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Catalytic Site Analysis and Characterization of a Solvent-Tolerant Aldo-Keto Reductase

WEI JIANG, RUI PEI, WEILIANG WU, PANPAN ZHAO, LIBING TIAN, AND SHU-FENG ZHOU

ABSTRACT

Chiral alcohols are important intermediates of various drugs. Compared with traditional chemical methods, the biocatalytic methods used for the synthesis of chiral alcohols exhibits many advantages, such as mild conditions and high enantioselectivity. Aldo-keto reductases are regarded as promising enzymes that can be potentially applied in the biocatalytic synthesis of chiral alcohols. In this study, a novel aldo-keto reductase, AKR7-2-1, was cloned and purified, and its important conserved sites were analyzed. In addition, this study analyzed the catalytic potential of AKR7-2-1. The optimum reaction conditions were studied; AKR7-2-1 showed excellent thermal stability and pH stability even when the temperature reached 80 °C or pH reached 9.0. Furthermore, AKR7-2-1 has strong enzymatic activity when 11 ketone-containing compounds are used as substrates, indicating the broad substrate spectrum of the enzyme. Most importantly, AKR7-2-1 has superior organic solvent tolerance even in an organic solvent of 30% volume per volume (V/V) or 10 hours in a 10% V/V organic solvent, where 60% enzyme activity was retained. It is worth mentioning that AKR7-2-1 can catalyze the reduction of N,N-dimethyl-3-keto-3-(2-thienyl)-1-propanamine to (S)-N,N-dimethyl-3-hydroxy-3-(2-thienyl)-1-propanamine, an intermediate of the antidepressant drug duloxetine. All this shows that AKR7-2-1 has broad application prospects in the field of biomedicine.

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PEER-REVIEWED

Article submitted: Feb. 2, 2019
Article accepted: April 4, 2019

Chiral alcohols are a class of compounds having a hydroxyl group attached to a chiral carbon atom. The compounds are structurally stable and are important intermediates for the synthesis of chiral drugs, perfume flavors, and agricultural chemicals (1, 2). Compared with traditional chemical methods, biochemical synthesis provides a promising strategy for the production of chiral alcohols with the advantages of high efficiency and enantioselectivity and mild reaction conditions. Enzymes and microorganisms are used to catalyze the production of various enantiomerically pure alcohols (3).

The aldo-keto reductase (AKR) is a class of nicotinamide adenine dinucleotide cofactor (NAD(P)H)-dependent enzymes belonging to the oxidoreductase family (4). AKRs are widely found in nature, such as bacteria (5), plants (6), and animal tissues (7). AKRs show great application prospects in the field of biomedicine due to their ability to catalyze the reacton of prochiral ketones to form chiral alcohols. AKR, for example, can be used for the asymmetric reduction of ethyl 4-chloro-3-oxobutanoate (COBE) to ethyl (R)-4-chloro-3-hydroxybutanoate (CHBE), known as an important intermediate in organic synthesis, which can be applied in synthesis of L-carnitine, HMGCOA reductase inhibitor (8, 9). Catalytic reduction of ethyl 2-oxo-4-phenylbutanoate with *Bacillus subtilis*-derived AKR is an

important way to obtain ethyl (R)-2-hydroxy-4-phenylbutanoate, which can be used as an intermediate for anti-hypertension drugs (10). Only a limited number of AKRs have been obtained and applied to the synthesis of chiral alcohols, however. Five AKRs were cloned for highly stereoselective reduction of bulky ketones by C. Liang *et al.* through genetic mining from microorganisms such as *Candida albicans* (CaCR), *Saccharomyces cerevisiae* (ScCR), *Kluyveromyces marxianus* (KmCR), and *Candida parapsilosis* (CPR-C1, CPR-C2) (11). X. Luo *et al.* cloned an AKR from *Kluyveromyces lactis* XP1461, which can be used for the synthesis of chiral alcohols (12). Y.H. Ma *et al.* cloned a stong heat-resistant AKR from thermophilic bacteria, Tm1743 (13). C. Ning *et al.* cloned three kinds of AKRs from *Lodderomyces elongisporus*—LEAKR 48, LEAKR 49, and LEAKR 50—which can be applied to synthesize ethyl 4-chloroacetoacetate (14). In addition, AKR from *S. cerevisiae*, YOL151W, can be used for asymmetric synthesis of (S)-3-chloro-1-phenyl-1-propanol (15). The new AKR from *L. elongisporus*, NRRL YB-4239, can be used to synthesize ethyl (R)-4-chloro-3-hydroxybutanoate (16). So far, chiral compounds have been among the top 10 drugs in history, and it is expected that by 2020 chiral compounds will still dominate the best-selling drug list (17). Although the demand for chiral drugs in the medical field is extremely large, there are still few studies on biocatalytic synthesis of chiral alcohols, which cannot meet the demand for chiral drugs in the medical field. Therefore, it is necessary to continue to excavate new types of AKRs to meet the synthetic needs of chiral drugs.

