L-Ascorbic Acid α -Glucoside Formed by Regioselective Transglucosylation with Rat Intestinal and Rice Seed α -Glucosidases: Its Improved Stability and Structure Determination

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The definite structure and chemical stability of a new glucoside of L-ascorbic acid (AA) which was enzymatically glucosylated with rat intestinal and rice seed α -glucosidases were reported. The stability of this AA derivative in water under aerobic conditions was proved by its remarkable resistance against enhanced oxidative degradation by heat, Cu^{2+} ion or ascorbate oxidase, and it was found to have no reducing activity toward radicals. These properties were obviously distinguishable from those of AA. This glucoside was effectively hydrolyzed by α -glucosidases which possessed the ability to synthesize itself, resulting in the liberation of AA activity.

The conjugate was composed of equimoles of AA and glucose. Nuclear magnetic resonance spectra, mass spectra, pH profiles of ultraviolet spectra and pK_a value of 3.10 supported the coupling of α -glucose to the 2-position of AA. From these results, its structure was assigned 2-O- α -D-glucopyranosyl-L-ascorbic acid, being distinct from 6-O- α -D-glucopyranosyl-L-ascorbic acid formed with *Aspergillus niger* α -glucosidase.

These findings indicate that the 2-O-glucoside formed by regioselective transglucosylation withstands oxidative degradation even in aqueous solutions and it can be used as an available active AA source for multicomponent liquid products.

Keywords L-ascorbic acid; L-ascorbic acid 2-glucoside; structural determination; chemical stability; regioselective transglucosylation; enzymatic hydrolysis; α -glucosidase

Introduction

Maintaining the periodical stability of L-ascorbic acid (AA) in multicomponent liquid pharmaceuticals, foods and cosmetics has been a large problem. Because of such a need for chemically stable forms of AA, various chemical methods have been devised for their production.^{1–4)} However, an enzymatic method is generally considered to be more advantageous than a chemical method not only in its simplicity of reaction process but also in its high regioselectivity.

While searching for a naturally-occurring AA glucoside in plants and mammals, we found a unique form of AA conjugate which has been effectively synthesized by enzymatic transglucosylation with rat intestinal^{5,6)} and rice seed α -glucosidases.⁷⁾ So far, the enzymatic glucosylation of AA was reported only by Suzuki *et al.*⁸⁾ by using *Aspergillus niger* α -glucosidase (transglucosidase) and the conjugate formed was very recently identified as 6-O- α -D-glucopyranosyl-L-ascorbic acid (AA-6G).⁹⁾ However, this site-directed glucosylation of AA by *Asp. niger* α -glucosidase has not satisfactorily improved the chemical stability of AA in aqueous solutions. The new glucoside formed with some specific α -glucosidases in the place of *Asp. niger* α -glucosidase showed remarkable stability in an aqueous state and nonreducibility toward cytochrome c and a redox dye,⁵⁾ suggesting its structural difference from AA-6G.

We herein present the structural assignment of 2-O- α -D-glucopyranosyl-L-ascorbic acid (AA-2G) to this new AA glucoside. In addition, we further describe the difference in the stability to oxidative degradation and the liability to enzymatic hydrolysis between AA-2G and AA-6G.

Materials and Methods

Materials AA, its sodium salt (AA·Na) and pyrogallol were purchased from Ishizu Pharmaceutical Co. (Osaka). 2,6-Dichloroindophenol (DCIP) was obtained from Tokyo Kasei Kogyo (Tokyo). Dowex 1 $\times$ 8 resin was purchased from Muromachi Kagaku Kogyo (Tokyo) and Bio-Gel P-2 was from Bio-Rad Labs. (Richmond, CA). *Asp. niger* α -glucosidase was a

product of Amano Pharmaceutical Co. (Osaka). Yeast and rice seed α -glucosidases and L-ascorbic acid 2-O-sulfate dipotassium salt (AAS) were purchased from Sigma Chemical Co. (St. Louis, MO). Almond β -glucosidase and ascorbate oxidase were obtained from Boehringer Mannheim Yamanouchi (Tokyo) and L-ascorbic acid 2-O-phosphate magnesium salt (AAP) was from Wako Pure Chemical Inds. (Osaka). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was a product of Nakarai Chemicals Co. (Kyoto). Maltose and AA-6G were generous gifts from Hayashibara Biochemical Labs. (Okayama). Rat intestinal α -glucosidase (maltase) was purified by the method previously described.⁶⁾ Other reagents used were of analytical grade.

