JOC The Journal of Organic Chemistry

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J. Org. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.joc.6b01132 • Publication Date (Web): 27 Jun 2016

Enzyme-catalyzed asymmetric domino thia-Michael/aldol condensation using pepsin

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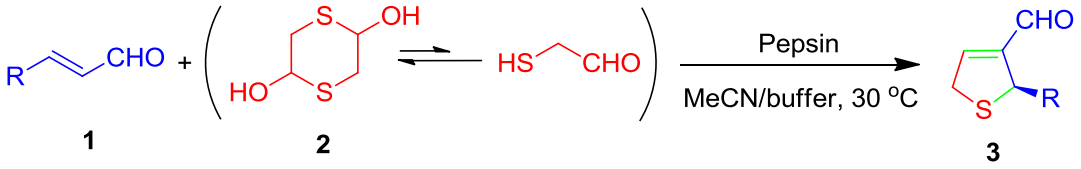


Pepsin: pepsin from porcine gastric mucosa; 13 examples, yields: 35-53%, ee: 40-84%.

Abstract: The novel catalytic promiscuity of pepsin from porcine gastric mucosa for the asymmetric catalysis of domino thia-Michael/aldol condensation reaction in MeCN/buffer was discovered for the first time. Broad substrate specificity was tested and a series of corresponding products were obtained with enantioselectivities of up to 84% ee. This specific catalysis was demonstrated by using recombinant pepsin and the control experiments with denatured and inhibited pepsin. The reaction was also proved to occur in the active site by site-directed mutagenesis (the Asp32Ala mutant of pepsin) and a possible mechanism was proposed.

Key words: pepsin; enzyme catalytic promiscuity; domino reaction; asymmetric catalysis; enzyme catalysis

Introduction

Enzymes, as green and efficient biocatalysts, have huge superiority in many important respects such as mild reaction conditions, good stereoselectivity, easy processing, economically and ecologically advantageous, etc.[1] Enzymes have specific substrate recognition in metabolism, which are optimized through evolution of a specific chemical transformation. Despite this, more and more enzymes have been found to have the ability to catalyze reactions, or act on substrates, other than those for which they evolved; this phenomenon is called enzyme promiscuity.[2] Until recently, the wider implications of the "darker" side of enzyme promiscuity were largely ignored. Actually, promiscuous activities are not rare exceptions but are rather widely spread, inherent features of enzymes and proteins in general.[3] It is believed that promiscuous activities serve as starting points for the divergence of new enzymes in natural evolution. Broad-specificity enzymes acted as progenitors for today's specialized enzymes.[4] Thus, enzyme catalytic promiscuity is a key factor in the evolution of new enzyme functions. Many examples of enzyme catalytic promiscuity have been reported,[2c,5] such as the aldol reactions,[6] Henry reactions,[7] Markovnikov additions,[8] Michael additions,[9] Mannich reactions,[10] the asymmetric synthesis of α-aminonitrile amides,[11] multi-component cascade or domino reactions,[1a-c,12] etc. However, only very few mechanisms of promiscuous functions have been proved by site-directed mutation of enzymes.[6a,13] It has been discovered that valuable insights regarding the catalytic mechanisms can be provided by a systematic research of the hidden skills of enzymes.[14] Thus, exploring more reaction types and verification of catalytic mechanism are still greatly in demand.

Dihydrothiophenes as valuable S-heterocycles have attracted great attention because of their

special values in biological and medical properties,[15] versatile synthetic intermediates[16] and material science.[17] Thus, many efficient synthetic approaches have been devoted to the development of these compounds.[16c,18] However, only few successful procedures achieved the enantioselective formation of dihydrothiophenes. In 2009, the De Risi[19] group reported the synthesis of chiral 4,5-dihydrothiophene; in 2010, the Xu[20] group developed the enantioselective formation of 2,5-dihydrothiophenes by domino thia-Michael/aldol condensation between 1,4-dithiane-2,5-diol and α,β-unsaturated aldehydes using chiral diphenylprolinol TMS ether as an organocatalyst. Based on the work of Xu[20] group, in 2015, De Risi[21] group developed a one-pot, four-step organocatalytic process catalyzed by (S)-diphenylprolinol TMS ether. This process consists of the domino thia-Michael/aldol condensation between 1,4-dithiane-2,5-diol and α,β-unsaturated aldehydes, and the derived chiral dihydrothiophene adducts subsequently react with bromonitromethane via the domino Michael/α-alkylation reaction to obtain chiral nitrocyclopropanes. Since the biological activities and pharmacological activities are often related to the configurations of most natural products and pharmaceuticals, the enantioselective syntheses of dihydrothiophenes is of great importance. Therefore, the development of novel catalysts that are environmentally friendly, sustainable and cheap toward chiral synthesis of dihydrothiophenes is still highly desired.

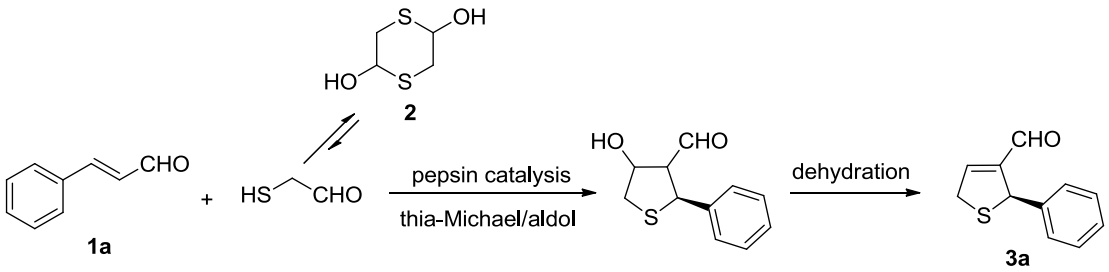
Pepsin is an aspartic protease that acts in food digestion in the mammal stomach. In recent years, some promiscuous activities of pepsin from porcine gastric mucosa have been disclosed gradually. In 2010, the Yu group reported the pepsin-catalyzed aldol reaction between acetone and substituted benzaldehydes in which enantioselectivity of up to 44% ee was observed.[22] Our group

