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Chemoselective One-Step Purification Method for Peptides Synthesized by the Solid Phase Technique

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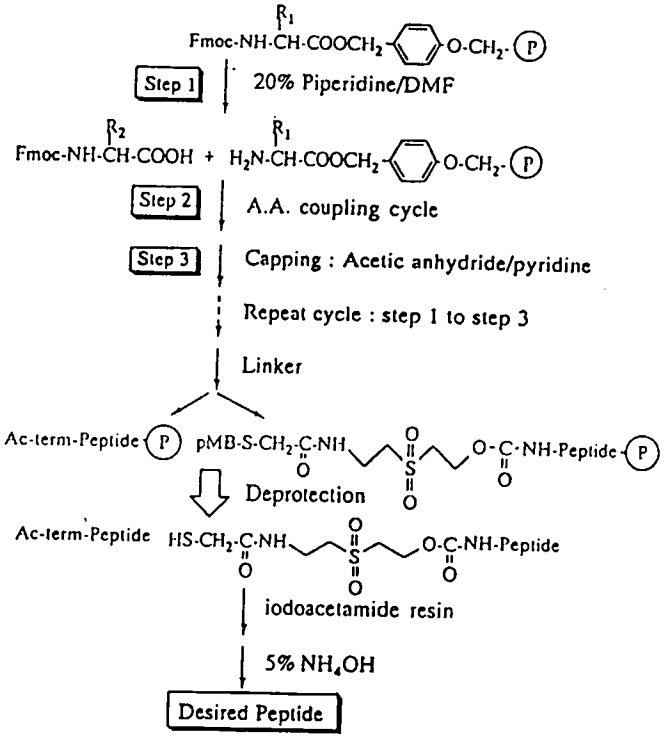
Abstract

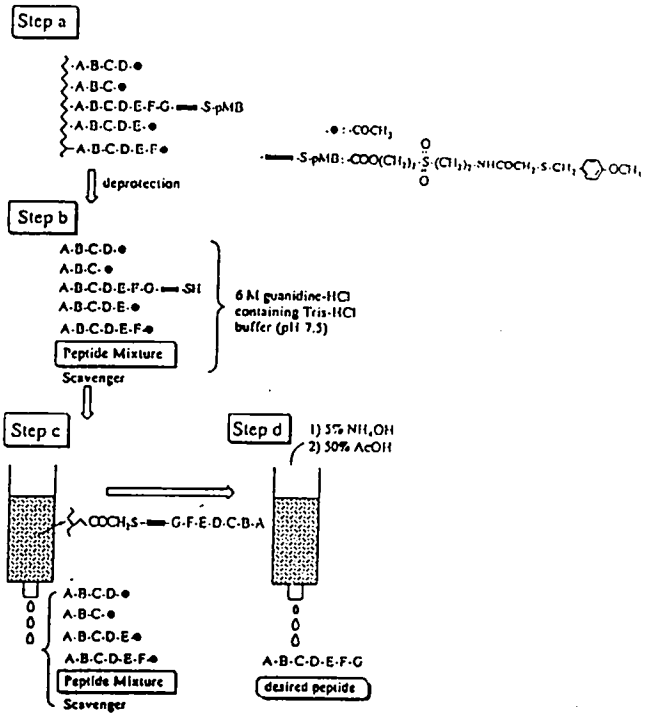
Based upon the specific reaction between SH and iodoacetamide groups, we have explored a new affinity-type purification procedure for peptides synthesized by the solid-phase technique. For this affinity-type purification procedure, we synthesized a new SH precursor reagent bearing an acid-labile S-protecting group, pMB-S-CH_2CO-$NHCH_2CH_2SO_2CH_2CH_2OCOOpNP$ [1]. Using this reagent, the procedure involves the following sequence of 4 reactions. 1. Attachment of the SH function of [1] to the α-amino group of a peptide-resin through a base-labile sulfonylethoxycarbonyl linkage in the final step of solid-phase peptide synthesis. 2. Acid treatment to remove the S-pMB and side chain protecting groups employed and cleave the modified peptide from the resin. 3. Immobilization of the derived SH-peptide on an iodoacetamide resin column. 4. Base (5% NH_4OH) treatment to release the desired peptide from the resin in nearly pure form. To facilitate this purification procedure, unreacted amino groups were acetylated in each step during solid-phase synthesis. The usefulness of this method was demonstrated by the purification of several peptides (18–44 amino acids in length) synthesized by the Fmoc-based solid-phase technique. The principle of this affinity-type purification procedure may also be applied to Boc-based solid-phase technique.

