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Promiscuous protease-catalyzed aldol reactions: A facile biocatalytic protocol for carbon–carbon bond formation in aqueous media

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ABSTRACT

Several proteases, especially pepsin, were observed to directly catalyze asymmetric aldol reactions. Pepsin, which displays well-documented proteolytic activity under acidic conditions, exhibited distinct catalytic activity in a crossed aldol reaction between acetone and 4-nitrobenzaldehyde with high yield and moderate enantioselectivity. Fluorescence experiments indicated that under neutral pH conditions, pepsin maintains its native conformation and that the natural structure plays an important role in biocatalytic promiscuity. Moreover, no significant loss of enantioselectivity was found even after four cycles of catalyst recycling, showing the high stability of pepsin under the selected aqueous reaction conditions. This case of biocatalytic promiscuity not only expands the application of proteases to new chemical transformations, but also could be developed into a potentially valuable method for green organic synthesis.

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1. Introduction

Biocatalysis has developed into a powerful tool for organic synthesis due to its high efficiency, good selectivity and environmental acceptability (García-Urdiales et al., 2005; Reetz and Hauer, 2007; Sukumaran and Hanefeld, 2005). Although it is well known that a given enzyme is able to catalyze a specific reaction efficiently, some unexpected experimental results have indicated that many enzymes are catalytically promiscuous; i.e., they have the ability to catalyze distinctly different reactions (Copley, 2003; Khersonsky et al., 2006; Hult and Berglund, 2007). Many instances of this phenomenon have recently been reported (Babtie et al., 2009; Feng et al., 2009; Hasnaoui-Dijoux et al., 2008; Lou et al., 2008; Olguin et al., 2008; Sharma et al., 2009; Svedendahl et al., 2008; Taglieber et al., 2007; Xu et al., 2007).

Among those promiscuous enzymes capable of catalyzing carbon–carbon bond formation, hydrolases are considered to be some of the most useful due to their good stability, broad range of substrate compatibility and high efficiency of forming various chemical bonds (Bornscheuer and Kazlauskas, 2004). Although instances of several important addition reactions (e.g., Michael additions, Markovnikov additions, and direct Mannich reactions) have been frequently reported (Svedendahl et al., 2005; Torre et al., 2004; Wu et al., 2005; Wu et al., 2006; Li et al., 2009), the aldol reaction—widely considered to be one of the most basic and powerful tools for organic synthesis—has been seldom mentioned in published studies of promiscuous biocatalysts (Branneby et al., 2003; Li et al., 2008).

The asymmetric aldol reaction is one of the most important C–C bond-forming reactions in organic synthesis (Mlynarski and Paradowska, 2008). The development of asymmetric aldol reaction catalysts remains an active area of research, and numerous successful organocatalysts and aldolases have been described in recent years (Garrabou et al., 2009; Mase et al., 2006). Although traditional catalysts exhibit high efficiency and enantioselectivity, the development of environmentally friendly and cost-efficient methods for C–C bond formation still presents a significant challenge (Mestres, 2004). Berglund et al. reported an aldol reaction catalyzed by a promiscuous biocatalyst in which a mutant CAL-B lipase had better activity than the wild-type lipase (Scheme 1) (Branneby et al., 2003). Recently, we found that several lipases have the ability to catalyze asymmetric aldol reactions under "wet" reaction conditions (Li et al., 2008). However, the enantioselectivities of PPL-catalyzed aldol reactions in aqueous media are quite low (less than 20% in most cases) and cannot readily compete with those obtained by conventional methods.

In an extension of this work, a wide variety of hydrolases, including proteases, lipases and acylases, were assessed as promiscuous biocatalysts of an aldol reaction—specifically, the aldol reaction between acetone and 4-nitrobenzaldehyde. Within these enzymes, we discovered that pepsin, a digestive enzyme, can catalyze the asymmetric aldol reaction with superior enantioselectivity (about 50% in most cases). Fluorescence analysis reveals that pepsin largely maintains its native conformation under neutral pH reaction condi-

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Scheme 1. Mutant lipase-catalyzed aldol reaction.

tions, indicating that the natural conformation of pepsin is essential to its catalysis of the aldol reaction. Moreover, a variety of substrates were found to undergo pepsin-catalyzed aldol reaction, showing a wide substrate tolerance for this reaction pathway. Of those hydrolases screened for promiscuous biocatalysis, pepsin also proved to be the most enantioselective. The promotion of C–C bond formation by pepsin could be regarded as not only a potential method for green organic synthesis, but also as a guide to the directed evolution of enzymes with more efficient or selective catalytic activity (Reetz et al., 2009).

