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Trypsin-catalyzed direct asymmetric aldol reaction

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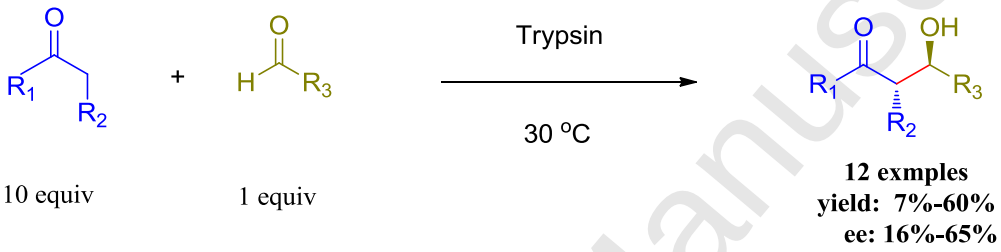

10 equiv 1 equiv

12 exmples
yield: 7%-60%
ee: 16%-65%

Trypsin-catalyzed direct asymmetric aldol reaction

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Abstract: Unnatural catalytic activity of trypsin from porcine pancreas for direct asymmetric aldol reaction was discovered. The reactions between aromatic aldehydes and various ketones gave products in moderate yields and enantioselectivities in the presence of a small amount of water. The influences of solvent, water content, temperature, mole ratio of substrates, and enzyme concentration were investigated. The mechanism of trypsin-catalyzed aldol reaction was discussed. This enzymatic promiscuity widens the application of trypsin to new chemical transformations.

Keywords: enzymatic promiscuity; trypsin; aldol reaction; asymmetric synthesis; biocatalysis

1. Introduction

As one of the most powerful C-C bond forming reactions for the production of chiral 1,3-dioxygenated compounds, the asymmetric direct aldol reaction is one of the central study issues in the field of asymmetric synthesis [1]. Since the first proline-catalyzed aldol reactions obtained the breakthrough, there has been a blossoming of general asymmetric organocatalyzed reactions. Though (S)-proline is readily available at low price, this merit is not a comment to all organocatalysts, let alone ecological sustainability of organocatalysts [3,4]. On the other hand,

enzymes as potential environmentally-friendly alternatives to chemical catalysts in organic synthesis have attracted more and more attention recently. Since enzymes were discovered to be active in nearly anhydrous organic media by Klibanov in the early 1980s [5], chemists and biochemists have focused on the notion of enzymatic promiscuity, which means that one single active site of a given enzyme can catalyze different chemical transformations of natural or non-natural substrates [6,7]. Enzyme catalytic promiscuity largely expands the application of biocatalysts and provides a useful approach for organic synthesis. Some elegant works on enzymatic promiscuity have been reported [8-10], and several aldol reactions catalyzed by promiscuous enzymes were available. Berglund and co-workers used mutant CAL-B (lipase from *Candida antarctica*) to catalyze aldol additions in cyclohexane in 2003 [11]. Wang and Yu reported the asymmetric aldol reactions catalyzed by lipase in "wet" acetone in 2008 [12], and they also found pepsin-catalyzed aldol reactions in aqueous media in 2010 [13]. More recently, our group found that nuclease p1 from *Penicillium citrinum*, alkaline protease from *Bacillus licheniformis*, chymopapain from *Carica papaya*, acidic protease from *Aspergillus usamii* and lipase from porcine pancreas could catalyze direct asymmetric aldol reactions with good to excellent selectivities [14-18]. However, since enzymatic promiscuity is still in its exploratory stage, no general methods are available to profile enzyme catalytic promiscuity [19]. Accordingly, it is still a formidable and significant task to discover enzyme-catalyzed asymmetric aldol and other reactions as many as possible. Herein, we wish to report the trypsin (from porcine pancreas)-catalyzed asymmetric direct aldol reaction in the presence of a small amount of water.

