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Research paper

Enzyme catalytic promiscuity: The papain-catalyzed Knoevenagel reaction

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ABSTRACT

Papain as a sustainable and inexpensive biocatalyst was used for the first time to catalyze the Knoevenagel reactions in DMSO/water. A wide range of aromatic, hetero-aromatic and α,β-unsaturated aldehydes could react with less active methylene compounds acetylacetone and ethyl acetoacetate. The products were obtained in moderate to excellent yields with Z/E selectivities of up to 100:0. This case of biocatalytic promiscuity not only widens the application of papain to new chemical transformations, but also could be developed into a potentially valuable method for organic synthesis.

1. Introduction

Knoevenagel reaction, as a facile and versatile method for the formation of carbon–carbon bond [1], has been commonly applied in the synthesis of chemicals and chemical intermediates such as carbocyclic as well as heterocyclic compounds of biological significance [2], coumarin derivatives, cosmetics, perfumes and pharmaceuticals [3]. Great efforts have been devoted to explore the effective catalysts, and elegant works have been described with high efficiency. Generally, these catalysts include bases [3], zeolites [4], ionic liquids [5], amino acids [6,7] and some metal based Lewis acids [8–10]. Moreover, the Knoevenagel reactions involving 1,3-diketones have often been subjected to low yields or drastic reaction conditions, due to less activity of 1,3-diketones which tend to form stable six-membered cyclic enols [11,12]. Therefore, the development of environmentally benign and cost-efficient catalysts for the Knoevenagel reactions of 1,3-diketones still maintains a significant challenge.

Over last three decades, enzymes as practical catalysts have been increasingly exploited for organic synthesis for their simple processing requirements, high selectivity and mild reaction conditions. Enzyme promiscuity means, in the broadest terms, one single active site of a given enzyme can catalyze different chemical transformation of natural or non-natural substrates [13–16]. Although the promiscuous behaviors were originally thought to be rare events, a growing number of enzymes have been used to catalyze the formation of carbon–carbon and carbon–heteroatom bonds through some classic and widely used organic reactions [17–19]. Recently a lipase-catalyzed decarboxylative Knoevenagel reaction has been reported, which was the only example of enzymatic Knoevenagel reaction [20]. CAL-B (acrylic resin immobilized *Candida antarctica* lipase B) was used to catalyze decarboxylative Knoevenagel reaction of substituted aromatic aldehydes and β-ketoesters in CH_3CN/H_2O, and a primary amine was used as an additive to form a Schiff base in the course of the reaction. But very recently, the mechanism of lipase-catalyzed Knoevenagel reaction has been challenged [21]. Herein, we wish to report a novel discovery that papain could promote the direct Knoevenagel reaction without using additives, and aromatic, hetero-aromatic and α,β-unsaturated aldehydes could react with less active methylene compounds acetylacetone and ethyl acetoacetate resulting in moderate to good yields. Papain (EC 3.4.22.2) is a powerful proteolytic enzyme belonging to the cysteine protease family. Its enzymatic and physiological properties have been extensively studied. Papain is mainly produced from the extraction of *Carica papaya* latex. Nowadays, some protocols for the cloning and overexpression of papain using baculovirus/insect [22], yeast [23,24] and bacteria [25–27] as expression host organisms have been reported. It is possible to obtain recombinant papain in substantial quantities for both basic research and industrial use. In this paper, papain was used for the first time to catalyze Knoevenagel reaction. It provides a novel case of enzyme catalytic promiscuity and might be a potential synthetic method for organic chemistry.

