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Biochemical Characterization of the Phospholipase A_2 Purified from the Venom of the Mexican Beaded Lizard (*Heloderma horridum horridum* Wiegmann)†

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ABSTRACT: A phospholipase A_2 was isolated from the venom of the mexican beaded lizard (*Heloderma horridum horridum*) by phenyl-Sepharose chromatography followed by Sephadex G-75 gel filtration and two additional steps on ion exchange resins (DE-32 cellulose). The affinity chromatographic method (PC–Sepharose 4B) reported for the isolation of other phospholipases [Rock, Ch. O., & Snyder, F. (1975) *J. Biol. Chem. 250*, 2564–2566; King, T. P., Alagon, A. C., Kwan, J., Sobotka, A. K., & Lichteinstein, L. M. (1983) *Mol. Immunol. 20*, 297–308; King, T. P., Kochoumian, L., & Joslyn, A. (1984) *Arch. Biochem. Biophys. 230*, 1–12] was uneffective for the separation of this enzyme. The monomeric form of the *Heloderma* phospholipase has an apparent M_r of 18 000 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and 19 060 as calculated from amino acid analysis. It also contains on the order of 7% carbohydrates per mole of enzyme. The N-terminal amino acid sequence was shown to be very different from that of phospholipases isolated from mammalian pancreas and crotalids and elapids snake venoms. The first 39 amino acid residues at the N-terminal region have 56% homology with bee venom phospholipase but differ from the bee phospholipase in that its isoelectric point is acidic (pI = 4.5), instead of basic, and it has approximately 50 amino acid residues more in the molecule. The specificity of the enzyme is mainly A_2 type with possible residual B-type activity. The enzymatic activity is Ca^{2+}-dependent. Half-cystine alignment of the *Heloderma* phospholipase sequence with those of other known phospholipases shows the lack of an octadecapeptide at the N-terminal region, the existence of an extra hexapeptide at positions 42–47, and an exact correspondence of *Heloderma* Gly-12, Gly-14, His-36, and Asp-37 with Gly-30, Gly-32, His-48, and Asp-49 from other phospholipases shown to be important for Ca^{2+} binding [Dijkstra, B. W., Drenth, J., Kalk, K. H., & Vandermaalen, P. J. (1978) *J. Mol. Biol. 124*, 53–60]. The *Heloderma* phospholipase like the bee phospholipase has a Trp at position 10 corresponding to Tyr-28 of other phospholipases, also claimed to be important for calcium binding. Although the present enzyme is structurally very different, it could be sharing similar peptide sequences around the Ca^{2+} binding site of other phospholipases A_2 reported, thus far.

The enzymes with phospholipase A_2 (EC 3.1.1.4) activity are calcium-dependent esterases. They hydrolyze the 2-acyl bond of 3-*n*-phosphoglycerides. Most of the phospholipases A_2 isolated so far, from pancreas, snake venoms, and bee venoms, are relatively small and rigid molecules, having 6–7 disulfide bridges in a protein approximately 125 residues in length (Verheij et al., 1981; Heinrikson, 1982).

Comparison of the primary structure of more than 30 phospholipases has revealed a high degree of homology in the amino acid sequence of these enzymes. Two classes of phospholipases have been proposed: group I comprises phospholipases from pancreatic juice, elapids (cobras), and hydrophids (sea snakes) and group II is composed by phospholipases from many crotalids (rattlesnakes) (Heinrikson et al., 1977). The two different structural groups were separated on the basis of specific disulfide bridges of the molecules.

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Crystallographic studies of the bovine pancreatic enzyme, group I (Dijkstra et al., 1978), and a phospholipase from the western diamondback rattlesnake (*Crotalus atrox*) (Keith et al., 1981) have shown great similarities in their three-dimensional structures. The phospholipase A_2 enzymes are Ca^{2+}-dependent enzymes. Among the amino acid residues shown to be important for the Ca^{2+} binding site are Tyr-28, Gly-30, Gly-32, His-38, and Asp-49, as shown by a X-ray crystallographic analysis of a Ca^{2+}–enzyme complex in bovine phospholipase (Dijkstra et al., 1978).

Despite the structural similarities and the knowledge of the Ca^{2+} binding site of phospholipases, the actual catalytic mechanism of these enzymes is yet to be established (Maraganore et al., 1984). The discovery of new structural types of phospholipases A_2 will certainly help in understanding the important characteristics of the structure of the enzyme that could be related to the catalytic activity.

The purpose of this paper is to report the purification and an extended characterization of a phospholipase enzyme present in the venom of the mexican beaded lizard. The N-terminal amino acid sequence of the *Heloderma horridum horridum* phospholipase A_2 reveals the uniqueness of the venom of these animals, considered to be "fossil animals" in danger of extinction (Tu, 1977), and the key role they might play in understanding comparative biochemical and structural features of certain types of proteins during evolution of the animal kingdom.

