

REGULAR ARTICLE

Towards royal jelly proteome

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The recent availability of the honey-bee *Apis mellifera* genome and transcriptome of both the female castes, has stimulated new efforts in investigating the protein composition of royal jelly (RJ), its role in caste differentiation and its quality and typicality by a proteomic approach. This study is aimed both to separate and identify proteins of royal jelly and to detect some of them in honey-bee pollen-bread by using two-dimensional gel electrophoresis, mass spectrometry and by *de novo* sequencing. All the identified proteins belonged to the *Apis mellifera* genome. Apalbumin 1 was also confirmed to be present in honey-bee pollen-bread where the presence of apalbumin 2 was also found. In addition several fragments of apalbumin 1 and apalbumin 3 were also found in RJ. These could be the result of protease activity other than that of serine-protease. This study is a contribution to the description of royal jelly proteome.

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1 Introduction

Larval diet plays a key role in honey-bee polyphenism [1–5]. Caste determination in honey-bees is due by larval diet rather than by a genetic predisposition of some of them to become queens or workers [3]. Nurse bees produce royal jelly (RJ) in their glandular cephalic system (hypopharyngeal and mandibular glands) between the sixth and twelfth day of their image life [6–8]. All the larvae within three days after hatching are fed with RJ by nurse bees; after the first three days of larval development the queen-designate larvae continue to be fed with RJ throughout development, while those designated to be workers undergo a diet made up of a mixture of the nurse bees mandibular secretion, honey and pollen (worker jelly) [9–12].

Diet (RJ or pollen-based food) determines the molecular events of cell differentiation of young honey-bee larvae into queens or workers during the two days (fourth and fifth day after hatching) before cell sealing by workers. Although molecular mechanisms involved in diet-based differentiation are still unknown [1, 2, 6, 13], several genes that are expressed differently by worker- and queen-designate larvae during the larval development have been identified and their transcripts characterized by Evans and Wheeler [3–5]. In particular, larvae to become queens overexpress several metabolic enzymes, while workers show growing expression of dihydrodiol dehydrogenase, of a cytochrome and exameric storage proteins [4]. Many of the differently expressed genes have been tied to metabolic rates and cellular responses to hormones [4].

RJ is a white-yellow colloid with a pH between 3.6–4.2. Its composition is variable depending on the metabolic and physiologic state of nurse bees as well as on the larval age [12, 14], on the honey-bee race [15] and on the seasonal and regional conditions [16, 17]. Commercial, fresh RJ is usually sampled from cells containing 72 h old queen-designate larvae, and is mainly composed of water (60–70%), proteins

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Abbreviations: RJ, royal jelly; RJP, royal jelly protein

(12–15%), carbohydrates (10–12%), lipids (3–7%) and traces of mineral salts and vitamins [18–22]. Moreover, the sampled RJ is always contaminated by pollen grains [16, 23].

In the last 15 years several proteins of RJ have been identified and described [15, 16, 18]. The biological function of some of the identified constituent proteins of RJ have been reported previously [18]. Royalisin is an antimicrobial peptide against Gram-positive bacteria and fungi [24, 25], the Jelleines are an antimicrobial family of peptides against Gram-positive, Gram-negative bacteria and yeasts [26]. Apisimin is a 54 amino acid peptide highly expressed in the head of the honey-bee and is able to strongly bind apalbumin 1 [27] and apisin is a 350 kDa glycoprotein that stimulates the proliferation of human monocytes [28–30].