2. Experimental section

2.1 Materials

The trypsin from porcine pancreas was purchased from Sigma-Aldrich, Shanghai, China (catalogue number: 93615, ~1500 U/mg). 1 U corresponds to the amount of enzyme which increases the absorbance at 253 nm by 0.001 per minute at pH 7.6 and 25°C (N-benzoyl-L-arginine ethyl ester, Fluka No. 12880, as substrate). Unless otherwise noted, all reagents were purchased from commercial suppliers and used without further purification.

2.2 Analytical methods

Reactions were monitored by thin-layer chromatography (TLC) with Haiyang GF254 silica gel plates (Qingdao Haiyang chemical industry Co Ltd, Qingdao, China) using UV light and vanillic aldehyde as visualizing agents. Flash column chromatography was performed using 100-200 mesh silica gel at increased pressure. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker-AM 300 (300 MHz) (Bruker BioSpin AG Ltd., Beijing, China). Chemical shifts were reported in ppm from TMS with the solvent resonance as the internal standard. Data were reported as follows: chemical shifts (δ) in ppm, coupling constants (J) in Hz, and solvent ($CDCl_3$). The following abbreviations were used to indicate the multiplicity: s, singlet; d, doublet; m, multiplet; br, broad signal. The enantiomeric excess (ee) of aldol products was determined by chiral HPLC analysis performed using Chiralcel OD-H, Chiralpak AD-H and AS-H columns (Daicel Chiral Technologies CO., LTD., Shanghai, China). Relative and absolute configurations of the products were determined by comparison with the known ^{1}H NMR, ^{13}C NMR and chiral HPLC analysis. All the aldol reaction products are known compounds (for details about references, please see the Supporting Information).

2.3 General procedure for the trypsin-catalyzed aldol reactions:

A round-bottom flask was charged with trypsin (100 mg), aldehyde (0.5 mmol), and ketone (5 mmol), to which deionized water (0.15 mL) was introduced. The resultant mixture was stirred at 30 °C for the specified reaction time and monitored by TLC. The reaction was terminated by filtering the enzyme (with 40 mm buchner funnel and qualitative filter paper), and ethyl acetate (20 mL) was employed 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 residue was purified by silica gel flash column chromatography with petroleum ether/ethyl acetate as eluent to afford the product**.**

3. Results and Discussion

Initially, the aldol reaction of 4-nitrobenzaldehyde and cyclohexanone was chosen as the model reaction. Since medium is one of the most important factors influencing the enzymatic reactions, in particular, on enantioselectivity and regioselectivity of enzymes [20], we explored the effects of various solvents on the trypsin-catalyzed model aldol reaction. As shown in **Table 1**, the reaction medium played an important role in this enzymatic reaction. The best enantioselectivity of 76 % ee was obtained with a low yield of 15 % in CH_2Cl_2 (**Table 1**, entry 1). The best yield of 51 % was received in DMF with 50 % ee (**Table 1**, entry 13). The reaction gave the product in a yield of 41 % with 61 % ee under organic solvent-free conditions in the presence of a small amount of water (0.10 mL) (**Table 1**, entry 3). However, when 1.0 mL of water was used as a solvent, only 15 % yield was obtained with 61 % ee (**Table 1**, entry 4). Thus, considering both enantioselectivity and yield of the reaction, organic solvent-free conditions (with a small amount

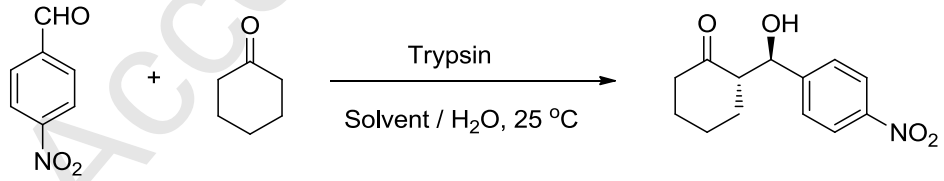
of water (0.10 mL)) was selected for the further study.

