Food Sci. Biotechnol. 23(6): 2007-2012 (2014)
DOI 10.1007/s10068-014-0273-3

RESEARCH NOTE

Molecular Cloning and Expression of Amylosucrase from Highly Radiation-resistant *Deinococcus radiopugnans*

Myo-Deok Kim, Dong-Ho Seo, Jong-Hyun Jung, Dong-Hyun Jung, Min-Ho Joe, Sangyong Lim, Ju-Hoon Lee, and Cheon-Seok Park

Received May 11, 2014; revised June 11, 2014; accepted July 1, 2014; published online December 31, 2014

Abstract Amylosucrase (AS), a glucosyltransferase of the glycoside hydrolase 13 family, does not require a nucleotide-activated sugar as a glucosyl-donor. A gene (*drpas*) of *Deinococcus radiopugnans* encoding a putative AS was identified and cloned for characterization. The amino acid sequence revealed that *drpas* exhibited 76 and 74% identities with AS genes from *D. geothermalis* and *D. radiodurans*, respectively. Recombinant DRpAS exhibited AS activities. The ratios of hydrolysis, polymerization, and isomerization reactions were 5.8:82.7:11.5 at 40ºC. The DRpAS was highly thermostable and produced more polymerization products than AS from *D. radiodurans* although the optimum growth temperature of both strains is 30ºC.

Keywords: amylosucrase, *Deinococcus radiopugnans*, glycoside hydrolase 13 family (GH13), insoluble glucan, transglycosylation

Introduction

Enzymes are important biological catalysts responsible for maintaining metabolic processes. Enzymes exhibit many general features; however, catalytic specificity is one of the most distinguishing and essential characteristics of these crucial biological molecules. Many enzymes exhibit different levels of specificity in the reactions they catalyze, including substrate, structural, linkage (or bond), and/or stereochemical specificity. However, some enzymes are known to perform multiple catalytic reactions with relaxed specificity.

Amylosucrase (AS, EC 2. 4. 1. 4) is a versatile enzyme that carries out 3 different catalytic reactions: 1) hydrolysis of sucrose to release a glucose molecule and a fructose molecule; 2) synthesis of glucose polymers from liberated glucose molecules; and 3) production of the sucrose isomers turanose and isomaltulose through an isomerization reaction (1). In addition, AS can attach glucose molecules to an atypical substrate, thereby generating unnatural glucan-conjugates (2). These unique enzymatic properties of AS have been used to produce modified starch, hyperbranched glycopolymers, turanose, and other functional glucan-conjugates (3-5).

This distinctive enzyme is not found in a broad range of microorganisms, unlike other members of the glucoside hydrolase family (GH) of enzymes. So far, *Alteromonas macleodii* (6), *Arthrobacter chlorohenolicus* (7), *Deinococcus geothermalis* (DGAS) (1), *D. radiodurans* (DRAS) (8), *Methylobacillus flagellatus* (9), and *Neisseria polysaccharea* (10) have been shown to contain AS in the genome. The enzymatic properties of these enzymes have also been investigated. Interestingly, 2 varieties of AS from *Deinococcus* (DGAS and DRAS) showed different thermostability, product ratio, and active site topology properties (1,8). Compared with the AS of DGAS, the AS of DRAS was shown to be relatively unstable. Furthermore, the crystal structure of DRAS revealed that subsite-1 of the DRAS active-site, which is a deep pocket involved in trans-

Myo-Deok Kim, Dong-Ho Seo, Jong-Hyun Jung, Dong-Hyun Jung, Ju-Hoon Lee, Cheon-Seok Park (✉)
Graduate School of Biotechnology and Institute of Life Science and Resources, Kyung Hee University, Yongin, Gyeonggi 446-701, Korea
Tel: +82-31-201-2631; Fax: +82-31-204-8116
E-mail: cspark@khu.ac.kr

Dong-Ho Seo
Korea Food Research Institute, Seongnam, Gyeonggi 463-746, Korea

Jong-Hyun Jung, Min-Ho Joe, Sangyong Lim
Korea Atomic Energy Research Institute, Jeongeup, Jeonbuk 580-185, Korea

glucosylation, was different from the corresponding site in DGAS. Skov *et al.* (11) suggested that these differences in the active site pocket might indicate that AS operates through an elongation mechanism that involves considerable conformational change that modulates accessibility to the sucrose-binding site and, thereby, allows successive cycles of glucosyl-moiety transfer to a growing glucan chain (11).

