Toxicon, Vol. 30, No. 9, pp. 1027–1036, 1992.
Printed in Great Britain.

Pergamon Press Ltd

MOLECULAR PROPERTIES AND STRUCTURE–FUNCTION RELATIONSHIPS OF LETHAL PEPTIDES FROM VENOM OF WAGLER'S PIT VIPER, *TRIMERESURUS WAGLERI*

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(*Received* 15 *January* 1992; *accepted* 19 *March* 1992)

J. J. SCHMIDT, S. A. WEINSTEIN and L. A. SMITH. Molecular properties and structure–function relationships of lethal peptides from venom of Wagler's pit viper, *Trimeresurus wagleri*. *Toxicon* **30**, 1027–1036, 1992.—Two new lethal peptides (waglerins) were purified from the venom of *Trimeresurus wagleri*, and sequenced. We found them to be analogs of lethal peptides (waglerins) I and II reported previously (WEINSTEIN *et al.*, *Toxicon* **29**, 227–236, 1991), with an additional Ser–Leu on the amino terminus. Three of the four waglerins were synthesized and the products were chemically and biologically equivalent to the naturally occurring counterparts in venom. Murine i.p. LD_{50} for synthetic waglerins I, SL-I and II were 0.33, 0.22, and 0.51 mg/kg, respectively. The single, intramolecular disulfide bond in each synthetic peptide formed rapidly in high yield. The reduced (cysteine-containing) forms of the peptides appeared to have significant toxicities, even without prior disulfide bond formation, but synthetic analogs with serine substituted for cysteine were not toxic. The synthetic dimer of waglerin I, formed by two intermolecular disulfide bonds, was not toxic, but rapidly rearranged to lethal, monomeric waglerin I at alkaline pH upon the addition of 5 mM β-mercaptoethanol. Waglerin I was inactivated by cleavage at Tyr-15 with chymotrypsin.

INTRODUCTION

WAGLER'S pit viper, *Trimeresurus* (= *Tropidolaemus*, BRATTSTROM, 1964) *wagleri* (LEVITON, 1964) is an arboreal crotaline viperid which ranges through parts of Thailand, Malaysia, Indonesia, the Philippines and the Indo-Australian archipelago. Investigators have noted the high lethal index of *T. wagleri* venom compared to other *Trimeresurus* venoms (SMITH and HINDLE, 1931; MINTON, 1968). Recently, we described purification and structural analyses of the two main lethal components of *T. wagleri* venom (WEINSTEIN *et al.*, 1991). These toxins were highly basic, thermostable 22-residue peptides. The more toxic peptide I had the amino acid sequence: GGKPDLRPCHPPCHYIPRPKPR. There was one intramolecular disulfide bond. The second toxin, peptide II, had the same sequence, except for tyrosine instead of histidine at residue 10. Here we report the isolation of two more lethal peptides from *T. wagleri* venom. We also describe the chemical syntheses of three of the four lethal peptides, compare the chemical and

biological properties of the synthetic and natural products, and characterize the effects of certain structural changes on toxicity. We propose to call the lethal peptides from *T. wagleri* venom 'waglerins'.

MATERIALS AND METHODS

High pressure liquid chromatography (HPLC)

Equipment was from Waters Division of Millipore (Milford, MA, U.S.A.). We used a Hi-Pore RP318 (C18) column, 0.46 × 25 cm, and a Hi-Pore RP304 (C4) column, 1.0 × 25 cm, both from Bio-Rad Laboratories, Richmond, CA, U.S.A. The larger column was used for preparative runs. Peptides were eluted with various gradients of acetonitrile in 0.09% (v/v) trifluoroacetic acid (TFA). Peaks in chromatograms with retention times less than 7 min were due to solvents and or reagents in the samples, and these contained no peptides.

Purification of lethal peptides from venom

Crude *T. wagleri* venom was obtained from a single female specimen collected in the Philippines, or was purchased from Ventoxin Laboratories (Frederick, MD, U.S.A.). The latter supplied venom from three female specimens collected in Thailand.

Venom was fractionated by size exclusion chromatography followed by reverse phase HPLC as described (WEINSTEIN *et al.*, 1991). Fractions were tested for lethality in mice. In the present study, HPLC fractions were reduced in volume with a stream of dry nitrogen, so that approximately 25–30% of a fraction could be administered to each of two mice. With this method, two previously unreported lethal peptides were found in this venom.

