A High Activity of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase in Chloroplasts of Stevia rebaudiana Bertoni

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Stevia leaves (Stevia rebaudiana Bertoni; Compositeae) accumulate sweet glycosides, the aglycone of which is a diterpene derivative, steviol. The activity of the rate-limiting enzyme of the isoprenoid pathway leading to steviol, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase was found to be extremely (over 150-fold in the specific activity) higher in chloroplasts of stevia leaves than the activity of Spinacia oleracea and about 10-fold higher than that of Solidago altissima L., which also belongs to the Compositeae family. There was no difference in apparent K_m values for HMG-CoA and inhibitions by mevastatin between the activities of thylakoid membranes from S. rebaudiana and S. altissima L. Those activities were almost the same level in microsomal fractions of the three plant leaves.

Key words: HMG-CoA reductase; isoprenoid; secondary metabolite; stevia sweetener

The leaves of Stevia (St.) rebaudiana Bertoni, a wild shrub belonging to the Compositeae family, accumulate the stevia sweeteners, stevioside and Reb-A, as the main constituents, for up to 5-10% of dry weight. Stevioside (13-O-beta-sophorosyl-19-O-beta-glucosyl steviol; triglucosylated form) and Reb-A (2’-O-beta-glucosyl-13-O-beta-sophorosyl-19-O-beta-glucosyl steviol; tetraglucosylated form) are the glycosides of the common aglycone, steviol (ent-13-hydroxy kaurn-en-19-ol acid). The diterpene derivative, ent-kaurenolic acid, is believed to be the precursor for steviol as the case for GA compounds. We detected the activities catalyzing the glucosylation from UDP-glucose to steviol and to its glycosides, and recently isolated and characterized three different kinds of GTase acting on steviol and steviol-glycosides from stevia leaves. On the other hand, the characterization of steviol synthetizes remains to be solved.

The enzyme HMGR (EC 1.1.1.34) catalyzes the conversion of HMG to mevalonic acid, which is a precursor to all isoprenoid compounds. In plants, these are antimicrobial terpenoid phytoalexins, toxic steroids, glycoalkaloids, sterols, plant growth regulators such as GA and abscisic acid, electron transfer components such as plastoquinone and ubiquinone, carotenoids, the phytol moiety of Chl and natural rubber. These compounds seem to be vital physiologically except for natural rubber. The sites of HMGR activity in plants appear to be cytosolic membrane recovered as microsomal fraction, mitochondria, and plastid membranes.

As steviol is the diterpene derivative synthesized via the isoprenoid pathway, we examined the activities of HMGR in chloroplasts and microsomal fractions of stevia leaves. The crude mitochondrial fraction obtained from the post-chloroplast fraction by centrifugation at 15,000 x g for 15 min showed a negligible activity, as compared with those observed in other chloroplastic and microsomal fractions. HMGR activities were examined with those of several agriculural species, Spinacia (Sp.) oleracea, and of wild Compositeae species, Solidago (So.) altissima L. (Table I). Although there was no difference in the microsomal activities among three plant species, HMGR in chloroplasts from stevia leaves was found to be extremely high even in stationary phase without any treatment that induces this enzyme, such as phytoalexin elicitor and slicing or chemical treatment. The activity was over 150-fold (specific activity) and 300-fold (on a mg Chl basis) higher than that found in chloroplasts of spinach leaves. The increased activity was 9.6-fold (specific activity) and 8.5-fold (on a mg Chl basis) over the activity of So. altissima. The chloroplastic HMGR was found to be a membrane-bound enzyme as is the case with

Table I. HMG-CoA Reductase Activities in Isolated Chloroplasts and Microsome Fractions from Stevia, Common Goldenrod, or Spinach Leaves

<table>
<thead>
<tr>
<th>Plant leaves</th>
<th>HMGR-CoA reductase activity in chloroplasts</th>
<th>HMGR-CoA reductase activity in microsome</th>
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<tbody>
<tr>
<td></td>
<td>nmol h^-1 mg^-1 protein</td>
<td>nmol h^-1 mg^-1 Chl</td>
</tr>
<tr>
<td>St. rebaudiana</td>
<td>8.41 ± 0.56 (168.7)</td>
<td>133.0 ± 13 (317.1)</td>
</tr>
<tr>
<td>So. altissima</td>
<td>0.87 ± 0.09 (17.5)</td>
<td>15.6 ± 0.2 (37.1)</td>
</tr>
<tr>
<td>Sp. oleracea</td>
<td>0.05 ± 0.01 (1.0)</td>
<td>0.4 ± 0.02 (1.0)</td>
</tr>
</tbody>
</table>

Stevia leaves were harvested in early summer from fully expanded shoots. The leaves of common goldenrod (So. altissima) were cut from the field-grown shoots. Spinach plants were obtained from local market. The leaves supplemented with 10% (v/v) polyvinylpolyoxolone and 1% sodium iso-ascorbate were homogenized in the isotonic buffer (50 mm potassium phosphate, pH 7.8 containing 5 mm MgCl_2, 25 mm 2-ME, 1 mm EDTA, 1% BSA and 0.33 mm sorbitol), then the precipitate obtained by centrifugation between 150 x g for 2 min and 1500 x g for 20 min was further purified by Percoll density gradient centrifugation to obtain chloroplasts. The post-chloroplast supernatant was centrifuged at 15,000 x g for 15 min, then 105,000 x g for 1 h to precipitate mitochondrial and microsomal fraction, respectively. HMGR-CoA reductase activities were measured radiochemically, but silica gel was scraped off both upper and lower halves of the TLC, and the measuring was done using three independent preparations. Numbers in parentheses indicate the increase fold of activity over the observed activity in chloroplasts prepared from spinach leaves.

