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Formation of a L-Ascorbic Acid 2-*O*-α-Glucoside during Kimchi Fermentation

Hong-Ki Jun†, Kyung-Mi Bae, Young-Hee Kim and Hong-Sik Cheigh*

Dept. of Microbiology, Pusan National University, Pusan 609-735, Korea
**Dept. of Food Science and Nutrition, and Kimchi Research Institute, Pusan National University, Pusan 609-735, Korea*

Abstract

Ascorbic acid 2-*O*-α-glucoside (AA-2G) is a chemically stable derivative of ascorbate that shows a vitamin C activity *in vitro* as well as *in vivo*. We studied whether ascorbic acid (AA) and AA-2G are formed in baechu kimchi during fermentation at 4°C or 18°C. To determine the formation of AA and AA-2G during fermentation of kimchi, wheat flour (as a carbohydrate source) added baechu kimchi (WBK) and control baechu kimchi (CBK) were prepared and fermented at 4°C or 18°C. A substance like AA-2G was detected by HPLC from WBK fermented at 18°C for 26 days in fall season and confirmed later to be the AA-2G showing distinctive characteristics of heat stability and resistance to ascorbate oxidase catalase. However, none of the kimchi formed AA-2G when the kimchi were fermented under a different temperature condition such as 4°C instead of 18°C or a different season such as summer instead of fall even if they were fermented at 18°C. The pH of kimchi was decreased rapidly during the first 3 days, and then decreased slowly after 4 days when the kimchi were fermented at 18°C. However, there were slight changes of pH in both CBK and WBK fermented at 4°C for 30 days. Therefore, the AA-2G-forming activity in kimchi seems to be correlated with the fermentation temperature, the microorganisms involved in kimchi fermentation and a suitable glycosyl donor for AA as provided by wheat flour in this study.

Key words: ascorbic acid 2-*O*-α-glucoside (AA-2G; 2-*O*-α-glucopyranosyl L-ascorbic acid), L-ascorbic acid (AA), cyclodextrin glucanotransferase (CGTase), kimchi, transglucosylation

INTRODUCTION

L-Ascorbic acid 2-*O*-α-glucoside (AA-2G) could be synthesized by regioselective transglucosylation by α-glucosidase and cyclodextrin glucanotransferase (CGTase) [EC 2.4.1.19] (1-4). Ascorbic acid (AA) is a very effective antiscorbutic reagent and has multiple other functions in various kinds of cells (5). However, its instability under oxidative conditions limits its usage. On the other hand, derivatives of AA such as AA-2G, L-ascorbic acid 2-*O*-phosphate (AA-2P) and L-ascorbic acid 2-*O*-sulfate (AA-2S) are chemically stable. Among them, AA-2G is easily hydrolyzed to AA and glucose by α-glucosidase (3), and shows biological activities like AA *in vivo* (6) and in tissue cultures (7). AA-2G stimulates collagen synthesis in cultured fibroblasts (8) and enhances antibody production in cultured splenocytes (9).

Kimchi is a fermented vegetable product that has sour, hot, salty, somewhat sweet and carbonated tastes. The fermentation of kimchi occurs mainly by the microorganisms naturally present in ingredients which might contain numerous and various microflora including lactic acid bacteria. The important factors for kimchi fermentation are microorganisms, salt concentration, fermentable carbohydrates, and other available nutrients as well as physical conditions such as oxygen, pH, and temperature. Nutritionally, kimchi has been considered as an important source of vitamins, minerals, dietary fiber and other nutrients (10). Especially, kimchi is known to be a good source of vitamin C and A. In addition, it could help digestion, prevent constipation, control intestinal flora, and it was reported to have anticarcinogenic and other healthful functions (11,12).

In this study, we have investigated by HPLC analysis the various conditions in which AA-2G can be formed during kimchi fermentation. In addition, on the basis of the possibility of the formation of AA-2G via an enzymatic transglucosylation reaction by the microorganisms present in fermented kimchi, we have examined to compare the formation of AA-2G in kimchi fermented in summer and fall seasons.

MATERIALS AND METHOD

Materials

The materials used in this study were obtained as follows : AA from Junsei Chemicals Co. (Japan), AA-2G from professor Sakai, T. at Kinki University, Japan, ascorbate oxidase (ASOD, EC 1.10.3.3) and rice seed α-glucosidase from Sigma Co., Ltd. (St. Louis, MO, USA), galic, ginger, red pepper powder, leek, sugar, green onion and wheat flour from local markets.

