Apisimin, a new serine–valine-rich peptide from honeybee (*Apis mellifera* L.) royal jelly: purification and molecular characterization[1]

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Abstract A peptide named apisimin was found in honeybee (*Apis mellifera* L.) royal jelly (RJ). N-terminal sequencing showed that this peptide corresponded to the sequence of a cDNA clone isolated from an expression cDNA library prepared from heads of nurse honeybees. No homology was found between the protein sequence of apisimin with a molecular mass of 5540.4 Da and sequences deposited in the Swiss-Prot database. The 54 amino acids of apisimin do not include Cys, Met, Pro, Arg, His, Tyr, and Trp residues. The peptide shows a well-defined secondary structure as observed by CD spectroscopy, and has the tendency to form oligomers. Isoelectrofocusing showed apisimin to be an acidic peptide.

Key words: Honeybee (*Apis mellifera* L.); Royal jelly; Apisimin; Serine–valine-rich peptide; Honeybee peptide

1. Introduction

An interesting property of the honeybee (*Apis mellifera* L.) is that young female larvae fed on royal jelly (RJ) develop into queen bees while genetically identical female larvae fed on worker jelly develop into worker bees. This is a case of insect polyphenism regulated by differential nourishment (for reviews see [1–3]). A substantial part of RJ is made of proteins, which form about 50% of the dry mass of RJ [4,5]. The major RJ proteins (MRJPs) belong to a larger family, of which nine members with molecular masses in the range of 49–87 kDa have been identified until now. The MRJP1, MRJP2, MRJP3 (that exists in five isoforms), MRJP4, and MRJP5 represent about 82% of total protein content of RJ [6–9]. With the exception of the yellow proteins of *Drosophila melanogaster*, which are associated with the polymerization of melanin precursors in adult cuticular structures [10], these MRJPs do not have any relatives in other non-insect metazoan species [9]. It is generally accepted that they play a role as a source of essential amino acids [11] and nitrogen in the nutrition of honeybee larvae. Until now there has not been any evidence provided for the function of individual proteins and peptides in the developmental processes of honeybee larvae.

On the other hand, mRNAs of apalbumin (MRJP1) [12] and MRJP2 [13] are expressed in mushroom bodies of the brain that are located in the center of learning and memory of adult honeybees. An interesting observation made in our previous study is that mRNA coding for a small unknown peptide is found in the heads of nurse and forager honeybees [14] and that it is expressed in honeybee heads at the same level as apalbumin-mRNA. This small peptide, for which we propose the name *apisimin* (derived from *Apis mellifera*), might play a physiological role in honeybee colonies because a relatively high level expression of its small (approximately 500 bp) mRNA was observed during the whole life span of the honeybee.

The aim of the present work was to use RJ for the isolation and purification of apisimin and its characterization on molecular and proteomic levels.

2. Materials and methods

2.1. Biological material

Honeybee foragers, 8-day-old nurses (*Apis mellifera* L.) and RJ were purchased from the Institute of Apicultural Research, Liptovský Hrádok, Slovak Republic.

2.2. Screening of cDNA library, DNA sequencing, Northern-blot analysis

A λUni-ZAP XR cDNA expression library was prepared from heads of nurse honeybees [7]. Plasmids were derived from random phage clones of a cDNA library by in vivo excision procedures (Stratagene manual). The isolation of recombinant plasmid DNAs was performed according to Lee and Rasheed [15]. The size of *Bam*HI and *Xho*I cDNA inserts were determined by agarose gel electrophoresis [7].

The clones containing around 500-bp-long cDNA inserts were subjected to the cycle-sequencing method employing the Prism Ready Reaction Dideoxy Terminator kit (Perkin Elmer, USA) on a 373A-DNA-sequencing device according to the manufacturer's instructions. DNA- and protein-sequence analyses were performed using computer

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Abbreviations: CD, circular dichroism; FPLC, fast flow protein liquid chromatography; IEF, isoelectrocusing; MALDI-TOF, matrix-assisted laser-desorption ionization time-of-flight; MBP, maltose-binding protein; MBP–apisimin, recombinant fusion of maltose-binding protein with apisimin; MRJPs, major royal jelly proteins; MRJP1, apalbumin; pI, isoelectric point; RJ, royal jelly; Tricine–SDS–PAGE, tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis

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programs of the University of Wisconsin Genetic Computer Group (Devereux, PC GENE 6.70) and the Heidelberg Unix Sequence Analysis Resources.

