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Enzymatic Synthesis of a CCK-8 Tripeptide Fragment

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A process for the synthesis of CCK-8 tripeptide H-Gly-Trp-Met-OH catalyzed by immobilized enzyme was reported. Enzymes were used for the formation of peptide bonds and the removal of protecting group. Starting with phenylacetyl (PhAc) glycin, *N*-protected dipeptide PhAc-Gly-Trp-OMe was obtained by coupling PhAc-protected glycine carboxamidomethyl ester (OCam) with Trp-OMe catalyzed by immobilized papain in buffered ethyl acetate. Then the condensation between PhAc-Gly-Trp-OMe and Met-OEt•HCl was carried out by immobilized α-chymotrypsin catalysis in solvent free system. Basic hydrolysis was followed getting PhAc-Gly-Trp-Met-OH. The PhAc-group was removed with penicillin G amidase and H-Gly-Trp-Met-OH was obtained in an overall yield of 43.9%. The reaction conversion of tripeptide in solvent free system was strongly affected by the system of basic salts added. The influence of the support materials used to deposit enzymes and structures of acyl donor and nucleophile on the reaction was also investigated.

Keywords enzymatic peptide synthesis, immobilized enzyme, solvent free system, tripeptide fragment

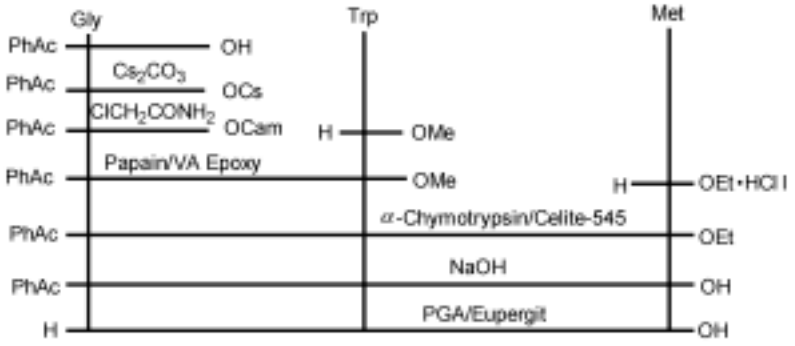
Introduction

The syntheses of biologically active peptides have been widely investigated because the market for peptides is continuously growing, especially in the pharmaceutical and food industries. Cholecystokinin octapeptide [CCK-8, Asp-Tyr(SO_3)-Met-Gly-Trp-Met-Asp-Phe-NH_2] is a biologically active peptide and a potential therapeutic agent in the control of gastrointestinal function.[1-3] Cholecystokinin pentapeptide is a commercially available therapeutical drug pentagastrin (Gly-Trp-Met-Asp-Phe-NH_2) because CCK and gastrin have the same five amino acids in the *C*-terminal sequence.

In this work we described the enzymatic synthesis of the tripeptide H-Gly-Trp-Met-OH which is a key intermediate of the syntheses of CCK-8 and cholecystokinin pentapeptide. The synthetic strategy, which is outlined in Scheme 1, involved three steps by enzymatic catalysis. As *N*-terminal protecting group the phenylacetyl group (PhAc) was chosen as an alternative to the conventional benzyloxycarbonyl group (Z) or *tert*-butyloxycarbonyl group (Boc),[4] because PhAc can be removed enzymatically by penicillin G amidase (PGA).[5] Beyond that, we investigated the immobilization of α-chymotrypsin on two commercially available carriers. It might be worthwhile in terms of quantity and value to mass industrial production.[6]

The key step in the synthesis of H-Gly-Trp-Met-OH is the reaction between PhAc-Gly-Trp-OMe and Met-OEt•HCl catalyzed by immobilized α-chymotrypsin in solvent free system. Enzymatic peptide synthesis in solvent free system, first reported in the early 1990s, is one of these new methods.[7,8] Because of its high substrate concentration, the synthesis in solvent free system

Scheme 1 Enzymatic synthesis of the tripeptide H-Gly-Trp-Met-OH



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offers attractive initial rate and yield. Capellas *et al*.[9] reported the enzymatic coupling between Z-Gly-Trp-OCam and Met-OEt catalyzed by α-chymotrypsin in organic solvent media and tested the reactivity of three *C*-terminal ester derivatives. Benzyl- (OBzl) and carboxamidomethyl- (OCam) esters are more active than the methyl ester. In this work we were successful in coupling the dipeptide methyl ester directly with Met-OEt hydrochloride in solvent free system. The pure tripeptide ester was obtained efficiently in 71.4% yield.

