DOI: 10.1002/adsc.201100555

Chymopapain-Catalyzed Direct Asymmetric Aldol Reaction

Yan-Hong He,[a,b] Hai-Hong Li,[a,b] Yan-Li Chen,[a] Yang Xue,[a] Yi Yuan,[a] and Zhi Guan[a,*]

[a] School of Chemistry and Chemical Engineering, Southwest University, Chongqing 400715, People's Republic of China
Fax: (+86)-23-6825-4091; e-mail: guanzhi@swu.edu.cn
[b] Yan-Hong He and Hai-Hong Li contributed equally to this work

Received: July 14, 2011; Revised: October 27, 2011; Published online: February 23, 2012

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/adsc.201100555.

Abstract: Chymopapain, a cysteine proteinase isolated from the latex of the unripe fruits of *Carica papaya*, displays a promiscuous activity to catalyze the direct asymmetric aldol reactions of aromatic and heteroaromatic aldehydes with cyclic and acyclic ketones in acetonitrile in the presence of a phosphate buffer. The excellent enantioselectivities of up to 96% *ee* and high diastereoselectivities of up to >99:1 (*anti*/*syn*) were achieved. The novel catalytic promiscuity of chymopapain widens the applicability of this biocatalyst in organic synthesis.

Keywords: aldol reaction; asymmetric synthesis; chymopapain; enzyme catalysis; promiscuity

Introduction

Since enzymes were discovered to be active in nearly anhydrous organic media by Klibanov in the early 1980s,[1] particularly as it has been found that the nature of the solvent can influence enzyme activity and selectivity,[2] enzymes as biocatalysts in organic media have attracted significant attention due to their high selectivity, mild reaction conditions and environmental benign quality.[3] However, there is only a limited number of enzymes existing in nature, and the commercial availability of enzymes is also limited.[4] Thus, it is a significant challenge to discover the enzymatic promiscuity of each available enzyme. In recent years, a growing number of enzymes have demonstrated their new activities with unnatural substrates in organic media.[5] Among those promiscuous enzymes, 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.[6] Several novel examples of catalytic promiscuity of hydrolases have been reported, such as Michael additions,[7] Markovnikov additions,[8] direct Mannich reactions,[9] and Henry reactions.[10]

The asymmetric aldol reaction is one of the most important carbon-carbon bond-forming reactions in organic synthesis. In 1997, the first chemocatalytic direct asymmetric aldol reaction of aldehydes with unmodified ketones was realized by the Shibasaki group using heterobimetallic multifunctional catalysts of the type $LnLi_3$tris[(*R*)-binaphthoxide].[11] In recent years, numerous successful organocatalysts and metal catalysts for direct asymmetric aldol reactions have been described with high efficiency and enantioselectivity,[12] yet the development of sustainable and cost-efficient catalysts for the asymmetric aldol reaction still remains a significant challenge.[13] It is well known that a number of aldol reactions occur in the living cells catalyzed by aldolases. There are some elegant reports about aldolase-catalyzed aldol reactions.[14] However, there are only a few reports of aldol reactions catalyzed by enzymes besides aldolases. Berglund and co-workers reported the first example of hydrolase-catalyzed aldol reaction in 2003.[15] They used CAL-B (lipase from *Candida antarctica*) and the Ser105Ala mutant CAL-B to catalyze aldol additions of aliphatic aldehydes (propanal or hexanal). They found that the mutant had better activity than the wild-type lipase, but both of them showed quite low activities (the reaction took almost 2 months) and the enzymatic process was not enantioselective. Wang and Yu et al. reported the first lipase-catalyzed asymmetric aldol reaction in 2008.[16] They used PPL (lipase from porcine pancreas) to catalyze the asymmetric aldol reaction between acetone and 4-nitrobenzaldehyde in the presence of water, and the best enantioselectivity obtained was 43.6% *ee*. Again in 2010, Wang and Yu et al. reported pepsin-catalyzed asymmetric aldol reactions of acetone and 4-nitroben-

zaldehyde in the presence of water, which gave an enantioselectivity of 44% *ee*.[17] Recently, we reported that the nuclease p1 from *Penicillium citrinum* catalyzed the direct asymmetric aldol reaction between aromatic aldehydes and cyclic ketones under solvent-free conditions. The products were obtained in yields of 17–55% with 49–99% *ee*.[18] We also found that BLAP (alkaline protease from *Bacillus licheniformis*) could catalyze the direct asymmetric aldol reactions between aromatic aldehydes and cyclic ketones in DMSO in the presence of water. The products were obtained in yields of 28–92% with 22–99% *ee*.[19]

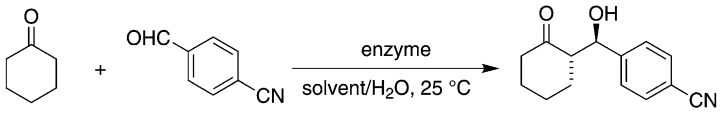
In continuation of our work with enzymatic synthetic promiscuity, we report herein another new biocatalyst for the direct asymmetric aldol reaction. Chymopapain (EC 3.4.22.6) is the most abundant cysteine proteinase, and indeed protein, in the latex of the unripe fruit of *Carica papaya*.[20] It is a medication used to treat slipped (herniated) lower lumbar discs in the spine. We have now found that chymopapain also has the ability to catalyze the direct asymmetric aldol reactions of aromatic or heteroaromatic aldehydes with cyclic ketones or acetone giving moderate to good enantioselectivities and diastereoselectivities. Wide substrate scopes were also investigated. This finding provides a novel example of enzymatic promiscuity.

