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Is α-endorphin an uremic toxin? α-Endorphin isolated from filtrate of uremic patients with carbohydrate intolerance

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Summary. A 16-residue peptide was isolated from filtrate of uremic patients with carbohydrate intolerance by ultrafiltration with an Amicon Centriflo DM-5 membrane, followed by gel-filtration on Sephadex G-50 and Sephadex G-25, droplet countercurrent chromatography and high performance liquid chromatography. The hexadecapeptide thus obtained was identical as the entire amino acid sequence of α-endorphin by amino acid analysis, application of the Edman degradation analysis and measurement of physical constants and analytical data of the synthetic hexadecapeptide. This result seems to suggest that an accumulation of α-endorphin in uremic patients might cause carbohydrate intolerance.

Keywords: Amino acids – α-Endorphin accumulation – Uremic toxin – Uremic patient – Carbohydrate intolerance

Introduction

According to the middle molecule hypothesis, solutes with molecular weight of 500 to 5000 daltons, so-called middle molecules (MM) are toxic and may cause or contribute to uremic symptoms (Babb et al., 1972). However identification of MM has not been accomplished yet.

Following our research on separations and identifications of six peptides (Abiko et al., 1978a; Abiko et al., 1978b; Abiko et al., 1979a; Abiko et al., 1979b; Abiko et al., 1980; Abiko et al., 1981) from dialysates of uremic patients, we describe here isolation and structural analysis of a peptide from filtrate of uremic patients with carbohydrate intolerance. The isolation process of the peptide is summerized in Fig. 1.

The peptide we purified was identical as a hexadecapeptide, α-endorphin, by amino acid composition and Edman degradation analysis. According to the deduced structure of the peptide isolated from the filtrate of the uremic patients,

we have synthesized the peptide by a solid phase method. The peptide isolated from the filtrate of the uremic patients found to be identical to our synthetic α-endorphin by paper chromatography, HPLC, optical rotation and FAB-MS.

Discussion

MM accumulate in uremia was shown to influence *Hb** synthesis (Goubead et al., 1976), neuropathy (Fürst et al., 1975) and inhibition of erythrocyte glycolysis (Gajdos and Dzürick 1973). MM are probably peptides and have molecular weight 500–5000 daltons (Asaba et al., 1979).

On the other hand, carbohydrate intolerance was found to a lot of patients with uremia (Westermelt et al., 1962). Recently circulating endorphins, endogenous opioids, have been found to be elevated in uremic patients (Nokao et al., 1980). An excess of endorphins may cause insulin deficiency, impairing its secretion mechanism (Rosemblatt, 1987). This might contribute to explain our result, of impairment of glucose tolerance in uremic patients, since high serum level of endogenous opioids are known to stimulate the secretion of parathyroid hormone, growth hormone, pituitary TSH, gonadotropins, prolactin and to cause glucose intolerance (Grzeszczak et al., 1987).

Experimental

Resins, amino acid derivatives and reagents

The chloro-methylated polystyrene resin cross-linked with 2% divinylbenzene (Cl content 2 mmol/g) and Boc-amino acids, Boc-Thr (Bzl)-OH, Boc-Val-OH, Boc-Leu-OH, Boc-Pro-OH, Boc-Gln-OH, Boc-Ser(Bzl)-OH, Boc-Lys(Z)-OH, Boc-Glu(OBzl)-OH, Boc-Met(O)-OH, Boc-Phe-OH, Boc-Gly-OH and Boc-Tyr(Cl_2-Bzl)-OH, were purchased from Protein Research Inc. (Mino, Osaka) and Kokusan Chemical Works, Ltd. (Kyoto). Solvents were freshly distilled. The purity and identity of the purified synthetic α-endorphin and the purified peptide isolated from the filtrate of uremic patients were chromatographed on filter papers, Toyo Roshi No. 51, at room temperature. Rf^1 values refer to Partridge system (Partridge, 1948) and Rf^2 values refer to BuOH-pyridine-AcOH-H_2O (30:20:6:24) (Waley and Watson, 1953). Amino acid analysis was performed by a JLC-8AH amino acid analyzer (one-column system). Filtrate was obtained by ECUM from two uremic patients with carbohydrate intolerance and two uremic patients with noncarbohydrate intolerance. Synthetic α-endorphin was prepared by a manual solid phase tequnigue. HPLC was