Important protein components of RJ are those belonging to a family named Major Royal Jelly Proteins (MRJPs) now named apalbumins [31, 32]. In this family five species of proteins (apalbumin 1–5) with molecular masses ranging from 49–87 kDa have been identified [33]. Apalbumin 3 exists in five isoforms. Apalbumin 5 and apalbumin 4 both supply nutritive components as essential amino acids; in particular apalbumin 5 is a storage of biologically accessible nitrogen required for the fast developing organism [34]. Additionally, apalbumin 3, with its five isoforms, and apalbumin 2, seem to function as a store of processable form of nitrogen [34]. Apalbumin 1 [35, 36], shows a mass of 55 kDa and self-assembling properties. In addition to nutrition function, this protein could also play a role in processing honey-bee products, in particular it may participate in the formation of pollen-pellet and pollen-bread [35]. Apalbumin 1, representing 48% of water soluble proteins of RJ, has been found also in honey, in the honey-bee pollen-pellet and in the honey-bee pollen-bread. Its mRNA has been found expressed in the mushroom bodies of the adult honey-bee brain [37] and in the hypopharyngeal glands of both nurse bees and foragers [18, 36, 38]. Apalbumin 1 has been investigated by using cDNA cloning, immunoblotting, SDS-PAGE and 2-DE electrophoresis [15, 18, 33, 35].

The availability of the *Apis mellifera* genome (<http://www.hgsc.bcm.tmc.edu/projects/honeybee/>) and the recent observation that differential expression of genes are involved with larval fate encourage a proteomic approach for the investigation of RJ proteins. This approach could help in understanding the role that RJ plays in the physiological mechanism of honey-bee larval female caste differentiation.

2 Materials and methods

2.1 Chemicals

IPG strips, IPG buffer, Pharmalyte, acrylamide, and Coomassie Brilliant Blue R-250 were purchased from Amersham Biosciences (San Francisco, CA, USA). Trypsin was purchased from Promega (Madison, WI, USA). Molecular weight marker proteins and all other chemicals were from Sigma (St. Louis, MO, USA).

2.2 Biological samples

An amount of 1.25 g of RJ was sampled from the University of Pisa experimental apiary of the laboratory of Apidology of the Cultivation and Protection of Wood Species Department (lots of 0.25 g *per* hive). Each lot of RJ was sampled from 10 caps containing 72 h old queen-designate larvae. An amount of 0.25 g of pollen-bread was sampled from the hive frames that contained stored pollen.

2.3 2-DE

250 mg of RJ and 250 mg of pollen-bread were each suspended in 1 mL of 50 mM Tris-HCl, pH 8 and in 10 μ L of Inhibitor Cocktail Solution (Sigma). After centrifugation at 19 000 rpm for 60 min at 4°C, the supernatant was recovered. Protein concentration was estimated according to Bradford [39] using ovalbumin as reference. 12.4 μ L of supernatant (170 μ g of protein) were mixed with 227.6 μ L of rehydration buffer containing 8 M urea, 2% CHAPS, 1 mg DTT, 0.8% IPG buffer, 0.8% Pharmalyte and a trace of bromophenol blue. The mixture was loaded on an 11 cm Immobiline DryStrip, pH 3–10. IEF was performed at 14°C on a Multiphor II apparatus (Amersham Biosciences) according to the following schedule: 1 min at 500 V, 5 h at 2500 V, 5 h of a linear gradient to 3500 V, and 9.5 h at 3500 V. Prior to SDS-PAGE the IPG strips were equilibrated in 6 M urea, 30% glycerol, 2% SDS, 50 mM Tris-HCl, pH 8.4 and 2% DTT, for 12 min and afterwards in 6 M urea, 30% glycerol, 2% SDS, 50 mM Tris-HCl pH 6, 2.5% iodoacetamide and a trace of bromophenol blue for 5 min. SDS-PAGE was performed using 15%T, 2.6%C separating polyacrylamide gels (1.5 mm thick) according to Laemmli [40] but without stacking gel, using a Hoefer SE 600 system (Amersham Biosciences). The second dimension was carried out at 40 mA/gel at 8°C for 3 h. The gels were stained with Coomassie Brilliant Blue G-colloidal, scanned with an Epson Expression 1680 Pro scanner and images were analyzed with PDQuest V 6.2.1 (Bio-Rad, Hercules, CA, USA).

2.4 Tryptic digestion of 2-DE spots

Spots of interest were cut from the gel and destained overnight with a solution of 25 mM ammonium bicarbonate, 50% acetonitrile. The proteins were digested in-gel with trypsin as described by Hellmann *et al.* [41].

