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ORIGINAL ARTICLE

The role of 10-hydroxy-Δ²-decenoic acid in the formation of fibrils of the major royal jelly protein 1/apisimin/24-methylenecholesterol complex isolated from honey bee (*Apis mellifera*) royal jelly

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Abstract. Queen larvae of the honey bee (*Apis mellifera*) are fed with royal jelly, a glandular secretion produced by the hypopharyngeal and mandibular glands of worker honey bees. The necessary consistency of royal jelly is dependent on a protein-sterol complex ($MRJP1_4$/$apisimin_4$/$24MC_8$). At low pH, this complex forms fibrillar structures, which increase the viscosity of royal jelly. While the proteins in this complex are produced in the hypopharyngeal gland, the low pH is achieved by the secretion of the mandibular gland, which contains fatty acids. It is shown for the first time that fibril formation of $MRJP1_4$/$apisimin_4$/$24MC_8$ is not only induced by low pH via a buffer system, but also by the addition of the major fatty acid 10-hydroxy-Δ²-decenoic acid (10-HDA) of the mandibular gland secretion. This result further substantiates that fibril formation of the $MRJP1_4$/$apisimin_4$/$24MC_8$ complex only occurs after mixing the hypopharyngeal and mandibular gland secretions.

INTRODUCTION

Queen larvae of the honey bee (*Apis mellifera*) are fed a composite secretion (named royal jelly, RJ) that is produced in two different head glands of brood raising worker bees; the hypopharyngeal (HPGs) and mandibular glands (MDGs) (Schiemenz, 1883; von Planta, 1888; Kratky, 1931; Rembold, 1983). RJ provides the nutrients and the perfect viscosity to ensure that developing queen larvae are held in place in the queen cells, which are vertically oriented and open downwards (Aristotle, 350 BCE; Buttstedt et al., 2018; Pirk, 2018). This is achieved by a complex of two proteins (major royal jelly protein 1 (MRJP1) and apisimin) and a sterol (24-methylenecholesterol, 24MC) in which four MRJP1 molecules surround four central molecules of apisimin and eight of 24MC ($MRJP1_4$/$apisimin_4$/$24MC_8$) (Mandacaru et al., 2017; Tian et al., 2018). This $MRJP1_4$/$apisimin_4$/$24MC_8$ complex pH-dependently forms fibrils thereby increasing the viscosity of RJ (Buttstedt et al., 2018; Kurth et al., 2019). At its native pH of around 4.0, RJ has a complex tissue-like organization with proteinaceous fibril bundles that fall apart when the pH is exogenously raised to 7.0 (Kurth et al., 2019). Within the fibrils, the individual $MRJP1_4$/$apisimin_4$/$24MC_8$ complexes are simply stacked on top of each other involving hydrophobic and electrostatic interactions between MRJP1 molecules of neighbouring complexes (Mattei et al., 2020). Fibril formation and assembly of purified $MRJP1_4$/$apisimin_4$/$24MC_8$ can be observed *in vitro* using buffers with different pH (Buttstedt et al., 2016; Buttstedt et al., 2018; Tian et al., 2018). However, RJ is not a buffered system. Thus, it is so far only assumed how and when fibril formation occurs during RJ production, which is split between the HPGs producing the proteins (Patel et al., 1960; Schiemenz, 1883) and the MDGs contributing fatty acids (Callow et al., 1959; Kratky, 1931). It is hypothesized that fibril formation of the $MRJP1_4$/$apisimin_4$/$24MC_8$ complex only takes place after mixing HPG and MDG secretions due to the following: (1) The pure protein containing HPG secretion has a pH of 5.1 ± 0.1 (Hoffmann, 1960) and fibril formation only starts below a pH of 5.0 (Buttstedt et al., 2018; Tian et al., 2018). (2) Electron micrographs of HPGs do not show any fibrous material (Liu, 1990; Deseyn & Billen, 2005). (3) The inner diameter of the microtubes that transport the proteinaceous secretion within HPGs have a diameter of 1.2 µm, too small to smoothly transport pre-existing fibril bundles with diameters of up to 1 µm (Kurth et al., 2019).

