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Solution Synthesis of Human Midkine, a Novel Heparin-binding Neurotrophic Factor Consisting of 121 Amino Acid Residues with Five Disulphide Bonds

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Human midkine (hMK), a novel heparin-binding neurotrophic factor consisting of 121 amino acid residues with five intramolecular disulphide bonds, was synthesized by solution procedure in order to demonstrate the usefulness of our newly developed solvent system, a mixture of dichloromethane or chloroform and trifluoroethanol. The final protected 121-residue peptide was assembled from two large fully protected intermediates, Boc-(1–59)-OH and H-(60–121)-OBzl, in CHL/TFE (3:1, v/v) using water-soluble carbodiimide in the presence of HOOBt as coupling reagents. After removal of the protecting groups by HF followed by treatment with $Hg(OAc)_2$ in 50% acetic acid, the fully deprotected peptide was subjected to the oxidative folding reaction. The final product was confirmed to have the correct disulphide structure from its tryptic peptide mapping and to possess the same biological activities as those of the natural product. In order to clarify the active region of the hMK molecule, the N-terminal and C-terminal half domains [(1–59) and (60–121)] were also synthesized by the same procedure used for the hMK synthesis. The C-half domain was confirmed to show the full pattern of bioactivities except for the neuronal cell survival activity, while the N-half one showed much less activity in general.

Keywords: Solution synthesis; human midkine; powerful solvent system; disulphide structure; active region

INTRODUCTION

Chemical synthesis of proteins, containing more than 100 residues with a specific folded structure, is still a challenging target for peptide chemists. Today, numerous peptides can be synthesized by the solid-phase method owing to the simplicity of its strategy and convenience. However, the difficulty of obtaining homogeneous products increases rapidly as the size of the target peptide increases because contaminants cannot be completely removed even by applying the most effective purification techniques. Recently, a combination of solid-phase synthesis of

Abbreviations: RP-HPLC, reversed-phase high-performance liquid chromatography; CZE, capillary zone electrophoresis; CHL, chloroform; DCM, dichloromethane; NMP, *N*-methylpyrrolidone; HOOBt, 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine; HOBt, 1-hydroxybenzotriazole; hMK, human midkine; TFE, 2,2,2-trifluoroethanol; WSCI, water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; BAEC, bovine aortic endothelial cell.

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peptides with a technique for their chemical ligation has been introduced for the synthesis of large peptides. However, the method is still limited by the availability of homogeneous peptides for ligation [1].

In terms of product homogeneity, solution synthesis of large peptides is much more advantageous than solid-phase synthesis because product purity can be checked at the stage of the small intermediates, and, if necessary, the intermediates can be purified before being used to assemble large peptides. Among the principles proposed for conventional solution synthesis, the segment condensation method combined with the maximum protection strategy is ideal from the standpoint of minimizing the formation of side products and maximizing the convenience of elongating the peptide chains. After development of such a maximum protection strategy in our laboratory in 1981 [2], we have synthesized various biologically active peptides using HF as the final deprotection procedure, and in 1990, we demonstrated the usefulness of our strategy for the synthesis of proteins using angiogenin, a 123-residue peptide containing four disulphide bonds [3]. The major problem encountered during the synthesis of such large peptides was the insolubility of the intermediates. The insolubility of protected peptides may be caused by a combination of β-sheet aggregation resulting from inter- or intrachain hydrogen bonds and van der Waals interaction between non-polar side chains [4,5]. In order to overcome the insolubility of the protected peptides, we have recently demonstrated the usefulness of a powerful solvent system which is suitable for the segment condensation reactions, a mixture of TFE and CHL or DCM [6]. This solvent system not only dissolves almost all of the sparingly soluble protected peptides but also suppresses the racemization during the coupling reaction of peptide segments using WSCI as the coupling reagent in the presence of 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (HOOBt). Applying the present techniques, we have succeeded in the synthesis of various medium-sized peptides such as ω-agatoxin IVA, 48-residue peptide with four disulphide bonds [7], and calciseptine, 60-residue peptide with three disulphide bonds [8, 9].

In order to demonstrate the usefulness of our present strategy further, we carried out the total synthesis of human midkine (hMK), a basic 121-residue protein containing five intramolecular disulphide bonds [10, 11]. hMK, a retinoic acid-inducible gene product, is a heparin-binding neurotrophic factor which induces neurite outgrowth and supports cell survival of mammalian embryonic brain cells [11, 12]. The hMK molecules are also known to be expressed strongly in human cancer cells [13] and in senile plaques of Alzheimer's disease [14]. More recently, hMK was found to raise the level of plasminogen activator in bovine aortic endothelial cells [15, 16]. The MK molecule consists of an N-terminal half domain, containing three intradomain disulfide bonds, which is linked to a C-terminal one, containing the remaining two disulphide bonds, through a short peptide bridge as shown in Figure 1. However, as nothing is known about the function of each domain, each domain was also synthesized and its role was examined in the biological function of hMK.

MATERIALS AND METHODS

Materials and Instruments

Boc-amino acids and other reagents for peptide synthesis were obtained from Peptide Institute Inc. (Osaka, Japan). TLC was performed on Merck (Darmstadt, Germany) Kieselgel 60F-254 precoated plate. The compounds were visualized with UV light (254 nm) and/or a ninhydrin reagent using the following solvent systems: (1) CHL/MeOH/AcOH, 85:10:5; (2) CHL/MeOH/AcOH, 85:15:5; (3) CHL/80% AcOH/TFE, 6:1:1. HPLC analysis was performed on a Shimadzu liquid chromatography Model LC-6A or LC-8A. Capillary zone electrophoresis (CZE) was performed on Model 270A apparatus (Applied Biosystems, Foster City, CA). Molecular weights were measured with an electrospray ionization mass spectrometer (Finigan MAT TSQ 700).

Figure 1 Structure of human midkine (hMK) showing disulphide bond linkages.

Amino acid analyses were carried out on a JEOL amino acid analyser Model JLC-300 (Tokyo, Japan), after hydrolysis of the peptides with 6 M HCl in the presence or absence of 4% thioglycolic acid at 110°C for 22 h.

