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IDENTIFICATION AND SYNTHESIS OF A TRIPEPTIDE IN ECUM FLUID OF AN UPEMIC PATIENT

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Summary: An unidentified ninhydrin and Pauly reaction positive substance of basic nature was found in the ECUM fluid of an uremic patient. This substance was isolated from ECUM fluid by the methods of ultrafiltration method and gel-filtration, and identified as H-His-Gly-Lys-OH by amino acid analysis, manual Edman degradation method and physical constants and analytical data of synthetic tripeptide.

INTRODUCTION

Several investigators (1-3) have confiremed the increased quantities of ninhydrin positive substances resembling polypeptides in the plasma of uremic patients. However, these substances have not been identified chemically, and their biological roles. Biochemical characterization of these substances might be important and valuable for elucidating the nature of uremic toxins.

This communication reports the identification and synthesis of H-His-Gly-Lys-OH in extracorporeal ultrafiltration method (ECUM) fluid of an uremic patient. The isolated substance had homogeneous material as described by J. C. Lote (4). The tripeptide has inhibitory effects on some neurones in the dorsal horn of the spinal cord (4).

MATERIAL AND METHODS

Patient selection

An uremic patient with following measurement values was selected

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for study: B. P. 165/115, B. U. N. 80mg/dl, creatinine 10.1mg/dl and a 24 hr creatinine clearance of 5.6ml/min with an urinary output of 600ml.

Separation and synthetic procedures

Melting points are uncorrected. Rotation was determined with an Atago Polax. Amino acid analysis was performed 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 and synthesized products were by paper chromatography using Toyo Roshi No. 51, at room temperature. Z-group of the protected peptide was deblocked with HBr in AcOH or catalytic hydrogenation in the presence of AcOH and Boc-group of the protected peptide was deblocked with trifluoroacetic acid (TFA) and the resulting amino components were chromatographed. Rf^1 values refer to Partridge system (5) and Rf^2 values refer to the system of BuOH-pyridine-AcOH-H_2O (30:20:6:24) (6). The brief details of the separation of ECUM fluid was summerized in Chart I.

1 Ultrafiltration

ECUM fluid (1500ml) was ultrafiltered using an Amicon Centriflo membrane DM-5 which has a molecular cut off at approximately 5000 dalton.

2 Sephadex G-25 gel filtration

4ml of the concentrated filtrate of 1 was fractioned on a column (92.0X2.6cm) of Sephadex G-25 with 1% AcOH at a flow rate of 1.3ml/min. 4ml of each samples were collected and their absorption at 230 nm was measured (Fig.1). The each fractions were located by ninhydrin and Pauly reaction. The eluates in tubes No. 91 to 134 were pooled, evaporated to dryness in vacuum, and lyophilized. Fraction I. Yield 400mg.

3 Sephadex G-15 gel filtration

The crude material (300mg) in 1% AcOH (4ml) was added to a Sephadex G-15 column (104.0X2.6cm) was eluted with 1% AcOH. Fractions of 4ml

ECUM fluid (1500ml)

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1. Amicon Centriflo membrane DM-5

cut-off at approximately 5000 dalton

↓

2. Sephadex G-25

column size: 92.0X2.6 cm

elution: 1% AcOH

↓

3. Sephadex G-15

column size: 104.0X2.6 cm

elution: 1% AcOH

↓

His-Gly-Lys-OH

Chart 1. Purification of N-His-Gly-Lys-OH from ECUM fluid

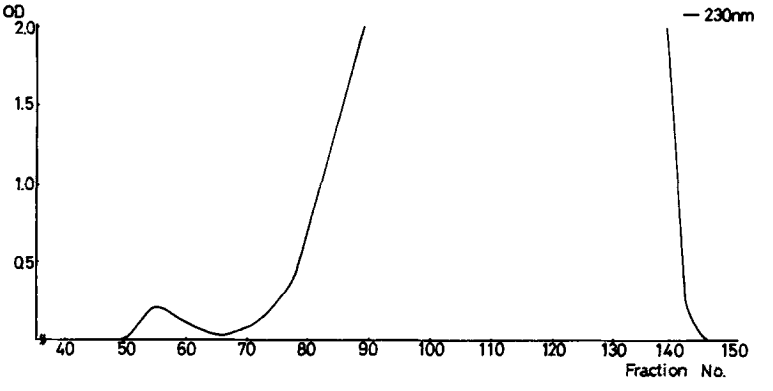

Fig.1. Elution pattern of Sephadex G-25 Fine.

each were collected at a flow rate of 1.0ml/min and absorbance of each fraction was determined at 230 nm (Fig. 3). The each fractions were located by ninhydrin and Pauly reaction. The eluates in tubes No. 130