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* Abbreviations used: *Hb* hemoglobin; *Et_3N* triethylamine; *TFA* trifluoroacetic acid; *MeCN* racetonitrile; *DCC* dicyclohexyl-carbodiimide; *HOBT* N-hydroxybenzotriazole; *OBzl* benzyl ester; *Bzl* benzyl; *Tyr(Cl_2–Bzl)* O–2,6-dichlorobenzyl-tyrosine; *DMF* dimethylformamide; *HF* hydrogen fluoride; *ECUM* extracorporeal untrafiltration method; *FAB-MS* fast atom bombardment mass spectrometry; *HPLC* high-performance liquid chromatography; *AcOH* acetic acid; *PTH* phenylthiohydantoin; *TSH* thyrotropic stimulating hormone; *EtOAc* ethyl acetate.

conducted with Shimadzu LC-9A 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 477A) coupled with PTH-amino acid analyzer (Applied Biosystems 120A).

Separation procedure

The highly purified hexadecapeptide was isolated from the filtrate (100 l) by ECUM of two uremic patients suffering from carbohydrate intolerance. The procedure for the isolation of the hexadecapeptide is summerized in Fig. 1.

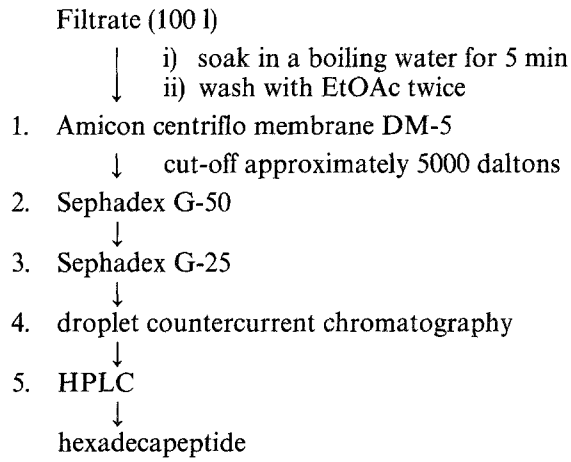

Fig. 1. Purification of the hexadecapeptide from filtrate of uremic patients with carbohydrate intolerance

1. Ultrafiltration

Filtrate (100 l containing 0.02% NaN_3) were soaked in a boiling water for 5 min to inactivate proteolytic enzymes and extracted with EtOAc twice. The water layer was ultrafiltered using an Amicon Centriflo DM-5 membrane, which has a molecular cut off at approximately 5000 daltons.

2. Sephadex G-50 gel-filtration

The concentrated filtrate was applied to a column of Sephadex G-50 (120 × 3.6 cm) and eluted with 1% AcOH. Fractions of 6 ml were collected at a flow rate of 6 ml/15 min and assayed for absorbance at 230 nm. Clear differences were detected between carbohydrate intolerance filtrate and noncarbohydrate intolerance filtrate (Fig. 2). The fractions corresponding to tubes 122 through 138 were present in the filtrate from carbohydrate intolerance patients, but were not detectable in noncarbohydrate intolerance filtrate. Yield 72 mg.

