한국농화학회지 제 40 권 제 6 호(1997)
Agricultural Chemistry and Biotechnology
Vol. 40, No. 6, pp. 501~507(1997)

Characterization of *ent*-Kaurenoic Acid 13-Hydroxylase in Steviol Biosynthesis of *Stevia rebaudiana* Bertoni

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Abstract : Chloroplasts isolated from *Stevia rebaudiana* Bertoni leaves contained an enzyme activity which catalyzed hydroxylation of *ent*-kaurenoic acid (*ent*-kaur-16-en-19-oic acid; *ent*-KA) to steviol (*ent*-13-hydroxy kaur-16-en-19-oic acid), the diterpenoid carboxylic alcohol which is the aglycone of sweet stevioside-related glycosides. [^{14}C]-methylated *ent*-KA was used to localize *ent*-KA hydroxylase. [^{14}C]-methyl-KA was most actively was transformed into methyl-steviol in chloroplast. The enzymatic activity was found in stroma fraction but not in thylakoid membrane in *Stevia rebaudiana* Bertoni. However, *ent*-KA 13-hydroxylase activity was not detected in stroma fraction of either *Spinacia oleracea* and *Solidago altissima*. The reaction products using [^{14}C]-methyl-KA were purified and identified on TLC autoradiogram. The hydroxylation of *ent*-KA from stromal protein to form steviol required NADPH and oxygen. FAD and riboflavin stimulated the enzyme activity 1.5-and 1.7-fold, respectively. It also turned out that the activity of this enzyme using methyl-KA as a substrate was 16.7% that of *ent*-KA. The purified *ent*-KA 13-hydroxylase did not act on *t*-cinnamic acid, 4-hydroxyphenyl acetic acid, choline and resorcinol, known as monooxygenase and hydroxylase substrates.(Received November 26, 1997; accepted December 11, 1997)

Introduction

Only one plant species, *Stevia rebaudiana* Bertoni belonging to Composite family, accumulates sweet glycosides, stevioside and rebaudioside-A.[1-4] The physiological role and the significance of accumulation in a high quantity of sweet glycosides (5-10% on the basis of dry matter),[2,3] have not yet been elucidated. Extract of the stevia leaves and isolated sweet glycosides are commercially available in Japan, Korea, and several countries where they are used as the noncaloric sugar substitute to sweeten a variety of foods including pickled vegetables, dessert items, soft drinks, and confectioneries.

The aglycone of these sweet glycosides, steviol, is diterpenoid compound (Fig. 1). [^{14}C]-Kaurene is reportedly incorporated into steviol skeleton,[5] thus steviol is believed to be synthesized from *ent*-kaurenoic acid (*ent*-KA).[6] *ent*-KA is also the precursor of gibberellins (GAs).[6] Accordingly, the step of steviol synthesis from *ent*-KA is the important diverging point from the biosynthetic route of GAs.

In several plants, the sub-cellular location of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a rate-limiting enzyme of the mevalonate pathway,[7] appears to be bound to the membranes of chloroplasts, endoplasmic reticulum, and mitochondria.[8] The synthesis of β-carotene and other chloroplast terpenoids from acetyl-CoA via acetoacetyl-CoA, HMG-CoA, and mevalonate is established from experiments using isolated chloroplasts.[9] Railton *et al.*[10] showed the synthesis of *ent*-kaurene in chloroplasts from higher plants. Stoddart[11] reported the incorporation of *ent*-KA into GAs by chloroplasts of *Brassica oleracea*. Moreover, metabolism of *ent*-kaurenol, *ent*-kaurenal, and *ent*-KA to GAs and related compounds was

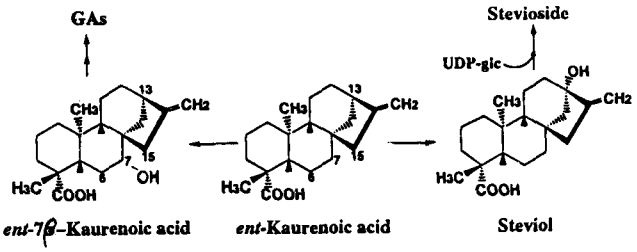

Fig. 1. Biosynthetic route of *ent*-kaurenoic acid to stevioside and gibberellins.

