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Modification of Ascorbic Acid Using Transglycosylation Activity of *Bacillus stearothermophilus* Maltogenic Amylase to Enhance Its Oxidative Stability

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Ascorbic acid (**1**), a natural antioxidant, was modified by employing transglycosylation activity of *Bacillus stearothermophilus* maltogenic amylase with maltotriose and acarbose as donor molecules to enhance its oxidative stability. The transglycosylation reaction with maltotriose as donor created mono- and di-glycosyl transfer products with an α-(1,6)-glycosidic linkage. In addition, two acarviosine-glucosyl transfer products were generated when transglycosylation was performed with acarbose as a donor. All transfer products were observed by TLC and HPLC, and purified by Q-sepharose anion exchange and Biogel P-2 gel permeation chromatographies. LC/MS and ^{13}C NMR analyses revealed that the structures of the transfer products were 6-*O*-α-D-glucosyl- (**2**) and 6-*O*-α-D-maltosyl-ascorbic acids (**3**) in the reaction of maltotriose, and 6-*O*-α-acarviosine-D-glucosyl- (**4**) and 2-*O*-α-acarviosine-D-glucosyl ascorbic acids (**5**) in the reaction of acarbose. The stability of the transglycosylated ascorbic acid derivatives was greatly enhanced against oxidation by Cu^{2+} ion and ascorbate oxidase. Among them, compound **3** proved to be the most stable against in vitro oxidation. The antioxidant effects of glycosyl-derivatives of ascorbic acid on the lipid oxidation in cooked chicken breast meat patties indicated that they had antioxidant activities similar to that of ascorbic acid. It is suggested that the transglycosylated ascorbic acids can possibly be applied as effective antioxidants with improved stability in food, cosmetic, and other applications.

KEYWORDS: Acarbose; antioxidant; ascorbic acid; *Bacillus stearothermophilus* maltogenic amylase; maltosyl-ascorbic acid; transglycosylation; lipid oxidation

INTRODUCTION

Ascorbic acid (**1, Figure 1**), a well-known antioxidant, is commonly used in many food systems for maintaining organoleptic quality and protecting other components from oxidation (*1, 2*). It functions as both reducing agent and free radical scavenger by donating either one or two electrons to more oxidized neighboring compounds (*3, 4*). Ascorbic acid is also utilized as a cosmetic ingredient for skin care because of its beneficial role against skin aging, such as promotion of collagen biosynthesis and inhibition of melanogenesis (*5*). However, ascorbic acid easily undergoes oxidation to create 2,3-diketo-L-gulonic acid, a biologically inactive compound, or 2-hydroxyfurfural in conditions such as the presence of heat, transition metals, and ascorbate oxidase (*6, 7*), consequently losing its antioxidant activity. The instability against such oxidative environments hampers its application in food and other industries. To overcome this problem, much research has been performed to generate more stabilized ascorbic acid derivatives by chemical and enzymatic methods. L-Ascorbic acid-6-*O*-palmitate, -2-*O*-sulfate, and -2-*O*-phosphate have been chemically synthesized (*8*). In addition, 2-*O*-α-glucosyl ascorbic acid was enzymatically prepared by the transglycosylation reaction of α-glucosidase (*7*), and 6-*O*-α-galactosyl ascorbic acid was

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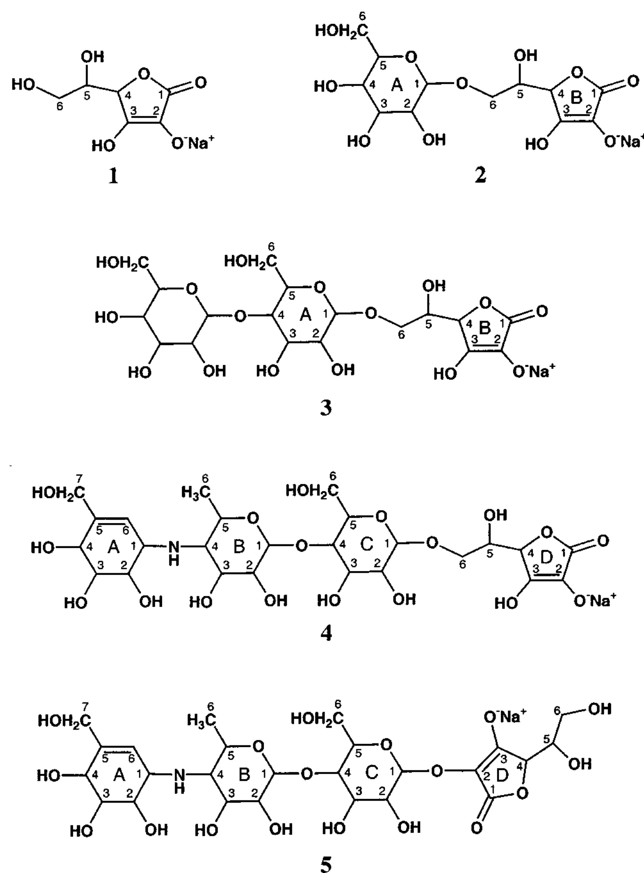


Figure 1. Chemical structures of ascorbic acid and its transglycosylated products. Ascorbic acid (**1**); 6-α-glucosyl ascorbic acid (**2**); 6-α-maltosyl ascorbic acid (**3**); 6-α-acarviosine-glucosyl ascorbic acid (**4**); and 2-α-acarviosine-glucosyl ascorbic acid (**5**).

produced by the transglycosylation reaction of α-galactosidase (*9*). In fact, 2-*O*-glycosyl ascorbic acid derivative showed enhanced stability against oxidative degradation, but lost its reducing capacity (*7*).

