Synthesis of Head Activator (HA)-Related Peptides and Development of HA-Radioimmunoassay

NAOKI SAKURA,* MIWAKO NISHIJIMA, YOSHIKI UCHIDA, TETSUHIRO TANI, and TADASHI HASHIMOTO

School of Pharmacy, Hokuriku University, Kanagawa-machi, Kanazawa 920–11, Japan

(Received November 20, 1986)

Synthesis of peptides related to hydra head activator (HA; pGlu–Pro–Pro–Gly–Gly–Ser–Lys–Val–Ile–Leu–Phe–OH) was performed by the solution method. The analogs synthesized were Tyr11-HA, des-Phe11-HA, Arg1-HA and Phe5-HA. Antisera were prepared by immunizing three rabbits with the synthetic HA. Two anti-HA-antisera with sufficient titer were obtained. An HA-radioimmunoassay system, developed by using an antiserum (ASH-04) and 125I-Tyr11-HA as a tracer, was found to be sensitive and specific to HA.

Keywords — head activator; synthesis; antibody preparation; 125I-Tyr11-head activator; radioimmunoassay; specificity; droplet counter current chromatography; HPLC

The hydra head activator (HA) is an undecapeptide isolated from the fresh water hydra and the sea anemone, and sequenced as pGlu–Pro–Pro–Gly–Gly–Ser–Lys–Val–Ile–Leu–Phe–OH1 (I). The peptide is a growth hormone-like factor in hydra stimulating head specific growth or bud formation, together with other morphogenous factors.2 A peptide with complete sequence homology occurs not only in the coelenterates but also in mammals—human, bovine and rat hypothalami, and intestine.3) Except for the induction of amylase release from rat pancreas in vitro but not in vivo, and a weak stimulatory effect on smooth muscle,4) no biological activity or physiological function of HA has been reported in mammals.

In the present paper we describe: a) the synthesis of four HA-related peptides (Fig. 1), and b) the development of an HA-radioimmunoassay (RIA).

Synthesis of HA-related peptides was carried out by a conventional solution method. The protected peptides prepared as units for successive azide fragment condensation to build up the peptide backbones were as follow; Z–pGlu–Pro–Pro–Gly–NHNHBoc (VI), Z–Arg(NO2)–Pro–Pro–Gly–NHNHBoc,5 Boc–Gly–Ser–N2H3,6 Boc–Phe–Ser–N2H3,7 Boc–Lys(Z)–Val–Ile–Leu–Tyr–OH (VII), Boc–Lys(Z)–Val–Ile–Leu–OH (VIII) and Boc–Lys(Z)–Val–Ile–Leu–Phe–OH (IX). In Fig. 2, the synthetic route is shown for an analog, Tyr11-HA (II), which

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
</table>

Fig. 1. Head Activator (HA) and Related Peptides
was designed as a labelled antigen preparation with minimum structural modification (replacing C-terminal Phe by Tyr). The C-terminal protected pentapeptide (VII) was prepared in a stepwise fashion by the active ester method. The starting material, H-Leu-Tyr-OH was coupled with Z-Ile-OSu to yield Z-Ile-Leu-Tyr-OH (X). Debenzyloxycarbonylation of X by catalytic hydrogenation followed by coupling with Z-Val-OSu gave Z-Val-Ile-Leu-Tyr-OH (XI), which was deprotected by the same procedure. Acylation of the resulting tetrapeptide with the active ester of Boc-Lys(Z)-OH gave VII. Removal of the N\textsuperscript{9}–Boc group of VII was carried out by treatment with trifluoroacetic acid (TFA) at 15 °C for 45 min to suppress NE-Z cleavage of Lys. The resulting pentapeptide H-Lys(Z)-Val-Ile-Leu-Tyr-OH\textsubscript{TFA} (VII') was condensed with Boc-Gly-Ser-N\textsubscript{N} by the azide method\textsuperscript{10} and the protected heptapeptide Boc-Gly-Ser-Lys(Z)-Val-Ile-Leu-Tyr-OH (XII) obtained was further subjected to tert-butyloxycarbonylation under the same conditions as described above.

