# บทที่ 2 วิธีวิจัย

This chapter describes how to generate OsBADH1 and OsBADH2 mutants using site-directed mutagenesis. Both wild-type and mutant OsBADHs were overexpressed in *E. coli* system. The recombinant proteins were then purified to high yield. Co-factor $NAD^+$ binding study was carried out as well as kinetic studies towards two substrates, Bet-ald and GAB-ald. Additionally, homology modeling, molecular docking and molecular dynamics simulations were described at the end of the chapter.

## 2.1. Site-directed mutagenesis of OsBADH1 and OsBADH2

Mutations in the wild-type *OsBADH1* and *OsBADH2* gene were generated using site-directed mutagenesis which was used to make a point mutation, switch amino acids and delete or insert single or multiple amino acids. The mutagenic primers were designed by Quikchange® Primer Design Program from Stratagene. The sense and antisense primers were purchased from Pacific Science. All primer sequences were shown in Table 1. The appropriate length of each primer is 25-45 nucleotides, with a melting temperature ($T_m$) of ≥78 °C. The primers have the desired mutation in the middle of each primer with 10-15 bases of correct sequence on both sides. The mutagenic primers have a minimum GC content of 40% and terminate in one C or G bases. Polymerase chain reaction (PCR) was performed using QuikChange® Lightning Site-Directed Mutagenesis kit (Stratagene, USA) according to the manufacturer's instruction. The reaction mixtures contained 1x reaction buffer, 100 ng of dsDNA template, 125 ng of oligonucleotide sense and antisense primer, 1 µl of dNTP mix, 1.5 µl of QuikSolution reagent, 1 µl of QuikChange Lightning enzyme and milliQ water to final volume 50 µl. The PCR condition was shown in Table 2. After PCR cycle, 2 µl of *Dpn*I was added to digest parental plasmids and the reaction was incubated at 37 °C for 30 min. Finally, the *Dpn*I-treated DNA was analyzed on 0.8% agarose gel.

**Table 1** Primer sequences for site-directed mutagenesis. The underlined represent the mutated codon. The mutagenic primers include mutations (underlined) at the corresponding triplets (bold).

| Set of primer | Sequence 5′-3′ |
|---|---|
| N164A | |
| Sense | GGA CTT ATC ACT CCC TGG **GCT** TAT CCT CTG ATG GC |
| Antisense | GCC ATC AGA GGA TAA **GCC** CAG GGA GTG ATA AGT CC |
| W172A | |
| Sense | TCT GCT GAT GGC TAC T**GC** GAA GGT TGC ACC TGC C |
| Antisense | GGC AGG TGC AAC CTT **CGC** AGT AGC CAT CAG CAG A |
| W172F | |
| Sense | CCT CTG CTG ATG GCT ACT **TTC** AAG GTT GCA CCT GC |
| Antisense | GCA GGT GCA ACC TT**G AA**A GTA GCC ATC AGC AGA GG |
| N162A | |
| Sense | GGT TGA TCA CAC CTT GG**G CC**T ATC CTC TCC TGA TGG C |
| Antisense | GCC ATC AGG AGA GGA TA**G GC**C CAA GGT GTG ATC AAC C |
| W170A | |
| Sense | TCT CCT GAT GGC AAC A**GC** GAA GGT AGC TCC TGC C |
| Antisense | GGC AGG AGC TAC CTT **CGC** TGT TGC CAT CAG GAG A |
| W170F | |
| Sense | CCT CTC CTG ATG GCA ACA **TTC** AAG GTA GCT CCT GC |
| Antisense | GCA GGA GCT ACC TT**G AA**T GTT GCC ATC AGG AGA GG |

**Table 2** PCR condition for site-directed mutagenesis

| Segment | Temperature | Time | Cycle |
|---|---|---|---|
| Pre-denaturation | 95 °C | 2 min | 1 |
| Denaturation | 95 °C | 20 sec | 18 |
| Annealing | 60 °C | 10 sec | |
| Extension | 68 °C | 3.5 min (30 sec / 1 kb) | |
| Final extension | 68 °C | 5 min | 1 |