Enzymatic transglycosylation has been used to modify natural bioactive compounds to improve their physicochemical properties (*10*−*14*). Recently, we found that *Bacillus stearothermophilus* maltogenic amylase (BSMA) exhibited a strong transglycosylation activity in addition to its hydrolytic activity (*15*). In the presence of various sugar molecules as acceptors, the enzyme can transfer mono- or di-saccharides to the acceptor by forming either α-(1,3)-, α-(1,4)-, or α-(1,6)-glycosidic linkage. The mechanism of transglycosylation was elucidated on the basis of three-dimensional structure of the enzyme (*16*). Notably, BSMA can hydrolyze acarbose, a potent pseudotetrasaccharide inhibitor of several carbohydrases such as α-glucosidase, α-amylase, and CGTase. Furthermore, it can not only cleave the first glycosidic linkage of acarbose, but also transfer acarviosine-glucose, a hydrolysis product, to various carbohydrate acceptors. It was also reported that BSMA had versatile acceptor specificity for glycosides (*12, 13*). This enzymatic property has been applied to create possible potent inhibitors against glycosidase (*17*).

In the present study, ascorbic acid has been modified by the transglycosylation activity of BSMA with maltotriose or acarbose as donor molecule. The presence of transfer products was confirmed by TLC and HPLC, and their molecular structures were determined by LC/MS and ^{13}C NMR analyses. Finally, the physicochemical properties of the resulting transglycosylated products against various oxidative conditions were studied.

MATERIALS AND METHODS

Materials. *Bacillus stearothermophilus* maltogenic amylase (BSMA) was prepared as reported previously (*15*). Ascorbic acid, maltotriose, and baker's yeast α-glucosidase were purchased from Sigma Chemical Co. (St. Louis, MO). Acarbose was kindly supplied by Bayer Korea (Seoul, Korea). All other chemicals used were of reagent grade.

Enzyme Assay. The activity of BSMA was assayed with 0.5% (w/v) β-cyclodextrin in 50 mM sodium citrate buffer (pH 6.0) at 55 °C using the 3,5-dinitrosalicylic acid method (*15*). Assay of α-glucosidase activity was carried out with 1% (w/v) maltose in 50 mM sodium acetate (pH 6.0) at 37 °C by the glucose oxidase/peroxidase method (*17*).

Transglycosylation of Ascorbic Acid by BSMA. The transglycosylation reaction was performed with 10% (w/v) maltotriose or acarbose as a donor and 60% (w/v) ascorbic acid as an acceptor in 25 mM sodium citrate buffer at pH 6.0. BSMA (0.2 U per mg of maltotriose; specific activity, 53.6 U/mg) or acarbose (1.0 U per mg; specific activity, 12.2 U/mg) was added into the reaction mixture. The reaction was carried out for 48 h at 55 °C in the dark and stopped by boiling for 5 min. After centrifugation at 6000*g* for 10 min, the supernatant was subjected to ultrafiltration using a Millipore Ultrafree (Millipore Co., Bedford, MA). The filtrate was applied to a Q-Sepharose anion exchange column (30 × 6 cm) equilibrated with 10 mM NaOH solution, and then eluted with a linear NaCl gradient (0−1.0 M) at a flow rate of 2 mL/min. The peak fractions of the transfer products were concentrated and loaded onto a Bio-Gel P-2 column (100 × 1.6 cm), followed by the elution with distilled water at a flow rate of 0.2 mL/min. After the presence of purified transfer compounds was confirmed by TLC, the products were lyophilized for further experiments. Absorption spectra of the transfer products were determined with a UV spectrometer (Ultraspec III Pharmacia LKB, Uppsala, Sweden).

Analysis of Transglycosylation Products by TLC and HPLC. The reaction products were analyzed by TLC on Whatman K6F silica gel plates (Fisher Scientific, Chicago, IL) with *n*-butyl alcohol/acetic acid/water (3:1:1, v/v/v). After irrigating twice, the TLC plate was dried and visualized either by dipping in a solution containing 0.3% (w/v) *N*-(1-naphthyl)-ethylenediamine and 5% (v/v) H_2SO_4 in methanol and heating at 110 °C for 10 min, or by a UV detector (CAMAG Reprostar 3, Muttenz, Switzerland) at 254 nm. HPLC analysis of the transfer products was carried out with a 150 × 3.9 mm i.d. Nova-Pak C_{18} reverse-phase analytical column (Waters, Milford, MA), using an isocratic solvent system at 0.7 mL/min of 0.1 M phosphate buffer (pH 2.0) as a mobile phase with UV detection (SLC 200, Samsung, Seoul, Korea) at 265 nm.

Liquid Chromatography/Mass Spectrometry (LC/MS). LC/MS analysis was performed using a JEOL LCmate mass spectrometer (JEOL Co., Tokyo, Japan) with atmospheric pressure chemical ionization (APCI) interface. Each 5 μL of the sample with the concentration of 100 μg/mL was directly injected to the instrument.

^1H and ^{13}C NMR Analysis. The ^{13}C NMR spectra of the transfer products were recorded with a JNM LA-400 FT-NMR spectrometer (JEOL) with heteronuclear multiple bond connectivity (HMBC) mode. The sample was dissolved in DMSO-d_6 at 24.9 °C with tetramethylsilane (TMS) as an internal reference.

Stability against Oxidation. The purified transfer products were dissolved (10 mM) in the solution containing 10 μM copper sulfate (*7*). The decrease in absorbance was monitored with a UV spectrophotometer at 265 nm (260 nm for **5**) and 25 °C. Oxidative degradation by ascorbate oxidase was investigated in 25 mM acetate buffer (pH 5.4) containing 100 mU of ascorbate oxidase (Sigma). The sample (100 mM) was dissolved and the decrease in absorbance was determined at 265 nm and 25 °C.

Determination of Radical Scavenging Activity. The reducing activities of the transfer products were determined using a stable radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH). Each 20 μL of the samples was added to 980 μL of 100 mM DPPH solution in ethanol, and the absorbance at 517 nm was measured after 20 min. As a control reaction, 20 μL of deionized distilled water was used instead. The difference in absorbance between the control and the sample was considered the radical scavenging (antioxidant) activity of the sample (*7*).