General scheme for Fmoc-based solid-phase peptide synthesis.



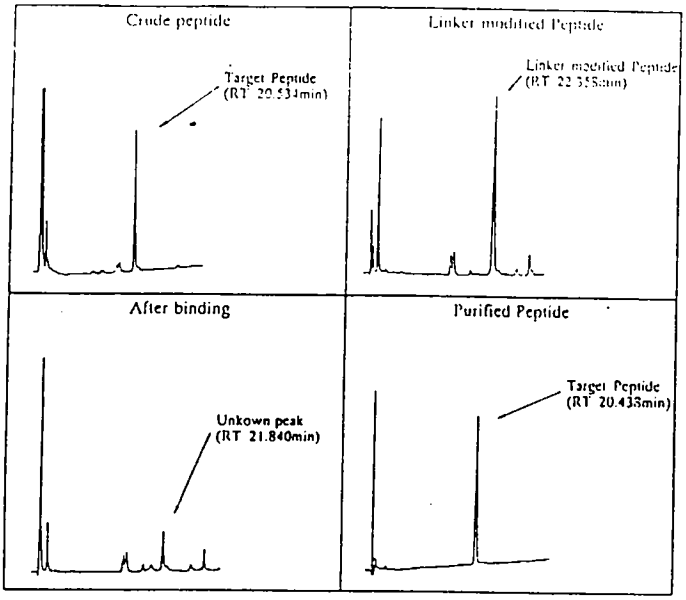
Synthesis was carried out automatically according to the principles of Sheppard *et al.*, using the following side chain protected Fmoc-amino acids: Arg(Mtr), Lys(tBoc), His(tBoc), Glu(O^{t}Bu), Asp(O^{t}Bu), Ser(^{t}Bu), Thr(^{t}Bu), and Tyr(^{t}Bu). Resin: polystyrene resin cross-linked with 1% divinyl benzene was used. Deprotection of the Fmoc group: resin was treated with 20% piperidine in DMF for 10 min. Coupling: coupling of protected amino acids was carried out in DMF for 30 min using several acylating reagents. Capping: capping of truncated peptides was performed with acetic anhydride (0.5 M)-pyridine (0.5 M) in DMF for 10 min. The S-protected base-labile SH linker was introduced manually by the *p*-nitrophenyl active ester-HOBt method.

Deprotection, and purification of peptides synthesized by the solid-phase technique.



The base-labile SH introducing reagent and an iodoacetamide column were used for the isolation of peptides synthesized by the solid-phase technique. After each step in which an Fmoc-amino acid group was added (A,B,C,D....), the residual unreacted amino groups were blocked with an acetyl group. In the last step of synthesis, the S-protected base-labile SH introducing reagent was added to the N-terminal residue (step a). After removal of the synthesized peptide from the solid support (step b), the mixture was passed through an iodoacetamide column to remove all of the truncated peptides and scavengers (step c). After washing the column, the resin was treated with 5% NH_4OH to release the desired peptide (step d).

