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ISOLATION, STRUCTURE AND BIOLOGICAL ACTIVITY OF THE Trp-CONTAINING PENTAPEPTIDE FROM UREMIC FLUID*

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SUMMARY

Trp-containing pentapeptide was isolated from uremic fluid of an uremic patient by ultrafiltration with Amicon membranes followed by gel filtrations. The peptide thus obtained was identified as H-Asp-Leu-Trp-Gln-Lys-OH by amino acid analysis, manual Edman degradation method, physical constants and analytical data of synthetic pentapeptide. Structural similarity was soon realized between this peptide and pentapeptide moiety corresponding to position 123 through 127 of β-chain of fibrinogen. E-rosettes inhibition test was shown this pentapeptide to have an inhibition activity by amount more than 1.0mg/ml.

INTRODUCTION

It was well known that MMS of a molecular weight of 300-5000 daltons are important factor in creating several symptoms on uremic syndrome (1,2). There is a rise in MMS level in uremic patients (3) and effective dialysis bring about a decrease in MMS level (3). On the other hand, it also known that cellular immunity is suppressed in patients with renal failure, although there is no certain information so far as to the nature of the factors responsible (4,5). The uremic toxic state is not well understood in regard to MMS. Fibrin-fibrinogen degradation products have also been found in the serum of many patients with renal disease (6). However, these

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* The abbreviations used to denote amino acid derivatives and peptides are those recommended by IUPAC-IUB Commission on Biochemical Nomenclature: (1972) Biochem. Biophys. Acta, 263, 205. Other abbreviations: MMS= middle molecular substance, E= sheep erythrocyte, FCS= fetal calf serum, GVB^{++}= gelatin veronal buffer, PBS= phosphate buffer ed saline, DMF= dimethylformamide, Et_3N= triethylamine, TFA= trifluoroacetic acid, HONB= N-hydroxy-5-norbornene-2,3-dicarboximide, WSCI= water soluble carbodiimide, Tos= p-toluenesulfonic acid, ECUM= extracorporeal ultrafiltration method.

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substances have not been identified chemically, and biological roles and serum levels in uremic patients are quite unknown.

In this paper, a Trp-containing peptide have been isolated from uremic fluid of an uremic patient and its entire amino acid sequence was disclosed as the linear pentapeptide. We have identified the pentapeptide as H-Asp-Leu-Trp-Gln-Lys-OH by amino acid composition in the methanesulfonic acid hydrolysate (7) and characteristics of its PTH-derivatives. Residue 4 is probably glutamine rather than glutamic acid in view of the Rf value of the PTH-derivative and the amino acid composition of AP-M digest (8). Structural similarity was soon realized between this peptide and the pentapeptide fragment corresponding to position 123 through 127 of β-chain of fibrinogen. The pentapeptide obtained from uremic fluid was found to be homogeneous to synthetic peptide by paper chromatography, paper electrophoresis and optical rotation. When E-rosettes inhibition test of normal lymphocytes, this peptide exhibited the inhibition activity by amount more than 1.0mg/ml. After incubation with amounts of pentapeptide varying from 0.1mg/ml to 2.0mg/ml of cell suspension, E-rosettes ranged from 76% to 49%.

PATIENT SELECTION

An uremic patient with following measurement values was selected for study: BUN 98mg/dl, creatinine 11mg/dl and a 24 hr creatinine clearance of 5.6ml/min with a urinary output of 600 ml.

EXPERIMENTAL

Melting points are uncorrected. Rotation was determined with a JEOL JLC-8AH amino acid analyzer. Evaporations were carried out in a rotary evaporator under reduce pressure at a temperature of 35°. The purity of the separated products were by paper chromatography using Toyo Roshi No. 51, at room temperature. Rf^1 values refer to Partridge system (9) and Rf^2 values refer to the system of BuOH-pyridine-AcOH-H_2O (30:20:6:24) (10). Peroxide free Et_2O used for the treatment of Trp-containing peptides were stored over Al_2O_3 powder. Boc-group of the protected peptides were deblocked with TFA and the resulting amino components were chromatopraphed on filter paper, Toyo Roshi No. 51, at room temperature.