## 2.2. Colony PCR screening of mutated plasmids

PCR screening was used to identify the desired mutation of *OsBADH1* and *OsBADH2*. The PCR amplification was performed using *Taq* DNA polymerase (Fermentas). A single colony of mutated plasmids was used as a template. The reaction mixtures were prepared containing 1x reaction buffer, 0.5 μM of screening primers and reverse primers (as shown in Table 3), 0.2 mM of dNTP mix, 2 mM $MgCl_2$, 0.5 μl *Taq* DNA polymerase (Fermentas) and milliQ water to final volume 25 μl. The PCR condition was shown in Table 4. Afterward, the PCR screening products were visualized using 0.8% agarose gel electrophoresis and photographed by gel documentation.

**Table 3** Primer sequences for screening the mutant plasmid. The underlined represent the mutated codon. The bold letters represent the restriction site.

| Set of primer | Sequence 5′-3′ |
|---|---|
| Screening N164A OsBADH1 | GGA CTT ATC ACT CCC TGG GC |
| Screening W172A OsBADH1 | TCT GCT GAT GGC TAC TGC |
| Screening W172F OsBADH1 | CCT CTG CTG ATG GCT ACT TTC |
| *Xho*I –Reverse OsBADH1 | CCG **CTC GAG** CTA CAG CTT GGA TGG AGG C |
| Screening N162A OsBADH2 | GGT TGA TCA CAC CTT GGG C |
| Screening W170A OsBADH2 | TCT CCT GAT GGC AAC AGC |
| Screening W170F OsBADH2 | CCT CTC CTG ATG GCA ACA TTC |
| *Xho*I –Reverse OsBADH2 | CCG **CTC GAG** TTA CAG CTT GGA AGG GGA TT |

**Table 4** PCR condition for colony PCR screening of mutant plasmid

| Segment | Temperature | Time | Cycle |
|---|---|---|---|
| Pre-denaturation | 95 °C | 5 min | 1 |
| Denaturation | 95 °C | 1 min | 30 |
| Annealing | 50 °C | 30 sec | |
| Extension | 72 °C | 1 min | |
| Final extension | 72 °C | 10 min | 1 |

## 2.3. Restriction enzyme analysis

The mutated plasmids were verified using restriction enzyme analysis. The mutated plasmids of OsBADH1 and OsBADH2 were digested by two restriction enzymes, *Nde*I and *Xho*I. The restriction site and their reaction buffers were shown in Table 5. The restriction enzyme analysis was performed with reaction mixtures containing 100 ng of mutated plasmids, 10 unit of *Xho*I, 10 unit of *Nde*I, 1 µl of 10x buffer and milliQ water to final volume 10 µl. Afterward, the reaction mixtures were incubated in a water bath at 37 °C for 3 hour. The digestion products were verified by 0.8% agarose gel electrophoresis. After electrophoresis, the gels were stained with ethidium bromide and photographed by gel documentation. The plasmid from colony PCR screening and restriction analysis is also confirmed by DNA sequencing.

**Table 5** List of restriction enzymes

| Enzyme | Recognition site | Recommended Fermentas buffer for 100% enzyme activity | Temperature |
|---|---|---|---|
| *Nde*I | CA↓TATG | 50 mM Tris-HCl (pH 7.5) 10 $MgCl_2$, 100 mM NaCl, 0.1 mg/ml BSA | 37 °C |
| *Xho*I | C↓TCGAG | 10 mM Tris-HCl (pH 8.5) 10 $MgCl_2$, 100 mM KCl, 0.1 mg/ml BSA | 37 °C |

## 2.4. SDS-PAGE analysis

Analysis of protein expression and purification were monitored by SDS-PAGE. 20 µl of loading samples consisting of a 1:1 ratio of protein sample and 2x loading buffer (100 mM Tris-Cl, pH 6.8, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol, 200 mM DTT) were boiled at 100 °C for 15 min and run to electrophoresis using running buffer (25 mM Tris pH 8.3, 250 mM glycine, 0.1% SDS). SDS-PAGE was run at 200 volts for 1 hour and 20 minutes at room temperature. After electrophoresis, protein bands were visualized by coomassie brilliant blue stain.

