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# Enzymatic Peptide Synthesis in Organic Solvent with Different Zeolites as Immobilization Matrixes

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**Abstract**—A series of zeolite immobilized α-chymotrypsin and thermolysin with microporous Y zeolites (HY, $NH_4Y$, NaY) and mesoporous dealuminized DAY zeolites (HDAY, $HNH_4DAY$) as matrixes have been prepared to catalyze peptide bond formation in organic solvents for the first time. The results indicated that most zeolite immobilized enzymes were active for peptide syntheses in organic media, and still had catalytic activity to some extent after being reused five times. According to the results, the immobilization effect of microporous Y zeolite was better than that of mesoporous DAY zeolite, suggesting that microporous Y zeolite can form more powerful hydrogen bonds with enzyme molecules since there are more hydroxyl groups on the Y zeolite than on the DAY zeolite. In addition, the influences of some reaction conditions such as reaction time and water content of the solvent on the enzymatic peptide synthesis were also studied and optimized. For the two kinds of proteases, $NH_4Y$ zeolite did not show its advantages for thermolysin, but was more suitable for α-chymotrypsin as an immobilization matrix. 

## Introduction[‡]

An immobilized enzyme has more stability and can be reused more times than a free enzyme during the enzymatic process. Utilizing a chemical or physical method, enzyme molecules are bound on the surface of an immobilization matrix. This usually allows the enzyme to maintain its catalytic activity while inhibiting other processes such as autolysis. The adsorptive binding method is a common method that is used in the preparation of immobilized enzymes on various supports such as ion-exchange polymers, celite, alumina, glass powder, etc.[1,2] In the pursuit of better carriers, zeolites have attracted much attention in recent years,[3–6] since they: (i) have unique structural characteristics and are resistant to biodegradation; (ii) possess novel properties such as high surface areas, hydrophobic or hydrophilic behavior and electrostatic interactions; (iii) can be readily prepared with cavities ranging from micropore (<20 Å) to mesopore (20–500 Å) according to the control of preparation technology.

To our knowledge, only a few papers[4–6] have reported that some enzymes such as papain, trypsin or cutinase, etc. have been trapped on zeolites and their catalytic activities measured. However, zeolite immobilized enzymes as catalysts for peptide synthesis have not been reported. The research on enzymatic peptide synthesis in organic solvents has made great progress since 1980s.[7–9] Based on our studies in this field,[10–13] in this article we report a new type of immobilized α-chymotrypsin and thermolysin with different kinds of zeolites as matrixes to catalyze peptide bond formation in organic solvents. The enzymatic peptide synthesis was performed according to Scheme 1. A tripeptide ZTyrGlyGlyOEt, the protected fragment of Leu-enkephalin, was synthesized by immobilized α-chymotrypsin from ZTyrOEt and GlyGlyOEt in dichloromethane. The other model peptide ZAspPheOMe, a precursor of aspartame, was prepared using immobilized thermolysin as catalyst in *tert*-amyl alcohol with ZAspOH and PheOMe as substrates. The preliminary results have previously been briefly reported.[14]

The purpose of the present study is to investigate the influence of zeolite properties such as acidity and pore diameter on the enzymatic peptide synthesis. In the previous papers,[10–13] the syntheses of these two model peptides by free enzymes have been reported. Herein, some immobilized enzyme-catalyzed reaction conditions including reaction time and optimum water content of the solvent

*Keywords*: enzymatic peptide synthesis; immobilization; organic solvent; zeolites.

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[‡] Abbreviations: The amino acid residues which were used in this paper are of L-configuration. Standard abbreviations for amino acid derivatives and peptides are according to the suggestions of the IUPAC-IUB Commission on Biochemical Nomenclature *Eur. J. Biochem.* **1984**, *138*, 9–37. Other abbreviations: Z, benzyloxycarbonyl; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

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$$\text{ZTyrOEt} + \text{GlyGlyOEt} \xrightarrow[\text{dichloromethane}]{\text{Immobilized } \alpha\text{-chymotrypsin}} \text{ZTyrGlyGlyOEt}$$

$$\text{ZAspOH} + \text{PheOMe} \xrightarrow[\textit{tert}\text{-amyl alcohol}]{\text{Immobilized thermolysin}} \text{ZAspPheOMe}$$