2.5 MALDI-TOF MS and database search

For MALDI-TOF MS, 0.5 μ L of each peptide mixture were applied to a target disk and allowed to air-dry. Subsequently, 0.5 μ L of matrix solution (1% CHCA in 30% ACN, 0.1% TFA) were applied to the dried sample and again allowed to dry. External calibration was performed with a peptide calibration standard (Bruker, Bremen, Germany) and internal calibration with trypsin autoprolytic fragments. Spectra were obtained using a Bruker ReflexIII MALDI-TOF spectrometer (Bremen, Germany). To interpret the MS spectra of

protein digests, MASCOT (http://www.matrixscience.com/search_form_select.html) and MS-Fit (<http://falcon.ludwig.ucl.ac.uk/MS-Fit.html>) software were used.

2.6 Zymography

250 mg of RJ and 250 mg of pollen-bread were suspended in 1 mL of 50 mM Tris-HCl, pH 8.0 and mechanically disrupted by a mini-potter homogenizer (Sigma, St. Louis, MO, USA). After centrifugation at 19 000 rpm for 60 min at 4°C, the supernatant was recovered. Protein concentration was estimated according to Bradford [39] using ovalbumin as reference. Zymography was performed in a MiniProtein II apparatus (Bio-Rad). Proteins in the supernatants of pollen-bread and RJ underwent proteolytic activity-staining electrophoresis [42], using 1% gelatine as a precast protein substrate in a discontinuous 15%T Laemmli electrophoresis system under semidenaturing conditions (before loading, samples were 1:2 diluted with 2% SDS without boiling and in the absence of β -mercaptoethanol). Five μ L aliquots of the clarified extracts ($\sim$ 10 mg of proteins) were loaded in each lane. After electrophoresis, gels were shaken gently at room temperature for 30 min in 100 mL 2% Triton X-100 in water to remove SDS and restore full enzyme activity. Gels were then transferred to a bath containing 100 mM Tris-HCl buffer, pH 8.0 and kept with gentle shaking at 37°C for 4 h, then stained with 0.1% Coomassie Brilliant Blue R-250 and destained with 40% methanol and 10% acetic acid.

3 Results and discussion

3.1 Protein identification

Figure 1 shows one representative RJ (a) and honey-bee pollen-bread gel (b). The two different 2-DE protein patterns show some spot series (in squares named A, B and C)

that match perfectly. Within square A, spot R7 belonging to the RJ gel and spots P1, P2 and P3 belonging to the honey-bee pollen-bread gel have been analyzed and identified; within square B, spot R8 belonging to the RJ gel and spots P4, P5, P6 and P7 belonging to the honey-bee pollen-bread gel have been analyzed and identified; within square C spot R21 belonging to the RJ gel and spots P8, P9 and P10 have been analyzed but not identified. Within squares A and B of the RJ gel, the single spots R7 and R8 have been chosen for MALDI-TOF analysis to confirm the identity of all spot series as already identified by Sano and colleagues [15] from the 2-DE. In the RJ other spots named R1–R6 and from R9 to R20 have been analyzed and identified by MALDI-TOF.

All the identified proteins belonged to the *Apis mellifera* genome. The results of the spot identification are reported in Table 1. R7 and R8 have been identified to be apalbumin 2 and apalbumin 1, respectively, indicating these spot series as those identified by Sano and colleagues [15]. In particular, the spot R7 identification revealed the presence in this spot series of at least two variants (O77061 and XP_393061) of apalbumin 2, that showed a pI 6.83 and 6.46, these values being lower than the 7.5–8.5 range reported by Bilikova and colleagues [43].

