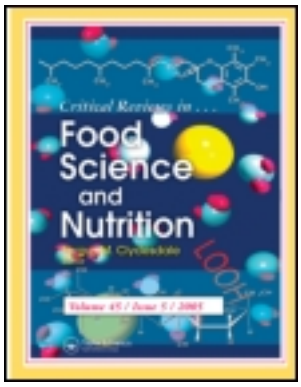

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Steviol Glycosides from Stevia: Biosynthesis Pathway Review and their Application in Foods and Medicine

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Stevia rebaudiana, a perennial herb from the Asteraceae family, is known to the scientific world for its sweetness and steviol glycosides (SGs). SGs are the secondary metabolites responsible for the sweetness of Stevia. They are synthesized by SG biosynthesis pathway operating in the leaves. Most of the genes encoding the enzymes of this pathway have been cloned and characterized from Stevia. Out of various SGs, stevioside and rebaudioside A are the major metabolites. SGs including stevioside have also been synthesized by enzymes and microbial agents. These are non-mutagenic, non-toxic, antimicrobial, and do not show any remarkable side-effects upon consumption. Stevioside has many medical applications and its role against diabetes is most important. SGs have made Stevia an important part of the medicinal world as well as the food and beverage industry. This article presents an overview on Stevia and the importance of SGs.

Keywords *Stevia rebaudiana*, steviol glycosides, sweetner, applications

INTRODUCTION

Stevia rebaudiana is a name that prevails in the medicinal world from the times of Ayurveda. It is a plant having applications in multifarious fields. It is commercially well known and has become an interesting area of research these days. It is a perennial, endemic shrub and a member among 950 genera of *Asteraceae* family. More than 150 species of *Stevia* are known but *Stevia rebaudiana* (Bertoni) stands out because of its sweetness (Debnath, 2008). Studies about *Stevia* have remained dormant for some time. Its existence was again felt when Dr. M.S Bertoni discovered this plant in 1888 at Paraguay. In 1905, the plant was scientifically named as *Stevia rebaudiana* after a Paraguayan chemist Dr. Rebaudi. *Stevia* is sweet in nature and a native of Paraguay, so it is called the "sweet herb of Paraguay." It is also known as honey leaf, sweet leaf, sweet herb, candy leaf, and honey yerba (Carakostas et al., 2008).

Stevia rebaudiana is a short day plant that grows up to a height of 1 m. It bears 2–3 cm long and elliptical leaves having alternate arrangement. It has a brittle stem and an extensive root system. Flowers are white in color with a pale purple throat. They are small in size and arranged in the form of small corymbs (Goettemoeller and Ching, 1999; Singh and Rao, 2005). *Stevia* is naturally present at semi-humid sub-tropical regions at a height of 200–400 meters above sea level. It requires an average rainfall of 1500–1800 mm and temperature conditions varying from −60 to +43°C. The very first crop of *Stevia* was domesticated at Japan in 1968 and by 1970s stevioside, a *Stevia* leaf extract, became a commercially important sweetener and a food supplement. Presently, *Stevia* has been adopted and commercialized by various countries such as Brazil, Korea, UK, China, and Malaysia (Geuns, 2003).

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CULTIVATION OF STEVIA

The methods adopted for large scale *Stevia* plant production includes seed germination (Fig. 1), vegetative propagation, and tissue culture techniques. *Stevia* has two types of seeds, black and tan. Black seeds have higher viability and more germination potential, while tan seeds are produced without fertilization and are non-viable. Few reports suggesting poor germination rate of black viable seeds are also present (Goettemoeller and Ching, 1999). These seeds produce a heterogeneous plant

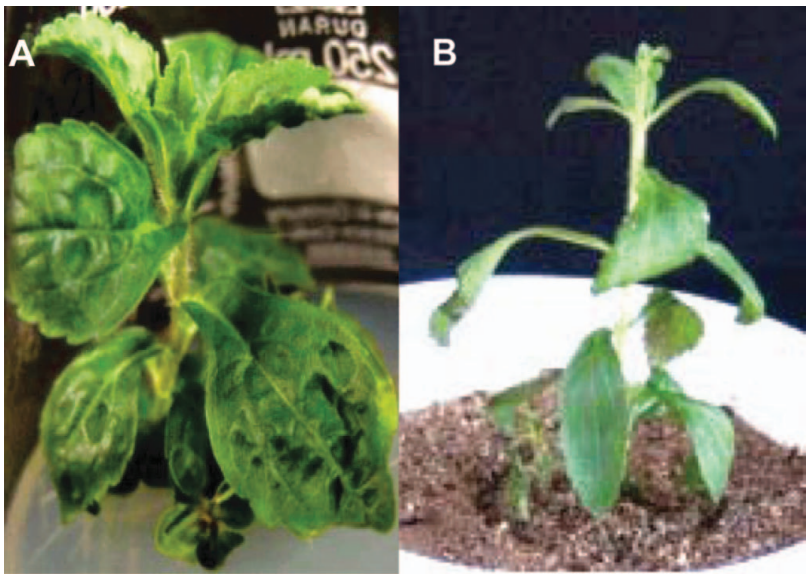

Figure 1 *Stevia rebaudiana* plant morphology. Seed raised *Stevia* seedlings in MS medium under aseptic conditions (A). Seedlings transferred to earthen pots in green house for hardening (B). (color figure available online.)

