Amino Acids and Peptides. VI.¹ Synthesis of the N-Terminal Pentapeptide of $\alpha_2$-Plasmin Inhibitor and Its Analogue

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The N-terminal pentapeptide of $\alpha_2$-plasmin inhibitor, H–Asn–Gln–Glu–Gln–Val–OH, and its analogue, H–Asp–Gln–Glu–Gln–Val–OH, were synthesized and their inhibitory effects on the cross-linking reaction of $\alpha_2$-plasmin inhibitor to fibrin mediated by factor XIII a were examined. The synthetic peptides were inhibitory at high concentration.

Keywords—$\alpha_2$-plasmin inhibitor; synthetic pentapeptide; cross-linking; fibrin

Aoki and Moroi isolated and characterized human $\alpha_2$-plasmin inhibitor ($\alpha_2$-PI), which cross-links to fibrin at Gln² in the presence of factor XIII a when blood coagulation takes place.² They demonstrated that the N-terminal dodecapeptide of $\alpha_2$-PI, H–Asn–Gln–Glu–

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**Fig. 1. Synthetic Scheme for I and II**

MA: Mixed anhydride method.
Gln–Val–Ser–Pro–Leu–Thr–Gly–Leu–Lys–NH₂, inhibited the cross-linking reaction of α₂-PI to fibrin.\(^3\)

To examine the effect of the N-terminal portion of α₂-PI on the cross-linking reaction, a smaller N-terminal peptide, H–Asn–Gln–Glu–Gln–Val–OH\(^1\) (I) and its analogue, H–Asp–Gln–Glu–Gln–Val–OH (II), were synthesized. The synthetic scheme is shown in Fig. 2. The carboxyl group of C-terminal valine was protected as the benzyl ester and the C-terminal tetrapeptide was synthesized stepwise by the mixed anhydride method.\(^5\) N-Protecting groups were removed by TFA-treatment at each step, and N-terminal asparagine was introduced onto the tetrapeptide benzyl ester by the p-nitrophenyl ester method.\(^6\) Introduction of the N-terminal asparagine by the mixed anhydride method afforded a by-product which was difficult to remove. All coupling reactions for preparation of the Asp\(^1\)-analogue (II) were done by the mixed anhydride method. The protecting groups on I and II were removed by catalytic hydrogenation to give the free pentapeptides. The purities I and II were confirmed by thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC).

The inhibitory effects of the synthetic peptides on the cross-linking reaction between α₂-PI and fibrin mediated by factor XIII\(_a\) were determined by measuring the amount of α₂-PI incorporated into a fibrin clot. A mixture of the synthetic peptide (I or II), human citrated plasma (containing α₂-PI, factor XIII and fibrinogen), calcium chloride and thrombin was incubated in Tris buffer and the resulting clot was squeezed and removed with a spatula. The resulting clot was insoluble in 1% monochloroacetic acid but a clot formed in a mixture containing ethylenediaminetetraacetic acid (EDTA) instead of calcium chloride was soluble. These results indicated that factor XIII was activated by thrombin in the presence of calcium and catalyzed the cross-linking reaction to form a covalent bond between α₂-PI and fibrin. The amount of α₂-PI in the supernatant was determined by the single radial immunodiffusion method.\(^7\) An aliquot of the supernatant was added to a well in agarose gel containing anti-α₂-PI IgG and the amount of α₂-PI was determined from the resulting precipitation ring. The results are summarized in Table I.

Both synthetic peptides inhibited the factor XIII-mediated cross-linking reaction between α₂-PI and fibrin, and the effects were dependent on the concentration of the synthetic peptide. The inhibitory potency of I was higher than that of II. The carboxyl group of Asp\(^1\) in II might decrease the affinity between II and fibrin or between II and factor XIII\(_a\).

