SHORT REPORT

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IMPROVED ISOLATION AND PURIFICATION OF STEVIOSIDE

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Abstract

Stevioside, a sweet-tasting glycoside, was isolated from Stevia (Stevia rebaudiana *Bertoni) in three main steps, namely hot water extraction, decolorization by electrolysis, and simultaneous decolorization and demineralization by ion exchange. Stevioside of 70-80 % purity was obtained in 8-10 % yield.*

Stevia *(Stevia rebaudiana* Bertoni) or "ya wan" (sweet grass) has been recently grown in Thailand. It is a tropical shrub indigenous to South America and is cultivated almost entirely for stevioside, the main sweet glycosidic diterpenoid substance contained in the leaves. The latter has a well-established structure and is said to be 100-300 times sweeter than sucrose. Presently, it is permitted as a non-nutritive sugar substitute, mainly in Japan. Numerous papers and patents have appeared in the literature on the methods of isolation, purification, analysis, safety and applications of this natural sweetener and related compounds. Of importance concering the safety aspects, a recent report[1] suggests that steviol, the aglycone part of stevioside, could be metabolically activated to yield a mutagenic form. Earlier studies[2] indicated that stevioside, along with the related compound rebaudioside A, could be degraded into steviol and efficiently absorbed in rat intestine. It therefore appears that similar metabolic conversion of stevioside to an active mutagenic species by human enzymic systems is a possibility. However, no reports have thus far appeared indicating that adverse effects have resulted from human use of Stevia products.

Meanwhile, the fact that steviol, derivable from stevioside[3;4] is involved in the biosynthesis of gibberellins[5-7] and itself has a gibberellin-like activity on certain plants[8-11], promises an attractive alternative outlet to the direct human consumption of stevioside. In fact, even stevioside itself, coupled with a sugar hydrolase enzyme, has been claimed to

be effective as a plant growth regulator[12]. Apparently, the key compound in this case is also steviol, so it comes as a surprise that stevioside, or its related glycosidic compounds alone, has also been reported to have similar effects on certain plants[13].

As the first step in this line of development, we are now reporting another simple, practical method of isolating stevioside in a relatively pure form from Stevia. The method is a modification of that of Kunihiko *et al*[14], who used electrolysis for partial purification of the crude aqueous Stevia extract. In our hands, this procedure for preliminary decolorization has proved to be the most efficient.

The typical procedure is described below.

Hot Water Extraction: 20 litres of water at 90-100° C was used to extract 1 kg of dry (10% moisture) Stevia leaves for 1 hour, whereupon, after one coarse filtration, 16-17 litres of the aqueous extract was obtained and then left standing at ambient temperature for 7 days.

Decolorization by electrolysis: DC current (30 amperes) was passed for 2 h via 2 pairs of aluminium plate electrodes (39 × 29 cm) through the aqueous extract in a high-form rectangular tank (34 × 31 × 21 cm) into which 0.02 mole of HCl/litre of extract had been added. The electrodes of each pair were 3 cm apart. The resulting mixture was then filtered and the aqueous solution (13 litres) obtained was subjected to a second electrolysis for 20-30 minutes under the same conditions. After filtration, a clear pale yellow solution (12 litres) was obtained.

Decolorization and Demineralization by Ion Exchange: The doubly-electrolysed solution was passed through a column (40 × 7 cm) of a mixed-bed ion-exchage resin e.g., Amberlite MB-1 (Amberlite IR-120 + Amberlite IRA-401), and the resulting clear, colourless eluate of conductivity < 50 μS was collected. The minimum volume of the mixed resin required to achieve this was 1 litre. After evporation to dryness, the eluate gave a white to pale yellow non-bitter solid in 8-10% yield. Analysis by HPLC indicated a stevioside content of 70-80 % in the extracted solid, which also contained small amounts of other related glycosides.

Operating conditions for HPLC were : detection wavelength - 210 nm; column — radial - pack L-C cartridge, 8 mm × 10 cm C_{18}; eluting solvent - $CH_3CN:H_2O$ (4:1) : retention time for stevioside - 4 min. A typical chromatogram of the extracted product is shown in Fig. 1 b. It was found that a linear relationship exists between amount of sample and peak height for the amount of sample tested (up to 70 μg).

