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Biocatalytic direct asymmetric aldol reaction using proteinase from *Aspergillus melleus*

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The direct asymmetric aldol reaction of aromatic aldehydes with cyclic or acyclic ketones was catalyzed by proteinase from *Aspergillus melleus* (AMP) in acetonitrile in the presence of water. A wide range of substrates could be transformed into the corresponding aldol products in yields up to 89%, enantioselectivities up to 91% ee and diastereoselectivities up to >99:1 (*anti*/*syn*). This work provided an example of enzyme catalytic promiscuity that widens the applicability of this biocatalyst in organic synthesis without the need for additional cofactors or special equipment.

enzyme catalysis, aldol reaction, proteinase, promiscuity, asymmetric synthesis

1 Introduction

Development of green catalytic methods for organic synthesis is a topic of continuing interest. Biocatalysts often perform an economically feasible, ecologically advantageous role which is more sustainable than current chemical technologies, and its high reaction selectivity, mild reaction conditions and potential use of inexpensive regenerable resources have often been proved [1]. Enzymes are efficient and green biocatalysts in synthetic chemistry. Both "natural" reactions and other alternative reactions can be catalyzed by enzymes [2]. The ability of enzymes to Catalyze unnatural reactions is known as enzymatic promiscuity. As one of the most outstanding concepts in biocatalysis, enzymatic promiscuity has caught researchers' attention [3]. Hydrolases are a group of enzymes, which conventionally catalyze bond cleavage using water as nucleophile. Due to their high stability and activity with a broad range of substrates, they have contributed significantly to the increasing use of enzymes in the industrial manufacture of agrochemicals, pharmaceuticals, and high value-added compounds [4]. Consequently, their catalytic activity with unnatural substrates in organic media is a realm attracting much attention [5]. Several examples with regard to the ability of hydrolytic enzymes to catalyze non-conventional synthetic organic reactions, which are a long way from their natural scope have recently been reported, such as Michael additions [6], Markovnikov additions [7], direct Mannich reactions [8], and Henry reactions [9].

Carbon-carbon bond formation is one of the most important keystone reactions in organic synthetic chemistry. Among the available methods, the direct asymmetric aldol reaction is a powerful strategy due to the concomitant creation and functionalization of stereogenic centers [10]. Thereby, developing catalysts for direct asymmetric aldol reaction is in great demand. Catalysts like aldolases [11], catalytic antibodies [12], and small molecules [13] have been used in the aldol reactions. However, there are only a few examples of aldol reactions catalyzed by hydrolases [14]. Recently, our group has reported some direct asymmetric aldol reactions using nuclease p1 [15], alkaline protease [16], acidic protease [17], and chymopapain [18] as catalysts. However, enzymatic promiscuity is still at the exploratory stage, and no general methods are available to profile enzyme catalytic promiscuity [19]. Therefore, it is

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still a formidable but significant task to explore enzyme catalyzed asymmetric aldol reactions and other reactions as much as possible. In continuation of our interest in hydrolase-catalyzed direct asymmetric aldol reaction, in this paper, we established another new synthetic possibility of aldol reaction by using a commercially available proteinase from *Aspergillus melleus*, type XXIII (AMP) as a catalyst without the need for additional cofactors or special equipment.

2 Experimental

2.1 Material

Proteinase from *Aspergillus melleus* (Type XXIII, ≥3 units/mg solid; one unit will hydrolyze casein to produce color equivalent to 1.0 μmol (181 μg) of tyrosine per min at pH 7.5 at 37 °C (color by Folin-Ciocalteu reagent)) was purchased from Sigma-Aldrich Co. LLC. Unless otherwise noted, all reagents were obtained from commercial suppliers and were used without further purification.

2.2 General procedure for preparation of products 3a–t by aldol reaction

A 10 mL round-bottomed flask was charged with AMP (50 mg), aldehyde (0.5 mmol), ketone (2.5 mmol), MeCN (0.90 mL) and deionized water (0.10 mL). The resulting mixture was stirred for the specified period of time at 30 °C. The reaction was terminated by filtering the enzyme. Ethyl acetate was used to wash the filter paper to assure that products obtained were all dissolved in the filtrate. Saturated brine (15 mL) was then added to the filtrate, and the filtrate was extracted three times with ethyl acetate (15 mL). The combined extracts were dried over anhydrous Na_2SO_4, and the solvents were then removed under reduced pressure. The crude products were purified by column chromatography with petroleum ether/ethyl acetate as the eluent.

