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Characterization and identification of three novel aldo–keto reductases from *Lodderomyces elongisporus* for reducing ethyl 4-chloroacetoacetate

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ABSTRACT

Lodderomyces elongisporus LH703 isolated from soil samples contained three novel aldo–keto reductases (AKRs) (LEAKR 48, LEAKR 49, and LEAKR 50). The three enzymes were cloned, expressed, and purified to homogeneity for characterization. These three AKRs shared <40% amino acid identity with each other. LEAKR 50 was identified as a member of AKR3 family, whereas the other two LEAKRs were identified as members of two novel AKR families, respectively. All the three AKRs required nicotinamide adenine dinucleotide phosphate as a cofactor. However, they showed diverse characteristics, including optimum catalyzing conditions, resistance to adverse reaction conditions, and substrate specificity. LEAKR 50 was estimated to be a promising biocatalyst that could reduce ethyl 4-chloroacetoacetate with high enantiomeric excess (98% e. e.) and high activity residue under adverse conditions.

Introduction

Aldo–keto reductases (AKRs)[1] belong to a growing oxidoreductase superfamily of proteins that bind to cofactor nicotinamide adenine dinucleotide phosphate (NADPH) without a Rossmann-fold motif [13]. They are present in various mammals, plants, yeast, and bacteria. Many mammalian AKRs are potential therapeutic targets. Moreover, structure-based drug designing has produced compounds with desired specificity and clinical efficacy [14]. Microbial AKRs catalyze the reduction of diverse substrates, including aliphatic and aromatic aldehydes, monosaccharides, steroids, prostaglandins, polycyclic aromatic hydrocarbons, and isoflavonoids, with excellent enantioselectivity and yield [5,20,21]. The resulting alcohols represent chiral building blocks that act as key intermediates of pharmaceutical and agricultural products [4]. Optically pure secondary alcohols have especially drawn a great deal of attention in introducing chiral groups into products [9].

Use of biocatalysts for manufacturing chiral chemicals offers major advantages in terms of enhanced reaction selectivity, reduced cost of raw materials, lowered energy cost, improved safety, and production sustainability [8]. Several enzymes are known to be attractive options for the industry. Of these, microbial AKRs have the potential to serve as versatile tools for producing various valuable optically pure compounds. Therefore, discovery of new AKRs is of a significant value.

In this study, we discovered, cloned, heterologously expressed, and characterized three novel AKR genes from *Lodderomyces elongisporus* LH703. These three AKR genes (*LEAKR 48*, *LEAKR 49*, and *LEAKR 50*) shared <50% identity in their gene sequences and <40% in their amino acid sequences. Although each AKR belonged to different AKR family, they shared the same conserved residues. Two of these AKRs (*LEAKR 48* and *LEAKR 49*) were identified as members of novel AKR families. All the three AKRs showed different thermostability, substrate specificity, and enantioselectivity. Detailed sequence analysis was used to investigate the different characteristics resulting from sequence diversity and to open the gate for AKR gene modification.

Materials and methods

Chemicals and enzymes

Prochiral ketones and (*R*)- or (*S*)-alcohols used in this study were purchased from Alfa Aesar (Karlsruhe, Germany),

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[1] *Abbreviations used:* AKRs, aldo–keto reductases; NADPH, nicotinamide adenine dinucleotide phosphate; GC, gas chromatography; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; MALDI-TOF-MS, Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry; GDH, D-glucose dehydrogenase; LEAKRs, *L. elongisporus* that encoded AKRs.

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Sigma-Aldrich (Schnelldorf, Germany), Acros Organics (New Jersey, USA), and Aladdin (Shanghai, China). Enzymes were obtained from Takara Biotechnology (Dalian, China) and Fermentas China (Shenzhen, China). Primers were obtained from Generay Biotechnology (Shanghai, China).

Microorganism cultivation

The medium for screening yeast included 0.6% (wt/vol) KH_2PO_4, 0.6% $(NH_4)_2SO_4$, 0.06% $MgSO_4{\cdot}7H_2O$, 0.006% $ZnSO_4{\cdot}7H_2O$, 0.006% $FeSO_4{\cdot}7H_2O$, 0.001% $CuSO_4{\cdot}5H_2O$, 0.001% $MnSO_4{\cdot}4H_2O$, 0.01% NaCl, 0.5% yeast extract, and 3% glucose (pH 7.0). Soil samples were collected from rich fields in Shanghai, China. Each soil sample (1 g) was added to a test tube containing 10 mL sterile distilled water and was shaken on a rotary shaker at 180 rpm for approximately 30 min. Next, 1 mL of the soil suspension was added to an Erlenmeyer flask containing 50 mL yeast screening medium. The flask was shaken on a rotary shaker at 28 °C, 150 rpm for 48 h. After 48-h incubation, 1 mL of culture was diluted appropriately and was spread onto a plate containing solid yeast screening medium. After 48 h of incubation at 28 °C, individual colonies on the plates were inoculated into a test tube (15 mmØ × 150 mm) containing 2 mL medium that was constantly shaken for 28 h at 28 °C.

Screening of ethyl 4-chloroacetoacetate-reducing strains

Cells grown in the test tubes were collected by centrifugation, and were washed. The cells were then suspended in 0.5 mL reaction mixture containing 100 mM sodium phosphate buffer (pH 7.0), 20 mM ethyl 4-chloroacetoacetate, and 500 mM D-glucose. After shaking for 24 h at 30 °C, the reaction mixture was extracted using 1 mL ethyl acetate, and was centrifuged. The organic layer was dried over anhydrous sodium sulfate and then analyzed to determine the conversion and optical purity by gas chromatography (GC) and high performance liquid chromatography (HPLC), respectively. Strains were identified using standard nucleotide sequence analysis.

Cloning

Genomic DNA was isolated from *L. elongisporus* LH703 (CCTCC AY 2014001) using a TaKaRa MiniBEST Bacterial Genomic DNA Extraction Kit (TaKaRa, China). Genes encoding the three LEAKRs were amplified using polymerase chain reaction (PCR; 48: forward primer, 5′-CGCCATATGGCATCCAACTT-3′ and reverse primer, 5′-CGCAAGCTTTTAAGCATCACCATC-3′; 49: forward primer, 5′-GC GCCATATGTCGTATAGAT-3′ and reverse primer, 5′-CAAGCTTTCATG GAGCATC-3′; 50: forward primer, 5′- GCGCCATATGTCTTCTCAA-3' and reverse primer, 5′-CGCAAGCTTTTAGGCATT-3′ [with *Nde*I and *Hind*III sites being underlined]). The primers were designed using sequences of conserved hypothetical protein partial mRNA with GenBank accession numbers: XM_001525808, XM_001526403, and XM_001526411. Each double-digested PCR product was inserted into pET-28a vector. Because of the chosen restriction sites, the N-terminal 6× His-tag sequence of the plasmid was retained. The resulting vector was transformed into *Escherichia coli* DH5α competent cells. After isolation from competent cells and DNA sequencing, the resulting plasmid was finally transformed into *E. coli* BL21 (DE3) cells.

