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Enzymatic Synthesis of Ascorbic Acid Fructoside by Transfructosylation Using Levan Fructotransferase

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Abstract To enhance the stability of ascorbic acid, the glycosylation of ascorbic acid was studied using the transfructosylation activity of levan fructotransferase. When levan was used as glycosyl donor, a novel fructoside (ascorbic acid 2-fructoside) was formed by the transfructosylation activity of the levan fructotransferase. The production of ascorbic acid 2-fructoside was highly affected by the concentration of the fructosyl acceptor (ascorbic acid). When 35% of ascorbic acid and 2% of levan were incubated with LFTase of 0.5 unit/g-levan at 37°C for 85 h, a maximum 52 g/l of AA-2F was produced.

Key words: Ascorbic acid 2-fructoside, fructoside, glycoside, glycosylation, levan fructotransferase

The enzyme-mediated synthesis of glycoconjugates has been widely investigated to enhance physico-chemical properties of biologically important compounds [2]. Researches have also been explored to enhance the activity, specificity, and selectivity of the enzyme [3, 7, 8]. L-ascorbic acid is a highly unstable bioactive substance that is easily decomposed or denatured by heat, light, oxygen, metal ions, etc. To overcome these drawbacks, many efforts have been made to convert the compound into its glycosyl derivatives. Typical glycosyl derivatives of ascorbic acid are ascorbic acid 2-glucoside (AA-2G) and ascorbic acid 6-glucoside (AA-6G), which can be synthesized through an enzymatic process using α-glucosidase [6, 13] or glucanotransferase [1, 12]. These processes have already been patented by a company [5] that is now the exclusive producer of AA-2G.

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However, apart from glucosides, there have been no reports on the production of other ascorbic acid-glycosides, such as ascorbic acid-fructoside. Levan fructotransferase (EC4.2.2.16) is a fructotransferase that produces di-β-D-fructofuranose 2,6':2',6-dianhydride (DFA IV) by successively eliminating the diminishing (2,6)-β-D-fructan (levan) chain from the terminal D-fructosyl-D-fructosyl disaccharide [11]. When levan and ascorbic acid are used as the fructosyl donor and fructosyl acceptor, respectively, levan fructotransferase (LFTase) catalyzes the production of a noble fructoside derivative with a β-configuration, ascorbic acid 2-fructoside (AA-2F). Based on this assumption, the current authors attempt to produce AA-2F using the

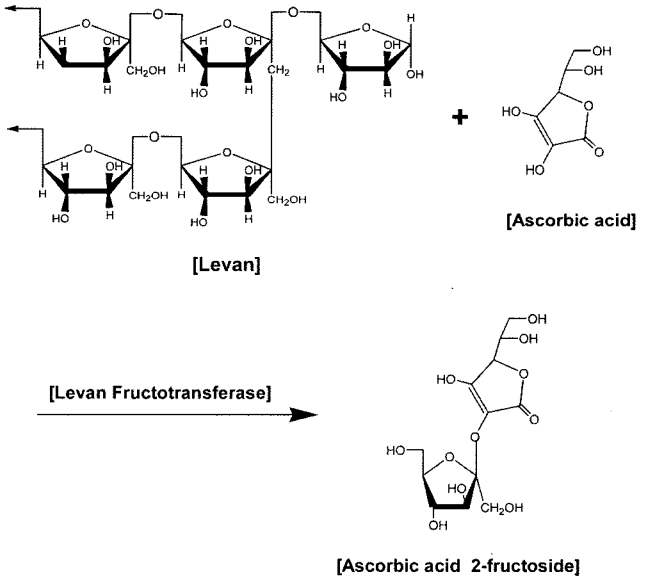

Fig. 1. Reaction scheme for enzymatic synthesis of ascorbic acid 2-fructoside.

fructotransferase activity of LFTase. Figure 1 illustrates the reaction scheme for transfructosylation by LFTase.

Accordingly, this study describes a novel enzymatic process that uses levan fructotransferase for the production of AA-2F, which has never previously reported and can be arbitrarily applied to cosmetics, food products, and pharmaceuticals.

