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Melanotropin-potentiating factor isolated from filtrate of uremic patients suffering from melanosis and carbohydrate intolerance

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Summary. Following the characterization of a hexadecapeptide, α-endorphin, melanotropin-potentiating factor (MPF) was isolated from the filtrate of uremic patients suffering from melanosis and carbohydrate intolerance. The structure of MPF has been determined on the bases of chemical and physicochemical examinations which included HPLC, Edman sequence analysis combined analysis of the amino acid composition and FAB-MS analysis. An accumulation of MPF might be a cause of melanosis in uremic patients.

Keywords: Amino acids – Melanotropin-potentiating factor – Melanosis – Uremic patient – Uremic toxin – Melanotropic activity

Abbreviations: Et_3N triethylamine; TFA trifluoroacetic acid; HF hydrogen fluoride; CH_3CN acetonitrile; DCC dicyclohexyl-carbidimide; HOBT N-hydroxybenzotriazole; OBzl benzyl ester; Boc tert-butoxycarbonyl; Z bebzyloxycarbonyl; EtOH ethanol; ECUM extracorporeal ultrafiltration method; FAB-MS fast atom bombardment spectrometry; HPLC high-performance liquid chromatography; TLC thin-layer chromatography; AcOH acetic acid; AP-M aminopeptidase-M; PTH phenylthiohydantoin.

Introduction

As a part of our continuing studies in search of uremic toxins from uremic patients, we have been investigating the chemical constituents of uremic patients. Dialysates of uremic patients contain a wide variety of middle molecules (MM) (Babb et al., 1972).

We have so far isolated seven peptides (Abiko et al., 1978a,b, 1979a,b, 1980, 1981; Abiko and Sekino, 1993) from dialysates and filtrate of uremic patients.

Following our research on separation and identification of α-endorphin from filtrate of uremic patients suffering from carbohydrate intolerance (Abiko and Sekino, 1993), we describe here isolation and structural analysis

of a peptide from the eluate corresponding to the second peak (retention time 17.69 min, detected by ultraviolet absorption measurement at 230 nm). However, judging from the data on the amino acid analysis of the acid hydrolysate of the second peak, we concluded that further purification was necessary to obtain a highly purified unknown peptide.

For further purification of the second peak, the crude peptide was purified by HPLC on an Asahipak ODP-90 column (21.5 × 300 mm). The highly purified peptide thus obtained found to be identical as MPF by amino acid analysis, application of the Edman degradation analysis and measurement of physical constants and analytical data of our synthetic tetrapeptide.

Results and discussion

Following the characterization of a peptide, α-endorphin from the first main peak on HPLC (Abiko and Sekino, 1993), we tried to isolate another peptide from the second peak on HPLC. However, judging from the data on the amino acid analysis of the acid hydrolysate of the second peak, we found that further purification was necessary to obtain a highly purified unknown peptide. So, the crude material from the second peak was further purified by HPLC on an Asahipak ODP-90 column. The structure of the highly purified peptide thus obtained has been determined on the bases of chemical and physicochemical examinations which included HPLC, Edman sequence analysis combined analysis of the amino acid composition and FAB-MS analysis.

From the accumulated evidence mentioned in the experimental, the structure of the highly purified peptide has been determined as MPF, H-Lys-Lys-Gly-Glu-OH.

In order to verify this presumption, MPF was synthesized by a solid-phase method. The peptide isolated from the filtrate of the uremic patients found to be identical to our synthetic MPF by TLC, HPLC and FAB-MS.

It is widely known that MPF potentiates the melanotropic activity of MSH and seems to be involved in other biological systems. It is also well-known that many uremic patients suffer from melanosis, but the cause of melanin-accumulation has not been found yet.

This is the first report that MPF was isolated from the filtrate of uremic patients. In our previous paper (Abiko and Sekino, 1993), we mentioned that the peak corresponding to tubes 122 through 138 on the elution profile on Sephadex G-50 is present in uremic plasma but not detected in plasma from nonuremic individuals. These results seem to suggest that the accumulation of MPF might be a cause of melanosis in these uremic patients.