MALDI-TOF analysis revealed that spots P1, P2 and P3 are all apalbumin 2 (O77061), the same result as for spot R7 in the RJ. P4, P5, P6 and P7 were identified as apalbumin 1, just like spot R8 in the RJ. The presence of apalbumin 1 in the RJ, as identified by MALDI-TOF, is in agreement with the findings of Hanes and Simuth [31, 35] obtained by SDS-PAGE and with those of Sano and colleagues [15] that identified apalbumin 1 by N-terminal sequencing in 2-D gel. The presence of apalbumin 1 in the honey-bee pollen-bread, confirms the immunoblotting and the N-terminal sequencing data published by Simuth *et al.* [18] during the preparation of this paper. Apalbumin 2 was also detected in the

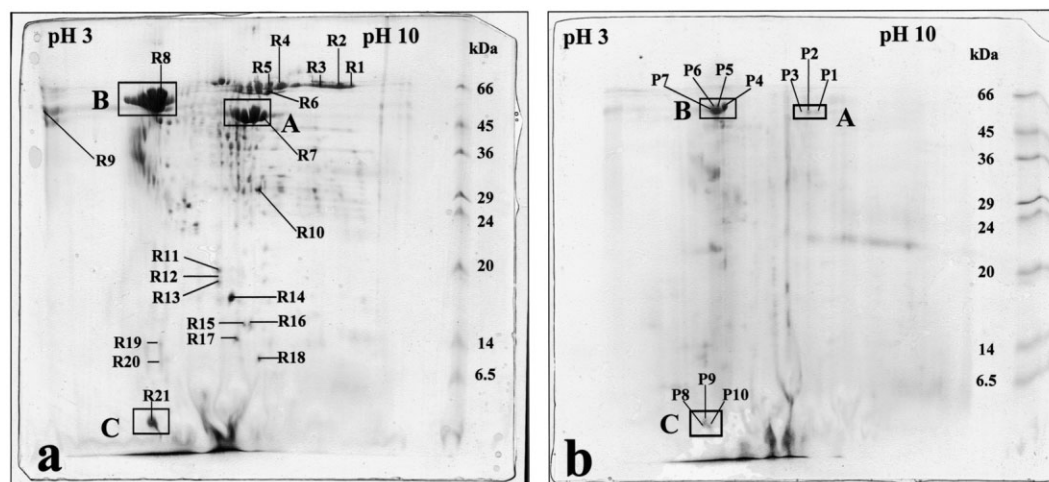


Figure 1. Coomassie-stained 2-D gels of proteins of RJ (a) and honey-bee pollen-bread (b). Spots in the squares were cut out and underwent tryptic digestion before MS analysis.

Table 1. Identification of proteins of RJ (R) and honey-bee pollen-bread (P)

Spot no.	Mass obs. (kDa)	pI obs.	PMF		Mass (Da)	pI	ID and name
			No. matched peptides/total signals	Sequence coverage			
P1	53	7	20/48	50%	51 441	6.83	Major royal jelly protein 2 precursor (Apalbumin-2) (Bee-milk protein) O77061
P2	53	7.2	25/60	60%	51 441	6.83	Major royal jelly protein 2 precursor (Apalbumin-2) (Bee-milk protein) O77061
P3	53	7.4	19/49	50%	51 441	6.83	Major royal jelly protein 2 precursor (Apalbumin-2) (Bee-milk protein) O77061
P4	53	5.2	27/68	62%	49 311	5.10	Major royal jelly protein 1 precursor (Apalbumin-1) (Bee-milk protein) O18330
P5	53	5	21/65	49%	49 311	5.10	Major royal jelly protein 1 precursor (Apalbumin-1) (Bee-milk protein) O18330
P6	53	4.9	25/87	63%	49 311	5.10	Major royal jelly protein 1 precursor (Apalbumin-1) (Bee-milk protein) O18330
P7	53	4.7	21/71	51%	49 311	5.10	Major royal jelly protein 1 precursor (Apalbumin-1) (Bee-milk protein) O18330
P8	Not determined	5					Not identified
P9	Not determined	5.1					Not identified
P10	Not determined	5.2					Not identified
R1	66	8.7	21/95	46%	62 056	6.47	Similar to royal jelly protein RJP57-1 [<i>Apis mellifera</i>] XP_391893
			19/95	46%	53 155	5.67	Royal jelly protein RJP57-1 – honey-bee S39193
R2	66	8.5	25/68	47%	62 056	6.47	Similar to royal jelly protein RJP57-1 XP_391893
R3	66	8.1	26/88	51%	62 056	6.47	Similar to royal jelly protein RJP57-1 XP_391893
R4	66	7.4	25/70	50%	62 056	6.47	Similar to royal jelly protein RJP57-1 XP_391893
R5	66	7.2	21/44	43%	62 056	6.47	Similar to royal jelly protein RJP57-1 XP_391893
			25/64	49%	62 056	6.47	Similar to royal jelly protein RJP57-1 XP_391893
R6	55	7.2	25/64	49%	61 966	6.47	Major royal jelly protein 3 precursor (Apalbumin-3) (Bee-milk protein) (Royal jelly protein RJP57-1) Q17060
			31/133	66%	51 441	6.83	Major royal jelly protein 2 precursor (Apalbumin-2) (Bee-milk protein) O77061
R7	53	7	28/133	69%	43 762	6.46	Major royal jelly protein 2 [<i>Apis mellifera</i>] XP_393061
R8	51.6	5.2	25/93	56%	49 311	5.10	Major royal jelly protein 1 precursor (Apalbumin-1) (Bee-milk protein) O18330
R9	51.6	3	25/100	62%	49 311	5.10	Major royal jelly protein 1 precursor (Apalbumin-1) (Bee-milk protein) O18330

