Synthesis of a Precursor of Bioactive Pentapeptide OGP-(10–14) and the Fragment of Enkephalin Catalyzed by MCM-22 Immobilized or Free Proteases in Organic Solvents

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Abstract: Full enzymatic synthesis of a precursor of osteogenic growth peptide fragment (10–14), *Z*-Tyr-Gly-Phe-Gly-Gly-OEt, was accomplished using immobilized proteases on molecular sieve MCM-22 by adsorption as catalyst via 3+2 synthetic route for the first time. The reuse time of immobilized enzyme was dependent on concrete reaction system. Compared with free enzyme, the reaction rate catalyzed by immobilized enzyme was remarkably enhanced and its synthetic yield was also increased in most cases. The effects of water content and the added amount of Et_3N on synthesis of fragments of bioactive peptides including OGP-(10–14) and enkephalin by immobilized enzymes were different from free enzyme.

Key words: immobilized enzyme, MCM-22, OGP-(10–14), enzymatic peptide synthesis, organic solvents

Peptide synthesis catalyzed by protease has attracted much attention in recent years.[1] The enzymatic method presents some noticeable advantages over the chemical coupling, such as minimal racemization, minimal side chain protection, high stereospecificity etc. Enzymatic peptide synthesis in organic media offers special merits. The enzymes are more stable in organic solvents where the formation of peptide bonds is more favorable than the hydrolysis of the peptide. Most organic compounds are soluble in organic solvents.[2,3] However, peptide synthesis by free enzyme exhibits some drawbacks including the cost of enzyme, the tendency of the enzyme to denature and aggregate. In addition to preventing autolysis, immobilized enzymes have more stability and can be reused more times than free enzymes.[4–8] The adsorptive binding method is a mild coupling and common method for immobilization of enzyme on various supports such as ion-exchange polymers, Celite, alumina, glass powder, etc. Molecular sieves are better inorganic carriers for immobilization of enzyme by adsorption because of its resistance of biodegradation, high surface areas, hydrophobic or hydrophilic behavior, electrostatic interactions, etc.[8–10] Some papers reported that molecular sieves were used for immobilization of enzymes and the catalytic activity of immobilized enzyme was measured,[11–15], but as we are aware, few papers deal with peptide synthesis by molecular sieve immobilized enzyme in organic solvents. Our group was the first to report on the enzymatic peptide synthesis of a tripeptide fragment of enkephalin and aspartame precursor in organic solvents with microporous Y zeolites and mesoporous dealuminized DAYzeolites as immobilization matrixes.[8]

Herein, we describe the synthesis of a precursor of bioactive pentapeptide OGP-(10–14), *Z*-Tyr-Gly-Phe-Gly-Gly-OEt, and the fragment of enkephalin using microporous molecular sieve MCM-22 immobilized proteases by adsorption or free proteases as catalyst in organic solvents. The osteogenic growth peptide (OGP) is a naturally occurring tetradecapeptide identical to the C-terminal amino acid sequence 89–102 (H-Ala-Leu-Lys-Arg-Gln-Gly-Arg-Thr-Leu-Tyr-Gly-Phe-Gly-Gly-OH) of histone H4.[16,17] Endogenous OGP is a proteolytic cleavage product of PreOGP translated from H4 mRNA via alternative translational initiation at a downstream initiation codon.[18] OGP increases bone formation and density when administered to rats.[16] OGP regulates proliferation, alkaline phosphatase activity, matrix mineralization and hematopoiesis.[16,19–22] The OGP C-terminal pentapeptide, H-Tyr-Gly-Phe-Gly-Gly-OH [OGP-(10–14)], shares the OGP-like in vitro mitogenic effect and in vivo stimulation of osteogenesis and hematopoiesis. It is the minimal OGP-derived sequence that retains the peptide's full proliferative activity.[16] In this paper, full enzymatic synthesis of OGP-(10–14) precursor by MCM-22 immobilized α-chymotrypsin and papain via 3+2 (Scheme 1) synthetic route in organic solvents was successfully achieved for the first time. Synthesis of fragments of bioactive peptides including OGP-(10–14) and enkephalin by immobilized enzyme was compared with free enzyme in regard to water content, the added amount of Et_3N, reaction yield and rate etc. The reuse time of immobilized enzyme was also investigated.

