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Cold-active and NaCl-tolerant exo-inulinase from a cold-adapted *Arthrobacter* sp. MN8 and its potential for use in the production of fructose at low temperatures

Junpei Zhou,[1,2,3,‡] Qian Lu,[2,‡] Mozhen Peng,[2] Rui Zhang,[1,2,3] Minghe Mo,[4] Xianghua Tang,[1,2,3] Junjun Li,[1,2,3] Bo Xu,[1,2,3] Junmei Ding,[1,2,3] and Zunxi Huang[1,2,3,*]

Engineering Research Center of Sustainable Development and Utilization of Biomass Energy, Ministry of Education, Yunnan Normal University, Kunming 650500, PR China,[1] College of Life Sciences, Yunnan Normal University, Kunming 650500, PR China,[2] Key Laboratory of Yunnan for Biomass Energy and Biotechnology of Environment, Yunnan, Kunming 650500, PR China,[3] and Laboratory for Conservation and Utilization of Bio-Resources & Key Laboratory for Microbial Resources of the Ministry of Education, Yunnan University, Kunming 650091, PR China[4]

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An exo-inulinase gene was cloned from *Arthrobacter* sp. MN8, a cold-adapted bacterium isolated from lead–zinc-rich soil. The gene was expressed in *Escherichia coli* BL21(DE3). The resultant 505-residue polypeptide (InuAMN8) showed the highest identity (81.1%) with the putative levanase from *Arthrobacter phenanthrenivorans* Sphe3 (ADX73279) and shared 57.8% identity with the exo-inulinase from *Bacillus* sp. snu-7 (AAK00768). The purified recombinant InuAMN8 (rInuAMN8) showed an apparently optimal activity at 35°C, and 75.3%, 39.4%, and 15.8% of its maximum activity at 20°C, 10°C, and 0°C, respectively. After pre-incubation for 60 min at 50°C and 55°C, the rInuAMN8 exhibited 69.8% and 17.7% of its initial activity, respectively. The apparent K_m values of rInuAMN8 towards inulin were 2.8, 1.5, 1.2, 5.3, and 8.2 mM at 0°C, 10°C, 20°C, 30°C, and 35°C, respectively. Inulin and Jerusalem artichoke tubers were effectively hydrolyzed to release fructose by rInuAMN8 at 0°C, 10°C, and 35°C. Compared with its hyperthermophilic and thermophilic counterparts, the exo-inulinase had less aromatic amino acid F and more hydrophobic amino acid A. In addition, the purified rInuAMN8 retained 127.9%–88.4% inulinase activity at 3.5%–15.0% (w/v) NaCl concentrations. Zn^{2+} and Pb^{2+} at 10 mM exhibited little or no effect on the enzyme activity. This paper is the first to report a cold-active and/or NaCl-tolerant exo-inulinase from the genus *Arthrobacter*. The exo-inulinase rInuAMN8 shows a potential for use in the production of fructose at low temperatures.

[**Key words:** Cold-active; NaCl; Exo-inulinase; *Arthrobacter*; Fructose]

Sucrose and starch are the primary storage carbohydrates in plants; however, approximately 15% of monocotyledons, dicotyledons, and bacteria also accumulate fructose polymers called fructans. Fructans are nonstructural polysaccharides composed of multiple fructose units, linked primarily by either β-2,1-glycosidic bonds (i.e., inulin) or β-2,6-glycosidic bonds (i.e., levan) (1). A number of plants store inulin in their roots, tubers, and bulbs, such as chicory, dahlia, and cama (up to 22% of the fresh weight and 80% of the dry weight) (2). Given the abundance of inulin, which can be used to produce fructose, inulin has high biotechnological value and is considered a prominent candidate as a renewable carbohydrate source.

Fructose is rapidly becoming an important ingredient in food, pharmaceutical, and energy industries for the production of high-fructose syrups, carbonated soft drinks, fruit beverages, yogurts, ice cream, bakery goods, puddings, dairy products, baby food, bioethanol, and excipients (2,3). Fructose has been produced via multi-step starch hydrolysis and glucose isomerization or acid hydrolysis of inulin. However, the two methods are unfavorable because of low yield (≤45%), undesirable coloring of inulin hydrolyzate, and the formation of by-products such as difructose anhydride (3). Unlike these techniques, a method utilizing exo-inulinase as an enzyme is capable of efficiently hydrolyzing inulin to produce fructose yields as high as 90%–95%. Moreover, this single-step method is environmentally friendly (i.e., avoids the use of inorganic acid for inulin hydrolysis) (3).

