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RESEARCH ARTICLE

Separation and Structural Characterization of Tri-Glucosyl-Stevioside

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Abstract

Stevioside is a non-cariogenic and low-calorigenic diterpenoid glycoside has slightly bitterness and bad after taste. Enzymatic modification by alternansucrase from *Leuconostoc citreum* SK24.002 was utilized in biotransformation of stevioside to remove the bitter taste fully or partially and an aftertaste of the stevioside, using sucrose as donor and stevioside as acceptor. Tri-glucosyl-stevioside produced during alternansucrase acceptor reaction of stevioside were successfully separated using adsorption separation technology of macroporous adsorption resin (AB-8) flowed by semipreparative high performance liquid chromatography. Structure of the product was characterized to be 13-{[α-D-glucopyranosyl-(1→3)-α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→3)-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl]oxy}kaur-16-en-19-oic acid β-D glucopyranosyl ester on the basis of extensive one dimension and two dimensional NMR and LC-ESI-MS in negative mode data.

Keywords: Stevioside, alternansucrase, acceptor reaction, structural characterization, spectral data.

Introduction

Stevioside, a natural sweetener and medical supplementary material has been widely applied to food and pharmaceutical industries (Chan *et al*., 2000). Due to its high sweetness and low calorie, stevioside is acknowledged as a natural sweetener resource and common with consumers and manufacturers (Carakostas *et al*., 2008). Stevioside also has some pharmacological effects, such as regulating blood sugar level, enhancing insulin secretion, decreasing blood pressure, adjusting or improving immunization and preventing chemical carcinogenesis (Yang *et al*., 2009). Furthermore, other subsidiary functions, such as preventing obesity, restraining bacterial growth and preventing cavities have also been demonstrated. All of these properties confer stevioside with broad application prospects and have been widely studied (Yoda *et al*., 2003; Chatsudthipong and Muanprasat, 2009).

The main component of Stevia leaf extracts is stevioside, which has slightly bitter taste and an aftertaste (Abelyan *et al*., 2004). In order to lessen these concerns, enzymatic saccharification of stevioside was studied (Fukunaga*et al*., 1989). Transglycosylation of stevioside with soluble starch using cyclodextrin glucosyltransferase was reported by many researchers (Kasai *et al*., 1981; Fukunaga *et al*., 1989; Lovov *et al*., 1991). In this enzymatic reaction, the transfer of one, two or more glucose units from the soluble starch to stevioside simultaneously occurred to form complex products. Adsorption separation technology of macroporous adsorption resin (MAR) is a relatively new separation method and displays an obvious superiority in industrial production since, MAR has a high adsorption capacity, certain selectivity, low cost, easy regeneration and has a good stability (Babic *et al*., 2006; Liu *et al*., 2006). These advantages of MAR have caught great attention of the researchers all over the world and MAR has been extensively used in the fields of chromatography, water treatment industry and extraction, isolation and purification of natural products (Ma *et al*., 2009). In our previous studies, we conducted the biotransformation of stevioside by *Leuconostoc citreum* SK24.002 alternansucrase acceptor reaction (Musa *et al*., 2014). As a continuation of this study, the present work reports the complete separation and structural characterization of the tri-glucosyl-stevioside.

Materials and methods

Chemicals and enzymes: Stevioside (purity 99%) was purchased from Wako Pure Chemical Industries, Ltd., Japan. Stevioside (purity >80%) was purchased from Aladdin Reagent Database Inc., Shanghai, China. All other chemicals were of analytical grade and were obtained from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China. Alternansucrase was prepared from *Leuconostoc citreum* SK24.002 as previously described by Musa *et al*. (2014).

Adsorbents: Macroporous resin AB-8 was obtained from a Chemical Plant of Nankai University (Tianjin, China), the resin was pretreated according to the manufacturer's recommendation to remove the monomers and porogenic agents trapped inside the pores during the synthesis process. In brief, before the adsorption experiments, the weighed resin was soaked in ethanol

overnight, subsequently washed with deionised water until the ethanol was thoroughly replaced by deionized water.

