Research Article

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Comparative study on amylosucrases derived from *Deinococcus* species and catalytic characterization and use of amylosucrase derived from *Deinococcus wulumuqiensis*

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Abstract: Amylosucrase (ASase; EC 2.4.1.4), a versatile enzyme, exhibits three characteristic activities: hydrolysis, isomerization, and transglycosylation. In this study, a novel ASase derived from *Deinococcus wulumuqiensis* (DWAS) was identified and expressed in *Escherichia coli*. The optimal reaction temperature and pH for the sucrose hydrolysis activity of DWAS were determined to be 45 °C and 9.0, respectively. DWAS displays relatively high thermostability compared with other ASases, as demonstrated by half-life of 96.7 and 4.7 min at 50 °C and 55 °C, respectively. DWAS fused with 6×His was successfully purified to apparent homogeneity with a molecular mass of approximately 72 kDa by Ni-NTA affinity chromatography and confirmed by SDS-PAGE. DWAS transglycosylation activity can be used to modify isovitexin, a representative flavone C-glucoside contained in buckwheat sprouts to increase its limited bioavailability, which is due to its low absorption rate and unstable structure in the human body. Using isovitexin as a substrate, the major transglycosylation product of DWAS was found to be isovitexin monoglucoside. The comparison of transglycosylation reaction products of DWAS with those of other ASases derived from *Deinococcus* species revealed that the low sequence homology of loop

8 in ASases may affect the acceptor specificity of ASases and result in a distinctive acceptor specificity of DWAS.

Keywords: amylosucrase; *Deinococcus*; isovitexin; transglycosylation.

Abbreviations

ASase, amylosucrase; CD, cyclodextrin; BCA, bicinchoninic acid; CR, conserved region; DNS, dinitrosalicylic acid; DSF, differential scanning fluorimetry; DGAS, amylosucrase from *Deinococcus geothermalis*; DRAS, amylosucrase from *Deinococcus radiodurans*; DRpAS, amylosucrase from *Deinococcus radiopugnans*; DWAS, amylosucrase from *Deinococcus wulumuqiensis*; GH, glycoside hydrolase; HPAEC, high-performance anion-exchange chromatography; HPLC, high-performance liquid chromatography; LC/MS liquid chromatography/mass spectrometry; MD, molecular dynamics; NPAS, amylosucrase from *Neisseria polysaccharea*; RT, retention time.

1 Introduction

Amylosucrase (ASase; EC 2.4.1.4) is a hydrolytic enzyme belonging to glycoside hydrolase (GH) family GH13. Evolutionary relationships between 152 GH13 enzymes revealed that ASase together with sucrose hydrolases and sucrose phosphorylases occupied a position in the tree between the ‘oligo-1,6-glucosidases’ and ‘neopullulanases’ [1]. Previous *in silico* analysis of GH13 enzymes assigned ASase to subfamily GH13_4 with having both sucrose hydrolysis activity and the “QpDln”

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sequence as a characteristic of the subfamily of oligo-1,6-glucosidase [1,2]. While its sucrose hydrolysis activity involves cleaving the α -1,6-glycosidic linkage in sucrose, the enzyme exhibits two additional characteristic catalytic activities, i.e., isomerase activity that produces sucrose isomers (turanose and trehalulose) and transglycosylation activity that produces α -1,4-glucans, which are amylose-like polymers [3-5]. In this so-called “transglycosylation” reaction, the enzyme transfers the glucosyl moiety from sucrose to glucose or fructose resulting from sucrose hydrolysis. Recently, ASase transglycosylation activity has been extensively studied for various biotechnological applications because the enzyme can transfer glucose from an inexpensive sucrose substrate without requiring the relatively expensive UDP-glucose as a substrate [6].