Exo-inulinases (EC 3.2.1.80; fructan β-fructosidases, exo-β-D-fructosidases) hydrolyze inulin, releasing fructose and a small fraction of glucose. In addition, exo-inulinases from bacteria usually hydrolyze levan and disaccharide sucrose as well (2). Based on their amino acid sequence similarities, endo-inulinases and exo-inulinases are classified into the glycosyl hydrolase (GH) family 32, which carries the consensus pattern H-x(2)-[PV]-x(4)-[LIVMA]-N-D-P-N-[GA] (http://prosite.expasy.org/PS00609) (4).

Although many exo-inulinases have been studied (5–18), no research on cold-active exo-inulinases has been reported. Cold-active enzymes are highly active at low temperatures and thermolabile at intermediate and high temperatures (19). The use of cold-active enzymes at the industrial level is important because biotechnological processes performed at low temperatures reduce

* Corresponding author at: College of Life Sciences, Yunnan Normal University, No. 1 Yuhua District, Chenggong, Kunming, Yunnan 650500, PR China. Tel.: +86 871 65920830; fax: +86 871 65920952.
E-mail addresses: huangzunxi@163.com, junpeizhou@126.com (Z. Huang).
‡ The first two authors contributed equally to this work.

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energy consumption and help avoid microbial development and fermentation, product denaturation, and alterations in the ingredient and product quality (19). Interestingly, over 80% of Earth's biosphere is classified as permanently cold, and the largest proportion of biomass on Earth is generated at low temperatures (19,20).

Sodium chloride is the world's oldest food additive and has key functions in food safety and preservation by delaying the growth of spoilage microorganisms. Enzymes that are active and stable at high NaCl concentrations have the potential for use in harsh industrial processes, such as food processing, biosynthetic processes, and washing (21). Furthermore, fermentation and material processing under high-NaCl conditions can also reduce the total cost by eliminating the sterilization process (21). Unlike NaCl-tolerant endoxylanases, which have been given considerable research interests (22–24), NaCl-tolerant inulinases have not been reported.

In this study, we isolated a cold-adapted strain, designated as *Arthrobacter* sp. MN8, from a soil collected from a lead–zinc mine. A novel GH 32 exo-inulinase gene was cloned from the strain and expressed in *Escherichia coli*. The purified recombinant enzyme was identified to be a cold-active and NaCl-tolerant exo-inulinase. The enzyme effectively hydrolyzed inulin and Jerusalem artichoke tubers to release fructose at 0°C, 10°C, and 35°C. To the best of our knowledge, this study is the first to report cold-active and/or NaCl-tolerant exo-inulinase, as well as the identification and characterization of an exo-inulinase isolated from the genus *Arthrobacter*.

MATERIALS AND METHODS

Vectors and reagents Genomic DNA isolation, DNA purification, and plasmid isolation kits were purchased from Tiangen (Beijing, China). DNA polymerases were purchased from TransGen (Beijing, China). The gene was cloned using a *pMD* 18-T vector (TaKaRa, Otsu, Japan) and *E. coli* Trans1-T1 (TransGen), and then expressed using a *pEASY*-E1 Expression Kit and *E. coli* BL21 (DE3) (TransGen). Isopropyl-β-D-1-thiogalactopyranoside (IPTG) was purchased from Amresco (Solon, OH, USA). The Ni-NTA agarose used to purify the recombinant protein (His-tagged) was purchased from Qiagen (Valencia, CA, USA). Inulin from dahlia tubers (I3754), D-fructose (F0127), raffinose (R0514), and starch (S9765) were purchased from Sigma (St. Louis, MO, USA). Levan from *Zymomonas mobilis* was purchased from Advanced Technology & Industrial (Hong Kong, China), stachyose from TCI (Shanghai, China), and silica gel G plate from Haiyang (Qingdao, China). All other reagents were analytical grade and commercially available.