Results and Discussion

Initially, the aldol reaction between 4-cyanobenzaldehyde and cyclohexanone was used as a model reaction. Since the reaction media have a great effect on the catalytic activity and the stability of an enzyme, in particular, on enzyme enantioselectivity and regioselectivity,[21] the catalytic activity of chymopapain in the aldol reaction was evaluated in different solvents (Table 1). It could be seen that the catalytic activity and the steroselectivity of chymopapain were obviously influenced by different media. Chymopapain exhibited the best catalytic activity and moderate stereoselectivity in DMSO (Table 1, entry 1), and the enzyme showed the best enantioselectivity of 79% *ee* in CH_2Cl_2 with low diastereoselectivity (Table 1, entry 7). In consideration of both diastereo- and enantioselectivities, we chose MeCN as a suitable solvent for the asymmetric direct aldol reaction, which gave the best *dr* of 77:23 and a moderate *ee* of 76% (Table 1, entry 5) among the tested solvents.

Next, we performed some control experiments to verify the specific catalytic effect of chymopapain on the aldol reaction (Table 1, entries 8–13). The reaction of 4-cyanobenzaldehyde with cyclohexanone in the absence of enzyme in MeCN at 25 °C only gave trace amounts of adduct even after 4 days (Table 1, entry 8). Then, the reactions catalyzed by the non-

Table 1. The effect of solvents on the chymopapain-catalyzed direct asymmetric aldol reaction and control experiments.[a]



Entry	Solvent	Time [h]	Yield [%][b]	*dr*[c]	*ee* [%][d]
1	DMSO	89	50	55:45	40
2	TBME	89	17	65:35	70
3	cyclohexane	89	19	57:43	70
4	THF	89	30	65:35	69
5	MeCN	89	21	77:23	76
6	H_2O	89	18	64:36	57
7	CH_2Cl_2	89	18	61:39	79
8	MeCN (no enzyme)	96	trace	–	–
9	MeCN (bovine serum albumin)	130	38	60:40	0
10	MeCN (egg white albumin)	130	34	60:40	0
11	MeCN (chymopapain denatured with urea[e])	96	trace	–	–
12	MeCN (chymopapain inhibited with MMTS[f])	96	trace	–	–
13	MeCN (papain)	89	7	66:34	52

[a] All reactions were carried out using 4-cyanobenzaldehyde (131 mg, 1.0 mmol), cyclohexanone (294 mg, 3 mmol,), enzyme (200 mg), H_2O (0.09 mL) and organic solvent (1.0 mL) at 25 °C.
[b] Yield of the isolated product after chromatography on silica gel.
[c] The *dr* is the *anti*/*syn* ratio, which was determined by HPLC analysis of the diastereomeric isomers.
[d] The *ee* (*anti*) was determined by HPLC analysis using a chiral column; relative and absolute configurations of the products were determined by comparison with the known ^{1}H NMR and chiral HPLC analysis results.
[e] Pre-treated with urea at 100 °C for 24 h.
[f] Pre-treated with MMTS at 25 °C for 24 h.

enzyme proteins bovine serum albumin (BSA) and egg white albumin were also conducted, which gave the products in yields of 38% and 34%, respectively, however, no enantioselectivity was observed in these two cases (Table 1, entries 9 and 10). This indicated that non-enzyme proteins also had the ability to catalyze the aldol reaction, but did not exhibit any enantioselectivity for aldol products. Next, the experiment with urea-denatured chymopapain only gave a trace of product after 4 days (Table 1, entry 11). This suggested that the tertiary structure of chymopapain was essential in the process. Besides, a complete inhibition of the catalytic activity of chymopapain in the aldol reaction was observed by using the cysteine protease inhibitor MMTS (methyl methanethiosulfonate),[22] and only a trace of product was observed on TLC (Table 1, entry 12). These experiments suggested that the direct asymmetric aldol reaction must take place on the catalytic site of chymopapain, and the catalysis did not simply arise from the amino acid residues on the surface of chymopapain. In addition, papain is also a cysteine protease present in the *Carica papaya* extracts, and similarly to chymopapain it is also inhibited by MMTS. In order to exclude the possibility that the chymopapain preparation had also papain activity that catalyzed the reaction, the experiment with papain in MeCN was conducted which gave a low yield of 7% with 66:34 *dr* and 52% *ee* (Table 1, entry 13). The result indicated that papain indeed had activity in aldol reaction, but much lower than chymopapain. Thus, we confirmed that chymopapain catalyzed the direct asymmetric aldol reaction.