Peptide synthesis

Peptides were synthesized with a Model 431A instrument from Applied Biosystems (Foster City, CA, U.S.A.). All relevant chemicals, supplies and protocols were obtained from the same company. The 'Fmoc' (*N*-(9-fluorenylmethyloxycarbonyl)) synthesis chemistry was used. Crude peptides were cleaved from the resin and deblocked with 93% TFA:3% ethyl methyl sulfide:3% anisole:1% ethanedithiol (v/v), and precipitated by adding cold anhydrous diethyl ether. Precipitates were recovered by filtration, washed with cold ether and dried under vacuum.

Crude peptides were suspended in 0.1% TFA at 5–10 mg/ml. Insoluble material was removed by centrifugation and filtration. Formation of the intramolecular disulfide bond was effected by diluting to 1 mg peptide per ml, adjusting the pH to 8.3 with Tris (free base), and incubating at 21°C. Purification was by reverse phase HPLC. Structures of all synthetic peptides, as well as purity, were assessed by amino acid sequencing.

Sequence analysis

Automated Edman degradation was done with a model 470A amino acid sequencer and an on-line model 120A liquid chromatography, both from Applied Biosystems (Foster City, CA, U.S.A.). Peptides containing cysteine or cystine were pyridylethylated before sequencing (CAVINS and FRIEDMAN, 1970). Approximately 200–500 pm of peptide was used in each run, and at least two sequencing determinations were done on each peptide.

Lethality determinations

The i.p. LD_{50} of each peptide was obtained by injecting male Swiss–Webster mice (14–16 g) in three to five groups of four mice each. Dosages were derived from peptide solutions (0.5–1.2 mg/ml) calibrated by absorbance at 276 nm (WEINSTEIN *et al.*, 1991) or by HPLC peak areas. Solutions of reduced (cysteine-containing) peptides were kept in 0.1% TFA until just before use, when they were neutralized with Tris (free base) or *N*-ethylmorpholine and injected into mice without delay. Appropriate solvent controls were also tested, and none was toxic. The i.p. LD_{50} and the 95% confidence limits for the LD_{50} were calculated by Thompson's moving average interpolation method (THOMPSON and WEIL, 1952), as modified by Spearman and Karber (W.H.O., 1981).

Chymotrypsin digestion of waglerin I

Synthetic, disulfide-bonded waglerin I, 0.74 mg, was dissolved in 0.40 ml of 0.10 M Tris–chloride, pH 8.0, and incubated at 35°C. Chymotrypsin (Sigma Chemical Co., St Louis, MO, U.S.A.) was added in 6 μg aliquots at 0, 1, 2.5, and 4.5 hr. After a further 2 hr at 35°C, the sample was adjusted to pH 2.5 with TFA. The final molar ratio of substrate to protease was approximately 290 : 1.

RESULTS

Isolation of new lethal peptides from T. wagleri *venom*

Reduction in volume of HPLC fractions before toxicity tests revealed the presence of two new lethal peptides, in addition to the previously reported peptides (waglerins) I and II (WEINSTEIN *et al.*, 1991). With reference to Fig. 3 in that report (not shown), the first of the new lethal peptides (waglerin SL-I) eluted with a retention time of 25.3 min, just ahead of waglerin II. The other (waglerin SL-II) was the last peak to emerge from the reverse phase column, with a retention time of 28.6 min. These peptides had the following sequences:

waglerin SL-I: SLGGKPDLRPCHPPCHYIPRPKPR

waglerin SL-II: SLGGKPDLRPCYPPCHYIPRPKPR.

Symptomology after administration of SL-I and SL-II to mice was identical to that seen with waglerins I and II. Mice receiving lethal doses showed a rapid onset of tachypnea, tremors, myoclonus and exophthalmus followed by prostration and death. Complete LD_{50} determinations for waglerins SL-I and SL-II could not be elucidated due to the very low amounts present in venom. These peptides were found in amounts less than 5% of the amounts of waglerins I and II, and therefore was less than 0.07–0.11% of crude venom protein. However, an LD_{50} was determined for synthetic SL-I (see below). Venom from *T. wagleri* specimens collected in Thailand had approximately the same relative and absolute amounts of waglerins I, II, SL-I and SL-II as found in venom from the female Philippine *T. wagleri* specimen.