ASase was first studied in the *Neisseria* genus, and studies on ASase have involved the synthesis of glycogen-like α -glucan from sucrose [6]. Tao et al. [7] reported the characterization of glycogen-like polysaccharides generated by *Neisseria perflava* ASase and ASase inhibition by sucrose derivatives. After performing cloning and characterization of the gene corresponding to ASase derived from *Neisseria polysaccharea* (NPAS), research mainly focused on its catalytic properties, three-dimensional structure, and product analysis [8-10] until the investigation of ASases in the *Deinococcus* genus [11]. Recently, ASases have been identified in many microorganisms including *Alteromonas macleodii*, *Arthrobacter chlorophenolicus*, *Cellulomonas carbonis*, *Methylobacillus flagellates*, *Methylobacterium alcaliphilum*, and *Synechococcus* sp. [12-21]. Among these, *Deinococcus* species have been thoroughly studied in the fields of biotechnology and bioremediation because of their exceptional ability to grow and metabolize under harsh conditions [22]. Recently, we identified ASases in various species of the *Deinococcus* genus and found that most species contain ASases in their genomes (data not shown).

Flavonoids are divided into several classes, i.e., flavones, flavonols, flavonones, isoflavones, flavan-3-ols, and anthocyanins, according to their backbone structure correlated to the locations of their B-ring and C-ring and the chemical structure based on the saturation, oxidation, and hydroxylation of C-rings [23-25]. Almost all natural flavonoids exist in the form of O-glycosides or C-glycosides rather than aglycones [25], with the most common flavonoid structures in plants being flavone glycosides and flavonol glycoside [26]. In nature, C-linked glycosylation is mostly found in the flavone group.

Among various flavone C-glycosides, isovitexin (apigenin 6-C-glucoside) has been more actively studied due to its biological functionality [27]. Isovitexin is one of

the major flavone C-glycosides present in mung bean and buckwheat sprouts [28-30]. It acts as a genetic mediator in innate and acquired immune mechanisms. Isovitexin has been found to exert anti-inflammatory and antioxidant activities by inhibiting the MAPK and NF- κ B pathways [31-33]. In addition to these activities, various health benefits, such as anti-cancer, anti-estrogenic, and anti-diabetes benefits have been reported [34-37]. However, isovitexin displays both low solubility and stability [38]. During the digestion process of flavonoids in the body, C-multiglucosides are constantly absorbed in the intestine and distributed to other tissues, whereas C-monoglucosides are not absorbed well and are unstable due to deglycosylation or degradation by the human intestinal bacteria present in the large intestine [33,39]. Therefore, the application of isovitexin in its native C-monoglucoside state remains limited in the pharmaceutical and food industries. Several strategies including the use of cyclodextrin (CD) [40], chemical synthesis of glycosides [41], and enzymatic transglycosylation [42], could be implemented to overcome this limitation. However, among these strategies, the use of CD is not favoured due to its poor water solubility, potential formation of crystalline complexes, and nephrotoxicity associated with parenteral administration [43]. Although chemical synthesis of glycosides is widely used, its drawback entails the protection and selective deprotection steps required to avoid the formation of side reactions [44-46]. On the other hand, enzyme glycosylation presents important advantages. It is an environmentally friendly method that typically does not require heavy metals and toxic components in the reaction. Furthermore, the biocatalytic reaction facilitates complete control over anomeric configuration and high regioselectivity without any protecting groups [47,48]. Therefore, the enzymatic glycosylation strategy has been intensively employed for the synthesis of glycosylated natural products.

In this study, we aimed to investigate and analyze the distribution of ASases in 30 *Deinococcus* species. Among the analyzed ASases, a putative ASase gene derived from *Deinococcus wulumuqiensis* (DWAS) was cloned and expressed in *Escherichia coli*. The enzymatic properties of recombinant DWAS were examined, and its transglycosylation activity was used to modify isovitexin. The product formation of DWAS was compared with those of other ASases derived from other *Deinococcus* species including ASases of *Deinococcus geothermalis* (DGAS) and *Deinococcus radiopugnans* (DRpAS). The expansive reactant and product profiles of *Deinococcus* ASases suggest that the enzyme has a broad acceptor specificity and potential applications for selective use in transglycosylation.