2. Experimental

2.1. Materials and methods

Pepsin, papain, protease from *Bacillus subtilis*, protease from *Bacillus polymyxa*, protease Type X VIII Fungal from *Rhizopus*, protease from *Aspergillus oryzae*, lipase acrylic resin from *Candida antarctica*, lipase-AY30, lipase from porcine pancreas, lipase from *Candida cylindracea*, lipase from *Mucor javanicus* and lipase from *Rhizopus oryzae* were purchased from Sigma. Lipases from *Mucor miehei* and *Penicillium camemberti* were purchased from Fluka. D-Aminoacylase, protease-S, lipase-PS and lipase-AK were obtained as gifts from Amano Enzyme China Ltd. (Shanghai, China). Trypsin and lipase from hog pancreas were purchased from Shanghai Kayon Biological Technology Co., Ltd. (China). Bovine serum albumin (BSA) was purchased from Shanghai Bio Life Co., Ltd. (China). The aldehydes were freshly distilled prior to use. Other chemical reagents and enzymes were used without further purification.

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker DMX 400 spectrometer. Chemical shifts are given in δ (ppm) relative to tetramethylsilane (TMS). HPLC was carried out on a Shimadzu LC-2010A HT system using a Daicel Chiralpak As-H column, a mobile phase of n-hexane/2-propanol (80:20, v/v) at a flow rate of 1.0 mL/min and UV detection at 254 nm. Electrospray ionization (ESI) mass spectrometry experiments were performed on a Bruker Daltonics Bio TOF mass spectrometer. Fluorescence spectroscopic experiments were carried out on a Horiba Jobin Yvon Fluoromax-4 spectrofluorometer with an excitation wavelength of 292 nm.

2.2. Hydrolase-catalyzed aldol reaction

In the cases of pepsin and PPL, 10 mL of acetone and 2.5 mL of deionized water (20% water content) were mixed in the presence of hydrolase (200 mg) and 4-nitrobenzaldehyde (181.2 mg, 1.2 mmol). The suspension was maintained at 30 °C and shaken at 200 rpm for 24 h. Product formation was monitored by TLC. The residue was then filtered off, and the solvent was evaporated. A single product was isolated by silica gel chromatography using an eluent consisting of hexane/ethyl acetate (2:1, v/v).

For other hydrolases, the reaction was initiated by adding hydrolase (20 mg) and 4-nitrobenzaldehyde (18.1 mg, 0.12 mmol) to a mixture of 1 mL of acetone and 0.25 mL of deionized water (20% water content). The suspension was maintained at 30 °C and shaken at 200 rpm for 24 h. Product formation was monitored by TLC. Aliquots were then taken and analyzed by HPLC. The retention time of the enantiomers are 16.7 min (major) and 22.8 min (minor).

2.3. Influence of the reaction media

Pepsin (20 mg) and 4-nitrobenzaldehyde (18.1 mg, 0.12 mmol) were added to a mixture of 1 mL of acetone and 0.11 mL of a second solvent (hexane, cyclohexane, *t*-butyl alcohol, ethanol, THF, DMSO, pyridine, toluene, dioxane, water, ethyl acetate, dichloromethane or chloroform). The suspension was maintained at 30 °C and shaken at 200 rpm for 48 h. Product formation was monitored by TLC. Aliquots were then taken for HPLC analysis.

2.4. Fluorescence spectroscopic experiments

Pepsin (5 mg) was added into a mixture containing 10 mL of acetone and 1.1 mL of a selected protic solvent (*t*-butyl alcohol, ethanol or water). Pepsin and denatured pepsin (5 mg each) were added into 11.1 mL portions of pH 2.2 phosphate buffer, respectively. The samples were then excited at 292 nm, and the resulting fluorescence spectra were recorded by the spectrofluorometer.

2.5. Influence of the water content

The reaction was initiated by adding pepsin (20 mg) and 4-nitrobenzaldehyde (18.1 mg, 0.12 mmol) to a mixture containing 1 mL of acetone and different amounts of deionized water (0 mL, 0.02 mL, 0.04 mL, 0.06 mL, 0.08 mL, 0.1 mL, 0.12 mL, 0.14 mL, 0.16 mL, 0.18 mL, 0.2 mL, 0.22 mL, 0.24 mL, 0.3 mL, 0.35 mL and 0.4 mL). The suspension was maintained at 30 °C and shaken at 200 rpm. Product formation was monitored by TLC. Aliquots of each reaction mixture were then taken after 27 h, 74 h and 121 h for HPLC analysis.

