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Acidic proteinase from *Aspergillus usamii* catalyzed Michael addition of ketones to nitroolefins

Ke-Ling Xu, Zhi Guan, Yan-Hong He*

School of Chemistry and Chemical Engineering, Southwest University, Chongqing 400715, PR China

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ABSTRACT

A new promiscuous activity of AUAP (acidic proteinase from *Aspergillus usamii*) was investigated in Michael addition of cyclic and acyclic ketones to aromatic and heteroaromatic nitroolefins in organic media in the presence of water. The yields were obtained from 38% to 84%.

1. Introduction

Biocatalysis as an efficient and green biotransformation tool in organic synthesis has attracted much attention of chemists and biochemists in recent years [1–3]. Especially, catalytic promiscuity in biocatalysis which means using old enzymes to form new bonds and follow new pathways, was greatly extended and expanded rapidly [4–7]. On the other hand, the Michael addition is a powerful, useful and atom economical reaction to form carbon–carbon and carbon–heteroatom bonds [8–12]. In recent years, some elegant works on enzyme catalyzed Michael-type reactions have been reported. Some lipases and proteases have been applied in the Michael-type addition to form the carbon-hetero bonds [13–29]. A few examples of enzyme-catalyzed C–C bond formations via Michael addition have also been reported. For instance, Berglund and co-workers gave the first example of C–C bond formation by lipase-catalyzed Michael addition between 1,3-dicarbonyls and α,β-unsaturated carbonyl compounds. They used lipase B from *Candida Antarctica* as a scaffold and increased its reaction specificity for Michael additions by the substitution of one amino acid (Ser105Ala) in the active site through rational design [8]. Recently they found that the CAL-B Ser105Ala variant was able to catalyze the Michael addition between methyl acrylate and acetyl acetone in aqueous media instead of the native hydrolytic process [30]. Lin and co-workers discovered that a zinc-dependent acylase from *Escherichia coli* displayed a promiscuous activity to catalyze the C–C bond formation reaction of 1,3-dicarbonyl compounds to methyl vinyl ketone in organic media [31,32]. Griengl and co-workers investigated lipase-catalyzed Michael addition with respect to the synthetic potential and stereochemistry of the products [33]. Very recently, we reported the immobilized lipase from *Thermomyces lanuginosus* catalyzed asymmetric C–C Michael addition for a wide range of 1,3-dicarbonyl compounds and cyclohexanone to aromatic, heteroaromatic nitroolefins and cyclohexenone, and the enantioselectivities up to 83% ee were obtained [34]. However, to the best of our knowledge, proteinase catalyzed C–C bond formation via Michael addition has not been reported yet. Herein, we wish to report the promiscuous activity of AUAP (acidic proteinase from *Aspergillus usamii* No 537) in Michael addition of ketones to nitroolefins in organic media in the presence of water.

2. Experimental

2.1. Materials

Acidic protease from *A. usamii* No 537 (50 U/mg), alkaline protease from *Bacillus licheniformis* No 2709 (200 U/mg), neutral protease from *Bacillus subtilis* A.S.1.398 (130 U/mg) and trypsin from porcine pancreas (4 U/mg) were purchased from Xuemei Enzyme Co. Ltd. (Wuxi, China). Pancreatin from porcine pancreas (4 U/mg), papain from papaya latex (650 U/mg), nuclease from *Penicillium citrinum* (5 U/mg), lysozyme from hen egg white (20,000 U/mg), bromelain from pineapple peduncle

* Corresponding author. Fax: +86 23 68254091.
E-mail address: heyh@swu.edu.cn (Y.-H. He).

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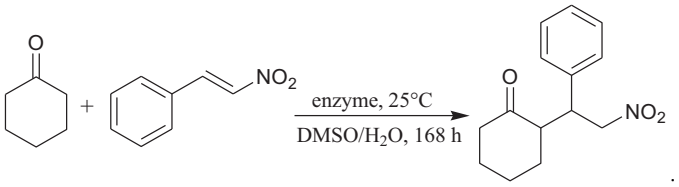
Table 1
The catalytic activities of different enzymes in Michael reaction[a].



