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Original article

Proteins in spermathecal gland secretion and spermathecal fluid and the properties of a 29 kDa protein in queens of *Apis mellifera*

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Abstract – One and two dimensional SDS-PAGE were used to characterize the protein pattern of the spermathecal gland secretion and spermathecal fluid in *Apis mellifera* queen pupae and emerged queens of different ages. The concentration of protein varied from 8.5 and 15.3 mg/mL in the spermathecal fluid, and from 5 to 8.5 mg/mL in the secretion. Development of the protein pattern of the gland secretion and spermathecal fluid was identical from pupae until the age of 3 days. In sexually mature queens (10 days or older) the gland secretion and spermathecal fluid each had one additional band at 79 kDa and at 29 kDa respectively. The 29 kDa protein was N-terminal blocked but several peptide fragments were sequenced after digestion with LysC protease. Only 2 of the sequences showed a distinct homology to the N-terminal half of the glycolytic enzyme triosephosphate isomerase (TPI). TPI antibody reacted with the 29 kDa protein, but the enzymatic activity was only 1/100 compared to TPI of hemolymph. The possible function of the protein is discussed.

spermatheca / sperm storage / protein pattern / 29 kDa protein / *Apis mellifera*

1. INTRODUCTION

Storage of spermatozoa in the spermatheca after mating is a widespread phenomenon in insects. Sperm storage enables females to fertilise and lay eggs independent of the presence of males. For fertilisation, only a few spermatozoa per egg are used. The duration of sperm storage has a wide range among insects. Especially in social Hymenoptera with perennial monogynous nests, queens can store spermatozoa for years. *Apis mellifera* L. queens are known to keep spermatozoa for the duration of their lives, which can be 3–5 years or sometimes longer (Butler, 1954). Furthermore, queens of all *Apis* species mate with many drones, 12 to 20 drones in *Apis mellifera* and up to 50 in *A. dorsata* Fabricius (review Palmer and Oldroyd, 2000). Queens store between 1×10^6 and 6×10^6 spermatozoa, the total number varying according to the species (review Koeniger and Koeniger, 2000).

In *Apis mellifera,* the spermatheca is a globular sac with a diameter of about 1.1 mm. It consists of a chitinous membrane with a one-layer epithelium, surrounded by a dense tracheal net (Bishop, 1920; Snodgrass, 1956; Ruttner et al., 1971). The lumen is filled with a transparent fluid and connected to a pair of tubular glands. About 2 days after the last mating,

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densely packed spermatozoa lay in bundles in the lumen giving the spermatheca a whitish marbled pattern. The spermatheca is separated from the lumen of the oviducts by a muscular system which keeps the spermathecal duct closed thus forming a separate spermathecal compartment. The muscles function as a pump for sperm transport (Bresslau's sperm pump, Bresslau, 1905).

The pH value of the spermathecal fluid is high (8.6). While the Na^+ concentrations in the hemolymph show the usual value for insect body fluids, the K^+ concentration in the spermathecal fluid is higher by about eightfold (Gessner and Gessner, 1976). The stored spermatozoa have a reduced metabolism (Verma, 1973) which is thought to depend on the high pH. Spermatozoa became immotile within 3 weeks after removal of only part of the dense tracheal net. After removal of the spermathecal gland the queen laid unfertilized eggs, even though a high percentage of the spermatozoa were motile for more than 90 days (Koeniger, 1970). Poole (1972) found after removal of some tracheae, the tall columnar cells of the spermathecal epithelium shortened at the tracheectomized areas. Thus, it is not clear if the infertility of these queens is caused directly by impairment of the oxygen supply to the spermatozoa or if it is caused by disturbing the isolating structure of the spermathecal complex.

The spermathecal complex contains several sugars (Alumot et al., 1969). Comparisons of proteins from hemolymph and the spermathecal complex by disk electrophoresis and immunological tests revealed that both are separate fluid systems (Lensky and Alumot, 1969). There is only little information on the amount and function of proteins in the spermathecal complex that may contribute to the survival of sperm during storage. Recently, antioxidases (CAT, SOD and GST) were found in the spermathecae of mated queens which may be involved with long-term storage by protecting the spermatozoa from oxidative stress (Weirich et al., 2002).

Satisfactory long-time preservation of *A. mellifera* sperm in vitro from ejaculate has not yet been achieved at room temperature or at a slightly cooler temperature (Verma, 1978; Verma, 1990; Collins, 2000). An addition of streptomycin and storage at 13–15 °C extended the sperm's ability to fertilize eggs to 35 weeks, still far less than the physiological duration of several years (Poole and Taber, 1969, 1970; Verma, 1978). After storage in liquid nitrogen for up to 2 years, spermatozoa had intermediate levels of survival. One level of partial survival was that only few spermatozoa reached the spermatheca (Bolten and Harbo, 1982), and of those that did, many did not fertilize eggs (resulting in non hatching eggs). A second level of partial viability was the production of mosaic males (Harbo, 1980), and a third level of survival was the production of few worker offspring (Harbo, 1983).