2 Materials and methods

2.1 Bacterial strains and cloning vectors

Genomic DNA of *D. wulumuqiensis* was obtained from the Korea Atomic Energy Research Institute (Jeongeup, Korea). *E. coli* DH10B [F^- *mcrA* Δ (*mrr-hsdRMS-mcrBC*) ϕ 80*lacZ* Δ M15 Δ *lacX74* *recA1* *araD139* Δ (*ara-leu*)7697 *galK* *rpsL*(Str^R) *endA1* *nupG*] and BL21(DE3)pLysS [F^- *ompT* *hsdSB* (rB⁻, mB⁻) *gal* *dcm* (DE3) pLysS (Cam^R)] were selected as strains for DNA manipulation and recombinant protein expression, respectively. The plasmids pGEM-T Easy Vector (Promega Co., Madison, WI, USA) and pET-21a(+) vector (Novagen, Darmstadt, Germany) were used to clone PCR products and construct an expression vector for the *dwas* gene, respectively.

2.2 PCR

Genomic DNA Prep Kit for Bacteria (Solgent, Seoul, Korea) was used to extract genomic DNA that originated from *D. wulumuqiensis*. PCR amplification was performed using the *D. wulumuqiensis* genomic DNA as a template and the following oligonucleotide primers: DWAS-F (5'-CAT ATG CTC ACG CCC GAC C-3') and DWAS-R (5'-CTC GAG AGC CTC GGC CCC C-3'). These primers contained *NdeI* and *XhoI* restriction enzyme sites (underlined) at their 5' ends. PCR amplification was conducted under the following conditions: initial denaturation step for 5 min at 95 °C followed by 25 cycles of 30 s at 95 °C, 30 s at 60 °C, and 2 min at 72 °C for amplification and a final elongation step for 5 min at 72 °C. The amplified *dwas* gene product was purified from agarose gel, and the absence of errors introduced in the PCR procedure was confirmed by DNA sequencing. The purified PCR amplification product was inserted into pGEM-T Easy Vector. Further, the plasmid was digested with *NdeI* and *XhoI*, and the resulting fragment corresponding to the *dwas* gene was subcloned into the pET-21(+) vector previously digested with the same restriction enzymes to obtain the pET-DWAS construct.

2.3 Recombinant protein expression and purification of DWAS

For efficient expression of the *dwas* gene, *E. coli* BL21-CodonPlus(DE3) cells containing pET-DWAS were grown in Luria-Bertani medium supplemented with 0.1 mg/mL ampicillin and 34 mg/mL chloramphenicol.

When the optical density at 600 nm reached 0.5–0.6 as measured using a spectrophotometer (Beckman DU 730; Beckman Coulter, Fullerton, CA, USA), 1 mM isopropyl β -D-thiogalactopyranoside was added to induce *dwas* gene expression. Cells were cultured in 250 mL of Luria-Bertani medium supplemented with ampicillin and chloramphenicol at 18 °C for 18 h. After induction, the cells in the pellet were harvested by centrifugation (Hanip Combi 514R, Hanip Centrifuge Co., Gimpo, Korea) at $7,000 \times g$ for 20 min. The pellet was then resuspended in 15 mL lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, and 10 mM imidazole; pH 7.5), and the cells were subjected to ultrasonication (Sonifier 450, Branson, Danbury, CT, USA; output 4, 6 times for 10 s, constant duty). The crude enzyme was further purified using Ni-NTA affinity chromatography (Ni-NTA Superflow; Qiagen Inc., Valencia, CA, USA). The eluted protein was analyzed by SDS-PAGE on a 10% (w/v) acrylamide gel.

2.4 Determination of protein concentration and enzyme activity

The bicinchoninic acid assay was used for protein quantification. The bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Agawam, MA, USA) was purchased for preparing the working reagent solution, and bovine serum albumin was used as a standard. ASase activity was determined by measuring sucrose hydrolysis activity using 3,5-dinitrosalicylic acid solution. The typical reaction mixture was composed of 50 mM Tris-HCl (pH 9.0), 5 g/L sucrose, and 1 unit of enzyme. One unit of hydrolysis activity was defined as the amount of enzyme that produces 1 μ M of fructose/min under the assay conditions.