A series of fragments of OGP-(10–14) were synthesized by the catalysis of adsorptively immobilized α-chymotrypsin on MCM-22 in cyclohexane (Table). The results indicated that the synthetic yield of products by immobilized α-chymotrypsin on MCM-22 was higher than those of free α-chymotrypsin in most cases. This could be ex-

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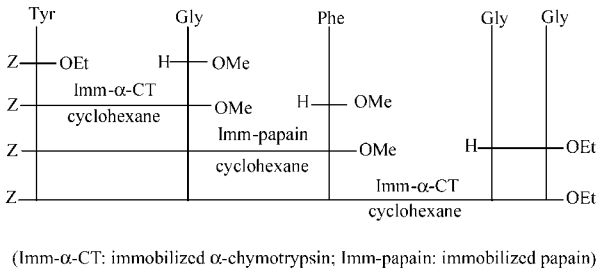


(Imm-α-CT: immobilized α-chymotrypsin; Imm-papain: immobilized papain)

Scheme 1 3+2 Synthetic route to the precursor of bioactive pentapeptide OGP-(10–14)

plained by the fact that α-chymotrypsin molecule was homogeneously dispersed on the surface of MCM-22, which increased the contact efficiency between enzymes and substrates. So the reaction efficiency by immobilized enzyme was enhanced and the yield was increased comparable to free α-chymotrypsin.[2] As for *Z*-Tyr-Gly-OMe, the synthetic yield by immobilized enzyme was lower than that by free enzyme probably because immobilized enzymes were easily gathered together and adsorbed on the bottom of reaction flask under reaction conditions, which prevented immobilized enzymes from efficiently contacting with substrates and decreased the yield. Moreover, the effect of water content on the synthetic yield of *Z*-Phe-Gly-Gly-OEt by MCM-22 immobilized α-chymotrypsin in cyclohexane was different from that of free α-chymotrypsin to some extent. When no water was added to the reaction system, the expected product was not obtained using free enzyme as catalyst because of lack of essential water that made the enzyme inactive; however, the corresponding product was obtained using immobilized enzyme as catalyst in 78% yield (Figure 1).

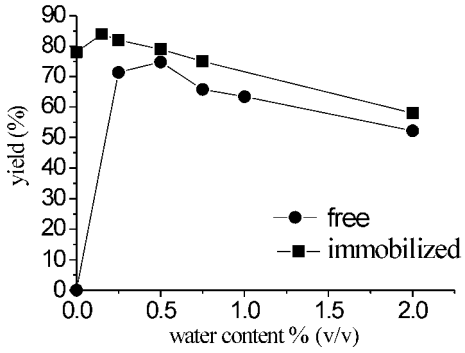


Figure 1 The effect of water content on the yield of *Z*-Phe-Gly-Gly-OEt catalyzed by free and MCM-22 immobilized α-chemotrypsin

Molecular sieves have a strong ability for adsorbing water, so the water from the immobilized enzyme could not be completely removed during the lyophilization for preparation of immobilized enzyme. Therefore, the immobilized enzyme itself still retains a small amount of water which serves as the essential water for the enzyme immobilized on MCM-22 to maintain its catalytic activity in organic solvents without addition of water.[2] The optimal water content (0.15%) for the reaction by immobilized enzyme was lower compared to free enzyme. This may also result from a small amount of water retained in immobilized enzyme. The synthetic yield by immobilized enzyme

Table Yields of OGP-(10–14) Precursor and its Fragments Synthesized by Free Proteases or Immobilized Proteases in Cyclohexane

No	Carboxyl Component	Amino Component	Product	Enzyme	Yield (%)
1	*Z*-Tyr-OEt	Gly-OEt	*Z*-Tyr-Gly-OEt	α-CT[a]	63
2	*Z*-Tyr-OEt	Gly-OEt	*Z*-Tyr-Gly-OEt	Imm-α-CT[b]	72
3	Boc-Phe-OCH_2CF_3	Gly-Gly-OEt	Boc-Phe-Gly-Gly-OEt	α-CT	75
4	Boc-Phe-OCH_2CF_3	Gly-Gly-OEt	Boc-Phe-Gly-Gly-OEt	Imm-α-CT	80
5	*Z*-Phe-OCH_2CF_3	Gly-Gly-OEt	*Z*-Phe-Gly-Gly-OEt	α-CT	75
6	*Z*-Phe-OCH_2CF_3	Gly-Gly-OEt	*Z*-Phe-Gly-Gly-OEt	Imm-α-CT	82
7	*Z*-Tyr-OEt	Gly-OMe	*Z*-Tyr-Gly-OMe	α-CT	59
8	*Z*-Tyr-OEt	Gly-OMe	*Z*-Tyr-Gly-OMe	Imm-α-CT	51
9	*Z*-Tyr-Gly-OMe	Phe-OMe	*Z*-Tyr-Gly-Phe-OMe	Pap[c]	62
10	*Z*-Tyr-Gly-OMe	Phe-OMe	*Z*-Tyr-Gly-Phe-OMe	Imm-Pap[d]	63
11	*Z*-Tyr-Gly-Phe-OMe	Gly-Gly-OEt	*Z*-Tyr-Gly-Phe-Gly-Gly-OEt	α-CT	51
12	*Z*-Tyr-Gly-Phe-OMe	Gly-Gly-OEt	*Z*-Tyr-Gly-Phe-Gly-Gly-OEt	Imm-α-CT	71

[a] α-CT: α-chymotrypsin.
[b] Imm-α-CT: MCM-22 immobilized α-chymotrypsin.
[c] Pap: papain.
[d] Imm-papain: MCM-22 immobilized papain.

was obviously decreased as well as that by free enzyme when the water content in the reaction system was beyond the optimal water content. In addition, the effect of the amount of Et_3N added to the reaction system for the synthesis of *Z*-Phe-Gly-Gly-OEt by MCM-22 immobilized α-chymotrypsin was also investigated (Figure 2).

