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INHIBITION EFFECT OF ROSETTE FORMATION BETWEEN HUMAN LYMPHOCYTES AND SHEEP ERYTHROCYTES BY SPECIFIC HEPTAPEPTIDE ISOLATED FROM UREMIC FLUID AND ITS ANALOGS*

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SUMMARY

One of middle molecular substances (H-His-Pro-Ala-Glu-Asn-Gly-Lys-OH) and its two analogs, in which the proline residue in position 2 was replaced by glycine and the alanine residue in position 3 was replaced by valine exert a inhibition effect on in vitro E-rosette formation. Its synthetic two analogs showed diminished biological activity compared to native heptapeptide.

INTRODUCTION

According to middle molecule hypothesis introduced by Babb et al (1), uremic solutes in the molecular range of 300-5000 daltons assumed to be toxic at least as far as uremic neuropathy is concerned. On the other hand, it is 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 peptides responsible (2,3,4).

After the primary structure of peptide isolated from uremic fluid was determined to be the heptapeptide, H-$\overset{1}{\text{His}}$-$\overset{2}{\text{Pro}}$-$\overset{3}{\text{Ala}}$-$\overset{4}{\text{Glu}}$-$\overset{5}{\text{Asn}}$-

* Symbols for amino acid derivatives and peptides used in this text are those recommended by IUPAC-IUB Commission on Biochemical Nomenclature: Biochem. J., 126, 773 (1972). Other abbreviations: WSCI= water soluble carbodiimide, DMF= dimethylformamide, TFA= trifluoroacetic acid, GVB^{++} = gelatin veronal buffer, FCS= fetal calf serum, E= sheep erythrocyte, HONB= N-hydroxy-5-norbornene-2,3-dicarbiximide, PBS= phosphate buffer saline.

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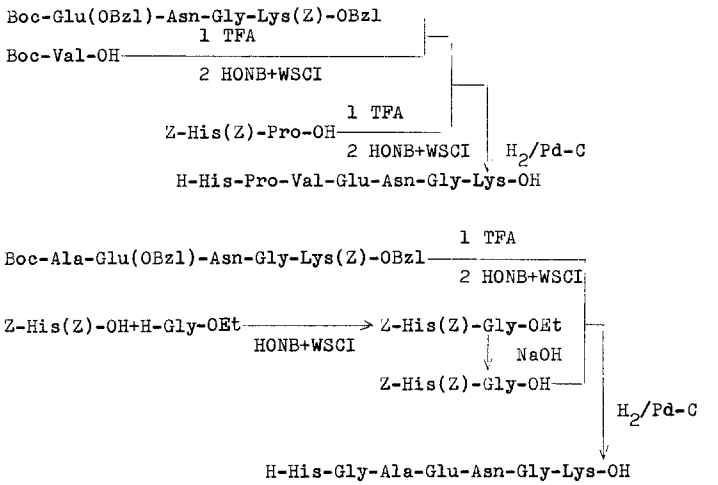

Fig. 1. Synthetic routes to (Val^3)- and (Gly^2)-analog.

^{6}Gly-^{7}Lys-OH (4), the biological property against E-rosette formation between human lymphocytes and sheep erythrocytes have been investigated by authors. Finally, E-rosette formation inhibiting activity on the heptapeptide and its two analogs was observed by authors. In the preceding paper (5), synthesis of the heptapeptide corresponding to positions 13 to 19 of human β_2-microglobulin was described.

In the present communication, the synthesis of two analogs of the heptapeptide fragment of the human β_2-microglobulin and the result of the E-rosette formation inhibiting activity of the synthetic peptides are described. The solution method for the peptide synthesis is used in this investigation. The synthetic routes for (Val^3)- and (Gly^2)-analog are illustrated in Fig. 1. (Val^3)-analog for purpose of the elucidation of the biological effect of alanine of position 3 of heptapeptide by substitution with valine which has a bulky and hydrophobic branched side chain, and which does not take the α-helix structure (6). The reason for study on (Gly^2)-analog is as follows: the difference on the structure feature between glucine

and proline in peptide is conformational flexibility of peptide bond. The biological activity of the two analogs is compared with that of heptapeptide on E-rosette formation inhibiting activity. Results of E-rosette formation inhibiting activities of these peptides are given in Table I. E-rosette formation inhibiting activity of its two analogs was lower than that of heptapeptide. E-rosette formation inhibiting activity of (Gly^2)-analog was fairly higher than that of (Val^3)-analog.

EXPERIMENTAL

Melting points were uncorrected. Optical rotations were measured on a Atago Polax. Unless otherwise mentioned, Z-group of the protected peptides were deblocked with catalytic hydroganation in the presence of 10% Pd-C and Boc group with TFA and the resulting peptides were chromatographed on the filter paper, Toyo Roshi No. 51, at room temperature. Rf^1 values refer to Partrdge system (7) and Rf^2 values refer to the system of nBuOH-pyridine-AcOH-H_2O (30:20:6:24) (8). The amino acid composition of the acid hydrolysates and aminopeptidase-M digest (9) were determined with JEOL JLC-8AH amino acid analyzer. Evaporations were carried out in a rotary evaporator under reduce pressure at a temperature of 35°.