2.3. Preparation of polyclonal antiserum against recombinant apisimin

The cDNA coding for apisimin (with six amino acids belonging to a signal peptide) was subcloned into the expression vector pMAL-c2 and expressed in *E. coli* as a C-terminal fusion protein to maltose-binding protein (MBP; unpublished results). The recombinant fusion protein formed by maltose-binding protein and mature apisimin plus six amino acids of signal peptide (MBP–apisimin) was purified by affinity chromatography using a maltose matrix according to the procedure described by manufacturer in catalogue No.10/1991 (BioLabs, USA), and finally purified by preparative sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). The recombinant MBP–apisimin was used for the preparation of rabbit polyclonal antiserum as described previously [16].

2.4. Purification of natural apisimin

The supernatant obtained by ultracentrifugation of RJ [5] was fractionated by size-exclusion chromatography on a Sephadex G-200 column (46 mm × 750 mm; Pharmacia, Sweden). A 30-ml sample of RJ supernatant containing 2 g of proteins was applied onto the column at a flow rate of 1 ml min^{-1}. Elution was performed with 50 mM Tris–HCl, pH 7.0, containing 50 mM NaCl, and the eluent was monitored by measuring the absorbance at 280 nm (spectrophotometer DU-20; Beckman, USA). Three distinct fractions were collected that corresponded to high molecular (HM), middle molecular (MM), and low molecular (LM) weight compounds. Apisimin was detected by dot-blot analysis using antiserum against MBP–apisimin (in the HM fraction) and was further purified by ion-exchange fast flow protein liquid chromatography (FPLC). 120 ml of HM fraction (0.8 mg ml^{-1}) in buffer 'A' (20 mM Tris–HCl, 100 mM glycine, pH 7.0) was loaded onto a DEAE Sepharose fast flow column (28 mm × 200 mm; Pharmacia Biotech, Sweden) at a flow rate of 2 ml min^{-1}. After washing the column with buffer 'A', 7 ml fractions were collected by stepwise elution with 0.1 M NaCl, 0.15 M NaCl and 0.2 M NaCl. The presence of apisimin was monitored by dot-blot analysis as described above. Fraction containing apisimin was concentrated by centrifugation (Sorvall Centrifuge, RC-5B, Germany) using Macrosep Centrifugal Concentrators with M.W. cut-off of 1 kDa (Pall Gelman Sciences, Germany) and then used for further studies. All procedures required to isolate apisimin were carried out at 4°C.

2.5. Protein quantification

The protein content of the samples was determined using the bicinchonionic acid protein assay [17]. Bovine serum albumin (Sigma, USA) was used as reference protein.

2.6. Polyacrylamide gel electrophoresis

Tricine–SDS–PAGE was carried out according to Schägger and Jagow [18]. Total acrylamide and bisacrylamide concentrations (%*T*) and the percentage of cross-linker relative to the total concentration (%*C*) were 16.5%*T*, 3%*C*, and 4%*T*, 3%*C*, for separating and stacking gels, respectively.

Native-PAGE was performed as described in Current Protocols in Molecular Biology [19] in 15% polyacrylamide separating and 4% stacking gels.

After electrophoresis, the proteins were visualized by Coomassie Blue G-250 staining (Serva, Electrophoresis, Heidelberg, Germany) in 10% acetic acid.