also has investigated the catalytic promiscuity of pepsin. In 2015, we found that pepsin can catalyze direct asymmetric aldol reactions for the synthesis of vicinal diol compounds, and products were obtained with the enantioselectivity of up to 75% ee.[23] In 2016, we reported the pepsin-catalyzed Morita–Baylis–Hillman (MBH) reaction between aromatic aldehydes and 2-cyclohexen-1-one or 2-cyclopenten-1-one, and the enantioselectivity of up to 38% ee was achieved.[24] Very recently, we found that pepsin can catalyze the domino Knoevenagel/Michael/Michael reaction for the synthesis of spirooxindole derivatives with diastereoselectivities of up to >99 : 1 dr without enantioselectivity.[12b] In view of the amazing catalytic versatility of pepsin and its stereoselectivity toward some synthetically useful molecules, it is necessary to gain more insights into its catalytic promiscuity. Herein, we report a novel activity of pepsin for the asymmetric domino thia-Michael/aldol condensation reaction of 1,4-dithiane-2,5-diol with α,β-unsaturated aldehydes. Broad substrate specificity was tested and a series of corresponding products were obtained with enantioselectivities of up to 84% ee. The specific catalytic effect of pepsin was clearly proved to occur in the active site by site-directed mutagenesis (the Asp32Ala mutant of pepsin). This is the first study utilizing site-directed mutagenesis to confirm the promiscuous activity of pepsin. And a possible mechanism was proposed.

Results and discussion

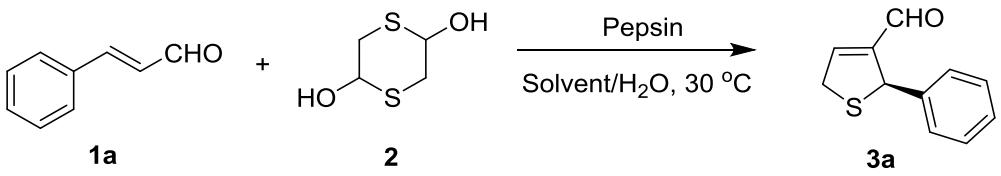
The asymmetric domino thia-Michael/aldol condensation reaction of cinnamaldehyde (**1a**) and 1,4-dithiane-2,5-diol (**2**) was chosen as a model reaction, and pepsin from porcine gastric mucosa was used as a catalyst. In this reaction 1,4-dithiane-2,5-diol (**2**), the mercaptoacetaldehyde dimer,

was used as a convenient and efficient synthon to provide an *in situ* generated mercaptoacetaldehyde (**Scheme 1**).[25] It is known that reaction media plays an important role in enzymatic reactions as the reaction media has a great effect on the stability and the catalytic activity of an enzyme, particularly on enantioselectivity and regioselectivity.[26] To optimize experimental conditions, we first examined the effect of solvent on the model reaction (**Table 1**). It could be seen that the catalytic effects of pepsin, especially the enantioselectivity, was obviously influenced by different media. The enzyme showed the best enantioselectivity of 41% ee in MeCN with a yield of 17% (**Table 1**, entry 1). Better yields of 20-21% were obtained in $CHCl_3$, PhMe and $ClCH_2CH_2Cl$, but with very low enantioselectivity (**Table 1**, entries 6-8). The reactions in the other tested solvents gave either lower yields or lower ees, and even only trace amounts of the product were observed in several solvents including water (**Table 1**, entries 2-5 and 9-16). These results may be attributed to the solubility of substrates and specific interactions between the solvent and pepsin. To get the best enantioselectivity, MeCN was chosen as a suitable solvent for the reaction. The absolute configuration of the product **3a** was determined as R-configuration by comparing with the known chiral HPLC analysis.[20]



Scheme 1 The model asymmetric domino thia-Michael/aldol condensation reaction

Table 1 Solvent screening for the model reaction [a]



1a **2** **3a**

Entry	Solvent	Yield (%)[b]	ee (%)[b]
1	MeCN	17[c]	41
2	MeOH	10	35
3	THF	11	15
4	EtOAc	11	11
5	CH_2Cl_2	17	6
6	$CHCl_3$	21	5
7	PhMe	20	4
8	$ClCH_2CH_2Cl$	21	3
9	Et_2O	11	3
10	PhOMe	Trace	
11	1,4-Dioxane	Trace	
12	EtOH	Trace	
13	DMF	Trace	
14	DMSO	Trace	
15	Xylene	Trace	
16	H_2O	Trace	

[a] Reaction conditions: a mixture of cinnamaldehyde **1a** (0.5 mmol), 1,4-dithiane-2,5-diol **2** (0.35 mmol), solvent (0.90 mL), deionized water (0.10 mL) and pepsin (6.5 kU) was stirred at 30 ℃ for 96 h.

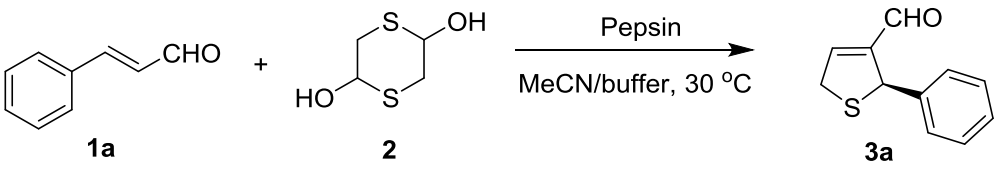
[b] Determined by chiral HPLC using a chiralpak AS-H column.

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

Since the pH value of the reaction medium significantly affects the stability and catalytic activity of enzymes,[27] phosphate buffer (NaH_2PO_4-Na_2HPO_4, 0.067 M, pH 4.7-8.7) was used to replace the water in the reaction system (buffer/MeCN = 1/9, v/v) to obtain the optimum reaction conditions (**Table 2**). It could be seen that addition of buffer obviously enhanced the enantioselectivity of the reaction. Pepsin showed the best enantioselectivity in the presence of

phosphate buffer (pH 6.5), giving the product in yield of 16% with 57% ee (**Table 2**, entry 4). However, the addition of phosphate buffer was unable to increase the yield. Thus, the phosphate buffer (pH 6.5, buffer/MeCN = 1/9, v/v) was selected as the optimum condition for the reaction.

