Tetrahedron 68 (2012) 3160–3164

Contents lists available at SciVerse ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

The enzymatic asymmetric aldol reaction using acidic protease from *Aspergillus usamii*

Bang-Hua Xie [a], Wei Li [b], Yan Liu [b], Hai-Hong Li [a], Zhi Guan [a,*], Yan-Hong He [a,*]

[a] School of Chemistry and Chemical Engineering, Southwest University, Chongqing 400715, China
[b] School of Life Science, Southwest University, Chongqing 400715, China

ARTICLE INFO

Article history:
Received 7 September 2011
Received in revised form 19 February 2012
Accepted 24 February 2012
Available online 1 March 2012

Keywords:
Biocatalytic promiscuity
Acidic protease
Enantioselectivity
Aldol reaction

ABSTRACT

AUAP (acidic protease from *Aspergillus usamii*) could catalyze the direct aldol reactions between aromatic aldehydes and cyclic ketones in acetonitrile (MeCN) in the presence of water. The enantioselectivities of up to 88% ee and diastereoselectivities of up to 97:3 (*anti*/*syn*) were achieved. This new activity of protease expands the application of the biocatalyst and provides a novel example of enzymatic promiscuity.

1. Introduction

New catalytic synthetic methods in organic chemistry that satisfy increasingly stringent environmental constraints are in great demand by the pharmaceutical and chemical industries. Enzyme-catalyzed chemical transformations are now widely recognized as practical alternatives to traditional (non-biological) organic synthesis, and as convenient solutions to certain intractable synthetic problems.[1] Biocatalytic promiscuity, a new frontier extending the use of enzymes in organic synthesis, has attracted much attention and expanded rapidly in recent years.[2,3] It means that enzymes were capable of catalyzing not only their natural reactions but also one or more alternative reactions. In the past few years, though some elegant works on enzymatic promiscuity have been reported,[2,3a,4–7] exploiting more reaction types catalyzed by each available enzyme is still necessary, due to the high chemo-, regio-, and stereoselectivity and non-toxicity of enzymes in organic synthesis.[8]

The asymmetric aldol reaction is one of the most useful methods for carbon–carbon bond formation in organic synthesis.[9,10] It is a useful approach for the preparation of pharmaceuticals, fine chemicals and natural products, but is also considered a powerful strategy for the preparation of complex molecules, since it allows the linking of two units to obtain a more complex structure. Although numerous successful organocatalysts for asymmetric aldol reaction have been described with high efficiency and enantioselectivity in recent years, the development of sustainable and cost-efficient biocatalysts for the asymmetric aldol reaction still remains a significant challenge. Wang and co-workers recently reported the first enzyme-catalyzed asymmetric aldol addition between acetone and different aromatic aldehydes with strong electron-withdrawing groups employing pig pancreas lipase (PPL) as catalyst in wet reaction media in 2008,[11] and they also found that pepsin could catalyze aldol reactions in aqueous media in 2010.[12] Very recently, our laboratory reported that nuclease p1 and alkaline protease could catalyze direct asymmetric aldol reactions.[13,14] As our continuous work on synthetic applications of enzymes, herein, we wish to report the discovery that AUAP (acidic protease from *Aspergillus usamii* No. 537) catalyzed direct asymmetric aldol reactions of aromatic aldehydes with some cyclic ketones in organic medium in the presence of water under mild reaction conditions.

2. Results and discussion

During the wide screening of promiscuous activities of enzymes, we found that AUAP could catalyze direct asymmetric aldol reaction. Thus, we further investigated this novel activity of AUAP. In our initial studies, the aldol reaction of cyclohexanone and 4-nitrobenzaldehyde was used as a model transformation. Since it is known that enzymes not only can maintain their activities in organic solvents, but also can acquire remarkable properties such as enhanced stability, altered substrate and enantiomeric specificities, and the ability to catalyze unusual reactions, which are impossible in aqueous media,[15] therefore, some conventional organic

* Corresponding authors. Tel./fax: +86 3 68254091; e-mail addresses: guanzhi@swu.edu.cn (Z. Guan), heyh@swu.edu.cn (Y.-H. He).