In this study, a novel AKR (AKR7-2-1) was cloned from *Bacillus megaterium*. The catalytic performance of AKR7-2-1 was subsequently investigated. The three-dimensional structure and important sites of AKR7-2-1 were also analyzed. The extensive substrate profile of AKR7-2-1 and strong organic solvent tolerance indicate the great potentials in the synthesis of high-value chiral alcohols.

MATERIALS AND METHODS

Strains, vectors, chemicals

Escherichia coli (*E. coli*) DH5α and BL21 (DE3) were cultured in Luria-Bertani (LB) medium. They were used for cloning and heterologous expression. Plasmid pET-28a was used in this study. All enzymes used in this work were from TaKaRa Co., Ltd. (Dalian, China). Plasmid Mini Kit I (200) and Gel Extraction Kit (200) were purchased from OMEGA Co. (United States). Nicotinamide adenine dinucleotide phosphate (NADPH) was purchased from Sigma-Aldrich Co. (Shanghai, China). All other chemicals were purchased from Aladdin (Germany). All chemicals used were chromatographically pure or analytical grades and therefore required no further purification in use.

Amino acid sequence analysis and 3D modeling

The National Center for Biotechnology Information (NCBI) basic local alignment search tool (BLAST) is commonly used in the analysis of bioinformatics (18). The amino acid sequence of AKR7-2-1 was blasted in NCBI, and nine sequences from other sources having similarity to the sequence of AKR7-1-2 were selected for multiple sequence alignment analysis. The AKR7-2-1 was constructed in a fully automated protein structure homology-modeling server (SWISS-MODEL) commonly used in the construction of three-dimensional models of proteins (19).

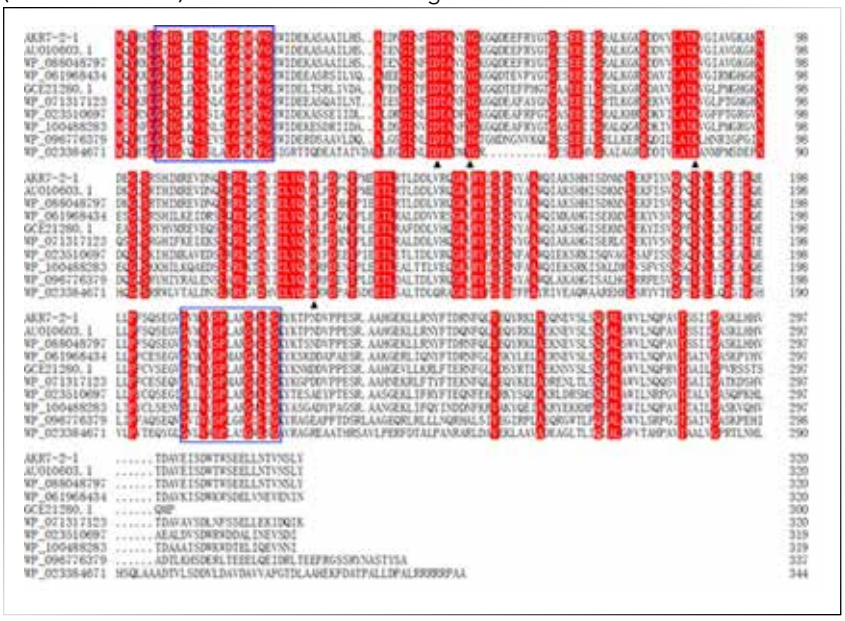
Cloning, expression, and purification of AKR7-2-1