population with great variability with respect to metabolite composition and degree of sweetness (Nakamura and Tamura, 1985). Later, it was observed that stem cuttings from *Stevia* rooted easily. So, vegetative propagation was adopted as an alternative method for plant propagation. But it required a huge amount of stem cuttings and was highly laborious (Carneiro et al., 1997; Goettemoeller and Ching, 1999). Now the focus is on the plant tissue culture techniques that offer a simple, rapid, economical, and efficient way of plant production, propagation, manipulation, and conservation (Moktaduzzaman and Rahman, 2009). Various studies have been carried out to make the best use of tissue culture techniques for *Stevia* propagation. In the earlier part of 1980s, somatic embryos from various explants of *Stevia* were analyzed for their ability of organogenesis but they failed to regenerate (Filho et al., 1993). Later in the twentieth century, various other explants like shoot apex, nodal, and leaf explants were used to find their potential for regeneration. A study in 2003 has reported a higher rate of shoot regeneration from these explants by making use of varying concentrations of 6-Benzyl adenine (BA) and Indole-3-acetic acid (IAA) (Sivaram and Mukundan, 2003). Various reports have recorded good regeneration from nodal explants. However, the success rate of regeneration from leaf and internodal explants was less (Mitra and Pal, 2007; Jain et al., 2009; Sadeak et al., 2009).

SECONDARY METABOLITES AND THEIR ROLE

Using various quantitative and qualitative analysis methods, *Stevia* leaves have been evaluated for their proximal composition and mineral contents. On dry weight basis, protein, fat, carbohydrate, and ash content has been estimated to be around 20.42, 4.34, 35.20, and 13.12%, respectively (Table 1). Mineral analysis has revealed that potassium, calcium, magnesium, phosphorus, sodium, and sulphur are present in higher amounts than copper, cobalt, manganese, zinc, selenium, and molybdenum. Data from phytochemical screening has shown that tannins are present in higher concentrations followed by alkaloids, glycosides, saponins, sterols, and triterpenes, anthraquinones, and other reducing compounds (Tadhani and Subash, 2006a). These chemical components are responsible for the medical and commercial importance of *Stevia*.

The role of various chemical components of *Stevia* leaf extract have been ascribed. Crude methanolic extract and flavonoid-rich ethyl acetate extract possess antioxidant and oxidative DNA damage preventive activity (Ghanta et al., 2007). The aqueous extract containing higher amounts of sesquiterpenes has antimicrobial and antifungal activity. Such extract is recommended as starting material for pheromone and

Table 1 Percentage of major biochemical constituents of *Stevia*

Components	Value (g/100 g dry leaf weight)
Carbohydrates	35.2
Proteins	12.0–20.42
Lipids	2.7–4.34
Ash	13.12
Stevioside	4–14%
Rebaudioside A	2–4%
Rebaudioside C	1–2%
Dulcoside A	0.4–0.7%
Rebaudioside D,E,F; steviolbioside; rubusoside	>0.4%

perfume synthesis (Markovic et al., 2008). In addition to steviol glycosides (SGs), other types of diterpenoids have also been reported from the leaf extract of *Stevia*. Manoyl oxide and labdane sclareol are such non-kaurenoid diterpenoids. Manoyl oxide has anti-inflammatory and anti-parasitic mode of action. The other, labdane sclareol, has anti-tumorous and cytotoxic properties (Kaushik et al., 2010). Phytosterols, present in the wax of *Stevia* leaves were found to respond against cardiovascular defects (Markovic et al., 2008). Various minerals were reported to maintain the metabolic mechanism and immune responses of the host (Tadhani and Subash, 2006a). These studies well suggest the contribution of secondary metabolites towards health-promoting potential of *Stevia* (Ghanta et al., 2007).

Stevia is highly sweet due to the presence of SGs. Additionally, *Stevia* has higher protein content that raises its water holding capacity and physical entrapment of oil and fats. These various features have made *Stevia* an important ingredient of various food stuffs (Savita et al., 2004). Of the various discussed components, the one responsible for the popularity of *Stevia rebaudiana* is SGs. Stevioside and rebaudioside A are the most important SGs as these render sweetness to *Stevia*. The composition of various SGs is shown in Table 1. The pathway responsible for the synthesis of stevioside and other SGs in *Stevia* has been described as SG biosynthesis pathway.

STEVIA AND MEDICINE

The name "*Stevia*" is not new to the medicinal world. The ancient Ayurvedic system of medicine has a long history regarding the use of *Stevia rebaudiana* (Megeji et al., 2005). SGs in *Stevia* are mainly contributing to its medicinal importance. In addition to SGs, other metabolites of *Stevia* are also adding to its medicinal value like β-carotene, riboflavin, thiamin, austroinullin, various terpenes, and flavanoids (Konoshima and Takasaki, 2002). *Stevia* has been recommended as a treatment against various diseases like diabetes, dental caries, obesity, and stomach infections (Debnath, 2008).

Role of Stevia against Diabetes

Type 2 Diabetes is a complex disorder involving dysfunctioning of islet cells and increased insulin sensitivity. Islet cell dysfunctioning hampers the activity of β-cells to compensate insulin resistance and decreased release of glucagon from α-cells. More and more deposition of fatty acids in non-adipose tissue is also an important factor causing diabetes. The ectopic deposition of fats in pancreatic islets, liver, heart, skeletal muscles, and blood vessels is found responsible for diabetic patients generally turning obese. To date, satisfactory treatment for diabetes is not available. Researchers are focusing on establishing a medicine that should fulfill two major conditions. First, it should enhance insulin secretion without affecting K^+-ATP channels and second, it should be easily and inexpensively available for people in developing nations.

It has been known that long term exposure of fatty acids to β-cells hault their activity causing diabetes. But little is known about the effects of fatty acid exposure on the activity of α-cells and fatty acid metabolism genes. In this regard, the role of stevioside has been studied and it has been concluded that stevioside not only counterbalanced the hypersecretion of α-cells caused by fatty acids but also upregulated the activity of fatty acid metabolism genes that is, oxidation, conversion, and disposal genes, thus suggesting that it can be a potent antidiabetic agent (Hong et al., 2006).

