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Cloning, Expression, and Purification of Exoinulinase from *Bacillus* sp. snu-7

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Abstract A gene encoding inulin-degrading enzyme of *Bacillus* sp. snu-7 with ORF of 1536 nucleotides was cloned. And it was overexpressed as His-tagged protein in *E. coli* BL21(DE3) pLysS using pRSET B vector containing mature enzyme sequence. Maximum enzyme production was achieved by IPTG (0.1 mM) induction at OD_{600} 1.2 and 30°C, followed by 6 h incubation. The expressed protein purified through immobilized metal affinity chromatography showed molecular mass of 60 kDa on SDS-PAGE. Results of thin-layer chromatography using inulin as a substrate showed the enzyme to be an exotype inulinase capable of producing only monomeric fructose as a product. K_m and k_{cat} for the hydrolyses of inulin and sucrose were 2.28±0.08 mM and 358.05± 20.38 min^{-1}, and 22.02±0.41 mM and 4619.11±215.12 min^{-1}, respectively. Optimal activity of the exoinulinase occurred at pH 7.0 and 50°C.

Key words: *Bacillus* sp. snu-7, exoinulinase, immobilized metal affinity chromatography

Inulin, a carbohydrate polymer composed of β-2,1-linked fructose with a terminal sucrose residue, serves as the main reserve of carbohydrate in several plants including Jerusalem artichoke (*Helianthus tuberosus* L.), dandelion (*Taraxacum officinale* Weber), dahlia (*Dahlia pinnata* Cav.), and chicory (*Cichorium intybus* L.) [19]. Inulin as a carbohydrate raw material can be used to produce pure fructose syrups, namely high-fructose inulin syrups, by enzymatic hydrolysis. In addition, direct fermentation of inulin can be used to produce alcohol products by employing inulinase-producing microbes [5].

Exoinulinase (2,1-β-D-fructan fructohydrolase, EC. 3.2.1.80), a family of 32 glycoside hydrolase, catalyzes the release of the terminal fructose from the non-reducing end of the inulin, thereby producing monomeric fructose units [1]. On the other hand, endoinulinase (2,1-β-D-fructan fructanohydrolase, EC. 3.2.1.7), another member of family of 32 glycosidase which uses inulin as a substrate, liberates inulooligosaccharides such as inulotriose, -tetraose, and -pentaose, and small amounts of fructooligosaccharides that have a terminal glucose residue [10]. Ingestion of the resulting inulooligosaccharides is expected to improve human health by increasing the population of intestinal bifidobacteria [24].

In a previous study, *Bacillus* sp. snu-7 was found to mainly secrete inulin fructotransferase (IFTase, EC. 2.4.1.93), which hydrolyzes inulin into di-D-fructofuranose-1,2':2,3'-dianhydride (DFA III), and also exoinulinase in the culture broth [11, 14]. Although the preliminary crystal structure of exoinulinase from *Aspergillus awamori* has been recently reported, the structure has yet to be resolved [1]. Therefore, to investigate the structures and activities of IFTase and exoinulinase, an attempt was made to isolate each gene. The cloning of the gene encoding the exoinulinase from *Bacillus* sp. snu-7, induction and purification of the gene product in *E. coli* BL21(DE3) pLysS are herein described. Subsequent characterization of the enzyme in terms of its kinetic parameters and substrate specificities was also performed.

Materials and Methods

Bacterial Strains

Bacillus sp. snu-7 [11, 14] was used for cloning of the exoinulinase gene. *E. coli* MC1061 and *E. coli* BL21(DE3) pLysS were used as hosts for plasmid preparations and gene expression, respectively. pBluescript SK(-) and pGEM T-easy, and pRSET B vectors were used as cloning and expression vectors, respectively.

