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Reduction of Benzaldehyde Catalyzed by Papain-Based Semisynthetic Enzymes

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Abstract Some features of native enzyme's active site were used to conjunction with a chemical reagent or modifying group, which would generate new functionality different from the natural enzyme. In order to obtain an efficient catalyst, we have designed four different molecular size N-derivatives of modifiers and introduced them into the active site of papain to obtain new semisynthetic enzymes, which were used as catalyst in reduction of benzaldehyde to yield benzyl alcohol respectively, and the reactions carried out with recycling agent in 0.1 M phosphate buffer pH 6.5 at 37 °C. The results had shown that a longer N-derivative of semisynthetic enzyme had higher catalytic activity. Furthermore, we propose a plausible model for the catalytic mechanism in the semisynthetic enzymes system.

Keywords Papain · Semisynthetic enzyme · Reduction

Introduction

Native enzymes are highly efficient catalysts. However, they are limited in terms of the reaction mechanisms and the substrates they catalyze, so a variety of methods have been developed to design tailor-made biocatalysts [1]. One method for designing a semisynthetic enzyme is to introduce a new modifier into naturally-occurring proteins by chemical modification. The semisynthetic enzyme would generate new functionality different from the natural proteins. The chemical modification of enzymes has been described in detail by Kaiser in 1985 [2]. The essence of Kaiser's strategy was to introduce a cofactor into the active site region of the protein through alkylation of a cysteine residue by flavins that can function as redox catalysts. In this way, several new semisynthetic enzymes had been obtained by modification of thiol-

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containing enzymes [3–10]. Distefano used proteins without any catalytic activity as templates to generalize the approach for creating new semisynthetic enzymes. The group prepared protein-based catalysts by covalent attachment of cofactors onto adipocyte lipid-binding protein and a mutant form of intestinal fatty acid-binding protein [11–17].

In the work described in this article, papain is used as a protein template for the construction of a semisynthetic oxidoreductase. Papain (EC 3.4.22.2) is the most studied plant cysteine protease, an endopeptidase containing a nucleophilic cysteine in the active site. The active site of papain contains a single group in Cys-25. There is enough space to introduce a suitably-sized chemical analogue to modify the active site residue Cys-25, therefore abolishing the proteolytic activity. The extended groove is approximately 25 Å long in the vicinity of the active site residue Cys-25 [18].

In earlier work, thiazolium salts and pyridinium salts have been introduced into the active site of papain [19]. The catalytic efficiency of semisynthetic enzyme depends on the geometry of substrate-template-cofactor complexes. Following the works of Kaiser and Suckling group, we further investigated the modified papain using pyridinium salts with different molecular size of modifiers. In order to improve the efficiency of these systems, we designed four longer side chain N-derivatives of modifiers, which were introduced into the active site of papain. The particular catalytic activity of the semisynthetic enzymes was determined in the reduction of the substrate benzaldehyde. And the catalytic activity of the semisynthetic enzyme increased about 3-fold in 10% ethanol-phosphate buffer compared with pure phosphate buffer.

Materials and Methods

Materials

Papain (EC 3.4.22.2), latex suspension, was purchased from Sigma Chemicals and purified through salt precipitation before use. A 3-acetylpyridine was obtained from Fluka. All other chemicals were of analytical grade and purchased from a local company. All measurements were made at room temperature unless mentioned otherwise.

