

Protease-Catalyzed Semisynthesis of Human Neuropeptide Y¹⁾

Kiyoshi SAKINA,^{*,a} Susumu FUNAKOSHI,^b and Haruaki YAJIMA^b

Kyoto Research Laboratories, Toho Pharmaceutical Ind. Co., Ltd.,^a Nagaokakyo-shi, Kyoto 617, Japan and Faculty of Pharmaceutical Sciences, Kyoto University,^b Sakyo-ku, Kyoto 606, Japan. Received August 29, 1988

Human neuropeptide Y was semisynthesized by enzymatic condensation of des-Tyr³⁶-NH₂ human neuropeptide Y and H-Tyr-NH₂ using *Pseudomonas aeruginosa* elastase, a metalloenzyme possessing a hydrolytic specificity for the imino side of hydrophobic amino acids. The optimum pH for this enzymatic synthesis was judged to be around 7 in a high concentration of an organic solvent.

Keywords peptide amide synthesis; human neuropeptide Y semisynthesis; des-Tyr³⁶-NH₂ human neuropeptide Y; protease-catalyzed synthesis; *Pseudomonas aeruginosa* elastase

In the previous paper,²⁾ we reported model experiments for the semisynthesis of several C-amidated peptides and showed that thermolysin catalyzed the condensation reaction of various hydrophobic amino acid amides with not only N^α-protected peptides but also unprotected peptides.

We have applied such an enzymatic method to the semisynthesis of human neuropeptide Y (hNPY), the structure of which was firmly established by the deoxyribonucleic acid (DNA) sequence analysis of its precursor by Minth *et al.*³⁾ in 1984. hNPY is a C-amidated peptide consisting of 36 amino acid residues. In the present synthesis, enzymatic condensation reactions between des-Tyr³⁶-NH₂ hNPY and H-Tyr-NH₂ were carried out in 90% DMF-EtOH (1:1, v/v) at pH 7 and room temperature for 4 h.

First, the starting material, des-Tyr³⁶-NH₂ hNPY, was prepared by the Fmoc-based solid-phase method,⁴⁾ followed by thioanisole-mediated deprotection with TMSOTf⁵⁾ as shown in Fig. 1. The peptide was purified to homogeneity by high-performance liquid chromatography (HPLC) and its purity was ascertained by amino acid analysis, after acid hydrolysis.

For condensation of des-Tyr³⁶-NH₂ hNPY with H-Tyr-NH₂, two metalloenzymes were selected. The reaction products were examined by HPLC in comparison with an authentic sample of hNPY. Under the above conditions, thermolysin did not give any product corresponding to hNPY. However, we could obtain the desired product in nearly 40% yield, when *Pseudomonas aeruginosa* elastase⁶⁾ was used as a catalyst (Fig. 2). The product was isolated and characterized by amino acid analysis, and incorporation of one residue of Tyr was confirmed.

Thermolysin and *P. aeruginosa* elastase are both metalloenzymes possessing a similar substrate specificity for the imino side of hydrophobic amino acids as the reaction site.

However, their affinities for hydrophobic amino acids are somewhat different. In the case of thermolysin,⁷⁾ the affinity for amino acid residues is in the order of Phe, Leu, Ile > Val > Ala > Tyr, and for *P. aeruginosa* elastase,⁸⁾ Phe > Tyr > Leu. Thus, high affinity of *P. aeruginosa* elastase for the Tyr residue was expected to make it possible to condense the two components employed in a relatively high yield.

Next, suitable reaction conditions for semisynthesis of hNPY by means of *P. aeruginosa* elastase were examined and the results are shown in Table I. At high pH values or low concentrations of organic solvents, undesirable hydrolysis of des-Tyr³⁶-NH₂ hNPY was observed. However, the substrate remained intact at relatively low pH values. Consequently, hNPY was produced in 40% yield at pH 7 in the presence of a high concentration of an organic solvent by suppressing undesirable hydrolysis of the peptide bond.

This enzymatic method may be a useful tool for semisynthesis of other biologically active peptide amides having a hydrophobic amino acid residue at the C-terminal position.

TABLE I. Effects of Reaction Conditions on Semisynthesis of hNPY

Organic solvent	Conc. (%)	pH	Enzyme conc. (mm)	Des-Tyr ³⁶ -NH ₂ hNPY remained (%)	hNPY yield (%)
DMF-EtOH (1:1)	90	7	1.8	40	40
	50	7	1.8	0	0
	90	5	1.8	100	0
	90	8	1.8	0	0
	90	7	0.65	80	10
EtOH	90	7	1.8	40	20

The reaction mixture, containing 5 mm des-Tyr³⁶-NH₂ hNPY, 250 mm H-Tyr-NH₂ and *Pseudomonas aeruginosa* elastase, was stirred at room temperature for 4 h.