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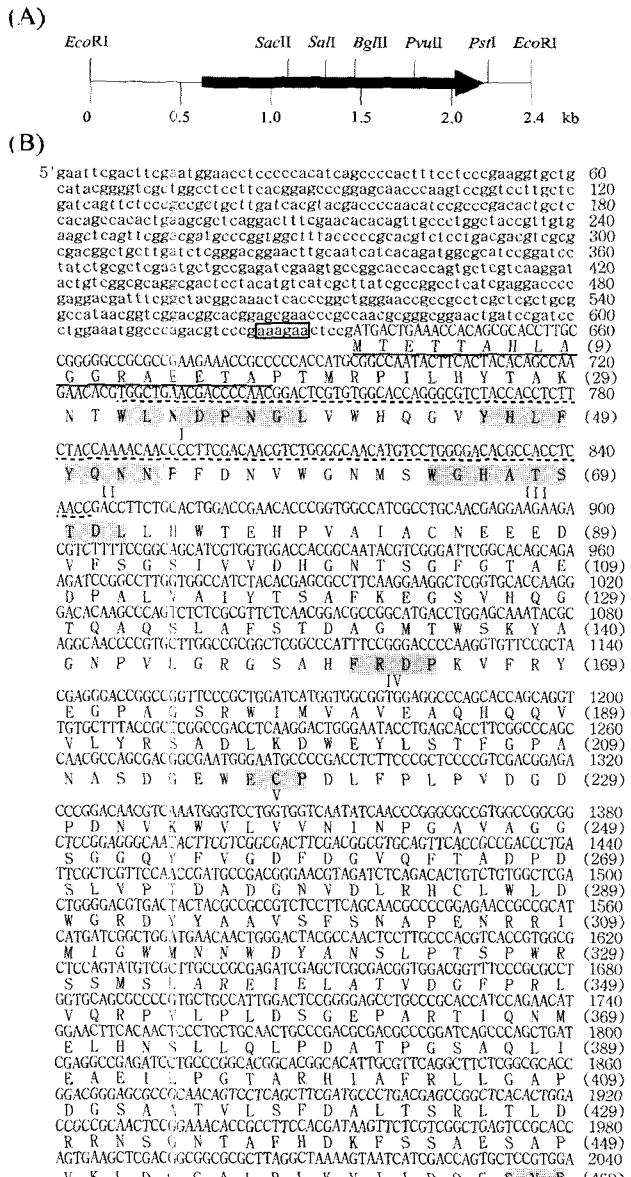

Fig. 1. Structure of exoinulinae gene from *Bacillus* sp. snu-7 and its nucleotide sequence (GenBank Accession number AF234992).

(A) Restriction map of the exoinulinase gene and positions of cloned DNA fragments in plamid pEX24. The arrow indicates the coding region and transcriptional direction of the exoinulinase gene. (B) Nucleotide and deduced amino acid sequences of *Bacillus* sp. snu-7 exoinulinase gene. The Shine-Dalgarno (SD) sequence is boxed. Six conserved domains (I, II, III, IV, V, and VI) of inulinases are boxed with shade. The solid underline and asterisk indicate putative signal peptide and stop codon, respectively. The dotted underline and two arrows indicate PCR amplified 117-bp DNA sequence for the probe preparation and catalytic amino acids of the exoinulinase, respectively.

Cloning of *Bacillus* sp. snu-7 Exoinulinase Gene

Genomic DNA of *Bacillus* sp. snu-7 was prepared following a method of Pospiech *et al.* [20]. Other molecular biological experiments were performed using the standard methods of Sambrook and Russell [23]. For cloning of the inulinase gene, 5'-primer Endo-N (5'-TGGACG/CAACG-AGCCC/GCACGG-3') and 3'-primer endo-C (5'-GTGCTG/CGTCATGTGG/CCCCCA-3') were synthesized based on the conserved amino acid sequences I (WTNEPHG) and III (WGHMTST) of endoinulinases from *Arthrobacter* sp. S37 [10]. Because the deduced amino acid sequence of the amplified PCR product, 117-bp DNA fragment, revealed a 52% identity with that of *Arthrobacter* sp. S37 endoinulinase [10] and possessed conserved domains I, II, and III (Fig. 1B), the DNA was labeled with [α-^{32}P] dATP by the random primer method [23] and used as a hybridization probe.