Recently, a homologue of AS in the genome of *D. radiopugnans* (ATCC 19172), a mesophilic and highly radiation-resistant microorganism, has been identified. In this study, the gene corresponding to AS (hereafter referred to as DRpAS) was cloned and expressed in *Escherichia coli*. Then, recombinant DRpAS was characterized and properties were compared with other varieties of AS, especially DGAS and DRAS, with the overall goal of elucidating the elongation mechanism of AS.

Materials and Methods

Bacterial strains *D. radiopugnans* (ATCC 19172) was obtained from the Korea Atomic Energy Research Institute (Jeongeup, Korea). Strains of *E. coli* were used for standard DNA manipulation and recombinant protein expression. *E. coli* DH10B [F^-Φ80*lacZ* M15 (*lacZ*YA-*arg*F) U169 *rec*A1*end*A1 *hsd*R17(r_k^+, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1λ$^-$] was used as a host for typical DNA manipulations, while BL21 [F^- *ompT hsdS*$_B$ (r_B^-, m_B^-) *gal dcm* λ(DE3)] was used for expression of recombinant proteins. All chemicals were of reagent grade and purchased from Sigma-Aldrich (St. Louis, MO, USA) or Duchefa Biochemie B.V (Haarlem, The Netherlands). DNA restriction and modifying enzymes were obtained from New England Biolabs (Beverly, MA, USA).

Cloning and recombinant expression of the putative AS gene from *D. radiopugnans* Genomic DNA was extracted from *D. radiopugnans* using a Genomic DNA Prep Kit for bacteria (Solgent, Seoul, Korea) according to the manufacturer's instructions. The gene corresponding to *drpas* was amplified using the 2 oligonucleotide primers, DRpAS-F (5'-CAT ATG CCC ACC ACC CTG CTG AC-3') and DRpAS-R (5'-CTC GAG TGC GTT CAC CTC CGC CGC AG-3'), designed based on corresponding genomic sequences. The PCR product was obtained using the thermocycling conditions described previously in Seo *et al.* (1). The resultant *drpas* gene product was cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) after confirming the absence of PCR-introduced errors using sequencing. The resultant fragments were subcloned into pET-21a(+) and digested using *Nde*I and *Xho*I, thus producing pETDRpAS. Transformed *E. coli* BL21 cells harboring pETDRpAS were grown in LB medium containing 0.1 mg/mL of ampicillin. Protein expression was induced using addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM when the optical density at 600 nm (OD_{600}) reached 0.5 to 0.6 with spectrophotometer (Beckman DU 730; Beckman Coulter, Fullerton, CA, USA), and cells were harvested with centrifugation (Hanil Combi 514R; Hanil Centrifuge Co., Incheon, Korea) after 3 h of induction. The resultant cell pellet was resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole, pH 7.0) and cells were disrupted by ultrasonication (output 4, six times for 30 s, constant duty, Sonifier 450; Branson, Danbury, CT, USA). Finally, the recombinant protein in the cell lysate was purified using Ni-NTA affinity chromatography (Ni-NTA Superflow; Qiagen Inc., Valencia, CA, USA) (1). The eluted protein was concentrated and dialyzed using a Vivaspin column (30,000 MWCO; Sartorius Stedim Biotech., GmbH, Göttingen, Germany) to remove imidazole, and the protein concentration and enzymatic activity were measured prior to further study.

Determination of enzymatic activity, optimal pH, temperature, and thermostability The enzymatic activity of DRpAS was measured using the 3,5-dinitrosalicylic acid (DNS) method (1). for detection of reducing sugar where 1 unit of DRpAS hydrolysis activity was defined as the amount of enzyme required to catalyze production of 1 μmol of fructose per min under the assay conditions. The effect of pH on the enzymatic activity of DRpAS was investigated within the range of pH 4.0 to pH 10.0 at 40°C. The effect of temperature on the activity was studied between 25 and 50°C at pH 8.0. The thermostability of purified DRpAS was assessed by incubation of the enzyme in the absence of substrate at 40, 45, and 50°C. The residual enzymatic activity was measured under standard assay conditions at certain time intervals. Differential scanning fluorimetry (DSF, ABI 7500 Fast Real-time PCR system; Applied Biosystems, Inc., Foster, CA, USA), which has been used to measure the melting temperature (T_m) of proteins, was performed using the procedures of Jeong *et al.* (9).