Further, insulinotropic activity of stevioside and steviol has also been reported to be dependent upon the concentration of prevailing glucose. Both compounds have shown long-lasting and reversible insulinotropic activity in the presence of 16.7 mmol/L glucose. Also, these SGs have an enhancing effect on insulin secretion by directly acting on β-cells without altering the K^+-ATP channel activity and cAMP level in the islets, thus documenting stevioside and steviol as potent antihyperglycemic agents (Jeppesen et al., 2000).

Stevioside has now been identified as an insulinotropic, glucogonostatic, and anti-hyperglycemic with its ability to lower the blood pressure effect. Similarly, a substance known as soy protein isolate has been reported to reduce serum cholesterol, LDL cholesterol, triglycerides, and arterial fatty acid streaks. So, an attempt was made to investigate the beneficial effects of a combination of stevioside and soy protein isolates on diabetes and associated cardiovascular risk factors. A long term exposure of such combination to diabetic host could efficiently balance blood glucose, blood pressure, lipid, and glucagon levels. Though, soy protein isolate was only reported to reduce plasma glucose levels but it significantly enhanced the insulinotropic and glucagonostatic ability of stevioside. Hence, the combination of these two could be a new potential treatment for diabetes. Clinical trials for this combination are under process (Jeppesen et al., 2006).

Role of **Stevia** *against Dental Caries*

In order to maintain oral health, natural sweeteners are the best substitute to sugar. It has been suggested that a sugar substitute should be non-toxic and non-carcinogenic. Controlled human and other animal studies have concluded that sugar substitutes play a significant role in preventing dental caries. In this regard, SGs are non-toxic, anti-microbial, and safe for use. Animal caries experiments have observed a significant difference in the sulcal caries scores caused by a sucrose group and a *Stevia* sweeteners group (Matsukubo and Takazoe, 2006). It has been reported that higher amount of stevioside and *Stevia* extract has the ability to reduce the bacterial growth. Interestingly, the required concentration of stevioside as sweetener is rather low compared to sugar. Thus, stevioside can be a substitute for a

certain cariogenic compound present in sucrose (Geuns, 2003). Various studies have concluded that stevioside as well as rebaudioside A are non-cariogenic sweeteners (Matsukubo and Takazoe, 2006).

Role of Stevia *against Diarrhea*

Diarrhea is a pathological state of increased watery content and increased frequency of stool causing intestinal damage and intestinal malfunctioning. It is mostly caused by bacterial and viral agents (Petri et al, 2008). SGs possess a number of features that make them potent antidiarrheal agents. Stevioside is an anti-bacterial as well as an anti-viral agent. It has an inhibitory role preventing the intestinal muscle contractions. Thus it helps against irritable bowel and inflammatory bowel disease. Steviol and its analogs have also been investigated for their role against diarrheal infections. During diarrhea, some of the bacterial enterotoxins enhance the hypersecretion of active chloride ions, resulting in dehydration and intestinal fluid loss. This chloride anion secretion is facilitated through specialized cAMP-activated chloride channels known as cystic fibrosis transmembrane conductance regulator (CFTR). Steviol and its various analogs have been reported to efficiently inhibit the CFTR. Out of the various analogs, dihydrosteviol was found to inhibit CFTR by directly targeting these chloride channels and hence decreased the loss of intestinal fluid (Chatsudthipong and Muanprasat, 2009). In China and Brazil, *Stevia* tea is used as an appetite stimulant, and as an aid to weight management and proper digestion (Singh and Rao, 2005).

Role of Stevia *in Blood Glucose Maintenance*

Stevioside has been reported to maintain blood glucose level by increasing glucose utilization. This was observed due to the enhancing effect of stevioside on insulin secretion. Secondly, stevioside has been found to downregulate the expression of phosphoenol pyruvate carboxylase (PEPCK) enzyme, a regulatory enzyme of gluconeogenesis. As a result, it reduces glucose production by downregulating the process of gluconeogenesis. But both these activities are stevioside-dose dependent. Daily stevioside treatment has produced no such effect; however, a higher stevioside dose was found to be a necessary condition for such conditions (Chen et al., 2005). A comparative study has been conducted to see the effect of dried powdered *Stevia* leaves and stevioside on glucose levels. Since *Stevia* leaves had a regulatory role on two enzymes of gluconeogenesis, pyruvate carboxylase (PC), and PEPCK, they could decrease glucose production and maintain blood glucose levels more efficiently than stevioside. *Stevia* leaves also contain various other metabolites in addition to SGs, such as non-glycosides diterpenoids, sterols, triterpenoids, flavonoids and volatile oils. So, the focus is on the determination of the particular hypoglycemic molecule among those present in the leaves (Ferreira et al., 2006).

Role of Stevia *against Myocardial Injury*

Isosteviol was found effective against myocardial injury caused by ischemia-reperfusion. Ischemia-reperfusion deteriorates the systolic-diastolic functions of left ventricle of heart, increases the activity of enzymes lactate dehydrogenase (LDH) and creatine kinase (CK) in coronary flow, and causes histopathological changes in myocardium and mitochondria. An in vitro study in this respect concluded that isosteviol has efficient ability to weaken the pathological changes caused by ischemia-reperfusion (Zhang and Xu, 2004–2006).

STEVIOL GLYCOSIDES

Steviol glycosides (SGs) are secondary metabolites, specifically tetracyclic diterpenoids. Diterpenoids are usually the least studied group among various secondary metabolites. Because of their fundamental and applied perspectives, they have gained great importance in recent times (Bondarev et al., 2003/04). *Stevia* is also popularly known as the "producer of diterpenoid steviol glycosides." SGs are highly sweet, non-toxic, and non-mutagenic diterpenoids (Bondarev et al., 2003).