Recombinant expression and purification of recombinant LEAKRs

A single colony was inoculated in a test tube (15 mmØ × 150 mm) containing 3 mL preculture medium and was constantly shaken (200 rpm) at 37 °C for 12 h. The cells were then cultured in 100 mL LB medium with kanamycin (final concentration, 30 mg l^{-1}) and were shaken (200 rpm) at 37 °C. Isopropyl-β-D-thiogalactopyranoside (final concentration 0.1 mM) was added for induction after the cells reached an optical density of 0.8 at 600 nm. Culturation continued at 20 °C for 20 h (200 rpm) until cells were collected by centrifugation. The centrifuged cells were suspended in 20 mL sodium phosphate buffer (100 mM, pH 7.0) and were disrupted using an ultrasonic oscillator (JY92-2D; Scienz Biotechnology Co. Ltd. Ningbo, China). The cell debris was removed by centrifugation (10 min, 15,000×*g*, 4 °C). The supernatant was used as cell-free extract, and was applied to a 5 mL His-Trap column assembled with Ni-NTA HisBind Resin (EMD Chemicals, Inc. NY, USA). The protein of interest that contained 6× His-tag was purified by binding and eluting with NPI elution buffers (50 mM NaH_2PO_4, 300 mM NaCl, and varied concentrations of imidazole [pH 8.0]). NPI-10, NPI-60, and NPI-200 (corresponding to 10 mM, 60 mM, and 200 mM of imidazole) were employed. The volume of elution buffers employed in this study was 10 times volume of Ni-NTA HisBind Resin. Binding procedure was carried out after balancing the His-Trap column with NPI-10. NPI-60 was then used to wash the His-Trap column. NPI-200 was employed to elute the protein of interest. The obtained protein solution was treated with dialysis overnight in sodium phosphate buffer (100 mM, pH 7.0), and then concentrated by ultrafiltration (molecular weight cutoff, 30 kDa). The 6× His-tag of the protein were cleaved by incubation with thrombin at 4 °C overnight, followed by further purification with His-Trap column again. The purified protein was examined using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE).

Protein analysis

Protein concentration was determined using Bradford assay with bovine serum albumin as the standard [3]. Protein purity was analyzed using SDS–PAGE. Protein samples for SDS-PAGE were prepared by heating the protein for 10 min at 100 °C in the presence of a loading buffer (Takara Biotechnology Co. Ltd). A protein marker (Fermentas China Co. Ltd) was used to estimate the molecular weight of the obtained proteins. Molecular mass of the purified enzymes and their subunits were determined using Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS).

Activity assay

The activity of the LEAKRs was determined using an assay mixture containing sodium phosphate buffer (100 mM, pH 7.0), 6 mM ethyl 4-chloroacetoacetate as substrate and 0.2 mM cofactor. A continuous assay using UV absorbance at 340 nm was employed to monitor the concentration of the cofactor during the reaction at 30 °C. One unit of activity was defined as the amount of LEAKR that catalyzed the oxidation of 1 μmol cofactor per minute under standard conditions [23].

Characterization of recombinant LEAKRs

Optimum temperature and thermal stability of the purified enzymes

The optimum temperature for each LEAKR was determined by incubating each enzyme in assay mixtures (pH 7.0) for 5 min at temperatures ranging from 15 to 65 °C. The subsequent reaction was started by adding ethyl 4-chloroacetoacetate as the substrate. For thermostability analysis, each enzyme was pre-incubated for 1 and 3 h at temperatures ranging from 15 to 65 °C, and then incubated at 30 °C for 15 min. The residual enzyme activity was determined at 30 °C under standard conditions.

Optimum pH and pH stability of the purified enzymes

The effect of pH on enzyme activity was determined using the following 0.1 M buffer systems: sodium citrate buffer (pH

3.0–6.0), sodium phosphate buffer (pH 6.0–8.0) and glycine–NaOH buffer (pH 8.0–11.0). For pH stability assay, each enzyme was pre-incubated in the buffer systems described above for 1 and 3 h at room temperature. The residual enzyme activity was assayed in sodium phosphate buffer system (pH 7.0) under standard conditions.

Cofactor and substrate specificity

Cofactor dependence of each LEAKR was analyzed by adding NADH or NADPH separately as cofactor into the assay mixtures and by determining LEAKR activity. Substrate specificity of each LEAKR was determined by employing different substrates (2 mM) and conducting activity assays under standard conditions.

Kinetic parameters

Kinetic parameters K_m and V_{max} were obtained from multiple determinations using Michaelis-Menten equation and program Origin (Origin Pro, version 8.0). Nonlinear Curve Fit from the analysis software program was used for least-squares parameter fitting on the basis of a Levenberg–Marquardt algorithm.

Effect of organic solvents on enzyme activity

The enzymes were pre-incubated for 60 min in a reaction mixture containing various organic solvents with increasing log *P* values. LEAKR activity in reaction systems with vol/vol 5% and 10% concentrations of the organic solvents was assayed under standard conditions, and reaction rates were compared for reactions with and without organic solvents.

Effect of metal ions, chelators, and detergents on enzyme activity

Various detergents, metal ions, and chelators were added to the reaction systems and were pre-incubated for 10 min at 30 °C. The residual activity of the enzymes was determined under standard conditions and was presented as percentage of enzyme activity without any additive.

Enantioselective reduction of ethyl 4-chloroacetoacetate

The enantioselectivity of LEAKRs was determined by employing NADPH regeneration system composed of D-glucose dehydrogenase (GDH) and D-glucose. The reaction mixture included 20 mM ethyl 4-chloroacetoacetate, 0.2 mM NADPH, 0.03 mg LEAKR (1.2 mg for LEAKR 49), 100 mM D-glucose, 0.1 mg GDH, and 100 mM sodium phosphate buffer (pH 7.0), with the final volume of 0.5 mL. After shaking constantly at 30 °C, the reaction mixture was extracted with 1 mL ethyl acetate. The organic layer collected by centrifugation and dried with anhydrous sodium sulfate was then analyzed to determine the conversion of the substrate and enantiomeric excess (e. e.) of the products by employing GC and HPLC as efficient and sensitive tools. A GC station (Agilent 6890N) with a capillary column (DB-5) was used to analyze the conversion of ethyl 4-chloroacetoacetate while an HPLC station (Agilent 1100) with a Chiralcel OB-H column was employed for the optical assay of the products.

The operating conditions for GC were as follows: flow rate was 1.3 mL/min, injector temperature was 250 °C, detector temperature was 250 °C and column temperature was programmed: 3 min at 90 °C, 10 °C/min to 180 °C and 2 min at 180 °C. Injection volume was 0.5 μL. Ethyl 4-oxopentanoate was employed as internal standard. Retention times were: ethyl 4-oxopentanoate, 6.1 min; ethyl 4-chloroacetoacetate, 6.9 min; and ethyl 4-chloro-3-hydroxybutyrate, 7.3 min.

Chiral detection of product ethyl 4-chloro-3-hydroxybutyrate was performed with a chiralcel OB-H column (Daicel Chemical Industries, Ltd.). The mobile phase was 90% hexane/10% isopropanol, flow rate was 0.8 mL/min, column temperature was 25 °C, detection was with a DAD set at 220 nm and injection volume was 10 μL. Retention times were: R-enantiomer, 11 min; S-enantiomer, 12 min; and ethyl 4-chloroacetoacetate, 19 min.

NCBI accession number for nucleotide sequence

LEAKR 48, GenBank accession number: JQ365664; LEAKR 49, GenBank accession number: JQ365665; LEAKR 50, GenBank accession number: JQ365666.

Results

Strains screening and genes selection

In the screening experiments, the method for detecting ethyl 4-chloroacetoacetate-reducing strains described above was performed efficiently. The resulting data showed that 41% of the isolated strains (approximately 400 strains) could catalyze the reduction of ethyl 4-chloroacetoacetate, however, most of these strains showed efficiency. One strain, identified by 18S rDNA as *L. elongisporus* (LH703) showed the best efficiency and high enantioselectivity in the screening reaction. *L. elongisporus* LH703 produced ethyl (*R*)-4-chloro-3-hydroxybutyrate, with a conversion rate of 92% and e. e. value of 80%.

The genome of *L. elongisporus* has already been analyzed for genes encoding hypothetical conserved proteins with oxidoreductase activity. Several potential genes encoding such proteins have been discovered. Of these, three genes were selected for further identification of their catalyzing activity to ethyl 4-chloroacetoacetate. These three genes shared <50% identity with each other.

