Production of 2-O-\(\alpha\)-D-glucopyranosyl L-ascorbic acid using cyclodextrin glucanotransferase from \textit{Paenibacillus} sp.

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Abstract

Transglycosylation to produce a 2-O-\(\alpha\)-D-glucopyranosyl L-ascorbic acid (AA-2G) was studied using cyclodextrin glucanotransferase (CGTase) from \textit{Paenibacillus} sp. A series of maltooligosaccharides substituted 2-O-derivatives of L-ascorbic acid (AA) were analyzed by HPLC. The maltooligosaccharides were hydrolyzed by glucoamylase to give AA-2G. CGTase also produced AA-2G using dextrin as a glycosyl donor and AA as an acceptor. CGTase utilized \(\alpha\)-, \(\beta\)-, and \(\gamma\)-CDs, amylose, soluble starch and corn starch as glycosyl donors but not glucose.

Introduction

The transglycosylation to synthesize AA-2G (2-O-\(\alpha\)-D-glucopyranosyl L-ascorbic acid) has been studied using commercially available enzymes for efficient mass production. Among various enzymes from microorganisms and plants, rice seed \(\alpha\)-glucosidase and CGTase (cyclodextrin glucanotransferase) are able to form AA-2G (Aga \textit{et al.} 1991, Tanaka \textit{et al.} 1991, Yamamoto \textit{et al.} 1990a). AA-2G is considered to be superior to other chemically-synthesized L-ascorbic acid derivatives in terms of the reaction specificity and efficiency in large-scale production. AA-2G is bioavailable as an ascorbate source for \textit{in vivo} and \textit{in vitro} (Kumano \textit{et al.} 1998, Yamamoto \textit{et al.} 1990c, 1992). The enzymatic production of AA-2G is more applicable than chemical synthesis of other AA derivatives such as L-ascorbic acid 2-phosphate (AA-2P) (Mima \textit{et al.} 1970), L-ascorbic acid 2-sulfate (AA-2S) (Mead \textit{et al.} 1969) or L-ascorbic acid 2-methylether (AA-2M) (Lu \textit{et al.} 1984) in terms of reaction steps, regiospecificity and production costs. However, the conventional methods using rice seed \(\alpha\)-glucosidase continue to have some problems in the purification of enzymes from these sources and a low yield of AA-2G. It is easier to purify CGTase from bacteria than \(\alpha\)-glucosidase from rice (Nam \textit{et al.} 2001, Shin \textit{et al.} 2000). CGTase is stable at a high temperature. Therefore, the enzymatic transglycosylation at high temperature using CGTase is suitable for the mass production of AA-2G. Although many studies are currently carried out on the biochemical properties, nutritional value and clinical effects of AA-2G, AA-2G is still not produced industrially. This is due to the lack of the research related to the safety of the human body and the method for efficient mass production. We found that Kimchi, a traditional Korean fermented cabbage, contains AA-2G (Jun \textit{et al.} 1998a). Furthermore, the optimal conditions for AA-2G formation in Kimchi were identified by analyzing the formation mechanism of AA-2G under the various conditions of Kimchi fermentation (Jun \textit{et al.} 1998b). These results indicate that AA-2G is safe for humans because Koreans have eaten Kimchi for more than a thousand years.

From the soil collected from several areas in Korea, we isolated \textit{Paenibacillus} sp. which produces large amount of CGTase with the transglycosylation activity for the C-2 position of AA. Furthermore, the purification method and some characteristics of the
CGTase from Paenibacillus sp. have been evaluated in our laboratory (Bae et al. 2001). In this study, we examine the enzymological properties of CGTase in AA-2-oligoglucosides production and specificity of glycosyl donor.