Steviol Glycoside Biosynthesis Pathway

SG biosynthesis pathway is presently an area of immense interest for researchers. The reason behind this is that at the genetic level very little is known about SG biosynthesis pathway (Richman et al., 1999). Secondly, this pathway shares four intermediate steps with Gibberellic acid (GA) biosynthesis pathway (Brandle and Telmer, 2007) which itself is holding an important role in plant growth and development.

SG biosynthesis pathway operates in the leaves, from where glycosides are transported to various plant parts. Studies have suggested that SGs are present mostly in the leaves, small amounts in the stem, and undetectable in the roots (Brandle and Rosa, 1992). The pathway has sixteen enzyme catalyzed steps as shown in Fig. 2. Initial seven steps synthesizing isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are common with the steps of methylerythritol-4-phosphate (MEP) pathway, known for isoprenoid biosynthesis (Wanke et al., 2001). The next four steps involving synthesis of kaurenoic acid from geranylgeranyl diphosphate (GGDP) share similarity with GA biosynthesis pathway. The remaining five steps are specific to SG biosynthesis pathway. The last formed kaurenoic acid undergoes hydroxylation via kaurenoic acid-13-hydroxylase (KAH) enzyme and produced steviol. At this step, the SG biosynthesis pathway diverges from the GA biosynthesis pathway (Kim et al., 1996). So, it is considered as the committed step of SGs biosynthesis (Brandle and Telmer, 2007).

The remaining four steps of SGs biosynthesis are catalyzed by UDP-glycosyltransferases (UGTs). Of these four UGTs, three of them, namely UGT85C2, UGT74G1, and UGT76G1 have been identified. UGT85C2 catalyzes the addition of

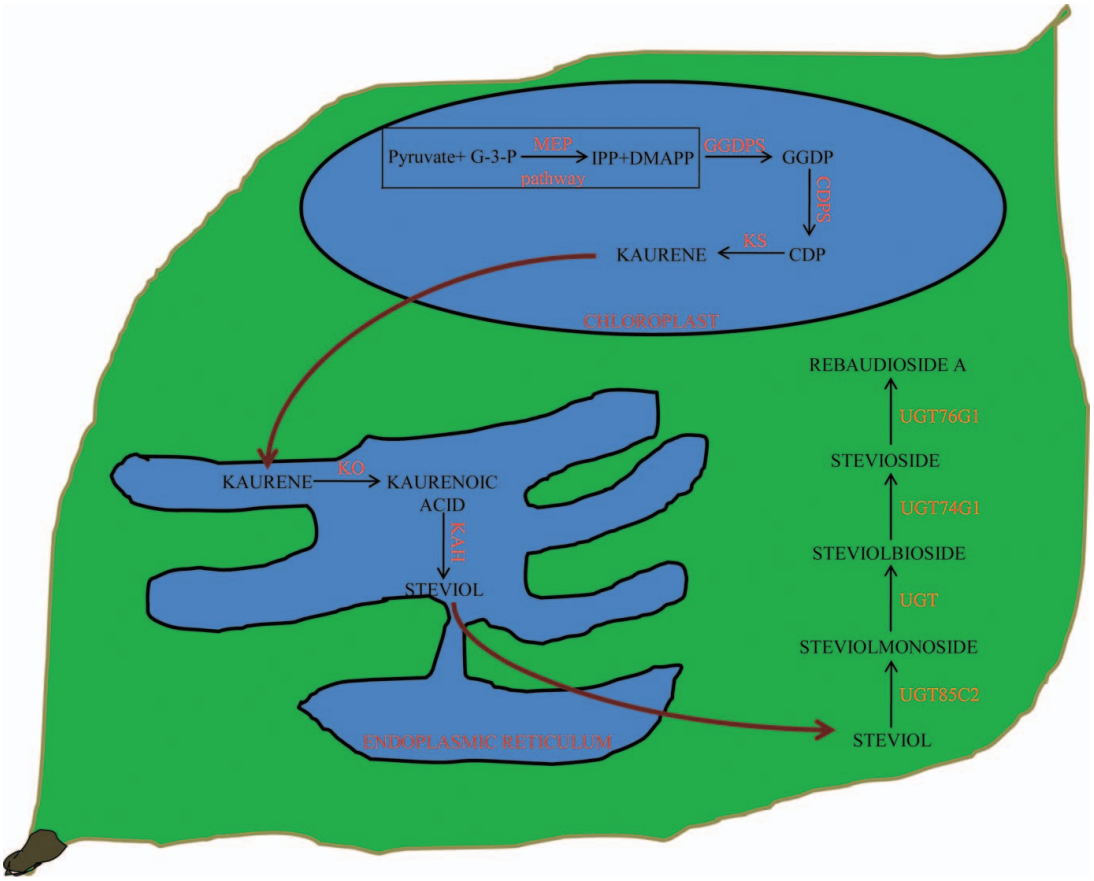

Figure 2 Steviol glycoside biosynthesis pathway operating in various compartments of *Stevia rebaudiana* leaf. Kaurene, the precursor of diterpenoids is synthesized in the chloroplast. It is converted to steviol by the activity of enzymes present at the membrane of endoplasmic reticulum. Steviol synthesizes various steviol glycosides in the cytosol that ultimately accumulate in the vacuole. (color figure available online.)

C13-glucose to steviol forming steviolmonoside. Steviolmonoside is then glycosylated to form steviolbioside. UGT catalyzing this step has not been identified yet. Steviolbioside further undergoes glycosylation at C-19 position by UGT74G1 and synthesizes stevioside. Rebaudioside A is formed by the glucosylation at C-13 stevioside by the activity of enzyme UGT76G1 (Brandle and Telmer, 2007).