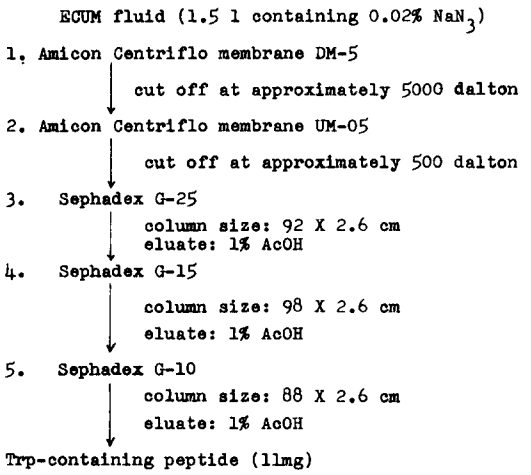

Chart 1 Purification of Trp-containing pentapeptide from ECUM fluid

SEPARATION PROCEDURE

The brief details of the separation of ECUM fluid was summerized in Chart 1.

1 Ultrafiltration

ECUM fluid (1.5l) was ultrafiltered using an Amicon Centriflo membrane DM-5 which has a molecular cut off at approximately 5000 dalton.

2 Ultrafiltration

The filtrate of 1 was ultrafiltered using an Amicon Centriflo membrane UM-05 which has a molecular cut off at approximately 500 dalton.

3 Sephadex G-25 gel filtration

4ml of the concentrated fluid of 2 was fractionated on a column (92 X 2.6 cm) of Sephadex G-25 with 1% AcOH at a flow rate of 1.3ml/min. 4ml of each fractions were collected and thier absorptions at 230 nm was measured (Fig.1). The each fractions were located by Ehrlich reaction. The eluates in tubes No. 128 to 160 containing Trp-containing peptide were pooled (Fraction I), evaporated to dryness in vacuum, and lyophilized (Fig. 2). The Fraction I is presented in ECUM fluid of an uremic patient but not detected in normal serum (Fig. 1). Yield 875mg.

4 Sephadex G-15 gel filtration

The crude material (800mg) in 1% AcOH (5ml) was added to a Sephadex G-15 column (98 X 2.6 cm) was eluted with 1% AcOH. Fractions of 4ml each were collected at a flow rate of 1.0ml/min and absorbance