Only after addition of the MDG secretion (pH 3.9 ± 0.1) (Hoffmann, 1960) to the proteinaceous HPG secretion does the pH of RJ decrease to values between 3.68 and 4.16

(Pavel et al., 2014; Mokaya et al., 2020) at which fibril formation is enabled (Buttstedt et al., 2018). The acidity of the MDG secretion and thus RJ is caused by high amounts of free organic acids with 10-hydroxy-Δ^2-decenoic acid (10-HDA) being by far the most abundant (50–70% of ether extracts of RJ) (Townsend & Lucas, 1940; Barker et al., 1959; Lercker et al., 1981; Isidirov et al., 2012). And indeed, the higher the amount of 10-HDA, the lower the pH of the RJ sample (Mokaya et al., 2020). Here it is shown that adding 10-HDA induces further assembly of the MRJP1$_4$/apisimin$_4$/24MC$_8$ complex supporting the hypothesis that fibril formation only occurs after the mixing of the HPG and MDG secretions.

MATERIALS AND METHODS

Protein isolation from royal jelly

Fibrillar MRJP1/apisimin/24MC from three different RJ (*Apis mellifera*) samples (RJF, RJG and RJH), acquired in 2018 from three different beekeepers in Romania, was purified based on the protocol published in Buttstedt et al. (2016). 5 g RJ were homogenized in 20 mM sodium citrate/citric acid, pH 4.0, the solution centrifuged at 6,500 × g for 10 min and the supernatant dialyzed two times for 3 h and once over night against 3 l of the same buffer (Spectra/Por® 6 Dialysis Membrane MWCO: 25 kDa, Spectrum Laboratories, Rancho Dominguez, CA, USA). The dialysate was subsequently centrifuged at 7,200 × g for 10 min and the supernatant was added to 5 ml Sulphopropyl (SP) Sepharose Fast Flow (GE Healthcare, Chicago, IL, USA) in 50 ml reaction tubes. The tubes were incubated for 2 h in a tube rotator during which all proteins except the fibrillar MRJP1/apisimin/24MC bound to the SP Sepharose. The slurry was centrifuged at 2,000 × g for 2 min, the supernatant was filtered through a 0.8 µm membrane and loaded onto a HiLoad 26/600 Superdex 200 pg gel filtration column (GE Healthcare, Chicago, IL, USA) equilibrated with 50 mM sodium citrate/citric acid, 150 mM NaCl, pH 4.0 at a flow rate of 2 ml/min with an ÄKTA FPLC system (GE Healthcare) (Fig. S1A). 15 ml of high molecular weight protein eluting in the void volume of the column was collected, dialysed three times for 3 h and once over night against 3 l double distilled water containing 150 mM NaCl (Spectra/Por® 6 Dialysis Membrane MWCO: 25 kDa, Spectrum Laboratories). The dialysate was analysed using SDS polyacrylamide gel electrophoresis (Laemmli, 1970) (Fig. S1B). Protein concentrations in the dialysate were assessed using UV spectroscopy as 0.680 mg/ml (RJF), 0.693 mg/ml (RJG) and 0.718 mg/ml (RJH) using a molar extinction coefficient of 224,740 M^{-1} cm^{-1} and a molecular weight of 209,477 Da (determined with ProtParam on the ExPASy server (Gasteiger et al., 2005) for the MRJP1$_4$/apisimin$_4$/24MC$_8$ complex).

Analytical size exclusion chromatography

All analytical experiments were carried out on an ÄKTA FPLC system (GE Healthcare) using a Superdex 200 Increase 10/300 GL (GE Healthcare) size exclusion column at a flow rate of 0.7 ml/min. The column was calibrated combining proteins from the gel filtration low molecular weight kit, the gel filtration high molecular weight kit (GE Healthcare) and the gel filtration marker kit for protein molecular weights 12,000–200,000 Da (Merck) (Fig. S2A) in 50 mM Tris/HCl, 150 mM NaCl, pH 7.5. Distribution coefficients (K_d) of calibration proteins and molecular weights (MW) of MRJP1/apisimin/24MC were determined using the following formula: $K_d = (V_e–V_0)/(V_t–V_0)$ and MW = $\exp((K_d–2.2129)/–0.155)$ (Fig. S2B, C). Even though size exclusion chromatography is not suitable for determining the precise molecular weight of the MRJP1$_4$/apisimin$_4$/24MC$_8$ complex, which is not globular (see discussion), it is suitable to detect the shift in elution volume that happens upon further assembly of the complex. Furthermore, size exclusion chromatography is widely used in the field of MRJP purification and characterization (Kamakura et al., 2001; Šimúth, 2001; Tamura et al., 2009; Majtan et al., 2010; Nozaki et al., 2012; Xu & Gao, 2013; Moriyama et al., 2015; Tian et al., 2018; Mattei et al., 2020) and thus it is necessary to know at which apparent molecular weights assemblies of the MRJP1/apisimin/24MC complex elute, even though this does not correspond to their precise molecular weights.