High performance anion exchange chromatography (HPAEC) analysis HPAEC analysis was carried out for carbohydrate detection using an analytical column. CarboPac PA1 and CarboPac PA200 columns (Dionex Co., Sunnyvale, CA, USA) were used for analysis of small oligosaccharides and relatively long polysaccharides, respectively. Filtered samples (Syringe filter, 0.45 μm; Whatman, Maidstone, Kent, UK) were eluted using a linear gradient ranging from 100% buffer A (100 mM NaOH in water) to 60% buffer B (500 mM sodium acetate in buffer A) over 70 min. The flow rate of the mobile phase was maintained at 1.0 mL/

min. Analytes were detected using a ED50 module (Dionex) and peak areas were calculated for quantitative analysis.

Results and Discussion

Whole genome sequence data from *D. radiopugnans* (ATCC 19172) were analyzed using the Rapid Annotation Subsystem Technology (RAST) server (12). A gene designated as a sucrose phosphorylase was identified. More comprehensive sequence analysis using the Basic Local Alignment Search Tool (BLAST, http://blast.st-va.ncbi.nlm.nih.gov/Blast.cgi) revealed that the deduced amino acid sequence of the corresponding protein shared 76 and 74% amino acid identity values with DGAS and DRAS, respectively. Furthermore, PredicProtein analysis (https://www.predictprotein.org/) predicted the product of this gene to function as an AS. Thus, the gene was incorrectly annotated as a sucrose phosphorylase as the gene actually encodes an AS (*drpas*). The putative *drpas* gene consisted of 1,956 nucleotides that encoded a protein of 651 amino acids with a calculated molecular weight (Mw) of 72.822 Da and an isoelectric point of 5.3. The deduced amino acid sequence of the putative protein product (DRpAS) exhibited strong homology to varieties of AS from other *Deinococcus* species. Sequence alignments revealed that DRpAS shared 4 consensus regions with proteins classified in GH family 13, and 2 important catalytic residues (Asp285 and Glu327) of AS varieties. Loops 3, 4, and 7 of AS have been shown to form an active-site pocket and to play an important role in transglycosylation activity (13). Loop 7 is located in the B'-domain, which is only found in AS. This loop is involved in the oligosaccharide-binding site at the acceptor-binding domain (11). The amino acid sequence of DRpAS loop 7 showed little homology with the loop 7 sequences of both DGAS and DRAS. However, loops 3 and 4 showed higher similarity values (Fig. 1). Recently, Champion *et al.* (2) constructed mutant variants of AS from *N. polysaccharea* (NPAS) that were able to glucosylate protected non-natural sugars (methyl α-L-rhamnopyranoside and allyl 2-acetoamindo-2-deoxy-α-D-glucopyranoside) as acceptors. These non-natural sugars were not used by wild-type NPAS. Thus, introduction of mutations into loop 4 influenced the acceptor specificity for the transglycosylation activity of AS (2,13). The active pocket topology of DRpAS was different from the topologies of DGAS and DRAS, indicating that the acceptor specificity of DRpAS was likely also different from other AS varieties.

Recombinant DRpAS was expressed in *E. coli* cells harboring the pETDRpAS expression plasmid. The recombinant protein was successfully purified to apparent homogeneity using Ni-NTA affinity chromatography and exhibited a Mw of approximately 72 kDa. The optimum pH for DRpAS activity was found to be 8.0, which was similar to the optimal pH values of other AS varieties (1,7,8,10) (Fig. 2A). The temperature profile of DRpAS activity was investigated in the range of 25 to 50°C (Fig. 2B), and the optimum temperature of DRpAS was determined to range from 40-45°C. The half-life of DRpAS was calculated as 421.4 h at 40°C and 3.3 h at and 45°C (Fig. 2C). The thermostability of DRpAS was dramatically decreased at 50°C compared with those at 40 and 45°C. The T_m value of DRpAS was calculated to be 50.7±0.2°C and the half-life of DRpAS was found to be 7.2 min at 50°C (Fig. 2D). Thus, the thermostability ranking of the AS varieties of different *Deinococcus* species was: DGAS >DRpAS>DRAS (Table 1) (1,7,8,10). Although the optimum growth temperature of both *D. radiopugnans* and *D. radiodurans* was 30°C, the thermostability values of some proteins from *D. radiopugnans* have been found to be higher than proteins from *D. radiodurans*. The half-life of *D. radiopugnans* single-stranded DNA-binding protein (SSB) was found to be 120 min at 90°C, whereas the half-life of *D. radiodurans* SSB was found to be 1 min at 60°C (14). Similar to SSB, DRpAS was more thermostable than DRAS. Most of the ions tested, with the exception of potassium, did not inhibit DRpAS activity. However, the presence of potassium ions increased DRpAS activity by 30% at both low (1 mM) and high (10 mM) concentrations (data not shown).

