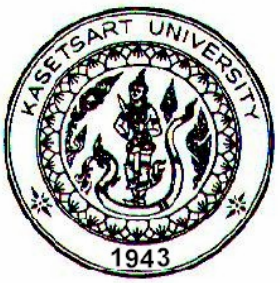

THESIS

GLYCONE SPECIFICITY ENGINEERING OF THAI ROSEWOOD β-GLUCOSIDASE FOR MANNOSE

KHUANJARAT CHOENGPANYA

GRADUATE SCHOOL, KASETSART UNIVERSITY
2008

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THESIS

GLYCONE SPECIFICITY ENGINEERING OF THAI ROSEWOOD β-GLUCOSIDASE FOR MANNOSE

KHUANJARAT CHOENGPANYA

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Master of Science (Genetic Engineering)
Graduate School, Kasetsart University
2008

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β-Glucosidases (EC 3.2.1.21) catalyze the hydrolysis of β-O-glucosidic linkages formed between D-glucose and aglycone group. The hydrolysis of glucoside substrates require the hydrogen bond interactions between glucose moiety and the active pocket amino acid residues for stabilization of transition state. Previous study has shown that the interaction of the hydroxyl group at C-2 position with amino acid residues in the glycone binding pocket was most important for transition state stabilization. Since glucose and mannose substrates are differ in the orientation of C-2 hydroxyl groups, which is equatorial in glucose and axial in mannose, it was interesting to study which amino acid residues are responsible for glycone specificity. Thai rosewood β-glucosidase or dalcochinase can hydrolyze *p*-nitrophenyl-β-D-glucopyranoside (*p*NP-Glc) with high efficiency, but show no activity toward *p*-nitrophenyl-β-D-mannopyranoside (*p*NP-Man), whereas most β-mannosidases show good hydrolytic activity toward mannoside substrate but cannot hydrolyze glucoside substrates. In this study, the amino acid residues that might be involved in glycone specificity of dalcochinase were studied. Molecular modeling and docking predicted that amino acid residues R90, W137, N181 and M369 of dalcochinase, corresponding to Q77, Q150, D206 and E374 of *Pyrococcus horikoshii* β-mannosidase, might be involved in glycone specificity. Therefore, four mutant forms of dalcochinase were generated, yielding R90Q, W137Q, N181D and M369E. The binding mode of sugar substrates, kinetic and transglycosylation properties of mutant enzymes were studied. The differences in K_m values of all mutant enzymes toward *p*NP-Glc were less than an order of magnitude (17.0, 24.0, 2.7, 11.9 mM for R90Q, W137Q, N181D and M369E, respectively) compared to wild-type recombinant dalcochinase (9.88 mM), suggesting that affinities of dalcochinase mutants for *p*NP-Glc substrate were not significantly affected by these mutations. The turnover rates of R90Q, W137Q and N181D indicated that residues R90, W137 and N181 of dalcochinase were important for catalysis activity. In agreement with the docking results, mutant M369E could hydrolyze *p*NP-Man, due to grater hydrogen-bond interactions between the residues and the mannose substrate, when compared to other mutants. In the transglucosylation studies, mutants N181D and M369E showed increased reactivity to ethanol, *n*-propanol and *n*-butanol acceptors, due to the low hydrolytic activities. In transmannosylation, wild-type dalcochinase could hydrolyze and transfer mannose to *n*-propanol and *n*-butanol. No alkyl-mannosides were obtained from all mutant enzymes. These results suggested that rearrangement of amino acid residues in the binding pocket of these mutant enzymes were not suitable for accommodation and hydrolysis of the mannoside substrates.

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20 / 5 / 2008

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Khuanjarat Choengpanya

May 2008

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GLYCONE SPECIFICITY ENGINEERING OF THAI ROSEWOOD β-GLUCOSIDASE FOR MANNOSE

INTRODUCTION

Glycoside hydrolases (E.C. 3.2.1.X) are hydrolytic enzymes that wildly occur in many organisms. They catalyze the hydrolysis of glycosidic linkages between glycone (sugars) and aglycone (aryl or alkyl) groups, or glycosidic linkages between carbohydrate moieties (e.g. cellobiose). Glycoside hydrolases are classified based on their primary and tertiary structures into 14 clans and 106 families (http://afmb.cnrs-mrs.fr/CAZY/fam/GH1.html).

Family 1 glycoside hydrolases are found in many organisms. Based on substrate specificity, enzymes in this family have been characterized as β-glucosidases (E.C. 3.2.1.21), β-mannosidases (E.C. 3.2.1.25), β-galactosidases (E.C. 3.2.1.23), β-glucuronidase (E.C. 3.2.1.31), β-D-fucosidase (E.C. 3.2.1.38), strictosidine β-glucosidase (E.C. 3.2.1.105), prunasin/raucaffricine β-glucosidase (E.C. 3.2.1.118/125), 6-phosphor-β-gluco/galactosidases (E.C. 3.2.1.86/85), lactase-phlorizin hydrolases (E.C. 3.2.1.108/62), β-primeverosidase (E.C. 3.2.1.149) and thioglucosidases (E.C. 3.2.3.147) (http://afmb.cnrs-mrs.fr/CAZY/fam/GH1.html). All family 1 glycoside hydrolases present the same tertiary structure, the $(\beta/\alpha)_8$ barrel, and act via a retaining mechanism. Their catalytic activities depend on the two glutamic acid residues positioned after the β-strand 4 (proton donor) and after the β-strand 7 (nucleophile) (Barrett *et al*., 1995; Czjzek *et al*., 2001; Marana *et al*., 2001).

β-Glucosidases (E.C. 3.2.1.21) hydrolyze β-O-glucosidic linkages, and are classified as members of glycoside hydrolase families 1 and 3. They are proposed to act via a retaining mechanism in which the anomeric configuration of product is the same as substrate. Almost all β-glucosidases have molecular weight of 55 to 65 kDa and optimal pHs between pH 5 to 6, and require β-glycosides as substrates. β-Glucosidases from different sources have similarity in substrate specificity for glycone (glucose) and some aglycones (e.g. nitrophenol and umbelliferone) (Esen,

1993). β-Glucosidases are expressed in many species, including animals, plants and microorganisms, and play various biological functions depending on their natural substrates (Eksittikul *et al*., 1988; Svasti *et al*., 1999; Chuankhayan *et al*., 2005). In human, acid β-glucosidase or glucocerebrosidase catalyzes hydrolysis of sphingosylglucosides and glucosylceramide. Accumulation of these substances in the absence of this enzyme is responsible for the Gaucher's disease. Plant β-glucosidases are implicated in growth regulation, cellobiose degradation, lignification and defense. In the xylophagous insects, they have β-glucosidases that hydrolyze cellulose (cellobiose) for energy supply (Nahrstedt and Mueller, 1993). In fungi and bacteria, β-glucosidases have an important role in the break down of cellulose to glucose or simpler oligosaccharides. Thus these enzymes appear as natural candidates for engineering ideal β-glucosidase to be used in many applications, such as in waste treatment process and in food industries.

β-Mannosidases (EC 3.2.1.25) belong to family 1 glycoside hydrolase, and catalyze the hydrolysis of terminal, non-reducing β-D mannose residue in β-D-mannosides or β-mannooligosaccharides such as β-mannan, glucomannan, galactomannan and galactoglucomannan to yield D-mannose. β-Mannosidases have molecular weight ranging between 56 and 150 kDa and optimal pHs of 2.5 to 7.4 (Oda *et al*., 1993; Bauer *et al*., 1996; Ademark *et al*., 1999; Kaper *et al*., 2002; Andreotti *et al*., 2005). β-Mannosidases are expressed in many organisms. In fungi, β-mannosidase is essential for the complete hydrolysis of plant manno-oligosaccharides such as galacto(gluco)mannans and mannan, which is a major component in plant cell wall, to yield mannose (Ademark *et al*., 1999). In addition, β-mannosidases play an important role in the lysosomal degradation process of N-linked glycoproteins in mammals, and the deficiency of β-mannosidase activity results in an autosomal recessive inherited disorder, β-mannosidosis (Chen *et al*., 1995). In bacteria and mollacs, they use β-mannosidases for nutrition requirement. Moreover, β-mannosidases have a transmannosylation activity. Thus, these enzymes are used in many industrial applications, such as therapeutic agents, bio-surfactants or in waste treatment process (Matsumura *et* al., 1990; Kobata, 1993; Sunna, 2000; Andreotti, 2005).

β-Glucosidases can hydrolyze glucoside substrates efficiently, but show no activity toward mannoside substrate, whereas β-mannosidase can hydrolyze mannoside substrates, but cannot cleave glucoside substrates. Glucose and mannose are epimers at C-2 position, which is equatorial in glucose and axial in mannose. It has been shown that hydrogen bond interaction of enzyme with C-2 hydroxyl group of sugar substrate contributes most to stabilization of the transition state. To investigate which amino acid residues were responsible for glycone specificity and catalysis in glycoside hydrolase family 1, the amino acid residues of β-glucosidase that might be involved in glycone specificity were changed to the corresponding amino acid residues of β-mannosidase. The mutant enzymes could serve as models for the investigation of structure-function relationships in family 1 glycoside hydrolases, particularly the residues responsible for difference in catalytic activities between β-glucosidase and β-mannosidase

Molecular modeling and docking techniques are useful tools for studying protein structure. The molecular modeling studies predict the three-dimensional structures and provide clues to the structure determinants of enzymatic function, substrate specificity and selectivity. The molecular docking approach provides structural basis for interpreting and predicting substrate selectivity, enantioselectivity and regioselectivity of enzymes. This approach consists of accurate prediction of the orientation of the bioactive conformation into the binding pocket and the estimation of the tightness of enzyme-substrate interactions (scoring) (Tyagi and Pleiss, 2006). Thus it is possible to search the geometric orientation of substrate-bound enzyme and the amino acids involve in this binding.

In this study, molecular modeling and docking approaches are used to predict the amino acids that are involved in substrate (glycone) binding of *Dalbergia cochinchinensis* Pierre β-glucosidase or dalcochinase. Site-directed mutagenesis is performed to create mutants, and the kinetic and transglycosylation properties of mutant enzymes are studied.

OBJECTIVES

1. To compare and predict the amino acid sequences between β-glucosidase from *Dalbergia cochinchinensis* Pierre (dalcochinase), β-glucosidase from *Pyrococcus furiosus* and β-mannosidase from *P. horikoshii* that may be responsible for difference in catalytic activities between β-glucosidase and β-mannosidase.

2. To make mutant forms of dalcochinase by replacing amino acid residues of the enzyme to amino acid residues of *P. horikoshii* β-mannosidase.

3. To study the kinetic properties (K_m, V_{max} and k_{cat}) of wild-type dalcochinase and its mutants.

4. To study the transglycosylation activity of wild-type dalcochinase and its mutants.

LITERATURE REVIEW

1. General features of β-glucosidases

β-Glucosidases (E.C. 3.2.1.21) are a group of enzymes that catalyze the hydrolysis of the β-O-glucosidic linkage between D-glucose and aglycone or other sugars. Most β-glucosidases have similar structures and functional properties, with subunit molecular weights between 55 to 65 kDa, and optimal pHs between pH 5 to 6 (Esen, 1993). These enzymes tend to have similar three-dimensional structures, consisting of $(\beta/\alpha)_8$ barrel structure with two catalytically active acidic amino acids (Figure 1).

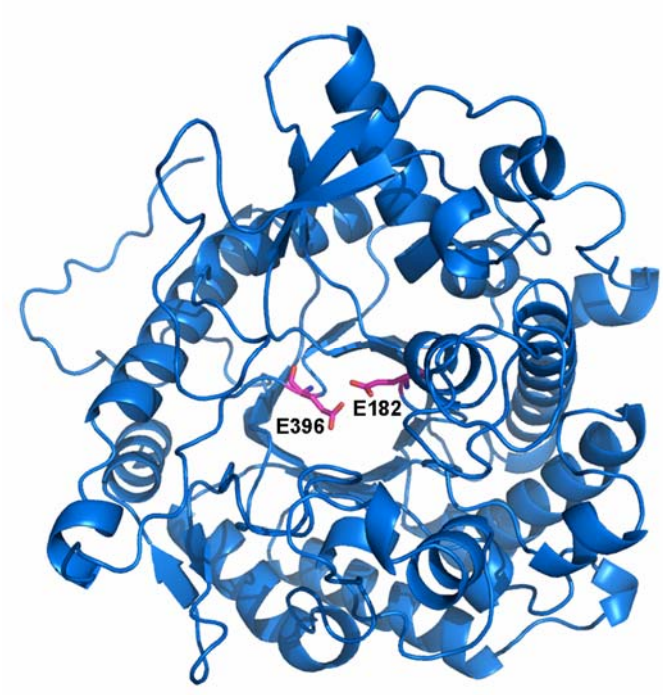


Figure 1 Three-dimensional model of *Dalbergia cochinchinensis* Pierre β-glucosidase (dalcochinase). This model is based on the crystal structure of white clover cyanogenic β-glucosidase (PDB: 1CBG), sorghum bicolour dhurrinase 1 myrosinase (PDB: 1V02) and sorghum dhurrinase (PDB: 1V03). The catalytic acid/base (E182) and nucleophile (E396) are shown as stick drawings with labels.

β-Glucosidases are expressed in many organisms, including animals, plants and microorganisms, and fulfill many biological functions depending on their natural substrates. In plants, β-glucosidases are involved in growth regulation, stress response, cellulose degradation, lignification and defense. For example, in defense, cyanogenic β-glucosidases, or linamarases, were found in white clover (*Trifolium repens* L.), flax (*Linum ussitatissinum*), birdfoot trefoil (*Lotus corniculatus* L.), butter bean (*Phaseolus lunatus*), rubber tree (*Hevea brasiliensis*) and cassava (*Manihot esculenta* Crantz), in which they play a role against herbivores through cyanogenesis (Eksittikul and Chulavatnatol, 1988). It was shown in model systems that the enzyme and substrate (cyanogenic glucosides) are present in different compartments. Cyanogenesis occurs when cells or tissues are injured by herbivores and/or pathogens, resulting in disruption of cellular compartments that allows the enzyme and substrates to come in contact and HCN released. This would suggest that cyanogenesis is a chemical defense. Moreover, other plant β-glucosidases catalyze the hydrolysis of conjugated phytohormones (e.g., glucosides of gibberellins, auxins, abscisic and cytokinins), which are key metabolic and growth-related compounds (Esen, 1993).

β-Glucosidase from *Dalbergia cochinchinensis* Pierre (dalcochinase)

D. cochinchinensis Pierre β-glucosidase or dalcochinase, has an apparent native molecular weight of 330 kDa, possibly consisting of 4-6 subunits of 66 kDa, and pH optimum of 5.5 (Srisomsap *et al*., 1996). This enzyme can hydrolyze both β-glucoside and β-fucoside substrates and has 12-dihydroamorphigenin-8'-O-β-D-glucoside, or dalcochinin-8'-O-β-D-glucoside, as its natural substrate (Figure 2) (Svasti *et al*., 1999).

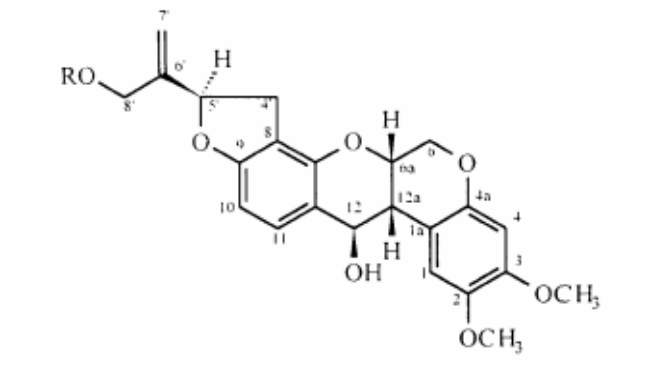


Figure 2 Dalcochinin-8'-O-β-D-glucoside (R = β-D-glucose)
Source: Svasti (1999)

The kinetic properties of dalchinase have been studied. It possesses K_m towards para-nitrophenyl-β-D-glucopyranoside (*p*NP-Glc) and para-nitrophenyl-β-D-fucopyranoside (*p*NP-Fuc) of 5.4 mM and 0.54 mM, respectively, and k_{cat} towards *p*NP-Glc and *p*NP-Fuc of 307 sec^{-1} and 151 sec^{-1}, respectively. The enzyme was strongly inhibited by mercuric compounds ($HgCl_2$ and *p*-chloromercuribenzoate), and mildly inhibited by δ-gluconolactone and conduritol epoxide (Srisomsap *et al*., 1996).

2. General features of β-mannosidases

β-Mannosidases (E.C. 3.2.1.25) are exo-acting enzymes, which catalyze the β-D-mannose removal from β-(1,4)-linked manno-oligosaccharides such as mannan, galactomannan or glucomannan. The molecular weight of these enzymes range from 56 to 150 kDa, and the optimal pHs are 6.5-7.4 (Oda *et al*., 1993; Bauer *et al*., 1996; Ademark *et al*., 1999; Kaper *et al*., 2002; Andreotti *et al*., 2005). β-Mannosidases from different sources have been purified and characterized. Fungal β-mannosidases are used for the complete hydrolysis of hemicellulose in plant cell wall (Ademark *et al*., 1999). In mammals and ruminants, deficiency in lysosomal β-mannosidases is responsible for β-mannosidosis. Ruminants affected by β-mannosidosis are recognized by the presence of severe neurological deficiencies associated with myelin abnormalities that lead to neonatal death, facial dysmorphism contractures, hyperextension of limb joints and deafness. Unlike the severe clinical features of the

disease in ruminants, the human disease is milder, with a wild range of symptoms and age of onset (Chen *et al*., 1995). In plants, activity of β-mannosidases may be involved in the weakening of the cell wall of endosperm to allow for radicle protrusion in the germination stage (Mo and Bewley, 2002).

The kinetic properties of β-mannosidases have been studied. They have good hydrolytic activity towards para-nitropheny-β-D-mannopyranoside (*p*NP-Man) as well as β-(1,4)-linked trisaccharide or disaccharide of mannose (Elbein *et al*., 1977; Andreotti *et al*., 2005). Since β-mannosidases can remove mannose residues from manno-oligosaccharides until they reach the non-mannose of polysaccharides, it is possible to use β-mannosidases in determining sequences of manno-oligosaccharides (Ademark *et al*., 1999). β-Mannosidases, together with β-mannanases, are wildly used in the saccharification of hemicellulose for further conversion to chemical and fuel, and in the biological bleaching of pulp and paper (Sunna *et al*., 2000). In addition to the hydrolytic activity, β-mannosidases are also good in transmannosylation activity. β-Mannosidase from *Aspergillus niger* can synthesize various alkyl β-mannosides from various alcohol acceptors. The result showed that the highest yield (81% of methyl β-mannoside) was obtained in the presence of 50% (v/v) methanol. Thus, there is a great interest in using β-mannosidases in the synthesis of oligosaccharides via transmannosylation for many purposes, such as manno-oligosaccharides synthesis for therapeutic applications (Kobata, 1993).

β-Mannosidase from *Pyrococcus horikoshii*

In the genome sequence of hyperthermophilic archeon *P. horikoshii*, two open reading frames have been annotated as family 1 glycoside hydrolases, PH0366 and PH0501. The PH0366 encoded a membrane-associated protein with high efficiency for the hydrolysis of long chain alkyl glycosides. The PH0501 has been annotated as β-mannosidase based on amino acid homology with the β-mannosidase BmnA from *P. furiosus* (Kaper *et al*., 2002). Multiple sequence alignment revealed that the *P. horikoshii* β-mannosidase represents a new subgroup of family 1 glycoside hydrolase,

characterized by two unique glutamine and aspartate residues in the active pocket (Figure 3).

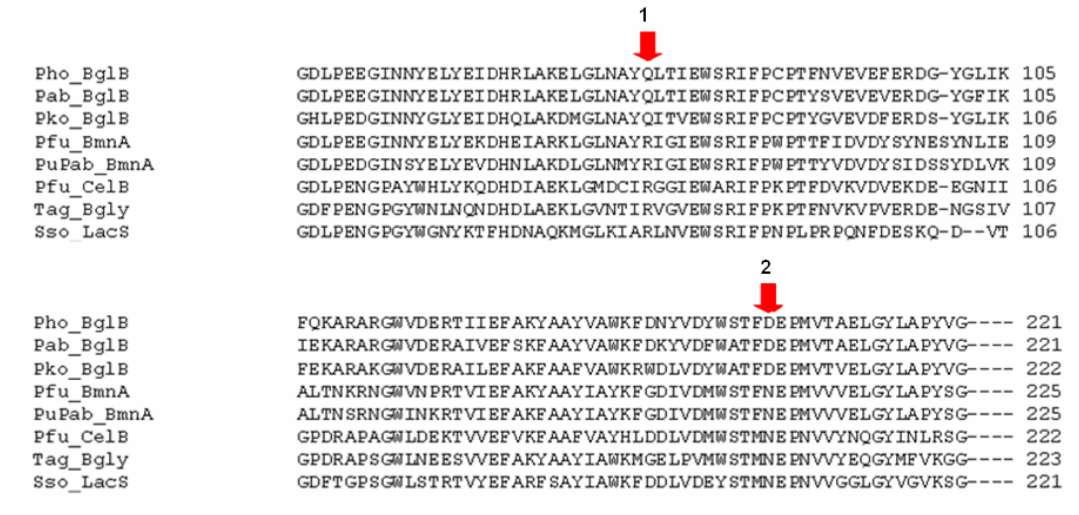


Figure 3 Multiple sequence alignment using ClustalW 1.83. Pho_BglB: *P. horikoshii* β-mannosidase (GenBank BA00002), Pab_BglB: putative *P. abyssi* β-mannosidase (GenBank AJ248288), Pko_BglB: putative *P. kodakaraensis* β-mannosidase (GenBank AB028601), Pfu_BmnA: *P. furiosus* β-mannosidase (GenBank U60214), PuPab_BmnA: putative *P. abyssi* β-mannosidase (GenBank AJ248285), Pfu_celB: *P. furiosus* β-glucosidase (GenBank AF013169), Tag_Bgly: *Thermococcus aggregans* β-glycosidase (GenBank AF053078), Sso_Lacs: *S. solfataricus* β-glycosidase LacS (M34696). "1" and "2" indicate positions of unique glutamine (Q) and aspartic acid (D) residue in *P. horikoshii* β-mannosidase, respectively.

The three-dimensional structure model of *P. horikoshii* β-mannosidase showed a $(\beta/\alpha)_8$ barrel with E399 and E207 act as the nucleophile and the catalytic acid/base, respectively, in the hydrolysis reaction (Figure 4). The active form of *P. horikoshii* β-mannosidase is a tetramer with a molecular weight of about 250 kDa. The optimal hydrolytic activity of *P. horikoshii* β-mannosidase at 90 °C was observed at pH 4.5. The kinetic study showed that *P. horikoshii* β-mannosidase was most specific for the hydrolysis of *p*NP-Man (K_m = 0.44 mM) with a low turnover rate (k_{cat} = 4.3 s^{-1}) (Kaper *et al*., 2002).

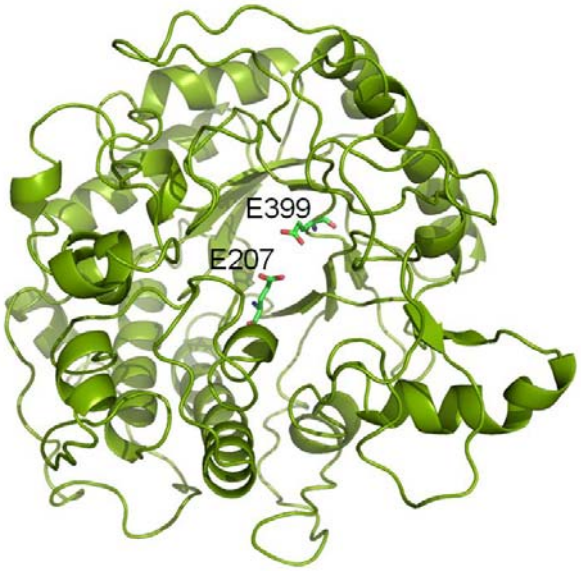


Figure 4 The tree-dimension model of *P. horikoshii* β-mannosidase. This model is based on the crystal structure of *Sulfolobus solfataricus* β-glycosidase (1UWS). The catalytic acid/base (E207) and nucleophile (E399) are shown as stick drawings with labels.

3. The hydrolytic mechanisms of glycosidase

There are two possible stereochemical outcomes of the hydrolysis of a glycosidic bond, namely inversion or retention of anomeric configuration. Both mechanisms (Figure 5) involve oxocarbenium ion-like transition states and a pair of carboxylic acids at the active site. In inverting glycosidases, these two residues are located approximately 10 Å (±2 Å) apart on average, and the reaction occurs via a single-displacement mechanism wherein one carboxylic acid acts as a general base and the other as a general acid (Figure 5a). In retaining enzymes, the two carboxylic acid residues are approximately 5.5 Å apart, and the reaction proceeds via a double displacement mechanism (Figure 5b) (Rye and Withers, 2000). In the first step, one of the carboxyl groups functions as a general acid catalyst, protonating the glycosidic oxygen with a concomitant bond cleavage. The other carboxyl group acts as a nucleophile, forming a covalent glycosyl-enzyme intermediate. In the second step, the

side-chain carboxylate deprotonates the incoming water molecule, which attacks at the anomeric center and displaces the sugar. Both steps occur via transition states with a substantial oxocarbenium ion character (Figure 5b).

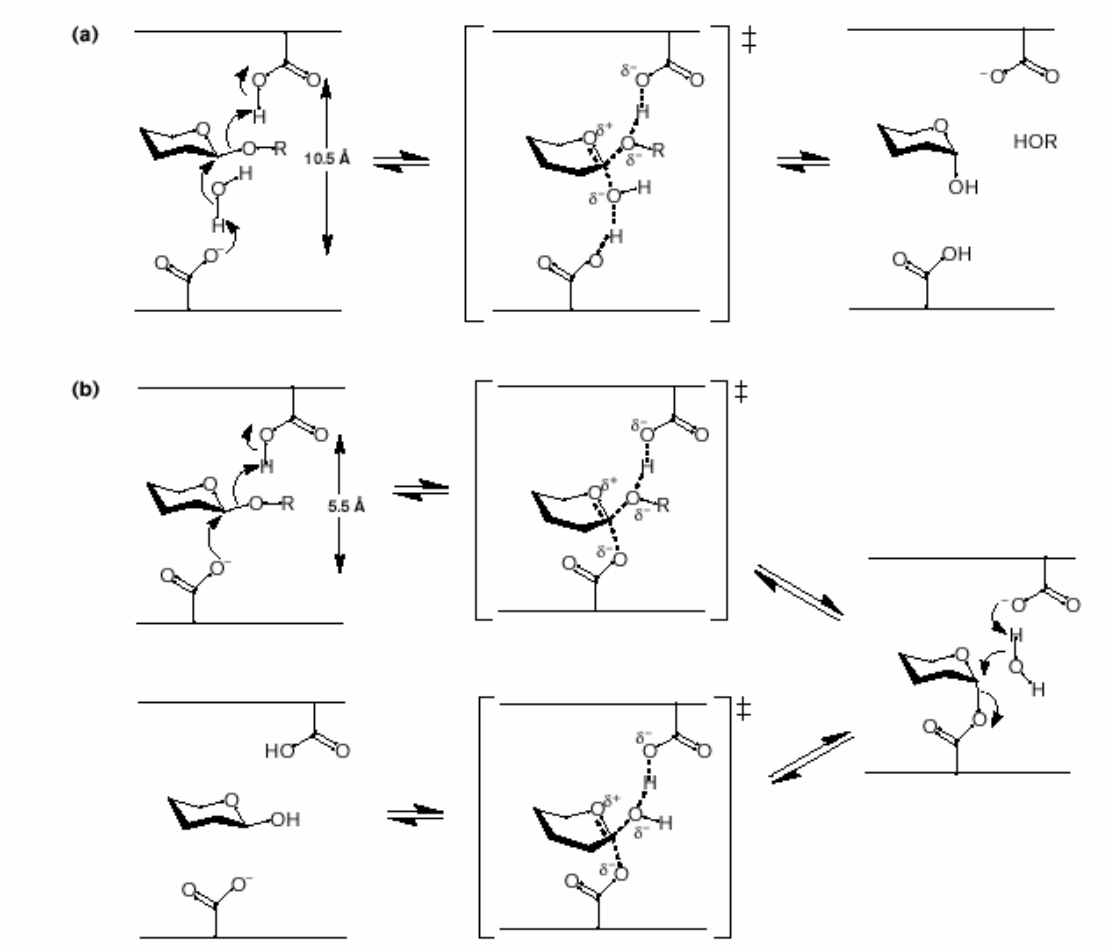


Figure 5 General mechanism for (a) an inverting β-glycosidase and (b) a retaining β-glycosidase proceeding through an intermediate with a $^4C_1$ conformation.

Source: Rye and Withers (2000)

4. Reverse hydrolysis and transglycosylation reaction

Apart from the hydrolytic activity, β-glycosidases from different sources can also catalyze the synthesis of oligosaccharides or glycosides. Under the suitable condition, with high concentration of reactants, low water activity and high temperature, β-glycosidases can synthesize oligosaccharides or glycosides via either reverse hydrolysis or transglycosylation reaction (Crout and Vic, 1998). Reverse hydrolysis is a thermodynamically controlled reaction which is favored by the increase of concentration of starting materials or enhanced temperature. Alternatively, transglycosylation reaction is controlled by the kinetics of the reaction (Mosan and Paul, 1995; Rantwijk *et al*., 1999).

Reverse hydrolysis comprises of the monosaccharide and the nucleophile, such as alcohol or another sugar, to produce the glycoside and water until the reaction equilibrium is reached. In this case, low water activity would be desirable to increase the yield. However, a small amount of water is added to activate the enzyme activity. Furthermore, a high concentration of substrate would be also desirable to push the equilibrium to the right (Rantwijk *et al*., 1999). The reaction is showed in Figure 6a.

The transglycosylation reaction comprises of the glycoside donor (oligosaccharides or para-nitrophenyl-β-D-glycopyranosides) and the glycoside acceptors (oligosaccharides or alcohols) in the reaction to yield alkyl glycosides or longer-chain oligosaccharides. The reaction efficiency is kinetically controlled (Figure 6b), so the final reaction yield could be increased by using efficient acceptor molecules, increasing donor and acceptor concentrations, decreasing water activity and removing the product from the reaction medium (Mosan and Paul, 1995).

$$\text{Glycosyl-OH} + R^1OH \leftrightarrow \text{Glycosyl-O}R^1 + H_2O \quad \text{(a)}$$

$$\text{Glycosyl-O}R^1 + R^2OH \leftrightarrow \text{Glycosyl-O}R^2 + R^1OH \quad \text{(b)}$$

Figure 6 The reverse hydrolysis (a) and transglycosylation (b) reaction, R^1 is (mono) saccharides and R^2 is alkyl groups.

Source: Rantwijk (1999)

Many β-glycosidases have been studied for reverse hydrolysis and transglycosylation activities. Ajisaka *et al*. (1987) reported that almond β-glucosidase could synthesize the glucose-oligosaccharides via the reverse hydrolysis reaction. They also found that the yield of glucose-oligosaccharides increased as the initial concentration of glucose increased and the reaction temperature was raised, with the maximum yield of about 30% (w/v). This result was in agreement with oligosaccharide synthesis via reverse hydrolysis by *Penicillium emersonii* β-glucanase. At a high glucose concentration and at an elevated temperature, the oligosaccharide production reached maximum yield of 17% (w/w) (Rastall, 1992). *Fusarium oxysporum* β-glucosidase catalyzed the reverse hydrolysis of cellobiose and gentiobiose to yield triose and higher oligosaccharides. The amount of triose formed in 20% (w/v) cellobiose and 40% (w/v) gentiobiose were five and eight times higher than total disaccharides synthesized by almond β-glucosidase (Christakopoulos *et al*., 1994). *A. niger* β-glucosidase also catalyzed the synthesis of cello-oligosaccharides from cellobiose via transglucosylation reaction. The maximum yield was about 30% (mol/mol) which was based on cellobiose consumed (15% w/v) (Yan and Liau, 1998).

β-Glucosidases from Thai plants have been screened for the synthesis ability (Svasti *et al*., 1996). Dalcochinase and cassava β-glucosidases showed interesting activities in oligosaccharide or alkyl glycoside synthesis. Dalcochinase synthesized disaccharides and trisaccharides from 40-70% (w/w) glucose, with gentiobiose (β 1-6-diglucose) being the major product (Srisomsap *et al*., 1999). It also catalyzed the transglucosylation of alcohol to synthesize alkyl glucosides. The good yield was obtained with primary alcohols, in which the yield of alkyl glucoside products was

over 90%. However, secondary alcohols gave poorer yield, and tertiary alcohols did not react (Lirdprapamongkol and Svasti, 2000). In contrast, cassava β-glucosidase showed good ability in transferring glucose to primary, secondary and tertiary alcohols. Cassava β-glucosidase is only β-glucosidase able to synthesis C_4, C_5 and C_6 tertiary alkyl glucosides with high yield of 50-90% (Svasti *et al*., 2003).

Moreover, β-mannosidases can also catalyze the synthesis of alkyl mannosides via transmannosylation reaction. *A. niger* β-mannosidase gave high yield of alkyl mannosides with C_1-C_6 alcohols (20-80%), but gave lower yield in longer chain alcohols (C_7-C_8) (Itoh and Kamiyama, 1995). Recently, transmannosylation by *A. awamori* K4 β-mannosidase was investigated by using mannobiose prepared from Konjak as a substrate. The enzyme could transmannosylate mannose to many acceptors including sugars, with fructose giving the highest yield (Kurakake and Komaki, 2001).

The mechanism of transglucosylation has been studied (Hommalai *et al*., 2005). The 2-deoxy-2-fluro-sugar analogues were used to form covalent glycosyl-enzyme intermediates. The trapped enzyme intermediates were used for investigating transglucosylation reaction specificity. The result showed that the reaction mechanism is a double-displacement type with the step involving the generation of reactive alkoxide being a major step governing transition state formation (Figure 7).

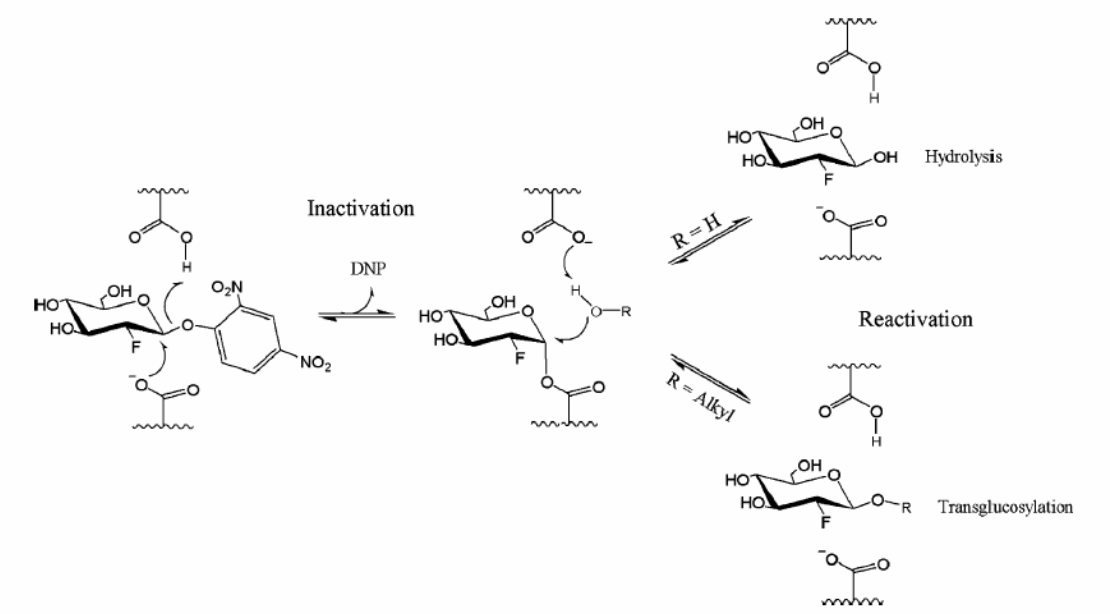


Figure 7 The reaction mechanism of transglucosylation reaction. The inactivator, 2-deoxy-2-fluoro-dinitrophenylglucoside (2FDNPG), was cleaved during the inactivation step (glycosylation of enzyme) resulting in the accumulation of glycosyl-enzyme intermediate. The enzyme activity was regained by transglucosylation, which requires the removal of proton from alcohol by active site Glu to generate a more reactive nucleophile, the alkoxide, to attack the C1-position of the glycosyl moiety.

Source: Hommalai (2005)

5. Glycone binding study

The amino acid residues involved in glycone binding were studied through the X-ray crystallography. The binding of glycone (glucose) moiety involves hydrogen bonding interactions with a set of universally conserved amino acids. In maize β-glucosidase ZmGlu1, these amino acid residues are Q38 (H-bonds to O4), H142 (H-bonds to O3), N190 (H-bonds to O3), E191 (H-bonds to O2) and E464 (H-bonds to O6) (Czjzek *et al.*, 2000) (Figure 8) These residues of maize β-glucosidase ZmGlu1 are corresponding to Q39, H143, N188, E189 and E460, respectively, of *Sorghum bicolor* β-glucosidase isoenzyme SbDhr1 (Verdoucq *et al.*, 2004). The binding interactions between these amino acid residues and glucose moiety are important for

the stabilization of transition state, which contribute up to 22 kJ mol^{-1} to both glycosylation and deglycosylation transition state stabilization (Zechel and Withers, 2000).