Purification of recombinant LEAKRs

The purified proteins were obtained using an optimized elution procedure. The typical yield of pure protein during the entire purification procedure was 0.12–0.14 mg/g wet cell weight. SDS–PAGE (Fig. 1) revealed a single band for each LEAKR. MALDI-TOF-MS assay showed that the molecular mass of each purified enzyme

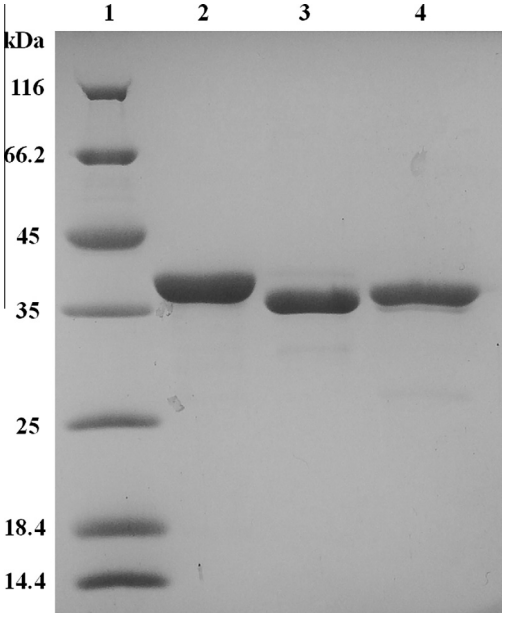

Fig. 1. SDS–PAGE analysis of each recombinant *Lodderomyces elongisporus* AKRs after purification step. Lane 1, protein marker; lane 2, purified LEAKR 48; lane 3, purified LEAKR 49; lane 4, purified LEAKR 50.

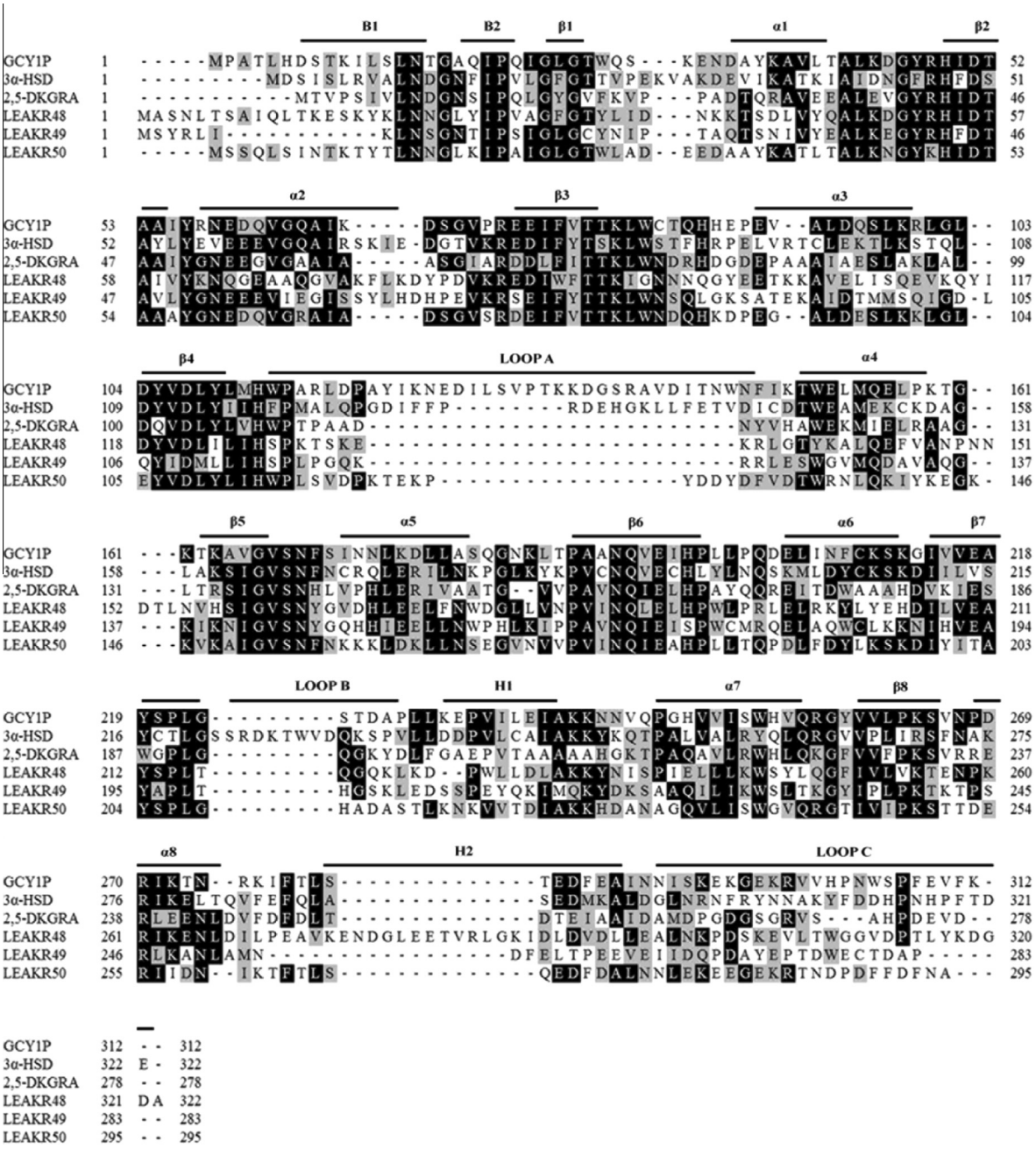

Fig. 2. Amino acid sequence alignment of three *Lodderomyces elongisporus* AKRs with members of the aldo–keto reductase superfamily. The abbreviations are used: GCY1P, GCY1 protein from *S. cerevisiae*; 3α-HSD, 3α-hydroxysteroid dehydrogenase from Rattus norvegicus liver; 2,5-DKGRA, 2,5-diketo-D-gluconic acid reductase from *Corynebacterium* sp.; LEAKR 48, aldo–keto reductase 48 from *Lodderomyces elongisporus*; LEAKR 49, aldo–keto reductase 49 from *Lodderomyces elongisporus*; LEAKR 50, aldo–keto reductase 50 from *Lodderomyces elongisporus*. The sequences were aligned using the CLUSTAL X program.

and its subunit was 156 kDa and 39 kDa for LEAKR 48, 204 kDa and 34 kDa for LEAKR 49, and 132 kDa and 33 kDa for LEAKR 50, respectively. LEAKR 48 and LEAKR 50 were found to be homotetramers while LEAKR 49 included six identical subunits. The activity of each LEAKR was determined under standard conditions. The activity of LEAKR 48 was 5.2 U/mg protein, LEAKR 49 was 0.14 U/mg protein, and LEAKR 50 was 7.6 U/mg protein.

Sequences analysis

Analysis of amino acid sequences of the enzymes encoded by the three genes showed differences among them. The identities of amino acid sequences of these enzymes (39.78% between LEAKR 48 and LEAKR 49; 29.97% between LEAKR 48 and LEAKR 50; and 27.34% between LEAKR 49 and LEAKR 50) are presented in Fig. 2. BLAST-P analysis (http://www.ncbi.nlm.nih.gov/BLAST/) was used to identify these enzymes, results showed that these enzymes shared high identities with many AKRs or putative members of AKR superfamily. GCY1 protein from *Saccharomyces. cerevisiae* (P14065), 3α-hydroxysteroid dehydrogenase from Rattus norvegicus liver (P23457), and 2,5-diketo-D-gluconic acid reductase from *Corynebacterium* sp. (AAA83534) belong to AKR3, AKR1, and AKR5 families, respectively, and have determined protein structures. These three AKRs along with the LEAKRs examined in this study were selected to obtain an alignment of six proteins for detailed analysis (Fig. 2).