The 2.4-kb *Eco*RI fragment of *Bacillus* sp. snu-7 genomic DNA, identified by southern hybridization with the probe, was purified and ligated at the *Eco*RI site of pBluescript SK(-) (named pEX24), and *E. coli* MC1061 was then transformed with the plasmid. The colony hybridization method was used to select positive clones containing the inulinase gene [23].

Nucleotide Sequencing and Data Analysis

Nucleotide sequencing reactions were performed using the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster, CA, U.S.A.) according to the manufacture's instructions, and run on an ABI Prism 377 DNA sequencer. The sequence data were analyzed using DNASIS program.

Construction of Expression Plasmid pREX12

A construction scheme of the expression plasmid pREX12 is summarized in Fig. 2. For the amplification of the exoinulinase gene, two separate PCRs were performed. The first PCR using n5' (5'-GGATCCCACCATGCGGC-CAATTAC-3') and n3' (5'-AGATCTACGTTCCCGTCGG-3') primers amplified the N-terminal region of the exoinulinase gene between the mature enzyme start position and the intrinsic *Bgl*II site, producing 800 bp of *Bam*HI/*Bgl*II fragment. The second PCR with c5' (5'-AGATCTCAGACACTGTCTGTGG-3) and c3' (5'-AAG-CTTAGGATAAGGACGTGACGG-3') primers amplified the C-terminal region of the exoinulinase gene between the intrinsic *Bgl*II site and the TAA termination codon, producing 697 bp of *Bgl*II/*Hin*dIII fragment. *Bam*HI and *Hin*dIII sites were introduced to facilitate the cloning of the exoinulinase gene for n5' and c3' primers. Each PCR fragment was ligated into pGEM T-easy vector (pTEX1 and pTEX2 for N- and C-terminal regions of the exoinulinase gene, respectively), and the sequences were determined. A *Bam*HI/*Eco*RI fragment harboring the N-

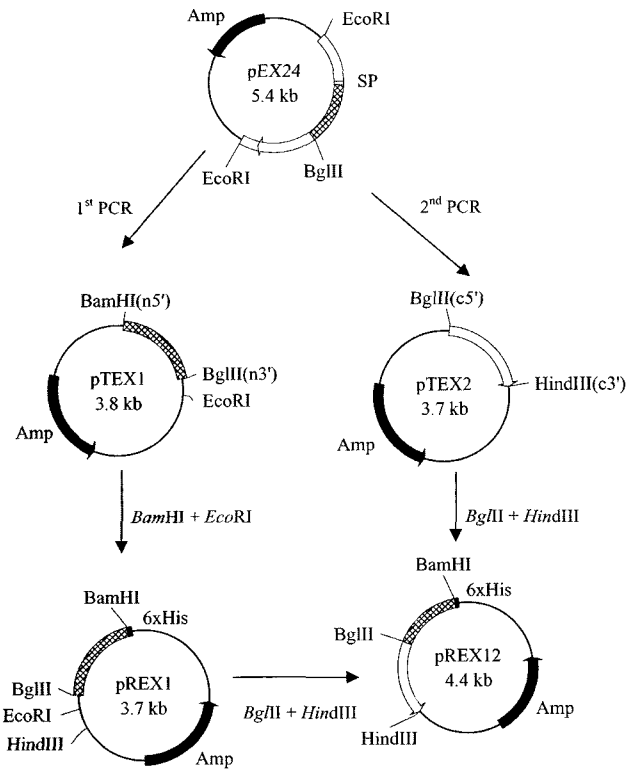

Fig. 2. Construction of plasmid pREX12. Mature exoinulinase gene of *Bacillus* sp. snu-7 was inserted into the pRSET B expression vector.
SP, putative signal peptide; bracketed passages, four primers synthesized for the gene amplification; Amp, Ampicillin-resistant gene; 6xHis, pRSET B-derived N-terminal six histidine tag for rapid purification with IMAC.

terminal region of exoinulinase gene, *Bam*HI/*Bgl*II fragment of pTEX1, was ligated into the expression vector pRSET B, and the resulting plasmid was named pREX1 (Fig. 2). *Bgl*II/*Hin*dIII fragment harboring the C-terminal region of exoinulinase gene and the same restriction enzyme fragment of pREX1 were ligated. The resulting plasmid containing a full DNA sequence of the mature exoinulinase gene was named pREX12.