Table 2 The influence of pH conditions (phosphate buffer) on the model reaction [a]



1a **2** **3a**

Entry	pH of phosphate buffer	Yield (%)[b]	ee (%)[b]
1	4.7	17	50
2	5.6	19	54
3	6.3	16	56
4	6.5	16	57
5	7.0	16	55
6	7.4	16	56
7	8.0	14	56
8	8.7	14	53

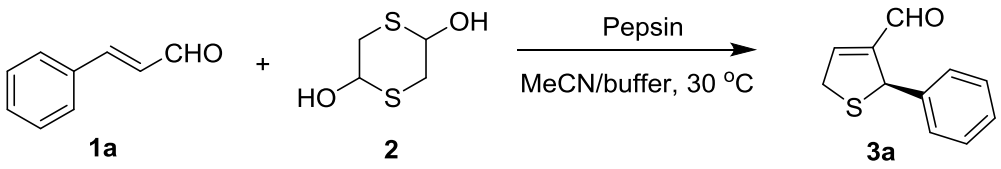
[a] Reaction conditions: a mixture of cinnamaldehyde **1a** (0.5 mmol), 1,4-dithiane-2,5-diol **2** (0.35 mmol), MeCN (0.90 mL), phosphate buffer (NaH_2PO_4-Na_2HPO_4, 0.067 M, pH 4.7-8.7, 0.10 mL) and pepsin (6.5 kU) was stirred at 30 ℃ for 96 h.

[b] Determined by chiral HPLC using a chiralpak AS-H column.

The effects of substrate molar ratios on the model reaction were investigated (**Table 3**). The results showed that the reaction was clearly influenced by changes in the molar ratio of substrates. As the amount of cinnamaldehyde (**1a**) was increased, the enhanced yields were obtained but with reduced ees (**Table 3**, entries 1-6). When increasing the amount of 1,4-dithiane-2,5-diol (**2**), a slight improvement was observed both in yield and ee (**Table 3**, entries 1 and 7-10). The best ee of

60% and yield of 19% were obtained at the molar ratio of **1a** : **2** = 1 : 3 (**Table 3**, entry 8), which was chosen as the optimal ratio for further studies.

Table 3 Effect of molar ratio of substrates on the model reaction [a]



1a **2** **3a**

Entry	Molar ratio (**1a** : **2**)	Yield (%)[b]	ee (%)[b]
1	1 : 1	15	56
2	1.4 : 1	16	57
3	2 : 1	23	46
4	3 : 1	29	36
5	4 : 1	29	33
6	5 : 1	28	28
7	1 : 2	18	56
8	1 : 3	19	60
9	1 : 4	19	59
10	1 : 5	19	60

[a] Reaction conditions: a mixture of cinnamaldehyde **1a** (0.35-1.75 mmol), 1,4-dithiane-2,5-diol **2** (0.35-1.75 mmol), MeCN (0.90 mL), phosphate buffer (NaH_2PO_4-Na_2HPO_4, 0.067 M, pH 6.5, 0.10 mL) and pepsin (6.5 kU) was stirred at 30 ℃ for 96 h.

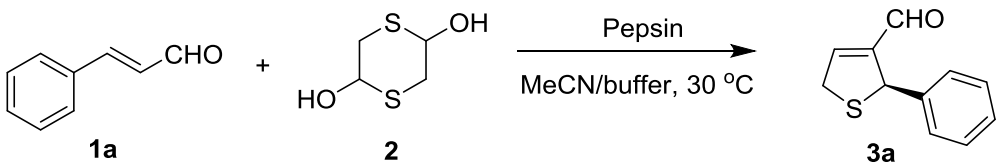
[b] Determined by chiral HPLC using a chiralpak AS-H column.

Next, to further optimize the reaction conditions, the influence of phosphate buffer content in the reaction system on the model reaction was investigated. The best yield of 24% with 59% ee was received in the mixed solvent of phosphate buffer (pH 6.5) and MeCN (buffer/MeCN = 1/4, v/v), which was chosen as the optimal condition for the reaction (for details, please see the Supporting Information). Then the influence of the mixed solvent volume on the model reaction was also

examined. To our delight, as the volume increased from 1.00 mL to 1.50 mL, the ee was raised from 59% to 70% with a slight decrease in yield from 24% to 18%. Thus, to get better enantioselectivity, a mixed solvent volume of 1.50 mL (buffer/MeCN = 1/4, v/v) was chosen as the optimal reaction condition (for details, please see the Supporting Information).

The influence of enzyme loading on the model reaction of cinnamaldehyde (0.35 mmol) and 1,4-dithiane-2,5-diol (1.05 mmol) was examined. The yield was visibly affected by enzyme loading (**Table 4**). A great enhancement in yield was obtained when the enzyme loading was increased from 3.9 kU to 16.9 kU (**Table 4**, entries 1-6) with ee remained about the same. Though there was a slight enhancement in yield with further increases in the amount of enzyme from 16.9 kU to 26.0 kU, the ee began to decline (**Table 4**, entries 6-9). Thus, we chose an enzyme loading of 16.9 kU (**Table 4**, entry 6) as the optimum condition for further studies.

Table 4 Effect of enzyme loading on the model reaction [a]



1a 2 3a

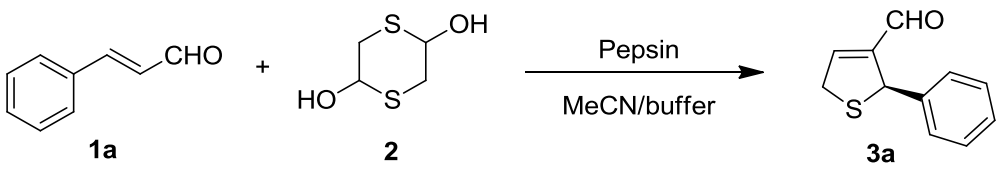
Entry	Enzyme loading (kU)	Yield (%)[b]	ee (%)[b]
1	3.9	11	70
2	6.5	18	70
3	9.1	28	70
4	11.7	30	72
5	14.3	37	71
6	16.9	46	70
7	19.5	46	69
8	22.1	53	65
9	26.0	55	63

[a] Reaction conditions: a mixture of cinnamaldehyde **1a** (0.35 mmol), 1,4-dithiane-2,5-diol **2** (1.05 mmol), MeCN (1.20 mL), phosphate buffer (NaH_2PO_4-Na_2HPO_4, 0.067 M, pH 6.5, 0.30 mL) and pepsin (3.9-26.0 kU) was stirred at 30 ℃ for 96 h.

[b] Determined by chiral HPLC using a chiralpak AS-H column.

