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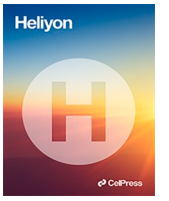

Research article

Vicia sativa subsp. *sativa* native to the Middle East comprises Pea Albumin1 b-like homologs: A promising natural biopesticide

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ABSTRACT

The extensive and indiscriminate use of chemical pesticides in agriculture has led to adverse effects on human health, environmental pollution, and the emergence of pesticide-resistant pests. To mitigate these challenges, the development of environmentally friendly alternatives is crucial, with biopesticides emerging as promising solutions such as peptides. Legume seeds naturally contain diverse insecticidal peptides or proteins to combat pest attacks. One such peptide is PA1b (Pea Albumin 1, subunit b), a 37 amino acid extracted from pea seeds (*Pisum sativum*). PA1b has shown significant potential in controlling cereal weevils (*Sitophilus* spp.), a major pest of stored cereals. Here, we screened PA1b-like peptides in five wild seeds of vetches (*Vicia sativa* subsp. *sativa)* from the Middle East. Using a comprehensive set of biochemical, biological, and molecular techniques, we characterized different PA1b homologs and assessed their toxicity and expression profiles. Our results reveal that PA1b homolog from *Vicia sativa* subsp. *sativa* originating from turkey displays outstanding insecticidal activity against *Sitophilus oryzae* through binding to the receptor site found in the midgut of the insect. Moreover, it exhibits a strong cytotoxic effect against Sf9 cells. This cysteine-rich peptide shows sequence identity and the same hydrophobic pole as AG41, a tenfold more toxic isoform of PA1b from *Medicago truncatula*. Such observations pave the way for the development of bioinsecticides, with PA1b-like peptides as lead compounds.

1. Background

Agriculture is characterized by a high proportion of cereal production, with yields reaching around 2.7 billion tons every year [1]. This production is important as food for the growing population and as animal feed. However, biotic and abiotic constraints affect this yield [2,3]. One of the most prominent biotic stresses affecting cereals during storage is the presence of insects. Cereal weevils (Coleoptera) are considered to be one of the most threatening insects that feed on intact whole grains [4]. In a survey conducted in Tanzania, Mihale et al. reported, that nearly 70% of the farmers experienced post-harvest losses of stored grain due to cereal weevils from the *Sitophilus* genus (Coleoptera: Curculionidae) [5]. Chemical treatments are used to mitigate the huge losses caused by these pests and to ensure that grain can be shipped between countries. Unfortunately, the misuse of chemicals leaves residues behind. In fact,

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these insecticide residues are of great concern to human health and components of the environment [6,7].

Reducing pesticide use while maintaining the sufficient quantity and good quality of food production is a common goal among different countries and a major insight for public and private policy instruments [8,9]. This is where the European Green Deal comes in defining a roadmap towards a sustainable agriculture through different applied strategies. At the center of this deal is farm-to-fork strategy which aims to reduce pesticide use to 50% by 2030. Current trends are not on track to achieve such a goal. New agronomic, technological, social and economic incentives must therefore be put in place in this perspective [10]. Alternatives to the unwise use of chemicals are available such as integrated pest management, cultural control, organic farming, transgenic applications and biological control [11].

To control pests of economically important crops, reduce the adverse effects of synthetic insecticide use, and preserve the ecosystem, new bioactive compounds of natural origin with much better target specificity are highly desirable. Plants are one of the most promising sources of naturally occurring compounds that could help in the development of biopesticides. Entomotoxic compounds derived from plants and in particular from edible ones are of great interest in the field of crop protection. Toxins produced by plants include chemical compounds such as tannins, terpenoids, flavonoids, alkaloids, quinones, phenols, and linomoides, molecules of proteinaceous nature such as thionins, defensins, proteinase inhibitors, lectins, and chitinase, and volatile compounds such as essential oils [12–14].

Legumes are members of the Fabaceae family (also known as Leguminosae), which is the third largest family of angiosperms with 800 genera and nearly 20,000 species [15]. Legumes contain defense compounds that have been investigated for their insecticidal potential. In 1986, Higgins et al. has identified a 37 amino acid peptideknown as pea albumin 1 subunit b (PA1b) from the seeds of peas *Pisum sativum*, the primary and gene structure of which was elucidated. The PA1 gene is reported to contain two exons separated by an intron. This gene is transcribed as a single mRNA encoding the preproprotein PA1(13.9 kDa, 153 a. a). PA1 undergoes post-translational cleavage to produce the mature form of PA1a (6 kDa, 53a.a) of unknown function and that of PA1b (3.8 kDa, 37a.a). Until 1998, the function of PA1b was known to be sulfur storage. Then, Delobel and his colleagues discovered and patented the insecticidal properties of this peptide. PA1b was identified as one of the few known plant peptides that is orally activated and has significant toxicity against cereal weevils (*Sitophilus oryzae*, *S zeamais*, and *S granarius*) [16], and other insects such as the pea aphid (*Acyrthosiphon pisum*) [16,17], and mosquitoes (*Culex pipiens* [17], *Aedes aegyptii* [18]), with no evidence of toxicity against beneficial insects such as bees (*Apis millifera*) and *Trichogramma* wasps (*Trichogramma pretiosum*) [17,19].

The three dimensional structure of PA1b, determined by NMR [20], shows that PA1b belongs to the inhibitor cystine knot (ICK) family. It contains six cystine residues interwoven with three disulfide bridges The tight packing of the cysteine residues in a knottin fold gives this peptide an outstanding stability against thermal and protease inactivation, extreme pH and mechanical stress[16,21].

In insects, PA1b acts by binding and inhibiting the activity of V-ATPase [22], a proton pump essential for nutrient absorption composed of 14 subunits organized into a V0 membrane complex and a V1 cytosolic complex [23–25]. Upon ingestion, PA1b binds to c and e subunits of the V0 complex of V-ATPase [26]. Once bound, PA1b stops ATP hydrolysis and disrupts the acidification of the lumen leading to the inhibition of nutrient absorption and ultimately causing the insect's death [22,26].