2.7. Western-blot analysis

For Western-blots, proteins were transferred after Tricine–SDS–PAGE to PVDF membrane ProBlott (Applied Biosystems, USA) using the tank method (MiniTrans-Blot Electrophoretic Transfer Cell, Bio-Rad Laboratories, USA). The membranes were first incubated overnight in buffer (20 mM NaCl, 50 mM Tris–HCl, pH 7.4) containing 10% powdered non-fat milk with polyclonal rabbit antiserum against recombinant MBP–apisimin at a dilution of 1:500, then 2 h in the same buffer with peroxidase-conjugated swine anti-rabbit IgG (SwAR Px, Institute of Sera and Vaccines, Prague, Czech Republic) at a dilution of 1:2000. Reaction of immunoactive peptide bands on the membrane was done by incubation with 0.33% 3,3-diaminobenzidine tetrahydrochloride and 30 μg ml^{-1} hydrogen peroxide (Fluka, Switzerland) in 50 mM Tris–HCl, pH 7.4, for 5 min.

2.8. N-Terminal amino acid sequencing

Proteins were separated by Tricine–SDS–PAGE and electroblotted onto PVDF membranes ProBlott as described above. The transfer was performed in electroblotting buffer consisting of 10 mM (3-[cyclohexylamino]-1-propanesulfonic acid) in 10% methanol according to the procedure recommended by manufacturer. Staining was performed using Coomassie Blue R 250 (Serva, Germany). The band of interest was excised and subjected to sequencing by automated Edman degradation on an LF3600D Protein Sequenator (Beckman, USA).

2.9. Matrix-assisted laser-desorption ionization time-of-flight mass spectrometry analysis

Matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF) mass spectra were acquired on a mass spectrometer (Bruker Scout MTP384 Reflex-III, Germany) using α-cyano-4-hydroxycinnamic acid as matrix. A 0.5 μl aliquot of purified peptide was prepared according to the procedure described by Roepstorff et al. [20]. Exclusively positively charged ions were analyzed in the reflector acquisition mode and 100 single-shot spectra were accumulated for improved signal-to-noise ratio. Spectra were processed using the software package XMASS 5.1 provided by the instrument manufacturer. For external and internal spectrum calibration, the singly and doubly charged molecular ions of horse heart cytochrome C (*m*/*z* 6181.1 and 12 361.2) were used.

2.10. Isoelectrofocusing

Isoelectrocusing (IEF) was performed using PhastGel Dry IEF pH 3–9 (PhastSystem, Pharmacia LKB, Sweden). Separation and silver staining were carried out according to the procedures recommended by IEF PhastSystem producer.

2.11. Circular dichroism and UV spectra

Circular dichroism (CD) measurements were made with a Jasco J-600 Spectrophotopolarimeter (Japan Spectroscopic, Japan) using 1-cm quartz cuvettes thermostated at 25°C. Spectra were measured from 190 to 250 nm, with three scans at 50 nm min^{-1}, time constant 1 s, bandwidth 1 nm. Ellipticity and photomultiplier voltage (HT) baselines for the protein were measured using deionized water in a 1-cm cuvette. The CD spectrum for apisimin in water (0.63 mg ml^{-1}) was obtained at peak ellipticity 50 mdeg and HT < 700 V. The spectra were evaluated with the software provided by the instrument manufacturer, Jasco.

The UV spectrum of native apisimin (0.48 mg ml^{-1}) in water was measured from 220 to 400 nm, in a 1-cm flow cell using a UV-Visible Recording Spectrophotometer UV-160A (Shimadzu, Japan).

3. Results and discussion

3.1. Molecular cloning and sequencing of cDNA coding for apisimin

The expression cDNA library prepared from heads of nurse honeybees [7] was screened for cDNA clones with a size around 500 bp which correspond to a small peptide with molecular mass below 10 kDa. Sequencing analysis revealed that eight clones contained identical full-length cDNAs. The complete nucleotide sequence and the deduced amino acid sequence of one of these clones, pRJP54, are shown in Fig. 1. This clone contains a cDNA insert of 356 bp, including the poly(A) tail. The consensus polyadenylation signal is located at nucleotides 335–340. RJP54 cDNA has an open reading frame encoding a precursor peptide of 78 amino acids. The N-terminal amino acid sequence of the natural peptide apisimin purified from RJ (see Section 3.2) is K-T-S-I-S-V-K, which corresponds to the amino acid sequence derived from the nucleotide sequence of cDNA clone RJP54 (Fig. 1). On the basis of these data it was possible to localize the cleavage site of the signal peptidase between Ala_{24} and Lys_{25}.