2.3 General method

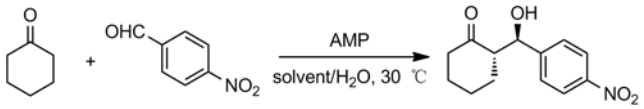
All NMR spectra were recorded on Bruker-AM 300 (300 MHz) spectrometer. Routine monitoring of reactions was performed by TLC using precoated Haiyang GF254 silica gel plates. Flash column chromatography was performed using silica gel (100–200 mesh) at increased pressure. The ee values of products were determined by chiral HPLC, with Chiralpak AD-H, AS-H and Chiralcel OD-H, OJ-H columns. Aldol adducts **3a–t** are all known compounds.

3 Results and discussion

In our initial investigation, the direct aldol reaction of cyclohexanone and 4-nitrobenzaldehyde was used as a model reaction. Since the reaction medium plays a crucial role in the maintenance of enzyme catalytic activity and stability [20], the influence of some common solvents on the AMP-catalyzed aldol reaction was investigated (Table 1). Among the tested solvents, satisfactory stereoselectivities of 84% ee (with 85:15 dr, 53% yield) and 83% ee (with 83:17 dr, 28% yield) were obtained in MeCN and $CHCl_3$, respectively (Table 1, entries 1 and 2). Satisfactory yields of 80% and 84% were achieved in ethanol and DMSO with low enantioselectivities of 35% ee and 33% ee, respectively (Table 1, entries 12 and 13). The reaction in other tested solvents including toluene, butyl acetate, THF, MTBE, cyclohexane, *i*-propyl ether and 1,4-dioxane gave moderate yields and selectivities (Table 1, entries 3–5, and 7–10). Moreover, it is noteworthy that AMP-catalyzed model aldol reaction in water only gave product in a yield of 47% with a low enantioselectivity of 48% ee (Table 1, entry 11). The reaction under solvent-free conditions was also investigated, which gave product in the best yield of 85% with 70% ee (80:20 dr) (Table 1, entry 6). The results clearly indicated that the catalytic activity and stereoselectivity of AMP were significantly influenced by the reaction media. It seems that the AMP-catalyzed model aldol reaction in high polar solvents was in favour of higher yield, however, the reaction in low to middle polar solvents appeared to give products in higher selectivity. Thus, considering stereoselectivity of the reaction, we chose MeCN as a solvent for the following study.

Next, in order to verify the specific catalytic effect of AMP on the aldol reaction, some control experiments were performed in MeCN (Table 1, entries 14–19). Just as we expected, the model aldol reaction in the absence of AMP only afforded a trace amount of product after 90 h (Table 1, entry 14). As comparison, a non-enzyme protein (bovine serum albumin) was also used to catalyze the model reaction, which gave the product in 47% yield without any enantioselectivity (Table 1, entry 19). This reaction suggested that protein without enzyme function also has the ability to catalyze the aldol reaction. Therefore, it is necessary to verify whether the catalytic activity of AMP for aldol reaction arose from unspecific protein-derived activation of the reagents by, for instance the surface of the enzyme. Thus, the experiments with denatured and inhibited AMP were conducted. The experiment with urea-denatured AMP only gave product in 11% yield with 9% ee (Table 1, entry 15). The experiment with phenylmethanesulfonyl fluoride (PMSF)-inhibited AMP provided the product only in 6% yield with 6% ee (Table 1, entry 17). Meantime, the contrast experiments were conducted using urea and PMSF to catalyze the reaction, and only 4% and 5% yields were obtained, respectively (Table 1, entries 16 and 18), which showed that urea or PMSF alone only had tiny effect on the reaction. Therefore, it was experimentally verified that inhibition and denaturation of AMP caused an almost complete disruption of the catalytic activity and selectivity of the enzyme. As a con-