To verify the specific catalytic effect of trypsin on the model aldol reaction, some control experiments were performed (**Table 1**, entries 15-21). In the absence of enzyme, the reaction only gave a trace amount of aldol product (**Table 1**, entry 15). When non-enzyme protein bovine serum albumin was used as a catalyst instead of trypsin, the aldol product was obtained in a yield of 68 % with 0 % ee (**Table 1,** entry 16), indicating that a non-enzyme protein also could catalyze aldol reaction, but it did not have any enantioselectivity for the aldol product. Moreover, urea-denatured trypsin was used to catalyse the model reaction, which only gave the product in 15 % yield with 0 % ee (**Table 1**, entry 17). To exclude the effect of extra urea on the reaction, the urea-pretreated trypsin was dialysed against water to remove the extra urea, and used to catalyze the reaction, which gave product in 10% yield with 24% ee (**Table 1**, entry 18). Meanwhile, urea alone was used to catalyze the reaction; it only produced the product in 4% yield, proving that urea nearly did not catalyse this transformation (**Table 1**, entry 19). These results demonstrated that the urea-denatured trypsin lost its most activity and selectivity towards aldol reaction. Thus, it can be inferred that the tertiary structure of trypsin is necessary for this reaction. Furthermore, when the reaction was performed with the trypsin inhibited by PMSF (phenylmethanesulfonyl fluoride, an irreversible serine protease inhibitor), only a trace amount of product was detected (**Table 1**, entry 20), which demonstrated that the reaction took place at the active site of trypsin.

Since the trypsin we used is a commercial enzyme preparation containing impurities, to further rule out the possibility of the catalysis of some impure proteins or other impurities, we

purified the enzyme (for the details of enzyme purification, please see the Supporting Information). From SDS-PAGE of the enzyme preparation (**Figure 1**), it can be seen that trypsin appeared as a clear band, but some protein impurities were also observed. After purification, only one band was obtained, which had the same molecular mass as trypsin (**Figure 1**). The protein solution was desalted and then lyophilized to give purified trypsin as white solid. The purified trypsin was used to catalyze the model aldol reaction under organic solvent-free conditions in the presence of a small amount of water (0.10 mL). The experiment with only 35 mg of purified trypsin gave aldol adduct in 41 % yield with 50 % ee (**Table 1**, entry 21), which was almost 3 times more active than commercial enzyme (as described above, 100 mg of commercial enzyme preparation of trypsin gave the product in a yield of 41 % with 61% ee (**Table 1,** entry 3)). The experiment with purified trypsin clearly confirmed that trypsin indeed has the promiscuity for the catalysis of asymmetric direct aldol reaction. Therefore, we continued using the commercially available enzyme preparation of trypsin in the following investigation.

Table 1 Solvent screening for the trypsin-catalyzed aldol reaction and control experiments [a]



Entry	Solvent	Yield (%) [b]	dr (*anti*:*syn*) [c]	ee % (*anti*) [c]
1	CH_2Cl_2	15	67:33	76
2	*i*-PrOH	14	70:30	69
3	organic solvent-free [d]	41	64:36	61
4	H_2O	15	71:29	61
5	MeCN	13	68:32	59
6	cyclohexane	26	70:30	57
7	$CHCl_3$	16	57:43	51
8	EtOH	40	72:28	55

9	dioxane	14	56:44	54
10	THF	25	64:36	53
11	*n*-hexane	14	57:43	51
12	MTBE	18	65:35	51
13	DMF	51	66:34	50
14	DMSO	40	62:38	40
15	organic solvent-free (no enzyme) [e]	trace	--	--
16	organic solvent-free (bovine serum albumin) [f]	68	53:47	0
17	organic solvent-free (urea-denatured trypsin) [g]	15	60:40	0
18	organic solvent-free (urea-denatured trypsin with extra urea removed) [h]	10	51:49	24
19	organic solvent-free (urea) [i]	4	44:56	0
20	organic solvent-free (PMSF-inhibited trypsin) [j]	trace	--	--
21	organic solvent-free (purified trypsin) [k]	41	66:34	50

[a] Reaction conditions (except the reactions under organic solvent-free conditions): 4-nitrobenzaldehyde (0.5 mmol), cyclohexanone (2.5 mmol), trypsin (100 mg), deionized water (0.10 mL) and solvent (0.90 mL) at 25 ˚C for 143 h.