2.5 Recombinant DWAS characterization

For characterization, 1 unit/mL of DWAS was used in each reaction. pH-dependent enzymatic activity was examined at 45 °C within pH 4.0–10.0 (50 mM sodium acetate buffer for pH 4.0 and 5.0; 50 mM sodium citrate buffer for pH 5.0 and 6.0; 50 mM Tris-HCl buffer for pH 7.0, 8.0, and 9.0; and 50 mM glycine-NaOH for pH 9.0 and 10.0). The effect of temperature on this activity was verified between 25 °C and 55 °C at pH 9.0 in 50 mM Tris-HCl buffer (pH 9.0). Enzyme thermostability was investigated after pre-incubation of the enzyme at specific temperatures including 45 °C, 50 °C, and 55 °C in 50 mM Tris-HCl buffer (pH 9.0) without a substrate. The residual activities were measured at 45 °C

with 50 g/L sucrose in 50 mM Tris-HCl buffer (pH 9.0) at various time intervals using the 3,5-dinitrosalicylic acid method to quantify the released reducing sugars. Further, protein melting temperature was determined using differential scanning fluorimetry (DSF) as described by Jeong et al. [17].

2.6 Bioconversion of isovitexin to isovitexin glycosides

Three ASases – DGAS, DRpAS and DWAS – were tested. To generate isovitexin glycosides, 1 unit/mL of each ASase was added to 50 mM Tris-HCl buffer (pH 9.0) containing 5% sucrose and 0.025% isovitexin. The reaction was left at 35 °C for 18 h and terminated by heat shock in boiling water for 10 min.

2.7 High-performance anion-exchange chromatography (HPAEC)

HPAEC analysis was conducted using an analytical column for carbohydrate detection and electrochemical detector (ED50, Dionex Co., Sunnyvale, CA, USA). Monosaccharide quantification was performed using a CarboPac PA1 column (0.4 × 25 cm, Dionex Co.) at a flow rate of 1.0 mL/min, whereas oligosaccharide analysis was performed using a CarboPac PA200 column (0.3 × 25 cm, Dionex Co.) at a flow rate of 0.5 mL/min and with an injection volume of 5 µL. Using eluent A (100 mM NaOH in water) and eluent B (100 mM NaOH in 500 mM sodium acetate), the filtered sample was eluted using a linear gradient from 100% eluent A to 60% eluent B.

2.8 High-performance liquid chromatography (HPLC)

The reaction mixture was measured at 360 nm using a ProntoSIL C18 column (120 Å, 5 µm, 4.6 × 250 mm) (Bischoff, Leonberg, Germany) connected to the Shimadzu LC20AD system (Shimadzu, Kyoto, Japan) and SPD-M20A PDA detector. Gradient elution was applied using solvent A (0.1% formic acid) and solvent B (acetonitrile) that had been subjected to filtration and degassing. The flow rate of the mobile phase was 1.0 mL/min with an injection volume of 5 µL.

2.9 Liquid chromatography/mass spectrometry (LC/MS)

The samples were analyzed by Waters alliance 2998 quaternary pump (Waters, Milford, MA, USA) equipped with an autosampler and a ProntoSIL C₁₈ column (120 Å, 5 µm, i.d. 4.6 × 250 mm) (Bischoff), and detection was performed at 360 nm using a photodiode array detector (Waters, Milford, MA, USA). Gradient elution was conducted with 0.1% formic acid in water (solvent A) and acetonitrile (solvent B). The flow rate of the mobile phase was 1.0 mL/min. All solvents were filtered, degassed, and maintained under pressure. An ACQUITY QDa™ Detector (Waters) was used to obtain the MS data. The QDa parameters were as follows: capillary voltage, 0.8 kV; cone voltage, 30 V; source temperature, 600 °C; and desolvation gas flow, 800 L/h. Further, full-scan data acquisition was performed by scanning from *m/z* 100 to 1,200 in the profile mode. Empower 3 (Waters) was used to control the LC-QDa system and analyze the obtained data.

3 Results and discussion

3.1 ASases derived from *Deinococcus* species

Advances in genome-wide research tools have confirmed the existence of ASases in numerous bacterial species. Among bacterial strains, those of the *Deinococcus* genus have gained substantial attention due to its biotechnological potential [6]. They demonstrate strong resistance to ionizing radiation, UV radiation, desiccation, and oxidizing agents. In addition, several *Deinococcus* spp. possess a set of genes required to eliminate toxic components of organic and inorganic cells [50-53]. Recently, Jeong et al. [53] reported a metabolically engineered *D. radiodurans* R1 strain to produce phytoene, a colourless C₄₀ carotenoid, as a promising antioxidant [53]. Therefore, the group of *Deinococcus* species is an attractive candidate for use in industrial biotechnology.