HPLC of synthetic waglerins I, SL-I, and II

After recovery from the synthesizer, peptides typically comprised about 60% of the dry weight of crude product. Most of the contaminants were insoluble in 0.1% TFA and were removed by centrifugation and filtration. Figure 1A shows reverse phase HPLC of crude synthetic waglerin I. Even at this stage, the peptide was relatively pure, with few contaminants.

The synthesis, deblocking and cleavage protocols produced peptides with cysteine residues, instead of the disulfide bond found in the native molecules. Cysteine-containing peptides were stable in 0.1% TFA at 4°C for at least 3 months. However, the intramolecular disulfide bond formed readily at pH 8.3 and 21°C, with a concomitant decrease in HPLC retention time (Fig. 1B). Fifty percent conversion to the disulfide bonded form occurred in 1.73 hr, and reached a maximum yield of 83% in 6 hr. Continued incubation for up to 24 hr did not significantly change the HPLC pattern seen in Fig. 1B. If the concentration of waglerin I was 1 mg/ml or less, then the intramolecular disulfide bond formed in very high yield. However, concentrations of peptide greater than about 1.5 mg/ml during oxidation led to the formation of byproducts.

In Fig. 1A, synthetic reduced waglerin I had a retention time of 17.30 min. After disulfide bond formation, the retention time decreased to 16.00 min (Fig. 1B), which was identical to that of native waglerin I purified from *T. wagleri* venom (Fig. 1C, 16.06 min).

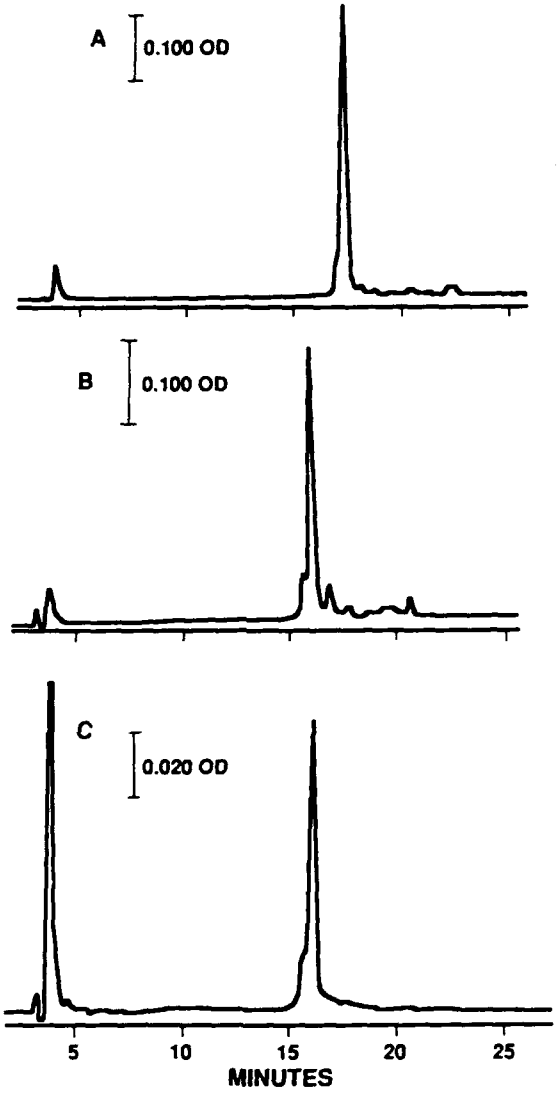

FIG. 1. REVERSE PHASE HPLC OF SYNTHETIC AND VENOM-DERIVED PREPARATIONS OF WAGLERIN I. The column was a Bio-Rad Hi-Pore RP318 (C18) column, 0.46 × 25 cm, operated at 30°C and 1.0 ml/min. Solvent A was 0.09% TFA and solvent B was 0.09% TFA/70% acetonitrile. The column was equilibrated with 18% B. After sample injection, the column was held at 18% B for 3 min, followed by a linear gradient to 35% B at 23 min. The column was then eluted with 100% B for 7 min. Optical density (OD) of the effluent was monitored at 210 nm. A: Crude synthetic waglerin I; B: synthetic waglerin I after 6 hr at pH 8.3, 21°C, and 1 mg/ml peptide; C: waglerin I purified from venom.

