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ENZYMATIC SYNTHESIS OF LEU- AND MET-ENKEPHALIN

Willi Kullmann

Max-Planck-Institut für biophysikalische Chemie,
D-3400 Göttingen, Am Faßberg, West Germany

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Summary: The protease-catalyzed synthesis of Leu- and Met-enkephalin is reported. Each peptide bond of the endogeneous opiate-pentapeptides was formed either by papain or α-chymotrypsin catalysis. N-acyl amino acids and peptides or their ester derivatives served as substrates whereas amino acid and peptide phenylhydrazides were used as nucleophiles. The free pentapeptides exhibited naloxone-reversible opiate-like activity in guinea-pig ileum and mouse vas deferens assays. The present study suggests the usefulness of enzymic peptide synthesis which allows rapid preparation of homogeneous compounds with high optical purity.

Peptide bond formation catalyzed by papain and α-chymotrypsin was first reported by Bergmann and Fraenkel-Conrat in 1937 and by Bergmann and Fruton in 1938, respectively (1,2). Investigations in this field were intensified in order to learn whether proteases were involved in protein biosynthesis (for a review see (3)).But the interest waned when the mechanism of in vivo protein synthesis was elucidated. A renewed interest in application of proteolytic enzymes to peptide synthesis arose during the last years (4,5,6,7,8). The enzymic synthesis takes advantage of products that are insoluble in biphasic aqueous organic reaction media thus shifting the chemical equilibrium towards peptide bond formation.

Since the elucidation of the structure of the enkephalins by Hughes et al. in 1975 (9), several syntheses of these peptides by the solid phase method, in solution, or by papain-catalyzed fragment condensation (10) have been reported.

In this paper I want to describe for the first time the preparation of biologically active enkephalins with all the peptide bonds established by protease-catalyzed reactions (Scheme 1).

Abbreviations used: Boc, t-butyloxycarbonyl; Bzl, benzyl; Ph, phenyl; OEt, ethyl ester; OTMB, trimethylbenzyl ester; HPLC, high pressure liquid chromatography.

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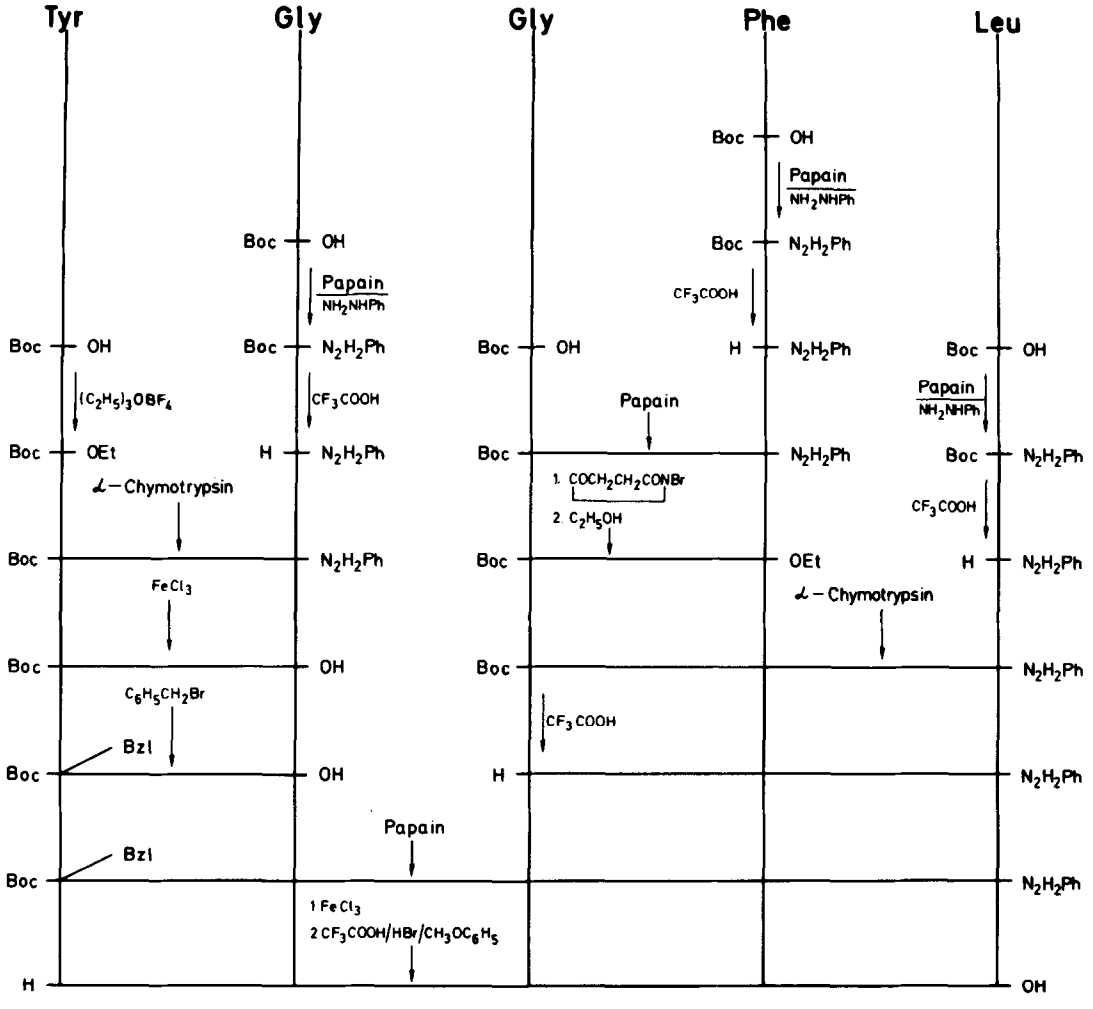