Boc-Val-Glu(OBzl)-Asn-Gly-Lys(Z)-OBzl (I)---- Boc-Glu(OBzl)-Asn-Gly-Lys(Z)-OBzl (1.100g) (5) was dissolved in TFA (2.0ml) and the solution was allowed to stand at room temperature for 20min and evaporated. The resulting residue was triturated with dry ether to give a powder, which was collected by filtration and dissolved in DMF (10.0ml) together with Et_3N (0.2ml) and to this were added Boc-Val-OH (0.330g), HONB (0.248g) (10) and WSCI (0.224g) at - 10°. The mixture was stirred at 0° for 18 hr. The mixture was diluted with EtOAc, and the solution was washed successively with 1N $NaHCO_3$, H_2O, 1N citric acid and H_2O and dried over $MgSO_4$. Then the solvent was evaporated. The residue was crystallized from hot EtOAc. Yield 0.700g (64%), mp 80-87°, $(\alpha)_D^{26}$ - 28.9° (c= 1.0, DMF). Anal. Calcd. for $C_{49}H_{65}O_{13}N_7$: C, 61.30; H, 6.83; N, 10.21. Found: C, 60.94; H, 7.21; N, 10.64. Rf^1 0.74, Rf^2 0.86 single ninhydrin positive spot.

Table I Inhibition Activity of E-Rosette Formation by The Heptapeptide and Its Analogs

Dose= mg/ml	H-Gly-Gly-His-OH[a] (%)	H-His-Pro-Ala-Glu-Asn-Gly-Lys-OH (%)	(Val^3)-analog (%)	(Gly^2)-analog (%)
0	70.0	70.0	70.0	70.0
1	67.0	69.0	68.0	69.0
3	68.0	27.1	50.7	42.0
5	70.0	18.8	40.0	30.0

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

Z-His(Z)-Pro-Val-Glu(OBzl)-Asn-Gly-Lys(Z)-OBzl (II)---- Compound I (481mg) was treated with TFA (1.0ml) in a similar manner as described above. The resulting pentapeptide ester was dissolved in DMF (5.0ml) together with Z-His-Pro-OH (85mg) (5) in the presence of Et_3N (0.08ml). To the solution were added HONB (100mg) and WSCI (90mg) at - 10° and mixture was stirred at 0° for 18 hr. The mixture was diluted with EtOAc and washed successively with 1N $NaHCO_3$, H_2O, 1N HCl and H_2O and dried over $MgSO_4$. Then the solution was evaporated to dryness. The residue was recrystallized from EtOAc and ether. Yield 410mg (64%), mp 110-115°, $(\alpha)_D^{26}$ - 21.4° (c= 1.0, DMF). Anal. Calcd. for $C_{69}H_{82}O_{15}N_{11}$: C, 67.79; H, 6.76; N, 12.60. Found: C, 67.31; H, 7.21; N, 12.07.

H-His-Pro-Val-Glu-Asn-Gly-Lys-OH (III)---- The fully protected heptapeptide II (200mg) was hydrogenated in 50% AcOH (12ml) over 10% Pd-C for 16 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 crude product in 1% AcOH (1.0ml) was added to a Sephadex G-15 (fine grade) column (1.8 X 40.0cm) which was eluted with 1% AcOH. Fractions of 4ml each were collected at a flow rate of 1ml/min with an automatic fraction collector and the absorbancy of each fraction was determined at 230 nm. The eluates in tubes No. 12 to 19 containing the heptapeptide were pooled, evaporated to dryness in vacuum and lyophilized. Yield 100mg (87%), mp 170-181° (decomp), $(\alpha)_D^{26}$ - 49.5° (c= 1.0, H_2O). Rf^1 0.07, Rf^2 0.23 single ninhydrin and Pauly positive spot. Amino acid ratios in the acid hydrolysate: His 0.91, Pro 0.96, Val 0.99, Glu 0.89, Asp 0.92, Gly 1.01, Lys 0.82.

Z-His(Z)-Gly-OEt (IV)---- The compound was prepared from H-GlyOEt HCl (1.4g), Z-His(Z)-OH (4.6g), HONB (2.0g) and WSCI (1.8g) essentially in the same manner as described in the preparation of I. The product was precipitated from EtOAc and petroleum ether. Yield 2.5g (68%),

mp 52-56°, $(\alpha)_D^{26}$ - 30.0° (c= 1.0, DMF). Anal. Calcd. for $C_{18}H_{23}O_5N_4$: C, 57.59; H, 6.18; N, 14.93. Found: C, 57.09; H, 6.61; N, 15.02. Rf^1 0.36, Rf^2 0.46 single ninhydrin and Pauly positive spot.