Entry	Enzyme	Yield (%)[b]
1	AUAP (acidic proteinase from *Aspergillus usamii* No 537)	48
2	Trypsin from porcine pancreas	26
3	Pancreatin from porcine pancreas	11
4	Alkaline proteinase from *Bacillus licheniformis* No 2709	8
5	Bromelain from pineapple peduncle	4
6	Cellulase from *Trichoderma*	3
7	Nuclease from *Penicillium citrinum*	5
8	Papain from papaya latex	Trace
9	Neutral proteinase from *Bacillus subtilis* A.S.1.398	Trace
10	Lysozyme from hen egg white	Trace
11	None	No reaction
12	AUAP denatured with SDS[c]	Trace
13	AUAP inhibited with NBS[d]	Trace

[a] All the reactions were carried out using *trans*-β-nitrostyrene (1.0 mmol), enzyme (200 mg), cyclohexanone (10.0 mmol), DMSO (5 mL) and deionized water (0.75 mL) at 25 °C for 168 h.
[b] Yield of the isolated product after chromatography on silica gel.
[c] Pre-treated with SDS at 100 °C in DMSO (5 mL) in the presence of deionized water (0.75 mL) for 24 h.
[d] Pre-treated with NBS at 25 °C in DMSO (5 mL) in the presence of deionized water (0.75 mL) for 24 h.

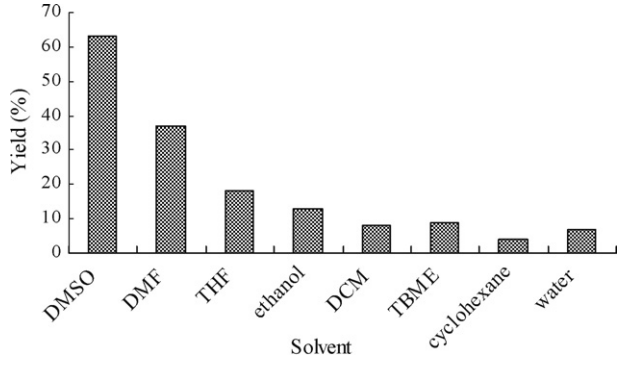


Fig. 1. Effects of solvent on AUAP-catalyzed Michael addition of cyclohexanone and *trans*-β-nitrostyrene. Conditions: *trans*-β-nitrostyrene (1.0 mmol), AUAP (200 mg), cyclohexanone (10.0 mmol), deionized water (0.60 mL), solvent (4 mL) at 30 °C for 144 h.

(500 U/mg) and cellulase from *Trichoderma* (10 U/mg) were purchased from Pangbo Biological Engineering Co. Ltd. (Nanning, China). Unless otherwise noted, all reagents were obtained from commercial suppliers and were used without further purification.

2.2. Analytical methods

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. The ^{1}H NMR spectra were recorded with TMS as internal standard on a Bruker AMX-300 MHz spectrometer. Chemical shifts were expressed in ppm and coupling constants (*J*) in Hz. All the known products were characterized by comparing the ^{1}H NMR with those reported in the literature.

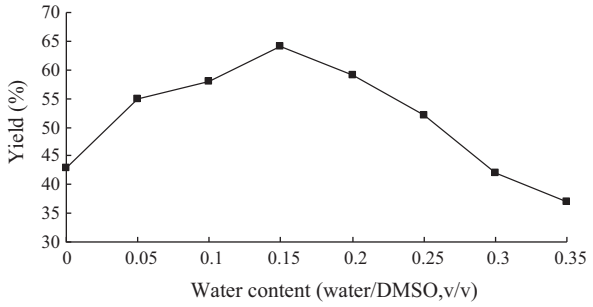


Fig. 2. Influence of water content in organic solvent on the yield of AUAP-catalyzed Michael reaction. Conditions: AUAP (200 mg), cyclohexanone (10.0 mmol), *trans*-β-nitrostyrene (1.0 mmol), and DMSO (4 mL) at 30 °C for 144 h. Deionized water was added from 0 to 0.35 (water/DMSO, v/v).

Table 2
Influence of temperature on the yield of the AUAP-catalyzed Michael addition[a].