Both the synthetic and venom-derived waglerin I had a characteristic shoulder on the front of the peak on reverse phase HPLC. Since it appeared in both the natural and synthetic peptides, it was unlikely to be a contaminant.

Similar results were found for synthetic waglerins SL-I and II. That is, crude reduced peptides, at alkaline pH and concentrations of 1 mg/ml or less, rapidly formed the intramolecular disulfide bond in very high yield. Waglerin SL-I, both natural and

TABLE 1. RETENTION TIMES OF SYNTHETIC AND NATURAL WAGLERINS

Waglerin	Source	Retention time (min.) Disulfide-bonded	Reduced
I	Venom	16.00	17.30
I	Synth.	16.06	17.30
SL-I	Venom	18.22	ND
SL-I	Synth.	18.13	19.26
II	Venom	19.14	ND
II	Synth.	19.23	21.20

ND: Not determined.

synthetic, exhibited a shoulder on the front of the peak on reverse phase HPLC, but waglerin II did not (not shown).

HPLC retention times of synthetic and natural waglerins I, SL-I and II are summarized in Table 1. Chromatography conditions were identical to those described in Fig. 1. The retention time of venom-derived, reduced waglerin I was obtained after 18 hr incubation with 40 mM dithiothreitol at pH 8.3 and 21 C. In each case, the synthetic peptide had a retention time virtually identical to the corresponding peptide isolated from venom.

Lethal potencies of synthetic waglerins I, SL-I and II

Specific lethalities of the synthetic and natural peptides are compared in Table 2. In all cases, symptomology produced by the synthetic peptides was identical to that reported previously for the peptides isolated from venom (WEINSTEIN *et al.*, 1991). For waglerins I and II, LD_{50} of the synthetic, disulfide-bonded peptides were equivalent to the naturally occurring counterparts. Furthermore, waglerin SL-I was more toxic than either I or II, and consistently elicited a more rapid onset of symptoms. Finally, we found that the reduced synthetic peptides were toxic even without prior incubation at alkaline pH to allow disulfide bond formation. A statistically significant difference in specific lethalities was found between reduced and disulfide-bonded forms of waglerin II, but differences between reduced and disulfide-bonded forms of waglerin I and of waglerin SL-I were not statistically significant. The S-pyridylethyl derivatives of these peptides were not toxic.

TABLE 2. LETHAL POTENCIES OF SYNTHETIC AND NATURAL WAGLERINS

Waglerin	Source	LD_{50}, mg/kg i.p. (95% confidence limits) Disulfide-bonded	Reduced
I	Venom	0.37 [0.30–0.41]	ND
I	Synthetic	0.33 [0.30–0.36]	0.38 [0.34–0.42]
SL-I	Synthetic	0.22 [0.20–0.24]	0.30 [0.27–0.34]
II	Venom	0.59 [0.51–0.66]	ND
II	Synthetic	0.51 [0.45–0.59]	1.34 [1.17–1.53]

ND: Not determined.