Homologs of PA1b have been identified in several legumes of the Faboideae subfamily. Louis and her colleagues discovered new albumin 1 (A1) genes from bean *Phaseolus vulgaris* and soybean (*Glycine max*) [14]. A study on 88 Fabaceae species revealed 19 PA1 genes from species of Faboideae but not from Caesalpinioideae or Mimosoideae. 53 A1 genes were also detected in the genome of *Medicago truncatula* showing the diversity of A1 peptides in this genus but none has been identified biochemically [27]. In addition, PA1b like amino acid sequence was revealed from the seeds of lentils (*Lens culinaris*) [28] as well as from the roots of *Astragalus membraneceous* [29].

In order to use PA1b on a large scale, several attempts have been made to mass-produce the peptide. However, the production of PA1b from peas has proven challenging in tested systems. Attempts to express the peptide in fungi or yeast have yielded nonfunctional forms, and expression in tobacco or baculovirus systems has resulted in low yields ([30]; Karaki, 2013). Chemical synthesis followed by in vitro folding remains the most viable approach [31]. However, it is costly and time-consuming. Consequently, the discovery of more potent homologs of Albumin 1b that exhibit higher toxicity at lower concentrations represents a promising solution.

This research aims to identify a novel potent PA1b homolog from a new source that could help overcome the challenges of mass production of such a peptide. The toxic variability and expression profiles of PA1b homologs in the seeds of five wild *Vicia sativa* subsp. *sativa,* belonging to different regions of the Middle East, were screened. Using a combination of biochemical, biological, and molecular approaches, several PA1b homologs were characterized. The PA1b homolog from *V. sativa* subsp. *sativa* from Turkey shows outstanding insecticidal activity against *Sitophilus oryzae* by binding to the receptor site located in the midgut of the insect. It also has a strong cytotoxic effect against cellular Sf9 cells. The genomic DNA sequence of the PA1b homolog from this seed previously characterized [32], shows sequence identity to AG41, a PA1b homolog from the alfalfa (*Medicago truncatula*) roots displaying high insecticidal activity against weevils [33]. The resulting structural modeling of the identified sequence also shows that it has the same hydrophobic pole as AG41. This study makes it possible for the potential use of more potent PA1b-like molecules, in the biological protection of cereal grains.

2. Materials and Methods

2.1. Biochemical characterization

2.1.1. Plant material

Two grams of seeds of each of *V. sativa* subsp. *sativa* species, originating from different regions of Middle East were provided by

ICARDA (International Center for Agricultural Research in the Dry Areas). To maximise the amount of fine powder collected, two seeds of each species were placed in an Eppendorf and mechanically crushed using a Tissue Lyser (Qiagen, Hilden, Germany) with two stainless steel beads (2.4 mm) shaken for 5 min at 20 Hz. Pea seed *P. sativum* (var. Isard) were provided in a batch of 5 Kg by Agri Obtentions (Filiale INRA, France). Pea seeds were ground in a Moulinex grinder and sieved through a 0.4 mm mesh to remove any remaining cuticle particles. A very fine powder was obtained which was used for peptide purification, and bait preparation.

2.1.2. Peptide extraction

Fine powder from each seed was subjected to H2O/EtOH extraction (40/60, 1 ml for 100 mg of powder) for 2 h at room temperature with constant stirring on a rotarymixer. The extract was then filtered through a 0.45 μm RC filter membrane (Phenomenex). The filter was washed twice with half the amount of ethanol added for extraction. Ethanol was then evaporated using speed vac (SPD111V, RVT400, Thermo Scientific). The resulting powder was re suspended in 60% ethanol (0.5 ml for 1 mg of powder) and then the suspension was passed through a 0.2 μm RC filter membrane (Phenomenex). The filter was also washed twice but this time with the same volume ethanol added for re suspending the powder.

2.1.3. HPLC

Reverse-phase High-Performance Liquid Chromatography (HPLC) was performed by injecting seed extract from each species onto a Jupiter® C18 LC column (250 × 4.6 mm, 5 μm particle size, 300 Å porosity, Phenomenex) on an Agilent 1200 HPLC instrument. PA1b like peptides were eluted at 0.5 ml/min with a 30 min linear gradient from 38% to 70% acetonitrile in water (0.04% TFA). The chromatographic program consisted of the following multistep gradient H2O+0.04%TFA (eluent A)/ACN+0.04%TFA (eluent B) 62/38 for 2min, 70% B for 14min and finally 38% B for 14 min. The elution was then monitored by UV diode-array detection at 210 nm. Quantification of the released peptides was based on HPLC peaks observed between 15 and 18 min retention time. PA1b-like fractions were purified, harvested, and lyophilized (Christ Alpha 1–2 LD plus Lyophilizer). Lyophilized products were then re suspended in EtOH 60% (1 ml for 1 mg of lyophilized product) for mass spectra analysis, binding assays, and in water for bait preparation.

2.1.4. LC/MS analysis

Purified PA1b-like peaks from all *Vicia* species were monitored by ESI-MS on a single quadrupole mass spectrometer (Alliance HPLC (Waters 2695) coupled to an ACQUITY QDa mass detector and 2998 PDa detector) in positive ion mode and scanning from *m/z* 100 to 10000. Program conditions and specifications were typically the same as those used for quantitative analysis, but 0.1% formic acid was used instead of 0.04% TFA. The most abundant peptide from *Vicia sativa* subsp. *sativa* (63977) from Turkey was analyzed using an Ultimate 3000 nano-RSLC (Thermo Scientific, San Jose California) coupled on line to a Q Exactive HF mass spectrometer via a nano-electrospray ionization source (Thermo Scientific, San Jose California) to obtain its monoisotopic mass.

