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Structural and Kinetic Insights into Amyloid-β (1–40) Aggregation and Fibril Formation

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ABSTRACT

Long-chain alkyl glucosides, such as octyl and decyl *β*-D-glucopyranosides (OG and DG, respectively), are regarded as a new generation of biodegradable, non-ionic surfactants. Previously, the mutants of *Dalbergia cochinchinensis* Pierre dalcochinase showed potential in the synthesis of oligosaccharides and alkyl glucosides. In this study, the N189F dalcochinase mutant gave the highest yields of OG and DG synthesis under reverse hydrolysis conditions. The optimized yield of OG (57.5 mol %) was obtained in the reactions containing 0.25 M glucose and 0.3 units of the N189F mutant in buffer-saturated octanol at 30 °C. The identity of OG and DG products was confirmed by high resolution mass spectrometry (HRMS) and NMR. Consistent with its capability for synthesis, the reactivation kinetics and ITC analysis revealed that the aglycone binding pocket of the N189F mutant was more favorable for long-chain alkyl alcohols than the wild-type dalcochinase, while their glycone binding pockets showed similar affinity for the glucosyl moiety. STD NMR revealed higher interactions at the aglycone sites than the glycone sites. Our results demonstrated a promising potential of the N189F dalcochinase mutant in the future commercial production of long-chain alkyl glucosides via reverse hydrolysis reactions.

Keywords:

alkyl glucoside; dalcochinase; decyl *β*-D-glucopyranoside; octyl *β*-D-glucopyranoside; reverse hydrolysis

Abbreviations:

2FDNPG, 2′,4′-dinitrophenyl 2-deoxy-2-fluoro-*β*-D-glucopyranoside; 4MU-Glc, 4-methylumbeliferyl *β*-D-glucopyranoside; 4NP-Glc, 4-nitrophenyl *β*-D-glucopyranoside; 4NP-S-Glc, 4-nitrophenyl *β*-D-thioglucopyranoside; DG, decyl *β*-D-glucopyranoside; GH1, glycoside hydrolase family 1; HG, hexyl *β*-D-glucopyranoside; HRMS, high resolution mass spectrometry; OG, octyl *β*-D-glucopyranoside.

1. Introduction

Long-chain alkyl glucosides, such as octyl and decyl *β*-D-glucopyranosides (OG and DG, respectively), represent a new generation of non-ionic, non-toxic, low irritating and biodegradable surfactants. They have found wide-ranging applications as food products, pharmaceuticals, cosmetic ingredients, cleaning products, drug carriers as well as solubilizing agents for dissolving biological membrane proteins [1,2]. The emulsifying properties of alkyl glucosides vary depending on the length of their hydrocarbon chains [3]. The enzymatic synthesis of alkyl glucosides has recently attracted considerable attention, since the specificity of enzymes allows mild reaction conditions, in addition to suitable regio- and stereo-selectivity that eliminates protection/deprotection steps. Some members of glycoside hydrolases and glycosyl transferases can catalyze synthesis reaction via reverse hydrolysis or transglucosylation reactions

that have potential applications for the synthesis of novel oligosaccharides and alkyl glucosides. Reactions by glycoside hydrolases are preferable over glycosyl transferases because they do not require expensive cofactors, such as uridine diphosphate. In addition, reverse hydrolysis reactions between free glucose and fatty alcohols are more cost effective than transglucosylation because glucose is naturally occurring and much cheaper than a glucosyl donor such as 4-nitrophenyl β-D-glucopyranoside (4NP-Glc) used in the transglucosylation reactions [4,5]. However, a drawback of reverse hydrolysis reactions is the presence of water, which is required to maintain enzymatic activity but also causes secondary product hydrolysis. Thus, many previous studies employed buffer-saturated alcohols in order to reduce water activity [6,7].