Table 1 Effect of solvents on the AMP-catalyzed asymmetric direct aldol reaction [a)]



Entry	Solvent	Yield (%) [b)]	dr (*anti*:*syn*) [c)]	ee (%)(*anti*) [c)]
1	MeCN	53	85:15	84
2	$CHCl_3$	28	83:17	83
3	toluene	31	72:28	70
4	butyl acetate	38	75:25	70
5	THF	74	82:18	70
6	solvent-free [d)]	85	80:20	70
7	MTBE	32	76:24	68
8	cyclohexane	37	73:27	67
9	*i*-propyl ether	43	68:32	61
10	1,4-dioxane	70	76:24	56
11	H_2O	47	67:33	48
12	EtOH	80	70:30	35
13	DMSO	84	60:40	33
14	MeCN (no enzyme)	trace	–	–
15	MeCN (urea-denatured AMP) [e)]	11	58:42	9
16	MeCN (urea) [f)]	4	37:63	–
17	MeCN (PMSF-inhibited AMP) [g)]	6	50:50	6
18	MeCN (PMSF) [h)]	5	47:53	–
19	MeCN (bovine serum albumin) [i)]	47	48:52	0

a) Reaction conditions: AMP (50 mg), 4-nitrobenzaldehyde (0.5 mmol), cyclohexanone (5 equiv), deionized water (0.10 mL) and solvent (0.90 mL) at 30 °C for 90 h; b) yield of the isolated product after silica gel chromatography; c) determined by chiral HPLC analysis; d) conditions: AMP (50 mg), 4-nitrobenzaldehyde (0.5 mmol), cyclohexanone (5 equiv), deionized water (0.10 mL) at 30 °C for 90 h; e) AMP (50 mg) was pre-treated with urea (50 mg) in deionized water (3.0 mL) at 100 °C for 48 h, and then water was removed under reduced pressure before reaction; f) urea (50 mg) was used instead of AMP; g) AMP (50 mg) was pre-treated with PMSF (200 mg) in dry THF (2.0 mL) at 30 °C for 48 h, and then THF was removed under reduced pressure before reaction; h) PMSF (200 mg) was used instead of AMP; i) bovine serum albumin (50 mg) was used instead of AMP.

sequence, it could be concluded that the native structure of AMP is responsible for its catalytic activity and selectivity for the aldol reaction. Thus, we validated that AMP catalyzed the asymmetric direct aldol reaction.

Water concentration in organic solvent has been considered as an important factor in enzymatic reactions [21]. Thus, we carried out a series of reactions in MeCN with different water content. The results are shown in Table 2. AMP exhibited the highest diastereoselectivity and enantioselectivity at water content of 10% [$H_2O/(H_2O+MeCN)$, *v*/*v*]. Under this condition, the enzymatic reaction gave the product in 60% yield with 84% ee (85:15 dr) (Table 2, entry 3). However, the best yield was obtained at water content of 30% which gave product in 83% yield (57% ee, 78:22 dr) (Table 2, entry 6). It can be seen that water content in organic solvent has an apparent influence on the enzymatic reaction. Therefore, to obtain the best enantioselectivity, we chose the water content of 10% as the optimal condition for the AMP-catalyzed aldol reaction.

Next, the effect of the molar ratio between two substrates was tested (Table 3). The increase of molar ratio of cyclohexanone to 4-nitrobenzaldehyde from 1:1 to 5:1 led to a remarkable increase of the yield (Table 3, entries 1 and 2). Further increase of the molar equivalents of cyclohexanone could not improve the yield evidently. Moreover, the molar ratio of the substrate did not show apparent effect on the selectivity of the reaction. Thus, in consideration of atom

Table 2 Effect of water content on the AMP-catalyzed asymmetric direct aldol reaction [a)]

Entry	H_2O (%)	Yield (%) [b)]	dr (*anti*:*syn*) [c)]	ee (%)(*anti*) [c)]
1	0	15	56:44	24
2	5	22	79:21	65
3	10	60	85:15	84
4	15	80	85:15	77
5	20	82	82:18	70
6	30	83	78:22	57
7	40	70	80:20	31
8	50	68	77:23	27

a) Conditions: AMP (50 mg), 4-nitrobenzaldehyde (0.5 mmol), cyclohexanone (5 equiv), deionized water (0–50%, $H_2O/(H_2O+MeCN)$, *v*/*v*) and $v_{H_2O+MeCN}$ = 1.0 mL at 30 °C for 96 h; b) yield of the isolated product after silica gel chromatography; c) determined by chiral HPLC analysis.

