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Cloning, Expression, and Characterization of *Bacillus* sp. snu-7 Inulin Fructotransferase

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Abstract A gene encoding inulin fructotransferase (di-D-fructofuranose 1,2': 2,3' dianhydride [DFA III]-producing IFTase, EC 4.2.2.18) from *Bacillus* sp. snu-7 was cloned. This gene was composed of a single, 1,353-bp open reading frame encoding a protein composed of a 40-amino acid signal peptide and a 410-amino acid mature protein. The deduced amino acid sequence was 98% identical to *Arthrobacter globiformis* C11-1 IFTase (DFA III-producing). The enzyme was successfully expressed in *E. coli* as a functionally active, His-tagged protein, and it was purified in a single step using immobilized metal affinity chromatography. The purified enzyme showed much higher specific activity (1,276 units/mg protein) than other DFA III-producing IFTases. The recombinant and native enzymes were optimally active in very similar pH and temperature conditions. With a 103-min half-life at 60°C, the recombinant enzyme was as stable as the native enzyme. Acidic residues and cysteines potentially involved in the catalytic mechanism are proposed based on an alignment with other IFTases and a DFA IIIase.

Key words: *Bacillus* sp. snu-7, inulin fructotransferase, difructofuranose anhydride

Inulin is a linear, β-2, 1-linked fructose polymer ending with a sucrose residue. Inulin is the main reserve carbohydrate in higher plants such as Jerusalem artichoke, dandelion, dahlia, and chicory [24], and expressions of heterogeneous inulin-degrading enzymes have been studied to produce oligofructose and ethanol from inulin [16, 17]. Inulin fructotransferase (IFTase), which catalyzes the depolymerization of inulin into di-D-fructofuranose 1,2':2,3' dianhydride (DFA III) and a small amount of oligosaccharides through intramolecular transfructosylation (DFA III-producing IFTase: EC 4.2.2.18), is a member of the family 91 glycoside hydrolases with IFTase producing di-D-fructofuranose 1,2':2,1' dianhydride (DFA I-producing IFTase) (EC 4.2.2.17). DFA III is a non-digestible and non-absorbable oligosaccharide that has half the sweetness of sucrose [26]. It enhances calcium absorption in the small and large intestines of rats by increasing the passage of tight junctions [21, 32]. IFTases reported so far have been studied mainly in *Arthrobacter* species [4, 7–9, 15, 23, 34, 36]. Three corresponding genes have been cloned, and the recombinant proteins were shown to have IFTase activity [5, 18, 28]. Two DFA I-producing IFTases have been reported from *Arthrobacter* species [30, 35], and a gene has been cloned [6]. Saito *et al.* [27] cloned a gene encoding the *Arthrobacter* sp. H65-7 DFA III hydrolysis enzyme (DFA IIIase) that converts DFA III into inulobiose.

We have been studying IFTase as well as other inulin-degrading enzymes including *Arthrobacter* sp. endo-inulinase [10, 12] and *Bacillus* sp. exo-inulinase [19]. We isolated and characterized IFTases from *Enterobacter* sp. S45 [11] and from *Bacillus* sp. snu-7 [13, 20]. To broaden our collection of bacterial IFTases with varying catalytic and stability properties, to develop DFA III mass-production methods, and to study the three-dimensional structure, we cloned the *Bacillus* sp. snu-7 IFTase gene, expressed it in *Escherichia coli* BL21(DE3) pLysS, and characterized it.

Materials and Methods

Bacterial Strains and Plasmids

Bacillus sp. snu-7 [13, 20] was the source of the IFTase gene. *E. coli* TOP10F' (Invitrogen, Carlsbad, CA, U.S.A.) and *E. coli* BL21(DE3) pLysS (Promega, Madison, WI, U.S.A.) were used as hosts for plasmid purification and for IFTase

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expression, respectively. pBluescript SK(−) (Stratagene, La Jolla, CA, U.S.A.) and pGEM T-easy (Promega, Madison, WI, U.S.A.) were used as cloning vectors, and pET15b (Novagene, La Jolla, CA, U.S.A.) was used as the expression vector.