2.6. A typical pepsin-catalyzed aldol reaction (Fig. 5)

The reaction was initiated by adding pepsin (400 mg) and 4-nitrobenzaldehyde (362.4 mg, 2.4 mmol) to a mixture containing 20 mL of anhydrous acetone and 2.24 mL of deionized water (10% water content). The suspension was maintained at 30 °C and shaken at 200 rpm. Aliquots were taken after 2 h, 4 h, 8 h, 12 h, 24 h, 48 h, 72 h, 96 h, 120 h, 168 h, 192 h, 216 h, 240 h and 264 h for HPLC analysis.

2.7. Study of enzyme recycling

To a mixture containing 5 mL of acetone and 0.56 mL of deionized water (10% water content), pepsin (100 mg) and 4-nitrobenzaldehyde (90.6 mg, 0.6 mmol) were added. The suspension was maintained at 30 °C and shaken at 200 rpm for 4 days. Product formation was monitored by TLC. Subsequently, aliquots were taken for HPLC analysis. Pepsin was filtered from the reaction mixture and washed with acetone. The recovered pepsin was used for the next reaction cycle using the procedure described above.

2.8. Characterization of substrate tolerance

Pepsin (20 mg) and 0.12 mmol of a suitable aldehyde were added to a mixture containing 1 mL of a selected ketone and 0.25 mL of deionized water (20% water content). The suspension was maintained at 30 °C and shaken at 200 rpm for 4 days. Product formation was monitored by TLC. Aliquots were then taken for HPLC analysis.

2.9. Characterization of products

2.9.1. 4-Hydroxy-4-(4-nitrophenyl)butan-2-one (1)

^{1}H NMR ($CDCl_3$, δ, ppm): 8.17 (m, 2H, Ph–H), 7.54 (m, 2H, Ph–H), 5.27 (m, 1H, Ph–CH–), 3.63 (s, 1H, –OH), 2.87 (m, 2H, $–CH_2–$), 2.23 (s, 3H, $–CH_3$). ^{13}C NMR ($CDCl_3$, δ, ppm): 208.46 (–C=O), 150.47, 147.14,

126.47, 123.63, 68.83 (–C–OH), 51.57 ($–CH_2–$), 30.67 ($–CH_3$). ESI-MS: 232.0 $[M+Na]^+$.

2.9.2. 4-Hydroxy-4-(3-nitrophenyl)butan-2-one (2)

^{1}H NMR ($CDCl_3$, δ, ppm): 8.25 (m, 1H, Ph–H), 8.14 (m, 1H, Ph–H), 7.72 (m, 1H, Ph–H), 7.53 (m, 1H, Ph–H), 5.26 (m, 1H, Ph–CH–), 3.68 (s, 1H, –OH), 2.89 (t, 2H, J = 4.0 Hz, $–CH_2–$), 2.24 (s, 3H, $–COCH_3$). ^{13}C NMR ($CDCl_3$, δ, ppm): 208.65 (–C=O), 148.39, 144.85, 131.84, 129.53, 122.59, 120.73, 68.79 (–CH–OH), 51.52 ($–CO–CH_2–$), 30.73 ($–CO–CH_3$). ESI-MS: 232.3 $[M+Na]^+$.

2.9.3. 4-Hydroxy-4-(2-nitrophenyl)butan-2-one (3)

^{1}H NMR ($CDCl_3$, δ, ppm): 7.96 (m, 1H, Ph–H), 7.90 (m, 1H, Ph–H), 7.67 (m, 1H, Ph–H), 7.45 (m, 1H, Ph–H), 5.68 (m, 1H, Ph–CH–), 3.57 (s, 1H, –OH), 3.13 (m, 1H, $–CH_2–$), 2.74 (m, 1H, $–CH_2–$), 2.24 (s, 3H, $–COCH_3$). ^{13}C NMR ($CDCl_3$, δ, ppm): 208.91 (–C=O), 147.11, 138.46, 133.86, 128.30, 128.20, 124.46, 65.61 (–CH–OH), 51.11 ($–CO–CH_2–$), 30.46 ($–CO–CH_3$). ESI-MS: 232.3 $[M+Na]^+$.