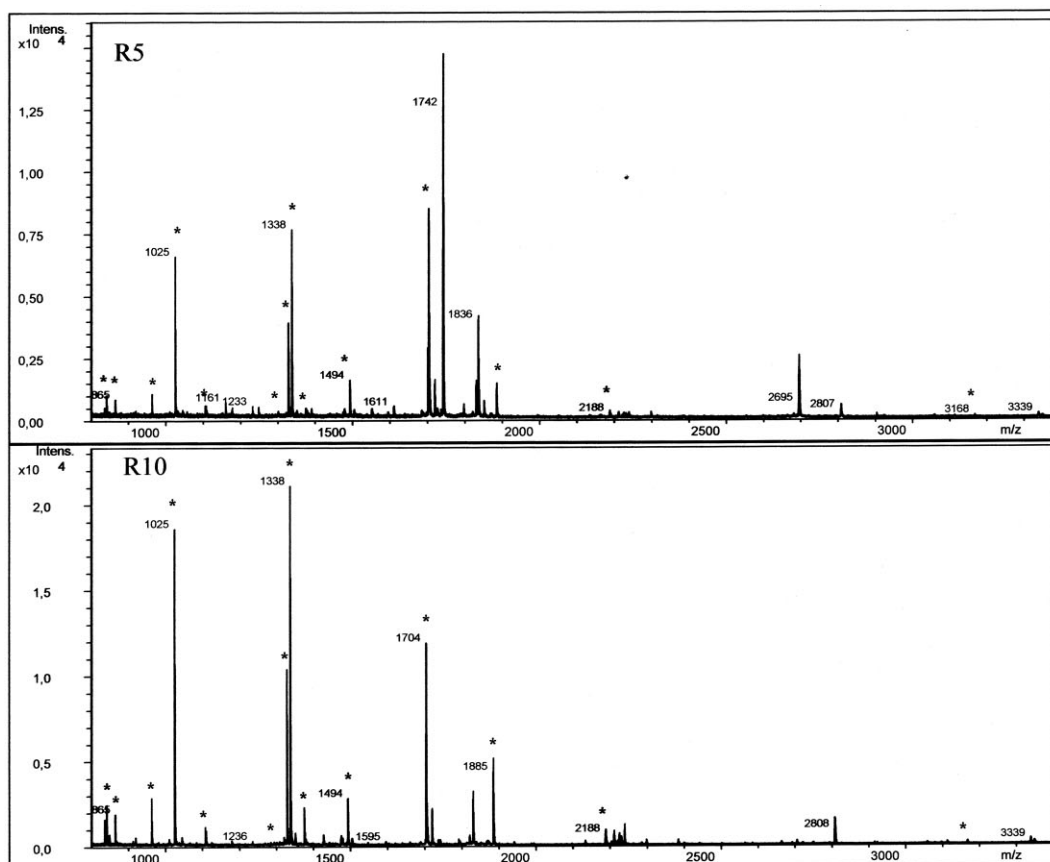
Table 1. Continued

Spot no.	Mass obs. (kDa)	pI obs.	PMF		Mass (Da)	pI	ID and name
			No. matched peptides/total signals	Sequence coverage			
			17/82	28%	62 056	6.47	Similar to royal jelly protein RJP57-1 [<i>Apis mellifera</i>] XP_391893
R10	30	7	16/82	31%	53 155	5.67	Royal jelly protein RJP57-1 – honey-bee S39193
R11	20	6.3	13/60	28%	49 311	5.10	Major royal jelly protein 1 precursor (Apalbumin-1) (Bee-milk protein) O18330
R12	19.7	6.3	11/71	29%	49 311	5.10	Major royal jelly protein 1 precursor (Apalbumin-1) (Bee-milk protein) O18330
R13	19.3	6.3	12/70	27%	49 311	5.10	Major royal jelly protein 1 precursor (Apalbumin-1) (Bee-milk protein) O18330
R14	18.8	6.5	9/74	21%	49 311	5.10	Major royal jelly protein 1 precursor (Apalbumin-1) (Bee-milk protein) O18330
R15	15.8	6.7	13/87	29%	49 311	5.10	Major royal jelly protein 1 precursor (Apalbumin-1) (Bee-milk protein) O18330
R16	15.5	6.8	7/42	19%	49 311	5.10	Major royal jelly protein 1 precursor (Apalbumin-1) (Bee-milk protein) O18330
R17	14	6.6	12/86	22%	49 311	5.10	Major royal jelly protein 1 precursor (Apalbumin-1) (Bee-milk protein) O18330
R18	13.1	7	12/71	22%	49 311	5.10	Major royal jelly protein 1 precursor (Apalbumin-1) (Bee-milk protein) O18330
R19	13.8	5.2					Not identified
R20	13.1	5.2					Not identified
R21	Not determined	5					Not identified

pollen-bread by this proteomic approach, suggesting a role other than the nutritional one for this protein, as already suggested for apalbumin 1 by Simuth *et al.* [18].