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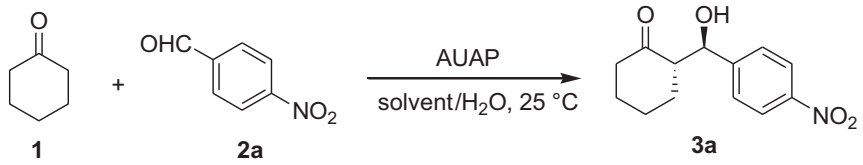
doi:10.1016/j.tet.2012.02.056

solvents were screened for the AUAP catalyzed aldol reaction (Table 1). Among the solvents used, AUAP exhibited the best catalytic activity and stereoselectivity in MeCN, which gave the corresponding aldol product in good yield of 63% with the enantioselectivity of 82% ee for *anti* isomer (*anti*/*syn* 83:17) (Table 1, entry 1). The reaction in CH_2Cl_2 afforded the aldol product with good ee of 80%, but in low yield of 33% (Table 1, entry 2). The other tested solvents gave the product in lower enantioselectivities (Table 1, entries 3–9). The reaction was also carried out under solvent-free conditions, which only gave a low yield of 37% with moderate stereoselectivity of 63% ee (Table 1, entry 10). Interestingly, when water was used as a solvent, the reaction only gave a low yield of 19% with moderate ee of 60% (Table 1, entry 11). The results clearly indicated that AUAP displayed better reaction activity and selectivity in most organic solvents than in water towards aldol reaction. So we chose MeCN as the optimum solvent for the AUAP catalyzed direct asymmetric aldol reaction.

Table 1
Solvent screening and control experiments[a]



Entry	Solvent	Yield[b] [%]	*anti*/*syn*[c]	ee[c] [%] (*anti*)
1	MeCN	63	83:17	82
2	CH_2Cl_2	33	71:29	80
3	DMSO	49	66:34	47
4	DMF	46	72:28	56
5	EtOH	53	68:32	57
6	THF	29	70:30	66
7	Toluene	23	67:33	73
8	Cyclohexane	26	67:33	76
9	1,4-Dioxane	39	72:28	67
10	Solvent-free	37	62:38	63
11	H_2O	19	67:33	60
12	MeCN (no enzyme)	Trace	—	—
13	MeCN (bovine serum albumin)	45	49:51	0
14	MeCN (enzyme denatured with NBS[d])	Trace	—	—
15	MeCN (enzyme denatured with urea[e])	Trace	—	—
16	MeCN (purified AUAP)[f]	45	80:20	74

[a] Reaction conditions: AUAP (100 mg), **1** (2.5 mmol), **2a** (0.5 mmol), deionized water (0.10 mL) and solvent (0.9 mL) at 25 °C for 144 h.
[b] Yield of the isolated product after chromatography on silica gel.
[c] Determined by chiral HPLC analysis (AD-H).
[d] Pre-treated with NBS at 25 °C for 24 h.
[e] Pre-treated with urea at 100 °C for 24 h.
[f] Reaction conditions: purified AUAP (5 mg), **1** (1.5 mmol), **2a** (0.3 mmol), deionized water (0.05 mL) and solvent (0.45 mL) at 25 °C for 144 h.

