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Effect of Synthetic Serum Thymic Factor Fragments on the Inhibition of Sheep Erythrocyte Rosette Formation by Uremic Toxin[1)]

TAKASHI ABIKO, IKUKO ONODERA, HIROSHI SEKINO,[2a)] and HIROKI HIGUCHI[2b)]

Kidney Center, Sendai Insurance Hospital,[2a)] and Kidney Research Laboratory, Kōjinkai[2b)]

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Three peptide fragments of STF, H–Ser–Gln–Gly–Gly–Ser–Asn–OH, H–Lys–Ser–Gln–Gly–Gly–Ser–Asn–OH and H–Ala–Lys–Ser–Gln–Gly–Gly–Ser–Asn–OH, were synthesized by the solution method and their biological activities were investigated. The minimum fragment of STF able to show antagonistic action towards the E-rosette formation inhibition activity of MMS appears to be H–Lys–Ser–Gln–Gly–Gly–Ser–Asn–OH.

Keywords——serum thymic factor; middle molecular substances; E-rosette formation; uremic toxin; lymphocytes

Bach *et al.*[3)] proposed the amino acid sequence (pGlu–Ala–Lys–Ser–Gln–Gly–Gly–Ser–Asn) for an STF isolated in their laboratory which showed high activity in the rosette test (used as a bioassay).[3)]

On the other hand, it is well known that MMS with molecular weights of 500—5000 daltons act as cellular immunosuppressing factors in patients with renal failure,[4)] although there is no clear information on the nature of the factors responsible.[5)] However, it was concluded that MMS are significant uremic toxins which might show activity for the inhibition of E-rosette formation if present at excessive blood concentration levels.[6)] In a previous report[7)] from this laboratory, we described the syntheses and some immunological properties of STF, [DAla^1]- and [Arg^3]-STF. We found[7)] increases in the percentage of E–RFC on incubation *in vitro* with MMS, lymphocytes and STF.

In the present communication, the syntheses of three fragments of STF and the results of E-rosettes formation inhibition tests of the synthetic peptides are described, and the basis of these findings the correlation between their structures and the immunological activity the uremic state is discussed. After removal of the Boc group of Boc–Ser(Bzl)–Gln–Gly–Gly–Ser(Bzl)–Asn–ONb[7)] with TFA, the resulting hexapeptide ester was hydrogenated over 10% Pd–C in 50% AcOH solution, and the hydrogenated product was purified through a Sephadex G-15 column to obtain H–Ser–Gln–Gly–Gly–Ser–Asn–OH (I). The other two peptides, H–Lys–Ser–Gln–Gly–Gly–Ser–Asn–OH (II) and H–Ala–Lys–Ser–Gln–Gly–Gly–Ser–Asn–OH (III), were prepared in essentially the same way. The hexa- (I), hepta- (II), and octapeptides (III) thus obtained were found to be homogeneous on the basis of paper chromatography using two different solvent systems, and the ratios of amino acids agreed well with the theoretical values. Table I shows the increases in the percentage of T cell rosettes on

1) Abbreviations used are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **11**, 1726 (1972). Other abbreviations: STF, serum thymic factors; MMS, middle molecular substances; sheep erythrocytes; RFC, rosette-forming cells; GVB^{2+}, gelatin-veronal buffer; PBS, phosphate-buffered saline; FCS, fetal calf serum; TFA, trifluoroacetic acid.

2) Location: *a) Tsutsumimachi 3-16-1, Sendai, 980, Japan*; *b) Higashishichibancho 84, Sendai, 980, Japan.*

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Table I. Effect of STF Fragments on the Inhibition of E-Rosette Formation by MMS (Mw 500—5000)

Materials	Dose (μg/ml)	E-rosettes (%)
Normal lymphocytes	—	77
Lymphocytes were incubated for 30 min at 37° with MMS	—	44
pGlu–Ala–Lys–Ser–Gln–Gly–Gly–Ser–Asn +MMS	10	74
Ser–Gln–Gly–Gly–Ser–Asn	10	42
	100	43
Lys–Ser–Gln–Gly–Gly–Ser–Asn	10	46
	100	60
Ala–Lys–Ser–Gln–Gly–Gly–Ser–Asn	10	45
	100	64