†Corresponding author

Preparation of kimchi

Baechu (Chinese cabbage) was throughly washed with tap water, then allowed to be drained. They were brined in 10% salt solution for 7 hours rinsed with fresh water, and then drained again. The recipe used for the preparation of kimchi is shown in Table 1. Two different kimchi, control baechu kimchi (CBK) and wheat flour added baechu kimchi (WBK), were prepared. The final salt concentration of the kimchi was 2.2~2.3%. Kimchi was put into a 1.7 L soy Jar and fermented at two different temperatures, that is, 4°C or 18°C for 30 days. To examine seasonal difference of raw materials in the production of AA-2G, the experiments were carried out in summer (1998. 06. 04 1998. 07. 04) and fall (1997. 11. 05-1997. 12. 05) seasons. The detailed time schedule is present in Table. 2. The kimchi soup was centrifuged at 12,000 rpm for 10 min at 4°C and the same volume of 2% HPO_3 solution was added to the supernatant. All samples were stored at -70°C until analyzed.

Analysis of pH, AA and AA-2G in kimchi

The kimchi samples were analyzed every 2 days during fermentation. The blended kimchi samples were filtered through disposable syringe equipped with sterilizing filter (0.45 μm). pH of the filtrate was determined immediately. AA and AA-2G produced during the kimchi fermentation were determined by HPLC. For the detection of AA and AA-2G, Waters 510 HPLC equipped with Waters UV spectrophotometric detector set at 254 nm and μBondapak C_{18} column (40×150 mm, i.d., Waters) were used. The elution was carried out with 0.1 M potassium phosphate - 0.1 M phosphoric acid (pH 2.0) at a flow rate of 0.5 ml/min.

Enzymatic and heat treatment of AA and AA-2G

Enzymatic degradation of AA and AA-2G isolated from kimchi samples was performed in the reaction mixture containing 20 μl kimchi sample, 5 unit of ASOD and 0.1 M acetate buffer (pH 5.5). The reaction was performed at 25°C for 20 min and the reaction mixture was analyzed by HPLC under the same condition described above. The heat stability of AA and AA-2G isolated from kimchi samples was also evaluated by performing HPLC after heating for 30 min at 100°C in 0.1 M acetate buffer (pH 5.5).

Table 1. Ingredients and their composition of kimchi

Ingredient	CBK[1]	WBK[2]
Chinese cabbage	950 g	950 g
Red pepper powder (dried)	20 g	20 g
Fermented anchovy juice	20 g	20 g
Crushed galic (fresh)	20 g	20 g
Crushed ginger (fresh)	30 g	30 g
Green onion	30 g	30 g
Leek	25 g	25 g
Sugar	15 g	15 g
Wheat flour	-	1/2 cup (35 g)

[1]Control baechu kimchi
[2]Wheat flour added baechu kimchi

Table 2. The detailed time schedule and conditions of kimchi preparation

Fermentation season	Fermentation period	Fermentation temperature
Fall kimchi (1997. 11. 05 - 1997. 12. 05)	30 days	4°C and 18°C
Summer kimchi (1998. 06. 04 1998. 07. 04)	30 days	18°C

RESULTS AND DISCUSSION

The pH of CBK and WBK prepared in fall season and fermented at 18°C decreased markedly until 3 and 5 days of fermentation (Fig. 1). After 5 days, the pH of those kimchi samples decreased gradually up to 20 days of fermentation. No further decrease in pH was seen up to 30 days of fermentation. This result was in accordance with that explained by Mheen et al. (13,14). However, there was no significant pH difference in CBK and WBK fermented at 4°C up to 30 days (Fig. 1).