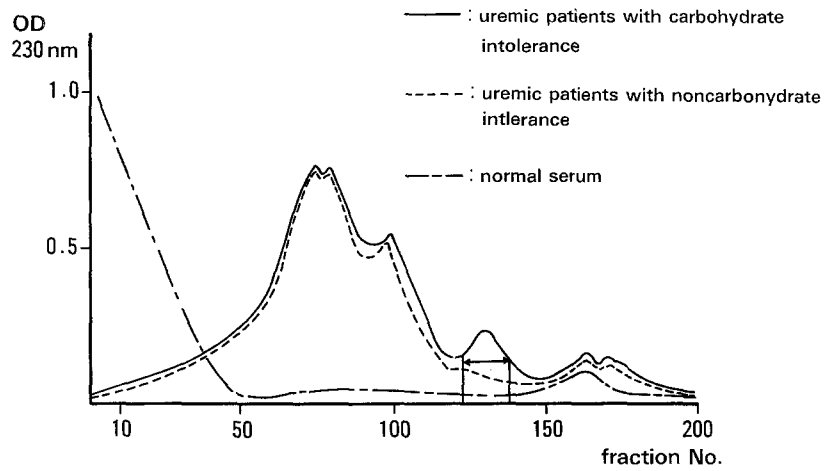

Fig. 2. Elution pattern on Sephadex G-50

3. Sephadex G-25 gel-filtration

The crude material (60 mg) from the step 2 was dissolved in a small amount of 1% AcOH and applied to a column of Sephadex G-25 (97 × 2.8 cm), then eluted with the same solvent. Fractions of 4 ml were collected at a flow rate of 4 ml/12 min and the absorbancy at 230 nm was determined (Fig. 3). Fractions of the main peak (tube Nos. 45–55) were combined and the solvent was removed by lyophilization. Yield 31 mg.

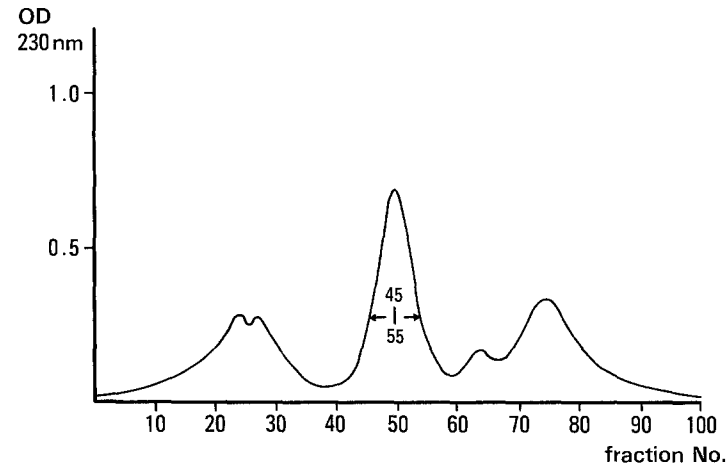

Fig. 3. Elution pattern on Sephadex G-25

4. Droplet countercurrent chromatography

The powder (28 mg) from the previous step was dissolved in 800 μl of the lower layer of the BuOH-AcOH-H_2O (4:1:5) and applied to a droplet countercurrent chromatography (Tokyo Rikakikai Co., S type, 75 transfer tubes, stationary

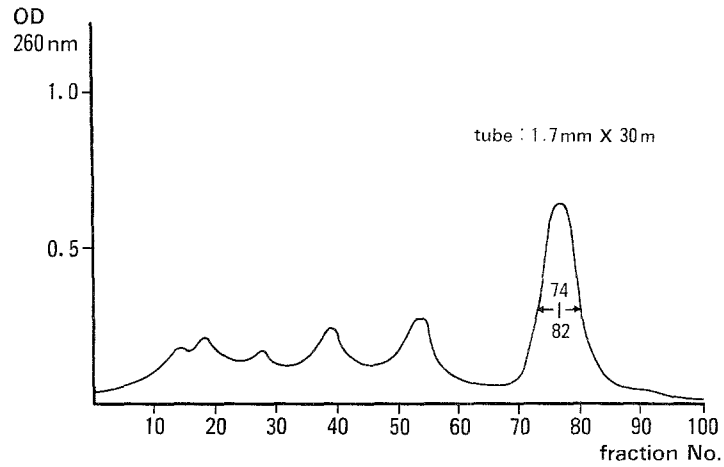

Fig. 4. Elution pattern on droplet countercurrent chromatogra chromatography

phase: upper layer), then eluted with the moving phase (lower layer). Fractions of 3 ml were collected at a flow rate of 3 ml/32 min, and the absorbancy at 260 nm was determined (Fig. 4). Fractions of the main single peak (tube Nos. 74-82) were combined, evaporated to dryness, and lyophilized. Yield 9 mg. Rf^1 0.20 (main), 0.31 (slight amount) and Rf^2 0.34 (main), 0.41 (slight amount), ninhydrin- and chlorine-tolidine-positive spots.

