Enzyme catalytic promiscuity: asymmetric aldol addition reaction catalyzed by a novel thermophilic esterase in organic solvent

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The asymmetric aldol addition of 2-butane and 4-nitrobenzaldehyde catalyzed by a novel thermophilic esterase (APE1547) from the archaeon \textit{Aeropyrum pernix} K1 was successfully conducted in organic solvents. APE1547 exhibited a good enzyme activity and enantioselectivity in the reaction. The effects of organic solvent, temperature, water content, and substrate concentration were investigated. The reaction provided optically active secondary alcohol with satisfying enantioselectivity (71.2 %ee) and enzyme activity (38.1 µmol/g/h) under the optimum conditions. A high yield (68.7%) could be obtained when the reaction time was approximately 120 h.

**Keywords:** catalytic promiscuity; asymmetric aldol addition; 2-butane; 4-nitrobenzaldehyde; enantioselectivity

**Introduction**

Enzyme catalytic promiscuity is the ability of the enzyme’s active site to catalyze chemically distinct chemical transformations (1–2). Exploiting enzyme catalytic promiscuity might lead to improvements in existing catalysts and provide novel synthesis pathways that are currently not available. The recent researches with enzyme catalytic promiscuity have shown that hydrolases, especially lipases or esterases, play a key role due to their broad specificity and relatively better stability (as compared to other enzymes) in media containing organic solvents (3–4). For example, the aldol reaction, Morita–Baylis–Hillman reaction, Mannich reaction, Henry reaction, Perhydrolysis, Markovnikov addition, Michael addition, addition of diethylzinc to aldehydes are typical and efficient promiscuous reactions for hydrolases (5–12). Though enzymes that are capable of performing the higher activity and enantioselectivity still remain to be discovered by creation with molecular biology techniques or bioengineering techniques, these findings open the door for many future applications in synthetic organic chemistry.

The asymmetric aldol addition reaction has attracted considerable interest over the years as the aldol reaction is one of the most fundamental tools for the construction of new carbon–carbon bonds (13). It’s very useful for the synthesis of pharmaceuticals, fine chemicals, and natural products (14). Aldol additions catalyzed by aldolases have been well established for synthetic reactions. However, the high cost of aldolases will undoubtedly limit its future application (15–16). Recently, it has been found that hydrolases can also catalyze the aldol addition. Lipase B from \textit{Candida antarctica} lipase B (CAL-B) could catalyze the aldol addition with substrates hexanal or propanal (17). However, the first hydrolase catalyzed asymmetric aldol addition reaction was reported by Li et al. (18) until 2008. They presented the asymmetric aldol addition between acetone and different aromatic aldehydes using porcine pancreatic lipase (PPL) with the ee% up to 44%, but the enzyme activity was very low in this reaction. He et al. reported that proteinase displayed a promiscuous activity to catalyze the direct asymmetric aldol reactions of aromatic and hetero-aromatic aldehydes with cyclic and acyclic ketones in acetonitrile in the presence of a phosphate buffer. The excellent enantioselectivities and high diastereoselectivities were achieved (19–20). However, there still remains relatively little research so far on the applications of hydrolyses in enzyme catalytic asymmetric aldol reactions. Recently, thermophilic enzymes have been recognized as having the potential for organic synthesis due to their high stability against organic solvents, high temperature, and chemical denaturants (21). Our group is focusing on the enzyme catalytic promiscuity of APE1547 which is a recombinant hyperthermophilic esterase from archaeon \textit{Aeropyrum pernix} K1 (22). It has been found that APE1547 showed moderate enzyme catalytic activities and enantioselectivity in Michael addition and asymmetric aldol reactions.
addition of diethylzinc to aldehydes compared with other commercial hydrolases (12, 23). These results encouraged us to assess the potential of APE1547 in the asymmetric aldol addition.

In our previous study, we have found APE1547 could catalyze the aldol addition of acetone and 4-nitrobenzaldehyde with low enzyme activity (data not shown) at room temperature. The low activity should be attributed to the special hyperthermophilic property of APE1547. Generally, the optimum reaction temperature of APE1547 is from 50 to 90°C (24). It is not very suitable for acetone to be selected as the substrate of the aldol addition catalyzed by APE1547. Therefore, we select 2-butanone and 4-nitrobenzaldehyde as the substrates of aldol addition in this study (Scheme 1). The reaction has been optimized to better understand the relationships between the factors (solvent, temperature, water content, and substrate concentration) and the performance of enzyme.