2.1.5. SDS-PAGE electrophoresis and Western Blotting

Purified peptides were separated on the basis of molecular weight by sodium dodecyl sulfate SDS polyacrylamide gel electrophoresis using NuPAGE™ 4–12% Bis-Tris Gel (1.00 mm ×10 well) gel (Invitrogen). Samples were stained with 3% Coomassie Blue solution. To validate our proteomic data, an unstained gel was electro transferred onto a nitrocellulose membrane (Novex iBlot Gel Transfer stacks nitrocellulose) using the iBlot® 7 min dry blotting system (Invitrogen) according to the manufacturer's instructions. Novex™ sharp pre stained ladder was used (Invitrogen), thesize range varies between 3.5 and 260 kDa was used. The membrane was blocked in 5% skimmed milk in Tris buffered saline (TBS: 50 mM Tris, 200 mM NaCl, pH 7.4) for 1 h, then rinsed twice with Tween 20 TBS (0.05% detergent in TBS (v/v)) and once with TBS for 5 min. Polyclonal anti-PA1*b* antibodies, provided by Julie Petit (Covalab, France), in milk were then used at 1/500 dilution for 1 h, and the membrane was then rinsed for 5 min twice with TTBS and once with TBS. Alkaline phosphatase conjugated secondary antibody (Pierce Goat Anti-Rabbit, Thermo Scientific) was then added in TBS at 1/50000 dilution for 1 h. Labeled PA1b-like peptides were revealed by 1-step^{TM}NBT/BCIP substrate solution (Thermo Scientific) after an incubation for 10 min in the dark. Finally, the membrane was rinsed with distilled water and left to dry. A photo was taken using Chemidoc Imaging System (Bio Rad).

2.2. Biological toxicity assays of PA1b and its homologs

2.2.1. Weevils

Toxicity bioassays were conducted on two strains *S. oryzae*. The susceptible strain "WAA42" was reared on wheat seeds (*Triticum aestivum*) whereas PA1b resistant strain "ISO3R2", housing the recessive pea-resistance allele [34], was reared on *P. sativum*. As described by Delobel et al. [16], 2 weeks old 30 adult weevils were released on wheat-based flour dumplings containing the desired seed flour or seed peptide fraction. Whole flours were tested at a concentration of 20% (w/w in wheat) (50 mg of seed flour+200 mg of wheat flour+160 μl ultrapure water). Purified peptide of the seed of interest Vs63977 was tested at a concentration of 400 μg per gram of food. Dumplings were left to dry overnight and then placed in cylindrical grilled plastic boxes (2 cm diameter, 4 cm height). Feeding insects were incubated at 27.5 ± 0.5 °C and 70 ± 0.5 % relative humidity in a ventilated incubator (Memmert) in the dark, and mortality was monitored for up to 14 days.

2.2.2. Sf9 cells

Ovarian Sf9 cells adherent cultures from the fall armyworm *Spodoptera frugirperda* were initiated from a frozen stock at BF2I. Cells were grown at 27 °C in Lonza's culture medium (Insect-XPRESS™ Protein-free Insect Cell Medium with L-Glutamine) (BioWhittaker),

supplemented with 5% fetal bovine serum (FBS, Carlsab, USA) and 0.1% antibiotic gentamicin solution at 10 mg.ml-1 (SIGMA). Cells were cultured in 4 ml of the medium at a rate between $15 \times 10^4$and 20×10^4 cells per 25 cm2 flask to facilitate their adhesion to the support (Nunc SoLo Flasks, Thermo Scientific). One week later, a confluent monolayer was observed where cells are in mid-log phase of growth. At this phase, cells were mechanically dislodged, counted, and sub cultured up until the 35th passage. The medium was changed once in each culture to ensure robust cell growth. Purified peptides from different origins of *Vicia sativa* species and PA1b purified from Pisum sativum were diluted with culture medium to obtain 11 different concentrations. Sf9 cells at 90% confluence were dissociated in a 2 ml of the culture medium, counted using a Molassez cell under an inverted microscope, and diluted to a concentration of 1×10^6 cells per ml of culture medium. Diluted Sf9 cells were seeded into a 96-well plates (2×10^4 cells/well) and were exposed to increasing concentrations of each purified peptide. For each concentration, 3 replicates were done. The cells were then incubated at 27 °C. An hour later, the phenotype of cells was observed. Three hours later, cell viability was determined using the CellTiter-Blue® kit (Promega, Madison, USA) where 20 μl of CellTiter-Blue reagent was added in each well according to the manufacturer's instructions. After the addition of the dye, cells are re incubated for 2 h and the absorbance, at 570 and 600 nm is measured using a microplate reader (MR 7000, DYnatech Laboratories Inc., USA) every 30 min.

2.2.3. Binding Assays

As described by Gressent and his colleagues [35], proteinaceous membranes from the susceptible strain of weevils 'WAA42' were isolated and a mixture of PA1b isoforms extracted from *P. sativum* var. Isard seeds was labeled with the radioactive iodine I-125. In a total volume of 204 μl, microsomal proteins (isolated quantity ranged between 1 and 3 μg) were incubated with 0.4 nM ^{125}I-labeled PA1b in binding buffer supplemented with 0.2%CHAPS. The nonspecific binding component was evaluated by the addition of 1 μM nonlabeled mixture of the PA1b isoforms (PA1b from *P. sativum* and PA1b like molecules from *Vicia* species). Samples were seeded into a 96 well microtiter plates and incubated for 2 h at room temperature, and then transferred onto Multiscreen filter plates containing GF/B filters previously treated with 20 mM Tris-HCL buffer pH 7.5 + 0.4% polyethylenimine PEI. Filters were then vacuum drained by means of Multiscreen system (Millipore, USA) and then washed with 200 μl of washing buffer (20 mM Tris-HCL buffer pH7.5). Finally, filters were placed in a gamma counter tubes where radioactivity was measured on a gamma counter (Riastar, Packard Instrument, USA) with each point being the mean of three readings.