Transglycosylation of stevioside: Acceptor reactions were carried out as described previously by Musa *et al*. (2014) using *Leuconostoc citreum* SK24.002 alternansucrase in 100 mL of 20 mM sodium acetate buffer (pH 5.4) with 2.5 g sucrose as a donor and 1.5 g stevioside as acceptor with shaking (125 rpm) at 25°C. The reaction was allowed to proceed until the sucrose was completely consumed.

Separation and purification: The reaction mixture was treated with the equal volume of ethanol and centrifuged at 10,000 rpm for 30 min (Eppendorf centrifuges 5804R, Germany). The supernatant was evaporated to remove ethanol using rotary evaporator (IKA RV 10 and HB 10 Basic, IKA-Werke GmbH and Co. KG, Staufen, Germany). Then, static adsorption was used to separate the modified stevioside from the solute. The collected eluate was condensed and lyophilized (LabconCo® stoppering tray dryer, Labconco Co., Kansas, MO, USA). The tri-glucosyl-stevioside was separated on a Shodex Asahipak NH2P-50 10E column (10 mm ID x 250 mm, 5 µm; Showa Denko K.K, Tokyo, Japan) fitted with a Shodex Asahipak NH2P-50 7G guard column (Showa Denko K.K, Tokyo, Japan) with aqueous acetonitrile solution (68 mL/100 mL) as mobile phase at the flow rate of 2.5 mL/min at 35°C. The static adsorption of modified stevioside was performed as follows: preweighed amounts of hydrated adsorbent AB-8 (equal to 3 g dry resin) were introduced into an airtight Erlenmeyer flask. Then, 15 mL of aqueous solution was added into the flask shaken (100 rpm) in a constant temperature water-bath at 25°C for 12 h. The selectivity and adsorption capacity of macroporous resin AB-8 toward modified stevioside was evaluated and desorption properties were obtained from desorption process using different ethanol/water ratios.

HPLC analysis: All samples were analyzed by HPLC on an Agilent 1200 series HPLC system (Agilent Technologies, USA). Analysis was performed using NH2 column (Shodex Asahipak, NH2P-50 4E, ID 4.6 x 250 mm, 5 µm, Showa Denko K. K, Tokyo, Japan). Acetonitrile: water (75:25) was used as the mobile phase. Flow rate, column temperature, injection volume and the wavelength of DAD detector were 1 mL/min, 30°C, 20 µL and 210 nm respectively.

LC-MS/MS analysis: LC-MS/MS (Waters Acquity UPLC and PDA; Waters Maldi Synapt Q-T of MS) was operated in a negative ion detection mode; ultra pure synthetic air was used as nebulisation solvation gas (flow rate = 500 L/h) and MS fragment ions were obtained with 20 eV collision energy. 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.

NMR analysis: The tri-glucosyl-stevioside was dissolved in pyridine-d5 (15 mg of sample was dissolved in 0.6 mL of pyridine-d5 at 25°C) and then introduced into an NMR spectrometer to determine; ^{1}H, ^{13}C NMR, correlation spectroscopy (COSY), Heteronuclear single-quantum correlation spectroscopy (HSQC) and Heteronuclear multiple-bond correlation spectroscopy (HMBC) spectra, using a Bruker Avance III Digital NMR Spectrometer (Bruker, Karlsruhe, Germany). Data acquisition and processing were done with Bruker Topspin 2.1 (Bruker, Karlsruhe, Germany). Coupling constants (J) are expressed in Hertz and chemical shifts are given on a δ (ppm) scale with TMS (tetramethylsilane) or solvent signals as an internal standard.

Results and discussion

Purification of modified stevioside: The adsorption and desorption efficiency of macroporous resin AB-8 for modified stevioside was studied. After adsorption equilibrium was reached, the modified stevioside desorbed for 30 min with shaking at 150 rpm and 25°C, using l0 mL of 0, 20, 40, 60, 80 and 95% aqueous ethanol solutions respectively. There is no detection of stevioside and modified stevioside after 12 h adsorption process indicate that the high selectivity and capacity of macroporous resin AB-8 toward stevioside and modified stevioside. The stevioside and modified stevioside was recovered using aqueous ethanol solutions between 40 and 80%.