β-Glucosidases (E.C. 3.2.1.21) are groups of enzymes that catalyze the hydrolysis of β-O-glucosidic linkages between D-glucose and an aglycone or another sugar. Most β-glucosidases belong to glycoside hydrolase family 1 (GH1), and share similar properties, such as a $(\beta/\alpha)_8$ barrel fold, optimal pH between 5-6, molecular weight about 55-65 kDa, and the double-displacement mechanism. However, β-glucosidases from various sources exhibit distinct substrate specificities and catalytic abilities [8]. Two GH1 β-glucosidases from *Dalbergia cochinchinensis* Pierre (dalcochinase) and *Manihot esculenta* Crantz (linamarase) share 47% sequence identity, but their aglycone specificities and synthesis abilities are very different [7,9-11]. In our previous studies, we probed for the structural determinants responsible for the catalytic differences between these two enzymes by generating a series of single, double and triple mutants of dalcochinase. Our results showed that the hydrolytic and transglucosylation activities of dalcochinase could be converted to those of linamarase by substitution of only 2-3 corresponding residues in the aglycone binding pocket [12,13]. In addition, the N189F mutant

exhibited a good ability to synthesize hexyl *β*-D-glucopyranoside (HG) via reverse hydrolysis reactions between free glucose and hexyl alcohol [13].

Therefore, this study aimed to investigate the potential application of these dalcochinase mutants in the enzymatic synthesis of long-chain alkyl glucosides, namely OG and DG, via reverse hydrolysis reactions (Scheme 1). To reduce secondary product hydrolysis, the buffer-saturated alcohol systems were employed. The yields of OG and DG were analyzed by using TLC, and optimized. The identity of the reaction products were further verified by NMR and high resolution mass spectrometry (HRMS). The thermodynamic and kinetic properties of these reactions were further characterized by the observed rate constants ($k_{re,obs}$) of reactivation, STD NMR and ITC. Altogether, these data expand the applicability of our novel *β*-glucosidases in synthesis of long-chain alkyl glucosides for various industries.

2. Materials and methods

2.1 Materials

2′,4′-Dinitrophenyl 2-deoxy-2-fluoro-*β*-D-glucopyranoside (2FDNPG), 4-methylumbeliferyl *β*-D-glucopyranoside (4MU-Glc), 4NP-Glc, 4-nitrophenyl *β*-D-thioglucopyranoside (4NP-S-Glc), HG and almond *β*-glucosidase were purchased from Sigma-Aldrich (St. Louis, MO, USA). Octanol, decanol, chloroform and TLC plates (silica gel 60 F_{254}) were obtained from Merck (Darmstadt, Germany). All other chemicals were of analytical grade. The wild-type dalcochinase, its three single mutants, namely I185A, N189F and V255F, as well as the ensuing double and triple mutants, namely I185A/N189F, I185/ V255F, N189F/V255F and I185A/N189F/V255F, were expressed and purified from their respective *P. pastoris* clones

as described previously [12,13]. Natural cassava linamarase was purified from its natural source as described previously [14]. The β-glucosidase activity was measured by incubating the purified enzyme with 15 mM 4NP-Glc in 0.1 M sodium acetate, pH 5.5, at 30 °C for 5 min. Then, the reaction was stopped by adding 2 M Na_2CO_3, pH 10. The absorbance of the released *p*-nitrophenol was measured at 405 nm and compared to its standard curve. One unit of enzyme is defined by the amount of enzyme required for the production of 1 µmol of *p*-nitrophenol from 4NP-Glc per min.

2.2 Alkyl glucoside synthesis via reverse hydrolysis reactions

OG and DG synthesis via reverse hydrolysis reactions was carried out at 40 °C with shaking at 300 rpm, in a 100 µL reaction mixture consisting of 0.25 M D-glucose and 0.1 unit β-glucosidase (in less than 2% of total volume) in alcohol (octanol or decanol), which was previously equilibrated with 0.1 M sodium acetate, pH 5.5 [7,13]. All reactions were performed in triplicate. The time-course of product formation was followed for 12 days. At intervals of 2 days, aliquots of 8 µL were taken, heated at 100 °C for 10 min, and evaporated under centrifugal vacuum concentrators. The resulting precipitates were re-dissolved in 5 µl of methanol:water (7:3 by volume) and spotted on a TLC plate, together with known amounts of HG and glucose as standard markers. The TLC plate was developed twice in chloroform:ethanol (15:5 by volume) for 5 cm, and twice in isopropanol:ethanol:water (5:1:2 by volume) for 2 cm. Chromatograms were visualized by spraying with 20% sulfuric acid in ethanol, and heating at 125 °C for 10 min, and quantitated by an image scanner (ChemiDoc XRS+, Bio-Rad, USA). Standard curves between density and amount of standard markers with correlation coefficient (r^2) of 0.95 or greater were used. The yields of alkyl glucosides were expressed as mole percent of the moles of

alkyl glucosides relative to the total moles of free glucose and alkyl glucosides present in each reaction.