When the inhibitory effect of I was compared with that of the N-terminal dodecapeptide of α₂-PI reported by Aoki et al.,\(^3\) I showed 55\% inhibition at 10 mmol/l, while the dodecapeptide exhibited 50\% inhibition at 350 to 1 μmol α₂-PI. Even though the molecular weight of I is lower than that of the dodecapeptide, the potency of I is lower than that of the

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<th>Table I. Inhibitory Effects of the Synthetic Peptides on the Cross-Linking Reaction of α₂-PI to Fibrin</th>
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\(a\) Inhibition = \(\frac{\text{amount of α₂-PI} - 72.5}{100 - 72.5} \times 100.\)
dodecapeptide on a weight per liter basis. Aoki et al. reported that 50% inhibition of the cross-linking reaction was achieved by addition of 1000-fold molar excess of the dodecapeptide to \( \alpha_2 \)-PI. Shortening the peptide chain of \( \alpha_2 \)-PI might cause a conformational change leading to a decrease of the binding affinity to factor XIIIa or to fibrin.

**Experimental**

Melting points are uncorrected. Solvent systems for ascending TLC on silica gel G (type 60, E. Merck) are indicated as follows: \( R_f^1 = n\)-BuOH–AcOH–H\(_2\)O (4:1:5, upper phase), \( R_f^2 = n\)-BuOH–AcOH–pyridine–H\(_2\)O (4:1:1:2), \( R_f^3 = \text{CHCl}_3–\text{MeOH}–\text{H}_2\text{O} \) (8:3:1, lower phase). Acid hydrolyses were performed in constant-boiling HCl at 110°C for 24 h in evacuated tubes.

**pMZ-Gln-Val-OBzl**—Triethylamine (1.93 ml) and isobutylchlororformate (1.84 ml) were added to a tetrahydrofuran (THF) solution of pMZ-Gln-OH (4.38 g) at -10°C and the reaction mixture was stirred for 10 min. The mixture was then combined with a solution of H-Val-OBzl tosylate (5.3 g) and triethylamine (1.93 ml), and the whole was stirred for 2 h. The solvent was evaporated off and the residue was washed successively with H\(_2\)O, 10% Na\(_2\)CO\(_3\), H\(_2\)O, 5% citric acid and H\(_2\)O in a mortar. The material was recrystallized from MeOH. Yield 5.72 g (82%), mp 171–174°C, \( [\alpha]_D^2 \) = -9.5° (c = 0.9, DMF), \( R_f^3 = 0.69 \). Anal. Calcd for C\(_{24}\)H\(_{33}\)N\(_6\)O\(_{11}\); C, 62.5; H, 6.7; N, 8.4. Found: C, 62.3; H, 6.7; N, 8.4. Amino acid ratios in an acid hydrolysate: Glu\(_{0.91}\), Val\(_{1.00}\) (average recovery 89%).

**Boc-Glu(OBzl)–Gln-Val-OBzl**—Boc-Glu(OBzl)–OH (2.95 g) dissolved in THF (30 ml) and H-Gln-Val-OBzl·TFA (prepared from 4.37 g of pMZ-Gln-Val-OBzl by TFA treatment) dissolved in DMF (30 ml) were coupled by the mixed anhydride method in the usual manner. The solvents were evaporated off and the residue was extracted with AcOEt. The AcOEt layer was washed successively with 10% Na\(_2\)CO\(_3\), H\(_2\)O, 5% citric acid and H\(_2\)O, then dried over Na\(_2\)SO\(_4\), and evaporated down. The residue was recrystallized from AcOEt–petroleum ether. Yield 3.92 g (69%), mp 125–126°C, \( [\alpha]_D^2 \) = -12.8° (c = 1.1, DMF), \( R_f^1 = 0.85 \), \( R_f^3 = 0.82 \). Anal. Calcd for C\(_{34}\)H\(_{46}\)N\(_6\)O\(_{12}\); C, 62.4; H, 7.1; N, 8.6. Found: C, 62.1; H, 7.2; N, 8.7. Amino acid ratio in an acid hydrolysate: Glu\(_{1.01}\), Val\(_{1.00}\) (average recovery 90%).