Cloning and Characterization of SGs Biosynthesis Pathway Genes

DXS (deoxyxylulose-5-phosphate synthase) converts pyruvate and glyceraldehyde-3-phosphate to deoxyxylulose-5-phosphate (DXP). Gene encoding DXS was first cloned and characterized in *Mentha* (peppermint) (Lange et al., 1998) and functionally validated by carrying out a complementation assay with *E. coli* strain MC4100 dxs::CAT. It has conserved amino acid sequence that has helped its cloning from *Stevia* via reverse transcriptase polymerase chain reaction (RT-PCR) using degenerate primers (Totte et al., 2003). DXR (DXP reductoisomerase) reduces DXP to 2-C-methyl-o-erythritol-4-phosphate (Brandle and Telmer, 2007). Gene encoding DXR has been cloned from *Arabidopsis* and *Mentha* (peppermint) (Lange and Croteau, 1999). Similar to DXS, DXR has also been cloned from *Stevia* and functionally characterized by complementation assay using *E. coli* strain MC4100 dxr::TET (Totte et al., 2003).

CMS (4-diphosphocytidyl-2-C-methyl-D-erythritol synthase), CMK (4-diphosphocytidyl-2-C-methyl-D-erythritol kinase), MCS (4-diphosphocytidyl-2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase), HDS (1-hydroxy-2-methyl-2(E)-butenyl 4-diphosphate synthase), HDR (1-hydroxy-2-methyl-2(E)-butenyl 4-diphosphate reductase) enzymes catalyses five steps synthesizing IPP and DMAPP. Genes encoding these enzymes have been cloned and characterized in *Arabidopsis* that has helped the cloning of these genes from other plants including *Stevia* (Rohdich et al., 2000; 2000; Querol et al., 2002; Guevara-Garcia et al., 2005; Hsieh and Goodman, 2006). These genes are still uncharacterized, but have been submitted to Genbank as putative genes from *Stevia* (Brandle and Telmer, 2007).

GGDPS (geranylgeranyl diphosphate synthase) is a plastidial prenyltransferase enzyme. Gene encoding this enzyme has been submitted from *Stevia* as putative (McGarvey and Croteau, 1995). CDPS (copalyl diphosphate synthase) and KS (kaurene synthase) catalyzed steps are considered committed for both GA and SG biosynthesis pathway. Genes encoding these

enzymes have been cloned and characterized from various plants including *Stevia*. Both CDPS and KS encoding genes are usually present in single copy, having low expression and that too, limited to rapidly growing tissues. In case of *Stevia*, southern and northern blot analysis have revealed the presence of single copy of CDPS and two copies of KS, with a comparatively higher expression in the leaves. GA content in *Stevia* is similar to that present in other plants, suggesting temporal and spatial separation of GA and SG biosynthesis pathways in *Stevia*. This in turn represents the site regulated expression of both CDPS and KS that keep the activities of these two enzymes separate in these two pathways. In situ hybridization studies of CDPS and KS have shown their presence only in leaf parenchyma (Richman et al., 1999; Hedden and Phillips, 2000). This has helped to conclude that early steps of SG biosynthesis pathway occur only in green tissues. It has later been confirmed that SGs are present only in chloroplast bearing tissues (Bondarev et al., 2003/04).

KO (kaurene oxidase) catalyzes the oxidation of kaurene to kaurenoic acid (Helliwell et al., 1999). Its expression is higher in leaves, flowers, succulent stems, and seedling shoots (Humphrey et al., 2006). In *Stevia* genome, genes encoding KO are present in duplicate functional forms (Brandle et al., 2002; Humphrey et al., 2006) and a third truncated non-functional form (Richman et al., 1999). KO is also a part of GA biosynthesis pathway. This has suggested the spatial and temporal expression of this gene (Fleet et al., 2003; Davidson et al., 2005). Using the prediction program PSORT, KO has been found to be localized at the membrane of endoplasmic reticulum (Nakai and Horton, 1999). Later, in vivo fusion studies have confirmed this prediction (Humphrey et al., 2006). KAH (kaurenoic acid-13-hydroxylase) is involved in the hydroxylation of kaurenoic acid to steviol (Brandle and Telmer, 2007). At this step, SG biosynthesis pathway diverges from GA biosynthesis pathway. KAH activity is observed in the stroma of chloroplast. It has been partially purified and its N-terminal sequence has been described. It is a heterotetramer of 39 kDa protein subunits. KAH is shown to have similarity with fructose biphosphate aldolase (FBPA), a homotetramer of 37 kDa protein subunits (Kim et al., 1996; Geuns, 2003).

UGTs (UDP-glycosyltransferases) are known to transfer single or multiple activated sugars from nucleotide sugar donor to variety of small molecular acceptors. Hydroxylated molecules are the most common acceptors of such sugars (Wang and Hou, 2009). The last four steps of SG biosynthesis pathway are catalyzed by UGTs. Among these, three UGTs have been identified and characterized. These are UGT85C2, UGT74G1, and UGT76G1 (Brandle and Telmer, 2007). GFP fusion and cell fractionation studies have suggested the presence of these three UGTs in the cytoplasm (Humphrey et al., 2006).

Physiochemical Features of Steviol Glycosides

SGs, namely steviol, steviolbioside, stevioside, rebaudioside A, B, C, D, E, and F, and dulcoside A are known to accumulate in the leaves (Brandle and Rosa, 1992; Bondarev et al., 2003). Among these, stevioside and rebaudioside A are present in higher concentrations. Stevioside is 110–270 times sweeter and rebaudioside A is 150–320 times sweeter than sucrose (Brandle et al., 2002). Increased stevioside content gives a metallic aftertaste. The presence of increased rebaudioside A ($> 50\%$) in glycosidal content reduces metallic aftertaste, thus improving the taste. All glycosides can be easily dissolved and extracted with aqueous solvents (Wanke et al., 2001). They show increased stability at higher pH (from 2 to 10) and exceptional thermostability. They are stable even at 200°C. These glycosides produced a synergistic effect upon addition to other sweeteners or flavors, thus enhancing sweetness and flavor (Sharma et al., 2009).