The N-terminal tetrapeptide (VI) was prepared by the reaction between Z-pGlu-Pro–OH\textsuperscript{11} and H-Pro-Gly-NHNNHBOc\textsuperscript{11} with the dicyclohexylcarbodiimide (DCC)–N-hydroxybenzotriazole (HOBT) procedure.\textsuperscript{12} The final coupling of Z-pGlu-Pro-Pro-Gly–N\textsubscript{3} derived from VI with H-Gly-Ser-Lys(Z)-Val-Ile-Leu-Tyr-OH (XII') yielded the protected undecapeptide Z-pGlu-Pro-Pro–Gly–Gly–Ser–Lys(Z)–Val–Ile-Leu–Tyr–OH, which was hydrogenated to give crude II. Purification of peptide II was performed by droplet counter current chromatography (DCCC),\textsuperscript{13} with a solvent system of n-butanol-acetic acid–water (4:1:5). The desired peptide emerged after the solvent front of the lower phase used as the mobile phase. A highly purified product was obtained after gel filtration on Sephadex G-25 using 2 M acetic acid as the solvent. The homogeneity of this peptide was proved by thin layer chromatography (TLC), high performance liquid chromatography (HPLC), elemental analysis and amino acid analysis of the acid hydrolysate.

The other three HA analogs, des-Phe\textsuperscript{11}–HA (III), Arg\textsuperscript{1}–HA (IV) and Phe\textsuperscript{5}–HA (V), were synthesized in the same manner as described above, starting from VIII or IX as the C-terminal fragment. HA itself was also prepared and extensively purified by DCCC employing two different solvent systems. The homogeneity of the synthetic peptides was proved by the same means as used for II. The synthetic HA prepared in this study had the same biological activity as the native peptide. It also cross-reacted with the HA-RIA system developed by Bodenmüller et al.\textsuperscript{14} Cross-reactivity and homogeneity data are summarized in Table I.

![Diagram of peptide synthesis](image-url)
To obtain a specific antiserum, we used HA-ascaris protein conjugates, prepared by using glutaraldehyde. Immunization of three rabbits with the conjugate gave antibodies with sufficient titer. 125I-Y10-HA was prepared by the Iodogen method. Purified labelled antigen was separated from the unlabelled antigen and other compounds by reversed-phase HPLC.

The specificity of these antisera was examined, and the antiserum ASH-04 was found to be more specific in binding to the undecapeptide HA than the others. In the HA-RIA using ASH-04 at a final dilution of 20000 fold, the cross-reactivities of the three HA analogs and bradykinin (BK) were examined. The dose-response curves of III, IV and V, which resemble BK, revealed that the minor change of the HA molecule resulted in a drastic decrease of the binding activity with the antisera (Table I). The Gly5 residue seemed to be important for binding with the antibody ASH-04, while BK did not cross-react at all. These results clearly indicate that the assay system is specific for the undecapeptide. In HA-RIA by the double antibody method, less than 10 pg of HA per tube was detectable; 2 N acetic acid extracts of rat whole intestine and whole brain displaced the tracer in a manner parallel to that of synthetic HA (Fig. 3). The content of immunoreactive HA, however, was found to be extremely low. The results suggest that the RIA system established here could be useful for further immunological studies on the head activator.

**Experimental**

Melting points are uncorrected. Optical rotations were measured in a Nippon Bunkoh DIP-4 polarimeter. Amino acid analyses were performed with a Hitachi KLA-5 amino acid analyzer. HPLC was performed on a system.
composed of two model 510 pumps, a model U6K injector, a model 680 gradient controller, a model 481 LC System, ARC-360 (Aloka). Acid hydrolysis of samples for amino acid analysis was conducted with twice-distilled 5.8 N HCl at 110 °C for 24 h and for 48 h in evacuated sealed tubes, and phenol was added thereto when the peptide contained tyrosine. Evaporation of solvents was carried out in vacuo below 45 °C in a rotary evaporator. The solvent systems used for TLC on silica gel (Merck) were n-BuOH-AcOH-H₂O (4:1:5, the upper phase, Rf₁) and n-BuOH-pyridine-AcOH-H₂O (30: 20: 6: 24, Rf₂).