## 2.5. Estimation of protein concentration

Concentration of protein was measured using Beer-lambert law, A = εbc where ε was the molar extinction coefficient, c was the molar concentration and b, the path length which was measured in centimeters. The molar extinction coefficients of either OsBADH1 or OsBADH2 were calculated using Protparam (Gasteiger *et al.*, 2005). Protparam computes various physicochemical properties that were deduced from a protein sequence including the molecular weight, theoretical pI, amino acid composition, atomic composition and extinction coefficient. Extinction coefficient of OsBADH1 and OsBADH2 were 80620 $M^{-1}cm^{-1}$ and 88765 $M^{-1}cm^{-1}$, respectively.

## 2.6. Binding study of OsBADH1 and OsBADH2

Fluorescence measurements were carried out according to the method described in a previous paper with some modifications (Gruez *et al.*, 2004). The measurements were performed to investigate the binding of $NAD^+$ to OsBADH proteins. Fluorescence binding between OsBADH proteins and $NAD^+$ was carried out by monitoring the intrinsic fluorescent intensity of Tryptophan residues. Fluorescence titration method was performed by adding microliter amounts of 5 mM $NAD^+$ to 400 µl of 1.5 µM OsBADH proteins in 50 mM HEPES-KOH buffer (pH 8.0). Excitation wavelength was 295 nm (slit 5 nm) and emission spectra were recorded between 300 nm and 450 nm (slit 5 nm). After addition of the cofactor, the sample was mixed and the spectrum was recorded. Titration results were corrected to account for ligand dilution. The apparent $K_d$ for $NAD^+$ binding was obtained by fitting the fluorescence changes against the concentration of $NAD^+$ using the following equation:

$$\%\triangle F_{obs} = \frac{\triangle F_{max}[L]_0}{K_d + [L]_0}$$

where $\%\Delta F_{obs}$ was the enhancement of fluorescence upon binding to proteins, $\Delta F_{max}$ was the maximum attainable change in fluorescence intensity, $[L]_0$ was the total molar concentration of the ligand, and $K_d$ was the dissociation constant for $NAD^+$ binding. Data were plotted as $\Delta F_{max}$ (the maximum attainable change in fluorescence intensity) at 350 nm versus the concentration of cofactors. The data were fitted and standard errors were calculated by non-linear regression analysis using the Microcal Origin 6.0 program.

## 2.7. Enzyme kinetic of OsBADH1 and OsBADH2

Enzyme kinetic assays of OsBADHs were measured spectrophotometrically by monitoring the oxidation of betaine aldehyde (Bet-ald) and γ-aminobutyraldehyde (GAB-ald) (Bradbury *et al.*, 2008). Betaine aldehyde chloride was dissolved in d.$H_2O$ and directly used in the enzymatic assay. γ-aminobutyraldehyde dimethyl acetal was used for GAB-ald. The diethylacetals of γ-aminobutyraldehyde were hydrolyzed with of 1M HCl and heated at 80 °C for 1 h. The HCl in the hydrolyzates was neutralized by adding equivalent volume of 1N NaOH. Both Bet-ald and GAB-ald were stored at -20 °C until used. The 100 mM stock solution of acetaldehyde was prepared and kept at 4 °C until used. All enzyme activities were determined using the reaction mixture containing 5 mM $NAD^+$ in 50 mM HEPES-KOH buffer (pH 8.0), 5 μM enzyme and various concentration of each substrate (between 8–1000 μM). One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of NADH per minute at 30 °C. The enzyme activities were monitored from the increase in absorbance at 340 nm corresponding to the extinction coefficient of NADH ($\varepsilon$ = 6220 $M^{-1}$ $cm^{-1}$) (Bradbury *et al.*, 2008). The $K_m$ and $V_{max}$ values were obtained by fitting the initial rates against the concentrations of each substrate to the Michaelis-Menten equation:

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

where $V$ was the initial rate, [$S$] were the substrate concentrations, $V_{max}$ was the initial rate achieved as [$S$] approach ∞, $K_m$ was Michaelis-Menten constant or the value of [$S$] giving $V_{max}/2$. The data were fitted and standard errors were calculated by non-linear regression using Microcal Origin 6 program.

## 2.8 Homology modeling and molecular docking of OsBADH1 and OsBADH2

Since the sequence identity between OsBADH1 and OsBADH2 is about 75%. The protein structure of OsBADH1 was modeled with residues ranging from 8 to 504 amino acids by SWISS-MODEL using the crystal structures of OsBADH2 as a template (Kuaprasert *et al.*, 2011). The structure of modeled OsBADH1 proteins was superimposed in order to compare the structural conformations using the program PyMol (Delano, 2002). The mutation in the protein was generated using Discovery Studio 2.5. Regarding for ligand, Bet-ald was taken from the crystal structure of *YdcW* (PDB entry 1WNB) (Gruez *et al.*, 2004) whereas GAB-ald was built using Discovery Studio 2.5. After both proteins and ligands were successfully constructed, docking analyses were performed on AutoDock 4.0 (Morris *et al.*, 1998) in

which, for ligand preparation, the rotational bonds of the side chain were treated as flexible whereas those of main chain were regarded as rigid. All hydrogen atoms were added into the OsBADH1 and OsBADH2 proteins and water molecules were removed from the structure. The grid boxes were created to cover substrate-binding domain of the protein. The size of grid box was set at 60 Å × 60 Å × 60 Å and the center of grid box was set at 7.545 Å (*x*), 3.238 Å (*y*), and 33.596 Å (*z*). The search parameter used was Lamarckian Genetic Algorithm (LGA) with 100 runs. Three-dimensional structures of OsBADHs–Bet-ald or OsBADHs–GAB-ald with the best docked conformation were observed. From the 100 running structure, one structure was chosen based on the lowest energy and the high population. The lowest energy and highest populations were visualized and analyzed by PyMol (Delano, 2002) and Discovery Studio 2.5. Therefore, the structures from the molecular docking analysis will be used as an initial model for molecular dynamic (MD) simulations.

## 2.9. Molecular dynamics simulations

The selected models of OsBADHs–Bet-ald or OsBADHs–GAB-ald complexes obtained from docking were used for MD simulations. MD simulations were performed using the AMBER10 simulation package with the Cornell force field (Cornell, 1995). OsBADH1 and OsBADH2 contained 505 and 503 amino acid residues, respectively. All complexes were immersed in an octahedral box of TIP3P water (Jorgensen, 1983) with the distance between solute surface and the edge of the box set at 10 Å. Minimization was achieved stepwise as follows: 2000 steps for hydrogen atoms, 2000 steps for solvent water molecules and 5000 steps for all atoms in the system. The equilibration was perfomed in the carononical ensemble (NVE) at 300 K; during the initial 100 ps all atoms in the protein were restrained while in the following 100 ps all atoms were set free. MD simulations were carried out under an isobaric–isothermal ensemble (NPT), at 1 atm and 300 K. Equilibration was achieved when the system was stable and this was followed by a production phase which was harvested during the last 500 ps of the trajectory. Root-mean-square displacement (RMSD) values for distances between interacting amino acid residues, hydrogen bonds and binding free energies were also calculated.