**Scheme 1.** Enzymatic strategy for syntheses of two model peptides by zeolite immobilized enzymes.

**Table 1.** Physical data of five kinds of zeolites

| Zeolite | Pore size (Å) | Pore volume (ml/g) | Specific surface ($m^2$/g) | Si/Al |
|---|---|---|---|---|
| HY, $NH_4Y$, NaY | 7.3 | 0.330–0.340 | 930–940 | 2.40–2.45 |
| HDAY, $HNH_4DAY$ | 40 | 0.460 | 840 | 6.10 |

were studied and compared with those of free enzyme-catalyzed reaction. In this work, zeolite immobilized α-chymotrypsin and thermolysin were successfully applied to peptide syntheses in organic solvents for the first time. The experiments indicated that most zeolite immobilized enzymes were active for the model peptides syntheses. Both the zeolite pore size and its acidity played important roles in enzyme adsorption and enzymatic reaction. The preference of immobilization support was distinguishable between the two enzymes used. Additionally, the reusability of zeolite immobilized enzyme was studied.

## Results and Discussion

Five kinds of zeolites were used for immobilization and some of their physical data are listed in Table 1. HY, $NH_4Y$ and NaY zeolites were three kinds of microporous Y zeolites and HDAY, $HNH_4DAY$ zeolites were mesoporous ones. DAY refers to dealuminized Y zeolite that was prepared by chemical and hydrothermal dealumination, and its Si/Al ratio was higher than that of Y zeolite.[15]

Firstly, we prepared five kinds of zeolite immobilized α-chymotrypsins by physical adsorption and studied their utilization in the synthesis of ZTyrGlyGlyOEt with dichloromethane as solvent.

### Reusability of zeolite immobilized α-chymotrypsin and effect of support properties

Fig. 1 illustrates the relationship between peptide yield and the reuse time of zeolite immobilized α-chymotrypsin. The results showed that all the immobilized α-chymotrypsins on different zeolites were active for peptide synthesis, and the catalytic yield declined to a varying extent after being reused 4–5 times. On the whole, the product yield using Y zeolites as matrixes dropped more slowly than using DAY zeolites with increasing the reuse time of immobilized enzyme. Moreover, there was no obvious difference in the reusability of the three kinds of Y zeolite immobilized α-chymotrypsins. When judging from the best yield that each immobilized enzyme obtained during the five times of reuse, HY zeolite was the best matrix since the yield of the second reuse reached the highest (78%) of all the immobilized α-chymotrypsin-catalyzed reactions.

The results indicated that the immobilization effect of microporous Y zeolites was better than that of mesoporous DAY zeolites; that is to say, Y zeolite was a more favorable type of immobilization matrix for α-chymotrypsin than DAY zeolite. Though the pore size of DAY zeolite (40 Å) is much larger than that of Y zeolite (7.3 Å), the enzyme cannot totally enter the inner channels or cages of DAY zeolite due to the large volume of the α-chymotrypsin molecule (50×40×40 $Å^3$). Accordingly, it was perhaps partly taken up or adsorbed on the surface of molecular sieves similarly to the case of Y zeolite. The interaction between enzyme molecules and zeolite is complicated. Apart from the electrostatic action among the charged groups on the surface of the enzyme and the cations from the framework of molecular sieves as well as the negatively charged oxygen atoms in the zeolite framework, there are a lot of hydrogen bonds between the H-bond acceptors on the enzyme and the hydroxyl groups on the zeolite. The cations and OH groups in the zeolite decrease with increasing the Si/Al ratio. After the Y zeolite was dealuminized to enlarge its pore diameter, the Si/Al ratio of DAY zeolite obviously increased. Therefore, there are more cations and OH groups in Y zeolite which can form more powerful hydrogen bonds with enzyme molecules to provide more stable zeolite–enzyme complexes. As a result, Y zeolite immobilized α-chymotrypsin possessed higher catalytic activity than

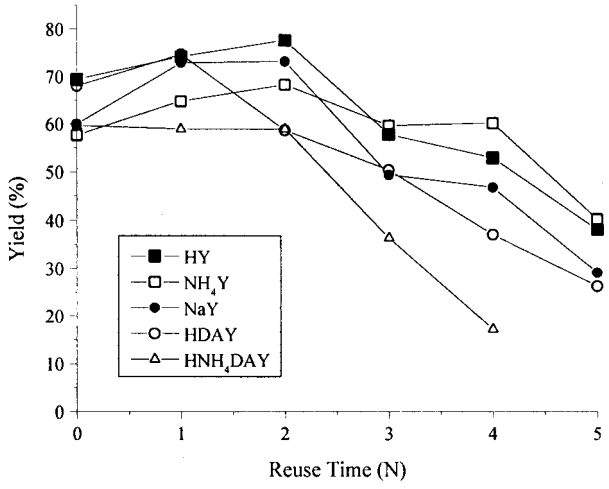


**Figure 1.** Reusability of immobilized α-chymotrypsins in the synthesis of ZTyrGlyGlyOEt. Reaction time: 1 day for the first use and 2 days for the first to fifth reuse.