Z-lys(Z)-Val-Ile-Leu-Tyr-CONH₂ (XVI) —— Z-lys(OH) (2.21 g, 6.1 mmol) was allowed to react at room temperature with H₂O (8 ml) containing triethylamine (TEA) (2.32 g, 0.04 mmol) at 25 °C for 3 h. The product was precipitated from n-BuOH-AcOH-H₂O (4: 1: 5, the upper phase, Rf₁) and triturated with ether. Yield 3.56 g (96.7%). mp 123-125 °C. Rf₁ 0.85, Rf₂ 0.82. [α]D²⁰ -2.9° (c = 0.3, DMF). Anal. Calcd for C₅₀H₇₂N₈O₁₄-½H₂O: C, 60.32; H, 7.99; N, 10.95. Found: C, 60.41; H, 7.85; N, 10.95.

Boc-Gly-Ser-Lys(Z)-Val-Ile-Leu-Tyr-OH (XIII) —— XIII (1.09 g, 1.9 mmol) was hydrogenated in 5% H₂ in the presence of palladium black under a pressure of 1 atm at 25 °C. The product was precipitated from n-BuOH-AcOH-H₂O (4: 1: 5, the upper phase, Rf₁) and triturated with ether-petrol ether and the solid was reprecipitated from the same solvents. Yield 1.10 g (95.1%). mp 123-125 °C. Rf₁ 0.85, Rf₂ 0.82. [α]D²⁰ -2.9° (c = 0.3, DMF). Anal. Calcd for C₅₀H₇₂N₈O₁₄-½H₂O: C, 60.32; H, 7.99; N, 10.95. Found: C, 60.41; H, 7.85; N, 10.95.

Z-pGlu-Pro-Pro-Glu-CONH₂ (XV) —— X (1.42 g, 4.1 mmol) was coupled with H-Ile-Leu-Tyr-OH (1.59 g, 3.4 mmol) obtained above in H₂O (6 ml) containing triethylamine (TEA) (0.31 g, 3.73 mmol), and DCC (0.563 g, 2.73 mmol) in DMF (1 ml)-THF (10 ml) as usual. The reaction mixture was kept for 1 h at -5 °C and for 24 h at 4 °C and then concentrated. The product was precipitated from n-BuOH-AcOH-H₂O (4: 1: 5, the upper phase, Rf₁) and triturated with ether-petrol ether and the solid was reprecipitated from the same solvents. Yield 0.48 g (73.8%). mp 284-285 °C (dec.). Rf' 0.85, Rf₂ 0.81. [α]D²⁰ -11.1° (c = 0.7, DMF). Anal. Calcd for C₄₅H₆₈N₆O₁₄: C, 60.32; H, 7.99; N, 10.95. Found: C, 60.41; H, 7.85; N, 10.95.

Z-pGlu-Pro-Pro-Gly-CONH₂ (XIV) —— XIV (1.32 g, 9.4 mmol) for 16 h. After evaporation of the solvents, the residue in AcOEt was washed successively with 1 N HCl and saturated NaCl solution. The organic phase was dried over Na₂SO₄ and evaporated. The residue was triturated with ether-petrol ether and the solid was reprecipitated from the same solvents. Yield 2.47 g (96.9%). mp 123-125 °C. Rf₁ 0.85, Rf₂ 0.82. [α]D²⁰ -2.9° (c = 0.3, DMF). Anal. Calcd for C₅₀H₇₂N₈O₁₄-½H₂O: C, 60.32; H, 7.99; N, 10.95. Found: C, 60.41; H, 7.85; N, 10.95.
lyophilized. Yield 65 mg. The crude material (20 mg) was dissolved in a mixture (2 ml) of the upper and lower phases of n-BuOH–AcOEt–H₂O (4:1:5) and subjected to DCCC which employed 200 tubes (0.2 x 30 cm each) filled with the upper phase. The lower phase (350 ml) was pumped through the tubes from top to bottom at a flow rate of 3 ml/h, followed by the upper phase (300 ml). The fractions (3 g each, No. 66–120) were gel-filtered and lyophilized. Yield 17 mg (48.4%). [α]D⁰ = −115.6° (c = 1, 3 M AcOH). *Anal. Calcd for C₃₉H₅₂N₄O₁₆·2AcOEt·2H₂O: C, 53.69; H, 7.46; N, 12.95. Found: C, 53.44; H, 6.90; N, 12.99. Amino acid ratios in an acid hydrolysate [24 h (48 h)]: Lys 0.94 (1.01), Ser 0.80 (0.85), Glu 0.97 (1.05), Pro 2.34 (2.04), Gly 2.00 (2.06), Val 0.58 (0.78), Ile 0.54 (0.77), Leu 1.01 (0.97), Tyr 0.95 (1.02), NH₃ trace (0.30).

