J. Pharm. Pharmacol. 1992, 44: 306–310
Received March 14, 1991

Comparison of Kinin-forming and Amidolytic Activities of Four Trimucases, Oedema-producing and Kinin-releasing Enzymes, from *Trimeresurus mucrosquamatus* Venom

CHE-MING TENG, MEI-FENG HSU* AND JIH-PYANG WANG†

*Pharmacological Institute, College of Medicine, National Taiwan University, Taipei, *Department of Biochemistry, School of Medicine, China Medical College, Taichung, and †Department of Medical Research, Taichung Veterans General Hospital, Taichung, Taiwan*

Abstract—Four kinin-releasing enzymes, trimucase I, II, III and IV, isolated from *Trimeresurus mucrosquamatus* venom (TMV) caused rat hind-paw swelling. Trimucase I and III were less potent than trimucase II and IV in this effect. Pretreatment with diphenhydramine or methysergide significantly reduced trimucase-induced paw swelling, while aspirin had no effect. Cellulose sulphate pretreatment suppressed the oedematous responses elicited by trimucases. The residual response was further depressed by diphenhydramine and methysergide. Trimucases also caused kinin generation in-vitro from rat plasma. This kinin-forming activity was in the order of trimucase II > IV ≥ III > I > TMV. All trimucases hydrolysed chromogenic peptides *N*-benzoyl-Pro-Phe-Arg *p*-nitroanilide, *N*-benzoyl-Phe-Val-Arg *p*-nitroanilide and DL-Val-Leu-Arg *p*-nitroanilide; the order of this amidolytic activity was trimucase I > II > III ≥ IV. These data indicate that the effects of venom kinin-releasing enzymes on plasma kininogen are not parallel to their amidolytic effects.

Many biological activities are displayed by the kinins. They are also involved in some pathophysiological effects. Kinins contribute to the inflammatory reaction by causing vasodilatation, local oedema and pain (Lewis 1970). The vasopermeating effect is not due to a nonspecific irritation, but rather to a weakening of the endothelial cell junctions through receptor activation (Gabbiani et al 1970; Marceau et al 1981, 1983). Kinin-releasing activity has been demonstrated in the venom of snakes belonging to *Crotalidae*, *Viperidae* and some *Elapidae* families (Oshima et al 1969). Some of these kinin-releasing enzymes have been purified, and their physicochemical properties characterized (Mebs 1969; Cohen et al 1970; Bjarnason et al 1983; Schwartz & Bieber 1985; Samel et al 1987). Recently, oedema-producing principles were isolated from *Trimeresurus mucrosquamatus* (Teng et al 1989). Four venom peptides possessed kinin-releasing activity, trimucase I–IV. Kinin was one of the major inflammatory mediators involved in the oedematous response induced by trimucase II (Wang & Teng 1988). In this study, the four trimucases were used to compare their kinin-releasing activities, hydrolysing activities on chromogenic substrates and paw swelling effects in rats.

Materials and Methods

Materials

Trimucase I–IV were isolated as previously described (Fractions X, XII, XIII and XIV (Teng et al 1989)). Diphenhydramine, EDTA, *o*-phenanthroline, soybean trypsin inhibitor (SBTI), chymotrypsin, atropine, propranolol, aspirin, indomethacin, DL-Val-Leu-Arg *p*-nitroanilide (V2628), *N*-benzoyl-Phe-Val-Arg *p*-nitroanilide (B7632), *N*-benzoyl-Pro-Phe-Arg *p*-nitroanilide (B2133) were purchased from Sigma Chemical Co., USA. Cellulose sulphate was obtained from Aldrich Chemical Co., USA. Methysergide was kindly supplied by Sandoz Pharmaceutical Ltd, Switzerland. Phenoxybenzamine was a gift from Smith Kline & French, USA.

Correspondence: C.-M. Teng, Pharmacological Institute, College of Medicine, National Taiwan University, Taipei, Taiwan.

