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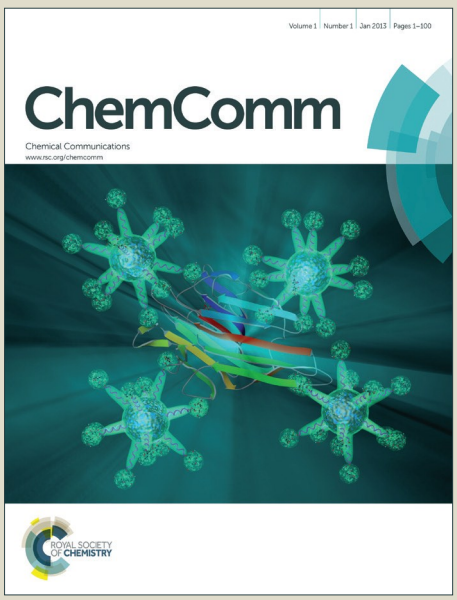

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Converting bulk sugars into prebiotics: semi-rational design of a transglucosylase with controlled selectivity

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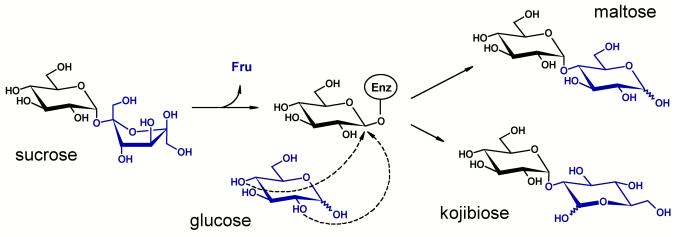
Despite the growing importance of prebiotics in nutrition and gastroenterology, their structural variety is currently still very limited. The lack of straightforward procedures to gain new products in sufficient amounts often hampers application testing and further development. Although the enzyme sucrose phosphorylase can be used to produce the rare disaccharide kojiobiose (α-1,2-glucobiose) from the bullk sugars sucrose and glucose, the target compound is only a side product that is difficult to isolate. Accordingly, for this biocatalyst to become economically attractive, the formation of other glucobioses should be avoided and therefore we applied semi-rational mutagenesis and low-throughput screening, which resulted in a double mutant (L341I_Q345S) with a selectivity of 95% for kojiobiose. That way, an efficient and scalable production process with a yield of 74% could be established, and with a simple yeast treatment and crystallization step over hundred grams of highly pure kojiobiose (>99.5%) was obtained.

One of the most fascinating scientific advances in recent years is the recognition of the intimate relation between the microbiome and our physiology. Commensals are not simply 'passengers', but play pivotal roles in our metabolism and immune response, as well as in several diseases like obesity, inflammatory bowel disease and type 2 diabetes.[1] The gut microbiome has accordingly become a therapeutic target, and prebiotics could be part of future multi-agent treatment regimens in gastroenterology or form components of new functional food formulations.[1-3] Unfortunately, the current menu of prebiotics is rather limited and expanding the scope is highly desired.[2, 3]

In that context, kojibiose (2-*O*-α-D-glucopyranosyl-D-glucopyranose) is a promising lead compound, since kojibiose and derived oligosaccharides can selectively stimulate beneficial gut populations and are largely resistant to digestive enzymes.[4-6] Kojibiose is moreover not metabolized by common oral bacteria and has therefore attracted attention as a low-calorie sweetener for the prevention of tooth decay.[6] Studies on the health-promoting properties are however hampered by the high price and limited availability.[4] Indeed, the amounts present in natural sources are far too low for practical isolation[7-10] and the numerous synthetic procedures all suffer from a poor yield, productivity and/or selectivity, or lack straightforward downstream processing.[11-17] Hence, we engineered a promising candidate enzyme, sucrose phosphorylase from *Bifidobacterium adolescentis*[18, 19], to enable a simple production procedure starting from cheap and readily available bulk sugars.

Sucrose phosphorylase (EC 2.4.1.7) is a member of the Glycoside Hydrolase family 13 (GH13)[20] and *in vivo* catalyzes the reversible phosphorolysis of sucrose into α-D-glucose 1-phosphate and D-fructose. Thanks to its double displacement mechanism, it can also be applied as a transglucosylase *in vitro* when presented with alternative acceptor substrates like glucose (Scheme 1).[21-23] Unfortunately, the *B. adolescentis* wild-type enzyme preferentially connects the two glucose units through an α-1,4-bond (forming maltose) and only generates kojibiose as a minor product (Fig. 1). We therefore decided to apply a semi-rational mutagenesis approach to shift the enzyme's specificity towards kojibiose formation.

First, all eleven positions in the acceptor site were fully



Sheme 1 Non-regioselective transglucosylation of glucose by wild-type sucrose phosphorylase.