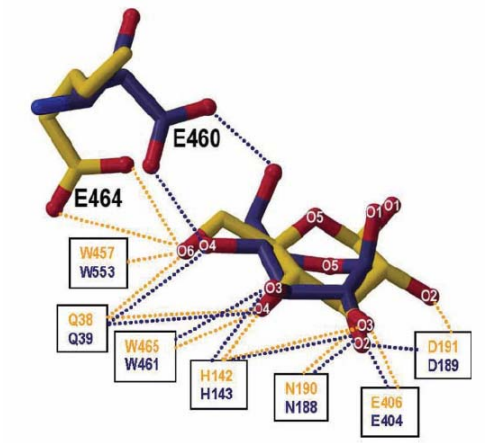


Figure 8 Superimposition of the glucose moieties of DIMBOA-Glc in ZmGlu1 and dhurrin in SbDhr1. The yellow and blue alphabets and a stick representation of amino acid residues indicate amino acid residues of ZmGlu1 and SbDhr1, respectively. The glucose rings of ZmGlu1 show in yellow and blue in SbDhr1.

Due to the lack of three-dimensional structure of dalcochinase, comparison of the primary sequence of dalcochinase with maize β-glucosidase ZmGlu1 indicated that Q36, H136, N181, E182 and E451 of dalcochinase might be involved in glucose binding (Figure 9). Beside these amino acid residues directly interacted with glucose moiety, the amino acid residues adjacent to the catalytic residues are also important for glycone binding. It was found that R77Q mutation of *P. furiosus* β-glucosidase reduced hydrolytic rate of about 10-fold (Kaper *et al*., 2002). This arginine residue is highly conserved in glycoside hydrolases. In *Cellvibrio mixtus* β-mannosidase 5A, R80, which is corresponding to R77 of *P. furiosus* β-glucosidase and R90 of dalcochinase, forms hydrogen bond with catalytic acid/base E215 (corresponding to E396 of dalcochinase), and this interaction are likely to contribute to both position and ionization state of the catalytic amino acids (Davies *et al*., 1998; Dias *et al*., 2004).

Figure 9 Sequence alignment of ZmGlu1 to SbDhr1 and dalcochinase. The bold and underlined alphabets indicate the amino acid involved in glycone binding.

6. Production of recombinant dalcochinase in *Pichia pastoris*

The production of recombinant protein usually uses *Escherichia coli* as a host because of *E. coli* is easy to manage, provides high growth rate, and also uses cheap medium. *E. coli* can secrete the recombinant protein to the culture medium which makes the secreted protein easy to be harvested. However, *E. coli* is prokaryotic cell that lacks the posttranslational modifications, which are critical to the structure and functions of some eukaryotic proteins. Due to this drawback of *E. coli* expression, *P. pastoris*, which is eukaryotic cell and possess the necessary modifications, was developed in order to be used for recombinant protein production.

P. pastoris is a methylotrophic yeast which can use methanol as its sole carbon source. The first step in metabolism of methanol is the oxidation of methanol to formaldehyde using enzyme alcohol oxidase. This reaction generates hydrogen peroxide which is toxic for yeast cells, so methanol metabolism takes place in peroxisome. Because alcohol oxidase has poor affinity to oxygen, *P. pastoris* compensates by generating large amount of enzyme. Thus the promoter regulation (*AOX1*) of alcohol oxidase is used to drive heterologous protein expression in *P. pastoris* (Invitrogen, n.d.).

To express dalcochinase in *P. pastoris*, dalcochinase gene was cloned into pPICZαB (Figure 10), which comprises of promoter *AOX1* for expression regulation followed by the alpha factor signal sequence for secretion. Due to posttranslational processing at both N- and C-termini, the recombinant plasmid, pPICZ-His_8-trncTRBG, was created to contain the N-terminally truncated form of dalcochinase (lacking the first 12 residues of the mature protein) following 8 histidine residues (Figure 11). The protein expressed by this construct could be purified well by immobilized metal-ion affinity chromatography (IMAC). The culture medium condition for expression was optimized. The highest β-glucosidase activity was obtained when cells were grown in BMGH media containing 1% (v/v) glycerol, and induced in BMMH media containing 0.5% (v/v) methanol and 0.5% (w/v) casamino acid. The presence of casamino acid in BMMH media appeared to help in pH stabilization and production of enzyme, since media lacking casamino acid became more acidic and the enzyme activity declined after 3-5 days of induction (Toonkool *et al*., 2006).

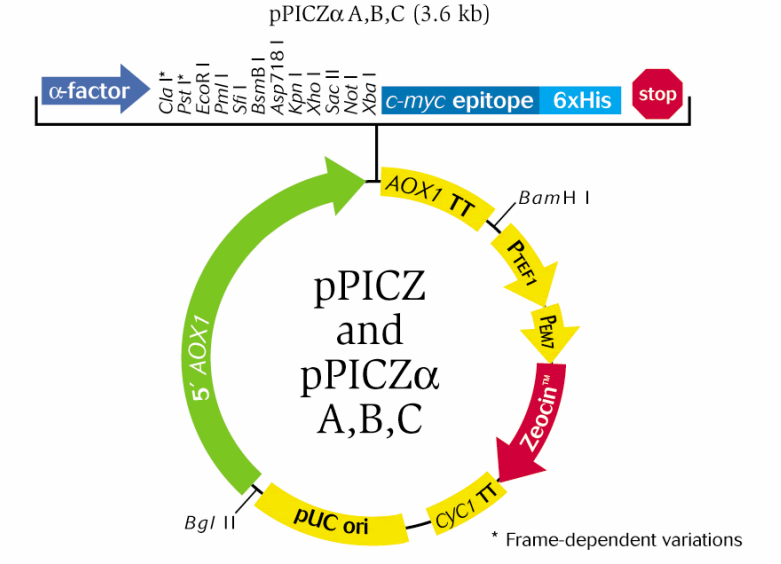


Figure 10 Plasmid pPICZα comprises of promoter *AOX1*, α-factor secretion signal for efficient secretion of heterologous protein, C-terminal *myc* epitope tag for detecting the fusion protein by Anti-*myc* antibody, polyhistidine tag for protein purification by IMAC, *AOX1* TT for transcription termination and polyadenylation signal, *TEF1* and EM7 promoters for driving expression of *Sh ble* gene conferring zeocin® resistance in *Pichia* and *E. coli*, respectively, *CYC1* TT for transcription termination of *Sh ble* gene, and pUC origin for plasmid replication in *E. coli*.

Source: Invitrogen (n.d.)

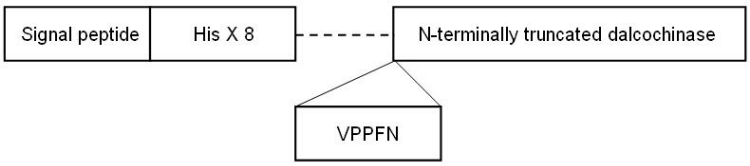


Figure 11 Construction of recombinant plasmids harboring coding sequence of dalcochinase, pPICZ-His_8-trncTRBG, in which first 12 amino acid residues of mature protein were truncated.

Source: Toonkool *et al.* (2006)

MATERIALS AND METHODS

Materials

1. Bacterial and yeast strains and plasmid

1.1 *Escherichia coli* (DH5α) genotype *supE*44Δ*lacU*169(ϕ80*lacZ*ΔM15) *hsdR*17*recA*1*endA*1*gyrA*96*thi*-1*relA*1

1.2 *Pichia pastoris* (GS115) genotype *his4*$^{-}$

1.3 *P. pastoris* (Y11430)

1.4 Plasmid pPICZ-His8-trncTRBG. Plasmid pPICZαB contains dalcochinase gene with an N-terminal polyhistidine tag (8 residues). The dalcochinase gene in the plasmid begins at amino acid residue VPPFN. This plasmid was from Assist. Prof. James Kedudat-Cairns, Suranaree University of Technology (Figure 12).

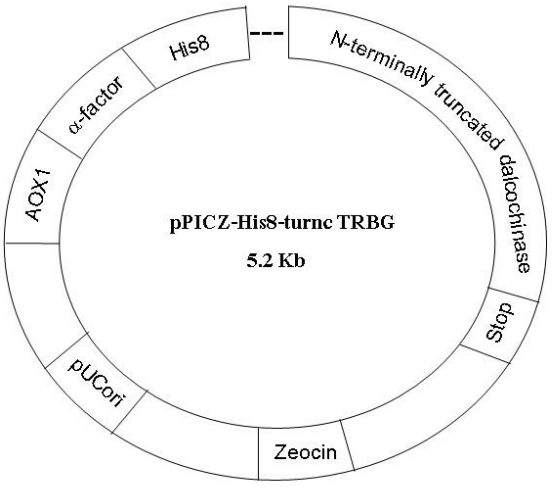


Figure 12 Plasmid construct of pPICZ-His$_8$-trncTRBG. The coding sequence of dalcochinase contains N-terminal truncation following the α-factor signal sequence and eight histidine residues.

2. Chemicals and enzymes

2.1 Acrylamide (Bio Basic, Canada)

2.2 Agarose (Research Organic, USA)

2.3 Ammonium hydroxide (Labscan, Thailand)

2.4 Ammonium sulfate (Ajax Finechem, Australia)

2.5 Biotin (Supelco, USA)

2.6 Bis-acrylamide (Bio Basic, Canada)

2.7 Casamino acid (Becton Dickinson, USA)

2.8 Dipotassium hydrogen phosphate (Ajax Finechem, Australia)

2.9 Disodium hydrogen phosphate (Ajax Finechem, Australia)

2.10 dNTP mix (Fermentas, USA)

2.11 Dynazyme (Finnzymes, Finland)

2.12 ECL plus Western Blotting Detection System (GE healthcare,Sweden)

2.13 *Eco*RI (Fermentas, USA)

2.14 Histidine (Bio Basic, Canada)

2.15 Imidazole (Fluka, Germany)

2.16 Methanol (Labscan, Thailand)

2.17 Mouse monoclonol antibody against natural dalcochinase

2.18 Natural β-glucosidase isolated from *Dalbergia cochinchinensis* Pierre, or dalcochinase (Laboratory of Prof. Dr. Jisnuson Svasti, Mahidol University)

2.19 *para*-Nitrophenol (*p*NP, Sigma, USA)

2.20 *para*-Nitrophenyl-β-D glucopyranoside (*p*NP-Glc, Sigma, USA)

2.21 *para*-Nitrophenyl-β-D mannopyranoside (*p*NP-Man, Sigma, USA)

2.22 *Pfu* DNA polymerase (Promega, USA)

2.23 Phenyl-sepharose CL4B (GE healthcare,UK)

2.24 Potassium dihydrogen phosphate (Ajax Finechem, Australia)

2.25 Rabbit Anti-Mouse Immunoglobulins/HRP (Dako Cytomation, Denmark)

2.26 Recombinant dalcochinase expressed and purified from *P. pastoris* containing pPICZ-His8-truncTRBG (Khakhanang Rattananikom, Kasetsart University)

2.27 *Sac*I (Fermentas, USA)

2.28 Sodium acetate (Merck, Germany)

2.29 Sodium carbonate (Fisher Scientific, UK)

2.30 Sodium chloride (Ajax Finechem, Australia)

2.31 Sodium dihydrogen phosphate (Ajax Finechem, Australia)

2.32 Sorbitol (Ajax Finechem, Australia)

2.33 Yeast nitrogen base without ammonium sulfate (Bio Basic, Canada)

2.34 Zeocin (Invitrogen, USA)

Note: All chemical are analytical grade.

Methods

1. Prediction of active amino acid residues for site-directed mutagenesis

1.1 Homology modeling and sequence alignment of wild-type enzymes

The three-dimensional models of dalcochinase and *Pyrococcus horikoshii* β-mannosidase were generated using Geno3D (geno3d-pbil.ibcp.fr). The crystal structure of maize β-glucosidase ZmGlu1 (PDB code 1E56A) and *Solfolobus salfataricus* β-glycosidase (PDB code 1UWSA) were used as templates for dalcochinase and *P. horikoshii* β-mannosidase, respectively. The overall models were checked The overall structures of mutated models were checked by Ramachandran plot and z-score using PROCHECK (Laskowski *et al*., 1993) and ProSA (Wiederstein *et al*., 2007), respectively. The selected three-dimensional models of dalcochinase and *P. horikoshii* β-mannosidase were used to predict the amino acid residues of dalcochinase that are involved in the glycone binding for site-directed mutagenesis. The primary protein sequence of dalcochinase was aligned to that of *P. horikoshii* β-mannosidase using ClustalW 1.83 (Higgins *et al*., 1994). The four amino acid residues of dalcochinase, which did not match to *P. horikoshii* β-mannosidase and were located in the glycone binding pocket of dalcochinase, were selected to be the targets for mutagenesis.

1.2 Homology modeling of dalcochinase mutants

The four targeted amino acid residues of dalcochinase were replaced with the corresponding residues of *P. horikoshii* β-mannosidase to create dalcochinase mutants. The four mutated protein sequences were submitted to Geno3D to create the mutant models. The crystal structure of maize β-glucosidase ZmGlu1 was used as a template. The overall structures of mutated models were checked The overall structures of mutated models were checked by Ramachandran plot and z-score using PROCHECK (Laskowski *et al*., 1993) and ProSA (Wiederstein *et al*., 2007), respectively.

1.3 Molecular docking of wild-type and mutant models of dalcochinase

To study the binding mode of enzymes and sugar substrates, the three-dimensional models of wild-type and mutant forms of dalcochinase were docked with *p*NP-Glc and *p*NP-Man using GOLD 3.1 program (Verdonk *et al.,* 2003). The *p*NP-Glc and *p*NP-Man molecules were generated and minimized using program SYBYL 7.3 (tripos Inc., USA). The enzyme models were fitted with the template structures, and the DIMBOAGlc molecule in the binding pocket of maize β-glucosidase ZmGlu1 was used to define the binding pockets of wild-type and mutant models of dalcochinase. Hydrogen atoms were added to the models using SYBYL 7.3 program. The *p*NP-Glc and *p*NP-Man were docked into the binding pockets of enzymes using GOLD 3.1 program. The hydrogen bonding interactions between the amino acid residues H136 and E452 and the C-3 and C-6 positions of sugar ring, respectively, were fixed during the GOLD docking process. The default genetic algorithm parameters were used; 100 for the population size, 1.1 for selection, 5 for number of island, 100,000 for number of genetic operations and 2 for the niche size. The annealing parameters for van der walls and hydrogen bonding were set to 4.0 and 2.5 Å, respectively. Fifteen genetic algorithm runs with default parameter setting were performed. The docked structures of *p*NP-Glc and *p*NP-Man in enzyme models were analyzed using DS ViewerPro 5.0 program (Accelrys Inc., USA).

1.4 Molecular docking of wild-type *P. horikoshii* β-mannosidase

To study the binding mode of sugar substrates in the binding pocket of wild-type *P. horikoshii* β-mannosidase, the *p*NP-Glc and *p*NP-Man were docked into the three-dimensional model of wild-type *P. horikoshii* β-mannosidase using GOLD 3.1 program. The *p*NP-Glc and *p*NP-Man molecules were generated as descripted above. The *P. horikoshii* β-mannosidase model was fitted with the template structures, and the glucose molecule in the binding pocket of *S. salfataricus* β-glycosidase was used to define the binding pockets of *P. horikoshii* β-mannosidase. Hydrogen atoms were added to the model using SYBYL 7.3 program. The hydrogen bonding interactions between the amino acid residues H149 and E443 and the C-3 and C-6 positions of sugar ring, respectively, were fixed during the GOLD docking process, and the default genetic algorithm parameters were used as mentioned above. The annealing parameters for van der walls and hydrogen bonding were set to 4.0 and 2.5 Å, respectively. Fifteen genetic algorithm runs with default parameter setting were performed. The docked structures of *p*NP-Glc and *p*NP-Man in enzyme model were analyzed using DS ViewerPro 5.0 program (Accelrys Inc., USA).

2. Generation of mutant constructs of dalcochinase

2.1 Site-directed mutagenesis of dalcochinase

Site-directed mutagenesis was performed using proof-reading *Pfu* DNA polymerase. The mutagenic oligonucleotide primers were designed, in which the mismatched bases were located at the center of the primers (Table 1). Primers were 25-45 bases in length, with the melting temperature (T_m) ≥ 78 ºC, and the GC content of at least 40%. The 3' end of the primers terminated with one or more G or C (Stratagene, n.d.). The 50 μl PCR reactions comprised of 50 ng of plasmid-governed wild-type dalcochinase gene as template, 0.2 mM dNTP mix, 125 ng of each primer and 2.5 units of *Pfu* polymerase in 1x *Pfu* buffer [20 mM Tris-HCl (pH 8.8 at 25 ºC), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton® X-100 and 0.1 mg/ml nuclease-free BSA]. The reactions were performed as follows: one cycle of 95 ºC for

30 seconds; twenty-five cycles of 95 ºC for 30 seconds, 55 ºC for 1 minute and 68 ºC for 12 minutes, respectively. Ten units of *Dpn*I were incubated with the PCR reaction to cleave the wild-type plasmid overnight at 37 ºC.

Table 1 Mutagenic primers used for creating mutant forms of dalcochinase

Mutant forms of dalcochinase	Names of primer	%GC	Primer sequences
R90Q	TeeR90Qfor	45.0	5' GGA TAT GAA CTT GGA TGC TTA TCA AAT GTC CAT CTC CTG GCC 3'
	TeeR90Qrev	45.0	5' GGC CAG GAG ATG GAC ATT TGA TAA GCA TCC AAG TTC ATA TCC 3'
W137Q	TeeW137Qfor	41.3	5' CCA TTT GTA ACC ATT TTT CAT CAA GAT CTT CCA CAA GCC TTG GAG G 3'
	TeeW137Qrev	41.3	5' CCT CCA AGGCTT GTG GAA GAT CTT GAT GAA AAA TGG TTA CAA ATG G 3'
N181D	TeeN181Dfor	48.6	5' GGA TTA CAC TAG ATG AGC CAT CAA TCT TCA CCG CG 3'
	TeeN181Drev	48.6	5' CGC GGT GAA GAT TGA TGG CTC ATC TAG TGT AAT CC 3'
M369E	TeeM369Efor	50.0	5' GGT CCA GTG ACT CCC TCA GGA TGG GAA TGC ATT TAT CCA AAA GG 3'
	TeeM369Erev	51.1	5' CCT TTT GGA TAA ATG CAT TCC CAT CCT GAG GGA GTC ACT GGA CC 3'

Note: The bold alphabets indicate the mutation sites.

2.2 Cloning of mutant dalcochinase plasmids into *E. coli* DH5α

One µl of *Dpn*I-treated mutagenesis reaction (from 2.1) was transformed into 40 µl of *E. coli* DH5α by electroporation. The electroporation device (Bio-Rad, USA) was set at 25 µF, 200 Ω and 2.5 kV. Mutants were grown on LB agar [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, pH 7.5, and 1.5% (w/v) agar] containing 25 µg/ml zeocin overnight at 37°C. Mutants were screened by restriction digestion and colony PCR. Colony PCR method used two primers: one contained the mutated sequences at its 3' end and a flanking primer (Table 2). The reactions were done in a total volume of 25 µl, which comprised of 10 µl of cell

suspension of *E. coli* containing mutant constructs of dalcochinase gene as template, 0.2 mM dNTP mix, 10 pmol of primers and 1 unit of Dynazyme in 1x Dynazyme buffer [20 mM Tris-HCl (pH 8.8 at 25 ºC), 1.5 mM $MgCl_2$, 50 mM KCl and 0.1% Triton® X-100]. The reactions were performed as follows: one cycle of 95 ºC for 5 minutes; sixteen cycles of 95 ºC for 1 minute, 55 ºC for 30 seconds and 72 ºC for 1 minute; and a final extension at 72 ºC for 8.5 minutes. The colony PCR product was checked with 1% (w/v) agarose gel electrophoresis.

Table 2 The sequences of oligonucleotide primers used for screening the mutant constructs of dalcochinase

Mutant forms of dalcochinase	Primers	Oligonucleotide sequence	Expected PCR product (bp)
R90Q	R90QRevSc	5' GGA TAT GAA CTT GGA TGC TTA TCA 3'	616
	AOXF	5' GAC TGG TTC CAA TTG ACA AG 3'	
W137Q	W137QforSc	5' CCA TTT GTA ACC ATT TTT CAT CAA 3'	1400
	AOXR	5' GCA AAT GGC ATT CTG ACA TC 3'	
N181D	N181DRevSc	5' GGT GAA GAT TGA TGG CTC ATC 3'	889
	AOXF	5' GAC TGG TTC CAA TTG ACA AG 3'	
M369E	M369ERevSc	5' GTC CTT TTG GAT AAA TGC ATT C 3'	1024
	TRF1	5' AAC CGA AGC TGT TTT CCT TC 3'	

2.3 Cloning of mutant dalcochinase plasmids into *P. pastoris*

All *E. coli* clones harboring mutant constructs of dalcochinase were grown on LB agar pH 7.5 containing 25 μg/ml zeocin overnight at 37 °C. Plasmid preparation (alkaline lysis method) and restriction digestion with *Eco*RI were done to confirm that the correct plasmid that has been extracted. *SacI* restriction digestion was, then, performed to make the linear plasmids. The electroporation device (Bio-Rad, USA) was set at 25 μF, 200 Ω and 1.5 kV to transform the 5 μg of linear plasmid into 80 μl (OD_{600} = 10) of competent *P. pastoris* (GS115 and Y11430) cells (Invitrogen, n.d.). The transformed clones were incubated on YPDS agar [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose and 1 M sorbitol] containing 100 μg/ml zeocin overnight at 30 °C.

3. Expression of mutant forms of dalcochinase

3.1 Expression screening of mutant forms of dalcochinase

All *P. pastoris* clones harboring mutant constructs of dalcochinase were grown in 5 mL BMGH medium [100 mM potassium phosphate, pH 6.0, 0.34% (w/v) Yeast Nitrogen Base, 1% (w/v) ammonium sulfate and 1% (v/v) glycerol] containing $4x10^{-5}$% (w/v) biotin and $4x10^{-3}$% (w/v) histidine overnight at 30°C and 180-200 rpm. Cell cultures were collected by centrifugation, washed in distilled water and regrown in 5 mL BMMH medium [100 mM potassium phosphate, pH 6.0, 0.34% (w/v) Yeast Nitrogen Base, 1% (w/v) ammonium sulfate, 0.5% methanol and 0.5% casamino acid] containing $4x10^{-5}$% (w/v) biotin and $4x10^{-3}$% (w/v) histidine. Methanol was added everyday to the final concentration of 0.5% (v/v) to induce protein expression. The β-glycosidase activities in the culture medium were assayed using *p*NP-Glc and *p*NP-Man after 7 days of induction for checking enzyme expression. The mutant clones with highest hydrolytic activity were selected for further study.

3.2 Large-scale expression of mutant forms of dalcochinase

For expression of mutant enzymes in the shake flask system, the selected clones of *P. pastoris* GS115 harboring mutant constructs of dalcochinase were grown in 20 ml BMGH containing $4x10^{-5}$% (w/v) biotin and $4x10^{-3}$% (w/v) histidine overnight at 30 ºC and 180-200 rpm. The starters were then inoculated into 130 ml BMGH containing $4x10^{-5}$% (w/v) biotin and $4x10^{-3}$% (w/v) histidine, and grown overnight at 30 ºC and 180-200 rpm. The cells were harvested by centrifugation and washed in sterile distilled water before being grown in 150 ml of BMMH medium containing $4x10^{-5}$% (w/v) biotin and $4x10^{-3}$ (w/v) histidine for enzyme expression. Methanol was added everyday to the final concentration of 0.5% (v/v). The β-glucosidase activities in the culture medium were assayed using *p*NP-Glc and *p*NP-Man until enzyme activities did not increase any further. SDS-PAGE and western blotting were performed to confirm the enzyme expression.

For expression in the fermenter system, the fermentation process was divided into four states, namely glycerol batch, glycerol exponential fed-batch, methanol exponential fed-batch and constant methanol fed-batch (Charoenrat *et al*., 2006). In the glycerol batch process, the selected clones of *P. pastoris* Y11430 containing mutant constructs of dalcochinase were grown in 20 ml YDP [1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) dextrose] containing 100 µg/ml zeocin overnight at 30 °C. The 20 ml YPD starter was, then, transferred into 80 ml BMGY medium [1% (w/v) yeast extract, 2% (w/v) peptone, 1% (w/v) ammonium sulfate, 0.34% (w/v) yeast nitrogen base, 100 mM potassium phosphate pH 6.0 and 1% (v/v) glycerol] overnight at 30 °C. The 100 ml starter was inoculated into the fermenter containing 3 l of glycerol basal salts medium [40.0 g l^{-1}glycerol, 26.7 ml l^{-1} of 85% H_3PO_4, 0.93 g l^{-1} $CaSO_4$, 18.2 g l^{-1} K_2SO_4, 14.9 g l^{-1} $MgSO_4 \cdot 7H_2O$ and 4.13 g l^{-1} KOH,] containing 4.35 ml l^{-1} PTM1 trace salts [6.0 g l^{-1} $CuSO_4 \cdot 5H_2O$, 0.08 g l^{-1} KI, 3.0 g l^{-1} $MnSO_4 \cdot H_2O$, 0.2 g l−1 $Na_2MoO_4 \cdot 2H_2O$, 0.02 g l^{-1} H_3BO_3, 20.0 g l^{-1} $ZnCl_2$, 13.7 g l^{-1} $FeCl_3$, 0.9 g l^{-1} $CoCl_2 \cdot 6H_2O$, 5.0 ml l^{-1} H_2SO_4 and 0.2 g l^{-1} biotin]. The temperature, aeration, agitation and pH were controlled at 30 °C, 6 l min^{-1} 1000 rpm and 5.0, respectively. The 25% (v/v) of NH_4OH was used as an alkaline to control the pH.

About 24 hours after inoculation when the glycerol was completely consumed, as indicated by the sudden increase of dissolved oxygen tension, the process was switched to the glycerol exponential fed-batch process by feeding 500 g l^{-1} glycerol containing 12 ml l^{-1} PTM1 trace salts to the culture medium at an exponential increasing rate of 0.18 h^{-1}. After 3 h, the process was switched to the methanol exponential fed-batch by placing the glycerol feed with a methanol feed containing 12 ml l^{-1} PTM1 trace salts for 2-3 h. In the constant methanol fed-batch process, the methanol was fed into the culture medium in the constant rate, by means of controlling the dissolved oxygen tension at 25% air saturation, to induce protein expression. After approximately a hundred hours of inoculation, the culture medium was collected for future protein purification process. SDS-PAGE and western blotting were performed to confirm the enzyme expression.

4. Purification of mutant forms of dalcochinase

The volume of culture medium of *P. pastoris* expressing mutant forms of dalcochinase was reduced using ultrafiltation with 30 kDa molecular weight cut-off regenerated cellulose membrane (Millipore, USA). The concentrated culture medium was added with 1 M ammonium sulfate, and applied to phenyl-sepharose chromatography, which had been pre-equilibrated with 3 column volumes of binding buffer [10 mM potassium phosphate pH 7.0 and 1 M ammonium sulfate]. The column was washed with 3 column volumes of phenyl-sepharose chromatography binding buffer, and eluted with a gradient of 1-0 M ammonium sulfate in 10 mM potassium phosphate, pH 7.0. The 2 ml fractions containing β-glucosidase activity were pooled and applied to a HiTrap FF desalting column (GE healthcare, UK), to remove ammonium sulfate and exchange buffer to the binding buffer [50 mM Sodium phosphate buffer pH 8.0 and 300 mM NaCl] for Ni^{2+} chromatography (Qiagen, Germany). The crude enzyme was applied to a Ni^{2+} chromatography which had been pre-equilibrated with 5 column volumes of Ni^{2+} chromatography binding buffer. The column was washed with 5 column volumes of the same binding buffer and eluted with 5 column volumes of elution buffer [50 mM sodium phosphate pH 8, 300 mM NaCl and 250 mM imidazole]. The 1 ml fractions containing β-glucosidase activity were pooled and reduced volume using 30 kDa molecular weight cut-off amicon ultrafiltration (Mollipore, USA). SDS-PAGE and western blotting were performed to confirm the enzyme purity.

5. Enzyme assay

5.1 Measurement of enzyme activity in the crude preparation

To detect the enzyme activity of mutant forms of dalcochinase in the culture medium and in the purification steps, 300 µl of culture medium was centrifuged to collect the crude enzyme. The aliquot 250 µl of centrifuged medium was added with 1 mM *p*NP-Glc (or 1 mM *p*NP-Man). The 0.1 M sodium acetate buffer pH 5.0 was added to adjust the final volume to 500 µl. The reaction mixture

was incubated at 30°C for 30 minutes and stopped the reaction by adding 1 ml of 2 M Na_2CO_3. The *p*NP released was measured at the absorbance of 400 nm (ABS400).

5.2 Measurement of enzyme activity in the purified preparation

The enzyme activity (unit) of wild-type and mutant forms of dalcochinase was measured by incubating 0-10 µl of purified enzymes with 15 mM *p*NP-Glc (or 15 mM *p*NP-Man) in 0.1 M sodium acetate buffer pH 5.0. The 50 µl of reaction mixture was incubated at 30 °C for 5 minutes, and the reaction was stopped by adding 100 µl of 2 M Na_2CO_3. The *p*NP released was measured at ABS400. The ABS400 values of enzymes were converted to the enzyme activity values (µmol *p*NP per min or unit) by dividing the ABS400 values with the slope value of standard *p*NP plot and reaction time (Appendix B).

5.3 Kinetic measurement for hydrolysis of *p*NP-Glc

The kinetic studies of purified enzymes were measured by incubating 0.05 unit of wild-type natural and recombinant dalcochinase, or 8 µl of mutant enzymes with various concentrations of *p*NP-Glc substrates. 0.1 M sodium acetate buffer pH 5.0 was added to adjust the final volume to 50 µl. The reaction mixture was incubated at 30 °C for 5 minutes and stopped the reaction by adding 100 µl of 2 M Na_2CO_3. The *p*NP released was measured at ABS400, and converted to the enzyme activity values (µmol *p*NP per min or unit). The kinetic parameters (K_m and V_{max}) were calculated by using the Michaelis-Menten equation. The Michaelis-Menten equation is expressed as:

$$V = \frac{V_{max}[S]}{K_m + [S]}$$

where K_m is Michaelis-Menten rate constant in mM, [S] is substrate concentration in mM, V is initial rate of production of product in µmol *p*NP per min (or unit), and V_{max} is maximum rate of production of product.

The turnover number (or k_{cat}) is the number of substrate molecules that can be converted to product per enzyme molecules per second. The value of k_{cat} can be calculated from:

$$k_{cat} = \frac{V_{max}}{60E}$$

where E is quantity of enzyme in μmol.

5.4 Hydrolytic activities toward *p*NP-Man

The hydrolytic activities of wild-type and mutant forms of dalcochinase toward of *p*NP-Man were measured by incubating 3 μg of purified enzymes with 15 mM *p*NP-Man. 0.1 M sodium acetate buffer pH 5.0 was added to adjust the final volume to 50 μl. The reaction mixture was incubated at 30 °C for 5 minutes and stopped the reaction by adding 100 μl of 2 M Na_2CO_3. The *p*NP released was measured at ABS400, and converted to the specific activity values (mU per μg protein). The specific activity of recombinant wild-type and mutant forms of dalcochinase were compared to that of natural dalcochinase.

6. Transglycosylation

To study the transglycosylation reaction, 5 μg of wild-type and mutant forms of dalcochinase were incubated with alcohols and sugar substrates. The 0.9 M of shot-chain alkyl alcohols (C_1-C_4), which were methanol, ethanol, *n*-propanol and *n*-butanol, were used as glycosyl acceptors, and 10 mM of *p*NP-Glc and *p*NP-Man were used as glycosyl donors. The reaction mixtures were adjusted with 0.1 M sodium acetate buffer pH 5.0 to the final volume of 100 μl, and incubated at 30 °C for 20 hours. The reactions were stopped by boiling for 2 minutes, and were analyzed using thin-layer chromatography (TLC).

For TLC analysis, the 8 µl of each reaction mixtures were spotted on silica gel 60 F_{254} aluminium sheet, developed twice with solvent A [ethyl acetate/methanol/water 16:6:1 by volume] for 4.25 cm and followed by developing twice with solvent P [2-propanol/ethanol/water 5:1:2 by volume] for 1.75 cm. The TLC sheets were visualized by spraying with 20% (v/v) sulfuric acid in ethanol and heating at 125 °C for 10 minutes. *p*NP-Glc, *p*NP-Man, glucose and methyl glucosides were used as standard markers. The TLC was scanned using the ImageScanner (GE healthcare, UK) to quantify the intensity of spots.

RESULTS AND DISCUSSION

1. Prediction of active amino acid residues for site-directed mutagenesis

1.1 Homology modeling and sequence alignment of wild-type enzymes

Because there are no three-dimensional structures of *Dalbergia cochinchinensis* Pierre β-glucosidase (or dalcochinase) and *Pyrococcus horikoshii* β-mannosidase, the amino acid sequences of dalcochinase and *P. horikoshii* β-mannosidase were submitted into Geno3D protein modeling server for generation of the three-dimensional models. The amino acid sequences of both enzymes were aligned with those of the known three-dimensional structures in the protein data bank (PDB), and the templates were chosen. The *Zea mays* β-glucosidase or maize β-glucosidase ZmGlu1 (PDB code 1E56) which shows 43% sequence identity to dalcochinase was selected as a template for dalcochinase modeling. The β-glycosidase from *Sulfolobus solfataricus* (PDB code 1UWS) which shows 40% sequence identity to *P. horikoshii* β-mannosidase was used as a template for *P. horikoshii* β-mannosidase modeling. The resolutions of maize β-glucosidase ZmGlu1 and *S. salfataricus* β-glycosidase were 2.10 and 1.95 Å which was good enough to be the templates for dalcochinase and *P. horikoshii* β-mannosidase modeling, respectively. Moreover, these template enzymes contained the ligand structures, which was natural substrate, DIMBOAGlc, in maize β-glucosidase ZmGlu1 and glucose in *S. solfataricus* β-glycosidase. The orientation of amino acid residues in the binding pocket of these two template enzymes with ligand structures might be imitated as the orientation of the amino acid residues in the binding pocket of dalcochinase and *P. horikoshii* β-mannosidase with *p*NP-Glc and *p*NP-Man substrates. The qualities of models were checked by Ramachandran plot and z-score using PROCHECK (Laskowski *et al*., 1993) and ProSA (Wiederstein *et al*., 2007), respectively. The Ramachandran plot showed that the majority of amino acid residues of dalcochinase were located in the most favorable region (76.2%), and the others located in the additional favorable region (30.8%), generously favorable region (1.5%) and disallowed region (1.5%) (Table 3 and Figure 13A). In *P. horikoshii* β-mannosidase,

the majority of amino acid residues were located in the most favorable regions (68.2%), and the others located in the additional favorable region (27.1%), generously favorable region (3.8%) and disallowed region (0.9%) (Table 3 and Figure 14A). Models which contained the amino acid residues in the disallowed region less than 2% might be chosen for further study since the reliable model should not contain amino acid residues more than 2% in this region (Kaper *et al.*, 2002). The z-scores of dalcochinase and *P. horikoshii* β-mannosidase (Figures 13B and 14B, respectively) were within the acceptable range. Moreover, the amino acid residues located in the disallowed region of Ramachandran plot of both enzyme models were not the amino acid residues in the binding pocket of enzymes, indicating the reliable models were obtained (Figures 13A and 14A).

Table 3 The quality of homology models.

Enzyme	Ramachandran Plot				z-score
	Most favorable region (%)	Additional favorable region (%)	Generously favorable region (%)	Disallowed region (%)	
Dalcochinase	76.2	30.8	1.5	1.5	-8.15
P. horikoshii β-mannosidase	68.2	27.1	3.8	0.9	-9.08

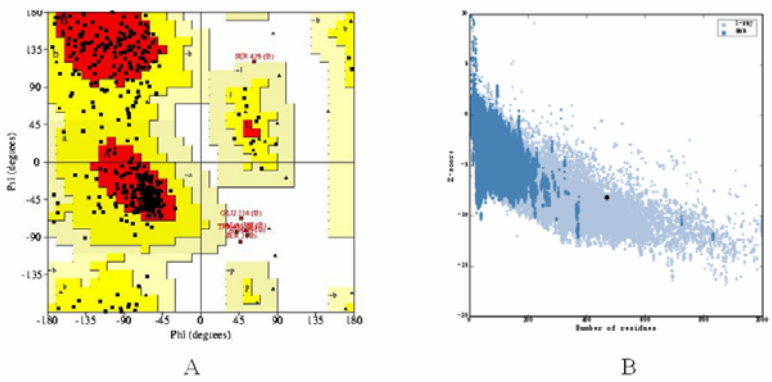


Figure 13 (A) The Ramachandran plot for the modeling of dalcochinase. The red region shows the most favorable region, the yellow region shows the additional allowed region, the light brown shows the generously allowed region and the white region shows the disallowed region. (B) The z-score plot. Black circle indicates the z-score of dalcochinase, which located in the acceptable range.

