Food Chemistry 169 (2015) 366–371

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Enzymatic synthesis of 3-*O*-α-maltosyl-L-ascorbate using an engineered cyclodextrin glucanotransferase

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ARTICLE INFO

Article history:
Received 9 April 2014
Received in revised form 17 June 2014
Accepted 22 July 2014
Available online 7 August 2014

Keywords:
L-Ascorbic acid
Cyclodextrin glucanotransferase
Acid/base mutant
3-*O*-α-Maltosyl-L-ascorbate
Transglycosylation

ABSTRACT

A mutant derived from a cyclodextrin glucantransferase with an alanine residue as its acid/base catalyst residue (CGT-E284A) catalyzed regioselective glycosylation at 3-OH of L-ascorbic acid using α-maltosyl fluoride (αG2F) and L-ascorbic acid as the donor and acceptor, respectively, yielding 3-*O*-α-maltosyl-L-ascorbate (AA3αG2). The optimum conditions were determined by high-performance liquid chromatography analysis with 20 mM αG2F and 40 mM L-ascorbic acid as the substrates at pH 7.5 and 25 °C with 1 mg/ml of the enzyme for 24 h. Calcium ions bound in CGT-E284A played an important role in the transglycosylation. CGT-E284A exhibited typical saturation kinetic behaviour for αG2F at a fixed acceptor concentration (40 mM), and substrate inhibition by L-ascorbic acid was observed at high L-ascorbic acid concentrations (>60 mM). AA3αG2 was isolated from a preparative scale reaction with a yield of 29%, and it showed extremely high stability under oxidative conditions.

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1. Introduction

L-Ascorbic acid, a well-known antioxidant, is commonly used in many foods and cosmetics as an anti-oxidizing agent (Padayatty et al., 2003). It functions as both a reducing agent and a free radical scavenger by donating either one or two electrons to more oxidized neighbouring compounds. However, L-ascorbic acid easily undergoes oxidation to a biologically inactive compound under conditions such as the presence of heat, transition metals, or ascorbate oxidase (Yamamoto, Nuto, Murakami, Suga, & Yamaguchi, 1990), consequently losing its antioxidant activity. To overcome this problem, many attempts have been made to generate more stabilized ascorbate derivatives, such as L-ascorbate-2-*O*-sulphate and L-ascorbate-2-*O*-phosphate, which have been synthesized by chemical and enzymatic methods (Lee, Seib, Liang, Hoseney, & Deyoe, 1978; Tolbert, Downing, Carlson, Knight, & Baker, 1975).

Alternatively, enzymatic glycosylation has been applied to synthesize ascorbate derivatives. Of the glycosyl ascorbate derivatives, 2-*O*-α-glucopyranosyl-L-ascorbate (AA2αG1) is commercially available and used in various cosmetic products (Kumano et al., 1998). Many enzymatic methods to synthesize AA2αG1 have been developed using the transglycosylation activity of retaining glycosidases (Fig. 1a), such as α-glucosidases (Yamamoto et al., 1990), cyclodextrin glucanotransferases (CGTases) (Han et al., 2013; Tanaka, Muto, & Yamamoto, 1991), a sucrose phosphorylase (Kwon, Kim, & Lee, 2007), and a glucansucrase (Kim et al., 2010). In addition, 6-*O*-α-glycosyl-L-ascorbic acid derivatives have been prepared by a α-glucosidase from *Aspergillus niger* (Suzuki, Miyake, Uchida, & Mino, 1973), an α-galactosidase from *Candida guilliermondii* (Kitahata et al., 1996), and a maltogenic amylase from *Bacillus stearothermophilus* (Bae et al., 2002). In contrast, the 3-OH position of L-ascorbic acid is less attractive than the previously mentioned two hydroxyl groups as a target for enzymatic glycosylation, and although 3-*O*-substituted L-ascorbic acid derivatives have recently been prepared through chemical synthesis (Li & Shi, 2007), their chemical synthesis is still challenging due to the chemical protection/deprotection processes and production of a mixture of stereochemical α/β-isomers. No reports are available about the enzymatic synthesis of 3-*O*-glycosyl-L-ascorbate derivatives.