To further optimize the chymopapain-catalyzed direct asymmetric aldol reaction, the other main factors which affect the enzymatic reactions such as water content of the reaction medium, temperature and addition of the buffer were investigated, respectively. The reaction of 4-cyanobenzaldehyde with cyclohexanone was still used as a model reaction.

The role of water content in the reaction medium is crucial, as it dramatically influences the activity, stability of the enzymes and, probably, their conformational flexibility.[23] Thus, the control of this parameter was proven to be vital. The water contents from 0–0.50 (water/solvent, v/v) in MeCN were screened for the chymopapain-catalyzed direct asymmetric aldol reaction (Table 2). It could be seen that the water content greatly affected the activity and selectivity of chymopapain for the aldol reaction. Chymopapain exhibited the best enantioselectivity and diastereoselectivity at the water content of 0.12 (water/MeCN, v/v), which gave the product in a yield of 28% with 73:27 *dr* (*anti*/*syn*) and 77% *ee* (for *anti* isomer) (Table 2, entry 5). However, the best enzyme activity was reached at the water content of 0.15, which gave a yield of 32% with lower selectivity (Table 2, entry 6). To obtain the best diastereoselectivity and enantioselectivity, the water content of 0.12 (water/MeCN, v/v) was chosen for the chymopapain-catalyzed aldol reaction.

Table 2. The influence of water content on the chymopapain-catalyzed direct asymmetric aldol reaction.[a]

Entry	Water content [water/MeCN, v/v]	Yield [%][b]	*dr*[c]	*ee* [%][d]
1	0	21	62:38	45
2	0.03	25	69:31	59
3	0.06	26	69:31	65
4	0.09	27	72:28	73
5	0.12	28	73:27	77
6	0.15	32	68:32	71
7	0.20	31	61:39	59
8	0.25	28	69:31	55
9	0.30	24	68:32	53
10	0.40	24	67:33	51
11	0.50	22	62:38	46

[a] All reactions were carried out using 4-cyanobenzaldehyde (131 mg, 1.0 mmol), cyclohexanone (294 mg, 3.0 mmol), chymopapain (200 mg), deionized water (0–0.50, water/MeCN, v/v) and MeCN (1.0 mL) at 25 °C for 98 h.

[b] Yield of the isolated product after chromatography on silica gel.

[c] The *dr* is the *anti*/*syn* ratio, which was determined by HPLC analysis of the diastereomeric isomers.

[d] The *ee* (*anti*) was determined by HPLC analysis using a chiral column.

Temperature is another important factor affecting the enzyme stability, selectivity and reaction rate. Then, to further characterize the activity and selectivity of chymopapain in the aldol reaction, the influence of temperature was investigated. As shown in Table 3, the chymopapain-catalyzed aldol reaction exhibited the best diastereoselectivity and enantioselectivity at 30 °C, which gave a yield of 27% with 76:24 *dr* (*anti*/*syn*) and 78% *ee* (for *anti* isomer) (Table 3, entry 4). However, a higher temperature (40 °C) was required for chymopapain to display its best activity for the aldol reaction. In order to get the best enantioselectivity, we chose 30 °C as the optimal temperature.

Next, we investigated the time course of the chymopapain-catalyzed aldol reaction between 4-cyanobenzaldehyde and cyclohexanone. As shown in Table 4, the enantioselectivity almost kept a constant value of approximately 74% *ee* during the whole phase of the reaction. Similarly the diastereoselectivity also almost kept a constant value of 75:25 *dr* aside from two exceptions. The reaction progressed at a nearly constant rate from 6 h to 28 h (Table 4, entries 1–3). After that the reaction rate decreased evidently. Only 26% yield was obtained after 101 h (Table 4, entry 7), and prolonging the reaction time to 125 h did not increase the yield (Table 4, entry 8). In order to verify whether a thermodynamic equilibrium

Table 3. The influence of temperature on the chymopapain-catalyzed direct asymmetric aldol reaction.[a]

Entry	Temperature [°C]	Yield [%][b]	*dr*[c]	*ee* [%][d]
1	15	6	66:34	67
2	20	11	72:28	71
3	25	25	67:33	70
4	30	27	76:24	78
5	35	35	74:26	72
6	40	38	70:30	66
7	50	25	60:40	45

[a] All reactions were carried out using 4-cyanobenzaldehyde (131 mg, 1.0 mmol), cyclohexanone (294 mg, 3.0 mmol), chymopapain (200 mg), deionized water (0.12 mL) and MeCN (1.0 mL) at temperature (15–50 °C) for 94 h.
[b] Yield of the isolated product after chromatography on silica gel.
[c] The *dr* is the *anti*/*syn* ratio, which was determined by HPLC analysis of the diastereomeric isomers.
[d] The *ee* (*anti*) was determined by HPLC analysis using a chiral column.