Entry	Temperature (°C)	Time (h)	Yield (%)[b]
1	30	120	60
2	35	120	63
3	40	120	68
4	45	120	75
5	50	120	69
6	55	120	65

[a] All reactions were carried out using *trans*-β-nitrostyrene (1.0 mmol), cyclohexanone (10.0 mmol), AUAP (200 mg), deionized water (0.60 mL), DMSO (4 mL) at specified temperature.
[b] Yield of the isolated product after chromatography on silica gel.

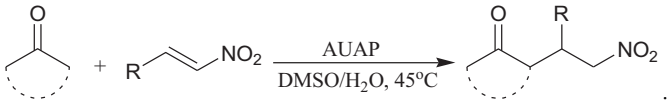
2.3. Typical experimental procedure for enzyme-catalyzed Michael reactions.

A mixture of nitroolefin (1.0 mmol), AUAP (200 mg) and ketone (20 mmol) in DMSO (4 mL) and deionized water (0.60 mL) was stirred for the specified time at 45 °C. The reaction was terminated by filtration to remove the enzyme. CH_2Cl_2 was used to wash the filter paper to assure that products obtained were all dissolved in the filtrate. Then the filtrate was washed three times with water. The organic phase was dried over anhydrous Na_2SO_4, and the solvents were removed under reduced pressure. The crude products were purified by column chromatography with petroleum ether/ethyl acetate as eluent.

3. Results and discussion

In our initial investigation, in order to ascertain the appropriate enzyme for Michael addition of ketones to nitroolefins, the Michael addition of cyclohexanone with *trans*-β-nitrostyrene was used as a model reaction, and 10 enzymes were screened. The results were shown in Table 1. The best result of 48% yield was achieved by using AUAP as a catalyst after 168 h (Table 1, entry 1). Trypsin, pancreatin and alkaline proteinase also showed catalytic activities which gave the product in yields of 26%, 11% and 8%, respectively (Table 1, entries 2–4). Bromelain, cellulase and nuclease presented low activities in this reaction (Table 1, entries 5–7). Moreover, in the presence of papain, neutral proteinase or lysozyme only trace product was observed on TLC (Table 1, entries 8–10). Just as we expected, the Michael addition of cyclohexanone to *trans*-β-nitrostyrene in the absence of enzyme showed no adduct after 168 h (Table 1, entry 11). Besides, in order to prove this reaction was catalyzed by APAU instead of the amino acids on the surface of the enzyme, the experiments using denatured enzyme and adding the inhibitor have been carried out. When the reactants were incubated with SDS-denatured AUAP, only trace adduct was observed (Table 1, entry 12). It suggested that the tertiary structure of AUAP

Table 3
Investigation of substrate scope in AUAP-catalyzed Michael addition[a].



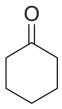
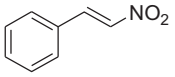
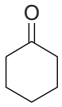
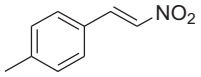
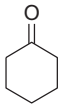
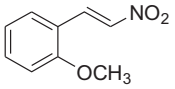
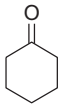
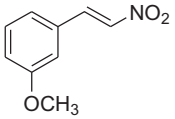
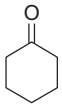
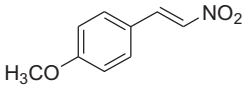
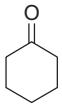
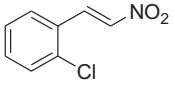
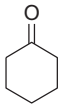
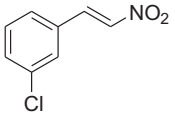
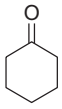
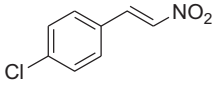
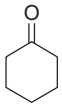
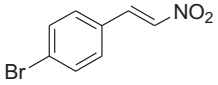
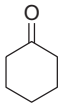
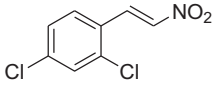
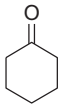
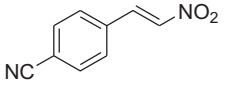
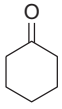
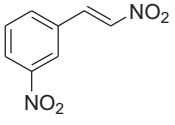
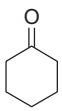
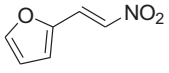
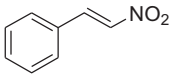
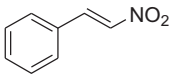
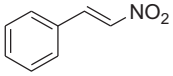
Entry	Donor	Acceptor	Time (h)	Yield (%)[b]	dr (*syn*/*anti*)[c]
1			120	80	94:6 (93:7)[d]
2			120	79	80:20 (85:15)[d]
3			120	78	77:23 (85:15)[d]
4			96	77	77:23
5			132	80	81:19 (82:18)[d]
6			108	81	94:6
7			120	78	92:8
8			72	80	92:8 (91:9)[d]
9			72	78	96:4 (89:11)[d]
10			72	77	93:7 (90:10)[d]
11			48	79	71:29 (80:20)[d]
12			72	77	79:21
13			120	69	87:13