2.3 Optimization of OG production in the buffer-saturated alcohols

OG synthesis was carried out in the buffer-saturated system catalyzed by the enzyme giving the highest yields for 12 days as described previously. Firstly, to find the optimum glucose concentration, the reactions were performed using 0.1 unit of enzyme and different glucose concentrations (namely 0.05, 0.1, 0.25 and 0.5 M) at 40 °C. Secondly, to find the optimum amount of enzyme, the reactions were performed using glucose (at the concentration that gave the highest yield of OG) and different amounts of enzyme (namely 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.8 unit) at 40 °C. Thirdly, to find the optimum temperature, the reactions were performed using glucose and enzyme (both at the concentrations that gave the highest yield of OG) at various temperatures (namely 20, 30, 40 and 50 °C). Then the amounts of products were detected by TLC and quantitated by an image scanner as described previously.

The stability of the N189F dalcochinase mutant in the optimized reverse hydrolysis reaction condition was tested by removing the enzyme portion from the total reaction at different time points, and reacting it with 15 mM 4NP-Glc in 0.1 M sodium acetate, pH 5.5, at 30 °C for 5 min. The percent relative activity was calculated from the remaining activity relative to the total activity before the synthesis reactions were initiated.

2.4 Preparative TLC and HRMS

OG and DC synthesis was carried out in the buffer-saturated system in a total volume of 400 µL under the optimized condition as determined previously. The reaction mixtures were

separated using preparative TLC (20 cm x 20 cm, 2.5 mm SiO_2 thickness), which was developed in dichloromethane:ethanol (15:2 by volume). A part of TLC plate was stained by spraying with 20% sulfuric acid in ethanol, and heating to visualize the expected band. Subsequently, the band of interest was scraped off with spatula, re-dissolved in methanol, filtered through the funnel with a cotton plug to remove silica, and dried by evaporator. Then the purified products were re-dissolved in 200 μL methanol, passed through a 0.22 μm pore size filter, and further analyzed by HRMS with electrospray ionization mode, on a Bruker microtof-Q III. The instrument was operated in a positive mode. The ion source was operated with the capillary voltage at 4500 V, nebulizer pressure at 0.3 bar, dry heater at 180 °C, and drying gas flow rate at 4.0 L/min.

2.5 Homology modeling and molecular docking

The three-dimensional models of the wild-type and N189F mutant forms of dalcochinase were generated by using SWISS-MODEL [15], using the structure of cyanogenic β-glucosidase from *Trifolium repens* L. (PDB code 1CBG) [16], with 60% sequence identity as a template. The quality of the models was checked by PROCHECK, ProSA and Verify-3D programs [17-19]. The active site was defined as 15 Å around the pseudo-atom which was located at the center of catalytic residues, E182 and E396, of dalcochinase. The structure of OG from PubChem (CID 548230) was docked into the binding pockets of both models by using GOLD [20]. The docked conformation was analyzed by using Accelrys DS Visualizer 3.0 (Accelrys Inc., San Diego, USA).

2.6 NMR and STD NMR spectroscopy

A solution of the N189F dalcochinase mutant in 0.1 M sodium acetate, pH 5.5, in D_2O (2% of the final volume) was diluted into octanol-d_{18} containing 0.25 M $^{13}C_1$-glucose, to achieve a final enzyme concentration of 0.37 μM (0.3 units) and 2% D_2O. The reaction mixtures were prepared in a 3 mm NMR tube, and monitored by acquiring ^{1}H-^{13}C HSQC spectra with spectral widths of 8 Hz for ^{1}H and 24 Hz for ^{13}C at 40 °C (313 K) for 150 h. Continuous measurements, which took approximately 10 min each, were acquired 5 min and 1.5 h after adding the enzyme, and then every 4 h. By integration of the corresponding peaks, the concentrations (mM) of each of the substances (α-glucose, β-glucose and OG) at different reaction times were estimated.