In order to verify the specific catalytic effect of acidic protease on the aldol reaction, we performed some control experiments (Table 1, entries 12–16). The aldol reaction between 4-nitrobenzaldehyde and cyclohexanone gave trace adduct in the absence of enzyme in MeCN after 144 h, which indicated that AUAP had a specific catalytic effect on the reaction (Table 1, entry 12). Furthermore, in order to prove that the catalytic activity of AUAP for aldol reaction did not arise from unspecific protein-derived activation, bovine serum albumin (BSA) was used to catalyze the model reaction in MeCN, which gave the product in yield of 45%, but no enantioselectivity and almost no diastereoselectivity were observed (Table 1, entry 13). The experiment indicated that the non-enzyme protein BSA also had the ability to catalyze the aldol reaction, but it did not display any selectivity. Besides, the experiments catalyzed by the denatured enzyme were conducted. NBS (*N*-bromosuccinimide) and urea were used to denature the enzyme. We found that the NBS-denatured and urea-denatured AUAP completely lost its catalytic activity for aldol reaction (Table 1, entries 14 and 15), which excluded the possibility that the reaction was caused by the catalysis simply arose from amino acids of the protein. On the other hand, it suggested that the tertiary structure of the enzyme is necessary to catalyze the reaction. In addition, since the enzyme AUAP we used was purchased as an industrial enzyme preparation, to further rule out the possibility of the catalysis of some impure protein or other impurities, we purified the enzyme (for the purification of enzyme, see Supplementary data), and used the purified AUAP to catalyze the model aldol reaction in MeCN. The purified AUAP showed high activity and good selectivity, and only 10 mg/ml of purified AUAP could give the product in yield of 45% with 74% ee for *anti* isomer (*anti*/*syn* 80:20) (Table 1, entry 16), which was almost 10 times more active than industrial enzyme preparation of AUAP (as described above, 100 mg/ml of industrial enzyme preparation of AUAP gave the product in yield of 63% with 82% ee for *anti* isomer (*anti*/*syn* 83:17) (Table 1, entry 1)). The experiment with purified AUAP clearly confirmed that AUAP indeed has the promiscuity for the catalysis of direct asymmetric aldol reaction. Thus, we continued using the commercially available industrial enzyme preparation of AUAP in the following investigation.

In previous studies of enzymatic promiscuity, water has been considered as a very important factor in enzymatic activity leading to an acceleration of the enzyme-catalyzed reaction.[16] Therefore, in order to confirm the optimal water content in the AUAP catalyzed aldol reaction, we decided to carry out the reaction with different water content in MeCN. The range of water concentration from 0 to 40% (water/[water+MeCN], v/v) was screened for the AUAP catalyzed aldol reaction (Fig. 1). As shown in Fig. 1, the activity and selectivity of the enzyme could be affected by the water content. When the reaction was performed with 10% water content, AUAP exhibited the highest activity and selectivity, and the best ee value

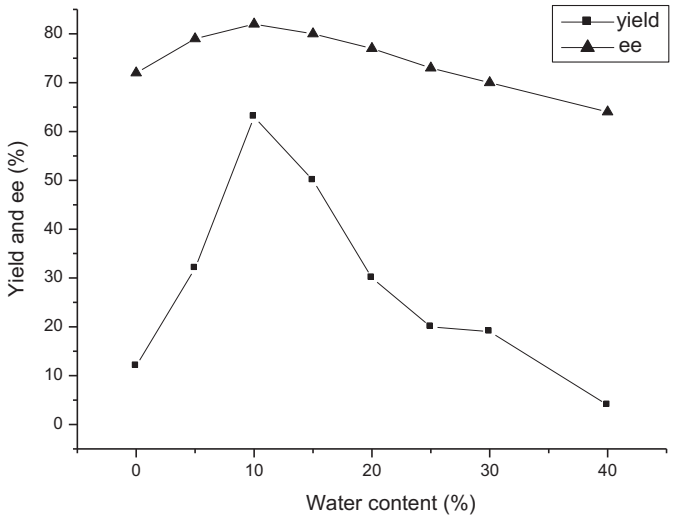


Fig. 1. Influence of water content on the AUAP catalyzed aldol reaction. Reaction conditions: AUAP (100 mg), 4-nitrobenzaldehyde (0.5 mmol), cyclohexanone (2.5 mmol), deionized water from 0 to 40% (water/[water+MeCN], v/v, [water+MeCN]=1 mL) at 25 °C for 144 h; yield of the isolated product after chromatography on silica gel; ee was determined by HPLC analysis on a Chiralpak AD-H column.

of 82% and yield of 63% were obtained. However, once the water content exceeded 10%, the yield and ee value decreased, which probably due to that excessive water leads to a serious decrease of the solubility of substrates and change of the special conformation of AUAP. Thus, 10% water content was chosen as the optimal condition for the following studies.

