Phytochemistry, Vol. 21, No. 8, pp. 1927–1930, 1982.
Printed in Great Britain.

0031-9422/82/081927-04$03.00/0

ENZYMATIC DETERMINATION OF STEVIOSIDE IN *STEVIA REBAUDIANA*

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(*Received* 23 *October* 1981)

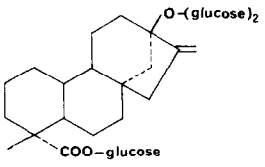
Key Word Index—*Stevia rebaudiana*; Compositae; enzymatic assay; diterpene glucoside; stevioside; natural sweetener.

Abstract—A simple enzymatic method is described for the determination of stevioside in *Stevia rebaudiana*. The method is based on the hydrolysis of stevioside with crude hesperidinase. The reaction is followed by monitoring the production of glucose with a glucose oxidase–peroxidase–2, 2′-azino-di-(3-ethylbenzothiazoline-6-sulfonic acid) system. The results for the stevioside content in *S. rebaudiana* leaves correlate with those obtained by other methods. The stevioside content in *S. rebaudiana* plants showed large variation.

INTRODUCTION

Stevioside (**1**), a diterpene glucoside isolated from *Stevia rebaudiana* Bertoni [1], is reported to be *ca* 300 times sweeter than sucrose [2] and has recently become of importance as a natural sweetener. It is expected that the demand for this valuable compound for use in the food industry will be met by large scale isolation of stevioside from *S. rebaudiana* plants. The breeding of high stevioside-producing strains of *S. rebaudiana* depends on the availability of rapid and simple analytical methods for stevioside which allow a large throughput of samples.

Although there have been reports on the quantitative assay of stevioside by TLC–colorimetric methods which employ either 2,4 - dinitrophenyl hydrazine [3] or anthrone [4], by GLC after acid [5] or enzymatic [6] hydrolysis, by TLC-densitometry [7] and by HPLC [8, 9], these methods are time consuming and/or require a special instrument such as TLC scanner or HPLC. In addition, the methods involving acid hydrolysis of stevioside are less specific for stevioside since rebaudioside A (**2**), an analogue of stevioside isolated from *S. rebaudiana* [10], is also hydrolysed to form isosteviol as is stevioside. It was desirable, therefore, to establish a simpler method for the quantitative assay of stevioside which was suitable for the rapid analysis of a large number of *Stevia* samples.



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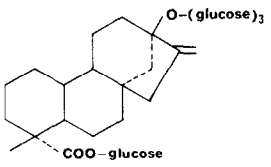

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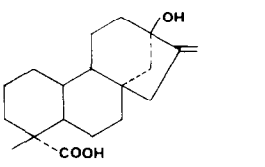

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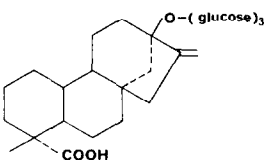

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An enzyme contained in crude hesperidinase prepared from a culture medium of *Aspergillus niger* [11] was reported to hydrolyse stevioside, affording steviol (**3**) and glucose [12]. Coupling of this enzyme with glucose oxidase makes it possible to quantify stevioside production by an enzymatic process, in which the hydrolysis of stevioside is followed by the production of hydrogen peroxide. The hydrogen peroxide generated by glucose oxidase is measured spectrophotometrically at 600 nm by the oxidation of 2, 2′-azino-di-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). The sequence of the reaction is as follows:

$$\text{Stevioside} + 3H_2O \xrightarrow{\text{Crude hesperidinase}} \text{Steviol} + 3\text{Glucose}$$

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{ABTS} \xrightarrow{\text{Peroxidase}} \text{Oxidized ABTS}$$

This report describes a simple colorimetric procedure for the enzymatic determination of stevioside in *S. rebaudiana* leaves based on the principle described above. The data presented suggest that in terms of simplicity the method is preferable to other methods.

RESULTS

Time course of the enzymatic hydrolysis

As shown in Fig. 1, the enzymatic hydrolysis of stevioside (up to 1 mM) was completed in 2 hr when incubated at 50° with 1 mg crude hesperidinase (0.25 units as stevioside hydrolysing enzyme and 7.2 units as hesperidinase) in a total volume of 1.0 ml citrate–phosphate buffer, pH 4.0 (an optimal pH reported by Sakamoto *et al.* [6]). The hydrolysis took 5 hr to go to

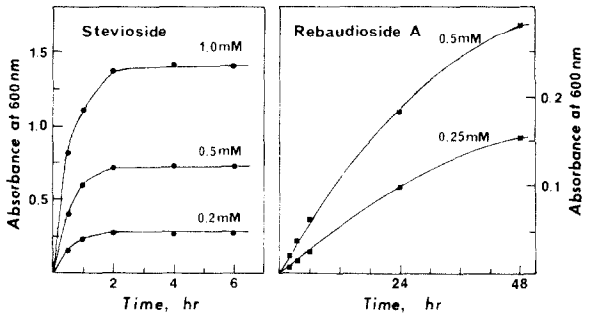

Fig. 1. Time course of the enzymatic hydrolysis of stevioside and of rebaudioside A.

completion at 40° and 2 hr at 60°. Therefore, the hydrolysis was carried out at 50° for 2 hr in subsequent experiments.