Temperature also plays an important role in enzyme-catalyzed reactions due to its effects on enzyme stability, as well as the rate and selectivity of the reaction.[28] To further characterize the activity and selectivity of pepsin in the model reaction, the influence of temperature was investigated (**Table 5**). When raising the temperature from 20 ℃ to 35 ℃, the yield improved from 33% to 47% with ee staying at about the same level (**Table 5**, entries 1-4). However, once the temperature surpassed 35 ℃, the yield and ee began to decrease (**Table 5**, entries 5-8). When the temperature was higher than 40 ℃, the yield and ee sharply declined (**Table 5**, entries 7 and 8), which we believe to be due to high temperature causing denaturation of pepsin. Overall, a relatively good result of 46% yield with 70% ee was exhibited at 30 ℃ (**Table 5**, entry 3). Thus, carrying out the reaction at 30 ℃ was proven to be the optimal reaction condition.

Table 5 Influence of temperature on the model reaction [a]



1a **2** **3a**

Entry	T (℃)	Yield (%)[b]	ee (%)[b]
1	20	33	69
2	25	44	69
3	30	46	70
4	35	47	69

5	38	45	65
6	40	38	60
7	50	7	29
8	60	7	19

[a] Reaction conditions: a mixture of cinnamaldehyde **1a** (0.35 mmol), 1,4-dithiane-2,5-diol **2** (1.05 mmol), MeCN (1.20 mL), phosphate buffer (NaH_2PO_4-Na_2HPO_4, 0.067 M, pH 6.5, 0.30 mL) and pepsin (16.9 kU) was stirred at 20-60 ℃ for 96 h.

[b] Determined by chiral HPLC using a chiralpak AS-H column.

Time course of the model reaction was investigated under the aforementioned optimal conditions. Generally, the yield increased as the reaction time went on at the early stage and finally kept at an almost constant level. No significant change of ee was detected during the whole time course (for details, please see the Supporting Information).

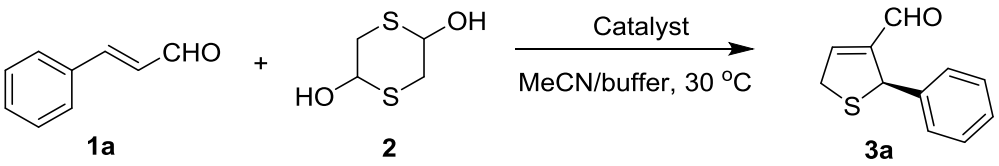
To verify the specific catalytic effect of pepsin on the domino reaction, some control experiments were performed (**Table 6**). In the absence of pepsin, the reaction only gave a trace amount of the product (**Table 6**, entry 2) which indicated that pepsin preparation had a catalytic effect on the domino reaction. The catalytic site of pepsin consists of two aspartate residues, Asp32 and Asp215. *N*,*N'*-carbonyldiimidazole (CDI) can be irreversibly covalently bound to carboxyl. Thus, CDI was used to pretreat the pepsin, and the reaction with CDI pretreated pepsin only gave the product in a low yield of 4% with 5% ee (**Table 6**, entry 3), which indicated that CDI strongly inhibited enzyme activity in the domino reaction. At the same time, CDI alone was verified to have no effect on the domino reaction (**Table 6**, entry 5). The results suggested that the enzymatic process may proceed in the active site. Moreover, urea as a denaturing agent can change the

conformational structure of enzymes, and ultimately denature the enzyme. Hence, urea was used to denature pepsin which nearly made pepsin lose its catalytic activity completely (**Table 6**, entry 4). Meanwhile, the blank experiment showed that urea itself did not catalyze the domino reaction (**Table 6**, entry 6). The above experiments indicated that the specific natural fold of pepsin was responsible for its activity in the domino reaction.

To further verify that the pepsin is truly the catalyst of the investigated domino reaction, we have carried out a series of studies involving the cloning, expression, purification and activation of recombinant porcine pepsinogen A,[29] and the obtained recombinant pepsin was used to catalyze the model domino reaction. A good yield of 60% with 43% ee was obtained from the recombinant pepsin catalyzed reaction (**Table 6**, entry 7). The result clearly confirmed that pepsin indeed catalyzed the domino reaction in an asymmetric manner.

Moreover, to validate if this observed promiscuous activity was arisen from the active site of the pepsin, a site-directed mutagenesis was conducted. The catalytic site of pepsin is formed by two aspartate residues, Asp32 and Asp215. We changed, by site-directed mutagenesis, pepsin active site Asp32 to an Ala32.[29] Only a trace amount of product was observed for the model domino reaction with mutant pepsin (Asp32Ala) (**Table 6**, entry 8). It can be inferred that the enzymatic process indeed proceeds in the active site, and the Asp residues located in the active centre are also crucial for this enzymatic domino reaction.

Table 6 Control experiments for the model reaction [a]



1a 2 3a

Entry	Catalyst	Yield (%)[b]	ee (%)[b]
1	Pepsin	46	70
2	None	Trace	-
3	Pepsin (pretreated with CDI)[c]	4	5
4	Pepsin (pretreated with urea)[d]	1	2
5	CDI (200 mg)	Trace	-
6	Urea (200 mg)	Trace	-
7	Recombinant pepsin[e]	60	43
8	Mutant pepsin (Asp32Ala)[e]	Trace	-

[a] Unless otherwise noted, reaction conditions: a mixture of cinnamaldehyde **1a** (0.35 mmol), 1,4-dithiane-2,5-diol **2** (1.05 mmol), MeCN (1.20 mL), phosphate buffer (NaH_2PO_4-Na_2HPO_4, 0.067 M, pH 6.5, 0.30 mL) and pepsin (16.9 kU) was stirred at 30 ℃ for 96 h.

[b] Determined by chiral HPLC using a chiralpak AS-H column.

[c] Pepsin (16.9 kU) in CDI solution (1.2 M in THF) (200 mg CDI in 1.0 mL THF) was stirred at rt for 4 h and THF was removed under reduced pressure before use.

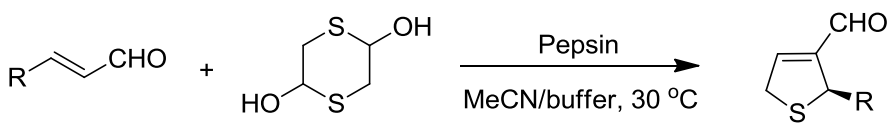
[d] Pepsin (16.9 kU) in urea solution (3.3 M) (200 mg urea in 1.0 mL deionized water) was stirred at rt for 24 h and water was removed by lyophilization before use.

