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Enzymatic production of prebiotic fructo-oligosteviol glycosides

Sebastian C. Spohner[a,b,c], Peter Czermak[a,b,c,d,*]

[a] Institute of Bioprocess Engineering and Pharmaceutical Technology, University of Applied Sciences Mittelhesse, Giessen, Germany
[b] Faculty of Biology and Chemistry, Justus Liebig University Giessen, Giessen, Germany
[c] Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Giessen, Germany
[d] Department of Chemical Engineering, Kansas State University, Manhattan, USA

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ABSTRACT

Fructo-oligosaccharides are low-caloric sweeteners with 30–50% of the sweetness of sucrose. They are also used as functional food ingredients due to their prebiotic properties, i.e. they stimulate the growth and activity of lactobacilli and bifidobacteria in the digestive tract. Such compounds are normally extracted from chicory, but they can also be produced enzymatically from sucrose using fructosyltransferases. Steviol glycosides are naturally sweet constituents of *Stevia rebaudiana*, a plant species native to subtropical and tropical regions of western North America and South America. But even highly purified steviol glycosides retain attributes such as bitterness, sweet aftertaste and liquorice flavour, which reflect their degree of glycosylation. Here we describe the enzymatic two-stage conversion of steviol glycosides to prebiotic fructo-oligosteviol glycosides. The steviol glycosides are initially transfructosylated by *Microbacterium saccharopilum* fructosyltransferase and in the second stage the fructosyl chain is elongated by *Aspergillus terreus* fructosyltransferase. The product combines the prebiotic functionality and palatability of fructo-oligosaccharides with the sweetness of native steviol glycosides.

1. Introduction

Fructo-oligosaccharides (FOS) are naturally sweet compounds that are valued as functional food ingredients due to their prebiotic properties, i.e. their ability to stimulate the growth and activity of lactobacilli and bifidobacteria in the digestive tract [1,2]. These short-chain sugar molecules are produced industrially using β-fructofuranosidases (EC 3.2.1.26), which are also known as invertases, and fructosyltransferases (FTases) (EC 2.4.1.9). The latter have been purified and characterized from higher plants, such as asparagus [3], onion [4] and Jerusalem artichoke [5], as well as various bacteria and fungi, such as *Aspergillus* spp. [6–8], *Bacillus macerans* [9], *Schwanniomyces occidentalis* [10], *Candida utilis* [11] and *Xanthophyllomyces dendrorhous* [12]. As shown in Fig. 1, four groups of FTases have been defined according to their specificity, namely 1-SST (sucrose:sucrose 1-fructosyltransferase), 6-SFT (sucrose:fructan 6-fructosyltransferase), 1-FFT (fructan:fructan 1-fructosyltransferase) and G^6-FFT (fructan:fructan 6G-fructosyltransferase). Although these enzyme types differ in their subunit structure, molecular weight, degree of glycosylation, chemical susceptibility and substrate specificity, they all display both hydrolase and transferase activities which require high concentrations of sucrose for the production of FOS. Recently, we demonstrated the Enzymatic production of fructo-oligosaccharides from inexpensive and abundant substrates with liquid commercial enzyme preparation Pectinex Ultra SP-L (Novozymes, Bagsværd, Denmark) using a membrane reactor system [13]. Pectinex Ultra SP-L from *A. aculeatus* is used to remove cellulose and pectin and from fruit juices, but also contains 4% of *A. aculeatus* 1-FFT [14,15]. For the production of FOS *A. aculeatus* 1-FFT from Pectinex Ultra SP-L can also be immobilized onto polymers [16,17]. The *A. japonicas* 1-FFT reaction mechanism has also been determined [18]. *Aspergillus* spp. FTases show remarkable thermal and pH stability as well as a high transferase to hydrolase activity ratio, making them ideal for the continuous production of FOS in a membrane reactor system [7,8,15,19–21]. The N-terminal sequence of the purified enzyme (LDTTAPPXFXLSTLPXXXLF) was determined by Edman degradation and is homologous to the N-terminus of *A. niger* β-fructofuranosidase [15]. Recently, we accomplished the successful secretory expression of *A. terreus* 1-FFT with *K. lactis* [22,23], which has been used to produce FOS from sucrose up to a degree of polymerization of seven. This enzyme is not capable to transfructosylate steviol glycosides. Therefore, a G^6-FFT is needed to transfructosylate the C6 of the terminal glucose moiety. A highly potent G^6-FFT has been purified from *Microbacterium*