A strategy for enzymatic synthesis of thioglycosidic linkages using engineered retaining glycosidases, named thioglycoligases, was developed in 2003 (Jahn, Marles, Warren, & Withers, 2003), and the general acid/base catalytic residue was replaced

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http://dx.doi.org/10.1016/j.foodchem.2014.07.110
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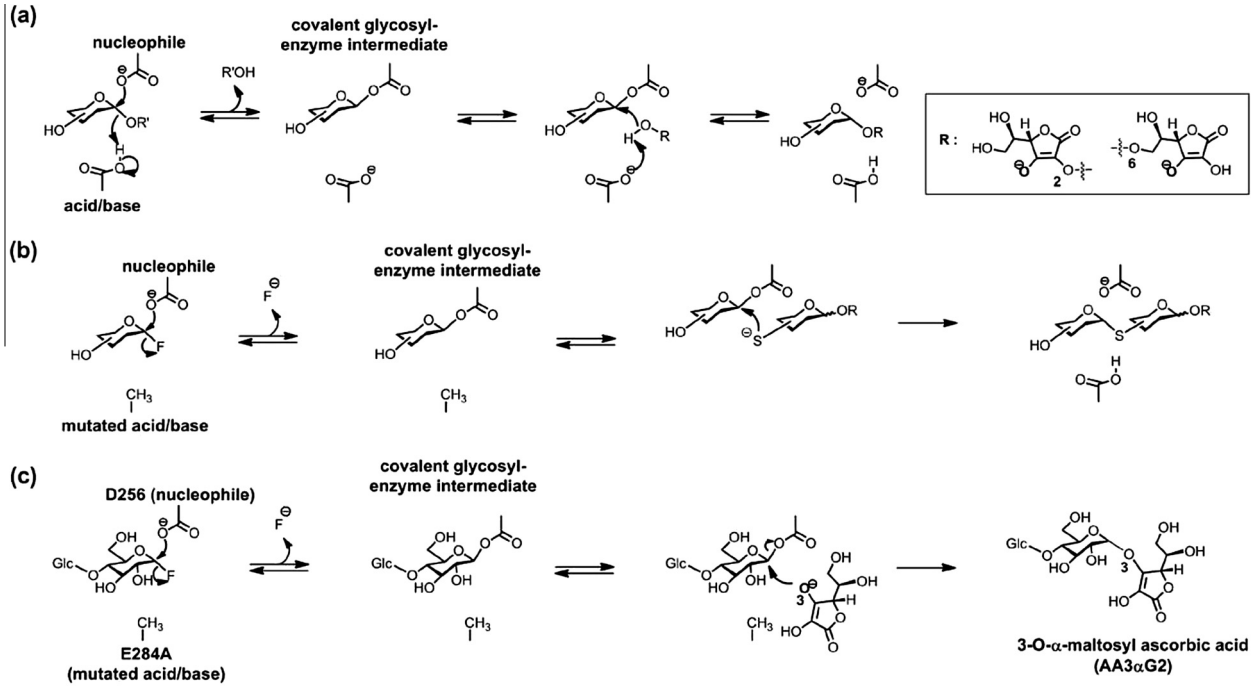

Fig. 1. (a) Mechanism of transglycosylation of the wild type retaining glycosidases for the synthesis of either 2-*O*- or 6-*O*-α-glycosyl-L-ascorbate derivatives, (b) mechanism of thioglycoligases derived from retaining glycosidases, and (c) mechanism of transglycosylation catalyzed by CGT-E284A to produce 3-*O*-α-maltosyl-L-ascorbate (AA3αG2).

