1339.