Three ASases derived from *Deinococcus* species including *D. radiodurans* [15], *D. geothermalis* [54], and *D. radiopugnans* [16] have been reported so far. These ASases from the same bacterial genus exhibit different thermostability and acceptor specificity and may differ in terms of their three-dimensional structures. Among these, DGAS has the highest thermostable characteristics with half-life of 26 h at 50 °C, whereas *D. radiodurans* ASase (DRAS) has half-life of 15 h at 30 °C. Recently, the

Figure 1: Phylogenetic analysis of amylosucrases in major *Deinococcus* species.

genomes of 30 different *Deinococcus* strains became accessible, and we examined the presence of ASases in these genomes (data not shown). Among the 30 strains, 28 carry a gene encoding ASase according to their DNA sequence alignment (Fig. 1). Thus, ASase is commonly found in *Deinococcus* strains although its physiological role remains poorly understood. Among *Deinococcus* strains, *D. radiodurans* possesses a unique ASase that shows low stability and presents a different active site conformation compared with other ASases of the genus [55]. Phylogenetic analysis revealed that DWAS is most related to DRAS. The deduced amino acid sequence analysis showed that the homologies between NPAS and ASases from *Deinococcus* (DGAS, DRAS, and DWAS) range from 42.4% to 44.0% (Fig. 2). DRAS and DWAS exhibited 83.6% homology, whereas DGAS and DWAS showed 75.8% homology. Four conserved regions (CR I, CR II, CR III, and CR IV) were identical in three ASases derived from *Deinococcus* (DGAS, DRAS, and DWAS). In addition, the key conserved sequence region (QpDln) of the GH13 subfamily of oligo-1,6-glucosidase was obviously found in DGAS, DRAS, and DWAS [1]. Interestingly, there are

several variations in loop 7, which might be associated with the active and ligand-binding sites (Fig. 2).

Along with the structural differences in ASases derived from *Deinococcus* (DGAS, DRAS, and DWAS), we investigated and compared the catalytic properties of the *dwas* gene expressed in *E. coli* to understand substrate preference of the ASase protein in transglycosylation.

3.2 Expression of the *dwas* gene and characterization of recombinant DWAS

The *dwas* gene encoding ASase was well expressed in *E. coli*. SDS-PAGE analysis revealed that DWAS tagged with six histidine molecules was successfully purified by Ni-NTA affinity chromatography with apparent homogeneity and a molecular mass of approximately 72 kDa (Fig. 3); this value corresponds to the theoretical molecular weight of recombinant DWAS (71.94 kDa). DWAS production in *E. coli* was typically in the range of 750-950 units/L, with the specific activity of DWAS being 9.8 U/mg. Because the specific activities of DGAS, NPAS, and DRAS at 50 °C were

Figure 2: Deduced amino acid sequence-based comparison among NPAS, DGAS, DWAS, and DRAS. Filled triangle represents residues of the catalytic active site (nucleophile, acid/base catalyst) and filled circle represents β -domain.

Figure 3: SDS-PAGE analysis of recombinant DWAS expressed in *E. coli*. M: molecular-weight size marker; lane 1: BL21-CodonPlus(DE3) crude enzyme; lane 2: crude passing through; lane 3: Debris; lane 4: purified enzyme.

Figure 4: Characterization of DWAS enzymatic properties. (A) The optimum pH of DWAS was measured based on sucrose hydrolyzing activity. Measurement was performed in different buffer solutions including sodium acetate pH 4.0 and 5.0 (●), sodium citrate pH 5.0 and 6.0 (○), sodium phosphate pH 6.0, 7.0, and 8.0 (▼), Tris-HCl pH 7.0, 8.0, and 9.0 (△), and glycine-NaOH pH 9.0 and 10.0 (■) at 45°C. (B) The optimum temperature of DWAS in the range of 25–55 °C was investigated in Tris-HCl pH 9.0. (C) The melting temperature of DWAS was measured using DSF. (D) Half-life of DWAS was confirmed by measuring residual activity after incubation at 45 °C (●), 50 °C (□), and 55 °C (▼) in the absence of a substrate.