Interestingly, the *Apis mellifera* ligustica RJ 2-DE obtained in this experiment revealed more spots than Sano and colleagues [15]. In particular, some spots are visible at both the acidic and alkaline edge of the gel, and other spots are detectable below the 29 kDa range. The spot named R9, present at the acidic edge (pI observed 3.0) of the gel obtained in this experiment, was identified as to be the glycoprotein apalbumin 1, that from the data bank is described as a protein with several potential glycosylation sites. The same protein has been identified in spot R8 (pI observed 5.2) present in gels obtained in both our experiment and in Sano *et al.* experiment [15]. The more acidic pI shown by R9 and R8 is in agreement with the behavior of glycoproteins that become more acidic according to the level of glycosylation (different degree of PTM).

At the alkaline edge of the gel the spots named R1, R2 and R3 are visible. Two proteins, similar to royal jelly protein RJP57-1 (XP_391893) and royal jelly protein RJP57-1 (S39193), contribute to spot R1, while both spots named R2 and R3 were identified as RJP57-1 (XP_391893) (see Table 1), the same as R4 and R5 (R5 spectrum is reported in Fig. 2) identified also by Sano and colleagues as apalbumin 3 [15]. Twenty-five of the 64 peptides resulting from digestion of spot R6 match perfectly with two proteins RJP57-1 (XP_391893) and apalbumin 3 (Q17060) available in the data bank. In this case, it was not possible to give a unique identification to spot R6. RJP57-1 is the original name of apalbumin 3, characterized by Albert and colleagues [34] and that turned out to be polymorphic, so the two proteins that contribute to the R1 spot and apalbumin 3 (Q17060) present in the R6 spot could be three isoforms of apalbumin 3. The apalbumin 3 form called RJP57-1 (XP_391893) that is