Abbreviations: GA, gibberellins; Chl, chlorophyll; HMG, 3-hydroxy-3-methylglutaryl CoA; HMGR, HMG reductase; IC_50, concentration required for 50% inhibition; 2-ME, 2-mercaptoethanol; Reb-A, rebaudioside-A.
Fig. The Double Reciprocal Plots of Initial HMGR Activities in Thylakoids of *St. rebaudiana* and *S. altissima* L.

The isolated chloroplast fraction was diluted with three volumes of the hypotonic buffer (50 mM potassium phosphate, pH 7.8 containing 5 mM MgCl₂, 25 mM 2-ME, and 1 mM EDTA), incubated for 15 min at 4 °C, then centrifuged at 25,000 g for 20 min to prepare thylakoids as the precipitate. The HMGR activities in the thylakoid fractions of *St. rebaudiana* (●) representing the ordinate is [1/v × 10⁻⁸], or of *S. altissima* L. (○) representing the ordinate is [1/v]. Activities were measured spectrophotometrically as in Fig. 11, as the difference between HMGR-dependent and HMGR-independent oxidations of NADPH. The concentrations of 2.5 mM-HMGR were varied from 5 to 200 μM, while NADPH maintained the concentration of 0.135 mM.

Table II. Comparison of Characteristics of HMGR-CoA Reductase in Thylakoids of Stevia or Common Goldenrod Leaves

<table>
<thead>
<tr>
<th>Properties</th>
<th><em>St. rebaudiana</em></th>
<th><em>S. altissima</em> L.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimum pH</td>
<td>7.5</td>
<td>7.5</td>
</tr>
<tr>
<td><em>Kₐ</em> for HMGR-CoA</td>
<td>8.62 μM</td>
<td>6.94 μM</td>
</tr>
<tr>
<td>Inhibition by mevastatin (IC₅₀)</td>
<td>24.8 μM</td>
<td>32.9 μM</td>
</tr>
</tbody>
</table>

HMGR-CoA reductase activities in thylakoid membranes were measured spectrophotometrically, as described in the legend for the figure.

other plant species,⁹ because almost all activity was recovered in the thylakoid fraction after separation of stroma and thylakoids by centrifugation in hypotonic buffer. Steviol, steviol-glycosides, and ent-kaurenoic acid had no effect on the HMGR activity in thylakoids from stevia leaves. In the substrate saturation experiments, HMGR in stevia thylakoids was inhibited by higher concentrations of HMGR (Fig.), but a similar characteristic is not observed in the case of the enzyme of *S. altissima* L. Other properties of the stevia enzyme in thylakoids such as optimum pH (7.5), apparent *Kₐ* for HMGR (8.62 μM), and inhibition by mevastatin (IC₅₀ = 24.8 μM) which is the specific inhibitor for HMGR, were almost the same levels or values of the enzyme in thylakoids from *S. altissima* L. (Table I).

Stevia HMGR activity in chloroplasts, was very much higher than that of other two plant species, which never accumulate steviol-glycosides, while those activities in microsomal fractions from three plant leaves were almost the same level (Table I). These results need be no matter for wonderment, because treatment of wounded potato tuber tissues with the sesquiterpenoid phytoalexin elicitor increased HMGR activity in the microsomal but not in the chloroplast (organelle) fractions,⁹ indicating the independent regulation or properties of HMGRs localized in different cellular compartments. The chloroplastic and microsomal HMGRs in pea seedlings appear to be different species and not simply compartmented forms of the same protein,¹² because these enzymes are different in pH optima and apparent *Kₐ* values for the substrate.

Higher plants including stevia should contain a scant amount of GA (for example; GA₄ + GA₉ + GA₂₀ = 27.4 ng g fresh weight in the ear of rice plant cv. Nihonbare)¹⁰ to function as a plant growth regulator. Stevia leaves accumulate the sweet steviol-related glycosides up to 5–10% of dry weight. Assuming that stevia leaves contain 90% water and accumulate an equal amount of both stevioside (5% of dry weight) and Reba-A (5% of dry weight), the weight of total steviol-skeleton would amount to 3.6 mg g fresh weight of stevia leaves. Accordingly stevia plants can synthesize a large amount of steviol depending on the mevalonate pathway.

There may be a consideration that steviol is an essential compound for *St. rebaudiana*, which has furnished evolutionarily a high activity of HMGR in chloroplasts, and that steviol-glycosides may serve as the reservoir of steviol. On the other hand, because there is no indication of the physiological role of steviol, it may be a waste or shunt product as the case of other plant secondary metabolites.¹³ The high HMGR activity would result in the production of a large amount of mevalonic acid, a precursor of many vital isoprenoid compounds: accordingly, the excess of mevalonic acid should be shunted to synthesize non-vital compounds. Stevia chloroplasts must metabolize the mevalonic acid to ent-kaurenoic acid, and convert it to steviol avoiding the synthesis of an excess of GA, the precursor of which is also ent-kaurenoic acid.¹⁰ Recently we detected an ent-kaurenoic acid 13-hydroxylase which converts ent-kaurenoic acid to steviol, in chloroplasts of stevia leaves but not in those of spinach and *S. altissima*.¹⁵

References