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Synthesis of 6-kestose using a highly efficient β -fructofuranosidase engineered by directed evolution

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Abstract. The β -fructofuranosidase (Ffase) from *Schwanniomyces occidentalis* (Ffase-Leu196 variant) was subjected to four cycles of directed evolution to enhance the transglycosylation activity for the synthesis of β -(2 $\rightarrow$ 6) linked fructooligosaccharides (FOS). With a 5.5-fold improvement in fructose transferase activity over the parental type and greater selectivity for the synthesis of 6-kestose (up to 73 % of the total FOS), the mutants doubled FOS synthesis to 168 g/L. Whilst the F523V and H510P mutations were located at the C-terminus of the protein, mutations Q78L and I203L were associated with the acidic catalytic triad where they modified its interactions with the surrounding residues, in turn varying the hydrolase and transferase rates.

Keywords: transglycosylation; 6-Kestose; β -fructofuranosidase; *Schwanniomyces occidentalis*; directed evolution.

Oligosaccharides play a pivotal role in cell interactions, recognition and metabolism. All cells in every living organism are covered by an array of different sugar chains, whose composition defines the cell type and phase of growth. In the last couple of decades, the selective sugar metabolism of colonizing microbial communities has aroused huge interest. It is well known that the intestinal microbiota plays an important role in the immune system, which has even led to a new physiological definition of a human being.^[1,2] Fructooligosaccharides (FOS) selectively stimulate the growth and activity of health-promoting Lactobacilli and Bifidobacteria, a so-called prebiotic

effect.^[3,4] Among prebiotic oligosaccharides, β -(2 $\rightarrow$ 6) linked FOS (e.g. 6-kestose) appears to be more beneficial than the traditionally commercialized β -(2 $\rightarrow$ 1) FOS (e.g. 1-kestose) typically produced by *Aspergillus* species.^[5,6] As second generation prebiotics, β -(2 $\rightarrow$ 6) linked FOS have enhanced properties and chemical stability,^[7,8] and they can be obtained by acid hydrolysis of levans polymers, albeit through a non-environmental friendly process. We previously identified a β -fructofuranosidase (Ffase) from the yeast *Schwanniomyces occidentalis* that is capable of synthesizing 6-kestose from sucrose, producing the highest yields and selectivity yet reported (a 3:1 molar ratio of 6-kestose:1-kestose).^[9] An analysis of the Ffase sequence revealed non-conventional codon usage by the native organism that provoked the L196S amino acid change. This non-universal decoding of the Leu codon (CUG) was studied by heterologous functional expression of the enzyme (Ffase-Ser196 and Ffase-Leu196 variants) in *Saccharomyces cerevisiae*. Surprisingly, the Ffase-Leu196 variant had both ~3-fold higher transglycosylation rates and it was ~7-fold more selective for the 6-kestose synthesis, reaching a 14:1 molar ratio of 6-kestose:1-kestose (more than four-fold that of its Ffase-Ser196 counterpart). Taking together, the Ffase-Leu196 variant produced an enriched mixture of FOS containing 65.7 g/L of 6-kestose.^[10] We also recently published the three-dimensional Ffase structure at 2.9 Å resolution, providing new insights in the mechanisms underlying

the transferase/hydrolase activity of family 32 glycosylhydrolases (GH32) to which our Ffase belongs.^[11,12]

Here, the remarkable synthetic capabilities of Ffase have been further optimized by directed evolution. The Ffase-Leu196 variant was used as the starting point for four cycles of random mutagenesis, *in vivo* DNA recombination and screening to tailor a Ffase that was more efficient and selective in synthesising 6-kestose. To explore the mutant libraries for transferase activity, we coupled two colorimetric assays with the framework of a single HTP-screening protocol, as published recently.^[13] *S. cerevisiae* was used as both a heterologous expression host and for the *in vivo* cloning and library construction.^[14] Since Ffase displays both hydrolytic and fructose-transglycosylation activities from its

natural substrate (*i.e.* sucrose acts as the donor and acceptor molecule), the glucose:fructose molar ratio was employed to discriminate between the most active clones, which were ultimately analyzed by anion-exchange chromatography coupled to pulsed amperometric detection (HPAEC-PAD).

The laboratory evolution strategy and the results obtained are summarized in **Fig. 1**. In short, the first three cycles involved random mutagenesis that adjusted the mutational rate to 1-2 amino acid changes per protein. The final round of evolution combined random mutagenesis with recombination by *in vivo* DNA shuffling. In this generation, the NR8 mutant was recovered for backcrossing recombination.