Preparation of Chemical Modifiers

3-α-Bromoacetyl-*N*-benzylpyridinium bromide 1. 3-Acetylpyridine (2 mL, 0.05 mol) and benzyl-bromide (2.184 mL, 0.05 mol) in dry acetonitrile (40 mL) were refluxed for 8 h. The solvent was removed under vacuum, leaving a light brown solid of *N*-benzyl-3-acetylpyridinium bromide (5.01 g, 93.9%). ^{1}HNMR (400 MHz D_2O) δ 8.2–9.3 (m, pyridine), 7.3 (m, C_6H_5), 5.75 (s, 2H), 2.58 (s, 3H). *N*-benzyl-3-acetylpyridinium bromide (1.44 g, 0.00628 mol) was dissolved in $CHCl_3$ (30 mL), and the solution was cooled in an ice–salt bath. Bromine (0.32 mL, 0.00628 mol) was added dropwise (very slowly) with vigorous stirring and cooling for 1.5 h and for 0.5 h at room temperature. The solvent was removed under vacuum, and the remaining powder was washed using diethyl ether (2.09 g, 90%). ^{1}HNMR (400 MHz D_2O) δ 7.7–8.69 (m, pyridine), 6.9–7.1 (m, C_6H_5), 5.73 (s, 2H), 3.32 (s, 2H).

3-α-Bromoacetyl-*N*-phenylethyl pyridinium bromide 2. 3-Acetylpyridine (0.5 mL, 0.0045 mol) and 2-phenylethyl bromide (0.625 mL, 0.0045 mol) in dry acetonitrile (15 mL) were refluxed 8 h. The solvent was removed under vacuum, leaving a light brown solid of *N*-phenylethyl-3-acetyl-pyridinium bromide (0.84 g, 60.4%). ^{1}HNMR (400 MHz D_2O) δ 7.9–8.8 (m, pyridine), 6.9–7.1 (m, C_6H_5), 4.65 (t, 2H), 3.20 (t, 2H), 2.46 (s, 3H). *N*-phenylethyl-3-acetylpyridinium bromide (0.89 g, 0.0029 mol) was dissolved in CH_3COOH

(15 mL), and the solution was cooled in an ice–salt bath. Bromine (0.15 ml, 0.0029 mol) was added dropwise (very slow) with vigorous stirring and cooling for 1.5 h and for 0.5 h at room temperature. The solvent was removed under vacuum and the remaining powder was washed with diethyl ether (0.76 g, 68%). ^{1}HNMR (400 MHz D_2O) δ 7.8–8.7 (m, pyridine), 6.9–7.2 (m, C_6H_5), 4.75 (t, 2H), 3.4 (t, 2H), 3.15 (s, 2H).

Similarly prepared was 3-α-bromoacetyl-*N*-amyl pyridinium bromide 3. Starting (35%) with 3-acetylpyridine and n-amyl bromide ^{1}HNMR (400 MHz D_2O) δ 8.8–9.2 (m, pyridine), 4.64 (t, 2H), 3.6 (s, 2H), 1.89 (m, 2H), 1.18 (m, 4H), 0.71 (m, 3H).

Similarly prepared was 3-α-bromoacetyl-*N*-hexane pyridinium bromide 4. (39%) starting with 3-acetylpyridine and 1-bromohexane ^{1}HNMR (400 MHz D_2O) δ 8.8–9.2 (m, pyridine), 4.76 (t, 2H), 3.66 (s, 2H), 2.95 (m, 2H), 2.79 (m, 2H), 2.11 (m, 2H), 1.79 (m, 2H), 0.73 (m, 3H).

Preparation of Recycling Agent

N-benzyl-4-dihydronicotinamide 5. Nicotinamide (3.66 g, 0.03 mol) and benzyl chloride (3.2 mL, 0.03 mol) in dry acetonitril (50 mL) were refluxed overnight. The solid was separated by filtration off, and the solvent was removed under vacuum. The white solid was recrystallised from ethanol (95%) and gave a solid of *N*-benzylnicotinamide bromide (7.26 g, 74%). This intermediate (2.0 g, 0.008 mol) was added into a solution of anhydrous sodium carbonate (2.76 g, 0.032 mol) and sodium dithionite (5.14 g, 0.032 mol) in double-distilled water (150 mL). The reaction mixture was heated to 50°C and then was stirred for 10 min. A yellow solid was collected by filtration and recrystallized from ethanol/water giving yellow crystals of *N*-benzyl-4-dihydronicotinamide (1.42 g, 71%). ^{1}HNMR (400 MHz $CDCl_3$) δ 7.3 (m, C_6H_5), 5.7 (m, 1H), 5.15 (s, 1H), 4.75 (m, 1H), 4.2 (s, 2H), 3.17 (m, 2H), 3.17 (s, 2H).