2.2.4. Statistical analyses

Data were analyzed by nonlinear regression model using the free software Simfit (http://www.simfit. man. ac.uk), where binding affinities (Kds) from binding assays and LT_{50} (lethal time 50%) and LC_{50} (lethal concentration 50%) from toxicity assays were calculated.

2.3. Molecular PA1b-like sequence from the most abundant and toxic seed

2.3.1. Sequence analysis

The genomic nucleotide sequences of partial A1 gene previously amplified using PCR approach from *Vicia sativa* subsp. *sativa* originating from Turkey were translated in the six open reading frames using the translate tool (https://web.expasy.org/translate) [32]. The peptide sequences with PA1b motifs were selected. The selected peptide sequences were blasted against the peptide sequence of PA1b (1P8B_A) using the protein blast in NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The theoretical average and monoisotopic molecular weight (Mw), as well as the isoelectric point (pI) of the obtained peptide sequences, were estimated using the software compute pI/Mw (https://web.expasy.org/compute_pi). The sequence that had a similar monoisotopic mass as that obtained experimentally by means of mass spectrometry was selected for comparative modeling.

2.3.2. Structural sequence alignments and comparative modeling

Sequence alignments (Fig. 4A) were performed using CLUSTAL OMEGA1 [36]. Comparative modeling was performed using ORCHESTRAR homology modeling program in the SYBYL-X 2.0 software package (TRIPOS Inc., St Louis, MO). AG41 NMR structure (Diya et al., 2023) was used as a template to build the 3D structure model of the peptide of interest from *Vicia sativa* subsp. *sativa* (63977).

3. Results and discussion

3.1. Variability of quantities and chromatographic profiles of PA1b-like molecules

PA1b like peptides from five seeds of *Vicia sativa* subsp. *sativa* belonging to different origin of the Middle East were extracted and detected. HPLC Chromatograms of each seed extract displayed an absorbance at a wavelength of 210 nm with slight absorbance at 280 nm signaling a peptidic nature. Additionally, all of the identified peaks were released in the PA1b zone area between 15 and 20 min retention time (Fig. 1A). Quantification of PA1b like molecules was performed based on the peak areas of the chromatographic profiles observed on HPLC. Table 1 shows the origin of each seed with its corresponding code number relative to its origin and the amount of PA1b like molecules found in each. The quantity of PA1b like molecules varied between geographical origins: the highest quantity (0.15%) was detected in seeds originating from Turkey 63977, followed by that originating from Syria 139275 and from another region in Turkey 61377 (0.12%) with the lowest amount (0.1%) found in that originating from Cyprus 60661 and that from Lebanon 60614. Further purification of the fractions corresponding to PA1b like molecules from the above studied seed selection were then

analyzed on mass spectrometer LC/MS. Electron spray ion mass spectrometer for all purified peptides revealed various expression profiles with *m/z* values between 960 and 1000 as shown in Fig. 1B corresponding to an PA1b like peptide. It seems that PA1b-like isoforms from species of *V. sativa* subsp. *sativa* are not as diverse as that in peas (3–4 copies). Putative different experimental masses of peptides ranged from 3.7 kDa to 4.0 kDa. This range is within the known masses of previously identified PA1b-like peptides from other legumes of the Faboideae subfamily [13]. The variation in the expression and quantification of PA1b-like peptides in populations of the same legume species coming from different origins could be attributed to the genetic variation, and environmental stressors [37] subjected to each seed. Given that Albumin 1 peptides are mainly expressed in the seeds [16,38], the expression of such a peptide family could be highly affected by the type of soil, availability of nutrient, and the developmental stage of the seed. For instance, sulfur, an essential macronutrient, is a critical element for grain yield and quality, sufficient accumulation and good composition of seed storage proteins in legumes [39]. In *P. sativum*, the expression of PA1b in developing seeds is negatively affected in sulfur deficient environment. It has been reported that this is due to reduced post-transcriptional stability of mRNA, resulting in decreased accumulation of PA1 peptides in mature seeds [38]. To gain insights into the molecular mechanisms occurring in developing seeds and which are involved in the regulation of the expression of A1 gene, a transcriptional analysis through the development of each studied seed could be carried out.

Since *V. sativa* subsp. *sativa* originating from Turkey contained the highest amount of peptide, the molecular weight of the peptide sequence of interest previously amplified using genomic DNA [32] was confirmed by high resolution mass spectrometry (Supplementary Fig. S1).

3.2. PA1b peptide characterization

Chromatographic fractions from all seeds were purified and analyzed by SDS-PAGE and Western Blotting. SDS PAGE identified peptides from all seed fractions of *Vicia sativa* subsp. *sativa* in the range of 3.7–4.0 KDa which are in the same range as PA1b from peas (Fig. 2 A). Proteomic data were confirmed with anti-pea PA1b polyclonal antibodies, designed on the highly conserved zones of the PA1b peptide. The designed antibodies recognized all the A1b like peptides purified from seed extract of all *V. sativa* subsp. *sativa* of different origin in the Middle East region (Fig. 2 B). The appearance of a double-sized band of the tested peptide is probably due to the its dimeric form.