Table 3 (*Continued*)

Entry	Donor	Acceptor	Time (h)	Yield (%)[b]	dr (*syn*/*anti*)[c]
14			96	84	80:20 (81:19)[d]
15			108	47	–
16			108	38	85:15

[a] All the reactions were carried out using nitroolefin (1.0 mmol), AUAP (200 mg), ketone (20.0 mmol), deionized water (0.60 mL) and DMSO (4 mL) at 45 °C.
[b] Yield of the isolated product after chromatography on silica gel.
[c] Determined by ^{1}H NMR spectroscopy.
[d] Numbers in parenthesis refer to the dr (*syn*/*anti*) obtained by chemocatalytic procedure using piperidine as a catalyst.

was essential in the process. Moreover, according to the literature [35] NBS (N-bromosuccinimide) is an inhibitor of AUAP. Thus, the control experiment with NBS-inhibited AUAP was performed, and only trace product was observed (Table 1, entry 13). The experiment suggested that the Michael addition was catalyzed by APAU instead of the amino acids on the surface of the enzyme. Thus, we confirmed that AUAP catalyzed the Michael addition.

Next, the volume of solvent directly related to the concentration of enzyme and substrates, and could influence the reaction yield. So we examined the effect of volume of solvent on the AUAP-catalyzed Michael addition. It was found that the best yield could be reached at 4 mL of DMSO (with 0.60 mL H_2O). Thus, we chose 4 mL solvent (with 0.60 mL H_2O) as the optimum solvent volume for the following study of AUAP-catalyzed Michael addition (for details see the Supplementary Information).

Solvents often play an important role in enzymatic reactions. Enzymes not only work in anhydrous organic media, but they acquire remarkable properties such as enhanced stability, altered substrate and enantiomeric specificities, and the ability to catalyze unusual reactions which are impossible in aqueous media [36–39]. Thus, a solvent screen was performed using cyclohexanone and *trans*-β-nitrostyrene as a model reaction. Some representative organic solvents were screened and the results were shown in Fig. 1. AUAP showed the good Michael addition activity in DMSO, and the best yield of 63% was obtained at 30 °C for 144 h. The reaction in DMF gave a yield of 37%, while both THF and ethanol provided the yields less than 20%. In the other tested solvents, including DCM, TBME (*tert*-butyl methyl ether), cyclohexane and H_2O, the activity of reaction was very poor and the yields were less than 10%. Hence, DMSO was chosen as the optimum solvent for the enzymatic Michael addition.

At the same time, enzymes require a specific amount of water bound to them to maintain activities, and water concentration affects the activity of enzymes [40–43]. Therefore, it is important to confirm the optimal water content in the reaction system. We analyzed the effects of water content on the AUAP-catalyzed Michael addition of cyclohexanone and *trans*-β-nitrostyrene in DMSO (Fig. 2). It was found that the yield of the enzymatic reaction could be raised by increasing the concentration of water, and the highest yield could be reached at water/DMSO = 0.15 (v/v). However, once the water content surpassed 0.15, the yield decreased evidently. Thus, the optimum water content for the reaction was 0.15 (water/DMSO, v/v), which was in coincidence with the amounts of water addition we used in the experiments of enzyme and solvent screen. The results indicated that water is obviously essential for the enzyme-catalyzed Michael addition in organic medium.