Temperature plays an important role in enzyme-catalyzed reactions, due to its effects on the selectivity and rate of the reaction, and the stability of the enzyme. Thus, we further investigated the effect of temperature on the AUAP catalyzed aldol reaction (Fig. 2). A noticeable increase of yield was obtained by raising the temperature from 10 to 40 °C, and AUAP exhibited the highest activity at 40 °C. However, the best enantioselectivity of the product was obtained at 25 °C, and higher temperature led to a remarkable decrease of the ee value. In order to get the best enantioselectivity, we chose 25 °C as the optimal temperature for the AUAP catalyzed aldol reaction.

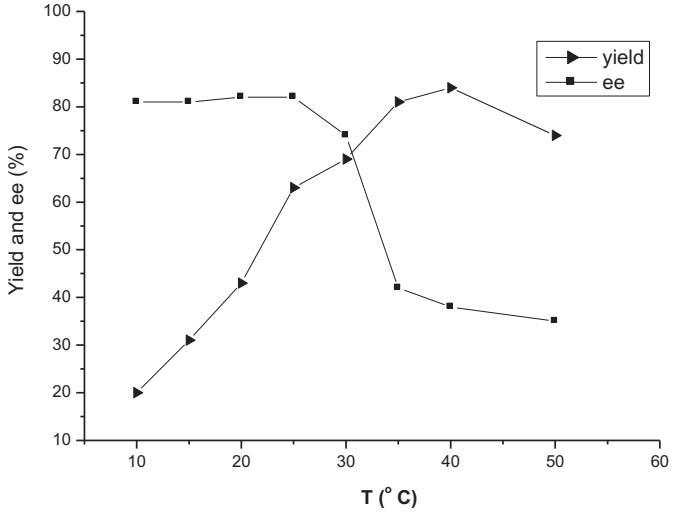


Fig. 2. Influence of temperature on the AUAP catalyzed aldol reaction. Reaction conditions: AUAP (100 mg), 4-nitrobenzaldehyde (0.5 mmol), cyclohexanone (2.5 mmol), temperature (10–50 °C), deionized water (0.10 mL) and MeCN (0.9 mL) for 144 h; yield of the isolated product after chromatography on silica gel; ee was determined by HPLC analysis on a Chiralpak AD-H column.

To further improve the AUAP catalyzed aldol reaction, we then examined the effects of molar ratio of substrates and enzyme concentration on the aldol reaction. As a result, 4-nitrobenzaldehyde/cyclohexanone=1:5 and enzyme concentration of 100 mg/ml were chosen as the optimal conditions (for details see Supplementary data).

Finally, we investigated the time course of the aldol reaction between 4-nitrobenzaldehyde and cyclohexanone catalyzed by AUAP under the optimal conditions (Fig. 3). It could be seen that the reaction progressed at a nearly constant rate for the first 8 days, and after that the yield did not increase. The best yield of 66% was observed. Moreover, there was a slight increase of ee value for the first 2 days, and after that the ee value almost kept invariably at about 80%.

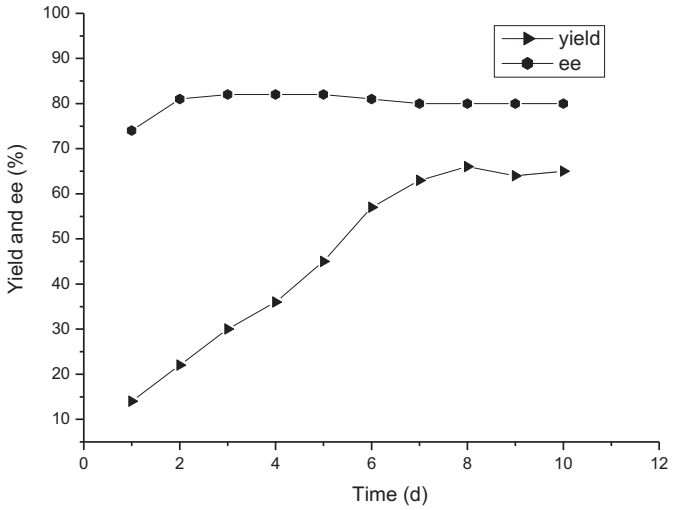
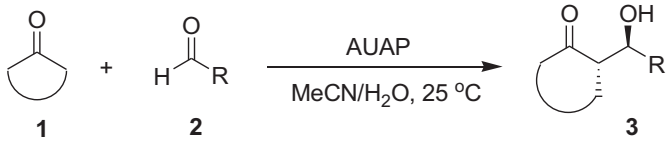