2. Material and methods

2.1. General information for the reagents

Papain (Sigma–Aldrich) from *C. papaya* (catalog number: 76220, ≥3 U/mg. 1 U corresponds to the amount of enzyme which

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hydrolyzes 1 μmol N-benzoyl-L-arginine ethyl ester (BAEE, Fluka No. 12880) per minute at pH 6.2 and 25 °C) was purchased from Sigma–Aldrich. Papain (Pangbo) from the latex of *C. 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 μg of tyrosine at 275 nm per minute at 37 °C and pH 7.0), and bromelain from pineapple peduncle (500 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 μg of tyrosine at 275 nm per minute at 37 °C and pH 7.0) were purchased from Guangxi Nanning Pangbo Biological Engineering Co. Ltd. (Nanning, China). Alkaline proteinase from *Bacillus licheniformis* No 2709 (200 U/mg. One unit of activity is the amount of enzyme that liberates 1 μg of tyrosine from casein per minute at 40 °C and pH 10.5), acidic proteinase from *Aspergillus usamii* No 537 (50 U/mg. One unit of activity is the amount of enzyme that liberates 1 μg of tyrosine from casein per minute at 40 °C and pH 3.0), and neutral proteinase from *Bacillus subtilis* A.S.1.398 (130 U/mg. One unit of activity is the amount of enzyme that liberates 1 μg of tyrosine from casein per minute at 30 °C and pH 7.5) were purchased from Wuxi Xuemei Enzyme Co. Ltd. (WuXi, China). Unless otherwise noted, all reagents were obtained from commercial suppliers and were used without further purification. All reactions were monitored by thin layer chromatography (TLC) with Haiyang GF254 silica gel plates. Flash column chromatography was carried out using 100–200 mesh silica gel at increased pressure.

2.2. Typical procedure for the papain-catalyzed Knoevenagel condensation

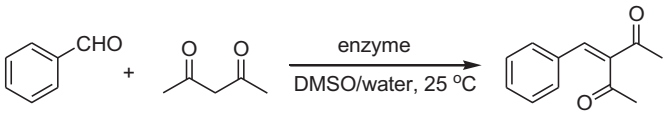
Papain (150 mg) was added to a 10 ml round-bottom flask containing benzaldehyde (212 mg, 2 mmol), acetylacetone (240 mg, 2.4 mmol), DMSO (3.75 ml) and deionized water (1.25 ml). The resulting mixture was stirred at 60 °C for specified time, and monitored by thin layer chromatography (TLC). The reaction was terminated by filtering the enzyme. The filtrate was diluted with ethyl acetate (10 ml) and washed with water (5 ml × 2). The aqueous phase was back-extracted with ethyl acetate (10 ml × 2). Combined organic phase was washed with water and concentrated in vacuo. The crude product was purified by flash column chromatography (ethyl acetate/petroleum ether).

3. Results and discussion

3.1. The catalytic activities of different proteinases in Knoevenagel reaction

Initial studies were undertaken using benzaldehyde and acetylacetone as a model reaction. We chose DMSO/water ($V_{water}/V_{DMSO} = 0.15$) as the medium, and the reaction was performed at 25 °C. The catalytic activities of 5 proteinases in Knoevenagel reaction were investigated using the model reaction (Table 1). The best result of 35% yield was achieved by using papain as the catalyst after 72 h (Table 1, entry 6). Other proteinases also exhibited varying degrees of catalytic activity in the experiment. It was worth mentioning that not all proteinases could catalyze the Knoevenagel reaction effectively such as acidic proteinase which only gave a yield of 9% (Table 1, entry 2). Moreover, the blank experiment was also performed under identical reaction conditions, and it only gave the product in yield of 6% after 110 h (Table 1, entry 1). It was obvious that papain was the most effective catalyst among the tested enzymes. Therefore, we chose papain as the catalyst for Knoevenagel condensation.

Table 1
The catalytic activities of different proteinases in Knoevenagel reaction.[a]



Entry	Enzyme	Time (h)	Yield (%)[b]
1	No enzyme	110	6
2	Acidic proteinase from *Aspergillus usamii* No 537	72	9
3	Alkaline proteinase from *Bacillus licheniformis* No 2709	72	19
4	Neutral proteinase from *Bacillus subtilis* A.S.1.398	72	20
5	Bromelain from pineapple peduncle	72	19
6	Papain from the latex of *Carica papaya* (Pangbo)	72	35

[a] All reactions were carried out using benzaldehyde (212 mg, 2 mmol), acetylacetone (240 mg, 2.4 mmol), enzyme (200 mg), deionized water (0.75 ml) and DMSO (5 ml) at 25 °C.
[b] Yield of the isolated product after chromatography on silica gel.