**Table 2.** Comparison of the one-day yields of ZTyrGlyGlyOEt synthesized by different zeolite immobilized α-chymotrypsins and free α-chymotrypsin

| Zeolite | HY | NaY | $NH_4Y$ | HDAY | $HNH_4DAY$ | Free enzyme |
|---|---|---|---|---|---|---|
| Yield (%) | 69 | 60 | 58 | 68 | 60 | 53 |

DAY zeolite immobilized enzyme when reused several times for the enzymatic reaction.

Table 2 describes the one-day yields of the model reaction catalyzed by different zeolite immobilized α-chymotrypsins and free α-chymotrypsin. After the free α-chymotrypsin was immobilized on zeolites, the yield increased to a varying degree. The increasing activity of HY zeolite immobilized enzyme was the highest among the five kinds of immobilized enzymes used. This showed that it was beneficial to have α-chymotrypsin immobilized using molecular sieves as matrix.

### Effect of reaction time on the zeolite immobilized α-chymotrypsin-catalyzed reaction

From Fig. 2, it can be seen that for the free α-chymotrypsin-catalyzed reaction, a good yield was obtained after carrying out the reaction for 2 days, while for the $NH_4Y$ zeolite immobilized α-chymotrypsin, the 1 day yield was nearly the same as the 3 day yield. Owing to the increase in contact efficiency between enzymes and substrates when α-chymotrypsin molecules were well dispersed on the surface of zeolite, the reaction efficiency was enhanced and therefore the good yield can be reached in less time in the immobilized enzyme-catalyzed reaction than in the free enzyme-catalyzed reaction.

### Effect of water content of dichloromethane on the zeolite immobilized α-chymotrypsin-catalyzed reaction

The water content of the organic solvent greatly influenced the activity of the immobilized enzyme and the product yield. With HY zeolite immobilized α-chymotrypsin as an example, different amounts of water were added to the reaction. Fig. 3 shows the changes that took place when free α-chymotrypsin was immobilized on HY zeolite. The effect of water content of dichloromethane on the free α-chymotrypsin-catalyzed reaction has been studied in our previous works,[10,13] and the optimized water content was 0.15% (v/v). Interestingly, in the HY zeolite immobilized

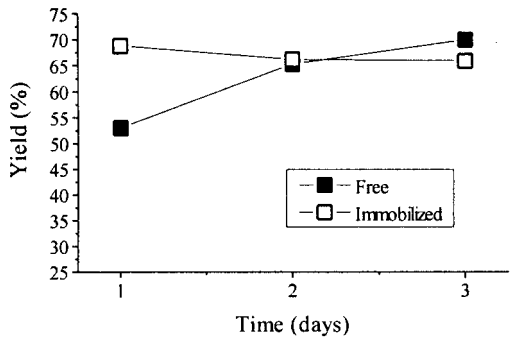


**Figure 2.** Relationship between reaction time and the product yield catalyzed by $NH_4Y$ zeolite immobilized or free α-chymotrypsin.

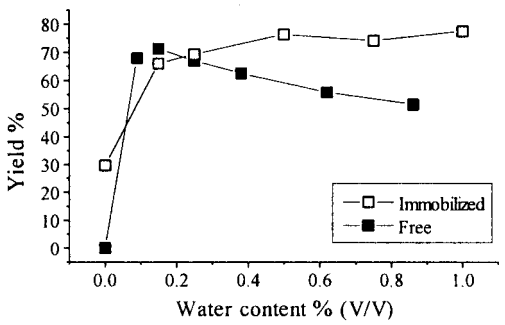


**Figure 3.** Influence of water content on the yield of ZTyrGlyGlyOEt catalyzed by HY zeolite immobilized or free α-chymotrypsin.

α-chymotrypsin-catalyzed peptide synthesis the optimum water content range was broader (0.25–1.0% (v/v)) than in the reaction using free enzyme. Even if no water was injected into the solvent, the model peptide was still obtained in 30% yield. While, in the free α-chymotrypsin-catalyzed model peptide synthesis there was no product obtained without water added, indicating that enzyme activity could not be shown in the dry solvent.