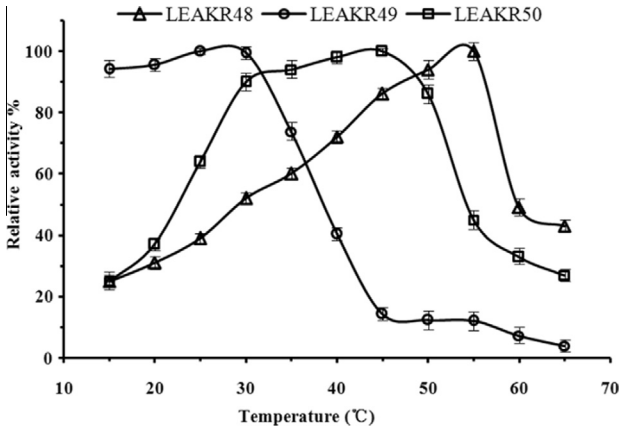

Fig. 3. Effect of reaction temperature on each purified *Lodderomyces elongisporus* AKR. Each of their activities at optimal reaction temperatures corresponds to 100% activity. The experiments were done in triplicate and the error bar represents the percentage error in each set of reading.

The AKR superfamily proteins share the characteristic $(\alpha/\beta)_8$-barrel structure [13] and conserved active site residues. Asp-52, Ala-54, Tyr-57, Lys-82, Leu-111, Ser-154, Asn-155, and Tyr-204 (the number of amino acid residues in this article corresponds to that of LEAKR 50) were found to be conserved in the three LEAKRs based on the alignment. Together with residue 28, which varied in different LEAKRs, they formed the bottom portion of the active site pocket. Residues comprising the rim of the pocket at 56, 84, 114, and 286, except His-113, were partially different among the three LEAKRs [18]. Of all the residues, Asp-52, Tyr-57, Lys-82 and His-113 were crucial for the catalytic activity. Tyr-57 is considered as the general acid in the reaction while Lys-82 and His-113 are thought to be involved in proton relay [10,7]. These residues are strictly conserved among AKRs. In contrast to short-chain dehydrogenase superfamily, AKRs bind to the cofactor NADPH (preferred by most AKRs) in an extended conformation without a Rossmann-fold motif [14,7,22]. Residues Asp-52, Ser-154, Asn-155, Gln-178, Leu-207, Lys-249, Arg-255, Asn-259, and Ser-271 of the three LEAKRs were involved in cofactor binding. Of these residues, three were a part of the active site pocket, as mentioned above. Asp-52 was highly conserved and played an important role in the binding mechanism. This was confirmed by a structure study of 3α-hydroxysteroid dehydrogenase from *R. norvegicus* [14]. The resulting sequence alignment indicated that there were no major gaps in the regions corresponding to the barrel structure. However, major gaps appeared in the C-terminal of the alignment, especially in the sequences of A-loop, B-loop, and H_1-helice. Loops of AKRs are known to vary near the active site to fit different substrates while maintaining the structure of the barrel scaffold [7]. The rest of conserved residues probably correspond to other protein characteristics. Based on the nomenclature of the AKR superfamily [15], LEAKR 50 was identified as a member of AKR3 family and shared >40% sequence identity with other members of this family. The other two LEAKRs (LEAKR 48 and LEAKR 49) were probably the members of two novel AKR families and shared <40% sequence identity with other AKR families identified thus far and with each other.

Optimum temperature and thermostability

Enzyme activity and thermostability were assayed for the three LEAKRs at various temperatures (Figs. 3 and 4). LEAKR 49 was

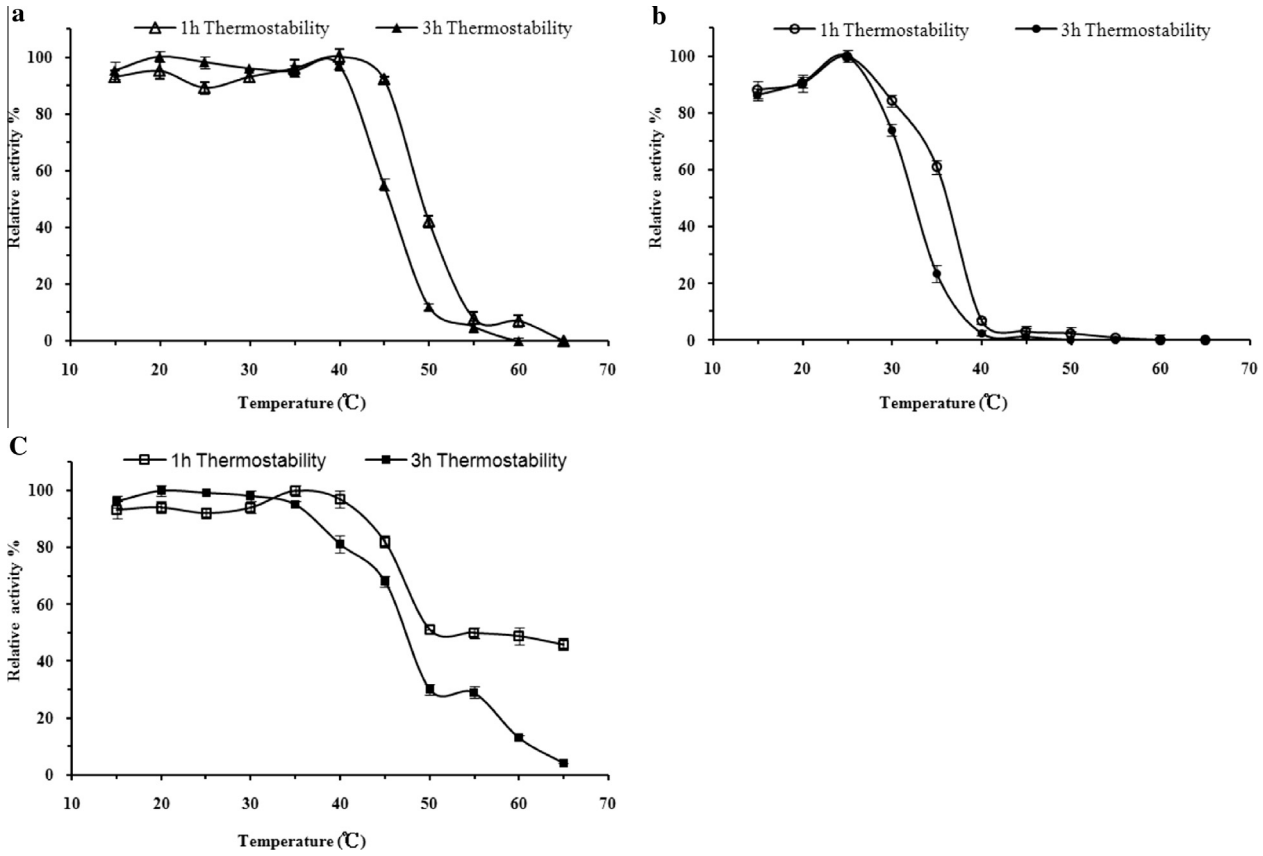

Fig. 4. Thermostability of each purified *Lodderomyces elongisporus* AKR: LEAKR 48 (a); LEAKR 49 (b); LEAKR 50 (c). Each of their activities at optimal temperatures corresponds to 100% activity. The experiments were done in triplicate and the error bar represents the percentage error in each set of reading.