MATERIALS AND METHODS

Materials

A bacterial levan from *Zymomonas mobilis* and recombinant levan fructotransferase from *Arthrobacter ureafaciens* were purchased from Real Biotech Co. (Daejeon, Korea). The ascorbic acid was purchased from Kanto Chemical Co. (Tokyo, Japan), and ascorbic acid 2-glucoside from Wako (Tokyo, Japan) was used as a standard. LiChroprep RP-18 (Merck, Germany) was used as a packing material for the purification of ascorbic acid 2-fructoside and all other solvents where the purification products were of extra pure grade.

Ascorbic Acid Fructoside Synthesis

The transfructosylation reactions were carried out in a 250 ml brown reagent bottle with a 100 ml reaction medium containing various concentrations of ascorbic acid, levan, and LFTase, which were dissolved in a 50 mM phosphate buffer (pH 6.5), at 37°C under mild agitation.

Analysis

The LFTase activity was assayed at 37°C in a 50 mM phosphate buffer pH 5.8. The amount of reducing sugar released from the levan was determined following the Somogyi-Nelson method [10]. One unit of LFTase activity was defined as the amount of enzyme that released 1 μmol of reducing sugar equivalent to fructose per minute. The quantitative determination of AA-2F was conducted by HPLC using UV detector (254 nm, SPD-M10A, Shimadzu, Japan) and Capcell Pak C18 column (Shiseido Co., Tokyo, Japan). AA-2G was used as the standard, and 0.05 M KH_2PO_4 (pH 2.2) was used in the mobile phase at a flow rate of 1 ml/min at room temperature.

RESULTS AND DISCUSSION

Formation of AA-2F

Two g of levan and 10 g of ascorbic acid were dissolved in 100 ml of 50 mM phosphate buffer (pH 6.5), and the solution set to pH 6.5 using phosphoric acid. This solution was allowed to react with the recombinant LFTase, which contained 20 U of LFTase, at 37°C for 72 h with mild mixing. The conversion of ascorbic acid to AA-2F was confirmed via HPLC (Fig. 2). A reaction product containing 2.9 g/l of AA-2F was obtained from the ascorbic acid and levan as a result of the fructosyltransferase activity of the recombinant LFTase. This reaction mixture was then subjected to a purification process to isolate the AA-2F and confirm its chemical structure using ^{1}H-NMR and ^{13}C-NMR spectroscopy.

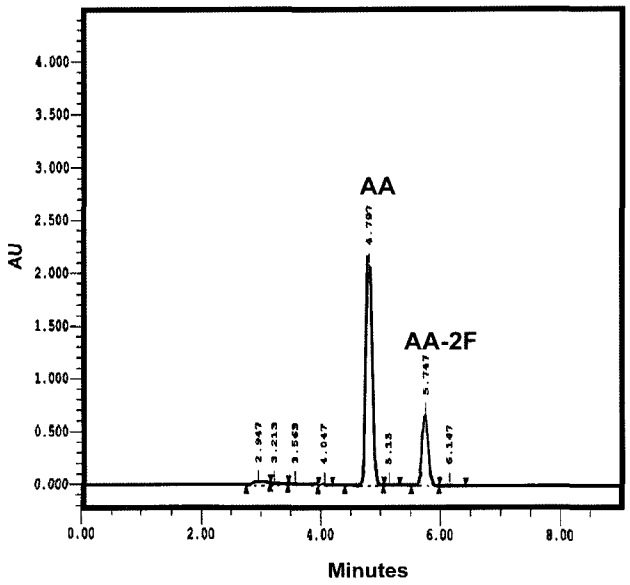

Fig. 2. HPLC spectrum of resulting AA-2F.

