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One-step synthesis of α-Gal epitope and globotriose derivatives by an engineered α-galactosidase

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Directed evolution of an α-galactosidase (Aga2) from *Bifidobacterium breve* 203 by random mutagenesis and subsequently by site-directed mutagenesis provided a mutant enzyme V564N that showed high α-transgalactosylation efficiency with unobserved hydrolysis towards transglycosylation products. Using this enzyme, a one-step reaction for the simultaneous synthesis of α-Gal epitope and globotriose derivatives was achieved.

Enzymatic synthesis of oligosaccharides has been a highly attractive approach as it possesses the advantages of high stereo- and regioselectivity, which can be achieved only through many protecting group manipulations in chemical synthesis.[1] Glycosyltransferases and glycosidases are two classes of enzymes responsible for this application. Typically, glycosyltransferases are effective and catalyze stereo/regioselective reactions, but they have strict substrate selectivity and require costly glycosyl donors in the one-step reactions.[2] Glycosidases, which catalyze the formation of glycosidic bonds via transglycosylation, use simple sugar donors and have broad substrate spectra.[3] This property of glycosidases make them extremely attractive for low cost, large-scale synthesis of oligosaccharides. However, the reaction yields catalyzed by glycosidases, which are basically hydrolases, are generally low or moderate, as the transglycosylation products are susceptible to glycosidase-promoted hydrolysis. Thereby, considerable effort has been put on modifying glycosidases so as to reduce or eliminate hydrolysis but retain their transglycosylation abilities. For example, directed evolution as an effective approach to modify the catalytic properties of an enzyme independent of enzyme structure and reaction mechanism has been successfully used to obtain glycosidase mutants with drastically improved transglycosylation efficiencies.[4] In the current work, directed evolution was employed to discover enzymes with high α-transgalactosylation activity.

α-Galacto-oligosaccharides are a class of molecules that have many important functions and applications. For example, α-Gal epitope oligosaccharides bearing the Galα1-3Galβ terminus, which are present on animal cells, have been extensively investigated in xenotransplantation, vaccine development and cancer therapy.[5] Globotriose, Galα1-4Galβ1-4Glc, is another important cell surface epitope, acting as the receptor for the Shiga toxins produced by *Shigella dysenteriae*. It can be used as an affinity inhibitor for the toxins in the prevention and treatment of related diseases.[6] Globotriose is also the core structure of Globo-H that is a tumor-associate glycan with great pharmaceutical potential, thus it can be used as a building block in the synthesis of Globo-H for antitumor vaccine development.[7]

We have recently derived an α-galactosidase, Aga2, from *Bifidobacterium breve* 203, which had a broad substrate spectrum and thus great potential of application to the synthesis of α-Gal-linked oligosaccharides.[8] For example, using *p*-nitrophenyl-α-D-galactopyranoside (*p*NPGal) as a donor, Aga2 was able to transfer galactose to a variety of acceptors including monosaccharides, disaccharides and sugar alcohols. In particular, Aga2 showed excellent Galα1-4 regioselectivity when melibiose was used as acceptor to give Galα1-4Galα1-6Glc as the sole product. However, the reaction yield was rather low (ca. 10.7%) and Aga2 catalyzed obvious product hydrolysis. In this work, directed evolution was applied to Aga2 to discover enzymes with decreased hydrolysis but increased transglycosylation efficiency.

The first round of directed evolution of Aga2 was carried out by random mutagenesis using error-prone PCR with the WT Aga2 as a template. After the blue-white spot screening with 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-Gal) and the detection of transglycosylation activity with melibiose as the substrate, two mutants RM70 and RM103 that exhibited elevated transglycosylation activity compared to WT Aga2 were selected from 335 blue clones. When melibiose is used as the sole substrate of α-galactosidase, the released galactose represents the hydrolysis efficiency, and the released glucose represents the sum of hydrolysis and transglycosylation efficiency. Therefore, the difference between the released glucose and galactose defines the transglycosylation efficiency.[9] The transglycosylation efficiencies of RM70 and RM103 were thus found to be 47% and 45%, which were 21% and 19% higher than that of the WT Aga2 (26%), respectively. Sequencing of these two mutants revealed the presence of three mutations (G218S, D457A and H729R) in RM70 and two mutations (V564E and

H573L) in RM103. According to the amino acid sequence alignment, Aga2 was classified into glycoside hydrolase family 36, and residues D537 and D471 were predicted to be the catalytic general acid/base and nucleophile residues, respectively (Fig. S1†). Thus, none of the above five mutation sites G218, D457, V564, H573 and H729 was the catalytic residue of Aga2.

