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Original Article

Chromatographic determination of stevioside in leaf parts of in vitro and in vivo regenerated plants of Stevia rebaudiana

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Abstract

An HPLC method for determination of stevioside from the leaf parts of in vitro and in vivo regenerated plants of Stevia rebaudiana was developed. Pre separation method consisted of solvent extraction of leaf powder using various solvents like petroleum ether, methanol, diethyl ether and butanol followed by its purification using high performance liquid chromatography. The chromatographic separation was realized using a C18 column, mobile phase consisting of methanol: water with UV detection at 210 nm. The limits of determination of stevioside were 28.17 µg/ml and 19.7 µg/ml in vitro and in vivo plants respectively.

Key words: Stevioside, in vitro, in vivo, Stevia rebaudiana

Introduction

Stevia rebaudiana Bertoni (Composite) is one of the most commercially successful plants, native to the valley of Rio in highlands of Paraguay [1]. Sweet tasting stevioside (STS) is a diterpene glycoside present in the leaves of the plant Stevia that is 300 times sweeter than sucrose and is non-caloric, non-fermentable, non discoloring with lengthy shelf life [2]. Different plant organs however, contained different amount of steviol glycosides (SGs) which declined in the order as leaves, flowers, stems, seeds and roots [3]. Along with these glycosides, plant also contains phenylpropanoids, caffeic acid, chlorogenic acid, scopoletin, umbelliferone, quercetin, avicularian, polystachoside and isoquercitrin extracted from leaves [4]. Extraction and refining of glycoside-based sweeteners from Stevia leaves involves many processes like subcritical fluid extraction [5]. Also, chromatographic separations using high performance liquid chromatography (HPLC), thin layer chromatography and spectroscopic methods like near infrared spectrometry, VIS spectrometry are commonly used methods for the determination of sweet tasting stevioside in the plant material [6,7]. Ahmed and Dobberstein [8] developed an HPLC method for determination of eight diterpene glycosides with satisfactory resolution on two protein columns in series after extraction with chloroform. The aim of the present work was to compare and determine the stevioside concentration in vitro raised and in vivo mother plants of Stevia rebaudiana using high performance liquid chromatography.

Materials and methods

In vitro plant regeneration

Leaves excised from healthy in vivo grown mother plants maintained at the University campus were washed under running tap water for half an hour, followed by washing in 1% (v/v) liquid detergent (Teepol) for five minutes. Explants were further sterilized using bevisitin (0.1% w/v) for 8 minutes followed by washings with tap water. Ultimately, the explants were disinfected with aqueous solution of mercuric chloride (0.05% w/v) for 2 minutes followed by 3-4 rinses in...
Fig. 1 In vitro plant regeneration of Stevia rebaudiana

A) Formation of callus on NAA (29.4 µM) and K (4.65 µM) supplemented MS medium. B) Shoot differentiation from callus on 18.6 µM of K medium forming innumerus multiple green leafy shoots C) Formation of roots on half strength basal MS medium D) A well established plant growing under green house conditions.

sterilized distilled water. Explants (4-5 mm) in size were excised, planted on variously supplemented Murashige and Skoog’s medium [9]. The media used in all the experiments consisted of MS basal salts supplemented with 2% sucrose (Himedia, Mumbai, India) and 1% agar. The pH of the medium was adjusted to 5.8 before the addition of agar. The media was autoclaved at 121°C for 15 minutes. An aliquot of 75 ml was dispensed into tissue culture bottles (Make Glasil, New Delhi) and all the cultures were maintained in the growth room at a temperature of 25 ± 2°C, 16 hr light/8 hr dark photoperiod and continuous illumination provided by cool white fluorescent tubes at 50µmolm⁻²s⁻¹. Thirty replicates were used for each treatment and each experiment was repeated thrice.

The leaf explants were placed horizontally in callus induction MS medium supplemented with varying concentrations of NAA and K. The callus raised on this medium was further transferred onto K supplemented medium for multiple shoot proliferation. The microshoots thus formed were rooted on half strength basal MS medium for root induction. For hardening, the in vitro raised plantlets were passed through different weaning stages. Initially, plantlets were acclimatized in moist cotton followed by their transfer to plastic cups containing potting mixtures of soil: vermicompost (1:1). Potted plants were then shifted to green house conditions from where they were moved to open field conditions for further growth.

Extraction of Stevioside from in vitro and in vivo plants

Leaves were collected from mature field grown healthy 2 yrs old in vitro raised and in vivo mother plants of Stevia rebaudiana. The collected leaf explants were washed under running tap water to remove dust and microbes sticking to the surface. Further leaves were air dried, crushed to fine powder using mortar and pestle. The grounded fine powder was used for the extraction of stevioside. 160gm of dried leaf powder was soaked in 500ml petroleum ether for 4-5 hrs. The extract was filtered using Whatmann paper no.1 and the resultant residue was then treated with hot methanol on boiling water
Fig. 2 HPLC chromatogram of stevioside *in vitro* and *in vivo* plants

Fig. 2 A) Chromatogram showing stevioside peak *in vitro* raised plants with 46.24 % peak area.  
B) Chromatogram showing stevioside peak *in vivo* mother plants with 28.97% peak area.  
C) Chromatogram showing stevioside peak in standard with 79.90% peak area.
Table 1. Showing the amount of stevioside in different samples