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

[c] Determined by chiral HPLC analysis using a Chiralpak AD-H column [21].

[d] Reaction conditions: 4-nitrobenzaldehyde (0.5 mmol), cyclohexanone (5 mmol), deionized water (0.10 mL) and trypsin (100 mg) at 25 ˚C for 143 h.

[e] Reaction conditions: 4-nitrobenzaldehyde (0.5 mmol), cyclohexanone (5 mmol), deionized water (0.10 mL) at 25 ˚C for 143 h.

[f] Bovine serum albumin (100 mg) was used instead of trypsin under organic solvent-free conditions.

[g] The trypsin was pre-treated with urea (100 mg) in water (3 ml) at 100 ˚C for 24 h, and the water was removed under reduced pressure before use.

[h] The trypsin was pre-treated with urea (100 mg) in water (3 ml) at 100 ˚C for 24 h; extra urea was removed by dialysis against water, and then water was removed under reduced pressure before use.

[i] Urea (100 mg) was used instead of trypsin under organic solvent-free conditions.

[j] The trypsin was pre-treated with PMSF (100 mg) in anhydrous MeCN (3 ml) at 25 ˚C for 24 h, and MeCN was

removed under reduced pressure before use.

[k] Reaction conditions: purified trypsin (35 mg), 4-nitrobenzaldehyde (0.5 mmol), cyclohexanone (5 mmol), deionized water (0.10 mL) at 25 ˚C for 144 h.

Enzymes require a specific amount of water bound to them to maintain activities, and water content affects both the stereoselectivity and activity of enzymes [22,23]. Therefore, to confirm the optimal water content in the reaction system, the trypsin-catalyzed model aldol reaction was carried out with different amounts of water under organic solvent-free conditions. As shown in **Table 2**, in the absence of water, the reaction gave the product in a relatively low yield of 40% with 54% ee and 64:36 dr (**Table 2,** entry 1). When the ratios of enzyme to water were 1:1.5 and 1:2.0 (enzyme/water, w/w), the reaction afforded the product in the best yield of 51 % with slightly better selectivity (**Table 2,** entries 4 and 5). Further increase of water caused a decrease of yield. Thus, we chose the ratio of enzyme to water 1:1.5 as the optimal condition for the following studies.

Table 2 Effect of water contents on the trypsin-catalyzed aldol reaction [a]

Entry	enzyme : water (w/w)	Yield (%) [b]	dr (*anti*:*syn*) [c]	ee % (*anti*) [c]
1	1:0	40	64:36	54
2	1:0.5	43	67:33	57
3	1:1.0	46	71:29	59
4	1:1.5	51	73:27	58
5	1:2.0	51	73:27	56
6	1:2.5	50	74:26	58
7	1:3.0	46	74:26	58

[a] Reaction conditions: 4-nitrobenzaldehyde (0.5 mmol), cyclohexanone (5 mmol), trypsin (100 mg) and deionized water (0-300 mg, water/enzyme (w/w) = 0-3.0) at 25 ˚C for 144 h.

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

[c] Determined by chiral HPLC analysis using a Chiralpak AD-H column [21].

Temperature has effects on enzymatic reaction rates, and the selectivity and stability of enzymes, so the influence of temperature on the trypsin-catalyzed model aldol reaction was investigated (**Table 3**). The increase of temperature from 10 °C to 40 °C led to a rise in the yield (from 18 % to 69 %) but a decrease in the diastereoselectivity (from 89:11 to 59:41) and enantioselectivity (from 84% ee to 42% ee) (**Table 3**, entries 1-6). As a compromise between yield and selectivity, we chose 30 °C for further studies.