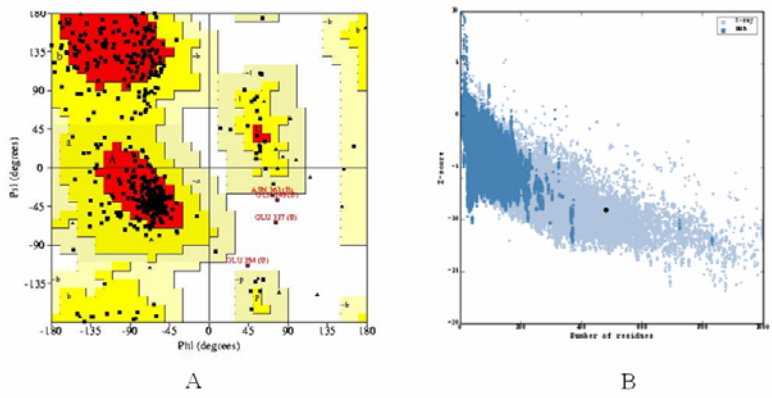


Figure 14 (A) The Ramachandran plot for the modeling of *P. horikoshii* β-mannosidase. The red region shows the most favorable region, the yellow region shows the additional allowed region, the light brown shows the generously allowed region and the white region shows the disallowed region. (B) The z-score plot. Black circle indicates the z-score of *P. horikoshii* β-mannosidase, which located in the acceptable range.

The three-dimensional models of dalcochinase (Figure 15) and *P. horikoshii* β-mannosidase (Figure 16) showed $(\beta/\alpha)_8$ barrel structures as expected for glycosyl hydrolase family 1.

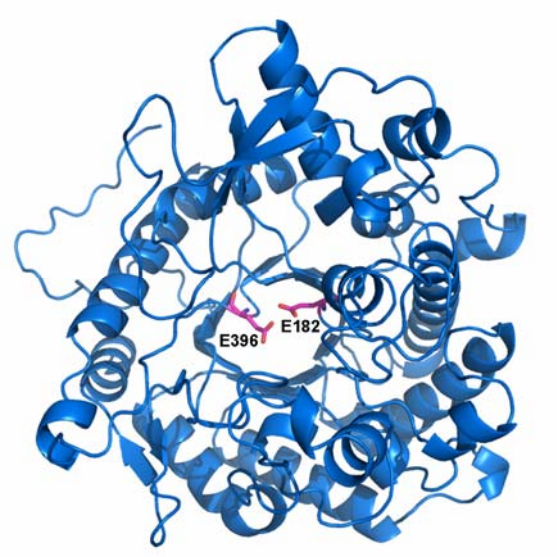


Figure 15 The three-dimensional model of dalcochinase. The maize β-glucosidase ZmGlu1 (1E56) was used as a template. The catalytic acid/base (E182) and nucleophile (E396) are shown as stick drawings with labels.

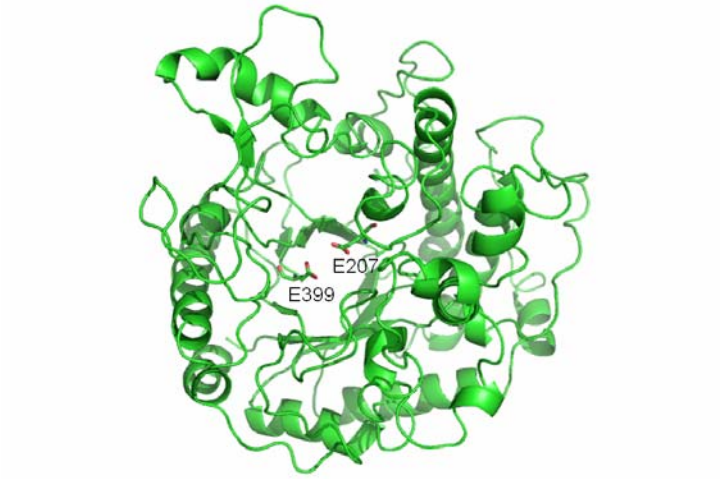


Figure 16 The three-dimensional model of *P. horikoshii* β-mannosidase. The *S. solfataricus* β-glycosidase (1UWS) was used as a template. The catalytic acid/base (E207) and nucleophile (E399) are shown as stick drawings with labels.

To predict which amino acid residues of dalcochinase are responsible for sugar substrate binding, the primary sequence of dalcochinase was aligned to those of *P. furiosus* β-glucosidase *and P. horikoshii* β-manosidase (Figure 17). Dalcochinase showed 24% and 20% sequence identity to *P. furiosus* β-glucosidase and *P. horikoshii* β-mannosidase, respectively. Only 104 amino acid residues of dalcochinase are identical to those of *P. horikoshii* β-mannosidase, while the other 420 residues were different. However, when focus on the sugar substrate binding pocket, most of amino acid residues of dalcochinase were the same as those of *P. horikoshii* β-mannosidase. These amino acid residues were Q36, H136, E182, E396, and E452, and Q13, H149, E207, E399 and E443 in dalcochinase and in *P. horikoshii* β-mannosidase, respectively. The different amino acid residues were R90, W137, N181 and M369 of dalcochinase, which were corresponding to Q77, Q150, D206 and E374 of *P. horikoshii* β-mannosidase, respectively.

Therefore, the amino acid residues that were located in the sugar binding pocket of dalcochinase and did not match to *P. horikoshii* β-manosidase were selected to be the target for mutation. Previous study conducted by Kaper and co-workers (2002) have identified residues R77 and N206 in *P. furiosus* β-glucosidase, corresponding to Q77 and D206 in *P. horikoshii* β-manosidase, respectively, as important residues for substrate specificity. These residues correspond to R90 and N181 in dalcochinase. Therefore R90 and N181 of dalcochinase were replaced with their corresponding residues Q and D of *P. horikoshii* β-manosidase, yielding R90Q and N181D dalcochinase mutants, respectively. The further two residues W137 and M369 in dalcochinase, corresponding to Q150 and E374 in *P. horikoshii* β-mannosidase, were also located in the sugar binding pocket of dalcochinase (Figure 18) and were selected for mutation, yielding W137Q and M369E dalcochinase mutants, respectively.

```
Dalcochinase      IDFAKEVRETITEVPPFNRSCFPSDFIFGTASSSYQYEGEGRVPSIWDNFTHQYPEKIAD 60
PyBGlu            -----------MFPEKFLWGVAQSGFQFEMGDKLRRNIDTNTDWWHWVRDKTNIEKGLVS 49
PyBglB            --------------MKFYWGVVQSAFQFEMGDPYRRNIDPRSDWWYWVRDPYNIKNDLVS 46

Dalcochinase      RSNGDVAVDQFHRYKKDIAIMKDMNLDAYRMSISWPRILPTG------------------ 102
PyBGlu            GDLPEEGINNYELYEKDHEIARKLGLNAYRIGIEWSRIFPWPTTFIDVDYSYNESYNLIE 109
PyBglB            GDLPEEGINNYELYEIDHRLAKELGLNAYQLTIEWSRIFPCPTFNVEVEFER-DGYGLIK 105

Dalcochinase      ----------RVSGGINQTGVDYYNRLINESLANGITPFVTIFHWDLPQALED------- 145
PyBGlu            DVKITKDTLEELDEIANKREVAYYRSVINSLRSKGFKVIVNLNHFTLPYWLHDPIEARER 169
PyBglB            KVKIEKEHLEELDKLANQKEVRHYLNVLRNLKKLGFTTFVTLNHQTNPIWIHDPIETRGN 165

Dalcochinase      ----EYGGFLNHSVVNDFQDYADLCFQLFGDRVKHWITLNEPSIFTANGY--AYGMFAPG 199
PyBGlu            ALTNKRNGWVNPRTVIEFAKYAAYIAYKFGDIVDMWSTFNEPMVVVELGYLAPYSGFPPG 229
PyBglB            FQKARARGWVDERTIIEFAKYAAYVAWKFDNYVDYWSTFDEPMVTAELGYLAPYVGWPPG 225

Dalcochinase      RCSPSYNPTCTGGDAGTETYLVAHNLILSHAATVQVYKRKYQEHQKGTIGISLH-VVWVI 258
PyBGlu            VLNP------EAAKLAILHMINAHALAYRQIKKFDTEKADKDSKEPAEVGIIYNNIGVAY 283
PyBglB            ILNP------SAAKKVIINQIVAHARAYDSIKKFSSK----------PVGVILN-IIPAY 268

Dalcochinase      PLS-NSTSDQNATQRYLDFTCGWFMDPLTAGRYPDSMQYLVGDRLPKFTTDQAKLVKGSF 317
PyBGlu            PKDPNDSKDVKAAENDNFFHSGLFFEAIHKGKLNIEFDGETFIDAPYLKGN--------- 334
PyBglB            PLDPNDSKSVRAAENYDLFHNRLFLEAVNRGNVDLDITGE-YTKIPHIKRN--------- 318

Dalcochinase      DFIGLNYYTTNYATKSDAS-TCCPPSYLTDPQVTLLQQRNGVFIG---PVTPSGWWCIYP 373
PyBGlu            DWIGVNYYTREVVTYQEPMFPSIPLITFKGVQGYGYACRPGTLSKDDRPVSDIGWE-LYP 393
PyBglB            DWIGNNYYTREVVKYVEPKYEELPLITFVGVEGYGYSGNPNSLSPDNNPTSDFGWE-VFP 377

Dalcochinase      KGLRDLLLYFKEKYNNPLVYITENGIDEKNDASLSLEESLIDTYRIDSYYRHLFYVRYAI 433
PyBGlu            EGMYDSIVEAHKYG--VPVYVTENGIADSK----------DILRPYYIASHIKMIEKAF 440
PyBglB            QGLYDSTLEAAEYN--KEVFITENGIADSK----------DILRPRYIIDHVNEVKKLI 424

Dalcochinase      RSGANVKGFFAWSLLDNFEWAEGYTSRFGLYFVNYTTLNRYP-KLSATWFKYFLARDQES 492
PyBGlu            EDGYEVKGYFHWALTDNFEWALGFRMRFGLYEVNLITKERIPREKSVSIFREIVANNGVT 500
PyBglB            ENGIKVGGYFHWALTDNYEWAMGFKIRFGLYEVDLITKERIPRRRSVEIYKKIVMEG--- 481

Dalcochinase      AKLEILAPKARWSLSTMIKEEKTKPKRGIEGF 524
PyBGlu            KKIEEELLRG---------------------- 510
PyBglB            --IE--------------------------- 483
```

Figure 17 Amino acid sequence alignment of dalcochinase to *P. furiosus* β-glucosidase (PyBGlu) and *P. horikoshii* β-mannosidase (PyBglB). The site of mutations are indicated by the white letters in the black background. The conserved motifs, L/FNEP and I/VTENG, containing two catalytic glutamate residues are underlined.

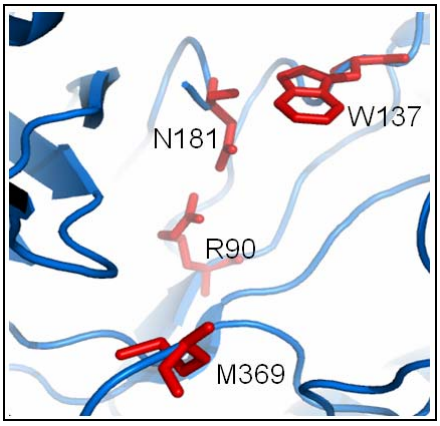


Figure 18 The amino acid residues of dalcochinase which were selected for site-directed mutagenesis. They are located in the sugar binding pocket of the dalcochinase (from homology modeling) and do not match to those of *P. horikoshii* β-manosidase.

1.2 Homology modeling of dalcochinase mutants

The three-dimensional models of dalcochinase mutants (R90Q, W137Q, N181D and M369E) were generated using Geno3D, using maize β-glucosidase zmGlu1 (PDB code 1E56A) as a template. The overall quality of three-dimensional models of mutant forms of dalcochinases was analyzed using Procheck (Laskowski *et al*., 1993) and ProSA (Wiederstein *et al*., 2007). The Ramachandran plot from PROCHECK revealed that the majority of amino acid residues of R90Q, W137Q, N181D and M369E were located in the most favorable regions (74.0%, 74.3%, 76.7% and 70.6%, respectively) and less than 2% of amino acid residues in the disallowed regions (0.5%, 1.2%, 1.5% and 0.5%, respectively), and the z-score from ProSA of all mutant models were located in the acceptable range, which make them reliable models (Table 4).

Table 4 The quality of homology models of mutant forms of dalcochinase.

Enzymes	Ramachandran Plot				z-score
	Most favorable region (%)	Generously favorable region (%)	Additional favorable region (%)	Disallowed region (%)	
R90Q	74.0	22.8	2.7	0.5	-7.91
W137Q	74.3	21.3	3.2	1.2	-7.85
N181D	76.7	18.4	3.4	1.5	-7.51
M369E	70.6	25.2	3.7	0.5	-7.62

1.3 Molecular docking of wild-type and mutant models of dalcochinase

To study the binding modes of *p*NP-Glc and *p*NP-Man to wild-type dalcochinase, the sugar substrates were docked into the binding pocket of the enzyme using GOLD 3.1 program. It was found that the catalytic acid/base (E182) and the catalytic nucleophile (E396) formed hydrogen bond with N181 and R90, respectively, both which are highly conserved in glycosyl hydrolase family 1. These interactions were likely to contribute to stabilization of both transition and ionization state of these critical amino acids (Sahz-Aparicio *et al*., 1998). The orientations and positions of glucose and mannose in the binding pocket of wild-type dalcochinase were different (Figure 19 and Table 5). The hydroxyl group at C-2 of glucose formed hydrogen bonds with E182 and E396, and the distance between the glycosidic oxygen and E182 and E396 were 3.47 and 4.23 Å, respectively. On the other hand, the hydroxyl group at C-2 of mannose formed a hydrogen bond only with E182, and the distance between the glycosidic oxygen and E182 and E396 were 3.87 and 5.59 Å, respectively, which was possibly too far for the formation of hydrogen bond between these two species in the first step of hydrolysis. The interactions between the hydroxyl groups at C-3, C-4, and C-6 with H136, W453 and W445, respectively, were present in glucose, but were lacking in mannose. Therefore, these differences in the interactions of glucose and mannose with the amino acid residues in the sugar binding pocket of dalcochinase may influence the catalytic activity of enzyme, such that wild-type dalcochinase can not hydrolyze *p*NP-Man.

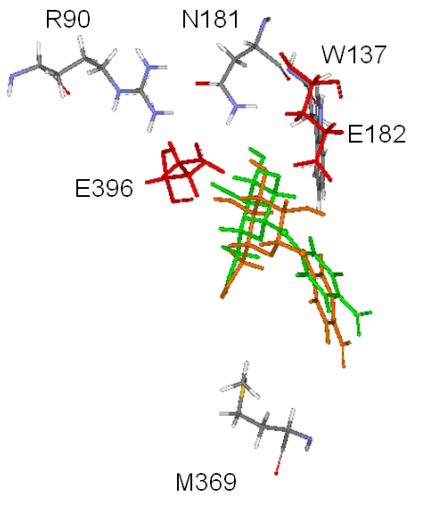


Figure 19 Comparison the orientation of *p*NP-Glc (green) and *p*NP-Man (orange) in the binding pocket of wild-type dalcochinase as predicted by molecular docking. The catalytic residues, E182 and E396, are showed in red. The glycosidic oxygens of *p*NP-Glc and *p*NP-Man are shown as ball-and-stick models.

Table 5 Distances between the substrates, *p*NP-Glc and *p*NP-Man, and the amino acid residues in the sugar binding pocket of wild-type dalcochinase model as predicted by molecular docking.

	Sugar position	Residue	Distance (Å)
*p*NP-Glc	Glycosidic oxygen	E182 Oε2	3.47
		E396 Oε1	4.23
	O-2	E182 Oε1	2.13
		E396 Oε1	1.92
	O-3	H136 Nε2	2.44
	O-4	W453 Hε1	1.12
	O-6	W445 Hε1	1.68
		E452 Oε1	2.03
*p*NP-Man	Glycosidic oxygen	E182 Oε2	3.87
		E396 Oε1	5.59
	O-2	E182 Oε2	2.31
	O-6	E452 Oε1	2.03

The docking study was performed to investigate the binding mode of *p*NP-Glc in the sugar binding pocket of models of dalcochinase mutants (Figure 20 and Table 6). Mutants R90Q and N181D lacked the necessary interactions between R90 with E396, and N181 with E182, respectively, probably resulting in lower catalytic activities of mutant enzymes. Moreover, the replacement of a longer side-chain of R with a shorter side-chain of Q resulted in the rearrangement of amino acid residues in the binding pocket. Especially, Y325 was displaced such that it could likely hinder the entry of the glucosyl moiety to the binding pocket of R90Q (Figure 21). Also in other mutants, rearrangements of amino acid residues in the binding pockets lead to alterations in the binding modes between glucose moiety of *p*NP-Glc and the active site residues, compared with the wild-type enzyme. In all cases, the glycosidic oxygen was placed much further away from the catalytic residues (Figure 20). In W137Q and N181D, the *p*NP group of *p*NP-Glc formed a stacking interaction with W368, which pulled the sugar ring out of the sugar binding pocket (Figures 20C, 22 and 23). The docking results suggested that all four dalcochinase mutants would probably be less efficient in hydrolysis of *p*NP-Glc.

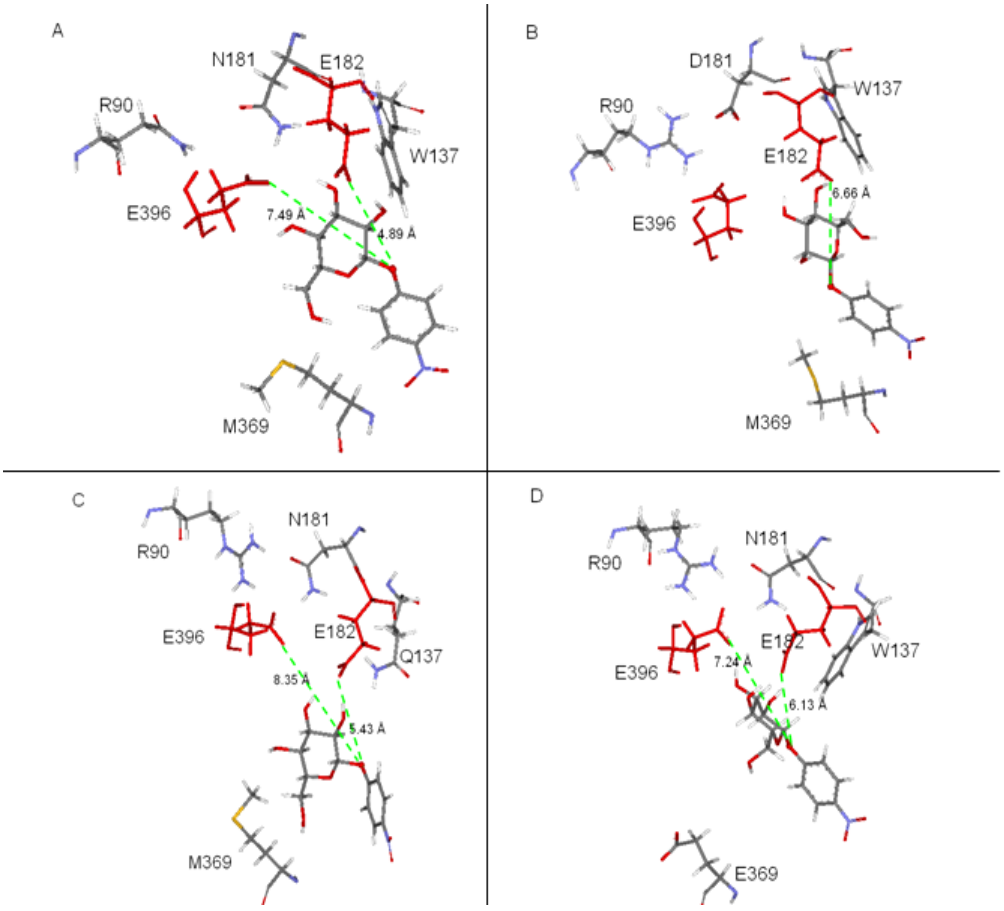


Figure 20 The binding modes of *p*NP-Glc in the binding pockets of mutant forms of dalcochinase, (A) R90Q, (B) N181D, (C) W137Q and (D) M369E. The catalytic residues, E182 and E396, are shown in red. The glycosidic oxygens of *p*NP-Glc are shown as ball-and-stick models, while the distances between the catalytic residues and the glycosidic oxygen are shown as the green dotted lines.

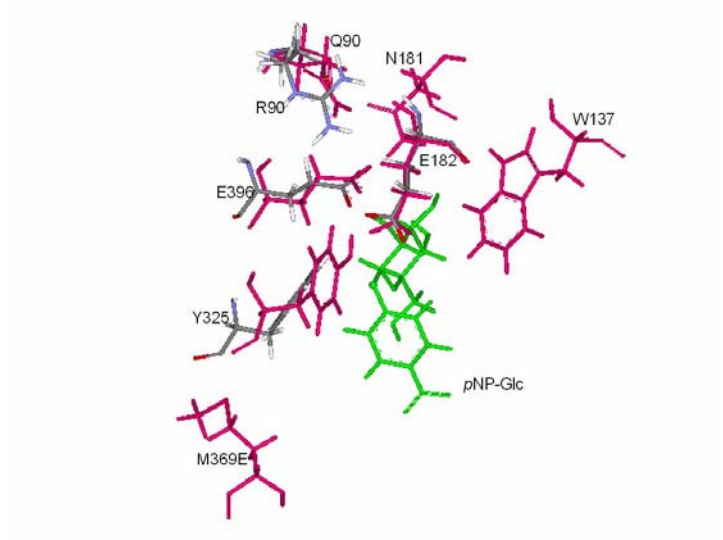


Figure 21 The change in the orientation of Y325 in dalcochinase mutant R90Q. The pink and grey stick-lines represent the orientation of amino acid residues in R90Q and wild-type dalcochinase, respectively. The molecule of *p*NP-Glc is shown in green, and its glycosidic oxygen as ball-and-stick models.

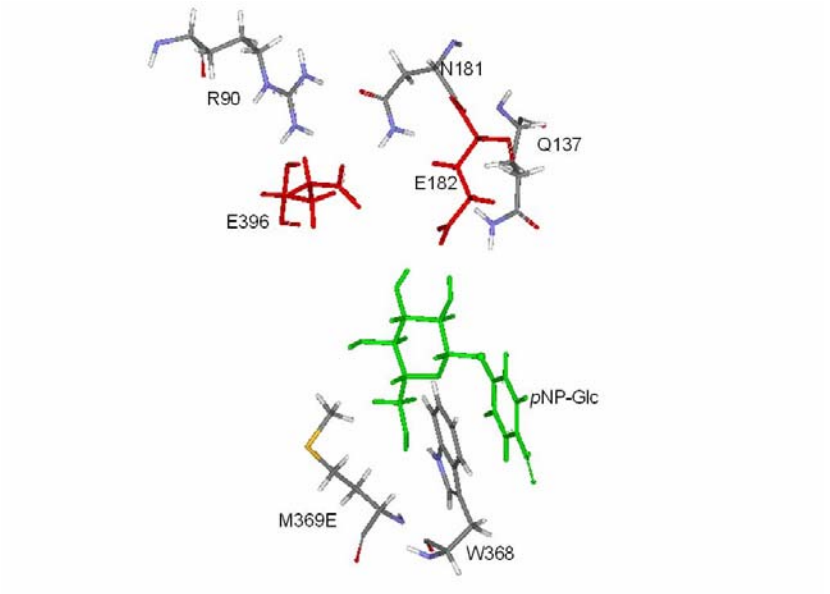


Figure 22 The stacking interaction between the *p*NP moiety of *p*NP-Glc substrate and amino acid residue W368 in the aglycone binding pocket of W137Q. The catalytic residues, E182 and E396, are showed in red. The molecule of *p*NP-Glc is shown in green, and its glycosidic oxygen as ball-and-stick models.

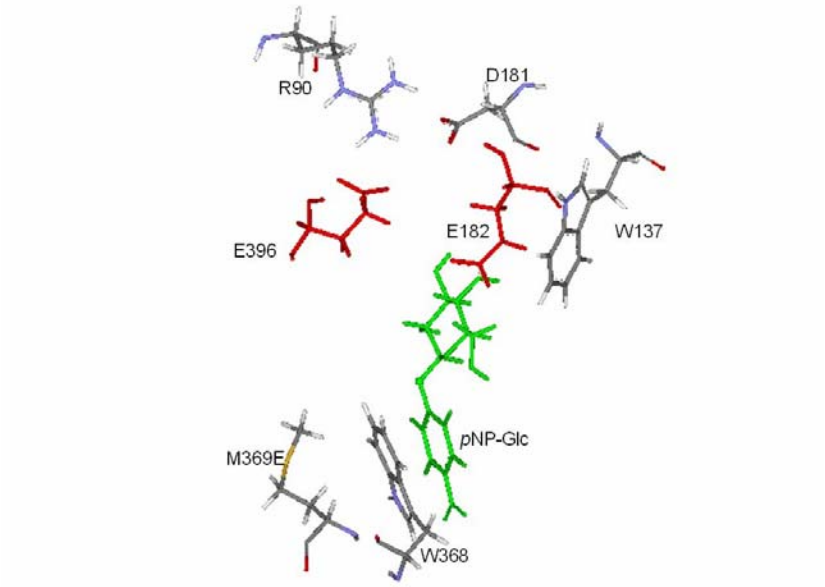


Figure 23 The stacking interaction between the *p*NP moiety of *p*NP-Glc substrate and amino acid residue W368 in the aglycone binding pocket of N181D. The catalytic residues, E182 and E396, are showed in red. The molecule of *p*NP-Glc is shown in green, and its glycosidic oxygen as ball-and-stick models.

Table 6 Distances between the substrate *p*NP-Glc and the amino acid residues in the sugar binding pocket of mutant dalcochinase models as predicted by molecular docking.

Sugar position	R90Q		W137Q		N181D		M369E	
	Site	Distance (Å)	Site	Distance (Å)	Site	Distance (Å)	Site	Distance (Å)
Glycosidic oxygen	E182 Oε1	4.89	E182 Oε2	5.43	E182 Oε1	6.66	E182 Oε2	6.13
	E396 Oε1	7.49	E396 Oε1	8.35			E396 Oε1	7.24
O-2	E182 Oε1	1.72	E182 Oε1	2.15	W445 Hε1	1.55	W453 Hε1	1.75
			E182 Oε2	2.29				
O-3	-	-	E182 Oε1	2.40	W453 Hε1	2.41	E396 Oε1	1.64
O-4	Y325 O	2.18	W445 Hε1	2.25	-	-	E182 Oε2	1.59
							Y325 H	1.37
O-6	W445 Hε1	2.40	W368 Hε1	2.37	-	-	-	-

To study the binding mode of *p*NP-Man substrate in mutant forms of dalcochinase, the molecule of *p*NP-Man was docked into the binding pocket of mutant enzymes, and the binding mode (Table 7) and the orientation of *p*NP-Man in binding pocket of mutant enzymes were predicted (Figure 24). The docking results showed that, in R90Q, W137Q and M369E mutant models, the glycosidic oxygens were located far from catalytic amino acid residues, except for mutant N181D. In mutants R90Q and W137Q, the mutant enzymes lacked the necessary hydrogen-bond interactions between the hydroxyl groups of sugar ring and the amino acid residues in the glycone binding pocket. Furthermore, the docking results also predicted that hydrogen at C-3 position on mannose ring might cause a steric clash with the side chain of amino acid residue W453 of mutant R90Q (Figure 25). In mutant W137Q, the *p*NP moiety of *p*NP-Man substrate formed a stacking interaction with amino acid residue W368, which might pull the sugar moiety further away from catalytic amino acid residues (Figure 24C). Thus, mutants R90Q and W137Q might not be able to hydrolyze *p*NP-Man. For mutant N181D, the glycosidic oxygen was placed close to the catalytic acid/base catalyst E182, but very far from the catalytic nucleophile, so this mutant might not be able to hydrolyze *p*NP-Man. Mutant M369E had more hydrogen-bond interactions than other mutant models, which might also contribute the mannose binding and mannose substrate hydrolysis.

Table 7 Distances between the substrate *p*NP-Man and the amino acid residues in the sugar binding pocket of mutant dalcochinase models as predicted by molecular docking.

Sugar position	R90Q		W137Q		N181D		M369E	
	Site	Distance (Å)	Site	Distance (Å)	Site	Distance (Å)	Site	Distance (Å)
Glycosidic	E182 Oε1	7.38	E182 Oε2	5.43	E182 Oε1	3.53	E182 Oε2	6.33
oxygen	E396 Oε1	7.68	E396 Oε1	8.44			E396 Oε1	7.50
O-2	-	-	-	-	-	-	E452 Oε1	1.84
O-3	-	-	E182 Oε1	2.31	W453 Hε1	1.70	E396 Oε1	1.87
O-4	E182 Oε1	1.79	-	-	-	-	E182 Oε2	1.70
O-6	-		W368 Hε1	2.11	W455 Hε1	2.44	-	-

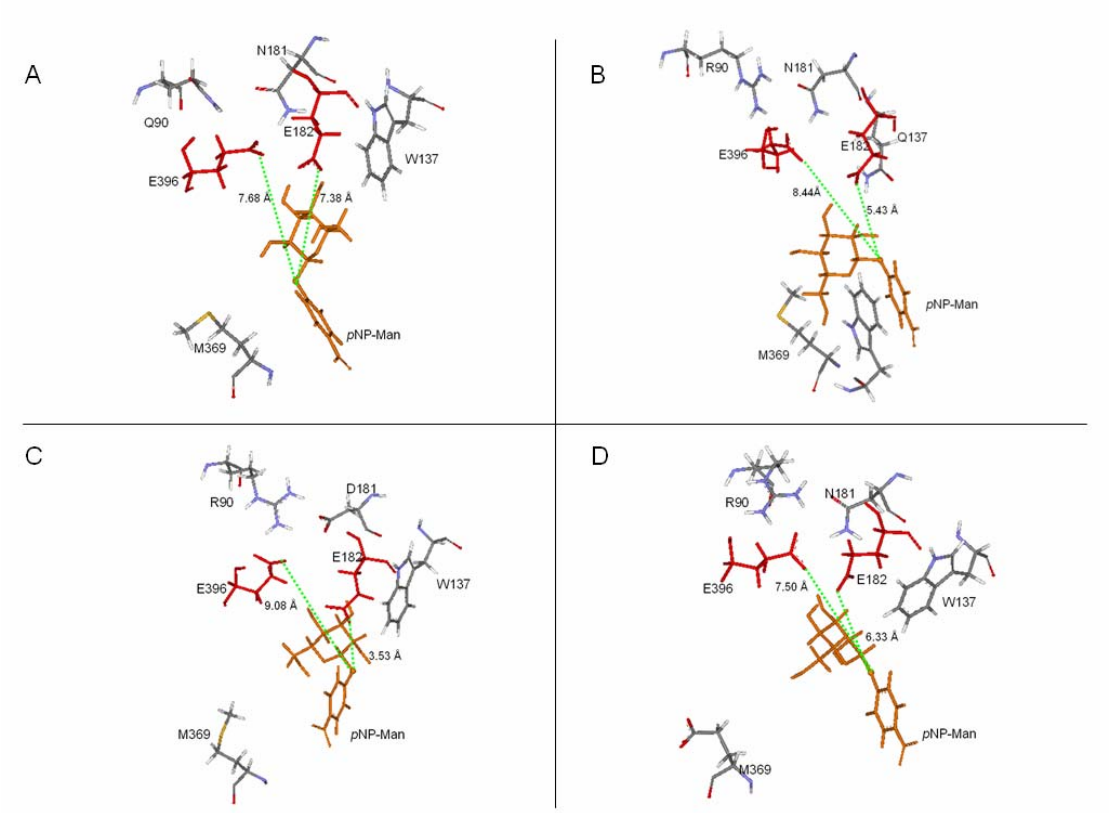


Figure 24 The binding modes of *p*NP-Man in the binding pockets of mutant forms of dalcochinase, (A) R90Q, (B) W137Q, (C) N181D and (D) M369E. The catalytic residues, E182 and E396, are showed in red. The glycosidic oxygens of *p*NP-Man are shown as ball-and-stick models, while the distances between the catalytic residues and the glycosidic oxygen are shown as the green dotted lines.

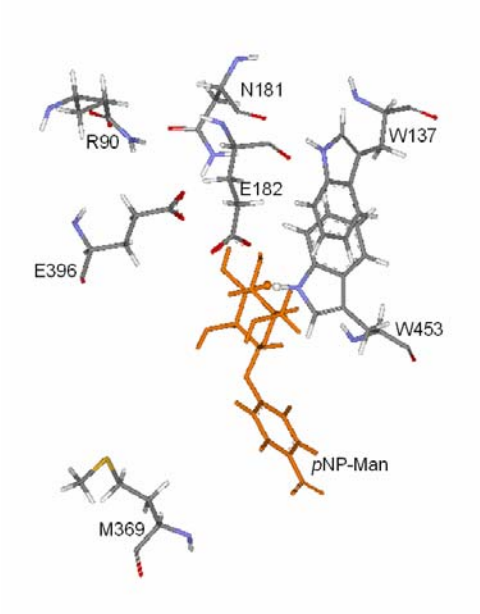


Figure 25 The steric clash between hydrogen at C-3 position of mannose and the side chain of W453 of mutant R90Q. The ball-and stick atoms represent the predicted clash site.

1.4 Molecular docking of *P. horikoshii* β-mannosidase

The orientation of *p*NP-Glc and *p*NP-Man in the binding pocket of *P. horikoshii* β-mannosidase were also studied. *P. horikoshii* β-mannosidase was first modeled, and the modeling showed that catalytic acid/base catalyst E207 and nucleophile E399 formed hydrogen bonding interactions with N264 and N324, respectively. The hydrogen-bonds formed in *P. horikoshii* β-mannosidase were different to dalcochinase, since these catalytic residues of dalcochinase formed hydrogen-bond with R90 and N181, corresponding to Q77 and D206 of *P. horikoshii* β-mannosidase, respectively. The difference of the orientation of catalytic amino acid residues between dalcochinase and *P. horikoshii* β-mannosidase might influence the different binding mode of these two enzymes. The binding mode between *P. horikoshii* β-mannosidase with *p*NP-Glc and *p*NP-Man were shown in Table 8 and Figure 26.

The docking results showed that the binding modes and the orientations of *p*NP-Glc was similar to that of *p*NP-Man, suggesting that *P. horikoshii* β-mannosidase could bind both *p*NP-Glc and *p*NP-Man substrates (Figure 26). From the previous study (Kaper *et al.,* 2002), *P. horikoshii* β-mannosidase showed hydrolytic activity toward both *p*NP-Glc and *p*NP-Man. *P. horikoshii* β-mannosidase bound *p*NP-Man tighter than *p*NP-Glc (K_m of 0.44 and 13.5 mM, respectively), this might be due to the additional hydrogen bonding interactions at C-4 position between mannose moiety and the amino acid residues in the binding pocket of enzyme (Figure 26).

Table 8 Distances between the sugar substrate and the amino acid residues in the sugar binding pocket of *P. horikoshii* β-mannosidase model as predicted by molecular docking.

	Sugar position	Residue	Distance (Å)
*p*NP-Glc	Glycosidic oxygen	E207 Oε2	5.33
		E399 Oε2	6.72
	O-2	E182 Oε1	2.07
	O-3	W445 Hε1	1.61
	O-6	E444 Oε2	3.55
*p*NP-Man	Glycosidic oxygen	E207 Oε1	4.70
		E399 Oε1	6.06
	O-2	E207 Oε2	3.80
	O-3	W445 Hε1	2.28
	O-4	W445 Hε1	3.41
	O-6	E452 Oε1	2.03

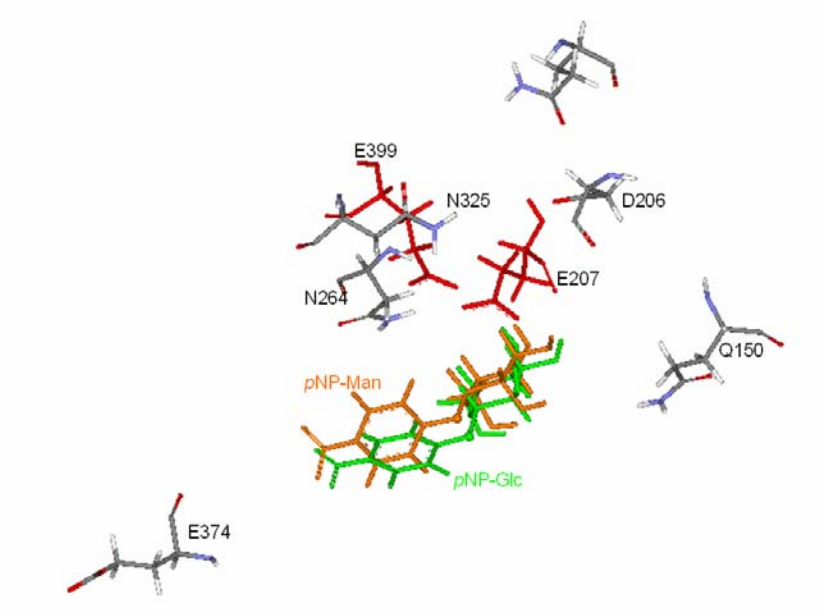


Figure 26 The orientation of *p*NP-Glc (green) and *p*NP-Man (orange) in the binding pocket of *P. horikoshii* β-mannosidase as predicted by molecular docking. The catalytic residues, E207 and E399, are showed in red. The green dotted lines represent the hydrogen-bonding interaction between catalytic residues and amino acid residues in the binding pocket. The glycosidic oxygens of *p*NP-Glc and *p*NP-Man are shown as ball-and-stick models.

2. Generation of mutant constructs of dalcochinase

2.1 Site-directed mutagenesis of dalcochinase

After the site for mutations have been selected using molecular modeling and docking approach, four pairs of site-directed mutagenic primers were designed and generated (Table 1). The PCR products were treated with *Dpn*I restriction digestion to remove the wild-type construct, and were checked with 1% (w/v) gel electrophoresis. The results showed a single band in each lane, indicating that the mutant forms of dalcochinase, which were R90Q, W137Q, N181D and M369E, in plasmid pPICZ-His_8-trncTRBG have been obtained (Figure 27).

