Protease-catalysed Direct Asymmetric Mannich Reaction in Organic Solvent

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We reported the first enzyme-catalysed, direct, three-component asymmetric Mannich reaction using protease type XIV from Streptomyces griseus (SGP) in acetonitrile. Yields of up to 92% with enantioselectivities of up to 88% e.e. and diastereoselectivities of up to 92:8 (syn:anti) were achieved under the optimised conditions. This enzyme’s catalytic promiscuity expands the application of this biocatalyst and provides a potential alternative method for asymmetric Mannich reactions.

Enzyme catalytic promiscuity, in which a single active site of a given enzyme can catalyse different chemical transformations of natural or non-natural substrates, has received widespread attention as more catalytic promiscuities of existing enzymes have been discovered. In fact, all enzymes are thought to have evolved as a result of promiscuous activities in the primordial, ancient enzymes. The relatively few rudimentary ancestral generalist enzymes acted on multiple substrates to afford a wider range of metabolic capabilities. The increased catalytic specificity and selectivity are thought to be a result of divergence and evolution. Thus, enzyme catalytic promiscuity is a key factor in the evolution of new enzyme functions. Moreover, promiscuous activity does not normally affect an organism if the promiscuous reaction does not affect the rate of the native activity or if the substrate for the promiscuous reaction is not natural to the enzyme. So there is no selective pressure to remove the promiscuous reaction. Catalytically promiscuous behaviour is often hidden behind a native catalytic transformation and only visible under non-natural conditions.

Many examples of enzyme catalytic promiscuity in organic synthesis have been reported recently. Several enzymes have been used in asymmetric syntheses in organic solvents, including asymmetric aldol reactions, C-C Michael additions, β-lactam opening, the asymmetric synthesis of α-aminonitrile amides and the preparation of chiral epoxides. An enzyme-catalysed asymmetric Mannich reaction has not yet been reported, even though some groups have reported enzyme-catalysed Mannich reactions without enantioselectivity. The asymmetric Mannich reaction is a powerful synthetic strategy to prepare chiral β-amino ketones and aldehydes with perfect atom economy through the loss of a molecule of water and the reaction products are versatile intermediates in the synthesis of chiral amines. This characteristic makes it important to develop an enzyme-catalysed asymmetric Mannich reaction as a more sustainable complement to chemical catalysis. In this context, we investigated the asymmetric one-pot Mannich reaction catalysed by the protease type XIV from Streptomyces griseus (SGP) without the need for additional cofactors or special equipment.

Results

Control experiments. Initially, the Mannich reaction of cyclohexanone, 4-nitrobenzaldehyde and aniline was used as a model reaction. We found that SGP was able to catalyse the model reaction in MeCN in the presence of water, which gave the product in a moderate yield of 66% with a good enantioselectivity of 82% e.e. for the syn-isomer and 85:15 dr (syn:anti) (Table 1, entry 1). To verify the specific catalytic effect of SGP on the Mannich reaction, some control experiments were performed (Table 1, entries 2–12). In the absence of the enzyme, the Mannich product was only obtained in a yield of 28% (Table 1, entry 2), indicating that SGP indeed had a catalytic effect on the Mannich reaction. To further confirm the effect of background and SGP on the reaction, we profiled both the blank reaction and the SGP-catalysed model Mannich reaction (for details, please see Supplementary Tables S1–S2). Next, the albumins from chicken egg white and from bovine serum were used separately in the
model reaction as non-enzyme proteins, which produced the Mannich products in 23% yield with 0% e.e. and 21% yield with 7% e.e., respectively (Table 1, entries 3 and 4). These reactions excluded the possibility of protein catalysis, meaning that catalysis was not simply a result of the amino acid residues on the surface of the protein. Furthermore, urea-denatured SGP was used to catalyse the model reaction, which gave a high yield of 87% with only 8% e.e. (Table 1, entry 5). The same amount of urea was then used to catalyse the reaction, but produced a result nearly indential to the blank, proving that urea alone did not catalyse this transformation (Table 1, entry 6). These results show that the urea-denatured SGP still had catalytic activity towards the Mannich reaction, but it almost completely lost its enantioselectivity. Metal-denatured SGP was also used to catalyse the model reaction to determine whether the metal ion could disrupt the bonds that hold the enzyme together and cause the enzyme to undergo a conformational change, disrupt the active site and ultimately denature the enzyme. SGP was pretreated with Cu$^{2+}$ and Ag$^{+}$ at different concentrations. A low concentration (2.5 mM) of Cu$^{2+}$ or Ag$^{+}$ did not have an obvious effect on the activity and selectivity of SGP towards the Mannich reaction (Table 1, entries 7 and 10), while a moderate concentration (25 mM) of Cu$^{2+}$ or Ag$^{+}$ caused a slight decrease in enantioselectivity (Table 1, entries 8 and 11). A higher concentration (250 mM) of Cu$^{2+}$ or Ag$^{+}$ almost completely destroyed the selectivity of SGP in the model Mannich reaction (Table 1, entries 9 and 12). From the above control experiments with urea or metal ion-denatured SGP, we determined that the denatured SGP still exhibited catalytic activity in the Mannich reaction, but it lost nearly all of its stereoselectivity, indicating that the specific natural fold of SGP is responsible for its stereoselectivity in Mannich reactions.