3.2. The effect of solvents

Based on the concept that enzymes can work in organic media, and they acquire remarkable properties such as enhanced stability, altered substrate specificity, and the ability to catalyze unusual reactions which was impossible in aqueous media [28], we investigated the effect of different solvents on the model reaction. We found that the catalytic activity of papain was remarkably influenced by solvents (Fig. 1). The reaction in DMSO gave the best yield of 35% while the reactions in water and toluene provided the yields of 28% and 24% respectively. The other tested solvents including DMF, chloroform, *tert*-butyl methyl ether (TBME), EtOH and THF gave the low yields. Moreover, only 10% yield was obtained under solvent-free conditions. No clear correlation between the solvent polarity and the enzyme activity was observed. This result may be attributed to specific interactions between the solvent and papain. Based on the results of solvent screen, DMSO was chosen as the optimum solvent for the papain-catalyzed Knoevenagel condensation.

3.3. The effect of water contents

Water plays a major role of "molecular lubricant" in enzyme resulting in conformational flexibility of enzyme, and the increased

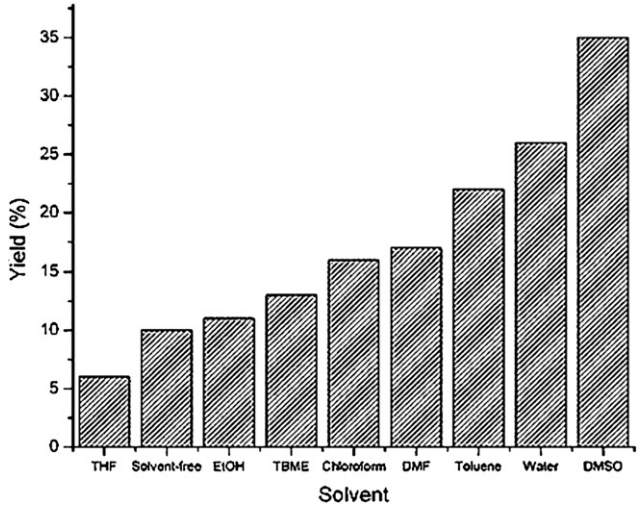


Fig. 1. The effect of solvents on the papain-catalyzed Knoevenagel reaction. Conditions: benzaldehyde (212 mg, 2 mmol), papain (Pangbo) (200 mg), acetylacetone (240 mg, 2.4 mmol), organic solvent (5 ml), deionized water (0.75 ml) at 25 °C for 72 h.

hydration leads to enhanced activity in non-aqueous solvents [29,30]. Thus, it is significant to confirm the optimal water content in reaction system. We found that the activity of papain in Knoevenagel reaction could be evidently affected by the water content in DMSO (Fig. 2). Generally, a hill-shaped curve could be used to describe the results. When water content was lower than 25% (water/water + DMSO, v/v), the yield of the Knoevenagel reaction could be enhanced by increasing the concentration of water. The reaction reached the highest yield of 57% at 25% water content after 120 h. However, once the water content surpassed 30%, the yield dropped sharply. All the results indicated that water is obviously essential in the papain-catalyzed Knoevenagel reaction. Thus, we chose 25% water content for the Knoevenagel reaction.

3.4. The effect of reaction temperature

Temperature also plays an important role in enzyme-catalyzed reactions, due to its effects on enzyme stability and reaction rate. Thus, a temperature screening was performed (Fig. 3). It was found that the activity of papain in Knoevenagel reaction could be increased by raising the temperature, and reached the best yield of 55% at 60 °C after 81 h. However, once the temperature surpassed 60 °C, the yield of the Knoevenagel product decreased sharply. In order to verify whether or not the decrease of the yield was caused by side-reactions, we performed the reactions at 70 °C and 80 °C twice, and the reaction mixtures were processed very carefully. No any by-product was detected, and the starting materials were left. So the obvious decrease in the yields at temperatures higher than 60 °C was probably due to the denaturation of papain caused by the high temperature. On the other hand, the influence of temperature implied that the catalytic behavior of papain was not simply caused by the amino acid distribution on the protein surface. The specific natural fold of papain was required for its ability to catalyze the Knoevenagel reaction. Based on the temperature screening, we chose 60 °C as the optimum temperature for the reaction.

