Abstract  β-hydroxybutyric acid is the most sensitive indicator in ketoacidosis detection, and accounts for nearly 78% of the ketone bodies. Diaphorase is commonly used to detect the β-hydroxybutyric acid in clinical diagnosis. However, the extraction of diaphorase from animal myocardium is complex and low-yield, which is not convenient for large-scale production. In this study, a diaphorase from *Geobacillus* sp. Y4.1MC1 was efficiently heterologous expressed and purified in *E. coli* with a yield of 110 mg/L culture. The optimal temperature and pH of this recombinant diaphorase (rDIA) were 55 °C and 6.5, respectively. It was proved that rDIA was a dual acid- and thermostable enzyme, and which showed much more accurate detection of β-hydroxybutyric acid than the commercial enzyme. Additionally, we also investigated the molecular interaction of rDIA with the substrate, and the conformation transition in different pH values by using homology modeling and molecular dynamics simulation. The results showed that 141–161 domain of rDIA played important role in the structure changes and conformations transmission at different pH values. Moreover, it was predicted that F105W, F105R, and M186R mutants were able to improve the binding affinity of rDIA, and A2Y, P35F, Q36D, N210L, F211Y mutants were benefit for the stability of rDIA.

Keywords  Diaphorase · Expression and purification · Enzymatic properties · Molecular dynamics simulation · Mutant prediction

Introduction

Ketone bodies are a class of metabolites that produced by fatty acid, including β-hydroxybutyric acid (~ 78%), acetoacetic acid (~ 20%) and acetone (~ 2%), and excess ketone bodies will exceed the utilization ability of liver (Yue et al. 2014). The
imbalance between ketone bodies and liver utilization plays a key role in the occurrence of ketoacidosis, such as acetonemia and acetonuria. In addition, type II diabetes mellitus complicated with ketosis or ketoacidosis also often occurs in clinical for the less insulin secretion in the body (Jerreat 2009). \(\beta\)-hydroxybutyric acid is the sensitive indicator for monitoring the ketoacidosis due to the rich distribution in the blood (Goldstein et al. 1995). Therefore, it is with great significance to efficiently and accurately detect the \(\beta\)-hydroxybutyric acid in the early detection and prevention ketoacidosis and type II diabetes (Sheikh-Ali et al. 2008).

Traditional method for the \(\beta\)-hydroxybutyric acid detection was to test acetone, the oxidation product of \(\beta\)-hydroxybutyric acid, by titration, specific gravity and colorimetry (Deng et al. 2004; Kalapos 2003). But it is a semi quantitative method, and the amount of acetone is affected by the experimental conditions. The quantitative analysis of acetone by gas chromatography can only determine the concentration of total ketones in serum that lack of specificity (Deng et al. 2004). Nowadays, enzymatic method by diaphorase and \(\beta\)-hydroxybutyrate dehydrogenase has been developed (Daniel et al. 1992). Under the specific oxidation of \(\beta\)-hydroxybutyric acid dehydrogenase and NAD\(^+\), \(\beta\)-hydroxybutyric acid produces acetoacetic acid and NADH. And in the presence of NADH and diaphorase, oxidized iodo nitro tetrazolium chloride blue (INT) can be catalyzed to reductive INT, which has absorbance at 505 nm (Huang et al. 2006; Cimen et al. 2010). There are lots of advantages of the enzymatic method, including high precision, high speed, low cost, wide linear range, and can be directly determined without pre-purification or chemical treatment (Parry et al. 2010).

Diaphorases are a class of flavin-containing enzymes, which have been widely used in biosensor design, biotransformation and in vitro diagnostic tests (Kianmehr et al. 2018). Various diaphorases have been identified from bacterial (Ahmed and Claiborne 1989), plant (Eftink and Bystrom 1986), and animal sources (Radi et al. 1993). Due to the difficulties on high amounts purification of diaphorase from pig heart muscle, heterologous expression has become the main method to produce diaphorase. Some studies have used genetic engineering methods and subsequent expression and purification steps to obtain various types of recombinant diaphorase (rDIA). For example, a diaphorase with molecular weight of 24 kDa was successfully expressed, purified and characterized from Clostridium kluyveri, and applied in the reduction of diphosphopyridine nucleotide and triphosphopyridine nucleotide (Chakraborty et al. 2008a; Madan et al. 2010). Both ferrocenium compounds and hexacyanoferrate (III) of this kind diaphorase could be used as electron transfer mediators and co-immobilization with several dehydrogenases in a membrane electrode format yielded sensors (Antiochia et al. 2015). Lipoamide dehydrogenase (DLD) with diaphorase activity, was successfully heterologous expressed and purified from E. coli, and the suitable kinetic features of DLD could make this biocatalyst useful for application as a diagnostic enzyme (Kianmehr et al. 2017). However, there are still some disadvantages such as low yield (Klyachko et al. 2005), low activity (Chakraborty et al. 2008b) and poor stability (Kianmehr et al. 2017).