Rat hind-paw oedema

Wistar rats, 180–220 g, were used. Oedema was induced as previously described (Wang & Teng 1989) by subplantar injection of 0·1 mL of irritant in 0·05 M phosphate-buffer saline (PBS, pH 7·4) or an equal volume of PBS into the right and left hind-paw, respectively. The volumes of both hind-paws of each rat were measured by means of a plethysmometer at the beginning and at various time intervals after the injection of irritants. Hind-paw swelling was calculated as a percentage as follows: hind-paw swelling (%) = ([(right paw volume − initial volume)/(right paw initial volume)] − [(left paw volume-initial volume)/(left paw initial volume)]) × 100. In some cases, the data were also used to compare the area under the time-swelling curve (AUC).

Kinin formation from plasma

Normal rat plasma was prepared by centrifugation of fresh blood containing 3·8% sodium citrate (1:9 to blood) at 4°C for 15 min at 800 *g*. The supernatant was incubated with trimucases or TMV in the presence of EDTA (5 mM) and *o*-phenanthroline (0·2 mM) at 37°C. The kinins formed were bioassayed on guinea-pig ileum in the presence of atropine, phenoxybenzamine, diphenhydramine, methysergide, indomethacin or propranolol (each 2 μg mL^{-1}). Bradykinin was used as standard. Kinin-forming activities were calculated and expressed as ng bradykinin formed min^{-1} (mg protein)$^{-1}$.

Amidolytic activities

The method of Lottenberg et al (1981) was used. Chromogenic substrates used were *N*-benzoyl-Pro-Phe-Arg *p*-nitroanilide (B2133), *N*-benzoyl-Phe-Val-Arg *p*-nitroanilide (B7632) and DL-Val-Leu-Arg *p*-nitroanilide (V2628). The rate of *p*-nitroanilide formation was monitored at 405 nm. At least four different substrate concentrations were used in order to calculate the K_m and V_{max} values of trimucases by the Michaelis–Menten equation.

Statistical evaluations

The statistical significances were determined by Student's *t*-test. $P<0.05$ was considered to be significant.

Results

Kinins involved in paw swelling caused by trimucases

Subplantar injection of trimucases caused swelling of the rat hind-paw (Fig. 1). This effect was dose-dependent (data not shown). Trimucase II and IV were more potent than trimucase I and III. Oedematous responses occurred rapidly and peaked by 1 h. Diphenhydramine (10 mg kg^{-1}, s.c.) pretreatment reduced paw swelling caused by trimucase I–IV by 32, 28, 17 and 38%, respectively, and methysergide (10 mg kg^{-1}, s.c.) reduced paw swelling by 36, 39, 34 and 45%, respectively. However, there was no significant difference between control and aspirin-pretreated groups for the trimucases-induced paw oedema (Table 1). Pretreatment with cellulose sulphate (3 mg kg^{-1}, i.v.) reduced paw swelling caused by trimucase II by about 48% (Fig. 2). In addition, trimucase II-induced paw oedema was decreased by 42% by pretreatment of the rat with diphenhydramine or methysergide (each 10 mg kg^{-1}, s.c.). The residual response was further reduced (75%) by cellulose sulphate pretreatment.

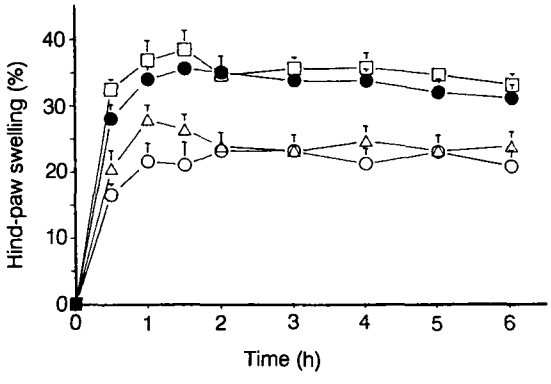

FIG. 1. The hind-paw swelling caused by trimucases. Rat hind-paw swelling was induced by subplantar injection of 10 μg of trimucase I, ○; trimucase II, ●; trimucase III, △; trimucase IV, □. Values are expressed as the means ± s.e.m. of 4–6 experiments.

