

Degradation study of stevioside using RP-HPLC and ESI-MS/MS

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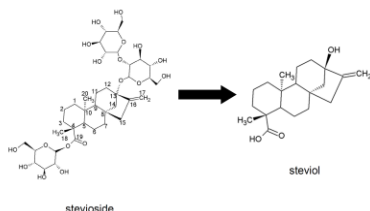
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Abstract

Stevioside is very potential to be an antidiabetic pro-drug. In processing, the active ingredient may be degraded. This research conducted a study of the degradation of stevioside on several stress factors such as acid and base hydrolysis, exposure to UV rays, thermal heating, and oxidation using a reversed phase-high performance liquid chromatography (RP-HPLC). The degradation products were identified using electrospray ionization tandem mass spectrometry (ESI-MS/MS). Hydrolysis of acid-base solution and exposure to UV254 nm rays caused the breakdown of glycoside bonds in the analyte. Stevioside was unstable in dry heating at 105 °C for 48 hours with degradation of 91%. Stevioside was oxidized by hydrogen peroxide (H₂O₂) for 48 hours. Based on the ESI-MS/MS analysis, the identified stevioside degradation products were [M-H]⁻ with the m/z = 803 as stevioside, [M-H]⁻ with the value of m/z = 641 as steviolbioside, [M-H]⁻ with the m/z = 479 as steviolmonoside, and [M-H]⁻ with the m/z = 317 as steviol. Termination of glucose was characterized by fragmentation [M-162]⁻. Our study provides a basic view of the stability and degradation characteristics of stevioside and demonstrates the formation of degradation products.

Keywords: degradation, ESI-MS/MS, HPLC, stevioside

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INTRODUCTION

Stevioside (Fig. 1) is a dominant diterpene glycoside compound contained in *Stevia rebaudiana* Bertoni. Stevioside is potentially developed as a candidate for type 2 antidiabetic drugs because it has significant antihyperglycemic bioactivity (Gregersen *et al.*, 2004). Stevioside also has some bioactivities as anticancer (Takasaki *et al.*, 2009), anti-diarrhea (Wang *et al.*, 2014), immunomodulatory (Sehar *et al.*, 2008), and antioxidant (Hajihashemi and Geuns, 2013; Kim *et al.*, 2011). Bioactivity of stevioside as antidiabetic is dose-dependent (Gregersen *et al.*, 2004; Jeppesen *et al.*, 2000; Kujur *et al.*, 2010; Melis and Sainati, 1991).

During the processing of raw materials into products, the analytes may be degraded. The stevioside compound in carbonated beverages is degraded into other compounds that are influenced by environment, temperature, and pH (Prakash *et al.*, 2012; Wölwer-Rieck *et al.*, 2010). In water, stevioside is stable at heating up to 120 °C and starts to degrade at heating of more than 140 °C. While stevioside is stable at the range pH of 2.0 - 10.0, it degraded at pH of 1.00 (Kroyer, 2010). However, stevioside is not degraded and affected by light (Clos *et al.*, 2008). Stevioside is also stable in semi-skim milk products, soy beverages, fermented milk, ice cream, yogurt, biscuits, and jams (Jooen *et al.*, 2012).

The study of the hydrolysis of stevioside standard in acid and base solutions by heating using reflux shows the breaking of glycoside bonds on C-19 atoms and one glycoside bond at C-13 (Chaturvedula and Prakash, 2011). Study of stevioside degradation in acid solution is carried out using liquid chromatography and mass spectrometry to identify its degradation product (Catharino and Santos, 2012; Musa *et al.*, 2014). Dry heating, oxidation, and UV light are also stressing factors that can be applied for forced degradation of a standard

compound instability study for biopharmaceuticals product development (Tamizi and Jouyban, 2016). Although studies about stevioside stability in some food and acid-base systems have been investigated, degradation studies of UV light stress factors, dry heating, oxidation and identification of degradation products are still limited. This study will explore the stevioside degradation against acid-base-neutral hydrolysis stressing factors, UV exposure, dry heating, and oxidation.