Optimisation of reaction conditions. Next, we explored the effects of different solvents on the SGP-catalysed model Mannich reaction (Table 2). The reaction medium played an important role in this enzymatic reaction. The highest selectivity of 82% e.e. for the syn isomer (85:15 dr, syn-anti) was obtained with a moderate yield in MeCN (Table 2, entry 1), whereas water gave the highest yield of 76% with low selectivity (Table 2, entry 12). The reaction in DMF gave the lowest yield of 16% (Table 2, entry 11) and the reaction in DMSO provided the lowest enantioselectivity of only 6% e.e. with reversed diastereoselectivity (36:64 dr, syn-anti) (Table 2, entry 15). Generally, no clear correlation was observed between solvent polarity and enzyme activity or selectivity. Thus, to optimise selectivity, we selected MeCN as the solvent for the SGP-catalysed Mannich reaction.
Table 3 | Investigation of substrate scope for the SGP-catalysed asymmetric Mannich reaction

| Entry | X     | R₁       | R₂     | Product No. | Time (h) | Yield (%) | dr (syn:anti) | e.e. [syn] [%] |
|-------|-------|----------|--------|-------------|----------|-----------|---------------|---------------|
| 1     | CH₂   | 4-NO₂C₆H₄ | H      | 4a          | 96       | 64        | 88:12         | 83            |
| 2     | CH₂   | 4CFC₆H₄   | H      | 4b          | 94       | 73        | 81:19         | 78            |
| 3     | CH₂   | 4BrC₆H₄   | H      | 4c          | 94       | 65        | 74:26         | 76            |
| 4     | CH₂   | 4ClC₆H₄   | H      | 4d          | 120      | 92        | 78:22         | 75            |
| 5     | CH₂   | 3FC₆H₄   | H      | 4e          | 94       | 72        | 70:30         | 74            |
| 6     | CH₂   | 4FC₆H₄   | H      | 4f          | 94       | 66        | 66:34         | 64            |
| 7     | CH₂   | 4CN₆H₄   | H      | 4g          | 120      | 61        | 58:42         | 68            |
| 8     | CH₂   | C₆H₅     | H      | 4h          | 100      | 62        | 60:40         | 61            |
| 9     | CH₂   | 4-CH₃C₆H₄ | H      | 4i          | 120      | 68        | 40:60         | 33            |
| 10    | CH₂   | 4-NO₂C₆H₄ | 3Br    | 4j          | 144      | 24        | 92:8          | 88            |
| 11    | CH₂   | 4-NO₂C₆H₄ | 3-CH₃  | 4k          | 123      | 54        | 91:9          | 83            |
| 12    | CH₂   | 4-NO₂C₆H₄ | 4-Cl   | 4l          | 144      | 47        | 89:11         | 83            |
| 13    | CH₂   | 4NO₂C₆H₄  | 4-CH₃  | 4m          | 120      | 81        | 90:10         | 82            |
| 14    | CH₂   | 4NO₂C₆H₄  | 4-OCH₃ | 4n          | 117      | 71        | 72:28         | 72            |
| 15    | CH₂   | 4BrC₆H₄   | 4-OCH₃ | 4o          | 165      | 66        | 52:48         | 40            |
| 16*   | S     | 4ClC₆H₄   | H      | 4p          | 168      | 81        | 57:43         | 58            |
| 17*   | S     | 4CF₆C₆H₄  | H      | 4q          | 142      | 80        | 44:56         | 52            |

*Reaction conditions: a mixture of aromatic aldehyde (0.5 mmol), arylamine (0.55 mmol), ketone (7.5 mmol), deionised water (0.10 mL), MeCN (0.9 mL) and SGP (50 mg) was stirred at 30°C.

**Yield of the isolated product after silica gel chromatography.

cDetermined by chiral HPLC analysis.

d Determined by chiral HPLC; the absolute configuration was assigned by comparison with the literature (for details, please see the Supplementary Information).

e.e. value of the syn-isomer, determined by chiral HPLC.
produce colour equivalent to 1.0 μmole (181 μg) of tyrosine per min at pH 7.5 at 37°C (colour by Folin-Ciocalteu reagent). Unless otherwise noted, all reagents were obtained from commercial suppliers and used without further purification.

**General procedure for the SGP-catalysed Mannich reaction.** SGP (50 mg) was added to a round-bottom flask containing an aromatic aldehyde (0.5 mmol), an aryamine (0.55 mmol), a ketone (7.5 mmol), MeCN (0.9 mL) and deionised water (0.1 mL). The resultant mixture was stirred at 30°C for the specified reaction time and monitored by TLC on Haiyang GF 254 silica gel plates. The reaction was terminated by filtering the enzyme. The filter cake was washed with ethyl acetate (10 mL). Then, the filtrate was concentrated in vacuo. The residue was purified by flash column chromatography (ethyl acetate/petroleum ether) to give the product.

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**Author contributions**

Guan and He designed and supervised the research. Xue and Li performed the experiments. Xue analysed data and drafted the paper. Guan and He reviewed and edited the manuscript. All the authors discussed the results and contributed extensively to the work presented in this paper.

**Additional information**

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

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