When kimchi was fermented at 18°C in fall season, the amounts of AA in CBK and WBK increased rapidly during the first 5 days of fermentation, decreased gradually after that and then disappeared completely after 26 days of fermentation (Fig. 2 and 3). This result was in agreement with the previous report demonstrating that the content of AA in kimchi increased during the initial stage of kimchi fermentation, and then decreased afterward (15). The content of AA in CBK was slightly higher than that in WBK. After 25 days of fermentation, a new peak at the same retention time of authentic AA-2G was detected only from WBK fermented at 18°C in fall season (Fig. 3). The highest concentration of AA-2G was observed after 27 days of fermentation. On the contrary, the AA-2G peak was not

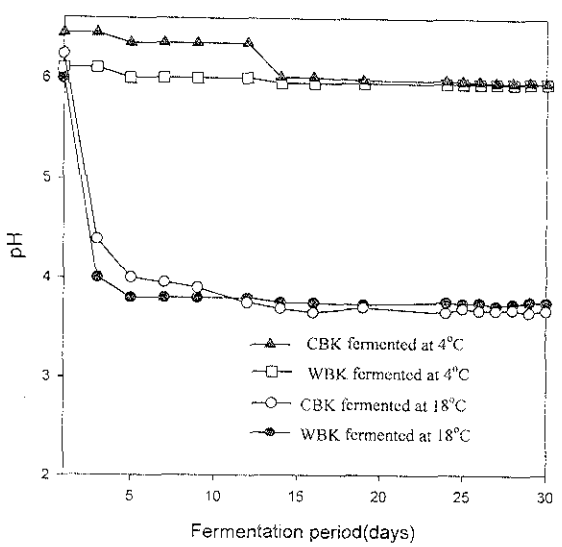

Fig. 1. pH changes during fermentation of CBK and WBK at 4°C and 18°C in fall season. CBK : Control baechu kimchi, WBK : Wheat flour-added baechu kimchi.

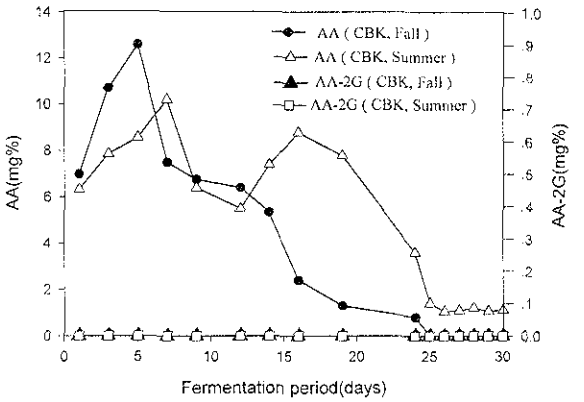

Fig. 2. Changes of AA and AA-2G contents during fermentation of CBK at 18°C in summer and fall seasons. AA: Ascorbic acid, AA-2G: Ascorbic acid 2-*O*-α-glucoside, CBK, WBK: See footnote in Fig. 1.

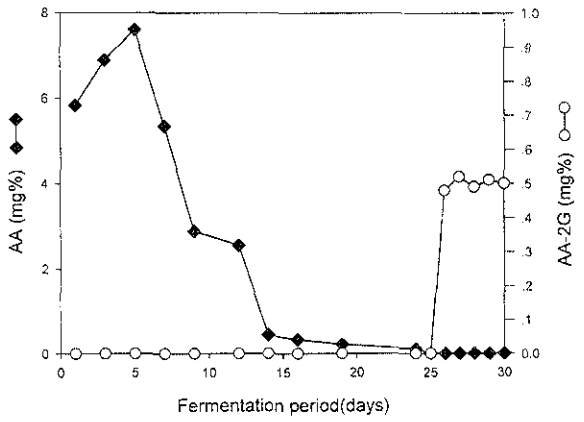

Fig. 3. Changes of AA and AA-2G contents during fermentation of WBK at 18°C in fall season. AA, AA-2G and WBK: See footnote in Fig. 1 and Fig. 2.

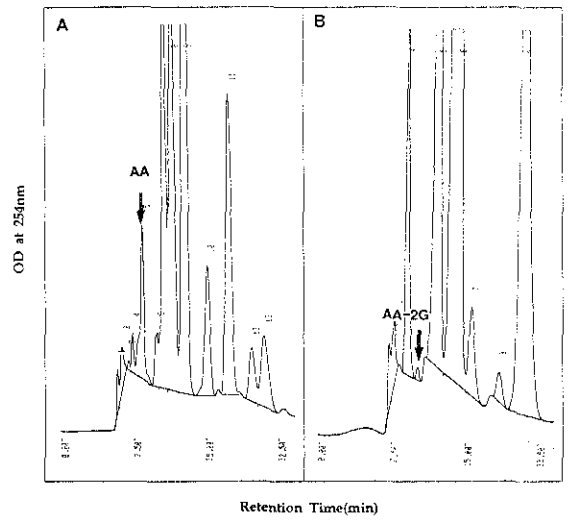

Fig. 4. HPLC profiles of WBK sample prepared before (A) and after (B) AA-2G formation. A 10 ul of filtered WBK sample was injected to the column. (A) and (B) was attained on the fermentation of 1st and 26th day, respectively. AA, AA-2G and WBK: See footnote in Fig. 1 and Fig. 2.