The gene of AKR7-2-1 was amplified by bacterial liquid polymerase chain reaction (PCR), and the primer used in this process was synthesized by Bioengineering Biotechnology (Shanghai) Co., Ltd. The DNA fragment of AKR7-2-1 was cloned into the pET28a vector by genetic engineering, and the recombinant vector pET-28a-akr7-2-1 was transformed into *E. coli* BL21 (DE3) cells for heterologous expression. The AKR7-2-1 was heterologously expressed, and 200 mL of LB medium was added with kanamycin at a concentration of 100 μg/mL. The temperature was set to 37 °C and the cells incubated at 200 rpm. *E. coli* BL21(DE3) was cultured for three to four hours until the optical density (OD) value reached 0.6–0.8. After that, the temperature was changed to 18 °C, and isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture medium with a final concentration of 0.1 mM. The *E. coli* BL21(DE3) cells were further cultured for 14 hours. Afterwards, *E. coli* BL21(DE3) cells were collected by centrifugation and washed three times with phosphate buffered saline (PBS). A sonicator was then used to lyse the cells, and the broken cell debris were centrifuged at 12,000 rpm for 30 minutes. The supernatant was collected and stored at 4 °C for further use. (The previous experimental steps were all performed on ice at all times). AKR7-2-9 was then purified using an AKTA Prime system equipped with a 10-mL Ni-IDA column (GE Healthcare, US). The purification results were subsequently detected using a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) gel. Finally, the protein concentration of AKR7-2-1 was measured using a protein assay kit (Bradford Protein Assay Kit, Sangon Biotech [Shanghai] Co., Ltd.).

Enzyme activity assays

The total volume of the reaction system was 220 μl, which contained 10 μl of methyl pyruvate, 170 μl of PBS, 10 μl of NADPH, and 30 μl of enzyme AKR 7-2-1. One unit of AKR 3-2-9 was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol NADPH per min. The rates of reduction were assayed at 37 °C by measuring the

Figure 1. Homologous comparison of AKR7-2-1 with the other nine amino acid sequences. Conserved sites are marked in red with a blue box labeled as a conserved region rich in glycine and a catalytically active tetrad (DX4YX34KX40H) labeled as a black triangle.



change in absorbance of NADPH at 340 nm (ε= 6.22 mM^{-1} cm^{-1}). Apparent kcat and Michaelis-Menten constant of AKR7-2-1 (Km) were calculated by using the Lineweaver-Burk double reciprocal plot.

CHARACTERIZATION OF THE AKR 7-2-1 ENZYME

Substrate specificity of AKR7-2-1

The substrate specificity of AKR7-2-1 was investigated under the standard assay conditons by using 11 ketone-containing compounds as substrates. The total volume of the reaction system was 220μl, in which the substrate, PBS buffer, cofactor NADPH, and AKR7-2-1 were set to 10 μL, 170 μL, 10 μL, and 30 μL, respectively. The maximum activity of AKR3-2-9 was defined as 100%. In this experiment, 11 substrates were used: 3-methylcyclohexanone, methyl pyruvate, phenoxyacetone, ethyl levulinate, 2-octanone, acetyl, acetone, 5-methyl-2-hexanone, 4-methyl-2-pentanone, phenylmethylketone, N-Boc-3-piperidone, and N, N-Dimethyl-3-keto-3-(2-thienyl)-1-propanamine (DKTP).

Effect of temperature and pH

Methyl pyruvate was used as a substrate to study the effect of temperature and pH on the reaction. To study the optimum temperature of AKR7-2-1, a gradient was set at a 2-°C interval between 30 °C and 50 °C. The reaction system without coenzyme NAPDH was incubated at the corresponding temperature for five minutes. After the completion of the incubation, NAPDH was added to rapidly measure the residual activity of AKR7-2-1 using a microplate reader. To determine the temperature stability of AKR7-2-1, a gradient was set every 10 °C from 30 °C to 80 °C. The AKR7-2-1 was allowed to stand at the corresponding temperature for one hour. After one hour, the residual activity of AKR7-2-1 was determined under standard methods.

To study the optimum pH of AKR7-2-1, CH3COOH-CH3COONa buffer with pH of 4.0–6.0, NaH2PO4-Na2HPO4 buffer with pH of 6.0–8.0, and Tris-HCl buffer with pH of 8.0–9.0 were used. The maximum enzyme activity was defined as 100%. Meanwhile, the pH stability of AKR7-2-1 was investigated by incubating it for 24 hours in the buffer with different pH ranges as described previously. After the incubation was completed, the residual enzyme activity of AKR7-2-1 was determined according to a standard measurement method. The maximum enzyme activity was defined as 100%.

Organic solvent tolerance of the AKR7-2-1 enzyme

Six industrially common organic solvents—including methanol, ethanol, isopropanol, ethyl acetate, acetonitrile, and dimethyl sulfoxide (DMSO)—were selected to study the organic solvent tolerance of AKR7-2-1. The concentration of organic solvents ranged from 10% to 30% V/V. The residual enzyme activity of AKR7-2-1 under different conditions was then determined according to standard methods. In addition, the organic solvent stability was also studied. The above six organic solvents were separately added to the reaction system. The concentration of organic solvent was set at 10% V/V. AKR7-2-1 was placed in the system (without NAPDH) in a 4 °C refrigerator for 10 hours. Every two hours, NAPDH was added to the reaction system and its residual activity was quickly determined by standard methods. The maximum enzyme activity was defined as 100%.