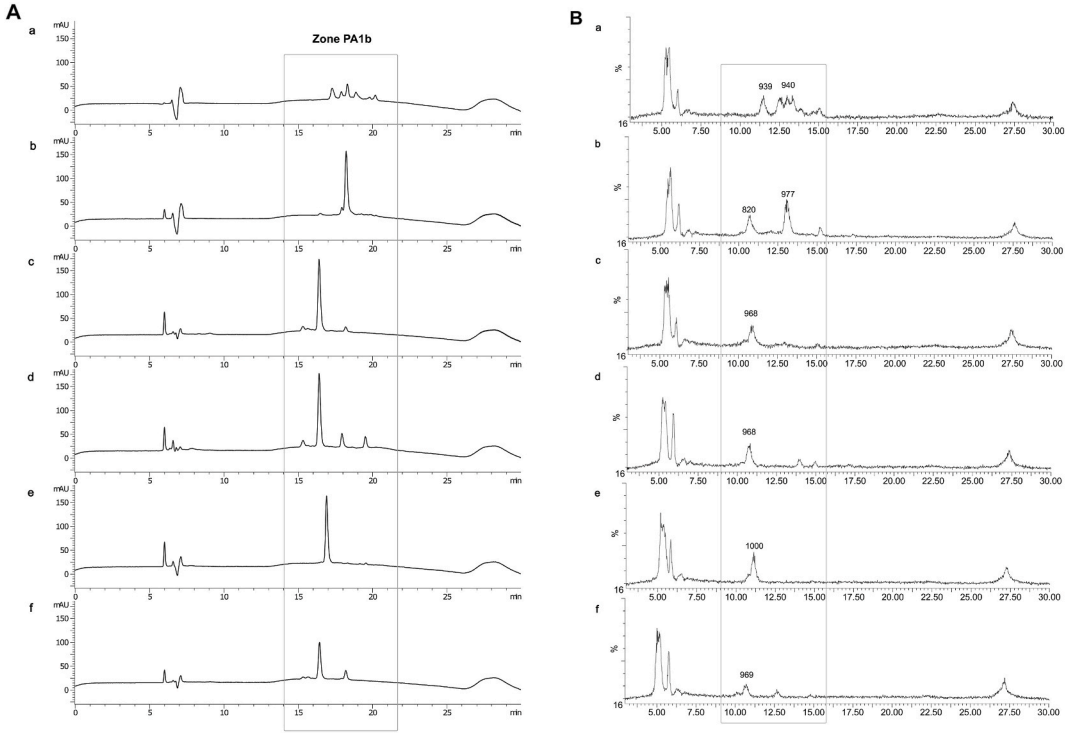

Fig. 1. Chromatographic profiles of purified peptides from seed extract of each of: (a) *Pisum sativum* (var. Isard), (b) *Vicia sativa* subsp. *sativa* 63977, (c) *Vicia sativa* subsp. *sativa* 139275, (d) *Vicia sativa* subsp. *sativa* 61377, (e) *Vicia sativa* subsp. *sativa* 60661, (f) *Vicia sativa* subsp. *sativa* 60614. (A) HPLC Chromatographic peaks from all *Vicia* species were observed in the zone of PA1b known from peas between 15 and 20 min retention time.(B) LC/MS ion chromatograms with *m/z* values corresponding to PA1b-like molecules.

Table 1
Geographic distribution of *Vicia sativa* subsp. *sativa* characterized in this study from the Middle East region and the quantity of PA1b like molecules obtained from each.

Seed	IG[a]	Origin	Quantity of PA1b-like (%)
Vicia sativa subsp. *sativa*	63977	Turkey	0.15
Vicia sativa subsp. *sativa*	139275	Syria	0.12
Vicia sativa subsp. *sativa*	61377	Turkey	0.12
Vicia sativa subsp. *sativa*	60661	Cyprus	0.1
Vicia sativa subsp. *sativa*	60614	Lebanon	0.1

For further information, please refer to supplementary Table 1
[a] Acession number issued by ICARDA (International Center for Agricultural Research in the Dry Areas).

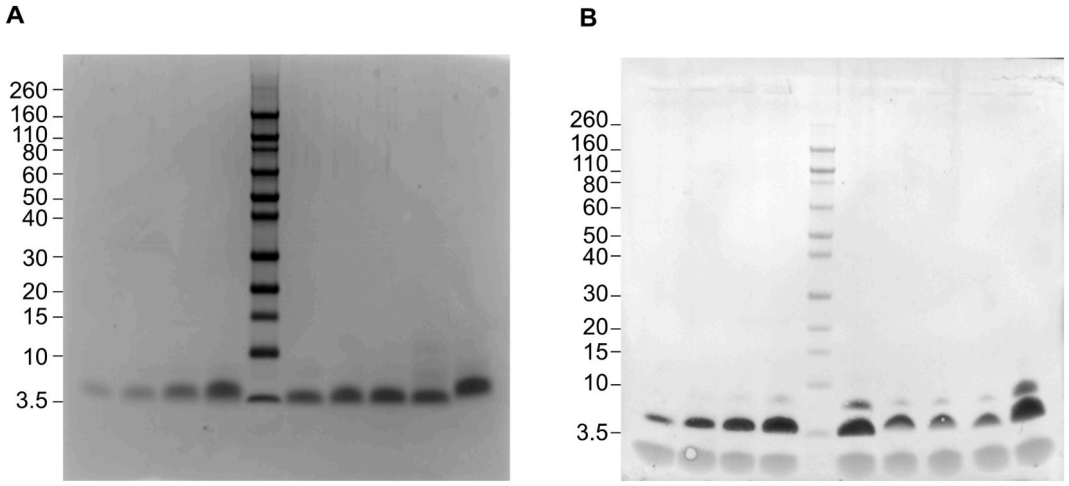

Fig. 2. SDS-PAGE electrophoresis and Western-blotting of purified peptides from seed extract of all studied *Vicia sativa* subsp. *sativa* from different regions of the Middle East. Lanes (protein load μg): (1,2,3,4): PA1b from *Pisum sativum* (0.25 μg, 0.5 μg, 1 μg, and 2 μg), (5) Ladder, (6) Purified peptide from *Vicia sativa* subsp. *sativa* 63977 5 μg, (7) Purified peptide from *Vicia sativa* subsp. *sativa* 63977 5 μg, (8) Purified peptide from *Vicia sativa* subsp. *sativa* 63977 5 μg, (9) Purified peptide from *Vicia sativa* subsp. *sativa* 63977 5 μg, (10) Purified peptide from *Vicia sativa* subsp. *sativa* 63977 5 μg. (A) Coomassie G stain (3%) of SDS PAGE electrophoresis gel, (B) Western blot with anti (pea) PA1b antibody and alkaline phosphatase detection.