Key words : *stevia rebaudiana* bertoni, stevia, *ent*-kaurenoic acid, steviol synthesis, chloroplasts, stroma
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detected in chloroplasts of germinating *Hordeum distichon* grains.[12] These results indicating the localization of mevalonate pathway and probably of *ent*-KA synthesis in chloroplasts of higher plants prompted us to investigate and characterize the steviol synthesizing activity from *ent*-KA in chloroplasts of stevia leaves.

In this study, we have detected the activity of steviol biosynthesis from *ent*-KA in stroma fraction, substrate specificity and cofactor requirement of *ent*-KA 13-hydroxylase purified from *S. rebaudiana*.

Materials and Methods

Plant material

Stevia (*S. rebaudiana*) plants were cultivated in field, and the leaves were harvested between early and late summer from fully grown shoots, frozen in liquid nitrogen and kept at -30℃.

Purification of *ent*-KA and steviol

The purification steps of *ent*-KA was followed by method of Metzger *et al.*,[13] and modified. Additional purification procedures of two cycles of TLC and repeated HPLC chromatographic separations were needed to obtain pure *ent*-KA.[2] The compound was identified as *ent*-KA by GC/MS analysis(Hitachi M80-B mass analyzer fitted with M-1010 data system), comparing with the published spectrum of the authentic *ent*-KA.[14] Steviol was prepared from stevioside and purified as reported previously.[3]

Preparation of [^{14}C]-methyl-KA, methyl-KA, and methyl-steviol.

[^{14}C]-methyl-KA was prepared by modification of the methods of Alvarez *et al.*[15] A solution of 13 mg of *ent*-KA in 500 m*l* of dimethyl acetamide was treated with 33 mg of sodium bicarbonate, 18.3 mg of methyl iodide, and of [^{14}C]-methyl iodide (558.70 MBq/mmol; Du Pont NEN Research Products) under anhydrous conditions. The mixture was stirred at room temperature in the dark for 72 hr, then poured into 1 m*l* of 10% aqueous sodium chloride solution. The crystalline material thus obtained was collected by centrifugation at 15,000 xg for 8 min. The pellet was washed with water, and dried in vacuo, 10.9 mg of [^{14}C]-methyl-KA (83% from *ent*-KA) was obtained. The product was taken up in MeOH and purified by prep. TLC on Kieselgel GF_{254} plates (20 × 20 × 0.25 mm) with petroleum ether : diethyl ether : EtOH (6 : 2 : 1) as solvent. The radioactivity of the reaction product was visualized and quantified using a AMBIS 4000 radio analytic imaging system. A zone at R_f 0.72-0.85 was removed and eluted with MeOH. The prepared [^{14}C]-methyl-KA was used for enzyme assays. *ent*-KA and steviol were also methylated with cold methyl iodide by the same procedure to obtain authentic methyl-KA and methyl-steviol. These samples were identified by CIMS(*iso*-butane, probe, 70 eV). They were used as the authentic standards.

Isolation of intact chloroplasts and preparation of thylakoid and stroma fractions.