Microorganism isolation and preservation The soil was collected from a 600-year-old lead–zinc mine located in Lancang County, Yunnan province, China. For the experiment, 2 g of soil was suspended in 0.7% (w/v) NaCl. The suspension was spread onto screening agar plates (w/v) that contain 0.5% soybean meal, 0.1% peptone, and 1.0% NaCl at room temperature and pH 7.0. The pure culture of the MN8 strain was obtained by repeated streaking on an inulin-inducing medium (w/v) that contains 0.5% inulin, 0.1% peptone, 1.0% NaCl, and 2.0% agar at 10°C and pH 7.0, and then deposited in the Strains Collection of Yunnan Institute of Microbiology under YMF 4.00006.

The 16S rDNA sequence of the strain was PCR-amplified using universal primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT). A 16S rDNA-based phylogenetic tree was constructed using MEGA 4.0 (25) with Kimura two-parameter model, neighbor-joining algorithm, and 1000 bootstrap replications.

Gene cloning The genomic DNA from the strain MN8 was extracted using the Tiangen genomic DNA isolation kit following the manufacturer's instructions. Two degenerate primers, eInu32F (CCCACAACTGGATGAACgayccnaaygg) and eInu32R (TCTGCTCGTGCCAGAACAcnttnggrtcnc), were designed corresponding to conserved blocks of H-N-W-M-N-D-P-N-G and R-D-P-K-V-F-W-H-E-Q-S (Fig. 1) of GH 32 exo-inulinases, respectively, using the web program, CODEHOP (http://blocks.fhcrc.org/codehop.html). A partial exo-inulinase gene was amplified via a touchdown-PCR method as follows: 94°C for 5 min; 11 touchdown cycles of 94°C for 30 s, 55°C for 30 s (decreased by 1°C in each cycle), and 72°C for 50 s; 30 cycles of 94°C for 30 s, 44°C for 30 s, and 72°C for 50 s; and one final extension at 72°C for 5 min. The PCR product obtained was directly sequenced by Beijing Genomics Institute (Guangzhou, China) using a specific primer, sInu32F (CCCACAACTGGATGAACga), which was designed based on the 5′ non-degenerate 19 bases of eInu32F.

Based on the partial exo-inulinase gene, we obtained the full-length gene *inuAMN8* via a time-saving and cost-efficient GC TAIL-PCR procedure (thermal asymmetric interlaced-PCR specific for GC-rich genes) (26) with four nested insertion-specific primers: *inuAMN8*uSP1 (ATACCGCCGAGCCGGAGAAGAT), *inuAMN8*uSP2 (GCTCGTCCCAGTGCAGCAGGTC), *inuAMN8*dSP1 (GTGCGACGAACA CGAGGCCATCT), and *inuAMN8*dSP2 (GCCTCGGCACAGCGGCCAATC). PCR products of the expected size, which appeared between the second and third rounds of amplifications, were gel purified and directly sequenced by Beijing Genomics Institute. All of the primers were synthesized by Generay (Shanghai, China).

Sequence analysis The signal peptide in the amino acid sequence (InuAMN8) (deduced from *inuAMN8*) was predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP/). The identity values of the protein sequences were calculated using BLASTP program (http://www.ncbi.nlm.nih.gov/BLAST/). The classification of InuAMN8 into the GH family was determined using the InterProScan online tool (http://www.ebi.ac.uk/Tools/pfa/iprscan/). All other sequence analyses were performed using Vector NTI 10.3 software (InforMax, Gaithersburg, MD, USA).

Expression of inuAMN8 in *E. coli* The gene *inuAMN8* was amplified via PCR using *TransTaq*-T DNA polymerase and the primer set r*inuAMN8*EF (ATGAATTCATT GACGACGGC) and r*inuAMN8*ER (TCAACGGCCGACGACGTCGA). The resulting PCR product was directly cloned into the vector *pEASY*-E1 through T-A ligation, and then the recombinant plasmid (*pEASY-inuAMN8*) was transformed into *E. coli* BL21 (DE3) competent cells. The positive transformant harboring *pEASY-inuAMN8* was identified by PCR analysis, confirmed by DNA sequencing, grown overnight at 37°C in LB medium with 100 μg ml^{-1} ampicillin, and then inoculated into 10-l fresh LB medium with ampicillin (1:100 dilution). For induction, we added IPTG at 0.7 mM final concentration when the A_{600} of the culture was approximately 0.7. The culture was then incubated for an additional 20 h at 20°C, and the cells were finally harvested by centrifugation at 12,000 ×*g* at 20°C for 5 min.