Results and discussion

Influence of inorganic salt systems

In the synthesis of PhAc-Gly-Trp-Met-OEt, different inorganic salt systems with crystalline water were investigated to optimize the pH of the reaction. Three systems of inorganic salts were studied. When the same moles of sodium sulfate or sodium carbonate were used as substitute of potassium hydrogen carbonate, the lowest conversion rate, 23.9%, was observed with the mixture of sodium sulfate and sodium carbonate decahydrate. A better yield, 40.2%, could be obtained with sodium carbonate and its decahydrate. With 96% yield the system potassium hydrogen carbonate/sodium carbonate decahydrate was clearly superior (Figure 1).

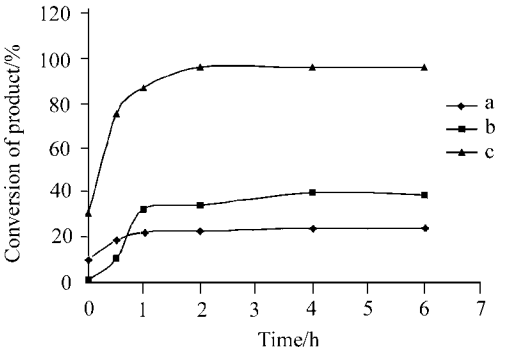

Figure 1 Effect of different inorganic salt systems (a) Na_2SO_4/Na_2CO_3•$10H_2O$, (b) Na_2CO_3/Na_2CO_3•$10H_2O$, (c) $KHCO_3$/Na_2CO_3•$10H_2O$.

Influence of immobilized enzymes and support materials

Immobilized enzymes can be used favorably for enzyme-catalyzed peptide synthesis, because they can be used continuously in a reactor and the product will not be contaminated with the enzymes. It is important to choose a support material that allows the immobilized enzyme to perform efficiently. The support materials must allow easy enzyme immobilizations without appreciable losses of activity and should be inexpensive. In the present work, we have investigated the immobilization of α-chymotrypsin on two commercially available support materials, Celite-545 and Eupergit C (Table 1). α-Chymotrypsin/Celite-545 is superior to α-chymotrypsin/Eupergit C for the enzymatic activity. The substrate Ac-Tyr-OEt was hydrolyzed with α-chymotrypsin/Celite-545 within 100 min whereas it took 150 min in case of α-chymotrypsin/Eupergit C (Figure 2). In the coupling reactin between PhAc-Gly-Trp-OMe and Met-OEt•HCl, α-chymotrypsin/Celite-545 was chosen as catalyst.

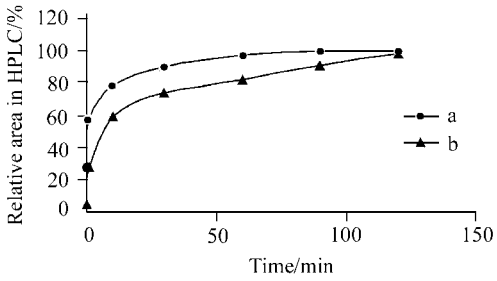

Figure 2 Activity tests of α-chymotrypsin/Celite-545 (a) and α-chymotrypsin/Eupergit C (b).

In the next coupling step the methyl ester of the dipeptide was active enough to be coupled with Met-OEt•HCl. The coupling in solvent free system was successful and the tripeptide could be isolated in 71.4% yield. With this strategy three reaction steps could be avoided in comparison to the published method by Capellas *et al*.[9]

Influence of the C-α carboxyl group of the nucleophiles

Generally, the selection of *C*-terminal protecting group was made taking into account its reactivity as well as the fact that the product of one reaction will be the acyl-donor for the next one. In the present work, Trp-OMe was a good nucleophile for the synthesis of PhAc-Gly-Trp-OMe with a very good yield of 91.0%, catalyzed by papain/VA Epoxy.

