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HYDROLYSIS OF SUBSTANCE P AND BRADYKININ BY BLACK WIDOW SPIDER VENOM GLAND EXTRACT

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Abstract—Black widow spider venom gland extract was found to contain significant peptidase activity. Aliquots of the venom gland extract incubated at 37° inactivated substance P (SP) and bradykinin but not angiotensin II or the enkephalins. The peptide inactivation was proportional to the duration of the incubation and the amount of extract used. Analysis of the peptides on high pressure liquid chromatography demonstrated that the loss in biological activity of SP and bradykinin in the longitudinal muscle of the guinea pig ileum was correlated with cleavage of the peptides into several fragments. Kinetic studies revealed that SP was initially split into two fragments but that these products underwent further degradation into smaller peptides. The optimal pH for the peptidase activity was 6.5. At 0° the enzymatic activity was undetectable, and it was irreversibly destroyed by incubation at 100° for 5 min or by pretreatment of the extract with 100 μM diisopropyl fluorophosphate. In addition, the gland extract preparation hydrolyzed artificial substrates designed to detect trypsin or chymotrypsin-like activity.

The toxicity caused by the bite of *Latrodectus mactans* (black widow spider) is due, in part, to a 130,000 molecular weight protein, α-latrotoxin [1–3]. The mode of action of the toxin is not known, but it is well documented that the crude venom or the purified toxin causes a marked increase in the frequency of the miniature end-plate potentials in the neuromuscular junction of frogs and mammals [4, 5]. In addition, the purified toxin is known to cause an increase in the release from brain slices of a variety of neurotransmitters such as acetylcholine, norepinephrine and γ-aminobutyric acid [6, 7]. The release of neurotransmitter substances has been further substantiated by electron microscopic studies showing the depletion of synaptic vesicles in the nerve endings of the tissues exposed to the venom (for a review, see Ref. 3). The main effect mediated through α-latrotoxin is to cause the release of neurotransmitters from excitatory or inhibitory nerve endings of central and peripheral synapses in vertebrates or invertebrates [8–16]. The precise site and molecular mechanism of action of the toxin in the nerve endings in causing such a widespread effect are not clearly understood. Recently, Tzeng and Siekevitz [2] described a high-affinity binding site in synaptosomal membranes obtained from mammalian brains. No peptidase activity has as yet been described in the black widow spider venom (BWSV).

In the course of our studies on the fading of the contractile response and tachyphylaxis to substance P (SP) in the guinea pig ileum, we found that BWSV extracts blocked the development of tachyphylaxis by rapidly inactivating the peptide [17]. Therefore, we decided to further investigate the nature of the peptidase activity present in the venom gland extract. We considered the studies necessary because the BWSV extract is a tool commonly used in neuropharmacology to obtain acutely denervated preparations or to release neurotransmitters.

METHODS

Preparation of the venom gland extract and protein determination. Ten pairs of venom glands from black widow spiders were homogenized in 3 ml of saline buffered with 50 mM sodium phosphate (pH 8.2), using a Polytron homogenizer (speed 8, three times during 30 sec) over ice, and centrifuged at 100,000 *g* for 1 hr. The supernatant fraction of these preparations is referred to as the extract; it was kept at 4° for over 3 months without loss of the peptidase activity. Total protein content in the extract was determined by the method of Lowry *et al.* [18] using bovine serum albumin as the standard. The concentration of proteins was 0.22 μg/μl of extract.

Conditions for the incubation of the gland extract with several peptides. To determine whether the gland extract contained peptidase activity, the extract was incubated with either SP, bradykinin, angiotensin II, methionine or leucine enkephalin. Aliquots of the reaction mixture were sampled at different time intervals to study the time course of the degradation of each peptide. The incubations were performed at 37° in Eppendorf test tubes for the times indicated in Results. The volume of each incubation was adjusted to 500 μl with 50 mM phosphate buffer (pH 7.5). All the reactions were started by addition of the gland homogenate. At the specified times, 50 μl aliquots of each incubation were sampled, and added to 15 μl of acetic acid, to give a final molarity of 0.5 M. The samples were bio-

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assayed immediately or kept frozen at −20° for 3–4 hr prior to high pressure liquid chromatography (HPLC).