^{1}H STD NMR were performed in order to study the interactions between the enzyme and ligands. The wild-type dalcochinase (9.4 μM) in 0.1 M sodium acetate, pH 5.5, in D_2O, was mixed with 940 μM small molecule ligands (glucose, OG, thiocellobiose, 4NP-Glc and 4NP-S-Glc) in 99.98% D_2O at 30 °C (303 K). ^{1}H STD NMR spectra were acquired 20 min after adding the ligand to the enzyme. For 4NP-S-Glc, a second experiment was acquired after overnight incubation.

All NMR experiments were performed on a Bruker AVANCE III 600MHz spectrometer. The resulting data were processed and analyzed by TopSpin 3.5 software.

2.7 Reactivation kinetics

The inactivated enzymes were prepared by incubating the purified enzymes with 50 μM 2FDNPG in 0.1 M sodium acetate, pH 5.5, at 30 °C for 30 min. The excess 2FDNPG was removed by centrifugal ultrafiltration. The trapped 2-deoxy-2-flouro-glucosyl-enzyme intermediate (E-2FGlc) was reactivated by incubating with 0.1 M sodium acetate, pH 5.5 (hydrolysis) or 0.5 M of various alcohols (transglucosylation) at 30 °C for 5 h. The 2-deoxy-2-

fluoroglucosyl molecule from the enzyme was transferred to water or alcohol acceptors resulting in the regain of enzyme activity, which was measured by using 200 µM 4MU-Glc in 0.1 M sodium acetate, pH 5.5, at 30 °C. The amount of 4-methylumbelliferone released was measured for 60 seconds at excitation 350 nm and emission 450 nm by using a luminescence spectrometer (Perkin Elmer LS50B, USA) with a slit width of 3 nm. The observed rate constants of reactivation ($k_{re,obs}$) were calculated from the linear slope of the plot of ln $[(V_0\text{-}V)/V_0]$ versus time, where V and V_0 represent enzyme activities in the presence and absence of inhibitor, respectively [21].

2.8 Isothermal Titration Calorimetry (ITC)

To investigate the interaction in the glycone binding pocket between δ-gluconolactone and the enzyme, ITC was carried out using a Microcal ITC200 at 25 °C (298 K). The purified enzyme was dialyzed with degassed 0.1 M sodium acetate, pH 5.5, overnight. Then the matching buffer was used to dissolve δ-gluconolactone. The calorimetric cell was filled with 77 µM of the wild-type dalcochinase or 45.5 µM of the N189F mutant, and the syringe was filled with 8 or 40 mM δ-gluconolactone, respectively. The titration was performed at a constant temperature. The first 0.4 µL injection was performed to minimize the volumetric error, and was discarded in the analysis. The subsequent titration was performed by 20 injections of ligand (2 µL each) into the sample cell at 500 rpm. In each case, the time between each injection was 150 s. The data obtained from isothermal titration were fitted to the one-site model with identical and independent binding sites, and the number of binding sites (n), the dissociation constant (K_D), and the enthalpy of binding (ΔH) were determined by using the Microcal ITC200 software. The

free energy (ΔG) and entropy (ΔS) of binding can be calculated from the Gibb's free energy relationship (Eq.1).

$$\Delta G = \Delta H - T\Delta S = RT \ln K_D \quad \text{(Eq. 1)}$$

where R is the gas constant, and T is the absolute temperature (303 K).