Preparation of New Glucoside The glucoside was synthesized according to the method described previously.^{5,6)} The reaction mixture (20 ml) containing 178 mM AA·Na, 178 mM maltose, 13 mM thiourea, 5 units (maltose hydrolysis) of rat intestinal or rice seed α -glucosidase and 0.1 M acetate buffer (pH 5.3) was incubated for 3 h at 50°C in the dark, and then directly applied to a column (1.6 $\times$ 10 cm) of Dowex 1 $\times$ 8 previously equilibrated with 0.01 M ammonium formate. After washing out nonadsorbed materials thoroughly, AA and its conjugate were subsequently eluted with 0.1 M ammonium formate. The appearance of the glucoside in the eluates was detected by high performance liquid chromatography (HPLC)⁵⁾ including a Shim-pack Octodecyl silica (ODS) column (6 $\times$ 150 mm), where elution was carried out with 0.1 M phosphate buffer (pH 2.0) at a flow rate of 0.7 ml/min and monitored spectrophotometrically at 240 nm. Crude fractions were collected, concentrated with a rotary evaporator and applied to a Bio-Gel P-2 column (1.2 $\times$ 90 cm), which was developed with distilled water. The glucoside was obtained with a purity of 99.6% by HPLC and then lyophilized. In this reaction 14.0% of AA used was converted to its glucoside. $[\alpha]_D^{20} + 189.6^\circ$ ($c = 5.0$, H_2O). Anal. Calcd for $\text{C}_{12}\text{H}_{18}\text{O}_{11}$: C, 42.6; H, 5.36. Found: C, 42.3; H, 5.38.

Spectrophotometric Analysis Ultraviolet (UV) absorption spectra were measured in various acidic solutions on a Shimadzu UV-150-02 double beam spectrophotometer. Field desorption mass spectrometry (FDMS) was taken with a Hitachi M-80B mass spectrometer. Infrared (IR) spectrum was recorded on a JEOL FTIR-40X spectrometer by beam condenser methods. Proton nuclear magnetic resonance (^1H -NMR) spectra (400 MHz) and carbon-13 NMR (^{13}C -NMR) spectra (100 MHz) were recorded on a JEOL JNM-GX400 spectrometer with sodium trimethylsilyl propionate-2,2,3,3- d_4 as an internal standard. The glucoside was dissolved in D_2O and its pH was 2.78.

Assay for Stability of Glucoside Autooxidation of AA in the presence of Cu^{2+} ion was determined in the solution containing 100 μM AA or its derivative, 2.5 to 10 μM copper sulfate and 0.1 M acetate buffer (pH 6.5). The decrease in absorbance at 260 nm for the new glucoside, 254 nm for

AAS, 258 nm for AAP and 265 nm for AA and AA-6G was monitored at 25 °C on a Shimadzu spectrophotometer. Enzymatic degradation of AA by ascorbate oxidase was estimated in the reaction mixture consisting of 100 μ M AA or its derivative, 30 to 120 mU of enzyme and 0.1 M acetate buffer (pH 6.5). The reaction was conducted at 25 °C and the decrease in absorbance at the same wavelength stated above was monitored. The thermal stability of AA was evaluated spectrophotometrically after heating for 30 min at 80 °C in 0.1 M acetate buffer (pH 6.5).

Determination of Reducing Activity The reducing activities of the new glucoside and AA-related compounds were determined by the use of a stable radical, DPPH.¹⁰ Twenty microliters of each sample were added to 980 μ l of 100 μ M DPPH solution in ethanol and the absorbance at 516 nm was measured after 20 min. The difference in absorbance between the sample and the control was taken as the reducing activity. Superoxide radical scavenging activity was estimated according to the method of Marklund and Marklund.¹¹ The compound in 0.05 M Tris-HCl buffer (pH 8.2) was incubated with 50 μ l of 8 mM pyrogallol solution at room temperature and the change in absorbance was monitored at 420 nm. The activity was evaluated by inhibition of the autooxidation of pyrogallol.