[e] Reaction conditions: a mixture of cinnamaldehyde **1a** (0.035 mmol), 1,4-dithiane-2,5-diol **2** (0.105 mmol), MeCN (0.24 mL), phosphate buffer (NaH_2PO_4-Na_2HPO_4, 0.067 M, pH 6.5, 0.06 mL) and recombinant pepsin (2.34 mg, 0.7 kU) (for entry 7) or mutant pepsin (Asp32Ala) (2.34 mg) (for entry 8) was stirred at 30 ℃ for 96 h.

With the optimal reaction conditions in hand, to investigate the generality and scope of the pepsin-catalyzed domino reaction, various α,β-unsaturated aldehydes were investigated. As shown in **Table 7**, aromatic α,β-unsaturated aldehydes bearing either electron-withdrawing or

electron-donating substituents could participate in this domino reaction smoothly. Substituents on the aromatic ring had a significant influence on the enantioselectivities. Generally, aromatic α,β-unsaturated aldehydes with electron-donating groups provided higher enantioselectivities than those with electron-withdrawing substituents (**Table 7**, entries 2-10). When the substituents on the aromatic ring were halogen atoms, the position of the substituents had effects on the enantioselectivity. Substituents at *para*-position gave higher ee values than those at *ortho*- or *meta*-position (**Table 7**, entries 5-9). The heteroaromatic α,β-unsaturated aldehyde, 3-(2-furyl)acrylaldehyde, could participate in the reaction as well, giving the best yield of 53% with 70% ee (**Table 7**, entry 11). Besides aromatic α,β-unsaturated aldehydes, aliphatic α,β-unsaturated aldehydes were also applicable to the reaction. The straight chain aliphatic α,β-unsaturated aldehyde gave a less efficient result of 35% yield and 40% ee (**Table 7**, entry 12), while the branched aliphatic α,β-unsaturated aldehyde exhibited the best ee of 84% with 38% yield (**Table 7**, entry 13). In general, the desired products were obtained with moderate to good enantioselectivities, but yields were not so high. In all cases, no obvious by-products were observed. Long reaction time was required in this investigated promiscuous activity of pepsin, which indicated that the reaction rates with substrates that are not natural to an enzyme are usually inefficiency and slower than those observed for native substrates.

Table 7 Investigation of substrate scope for the pepsin-catalyzed domino reaction [a]



1 **2** **3**

Entry	R	Product No.	Time (h)	Yield (%)[b]	ee (%)[c]

1	Ph	**3a**	96	46	70
2	4-MeC_6H_4	**3b**	132	36	72
3	2-$OMeC_6H_4$	**3c**	132	44	71
4	4-$OMeC_6H_4$	**3d**	132	50	70
5	4-ClC_6H_4	**3e**	144	42	66
6	3-ClC_6H_4	**3f**	120	41	56
7	2-ClC_6H_4	**3g**	120	40	50
8	4-BrC_6H_4	**3h**	144	39	65
9	2-BrC_6H_4	**3i**	120	36	55
10	4-$NO_2C_6H_4$	**3j**	144	40	60
11	2-furanyl	**3k**	144	53	70
12	propyl	**3l**	168	35	40
13	*i*-propyl	**3m**	168	38	84

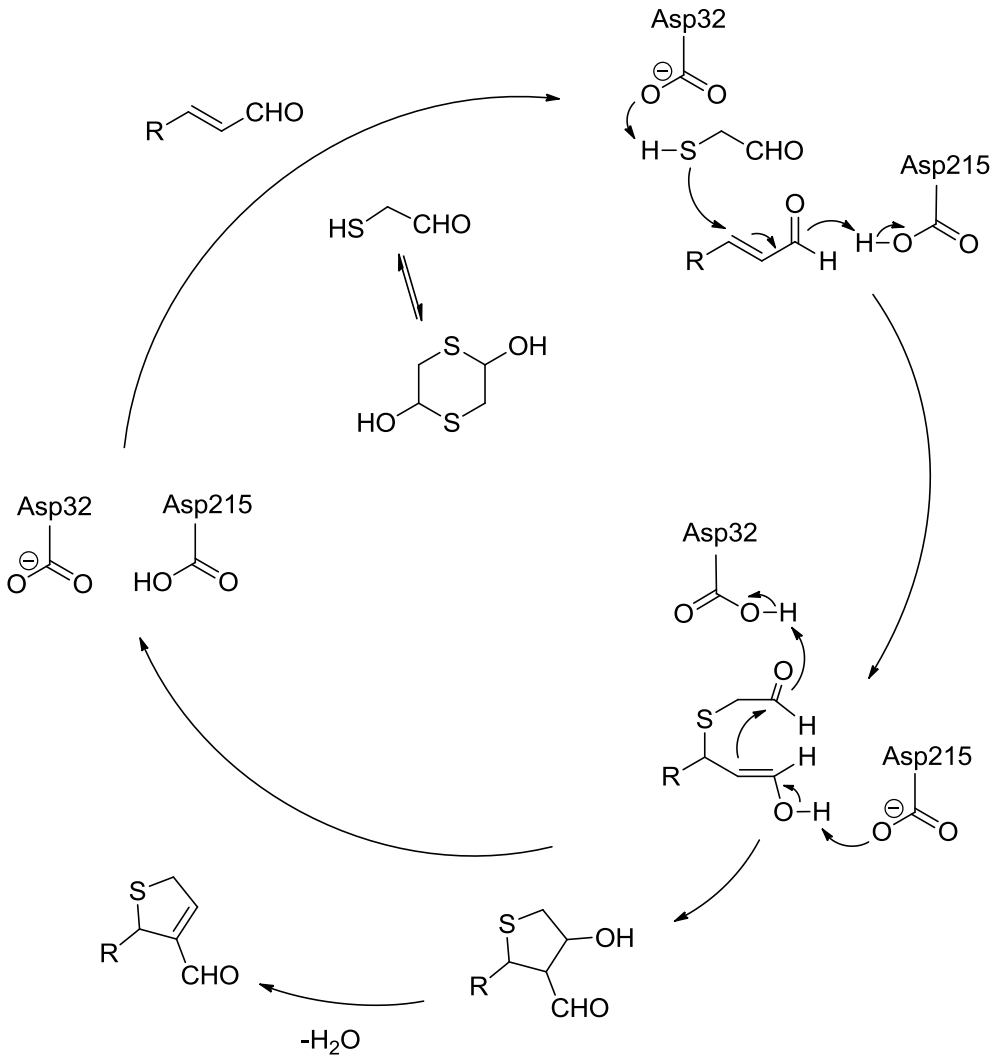
[a] Reaction conditions: a mixture of α,β-unsaturated aldehyde **1** (0.35 mmol), 1,4-dithiane-2,5-diol **2** (1.05 mmol), MeCN (1.20 mL), phosphate buffer (NaH_2PO_4-Na_2HPO_4, 0.067 M, pH 6.5, 0.30 mL) and pepsin (16.9 kU) was stirred at 30 ℃.