The quaternary structure of DGAS and DRAS was a dimer in solution. To investigate the oligomeric state of DRpAS in solution, GPC with Superdex-200 column (10×300 mm; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was performed with DRpAS. The DRpAS peak displayed a Mw of approximately 152.10 kDa, implying that DRpAS also exists as a dimer in solution (Table 1).

AS varieties produce glucose, fructose, soluble malto-oligosaccharide, insoluble glucan, and sucrose isomers (turanose and trehalulose) using only sucrose as a substrate (15). To validate the putative AS activity, DRpAS and sucrose were allowed to react for 48 h at 40°C. The sucrose was completely consumed after this incubation. The resultant products were analyzed using HPAEC (Fig. 2E-F, Table 1). DRpAS produced glucose and fructose as sucrose hydrolysates, maltooligosaccharides as transglycosylation products, and turanose and trehalulose as sucrose isomers. Moreover, the turbidity of the reaction mixture increased during the reaction time, and a white precipitate appeared. The insoluble glucans synthesized by AS have previously been shown to form a white precipitate (15). The ratio of hydrolysis, polymerization, and isomerization reactions was 5.8:82.7:11.5 at 40°C (Table 1). Thus, DRpAS efficiently synthesized more insoluble glucans and soluble maltooligo-saccharides than AS varieties from other *Deinococcus* species. The