Expression and Purification of Recombinant Protein

pREX12 was transformed into *E. coli* BL21(DE3) pLysS for overexpression [3]. *E. coli* cell was cultured in 100 ml LB medium with ampicillin (100 μg/ml) and chloramphenicol (100 μg/ml). Overnight bacterial culture was inoculated into 1-l LB medium with 100 ml inoculum and cultured at 37°C. When OD_{600} of the culture reached about 1.2, the expression was induced by the addition of isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 0.1 mM. The culture was then incubated at 30°C for 6 h. The induced *E. coli* cells were harvested by centrifugation, and suspended with 25 ml of buffer A [20 mM Tris-HCl (pH 7.5), 0.5 M NaCl] containing 1 mg/ml lysozyme, then disrupted by sonication. The disrupted cells were centrifuged at 15,000 rpm for 30 min at 4°C, and the collected supernatant was purified by immobilized metal affinity chromatography (IMAC) with HiTrap chelating HP 1 ml column (Amersham Pharmacia, Uppsala, Sweden) at a flow rate of 1 ml/min using ÄKTA purifier FPLC (Amersham Pharmacia, Uppsala, Sweden). The column was chelated with 0.1 M $CoCl_2$, and equilibrated with buffer A. The recombinant protein was linearly eluted with imidazole (0-0.15 M) in 15 ml of buffer A. Major fractions showing inulinase activity were pooled and dialyzed against buffer B [50 mM Tris-HCl (pH 7.5)].

Inulinase Activity Assay

Enzyme activity assay was performed using a modified method of Koo *et al.* [15]. Inulinase activity was assayed at 37°C for 5 min with a mixture of 40 μl of buffer B, 50 μl of 1 mM inulin (from Dahlia tubers, Sigma, St. Louis, MO; average molecular weight, 5,000) in buffer B, and 10 μl of the enzyme sample. For the assay of invertase activity, 20 mM sucrose was used as a substrate instead of 1 mM inulin. The reaction was terminated by the addition of 100 μl of 3,5-dinitrosalicylic acid. Then the reaction mixture was boiled for 5 min, and the liberated amount of reducing sugar was determined according to the method of Melius [17]. One unit of inulinase activity was defined as the amount of enzyme required to liberate 1 μmole of the reducing sugar per min at 37°C.

To analyze the kinetic properties of the enzyme, enzyme activities at various substrate concentrations were measured every minute for 5 min to determine the initial velocity. Substrate concentrations were set at 0.2-4 times the respective K_m values of inulin and sucrose. K_m and V_{max} were determined on a Lineweaver-Burk plot. k_{cat} was defined as V_{max} divided by the molecular weight of the enzyme [4].

Protein Analysis and Thin Layer Chromatography

Protein concentration was determined by the method of Bradford using a commercial protein assay kit (Bio-Rad, Hercules, U.S.A.) and bovine serum albumin as a standard [2]. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and native-PAGE were performed as described by Hames and Rickwood [9]. Activity staining for inulinase detection on native-PAGE gel was performed according to the method of Gabriel and Wang [6]. Enzyme reaction products were identified by thin layer chromatography (TLC) as previously described [8].