Synthesis of Segments

Segments **I** to **XII** in Figure 2 were synthesized step by step from their respective C-terminal amino acid phenacyl (Pac) esters using 1.05 eq. each of Boc-amino acid in DMF or NMP; WSCI (1.05 eq.) was used as the coupling reagent in the presence of HOBt (1.05 eq.). The functional side chains of Boc-amino acids were protected by the following groups: benzyl ether (Bzl) for Ser and Thr, cyclohexyl ester (cHx) for Asp and Glu, 2-chlorobenzyloxycarbonyl (ClZ) for Lys, 2-bromobenzyloxycarbonyl (BrZ) for Tyr, tosyl (Tos) for Arg, acetamidomethyl (Acm) for Cys, and formyl (For) for Trp. Segment **XIII** was synthesized similarly using Asp(cHx)-OBzl as the starting material instead of the Pac ester. The progress of each coupling reaction was carefully monitored by TLC using fluorescamine or ninhydrin. The final product was purified by recrystallization or reprecipitation from appropriate solvents to yield 17.5 g for segment **I**, 7.5 g for **II**, 8.2 g for **III**, 5.5 g for **IV**, 4.0 g for **V**, 7.5 g for **VI**, 16.1 g for **VII**, 15.8 g for **VIII**, 15 g for **IX**, 18.2 g for **X**, 21 g for **XI**, 12.9 g for **XII** and 18.4 g for **XIII**. The homogeneity of each segment was confirmed by amino acid analysis, TLC and RP-HPLC using gradient systems with CH_3CN (30% or 40–95%) in 0.1% TFA at a flow rate of 1 ml/min.

ClZ ClZ ClZ cHx ClZ ClZ ClZ
I: Boc-Lys-Lys-Lys-Asp-Lys-Val-Lys-Lys-Gly-Gly-Pro-OPac (1-11)

Bzl cHx Acm cHx For For
II: Boc-Gly-Ser-Glu-Cys-Ala-Glu-Trp-Ala-Trp-Gly-Pro-OPac (12-22)

Acm Bzl Bzl Bzl ClZ cHx Acm
III: Boc-Cys-Thr-Pro-Ser-Ser-Lys-Asp-Cys-Gly-OPac (23-31)

Tos cHx Bzl Acm
IV: Boc-Val-Gly-Phe-Arg-Glu-Gly-Thr-Cys-Gly-OPac (32-40)

Bzl Tos Tos Acm Tos
V:Boc-Ala-Gln-Thr-Gln-Arg-Ile-Arg-Cys-Arg-Val-Pro-OPac (41-51)

Acm For ClZ ClZ cHx
VI: Boc-Cys-Asn-Trp-Lys-Lys-Glu-Phe-Gly-OPac (52-59)

Removal of the Pac Ester From Each Segment

The Pac esters of segments **III** (8.2 g), **IV** (5.4 g), **VIII** (6.5 g), **IX** (7.5 g), **XI** (10 g) and **XII** (10 g), which were freely soluble in AcOH, were removed by treatment with zinc powder in AcOH at 40°C for 1 h as reported previously [2] to obtain protected segments possessing a free carboxylic acid at their C-termini; yields were 7 g for **III'**, 4.5 g for **IV'**, 5.2 g for **VIII'**, 6.8 g for **IX'**, 9.2 g for **XI'** and 8.0 g for **XII'**. Removal of the Pac ester from segments **I** (17.3 g) and **V** (3.5 g) was carried out by the same method after dissolving them in DCM/TFE (3:1) followed by adding excess AcOH as reported previously [6]; yields were 16.0 g for **I'** and 3.0 g for **V'**. Segments **II** (7.6 g), **VII** (7.2 g) and **X** (8.8 g) were dissolved in DCM/TFE. After adding 30 eq. of ammonium formate and 60 eq. of AcOH, the solution was treated with 50 eq. of Zn powder, the mixture was vigorously stirred at room temperature for 1 h under argon gas, and then the Zn powder was removed by filtration. The filtrate was evaporated to a residue, which was triturated with 1 M HCl, washed with water, MeOH, ethyl acetate and *n*-hexane, successively, and dried; yields were 5.9 g for **II'**, 6.5 g for **VII'** and 8.0 g for **X'**. The homogeneity of each segment was confirmed by amino acid analysis, TLC and RP-HPLC as described above. The R_F values on TLC and retention times on RP-HPLC as well as amino acid compositions are given in Table 1.

Protected Box-(104–121)-OBzl

Segment **XIII** (7.8 g, 3.76 mmol) was dissolved in TFA (50 ml) at − 10°C, and then allowed to react for

cHx Acm ClZ BrZ ClZ cHx For
VII: Boc-Ala-Asp-Cys-Lys-Tyr-Lys-Phe-Glu-Asn-Trp-Gly-OPac (60-70)

Acm cHx Bzl
VIII: Boc-Ala-Cys-Asp-Gly-Gly-Thr-Gly-OPac (71-77)

Bzl ClZ Tos
IX: Boc-Thr-Lys-Val-Arg-Gln-Gly-OPac (78-83)

Bzl ClZ ClZ Tos BrZ
X: Boc-Thr-Leu-Lys-Lys-Ala-Arg-Tyr-Asn-Ala-Gln-OPac (84-93)

Acm cHx Bzl Tos Bzl ClZ
XI: Boc-Cys-Gln-Glu-Thr-Ile-Arg-Val-Thr-Lys-Pro-OPac (94-103)

Acm Bzl ClZ Bzl ClZ ClZ
XII: Boc-Cys-Thr-Pro-Lys-Thr-Lys-Ala-Lys-Ala-OPac (104-112)

ClZ ClZ ClZ ClZ ClZ cHx
XIII: Boc-Lys-Ala-Lys-Lys-Gly-Lys-Gly-Lys-Asp-OBzl (113-121)

Figure 2 Structure of protected segments for the synthesis of hMK.

Table 1 Analytical Data for the Synthetic Segments

Segment	R_F[a]	Reaction time (min)[b]	Amino acid analysis
I	0.51 (1)	25.6 (A)	Asp 1.00 (1), Gly 1.93 (2), Val 0.95 (1), Lys 6.10 (6), Pro 0.99 (1)
II	0.53 (3)	17.5 (A)	Ser 0.95 (1), Glu 2.00 (2), Gly 2.04 (2), Ala 2.07 (2), 1/2(Cys)$_2$ 0.88 (1), Trp 0.64 (2), Pro 1.00 (1)
III	0.49 (1)	17.0 (A)	Asp 1.00 (1), Thr 0.97 (1), Ser 1.81 (2), Gly 0.96 (1), 1/2(Cys)$_2$ 1.59 (2), Lys 1.02 (1), Pro 1.03 (1)
IV	0.54 (3)	12.3 (A)	Thr 0.95 (1), Glu 1.01 (1), Gly 3.02 (3), 1/2(Cys)$_2$ 0.80 (1), Val 1.00 (1), Phe 1.03 (1), Arg 0.95 (1)
V	0.31 (1)	9.4 (A)	Thr 0.93 (1), Glu 2.01 (2), Ala 1.08 (1), 1/2(Cys)$_2$ 0.86 (1), Val 1.00 (1) Ile 0.91 (1), Arg 2.92 (3), Pro 0.98 (1)
VI	0.50 (1)	19.2 (A)	Asp 0.75[c] (1), Glu 1.01 (1), Gly 0.97 (1), 1/2(Cys)$_2$ 0.81 (1), Phe 1.00 (1) Lys 2.03 (2), Trp 0.36 (1)
VII	0.60 (3)	23.1 (B)	Asp 1.80[d] (2), Glu 1.00 (1), Gly 1.00 (1), Ala 1.01 (1), 1/2(Cys)$_2$ 0.70 (1), Tyr 0.97 (1), Phe 1.00 (1), Lys 2.02 (2), Trp 0.24 (1)
VIII	0.62 (1)	11.2 (B)	Asp 1.00 (1), Thr 0.99 (1), Gly 3.08 (3), Ala 1.03 (1), 1/2(Cys)$_2$ 0.71 (1)
IX	0.64 (3)	16.1 (B)	Thr 0.97 (1), Glu 1.02 (1), Gly 1.00 (1), Val 0.94 (1), Lys 1.00 (1), Arg 0.92 (1)
X	0.59 (2)	21.2 (A)	Asp 0.95 (1), Thr 0.94 (1), Glu 1.00 (1), Ala 2.08 (2), Leu 1.02 (1), Tyr 0.97 (1), Lys 2.05 (2), Arg 0.97 (1)
XI	0.70 (3)	17.2 (A)	Thr 1.87 (2), Glu 1.97 (2), 1/2(Cys)$_2$ 0.80 (1), Val 0.93 (1), Ile 0.94 (1), Lys 1.03 (1), Arg 0.98 (1), Pro 0.99 (1)
XII	0.61 (1)	19.2 (A)	Thr 1.86 (2), Ala 2.05 (2), 1/2(Cys)$_2$ 0.79 (1), Lys 3.02 (3), Pro 0.97 (1)
XIII	0.71 (3)	25.1 (A)	Asp 0.98 (1), Gly 2.03 (2), Ala 1.05 (1), Lys 4.98 (5)