Effect of diisopropyl fluorophosphate (DFP). To study if the peptidase activity in the venom gland was sensitive to inactivation by treatment with DFP, 3 μl of the gland extract was dissolved in 79 μl of 50 mM phosphate buffer (pH 7.5) plus 18.4 mg of DFP (1 mg/μl stock commercial solution). The mixture was incubated immediately at 37° for 15 min and then 10^{-4} M SP was added and incubated as usual at 37° to determine the degradation of the peptide. DFP was not added to the control samples. Aliquots were obtained at different intervals to determine SP by HPLC.

Hydrolysis of synthetic substrates. Two synthetic substrates were used: *N*-benzoyl-arginine-*p*-nitroanilide (BAPNA), to detect trypsin-like activity, and *N*-benzoyl-tyrosine ethyl ester (BTEE), to investigate for chymotrypsin-like enzyme activity. The conditions for the assay were as follows. A 2 mM concentration of each substrate (or 1.0 μmole in 500 μl) was incubated with 20 or 100 μl of the gland extract in 50 mM Tris plus 20 mM calcium chloride adjusted to pH 8.2. The volume of incubation was 500 μl. The incubations were prolonged up to 18 hr at room temperature. Product formation was determined on a spectrophotometer at 410 and 256 nm for BAPNA and BTEE respectively. Control experiments demonstrated that there was negligible spontaneous hydrolysis of the substrates overnight under the experimental conditions. Results are expressed as equivalents of trypsin and chymotrypsin hydrolytic activity in 18 hr.

Bioassay of the peptides and quantification of results. Substance P, bradykinin and angiotensin II were bioassayed in the myenteric plexus-longitudinal muscle (MPLM) preparation of the guinea pig ileum by their ability to contract the tissue. The enkephalins were tested on the same preparation but by their inhibitory action on the electrically evoked twitching of the longitudinal muscle [19]. The MPLM strips were prepared according to Puig *et al.* [19]; the tissues were given 0.3 g of tension and allowed to equilibrate for 1 hr prior to drug additions. Isometric muscular contractions were recorded on a Grass polygraph. Results are expressed as the percentage of the maximal response produced by the application of the peptides prior to and after the incubation with the venom extract.

Conditions for HPLC. Peptides were analyzed in a Waters liquid chromatograph with a model 720 system controller and a Bondapack C_{18} column. The column was equilibrated with solvent A [10 mM sodium phosphate (pH 3.0)–10% acetonitrile] and the peptides were eluted with a 25-min linear gradient with buffer B [10 mM sodium phosphate (pH 3.0)–40% acetonitrile]. The flow rate was 1 ml/min, and the peptides were detected with a variable wavelength spectrophotometer at 210 nm.

Results are expressed in all cases as the percentage of the area integrated under the elution peaks. The areas were all automatically integrated after appropriate calibration for each peptide. The results are generally plotted as a percentage of the initial concentration of SP, bradykinin or angiotensin II.

Materials. The venom glands from black widow spiders (catalog V 3626, lots 108C-0079-1 and 100F-0416) were purchased from the Sigma Chemical Co. (St. Louis, MO). The two artificial substrates for peptidase activity and the diisopropyl fluorophosphate were also obtained from the Sigma Chemical Co. Substance P, bradykinin and angiotensin II were from Peninsula Laboratories (San Carlos, CA). Methionine and leucine-enkephalin were obtained from CalBiochem (La Jolla, CA). TPCK*-treated trypsin (241 units/mg) and α-chymotrypsin (50 units/mg) were provided by Worthington Chemicals (Freehold, NJ). All the solvents used for HPLC were provided by Burdick & Jackson Laboratories (Muskegon, MI).

English short hair adult male guinea pigs (300–400 g) were used for the bioassay of peptides. The animals were bred by CAMM Laboratories and fed in our quarters with a Purina Chow diet plus fresh vegetables.