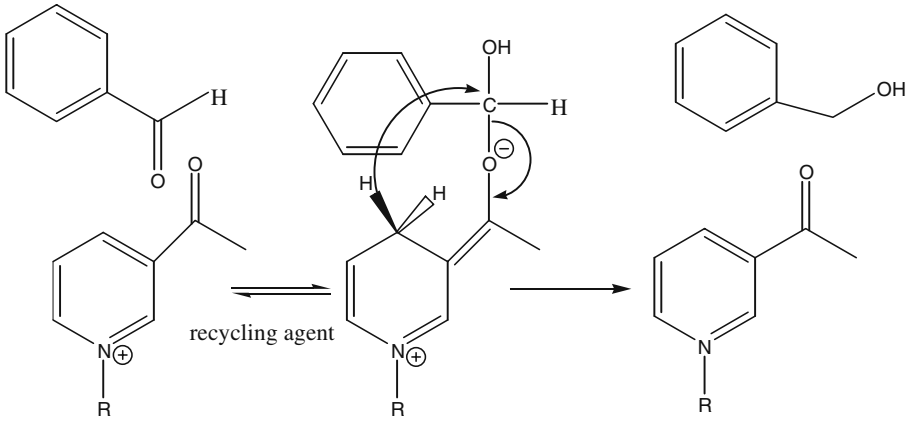
Synthesis of Semisynthetic Enzymes

The semisynthetic enzymes were obtained by alkylation of papain with active halogen derivatives (Scheme 1). Papain (200 mg, 8.6×10^{-6} mol), purified as above, was dissolved in phosphate buffer (4 mL, 0.01 M, pH 7) and mixed with a fivefold excess of pyridinium salts 1. The mixture was stirred for 5 h in ice-bath. Then a second fivefold excess of pyridinium salt 1 was added to solution with fivefold excess of cysteine (5.2 mg, 4.3×10^{-5} mol) and the mixture was stirred for overnight in an ice-bath. A third fivefold excess of pyridium salt 1 with fivefold excess of cysteine (5.2 mg, 4.3×10^{-5} mol) was added and was stirred for another 5 h in an ice-bath.

Similarly prepared were 3-α-bromoacetyl-*N*-phenylethyl-pyridinium modified papain 7 from 2, 3-α-bromoacetyl-*N*-amyl-pyridinium-modified papain 8 from 3, and 3-α-bromoacetyl-*N*-hexane-pyridinium-modified papain 9 from 4.

Purification of Semisynthetic Enzymes

To get the purified semisynthetic enzymes, the crude semisynthetic enzymes were purification with following three steps. Step 1, the semisynthetic enzymes were separated from excess pyridinium salts and cysteine by dialysis for 24–48 h against phosphate buffer (0.1 M, pH 7.0) at 4°C; step 2, the solutions were run in Sephadex G50 column (35× 2.5 cm) and eluted with 0.01 M phosphate buffer, pH 7.0, the fractions of enzymes pooled**;** In the third step, activated thiol-Sepharose (ATS) was chosen as the matrix to separate papain and semisynthetic enzymes. Semisynthetic enzymes were dissolved in phosphate



Scheme 1 Reaction mechanism of the *N*-R-3-acyl-pyridinium salt catalyzed reduction. R=$(CH_2)_4CH_3$, $(CH_2)_5CH_3$, C_7H_7, C_8H_9

buffer (0.01 M, pH 7.0) and elution with the same buffer, the solution freeze-dried (150 mg, 69% yield). After purification of semisynthetic enzymes, the activity of papain of each step measured by using BAEE as substrate.