Lymphocytes were incubated for 30 min at 37° with MMS and STF or an STF fragment.

incubation *in vitro* with MMS, lymphocytes and STF fragments. Increased activity for E-rosette formation was observed with H–Lys–Ser–Gln–Gly–Gly–Ser–Asn–OH (II) and H–Ala–Lys–Ser–Gln–Gly–Gly–Ser–Asn–OH (III) up to a concentration of 100 μg/ml, but the activity of these two fragments was lower than that of STF. H–Ser–Gln–Gly–Gly–Ser–Asn–OH (I) showed no effect on the E-rosette formation inhibition activity of MMS. Thus, H–Lys–Ser–Gln–Gly–Gly–Ser–Asn–OH appears to be the shortest fragment of STF which can exhibit antagonistic action towards the inhibition of E-rosette formation by MMS.

Experimental

Melting points are uncorrected. Rotation was determined with an Atago Polax. The peptides were chromatographed on filter paper, Toyo Roshi No. 51, at room temperature. Rf^1 values refer to the Partridge system[8] and Rf^2 values refer to the BuOH–pyridine–AcOH–H_2O (30: 20: 6:24) system.[9] Amino acid compositions of the acid hydrolysates and AP–M digest[10] were determined with a Hitachi 835-50 amino acid analyzer. MMS (Mw 500—5000) were isolated from the dialysate of an uremic patient by ultrafiltration using Amicon Centriflo membranes. STF was purchased from the Protein Research Foundation, Osaka.

H-Ser-Gln-Gly-Gly-Ser-Asn-OH (I)——Boc–Ser(Bzl)–Gln–Gly–Gly–Ser(Bzl)–Asn–ONb[7] (200 mg) was treated with TFA (1.0 ml) in the presence of anisole (0.1 ml) at room temperature for 30 min and dry ether was added. The resulting powder was hydrogenated in 50% AcOH (15 ml) over 10% Pd-C (300 mg) for 32 hr. The catalyst was removed with the aid of Cellite. The solution was evaporated to dryness and the residue was dried over KOH pellets *in vacuo*. A solution of the crude product in 1% AcOH (2.0 ml) was applied to a Sephadex G-15 column (2.8×60 cm), which was eluted with 1% AcOH. Fractions of 4 ml were collected at a flow rate of 1 ml/1.5 min and the absorbancy was determined at 230 nm. The eluates in tubes 45 to 52 (containing the hexapeptide) were pooled, evaporated to dryness *in vacuo* and lyophilized. Yield 63 mg (55%), mp 135—137°, $[\alpha]_D^{23}$ −33.0° (c=1.0, H_2O), Rf^1 0.04, Rf^2 0.10, single ninhydrin-positive spot. Amino acid ratios in the acid hydrolysate: Glu 0.85, Ser 1.82, Gly 2.00, Asp 0.90. Amino acid ratios in the AP-M digest: 0.95, Asn 0.87, Ser 1.90, Gly 2.00.

H-Lys-Ser-Gln-Gly-Gly-Ser-Asn-OH (II)——Boc–Lys(Z)–Ser(Bzl)–Gln–Gly–Gly–Ser(Bzl)–Asn–ONb[7] (100 mg) was treated with TFA (1.0 ml), and the resulting product in 50% AcOH (12 ml) was hydrogenated for 32 hr in the presence of 10% Pd-C (180 mg) as described above. The crude product in 1% AcOH (2.0 ml) was fractionated on a Sephadex G-15 column (2.6×60 cm) as described above. Fractions 41 to 53 (containing the heptapeptide) were pooled, evaporated to dryness *in vacuo* and lyophilized. Yield 46 mg (53%), mp 141—146°, $[\alpha]_D^{22}$ −40.1° (c=1.0, H_2O), Rf^1 0.05, Rf^2 0.06, single ninhydrin-positive spot. Amino acid hydrolysate: Gly 2.00, Glu 1.06, Ser 1.92, Asp 0.98, Lys 0.94. Amino acid ratios in the AP–M digest: Gln 0.97, Asn 0.88, Gly 2.00, Ser 1.92, Lys 0.88.