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

[c] Determined by chiral HPLC using a chiralpak AS-H or chiralcel OD-H column.

The control experiments with denatured and inhibited pepsin as well as the experiments with recombinant and mutant enzyme clearly demonstrated that the domino reaction occurs in the active site of pepsin, and the Asp residues located in the active centre are responsible for this promiscuous activity. According to the literature,[30] the catalytic site of pepsin is formed by two aspartate residues, Asp32 and Asp215, one of which (Asp215) has to be protonated, and the other (Asp32) deprotonated, for the protein to be active. Thus, based on the control experiments and literature, we attempted to proposed a possible mechanism for the pepsin-catalyzed domino reaction of α,β-unsaturated aldehydes and 1,4-dithiane-2,5-diol (**Scheme 2**). Firstly, Asp32 acts as a base to take away a proton from the mercaptoacetaldehyde which is generated *in situ* from

1,4-dithiane-2,5-diol. Secondly, the activated mercaptoacetaldehyde and α,β-unsaturated aldehyde undergo the intermolecular thia-Michael addition, and the derived adduct accepts the proton from Asp215 forming the enol. Thirdly, the tetrahydrothiophen scaffold is provided through a subsequent intramolecular aldol reaction. Finally, the dehydration process occurs forming the dihydrothiophen skeleton.



Scheme 2 The proposed mechanism for the pepsin-catalyzed domino reaction

Conclusion

In summary, we have shown a novel promiscuous pepsin-catalyzed asymmetric domino

thia-Michael/aldol condensation reaction of aromatic, heteroaromatic and aliphatic α,β-unsaturated aldehydes with 1,4-dithiane-2,5-diol in MeCN/buffer for the first time. The corresponding functionalized chiral dihydrothiophenes were obtained with 40-84% ee without additive. The specific catalytic effect of pepsin was demonstrated by combining some control experiments and site-directed mutagenesis. This is the first study utilizing site-directed mutagenesis to confirm the promiscuous activity of pepsin. This work broadens the scope of enzyme catalyzed transformations. The performance of pepsin in the present work as well as its other promiscuous activities reported previously demonstrate that pepsin has the considerable potential on broad catalytic promiscuity, which may enable organic chemists to rapidly develop new synthetic applications of pepsin to expand the repertoire of synthetic organic methodologies.

Experimental Section

Materials

Pepsin from porcine gastric mucosa [EC number 3.4.23.1, CAS number: 9001-75-6, product number: P7125-100G, Lot # SLBD7698V, 721 units per mg of protein, 18% protein (UV). One unit will produce a change in ΔA_{280nm} of 0.001 per minute at pH 2.0 at 37℃ measured as TCA-soluble products using hemoglobin as substrate] was purchased from Sigma-Aldrich. Unless otherwise noted, all reagents were purchased from commercial suppliers and used without further purification.

General procedure for the pepsin-catalyzed domino thia-Michael/aldol reaction

Pepsin (16.9 kU) was added to a 10 mL round-bottom flask containing α,β-unsaturated aldehyde

(0.35 mmol), 1,4-dithiane-2,5-diol (1.05 mmol), MeCN (1.20 mL) and phosphate buffer (NaH_2PO_4-Na_2HPO_4, 0.067 M, pH 6.5, 0.30 mL). The resultant mixture was stirred at 30 ℃ for the specified reaction time and monitored by thin-layer chromatography (TLC). 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 crude product was purified by flash column chromatography with petroleum ether/ethyl acetate (4:1 – 8:1) as eluent.

*(R)-2-phenyl-2,5-dihydrothiophene-3-carbaldehyde (**3a**) (Table 7, Entry 1).*[20] (30.6 mg, 46%, brown solid, mp 68-70 ℃) ^{1}H NMR (600 MHz, $CDCl_3$): 9.71 (s, 1H), 7.28-7.27 (m, 4H), 7.22-7.19 (m, 1H), 7.01-7.00 (m, 1H), 5.50 (d, J = 5.6 Hz, 1H), 4.23-4.19 (m, 1H), 4.02-3.98 (m, 1H); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 187.0, 148.8, 148.7, 142.3, 128.6, 127.5, 54.9, 38.8. HPLC analysis: Chiralpak AS-H, *n*-hexane / *i*-PrOH 90 : 10, flow rate 1.0 mL / min, λ = 240 nm, t_{major} = 15.4 min, t_{minor} = 19.3 min.

(R)-2-(p-tolyl)-2,5-dihydrothiophene-3-carbaldehyde (3b) (Table 7, Entry 2).[20] (25.7 mg, 36%, brown solid, mp 158-160 ℃) ^{1}H NMR (600 MHz, $CDCl_3$): δ = 9.73 (s, 1H), 7.17 (d, J = 8.1 Hz, 2H), 7.09 (d, J = 7.9 Hz, 2H), 7.02-7.01 (m, 1H), 5.49 (d, J = 5.5 Hz, 1H), 4.24-4.20 (m, 1H), 4.03-3.99 (m, 1H), 2.30 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 187.1, 148.9, 148.5, 139.3, 137.2, 129.3, 127.3, 54.7, 38.6, 21.1. HPLC analysis: Chiralcel OD-H, *n*-hexane / *i*-PrOH 95 : 5, flow rate 0.5 mL / min, λ = 244 nm, t_{major} = 23.0 min, t_{minor} = 25.8 min.