The mature apisimin is formed by 54 amino acids. It is rich in valine (18.5%) and serine (16.7%), and contains only one

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        10         20         30         40         50         60
GCACGAGGAACCGAGCTTTCTAAAAGCAATTCCAAACAGCACAAAAATCAAAATGAGCAA
                                                    M  S  K

        70         80         90        100        110        120
AATCGTTGCTGTCGTCGTCCTAGCTGCCTTCTGCGTAGCCATGTTGGTCAGCGATGTGTC
  I  V  A  V  V  V  L  A  A  F  C  V  A  M  L  V  S  D  V  S

       130        140        150        160        170        180
CGCCAAAACATCAATCAGTGTCAAAGGCGAATCGAACGTGGATGTCGTTTCCCAAATCAA
  A▲K  T  S  I  S  V  K  G  E  S  N  V  D  V  V  S  Q  I  N

       190        200        210        220        230        240
CAGTTTGGTTTCATCTATCGTGTCTGGTGCCAACGTGTCAGCAGTACTCCTAGCTCAAAC
  S  L  V  S  S  I  V  S  G  A  N  V  S  A  V  L  L  A  Q  T

       250        260        270        280        290        300
TTTAGTTAATATCCTGCAAATTCTTATCGACGCTAATGTTTTCGCTTAATTTATATATTC
  L  V  N  I  L  Q  I  L  I  D  A  N  V  F  A  *
       310        320        330        340        350        360
TTTAGCTTTGTATTGCGCGCATACAACGCATTCGAATAAAGTAATTAATAAAATTCAAAA
        370        380
AAAAAAAAAAAAAAAAAAAAA
```

Fig. 1. Nucleotide and encoded amino acid sequences of apisimin cDNA sequenced for the clone pRJP54. Translation initiation and termination codons are indicated in bold. The arrow shows the cleavage site for signal peptidase deduced from the N-terminal sequence of the natural peptide isolated from RJ.

aromatic amino acid, phenylalanine. Homology to the protein sequence of apisimin was found neither with proteins deposited in the Swiss-Prot database nor with known honeybee peptides possessing antimicrobial activities like royalisin [21], apideacin, hymenoptacin and abaecin [22], or with the peptides of honeybee venom, such as melittin, apamin and MCD-peptide [23].

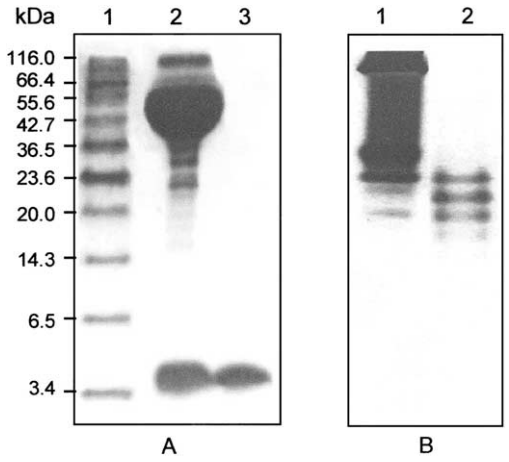

Fig. 2. Electrophoretic characterization of natural apisimin (see Section 2). Panel A, Tricine–SDS–PAGE: lane 1, molecular weight protein standard (BioLabs, USA); lane 2, HM fraction (100 μg); lane 3, apisimin (6.5 μg). Panel B, native-PAGE: lane 1, HM fraction (100 μg); lane 2, apisimin (6.5 μg). Detection made with Coomassie Blue G-250 staining.

3.2. Purification of natural apisimin

Size-exclusion chromatography of the supernatant obtained by ultracentrifugation of RJ showed that mature apisimin with a molecular mass of 5540.4 Da eluted in the HM weight fraction containing oligomeric apalbumin (previously named MRJP1) with a molecular mass around 420 kDa [5] (data not shown). This fraction was further separated in three fractions F1, F2 and F3, by ion-exchange FPLC using a stepwise elution (data not shown). Apisimin was immunochemically detected by dot-blot assay in fraction F1, which eluted as a single peak with 0.1 M NaCl in the presence of 100 mM gly-