In this study, a novel rDIA from Geobacillus sp. Y4.1MC1 was efficiently heterologous expressed and characterized in E. coli BL21(DE3). The enzymatic properties including optimum pH and temperature, thermo- and acid-stability were studied. And we also probed the molecular interaction of rDIA with \(\beta\)-hydroxybutyric acid, and investigated the mechanism of pH stability related with the rDIA structure by using homology modeling and molecular dynamics (MD) simulation. Finally, mutation evolution on pH stability was also performed by the interaction energy analysis.

Materials and methods

Materials

rDIA gene was codon-optimized and synthesized by Sangon Inc. (Shanghai, China). Both of the E. coli BL21(DE3) and expression vector pET28a were purchased from Invitrogen Inc. (Carlsbad, USA). Protein marker, gene marker and restriction enzymes were obtained from Tiangen Inc. (Beijing, China). Ni–NTA columns, AKTA Pure chromatography system and PD-10 Desalting Columns were purchased from GE Healthcare. Coomassie brilliant blue G250, peptone, glycine and SDS-PAGE were purchased from Solarbio Inc (Beijing, China). Monopotassium, sodium hydrogen phosphate, ammonia chloride,
sodium sulfate and magnesium sulfate were purchased from Tianjin Jiangtian chemical technology Co. Ltd. (Tianjin, China). Unless noted, all used reagents were of analytical grade.

Methods

Construction of the expression vector and engineered strains

The gene sequence of DIA (GenBank: CP002293.1) from Geobacillus sp. Y4.1MC1 was optimized according to the codon preference of E. coli, and the rDIA gene (Fig S1) was cloned into the pUC57 vector (pUC57-rDIA). Then the rDIA was subcloned into the pET28a expression vector by NdeI and XhoI restriction enzymes under the T7 promoter. After identified by gene sequencing, the plasmid was named pET28a-rDIA. Finally, the vector pET28a-rDIA was transformed into the expression host E. coli BL21(DE3) by chemical competence method to heterologous expression of rDIA. After identified by colony PCR, the engineered strain was named BL21-rDIA.

Expression and purification of rDIA

Single colony of engineered strain was picked and cultured in 20 mL LB medium at 37 °C overnight to prepare the seeds, and transferred to 1000 mL fresh TB medium with 5% inoculation. After OD600 of the inoculated broth reached to 0.6, the BL21-rDIA culture was incubated at 16 °C for 18–22 h. In the E. coli fermentation experiment, the heterologous expressed proteins are easily formed as inclusion bodies at high temperature induction conditions. And the purification procedure of proteins in inclusion bodies is time-consuming and inefficient, and maybe lead a substantial activity loss of target enzymes. Therefore, we chose a relative low induction temperature, 16 °C, for the soluble expression of rDIA, the method of which have been commonly used in E. coli system to express a lot of functional proteins (Jia et al. 2018).

After centrifugation of BL21-rDIA culture at 8000 rpm for 20 min, the supernatant was removed and cell pellets were collected. The bacterial pellets were resuspended in the 0.1 M potassium phosphate (pH 7.5, 0.05 mol/L K2HPO4, 0.05 mol/L KH2PO4, 5 mg/L FAD) and sonicated by 9 pulses (30 s sonication with the same intervals at 30% amplitude) (Damough et al. 2021), and the mixture was centrifuged at 12,000 rpm at 4 °C for 40 min. The supernatant was loaded on the Ni–NTA column, which has been equilibrated using the binding buffer (0.1 M potassium phosphate, 5 mg/L FAD; pH 7.5) in the AKTA Pure chromatography system (GE Healthcare). After washed by the buffer I (0.1 M potassium phosphate, 5 mg/L FAD, 10 mM Imidazole; pH 7.5), The rDIA were eluted using the elution buffer (0.1 M potassium phosphate, 5 mg/L FAD, 500 mM Imidazole; pH 7.5) under 1 ml/min flow rate. The imidazole in the rDIA solution was removed by using PD-10 Desalting Columns (GE Healthcare). Nanodrop 2000 (Thermo Fisher Scientific, Wilmington, USA) was used to measure protein concentration at 280 nm. At last, the product was lyophilized and stored at − 80 °C.