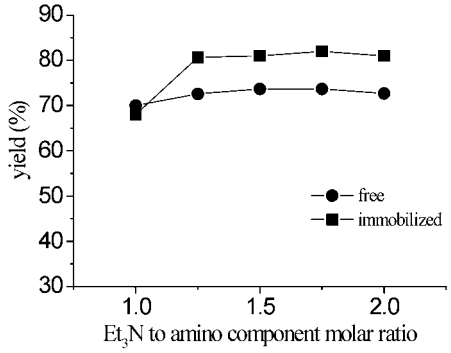
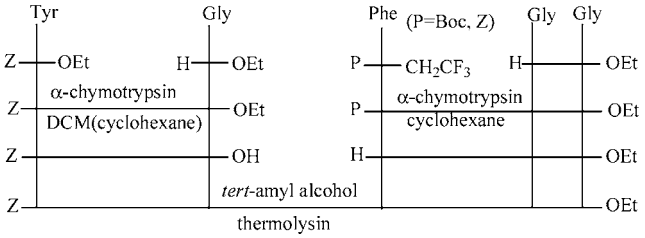


Figure 2 The effect of Et_3N on the yield of *Z*-Phe-Gly-Gly-OEt catalyzed by free or MCM-22 immobilized α-chymotrypsin

The observed pH value of reaction solution would be much higher than 7 even if the equivalent amount of Et_3N was added to the reaction system to neutralize the HCl of amino component. This was caused by the poor solubility of amino component (Gly-Gly-OEt·HCl) in cyclohexane. Therefore, it was difficult to accurately investigate the influence of pH value on the synthesis of *Z*-Phe-Gly-Gly-OEt. The result indicated that the synthetic yield by immobilized enzyme with a 1:1 molar ratio of Et_3N to amino component was clearly lower than that with a 1.25:1–2:1 molar ratio of Et_3N to amino component. However, the amount of Et_3N had little effect on the synthesis of *Z*-Phe-Gly-Gly-OEt by free enzyme when the amount of Et_3N was 1–2 times that of the amino component. This may contribute to the fact that the hydrochloride of amino component could not be completely neutralized by Et_3N when equimolar amounts of Et_3N and amino component were added, because the buffer solution with weak acidity (pH 6.0) for the preparation of immobilized enzyme neutralized a part of Et_3N.

Full enzymatic synthesis of the precursor of bioactive pentapeptide OGP-(10–14) was achieved by immobilized α-chymotrypsin and papain via 3+2 synthetic route (Scheme 1) in cyclohexane for the first time. *Z*-Tyr-Gly-OMe synthesized by MCM-22 immobilized α-chymotrypsin was coupled with Phe-OMe by MCM-22 immobilized papain in cyclohexane to give *Z*-Tyr-Gly-Phe-OMe. Then *Z*-Tyr-Gly-Phe-OMe was condensed with Gly-Gly-OEt to afford the precursor of OGP-(10–14), *Z*-Tyr-Gly-Phe-Gly-Gly-OEt (Table), by the catalysis of MCM-22 immobilized α-chymotrypsin via 3+2 synthetic route in cycohexane with 71% yield. The yield of the precursor by immobilized enzyme was markedly higher than the free enzyme. We also attempted to synthesize the precursor of OGP-(10–14) by thermodynamically controlled peptide synthesis via 2+3 synthetic route using full enzymatic method. Although the precursor of OGP-(10–14) was successfully obtained by α-chymotrypsin and thermolysin via 2+3 synthetic route (Scheme 2), we failed to synthesize the precursor by immobilized enzyme via 2+3 synthetic route because the amount of thermolysin immobilized on MCM-22 was poor.



Scheme 2 2+3 Synthetic route to the precursor of bioactive pentapeptide OGP-(10–14)

Synthesis of a protected tripeptide fragment of enkephalin, *Z*-Tyr-Gly-Gly-OEt, by MCM-22 immobilized α-chymotrypsin was studied. In contrast to free enzyme, water content of dichloromethane had lower effect on the yield of *Z*-Tyr-Gly-Gly-OEt synthesized by immobilized enzyme (Figure 3).