Freshly harvested stevia leaves (100 g, fr. wt) were cut into small segments and immediately homogenized at 4℃ by blending twice for 5 sec with 300 m*l* of 50 mM potassium phosphate buffer, pH 7.8 containing 10 mM EDTA, 5 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 330 mM sorbitol, and 20 g insoluble-PVP(grinding medium). The homogenate was briefly filtered through four layers of cheese cloth and the filtrate was centrifuged at 100 xg for 2 min at 4℃. Crude chloroplast fraction precipitated by centrifugation at 1,500 xg for 8 min was resuspended in 10 m*l* of grinding medium, then purified by Percoll density gradient centrifugation.[16] The post-mitochondrial fraction (supernatant of 20,000 xg for 20 min) was centrifuged at 150,000 xg for 1 hr, to precipitate microsomal raction. The purified chloroplasts were resuspended in the grinding medium and used as the intact chloroplasts. For the preparation of stroma fraction, the intact chloroplasts were resuspended in TE buffer (10 mM Tricine pH 7.5, containing 2 mM EDTA) containing 0.6 M sucrose.[17] The suspension was allowed to stand for 10 min in an ice bath, ruptured them in a glass homogenizer, then diluted with 2 volumes of TE buffer. The broken chloroplasts were centrifuged at 4,500 xg for 15 min to precipitate most of the thylakoid membranes, then the supernatant was recentrifuged at 100,000 xg for 1 hr, to obtain stroma fraction. Before the assay of the activity, this fraction was concentrated by ultra filtration(UP-20 filter, Advantec, Japan), and passed through Dowex-1(Cl^- form) equilibrated with TE buffer to remove endogenous steviol, *ent*-KA and related compounds.

Enzyme assay with [^{14}C]-methyl-KA.

Hydroxylation of [^{14}C]-methyl-KA was assayed using subcellular fraction prepared from stevia. The reaction mixtures contained 50 mM Tricine-KOH, pH 7.8, 10 mM 2-mercaptoethanol, 0.2 mM NADPH, 25 μM [^{14}C]-methyl-KA dissolved in 40 μ*l* of 20% EtOH, and 100 μg total protein of stevia thylakoid and stroma in a total

volume of 2 m*l*. Mixtures were incubated in 20 m*l* glass scintillation vials at 30℃ on a shaker, for 15 min, then extracted with organic solvent as mentioned before. The reaction products were dissolved in MeOH and applied to Kieselgel 60 GF_{254} plate (0.5 mm). The plates were developed with dichloromethan : MeOH (40 : 1). The radioactivity of the reaction product was visualized and quantified using a AMBIS 4000 radio analytic imaging system.

Enzyme assays for the conversion of *ent*-KA.

Steviol synthesis from *ent-KA was assayed in a reaction mixture containing 50 mM Tricine-KOH, pH 7.7, 10 mM 2-mercaptoethanol, 40* μM *ent*-KA dissolved in 40 μ*l* of 20% EtOH, 100 mM NaCl, 10 μM FAD, 0.2 mM NADPH, and intact chloroplasts or stroma fraction, equivalent to 200 μg chlorophyll or 100 μg protein, respectively, in a total volume of 2 m*l*. Activities were determined at 30℃ by the decrease in A_{340} and expressed as the amounts of oxidized NADPH. In the cases using crude or partially purified enzymes, activities were determined as the differences between *ent*-KA-dependent and -independent oxidations of NADPH.

Separation and identification of reaction product.

The incubated reaction mixtures were extracted twice with 3 m*l* of cyclohexane-acetone(3 : 1, v/v), then with 3 m*l* ethylacetate(acidified with H_3PO_4 to pH 3). The combined organic extract was dried under vacuum and the residue was dissolved in 100 μ*l* MeOH for TLC (Kieselgel 60 GF_{254}). The plates were developed in petroleum ether-diethyl ether-EtOH (6 : 2 : 1). MeOH-extract from the zones at R_f 0.40-0.52 and R_f 0.70-0.78 where authentic steviol (R_f 0.47) and *ent*-KA (R_f 0.74) were detected, respectively, were concentrated and dissolved in 20 m*l* MeOH, then each aliquot (10 μ*l*) was analyzed by HPLC on a Puresil 5 μ C_{18} column (Waters, 4.6 × 250 mm). Following injection, the solvent composition was maintained at 82% MeOH in H_2O previously acidified with H_3PO_4 to pH 3, for 18 min at a flow rate 0.6 m*l* min^{-1}. The percent of MeOH was then increased linearly to 100% within 4 min then maintained 100% for 30 min to elute *ent*-KA (R_t = 23.5 min). Steviol was eluted at 13.88 min (data not shown). Elution was monitored at A_{210} nm. The fraction containing the reaction product was pooled and dried under vacuum. This sample was identified by CIMS as above.

