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Protease-catalyzed peptide bond formation: Application to synthesis of the COOH-terminal octapeptide of cholecystokinin

(enzymatic synthesis/papain/α-chymotrypsin/thermolysin)

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ABSTRACT **This study of protease-catalyzed peptide synthesis reports the preparation of the COOH-terminal octapeptide amide of cholecystokinin. The octapeptide was assembled by chemical condensation of two tetrapeptide segments that had been synthesized through the concerted catalytic reactions of several proteases of different specificities. The resulting octapeptide derivative was subjected to catalytic transfer hydrogenation, followed by sulfation of its tyrosine residue and removal of the N^{α}-protecting group. The homogeneous target peptide was obtained after purification via partition chromatography, gel filtration, and ion-exchange chromatography. The synthetic octapeptide stimulated amylase release from pancreatic acinar cells.**

As early as 1898, van't Hoff considered the possibility of enzymatic peptide bond formation by reversal of the hydrolytic action of proteases into synthetic action (1). The first syntheses of well-defined peptides via papain and α-chymotrypsin catalysis were reported by Bergmann, Fraenkel–Conrat, and Fruton in 1938 (2, 3).

In recent years, there has been a renewed interest in protease-catalyzed peptide synthesis (4). Taking up Fruton's earlier suggestion (5), these investigations have aimed mainly at exploitation of the potential of proteolytic enzymes for preparative scale peptide synthesis. Several different synthetic pathways have been described (6–8) that led to the endogenous opioid peptides [Met]- and [Leu]enkephalin, all the peptide bonds of which were formed by protease-controlled catalysis. The advantage of the enzymatic approach to peptide synthetic chemistry lies mainly in the specificity of the proteolytic enzymes, which suppresses the formation of by-products frequently generated during conventional syntheses. In particular, the stereoselectivity of the enzymes rules out one of the principal side-reactions: racemization.

To explore further the proteosynthetic capabilities of several proteases, I decided to assemble the COOH-terminal octapeptide amide of cholecystokinin (CCK-8) as far as possible by enzymatic peptide bond formation. CCK-8, which has the amino acid sequence (9)

H-Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2,

elicits the cholecystokinetic and pancreozyminic activities of the entire tritriacontapeptide hormone (10). Cholecystokinin, a polypeptide of 33 amino acid residues, stimulates gallbladder contraction and pancreatic enzyme secretion.

In this paper, I report the synthesis of an octapeptide possessing the above primary structure. The protected CCK-8 derivative was assembled by chemical fragment coupling of two enzymatically prepared tetrapeptides. Protease-mediated peptide bond formation was achieved by papain, thermolysin, or α-chymotrypsin catalysis (Fig. 1).

EXPERIMENTAL PROCEDURES

Materials and Methods. *tert*-Butyloxycarbonyl (Boc)-amino acids were purchased from Bachem and were of the L-configuration. Papain (EC 3.4.22.2), N^{α}-tosyllysine chloromethyl ketone-treated α-chymotrypsin (EC 3.4.21.1), and arylsulfatase (EC 3.1.6.1) were obtained from Sigma; thermolysin (EC 3.4.24.2) and aminopeptidase M (EC 3.4.11.2) were purchased from Boehringer. The preparation of Boc-Met-OMe via acylation of H-Met-OMe followed the method of Moroder *et al.* (11). Boc-Tyr-N_2H_2Ph (Ph, phenyl), Boc-Gly-N_2H_2Ph, and Boc-Phe-N_2H_2Ph were synthesized by papain-catalyzed condensation in the presence of 2-mercaptoethanol as described by Milne and Carpenter (12). Deacylation of Boc-amino acid or peptide phenylhydrazides was achieved as reported (7). Thioanisole was added when methionine- or tryptophan-containing compounds were used. Removal of phenylhydrazide groups was accomplished with $FeCl_3$ as described (7). Acid hydrolyses (6 M HCl) were carried out at 110°C for 24 hr. Tryptophan-containing peptides were hydrolyzed in 4 M CH_3SO_3H/0.2% tryptamine (24 hr, 110°C). Quantitative peptide digestion was achieved by aminopeptidase M catalysis as described by Hofmann *et al.* (13) (5 units of enzyme/μmol of CCK-8; 30 hr). Amylase secretion by pancreatic acinar cells of guinea pigs was determined as reported by Peikin *et al.* (14). Thin-layer chromatograms were developed on precoated silica gel plates (Merck) using the following solvent systems (vol/vol): A, chloroform/methanol (3:1); B, chloroform/methanol/acetic acid (45:4:1); C, 1-butanol/pyridine/acetic acid/water (15:10:3:12); D, 2-butanol/5% ammonia (3:1). Electrophoreses were carried out as described by Gutte *et al.* (15).