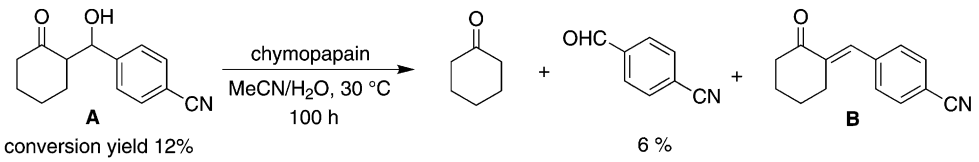
Table 4. The time course of the chymopapain-catalyzed aldol reaction.[a]

Entry	Time [h]	Yield [%][b]	*dr*[c]	*ee* [%][d]
1	6	5	60:40	73
2	17	11	75:25	72
3	28	17	74:26	74
4	41	20	76:24	74
5	53	22	68:32	75
6	77	24	76:24	74
7	101	26	75:25	75
8	125	26	75:25	74

[a] All reactions were carried out using 4-cyanobenzaldehyde (131 mg, 1.0 mmol), cyclohexanone (294 mg, 3.0 mmol), chymopapain (200 mg), deionized water (0.12 mL) and MeCN (1.0 mL) at 30 °C.
[b] Yield of the isolated product after chromatography on silica gel.
[c] The *dr* is the *anti*/*syn* ratio, which was determined by HPLC analysis of the diastereomeric isomers.
[d] The *ee* (*anti*) was determined by HPLC analysis using a chiral column.

was the reason for the limitation to 26% yield, the analogous retro-aldol reaction was conducted under the same reaction conditions starting from the aldol product (**A**) which was prepared by the aldol reaction of 4-cyanobenzaldehyde and cyclohexanone catalyzed by sodium bicarbonate. We found that chymopapain could catalyze the retro-aldol reaction (Scheme 1). However, only 12% of aldol product (**A**) was converted after 100 h, and only 6% yield of 4-cyanobenzaldehyde was obtained while a small amount of eliminated product (**B**) was observed. The *dr* for the aldol product (**A**) was 58:42 (*anti/syn*) before the retro-aldol reaction, but we did not check the *dr* of the remaining **A** after the reaction because some aldol product (**A**) was converted to eliminated product (**B**). The experiment clearly indicated that a thermodynamic equilibrium was not the reason for the limitation to 26% yield because the analogous retro-aldol reaction could not reach the same product/substrate mixture. Then the other potential explanations for the limitation in conversion might be deactivation and destabilization of the enzyme. To confirm these explanations we further conducted the experiment of chymopapain-catalyzed aldol reaction between 4-cyanobenzaldehyde and cyclohexanone. The reaction was first performed under the same reaction conditions as the experiment above (listed in Table 4, entry 7) for 100 h; after that another 100 mg of chymopapain as extra amount of catalyst were added to the reaction mixture, which then was stirred for another 100 h before terminating the reaction. In this case, the aldol product was obtained in a much better yield of 52% with 71:29 *dr* (*anti/syn*) and 75% *ee* (for *anti* isomer). The fact that addition of the catalyst could enhance the yield of the aldol reaction further demonstrated that a thermodynamic equilibrium was not the reason for the limitation in conversion. At the same time, it suggested that deactivation and destabilization of chymopapain may be the explanations.

Next, the pH value of the reaction medium plays a significant role in maintaining the stability and catalytic activity of enzymes. We then used phosphate buffer (pH from 4.60 to 7.84) to replace the optimized water content in the reaction system (buffer/MeCN = 0.12, v/v) to obtain the optimum reaction conditions (Table 5). It could be seen that pH had obvious effects on the chymopapain-catalyzed direct asymmetric



Scheme 1. The chymopapain-catalyzed analogous retro-aldol reaction. *Reaction conditions:* **A** (1.0 mmol, *anti/syn* 58:42,), chymopapain (200 mg), MeCN (1.0 mL) and deionized water (0.12 mL) at 30 °C for 100 h. Yield refers to the isolated product after chromatography on silica gel.

Table 5. The influence of pH conditions (phosphate buffer) on the chymopapain-catalyzed direct asymmetric aldol reaction.[a]

Entry	pH	Yield [%][b]	*dr*[c]	*ee* [%][d]
1	4.60	37	72:28	70
2	4.91	45	72:28	78
3	5.11	42	73:27	77
4	5.35	41	67:33	75
5	5.85	41	72:28	74
6	6.17	40	73:27	74
7	6.45	39	72:28	74
8	6.74	38	68:32	74
9	7.06	38	73:27	74
10	7.36	38	76:24	68
11	7.63	36	73:27	63
12	7.84	35	74:26	60

[a] All reactions were carried out using 4-cyanobenzaldehyde (131 mg, 1.0 mmol), cyclohexanone (294 mg, 3.0 mmol), chymopapain (200 mg), phosphate buffer (pH 4.60–7.84, 0.12 mL) and MeCN (1.0 mL) at 30 °C, for 131 h.

