Food Sci. Biotechnol. 19(3): 757-762 (2010)
DOI 10.1007/s10068-010-0106-y

RESEARCH ARTICLE

Biochemical Characterization of Dextranase from *Arthrobacter oxydans* and Its Cloning and Expression in *Escherichia coli*

Jin Ha Lee, Seong Hee Nam, Hyen Joung Park, Young-Min Kim, Nahyun Kim, Ghahyun Kim, Eun-Seong Seo, Seong-Soo Kang, and Doman Kim

Received: 13 January 2010 / Revised: 22 February 2010 / Accepted: 22 February 2010 / Published Online: 30 June 2010

Abstract Appreciably elevated levels of dextranase from *Arthrobacter oxydans* (AODex) isolated from sugar-cane farm soil was resulted from the culture on the Luria-Bertani (LB) medium containing 1%(w/v) soluble starch, glycerol, or dextran. The responsible gene (*aodex*) was cloned, its nucleotide sequence was determined, and expression of the encoded protein was achieved in *Escherichia coli*. An open reading frame was composed of 1,863 bp putatively encoding a 68.3 kDa protein. Recombinant *A. oxydans* dextranase (rAODex) was purified about 16 fold by nickel-nitrilotriacetic acid affinity column chromatography; K_m value for dextran T2000 was 0.85 mg/mL (w/v). AODex treatment of stale sugar cane juice resulted in a yield of square and light-colored sugar crystals.

Young-Min Kim, Eun-Seong Seo, Doman Kim (✉)
School of Biological Sciences and Technology and Research Institute for Catalysis, Chonnam National University, Gwangju 500-757, Korea
Tel: +82-62-530-1844; Fax: +82-62-530-0874
E-mail: dmkim@jnu.ac.kr

Jin Ha Lee
Dental Science Research Institute, School of Dentistry, 2nd Stage of Brain Korea 21 for School of Dentistry, Chonnam National University, Gwangju 500-757, Korea

Seong Hee Nam
Jeonnam Agricultural Research and Extension Services, Naju, Jeonnam 520-715, Korea

Hyen Joung Park
Cosmax Ltd., Hwaseung, Gyeonggi 445-746, Korea

Nahyun Kim
Korean Minjok Leadership Academy, Hoengseong, Gangwon 225-823, Korea

Ghahyun Kim
Major in Biochemistry and Cell Biology, University of California-San Diego, La Jolla, CA 92093, USA

Seong-Soo Kang
College of Veterinary Medicine, Chonnam National University, Gwangju 500-757, Korea

Keywords: dextranase, *Arthrobacter oxydans*, dextran, sugar, expression

Introduction

Dextranase (EC 3.2.1.11, 1,6-α-D-glucan 6-glucanohydrolase) hydrolyzes the α-1,6 glucosidic bond of dextran to release either glucose or isomaltose (exo-dextranase), or isomalto-oligosaccharides (endo-dextranase) produced by various microorganisms including bacteria, yeast, and fungi (1). On the basis of amino acid sequence similarity, dextranases have been classified into 2 glycoside hydrolase (GH) families, 49 and 66 (2), with no sequence similarity between them. In GH family 49, the nucleotide sequences and properties of endo-dextranases from *Arthrobacter* sp. (3,4), *Lipomyces starkeyi* KSM 22 (5), *Penicillium* sp. (6), and isomaltotrio-dextranase from *Brevibacterium fuscum* var. *dextranlyticum* strain (7) have been reported. Pullulan is hydrolyzed to isopanose (Glc-α-1,4-Glc-α-1,6-Glc) by iopullulanase (EC 3.2.1.57) from *Aspergillus niger* ATCC 9642. Although isopullulanase does not hydrolyze dextran at all, it belongs to GH family 49 with endo-dextranase (8). Industrial applications of dextranase include food preparation and processing, carbohydrate modification, and medicine (1). For the latter, dextranase is used for partial hydrolysis of native dextran in the preparation of blood substitutes or for preparation of appropriately sized clinical dextran suitable for use as a synthetic blood volume expander (9). With the aim of improving clinical dextran production, a mutant derepressed and partially constitutive strain, designated *L. starkeyi* ATCC 74054, was isolated (10). As well, a carbohydrase from a hyper-producing strain (*L. starkeyi* KSM 22) appeared to be effective in removal of soluble polysaccharides in sugar syrup (11). In seeking an

inexpensive and simple method of sugar syrup modification, bacteria in the genus *Arthrobacter* are potentially good sources of various dextran-hydrolyzing enzymes such as glucodextranase (3), isomalto-dextranase (IMDase) (12-14), and endo-dextranase (3,4,15).