Enzyme activity measurement

One unit of rDIA activity is defined as the amount of enzyme which oxidizes 1 μM of NADH to NADH⁺ per minute at 37 °C under the conditions specified in the assay procedure. Pipette accurately 1 mL of reaction mixture into a small test tube and preincubate it at 37 °C. Reaction mixture: 0.5 mL KH2PO4-NaOH buffer (0.2 M, pH 8.0), 0.1 mL 0.25% (W/V) nitrotrazolium blue solution, 0.1 mL 1% (W/V) BSA solution, 0.1 mL 10 mM NADH solution, 0.2 mL Distilled water. After 7 min, 100 µL of enzyme solution was added into the above reaction mixture to start the reaction at 37 °C. Absorbance of the sample at 505 nm was monitored per minute. At last, the enzyme activity was calculated according to the following equation:

\[
\text{Activity (U/L)} = \frac{(\Delta A \times V_t \times df \times 1000)}{(12.4 \times V_s)}
\]

in which, \(\Delta A\) means the change of absorbance in 1 min; \(V_t\) means final volume (mL); \(df\) means dilution times of enzyme solution; 12.4 means millimolar extinction coefficient of formazan dye at 505 nm; \(V_s\) means volume of enzyme solution added. The Michaelis constant was calculated by GraphPad prism 6 software. All experiments were performed in triplicate to ensure the reproducibility.
Effect of temperature on the activity and stability of rDIA

The optimum temperature of purified rDIA was determined by measuring its activity at a series of temperatures of 20, 25, 30, 35, 37, 40, 45, 50, 55, 60, 65, 70, 75, and 80 °C. For calculating its thermostability, rDIA was pre-incubated in 0.2 M potassium phosphate buffer (pH 6.5) at a series of gradient temperatures (treated by PCR instrument) of 30.3, 32.1, 34.4, 36.7, 40.1, 43.3, 46.7, 50.0, 53.4, 55.0, 58.4, 59.9, 65.0, and 70 °C for 40 min. After cooling on ice for 5 min, the relative residual activity was determined using the above method. For calculating its thermostability, the activity of un-treated enzyme was defined as 100%.

Effect of pH on the activity and stability of rDIA

In order to optimal pH, the enzymatic activity of the rDIA was measured in Acetate buffer (pH 5.0–6.0), Phosphate buffer (pH 6.0–8.5) and Glycine–NaOH buffer (pH 8.5–9.5) at 37 °C. pH stability of rDIA was determined by assaying its catalytic activity after treatment of rDIA in Citrate buffer (pH 3.0–6.0), Phosphate buffer (pH 6.0–9.0) and Glycine–NaOH buffer (pH 9.0–12.0) at 4 °C for 6 h, respectively. The maximum enzyme activity was set to 100%, and the relative enzyme activity was calculated. Each experiment was performed in triplicate to ensure the reproducibility.

Comparation of rDIA with commercial diaphorase

We compared the enzymatic property of the rDIA and commercial diaphorase (purchased from ToYoBo) by using the β-hydroxybutyric acid test kit (Abbexa, UK). There is a positive correlation of the activity of diaphorase and the dose of β-hydroxybutyric acid. In which, with the β-hydroxybutyric acid dehydrogenase and oxidized coenzyme I, β-hydroxybutyric acid can be oxidized to acetoacetic acid, and oxidized coenzyme I is changed to reduced coenzyme I. And in the presence of diaphorase, reduced coenzyme I and INT (iodo nitro tetrazolium chloride blue) will produce formazan. The absorbance of formazan was measured at 505 nm by Hitachi 7170A automatic biochemical analyzer (Hitachi, Japan), which was positively correlated with the enzyme activity. The detailed detection process of β-hydroxybutyric acid was as followed: 6 μL β-hydroxybutyric acid (1 mM) was added to 180 μL reagent 1 and gently mixed. After incubated at 37 °C for 5 min, 60 μL reagent 2 was added into the above mixture solution and incubated at 37 °C for 1 min. The absorbance of the test solution at 505 nm at 0 and 2-min were recorded, and then the absorbance change (ΔA) was calculated. Of note, the Reagent 1 including 100 mM Tris buffer, 4.1 kU/L β-hydroxybutyrate dehydrogenase, 1.8 kU/L commercial diaphorase or rDIA; Reagent 2 is 2.5 mM NAD+ and 1 mM INT (iodonitrotetrazolium chloride).