detected in despite of the presence of higher AA amount in CBK (Fig. 2). It suggested that wheat flour presented in WBK may be the glycosyl donor to AA. When the content of AA-2G was examined in CBK and WBK during the fermentation at 4°C for 30 days, AA-2G in both treatments was not detected by HPLC analysis (data not shown).

The HPLC profiles in Fig. 4 demonstrate the presence of a substance like AA-2G in WBK. This could be another type of glycosylated AA such as AA-6G. However, HPLC profile obtained from this experiment was not sufficient to distinguish between them. But if we have an additional data showing apparent differences in their heat stability and their response against ASOD catalyzed oxidation (16-18) then it

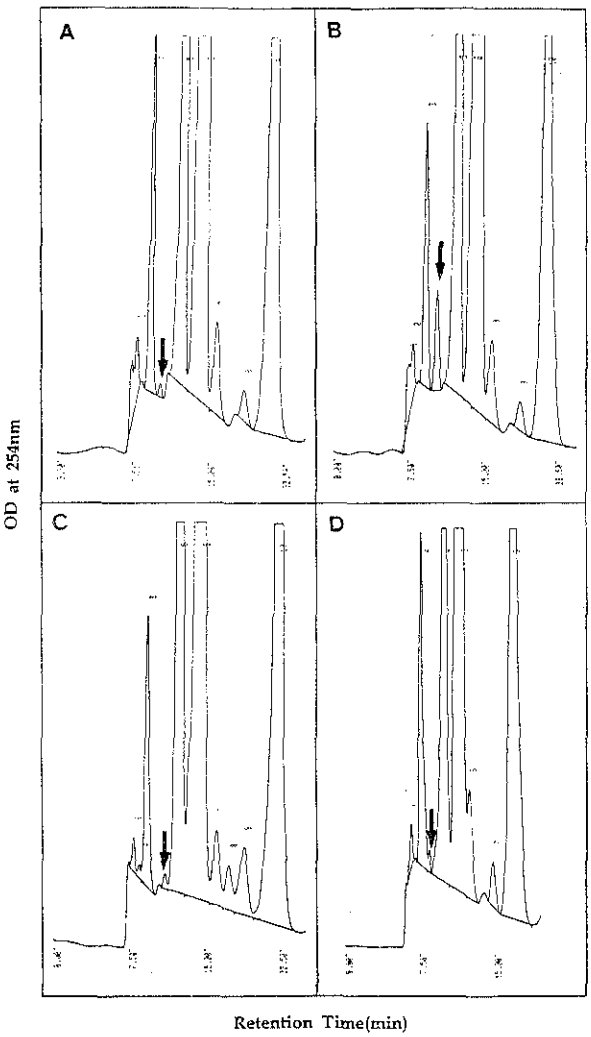

Fig. 5. HPLC chromatogram of co-injection analysis with standard AA-2G and stability of the AA-2G-like substance deteted in WBK sample to the heat and oxidation by ASOD. (A), (B): A 10 ul of filtered WBK sample was injected alone (A) and co-injected with standard AA-2G (B). (C), (D): Stability to the heat and oxidation by ASOD was examined by analysing AA-2G-like substance in HPLC after heat treatment in water (100°C, 30 min), and ASOD (5 unit) treatment in 0.1 M acetate buffer (pH 5.5) at 25°C for 20 min (D). The arrows indicate the elution positions of standard AA-2G. AA-2G and WBK: See footnote in Fig. 1 and Fig. 2.

may be possible to distinguish them. Whereas AA and AA-6G oxidize spontaneously and their degradation can be remarkably accelerated by ASOD treatment, AA-2G is completely resistant against the enzymatic oxidation. The AA-2G-like substance detected in WBK was completely resistant to ASOD treatment (Fig. 5D) indicating that it is the AA-2G.