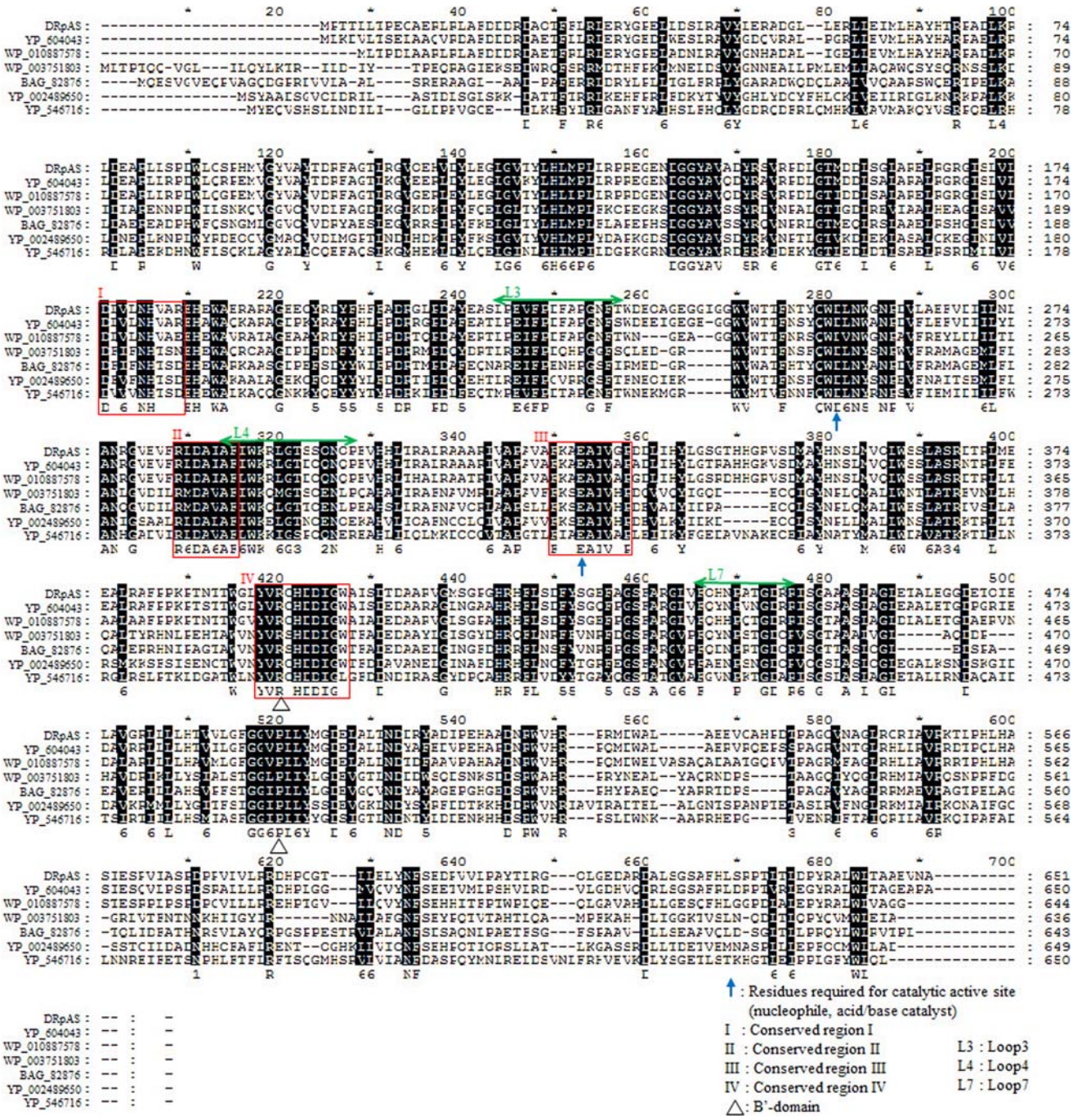

Fig. 1. Multiple amino acid sequence alignments of DRpAS with other AS varieties from different microorganisms. Fully conserved amino acid residues are designated in black, and the 4 conserved regions (I, II, III, and IV) are indicated with boxes. Blue arrows indicate the amino acid residues required for catalytic activity and green arrows indicate the 3 conserved loops (3, 4, and 7). Triangles indicate the B'-domain of amylosucrase. Sequences shown are from *Deinococcus radiopugnans* (DRpAS), *Deinococcus geothermalis* DSM 11300 (YP_604043), *Deinococcus radiodurans* (WP_010887578), *Neisseria polysaccharea* (WP_003751803), *Arteromonas macelodii* (BAG_82876), *Arthrobacter chlorophenolicus* A6 (YP_002489650), and *Methylobacillus flagellates* KT (YP_546716).

polymerization activity of DRpAS was comparable to the activity of NPAS, which is known to be the most effective transglucosidase among all the varieties of AS. In addition, this study found that DRpAS is more thermostable than NPAS. DRpAS has a strong potential for application as an efficient transglycosylation enzyme.