Determination of the Antioxidant Activity on Lipid Oxidation in Cooked Chicken Breast Meat. Hand-deboned and skinless breast meat (2 kg) was prepared from four chickens raised in the Poultry Research Farm at Iowa State University (*18*). The breast meat was ground twice through a 3-mm plate and used to make meat patties (30 g/patty). Four sets of patty samples with four replications were prepared by adding the solution of ascorbic acid (**1**) or glycosyl derivatives (**2** and **3**) of ascorbic acid to the ground meat (15%/patty, v/w). A patty made with only deionized distilled water was used as a control. The patties were cooked in a water bath of 80 °C for 15 min. Immediately after cooking, the patties were individually vacuum-packaged in oxygen-impermeable nylon/polyethylene bags and then stored at 4 °C. Thiobarbituric acid-reactive substances (TBARS) in the cooked meat patties, after exposing them to air, were determined every 6 h up to 18 h at 15 °C. Lipid peroxidation was determined by the modified method of Buege and Aust (*18*, *19*). Each sample of 1 g meat was placed in a 50-mL test tube and homogenized with 5 mL of deionized distilled water by using a homogenizer (type PT 10/35, Brinkman Instruments Inc., Westbury, NY) for 15 s at speed 7−8. Meat homogenate (1 mL) was transferred to a disposable test tube (10 × 13 mm) containing a butylated hydroxyanisole (50 μL, 7.2%) and thiobarbituric acid/trichloroacetic acid (TBA/TCA) solution (2 mL). The mixture was vortexed and then incubated in a boiling water bath for 15 min to develop color. After color development, the samples were cooled in cold water for 10 min and centrifuged for 15 min at 2000*g*. The absorbance of the supernatant in each sample was determined at 531 nm. The TBARS number was expressed as milligrams of malondialdehyde (MDA) per kilogram of meat (mg MDA/kg).

RESULTS AND DISCUSSION

Transglycosylation of Ascorbic Acid by BSMA. Transglycosylation activity of BSMA has been used to modify various natural chemicals, such as naringin, neohesperidin, and simmondsin, to change their physicochemical properties (*12*−*14*). Accordingly, transglycosylation activity of BSMA was employed to improve the stability of ascorbic acid in oxidative environments in this study. Maltotriose (or acarbose) and ascorbic acid were utilized as donor and acceptor molecules, respectively, in the BSMA transglycosylation reaction. In the reaction of maltotriose as a donor, two major transfer products were observed by TLC analysis, as shown in **Figure 2** (designated as **2** and **3** in lane 3 of **panel A**). Similarly, two transfer products (**4** and **5**) also appeared when acarbose was used as a donor (**Figure 2**, lane 3 of **panel B**). For the structural determination, transfer products of maltotriose or acarbose were purified from the reaction mixture using an anion exchange chromatography. After washing the column with distilled water to remove nonadsorbed carbohydrates, transfer products and ascorbic acid bound were eluted with 0.3 M NaCl. Although transfer products were eluted faster than ascorbic acid, they were not clearly separated from each other. Hence, each transfer product was further purified using Bio-Gel P-2 gel chromatography and its purity was determined by TLC and HPLC analyses (**Figures 2** and **3**). Finally, each of the transfer products were quantified as ascorbic acid equivalent using an ascorbic acid standard curve by spectrophotometer. The extinction coefficients of compounds, **2**, **3**, **4**, and **5** at 260 nm are 7700, 6750, 8720, and 10900 $M^{-1}\cdot cm^{-1}$, respectively, whereas at 265 nm they are 8160, 6920, 9180, and 9870 $M^{-1}\cdot cm^{-1}$. It was found that the yield of the reaction depends on various factors such as the concentrations of donor, acceptor, and enzyme. Typical yield of the tranglycosylation reaction was in the range of 30−50% on the basis of the concentration of donor molecule. Normally, transglycosylation reaction performed with acarbose as a donor molecule showed rather higher yield (50%) than that with maltotriose (30%).

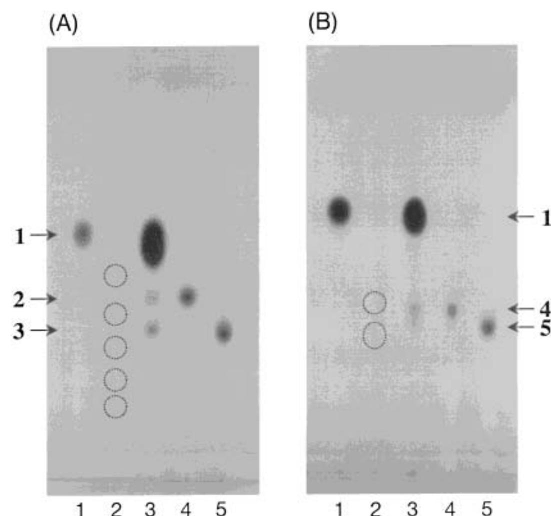


Figure 2. TLC analysis of transglycosylated products of ascorbic acid: (A) maltotriose; (B) acarbose as a donor, respectively. (A) lane 1, ascorbic acid (**1**); lane 2, maltodextrin standards of glucose, maltose, maltotriose, maltotetraose, and maltopentaose (from top to bottom) whose positions were visualized by sulfuric acid; lane 3, reaction mixture; lane 4, purified 6-α-glucosyl ascorbic acid (**2**); lane 5, purified 6-α-maltosyl ascorbic acid (**3**); (B) lane 1, ascorbic acid (**1**); lane 2, standard mixture of acarviosine-glucose (top) and acarbose (bottom); lane 3, reaction mixture; lane 4, purified 6-α-acarviosine-glucosyl ascorbic acid (**4**); lane 5, purified 2-α-acarviosine-glucosyl ascorbic acid (**5**). The TLC plates were visualized with UV detection at 254 nm. Locations of maltodextrins, (lane 2, A) acarviosine-glucose, and acarbose (lane 2, B), are designated as dotted circles because they are not visualized by UV, but only by chemical methods.