Separation of tri-glucosyl-stevioside: Separation of modified stevioside was carried out using semi-preparative HPLC and the modified stevioside product was successfully separated, while the modified stevioside deduced to be tri-glucosyl-stevioside, according to the result obtained from HPLC and LC-MS/MS analysis of modified stevioside as presented in Fig. 1 and 2.

Fig. 1. HPLC chromatogram of the purified tri-glucosyl-stevioside.

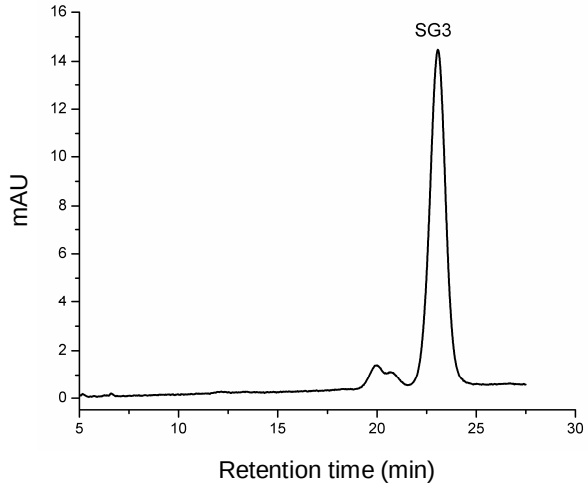


Fig. 2. Mass spectrum of the purified tri-glucosyl-stevioside.

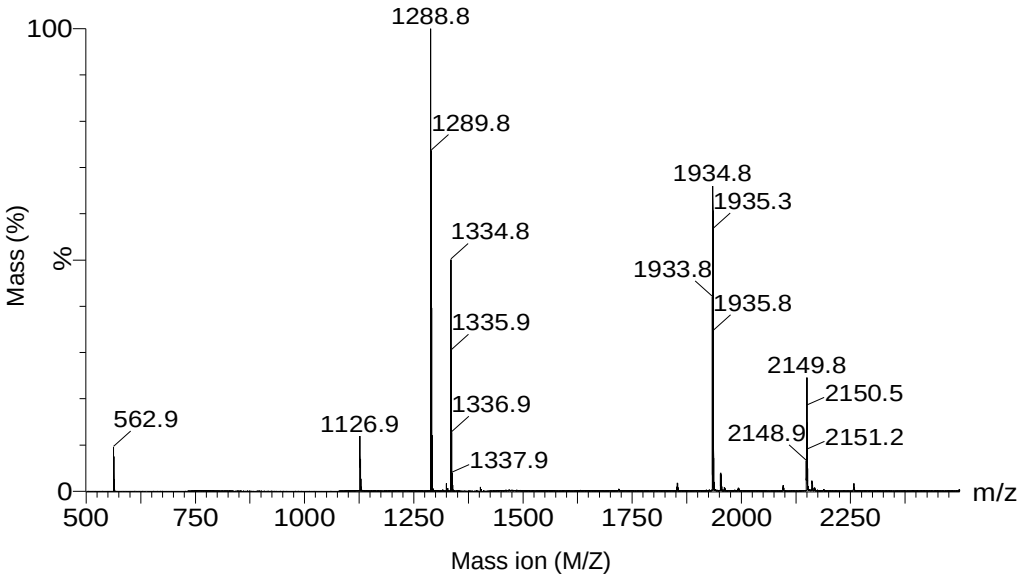


Fig. 3. ^{1}H and ^{13}C NMR spectrum of tri-glucosyl-stevioside (A and B).