H-Ala-Lys-Ser-Gln-Gly-Gly-Ser-Asn-OH (III)——Boc–Ala–Lys(Z)–Ser(Bzl)–Gln–Gly–Gly–Ser(Bzl)–Asn–ONb[7] (90 mg) was treated with TFA (1.0 ml), and the resulting product in 50% AcOH (12 ml) was hydrogenated for 32 hr in the presence of 10% Pd-C (130 mg) as described above. The hydrogenated product

8) S.M. Partridge, *Biochem. J.*, **42**, 238 (1948).
9) S.G. Waley and G. Watson, *Biochem. J.*, **55**, 328 (1953).
10) K. Hofman, F.M. Finn, M. Limetti, J. Montibeller, and G. Zanetti, *J. Am. Chem. Soc.*, **88**, 3633 (1966).

in 1% AcOH (2.0 ml) was fractionated on a Sephadex G-15 column (2.6×65 cm) as described above. Fractions 40 to 55 (containing the octapeptide) were pooled, and evaporated to dryness *in vacuo*. Paper chromatography revealed the presence of two ninhydrin-positive spots with Rf^1 0.06 (major), 0.23 (minor) and Rf^2 0.04 (major), 0.29 (minor). A solution of the crude product in Waley's solvent was applied to a cellulose powder column (2.6×50 cm) and eluted with the same solvent system. Fractions of 4 ml were collected at a flow rate of 4 ml/15 min and the peptide was located by ninhydrin reaction. Fractions No. 39 to 46 (containing the octapeptide) were pooled, evaporated to dryness and lyophilized. Yield 36 mg (58%), mp 151—158°, $[\alpha]_D^{24}$ −42.3° (c=1.0, H_2O), Rf^1 0.06, Rf^2 0.04, single ninhydrin-positive spot. Amino acid ratios in the acid hydrolysate: Glu 1.00, Asp 0.97, Gly 2.00, Ser 1.91, Lys 0.97, Ala 0.97. Amino acid ratios in the AP-M digest: Gln 1.00, Asn 0.90, Ala 1.02, Gly 2.00, Ser 1.96, Lys 0.99.

E-rosette Formation——Peripheral blood lymphocytes were isolated in a Hypaque-Ficoll gradient.[11] The lymphocytes were adjusted to a concentration of 5×10^5/ml with PBS. Contamination by monocytes and polymorphonuclear cells amounted to less than 5%. Sheep erythrocytes were washed with PBS and a suspension (1×10^6/ml) was prepared. Lymphocytes suspended in GVB^{2+} or FCS (1.0 ml) were incubated for 30 min at 37° with MMS (3 mg) and STF fragments. The lymphocytes were washed with GVB^{2+} and centrifuged for 10 min at 1500 rpm, then suspended in GVB^{2+} (1.0 ml). The suspension was mixed with sheep erythrocytes (0.5 ml) and then incubated for 18 hr at 4°. The mixture was centrifuged for 5 min 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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11) R. Haris and E.O. Ukaejiofo, *Brit. J. Haematol.*, **18**, 229 (1970).

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Fluorometric Determination of Acetaminophen and Its Conjugates in Whole Blood

Juichiro Shibasaki, Ryoji Konishi, and Keiji Yamada

Faculty of Pharmaceutical Sciences, Nagasaki University[1]

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A fluorometric procedure has been developed for the determination of acetaminophen and its conjugates, not only in blood plasma and serum, but also in whole blood. This method, in principle, is based on a method reported previously for the determination of acetaminophen in preparations, in which the drug is oxidized with potassium ferricyanide to give a fluorescent substance.

The present method has the advantage of being able to determine the drug without hydrolysis to *p*-aminophenol, in contrast to many of the published methods. The detection limit is about 4 μg/ml of acetaminophen in whole blood, which is comparable to that of spectrophotometric assays reported previously.

Keywords——fluorometric determination; acetaminophen; acetaminophen glucuronide; acetaminophen sulfate; *p*-aminophenol; potassium ferricyanide oxidation; determination in whole blood

Although many types of acetaminophen determination procedures in biological fluids are described in the literature, no fluorometric assay has been reported for this drug. We present here a fluorometric assay for acetaminophen and its conjugates in whole blood. This

1) Location: *1–14, Bunkyo-machi, Nagasaki 852, Japan.*