It seems possible that for the long time storage of many years, honey bee queens produce special proteins which might be necessary for survival of spermatozoa. We started to study the total amount of protein and the ontogenetic development of the protein pattern of glandular secretion and in spermathecal fluid and compared these to hemolymph. This research represents an initial step and should advance interest and research on functions of female secretions that may be important for sperm storage in vivo and in vitro.

2. MATERIALS AND METHODS

2.1. Producing and keeping the queens

Honey bee queens were reared in queenless colonies (Ruttner, 1983). Pupae were collected at different times after the queen cell was sealed, covering the pupal stages from day 1 until emergence. Adult queens were kept in nucleus colonies and were collected at different ages. Virgin queens were collected at the ages of 1, 2, 3 and 10 days. Egg laying queens were produced after they were allowed to mate naturally at the age of 10 days, or after they received two CO_2 narcosis on two successive days. The nuclei containing these queens were checked every third day for eggs and larvae. Egg laying virgin queens were sampled 6 days after the onset of oviposition, and mated queens at different ages.

2.2. Sample collection

Hemolymph was obtained by puncturing the second tergite. After anaesthesis with CO_2 the abdomen was dissected and the spermathecal complex was removed without loss of internal fluid (the spermaduct is naturally blocked by Bresslau's sperm pump, Bresslau, 1905). The spermatheca and its glands were rinsed 3 times in Hyes solution before transferring

Table I. Number of samples for one and two dimensional gels per different queen types and organs.

Queen sample	glandular secretion		spermathecal fluid		hemolymph	
	1D	2D	1D	2D	1D	2D
Pupae	22	3	24	4	5	5
Virgin, 1 day old	10	–	10	–	–	–
Virgin, 2 days old	6	1	6	1	–	–
Virgin, 3 days old	6	–	6	–	–	–
Virgin, 10 days old	22	3	25	3	2	2
Virgin, egg laying	15	1	20	2	–	–
Mated, egg laying	30	4	31	5	2	2
Mated, no eggs	9	2	12	2	–	–

into a vial containing 10 µL Hyes solution (Hyes solution contained 0.90% NaCl, 0.02% KCl, 0.02% $CaCl_2$ and 0.01% $NaHCO_3$, Ruttner, 1976). The spermathecal glands were separated, and the intact spermatheca was transferred into another vial with 10 µL Hyes solution. The spermathecal glands were cut into small pieces, and the spermatheca was punctured for extraction of the internal fluid. The contents of both vials were centrifuged at 14 000 *g* to precipitate the tissue, and in the case of mated queens, also the spermatozoa. The supernatant was frozen at –20 °C until use.

2.3. Determination of the protein amount

The amount of protein was measured with a commercial protein test solution (according to the method after Bradford, 1976). 200 µL Bradford solution was mixed with 800 µL of the test solution of hemolymph, gland extraction and spermathecal fluid. The absorption was measured at 595 nm (Beckmann, spectrophotometer DU640). Calibration curves were established from bovine serum albumin (Sigma Chem. Co), human γ-globulin (Boehringer, Mannheim), and samples of dialyzed and lyophilized hemolymph, all in the range of 0.05–5 mg/mL. The curves differed by less than 10% over this range.

2.4. Separation of soluble proteins by 1 and 2-dimensional SDS-PAGE

The protein was precipitated with methanol/chloroform solution after Wessel and Flügge (1984). Sodium dodecylsulfat-polyacrylamide gel electrophoreses (SDS-PAGE) were carried out using conventional methods in a minigel apparatus of Biorad, Miniprotean TM II (Laemmli, 1970). pH values were chosen either at 8.8 in the separation gels. Stacking gels always were at pH 6.8 and 5% acrylamide. Separation gels used 12.5% acrylamide, in the instance for band elution of the 29 kD protein an 18% acrylamid concentration was used for sharpening the band.

For 1-D-SDS-PAGE, 3 µg protein were applied per sample, which corresponds to 2–3 queens. For the two dimensional separations, the first dimension (isoelectric focussing) used a commercially available urea-ampholyte system (Sigma, Co) in the pH range between 5.0 and 8.0 in 2 mm diameter tubes (Hochstrasser et al., 1988). The second dimension followed the procedure of Laemmli as described above. For each 2-D-SDS-PAGE gel, 90 µg protein per sample was used which corresponds to more than 45 queens. The gels were stained with silver nitrate according to Blum, Beier and Gross (1987) and then dried (Biorad gel dryer model 453). The number of samples for the one and two dimensional gels are given in Table I.