Because molecular sieve itself can easily adsorb water molecules that cannot be completely eliminated during the lyophilization for preparing the immobilized enzyme, the obtained zeolite immobilized enzyme still retains a small amount of water which serves as the essential water for the enzyme in the organic solvent to maintain its catalytic activity in the enzymatic reaction. As a result, the sensitivity of immobilized α-chymotrypsin to the water content decreased. The zeolite immobilized α-chymotrypsin extended the range for the optimum water content of the solvent.

Although the HY zeolite immobilized α-chymotrypsin exhibited high catalytic activity as the water content varied from 0.25 to 1.0% (v/v), when more water was added into the solvent its reusability went down remarkably as Table 3 indicated. In the case of the water content being 0.25%, the HY zeolite immobilized α-chymotrypsin can be efficiently used several times and 58% yield obtained for the third reuse. Compared with this, when 1.0% water content was chosen in the reaction, the activity of immobilized enzyme decreased obviously from the second reuse and the tripeptide was synthesized in low yield (38%). The results showed that high water content can lead to a good yield in the first use, but at the same time the enzyme activity was lost rapidly. Taking both product yield and enzyme reusability into consideration, 0.25% water content of the dichloromethane was selected for the reaction.

**Table 3.** Reusability of HY immobilized α-chymotrypsin under different water contents

| Water content (%, v/v) | Product yield for different reuses of the catalyst (%) | | | |
|---|---|---|---|---|
| | 0 | 1 | 2 | 3 |
| 0.25 | 69 | 74 | 78 | 58 |
| 1.00 | 78 | 77 | 38 | 11 |

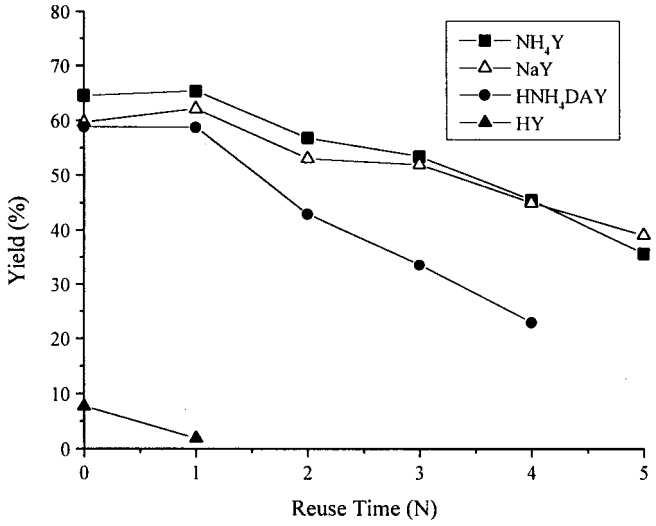


**Figure 4.** Reusability of immobilized thermolysin in the synthesis of ZAspPheOMe. Reaction conditions: ZAspOH was 100 mM and PheOMe was 150 mM in the reactions; water content was 6% (v/v); reaction time was 3 days in all cases; 100 mg immobilized thermolysin was added in the first use.

## Reusability of zeolite immobilized thermolysin and effect of support properties

In order to study the influence of different zeolites as matrixes on the activity of different enzymes, four kinds of zeolite immobilized thermolysins have also been prepared. They were microporous HY, $NH_4Y$, NaY and mesoporous $HNH_4DAY$ zeolite immobilized thermolysins. Their reusability was investigated with enzymatic synthesis of ZAspPheOMe as model reaction.

Unlike immobilized α-chymotrypsin, the catalytic effect of HY immobilized thermolysin was the worst of the four kinds of zeolite immobilized thermolysins used (Fig. 4). Thermolysin is a neutral protease which is stable at pH 6.0–9.0. However, owing to the strong acidity of HY zeolite the pH value of the solution decreased to 3.22 after the thermolysin buffer solution (pH 6.98) was stirred for immobilization over HY zeolite for an hour. As a consequence, the activity of HY zeolite immobilized thermolysin became very low and the yield of dipeptide ZAspPheOMe was poor. HY zeolite immobilized α-chymotrypsin had a high catalytic activity resulting from the broader stable pH range (pH 3–10) of α-chymotrypsin compared with that of thermolysin. With this provision, the acidity of HY zeolite had no noticeable influence on the enzyme activity. The above results indicated that different physical properties of enzymes can lead to them being more effectively immobilized on different matrixes. The exact pH value changes during the enzyme immobilization are shown in Tables 4 and 5.