The first processing step in the production of raw cane sugar is the shredding of the sugar cane, usually by knives and/or shredders, followed by milling to extract the juice that is then concentrated by vacuum evaporation (11). Crystallization is the final stage of the process. Delays between harvest and grinding, as well as damage to the sugarcane inflicted by freezing and subsequent shipping delays, allow more time for infection and deterioration of the cane. This can also result in a buildup of microorganisms, and an increase in dextran production. Dextranase can be economically utilized to alleviate many of the production problems associated with dextran.

Arthrobacter sp. with dextranase activity (AODex) was isolated using soluble starch as a readily available and inexpensive carbon source. In this study, the gene encoding AODex (*aodex*) was cloned, sequenced, and expressed in *Escherichia coli*, and biochemical aspects of the encoded dextranase were investigated.

Materials and Methods

Materials The reagents for analytical assays were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and reagents were of analytical grade.

Dextranase production from *A. oxydans* and purification *A. oxydans* was isolated from sugar-cane farm soil following growth of the bacteria in Luria-Bertani (LB) medium supplemented with 1%(w/v) dextran T2000, (Sigma-Aldrich) in an 8 L jar fermentor (Biotron, Bucheon, Korea) at 37°C for 24 hr. Various carbon sources (1%, w/v) including glucose, starch, mutan, sorbitol, dextran, glycerol, lactose, inulin, or levan were used to test efficient enzyme production in LB medium. The cells were removed by centrifugation (6,000×g for 10 min at 4°C). The culture supernatant was further concentrated from 8 L to 500 mL using a 30,000 cut-off hollow-fiber (Milipore-Amicon, Danvers, MA, USA). Dextranase was fractionated using a 2.5×35 cm DEAE-Sepharose column (Tosho, Tokyo, Japan) with a linear gradient of 0-1 M NaCl in 20 mM sodium phosphate buffer (pH 6.8).

Dextranase assay Dextranase activity was measured by determining the rate of hydrolysis of 1%(w/v) dextran T2000 (Sigma-Aldrich) at 37°C in 20 mM sodium phosphate buffer (pH 7.0). The amount of reducing sugars liberated was spectrophotometrically measured by a copper-bicinchoninate method (16), where 1 unit of enzyme activity constitutes the amount of enzyme that releases 1 μmol of isomaltose/min under the assay conditions. The protein concentration was determined by the Bradford method (17) using bovine serum albumin (Sigma-Aldrich) as the standard.

To determine the optimum temperature of dextranase activity, dextranase (0.2 U/mL) was incubated with 1%(w/v) dextran T2000 for 5 min at 4-60°C in 20 mM sodium phosphate buffer, pH 7.0. Enzyme thermo-stability was measured by incubation of 3 hr-treated enzyme at the aforementioned temperatures, before addition of dextran T2000. After being placed on ice, residual activity was measured as described above.

To ascertain the optimal pH, dextranase (0.2 U/mL) was incubated for 5 min with 1%(w/v) dextran T2000 in 100 mM imidazole-HCl buffer of pH 3.5-9.5. The pH stability was measured by incubation of enzyme for 3 hr in 20 mM imidazole buffers with various pH values and followed by addition of 1% dextran T2000 at 37°C. Residual activity was measured using copper-bicinconinate method (16).