To identify mutation site(s) that had a significant positive impact on the transglycosylation activity, we constructed five independent mutants G218S, D457A, V564E, H573L and H729R. As shown in Fig. 1, their transglycosylation efficiencies towards melibiose were 27%, 30%, 40%, 39% and 43%, respectively. Compared with the WT Aga2, little difference was observed with mutants G218S and D457A, while remarkable increases in transglycosylation efficiency were found with mutants H729R, V564E and H573L.

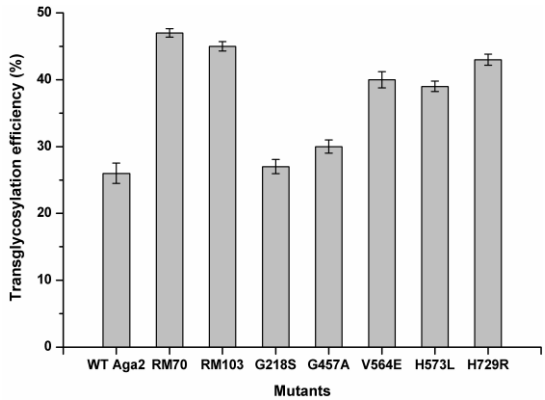


Fig. 1 Transglycosylation efficiencies of the WT Aga2, mutants RM70 (G218S/D457A/H729R), RM103 (V564E/H573L), G218S, D457A, V564E, H573L and H729R.

Since residues V564, H573 and H729 seemed to weigh more importance for the elevated transglycosylation efficiency of mutants RM70 and RM103, these three residues were chosen for the second round of directed evolution through site-directed mutagenesis with the WT Aga2 as template. Amino acid residues with aliphatic, hydroxyl, sulfur, polar, acidic, basic, aromatic and heterocyclic substitutions (I/V, S, M, N, D, R/K, Y, W, and H, respectively) were used to probe these sites. For site H573, the selected aliphatic amino acid was V instead of I because mutant H573L was obtained during the first round of directed evolution and L and I generally had the similar properties. Similarly, for site H729, the chosen basic amino acid was K rather than R because mutant H729R was also obtained in the first round of directed evolution. The transglycosylation efficiency of each mutant was determined by the protocol described above. The results (Fig. 2) showed that residue V564 exhibited a greater impact on improving the transglycosylation efficiency of Aga2 than residues H573 and H729. For residue V564, mutants with aromatic (V564W), acidic (V564D) and polar (V564N) substitutions presented higher transglycosylation efficiency than those of RM70 (47%) and RM103 (45%). Especially, the mutant V564N gave the highest transglycosylation efficiency of 58%, being 32% higher than that of WT Aga2. For residues H573 and H729, however, no transglycosylation activity was detected with mutants H573S, H729N and H729W, and other mutants did not show better activity than RM103 and RM70.

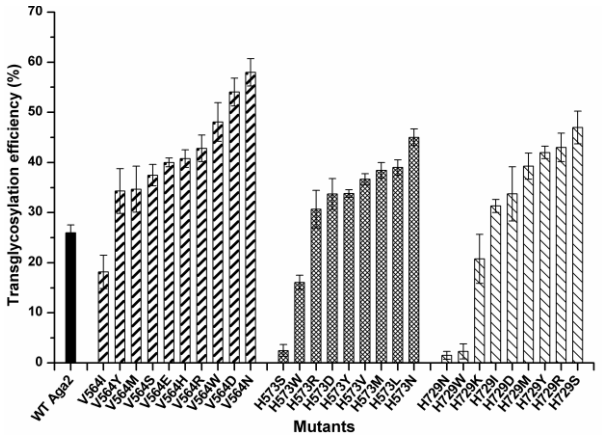


Fig. 2 Transglycosylation efficiencies of Aga2 mutants at residues V564, H573 and H729.