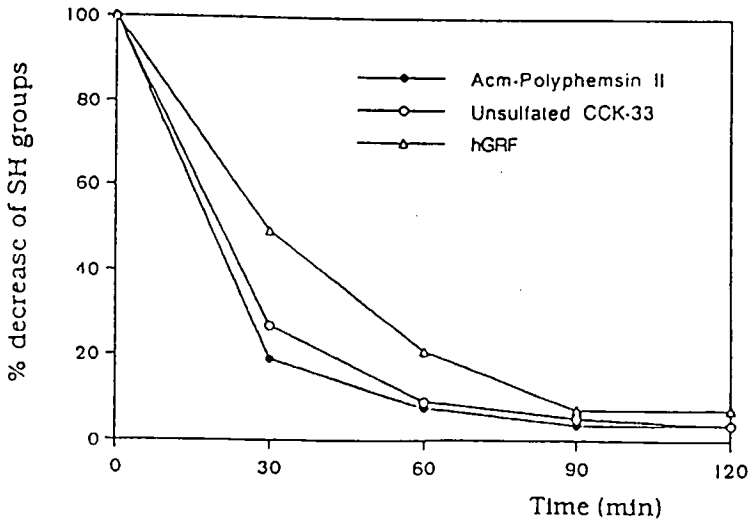
Identification of the target peptide during purification



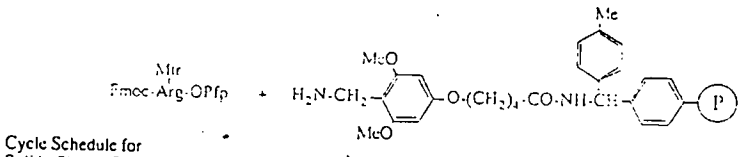
This new purification method can easily identified the target peptide by comparision of HPLC assays of the purified peptide, the linker modified peptide and the crude peptide. The linker modified peptide was characterized by its longer retention time due to the hydrophobicity of the linker.
Therefor the peak with its retention time extended by introduction of the linker can be identified as that of the target peptide.

HPLC was performed on a μ Bondasphere 5C18 (100Å) column (3.9 x 150mm), using a 0.1% TFA/acetonitril solvent system at a flow rate of 1.0ml/min. Absorbance was monitored at 220nm and the column was eluted with a linear gradient of 20-30% solvent B over 30min.

Binding of the linker-modified peptide to an iodoacetamide resin column monitered by the Ellman method.



Fmoc-Based Solid-Phase Synthesis of Acm-Polyphemusin II



Cycle Schedule for Solide Phases Synthesis

1) Condensation by Pfp ester or BOP + HOBt in DMF

2) Termination by Acetic Anhydride/DMF

3) Deprotection by 20% Piperidine/DMF

Mtr Mtr Acm Mtr Acm Bu Boc Acm Bu Mtr Boc AcmMtr
H-Arg-Arg-Trp-Cys-Phe-Arg-Val-Cys-Tyr-Lys-Gly-Phe-Cys-Tyr-Arg-Lys-Cys-Arg-PAL resin

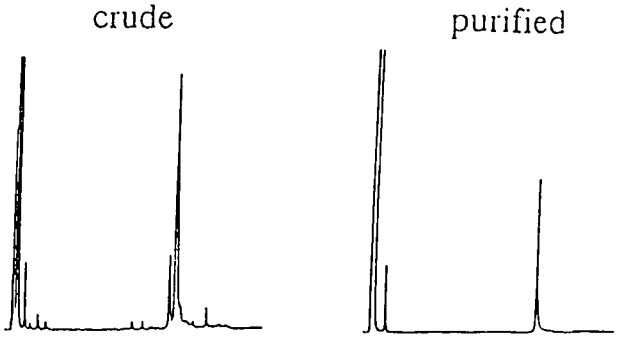
1) Purification Linker + HOBt in DMF

2) 1M TMSBr-thioanisole/TFA +EDT

3) Purification using Iodo-resin

Acm Acm Acm Acm
H-Arg-Arg-Trp-Cys-Phe-Arg-Val-Cys-Tyr-Lys-Gly-Phe-Cys-Tyr-Arg-Lys-Cys-Arg-NH_2

HPLC elution profile of synthetic Acm-polyphemsin II.