[b] Yield of the isolated product after chromatography on silica gel.

[c] The *dr* is the *anti*/*syn* ratio, which was determined by HPLC analysis of the diastereomeric isomers.

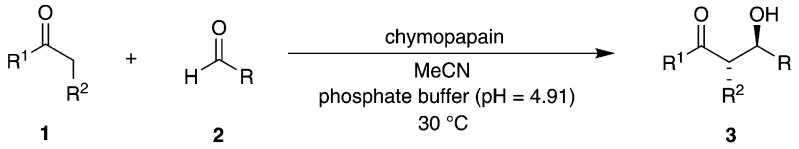
[d] the *ee* (*anti*) was determined by HPLC analysis using a chiral column.

aldol reaction. Chymopapain showed the best activity and enantioselectivity in the presence of phosphate buffer (pH 4.91, buffer/MeCN = 0.12, v/v), which gave the product in yield of 45% with 72:28 *dr* (*anti/syn*) and 78% *ee* (for *anti* isomer) (Table 5, entry 2). Although the addition of phosphate buffer could not enhance the enantioselectivity, it increased the yield obviously. Therefore, we chose the phosphate buffer (pH 4.91, buffer/MeCN = 0.12, v/v) as the optimum conditions for aldol reaction.

Subsequently, we studied the substrate scope and the generality of the chymopapain-catalyzed direct asymmetric aldol reaction. Various aromatic or heteroaromatic aldehydes and ketones were investigated in MeCN in the presence of the phosphate buffer (pH 4.91, buffer/MeCN = 0.12, v/v) at 30 °C (Table 6). 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 the enzyme. Generally, chymopapain exhibited better diastereoselectivity and enantioselectivity with cyclohexanone (Table 6, entries 1–11 and 15) than with cyclopentanone, cycloheptanone and acetone (Table 6, entries 12–14). The best diastereoselectivity of >99:1 *dr* (*anti/syn*) (Table 6, entry 7) and the best enantioselectivity of 96% *ee* (Table 6, entry 1) were achieved. Moreover, the aldehydes with election-withdrawing groups gave higher yields than those with electron-donating groups. Also, the effect of steric hindrance of substituents on benzaldehydes had a great impact on the diastereoselectivity and the yield of the reaction. When reacting with cyclohexanone, the benzaldehydes with a substituent in the *o*-position gave higher *dr* values but lower yields (Table 6, entries 4 and 10) than those with a substituent in the *m*- or *p*-position (Table 6, entries 3, 5, 9 and 11). The most hindered substrate 2,6-dichlorobenzaldehyde gave the best diastereoselectivity of >99:1 *dr* (Table 6, entry 7). The *anti*-isomers were obtained as the major products by using cyclohexanone as an aldol donor (Table 6, entries 1–11 and 15), but there was rarely any diastereoselectivity observed on using cyclopentanone and cycloheptanone as the donors (Table 6, entries 12 and 13). Besides, furfural was also accepted by chymopapain as a substrate, which gave a low yield with moderate diastereo- and enantioselectivity (Table 6, entry 15). However, chymopapain showed a poor activity and selectivity towards acetone (Table 6, entry 14). It seems likely that the chymopapain-catalyzed aldol reaction prefers cyclic ketones over acyclic ketones. It is also worthy to note that chymopapain had a different degree of enantioselectivity for *anti* isomers, but low or no enantioselectivity for *syn* isomers. These results implied that the catalytic site of chymopapain had a specific substrate selectivity as well as stereoselectivity in direct aldol reactions.

Based on the above results and the viewpoint, we attempted to propose a mechanism for the chymopapain-catalyzed aldol reaction. The experiments catalyzed by denatured enzyme indicated that the specific natural fold of chymopapain is responsible for its ability to catalyze the direct asymmetric aldol reaction. Also, the control experiment with MMTS-inhabited chymopapain implied that the aldol reaction must take place on the catalytic site of chymopapain. Furthermore, according to the X-ray structure of chymopapain determined by Maes and co-workers,[25] the polypeptide chain is folded into two domains of roughly the same size but with different conformations. One domain (the L-domain) is mainly α-helical; the other domain (the R-domain) essentially consists of extensive antiparallel β-sheet interactions. The active site is located at the interface between these domains. The active site Cys-25 residue is part of the L1 α-helix at the surface of the L-domain, while the His-159 is in a β-sheet at the surface of the R-domain of the enzyme. The active site residues (Gln-19, Cys-25, His-159 and Asn-179) are involved in the catalytic process. Their study also revealed that the structure of chymopapain is extremely similar to that of papain, and differences in backbone conformation were found only for two loops at the surface of the protein, far away from the active site cleft. Moreover, according to Hillier and co-workers' predictions about the catalytic mechanism of papain,[26] and based on Berglund and co-workers' mechanism of serine hydrolase