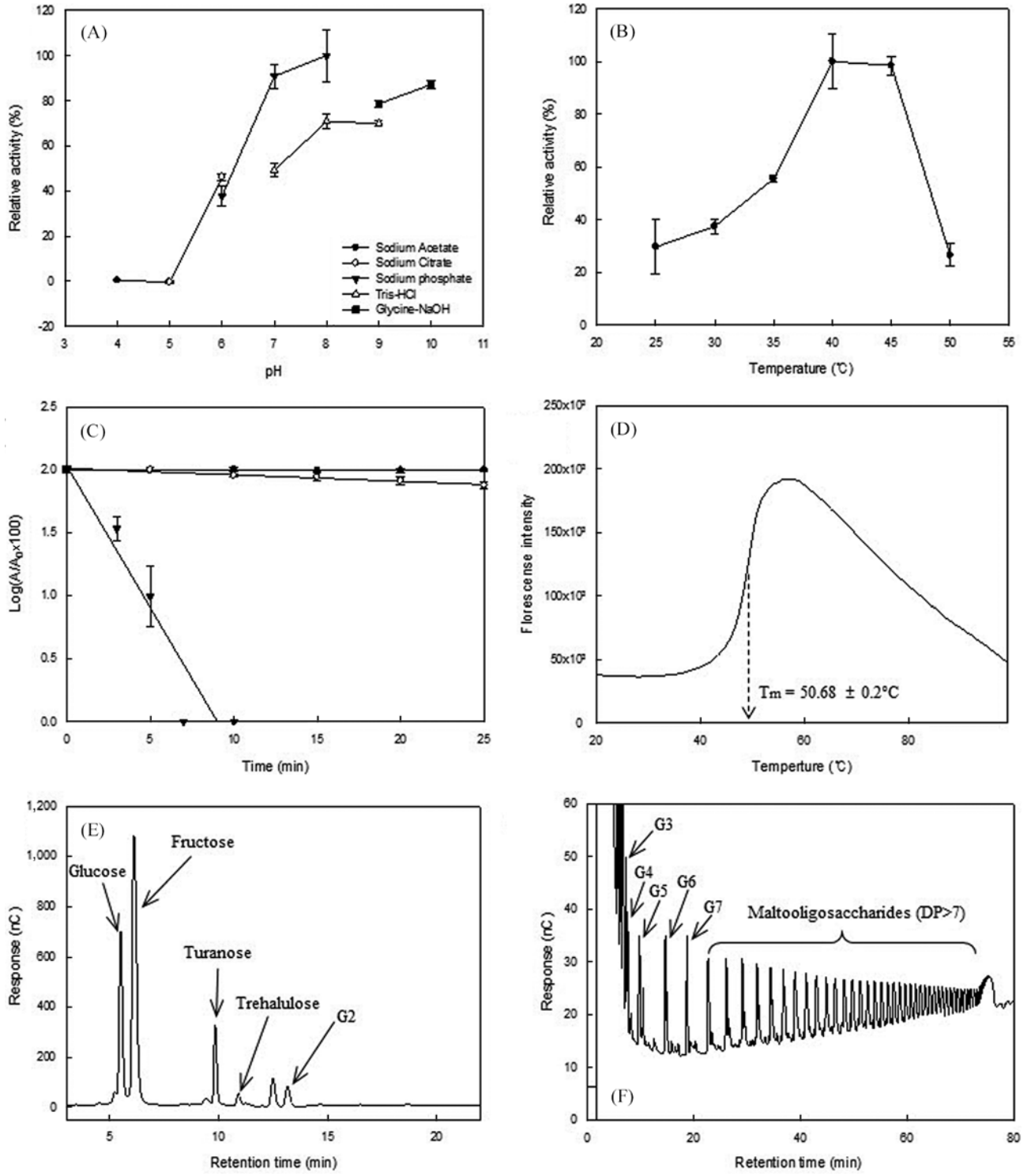

Fig. 2. Biochemical properties of DRpAS and high performance anion-exchange chromatography (HPAEC) analysis of the α-1,4 glucan synthesized using DRpAS. (A) Determination of the optimal pH for DRpAS activity. The following buffers and pH values were examined at 40°C: ●, sodium acetate pH 4.0-5.0; ○, sodium citrate pH 5.0-7.0; ▼, sodium phosphate pH 6.0-8.0; △, Tris-HCl pH 7.0-9.0; and ■, glycine-NaOH pH 9.0-10.0. (B) Determination of the optimal temperature for DRpAS activity. A temperature range of 25 to 50°C was investigated at pH 8.0. (C) Determination of the half-life of DRpAS. The half-life of DRpAS was measured at the following temperatures in 50 mM sodium phosphate, pH 8.0: ●, 40°C; ○, 45°C; ▼, 50°C. (D) Results from differential scanning fluorimetry (DSF) analysis to confirm the melting temperature. (E) HPAEC analysis of the DRpAS reaction products of sucrose isomers and short maltooligosaccharides. (F) HPAEC analysis of maltooligosaccharides. G2, maltose; G3, maltotriose; G4, maltotetraose; G5, maltopentaose; G6, maltohexaose; DP, degree of polymerization. The reaction was performed for 48 h at 40°C.

Table 1. Comparison of the enzymatic properties of DRpAS with the properties of other amylosucrases

	Optimal temperature (°C)	Optimal pH	Oligomeric state[1)]	Thermostability (T_m, °C)	Ratio of products (%)			References
					Hydrolysis	Polymerization (DP<33)	Isomerization	
DRpAS	40-45	8.0	Dimer	50.7	5.8	82.7	11.5	This study
NPAS	30-35	8.0	Monomer	49.5	5.4	80.1	14.5	De Montalk *et al.* (10)
DGAS	45-50	8.0	Dimer	60.8	7.0	71.0	22.0	Seo *et al.* (1)
DRAS	50	8.0	Dimer	-	9.6	56.9	33.5	Pizzut *et al*. (8)

[1)]Oligomeric state was determined using GPC. Protein molecular weight markers, including thyroglobulin (669 kDa), β-amylase (200 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa) were used to determine the apparent enzyme Mw.

Acknowledgments This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korean Government (MEST) (no. 2014-002744).