2.5. Sequencing

For sequencing, spermathecal fluid was applied to one-dimensional SDS-PAGE with an acrylamide concentration of 18% as a broad band. A “prestained SDS-PAGE standard, low range” from Biorad was used. The gel was run until the 20 kDa band of the standard migrated off the gel, allowing distinct separation of the 29 kDa band from the 28 kDa band. The 29 kDa band was then cut out. Elution of gel slices was carried out in a Schleicher & Schüll apparatus (Biotrap). Slices were suspended in ammonium bicarbonate buffer, pH 8.8, and electrophoresed against a PVP membrane (Schleicher & Schüll). The concentrated samples in a 0.8 mL compartment were then subjected to protein precipitation by the addition of a tenfold excess of ethanol-chloroform

mixture (3:1). The protein precipitate aggregated in an interlayer between to phases. The protein was then run on a second electrophoresis at pH 8.8, before blotting on PVDF membranes. For blotting the PVDF – membranes (Millipore) were rinsed shortly in methanol and then introduced into the TankBlot – apparatus (BioRad), according to the instruction by the firm. Blotting was carried out for 1 h at 300 mA with cooling. A 10 mM CHAPS buffer (Sigma Chem.Co), pH 10.7, containing 10% of methanol, was used.

The PVDF – membranes were then stained at room temperature with 0.25 g Coomassie Blue R-250 in a 45% methanol –10% acetic acid solution for 30 seconds, and then destained in 45% methanol – 10% acetic acid until the proteins were just clearly visible. The bands were then submitted to sequencing.

The first trial runs showed the protein to be N-terminal blocked. The protein was therefore digested with LysC protease (Boehringer Mannheim, specific activity 3000 U/mg). Incubation was for 12 h at pH 8.8 (ammoniumbicarbonate, 0.01 mM) with 0.01 mg of the protease. The same amount of the enzyme was then added, a further incubation lasted 8 h. The fragments were isolated as described above, and subjected to gas phase sequencing. 3 fragments could be sequenced.

2.6. Immunological tests

TPI (rabbit crystalline) from Sigma Chem. Co. and Boehringer, Mannheim were used for immunisation. Both gave identical results. Three rabbits were injected by a scheme of 1 mg/week with the aid of phosphoryl lipid A and synthetic trehalose dicorynomycolate in squalene and 0.2% Tween 80 adjuvans (Sigma). After 3 weeks, the appearance of specific antibodies was monitored by an Ouchterlony test, with a dilution series of 1 (1:10 original serum) to 1×10^6. Additional tests against hemolymph showed no cross reaction.

For Western blotting, SDS-PAGE gels were partially destained and blotted on PVDF membranes (Amicon Co, see above). The weakly stained membranes were treated with a 1:10 000 dilution of the TPI antiserum in skimmed milk solution and rinsed after 1 h in 0.001 Tris-HCL buffer, pH 7.8. After 5 rinses, the membranes were incubate with peroxidase-conjugated goat-anti-rabbit IgG (Behring, Marburg, now Aventis).The commercial solution was diluted 1:50 000 in the buffer indicated, supplemented with 1% of skimmed milk powder. After incubation at 4 °C overnight, the supernatant solution was removed and the membranes were again rinsed thoroughly. The bands recognized by the TPI antibody were then displayed by the enhanced chemoluminescence system (Amersham Co).

2.7. Determination of the amount of a specific protein

50 µL samples of spermathecal fluid with a protein concentration of 2.02 mg/mL were diluted tenfold and iodinated (Schlatter et al., 2002) to a specific activity of 150 cpm/ng. The dialyzed solutions were treated with 0.2 mL of the TPI antiserum, incubated at 4 °C overnight, and then 1 mL of anti-rabbit antibody solution (Behring, Marburg) was added. The precipitate was centrifuged at 3000 *g*, and washed twice in 0.2 mL of phosphate-buffered saline with centrifugation. The final pellet was dissolved in 1% SDS solution. Counting of radioactivity was carried out in a Beckman LS 6500 β-Counter. For electrophoresis, at least 200 000 cpm were applied to SDS-PAGE gels. For autoradiography, the gels were dried in a gel vacuum drier, before exposure to X-OMAT-AR.KODAK film in a wooden press of adequate size.

2.8. Enzymological tests

TPI enzymatic activity was determined by two independent assays. The first contained, in final concentrations, 50 µg enolase, 50 µg pyruvate kinase, 50 µg hexokinase, 100 µg glucose-1-phosphate dehydrogenase, 5 mM ADP, 10 mM 3-phosphoglycerate and 10 mM NADH (Boehringer, Sigma) in 2.8 mL of 10 mM potassium carbonate – potassium phosphate buffer, pH 7.4 or 9.6. The reaction was started by addition of spermathecal fluid, or TPI from yeast, at final concentrations between 0.01 and 5 µg in the assay solution. The second assay contained 50 µg 3-phosphate glycerate kinase, 50 µg 3-phosphoglyceraldehyde dehydrogenase, 10 mM 2-phosphoglyceraldehyde, and 5 mM ATP in the buffers named above. Again, the test was started by the addition of spermathecal fluid or TPI from yeast (Sigma). Both tests reliably detected the presence of 1 ng of yeast TPI.