5. HPLC

1.5 mg sample from the previous step was further purified by HPLC on a 4 mm × 30 cm μBondapak/C18 column using solvent MeCN (25–45%, 30 min)

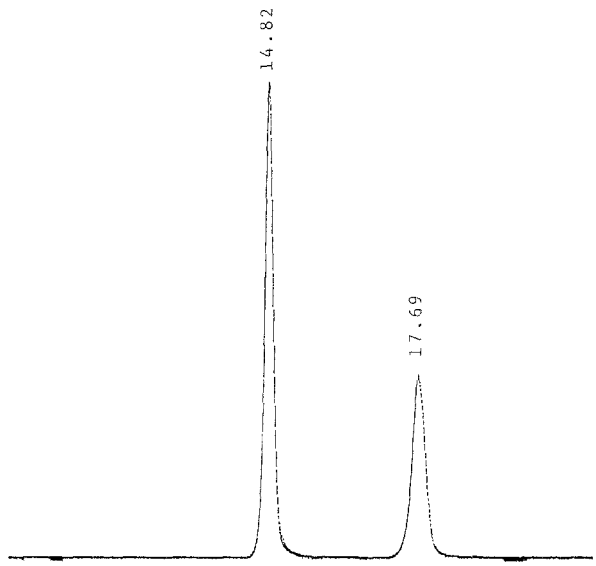

Fig. 5. HPLC profile of the main fraction obtained from droplet countercurrent chromatography

in 0.1% aqueous TFA at the flow rate of 1.0 min/min (Fig. 5). The eluate corresponding to the first main peak (retention time 14.82 min, detected by ultraviolet absorption measurement at 260 nm) was collected and the solvent was removed by lyophilization to give a fluffy powder. The rest of the sample was similarly purified. Yield 2.1 mg. Rf^1 0.20, Rf^2 0.34, single ninhydrin- and chlorine-tolidine-positive spot. $[\alpha]_D^{21} - 75.4°$ ($c = 0.2$, 1% AcOH). Amino acid composition in the acid hydrolysate: Leu 1.00, Val 1.03, Gly 2.03, Phe 0.96, Tyr 0.92, Met 0.93, Ser 1.89, Thr 2.94, Pro 0.95, Glu 1.95, Lys 1.02 (average recovery 86%); amino acid composition in the AP-M digest: Leu 1.00, Val 1.02, Gly 1.98, Phe 1.04, Tyr 0.97, Met 0.94, Ser 1.92, Gln + Thr 3.91 (calculated Thr + Gln as Thr since Gln emerged at the same position as Thr and was calculated as Thr), Pro 0.94, Glu 1.02, Lys 1.01 (average recovery 84%). The peptide exhibited a single peak on HPLC using a VYDAC 218 TP 54/C18 column (4.6 × 250 mm) at a tetention time of 24.339 min (Fig. 6) when eluted with a gradient of MeCN (5 → 30%) in 0.1% TFA at a flow rate of 0.8 ml/min. FAB-MS m/z 1746, Fig. 7).