Syntheses. *Boc-Asp(Bzl)-Tyr-N_2H_2Ph (I).* A solution of Boc-Asp(Bzl)-OH (Bzl, benzyl; 3.22 g, 10 mmol) and H-Tyr-N_2H_2Ph·CF_3COOH (23.2 g, 60 mmol) in 150 ml of methanol/McIlvain buffer, 1:3 (vol/vol), pH 6.3, was incubated with papain (2.5 g) in the presence of 2-mercaptoethanol (1.8 ml) at 40°C. After 50 hr, the resulting suspension was poured into 350 ml of ethyl acetate and the organic layer was washed in succession with 0.1 M HCl, water, 0.5 M $NaHCO_3$, and water, and dried over Na_2SO_4. On evaporation, an oil was obtained that was further purified by adsorption chromatography on a silica gel 60 column. The pure dipeptide (**I**) was eluted with chloroform/methanol, 90:1 (vol/vol). Crystallization from ethyl acetate/ether gave 1.90 g of **I** [3.3 mmol, 33% based on Boc-

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Abbreviations: CCK-8, COOH-terminal octapeptide amide of cholecystokinin; Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; Ph, phenyl.

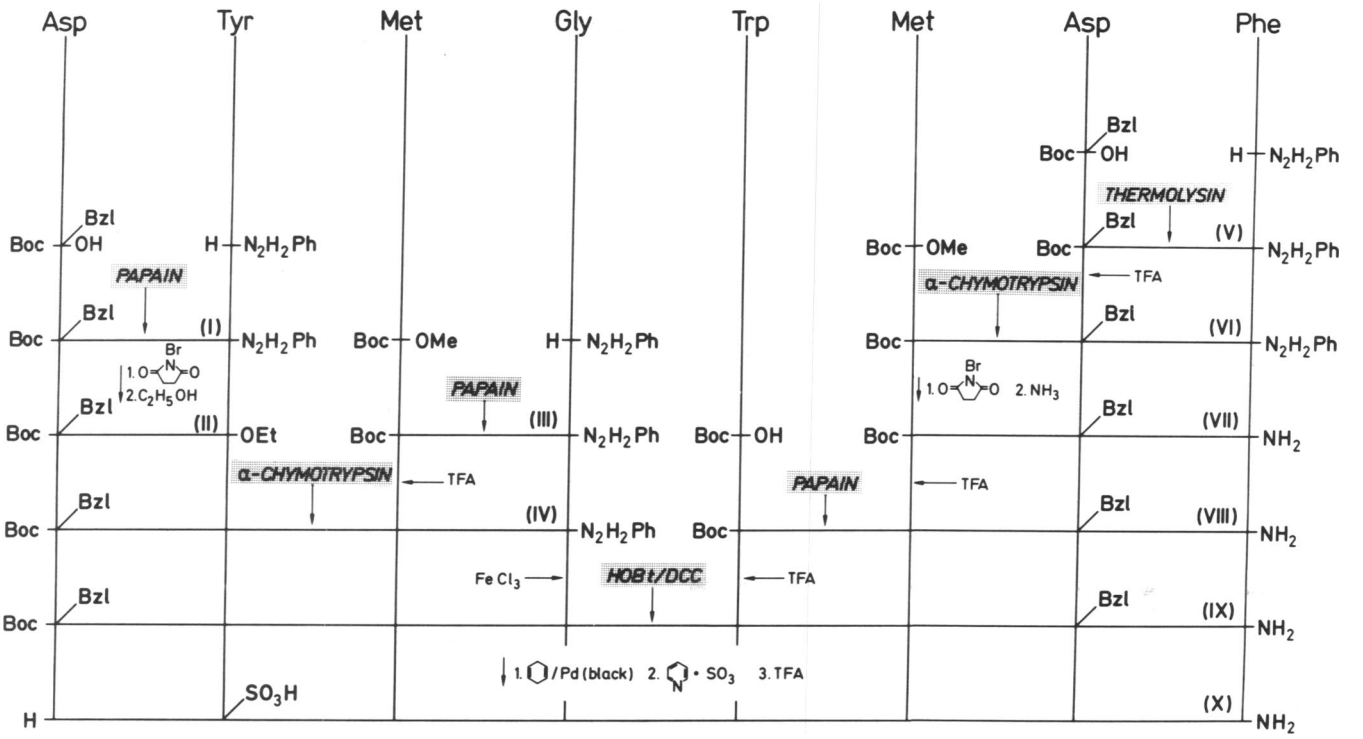

FIG. 1. Synthesis of the COOH-terminal octapeptide amide of cholecystokinin. TFA, CF_3COOH; HOBt, *N*-hydroxybenzotriazole; DCC, dicyclohexylcarbodiimide.

Asp(Bzl)-OH]. The product was chromatographically homogeneous in solvent systems A and B; mp 183–185°C; $[\alpha]_D^{22}$ −29.8° [*c* 1.0, dimethylformamide (Me_2CONH_2)]: Analysis. Calculated for $C_{31}H_{36}N_4O_7$: C, 64.57; H, 6.29; N, 9.72. Found: C, 64.29; H, 6.67; N, 9.62.

Boc-Asp(Bzl)-Tyr-OEt (II). The dipeptide phenylhydrazide (I) (1.73 g, 3.00 mmol) was oxidized with *N*-bromosuccinimide (587 mg, 3.30 mmol) and the resulting dipeptide diimide was then treated with absolute ethanol (10 ml). The preparation of II and the work-up conditions followed those described for Boc-Gly-Phe-OEt (7). II crystallized from ethyl acetate and was chromatographically homogeneous in systems A and B; yield, 638 mg (1.24 mmol, 41%); mp 111–113°C; $[\alpha]_D^{22}$ −11.2° (*c* 1, Me_2CONH_2): Analysis. Calculated for $C_{27}H_{34}N_2O_8$: C, 63.03, H, 6.66; N, 5.44. Found: C, 63.00; H, 6.70; N, 5.37.

