

Analysis of the cDNA for phospholipase A₂ from honeybee venom glands

The deduced amino acid sequence reveals homology to the corresponding vertebrate enzymes

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A cDNA expression library was constructed from worker bee venom glands and screened with an antibody against phospholipase A₂. The nucleotide sequence of a positive clone with the largest insert showed an open reading frame that codes for part of the signal peptide, the pro-region and the entire mature enzyme of the bee venom phospholipase A₂ precursor. This sequence differs in the central region from the one determined by Shipolini et al. [*FEBS Lett.* 17, 39–40 (1971)] in showing, among other exchanges, two additional cysteines. The revised sequence of bee venom phospholipase is similar to the pancreatic enzyme in the spacing of cysteines and the presence of several amino acids known to be part of the active site or the Ca²⁺-binding region in identical positions. Moreover, these parts of the bee protein can be fitted into the three-dimensional structure determined for the bovine pancreatic phospholipase A₂ [Dijkstra et al. (1981) *Nature* 289, 604–606]. Contrary to earlier suggestions, we therefore conclude that the bee venom enzyme shows some homology to phospholipases from mammalian pancreas and snake venoms.

Phospholipase A₂ catalyzes the specific hydrolysis of ester bonds at the C2 position of 1,2-diacyl-3-*sn*-glycerophospholipids [1]. Secreted forms of this enzyme have been isolated from different sources, mainly from mammalian pancreas and from snake venoms. In the pancreas, the enzyme is present as a zymogen which is activated upon secretion into the duodenum by tryptic cleavage of a small pro-peptide from the amino-terminus [2]. Comparisons of the amino acid sequences of these enzymes have revealed a close relationship typical for proteins with a common evolutionary origin [1, 3]. Conserved residues include a histidine–aspartic acid pair which forms part of the active site [4], several residues involved in the binding of Ca²⁺ ions, as well as a number of disulfide bridges. The similarity between these enzymes of different origin has been corroborated by an analysis of the three dimensional structure of phospholipase A₂ from bovine pancreas [5, 6] and from the venom of the rattlesnake *Crotalus atrox* [7]. More recently, the structure of several prepro-phospholipases from mammalian pancreas as well as snake venom has been determined using recombinant DNA techniques [8–11]. Interestingly, the structure of the pro-region and hence the mechanism of activation is different in the mammalian and reptilian enzymes.

A phospholipase A₂ is one of the main components of the venom of the honeybee, *Apis mellifera* [12]. It is a glycoprotein containing one asparagine-linked oligosaccharide [13, 14]. Determination of the amino acid sequence of this enzyme [13, 15] as well as the assignment of the disulfide bridges [16], led to the conclusion that the bee phospholipase A₂ was unrelated to the vertebrate enzymes [1]. However, a phospholipase A₂ from the venom of a Mexican lizard was

shown by Sosa et al. [17] to possess an amino-terminal sequence similar to the bee venom enzyme.

In previous work, the structure of the precursors of the two bee venom peptides melittin and secapin were determined [18, 19] through the use of cDNA libraries from queen bee venom glands. As phospholipase A₂ is virtually absent from the venom of this caste [20], we have constructed a cDNA library from worker bee venom glands and screened this with a phospholipase A₂ antibody. From the nucleotide sequence of positive clones, the amino acid sequence of the bee venom phospholipase A₂ could be deduced. The sequence and the analysis presented here indicate homology to the vertebrate enzymes.

MATERIALS AND METHODS

Enzymes and reagents

DNA-modifying enzymes and restriction endonucleases were obtained from New England Biolabs (Schwalbach/Frankfurt, FRG), Bethesda Research Laboratories (Vienna), Stratagene (Heidelberg, FRG) and Boehringer Mannheim (Vienna). All radiochemicals were purchased from Amersham International (Amersham, GB). Oligonucleotides were kindly supplied by Dr G. Högenauer (University of Graz). All biochemicals and reagents were of highest purity commercially available.

RNA and DNA isolation

Total RNA was isolated from the venom glands of newly emerged queen bees and of worker bees of different ages using published procedures [19] with minor modifications. Poly(A)-rich RNA was then obtained by chromatography on

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Enzyme. Phospholipase A₂ (EC 3.2.2.4).