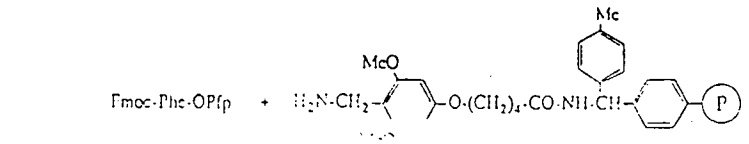
Structure of Acm-polyphemusin II

H-Arg-Arg-Trp-Cys(Acm)-Phe-Arg-Val-Cys(Acm)-Tyr-Lys-Gly-Phe-Cys(Acm)-Tyr-Arg-Lys-Cys(Acm)-Arg-NH_2

FAB-MS: cal. 2712.3 ($C_{120}H_{185}N_{41}O_{24}S_4$), ob. 2714.1 ($M+H^+$)

HPLC was performed on a μBondasphere 5C18 (100 Å) column (3.9 x 150 mm), using a 0.1% TFA/acetonitrile solvent system at a flow rate of 1.0 ml/min. Absorbance was monitored at 220 nm and the column was eluted with a linear gradient of 10-40% solvent B over 30 min.

Fmoc-Based Solid-Phase Synthesis of unsulfated Human CCK-33



Cycle Schedule for Solide Phase Synthesis

1) Condensation by Pfp ester or BOP + HOBt in DMF

2) Termination by Acetic Anhydride/DMF

3) Deprotection by 20% Piperidine/DMF

Boc Bu Mtr Bu Boc OBu Bu
H-Lys-Ala-Pro-Ser-Gly-Arg-Met-Ser-Ile-Val-Lys-Asn-Leu-Gln-Asn-Leu-Asp-Pro-Ser-

Boc Mtr Bu OBu Mtr OBu Bu OBu
His-Arg-Ile-Ser-Asp-Arg-Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-PAL resin

1) Purification Linker + HOBt in DMF

2) 1M TMSBr-thioanisole/TFA +EDT

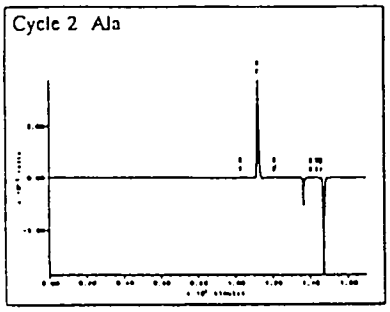
3) Purification using Iodo-resin

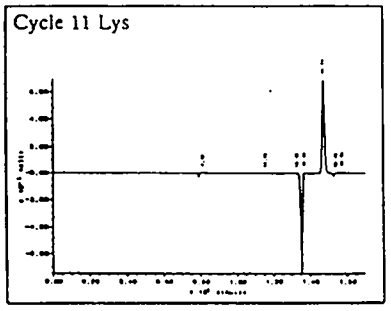
H-Lys-Ala-Pro-Ser-Gly-Arg-Met-Ser-Ile-Val-Lys-Asn-Leu-Gln-Asn-Leu-Asp-Pro-Ser-

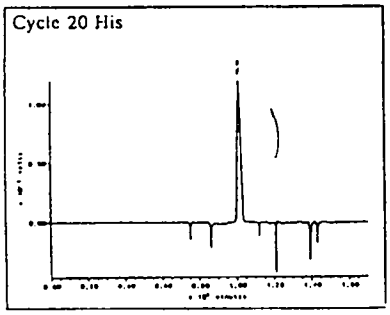
His-Arg-Ile-Ser-Asp-Arg-Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2

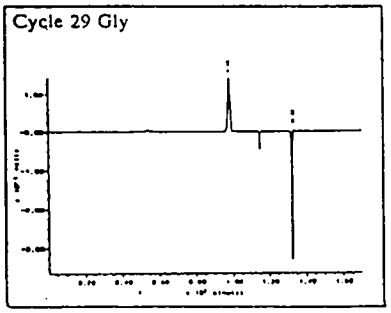
Amino Acid Sequence Analysis of unsulfated Human CCK- 33