Figure 1. Directed evolution of the Ffase. New mutations are depicted as stars and the mutations accumulated are shown as squares. The parental types used in each cycle are represented in green and the discarded mutants in grey. The NR8 mutant (1G) was recovered for backcrossing recombination in the 4th generation (4G). ¹Improvements in transglycosylation activity (in fold) were determined by HPAEC-PAD, estimating the [transferred fructose]/[total fructose in the reaction mixture], and normalized to the Ffase-Leu196 parental type after 16 h (sucrose conversion 15-30%). The concentration of transferred fructose refers to the fructose contained in the FOS synthesized. ²Reaction time 136 hours.

After screening only ~4000 clones, we identified two variants (11H8 and 8G9) that harboured three amino acid changes, as well as the S196L alteration naturally incorporated by *S. cerevisiae*. Evolved mutants were purified and their activities were analyzed, identifying the main products synthesized as described previously using a combination of ^1H , ^{13}C and 2D-NMR (see **Supplementary Material**, online).^[9,13] From the HPAEC-PAD analysis (**Fig. 2**) it was evident that the high selectivity shown by the Ffase-Leu196 variant for 6-kestose synthesis remained fairly constant throughout the evolution process, while the transglycosylation ratio gradually increased (**Fig. 3**), an effect that was much enhanced over shorter reaction times (the 8G9 mutant showing improvements of ~ 5.5-fold at 16 h vs 2.2-fold at 136 h). This data indicates that the evolved Ffase mutants are capable of producing 6-kestose in sufficiently high amounts as to act as an acceptor, thereby enhancing the size of their homologous series (**Fig. 2**).

Figure 2. HPAEC-PAD chromatograms corresponding to the reaction with sucrose after 136 h. The peaks correspond to sucrose (1), glucose (2), 1-kestose (3), fructose (4), 6-kestose (5), neokestose (6) and tetrasaccharide (7).

As a final result of the evolution process the 11H8 and 8G9 mutants doubled the net FOS production after 136 h over the Ffase-Leu196 parental type: 168.3 g/L for 11H8 and 158.2 g/L for 8G9, of which 70 to 73 % corresponded to 6-kestose (**Fig. 4**).

Figure 3. Transglycosylation activity for Ffase variants. The sugar concentrations were determined from the HPAEC-PAD chromatograms corresponding to the reaction with sucrose after 16 and 136 h.

Figure 4. FOS produced by the final evolved variants. The sugars were evaluated from HPAEC-PAD chromatograms corresponding to reactions over 16 and 136 h.

In addition, the hydrolytic activity of the 11H8 and 8G9 mutants was improved, with the catalytic efficiency (k_{cat}/K_m) enhanced 6 to 8-fold for the three substrates tested (**Table 1**).

Substrate	Variant	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{mM}^{-1}\text{s}^{-1}$)
Sucrose	Parental	39.1 ± 5.1	0.46 ± 0.03	0.012
	11H8	24.2 ± 1.7	2.42 ± 0.3	0.100
	8G9	20.7 ± 2.9	1.39 ± 0.25	0.067
Nystose	Parental	9.8 ± 2.6	0.11 ± 0.02	0.011
	11H8	7.3 ± 1.3	0.69 ± 0.05	0.094
	8G9	6.5 ± 1.9	0.47 ± 0.07	0.072
Raffinose	Parental	13.2 ± 1.7	0.13 ± 0.01	0.010
	11H8	10.4 ± 1.3	0.72 ± 0.04	0.069
	8G9	8.2 ± 1.6	0.50 ± 0.09	0.061

Table 1. Kinetic parameters of the Ffase variants. Parental, Ffase-Leu196. The $\pm$ refers to the standard errors based on the curve fitting using SigmaPlot software.

The 11H8 and 8G9 mutants share three substitutions: S196L, Q78L and I203V (**Fig. 1**). Moreover, they had two independent mutations located at the C-end of the protein (F523V and H510P), in a region far from the Ffase active site that could not have been anticipated by rational design. Indeed, we cannot find a reasonable justification for the effect that such substitutions have on Ffase activity. Concerning the other three substitutions, Gln78 and Ile 203, together with Ser196, are all located at relevant positions of the active site in the Ffase 3D-structure (**Fig. 5A**). As recently reported^[10], the natural S196L change improved transglycosylation activity, whereas Gln78 lies close to Asp50, the catalytic residue that acts as a nucleophile in the enzymatic reaction. Furthermore, this residue is involved in atomic interactions that might help to establish the conformation of the active site. The Q78L mutation may provoke some rearrangements in key residues (Phe110 and Trp76)

that favour substrate binding or the release of certain products, making Ffase more efficient (**Fig. 5B**). Furthermore, this mutation might also alter the transfructosylating product specificity (**Fig. 5C**). Conversely, Ile203 lies close to Glu230 (**Fig. 5B**), the acid base catalyst, and it is also next to Ser196. The I203V-S196L substitutions might introduce local structural modifications in the catalytic Glu230 environment (that includes Gln228 and Asn254, **Fig. 5C**), which may have an important influence on enzyme kinetics. Significantly, a similar effect has previously been reported for the P232V mutation.^[10]