All analyses of the oligomeric state of MRJP/apisimin/24MC were done in double distilled water containing 150 mM NaCl supplemented with different amounts of 10-HDA (0–150 µg/ml, 0–805.2 µM) (Cayman Chemical, Ann Arbor, MI, USA). The initial trials to perform the analytical size exclusion runs in water only failed as 10-HDA in water eluted as micelles in the void volume of the size exclusion column even at a concentration as low as 5.4 µM (0.001 mg/ml), which interferes with fibril elution. The addition of 150 mM NaCl prevented hydrophobic interactions and thus micelle formation up to a concentration of 5.4 mM (1 mg/ml) 10-HDA (Fig. S3). For each run, the column was equilibrated using two column volumes of 150 mM NaCl containing the respective 10-HDA concentration (Table 1). 500 µl of 500 µg/ml (2.39 µM) MRJP1$_4$/apisimin$_4$/24MC$_8$ (purified from three different RJs) supplemented with 10-HDA (0–150 µg/ml, 0–805.2 µM) were injected into the column and the runs performed at a flow rate of 0.7 ml/min. To detect the proteins, but not 10-HDA, absorption was measured at 295 nm. In addition to the runs in 150 mM NaCl, the oligomeric state of MRJP1/apisimin/24MC was assessed in pH adjusted buffers (50 mM Tris/HCl, 150 mM NaCl, pH 7.5, and 50 mM sodium acetate/acetic acid, 150 mM NaCl, pH 4.0) in order to confirm the fibrillar state at pH 4.0 and the oligomeric state (MRJP1$_4$/apisimin$_4$/24MC$_8$) at pH 7.5 as reported earlier (Buttstedt et al., 2018; Tian et al., 2018).

Table 1. Concentration and proportion of 10-HDA and MRJP1/apisimin/24MC in the sample as well as elution volume (V_e) (median ± IQR) and calculated molecular weight (MW) (median ± IQR) of MRJP1/apisimin/24MC.

10-HDA (µM) in sample and running buffer	Protein (µM) in sample	Proportion 10-HDA : protein in sample	V_e (ml)	MW (kDa)
0.0	2.39	0.0 : 1	10.4 ± 0.1	485 ± 17
26.8	2.39	11.2 : 1	10.3 ± 0.2	517 ± 45
134.2	2.39	56.2 : 1	9.9 ± 0.8	622 ± 253
201.3	2.39	84.2 : 1	8.2 ± 0.1	> 1000
268.4	2.39	112.3 : 1	8.3 ± 0.2	> 1000
536.8	2.39	224.6 : 1	8.2 ± 0.1	> 1000
805.2	2.39	336.9 : 1	8.2 ± 0.1	> 1000