BSMA not only hydrolyzes various carbohydrates such as starch, cyclomaltodextrins, and pullulan, to produce maltose, glucose, and panose, but also transfers mono- or di-saccharides created from carbohydrate donors to numerous acceptors such as D-glucose, maltose, cellobiose, lactose, and sugar alcohols (*15*). Strikingly, when acarbose, a potent amylase inhibitor, is used as a donor, BSMA cleaves its first glycosidic linkage and transfers the resulting acarviosine-glucose to acceptors (*15*). When naringin and simmondsin were employed as acceptors, the transglycosylation reaction by BSMA produced the transfer products of naringin and simmondsin with α-(1,3)-, α-(1,4)-, and α-(1,6)-glycosidic linkages (*12, 13*). Therefore, it is presumed that the transfer products of ascorbic acid observed in the present study were most likely ascorbic acid glycosides or ascorbic acid acarviosine-glucosides.

Structure of the Transfer Products. To determine the structure of purified transfer products, LC/MS and ^{13}C NMR analyses were performed. LC/MS analysis revealed that the molecular weight of **2** was 360 Da as two peaks appeared at m/z 361 ($[M + H]^+$) and m/z 383 ($[M + Na]^+$), corresponding to the calculated molecular masses of hydrogen and sodium adducts of glucosyl ascorbic acid, respectively. Likewise, the molecular weight of **3** was calculated as 522 Da from two peaks of m/z 523 ($[M + H]^+$) and m/z 545 ($[M + Na]^+$), matching the expected molecular masses of hydrogen and sodium adducts of maltosyl ascorbic acid. Interestingly, both acarbose transfer products exhibited the same peaks at m/z 642 ($[M + H]^+$) and m/z 664 ($[M + Na]^+$), being equivalent to the calculated molecular masses of hydrogen and sodium adducts of acarviosine-glucosyl ascorbic acid, respectively. Thus, the molecular weight of two transfer products, **4** and **5**, was determined as 643 Da. Hence, these results implied that two transfer products,

Table 1. ^{13}C NMR Signals (Chemical Shifts) of 6-α-Glucosyl Ascorbic Acid (**2**) and 6-α-Maltosyl Ascorbic Acid (**3**) Purified from the Reaction with Maltotriose (units: ppm)

		glucose	ascorbic acid	2	3	Δδ (2)	Δδ (3)
ring A	1	100.550		99.428	99.214	1.122	1.336
	2	72.650		72.842	72.608	0.192	0.042
	3	73.913		73.913	73.718	0.015	0.195
	4	70.375		70.375	70.214	0.094	0.161
	5	72.328		72.328	72.266	0.021	0.062
	6	61.476		61.476	61.332	0.049	0.144
ring B	1		178.384	177.783	177.676	0.601	0.708
	2		113.929	114.583	114.677	0.654	0.748
	3		176.636	173.748	173.234	2.888	3.402
	4		79.264	78.984	78.889	0.280	0.375
	5		70.440	68.634	68.560	1.806	1.880
	6		**63.426**	**69.169**	**69.181**	**5.743**	**5.755**

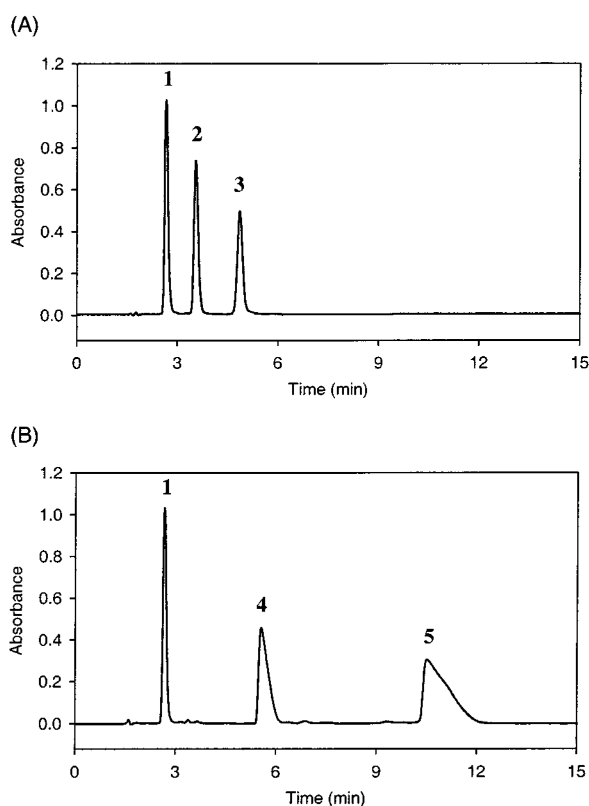


Figure 3. HPLC analysis of transglycosylated products of ascorbic acid: (A) with maltotriose; (B) with acarbose. A Nova-Pak C_{18} reverse-phase column was used with 0.1 M phosphate buffer at a flow rate of 0.7 mL/min. The absorbance was measured at 265 nm. Ascorbic acid (**1**); 6-α-glucosyl ascorbic acid (**2**); 6-α-maltosyl ascorbic acid (**3**); 6-α-acarviosine-glucosyl ascorbic acid (**4**); and 2-α-acarviosine-glucosyl ascorbic acid (**5**).

2 and **3**, produced by BSMA with maltotriose as a donor were glucosyl- and maltosyl-ascorbic acid, respectively, whereas two transfer products, **4** and **5**, created from acarbose were acarviosine-glucosyl ascorbic acids.