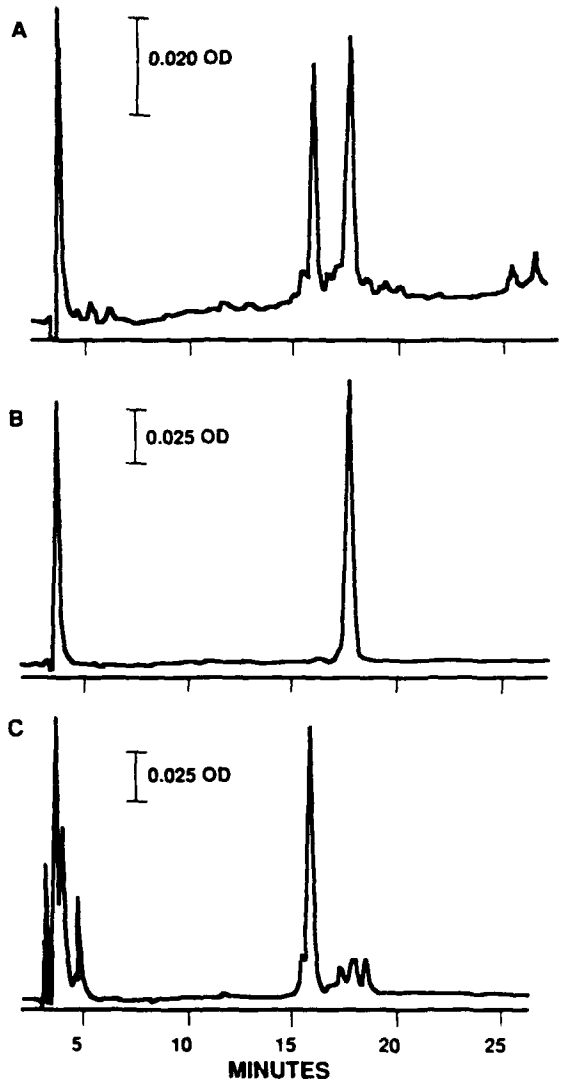

FIG. 2. REVERSE PHASE HPLC OF SYNTHETIC WAGLERIN I, AFTER INCUBATION AT ALKALINE pH AND 12 mg/ml PEPTIDE.
Run conditions were identical to those in Fig. 1. A: Synthetic waglerin I incubated 6 hr at pH 8.3, 21°C, and 12 mg/ml peptide; B: purified preparation of the second (later-eluting) peak in A; C: the second peak, after incubation for 5 hr at pH 8.3, 21°C, 1 mg/ml peptide, and 5 mM β-mercaptoethanol.

Peptide analogs of waglerin I

To investigate the role of the intramolecular disulfide bond in toxicity of waglerin peptides, an analog of waglerin I was synthesized with serine substituted for both cysteine residues 9 and 13. This peptide was purified, incubated overnight at pH 8.3 and 21°C and injected into mice. There were no deaths or signs of intoxication at doses up to 7 mg/kg.

Another analog was synthesized, with serine substituted for cysteine at residue 13 only. This peptide was tested in mice in the reduced form, and produced no deaths or symptoms at doses up to 10 mg/kg.

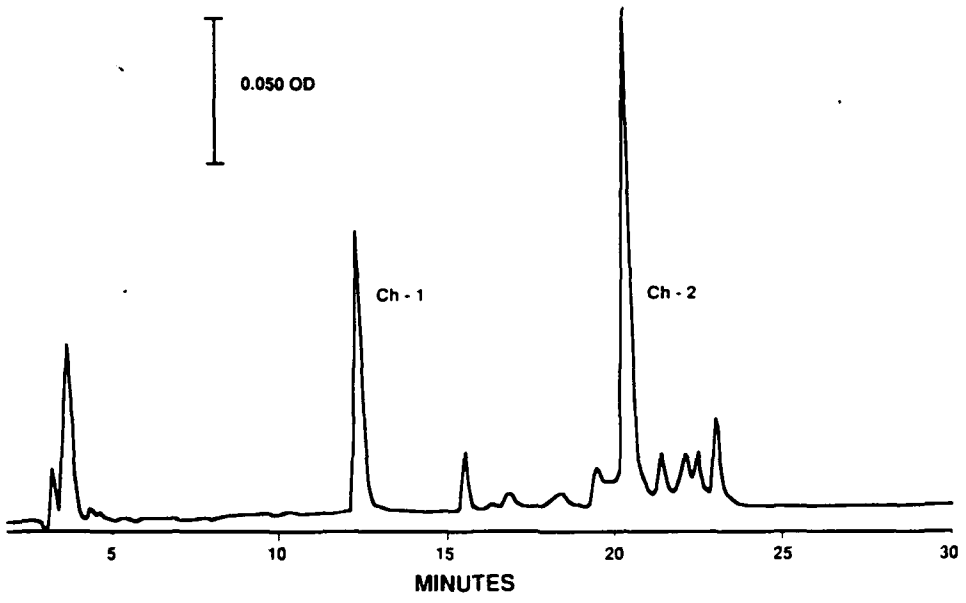

FIG. 3. REVERSE PHASE HPLC OF WAGLERIN I CHYMOTRYPSIN DIGEST.
The column was equilibrated with 10% B. After sample injection, the column was held at 10% B for 3 min, followed by a linear gradient to 35% B at 28 min. The column was then eluted with 100% B for 7 min. Other run conditions were identical to those in Fig. 1. The main peptides resulting from chymotrypsin hydrolysis of waglerin I are labeled Ch-1 and Ch-2.