The dipeptide methyl ester was a tolerable acyl-donor for the following reaction with Met-OEt•HCl as a nucleophile. PhAc-Gly-Trp-Met-OEt was obtained in a good yield of 71.4% within 2 h. The by-product PhAc-Gly-Trp-$(Met)_n$-OEt, $n=2$, could be kept below 5%.

Table 1 Condition of immobilization and activity test of α-chymotrypsin

Enzyme	Support	Enzyme/Carrier/($mg•g^{-1}$)	Condition of immobilization	Substrate	Condition of activity test
α-Chymotrypsin	Celite-545	10	Tris-HCl buffer (0.05 moL/L, pH 9.0)	Ac-Tyr-OEt	Tris-HCl buffer (0.01 moL/L, pH 8.1)
α-Chymotrypsin	Eupergit C	60	Tris-HCl buffer (0.05 moL/L, pH 9.0)	Ac-Tyr-OEt	Tris-HCl buffer (0.01 moL/L, pH 8.1)

Experimental

Materials

Silica gel plates, Papain (EC 3.4.22.2) from carica papaya (water-soluble, 30000 USP-U/mg using casein as substrate) and α-chymotrypsin (EC 3.4.21.1) from bovine pancreas [crystallized, lyophilized powder, 350 U/mg using *N*-acetyl-*L*-tyrosine ethyl ester (ATEE) as substrate] were obtained from Merck (Darmstadt, Germany). Penicillin G Amidase (PGA) (EC 3.5.1.11), immobilized on Eupergit (powder, 109 U/g), and Celite-545 (particle size 20—45 μm) were obtained from Fluka (Buchs, Switzerland). The polymers were donated by Riedel-de Haen AG (VA Epoxy) and Roehm Pharm GmbH (Eupergit C). The amino acid derivatives were prepared by standard procedures. All other chemicals and solvents used were of analytical grade.

Enzyme immobilization

The enzymes were immobilized by deposition onto solid supports.[10-12] The general procedure for enzyme immobilization was as follows. A solution of enzyme in the proper buffer (10 mL) was mixed with the support (1 g). After thorough mixing at room temperature for 24 h, the enzyme-support preparation was lyophilized overnight and then stored at −20 ℃. Papain (300 mg) was dissolved in 0.2 moL/L borax buffer (pH 8.5, 10 mL). The solution of papain was mixed with VA Epoxy (1 g). α-Chymotrypsin (10 mg) was dissolved in 0.05 moL/L Tris-HCl buffer (pH 9.0, 10 mL). The solution of α-chymotrypsin was mixed with Celite-545 (1 g). α-Chymotrypsin (60 mg) was dissolved in 0.05 moL/L Tris-HCl buffer (pH 9.0, 10 mL). The solution of α-chymotrypsin was mixed with Eupergit C (1 g).

Activity tests of immobilized enzymes

Papain/VA Epoxy: The activity was tested with Bz-Arg-OEt (BAEE) as substrate and with 2-mercaptoethanol as alternative activation agent. The reaction product Bz-Arg-OH was determined by HPLC.

α-Chymotrypsin/Celite-545 and α-chymotrypsin/Eupergit C: The activities of the enzymes prepared were tested with Ac-Tyr-OEt (ATEE) as substrate. The conversions of ATEE to Ac-Tyr-OH were determined by HPLC.

HPLC analysis

The amounts of substrates, products and byproducts produced in the enzymatic reactions were determined by HPLC (Gilson pumps 305/307) analysis using a Nucleosil C18, 5 μm and 100 mm×2 mm column. The solvent system was as follows: (A) 0.1% aqueous TFA, (B) 80% ACN/0.1% TFA; Gradient elution from 30% B to 70% B over 12 min: 0—3 min, 30% B; 3—5 min, 30% B to 70% B; 5—9 min, 70% B; 9—9.5 min, 70% B to 30% B; 9.5—12 min, 30% B. A flow rate of 0.3 mL/min was used. UV detection (Merck LaChrom-7400 detector) was set to 260 nm.