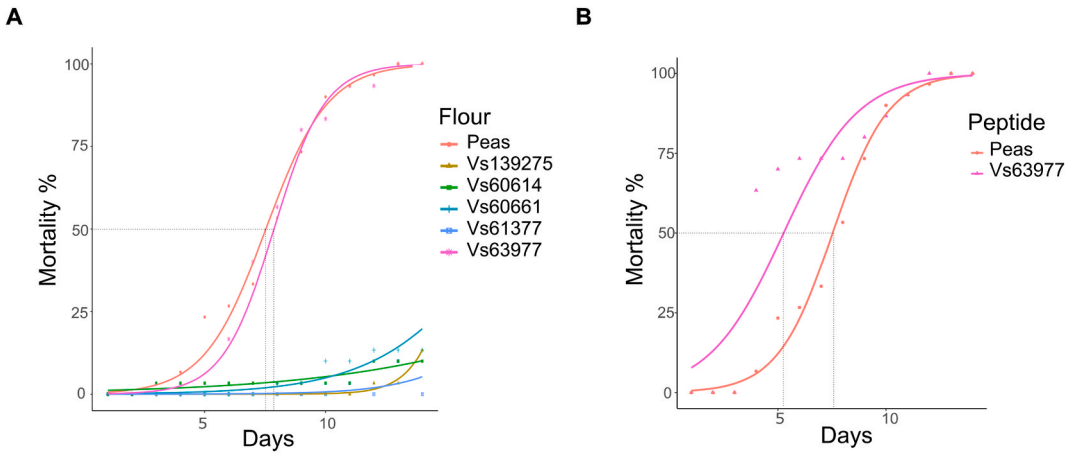

Fig. 3. Acute toxicity to the susceptible strain of weevils (WAA42) following 14 days after exposure to: (A) 20% of the seed flour tested; (B)100 μg of the peptide fraction tested.

3.3. Insecticidal PA1b activity on rice weevils

Toxicity bioassays were conducted on weevils to determine the in vivo activity of the putative PA1b-like peptides present in seeds of *Vicia sativa* subsp. *sativa*. A total differential toxicity of seed flour from the five studied seeds was observed between the susceptible (WAA42) and the resistant (ISO3R2) *S. oryzae* strains. The results of whole seed toxicity bioassays at a meal concentration of 20% are reported in Fig. 3A, showing that only seed flour from *V. sativa* subsp. *sativa* (63977) from Turkey demonstrates potential toxicity to the susceptible strain where LT_{50} calculated is 8.0± 0.4 days. Pea flour is used as a positive control where LT_{50} calculated is 7.6 ± 0.4 days. No toxicity was found in the resistant strain. The acute toxicity of weevils feeding on pea seeds was first discovered due to the presence of PA1b [16]. Since the resistance of weevils is due to a monogenetic trait [34], therefore, the mortality observed only on the susceptible strain is due to the presence of PA1b molecules. To confirm that, 100 μg of the seed peptide fraction (zone PA1b), from *V. sativa* subsp. *sativa* (Vs63977) was collected and tested. Results reported in Fig. 3B shows that the purified peptide fraction is highly toxic to the susceptible weevil strain where LT_{50} calculated is 5.09± 0.5 less than that recorded upon exposure to a mixture of pea toxin isoforms (LT_{50} = 8.91 ± 0.5). A similar total differential toxicity has been observed in almost all tested seed extract from species belonging to the tribe Fabaeae such as *Lens culinaris*, *Vicia hirsuta*, *Lathyrus latifolius* and *P. sativum* [14]. This confirms the high conservation of Albumin 1 family in species of this tribe. Given that 20% of the seed flour was tested the absence of toxicity could be