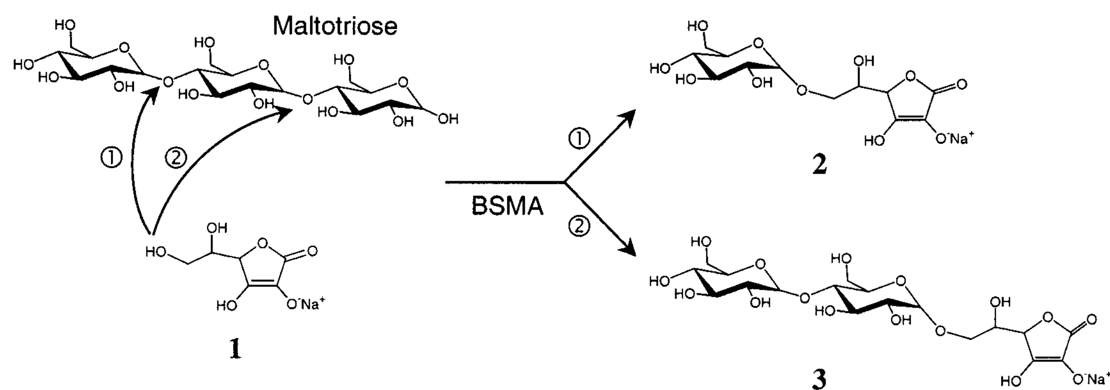
^{1}H and ^{13}C NMR analyses were carried out to determine the glycosidic linkages between glucosyl, maltosyl, or acarviosine-glucosyl units and ascorbic acid. Chemical shifts of **2** and **3** were compared with those of authentic ascorbic acid in ^{13}C NMR spectra, as shown in **Table 1**. Additional carbon signals were observed in ^{13}C NMR spectra of both **2** and **3**. Unquestionably, those signals resulted from the transfer of glucose or maltose moiety to ascorbic acid. In addition, there was a large chemical shift of C-6 in ascorbic acid from 63.426 to 69.169 and 69.181 for **2** and **3**, respectively, confirming that the transferred glucosyl or maltosyl group was attached to C-6 of ascorbic acid. Combining these results together, the two transglycosylation products, **2** and **3**, were defined as α-(1,6)-glucosyl ascorbic acid and α-(1,6)-maltosyl ascorbic acid, respectively.

The chemical shifts of **4** and **5** were compared with those of authentic ascorbic acid and acarviosine-glucose in ^{13}C NMR spectra. As shown in **Table 2**, there was a large chemical shift of C-6 in ascorbic acid from 63.426 to 69.107, and C-1 in glucose moiety of acarviosine-glucose from 92.731 and 96.610 to 99.186 for **4**, indicating that the transferred acarviosine-glucose was linked to C-6 of ascorbic acid in **4**. From this result, the compound **4** was defined as α-(1,6)-acarviosine-glucosyl ascorbic acid. Unlike the compound **4**, the chemical shift of ascorbic acid moiety of **5** was similar to those of authentic ascorbic acid, whereas the significant chemical shift of C-1 in glucose moiety of acarviosine-glucose was observed as in **4**. It was reported that the chemical shift of the ascorbic acid moiety of α-(1,2)-glucosyl ascorbic acid was not meaningfully changed from that of ascorbic acid in the ^{13}C NMR spectrum (*7*). Also the wavelength of maximum absorbance was 260 nm for the α-(1,2)-linkage product, whereas it was 265 nm for the α-(1,6)-linkage product of ascorbic acid (*7*). In our experiment the compounds **4** and **5** exhibited their maximum absorbances at 265 and 260 nm, respectively. Judging from these results, the structure of **5** was presumed to be α-(1,2)-acarviosine-glucosyl ascorbic acid.

Previously, the transglycosylation activity of BSMA was employed to modify various compounds such as naringin, simmondsin, and neohesperidin to improve their physicochemical properties (*12*−*14*). It was found that the resulting transglycosylation products were mono- and di-glycosyl derivatives, in which the transferred glycosyl unit was attached to the glucose moiety of the acceptor compounds at C-6 rather than at C-3 or C-4. Kim et al. (*20*) determined the hydrolysis and transglycosylation activities of maltogenic amylase from *Thermus* sp. It was found that the α-(1,4)-linked transglycosylation product was rapidly hydrolyzed and almost disappeared although it was predominant at the beginning of the reaction. On the contrary, α-(1,6)-linked transglycosylation product increased and steadily maintained during the reaction. From this result, it can be assumed that α-(1,4)-linked transglycosylation product is a kinetically controlled compound, whereas α-(1,6)-linked transglycosylation product is a thermodynamically controlled compound. Likewise, the transferred moieties (glucose, maltose, or acarviosine-glucose) in this study were linked mainly to C-6 of ascorbic acid, resulting in α-(1,6)-glycosidic linked products which might be the thermodynamically controlled compounds and accumulated as a main transglycosylation product during

Table 2. ^{13}C NMR Signals (Chemical Shifts) of 6-α-Acarviosine-Glucosyl Ascorbic Acid (**4**) and 2-α-Acarviosine-Glucosyl Ascorbic Acid (**5**) Purified from the Reaction with Acarbose (units: ppm)

		acarviosine glucose	ascorbic acid	**4**	**5**	$\Delta\delta$ (**4**)	$\Delta\delta$ (**5**)
ring A	1	56.845		56.816	57.182	0.029	0.337
	2	72.098		72.110	72.028	0.012	0.070
	3	73.673		73.632	73.315	0.041	0.358
	4	71.950		71.987	71.789	0.037	0.161
	5	124.151		123.114	117.293	1.037	6.858
	6	140.148		141.185	146.014	1.037	5.866
	7	62.410		62.418	62.007	0.008	0.403
ring B	1	100.559, 100.675		100.798	100.477	0.181	0.140
	2	70.835		71.024	70.312	0.189	0.523
	3	73.443, 73.537		73.595	73.315	0.105	0.175
	4	65.631		65.479	64.167	0.152	1.464
	5	70.008		69.535	70.020	0.473	0.012
	6	18.169		18.210	18.202	0.041	0.033
ring C	**1**	**92.731, 96.610**		**99.186**	**99.441**	**4.515**	**4.770**
	2	74.093, 74.817		74.344	74.207	0.111	0.248
	3	75.413, 77.067		-	-	-	-
	4	77.910, 78.165		78.441	77.873	0.403	0.164
	5	71.304		71.386	71.378	0.082	0.074
	6	61.476, 61.588		61.456	61.061	0.076	0.471
ring D	1		178.384	178.293	179.667	0.091	1.283
	2		113.929	114.023	114.706	0.094	0.777
	3		176.636	176.204	177.520	0.432	0.884
	4		79.264	79.185	79.412	0.079	0.148
	5		70.440	68.651	70.312	1.789	0.123
	6		**63.426**	**69.107**	63.295	**5.681**	0.131



