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NOTE

Identification of L-Ascorbic Acid 2-*O*-α-Glucoside, a Stable Form of Ascorbic Acid, in Kimchi

JUN, HONG-KI*, KYUNG-MI BAE, AND YOUNG-HEE KIM

Department of Microbiology, Pusan National University, Pusan 609-735, Korea

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Abstract A material with the same high performance liquid chromatography (HPLC) retention profile as authentic ascorbic acid 2-*O*-α-glucoside (AA-2G) was detected in kimchi. This material was identified as AA-2G by testing its susceptibility to α-glucosidase hydrolysis, the HPLC profile, and through the elementary analysis. Among several strains of bacteria isolated from fermented kimchi, four strains could produce cyclodextrin glucanotransferase (CGTase) which catalyzes the transglucosylation reaction of ascorbic acid. By using starch as the glycosyl donor, AA-2G was produced as the major product through this reaction.

Key words: L-Ascorbic acid 2-*O*-α-glucoside (AA-2G), cyclodextrin glucano transferase (CGTase), kimchi, transglucosylation

Kimchi is one of the most popular Korean traditional fermented foods made of fermented vegetables. Various microorganisms, especially lactic acid bacteria, are involved in kimchi fermentation and these are probably originated by the raw materials [9]. Numerous physiological and biological factors could affect the growth and sequential fluctuation of the principal microorganisms involved in the fermentation [2]. Nutritionally, kimchi contains a high level of vitamins (ascorbic acid, carotene, and vitamin B-complex), minerals (calcium, iron, potassium, etc.) and dietary fibers [15, 17]. In addition, kimchi is known to have antioxidative, antimutagenic, and anticancer activities [13, 14]. As the process of kimchi preparation is rather complex and requires the involvement of several ingredients, systematic approaches to investigate the entire process of kimchi production is difficult. Nevertheless, several preliminary studies on the kimchi production have been reported [3, 10].

Ascorbic acid (Fig. 1A) is necessary for all living organisms, most importantly as an antioxidant [11] and a cofactor in hydroxylation reactions [5, 16]. It has been proved to be the most effective aqueous phase antioxidant in human blood plasma [4]. However, the instability of ascorbic acid limits its usage in the fields of multicomponent liquid pharmaceuticals, food, and cosmetics. In contrast to ascorbic acid, AA-2G (Fig. 1B) is characterized by its high stability towards thermal and oxidative degradation in aqueous solution and its non-reducibility, therefore it could replace ascorbic acid in several usages [12, 19, 20, 23]. AA-2G is also able to stimulate the collagen synthesis in cultures of human skin fibroblasts [21].

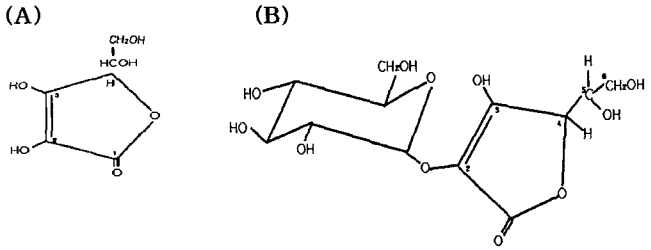

Fig. 1. Structure formula of L-ascorbic acid (A) and L-ascorbic acid 2-*O*-α-glucoside (B).

In this study, we analyzed the levels of ascorbic acid and its derivatives in kimchi samples. For this purpose, several kimchi samples were prepared and processed as follows. Kimjang kimchi (kimchi for the winter) was mainly used throughout this study. After the whole components were ground, the samples were filtered through the sterilized gauze. Aliqouts of kimchi samples were centrifuged (5,000×g, 10 min) and an equal volume of 2% HPO_3 solution was added to the supernatant. When we performed HPLC to analyze the level of ascorbic acid and its derivatives in kimchi, a peak with the same retention time of the standard AA-2G was detected (Fig. 2A). Furthermore, this peak did not disappear even after heating for 30 min in boiling water (Fig. 2B), suggesting the material detected in kimchi could be AA-2G with a high heat stability. The peak was further confirmed by