Z-Val-Ile-Leu-OH (XIII) — Z-Val-OsU (2.68 g, 7.7 mmol) was allowed to react with H-Ile-Leu-OH·HCl (1.97 g, 7.0 mmol) in DMF (20 ml) containing TEA (2.66 ml, 19 mmol) at room temperature for 18 h. The product was worked up in the same manner as described for X and precipitated from AcOEt–ether. The solid was reprecipitated from the same solvents. Yield 3.13 g, (93.7%). mp 214–216°C (dec.). Rf 0.89, Rf₂ 0.74. RI 5  15.7° (c= 1.0, DMF). *Anal. Calcd for C₃₅H₄₉N₄O₇·1/2H₂O: C, 64.77; H, 7.89; N, 8.94. Amino acid ratios in an acid hydrolysate [24 h (48 h)]: Lys 0.93 (1.08), Ser 0.95 (0.85), Gly 1.06 (1.07), Val 0.80 (1.02), Ile 0.74 (0.97), Leu 0.97 (1.10), NH₃ 0.33 (0.26).

Boc-Lys(Z)-Val-Ile-Leu-OH (VIII) — XIII (995 mg, 2 mmol) was hydrogenated in MeOH (100 ml)–5% AcOH (50 ml) in the same manner as described for XI. The product (Rf⁺ 0.61, Rf² 0.73) was reacted with Boc-Lys(Z)-OsU (2.6 mmol) in DMF (20 ml) containing TEA (0.433 ml, 3.1 mmol) at room temperature for 20 h. The product was worked up in the same manner as described for XI and precipitated from AcOEt–ether. Yield 3.48 g (78.0%). mp 164–167°C. Rf 0.91, Rf² 0.86. [α]D⁰ = −22.4° (c= 1.0, DMF). *Anal. Calcd for C₃₅H₃₁N₉O₁₆·2AcOEt: C, 61.26; H, 8.42; N, 9.92. Found: C, 60.97; H, 8.53; N, 10.02. Amino acid ratios in an acid hydrolysate [24 h (48 h)]: Lys 1.03 (0.90), Ser 0.80 (1.02), Gly 0.74 (0.97), Leu 0.97 (1.10), NH₃ 0.33 (0.26).

Boc-Gly-Ser-Lys(Z)-Val-Ile-Leu-OH (XIV) — VIII (1.19 g, 1.4 mmol) was partially deblocked with ice-chilled TFA (5 ml–anisole (0.5 ml) in the same manner as described for XII to give H-Gly–Ser-Lys(Z)-Val–Ile–Leu–OH–TFA (XIV). RI 0.53, RI² 0.74. The azide [prepared from VI (0.22 mmol), 6 N HC1 in dioxane (1.10 ml, 10.2 mmol) and isoamyl nitrite (30/21, 0.58 g, 2.1 mmol)] in DMSO (30 ml)–DMF (20 ml) at 4°C for 18 h. The desired product was isolated in the same manner as described for XI and precipitated from MeOH (80 ml). Yield 1.10 g (78.0%). mp 164–167°C. Rf 0.91, Rf² 0.86. [α]D⁰ = −22.4° (c= 1.0, DMF). *Anal. Calcd for C₃₅H₃₄N₉O₁₆·7H₂O: C, 61.26; H, 8.42; N, 9.92. Found: C, 60.97; H, 8.53; N, 10.02. Amino acid ratios in an acid hydrolysate [24 h (48 h)]: Lys 0.93 (1.08), Ser 0.95 (0.85), Gly 1.06 (1.07), Val 0.69 (1.01), Ile 0.80 (0.92), Leu 1.06 (1.05), NH₃ 0.49 (0.45).