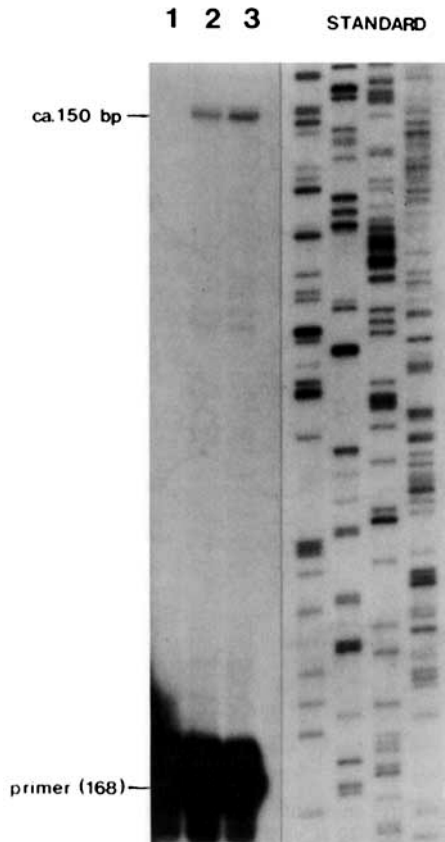


Fig. 2. *Primer extension experiment.* A radioactive single-stranded primer was isolated as described in Methods, annealed to different amounts of poly(A)-rich RNA from worker bee venom glands, and extended with reverse transcriptase. Products were analyzed on a sequencing gel using a sequencing ladder as size marker. Lane 1, no RNA; lane 2, 5 μ g RNA; lane 3, 10 μ g RNA

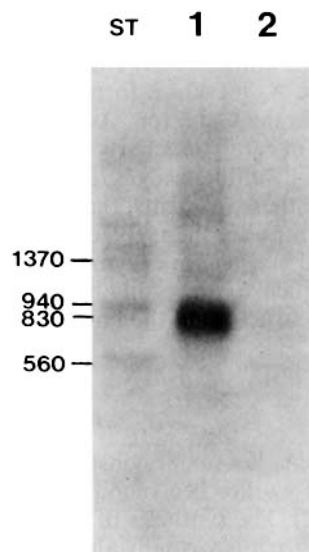


Fig. 3. *Northern blot analysis of RNA from queen and worker bee venom glands.* Poly(A)-rich RNA from worker bee (lane 1) and queen bee (lane 2) venom glands were fractionated in agarose/formaldehyde gels (see Methods) and hybridized with the nick-translated phospholipase cDNA insert. Size markers were end-labeled λ DNA fragments (ST)

50

a) -L-T-D-T-A-S- -R-L-S-C-N-N-N-D- -F-Y- -K-N-S-A-D-

b) -L-T-N-T-A-S-H-T-R-L-S-C-D-C-D-K-F-Y-D-C-L-K-N-S-A-D-

Fig. 4. *Comparison of the central region of the amino acid sequence of bee venom phospholipase A_2 taken from (a) Shipolini et al. [13, 15] and (b) the present work.* Differences are underlined

1 10 20

a) 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

b) I I Y P G T L W

1

*30 40 # * 50 +

C G L G G S G T P V D D L D R C C Q T H D N C Y

C 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 P

10 20 30

60 70 80

K Q A K K L D S C K F L V D N P Y T N N Y S Y S C S N N E I

D V M S A G E S K H G L

40 50

§ 90 § + 100 # 110

T C N S E N N A C G A F I C N C D R N A A I C F S K V P Y N

T N T A S H T R L S C D C D D K F Y D C L K N S A D T

60 70

120

K E H K N L D K K N C

I S S Y F V G K M Y F N L I D T K C Y K L E H P V T G C G

80 90 100

E R T E G R C L H Y T V D K S K P K V Y Q W F D L R K Y (b)

110 120 130

Fig. 5. *Comparison of the amino acid sequence of phospholipase A_2 from bovine pancreas (a) and honeybee venom (b).* The sequences are numbered above and below, respectively. Common cysteines and residues known to be part of the active site or the Ca^{2+} -binding region of the bovine enzyme are underlined. Pairs of cysteines marked with the same symbol (*, +, §, #) form disulfide bridges in the bovine enzyme