RESULTS

Effect of the BWSV gland extract on the biological activity of several peptides. BWSV gland extract rapidly destroyed the biological activity of a 10 μM concentration of SP and bradykinin as assayed by the contractile effects of the peptides on the guinea pig ileum. The contractile effect of bradykinin was completely lost after a 15-min incubation whereas the action of SP was only slightly reduced in the same period (Fig. 1). In contrast, methionine and leucine-enkephalin were found to retain full biological activity after incubations under the conditions described in Fig. 1. In other experiments in which the amount of extract was increased 10-fold, the potency of the enkephalins was not reduced by more than 20% after a 1-hr incubation. When the concentration of the peptides was reduced to 1 μM, the inactivation of SP and bradykinin was much faster while angiotensin II proved to be relatively resistant to inactivation. Only 40% of the activity of angiotensin II was lost after a 1-hr incubation with 30 μl

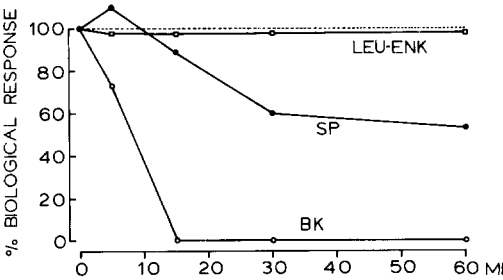

Fig. 1. Effect of BWSV gland extract on the biological activity of several peptides. The peptides (10 μM) were incubated at 37° with 3 μl of gland extract. Aliquots of the incubation were removed at the times indicated and assayed on the guinea pig ileum MPLM preparation. The biological activity of the peptides was determined as described in Methods. Abbreviations: LEU-ENK, Leu-enkephalin; SP, substance P; and BK, bradykinin. The biological activity of the peptides remaining after incubation with gland extract for different times is indicated on the ordinate as a percentage of the activity detected at zero time.

* L-1-Tosylamide-2-phenylethyl chloromethyl ketone.

Table 1. Biological activity of several peptides after incubation with BWSV extract*

Peptide	BWSV (μl)	Biological activity (% of zero time value) Incubation time (min) 1	5	15	30	60
Substance P	3	64	5	0	0	0
Bradykinin	3	8	0	0	0	0
Angiotensin II	3		82	91	84	86
Angiotensin II	30		83	80	66	58

* Results are the average of two independent bioassays. In all cases, a control sample of the peptide incubated at 37° without the BWSV extract gave a biological effect ranging in potency between 95 and 100% of the zero time value.

of extract, which is 10-fold more than what is required to inactivate SP or bradykinin in less than 15 min. The results of these experiments are presented in Table 1.

Analysis of the degradation of SP by HPLC. To establish whether the loss in biological activity of SP was due to enzymatic degradation of the peptide, SP was incubated with BWSV gland extract and aliquots were removed at different times and subjected to HPLC. The retention time (RT) for SP and all the peptide fragments obtained was remarkably constant with variations of less than 1.0%. The RT for SP under the conditions of the experiment was 23.9 min. As can be appreciated in Fig. 2A, SP had no detectable impurities. After a 5-min incubation with BWSV gland extract, the SP peak was reduced markedly and two other peaks emerged: peak I, with a RT of 16.5 min, and peak II, 26.7 min. As the reaction proceeded, SP completely disappeared and peaks I and II were gradually reduced, whereas a new fragment, peak III, with an RT of 14.5 min became increasingly more apparent; additional minor peaks appeared near the solvent peak.

We also studied the HPLC profile of SP digestion with chymotrypsin and found it quite different, with three main peaks with RTs of 18.6, 7.5 and 20.9 min. The SP peak progressively decreased, whereas the area of the three degradation products increased with further degradation. These experiments indicated that the peptidase(s) contained in the BWSV gland homogenate is not chymotrypsin-like.