*Corresponding author
Phone: 82-51-510-2270; Fax: 82-51-513-4532;
E-mail: hgjun@hyowon.cc.pusan.ac.kr

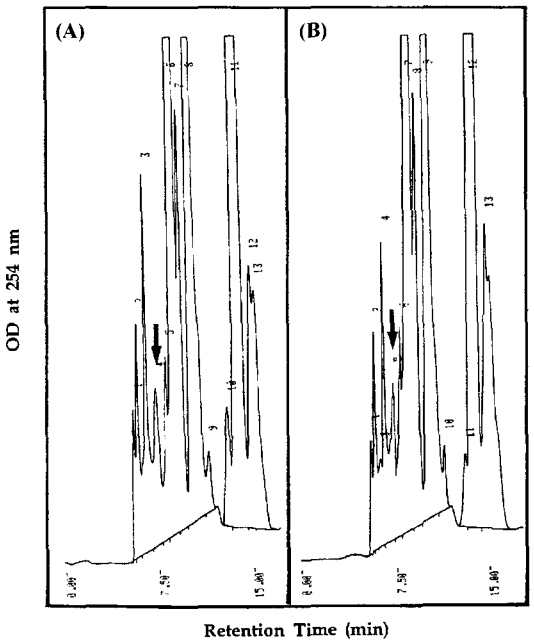

Fig. 2. HPLC chromatogram of L-ascorbic acid 2-*O*-α-glucoside detected in kimchi.

(A) No treatment, (B) Heat treatment (100°C, 30 min). The arrow indicates the elution position of authentic AA-2G. The detection of AA-2G was performed by HPLC, using a model 510 liquid chromatograph (Waters, U.S.A.) with a UV spectrophotometric detector set at 254 nm, and a μBondapak C_{18} column (40×150 mm, i.d., Waters, U.S.A.). The mobile phase was 0.1 M potassium phosphate-0.1 M phosphoric acid (pH 2.0) and the flow rate was 0.5 ml/min.

co-injection analysis of kimchi samples and authentic AA-2G (Fig. 3B).

Whereas ascorbic acid is susceptible to enzymatical oxidation in water under aerobic condition, AA-2G is stable to ascorbate oxidase (ASOD, EC 1.10.3.3)-catalyzed oxidation [22]. Therefore, the resistance of the kimchi sample against ASOD-catalyzed oxidation was evaluated. When kimchi was treated with 5 units of ASOD at 25°C, pH 5.6, for 10 min, it was completely resistant to oxidation as shown in Fig. 3C. Furthermore, as AA-2G can be effectively hydrolyzed *in vitro* by rice seed α-glucosidase [22], the HPLC retention behavior before and after α-glucosidase hydrolysis of kimchi samples was examined. After treatment of kimchi samples with 1 unit of rice seed α-glucosidase treatment in 0.02 M acetate buffer (pH 5.5) at 37°C for 30 min, two peaks corresponding to ascorbic acid and glucose were produced, while the peak corresponding to AA-2G disappeared completely (Fig. 4).

The material obtained from kimchi could be one of the other ascorbic acid derivatives such as ascorbic acid 2-*O*-sulfate (AA-2S), ascorbic acid 2-*O*-phosphate (AA-2P), ascorbic acid 2-*O*-methyl ether (AA-2M), and ascorbic acid 6-*O*-α-glucoside (AA-6G). In particular, as AA-2G and AA-6G have similar characteristics and could be eluted at a similar retention time, it is difficult to distinguish them. However, if considering our results, the material detected in the kimchi sample is AA-2G, because AA-6G is rather unstable against heat treatment