Molecular dynamics (MD) simulations

We selected the crystal structure of Bacillus smithii FMN-dependent NADH-azoreductase (PDB entry 6JXS) as the template for homology modeling. MD simulations were performed using the program GROMACS 2018.4 with Amber99SB force field parameters (Hornak et al. 2006). Firstly, the diaphorase was placed in a simulation box, filled with water molecules and equilibrium ions, at least 12 Å distances from any atom of the enzyme. To remove possible steric stresses, system energy minimization was then performed for 50,000 steps. The six polypyrrole-pyrethrin configurations were energy minimized and subjected to 100 ps NVT (N for the number of atoms, V for volume, and T for temperature) equilibration at 300 K. Then, the six systems were respectively run for 50 ns of NPT (N for the number of atoms, P for pressure, and T for temperature) production. In this study, we carried out a series of all-atom MD simulations using the standard AMBER99SB force field (Hornak et al. 2006; Gao et al. 2017) at pH 3.0 and 6.5. Under the two pH values of mimic environment, the side chains of the Asp55, Glu75 and Glu194 were modeled to take different charge states: neutral at pH 3 and negatively charged at pH 6.5 (Rostkowski et al. 2011). The same strategy is often applied in MD simulations to investigate the pH-dependent behaviors of protein folding/aggregation (Khandogin and Brooks 2007; Li et al. 2013). A substrate receptor-binding model was proposed to identify key residues that bind to DIA. The simulation trajectories were analyzed using several auxiliary programs provided with the GROMACS 2018.4 package. Secondary structure analyses were carried out using DSSP (Kabsch and Sander 1983) and Dm (Chen et al. 2012).
out using the dictionary secondary structure of proteins (DSSP) (Kabsch and Sander 1983) method and examined visually using VMD software (Humphrey et al. 1996).

Results and discussion

Construction and identification of the expression vector

According to the protein sequence of DIA (GenBank: CP002293.1) in NCBI, the gene sequence was codon optimized for the preference of E. coli. The optimized gene was synthesized, and cloned into the pUC57 vector. The rDIA fragment was double-digested using NdeI/XhoI and sub-cloned into the pET28a expression vector. Schematic of the construction of recombinant expression plasmid pET28a-rDIA was shown in Fig. S2A. The recombinant expression vector was identified by PCR using the primer pairs of T7-F (5’TATAATACGACTCACTATAGGG-3’) and T7-R (5’GCCTAGTTATTGCTCAGCGG-3’). As shown in Fig. S2B, the target fragment of rDIA was shown to be approximately 1000 bp, which is almost identical to the theoretical value. We also identified the pET28a-rDIA plasmid by gene sequencing from GENEWIZ Inc. (Suzhou, China).

Expression and purification of the rDIA

The flow chart of expression and purification process of rDIA was shown in Fig. 1A. As shown in Fig. 1B, the target protein band was found in both bacterial lysate of BL21-rDIA and supernatant in TB culture, which indicated that the rDIA was successfully soluble expressed. Figure 1B also indicated the molecular mass of recombinant rDIA consistent with deduced molecular mass of 29 kDa. Large scale protein expression was performed under the auto-induction fermentation medium. After purified by using HisTrap FF column, the protein samples were identified using SDS-PAGE (Fig. 1C). The rDIA was over 95% pure and with a yield of 110 mg/L culture. The molecule weight of rDIA was similar to the thermophilic alcohol dehydrogenase (hADH) (Liang et al. 2004), an intracellular diaphorase, which was isolated from a strain of Bacillus stearothermophilus with the molecular weight of 30 kDa. Chakraborty et al. expressed and characterized a diaphorase, rBfmBC, from Clostridium kluyveri using E. coli expression system, the induction temperature of which is 37 °C, and induction time is 5 h, but with a very low yield of production of ~ 10 mg per liter of the culture (Chakraborty et al. 2008b). Due to the low yield of rDIA, several strategies can be tried to improve the production, including optimize of the fermentation conditions, optimize the dose of IPTG, change to other expression systems, and so on.