Gene Cloning

Bacillus sp. snu-7 genomic DNA was prepared by following the method of Pospiech and Neumann [25]. DNA manipulations were performed as described by Sambrook and Russell [29]. Two degenerate primers, BaF1 (5'-GACAT(A/C/T)GGNGCNGTNAT(A/C/T)AA-3') and BaR3 (5'-ACGCC(A/G)TCNA(A/G)(A/G)CA(A/G)AA-3'), which encode DIGNVIN and FCLDGV (Fig. 1), respectively, were used to clone an internal segment of the IFTase gene. PCR was conducted in a Thermal Cycler DICE standard (TaKaRa, Shiga, Japan) with the following program: denaturation at 96°C for 1 min, annealing at 60°C for 1 min

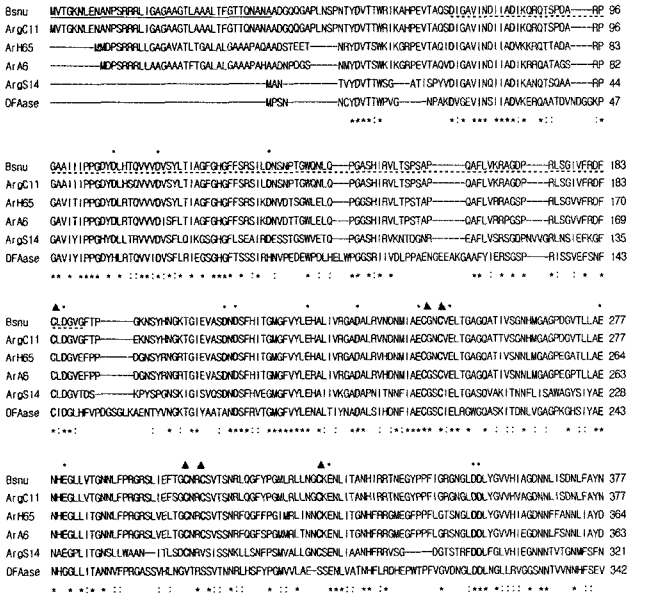

Fig. 1. Alignment of *Bacillus* sp. snu-7 DFA III-producing IFTase (Bsnu) with other IFTases and DFAIIIase.

ArgC11, *A. globiformis* C11-1 DFAIII-producing IFTase [5]; ArH65, *Arthrobacter* sp. H65-7 DFAIII-producing IFTase [28]; ArA6, *Arthrobacter* sp. A-6 DFAIII-producing IFTase [18]; ArgS14, *A. globiformis* S14-3 DFAI-producing IFTase [6]; DFAase, *Arthrobacter* sp. H65-7 DFAIIIase [27]. The signal peptide sequence is underlined. Dotted underline indicates the deduced amino acid of the PCR-amplified 348-bp DNA fragment used as probe for Southern and colony hybridizations. Asterisks indicate identical residues; functionally similar residues are shown by colons. Gaps are introduced to improve the alignment. The symbols ▲ and • indicate highly conserved cysteines and acidic residues (Asp and Glu), respectively. Residue numbers are indicated on the right.

for the first 10 cycles, at 55°C for 1 min for the next 15 cycles, and at 50°C for 1 min for the last 20 cycles, and extension at 72°C for 1 min. The PCR product was extracted from an agarose gel with a MEGA-spin Agarose Gel Extraction Kit (iNtRON, Sungnam, Korea) and subcloned into the pGEM T-Easy vector, resulting in plasmid pGEMF1R3. The gel-purified PCR fragment was labeled with [α-^{32}P] dCTP by the random priming method and used as a probe for Southern hybridization.

Bacillus sp. snu-7 genomic DNA was digested with PstI, and Southern hybridization was performed to identify the size of the fragment containing the full IFTase gene. *Bacillus* sp. snu-7 2.5-kb PstI DNA fragments were purified from an agarose gel and ligated into the pBluescript SK(−) PstI site. Finally, the transformants harboring the IFTase gene (in plasmid pIF25) were selected by colony hybridization.

Nucleotide Sequencing and Data Analysis

Nucleotide sequences were determined by the dideoxynucleotide chain termination method using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA, U.S.A.) according to the manufacturer's instructions, and run on an ABI Prism 3730 XL DNA Analyzer.

Construction of Expression Vector

A DNA fragment encoding the mature IFTase was amplified using the following primers: IFTEX-F (5'-GGGAATTC-CATATGGCCGACGGCCAGCAAGG-3') and IFTEX-R (5'-AGACGCGTCGACCGCGTTGGTCTGTCAGGG-3'). IFTEX-F and IFTEX-R contain an NdeI site (underline) and a SalI site (double underline), respectively. The PCR product was digested with NdeI and SalI and ligated into the NdeI and SalI sites of a modified pET15b. This modified pET15b contains a SalI site coming from part of an endo-inulinase gene [12]. The final expression plasmid was designated as pEIF.