Scheme 1: Synthesis of Leu-enkephalin

Enzymic as well as chemical peptide synthesis require easily and selectively removable protecting groups. Furthermore, the blocking groups chosen for the enzymatic synthesis should render the reaction products insoluble in aqueous medium in order to give high yields. On the other hand, the reactants must at least be partially soluble as to allow the coupling reaction to take place at all. Boc groups meet these requirements for N^{α}-protection as they are readily removable by mild acidolysis and Boc-amino acids were found to be quite soluble in the pH-range applied during this work.

Carboxyl groups were protected as phenylhydrazides since the latter were easily available from Boc-amino acids by papain-catalyzed condensation (11) and were rapidly cleaved by oxidation using $FeCl_3$ (12). They also ensured high yields of the α-chymotrypsin-mediated coupling reactions since α-chymotrypsin prefers amides or hydrazides of amino acids or peptides as acceptor nucleophiles over esters (6). With respect to the papain-catalyzed peptide bond formation, phenylhydrazides have been described as suitable amino components due to their low basicity as compared with amides (13).

Although all the dipeptide sequences contained in the pentapeptides could be synthetized enzymatically as their Boc-amino acid phenylhydrazides, elongation of the peptide chain was impossible in many cases because the reactants, partially deprotected to enable peptide bond formation, had enhanced solubility in aqueous solutions and thus were more susceptible to proteolytic cleavage.

The synthetic pathway outlined in scheme 1 was found to be a practicable procedure whereas an initial attempt to couple enzymatically prepared Boc-Tyr(Bzl)-Gly-Gly-OH and H-Phe-Leu-OTMB using papain as catalyst resulted in a product the amino acid composition of which was found to be: Tyr, 0.81; Gly, 1.00; Phe, 1.03; Leu, 1.00. Further evidence for the formation of the tetrapeptide Boc-Tyr(Bzl)-Gly-Phe-Leu-OTMB was given by chromatograms of the reaction mixture indicating cleavage of the Gly-Gly bond followed by papain-mediated coupling of Boc-Tyr(Bzl)-Gly-OH with H-Phe-Leu-OTMB. This may be explained by the earlier finding(14) that peptides containing a phenylalanine residue preceding the residue which contributes the carboxyl group to the bond to be split are very susceptible to papain action. As this specificity of papain catalysis may also apply to a tyrosine instead of a phenylalanine residue, I decided to couple the protected fragments Boc-Tyr(Bzl)-Gly-OH and H-Gly-Phe-Leu-N_2H_2Ph. Papain-catalyzed condensation using McIlvain buffer-ethanol (6:4), pH 6.1, in the presence of 2-mercaptoethanol gave the desired protected pentapeptide which readily precipitated (yield,82%).