[a]TLC See experimental part for the solvent systems.
[b]RP-HPLC solvent systems: A, 40–95% CH_3CN in 0.1% TFA (25 min grad.). B, 30–95% CH_3CN in 0.1% TFA (25 min grad.).
[c] Asp 0.96 and [d] Asp 1.93 when hydrolysed with 3 M p-toluene sulphonic acid at 110°C for 22 h.

40 min at room temperature. After removal of excess TFA *in vacuo*, the residue was triturated with 5.2 M HCl solution in dioxane (0.94 ml, 4.9 mmol) to convert the TFA salt to the HCl salt, the product was precipitated with ether, and dried over NaOH *in vacuo*. The dried product and segment **XII'** (7.2 g, 3.95 mmol) were dissolved in DMF (60 ml). To the solution, HOOBt (0.64 g, 3.95 mmol) and WSCI (0.72 ml, 3.95 mmol) were added at − 10°C, and the whole mixture was allowed to react under stirring for 16 h at room temperature. The product was precipitated by adding excess chilled water, and the precipitates were collected by filtration, then washed with water and with MeOH. The product was further purified by reprecipitation from DMF and ethyl acetate; yield 13.7 g (97.2%); $R_F(1) = 0.75$; amino acid analysis: Asp 0.98 (1), Thr 1.86 (2), Gly 2.03 (2), Ala 3.10 (3), 1/2 (Cys)$_2$0.93 (1), Lys 8.00 (8), Pro 0.97 (1).

Protected Boc-(94–121)-OBzl

Boc-(104–121)-OBzl obtained above (11.3 g, 3 mmol) was treated with TFA (60 ml) and the product was converted to the HCl salt as described above. The HCl salt and segment **XI'** (6.08 g, 3.15 mmol) were dissolved in a mixture of DMF (70 ml) and NMP (60 ml). To the solution, HOBt (0.43 g, 3.15 mmol) and WSCI (0.58 ml, 3.15 mmol) were added at − 10°C, and the whole mixture was allowed to react by stirring at room temperature for 16 h. The product was precipitated by adding excess water, and the precipitates were collected by filtration and successively washed with MeOH. The product was further purified by reprecipitation from CHL/TFE (1:1, v/v) and CH_3CN, and dried; yield 16.1 g (96.4%); $R_F(1) = 0.35$, $R_F(3) = 0.51$; amino acid analysis: Asp 0.96(1), Thr 3.95 (4), Glu 2.10 (2), Gly 2.00 (2), Ala 3.02 (3), 1/2(Cys)$_2$1.42 (2), Val 1.11 (1), Ile 1.04 (1), Lys 9.19 (9), Arg 1.05 (1), Pro 2.05 (2).

Protected Boc-(84–121)-OBzl

Boc-(94–121)-OBzl (13.9 g, 2.5 mmol) obtained above was treated with TFA (60 ml), and the product was converted to the HCl salt as described before. The HCl salt and segment **X'** (5.7 g, 2.75 mmol) were dissolved in a mixture of CHL/TFE (3:1, v/v) (150 ml).

To the solution, HOOBt (0.45 g, 2.75 mmol) and WSCI (0.50 ml, 2.75 mmol) were added at − 10°C, and the two segments were allowed to react for 4 h at room temperature. After evaporating the solvents, the product was precipitated by adding excess water, the precipitates were collected by filtration, and washed with MeOH. The product was further purified by reprecipitation from CHL/TFE and ethyl acetate; yield 17.9 g (95.0%); $R_F(3) = 0.55$; amino acid analysis: Asp 1.96 (2), Thr 4.76 (5), Glu 3.02 (3), Gly 2.04 (2), Ala 5.04 (5), 1/2(Cys)$_2$ 1.87 (2), Val 0.92 (1), Ile 0.88 (1), Leu 0.96 (1), Tyr 0.95 (1), Lys 11.10 (11), Arg 1.93 (2), Pro 1.88 (2).

Protected Boc-(78–121)-OBzl

Protected Boc-(84–121)-OBzl obtained above (13.2 g, 1.75 mmol) was treated with TFA (70 ml) and the product was converted to the HCl salt as described above. To a solution of the dried HCl salt and segment **IX′** (2.30 g, 1.93 mmol) in a mixture of CHL/TFE (3:1, v/v) (150 ml), HOOBt (0.31 g, 1.93 mmol) and WSCI (0.35 ml, 1.93 mmol) were added at 10°C, and the two components were allowed to react for 8 h at room temperature. The product was precipitated by adding excess CH_3CN, and the precipitates were collected by filtration, washed with water and then with MeOH. The product was further purified by reprecipitation from CHL/TFE and ethyl acetate, yield 14.7 g (97.5%); $R_F(3) = 0.49$; amino acid analysis: Asp 1.95 (2), Thr 5.67 (6), Glu 4.03 (4), Gly 3.08 (3), Ala 5.02 (5), 1/2(Cys)$_2$ 1.86 (2), Val 1.89 (2), Ile 0.87 (1), Leu 0.95 (1), Tyr 0.94 (1), Lys 11.98 (12), Arg 2.78 (3), Pro 1.89 (2).