Reduction of Benzaldehyde by Semisynthetic Enzymes

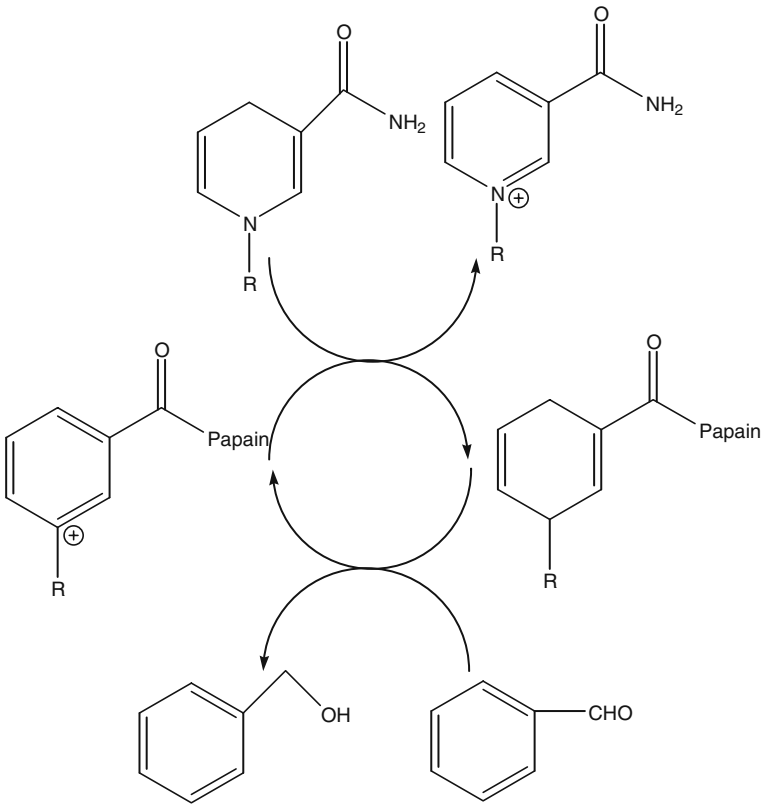
Reactions between benzaldehyde (100 mg) and each of the semisynthetic enzymes (10 mg, 4×10^{-7} mol), papain control and control of mixture (free modifiers and recycling agent) were performed in phosphate buffer (10 mL, 0.1 M, pH 7). *N*-benzyl-4-dihydronicotinamide (150 mg) was added to the solution and stirred for 1.5 h. The substrate was added and the solution incubated for 4 days. Control reactions were performed with the mixture of recycling agent and modifier alone. The reduction of benzaldehyde by semisynthetic enzymes is illustrated in Schemes 1 and 2.

Gas Chromatography Analysis

The ratio of benzaldehyde and benzyl alcohol in reaction mixture was determined by gas chromatography (7890Π, Tianmei Instrument, China) equipped with a flame-ionization detector and a capillary column (FFAP, 30 m×0.25 mm). All reaction mixtures taken from each reaction were timed samples, and the products were determined by GC (start temperature, 80°C; initial time, 1 min; rate, 10°C/min; final temperature, 200°C; final time, 1 min). The retention times for benzaldehyde and benzyl alcohol were 6.59 min and 10.75 min, respectively. Conversions of reduction reactions catalyzed by four semisynthetic enzymes were showed in Table 2.

Results

The pyridine-dependent reductase is established [20], and the intention of this study was not simply to replicate such chemistry but to further investigate about the behavior of chemical modifiers in the active site of papain and the interaction between them which would affect the catalytic activity. The final purpose is to extend the range of chemical modifiers available and to optimize the reaction conditions.



Scheme 2 Recycling strategy using *N*-benzyl-4-dihydronicotinamide as a recycling agent. R=$(CH_2)_4CH_3$, $(CH_2)_5CH_3$, C_7H_7, C_8H_9

Results of Purification

As shown in Table 1, most of unreacted modifiers had been removed by dialysis; Sephadex G50 column was used for remove the trace of unreacted modifier; in the third step, ATS was used to remove unreacted papain. The SH groups in unreacted papain that have not be modified by modifier would tightly bound on ATS, but semisynthetic enzymes were left in solution. No hydrolytic activity had been observed in the final step of purification which was due to two reasons: (1) the semisynthetic enzymes were completely purified; (2) the Cys residue in the active site of papain was modified, and the semisynthetic enzymes had no hydrolytic activity.