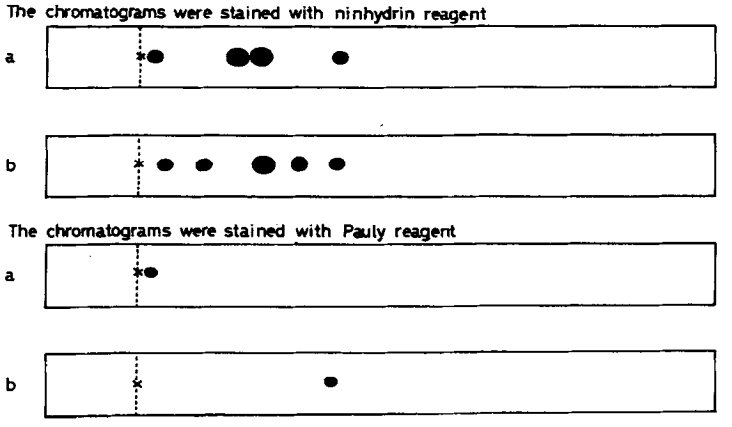

Fig. 2. Paper chromatogram of Fraction I: Toyo Roshi No. 51, Solvent: a=Partridge system, b=Waley system.

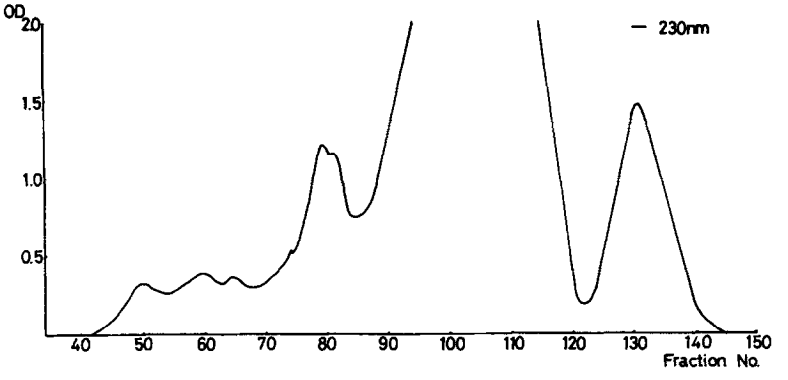

Fig. 3. Elution patten of Sephadex G-15 Fine.

to 140 were pooled, evaporated to dryness in vacuum, and lyophilized. Fraction II. Yield 16mg, $[\alpha]_D^{21}$ +5.6° (c=0.3, H_2O), Rf^1 0.01, Rf^2 0.15, single ninhydrin and Pauly positive spot. Amino acid ratios in the AP-M digest (7); His 0.89, Gly 0.90, Lys 0.91.

4 Edman sequence analysis

For the sequence analysis of Fraction II manual Edman procedure (8) was used. The peptide sample (0.5μmoles) was dissolved in 0.2ml of pyridine-water (3:2 V/V) containing dimethylallylamine and adjusted to

PH 9.5 with TFA. After addition of 10 μl phenylisothiocyanate, the tube was flushed with a gentle stream of N_2 for 5-10 sec, stoppered and left at 40° for 1 hr. The solution was then extracted 3 times with 1.2 ml of benzene. The aqueous phase was subsequently freeze-dried and remaining reagents removed by sublimation in vacuum at 50° for 15 min with solid CO_2-ethanol as cold trap. Cleavage was performed in 10 μl of TFA for 20 min at 40°. The residual peptide was precipitated with 0.6 ml of CH_2Cl_2 and washed with 0.6 ml of CH_2Cl_2. The precipitate was dried over night in vacuum over P_2O_5 and KOH and used for the next degradation cycle. The intermadiate thiazolinone contained in the CH_2Cl_2 phase was without delay brought to dryness and converted to the corresponding thiohydantoin by treatment with 0.3ml of 1N HCl at 80° for 10min. The thiohydantoin derivative was extracted two times with 0.5ml of EtOAc. The extract was brought to dryness and dissolved in EtOAc or 90% AcOH. The amino acid phenylthiohydantoins contained in the organic phase were routinely identified by thin-layer chromatography in silica gel (containing 1% starch, 0.1% EDTA and fluorescence indicator) in CH_3Cl:MeOH (90:10) solvent system.

The results of this degradation study indicated the following sequence: H-His-Gly-Lys-OH.

Synthesis of H-His-Gly-Lys-OH

H-His-Gly-Lys-OH was synthesized as authentic specimen for identification of the isolated peptide (Fig. 4).