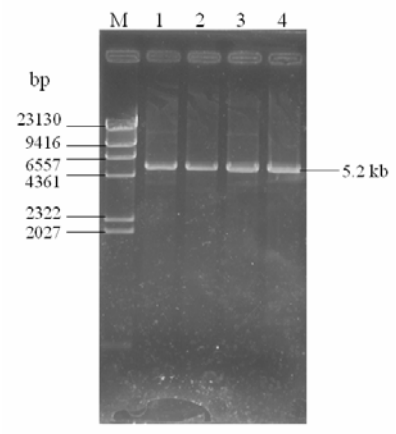


Figure 27 1% Gel electroporesis for detection of site-directed mutagenic products. Lane M, DNA marker; lane 1, R90Q; lane 2, W137Q; lane 3, N181D; and lane 4, M369E.

2.2 Cloning of mutant constructs of dalcochinase into *Escherichia coli* DH5α and *Pichia pastoris*

All *Dpn*I-treated PCR products were transformed into *E. coli* DH5α using electroporation method, giving many single colonies. Plasmids harboring expected mutant forms of dalcochinase were checked by restriction digestion with *Eco*RI and colony PCR method, and confirmed by DNA sequencing. The plasmids were extracted from *E. coli* clones, and digested by *Eco*RI to yield the fragments of about 3.8 kb and 1.4 kb. The results showed that all selected clones, excepted colonies 1 of dalcochinase mutant M369E, contained plasmids with dalcochinase sequence (Figure 28).

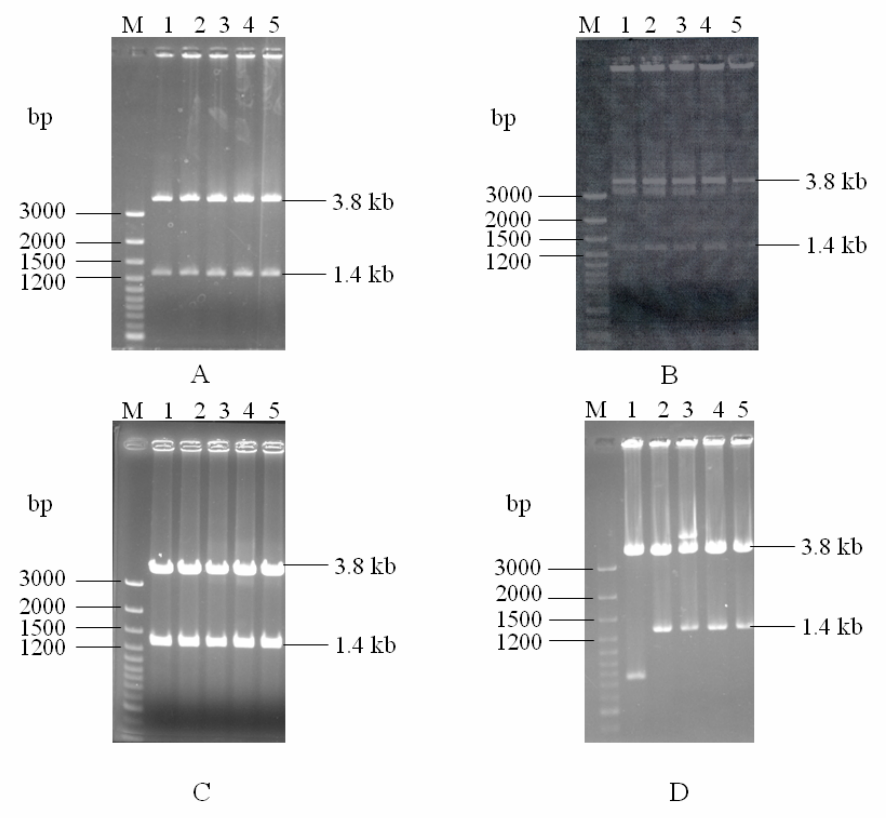


Figure 28 *Eco*RI-digestion of the recombinant plasmids containing mutant constructs of dalcochinase from the selected clones of *E. coli*. (A) R90Q, (B) N181D, (C) W137Q and (D) M369E. M indicates DNA standard marker, and the numbers above the figures indicate colony number.

These clones were also checked using colony PCR method in which the colony PCR products could only be obtained from the mutant constructs. It was found that all selected clones gave PCR products with different product sizes depending on the type of mutant constructs (Figure 29).

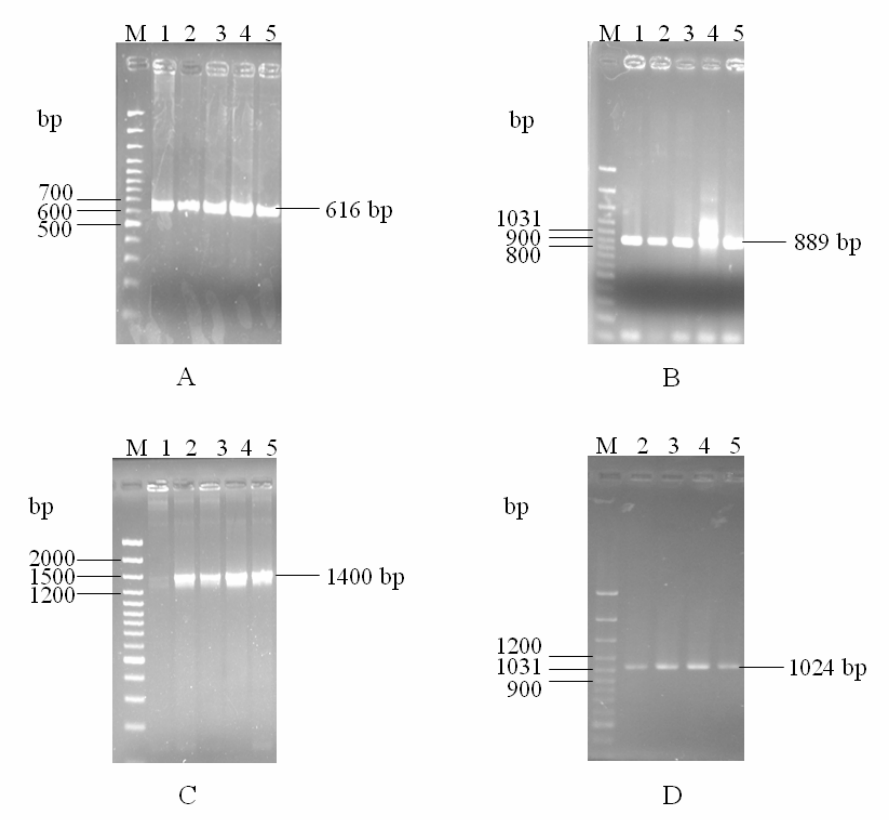


Figure 29 Products from colony PCR reactions of *E. coli* containing mutant constructs of dalcochinase. (A) R90Q, (B) N181D, (C) W137Q and (D) M369E. M indicates DNA standard marker, and the numbers above the figures indicate colony number.

From these results, colony number 3, 3, 3 and 5 of dalcochinase mutants R90Q, W137Q, N181D and M369E, respectively, were chosen for DNA sequencing to confirm the results. The DNA sequencing results showed that all selected clones were mutated at the desired sites. The DNA sequences of dalcochinase mutant R90Q were changed from AGA to CAA, resulting in the replacement of amino acid residues from R to Q (Figure 30). The DNA sequences were mutated from TGG to CAA, from AAT to GAT, and from ATG to GAA in dalcochinase mutants W137Q (Figure 31), N181D (Figure 32) and M369E (Figure 33), respectively. The entire coding sequences of all four dalcochinase mutants were also checked to confirm that there were no undesired mutations at other sites (shown in appendix C-F).

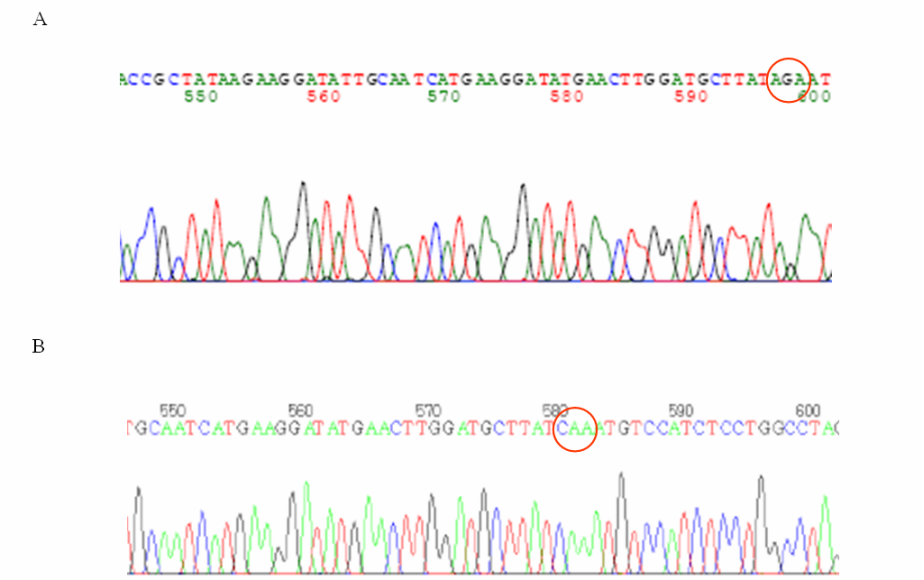


Figure 30 Comparison of partial DNA sequences of (A) wild-type and (B) mutant R90Q of dalcochinase. The red circles indicate the site that has been changed.

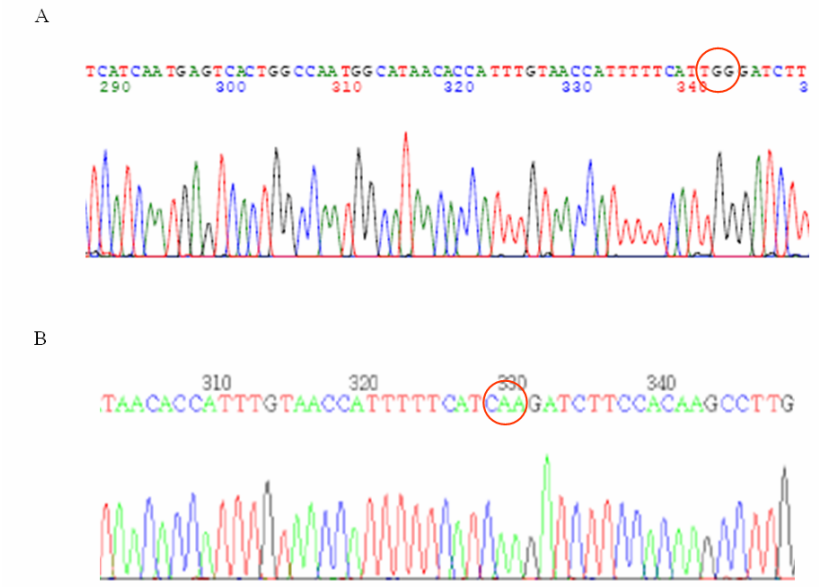


Figure 31 Comparison of partial DNA sequence of (A) wild-type and (B) mutant W137Q of dalcochinase. The red circles indicate the site that has been changed.

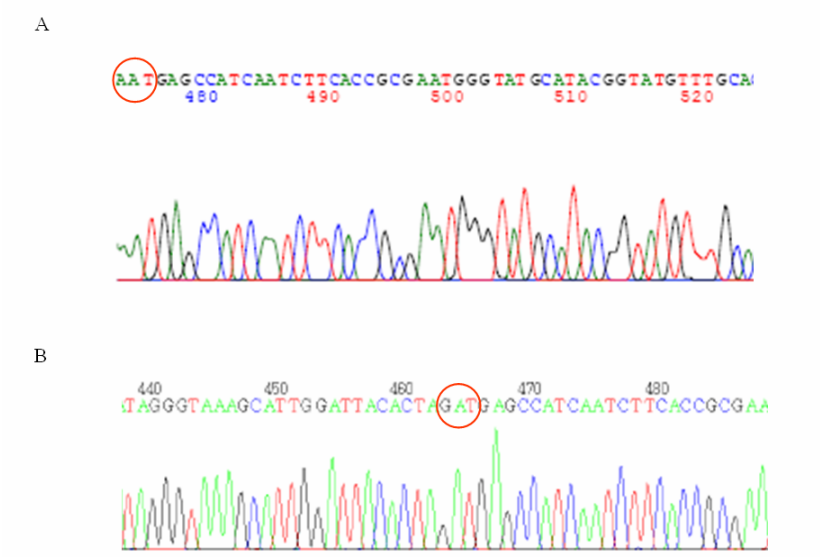


Figure 32 Comparison of partial DNA sequence of (A) wild-type and (B) mutant N181D of dalcochinase. The red circles indicate the site that has been changed.

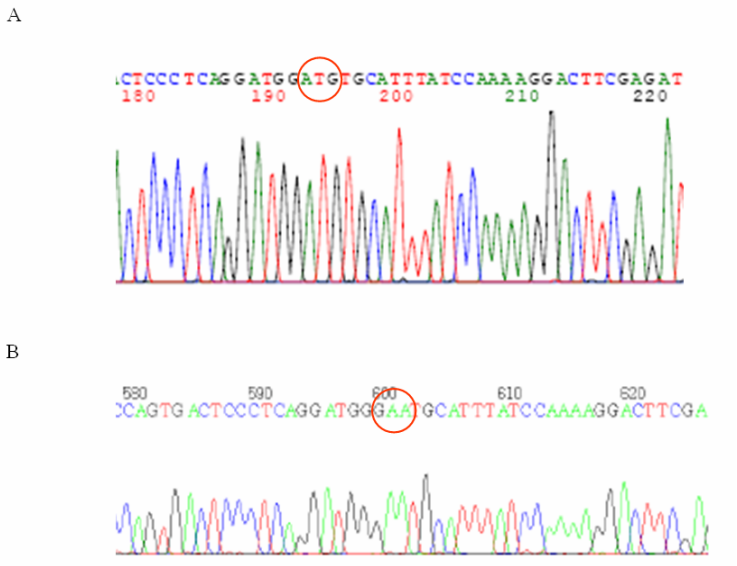


Figure 33 Comparison of partial DNA sequence of (A) wild-type and (B) mutant M369E of dalcochinase. The red circles indicate the site that has been changed.

The plasmids harboring mutant constructs of dalcochinase were extracted from the selected *E. coli* clones, and digested with *Eco*RI to confirm that the correct plasmid were obtained. *Sac*I restriction digestion was then performed to make the linear plasmids. All mutant constructs were transformed into *P. pastoris* strains GS115 and Y11430 for production of mutant enzymes in shake flask and fermenter systems, respectively. The transformed clones were grown on YDPS agar containing 100 μg/ml zeocin for clone selection.

3. Expression of mutant forms of dalcochinase

All transformed clones were screened for enzyme production. Cells were grown in BMGH and BMMH media, and methanol was added every 24 hours for induction of enzyme expression. At day 7, the culture media was analyzed for enzyme activity using *p*NP-Glc as substrate. Therefore, the yeast colony that gave highest enzyme activity in each mutant was selected for large-scale enzyme expression. The results of activity screening were shown in Tables 9, 10, 11 and 12 for dalcochinase mutants R90Q, W137Q, N181D and M369E, respectively.

Table 9 Activity screening of dalcochinase mutant R90Q.

P. pastoris GS115		*P. pastoris* Y11430	
Colony number	ABS400	Colony number	ABS400
1	0.086	1	0.040
2	0.104	2	0.050
3	0.129	3	0.019
4	0.098	4	0.022
5	0.115	5	0.030
6	0.157	6	0.021
7	0.093	7	0.024
12	0.202	26	0.090
23	0.173	27	0.256
24	0.203	28	0.257

Table 10 Activity screening of dalcochinase mutant W137Q.

P. pastoris GS115		*P. pastoris* Y11430	
Colony number	ABS400	Colony number	ABS400
1	0.130	1	0.138
2	0.163	2	0.128
3	0.198	11	0.039
4	0.152	12	0.039
5	0.124	21	0.077
6	0.231	22	0.090
7	0.197	31	0.069
8	0.203	41	0.142
9	0.027	50	0.406
		54	0.403

Table 11 Activity screening of dalcochinase mutant N181D.

P. pastoris GS115		*P. pastoris* Y11430	
Colony number	ABS400	Colony number	ABS400
1	0.120	1	0.117
2	0.169	2	0.154
3	0.123	3	0.089
4	0.134	11	0.249
5	0.175	13	0.231
14	0.096	16	0.231
15	0.134	17	0.243
16	0.186	18	0.289
17	0.096	19	0.278
18	0.198	20	0.189

Table 12 Activity screening of dalcochinase mutant M369E.

P. pastoris GS115		*P. pastoris* Y11430	
Colony number	ABS400	Colony number	ABS400
3	0.572	1	0.429
6	0.650	5	0.498
7	0.547	11	0.019
10	0.833	16	0.567
11	0.604	21	0.567
15	0.521	24	0.593
16	0.598	33	0.213
23	0.493	35	0.151
29	0.547	45	0.340
32	0.530	50	0.269

Colony number 24, 6, 18 and 10, and colony number 28, 50, 18 and 24 of dalcochinase mutants R90Q, W137Q, N181D and M369E in yeast *P. pastoris* GS115 and Y11430 were selected for large-scale expression, respectively.

4. Large-scale expression and purification of mutant forms of dalcochinase

The selected yeast colonies containing mutant constructs of dalcochinase were grown in 150 ml of BMGH and BMMH media, and methanol was added every 24 hours for enzyme induction. The mutant enzymes were secreted into the culture media because of the α-factor secretion signal at the N-terminus of the mutant constructs, so the culture media were centrifuged and kept for purification. Due to the very low enzyme activities of dalcochinase mutants R90Q and N181D, these two mutant enzymes were produced in the fermenter, and culture media were also kept.

The volume of crude enzymes were reduced by ultrafiltration through the 30 kDa molecular weight cut-off regenerated cellulose membrane, and applied to the phenyl-sepharose chromatography and immobilized Ni^{2+} affinity chromatography, respectively. Amicon ultrafiltration was performed to remove imidazole and exchange the buffer from sodium phosphate buffer pH 8.0 to acetate buffer pH 5.0, which was

suitable for keeping enzyme activity. At each step of purification, enzyme activity and quantity of protein was measured, as shown in Tables 13, 14, 15 and 16 for dalcochinase mutants R90Q, W137Q, N181D and M369E, respectively.

Table 13 Purification of dalcochinase mutant R90Q per 1 liter of culture medium

Steps	Total activity (unit)	Total protein (mg)	Specific activity (unit/mg)	Purification fold	Yield (%)
Culture medium	1.80	23.75	0.076	1.00	100.00
Ultrafiltration	1.75	11.02	0.159	2.09	97.22
Phenyl-sepharose	1.20	3.13	0.383	5.04	66.67
Buffer exchange	0.07	2.07	0.034	0.45	3.89
Ni^{2+} chromatography	0.05	1.43	0.035	0.46	2.78
Amicon ultrafiltration	0.02	0.20	0.100	1.32	1.11

Table 14 Purification of dalcochinase mutant W137Q per 1 liter of culture medium.

Steps	Total activity (unit)	Total protein (mg)	Specific activity (unit/mg)	Purification fold	Yield (%)
Culture medium	6.456	448.7	0.014	1.00	100.00
Ultrafiltration	0.767	64.81	0.012	0.82	11.88
Phenyl-sepharose	0.412	12.82	0.032	2.23	6.38
Buffer exchange	0.029	7.69	0.004	0.26	0.45
Ni^{2+} chromatography	0.026	2.85	0.009	0.93	0.40
Amicon ultrafiltration	0.022	0.54	0.041	2.83	0.35

Table 15 Purification of dalcochinase mutant N181D per 1 liter of culture medium.

Steps	Total activity (unit)	Total protein (mg)	Specific activity (unit/mg)	Purification fold	Yield (%)
Culture medium	2.12	29.75	0.07	1.00	100.00
Ultrafiltration	1.93	17.69	0.11	1.57	91.04
Buffer exchange	0.33	3.10	0.11	1.57	15.57
Ni^{2+} chromatography	0.12	1.71	0.07	1.00	5.66
Amicon ultrafiltration	0.08	0.66	0.12	1.71	3.77

Table 16 Purification of dalcochinase mutant M369E per 1 liter of culture medium.

Steps	Total activity (unit)	Total protein (mg)	Specific activity (unit/mg)	Purification fold	Yield (%)
Culture medium	5.79	24.71	0.24	1.00	100.00
Ultrafiltration	6.72	17.41	0.39	1.64	115.94
Buffer exchange	3.93	5.18	0.76	3.17	67.88
Ni^{2+} chromatography	1.51	1.48	1.02	4.34	26.08
Amicon ultrafiltration	0.66	0.54	1.22	5.19	11.40

The results showed that dalcochinase mutant R90Q was purified 1.32-fold purification with 0.10 unit/mg specific activity and 1.11% activity recovery. In dalcochinase mutant W137Q, enzyme was purified 2.83-fold with 0.04 unit/mg specific activity and 0.35% activity recovery. Dalcochinase mutants N181D and M369E were purified 1.71 and 5.19-fold with specific activities of 0.12 and 1.22 unit/mg protein, and 3.77 and 11.40% activity recovery, respectively. Note that, in the purification of dalcochinase mutants N181D and M369E, phenyl-sepharose chromatography was not performed in order to reduce purification steps and improve protein yield.

The total activities of all dalcochinase mutants were greatly reduced in the buffer exchange step: from 1.20 to 0.07 unit for R90Q (94%), from 0.41 to 0.03 unit for W137Q (93%), from 1.93 to 0.33 unit for N181D (83%), and 6.72 to 3.93 unit for M369E (42%). These activity losses are accompanied by the loss of proteins: from 3.13 to 2.07 mg for R90Q (34%), from 12.82 to 7.69 mg for W137Q (40%), from

17.69 to 3.10 mg for N181D (83%), and 17.41 to 5.18 mg for M369E (70%). This step was performed to exchange the buffer from 10 mM potassium phosphate pH 7.0 to 50 mM sodium phosphate buffer pH 8.0, using 30 kDa molecular weight cut-off amicon ultrafiltration (Millipore, USA), and it was an essential step prior to Ni^{2+} chromatography, which required the protein to be in pH 8.0 to ensure the deprotonated state of side chain histidines in the polyhistidine tag for binding to immobilized Ni^{2+}-ion resin. For all mutants, the loss of protein quantity, which was possibly caused by absorption to amicon ultrafiltration membrane or membrane leakage, led to the reduction in total activities. Moreover, the temperature in the buffer exchange step may lead to the denaturation of enzyme which resulted in the loss of total activity. For mutants R90Q and W137Q, the percentage of activity loss are greater than the percents of protein loss, suggesting that these two mutants had low stability in high pH condition. For mutant M369E, the percentage loss of enzyme activity was smaller than the percentage of protein loss, implying that M369E was less sensitive to high pH or not involve in protonation/deprotonation state of catalytic residues. The sensitivity to high pH condition has not been observed with wild-type dalcochinase or other constructs of dalcochinase with mutations in other regions. So, this pH sensitivity suggested that the mutations may affected the protonation state of the catalytic residues, and can be confirmed by testing the effect of pH on enzymatic activities.

Another step of activity loss occurred during the amicon ultrafiltration step of mutants R90Q, N181D and M369E: from 0.05 to 0.02 unit for R90Q (60%), from 0.026 to 0.022 unit for W137Q (15%), from 0.12 to 0.08 unit for N181D (33%), and 1.51 to 0.66 unit for M369E (56%). These activity losses are again accompanied by the loss of proteins: from 1.43 to 0.20 mg for R90Q (86%), from 2.85 to 0.54 mg for W137Q (81%), from 1.71 to 0.66 mg for N181D (61%), and 1.48 to 0.54 mg for M369E (64%). This step was performed to remove imidazole from the Ni^{2+} chromatography and to reduce the volume of the protein samples using 30 kDa molecular weight cut-off amicon ultrafiltration (Millipore, USA). The loss of activities was most likely due to the loss of protein, possibly by protein absorption to

amicon ultrafiltration membrane or membrane leakage during several rounds of centrifugation.

Furthermore for mutant W137Q, the total activity was also lost in the ultrafiltration step (from 6.456 to 0.767 unit, or 88%), possibly because the culture medium from the fermentation process was kept in the 4 °C refrigerator for too long. Moreover, the sticky crude enzyme was hard to purify through the phenyl-sepharose chromatography. These also cause the reduction of the total activity, specific activity and the purification fold of mutant W137Q.

From these results, it seem likely that the activities of dalcochinase mutants R90Q, W137Q and N181D was sensitive to pH alteration, as seen in the buffer exchange and Ni^{2+} chromatography steps. Thus, for improving the activity recovery, the purification step which may involve pH alteration should be cautioned. An alternative purification method, such as gel filtration or intein mediated purification (Chong *et al.*, 1998), may be considered.

The purity of mutant enzymes was checked using SDS-PAGE. Western blot was performed with mouse monoclonal antibody against natural dalcochinase as a primary antibody and rabbit anti-mouse immunoglobulins/HRP as a secondary antibody to confirm the identity of the purified enzymes. All mutant enzymes showed a molecular weight of 66 kD which was the same as the molecular weight of the recombinant wild-type enzyme (Figure 34).

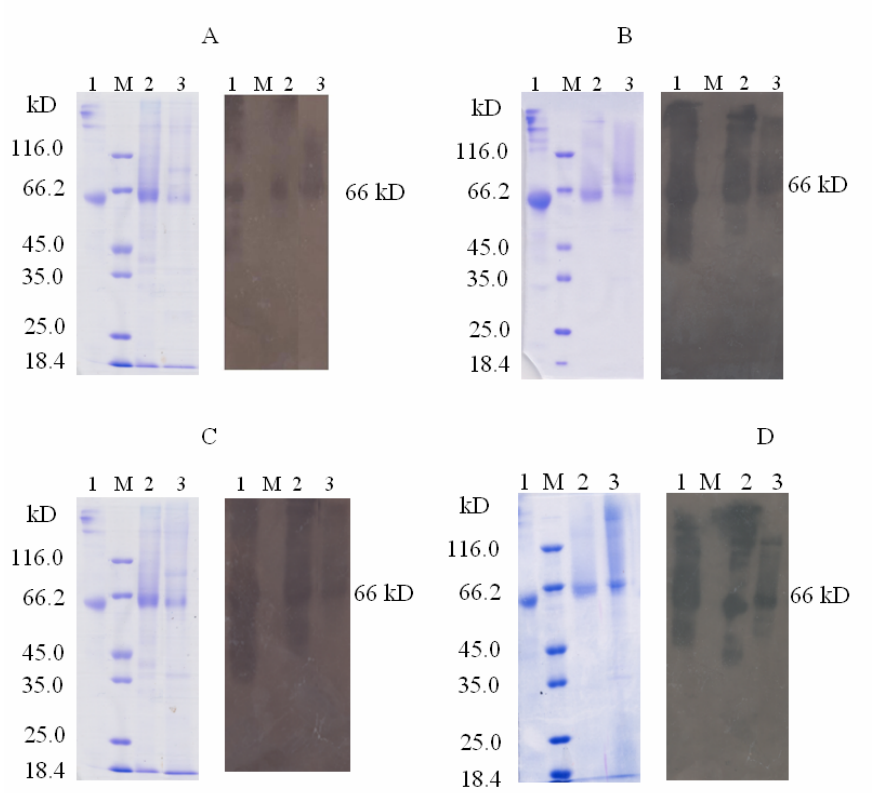


Figure 34 The quality of purified mutant forms of dalcochinase. (A) R90Q, (B) W137Q, (C) N181D, (D) M369E. The left panel shows SDS-PAGE stained with Coomassie brilliant blue and the right panel shows western blot. Lane M, protein marker; lane 1, natural dalcochinase; lane 2, recombinant wild-type dalcochinase; and lane 3, mutant dalcochinase.

However, the recombinant wild-type dalcochinase and mutant enzymes (which were produced in yeast) had a higher molecular weight than the natural enzyme (which was produced in plant). The higher molecular weight of recombinant and mutant enzymes were due to the difference in the post-translational modifications between plant and yeast. It was found that yeast could perform both O- and N-linked glycosylation, resulting in the production of a hyperglycosylated protein (up to 150 mannose residues in glycan structure) and shift in apparent molecular weight in SDS-PAGE (Cregg *et al.*, 2000). Ketudat-Cairns *et al.*, (2000) reported that dalcochinase is N-glycosylated protein with eight potential sites for N-glycosylation (N-X-S/T). Dubois's phenol-sulfuric acid assay was also performed for a direct total sugar

estimation, showing that natural and recombinant dalcochinase contained approximately 13.6 and 16.1% by weight, respectively (Toonkool *et al.,* 2006).

5. Enzyme assay

5.1 Hydrolysis of *p*NP-Glc

The kinetic parameters (K_m, V_{max}, k_{cat}) of mutant forms of dalcochinase toward *p*NP-Glc were studied, and compared with those of wild-type natural and recombinant dalcochinase. The purified enzymes were incubated with various *p*NP-Glc concentrations at 30 °C for 5 minutes in 0.1 M sodium acetate buffer pH 5.0. The kinetic parameters of enzymes and the standard error values (S.E.) toward *p*NP-Glc were shown in Table 17. The Michaelis-Menten plots of these enzymes were shown in Appendix G.

Table 17 The kinetic parameters of wild-type and mutant forms of dalcochinase for hydrolysis of *p*NP-Glc.

Enzyme	K_m±S.E. (mM)	V_{max}±S.E. (mU)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)
Natural dalcochinase	12.13±0.62	89.37±1.68	240.27	19.81
Recombinant dalcochinase	9.88±1.18	92.16±4.07	168.96	17.10
R90Q	17.04±2.34	1.13±0.07	0.91	0.05
W137Q	24.05±2.50	1.67±0.08	0.21	0.01
N181D	2.76±0.23	2.55±0.05	0.38	0.14
M369E	11.95±0.70	41.61±0.91	8.94	0.91

In this study, 0.05 unit of wild-type enzymes were incubated with various concentrations of *p*NP-Glc. The K_m values of natural and recombinant dalcochinase were different from those reported previously (5.37 mM and 5.78 mM, respectively) (Srisomsap *et al.,* 1996 and Toonkool *et al*., 2006). The higher K_m values in this study might be due to less skill and accuracy of the experimenter. In the case of recombinant dalcochinase, the error in the K_m value is approximately 12%, which is too high to be accurate (the error should be less than 10%, or preferably less than 5%).

The difference in the quantity of protein used in the reaction, which were 0.4 µg in the natural dalcochinase and 0.6 µg in the recombinant dalcochinase, resulted in the different k_{cat} values. However, both wild-type natural and recombinant enzymes showed similar k_{cat}/K_m values, which indicate similar hydrolytic efficiencies toward *p*NP-Glc substrate.

Due to the very low activity and limited amount of mutant enzymes, only 8 µl of each mutant enzyme was used in the kinetic studies. The K_m values of mutants R90Q and W137Q were 1.7- and 2.4-fold grater than the wild-type recombinant enzyme, respectively. Mutant N181D showed a 3.6-fold increased affinity for *p*NP-Glc. Mutant M369E showed the similar K_m value to wild-type enzyme. Again, the K_m values of R90Q and W137Q contained greater than 10% errors, which made them inaccurate and must be improved. While the K_m values of these dalcochinase mutants were not the same as those found for the wild-type enzymes, these differences were less than an order of magnitude (less than 10-fold difference). So, it can be said that the affinities of dalcochinase mutants for *p*NP-Glc substrate were not significantly affected by these mutations.

The low values of V_{max}, k_{cat} and k_{cat}/K_m of all dalcochinase mutants are in agreement with the prediction from the docking experiments (Table 6). While the exact interactions between the amino acid residues of the enzyme and the molecule of the substrate are not known, the docking results suggested that dalcochinase mutants may not be able to hydrolyze *p*NP-Glc substrate due to the lack of many important hydrogen bonds between the sugar binding residues and glucose moiety as well as the extended distance between the catalytic residues and the glycosidic oxygen. In the case of mutants R90Q, W137Q and N181D, the turnover rates were reduced by 186-, 804- and 445-fold compared with the wild-type recombinant enzyme, respectively, indicating significant effects on the catalytic activities of the mutant enzymes. Thus, these results indicated the important roles of residues R90, W137 and N181 of dalcochinase in catalysis. On the other hand, the turnover rate of mutant M369E was reduced by 19-fold compared with the wild-type recombinant enzyme (while their K_m values were similar). So, the role of residue M369 of dalcochinase may be less

important in catalysis than the other three residues, which is possibly due to the further distance form the two catalytic glutamates (Figure 20D).

From these results, it might be suggested that amino acid residues R90, W137 and N181 of dalcochinase were important for catalytic activity toward glucoside substrates. These mutant enzymes might also show low catalytic activity toward natural substrate, dalcochinin glucoside, which is a glucose derivative. Marana *et al*., (2003) reported that amino acid residue R97 of *Spodoptera frugiperda* β-glycosidase, which was corresponding to R90 of dalcochinase, was important for modulating the ionization state of catalytic nucleophile, by stabilizing its charge and enabling nucleophilic function. The mutation of this residue resulted in the shift in pH optimum of mutant enzyme. Since amino acid residue R97 of *S. frugiperda* β-glycosidase (or R90 of dalcochinase) was strictly conserved in all family 1 β-glycosidases from different organisms, this residue may have the same essential function in all those β-glycosidases, especially modulating pH optimum in this case. Therefore, the pH optima of wild-type dalcochinase and mutant R90Q might not be the same. Moreover, Davies *et al*., (1998) suggested that this R residue was probably crucial for correct orientation of nucleophile for the glycosylation step. Amino acid residue N214 of *Cellvibrio mixtus* mannosidase 5A (corresponding to N181 of dalcochinase) was also highly conserved in family 1 β-glycosidases, suggesting its important role for catalysis. This N residue makes a hydrogen bond with the oxygen atom of C2-OH of sugar ring at glycone binding subsite (Dias *et al*., 2004). Therefore, in agreement with previous report, the result from this study indicated that amino acid residues R90 and N181 of dalcochinase might play important roles in the orientation and ionization state of catalytic residue, as these mutant enzymes showed 200-400-fold lower turnover rates.

The roles of amino acid residues W137 and M369 in hydrolytic activity have not been previously reported in literature. The replacement of indole side-chain of W with short linear side-chain of Q might result in the rearrangement of amino acid residues in the binding pocket of mutant enzyme and affect the orientation of catalytic residues. The role of W residue on the ionization state, transition state stabilization

and stability will be further studied using various glucose-derived structures. Modeling result predicted that amino acid residue M369 of dalcochinase was placed far from catalytic amino acid residues, and it seemed likely that M369 was located in the aglycone binding pocket. Therefore, residue M369 showed less effect on binding and hydrolytic activity toward glucoside substrate compared with other residues.

5.2 Hydrolysis of *p*NP-Man

The activities of recombinant and mutant forms of dalcochinase for the hydrolysis of *p*NP-Man were shown in Table 18. Due to the very low activity of these enzymes toward *p*NP-Man and the limited stock of purified enzymes, the full kinetic analysis could not be performed. Therefore, in this study, 3 µg of each purified enzyme was incubated with 15 mM *p*NP-Man, and the specific activities were obtained.

Table 18 The specific activities of wild-type and mutant forms of dalcochinase for hydrolysis of *p*NP-Man.

Enzyme	Specific activity (mU/µg protein)	Relative specific activity (%)
Natural dalcochinase	0.086	100.00
Recombinant dalcochinase	0.122	142.03
R90Q	0.014	15.94
W137Q	0.015	17.39
N181D	0.016	18.84
M369E	0.035	40.58

Recombinant dalcochinase show higher relative specific activity toward *p*NP-Man substrate, which has been observed previously by another group but their difference was much greater than this study (23-fold) (Chuankhayan *et al*., 2007). All dalcochinase mutants, R90Q, W137Q, N181D and M369E, showed lower specific activities than that of natural and recombinant dalcochinase. It seemed likely that the rearrangement of amino acid residues in the binding pocket of these mutant enzymes were not suitable for accommodation and hydrolysis of the mannoside substrate.

These results are in agreement with the docking results, which predicted that mutants R90Q, W137Q and N181D might not be able to hydrolyze *p*NP-Man, while mutant M369E might show better hydrolytic activity than other mutants due to greater hydrogen-bond interactions between the residues and the mannose substrate.

6. Transglycosylation

Purified enzymes (5 μg) were incubated with 0.9 M of short-chain primary alcohols, which were methanol, ethanol, *n*-propanol and *n*-butanol, and 10 mM *p*NP-Glc or *p*NP-Man substrates. The reactions were performed at 30 °C for 20 h, and analyzed using Thin-layer chromatography. Standards were made up of 40, 80, 120 and 160 nmol of sugar substrates, methyl-glucoside and glucose. The results were shown in Figures 35-36 and Table 19.