2.9.4. 4-Hydroxy-4-(4-cyanophenyl)butan-2-one (4)

^{1}H NMR ($CDCl_3$, δ, ppm): 7.64 (m, 2H, Ph–H), 7.48 (m, 2H, Ph–H), 5.22 (m, 1H, Ph–CH–), 3.62 (s, 1H, –OH), 2.84 (m, 2H, $–CH_2–$), 2.22 (s, 3H, $–CH_3$). ^{13}C NMR ($CDCl_3$, δ, ppm): 208.59 (–C=O), 148.06, 132.38, 126.35, 118.76 (–CN), 111.34, 69.06 (–C–OH), 51.54 ($–CH_2–$), 30.75 ($–CH_3$). ESI-MS: 190.2 $[M+H]^+$.

3. Results and discussion

We performed a series of experiments to investigate the promiscuous catalytic activity of hydrolases. Several important factors were examined to optimize the biocatalytic process, including the type of hydrolase, the type of reaction co-solvent, the water content of the reaction medium and the efficiency of enzyme recycling. Fluorescence experiments were also employed to investigate the conformation of pepsin under neutral pH reaction conditions. In addition, a number of substrates were examined to elucidate the substrate tolerance of the biocatalytic aldol reaction.

3.1. Promiscuity of the biocatalyst

Firstly, experiments were carried out in order to investigate the promiscuity of hydrolase catalytic activity, focusing on the ability of selected enzymes to catalyze an aldol reaction between acetone and 4-nitrobenzaldehyde (Table 1). Within this screen, various hydrolases presented a range of catalytic activities. Pepsin (a gastric aspartic proteinase, EC 3.4.23.1) showed obvious asymmetric catalysis of the aldol reaction between 4-nitrobenzaldehyde and acetone with a yield up to 22% and an e.e. value up to 43% at 24 h. This enzyme displayed the highest enantioselectivity of all the tested hydrolases (entry 5). D-Aminoacylase showed the best overall catalytic activity, with yields up to 35%; however, this transformation exhibited poor enantioselectivity (entry 10). Other hydrolases, such as protease-S and papain exhibited low rate enhancements and enantioselectivities (entries 2 and 3). Thus, out of the panel of screened hydrolases, pepsin appears to be the best promiscuous aldol reaction catalyst. As a control, the blank reaction (i.e., without any biocatalyst) was carried out under otherwise identical reaction conditions, and few products were detected after 24 h (entry 1). Notably, BSA (bovine serum albumin), which has a surface amino acid distribution similar to many soluble enzymes, showed very little activity in a second control experiment (entry 22). To further elucidate the promiscuous catalytic ability of certain hydrolases, denaturation experiments were conducted. In comparison to their well-folded counterparts, four denatured enzymes barely catalyzed the aldol reaction (entries 23–26). All of

Table 1
The activities and stereoselectivities of hydrolase-catalyzed aldol reaction[a]

4-nitrobenzaldehyde (CHO, O_2N) + acetone → (Hydrolase, Water) → 4-hydroxy-4-(4-nitrophenyl)butan-2-one (OH, O, *, O_2N).

Entry	Biocatalyst	Yield[b] (%)	e.e. (%)
1	No enzyme	<1	–[c]
2	Protease-S	<1	–
3	Papain	18	11
4	Protease from *Bacillus subtilis*	<1	–
5	Pepsin	22	43
6	Trypsin	23	19
7	Protease from *Bacillus polymyxa*	< 1	–
8	Protease Type X VIII	<1	–
9	Protease from *Aspergillus oryzae*	<1	–
10	D-Aminoacylase	35	7
11	Lipase from *Mucor miehei*	9	9
12	Lipase from acrylic resin *Candida antarctica*	2	9
13	Lipase-AY30	<1	–
14	Lipase from *Penicillium camemberti*	<1	–
15	Lipase from porcine pancreas	24	15
16	Lipase-PS	<1	–
17	Lipase from *Rhizopus oryzae*	7	4
18	Lipase from *Candida cylindracea*	<1	–
19	Lipase from hog pancreas	23	10
20	Lipase from *Mucor javanicus*	15	12
21	Lipase-AK	11	6
22	BSA	<1	–
23	Denatured pepsin[d]	<1	–
24	Denatured trypsin	<1	–
25	Denatured lipase from	<1	–
26	Denatured pepsin	<1	–

[a] Reaction conditions: hydrolase 20 mg, 4-nitrobenzaldehyde 0.12 mmol, acetone 1 mL, deionized water 0.25 mL, 30 °C for 24 h.
[b] Values in entries 5 and 15 are isolated yields and others were measured by HPLC.
[c] Not determined.
[d] Pretreated with urea at 100 °C for 10 h.

these results indicate that the specific natural folds of these hydrolases are responsible for their ability to catalyze the aldol reaction. Because of its catalytic efficiency, enantioselectivity and ease of sourcing, pepsin was chosen as the biocatalyst for subsequent studies.