Biological Features of Steviol Glycosides

Various in vivo and in vitro studies have been carried out to determine the safety aspects of *Stevia* and SGs. In 2006, a study was carried out to determine the cytotoxic and genotoxic behavior of stevioside. *E. coli* strains were incubated with varying concentrations of stevioside followed by agarose electrophoresis and bacterial transformation assays for survival assays. A drop in the survival rate of *E. coli* was observed. This has suggested that stevioside being cytotoxic has the ability to cause lesions in DNA and destroy lesion recovery pathways. Metabolization of stevioside to steviol has actually been responsible for its DNA damage capability (Nunes et al., 2006). The leaf extract of *Stevia* was also investigated for its antimicrobial activity. Isolation of leaf extract was carried out with different solvents and each extract was examined against certain microbial species. Different extracts showed differential inhibitory activity against various microbes. This experimentation confirmed the antibacterial as well as antifungal potential of *Stevia* leaf extract (Tadhani and Subash, 2006b) and documented that *Stevia* might be a source of new non-antibiotic antibacterial and antifungal agent. Its antifungal activity was estimated to be higher than the standard fungicide usually used against plant pathogens. Such extraordinary antimicrobial activity of *Stevia* has presented it as a potent non-antibiotic pharmaceutical and an efficient food preservative (Ghosh et al., 2008). Stevioside alone has been observed to significantly reduce the amount of inflammation mediators and activate cytotoxic cells of the host. These activities suggested that stevioside might play a synergistic role with the innate immunity of the host. Thus stevioside is antibacterial, antifungal, anti-inflammatory, anti-tumorous, and safe for use (Boonkaewwan et al., 2006). While at the same time rebaudioside A has been reported to be clinically insignificant (Nikiforov and Eapen, 2008).

Isolation and Analysis of Steviol Glycosides

Various techniques have been used to determine the concentration of SGs in the *Stevia* leaves. In 1996, a study had

successfully described capillary electrophoresis for the analysis of glycosides. Rebaudioside A and steviolbioside were isolated using semi-preparative high performance liquid chromatography (HPLC) and their structure was elucidated via mass spectrometry. In addition, the effect of organic solvent, used while injecting the sample, was studied on the electrophoretic solution (Mauri et al., 1996). Later in 2001, an improved analytical method was introduced that saved time during sample preparation, chromatographic analysis, and simplified sample handling. Stevioside and rebaudioside A were isolated by solvent extractions technique and were analyzed by isocratic HPLC (Kolb et al., 2001). In mid-2004, reverse phase HPLC was introduced to determine the amount of steviol on the picomole scale (Minne et al., 2004). In 2009, it was found that the raw material used for isolation and evaluation of SGs lost its quality due to the presence of organic and inorganic compounds. Therefore, a purification process using ceramic membrane was introduced to enhance the product quality. The sweetener finally obtained was highly pure with $> 90\%$ purity (Reis et al., 2009). In the same year, desorption electrospray ionization (DESI) mass spectrometry (Jackson et al., 2009) and near infrared reflectance spectroscopy (NIRS) (Hearn and Subedi, 2009) were judged for their potential to determine the level of SGs. DESI was found as a rapid, qualitative, and semi-quantitative method that does not require sample preparation for SGs estimation in *Stevia* leaves. In 2010, a study has concluded that ultra-HPLC methods with electrospray ionization mass spectrometry (UHPLC-MS) can be used for the routine evaluations of SGs (Gardana et al., 2010). Most recently, ultrasonically assisted extraction method has been investigated that reported an increase in the yield of rebaudioside A by a factor of 1.5 (Liu et al., 2010). Since the year 2000, various modified techniques are being explored for glycoside extraction and evaluations.

Derivatives of Steviol Glycosides

Few reports are present about the chemical synthesis of SGs. Researchers are keenly working on this aspect. Earlier in 1991, cyclodextrin glucanotransferase-starch system was utilized to synthesize a mixture of products from stevioside and rubusoside. The obtained products were sweeter than stevioside itself. This study also reported the solubilization of steviolmonoside and steviolbioside in an acetate buffer in the presence of gamma-cyclodextrin (Ohtani et al., 1991). Later in the same year, two α-glucosidases (pullulanase and pullulan) and Biozyme L and maltose were used for the transglucosylation of stevioside. Products synthesized by use of these could give new products having high quality taste (Lobov et al., 1991). In 2004, a diterpenoid obtained from stevioside called isosteviol was hydrolyzed by fungus *Aspergillus niger* that produced five metabolites. All the metabolites had potent inhibitory effect on Epstein-Barr virus activation (Akihisa et al., 2004). At the end of 2004, amide dimers of steviolbioside, steviol, and isosteviol were synthesized by reacting aliphatic alkylamine and alkyldiamines with benzotriazol-1-yloxtri (pyrrolidinol) phosphonium hexofluoro phosphate (PyBOP) and diisopropylethylethylamine (DIEA). The synthesized products had cytotoxic effects on human embryonic lung cells and cancer cells (Lin et al., 2004). In 2009, a comparative study has been carried out in order to find a potent biological agent and enzyme for the synthesis of aglycon steviol and its derivative isosteviol in higher amounts. During this effort, *Aspergillus niger* and pancreatin have shown best results.

Microbial synthesis of glycosides mostly occur at mild reaction conditions and results in the production of metabolites at varying concentration. This has suggested that the reaction yield can be improved by altering reaction conditions and time, chemical composition of the medium, substrate feeding procedures, and bioreactors. Presently, microbial reactions are being highly explored for the synthesis of glycosides and their derivatives because their mild reaction conditions are in accordance with the objectives of green chemistry (Milagre et al., 2009).