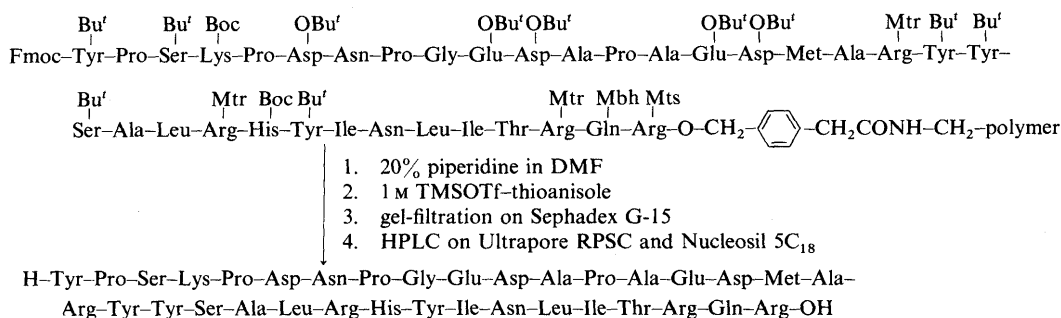


Fig. 1. Synthetic Route to Des-Tyr³⁶-NH₂ hNPY

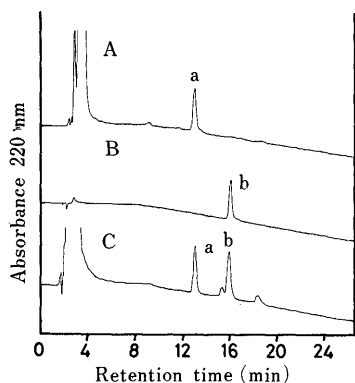


Fig. 2. HPLC Profile of Des-Tyr³⁶-NH₂ hNPY (A), hNPY (B), and the Coupling Product Formed by *Pseudomonas aeruginosa* Elastase (C)

Column: Nucleosil 5C₁₈ (4.0 × 200 mm). Solvent: gradient of CH₃CN (30 → 45%/20 min) in 50 mM AcONH₄ (pH 7.0). Flow rate: 1.0 ml/min. a, des-Tyr³⁶-NH₂ hNPY; b, hNPY.

Experimental

Thermolysin and *P. aeruginosa* elastase (81.2 mPU/mg protein) were obtained from Wako Pure Chemicals, Osaka, and Nagase Biochemicals, Kyoto, respectively. hNPY was purchased from Peptide Institute, Inc., Osaka. HPLC was conducted with a Shimadzu LC-4A model. Acid hydrolysis (6N HCl) was carried out at 110 °C for 18 h, and amino acids were determined with a Hitachi 835 analyzer.

Solid-Phase Synthesis of Des-Tyr³⁶-NH₂ hNPY The solid-phase synthesis was carried out manually by means of an Fmoc-based strategy according to the principle of Sheppard *et al.*⁴⁾ using the following side-chain-protected Fmoc-amino acids; Arg(Mts),⁹⁾ Arg(Mtr),¹⁰⁾ His-(Boc),¹¹⁾ Glu(OBu^t), Asp(OBu^t), Tyr(Bu^t), Ser(Bu^t), and Gln(Mbh).¹²⁾ First, Boc-Arg(Mts)-Pam-resin (323 mg, amine content 0.62 mmol/g polystyrene resin cross linked with 1% divinylbenzene, purchased from Applied Biosystems, California, U.S.A.) was treated twice (5 and 15 min) with 60% TFA in CH₂Cl₂ to remove the Boc group and then treated with 5% diisopropylamine in CH₂Cl₂. At each cycle, the Fmoc group was removed by treatment with 20% piperidine in DMF (twice, 2 ml each, 5 and 15 min), then the resin was washed with DMF (6 times, 5 ml each). Each Fmoc-amino acid (2.5 eq) in *N*-methyl-2-pyrrolidone (2 ml) was condensed successively by means of DIPCD-HOBt (2.5 eq each). The container was shaken until the resin became negative to the Kaiser test¹³⁾ (usually 1 h). For introduction of Arg(Mtr), Gln(Mbh), and Tyr(Bu^t) (positions 15 and 16), double couplings were necessary to complete the reaction.