Materials and methods

Materials

α-CD, β-CD, γ-CD, amyllose, amylopectin, and maltotriose were obtained from Sigma (St. Louis, USA). L-Ascorbic acid, sodium ascorbate, glucose, dextrin, soluble starch, potato starch and corn starch were purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan). Paenibacillus sp. was isolated from a soil sample and produced CGTase with high activity. α-Glucosidase from rice seed, glucoamylase from Rhizopus mold and ascorbate oxidase were obtained from Sigma (St. Louis, USA). AA-2G was obtained from Prof T. Sakai at Kinki University, Japan. Other reagents used were of analytical grade of commercial sources.

Assay for AA-2G production

The standard reaction mixture contained sodium ascorbate, dextrin or other saccharides, and CGTase from Paenibacillus sp. The reaction mixture was incubated at 37 °C for 24 h (pH 6.5). To hydrolyze AA-2-oligosaccharides produced by CGTase, glucoamylase (20 units) was added to the reaction mixture and incubated at 55 °C, pH 4.5, for 24 h. The determination of AA-2G content was carried out by HPLC with µBondapak C18 (3.9 × 300 mm). The assay conditions were as follows: detection at 238 nm; mobile phase was 0.1 M KH₂PO₄/0.1 M H₃PO₄ (pH 2) at 0.5 ml min⁻¹. The amount of AA-2G was calculated on the basis of the peak area.

Assay for glucose-releasing

Glucose-releasing activity was determined by measuring the rate of glucose release from α-CD or other saccharides. The reaction mixture consisted of 1% α-CD or other saccharides in a 0.1 M sodium citrate buffer (pH 6.5) and 2500 units/CGTase ml, and was incubated at 37 °C for 2 h. The determination of glucose content was carried out by HPLC with Waters Sugar-pak I (6.5 × 300 mm). The assay conditions were as follows: mobile phase was acetonitrile/water (65:35, v/v) at 1 ml min⁻¹. The amount of glucose was calculated on the basis of its standard curve of the peak area.

Preparation of enzyme solution

After the bacteria were cultured at 37 °C for 48 h in 100 ml of the optimal medium containing 1% soluble starch, 1% yeast extract, 1.5% Na₂CO₃, 0.1% NaH₂PO₄, and 0.02% MgSO₄ · 7H₂O with an initial pH of 7, the culture broth was centrifuged at 20000 × g for 20 min and the supernatant was collected. CGTase in the supernatant was precipitated by ammonium sulfate at 10–50% saturation. The precipitate was dissolved in a 50 mM phosphate buffer (pH 7) and dialyzed against the same buffer for 24 h.

Results and discussion

Enzymatic production of glycosylated AA

The degree of glycosylation to AA by CGTase from Paenibacillus sp. was determined. As shown in Figure 1A, CGTase produced a series of transglycosylation products of AA. The peak of AA-2G was observed just after the peak of AA, with the same retention time as that of the standard AA-2G. The transglycosylation products were treated with either α-glucosidase (Figure 1B), ascorbate oxidase (Figure 1C) or glucoamylase (Figure 1D). All AA-2Gₙ except AA-2G were hydrolyzed by α-glucosidase (Figure 1B). All of the glucose-attached derivatives (AA-2Gₙ) were stable while the ascorbic acid was degraded by the ascorbate oxidase (Figure 1C). After the addition of glucoamylase to the reaction mixtures and the incubation at 55 °C for 12 h, peaks of AA-2G₂ to AA-2G₆ disappeared and the peaks of AA-2G and AA remained (Figure 1D). These results confirmed that these peaks were the conjugates of AA and oligosaccharides, corresponding to AA-2-oligosaccharides(AA-2G₂ to AA-2G₆) from the hydrolysis patterns by glucoamylase and α-glucosidase and the stability to the oxidation by ascorbate oxidase. The amounts of these products in the reaction mixture increased gradually with the incubation time, although the increase of AA-2Gₙ with long chain oligosaccharides was slow (Figure 2). The production of AA-2G increased as the incubation time increased, while the amounts of AA-2Gₙ and AA-2G₆ did not increase during prolonged incubation. From