Boc-Gly-Ser-Lys(Z)-Val-Ile-Leu-OH (III) — XIV (128 mg, 0.15 mmol) was partially deblocked with ice-chilled TFA (5 ml–anisole (0.5 ml) in the same manner as described for XII to give H-Gly–Ser-Lys(Z)-Val–Ile–Leu–OH–TFA (XIV). RI 0.53, RI² 0.74. The azide [prepared from VI (0.22 mmol), 6 N HC1 in dioxane (178 µl, 8.0 mmol) and isoamyl nitrite (0.28 ml, 2.1 mmol)] in DMSO (30 ml)–DMF (20 ml) at 4°C for 18 h. The desired product was isolated in the same manner as described for XI and precipitated from MeOH (40 ml)–AcOEt (80 ml). Yield 1.04 g (87.5%). mp 226–227°C (dec.). Rf 0.85, Rf² 0.85. [α]D⁰ = −14.5° (c= 1.0, DMF). *Anal. Calcd for C₃₅H₃₄N₉O₁₆: C, 65.15; H, 7.50; N, 7.73. Found: C, 65.15; H, 7.50; N, 7.73. Amino acid ratios in an acid hydrolysate [24 h (48 h)]: Lys 1.01 (1.08), Ser 0.91 (0.82), Gly 1.06 (1.05), Pro 2.02 (2.02), Gly 2.06 (2.07), Val 0.79 (0.98), Ile 0.74 (0.90), Leu 0.95 (0.95), NH₃ 0.54 (0.65).

Boc-Gly-Ser-Lys(Z)-Val-Ile-Leu-OH (IV) — XIII (6.12 g, 16.9 mmol) was allowed to react at room temperature for 20 h with H-Leu–Phe–OEt (1.02 g, 3.6 mmol) in DMF (25 ml)–H₂O (10 ml) containing TEA (3.09 ml, 22.1 mmol). The mixture was treated in the same manner as described for XI. The residue was triturated with ether–petr. ether. The solid was reprecipitated from MeOH–AcOEt. Yield 5.34 g (76.8%). mp 156–158°C. Rf 0.88, Rf² 0.78. [α]D⁰ = −5.7° (c= 1.0, DMF). *Anal. Calcd for C₃₅H₃₄N₉O₁₆·1/2H₂O: C, 64.43; H, 7.79; N, 8.84. Found: C, 64.77; H, 7.89; N, 8.94. Amino acid ratios in an acid hydrolysate [24 h (48 h)]: Lys 1.01 (1.08), Ser 0.91 (0.82), Gly 1.06 (1.05), Pro 2.02 (2.02), Gly 2.06 (2.07), Val 0.79 (0.98), Ile 0.74 (0.90), Leu 0.95 (0.95), NH₃ 0.54 (0.65).

Z-Val-Ile-Leu-Phe-OH (XX) — XI-Val–O–SO₃H (1.11 g, 1.3 mmol) was partially deblocked with ice-chilled TFA (10 ml–anisole (1.3 ml) in the same manner as described for XII to give H-Lys(Z)-Val–Ile–Leu–Phe–OEt·TFA (IX). Rf 0.75, Rf² 0.73. IX' in DMSO (30 ml)–DMF (15 ml) was allowed to react with azide [prepared...
from Boc-Gly-Ser-N\textsubscript{3}H\textsubscript{4} (0.72 g, 2.6 mmol), 6 N HCl in dioxane (1.73 ml, 10.4 mmol) and isoamyl nitrite (0.35 ml, 2.6 mmol) at 4 °C for 20 h. The mixture was treated in the same manner as described for XI and the product was washed with MeOH. Yield 1.0 g (76.8%), mp 275–279 °C (dec.). \( R_f^T \) 0.86, \( R_f^T \) 0.80. \([\alpha]_{D}^{20} = -13.9 \) (c = 1.0, DMF).