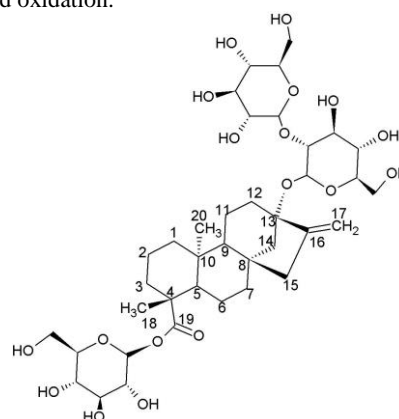


Fig. 1 Molecular structure of stevioside.

EXPERIMENTAL

Materials

A standard of stevioside used was purchased from WAKO, Japan that has purity grade of 99.0% based on Certificates of Analysis (CoA).

Solvents for mobile phase of high performance liquid chromatography (HPLC) were acetonitrile (LC grade) and methanol (LC grade). Solvents for acid-base degradation were hydrochloric acid (HCl), sulfate acid (H₂SO₄), phosphoric acid (H₃PO₄), and sodium hydroxide (NaOH) (pro analysis grade). All these solvents were purchased from E. Merck, Germany. Oxidation agent used was hydrogen peroxide (H₂O₂) (pro analysis grade), which was purchased from E. Merck, Germany.

Forced Degradation of Stevioside

Acid-base Degradation

Stevioside standard compound of 500 µg/mL was dissolved in distilled water, HCl 0.1 M, NaOH 0.1 M, H₃PO₄ 0.1 M, and citric acid 0.1 M. Each solution was heated using water bath at 80 °C for 8 hours. Each solution was cooled, neutralized and added to 10 mL of volume using water. Each solution was injected into HPLC system for degradation profile identification.

Dry Heating Degradation

A total of 2.5 mg of stevioside was heated in an oven at 105 °C for 48 hours. Afterward, the stevioside degraded product was dissolved in 10 mL of distilled water prior to HPLC analysis.

Ultraviolet (UV) Exposure Degradation

Each of stevioside standard of 500 µg/mL dissolved in distilled water; HCl 0.1 M; and NaOH 0.1 M. Each standard solution of stevioside was placed evenly forming a thin layer in a petri dish and closed. Each standard solution of stevioside was irradiated with a UV_{254 nm} lamp for 48 hours. Then, each standard solution of stevioside was removed and neutralized with trifluoroacetic acid (TFA) and triethylamine (TEA). Afterward, each standard solution of stevioside was fulfilled in volume up to 10 mL using water. Each solution was injected into HPLC system for degradation profile identification.

Oxidation Degradation

A total of stevioside of 250 µg/mL was dissolved in H₂O₂ 10% for 72 hours. The stevioside solution was placed at 25 ± 5 °C in dark for 72 hours for oxidative degradation.

Reversed Phase-HPLC (RP-HPLC) Condition

RP-HPLC conditions were adjusted according to the previous study (Martono *et al.*, 2016). HPLC used was Knauer, GmbH, Germany. Stationary phase used was Eourosphere C-18 (250 × 4.6 mm, 5 µm) with a guard column. The mobile phase was a mixture of methanol 10% in water: acetonitrile (65: 35, v/v). TFA 0,01% (v/v) was added into a mixture. The mobile phase was homogenized and degassed using an ultrasonic bath. A flow rate of mobile phase applied was 0.6 mL/min. Separation was detected using the UV detector at 210 nm. Sample volume injected was 20 µl using Rheodyne 7726i injector.

Electrospray Ionization Tandem Mass Spectrometry (ESI-MS/MS)

Stevioside standard and stably degraded hydrolysis of citric acid was injected directly (direct injection) in mass spectrometer system (Waters Xevo TQ-S) with electrospray ionization (ESI) method. Operational conditions used were a capillary voltage of 2.5 kV, cone voltage of 80 V, a source voltage of 60 V. Gas desolvation temperature was 450 °C. Gas desolvation flow rate was 800 L/h, cone of 150 L/hr and nebulizer of 7.0 bar pressure. The conditions of the analyzer were low mass (LM) resolution 1 and 2 was 3.00 and high mas (HM) resolution 1 and 2 was 15.00. Energy ion 1 was 1.0 V and ion 2 was 2.0 V. Collision gas flow used was 15 mL/min. The m/z values analyzed by ESI-MS were in the range of 50-1250 amu.