The temperature is another important factor for the enzyme-catalyzed reactions, due to their effects on the enzyme stability and reaction rate. We further studied the temperature effect on the AUAP-catalyzed Michael addition using cyclohexanone and *trans*-β-nitrostyrene as a model reaction (Table 2). It was found that the yield of the enzymatic reaction could be increased by raising the temperature, and the highest yield of 75% was reached at 45 °C. However, when the temperature was higher than 45 °C, the yield of product decreased, probably due to the gradual denaturation of the enzyme. Thus, the optimum temperature for the reaction was 45 °C.

Next, to further optimize the experimental conditions, we then examined the effect of cyclohexanone stoichiometry on the reaction. The best result was achieved by using 20 equivalents of cyclohexanone. Thus, we chose 20 equivalents of cyclohexanone as the optimum molar equivalent for the Michael addition (for details see the Supplementary Information).

Finally, in order to explore the scopes and limitations of the method, the reactions of cyclohexanone, cyclopentanone, acetone and butan-2-one with different aromatic and heteroaromatic nitroalkenes were carried out under optimal conditions (Table 3). It can be seen that the AUAP-catalyzed Michael addition of cyclohexanone with aromatic nitroalkenes gave the desired products in good yields (77–81%) (Table 3, entries 1–12). The electron-withdrawing groups on aromatic nitroalkenes could enhance the reactivity of the substrates. For example, 4-cyano-β-nitrostyrene reacted with cyclohexanone to give the corresponding product in satisfied yield after only 48 h (Table 3, entry 11). 4-Chloro, 4-bromo, 2,4-dichloro and 3-nitro substituted β-nitrostyrenes gave good yields after 72 h (Table 3, entries 8–10, 12). On the contrary, when there were electron-donating groups on the aromatic nitroalkenes, the reactions were slow, and relatively longer reaction times were required to reach the satisfied yields (Table 3, entries 2–5). For instance, 4-methoxy-β-nitrostyrene reacted with cyclohexanone to give the product in yield of 80% after 132 h. (Table 3, entry 5). Moreover, the largest electronic effect was found for substitutents in the *para* position for both electron-withdrawing and electron-donating groups. Besides, the AUAP-catalyzed Michael reaction between heteroaromatic nitroalkene and cyclohexanone was also performed, which gave the corresponding product in moderate yield (Table 3, entry 13). Furthermore, the enzymatic Michael addition of cyclopentanone with *trans*-β-nitrostyrene could obtain a good yield of 84% after 96 h (Table 3, entry 14). In addition, acyclic ketones as Michael donors were also accepted by the enzyme. The reaction of acetone with *trans*-β-nitrostyrene gave a low yield of 47% (Table 3, entry 15). Also, the reaction between butan-2-one and *trans*-β-

nitrostyrene only gave 3-methyl-5-nitro-4-phenylpentan-2-one in yield of 38% after 108 h (Table 3, entry 16), and a small amount of 6-nitro-5-phenylhexan-3-one was obtained as well.

It was also worthy to note that this enzymatic Michael addition had a moderate to excellent diastereoselectivity (*syn/anti* 71:29–96:4) in favor of the *syn*-isomers. In order to verify whether the diastereoselectivity observed was contributed by the enzyme or just the result of thermodynanmic control, chemocatalytic Michael additions were also conducted using piperidine as a catalyst. The similar results of diastereoselectivity were obtained from the chemocatalytic procedure, which indicated that the diastereoselectivity observed was the result of thermodynanmic control (Table 3, entries 1–3, 5, 8–11 and 14). Finally, we examined the ee value of the products by chiral HPLC, but to our disappointment, no enantioselectivity could be observed. Similar results were reported for the enzymatic Michael addition catalyzed by other enzymes [19,21]. The reason will be further investigated.

4. Conclusion

In conclusion, we describe here the readily available AUAP catalyzed Michael addition of ketones to nitroolefins in DMSO in the presence of water. The reaction conditions including organic solvents, solvent volume, water content, temperature, substrate stoichiometry were optimized. The scope of the reaction was tested by varying the nitroalkenes and ketones. For most of aromatic and heteroaromatic nitroalkenes, satisfied yields were obtained. This acidic proteinase catalyzed Michael reaction provides a novel case of catalytic promiscuity and might be a useful synthetic method for application.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2011.04.005.

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