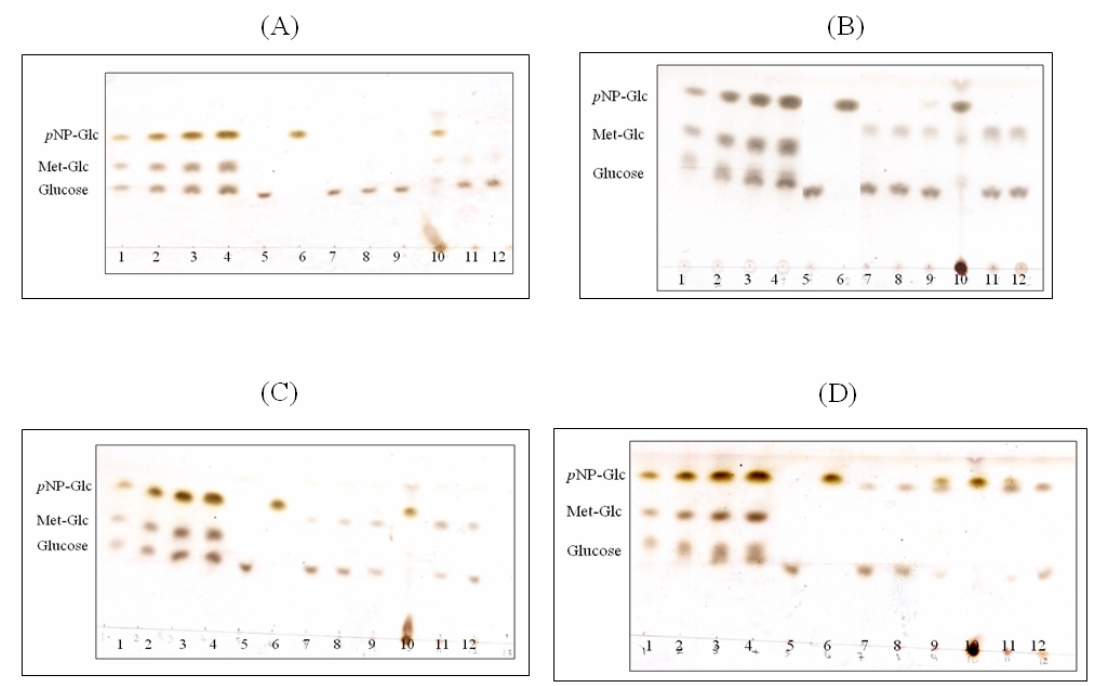


Figure 35 The TLC analysis for the transglucosylation of wild-type and mutant forms of dalcochinase to various primary alcohols. (A) Methanol, (B) Ethanol, (C) *n*-Propanol and (D) *n*-Butanol. Number 1, 40 nmol of standards; 2, 80 nmol of standards; 3, 120 nmol of standards; 4, 160 nmol of standards; 5, no alcohol in the reaction; 6, no enzyme in the reaction; 7, natural dalcochinase; 8, recombinant dalcochinase; 9, R90Q; 10, W137Q; 11, N181D and 12, M369E.

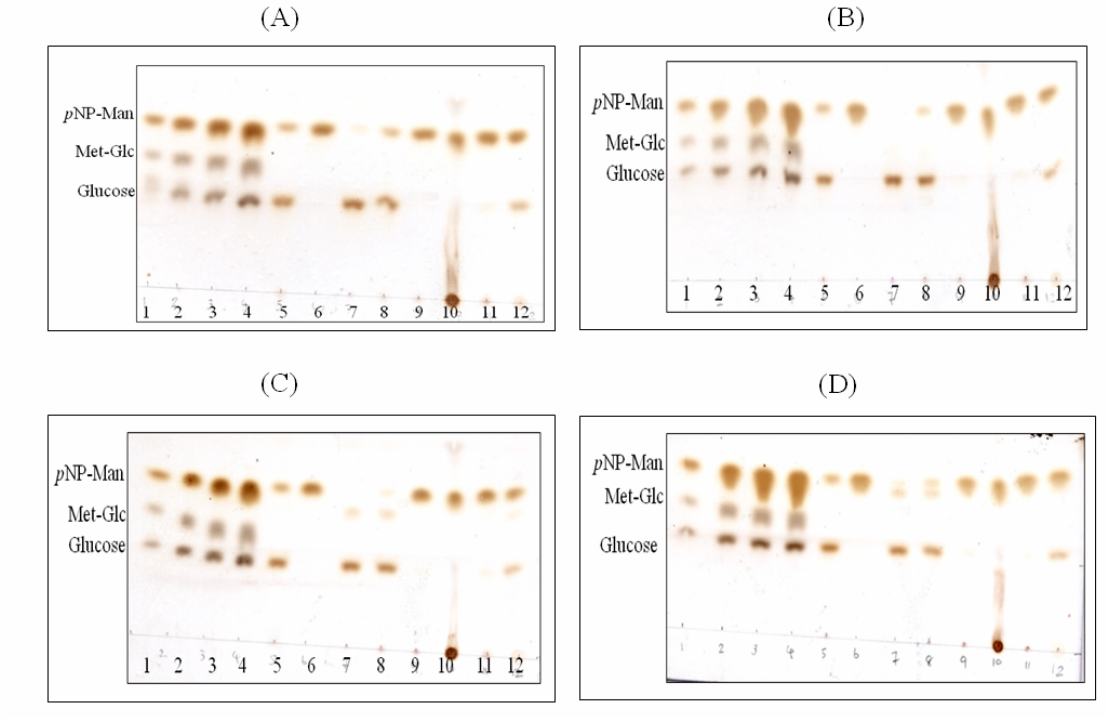


Figure 36 The TLC analysis for the transmannosylation of wild-type and mutant forms of dalcochinase to various primary alcohols. (A) Methanol, (B) Ethanol, (C) *n*-Propanol and (D) *n*-Butanol. Number 1, 40 nmol of standards; 2, 80 nmol of standards; 3, 120 nmol of standards; 4, 160 nmol of standards; 5, no alcohol in the reaction; 6, no enzyme in the reaction; 7, natural dalcochinase; 8, recombinant dalcochinase; 9, R90Q; 10, W137Q; 11, N181D and 12, M369E.

Table 19 Appearance of alkyl-glycosides in transglycosylation reaction.

Glycosyl donor	Glycosyl acceptor	Natural dalcochinase	Recombinant dalcochinase	R90Q	W137Q	N181D	M369E
*p*NP-Glc	Methanol	-a	-a	-a	+c	+a	+a
	Ethanol	++a	++a	++ac	-c	+++a	+++a
	n-Propanol	+a	+a	+a	-c	++a	+a
	n-Butanol	+a	+a	+ac	-c	++ac	++a
*p*NP-Man	Methanol	-bc	-bc	-c	-c	-c	-bc
	Ethanol	-b	-bc	-c	-c	-c	-bc
	n-Propanol	+b	+bc	-c	-c	-c	-bc
	n-Butanol	+b	+bc	-c	-c	-c	-bc

Note: - = no alkyl-glycoside
+ = small amount of alkyl-glycoside
++ = moderate amount of alkyl-glycoside
+++ = high amount of alkyl-glycoside
a = glucose remaining in the reaction
b = mannose remaining in the reaction
c = *p*NP-glycoside remaining in the reaction

In the transglucosylation reaction to methanol acceptor, the activities of wild-type and mutant forms of dalcochinase were different. Only glucose was obtained as a product from the reactions catalyzed by wild-type natural and recombinant dalcochinase and mutant R90Q, while small amounts of methyl-glucoside were obtained from those catalyzed by mutants W137Q, N181D and M369E. For wild-type natural and recombinant dalcochinase, which are capable of transferring glucose to methanol, the methyl-glucoside product might be formed and subsequently cleaved by the enzyme since the wild-type enzymes showed high hydrolytic activity toward glucoside substrates. For mutant R90Q, the *p*NP-Glc substrate was completely cleaved, but whether the methyl-glucoside product was also formed was not known. Reaction catalyzed by mutant W137Q showed *p*NP-Glc remaining in the reaction, possibly due to the very low specific activity of this enzyme (Table 17). The appearance of small amounts of methyl-glucoside product in reactions catalyzed by

mutants W137Q, N181D and M369E, suggested that methyl-glucoside was formed but was not efficiently hydrolyzed by mutant enzymes due to their low hydrolytic activities.

In the transglucosylation reactions to ethanol, *n*-proponal and *n*-butanol acceptors, small amounts of alkyl-glucoside were obtained from those catalyzed by wild-type natural and recombinant dalcochinase and mutant R90Q, while higher yields of alkyl-glucoside were obtained from mutants N181D and M369E. The greater transglucosylation activities of mutants N181D and M369E may be due to their lower hydrolytic activities compared with the wild-type enzyme. Reaction catalyzed by mutant W137Q showed no alkyl-glucoside or glucose, with only *p*NP-Glc remaining in the reaction, suggesting that the hydrolytic activity of this mutant is too low to cleave the *p*NP-Glc substrate.

In the transmannosylation reactions by wild-type natural and recombinant dalcochinase, small amounts of alkyl-mannoside product were obtained in the transmannosylation to *n*-propanal and *n*-butanol aceeptors, but not to methanol and ethanol acceptors. It is surprising that the wild-type enzymes can hydrolyze *p*NP-Man to yield mannose since the activity of natural enzyme for *p*NP-Man hydrolysis was reported to be only 0.26% relative the hydrolysis of *p*NP-Glc (Srisomsap *et al*., 1996). Hence, this is the first report for the alkyl-mannoside synthesis using β-glucosidase enzyme. In the transmannosylation reactions by all dalcochinase mutants, no alkyl-mannoside product could be obtained with any tested primary alcohol acceptors, while some *p*NP-Man substrate remained in the reactions as a result of very low hydrolytic activities of these mutants. Mutant M369E also hydrolyzed *p*NP-Man to yield mannose, in agreement with the 40% relative specific activity compared to natural dalcochinase. However, mutant M369E could not transfer mannose to the alcohol acceptors. As the position 369 is close to the aglycone binding site, the replacement of nonpolar amino acid of M to negatively charged amino acid of E might increase the chance of water molecule (which is polar) to react with the mannose moiety in the glycone binding pocket, instead of the alkyl group of the alcohol acceptor (which is hydrophobic).

CONCLUSION

1. To predict the amino acid residues of *Dalbergia cochinchinensis* Pierre β-glucosidase or dalcochinase that may be responsible for difference in catalytic activities between β-glucosidase and β-mannosidase, sequence alignment together with molecular modeling and docking were performed to predict the important amino acid residues in glycone binding pocket. Dalcochinase showed 20% sequence identity to *Pyrococcus horikoshii* β-mannosidase. The results revealed that most amino acid residues that might be involved in glycone binding of these enzymes were similar. These amino acid residues were Q36, H136, E182, E396 and E452 in dalcochinase, corresponding to Q13, H149, E207, E399 and E443 in *P. horikoshii* β-mannosidase, respectively. These amino acid residues were highly conserved in glycoside hydrolase 1. However, there were different amino acid residues in this region. These residues were R90, N181, W137Q and M369 in dalcochinase, corresponding to Q77, Q150, D206 and E374 in *P. horikoshii* β-mannosidase. The different amino acid residues between these two enzymes in the glycon binding pocket might be responsible for the glycone specificity, thus these residues were chosen to be the targets for this study

2 Amino acid residues R90, W137, N181 and M369 of dalcochinase were replaced with the corresponding amino acid residues in *P. horikoshii* β-mannosidase, which were Q77, Q150, D206 and E374, respectively, to yield mutants R90Q, W137Q, N181D and M369E of dalcochinase. Mutant enzymes were generated using site-directed mutagenesis method. The mutant enzymes were expressed in *Pichia pastoris*, and could be purified to homogeneity.

3. The kinetic properties of mutant forms of dalcochinase toward *p*NP-Glc substrate were studied, and compared to that of wild-type enzyme. The K_m values of all dalcochinase mutants differ from the wild-type enzymes less than 10-fold, indicating that these mutations did not improve the affinity for *p*NP-Glc substrate.

Mutants R90Q, W137Q and N181D of dalcochinase showed lower turnover rate of about 200-400-fold when compared to wild-type enzymes, indicating the importance of residues R90, W137 and N181 of dalcochinase for hydrolysis of pNP-Glc substrate. On the other hand, the turnover rate of mutant M369E was reduced by 19-fold compared with the wild-type recombinant enzyme, Therefore, the role of residue M369 of dalcochinase may be less important in catalysis than the other three residues.

The low values of V_{max}, k_{cat} and k_{cat}/K_m of all dalcochinase mutants were in agreement with the prediction results from the docking study. Mutant R90Q, W137Q and N181D might lack the important hydrogen bonds between the sugar binding residues and glucose moiety as well as the extended distance between the catalytic residues and the glycosidic oxygen. In mutant M369E, this residue was located further away form the catalytic glutamates, resulting in the less effect on the catalytic activity compared to other three residues.

The hydrolytic activities of dalcochinase mutants toward *p*NP-Man were also studied. It was surprising that all dalcochinase mutants showed lower specific activities than that of wild-type enzymes. It seemed likely that the rearrangement of amino acid residues in the binding pocket of these mutant enzymes were not suitable for accommodation and hydrolysis of the mannoside substrate. These results also in agreement with docking study. Mutant M369E might show better hydrolytic activity toward *p*NP-Man than other mutants due to greater hydrogen-bond interactions between the residues and the mannose substrate.

4. The transglycosylation abilities of dalcochinase mutants were studied and compared to wild-type enzymes. Mutant N181D and M369E showed higher abilities to transfer glucose molecules to ethanol, *n*-propanol and *n*-butanol acceptors compared to wild-type natural and recombinant dalcochinase. These might be due to the low hydrolytic activity of mutant enzymes toward glucoside substrates. It was surprising that wild-type enzymes could hydrolyze and transfer mannose to alcohol acceptors, yielding propyl- and butyl-mannoside. It was the first report on the alkyl-mannoside synthesis using β-glucosidase enzyme. Mutant M369E could hydrolyze *p*NP-Man but could not transmannosylate to alcohol acceptors. As the position 369 was located close to the aglycone binding site, the replacement of nonpolar amino acid of M to negatively charged amino acid of E might hindered the incoming acceptor molecules, resulted in increase the chance of water molecule to react with the mannose moiety in the glycone binding pocket, instead of the alkyl group of the alcohol acceptor.

LITERATURE CITED

Ademark, P., J. Lundqvist, P. Hagglund, M. Tenkanen, N. Torto, F. Tjerneld and H. Stalbrand. 1999. Hydrolytic properties of β-mannosidase purified from *Aspergillus niger*. **J. Biotechnol.** 75: 281-289.

Ajisaka, K., H. Nishida and H. Fujimoto. 1987. The synthesis of oligosaccharides by the reversed hydrolysis reaction of β-glucosidase at high substrate concentration and at high temperature. **Biotechnol. Lett.** 8: 421-424.

Anderotti, G., A. Giordano, A. Tramice, E. Mollo and A. Trincone. 2005. Purification and characterization of a β-D-mannosidase from marine anaspidean *Aplysia fasciata*. **J. Biotechnol.** 26-35.

Barret, T., C.G. Suresh, SP. Tolley, EJ. Dotson and M.A. Hughes. 1995. The crystal structure of a cyanogenic β-glucosidase from white clover, a family 1 glycosyl hydrolase. **Structure** 3: 951-960.

Bauer, M.W., E.J. Bylina, R.V. Swanson and R.M. Kelly. 1996. Comparison of a β-glucosidase and a β-mannosidase from the hyperthermophilic archaeon *Pyrococcus furiosus*: Purification, characterization, gene cloning and sequence analysis. **J. Biol. Chem.** 271: 23749-23755.

Chen, H., J.R. Leipprandt, C.E. Traviss, B.L. Sopher, M.Z. Jones, K.T. Cavanagh and K.H. Friderici. 1995. Molecular cloning and characterization of bovine β-mannosidase. **J. Biol. Chem.** 270: 3841-3848.

Chong, A., G.E. Montello, A. Zhang, E.J. Cantor, W. Liao. M. -Q. Xu and J. Benner. 1998. Utilizing the C-terminal cleavage activity of a protein splicing element to purify recombinant proteins in a single chromatographic step. **Nucl. Acids. Res.** 26: 5109-5115.

Christakpoulos, P., D. Kekos, B.J. Maceis, P.W. Goodenough and M.K. Bhat. 1994. Optimization of β-glucosidase catalysed the synthesis of trisaccharides from cellobiose and gentiobiose. **Biotechnol. Lett.** 16: 587-592.

Chuankhayan, P., T. Rimlumduan, W. Tantanuch, N. Mothong, P.T. Kongsaeree, P. Metheenukul, J. Svasti, O.N. Jensen and J.R. Ketudat Cairns. 2007. Functional and structural differences between isoflavonoid β-glycosidases from *Dalbergia* sp. **Arch. Biochem. Biophys**. 468: 205–216.

_____, Y. Hua, J. Svasti, S. Sakdarat, P.A. Sullivan and J.R. Ketudat-Cairns. 2005. Purification of an isoflavonoid 7-O-β-apiosyl-glucoside β-glucosidase and its substrates from *Dalbergia nigrescens* Kurz. **Phytochem.** 66: 1880-1889.

Coutinho, P.M. and B. Henrissat. 2000. **Carbohydrate active enzymes server**, Available Source: http://afmb.cnrs-mrs.fr/~pedro/CAZY, Fabuary 14, 2007.

Cregg, J.M., J.L. Cereghino, J. Shi and D.R. Higgins. 2000. Recombinant protein expression in *Pichia pastoris.* **Mol. Biotechnol.** 16: 23-52.

Crout, D.H.G. and G. Vic. 1998. Glycosidases and glycosyl transferases in glycoside and oligosaccharide synthesis. **Curr. Opin. Chem. Biol.** 2: 98-111.

Czjzek, M., M. Cicek, V. Zamboni, D.R. Bevan, B. Henrissat and A. Esen. 2000. The mechanism of substrate (aglycone) specificity in β-glucosidase is revealed by crystal structures of mutant maize β-glucosidase-DIMBOA, -DIMBOAGlc, and -dhurrin complexes. **Proc. Natl. Acad. Sci.** 97: 13555-13560.

_____, _____, V. Zamboni, W.P. Burmeister, D.R. Bevan, B. Henrissat and A. Esen. 2001. Crystal structure of a monocotyledon (maize ZMGlu1) β-glucosidase and a model of its complex with p-nitrophenyl β-D-thioglucoside. **Biochem. J.** 354: 37-46.

Davies, G.J., L. Mackenzie, A. Varrot, M. Dauter, A.M. Brzozowski, M. Schulein and S.G. Withers. 1998. Snapshot along the enzymatic reaction coordinate: Analysis of a retaining β-glycoside hydrolase. **Biochemistry** 37: 11707-11713.

Dias, F.M.V., F. Vincent, G. Pell, J.A.M. Prates, M.S.J. Centeno, L.E. Tailford, L.M.A. Ferreira, C.M.G.A. Fontes, G.J. Davies and H.J. Gilbert. 2004. Insights into the molecular determinants of substrate specificity in glycoside hydrolase family 5 revealed by the crystal structure and kinetics of *Cellvibrio mixtus* mannosidase 5A. **J. Biol. Chem.** 279: 25517–25526.

Elbein, A.D., S. Adya and Y.C. Lee. 1977. Purification and properties of a β-mannosidase from *Aspergillus niger*. **J. Biol. Chem.** 252: 2026-2031.

Eksittikul, T. and M. Chilavatnatol. 1988. Characterization of cyanogenic β-glucosidase (linamarase) from Cassava (*Manihot eaculenta* Crantz). **Arch. Biochem. Biophy.** 266: 263-269.

Esen, A. 1993. β-glucosidase: overview, pp 1-14. In A. Esen, Ed. **β-glucosidase Biochemistry and Molecular Biology**. American Chemical Society, Washington D.C.

Higgins, D.G., Thompson J.D., and Gibson T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. **Nuc. Acids Res.** 22: 4673-4680.

Hommalai, G., P. Chaiyen and J. Svasti. 2005. Studies on the transglucosylatio reaction of cassava and Thai rosewood β-glucosidases using 2-deoxy-2-fluor-glycosyl-enzyme intermediates. **Arch. Biochem. Biophys.** 442: 11-20.

Invitrogen. n.d. **A Manual of Method for Expression of Recombiant Protein Using pPICZ and pPICZα in *Pichia pastoris* Easyselect™ *Pichia* Expression Kit**. Available source: www.invitrogen.com, January 29, 2007.

Itoh, H and Y. Kamiyama. 1995. Synthesis of alkyl β-mannosides from mannobiose by *Aspergillus niger* β-mannosidase. **J. Ferment. Bioeng.** 80: 510-512.

Kaper, T., H.H. van Heusden, B. van Loo, A. Vasella, J. van der Oost and W.M. de Vos. 2002. Substrate specificity engineering of β-mannosidase and β-glucosidase from *Pyrococcus* by exchange of unique active site residues. **Biochemistry** 41: 4147-4155.

Kobata, A. 1993. Glycobioogy: An expanding research area in carbohydrate chemistry. **Acc. Chem. Res.** 26: 319-324.

Kurakake, M. and T. Komaki. 2001. Production of β-mannanase and β-mannosidase from *Aspergillus awamori* K4 and their properties. **Curr. Microbiol.** 42: 377-380.

Laskowski, R.A., M.W. MacArthur, D.S.Moss, J.M. Thronton. 1993. Procheck: a program to check the stereochemical quality of protein structures. **J. Appl. Crystallogr.** 26: 283-291.

Lirdprapamongkol, K. and J. Svasti. 2000. Alkyl glucoside synthesis using Thai rosewood β-glucosidase. **Biotechnol. Lett.** 22: 1889-1894.

Marana, S.R., Jacobs-Lorena, M., Terra, W.R. and Ferreira, Clelia. 2001. Amino acid residues involve in substrate binding and catalysis in an insect digestive β-glycosidase. **Biochim. Biophys. Acta** 1545: 41-52.

Marana, S.R, L.M.F. Mendonca, E.H.P. Andrade, W.R. Terra and C. Ferreira. 2003. The role of residues R97 and Y331 in modulating the pH optimum of an insect β-glycosidase of family 1. **Eur. J. Biochem.** 270: 4866-4875.

Matsumura, S., K. Imao, S. Yoshikawa, K. Kawada and T. Uchibor. 1990. Surface activities, biodegradability and antimicrobial properties of n-alkyl glucosides, mannosides and galactosides. **J. Am. Oil Chem. Soc.** 67: 996-1001.

Mo, B. and J.D. Bewley. 2002. β-Mannosidase (EC. 3.2.1.25) activity during and following germination of tomato (*Lycopersicon esculantum* Mill.) seeds. Purification, cloning and characterization. **Planta** 2002: 141-152.

Mosan, P. and F. Paul. 1995. Enzymatic synthesis of oligosaccharides. **FEMS Microbiol. Rev.** 16: 187-192.

Nahrstedt, A. and E. Mueller. 1993. β-Glucosidase (linamarase) of the larvae of the moth *Zygaena trifolii* and its inhibition by some alkaline earth metal ions. In **β-glucosidase Biochemistry and Molecular Biology**. (A. Esen, Ed.) pp. 132-144. American Chemical Society, Washington D.C.

Oda, Y., T. Komaki, and K. Tonomura. 1993. Production of β-mannanase and β-mannosidase by *Enterococcus casseliflavus* FL2121 isolated from decayed Konjac. **Food Microbiol**. 10: 353-358.

Rantwijk, F. van, M. Woudenberg-van Oosterom and R. A. Sheldon. 1999. Glycosidase-catalysed synthesis of alkyl glycosides. **J. Mol. Catal. B: Enz.** 6: 511-532.

Rastall, R.A., S.F. Pikett, M.W. Adlard and C. Bucke. 1992. Synthesis of oligosaccharides by reversal of a fungal β-glucanase. **Biotechnol. Lett.** 14: 373-378.

Rye, C.S. and S.G. Withers. 2000. Glycosidase mechanisms**. Curr. Opin. Chem. Biol.** 4: 573–580.

Sahz-Aparicio, J., J.A. Hermoso, M. Martinez-Ripoll, J.L. Lequerica and J. Polaina. 1998. Crystal structure of β-glucosidase A from *Bacillus polymyxa*: insights into the catalytic activity in family 1 glycosyl hydrolases**. J. Mol. Biol.** 275: 491-502.

Srisomsap, C., J. Svasti, R. Surarit, V. Champattanachai, P. Sawangareetrakul, K. Boonpuan, P. Subhasitanont and D. Chokchaichamnankit. 1996. Isolation and characterization of an enzyme with β-glucosidase and β-fucosidase activities from *Dalbergia cochincinensis* Pierre. **J. Biochem.** 119: 585-590.

_____, P. Subhasitanont, S. Techasakul, R. Surarit and J. Svasti. 1999. Synthesis of homo- and hetero-oligosaccharides by Thai rosewood β-glucosidase. **Biotechnol Lett.** 21: 947-951.

Sunna, W., M.D. Gibbs, C.W.J. Chin, P.J. Nelson and P.L. Bergquist. 2001. A gene encodind a novel multidomain β-1,4-mannanase from *Caldibacillus cellulovorans* and action of the recombinant enzyme on kraft pulp. **App. Environ. Microbiol.** 66: 664-670.

Svasti, J., C. Srisomsap, R. Surarit, E. Benjavongkulchai, W. Suginta, S. Khunyoshyeng, V. Champattanachai, S. Nilwarangkoon and S. Rungvirayudx. 1996. Potential application of plant glycoydrolases for oligosaccharide synthesis. In **Protein Structure-Function Relationship**.(Zaidi and Smith, Ed.) pp. 249-257. Plenum Press, New York.

_____, _____, S. Techasakul and R. Surarit. 1999. Dalcochinin-8'-O-β-D-glucoside and its β-glucosidase enzyme from *Dalbergia cochinchinensis*. **Phytochem.** 50: 739-743.

Svasti, J., T. Phongsak and R. Sarnthima. 2003. Transglucosylation of tertiary alcohol using cassava β-glucosidase. **Biochem. Biophys. Res. Commun.** 305: 470-475.

Toonkool, P., P. Metheenukul, P. Sujiwattanarat, P. Paiboon, N. Tongtubtim, M. Ketudat-Cairns, J. Ketudat-Cairns and J. Svasti. 2006. Expression and purification of dalchochinase, a β-glucosidase from *Dalbergia cochinchinensis* Pierre, in yeast and bacterial hosts. **Protein Exp. Purif.** 48: 195-204.

Tyagi. S., J. Pleiss. 2006. Biochemical profiling in silico-predicting substrate specificities of large enzyme families. **J. Biotechnol.** 124: 108-116.

Verdonk, M.L., J.C. Cole, M.J. Hartshorn, C.W. Murray and R.D. Taylor. 2003. Improved protein-ligand docking using GOLD. **Proteins** 52: 609-623.

Verdoucg, L., J. Moriniere, D.R. Bevan, A. Esen, A. Vesella, B. Henrissart and M. Czjzek. 2004. Structural determinants of substrate specificity in family 1 β-glucosidases. **J. Biol. Chem.** 279: 31796-31803.

Wiederstein, M. and M.J. Sippl. 2007**.** ProSA-web: web service for the recognition of errors in three-dimensional structures of proteins. **Nucl. Acids Res.** 35: 407-410.

Yan, T.-R. and J.-C. Liau. 1998. Synthesis of cello-oligosaccharides from cellobiose with β-glucosidase II from *Aspergillus niger*. **Biotechnol. Lett.** 20: 591-594.

Zechel, D.L. and S.G. Withers. 2000. Glycosidase mechanism: Anatomy of a finely tuned catalyst. **Acc. Chem. Res.** 33: 11-18.

APPENDICES

APPENDIX A

DNA and Protein Techniques

1. Agarose gel electrophoresis

One gram of agarose powder is weighted and dissolved in 1X TAE buffer [40 mM Tris, 0.1142% (v/v) acetic acid and 2 mM EDTA pH 8.5]. Agarose powder is melted using microwave oven and poured into plate. Loading wells are formed by placing a plastic well-forming template or comb in the poured gel solution, and removing the comb when the gel has set. The gel is placed in the electrophoresis tank which covered with 1X TAE buffer. DNA samples are mixed with loading buffer [50 mM EDTA, pH 8.0, 0.4% (w/v) bromphenol and 5% (w/v) glycerol] before injected in the gel. Gel is run approximately 3 in 4 of length of the gel, at 100 volts. Finally, gel is stained in 0.5 μg/ml ethidium bromide and then viewed under ultraviolet light (300 nm wavelenght).

2. Preparation of competent *Escherichia coli* DH5α

E. coli DH5α cells are grown in 3 ml of LB broth [1% (w/v) tryptone, 0.5% (w/v) yeast extract and 0.5% (w/v) NaCl] and incubated at 37°C, 250 rpm overnight. 2 ml of cell suspension is grown in 200 ml of LB broth at 37 °C, 250 rpm and incubated in the shaker until the OD 600 nm reach 0.5-1.0. Cell suspension is then incubated on ice for 30 minutes. Cells are harvested by centrifugation at 4,000 rpm, 4 °C for 15 minutes. Cells are washed twice in 200 ml and 100 ml of cold and steriled water and 40 ml of cold 10 % (v/v) glycerol, respectively. Cells are harvested by centrifugation at 4,000 rpm, 4 °C for 15 minutes. Finally, cells are re-suspended in 2.5 ml of cold 10 % (v/v) glycerol. The competent cells are kept at -80 °C before use.

3. Preparation of competent *Pichia pastoris* strain GS115 and Y11430

P. pastoris strain GS115 and Y11430 are grown in 5ml of YPD broth [1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) dextrose] at 200 rpm, 30 °C overnight. One milliliter of cell suspension is the grown in 50 ml YPD at 200 rpm, 30 °C overnight. Cells are harvested by centrifugation at 4 °C, 5,000 rpm for 5 minutes. Cells are washed twice with 50 ml steriled water and 50 ml of 1 M sorbital,

respectively, and harvested by centrifugation at 4 °C, 5,000 rpm for 10 minutes. Cells are re-suspended in 1 or 2 ml of 1 M sorbital to adjust the OD 600 nm of 100.

4. Plasmid extraction (miniprep or alkaline lysis)

E. coli harboring plasmid constructs are grown in LB broth containing 25 µg/ml zeocin at 200 rpm, 30 °C overnight. Cells are harvested by centrifugation at 13,000 rpm for 10 minutes, and discarded the supernatant. 100 µl of solution I [50 mM glucose, 25 mM Tris pH 8 and 10 mM EDTA] is added into harvested cells and mixed. After incubation for 5 minutes, 200 µl of solution II [1% (w/v) sodium dodecyl sulfate and 0.2 M sodium hydroxide] is added into the reaction mixture, mixed gently and incubated for 5 minutes. 150 µl of cold solution III [3 M potassium acetate and 2 M acetic acid] is added, mixed gently, and incubated on ice for 5 minutes. DNA is separated from the reaction mixture by centrifugation at 13,000 rpm for 1 minute. The supernatant which contained plasmid DNA was mixed 2 volumes of absolute ethanol to precipitate DNA. The precipitated DNA is washed with 70% (v/v) ethanol, and dried. The dried DNA is dissolved in 20 µl TE buffer pH 8 [10 mM Tris-HCl, pH 8.0 and 1 mM EDTA].

5. DNA precipitation

The DNA solution is added with 0.1 volume of DNA solution of 3 M sodium acetate, and 2.5 volume of DNA solution of 100% ethanol. DNA is precipitated by centrifugation at 13,000 rpm for 1 minute. Precipitated DNA is wash with 150 µl 70% (v/v) ethanol. The DNA is dissolved in sterile water.

6. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Appendix Table A1 Quantity of reagent used for preparation of SDS-PAGE.

Reagents	7.5% Resolving gel	4% Stacking gel
Steriled water	2050 µl	3050 µl
30% T, 2,67% C Polyacrylamide	1650 µl	650 µl
1.5 M Tris-HCl pH 8.8	1250 µl	-
0.5 M Tris-HCl pH 6.8	-	1250 µl
10% (w/v) SDS	50 µl	50 µl
10% (w/v) Ammonium persulfate	50 µl	50 µl
TEMED	15 µl	10 µl

The protein samples are mixed with 1X sample buffer [1% (w/v) SDS, 5% (v/v) glycerol, 0.0025% (w/v) coomasie blue G and 1.25mM Tris pH 6.8], and boiled for 10 minutes. The electrophoresis is performed in 1X running buffer [25 mM Tris pH 8.3, 0.2 M glycine and 0.1% (w/v) SDS], at 30 mA for an hour. The protein gel is stained with staining solution [0.125% (w/v) coomasie blue R, 45% (v/v) and 10% (v/v)], and de-stained using de-staining solution [25% (v/v) methanol and 10% (v/v) acetic acid].

7. Western blot

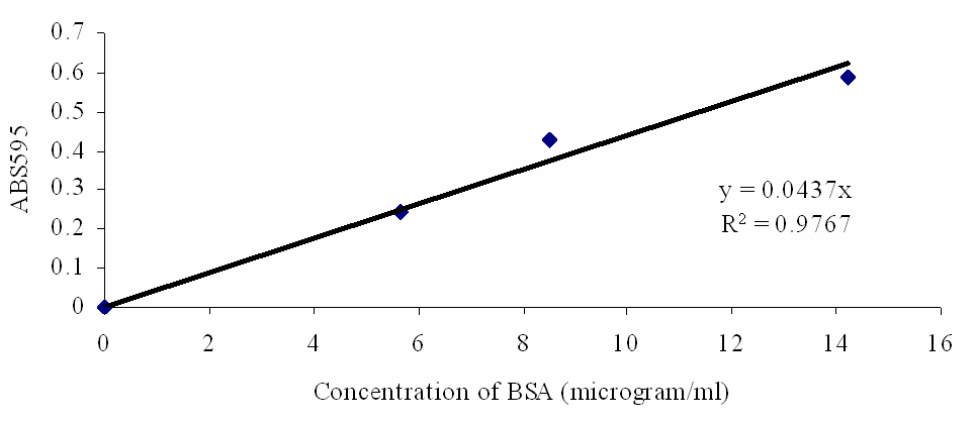
To detect the protein of interest on the SDS-PAGE gel using antibody, the protein gel is soaked in transfer buffer [10 mM Tris and 100 mM glycine] for 20 minutes. Polyvinylidene fluoride membrane and 6 sheets of filter papers are cut to the same size as the protein gel. Polyvinylidene fluoride membrane is prepared by soaking in methanol and transfer buffer for 5 seconds and 10 minutes, respectively, before use. Filter papers are also soaked in transfer buffer for 5 minutes. The 3 sheets of filter papers, polyvinylidene fluoride membrane, SDS-PAGE, and 3 sheets of filter papers are arranged in order on the TransBlot®Semi-Dry (Bio-Rad, USA). The transfer reaction is performed at 10 volts for 30 minutes. The transferred polyvinylidene fluoride membrane is then soaked in 5% (w/v) skim milk in 1X PBST [80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 1M Nacl, pH 7.5 and 0.1% (v/v) tween 20], 1 in 5,000 dilution of primary antibody (mouse monoclonal antibody against natural dalcochinase) and 1 in 5,000 dilution of secondary antibody (rabbit anti-mouse immunoglobulins/HRP), respectively, for an hour. In all steps, membrane is washed with 1X PBST before changing antibody. After soaking membrane in the secondary antibody, membrane is incubated with ECL plus western blotting detection reagent for 5 minutes, and exposed on film for 30 seconds. The film is soaked in developer and fixer reagent, and washed with water.

APPENDIX B

Standard Curves

1. Standard curve for protein measurement of bovine serum albumin (BSA)

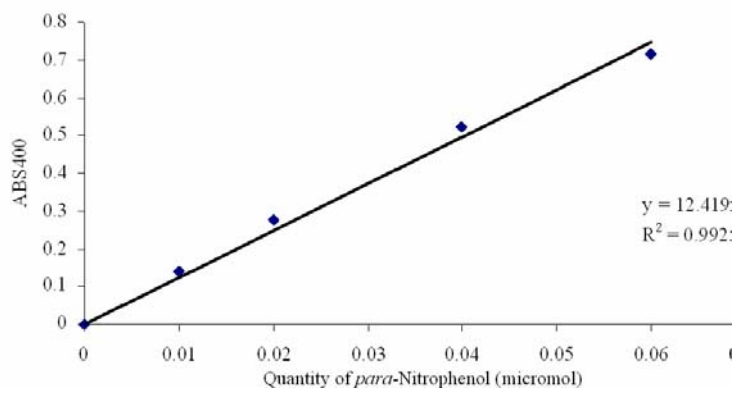
To measure the protein concentration in samples, BSA is used as a standard protein for determining the quantity of protein. BSA is prepared in various concentrations (0-25 µg/ml) to perform the reaction with coomasie reagent (Bio-Rad, USA). The 800 µl of protein samples with various BSA concentrations are incubated with 200 µl coomasie reagent for 5 minutes. The protein sample in complexed with coomasie reagent is measured at the absorbance of 595 nm (ABS595).



Appendix Figure B1 The standard curve of BSA.

2. Standard curve for measurement of *para*-nitrophenol (*p*NP)

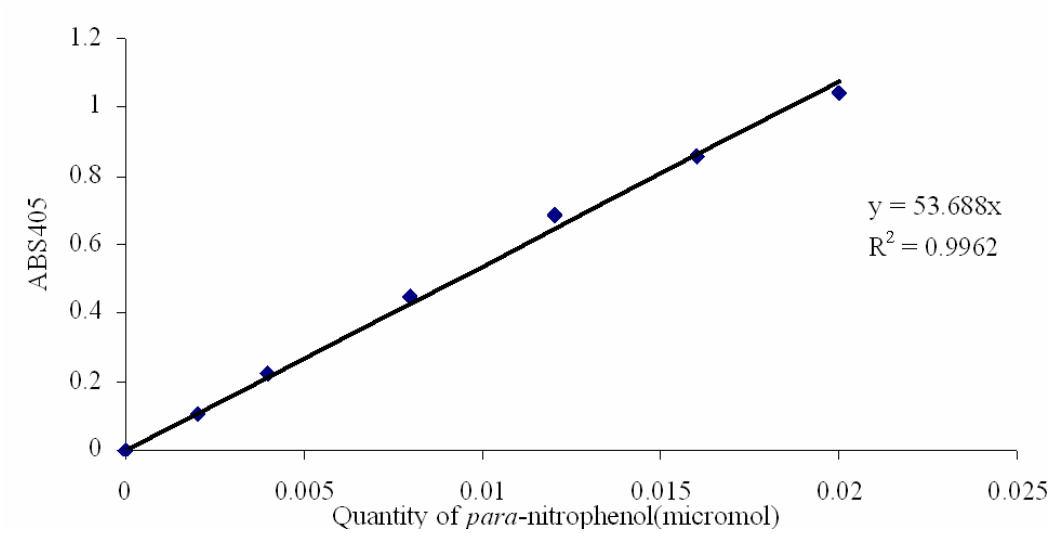
To detect the enzyme activity of β-glucosidase in the culture medium and in the purification steps, the *p*NP standard curve is constructed. *p*NP is prepared in various quantities (0-0.1 µmol). The 0.1 M sodium acetate buffer pH 5.0 is added to adjust the reaction volume to 500 µl. The reactions are incubated at 30 °C for 30 minutes, and added with 1 ml of 2 M sodium carbonate. The *p*NP is measured at the absorbance of 400 nm (ABS400).



