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Transglycosylation specificity of glycosyl donors in transglycosylation of stevioside catalysed by cyclodextrin glucanotransferase

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ABSTRACT

Specificity of glycosyl donors is a critical issue in transglycosylation of stevioside, the main methodology to improve edulcorant quality of stevioside. The most popular glucanotransferase applied in this reaction is cyclodextrin glucanotransferase (CGTase) that catalyses cyclisation, coupling, hydrolysis and disproportionation simultaneously; which results a crosstalk in the glycosyl donors that comes from initial reactants, reaction intermediates as well as side products in parallel reactions. In this work, the specificity of glycosyl donors was studied to understand the transglucosylation pathways with the designed experiments and material balance analysis on the products using a commercial CGTase. It has been found that cyclodextrins and starches provided the best transglucosylation yield, while the assayed mono- and disaccharides were not effective glycosyl donors to stevioside with the CGTase. It is proposed that α- and β-cyclodextrins performed transglycosylation via coupling to produce intermediates of reducing sugar and followed by disproportionation with stevioside; while starches may perform the transglycosylation combined the cyclodextrins pathway and hydrolysis pathway of starches.

1. Introduction

Enzymatic transglycosylation has been a commonly used methodology in food and medicine chemistry for green synthesis of oligosaccharides, polysaccharides or saccharide derivatives, because glycosylation can endow glycosyl acceptors more hydrophilicity or different taste safely. For instance, stevioside (13-O-β-sophorosyl-19-O-β-D-glucosyl-steviol) is an immersing sweetener with bitter aftertaste; while the transglucosylation of stevioside catalysed by cyclodextrin glucanotransferases (CGTase, EC2.4.1.19) can eliminate the bitter aftertaste and make the transglucosylized stevioside a popular sweetener in Southeast Asia (Jaitak et al., 2009; Jung, Kim, Lee, & Lee, 2007; Li, Li, Xiao, & Xia, 2013; Lobov, Kasai, Ohtani, Tanaka, & Yamasaki, 1991). Stevioside has three glucosyl groups, in which the glucosyl at its C19 position is essential to the sweetness, while the glycosyls on its C13 position decide the taste. The effective glycosyls grafted for this taste modification are glucosyl, fructosyl, sucrosyl and sophorosyl (Lobov et al., 1991). The most popular glycosyl donors used in transglucosylation of stevioside were cyclodextrins (Abelian, Balaian, Kochikian, & Markosian, 2004; In, Kim, Chae, Choi, & Kim, 1997; Jaitak et al., 2009; Jung et al., 2007; Ohtani et al., 1991) or starches (Kochikian, Markosian, Abelian, Balaian, & Abelian, 2006; Li et al., 2013; Park, Kim, & Lee, 1998) when CGTases were applied.

CGTase is one of the most popular catalyst in enzymatic synthesis of many food additives (Li et al., 2013; Ng et al., 2013; Park, Choi, Eom, & Choi, 2013; Shahrazi, Saallah, Mokhtar Noriznan, Samsu Baharuddin, & Md. Yunos, 2013; Tai, Iwaoka, & Ito, 2013; van der Veen, Uitdehaag, Dijkstra, & Dijkhuizen, 2000; van der Veen, van Alebeek, Uitdehaag, Dijkstra, & Dijkhuizen, 2000) in addition to their traditional catalysed synthetic product, cyclodextrins; it is a multi-functional enzyme, which could catalyse transglycosylation (cyclisation, coupling and disproportionation) and hydrolysis separately (Uitdehaag, van der Veen, Dijkhuizen, & Dijkstra, 2002; van der Veen, van Alebeek, et al., 2000) or simultaneously (Li et al., 2013; van der Veen, Uitdehaag, et al., 2000).

Obviously, the substrate specificity of CGTase becomes a very important issue that decides the product category with multiple reactions. Some efforts have been paid on this topic, mainly for finding suitable CGTases. For example, a β-CGTase from alkalophilic *Bacillus* sp. BL-12 presented the glycosyl donor specificity for transglycosylation of stevioside, in which glucose, maltose, maltriose showed few transglycosylation activity while starches and cyclodextrins performed well (Jung et al., 2007). β-Cyclodextrin was reported to be an effective glycosyl donor to stevioside with another β-CGTase from an alkalophilic strain of *Bacillus firmus* (Jaitak et al., 2009).

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Toruzyme 3.0L is a popular commercial CGTase, an enzyme belong to both α-amylase and β-amylase family (Calsavara, Dias Da Cunha, Balbino, Zanin, & De Moraes, 2011; Mathew & Adlercreutz, 2013). It was found that Toruzyme 3.0L acted as an α-amylase in glucosylation of steroidal saponins with the glucosyl group as only acceptor (Wang et al., 2010); it also presented similar catalytic activity as the other two commercial CGTases in the transglycosylation of stevioside (Li et al., 2013), in which starch hydrate was applied as glucosyl donor.

However, since starch can also be cyclised to cyclodextrins in the presence of CGTase, yet the hydrolysis of starch or cyclodextrins might make the hydrolysis products become endogenous glycosyl donors; in addition, stevioside could also be hydrolysed to produce glycosyl donors, all of these remain us an interesting question: oligoglucoses or cyclodextrins produced from starches, or their intermediates, which one is the real glucosyl donor in transglucosyaltion? It is still obscure that how cyclodextrin or starch acts as effective glycosyl donors. The specificity of glycosyl donor and the corresponding pathway still lack of study.

