Structure of Potentiator A, one of the Five Bradykinin Potentiating Peptides from the Venom of *Agkistrodon halys blomhoffii*

In previous papers[1–3] we reported on the isolation of 5 bradykinin-potentiating peptides (potentiators A, B, C, D and E). Amino acid sequences of B, C nad E were determined by conventional degradation methods and confirmed by chemical syntheses[4] and mass spectrometry[5]. Now we report the amino acid sequence of potentiator A, determined by a combination of the dansyl-Edman method and mass spectrometry. The sequence was confirmed by chemical synthesis.

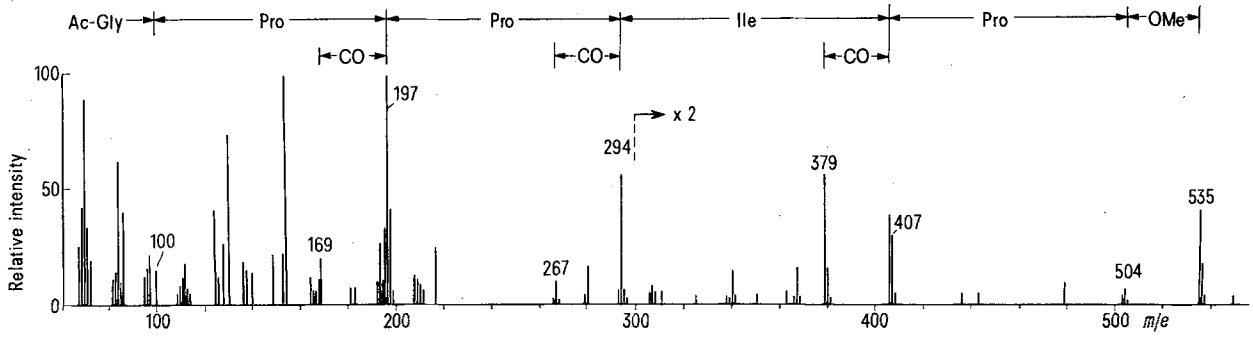
Potentiator A consisted of Arg (1), Glu (1), Pro (5), Gly (2), and Ile (1), and, like the other potentiators, it seemed to have no N-terminal amino acid because it did not give a positive reaction with ninhydrin reagent. Previously, it was reported that potentiator A contained 6 moles of proline residues[2]. After that, it was found that the potentiator A preparation was contaminated with a heptapeptide, which was not separated from potentiator A on paper electrophoresis and paper chromatography, but was separated from potentiator A by column chromatography on Dowex 50. From the reexamination of amino acid composition of potentiator A, it was revealed that potentiator A should contain 5 moles of proline residues.

From the mass spectrum of the derivative obtained by consecutive treatments of potentiator A (ca. 1 mg) with hydrazine[6], acetic anhydride and diazomethane, the sequence of the first 5 N-terminal residues was deduced to be as follows: Pyr-Gly-Arg-Pro-Pro.

To obtain further information on the sequence by mass spectrometry, the above derivative was permethylated using a published procedure[7]. The spectrum contained a set of sequence ion peaks together with accompanying peaks due to C-methylation at the glycine residue[8], and from it the sequence of the 9 amino acids from the N-terminus was concluded to be Pyr-Gly-Arg-Pro-Pro-Gly-Pro-Pro-Ile-. The presence of N-terminal pyroglutamic acid, as expected, was clearly indicated by the intense *m/e* 98 (N-methyl-pyrrolidone ion) and 126 peaks (N-methyl-pyrrolidone carbonyl ion)[9]. Hydrolysis of potentiator A with collagenase produced 3 peptide fragments, A–C-1, A–C-2 and A–C-3, which were separated from each other by paper electrophoresis at pH 3.5 (Pyridine:acetic acid:watre = 1:10:289, v/v). Quantitative amino acid analyses showed that A–C-1 contained Glu (1), Gly (1) and Arg (1), A–C-3, Ile (1), Gly (1) and Pro (3), and A–C-2, Pro only. Carboxypeptidase B hydrolyzed A–C-1 to arginine and pyroglutamylglycine. Thus, A–C-1 must have the amino acid sequence Pyr-Gly-Arg. A–C-2 was identified as Pro-Pro by the dansyl method, and compared with synthetic Pro-Pro and Pro-Pro-Pro by thin layer chromatography (chloroform:benzylalcohol:acetic acid = 70:30:3, v/v). The fragment A–C-3 was acetylated and then esterified with diazo-methane. The mass spectum (Figure) of the resulting derivative showed a molecular ion (*m/e* 535) and a complete series of sequence ions, from which the entire sequence was deduced as Gly-Pro-Pro-Ile-Pro, in good agreement with that deduced using the dansyl-Edman method[10]. The amino acid sequence was confirmed by comparing with synthetic peptide, Gly-Pro-Pro-Ile-Pro, on paper chromatography (*n*-butanol:pyridine:acetic acid:water = 150:10:3:12, v/v). Based on these results and the amino acid composition of potentiator A, it is concluded that the full structure of the potentiator A is as follows:

Pyr–Gly–Arg–Pro–Pro–Gly–Pro–Pro–Ile–Pro.