The model structure of mutant V564N showed a significant difference from WT Aga2. The substitution of V564 by asparagine induced remarkable adjustment of the enzyme structure. As shown in Fig. 3, the catalytic cavity of V564N became shallow and wide. This alteration might be responsible for the improved transglycosylation efficiency as it led to easier access or separation of the enzyme from substrate or product. Furthermore, Fig. S2† showed that the residue V564 located at the end of one β-strand segment that took part in the formation of the catalytic cavity. The residue V564 was farther from the active center of the catalytic cavity, when compared with the residues that were adjacent to the catalytic residues or participated in the interaction with substrate. Those residues were usually considered to play positive impact on the transglycosylation efficiency.[9-10] Thusly for α-galactosidase, residues which located in the catalytic cavity but far away from the activity center could influence the structure of the catalytic cavity and also acted as the good alternatives for modification to increase the transglycosylation efficiency.

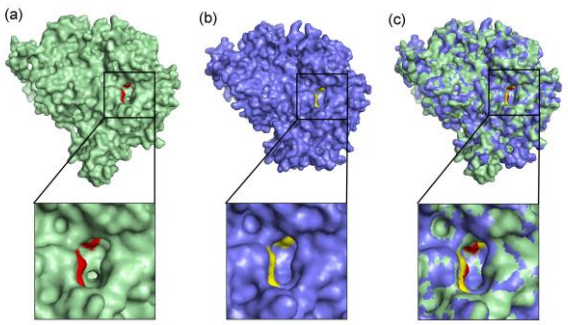


Fig. 3 The model structures of WT Aga2 (a) and V564N (b) as well as their alignment (c). The magnified region includes catalytic cavities. Catalytic residues in WT Aga2 and V564N are red and yellow, respectively.

After an optimized enzyme V564N was discovered, its catalytic property and application to α-galacto-oligosaccharide synthesis were examined with *p*NPGal as donor and methyl β-lactoside as acceptor. Fig. 4 showed the time course of transglycosylation reaction catalyzed by WT Aga2 and V564N. WT Aga2 attained the maximal product yield of 28% at 7.5 min. Then a rapid hydrolysis of the transglycosylation products occurred, which prevented the accumulation of transglycosylation products. For V564N, the maximal total product yield was 38% (at 10 min), which was 10%

higher than that of WT Aga2. Interestingly, the product yield was stable after reaching the maximum in the presence of V564N, and there was hardly any hydrolysis of the products during the testing time (6 h). These results indicated that V564N acted as a transgalactosidase without apparent hydrolysis of the transglycosylation products. This property made V564N significant and useful for the practical application in the oligosaccharides synthesis.

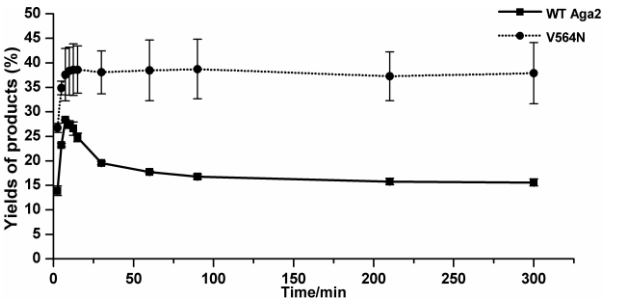
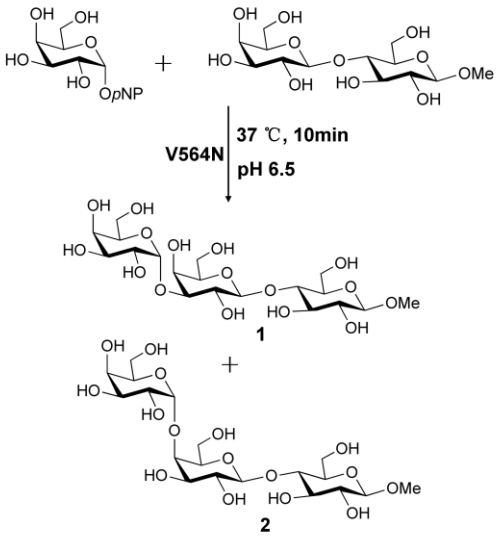


Fig. 4 Time courses of product formation from transgalactosylation reactions catalyzed by the WT Aga2 and V564N with *p*NPGal as donor and methyl β-lactoside as acceptor.