Experimental Procedures

Materials. Lyophilized whole venom was obtained as previously described (Alagón et al., 1982). Sephadex G-75, phenyl-Sepharose 4B, and AH-Sepharose 4B were from Pharmacia Fine Chemicals. DE-32 cellulose was from Whatman, Inc. Synthetic phospholipids were from Sigma Chemical Co. Lecithin analogue PC[1] was obtained from Berchtold Chemical Laboratory, Bern, Switzerland. Bee venom phospholipase was purified according to King et al. (1976). Specific anti-venom sera were prepared in three sheep as previously described (Alagón et al., 1982).

Methods. The quantitation of the phospholipase activity was determined by the titrimetric procedure of Shiloah et al. (1973). One unit of enzymic activity was defined as the amount of enzyme releasing 1 μmol of acid per minute at pH 8.0 and 25 °C in 3.0 mL of a 10% (w/v) solution of egg yolk in 0.1 M NaCl. When required, the phospholipase activity in slab gels was detected by clearing of egg yolk suspension with erythrocytes in 1.2% agarose gel (Haberman & Hardt, 1972).

For the purpose of studying the products formed on phospholipase digestion, we followed the standard procedure described by King et al. (1984), with some minor modifications. Synthetic phospholipids 1-stearoyl-2-oleoyl-3-*sn*-glycerophosphocholine and 1-oleoyl-2-stearoyl-3-*sn*-glycerophosphocholine were used as substrates. Fine suspensions of phospholipids (4 mg/ mL) were prepared by sonication in 10 mM Tris-HCl buffer (pH 8) containing 10 mM $CaCl_2$ and 0.5% Triton X-100, in an ultrasonic cleaner bath. The digests were examined by thin-layer chromatography on silica gel plates (DC Alufolien Kieselgel 60, Merck); 10 μL of digest was applied to the plate and developed in a system of chloroform–methanol–0.1 N HCl (70:30:5). Spots were visualized after exposure to iodine vapor. They were also examined directly for saturated and unsaturated fatty acids by chromatography on silica gel plates previously soaked by capillarity in 10% $AgNO_3$ dissolved in 82% methanol and dried at 100 °C for 30 min. After the plates were developed in a system of hexane–diethyl ether–acetic acid (80:20:1), the spots were visualized under UV light after being sprayed with 0.2% dichlorofluorescein in ethanol. R_f values for saturated and unsaturated fatty acids were 0.6 and 0.47, respectively.

The absorbent for affinity chromatography of phospholipase (Rock & Snyder, 1975) was prepared by a carbodiimide-promoted coupling of 240 mg of PC to 6 g of AH-Sepharose 4B. Protein solutions were concentrated by ultrafiltration with an Amicon PM-10 membrane. Dialysis was carried out in a Visking 8/32 membrane. Protein concentration was measured spectrophotometrically by assuming that 1 A^{1cm}_{280nm} unit = 1 mg/mL.

Electrophoresis was carried out in 7.5% polyacrylamide slab gels (0.1 × 10 × 12 cm) containing Tris buffer (Jovin et al., 1964) or in 15% gels containing Tris–SDS buffer (Laemmli, 1970). Proteins were visualized by staining with Coomassie Brilliant Blue R-250 (Swank & Munkres, 1971). Isoelectrofocusing was performed in 5.6% polyacrylamide gels with 2% ampholytes (pH 3–10) and 6 M urea (Awdeh et al., 1968).

Indirect hemolytic activity was measured as previously described (Sosa et al., 1979). The 50% hemolytic unit (HU_{50}) was graphically determined following Mayer's immune hemolytic technique as modified by De Hurtado and Layrisse (1964).

Immunodiffusion and immunoelectrophoresis were carried out in 1% agarose gels (King et al., 1978), and the precipitates were visualized after dye staining of dried and washed gels (Axelsen et al., 1973).

Amino acid composition was obtained by the time course hydrolysis method described by Moore (1963). Duplicate samples of protein were hydrolyzed under vacuum in individually sealed tubes with 6 M HCl containing 0.5% phenol, at 110 °C for 20, 48, and 72 h. Half-cystine content was determined as cysteic acid after performic acid oxidation. Approximately 1 nmol of protein was treated with 50 μL of 3% H_2O_2 in 90% formic acid for 3 h at 25 °C. The reagent was blown off under nitrogen at 50 °C. After being dried in vacuo, the sample was hydrolyzed as usual. The analysis of the hydrolysates was carried out on a Durrum D-500 amino acid analyzer.

The glucosamine and galactosamine contents were determined from the amino acid analysis after calibration with the proper standards. Carbohydrate content, excluding amino sugars, was measured by the orcinol–sulfuric acid method (Tsugita & Akabori, 1959).