3. Results and discussion

3.1 Synthesis of alkyl glucosides

All 10 β-glucosidase enzymes, including the wild-type dalcochinase, 7 constructs of dalcochinase mutants, cassava linamarase and almond β-glucosidase, were used to catalyze the reverse hydrolysis reactions between 0.25 M glucose and buffer-saturated octanol or decanol to produce OG and DG. The amounts of OG and DG increased significantly during the first 4 days of the reactions, and then only slightly up to day 12 (Fig. 1). The yields of OG on day 12 of the reactions of all dalcochinase mutants were similar to that of almond β-glucosidase, but higher than those of the wild-type dalcochinase and cassava linamarase (Table 1). For DG production, all dalcochinase mutants (except the V255F mutant) gave similar or higher production yields than the wild-type dalcochinase, cassava linamarase and almond β-glucosidase. In particular, the N189F mutant exhibited the highest product yields, which were more than 2-fold higher than the wild-type dalcochinase, and similar (for OG) and 3-fold higher (for DG) than almond β-glucosidase. Our results agree well with our previous report of the highest yield of HG production using the N189F mutant under similar conditions compared with the wild-type dalcochinase and other dalcochinase mutants [13]. Therefore the N189F substitution in dalcochinase provided suitable geometry and hydrophobicity to accommodate long-chain alkyl

alcohols in the aglycone binding pocket, while maintaining the ability to catalyze the reverse hydrolysis reaction. In support of our experimental finding, we also performed *in silico* analysis to assess the possible occupation of OG in the binding pockets of both the wild-type dalcochinase and the N189F mutant. Docking of OG in the binding pockets of both enzyme models showed that OG were located at similar positions (Fig. 2 and Table S1). The octyl chain of OG could form close hydrophobic interaction with the sidechain of F189 in the N189F mutant, but face the polar sidechain of the N189 of the wild-type enzyme. Consequently, the N189F dalcochinase mutant, which exhibited the highest potential of long-chain alkyl glucoside synthesis, was selected for the following optimization studies.

To find the optimum glucose concentration for OG production, the reactions were performed using 0.1 unit of the N189F dalcochinase mutant and different glucose concentrations at 40 °C. The highest yield of OG was found in the reaction with 0.25 M glucose, followed by 0.1, 0.5 and 0.05 M glucose (Table 2). It might be expected that greater starting concentrations would drive the reaction forward, resulting in higher product yields at equilibrium. However, high glucose concentration (in this case, 0.5 M) may have an inhibitory effect since glucose is a well-known competitive inhibitor of β-glucosidases [22]. Moreover, the solubility of glucose in alcohol is a factor affecting alkyl glucoside synthesis [23]. Therefore, 0.25 M was the optimal glucose concentration to be used in further optimization experiments.

To find the optimum amount of enzyme for OG production, the reactions were performed using 0.25 M glucose and different amounts of the N189F dalcochinase mutant at 40 °C. The production of OG increased with increasing amount of enzyme as the enzyme became denatured over the course of reaction, and reached the highest yield in the reaction with 0.3 unit of enzyme (Table 2). However, increasing the amounts of enzyme above 0.3 unit was not beneficial for OG

production, probably because higher amounts of enzyme could also lead to hydrolysis of the alkyl glucoside products to glucose and alcohol. Therefore, 0.25 M glucose and 0.3 unit of the N189F mutant were the optimal conditions to be used in further optimization experiment.

To find the optimum temperature for OG production, the reactions were performed using 0.25 M glucose and 0.3 unit of the N189F dalcochinase mutant at various temperatures. The highest yield of OG was found at 30 °C (57.5 mol % or about 42 g/L), followed by 20, 40 and 50 °C (Table 2), presumably because higher reaction temperature may cause enzyme denaturation during prolonged incubation, resulting in lower product yields. Lowering the temperature to 20 °C was not beneficial for OG production because the elevated temperature is needed to provide kinetic energy for productive collision between the reacting species, and the solubility of glucose in water solution decreased when the temperature decreased (up to 0.45 M glucose at 20 °C) [24]. Therefore, 0.25 M glucose, 0.3 unit of the N189F mutant and 30 °C were the optimal conditions for synthesis of OG via reverse hydrolysis reactions in the buffer-saturated system. Comparison with previously reported data reveals that our OG production using the N189F mutant under the optimized condition was higher than those using other β-glucosidases under similar reverse hydrolysis reaction conditions (Table S2), and also higher than those in other non-aqueous reverse hydrolysis reaction systems (Table S3). The optimized reaction conditions described here can be used as a starting point for further optimization by via a well-developed statistical approach such as the response surface methodology.