Appendix Figure B2 Standard curve of *p*NP for measurement enzyme activity.

3. Standard curve of *para*-nitrophenol (*p*NP) for enzyme characterization

*p*NP is prepared in various quantities (0-0.02 µmol). 0.1 M Sodium acetate buffer pH 5.0 is added to adjust the reaction volume to 50 µl. The reactions are incubated at 30 °C for 5 minutes, and added with 100 µl of 2 M sodium carbonate. The *p*NP is measured at the absorbance of 405 nm (ABS405).



Appendix Figure B3 Standard curve of *p*NP at total reaction of 50 µl.

APPENDIX C

Sequencing result of dalcochinase mutant R90Q

1. Sequencing result of dalcochinase mutant R90Q using primer AOXF

NNAACTTAACTTACACACTTGAGAGATCAAAAACAACTAATTATTCGAAACGATGAGATTTCCTTCAAT
TTTTACTGCTGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTACAACAGAAGATGA
AACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTCGATGTTGCTGT
TTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAATACTACTATTGCCAGCATTGCTGC
TAAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGGCTGAAGCTGCACATCACCATCACCATCATCACCA
TGCTGCAGTTCCTCCATTCAACCGAAGCTGTTTTCCTTCAGATTTCATTTTTGGGACAGCATCCTCCTC
GTACCAGTATGAAGGTGAGGGCAGAGTACCAAGTATATGGGATAACTTCACCCACCAATATCCAGAAAA
GATAGCGGATAGAAGCAACGGAGATGTTGCAGTTGACCAATTTCACCGCTATAAGAAGGATATTGCAAT
CATGAAGGATATGAACTTGGATGCTTATCAAATGTCCATCTCCTGGCCTAGAATTCTCCCAACGGGTAG
GGTTAGTGGAGGCATAAACCAAACAGGAGTTGACTACTACAACAGGCTCATCAATGAGTCACTGGCCAA
TGGCATAACACCATTTGTAACCATTTTTCATTGGGATCTTCCACAAGCCTTGGAGGATGAGTACGGTGG
CTTCTTAAATCATAGCGTTGTAAATGATTTCCAAGACTATGCGGATCTTTGCTTCCAATTATTTGGAGA
TAGGGTAAAGCATTGGATTAC

Expected sequence : The expected DNA sequence of dalcochinase mutant R90Q

R90Q_D2nd13-AOXF : The sequencing result of dalcochinase mutant R90Q using primer AOXF

```
Expected sequence    ACTTTCATAATTGCGACTGGTTCCAATTGACAAGCTTTTGATTTTAACGACTTTTAACGA 900
R90Q_D2nd13-AOXF.    ----------------------------------------------NNAACTTAACTTA 13

Expected sequence    CA-ACTTGAGAAGATCAAAAAACAACTAATTATTCGAAACGATGAGATTTCCTTCAATTT 959
R90Q_D2nd13-AOXF.    CACACTTGAGA-GATCAAAAA-CAACTAATTATTCGAAACGATGAGATTTCCTTCAATTT 71

Expected sequence    TTACTGCTGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTACAACAG 1019
R90Q_D2nd13-AOXF.    TTACTGCTGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTACAACAG 131

Expected sequence    AAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGG 1079
R90Q_D2nd13-AOXF.    AAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGG 191

Expected sequence    ATTTCGATGTTGCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAA 1139
R90Q_D2nd13-AOXF.    ATTTCGATGTTGCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAA 251

Expected sequence    ATACTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGG 1199
R90Q_D2nd13-AOXF.    ATACTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGG 311

Expected sequence    CTGAAGCTGCACATCACCATCACCATCATCACCATGCTGCAGTTCCTCCATTCAACCGAA 1259
R90Q_D2nd13-AOXF.    CTGAAGCTGCACATCACCATCACCATCATCACCATGCTGCAGTTCCTCCATTCAACCGAA 371

Expected sequence    GCTGTTTTCCTTCAGATTTCATTTTTGGGACAGCATCCTCCTCGTACCAGTATGAAGGTG 1319
R90Q_D2nd13-AOXF.    GCTGTTTTCCTTCAGATTTCATTTTTGGGACAGCATCCTCCTCGTACCAGTATGAAGGTG 431

Expected sequence    AGGGCAGAGTACCAAGTATATGGGATAACTTCACCCACCAATATCCAGAAAAGATAGCGG 1379
R90Q_D2nd13-AOXF.    AGGGCAGAGTACCAAGTATATGGGATAACTTCACCCACCAATATCCAGAAAAGATAGCGG 491

Expected sequence    ATAGAAGCAACGGAGATGTTGCAGTTGACCAATTTCACCGCTATAAGAAGGATATTGCAA 1439
R90Q_D2nd13-AOXF.    ATAGAAGCAACGGAGATGTTGCAGTTGACCAATTTCACCGCTATAAGAAGGATATTGCAA 551

Expected sequence    TCATGAAGGATATGAACTTGGATGCTTATCAAATGTCCATCTCCTGGCCTAGAATTCTCC 1499
R90Q_D2nd13-AOXF.    TCATGAAGGATATGAACTTGGATGCTTATCAAATGTCCATCTCCTGGCCTAGAATTCTCC 611

Expected sequence    CAACGGGTAGGGTTAGTGGAGGCATAAACCAAACAGGAGTTGACTACTACAACAGGCTCA 1559
R90Q_D2nd13-AOXF.    CAACGGGTAGGGTTAGTGGAGGCATAAACCAAACAGGAGTTGACTACTACAACAGGCTCA 671

Expected sequence    TCAATGAGTCACTGGCCAATGGCATAACACCATTTGTAACCATTTTTCATTGGGATCTTC 1619
R90Q_D2nd13-AOXF.    TCAATGAGTCACTGGCCAATGGCATAACACCATTTGTAACCATTTTTCATTGGGATCTTC 731

Expected sequence    CACAAGCCTTGGAGGATGAGTACGGTGGCTTCTTAAATCATAGCGTTGTAAATGATTTCC 1679
R90Q_D2nd13-AOXF.    CACAAGCCTTGGAGGATGAGTACGGTGGCTTCTTAAATCATAGCGTTGTAAATGATTTCC 791
Expected sequence    AAGACTATGCGGATCTTTGCTTCCAATTATTTGGAGATAGGGTAAAGCATTGGATTACAC 1739
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R90Q_D2nd13-AOXF.    AAGACTATGCGGATCTTTGCTTCCAATTATTTGGAGATAGGGTAAAGCATTGGATTAC—  849
```

2. Sequencing result of dalcochinase mutant R90Q using primer TRF1

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NNGGCTGAGGGAGCATCCTCCTCGTACAGTATGAAGGTGAGGGCAGAGTACCAAGTATATGGGA
TAACTTCACCCACCAATATCCAGAAAAGATAGCGGATAGAAGCAACGGAGATGTTGCAGTTGAC
CAATTTCACCGCTATAAGAAGGATATTGCAATCATGAAGGATATGAACTTGGATGCTTATCAAA
TGTCCATCTCCTGGCCTAGAATTCTCCCAACGGGTAGGGTTAGTGGAGGCATAAACCAAACAGG
AGTTGACTACTACAACAGGCTCATCAATGAGTCACTGGCCAATGGCATAACACCATTTGTAACC
ATTTTTCATTGGGATCTTCCACAAGCCTTGGAGGATGAGTACGGTGGCTTCTTAAATCATAGCG
TTGTAAATGATTTCCAAGACTATGCGGATCTTTGCTTCCAATTATTTGGAGATAGGGTAAAGCA
TTGGATTACACTAAATGAGCCATCAATCTTCACCGCGAATGGGTATGCATACGGTATGTTTGCA
CCAGGTCGATGTTCTCCATCGTACAATCCAACTTGCACAGGTGGGGATGCAGGAACAGAGACTT
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TCAGGAACATCAGAAAGGTACAATAGGCATTTCCTTGCACGTAGTTTGGGTTATACCGCTTTCA
AATAGCACATCAGATCAAAATGCTACCCAGCGATATCTTGACTTCACATGTGGATGGTTTATGG
ACCCACTTACAGCAGGAAGGTATCCAGATAGCATGCAATATCTAGTTGGAGATCGATTGCCTAA
GTTTACTACAGATCAAGCCAA
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Expected sequence : The expected DNA sequence of dalcochinase mutant R90Q

R90Q_D2nd13-TRF1 : The sequencing result of dalcochinase mutant R90Q using primer TRF1

```
Expected sequence              TCAACCGAAGCTGTTTTCCTTCAGATTTCATTTTTGGGACAGCATCCTCC 1300
R90Q_2nd13-TRF1                ----------------------------NNGGCTGAGGGAGCATCCTCC 21

Expected sequence              TCGTACCAGTATGAAGGTGAGGGCAGAGTACCAAGTATATGGGATAACTT 1350
R90Q_D2nd13-TRF1               TCGTAC-AGTATGAAGGTGAGGGCAGAGTACCAAGTATATGGGATAACTT 70

Expected sequence              CACCCACCAATATCCAGAAAAGATAGCGGATAGAAGCAACGGAGATGTTG 1400
R90Q_D2nd13-TRF1               CACCCACCAATATCCAGAAAAGATAGCGGATAGAAGCAACGGAGATGTTG 120

Expected sequence              CAGTTGACCAATTTCACCGCTATAAGAAGGATATTGCAATCATGAAGGAT 1450
R90Q_D2nd13-TRF1               CAGTTGACCAATTTCACCGCTATAAGAAGGATATTGCAATCATGAAGGAT 170

Expected sequence              ATGAACTTGGATGCTTATCAAATGTCCATCTCCTGGCCTAGAATTCTCCC 1500
R90Q_D2nd13-TRF1               ATGAACTTGGATGCTTATCAAATGTCCATCTCCTGGCCTAGAATTCTCCC 220

Expected sequence              AACGGGTAGGGTTAGTGGAGGCATAAACCAAACAGGAGTTGACTACTACA 1550
R90Q_D2nd13-TRF1               AACGGGTAGGGTTAGTGGAGGCATAAACCAAACAGGAGTTGACTACTACA 270

Expected sequence              ACAGGCTCATCAATGAGTCACTGGCCAATGGCATAACACCATTTGTAACC 1600
R90Q_D2nd13-TRF1               ACAGGCTCATCAATGAGTCACTGGCCAATGGCATAACACCATTTGTAACC 320

Expected sequence              ATTTTTCATTGGGATCTTCCACAAGCCTTGGAGGATGAGTACGGTGGCTT 1650
R90Q_D2nd13-TRF1               ATTTTTCATTGGGATCTTCCACAAGCCTTGGAGGATGAGTACGGTGGCTT 370

Expected sequence              CTTAAATCATAGCGTTGTAAATGATTTCCAAGACTATGCGGATCTTTGCT 1700
R90Q_D2nd13-TRF1               CTTAAATCATAGCGTTGTAAATGATTTCCAAGACTATGCGGATCTTTGCT 420

Expected sequence              TCCAATTATTTGGAGATAGGGTAAAGCATTGGATTACACTAAATGAGCCA 1750
R90Q_D2nd13-TRF1               TCCAATTATTTGGAGATAGGGTAAAGCATTGGATTACACTAAATGAGCCA 470

Expected sequence              TCAATCTTCACCGCGAATGGGTATGCATACGGTATGTTTGCACCAGGTCG 1800
R90Q_D2nd13-TRF1               TCAATCTTCACCGCGAATGGGTATGCATACGGTATGTTTGCACCAGGTCG 520

Expected sequence              ATGTTCTCCATCGTACAATCCAACTTGCACAGGTGGGGATGCAGGAACAG 1850
R90Q_D2nd13-TRF1               ATGTTCTCCATCGTACAATCCAACTTGCACAGGTGGGGATGCAGGAACAG 570
Expected sequence              AGACTTATCTGGTTGCGCACAACCTGATCCTTTCTCATGCAGCAACTGTC 1900
R90Q_D2nd13-TRF1               AGACTTATCTGGTTGCGCACAACCTGATCCTTTCTCATGCAGCAACTGTC 620
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Expected sequence              CAAGTGTACAAAAGGAAGTATCAGGAACATCAGAAAGGTACAATAGGCAT 1950
R90Q_D2nd13-TRF1               CAAGTGTACAAAAGGAAGTATCAGGAACATCAGAAAGGTACAATAGGCAT 670

Expected sequence              TTCCTTGCACGTAGTTTGGGTTATACCGCTTTCAAATAGCACATCAGATC 2000
R90Q_D2nd13-TRF1               TTCCTTGCACGTAGTTTGGGTTATACCGCTTTCAAATAGCACATCAGATC 720

Expected sequence              AAAATGCTACCCAGCGATATCTTGACTTCACATGTGGATGGTTTATGGAC 2050
R90Q_D2nd13-TRF1               AAAATGCTACCCAGCGATATCTTGACTTCACATGTGGATGGTTTATGGAC 770

Expected sequence              CCACTTACAGCAGGAAGGTATCCAGATAGCATGCAATATCTAGTTGGAGA 2100
R90Q_D2nd13-TRF1               CCACTTACAGCAGGAAGGTATCCAGATAGCATGCAATATCTAGTTGGAGA 820

Expected sequence              TCGATTGCCTAAGTTTACTACAGATCAAGCCAAATTAGTTAAGGGTTCAT 2150
R90Q_D2nd13-TRF1               TCGATTGCCTAAGTTTACTACAGATCAAGCCAA---------------- 853
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3. Sequencing result of dalcochinase mutant R90Q using primer TRF2

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NCCTTCATATTGGAGATNGGTAAGCATTGGATTACACTAAATGAGCCATCAATCTTCACCGCGAATGGG
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AAAAGGAAGTATCAGGAACATCAGAAAGGTACAATAGGCATTTCCTTGCACGTAGTTTGGGTTATACCG
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ACTACAGATCAAGCCAAATTAGTTAAGGGTTCATTTGATTTTATTGGACTAAACTATTACACCACTAAC
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AGTTATTATCGTCATCTCTTTTATGTTCGATATGCAATTAGGTCTGGCGCANATGTGAAAGGATTTTTT
GCATGGTCATTGTTGGACAACTTTGA
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Expected sequence : The expected DNA sequence of dalcochinase mutant R90Q

R90Q_D2nd13-TRF2 : The sequencing result of dalcochinase mutant R90Q using primer TRF2

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Expected sequence    AGACTATGCGGATCTTTGCTTCCAATTATTTGGAGATAGGGTAAAGCATTGGATTACACT 1740
R90Q_D2nd13-TRF2     ----------------NCCTTCA--TATT-GGAGATNGGT--AAGCATTGGATTACACT 38

Expected sequence    AAATGAGCCATCAATCTTCACCGCGAATGGGTATGCATACGGTATGTTTGCACCAGGTCG 1800
R90Q_D2nd13-TRF2     AAATGAGCCATCAATCTTCACCGCGAATGGGTATGCATACGGTATGTTTGCACCAGGTCG 98

Expected sequence    ATGTTCTCCATCGTACAATCCAACTTGCACAGGTGGGGATGCAGGAACAGAGACTTATCT 1860
R90Q_D2nd13-TRF2     ATGTTCTCCATCGTACAATCCAACTTGCACAGGTGGGGATGCAGGAACAGAGACTTATCT 158

Expected sequence    GGTTGCGCACAACCTGATCCTTTCTCATGCAGCAACTGTCCAAGTGTACAAAAGGAAGTA 1920
R90Q_D2nd13-TRF2     GGTTGCGCACAACCTGATCCTTTCTCATGCAGCAACTGTCCAAGTGTACAAAAGGAAGTA 218

Expected sequence    TCAGGAACATCAGAAAGGTACAATAGGCATTTCCTTGCACGTAGTTTGGGTTATACCGCT 1980
R90Q_D2nd13-TRF2     TCAGGAACATCAGAAAGGTACAATAGGCATTTCCTTGCACGTAGTTTGGGTTATACCGCT 278

Expected sequence    TTCAAATAGCACATCAGATCAAAATGCTACCCAGCGATATCTTGACTTCACATGTGGATG 2040
R90Q_D2nd13-TRF2     TTCAAATAGCACATCAGATCAAAATGCTACCCAGCGATATCTTGACTTCACATGTGGATG 338

Expected sequence    GTTTATGGACCCACTTACAGCAGGAAGGTATCCAGATAGCATGCAATATCTAGTTGGAGA 2100
R90Q_D2nd13-TRF2     GTTTATGGACCCACTTACAGCAGGAAGGTATCCAGATAGCATGCAATATCTAGTTGGAGA 398

Expected sequence    TCGATTGCCTAAGTTTACTACAGATCAAGCCAAATTAGTTAAGGGTTCATTTGATTTTAT 2160
R90Q_D2nd13-TRF2     TCGATTGCCTAAGTTTACTACAGATCAAGCCAAATTAGTTAAGGGTTCATTTGATTTTAT 458
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Expected sequence    TGGACTAAACTATTACACCACTAACTATGCTACCAAATCAGATGCGTCAACATGCTGCCC 2220
R90Q_D2nd13-TRF2     TGGACTAAACTATTACACCACTAACTATGCTACCAAATCAGATGCGTCAACATGCTGCCC 518

Expected sequence    ACCTAGTTACCTCACAGATCCTCAAGTCACTCTCTTACAGCAACGCAATGGGGTCTTTAT 2280
R90Q_D2nd13-TRF2     ACCTAGTTACCTCACAGATCCTCAAGTCACTCTCTTACAGCAACGCAATGGGGTCTTTAT 578

Expected sequence    AGGTCCAGTGACTCCCTCAGGATGGATGTGCATTTATCCAAAAGGACTTCGAGATTTGTT 2340
R90Q_D2nd13-TRF2     AGGTCCAGTGACTCCCTCAGGATGGATGTGCATTTATCCAAAAGGACTTCGAGATTTGTT 638

Expected sequence    GCTTTACTTCAAGGAAAAGTATAACAATCCTTTGGTTTACATCACTGAAAATGGTATAGA 2400
R90Q_D2nd13-TRF2     GCTTTACTTCAAGGAAAAGTATAACAATCCTTTGGTTTACATCACTGAAAATGGTATAGA 698

Expected sequence    TGAGAAGAATGATGCATCACTATCACTTGAGGAATCCTTGATAGACACTTATAGAATTGA 2460
R90Q_D2nd13-TRF2     TGAGAAGAATGATGCATCACTATCACTTGAGGAATCCTTGATAGACACTTATAGAATTGA 758

Expected sequence    TAGTTATTATCGTCATCTCTTTTATGTTCGATATGCAATTAGGTCTGGCGCAAATGTGAA 2520
R90Q_D2nd13-TRF2     TAGTTATTATCGTCATCTCTTTTATGTTCGATATGCAATTAGGTCTGGCGCANATGTGAA 818

Expected sequence    AGGATTTTTTGCATGGTCATTGTTGGACAACTTTGAATGGGCTGAGGGTTATACATCACG 2580
R90Q_D2nd13-TRF2     AGGATTTTTTGCATGGTCATTGTTGGACAACTTTGA---------------------- 854
```

4. Sequencing result of dalcochinase mutant R90Q using primer TRF3

```
NNGGTCAGCAATTGTTAGGGTTCATTTGATTTTATTGGACTAAACTATTACACCACTAACTATGCTACC
AAATCAGATGCGTCAACATGCTGCCCACCTAGTTACCTCACAGATCCTCAAGTCACTCTCTTACAGCAA
CGCAATGGGGTCTTTATAGGTCCAGTGACTCCCTCAGGATGGATGTGCATTTATCCAAAAGGACTTCGA
GATTTGTTGCTTTACTTCAAGGAAAAGTATAACAATCCTTTGGTTTACATCACTGAAAATGGTATAGAT
GAGAAGAATGATGCATCACTATCACTTGAGGAATCCTTGATAGACACTTATAGAATTGATAGTTATTAT
CGTCATCTCTTTTATGTTCGATATGCAATTAGGTCTGGCGCAAATGTGAAAGGATTTTTTGCATGGTCA
TTGTTGGACAACTTTGAATGGGCTGAGGGTTATACATCACGATTTGGATTATATTTTGTGAACTACACT
ACTTTGAATAGATATCCCAAGCTCTCTGCAACATGGTTCAAGTATTTTCTGGCACGTGATCAAGAGAGT
GCTAAATTGGAAATTTTAGCACCAAAGGCAAGATGGAGCTTATCAACGATGATCAAGGAAGAAAAGACA
AAACCCAAGTGGGGCATTGAAGGCTTTTGATCTAGATCTTAATCACTAGTGAATTCGCGGCCGCCTGCA
GTACGTAGAATTCACGTGGCCCAGCCGGCCGTCTCGGATCGGTACCTCGAGCCGCGGCGGCCGCCAGCT
TTCTAGAACAAAAACTCATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCATCATCATCATCATT
GAGTTTGTAGCCTTAGACATGACTGTTCC
```

Expected sequence : The expected DNA sequence of dalcochinase mutant R90Q

R90Q_D2nd13-TRF3 : The sequencing result of dalcochinase mutant R90Q using primer TRF3

```
Expected sequence    TCGATTGCCTAAGTTTACTACAGATCAAGCCAA-ATTAGTTAAGGGTTCATTTGATTTTA 2159
R90Q_D2nd13-TRF3     ------------------------NNGGTCAGCAATTGTTA-GGGTTCATTTGATTTTA 34

Expected sequence    TTGGACTAAACTATTACACCACTAACTATGCTACCAAATCAGATGCGTCAACATGCTGCC 2219
R90Q_D2nd13-TRF3     TTGGACTAAACTATTACACCACTAACTATGCTACCAAATCAGATGCGTCAACATGCTGCC 94


Expected sequence    CACCTAGTTACCTCACAGATCCTCAAGTCACTCTCTTACAGCAACGCAATGGGGTCTTTA 2279
R90Q_D2nd13-TRF3     CACCTAGTTACCTCACAGATCCTCAAGTCACTCTCTTACAGCAACGCAATGGGGTCTTTA 154

Expected sequence    TAGGTCCAGTGACTCCCTCAGGATGGATGTGCATTTATCCAAAAGGACTTCGAGATTTGT 2339
R90Q_D2nd13-TRF3     TAGGTCCAGTGACTCCCTCAGGATGGATGTGCATTTATCCAAAAGGACTTCGAGATTTGT 214

Expected sequence    TGCTTTACTTCAAGGAAAAGTATAACAATCCTTTGGTTTACATCACTGAAAATGGTATAG 2399
R90Q_D2nd13-TRF3     TGCTTTACTTCAAGGAAAAGTATAACAATCCTTTGGTTTACATCACTGAAAATGGTATAG 274

Expected sequence    ATGAGAAGAATGATGCATCACTATCACTTGAGGAATCCTTGATAGACACTTATAGAATTG 2459
R90Q_D2nd13-TRF3     ATGAGAAGAATGATGCATCACTATCACTTGAGGAATCCTTGATAGACACTTATAGAATTG 334
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Expected sequence    ATAGTTATTATCGTCATCTCTTTTATGTTCGATATGCAATTAGGTCTGGCGCAAATGTGA 2519
R90Q_D2nd13-TRF3     ATAGTTATTATCGTCATCTCTTTTATGTTCGATATGCAATTAGGTCTGGCGCAAATGTGA 394

Expected sequence    AAGGATTTTTTGCATGGTCATTGTTGGACAACTTTGAATGGGCTGAGGGTTATACATCAC 2579
R90Q_D2nd13-TRF3     AAGGATTTTTTGCATGGTCATTGTTGGACAACTTTGAATGGGCTGAGGGTTATACATCAC 454

Expected sequence    GATTTGGATTATATTTTGTGAACTACACTACTTTGAATAGATATCCCAAGCTCTCTGCAA 2639
R90Q_D2nd13-TRF3      GATTTGGATTATATTTTGTGAACTACACTACTTTGAATAGATATCCCAAGCTCTCTGCAA 514

Expected sequence    CATGGTTCAAGTATTTTCTGGCACGTGATCAAGAGAGTGCTAAATTGGAAATTTTAGCAC 2699
R90Q_D2nd13-TRF3     CATGGTTCAAGTATTTTCTGGCACGTGATCAAGAGAGTGCTAAATTGGAAATTTTAGCAC 574

Expected sequence    CAAAGGCAAGATGGAGCTTATCAACGATGATCAAGGAAGAAAAGACAAAACCCAAGTGGG 2759
R90Q_D2nd13-TRF3     CAAAGGCAAGATGGAGCTTATCAACGATGATCAAGGAAGAAAAGACAAAACCCAAGTGGG 634

Expected sequence    GCATTGAAGGCTTTTGATCTAGATCTTAATCACTAGTGAATTCGCGGCCGCCTGCAGTAC 2819
R90Q_D2nd13-TRF3     GCATTGAAGGCTTTTGATCTAGATCTTAATCACTAGTGAATTCGCGGCCGCCTGCAGTAC 694

Expected sequence    GTAGAATTCACGTGGCCCAGCCGGCCGTCTCGGATCGGTACCTCGAGCCGCGGCGGCCGC 2879
R90Q_D2nd13-TRF3     GTAGAATTCACGTGGCCCAGCCGGCCGTCTCGGATCGGTACCTCGAGCCGCGGCGGCCGC 754

Expected sequence    CAGCTTTCTAGAACAAAAACTCATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCA 2939
R90Q_D2nd13-TRF3     CAGCTTTCTAGAACAAAAACTCATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCA 814

Expected sequence    TCATCATCATCATTGAGTTTGTAGCCTTAGACATGACTGTTCCTCAGTTCAAGTTGGGCA 2999
R90Q_D2nd13-TRF3     TCATCATCATCATTGAGTTTGTAGCCTTAGACATGACTGTTCC---------------- 857
```

APPENDIX D

Sequencing result of dalcochinase mutant W137Q

1. Sequencing result of dalcochinase mutant W137Q using primer AOXF

NNNCCTTAACTTACGACACTTGAGAGATCAAAAAACAACTAATTATTCGAAACGATGAGATTTCCTTCA
ATTTTTACTGCTGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTACAACAGAAGAT
GAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTCGATGTTGCT
GTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAATACTACTATTGCCAGCATTGCT
GCTAAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGGCTGAAGCTGCACATCACCATCACCATCATCAC
CATGCTGCAGTTCCTCCATTCAACCGAAGCTGTTTTCCTTCAGATTTCATTTTTGGGACAGCATCCTCC
TCGTACCAGTATGAAGGTGAGGGCAGAGTACCAAGTATATGGGATAACTTCACCCACCAATATCCAGAA
AAGATAGCGGATAGAAGCAACGGAGATGTTGCAGTTGACCAATTTCACCGCTATAAGAAGGATATTGCA
ATCATGAAGGATATGAACTTGGATGCTTATAGAATGTCCATCTCCTGGCCTAGAATTCTCCCAACGGGT
AGGGTTAGTGGAGGCATAAACCAAACAGGAGTTGACTACTACAACAGGCTCATCAATGAGTCACTGGCC
AATGGCATAACACCATTTGTAACCATTTTTCATCAAGATCTTCCACAAGCCTTGGAGGATGAGTACGGT
GGCTTCTTAAATCATAGCGTTGTAAATGATTTCCAAGACTATGCGGATCTTTGCTTCCAATTATTTTGG
AGATAGGGTAAAAGCATTGGATTACA

Expected sequence : The expected DNA sequence of dalcochinase mutant W137Q

W137Q_D1st23-AOXF : The sequencing result of dalcochinase mutant W137Q using primer AOXF

```
Expected sequence    ACTTTCATAATTGCGACTGGTTCCAATTGACAAGCTTTTGATTTTAACGACTTTTAACGA 900
W137Q_D1st23-AOXF    ------------------------------------NNNCCTTAAC------TTACGA 16

Expected sequence    CAACTTGAGAAGATCAAAAAACAACTAATTATTCGAAACGATGAGATTTCCTTCAATTTT 960
W137Q_D1st23-AOXF    CA-CTTGAGA-GATCAAAAAACAACTAATTATTCGAAACGATGAGATTTCCTTCAATTTT 74

Expected sequence    TACTGCTGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTACAACAGA 1020
W137Q_D1st23-AOXF    TACTGCTGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTACAACAGA 134

Expected sequence    AGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGA 1080
W137Q_D1st23-AOXF    AGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGA 194

Expected sequence    TTTCGATGTTGCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAA 1140
W137Q_D1st23-AOXF     TTTCGATGTTGCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAA 254

Expected sequence    TACTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGGC 1200
W137Q_D1st23-AOXF    TACTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGGC 314

Expected sequence    TGAAGCTGCACATCACCATCACCATCATCACCATGCTGCAGTTCCTCCATTCAACCGAAG 1260
W137Q_D1st23-AOXF    TGAAGCTGCACATCACCATCACCATCATCACCATGCTGCAGTTCCTCCATTCAACCGAAG 374

Expected sequence    CTGTTTTCCTTCAGATTTCATTTTTGGGACAGCATCCTCCTCGTACCAGTATGAAGGTGA 1320
W137Q_D1st23-AOXF    CTGTTTTCCTTCAGATTTCATTTTTGGGACAGCATCCTCCTCGTACCAGTATGAAGGTGA 434

Expected sequence    GGGCAGAGTACCAAGTATATGGGATAACTTCACCCACCAATATCCAGAAAAGATAGCGGA 1380
W137Q_D1st23-AOXF    GGGCAGAGTACCAAGTATATGGGATAACTTCACCCACCAATATCCAGAAAAGATAGCGGA 494

Expected sequence    TAGAAGCAACGGAGATGTTGCAGTTGACCAATTTCACCGCTATAAGAAGGATATTGCAAT 1440
W137Q_D1st23-AOXF    TAGAAGCAACGGAGATGTTGCAGTTGACCAATTTCACCGCTATAAGAAGGATATTGCAAT 554

Expected sequence    CATGAAGGATATGAACTTGGATGCTTATAGAATGTCCATCTCCTGGCCTAGAATTCTCCC 1500
W137Q_D1st23-AOXF    CATGAAGGATATGAACTTGGATGCTTATAGAATGTCCATCTCCTGGCCTAGAATTCTCCC 614

Expected sequence    AACGGGTAGGGTTAGTGGAGGCATAAACCAAACAGGAGTTGACTACTACAACAGGCTCAT 1560
W137Q_D1st23-AOXF    AACGGGTAGGGTTAGTGGAGGCATAAACCAAACAGGAGTTGACTACTACAACAGGCTCAT 674

Expected sequence    CAATGAGTCACTGGCCAATGGCATAACACCATTTGTAACCATTTTTCATCAAGATCTTCC 1620
W137Q_D1st23-AOXF    CAATGAGTCACTGGCCAATGGCATAACACCATTTGTAACCATTTTTCATCAAGATCTTCC 734
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Expected sequence    ACAAGCCTTGGAGGATGAGTACGGTGGCTTCTTAAATCATAGCGTTGTAAATGATTTCCA 1680
W137Q_D1st23-AOXF    ACAAGCCTTGGAGGATGAGTACGGTGGCTTCTTAAATCATAGCGTTGTAAATGATTTCCA 794

Expected sequence    AGACTATGCGGATCTTTGCTTCCAATTATTT-GGAGATAGGGTAAA-GCATTGGATTACA 1738
W137Q_D1st23-AOXF    AGACTATGCGGATCTTTGCTTCCAATTATTTTGGAGATAGGGTAAAAGCATTGGATTACA 854
```

2. Sequencing result of dalcochinase mutant W137Q using primer TRF1

```
NNGGGATTTGGCACACCTCCTCGTACAGTATGAAGGTGAGGGCAGAGTACCAAGTATATGGGATAACTT
CACCCACCAATATCCAGAAAAGATAGCGGATAGAAGCAACGGAGATGTTGCAGTTGACCAATTTCACCG
CTATAAGAAGGATATTGCAATCATGAAGGATATGAACTTGGATGCTTATAGAATGTCCATCTCCTGGCC
TAGAATTCTCCCAACGGGTAGGGTTAGTGGAGGCATAAACCAAACAGGAGTTGACTACTACAACAGGCT
CATCAATGAGTCACTGGCCAATGGCATAACACCATTTGTAACCATTTTTCATCAAGATCTTCCACAAGC
CTTGGAGGATGAGTACGGTGGCTTCTTAAATCATAGCGTTGTAAATGATTTCCAAGACTATGCGGATCT
TTGCTTCCAATTATTTGGAGATAGGGTAAAGCATTGGATTACACTAAATGAGCCATCAATCTTCACCGC
GAATGGGTATGCATACGGTATGTTTGCACCAGGTCGATGTTCTCCATCGTACAATCCAACTTGCACAGG
TGGGGATGCAGGAACAGAGACTTATCTGGTTGCGCACAACCTGATCCTTTCTCATGCAGCAACTGTCCA
AGTGTACAAAAGGAAGTATCAGGAACATCAGAAAGGTACAATAGGCATTTCCTTGCACGTAGTTTGGGT
TATACCGCTTTCAAATAGCACATCAGATCAAAATGCTACCCAGCGATATCTTGACTTCACATGTGGATG
GTTTATGGACCCACTTACAGCAGGAAGGTATCCAGATAGCATGCAATATCTAGTTGGAGATCGATTGCC
TAAGTTTACTACAGATCAAGCCAAATTA
```

Expected sequence : The expected DNA sequence of dalcochinase mutant W137Q

W137Q_D1st23-TRF1 : The sequencing result of dalcochinase mutant W137Q using primer TRF1

```
Expected sequence    TGTTTTCCTTCAGATTTCATTTTTGGGACAGCATCCTCCTCGTACCAGTATGAAGGTGAG 1320
W137Q_D1st23-TRF1    --------------NNGGGATTTGGCACA----CCTCCTCGTAC-AGTATGAAGGTGAG 40

Expected sequence    GGCAGAGTACCAAGTATATGGGATAACTTCACCCACCAATATCCAGAAAAGATAGCGGAT 1380
W137Q_D1st23-TRF1    GGCAGAGTACCAAGTATATGGGATAACTTCACCCACCAATATCCAGAAAAGATAGCGGAT 100

Expected sequence    AGAAGCAACGGAGATGTTGCAGTTGACCAATTTCACCGCTATAAGAAGGATATTGCAATC 1440
W137Q_D1st23-TRF1    AGAAGCAACGGAGATGTTGCAGTTGACCAATTTCACCGCTATAAGAAGGATATTGCAATC 160

Expected sequence    ATGAAGGATATGAACTTGGATGCTTATAGAATGTCCATCTCCTGGCCTAGAATTCTCCCA 1500
W137Q_D1st23-TRF1    ATGAAGGATATGAACTTGGATGCTTATAGAATGTCCATCTCCTGGCCTAGAATTCTCCCA 220

Expected sequence    ACGGGTAGGGTTAGTGGAGGCATAAACCAAACAGGAGTTGACTACTACAACAGGCTCATC 1560
W137Q_D1st23-TRF1    ACGGGTAGGGTTAGTGGAGGCATAAACCAAACAGGAGTTGACTACTACAACAGGCTCATC 280

Expected sequence    AATGAGTCACTGGCCAATGGCATAACACCATTTGTAACCATTTTTCATCAAGATCTTCCA 1620
W137Q_D1st23-TRF1    AATGAGTCACTGGCCAATGGCATAACACCATTTGTAACCATTTTTCATCAAGATCTTCCA 340

Expected sequence    CAAGCCTTGGAGGATGAGTACGGTGGCTTCTTAAATCATAGCGTTGTAAATGATTTCCAA 1680
W137Q_D1st23-TRF1    CAAGCCTTGGAGGATGAGTACGGTGGCTTCTTAAATCATAGCGTTGTAAATGATTTCCAA 400

Expected sequence    GACTATGCGGATCTTTGCTTCCAATTATTTGGAGATAGGGTAAAGCATTGGATTACACTA 1740
W137Q_D1st23-TRF1    GACTATGCGGATCTTTGCTTCCAATTATTTGGAGATAGGGTAAAGCATTGGATTACACTA 460

Expected sequence    AATGAGCCATCAATCTTCACCGCGAATGGGTATGCATACGGTATGTTTGCACCAGGTCGA 1800
W137Q_D1st23-TRF1    AATGAGCCATCAATCTTCACCGCGAATGGGTATGCATACGGTATGTTTGCACCAGGTCGA 520

Expected sequence    TGTTCTCCATCGTACAATCCAACTTGCACAGGTGGGGATGCAGGAACAGAGACTTATCTG 1860
W137Q_D1st23-TRF1    TGTTCTCCATCGTACAATCCAACTTGCACAGGTGGGGATGCAGGAACAGAGACTTATCTG 580

Expected sequence    GTTGCGCACAACCTGATCCTTTCTCATGCAGCAACTGTCCAAGTGTACAAAAGGAAGTAT 1920
W137Q_D1st23-TRF1    GTTGCGCACAACCTGATCCTTTCTCATGCAGCAACTGTCCAAGTGTACAAAAGGAAGTAT 640
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Expected sequence    CAGGAACATCAGAAAGGTACAATAGGCATTTCCTTGCACGTAGTTTGGGTTATACCGCTT 1980
W137Q_D1st23-TRF1    CAGGAACATCAGAAAGGTACAATAGGCATTTCCTTGCACGTAGTTTGGGTTATACCGCTT 700

Expected sequence    TCAAATAGCACATCAGATCAAAATGCTACCCAGCGATATCTTGACTTCACATGTGGATGG 2040
W137Q_D1st23-TRF1    TCAAATAGCACATCAGATCAAAATGCTACCCAGCGATATCTTGACTTCACATGTGGATGG 760

Expected sequence    TTTATGGACCCACTTACAGCAGGAAGGTATCCAGATAGCATGCAATATCTAGTTGGAGAT 2100
W137Q_D1st23-TRF1    TTTATGGACCCACTTACAGCAGGAAGGTATCCAGATAGCATGCAATATCTAGTTGGAGAT 820

Expected sequence    CGATTGCCTAAGTTTACTACAGATCAAGCCAAATTAGTTAAGGGTTCATTTGATTTTATT 2160
W137Q_D1st23-TRF1   CGATTGCCTAAGTTTACTACAGATCAAGCCAAATTA---------------------- 856
```