3.5. The effect of papain loading

Next, we investigated the effect of papain loading on the reaction (Fig. 4). The results showed that 150 mg of papain (650 U/mg) was the optimum quantity for the Knoevenagel reaction between 2 mmol of benzaldehyde and 2.4 mmol of acetylacetone in 5 ml of DMSO/H_2O. When higher papain loading was used, the reaction yield became saturated.

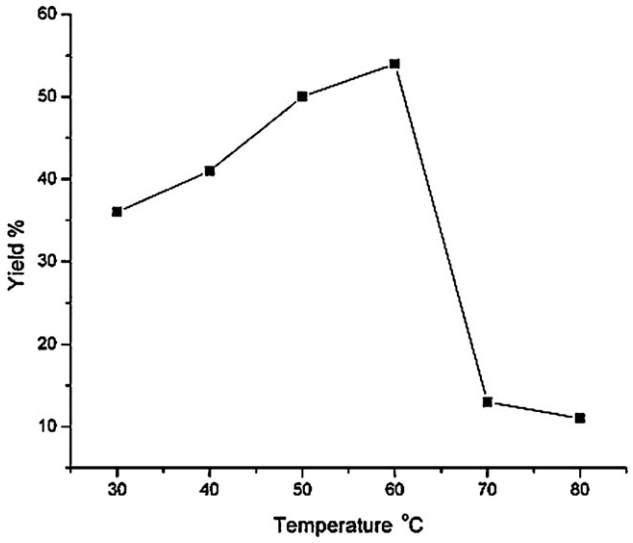


Fig. 3. The effect of temperature on the papain-catalyzed Knoevenagel reaction. Conditions: benzaldehyde (212 mg, 2 mmol), papain (Pangbo) (200 mg), acetylacetone (240 mg, 2.4 mmol), deionized water (1.25 ml), DMSO (3.75 ml) at specified temperature for 81 h.

3.6. The control experiments

To further demonstrate the specific catalytic effect of papain under the optimized conditions, some control experiments have been performed (Table 2). Because we used the papain purchased from Pangbo Biological Engineering Co. Ltd without further purification, it was necessary to exclude the possibility that the reaction was catalyzed by other proteins or impurities in the mixture instead of papain itself. So the papain purchased from Sigma–Aldrich was also used to conduct the model reaction and

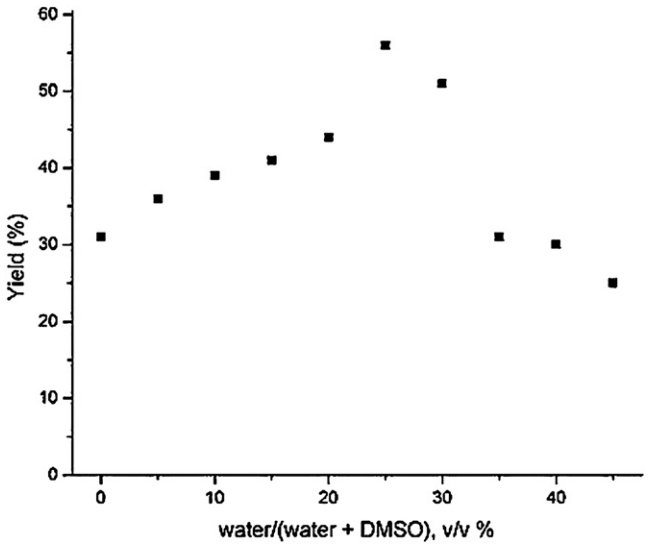


Fig. 2. The effect of water contents on the papain-catalyzed Knoevenagel reaction. Conditions: benzaldehyde (212 mg, 2 mmol), papain (Pangbo) (200 mg), acetylacetone (240 mg, 2.4 mmol) deionized water from 0% to 45% [water/(water + DMSO), v/v] at 25 °C for 120 h.