For N-terminal sequence determination, phospholipase (10 nmol) was reduced, pyridylethylated (Friedman et al., 1970), exhaustively dialyzed against double-distilled water, and lyophilized. Two nanomoles in 0.2 mL of 20% acetic acid was evaporated to dryness in the spinning cup (previously treated with 3.0 mg of polybrene) of a Beckman Model 890 M sequencer, using program 3 (Possani et al., 1985). PTH-amino acid derivatives were identified and quantitated by high-performance liquid chromatography on a Hewlett-Packard 1084 B equipped with a variable-wavelength detector set at 269 nm.

Results

Purification of Phospholipase. Lyophilized whole venom was dissolved in 10 mM phosphate buffer (pH 6.8) and applied

[1] Abbreviations: AH, aminohexyl; DE, diethylaminoethyl; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PC, 1-(11-carboxyundecyl)-2-hexadecyl-*rac*-glycero-3-phosphocholine; PTH, phenylthiohydantoin; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

Table I: Summary of the Purification Procedure of the Phospholipase A_2 from *H. horridum horridum* Venom

fraction	source of fraction	protein (mg)[a]	total act. (units)[b]	sp act. (units/mg)	recovery (%)	ratio to sp act. of whole venom
whole venom	lyophilized venom	100.0	12 500	125	100.0	1.0
V	phenyl-Sepharose	8.0	5160	645	41.3	5.2
V.2	Sephadex G-75	4.8	3070	640	24.6	5.1
V.2.2	DE-32 cellulose	0.6	1225	2040	9.8	16.3
V.2.2.1	DE-32 cellulose	0.5	1295	2590	10.4	20.7

[a] Assuming 1 absorbancy unit at 280 nm equal to 1 mg/mL. [b] One unit is the amount of enzyme that releases 1 μmol of acid per minute in the conditions described under Experimental Procedures.

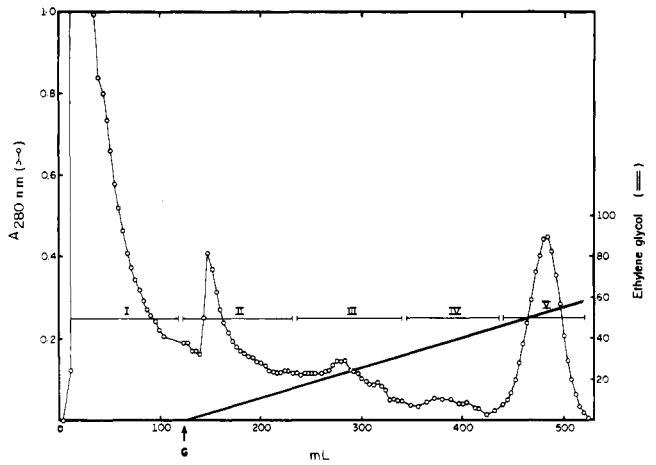

FIGURE 1: Fractionation of soluble venom. Soluble venom (8.0 mL; 158.8 A_{280}^{1cm} units) in 0.01 M sodium phosphate buffer (pH 6.8) was applied to a phenyl-Sepharose column (41.5 × 7.0 cm). The column was eluted with a linear gradient (200 mL each) from 0 to 58% ethylene glycol in the same buffer, at 60 mL/h. Fractions (4 mL/tube) were collected and pooled as indicated (horizontal bars; I–V). G indicates the starting of the gradient. Fraction V (with the phospholipase activity) was dialyzed against the buffer of Figure 2 and concentrated by ultrafiltration.

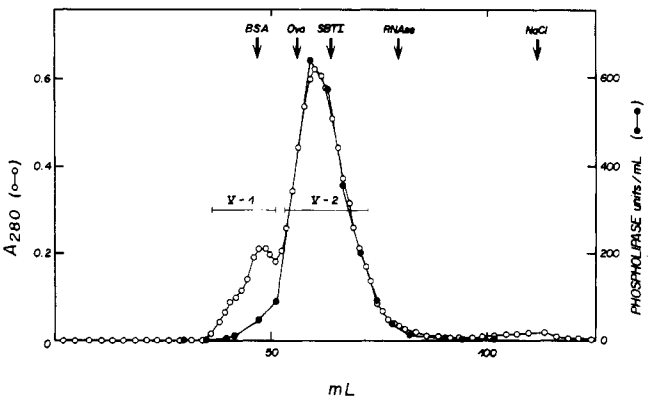

FIGURE 2: Gel filtration of fraction V. Fraction V (4.5 mL; 14.5 A_{280}^{1cm} units) in 0.05 M sodium phosphate buffer (pH 8) was applied to a Sephadex G-75 column (0.9 × 200 cm) and eluted with this same buffer, at 15 mL/h. Fractions (2 mL/tube) were collected, pooled as indicated (horizontal bars), and concentrated. Vertical arrows indicate the elution volume of BSA (bovine serum albumin, M_r 65 000), Ova (ovalbumin, M_r 45 000), SBTI (soybean trypsin inhibitor, M_r 21 500), RNase (ribonuclease, M_r 13 500), and NaCl (sodium chloride), used as molecular weight markers.