Characterization of the peptidase activity in the venom gland extract as analyzed by HPLC using SP as substrate. The kinetics of the degradation of SP was studied at 37° with different concentrations of the gland extract. In all cases, the disappearance of SP was linear with time and proportional to the amount of extract used. As seen in Fig. 3, after a 5- to 15-min incubation of 100 μM SP with 10 μl of extract (approximately 1/30 of a set of glands =

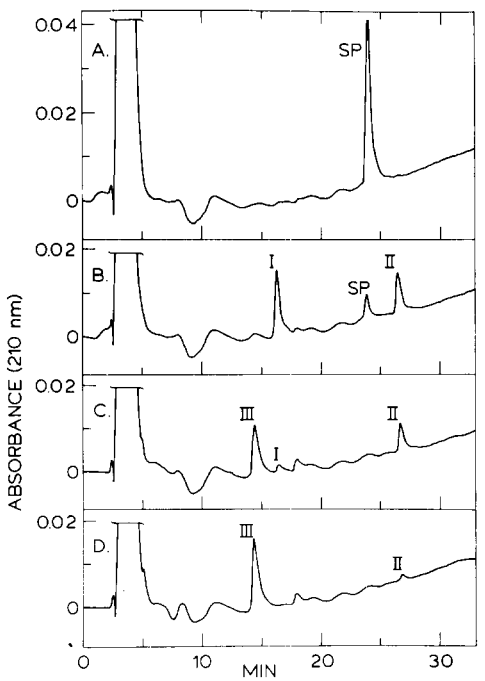

Fig. 2. HPLC of SP after incubation with BWSV gland extract. Substance P (100 μM) was incubated with 3 μl of gland extract for different times at 37°. Aliquots of the incubation mixture were removed at the times indicated and the equivalent of 1 nmole of SP was analyzed by HPLC as described in Methods. (A) Chromatogram of SP obtained at zero time, before the addition of the BWSV gland extract. (B) Sample obtained after a 5-min incubation. (C) Sample obtained after a 30-min incubation. (D) Sample obtained after a 60-min incubation. Abscissa: retention time, in minutes. Ordinate: absorbancy units at 210 nm. SP: substance P; peaks I, II and III are indicated by Roman numerals.

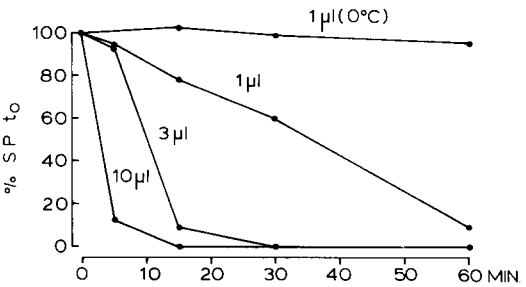

Fig. 3. Effect of the concentration of BWSV gland extract on the degradation of SP. Samples of 500 μl containing 100 μM SP were incubated with either 1, 3 or 10 μl of the gland extract at 37° or with 1 μl at 0°. Aliquots from each incubation mixture were removed at the times indicated and were analyzed for the content of SP by HPLC. Results are expressed as the percentage of SP initial concentration left in the reaction mixture at the different intervals.

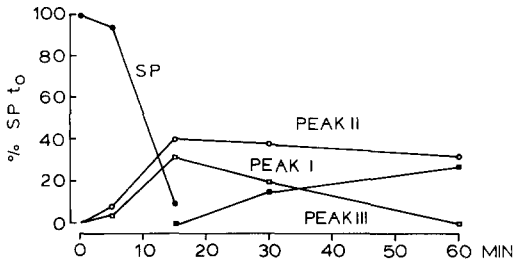

Fig. 4. Time course of the formation of the degradation products of SP incubated with BWSV gland extract. Substance P (100 μM) was incubated at 37° as described. Aliquots were removed at the times indicated and were analyzed by HPLC for SP and degradation products. Results are expressed as the percentage of the area under the SP peak in the zero time sample.

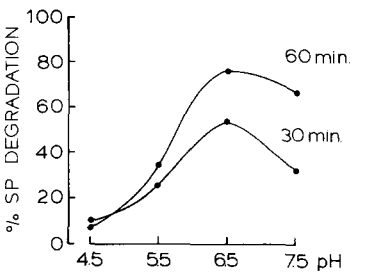

Fig. 5. Effect of pH on the rate of degradation of SP. Samples of 500 μl containing 100 μM SP were incubated with 1 μl of the venom gland extract at 37° at pH 4.5, 5.5, 6.5 and 7.5. Aliquots of the reaction mixture were sampled at different intervals and were analyzed by HPLC for the amount of SP left. Results are expressed as the percentage of the area under the SP peak in the sample obtained at time zero of the reaction.