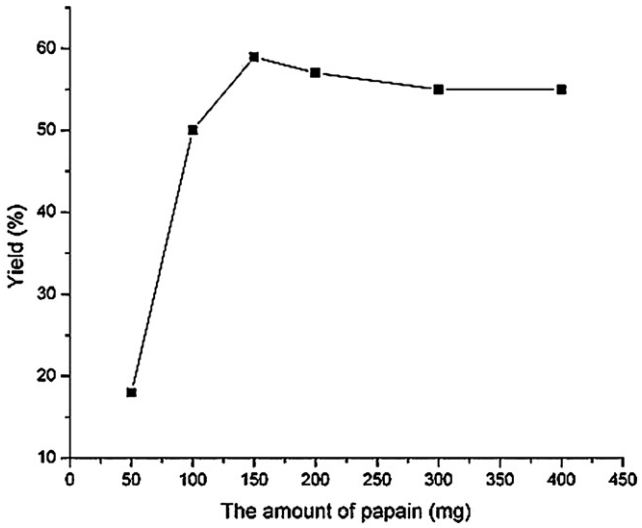


Fig. 4. The effect of papain loading on the yield of Knoevenagel reaction. Conditions: benzaldehyde (212 mg, 2 mmol), acetylacetone (240 mg, 2.4 mmol), deionized water (1.25 ml), DMSO (3.75 ml) and specified amount of papain (Pangbo) (650 U/mg) at 60 °C for 120 h.

Table 2
The control experiments.[a]

Entry	Catalyst	Yield (%)[b]
1	Papain (Pangbo)	59
2	Papain (Sigma–Aldrich)	54
3	No enzyme	6
4	Papain (Pangbo) inhibited with MMTS[c]	10
5	Papain (Sigma–Aldrich) inhibited with MMTS[c]	11
6	Papain (Pangbo) denatured by high temperature[d]	6
7	Papain (Sigma–Aldrich) denatured by high temperature[d]	7
8	Papain (Pangbo) denatured with urea[e]	12
9	Papain (Sigma–Aldrich) denatured with urea[e]	7
10	Albumin egg	10

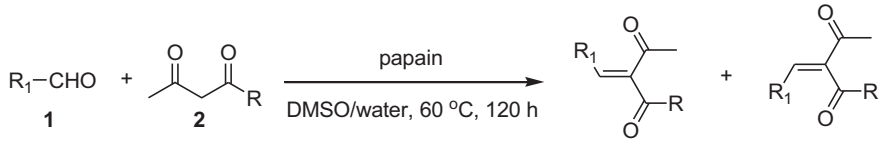
[a] Conditions: benzaldehyde (2 mmol), acetylacetone (2.4 mmol), deionized water (1.25 ml), DMSO (3.75 ml) and enzyme (150 mg) at 60 °C for 120 h.
[b] Yield of the isolated product after chromatography on silica gel.
[c] The mixture of papain (150 mg), DMSO (3.75 ml), deionized water (1.25 ml) and MMTS (50 μL) was stirred at r.t. for 48 h before use.
[d] The mixture of papain (150 mg), DMSO (3.75 ml) and deionized water (1.25 ml) was stirred at 100 °C for 48 h before use.
[e] The mixture of papain (150 mg), DMSO (3.75 ml), deionized water (1.25 ml) and urea (50 mg) was stirred at r.t. for 48 h before use.