Boc-Met-Gly-N_2H_2Ph (III). Boc-Met-OMe (1.84 g, 7 mmol) and H-Gly-N_2H_2Ph·CF_3COOH (3.91 g, 14 mmol) in 80 ml of methanol/McIlvain buffer (1:4), pH 5.6, were incubated with stirring with 0.8 ml of 2-mercaptoethanol and 1.1 g of papain at 40°C for 48 hr. The reaction mixture was extensively extracted with ethyl acetate and the organic layer was worked up as outlined for I. The resulting oil was fractionated on a silica gel 60 column. Elution with chloroform/methanol (75:1) gave the pure dipeptide (III), which was crystallized from ether/petroleum ether, and chromatographically homogeneous in systems A and B; yield, 1.69 g (4.27 mmol, 61% based on Boc-Met-OMe); mp 79–81°C; $[\alpha]_D^{22}$ −13.8° (*c* 0.7, Me_2CONH_2): Analysis. Calculated for $C_{18}H_{28}N_4O_4S$: C, 54.53; H, 7.12; N, 14.13; S, 8.09. Found: C, 54.23; H, 7.30; N, 14.39; S, 7.71.

Boc-Asp(Bzl)-Tyr-Met-Gly-N_2H_2Ph (IV). α-Chymotrypsin (60 mg) was added with stirring to a suspension of Boc-Asp(Bzl)-Tyr-OEt (II) (412 mg, 0.8 mmol) and H-Met-Gly-N_2H_2Ph·CF_3COOH (656 mg, 1.6 mmol) in 10 ml of Me_2CONH_2/0.2 M carbonate buffer, 2:3 (vol/vol) (pH 10.1). The reaction was allowed to proceed for 15 min at room temperature and then terminated by acidification to pH 3.0 with 1 M HCl. The resulting semisolid product was extracted into ethyl acetate and the organic layer was worked up as described for I. The resulting oil was applied to a silica gel 60 column and the pure tetrapeptide (IV) was eluted with chloroform/methanol, 85:1 (vol/vol). Crystallization from methanol/ether gave 296 mg of IV (0.39 mmol, 48% based on II). The product was chromatographically homogeneous in systems A and B; mp 167–169°C; $[\alpha]_D^{22}$ −18.5° (*c* 1, Me_2CONH_2): Analysis. Calculated for $C_{38}H_{48}N_6O_9S$: C, 59.68; H, 6.29; N, 10.99; S, 4.19. Found: C, 59.71; H, 6.09; N, 11.18; S, 4.37. Amino acid analysis: Asp, 1.04 (1); Gly, 1.00 (1); Met, 0.96 (1); Tyr, 0.91 (1).