Fig. 3. Time course of the AUAP catalyzed aldol reaction. Reaction conditions: AUAP (100 mg), 4-nitrobenzaldehyde (0.5 mmol), cyclohexanone (2.5 mmol), deionized water (0.10 mL) and MeCN (0.9 mL) at 25 °C for 1–10 days; yield of the isolated product after chromatography on silica gel; ee was determined by HPLC analysis on a Chiralpak AD-H column.

Finally, with the optimal reaction conditions in hand, we further studied the generality of the AUAP catalyzed direct asymmetric aldol reaction (Table 2). It could be seen that the aldol reactions of cyclic ketones to a number of aromatic and heteroaromatic aldehydes proceeded smoothly to afford aldol adducts in moderate to good enantioselectivities with good to excellent diastereoselectivities. In general, the aromatic aldehydes bearing electron-withdrawing substituents (Table 2, entries 1–11) were converted to the corresponding aldol products in higher yields than the aromatic aldehydes containing electron-donating groups (Table 2, entry 13). Moreover, the substituent positions on the benzene ring of aromatic aldehydes had a great impact on the enantio- and diastereoselectivity of the reaction. For example, the aldol reaction of *m*-chlorobenzaldehyde with cyclohexanone generated the aldol product with higher dr and ee values than *o*- and *p*-chlorobenzaldehydes (Table 2, entries 3–5). To our surprise, the reaction appeared quite tolerant with respect to the steric contribution of the substituents in substituted benzaldehydes, and the most sterically hindered 2,6-dichlorobenzaldehyde provided the product in an acceptable yield of 59% with the best diastereoselectivity (97:3) and moderate enantioselectivity (52% ee) (Table 2, entry 11). In contrast, the reaction of the least sterically hindered benzaldehyde with cyclohexanone afforded the aldol product only in 26% yield with moderate diastereoselectivity (72:28) and higher enantioselectivity (77% ee) (Table 2, entry 12). Furthermore, heteroaromatic aldehydes 2-furaldehyde and 2-thiophenaldehyde were also used as the substrates, which gave the corresponding aldol products in low yields

Table 2
Scope of the AUAP catalyzed direct asymmetric aldol reaction under optimal conditions[a]



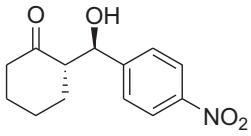
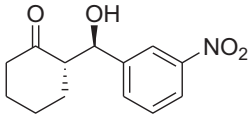
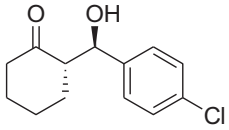
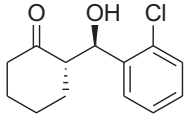
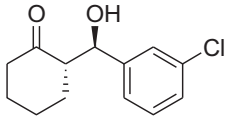
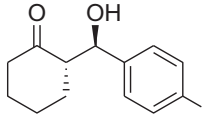
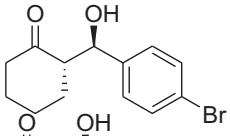
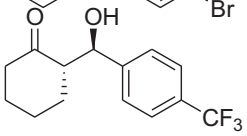
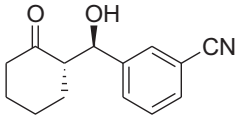
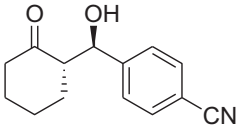
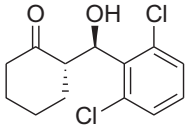
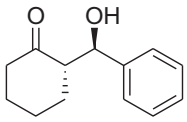
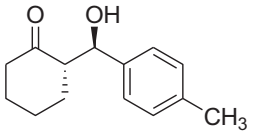
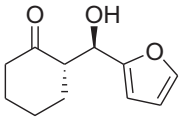
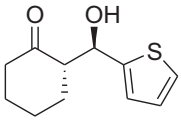
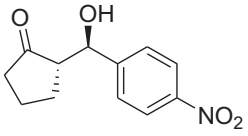
Entry	Product	No.	Time [h]	Yield[b] [%]	*anti*/*syn*[c]	ee[c] [%] (*anti*)
1		**3a**	144	63	83:17	82
2		**3b**	168	61	74:26	85
3		**3c**	168	20	82:18	76
4		**3d**	168	48	87:13	62
5		**3e**	168	29	92:8	88
6		**3f**	168	20	72:28	70
7		**3g**	168	32	82:18	88
8		**3h**	168	47	75:25	80
9		**3i**	168	57	84:16	88
10		**3j**	168	59	79:21	74
11		**3k**	168	59	97:3	52