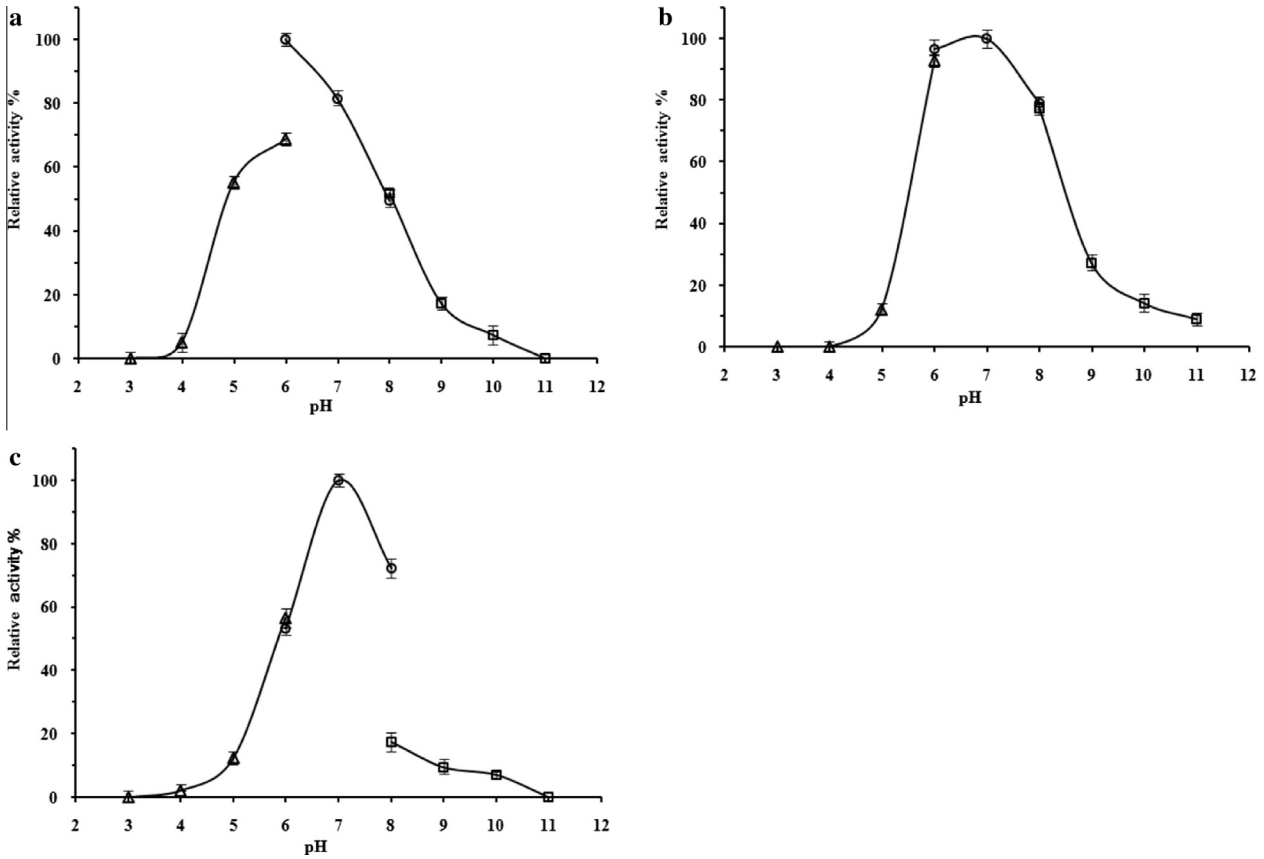

Fig. 5. Effect of pH on each purified *Lodderomyces elongisporus* AKR: LEAKR 48 (a); LEAKR 49 (b); LEAKR 50 (c). The activity was measured in the following 0.1 M buffers: sodium citrate buffer (pH 3.0–6.0), sodium phosphate buffer (pH 6.0–8.0) and glycine–NaOH buffer (pH 8.0–11.0). The experiments were done in triplicate and the error bar represents the percentage error in each set of reading.

found to be heat sensitive with an optimum temperature of 30 °C, and showed a sharp decline in activity when it was pre-incubated at higher temperatures. In contrast, LEAKR 48 and LEAKR 50 showed stronger heat resistance with maximal activity at 55 °C (10 U/mg) and 45 °C (4.2 U/mg), respectively. LEAKR 48 was stable at temperature <40 °C for 3 h and showed an activity of 92% after pre-incubation at 45 °C for 1 h. LEAKR 50 showed a high activity of >86% at a wide temperature range from 30 to 50 °C.

Optimum pH and stability

The effect of pH on enzyme activity was measured using buffer systems with various pH. Optimum pH was determined for three LEAKRs separately: pH 6.0 for LEAKR 48, pH 7.0 for LEAKR 49 and LEAKR 50 (Fig. 5). As for pH stability, LEAKR 49 was stable at pH from 6.0 to 7.0, however, it lost most of its activity at pH 5.0. LEAKR 48 and LEAKR 50 retained most of their activity at extreme pH ranging from 3.0 to 10.0 for 3 h. In addition, LEAKR 48 retained 75% of its maximal activity at pH 11.0 (Fig. 6).

Cofactor and substrate specificity

To determine the cofactor specificity of each LEAKR, 0.2 mM NADPH or NADH was used as the cofactor in a standard reaction mixture. The ability to catalyze various substrates was negligible in the absence of NADPH. Therefore, NADPH was considered as the only available cofactor for reactions catalyzed by these three LEAKRs.

Several substrates of keto esters and aromatic ketones were chosen to determine the substrate specificity of the LEAKRs. By considering the catalyzing activity of LEAKRs to propiophenone as 100, the activities to other substrates were determined relatively (Table 1). Ethyl 4-chloroacetoacetate was found to be the preferred substrate by LEAKR 48 (39,870% compared to the activity against propiophenone) and LEAKR 50 (36,735% compared to the activity against propiophenone) while 3-chloropropiophenone was found to be the preferred substrate for LEAKR 49 (2,143% compared to the activity against propiophenone). The size of substituent group and the length of carbon chain were found to affect the activity considerably. Among the keto esters tested, ethyl 4-chloroacetoacetate was considered to possess the most suitable structure as the substrate for LEAKR 48 and LEAKR 50. We also observed that all the three LEAKRs showed poor activities against heterocyclic ketones. As for aromatic ketones with phenyl group, various electron-donating and electron-withdrawing substituents were confirmed to have a great influence on the catalyzing activity of the LEAKRs.

Enzyme kinetics

The kinetic parameters of the three LEAKRs were determined for substrates in Table 1 and cofactor NADPH. Curves of activity versus substrate concentration were obtained. The three enzymes showed large differences in kinetic properties. LEAKR 49 showed a distinctly low affinity for ethyl 4-chloroacetoacetate and NADPH, with *Km* values of 18.79 mM and 270 μM, respectively, compared with the other two enzymes. LEAKR 48 and LEAKR 50 showed