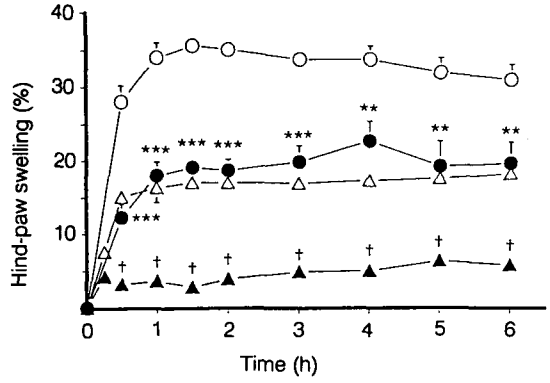

FIG. 2. Effect of diphenhydramine, methysergide and cellulose sulphate on the hind-paw swelling caused by trimucase II. Oedematous response was induced by subplantar injection of trimucase II (10 μg) in control, ○; cellulose sulphate- (total 3 mg kg^{-1}, i.v.) pretreated, △; diphenhydramine/methysergide- (each 10 mg kg^{-1}, s.c.) pretreated, ●; diphenhydramine/methysergide- and cellulose sulphate-pretreated rat, ▲. Values are expressed as the means ± s.e.m. of 5 experiments. Statistically significant difference from the corresponding values in the control are noted as $^{**}P<0.01$, $^{***}P<0.001$ and from the corresponding values the cellulose sulphate treated group are noted as $\dagger P<0.001$.

Kinin-forming activity of trimucases

Like bradykinin, the kinins formed from normal rat plasma by trimucases caused contraction of guinea-pig ileum (Fig. 3). The kinin-forming effect was inhibited by SBTI and was absent in cellulose sulphate-pretreated plasma. The kinins formed were destroyed by chymotrypsin. The kinin-forming activity of trimucases and TMV crude venom (4·7 ± 0·5, 9·0 ± 1·0, 612·1 ± 45·6, 126·8 ± 23·6 and 135·6 ± 24·4 ng bradykinin formed min^{-1} (mg protein)$^{-1}$ for TMV crude venom and trimucase I-IV, respectively) was in the order of trimucase II > IV ≥ III > I > TMV (Fig. 4).

Table 1. Effect of diphenydramine, methysergide and aspirin on the hind-paw oedema caused by trimucases I, II, III and IV.

	Oedema (AUC)[b]			
	Trimucase I (15 μg)	Trimucase II (10 μg)	Trimucase III (15 μg)	Trimucase IV (10 μg)
Control	186·6 ± 5·5	187·2 ± 6·8	211·4 ± 11·6	200·9 ± 11·2
Pretreatment with[a]				
Diphenhydramine	125·5 ± 8·8***[c]	135·7 ± 12·9**	176·5 ± 10·6*	125·0 ± 9·9***
Methysergide	119·9 ± 9·9***	115·2 ± 14·9***	140·1 ± 11·6***	110·8 ± 9·9***
Aspirin	192·6 ± 18·8	181·4 ± 21·0	214·7 ± 9·2	206·1 ± 12·9

[a] Diphenhydramine (10 mg kg^{-1}, s.c.), methysergide (10 mg kg^{-1}, s.c.) or aspirin (180 mg kg^{-1}, s.c.) was given 1 h before the trimucase injection in the paw. [b] Values are expressed as the means ± s.e.m. of 5–6 experiments. [c] Statistically significant differences from the corresponding control values are noted as $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

Table 2. Amidolytic activities of trimucases on some chromogenic peptides.