The protected fragment Boc-Tyr(Bzl)-Gly-OH (Scheme 1) was prepared by incubation of Boc-Tyr-OEt and H-Gly-N_2H_2Ph in 0.2 M carbonate buffer-dimethylformamide (2:1),pH 10.1, in the presence of α-chymotrypsin to give Boc-Tyr-Gly-N_2H_2Ph (yield, 72%) which was subsequently treated with $FeCl_3$ to cleave the phenylhydrazide and with benzyl bromide to benzylate the tyrosine side chain. The synthetic procedures described above were chosen because Boc-Tyr(Bzl)-OEt could not be coupled with H-Gly-N_2H_2Ph when incubated in the presence of α-chymotrypsin. On the other hand, the benzyl ether was indispensable because the desired pentapeptide could not be obtained using Boc-Tyr-Gly-OH as substrate, probably due to increased solubility of the latter.

All attempts to prepare Boc-Gly-Phe-Leu as the t-butyl or trimethylbenzyl ester or phenylhydrazide by papain or thermolysine catalysis failed because of hydrolytic cleavage of the Phe-Leu bond. However, this tripeptide (Scheme 1) could be synthetized by incubation of Boc-Gly-OH and H-Phe-N_2H_2Ph in a 3 M acetate buffer,pH 4.8, in the presence of papain and 2-mercaptoethanol (yield, 80%), subsequent replacement of the phenylhydrazide by an ethyl ester (15) and α-chymotrypsin-catalyzed condensation of the protected dipeptide with H-Leu-N_2H_2Ph in 0.2 M carbonate buffer-dimethylformamide (2:1),pH 9.95, (yield, 70%). Met-enkephalin was prepared in a manner similiar to the synthesis of the leucine analogue. A detailed description of the enzymatic syntheses of these pentapeptides will be given elsewhere.

The enzymatically derived protected fragments were worked up as usual and were further purified by preparative HPLC on prepacked silica gel 60 columns. Assignment of the products obtained by catalytic synthesis was achieved by comparison with the corresponding peptides prepared by conventional methods in solution.

The fully protected pentapeptides were treated with $FeCl_3$ followed by CF_3COOH/HBr in the presence of anisole to cleave the phenylhydrazide and the Boc and Bzl groups,respectively. The free peptides were obtained in homogeneous form after chromatography on Bio-Gel P-2 (0.05 M NH_4HCO_3), silica gel

60 (n-butanol-acetic acid-water, 4:1:1), and gel filtration on sephadex LH-20 using methanol-water (95:5) as eluant.

The chromatographic behaviour of the pentapeptides was identical to that of Leu- and Met-enkephalin prepared by solution methods.
Amino acid analyses of acid hydrolysates gave: Gly, 2.00; Leu, 0.98; Tyr, 0.89; Phe, o.95; and Gly, 2.00; Met, 0.98; Tyr, 0.91; Phe, 0.96.
Elementary analyses: Found: C, 60.23; H, 6.88; N, 12.35; $C_{28}H_{37}N_5O_7$ (555.6) requires: C, 60.52; H, 6.71; N, 12.60 ; found: C, 56.25; H, 6.37; N, 11,92; S, 5.88; $C_{27}H_{35}N_5O_7S$ (573.7) requires: C, 56.55; H, 6.15; N, 12.21; S, 5.59. $[\alpha]_D^{25}$= + 32.5° (c=0.9 in methanol) for Leu-enkephalin; $[\alpha]_D^{25}$= + 31.6° (c=1.0 in methanol) for Met-enkephalin.

The biological activities of the enzymatically prepared peptides as determined by the naloxone-reversible inhibition of electrically induced contractions of the guinea-pig ileum (GPI) and of the mouse vas deferens (MVD) were found to be: ED_{50}(M) 0.86 x 10^{-7} (GPI) and 1.4 x 10^{-8} (MVD) (Met-enkephalin);
ED_{50}(M) 4.45 x 10^{-7} (GPI) and 0.85 x 10^{-8}(MVD) (Leu-enkephalin).
These results were in close agreement with those reported by Waterfield et al. (16). Enzymatically and chemically prepared Leu-enkephalins were shown to be nearly equipotent with respect to the abovementioned assays, whereas an all-D-enantiomer of Leu-enkephalin, synthetized in solution, was less effective by a factor of ca. 200 in the mouse vas deferens assay.

The results of this study demonstrate the usefulness of proteolytic enzymes as catalysts in peptide synthesis. The products are readily available by simple mixing of the reactants in aqueous buffers at room temperature or 37°C. This method provides optically pure products due to the stereospecific action of the enzymes whereas activation of the carboxyl groups of N^{α}-acyl amino acids or peptides during chemical synthesis may often result in partial racemization.

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