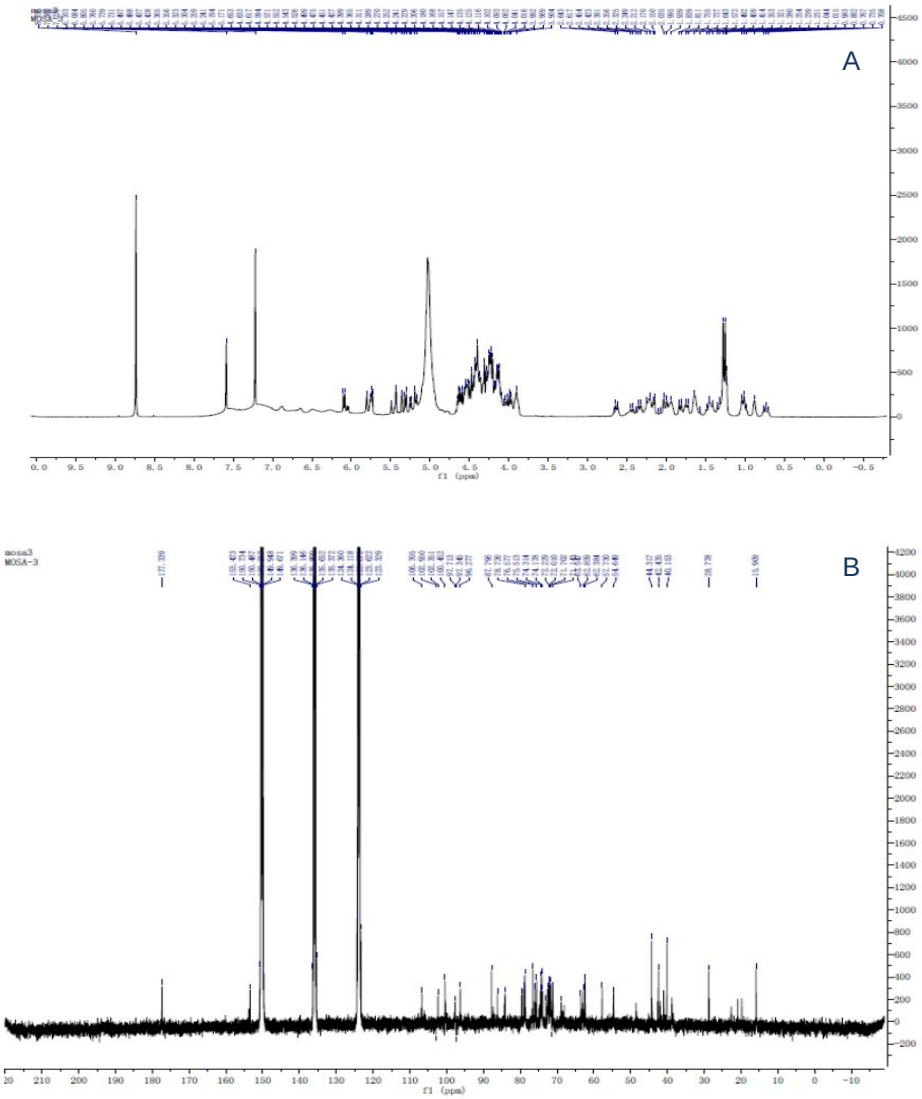


Fig. 4. COSY H-H spectrum of the tri-glucosyl-stevioside.