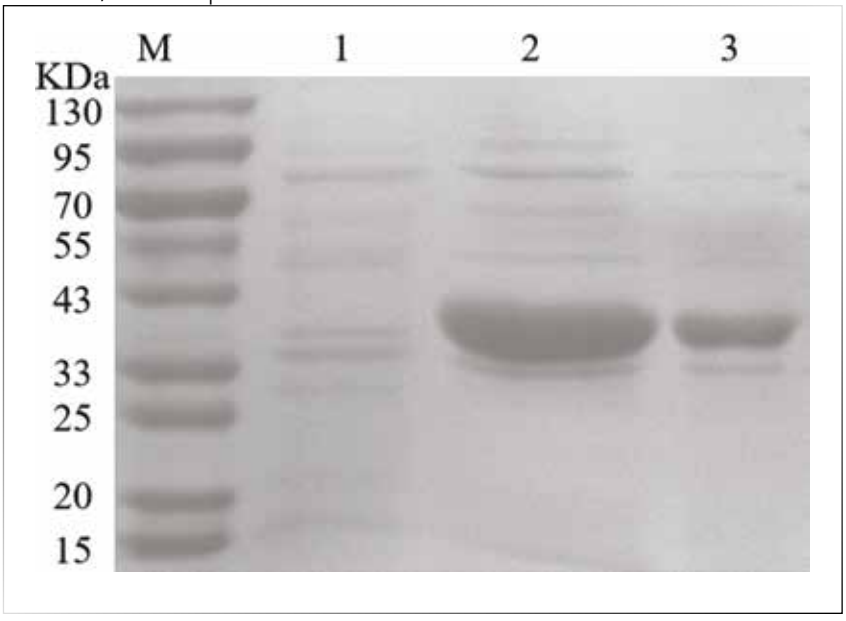
RESULT AND DISCUSSION

Gene cloning and sequencing analysis of AKR3-2-9

In the present study, the cloned DNA fragment of AKR7-2-1 was 963 bp in length. The DNA sequence was translated into an amino acid sequence with a length of 320 bp, which was then subjected to BLAST in NCBI. Nine sequences with different similarities were selected, and multiple sequence alignment

analysis was performed. The result is shown in **Figure 1**. AKR7-2-1 is 97% similar to aldo/keto reductase from *Bacillus megaterium* (NO.:AUO10603.1), 96% similarity to aldo/keto reductase from *Bacillus aryabhattai* (NO.:WP_088048797.1), 76% similarity to aldo/keto reductase from *Fictibacillus enclensis* (NO.:WP_061968434.1), 73% similarity to the aldo/keto reductase from *Ktedonobacterales bacterium Uno11* (NO.:GCE21280.1), 72% similarity to aldo/keto reductase from *Anaerobacillus isosaccharinicus* (NO.:WP_071317123.1), 68% similarity to the aldo/keto reductase from *Youngiibacter fragilis* (NO.:WP_023384671.1), 64% similarity to aldo/keto reductase from *Sporolactobacillus laevolacticus* (NO.:WP_023510697.1), 63% similarity to aldo/keto reductase from *Sporolactobacillus pectinivorans* (WP_100488283.1), 58% similarity to aldo/keto reductase from *Paenibacillus cellulosilyticus* (NO.:WP_110042924.1), 57% similarity to aldo/keto reductase from *Paenibacillus lautus* (NO.:WP_096776379.1). In addition, in the N-terminal and middle (blue box, see **Figure 1**) of the amino acid sequence of AKR7-2-1, a glycine-rich sequence was present, indicating that this motif is highly conserved. The amino acid sequence of AKR7-2-1 also has four highly conserved catalytically active sites located at Asp48, Tyr53, Lys88, and His129 (20). So far, the specific mechanism of action for catalytically active tetrads is not well understood. A limited number of studies have shown that Lys (21) is stable in binding to the coenzyme NAD(P)H by enhancing the stability of the charge interaction and participating in the catalytic reaction; Tyr (22, 23) contains a phenolic hydroxyl group, which binds to the substrate and participates in the catalytic reaction; His (24) is a proton donor in the catalytic process and also relevant to the reduction reaction in combination with the substrate; and Asp (25) plays a vital role in the spatial conformation of proteins.

Figure 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of expression products. Lane M = marker; Lane 1 = not induced with isopropyl β-D-1-thiogalactopyranoside (IPTG); Lane 2 = crude enzyme solution after IPTG induction; Lane 3 = purified AKR3-2-9.