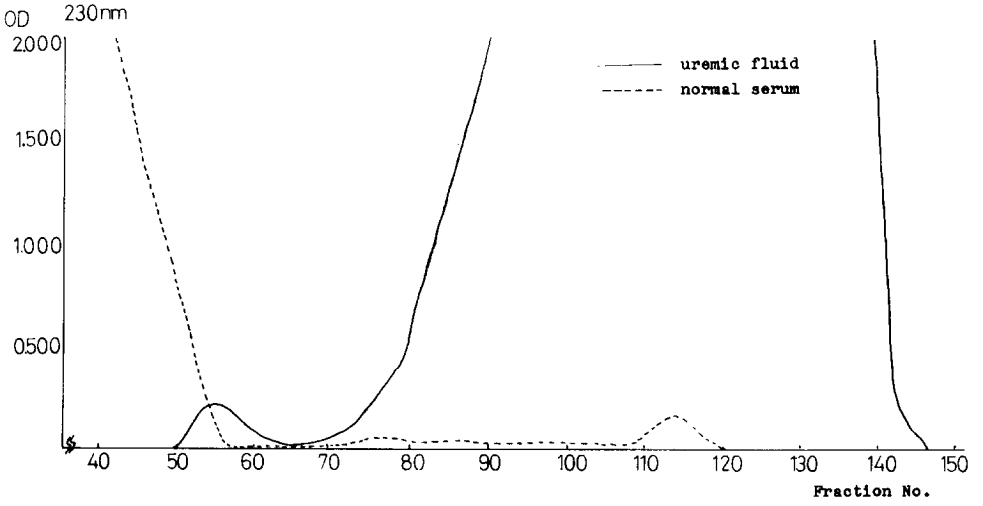

Fig. 1 Elution pattern of Sephaex G-25 fine

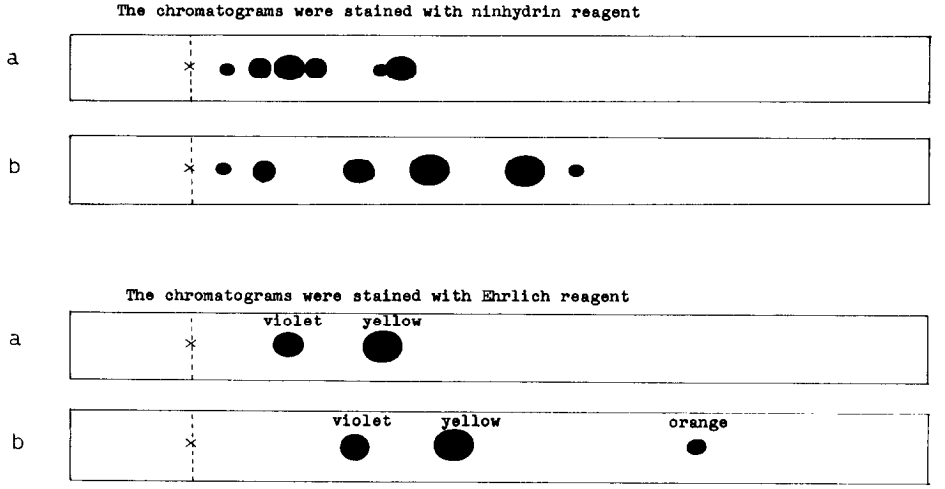

Fig. 2 Paper chromatograms of Fraction I; Toyo Roshi No. 51,
Solvent: a= Partridge system
b= Waley system

of each fraction was determined at 280 and 230 nm (Fig. 3). The each fractions were located by Ehrlich reaction. The eluates in tubes No. 86 to 124 containing peptide were pooled (Fraction II) (Fig. 4). Yield 200mg.

5 Sephadex G-10 gel filtration

The 1% acetate solution(4ml) of Fraction II (180mg) was added to Sephadex G-10 column (88 X 2.6 cm) which was eluted with 1% AcOH.