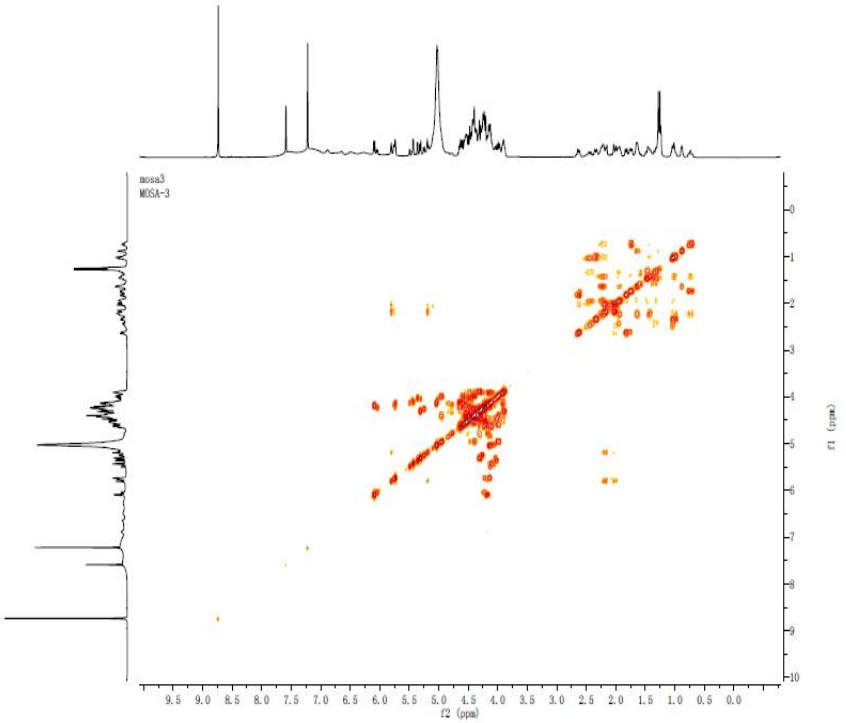
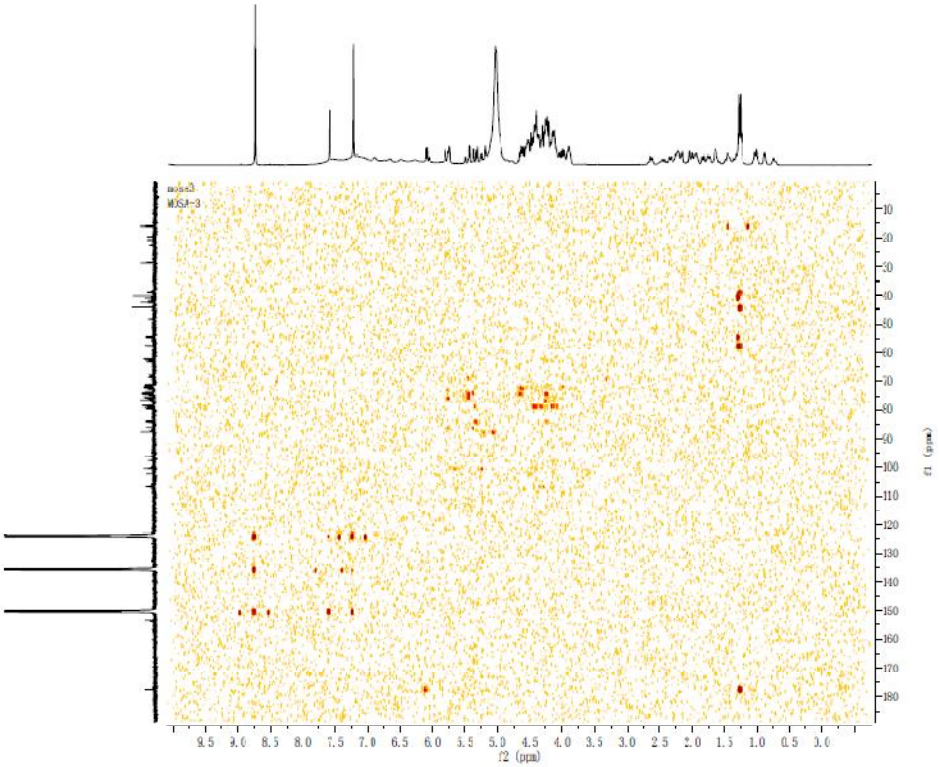


Fig. 5. HMBC spectrum of the tri-glucosyl-stevioside.