Boc-Asp(Bzl)-Phe-N_2H_2Ph (V). Boc-Asp(Bzl)-OH (9.66 g, 30 mmol) and H-Phe-N_2H_2Ph·CF_3COOH (44.3 g, 120 mmol) in 200 ml of Me_2CONH_2/methanol/0.2 M Tris·HCl buffer/50 mM containing $Ca(OAc)_2$ (1:2:7), pH 8.0, were incubated at 46°C in the presence of thermolysin (1.5 g). After 24 hr, the resulting suspension was worked up as described for I. Crystallization from methanol/water gave 8.74 g of V [15.6 mmol, 52% based on Boc-Asp(Bzl)-OH]. The product was chromatographically homogeneous in systems A and B; mp 138–140°C; $[\alpha]_D^{22}$ −33.5° (*c* 0.9, MeOH): Analysis. Calculated for $C_{31}H_{36}N_4O_6$: C, 66.41; H, 6.47. N, 9.99. Found: C, 66.32; H, 6.59; N, 9.71.

Boc-Met-Asp(Bzl)-Phe-N_2H_2Ph (VI). To a stirred solution of Boc-Met-OMe (1.62 g, 6.15 mmol) and H-Asp(Bzl)-Phe-N_2H_2Ph·CF_3COOH (8.62 g, 15 mmol) in 24 ml of Me_2CONH_2 was added 40 ml of 0.2 M carbonate buffer until the solution became slightly turbid. The reaction mixture (pH 9.9) was incubated with α-chymotrypsin (500 mg) at 37°C with vigorous stirring. After 12 hr, the reaction was stopped by acidification to pH 3.2 with 1 M HCl. The resulting heavy suspension was worked up as described for I. Crystallization from chloroform/ether gave 1.99 g of VI (2.89 mmol, 47% based on Boc-Met-OMe). The product was chromatographically homogeneous in systems A and B; mp 168–170°C; $[\alpha]_D^{22}$ −36.8° (*c* 1, Me_2CONH_2): Analysis. Calculated for $C_{36}H_{45}N_5O_7S$: C, 62.50; H, 6.55; N, 10.12; S, 4.63. Found: C, 62.18; H, 6.42; N, 9.94; S, 4.58.

Boc-Met-Asp(Bzl)-Phe-NH_2(VII). The tripeptide phenylhydrazide (**VI**) (1.82 g, 2.63 mmol) was oxidized with *N*-bromosuccinimide (516 mg, 2.90 mmole) as described for **II**. The resulting tripeptide diimide was dissolved in 10 ml of a saturated solution (prepared at 0°C) of ammonia in methylene chloride and stored in the dark. After 1 hr at 0°C and 5 hr at room temperature, the solvent was evaporated and the residue was dissolved in ethyl acetate. The organic layer was worked up as described for **I**. The resulting oil was applied to a silica gel 60 column and the pure tripeptide (**VII**) was eluted with chloroform/methanol, 70:1 (vol/vol). Crystallization from ethyl acetate/ether gave 850 mg of **VII** (1.42 mmol, 54%). The product was chromatographically homogeneous in systems A and B; mp 171–172°C; $[\alpha]_D^{22}$ −34.5° (*c* 1, Me_2CONH_2): Analysis. Calculated for $C_{30}H_{40}N_4O_7S$: C, 59.99; H, 6.71; N, 9.33; S, 5.34. Found: C, 59.97; H, 6.46; N, 9.17; S, 5.62.