Cloning of AODex Chromosomal DNA of *A. oxydans* was isolated to be used as a template for polymerase chain reaction (PCR) (9) with some modification. To amplify genomic DNA and plasmid, PCR mixtures (25 mL) contained 2.5 mL reaction buffer (500 mM KCl, 100 mM Tris-HCl, pH 9, 1% Triton X-100), 1.5 mL of 2.5 mM $MgCl_2$, 250 mM dNTP, 50-100 ng of template DNA, 10 *p*mol of each primer (DEX2-OF; 5'-GAACAGGGGAAA GCAATGAAGC-3' and DEX2-OR; 5'-AGAGTCACCAC GCGTTCCAAGT-3' based on conserved amino acid sequences from *A. globiformis* dextranase gene) and 1.2 U of Ex *Taq* DNA polymerase (Takara Bio, Kyoto, Japan). *E. coli* BL21 (DE3) and pRSETC vector (Invitrogen, Carlsbad, CA, USA) were used as the host strain and vector for dextranase gene expression. LB agar supplemented with 0.3%(w/v) blue dextran was used to select for elevated activity of dextranase gene (18).

Expression and purification of recombinant enzyme Expression of *aodex* in *E. coli* was confirmed by transparent halos around colonies on blue dextran plates (18). Overnight bacterial culture was inoculated in 100 mL of LB broth supplemented with ampicillin (50 mg/mL), and grown with shaking at 37°C until the optical density at 500 nm reached 0.5. The cells were incubated with 1 mM isopropyl-α-D-thiogalactopyranoside (IPTG) or 1 mM lactose for 6 hr at 28°C (7,12), harvested by centrifugation, and washed with 20 mM sodium phosphate buffer (pH 7.0). The cell pellets were disrupted by ultrasonication with a Sonifier apparatus (Branson Ultrasonics, Danbury, CT, USA) at 4°C for 5 min. Lysate supernatant recovered

Table 1. 16S rRNA sequence comparison of *A. oxydans*

Strain	Accession No.	Similarity	Differences/Compared
Arthrobacter oxydans DSM 20119T	X83408	100.00	0/808
Arthrobacter polychromogenes DSM 20136T	X80741	99.88	1/807
Arthrobacter globiformis DSM 20546T	M23411	98.39	13/807
Arthrobacter ramosus DSM 20546T	X80742	98.39	13/806
Arthrobacter pascens DSM 20545T	X80740	98.39	13/806
Arthrobacter nicotinovorans DSM 420T	X80743	97.26	22/804
Arthrobacter histinolovorans DSM 20115T	X83406	96.76	26/802

following centrifugation was used as the cell-free extract solution (14,19). Ten mL of supernatant containing 25 mg protein was collected and the recombinant protein was purified by nickel-nitrilotriacetic acid (Ni-NTA) affinity column chromatography (Qiagen, Valencia, CA, USA). Dextranase was eluted with 3 mL of 250 mM imidazole buffer (pH 8.0) after washing column 3 times with 4 mL of 20 mM imidazole buffer (pH 8.0). Purified protein was concentrated by ultrafiltration (Millipore, Bedford, MA, USA) after dialysis against 20 mM sodium phosphate buffer (pH 7.0). The purity and molecular weight of the recombinant AODex (rAODex) were estimated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of 1% blue dextran (18). After electrophoresis, SDS was removed by washing the gel with 50 mM Tris-HCl (pH 8.0) with gentle shaking. Dextranase activity was apparent as a white band on the blue background.

Effects of dextranase on removal of soluble polysaccharides from sugar syrup Sugar syrup was generously supplied by the Midland Co. (Kansas City, MO, USA). The final polysaccharide concentration in the membrane-filtered and concentrated sugar syrup was 13.3%. Syrup polysaccharide without monosaccharide or sucrose contamination was prepared as previously described (11) and treated with the equivalent of 0.5 U AODex. The final pH of the enzyme reaction mixture was 6.2, and the mixture was incubated for 4 hr at 37°C. After 24 hr hydrolysis, the end products were identified using thin-layer chromatography, as previously described (11).

Results and Discussion

Isolation and induction of AODex A dextranase producing bacterial isolate from sugar farm soil was identified as *A. oxydans*, based on 100% 16S rRNA sequence identity (Table 1). Various carbon sources were applied in LB medium at 1%(w/v) to enhance dextranase from *A. oxydans*. *A. oxydans* was grown with 1%(w/v) dextran, inulin, soluble starch, glycerol, levan, glucose, lactose, and raffinose as carbon sources. Culture supernatants were sampled for dextran activity. Cell growth on all the above substrates was similar except for inulin and levan on which growth was very poor.