Determination of Enzymatic Hydrolysis by Glucosidase To compare the ability of various glycosidases in hydrolyzing the new glucoside, the liberation of AA was measured by means of the decrease in the 522 nm band of DCIP,⁵ because the glucoside used as a substrate showed no reducibility toward DCIP. The reaction mixture contained 27.6 μ M DCIP, 415 μ M of the new glucoside and a sample of enzyme solution in a total volume of 1 ml of 0.02 M acetate buffer (pH 5.3). Incubation was performed at 37 °C and the absorbance was recorded on a Shimadzu spectrophotometer. The AA content released in the reaction mixture was estimated on the basis of the standard curve of AA.

Results and Discussion

Chemical Structure of New AA Glucoside The pH profile of UV spectra of the new AA glucoside exhibited a bathochromic shift (λ_{max} shifts toward longer wavelength) accompanied by a hyperchromic effect upon increasing the pH of the solvent (Fig. 1). Although such profiles resembled those of AA and AA-6G, there was an apparent difference in their absorption maxima. The absorption maxima of this new glucoside were 260 nm ($\epsilon = 12900$) at pH 7.0 and 238 nm ($\epsilon = 8100$) at pH 2.0, both shifting to the lower wavelength by about 5 nm compared to those of AA and AA-6G. Such a shift at both pH was commonly observed in the 2-*O*-substituted AA derivatives.^{4,5,12-14} This hyperchro-

Fig. 1. UV Spectra of AA Glucoside at Various Acidities

The glucoside was prepared in diluted HCl or 0.1 M phosphate buffer (pH 7.0) at a concentration of 50 μ M and each UV spectrum was scanned immediately.

Curve 1, pH 1.13; 2, 1.58; 3, 2.10; 4, 2.57; 5, 3.08; 6, 3.52; 7, 7.00.

mic effect is known to be characteristic only to the 2-*O*-substituted forms, such as 2-*O*-methyl-AA⁴) and AAP and AAS,¹²) whereas the hypochromic effect was observed in 3-*O*-methyl-AA⁴) and 3-*O*-phosphinyl-AA.¹²) These spectrophotometric analyses showed its pK_a to be 3.10 (Fig. 2). AA displays two acidic protons of pK_a values of 4.17 and 11.79 for the 3- and 2-hydroxyls, respectively.¹⁵ Lu *et al.*⁴) and Jernow *et al.*¹²) have already reported that the substitution of the 2-OH of AA enhanced the acidity of the 3-OH to pK_a of 3.11–3.40, whereas the substitution at the 3-position caused a drop in the pK_a value of the 2-OH to 6.22–7.90. Thus, the pK_a value of 3.10 for the new AA glucoside is obviously compatible with the 2-*O*-glucoside structure.

Its structure was further confirmed by measuring FDMS, IR, ¹H-NMR and ¹³C-NMR spectra. The molecular weight was determined to be 338 by FDMS spectra, supporting the conjugate structure of equimoles of AA and glucose. In IR spectra, characteristic absorption bands at 1700 and 1755 cm^{-1} were demonstrated, which corresponded to the double bond at C₂–C₃ and the carbonyl group, respectively.

Fig. 2. Change in Absorbance at Absorption Maxima as a Function of pH

UV absorption of the glucoside at 238 and 260 nm was measured as described in Fig. 1 and each absorbance was plotted as a function of pH.

Fig. 3. ¹³C-NMR Spectrum of AA Glucoside in D₂O Referenced to Internal Sodium Trimethylsilyl Propionate-2,2,3,3-*d*₄ at 100 MHz