Protected Boc-(71–121)-OBzl

Protected Boc-(78–121)-OBzl (14.7 g, 1.7 mmol) obtained above was treated with TFA (70 ml) and the product was converted to the HCl salt as described above. To a solution of the HCl salt and segment **VIII′** (1.7 g, 1.87 mmol) in a mixture of CHL/TFE (3:1, v/v) (200 ml), HOOBt (0.31 g, 1.87 mmol) and WSCI (0.34 ml, 1.87 mmol) were added at − 10°C, and the two components were allowed to react at room temperature for 8 h. The product was precipitated by adding excess CH_3CN, and the precipitates were washed with water and MeOH, successively, and further purified by reprecipitation from CHL/TFE and CH_3CN; yield 15.0 g (93.6%); $R_F(3) = 0.43$; amino acid analysis: Asp 2.94 (3), Thr 6.58 (7), Glu 4.02 (4), Gly 6.02 (6), Ala 6.05 (6), 1/2(Cys)$_2$ 2.80 (3), Val 1.96 (2), Ile 0.86 (1), Leu 0.95 (1), Tyr 1.92 (1), Lys 12.05 (12), Arg 2.81 (3), Pro 1.92 (2).

Protected Boc-(60–121)-OBzl (XV)

Protected Boc-(71–121)-OBzl (15.0 g, 1.6 mmol) obtained above was treated with TFA (80 ml), and the product was converted to the HCl salt as described before. To a solution of the HCl salt and segment **VII′** (4.0 g, 1.76 mmol) in a mixture of CHL/TFE (3:1, v/v) (200 ml), HOOBt (0.29 g, 1.76 mmol) and WSCI (0.32 ml, 1.76 mmol) were added at − 10°C, and the whole mixture was allowed to react at room temperature for 2 h. The product was precipitated by adding excess CH_3CN, the precipitates were washed with water, MeOH and DMF, successively, and further purified by reprecipitation from CHL/TFE and CH_3CN; yield 17.0 g (91.8%); $R_F(3) = 0.53$; amino acid analysis: Asp 4.68 (5), Thr 6.59 (7), Glu 5.04 (5), Gly 7.00 (7), Ala 7.20 (7), 1/2(Cys)$_2$ 3.80 (4), Val 1.97 (2), Ile 0.88 (1), Leu 0.96 (1), Tyr 1.93 (2), Phe 0.99 (1), Lys 14.00 (14), Trp 0.36 (1), Arg 2.86 (3), Pro 1.86 (2).

Protected Boc-(41–59)-OPac

Protected Boc-(52–59)-OPac (**VI**) (2.44 g, 1.4 mmol) was treated with TFA (50 ml) and the product was converted to the HCl salt as described above. To a solution of the HCl salt and segment **V′** (3.0 g, 1.47 mmol) in DMF (100 ml), HOOBt (0.24 g, 1.47 mmol) and WSCI (0.27 ml, 1.47 mmol) were added at − 10°C and the whole mixture was allowed to react at room temperature for 8 h. The product was precipitated by adding excess water, and the precipitates were washed with MeOH, ethyl acetate and *n*-hexane, successively, and dried; yield 4.5 g (87.4%); $R_F(3) = 0.41$; amino acid analysis: Asp 0.73 (1), Thr 0.93 (1), Glu 3.00 (3), Gly 0.96 (1), Ala 1.04 (1), 1/2(Cys)$_2$ 1.48 (2), Val 1.03 (1), Ile 0.92 (1), Phe 0.97 (1), Lys 2.03 (2), Trp 0.36 (1), Arg 2.85 (3), Pro 1.00 (1).

Protected Boc-(32–59)-OPac

Protected Boc-(41–59)-OPac (4.48 g, 1.22 mmol) obtained above was treated with TFA (50 ml) and the product was converted to the HCl salt as described above. To a solution of the HCl salt and segment **IV′** (1.92 g, 1.28 mmol) in a mixture of DMF (70 ml) and NMP (100 ml), HOBt (0.17 g, 1.28 mmol) and WSCI (0.23 ml, 1.28 mmol) were added at − 10°C, and the whole mixture was allowed to react at room temperature for 16 h. The product was precipitated by adding

excess water, the precipitates were washed with MeOH, ethyl acetate and *n*-hexane, successively, and dried; yield 5.33 g (87.7%); $R_F(3) = 0.60$; amino acid analysis: Asp 0.75 (1), Thr 1.89 (2), Glu 4.06 (4), Gly 3.90 (4), Ala 1.02 (1), 1/2(Cys)$_2$ 2.35 (3), Val 2.04 (2), Ile 0.91 (1), Phe 2.00 (2), Lys 2.02 (2), Trp 0.35 (1), Arg 3.86 (4), Pro 1.02 (1).

Protected Boc-(23–59)-OPac

Protected Boc-(32–59)-OPac obtained above (5.3 g, 1.06 mmol) was dissolved in TFA (50 ml) and the product was converted to the HCl salt as shown above. To a solution of the HCl salt and segment **III'** (1.85 g, 1.11 mmol) in a mixture of DMF (50 ml) and NMP (100 ml), HOBt (0.15 g, 1.11 mmol) and WSCI (0.20 ml, 1.11 mmol) were added at − 10°C and the whole mixture was allowed to react at room temperature for 16 h. The product was precipitated by adding excess water, and the precipitates were washed with MeOH and purified by reprecipitation from CHL/TFE and ethyl acetate; yield 6.18 g (90.2%); $R_F(3) = 0.61$; amino acid analysis: Asp 1.70 (2), Thr 2.81 (3), Ser 1.82 (2), Glu 4.10 (4), Gly 4.99 (5), Ala 1.02 (1), 1/2(Cys)$_2$ 4.45 (5), Val 2.08 (2), Ile 0.93 (1), Phe 2.00 (2), Lys 3.10 (3), Trp 0.38 (1), Arg 3.92 (4), Pro 2.05 (2).

Protected Boc-(12–59)-OPac

Protected Boc-(23–59)-OPac obtained above (6.11 g, 0.95 mmol) was treated with TFA (50 ml) and the product was converted to the HCl salt as described above. To a solution of the HCl salt and segment **II'** (1.78 g, 1.05 mmol) in a mixture of CHL/TFE (3:1, v/v) (100 ml), HOOBt (0.17 g, 1.05 mmol) and WSCI (0.19 ml, 1.05 mmol) were added at − 10°C and the whole mixture was allowed to react for 4 h at room temperature. The product was precipitated by adding excess CH_3CN, and the precipitates were washed with water and MeOH, successively, and purified by reprecipitation from CHL/TFE and CH_3CN; yield 6.48 g (85.0%); $R_F(3) = 0.50$; amino acid analysis: Asp 1.68 (2), Thr 2.80 (3), Ser 2.77 (3), Glu 6.10 (6), Gly 7.01 (7), Ala 3.10 (3), 1/2(Cys)$_2$ 5.16 (6), Val 2.00 (2), Ile 0.90 (1), Phe 1.89 (2), Lys 3.07 (3), Trp 1.13 (3), Arg 3.94 (4), Pro 3.05 (3).