**Table 4.** The pH value changes during α-chymotrypsin immobilization on different zeolites

| Zeolite | HY | NaY | $NH_4Y$ | HDAY | $HNH_4DAY$ |
|---|---|---|---|---|---|
| Starting pH value of the enzyme solution | 7.95 | 7.95 | 7.95 | 7.95 | 7.95 |
| pH value of the water phase after adsorption on zeolite for 1 h | 4.07 | 7.74 | 7.55 | 6.69 | 7.31 |

**Table 5.** The pH value changes during thermolysin immobilization on different zeolites

| Zeolite | HY | NaY | $NH_4Y$ | $HNH_4DAY$ |
|---|---|---|---|---|
| Starting pH value of the enzyme solution | 6.98 | 6.98 | 6.98 | 6.98 |
| pH value of the water phase after adsorption on zeolite for 1 h | 3.22 | 7.10 | 6.76 | 6.84 |

In the case of the other three zeolites $NH_4Y$, NaY and $HNH_4DAY$, their pH values all approached neutral and were located in the stable pH scope of thermolysin. Thus, with them as immobilization matrixes, thermolysin presented catalytic activity to a certain extent in the reaction. However, it was worth noting that the activity of dealuminized mesoporous $HNH_4DAY$ zeolite immobilized thermolysin was lower than that of $NH_4Y$ or NaY zeolite immobilized enzyme. This observation is similar to that using different zeolite immobilized α-chymotrypsins as catalysts which has been analyzed and described above.

## Effect of reaction time on the zeolite immobilized thermolysin-catalyzed reaction

The influence of reaction time on the synthesis of ZAspPheOMe catalyzed by $NH_4Y$ zeolite immobilized thermolysin is the same as free thermolysin (Fig. 5). In both cases, the model peptide yields increased with increasing the reaction time, and during the same period, the corresponding yields were close to each other. When compared with the influence of reaction time on the zeolite immobilized α-chymotrypsin-catalyzed reaction, the results suggest that for the two kinds of proteases, the zeolite material did not show its advantages for thermolysin, but was more suitable for α-chymotrypsin as an immobilization matrix.

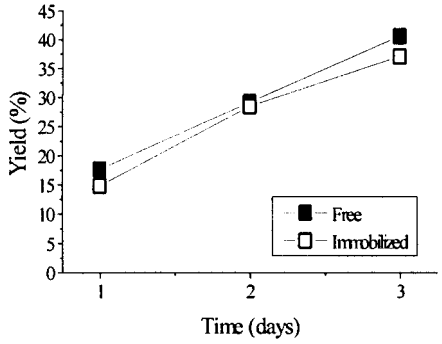


**Figure 5.** Relationship between reaction time and the product yield catalyzed by $NH_4Y$ zeolite immobilized or free thermolysin.

## Effect of water content of *tert*-amyl alcohol on the zeolite immobilized thermolysin-catalyzed reaction

Fig. 6 illustrates the influence of water content of *tert*-amyl alcohol on the peptide yield when $NH_4Y$ zeolite immobilized thermolysin was used. The optimum water content in the $NH_4Y$ zeolite immobilized thermolysin-catalyzed reaction was about 6% (v/v), and it was the same as that (6~8%) in the free thermolysin-catalyzed model peptide synthesis which was reported in the previous articles.[10,11] This result differed from the situation using immobilized and free α-chymotrypsin as catalysts. Compared with *tert*-amyl alcohol (log *P* 0.89),[16] dichloromethane (log *P* 1.25)[16] is a more hydrophobic organic solvent. Thus, adding only a small amount of water into dichloromethane (the optimum water content <1% (v/v)) can provide enough essential water to maintain the activity of α-chymotrypsin. In contrast, more water must be added to *tert*-amyl alcohol (the optimum water content >1% (v/v)) for thermolysin to show its catalytic activity. In addition, due to the optimum water content being low (0.25%) in the immobilized α-chymotrypsin-catalyzed synthesis, a little residual water in the zeolite carriers can maintain the activity of enzyme even when no water was added into dichloromethane. In the case of immobilized thermolysin-catalyzed reaction, when no water was injected, no product was obtained, since the optimum water content of *tert*-amyl alcohol system was high (6%) and the water remaining in the immobilization support was not enough to enable the thermolysin-catalyzed peptide synthesis.