Results

Purification of *ent*-KA from sunflower.

Sunflower florets are reportedly to be a rich source of *ent*-KA.[13] This was proved to be the case in our initial attempts to purify *ent*-KA from this source. Typically, about 350 mg purified *ent*-KA were obtained from 100 g of dried sunflower immature seeds (Table 1). Fig. 2 shows that *ent*-KA-containing fractions were separated after three cycle reverse phase HPLC separations at retention times of 16.37 min (A), 17.69 min (B), and 8.89 min (C), respectively. The peak A (or B, C) was identified as *ent*-KA by GC/MS analysis(Fig. 5. A), comparing with the

Table 1. Sequential purification of *ent*-KA from 100 g of dried sunflower immature seeds.

Procedure	Mass of Dried Fraction	Mass Reduction from Previous Step
	g	%
Lyophilized seeds	100.00	
Acetone extract	11.13	88.9
Saponification	3.92	64.8
Clatharization	1.87	52.3
Sep-Pak column chro.	0.94	49.7
TLC I, II	0.52	44.7
HPLC	0.53	32.7

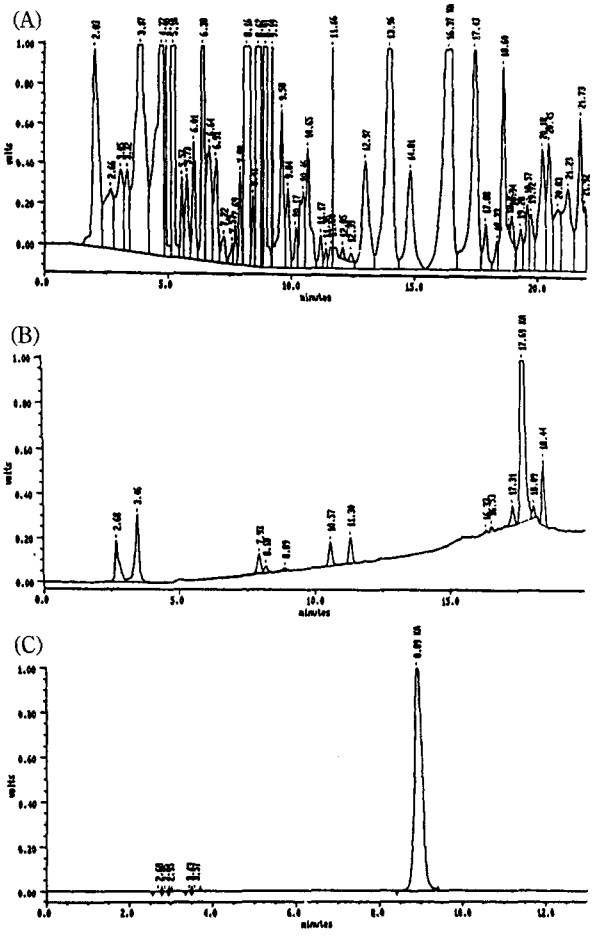

Fig. 2. Reverse-phase HPLC chromatograms, showing purification of a *ent*-KA from arid sunflower immature seeds. After 1st(A), 2nd (B), and third cycle (C)of purification.

published spectrum of the authentic *ent*-KA.[14] The characteristic ions on CIMS; m/z: 303 $[M+H]^+$ (96.7), 287 $[M-Me]^+$(4), and 116 $[M-187]^+$(11), and on EIMS(data not shown); m/z: 302$[M]^+$(94), 287$[M-Me]^+$(73), 259$[M-43]^+$ (37), 256$[M-46]^+$(53), 241$[M-61]^+$(57), and 213$[M-89]^+$ (29), were the same as those reported.