Gel Filtration Chromatography

Gel filtration chromatography was performed using an FPLC system (Pharmacia) and a Hitrap™ SPFF (1 mL) column. Samples of semisynthetic enzymes (0.5 mL) were eluted with 0.05 M NaAc (pH 4.5) at a flow rate of 0.5 mL/min for 2 mL, adjusted the flow rate to 1 mL/min for 5 mL. Then flowed by a linear gradient 0–1.5 M NaCl (total volume, 15 mL). Each chromatographic fraction was analyzed by measurement of A_{280} and concentration of NaCl.

Gel filtration chromatography (Fig. 1) of semisynthetic papain 7 and papain under native conditions gave identical retention times suggesting that semisynthetic papain 7 maintains

Table 1 Purification of semisynthetic enzyme.

Enzyme	Steps[a]	A (λ=253 nm)[b]	Activity (%)	Yield (%)
Papain-control	/	0.514	100[c]	/
Semisynthetic enzyme 6	1	0.117	23	75
	2	0.034	5	70
	3	0	0	69
Semisynthetic enzyme 7	1	0.126	24	78
	2	0.032	6	75
	3	0	0	72
Semisynthetic enzyme 8	1	0.099	19	70
	2	0.026	5	69
	3	0	0	65
Semisynthetic enzyme 9	1	0.085	17	71
	2	0.025	5	69
	3	0	0	63

[a] Three steps for purification of semisynthetic enzymes: step 1, dialysis for 24–48 h against phosphate buffer (0.01 M, pH 7.0) at 4 °C; step 2, run in Sephadex G50 column to remove small molecules; step 3, run in activated thiol-Sepharose to remove unreacted papain

[b] The residual catalytic activity of papain was measured by using BAEE as substrate (λ=253 nm)

[c] The activity of papain control was defined as 100%, the remain activity of unreacted papain was measured

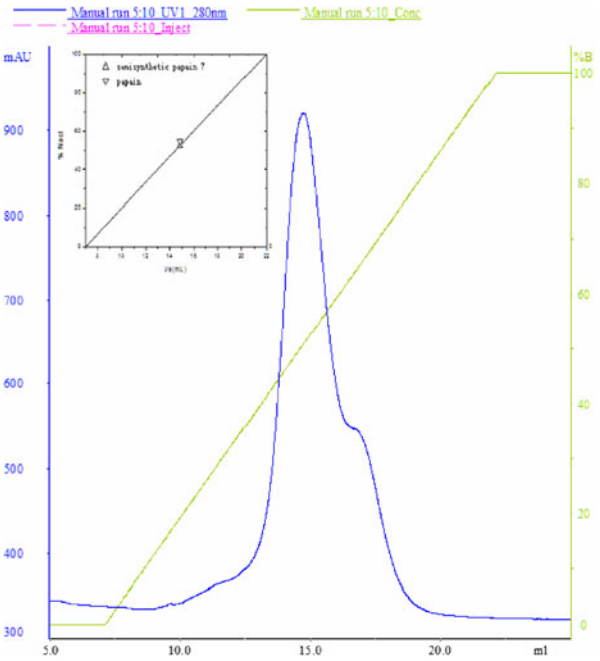


Fig. 1 Gel filtration chromatography of semisynthetic papain 7 using a HitrapTM SSPF(1 mL) column. *Inset*: Elution volume versus concentration of NaCl of standard papain (*empty inverted triangle*) used for the molecular mass determination of semisynthetic papain 7 (*empty triangle*)

the folded structure present in the unmodified protein, the modification of Cys-25 does not result in large perturbations of the protein structure.

