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Biotechnological production of natural zero-calorie sweeteners

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The increasing public awareness of adverse health impacts from excessive sugar consumption has created increasing interest in plant-derived, natural low-calorie or zero-calorie sweeteners. Two plant species which contain natural sweeteners, *Stevia rebaudiana* and *Siraitia grosvenorii*, have been extensively profiled to identify molecules with high intensity sweetening properties. However, sweetening ability does not necessarily make a product viable for commercial applications. Some criteria for product success are proposed to identify which targets are likely to be accepted by consumers. Limitations of plant-based production are discussed, and a case is put forward for the necessity of biotechnological production methods such as plant cell culture or microbial fermentation to meet needs for commercial-scale production of natural sweeteners.

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Introduction

Global demand for naturally sourced, zero-calorie sweeteners has increased significantly over the last decade as consumers have become increasingly health conscious. The popular press publishes frequent articles about health impacts of sugars and sugar substitutes, creating public or personal pressures that steer consumers away from sugar to natural low-calorie or zero-calorie alternatives. Perhaps more importantly, demonstrated health issues related to excessive sucrose consumption have helped propel growth of plant-derived natural sweeteners. However, further growth of natural sweeteners is potentially limited by agricultural sustainability, undesirable taste qualities, perceived safety and commercial viability. Biotechnological production platforms for natural sweeteners can address supply scalability limitations associated with plant-based production while increasing sustainability of the overall endeavor. In addition, the development of plant or microbial cell-based production platforms can allow for the rapid modification of pathway enzymes to generate novel sweeteners with fewer or no negative taste attributes.

Given the explosive growth in interest in natural zero-calorie sweeteners in the past few years, we will endeavor to provide a solid background on sweetener science from the past twenty years while emphasizing the recent resurgence in research focused on naturally derived zero-calorie sweeteners. A brief exploration of some of the chemistry and biology underlying natural sweeteners will be followed by a short analysis of the potential for biotechnological methods to tackle some sustainability issues with plant-based production methods. Finally, we identify some potential challenges that must be addressed to ensure the successful development of a biotechnologically produced sweetener product.

Discovery of zero-calorie sweeteners

Many synthetic and natural sweet-tasting compounds have been identified since the early 1800s. Most of these compounds are much more potent than commonly used sucrose and are generally referred to as high-potency (HP) sweeteners. Sweeteners can be found in many areas of chemical space, with at least 50 structural classes of organic compounds represented [1,2]. Despite centuries-long usage of plants traditionally known for their sweetening potential by certain societies, the original non-sucrose sweeteners developed were artificially synthesized molecules, not natural products. This is in part due to their discovery at a time when taste and smell were key methods employed for the characterization of newly synthesized compounds. The first artificial sweetener to be commercialized is saccharin, discovered in 1878 by Fahlberg and Remsen [2]. This was followed by cyclamate, neohesperidin dihydrochalcone, aspartame, acesulfame K, sucralose, neotame, and advantame [2]. All of these artificial sweeteners are approved for use in various countries, but not all are approved everywhere due to differences in public health administrations. For example, cyclamate is not approved for use in the United States due to health concerns [3], while it is approved in Canada, Europe, Central and South America, and Asia.

Identification of the sweetness receptor first in rats [4] and then in humans [5] has enabled high-throughput screening (HTS) of large compound libraries via cell-based

assays, and promises to speed the discovery of even more compounds with sweetening potential. This technology has also enabled the identification of sweetness enhancers named positive allosteric modulators (PAMs), compounds that while not necessarily sweet themselves are capable of enhancing the perception of sweetness from other compounds [6••,7]. These technologies have resulted in faster and safer methods for the discovery of novel sweetening compounds.