Purification of AA-2F

Fifty g of the reaction mixture containing AA-2F was freeze-dried and dissolved in 15 ml of distilled water. This aqueous solution was then discolored with activated charcoal, loaded on a column (25 mm×300 mm) packed with LiChroprep RP-18, and eluted with aqueous methanol (MeOH:H_2O=10:90 (v/v)) to give 5 fractions. Fraction 3 was eluted with the same solvent 3 times to obtain 30 mg of AA-2F. The ^{1}H-NMR spectrum of the AA-2F is shown in Fig. 3, and the chemical shifts recorded as ppm in D_2O were as follows: 3.50-4.20 (m, 5H), 3.73 (dd, 2H, J= 14Hz, 4Hz), 3.93(dd, 1H, 12Hz, 2Hz), and 4.81 (m, 3H). Plus, the ^{13}C-NMR spectral data (spectrum not shown) in D_2O (ppm) were as follows: 51.2, 57.7, 60.2, 60.4, 61.6, 67.3, 70.7, 74.6, 75.7, 116.2, 153.7, and 171.8.

Effect of Levan Concentration on AA-2F Production

Although levan is highly soluble in water at room temperature, a levan solution is viscous at high concentrations [9]. The viscosity of the levan from *Z. mobilis* at concentrations of 1%, 2%, and 4% has been reported as 1.02 cp, 1.80 cp, and 6.85 cp, respectively, at 40°C [4]. Thus, at a concentration above 6.0%, it was difficult to homogeneously mix a reaction mixture containing 10% ascorbic acid and 0.15 unit/g-levan of LFTase. The effect

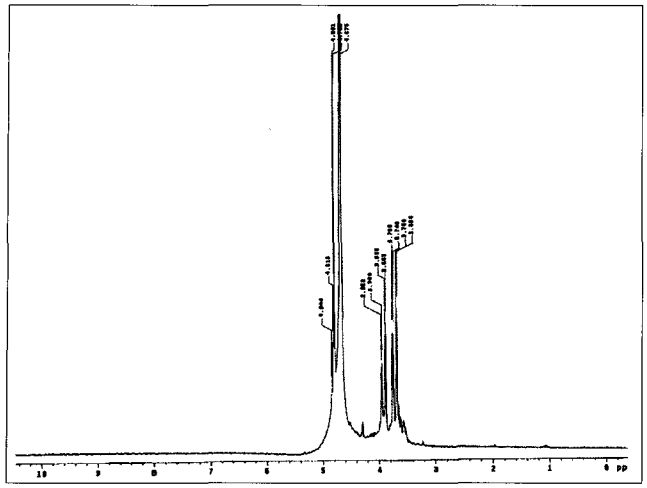

Fig. 3. ^{1}H-NMR spectrum of ascorbic acid 2-fructoside.

of the levan concentration on the production of AA-2F was investigated between concentrations of 1 and 4%. As shown in Fig. 4, the highest production of AA-2F at 18 g/l was obtained with a levan concentration of 2%, and then decreased as the concentration increased above this point.

Effect of LFTase Dosage on AA-2F Production

The effect of the LFTase dosage on the production of AA-2F was investigated between 0.3 and 1.0 unit/g-levan using a reaction mixture containing 2% levan and 10% ascorbic acid. The production of AA-2F increased with a higher dosage of LFTase, and about 20 g/l of AA-2F was produced at the dosage above 0.5 unit/g-levan of LFTase (Fig. 5).

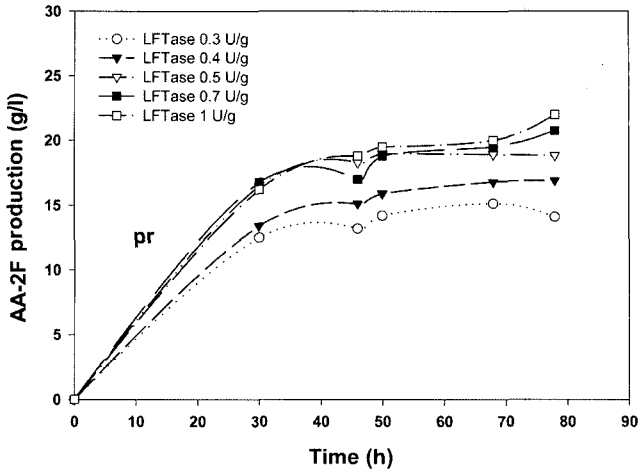

Fig. 5. Effect of LFTase dosage on production of ascorbic acid 2-fructoside.
The reactions were carried out in a 250 ml brown reagent bottle with a 100 ml reaction medium containing 10% ascorbic acid and 2% levan at pH 6.5 and 37°C.