3. RESULTS

3.1. Queens 10 or more days of age

3.1.1. Secretion of the spermathecal gland

Figure 1 shows the protein bands of the secretion of the spermathecal gland (lanes 3, 4 and 5) fractionated in a 12.5% gel. 28 bands are clearly visible, 68% of them have a molecular weight from 21.5 and 66.2 kDa, only 7 bands have a higher weight. In all 76 gels the pattern

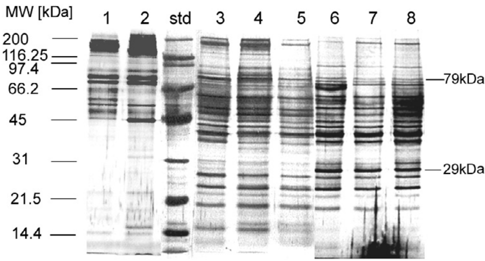

Figure 1. 1D SDS-PAGE with 12.5% polyacrylamide of queens older than 10 days. Hemolymph: 1 virgin ♀ no oviposition, 2 virgin ♀ in oviposition; gland secretion: 3 virgin ♀ no oviposition, 4 virgin ♀ in oviposition, 5 mated ♀ in oviposition; spermathecal fluid: 6 virgin ♀ no oviposition, 7 virgin ♀ in oviposition, 8 mated ♀ in oviposition.

of the protein spectrum show little intragroup variation in concentration, for all age groups above 10 days and independent of physiological status. The 2-D gel (n = 10) showed 96 or 97 proteins (Fig. 2B).

The amount of protein did not differ between non-laying or egg-laying virgin queens (5.02 ± 1.59 mg/mL and 5.49 ± 2.61 mg/mL, n = 37) and was only little higher in mated egg-laying queens (6.38 ± 2.19 mg/mL, n = 30). Significantly higher amounts were found in mated queens which did not lay eggs (8.45 ± 3.02 mg/mL, n = 9, P < 0.0001).

3.1.2. Spermathecal fluid

The 1D gels of the spermathecal fluid (Fig. 1, lanes 6, 7 and 8) also showed little intragroup variation. There were only two distinct differences in the protein pattern between the glandular secretion and spermathecal fluid. The amount of 79 kDa protein was much lower in the spermathecal fluid while the amount of the 29 kDa protein was much higher in all gels (n = 88).

Also the 2 D-gels from spermathecal fluids of virgin and mated queens showed similar profiles. The band at 29 kDa was situated just above the 28 kDa line and was split into three spots, a feature occurring frequently (Fig. 2C). Other fluids did not show these spots. This finding is in agreement with the absence of the band at 29 kDa in all other samples (Figs. 1 and 2A, B). The 29 kDa proteins therefore, only appeared in the spermathecal fluid of the queen.

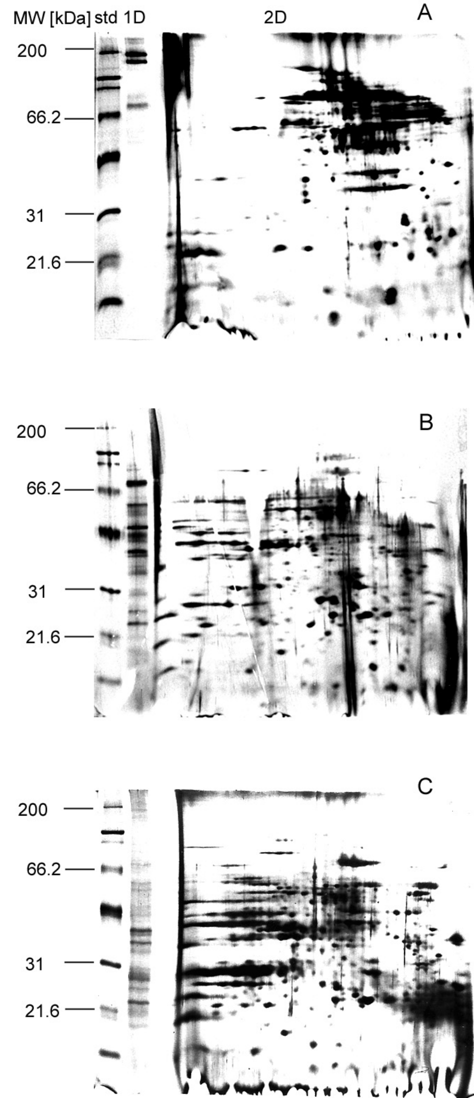

Figure 2. 2D SDS-PAGE (first separation by isoelectric focussing) of queens older than 10 days. A: hemolymph; B: gland secretion; C: spermathecal fluid.

The concentration of proteins in virgin queens was independent of oviposition (8.56 ± 2.14 mg/mL, n = 25 and 8.56 ± 2.3 mg/mL, n = 20). It was significantly higher in mated, egg-laying queens (11.27 ± 4.69 mg/mL, n = 31, P < 0.005). The highest concentration was found in the spermathecal fluid of mated queens which had stopped oviposition because of confinement (15.39 ± 3.49 mg/mL, n = 12, P < 0.001).