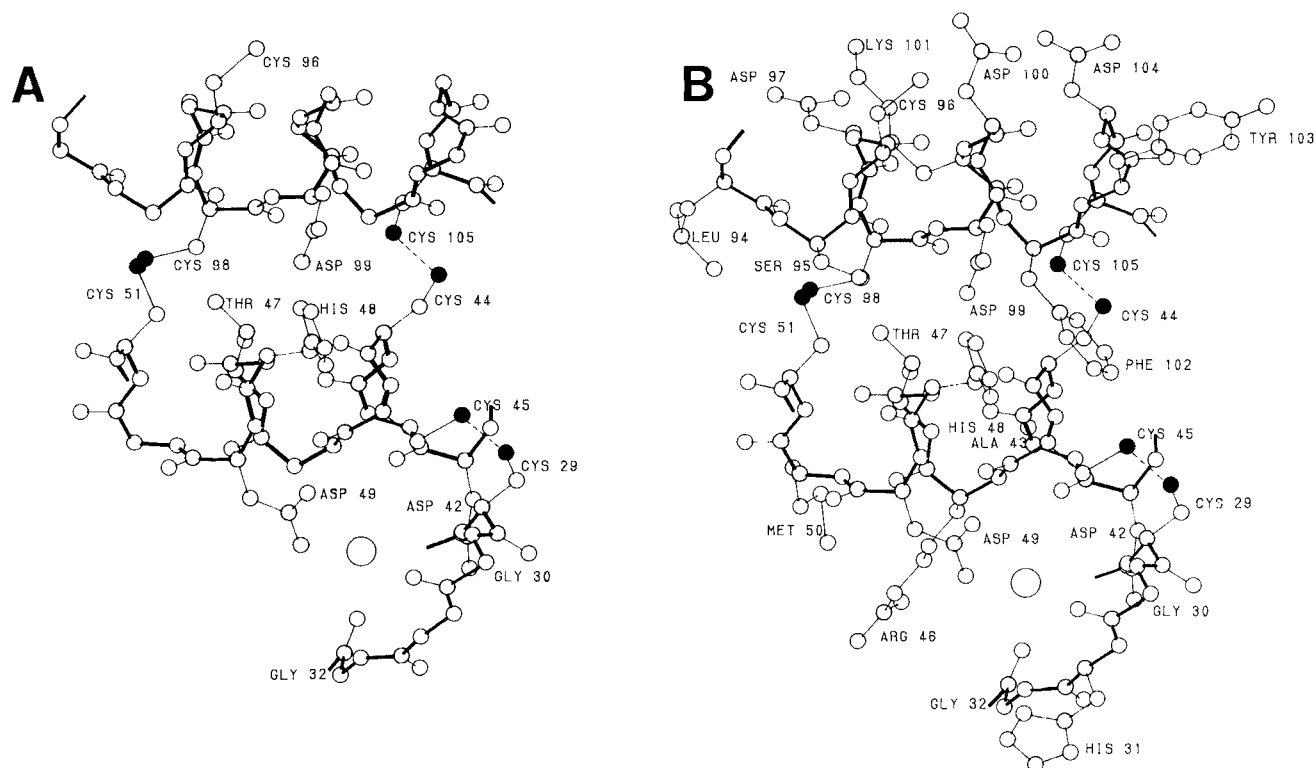


Fig. 6. Partial view of the X-ray structure of bovine phospholipase A₂ (taken from [6]). Three segments showing similarity to the bee enzyme are shown (numbers in parentheses refer to the sequence of the bee enzyme): Cys-29 — Gly-32 (Cys-9 — Gly-12), Asp-42 — Cys-51 (Asp-28 — Cys-37) and Phe-94 — Cys-105 (Leu-59 — Cys-70). Disulfide bridges are represented by dotted lines and the participating sulfur atoms by filled circles. The larger circle represents the Ca²⁺ ion. Bonds along the peptide backbone are emphasized by thick lines. In (A) the side chains of residues that are identical in the bovine and the bee enzymes are shown (numbers refer to the bovine sequence). Residues that are important for catalysis (His-48, Asp-99), ion binding (Asp-49) and the three disulfide bridges (Cys-29 — Cys-45, Cys-44 — Cys-105 and Cys-51 — Cys-98) are conserved in the two structures. In (B), the side chains of all the residues of the bovine enzyme in these segments are replaced by those of the bee sequence. Most of these replacements point away from the active site. Ala-102, Ala-103, and Phe-106 are replaced by Phe, Tyr, and Leu (not shown in the figure) in the bee enzyme. These replacements are not conservative, yet they provide a strong hydrophobic environment around the active site in the bee enzyme.

dicating that a single, rather small gene for this enzyme exists in the genome of *A. mellifera* (data not shown).