Purification and properties of a dimer of waglerin I

As noted above, incubating reduced, synthetic waglerin I at alkaline pH promoted intramolecular disulfide bond formation, but also led to other molecular species if the peptide concentration was appreciably greater than approximately 1 mg/ml. The latter situation is illustrated in Fig. 2A, where the concentration of peptide during disulfide bond formation was approximately 12 mg/ml. Two main peaks were found and individually purified. The first peak was the desired product, disulfide-bonded waglerin I. The second peak (Fig. 2B) had a retention time of 17.73 min (later than reduced waglerin I), no free sulfhydryl groups and had no effect on mice at doses up to 9 mg/kg. However, the amino acid sequence of this peak was identical to waglerin I. Futhermore, on dilution to 1 mg/ml, addition of β-mercaptoethanol to 5 mM and incubation for 5 hr at pH 8.3, the second peak rearranged to a form with a retention time (Fig. 2C) and a specific lethality identical to those of disulfide-bonded waglerin I. In sum, the evidence strongly suggests that this non-toxic second peak was a dimer of waglerin I, formed by two intermolecular disulfide bonds.

Effect of chymotrypsin digestion on toxicity of waglerin I

Chymotrypsin cleaved disulfide-bonded waglerin I into two smaller peptides (Fig. 3), which were purified and sequenced. With reference to the sequence of waglerin I, the first major chymotrypsin peptide (Ch-1) contained residues 16–22, while the second (Ch-2) contained residues 1–15. These peptides, administered individually or in combination, were not toxic to mice at doses up to 10 mg/kg. Therefore, neither the first 15 residues alone of waglerin I, nor the last seven residues alone, were sufficient for toxicity.

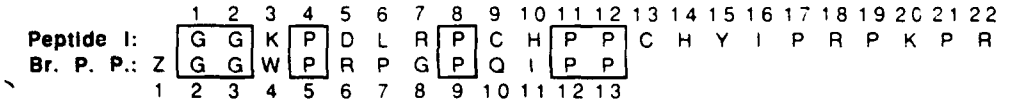

FIG. 4. SEQUENCE HOMOLOGY BETWEEN WAGLERIN I AND A BRADYKININ-POTENTIATING PEPTIDE. Br.P.P., a bradykinin-potentiating peptide from *Bothrops jararaca* (ONDETTI *et al.*, 1971). Z, Nα-(pyrrolidonecarboxyl).

Homology of waglerin I with a bradykinin-potentiating peptide

The sequences of waglerin I and a bradykinin-potentiating peptide from *Bothrops jararaca* (ONDETTI *et al.*, 1971) are compared in Fig. 4. Significant (50%) homology was found for the aligned residues. Further, like the waglerins, the bradykinin-potentiating peptides have an unusually high proline content. In contrast, the latter have no disulfide bonds, contain significantly fewer residues than the waglerins, and have pyrrolidonecarboxylic acid as the amino-terminal residue (that is, a 'blocked' amino terminus).

DISCUSSION

The structures and biological properties of the two principal toxic components from *Trimeresurus wagleri* venom, described by WEINSTEIN *et al.* (1991), have been confirmed by complete chemical synthesis. The chromatographic behavior, specific lethalities, and symptomology of the natural and synthetic products were essentially identical. Furthermore, we report the isolation and amino acid sequences of two new lethal toxic peptides from *T. wagleri* venom, SL-I and SL-II. We determined these to be naturally occurring analogs of waglerins I and II, respectively, with an additional Ser–Leu at the amino terminus. Due to the very small amounts of these analogs in the venom, it was not possible to purify enough material for accurate toxicity tests. However, waglerin SL-I was synthesized, and proved to be more toxic than waglerins I and II. Because synthetic waglerins I and II had specific lethalities equivalent to the natural counterparts, it was reasonable to expect that the same would hold true for SL-I.

Synthetic waglerins I, SL-I and II at first contained cysteine residues, rather than the intramolecular disulfide bond of the natural products from venom. However, formation of the disulfide bond occurred very rapidly in high yield at alkaline pH, with a concomitant decrease in retention time on reverse phase HPLC. These observations suggest that both the reduced and the disulfide-bonded forms of the peptides have very similar conformations, possibly due in part to the unusually high proportion of proline in the sequences, and that disulfide bond formation produced slightly more compact and/or more hydrophilic molecules.