Substrate specificity of *ent*-KA hydroxylase.

To characterize whether the activity localized in chloroplast membranes as well as in stroma, [^{14}C]-methyl-KA was incubated with stroma or thylakoid fraction, in the presence of NADPH and FAD. A new [^{14}C]-labeled spot (R_f 0.41) corresponding to the developed position of methyl-steviol appeared after the incubation of [^{14}C]-methyl-KA in stroma (Fig. 3, A, lane 3). From the total [^{14}C] (25,000 cpm) added to the mixture containing stroma (100 μg protein), [^{14}C] recovered in the product was 12.3%. The conversion by the thylakoid fraction was negligible (Fig. 3, A, lane 2), and by the control fraction without chloroplast was not observed (Fig. 3, A, lane 1). The [^{14}C]-methyl steviol spot was identified by methylated steviol (Fig. 3, B). The methylated KA and steviol were developed with prep TLC, and these compounds visualized by I_2 vapor. And then the purified standards were identified with GC/MS. The CIMS chracteristic ions were; 317$[M+H]^+$(100), 299(7), 273(12), and 257$[M-COOH-Me]^+$(40) for methyl-KA (Fig. 4. A); 333$[M+H]^+$ (100), 315$[M-OH]^+$(94), 300$[M-OH-Me]^+$(10), 273 (27), and 121 (77), for methyl-steviol (Fig. 4. B). The specific activity was 5.4 nmol min^{-1} mg $protein^{-1}$(lasser activity about 16.7% than *ent*-KA). These results also revealed that enzyme is a soluble stroma enzyme. The purified *ent*-KA 13-hydroxylase did not act on *t*-cinnamic acid, 4-hydroxyphenyl acetate, choline and resorcinol, which are the well known substrates for soluble-type hydroxylases. From these results we concluded that the steviol-synthesizing activity is not membrane-bound but localizes in stroma of stevia leaves.

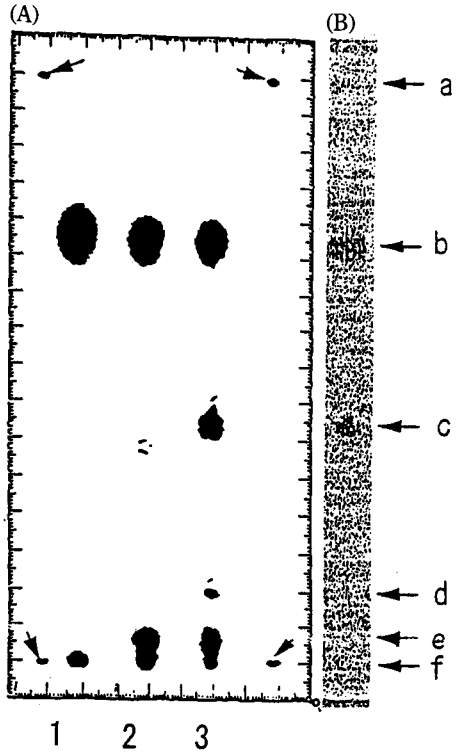

Fig. 3. Autoradiogram of the reaction product from [^{14}C]-methyl-kaurenoic acid by the incubation with stroma prepared from stevia chloroplasts. (A): lane 1, [^{14}C]-methyl-KA (25,000 cpm) incubated without chloroplast fraction; lane 2, [^{14}C]-methyl-KA was incubated with thylakoid; and lane 3, [^{14}C]-methyl-KA was incubated with stroma. The combined organic extract was concentrated and applied to TLC. The four spots indicated by arrows are the radiolabeled markers indicating the four edges of the developed TLC plate. (B): The developed methylated standards visualized by I_2 vapor. The attached symbols indicate. a, front; b, methyl-KA; c, methyl-steviol, d and f, unknown products; and f, origin.