Enzymatic characterization of rDIA

As the temperature have great influence on the enzymatic reaction, the optimum temperature of purified rDIA was determined by measuring its activity at a series of temperatures of 20, 25, 30, 35, 37, 40, 45, 50, 55, 60, 65, 70, 75, and 80 °C at pH 6.5. As shown in the Fig. 2A, rDIA displayed maximum activity when the temperature at 55 °C, and maintained good catalytic activity at broad temperature range from 30 °C to 80 °C. Nearly 60% of the maximum activity was remained when the temperature increased to 80 °C. We also measured the thermostability of rDIA by heating the enzyme at different temperatures for 40 min before the measurement. The rDIA showed good activity at temperatures of 30–60 °C (Fig. 2B), and nearly 85% enzymatic activity was still remained after treated at 60 °C. These results indicated that the optimum temperature of this rDIA was 55 °C and possessed outstanding thermostability from 30 to 60 °C. rDIA showed more thermal stability than DLD, which has been heterologous expressed and purified from E. coli with a sharp decrease in enzyme activity above 30 °C and completely inactivated at 70 °C (Kianmehr et al. 2017).

The effects of pH on the rDIA activity were evaluated after exposure to pH ranged from 5.0 to 9.5 at 37 °C, and the results were shown in Fig. 2C. The enzyme activity in all pH conditions were all above 40%, and the optimum pH was 6.5. Besides, the stability of this enzyme at different pH was probed by treating the rDIA with different pH buffer ranging from 3 to 12 at 4 °C for 6 h before the enzymatic measurement (Fig. 2D). The rDIA showed excellent pH stability with a broad range from 5.0 and 9.0, which remain over 85% activity. Even at pH 10.0, the enzyme maintained approximately 60% catalytic activity. These results proved that the optimum pH
of rDIA was 6.5, and possessed good pH stability. In this study, the optimum pH and stability of the rDIA were similar to rBfmBC, encoded by *Clostridium kluveri* rBfmBC gene (Chakraborty et al. 2008b). And the optimum pH of the recombinant enzyme rBfmBC was found to be about 7.0, while it showed high activity only in a narrow pH range of 6.0–8.0 in diaphorase assays (Chakraborty et al. 2008b). Therefore, the optimum temperature and pH of rDIA were 55 °C and 6.5, respectively, and the maximum activity of the purified rDIA was ~ 40 U/mL, and corresponding specific activity was ~ 364 U/mg.

We also probed the effect of different metal ions and chemical compounds on the enzymatic activity of rDIA. The rDIA (5 U/mL) was soluable expressed in 0.1 M potassium phosphate (pH 6.5) was incubated with each chemical at 37 °C for 30 min, and the catalysis activity were measured. As can be seen in Table 1, all of the regents showed negligible effect on the enzyme activity, which was similar to the results of other diaphorases (Kianmehr et al. 2017; Dietrichs et al. 1990), which indicated that the rDIA presented promising stable enzymatic property. Moreover, to calculate the Michaelis constant, the substrate NADH was diluted with 2 times gradient to determine the activity of diaphorase at different concentrations of NADH. The kinetic curve of rDIA was drawn based on GraphPad prism 6 software. The results calculated by software of Michaelis constant showed Km = 0.09 mM and Vmax = 81.40 lM/min (Fig. 2E).

**Fig. 1** Expression and purification of rDIA. A Flow chart of the expression, purification and characterization of the rDIA. B rDIA was soluable expressed in TB medium. Lane M: protein marker; Lane 1: bacterial lysate of BL21-rDIA in LB culture; Lane 2: bacterial lysate of BL21-rDIA in TB culture; Lane 3: supernatant of BL21-rDIA in LB culture; Lane 4: supernatant of BL21-rDIA in TB culture. C Lane M: protein marker; Lane 1, 2: sample 1 and 2 of the purified rDIA; Lane 3, 4: sample 1 and 2 of the rDIA after desalination

**Comparation of rDIA with commercial diaphorase**

We compared the enzymatic property of the rDIA with commercial diaphorase by using the β-hydroxybutyric acid test kit (Abbexa, UK), according to the positive correlation of the activity of diaphorase and the dose of β-hydroxybutyric acid in this measurement. The absorbance changes of test samples were shown in Fig. 3A, which demonstrated that real time enzymatic activity of rDIA was similar to the commercial diaphorase. Moreover, we evaluated the enzyme stability of rDIA and commercial diaphorase by storing at 4 °C and 37 °C for 14 days. As shown in
Fig. 3B, the absorbance change of rDIA was similar to the commercial enzyme after stored at 4 °C, but significantly higher than the other one at 37 °C, which indicated that the enzymatic of rDIA was more stable than commercial enzyme.