Results and discussion

Enzyme catalytic performance mainly depends on the type and origin of the enzyme (25). In this study, five kinds of hydrolases were selected to carry out the aldol addition of 2-butanone and 4-nitrobenzaldehyde. It could be found (Table 1) that these selected hydrolase exhibited various enzyme performances. Among the tested hydrolases, CAL-B (Candida antarctica lipase), BSL2 (Bacillus subtilis lipase), and PSL (Pseudomonas sp. lipase) showed very low catalytic performances. In the case of PPL and APE1547, good activities and enantioselectivities were obtained. APE1547 showed the highest enantioselectivity and moderate activity in this addition at room temperature. These results suggest that APE1547 has a great potential as a catalyst for the asymmetric aldol addition.

In order to optimize the reaction conditions, the effect of several factors, including solvent, temperature, water content and substrate concentration on the reaction, were studied.

It is well known that a proper selection of organic solvent is essential in biocatalytic reactions because the activity of enzymes may be affected by the polarity of organic media (26). The polarity of the organic solvents can be quantitatively measured by the value of log $P$, the logarithm of the partition coefficient of a given solvent between water and 1-octanol. In the present study, the effects of the solvents with different log $P$ in the asymmetric aldol addition were displayed in Table 2. As shown in Table 2, it could be found that APE1547 shows the highest activity and enantioselectivity in $n$-heptane and exhibits moderate activities and enantioselectivities in polar solvents (dimethyl sulfoxide and acetonitrile). These results were inconsistent with previous reports (18–20). It should be attributed to the unique enzyme structure of thermophilic esterase APE1547. APE1547 has a $\beta$-propeller domain which is connected to the catalytic domain via two polypeptides. The two domains are stabilized by hydrogen bonds and salt bridges, with additional stability provided by hydrophobic forces (27). Therefore, APE1547 are preferred to exhibit higher catalytic performance in $n$-heptane. From the solvents studied, $n$-heptane was selected as the appropriate solvent in this reaction.

The effect of temperature on the activity and enantioselectivity of APE1547 in the addition of 4-nitrobenzaldehyde and 2-butanone has been optimized (Table 2).

Table 1. Effect of enzyme sources on the asymmetric aldol addition of 4-nitrobenzaldehyde and 2-butanone.

| Enzyme | Enzyme activity ($\mu$mol/g/h) | %ee | Stereoselectivity |
|--------|--------------------------------|-----|-------------------|
| CAL-B  | 1.7                            | 10.5| S                 |
| BSL    | 8.5                            | 5.8 | S                 |
| PSL    | 2.6                            | 16.8| S                 |
| PPL$^a$| 20.7                           | 46.9| S                 |
| APE1547$^a$ | 11.1                  | 53.3| S                 |
| No enzyme | Trace                     | –   | –                 |

$^a$The reactions were carried out in $n$-heptane (2 ml) with 4-nitrobenzaldehyde (0.05 mmol/mL), 2-butanone (0.2 mmol/mL), water content (8.4% v/v) and enzyme (20 mg) at 30°C.

Table 2. Effect of solvent on the asymmetric aldol addition of 4-nitrobenzaldehyde and 2-butanone catalyzed by APE1547.$^a$

| Solvent         | Log $P$ | Enzyme activity ($\mu$mol/g/h) | %ee |
|-----------------|---------|---------------------------------|-----|
| Dimethyl sulfoxide | −1.4   | 7.4                             | 42.4|
| Acetonitrile    | −0.33   | 6.1                             | 35.8|
| Tetrahydrofuran | 0.49    | 2.3                             | 25.8|
| Diisopropyl ether | 1.9    | 5.3                             | 37.6|
| Toluene         | 2.5     | 5.7                             | 41.0|
| n-Hexane        | 3.5     | 10.8                            | 46.3|
| n-Heptane       | 4.66    | 11.1                            | 53.3|

$^a$The reactions were carried out in organic solvent (2 ml) with 4-nitrobenzaldehyde (0.05 mmol/mL), 2-butanone (0.2 mmol/mL), water content (8.4% v/v), and APE1547 (20 mg) at 30°C.