Table 2. Influence of carbon sources on AODex production

Carbon source	Activity (U/mL)
LB[1)]	0.01
LB+Dextran	0.3
LB+Inulin	ND[2)]
LB+Soluble starch	0.18
LB+Glycerol	0.25
LB+Levan	ND
LB+Glucose	0.02
LB+Lactose	0.06
LB+Raffinose	0.05

[1)]LB, Luria-Bertani medium
[2)]Not detectable

Soluble starch, glycerol, and dextran resulted in 18-30 times higher enzyme activity, compared to free carbon source. Glucose, lactose, or raffinose showed dextranase activity 2-5 fold, whereas inulin and levan were repressing carbon source (Table 2). The result showed that soluble starch was presumably useful, since it is a readily available and inexpensive compound.

AODex characterization Over 80% of initial AODex activity was retained at temperatures ranging from 37 to 50°C, but activity was 50% of initial activity at temperatures below 25°C or above 60°C (Fig. 1). AODex was stable at pH 6.0-8.0, showing 70-100% of initial activity (Fig. 2). However, the dextranase activity was ≤50% of initial activity at a pH <5.5 and >8.3. Consistent with these results, *Arthrobacter* sp. CB-8 endo-dextranase is stable at temperatures below 40°C and displays optimal activity at pH 6 (4).

Cloning of AODex The gene encoding AODex (*aodex*) was cloned from *A. oxydans* and sequenced (Genbank accession number; AY769086). The 1 open reading frame (ORF) of *aodex* was determined to be a 1,863 bp nucleotide region encoding a 621 amino acid putative

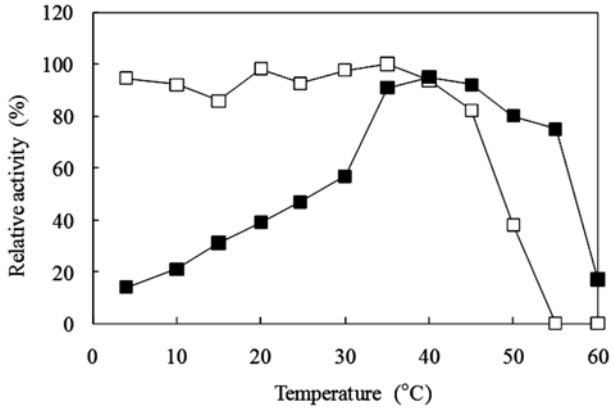

Fig. 1. Temperature effect on purified AODex. -■- optimum temperature; -□- thermostability

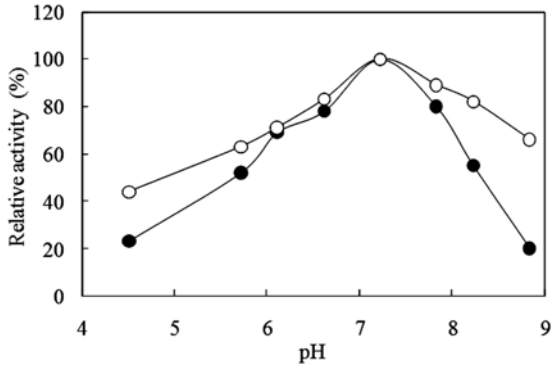

Fig. 2. pH effect on purified AODex. -●- optimum pH; -○- pH stability

protein with a calculated molecular weight (Mw) of 69.16 kDa. A multiple amino acid sequence alignment (http://www.ncbi.nlm.nih.gov/BLAST) of AODex revealed a high homology with endo-dextranases in the GH family 49 (3-7). The deduced amino acid sequence of AODex shared 92% sequence identity with the endo-dextranase from *A. globiformis* (Swiss-Prot Accession Number P77813) (3), 68% identity with isomaltotrio-dextranase from *Brevibacterium fuscum* var. *dextranlyticum* strain 0407 (Q9WXI3) (7), 67% identity with endo-dextranase from *A. globiformis* (P70744) (3), and 67% identity with endo-dextranase from *Arthrobacter* sp. CB-8 (P39652) (4).