The target product was separated by HPLC (Gilson pumps 305/307) using a Nucleosil C18, 7 μm and 250 mm×8 mm column. The solvent systems (A) and (B) were the same as above. Isocratic elution 23% B; flow rate 5 mL/min; UV detection (ISCO UA-6 detector) 260 nm.

Peptide syntheses

PhAc-Gly-OCam: PhAc-Gly-OCam was synthesized according to Martinez *et al.*[13,14] PhAc-Gly-OH (5.0 g, 26 mmol) were dissolved in the mixture of 40 mL of MeOH and 10 mL of H_2O, and the pH was adjusted to 7.0 by adding 20 mL of 20% Cs_2CO_3 dropwise. The solution was evaporated to dryness under vacuum below 45 ℃. After evaporation to dryness with tolune (3×50 mL), PhAc-Gly-OCs was dried over night under high vacuum. PhAc-Gly-OCs (9.8 g) was dissolved in 60 mL of anhydrous DMF, and 2-chloroacetamide added (3.5 g). The mixture was stirred at 35 ℃. After 72 h the reaction was complete and no starting material was detected by TLC. The mixture was evaporated to dryness below 50 ℃, and the residue washed with 500 mL of H_2O. The product was collected by filtration, transferred to 100 mL of ethyl acetate and extracted with H_2O (3×100 mL) and saturated sodium chloride (3×100 mL). The organic phase was dried over anhydrous sodium sulfate and concentrated under vacuum. The crystallization was performed in a mixture of ethyl acetate/petroleum ether yielding a white solid (5.55 g, 85.6%). m.p. 128—129 ℃; FABMS *m/z*: 250.9 $[M+H]^+$, $C_{12}H_{14}N_2O_4$ requires 250.2. TLC data (DCM∶MeOH＝9∶1): R_f＝0.2 (PhAc-Gly-OCs), R_f＝0.65 (Ph-Ac-Gly-OCam).

PhAc-Gly-Trp-OMe: To synthesize PhAc-Gly-Trp-OMe, 125 mg (0.5 mmol) of PhAc-Gly-OCam and 164 mg (0.75 mmol) of H-Trp-OMe were dissolved in 50 mL of ethyl acetate containing 270 μL of 0.2 moL/L borax buffer (pH＝8.5), 30 μL of β-mercaptoethanol, and 1 mg of EDTA. To this solution 300 mg of immobilized papain were added. After 3 h the reaction was complete and the HPLC yield was 97%. The mixture was filtered to remove the immobilized enzyme which was washed with a mixture of 40 mL of ethyl acetate and 10 mL of water. The combined filtrates were extracted successively with 1 moL/L citric acid (3×100 mL), 5% $NaHCO_3$ (3×100 mL), and saturated sodium chloride (1×100 mL). The organic phase was dried over anhydrous sodium sulfate and concentrated under vacuum. The residue was lyophilized yielding a white solid (174 mg, 91.0%). m.p. 126—128 ℃; $[\alpha]_D^{20}$ 8.7 (*c* 1.0, DMF); FABMS *m/z*: 394.2 $[M+H]^+$; MS *m/z* (%): 416.2 $[M+Na]^+$, $C_{22}H_{23}N_3O_4$ requires 393.5. HPLC data: R_t＝6.95 (PhAc-Gly-Trp-OMe), R_t＝1.66 (PhAc-Gly-OCam).

PhAc-Gly-Trp-Met-OEt: The synthesis of tripeptide was carried out under a solvent free condition.[7] In a beaker, PhAc-Gly-Trp-OMe (1.97 g, 5.0 mmol), Met-OEt•HCl (4.28 g, 20.0 mmol), $KHCO_3$ (10 g, 100 mmol) and Na_2CO_3•$10H_2O$ (5.72 g, 20.0 mmol) were mixed. Immobilized α-chymotrypsin (2 g) (10 mg α-chy-