2.2 μg of protein) virtually all the SP was degraded, as detected by HPLC. The rate of formation of reaction products was proportional to the degradation of SP. Peak III is the major metabolite observed after prolonged incubation of SP with the venom gland extract. Consistent with Fig. 2, this reaction product appeared after a lag period that was inversely proportional to the amount of gland extract used. The appearance of this peak was dependent on the degree of degradation of SP (Figs. 3 and 4). The relative size of peak I (RT 16.5 min) and peak II (RT 26.7 min) was variable depending on the amount of BWSV added (Table 2). Peak I was degraded shortly after its formation and peak II had a relatively longer life although it was also completely digested after a prolonged incubation with the extract (Table 2). Control experiments in which SP was left out of the incubation failed to develop any peak, indicating that the peaks were products of the hydrolysis of the peptide and did not originate in the BWSV extract (results not shown).

The activity of the extract was temperature dependent; when the incubations were done at 0°, SP was not degraded (Fig. 3) and obviously no metabolites were formed. The pH optimum for the degradation of SP by the gland homogenate was about 6.5; at pH 4.5 there was virtually no degradation of SP as determined by HPLC analysis. Figure 5 shows the degradation of SP at the various pH values.

Table 2. Time course of the degradation of substance P by different concentrations of the BWSV gland extract. Appearance and disappearance of two intermediate metabolites (peaks I and II) as detected by high pressure liquid chromatography*

BWSV (μl)	Peak I				Peak II			
	Incubation time (min)							
	5	15	30	60	5	15	30	60
1	0	12	16	35	0	4	11	28
3	4	32	21	2	9	40	38	30
10	45	25	0	0	21	22	15	0

* Results are expressed as the percentage of the area under the substance P peak at zero time and were calculated as described in Methods. The initial concentration of SP was 100 μM or 50 nmoles in 500 μl.

The hydrolytic activity of the venom gland homogenate was completely destroyed by heating to 100° for 5 min; we found that 92–100% of the original SP was recovered after incubation with boiled extract for 30 min at 37°. In contrast, the control samples completely destroyed SP in the same period. When the BWSV gland homogenate was preincubated with DFP as described in Methods, the hydrolytic activity of the extract was completely inactivated (results not shown).

Degradation of other peptides as analyzed by HPLC. In support of the results obtained using the bioassay, it was demonstrated that the biological inactivation of bradykinin by the venom gland homogenate was due to cleavage of the bradykinin molecule. Three microliters of the extract hydrolyzed the nonapeptide, producing two fragments. The rate of degradation of bradykinin and the appearance of the two fragments are shown in Fig. 6. One of the products (peak I) eluted from the HPLC column with a retention time of 13.3 min, while the other fragment (peak II) was retained in the column for 16.5 min. The retention time for bradykinin was 18.2 min.

Angiotensin II was resistant to degradation by the gland extract as detected by HPLC in agreement with the negligible loss in biological potency when tested on the guinea pig ileum bioassay. In a time

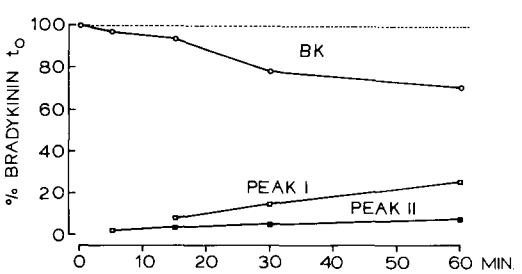

Fig. 6. Degradation of bradykinin by BWSV gland extract. Three microliters of the venom gland extract was incubated at 37° with 50 μl of 100 μM bradykinin. The time course of the reaction was followed using HPLC. BK: bradykinin. The amounts of bradykinin remaining, as well as the metabolites formed (peaks I and II), are expressed as a percentage of the area of the bradykinin peak obtained at zero time.

course experiment designed to study the effect of the gland homogenate on the octapeptide, we did not observe digestion of the peptide after 60 min of incubation. In a matched control experiment, 90% of the SP applied was hydrolyzed in less than 15 min by the extract. The retention time for angiotensin II was 19.9 min.