Z-His(Z)-Gly-OH (V)---- The fully protected dipeptide ester IV (1.9g) was saponified in dioxane (15.0ml) with 1N NaOH (5.0ml) at room temperature for 1 hr. The solution was evaporated to small volume and diluted with H_2O (15.0ml) and washed with EtOAc 2 times. The aqueous layer was acidified to Congo red with 3N HCl at 0° and then saturated with NaCl. The oily product was extracted with EtOAc and the solution was washed with H_2O and dried over $MgSO_4$. Then the solvent was evaporated to dryness. The residue was reprecipitated from EtOAc and ether. Yield 1.0g (56%), mp 58-60°, $(\alpha)_D^{26}$ - 36.6° (c= 1.0, DMF). Anal. Calcd. for $C_{16}H_{19}O_5N_4$: C, 55.32; H, 5.51; N, 16.13. Found: C, 54.91; H, 5.89; N, 15.91. Rf^1 0.10, Rf^2 0.14 single ninhydrin and Pauly positive spot.

Z-His(Z)-Gly-Ala-Glu(OBzl)-Asn-Gly-Lys(Z)-OBzl (VI)---- The compound was prepared from Boc-Ala-Glu(OBzl)-Asn-Gly-Lys(Z)-OBzl (466mg) (5), V (191mg), HONB (100mg) and WSCI (154mg) essentially in the same manner as described in the preparation of II. The product was crystallized from EtOAc and ether. Yield 480mg (78%), mp 85-89°, $(\alpha)_D^{26}$ - 31.1° (c= 1.0, DMF). Anal. Calcd. for $C_{66}H_{75}O_{17}N_{11}$: C, 61.24; H, 5.84; N, 11.91. Found: C, 61.24; H, 5.49; N, 9.96.

H-His-Gly-Ala-Glu-Asn-Gly-Lys-OH (VII)---- VI (200mg) in 50% AcOH was hydrogenated in the usual manner for 15 hr. The hydrogenated product in 1% AcOH (1.0ml) was added to a Sephadex G-15 (fine grade) column (1.8 X 40.0 cm) which was eluted with 1% AcOH. Fractions of 4ml each were collected at a flow rate of 1ml/min with an automatic fraction collector and the absorbancy of each fraction was determined at 230 nm. The eluates in tubes No. 12 to 19 containing the heptapeptide were pooled, evaporated to dryness in vacuum and

lyophilized. Analysis by paper chromatography revealed the presence of two ninhydrin positive spots with Rf^1 0.14 (major), 0.10 (minor) and Rf^2 0.26 (major), 0.36 (minor). A solution of the crude product in H_2O (5.0ml) was added to a CM-Sephadex C-25 column (1.8 X 40.0 cm) which was eluted with a linear gradient elution from H_2O (100ml) in mixing chamber to 0.06 M NH_4OAc buffer (PH 6.5, 100ml) in reservoir. Fractions of 4ml each were collected at a flow rate of 0.5ml/min with an automatic fraction collector and the absorbancy of each fraction was determined at 230 nm. The eluates in tubes No. 19 to 24 containing the heptapeptide were pooled, evaporated to dryness in vacuum and lyophilized. Yield 70mg (74%), mp 165-178° (decomp), $(\alpha)_D^{26}$ - 35.8° (c= 1.0, H_2O). Rf^1 0.14, Rf^2 0.26 single ninhydrin and Pauly positive spot. Amino acid ratios in acid hydrolysate: His 0.99, Gly 1.89, Ala 1.00, Glu 0.81, Asp 0.91, Lys 0.93; amino acid ratios in the aminopeptidase-M digest: His 0.91, Gly 1.78, Ala 0.98, Glu 0.84, Asn 0.89, Lys 0.90.

E-ROSETTE FORMATION INHIBITION TEST

Normal peripheral blood lymphocytes were isolated by Hypaque-Ficoll gradient for T cell rosette formation. For T cell rosette formation, isolated lymphocytes were adjusted to a concentration of 5 X 10^5/ml with PBS. Contamination with monocytes and polymorphonuclear cell was less than 5% sheep erythrocytes were washed with PBS and suspension (1 X 10^6/ml) was prepared. Lymphocytes were suspended in GVB^{++} or FCS (1.0ml) and incubated for 30 min at 37° heptapeptide analogs at concentrations of 1, 3 and 5mg respectively. Lymphocytes were washed with GVB^{++} and centrifuged for 10 min at 1500 rpm and then suspended with GVB^{++} or FCS (1ml). The suspension was mixed with sheep erythrocytes (0.5ml) and then incubated for 18 hr at 4°. The mixture was centrifuged for 5 min at 900 rpm. Triplicate wet-cell preparations were under phase microscopy.

For each preparation, 200 lymphocytes were counted, and the proportion binding three more sheep erythrocytes was determined (normal, 70%). Monocyte or polymorphonuclear cells forming rosette were excluded (Table I).

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