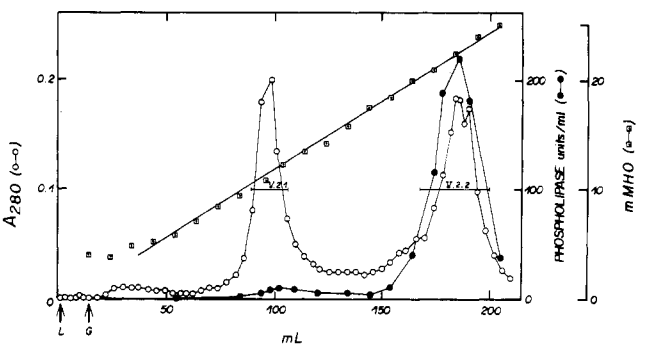

FIGURE 3: Ion-exchange chromatography of fraction V.2. Fraction V.2 (5.0 mL; 7.05 A_{280}^{1cm} units) in 0.05 M sodium phosphate buffer (pH 8) was applied to a DE-32 cellulose column (0.9 × 30 cm) and eluted with a linear NaCl gradient (200 mL each) from 0 to 0.5 M in the same buffer, at 60 mL/h. Fractions (2 mL/tube) were collected as indicated by the horizontal bars. Fractions V.2.2. was dialyzed against the starting buffer, concentrated, and rechromatographed in this column under the same conditions. L and G indicate loading of the sample and the starting of the gradient, respectively.

to a phenyl-Sepharose CL-4B column (Figure 1). Proteins were eluted with a linear ethylene glycol gradient to yield five fractions. Fraction V, which contained the phospholipase activity, was concentrated and used for gel filtration on Sephadex G-75 (Figure 2). Tubes containing phospholipase were applied to a DE-32 cellulose column eluted with a linear salt gradient; two fractions were collected (Figure 3). Fraction V.2.2, which accounted for most of the phospholipase activity, was dialyzed and rechromatographed in the same DE-32 cellulose column under identical conditions (results not shown). Tubes containing phospholipase activity (fraction V.2.2.1) were pooled, dialyzed against 5 mM ammonium acetate buffer (pH 8.3), concentrated, and stored at −22 °C until used. Table I summarizes the isolation procedures and recoveries of protein and phospholipase activity.

Characterization of Purified Phospholipase. The gel electrophoretic pattern in the presence of SDS of the purified enzyme (fraction V.2.2.1) showed a major protein band with an apparent M_r of 18 000 and a minor contaminant of 16 000; identical results were obtained after reduction with 1% 2-mercaptoethanol (data not shown). Under nondenaturating conditions, the above sample showed three protein bands. In order to identify the protein containing phospholipase activity, unstained gels without SDS were placed on the surface of agarose gels containing egg yolk suspension with erythrocytes. Two bands with clearing activity were located, either with crude venom or with purified phospholipase. Most of the activity matched the major band detected with Coomassie Blue, while the rest of the activity corresponded to a very faint band of slower mobility. This band is apparently a dimer of phospholipase, noncovalently bound, since it did not appear in SDS gels electrophoresis, even when higher concentrations of the sample were used.

The isoelectric point determined for the purified phospholipase was 4.5, and that of the small contaminant was 4.6 (data not shown). Immunodiffusion of the enzyme showed a single, rather broad, precipitin line (Figure 4A), while immunoelectrophoresis of the same sample gave one main arc and a smaller one, farther, in the direction of the anode (Figure 4B).

The enzymatic activity of the purified protein is Ca^{2+}-dependent. Preincubation of the enzyme, for 10 min, with 10 mM EDTA completely abolished its activity, when measured