STEVIA AND PLANT DEVELOPMENT

Studies have also shown the role of *Stevia* in plant development. It has been reported that aerial parts of *Stevia* contain plant growth promoting substances. In 2005, plant growth promoters were isolated from *Stevia* for the first time. Isolation and purification was done by bioactivity guided extraction and chromatographic techniques. It was observed that one of the isolated compounds enhanced the growth of cucumber and lettuce by 147% and 140%, respectively. The isolated compound has not yet been characterized (Nasir et al., 2005). Another report suggests the role of *Stevia* in delayed senescence of rice. Steviol glycoside blending liquid (SBL), composed of stevioside and some other nutrient factors, could efficiently increase the activity of superoxide dismutase (SOD) and catalase (CAT). SOD and CAT prevents injury due to reactive oxygen species and thus delays senescence, maintaining normal growth and development of the plant (Congmin et al., 2009).

ASSOCIATED RISKS WITH SGS USE

SGs are generally considered safe for consumption. However, steviol consumption by the animal system has resulted in adverse effects (Gardana et al., 2010). There are few reports suggesting harmful effects of SGs on the host system. In 1985, a study investigated the inhibitory role of various glycosides on oxidative phosphorylation and on certain enzymes such as ATPase, NADH-oxidase, succinate oxidase, and succinate dehydrogenase. However, stevioside had not been reported for inhibitory activities (Bracht et al., 1985). Stevioside and steviol has been tested for mutagenicity and chromosomal effects on the strains of *Salmonella typhimurium* and cultured human lymphocytes. Both glycosides were neither mutagenic nor clastogenic at limited doses. However, increased dosage of stevioside has shown direct mutagenicity (Suttajit et al., 1993). SGs

have also been tested for effects on glucose absorption. Steviol was reported to inhibit glucose absorption by altering the morphology of intestinal absorptive cells (Toskulkao et al., 1995). But afterwards, various studies have experimentally proved and concluded that *Stevia* and SGs are safe (Geuns, 2002).

STEVIA AND COMMERCIALIZATION

Earlier in the fourteenth century, sugar was considered an important food ingredient. No doubt sugar had sweetening ability but it contributed a huge amount of calories, giving rise to several medical problems. As a result various alternative sweeteners, natural as well as artificial, that provide less calories and more sweetness as a replacement of sugar, are available. Such sweeteners include aspartame, saccharin, cyclamate, glycyrrhizin, and *Stevia* and its glycosides. It was soon found that most of these sweeteners have associated health risks. Aspartame raised the possibilities of the disease phenylketonuria as it produced phenylalanine as a major metabolite during metabolism. Saccharin was found to cause bladder cancer in laboratory animals and hence was reported carcinogenic. Cyclamate produced cyclohexamine, a toxic metabolite to heart and testes. Similarly, glycyrrhizin was reported to cause pseudoaldosteronism (Kim and Kinghorn, 2002). In case of *Stevia*, initial few studies have reported it as mutagenic, carcinogenic, and highly unsafe. However, later reports have proved that it is safe for consumption without any health risks. Thereafter, *Stevia* emerged as one of the best alternative sources of sweetners (Savita et al., 2004).

Presently, *Stevia* and its glycosides comprise an important ingredient of various food items. In addition to the abovementioned features, there are some more properties that make *Stevia* an attractive compound for industrialists. Sweetness caused by *Stevia* lasts longer, for more than 40 seconds in comparison to sugar. Its high water absorption capacity makes it an advantageous ingredient of viscous food stuffs. Similarly, it has a higher tendency of fat absorption that retains flavor for a longer duration. Products containing *Stevia* have lower glycemic index that is beneficial for diabetic persons (Savita et al., 2004). Various investigations have reported that the most acceptable quantity of *Stevia* for confectionary products is around 0.25–1 g and for dairy products is 25 mg (Agarwal et al., 2010).

Because of these features, *Stevia* and its SGs are available in the market as dietary supplements. Most of the marketed SG products primarily consist of stevioside and rebaudioside A (Carakostas et al., 2008). Rebiana is a common term for the products containing a higher amount of rebaudioside A than other SGs. The Coca-Cola Company and Cargill, Inc. have started a joint venture for the development and commercialization of sweetener rebaudioside A. Their main focus has been the introduction of Rebiana to the market (Clos et al., 2008). Tea, coffee, ice creams, cakes, soft drinks, fruit juices, and color enhancers are some of the commercialized products using *Stevia* extracts.

Stevia thus holds an important place in the commercial market. In June 2008, the WHO/FAO expert committee on food additives (JECFA) has concluded that SGs use is safe for the food and beverage industry. Still few nations have not approved *Stevia* as a food product because of the controversies regarding toxicity of SGs. Presently, Japan and China have the biggest market for the production and utilization of SGs (FAO Fact Sheet, 2008). In addition to Japan and China, various other nations such as Korea and Brazil have accepted SGs as natural plant products and marketed as low calorie sweetener (Kim et al., 2002; Mizutani and Tanaka, 2002). In 2008, Australia and New Zealand have also approved the use of SGs in the food industry but to a defined range of products. In Australia, these SGs constitute a small domestic market that comprises of unprocessed and crystal forms of SGs only. At the end of the same year, the Food and Drug Administration in the US has granted permission for the use of *Stevia* in the food and the beverage industry. In 2009, France has authorized the use of rebaudioside A as a sweetener. In Europe, SGs have not been approved for human consumption yet. European Food Safety Authority is currently conducting general safety assessments to grant approval for the marketing of SGs (FAO Fact Sheet, 2008).