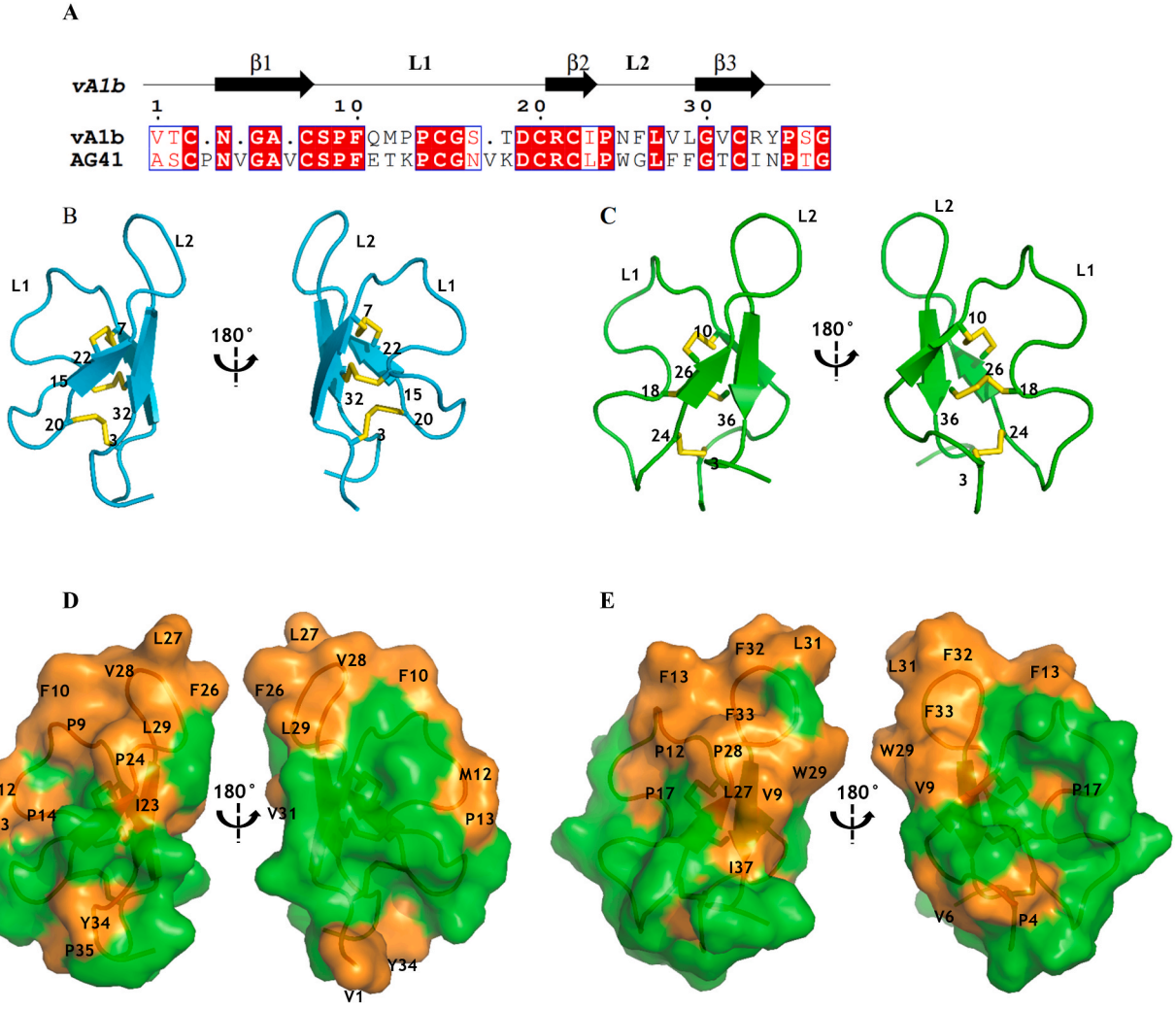

Fig. 4. A: Alignment of vA1b and AG41 sequences. Identical residues are shown on a red background and similar residues are shown in red, the triple-stranded antiparallel β-sheet are indicated by black arrows, the loop L1 and L2 are also annoted. Ribbon representations of vA1b (cyan) panel B and AG41 (green) panel C. The cystine knot motif is characterized by three disulfide bonds, shown as yellow sticks, and by the triple-stranded antiparallel β-sheet (arrows). The numbers of the cysteine residues and the two loops L1 and L2 are also indicated. D and E: Surface representations showing the localization of the hydrophobic residues of vA1b panel D and AG41 panel E. The hydrophobic residues of vA1b and PA1b are highlighted in orange. The figures on the right are rotated 180° relative to those on the left. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

explained by the fact that the amount of PA1b-like molecules in other seed flour is insufficient to induce death. Through the quantification of potential PA1b-like molecules in these seeds, it was seen that the amount present is less than that in Vs63977 (Table 1). It is possible that the absence of toxicity could be also due to the presence of non-toxic isoforms in the seed flour.

3.4. Insecticidal PA1b activity on Sf9 cells

Bioassays were also carried out on Sf9 cells to assess the in vitro activity of the purified peptides in which cells were incubated with different concentrations of the collected peptides. After 1 h of incubation, the phenotype of the cells is observed. Dead cells adopted an almond shape. On the contrary, healthy cells remained rounded. Three hours later, the mortality rate was determined. Sf9 cells were sensitive to peptides extracted from *Vicia sativa* subsp. *sativa* (63977) and from Vs (60614) but not to other peptides tested as reported in Table 2. Cell viability decreased in a dose-dependent manner. The calculated LC_{50} is 57.8±15.9 nM and 123 ± 49 nM when cells were incubated with the peptide extracted from Vs 63977 and Vs60614 respectively. On the other hand, LC_{50} recorded 155.48± 55 nM when using a mixture of PA1b isoforms extracted from *Pisum sativum* var. (Isard). Since Sf9 cells contain a high affinity binding site to PA1b [40], we suggested a similar insecticidal activity for the toxic isoform isolated from species of *V. sativa* subsp. *sativa*. The absence of cytotoxic activity could be attributed to mutations in the key residues rending the isoform inactive [30,31], or insufficient amount of functional PA1b-like molecules.

3.5. Variation in the affinity of PA1b peptides

So far, limited number of plant cystine knot peptides have been identified to exhibit insecticidal activity with different reported modes of action. Cyclotides, such as Kalata B1 and B2 isolated from the plant *Oldenlandia affinis*, exhibit insecticidal activity against *Helicoverpa* caterpillar species [41,42] through membrane pore formation, a mechanism similar to that of *Bacillus thuriengiensis* bacterial delta-endotoxin [43]. Amaranthus α-amylase inhibitor (AAI) derived from *Amaranthus hypocondriacus* strongly inhibits the larval α-amylase activity in the red flour beetle *Tribolium castaneum* and the larger grain borer *Prostephanus truncatus* [44]. In a diverse manner, PA1b targets the cytoplasmic domain of the insect's V-ATPase, a transmembrane protein complex. Binding assays performed on the membrane protein extract of the susceptible strain of weevils revealed a high binding activity of the purified peptide from seed extract of Vs63977 (Kd = 37.5 nM ± 5 nM) as well as that from Vs 60614 (Kd = 59.0 nM + 13.9 nM), which assures an activity similar to that of PA1b. However, binding activity was low with the purified peptide from Vs60661 (Kd = 107.4 nM ± 50 nM) as shown in Table 2. No binding was detected with the other purified peptides from Vs 139275 and Vs 61377. Binding Assays and in vitro toxicity bioassays previously reported, were in consistent with the enhanced PA1b activity in Vs63977. Besides, peptide from Vs60614 showing a similar PA1b activity as that of PA1b means that the absence of toxicity in seed flour is due to the low concentration of PA1b-like peptides (0.1%). Other seeds (Vs139275, and Vs61377, Vs60661) seems to contain very few amounts of functional PA1b-like and/or some non-functional/non-toxic isoforms of PA1b.