Figure 5: Stability of DWAS exposed to organic solvent based on concentration. One unit/mL of DWAS was exposed to each organic solvent. DWAS activity is maintained until exposure to DMSO (●) at a concentration of 30%, whereas the activity in other organic solvents, such as EtOH (○), DMF (▲), isopropanol (s), and ACN (■) is significantly reduced at concentrations above 10%.

19, 9.6, and 4 U/mg, respectively [55], the specific activity of DWAS was comparable to them.

The optimal temperature and pH of DWAS for sucrose hydrolyzing activity were determined to be 45 °C and 9.0, respectively. Its melting temperature was calculated to be 56.2 ± 0.2 °C by DSF because the thermostability of DWAS dramatically decreased at 55 °C (Fig. 4). The half-life of DWAS at 45 °C, 50 °C, and 55 °C was 519, 96.7, and 4.7 min, respectively.

The exposure to dimethylsulfoxide at a concentration of up to 30% (v/v) did not reduce DWAS activity, whereas that to other organic solvents (at a concentration of 20%) including ethanol, isopropanol, dimethylformamide, and acetonitrile drastically inhibited DWAS activity (Fig. 5).

To confirm the three different ASase activities of DWAS, sucrose was used as a sole substrate and reaction was conducted at 45 °C for 48 h. The composition of the reaction product was analyzed by HPAEC. The reaction products included glucose, fructose, maltose, turanose, trehalulose, soluble maltooligosaccharides, and insoluble

Figure 6: HPAEC analysis of reactants produced by DWAS using sucrose as a sole substrate. The reaction was performed with 5% sucrose and 1 unit/mL of DWAS in 50 mM Tris-HCl (pH 9.0) at 37 °C for 18 h. (A) Fructose and glucose formation confirmed the hydrolysis activity of DWAS. Similarly, the isomerization activity was confirmed by the formation of the sucrose isomers turanose and trehalulose. (B) Polymers with a DP length of ≥ 7 produced by polymerization activity were identified.

Table 1: Comparison of the characteristics of DWAS and other ASases.

ASase	Length	Similarity (%)	T_{opt}	pH_{opt}	T_m	Ratio of products (%)			Reference
						Hydrolysis	Isomerization	Polymerization (DP<33)	
DWAS	640	100	45	9.0	56.2	9.7	8.6	81.7	This work
DRAS	644	83	30	8.0	-	9.6	33.5	56.9	[15]
DRpAS	651	76	40-45	8.0	50.7	5.8	11.5	82.7	[16]
DGAS	650	75	45-50	8.0	60.8	7.0	22.0	71.0	[54]
NPAS	636	42	37	8.0	49.6	5.4	14.5	80.1	[19]
AMAS	649	40	45	7.0	-	-	-	-	[12]
ACAS	643	44	45	8.0	-	1.9	19.7	78.4	[13]
MFAS	650	39	45	8.5	50.6	9.5	15.0	75.5	[17]
MAAS	651	38	30	8.0	-	-	-	-	[18]
CCAS	644	45	40	7.0	47.8	5.8	10.2	84.0	[14]
<i>Synechococcus</i> sp. AS	661	39	30	6.5-7.0	-	-	-	-	[21]

glucans (Fig. 6). Depending on the activity of DWAS, the reaction products were divided into three groups: hydrolysis (glucose and fructose), isomerization (turanose and trehalulose), and polymerization (maltose, soluble maltooligosaccharides, and insoluble glucans).

The ratio of the reaction products of hydrolysis, isomerization, and polymerization was 9.7:8.6:81.7 (Table 1), indicating that DWAS has a lower isomerization activity than other ASases derived from *Deinococcus* species, but it has a relatively strong hydrolysis activity.