The shoulder on the front of the peak in Fig. 1C was a persistent feature of waglerin I purified from venom, and could not be eliminated by employing shallow gradients and selectively collecting fractions from the center or rear of the main peak. Consequently it is not likely to be a contaminant. This view is strengthened by the finding that synthetic waglerin I also displayed this feature before and after formation of the disulfide bond (Fig. 1A, B), as did waglerin SL-I (not shown). This might be due to different degrees of protonation of the imidazole ring of histidine-10, as waglerin II, which had tyrosine instead of histidine at residue 10, produced a relatively symmetrical peak without a shoulder on reverse phase HPLC.

The observation that the reduced forms of the peptides appeared to have significant toxicities (Table 2) prompted us to investigate the role of the disulfide bond in expression of lethality. Serine is considered to be a very conservative replacement for cysteine in polypeptides (HE and QUIOCHO, 1991). However, synthetic peptides containing serine instead of cysteine at residues 9 and 13, or serine instead of cysteine at residue 13 only, were not toxic. Therefore, either serine disturbed the conformation of the peptide enough to inactivate it, or the disulfide bond was required for toxicity. Although the reduced peptides were stable indefinitely in dilute TFA, and solutions for testing were neutralized just before injection, the apparent lethality of the reduced peptides could be the result of *in vivo* disulfide bond formation. That is, the ease and rapidity of disulfide bond formation might effectively preclude toxicity assessment of waglerins that contain free sulfhydryl groups.

The importance of the intramolecular disulfide bond was also demonstrated by finding that the dimer of waglerin I, formed by two intermolecular disulfide bonds, was non-toxic. However, rearrangement to the monomeric, lethal waglerin I occurred rapidly upon addition of 5 mM β-mercaptoethanol (molar ratio of reductant to peptide approximately 13:1). That the intramolecular disulfide-bonded form and not the reduced form of the peptide was by far the predominant product in the presence of excess reductant suggests that the former was the most energetically favored conformation for this molecule.

The effect of proteolysis on toxicity of waglerin I was studied. Although this peptide was moderately resistant to trypsin-catalyzed hydrolysis (not shown), cleavage by chymotrypsin at tyrosine-15 occurred readily, with low rates of hydrolysis at other sites. The peptide products of this digestion, waglerin residues 1–15 and 16–22, alone or in combination, had no effect on mice at the tested doses. Proteolysis at tyrosine-15 could play a role in detoxification of waglerins *in vivo*.

The mechanism of action of the waglerins has not yet been elucidated. One approach to this problem is to search for structural homologies among other polypeptides with known pharmacological properties. An earlier report, WEINSTEIN *et al.* (1991), stated that a search of two protein sequence databases found no significant homologies with the sequences of waglerins I or II. However, after publication of that manuscript, we noticed a degree of homology between waglerin I and a bradykinin-potentiating peptide from *Bothrops jararaca* (ONDETTI *et al.*, 1971) (Fig. 4). This could be relevant, since a vasoactive mechanism has been suggested for the waglerins (WEINSTEIN *et al.*, 1991). Nonetheless, significant structural differences also exist. Furthermore, inactivation of waglerin I by chymotrypsin might argue against a bradykinin potentiator mode of action, as the region of waglerin I that is homologous with *B. jararaca* bradykinin-potentiating peptide remains intact after chymotrypsin cleavage, but has no effect on mice. Finally, a preliminary report has appeared (AIKEN *et al.*, 1991), describing both pre- and post-synaptic inhibitory effects of waglerin I at the rat neuromuscular junction. Thus, any hypotensive effect could be secondary to peripheral neurotoxicity.

The origin of the multiple forms of toxic peptides in *T. wagleri* is open to speculation. They could result from proteolysis of precursor molecules, although the proteolytic index of this venom is relatively low (WEINSTEIN *et al.*, 1991). Alternatively, they could be the products of individual genes. A precedent for the latter situation was found in a study of crotamine, a relatively small (42 residue) membrane-damaging peptide toxin from the venom of the South American rattlesnake, *Crotalus durissus terrificus* (SMITH and SCHMIDT, 1990). Several sequence variants of this toxin were found in the venom, which were encoded by individual genes.

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