3.1.3. Hemolymph

The protein pattern of the hemolymph was clearly different from the spermathecal complex (Fig. 1, lane 1 and 2). Fifteen of the 20 protein bands in the 1-D gel had a molecular weight between 45 and 116 kDa, and 5 bands were even heavier.

In the 2 D gels most of the proteins had an isoelectric point in the acidic region. There were only quantitative but no qualitative differences in the pattern between the various queen types.

3.2. Ontogenesis of spermathecal fluid, spermathecal gland secretion and hemolymph

The spermatheca and its glands were big enough to be isolated only in 3 day old pupae. The spectra of the 3 fluids, hemolymph, spermathecal fluid and glandular secretion were similar in the 1-D gel, showing only 9 distinct bands. In 5 day old pupae (2 days before emergence) the growth of spermathecal complex is completed. The 1-D and 2-D protein spectra of the 3 fluids still showed similarities but in the hemolymph proteins with higher molecular weight dominated. Until the age of 3 days, in all 3 fluids, there was a prominent band at 23 kDa and at 80 kDa (Fig. 3).This finding differed from sexually mature queens (10 days) in which the 80 kDa disappeared from the spermathecal complex. In 3 day old queens 97% of the spermathecal fluid pattern and 94% of the glandular secretion correspond with sexually mature queens. But neither the 79 kDa nor the 29 kDa proteins were detectable yet (Fig. 3).

The 29 kDa protein was further characterized because it occurred only in the spermatheca of all sexually mature queens and of all mated queens.

3.3. Characteristics of the 29 kDa protein

3.3.1. Sequencing

As the first trial runs showed the protein to be N-terminal blocked only fragments of the protein could be sequenced after digestion with LysC protease. After further purification of the peptide fragments by HPLC and separation of the resulting peptides 3 fragments were sequenced. The positions of the amino acids of the fragments of the 29 kDa-protein are shown in Table II and compared with corresponding parts of TPI protein sequences from various sources. The number of amino acids in TPI of other animals, which are identical to the specific 29 kDa protein, is high in the first 29 kDa sequence (87.5 to 100%), while in the 2nd fragment (position between 52 and 67 AA) it is much lower. Only in *Drosophila* TPI there is more than 40% identity, the others show 31.25% or less. The third fragment of the 29 kDa protein ILRSELK could neither be found in the other TPI samples nor in other known proteins. The various peptides which were eluted by HPLC were obtained in exactly the same amounts, with less than 5% difference in the amounts of amino acids identified in the first sequencing step. We ascribe this to the sequencing of a homogenous protein.

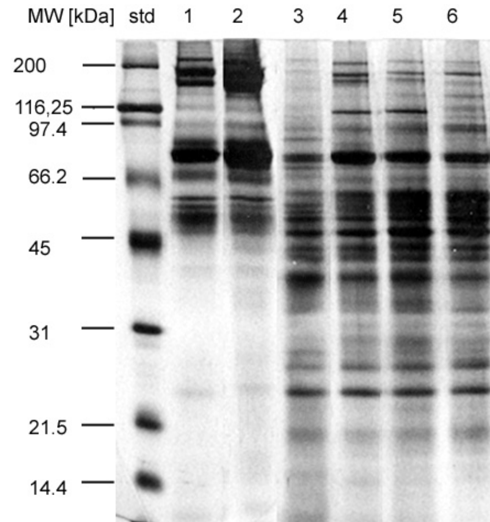

Figure 3. 1D SDS-PAGE with 12.5% polyacrylamide of queens 3 days old. 1 and 2: hemolymph; 3 and 4: gland secretion; 5 and 6: spermathecal fluid.

3.3.2. Immunological experiments

The immunoblot of antibodies against yeast TPI from rabbits shows that these antibodies recognized the purified 29 kDa honey bee protein

Table II. Comparison of selected sequences of TPI (source: SwissProt).

Apis mellifera	F F V G G **N** W K	//	I L P N N I S I I **A** G Q N T Y K
	??		**??**
Drosphila melanogaster	F C V G G **N** W K	//	L L P C E L G L **A** G Q N A Y K
	10		**61**
Culex tarsalis	F C V G G **N** W K	//	Q L P D DS V C V **A** A Q N C Y K
	10		**60**
chicken	F F V G G **N** W K	//	Q K L D A K I G V **A** A Q N C Y K
	10		**61**
Caenorhabdis elegans	F F V G G **N** W K	//	S K L K A G V L V **A** A Q N C Y K
	10		**61**
Arabidopsis thaliana	F F V G G **N** W K	//	S T L R S D F F V **A** A Q N C W V K
	10	//	**63**
Mus musculus	F F V G G **N** W K	//	Q K L D P K I A V **A** A Q N C Y K
	12		**62**
Giardia lamblia	P F I G G **N** F K	//	R L S T A I **A** A N T S K Q L R I
	11		**53**
Aedes togoi	C K V L T **T** G P	//	Q L P D S V G V **A** A Q N C Y K V
	10		**43**

The third 29 kDa sequence, ILRSELK, could not be found in the protein sequence libraries.