Experimental

Solvent systems for ascending TLC on cellulose plates (Merck) are indicated as follow: Rf^1 = BuOH-AcOH-H_2O (4:1:5, upper phase), Rf^2 = BuOH-pyridine-AcOH-H_2O (4:1:1:2). Isolated MPF from uremic patients and synthetic MPF were hydrolyzed in 6 N HCl at 110°C for 16 h. Amino acid compositions of acid hydrolysates were determined with a Hitachi 835 amino acid analyzer. HPLC was conducted with Shimadzu LC-6A

apparatus. FAB-MS spectrum were obtained on a Auto Spec Q instrument (UQ Analytical Co., England) mass spectrometer equipped with an OPUS data processor. Amino acid sequence analysis of the purified peptide was performed by automated Edman degradation with a gas phase sequencer (Applied Biosystems 477 A) coupled with PTH-amino acid analyzer (Applied Biosystems 120 A). Filtrate was obtained by ECUM from uremic patients suffering from melanosis and carbohydrate intolerance.

Separation procedure

The highly purified tetrapeptide was isolated from the filtrate (150 l) by ECUM of two uremic patients suffering from melanosis and carbohydrate intolerance. The procedure for the isolation of the tetrapeptide is summerized in Fig. 1.

Isolation and purification procedures from the ultrafiltration on an Amicon Centriflo DM-5 membrane to HPLC on a 4 mm × 30 cm as μBondapak C18 column we employed here are essentially the same as employed for our previous isolation of a α-endorphin from the first peak (retention time 14.82 min) (Abiko and Sekino, 1993).

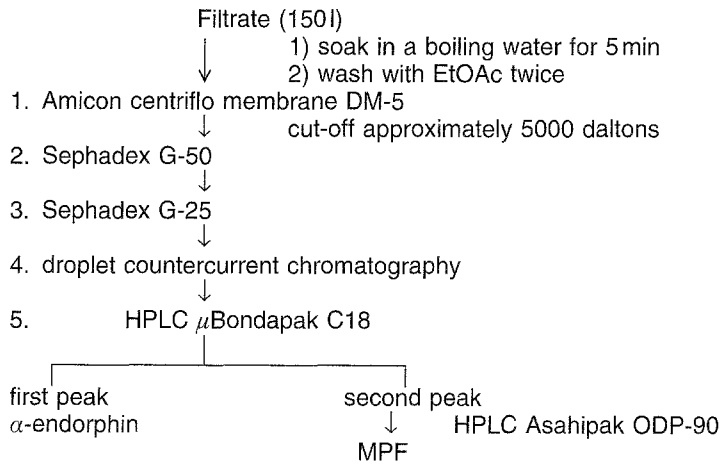

Fig. 1. Purification of the tetrapeptide from filtrate of uremic patients with carbohydrate intolerance