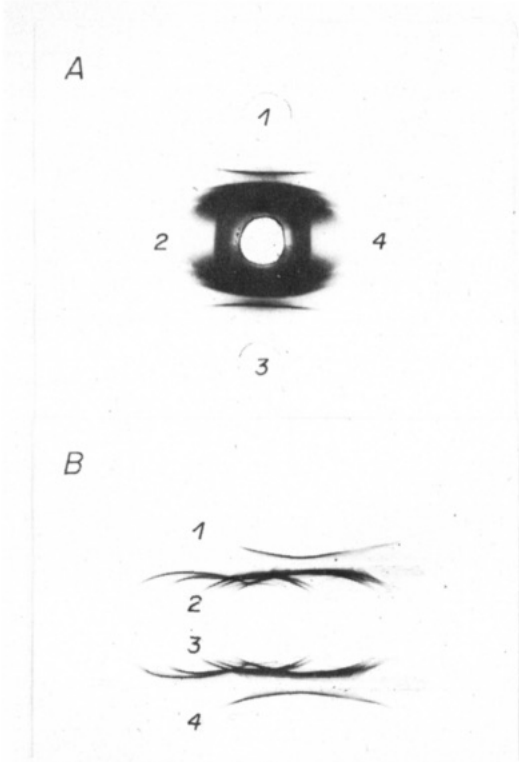

FIGURE 4: Immunodiffusion and immunoelectrophoresis tests. (A) Immunodiffusion test was carried out in 1% agarose containing 0.1 M Tris-HCl buffer, pH 7.2. The central well contained sheep anti-venom serum (20 μL); wells 2 and 4 contained 20 μg of purified phospholipase; wells 1 and 3 contained 200 μg of *H. horridum horridum* venom. (B) Immunoelectrophoresis in 1% agarose containing 0.025 M Tris-Hcl, pH 8.65, with sheep anti-venom serum (50 μL). (1 and 4) Purified phospholipase (20 μg); (2 and 3) *H. horridum horridum* venom (200 μg). The anode was at the right side.

titrimetrically with a suspension of egg yolk containing 10 mM EDTA. The activity is restored upon addition of calcium chloride to a final concentration of 25 mM.

The specificity of *H. horridum horridum* phospholipase was examined by thin-layer chromatography. Two synthetic phospholipids (4 mg/mL) were digested by phospholipase at 1.4 μg/mL final concentration, at pH 8 and 25 °C. Under these conditions, both 1-stearoyl-2-oleoyl-3-*sn*-glycerophosphocholine and 1-oleoyl-2-stearoyl-3-*sn*-glycerophosphocholine were completely converted to their respective monoacyl phospholipid in 2–5 min. The second substrate was also converted to glycerophosphocholine in about 6 h. Qualitative estimation of $AgNO_3$-treated chromatograms showed that after 10-min digestion only fatty acids at position 2 were released from the two substrates. After 6-h digestion, oleic acid was also released from 1-oleoyl-2-stearoyl-3-*sn*-glycerophosphocholine. Since the specificity of bee venom phospholipase A_2 is well-known (Shipolini, 1971), digests of this phospholipase at a final concentration 10 μg/mL were also examined in control experiments; only products corresponding to the hydrolysis of the acyl group at the 2-position were obtained, even after 6-h digestion.

The graphical determination of the 50% hemolytic unit for crude venom and for purified phospholipase A_2 gave 210 and 760 HU_{50}/mg, respectively. The hemolytic potency was only 3.6 times higher for the purified enzyme than for the unfractionated venom, which is less than the calculated ratio between the specific activity of the two samples (20.7 times; Table I). The above results suggest the presence of some other factors in the crude venom, which synergistically potentiate the hemolytic effect of phospholipase A_2, as previously discussed (Sosa et al., 1979).

Table II: Amino Acid Composition of Phospholipase A_2 from *H. horridum horridum* Venom[a]

amino acid	found[b]	nearest integer
aspartic acid	16.1 ± 0.4	16
threonine	9.0 ± 0.2	9
serine	11.9 ± 0.2	12
glutamic acid	21.2 ± 0.0	21
proline	5.4 ± 0.0	5
glycine	16.5 ± 0.6	16
alanine	12.8 ± 0.1	13
half-cystine	10.5 ± 0.6	10
valine	5.8 ± 0.4	6
methionine	5.2 ± 0.2	5
isoleucine	6.0 ± 0.4	6
leucine	11.7 ± 0.5	12
tyrosine	8.9 ± 0.4	9
phenylalanine	6.0 ± 0.5	6
histidine	5.7 ± 0.3	6
lysine	9.3 ± 0.4	9
arginine	3.8 ± 0.3	4
tryptophan	5.1	5
total		170
calculated M_r		19060

[a] Calculated on the assumption that the number of aspartic acid, glutamic acid, alanine, and leucine are 16, 21, 13, and 12 per mole of phospholipase A_2, respectively. [b] Average values ± SEM of duplicate hydrolysates at 20, 48, and 72 h. The serine and threonine contents were estimated by extrapolation to zero time. The value for half-cysteine was determined as cyteic acid after performic acid oxidation. Tryptophan was estimated from its molar extinction coefficient, according to King and Spencer (1970).

The amino acid composition of phospholipase A_2 from *H. horridum horridum* shown in Table II suggests that the enzyme is composed of 170 residue with a calculated M_r of 19060 in agreement with the apparent M_r of 18000 calculated by SDS gel electrophoresis. Phospholipase A_2 contained three residues of galactosamine (possibly acetylated) per mole of protein; glucosamine was not detected. The carbohydrate content of the enzyme by the method used (Tsugita & Akabori, 1959), which does not detected amino sugars, gave a value of 3.77 ± 0.32%. Thus, the peptide molecular weight (19060) is increased to 20420 if the total carbohydrate content is taken into account.