Expression and purification of AKR7-2-1

After induction with 0.1 mM IPTG, the recombinant vector pET-28a-akr7-2-1 was successfully heterologously expressed in *E. coli* BL21 (DE3). The sonicated crude enzyme solution was purified. The SDS–PAGE results can be seen in **Figure 2**.

Substrate specificity

To detect the substrate specificity of AKR7-2-1, 11 substrates with ketone groups were selected in this study, and the results shown in **Figure 3** demonstrate the different degrees of catalytic activity on these substrates by AKR7-2-1. Among them, AKR7-2-1 exhibited the highest catalytic activity when methyl pyruvate, which is an important raw material in pharmaceutical production, is used as a substrate (26). When acetylacetone and DKTP were used as substrates, the catalytic activity of AKR7-2-1 is also relatively high. It is worth mentioning that the reduction of DKTP to DHTP is a key step in the synthesis of the antidepressant duloxetine (26, 27). These results indicate that AKR7-2-1 has a good application prospect in the field of biomedicine because of its broad substrate spectrum.

Effect of temperature and pH

Temperature and pH are important factors influencing enzyme activity, and their effects on AKR7-2-1 were investigated in this study (see **Figure 4**). **Figure 4A** shows that the optimum temperature for AKR7-2-1 is 37 °C, at which point enzyme activity was the highest. Enzyme activity was shown to increase at 30 °C and then decrease as the temperature approached 50 °C. Further, enzyme activity slowly decreased as the temperature exceeded 37 °C, but still retained 70% or more relative activity even when it reached 50 °C. Previous studies by other researchers had reported an optimum temperature of 30 °C for an aldo-keto reductase cloned from Kluyveromyces lactis XP1461 with enzyme activity decreasing sharply when the tempera-

Figure 3. Substrate spectrum of AKR7-2-1. The number 1 represents 3-methylcyclohexanone; the number 2 represents methyl pyruvate; the number 3 represents phenoxyacetone; the number 4 represents ethyl levulinate; the number 5 represents 2-octanone; the number 6 represents acetylacetone; the number 7 represents 5-methyl-2-hexanone; the number 8 represents 5-methyl-2-hexanone, 4-methyl-2-pentanone; the number 9 represents phenylmethylketone; the number 10 represents N-Boc-3-piperidone; and the number 11 represents N,N-dimethyl-3-keto-3-(2-thienyl)-1-propanamine (DKTP). This experiment was repeated three times, and the error bars represent the standard error of the mean. The maximum enzyme activity of AKR7-2-1 was defined as 100%.

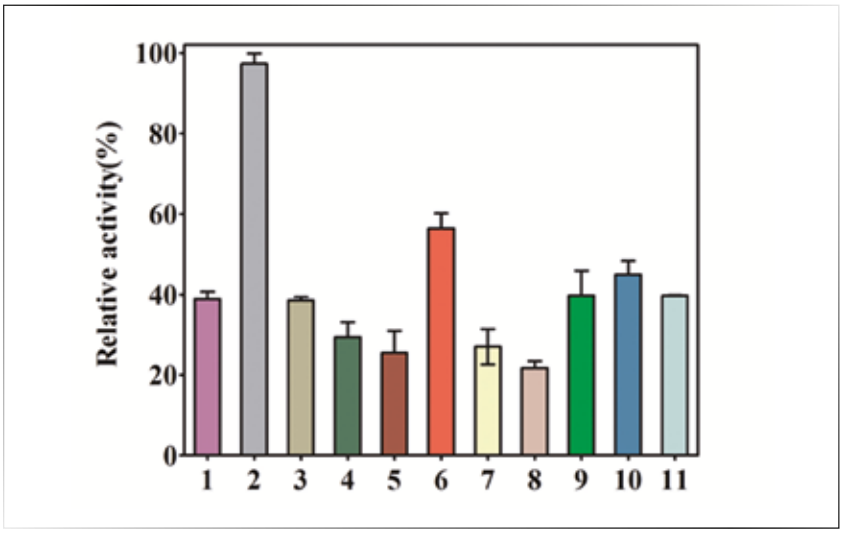
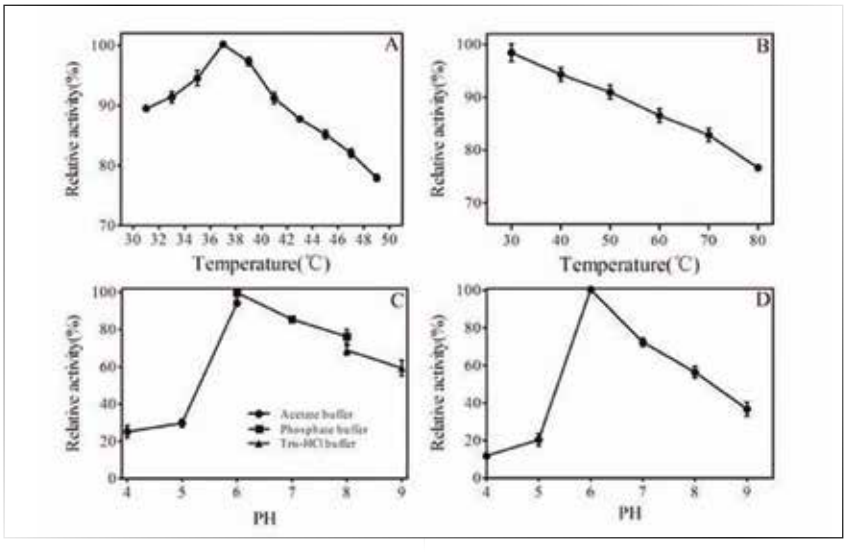