	Amidolytic activities[a]	
	V_{max} (μmol min^{-1} mg^{-1})	K_m ($\times 10^{-4}$ M)
N-Benzoyl-Pro-Phe-Arg *p*-nitroanilide (B2133)		
Trimucase		
I	43·20 ± 4·38	1·19 ± 0·13
II	2·21 ± 0·09	0·57 ± 0·15
III	1·02 ± 0·08	0·62 ± 0·17
IV	0·43 ± 0·04	0·71 ± 0·08
N-Benzoyl-Phe-Val-Arg *p*-nitroanilide (B7632)		
Trimucase		
I	1·37 ± 0·16	2·30 ± 0·32
II	0·09 ± 0·01	1·08 ± 0·18
III	0·03 ± 0·01	2·02 ± 0·77
IV	0·02 ± 0·01	3·76 ± 0·65
DL-Val-Leu-Arg *p*-nitroanilide (V2628)		
Trimucase		
I	8·20 ± 1·09	2·75 ± 0·37
II	0·84 ± 0·02	1·71 ± 0·19
III	0·26 ± 0·02	1·41 ± 0·10
IV	0·21 ± 0·02	2·05 ± 0·28

[a] Values are expressed as the means ± s.e.m. of at least three separate experiments.

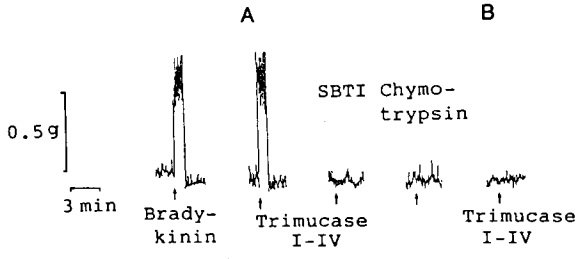

FIG. 3. Contraction of guinea-pig ileum by kinins formed by trimucases. Ileum contraction was induced by bradykinin (3 ng mL^{-1}). A. Rat plasma was incubated with trimucases (50–150 μg mL^{-1}) in the presence or absence of enzyme inhibitors (SBTI 0·3 mg mL^{-1}) at 37°C for 45 min, or the reaction mixture obtained after incubation (without SBTI) was further incubated with chymotrypsin (50 units mL^{-1}) for 10 min. B. Plasma from cellulose sulphate-pretreated rats was incubated with trimucase. After incubation, a sample of the reaction mixture was added to the organ bath for testing ileum contraction. Similar results were obtained in at least three other experiments.

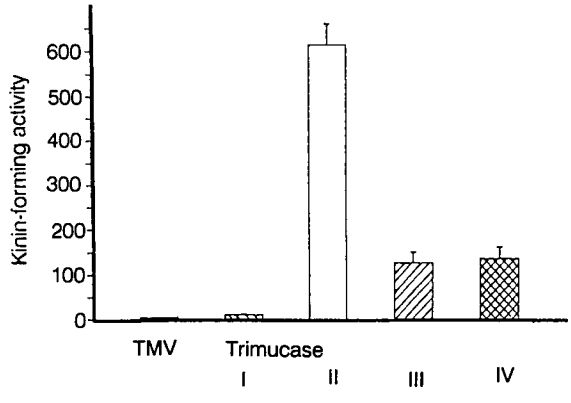

FIG. 4. Comparison of the kinin-forming activities of trimucases and the crude venom of *Trimeresurus mucrosquamatus* (TMV). The kinin formed in the reaction mixture was assayed by ileum contraction and presented as ng bradykinin formed min^{-1} (mg protein)$^{-1}$. Values are expressed as the means ± s.e.m. of 4–5 experiments.

Amidolytic activity of trimucases on chromogenic substrates

Three chromogenic substrates, B2133(substrate for plasma kallikrein), B7632 (substrate for trypsin) and V2628 (substrate for glandular kallikrein), were used in this study. Trimucases hydrolysed these three chromogenic substrates in the order of B2133 > V2628 > B7632. In general, the amidolytic activity of trimucases was in the order of trimucase I > II > III ≥ IV (Table 2).