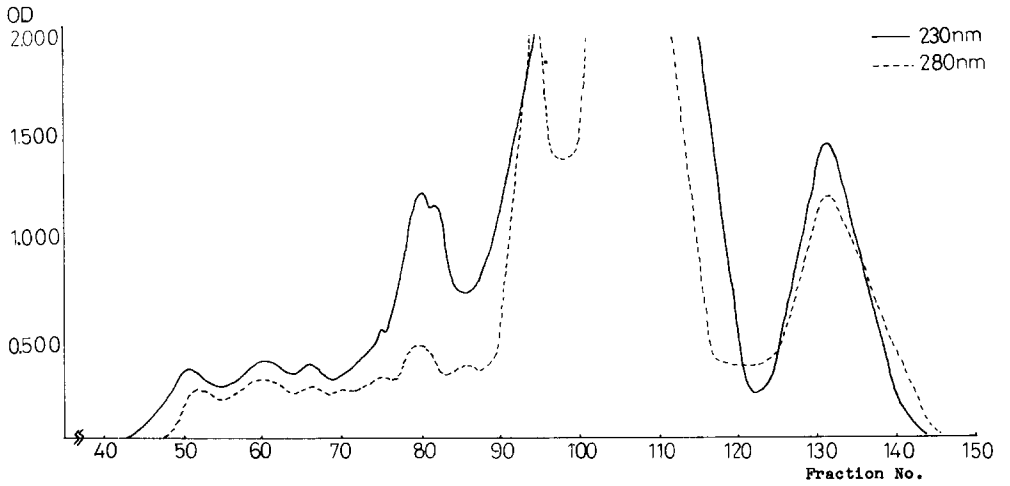

Fig. 3 Elution pattern of Sephadex G-15 fine

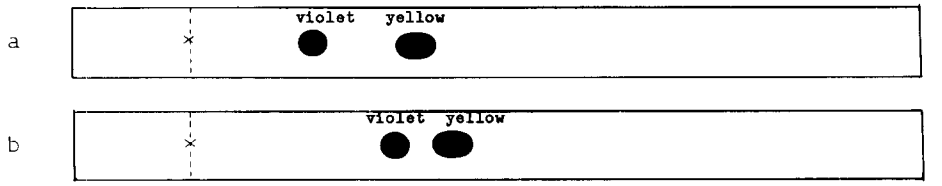

Fig. 4 Paper chromatograms of Fraction II; Toyo Roshi No. 51,
Solvent: a= Partridge system
b= Waley system

Fractions of 4ml each were collected at a flow rate of 0.5ml/min and the absorbancy of each fraction was determined at 280 nm. The each fraction was also located by Ehrlich reaction. The eluates in tubes No. 80 to 88 containing Trp-containing peptide were pooled (Fig. 5), evaporated to dryness in vacuum, and lyophilized. Yield 11mg, mp 136-143°, $(\alpha)_D^{29}$ - 47.7° (c= 0.3, H_2O), Rf^1 0.11, Rf^2 0.12, single ninhydrin and Ehrlich positive spot. Amino acid ratios in the acid hydrolysate by 4N methanesulfonic acid containing 0.02% 3-(2-aminoethyl)indole (7): Leu 1.01, Trp 0.91, Asp 0.96, Glu 0.90, Lys 0.94. Amino acid ratios in the AP-M digest (8): Leu 0.98, Trp 1.01, Asp 0.98, Gln 0.91, Lys 0.99.

Edman sequence analysis

The pentapeptide was sequenced by manual Edman degradation procedure as described previous paper (11).
The results of this degradation study indicated the following sequence: H-Asp-Leu-Trp-Gln-Lys-OH.

Synthesis of H-Asp-Leu-Trp-Gln-Lys-OH

The pentapeptide was synthesized as authentic specimen for identification of the isolated peptide (Fig. 6).