* Corresponding author at: University of Applied Sciences Mittelhesse, Wiesenstrasse14, 35390, Giessen, Germany.
E-mail address: peter.czermak@kmub.thm.de (P. Czermak).

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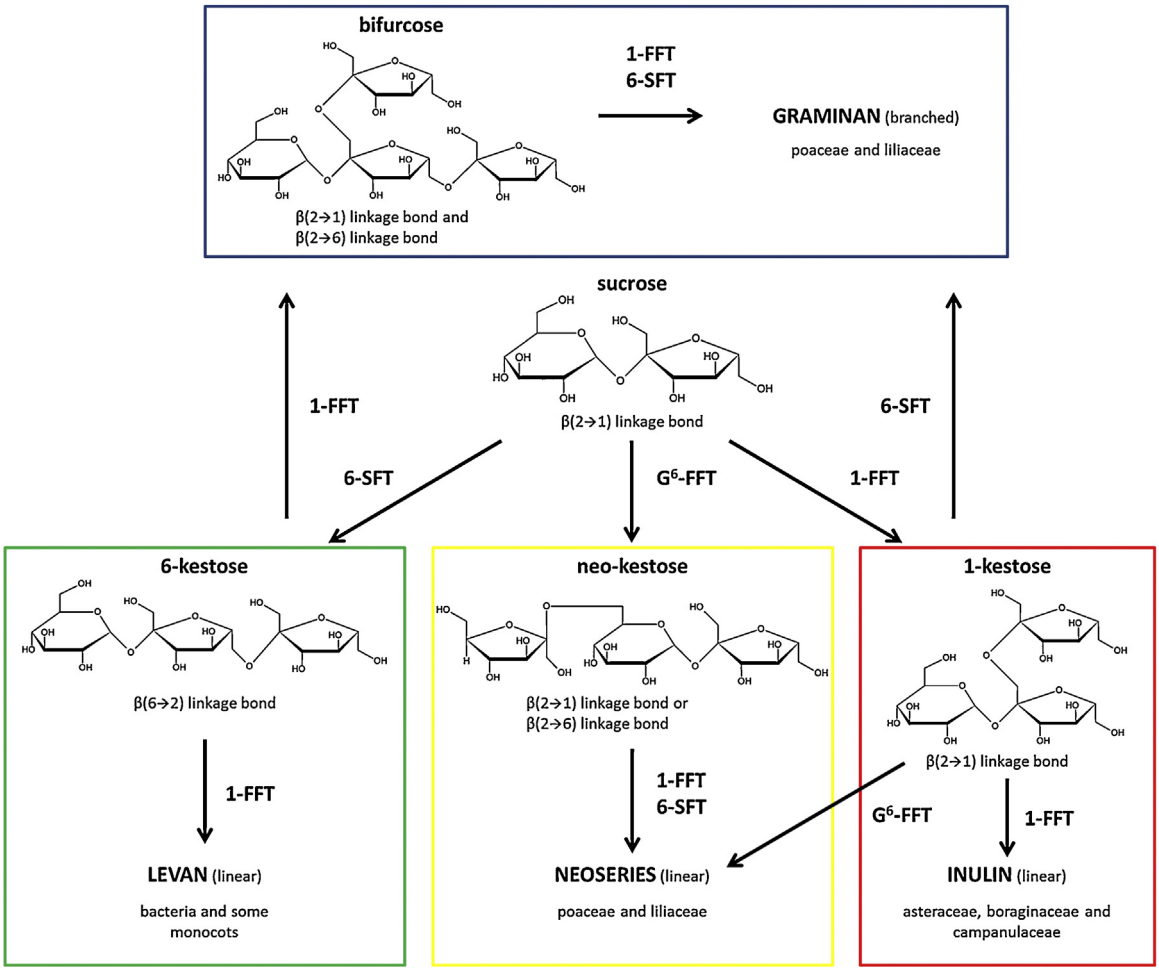

