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Multiple mutations in the aglycone binding pocket to convert the substrate specificity of dalcochinase to linamarase

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ABSTRACT

Dalcochinase from *Dalbergia cochinchinensis* Pierre and linamarase from *Manihot esculenta* Crantz are β-glucosidases which share 47% sequence identity, but show distinct substrate specificities in hydrolysis and transglucosylation. Previously, three amino acid residues of dalcochinase, namely I185, N189 and V255, were identified as being important for determining substrate specificity. In this study, kinetic analysis of the ensuing double and triple mutants of dalcochinase showed that only those containing the 185A mutation could appreciably hydrolyze linamarin as well as transfer glucose to 2-methyl-2-propanol. So, the space provided by the I185A mutation appeared to be a prerequisite for accommodation of the aglycone moiety containing three substituents at the carbinol carbon. However, quantitative analysis of the energy parameters revealed mostly antagonistic interactions between these mutations. In addition, the N189F mutant showed a potential for use in enzymatic synthesis of alkyl glucosides via transglucosylation and reverse hydrolysis reactions. Thus, substitution of only 2–3 key residues in the aglycone binding pocket of dalcochinase could convert its specificities to that of linamarase, as well as to be suitable for any chosen hydrolytic or synthetic applications.

1. Introduction

β-Glucosidases (EC 3.2.1.21) are a group of glycoside hydrolases (GH) that catalyze the hydrolysis of β-O-glucosidic bond between D-glucose and an aglycone (alkyl or aryl) group or another sugar (Reaction 1). β-Glucosidases are classified on the basis of their amino acid sequence similarities as members of GH families 1, 3, 5, 9, 30, 116, and non-classified (CAZy: http://www.cazy.org/). Most plant β-glucosidases belong to GH1, with a common $(\beta/\alpha)_8$ barrel structure, pH optima at 5 to 6, subunit molecular weights of 55–65 kD, and the double-displacement mechanism. β-Glucosidases from different sources exhibit similar specificities for the glycone group (glucose), but show distinct specificities for the aglycone moieties, reflecting their diverse biological functions [1]. Beside hydrolysis, some β-glucosidases may synthesize oligosaccharides or alkyl glucosides via reverse hydrolysis (Reaction 1) or transglycosylation reactions (Reaction 2) [2–4].

$$\text{Glucosyl}{-}\text{OR}^1 + \text{H}_2\text{O} \leftrightarrows \text{Glucosyl}{-}\text{OH} + \text{R}^1\text{OH} \qquad \text{(Reaction 1)}$$

$$\text{Glucosyl}{-}\text{OR}^1 + \text{R}^2{-}\text{OH} \rightarrow \text{Glucosyl}{-}\text{OR}^2 + \text{R}^1\text{OH} \qquad \text{(Reaction 2)}$$

Dalcochinase and linamarase are GH1 β-glucosidases from *Dalbergia cochinchinensis* Pierre (Thai rosewood) and *Manihot esculenta* Crantz (cassava), respectively [5,6]. While their crystal structures are still lacking, two glutamate residues within the conserved sequences TL/FNEP and I/VTENG are predicted to act as a catalytic acid/base and a catalytic nucleophile, respectively [7,8]. Dalcochinase and linamarase share 47% protein sequence identity, but their substrate specificities and synthetic abilities are distinct. In hydrolysis, they could hydrolyze their own natural substrates, which are dalcochinin-8′-O-β-D-glucoside (Dal-Glc) for dalcochinase, and linamarin for linamarase (Fig. 1), but not the natural substrate of the other enzyme [9,10]. In reverse hydrolysis, dalcochinase could use free glucose to synthesize disaccharides,

Abbreviations: Dal-Glc, dalcochinin-8′-O-β-D-glucoside; GH, glycoside hydrolase; *p*NP-Glc, *p*-nitrophenyl-β-D-glucoside.

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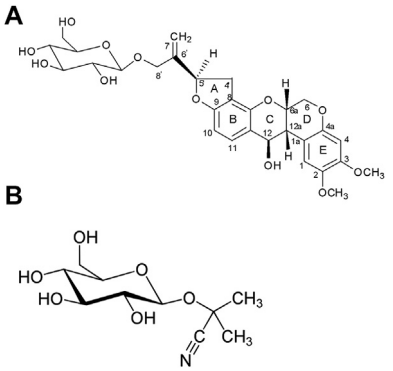

Fig. 1. Structures of Dal-Glc (A) and linamarin (B).

trisaccharides and oligosaccharides, whereas linamarase showed low capability in the same reaction [2]. On the other hand, in the transglucosylation reaction, dalcochinase could moderately use primary and secondary alcohols as glucolsyl acceptors, whereas linamarase could effectively use primary, secondary and tertiary alcohols as glucosyl acceptors [2,3]. In order to probe for the structural elements responsible for their catalytic differences, we previously generated a series of single mutations by replacing the amino acid residues in the aglycone binding pocket of dalcochinase with the corresponding residues of linamarase, and identified three mutants, namely I185A, N189F and V255F, with significant effects on both hydrolytic and transglucosylation specificities [10]. In particular, the I185A and V255F mutants of dalcochinase exhibited lowered substrate specificities toward Dal-Glc, which is its natural substrate, while all three single mutants showed improved transglucosylation activities using primary and secondary alcohols as acceptors. However, none of these mutants could mimic the substrate specificity of linamarase in hydrolyzing linamarin or transferring glucose to tertiary alcohol. Thus, the question arose whether dalcochinase could be further mutated in order to gain the substrate specificities of linamarase in hydrolysis and transglucosylation reactions, and whether the capability to catalyze reverse hydrolysis reactions could be retained in the dalcochinase mutants.