The absorption band at about 1700 cm^{-1} has been shown by Cabral and Haake¹⁶⁾ to be assigned to a Δ^2 stretching bond in various AA derivatives. In ^1H -NMR spectrum of the AA glucoside, proton signals appeared at 3.50 (1H, dd, $J=9.7, 9.5\text{ Hz}$), 3.66 (1H, dd, $J=9.5, 3.4\text{ Hz}$), 3.75 (2H, d, $J=6.4\text{ Hz}$), 3.78 (2H, d, $J=3.0\text{ Hz}$), 3.86 (1H, dd, $J=9.5, 9.5\text{ Hz}$), 4.02 (1H, dt, $J=9.7, 3.0\text{ Hz}$), 4.08 (1H, td, $J=6.4, 1.5\text{ Hz}$), 4.91 (1H, d, $J=1.5\text{ Hz}$) and 5.52 (1H, d, $J=3.4\text{ Hz}$). Particularly, the signals at 3.75 and 4.08 ppm due to the protons on the side chain in AA moiety showed similar chemical shifts and the same splitting patterns with similar coupling constants to those of the standard AA, supporting the absence of glucose moiety at the side chain. Other proton signals could be assigned reasonably to the glucose moiety in comparison with those of the standard α -D-glucose. A doublet signal ($J=3.4\text{ Hz}$) at 5.52 ppm was assigned to the anomeric proton of glucose moiety. This anomeric proton signal showed a downfield shift by 0.03 ppm from that of the standard α -D-glucose and also showed a smaller coupling constant in comparison with that of the standard β -D-glucose ($J=8\text{ Hz}$), indicating the presence of an α -D-glucopyranosyl bond at C_2 or C_3 on the AA moiety. The structure of the AA glucoside was further verified by a ^{13}C -NMR spectrum (Fig. 3). The signals of C-1 (176.9 ppm), C-2 (119.4 ppm), C-3 (171.6 ppm), C-4 (80.2 ppm), C-5 (72.0 ppm) and C-6 (65.2 ppm) in the AA moiety were similar to those of the standard AA monoanion and, moreover, their chemical shifts were close to those of 2-*O*-substituted AA, but not 3-*O*-substituted AA.^{4,17)} The remaining signals observed were assigned to the carbons in glucose moiety: C-1', 101.9 ppm; C-2', 75.6 ppm; C-3', 75.8 ppm; C-4', 72.0 ppm; C-5', 74.2 ppm; C-6', 63.1 ppm. Thus, these results support its formulation

Fig. 4. Proposed Structure of AA Glucoside

Fig. 5. Stability of AA-2G to the Oxidative Degradation in the Presence of Cu^{2+}

The amount of AA (A), AA-6G (B) and AA-2G (C) in the reaction mixture was continuously monitored at 265 nm for AA and AA-6G or 260 nm for AA-2G after mixing the components. The reaction mixture contained 0 (●), 2.5 (○), 5 (△) and $10\text{ }\mu\text{M}$ CuSO_4 (□).

as 2-*O*-glucoside of AA.

Both glucosides synthesized with rat intestinal and rice seed α -glucosidases showed the same spectral profiles, being identical. In addition, these enzymes could not glucosylate the 2-*O*-substituted forms such as AAP and AAS.⁵⁾ From these findings, the new AA glucoside could be herein assigned 2-*O*- α -D-glucopyranosyl-L-ascorbic acid (AA-2G, Fig. 4). The definitive structure, however, should be confirmed by its X-ray crystallographic analysis.

Chemical Stability of AA-2G AA is susceptible to chemical and enzymatic oxidation in water under aerobic conditions. Therefore, the resistance of AA, AA-6G and AA-2G against oxidative degradation in the presence of Cu^{2+} ion was evaluated (Fig. 5). Although AA and AA-6G decomposed spontaneously even at pH 6.5 without the addition of Cu^{2+} ion, their degradation rates were remarkably accelerated by the addition of $2.5\text{ }\mu\text{M}$ Cu^{2+} . AA-6G was somewhat resistant to this oxidative degradation compared to AA. On the contrary, AA-2G was found to be extremely stable irrespective of the presence of Cu^{2+} ion. Similar phenomena were demonstrated in ascorbate oxidase-catalyzed oxidation (Fig. 6). Although AA-6G showed some improved stability to the enzymatic oxidation, AA-2G was completely resistant against it. In addition, AA-2G maintained its spectrophotometric activity completely after heat treatment at 80°C for 30 min, whereas AA and AA-6G were completely degraded by this treatment (data not shown). The same chemical stability was also observed in other 2-*O*-esters, AAP and AAS, indicating that it is a common property of the 2-*O*-substituted forms of AA.

Such stability of AA-2G as described here may provide many advantages for its use in the place of AA. Autooxidation of AA can generate oxygen radicals which bring about various oxidative reactions to reduce the content of biologically available agents¹⁸⁻²⁰⁾ and this autooxidation can be remarkably enhanced in the presence of free transition metal catalysts.²¹⁾ On the other hand, AA is abundant in a variety of plant sources, which concomitantly contain ascorbate oxidase.²²⁾ Thus, the content of AA tends to reduce during storage, processing and cooking. It is, therefore, considered that AA-2G can be used as a biologically available AA source due to its significant stability.