Boc-Trp-Met-Asp(Bzl)-Phe-NH_2(VIII). Boc-Trp-OH (170 mg, 0.56 mmol) and H-Met-Asp(Bzl)-Phe-NH_2·CF_3COOH (690 mg, 1.12 mmol) were dissolved in 7 ml of methanol/3 M sodium acetate buffer (1:3), pH 5.0. 2-Mercaptoethanol (0.07 ml) was added and the solution was incubated with stirring in the presence of papain (100 mg) at 40°C for 28 hr. The resulting suspension was poured into ethyl acetate (40 ml). The organic layer was worked up as described for **VII**. Crystallization from ethanol/ether gave 236 mg (0.30 mmol, 54%). The product was chromatographically homogeneous in systems A and B; mp 168–170°C; $[\alpha]_D^{22}$ −20.5° (*c* 1, MeOH): Analysis. Calculated for $C_{41}H_{50}N_6O_8S$: C, 62.58; H, 6.40; N, 10.68; S, 4.07. Found: C, 62.69; H, 6.45; N, 10.78; S, 3.91. Amino acid analysis: Asp, 1.03 (1); Met, 0.98 (1); Phe, 1.00 (1); Trp, 0.90 (1).

Boc-Asp(Bzl)-Tyr-Met-Gly-Trp-Met-Asp(Bzl)-Phe-NH_2(IX). Boc-Asp(Bzl)-Tyr-Met-Gly-OH (135 mg, 0.20 mmol) and H-Trp-Met-Asp(Bzl)-Phe-NH_2·CF_3COOH (160 mg, 0.20 mmol) in 3 ml of Me_2CONH_2 was treated with stirring with *N*-hydroxybenzotriazole (40 mg, 0.27 mmol) and dicyclohexylcarbodiimide (41 mg, 0.20 mmol) in the presence of 4-methylmorpholine (0.023 ml, 0.20 mmol). After 2 hr at 0°C and 20 hr at room temperature, the reaction mixture was diluted by addition of 30 ml of ethyl acetate, the insoluble by-product was removed by filtration, and the filtrate was worked up as described for **VIII**. The resulting oil was chromatographed on a silica gel 60 column and the octapeptide (**IX**) was eluted with chloroform/methanol (50:1). Crystallization from methanol/diisopropyl ether gave 161 mg of **IX** (0.12 mmol, 60%). The product was homogeneous in systems A and B; mp 202–205°C; $[\alpha]_D^{22}$ −33.6° (*c* 0.8, Me_2CONH_2): Analysis. Calculated for $C_{68}H_{82}N_{10}O_{15}S_2$: C, 60.79; H, 6.15; N, 10.43; S, 4.77. Found: C, 60.42; H, 6.43; N, 10.62; S, 4.71. Amino acid analysis: Asp, 2.04 (2); Gly, 1.00 (1); Met, 1.95 (2); Tyr, 0.92 (1); Phe, 1.02 (1); Trp, 0.93 (1).

H-Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2(X). Catalytic transfer hydrogenolysis followed the methods described by Felix *et al.* (16). The protected octapeptide (**IX**) (134 mg, 0.1 mmol) was dissolved in 2 ml of Me_2CONH_2/acetic acid, 1:2 (vol/vol) and treated with 1, 4-cyclohexadiene (0.19 ml, 2 mmol) in the presence of palladium black (270 mg) at 25°C. After 15 hr, the mixture was filtered and evaporated at reduced pressure. The residue was dissolved in dry Me_2CONH_2 (4 ml)/pyridine (2 ml). After addition of pyridine–sulfur trioxide complex (312 mg, 2 mmol), the mixture was incubated for 20 hr at 23°C. The solvents were removed at reduced pressure and the residue was washed with cold water and was again dried at reduced pressure. The crude sulfated peptide was fractionated by partition chromatography on a silica gel 60 column. Elution with the organic layer of the solvent system 1-butanol/acetic acid/water (4:1:5) allowed separation of a faster and a slower moving by-product. The fractions containing the desired peptide were pooled and evaporated to dryness at reduced pressure. To remove the N^{α}-protecting group, the residue from evaporation was treated with trifluoroacetic acid in the presence of thioanisole. After 20 min, the solvents were removed by evaporation at reduced pressure and the remaining octapeptide sulfate was precipitated with ether. The precipitate was filtered off, dried at reduced pressure, and fractionated on a Bio-Gel P-6 column using 0.05 M ammonium bicarbonate as eluant. The appropriate fractions were pooled and lyophilized. Further purification was achieved by ion-exchange chromatography on a DEAE-Sephadex A-25 column, as described by Ondetti *et al.* (17). The synthetic octapeptide (**X**) was chromatographically and electrophoretically homogeneous in systems C and D and in 0.1 M pyridinium acetate buffer (pH 5.8), respectively; yield, 37 mg of **X** (0.032 mmol, 32%); $[\alpha]_D^{22}$ −19.7° (*c* 0.8, 1 M NH_4OH) [lit.: $[\alpha]_D^{23}$ −18.4° (*c* 0.7, 1 M NH_4OH) (17); $[\alpha]_D^{25}$ −20.0° (*c* 0.5, 1 M NH_4OH) (18)]. Amino acid analysis: Asp, 2.03 (2); Gly 1.00 (1); Met, 1.96 (2); Tyr, 0.94 (1); Phe, 1.01 (1); Trp, 0.95 (1). Aminopeptidase M digest: Tyr(SO_3H), 0.96 (1); Asp, 1.98 (2); Gly, 1.00 (1); Met, 1.97 (2); Phe, 1.02 (1); Trp, 0.97 (1).