Effect of Ascorbic Acid Concentration on AA-2F Production

To increase the production of AA-2F, the transfructosylation reaction was performed in reaction media that contained 0.5 unit/g-levan of LFTase, 2% levan, and an excess of the acceptor (ascorbic acid) at concentrations of 6% to 50%. The production of AA-2F increased with an increase in the concentration of ascorbic acid up to 35%, while the production of AA-2F decreased with a concentration above 40% (Fig. 6). 52 g/l of AA-2F was produced with a 35% concentration of ascorbic acid.

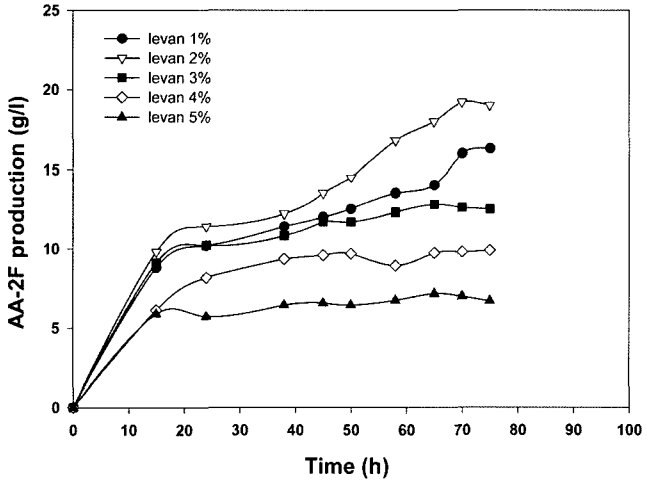

Fig. 4. Effect of levan concentration on production of ascorbic acid 2-fructoside.
The reactions were carried out in a 250 ml brown reagent bottle with a 100 ml reaction medium containing 10% ascorbic acid and 0.15 unit/g-levan of LFTase at pH 6.5 and 37°C.

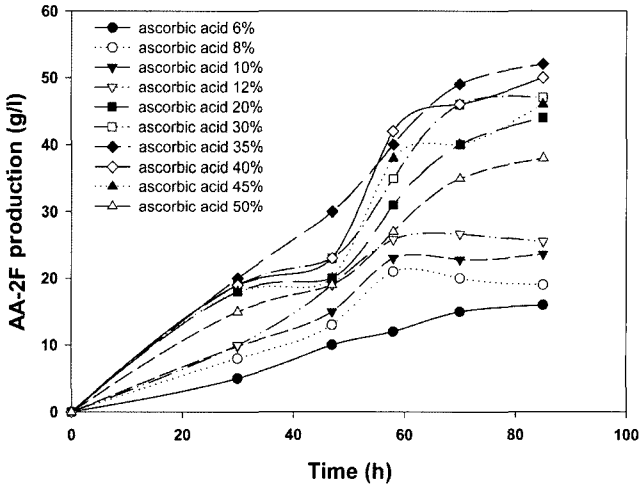

Fig. 6. Effect of ascorbic acid concentration on production of ascorbic acid 2-fructoside.
The reactions were carried out in a 250 ml brown reagent bottle with a 100 ml reaction medium containing 2% levan and 0.5 unit/g-levan of LFTase at pH 6.5 and 37°C.

In conclusion, ascorbic acid was transformed to a stable glycoside, ascorbic acid 2-fructoside, by the transfructosylation activity of levan fructotransferase when levan was used as fructosyl donor. This kind of glycosylation reaction could also be applied to increase the stability, solubility and/or biological activity of certain kinds of aglycones.

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