Discussion

Four oedema-producing and kinin-releasing enzymes were isolated from *Trimeresurus mucrosquamatus* (Teng et al 1989). They induced rat hind-paw oedema in a similar pattern, occurring rapidly and peaking by 1 h. These oedematous responses were suppressed by diphenhydramine and methysergide to mean values of about 28 and 38%, respectively, of the control value. These results indicate that beside histamine and 5-hydroxytryptamine, other mediators may play important roles in this oedematous response. Aspirin had no effect on trimucases-induced paw oedema whether it was given alone or in combination with diphenhydramine/methysergide (data not shown). The residual response was further suppressed (more than 70%) by the cellulose sulphate pretreatment as in the case of trimucase II. Depletion of kinin precursor, kininogen, was produced by the intravenous injection of cellulose sulphate (Rothschild & Castania 1968; Eisen & Loveday 1971) which provided its negative surface charge to activate Factor XII (Kellermeyer & Kellermeyer 1969). This residual response was also decreased by 40 and 90% by trasylol and [Thi^{5,8}, D-Phe^{7}] bradykinin, respectively, as reported by Wang & Teng (1988). Thus, kinins act as important mediators of trimucase-induced paw oedema. Arachidonate metabolites are closely involved in inflammation. Prostaglandin E has been found to induce vasodilation and to potentiate the action of histamine to increase vascular permeability (Higgs et al 1980). Prostaglandins are involved in carrageenan-, cardiotoxin- and

Table 3. Properties of kinin-releasing enzymes isolated from some snake venoms.

	Molecular weight (kDa)	Kinin-releasing activity ($\mu g\ min^{-1}$ (mg protein)$^{-1}$)	Inhibitor		References
			SBTI	Trasylol	
Viperidae					
B. gabonica	32	17			Viljoen et al (1979)
B. gavonica	33·5	30·3	−		Mebs (1969)
E. coloratus	22	2·5	−	−	Cohen et al (1970)
V. ammodytes ammodytes	40·5	43·2			Bailey & Shipolini (1976)
V. berus berus	41	34·2	+	+	Samel et al (1987)
Crotalidae					
A. contortrix laticinctus	31		+		Toom et al (1970)
A. halys blomhoffii		4·7	−		Sato et al (1965)
C. atrox	27·5			+	Bjarnason et al (1983)
	29·2			+	
C. scutulatus scutulatus	33·4	23·2	−		Schwartz & Bieber (1985)
	34·3	22·6	−		
T. mucrosquamatus	23	0·6	+	+	

phospholipase A_2-induced paw oedema (Di Rosa et al 1971; Chiu et al 1989; Wang & Teng 1990). However, bradykinin-induced oedematous response was not affected by aspirin (Wang et al 1989). In this study, kinins play an important role as shown in the results and this could explain the ineffectiveness of aspirin in trimucase-induced paw oedema.

The kinin-forming effects of trimucase were also demonstrated in-vitro. This kinin-forming effect was inhibited by SBTI and greatly suppressed in plasma of rats pretreated with cellulose sulphate. Kinin formation was also inhibited by trasylol (Wang & Teng 1988). The kinins formed were destroyed by incubation with chymotrypsin. These properties were similar to those of kinin-releasing enzymes from *V. berus berus*, *A. contortrix laticinctus* and *C. atrox* (Toom et al 1970; Bjarnason et al 1983; Samel et al 1987) (Table 3). The kinin-forming activity was in the order of trimucase II > IV $\geqslant$ III > I comparable with the order of oedematous response caused by trimucases. Kinin acted as an important, but not the only, mediator in trimucase-induced paw swelling; other mediators such as histamine, 5-hydroxytryptamine and superoxide radical were also involved in trimucases-induced paw oedema (data not shown).