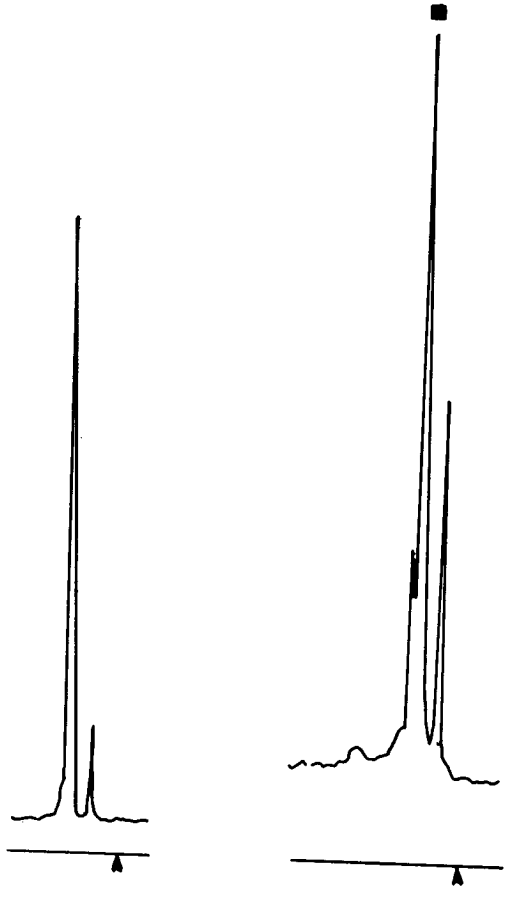

Fig 1. a. Standard sample of stevioside (small peak is impurity)
b. Extracted product (■ = stevioside)

The above method of isolation of stevioside has an important advantage of forgoing the use of expensive organic solvents, as well as using a minimum number of expendable common chemicals namely hydrochloric acid (for electrolysis and resin regeneration) and sodium hydroxide (for resin regeneration). Ordinary aluminium sheets, used as electrodes in the electrolysis step, can also be reused for a few successive operations, during which time they are slowly worn out and finally replaced. The crucial factor in the whole operation is the life-time of the expensive ion-exchange resins used for the maximum purification of the Stevia extract. However, pretreatment of the extract by electrolysis twice efficiently precipitates out most impurities from the extract by adsorption on the gelatinous

aluminium hydroxide formed, with minimum loss of the sweet components. The degree of decolorization achieved in this partial purification step turns out to be more than 99%. (This was calculated from absorbances measured at 420nm and 670nm as suggested by Cheng and Chang.[16]) Consequently the decolorizing resin used in the next step does not suffer from undue degeneration. The exhausted mixed resin was also capable of being reused at least 17 times after regenerations by standard procedures[15] without appreciable loss of capacity and efficiency. Furthermore, the use of HCl as opposed to NaCl in the electrolysis step, considerably reduces the amount of electrolyte that must be added for efficient flow of current through the extract, with a consequent reduction of the amount of resin needed to remove it in final step. Apparently, the acid concentration (about 0.07%) in the extract (pH 4) is too low for any undesirable hydrolysis of stevioside to take place, but conveniently allows a single step pass of the solution through a decolorizing and deionising mixed-bed resin, which is quite sufficient for the final purification. (Semi-quantitative test with morin[17] indicated not more than 200 ppm of aluminium III in the final product.) Finally, the 7-day incubation period of the extract before electrolysis was accidently found to be somewhat beneficial for decolorizing efficiency, presumably because of partial decomposition of the pigments, with no effect on the quality of the final product. The overall process, however, still has a small drawback in the rather large volumes of aqueous solutions that must be handled all through. However, we believe some further reduction of the extracting solvent initially added should be possible without serious loss in yield or quality of the final product.

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บทคัดย่อ

ได้แยกสตีเวียไซด์ซึ่งเป็นกลัยโคไซด์ที่มีรสหวานจากสตีเวีย โดยการแยกแบ่งเป็นสามขั้นตอนคือ การสกัดด้วยน้ำร้อน การฟอกสีด้วยไฟฟ้า การฟอกสีควบกับการกำจัดไอออนโดยกระบวนการแลกเปลี่ยนไอออน จะได้สตีเวียไซด์บริสุทธิ์ 70-80% ในปริมาณ 8-10% ของวัตถุดิบ