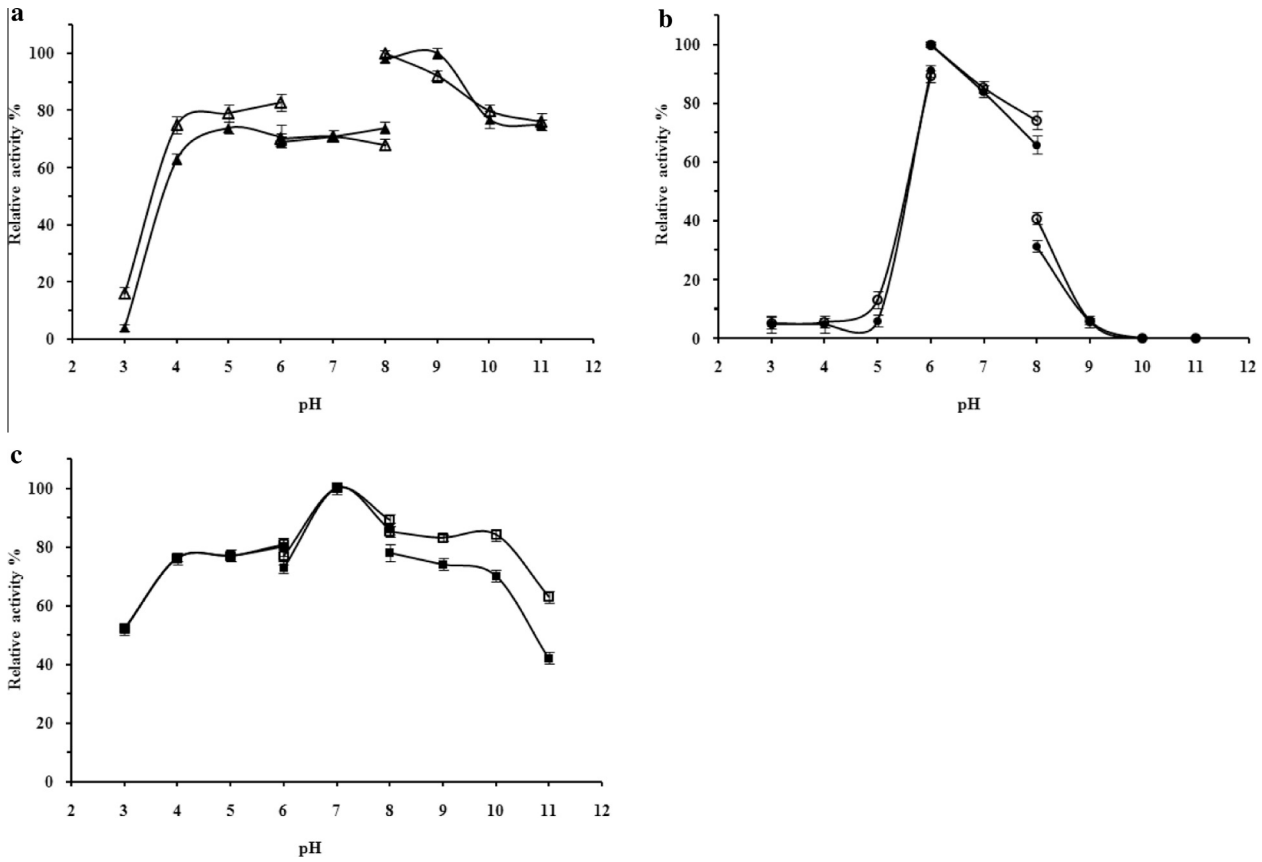

Fig. 6. 1 h (blank) and 3 h (solid) pH stability of each purified *Lodderomyces elongisporus* AKR: LEAKR 48 (a); LEAKR 49 (b); LEAKR 50 (c). The activity was measured in the following 0.1 M buffers: sodium citrate buffer (pH 3.0–6.0), sodium phosphate buffer (pH 6.0–8.0) and glycine–NaOH buffer (pH 8.0–11.0). Each of their activities at optimal pH corresponds to 100% activity. The experiments were done in triplicate and the error bar represents the percentage error in each set of reading.

Table 1
Substrate specificity of the three purified *Lodderomyces elongisporus* AKRs.

Substrate[a]	Relative activity[b] (%)		
	LEAKR 48	LEAKR 49	LEAKR 50
Aromatic ketones			
4′-Methoxyacetophenone	54 ± 0.2	73 ± 0.3	23 ± 0.0
4′-Chloroacetophenone	0 ± 0.0	1498 ± 5.0	595 ± 2.0
2′-Bromoacetophenone	96 ± 0.1	632 ± 2.0	472 ± 4.0
4′-Bromoacetophenone	1103 ± 5.0	724 ± 4.0	722 ± 4.0
4′- Fluoroacetophenone	96 ± 0.2	105 ± 0.2	54 ± 0.1
Propiophenone	100 ± 0.0[c]	100 ± 0.0	100 ± 0.0
3-Chloropropiophenone	200 ± 3.0	2143 ± 0.0	177 ± 0.1
2-Acetylpyridine	1168 ± 3.0	128 ± 0.3	98 ± 0.4
3-Acetylpyridine	393 ± 1.0	89 ± 0.3	106 ± 0.2
4-Acetylpyridine	1382 ± 4.0	244 ± 0.0	102 ± 0.3
2-Acetylthiophene	64 ± 0.6	110 ± 0.3	79 ± 0.1
Keto esters			
Ethyl acetoacetate	1193 ± 3.0	133 ± 0.1	2213 ± 3.0
Ethyl 3-oxohexanoate	150 ± 0.4	87 ± 0.2	56 ± 0.0
Ethyl 4-methyl-3-oxopentanoate	50 ± 0.4	132 ± 0.2	65 ± 0.0
Ethyl benzoylacetate	276 ± 2.0	473 ± 3.0	184 ± 0.3
Ethyl 4-oxopentanoate	87 ± 0.1	95 ± 0.3	39 ± 0.0
Ethyl 4-chloroacetoacetate	39,870 ± 9	210 ± 0.0	36,735 ± 7.0

[a] Concentration of each substrate was 2.0 mM.
[b] The activity of each LEAKR with propiophenone was considered as 100%.
[c] The experiments were done in triplicate and the error bar represents the standard error of the mean.

V_{max}/K_m values of 8.632 and 2.028 U mg^{-1} mM^{-1} against ethyl 4-chloroacetoacetate, much higher than those against other compounds (Table 2). The preference to ethyl 4-chloroacetoacetate over other substrates obtained from kinetic study confirmed the substrate specificities of LEAKR 48 and LEAKR 50 (Table 1).

Effect of organic solvents on enzyme activity

A series of organic solvents were used in this study to test their effect on the catalyzing activity of the three LEAKRs. Hydrophobicity of the organic solvents was indicated using log *P* values (Table 3). Different amounts of organic solvents were used in the assay. All the three LEAKRs showed low sensitivity to: dimethyl sulfoxide and methanol (low log *P* values of −1.3 and −0.76), ethyl acetate (medium log *P* value of 0.68), and *n*-hexane and isooctane (high log *P* values of 3.5 and 4.5). Benzene, which is toxic toward many proteins, strongly impaired the activity of LEAKR 48. Octanol also had a large effect on the activities of LEAKR 48 and LEAKR 49. Their activities sharply declined with the increasing amount of octanol added. LEAKR 50 generally showed a strong resistance to all the organic solvents, except isopropyl alcohol which reduced the activity by 42% with 10% added.

Effect of metal ions, chelators, and detergents on enzyme activity

The effect of various compounds on the activity of LEAKR was assayed by adding each of these to the reaction system. Metal ions highly affected the enzyme activity (Table 4). The effects that were mostly negative differed thoroughly among the three LEAKRs. Chelators reduced the activity of LEAKR 49, but, slightly accelerated the reaction catalyzed by LEAKR 48 and LEAKR 50. Similarly, detergents, except SDS, reduced the activity of LEAKR 49 but increased the activity of LEAKR 48 and LEAKR 50. This indicated that LEAKR

Table 2
Steady-state kinetic constants of three *Lodderomyces elongisporus* AKRs.