Fig. 6. Stability of AA-2G to the Oxidation by Ascorbate Oxidase

The amount of AA (A), AA-6G (B) and AA-2G (C) in the reaction mixture was continuously measured at 265 nm for AA and AA-6G or 260 nm for AA-2G after adding 0 (●), 30 (○), 60 (△) and 120 mU of ascorbate oxidase.

TABLE I. Effect of Various Glucosidases on AA Release from AA-2G

Enzyme	Enzyme amount ^{a)} (mU/tube)	AA released ^{b)}	
		(nmol/min/ tube)	(pmol/min/ mU)
Rice seed α -glucosidase	8.2	1.76	214
Rat intestinal α -glucosidase	4.2	0.377	89.7
<i>Asp. niger</i> α -glucosidase	70	0.574	8.2
Yeast α -glucosidase	500	0.700	1.4
Almond β -glucosidase	500	0	0

a) Enzyme activity of α - or β -glucosidase was determined toward maltose or cellobiose, respectively, and the amount indicated was added to the reaction mixture containing AA-2G. b) The content of AA released in the reaction mixture was determined on the basis of the reduction of DCIP by AA and expressed in nmol/min/assay tube and pmol/min/mU of α -glucosidase activity.

Reducing Activity of AA-2G The reducing abilities of AA-2G and other AA-related agents were determined by the use of a stable radical DPPH. Although AA-6G exhibited nearly the same reducing potency as AA, AA-2G, as well as AAP and AAS, completely lost their reducing activity (data not shown). However, Kato *et al.*²³⁾ have shown that 2-*O*- and 3-*O*-monoalkylascorbic acid had the same electron-donating potency as AA under similar assay conditions. This discrepancy that the AA derivatives substituted at the same 2-position of AA show a distinct reducibility toward DPPH has remained uncertain. Radical scavenging activity of AA-2G was also determined by the pyrogallol method. As AAP and AAS, AA-2G failed to scavenge the superoxide radicals generated by the autooxidation of pyrogallol at a concentration of 1 mM, whereas AA and AA-6G were both effective at 0.1 mM (data not shown). The present results show that the intact form of AA-2G has no reducing activity toward some radicals, as demonstrated previously by using cytochrome c and a redox dye.⁵⁾

Enzymatic Hydrolysis of AA-2G In our previous paper,⁵⁾ we described that AA-2G formed by rat intestinal α -glucosidase can be effectively hydrolyzed *in vitro* by the same enzyme. We further examined the ability of various glucosidases to hydrolyze AA-2G (Table I). The effective release of AA from AA-2G was observed only in cases where some specific α -glucosidases were employed. The order of hydrolytic activity was rice seed α -glucosidase > rat intestinal α -glucosidase > *Asp. niger* α -glucosidase > yeast α -glucosidase. Since the first two α -glucosidases had the high regioselectivity to form AA-2G and the following two had little or no activity,⁷⁾ the specificity for hydrolysis and synthesis is almost in parallel. The significant difference observed in the hydrolytic activity of α - and β -glucosidases also demonstrates that the structure of glucose in AA-2G is really in the α -configuration. The results obtained suggest the *in vivo* generation of free AA through enzymatic cleavage after the ingestion of AA-2G. In fact, its oral administration to experimental animals significantly increased the serum AA levels and exhibited potent antiscorbutic activity in

guinea pigs.²⁴⁾ We have also obtained a result indicating that AA-2G, as well as AA, is effective in collagen synthesis in human skin fibroblasts, where an α -glucosidase inhibitor, castanospermine, abolishes the AA-2G-induced collagen synthesis.²⁵⁾ This indicates that AA released from AA-2G by α -glucosidase in the cell acts as an active moiety.

In conclusion, the chemical structure of the new AA glucoside which was synthesized by regioselective transglucosylation with rat intestinal and rice seed α -glucosidases was determined to be 2-*O*- α -D-glucopyranosyl-L-ascorbic acid. This glucoside showed satisfactory stability to chemical and enzymatic oxidation and it liberated AA effectively by enzymatic hydrolysis with a limited number of α -glucosidases including mammalian enzyme. This evidence indicates that this new AA glucoside can be made available for *in vivo* AA supplementation and for various multicomponent liquid products such as pharmaceuticals, foods and cosmetics.

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