The Ffase evolved variants described here constitute a suitable point of departure for further engineering. Easily secreted by *S. cerevisiae*, highly selective for the synthesis of β -(2 $\rightarrow$ 6) linked FOS and with improved transglycosylation activity, these variants are a good platform to tailor more robust Ffases for synthetic applications.^[15]

Experimental Section

Laboratory evolution of Ffase

The β -fructofuranosidase gene (SoINV) from *Sw. occidentalis* was linearized with *Bam*HI-*Xba*I, cloned with its own signal peptide (1,608 bp) into the pYES2 (Invitrogen) plasmid and functionally expressed in *S. cerevisiae* under the control of *GAL1p*, as described previously.^[11,13] Mutant libraries of the SoINV gene were created by error-prone PCR, testing different $MnCl_2$ concentrations (from 0 to 0.2 mM) to adjust the mutation rate to 1-2 amino acid changes per protein as indicated previously.^[13] In each generation, PCR fragments were cleaned, concentrated and purified in a low melting point preparative agarose gel using the Zymoclean gel DNA recovery kit (Zymo Research). The last cycle of evolution was a combination of random mutagenesis and DNA-shuffling. Mutants 6B12, 1A12 (3G) and NR8 (1G) were employed to construct three mutant libraries that were each recombined *in vivo* by engineering overhangs with 40 bp homology with the *Bam*HI-*Xba*I linearized pYES2 plasmid, allowing the cloning and shuffling of the parental types during the transformation.

The high-throughput (HTP)-screening was based on two colorimetric assays that evaluated glucose and fructose content, and it was adapted to a 96-well microplate format as reported elsewhere.^[13]

Protein purification, enzyme detection and kinetic analysis

Proteins expressed in *S. cerevisiae* were purified as described elsewhere.^[10] Yeast was grown in YPGal medium (1L), the culture filtrates were concentrated and applied to a DEAE-Sephacel chromatography column, and then eluted using a 0-0.5M NaCl gradient. Samples containing fructofuranosidase activity were eluted in 0.1M NaCl and then analysed by Coomassie-stained SDS-PAGE (8%). Only those containing apparently pure proteins were used. Protein concentrations were estimated photometrically at 280 nm (NanoDrop spectrophotometer ND-1000).

Ffase hydrolytic activity was quantified by the dinitrosalicylic acid (DNS) method adapted to a 96-well microplate as described previously.^[9] Enzymatic assays were performed in 50 mM sodium acetate buffer (pH 5.5) at 45 °C. One unit of activity was defined as that catalyzing the formation of 1 μ mol of reducing sugar $min^{-1}ml^{-1}$. For kinetic analysis, the initial velocity was measured in triplicate using 0.3-1 $\mu g\ ml^{-1}$ of the enzyme, and sucrose, nystose or raffinose (0-250 mM) over a 25 min reaction time. The resulting curves were plotted and analysed using SigmaPlot software (version 7.101) and the kinetic

parameters were calculated by fitting the initial rate values to the Michaelis-Menten equation.

Batch production of fructooligosaccharides and HPAEC-PAD analysis

Reactions were carried out and analysed as described elsewhere, with minor modifications.^[13] In short, selected enzymes (0.1 U) were added to 1 mL of 600 g/L sucrose in 50 mM sodium phosphate buffer (pH 5.5) and the mixture was incubated at 45 °C (225 rpm). Aliquots were removed at different times, filtered through 0.45 μm Durapore® membranes and analyzed by high performance anion-exchange chromatography coupled to pulsed amperometric detection (HPAEC-PAD). The concentration of sugars were measured on a ICS3000 Dionex system consisting of a SP gradient pump, an AS-HV autosampler and an electrochemical detector with a gold working electrode and Ag/AgCl as the reference electrode. All the eluents were degassed by flushing with helium and assayed on a pellicular anion-exchange column at 30 °C (4 x 250 mm Carbo-Pack PA-1 column: Dionex) connected to a 4 x 50 CarboPac PA-1 guard column. The peaks were analyzed using Chromeleon software and the different carbohydrates were identified based on known standards. 6-Kestose was purified by preparative HPLC and identified by bidimensional NMR as reported previously.^[9]

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Graphical Abstract

Detail of the active site in the evolved Ffase modelled with 6-kestose (in yellow). The catalytic triad is in pink and the mutated residues are underlined.

Title Text

Adv. Synth. Catal. **Year**, *Volume*, Page – Page

TOC Graphic

Author(s), Corresponding Author(s)*