1 MTKWLLLVVC LGIACQDVTS AAVNHQRKSA>NNLAHSMKVI **YEWKHIDFDF**
 51 **GSDERRDAAI** **KSGEFDHTKN** **YFPDVRWRD** **KTFVTIERYN** **GVPSSLNVVT**
 101 **NKKGKGGPLL** **RPYPDWSFAK** **YEDCSGIVSA** **FKIAVDKFRD** **LWVLDSGLVN**
 151 **NNQPMCSPKL** **LTFDLKTSL** VKQVEIPHNI AVNATTGMGE LVSLAVQAID
 201 **RTNTMVIYIAD** **EKGEGLIMYQ** **NSDDSFHRLT** **SNTFDYDPRY** TKLTVAGESF
 251 TVKNGICGIA LSPVTNNLYY SPLSSHGLYY VDTEQFRDPQ YEENNVQYEG
 301 SQDILNTQSF GKVVSKNQVL FLGLVGNQSGI ACVNEHQVLQ RESFDVVAQN
 351 EETLQMIVSM KIMENLPQSG RINDPEGNEY MLALSNRMQK IINNDNFND
 401 VNFRILGANV DDLMRNTRCG RYHNQNAQNG NADNQADNQ NANNQNADNQ
 451 NANKQNGNRQ NDNRQNDNKQ NGNRQNDNKQ NGNRQNDNKQ NGNRQNGNKQ
 501 NDNKQNGNRQ NDNKRNGNRQ NDNQNNQNDN NRNDNQVHHS SKLH

Figure 2. MALDI-TOF spectra of apalbumin 3 RJP57-1 (XP_391893) (spot R5 (whole protein) and R10 (N-terminal part)). Sequence coverage by PMF of spot R5 (underlined) and spot R10 (in bold). Asterisks indicate peptides belonging to the N-terminal part of RJP57-1 present in both spectra.

detected in the alkaline spots R1, R2 and R3 could be less glycosylated than the same protein detected in spots R4, R5 and probably in spot R6.

The presence of proteins in the alkaline and acidic range of the RJ 2-DE performed in our experiment and that were not detected in that of Sano and colleagues [15] could be the result of a difference in the larval age from which the RJ is sampled, as well as in a difference between the honey-bee races. This hypothesis is supported by the finding of Sano

and colleagues [15] who reported differences in the 2-DE protein pattern between the European honey-bee and the Africanized one. Unfortunately, neither the larval age nor the honey-bee race have been reported in [15].

Figure 1a shows also several spots (from R10 to R21) with a mass below 29 kDa that have not been detected in the 2-D gel by Sano *et al.* [15]. This higher resolution of low M_r proteins is due to the higher percentage of polyacrylamide (15% instead of 10%T).

The two proteins identified as similar to royal jelly protein RJP57-1 (XP_391893), and royal jelly protein RJP57-1 (S39193), both isoforms of apalbumin 3, were found to contribute to spot R10. The sequence coverage and peptide distribution at the N-terminal part of the protein (Fig. 2) suggest that the two identified proteins could be fragments of apalbumin 3, as a consequence of a degradation process.

Spots numbered from R11–R18, after MALDI-TOF analysis, turned out to be C-terminal fragments of apalbumin 1, confirmed also by the peptide distribution in the sequence coverage. The presence in the RJ of the two isoforms of apalbumin 3 fragments, to the best of our knowledge, has not been reported previously in the literature. The presence of fragments of apalbumin 1 in the RJ gel is in agreement with the presence of immunoactive peptides with a mass under 55 kDa against apalbumin 1 polyclonal antibodies, reported by Simuth *et al.* [18] and is in agreement also with Funakoshi *et al.* [44], with Chen and Chen [17] that reported the presence of some proteinase(s) in the RJ and with Kamakura and colleagues [45, 46] who found the degradation of apalbumin 1 was proportional to storage temperature and storage period and due to a metalloproteinase.