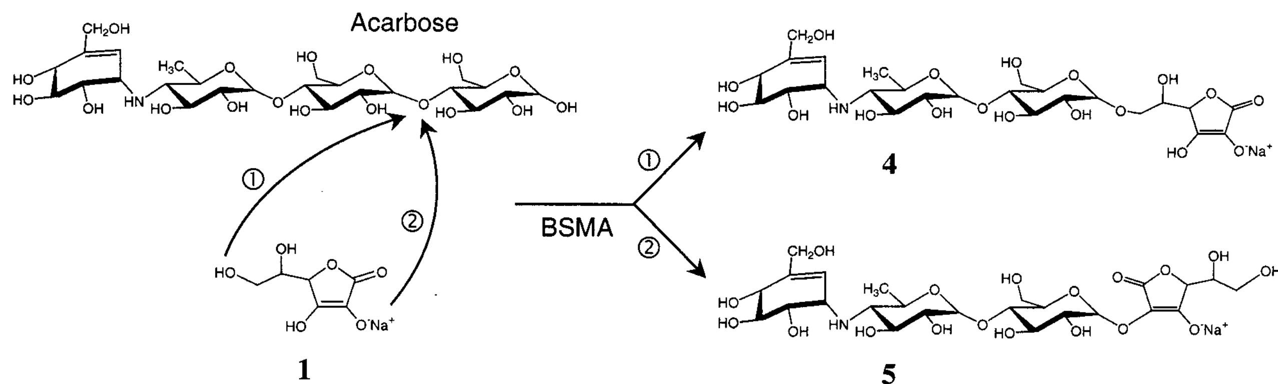


Figure 4. Proposed reaction mode of transglycosylation by *Bacillus stearothermophilus* maltogenic amylase (BSMA): (A) with maltotriose; (B) with acarbose. Ascorbic acid (**1**); 6-α-glucosyl ascorbic acid (**2**); 6-α-maltosyl ascorbic acid (**3**); 6-α-acarviosine-glucosyl ascorbic acid (**4**); and 2-α-acarviosine-glucosyl ascorbic acid (**5**).

the reaction. A proposed BSMA transglycosylation reaction of ascorbic acid is summarized in **Figure 4**. In conclusion, BSMA preferentially creates the transglycosylation products by forming α-(1,6)-glycosidic linkages, which is different from other enzymes such as CGTase which mainly produces α-(1,4) or α-(1,3)-glycosidic linkages (*11*).

Table 3. Relative Scavenging Activity of the Transfer Products with DPPH[a]

compound	absorbance at 517 nm	percent (%) of[b] radical scavenging	relative activity
control	1.09 ± 0.01	0	
ascorbic acid (**1**)	0.71 ± 0.02	34.31 ± 2.48	100.0
6-α-glucosyl ascorbic acid (**2**)	0.71 ± 0.01	34.17 ± 1.99	99.6
6-α-maltosyl ascorbic acid (**3**)	0.71 ± 0.02	34.00 ± 1.32	99.1
6-α-acarviosine-glucosyl ascorbic acid (**4**)	0.79 ± 0.02	32.69 ± 1.98	95.2
2-α-acarviosine-glucosyl ascorbic acid (**5**)	1.04 ± 0.02	5.56 ± 1.46	16.2

[a] DPPH is a stable radical of 1,1-diphenyl-2-picrylhydrazyl. [b] Percent (%) of radical scavenging was calculated by 1000 × (absorbance of control − absorbance of sample)/absorbance of control.

Table 4. Effects of Ascorbic Acid and Glycosyl Derivatives of Ascorbic Acid on Lipid Oxidation of Cooked Chicken Breast Meat Patties during Storage

storage time (hr)	TBARS (mg MDA/kg meat)[a]			
	control	ascorbic acid (**1**)	6-α-glucosyl ascorbic acid (**2**)	6-α-maltosyl ascorbic acid (**3**)
0	0.973 ± 0.022[b]	0.521 ± 0.093	0.694 ± 0.069	0.712 ± 0.004
6	2.834 ± 0.067	0.831 ± 0.044	1.083 ± 0.191	0.986 ± 0.022
12	4.001 ± 0.240	1.122 ± 0.032	1.444 ± 0.045	1.339 ± 0.047
18	5.542 ± 0.124	2.021 ± 0.055	2.667 ± 0.037	2.376 ± 0.078

[a] Thiobarbituric acid-reactive substances (TBARS) values expressed as milligrams of malon-dialdehyde (MDA) per kilogram of meat. [b] Standard deviation from four independent experiments.

Antioxidant Activity of the Transfer Products. The antioxidant activity of the transglycosylated products was examined by exposure to oxidation conditions. Generally, free radical scavenging activity can be used to evaluate an antioxidant effect of certain chemicals (*21*). Therefore, the radical scavenging activity of transfer products was determined using a stable free radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH). The DPPH radical is scavenged by the electron donated from the antioxidant, resulting in decolorization and decrease in absorbance. As shown in **Table 3**, the absorption of DPPH significantly decreased by the addition of **2**, **3**, and **4** at the same level as ascorbic acid. However, the change in the absorbance was negligible in the reaction of **5**. Hence, it was inferred that the antioxidant activity of the transfer products with α-1,6-glycosidic linkages was essentially maintained, regardless of their transferred groups.