Expression and Purification of Recombinant IFTase

BL21(DE3) pLysS cells harboring pEIF were grown at 37°C for 16 h with shaking in 200 ml of LB medium containing ampicillin (100 μg/ml) and chloramphenicol (100 μg/ml). The 30-ml culture was used to inoculate 3 l of LB medium, and the cells were grown at 37°C until the OD_{600} reached 1.0. IFTase expression was induced by adding isopropyl β-D-thiogalactoside (IPTG) at a final concentration of 1 mM, and cultivation was continued for an additional 6 h at 23°C. Cells were harvested by centrifugation (9,000 ×*g* for 10 min) and resuspended with 98 ml of buffer A [20 mM Tris-HCl (pH 7.5) containing 500 mM NaCl]. Cells were broken by sonication, and cell debris was removed by centrifugation (17,000 ×*g* for 1 h). The supernatant was purified on an ÄKTA purifier FPLC (Amersham Pharmacia, Uppsala, Sweden) equipped with a

1-ml HiTrap chelating HP column (Amersham Pharmacia) at a flow rate of 1 ml/min. The column was chelated with 0.1 M $CoCl_2$ and equilibrated with buffer A. The recombinant protein was eluted with a 12-ml linear 0–0.5 M imidazole gradient, and fractions showing IFTase activity were collected. Protein concentration was determined by the method of Bradford [1] using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, U.S.A.) and bovine serum albumin as a standard. SDS-PAGE and native PAGE were performed as described by Hames and Rickwood [3].

Enzyme Assays

Inulin fructotransferase activity was determined by modifying the method of Kang *et al.* [13]. A mixture of 40 μl of 50 mM sodium acetate (pH 5.5) (buffer B), 50 μl of 1% inulin (from dahlia tubers, Sigma, St. Louis, MO, U.S.A.) in buffer B, and 10 μl of the purified enzyme were incubated at 37°C for 5 min. The reaction was stopped by boiling for 10 min, and the mixture was centrifuged at 16,000 ×*g* for 10 min. The amount of DFA III produced in the supernatant was analyzed by high-pH anion-exchange chromatography with a pulsed amperometric detector (HPAEC-PAD) equipped with a 4×250 mm CarboPac PA1 column (Dionex, Sunnyvale, CA, U.S.A.). One unit of IFTase activity was defined as the amount of enzyme that produces 1 μmole of DFA III per min under the assay conditions described above. To determine the hydrolysis pattern of recombinant IFTase on inulooligosaccharides, 50 μl of 1.5 mM substrate [inulobiose (F_2), inulotriose (F_3), inulotetraose (F_4), and inulopentaose (F_5)] in 50 mM Tris-HCl (pH 7.5) (Buffer C) were incubated with 50 μl of enzyme solution in buffer C at 37°C for 12 h. Enzyme reaction products were analyzed by TLC as described by Ha and Kim [2].

Effect of pH and Temperature on Enzyme Activity

The effect of pH on enzyme activity was investigated from pH 3.0 to pH 10 (0.1 M citric acid-Na_2HPO_4 for pH 3–7, 0.1 M Tris-HCl for pH 8, and 0.1 M glycine-NaOH for pH 10) at 40°C. The effect of temperature on enzyme activity was investigated between 30°C and 80°C at pH 5.5. Other reaction conditions were the same as the standard enzyme assay method described in Materials and Methods.

Thermal and pH Stability

To determine the effect of temperature on stability, the enzyme was incubated at various temperatures for 10 min at pH 5.5, and residual activity was measured using the standard enzyme assay method. To determine the effect of pH on stability, the enzyme was incubated at various pHs for 24 h at 4°C, and residual activity was measured.

Nucleotide Sequence Accession Number

The nucleotide sequence reported here has been deposited in the GenBank database under accession number DQ112363.