Structure changes and conformations transmission of rDIA at different pH were probed by MD simulations.

Table 1 Effects of various chemicals on rDIA activity

| Reagents | Conc. (mM) | Residual activity (%) |
|----------|-----------|-----------------------|
| None     | –         | 97.3 ± 3.5            |
| Ca²⁺     | 2.0       | 96.8 ± 3.4            |
| Zn²⁺     | 2.0       | 99.2 ± 2.3            |
| Fe²⁺     | 2.0       | 102.4 ± 2.7           |
| Mg²⁺     | 2.0       | 98.5 ± 3.9            |
| NaN₃     | 2.0       | 107 ± 1.8             |
| PMSF     | 2.0       | 97.8 ± 3.4            |
| EDTA     | 2.0       | 97.3 ± 3.5            |

The enzyme activity without any reagents was defined as 100%, and results represent means ± SD (n = 3).

As the pH was important in the application of diaphorase, we investigated the structure and conformation transitions of rDIA at different pH solutions by using homologous modeling and MD simulations. Firstly, the Cα-RMSD and atom–atom contacts of the whole protein were used to represent the enzyme activity at two pH values of 3.0 and 6.5. Currently, Bacillus smithii’s FMN-dependent NADH-azoreductase (PDB code: 6JXS, resolution: 1.95 Å) has been crystallized, and has a sequence identity of 60.66% with rDIA (Fig. S3). The residue completeness and crystal structure resolution were also proved FMN-dependent NADH-azoreductase could be considered as the homology model of rDIA in the following MD simulations.

The Cα-RMSD and atom–atom contacts were displayed as a function of time in Fig. 4. As shown in Fig. 4A, the Cα-RMSD value at pH 3.0 was higher than pH 6.5 during the last 25 ns. And atom–atom contacts at pH 6.5 was generally larger than pH 3.0 during the whole 50 ns (Fig. 4B). Therefore, the structure of rDIA was more stable at pH 6.5 than pH 3.0 based on the results of the MD simulation.
Fig. 3 Comparison of rDIA with a commercial enzyme. A Control: water; Enzyme: detection of enzyme activity of rDIA and commercial enzyme using equal catalytic capacity at 505 nm. B After storage at 4 °C and 37 °C for 14 days, detection of enzyme activity of rDIA and commercial enzyme using equal catalytic capacity at 505 nm.

Fig. 4 Structure changes of rDIA at different pH were probed by MD simulations. A Time dependence of the RMSD of the backbone Cα-atoms with respect to their initial structure at pH 3.0 (Red) and pH 6.5 (Blue). B The number of atom–atom contacts as a function of time for the MD simulations at pH 3.0 (Red) and pH 6.5 (Blue). C The Cα atom root-mean-square fluctuation (RMSF) as a function of residue number for rDIA at pH 3.0 (Red) and pH 6.5 (Blue).
Table 2  Average content of secondary structures of complete sequence and fragment 141–161 of rDIA at different pH values

| Secondary structures | pH 3.0 | Helix (%) | Coil (%) | Turn (%) | β-sheet (%) | 3_{10}-helix (%) |
|----------------------|--------|-----------|----------|----------|-------------|-----------------|
| Total                | pH 3.0 | 42.36     | 24.75    | 10.89    | 21.66       | 0.34            |
|                      | pH 6.5 | 42.93     | 23.83    | 11.45    | 21.24       | 0.55            |
| 141–161 domain       | pH 3.0 | 1.76      | 41.70    | 18.91    | 33.10       | 4.52            |
|                      | pH 6.5 | 0         | 36.87    | 27.66    | 33          | 2.46            |

Fig. 5  The secondary structure evolutions of residues as a function of simulation time. A Simulations were carried out at pH 3.0. Under the condition of pH 3.0, the part marked by black wire frame indicated the transition from spiral structure to turns. B Simulations were carried out at pH 6.5. The assignment of secondary structures is made with DSSP.
We also analyzed the changes of RMSF, which represented the flexibilities of the individual residues in the protein (Sun et al. 2016), at pH 3.0 and 6.5. Time dependence of the RMSF values of C\textalpha\ atoms of rDIA were calculated and displayed as shown in Fig. 4C. Two residue regions of 125–246 and 150–170 present higher RMSF values at pH 3.0 than 6.5, indicating the structure of these two regions were flexible and easily influenced by the pH changes. Of note, majority residues in 125–246 and 150–170 regions were negatively charged, we speculated that the negatively charged residues might contributed most in the rDIA structure changes at different pH.