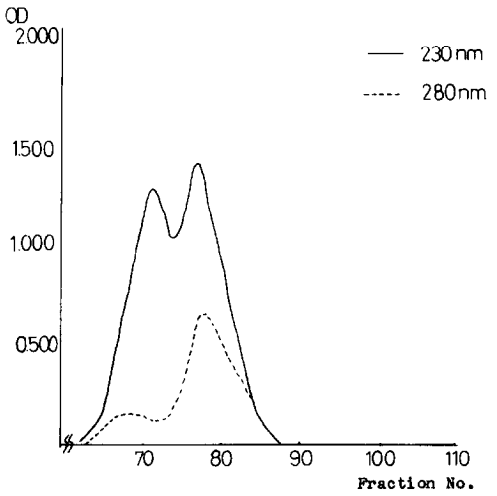

Fig. 5 Elution pattern of Sephadex G-10 fine

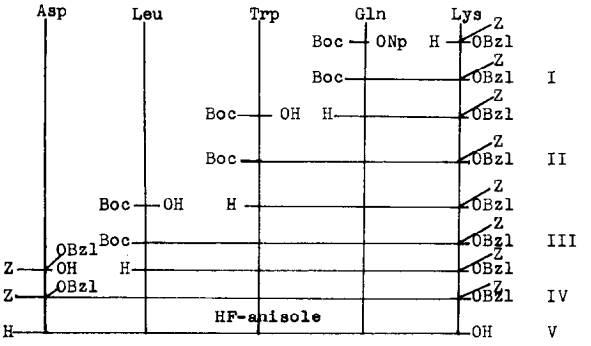

Fig. 6 Synthetic scheme of the pentapeptide

Boc-Gln-Lys(Z)-OBzl (I)---- To a solution of H-Lys(Z)-OBzl Tos (2.2g) in DMF (20.0ml), Boc-Gln-ONp (1.9g) was added, followed by Et_3N to keep the solution slightly alkaline. After 24 hr at room temperature, the reaction mixture was diluted with 1N NH_4OH (3.0ml) with stirring. After 1 hr, the mixture was extracted with EtOAc and the solution was washed successively with 1N NH_4OH, H_2O, 1N citric acid and H_2O. The solution was dried over $MgSO_4$ and concentrated to small volume and petroleum ether was added to the residue. The precipitate was reprecipitated from EtOAc and petroleum ether. Yield 1.9g (79%), mp 123-124°, $(\alpha)_D^{24}$ - 15.1° (c= 1.0, DMF), Anal. Calcd. for $C_{31}H_{42}N_4O_8$: C, 62.19; H, 7.07; N, 9.36. Found: C, 61.82; H, 6.96; N, 9.27. Rf^1 0.56, Rf^2 0.64, single ninhydrin positive spot.

Boc-Trp-Gln-Lys(Z)-OBzl (II)---- I (1.2g) was dissolved in TFA (4.0ml) and the solution was kept at room temperature for 30 min. The mixture was evaporated in vacuum and dried over KOH pellets in vacuum.

To an ice-cold solution of the resulting dipeptide ester, Boc-Trp-OH (0.669g) and Et_3N (0.3ml) in DMF (12.0ml) were added HONB (12) (0.394g) and WSCI (0.341g) with stirring and the mixture was stirred at 0° for 18 hr. The mixture was diluted with EtOAc and washed successively with 1N $NaHCO_3$, H_2O, 1N citric acid and H_2O and dried over $MgSO_4$. Then the solution was evaporated to dryness. The residue was precipitated from EtOAc and Et_2O. Yield 0.9g (56%), mp 65-71°, $(\alpha)_D^{24}$ - 12.3° (c= 1.0, DMF), Anal. Calcd. for $C_{42}H_{52}N_6O_9$: C, 64.27; H, 6.68; N, 10.71. Found: C, 63.89; H, 6.87; N, 10.45. Rf^1 0.64, Rf^2 0.78, single ninhydrin and Ehrlich positive spot.

Boc-Leu-Trp-Gln-Lys(Z)-OBzl (III)---- The compound was prepared from II (329mg), Boc-Leu-OH (132mg), HONB (100mg) and WSCI (86mg) essentially in the same manner as described in the preparation of II. The product was precipitated from EtOAc and Et_2O. Yield 291mg (74%), mp 101-106°, $(\alpha)_D^{23}$ - 21.0° (c= 1.0, DMF), Anal. Calcd. for $C_{40}H_{57}N_7O_8 \cdot H_2O$: C, 61.44; H, 7.61; N, 12.54. Found: C, 61.51; H, 8.01; N, 12.38. Rf^1 0.77, Rf^2 0.88, single ninhydrin and Ehrlich positive spot.

Z-Asp(OBzl)-Leu-Trp-Gln-Lys(Z)-OBzl (IV)---- The compound was prepared from III (255mg), Z-Asp(OBzl)-OH (131mg), HONB (66mg) and WSCI (57mg) essentially in the same manner as described in the preparation of II. The product was precipitated from EtOAc and Et_2O. Yield 210mg (55%), mp 126-131°, $(\alpha)_D^{23}$ - 33.1° (c= 1.0, DMF), Anal. Calcd. for $C_{62}H_{72}N_8O_{13}$: C, 65.48; H, 6.40; N, 9.85. Found: C, 65.66; H, 6.82; N, 9.46.