Substrate	K_m (mM)[a]			V_{max} (U mg^{-1})			V_{max}/K_m (U mg^{-1} mM^{-1})		
	LEAKR 48	LEAKR 49	LEAKR 50	LEAKR 48	LEARK 49	LEAKR 50	LEAKR 48	LEAKR 49	LEAKR 50
Aromatic ketones									
4′-Methoxyacetophenone	1.26	10.32	8.47	0.01	0.129	0.01	0.008	0.012	0.001
4′-Chloroacetophenone	–[b]	7.64	14	–	1.87	0.41	–	0.245	0.029
2′-Bromoacetophenone	6.6	0.49	1.92	0.051	0.209	0.08	0.008	0.426	0.042
4′-Bromoacetophenone	20.9	1.98	8.89	1.56	0.383	0.345	0.075	0.193	0.039
4′- Fluoroacetophenone	3.4	7.14	45.1	0.032	0.128	0.118	0.009	0.018	0.003
Propiophenone	89.3	4.38	5.28	0.545	0.086	0.033	0.006	0.02	0.006
3-Chloropropiophenone	1.05	1.78	1.12	0.038	1.08	0.025	0.036	0.607	0.022
2-Acetylpyridine	4.54	1.15	39.8	0.473	0.053	0.167	0.104	0.046	0.004
3-Acetylpyridine	15.3	18.9	0.45	0.42	0.25	0.011	0.027	0.013	0.024
4-Acetylpyridine	30.4	5.04	183.2	2.78	0.23	0.63	0.091	0.046	0.003
2-Acetylthiophene	11.68	6.6	1.09	0.054	0.124	0.011	0.005	0.019	0.01
Keto esters									
Ethyl acetoacetate	6.8	6.81	0.49	0.65	0.154	0.24	0.096	0.023	0.49
Ethyl 3-oxohexanoate	0.93	2.14	13.7	0.028	0.047	0.039	0.03	0.022	0.003
Ethyl 4-methyl-3-oxopentanoate	0.97	6.54	5.92	0.009	0.149	0.024	0.009	0.023	0.004
Ethyl benzoylacetate	1.21	158.8	0.26	0.054	10.1	0.018	0.045	0.064	0.069
Ethyl 4-oxopentanoate	1.1	28.66	18.1	0.017	0.381	0.03	0.015	0.013	0.002
Ethyl 4-chloroacetoacetate	0.76	18.79	10.7	6.56	0.58	21.7	8.632	0.031	2.028
Cofactor									
NADPH	0.08	0.27	0.07						

[a] The activities were measured at pH 7.0 and 30 °C in all reactions. The experiments were done in triplicate and the average values were given.
[b] LEAKR 48 showed no activity on 4′-chloroacetophenone.

Table 3
Effect of organic solvents on the activity of each purified *Lodderomyces elongisporus* AKR.

Oragnic solvents	Concentration (v/v, %)	Relative activity (%)		
		LEAKR 48	LEAKR 49	LEAKR 50
None		100 ± 0.0[a]	100 ± 0.0	100 ± 0.0
DMSO[b]	5	95 ± 0.1	98 ± 0.2	90 ± 0.1
	10	93 ± 0.2	85 ± 0.4	95 ± 0.1
Methanol	5	100 ± 0.4	99 ± 0.8	100 ± 0.4
	10	92 ± 0.3	92 ± 0.5	90 ± 0.2
Acetonitrile	5	100 ± 0.6	98 ± 0.5	95 ± 0.2
	10	76 ± 0.2	66 ± 0.0	83 ± 0.2
Isopropanol	5	92 ± 0.0	90 ± 0.3	94 ± 0.2
	10	60 ± 0.1	72 ± 0.6	58 ± 0.1
Ethyl acetate	5	100 ± 0.0	95 ± 0.1	100 ± 0.0
	10	82 ± 0.4	93 ± 0.1	95 ± 0.4
Benzene	5	83 ± 0.3	80 ± 0.7	94 ± 0.2
	10	57 ± 0.6	69 ± 0.2	83 ± 0.0
Toluene	5	96 ± 0.3	85 ± 0.0	96 ± 0.5
	10	92 ± 0.1	81 ± 0.1	83 ± 0.8
1-Octanol	5	92 ± 0.3	47 ± 0.0	85 ± 0.9
	10	55 ± 0.2	34 ± 0.0	79 ± 0.2
n-Hexane	5	100 ± 0	97 ± 0.5	100 ± 0.1
	10	91 ± 0.5	96 ± 0.2	95 ± 0.1
Isooctane	5	100 ± 0.6	100 ± 0.1	92 ± 0.5
	10	94 ± 0.6	99 ± 0.1	90 ± 0.4

[a] The experiments were done in triplicate and the error bar represents the standard error of the mean.
[b] DMSO is short for dimethyl sulfoxide.

49, unlike LEAKR 48 and LEAKR 50, might need metal ions for its catalyzing activity.

Enantioselective reduction of ethyl 4-chloroacetoacetate

The enantioselectivity of each LEAKR was tested using ethyl 4-chloroacetoacetate as substrate. Absolute configurations of product ethyl 4-chloro-3-hydroxybutyrate were measured. All the LEAKRs showed 100% conversion with different e. e. values (70% for LEAKR 48, 58% for LEAKR 49, and 98% for LEAKR 50). Furthermore, all the LEAKRs showed different optical configuration preferences, as indicated in Table 5.

Discussion

To the best of our knowledge, no enzyme from *L. elongisporus* has been reported to have explicit AKR activity. These gaps were filled in this study. Three genes from *L. elongisporus* that encoded AKRs (LEAKRs) were characterized, cloned, and heterologously overexpressed. The three LEAKRs showed <40% amino acid identity with each other but shared highly conserved residues with other members of the AKR superfamily. According to sequence analysis, LEAKR 50 was identified as a member of the AKR3 family while the other two LEAKRs were identified as the members of two novel AKR families based on the latest AKR family list. Considering their novelty, a detailed sequence analysis was performed in this study by using sequence comparison and structure relativity. LEAKRs shared the same crucial part of the active site and similar NADPH binding site. This might explain why they all catalyzed the same ketone substrates and preferred NADPH as cofactor. Furthermore, major gaps appeared in loops of C-terminal which were considered to have a large effect on the substrate specificity of AKRs. This was confirmed from the data acquired in this study.

The members of the AKR superfamily, which are found in diverse sources, catalyze numerous metabolic pathways involving a wide range of substrates. Some of the identified AKRs are excellent industrial catalysts with outstanding characteristics [11,17,12,16]. For example, an alcohol dehydrogenase belonging to the AKR superfamily identified from a hyperthermophilic archaeon *Pyrococcus furiosus* showed increasing catalyzing activity at up to 100 °C [19]. Over 150 AKRs have been identified thus far, however, these only represent a small part of the entire AKR superfamily. In this study, we identified novel members of the AKR family from a new source. It is notable that the three enzymes showed different characteristics in this study. LEAKR 48 and LEAKR 50 showed particularly high activities toward ethyl 4-chloroacetoacetate, which probably resulted from the similarity of ethyl 4-chloroacetoacetate to their natural substrates. Compared to LEAKR 49, the

Table 4
Effect of metal ions, chelators and detergents on the activity of each purified *Lodderomyces elongisporus* AKR.