Protected Boc-(1–59)-OPac (XIV)

Protected Boc-(12–59)-OPac obtained above (6.45 g, 0.81 mmol) was treated in TFA (50 ml) and the product was converted to the HCl salt as described above. To a solution of the HCl salt and segment **I'** (2.12 g, 0.88 mmol) in a mixture of CHL/TFE (3:1, v/v) (150 ml), HOOBt (0.14 g, 0.88 mmol) and WSCI (0.16 ml, 0.88 mmol) were added at − 10°C, and the whole mixture was allowed to react at room temperature for 3 h. The product was precipitated by adding excess CH_3CN, and the precipitates were washed with water and MeOH, and further purified by reprecipitation from CHL/TFE and ethyl acetate; yield 7.88 g (96.1%); $R_F(3) = 0.48$; amino acid analysis: Asp 2.65 (3), Thr 2.87 (3), Ser 2.73 (3), Glu 5.99 (6), Gly 9.00 (9), Ala 3.05 (3), 1/2(Cys)$_2$ 5.18 (6), Val 3.02 (3), Ile 0.90 (1), Phe 1.91 (2), Lys 9.21 (9), Trp 1.24 (3), Arg 3.95 (4), Pro 4.09 (4).

Protected Boc-(1–121)-OBzl (XVI)

Ammonium formate (1.45 g, 23.1 mmol) and AcOH (2.77 ml, 46.2 mmol) were added to a solution of segment **XIV**, Boc-(1–59)-OPac, (7.85 g, 0.77 mmol) in a mixture of DCM and TFE (3:1, v/v) (150 ml), zinc powder (2.69 g) was added to the solution, and the whole mixture was vigorously stirred under an argon atmosphere at room temperature for 1 h. Next, the zinc dust was removed by filtration, the filtrate was concentrated to dryness, and the residue was washed successively with 1 M HCl, water, MeOH, ethyl acetate and *n*-hexane, and dried; yield of Boc-(1–59)-OH (**XIV'**) was 7.1 g (93.4%); $R_F(3) = 0.41$; amino acid analysis: Asp 2.70 (3), Thr 2.85 (3), Ser 2.62 (3), Glu 5.95 (6), Gly 8.95 (9), Ala 3.00 (3), 1/2(Cys)$_2$ 5.25 (6), Val 2.93 (3), Ile 0.93 (1), Phe 1.90 (2), Lys 9.20 (9), Trp 1.23 (3), Arg 3.96 (4), Pro 4.05 (4).

Segment **XV**, Boc-(60–121)-OBzl, (6.03 g, 0.52 mmol) was treated with TFA (50 ml) at − 10°C for 10 min and then at room temperature for 50 min. After removal of excess TFA *in vacuo*, 5.2 M HCl in dioxane (0.12 ml, 0.63 mmol) was added to the residue, and the product was precipitated with ether and dried over NaOH *in vacuo*. The dried product, HCl salt of H-(60–121)-OBzl, **XIV'** obtained above (5.87 g, 0.57 mmol), and HOOBt (0.09 g, 0.57 mmol) were dissolved together in a mixture of CHL/TFE (200 ml), and then WSCI (0.10 ml, 0.57 mmol) was added to the solution at − 10°C. After allowing the reaction to proceed for 1 h at room temperature, the product was precipitated by adding excess CH_3CN, and the precipitates were washed successively with water, MeOH, ethyl acetate and *n*-hexane. Next, the product was reprecipitated from CHL/TFE and CH_3CN; yield 11.0 g (96.6%); $R_F(3) = 0.40$; amino acid analysis; Asp 7.54 (8), Thr 9.65 (10), Ser 2.78

(3), Glu 11.00 (11), Gly 15.99 (16), Ala 10.29 (10), 1/2(Cys)$_2$ 9.30 (10), Val 4.73 (5), Ile 1.70 (2), Leu 1.07 (1), Tyr 2.05 (2), Phe 2.99 (3), Lys 22.90 (23), Trp 1.73 (4), Arg 6.80 (7), Pro 6.07 (6).

Removal of the Protecting Groups from XVI

Fully protected hMK, Boc-(1–121)-OBzl (**XVI**) (2 g, 0.09 mmol) was treated with TFA (50 ml) as described above, and the N$^{\alpha}$-deprotected product was treated by HF (72 ml) in the presence of anisole (8 ml) at -5°C for 1 h. After evaporation of HF *in vacuo*, ether was added to the residue. The precipitate was washed with ether in the HF-reaction cylinder, and then reacted again with HF (28 ml) in the presence of butanedithiol (12 ml) at -5°C for 30 min. After evaporation of HF, the residue was triturated with ether, and collected by filtration, the yield of the crude product was 1.30 g. The crude product was purified by RP-HPLC using 0.1% TFA/H_2O as buffer A and 0.1% TFA/CH_3CN as buffer B. The product was further purified by CM–cellulose chromatography using a linear gradient of 0.2 M (pH 5.0) to 0.7 M ammonium acetate buffer (pH 6.0) in 3 M urea followed by RP-HPLC as described above to obtain 100 mg of the purified 10 Cys(Acm)-containing peptide.

Removal of Acm Groups

A solution of the 10 Acm-peptide(1–121) (100 mg, 7.16 μmol) in 50% AcOH (14.3 ml) was treated with $Hg(OAc)_2$ (25.1 mg, 78.76 mmol) at room temperature for 2 h. After adding β-mercaptoethanol (0.17 ml), the solution was stirred for 2 h at room temperature, then the solution was applied to a Sephadex G-25 column, and the product was chromatographed by gel filtration with 1 M AcOH. The principal fraction was collected and lyophilized to obtain 77 mg of 10SH-peptide (1–121).

Oxidative Folding of 10SH-peptide (1–121)

The 10SH-peptide (1–121) thus obtained (75 mg, 5.66 μmol) was dissolved in 50 mM $AcONH_4$ buffer at pH 7.7 (566 ml) containing 1 mM EDTA, 2 M $(NH_4)_2SO_4$ and reduced (174 mg) and oxidized gluthatione (35 mg). The ratio of peptide and redox reagents was 1:100:10, and the peptide concentration was 10^{-5}M. The oxidation reaction was kept for two days at 5°C under gentle stirring. The mixture was acidified to pH 3 by adding TFA and the folded peptide was desalted and purified by RP-HPLC using a linear gradient (16–30%) of CH_3CN in 0.1% TFA to obtain 48 mg. Amino acid analysis: Asp 7.52 (8), Thr 9.51 (10), Ser 2.86 (3), Glu 11.12 (11), Gly 16.00 (16), Ala 10.73 (10), 1/2(Cys)$_2$ 8.41 (10), Val 4.87 (5), Ile 1.83 (2), Leu 1.02 (1), Tyr 2.01 (2), Phe 3.10 (3), Lys 23.32 (23), Trp 2.88 (4), Arg 6.90 (7), Pro 5.98 (6). The molecular weight measured by electrospray ionization mass spectrometry (ESI-MS) was 13,240.9, which agreed well with the theoretical value of 13,240.2.