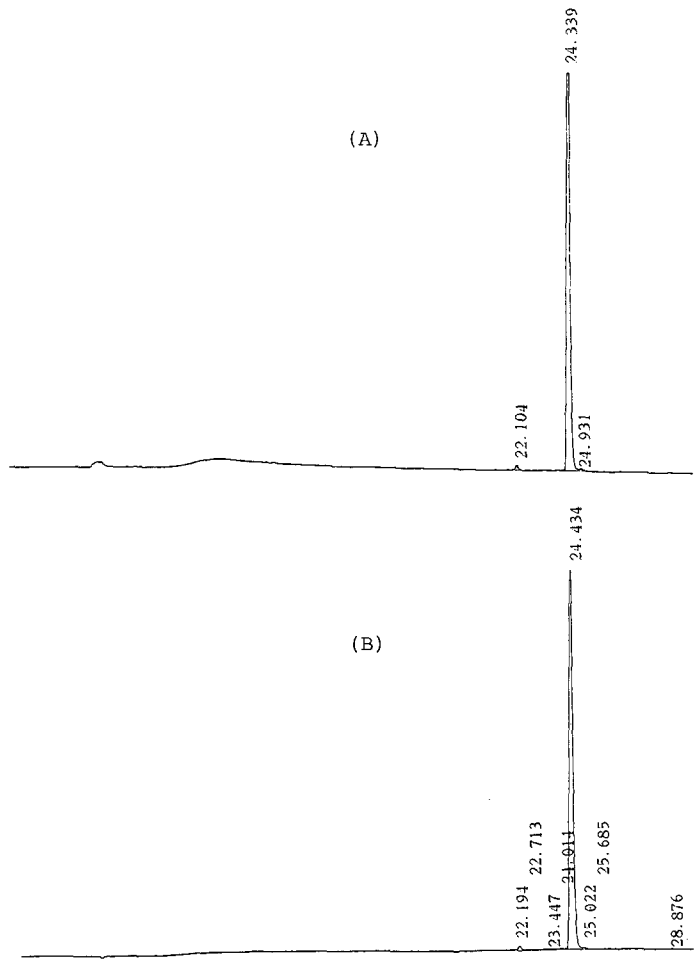

Fig. 6. Results of analytical HPLC of the purified hexadecapeptide **(A)** isolated from uremic filtrate and the synthetic α-endorphin **(B)**