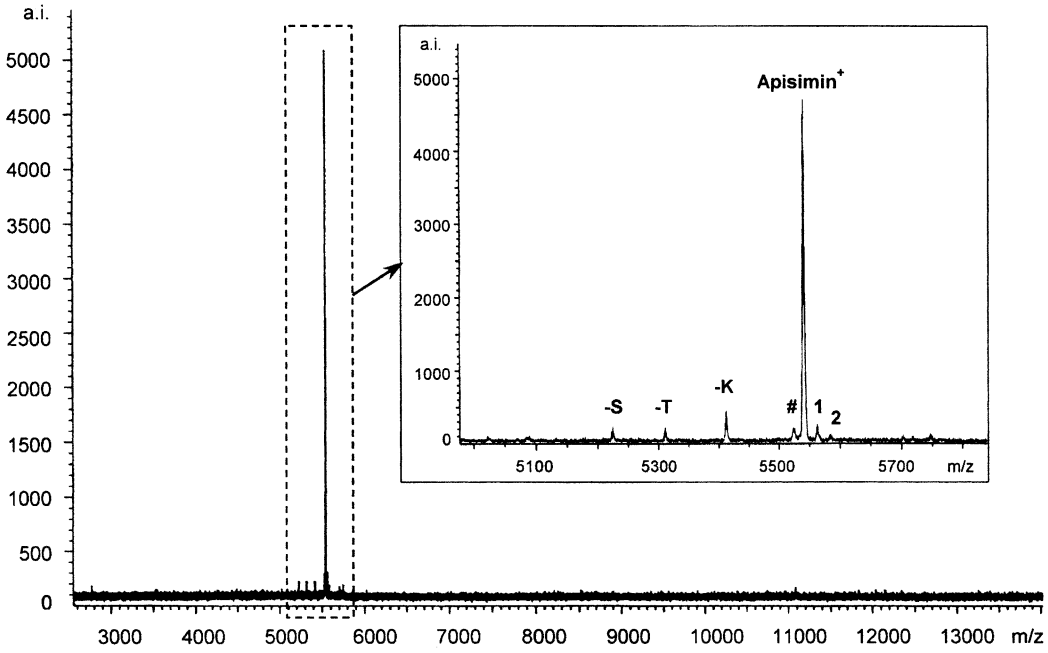

Fig. 3. MALDI-TOF mass spectrum of apisimin purified from RJ fraction using ion-exchange FPLC. For conditions see Section 2. Apisimin$^+$: singly protonated molecular ions of apisimin. Based on this signal, the M.W. of apisimin was determined to be 5540.5 Da (expected 5540.4 Da). -K, -T, -S: subsequent loss of a lysine, threonine and serine residue. These signals match the partial amino acid sequences of apisimin 2-54 (M.W. determined: 5412.2, expected: 5412.2), 3-54 (M.W. determined: 5311.0, expected: 5311.1) and 4-54 (M.W. determined: 5224.3, expected: 5224.0), indicating partial N-terminal degradation of the isolated peptide. 1, 2: singly sodiated molecular ions of apisimin (apisimin+Na$^+$) and the disodium salt of apisimin (apisimin–H$^+$+2Na$^+$). #: apisimin$^+$- 16 Da.

cine, whereas MRJP1 eluted with 0.15 M NaCl and 0.2 M NaCl in fractions F2 and F3. Apisimin in the fraction F1 appeared to be homogeneous by Tricine–SDS–PAGE analysis (Fig. 2, panel A, lane 3).

An interesting observation is that apisimin was found in the HM weight fraction, where apalbumin was also present. Apalbumin is the most abundant protein of RJ [6,24] and occurs in RJ as monomer (55 kDa), as oligomer (ca 420 kDa), and as water insoluble aggregates formed after interaction with fatty acids [5]. Apalbumin tends to self-assemble and to form gel. It appears that apisimin interacts with apalbumin and forms an oligomeric [apalbumin:apisimin] complex of yet unknown stoichiometry and structure. This complex is stable under the conditions used during size-exclusion and ion-exchange column chromatography.

We have obtained natural apisimin by separation of the HM weight fraction using ion-exchange FPLC in the presence of 100 mM glycine. In the absence of glycine, apisimin eluted together with apalbumin at 0.2 M NaCl. From 2 g proteins of the supernatant fraction of RJ we have obtained approximately 23 mg of pure apisimin.

It seems that apisimin binds to apalbumin strongly, because it was not found in the dialysate when RJ was dialyzed at pH 2.0 for 24 h at 8°C against water (data not shown). Under these conditions, we found in the dialysate of RJ the 52-amino acids-long antimicrobial peptide royalisin [21] that is active against *Paenibacillus larvae larvae* (*P. l. larvae*) [25], the inducer of microbial epidemy of honeybee larvae. We have not observed any antimicrobial activity of purified apisimin tested against *E. coli*, *B. subtilis,* or *P. l. larvae* (data not shown).