A comparison of the amino acid sequence of bee venom phospholipase A₂ determined by Shipolini et al. [13, 15] and the sequence deduced from the cloned cDNA shows that the two are identical in the amino- and carboxy-terminal regions, except that Asn-39 and Asn-92 are both Asp in the cloned cDNA. However, in the middle region ten differences were found and these parts of the two sequences are compared in Fig. 4. After residue 50, the cDNA sequence contains six additional codons including one for cysteine. A second cysteine codon was found at a position where Asn was detected in the published amino acid sequence.

The revised sequence deduced from the cloned cDNA demonstrates notable similarities between the insect and the vertebrate enzymes. Moreover, the extra cysteine residues call into question the disulfide bridges previously determined at the protein level [16]. Rather, by comparison with the amino acid sequences of vertebrate phospholipases A₂, alternative disulfide bridges seem to be more likely. The similarity between the insect and vertebrate enzymes is outlined in Fig. 5. This comparison reveals several common elements in the vicinity of the active site but also substantial differences (see Discussion).

In order to assess this similarity further, we tried to fit parts of the amino acid sequence of the bee enzyme into the

three-dimensional structure of the pancreatic enzyme. The results of such an attempt are shown in Fig. 6. Three segments were taken from the known X-ray structure of bovine pancreatic phospholipase A₂ [6] which form the active site and the region which binds the Ca²⁺ ion. As shown in Fig. 6A, the residues essential for catalytic activity and binding of the Ca²⁺ ion, as well as the disulfide bridges, are conserved in the bee venom enzyme. In these segments, the amino acid side chains of the bovine enzyme were then replaced with the corresponding residues of the bee venom enzyme (see Fig. 6B). Indeed, the bee venom sequence can readily be accommodated in the three dimensional structure of the bovine enzyme.

DISCUSSION

Phospholipase A₂ is a major constituent and the principal allergen [14,30] of worker bee venom constituting 10–15% of its dry mass [12]. The synthesis of this enzyme appears to be caste specific since it is virtually absent from the venom of queen bees [20]. According to our cDNA sequence data, the bee venom enzyme is derived from a precursor starting with a signal peptide typical for secreted polypeptides. Based on the predictions derived by von Heijne [31], signal peptidase most likely cleaves after Ser-11 or Gly-13 of the sequence shown in Fig. 1. The pro-region would then comprise 17 or

15 amino acids and thus be more than twice as long as that of pancreatic phospholipases [1]. In the former case, the amino-terminal sequence of bee venom pro-phospholipase A₂, i.e. His-Gly-Trp-Gln-Ile-Arg-Asp-Arg-Ile-, would in fact show some similarity to the amino-terminal end of the mature porcine pancreatic enzyme Ala-Leu-Trp-Gln-Phe-Arg-Ser-Met-Ile- (common residues are underlined) (see [1, 3]). Activation of the bee pro-phospholipase involves hydrolysis after the single arginine residue at the COOH-terminal of the pro-peptide. In this respect, it thus resembles the mammalian rather than the reptilian enzymes [1, 2, 10, 11]. It is noteworthy that the liberation of the bee venom peptide scapin from its precursor [19] also involves cleavage after a single arginine residue. Conversely, the biosynthesis of melittin, the main component of honeybee venom, proceeds via the stepwise cleavage of dipeptides from the amino end [32]. At least two different activation mechanisms for pro-polypeptides thus appear to exist in these glands.