Potentiator A had the same Rf-value as the synthetic peptide with the above amino acid sequence ($[\alpha]_D^{20}$-201.3 (c 0.4 H_2O)) on paper chromatography (*n*-butanol:pyridine:acetic acid:water = 15:10:3:12, v/v). Collagenase



Mass spectrum of acetyl A–C-3 methyl ester.

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digests of the potentiator A and the synthetic peptide showed similar patterns on the paper chromatography.

Of the 5 peptides isolated from the venom of *Agkistrodon halys, blomhoffii,* this peptide had the weakest bradykinin-potentiating activity on guinea-pig ileum[2]. This weak activity may be because, unlike potentiators B, C and E, it lacks prolylprolyl sequence at the C-terminus. This was supported by the fact that the synthetic peptide, Pyr-Gly-Arg-Pro-Pro-Gly-Pro-Pro-Ile-Pro-Pro, has strong bradykinin-potentiating activity on guinea-pig ileum. The amount of the synthetic peptide to potentiate 2-fold action of bradykinin on guinea-pig ileum was 0.16 nmole/ml, while that of synthetic potentiator A was than 33 nmole/ml.

Zusammenfassung. Strukturaufklärung eines Bradykinin-potenzierenden Peptids, Peptids A, aus dem Gift von *Agkistrodon halys blomhoffii.*

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Increased Urinary Excretion of a Basement Membrane Like Glycoprotein in Acute Uranium Nephropathy*

Urinary glycoproteins which cross react immunologically with antigens in glomerular basement membranes (GBM) have been isolated in several species[1-4]. Quantitative and qualitative alterations in these proteins have been described in immunologic and chemical injury and appear to reflect GBM damage[5,6]. In previous communications we have purified and immunologically characterized a major protein of rat urine (MUP), a GBM like glycoprotein, and demonstrated alterations in this protein by drugs and chemicals[7,8].

Uranium poisoning is a model of non-immunologic mediated renal injury. As well as acute tubular injury, uranium produces glomerular lesions with deposits of randomly oriented fibers on the endothelial side of the lamina densa[9,10]. This study was done to examine rat MUP during acute uranium nephropathy.

Materials and methods. Fifteen Sprague Dawley rats, 300 g, were used in this study. On day 0 baseline 24 h urine samples were collected from each animal. 10 animals received 14 mg/kg of uranyl nitrate hexahydrate as a single s.c. dose 24-h urine specimens were collected daily over the next 3 days. Urine protein excretion was determined using sulfosalicylic acid. Daily excretion of MUP was quantitated by radial immunodiffusion using concentrated urine samples. On day 5 all animals were sacrificed and serum creatinine determinations performed. Sections of kidney were fixed in formalin for light microscopy. Rat MUP was isolated and purified as previously described[7], and used to prepare standards for radial immunodiffusion.

Antisera to rat MUP was prepared in rabbits by weekly immunization. The antiserum was absorbed with lyophylized rat serum and its monospecificity confirmed by immunoelectrophoresis against concentrated rat urine. A single precipitin band was obtained against purified rat MUP and against concentrated rat urine. No precipitin band occurred with rat serum. The antisera was incorporated into agar and radial immunodiffusion plates prepared.

One ml concentrated normal rat urine and concentrated uranium rat urine containing a total protein level of 20 mg/ml was used for chromatography. Sephadex G 200 gel chromatography was performed on a Pharmacia column, 0.9×85 cm, and eluted at a flow rate of 5 ml/h. 2 ml fractions were collected and read at 280 nm in a Leitz spectrophotometer. Agarose gel and immunoelectrophoresis were performed on urine from normal and uranium treated animals.

Results. The uranium treated animals showed marked renal morphologic alterations varying from acute tubular necrosis with moderate to severe diffuse mesangial proliferation to cortical necrosis. Mean creatinine levels in control animals was 0.8 mg/100 ml S.D. $\pm$ 0.1 as compared to 12.2 mg/100 ml S.D. $\pm$ 3 in diseased animals.

Daily urine volume, urine protein and MUP excretion are shown in the Table. Excretion of MUP increased almost 50% during the first 24 h after uranium injection. This increase was significantly different from the controls at the 95% confidence limit. Over the next 2 days MUP excretion returned to normal. As the animals became moribund and oliguric on day 5, MUP excretion dropped. The control animals showed no significant alterations in protein, volume or MUP during the course of the study.

No unique MUP fragments were recognized in the urine of uranium rats as compared to normals by immunoelectrophoresis using antisera to rat MUP. Migration of

Average total daily urine protein, MUP and volume in baseline and uranium treated rats.

		Day 0	Day 1	Day 2	Day 3	Day 4
Protein	Mean (mg)	9.3	36[a]	89[a]	111[a]	10
	Std. Error	1.6	45	18	18	10
Volume	Mean (ml)	9.7	24[a]	31[a]	24[a]	10
	Std. Error	1.5	2.7	7.0	4.8	3.5
MUP	Mean (mg)	7.0	11[a]	7.8	6.8	2.8[a]
	Std. Error	1.2	1.4	0.8	1.0	1.5

[a] Denotes statistically significant at 95% compared to control period and to control animals.

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