Cofactor requirements of *ent*-KA hydroxylase.

Cofactor dependency is shown in Table 2. As compared with the activity detected in the air-saturated mixture that contained NADPH, NADH also could work as the electron donor but with a lower (two-thirds of NADPH) efficiency. When flavins were added to the stromal protein, about 1.5- and 1.7 fold enhancement of

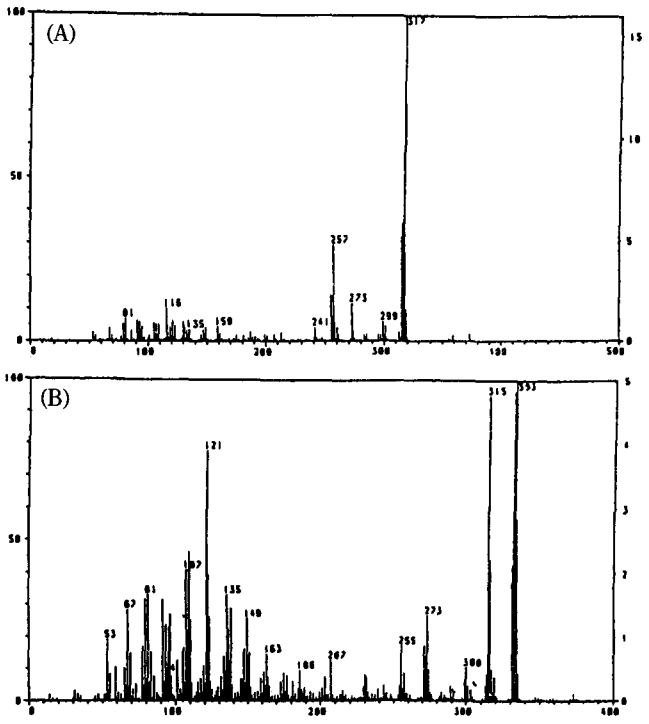

Fig. 4. Mass spectra of the prepared methyl-KA (A) and methyl-steviol (B).

Table 2. Cofactor requirements for the enzymic conversion of *ent*-KA to steviol using stroma prepared from stevia chloroplasts. Complete enzyme assays contained 40 μM *ent*-KA, 10 μM FAD, 0.4 mM NADPH, and 100 μg of stromal protein in 50 mM Tricine/10 mM 2-mercaptoethanol.

Reaction Mixture	Products(S±E) (n mol)	Relative activity(%)
Complete	21.86±0.5	1.00
Minus *ent*-KA	1.45±1.2	0.07
Minus NADPH	3.15±0.3	0.15
+NADH	15.08±0.4	0.69
Minus FAD	14.36±0.7	0.67
+Riboflavin[1]	24.48±0.2	1.12
+FMN[1]	15.96±0.2	0.73
Anaerobic[2]	8.21±0.9	0.38

[1]Adding concentration was 10 μM.
[2]Before the incubation, reaction mixture was evacuated and then argon gas was introduced three times. The tubes were sealed with parafilm.

the activity was observed in the precence of 10 μM FAD and riboflavin, respectively. To identify the product from the hydroxylation to *ent*-KA, after the incubation conditions as indicated, the reaction mixture were extracted with organic phase, then purified with HPLC. The product was identified on CIMS, m/z; 319[M+H]$^+$(91), 301[M-OH]$^+$(100), 273[M-COOH]$^+$(6), and 255[M-COOH-OH]$^+$(10), for steviol(Fig. 5. B). The slightly enhanced activity of the purified *ent*-KA 13-hydroxylase by flavins addition was due to the binding site