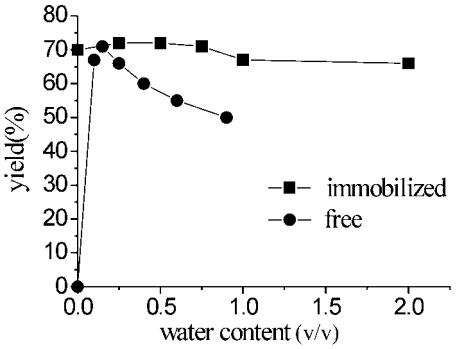


Figure 3 The effect of water content on the yield of *Z*-Tyr-Gly-Gly-OEt catalyzed by free or MCM-22 immobilized α-chymotrypsin

The expected product was obtained in about 70% yield using immobilized enzyme as catalyst whereas the corresponding product was not obtained using free enzyme as catalyst in dichloromethane without addition of water. The reason was same as mentioned above. The yield of product by immobilized enzyme increased slightly (70–72%) when water content varied from 0% to 0.75%. Nevertheless, there was an obvious change for the yield of product by free enzyme. The yield of product increased from 0 to 71% with increasing water content from 0 to 0.15% and distinctly decreased (71–54%) when water content continuously increased from 0.15% to 0.75%.

The yield of product by immobilized enzyme decreased only by 5% even if water content increased from 0.75 to 2%. It showed that water content (0–2%) had low effect on the yield of *Z*-Tyr-Gly-Gly-OEt by immobilized enzyme. However, addition of a little water in reaction system obviously enhanced the reaction rate of synthesis of

Z-Tyr-Gly-Gly-OEt by immobilized enzyme in dichloromethane.[2]

The expected peptide quickly precipitated from reaction solution when a little water was added whereas it took much more time to observe the precipitate of product without the addition of water. Additionally, the reaction rate by immobilized enzyme was much faster than that by free enzyme. The reaction by immobilized enzyme nearly reached equilibrium within about one day while it took 2–3 days for the reaction catalyzed by free enzyme to reach equilibrium because of the increasing contact efficiency between immobilized enzymes and substrates (Figure 4).

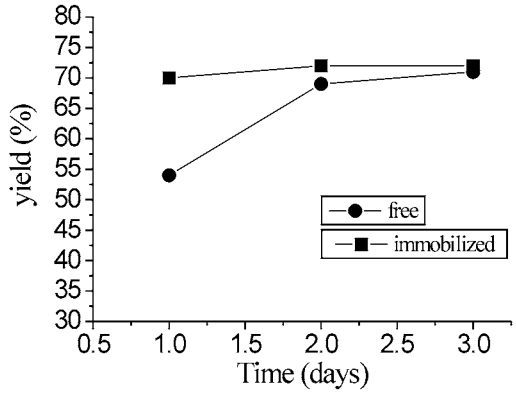


Figure 4 The effect of reaction time on the yield of *Z*-Tyr-Gly-Gly-OEt catalyzed by free or MCM-22 immobilized α-chymotrypsin

Reuse time of immobilized enzyme plays an important role in its practical application. The more reuse times of immobilized enzyme are, the less the amount of immobilized enzyme is consumed, and the lower the cost of enzyme is. The reuse times of MCM-22 adsorptively immobilized α-chymotrypsin were evaluated by the synthesis of *Z*-Tyr-Gly-Gly-OEt and *Z*-Phe-Gly-Gly-OEt in organic solvents (Figure 5). As for synthesis of *Z*-Tyr-Gly-Gly-OEt in dichloromethane, the synthetic yield for the first use of immobilized enzyme was comparable to that of the first reuse. Then the yield of product was gradually declined with increasing of the reuse times of immobilized enzyme. The immobilized enzyme could be repeatedly used for five times. As for synthesis of *Z*-Phe-Gly-Gly-OEt in cyclohexane, there was obviously no difference among the synthetic yields for the first use to the second reuse of immobilized enzyme. The synthetic yield was markedly dropped to 23% for the third reuse of immobilized enzyme. These results indicate that the reuse times of immobilized enzyme were dependent on concrete reaction probably because some factors influencing the activity of immobilized enzyme including substrates, organic solvents and pH value etc., were discrepant in different reactions. Additionally, the yield of product for the first reuse of immobilized enzyme was slightly higher than that for the first use of immobilized enzyme possibly because the immobilized enzyme still retained some amino component that could not be completely eliminated by washing with acetone after the first use. This resulted in increasing the amount of amino component for the next use of immobilized enzyme to catalyze the reaction.