Antioxidant Activity of the Transfer Products in Cooked Chicken Breast Meat. The antioxidant activities of the glycosyl derivatives (**2** and **3**) were practically evaluated and compared with that of ascorbic acid (**1**) by examining the lipid oxidation in cooked meat of chicken breast. Hydroxyl radicals and other reactive oxygen species can interact with lipids in meat to form lipid hydroperoxides. The resulting lipid hydroperoxides are broken down to generate lipid alkoxyl radicals which consequently initiate and propagate the chain reactions of lipid peroxidation. The extent of lipid peroxidation can be represented as the TBARS number which indicates the amount of malondialdehyde (mg/kg lipid) produced from lipid peroxidation (*18*).

The antioxidant effects of compounds **1**, **2**, and **3** were evaluated as the TBARS values on lipid oxidation in cooked meat of chicken breast (**Table 4**). The result is the average of four determinations. In the control sample (the cooked chicken breast meat without any treatment) the TBARS value of meat samples held at 15 °C dramatically increased during the storage period up to 18 h. On the other hand, the samples containing ascorbic acid or its transglycosylated products (**2** and **3**) exhibit lower TBARS values than that of the control sample (**Table 4**). In cooked meat patties, the development of lipid oxidation was reported to be very rapid after exposure to air. Cooking not only disrupts the membrane structure and consequently destroys the endogenous antioxidant system, but also facilitates the release of iron from carrier or storage proteins in food.

The increments of TBARS per hour were calculated as follows: 0.248 (correlation coefficient, $r^2 = 0.993$) for control, 0.080 ($r^2 = 0.915$) for **1**, 0.104 ($r^2 = 0.902$) for **2**, and 0.0891 ($r^2 = 0.897$) for **3**. The results implied that ascorbic acid and its glycosyl derivatives functioned as antioxidants and suppressed the lipid oxidation in cooked chicken breast meat. Also, it was suggested that the antioxidant effect of ascorbic acid and its glycosyl derivatives on lipid oxidation in the cooked meat were almost at the same level. This result was in agreement with the report that ascorbic acid was effective in preventing lipid peroxidation in plasma and low-density lipoprotein (*22*) and cooked pork (*23*).

Stability of 6-α-Glucosyl and 6-α-Maltosyl Ascorbic Acids against Chemical and Enzymatic Oxidative Degradation. The stability of compounds **2** and **3** against an oxidative environment was measured in the presence of Cu^{2+} ion and ascorbate oxidase. As shown in **Figure 5** (**panel A**), the compound **3** exhibited its stability against Cu^{2+} ion as 70% was maintained even after 24 h in 10 μM of Cu^{2+} ion, whereas ascorbic acid was almost completely degraded by oxidation. Likewise, compound **2** was more stable than ascorbic acid, although its stability was relatively less than that of **3** in the presence of Cu^{2+} ion. This result has been confirmed by examining the susceptibility of transfer products against enzymatic degradation. Similar to the case of Cu^{2+} ion, compound **3** showed much higher stability against enzymatic degradation than **2** and ascorbic acid (**Figure 5**, **panel B**). In fact, only 30% of **3** was degraded when 100 mM of **3** was incubated with 100 mU of ascorbate oxidase for 5 min, but less than 10% and 40% of ascorbic acid and **2** remained, respectively. The oxidative degradation of the transfer products and ascorbic acid by Cu^{2+} ion and ascorbate oxidase was also confirmed on TLC analysis after the oxidation treatments (data not shown). In addition, when compound **3** was digested by α-glucosidase, it was hydrolyzed to **2** and glucose. After the digestion of **3** by α-glucosidase, the stability of the reaction product against Cu^{2+} ion was dramatically decreased as much as that of **2**. This result clearly indicated that the maltosyl moiety of the glycosylated ascorbic acid more ef-

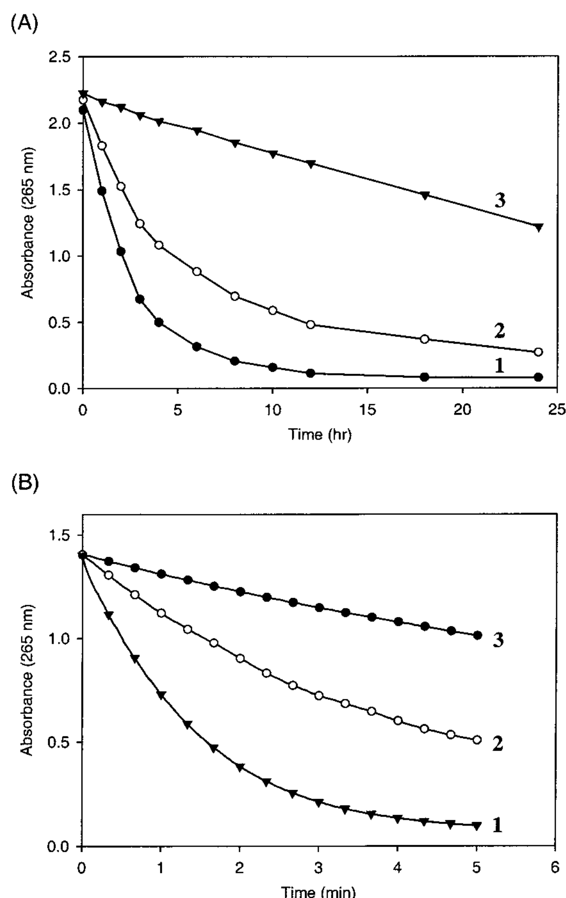


Figure 5. Stability of 6-α-glucosyl ascorbic acid (**2**) and 6-α-maltosyl ascorbic acid (**3**) against oxidation: (A) in the presence of 10 μM Cu^{2+} ion; (B) in the presence of 100 mU ascorbate oxidase. Closed circle (●), ascorbic acid (**1**); open circle (○), **2**; and closed triangle (▼), **3**. Absorbance was monitored at 265 nm.