RESULTS AND DISCUSSION

Cloning and Sequence Analysis of Exoinulinase Gene

PCR amplification of *Bacillus* sp. snu-7 for the probe preparation yielded a 117-bp genomic DNA fragment

possessing conserved sequences I, II, and III (Fig. 1B) and this indicated that the PCR product was a partial fragment of inulinase gene. Therefore, the product was used as a probe for cloning of the exoinulinase gene. Southern hybridization with ^{32}P-labeled probe gave a positive band of 2.4-kb for *Eco*RI digests of *Bacillus* sp. snu-7 genomic DNA (Fig. 1A), and the positive DNA fragment was purified and ligated into pBluescript SK(-). The plasmid, named pEX24, from this transformant was prepared and sequenced. The 2,449-bp sequence contained a 1,536-bp open reading frame encoding a protein of 512 amino acids with a molecular mass of 55,783.1 Da (Fig. 1B). The deduced amino acid sequence of *Bacillus* sp. snu-7 exoinulinase showed 42%, 37%, and 26% identities with those of *Bacillus subtilis* levanase (GeneBank Accession No.X05649), *Aspergillus awamori* exoinulinase [1], and *Arthrobacter* sp. S37 endoinulinase, respectively [10].

The catalytic site of family 32 glycosidase was proposed for using the invertase of *Saccaromyces cerevisiae* as a model enzyme [16, 21, 22] and the examples are as follows: catalysis of *Saccharomyces cerevisiae* invertase involves Asp-23 in the conserved domain I as a nucleophile and Glu-204 in domain V as an acid/base catalyst [21, 22]. As revealed by the alignment result, Asp-35 in domain I and Glu-217 in domain V of exoinulinase coincide with Asp-23 in domain I and Glu-204 in domain V of invertase of *Saccharomyces cerevisiae*, respectively (Fig. 1B) [21, 22]. RDP motif in the conserved domain IV (Fig. 1B) was also conserved in family 32 glycosidases, appearing to coordinate the role of acid/base catalyst.

The conserved domain VI consists of SVEVF sequence in the C-terminal part of the enzyme, which is reported to be characteristic of bacterial levanases, fungal endoinulinases, and fructosyltransferase, but not that of the yeast invertases and exoinulinases. This was also found in *Bacillus* sp. snu-7 exoinulinase [18]. The SVEVF sequence might play an important role in the binding of high molecular weight fructans [18].

Expression and Purification of Exoinulinase

The expression vector pREX12 provided a high expression level of *Bacillus* sp. snu-7 exoinulinase gene in *E. coli*. The expressed enzyme was purified by IMAC using a HiTrap chelating HP 1 ml column. The major peak of inulinase activity was eluted with approximately 0.1 M imidazole. Purification of the enzyme is summarized in Table 1. The enzyme was purified 2.16-fold, as determined by SDS-PAGE (Fig. 3A) with an 85% recovery from the

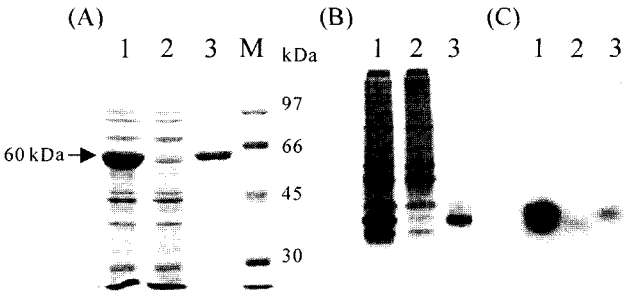

Fig. 3. SDS- and Native-PAGE of exoinulinase expressed in *E. coli* BL21(DE3) pLysS.
(A) 10% SDS-PAGE of coomassie blue staining; (B) and (C) 10% Native-PAGE of coomassie blue staining and activity staining, respectively. Lane 1, exoinulinase with 0.1 mM IPTG induction; Lane 2, IMAC-unbound fraction of pREX12; Lane 3, IMAC-bound fraction of pREX12; Lane M, molecular weight markers.

supernatant of the sonicated cell extract. SDS-PAGE results showed that the His-tagged enzyme was well separated from the unbound fractions. Theoretical molecular weight of the expressed exoinulinase in *E. coli* was 57,668.16 Da, which coincides with about 60 kDa molecular mass of the exoinulinase band determined by the SDS-PAGE (Fig. 3A). In native-PAGE, the enzyme showed a clear band revealed by coomassie blue (Fig. 3B) and activity (Fig. 3C) stainings. The faint activity band detected in the IMAC-unbound fraction was probably formed as a result of diffusion of the crude extract during loading or staining of the protein (Fig. 3C).