Disclosure The authors declare no conflict of interest.

References

1. Seo DH, Jung JH, Ha SJ, Yoo SH, Kim TJ, Cha J, Park CS. Molecular Cloning of the Amylosucrase Gene from a Moderate Thermophilic Bacterium *Deinococcus geothermalis* and Analysis of its Dual Enzyme Activity. Woodhead Publiching Limited, Cambridge, UK. pp. 125-140 (2008)
2. Champion E, André I, Moulis C, Boutet J, Descroix K, Morel S, Monsan P, Mulard LA, Remaud Siméon M. Design of α-transglucosidases of controlled specificity for programmed chemoenzymatic synthesis of antigenic oligosaccharides. J. Am. Chem. Soc. 131: 7379-7389 (2009)
3. Ryu JH, Lee BH, Seo DH, Baik MY, Park CS, Wang R, Yoo SH. Production and characterization of digestionresistant starch by the reaction of *Neisseria polysaccharea* amylosucrase. Starch-Starke 62: 221-228 (2010)
4. Wang R, Bae JS, Kim JH, Kim BS, Yoon SH, Park CS, Yoo SH. Development of an efficient bioprocess for turanose production by sucrose isomerisation reaction of amylosucrase. Food Chem. 132: 773-779 (2012)
5. Albenne C, Skov LK, Tran V, Gajhede M, Monsan P, Remaud Siméon M, André Leroux G. Towards the molecular understanding of glycogen elongation by amylosucrase. Proteins 66: 118-126 (2007)
6. Ha SJ, Seo DH, Jung JH, Cha J, Kim TJ, Kim YW, Park CS. Molecular cloning and functional expression of a new amylosucrase from *Alteromonas macleodii*. Biosci. Biotech. Bioch. 73: 1505-1512 (2009)
7. Seo DH, Choi HC, Kim HH, Yoo SH, Park CS. Functional expression of amylosucrase, a glucan-synthesizing enzyme, from *Arthrobacter chlorophenolicus* A6. J. Microbiol. Biotechn. 22: 1253-1257 (2012)
8. Pizzut Serin S, Potocki Véronèse G, van der Veen BA, Albenne C, Monsan P, Remaud Simeon M. Characterisation of a novel amylosucrase from *Deinococcus radiodurans*. FEBS lett. 579: 1405-1410 (2005)
9. Jeong JW, Seo DH, Jung JH, Park JH, Baek NI, Kim MJ, Park CS. Biosynthesis of glucosyl glycerol, a compatible solute, using intermolecular transglycosylation activity of amylosucrase from *Methylobacillus flagellatus* KT. Appl. Biochem. Biotech. 173: 904-917 (2014)
10. De Montalk GP, Remaud Simeon M, Willemot R, Planchot V, Monsan P. Sequence analysis of the gene encoding amylosucrase from *Neisseria polysaccharea* and characterization of the recombinant enzyme. J. Bacteriol. 181: 375-381 (1999)
11. Skov LK, Mirza O, Sprogøe D, Dar I, Remaud Simeon M, Albenne C, Monsan P, Gajhede M. Oligosaccharide and sucrose complexes of amylosucrase structural implications for the polymerase activity. J. Biol. Chem. 277: 47741-47747 (2002)
12. Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, Formsma K, Gerdes S, Glass EM, Kubal M. The RAST Server: Rapid annotations using subsystems technology. BMC Genomics 9: 75 (2008)
13. Champion E, Gueìrin Fdr, Moulis C, Barbe S, Tran TH, Morel S, Descroix K, Monsan P, Mourey L, Mulard LA. Applying pairwise combinations of amino acid mutations for sorting out highly efficient glucosylation tools for chemo-enzymatic synthesis of bacterial oligosaccharides. J. Am. Chem. Soc. 134: 18677-18688 (2012)
14. Filipkowski P, Koziatek M, Kur J. A highly thermostable, homodimeric single-stranded DNA-binding protein from *Deinococcus radiopugnans*. Extremophiles 10: 607-614 (2006)
15. Albenne C, Skov LK, Mirza O, Gajhede M, Feller G, D'Amico S, André G, Potocki Véronèse G, van der Veen BA, Monsan P. Molecular basis of the amylose-like polymer formation catalyzed by *Neisseria polysaccharea* amylosucrase. J. Biol. Chem. 279: 726-734 (2004)