The changes of secondary structures of rDIA at pH 3.0 and pH 6.5 was also investigated. The total contents of secondary structures of rDIA during the simulations at pH 3.0 and 6.5 were similar, but in the 141–161 domain, there were obvious differences in the secondary structure composition between these two pH (Table 2). Real time simulations of the secondary structure changes 50 ns at pH 3.0 and 6.5 were also monitored in Fig. 5. Of note, in the 141–161 domain that labeled by black wire frame, the structure of helix changed into turns with the simulation time under the condition of pH 3.0 (Fig. 5A), while there were not significant changes in the same fragment at pH 6.5 (Fig. 5B). It was indicated that the secondary

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**Fig. 6** Mutation screening results by MD simulations. A Energy change of before and after mutation under the alanine-scanning mutation and site directed full mutation; A: chain A, B: chain B. B Representative snapshots of rDIA at pH 6.5 with the mutation sites, which was carried out by Alanine-scanning mutagenesis and site directed full mutation scanning. C Energy change of before and after mutation under stability mutation. D Representative snapshots of rDIA stability mutation at pH 6.5 with the mutation sites.
structures of rDIA easily changed at pH 3.0. Representative snapshots of rDIA at pH 3.0 and pH 6.5 values (Fig SI 4) also proved that pH can affect the structure of rDIA.

**Mutation prediction of rDIA by MD simulations**

The above results proved that pH can affect the structure of rDIA through different charged states of amino acids, especially of the 141–161 domain. Based on these results, we furtherly investigated the key residues on maintaining the enzymatic stability by using MD simulations, which was helpful for the direction evolution to improve the enzyme activity and stability. According to the homology modeling and MD simulations, the binding sites were formed from positions of 17–20, 52–60, 101–105, 146–150, 172, 186–187, which were related to enzyme catalysis pocket (Fig. 6). In order to improve the binding affinity, we did alanine-scanning mutagenesis for activity domain of rDIA and screened out the amino acid positions that had great energy changes, and then made site directed full mutation scanning of these amino acids. F105, M186 and W60 were found that there was a large energy change after alanine-scanning mutagenesis. Then site directed full mutation scanning was carried out for F105, M186 and W60, and the energy of F105W, F105R and M186R was negative (Fig. 6A), which indicated that the energy was reduced. Due to the reduction in energy, these mutants were able to improve the binding affinity of rDIA (Fig. 6B). We did mutation-scanning far away from the activity domain to screen for stability mutations under the optimal pH 6.5. The results of mutation-scanning showed that the energy change of A2Y, P35F, Q36D, N210L, F211Y was negative, especially A2Y and Q36D (Fig. 6C). Due to the reduction in energy, these mutants were able to improve the stability of rDIA (Fig. 6D). In summary, F105W, F105R and M186R mutants were able to improve the binding affinity of rDIA, and A2Y, P35F, Q36D, N210L, F211Y mutants were benefit for the stability of rDIA.

**Conclusion**

In this work, we successfully expressed and purified a novel diaphorase, rDIA from *Geobacillus* sp. Y4.1MC1 in *E. coli* with a high purity over 95% and yield of 110 mg/L culture. Optimal temperature for the rDIA was ~55 °C, and optimum pH was 6.5. The rDIA showed temperature stability at 30–60 °C, and possessed high activity at a broad pH range from 5.0 to 9.0. MD simulations results showed the 141–161 domain of rDIA played important role in the structure changes and conformations transmission at different pH values. Moreover, we recommended that F105W, F105R and M186R mutants were able to improve the binding affinity of rDIA, and A2Y, P35F, Q36D, N210L, F211Y mutants were benefit for the stability of rDIA.

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**Declarations**

**Conflict of interest** The authors declare that they have no competing interests.

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