Table 6. Substrate scope of the chymopapain-catalyzed direct asymmetric aldol reactions.[a]



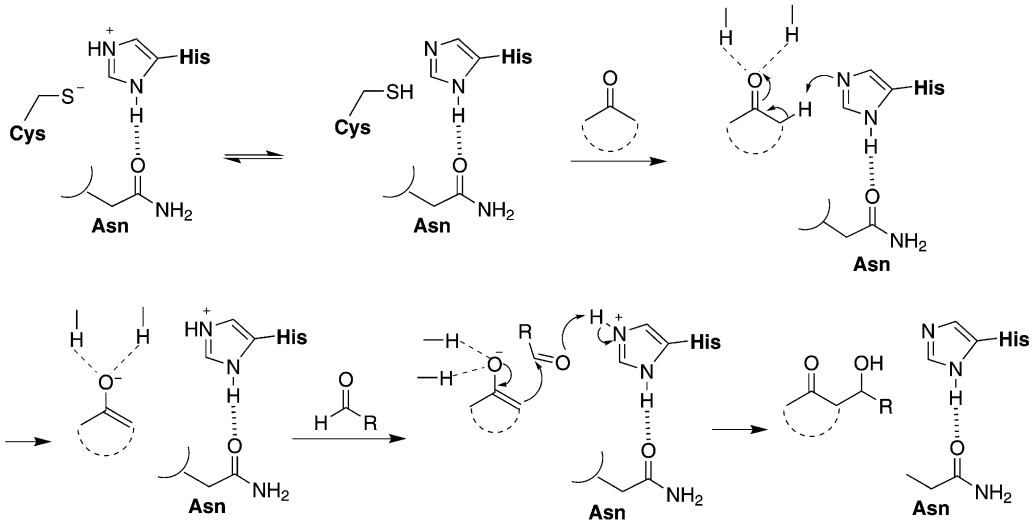
1 2 3

Entry	R	R^1, R^2	No.	Time [h]	Yield [%][b]	*dr* (*anti*/*syn*)[c]	*ee* [%][d] (*anti*)
1	4-MeC_6H_4	$(CH_2)_4$	**3a**	240	23	63:37	96
2	4-BrC_6H_4	$(CH_2)_4$	**3b**	240	29	81:19	84
3	4-ClC_6H_4	$(CH_2)_4$	**3c**	240	47	69:31	75
4	2-ClC_6H_4	$(CH_2)_4$	**3d**	168	30	86:14	79
5	3-ClC_6H_4	$(CH_2)_4$	**3e**	168	32	65:35	93 (51)[e]
6	2,4-$Cl_2C_6H_3$	$(CH_2)_4$	**3f**	240	43	80:20	81
7	2,6-$Cl_2C_6H_3$	$(CH_2)_4$	**3g**	168	39	>99:1	58
8	4-CNC_6H_4	$(CH_2)_4$	**3h**	240	60	72:28	76
9	4-$NO_2C_6H_4$	$(CH_2)_4$	**3i**	240	69	63:37	78 (70)[f]
10	2-$NO_2C_6H_4$	$(CH_2)_4$	**3j**	168	24	87:13	82
11	3-$NO_2C_6H_4$	$(CH_2)_4$	**3k**	168	41	87:13	86
12	4-$NO_2C_6H_4$	$(CH_2)_3$	**3l**	240	73	56:44	52 (34)[g]
13	4-$NO_2C_6H_4$	$(CH_2)_5$	**3m**	240	19	47:53	26
14	4-$NO_2C_6H_4$	CH_3, H	**3n**	120	12	–	14
15	2-furanyl	$(CH_2)_4$	**3o**	168	16	63:37	61 (44)[h]

[a] For the general procedure see Experimental Section.
[b] Yield of the isolated product (*anti*+*syn*) after chromatography on silica gel.
[c] The *dr* was determined by HPLC analysis of the diastereomeric isomers.
[d] The *ee* was determined by HPLC analysis using a chiral column; absolute configurations of the products were determined by comparison with the known ^{1}H NMR and chiral HPLC analysis results[24] (see the Supporting Information).
[e] *anti* (93% *ee*), *syn* (51% *ee*).
[f] *anti* (78% *ee*), *syn* (70% *ee*).
[g] *anti* (52% *ee*), *syn* (34% *ee*).
[h] *anti* (61% *ee*), *syn* (44% *ee*).

CALB-catalyzed aldol reaction,[15] we hypothesized the following mechanism of the chymopapain-catalyzed direct aldol reaction (Scheme 2).

The active site of chymopapain consists of a catalytic triad formed by Cys, His and Asn, and there is an equilibrium between the neutral (thiol-imidazole) and the ion-pair (thiolate-imidazolium) of the Cys-His couple. Firstly, the carbonyl of the substrate ketone is coordinated in the Asn-His dyad and the oxyanion hole in the active site. Secondly, a proton is trans-



Scheme 2. Proposed mechanism for the chymopapain-catalyzed aldol reaction.

ferred from the ketone to the His residue and an enolate ion is formed. Thirdly, another substrate aldehyde accepts the proton from imidazolium cation and combines the ketone forming a new carbon-carbon bond. Eventually, the product is released from the oxyanion hole, and separates from the active site.