TLC analysis of the above reactions revealed that WT Aga2 and V564N gave the same product pattern, namely, forming two products from each reaction (Fig. S4†). The MS result showed the two products were methyl trisaccharides. The higher polymerization degree galactosyl-oligosaccharides were not detected in the reaction. It is common that glycosidases could generate regioisomers in the same transglycosylation reaction. The flexibility in the regioselectivity of the glycosidases permits the production of diverse glycosides in one-step reaction with low cost. In this work, the two products synthesized by WT Aga2 and V564N could be easily isolated via silica column by HPLC. This facile purification method promoted the availability of this reaction. After purification, the two products were identified as methyl glycoside of the α-Gal epitope (1, 22%, 27.35 g/L ħ) and methyl globotrioside (2, 16%, 19.89 g/L ħ) (Scheme 1). Surprisingly, the two valuable α-galacto-oligosaccharides were available at the same time through one-step enzyme-catalyzed reaction. It is worth noting that the globotriose derivative was obtained by the glycosidase for the first time. According to the report, the globotriose and its derivatives have been achieved through chemical and glycosyltransferase-mediated synthesis. Dohi et al. reported 11-step laborious reactions for the chemical synthesis of globotrioside from methyl D-galactopyranoside and *p*NP β-lactoside as staring materials.[11] And of course, use of galactotransferase is able to synthesize the globotriose in one step with UDP-galactose (UDP-Gal) and lactose as substrates. However, the glycosyl donor UDP-Gal is high cost. Zhao et al. recently reported a multi-enzyme one-pot system in which the UDP-Gal could be produced by three enzymes SpGalK (E.C.2.7.1.6), SpGalU (E.C.2.7.7.9) and PPase (E.C.3.6.1.1) from relatively cheap substrates (Gal, ATP and UTP), combined with the α-1,4 galactosyl transfer catalyzed by galactotransferase.[12] Nevertheless, the preparation of multiple enzymes was laborious and introduced extra cost. In this work, the globotriose derivative was obtained through a novel method using the glycosidase. The methyl globotrioside could be synthesized by an engineered galactosidase using simple and low-cost substrates. Additionally, the α-Gal epitope that had been previously synthesized by glycosidases[13] was also produced by the enzyme along with the globotrioside. In this work, one-step reaction was enough to attain these two valuable α-galacto-oligosaccharides.



Scheme 1 Synthesis of α-Gal epitope and globotriose derivatives by V564N.

Conclusions

In summary, we have established a novel facile method for the simultaneous synthesis of α-Gal epitope and globotriose derivatives in the same one-step reaction by an engineered glycosidase. The α-galactosidase (Aga2) from *B. breve* was firstly subjected to directed evolution and generated a transgalactosidase (V564N) with improved transglycosylation property. The mutant enzyme V564N was subsequently used for the synthesis of α-Gal epitopes and globotriose derivatives from the simple substrates *p*NPGal and methyl β-lactoside. The two products were readily separated from each other by HPLC. The successful access to the α-Gal epitopes and globotriose derivatives in a one-enzyme one-step reaction suggested that the V564N would be a powerful tool for the synthesis of medically and pharmaceutically important α-galacto-oligosaccharides, and this method would be of practical meaning as it significantly reduced the time and cost for the oligosaccharide production.

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Notes and references

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† Electronic supplementary information (ESI) available: Experimental details for directed evolution of Aga2 and enzymatic synthesis; NMR spectra of final products. See DOI: 10.1039/c000000x/

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