RESULTS AND DISCUSSION

Considering the limited availability of proteolytic enzymes exhibiting narrowly restricted specificity, one can hardly expect to compile a collection of seven different proteases each of which solely acts on the particular peptide bond of the CCK-8 molecule that actually is to be formed. Therefore, I resorted to proteases of broader specificity that enable the synthesis of various peptide bonds. Consequently, enzymatic chain elongation by stepwise coupling of successive amino acid units could jeopardize preexisting scissile bonds. Preliminary results indicated that fragments containing more than four amino acid units were not obtainable by protease-controlled synthesis. This result, together with the fact that a glycine residue is present in the middle of the CCK-8 molecule (see above), suggested assembly of the desired octapeptide from two separately prepared tetrapeptides. In case the above fragments could not be condensed enzymatically, they could nevertheless be coupled by chemical means without endangering the chiral integrity of the resulting octapeptide.

As shown in the scheme of the synthesis (Fig. 1), the key intermediates for the preparation of the octapeptide (**IX**) were the protected peptides Boc-Asp(Bzl)-Tyr-Met-Gly-N_2H_2Ph (**IV**) and Boc-Trp-Met-Asp(Bzl)Phe-NH_2 (**VIII**). **IV** was synthesized in 48% yield via α-chymotrypsin-catalyzed coupling of Boc-Asp(Bzl)-Tyr-OEt (**II**) and H-Met-Gly-N_2H_2Ph. **II** was obtained by incubation of Boc-Asp(Bzl)-OH and H-Tyr-N_2H_2Ph with papain to give Boc-Asp(Bzl)-Tyr-N_2H_2Ph (**I**) (33%). Subsequent replacement of the phenylhydrazide group of **I** by an ethyl ester using *N*-bromosuccinimide and ethanol (19) gave **II** (41%). Although the thermolysin-catalyzed coupling of Boc-Asp(Bzl)-OH and H-Phe-N_2H_2Ph was quite efficient (see below), formation of **I** in the presence of thermolysin did not work. Similar observations have been reported by Isowa and Ichikawa (20), who found that tyrosine derivatives were inappropriate acceptor nucleophiles for thermolysin catalysis. Boc-Met-Gly-N_2H_2Ph (**III**), the deacylated form of which served as acceptor nucleophile during the α-chymotrypsin-catalyzed formation of **IV**, was obtained by papain-mediated coupling of either Boc-Met-OMe or Boc-Met-OH and H-Gly-N_2H_2Ph. I preferred Boc-Met-OMe as acyl group donor (yield, 61%) to Boc-Met-OH (51%).

A previous attempt to prepare **IV** by protease-catalyzed stepwise coupling of successive amino acid residues had failed. Although the tripeptide Boc-Tyr-Met-Gly-N_2H_2Ph could be ob-

tained by α-chymotrypsin-controlled reaction between Boc-Tyr-OEt and H-Met-Gly-N_2H_2Ph, enzymatic acylation of H-Tyr-Met-Gly-N_2H_2Ph with Boc-Asp(Bzl)-OH could not be achieved. Neither thermolysin nor papain catalyzed the projected reaction. In the presence of papain, the Met-Gly bond of the amino component was split. To prevent proteolytic cleavage of preexisting peptide bonds, the synthetic pathway shown in Fig. 1 was chosen. α-Chymotrypsin-mediated fragment coupling of two dipeptides, separately prepared via papain catalysis, enabled the successful synthesis of **IV**.