The N-terminal amino acid sequence of a reduced and pyridylethylated sample of the enzyme showed the following sequence: H_2N-Gly-Ala-Phe-Ile-Met-Pro-Gly-Thr-Leu-Trp-Cys-Gly-Ala-Gly-Asn-Ala-Ala-Ser-Asp-Tyr-Ser-Gln-Leu-Gly-Thr-Glu-Lys-Asp-Thr-Asp-Met-Cys-Cys-Arg-Asp-His-Asp-His-Cys. Only one amino acid was identified per cycle, and the repetitive yield was 95%. From this, we conclude that either the minor contaminant is blocked at the N-terminal position or more likely its concentration is too low to be detected.

Discussion

Many phospholipase enzymes have been purified from various sources: pancreatic tissue (Drenth et al., 1976; Evenberg et al., 1977; Fleer et al., 1978), honey bee venom (Shipolini, 1971), and numerous snake venoms (Randolph & Heirikson, 1982). The procedure described above for purification of the phospholipase from the *H. horridum horridum* venom was not a straightforward procedure. Initially, we have used the affinity column method described by Rock and Synder (1975) and King et al. (1983, 1984) in order to purify this enzyme. For comparative purposes and with the aim to stress the point, we are including in Figure 5 the chromatographic behavior of *Heloderma* venom in a PC–Sepharose 4B column prepared as described by the above-refered authors.

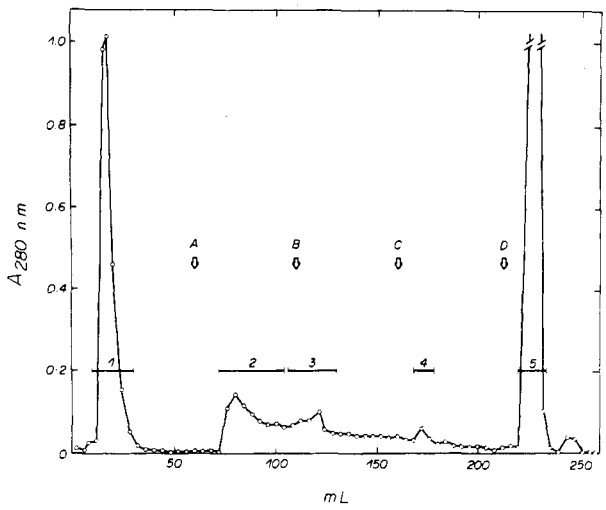

FIGURE 5: Affinity chromatography of crude venom on a column of Sepharose 4B with a bound substrate analogue of phosphatidylcholine. A total of 48 A_{280}^{1cm} units of soluble venom in 4 mL of 25 mM Tris-HCl buffer (pH 7.95) containing 25 mM calcium chloride was applied into a column of PC–Sepharose 4B (0.9 × 7.5 cm) and washed with 50 mL of the same buffer. Proteins fractions were eluted successively with 50 mL of each of the following solutions: (A) 0.05 M Tris-HCl buffer (pH 7.95) containing 0.5 M NaCl, (B) 0.05 M Tris-HCl buffer (pH 7.45) containing 50 mM EDTA, (C) 0.1 M acetic acid containing 0.5 M NaCl, and (D) 25% 2-propanol in 0.05 M Tris-HCl buffer (pH 7.95). Tubes of fraction 4 contained 0.3 mL of 1 M Tris-HCl buffer (pH 7.95). The column was eluted at 60 mL/h, and fractions of 2 mL were collected.

A control for the efficacy and goodness of the PC–Sepharose 4B resin that we have synthesized was established by isolating a wasp phospholipase from the venom of *Polistes dorsalis* (Amezcua & Alagon, 1984). As shown in Figure 5, several conditions were used to elute the enzyme from the column. However, no phospholipase activity could be detected in fractions 1–4. Fraction 5 was obtained with a more severe step and represented 20.3% of the protein applied to the absorbent. Fractions 5 from repetitive experiments contained from 2 to 60% of the initial phospholipase activity, when measured after dialysis against 25 mM Tris-HCl buffer (pH 7.95) containing 25 mM calcium chloride. The highest value was obtained when the protein was dialyzed immediately after its elution from the column. Electrophoretic analysis showed that fraction 5 was still a rather complex mixture of proteins with six major and five minor bands (data not shown). The average protein recovery was 75%. Attempts to purify the enzyme by means of increasing linear gradients of 2-propanol also failed to separate it from other proteins.