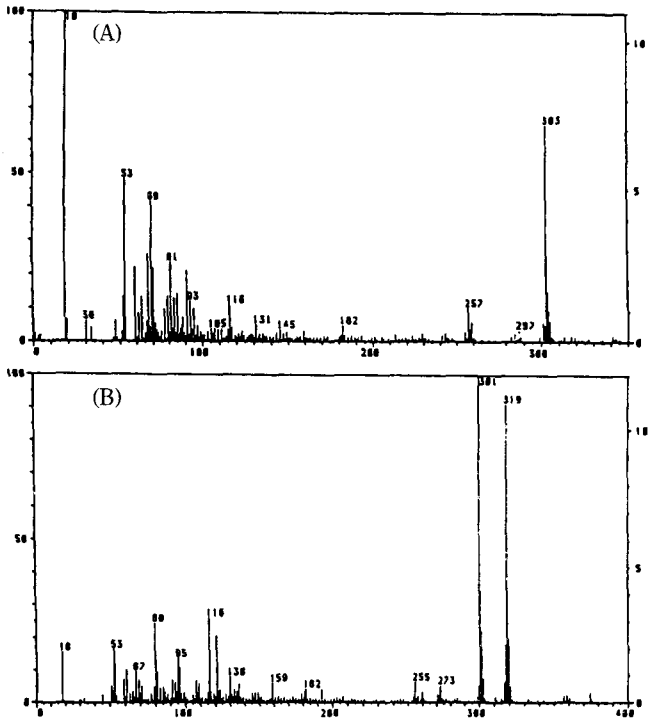

Fig. 5. Mass spectra of the purified *ent*-kaurenoic acid (A) and of the product (B) converted from *ent*-kaurenoic acid by incubating with stevia chloroplasts, after the isolation by TLC followed by the purification by HPLC as described in the text.

present in the *ent*-KA 13-hydroxylase, similar to the flavin site in the tobacco oxidoreductase.[18] A little activity was detected under anaerobic condition. These results indicate that this enzyme requires NADPH and O_2 for full activity.

Discussion

Steviol is the monooxygenated or hydroxylated form of *ent*-KA (Fig. 1). A key step in biosynthesis of GAs may be the conversion of *ent*-KA to *ent*-7β-hydroxy-KA, mediated by *ent*-KA hydroxylase. *ent*-KA-7β-hydroxylase was characterized as cytochrome P-450 constituent bound to microsome membrane in *G. fujikuroi*,[19] *Citrus sinensis*,[20] and *Marah macrocarpus*,[21] which is a precursor of many GA-related compounds.[6] *G. fujikuroi* can convert also steviol to many GA-related compounds (GA_1, GA_{18}, GA_{19}, and GA_{53}), 7β-hydroxy- and 6β, 7β-dihydroxysteviol and 7β, 13-dihydroxy-kaurenoids.[22] Kleinig[23] has pointed out that the true cytochrome P-450 reaction is localized in endoplasmic reticulum rather than chloroplasts. Recent review[24] also indicated that plant cytochrome P-450 systems are found in various subcellular locations, including endoplasmic reticulum, plasma membranes, glyoxysomes and perhaps mitochondria, but not chloroplasts. In preliminary experiments, we compared *ent*-KA metabolizing activity by monitoring the decrease in the amount of *ent*-KA added to an oxygen-saturated reaction mixture containing NADPH, FAD and chloroplast or microsomal fractions prepared from stevia leaves. We could detect a significant activity of *ent*-KA metabolism in chloroplasts. Microsomal fraction, however, showed only a slight activity.

Table 3. Substrate specifiity of *ent*-KA 13-Hydroxylase isolated from Stevial rebaudiana Bertoni.

Condition	Specific activity (nKatal/mg protein)	Relative activity(%)
Complete[a]	210.9	1.00
- NADPH + 0.2 mM NADH	139.2	0.66
- *ent*-KA + t-cinnamic acid	0	0
- *ent*-KA + 4-hydroxyphenyl acetic acid	0	0
- *ent*-KA + choline	0	0
- *ent*-KA + resorcinol	0	0
Anaerobic[b]	4.4	0.02

[a]The reaction was conducted in an air-saturated mixture containing 50 mM Tricine-KOH, pH 7.8, 40 μM *ent*-KA, 10 mM 2-ME, 0.2 mM NADPH and the purified enzyme.
[b]Anaerobic condition was made by the addition of 100 mM glucose and 5 units of glucose oxidase(Oriental Yeast, Japan) to the reaction mixture which was covered with a layer(5 mm) of liquid paraffin. The *ent*-KA solution evacuated and then purged with argon.