Structural characterization of tri-glucosyl-stevioside: Tri-glucosyl-stevioside was isolated as a white powder and its molecular formula has been deduced to be $C_{56}H_{90}O_{33}$ on the basis of its negative ESI data of LC-MS analysis which showed the presence of a $[M-H]^-$ ion at m/z 1289.8. This was supported by the ^{13}C NMR spectral data (Clos *et al*., 2008). The ^{1}H and ^{13}C NMR spectrum of tri-glucosyl-stevioside was shown in Fig. 3. The presence of two methyl singlets at δ1.22 and 1.23, two olefinic protons as singlets at δ5.086 and 5.731 of an exocyclic double bond, nine methylene and two methine protons between δ0.97-2.45 characteristic for the diterpenes belongs to the class of ent-kaurenes isolated earlier from the genus Stevia (Kohda *et al*., 1976; Clos *et al*., 2008; Ohta *et al*., 2010). The basic skeleton of ent-kaurene diterpenoids was supported by the COSY (H-1/H-2; H-2/H-3; H-5/H-6; H-6/H-7; H-9/H-11; H-11/H-12) and HMBC (H-1/C-2, C-10; H-3/C-1, C-2, C-4, C-5, C-18, C-19; H-5/C-4, C-6, C-7, C-9, C-10, C-18, C-19, C-20; H-9/C-8, C-10, C-11, C-12, C 14, C-15; H-14/C-8, C-9, C-13, C-15, C-16 and H-17/C-13, C-15, C-16) correlations (Fig. 4 and 5).

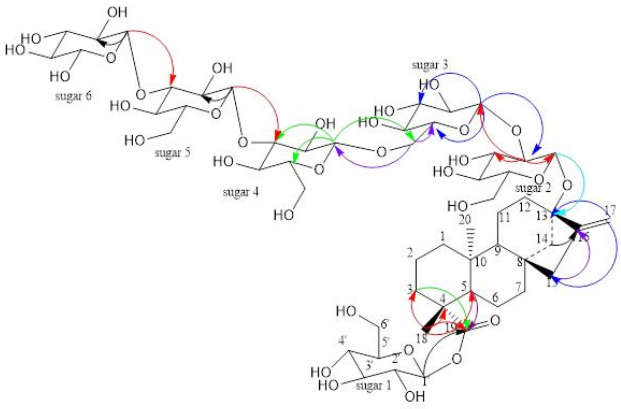
The ^{1}H NMR spectrum of tri-glucosyl-stevioside also indicated the presence of six anomeric protons at δ5.013, 5.243, 5.485, 5.796, 5.857 and 6.069 ppm, suggesting the presence of six sugar molecules in the structure. This was further supported by the fragment ions observed in the negative ESI mode MS spectrum of tri-glucosyl-stevioside which provided fragment ion corresponding to the successive losses of glucose residue $[M-H-glu]^-$ to yield the fragment ion at m/z 1126.9. A close comparison of the ^{1}H and ^{13}C NMR spectral data of tri-glucosyl-stevioside with stevioside suggested that tri-glucosyl-stevioside is also a steviol glycoside. This has a β-D-glucosyl substituent at C-19 and 2 substituted β-D-glucotriosyl at C-13 leaving the assignment of an additional 3 D-glucose units. The complete structure was further supported by the key HMBC correlation as shown in Fig. 6. Since the coupling constants of the six anomeric protons, it was confirmed that the three glucosyl moieties are β-orientation and three glucosyl units have α-orientation. Thus, structure of tri-glucosyl-stevioside was established as:

13-{[α-D-glucopyranosyl-(1→3)-α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→3)-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl]oxy}kaur-16-en-19-oic acid β-D glucopyranosyl ester.

Conclusion

The separation process of tri-glucosyl-stevioside using macropours resin AB-8 flowed by semi-preparative HPLC was established. Macropours AB-8 resin exhibited higher separation efficiency of steviol glucosyl. The tri-glucosyl-stevioside was successfully separated and characterized on the basis of NMR (1D and 2D) and mass spectral data.

Fig. 6. Key HMBC correlations of tri-glucosyl-stevioside.



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