Synthesis of the N- and C-half domains of hMK, (1–59) and (60–121), was carried out similarly to the hMK(1–121) synthesis. The retention time of the folded N-half domain on ODS-column (YMC-pak) was 13.9 min; elution, 10–40% CH_3CN in 0.1% TFA (25 min) at 40°C. Amino acid analysis after 6 M HCl hydrolysis: Asp 2.70 (3), Thr 2.88 (3), Ser 2.81 (3), Glu 6.08 (6), Gly 9.00 (9), Ala 3.16 (3), 1/2(Cys)$_2$ 5.83 (6), Val 2.91 (3), Ile 0.94 (1), Phe 2.04 (2), Lys 9.16 (9), Trp 0.89 (3), Arg 4.00 (4), Pro 4.08 (4). The retention time of the folded C-half domain on ODS-column (YMC-pak) was 12.3 min; elution, 10–40% CH_3CN in 0.1% TFA (25 min) at 40°C. Amino acid analysis after 6 M HCl hydrolysis: Asp 4.71 (5), Thr 6.59 (7), Glu 5.04 (5), Gly 7.00 (7), Ala 7.26 (7), 1/2(Cys)$_2$ 3.80 (4), Val 1.97 (2), Ile 0.88 (1), Phe 0.99 (1), Lys 14.00 (14), Trp 0.26 (1), Arg 2.86 (3), Pro 1.86 (2).

Disulphide Structure Determination

Synthetic hMK was digested with trypsin in a 0.1 M ammonium acetate buffer (pH 6.0) at 37°C for 15 h (E/S = 1/50 at a peptide concentration of 0.1 mg/ml), and the product was analysed by RP-HPLC. All peaks separated as shown in Figure 8 were isolated and their structures were analysed by amino acid and mass analyses. The data are given in Figure 8. Disulphide structures of the N- and C-half domains were determined by the identical procedure.

Biological Activities

The neurite outgrowth, cell survival activities and heparin-binding activities of synthetic hMK and its N- and C-half domains were measured as described in the literature [12, 17]. For neurite outgrowth and cell survival assay, samples to be tested were dissolved in PBS at 10 μg/ml and coated on the Falcon tissue culture dish by incubation at room temperature for 2 h. Trypsin-dissociated embryonic brain cells (SD rat, the 17th day of gestation) were in the dish at the density of 1×10^5 cells/cm^2, and were cultured as described previously [18]. For heparin

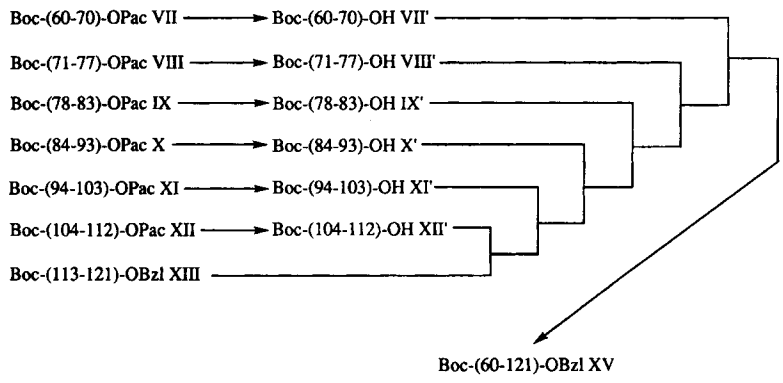

Figure 3 Coupling route for the synthesis of protected hMK (60–121).

affinity column chromatography (50 μg) were applied to a column of heparin Sepharose (Pharmacia, 0.5 ml) equilibrated with 50 mM Tris–HCl, pH 6.8, containing 0.2 M NaCl. Next, the column was successively washed with 2.5 ml of buffers with increasing NaCl concentration. For plasminogen activator assay, cell lysates were prepared from BAECs after treatment with the sample to be tested. Plasminogen activator activity levels in the lysates were measured using the chromogenic substrate S-2251 [15, 16].

RESULTS AND DISCUSSION

The synthesis of hMK was designed to couple two large segments to produce the final fully protected molecule as shown in Figure 6. By this procedure, we expected to be able to synthesize the N-terminal half-domain and the C-terminal one simultaneously. The large segments **XV** and **XIV** were constructed from seven and six small segments, respectively, as shown in Figures 3 and 4. These small segments were prepared by the stepwise elongation method in ordinary organic solvents such as DMF or NMP using WSCI as the coupling reagent in the presence of HOBt. Removal of the Pac ester from each segment was carried out using Zn powder in AcOH when each segment was soluble in AcOH. However, for the segments that were sparingly soluble in AcOH (segments **I** and **V**), a mixture of DCM and TFE (3:1) was used as solvent for the removal of their Pac esters [6]. Segments **II**, **VII** and **X** were highly soluble in DCM/TFE, but precipitated easily upon addition of excess AcOH. Therefore, a new condition was introduced for removing their Pac esters, in which 30 eq. of ammonium formate was added as a proton source for the Zn-reduction reaction [19]. The reaction proceeded smoothly within 1 h at room temperature. During this procedure AcOH must be added to maintain the reaction mixture at around pH 4. The homogeneity of each segment thus obtained was analysed by amino acid analysis, TLC and RP-HPLC, and compounds were found to have more than 95% purity on HPLC (Table 1). During the amino acid analysis of peptides having an Asn-Trp sequence, which were hydrolysed with 6 M HCl, the recovery of Asp was frequently lower than calculated. This phenomenon is specific for this dipeptide sequence as can be seen with segments **VI** and **VII** and the larger ones containing **VI** and/or **VII**. However, if the peptide was hydrolysed with *p*-toluene sulphonic acid, the recovery of Asp returned to normal as shown in Table 1.