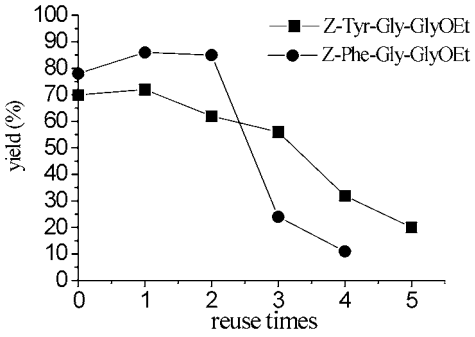


Figure 5 Reusabilitiy of MCM-22 immobilized α-chymotrypsin. Reaction time: 1 day for the first and second use and 2 days for the others

In conclusion, full enzymatic synthesis of a precursor of osteogenic growth peptide (10–14), *Z*-Tyr-Gly-Phe-Gly-Gly-OEt, was synthesized by adsorptively immobilized proteases on molecular sieve MCM-22 via 3+2 synthetic route for the first time. Immobilized enzyme could be used for several times and its reuse time was relevant to concrete reaction system. In comparison with free enzyme, the reaction rate catalyzed by immobilized enzyme was remarkably enhanced and its synthetic yield was also increased in most cases. The effects of water content and the added amount of Et_3N on synthesis of fragments of bioactive peptides including OGP-(10–14) and enkephalin by immobilized enzymes were different from free enzyme. Without the addition of water to reaction system, immobilized enzyme was still active for catalyzing peptide synthesis while no product was obtained using free enzyme as catalyst.

α-Chymotrypsin (EC 3.4.21.1, from bovine pancreas) and thermolysin (EC 3.4.24.4 from bacillus thermoprotedyticus rokko) were purchased from Sigma Chemical Company. Papain (EC 3.4.22.2, 2000 units/mg) was prepared by Sino-America Biotech. All amino acids including N-protected amino acids are L-configuration and commercial reagents. CH_2Cl_2 was dried ($MgSO_4$) and redistilled. Cyclohexane was redistilled from Na/benzophenone. *tert*-Amyl alcohol was dried over K_2CO_3 and redistilled. Microporous molecular sieve MCM-22 (pore volume: 0.16 cm^3/g; Si/Al = 14; pore size: 4.0 × 5.9Å, 4.0 × 5.4 Å; specific surface: 400 m^2/g) was provided by Research Institute of Petroleum Processing, SINOPEC. HCl·Gly-OMe, HCl·GlyOEt, HCl·GlyGlyOEt were synthesized by $SOCl_2$ method. *Z*-Tyr-OEt, *Z*-Phe-OCH_2CF_3, Boc-Phe-OCH_2CF_3 were synthesized by DCC/HOBt coupling method with addition of 10% (molar ratio) 4-dimethylaminopyridine (DMAP) as catalyst.[23] Petroleum ether used had bp 30–60 °C.

The melting points were determined on aYanaco micromelting point apparatus and are uncorrected. Mass spectra were recorded on VG-ZAB-HS and Bruker APEX™II spectrometers. ^1H NMR was recorded on a Bruker ARX-200 spectrometer. Elemental analysis was performed on a Elementar Vario EL appartus (Germany). Optical rotations were measured using a Perkin-Elmer 341LC polarimeter.

Standard abbreviations for amino acid derivatives and peptides are according to the suggestions of the IUPAC-IUB Commission on Biochemical Nomenclature (1984).[24] Other abbreviations: Z (benzyloxycarbonyl), Boc (*tert*-butyloxycarbonyl), PBS (phosphorus buffer solution).

Synthesis of *Z*-Tyr-Gly-OEt, *Z*-Tyr-Gly-OMe, *Z*-Phe-Gly-Gly-OEt, Boc-Phe-Gly-Gly-OEt Catalyzed by α-Chymotrypsin in Cyclohexane; General Procedure

To a suspension of the carboxyl component *Z*-Tyr-OEt (or *Z*-Phe-OCH_2CF_3, Boc-Phe-OCH_2CF_3) (0.2 mmol) and the amino component HCl·Gly-OMe (or HCl·Gly-OEt, HCl·Gly-Gly-OEt) (0.2 mmol) in cyclohexane (3 mL) were added Et_3N (35 μL, 0.25 mmol), H_2O (7.5 μL, 0.25%, v/v) and α-chymotrypsin (3 mg). The reaction mixture was stirred at 17–20 °C for about 3 d and monitored by TLC. At the end of the reaction, the solvent was evaporated under vacuum and the residue was dissolved in EtOAc (30 mL). Then it was successively washed with aq 1 M HCl (5% citric acid was used for Boc-protected product) (3 × 7 mL), H_2O (3 × 7 mL), aq 5% Na_2CO_3 (3 × 7 mL), H_2O (3 × 7 mL) and brine. The organic layer was dried ($MgSO_4$) and filtered. The solvent was removed and the crude product was purified by silica gel chromatography ($CHCl_3$–MeOH, 35:1→60:1) to afford the pure product. As for the amino component HCl·Gly-OEt, the product (*Z*-Tyr-Gly-OEt) was purified as follows: at the end of the reaction, the solvent was evaporated under vacuum. The residue was diluted with CH_2Cl_2 (4 mL) and filtered. The precipitate was successively washed by 1 M HCl and distilled H_2O. The crude product was dried at r.t. and recrystallized from acetone to give the pure product as a white solid.