Expression and purification of 6 his-tagged dextranase The *Bam*HI and *Kpn*I fragments of *aodex* were ligated with pRSET C vector and transformed into *E. coli* BL21 (DE3). AODex production was induced by 1 mM IPTG or 1 mM lactose. The expressed recombinant dextranase activities were confirmed as colonies surrounded by transparent halos on 0.3% blue dextran plates. Lactose-induced production of AODex was 1.3 times greater than that by IPTG. The expressed dextranase purified using a Ni-NTA affinity column displayed a single band on 10%

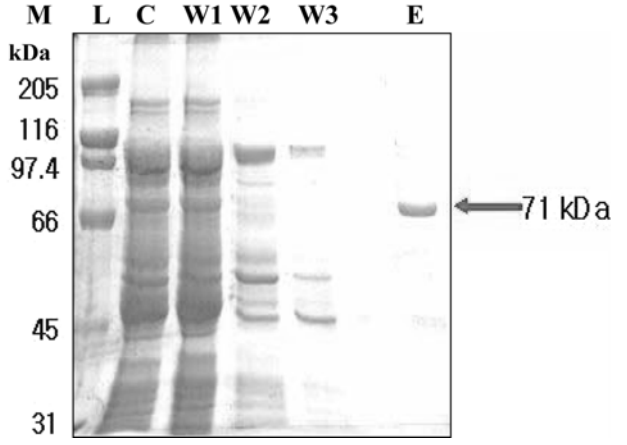

Fig. 3. SDS-PAGE analysis of purified rAODex. M, protein size marker; L, cell free lysate; C, concentrated cell free lysate by Centricon YM-30; W1-3, 3 times washing with 20 mM imidazole buffer, pH 8; E, elution with 250 mM imidazole buffer, pH 8

SDS-PAGE, which migrated with an apparent Mw of 71 kDa and which was obtained at 90% purity (Fig. 3); the electrophoretic Mw was consistent with the calculated Mw of AODex plus a 6×his tag. Previously, recombinant dextranase from *Arthrobacter* sp. CB-8 (4) or *Brevibacterium* sp. (7) were purified using CM and/or DEAE-Sepharose chromatography. Several combined purification steps were used to obtain dextranase with high purity (90%) or highly increased specific activity (24 fold) (7,20). Here, AODex was purified in a simple and efficient single-step procedure using a Ni-NTA column. Specific activity of purified dextranase was increased by 16 times to 2.6 U/mg, compared to crude enzyme (0.16 U/mg).

AODex kinetics The Michaelis constant (K_m) value for dextran hydrolysis was obtained by Lineweaver-Burk plot using dextran T2000, ranging from 0.02-5% (Fig. 4). The K_m of AODex dextranase with 0.85 mg/mL (w/v) was 6 times lower than that from *Arthrobacter* sp. with 5.4 mg/mL (w/v) (3), indicative of a higher dextran affinity of AODex than that from *Arthrobacter* sp. Generally dextran and oligosaccharides are known to stimulate the elongation of sugar crystals [11]. The commercial enzyme treatment is a suboptimal solution to the dextran and starch problem, whereas the dextranase from *Arthrobacter* sp. may possibly reduce the degree to which dextran is formed in sugar.

Residual sugar concentration in sugar syrup after sugar crystal formation was decreased by about 47% (data not shown) as sugar syrup was treated with AODex (Fig. 5C2, 5C4, and 5C6). Prior to AODex treatment sugar crystals were slightly elongated (Fig. 5A), whereas AODex-treated sugar crystals were square (Fig. 5B) and formed many crystals (Fig. 5C6). In sugar production, the presence of dextran increases the viscosity of sugar syrup, resulting in

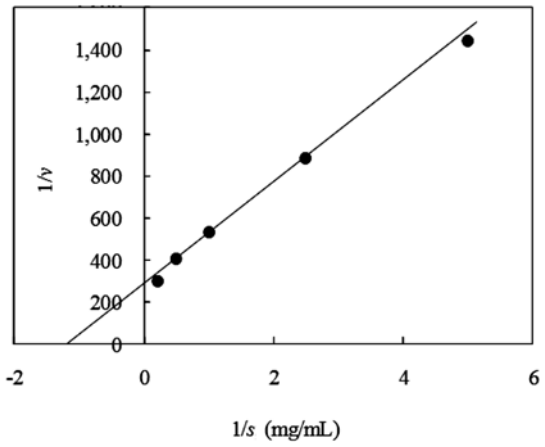

Fig. 4. Lineweaver-Burk plot with various dextran concentrations.