Theoretically, in the absence of stevioside and presence of the CGTase and water, starch or cyclodextrin may produce oligoglucoses and subsequently yield other oligoglucoses or cyclodextrin (hydrolysis, cyclisation, disproportionation and transglycosylation on sugars, Scheme 1); while in the presence of both stevioside and the CGTase, except the aforementioned four reactions, transglycosylation on stevioside will be an add-on. Unfortunately, in most researches, the hydrolysis of stevioside or cyclodextrin itself in the transglycosylation has been ignored; subsequently, the endogenous glycosyl donors have been ignored as well that would remain the reaction process many questions.

Luckily, by careful experiment design and simple whole material analysis, the reactions along with the glycosyl donor specificity could be disclosed. Therefore, in this experiment, Toruzyme 3.0L is used as a model CGTase to study the glycosyl donor specificity in transglycosylation of stevioside. Different saccharides, cyclodextrins and starches will be served as the glycosyl donors. The change of saccharide level in the transglycosylation will be observed to monitor the side reactions and hence help to understand the reaction pathway.

2. Materials and methods

2.1. Materials and chemicals

Raw stevioside (90% stevioside and 10% rebauside A, HPLC) and purified stevioside (99.5%, HPLC) were provided by Niutang Chemicals. Glucose, fructose, maltose, sucrose were purchased from Sinopharm Chemical Reagent Co., Ltd. α-Cyclodextrin (α-CD, 98.0%, HPLC) and β-cyclodextrin (β-CD, 98.0%, HPLC) were purchased from Tokyo Chemical Industry. Corn starch was produced from Shang dong province, China. CGTase Toruzyme 3.0L (coupling activity, 114 U/mL) from *Thermoanaerobacter* sp. was presented by Novozymes (China). The mesophilic α-amylase (hydrolysis activity, 4000 U/g) from *Bacillus Subtilis* was from Wuxi Xuemei Co. Ltd. All other reagents were of analytical grade and used as received. DI water was used in all experiments unless otherwise stated.

2.2. Preparation of the gelatinized starch and hydrolysed starch

Gelatinized starch: 2 g Cornstarch was mixed with 100 mL water, boiled in a water bath until a transparent solution formed to get gelatinized starch (20 g/L).

Hydrolysed starch: The aforementioned gelatinized starch solution (200 g/L) was cooled to room temperature and then made up to 100 mL with DI water; followed by hydrolysis using 2 mL α-amylase (4 U/mL) at 70 °C for 30 min, by then the reducing sugar content reached a constant level. The α-amylase was deactivated by boiling the solution for another 10 min.

The reducing sugar content was measured using dinitrosalicylic acid (DNS) assay (Sengupta, Jana, Sengupta, Naskar, 2000). The glucose content in the reaction solution was recorded on a glucose biosensor (SBA-40C, Biology Research Institute of Shandong Province).

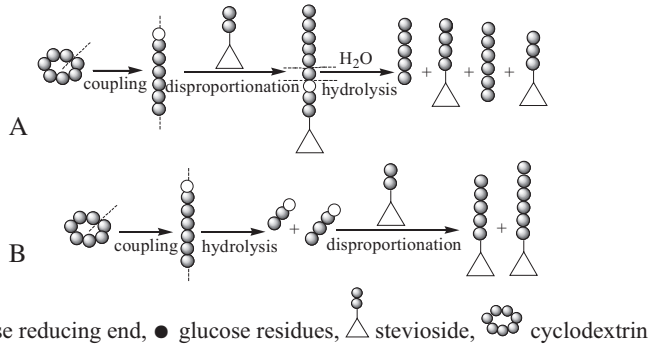
2.3. The transglycosylation of stevioside

In a typical reaction, 10 mL stevioside (20 g/L), 10 mL cornstarch (20 g/L) and 10 U CGTase /g stevioside were mixed in a 50 mL Elenmer flask, shaken at 60 °C for 2 h.

Composition of the raw products was determined with HPLC (Waters 1525, United States) using an APS-2 HYPERSIL column (Thermo Scientific Co. Ltd) equipped with a UV detector and recorded at 210 nm. A mixture of acetonitrile and water was used as the eluent, gradient from 75:25 *v/v* (2 min) to 50:50 *v/v* (30 min), the flow rate was 0.8 mL/min. HPLC-MS-MS (Waters Acquity UPLC and PDA; Wates Maldi Synapt Q-T of MS) was operated in negative ion detection mode; ultra pure synthetic air was used with flow rate of 500 L/h; MS fragment ions were obtained with 20 eV of collision energy. The stevioside conversion was calculated as following:

$$\text{St conversion}(\%) = \frac{C - C(\text{St})}{C}$$

There C represents the initial stevioside concentration; C (St) represents the detected stevioside concentration during the reaction. The stevioside concentration was determined using HPLC with a standard calibration curve. And yields of the glucosylated products were decided according to the corresponding percentages of chromatographic area, calibrated with that of stevioside.



Scheme 1. The possible pathway of transglucosylation of stevioside with cyclodextrins. (A) Coupling pathway; (B) hydrolysis-disproportionation pathway.