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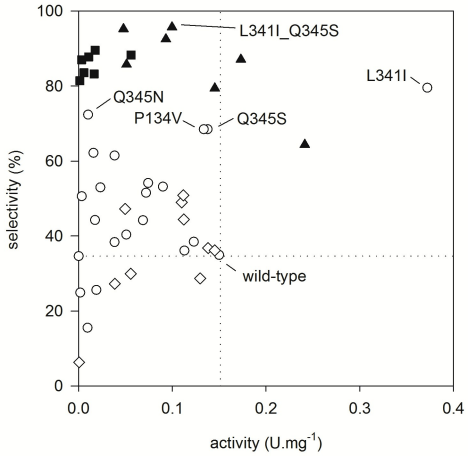

Fig. 1 Activity and selectivity of single mutants obtained from initial screening (○), single alanine mutants (◇) and mutants from statistical (■) and rational recombination (▲) (selectivity: fraction of kojibiose in total product formation; activity: formation of kojibiose; all reactions performed with His-tag purified enzyme, 100 mM sucrose, 200 mM glucose, 55°C, pH 7.0) (more details in Table S1).

randomized one by one (Fig. S1 and Experimental details) and libraries were screened with high-performance anion-exchange chromatography to unequivocally detect kojibiose in the presence of maltose (and other disaccharides that could potentially be formed by mutants) (Fig. S2). From this initial screen on crude cell extracts (~2200 clones), several hits were obtained, which were then purified and characterized to verify the screening results (Fig. 1, Table S1). The best single mutant L341I displayed already an increase in selectivity from 35% (wild-type) to 79%, while its specific activity was 2.5 times higher.

To guide the choice of mutations to recombine for a further uphill walk, a statistical model that links protein sequence to activity/selectivity was applied (see ESI for details).[24] Data from all characterized single mutants (improved as well as some neutral and deleterious) and previously created alanine mutants[25] was used as the training set (Table S1), and predictions for selectivity and activity were made for all possible combinations (~60500). The top ten, predicted to have the highest selectivity and an activity higher than the wild-type enzyme were subsequently retained for *in vitro* testing (Fig. 1, Table S1). Three of these did not express, but for the other variants the selectivity was indeed further enhanced (up to 91%), albeit at the cost of activity. They all contained a triple combination of the most favorable mutations for selectivity (P134V, L341I and Q345N/S) with in addition one or more mutations from variants with very low activity. Since accurate prediction of the activity was difficult (Fig. S3) and an excess of mutations seemed to mask positive synergies, all possible combinations of only P134V, L341I and Q345N/S were evaluated as well (Fig. 1). That way, a double mutant (L341I_Q345S) was obtained with a selectivity of 95% and a specific activity that is still two thirds of the wild-type enzyme. Kinetic characterization revealed that the shift in selectivity is mainly caused by a lowered formation of maltose, rather than an increased production of kojibiose (Table 1; Fig. S4). Nevertheless, the K_M for glucose in the kojibiose forming mode did decrease from about 0.3 M to 0.1 M, which is useful to maintain enzyme saturation during production. Notably, this improved affinity seems to be realized through a synergistic effect, as the mutation Q345S has a negative influence on binding on its own, but further enhances the positive effect of the L341I mutation when they are introduced together. Docking of D-glucose into the wild-type enzyme and its variants could regrettably not provide a rationale for these findings (Fig. S5). It is possible that the active site topology severely changes to accommodate the mutations and/or the glucose molecule, especially since it is known that SP is highly dynamic, with several loop movements and structural rearrangements induced upon substrate binding or mutation.[18, 26]

Interestingly, when presented with the *in vivo* substrates (sucrose and phosphate), the activity of the L341I mutant is the same as the wild-type enzyme, while in the Q345S and the double mutant it is decreased to only 2-3% (data not shown). It thus seems that our final mutant bears a mutation that improves the promiscuous function without abolishing the primary, native activity and another that narrows down the specificity, consistent with the prevailing view on enzyme evolution.[27, 28] Likewise, function-switching mutations often tend to negatively affect protein integrity[29, 30] and therefore the half-life (t_{50}) of the mutants was also determined. The double mutant had a 25% decreased half-life at 60°C ($t_{50,\ L341I\_Q345S}$ = 36 h; $t_{50,\ wild\text{-}type}$ = 49 h), entirely due to the Q345S mutation ($t_{50,\ Q345S}$ = 35 h; $t_{50,\ L341I}$ = 47 h). Yet, no loss in activity was observed after one week of incubation at 55°C, which makes the mutant enzyme a suitable catalyst for industrial processes.