Fig. 1. Fructan biosynthesis from sucrose. Depending on the position of the fructosyl unit and the type of glycosidic linkage, fructans are assigned to four major categories: the graminan, inulin, levan, and neoseries fructans. Their biosynthesis is mediated by S-type and F-type FTases: 1-SST (sucrose:sucrose 1-fructosyltransferase); 6-SFT (sucrose:fructan 6-fructosyltransferase); 1-FFT (fructan:fructan 1-fructosyltransferase) and G^6-FFT (fructan:fructan 6G-fructosyltransferase).

saccharophilum (formerly *Arthrobacter* sp. K-1) cultures grown on sucrose. Unlike other FTases, this enzyme hydrolyses sucrose, erlose, raffinose, fructosylxyloside and neo-kestose, but is largely inactive against inulin-type fructans [24]. This enzyme can therefore be used to fructosylate steviol glycosides such as rebaudioside A (RebA) and thus improve the taste quality of the fructosylated derivatives [25,26].

Steviol glycosides are naturally sweet compounds extracted from the plant *Stevia rebaudiana*. Stevioside is the most abundant steviol glycoside in the leaves of this species and it represents 5–10% of the wet leaf weight. Other sweet compounds in the leaf include rebaudioside A (2–6%), dulcosid A (0.2–2%) and dulcoside B, also known as rebaudioside C (1–2%). The structures of these compounds are shown in Fig. 2. The taste of steviol glycosides strongly depends on the nature and degree of glycosylation [27]. Several approaches have therefore been developed to modify the glycosylation of steviol glycosides [28–40]. A combination of two different FTases can be used not only to improve the taste of steviol glycosides, but also to functionalize them. Fructo-oligosteviol glycosides (FOSGs) combine the prebiotic functionality and palatability of FOS with the intense sweetness of steviol glycosides. Here we describe the two-stage enzymatic conversion of steviol glycosides to produce a novel prebiotic sweetener with intense sweetness and a palatable taste.

2. Materials and methods

2.1. Strains and media

M. saccharophilum strain DSM 28107 producing endogenous G^6-FFT was grown in medium comprising 5.0 g/L NaCl, 2.5 g/L K_2HPO_4, 20 g/L Trypton, 3.0 g/L yeast extract and 40 g/L sucrose (pH 7.0). A recombinant *Kluyveromyces lactis* GG799 strain was used for the secretory expression of *A. terreus* 1-FFT. The strain was pre-cultured on FM22 medium supplemented with 3% glucose. The main culture was supplemented with 1% galactose to induce recombinant protein expression.

2.2. Production of M. saccharophilum G^6-FFT

The main culture was cultivated in a 7-L Applikon bioreactor (working volume 5 L) in the medium described above with control modules for pH, temperature, dissolved oxygen (DO) and air flow. The antifoaming agent J673A Struktol (Schill + Seilacher "Struktol" GmbH, Hamburg, Germany) was used at 5% (v/v) to control foaming. A pre-culture of 100 mL was inoculated to an OD_{600} of 0.01 from a cryostock culture and incubated until the OD_{600} reached 1.0. The entire pre-culture was used to inoculate the main culture to an OD_{600} of approximately 0.02. The temperature was maintained at 23 °C and the pH at 7.0 by automatic titration with NH_3 and 5 M