Results

Cloning and Sequence Analysis of the IFTase Gene

A 348-bp PCR product was amplified from *Bacillus* sp. snu-7 genomic DNA using degenerate primers designed on the basis of conserved amino acid sequences (Fig. 1) in known IFTases (data not shown). The 348-bp DNA fragment contained both primer sequences, and it encoded a peptide that is highly similar to IFTase sequences (Fig. 1). The 348-bp PCR product was used as a probe for genomic Southern hybridization, and finally, a 2.5-kb PstI fragment was identified (data not shown). Through colony hybridization, a plasmid pIF25 harboring the 2.5-kb PstI fragment was obtained. The pIF25 insert was 2,522-bp long and contained a single, 1,353-bp open reading frame encoding a protein composed of a putative 40-amino acid signal peptide and a 410-amino acid mature protein (Fig. 1). Residues A_{41}DGQ**QG**APLN**SP**NTYD_{56} were almost identical to sequence ADGQ**D**GAPLN**QV**NTYD previously identified as the N-terminal sequence in the native IFTase (bold type indicates different residues between two data) [13]. This result confirms that the 40 N-terminal amino acid residues correspond to a signal peptide (Fig. 1). Very similar retention time (Q and S) and simultaneous increase of height of several peaks during protein sequencing could lead to wrong sequence assignment. The deduced amino acid sequence of *Bacillus* sp. snu-7 IFTase showed 98%, 77%, and 75% identity with the DFAIII-producing IFTases from *Arthrobacter globiformis* C11-1 [5], *Arthrobacter* sp. H65-7 [28], and *Arthrobacter* sp. A-6 [18], respectively (Fig. 1). Interestingly, *Bacillus* sp. snu-7 IFTase had the same number of residues as *A. globiformis* C11-1 DFA III-producing IFTase [5]. The two enzymes' signal peptide sequences were identical, and only five residues were different in the two enzymes (Fig. 1). It also showed 51% identity with *A. globiformis* S14-3 DFA I-producing IFTase [6], and 44% identity with *Arthrobacter* sp. H65-7 DFA IIIase [27] (Fig. 1). *Bacillus* sp. snu-7 IFTase, however, shared very low identity (5–6%) with other inulin-degrading enzymes such as exo-inulinase and endo-inulinase (data not shown).

The AGGAG sequence, identified 11 bases upstream from the start codon, was considered as the putative ribosomal binding sequence, and bacterial promoter sequences were found upstream of the ribosomal binding sequence (data not shown) [31]. The initiation codon was identified as GTG, instead of ATG. A GTG start codon has also been reported for *A. globiformis* C11-1 DFA III-producing IFTase [5] and for *Arthrobacter* sp. FB24 putative DFA I-producing IFTase (GenBank accession no. AAHG01000002).

Expression and Purification of Recombinant IFTase

The recombinant *Bacillus* sp. snu-7 putative IFTase was expressed in *E. coli* BL21(DE3) pLysS by IPTG induction

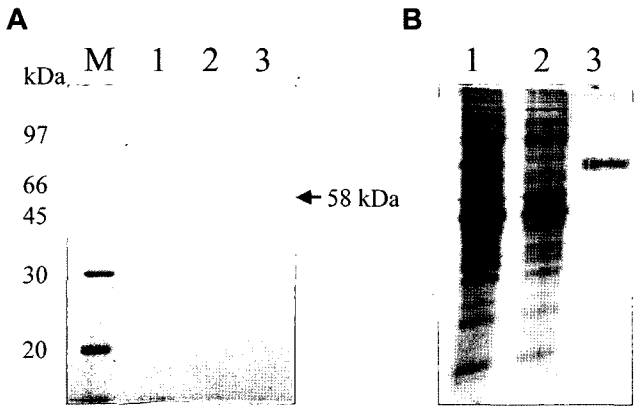

Fig. 2. SDS- (**A**) and native (**B**) PAGE of *Bacillus* sp. snu-7 IFTase expressed in *E. coli* BL21(DE3) pLysS.
M, molecular weight markers; 1, crude extract; 2, IMAC flow-through; 3, elutate from IMAC.

for 6 h at 23°C. The enzyme was purified by immobilized metal affinity chromatography (IMAC) using a 1-ml HiTrap chelating HP column. The enzyme was eluted at approximately 250 mM imidazole concentration (data not shown). The purified enzyme gave a single band on SDS- and native PAGE (Figs. 2A and 2B). The molecular mass of the purified enzyme was estimated to be 58 kDa by SDS-PAGE (Fig. 2A) and 88 kDa by gel filtration (data not shown). With a calculated molecular mass of 43 kDa, these results suggest that *Bacillus* sp. snu-7 IFTase exists as a dimer. All the available DFA III-producing IFTases [7–9, 15, 36], except that of *A. globiformis* C11-1 [4, 5], were also considered to be dimers. Although the native *A. globiformis* C11-1 IFTase was considered to be a monomer [4], the recombinant enzyme was estimated to be a trimer, and preliminary X-ray crystallographic result supported that estimation [22]. The specific activity of the purified IFTase was 1,276 units/mg, and the enzyme was purified 4.3-fold with a yield of 41.8% from the crude extract (Table 1).