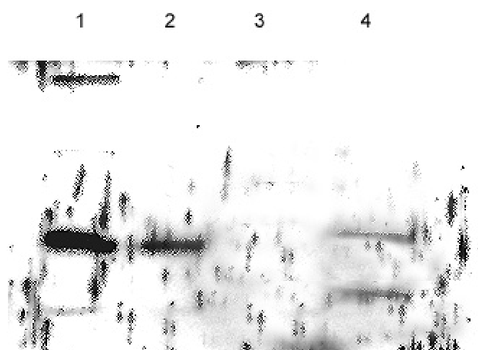

Figure 4. Immunological cross reaction with antibodies against iodine labeled TPI. 1: yeast 3 µg; 2: yeast 1 µg; 3: gland secretion; 4: spermathecal fluid.

from the preparation that was used for sequencing (Fig. 4). This further verified the similarity between 29 kDa protein and TPI.

3.3.3. Determination of the amount of the 29 kDa protein

After labeling samples of the spermathecal fluid with ^{125}Iodine as described in Methods (2.7) to an average radioactivity of 150 cpm/ng and an immune precipitation of the specific protein, a concentration of 25 ng/mL ± 10% could be calculated for the spermathecal fluid. The 2D PAGE of the redissolved immune precipitate in electrophoresis buffer showed only 2 radioactive spots for the 29 kDa spermatheca protein in the silver stain and autoradiography. No other spots except the antibody spots could be detected.

3.3.4. Enzymological determinations

Samples of the various physiological fluids (hemolymph, glandular secretion, spermathecal fluid) were tested for TPI activity. Using the specific activity of yeast TPI as a standard (10 000 U/mg protein) the concentrations of TPI in hemolymph and in whole body homogenate fluid after homogenization and centrifugation at 30 000 rpm for 2 h was determined to be 5 and 7 µg/mL ± 15% respectively. But the enzymatic activity for the spermathecal fluid was too low for a reproducible determination, certainly less than 0.2 ng of TPI/mL. This amount does not correspond to the previous calculated amount of the specific 29 kDa protein of 25 ng/mL ± 10%. Thus, the relation

of enzyme activity of the 29 kDa protein and TPI is only about 1 to 100.

4. DISCUSSION

This study shows for the first time that a high concentration of protein is found in the spermathecal fluid in *Apis mellifera* (from 8.5 mg/mL to 15.3 mg/mL). The secretion of the spermathecal gland has about half the concentration. Mated, egg laying queens had a concentration of 6.4 mg/mL in the gland secretion and 11.3 mg/mL in the spermathecal fluid. Significantly higher amount of proteins occurred in queens in which oviposition was interrupted in both in the gland secretion and in the fluid. Harbo (1979) calculated that a queen, laying and fertilizing 1000 eggs per day depletes about 1/530 of the spermatheca's contents independent of the number of spermatozoa. This seems to be constantly replaced by gland secretion during oviposition, especially in 2 year old queens in which half of the spermatozoa were already depleted after laying 60 000 eggs (Harbo, 1979). The lack of the constant depletion of the content in the spermatheca in non-ovipositing queens may explain the higher concentration in the spermathecal compartment.

During ontogenesis, the 1D gels of glandular secretion and spermathecal fluid showed similar protein patterns, and also the 2D gels revealed only few differences. In young pupae hemolymph had a similar pattern as the spermathecal fluid and the gland secretion. The protein pattern became increasingly different during the process of maturation. This result suggests that in mature queens the spermathecal fluid is produced by the gland cells.

Two additional prominent bands appeared in different concentrations in the spermathecal complex in sexually mature virgin and mated queens: a band at 79 kDa was prominent in the glandular secretion, and one at 29 kDa was prominent in the spermathecal fluid. As the 29 kDa protein was limited to the spermatheca of sexually mature queens we hypothesized that it may play a role in sperm storage and characterized it in detail.

Reaction of 29 kDa proteins with antibodies against yeast TPI raised in rabbits could be achieved.

But sequencing resulted only in similarity of 2 fragments with TPI of various insects. The third fragment of the 29 kDa protein could not be traced in other TPI samples. Further the enzymatic activity of the specific 29 kDa protein was much lower (about 1/100) than the activity of TPI in hemolymph and whole body homogenate fluid of queens. Actually it was too low for a reproducible determination. We therefore tentatively follow the hypothesis that this protein, which is not bound to cell structures but distributed in the spermathecal fluid, is different from TPI of the glycolytic pathway. Thus we assume that it has no enzymatic but may have other functions necessary for storage.