Although the cofactor dependency was similar to the cytochrome P-450 system,[19] many chloroplast-localized reactions are known to require O_2 and NADPH, as 2-hydroxylation of *t*-cinnamic acid,[25] monooxygenation of choline,[26] desaturation of stearoyl-acyl-carrier protein to oleoyl-acyl-carrier protein,[27] and cyclation of Mg-protoporphyrin IX monomethyl ester.[28] The former two enzymes are soluble stroma enzymes.

Thus, we assayed cofactor requirement for the enzyme activity (Table 2) using stroma fraction. Using partially purified enzyme we could detect *ent*-KA-dependent oxygen consumption using oxygen electrode, supporting the O_2 requirement for the activity.[2] The stimulation of its activity by the flavins might be explained by the replacement of flavins that had been dissociated from the enzyme. Several carboxylic acids were characterized as substrates for monooxygenase and hydroxylase. *ent*-KA was high specific substrate, while *t*-cinnamic acid, 4-hydroxyphenyl acetate, choline and resorcinol were not substrates. The enzymatic conversion of *ent*-KA to steviol appeared to have an absolute requirement for NADPH and O_2. These results indicated again that the enzyme was a soluble form. This work described in the present report will be a starting point for the further elucidation of the properties of steviol-synthesizing enzyme in stevia leaves.

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Stevia rebaudiana Bertoni의 Steviol 생합성 효소 *ent*-Kaurenoic Acid 13-Hydroxylase의 특성

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초 록 : 파라과이 원산의 국화과 식물 *Stevia rebaudiana* Bertoni는 steviol (*ent*-13-hydroxy kaur-16-en-19-oic acid)을 기본 골격으로 하는 stevia 감미료를 건물 중량의 5-10%를 생합성하여 축적을 한다. 감미성분은 Gibberellin 생합성의 전구체로 알려진 ent-kaurenoic acid (*ent*-kaur-16-en-19-oic acid; *ent*-KA)의 7번과 13번의 어느 탄소에 hydroxylation을 시키느냐에 따라 gibberellin 대사와 steviol 대사로 나뉘어진다. *ent*-KA 13-hydroxylase를 stevia 엽록체의 stroma에 존재하는 것을 확인했다. Stroma 분획 단백질 100 μg으로 효소 반응의 cofactor 요구성을 조사해 본 결과, 반응 혼합액에 NADPH를 첨가하지 않았을 때는 85%의 효소활성의 감소를 보였고, NADPH 대신 NADH를 첨가해도 34%의 감소를 보였다. anaerobic 조건에서는 2.1%의 활성을 띄었다. 이 결과로 *ent*-KA 13-hydroxylase는 NADPH와 O_2 요구성임을 밝혔다. FAD, FMN, riboflavin을 첨가함으로써 FAD는 1.5배, riboflavin은 1.7배의 효소활성 증가 효과가 나타났다. 효소의 기질 특이성을 조사한 결과, *t*-cinnamic acid, 4-hydroxyphenyl acetic acid, choline과 resorcinol에는 전혀 활성이 검출되지 않았으며, [^{14}C]-methyl-KA를 *ent*-KA 대신에 기질로 사용했을 때는 16.7% 의 활성이 검출되어 *ent*-KA 13-hydroxylase는 기질특이성이 높은것으로 확인되었다.

찾는말 : *Stevia rebaudiana* Bertoni, Stevia, *ent*-Kaurenoic acid, Steviol synthesis, Chloroplasts, Stroma
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