the same set control experiments in Table 2. The Knoevenagel condensation between benzaldehyde and acetylacetone under optimized conditions gave satisfied yields of 59% (using papain from Pangbo) (Table 2, entry 1) and 54% (using papain from Sigma–Aldrich) (Table 2, entry 2). The reaction in the absence of enzyme under identical reaction conditions only gave the product in yield of 6% (Table 2, entry 3). Furthermore, an almost complete inhibition of the catalytic activity of papain in the Knoevenagel condensation was observed by using serine protease inhibitor methyl methanethiosulfonate (MMTS) [31], which gave the yields of 10% (Pangbo) and 11% (Sigma–Aldrich) (Table 2, entries 4 and 5). Moreover, the high temperature (100 °C) denatured papain (from both companies) completely lost its activity in Knoevenagel condensation giving the results equal to the blank (Table 2, entries 6 and 7), which was in agreement with the observation in the temperature screening that high temperature could cause denaturation of papain under employed reaction conditions. Next, when the reactants were incubated with urea-denatured papain, only 12% (Pangbo) and 7% (Sigma–Aldrich) yields were obtained (Table 2, entries 8 and 9). In addition, non-enzyme protein albumin egg almost did not show catalytic activity for Knoevenagel condensation, which only gave a yield of 10% (Table 2, entry 10). The facts that papain from different companies had the same effect on the enzymatic Knoevenagel reaction, and the results from the experiments using denatured and inhibited papain (from both companies) indirectly excluded the possibility of other protein or impurity catalysis. These results also indicated that the specific natural fold and the native proteolytic active site of papain were responsible for its activity in Knoevenagel condensation.

3.7. Scope of the papain-catalyzed Knoevenagel reactions

Finally, with the optimized conditions in hand, some other aldehydes and methylene compounds were used to expand upon the papain-catalyzed Knoevenagel reaction to test the generality and scope of this new enzymatic promiscuity. Aromatic and hetero-aromatic aldehydes could react with acetylacetone and ethyl acetoacetate smoothly to give the corresponding products under optimized conditions (Table 3). In general, acetylacetone was more reactive than ethyl acetoacetate under employed reaction conditions. This observation was contrary to the reported amino acid catalyzed Knoevenagel reactions [7], probably due to the special catalytic effect of papain. For example, 4-nitrobenzaldehyde gave the best yield of 87% with acetylacetone (Table 3, entry 1), but it gave a very low yield of 30% with ethyl acetoacetate and only Z stereoisomer was obtained (Table 3, entry 2). In order to verify this low yield and the stereoselectivity of the products, the reaction of 4-nitrobenzaldehyde with ethyl acetoacetate was repeated, and the reaction mixture was processed carefully. Although there were two product spots observed on TLC plate, one of them was too rare to be isolated. Only one product was isolated which was determined as Z isomer after analysis of spectroscopic data. Similarly, 2-chlorobenzaldehyde gave a good yield of 71% with acetylacetone (Table 3, entry 7), but it gave a low yield of 32% with ethyl acetoacetate (Table 3, entry 8). Moreover, both electron-donating and electron-withdrawing substituents of aromatic aldehydes were compatible (Table 3, entries 1–10), but aromatic aldehydes containing electron-withdrawing substituent generally gave better yields than those with electron-donating substituent. For instance, the reactions of 4-chlorobenzaldehyde with acetylacetone and ethyl acetoacetate gave good yields of 82% and 62% respectively (Table 3, entries 3 and 4). However, 4-methoxybenzaldehyde gave a low yield of 30% with acetylacetone

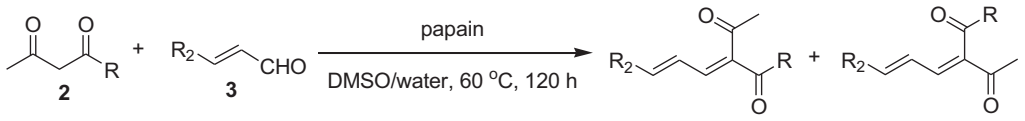
Table 3
The papain-catalyzed Knoevenagel reactions of aromatic and hetero-aromatic aldehydes with acetylacetone and ethyl acetoacetate.[a]



Entry	R	R_1	Yield (%)[b]	[Z/E][c]
1	Me	4-$NO_2C_6H_4$	87	
2	OEt	4–$NO_2C_6H_4$	30	100:0
3	Me	4-ClC_6H_4	82	
4	OEt	4-ClC_6H_4	62	72:28
5	Me	3-ClC_6H_4	84	
6	OEt	3-ClC_6H_4	60	75:25
7	Me	2-ClC_6H_4	71	
8	OEt	2-ClC_6H_4	32	90:10
9	Me	4-$MeOC_6H_4$	30	
10	OEt	4-$MeOC_6H_4$	25	100:0
11	Me	Furan-2-yl	77	
12	OEt	Furan-2-yl	70	18:82
13	Me	Thien-2-yl	51	