Sequence ; H-KAPSGRMSI^{10}VKNLQNLDPS^{20}HRISDRDYMG^{30}WMDF-NH_2

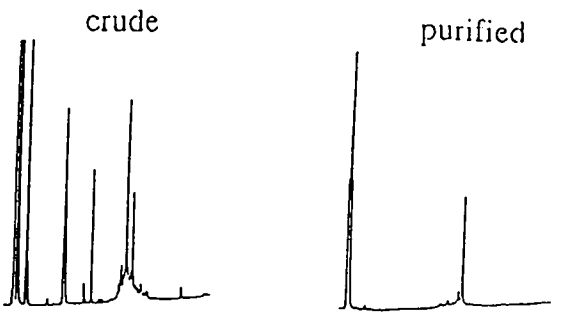








HPLC elution profile of synthetic unsulfated CCK-33.



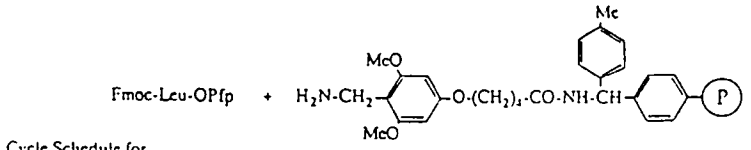
Structure of unsulfated human CCK-33

H-Lys-Ala-Pro-Ser-Gly-Arg-Met-Ser-Ile-Val-Lys-Asn-Leu-Gln-Asn-Leu-Asp-Pro-Ser-His-Arg-Ile-Ser-Asp-Arg-Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2

FAB-MS: cal. 3862.9 ($C_{167}H_{263}N_{51}O_{49}S_3$), ob. 3865.2 ($M+H^+$)

HPLC was performed on a μBondasphere 5C18 (100Å) column (3.9 x 150 mm), using a 0.1% TFA/acetonitrile solvent system at a flow rate of 1.0 ml/min. Absorbance was monitored at 220 nm and the column was eluted with a linear gradient of 10-60% solvent B over 30 min.

Fmoc-Based Solid-Phase Synthesis of Human Growth hormone releasing Factor



Cycle Schedule for Solide Phases Synthesis

1) Condensation by Pfp ester or BOP + HOBt in DMF

2) Termination by Acetic Anhydride/DMF

3) Deprotection by 20% Piperidine/DMF

Bu OBu Bu Bu Bu PmcBoc Bu Pmc Boc
H-Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Gly-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-

OBu Bu Pmc OBu Bu OBu Pmc Pmc Pmc
Asp-Ile-Met-Ser-Arg-Gln-Gln-Gly-Glu-Ser-Asn-Gln-Glu-Arg-Gly-Ala-Arg-Ala-Arg-Leu-PAL resin

1) Purification Linker + HOBt in DMF

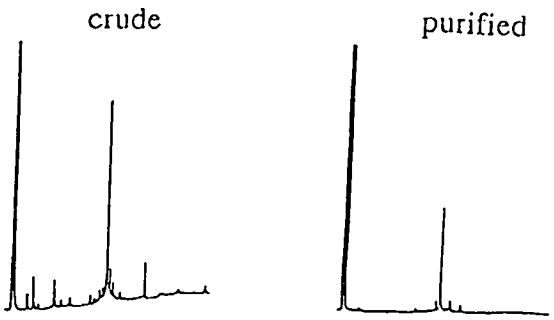
2) 1M TMSBr-thioanisole/TFA +EDT

3) Purification using Iodo-resin

H-Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Gly-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-

Asp-Ile-Met-Ser-Arg-Gln-Gln-Gly-Glu-Ser-Asn-Gln-Glu-Arg-Gly-Ala-Arg-Ala-Arg-Leu-NH_2

HPLC elution profile of synthetic human growth hormone releasing factor.