Table 3 Effect of temperature on the trypsin-catalyzed aldol reaction [a]

Entry	Temp. (˚C)	Time (h)	Yield (%) [b]	dr (*anti*:*syn*) [c]	ee % (*anti*) [c]
1	10	168	18	89:11	84
2	20	144	41	78:22	62
3	25	144	49	74:26	60
4	30	144	60	73:27	59
5	35	144	63	67:33	54
6	40	144	69	59:41	42

[a] Reaction conditions: 4-nitrobenzaldehyde (0.5 mmol), cyclohexanone (5 mmol), trypsin (100 mg), and deionized water (0.15 mL) at temperature (10-40 ˚C)

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

[c] Determined by chiral HPLC analysis using a Chiralpak AD-H column [21].

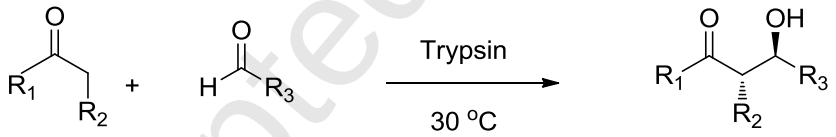
Molar ratio of substrates and enzyme loading are also important factors in enzymatic reactions, and their influence on the trypsin-catalyzed model aldol reaction was investigated next. When the molar ratio of 4-nitrobenzaldehyde to cyclohexanone was 1:10, the best yield of 46 % was obtained.

Higher ratio led to a slight decrease of the yield. Molar ratio of substrates nearly had no effect on the selectivity of the reaction. Thus, a molar ratio of 1:10 (4-nitrobenzaldehyde to cyclohexanone) was selected as the optimal condition. The influence of catalyst loading on the aldol reaction between 4-nitrobenzaldehyde (0.5 mmol) and cyclohexanone (5 mmol) showed that an increase of trypsin loading (from 25 mg to 150 mg) led to a rise in the yield (from 23% to 55%) with a slight increase of selectivity (from 68:32 to 73:27 dr, and from 50% to 60% ee). When the dosage surpassed 100 mg, no increase of the ee and dr values was observed. So we chose enzyme loading of 100 mg as the optimal condition. Finally, the time course of the trypsin-catalyzed model aldol reaction was also investigated. The yield increased as the reaction time prolonged and reached the highest (59%) at 144 h, while the ee value reached the peak (61% ee) at 120 h and then decreased (for details of molar ratio, enzyme loading and time course, please see the Supporting Information).

To investigate the generality and scope of this biocatalytic promiscuity, several other substrates were tested to expand upon trypsin-catalyzed asymmetric direct aldol reaction (**Table 4**, entries 1-12). Generally, trypsin showed the ability to tolerate five-, six- cyclic ketones and acetone as aldol donors. The effect of substituents on benzaldehyde had a great impact on both the yield and selectivity of the trypsin-catalyzed aldol reactions. For instance, reactions of the aromatic aldehydes with electron-withdrawing substituents provided products in higher yields (**Table 4**, entries 1-9) than those with electron-donating substituents (**Table 4**, entry 10). 4-Nitrobenzaldehyde (**Table 4**, entry 1) gave a better yield, and better dr and ee values than 4-halogen aromatic aldhydes (**Table 4**, entries 4-6) when reacting with cyclohexanone. Moreover, the reaction of 4-(trifluoromethyl)benzaldehyde with cyclohexanone gave the best ee value of

65 % with a low yield of 34 % (**Table 4**, entry 7). However, the best diastereoselectivity of 89:11 was achieved by using the most sterically hindered substrate 2,6-dichlorobenzaldehyde (**Table 4**, entry 9). Besides, when reacting with 4-nitrobenzaldehyde, the moderate yield and ee value were received by using cyclopentanone (**Table 4**, entry 11), but only low yield and poor ee value were gained by using acetone as an aldol donor (**Table 4**, entry 12). Surprisingly, when reacting with cyclohexanone, 4-tolualdehyde gave diastereoselectivity for syn-isomer (**Table 4**, entry 10). Otherwise, trypsin displayed different degrees of diastereoselectivities for *anti*-isomers. Most of the reactions we investigated showed enantioselectivities for *anti*-isomers, but low or no enantioselectivities for *syn*-isomers, potentially indicating that the catalytic site of trypsin had a specific selectivity for the aldol reaction.