In contrast, the enzymatic hydrolysis of rebaudioside A took 48 hr to go to completion and less than 10% of rebaudioside A was hydrolysed within 2 hr.

Stoichiometry of the enzymatic hydrolysis

Table 1 shows the result of stoichiometric study of the enzymatic hydrolysis of stevioside and rebaudioside A. Three mol of glucose were generated from 1 mol of stevioside, showing that stevioside is completely hydrolysed to its aglucone, steviol. Only 1 mol of glucose, in contrast, was produced from 1 mol of rebaudioside A which contains four glucose molecules in its molecule.

Calibration curve

The calibration curves of authentic stevioside and the methanol extract of *Stevia* leaves were both linear in the range 0.1–1.0 μmol and 50–300 μl., respectively, and both lines passed through the origin.

Precision of the method

The mean recovery of 40, 50, and 80 μg of stevioside added to 0.1 ml of a methanol extract of *S. rebaudiana* leaves which contained 116 μg stevioside was 95.0, 98.8, and 96.7%, respectively.

Using the methanol extract of *S. rebaudiana*, we found a within-day precision (CV) of 1.7% for a stevioside content with a mean value ± s.d. (10) of 111 ± 1.90 mg/g dry wt leaves. Analysis of a sample of *Stevia* extract over a 6 day period showed a between-day precision (CV) of 5.8% (75.6 ± 4.4 mg/g dry wt, mean ± s.d.).

Specificity of the present method

A methanol extract of *S. rebaudiana* leaves was subjected to TLC (0.2 ml/plate) in two different solvent systems. The developed plates were each divided into 13 equal zones and each zone was extracted with methanol or water. An aliquot of the each eluate was then assayed for sterioside by the present method. The results obtained clearly showed both the presence and the location of stevioside, and suggested that the extract contained no other compounds which produce glucose as a result of enzymatic hydrolysis with crude hesperidinase (Fig. 2). The same experiment was performed using the methanol extracts prepared from 12 different plants of *S. rebaudiana* and the same results were obtained.

Comparison with other methods

Six randomly selected samples of *S. rebaudiana* leaves were analysed by the present method and by TLC-densitometry[7] and GLC[6]. The results are compared in Table 2. In most cases the stevioside content obtained by the present method was consistent with those obtained by the other methods. Using more *Stevia* samples, similar comparisons of the method with the TLC-densitometric method gave a correlation coefficient of $r = 0.965$ ($P = 0.01$) and a regression curve of $y = 1.10x - 7.99$ (Fig. 3).

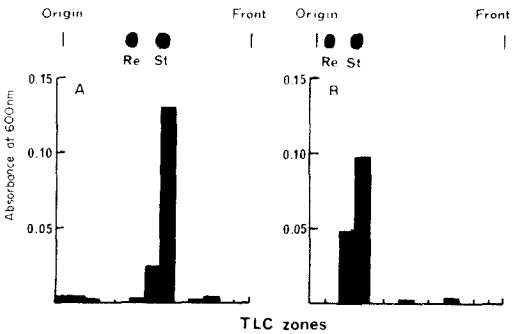

Fig. 2. Distribution of the compounds in the methanol extract of *Stevia* leaves which produce glucose on incubation with crude hesperidinase. (A) TLC (Si gel) developed with $CHCl_3$–MeOH–H_2O (90 : 65 : 9). (B) TLC (Si gel) developed with EtOAc–MeCOEt–HCO_2H–H_2O (5 : 3 : 1 : 1). Re, rebaudioside A; St, stevioside.

Table 1. Stoichiometry of the enzymatic hydrolysis of stevioside and rebaudoside A

Substrate added (μmol)	Glucose produced (μmol)
Stevioside*	
0.5	1.43
1.0	2.76
Rebaudioside A†	
0.25	0.29
0.5	0.53

*Incubation performed at 50° for 2 hr.
†Incubation performed at 50° for 48 hr.