Table 3 Influence of molar equivalents of cyclohexanone on the model aldol reaction [a)]

Entry	**1a:2a**	Yield (%) [b)]	dr (*anti*:*syn*) [c)]	ee (%)(*anti*) [c)]
1	1:1	15	87:13	82
2	5:1	70	88:12	84
3	10:1	75	88:12	83
4	15:1	78	88:12	82
5	20:1	77	87:13	81
6	30:1	75	88:12	81

a) Reaction conditions: AMP (50 mg), 4-nitrobenzaldehyde (0.5 mmol), cyclohexanone (1–30 equiv), deionized water (0.10 mL) and MeCN (0.90 mL) at 30 °C for 100 h; b) yield of the isolated product after silica gel chromatography; c) determined by chiral HPLC analysis.

economy, the 5:1 molar ratio of ketone to aldehyde was chosen as the optimal ratio for further studies.

To further optimize the experimental conditions, we investigated the effect of enzyme concentration on the AMP-catalyzed aldol reaction (Table 4). It was found that both yield and ee value had an increase when the enzyme concentration increased from 25 mg/mL to 50 mg/mL. Further increase of enzyme concentration could not give better results. Therefore, we chose 50 mg/mL as the optimal enzyme concentration for further study.

Then we examined the effect of temperature on the AMP-catalyzed aldol reaction (Table 5). The yield increased apparently with the temperature ranging from 15 to 30 °C (Table 5, entries 1–4). Once the temperature surpassed 30 °C, the yield increased slightly but the ee value decreased. Thus, 30 °C was chosen as the optimal temperature for the reaction.

Time course of the AMP-catalyzed model aldol reaction under optimal conditions was also tested. As shown in Table 6, the selectivity was nearly constant while the yield had been increasing to 86% and plateaued at 144 h.

With the optimized general procedure in hand, we explored the substrate scope and the generality of the AMP-catalyzed asymmetric direct aldol reaction. Various ketones and substituted benzaldehydes were investigated (Table 7). A wide range of substrates could participate in the reaction. Five-, six- and seven-membered cyclic ketones and acetone as aldol donors could be accepted by AMP. In general, AMP showed better diastereoselectivity and enantioselectivity with cyclohexanone (Table 7, entries 1–16) than with cyclopentanone, cycloheptanone and acetone (Table 7, entries 17–20). The best diastereoselectivity of >99:1 (*anti/syn*) (Table 7, entry 12) and the best enantioselectivity of 91% ee (Table 7, entry 3) were achieved. Aromatic aldehydes with electron-withdrawing substituents generally gave higher yields than aromatic aldehydes with electron-donating substituents. Meanwhile, the effect of steric hindrance of substituents on benzaldehydes also had a great impact on the diastereoselectivity and yield of the reaction. For instance, when reacting with cyclohexanone, 4-nitrobenzaldehyde gave the highest yield but the lowest dr and ee value among 2-, 3-, and 4-nitrobenzaldehyde. By contrast, 2-nitrobenzaldehyde gave the best dr and ee value but the lowest yield (Table 7, entries 1–3). The major products were obtained as *anti*-isomers when the aldol donor was cyclohexanone. However, the diastereoselectivity was hardly observed by using cyclopentanone and cycloheptanone as donors (Table 7, entries 17–19). Besides, acetone was also accepted by AMP as a substrate, which gave a low yield with low enantioselectivity (Table 7, entry 20). We also attempted to use some aliphatic aldehyde as the aldol acceptors, but nearly no desired products were obtained. The results illustrated that AMP had a specific substrate selectivity and stereoselectivity in direct asymmetry aldol reaction.