Table 4 Substrate scope of the trypsin-catalyzed direct asymmetric aldol reactions [a]



1 **2** **3**

Entry	R_1 R_2	R_3	Prod.	Time (h)	Yield (%) [b]	dr (*anti*:*syn*) [c]	ee % (*anti*) [c]
1	-$(CH_2)_4$-	4-$NO_2C_6H_4$	**3a**	144	60	73:27	60
2	-$(CH_2)_4$-	3-$NO_2C_6H_4$	**3b**	192	58	70:30	51
3	-$(CH_2)_4$-	4-CNC_6H_4	**3c**	162	47	55:45	54
4	-$(CH_2)_4$-	4-FC_6H_4	**3d**	192	14	52:48	33
5	-$(CH_2)_4$-	4-ClC_6H_4	**3e**	192	19	50:50	48
6	-$(CH_2)_4$-	4-BrC_6H_4	**3f**	192	19	58:42	55
7	-$(CH_2)_4$-	4-$CF_3C_6H_4$	**3g**	144	34	59:41	65
8	-$(CH_2)_4$-	2,4-$Cl_2C_6H_3$	**3h**	192	37	60:40	40
9	-$(CH_2)_4$-	2,6-$Cl_2C_6H_3$	**3i**	192	58	89:11	33
10	-$(CH_2)_4$-	4-MeC_6H_4	**3j**	187	7	11:89	45 (7) [d]
11	-$(CH_2)_3$-	4-$NO_2C_6H_4$	**3k**	71	47	62:38	42
12	Me, H	4-$NO_2C_6H_4$	**3l**	46	28	---	16

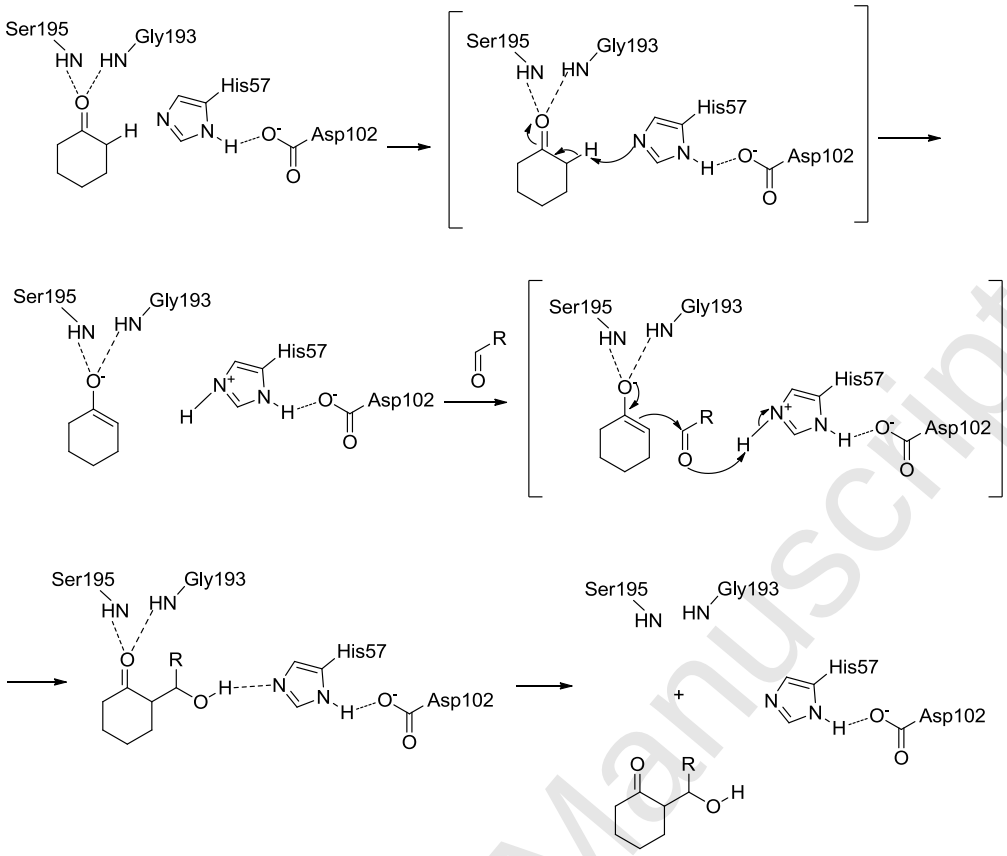
[a] Reaction conditions: aldehyde (0.5 mmol), ketone (5 mmol), trypsin (100 mg) and deionized water (0.15 mL) at 30 °C.