sucrose loss and slowing the process, which is an industrial economic impediment. The use of dextranase offers an efficient remedy. With dextran concentrations of 250 ppm or higher, a factory grinding 6,000 tons of cane/day could potentially realize an additional sugar recovery of between $800 and $2,500, as well as increased molasses purity (11). Other potential savings include efficient production flow and energy savings, both of which can significantly add to the overall savings. Of additional importance is the fact that a raw sugar producer can avoid problems associated with excessive levels of dextran (11).

In this study, *Arthrobacter* dextranase can hydrolyze amylopectin as well as dextran (data are not shown). The use of dextranase from *Arthrobacter* sp. exhibited both dextranase and amylase and it hydrolyzed the soluble polysaccharides in sugar syrup more efficiently than treatment of both commercial dextranase and amylase. The single use of the AODex can hydrolyze the soluble polysaccharides in sugar syrup efficiently and may also renders the entire process far more convenient than the current sugar process in sugar mills and refineries. The development of AODex with increased thermostability and a pilot scale experiment using the AODex treatment at sugar mill are both currently underway.

Acknowledgments This work was supported by 21C Frontier Microbial Genomics and the Applications Center Program. We thank the Korea Basic Science Institute Gwangju Branch for the NMR analysis.

References

1. Khalikova E, Susi P, Korpela T. Microbial dextran-hydrolyzing enzymes: Fundamentals and applications. Microbiol. Mol. Biol. R. 69: 306-325 (2005)
2. Coutinho PM, Henrissat B. Carbhydrate-active enzymes: An integrated database approach. pp. 3-12. In: Recent Advances in Carbohydrate Bioengineering. Gilbert HJ, Davies GJ, Henrissat B, Svensson B (eds). The Royal Society of Chemistry, Cambridge, UK (1999)
3. Oguma T, Kurokawa T, Tobe K, Kitao S, Kobayashi M. Cloning and sequence analysis of the gene for glucodextranase from *Arthrobacter globiformis* T-3044 and expression in *E. coli* cells. Biosci. Biotech. Bioch. 63: 2174-2182 (1999)
4. Okushima M, Sugino D, Kouno Y, Nakano S, Miyahara J, Toda H, Kubo S, Matsushiro A. Molecular cloning and nucleotide sequencing of the *Arthrobacter* dextranase gene and its expression in *Escherichia coli* and *Streptococcus sanguis*. Jpn. J. Genet. 66: 173-187 (1991)
5. Kang HK, Kim SH, Park JY, Jin XJ, Oh DK, Kang SS, Kim D. Cloning and characterization of dextranase gene (LSD1) from *Lipomyces starkeyi* and its expression in *Saccharomyces cerevisae*. Yeast 22: 1239-1248 (2005)
6. Garcia B, Margolles E, Roca H, Mateu D, Raices M, Gonzales ME, Herrera L, Delgado J. Cloning and sequencing of dextranase-encoding cDNA from *Penicillium minioluteum*. FEBS Microbiol. Lett. 143: 175-183 (1996)
7. Mizuno T, Mori H, Ito H, Matsui H, Kimura A, Chiba S. Molecular cloning of isomaltotrio-dextranase gene from *Brevibacterium fuscum* var. dextranlyticum strain 0407 and its expression in *E. coli*. Biosci. Biotech. Bioch. 63: 1582-1588 (1999)
8. Tonozuka T, Suzuki S, Ikehara Y, Mizuno M, Kim YK, Nishikawa A, Sakano Y. Heterogonous production and characterization of *Arthrobacter globiformis* T6 isomalto-dextranase. J. Appl. Glycosci. 51: 27-32 (2004)
9. Molodova GA, Danilova TI, Maksimov VI, Kozhevnikova NP. Methods of purifying dextranase from *Penicillium funiculosum*. Prikl. Biokhim. Mikrobiol. 16: 909-914 (1980)