Optimal Reaction Conditions

Reactions catalyzed by semisynthetic enzymes were studied also at different pH values (Table 2). The pH value did not significantly influence the outcome of benzyl alcohol. However, we found higher benzyl alcohol concentrations at pH 6.5 compared with reactions at pH of 6.0 and 7.0. Effect of reaction temperature on reactions was investigated. The temperature was set at 30, 35, 37, 40, 45°C, respectively. Total conversion increased with the temperature rising, and it reached the top point at 48 h when the temperature was 37°C, but it decreased at 40°C (data had not been shown). Based on results above, the reaction temperature was fixed at 37°C in the following reactions. As shown in (Fig. 2), the conversion of reactions nearly kept the same when the incubation time was above 96 h. So the optimum time of incubation for semisynthetic enzymes was 96 h.

The conversion degrees of benzaldehyde in phosphate buffer were poor giving from 2.15% to 7.9% yields, respectively, and the results shown in Table 3. These semisynthetic enzymes accelerate the reaction relative to free chemical modifiers. Comparing with the results of Suckling, the catalytic activity of these semisynthetic enzymes is too low. It is probably due to the following two reasons: Firstly, the substrate of benzaldehyde is macro-soluble in water; secondly, in the study of Suckling, the strongly electrophilic carbonyl groups such as ethyl pyruvate and α, α, α-trifluoroacetophenone had been used as substrate, which were easier to be reducted than benzaldehyde.

The low conversion of benzaldehyde is also probably due to both the lack of specific interactions between the substrate of benzaldehyde and conjugate, and the absence of rate-accelerating functional groups within the cavity. The low affinity exhibited by semisynthetic enzymes for benzaldehyde appears to be mirrored in its product interactions. So the development of suitable reaction condition for higher catalytic activity is viable target for this research.

Effect of Solvent

An ideal solvent for biocatalysis should not only maintain the enzyme activity, but also can dissolve substrate as well. Under above-mentioned optimal reaction conditions, the semisynthetic enzymes reacted with benzaldehyde in the presence of recycling agent (*N*-benzyl-4-dihydronicotinamide) in 0.1 M phosphate buffers (pH 6.5) at 37°C for 4 days, but the conversions of reactions were poor in phosphate buffer. The substrate of benzaldehyde

Table 2 Effect of pH on the conversion of reactions.

Semisynthetic enzymes	Conversion (%)[a]		
	pH 6.0	pH 6.5	pH 7.0
6	0.6	2.1	0.5
7	0.4	1.4	0.3

The reactions were performed in 0.1 M phosphate buffer at 37°C with buffers adjusted to pH 6.0, 6.5, 7.0, and stopped after incubation for 48 h. The formation of benzyl alcohol was monitored by GC. But the conversion of semisynthetic enzymes 8 and 9 were too low, the data did not shown in table

[a] The conversion of benzaldehyde in control experiment had been subtracted in total conversion

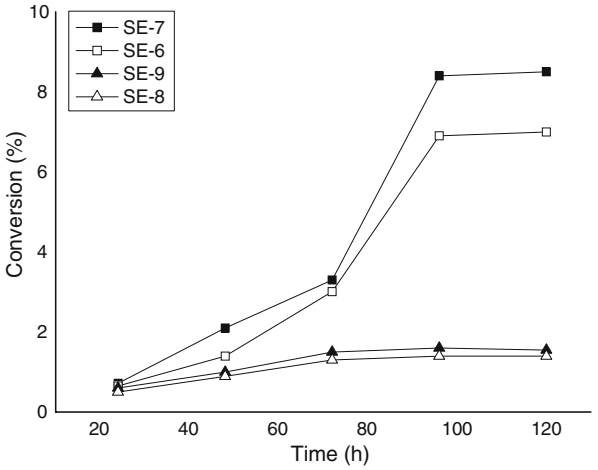


Fig. 2 Effect of time on catalytic activity of semisynthetic enzyme. *SE6* semisynthetic enzyme **b**, *SE7* semisynthetic enzyme 7, *SE8* semisynthetic enzyme 8, *SE9* semisynthetic enzyme 9

is macro-soluble in water, but the product benzyl alcohol is soluble in water. To improve the conversion of reactions, the ethanol-phosphate buffer was chosen as the reaction media based on the findings of Simon [21]. So a certain concentration of ethanol was used in the buffer. As shown in Table 3, the catalytic activity of the semisynthetic enzymes increased about threefold in 10% ethanol-phosphate buffer compared with phosphate buffer. The degree of conversion of the substrate catalyzed by the four enzymes ranged from 6.2% to 21.9%. The best result was obtained from semisynthetic enzyme 7, and semisynthetic enzyme 8 showed the lowest catalysis activity.