Table 2. Comparison of the present method with the published methods for the determination of stevioside contents

Sample	Stevioside content (mg/g dry wt)		
	Present method	TLC-densitometric method[7]	GLC method[6]
1	107.0	106.0	96.2
2	116.0	110.0	97.2
3	86.1	85.0	89.9
4	93.1	96.8	89.7
5	85.5	83.4	95.0
6	95.0	105.0	93.1

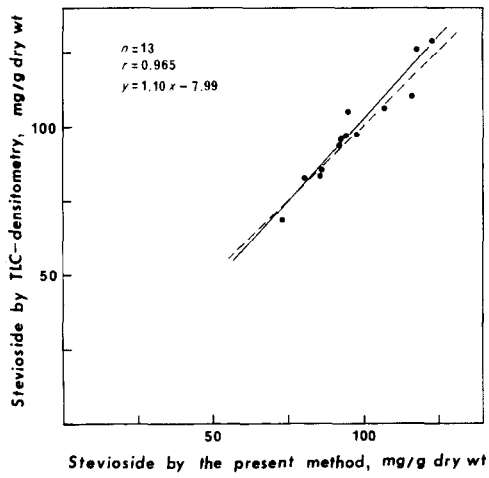

Fig. 3. Comparison of stevioside content measured by the present method and by the TLC-densitometric method. (—), Regression line; (----), $y = x$ line.

Variation of stevioside content in S. rebaudiana

By using the present method, the stevioside content of *S. rebaudiana* plants cultivated under the same conditions were analysed. As shown in Fig. 4, a large variation of stevioside content in *S. rebaudiana* leaves was observed. The average stevioside content of the leaves of S. *rebaudiana* was 92.7 mg/g dry wt with a maximum stevioside content of 123 mg/g dry wt and a minimum content of 63.0 mg/g dry wt ($n = 43$).

DISCUSSION

Crude hesperidinase was first reported to contain β-1,4, - rhamnoglucosidase (hesperidinase) and flavonoid-β-glucosidase[13]. Kohda and Tanaka[12] found that this crude enzyme preparation also had the activity to hydrolyse saponins and stevioside but that purified hesperidinase did not show any activity to hydrolyse these compounds. Stevioside was also reported[14] to be stable to normal β-glucosidases. These results suggest that a β-glucosidase which is highly specific for diterpene glucosides such as stevioside is present in the crude hesperidinase preparation.

As a crude enzyme preparation containing various β-glucosidases is used in the present method, some compounds such as oligosaccharides, polysaccharides and flavonoid glucosides which also yield glucose by hydrolysis with crude hesperidinase may interfere with the present method if present in large quantities in the methanol extract of *S. rebaudiana* leaves. However, the specificity test of the present method has demonstrated (Fig. 2) that such compounds are not present in the leaf extracts. In addition, the data obtained by the present method are in good agreement with those obtained by the methods specific for stevioside.

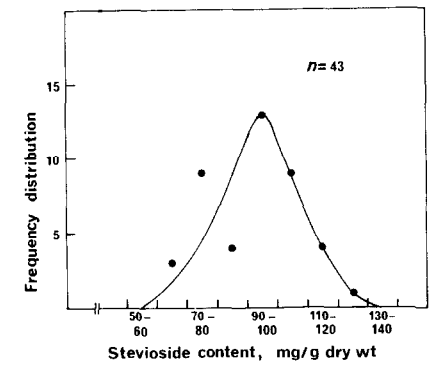

Fig. 4. Frequency destribution of stevioside content in *S. rebaudiana* plants.

The enzymatic hydrolysis of stevioside was completed within 2 hr at 50° if incubated with 0.25 units of crude hesperidinase. Sakamoto *et al.*[6] reported that hydrolysis of stevioside took 48 hr to complete at 40° when 3 mg of stevioside in 12 ml buffered solution (*ca* 0.3 mM stevioside), pH 4.0, was incubated with 6 mg crude hesperidinase. It is difficult to compare these two results since the specific activity of the enzyme preparation used by Sakamoto *et al.* was not indicated in their report. It may be that the enzyme preparation used in this study had a higher specific activity.

The rate of the enzymatic hydrolysis of rebaudioside A is less than 10% when compared with that of stevioside. In addition, only 1 mol of glucose is produced from 1 mol of rebaudioside A while 3 mol of glucose are generated from 1 mol of stevioside, which is consistent with the report by Sakamoto *et al.*[16] who showed that stevioside was hydrolysed to its aglucone, steviol (**3**) with crude hesperidinase but that rebaudioside A was enzymatically hydrolysed to form rebaudioside B (**4**). These results show that the hydrolase is much more specific for stevioside than for rebaudioside A and that the amount of glucose produced from rebaudioside A was *ca* 3% of that produced from stevioside even if rebaudioside A and stevioside are present in equal amount in the methanol extract of leaves of *S. rebaudiana*. The content of rebaudioside A in *S. rebaudiana* leaves was reported to be usually 30–50% of that of *stevioside*.