Boc-Phe-Gly-Gly-OEt

Yield: 61 mg (75%); mp 98.5–100 °C; $[\alpha]_D^{20}$ +8.9 (*c* = 1, MeOH).

FAB-MS: *m*/*z* = 408 $(M + H)^+$.

Anal. Calcd for $C_{20}H_{29}N_3O_6$: C, 58.97; H, 7.12; N, 10.32. Found C, 59.06; H, 7.25; N, 10.27.

Z-Phe-Gly-Gly-OEt

Yield: 66 mg (75%); mp 92–93.5 °C (Lit.[25] mp 90–92 °C, Lit.[26] mp 88–90 °C); $[\alpha]_D^{20}$ +4.6 (*c* = 2, $CHCl_3$–MeOH, 4:1) {Lit.[26] $[\alpha]_D^{20}$ +3.0 (*c* = 2, $CHCl_3$–MeOH, 4:1)}.

FAB-MS: *m*/*z* = 442 $(M + H)^+$

Z-Tyr-Gly-OEt

Yield: 50 mg (63%); mp 168–170 °C; $[\alpha]_D^{20}$ –24.5 (*c* = 1, DMF); [Lit. [27] mp 168–170 °C; $[\alpha]_D^{20}$ –23.5 (*c* = 5.0, DMF)].

Z-Tyr-Gly-OMe

Yield: 46 mg (59%); mp 138–140 °C; $[\alpha]_D^{20}$ –24.5 (*c* = 1, DMF). [Lit.[28] mp 141–143 °C; $[\alpha]_D^{20}$ –22.2 (*c* = 1.3, DMF)].

Synthesis of *Z*-Tyr-Gly-Gly-OEt Catalyzed by α-Chymotrypsin in CH_2Cl_2

This compound was prepared according to Ref.[2]

Synthesis of *Z*-Tyr-Gly-Phe-OMe Catalyzed by Papain in Cyclohexane

To a mixture of *Z*-Tyr-Gly-OMe (77.2 mg, 0.2 mmol) and HCl·Phe-OMe (64.8 mg, 0.3 mmol) in cyclohexane (3 mL) were added Et_3N (50 μL, 3.6 mmol), H_2O (7.5 μL, 0.25%, v/v), $HOCH_2CH_2SH$ (50 μL) and papain (3.5 mg). The reaction mixture was stirred at 37 °C for about 3 d and monitored by TLC. The workup procedure was the same as described under the general procedure for the purification of *Z*-Tyr-Gly-OMe. The resulting crude product was purified by silica gel chromatograph (EtOAc–petroleum ether, 1.5:1; $CHCl_3$–MeOH, 30:1) to give the pure product as a white solid; yield: 66 mg (62%); mp 79–81 °C; $[\alpha]_D^{20}$ +20.5 (*c* = 1, $CHCl_3$).

High Resolution-SIMS: *m*/*z* calcd for $C_{29}H_{31}O_7N_3$ $[M + H]^+$: 534.2234; found $[M + H]^+$ 534.2238.

^{1}H NMR (200 MHz, $CDCl_3$): δ = 2.90–3.10 (m, 4 H, 2 CH_2) 3.66 (s, 3 H, CH_3), 3.84–4.00 (m, 2 H, CH_2), 4.31 (m, 1 H, CH), 4.78 (m, 1 H, CH), 5.03 (d, 2 H, *J* = 2.8 Hz, CH_2O), 5.60 (d, 1 H, *J* = 6.8 Hz, CONH), 6.66 (d, 2 H, *J* = 8.2 Hz, ArH), 6.91 (d, 2 H, *J* = 8.6 Hz, ArH), 6.75–7.29 (m, 12 H, 10 H_{arom} + 2 CONH).

HCl·Phe-Gly-Gly-OEt

To a stirred solution of Boc-PheGlyGlyOEt (203.5 mg, 0.5 mmol) in EtOAc (1.5 mL) was added a 5 M solution of HCl in EtOAc (4 mL) under ice cooling with stirring. After stirring for 2 h under ice cooling, the solution was stirred overnight at r.t. The resulting precipitate was filtered and crystallized from MeOH–EtOAc to give the pure product as a white solid; yield: 149 mg (87%); mp 125–127 °C.