Peptidase activity of the venom gland homogenate detected with model substrates. BAPNA was chosen as a prototype substrate for the identification of trypsin-like enzymes. The venom gland extract revealed significant trypsin-like activity when incubated with 1 mM BAPNA. BTEE, the model substrate for chymotrypsin-like activity, was substantially hydrolyzed by the gland preparation. The enzymatic activity of the extract was clearly evident after 18 hr of incubation, and it was found to be linearly related to the amount of extract used. A 100 μl aliquot of BWSV homogenate hydrolyzed BAPNA at the same rate as 0.295 μg of trypsin and hydrolyzed BTEE at the same rate as 43.15 μg of chymotrypsin.

DISCUSSION

The main aim of this study was to determine whether extracts from the venom gland of the black widow spider contained any enzymatic activity that could hydrolyze biologically active peptides. We concluded that they could, for the following reasons. The BWSV gland extracts inactivated the biological activity of SP and bradykinin; the rate of inactivation was proportional to the amount of extract added and it was time and temperature dependent. The hydrolytic activity could not be detected at 0° and was inhibited by DFP and by preincubation at 100° for 5 min. These results demonstrate that SP and bradykinin were inactivated by an enzyme. Furthermore, the results of the HPLC demonstrate that the extract hydrolyzed both peptides into several different fragments.

The peptidase activity had a pH optimum around 6.5. It is tempting to speculate that this is an indication that the peptidase activity was not derived from the gland lysosomes. However, we cannot assume that the proteinases in spider lysosomes had a low pH optimum similar to those of mammalian lysosomes. On the other hand, the relative specificity of the peptidase activity that did not hydrolyze either angiotensin II or enkephalin could be taken as an indication that the enzymes contained in the BWSV extract probably were not lysosomal hydrolases. The proteolytic activity in the gland extract belongs to the family of the serine proteases, since the hydrolytic activity of the venom gland extract was completely abolished by pretreatment with DFP. The cleavage of SP by the venom gland homogenates bears some resemblance to the digestion of SP by tonin, a newly discovered proteolytic enzyme present in the submaxillary gland of the rat [20].

Our results are at variance with previous studies indicating that the venom gland does not have significant proteolytic activity. Studies by Frontali *et al.* [1], using an Italian variety of the black widow spider, did not detect proteolytic activity in the whole, crude extract of the gland or in the purified B5 fraction of the extract. Both of these preparations are well characterized for the presence of the active toxin of the venom. Several reasons could account for these discrepancies. One may be related to the different species of black widow spiders used in these studies. It is possible that the European black widow spiders contain much less enzymatic activity in their venom gland than the American spiders used in our preparations. Another explanation could derive from the much broader selection of natural and model substrates that we used to detect and characterize the peptidase activity.

Primor [21] has reported that the BWSV extract obtained from spiders (*Latrodectus tredecimguttatus*) collected in the area of Jerusalem (Israel) produced changes in the response of the guinea pig ileum to several pharmacological agents: the extract itself first produced a prolonged tonic contraction which was followed by a loss of the nicotine-induced contraction. The stimulation produced by 5-hydroxytryptamine, but not that of acetylcholine, was also abolished. It is of interest to mention that we did not find the expected venom activity in the venom gland homogenate. It is possible that our failure to detect the spider venom activity *per se*, as originally intended, could be entirely related to the presence of this proteolytic activity. It is conceivable that the peptidases, once in contact with the active principle of the venom, digested the α-latrotoxin and rendered it inactive. However, this interpretation is unlikely because it is not evident why the gland would contain a peptidase that will destroy its own venom. It may be speculated that the peptidase may contribute to the toxic effects of the venom. Whatever the biological significance of the peptidase activity in the venom gland extract is, the presence of the peptidase(s) should be taken into consideration when trying to interpret the results of experiments in which gland extracts are used. It is not known at the present time whether there is any peptidase activity in more purified preparations of the venom. Researchers should be aware that proteolytic activity may be found in extracts of the BWSV glands and that this activity may introduce an additional variable in their experiments.

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