The absence of antimicrobial activity of apisimin is probably not associated with the lack of cysteine, an important structural component of most antimicrobial insect peptides [26]. This is because other antimicrobial peptides detected in honeybee hemolymph [22] (except for apis-defensin [22] and the peptide royalisin that is present in RJ [21]) are also devoid of cysteine.

3.3. Physico-chemical characterization of apisimin

The sequence of the N-terminal peptide K-T-S-I-S-V-K determined by N-terminal amino acid-sequence analysis of apisimin allowed the localization of the cleavage site of the signal peptidase between Ala_{24} and Lys_{25} (Fig. 1). The molecular masses of non-mature and mature apisimin (with or without signal peptide) were calculated to be 7943.4 Da and 5540.4 Da, respectively. The molecular mass of natural apisimin was determined by MALDI-TOF mass spectrometry analysis (Fig. 3) to be $[M+H]^+ = m/z$ 5540.5, which is in perfect agreement with 5540.4 Da calculated from the deduced amino acid sequence of mature apisimin without signal peptide (Fig. 1). This indicates that apisimin is not post-translationally modified. The three satellite signals at 5413.2, 5312.0 and 5225.3 (Fig. 3, inset) correspond to a consecutive loss of the N-terminal amino acids: Lys, Thr and Ser. Therefore, besides the precise molecular mass determined in this experiment, also the data on the three N-terminal amino acid residues confirm the identity of apisimin.

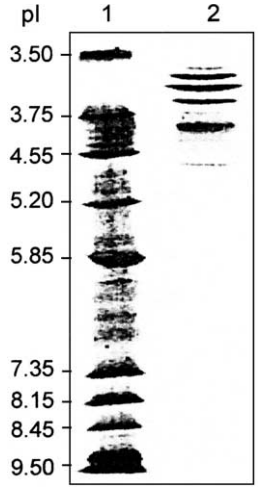

Fig. 4. IEF profile of apisimin using PhastGel IEF pH 3–9 (Pharmacia). Lane 1, pI protein marker (Pharmacia); lane 2, apisimin (1 μg). Detection made with silver staining.

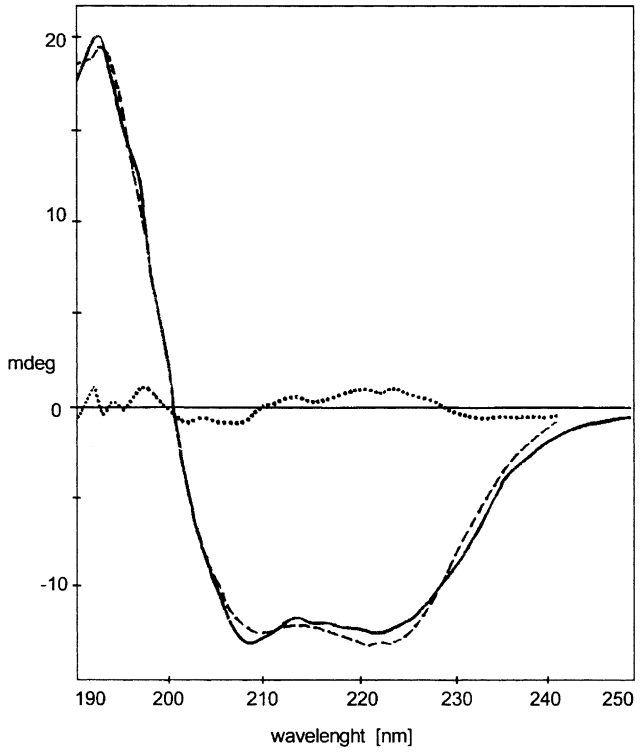

Fig. 5. CD spectrum of natural apisimin. For details see Section 2. — measured CD spectrum of natural apisimin; – – –, calculated CD spectrum according to the software protein standards with known secondary structure; ⋯, differences between measured and calculated CD data.

The purified apisimin appeared to be homogeneous by Tricine–SDS–PAGE analysis (Fig. 2, panel A, lane 3). However, native-PAGE (Fig. 2, panel B, lane 2) and IEF (Fig. 4) showed several distinct bands. The isoelectric point (pI) is in the acidic range 3.55–4.55 because apisimin contains two Glu and one Asp that are counterbalanced by only two Lys. The observed microheterogeneity of the apisimin composition indicates the presence of oligomers, i.e. natural apisimin occurs as monomer and as a variety of oligomers. The different forms of apisimin resemble oligomers of the antimicrobial peptides magainin [27] and dermaseptin [28] isolated from frog skin.