Investigations on the spermathecal proteins produced by females are still rare. For example in *Drosophila melanogaster* data on proteins from the accessory glands, ejaculatory duct and ejaculatory bulb of males (Acps) and their encoding genes are rapidly accumulating (review Chapman, 2001). Acps comprise diverse molecules, ranging from small peptides to large glycoproteins. These proteins affect female reproductive processes after mating, such as sperm storage, receptivity, ovulation and oogenesis. Since in *Drosophila melanogaster* there is only a short time of sperm storage, Acps may be stable for this period. But in honey bees, proteins of male accessory glands seem to be insignificant. Instrumentally inseminated honey bee queens with semen, washed in an adequate diluent and subsequently concentrated again by centrifugation, showed no significant difference to queens inseminated with ejaculate (Kaftanoglu and Peng, 1980). Moritz (1984) found effects of different semen diluents on the onset of oviposition but using an appropriate diluent there was no significant difference to ejaculate. Also, brood pattern and colony development in test and control queens were the same. Thus in honey bees, female derived proteins seem to play a major role in sperm storage.

Studies of spermathecal extracts in the Orthoptera, *Eyprepocmemis plorans*, revealed a change in the sperm tail resulting from complete removal of the glycocalyx in vivo and vitro (Giuffrida and Rosati, 1993; Giuffrida et al., 1996). The biological activity is achieved during the course of sexual maturation (Giuffrida et al., 1996). A glycoprotein of 35 kDa, whose N-linked Glycan chains proved to be the active parts, induced this modification of the sperm

tail (Giuffrida et al., 1997). In *Drosophila melanogaster* a male protein Acp36DE is transferred to the female reproductive tract after mating (Lung and Wolfner, 1999). It is tightly bound to the spermatozoa and possibly helping them to assume or retain an orderly parallel arrangement and prevent their premature loss from the organs (Neubaum and Wolfner, 1999). Further sex peptides (a 36 aa and a glycolysated 31 aa peptide (DUP99B) were shown to bind to the tip of sperm heads by immunolocalisation (Peng et al., 2000).

In the honey bee queen, the densely packed spermatozoa, arranged in parallel bundles, fill the whole volume. It is surrounded by spermathecal fluid. The amount of spermathecal fluid increases with the depletion of spermatozoa for fertilizing the eggs. Queens, whose spermatozoa have curled tails start to lay unfertilized eggs (Fyg, 1960). Thus, properties of the proteins may influence the activity of sperm tail movement.

We hypothesize that in *Apis mellifera,* in addition to the high pH, many proteins are produced by the queen which have a function in long sperm storage. The present report is just a beginning. For functional studies the 29 kDa and other proteins have to be further characterized. From cDNA banks, the genes coding for the respective proteins should be identified to enable a multiplication for in vitro and in vivo sperm tests. Considering the economical importance of breeding programs, the storage of sperm of successful lines is desirable. More knowledge of the function of proteins in the spermatheca may provide a step towards this goal.

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Résumé – Les protéines de la sécrétion de la glande spermathécale et du liquide spermathécal et les propriétés d'une protéine de 29 kDa chez les reines d'*Apis mellifera*. Le stockage des spermatozoïdes dans la spermathèque après l'accouplement est un phénomène largement répandu chez les insectes. La durée du stockage varie entre quelques jours et plusieurs années. En particulier, les reines des colonies d'Hyménoptères sociaux qui ont des nids monogynes pérennes peuvent conserver les spermatozoïdes fonctionnels pendant plusieurs années. Les reines d'*Apis mellifera* pondent des œufs fécondés durant 3 à 5 ans, chez les fourmis cela peut aller jusqu'à 20 ans.

Alors qu'il y a eu de nombreuses recherches sur la fonction des protéines des glandes accessoires mâles (acp) et sur leurs séquences de gènes principalement chez *Drosophila melanogaster*, on connaît peu de choses en ce qui concerne les protéines femelles. Le comportement de ponte de reines inséminées artificiellement avec des spermatozoïdes sans liquide spermathécal n'est pas significativement différent de celui de reines inséminées avec du sperme éjaculé. C'est pourquoi on pense que les protéines du tractus génital femelle jouent un rôle. Puisqu'on n'est pas parvenu jusqu'à présent à conserver des spermatozoïdes dans de l'azote liquide en vue d'élevage, nous avons commencé à décrire les protéines susceptibles de jouer un rôle dans le stockage.

La concentration des protéines de reines adultes de différents âges a varié entre 5 et 8,5 mg/mL dans la sécrétion glandulaire et entre 8,5 et 15,3 mg/mL dans le liquide spermathécal. Une analyse par PAGE-SDS uni- ou bidimensionnelle a permis de caractériser leur profil protéinique et de les comparer à celui de l'hémolymphe. Chez les jeunes nymphes les profils ressemblent à celui de l'hémolymphe, mais la différenciation se met en place dès deux jours avant l'émergence. A partir de ce moment les gels PAGE-SDS unidimensionnels du complexe spermathécal présentent 28 bandes nettes et 70 % d'entre elles possèdent un poids moléculaire compris entre 66,2 et 21,5 kDa. Elles se distinguent ainsi clairement des protéines de l'hémolymphe, dont le poids moléculaire est majoritairement compris entre 50 et 116 kDa (Figs. 1 et 3).