3.2 Proteinase activity

RJ is a honey-bee secretion, in which contaminant pollen and maybe microorganisms can be found. Both, microorganisms and pollen grains, if disrupted, could potentially release proteinases that could digest some of the RJ proteins. Figure 3a shows a zymography where an intense protease activity band in the pollen-bread extract is detectable. The activity of this proteinase could be, in principle, responsible for apalbumin 1 and apalbumin 3 degradation in the RJ. However, the zymogram of RJ did not show any proteinase activities (Fig. 3b) revealing that pollen content of day 1 sampled RJ (physiologically useful for the 72 h old honey-bee) does not contribute towards protease activity. The same results have been achieved by Kamakura and Fukushima [46] using casein as a substrate. The absence of proteinase activity in the RJ zymograms suggests that in RJ there are no serine-proteinase activity. The lack of detection of proteinase activity could also be the result of a short period of incubation and a low concentration of disrupted pollen. Degradation of apalbumin 1 and apalbumin 3 could also be the result of proteinases other than serine ones, such as metalloproteinase, as suggested by Kamakura and Fukushima [46].

We need to keep in mind that in six days the newly hatched honey-bee larva will reach the last instar after which it will be sealed by the worker bees, the larva designated to be a queen will be fed daily with new RJ by the nurse bees. In this investigation, only fresh RJ has been analyzed. The RJ has been sampled from cups containing 72 h old queen-designate larvae, so at the sampling moment the larvae food had been stored for at least a few hours. Moreover, Chen and Chen [17] found first symptoms of protein degradation of RJ after one month's storage at room temperature, while

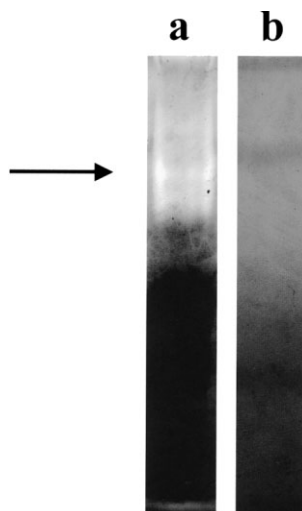


Figure 3. Gelatine-precast proteinase activity staining PAGE of RJ (a) and honey-bee pollen-bread (b). Proteins were separated in 10%T polyacrylamide gel containing 1% gelatine and incubated in 100 mM Tris-HCl, pH8 at 37°C for 4 h. The slabs were stained with Coomassie Blue and destained. 10 µg of protein extracts were loaded *per* lane. The arrow indicates the activity band.

Kamakura *et al.* [45] reported that apalbumin 1 degradation starts to become consistent over 30°C. This fact associated with the presence of fragments only belonging to apalbumin 1 and apalbumin 3 and not from others, could suggest a physiological role of these peptides in the development of the larvae. It is quite interesting to note that while apalbumin 2 is reported to be readily degraded in proportion to storage period and that degradation starts immediately after sampling [45], up till now in this investigation no fragments of apalbumin 2 have been detected.

4 Concluding remarks

This proteomic approach allows the identification of two apalbumin 2 isoforms in the RJ and the presence of the apalbumin 2 and apalbumin 1 also in the honey-bee pollen-bread. The presence of apalbumin 2 in the honey-bee pollen-bread reinforces the idea that also this protein could play a role other than the nutritional one, although more investigations aimed to outline the possible functional role of apalbumin 2 are needed.

Differences concerning the presence of proteins in the alkaline and acidic range between the 2-DE performed in this investigation and that of Sano and colleagues [15], could be the result of differences concerning the honey-bee race, as well the larval age from which the RJ is sampled.

RJ 2-DE with a 15%T revealed spots at masses below 29 kDa suggesting that the two proteins identified in spot R10 are degradation products of apalbumin 3; this would be the first case of detection of the hydrolysis of a protein other than apalbumin 1. Spots numbered R11–R18 have been identified as apalbumin 1 fragments. The presence of apalbumin 1 and apalbumin 3 fragments could suggest a degradation process due to a proteinase activity. The RJ zymogram shows the absence of serine-proteinase activity, while the pollen-bread zymogram shows an intense serine-protease

activity, so apalbumin 1 and apalbumin 3 fragments could be the result of metalloproteinase activity that is present in the RJ. Nevertheless, it is not possible to completely exclude the presence of serine-proteases. They could be the result of the disruption or germination of the pollen grains that contaminate the RJ, or due to the possible low concentration of the serine-protease activity that could not be detected in our experimental conditions.

5 References

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