[a] Conditions: **1** (2 mmol), **2** (2.4 mmol), papain (Pangbo) (150 mg), deionized water (1.25 ml), DMSO (3.75 ml) at 60 °C for 120 h.
[b] Yield of the isolated product after chromatography on silica gel.
[c] The Z/E geometry was determined by ^{1}HNMR and the complete spectroscopic data is provided in the Supporting Information.

Table 4
The papain-catalyzed Knoevenagel reactions of α,β-unsaturated aromatic aldehydes with acetylacetone and ethyl acetoacetate.[a]



Entry	R	R_2	Yield (%)[b]	[Z/E][c]
1	Me	4-$CH_3C_6H_4$	76	
2	OEt	4-$CH_3C_6H_4$	68	55:45
3	Me	4-$MeOC_6H_4$	81	
4	OEt	4-$MeOC_6H_4$	86	56:44
5	Me	Ph	70	
6	OEt	Ph	42	58:42
7	Me	4-ClC_6H_4	74	
8	OEt	4-ClC_6H_4	62	51:49
9	Me	4-FC_6H_4	66	

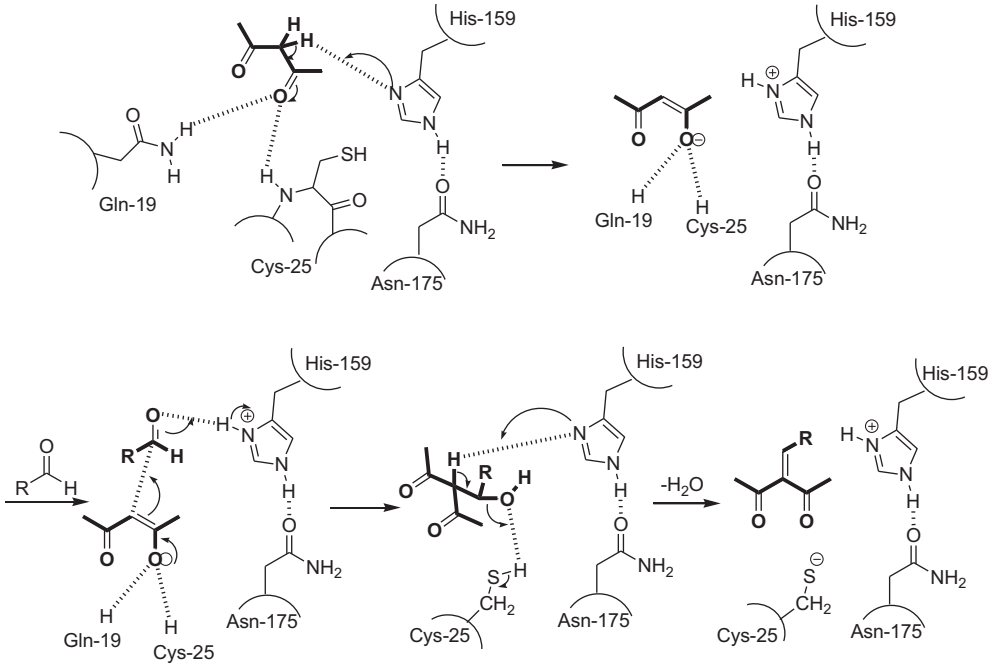
[a] Conditions: **2** (2.4 mmol), **3** (2 mmol), papain (Pangbo) (150 mg), deionized water (1.25 ml), DMSO (3.75 ml) at 60 °C for 120 h.
[b] Yield of the isolated product after chromatography on silica gel.
[c] The Z/E geometry was determined by ^{1}HNMR and the complete spectroscopic data is provided in the Supporting Information.