Structure of human growth hormone releasing factor

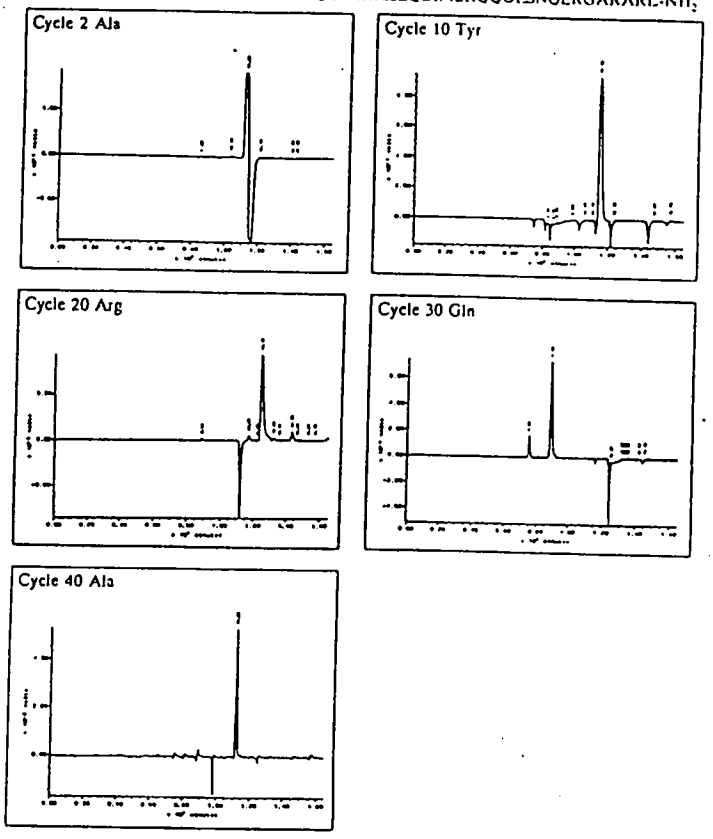
H-Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Gly-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Met-Ser-Arg-Gln-Gln-Gly-Glu-Ser-Asn-Gln-Glu-Arg-Gly-Ala-Arg-Ala-Arg-Leu-NH_2

FAB-MS: cal. 5036.7 ($C_{215}H_{358}N_{72}O_{66}S$), ob. 5037.8 ($M+H^+$)

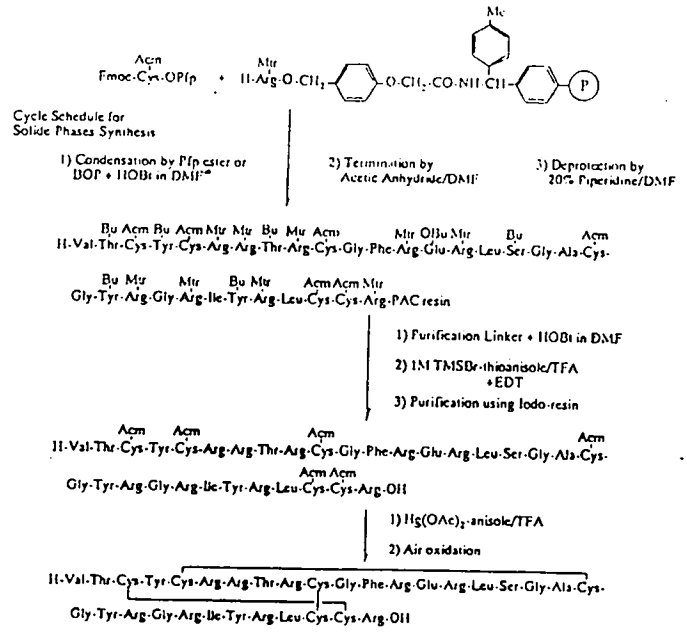
HPLC was performed on a μBondasphere 5C18 (100Å) column (3.9 x 150 mm), using a 0.1% TFA/acetonitrile solvent system at a flow rate of 1.0 ml/min. Absorbance was monitored at 220 nm and the column was eluted with a linear gradient of (a) 23-40% solvent B over 30 min or (b) 25-40% solvent B over 30 min.