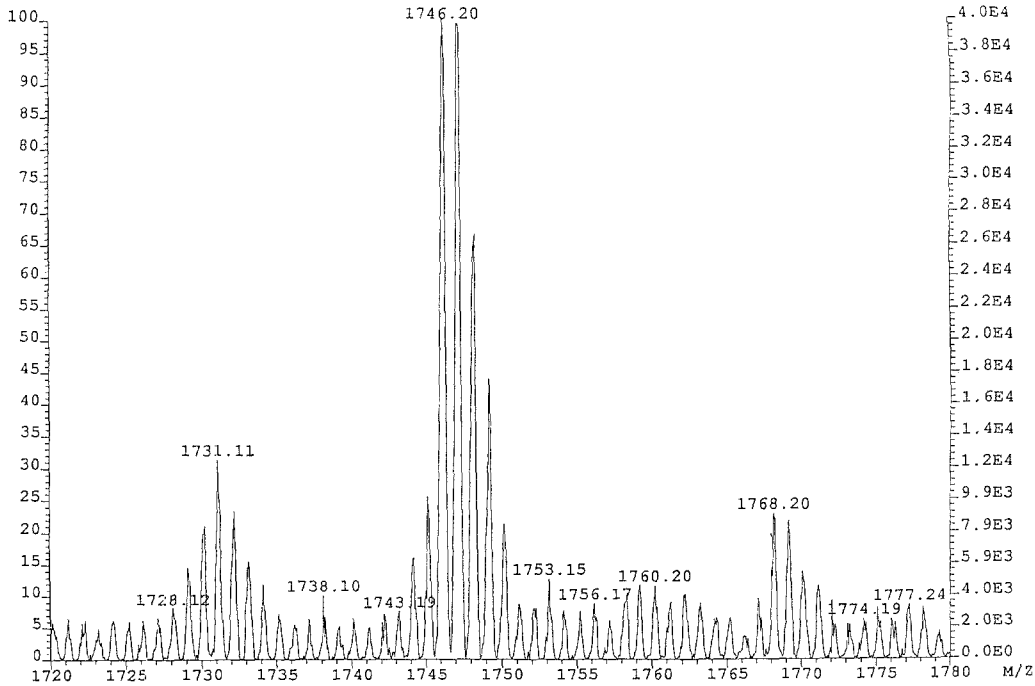

Fig. 7. FAB-MS of the purified hexadecapeptide isolated from uremic filtrate

Edman sequence analysis

The peptide was subjected to amino acid sequence analysis by automated Edman degradation with gas-phase sequencer (Applied Biosystems 477A) coupled with a PTH-amino acid analyzer (Applied Biosystems 120 A). From the results of the amino acid analyses and the amino acid sequence analysis, the structure of the peptide was speculated as follows: H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-OH corresponding to α-endorphin.

Solid phase synthesis of the hexadecapeptide corresponding to α-endorphin

The hexadecapeptide was synthesized as an authentic specimen to confirm the identification of the isolated peptide. Solid phase peptide synthesis of α-endorphin was carried out manually in a glass vessel by a stepwise strategy starting with Boc-Thr(Bzl)-resin (0.12 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) prewashings with 10% Et_3N in CH_2Cl_2; (vi) neutralization 10 min with 10% Et_3N in CH_2Cl_2; (vii) three washings with CH_2Cl_2; (viii) addition of 4 eq of Boc-amino acid 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. In the synthetic cycle that involves Gln residue, 4.5 eq of HOBT was added in step viii in order to minimize the side reaction (Barany and Merrifield, 1980). Coupling efficiency was monitored after every cycle by the

ninhydrin test (Kaiser et al., 1970). Double couplings were done when necessary as judged by this test and then acetylations with Ac_2O-pyridine were done. The hexadecapeptide-resin was then cleaved with anhydrous HF (10 ml) at 0°C for 60 min in the presence of anisole (2 ml) and thioanisole (0.5 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 resulting powder was dissolved in H_2O (5 ml) and was adjusted to pH 8.0 with 1 N NH_4OH and stirred in an ice-bath for 30 min to reverse a possible N → O shift at the Ser and Thr residues as usual (Sakakibara, 1971). The pH of the solution was adjusted to pH 6.5 with a few drops of AcOH. The solution, after addition of dithiothreitol (20 mg), was incubated at 60°C under N_2 gas for 36 h to reduce back the Met(O) into Met. The solvent was evaporated off in vacuo and the residue was dissolved in a small amount of 1% AcOH and then applied to a column of Sephadex G-25 (2.8 × 96 cm), which was eluted with the same solvent. Individual fractions (5 ml each) were collected and the absorbancy at 260 nm was determined for each fraction. The fractions corresponding to the front main peak (tube Nos. 48–56) were combined and the solvent was evaporated off and the residue was further purified by HPLC as described for the purification of the hexadecapeptide isolated from filtrate of uremic patients with carbohydrate intolerance. Yield 36 mg (18% based on the Thr loaded on the resin). Rf^1 0.20, Rf^2 0.34, single ninhydrin- and chlorine-tolidine-positive spot. $[\alpha]_D^{21}$ − 76.2° (c = 1.0, 1% AcOH). Amino acid composition in the acid hydrolysate: Leu 1.00, Val 0.96, Gly 2.03, Phe 1.02, Tyr 0.95, Met 0.92, Ser 1.87, Thr 2.91, Pro 0.91, Glu 2.01, Lys 0.98 (average recovery 84%); amino acid composition in the AP-M digest: Leu 1.00, Val 1.03, Gly 2.01, Gln + Thr 3.90 (calculated Thr + Gln as Thr since Gln emerged at the same position as Thr and was calculated as Thr), Pro 0.92, Glu 0.94, Lys 1.03 (average recovery 85%). The peptide exhibited a single peak on HPLC using a VYDAC 218 TP 54/C18 column (4.6 × 250 mm) at a retention time of 24.434 min (Fig. 6) when eluted with a gradient of MeCN (5 → 30%) in 0.1% TFA at a flow rate of 0.8 ml/min. FAB-MS m/z 1746.

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