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Peak number</th>
<th>Retention Time</th>
<th>Area</th>
<th>Area Percent</th>
<th>Stevioside (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11</td>
<td>15.947</td>
<td>2102806</td>
<td>46.24</td>
<td>28.17</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>15.861</td>
<td>2994173</td>
<td>28.97</td>
<td>19.7</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>16.0</td>
<td>2125769</td>
<td>79.90</td>
<td>------</td>
</tr>
</tbody>
</table>

residue was then treated with hot methanol on boiling water bath for 2hrs. The treated extract was filtered, washed with 50ml methanol three times. The filtrate was further concentrated in rotary flash evaporator at 60°C and to this dried extract 500ml of distilled water was added. Extract was further separated using separatory funnel with 100 ml of diethyl ether to remove green colour of the extract. Lower transparent layer was collected and treated with Butanol. Finally the butanol layer was collected and refrigerated at 4°C to get fine crystals. Crystals were resuspended in methanol and used for further purification of stevioside.

Chromatographic analysis

The Waters HPLC system was used for the analysis of stevioside. Operating conditions for HPLC includes C18 column, mobile phase methanol: water (8:2), ambient temperature, flow rate of mobile phase was 0.9 ml/min and UV detection at 210nm.

Standard preparation

Standard Stevioside (Make Sigma Aldrich) was prepared by taking 5 mg standard in 2.5ml acetonitrile: water (8:2). Stock standard concentration of 2mg/ml.

Results and discussion

The present investigation was aimed to compare the concentration levels of stevioside in vitro raised and in vivo mother plants of Stevia rebaudiana. For in vitro plant regeneration, leaf explants were cultured on NAA (29.4 µM) and K (4.65 µM) supplemented MS medium for callus induction. Good callus growth occurred from the cut ends as well as entire surface of explant after 7-8 days of culturing and within 3-4 weeks, large friable light brown calli was formed in nearly 95% of the cultures. High frequency shoot differentiation from the leaf callus occurred when the latter was transferred to MS medium supplemented with different concentrations of K (4.65 – 23.25 µM). However, 18.6 µM of K was most effective for inducing 90% of the cultures to form shoots and induced greatest number of shoots (too numerous to count) and highest average length of the shoot (8.8 ± 0.05 cm). The microshoots thus formed were cultured on half strength basal MS medium for root induction. Roots appeared in the form of cluster after 12 days of culturing. Initially roots formed were shorter in length, but with passage of time length and number increased further. Growth of roots was observed in 88% of the cultures. The plantlets were further transferred to the culture bottles containing moist cotton covered with perforated plastic covers and were kept for a period of 20 days under growth room conditions for acclimatization. After that the regenerants were shifted to plastic cups containing potting mixture of soil: vermicompost (1:1) and were kept inside the growth room for another 15 days. The plants were thoroughly monitored and watered and after 35 days of initial acclimatization, the plants with newly formed leaves were established in soil in earthen pots and were shifted to green house. Fig. 1 shows different stages of in vitro plant regeneration from leaf explants. Similar reports of plants propagation through in vitro culturing of explants like leaf, nodal segments, internodal and roots for the extraction of steviol glycosides are also available in the literature [2, 10].

The first step of pre-separation was to extract stevioside from the excised leaf explants using different organic solvents. For this powdered Stevia leaves from both in vitro and in vivo plants were soaked in petroleum ether followed by extraction with methanol and then diethyl ether. Further extraction was carried out with butanol, which upon evaporation in vacuum and resuspension in methanol yielded crystals at 4°C and the methanolic mother liquor was separated and loaded on HPLC system. Various workers used mixtures of solvents for extraction of sweet tasting glycosides from Stevia rebaudiana like a mixture of water, ethanal, ethyl acetate and cyclohexane [11]. Similarly, for quantitative analysis of sweet tasting glycosides, techniques like HPLC and near-infra red spectroscopy are widely used [12, 13].

For HPLC analysis, the system was scanned over a range 200-400nm however, the optimum wavelength selected was 210 nm. The HPTLC scan of both the samples along with the standard is presented in Figure 2. A similar retention time of stevioside was observed in all the test samples as compared with the standard. Standard peak showed retention time of 16 with 79.90 % peak area whereas for in vitro and in vivo plants retention peaks were observed at 15.9 and 15.8 with 46.24 and 28.94 % peak area respectively (Table 1). The linearity was determined in the concentration range of 10-250 µg/ml. A linear calibration curve was obtained with a correlation coefficient of 0.99. The concentration of stevioside as calculated was 28.17 µg/ml and 19.7 µg/ml in vitro and in vivo plants respectively. Bovanova et al [14] also used HPLC based chromatographic method for determination of stevioside in the plant material and food samples prepared from Stevia rebaudiana. The limits of determination of glycosides were found to be 5µg/ml and 8µg/ml for leaf extracts and juice samples respectively. Although numbers of reports are available in literature regarding the estimation of
stevioside in plant and food samples however, not much is available regarding a comparative study of stevioside content in vitro and in vivo plants. This is a first of its kind report where stevioside content was found to be higher in vitro raised plants as compared to in vivo mother plants. Another study by Bondarev et al [15] reports that the quantitative composition of SG in in vitro plants was identical to that of intact plants and even after a long-term cultivation of in vitro plants, the significant difference in SGs was not observed.

Conclusion

The present investigation reports for the first time a comparative analysis for the occurrence and amount of stevioside in in vitro raised and in vivo mother plants. The stevioside content was found to be higher in vitro raised plants as compared to in vivo grown mother plants when leaf explants from both were analyzed using HPLC.

References


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