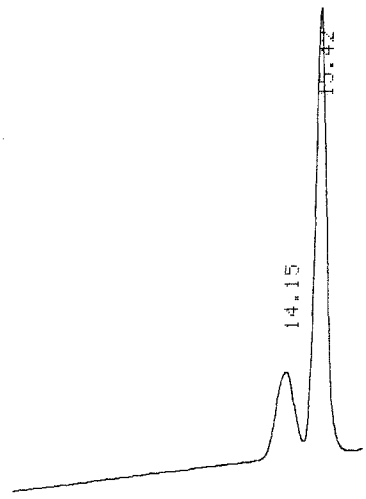

Fig. 2. HPLC profile of the crude tetrapeptide

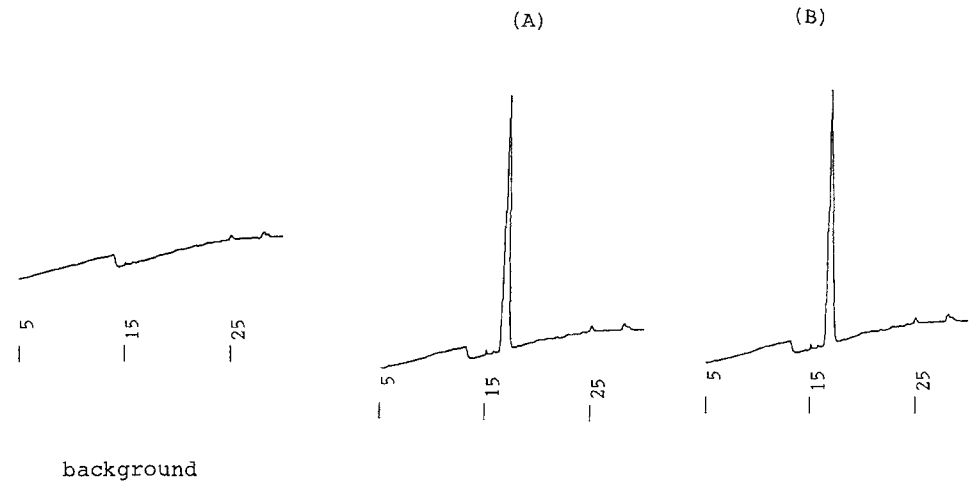

Fig. 3. Results of analytical HPLC of the purified tetrapeptide (**A**) isolated from uremic filtrate and the synthetic MPF (**B**)