Various artificial sweeteners constitute the global high intensity sweetener market. *Stevia* comprises of only 1% of this market. The market grows at a rate of 4% per annum and has a business of around 1.3 billion US dollar. Japan alone invests 40% to the international sweetener market. The total market value of *Stevia* sweetener in Japan has been estimated to be around 25–35 million US dollars per year (Megeji et al., 2005). Worldwide, 80,000 acres of land is under *Stevia* cultivation, 75% of which lies in China alone (TIMEIS, 2009). The *Stevia* industry in China has witnessed a significant increment in the yield from 2,073 tons in 2007 to 3,096 tons in 2009 and 80% of this has been exported. Also, the *Stevia* sweetener capacity has been estimated to enhance from 5,000 tons/annum in 2007 to 11,789 tons/annum in 2009 (Hospitality Trends, 2009). The United States and Europe are the major consumers (about 65%) of global high intensity sweetener market. Both of these nations have not accepted *Stevia* as a food ingredient (Analysts/Media Conference Call, 2008). However, FDA approved the safe use of rebaudioside A in 2008 and thereafter the *Stevia* market erupted in the US. In addition to Cargill and Merisant, Blue California became the third company to commercialize *Stevia* in the US market (Caroline Scott-Thomas, 2009). In 2008, a business of 21 million US dollar was observed that substantially rose to 95 million US dollar by July 2009. It has been predicted that the US *Stevia* market would exceed 2 billion US dollar by the end of 2011 (Mintel Oxygen Reports, 2009). Global production of *Stevia* is estimated to be around 40,000 million tons. Two international business groups, Wilmar and Olam, have started a joint venture to invest 106.2 million US dollar to globally enhance the production and consumption of *Stevia* and to raise the market of *Stevia*-based sweeteners (Analysts/Media Conference Call, 2008).

India is supposed to have suitable conditions for the cultivation of *Stevia*. It has been found that Indian *Stevia* plant gives a higher stevioside yield of 10–18 percent in comparison

to the reported 8–12 percent from other countries (Medicinal Plants, Agriculture and Industrial Survey, 2005). But still the cultivation and commercialization of *Stevia* has not achieved the expected heights. The current annual production of *Stevia* in India is estimated to be around 600 tonnes (TIMEIS, 2009). Lack of support and awareness by the Indian Government about the medicinal and commercial importance of *Stevia* among the farming fraternity has adversely affected its market (Medicinal Plants, Agriculture and Industrial Survey, 2005).

An International *Stevia* supplier called GLG Life Tech has taken the initiative of introducing *Stevia* to the Indian market and to facilitate its production and extraction in India. India itself is also stepping forward to compete in the *Stevia* sweetener international market (Caroline Scott-Thomas, 2010). Initially, 200 acres of land was utilized for *Stevia* cultivation which has now expanded to 1,000 acres. Further, it is expected that the total area will scale up to 10,000 acres by 2011 (Business Standard, 2008). The central government has extended a 30% subsidy on this plant. The NABARD has sanctioned loans of up to Rs. 2.40 lakhs per acre for its cultivation. Even the Chattisgarh state government has given a subsidy of 20 percent on this plant to the farmers. It is believed that similar steps by other states will help the country to evolve as an emerging power in the global *Stevia* market. It has been estimated that *Stevia* cultivation in India requires an investment of Rs. 2.35 lakhs per acre to grow at the rate of 30,000 plants per acre. The same crop can be harvested continuously for seven years, giving an output of 2,500 kg stevioside per acre in the first year and over 3,000 kg per acre the second year that declines in the subsequent years (Medicinal Plants, Agriculture, and Industrial Survey, 2005). *Stevia* Global Forum has organized "The *Stevia* Technology, Innovation and Safety Summit India 2010" in New Delhi on July 11. The FAO/WHO Expert Committee on Food Additives (JECFA), Food Standards Australia New Zealand (FSANZ), and European Food Safety Authority (EFSA) have made a collective effort to revolutionize the next generation of the sweetener market in India. The main aim of this Summit was to converge the leading R&D, innovation, new product development (NPD), marketing and food safety, and quality assurance professionals to share the knowledge and shape the future of the *Stevia* industry in India (*Stevia* Global Forum, 2010).

CONCLUSION

Stevia, the "sweet herb of Paraguay," is emerging as the best alternative source of sugar. Not only a dietary supplement, it has wide applications in medicine and the commercial world as well. In medicine, *Stevia* is a well known therapeutic agent. It is an efficient medication for diabetes, hypertension, myocardial and antimicrobial infections, dental troubles, and tumors. In the food and beverage industry, various products such as jams, jellies, chocolates, juices, and other food stuffs contain *Stevia* as sweetener additives. *Stevia* is now not only a seasonal crop of a field but also an important component of commercial business. Thus, commercialization of *Stevia* in one way or the other affects initially its cultivation, followed by influence on medical field and business, and ultimately affecting the economy of the concerned area on a small or a large scale.

Europe and other countries have controversies on the use of *Stevia* for human consumption. This is due to the lack of scientific data on the consequences of *Stevia* constituents on the human body. Despite the controversies, these countries are also making their market for *Stevia*. From this, it seems that these countries know that sooner or later *Stevia* will find a place as a sweetner for human consumption. India has found suitable weather for *Stevia* cultivation and has already taken steps towards increasing the production of *Stevia*. Till now, the lack of awareness in India has not raised much controversy on its consumption. It seems that India will soon find a comfortable position in the *Stevia* market.

The multifarious applications of *Stevia* have brought the attention of researchers to initiate research in various fields. To understand the structure, behavior, and role of these SGs, chemistry and biochemistry have played their roles as well. In the field of immunology, researchers have been working for a long time to elucidate the biological nature and safety aspects of SGs. Similarly, agronomists are keenly involved in plant growth and quality improvement. In this way, a single plant has converged multidisciplinary fields. In the present world, where there is a need to enhance the use of plants in various fields, *Stevia* is playing its part efficiently. Though researchers have been working on *Stevia* for a long time, it is yet to be explored for its full potential.

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