Jusqu'à l'âge de trois jours il n'y a aucune différence entre la sécrétion glandulaire et le liquide spermathécal. Chez les reines sexuellement mûres il apparaît dans la sécrétion glandulaire une bande protéinique supplémentaire à 79 kDa et dans le liquide spermathécal une bande à 29 kDa (Fig. 1). Cette protéine de 29 kDa a été plus précisément caractérisée. Comme les premiers essais ont montré qu'elle était bloquée en position N terminale, seuls quelques fragments ont pu être séquencés après digestion par la protéase LysC. Deux des séquences ont montré une nette homologie avec la partie bloquée en position N terminale de la triose phosphate isomérase (TPI). La concentration de cette protéine a atteint 25 ng/mL mais l'activité enzymatique correspondait à moins de 0,2 ng/mL de TPI, donc à moins de 1/100. On discute de la fonction de cette protéine de 29 kDA, non liée à une cellule mais en solution dans le liquide spermathécal, qui doit être différente de celle de la TPI de la voie glycolytique.

***Apis mellifera* / spermathèque / stockage des spermatozoïdes / profil protéinique / protéine de 29 kD**

Zusammenfassung – Proteine des Sekrets der Spermathekaldrüse und der Spermathekalflüssigkeit und die Eigenschaften eines 29 kDa Proteins bei *Apis mellifera* Königinnen. Die Speicherung von Spermatozoen nach der Paarung in Spermatheken kommt bei Insekten häufig vor. Die Dauer der Speicherung variiert zwischen einigen Tagen und mehreren Jahren. Besonders Königinnen von sozialen Hymenopteren mit mehrjährigen monogynen Nestern können Spermatozoen jahrelang voll funktionsfähig speichern. Königinnen von *Apis mellifera* legen 3–5 Jahre lang befruchtete Eier, bei Ameisenarten gibt es Berichte von mehr als 20 Jahren.

Während es über die Funktion von Proteinen der männlichen Anhangsdrüsen (acp) und ihre Gensequenzen vor allem bei *Drosophila melanogaster* viele Untersuchungen gibt, ist von den weiblichen Proteinen wenig bekannt. Das Eilegeverhalten von Königinnen, die mit Spermatozoen ohne Spermaflüssigkeit instrumentell besamt wurden, unterschieden sich nicht signifikant von Königinnen, die mit Ejakulat besamt wurden. Deshalb ist zu vermuten, dass Proteine des weiblichen Genitaltraktes bei der Speicherung eine Rolle spielen. Da eine Speicherung von Spermatozoen in flüssigem Stickstoff für Zuchtzwecke bisher nicht erfolgreich war, soll hier begonnen werden Proteine zu beschreiben, die eine Rolle bei der Speicherung spielen könnten.

Die Konzentration der Proteine adulter Königinnen verschiedenen Alters variierte von 5 bis 8,5 mg/mL im Drüsensekret und von 8,5 bis 15,3 mg/mL in der Spermathekalflüssigkeit. Ein- und zweidimensionale SDS-PAGE wurden durchgeführt, um das Proteinmuster beider Systeme zu charakterisieren. In jungen Puppen ähnelten sie dem Muster der Hämolymphe, aber bereits 2 Tage vor dem Schlupf begann eine Differenzierung. Ab dem Schlupf zeigten eindimensionale SDS-PAGE Gele des Spermathekal-Komplexes 28 deutliche Banden, von denen 70 % ein Molekulargewicht zwischen 66,2 und 21,5 kDa besaßen. Die Proteine der Hämolymphe unterschieden sich deutlich, ihr Gewicht lag meist zwischen 50 und 116 kDa (Abb. 1 und 3).

Bis zum Alter von 3 Tagen fand sich kein Unterschied zwischen Drüsensekret und Spermathekalflüssigkeit. Bei sexuell reifen Königinnen erschien im Drüsensekret eine zusätzliche Proteinbande bei 79 kDa und in der Spermathekalflüssigkeit bei 29 kDa (Abb. 1). Dieses 29 kDa Protein wurde genauer charakterisiert. Es erwies sich als N-terminal blockiert, aber einige Peptidfragmente konnten nach Verdauung durch LysC Protease sequenziert werden. Zwei der Sequenzen zeigten eine deutliche Homologie mit dem N-terminalen Teil von Triosephosphat Isomerase (TPI). Die Konzentration dieses Proteins betrug 25 ng/mL, aber die enzymatische Aktivität entsprach weniger als 0,2 ng/mL TPI, also weniger als 1/100. Es wird diskutiert, dass das in der Spermathekalflüssigkeit gelöste 29 kDa Protein eine andere Funktion hat als bei der Glykolyse in einer Zelle.

Spermatheka / Spermaspeicherung / Proteinmuster / 29 kDa Protein / *Apis mellifera*

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