Since the PC–Sepharose 4B resin has large alkyl groups, it is conceivable that strong hydrophobic interactions between *Heloderma* proteins and the matrix of the affinity column prevent successful isolation of the phospholipase. For this reason, instead of the affinity PC–Sepharose 4B resin we have used a hydrophobic support (phenyl-Sepharose, see Figure 1) as the first step in the purification procedure. Three additional purification steps were included that used molecular weight sieving properties of the Sephadex gel (Figure 2) and ion exchanger resin (Figure 3) in order to obtain this enzyme at a degree of homogeneity that would allow further appropriate characterization. It was necessary to include the Sephadex G-75 step because the phospholipase activity was lost when the phenyl-Sepharose-eluted fractions were directly applied to a DE-32 cellulose column, at pH 8.0. This was interpreted as due to proteolytic cleavage from contaminants present in the venom at this stage of the purification procedure. After the last chromatographic step a highly purified fraction was obtained as mentioned under Results, containing a monomeric and a dimeric form of the enzyme as shown by the combined results of slab gel in nondenaturing and denaturing conditions. Because of similar molecular weights and isoelectric points of the phospholipase and the minor contaminant, it was impossible to separate them by these methods. In a separate experiment (results not shown), a slab gel run on this purified material was cut in the position of the main band corresponding to the phospholipase activity, and after this was eluted from the gel, it was used for amino acid analysis determination. In the sample obtained after the column shown in Figure 3, no proteinase activity was detected by the method described by Alagon et al. (1982), and since the main component (over 95%) was shown to have the enzyme activity, we decided to proceed with the chemical and enzymatic characterization of the phospholipase A_2.

The amino acid analysis of the phospholipase was obtained with samples purified independently from different batches of venom and gave a minimum M_r of 19060. The molecule contained 170 amino acid residues. Because it has five methionines, a sample of the enzyme was reduced, alkylated, and cleaved with cyanogen bromide. The six resulting peptides purified by HPLC (results not shown) are consistent with the calculated M_r of 19060. The high content of hydrophobic amino acids (13 Ala, 6 Val, 12 Leu, 6 Ile, 6 Phe, 9 Tyr, 6 Trp) is also consistent with the chromatographic behavior of the enzyme on solid supports containing alkyl or aryl groups (PC–Sepharose, phenyl-Sepharose). The immunodifusion and immunoelectrophoretic results obtained with both crude venom and purified enzyme (Figure 4) confirmed the results obtained by gel electrophoresis.

The specificity of the phospholipase activity assayed with two synthetic substrates indicates that this enzyme predominantly hydrolyzes the fatty acids at position 2 in the phospholipid molecule (type A_2 activity); also, when fatty acid at the 1-position in the monoacylglycerophosphocholine molecule is unsaturated, it can be hydrolyzed although at a slower rate (more than 6 h; type B activity). This does not occur with some other phospholipases A_2, as is the case for bee venom enzyme, which was used in these experiments as a control for type A_2 activity. The selective preference of purified phospholipase to hydrolyze unsaturated fatty acids has been reported previously for wasp venom phospholipase A_1 by King et al. (1984). Since the purified phospholipase still had a minor contaminant of lower molecular weight, we cannot eliminate the possibility that A_2 and B types of activities are in different protein molecules.

The activity of the phospholipase from *H. horridum horridum* is possibly related to a similar protein in the venom of the Gila monster (*Heloderma suspectum suspectum*) reported by Dehaye et al. (1984). These authors have described a pancreatic secretory protein (M_r 17500), which also displays phospholipase activity. Thus far, no chemical data are available on the Gila monster secretory protein molecule.

Sequence comparison of the N-terminal regions from bee venom, bovine pancreatic, and other snake venom phospholipases shows *H. horridum horridum* to be more homologous to the bee venom phospholipase A_2 than to the others (Figure 6). The amino-terminal amino acid sequence of phospholipase A_2 from hornet venom is also very different from the *Heloderma* phospholipase (Tuichibaev et al., 1985). Direct sequence comparison of the first 39 amino acid residues at the N-terminal region of *Heloderma* and bee venom phospholipase (Figure 7) shows a 56% homology. This somehow unexpected result makes the venom of *Heloderma* a more interesting one,

	1								10										20										
B. pancreas	A	L	W	Q	F	N	G	M	I	K	C	K	I	P	S	S	E	P	L	L	D	F	N	N	Y	G	C	Y	C
H. h. horridum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	A	F	I	M	P	G	T	L	W	C
A. mellifera	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	I	Y	P	G	T	L	W	C
N. melanoleuca (group I)	N	L	Y	E	F	K	N	M	I	Q	C	T	V	P	N	R	S	W	W	H	-	F	A	N	Y	G	C	Y	C
C. adamanteus (group II)	S	L	V	Q	F	E	T	L	I	M	K	V	A	K	R	S	G	L	L	W	-	Y	S	A	Y	G	C	Y	C