Hydrolysis of Inulin with Recombinant IFTase

After exhaustive digestion, the purified recombinant enzyme produced DFA III and a small amount of fructooligosaccharides (*i.e.*, 1-kestose [GF_2] and 1-nystose [GF_3]) from inulin, as did the native IFTase (Fig. 3) [13]. The purified enzyme (1.28 units) converted 63% of inulin (5 mg/ml) into DFA III within 10 min. After that, DFA III production increased very slowly and reached up to 70% after 1 h (data not shown).

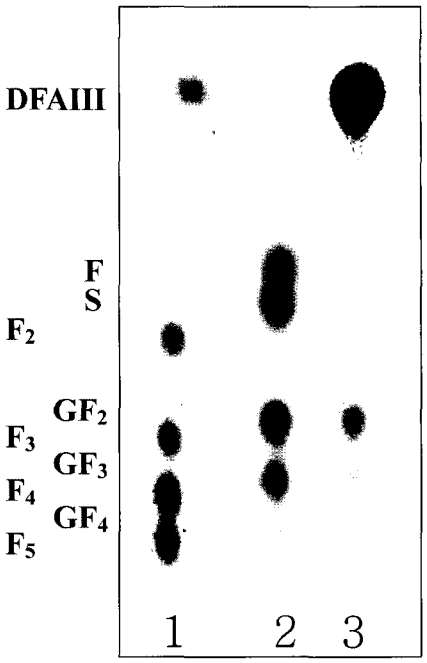

Fig. 3. Thin-layer chromatogram of inulin hydrolysate by the purified recombinant *Bacillus* sp. snu-7 IFTase.
1, DFA III and inulooligosaccharide standards (inulobiose [F_2], -triose [F_3], -tetraose [F_4], and -pentaose [F_5]); 2, fructose (F), sucrose (S), and fructooligosaccharide standards (1-kestose [GF_2], 1-nystose [GF_3], and 1-F-1-β-D-fructofuranosyl-nystose [GF_4]); 3, inulin hydrolysate reacted for 96 h.

Action Mode of Purified IFTase on Inulooligosaccharides

The hydrolysis pattern of purified IFTase on inulooligosaccharides (*i.e.*, F_2, F_3, F_4, and F_5) by the IFTase was investigated. Those inulooligosaccharides were prepared from inulin hydrolysates by endo-inulinase [12]. While F_2 and F_3 were not hydrolyzed by IFTase, F_4 and F_5 were converted into DFA III and F_2 and DFA III and F_3, respectively (Fig. 4). The substrate specificity for inulooligosaccharides has been studied only for *Arthrobacter ureafaciens* IFTase, which hydrolyzed F_3 as well as F_4 to produce DFA III [33]. According to the hydrolysis pattern of inulin and inulooligosaccharides, the smallest substrates for *Bacillus* sp. snu-7 IFTase were 1-F-fructosylnystose (GF_4) [13] and F_4.

Effects of pH and Temperature on Enzyme Activity and Stability

Both the recombinant and native *Bacillus* sp. snu-7 IFTases had very similar optimal conditions for activity: 40°C and between pH 5.0 and 6.0 (Figs. 5A and 5B) [13].

Table 1. Purification of *Bacillus* sp. snu-7 IFTase expressed in *E. coli* BL21(DE3) pLysS.