Table 2 (*continued*)

Entry	Product	No.	Time [h]	Yield[b] [%]	*anti*/*syn*[c]	ee[c] [%] (*anti*)
12		**3l**	168	26	72:28	77
13		**3m**	168	11	86:14	82
14		**3n**	168	16	67:33	70
15		**3o**	168	10	75:25	75
16		**3p**	144	78	53:47	60:40[d]

[a] Reaction conditions: AUAP (100 mg), ketone **1** (2.5 mmol), aldehyde **2** (0.5 mmol), MeCN (0.9 mL) and deionized water (0.10 mL) at 25 °C.
[b] Yield of the isolated product after chromatography on silica gel.
[c] Determined by chiral HPLC analysis (AD-H, OD-H, AS-H).
[d] *anti* (60% ee), and *syn* (40% ee).

with moderate diastereoselectivities and enantioselectivities (Table 2, entries 14 and 15). In addition, to further examine the generality of this catalytic system, cyclopentanone was also used as the aldol donor. The aldol reaction of cyclopentanone with 4-nitrobenzaldehyde gave the corresponding product in good yield of 78% but with low enantioselectivity (60% ee) and diastereoselectivity (53:47) (Table 2, entry 16). It is worthy to note that the AUAP catalyzed aldol reaction mainly provided *anti*-isomers as the major products with moderate to high enantioselectivities, but low or no enantioselectivity for *syn*-isomers. The yield of the aldol reaction catalyzed by AUAP is still low and the reaction mechanism is unclear at the moment. Further efforts to deal with these problems are currently undertaken.

3. Conclusion

In summary, we described a novel example of biocatalytic promiscuity, in which AUAP was used to catalyze the direct aldol reaction of aromatic and heteroaromatic aldehydes with cyclic ketones under mild reaction conditions. The reaction was carried out in MeCN/H_2O system, and the corresponding aldol products were obtained in moderate to excellent diastereoselectivities (up to 97:3) with moderate to high enantioselectivities (up to 88% ee). The influence of some parameters including solvents, water contents, temperature, molar ratio of substrates and enzyme concentration were also investigated. This AUAP catalyzed direct asymmetric aldol reaction expands the application of the biocatalyst in organic synthesis.

4. Experimental section

4.1. General procedure for the AUAP catalyzed aldol reactions (products, 3a–p)

A 25 mL round-bottomed flask was charged with AUAP (100 mg), MeCN (0.90 mL) and aldehyde (0.5 mmol), to which the deionized water (0.10 mL) and ketone (2.5 mmol) were introduced. The resulting mixture was stirred for a specified amount of time at 25 °C. The reaction was terminated by filtering the enzyme, and ethyl acetate was used to wash the filter paper and the residue to assure that the products were dissolved in the filtrate. The solvents were then removed under reduced pressure. The crude products were purified by column chromatography with petroleum ether/ethyl acetate as eluent.

Acknowledgements

Financial support from the Natural Science Foundation Project of CQ CSTC (2009BA5051) is gratefully acknowledged.

Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.tet.2012.02.056.

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