motrypsin/g Celite-545) were added and stirred manually and circularly every 20 min. The reaction was monitored by HPLC. After 2 h the reaction was complete and the HPLC yield was 96%. The mixture was washed with water until a pH of 7.0 was reached, diluted with 100 mL of 80% EtOH and then sonicated to extract the tripeptide for 5 min. After removal of the immobilized enzyme by filtration, the solvent was evaporated to dryness under vacuum. The tripeptide ester was obtained by recrystallization with EtOH as a white solid (1.92 g, 71.4%). m.p. 174—176 ℃; $[\alpha]_D^{20}$ −24.9 (*c* 1.0, DMF); FABMS *m/z*: 539.1 $[M+H]^+$; MS *m/z* (%): 561.2 $[M+Na]^+$, $C_{28}H_{34}N_4O_5S_1$ requires 538.6. HPLC data: R_t=6.96 (PhAc-Gly-Trp-OMe), R_t=7.89 (Ph-Ac-Gly-Trp-Met-OEt), R_t = 9.38 (PhAc-Gly-Trp-Met-Met- OEt).

PhAc-Gly-Trp-Met-OH: To obtain Phac-Gly-Trp-Met-OH, 3.8 g (0.7 mmol) of PhAc-Gly-Trp-Met-OEt were dissolved in 150 mL of H_2O ∶ acetone (1 ∶ 1) and 5 mL of 4 moL/L NaOH were added. After stirring for 1 h, no starting material was detected by TLC. The acetone was removed under vacuum. The product was precipitated by acidification with 1 moL/L HCl to pH 3 and filtered. The residue was washed with 100 mL of water and dried. The crystallization was performed in a mixture of ethyl acetate/petroleum ether yielding a white solid (3.45 g, 98.4%). m.p. 180—182 ℃; $[\alpha]_D^{20}$ −16.2 (*c* 1.0, DMF); FABMS *m/z*: 511.1 $[M+H]^+$; MS *m/z* (%): 533.2 $[M+Na]^+$, $C_{26}H_{30}N_4O_5S_1$ requires 510.6. TLC data (DCM ∶ MeOH=9 ∶ 1): R_f=0.15 (Ph-Ac-Gly-Trp-Met-OH), R_f=0.8 (PhAc-Gly-Trp-Met-OEt).

H-Gly-Trp-Met-OH: To remove the phenylacetyl group, 0.2g (0.4 mmol) of PhAc-Gly-Trp-Met-OH was suspended in 20 mL of H_2O and the pH was adjusted with 1 moL/L NaOH to 7.6. After addition of 30 mg of immobilized PGA, the mixture was stirred at 35 ℃ for 3 h. To extract the product, the mixture was diluted with 20 mL of 80% EtOH, sonicated, and filtered. The filtrate was evaporated to dryness under vacuum. The residue was separated by preparative HPLC with an eluent of 23% B. The residue was dissolved in 5 mL of 23% B and the desired product was eluted as the second peak. The pooled fractions were lyophilized twice yielding a white powder (124 mg, 80.3%). m.p. 182—184 ℃; $[\alpha]_D^{20}$ −26.4 (*c* 1.0, DMF); FABMS *m/z*: 393.1 $[M+H]^+$; MS *m/z* (%): 415.1 $[M+Na]^+$, $C_{18}H_{24}N_4O_4S_1$ requires 392.5. HPLC data: R_t=1.77 (H-Gly-Trp-Met-OH), R_t = 7.60 (PhAc-Gly-Trp-Met-OH).

Conclusion

The enzymatic synthesis of H-Gly-Trp-Met-OH was studied avoiding chemical reaction steps, whenever it was possible. In our work, two steps of peptide couplings could be performed with immobilized enzymes. Applying the solvent free system, we could use the dipeptide methyl ester directly for the coupling of PhAc-Gly-Trp-OMe as acyl-donor and Met-OEt•HCl as nucleophile. Thus three more reaction steps of preparing an OCam ester could be avoided. At the end of the synthesis the amino protecting group could also be removed enzymatically from PhAc-Gly-Trp-Met-OH with immobilized PGA. All the overall yield was 43.9%.

In this paper, we could again demonstrated that "green chemistry", the application of the enzymatic technology, is indeed a good and versatile alternative to the "chemical" peptide synthesis.

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