Deprotection with TMSOTf-Thioanisole/TFA The protected peptide-resin (100 mg) was treated with 1 M TMSOTf-thioanisole/TFA⁵⁾ (10 ml) in the presence of *m*-cresol (200 μl) and ethanedithiol (568 μl) in an ice bath for 2 h. Dry ether was added to precipitate the product, and the residue was dissolved in 0.2 M Tris-HCl buffer (pH 8.0) containing 6 M guanidine hydrochloride (3 ml). The resin was removed by filtration and washed with the same buffer (three times, 1 ml each). The filtrate and washing were combined and then 1 M NH₄F (100 μl) and 2-mercaptoethanol (200 μl) were added. The solution was kept in an ice bath for 30 min, then the pH was adjusted to 6.5 with AcOH. After being incubated at 37 °C for 12 h, the solution was applied to column of Sephadex G-15 (3.8 × 50 cm), which was eluted with 1 N AcOH. The fractions corresponding to the front peak (tube Nos. 11–14, 10 ml each, monitored by ultraviolet (UV) absorption measurement at 280 nm) were combined and the solution was lyophilized to give a powder; yield 35.4 mg (yield 61.1% based on the Arg (Mts) loaded on the resin).

The crude peptide thus obtained was purified by HPLC in 2 steps. First, the sample was applied to a Ultrapore RPSC column (10 × 250 mm), which was eluted with a gradient of CH₃CN (20 → 40%/60 min) in 0.1% TFA at a flow rate of 2.0 ml/min. The eluate corresponding to the main peak

(retention time 45 min, detected by UV absorption measurement at 230 nm) was collected and lyophilized. Second, the residue was applied to a Nucleosil 5C₁₈ column (4 × 200 mm), which was eluted with a gradient of CH₃CN (30 → 45%/20 min) in 50 mM AcONH₄ (pH 7.0) at a flow rate of 1.0 ml/min. The eluate corresponding to the main peak (retention time 14 min, detected by UV absorption measurement at 220 nm) was collected and lyophilized to give a white powder; yield 1.2 mg (2.1%). Amino acid ratios in a 6N HCl hydrolysate (numbers in parentheses are theoretical): Asp 5.29 (5), Thr 1.00 (1), Ser 1.89 (2), Glu 3.10 (3), Pro 4.22 (4), Gly 1.07 (1), Ala 4.00 (4), Met 0.95 (1), Ile 1.91 (2), Leu 1.90 (2), Tyr 3.76 (4), Lys 1.14 (1), His 0.99 (1), Arg 3.71 (4) (recovery of Ala, 75%).

Enzymatic Synthesis of hNPY A suspension of *P. aeruginosa* elastase (36 nmol) in H₂O (40 μl) containing 3.0 M (NH₄)₂SO₄, 10 mM AcONa, 2 mM CaCl₂, and 0.05 mM ZnCl₂, was centrifuged (2000 rpm, 5 min), and the pellet was suspended in an appropriate solvent (see Table I, 18 μl) together with des-Tyr³⁶-NH₂hNPY (400 μg, 100 nmol) and H-Tyr-NH₂·HCl (1.08 mg, 5.0 μmol). After pH adjustment (see Table I) by addition of 5% NH₄OH and H₂O (2 μl), the mixture was stirred at room temperature for 4 h, then diluted with 2% TFA (180 μl). After centrifugation (2000 rpm, 5 min), an aliquot (5 μl) of the supernatant was subjected to HPLC analysis using a Nucleosil 5C₁₈ column (4 × 200 mm), which was eluted with gradient of CH₃CN (30 → 45%/20 min) in 50 mM AcONH₄ (pH 7.0) at a flow rate of 1.0 ml/min (detected by UV absorption measurement at 220 nm). A typical elution pattern is shown in Fig. 2C, and the yields calculated from the peak areas are listed in Table I.

Identification of Product The desired product obtained by HPLC purification exhibited a retention time identical with that of authentic hNPY in HPLC (16 min) under the conditions stated above. Its amino acid ratios in a 6N HCl hydrolysis were (numbers in parentheses are theoretical): Asp 4.94 (5), Thr 1.11 (1), Ser 1.89 (2), Glu 2.88 (3), Pro 4.04 (4), Gly 1.07 (1), Ala 4.00 (4), Met 0.94 (1), Ile 1.85 (2), Leu 1.83 (2), Tyr 4.63 (5), Lys 1.10 (1), His 1.06 (1), Arg 3.84 (4).

References and Notes

- 1) Amino acids used in this paper are of L-configuration. The following abbreviations are used: Fmoc = 9-fluorenylmethyloxycarbonyl, Bu^t = *tert*-butyl, Boc = *tert*-butoxycarbonyl, Mts = mesitylene-2-sulfonyl, Mtr = 4-methoxy-2,3,6-trimethylbenzenesulfonyl, Mbh = 4,4'-dimethoxybenzhydryl, TMSOTf = trimethylsilyl trifluoromethanesulfonate, TFA = trifluoroacetic acid, HOBt = *N*-hydroxybenzotriazole, DMF = dimethylformamide, DIPCD = 1,3-diisopropylcarbodiimide, Pam = (4-oxymethyl)phenylacetamidomethyl.
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