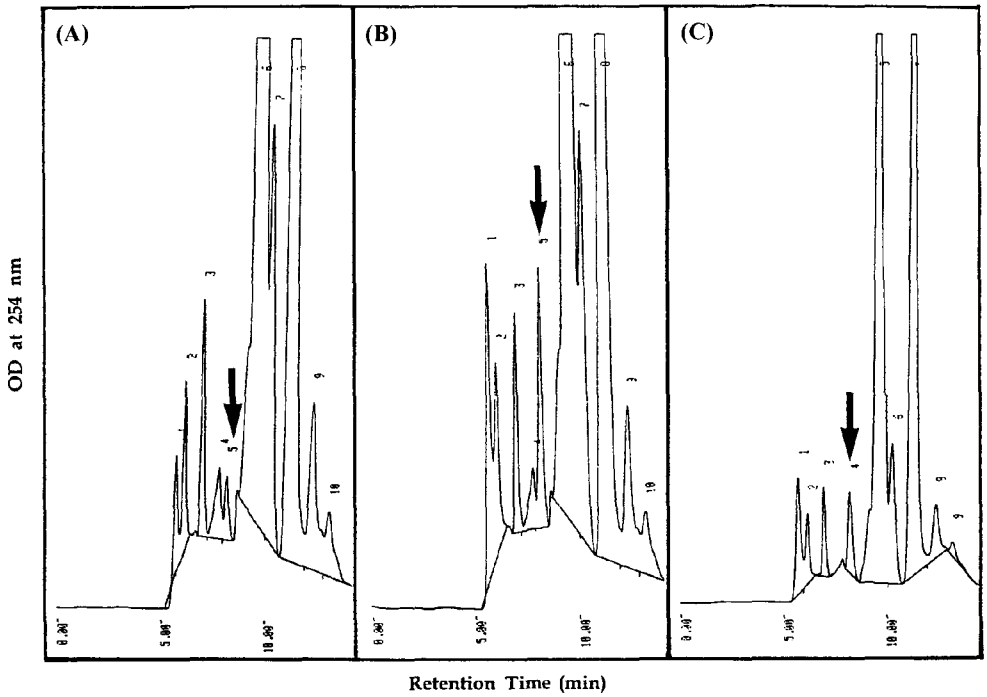

Fig. 3. HPLC profiles of AA-2G detected in kimchi samples.

A 10 μl aliquot of the two-fold diluted kimchi sample was injected alone (A) or co-injected with authentic AA-2G (B). The sample was pretreated with ascorbate oxidase before injection into the column (C). The arrow indicates the elution position of AA-2G.

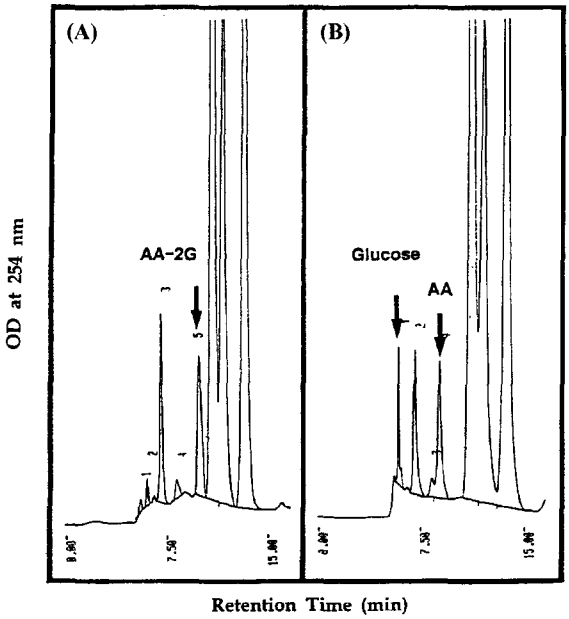

Fig. 4. HPLC profiles of kimchi sample before (A) and after (B) treatment with rice seed α-glucosidase; AA, ascorbic acid.

or ASOD-catalyzed oxidation [20]. The content of AA-2G calculated on the basis of the standard plot peak of area and height was about 6 μg/ml.