3.3 Bioconversion of isovitexin to isovitexin glycoside by DWAS

Isovitexin was modified by transglycosylation activity of ASases derived from *Deinococcus* species (DGAS, DRpAS and DWAS) and the reaction products were determined. HPLC analysis showed that similar reaction products were generated in different ratios. A total of four major reaction products were observed at retention time (RT) of 22.8, 22.3, 20.8, and 19.9 min in addition to the original isovitexin

Figure 7: HPLC chromatogram of transglycosylated products obtained using isovitexin as a substrate. Under the same conditions, (A) DWAS, (B) DRpAS, and (C) DGAS were reacted with isovitexin and sucrose as acceptors and donors, respectively, and the reaction products were analyzed by HPLC performed at 360 nm. The peak at RT of 19.9 min corresponds to the isovitexin triglucoside anion, those at 20.8 and 22.3 min correspond to isovitexin diglucoside, and that at 22.8 min corresponds to isovitexin monoglucoside. The molecular weight of each isovitexin transglycosylation derivative was confirmed by LC/MS.

peak at RT of 45.0 min. While the peak at RT of 22.8 min was the highest in the DWAS reaction, those at RT of 20.8 and 19.9 min were the main peaks in DRpAS and DGAS reactions, respectively (Fig. 7).

The molecular weights of the isovitexin transglycosylation derivatives were confirmed by LC/MS (Fig. 8). The peak data at the top of the chromatogram were obtained by HPLC using the reactants formed under optimal conditions, in which the bioconversion to isovitexin glucoside was the highest. The molecular masses of four transglycosylated products were determined by the ACQUITY QDa detector. The MS spectra peaks at RT of 22.8, 22.3, 20.8, and 19.9 min corresponded

to the calculated molecular masses of transglycosylated isovitexin derivatives, including the isovitexin substrate. The HPLC peak at RT of 19.9 min corresponded to the calculated molecular mass of the isovitexin triglucoside anion ($[M-H]^-$: 917.80). The HPLC peaks at RT of 20.8 and 22.3 min are in accordance with the characterized values of the isovitexin diglucoside anion ($[M-H]^-$: 755.66). The HPLC peak at RT of 22.8 min was equal to the estimated molecular mass of the isovitexin monoglucoside anion ($[M-H]^-$: 593.52), demonstrating that the compound at the HPLC peak obtained at RT of 22.8 min is isovitexin monoglucoside.

Figure 8: LC/MS analysis of the DWAS reaction mixture. The four major peaks were identified as isovitexin monoglucoside, two isovitexin diglucosides, and isovitexin triglucoside.

Isovitexin is apigenin 6-C-glucoside, in which the glucose residue is covalently attached to C₆ of apigenin. There are four hydroxyl groups on the glucose residue and three hydroxyl groups on the apigenin flavone backbone. Because ASase typically prefers free hydroxyl groups as acceptor residues, there are many possibilities of making transglycosylation products. However, in our experiment, four major transglycosylation products were observed, indicating selectivity for the hydroxyl groups of acceptor molecules. A further study on the structures of isovitexin transglycosylation products would provide clues on the underlying reason for the free hydroxyl group preference in the transglycosylation reaction of ASase.

The active pocket of ASase is structurally formed by the A, B, and B' domains [21,56]. Three-dimensional structural and molecular dynamics simulation analyses have found that loops 3, 4, 7, and 8 play important roles in the formation of the active site topology. In particular, loops 7 and 8 block the oligosaccharide-binding site from the solvent and promote the glucose transfer reaction [57,58]. These structural differences could affect

Table 2: Comparison of loop homology among the ASases present in DGAS, DRAS and DRpAS.^a

ASases	Loop3	Loop4	Loop7	Loop8
DGAS	92.3	95.0	82.4	72.4
DRAS	92.3	100.0	94.1	70.2
DRpAS	92.3	85.0	94.1	74.5

^a Amino acid sequence of DWAS is set at 100% and the relative homology of DGAS and DRpAS is compared with this standard.

transglycosylation activity and acceptor specificity of ASase [4,59]. When the deduced amino acid sequences of loops 3, 4, 7, and 8 were compared among DGAS, DRAS, and DRpAS, loop 8 showed relatively low sequence similarity with those of other ASases. While the sequence homologies of loops 3, 4, and 7 were above 90%, those of loop 8 were less than 75%, suggesting that loop 8 plays an important role in acceptor specificity in the transglycosylation reaction of ASases (Table 2). Because

loop 8 may not be the only factor involved in the variation of the product profiles, structural determination and mutant analysis will elucidate the specific role of loop 8 in ASases in the future.

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