In addition, it is well known that AA-2G maintains its complete spectrophotometric activity after heat treatment at 80°C for 30 min, whereas AA and AA-6G are degraded by the same treatment (2,4,16,18). The AA-2G-like substance detected in WBK maintained its spectrophotometric activity completely after heat treatment at 100°C for 30 min (Fig. 5C). This chemical stability is the common property of the 2-*O*-substituted forms of AA such as other 2-*O*-esters, AA-2P (19) and AA-2S (20,21). Additional evidence was obtained when co-injected with pure AA-2G (Fig. 5B). Thus, these results support that the substance found in WBK is the AA-2G instead of AA-6G. AA-6G was not detected from any of experimental kimchi samples (data not shown).

Fig. 2 and Fig. 6 show the contents of AA and AA-2G in CBK and WBK fermented in other seasons. Changing pattern of AA in WBK and CBK when fermented at 18°C for 30 days in summer season was different to that of AA in WBK and CBK fermented in fall season. The amount of AA in CBK and WBK was fluctuated as the fermentation progressed. It was increased during the initial fermentation, decreased during 7 to 14 days of fermentation, increased again up to 17 days, and then decreased after that. This result strongly indicates that the production of AA in kimchi could be changed by the seasonal condition. The result of pH changes in kimchi experimented in summer season was similar to that of WBK and CBK fermented in fall season (data not shown). The immediate pH value after preparation was in the range of 6.48~6.45 in both WBK and CBK and decreased as fermentation progressed.

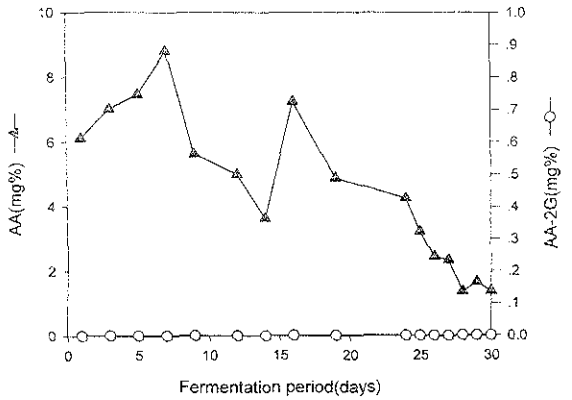

Fig. 6. Changes of AA and AA-2G contents during fermentation of WBK at 18°C in summer season. AA, AA-2G and WBK: See footnote in Fig. 1. and Fig. 2.

AA-2G-like substance detected in WBK fermented at 18°C in fall season was not present in WBK and CBK fermented at 18°C in summer season (Fig. 6). This may be due to the differences in microbial flora species involved in kimchi fermentation. Kimchi fermentation may be initiated by various microorganisms originally presented in the raw materials (22,23). During the fermentation at room temperature, microflora dominate in the order of Gram positive bacteria, yeast, and Gram negative bacteria (24). However, the number and species of the microorganisms involved in kimchi fermentation vary widely, and influenced by the sources of baechu and other ingredients. Since the level of microorganisms in raw materials could be different depending on the season, a great microbial changes during kimchi fermentation could occur.

CGTase which is able to synthesize AA-2G more efficiently than rat and rice α-glucosidase (1) is mainly produced by certain species of *Bacillus*, i.e., *B. macerans*, *B. circulans*, *B. stearothermophilus*, *B. megaterium*, and *B. ohbensis* (25-32). Therefore, it is possible to speculate that AA-2G is produced via enzymatic transglucosylation by CGTase provided by certain species of *Bacillus* sp. in kimchi. According to this study, it could be assumed that numerous and complex biochemical and microbiological changes occur during kimchi fermentation and these are associated with nutritional enhancement of the products. Interestingly, according to this study, it is clear that the addition of wheat flour to kimchi induces the synthesis of AA-2G.

In conclusion, we found that AA-2G could be produced by the microorganisms having enzymatic transglucosylation activity in kimchi prepared in fall season during fermentation at 18°C in the presence of wheat flour. The WBK fermented at 18°C in fall season might provide the most favorable conditions to grow microorganisms producing AA-2G throughout the whole fermentation period. Especially, the presence of AA-2G which is a stable derivative of AA in kimchi made the Korean traditional food to be reevaluated.

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