For the synthesis of Boc-(60–121)-OBzl (**XV**), the C-terminal segment **XIII** was elongated in sequence manner with the segments having a free carboxylic acid at the C-terminus by applying the WSCI/HOBt or WSCI/HOOBt method in DMF or NMP as shown in Figure 3. However, a significant difference in the solubility was observed in the coupling reaction between segment **X'** and H-(94–121)-OBzl; if NMP was used as the solvent, the reaction mixture turned to a gel during the coupling reaction, but if CHL/TFE was used, the reaction mixture remained a clear solution until the coupling reaction was over. Therefore, the subsequent acylation steps with **IX'**, **VIII'** and **VII'** were performed in CHL/TFE. However, quantitative couplings required longer reaction times when segment **IX'** or **VIII'** was coupled to H-(84–121)-OBzl or H-(78–121)-OBzl, respectively, because of the location of a hindered amino acid, i.e. Thr(Bzl), at the N-terminus of the amino component [9]. On the other hand, the coupling reaction between **VII'** and H-(71–121)-OBzl proceeded smoothly within 2 h to give the final large segment **XV** in a satisfactory yield. The same solvent system was employed for the successive

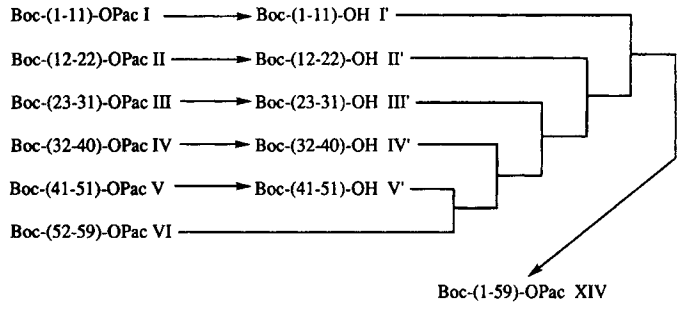

Figure 4 Coupling route for the synthesis of protected hMK (1–59).

coupling of segments **II'** and **I'** with H-(23–59)-OPac, and the large segment **XIV** was obtained smoothly following the route shown in Figure 4. During the segment coupling reactions in CHL/TFE, 1.1 eq. of the carboxyl component against the amino component was used. The excess carbonyl component after completion of the reaction was easily removed by reprecipitation from CHL/TFE and CH_3CN.

Boc-(1–59)-OPac (**XIV**) was hardly soluble even in DMSO, but easily soluble in DCM/TFE; thus, the Pac group could be removed with the solvent system within 1 h as shown in the experimental section. To examine the homogeneity of both Boc-(1–59)-OH (**XIV'**) and Boc-(60–121)-OBzl (**XV**), they were treated with a two-step (high–low) HF procedure [20, 21] and the deprotected products were analysed by HPLC. As can be seen in Figure 5, these two components were judged to be sufficiently homogeneous for the synthesis of the final fully protected peptide, Boc-(1–121)-OBzl.

The two large segments **XIV'** and **XV** were insoluble even in DMSO but highly soluble in CHL/TFE. Therefore, their coupling reaction was carried out in CHL/TFE under the conditions described above. It is noteworthy that the coupling reaction between such large molecules was completed within 1 h, and the yield was almost quantitative. This phenomenon contrasts results reported for the synthesis of ribonuclease A by Fujii and Yajima; they had to use 30-fold excess of Z-(1–8)-N_3, and it took one week for the final coupling reaction with H-(9–124)-OBzl [22]. The use of one component

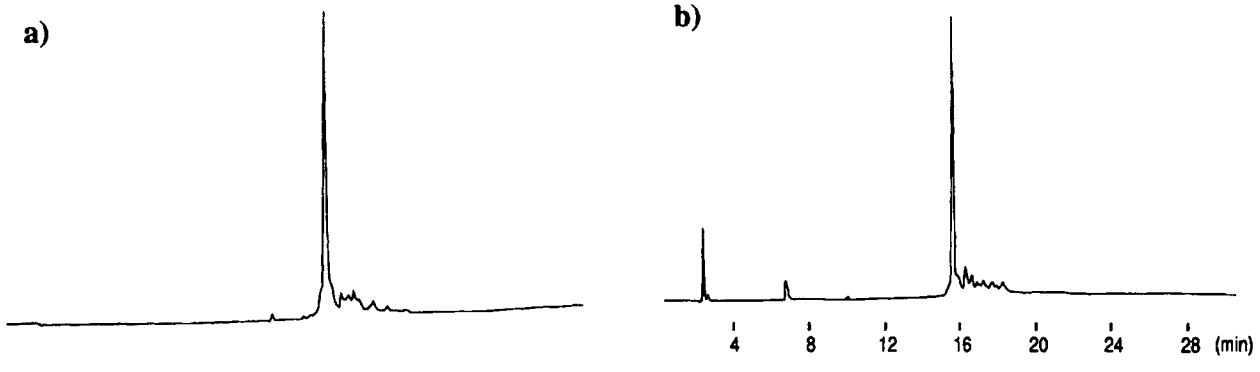

Figure 5 HPLC profile of the crude HF-deprotection products of protected hMK (1–59) and (60–121): (a) hMk (1–59); (b) hMK (60–121). Column: YMC-Pak ODS (4.6 × 150 mm). Elution: 10–40% CH_3CN in 0.1% TFA (25 min) at 40°C. Flow rate: 1.0 ml/min. Absorbance: 220 nm.

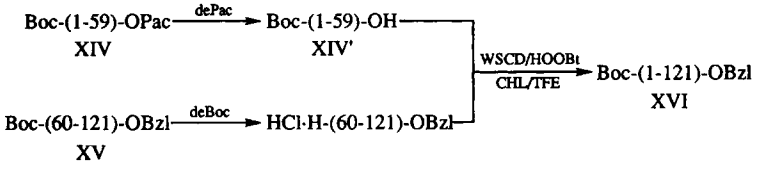

Figure 6 Final fragment condensation reaction between protected hMK (1–59) and hMK (60–121).