Scheme 1. Asymmetric aldol reaction of 2-butanone and 4-nitrobenzaldehyde catalyzed by APE1547.
investigated over the temperature range of 30–80°C. As shown in Figure 1, the activity increased as the reaction temperature increased from 30°C to 65°C. The maximal enzyme activity of 38.1 µmol/g/h was obtained at 65°C and decreased rapidly by further increasing the temperature (65–80°C). The collision chance between enzyme and substrate molecules increased on the higher temperature, which might help to form enzyme–substrate complexes and then improve the reaction rate (28). Further increasing of temperature might destruct the enzyme conformation and decrease the enzyme activity (29). Furthermore, the side reaction (condensation of target product) would occur at higher temperature (>65°C), which decreased the yield of the target product. The enantioselectivity of APE1547 only presented gradually increase with increasing the temperature (30–70°C). A further increase in the temperature resulted in an obvious decrease in enzyme enantioselectivity. Since enzyme activity was found to be highest (38.1 µmol/g/h) at 65°C while maintaining a higher enantioselectivity (71.2 %ee), 65°C was selected as the optimal temperature for this reaction.

Water plays an important role for the enzyme to maintain its proper conformation so as to keep its catalytic activity in nonaqueous media (30). In the present study, the reaction catalyzed by APE1547 was conducted in a wide range of water content (2.1–12.3% v/v), and the results are shown in Figure 2. The maximum enzyme activity (38.1 µmol/g/h) was obtained at high water content (8.4% v/v). The conformation of APE1547 was excessively rigid at lower water content, which could disturb the “induced-fit” process of APE1547 and decrease the enzyme activity. At higher water content, the excessively flexible conformation of APE1547 may also decrease the enzyme activity (31). Concerning the effect of water content on enantioselectivity, the results in this study indicate that the enantioselectivity changed with the variation of water contents, and the maximum enantioselectivity (71.2%ee) was found at water content of 8.4% v/v. The possible explanation may be that the microenvironment of the active site in APE1547 was affected by the water, and then the enzyme conformation made a tremendous change which altered the enantioselectivity of APE1547.

The effect of substrate concentration on the aldol addition was investigated at a fixed enzyme amount and substrate ratio (2-butanone: 4-nitrobenzaldehyde = 4:1). It could be found from Figure 3 that if the concentration of 4-nitrobenzaldehyde was increased, the enzyme activity steeply increased until it reached a maximum at the point of 0.05 mmol/mL. The enzyme activity would be changed slightly with further increasing of the concentration. The beneficial effect of high substrate concentration on the enzyme activity may be due to an increase in the interaction between substrate and enzyme (32). The enzyme molecules could be saturated when the substrate concentration was 0.05 mmol/mL. When the saturation point was reached, adding extra substrate would make no difference on the enzyme activity. On the other hand, the enantioselectivity of APE1547 was not affected by increasing the concentration of 4-nitrobenzaldehyde.

The time course of the aldol addition catalyzed by APE1547 is shown in Figure 4. As can be observed,
the yield of 4-nitrobenzaldehyde increased gradually with the reaction time extending. However, the yield of 4-nitrobenzaldehyde was increased very slowly after 120 h, and we found the enzyme was deactivated partly in the organic solvent. Furthermore, the rate of reaction could be slow down with the reducing of the concentration of substrates. In contrast, the enantiomeric excess of the obtained alcohol remained almost the same (71.2–70.1%ee). A yield of 68.7% was obtained in this aldol addition under the optimum reaction when the reaction time was approximately 120 h.

**Experimental**

**Materials**

Porcine pancreas lipase (lipase Type II) was purchased from Sigma (Beijing, China). PSL was purchased from Amano Pharmaceutical Co. Ltd. (Japan). CAL-B was kindly donated by Novo Nordisk Industries (China). BSL2 was overexpressed from *B. subtilis* strain IFFI10210 in our laboratory according to the method we have reported (33). *Aeropyrum pernix* esterase (APE1547) was produced from a hyperthermophilic archaeon strain, then purified and lyophilized (22). These enzymes were used after lyophilization for enzymatic reaction without further purification. 4-Nitrobenzaldehyde was purchased from Sigma. Other reagents were purchased from Shanghai Chemical Reagent Company (Shanghai, China). Commercially available reagents and solvents were used without further purification. Nuclear magnetic resonance (NMR) spectra were recorded on an Inova 500 (1H, 500 MHz) spectrometer.