H-Asp-Leu-Trp-Gln-Lys-OH (V)---- IV (100mg) was placed in an HF-reaction cylinder together with anisole (0.5ml). HF (5ml) was added into the cylinder using an HF-reaction apparatus and the mixture was allowed to reacted at 0° for 30 min. The residue was diluted with H_2O (10ml) and the solution was washed with EtOAc three times. The aqueous solution thus obtained was passed through a column of Amberlite IRA-410 (acetate form, 2.6 X 10 cm). The column was washed with H_2O and Ehrlich positive eluates were collected. The solution was lyophilized. The solution of crude product in 1% AcOH (4ml) was added to a Sephadex G-10 column (2.6 X 84 cm) which was eluted with 1% AcOH. Fractions of 5ml each were collected at a flow rate of 2ml/min with an automatic fraction collector and the absorbancy of each fraction was determined at 280 nm. The eluates in tubes No. 79 to 85 containing the pentapeptide were pooled, evaporated to dryness in vacuum and lyophilized. Yield 34mg (60%), mp 132-142°, $(\alpha)_D^{23}$ - 45.1° (c= 1.0, H_2O), Rf^1 0.10, Rf^2 0.12, single ninhydrin and Ehrlich positive spot. Amino acid ratios in the acid hydrilysate by 4N methanesulfonic acid containing 0.02% 3-(2-aminoethyl)indole: Leu 0.98, Trp 0.89, Asp 1.02, Glu 0.96, Lys 0.88. Amino acid ratios in the AP-M digest: Leu 1.00, Trp 0.89, Asp 0.98, Gln 0.87, Lys 0.95.

High voltage paper electrophoresis

The pentapeptide obtained by gel filtration on Sephadex G-10 was subjected to paper electrophoresis. The electrogram of pentapeptide was compared with that of synthetic pentapeptide (Fig. 7). The isolated peptide and the synthetic peptide had identical movement values.

E-rosettes formation inhibition activity

Peripheral blood lymphocytes were isolated in a Hypaque-Ficoll gradient for T cell rosettes formation. Isolated lymphocytes were adjusted to a concentration of 5 X 10^5/ml with PBS. Contamination by

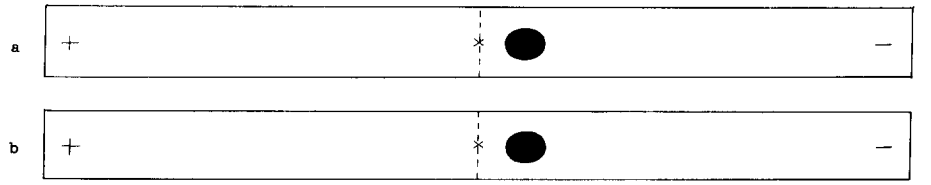

Fig. 7 Paper electrophoresis of pentapeptide (a) and synthetic pentapeptide (b).

Electrophoresis was carried out on Toyo Roshi No. 51 (2 X 40 cm) using acetate buffer (PH 2.8), at a potential gradient of 60 V/cm for 90 min, ninhydrin and Ehrlich positive spot.

Table I Inhibition activity of E-rosettes formation by the pentapeptide

Dose= mg/ml	H-Gly-Gly-His-OH[a)] (%)	H-Asp-Leu-Trp-Gln-Lys-OH (%)
0	77	77
0.1	76	75
0.5	74	70
1.0	76	67
1.5	75	56
2.0	75	49

a) Control: the tripeptide was purchased from the Protein Research Foundation, Minoh, Osaka, Japan

monocytes and polymorphonuclear cells amounted less than 5%. Sheep erythrocytes were washed with PBS and a suspension (1 X 10^6/ml) was prepared . Lymphocytes were suspended in GVB^{++} or FCS (1.0ml) and incubated for 90min at 37° with the pentapeptide. Lymphocytes were washed with GVB^{++} and centrifuged for 10min at 1500 rpm, then suspended in GVB^{++} (1.0ml). The suspension was mixed with sheep erythrocytes (0.5ml) and then incubated for 18 hr at 4°. The mixture was centrifuged for 5min at 900 rpm. Triplicate wet-cell preparations were checked by phase contrast microscopy. For each preparation, 200 lymphocytes were counted, and the proportion binding three more sheep erythrocytes was determined. Monocytes or polymorphonuclear cells forming rosettes were excluded (Table I).

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