3. Sequencing result of dalcochinase mutant W137Q using primer TRF2

```
NTCCTCATTTTGAATAGGTAAGCATTGGATTACACTAAATGAGCCATCAATCTTCACCGCGAATGGGTA
TGCATACGGTATGTTTGCACCAGGTCGATGTTCTCCATCGTACAATCCAACTTGCACAGGTGGGGATGC
AGGAACAGAGACTTATCTGGTTGCGCACAACCTGATCCTTTCTCATGCAGCAACTGTCCAAGTGTACAA
AAGGAAGTATCAGGAACATCAGAAAGGTACAATAGGCATTTCCTTGCACGTAGTTTGGGTTATACCGCT
TTCAAATAGCACATCAGATCAAAATGCTACCCAGCGATATCTTGACTTCACATGTGGATGGTTTATGGA
CCCACTTACAGCAGGAAGGTATCCAGATAGCATGCAATATCTAGTTGGAGATCGATTGCCTAAGTTTAC
TACAGATCAAGCCAAATTAGTTAAGGGTTCATTTGATTTTATTGGACTAAACTATTACACCACTAACTA
TGCTACCAAATCAGATGCGTCAACATGCTGCCCACCTAGTTACCTCACAGATCCTCAAGTCACTCTCTT
ACAGCAACGCAATGGGGTCTTTATAGGTCCAGTGACTCCCTCAGGATGGATGTGCATTTATCCAAAAGG
ACTTCGAGATTTGTTGCTTTACTTCAAGGAAAAGTATAACAATCCTTTGGTTTACATCACTGAAAATGG
TATAGATGAGAAGAATGATGCATCACTATCACTTGAGGAATCCTTGATAGACACTTATAGAATTGATAG
TTATTATCGTCATCTCTTTTATGTTCGATATGCAATTAGGTCTGGCGCAAATGTGAAAGGATTTTTTGC
ATGGTCATTGNTGGACAACT
```

Expected sequence : The expected DNA sequence of dalcochinase mutant W137Q

W137Q_D1st23-TRF2 : The sequencing result of dalcochinase mutant W137Q using primer TRF2

```
Expected sequence    AGACTATGCGGATCTTTGCTTCCAATTATTTGGAGATAGGGTAAAGCATTGGATTACACT 1740
W137Q_D1st23-TRF2    ------------------NTCC--TCATTTTGA-ATAGGT--AAGCATTGGATTACACT 36

Expected sequence    AAATGAGCCATCAATCTTCACCGCGAATGGGTATGCATACGGTATGTTTGCACCAGGTCG 1800
W137Q_D1st23-TRF2    AAATGAGCCATCAATCTTCACCGCGAATGGGTATGCATACGGTATGTTTGCACCAGGTCG 96

Expected sequence    ATGTTCTCCATCGTACAATCCAACTTGCACAGGTGGGGATGCAGGAACAGAGACTTATCT 1860
W137Q_D1st23-TRF2    ATGTTCTCCATCGTACAATCCAACTTGCACAGGTGGGGATGCAGGAACAGAGACTTATCT 156

Expected sequence    GGTTGCGCACAACCTGATCCTTTCTCATGCAGCAACTGTCCAAGTGTACAAAAGGAAGTA 1920
W137Q_D1st23-TRF2    GGTTGCGCACAACCTGATCCTTTCTCATGCAGCAACTGTCCAAGTGTACAAAAGGAAGTA 216

Expected sequence    TCAGGAACATCAGAAAGGTACAATAGGCATTTCCTTGCACGTAGTTTGGGTTATACCGCT 1980
W137Q_D1st23-TRF2    TCAGGAACATCAGAAAGGTACAATAGGCATTTCCTTGCACGTAGTTTGGGTTATACCGCT 276

Expected sequence    TTCAAATAGCACATCAGATCAAAATGCTACCCAGCGATATCTTGACTTCACATGTGGATG 2040
W137Q_D1st23-TRF2     TCAAATAGCACATCAGATCAAAATGCTACCCAGCGATATCTTGACTTCACATGTGGATG 336

Expected sequence    GTTTATGGACCCACTTACAGCAGGAAGGTATCCAGATAGCATGCAATATCTAGTTGGAGA 2100
W137Q_D1st23-TRF2    GTTTATGGACCCACTTACAGCAGGAAGGTATCCAGATAGCATGCAATATCTAGTTGGAGA 396

Expected sequence    TCGATTGCCTAAGTTTACTACAGATCAAGCCAAATTAGTTAAGGGTTCATTTGATTTTAT 2160
W137Q_D1st23-TRF2    TCGATTGCCTAAGTTTACTACAGATCAAGCCAAATTAGTTAAGGGTTCATTTGATTTTAT 456

Expected sequence    TGGACTAAACTATTACACCACTAACTATGCTACCAAATCAGATGCGTCAACATGCTGCCC 2220
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W137Q_D1st23-TRF2    TGGACTAAACTATTACACCACTAACTATGCTACCAAATCAGATGCGTCAACATGCTGCCC 516

Expected sequence    ACCTAGTTACCTCACAGATCCTCAAGTCACTCTCTTACAGCAACGCAATGGGGTCTTTAT 2280
W137Q_D1st23-TRF2    ACCTAGTTACCTCACAGATCCTCAAGTCACTCTCTTACAGCAACGCAATGGGGTCTTTAT 576

Expected sequence    AGGTCCAGTGACTCCCTCAGGATGGATGTGCATTTATCCAAAAGGACTTCGAGATTTGTT 2340
W137Q_D1st23-TRF2    AGGTCCAGTGACTCCCTCAGGATGGATGTGCATTTATCCAAAAGGACTTCGAGATTTGTT 636

Expected sequence    GCTTTACTTCAAGGAAAAGTATAACAATCCTTTGGTTTACATCACTGAAAATGGTATAGA 2400
W137Q_D1st23-TRF2    GCTTTACTTCAAGGAAAAGTATAACAATCCTTTGGTTTACATCACTGAAAATGGTATAGA 696

Expected sequence    TGAGAAGAATGATGCATCACTATCACTTGAGGAATCCTTGATAGACACTTATAGAATTGA 2460
W137Q_D1st23-TRF2    TGAGAAGAATGATGCATCACTATCACTTGAGGAATCCTTGATAGACACTTATAGAATTGA 756

Expected sequence    TAGTTATTATCGTCATCTCTTTTATGTTCGATATGCAATTAGGTCTGGCGCAAATGTGAA 2520
W137Q_D1st23-TRF2    TAGTTATTATCGTCATCTCTTTTATGTTCGATATGCAATTAGGTCTGGCGCAAATGTGAA 816

Expected sequence    AGGATTTTTTGCATGGTCATTGTTGGACAACTTTGAATGGGCTGAGGGTTATACATCACG 2580
W137Q_D1st23-TRF2    AGGATTTTTTGCATGGTCATTGNTGGACAACT--------------------------- 848
```

4. Sequencing result of dalcochinase mutant W137Q using primer TRF2

```
NNNCCAGATTAGCATAGTTAGGGTTCATTTGATTTTATTGGACTAAACTATTACACCACTAACTATGCT
ACCAAATCAGATGCGTCAACATGCTGCCCACCTAGTTACCTCACAGATCCTCAAGTCACTCTCTTACAG
CAACGCAATGGGGTCTTTATAGGTCCAGTGACTCCCTCAGGATGGATGTGCATTTATCCAAAAGGACTT
CGAGATTTGTTGCTTTACTTCAAGGAAAAGTATAACAATCCTTTGGTTTACATCACTGAAAATGGTATA
GATGAGAAGAATGATGCATCACTATCACTTGAGGAATCCTTGATAGACACTTATAGAATTGATAGTTAT
TATCGTCATCTCTTTTATGTTCGATATGCAATTAGGTCTGGCGCAAATGTGAAAGGATTTTTTGCATGG
TCATTGTTGGACAACTTTGAATGGGCTGAGGGTTATACATCACGATTTGGATTATATTTTGTGAACTAC
ACTACTTTGAATAGATATCCCAAGCTCTCTGCAACATGGTTCAAGTATTTTCTGGCACGTGATCAAGAG
AGTGCTAAATTGGAAATTTTAGCACCAAAGGCAAGATGGAGCTTATCAACGATGATCAAGGAAGAAAAG
ACAAAACCCAAGTGGGGCATTGAAGGCTTTTGATCTAGATCTTAATCACTAGTGAATTCGCGGCCGCCT
GCAGTACGTAGAATTCACGTGGCCCAGCCGGCCGTCTCGGATCGGTACCTCGAGCCGCGGCGGCCGCCA
GCTTTCTAGAACAAAAACTCATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCATCATCATCATC
ATTGAGTTTGTAGCCTTAGACATGACTGTCCN
```

Expected sequence : The expected DNA sequence of dalcochinase mutant W137Q

W137Q_D1st23-TRF3 : The sequencing result of dalcochinase mutant W137Q using primer TRF3

```
Expected sequence    TCGATTGCCTAAGTTTACTACAGATCAAGCCAAATTAGTTAAGGGTTCATTTGATTTTAT 2160
W137Q_D1st23-TRF3    --------------------NNNCCAGATTAGCATAGTTA-GGGTTCATTTGATTTTAT 38

Expected sequence    TGGACTAAACTATTACACCACTAACTATGCTACCAAATCAGATGCGTCAACATGCTGCCC 2220
W137Q_D1st23-TRF3    TGGACTAAACTATTACACCACTAACTATGCTACCAAATCAGATGCGTCAACATGCTGCCC 98

Expected sequence    ACCTAGTTACCTCACAGATCCTCAAGTCACTCTCTTACAGCAACGCAATGGGGTCTTTAT 2280
W137Q_D1st23-TRF3    ACCTAGTTACCTCACAGATCCTCAAGTCACTCTCTTACAGCAACGCAATGGGGTCTTTAT 158

Expected sequence    AGGTCCAGTGACTCCCTCAGGATGGATGTGCATTTATCCAAAAGGACTTCGAGATTTGTT 2340
W137Q_D1st23-TRF3    AGGTCCAGTGACTCCCTCAGGATGGATGTGCATTTATCCAAAAGGACTTCGAGATTTGTT 218

Expected sequence    GCTTTACTTCAAGGAAAAGTATAACAATCCTTTGGTTTACATCACTGAAAATGGTATAGA 2400
W137Q_D1st23-TRF3    GCTTTACTTCAAGGAAAAGTATAACAATCCTTTGGTTTACATCACTGAAAATGGTATAGA 278

Expected sequence    TGAGAAGAATGATGCATCACTATCACTTGAGGAATCCTTGATAGACACTTATAGAATTGA 2460
W137Q_D1st23-TRF3    TGAGAAGAATGATGCATCACTATCACTTGAGGAATCCTTGATAGACACTTATAGAATTGA 338
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Expected sequence    TAGTTATTATCGTCATCTCTTTTATGTTCGATATGCAATTAGGTCTGGCGCAAATGTGAA 2520
W137Q_D1st23-TRF3    TAGTTATTATCGTCATCTCTTTTATGTTCGATATGCAATTAGGTCTGGCGCAAATGTGAA 398

Expected sequence    AGGATTTTTTGCATGGTCATTGTTGGACAACTTTGAATGGGCTGAGGGTTATACATCACG 2580
W137Q_D1st23-TRF3    AGGATTTTTTGCATGGTCATTGTTGGACAACTTTGAATGGGCTGAGGGTTATACATCACG 458

Expected sequence    ATTTGGATTATATTTTGTGAACTACACTACTTTGAATAGATATCCCAAGCTCTCTGCAAC 2640
W137Q_D1st23-TRF3    ATTTGGATTATATTTTGTGAACTACACTACTTTGAATAGATATCCCAAGCTCTCTGCAAC 518

Expected sequence    ATGGTTCAAGTATTTTCTGGCACGTGATCAAGAGAGTGCTAAATTGGAAATTTTAGCACC 2700
W137Q_D1st23-TRF3    ATGGTTCAAGTATTTTCTGGCACGTGATCAAGAGAGTGCTAAATTGGAAATTTTAGCACC 578

Expected sequence    AAAGGCAAGATGGAGCTTATCAACGATGATCAAGGAAGAAAAGACAAAACCCAAGTGGGG 2760
W137Q_D1st23-TRF3    AAAGGCAAGATGGAGCTTATCAACGATGATCAAGGAAGAAAAGACAAAACCCAAGTGGGG 638

Expected sequence    CATTGAAGGCTTTTGATCTAGATCTTAATCACTAGTGAATTCGCGGCCGCCTGCAGTACG 2820
W137Q_D1st23-TRF3    CATTGAAGGCTTTTGATCTAGATCTTAATCACTAGTGAATTCGCGGCCGCCTGCAGTACG 698

Expected sequence    TAGAATTCACGTGGCCCAGCCGGCCGTCTCGGATCGGTACCTCGAGCCGCGGCGGCCGCC 2880
W137Q_D1st23-TRF3    TAGAATTCACGTGGCCCAGCCGGCCGTCTCGGATCGGTACCTCGAGCCGCGGCGGCCGCC 758

Expected sequence    AGCTTTCTAGAACAAAAACTCATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCAT 2940
W137Q_D1st23-TRF3    AGCTTTCTAGAACAAAAACTCATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCAT 818

Expected sequence    CATCATCATCATTGAGTTTGTAGCCTTAGACATGACTGTTCCTCAGTTCAAGTTGGGCAC 3000
W137Q_D1st23-TRF3    CATCATCATCATTGAGTTTGTAGCCTTAGACATGACTGTCCN---------------- 860
```

APPENDIX E

Sequencing result of dalcochinase mutant N181D

1. Sequencing result of dalcochinase mutant N181D using primer AOXF

NNAAACCTTGACTTACACACTTGAGAGATCAAAAACAACTAATTATTCGAAACGATGAGATTTCCTTCA
ATTTTTACTGCTGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTACAACAGAAGAT
GAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTCGATGTTGCT
GTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAATACTACTATTGCCAGCATTGCT
GCTAAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGGCTGAAGCTGCACATCACCATCACCATCATCAC
CATGCTGCAGTTCCTCCATTCAACCGAAGCTGTTTTCCTTCAGATTTCATTTTTGGGACAGCATCCTCC
TCGTACCAGTATGAAGGTGAGGGCAGAGTACCAAGTATATGGGATAACTTCACCCACCAATATCCAGAA
AAGATAGCGGATAGAAGCAACGGAGATGTTGCAGTTGACCAATTTCACCGCTATAAGAAGGATATTGCA
ATCATGAAGGATATGAACTTGGATGCTTATAGAATGTCCATCTCCTGGCCTAGAATTCTCCCAACGGGT
AGGGTTAGTGGAGGCATAAACCAAACAGGAGTTGACTACTACAACAGGCTCATCAATGAGTCACTGGCC
AATGGCATAACACCATTTGTAACCATTTTTCATTGGGATCTTCCACAAGCCTTGGAGGATGAGTACGGT
GGCTTCTTAAATCATAGCGTTGTAAATGATTTCCAAGACTATGCGGATCTTTGCTTCCAATTATTTGGA
GATAGGGTAAAGCATTGGATTACA

Expected sequence : The expected DNA sequence of dalcochinase mutant N181D

N181D_D1st13-AOXF : The sequencing result of dalcochinase mutant N181D using primer AOXF

```
Expected sequence              TTGCGACTGGTTCCAATTGACAAGCTTTTGATTTTAACGACTTTTAACGA 900
N181D_D1st13-AOXF              ----------------------------------NNAAACCTTGACTTA 15

Expected sequence              CA-ACTTGAGAAGATCAAAAAACAACTAATTATTCGAAACGATGAGATTT 949
N181D_D1st13-AOXF              CACACTTGAGA-GATCAAAAA-CAACTAATTATTCGAAACGATGAGATTT 63

Expected sequence              CCTTCAATTTTTACTGCTGTTTTATTCGCAGCATCCTCCGCATTAGCTGC 999
N181D_D1st13-AOXF              CCTTCAATTTTTACTGCTGTTTTATTCGCAGCATCCTCCGCATTAGCTGC 113

Expected sequence              TCCAGTCAACACTACAACAGAAGATGAAACGGCACAAATTCCGGCTGAAG 1049
N181D_D1st13-AOXF              TCCAGTCAACACTACAACAGAAGATGAAACGGCACAAATTCCGGCTGAAG 163

Expected sequence              CTGTCATCGGTTACTCAGATTTAGAAGGGGATTTCGATGTTGCTGTTTTG 1099
N181D_D1st13-AOXF              CTGTCATCGGTTACTCAGATTTAGAAGGGGATTTCGATGTTGCTGTTTTG 213

Expected sequence              CCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAATACTACTAT 1149
N181D_D1st13-AOXF              CCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAATACTACTAT 263

Expected sequence              TGCCAGCATTGCTGCTAAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGG 1199
N181D_D1st13-AOXF              TGCCAGCATTGCTGCTAAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGG 313

Expected sequence              CTGAAGCTGCACATCACCATCACCATCATCACCATGCTGCAGTTCCTCCA 1249
N181D_D1st13-AOXF              CTGAAGCTGCACATCACCATCACCATCATCACCATGCTGCAGTTCCTCCA 363

Expected sequence              TTCAACCGAAGCTGTTTTCCTTCAGATTTCATTTTTGGGACAGCATCCTC 1299
N181D_D1st13-AOXF              TTCAACCGAAGCTGTTTTCCTTCAGATTTCATTTTTGGGACAGCATCCTC 413

Expected sequence              CTCGTACCAGTATGAAGGTGAGGGCAGAGTACCAAGTATATGGGATAACT 1349
N181D_D1st13-AOXF              CTCGTACCAGTATGAAGGTGAGGGCAGAGTACCAAGTATATGGGATAACT 463

Expected sequence              TCACCCACCAATATCCAGAAAAGATAGCGGATAGAAGCAACGGAGATGTT 1399
N181D_D1st13-AOXF              TCACCCACCAATATCCAGAAAAGATAGCGGATAGAAGCAACGGAGATGTT 513

Expected sequence              GCAGTTGACCAATTTCACCGCTATAAGAAGGATATTGCAATCATGAAGGA 1449
N181D_D1st13-AOXF              GCAGTTGACCAATTTCACCGCTATAAGAAGGATATTGCAATCATGAAGGA 563

Expected sequence              TATGAACTTGGATGCTTATAGAATGTCCATCTCCTGGCCTAGAATTCTCC 1499
N181D_D1st13-AOXF              TATGAACTTGGATGCTTATAGAATGTCCATCTCCTGGCCTAGAATTCTCC 613
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Expected sequence              CAACGGGTAGGGTTAGTGGAGGCATAAACCAAACAGGAGTTGACTACTAC 1549
N181D_D1st13-AOXF              CAACGGGTAGGGTTAGTGGAGGCATAAACCAAACAGGAGTTGACTACTAC 663

Expected sequence              AACAGGCTCATCAATGAGTCACTGGCCAATGGCATAACACCATTTGTAAC 1599
N181D_D1st13-AOXF              AACAGGCTCATCAATGAGTCACTGGCCAATGGCATAACACCATTTGTAAC 713

Expected sequence              CATTTTTCATTGGGATCTTCCACAAGCCTTGGAGGATGAGTACGGTGGCT 1649
N181D_D1st13-AOXF              CATTTTTCATTGGGATCTTCCACAAGCCTTGGAGGATGAGTACGGTGGCT 763

Expected sequence              TCTTAAATCATAGCGTTGTAAATGATTTCCAAGACTATGCGGATCTTTGC 1699
N181D_D1st13-AOXF              TCTTAAATCATAGCGTTGTAAATGATTTCCAAGACTATGCGGATCTTTGC 813

Expected sequence              TTCCAATTATTTGGAGATAGGGTAAAGCATTGGATTACACTAGATGAGCC 1749
N181D_D1st13-AOXF              TTCCAATTATTTGGAGATAGGGTAAAGCATTGGATTACA---------- 852
```

2. Sequencing result of dalcochinase mutant N181D using primer TRF1

```
NNNNNGGGATTTGGCACATCTCCTCNTACAGTATGAAGGTGAGGGCAGAGTACCAAGTATATGGGATAA
CTTCACCCACCAATATCCAGAAAAGATAGCGGATAGAAGCAACGGAGATGTTGCAGTTGACCAATTTCA
CCGCTATAAGAAGGATATTGCAATCATGAAGGATATGAACTTGGATGCTTATAGAATGTCCATCTCCTG
GCCTAGAATTCTCCCAACGGGTAGGGTTAGTGGAGGCATAAACCAAACAGGAGTTGACTACTACAACAG
GCTCATCAATGAGTCACTGGCCAATGGCATAACACCATTTGTAACCATTTTTCATTGGGATCTTCCACA
AGCCTTGGAGGATGAGTACGGTGGCTTCTTAAATCATAGCGTTGTAAATGATTTCCAAGACTATGCGGA
TCTTTGCTTCCAATTATTTGGAGATAGGGTAAAGCATTGGATTACACTAGATGAGCCATCAATCTTCAC
CGCGAATGGGTATGCATACGGTATGTTTGCACCAGGTCGATGTTCTCCATCGTACAATCCAACTTGCAC
AGGTGGGGATGCAGGAACAGAGACTTATCTGGTTGCGCACAACCTGATCCTTTCTCATGCAGCAACTGT
CCAAGTGTACAAAAGGAAGTATCAGGAACATCAGAAAGGTACAATAGGCATTTCCTTGCACGTAGTTTG
GGTTATACCGCTTTCAAATAGCACATCAGATCAAAATGCTACCCAGCGATATCTTGACTTCACATGTGG
ATGGTTTATGGACCCACTTACAGCAGGAAGGTATCCAGATAGCATGCAATATCTAGTTGGAGATCGATT
GCCTAAGTTTACTACAGATCAAGCCAAA
```

Expected sequence : The expected DNA sequence of dalcochinase mutant N181D

N181D_D1st13-TRF1 : The sequencing result of dalcochinase mutant N181D using primer TRF1

```
Expected sequence              TCAACCGAAGCTGTTTTCCTTCAGATTTCATTTTTGGGACAGCATCCTCC 1300
N181D_D1st13-TRF1              ----------------------NNNNNGGGATTTGGCACA----TCTCC 23
                                                      ***** ***     ****

Expected sequence              TCGTACCAGTATGAAGGTGAGGGCAGAGTACCAAGTATATGGGATAACTT 1350
N181D_D1st13-TRF1              TCNTAC-AGTATGAAGGTGAGGGCAGAGTACCAAGTATATGGGATAACTT 72

Expected sequence              CACCCACCAATATCCAGAAAAGATAGCGGATAGAAGCAACGGAGATGTTG 1400
N181D_D1st13-TRF1              CACCCACCAATATCCAGAAAAGATAGCGGATAGAAGCAACGGAGATGTTG 122

Expected sequence              CAGTTGACCAATTTCACCGCTATAAGAAGGATATTGCAATCATGAAGGAT 1450
N181D_D1st13-TRF1              CAGTTGACCAATTTCACCGCTATAAGAAGGATATTGCAATCATGAAGGAT 172

Expected sequence              ATGAACTTGGATGCTTATAGAATGTCCATCTCCTGGCCTAGAATTCTCCC 1500
N181D_D1st13-TRF1              ATGAACTTGGATGCTTATAGAATGTCCATCTCCTGGCCTAGAATTCTCCC 222

Expected sequence              AACGGGTAGGGTTAGTGGAGGCATAAACCAAACAGGAGTTGACTACTACA 1550
N181D_D1st13-TRF1              AACGGGTAGGGTTAGTGGAGGCATAAACCAAACAGGAGTTGACTACTACA 272

Expected sequence              ACAGGCTCATCAATGAGTCACTGGCCAATGGCATAACACCATTTGTAACC 1600
N181D_D1st13-TRF1              ACAGGCTCATCAATGAGTCACTGGCCAATGGCATAACACCATTTGTAACC 322

Expected sequence              ATTTTTCATTGGGATCTTCCACAAGCCTTGGAGGATGAGTACGGTGGCTT 1650
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N181D_D1st13-TRF1              ATTTTTCATTGGGATCTTCCACAAGCCTTGGAGGATGAGTACGGTGGCTT 372

Expected sequence              CTTAAATCATAGCGTTGTAAATGATTTCCAAGACTATGCGGATCTTTGCT 1700
N181D_D1st13-TRF1              CTTAAATCATAGCGTTGTAAATGATTTCCAAGACTATGCGGATCTTTGCT 422

Expected sequence              TCCAATTATTTGGAGATAGGGTAAAGCATTGGATTACACTAGATGAGCCA 1750
N181D_D1st13-TRF1              TCCAATTATTTGGAGATAGGGTAAAGCATTGGATTACACTAGATGAGCCA 472

Expected sequence              TCAATCTTCACCGCGAATGGGTATGCATACGGTATGTTTGCACCAGGTCG 1800
N181D_D1st13-TRF1              TCAATCTTCACCGCGAATGGGTATGCATACGGTATGTTTGCACCAGGTCG 522

Expected sequence              ATGTTCTCCATCGTACAATCCAACTTGCACAGGTGGGGATGCAGGAACAG 1850
N181D_D1st13-TRF1              ATGTTCTCCATCGTACAATCCAACTTGCACAGGTGGGGATGCAGGAACAG 572

Expected sequence              AGACTTATCTGGTTGCGCACAACCTGATCCTTTCTCATGCAGCAACTGTC 1900
N181D_D1st13-TRF1              AGACTTATCTGGTTGCGCACAACCTGATCCTTTCTCATGCAGCAACTGTC 622

Expected sequence              CAAGTGTACAAAAGGAAGTATCAGGAACATCAGAAAGGTACAATAGGCAT 1950
N181D_D1st13-TRF1              CAAGTGTACAAAAGGAAGTATCAGGAACATCAGAAAGGTACAATAGGCAT 672

Expected sequence              TTCCTTGCACGTAGTTTGGGTTATACCGCTTTCAAATAGCACATCAGATC 2000
N181D_D1st13-TRF1              TTCCTTGCACGTAGTTTGGGTTATACCGCTTTCAAATAGCACATCAGATC 722

Expected sequence              AAAATGCTACCCAGCGATATCTTGACTTCACATGTGGATGGTTTATGGAC 2050
N181D_D1st13-TRF1              AAAATGCTACCCAGCGATATCTTGACTTCACATGTGGATGGTTTATGGAC 772

Expected sequence              CCACTTACAGCAGGAAGGTATCCAGATAGCATGCAATATCTAGTTGGAGA 2100
N181D_D1st13-TRF1              CCACTTACAGCAGGAAGGTATCCAGATAGCATGCAATATCTAGTTGGAGA 822

Expected sequence              TCGATTGCCTAAGTTTACTACAGATCAAGCCAAATTAGTTAAGGGTTCAT 2150
N181D_D1st13-TRF1              TCGATTGCCTAAGTTTACTACAGATCAAGCCAAA---------------- 856
```

3. Sequencing result of dalcochinase mutant N181D using primer TRF2

```
NNNCCCGTCATTTGGAATAGGTAAGCATTGGATTAACTAGATGAGCCATCAATCTTCACCGCGAATGGG
TATGCATACGGTATGTTTGCACCAGGTCGATGTTCTCCATCGTACAATCCAACTTGCACAGGTGGGGAT
GCAGGAACAGAGACTTATCTGGTTGCGCACAACCTGATCCTTTCTCATGCAGCAACTGTCCAAGTGTAC
AAAAGGAAGTATCAGGAACATCAGAAAGGTACAATAGGCATTTCCTTGCACGTAGTTTGGGTTATACCG
CTTTCAAATAGCACATCAGATCAAAATGCTACCCAGCGATATCTTGACTTCACATGTGGATGGTTTATG
GACCCACTTACAGCAGGAAGGTATCCAGATAGCATGCAATATCTAGTTGGAGATCGATTGCCTAAGTTT
ACTACAGATCAAGCCAAATTAGTTAAGGGTTCATTTGATTTTATTGGACTAAACTATTACACCACTAAC
TATGCTACCAAATCAGATGCGTCAACATGCTGCCCACCTAGTTACCTCACAGATCCTCAAGTCACTCTC
TTACAGCAACGCAATGGGGTCTTTATAGGTCCAGTGACTCCCTCAGGATGGATGTGCATTTATCCAAAA
GGACTTCGAGATTTGTTGCTTTACTTCAAGGAAAAGTATAACAATCCTTTGGTTTACATCACTGAAAAT
GGTATAGATGAGAAGAATGATGCATCACTATCACTTGAGGAATCCTTGATAGACACTTATAGAATTGAT
AGTTATTATCGTCATCTCTTTTATGTTCGATATGCAATTAGGTCTGGCGCAAATGTGAAAGGATTTTTT
GCATGGTCATTGTTGGACAACTTTGA
```

Expected sequence : The expected DNA sequence of dalcochinase mutant N181D

N181D_D1st13-TRF2 : The sequencing result of dalcochinase mutant N181D using primer TRF2

```
Expected sequence              CTTAAATCATAGCGTTGTAAATGATTTCCAAGACTATGCGGATCTTTGCT 1700
N181D_D1st13-TRF2              ----------------------------------------------NN 2

Expected sequence              TCCAATTATTTGGAGATAGGGTAAAGCATTGGATTACACTAGATGAGCCA 1750
N181D_D1st13-TRF2              NCCCGTCATTTGGA-ATAGGT--AAGCATTGGATTA-ACTAGATGAGCCA 48
```

```
Expected sequence              TCAATCTTCACCGCGAATGGGTATGCATACGGTATGTTTGCACCAGGTCG 1800
N181D_D1st13-TRF2              TCAATCTTCACCGCGAATGGGTATGCATACGGTATGTTTGCACCAGGTCG 98

Expected sequence              ATGTTCTCCATCGTACAATCCAACTTGCACAGGTGGGGATGCAGGAACAG 1850
N181D_D1st13-TRF2              ATGTTCTCCATCGTACAATCCAACTTGCACAGGTGGGGATGCAGGAACAG 148

Expected sequence              AGACTTATCTGGTTGCGCACAACCTGATCCTTTCTCATGCAGCAACTGTC 1900
N181D_D1st13-TRF2              AGACTTATCTGGTTGCGCACAACCTGATCCTTTCTCATGCAGCAACTGTC 198

Expected sequence              CAAGTGTACAAAAGGAAGTATCAGGAACATCAGAAAGGTACAATAGGCAT 1950
N181D_D1st13-TRF2              CAAGTGTACAAAAGGAAGTATCAGGAACATCAGAAAGGTACAATAGGCAT 248

Expected sequence              TTCCTTGCACGTAGTTTGGGTTATACCGCTTTCAAATAGCACATCAGATC 2000
N181D_D1st13-TRF2              TTCCTTGCACGTAGTTTGGGTTATACCGCTTTCAAATAGCACATCAGATC 298

Expected sequence              AAAATGCTACCCAGCGATATCTTGACTTCACATGTGGATGGTTTATGGAC 2050
N181D_D1st13-TRF2              AAAATGCTACCCAGCGATATCTTGACTTCACATGTGGATGGTTTATGGAC 348

Expected sequence              CCACTTACAGCAGGAAGGTATCCAGATAGCATGCAATATCTAGTTGGAGA 2100
N181D_D1st13-TRF2              CCACTTACAGCAGGAAGGTATCCAGATAGCATGCAATATCTAGTTGGAGA 398

Expected sequence              TCGATTGCCTAAGTTTACTACAGATCAAGCCAAATTAGTTAAGGGTTCAT 2150
N181D_D1st13-TRF2              TCGATTGCCTAAGTTTACTACAGATCAAGCCAAATTAGTTAAGGGTTCAT 448

Expected sequence              TTGATTTTATTGGACTAAACTATTACACCACTAACTATGCTACCAAATCA 2200
N181D_D1st13-TRF2              TTGATTTTATTGGACTAAACTATTACACCACTAACTATGCTACCAAATCA 498

Expected sequence              GATGCGTCAACATGCTGCCCACCTAGTTACCTCACAGATCCTCAAGTCAC 2250
N181D_D1st13-TRF2              GATGCGTCAACATGCTGCCCACCTAGTTACCTCACAGATCCTCAAGTCAC 548

Expected sequence              TCTCTTACAGCAACGCAATGGGGTCTTTATAGGTCCAGTGACTCCCTCAG 2300
N181D_D1st13-TRF2              TCTCTTACAGCAACGCAATGGGGTCTTTATAGGTCCAGTGACTCCCTCAG 598

Expected sequence              GATGGATGTGCATTTATCCAAAAGGACTTCGAGATTTGTTGCTTTACTTC 2350
N181D_D1st13-TRF2              GATGGATGTGCATTTATCCAAAAGGACTTCGAGATTTGTTGCTTTACTTC 648

Expected sequence              AAGGAAAAGTATAACAATCCTTTGGTTTACATCACTGAAAATGGTATAGA 2400
N181D_D1st13-TRF2              AAGGAAAAGTATAACAATCCTTTGGTTTACATCACTGAAAATGGTATAGA 698

Expected sequence              TGAGAAGAATGATGCATCACTATCACTTGAGGAATCCTTGATAGACACTT 2450
N181D_D1st13-TRF2              TGAGAAGAATGATGCATCACTATCACTTGAGGAATCCTTGATAGACACTT 748

Expected sequence              ATAGAATTGATAGTTATTATCGTCATCTCTTTTATGTTCGATATGCAATT 2500
N181D_D1st13-TRF2              ATAGAATTGATAGTTATTATCGTCATCTCTTTTATGTTCGATATGCAATT 798

Expected sequence              AGGTCTGGCGCAAATGTGAAAGGATTTTTTGCATGGTCATTGTTGGACAA 2550
N181D_D1st13-TRF2              AGGTCTGGCGCAAATGTGAAAGGATTTTTTGCATGGTCATTGTTGGACAA 848

Expected sequence              CTTTGAATGGGCTGAGGGTTATACATCACGATTTGGATTATATTTTGTGA 2600
N181D_D1st13-TRF2              CTTTGA---------------------------------------------- 854
```

4. Sequencing result of dalcochinase mutant N181D using primer TRF3

NNNNNNAATAAGCAATAGTTAGGGTCATTTGATTTTATTGGACTAAACTATTACACCACTAACTATGCT
ACCAAATCAGATGCGTCAACATGCTGCCCACCTAGTTACCTCACAGATCCTCAAGTCACTCTCTTACAG
CAACGCAATGGGGTCTTTATAGGTCCAGTGACTCCCTCAGGATGGATGTGCATTTATCCAAAAGGACTT
CGAGATTTGTTGCTTTACTTCAAGGAAAAGTATAACAATCCTTTGGTTTACATCACTGAAAATGGTATA
GATGAGAAGAATGATGCATCACTATCACTTGAGGAATCCTTGATAGACACTTATAGAATTGATAGTTAT
TATCGTCATCTCTTTTATGTTCGATATGCAATTAGGTCTGGCGCAAATGTGAAAGGATTTTTTGCATGG
TCATTGTTGGACAACTTTGAATGGGCTGAGGGTTATACATCACGATTTGGATTATATTTTGTGAACTAC
ACTACTTTGAATAGATATCCCAAGCTCTCTGCAACATGGTTCAAGTATTTTCTGGCACGTGATCAAGAG
AGTGCTAAATTGGAAATTTTAGCACCAAAGGCAAGATGGAGCTTATCAACGATGATCAAGGAAGAAAAG
ACAAAACCCAAGTGGGGCATTGAAGGCTTTTGATCTAGATCTTAATCACTAGTGAATTCGCGGCCGCCT
GCAGTACGTAGAATTCACGTGGCCCAGCCGGCCGTCTCGGATCGGTACCTCGAGCCGCGGCGGCCGCCA
GCTTTCTAGAACAAAAACTCATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCATCATCATCATC
ATTGAGTTTGTAGCCTTAGACATGACT