Conclusions

In summary, here we have reported a chymopapain-catalyzed direct asymmetric aldol reaction. Several important factors including solvent, water content, temperature and the addition of phosphate buffer were examined to optimize the biocatalytic process. A wide range of ketones including five-, six- and seven-membered cyclic ketones and acetone as aldol donors could be accepted by the enzyme to react with different aromatic or heteroaromatic aldehydes. In most cases, chymopapain showed a moderate to good enantioselectivity and diastereoselectivity. Compared with current chemical technologies, the chymopapain-catalyzed direct asymmetric aldol reaction is more economically feasible and sustainable by using inexpensive regenerable resources. This case of biocatalytic promiscuity not only expands the application of chymopapain to new chemical transformations, but also could be developed into a potentially valuable method for organic synthesis.

Experimental Section

General Procedure for the Chymopapain-Catalyzed Direct Aldol Reactions (Products 3a–o)

A 25-mL round-bottomed flask was charged with chymopapain (20 U/mg, 200 mg), aldehyde (1.0 mmol) and MeCN (1.0 mL), to which the phosphate buffer (pH 4.91) (0.12 mL) and ketone (3.0 mmol) were introduced. 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. 20 mL of water were then added to the filtrate, and the filtrate was extracted three times with 20 mL of ethyl acetate. 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 eluent.

Materials

Chymopapain isolated from the latex of the unripe fruits of *Carica papaya* (20 U/mg, one unit of activity was defined as the amount of enzyme to produce TCA-soluble hydrolyzed products from casein, which gives an absorbance value equivalent to 1.0 μg of tyrosine at 275 nm/min at 30 °C and pH 7.5), and papain from the latex of the unripe fruits of *Carica papaya* (650 U/mg, one unit of activity was defined as the amount of the enzyme to produce TCA-soluble hydrolysis products from casein, which gives an absorbance value equivalent to 1.0 μg of tyrosine at 275 nm min^{-1} at 37 °C and pH 7.0) were purchased from Guangxi Nanning Pangbo Biological Engineering Co. Ltd. (Nanning, China). Unless otherwise noted, all reagents were obtained from commercial suppliers and were used without further purification.

Supporting Information

General methods, the influence of some reaction conditions on the chymopapain-catalyzed direct asymmetric aldol reaction shown in figures, HPLC data, ^{1}H NMR and ^{13}C NMR are available as Supporting Information.

Acknowledgements

Financial support from Natural Science Foundation Project of CQ CSTC (2009A5051) is gratefully acknowledged.

References

[1] a) A. Zaks, A. M. Klibanov, *Proc. Natl. Acad. Sci. USA* **1985**, *82*, 3192–3196; b) A. M. Klibanov, *Chemtech* **1986**, *16*, 354–359.
[2] G. Carrea, G. Ottolina, S. Riva, *Tibtech* **1995**, *13*, 63–70.
[3] a) D. J. Pollard, J. M. Woodley, *Trends Biotechnol.* **2007**, *25*, 66–73; b) J. Aleu, A. J. Bustillo, R. Hernandez-Galan, I. G. Collado, *Curr. Org. Chem.* **2006**, *10*, 2037–2054; c) A. Schmid, J. S. Dordick, B. Hauer, A. Kiener, M. Wubbolts, B. Witholt, *Nature* **2001**, *409*, 258–268; d) J. R. Knowles, *Nature* **1991**, *350*, 121–124.
[4] A. S. Bommarius, B. R. Riebel, in: *Enzyme Biocatalysis: Principles and Applications*, Wiley-VCH Verlag, Weinheim, **2004**, pp 1–2.
[5] a) E. Busto, V. Gotor-Fernández, V. Gotor, *Chem. Soc. Rev.* **2010**, *39*, 4504–4523; b) Q. Wu, B.-K. Liu, X.-F. Lin, *Curr. Org. Chem.* **2010**, *14*, 1966–1988.
[6] U. T. Bornscheuer, R. J. Kazlauskas, *Angew. Chem.* **2004**, *116*, 6156–6165; *Angew. Chem. Int. Ed.* **2004**, *43*, 6032–6040.
[7] a) M. Svedendahl, K. Hult, P. Berglund, *J. Am. Chem. Soc.* **2005**, *127*, 17988–17989; b) O. Torre, I. Alfonso, V. Gotor, *Chem. Commun.* **2004**, 1724–1725; c) J.-F. Cai, Z. Guan, Y.-H. He, *J. Mol. Catal. B: Enzym.* **2011**, *68*, 240–244.
[8] a) W.-B. Wu, N. Wang, J.-M. Xu, Q. Wu, X.-F. Lin, *Chem. Commun.* **2005**, 2348–2350; b) W.-B. Wu, J.-M. Xu, Q. Wu, D.-S. Lv, X.-F. Lin, *Adv. Synth. Catal.* **2006**, *348*, 487–492.
[9] K. Li, T. He, C. Li, X.-W. Feng, N. Wang, X.-Q. Yu, *Green Chem.* **2009**, *11*, 777–779.
[10] J.-L. Wang, X. Li, H.-Y. Xie, B.-K. Liu, X.-F. Lin, *J. Biotechnol.* **2010**, *145*, 240–243.
[11] Y. M. A. Yamada, N. Yoshikawa, H. Sasai, M. Shibasaki, *Angew. Chem.* **1997**, *109*, 1942–1944; *Angew. Chem. Int. Ed. Engl.* **1997**, *36*, 1871–1873.