Amino Acid Sequence Analysis of Human Growth hormone releasing Factor

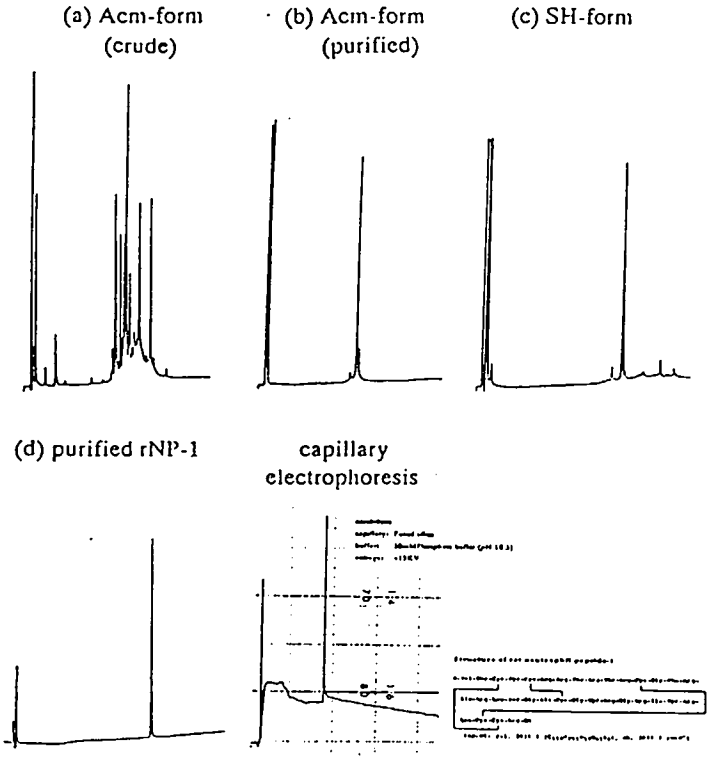
Sequence ; H-YADAIFTNSYRKVLGQLSARKLLQDIMSRQQGESNQERGARARL-NH_2



Fmoc-Based Solid-Phase Synthesis of Rat Neutrophil Peptide-1



HPLC elution profile and capillary electrophoresis of synthetic rat neutrophil peptide-1 (rNP-1)



HPLC was performed on a μBondasphere 5C18 (100Å) column (3.9 x 150 mm), using a 0.1% TFA/acetonitrile solvent system at a flow rate of 1.0 ml/min. Absorbance was monitored at 220 nm and the column was eluted with a linear gradient of (a,b,d) 10-40% solvent B over 30 min (c) 15-25% solvent B over 40 min.

Conclusion

The advantages of our method consists of the purity of the product and its easiness. Even peptides which have shoulder peaks can be purified with this method. Compared with conventional HPLC purification, this method can make it possible for any researcher without special technique to obtain pure peptides easily at one step in short time by solid-phase technique. The easily prepared cross-linking reagent introduced here will be of wide applicability for the one-step purification of peptides synthesized by the solid-phase technique. Additionally, a more readily obtainable supply of high-purity or constant-purity peptides should facilitate research in the fields of biochemistry, physiology, and medicine with respect to the physiological activities and mechanisms of action of peptides and proteins.

Acknowledgement

We are grateful to Dr. H. Tanaka (Fujisawa Pharmaceutical Co.) for FAB-MS, and Mrs. S. Funakoshi and Mr. M. Ono for ^{1}H-NMR. We express our sincere appreciation to Professor H. Yajima (Niigata Pharmaceutical College), Professor M. Fridkin, Professor A. Patchornik and Professor M. Wilchek (The Weizmann Institute of Science, Rehovot, Israel) for valuable discussions.