Figure 4. Effect of temperature and pH on AKR7-2-1. (A) Optimum temperature of AKR7-2-1 (B) Temperature stability of AKR7-2-1. (C) Optimum pH of AKR7-2-1 50 mM acetate buffer (•), 50 mM potassium phosphate (■) and 50 mM Tris-HCl (▲) were used. (D) pH stability of AKR3-2-9. All of the above experiments evaluated residual enzyme activity under standard assay conditions. The maximum enzyme activity was defined as 100%. All experiments were performed in triplicate, and the error bars represent the standard error of the mean.



ture exceeded 35 °C. The reductase in those previous studies kept less than 20% activity when the temperature was set to 50 °C (12). Compared with the aldo-keto reductase in previous studies, AKR7-2-1 was well resistant to high temperature. The thermal stability of AKR7-2-1 was investigated by incubating the enzyme at a temperature range of 30 °C to 80 °C (**Figure 4B**). It was shown that relative enzyme activity decreased with the incremental increase in temperature; however, more than 70% activity remained when the temperature reached 80 °C, showing superior thermal stability. In comparison, an aldo-keto reductase cloned from Lodderomyces elongisporus NRRL YB-4239 by Q. Wang *et al.* completely lost activity when incubated for 30 minutes at 45 °C (28).

As can be seen in **Figure 4C**, the optimum pH of AKR7-2-1 is 6.0. When the pH is lower than 6.0, the enzyme activity of AKR7-2-1 is drastically decreased, with activity falling to 20% when pH is reduced to 5.0. Interestingly, as the pH dropped from 5.0 to 4.0, it decelerated the reduction of enzyme acitivity. On the other hand, the activity of AKR7-2-1 decreased only mildly when the pH was above 6.0, maintaining 60% activity even when the pH reached 9.0. In addition, there is an interesting phenomenon that even under the same pH conditions, the enzyme activity of AKR7-2-1 in the phosphate buffer was higher than in the acetate buffer or the Tris-HCl buffer. This is similar to the results studied by Y. Wang *et al.* (29). AKR7-2-1 exhibited the best stability in a buffer with pH of 6.0. as shown in **Figure 4D**. Its activity decreased to less than 20% when incubated in buffer at a pH below 5.0 for 24 hours. The relative enzyme activity also decreased at a pH ranging from 6.0 to 9.0, although it still retained 40% activity when the pH reached 9.0, which indicates an ability to resist extreme pH. In a similar study by Q. Wang *et al.* (28), the enzyme retained less than 30% of activity even when it

was only incubated in a buffer of pH 8.0 for 60 minutes.