**Water content control and measurement**

All the reaction mixture components were previously dried in a vacuum of 1 mm Hg for 10 h. Following that, all reaction mixtures with different water content were prepared by adding a specific amount of water. The water content was measured with a coulometric Karl Fisher apparatus (Denver Instrument, Model 260).

**Enzymatic aldol reaction of 2-butanone and 4-nitrobenzaldehyde**

4-Nitrobenzaldehyde (0.05 mmol/mL) and 2-butanone (0.2 mmol/mL) were dissolved in *n*-heptane (2 ml). APE1547 (20 mg) was added into the mixture, and then the reaction was performed at 65°C and water content (8.4% v/v). The enzyme activity (µmol/g/h) was defined as the amount (in micromoles) of 4-nitrobenzaldehyde converted per hour per gram of enzyme. The yield of 4-nitrobenzaldehyde and the enantiomeric excess of the obtained alcohol was determined by HPLC in 24 h (34). Chiral HPLC analyses were performed with a Shimadzu LC-10AD apparatus equipped with a SPD-M10A UV detector (λ = 254 nm) and Daicel column (AS-H) with hexane/isopropyl alcohol (70:30). The retention times of the two enantiomers were 9.65 min and 7.52 min, respectively. 1H NMR (CDCl3): δ = 8.26 (d, J = 8.5 Hz, 2H), 7.59 (d, J = 8.5 Hz, 2H), 5.28 (s, 1H), 3.71 (s, 1H), 2.85 (dd, J = 6.0, 2.5 Hz, 1H), 2.83 (dd, J = 6.0, 2.5 Hz, 1H), 2.48 (q, J = 7.0 Hz, 2H), 1.12 (t, J = 7.0 Hz, 3H) ppm. The experiments were performed triplicate, and all data were obtained based on the average values.
Conclusion
In conclusion, APE1547 was successfully used in the asymmetric aldol addition of 2-butane and 4-nitrobenzaldehyde. Under the optimum conditions, the highest enzyme activity (38.1 µmol/g/h) was obtained with a high enantioselectivity (71.2%ee). Furthermore, the time course of the reaction showed that a high yield (68.7%) could be obtained. The results illustrate that APE1547 exhibits the greatest application in asymmetric aldol addition. Studies of synthesizing complicated structures, especially chiral drug derivatives, are currently underway.