**pMZ-Gln-Glu(OBzl)–Glu-Val-OBzl**—pMZ-Gln-OH (1.85 g) and H-Glu(OBzl)–Gln-Val-OBzl·TFA (prepared from 3.9 g of Boc-Glu(OBzl)–Gln-Val-OBzl by TFA treatment) were coupled by the mixed anhydride method in DMF (30 ml). The solvent was evaporated off and the resulting residue was washed successively with H\(_2\)O, 5% Na\(_2\)CO\(_3\), 10% citric acid, H\(_2\)O and AcOEt in a mortar. Yield 3.77 g (75%), mp 239–245°C, \( [\alpha]_D^2 \) = -14.6° (c = 1.0, DMF), \( R_f^3 = 0.71 \). Anal. Calcd for C\(_{48}\)H\(_{64}\)N\(_{13}\)O\(_{12}\); C, 61.0; H, 6.4; N, 9.9. Found: C, 60.9; H, 6.5; N, 9.9. Amino acid ratio in an acid hydrolysate: Glu\(_{0.99}\), Val\(_{1.00}\) (average recovery 89%).

**Z-Asn-Gln(Glu(OBzl))–Gln-Val-OBzl**—Z-Asn-ONp (358 mg) was added to a solution of H-Gln(Glu(OBzl))–Gln-Val-OBzl·TFA (prepared from 627 mg of pMZ-Gln-Glu(OBzl)–Gln-Val-OBzl by TFA treatment) in DMF (10 ml) and the mixture was adjusted to pH 8 with triethylamine. The reaction mixture was stirred in a cold room overnight, then the solvent was evaporated off. The residue was washed successively with 10% Na\(_2\)CO\(_3\), H\(_2\)O, 5% citric acid, H\(_2\)O and MeOH in a mortar. Yield 427 mg (62%) mp 263–267°C, \( [\alpha]_D^2 \) = -20.8° (c = 1.0, DMF), \( R_f^2 = 0.85 \), \( R_f^3 = 0.84 \). Anal. Calcd for C\(_{46}\)H\(_{59}\)N\(_{12}\)O\(_{13}\); C, 58.8; H, 6.3; N, 11.9. Found: C, 58.6; H, 6.2; N, 11.9. Amino acid ratios in an acid hydrolysate: Glu\(_{0.99}\), Val\(_{1.00}\) (average recovery 94%).

**H-Asn-Gln-Gln-Val-OH (I)**—Z-Asn-Gln(Glu(OBzl))–Gln-Val-OBzl (198 mg) was hydrogenated over Pd catalyst in 90% AcOH (30 ml) for 5 h. The reaction mixture was concentrated and lyophilized to afford a hygroscopic fluffy powder. Yield 125 mg (100%), \( [\alpha]_D^2 \) = -18.3° (c = 1.0, AcOH), \( R_f^2 = 0.28 \). Anal. Calcd for C\(_{24}\)H\(_{40}\)N\(_{4}\)O\(_{2}\); C, 43.6; H, 6.9; N, 16.9. Found: C, 43.8; H, 7.1; N, 17.0. Amino acid ratios in an acid hydrolysate: Asp\(_{0.96}\), Glu\(_{0.99}\), Val\(_{1.00}\) (average recovery 78%).

**Z-Asp(OBzl)–Gln-Glu(OBzl)–Gln-Val-OBzl**—Z-Asp(OBzl)–OH (597 mg) dissolved in 10 ml of THF was coupled with H-Gln-Glu(OBzl)–Gln-OBzl·TFA (prepared from 1.41 g of pMZ-Gln-Glu(OBzl)–Gln-Val-OBzl by TFA treatment) in DMF (10 ml) by the mixed anhydride method in the usual manner. The solvents were evaporated off and the residue was washed successively with H\(_2\)O, 10% Na\(_2\)CO\(_3\), H\(_2\)O, 5% citric acid, H\(_2\)O and AcOEt in a mortar. Yield 1.3 g (76%), mp 230–239°C, \( [\alpha]_D^2 \) = -17.5° (c = 1.0, DMF), \( R_f^3 = 0.75 \). Anal. Calcd for C\(_{35}\)H\(_{53}\)N\(_{14}\)O\(_{2}\); C, 61.7; H, 6.3; N, 9.5. Found: C, 61.7; H, 6.2; N, 9.6. Amino acid ratios in an acid hydrolysate: Asp\(_{0.96}\), Glu\(_{0.99}\), Val\(_{1.00}\) (average recovery 99%).