The UV spectrum of apisimin (not shown) does not show the typical protein profile. There is no maximum at 280 nm

and no minimum at 240 nm because only one aromatic amino acid (Phe) is present in the peptide.

The CD spectrum of apisimin (Fig. 5) is in good agreement with the theoretically calculated parameters which are characteristic for proteins with a predominantly helical structure. The analysis of the spectrum as performed by the Jasco software showed that apisimin contains the following percentages of structural elements: 34% α-helical, 20% β-sheet, 11% β-turn, 30% random structure (the root-mean-square deviation is 6%). The occurrence of 65% of well-defined secondary structure in such a small peptide with only one aromatic amino acid and without disulfide bridges is surprising and requires further structural studies.

We have detected a relatively high amount of apisimin-mRNA and a high amount of apalbumin-mRNA in the heads of honeybees [14]. Apisimin and apalbumin are synthesized during the whole life span of honeybees, whereas technological enzymes (α-glucosidase, glucose oxidase, and amylase) involved in processing nectar to honey are produced by foragers only [29,30]. Thus, the role of apisimin in honeybee colonies could not only be associated with larval development, because a high level of apisimin-mRNA was found in the heads of both nurse and forager honeybees [14].

Recent experiments have shown that some RJ proteins have polyfunctional properties. An exclusive position among MRJPs belongs to acidic apalbumin, the most abundant protein of RJ [5]. From a physiological point of view, it is interesting that the 350-kDa protein with N-terminal sequence identical to apalbumin [6,8] isolated from RJ exerted proliferation stimulating activity on human monocytic cell lines [31]. The 57-kDa RJ protein with N-terminal sequence identical to apalbumin enhanced the proliferation of primary cultured rat hepatocytes [32]. This may also suggest that apisimin alone and/or in complex with apalbumin could be involved in the activation of different cellular processes.

The mechanisms of physiological functions of RJ peptides and proteins have not yet been defined because fundamental structural data are not available thus far. The herein described results provide for the first time the molecular characterization of individual proteinaceous components of RJ.

The structural properties, protein–protein interactions with apalbumin and formation of natural oligomers render apisimin an interesting object for investigations, not only in the context of insect studies but also from a basic molecular standpoint.

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