*(R)-2-(2-methoxyphenyl)-2,5-dihydrothiophene-3-carbaldehyde (**3c**) (Table 7, Entry 3).*[20] (33.9 mg, 44%, brown solid, mp 152-154 ℃) ^{1}H NMR (600 MHz, $CDCl_3$): δ = 9.80 (s, 1H), 7.20 (ddd, J =

1.6, 1.1, 1.6 Hz, 1H), 7.12-7.10 (m, 1H), 6.97 (dd, *J* = 1.7, 1.5 Hz, 1H), 6.87-6.85 (m, 2H), 5.88 (d, *J* = 5.6 Hz, 1H), 4.13-4.09 (m, 1H), 3.98-3.94 (m, 1H), 3.86 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 187.2, 156.5, 150.1, 148.0, 130.5, 128.5, 127.0, 120.7, 111.0, 55.7, 47.9, 38.2. HPLC analysis: Chiralcel OD-H, *n*-hexane / *i*-PrOH 90 : 10, flow rate 1.0 mL / min, λ = 220 nm, t_{major} = 18.8 min, t_{minor} = 15.1 min.

*(R)-2-(4-methoxyphenyl)-2,5-dihydrothiophene-3-carbaldehyde (**3d**) (Table 7, Entry 4).*[20] (38.5 mg, 50%, brown solid, mp 134-136 ℃) ^{1}H NMR (600 MHz, $CDCl_3$): δ = 9.73 (s, 1.0 Hz), 7.22-7.20 (m, 2H), 7.01-7.00 (m, 1H), 6.83-6.81 (m, 2H), 5.49 (d, *J* = 5.5 Hz, 1H), 4.24-4.20 (m, 1H), 4.03-4.00 (m, 1H), 3.77 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 187.1, 159.0, 148.9, 148.3, 134.4, 128.6, 114.0, 55.3, 54.5, 38.6. HPLC analysis: Chiralcel OD-H, *n*-hexane / *i*-PrOH 90 : 10, flow rate 1.0 mL / min, λ = 244 nm, t_{major} = 14.9 min, t_{minor} = 19.8 min.

*(R)-2-(4-chlorophenyl)-2,5-dihydrothiophene-3-carbaldehyde (**3e**) (Table 7, Entry 5).*[20] (33.0 mg, 42%, brown solid, mp 145-146 ℃) ^{1}H NMR (600 MHz, $CDCl_3$): δ = 9.73 (s, 1H), 7.26-7.24 (m, 4H), 7.04-7.03 (m, 1H), 5.48-5.46 (m, 1H), 4.25-4.21 (m, 1H), 4.05-4.02 (m, 1H); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 186.8, 148.9, 148.6, 140.8, 133.2, 128.9, 128.7, 54.3, 38.8. HPLC analysis: Chiralcel OD-H, *n*-hexane / *i*-PrOH 90 : 10, flow rate 1.0 mL / min, λ = 240 nm, t_{major} = 11.4 min, t_{minor} = 13.1 min.

*(R)-2-(3-chlorophenyl)-2,5-dihydrothiophene-3-carbaldehyde (**3f**) (Table 7, Entry 6).*[20] (32.2 mg,

41%, brown solid, mp 140-142 ℃) ^{1}H NMR (600 MHz, $CDCl_3$): δ = 9.74 (s, 1H), 7.26 (s, 1H), 7.22-7.17 (m, 3H), 7.07-7.05 (m, 1H), 5.45 (d, *J* = 5.4 Hz, 1H), 4.26-4.22 (m, 1H), 4.06-4.02 (m, 1H); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 186.8, 149.2, 148.4, 144.3, 134.4, 129.8, 127.7, 127.6, 125.9, 54.4, 38.9. HPLC analysis: Chiralcel OD-H, *n*-hexane / *i*-PrOH 90 : 10, flow rate 1.0 mL / min, λ = 240 nm, t_{major} = 11.3 min, t_{minor} = 12.9 min.

*(S)-2-(2-chlorophenyl)-2,5-dihydrothiophene-3-carbaldehyde (**3g**) (Table 7, Entry 7).*[20] (31.5 mg, 40%, brown solid, mp 179-182 ℃) ^{1}H NMR (600 MHz, $CDCl_3$): δ = 9.74 (s, 1H), 7.26 (s, 1H), 7.22-7.17 (m, 3H), 7.07-7.05 (m, 1H), 5.45 (d, *J* = 5.3 Hz, 1H), 4.26-4.22 (m, 1H), 4.06-4.02 (m, 1H); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 186.8, 149.2, 148.4, 144.3, 134.4, 129.8, 127.7, 127.6, 125.9, 54.4, 38.9. HPLC analysis: Chiralcel OD-H, *n*-hexane / *i*-PrOH 90 : 10, flow rate 1.0 mL / min, λ = 228 nm, t_{major} = 15.7 min, t_{minor} = 19.2 min.

*(R)-2-(4-bromophenyl)-2,5-dihydrothiophene-3-carbaldehyde (**3h**) (Table 7, Entry 8).*[20] (36.7 mg, 39%, brown solid, mp 107-110 ℃) ^{1}H NMR (600 MHz, $CDCl_3$): δ = 9.73 (s, 1H), 7.41-7.40 (m, 2H), 7.18-7.16 (m, 2H), 7.04-7.03 (m, 1H), 5.45 (d, *J* = 5.5 Hz, 1H), 4.25-4.20 (m, 1H), 4.05-4.01 (m, 1H); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 186.8, 149.0, 148.5, 141.3, 131.7, 129.3, 121.3, 54.4, 38.8. HPLC analysis: Chiralcel OD-H, *n*-hexane / *i*-PrOH 90 : 10, flow rate 1.0 mL / min, λ = 236 nm, t_{major} = 12.5 min, t_{minor} = 13.9 min.

*(S)-2-(2-bromophenyl)-2,5-dihydrothiophene-3-carbaldehyde (**3i**) (Table 7, Entry 9).*[20] (33.9 mg, 36%, brown solid, mp 152-154 ℃) ^{1}H NMR (600 MHz, $CDCl_3$): δ = 9.81 (s, 1H), 7.55 (d, *J* =

10.0 Hz, 1H), 7.24-7.20 (m, 2H), 7.09-7.05 (m, 2H), 5.93 (d, *J* = 5.5 Hz, 1H), 4.17-4.12 (m, 1H), 4.04-4.00 (m, 1H); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 186.8, 150.6, 147.6, 141.4, 133.1, 128.8, 127.8, 123.8, 53.9, 38.3. HPLC analysis: Chiralcel OD-H, *n*-hexane / *i*-PrOH 90 : 10, flow rate 1.0 mL / min, λ = 236 nm, t_{major} = 19.0 min, t_{minor} = 23.5 min.