Kinetic analysis

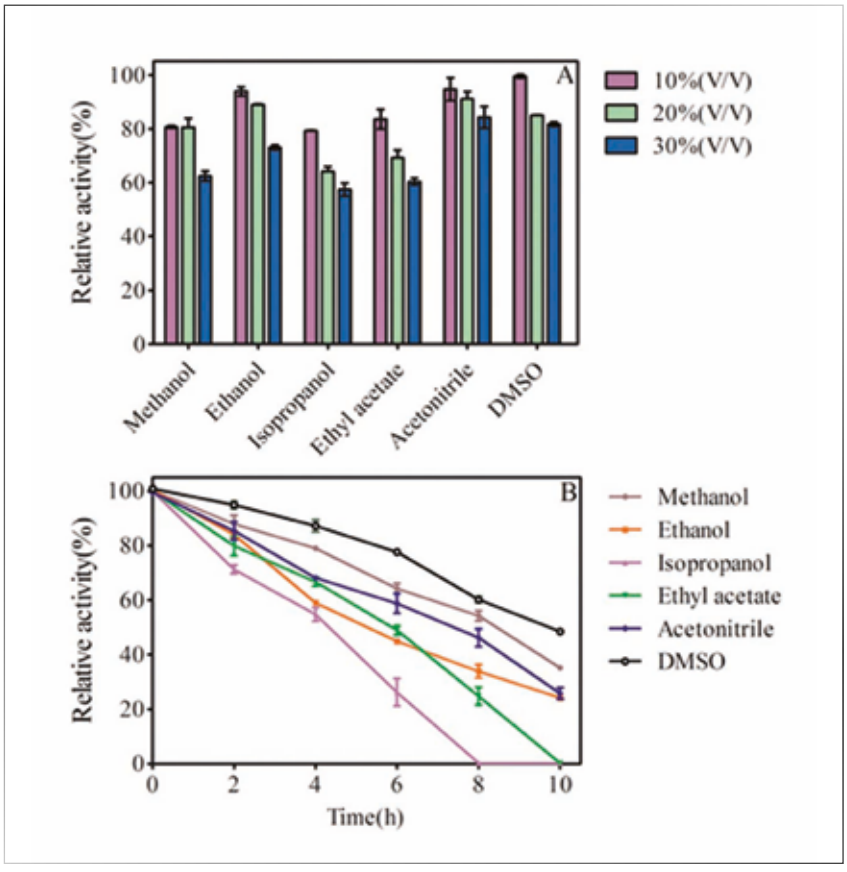
In this study, the kinetic analysis of purified AKR7-2-1 was also performed. The results of various kinetic parameters of AKR7-2-1 show that *Vmax*, *Km*, *Kcat*, and *Kcat/Km* of the enzyme was 110.0 uM/min, 4.380 uM, 57.29 min^{-1}, and 13.04 $min^{-1}uM^{-1}$, respectively. These kinetic parameter data indicate the strong binding ability to the substrate (methyl pyruvate). In addition, the Kcat/Km of AKR7-2-1 is 13.04 $min^{-1}uM^{-1}$ (218 $s^{-1}mM^{-1}$), demonstrating an extremely high catalytic efficiency compared to other reported Kcat/Km of AKRs. For example, the Kcat/Km of kmAKR-W297 is 60.97 $s^{-1}mM^{-1}$ (29), Tm1743 is 54.6 $s^{-1}mM^{-1}$ (13), and CgKR1-F92L is 33.2 $s^{-1}mM^{-1}$ (30).

Detection of AKR3-2-9-tolerant organic solvent properties

Enzyme catalytic systems containing organic solvents have many advantages in industrial production. Many natural enzymes, however, are poorly resistant to organic solvents (31). As shown in **Figure 5A**, methanol, ethanol, isopropanol, ethyl acetate, acetonitrile, and DMSO were used to study the organic solvent tolerance of AKR7-2-1, which was able to survive to 10% V/V of organic solvents with more than 80% residual activity. When the concentration of the organic solvent in the enzymatic reaction increased from 10% V/V to 30% V/V, the enzyme activity of AKR7-2-1 began to gradually decrease but still retained more than 60% activity.

Furthermore, the organic solvent stability of AKR7-2-1 was also studied by incubating the enzyme in methanol, ethanol, isopropanol, ethyl acetate, acetonitrile, or DMSO with the concentration of 10% V/V for 10 hours, and the enzyme activity of AKR7-2 was detected every two hours. As shown by **Figure 5B**, AKR7-2-1 retained more than 50% enzyme activity in DMSO after 10 hours of incubation, while it retained approximately 40% activity in methanol, ethanol, and acetonitrile under the same reaction conditions. An aldo-keto reductase cloned by X. Luo *et al.* from Kluyveromyces lactis XP1461 was only able to tolerate up to 5% V/V of organic solvents such as isopropanol and ethyl acetate (12). In addition, an aldo-keto reductase (Tm1743) cloned from Thermotoga maritima has been reported to withstand up to 10% V/V of organic solvent (13). Compared with the aldo-keto reductase reported, AKR7-2-1 showed superior organic tolerance.