So, in this study, the roles of these residues in determining substrate specificities in hydrolysis, transglucosylation and reverse hydrolysis reactions were defined. We showed that combined mutations at the key amino acid positions of dalcochinase could create a binding pocket that could fit the tri-functional aglycone moieties of linamarin and tertiary alcohol. Furthermore, some of these dalcohinase mutants exhibited potential applications in synthesis of alkyl glucosides via reverse hydrolysis, eliminating the need for an expensive synthetic glucosyl donor in the synthetic reactions. Our data therefore demonstrated that the activities and specificities of β-glucosidases could be tailored to suit desired applications.

2. Materials and methods

2.1. Homology modeling and molecular docking

The three-dimensional models of the I185A/N189F/V255F mutant of dalcochinase [7] and the wild-type linamarase [8] were generated by using MODELLER9v4 [11], using the structure of cyanogenic β-glucosidase from *Trifolium repens* L. (PDB code 1CBG), with 60% and 51% sequence identities, respectively, as a template [12]. The quality of the models was checked by PROCHECK, ProSA and Verify-3D programs [13–15]. 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 linamarin from PubChem (CID 11128) was docked into the binding pockets of both models by using AutoDock version 4.2 [16]. The docked conformation was analyzed by using Accelrys DS Visualizer 3.0 (Accelrys Inc., San Diego, USA).

2.2. Construction, expression and purification of dalcochinase mutants

The double and triple mutants of dalcochinase (I185A/N189F, I185A/V255F, N189F/V255F and I185A/N189F/V255F) were generated by site-directed mutagenesis method published previously [17]. The plasmids harboring the single mutations, namely I185A, N189F and V255F, were used as template for generating double mutations [10]. The plasmid harboring the I185A/N189F double mutations was then used as a template for generating the I185A/N189F/V255F triple mutation. The sequences of the sense/antisense mutagenic primer pairs were as reported previously [10]. The plasmids containing correctly mutated dalcochinase sequences, as verified by DNA sequencing, were linearized with *Sac*I, transformed into *P. pastoris* by electroporation, and selected on YPDS plates with 100 μg/mL zeocin at 30 °C, following the protocols from Invitrogen.

The wild-type and mutant forms of dalcochinase were expressed from *P. pastoris* and purified from the culture media via phenyl-sepharose followed by immobilized metal-ion affinity chromatography as described previously [10]. Their β-glucosidase activities were measured against 1 mM *p*-nitrophenyl-β-D-glucoside (*p*NP-Glc) in 0.1 M sodium acetate, pH 5.0, in a 0.5 mL reaction at 30 °C for 30 min, and stopped by adding 2 M Na_2CO_3. Total protein concentration was estimated by Bradford assay (Bio-Rad, Hercules, CA, USA), using bovine serum albumin as standard. The purity and identity of the purified enzymes was confirmed by 10% SDS-PAGE and Western blot analysis using mouse monoclonol antibody against natural dalcochinase as a primary antibody (a gift from Dr. Watchara Kasinrerk, Chiangmai University, Thailand). Deglycosylation was performed by treatment of the purified enzymes with endoglycosidase H (New England BioLabs, USA) under denaturing conditions at 37 °C for 1 h. The activities of the purified enzymes were determined against 15 mM *p*NP-Glc in 0.1 M sodium acetate, pH 5.0, in a 50 μL reaction at 30 °C for 5 min. One unit of activity is defined as the amount of the purified enzymes used to release 1 μmol of *p*-nitrophenol in 1 min.

2.3. Kinetic measurement in hydrolysis

Natural dalcochinase, Dal-Glc, cassava linamarase and linamarin were purified from their natural sources as described previously [5,6,9,18]. Kinetic parameters of the purified enzymes were determined by incubating 0.1 unit of the purified enzyme with substrates at various concentrations in 0.1 M sodium acetate, pH 5.0, in a 50 μL reaction at 30 °C for 5 min. The hydrolysis was stopped by the boiling for 5 min. The amount of glucose released was estimated by reaction with 50 μl of 2 mg/mL 2,2′-azonobis-3-ethylbenz-thiazolinesulfonic acid and 100 μL of 5 unit/mL glucose oxidase reagent at 37 °C for 15 min. The absorbance of product was measured at 405 nm, and compared with a glucose standard curve. The kinetic parameters were obtained by nonlinear regression of the Michaelis-Menten plots using the program KaleidaGraph (Synergy Software, Reading, PA, USA) [10].

The binding free energy (ΔG_b) associated with the K_m value, and the activation free energy ($\Delta G^{\ddagger}$) associated with the k_{cat} value were calculated from the following relationships [19,20]:

$$\Delta G_b = -RT\,[\ln(1/K_m)] \qquad \text{(Equation 1)}$$