Boc-Gly-Lys(Z)-OBzl (I)——— To a solution of H-Lys(Z)-OBzl p-toluenesulfonate (1.100g) (9) and Boc-Gly-OH (0.380g) (10) in dimethylformamide (DMF) (10.00ml) were added Et_3N (0.28ml), N-hydroxy-5-norbornene-2,3-dicarboximide (HONB) (0.394g) (11) and dicyclohexylcarbodiimide (DCC) (0.453g) at 0°. The mixture was stirred at 0° for 20 hr and then filtered to remove the formed dicyclohexyl urea (DCU). The filtrate was diluted with EtOAc, washed with 1N $NaHCO_3$, H_2O, 1N citric acid and H_2O, dried over

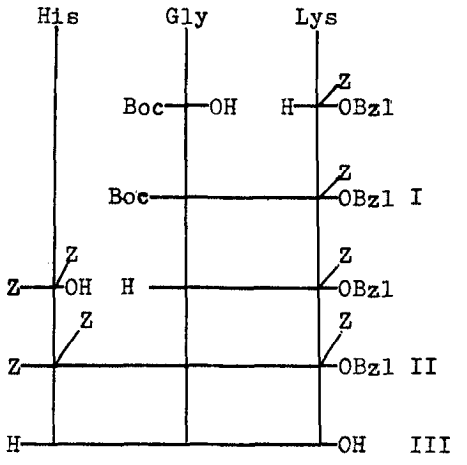

Fig. 4 Synthetic route for H-His-Gly-Lys-OH.
Abbreviations: Boc= t-butoxycarbonyl, Z= benzyloxycarbonyl, OBzl= benzyloxy.

anhydr. $MgSO_4$ and then the solvent was evaporated. The residue was precipitated from EtOAc and petroleum ether. Yield 0.900g (81.8%), mp 71-77°, $[\alpha]_D^{21}$ + 8.9° (c= 0.40, DMF), Anal. Calcd. for $C_{27}H_{37}O_5N_3$: C, 67.06; H, 7.71; N, 8.69. Found: C, 66.65; H, 8.08; N, 8.46. Rf^1 0.79 Rf^2 0.91, single ninhydrin positive spot.

Z-His(Z)-Gly-Lys(Z)-OBzl (II)——— I (1.000g) was treated with TFA (6.0ml) and the free base obtained was dissolved in DMF (10.0ml) together with Et_3N (0.3ml). To this solution were added Z-His(Z)-OH (0.838g) (12), HONB (0.374g) and DCC (0.429g) with stirring. After the same manner as described in the preparation of I the product was crystallized from EtOAc and Et_2O. Yield 0.770g (51.0%), mp 86-90°, $[\alpha]_D^{23}$ - 8.2° (c= 1.0, DMF), Anal. Calcd. for $C_{45}H_{48}O_{10}N_6$: C, 64.49; H, 5.81; N, 10.09. Found: C, 64.92; H, 6.30; N, 10.02. Rf^1 0.11, Rf^2 0.45, single ninhydrin positive spot.

H-His-Gly-Lys-OH (III)——— II (300mg) was hydrogenated in 50% AcOH (15ml) over 10% Pd-C for 18 hr. The catalyst was removed by the aid of Cellite. The solution was evaporated to dryness and the residue was dried over KOH pellets in vacuum. The solution of the product in H_2O (6ml) was added

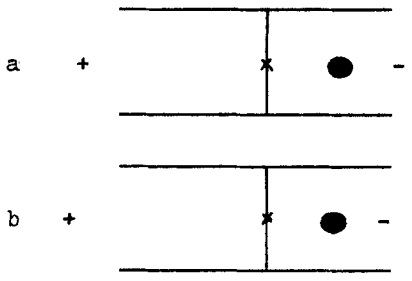

Fig. 5 Paper electrophoresis of Fraction II (a) and synthetic tripeptide (b). Electrophoresis was carried out on Toyo Roshi No. 51 (2.0X 40.0 cm) using acetate buffer PH 2.8, at a potential gradient of 60V/cm for 90 min. Ninhydrin and Pauly positive spot.

to a CM-cellulose column (1.6X12.0cm) which was eluted with a linear gradient elution from H_2O (300ml) in mixing chamber to 0.15M NH_4OAc buffer (PH 6.50, 300ml) in reservoir. Fractions of 5ml each were collected at a flow rate of 2ml/min with an the absorbancy of each fraction was determined at 230 nm. The eluates in tubes No. 51 to 64 containing the tripeptide were pooled, evaporated to dryness in vacuum and lyophilized. NH_4OAc was removed by repeated lyophilization to constant weight. Yield 153mg (67.1%), $[\alpha]_D^{20}$ + 6.1° (c= 0.5, H_2O), amino acid ratios in the AP-M digest: His 0.89, Gly 0.92, Lys 0.93. Rf^1 0.01, Rf^2 0.14, single ninhydrin and Pauly positive spot.

High voltage paper electrophoresis

The fraction II obtained by gel-filtration on Sephadex G-15 was subjected to paper eletrophoresis. The electrogram of Fraction II was compared with that of synthetic tripeptide (Fig.5). The isolated product and the synthetic peptide had identical movement values in the electrograms used (Fig. 5).

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