Moreover, tributyrin was used as substrate to further determine the promiscuous catalytic activity. In the same reaction condition after 2 days, butyric acid can be obviously detected by means of copper soap method. This suggests the pepsin might keep its nature feature in catalytic site.

3.2. Influence of the reaction media

The reaction medium plays a significant role in maintaining the catalytic activity and stability of an enzyme. To further characterize the activity of pepsin in the aldol reaction, the influence of various reaction co-solvents was investigated. These experiments were performed in a co-solvent/acetone (1:10, v/v) mixed solvent system, a homogeneous mixture. The co-solvents had been chosen according to the log P value, which is very important to enzymatic reactions. The catalytic activity and enantioselectivity of pepsin were remarkably influenced by different media (Fig. 1). When compared with other solvent systems, those aldol reactions performed in the presence of t-butyl alcohol, ethanol and DMSO presented high enantioselectivities, but quite low activities (less than 5% yield after 2 days). On the other hand, water greatly promoted promiscuous catalysis with notable enantioselectivity. Indeed, it appears to be required for biocatalytic promiscuity, partly because water can act as a molecular lubricant, allowing for a high degree of structural flexibility. These results suggest that protic solvents, such

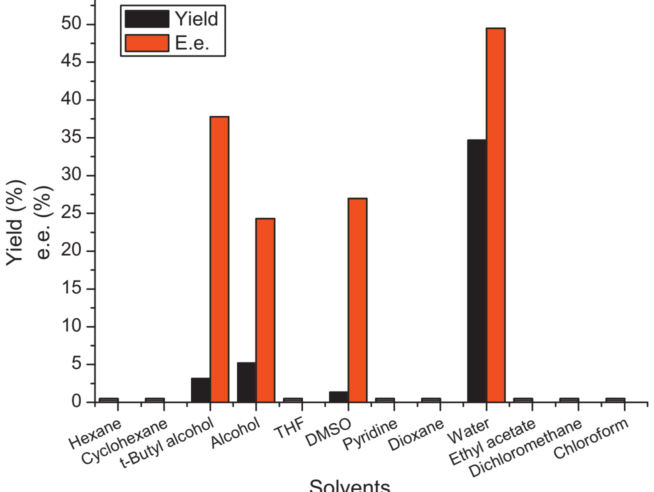

Fig. 1. The influence of the reaction media on pepsin-catalyzed aldol reaction, 30 °C for 2 days.

as *t*-butyl alcohol, ethanol and water, favor the pepsin-catalyzed aldol reaction. Therefore, subsequent reactions were carried out in water/acetone-mixed solvent systems.

3.3. Fluorescence spectroscopic experiments

To explore the effects of various reaction media in more detail, fluorescence experiments were employed to characterize the conformation of pepsin in different reaction solvents. Structural variations of enzymes that contain fluorescent residues (such as Phe, Tyr or Trp) are reflected by changes in the maximal emission wavelength (Lou et al., 2006). We investigated the fluorescence emission spectrum of pepsin in various protic reaction solvents. Denatured pepsin was also investigated under similar conditions as a comparison.

As shown in Fig. 2, two fluorescence curves were obtained from pepsin dissolved in our reaction system (neutral pH) and a pH 2.2 buffer, respectively. The acidic buffer was chosen to mimic gastric fluids—the physiological environment of maximum pepsin proteolysis. Interestingly, both curves are similar in shape, and their maximal fluorescence emission wavelengths (λ_{max}) are very similar. The curves obtained from solvent systems containing ethanol or *t*-butyl alcohol nearly overlap with each other, while their λ_{max} values were red-shifted approximately 20 nm from those obtained in aqueous media. Interestingly, the λ_{max} values obtained with denatured pepsin in these reaction media are also different. Although it is well accepted that pepsin is unstable under neutral pH conditions (Simon et al., 2007), this fluorescence analysis suggests that pepsin can maintain many aspects of its native conformation in the water/acetone reaction system (neutral conditions). Thus, there is reason to believe that pepsin is properly folded and stable under the neutral conditions favorable to aldol catalysis as well as the highly acidic conditions that favor proteolysis. Combining the reaction media experiments with the fluorescence results, it is evident that the ability of pepsin to adopt an appropriate conformation in a given reaction solvent is significant to its ability to catalyze aldol reactions. This conclusion is similar to previous assessments made by near-UV circular dichroism spectroscopy (Campos and Sancho, 2003). Generally, the fluorescence experiments provided important evidence for a relationship between the promiscuous catalytic activity of pepsin in the aldol reaction and its spatial conformation.