The stability of the N189F dalcochinase mutant in the optimized reaction condition was tested at different time points. The relative activities in the reaction containing buffer-saturated octanol, which were about 60% and 10% after 3 and 12 days of reaction, respectively, were lower than those in the buffer, which were about 90% and 60%, respectively (Table S4). In

comparison, almond β-glucosidase retained 48% relative activity after 2 days in pure octanol [25]. The decreased activity of the N189F dalcochinase mutant suggested that further incubation beyond day 12 of the reaction would produce little more OG.

3.2 Identification of alkyl glucoside products

OG and DG were prepared via the reverse hydrolysis reactions under the optimization conditions described above, resulting in one major spot of each alkyl glucoside on TLC. Each alkyl glucoside was further isolated on preparative TLC to yield alkyl glucosides with higher purity (0.0037 g for OG and 0.0021 g for DG). These purified products were determined as OG and DG by HRMS (Fig. 3). The observed masses of OG and DG correspond to the mass of their sodium adducts. A previous research has reported two alternative mass spectra of OG, which were m/z =294 $[M+2]^+$ and m/z = 317 $[M+2+Na]^+$ [26].

The synthesis of OG catalyzed by the N189F dalcochinase mutant in octanol-d_{18} was followed by ^1H-^{13}C HSQC NMR using ^{13}C-labelled glucose at position C_1 as starting material. The increasing signals of the product were observed at 4.13 ppm for ^1H and 102.8 ppm for ^{13}C (Fig. 4A), corresponding to the anomeric $^1H/^{13}C$ pair of the β-linkage of OG [27]. The corresponding signals of the starting materials, which were 4.99/92.29 ppm for the α-glucose and 4.37/96.53 ppm for the β-glucose slowly decreased with time [28]. Integration of the corresponding peaks on the ^1H-^{13}C HSQC spectra revealed that the OG yield increased over incubation time, while the concentrations of the starting materials (α-glucose and β-glucose) decreased slowly (Fig. 4B). The highest yield of OG, approximately 185 mM (74 mol % or 54 g/L), was obtained after 7 days of reaction. The discrepancy between the OG yields estimated by

TLC and NMR was likely due to the possible loss of OG during sample handling when analyzed by TLC and also the higher sensitivity of the NMR technique.

3.3 Reactivation kinetics

All tested enzymes were reversibly inactivated by 2FDNPG, forming the trapped glucosyl-enzyme intermediates, which could be rescued by a transfer of the trapped glucosyl moiety to alcohol (transglucosylation) or water (hydrolysis) acceptors. The observed rate constants of reactivation ($k_{re,obs}$) of the inactivated enzyme were used as a quantitative measure of the rates of transglucosylation or hydrolysis reactions in the presence or absence of alcohol, respectively. The plots of $\ln[(V_0\text{-}V)/V_0]$ versus incubation time were linear (data not shown), suggesting the reactions follow pseudo-first-order kinetics, and the slopes of which were taken as the $k_{re,ob}$ values (Table 3).

It is noted that only a few dalcochinase mutants exhibited increased $k_{re,obs}$ values when compared with the wild-type enzyme, even though they were supposed to provide better hydrophobic interaction with the aliphatic chain of alcohols while retaining reverse hydrolysis capability [13]. More importantly, however, most dalcochinase mutants showed higher $k_{re,obs}$ values with the 2 alcohol acceptors than with water, indicating a preference for alcohol as glycosyl acceptors (transglucosylation) over water (hydrolysis), whereas the wild-type dalcochinase showed similar $k_{re,obs}$ values with all acceptor moieties. So, these results are in agreement with the higher OD and DG yields from most dalcochinase mutants than from the wild-type enzyme (Table 1). Almond β-glucosidase exhibited similar $k_{re,obs}$ values with decanol and buffer, indicating comparable preferences for both acceptors and in agreement with its low DG production. Cassava linamarase, on the other hand, showed the greatest increases in the $k_{re,obs}$

values with the 2 alcohol acceptors relative to water but produced low alkyl glucoside yields, which are consistent with its high transglucosylation activity but low reverse hydrolysis capability [7]. There were some additional disagreements between the alkyl glucoside yields and the $k_{re,obs}$ values (such as the significant increase and decrease in the $k_{re,obs}$ values of the V255F and the I185A/V255F mutants, respectively, with decanol), which could be due to secondary product hydrolysis during the 12-day incubation.