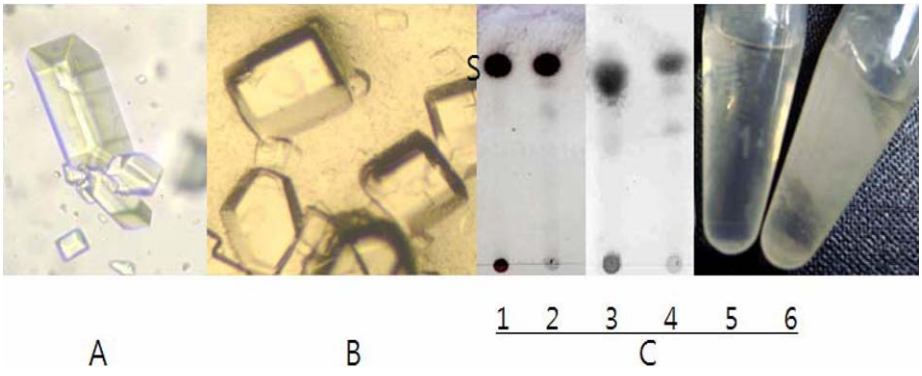

Fig. 5. Sugar crystals acquired from untreated sugar syrup (A, C5) and AODex-treated sugar syrup (B, C6). C1&C2, before sugar crystal formation-sucrose left in sugar syrup without and with AODex treated sample, respectively, C3&C4, after sugar crystal formation-sucrose left in sugar syrup without and with AODex treated sample, respectively.

10. Kim D, Day DF. A new process for the production of clinical dextran by mixed-culture fermentation of *Lipomyces starkeyi* and *Leuconostoc mesenteroides*. Enzyme Microb. Tech. 16: 844-848 (1994)
11. Lee JH, Kim GH, Kim SH, Cho DL, Kim DW, Day DF, Kim D. Treatment with glucanohydrolase from *Lipomyces starkeyi* for removal of soluble polysaccharides in sugar processing. J. Microbiol. Biotechn. 16: 983-987 (2006)
12. Hatada Y, Hidaka Y, Nogi Y, Uchimura K, Katayama K, Li Z, Akita M, Ohta Y, Goda S, Ito H, Matsui H, Ito S, Horikoshi K. Hyper-production of an isomalto-dextranase of an *Arthrobacter* sp. by a protease-deficient *Bacilllus subtilis*: Sequencing, properties, and crystallization of the recombinant enzyme. Appl. Microbiol. Biotechnol. 65: 583-592 (2004)
13. Iwai A, Ito H, Mizuno T, Mori H, Matsui H, Honma M, Okada G, Chiba S. Molecular cloning and expression of an isomalto-dextranase gene from *Arthrobacter globiformis* T6. J. Bacteriol. 176: 7730-7734 (1994)
14. Okada G, Takayanagi T, Sawai T. Improved purification and further characterization of an isomaltodextranase from *Arthrobacter globiformis* T6. Agr. Biol. Chem. Tokyo 52: 495-501 (1988)
15. Kubo S, Kubota H, Ohnishi Y, Morita T, Matsuya T, Matsushiro A. Expression and secretion of an *Arthrobacter* dextranase in the oral bacterium *Streptococcus gordonii*. Infect. Immun. 61: 4375-4381 (1993)
16. Fox JD, Robyt J. Miniaturization of three carbohydrate analyses using a microsample plate reader. Anal. Biochem. 195: 93-96 (1991)
17. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254 (1976)
18. Ohnishi Y, Kubo S, Ono Y, Nozaki M, Gonda Y, Okano H, Matsuya T, Matsushiro A, Morita T. Cloning and sequencing of the gene coding for dextranase from *Streptococcus salivarius*. Gene 14: 93-96 (1995)
19. Ryu HJ, Kim D, Kim DW, Moon YY, Robyt F. Cloning of a dextransucrase gene (fmcmds) from a constitutive dextransucrase hyper-producing *Leuconostoc mesenteroides* B-512FMCM developed using VUV. Biotechnol. Lett. 22: 421-425 (2000)
20. Igarashi T, Morisaki H, Goto N. Molecular characterization of dextranase from *Streptococcus rattus*. Microbiol. Immunol. 48: 155-162 (2004)