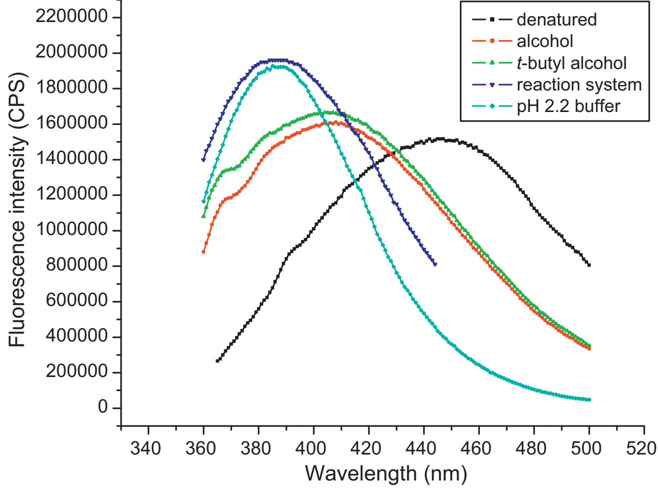

Fig. 2. Fluorescence emission spectra of pepsin in different reaction media under the fluorescence condition: excitation wavelength 292 nm and emission wavelengths from 360 nm to 500 nm with various slit widths.

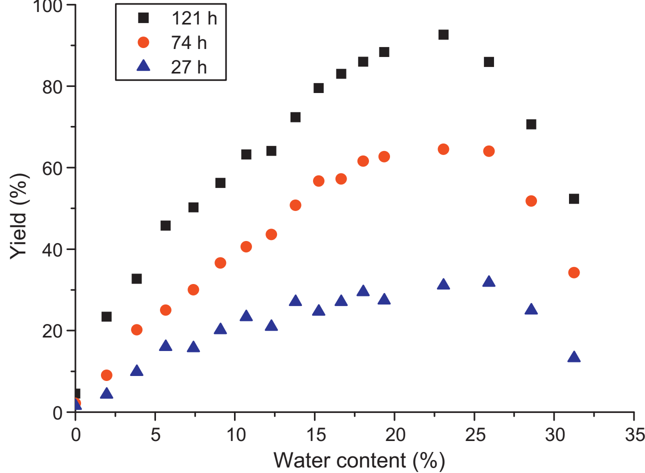

Fig. 3. The influence of water content on the yield of pepsin-catalyzed aldol reaction at 30 °C.

It is important to note that the optimized reaction system presented a cloudy mixture, as powdered pepsin hardly dissolved in the water/acetone reaction medium (10%, v/v). This observation is significant with respect to the enzyme-recycling experiments discussed below.

3.4. Influence of water content in the reaction medium

After establishing water as an optimal aldol reaction co-solvent, the water content of the reaction medium was varied in order to optimize promiscuous biocatalysis. Solvent mixtures containing 0–35% water were screened in the pepsin-catalyzed aldol reaction. As expected, the water content greatly altered the activity of pepsin in the aldol reaction, and the control of this parameter proved to be crucial for efficient biocatalysis. The results are shown in Fig. 3. By monitoring reaction progress at three time points as a function of water content, three distinct curves can be obtained, showing that water content plays an important role in the acceleration of the reaction rate. The figure shows that a water content of 23% attained the fastest reaction rate, with a yield of up to 93% after 121 h. In contrast, other water contents proved to be less efficient; for example, a 5% water content gave a reduced yield of 40% after 121 h. These experimental results indicate that an optimized water content is crucial for the promiscuous catalytic activity of pepsin.