**H-Asp-Gln-Gln-Val-OH (II)**—Z-Asp(OBzl)–Gln-Glu(OBzl)–Gln-Val-OBzl (200 mg) was hydrogenated over Pd catalyst in 90% AcOH (50 ml) for 5 h. The reaction mixture was evaporated and lyophilized to afford a hygroscopic fluffy powder. Yield 123 mg (96%), \( [\alpha]_D^2 \) = -20.0° (c = 0.9, DMF), \( R_f^2 = 0.22 \). Anal. Calcd for C\(_{24}\)H\(_{39}\)N\(_{14}\)O\(_{4}\); C, 44.1; H, 6.6; N, 15.0. Found: C, 43.8; H, 6.5; N, 14.9. Amino acid ratios in an acid hydrolysate: Asp\(_{0.96}\), Glu\(_{0.99}\), Val\(_{1.00}\) (average recovery 89%).

**Inhibitory Effects of the Synthetic Peptides on the Cross-Linking Reaction of \( \alpha_2 \)-PI to Fibrin**—Human blood was collected from the antecubital vein of normal subjects into a syringe containing 0.1 volume of 3.8% sodium citrate, and centrifuged at 1800 g for 20 min to prepare platelet-poor plasma. This plasma (0.41 ml) was mixed with a synthetic peptide (I or II) dissolved in 20 mM Tris buffer (0.05 ml, pH 7.4) containing 0.8% NaCl and with 0.5 mM CaCl\(_2\).
(0.02 ml) or 0.05 m EDTA (0.02 ml) instead of CaCl₂. The mixture was incubated with thrombin (0.02 ml of 40 U/ml, Parke Davis Co.) at 37 °C for 1 h. After incubation, the plasma clot was squeezed and removed with a spatula and the concentration of α₂-PI in the supernatant was measured by the single radial immunodiffusion method as follows. A 1% solution of agarose in barbitone buffer (16 ml, pH 8.6, ionic strength 0.05, Daiichi Chemical Co., Ltd.) was mixed with rabbit immunoglobulin G (IgG 0.11 ml) purified from rabbit anti-human α₂-PI serum by using protein A-Sepharose (Sigma Chemical Co., Ltd.). The agarose solution containing IgG was layered onto a slide (11 × 7.5 cm) and 3 mm diameter wells were cut out with a gel punch. The supernatant (0.02 ml) of the plasma clot was added to a well, and diluted human plasma was used as a standard. The gel slide was incubated for 30 h at 37 °C under a humid atmosphere and the diameter of each precipitation ring was measured. The concentration of α₂-PI in the supernatant was determined from a standard curve. The results are shown in Table I.

**HPLC**—The purities of the synthetic peptides were checked by chromatography on a Cosmosil 5C18 column (4.6 × 150 mm, Nakarai Chem. Co.) with the following eluents at a flow rate of 0.1 ml/min. The eluents: MeOH–H₂O (6:4); CH₃CN–H₂O (7:3); 0.05% H₃PO₄–CH₃CN (4:6); 0.1% TFA.

**References and Notes**

1) Amino acids and peptides and their derivatives mentioned in this paper are of L-configuration. Abbreviations used in this paper are: Z=benzyloxy carbonyl, Boc=tert-butoxy carbonyl, pMZ=p-methoxybenzyl oxycarbonyl, OBzl=benzyl ester, ONp=p-nitrophenyl ester, TFA=trifluoroacetic acid, DMF=dimethylformamide.