Compound	Concentration	Relative activity (%)		
		LEAKR 48	LEAKR 49	LEAKR 50
None		100 ± 0[a]	100 ± 0	100 ± 0
Metal ions				
Ca^{2+}	1.0(mM)	87 ± 0.2	62 ± 0.5	86 ± 0.4
Mg^{2+}	1.0 (mM)	82 ± 0.7	69 ± 0.1	67 ± 0.5
Zn^{2+}	1.0 (mM)	10 ± 0.1	45 ± 0.1	5 ± 0.0
Cu^{2+}	1.0 (mM)	84 ± 0.1	10 ± 0.0	73 ± 0.0
Fe^{2+}	1.0 (mM)	58 ± 0.3	24 ± 0.2	54 ± 0.1
Mn^{2+}	1.0 (mM)	41 ± 0.0	119 ± 0.4	13 ± 0.1
Ni^{2+}	1.0 (mM)	94 ± 0.3	98 ± 0.5	34 ± 0.2
Co^{2+}	1.0 (mM)	80 ± 0.6	81 ± 0.0	43 ± 0.1
Li^{1+}	1.0 (mM)	97 ± 0.1	95 ± 0.1	96 ± 0.1
Chelators and detergents				
EDTA·2Na	0.005% (wt/v)	113 ± 2.0	69 ± 0.5	105 ± 2.0
SDS	0.005% (wt/v)	0 ± 0.0	0 ± 0.0	18 ± 0.2
Triton X-100	0.005% (wt/v)	136 ± 0.2	91 ± 0.1	132 ± 0.0
Tween-20	0.005% (wt/v)	182 ± 1.0	93 ± 0.3	165 ± 0.5
Tween-60	0.005% (wt/v)	166 ± 0.3	82 ± 0.6	156 ± 0.8
Tween-80	0.005% (wt/v)	154 ± 0.7	89 ± 0.2	160 ± 1.0

[a] The experiments were done in triplicate and the error bar represents the standard error of the mean.

Table 5
Optical preference of each purified *Lodderomyces elongisporus* AKR.

	LEAKR 48	LEAKR 49	LEAKR 50
Optical configuration[a]	R	S	R
e. e. (%)	70 ± 0.5[b]	58 ± 0.5	98 ± 0

[a] Ethyl 4-chloroacetoacetate was used as substrate and the absolute configurations of the ethyl 4-chloro-3-hydroxybutyrate were measured.
[b] The experiments were done in triplicate and the error bar represents the standard error of the mean.

other two LEAKRs showed superior characteristics of resistance to high temperatures and extreme pH conditions. Use of ethyl 4-chloroacetoacetate as the substrate showed great disparity in the optical purities of products. It is also worth mentioning that LEAKR 50 was found to be outstanding among the three LEAKRs, with high activity of >86% at a wide range of reaction temperatures (30 to 50 °C), strong resistance to adverse pH environments (pH 3.0–10.0), and high (*R*)-enantioselectivity (e. e., 98%), thus making it a potential catalyst for industry.

The diverse characteristics of the three LEAKRs provided us a pathway to partially identify the roles of some key amino acids that are important for protein modifications. In the past, processes have been designed and improved around the limitations of an enzyme. Nowadays, an enzyme is engineered to fit process specifications [2]. Directed evolution of proteins has arisen as an efficient way to modify proteins and obtain enzymes with superior stability, substrate specificity, and enantioselectivity. Numerous successful attempts have drawn more attention of researchers. For instance, site-saturation mutagenesis methods were applied in a semi-rational approach, to alter an amylosucrase (α-transglucosidase) from *Neisseria polysaccharea*. The best mutant showed 400-fold enhanced catalytic efficiency toward the donor substrate sucrose and a non-natural acceptor substrate allyl 2-acetamido-2-deoxy-alpha-D-glucopyranoside [6]. A structure study of the three LEAKRs identified in this study is underway for modification purposes. Identification of diverse characteristics and comparison of sequences are expected to identify some valuable residues, thus contributing to structure-guided rational engineering of AKRs.

It is possible that more than one enzyme that can catalyze the reaction of one particular substrate may exist in a single cell. Characterization of the three AKRs from the same microorganism has also created confusion about the overlap in activity between several enzymes within a single cell. Microbial genome data have predicted that a common prokaryote such as *E. coli* may possess six AKRs [1]. Although it is believed that each enzyme has evolved for a particular metabolic purpose, its ability to act on a range of substrates stays apparent. For example, LEAKR 48 and LEAKR 50 were found to prefer the same substrate but catalyzed its reaction at different optimum reaction conditions. One prediction was that the metabolic functions performed by these enzymes were similar in reaction type but differed with the changing conditions. Determining and observing different AKRs from the same microbial source may also reveal their physiological roles.

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References

[1] F.R. Blattner, G. Plunkett III, C.A. Bloch, N.T. Perna, V. Burland, M. Riley, J.C. Vides, J.D. Glasner, C.K. Rode, G.F. Mayhew, J. Gregor, N.W. Davis, H.A. Kirkpatrick, M.A. Goeden, D.J. Rose, B. Mau, Y. Shao, Science 277 (1997) 1453–1462.
[2] U.T. Bornscheuer, G.W. Huisman, R.J. Kazlauskas, S. Lutz, J.C. Moore, K. Robins, Nature 485 (2012) 185–194.
[3] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[4] M. Breuer, K. Ditrich, T. Habicher, B. Hauer, M. Keßeler, R. Stürmer, T. Zelinski, Angew. Chem. Int. Ed. 43 (2004) 788–824.
[5] N.C. Bruce, D.L. Willey, A.F. Coulson, J. Jeffery, Biochem. J. 299 (1994) 805–811.
[6] E. Champion, F. Guérin, C. Moulis, S. Barbe, T.H. Tran, S. Morel, K. Descroix, P. Monsan, L. Mourey, L.A. Mulard, S. Tranier, M.R. Siméon, I. André, J. Am. Chem. Soc. 134 (2012) 18677–18688.
[7] E.M. Ellis, FEMS Microbiol. Lett. 216 (2002) 123–131.
[8] W.D. Fessner, N.J. Turner, M.X. Wang, Adv. Synth. Catal. 353 (2011) 2189–2190.
[9] K. Goldberg, K. Schroer, S. Lütz, A. Liese, Appl. Microbiol. Biotechnol. 76 (2007) 237–248.
[10] E. Hur, D.K. Wilson, Chem-Biol. Interact. 130 (2001) 527–536.
[11] K. Ishihara, H. Yamaguchi, H. Hamada, K. Nakamura, N. Nakajima, J. Mol. Catal. B-Enzym. 10 (2000) 419–428.
[12] N. Itoh, H. Asako, K. Banno, Y. Makino, M. Shinohara, T. Dairi, R. Wakita, M. Shimizu, Appl. Microbiol. Biotechnol. 66 (2004) 53–62.
[13] J.M. Jez, M.J. Bennett, B.P. Schlegel, M. Lewis, T.M. Penning, Biochem. J. 326 (1997) 625–636.
[14] J.M. Jez, T.G. Flynn, T.M. Penning, Biochem. Pharmacol. 54 (1997) 639–647.
[15] J.M. Jez, T.M. Penning, Chem-Biol. Interact. 130 (2001) 499–525.

[16] K.J. Jing, Z.N. Xu, Y. Liu, X.X. Jiang, L. Peng, P.L. Cen, Prep. Biochem. Biotechnol. 35 (2005) 203–215.
[17] M. Kataoka, K. Yamamoto, H. Kawabata, M. Wada, K. Kita, H. Yanase, S. Shimizu, Appl. Microbiol. Biotechnol. 51 (1999) 486–490.
[18] S. Khurana, D.B. Powers, S. Anderson, M. Blaber, Proc. Natl. Acad. Sci. USA 95 (1998) 6768–6773.
[19] R. Machielsen, A.R. Uria, S.W.M. Kengen, J.V.D. Oost, Appl. Environ. Microbiol. 72 (2006) 233–238.
[20] T.M. Penning, J.E. Pawlowski, B.P. Schlegel, J.M. Jez, H.K. Lin, S.S. Hoog, M.J. Bennett, M. Lewis, Steroids 61 (1996) 508–523.
[21] J.M. Petrash, I. Tarle, D.K. Wilson, F.A. Quiocho, Diabetes 43 (1994) 955–959.
[22] G. Sanli, M. Blaber, J. Mol. Biol. 309 (2001) 1209–1218.
[23] S. Shimizu, M. Kataoka, M. Katoh, T. Morikawa, T. Miyoshi, H. Yamada, Appl. Environ. Microbiol. 56 (1990) 2374–2377.