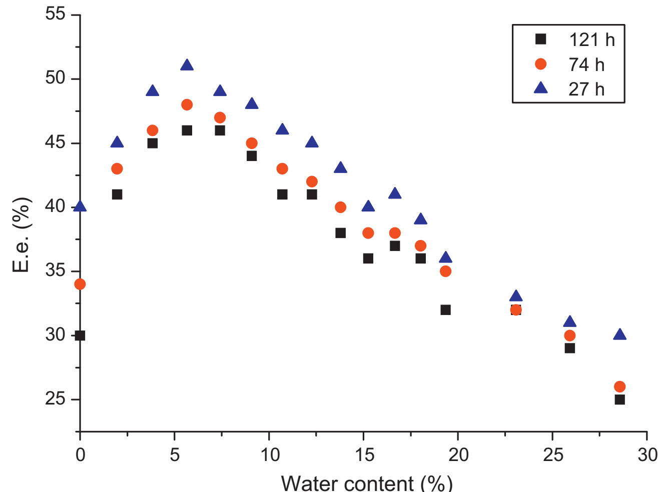

Fig. 4. The influence of water content on the enantioselectivities of pepsin-catalyzed aldol reaction.

The influence of water content on the enantioselectivity of pepsin-catalyzed aldol reactions has also been studied in our experiments (Fig. 4). By monitoring e.e. values at three separate time points, three curves can be obtained that nearly overlap each other, showing that enantioselectivity decreases only slightly as the reaction progresses. While the optimal water content for enantioselectivity seems to be approximately 5% (53% e.e. after 27 h), a solvent system containing 10% water was chosen for subsequent experiments due to its combination of adequate enantioselectivity and enhanced rate of catalysis.

3.5. A typical pepsin-catalyzed aldol reaction

Based on the results of the above experiments, a typical enzymatic aldol reaction was carried out to further investigate the biocatalytic process. The water content of the reaction mixture was maintained at 10% for the reasons described above. The yields and product enantioselectivities were monitored as a function of time. As shown in Fig. 5, the reaction progressed at a nearly constant rate for the first 120 h. At this point, the reaction reached equilibrium with a product yield up to 89%. Notably, product enantioselectivity dropped from a high of approximately 61% during the initial phase of the reaction (i.e., in the first 20 h) to a constant moderate value of approximately 45%.

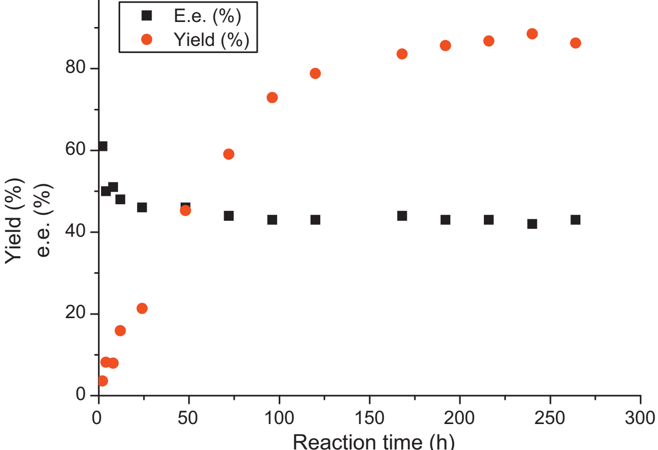

Fig. 5. Time course of the pepsin-catalyzed aldol reaction at 30 °C.

Table 2
Study of enzyme recycling in the pepsin-catalyzed aldol reaction[a].

Entry	Cycle	Yield (%)[b]	e.e. (%)
1	1	54	44
2	2	41	44
3	3	23	42

[a] Reaction condition: pepsin 100 mg, 4-nitrobenzaldehyde 0.66 mmol, acetone 5 mL, deionized water 0.56 mL, 30 °C for 4 days.
[b] Calculated by HPLC.

To obtain k_{cat} and K_M estimates for pepsin, we investigated the initial reaction rate and hypothesized simple Michaelis–Menten kinetics. The k_{cat} and K_M values are $1.2 \times 10^3\ \text{min}^{-1}$ and 0.11 M, which show the low reactivity of this biocatalytic promiscuity. The slow reaction rate is probably associated with the low activity of acetone.

3.6. Study of enzyme recycling

The stability of an enzyme under given reaction conditions is crucial because this factor will determine the ultimate cost and acceptability of an enzymatic process. As shown in Table 2, three recycling steps were carried out, and in each step, the enzymatic process persisted for 4 days and generated a high yield of product. Although the yield decreased after each subsequent reaction, noticeable activity remained after three recoveries (entry 3), showing that pepsin has considerable stability under the reaction conditions. Recycled pepsin also produced good product enantioselectivities without a significant decrease over four cycles. The robust catalytic activity of pepsin suggests that the pepsin-catalyzed aldol reaction could become a practical method for organic synthesis due to its low cost and simplicity.