3.6. Molecular sequence of PA1b-like (Vs63977) from Vicia sativa subsp. sativa

Through our previous molecular PA1b-like characterization from different species of the Faboideae subfamily by PCR approach using degenerate primers [32], we selected *Vicia sativa* subsp. *sativa* as a natural source of which potential toxic PA1b-like molecules are present. The gDNA of the PA1b homologs from the most abundant and toxic population of *V. sativa* subsp. *sativa* (Vs63977) was analyzed. The three uncovered amino acid sequences consisted of 37 amino acids. The theoretical average and monoisotopic molecular weight (Da) with the isoelectric point of the putative sequences is reported in Table 3. The analysis of the putative PA1b-like molecule in Vs63977 though high resolution mass spectrometry yielded a monoisotopic mass [M+H] of 3903,7 (Supplementary Fig. S1.) corresponding to the theoretical monoisotopic mass [M+H] of vA1b of Vs63977 (Table 3) plus addition of 16 Da corresponding to an oxidation of the methionine residue already observed in various PA1b like peptide [45]. The amino acid sequence confirmed biochemically and molecularly was used for comparative modeling. Pairwise sequence alignment showed that recovered sequences displayed high identity (60–78%) to PA1b which confirms their belonging to the pea albumin 1 subfamily.

3.7. Molecular modeling of Vs63977 peptide

The 3D structural model of the 63977 peptide (Fig. 4 A) was constructed according to the procedure described in the Materials and Methods section. As expected, the modelled 3D structure of the 63977 peptide adopted the typical PA1b knottin fold consisting of a three-stranded β-sheet (Fig. 4 B, C). A long L1 loop retains the well-conserved CSPFE motif among the insecticidal homologs of PA1b, of which phenylalanine has been shown to be a key residue for entomotoxic activity ([31]; Diya et al., 2023) (Fig. 4). To extend our comparison, we plotted the hydrophobic residues on the Connolly surfaces of the peptides (Fig. 4 D, E). The surface of peptide Vs63977 shows a large hydrophobic surface formed by the residues of the L2 hydrophobic loop: Phe26, Leu27, Val28 and Leu29, but also the Phe10 residue of L1. Like AG41, the surface of vA1b (Fig. 4D and E), appears to be predominantly hydrophobic, which may be related to its insecticidal activity, as the hydrophobicity of the pore has been identified as a critical determinant of insecticidal activity ([31]; Diya et al., 2023).

Table 2
Binding affinity to membrane proteins of the susceptible strain of weevils and Sf9 cellular toxicity of the purified peptides from seeds of *Pisum sativum* (positive control) and five wild *Vicia sativa* subsp. *sativa* species.

Seed peptide fraction	Binding Kd±SEM (nM)	LC50 ± SEM (nM)
Pisum sativum var. Isard	39.6±5.0	155.48±55.0
Vs 63977	37.5±4.5	57.8±15.9
Vs 139275	No Binding	Not toxic
Vs61377	No binding	Not toxic
Vs60661	107.4±50	Not toxic
Vs60614	59.0±13.9	123±49.0

Table 3
Primary sequence of putative PA1b-like recovered from genomic DNA of *Vicia sativa* subsp. *sativa* from Turkey. Percentage of identity for each sequence was done based on the Pea albumin 1 b reference from *Pisum sativum* (1P8B_A). The average and monoisotipic mass were calculated and the isoelectric point for each peptide sequence is indicated.

Sample	Code	Sequence of the putative PA1b-like recovered from gDNA	Average Mw (Da)	Monoisotopic Mw (Da)	pI	Identity %
PA1b	1P8B_A	ASCNGVCSPF EMPPCGTSAC RCIPVGLVIG YCRNPSG	3742.39	3739.65	7.81	
Vs63977	vA1b	VTCNGACSPF QMPPCGSTDC RCIPNFLVLG VCRYPSG	3889.56	3886.72	7.75	67.57
	vA1b_A	VQCNGACSAF EMPPCGFTDC RCIPTGLIVG YCRYPSG	3912.55	3909.68	6.00	70.27
	vA1b_B	TDCSGACSPF EMPPCRSLDF RCIPVALFGG YCTYPSS	3974.56	3971.71	4.56	61.11

4. Conclusion

PA1b is an insecticidal toxin extracted from *P. sativum* seeds. It exhibits a strong insecticidal effect against cereal weevils, one of the major storage pests. However, the mass production of this peptide from its natural source, peas, has not been successful thus far. Finding homologs of PA1b from another legume source such as *Vicia sativa* subsp. *sativa* with higher toxicity could potentially overcome the challenges associated with mass production. Screening of five wild seeds of *Vicia sativa* subsp. *sativa,* native to the Middle East, allowed us to identifiy *Vicia sativa* subsp. *sativa* originating from Turkey as a promising seed candidate housing potent PA1b homologs. A comprehensive set of biochemical, molecular, and biological techniques was used to characterize PA1b homologs from this seed. The latter showed an enhanced entomotoxic activity against cereal weevils by binding to their V-ATPase receptor through hydrophobic interactions. This finding could be an exciting prospect for the development of potent PA1b-like molecules in a sustainable strategy to combat storage pests such as weevils.

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CRediT authorship contribution statement

F. Diya: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **I. Rahioui:** Writing – review & editing, Resources, Investigation. **A. Vallier:** Writing – review & editing, Investigation, Formal analysis. **S. Benhamou:** Writing – review & editing, Investigation. **C. Sivignon:** Writing – review & editing, Resources, Investigation. **L. Kfoury:** Writing – review & editing, Supervision, Investigation, Conceptualization. **F. Rizk:** Writing – review & editing, Supervision, Conceptualization. **P. Da Silva:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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