Except for replacement of the third amino acid residue of its peptide chain (methionine) by threonine, CCK-8 is identical to the COOH-terminal octapeptide of cerulein (21), which exhibits biological functions similar to cholecystokinin (22). To study the applicability of the synthetic pathway leading to **IV** to the synthesis of the corresponding cerulein fragment, we synthesized the tetrapeptide Boc-Asp(Bzl)-Tyr-Thr(Bzl)-Gly-N_2H_2Ph by α-chymotrypsin-catalyzed condensation of **II** with H-Thr(Bzl)-Gly-N_2H_2Ph. The dipeptide Boc-Thr(Bzl)-Gly-N_2H_2Ph was prepared by incubation of Boc-Thr(Bzl)-OH and H-Gly-N_2H_2Ph in the presence of papain. The threonine benzyl ether was incorporated into the tetrapeptide so as to leave the possibility of eventual sulfation of the phenolic function of tyrosine without affecting the alcoholic moiety of threonine.

VIII was prepared by a stepwise procedure through the concerted action of three proteases of different specificities. Boc-Asp(Bzl)-Phe-N_2H_2Ph (**V**) was obtained in 52% yield by incubating Boc-Asp(Bzl)-OH and H-Phe-N_2H_2Ph in the presence of thermolysin. The tripeptide Boc-Met-Asp(Bzl)-Phe-N_2H_2Ph (**VI**) (47%) was synthesized by α-chymotrypsin-mediated reaction of Boc-Met-OMe with H-Asp(Bzl)-Phe-N_2H_2Ph. The phenylhydrazide-protected amino components were chosen to enable the synthesis of the Met-Asp bond, which could not be formed via incubation of Boc-Met-OMe or Boc-Met-OH and H-Asp(Bzl)-Phe-NH_2 in the presence of α-chymotrypsin or papain. [Boc-Asp(Bzl)-Phe-NH_2 was easily available by thermolysin catalysis.] The phenylhydrazide group of **VI** was replaced by an amide group by using *N*-bromosuccinimide and then ammonia. The resulting tripeptide amide (**VII**) was obtained in 54% yield. This rearrangement was carried out prior to the incorporation of a tryptophan residue into the growing peptide chain so as to prevent any undesired reaction of *N*-bromosuccinimide with the indole moiety of tryptophan (23). **VIII** was obtained by acylation of H-Met-Asp(Bzl)-Phe-NH_2 with Boc-Trp-OH in the presence of papain (54%); it could not be prepared by α-chymotrypsin-controlled coupling of Boc-Trp-OEt and H-Met-Asp(Bzl)-Phe-NH_2 because the donor ester was not cleaved by the enzyme.

VIII represents a derivatized form of the COOH-terminal tetrapeptide (tetragastrin) of gastrin, a heptadecapeptide hormone (24) that controls secretion of gastric acid. The tetragastrin molecule is sufficient to display the physiological activities of the complete gastrin molecule (25).

The target octapeptide derivative (**IX**) was finally obtained in 60% yield by condensation of Boc-Asp(Bzl)-Tyr-Met-Gly-OH and H-Trp-Met-Asp(Bzl)-Phe-NH_2 in the presence of *N*-hydroxybenzotriazole and dicyclohexylcarbodiimide; attempts to couple the two tetrapeptides via papain, thermolysin, ficin, or bromelain catalysis did not provide **IX**. These failures can be explained by the finding that, in all cases, at least one of the reactants was degraded by the protease. **IX** was subjected to catalytic transfer hydrogenation to remove the benzyl ester groups from the aspartic acid side chains, after which the phenolic hydroxyl group of the tyrosine residue was esterified with the aid of a pyridine–sulfur trioxide complex (26). Removal of the N^{α}-*tert*-butyloxycarbonyl group by using trifluoroacetic acid in the presence of thioanisole completed the synthesis. After purification via gel filtration and ion-exchange and partition chromatography, the COOH-terminal cholecystokinin octapeptide (**X**) was obtained in homogeneous form. The stereochemical homogeneity of the octapeptide amide and the presence of the tyrosine unit as *O*-sulfate were confirmed by amino acid analysis of aminopeptidase M digests. Arylsulfatase-catalyzed desulfation of tyrosine-*O*-sulfate (27)—recovered from the aminopeptidase M hydrolysate—gave the free tyrosine. The biological activity of the synthetic **X** with respect to stimulation of amylase release was tested on isolated pancreatic cells of guinea pigs. The secretion of amylase by dispersed pancreatic acini was maximal at a concentration of **X** of 0.3 nM (Fig. 2).