3.4 ITC analysis

ITC analysis was performed to investigate the interaction between the glycone binding pocket of enzyme and δ-gluconolactone, which mimics the proposed $^4H_3$ half chair transition-state conformation of the glucose substrate and thus represents the substrate glycone moiety [29]. The isothermal titration curves and the heat of interaction per injection (Fig. S1) exhibited downward peaks, consistent with exothermic reaction [30]. The number of binding sites on the wild-type dalcochinase was determined to be about 4 sites, which is consistent with the estimated 4-6 subunits constituting the native enzyme [31]. So, the number of binding sites on the N189F mutant was fixed at 4. The binding parameters of δ-gluconolactone to the wild-type dalcochinase and the N189F mutant were compared with the corresponding parameters of almond β-glucosidase reported previously (Table 4) [32]. The K_D values of the wild-type enzyme and the N189F mutant were 26- and 75-fold higher, respectively, than that of almond β-glucosidase, indicating a higher affinity of the latter enzyme for the glucosyl moiety and consistent with its high reverse hydrolysis capability [7]. The higher K_D values of the two dalcochinase enzymes resulted in smaller decreases in ΔG when compared with almond β-glucosidase. Therefore, the binding of δ-gluconolactone to both enzymes resulted in less favorable changes in enthalpy

(negative ΔH) and entropy (positive ΔS) compared with almond β-glucosidase. Nonetheless, the K_D value of the N189F mutant was only 3-fold higher than the wild-type dalcochinase, suggesting similar binding affinity to the glycone moiety and providing evidence for their similar reverse hydrolysis capability.

3.5 STD NMR

^{1}H STD NMR experiments were performed to analyze the binding epitopes of various ligands when binding to the wild-type dalcochinase. 4NP-Glc and OG were completely hydrolyzed within 20 min of incubation, and thus were not suitable for studying recognition mode (results not shown). In addition, the wild-type dalcochinase showed no interaction with free glucose, and only extremely weak STD signals with thiocellobiose after overnight incubation (results not shown). On the other hand, the STD experiments performed on a 1:100 molar ratio of the wild-type dalcochinase and 4NP-S-Glc after 20 min of incubation demonstrated the interaction of this ligand with the enzyme (Fig. 5A). The highest STD signals were observed for the aromatic aglycone, suggesting its major involvement in binding, while additional STD signals for the sugar protons were also deduced. Moreover, additional signals were observed after overnight reaction incubation, also indicating that the hydrolysis of 4NP-S-Glc took place, and free aglycone was released (Fig. 5B).

3.6 General discussions

In order to prevent secondary product hydrolysis in the presence of water, the synthesis of OG and DG was performed via the reverse hydrolysis reaction using 0.25 M glucose and buffer-saturated alcohol at pH 5.5, 40 °C, in the presence of 0.1 unit enzyme, which was the same

condition as previously reported [7,13]. The highest yields of both products were obtained from the reactions catalyzed by the N189F dalcochinase mutant, which was also the best enzyme for the synthesis of HG under similar reaction conditions [13]. After optimization, the highest yield of OG was about 57.5 mol% (or 42 g/L) from the reaction containing 0.25 M glucose and 0.3 unit of the N189F mutant at 30 °C. Almond β-glucosidase has a higher affinity for the glucosyl moiety than dalcochinase, as shown by its low K_D value for δ-gluconolactone (Table 4), but its aglycone binding pocket was not suitable for the long, hydrophobic chain of the alkyl alcohol acceptors, as shown by the similar $k_{re,obs}$ values with alcohols and buffer (Table 3), which was consistent with its low DG production (Table 1). On the other hand, cassava linamarase has a hydrophobic aglycone binding pocket suitable for the alkyl alcohol acceptor so that it was very efficient in transglucosylation of alkyl alcohols [7,12,13], and its $k_{re,obs}$ values with short- and long-chain alcohols were much higher than water (Table 3) [13,21]. However, cassava linamarase catalyzes reverse hydrolysis poorly [7], as reflected by its low yields of OG and DG (Table 1). In comparison, the aglycone binding pocket of the N189F mutant has suitable geometry and hydrophobicity to accommodate long-chain alkyl alcohols as reflected by its higher $k_{re,obs}$ values with the 2 alcohol acceptors than with water (Table 3), while its glycone binding pocket maintains potency to catalyze reverse hydrolysis reaction of the wild-type dalcochinase as shown by ITC (Table 4). The *key* interactions between the aglycone moiety and the enzyme as demonstrated by STD NMR (Fig. 5) provide further support for the important roles played by the residues in the aglycone binding pocket in determining substrate specificity.