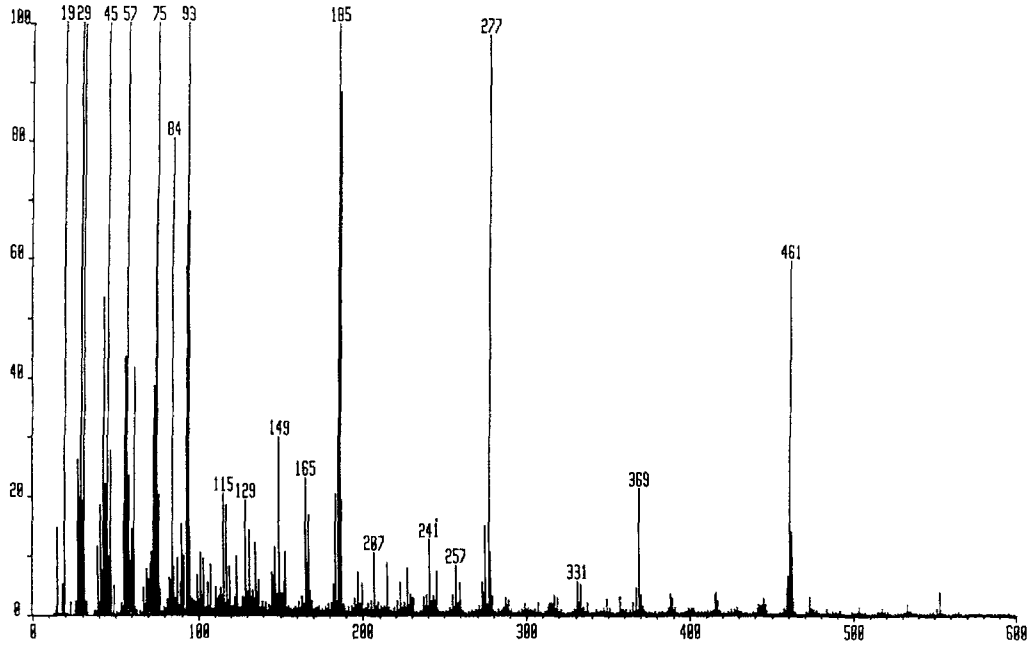

Fig. 4. FAB-MS of the purified tetrapeptide isolated from uremic filtrate

0.5 mg sample from the second peak (retention time 17.69 min) was further purified by HPLC on a 21.5 × 300 mm Asahipak ODP-90 column, which was eluted with a gradient of CH_3CN (25 → 45%, 30 min) in aqueous 0.1% TFA at a flow rate of 2 ml/min. The eluate corresponding to the main peak (retention time 15.42 min, determined by ultraviolet absorption measurement at 230 mn) was collected and the solvent was removed by lyophilization to give a fluffy powder. Yield 263 μg. Rf^1 0.08, Rf^2 0.12, single ninhydrin- and chlorine-tolidine-positive spot. Amino acid composition in the acid hydrolysate: Gly 1.00, Lys 1.98, Glu 0.97 (average recovery 88%); amino acid composition in the AP-M digest: Gly 1.00, Lys 2.01, Glu 0.95 (average recovery 90%). The peptide exhibited a single peak on HPLC using a YMC-PACK (R-ODS-5 120Å 4.6 mm × 250 mm) at a retention time of 17.04 min (Fig. 3) when eluted with a gradient of CH_3CN (5 → 65%) in 0.1% TFA (30 min) at a flow rate of 0.5 ml/min. FAB-MS ($M + H^+$) m/z 461 (Fig. 4).

Edman sequence analysis

The peptide was subjected to amino acid sequence analysis by automated Edman degradation with gas-phase sequencer (Applied Biosystems 477 A) coupled with a PTH-amino acid analyzer (Applied Biosystems 120 A). From the results of the amino acid analysis and the amino acid sequence analysis, the structure of the peptide was speculated as follows: H-Lys-Lys-Gly-Glu-OH corresponding to MPF.

Solid-phase synthesis of the tetrapeptide corresponding to MPF

The tetrapeptide was synthesized as an authentic specimen to confirm the identification of the isolated peptide. The following amino acid derivatives were used; Boc-Lys (Z)-OH, Boc-Gly-OH and Boc-Glu (OBzl)-OH-resin. Solid-phase peptide synthesis of MPF was carried out manually in a glass vessel by a stepwise strategy starting with Boc-Glu (OBzl)-resin (0.25 mmol/g, 1 g). The general procedure for each synthetic cycle was: (I) three washings with CH_2Cl_2; (II) prewashing with 40% TFA in CH_2Cl_2; (III) deprotection for 30 min with 40% TFA in CH_2Cl_2; (IV) three washings with CH_2Cl_2; (V) prewashing with 10% Et_3N in CH_2Cl_2; (VI) neutralization for 10 min with 10% Et_3N in CH_2Cl_2; (VII) three washings with CH_2Cl_2; (VIII) addition of 3 eq of Boc-amino acid, HOBT and DCC; (IX) reaction for 120 min; (X) three washings each with CH_2Cl_2, 50% EtOH in CH_2Cl_2 and then CH_2Cl_2. Coupling efficiency was monitored after every cycle by the ninhydrin test and then acetylations with Ac_2O-pyridine were done after each coupling reaction. The tetrapeptide-resin was then cleaved with anhydrous HF (10 ml) at 0°C for 60 min in the presence of anisole (1 ml). Evaporation of the acid and extraction of the residue with 5% AcOH (20 ml) gave a solution which on lyophilization yield the crude peptide. The residue was dissolved in a small amount of 1% AcOH and then applied to a column of a Sephadex G-10 (2.8 × 94 cm), which was eluted with the same solvent. Individual fractions (5 ml each) were collected and the absorbancy at 230 nm was determined for each fraction. The fractions corresponding to the front main peak (tube Nos. 45–52) were combined and the solvent was evaporated off and the residue was furhter purified by HPLC as described for the purification of the tetrapeptide isolated from the fitrate of the uremic patients. Yeild 19 mg (36%), Rf^1 0.08, Re^2 0.12, single ninhydrin- and chlorine-tolidine-positive spot. Amino acid composition in the acid hydrolysate: Gly 100, Lys 2.03, Glu 0.98 (average recovery 89%); amino acid composition in the AP-M digest: Gly 1.00, Lys 1.96, Glu 0.97 (average recovery 87%). The peptide exhibited a single peak on HPLC using a YMC-PACK (R-ODS-5, 120 Å, 4.6 mm × 250 mm) at a retention time of 17.04 min when eluted with a gradient of CH_3CN (5 → 65%) in 0.1% TFA (30 min) at a flow rate of 0.5 ml/min. FAB-MS ($M + H^+$) m/z 461.

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