To demonstrate the practical usefulness of the obtained variant, the production of kojibiose was performed at a larger scale (1 liter) and with higher substrate concentrations (0.5 M sucrose and 0.5 M glucose), as preferred by the industry (Fig. 2a). A maximal kojibiose yield of 74% (n/n), relative to the

Table 1 Apparent kinetic parameters for the wild-type enzyme and improved variants.

enzyme	Glc_{koji} [a]			Glc_{malt} [b]		
	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1}.M^{-1}$)	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1}.M^{-1}$)
wild-type	305 ± 48	0.7 ± 0.1	2.4	323 ± 53	1.4 ± 0.1	4.4
L341I	193 ± 23	1.3 ± 0.1	6.7	347 ± 133	0.3 ± 0.1	0.8
Q345S	350 ± 43	0.5 ± 0.1	1.5	406 ± 52	0.3 ± 0.1	0.7
L341I_Q345S	96 ± 6	0.3 ± 0.1	3.0	- [c]	- [c]	- [c]

[a] D-glucose in binding mode that leads to formation of kojibiose; [b] D-glucose in binding mode that leads to formation of maltose; [c] values could not be calculated, because initial reaction rates could not be quantified accurately.

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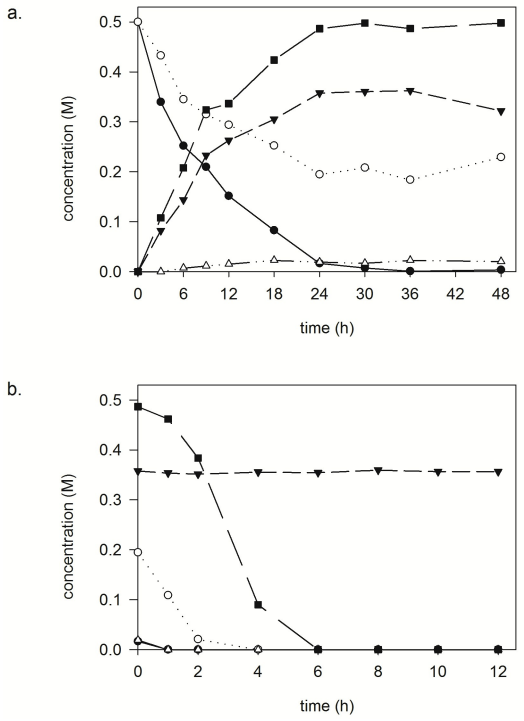

Fig. 2 Synthesis and purification of kojibiose at industrially relevant conditions. a. Enzymatic reaction (0.5 M sucrose, 0.5 M D-glucose, 2 mg/ml heat purified enzyme, 55°C, pH 7.0, buffer-free) and b. yeast treatment to remove contaminating sugars from a 24-hour reaction (lyophilized *S. cerevisiae*, 30 g/l, 30°C) (● sucrose, ○ D-glucose, ▼ kojibiose, △ maltose and ■ D-fructose).

donor substrate, was obtained from the enzymatic reaction (24 h) and kojibiose was only very slowly broken down again upon prolonged incubation. Note that some sucrose donor substrate is lost due to hydrolysis (inherent to the enzyme's mechanism), albeit to a lesser extent than with the wild-type enzyme (~20% compared to ~40%). After reaction, the other sugars were readily removed by a simple yeast treatment that leaves the kojibiose completely untouched (Fig. 2b). The treated solution was finally concentrated and slowly cooled to induce crystallization, yielding 124 g of crystalline kojibiose with a purity exceeding 99.5% (confirmed by HPLC, HPAEC and NMR spectroscopy; Fig. S6 and Fig. S7).

The necessity of our engineering effort can be demonstrated by the fact that we were unable to obtain crystalline kojibiose when this process was performed with the wild-type enzyme. Most likely, the lower kojibiose yield (and higher concentrations of contaminating sugars) complicated both the yeast treatment and the crystallization step.

To put the efficiency of our process in perspective, it can also be compared to a recently developed procedure that makes use of dextransucrase and galactosidase as biocatalysts, and sucrose and lactose as substrates.[16] Although it certainly was the most efficient and sustainable way to obtain kojibiose up to that time, it requires multiple enzymatic and yeast treatments steps with a yield of 19% ($w_{product}/w_{all\_substrates}$) and a kojibiose purity of 65% (as a solution). Higher purities could only be achieved by preparative liquid chromatography, while freeze-drying was applied to obtain a powdered product. In contrast, we reached a conversion yield of 48% ($w_{product}/w_{all\_substrates}$) and could easily crystallize the product, to a purity of >99.5%.

In conclusion, our engineered transglucosylase paved the way for a sustainable, cost-effective and scalable biocatalytic process for the production of highly pure kojibiose, starting from renewable, cheap and readily available bulk sugars. The problem of limited availability and high price has thus been overcome and we foresee that this will allow the potential of kojibiose to be fully exploited and provide opportunities for new applications.

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