Trimucases II, III and IV possessed only weak esterase activity toward tosyl-L-arginine methyl ester (TAME) (Teng et al 1989). B2133, V2628 and B7632 are widely used for the determination of the enzyme activities of plasma kallikrein, glandular kallikrein and trypsin, respectively. In this study, all four trimucases hydrolysed these three chromogenic substrates in the order of B2133 > V2628 > B7632. The substrate specificities of the trimucases are similar to that of plasma kallikrein. The kinin-releasing enzymes from *C. adamateus* and *C. atrox* (Markland et al 1982; Bjarnason et al 1983) also hydrolysed B2133 and V2628. However, a snake venom protease isolated from *A. caliginosus* (Ohtani & Takahashi 1988) hydrolysed B2133 and V2628 without any kinin-forming activity (Ohtani et al 1985). In general, the kinin-releasing enzymes from the venom of snakes belonging to the *Viperidae* or *Crotalidae* possessed arginine esterase activity (Rothschild & Rothschild 1979), but the esterase enzyme activity of trimucases was much weaker than that of thrombin-like enzymes or arginine ester hydrolases. In this study, the amidolytic activity of trimucases was in the order of I > II > III $\geqslant$ IV and did not parallel that of their kinin-forming activity.

Acknowledgements

This work was supported by research grants from the National Science Council of the Republic of China (NSC 77-0412-B002-92 and NSC 78-0412-B002-60).

References

Bailey, G. S., Shipolini, R. A. (1976) Purification and properties of a kininogenin from the venom of *Vipera ammodytes ammodytes*. Biochem. J. 153: 409–414

Bjarnason, J. B., Barish, A., Direnzo, G. S., Campbell, R., Fox, J. W. (1983) Kallikrein-like enzymes from *Crotalus atrox* venom. J. Biol. Chem. 258: 12566–12573

Chiu, H. F., Chen, I. J., Teng, C. M. (1989) Edema formation and degranulation of mast cells by a basic phospholipase A_2 purified from *Trimeresurus mucrosquamatus* snake venom. Toxicon 27: 115–125

Cohen, I., Zur, M., Kaminsky, E., De Varies, A. (1970) Isolation and characterization of kinin-releasing enzyme. Biochem. Pharmacol. 19: 785–793

Di Rosa, M., Giroud, J. P., Willoughby, D. A. (1971) Studies of the mediators of the acute inflammatory response induced in rats in different sites by carrageenan and terpentine. J. Pathol. 104: 15–29

Eisen, V., Loveday, C. (1971) In vitro effects of cellulose sulfate on plasma kininogen, complement and inflammation. Br. J. Pharmacol. 42: 383–391

Gabbiani, G., Baddonel, M. C., Majno, G. (1970) Vascular leakage induced by intra-arterial histamine, serotonin or bradykinin. Proc. Soc. Exp. Biol. Med. 135: 447–452

Higgs, G. A., Moncada, S., Vane, J. R. (1980) The mode of action of anti-inflammatory drugs which prevent the peroxidation of arachidonic acid. Clin. Rheum. Dis. 6: 675–693

Kellermeyer, W. F., Kellermeyer, R. W. (1969) Hageman factor activation and kinin formation in human plasma induced by cellulose sulfate solution. Proc. Soc. Exp. Biol. Med. 130: 1310–1314

Lewis, G. P. (1970) Kinins in inflammatory and tissue injury. Handb. Exp. Pharmak. 25: 516–520

Lottenberg, R., Christensen, V., Jackson, C. M., Coleman, P. L. (1981) Assay of coagulation proteases using peptide chromogenic and fluorogenic substrates. Method Enzymol. 80: 341–361

Marceau, F., Knap, M., Regoli, D. (1981) Pharmacological characterization of the vascular permeability-enhancing effects of kinins in the rabbit skin. Can. J. Physiol. Pharmacol. 59: 921–926

Marceau, F., Lussier, A., Regoli, D., Giroud, J. P. (1983) Pharma-

cology of kinins: their relevance to tissue injury and inflammation. Gen. Pharmacol. 14: 209–229