Steps	Protein (mg)	Total activity (units)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude extract (87 ml)	513	150,945	294	100.0	1.0
IMAC (35 ml)	49.4	6,321	1,276	41.8	4.3

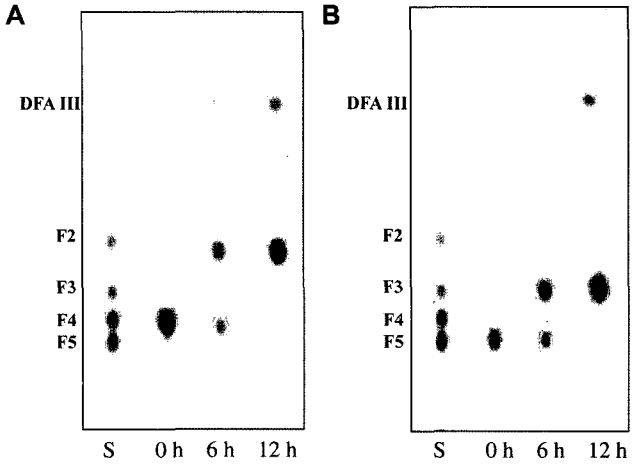

Fig. 4. Thin-layer chromatogram of hydrolysates of (**A**) inulotetraose (F_4) and (**B**) inulopentaose (F_5) reacted with recombinant IFTase.
S, inulooligosaccharide standards (inulobiose [F_2], -triose [F_3], -tetraose [F_4], and -pentaose [F_5]).

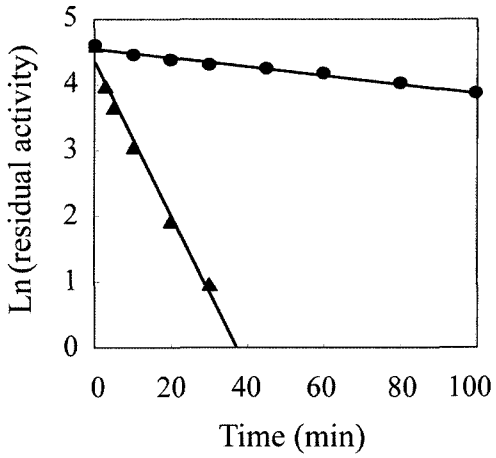

Fig. 6. Thermostability of the purified recombinant *Bacillus* sp. snu-7 IFTase at 60°C (●) and 70°C (▲).

The recombinant IFTase was stable between pH 5.0 and pH 10.0, and remained stable up to 60 °C (Figs. 5C and 5D). The pH stability range was broader than that (pH 4.0–7.0) of the native IFTase (Fig. 5C) [13]. The recombinant enzyme retained 86.2% of maximal activity after a 10-min heat treatment at 60°C, and its half-life at 60°C was 103 min (Fig. 6). It was rapidly inactivated at 70°C, with half-life of 6.6 min (Fig. 6). The recombinant *Bacillus* sp. snu-7 IFTase was less thermostable than other IFTases [9] and its thermostability would need improvement for industrial application.