(Table 3, entry 9), while it gave a yield of 25% with ethyl acetoacetate (Table 3, entry 10) and only Z stereoisomer was obtained. We have carefully checked the reaction of 4-methoxybenzaldehyde and ethyl acetoacetate, and only one product spot could be observed on TLC plate which was determined as Z isomer after isolation and analysis of spectroscopic data. In addition, hetero-aromatic aldehydes 2-furaldehyde and 2-thienylaldehyde could react with acetylacetone and ethyl acetoacetate to provide products in good yields (Table 3, entries 11–13). Particularly noteworthy are the configurations of the newly formed double bonds when ethyl acetoacetate was used as the active methylene compound. This enzymatic reaction showed good to excellent Z/E selectivities with the best Z/E ratios of up to 100:0. The Z-selectivity was observed for aromatic aldehydes (Table 3, entries 2, 4, 6, 8 and 10), however a good E-selectivity was obtained for hetero-aromatic aldehyde (Table 3, entry 12).

This biocatalytic transformation was also applicable to α,β-unsaturated aromatic aldehydes (Table 4). It was found that the electronic effects of substituents had some impact on the reaction yields. α,β-Unsaturated aromatic aldehydes bearing strong electron-donating substituents gave higher yields than those containing strong electron-withdrawing substituents. For instance, when reacting with acetylacetone, 4-methoxy cinnamaldehyde gave the product in a good yield of 81% (Table 4, entry 3), but 4-fluorocinnamaldehyde only gave a yield of 66% (Table 4, entry 9). Moreover, low Z/E selectivities were observed for the products from α,β-unsaturated aromatic aldehydes (Table 4, entries 2, 4, 6 and 8), probably due to the less steric hindrance of –CHO in α,β-unsaturated aromatic aldehydes than in aromatic aldehydes. Besides, when aliphatic aldehydes such as isobutylaldehyde and 3-methyl butanal were used to react with acetylacetone respectively, no Knoevenagel adducts were detected under employed reaction conditions.

3.8. The proposed catalytic mechanism for the papain-catalyzed Knoevenagel reaction

Papain is a globular protein consisting of a single polypeptide chain of 212 residues, folded to form two domains with a deep cleft between them [32,33]. The active site cysteine residue (Cys-25) is



Scheme 1. The proposed catalytic mechanism for the papain-catalyzed Knoevenagel reaction.

part of the L1 α-helix at the surface of the left domain, while the histidine (His-159) is in a β sheet at the surface of the right domain of the enzyme. From the control experiments using inhibited papain and denatured papain, it was inferred that the catalysis of the Knoevenagel reaction depends on the native proteolytic active site in papain. Based on Hillier and co-worker's predictions about the catalytic mechanism of papain [34], we hypothesized the mechanism of the papain-catalyzed Knoevenagel reaction (Scheme 1). Firstly, the substrate acetylacetone was deprotonated by His-159 forming an enolate anion, which was stabilized by the oxyanion hole formed by the side chain of Gln-19 and the backbone NH of Cys-25 [35,36]. Secondly, another substrate aldehyde accepted the proton from the imidazolium cation, and simultaneously connected the enolate anion with the formation of a carbon–carbon bond. Finally, the dehydration of the resulted adduct took place under the catalysis of Cys and Asn–His dyad to gave the Knoevenagel product. Meanwhile, the Cys–His ion pair formed which was stabilized by Asn-175 via keeping the imidazole ring of His-159 in a favorable orientation [34].

4. Conclusion

We describe here the first papain-catalyzed Knoevenagel reaction. The catalyst, with a safe, economical and environmentally benign quality, can catalyze the Knoevenagel reactions of a wide range of aromatic, hetero-aromatic and α,β-unsaturated aldehydes with less active methylene compounds acetylacetone and ethyl acetoacetate to give moderate to good yields. The influence of reaction conditions including solvents, water content, temperature and enzyme loading was investigated. The control experiments were also conducted to demonstrate the specific catalysis of papain. This papain-catalyzed Knoevenagel reaction provides a novel case of catalytic promiscuity which widens the applicability of papain in organic synthesis.

Acknowledgments

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Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.biochi.2011.09.018.

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