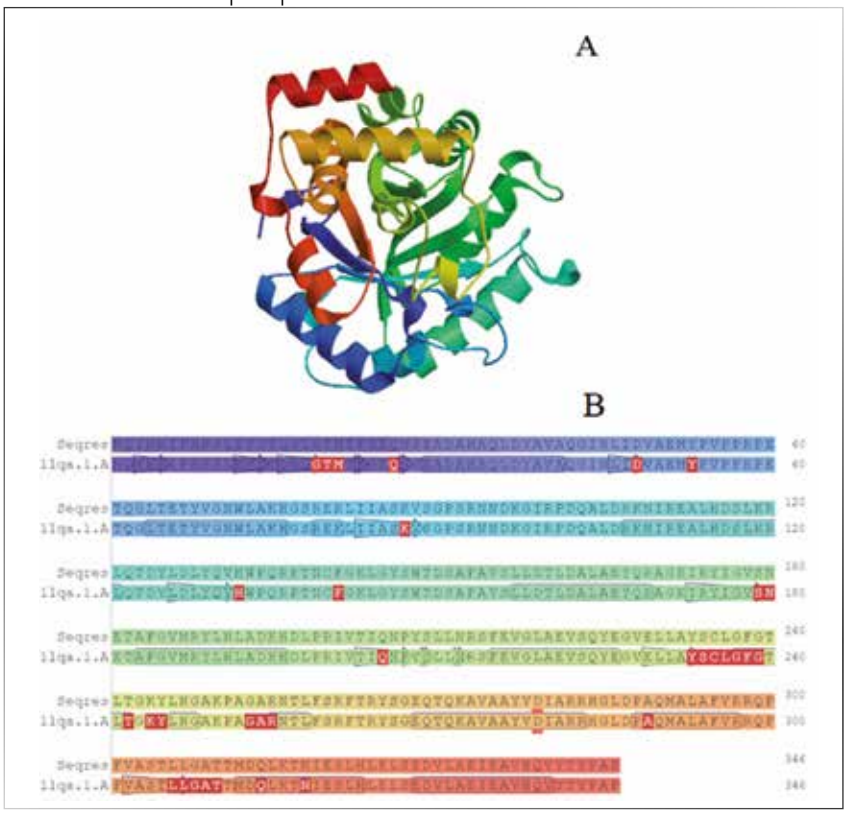
Figure 5. (A) Detection of the resistance of AKR7-2-1 to organic solvents. The six organic solvents were methanol, ethanol, isopropanol, ethyl acetate, acetonitrile, and d imethyl sulfoxide (DMSO). Each organic solvent was set to a concentration gradient of 10% V/V to 30% v/v. (B) Organic solvent stability of AKR7-2-1. Incubate for 10 hours in six organic solvents at 10% V/V and test once every two hours. The enzyme activity of AKR3-2-9 was measured using standard methods. The experiment was performed in triplicate and the error bars represent the standard error of the mean.



Molecular modeling

A three-dimensional model of AKR7-2-1 that was constructed using SWISS-MODEL, AKR7-2-1, and the Crystal structure of the *E. coli* Tas protein (SMTL ID: 1lqa.1) were shown to be structurally similar (**Figure 6A**). This protein is an NADP(H)-dependent aldo-keto reductase (32). The amino acid sequence of AKR7-2-1 is compared with the amino acid sequence of 1lqa.1 as shown in **Figure 6B**. The homology modeling results of AKR7-2-1 illustrated that it may have a binding site for NADPH coenzyme with a high conservative. As shown in **Figure 6B**, the amino acid positions marked with white letters on a red background are possible binding sites for NADPH and AKR7-2-1.

Figure 6. (A) The 3D model of AKR7-2-1 and (B) alignment of the sequence of AKR7-2-1 with the sequence of 1lqa.1. The amino acid positions marked with white letters on a red background are possible binding sites for nicotinamide adenine dinucleotide phosphate and AKR7-2-1.



CONCLUSION

In this study, a novel aldo-keto reductase AKR7-2-1 with conservative site was excavated. The broad substrate spectrum showed that the enzyme was especially able to catalyze DKTP to DHTP, an important precursor of the antidepressant drug duloxetine. In addition, the enzyme was shown to have superior thermal stability, pH stability, and excellent tolerance to a wide range of organic solvents. These characteristics make AKR7-2-1 a promising enzyme to be applied in the medicine field.

ACKNOWLEDGMENTS

The work was supported by the Natural Science Foundation of China (No. 21808073), the high-level personnel activation fee of Huaqiao University (No. 600005-Z17Y0072), and Quanzhou City Science & Technology Program of China (No. 2018C008).

AUTHOR CONTRIBUTIONS

Rui Pei did experiments, Weiliang Wu, PanPan Zhao, and Libing Tian provided help for the experiment. Pei and Wei Jiang wrote and modified the article, and Jiang and Shu-Feng Zhou designed and supervised the work.

CONFLICT OF INTEREST

The authors state that they have no competing interests.

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