The conversion degrees in organic-phosphate buffer are higher than in pure phosphate buffer, because they can dissolve the organic substrate benzaldehyde and recycling agent, and according to the findings of Simon, addition of ethanol would cause the alterations in the secondary and tertiarystructures of the main scaffold of papain, and the catalytic activity of papain increased in low concentration of ethanol. Further studies about the influence of organic-phosphate buffer are in progress.

Table 3 Conversion of reductive reactions catalyzed by different semisynthetic enzymes.

Modifer	Semisynthetic enzyme	Conversion (%)[a]	
		I	II
	Papain control[c]	0	0
3-α-bromoacetyl-*N*-benzylpyridinium bromide 1	6	5.6	15,9
3-α-bromoacetyl-*N*-phenylethyl pyridinium bromide 2	7	7.9	21.9
3-α-bromoacetyl-*N*-amyl pyridinium bromide 3	8	2.15	6.2
3-α-bromoacetyl-*N*-hexane pyridinium bromide 4	9	2.25	6.3

[a] The reactions were stopped after 4 days of incubation, and the production of benzyl alcohol analyzed by GC. The substrate can be reduced in the control experiment of free modifiers and recycling agent, and the conversion of control experiment had been subtracted in total conversion. Solvent 1 is the phosphate buffer. The reactions were performed in 0.1 M phosphate buffer pH 6.5 at 37°C

[b] Papain control after the incubation for 4 days in the similar incubation conditions

Model Explanation

From the experiments performed, there appears to be more significant levels of turnover were obtained using semisynthetic enzymes possessing aromatic side chains modifying group. A possible explanation for this behavior is likely due to the interaction among modifying group and the active site of papain. The catalytic activity depends on the nature of the modifying group, and this suggests that the modifier is conformationally locked due