3.7. Exploring substrate tolerance by pepsin

Other aldol-type substrates were subjected to pepsin catalysis to determine the degree of substrate tolerance by the enzyme and to expand the practical scope of our methodology. In these experiments, selected substrates were incubated under typical reaction conditions as described above. The results are shown in Table 3. In our experiments, different aldehydes were selected to react with acetone, whereas 4-nitrobenzaldehyde was selected to react with different ketones. Among aldehyde substrates, those bearing strong electron-withdrawing functional groups, such as 4-nitrobenzaldehyde, 3-nitrobenzaldehyde, 2-nitrobenzaldehyde and 4-cyanobenzaldehyde (entries 1–4), exhibited high reactivity in the enzymatic aldol reaction, as expected. Other aldehydes, such as benzaldehyde, 2-methoxybenzaldehyde, 4-bromobenzaldehyde and even 4-chlorobenzaldehyde (entries 5, 6, 9 and 11), reacted sluggishly. With respect to different ketone reactants, 2-butanone, cyclohexanone and acetylacetone showed relatively high aldol-type reactivity (entries 7, 8 and 10). However, acetophenone—the bulkiest of the tested ketones—produced a low product yield, possibly due to steric congestion within the catalytic site of the enzyme (entry 12). These results imply that aldol biocatalysis by pepsin is favored by the use of aldehydes that possess strong electron-withdrawing groups and ketones that present minimal steric bulk to the active site.

3.8. Discussion of the catalytic mechanism of the aldol reaction

The structure and catalytic properties of pepsin have been well studied. As an aspartic proteinase, proteolysis is thought to occur through an acid/base mechanism involving two aspartate residues (Asp32 and Asp215). These residues are located at the bottom of

Table 3

Promiscuous pepsin-catalyzed aldol reaction of aldehydes with ketones[a].

Entry	Product	Yield (%)[b]	Entry	Product	Yield (%)
1	**1**	69	7	**7**	37
2	**2**	57	8	**8**	25
3	**3**	59	9	**9**	<5
4	**4**	43	10	**10**	66
5	**5**	<5	11	**11**	6
6	**6**	6	12	**12**	<5

[a] Reaction condition: pepsin 20 mg, aldehyde 0.12 mmol, ketone 1 mL, deionized water 0.25 mL, 30 °C for 4 days.
[b] Calculated by HPLC.

a deep cleft between two pseudosymmetrical lobes in pepsin's structure. It is hard to determine the mechanism of aldol biocatalysis without precise knowledge of enzyme-bound water molecules and the active site conformation within the water/acetone reaction medium. However, some clues are provided by experiments with denatured enzymes as well as fluorescence spectroscopy. It seems likely that the catalysis of the aldol reaction also depends on the native proteolytic active site in pepsin. More work is required to elucidate this mechanism of promiscuous biocatalysis.

4. Conclusion

In summary, a protease-catalyzed asymmetric aldol reaction has been developed that occurs under mild and environmentally benign conditions. Several parameters were optimized to achieve optimal biocatalysis. Pepsin exhibits the greatest degree of catalytic promiscuity, with high product yields and significant enantioselectivity. Notably, water seems to be more suitable as a reaction co-solvent than several tested organic solvents. This finding is consistent with previous observations that the water content of the reaction medium also greatly influences aldol catalysis by lipase PPL. Fluorescence experiments have revealed that pepsin can maintain its native conformation under non-acidic reaction conditions, and that this conformation plays a critical role in pepsin's ability to catalyze aldol reactions. Additionally, a number of different aldol substrates were successfully reacted under pepsin catalysis to reveal the scope of substrate tolerance by the enzyme. Compared with the lipase PPL-catalyzed aldol reaction reported previously, the enantioselectivity was increased using a protease, pepsin. Finally, some mechanistic aspects of this asymmetric aldol

reaction have been preliminarily discussed. The facile biocatalysis of asymmetric aldol reactions by pepsin could be regarded as a potential alternative method for green organic synthesis. Future work in our laboratory will include the use of directed evolution to develop highly promiscuous mutant pepsins that catalyze a range of asymmetric aldol reactions with even greater enantioselectivity.

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