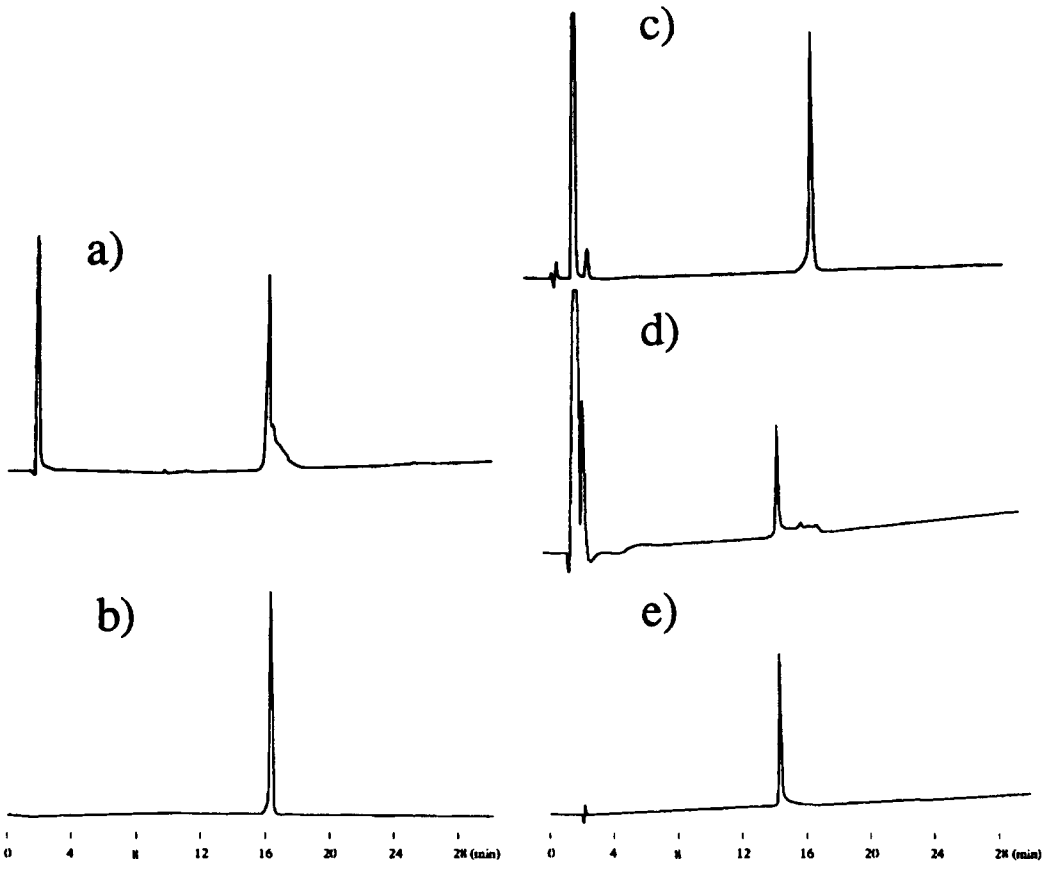

Figure 7 Deprotection and folding of hMK: (a) crude product obtained after HF treatment; (b) purified (10 Acm)-peptide; (c) (10 SH)-peptide; (d) after folding reaction; (e) purified hMK. Column: YMC-Pak ODS (4.6 × 150 mm). Elution: 10–40% CH_3CN in 0.1% TFA (25 min) at 40°C. Flow rate: 1.0 ml/min. Absorbance: 220 nm.

in such a large excess in every coupling reaction must have been a major cause of difficulty in the purification steps.

The fully protected peptide, Boc-(1–121)-OBzl, thus obtained was subjected to the two-step reaction (high–low) to remove all of the protecting groups except Acm groups. The crude product (Figure 7(a)) was purified by CM-cellulose chromatography followed by RP-HPLC. The homogeneity of the (10Acm)-peptide thus obtained was satisfactory as shown in Figure 7(b), which was further treated with $Hg(OAc)_2$ in 50% AcOH to remove the remaining Acm groups [23].

After removal of Hg ions by treatment with β-mercaptoethanol followed by gel filtration on Sephadex G-25, the reduced peptide (Figure 7(c)) was subjected to the oxidative folding reaction. We examined various conditions for optimizing the folding reaction. After a two day reaction (Figure 7(d)) under the conditions described in the experimental section, the product was isolated by RP-HPLC in 34% yield calculated from the reduced peptide. The final product showed a single peak not only on RP-HPLC (Figure 7(e)) but also on CZE (data not shown). The amino acid composition after acid hydrolysis and molecular weight measured by electrospray ionization mass spectrometry of the final product were in good agreement with the theoretical values.

In order to determine the disulphide structure, the product was digested with trypsin, and the hydrolyzate was analysed on RP-HPLC as shown in Figure 8. Each peak was isolated and their structures were determined by amino acid analysis and the molecular weight measurement by mass spectrometry. The results summarized in Figure 8, demonstrate that the disulphide structure of the final product was the same as that reported for the native form [10]. Peaks

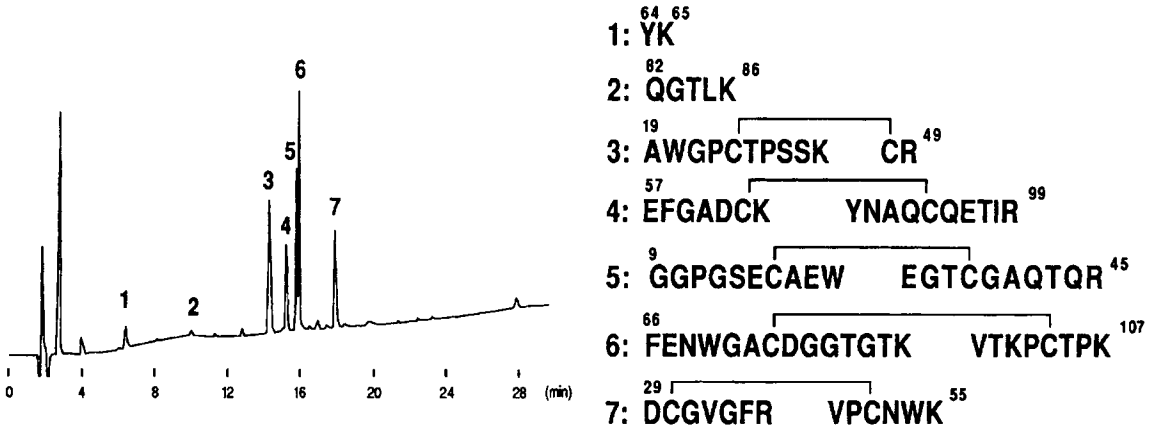

Figure 8 Tryptic peptide mapping of synthetic hMK and assigned structure for peptides in the separated peaks. Column: YMC-Pak ODS (4.6 × 150 mm). Elution: 1–40% CH_3CN in 0.1% TFA (25 min) at 40°C. Flow rate: 1.0 ml/min. Absorbance: 220 nm.

3 and 5 in tryptic peptide mapping were derived from the cleavage reaction between Trp^{18} and Ala^{19} by a chymotrypsin-like enzyme.

The biological activities of the synthetic products were measured with embryonic rat brain cell, and the product was found to show the same neurite extension and neuronal cell survival activities as those of the natural product reported previously [24]

To clarify the roles of the N- and C-half domains, we compared their biological activities with those of hMK. As has been reported previously, the C-terminal half of hMK possessed full neurite extension and heparin-binding activities except for neuronal cell survival activity, for which the full-length hMK molecule was necessary; the N-terminal domain showed much less activity in all aspects [24]. The importance of the C-terminal half of the hMK molecule was also confirmed by the fact that this portion showed almost the same plasminogen activator-enhancing activity as that of intact hMK while the N-terminal portion was inactive [15, 16]. These results confirmed that the active region in the MK molecule is located in the C-terminal domain. Although the role of the N-terminal domain was not clarified, we expect that this part should have other MK functions which remain to be clarified.

CONCLUSIONS

The present study clearly demonstrates that human MK, a 121-residue protein containing five intramolecular disulphide bonds, can be synthesized by the solution procedure without particular difficulty. The principle applied for the synthesis, the segment condensation method in combination with the maximum protection strategy, should be the method of choice for the protein synthesis with CHL/TFE as the solvent system for coupling sparingly soluble segments and liquid anhydrous HF as the reagent for removing the protecting groups at the final stage of the synthesis. This procedure makes it possible to target proteins having molecular weights of 10,000 to 20,000 for routine synthesis.

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