Anal. Calc'd for C\textsubscript{54}H\textsubscript{87}N\textsubscript{12}O\textsubscript{14} • 3AcOH • 3H\textsubscript{2}O: C, 57.47; H, 7.26; N, 13.03. Amino acid ratios in an acid hydrolysate [24 h (48 h)]: Ser 0.69 (0.75), Pro 1.86 (2.19), Val 0.61 (0.73). Lys 0.96 (1.06). Phe 0.89 (1.04), Lys 0.93 (1.15), NH\textsubscript{3} 0.25 (0.43).

**Immunocquial Experiments**

**Preparation of Conjugate** — A 2% aqueous solution of glutaraldehyde (1 ml) was added dropwise to an ice-cold solution of HA (7.5 mg) and crude protein extract of Ascaris suilla (15 mg) in H\textsubscript{2}O (7.5 ml) during 30 min. The mixture was stirred overnight at 4 °C and taken into dialysis tubing (Spectraper, MW cut-off 1000). The dialysis was continued at 4 °C for 20 h with two changes of H\textsubscript{2}O (1 each) and the dialysate was lyophilized. Yield 13 mg.

**Immunization** — The conjugate (3 mg) was dissolved in saline (1.5 ml) and the solution was emulsified with complete Freund's adjuvant (1.5 ml, Calbiochem-Behring) in a Sorval Omni-Mixer at 50000 rpm. The emulsion was injected subcutaneously at multiple sites on three mixed-bred male rabbits. The rabbits were boosted three times at
biweekly intervals and monthly thereafter using a half amount of the conjugate. Blood was taken at 10 d after the injection. All rabbits produced antibodies after the 5th injection.

**Labelled Antigen Preparation** — A solution of II (11 µg, 9.2 nmol) in H2O (22 µl) was added to a solution of 125I Na (250 µCi, 0.115 nmol) in 0.25 M phosphate buffer (50 µl, pH 7.40) in a glass tube, which had been filmed with 1,3,4,6-tetrachloro-3,6-diphenyl-glycouril (0.99 µg, 2.3 nmol, Iodogen, Pierce) at the bottom. The mixture was stirred on a test tube mixer and allowed to react for 5 min. The product was purified on a column (3.9 x 30 mm) of Chemcosorb 7-ODS-H with a linear gradient of acetonitrile from 10% to 30% over a period of 15 min in 20 mm phosphate buffer (pH 3.0), at flow rate of 0.8 ml/min. Fractions of the highest radioactivity (retention time: 13.5 min) were used as the labelled antigen in HA-RIA.

**Radioimmunoassay (RIA)** — The standard diluent (SD) used was 10 mm phosphate buffer (pH 7.40), containing 0.5% bovine serum albumin (BSA), 0.025 M ethylenediaminetetraacetic acid (EDTA) and 0.15 M NaCl. The mixture in each assay tube consisted of SD (0.5 ml), peptide or tissue extract (0.1 ml), diluted antiserum (0.1 ml) and labelled antigen (0.1 ml). 10% goat anti-rabbit γ-globulin serum (0.1 ml) and 30% polyethylene glycol 1190 in BSA free SD were added. After an additional incubation for 1 h at 4 °C, the mixture was centrifuged at 3000 rpm at 4 °C. The supernatant was removed and the radioactivity in the tube was counted.

**Tissue Extraction** — Rat whole intestine and whole brain were diced, immersed in a small amount of 2 M AcOH and then plunged into a boiling water bath for 5 min. The mixture was cooled and homogenized in a ten fold excess of ice-cold 2 M AcOH. A supernatant was obtained by centrifugation at 30000 g for 20 min, and the precipitate was re-extracted in the same manner. The combined supernatant was washed with three portions of CHCl3 and lyophilized.

**Acknowledgements** The authors wish to express their gratitude to Professor N. Yanaihara, Shizuoka College of Pharmacy, for his valuable advice during the preparation of this manuscript. The authors are also grateful to Drs. H.C. Schaller and H. Bodenmüller for the bioassay and radioimmunoassay of our synthetic peptides.

**References**