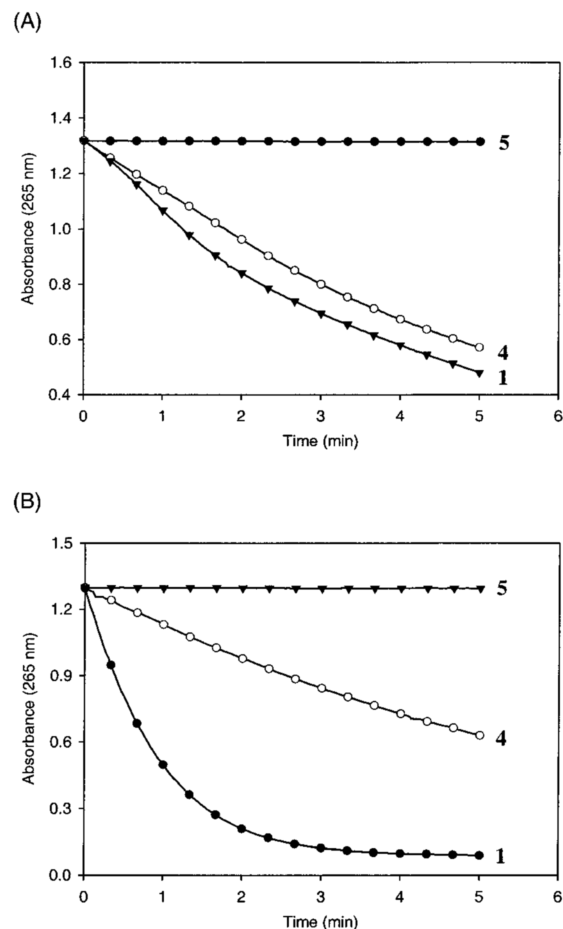


Figure 6. Stability of 6-α-acarviosine-glucosyl ascorbic acid (**4**) and 2-α-acarviosine-glucosyl ascorbic acid (**5**) against oxidation: (A) in the presence of 5 μM Cu^{2+} ion; (B) in the presence of 100 mU ascorbate oxidase. Closed circle (●), ascorbic acid (**1**); open circle (○), **4**; and closed triangle (▼), **5**. Absorbance was monitored at 265 nm for **4** and at 260 nm for **5**.

fectively stabilized ascorbic acid against the oxidation than the glucosyl unit of ascorbic acid. In general, it has been known that transition metal ions, including Cu^{2+} and Fe^{3+}, bind ascorbic acid creating metal ion−ascorbic acid complexes (*3, 24*). As a result of the metal ion−ascorbic acid complex, the oxidation of ascorbic acid is promptly stimulated. However, in the present study, the Cu^{2+}-catalyzed oxidation of ascorbic acid was considerably inhibited by the glycosylation of the C-6 primary alcohol in ascorbic acid. It is most likely that the glycosyl sugar groups attached to ascorbic acid have a Cu^{2+}-binding affinity (*25*) which may be involved in the inhibition on the accessibility of Cu^{2+} to ascorbic acid. Hence, the glycosyl group introduced into C-6 of ascorbic acid was proposed to sterically hinder the access or binding of metal ion to ascorbic acid, in which the maltose unit was suggested to be more effective in the inhibition than the glucose moiety. This structural hindrance by the glycosyl group is assumed to cause the retardation in the Cu^{2+}-catalyzed oxidation of ascorbic acid. Likewise, the glycosyl groups of the transfer products are also considered to bind to Cu^{2+} ion in the catalytic site of ascorbate oxidase, resulting in the slowing of enzymatic oxidation catalysis.

Stability of 6-α-Acarviosine-Glucosyl and 2-α-Acarviosine-Glucosyl Ascorbic Acids against Chemical and Enzymatic Oxidative Degradation. The stability of two acarviosine-glucose-transfer products, **4** and **5**, against oxidative degradation by Cu^{2+} ion and ascorbate oxidase was also compared to that of ascorbic acid (**Figure 6**). Interestingly, compound **5** was remarkably stable against Cu^{2+} ion and ascorbate oxidase. However, compound **4** exhibited marginal stability against Cu^{2+} ion, although its stability against ascorbate oxidase was significant. It has been previously reported that the α-(1,2)-linkage product of ascorbic acid such as 2-α-glucosyl ascorbic acid was stable, but possessed no antioxidant capability (*7*). Thus, it seems that the glycosidic linkage formation between C-2 of ascorbic acid and C-1 of the glucose unit prevents ascorbic acid from losing an electron and forms a stable compound. Recently we reported that acarviosine-glucose and acarviosine-glucosyl simmondsin were potent inhibitors against α-glucosidase and α-amylase (*13, 17*). Similarly, compounds **4** and **5** may possess a potent inhibition capacity against those enzymes. If so, there is a strong possibility that the acarviosine-glucose transfer product might be a powerful compound having a stable antioxidant activity, as well as an inhibition capacity against various amylases. The study of inhibition kinetics of acarviosine-glucose transfer product against amylases is under investigation.

CONCLUSIONS

Ascorbic acid is susceptible to chemical and enzymatic oxidation in solution under aerobic conditions. The susceptibility of ascorbic acid to oxidation limits its usage in the fields of pharmaceuticals, cosmetics, and foods (*2, 26*). Hence, the enhancement of oxidative stability of ascorbic acid is practically beneficial in industrial applications. In fact, ascorbic acid oxidized promptly during the storage of raw milk. The oxidation

of ascorbic acid in raw milk has been induced by enzymes and Cu (II) (*27*). Ascorbic acid in fruits and vegetables is easily exposed to oxidative environments during processing, such as free access of oxygen, transition metal ions, and oxidases excreted in solution. As shown in the prevention of lipid oxidation in cooked chicken breast meat, it is suggested that the various transfer products created by BSMA, particularly 6-α-maltosyl ascorbic acid, could be utilized as effective antioxidants with better stability than ascorbic acid in the food industry and other applications.

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