Properties of Purified Enzyme

Mode of action and substrate specificity of the exoinulinase were investigated. In addition, the enzyme reaction product was analyzed by TLC. Inulin was hydrolyzed to fructose by the enzyme, which indicates that it has the exo-type depolymerization activity of the enzyme (Fig. 4). Furthermore, the enzyme also hydrolyzed sucrose and raffinose, indicating that it can hydrolyze not only β-2,1-linkage but also β-2,6-linkage (data not shown).

Initial velocity of the enzyme reaction was measured at different concentrations of inulin and sucrose, ranging from 0.4 to 12 mM and 2 to 80 mM, respectively. Kinetic parameters of the hydrolysis are shown in Table 2. Based on the Lineweaver-Burk plot, K_m of the purified enzyme for inulin was calculated as 2.28±0.08 mM. V_{max} of sucrose was comparable with that of inulin, in contrast to the K_m values, which differed significantly. K_m values of

Table 1. Purification of exoinulinase from pREX12 produced in *E. coli* BL21(DE3) pLysS.

Step	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude extract	97.52	43750	450	100	1
IMAC	38.36	37140	970	84.9	2.16

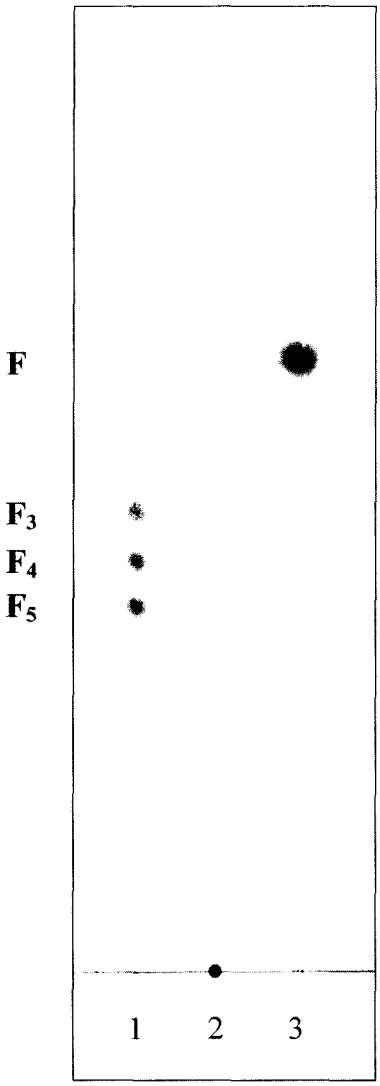

Fig. 4. Thin layer chromatogram of inulin hydrolysate of the purified enzyme.
Lane 1: fructose (F) and oligosaccharides standards, inulo-triose (F_3), inulotetraose (F_4), inulo-pentaose (F_5). Lane 2: pRSET B vector control. Lane 3: exoinulinase hydrolysate.

intracellular and extracellular fractions of *Kluyveromyces marxianus* were 16- 19 and 9- 11 mM for inulin, and 13- 14 and 12- 15 mM for sucrose, respectively [13]. Upon comparison, the *Bacillus* sp. snu-7 exoinulinase showed 4- to 8-fold higher affinity for inulin, whereas 0.5- to 0.7-fold lower affinity for sucrose than *K. marxianus* exoinulinases [13].