Despite its promising features, protease-catalyzed peptide synthesis is not yet generally applicable. This drawback could be overcome most efficiently by a set of proteases whose stringent specificity would enable the synthetic peptide chemist to prepare any conceivable peptide linkage without interfering with preexisting bonds. But it is rather unlikely that nature can satisfy this requirement. Nevertheless, the incomplete collection can be supplemented by proteases whose broader specificities enable the synthesis of a variety of peptide bonds. However, the bright side of the picture may be darkened by the lack of narrowly restricted specificity of the enzymes used that jeopardizes preexisting peptide linkages (28). To prevent these undesired side reactions, smaller peptides can be synthesized by the action of proteases of different noninterfering specificities. By using this procedure, I prepared **VIII** with the aid of thermolysin, α-chymotrypsin, and papain. In case the synthesis of a peptide requires the use of a particular protease more than once, the target peptide can be divided into subunits that are prepared separately. The fragments thus obtained are coupled by a protease that does not endanger the preformed peptide bonds. **IV** was synthesized according to this procedure via papain and α-chymotrypsin catalysis.

In the condensation reactions discussed above, relatively

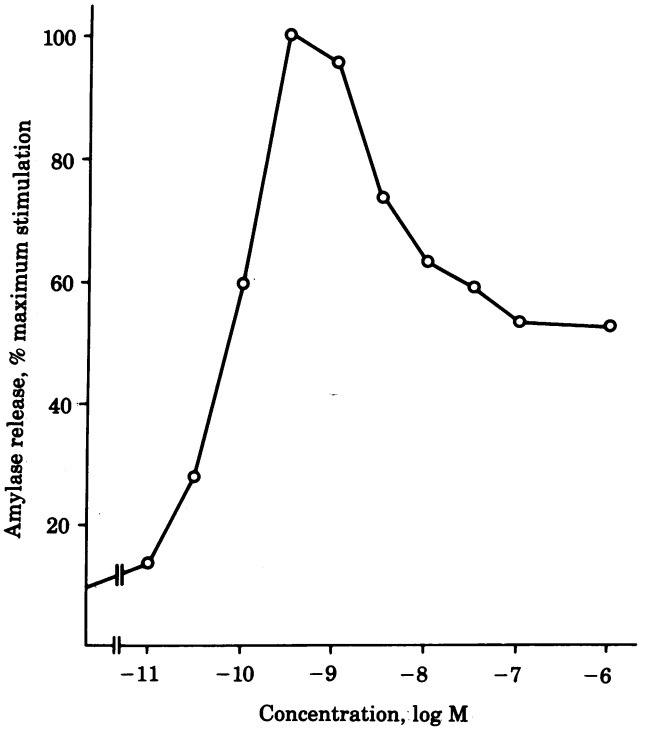

FIG. 2. Stimulation of amylase release by synthetic CCK-8 from dispersed acinar cells of guinea pig pancreas.

high concentrations of proteases were required to obtain reasonably rapid rates of peptide bond synthesis. This finding is consistent with earlier observations (4). Fruton (4) attributed this disadvantage of the enzymic method to the unfavorable equilibrium of the process

$$RCOO^- + {}^+NH_3R' + EH \rightleftarrows [RCO]E[NH_2R'] + H_2O.$$

The susceptibility of preexisting peptide linkages was responsible for the failure to assemble **IX** enzymatically. Unfortunately, a protease whose substrate specificity is limited to bonds in which the carbonyl group is contributed by a glycine residue was not available. Therefore, I used enzymes that are known to act on a variety of different peptidic substrates. As a result, at least one of the reactants was degraded by each of the proteolytic enzymes. On the other hand, a carboxyl component possessing a COOH-terminal glycine residue allows a chemical coupling step without impairing one of the most significant merits of the enzymatic synthesis: preservation of chiral integrity.

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