	30								40										50											
B. pancreas	G	L	G	G	S	G	T	P	V	D	D	L	-	-	-	-	-	-	D	R	C	C	Q	T	H	D	N	C	...
H. h. horridum	G	A	G	N	A	A	S	D	Y	S	Q	L	G	T	E	K	D	T	D	M	C	C	R	D	H	D	H	C	...
A. mellifera	G	H	G	N	K	S	S	G	P	N	E	L	G	R	F	K	H	T	D	A	C	C	R	T	H	D	M	C	...
N. melanoleuca	G	R	G	G	S	G	T	P	V	D	D	L	-	-	-	-	-	-	D	R	C	C	Q	I	H	D	N	C	...
C. adamanteus	G	W	G	G	H	G	R	P	Q	D	A	T	-	-	-	-	-	-	D	R	C	C	F	V	H	D	C	C	...

FIGURE 6: Comparison of amino-terminal sequences for phospholipases A_2 from bovine pancreas with others from the venom of *H. horridum horridum*, *A. mellifera*, *Naja melanoleuca*, and *Crotalus ademanteus* [these two last are representative of group I and II phospholipases A_2, respectively, according to Heinrikson et al. (1977)]. Gaps (–) are introduced to provide proper alignment of half-cystine residues and the greatest homology. Invariant residues are enclosed in boxes.

	1									10									20			
Heloderma	G	A	F	I	M	P	G	T	L	W	C	G	A	G	N	A	A	S	D	Y		11/20
Bee			I	I	Y	P	G	T	L	W	C	G	H	G	N	K	S	S	G	P		0.55
	*	*	*		*								*			*	*		*	*		

	31									30									40			
Heloderma	S	Q	L	G	T	E	K	D	T	D	M	C	C	R	D	H	D	H	C...			11/19
Bee	N	E	L	G	R	F	K	H	T	D	A	C	C	R	T	H	D	M	C...			0.58
	*	*			*	*		*			*				*			*				

	Total	22/39
		0.56

FIGURE 7: Comparison of the N-terminal amino acid sequences of the phospholipases A_2 from bee and *Heloderma* venoms. A Beckman program (Genes) was used to compare both sequences. Asterisks (*) mean different amino acids. The values on the right hand of the figure mean identical amino acids/total amino acids compared in that line, and the fractional number means the proportion of homology.

as mentioned in the introduction. The N-terminal sequence of the phospholipase from the venom of *Heloderma* is similar to that of the enzyme from been venom. It would be more logical to find greater homologies in the sequences of proteins coming from animals that belong to the same taxonomic group, like *Heloderma* and snakes (both from the class Reptilia, order Squamata), than to the bees (from the class Insecta, order Hemiptera). However, if we compare some properties of bee and *Heloderma* venom phospholipases, the bee phospholipase A_2 has a basic isoelectric point (p*I* = 10), and the *Heloderma* has an acidic phospholipase (p*I* = 4.5). The amino acid composition of *Heloderma* phospholipase (Table II) shows that it is rich in Asx (16 residues) and Glx (26 residues). Most of the Asx and Glx residues in bee venom phospholipase as reported by Shipolini (1971) are in the amidated form. It could well be that in the *Heloderma* the Asx and Glx residues are in the acidic form. Bee venom phospholipase A_2 contains four residues of glucosamine (presumed to be N-acetylated) per mole of protein (Shipolini et al., 1974), while *Heloderma* venom phospholipase A_2 has three residues of galactosamine per molecule. The total carbohydrate content is 7.88 and 6.66% for the bee and *Heloderma* venom phospholipases, respectively. These differences and possible differences at the C-terminal sequence (unknown so far) of the *Heloderma* phospholipase will certainly shed light on this interesting structural feature of phospholipases from different origins. It will also probably aid in the classification of phospholipase groups proposed by Heinrickson et al. (1977) and Maraganore et al. (1984).

Another interesting aspect of the results shown in Figure 6 is the positions of invariant amino acids. In this figure we have compared the two well-described groups of phospholipases with that of *Heloderma* and *Apis melifera* (bee). The rectangular boxes in the figure show that Gly-30, Gly-32, His-48, and Asp-49 are found in all sequences. Since the catalytic activity of *Heloderma* phospholipase, as well the others, is Ca^{2+}-dependent and the above-mentioned amino acids were shown to be involved at the Ca^{2+} binding sites of bovine

pancreatic phospholipase (Dijkstra et al., 1978), it is conceivable that both the *Heloderma* and the bee venom phospholipases also share the same type of amino acid at a similar Ca^{2+} binding site or catalytic active site (Dijkstra et al., 1981) of group I and II phospholipases from pancreatic juice and snake venoms. We are presently completing the amino acid sequence of the *Heloderma* phospholipase A_2 and expect this to contribute to a better understanding of this interesting enzyme.

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