RESULTS AND DISCUSSION

Stevioside degradation evaluation using RP-HPLC

Stevioside stability is influenced by stressor applied to the compounds. Percentage of stevioside degradation was calculated

based on the remaining stevioside peak area after treatments of degradation by the stressor. The result of this study demonstrated that stevioside was hydrolyzed in distilled water at 80 °C for 8 hours with degradation of 25%, whereas at the same hydrolysis condition in 0.1 M NaOH solution caused total degradation of the stevioside compounds. The stevioside compound was more stable to acid hydrolysis than in basic solutions, in which stevioside degradation of HCl hydrolysis reached 81% (Table 1 and Fig. 2). The results of this study were consistent with the results of reported studies (Musa *et al.*, 2014; Wölwer-Rieck *et al.*, 2010), which showed that the stevioside was more stable in the hydrolysis of distilled water.

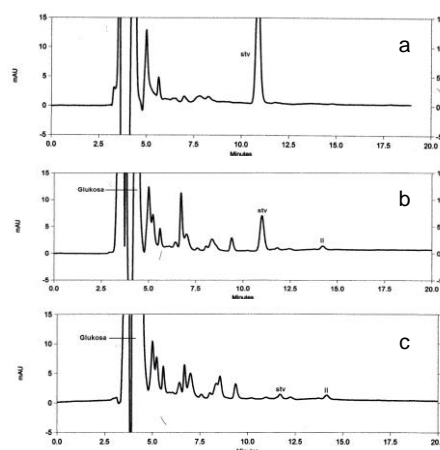


Fig. 2 RP-HPLC chromatograms of stevioside degradation in (a) distilled water, (b) 0.1 M HCl, and (c) 0.1 M NaOH for 8 hours at 80 °C. Stv = stevioside.

As shown in Table 1, the stevioside compound was more degraded in phosphoric acid solution than in citric acid solution under hydrolysis conditions at 80 °C for 8 hours. Phosphoric acid degraded stevioside by 98%, while citric acid degraded stevioside by 86% (Fig. 3). The results of this study were consistent with the results of research Kroyer (2010), suggesting that stevioside was more degraded in phosphoric acid.

Table 1 Degradation percentage of stevioside in several stressors.

Stressor	Stevioside	
	Concentration (µg/mL)	Percentage of degradation (%)
Standard	134.20	-
Distilled water hydrolysis	121.85	25.86
0.1 M HCl hydrolysis	31.26	80.98
0.1 NaOH hydrolysis	n.q.	100
0.1 M citric acid hydrolysis	22.24	86.47
0.1 M phosphoric acid hydrolysis	1.90	98.84
Dry heating	14.87	90.96
UV ₂₅₄ exposure in distilled water	76.42	53.51
UV ₂₅₄ exposure in 0.1 M HCl	74.90	54.43
UV ₂₅₄ exposure in 0.1 M NaOH	14.65	91.09
H ₂ O ₂ oxidation	n.q.	100

n.q. = not quantified

The results also showed that stevioside was unstable in dry heating at 105 °C for 48 hours. Stevioside degradation by dry heating was 91%. Stevioside was fully degraded by oxidation (Fig. 4). Stevioside was more stable in distilled water and HCl 0.1 M solutions

than in NaOH 0.1 M solution by UV₂₅₄ nm exposure. Levels of stevioside degradation to UV₂₅₄ nm exposure in distilled water and HCl 0.1 M solutions were 53.5% and 54.4% respectively, whereas in NaOH 0.1M solution was 91.1% (Table 1 and Fig. 5). Product degradations were varied among stressor factor. Each stressor factor has specific energy to breakdown molecular bond of interested compound. In addition, the type of acid and base solution used to dissolve also affected the stability of the stevioside. It reflected that the stevioside degradation was influenced by stressor and environment factors.