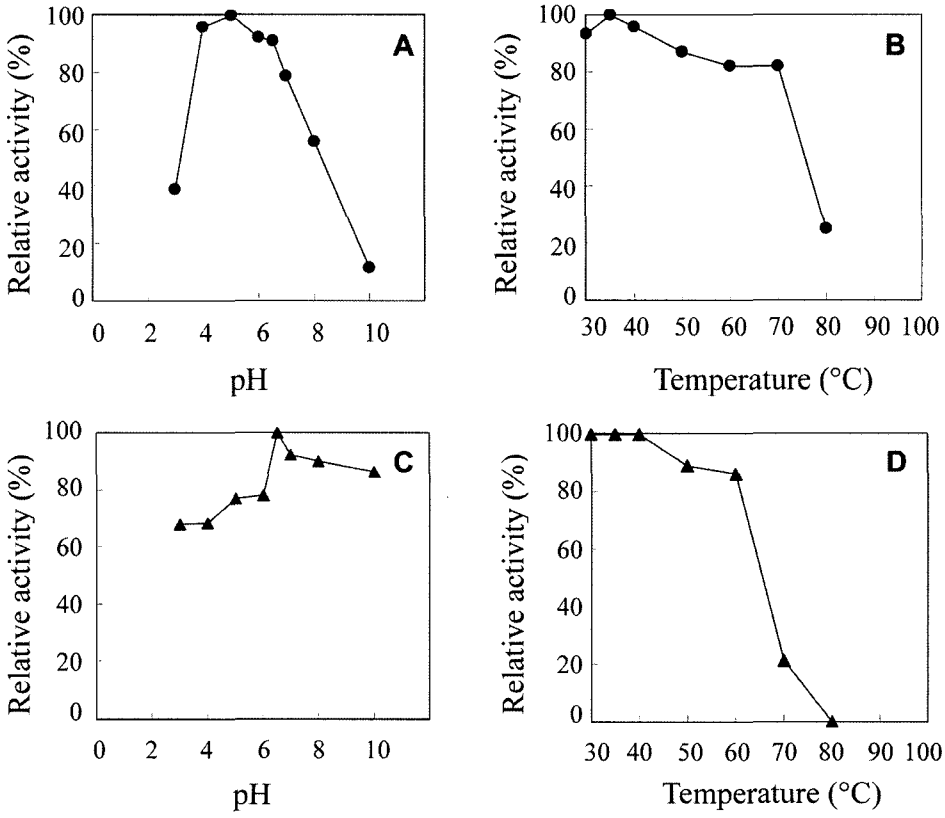

Fig. 5. Effects of pH and temperature on IFTase activity and stability.
A. Optimal pH. **B**. Optimal temperature. **C**. pH stability. **D**. Thermal stability.

DISCUSSION

We cloned a *Bacillus* sp. snu-7 gene encoding DFA III-producing IFTase. The recombinant enzyme was purified from a crude extract in a single-step using IMAC with a 41.8% yield, and the purified enzyme had very high specific activity (1,276 units/mg protein). The specific activity was much higher than that of purified native IFTases having from 240 to 933 units/mg protein [7, 8, 15, 36] and that (259 units/mg protein) of purified recombinant IFTase from *A. globiformis* C11-1 [5]. The purification yield (41.8%) was also much better than those (11 to 16%) of purified native IFTases and higher than that (32.9%) of purified recombinant IFTase from *A. globiformis* C11-1 [5].

Inulin conversion into DFA III by recombinant IFTase reached up to 63% within 10 min with the final concentration of 5 mg/ml of inulin. Because 1-fructosylnystose (GF_4) is the smallest substrate and IFTase hydrolyzed GF_4 very slowly, GF_4 conversion into DFA III and GF_2 mainly contributed to the slight increase of DFA III production during exhaustive digestion [13]. Coexisting fructooligosaccharide products such as 1-kestose (GF_2), 1-nystose (GF_3), and 1-fructosylnystose (GF_4) can be removed completely by incubating with Baker's yeast, because DFA III is non-fermentative [26]. Therefore, both high specific activity and yield and fast and high conversion of inulin within short period are very promising for further structural analysis of IFTase and for mass production of DFA III from natural inulin stored in Jerusalem artichoke tubers and in chicory roots.

In spite of 98% identity with the DFAIII-producing IFTases from *Arthrobacter globiformis* C11-1 [5], the two enzymes had several different properties, including optimal temperature, thermal stability, minor products of inulin, and specific activity (259 units/mg protein for the *A. globiformis* C11-1 enzyme versus 1,276 units/mg protein for the *Bacillus* sp. snu-7 IFTase) [4, 5]. It is unclear at this point what causes such a large difference in activity between the two enzymes.

According to the amino acid alignment (Fig. 1), 15 acidic residues (10 Asp and 5 Glu) were conserved in all six enzymes, and 4 acidic residues (3 Asp and 1 Glu) were conserved in the five IFTases but not in DFA IIIase (• in Fig. 1). Because IFTases and DFA IIIase shared no sequence similarity with exo-inulinases and endo-inulinases, we intend to mutagenize the conserved acidic residues to identify IFTase catalytic residues. Such approach has successfully been applied to the identification of catalytic residues of *Pyrococcus furiosus* amylopullulanase, a member of the family 57 glycosyl hydrolases [14].

Three cysteines (C184, C245, and C248 in *Bacillus* sp. snu-7 IFTase) are conserved in all six enzymes, two cysteines (C302 and C326) are conserved in the five IFTases but not in DFA IIIase, and one cysteine (C305) is conserved only in DFAIII-producing IFTases (▲ in Fig. 1). DFA IIIase has five cysteines, but two of them are at the N-terminus and in an extra insertion in the C-terminal region (Fig. 1). The native *Bacillus* sp. snu-7 IFTase was totally inactivated by Hg^{2+} ions; therefore, we suggested that an SH group might be essential for catalytic activity [13]. We will elucidate the roles of cysteines in IFTase catalytic activity and stability by mutagenizing the five completely conserved cysteines among the IFTases.

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