by an amino acid that has no negative charge (Fig. 1b). These thioglycoligases show very low rates of formation and hydrolysis of a covalent glycosyl–enzyme intermediate due to the lack of a general acid/base catalytic residue. However, the rate of formation of the intermediate can be accelerated by incubating with substrates bearing a good leaving group, such as dinitrophenol or fluoride (Jahn et al., 2003; Kim et al., 2006). Although intermediates form, their turnover through either hydrolysis or transglycosylation occurs extremely slowly due to the absence of a general base catalyst to activate the hydroxyl group of a water molecule or sugar acceptors. However, after employing acceptors bearing a suitably positioned thiol group, the glycosyl moieties of the intermediates transfer efficiently to the thiol sugar acceptors, as the thiol group is much more nucleophilic and requires no general base catalytic assistance, resulting in the formation of a new thioglycosidic linkage. This thioglycoligase strategy has been expanded to various retaining glycosidase families (Armstrong, Reitinger, Kantner, & Withers, 2010; Kim, Chen, Kim, & Withers, 2006; Kim et al., 2006).

Our group has recently expanded the application of thioglycoligases to the synthesis of aryl *O*-glycosides using aryl acceptors bearing a hydroxyl group with pK_a values <8 (Li, Kim, & Kim, 2013). The deprotonated hydroxyl group of the aryl compounds takes the place of the thiol group of the thiol-sugar acceptors in the thioglycoligase reactions. L-Ascorbic acid has two deprotonatable hydroxyl groups, 2-OH (pK_a = 11.57) and 3-OH (pK_a = 4.17) (O'Neil, 2006). Therefore, the singly deprotonated ascorbate at a neutral pH should be able to function as a sugar acceptor for thioglycoligases, resulting in the formation of 3-*O*-glycosyl-L-ascorbate derivatives. Here, we describe the regioselective glycosylation of the 3-OH of L-ascorbic acid using an engineered cyclodextrin glucantransferase from *Bacillus* sp. I-5 (CGT-E284A) in which the acid/base catalyst (Glu284) was mutated to an alanine residue (Li, Ahn, Kim, & Kim, 2014) (Fig. 1c). The reaction conditions catalyzed by CGT-E284A were optimized, and the resulting transglycosylated product was characterized.

2. Methods

2.1. General experiments

CGT-E284A was obtained, as described previously, (Li et al., 2014). α-Maltosyl fluoride (αG2F) was synthesized, as described previously, (Hayashi, Hashimoto, & Noyori, 1984). L-Ascorbic acid and silica gel (200–425 mesh) for flash chromatography were purchased from Sigma–Aldrich Co., Ltd. High-performance liquid chromatography (HPLC) was carried out using a YL9100 HPLC system (Younglin, Anyang, Korea) equipped with a Sunfire™ C18 column (3.9 × 150 mm, Waters). The products were eluted at 0.7 ml/min with 0.1 M phosphate buffer pH 2.0 and analyzed using an ultraviolet (UV) detector at 243 nm. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a 400 MHz spectrometer from JEOL Ltd. using D_2O as a solvent. Mass spectra for the ascorbate derivative were recorded using a LTQ XL linear ion trap mass spectrometer in the negative ion mode (Thermo Scientific Inc., USA).

2.2. Transglycosylation of L-ascorbic acid using CGT-284A

The transglycosylation reactions were carried out using 20 mM αG2F and 40 mM L-ascorbic acid as the substrates in 0.1 M potassium-phosphate buffer (pH 7.0) with CGT-E284A (final concentration, 1 mg/ml). The mixture was incubated at 25 °C for 24 h. After determining the optimum temperature for CGT-E284A, the reaction mixtures were incubated at temperatures of 20–50 °C and a fixed pH of 7.5. The reactions were carried out at various pH values of 6.5–9.0 at 25 °C to identify the optimal pH, and the L-ascorbic acid concentration was varied from 10 to 100 mM at a fixed donor concentration (20 mM) to determine the ratio of L-ascorbic acid to αG2F. Upon investigating the effect of Ca^{2+} ions on transglycosylation, reaction mixtures containing 20 mM αG2F, 40 mM L-ascorbic acid, and CGT-E284A in 100 mM potassium phosphate buffer (pH 7.5) were incubated at 25 °C for 24 h in the presence of either EDTA or $CaCl_2$ (1 and 5 mM). Twenty-microlitre aliquots of the reaction mixtures were removed at the indicated time points for

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