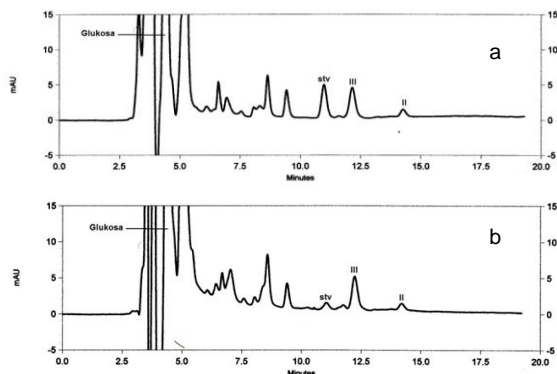


Fig. 3 RP-HPLC chromatograms of stevioside degradation in (a) 0.1 M citric acid and (b) 0.1 M phosphoric acid for 8 hours at 80 °C. Stv = stevioside.

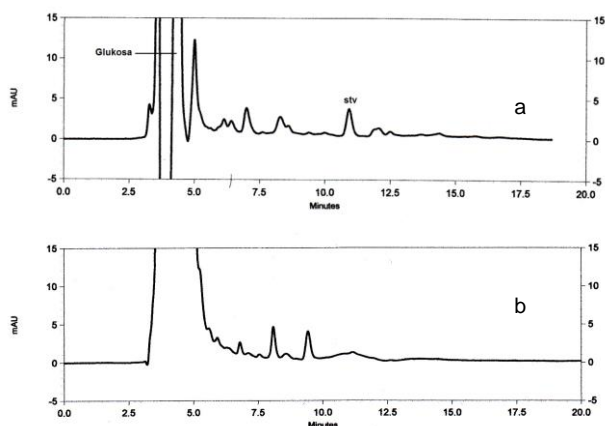


Fig. 4 RP-HPLC chromatograms of stevioside degradation in (a) dry heating of 105 °C for 48 hours and (b) oxidation by H₂O₂ 10% for 72 hours. Stv = stevioside.

Identification of stevioside degradation product using ESI-MS/MS

The identification of stevioside compound degradation product using ESI-MS/MS by direct inlet injection showed m/z value as a basis for determining the possible structure of the degradation product. The residual stevioside compound was detected as [MH]⁻ at m/z = 803 (Fig. 6). Stevioside degradation causes glycoside bond termination. The m/z value [M-162]⁻ corresponded to the release of the sugar substituent (Musa *et al.*, 2014). Stevioside lost one glucose molecule, resulting in a steviolbioside compound and was detected as [MH]⁻ at m/z = 641 (Fig. 7). One glucose molecule was terminated from steviolbioside compound and formed a steviolmonoside compound. Steviolmonoside was identified as [MH]⁻ at m/z = 479 (Fig. 8). Further analysis showed that the steviol compound was also formed from the degradation of stevioside and was detected as [MH]⁻ at m/z = 317 (Fig. 9). The results of this study were consistent with the results of the reported studies (Catharino and Santos, 2012; Musa *et al.*, 2014), which showed that stevioside would be degraded into

various products by terminating glycoside bond and producing steviolbioside, steviolmonoside, and steviol.

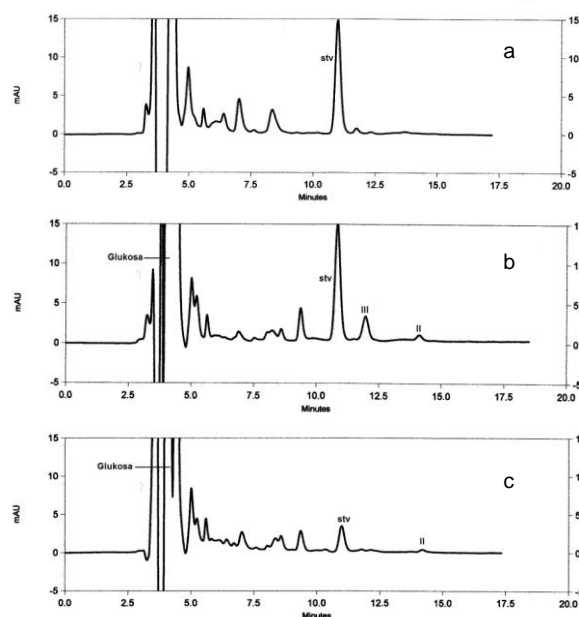


Fig. 5 RP-HPLC chromatograms of stevioside photodegradation in (a) distilled water; (b) 0.1 M HCl; (c) 0.1 M NaOH by UV₂₅₄ nm exposure. Stv = stevioside.