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

[c] Determined by chiral HPLC analysis (for details, please see the Supporting Information).

[d] *anti* (45% ee), *syn* (7% ee)

Finally, we attempted to propose the mechanism of the trypsin-catalyzed aldol reaction. As one of the important serine proteases, the active center of trypsin is the catalytic triad composed of histidine-57, aspartate-102, and serine-195 [24]. In addition, trypsin contains an "oxyanion hole" formed by the backbone amide hydrogen atoms of Gly-193 and Ser-195, in the native activity of trypsin, which serves to stabilize the developing negative charge on the carbonyl oxygen atom of the cleaved amides [25,26]. Based on the mechanism proposed by Berglund [11], we hypothesized a tentative mechanism of trypsin-catalyzed aldol reaction of aldehyde with cyclohexanone shown in **Scheme 1**. Firstly, the Asp-His dyad and the oxyanion hole in the active site stabilizes the substrate cyclohexanone. Secondly, a proton is transferred from the cyclohexanone to the His residue and enolate ion is formed. Thirdly, another substrate aldehyde accepts the proton from imidazolium cation and combines the cyclohexanone forming a new carbon-carbon bond. Eventually, the product is released from the oxyanion hole.



Scheme 1 Proposed mechanism for the trypsin-catalyzed aldol reaction of aldehyde with cyclohexanone

4. Conclusion

In summary, we reported the trypsin-catalyzed asymmetric direct aldol reactions under organic solvent-free conditions in the presence of a small amount of water. This was a safe, economical, and environmentally benign method to synthesize aldol products with a wide range of substrates. It is interesting that trypsin from porcine pancreas possesses the function of aldolase under the present conditions. This methodology also expanded the application of trypsin in the asymmetric reaction.

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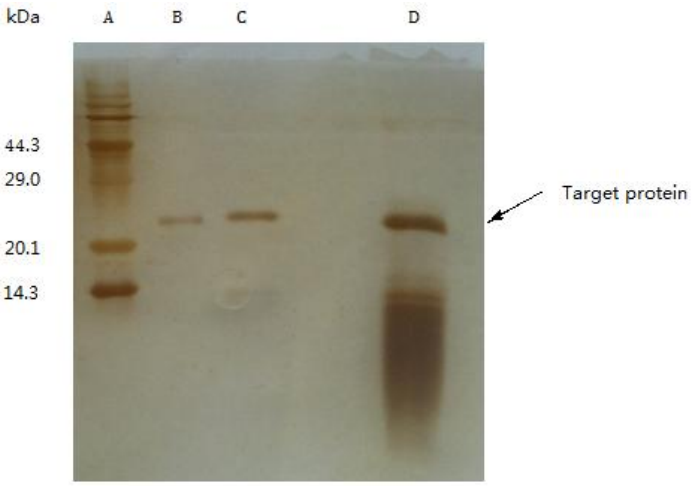

Fig. 1 SDS-PAGE analysis of trypsin

A: Premixed Protein Marker (Low)

B,C: Purified protein (23.8 kDa)

D: Trypsin preparation

Trypsin-catalyzed direct asymmetric aldol reaction

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Highlights

► Unnatural catalytic activity of trypsin from porcine pancreas for direct asymmetric aldol reaction was discovered. ► Mechanism of trypsin-catalyzed aldol reaction was hypothesized.► This enzymatic promiscuity widens the application of trypsin to new chemical transformations.