Expected sequence : The expected DNA sequence of dalcochinase mutant N181D

N181D_D1st13-TRF3 : The sequencing result of dalcochinase mutant N181D using primer TRF3

```
Expected sequence            TCGATTGCCTAAGTTTACTACAGATCAAGCCAAATTAGTTAAGGGTTCAT 2150
N181D_D1st13-TRF3            ----------------NNNNNNAATAAGCAA---TAGTTAGGG--TCAT 28

Expected sequence            TTGATTTTATTGGACTAAACTATTACACCACTAACTATGCTACCAAATCA 2200
N181D_D1st13-TRF3            TTGATTTTATTGGACTAAACTATTACACCACTAACTATGCTACCAAATCA 78

Expected sequence            GATGCGTCAACATGCTGCCCACCTAGTTACCTCACAGATCCTCAAGTCAC 2250
N181D_D1st13-TRF3            GATGCGTCAACATGCTGCCCACCTAGTTACCTCACAGATCCTCAAGTCAC 128

Expected sequence            TCTCTTACAGCAACGCAATGGGGTCTTTATAGGTCCAGTGACTCCCTCAG 2300
N181D_D1st13-TRF3            TCTCTTACAGCAACGCAATGGGGTCTTTATAGGTCCAGTGACTCCCTCAG 178

Expected sequence            GATGGATGTGCATTTATCCAAAAGGACTTCGAGATTTGTTGCTTTACTTC 2350
N181D_D1st13-TRF3            GATGGATGTGCATTTATCCAAAAGGACTTCGAGATTTGTTGCTTTACTTC 228

Expected sequence            AAGGAAAAGTATAACAATCCTTTGGTTTACATCACTGAAAATGGTATAGA 2400
N181D_D1st13-TRF3            AAGGAAAAGTATAACAATCCTTTGGTTTACATCACTGAAAATGGTATAGA 278

Expected sequence            TGAGAAGAATGATGCATCACTATCACTTGAGGAATCCTTGATAGACACTT 2450
N181D_D1st13-TRF3            TGAGAAGAATGATGCATCACTATCACTTGAGGAATCCTTGATAGACACTT 328

Expected sequence            ATAGAATTGATAGTTATTATCGTCATCTCTTTTATGTTCGATATGCAATT 2500
N181D_D1st13-TRF3            ATAGAATTGATAGTTATTATCGTCATCTCTTTTATGTTCGATATGCAATT 378

Expected sequence            AGGTCTGGCGCAAATGTGAAAGGATTTTTTGCATGGTCATTGTTGGACAA 2550
N181D_D1st13-TRF3            AGGTCTGGCGCAAATGTGAAAGGATTTTTTGCATGGTCATTGTTGGACAA 428

Expected sequence            CTTTGAATGGGCTGAGGGTTATACATCACGATTTGGATTATATTTTGTGA 2600
N181D_D1st13-TRF3            CTTTGAATGGGCTGAGGGTTATACATCACGATTTGGATTATATTTTGTGA 478

Expected sequence            ACTACACTACTTTGAATAGATATCCCAAGCTCTCTGCAACATGGTTCAAG 2650
N181D_D1st13-TRF3            ACTACACTACTTTGAATAGATATCCCAAGCTCTCTGCAACATGGTTCAAG 528

Expected sequence            TATTTTCTGGCACGTGATCAAGAGAGTGCTAAATTGGAAATTTTAGCACC 2700
N181D_D1st13-TRF3            TATTTTCTGGCACGTGATCAAGAGAGTGCTAAATTGGAAATTTTAGCACC 578

Expected sequence            AAAGGCAAGATGGAGCTTATCAACGATGATCAAGGAAGAAAAGACAAAAC 2750
N181D_D1st13-TRF3            AAAGGCAAGATGGAGCTTATCAACGATGATCAAGGAAGAAAAGACAAAAC 628

Expected sequence            CCAAGTGGGGCATTGAAGGCTTTTGATCTAGATCTTAATCACTAGTGAAT 2800
N181D_D1st13-TRF3            CCAAGTGGGGCATTGAAGGCTTTTGATCTAGATCTTAATCACTAGTGAAT 678

Expected sequence            TCGCGGCCGCCTGCAGTACGTAGAATTCACGTGGCCCAGCCGGCCGTCTC 2850
N181D_D1st13-TRF3            TCGCGGCCGCCTGCAGTACGTAGAATTCACGTGGCCCAGCCGGCCGTCTC 728

Expected sequence            GGATCGGTACCTCGAGCCGCGGCGGCCGCCAGCTTTCTAGAACAAAAACT 2900
N181D_D1st13-TRF3            GGATCGGTACCTCGAGCCGCGGCGGCCGCCAGCTTTCTAGAACAAAAACT 778

Expected sequence            CATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCATCATCATCATC 2950
N181D_D1st13-TRF3            CATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCATCATCATCATC 828

Expected sequence            ATTGAGTTTGTAGCCTTAGACATGACTGTTCCTCAGTTCAAGTTGGGCAC 3000
N181D_D1st13-TRF3            ATTGAGTTTGTAGCCTTAGACATGACT---------------------- 855
```

APPENDIX F

Sequencing result of dalcochinase mutant M369E

1. Sequencing result of dalcochinase mutant M369E using primer AOXF

ATTTAATTTAGCACTTGAGAGATCAAAAACAACTAATTATTCGAAACGATGAGATTTCCTTCACTTTTT
ACTGCTGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTACAACAGAAGATGAAACG
GCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTCGATGTTGCTGTTTTG
CCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAATACTACTATTGCCAGCATTGCTGCTAAA
GAAGAAGGGGTATCTCTCGAGAAAAGAGAGGCTGAAGCTGCACATCACCATCACCATCATCACCATGCT
GCAGTTCCTCCATTCAACCGAAGCTGTTTTCCTTCAGATTTCATTTTTGGGACAGCATCCTCCTCGTAC
CAGTATGAAGGTGAGGGCAGAGTACCAAGTATATGGGATAACTTCACCCACCAATATCCAGAAAAGATA
GCGGATAGAAGCAACGGAGATGTTGCAGTTGACCAATTTCACCGCTATAAGAAGGATATTGCAATCATG
AAGGATATGAACTTGGATGCTTATAGAATGTCCATCTCCTGGCCTAGAATTCTCCCAACGGGTAGGGTT
AGTGGAGGCATAAACCAAACAGGAGTTGACTACTACAACAGGCTCATCAATGAGTCACTGGCCAATGGC
ATAACACCATTTGTAACCATTTTTCATTGGGATCTTCCACAAGCCTTGGAGGATGAGTACGGTGGCTTC
TTAAATCATAGCGTTGTAAATGATTTCCAGACTATGCGGATCTTTGCTTCCCATTATTTGGAGATAGGG
TAAAGCATTGG

Expected sequence : The expected DNA sequence of dalcochinase mutant M369E

M369E_D1st23-AOXF : The sequencing result of dalcochinase mutant M369E using primer AOXF

```
Expected sequence    ACTTTCATAATTGCGACTGGTTCCAATTGACAAGCTTTTGATTTTAACGACTTTTAACGA 900
M369E_D1st23-AOXF    ----------------------------------------------------ATTTAATTTA 10

Expected sequence    CAACTTGAGAAGATCAAAAAACAACTAATTATTCGAAACGATGAGATTTCCTTCAATTTT 960
M369E_D1st23-AOXF    GCACTTGAGA-GATCAAAAA-CAACTAATTATTCGAAACGATGAGATTTCCTTCACTTTT 68

Expected sequence    TACTGCTGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTACAACAGA 1020
M369E_D1st23-AOXF    TACTGCTGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTACAACAGA 128

Expected sequence    AGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGA 1080
M369E_D1st23-AOXF    AGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGA 188

Expected sequence    TTTCGATGTTGCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAA 1140
M369E_D1st23-AOXF    TTTCGATGTTGCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAA 248

Expected sequence    TACTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGGC 1200
M369E_D1st23-AOXF    TACTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGGC 308

Expected sequence    TGAAGCTGCACATCACCATCACCATCATCACCATGCTGCAGTTCCTCCATTCAACCGAAG 1260
M369E_D1st23-AOXF    TGAAGCTGCACATCACCATCACCATCATCACCATGCTGCAGTTCCTCCATTCAACCGAAG 368

Expected sequence    CTGTTTTCCTTCAGATTTCATTTTTGGGACAGCATCCTCCTCGTACCAGTATGAAGGTGA 1320
M369E_D1st23-AOXF    CTGTTTTCCTTCAGATTTCATTTTTGGGACAGCATCCTCCTCGTACCAGTATGAAGGTGA 428

Expected sequence    GGGCAGAGTACCAAGTATATGGGATAACTTCACCCACCAATATCCAGAAAAGATAGCGGA 1380
M369E_D1st23-AOXF    GGGCAGAGTACCAAGTATATGGGATAACTTCACCCACCAATATCCAGAAAAGATAGCGGA 488

Expected sequence    TAGAAGCAACGGAGATGTTGCAGTTGACCAATTTCACCGCTATAAGAAGGATATTGCAAT 1440
M369E_D1st23-AOXF    TAGAAGCAACGGAGATGTTGCAGTTGACCAATTTCACCGCTATAAGAAGGATATTGCAAT 548

Expected sequence    CATGAAGGATATGAACTTGGATGCTTATAGAATGTCCATCTCCTGGCCTAGAATTCTCCC 1500
M369E_D1st23-AOXF    CATGAAGGATATGAACTTGGATGCTTATAGAATGTCCATCTCCTGGCCTAGAATTCTCCC 608

Expected sequence    AACGGGTAGGGTTAGTGGAGGCATAAACCAAACAGGAGTTGACTACTACAACAGGCTCAT 1560
M369E_D1st23-AOXF    AACGGGTAGGGTTAGTGGAGGCATAAACCAAACAGGAGTTGACTACTACAACAGGCTCAT 668

Expected sequence    CAATGAGTCACTGGCCAATGGCATAACACCATTTGTAACCATTTTTCATTGGGATCTTCC 1620
M369E_D1st23-AOXF    CAATGAGTCACTGGCCAATGGCATAACACCATTTGTAACCATTTTTCATTGGGATCTTCC 728
```

```
Expected sequence    ACAAGCCTTGGAGGATGAGTACGGTGGCTTCTTAAATCATAGCGTTGTAAATGATTTCCA 1680
M369E_D1st23-AOXF    ACAAGCCTTGGAGGATGAGTACGGTGGCTTCTTAAATCATAGCGTTGTAAATGATTTCCA 788

Expected sequence    AGACTATGCGGATCTTTGCTTCCAATTATTTGGAGATAGGGTAAAGCATTGGATTACACT 1740
M369E_D1st23-AOXF    -GACTATGCGGATCTTTGCTTCCCATTATTTGGAGATAGGGTAAAGCATTGG-------- 839
```

2. Sequencing result of dalcochinase mutant M369E using primer TRF1

AATTGGCACATCTCCTCGTACACTATGAAGGTGAGGGCAGAGTACCAGTATATGGGATAACTTCACCCA
CCAATATCCAGAAAAGATAGTCGCATAGAAGCAACGGAGATGTTGCAGTTGACCAATTTCACCGCTATA
AGAAGGATATTGCAATCATGAAGGATATGAACTTGGATGCTTATAGAATGTCCATCTCCTGGCCTAGAA
TTCTCCCAACGGGTAGGGTTAGTGGAGGCATAAACCAAACAGGAGTTGACTACTACAACAGGCTCATCA
ATGAGTCACTGGCCAATGGCATAACACCATTTGTAACCATTTTTCATTGGGATCTTCCACAAGCCTTGG
AGGATGAGTACGGTGGCTTCTTAAATCATAGCGTTGTAAATGATTTCCAAGACTATGCGGATCTTTGCT
TCCAATTATTTGGAGATAGGGTAAAGCATTGGATTACACTAAATGAGCCATCAATCTTCACCGCCAATG
GGTATGCATACGGTATGTTTGCACCAGGTCGATGTTCTCCATCGTACAATCCAACTTGCACAGGTGGGG
ATGCATGAACAGACACTTATCTGGTTGCGCACAACCTGATCCTTTCTCATGCAGCAACTGTCCAAGTGT
ACAATACGAAGTATCATGAACCTCCCAAAGGTACAATAGGCAATTCCTTGCACGTATTCTGGGTTATAC
CGCTTTCAAATATCCCATTCCAATCAAAATGCTACCCCAACGATATCTTGGAATTCACCTGTGGGATGG
GTTTAGGGACCCCCCCTACAACCAGAAAGTAATCCAATAACACGCCAAATCTAGTTGGAAA

Expected sequence : The expected DNA sequence of dalcochinase mutant M369E

M369E_D1st23-TRF1 : The sequencing result of dalcochinase mutant M369E using primer TRF1

```
Expected sequence    CTGTTTTCCTTCAGATTTCATTTTTGGGACAGCATCCTCCTCGTACCAGTATGAAGGTGA 1320
M369E_D1st23-TRF1    --------------------AATTGGCACA----TCTCCTCGTAC-ACTATGAAGGTGA 34

Expected sequence    GGGCAGAGTACCAAGTATATGGGATAACTTCACCCACCAATATCCAGAAAAGATAG-CGG 1379
M369E_D1st23-TRF1    GGGCAGAGTACCA-GTATATGGGATAACTTCACCCACCAATATCCAGAAAAGATAGTCGC 93

Expected sequence    ATAGAAGCAACGGAGATGTTGCAGTTGACCAATTTCACCGCTATAAGAAGGATATTGCAA 1439
M369E_D1st23-TRF1    ATAGAAGCAACGGAGATGTTGCAGTTGACCAATTTCACCGCTATAAGAAGGATATTGCAA 153

Expected sequence    TCATGAAGGATATGAACTTGGATGCTTATAGAATGTCCATCTCCTGGCCTAGAATTCTCC 1499
M369E_D1st23-TRF1    TCATGAAGGATATGAACTTGGATGCTTATAGAATGTCCATCTCCTGGCCTAGAATTCTCC 213

Expected sequence    CAACGGGTAGGGTTAGTGGAGGCATAAACCAAACAGGAGTTGACTACTACAACAGGCTCA 1559
M369E_D1st23-TRF1    CAACGGGTAGGGTTAGTGGAGGCATAAACCAAACAGGAGTTGACTACTACAACAGGCTCA 273

Expected sequence    TCAATGAGTCACTGGCCAATGGCATAACACCATTTGTAACCATTTTTCATTGGGATCTTC 1619
M369E_D1st23-TRF1    TCAATGAGTCACTGGCCAATGGCATAACACCATTTGTAACCATTTTTCATTGGGATCTTC 333

Expected sequence    CACAAGCCTTGGAGGATGAGTACGGTGGCTTCTTAAATCATAGCGTTGTAAATGATTTCC 1679
M369E_D1st23-TRF1    CACAAGCCTTGGAGGATGAGTACGGTGGCTTCTTAAATCATAGCGTTGTAAATGATTTCC 393

Expected sequence    AAGACTATGCGGATCTTTGCTTCCAATTATTTGGAGATAGGGTAAAGCATTGGATTACAC 1739
M369E_D1st23-TRF1    AAGACTATGCGGATCTTTGCTTCCAATTATTTGGAGATAGGGTAAAGCATTGGATTACAC 453

Expected sequence    TAAATGAGCCATCAATCTTCACCGCGAATGGGTATGCATACGGTATGTTTGCACCAGGTC 1799
M369E_D1st23-TRF1    TAAATGAGCCATCAATCTTCACCGCCAATGGGTATGCATACGGTATGTTTGCACCAGGTC 513

Expected sequence    GATGTTCTCCATCGTACAATCCAACTTGCACAGGTGGGGATGCAGGAACAGAGACTTATC 1859
M369E_D1st23-TRF1    GATGTTCTCCATCGTACAATCCAACTTGCACAGGTGGGGATGCATGAACAGACACTTATC 573


Expected sequence    TGGTTGCGCACAACCTGATCCTTTCTCATGCAGCAACTGTCCAAGTGTACAAAAGGAAGT 1919
M369E_D1st23-TRF1    TGGTTGCGCACAACCTGATCCTTTCTCATGCAGCAACTGTCCAAGTGTACAATACGAAGT 633
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Expected sequence    ATCAGGAACATCAGAAAGGTACAATAGGCATTTCCTTGCACGTAGTTTGGGTTATACCGC 1979
M369E_D1st23-TRF1    ATCATGAACCTCCCAAAGGTACAATAGGCAATTCCTTGCACGTATTCTGGGTTATACCGC 693

Expected sequence    TTTCAAATAGCACAT-CAGATCAAAATGCTACCC-AGCGATATCTTG-ACTTCACATGTG 2036
M369E_D1st23-TRF1    TTTCAAATATCCCATTCCAATCAAAATGCTACCCCAACGATATCTTGGAATTCACCTGTG 753

Expected sequence    GAT--GGTTTATGGACCCACT-TACAGCAGGAAGGTATCCAGATAGCATGCAATATCTAG 2093
M369E_D1st23-TRF1    GGATGGGTTTAGGGACCCCCCCTACAACCAGAAAGTAATCCAATAACACGCCAAATCTAG 813

Expected sequence    TTGGAGATCGATTGCCTAAGTTTACTACAGATCAAGCCAAATTAGTTAAGGGTTCATTTG 2153
M369E_D1st23-TRF1    TTGGAAA------------------------------------------------------ 820
```

3. Sequencing result of dalcochinase mutant M369E using primer TRF2

```
CATATATTGGAATAGGTAAGCATTGGATTACACTAAATGAGCCATCAATCTTCACCGCGAATGGGTATG
CATACGGTATGTTTGCACCAGGTCGATGTTCTCCATCGTACAATCCAACTTGCACAGGTGGGGATGCAC
GAACAGAGACTTATCTGGTTGCGCACAACCTGATCCTTTCTCATGCAGCAACTGTCCAAGTGTACAAAA
GGAAGTATCAGGAACATCAGAAAGGTACAATAGGCATTTCCTTGCACGTACTTTGGGTTATACCGCTTT
CAAATAGCACATCAGATCAAAATGCTACCCAGCGATATCTTGACTTCACATGTGGATGGTTTATGGACC
CACTTACATCACGAAGGTATCCAGATAGCCATGCAATATCTAGTTGGAGATCGATTGCCTAACTTTACT
ACAGATCAAGCCAAATTAGTTAAGGGTTCATTTGATTTTATTGGACTAAACTATTACACCACTAACTAT
GCTACCAAATCAGATGCGTCAACATGCTGCCCACCTAGTTACCTCACAGATCCTCAAGTCACTCTCTTA
CAGCAACACAATGGGGTCTTTATAGGTCCAGTGACTCCCTCAGGATGGGAATGCATTTATCCTAAAGGA
CTTCCAGATTTGTTGCTTTTACTTCAAGGAAAAGTATAACACTCCTTTGGGTTACATCACTGACAATGG
TATTAGATGAGAAGAAAGATGCATCACTATCCCTTGAAGAATCCTTTGAAAGACACCTACTAAAATTGA
AAGCTATTTACCCCACCCCTTTTATGCTCCCAAAATGAATTTAGGCTTGGCTCAAAATGTGAAAGGATC
```

Expected sequence : The expected DNA sequence of dalcochinase mutant M369E

M369E_D1st23-TRF2 : The sequencing result of dalcochinase mutant M369E using primer TRF2

```
Expected sequence    AGACTATGCGGATCTTTGCTTCCAATTATTTGGAGATAGGGTAAAGCATTGGATTACACT 1740
M369E_D1st23-TRF2    ----------------------CATATATTGGAATAGGT--AAGCATTGGATTACACT 34

Expected sequence    AAATGAGCCATCAATCTTCACCGCGAATGGGTATGCATACGGTATGTTTGCACCAGGTCG 1800
M369E_D1st23-TRF2    AAATGAGCCATCAATCTTCACCGCGAATGGGTATGCATACGGTATGTTTGCACCAGGTCG 94

Expected sequence    ATGTTCTCCATCGTACAATCCAACTTGCACAGGTGGGGATGCAGGAACAGAGACTTATCT 1860
M369E_D1st23-TRF2    ATGTTCTCCATCGTACAATCCAACTTGCACAGGTGGGGATGCACGAACAGAGACTTATCT 154

Expected sequence    GGTTGCGCACAACCTGATCCTTTCTCATGCAGCAACTGTCCAAGTGTACAAAAGGAAGTA 1920
M369E_D1st23-TRF2    GGTTGCGCACAACCTGATCCTTTCTCATGCAGCAACTGTCCAAGTGTACAAAAGGAAGTA 214

Expected sequence    TCAGGAACATCAGAAAGGTACAATAGGCATTTCCTTGCACGTAGTTTGGGTTATACCGCT 1980
M369E_D1st23-TRF2    TCAGGAACATCAGAAAGGTACAATAGGCATTTCCTTGCACGTACTTTGGGTTATACCGCT 274

Expected sequence    TTCAAATAGCACATCAGATCAAAATGCTACCCAGCGATATCTTGACTTCACATGTGGATG 2040
M369E_D1st23-TRF2    TTCAAATAGCACATCAGATCAAAATGCTACCCAGCGATATCTTGACTTCACATGTGGATG 334

Expected sequence    GTTTATGGACCCACTTACAGCAGGAAGGTATCCAGATAGC-ATGCAATATCTAGTTGGAG 2099
M369E_D1st23-TRF2    GTTTATGGACCCACTTACATCACGAAGGTATCCAGATAGCCATGCAATATCTAGTTGGAG 394

Expected sequence    ATCGATTGCCTAAGTTTACTACAGATCAAGCCAAATTAGTTAAGGGTTCATTTGATTTTA 2159
M369E_D1st23-TRF2    ATCGATTGCCTAACTTTACTACAGATCAAGCCAAATTAGTTAAGGGTTCATTTGATTTTA 454
Expected sequence    TTGGACTAAACTATTACACCACTAACTATGCTACCAAATCAGATGCGTCAACATGCTGCC 2219
M369E_D1st23-TRF2    TTGGACTAAACTATTACACCACTAACTATGCTACCAAATCAGATGCGTCAACATGCTGCC 514
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Expected sequence    CACCTAGTTACCTCACAGATCCTCAAGTCACTCTCTTACAGCAACGCAATGGGGTCTTTA 2279
M369E_D1st23-TRF2    CACCTAGTTACCTCACAGATCCTCAAGTCACTCTCTTACAGCAACACAATGGGGTCTTTA 574

Expected sequence    TAGGTCCAGTGACTCCCTCAGGATGGGAATGCATTTATCCAAAAGGACTTCGAGATTTGT 2339
M369E_D1st23-TRF2    TAGGTCCAGTGACTCCCTCAGGATGGGAATGCATTTATCCTAAAGGACTTCCAGATTTGT 634

Expected sequence    TGCTTT-ACTTCAAGGAAAAGTATAACAATCCTTTGGTTTACATCACTGAAAATGGTAT- 2397
M369E_D1st23-TRF2    TGCTTTTACTTCAAGGAAAAGTATAACACTCCTTTGGGTTACATCACTGACAATGGTATT 694

Expected sequence    AGATGAGAAGAATGATGCATCACTATCACTTGAGGAATCCTT-GATAGACACTTA-TAGA 2455
M369E_D1st23-TRF2    AGATGAGAAGAAAGATGCATCACTATCCCTTGAAGAATCCTTTGAAAGACACCTACTAAA 754

Expected sequence    ATTGATAGTTATTATCGTCATCTCTTTTATGTT--CGATATGCAATTAGGTCTGGCGCAA 2513
M369E_D1st23-TRF2    ATTGAAAGCTATTTACCCCACCCCTTTTATGCTCCCAAAATGAATTTAGGCTTGGCTCAA 814

Expected sequence    A-TGTGAAAGGATTTTTTGCATGGTCATTGTTGGACAACTTTGAATGGGCTGAGGGTTAT 2572
M369E_D1st23-TRF2    AATGTGAAAGGATC---------------------------------------------- 828
```

4. Sequencing result of dalcochinase mutant M369E using primer TRF3

```
NTATTAGCAATAGTTAGGGTTCATTTGATTTTATTGGACTAAACTATTACACCACTAACTATGCTACCA
AATCAGATGCGTCAACATGCTGCCCACCTAGTTACCTCACAGATCCTCAAGTCACTCTCTTACAGCAAC
GCAATGGGGTCTTTATAGGTCCAGTGACTCCCTCAGGATGGGAATGCATTTATCCAAAAGGACTTCGAG
ATTTGTTGCTTTACTTCAAGGAAAAGTATAACAATCCTTTGGTTTACATCACTGAAAATGGTATAGATG
AGAAGAATGATGCATCACTATCACTTGAGGAATCCTTGATAGACACTTATAGAATTGATAGTTATTATC
GTCATCTCTTTTATGTTCGATATGCAATTAGGTCTGGCGCAAATGTGAAAGGATTTTTTGCATGGTCAT
TGTTGGACAACTTTGAATGGGCTGAGGGTTATACATCACGATTTGGATTATATTTTGTGAACTACACTA
CTTTGAATAGATATCCCAAGCTCTCTGCAACATGGTTCAAGTATTTTCTGGCACGTGATCAAGAGAGTG
CTAAATTGGAAATTTTAGCACCAAAGGCAAGATGGAGCTTATCAACGATGATCAAGGAAGAAAAGACAA
AACCCAAGTGGGGCATTGAAGGCTTTTGATCTAGATCTTAATCACTAGTGAATTCGCGGCCGCCTGCAG
TACGTAGAATTCACGTGGCCCAGCCGGCCGTCTCGGATCGGTACCTCGAGCCGCGGCGGCCGCCAGCTT
TCTAGAACAAAAACTCATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCATCATCATCATCATTG
AGTTTGTAGCCTTAGACATGACC
```

Expected sequence : The expected DNA sequence of dalcochinase mutant M369E

M369E_D1st23-TRF3 : The sequencing result of dalcochinase mutant M369E using primer TRF3

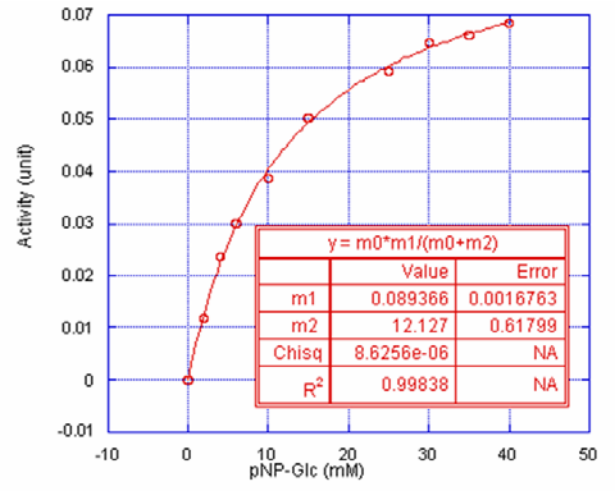
```
Expected sequence    TCGATTGCCTAAGTTTACTACAGATCAAGCCAAATTAGTTAAGGGTTCATTTGATTTTAT 2160
M369E_D1st23-TRF3    ------------------------NTATTAGCAATAGTTA-GGGTTCATTTGATTTTAT 34

Expected sequence    TGGACTAAACTATTACACCACTAACTATGCTACCAAATCAGATGCGTCAACATGCTGCCC 2220
M369E_D1st23-TRF3    TGGACTAAACTATTACACCACTAACTATGCTACCAAATCAGATGCGTCAACATGCTGCCC 94

Expected sequence    ACCTAGTTACCTCACAGATCCTCAAGTCACTCTCTTACAGCAACGCAATGGGGTCTTTAT 2280
M369E_D1st23-TRF3    ACCTAGTTACCTCACAGATCCTCAAGTCACTCTCTTACAGCAACGCAATGGGGTCTTTAT 154

Expected sequence    AGGTCCAGTGACTCCCTCAGGATGGGAATGCATTTATCCAAAAGGACTTCGAGATTTGTT 2340
M369E_D1st23-TRF3    AGGTCCAGTGACTCCCTCAGGATGGGAATGCATTTATCCAAAAGGACTTCGAGATTTGTT 214

Expected sequence    GCTTTACTTCAAGGAAAAGTATAACAATCCTTTGGTTTACATCACTGAAAATGGTATAGA 2400
M369E_D1st23-TRF3    GCTTTACTTCAAGGAAAAGTATAACAATCCTTTGGTTTACATCACTGAAAATGGTATAGA 274

Expected sequence    TGAGAAGAATGATGCATCACTATCACTTGAGGAATCCTTGATAGACACTTATAGAATTGA 2460
M369E_D1st23-TRF3    TGAGAAGAATGATGCATCACTATCACTTGAGGAATCCTTGATAGACACTTATAGAATTGA 334

Expected sequence    TAGTTATTATCGTCATCTCTTTTATGTTCGATATGCAATTAGGTCTGGCGCAAATGTGAA 2520
M369E_D1st23-TRF3    TAGTTATTATCGTCATCTCTTTTATGTTCGATATGCAATTAGGTCTGGCGCAAATGTGAA 394
```

```
Expected sequence    AGGATTTTTTGCATGGTCATTGTTGGACAACTTTGAATGGGCTGAGGGTTATACATCACG 2580
M369E_D1st23-TRF3    AGGATTTTTTGCATGGTCATTGTTGGACAACTTTGAATGGGCTGAGGGTTATACATCACG 454

Expected sequence    ATTTGGATTATATTTTGTGAACTACACTACTTTGAATAGATATCCCAAGCTCTCTGCAAC 2640
M369E_D1st23-TRF3    ATTTGGATTATATTTTGTGAACTACACTACTTTGAATAGATATCCCAAGCTCTCTGCAAC 514

Expected sequence    ATGGTTCAAGTATTTTCTGGCACGTGATCAAGAGAGTGCTAAATTGGAAATTTTAGCACC 2700
M369E_D1st23-TRF3    ATGGTTCAAGTATTTTCTGGCACGTGATCAAGAGAGTGCTAAATTGGAAATTTTAGCACC 574

Expected sequence    AAAGGCAAGATGGAGCTTATCAACGATGATCAAGGAAGAAAAGACAAAACCCAAGTGGGG 2760
M369E_D1st23-TRF3    AAAGGCAAGATGGAGCTTATCAACGATGATCAAGGAAGAAAAGACAAAACCCAAGTGGGG 634

Expected sequence    CATTGAAGGCTTTTGATCTAGATCTTAATCACTAGTGAATTCGCGGCCGCCTGCAGTACG 2820
M369E_D1st23-TRF3    CATTGAAGGCTTTTGATCTAGATCTTAATCACTAGTGAATTCGCGGCCGCCTGCAGTACG 694

Expected sequence    TAGAATTCACGTGGCCCAGCCGGCCGTCTCGGATCGGTACCTCGAGCCGCGGCGGCCGCC 2880
M369E_D1st23-TRF3    TAGAATTCACGTGGCCCAGCCGGCCGTCTCGGATCGGTACCTCGAGCCGCGGCGGCCGCC 754

Expected sequence    AGCTTTCTAGAACAAAAACTCATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCAT 2940
M369E_D1st23-TRF3    AGCTTTCTAGAACAAAAACTCATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCAT 814

Expected sequence    CATCATCATCATTGAGTTTGTAGCCTTAGACATGACTGTTCCTCAGTTCAAGTTGGGCAC 3000
M369E_D1st23-TRF3    CATCATCATCATTGAGTTTGTAGCCTTAGACATGACC---------------------- 851
```

APPENDIX G

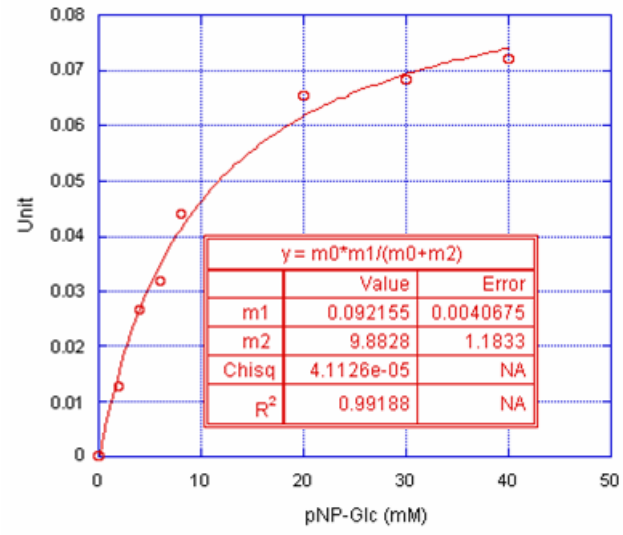
Michaelis-Menten plots

1. The Michaelis-Menten plot of natural dalcochinase toward *p*NP-Glc



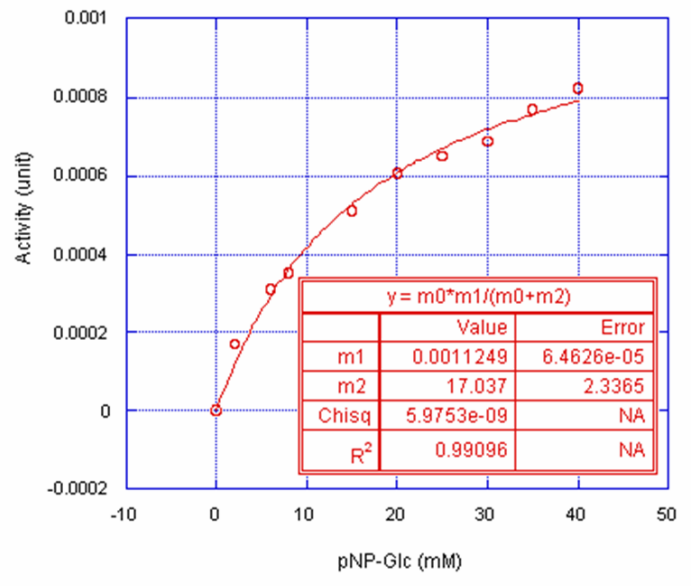
Appendix Figure G1 The Michaelis-Menten curve of natural dalcochinase toward *p*NP-Glc. The plot shows K_m and V_{max} of enzyme.

2. The Michaelis-Menten plot of recombinant dalcochinase toward *p*NP-Glc



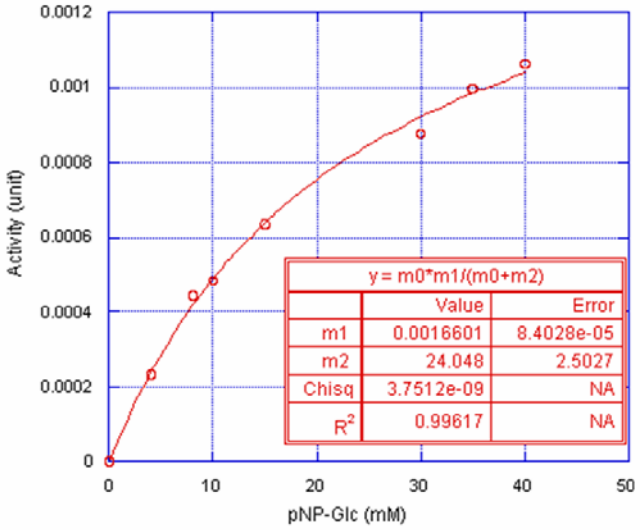
Appendix Figure G2 The Michaelis-Menten curve of recombinant dalcochinase toward *p*NP-Glc. The plot shows K_m and V_{max} of enzyme.

3. The Michaelis-Menten plot of dalcochinase mutant R90Q toward *p*NP-Glc



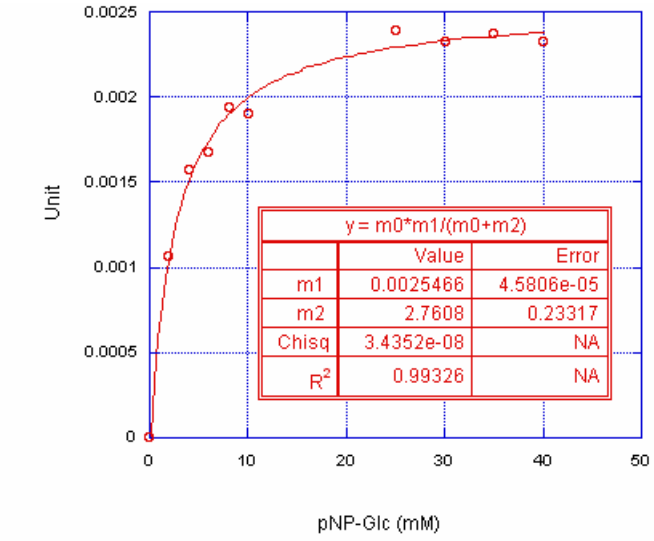
Appendix Figure G3 The Michaelis-Menten curve of dalcochinase mutant R90Q toward *p*NP-Glc. The plot shows K_m and V_{max} of enzyme.

4. The Michaelis-Menten plot of dalcochinase mutant W137Q toward *p*NP-Glc



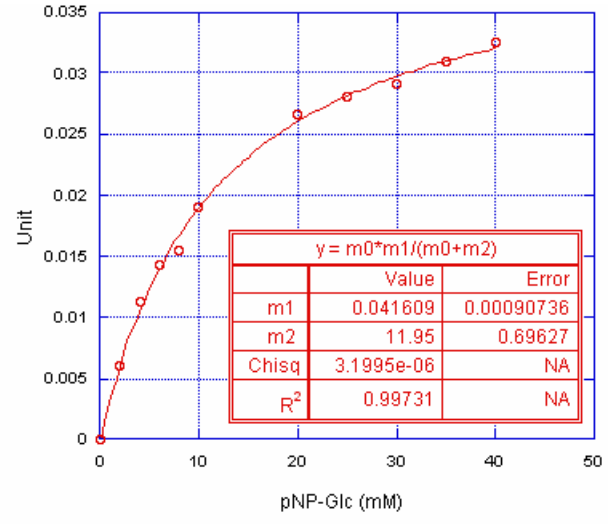
Appendix Figure G4 The Michaelis-Menten curve of dalcochinase mutant W137Q toward pNP-Glc. The plot shows K_m and V_{max} of enzyme.

5. The Michaelis-Menten plot of dalcochinase mutant N181D toward *p*NP-Glc



Appendix Figure G5 The Michaelis-Menten curve of dalcochinase mutant N181D toward *p*NP-Glc. The plot shows K_m and V_{max} of enzyme.

6. The Michaelis-Menten plot of dalcochinase mutant M369E toward *p*NP-Glc



Appendix Figure G6 The Michaelis-Menten curve of dalcochinase mutant M369E toward *p*NP-Glc. The plot shows K_m and V_{max} of enzyme.

BIOGRAPHY

NAME : Miss Khuanjarat Choengpanya

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WORK PLACE : -

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