*(R)-2-(4-nitrophenyl)-2,5-dihydrothiophene-3-carbaldehyde (**3j**) (Table 7, Entry 10).*[20] (32.9 mg, 40%, brown solid, mp 136-137 ℃) ^{1}H NMR (600 MHz, $CDCl_3$): δ = 9.75 (s, 1H), 8.15 (d, J = 8.7 Hz, 2H), 7.46 (d, J = 8.6 Hz, 2H), 7.13 (s, 1H), 5.55 (d, J = 5.8 Hz, 1H), 4.31-4.26 (m, 1H), 4.13-4.09 (m, 1H); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 186.7, 149.8, 149.5, 148.0, 128.5, 123.9, 54.2, 39.1. HPLC analysis: Chiralcel OD-H, *n*-hexane / *i*-PrOH 90 : 10, flow rate 1.0 mL / min, λ = 220 nm, t_{major} = 31.0 min, t_{minor} = 33.9 min.

*(S)-2-(furan-2-yl)-2,5-dihydrothiophene-3-carbaldehyde (**3k**) (Table 7, Entry 11).*[20] (33.4 mg, 53%, brown oil) ^{1}H NMR (600 MHz, $CDCl_3$): δ = 9.79 (s, 1H), 7.31 (d, *J* = 1.1 Hz, 1H), 7.08-7.06 (m, 1H), 6.30-6.29 (m, 1H), 6.15 (d, *J* = 3.2 Hz, 1H), 5.60 (d, *J* = 5.3 Hz, 1H), 4.23-4.19 (m, 1H), 4.00-3.95 (m, 1H); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 186.8, 153.4, 149.8, 146.0, 142.1, 110.7, 106.6, 47.3, 38.3. HPLC analysis: Chiralcel OD-H, *n*-hexane / *i*-PrOH 90 : 10, flow rate 1.0 mL / min, λ = 232 nm, t_{major} = 16.2 min, t_{minor} = 14.1 min.

*(R)-2-propyl-2,5-dihydrothiophene-3-carbaldehyde (**3l**) (Table 7, Entry 12).*[20] (19.1 mg, 35%, brown oil) ^{1}H NMR (600 MHz, $CDCl_3$): δ = 9.75 (s, 1H), 6.85 (dd, *J* = 4.1, 2.9 Hz, 1H), 3.97 – 3.93 (m, 1H), 3.85 – 3.81 (m, 1H), 2.01 – 1.95 (m, 1H), 1.57 – 1.51 (m, 2H), 1.43 – 1.35 (m, 2H), 0.90 (t, *J*

= 7.4 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 187.9, 149.6, 149.4, 52.1, 38.3, 37.5, 20.5, 13.7. HPLC analysis: Chiralpak AS-H, *n*-hexane / *i*-PrOH 90 : 10, flow rate 1.0 mL / min, λ = 236 nm, t_{major} = 6.6 min, t_{minor} = 7.4 min.

*(R)-2-isopropyl-2,5-dihydrothiophene-3-carbaldehyde (**3m**) (Table 7, Entry 13).*[20] (20.7 mg, 38%, brown oil) ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.76 (s, 1H), 6.92 – 6.90 (m, 1H), 4.54 – 4.53 (m, 1H), 3.83 – 3.80 (m, 2H), 2.42 – 2.39 (m, 1H), 0.95 (d, *J* = 6.8 Hz, 3H), 0.77 (d, *J* = 6.7 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 187.8, 150.5, 148.6, 59.5, 37.7, 30.3, 21.8, 15.2. HPLC analysis: Chiralpak AS-H, *n*-hexane / *i*-PrOH 90 : 10, flow rate 1.0 mL / min, λ = 244 nm, t_{major} = 7.0 min, t_{minor} = 7.9 min.

Construction of the expression vector for the production of pepsinogen A and Asp91Ala mutant (See the Supporting Information for details.)

The cDNA for swine pepsinogen A precursor (accession number GI: 164603) was synthesized from GenScript Corporation (Nanjing, China). The fragment of pepsinogen A which coding sequence begins at amino acid position L16 (and thereby lacking the N-terminal signal peptide) was generated by PCR amplification, by using primers 5'-GGAATTCCATATGTTGGTAAAAGTTCCATTA-3' and 5'-CCCAAGCTTAGCGACTGGGGCTAAA-3' to introduce *Nde*I and *Hind*III restriction sites (underlined sequence) at the 5' and 3' ends, respectively. The PCR products were cloned into the pET28a (+) vector. The Asp91Ala point mutant construct was generated by QuikChange™ Site-Directed Mutagenesis Kit (Stratagene). All constructs were confirmed by DNA sequencing.

Expression and purification of proteins (See the Supporting Information for details.)

Wild type pepsinogen A and Asp91Ala mutant were produced in *E. coli* BL21 (DE3) by using the pET28a (+) expression system. Protein expression was overnight induced at 30 ℃ with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) after OD600 reached 0.6-0.8. A cell pellet was harvested by centrifugation, resuspended and lysed by high-pressure homogenization, and soluble protein was fractionated from insoluble material by centrifugation. The targeted protein was purified by Ni-NTA affinity chromatography. 25 mg purified wild type pepsinogen A and 20 mg purified Asp91Ala mutant were obtained from one liter cell culture, respectively.

Activation of the pepsinogen A and Asp91Ala mutant

The pepsinogen A and Asp91Ala mutant were activated according to the literature.[29] After activation, the 60-385 amino acid residues were retained to get the active pepsin and Asp32Ala mutant (numbering for porcine pepsin A). Enzyme assay was conducted according to the reference.[31] The activity of the pepsin was 299 U/mg protein (Unit definition: one unit will produce a change in ΔA_{280nm} of 0.001 per minute at pH 2.0 at 37 ℃ measured as TCA-soluble products using hemoglobin as substrate); no activity was detected for Asp32Ala mutant pepsin.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (No. 21276211, No. 21472152 and No. 21542014) and the Basic and Frontier Research Project of Chongqing (cstc2015jcyjBX0106). We thank professor Hong-Tao Li from School of Life Science, Southwest University for kind help with manuscript preparation.

Supporting Information:

General methods, Extra information for optimization of the reaction conditions, The detailed information for construction of the expression vector for the production of swine pepsinogen A and Asp91Ala mutant, and expression and purification of proteins, ^{1}H NMR and ^{13}C NMR spectra of the products, HPLC charts of the products.

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