In order to adapt the current synthetic reaction for future commercial production, various cost-consuming parameters need to be carefully considered. For example, the cost of enzyme production may be reduced by using an immobilized enzyme. A previous study showed that

immobilization of bitter almond β-glycosidase led to a higher yield of HG (2 g/L) than a free enzyme (1.6 g/L) after 90 h of incubation [33]. Another study demonstrated that immobilization of bitter almond β-glucosidase enhanced enzyme stability by retaining about 40% relative activity after incubating at 80 °C for 2 days, while the free enzyme nearly lost all activity [25]. In addition, the OG synthesis catalyzed by the immobilized β-glucosidase increased 2.9-fold when compared with the free enzyme. It was suggested that immobilization may improve the interaction between enzyme and substrate, as well as the thermal stability and solvent tolerance of enzyme [25,33]. Uses of carriers for β-glucosidase immobilization, such as polyamine microspheres, chitosan and Eupergit C resin, were found to enhance thermal stability of enzyme [34,35].

In addition, the methods for separating alkyl glucoside from synthesis reaction need to be developed for future commercial production. However, alkyl glucoside separation has currently only been successful at the laboratory scale level. Previously, separation of OG by using short-path chromatography through a silica bed resulted in 40.8% yield and 96.6% purity, while other methods could achieved less than 3% yield and 10% purity [36]. Therefore, the methods for product isolation need to be further explored for future commercial production.

Unfortunately, however, the information on the 3-dimensional structure of dalcochinase is still lacking, despite extensive efforts form our group and others. This difficulty could be due to the heterogeneity in the protein glycosylation of both the wild-type and mutant forms of dalcochinase. Therefore, in the future, the crystal structures of both dalcochinase and linamarase will be very helpful in the understanding of the protein-substrate interactions, and for designing novel β-glucosidases with efficient synthetic capability.

Conclusion

OG and DG have been widely used as biodegradable, non-ionic surfactants. In this study, they were synthesized via reverse hydrolysis reactions between glucose and buffer-saturated octanol and decanol, respectively, in order to reduce water activity. Among all 10 β-glucosidases tested, the N189F mutant of dalcochinase, a β-glucosidase from *D. cochinchinensis* Pierre, showed the highest potential in OG and DG synthesis (39 and 40 mol %, respectively) under reverse hydrolysis conditions. The highest yield of OG was obtained at 57 mol % in the reactions containing 0.25 M glucose and 0.3 units of the N189F mutant in buffer-saturated octanol at 30 °C. The reactivation kinetics showed that most dalcochinase mutants had higher $k_{re,obs}$ values with octanol and decanol than with water, while the wild-type dalcochinase showed similar values with all acceptor moieties, consistent with the higher alkyl glucoside yields from most dalcochinase mutants compared to the wild-type enzyme. ITC analysis revealed similar binding affinities of δ-gluconolactone to the wild-type dalcochinase and the N189F mutant, supporting the view that the reverse hydrolysis capability was retained in the mutant enzyme. STD NMR experiments showed that the wild-type dalcochinase strongly interacts with the aglycone sites of the ligand. Taken together, this study has shown the potential of using the N189F dalcochinase mutant to catalyze the synthesis of long-chain alkyl glucosides via reverse hydrolysis reactions in buffer-saturated alcohols.

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