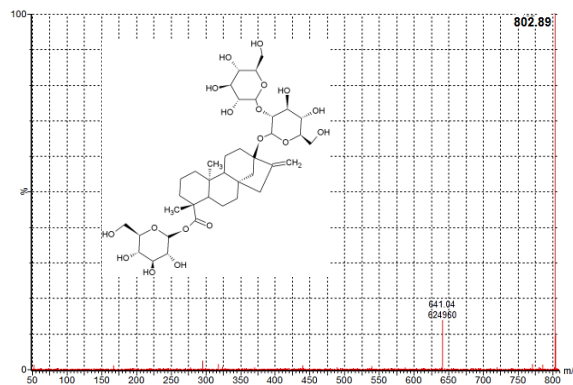


Fig. 6 Identification of stevioside [M-H]⁻ (m/z = 803) using ESI-MS/MS and its molecular structure.

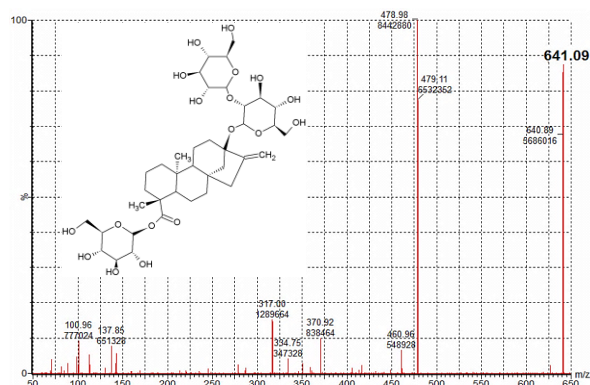


Fig. 7 Identification of steviolbioside [M-H]⁻ (m/z = 641) using ESI-MS/MS and its possible molecular structure.

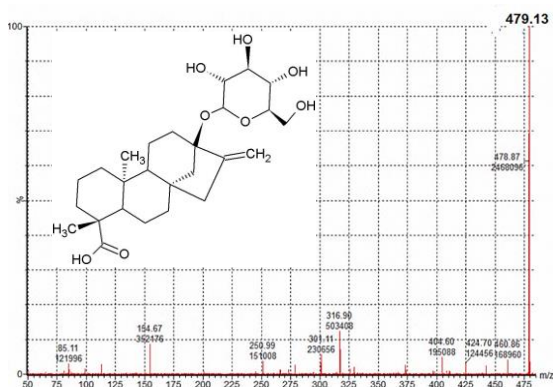


Fig. 8 Identification of steviolmonoside $[M-H]^-$ ($m/z=479$) using ESI-MS/MS and its possible molecular structure.

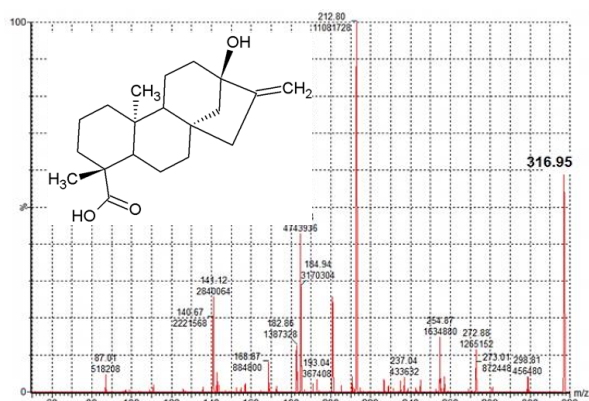


Fig. 9 Identification of steviol $[M-H]^-$ ($m/z=317$) using ESI-MS/MS and possible its molecular structure.

CONCLUSION

Stevioside degradation was influenced by acid-base hydrolysis, thermal, UV rays, and oxidation. The stevioside lost glucose molecule in degradation process and finally produced steviol. Pharmaceutical products containing stevioside were more stable in neutral solutions and shall be protected from UV exposure and possibly oxidation.

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