Markland, F. S., Kettner, C., Schiffman, S., Shaw, E., Bajwa, S. S., Reddy, K. N. N., Kirakossian, H., Patkos, G. B., Theodor, I., Pirkle, H. (1982) Kallikrein-like activity of crotalase, a snake venom enzyme that clots fibrinogen. Proc. Natl. Acad. Sci. USA 79: 1688–1692

Mebs, D. (1969) Uber Schlangengift-kallikreine: Reinigung und Eigenschaften eines Kinin-freisetzenden Enzymes aus dem Gift der Viper *Bitis gavonica*. Hoppe-Seylers Z. Physiol. Chem. 350: 1563–1569

Ohtani, Y., Takahashi, H. (1988) Some properties of a proteolytic enzyme from the venom of *Agkistrodon caliginosus* (Kankoku-mamushi) which increase capillary permeability. Toxicon 26: 181–190

Ohtani, Y., Suda, T., Takahashi, H. (1985) Capillary permeability-increasing enzyme from the venom of *Agkistrodon caliginosus* (Kankoku-mamushi): activity due to the release of peptide material from a protein in bovine plasma. Ibid. 23: 53–61

Oshima, G., Omori-Satoh, T., Suzuki, T. (1969) Distribution of proteinase, arginine ester hydrolase and a kinin releasing enzyme in various kinds of snake venoms. Ibid. 7: 229–233

Rothschild, A. M., Castania, A. (1968) Endotoxin shock in dogs pretreated with cellulose sulphate, an agent causing partial plasma kininogen depletion. J. Pharm. Pharmacol. 20: 77–78

Rothschild, A. M., Rothschild, Z. (1979) Liberation of pharmacologically active substances by snake venoms. Handb. Exp. Pharmacol. 52: 591–628

Samel, M., Siigur, E., Siigur, J. (1987) Purification and characterization of two arginine ester hydrolases from *Viper berus berus* (Common Viper). Toxicon 25: 379–388

Sato, T., Iwanaga, S., Mizushima, Y., Suzuki, T. (1965) Studies on snake venom XV. Separation of arginine ester hydrolase of *Agkistrodon halys blomhoffii* venom into three enzymatic entities: "bradykinin releasing", "clotting" and "permeability increasing". J. Biochem. (Tokyo) 57: 380–391

Schwartz, M. W., Bieber, A. L. (1985) Characterization of two arginine ester hydrolases from Mojave rattlesnake (*Crotalus scutulatus scutulatus*) venom. Toxicon 23: 255–269

Teng, C. M., Wang, J. P., Peng, H. C., Ouyang, C. (1989) Edema-producing proteins isolated from *Trimeresurus mucrosquamatus* snake venom. Ibid. 27: 899–905

Toom, P. M., Solie, T. N., Tu, A. T. (1970) Characterization of a nonproteolytic arginine ester-hydrolyzing enzyme from snake venom. J. Biol. Chem. 245: 2549–2555

Viljoen, C. C., Meehan, C. M., Botes, D. P. (1979) Separation of *Bitis gabonica* (Gaboon adder) venom arginine esterase into kinin-releasing, clotting and fibrinolytic factors. Toxicon 17: 145–154

Wang, J. P., Teng, C. M. (1988) Rat paw edema induced by trimucase II, a kinin-releasing enzyme from *Trimeresurus mucrosquamatus* venom. Eur. J. Pharmacol. 157: 61–66

Wang, J. P., Teng, C. M. (1989) Roles of mast cells and PMN leukocytes in cardiotoxin-induced rat paw edema. Ibid. 161: 9–18

Wang, J. P., Teng, C. M. (1990) Effect of anti-inflammatory drugs on the cardiotoxin-induced hind-paw oedema in rats. J. Pharm. Pharmacol. 42: 842–845

Wang, J. P., Hsu, M. F., Ouyang, C., Teng, C. M. (1989) Edematous response caused by [Thi^{5,8},D-Phe^{7}]bradykinin, a B_2 receptor antagonist, is due to mast cell degranulation. Eur. J. Pharmacol. 161: 143–149