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References
(1) Nobeli, I.; Favia, A.D.; Thornton, J.M. Nat. Biotechnol. 2009, 27, 157–167.
(2) Kazlauskas, R.J. Curr. Opin. Chem. Biol. 2005, 9, 195–201.
(3) Hult, K.; Berglund, P. Trends. Biotechnol. 2007, 25, 231–238.
(4) Manali, K.; Gupta, M.N. Lipase Promiscuity and Its Biochemical Applications. Process Biochem. 2012, 47, 555–569.
(5) Majumder, A.B.; Ramesh, N.G.; Gupta, M.N. Tetrahedron. Lett. 2009, 50, 5199–5203.
(6) Reetz, M.T.; Mondiere, R.; Carballera, J.D. Tetrahedron. Lett. 2007, 48, 1679–1681.
(7) He, T.; Li, K.; Wu, M.Y.; Feng, X.W.; Wang, N.; Wang, H.Y.; Li, C.; Yu, X.Q. J. Mol. Catal. B: Enzym. 2010, 67, 189–194.
(8) Tang, R.C.; Guan, Z.; He, Y.H.; Zhu, W. J. Mol. Catal. B: Enzym. 2010, 63, 62–67.
(9) Hernandez, K.; Berenguer-Murcia, A.; Rodrigues, R.C.; Fernandez-Lafuente, R. Curr. Org. Chem. 2012, 16, 2652–2672.
(10) Lou, F.W.; Liu, B.K.; Wu, Q.; Lv, D.S.; Lin, X.F. Adv. Synth. Catal. 2008, 350, 1959–1962.
(11) Svedendahl, M.; Hult, K.; Berglund, P. J. Am. Chem. Soc. 2005, 127, 17988–17990.
(12) Wei, X.F.; Zheng, Q.C.; Ji, T.F.; Zhao, B.; Li, C.Y.; Wang, P.; Cao, S.G.; Wang, Z.; Wang, L. Chin. J. Catal. 2009, 30, 396–400.
(13) Palomo, C.; Oiarbide, M.; Garcia, J.M. Chem. Soc. Rev. 2004, 33, 65–75.
(14) Hayashi, Y.; Sumiya, T.; Takahashi, J.; Gotoh, H.; Urushima, T.; Shoji, M. Angew. Chem. Int. Edit. 2006, 45, 958–961.
(15) Durany, O.; Mas, C.; Lopez-Santin, J. Process. Biochem. 2005, 40, 707–716.
(16) Garcia-Junceda, E.; Iturrate, L.; Sanchez-Moreno, I. New. Biotechnol. 2009, 25, S84–S85.
(17) Branneby, C.; Carlqvist, P.; Magnusson, A.; Hult, K.; Brinck, T.; Berglund, P. J. Am. Chem. Soc. 2003, 125, 874–875.
(18) Li, C.; Feng, X.W.; Wang, N.; Zhou, Y.J.; Yu, X.Q. Green Chem. 2008, 10, 616–618.
(19) He, Y.H.; Li, H.H.; Chen, Y.L.; Xue, Y.; Yuan, Y.; Guan, Z. Adv. Synth. Catal. 2012, 354, 712–719.
(20) Yuan, Y.; Guan, Z.; He, Y.H. Sci. China. Chem. 2013, 56, 939–944.
(21) Bouzas, T.D.; Barros-Velazquez, J.; Villa, T.G. Protein. Peptide. Lett. 2006, 13, 645–651.
(22) Tian, R.; Yang, C.H.; Wei, X.F.; Xun, E.N.; Wang, R.; Cao, S.G.; Wang, Z.; Lang. L. Biotechnol. Bioproc. E. 2011, 16, 337–342.
(23) Wang, L.; Du, C.; Ji, T.F.; Wang, Z.; Cao, S.G. J. Mol. Catal. (China) 2008, 22, 172–176.
(24) Gao, R.J.; Feng, Y.; Ishikawa, K.; Ishida, H.; Ando, S.; Kosugi, Y.; Cao, S.G. J. Mol. Catal. B: Enzym. 2003, 24–25, 1–8.
(25) Filice, M.; Fernandez-Lafuente, R.; Terreni, M.; Guisan, J.M., Palomo, J.M. J. Mol. Catal. B: Enzym. 2007, 49, 12–17.
(26) Wu, A.C.; Wang, P.Y.; Lin, Y.S.; Kao, M.F.; Chen, J.R.; Ciou, J.F.; Tsai, S.W. J. Mol. Catal. B: Enzym. 2010, 62, 235–241.
(27) Bartlam, M.; Wang, G.G.; Yang, H.T.; Gao, R.J.; Zhao, X.D.; Xie, G.Q.; Cao, S.G.; Feng, Y.; Rao, Z.H. Stucture. 2004, 12, 1481–1488.
(28) Chen, C.C.; Tsai, S.W. Enzyme. Microb. Tech. 2005, 36, 127–132.
(29) Graebin, N.G.; Martins, A.B.; Lorenzoni, A.S.; Garcia-Galan, C.; Fernandez-Lafuente, R.; Ayub, M.A.; Rodrigues, R.C. Biotechnol. Prog. 2012, 28, 406–412.
(30) Leonard-Nevers, V.; Marton, Z.; Lamare, S.; Hult, K.; Graber, M. J. Mol. Catal. B: Enzym. 2009, 59, 90–95.
(31) Halling, P.J. In Handbook of Enzyme Catalysis in Organic Synthesis; Drazu, K., Waldmann, H., Eds.; Wiley-VCH: Germany, 2002; pp. 259–286.
(32) Ulker, S.; Karaoglu, S.A. J. Biosc. Bioeng. 2012, 114, 385–390.
(33) Wang, L.; Tai, J.D.; Wang, R.; Xun, E.N.; Wei, X.F.; Wang, Z. Biotechnol. Appl. Biochem. 2010, 56, 1–6.
(34) Chen, F.F.; Huang, S.; Zhang, H.; Liu, F.Y.; Peng, Y.G. Tetrahedron. 2008, 64, 9585–9591.