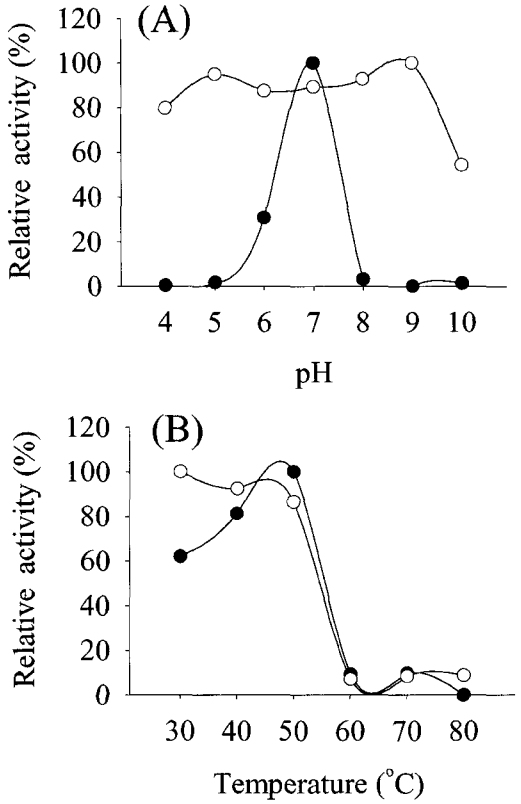

Fig. 5. Effects of pH and temperature on enzyme activity and stability.
(A) Optimum pH (●): enzyme activity was measured between pH 4- 10 [0.2 M sodium acetate (pH 4- 5), 0.2 M sodium phosphate (pH 6- 8), 0.2 M glycine-NaOH (pH 9- 10)]; pH stability (○): the enzyme was treated for 24 h at 4°C at various pHs. (B) Optimum temperature (●): enzyme activity at pH 7 was measured between 30- 80°C; thermal stability (○): the enzyme was treated at pH 7 for 10 min. at various temperatures.

The optimum conditions for enzyme reaction were at pH 7 and 50° C (Fig. 5). The enzyme was stable at pH 4- 9 and 30- 50° C (Fig. 5). Effects of pH and temperature on the enzyme activity of *Bacillus* sp. snu-7 were compared with those of *K. marxianus* [13], *Streptomyces* sp. S34 [7], *Streptomyces* sp. S56 [8], and *Arthrobacter* sp. S37 [12] (Table 3). The purified enzyme was relatively stable between weak acidic and basic pH and showed temperature effects similar to those of other inulinases. In addition, the inulinase to invertase activity (I/S) ratio of *Bacillus* sp. snu-7 exoinulinase was much lower than that of other exoinulinases. Although the substrate affinity (K_m) of inulin was 10-fold higher than that of sucrose, the

Table 2. Determination of enzyme kinetic parameters.

Substrate	Mol. wt.	K_m (mM)	V_{max} (mM min^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
Inulin[a]	5000	2.28±0.08	3.30±0.14	358.05±20.38	156.99±3.29
Sucrose[b]	342.3	22.02±0.41	2.26±0.11	4,619.11±215.12	209.84±12.33

[a]Inulin and [b]sucrose concentrations were 0.4- 12 and 2- 80 mM for exoinulinase, respectively.

Table 3. Comparison of inulinases from different microorganisms.

Microorganism	Optimum pH	Optimum temperature	pH stability	Thermal stability	I/S ratio	Reference
Kluyveromyces marxianus	5.0	40°C	4.0-5.5	30-55°C	1.62-2.16	[13]
Streptomyces sp. S34	5.5-6.0	50°C	-	30-50°C	1.92	[7]
Streptomyces sp. S56	5.5-6.0	50°C	-	-	-	[8]
Arthrobacter sp. S37	7.5	50°C	5.0-10.5	30-40°C	-	[12]
Bacillus sp. snu-7	7.0	50°C	4.0-9.0	30-50°C	0.75	This work

turnover number (k_{cat}) of inulin was 13-fold lower than that of sucrose, which indicates that exoinulinase degrades sucrose more efficiently than inulin.

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