Aga *et al.* [1] reported that AA-2G is efficiently synthesized by regioselective transglucosylation with CGTase from *Bacillus stearothermophilus*. CGTase is a unique enzyme that catalyzes the conversion of starch to cyclodextrin by intramolecular transglucosylation [6]. In the presence of a suitable acceptor (such as glucose or sucrose), glycosyl residues are transferred from starch or cyclodextrin to the acceptor to form an α-1,4-glycosidic linkage [8, 18]. Therefore, we attempted to isolate the CGTase-producing bacteria from kimchi samples. CGTase activity was determined by the incorporation of dyes such as congo red and methyl orange into cyclodextrin molecules, thereby producing a hollow area on the plate containing the dyes [7]. For the isolation of microorganisms producing CGTase, the ground kimchi filtrates diluted to 10^6~10^8 folds were plated on the basal medium composed of 1.0% soluble starch, 0.5% polypeptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.8% Na_2CO_3, 1.8% agar, 0.03% congo red, and 0.02% methyl orange. When the plates were cultured at 37°C for 2~3 days, several colonies which formed clear and transparent zones were isolated (Fig. 5).

Among several strains of bacteria isolated, four strains were selected and tested for their ability to produce AA-2G through the transglucosylation reaction. The reaction mixture composed of 1.0% ascorbic acid, 1.0% starch, and bacteria culture supernatant in distilled water (pH 5.5) was incubated at 55°C for 24 hrs. Subsequently, 5 units

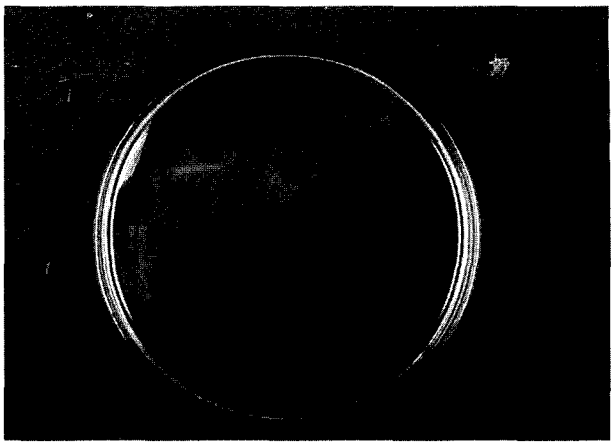

Fig. 5. Formation of clear zones by CGTase-producing strains on the basal medium.

of glucoamylase was added to the reaction mixture and incubated at 55°C for 24 hrs. When the reaction mixtures were analyzed by HPLC, a peak corresponding to AA-2G was detected (Fig. 6) along with the unreacted ascorbic acid. Therefore, the CGTase produced by the isolated strains could catalyze the transglucosylation reaction of ascorbic acid.

This is the first report demonstrating the occurrence of AA-2G in food. Therefore, we could assume that AA-2G is not harmful for humans because Koreans have consumed

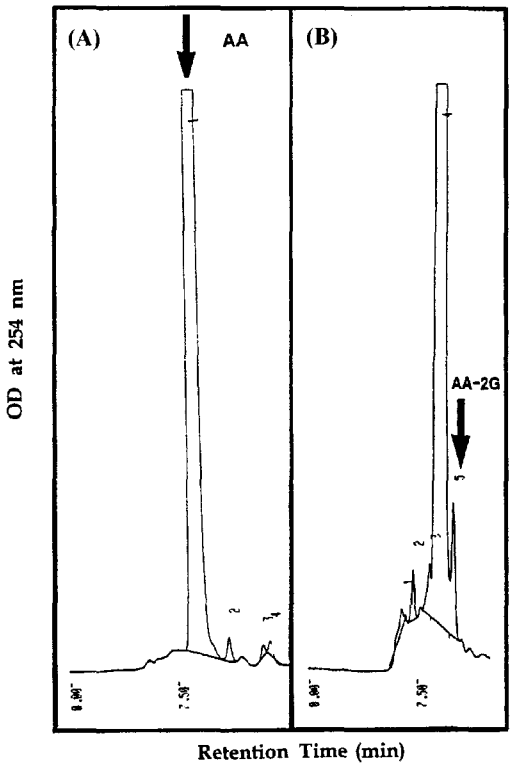

Fig. 6. A reaction mixture either before (A) or after (B) treatment with CGTase and injection with glucoamylase.

kimchi for more than one thousand years without any side effects. Therefore, the AA-2G produced during kimchi fermentation could replace ascorbic acid in many commercial purposes. Also, the CGTase produced by the bacterial strain isolated through this study could be used for the large-scale production of AA-2G.

Acknowledgments

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