Z-Tyr-Gly-OH

To a stirred solution of *Z*-Tyr-Gly-OEt (200 mg, 0.5 mmol) in DMF (1.5 mL) and MeOH (2 mL) was added dropwise an aq 1 M LiOH soln (1.5 mL) over ca. 15 min at 30 °C. The solution was continuously stirred for ca. 2–3 h. Then aq 1 M HCl was added to adjust pH to 7–8. After removal of a part of solvent under vacuum, aq 2 M HCl was added to bring the pH to 2. The solution was concentrated, the residue was diluted with brine (10 mL) and extracted with EtOAc (3 × 20 mL). After successive washings with aq 1 M HCl, H_2O, aq 5% $NaCO_3$, H_2O and brine, the organic layer was dried ($MgSO_4$) and filtered. The solvent was removed and the crude product was recrystallized from $CHCl_3$–petroleum ether to yield the pure product as a white solid; yield: 173 mg (93%); mp 99–101 °C; $[\alpha]_D^{20}$ –13.9 (*c* = 1, MeOH).

FAB-MS: *m*/*z* = 373 $(M + H)^+$.

Synthesis of *Z*-Tyr-Gly-Phe-Gly-Gly-OEt by Kinetic Control via 3+2 Synthetic Route

To a mixture of *Z*-Tyr-Gly-Phe-OMe (53.3 mg, 0.1 mmol) and HCl·Gly-Gly-OEt (54.2 mg, 0.3 mmol) in cyclohexane (3 mL) were added Et_3N (52 μL, 0.37 mmol), H_2O (7.5 μL, 0.25%, v/v) and α-chymotrypsin (3 mg). The reaction mixture was stirred at 17–20 °C for about 3 d. Then the solvent was evaporated under vacuum and the residue was dissolved in EtOAc (70 mL). After successively washing with aq 1 M HCl, H_2O, aq 5% Na_2CO_3, H_2O and brine, the organic layer was dried ($MgSO_4$) and filtered. The solvent was removed and the crude product was purified by silica gel chromatography (gradient elution: $CHCl_3$–MeOH, 30:1→15:1) and recrystallized from acetone–petroleum ether to give the pure product as a white solid; yield: 33.8 mg (51%); mp 161–163 °C; $[\alpha]_D^{20}$ –23.6 (c = 0.5, DMF).

FAB-MS: *m*/*z* = 662 $(M + H)^+$.

Anal. Calcd for $C_{34}H_{39}N_5O_9$: C, 61.72; H, 5.90; N, 10.59. Found C, 61.40; H, 5.58; N 10.37.

Synthesis of *Z*-Tyr-Gly-Phe-Gly-Gly-OEt by Thermodynamic Control via 2+3 Synthetic Route

To a suspension of *Z*-Tyr-Gly-OH (37.2 mg, 0.1 mmol) and HCl·Phe-Gly-Gly-OEt (103.0 mg, 0.3 mmol) in *tert*-amyl alcohol (2 mL) were added Et_3N (70 μL, 0.5 mmol) and 10 mM $CaAc_2$ buffer (0.12 mL, 6%, v/v). The substrates dissolved completely on stirring. Then thermolysin (5 mg) was added and the reaction mixture was stirred at 40 °C for about 3 d. The workup procedure was the same as described for the 3+2 synthetic route. The resulting crude product was recrystallized from acetone–petroleum ether to give the pure product as a white solid; yield: 46 mg (70%); mp 159–161 °C; $[\alpha]_D^{20}$ –23.8 (*c* = 0.5, DMF).

FAB-MS: *m*/*z* = 662 $(M + H)^+$.

Preparation of Immobilized α-Chymotrypsin and Papain on MCM-22 by Adsorption.

Immobilized α-Chymotrypsin on MCM-22

To a solution of α-chymotrypsin (21.8 mg) in PBS (10 mL, pH 6.0, 0.05M) was added MCM-22 (600 mg) under ice-water cooling and the mixture was stirred for 2 h. The mixture was filtered and washed with PBS (5 mL). The resulting immobilized enzyme was suspended in PBS (4 mL) and lyophilized for 8 h to give the expected immobilized enzyme that was stored at –2 °C.

Immobilized Papain on MCM-22

To a stirred solution of papain (18 mg) in PBS (10 mL, pH 6.0, 0.05 M) was added MCM-22 (500 mg) under ice-water cooling and the mixture was stirred for 2 h. The workup procedure was the same as above.

Synthesis of *Z*-Tyr-Gly-Gly-OEt Catalyzed by MCM-22 Immobilized α-Chymotrypsin

To a mixture of *Z*-Tyr-OEt (102.9 mg, 0.3 mmol) and HCl·Gly-Gly-OEt (59.0 mg, 0.3 mmol) in CH_2Cl_2 (3 mL) was added Et_3N (84 μL, 0.6 mmol). The mixture was stirred to completely dissolve the substrate. The observed pH value was about 10. Then H_2O (7.5 μL, 0.25%, v/v) and MCM-22 immobilized α-chymotrypsin (100 mg containing about 2.6 mg of enzyme) were added and the mixture was continuously stirred for 2 d at 20 °C. The precipitate from the reaction solution and the immobilized enzyme were filtered and washed with CH_2Cl_2 (4 mL). Then the precipitate was thoroughly dissolved in acetone by several washings. The expected product was obtained by evaporation of acetone and crystallized from acetone. The immobilized enzyme was stored –2 °C for reuse; yield: 98.7 mg (72%); mp 167–168 °C; $[\alpha]_D^{20}$ +3.2 (*c* = 2, AcOH) [Lit.[2] mp 164–165 °C; $[\alpha]_D^{20}$ +2.9 (*c* = 2, AcOH)].