Natural high-potency sweeteners

Given this variability in acceptance of artificial sweeteners, an increasing realization of the effects of excessive sugar consumption, and growing interest for natural products by consumers in general, the demand for natural zero-calorie sweeteners has increased significantly. A wide range of plant derived natural products can elicit sweet responses or can modulate sweetness, including terpenoids, phenylpropanoids, dihydroisocoumarins, flavonoids, steroids, proanthocyanidins, amino acids, and proteins. Numerous excellent reviews are available on this subject [7,8•,9], with ongoing work highlighting the potential of biotechnological production of proteins with sweetening or sweetness enhancing ability — thaumatin, mabinlin, monellin, neoculin, brazzein, and miraculin [10–12]. Relatively few sweet-tasting plant-derived natural products have been commercially launched to date, but those numbers are rapidly increasing. These include thaumatin, glycyrrhizic acid, monatin, brazzein, mogroside V, stevioside, and rebaudioside A [13]. The latter two compounds are *ent*-kaurene glycosides from *Stevia rebaudiana*. Along with the mogrosides from *Siraitia grosvenorii*, these glycosylated terpenoid compounds have become increasingly interesting targets for commercial production. The two plant sources that produce the former two molecules are discussed in more detail below.

S. rebaudiana (Bertoni)

S. rebaudiana (Bertoni) (stevia) is a sweet herb native to Paraguay and Brazil [14]. The sweet taste of the leaves (Figure 1a) is due to *ent*-kaurane diterpenoid glycosides, which contain the steviol aglycone core in common and differ in the number and type of sugars attached to C-13 and C-19 (Figure 1b). There are more than a dozen steviol glycosides (SGs) identified in *S. rebaudiana*, including stevioside and rebaudioside A [15–21]. These latter two form the majority of SGs found in stevia, which all told can accumulate up to 20% of dry leaf weight in some strains, and are approximately 200 and 300 times sweeter than sucrose, respectively [16,17]. More highly branched sugar chains at the C-13 and C-19 positions result in increasing sweetness [22], while reduction of the C-16 exocyclic double bond greatly reduces sweetness [23•]. SGs can be perceived as bitter or 'metallic'; SGs with fewer glycosylations appear more so [16,17]. Pawar and colleagues have provided a timely review of analytical methods for SGs [24].

While earlier studies reported mutagenic potential for steviol [25], subsequent studies have shown that steviol is not absorbed by humans [26]. Steviol glycosides have since been demonstrated as safe [27] and have received Generally Regarded as Safe (GRAS) status from the FDA in the United States. Additional information on the nutritional aspects of stevia is available [21,28].

S. grosvenorii (Swingle)

The fruit of *S. grosvenorii* (Swingle), or Luo Han Guo (LHG) (Figure 1c), has a long history of usage as a sweetener and in traditional Chinese medicine for the treatment of colds, dry cough, sore throat, and minor stomach or intestinal discomfort [8]. The major sweet component of LHG fruit was identified as mogroside V (Figure 1d), with further work leading to the identification of seven additional sweet-tasting mogrol glycosides (MGs) [29]. These MGs have the mogrol triterpenoid aglycone in common and differ in the sugars attached, requiring at least three glycosylations for sweetness [30]. The commercial extract mixture of mogrosides IV, V, and VI, siamenoside I, and 11-oxomogroside-V is ~300 times sweeter than sucrose [31,32]. LHG extract and mogroside V have been acknowledged as GRAS by the FDA. As with the SGs, MGs with fewer glycosylations such as mogrosides II and IIIE, found in unripe fruit, can have a bitter or metallic taste [29,30,33], necessitating careful agricultural production to maximize the quality of the plant extract produced.

Criteria for sweetener development

While numerous chemical entities possess sweet or sweetness-enhancing properties, very few prove to be suitable for development and application in food and beverage products. Given the cost-effectiveness of sucrose or artificial sweeteners, high commercial potential is required to justify the major investments required to develop a new natural sweetener product. To have high commercial potential, a sweetener must score highly in a variety of metrics, including the availability in scale, taste quality, safety, stability, solubility, cost, and patentability (Figure 2) [13,34]. Very few molecules with sweetening potential can satisfy all these metrics [8]. Moreover, taste quality is absolutely critical. Consumers strongly prefer the taste of sucrose, and HP sweeteners that perfectly mimic the taste of sucrose have not yet been identified. HP sweeteners commonly show (a) low maximal sweetness intensities, (b) undesirable tastes such as bitter, metallic, and licorice-like, (c) slow-onset sweetness that lingers, and (d) an ability to desensitize perception of sweetness [13]. Blending HP sweeteners has been shown to alleviate some of these issues [9]. To be a successful product, a HP sweetener should be soluble enough to sweeten to an equivalent of 10% sucrose with a clean taste that develops quickly and does not linger. It needs to be safe to the consumer and not exhibit toxicity or mutagenicity. It needs to be pH,