Purification and identification of recombinant InuAMN8 The harvested cells were washed with sterile distilled H_2O and re-suspended in sterilized ice-cold buffer A (20 mM Tris–HCl, 0.5 M NaCl, pH 7.2). The cells were disrupted by sonication (20 kHz–24 kHz) on ice and then centrifuged at 12,000 ×*g* for 10 min at 20°C. The recombinant InuAMN8 (rInuAMN8; His_6-tag at N′ terminal) from the supernatant (~100 ml) was then purified with a Ni-NTA agarose gel column. The supernatant (2.5 ml) was loaded onto the column, left undisturbed for 5 min, washed with three column volumes of buffer A, and eluted with one column volume of a linear imidazole gradient of 20 mM–500 mM in buffer A. The protein (~100 ml, eluted by 300 mM imidazole) was used for enzyme characterization.

The purified enzyme (denatured at 90°C for 5 min) was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 12% running gel, 5% stacking gel). The gel was stained with Coomassie brilliant blue R-250, and then destained with 10% (v/v) acetic acid. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed by Tianjin Biochip (Tianjin, China) to confirm that the purified enzyme was rInuAMN8. Finally, Bradford method was employed to determine the protein concentration, using bovine serum albumin as a standard (27).

Enzyme assay We measured the release of reducing sugars from the substrate hydrolyzed by the purified rInuAMN8 using 3,5-dinitrosalicylic acid (DNS) reagent. The standard reaction contained 0.1 ml of diluted enzyme and 0.9 ml of McIlvaine buffer (pH 7.0) containing 0.5% (w/v) substrate. The mixture was incubated in a water bath at 37°C for 10 min. The reaction was stopped using 1.5 ml of DNS reagent and subsequently boiled in a water bath for 5 min to produce a measurable reddish brown (540 nm) product. One unit (U) of exo-inulinase activity was defined as the amount of enzyme that released 1 μmol of reducing sugar equivalent to D-fructose per minute. The enzyme activity was assayed by following this standard procedure unless otherwise noted.

Biochemical characterization To identify the substrate specificity of the purified rInuAMN8, 0.5% (w/v) inulin, levan, raffinose, stachyose, sucrose, or starch (determined at pH 7.0 and 35°C) was added to each reaction solution. Any further biochemical characterizations were determined using inulin (dissolved at approximately 90°C and then cooled down to room temperature) as the substrate.

The optimal pH for the exo-inulinase activity of the purified rInuAMN8 was determined at 37°C in various buffers [McIlvaine buffer (pH 4.0 to pH 8.0) and 0.1 M glycine–NaOH (pH 9.0 to pH 10.0)]. The enzyme stability was estimated by measuring the residual enzyme activity after incubating the enzyme solution in various buffers at 37°C for 60 min, with the untreated enzyme defining 100% activity.

The thermoactivity of the purified rInuAMN8 was determined over 0°C–60°C temperature range in McIlvaine buffer (pH 7.0). The thermostability of the purified rInuAMN8 was determined at 37°C following pre-incubation of the enzyme in McIlvaine buffer (pH 7.0) without substrate for 60 min at 30°C–55°C, with the untreated enzyme defining 100% activity.

The effects of various chemical reagents and metal ions on rInuAMN8 activity were analyzed in McIlvaine buffer (pH 7.0) at 37°C. Solutions included one of the following: 10 mM (final concentration) EDTA, β-mercaptoethanol SDS, NaCl, KCl, $CaCl_2$, $CoCl_2$, $NiSO_4$, $CuSO_4$, $MgSO_4$, $ZnSO_4$, $Pb(CH_3COO)_2$, $HgCl_2$, $FeCl_3$, and $AlCl_3$.

The effect of 3.5%–30.0% (w/v) NaCl on the purified rInuAMN8 was determined at pH 7.0 and 37°C. The enzyme stability in NaCl was estimated by measuring the residual enzyme activity following pre-incubation of the enzyme for 60 min; the untreated enzyme was used to define 100% activity.

K_m, V_{max}, and k_{cat} of rInuAMN8 were determined using 0.1 mM–10.0 mM inulin as the substrate in McIlvaine buffer (pH 7.0) at 0°C, 10°C, 20°C, 30°C, and 35°C. The data were plotted according to Lineweaver–Burk method (28).