In summary, five kinds of zeolite immobilized α-chymotrypsins and four kinds of zeolite immobilized thermolysins were prepared, and the peptide formation reactions catalyzed by them in organic solvents were investigated and compared. The results demonstrate that it is feasible to use zeolites as supports for enzymatic peptide synthesis. The effects of immobilization matrix and the reaction conditions on the model reactions are complicated. Some problems such as the identification of hydrogen bonds formed between enzyme and zeolite are currently being investigated. The study presented here will be helpful for the investigation of this type of zeolite immobilized enzyme and in finding more valuable and promising immobilization matrixes.

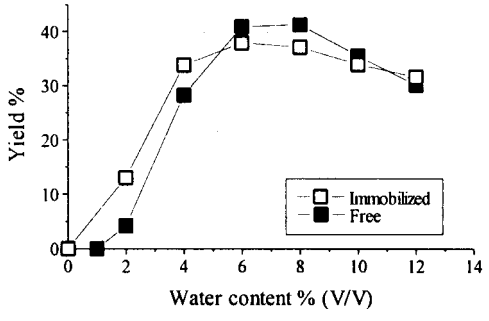


**Figure 6.** Influence of water content on the yield of ZAspPheOMe catalyzed by $NH_4Y$ zeolite immobilized or free thermolysin.

## Experimental

α-Chymotrypsin (EC 3.4.21.1 Type II, from bovine pancreas) and thermolysin (EC 3.4.24.4 from bacillus thermoprotedyticus rokko) were purchased from Sigma Chemical Company. All the zeolites (HY, $NH_4Y$, NaY, HDAY, $HNH_4DAY$) were prepared by our laboratory as described in the literature.[15] ZAspOH, HCl·PheOMe, ZTyrOEt and HCl·GlyGlyOEt were prepared according to the standard method.[17] Dichloromethane and *tert*-amyl alcohol were from the local chemical factory, they were dried over $K_2CO_3$ and distilled before use.

### Preparation of immobilized α-chymotrypsin and thermolysin on different zeolites

α-Chymotrypsin (20 mg) was dissolved in phosphate buffer (4 ml, pH 7.95, 50 mM), or [thermolysin (20 mg) was dissolved in MOPS($Na^+$) buffer (4 ml, pH 6.98, 50 mM)], then zeolite (200 mg) was added at room temperature under stirring. After about an hour, the pH value of the mixture was measured by pH meter and the suspension was lyophilized overnight to obtain the zeolite immobilized α-chymotrypsin or thermolysin.

### Synthesis of ZTyrGlyGlyOEt by different zeolite immobilized α-chymotrypsins (general procedure)

ZTyrOEt (0.5 mmol) and HCl·GlyGlyOEt (0.5 mmol) were suspended in dichloromethane (5 ml), then triethylamine (140 μl) was added and the mixture was shaken thoroughly to make the substrates dissolve completely. After zeolite immobilized α-chymotrypsin (50 mg), water (12.5 μl, $H_2O/CH_2Cl_2$=0.25% (v/v)) were added, the mixture was stirred at room temperature for 1–3 days. At the end of the reaction, the precipitate from the reaction solution and the immobilized enzyme were filtered and washed thoroughly with acetone. Then the immobilized α-chymotrypsin was dried for storage or reuse, and the acetone solution was evaporated to obtain ZTyrGlyGlyOEt.

### Synthesis of ZAspPheOMe by different zeolite immobilized thermolysins (general procedure)

ZAspOH (0.5 mmol) and HCl·PheOMe (0.5–0.75 mmol) were suspended in *tert*-amyl alcohol (5 ml), then triethylamine (220–255 μl) was added and the mixture was shaken thoroughly. After zeolite immobilized thermolysin (50–

100 mg) and 10 mM calcium acetate (0.3–0.4 ml) were added, the solution was stirred at 40°C for 1–3 days. At the end of the reaction, the immobilized enzyme were filtered and washed thoroughly with ethyl acetate. Then the immobilized thermolysin was dried for storage or reuse, and the ethyl acetate solution as well as the filtrate were evaporated under reduced pressure. The oily residue was diluted by ethyl acetate (50 ml), and then washed with 1 M HCl and saturated NaCl, dried over anhydrous $Na_2SO_4$. After removal of solvent, the crude product was recrystallized from methanol–water to give pure ZAspPheOMe.

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