The present method may not be used for other *Stevia* species which contain stevioside since these plants may contain β-glucosidic oligosaccharides, polysaccharides or flavonoid glucosides in large amounts, although no other species belonging to the genus *Stevia* has been reported to contain stevioside until now. In spite of this, the results presented in this paper show that the present method can be used to evaluate the stevioside content of *S. rebaudiana* leaves. Because of its simplicity the method is especially applicable when many samples are to be analysed.

The large variation in the stevioside content in *S. rebaudiana* plants (Fig. 4) may allow us to select and establish high stevioside producing strains of *S. rebaudiana*. The breeding of such strains is now underway.

EXPERIMENTAL

Chemicals. A crude hesperidinase preparation and authentic samples of stevioside and rebaudioside A were obtained as gifts (see Acknowledgements). The sp. act. of the enzyme preparation was 7.2 units/mg as hesperidinase and 0.25 units/mg as stevioside hydrolysing enzyme. [1 unit hesperidinase is the amount of enzyme producing 1 μmol reduc-

ing sugar (measured as glucose) from hesperidine per min at 40°; 1 unit sterioside hydrolysing enzyme is the amount of enzyme producing 1 μmol glucose from stevioside per min at 50°.] A glucose assay kit (Boehringer No. 124028) containing glucose oxidase, peroxidase and ABTS was purchased from Boehringer–Mannheim–Yamanouchi, Tokyo. All other chemicals were commercially available and of reagent grade.

Plant material. Leaves of *S. rebaudiana* Bertoni were obtained from plants cultivated in the Medicinal Plant Garden of this University.

Enzymatic assay of stevioside and rebaudioside A. The reaction mixture containing an appropriate amount of stevioside or rebaudioside A (0.1–1.0 μmol) and 1.0 mg crude hesperidinase (0.25 units as stevioside hydrolysing enzyme) in 1.0 ml citrate–phosphate buffer, pH 4.0, was incubated at 50° for 2 hr and the reaction was terminated by heating the mixture in a boiling water bath for 3 min. The reaction mixture was then centrifuged at 700*g* for 2 min. 0.2 ml of the supernatant was mixed with 5.0 ml 0.1 M NaPi buffer, pH 7.0, containing glucose oxidase (50 units), peroxidase (4 units) and ABTS (5.0 mg) and incubated for 50 min at room temp. and the $A_{600\,nm}$ measured spectrophotometrically.

Enzymatic assay of the plant extract. Dried leaves (*ca* 1 g) of *S. rebaudiana* were refluxed twice with 30 ml MeOH for 1 hr. The combined filtrate was made up to 100 ml with MeOH. An aliquot (usually 0.2 ml) of the extract was put into a test tube and evaporated to dryness. The residue was dissolved in 1.0 ml citrate–phosphate buffer (pH 4.0) containing 1.0 mg crude hesperidinase and was incubated at 50° for 2 hr. In the blank, the residue was incubated without the enzyme preparation.

The reaction was terminated by heating the mixture in a boiling water bath for 3 min and 25 mg of PVP was added to the mixture to remove compounds which otherwise interfere with the glucose assay. After centrifugation at *ca* 700 *g* for 2 min, 0.2 ml of the supernatant was assayed for glucose.

Determination of stevioside content by TLC-densitometry. Determination of stevioside content using TLC-densitometry was carried out according to ref.[7]. An aliquot (5.0–10.0 μl) of the plant extract as well as authentic stevioside (4.1 and 8.2 μg) were applied to a pre-coated Si gel plate (Merck). After development with $CHCl_3$–MeOH–H_2O (90 : 65 : 9), the plate was sprayed with 10% H_2SO_4 soln and was heated at 120° for 10 min. The dark-grey spot of stevioside was scanned at 450 nm with a chromatoscanner (Shimadzu CS-920).

Determination of stevioside content by GLC. Quantitative analysis of stevioside was performed according to the method of Sakamote *et al.*[6].

Acknowledgement—We wish to thank Dr. M. Kawanishi, Tanabe Seiyaku Co. Ltd. and Professor O. Tanaka, Hiroshima University, for their generous gifts of crude hesperidinase and authentic samples of stevioside and rebaudioside A, respectively. Thanks are also due to Dr. S. L. Nickel, University of Toledo, U.S.A. and Dr. N. Hiraoka, Niigata College of Pharmacy for reviewing the manuscript.

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