Finally, we compared the performance of different proteases which our group has reported on asymmetric aldol reactions (Table 8). It elucidates that AMP-catalyzed aldol reaction could obtain higher yields than that of acidic protease from *Aspergillus usamii* (AUAP) and chymopapain. AMP also showed better diastereoselectivities and enantioselectivities than alkaline protease from *Bcaillus licheniformis* (BALP) and chymopapain. Generally, AMP is apparently superior than other reported proteases in activity and selectivity towards asymmetric aldol reactions between aromatic aldehydes and cyclic ketones.

Table 4 Effect of enzyme concentration on the AMP-catalyzed aldol reaction [a)]

Entry	Enzyme concentration (mg/mL)	Yield (%) [b)]	dr (*anti*:*syn*) [c)]	ee (%)(*anti*) [c)]
1	25	51	88:12	82
2	50	68	88:12	84
3	75	66	87:13	83
4	100	69	86:14	82
5	200	70	86:14	82

a) Reaction conditions: AMP (25–200 mg), 4-nitrobenzaldehyde (0.5 mmol), cyclohexanone (5 equiv), deionized water (0.10 mL) and MeCN (0.90 mL) at 30 °C for 96 h; b) yield of the isolated product after silica gel chromatography; c) determined by chiral HPLC analysis.

Table 5 Effect of temperature on the AMP-catalyzed aldol reaction [a)]

Entry	*T* (°C)	Yield (%) [b)]	dr (*anti*:*syn*) [c)]	ee (%)(*anti*) [c)]
1	15	28	86:14	78
2	20	35	85:15	80
3	25	77	85:15	78
4	30	88	85:15	84
5	35	90	78:22	77
6	40	91	71:29	70

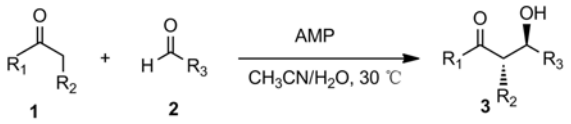
a) Reaction conditions: AMP (50 mg), 4-nitrobenzaldehyde (0.5 mmol), cyclohexanone (5 equiv), deionized water (0.10 mL), and MeCN (0.90 mL) for 144 h; b) yield of the isolated product after silica gel chromatography; c) determined by chiral HPLC analysis.

Table 6 Time curve in the process of AMP catalyzed asymmetric direct aldol reaction [a)]

Entry	Time (h)	Yield (%) [b)]	dr (*anti:syn*) [c)]	ee (%)(*anti*) [c)]
1	6	7	86:14	84
2	12	13	89:11	84
3	24	25	88:12	85
4	48	37	88:12	84
5	72	51	90:10	84
6	96	68	90:10	84
7	120	80	89:11	84
8	144	86	88:12	85
9	168	86	88:12	84

a) Conditions: 4-nitrobenzaldehyde (0.5 mmol), cyclohexanone (5 equiv), AMP (50 mg), deionized water (0.10 mL) and MeCN (0.90 mL) at 30 °C; b) yield of the isolated product after silica gel chromatography; c) determined by chiral HPLC analysis.

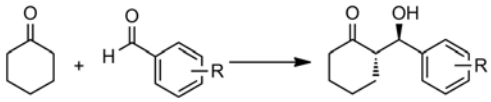
Table 7 Scope of the AMP-catalyzed aldol reaction [a)]