Synthesis of *Z*-Tyr-Gly-OEt, *Z*-Tyr-Gly-OMe, *Z*-Phe-Gly-Gly-OEt, Boc-Phe-Gly-Gly-OEt Catalyzed by MCM-22 Immobilized α-Chymotrypsin in Cyclohexane; General Procedure

To a suspension of *Z*-Tyr-OEt (or *Z*-Phe-OCH_2CF_3, Boc-Phe-OCH_2CF_3) (0.2 mmol) and HCl·Gly-OMe (or HCl·Gly-OEt, HCl·Gly-Gly-OEt) (0.2 mmol) in cyclohexane (3 mL) were added Et_3N (35 μL, 0.25 mmol), H_2O (7.5 μL, 0.25%, V/V) and MCM-22 immobilized α-chymotrypsin (100 mg). The mixture was stirred at 17–20 °C for about 2 d and monitored by TLC. Then a larger amount of EtOAc was added to the solution to make the product dissolve completely. The solution was filtered and the residue was washed several times with EtOAc. The immobilized enzyme was dried under reduced pressure at r.t. for about 5 min and stored at –2 °C for reuse. The filtrate was evaporated under vacuum and the residue was dissolved in EtOAc (30 mL). The workup procedure was the same as described for the corresponding product from free enzyme. The resulting crude product was purified by silica gel chromatography ($CHCl_3$–MeOH, 35:1→60:1) to afford pure products. As for the amino component HCl·Gly-OEt, the product (Z-Tyr-Gly-OEt) was purified as follows: At the end of reaction, the reaction mixture was diluted with acetone, filtered and washed with several portions of acetone to make the product completely dissolve in acetone. The immobilized enzyme was dried as described above and stored at –2 °C for reuse. The solvent was removed and the residue was diluted with CH_2Cl_2 (4 mL) and filtered. The precipitate was successively washed by aq 1 M HCl (3 × 7 mL) and distilled H_2O (3 × 7 mL). The crude product was dried at r.t. and recrystallized from acetone to yield the pure product as a white solid. The yields of Boc-Phe-Gly-Gly-OEt, *Z*-Phe-Gly-Gly-OEt, *Z*-Tyr-Gly-OEt and *Z*-Tyr-Gly-OMe were 65 mg (80%), 72 mg (82%), 58 mg (72%) and 39 mg (51%), respectively. Their physical data were identical with those of corresponding products by free enzyme.

Synthesis of *Z*-Tyr-Gly-Phe-OMe Catalyzed by MCM-22 Immobilized Papain in Cyclohexane

To a mixture of *Z*-Tyr-Gly-OMe (77.2 mg, 0.2 mmol) and HCl·Phe-OMe (64.7 mg, 0.3 mmol) in cyclohexane (3 mL) were added Et_3N (50 μL, 3.6 mmol), H_2O (7.5 μL, 0.25%, v/v), $HOCH_2CH_2SH$ (50 μL) and MCM-22 immobilized papain (100 mg). The mixture was stirred at 37 °C for about 2 d and detected by TLC. Then a larger amount of EtOAc was added to make the product dissolve completely. The reaction solution was filtered and washed with several portions of EtOAc. The immobilized enzyme was dried as described above and stored at –2 °C for reuse. The workup and purification procedure were the same as for the corresponding product from free enzyme; yield: 67 mg (63%). Its physical data were identical to that of the corresponding product from free enzyme.

Synthesis of Protected Pentapeptide OGP-(10–14) (*Z*-Tyr-Gly-Phe-Gly-Gly-OEt) by Kinetic Control via 3+2 Synthetic Route

To a mixture of *Z*-Tyr-Gly-Phe-OMe (53.3 mg, 0.1 mmol) and HCl·Gly-Gly-OEt (54.2 mg, 0.3 mmol) in cyclohexane (3 mL) were added Et_3N (52 μL, 0.37 mmol) and H_2O (7.5 μL, 0.25%, v/v) and MCM-22 immobilized α-chymotrypsin (100 mg). The reaction mixture was stirred at 17–20 °C for about 2 d. Then a larger amount of MeOH was added to make the product dissolve completely. The mixture was filtered with several washings of MeOH. The solvent of the filtrate was evaporated under vaccum and the residue was dissolved in EtOAc (70 mL). The workup and purification procedure were the same as for the corresponding product from free enzyme via the 3+2 synthetic route; yield: 47 mg (71%). The physical data were identical with that of the corresponding product by free enzyme.

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