The sequence of the mature bee venom enzyme as deduced from the cloned cDNA sequence comprises 134 amino acids. Apart from two Asn-Asp replacements, it is identical to the amino acid sequence determined almost 20 years ago by Shipolini et al. [13, 15] in the amino- and carboxy-terminal parts. However, as summarized in Fig. 4, several differences were detected in the central region. The earlier conclusions that no similarity exists between the vertebrate and the honeybee enzyme have to be reconsidered on the basis of our revised structure. It is noteworthy that several key features of the two central helices surrounding the active site of vertebrate enzymes [1, 5–7] are also present in the bee enzyme. These include the conserved sequences Cys-Gly-Xaa-Gly, Cys-Cys-Xaa-Xaa-His-Asp-Xaa-Cys and Cys-Xaa-Cys-Asp-Xaa-Xaa-Xaa-Xaa-Xaa-Cys (where Xaa stands for any of the 20 amino acids). In bovine pancreatic phospholipase A₂, six of the cysteines present in these fragments are known to form three disulfide bridges. Moreover, the histidine in the second and the aspartic acid residue in the third fragment have been shown to be part of the active site, while the two glycine residues in the first and the aspartic acid in the second peptide are involved in binding Ca²⁺ ions. We thus consider it likely that the same functional roles can be assigned to the corresponding amino acids in the bee venom enzyme. Indeed, as shown in Fig. 6, these parts of the bee enzyme sequence can readily be accommodated in the central helices previously determined for a pancreatic phospholipase A₂ [6]. These similarities in the first two segments mentioned above have in fact previously been noted by Maraganore et al. [33], who have already questioned the arrangement of disulfide bridges as determined by Shipolini et al. [16] and speculated about a possible similarity between the bee venom and the vertebrate enzymes [33].

Several other amino acids which are invariant in the vertebrate enzymes are, however, changed or absent in the honeybee enzyme. Of the proton-relay system proposed for the active site of pancreatic phospholipase A₂ [1, 5, 6], only His-48 and Asp-99 are present in the bee enzyme in apparently identical positions. Of the other residues in this structure, Tyr-52 is replaced by proline and no counterparts for Ala-1, Gln-4, Pro-68 and Tyr-73 are found in the primary sequence of the bee enzyme. On the other hand, of the four amino acids forming the proposed Ca²⁺-binding site, only Tyr-28 of the vertebrate enzymes, which supplies one carbonyl group, is replaced by tryptophan.

Compared to the mammalian and snake enzymes, the honeybee enzyme is shorter by 20 amino acids at the amino

end. Interestingly, in the sequence of the first 39 amino acids of the phospholipase A₂ from the venom of a lizard, 22 are identical to the bee venom sequence [17]. It would clearly be of some interest to know whether the other parts of the sequence of this phospholipase are more similar to other vertebrate enzymes or to the bee enzyme.

In the middle region, the insect enzyme lacks a segment of 21 amino acids which forms a loop on the surface of the three-dimensional structure of bovine phospholipase A₂ [6, 7]. Conversely, the honeybee enzyme has an insert of six residues between the region involved in the binding of Ca²⁺ ions (Cys-Gly-Xaa-Gly) and the active site histidine, and has a tail of 44 additional amino acids at the carboxyl end. This latter region also contains three cysteines, of which one must make a disulfide bridge with the first cysteine in the conserved Cys-Xaa-Cys-Asp sequence.

In our view, this comparison of vertebrate with an invertebrate phospholipase A₂ reveals common features typical for proteins having a common evolutionary origin. It would be most unlikely that the identical spacing of cysteines and the adjacent essential amino acids as outlined above could have been formed by convergent evolution. Rather, we favor the view that these parts in these enzymes are the products of divergent evolution originating from a common ancestor. As for the observed similarity between the amino-terminal sequences of the bee and lizard venom phospholipases, several explanations could be proposed. One possibility is that, early in the evolution of the corresponding genes, a duplication occurred with independent lines leading to the bee/lizard and the snake/mammalian pancreas types, respectively. On the other hand, the lizard enzyme may in fact be closely related to the other vertebrate enzymes in all parts but the amino-terminal region. Other reasons, like exon shuffling may then account for this localized similarity between two enzymes from widely different sources.

It should, however, be noted that in some parts of the primary sequence the differences between the honeybee and the typical vertebrate phospholipases are substantial indeed, much larger than usually encountered when comparing cytochromes *c*, glycolytic enzymes etc. from different phyla [34]. As mentioned above, several amino acids which are part of the charge relay system surrounding the active site of vertebrate phospholipases are absent in the structure of the insect enzyme. Current evidence is insufficient to decide whether in the bee enzyme a different active site is present or whether residues from other parts, particularly the carboxy-terminal extension, perform analogous functions.

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