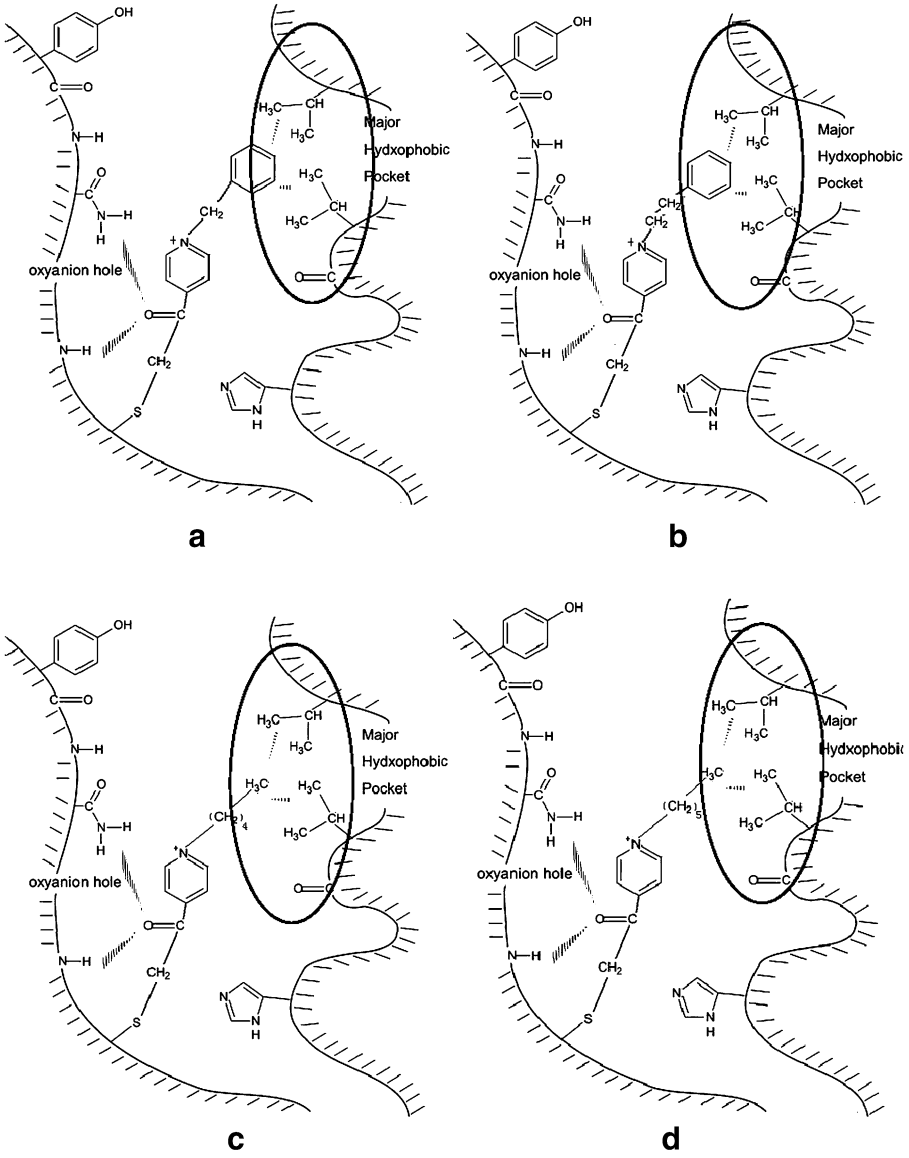


Fig. 3 The model for the interaction of modifiers in the active site of semisynthetic enzymes. The interaction between the modifiers and the hydrophobic pocket of active site of papain were focused in the oval circle. **a** Semisynthetic papain 6. **b** Semisynthetic papain 7. **c** Semisynthetic papain 8. **d** Semisynthetic papain 9

to interaction with the hydrophobic pocket in the active site of papain. We propose a model to show these modifying agents might form different contact with hydrophobic pocket in the active site of papain. These results provide important information about the relation between the active site of papain and chemical modifiers. A direct comparison of conversion for two series of semisynthetic enzymes clearly shows the increase in efficiency for the aromatic side chain of semisynthetic enzymes 6 and 7. As shown in Fig. 3, it is quite clear that *N*-benzyl derivatives appear to be superior to *N*-methyl analogues, the *N*-benzyl derivatives of modifiers would bound more tightly to the hydrophobic pocket in the active site of papain due to a longer aromatic side chain, which has led to higher catalytic activity. The conversion of semisynthetic enzyme 7 is much higher than semisynthetic enzyme 6. Because the introduced *N*-benzyl group is shorter than *N*-phenylethyl derivative, and which leads to a weaker interaction with hydrophobic pocket in the active site of papain. The results are consistent with the conclusion made by Zhu [22] that longer side chain N-derivatives of modifiers are more favorable bonding in the hydrophobic site of papain.

Conclusions

In this paper, four semisynthetic enzymes were successfully made by insertion of different modifiers in the active site of papain, respectively. The result provided additional evidence that chemical modification of existing proteins is certainly a viable method for producing new catalysts. It appears that the semisynthetic enzymes of different modifying groups including 3-bromoacetyl-*N*-benzylpyridinium bromide (1) or 3-bromoacetyl-*N*-phenylethyl pyridinium bromide (2) all gave higher turnovers than other semisynthetic enzymes (8 and 9). Because the longer side chain N-derivatives of modifiers led to higher bonding in the hydrophobic site of papain. The better understanding of the relation between the active site of template enzyme and chemical modification would provide more efficient evidence for optimizing the design of semisynthetic enzymes. The semisynthetic enzymes showed higher catalytic activity in 10% ethanol-phosphate buffer than in pure phosphate buffer. More organic-phosphate buffer would be used in the next step of our further study. And more efforts to introduce additional functionality using chemical modification to increase the rate of reaction are currently in progress.

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