Entry	R_1 R_2	R_3	No.	Time (h)	Yield (%) [b)]	dr (*anti*:*syn*) [c)]	ee (%)(*anti*) [c)]
1	-$(CH_2)_4$-	4-$NO_2C_6H_4$	**3a**	143	87	83:17	84
2	-$(CH_2)_4$-	3-$NO_2C_6H_4$	**3b**	143	79	90:10	90
3	-$(CH_2)_4$-	2-$NO_2C_6H_4$	**3c**	143	52	92:8	91
4	-$(CH_2)_4$-	4-CNC_6H_4	**3d**	130	74	84:16	89
5	-$(CH_2)_4$-	3-CNC_6H_4	**3e**	130	53	91:9	90
6	-$(CH_2)_4$-	4-FC_6H_4	**3f**	192	27	84:16	88
7	-$(CH_2)_4$-	4-ClC_6H_4	**3g**	192	57	84:16	86
8	-$(CH_2)_4$-	3-ClC_6H_4	**3h**	192	84	72:28	86 (*anti*)/55 (*syn*)
9	-$(CH_2)_4$-	4-BrC_6H_4	**3i**	192	57	86:14	88
10	-$(CH_2)_4$-	4-$CF_3C_6H_4$	**3j**	180	73	80:20	87
11	-$(CH_2)_4$-	2,4-$Cl_2C_6H_3$	**3k**	192	70	93:7	89
12	-$(CH_2)_4$-	2,6-$Cl_2C_6H_3$	**3l**	192	89	> 99:1	53
13	-$(CH_2)_4$-	4-MeC_6H_4	**3m**	216	24	86:14	87
14	-$(CH_2)_4$-	4-$MeOC_6H_4$	**3n**	216	11	90:10	87
15	-$(CH_2)_4$-	3-$MeOC_6H_4$	**3o**	204	40	87:13	84
16	-$(CH_2)_4$-	2-$MeOC_6H_4$	**3p**	204	38	93:7	57
17	-$(CH_2)_3$-	4-$NO_2C_6H_4$	**3q**	96	78	47:53	73 (*anti*)/53 (*syn*)
18	-$(CH_2)_3$-	3-$NO_2C_6H_4$	**3r**	96	84	53:47	80 (*anti*)/59 (*syn*)
19	-$(CH_2)_5$-	4-$NO_2C_6H_4$	**3s**	210	23	55:45	40
20	Me, H	4-$NO_2C_6H_4$	**3t**	192	20	–	32

a) Reaction conditions: AMP (50 mg), aldehyde (0.5 mmol), ketone (5 equiv), MeCN (0.90 mL) and deionized water (0.10 mL) at 30 °C; b) yield of the isolated product after silica gel chromatography; c) determined by chiral HPLC analysis, and absolute configuration was assigned by comparison with literature (for details, see the Supporting Information).

Table 8 The comparison of some reported proteases with AMP on the performance in asymmetric aldol reactions



Entry	R	Yield (%)				dr (*anti*:*syn*)				ee (%) (*anti*)			
		AMP	AUAP [a)][17]	BLAP [b)][16]	Chymo-papain[c)][18]	AMP	AUAP	BLAP	Chymo-papain	AMP	AUAP	BLAP	Chymo-papain
1	4-NO_2	87	63	91	69	83:17	83:17	68:32	63:37	84	82	70	78
2	3-NO_2	79	61	87	41	90:10	74:26	58:42	87:13	90	85	51	86
3	4-CN	74	59	59	60	84:16	79:21	75:25	72:28	89	74	51	76
4	4-Cl	57	20	74	47	84:16	82:18	75:25	69:31	86	76	55	75
5	3-Cl	84	29	73	32	72:28	92:8	72:28	65:35	86	88	50	93
6	4-Br	57	32	65	29	84:16	82:18	73:27	81:19	88	88	53	84
7	2,6-Cl_2	89	59	92	39	>99:1	97:3	95:5	>99:1	53	52	36	58

a) AUAP: acidic protease from *Aspergillus usamii*; b) BLAP: alkaline protease from *Bcaillus licheniformis*; c) chymopapain is in the latex of the unripe fruit of *Carica papaya*.

4 Conclusions

In summary, we showed that AMP had the ability to catalyze asymmetric direct aldol reaction in organic media. The influences of reaction conditions including solvent, water content, molar ratio of substrates, enzyme concentration, and temperature were investigated. The AMP can catalyze direct aldol reaction with a wide range of substrates resulting in moderate to high yields, with moderate to excellent diastereoselectivity and enantioselectivity under mild reaction conditions, and not any additional cofactors or special equipment were required. These features are expected to make AMP as an attractive tool in synthesis. This activity of AMP greatly expands the application of this biocatalyst.

Supporting Information Details of aldol products **3a–3t**, and copies of the ^{1}H and ^{13}C NMR, as well as HPLC spectra

for all final products.

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