[12] a) S. Mukherjee, J.-W. Yang, S. Hoffmann, B. List, *Chem. Rev.* **2007**, *107*, 5471–5569; b) A. Dondoni, A. Massi, *Angew. Chem.* **2008**, *120*, 4716–4739; *Angew. Chem. Int. Ed.* **2008**, *47*, 4638–4660.
[13] R. Mestres, *Green Chem.* **2004**, *6*, 583–603.
[14] a) O. Eyrisch, W.-D. Fessner, *Angew. Chem.* **1995**, *107*, 1738–1740; *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 1639–1641; b) T. Kimura, V. P. Vassilev, G.-J. Shen, C.-H. Wong, *J. Am. Chem. Soc.* **1997**, *119*, 11734–11742; c) T. D. Machajewski, C. H. Wong, *Angew. Chem. Int. Ed.* **2000**, *39*, 1353–1374; d) A. Heine, G. DeSantis, J. G. Luz, M. Mitchell, C.-H. Wong, I. A. Wilson, *Science* **2001**, *294*, 369–374; e) W. D. Fessner, V. Helaine, *Curr. Opin. Biotechnol.* **2001**, *12*, 574–586, and references cited therein; f) W. A. Greenberg, A. Varvak, S. R. Hanson, K. Wong, H. J. Huang, P. Chen, M. J. Burk, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 5788–5793.
[15] C. Branneby, P. Carlqvist, A. Magnusson, K. Hult, T. Brinck, P. Berglund, *J. Am. Chem. Soc.* **2003**, *125*, 874–875.
[16] C. Li, X.-W. Feng, N. Wang, Y.-J. Zhou, X.-Q. Yu, *Green Chem.* **2008**, *10*, 616–618.
[17] C. Li, Y.-J. Zhou, N. Wang, X.-W. Feng, K. Li, X.-Q. Yu, *J. Biotechnol.* **2010**, *150*, 539–545.
[18] H.-H. Li, Y.-H. He, Y. Yuan, Z. Guan, *Green Chem.* **2011**, *13*, 185–189.
[19] H.-H. Li Y.-H. He, Z. Guan, *Catal. Commun.* **2011**, *12*, 580–582.
[20] S. Zucker, D. J. Buttle, M. J. H. Nicklin, A. J. Barrett, *Biochim. Biophys. Acta* **1985**, *828*, 196–204.
[21] C. R. Wescott, A. M. Klibanov, *Biochim. Biophys. Acta* **1994**, *1206*, 1–9.
[22] M. St-Vincent, M. Dickman, *J. Chem. Educ.* **2004**, *81*, 1048–1050.
[23] a) A. M. Klibanov, *Trends Biochem. Sci.* **1989**, *14*, 141–144; b) J. S. Dordick, *Enzyme Microb. Technol.* **1989**, *11*, 194–211; c) P. J. Halling, *Enzyme Microb. Technol.* **1994**, *16*, 178–206.
[24] a) J. R Chen, H. H. Lu, X. Y. Li, L. Cheng, J. Wan, W. J. Xiao, *Org. Lett.* **2005**, *7*, 4543–4545; b) Y. Y. Wu, Y. Z Zhang, M. L. Yu, G. Zhao, S. W. Wang, *Org. Lett.* **2006**, *8*, 4417–4420; c) N. Mase, Y. Nakai, N. Ohara, H. Yoda, K. Takabe, F. Tanaka, C. F. Barbas, *J. Am. Chem. Soc.* **2006**, *128*, 734–735; d) M. Gruttadauria, F. Giacalone, A. M. Marculescu, P. L. Meo, S. Riela, R. Noto, *Eur. J. Org. Chem.* **2007**, 4688–4698; e) H. Yang, R. G. Carter, *Org. Lett.* **2008**, *10*, 4649–4652.
[25] D. Maes, J. Bouckaert, F. Poortmans, L. Wyns, Y. Looze, *Biochemistry* **1996**, *35*, 16292–16298.
[26] M. J. Harrison, N. A. Burton, I. H. Hillier, *J. Am. Chem. Soc.* **1997**, *119*, 12285–12291.