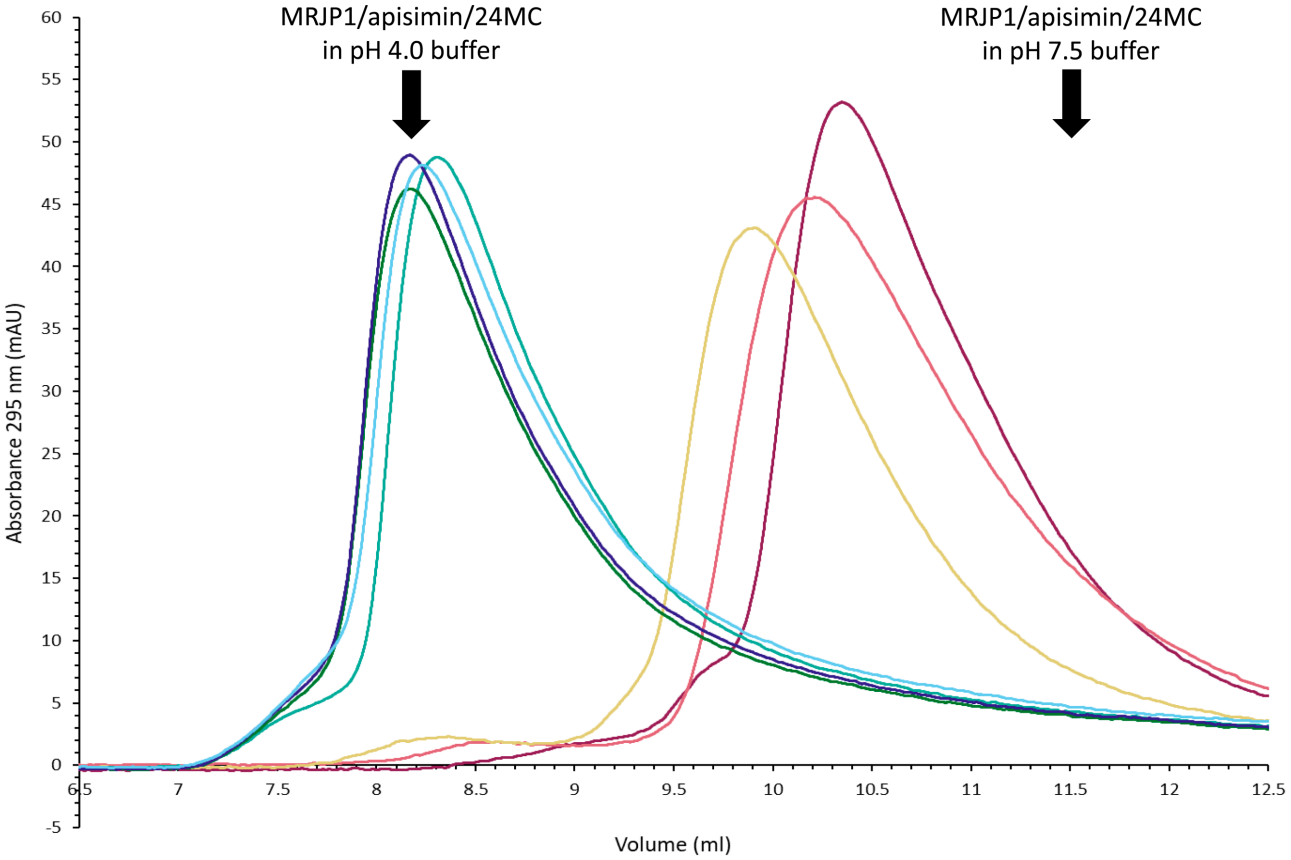

Fig. 1. Gel filtration analysis of 500 µg/ml MRJP1/apisimin/24MC in 150 mM NaCl supplemented with different concentrations of 10-HDA (0–805.20 µM) (column: Superdex 200 Increase 10/300 GL; flow rate: 0.7 ml/min; injection volume: 500 µl). 10-HDA concentrations: Purple – 0 µM; rose – 26.8 µM; sand – 134.2 µM; green – 201.3 µM; teal – 268.4 µM; cyan – 536.8 µM; indigo – 805.2 µM. Lines show exemplarily RJG (see Fig. S4 for all three RJ samples).

Statistics

All values are medians ± interquartile range (IQR). Statistical analysis was done using Statistica 8.0 (StatSoft, Tulsa, OK, USA). Data diverged from a normal distribution and were analysed using Kruskal Wallis analysis of variance (ANOVA).

RESULTS

When pH was adjusted via buffers, MRJP1/apisimin/24MC eluted at pH 7.5 (50 mM Tris/HCl, 150 mM NaCl) after 11.5 ml (all three RJ samples), which corresponds to a K_d of 0.267 and a molecular weight of 283 kDa. At pH 4.0 (50 mM sodium acetate/acetic acid, 150 mM NaCl), the peak eluted close to the void volume of the column after 8.2 ml (all three RJ samples) and thus outside of the fractionation range of the column (> 1,000 kDa), which is typical for MRJP1/apisimin/24MC fibrils (Buttstedt et al., 2016, 2018).

Without buffer (in 150 mM NaCl) MRJP1/apisimin/24-MC eluted after 10.4 ± 0.1 ml corresponding to a molecular weight of 485 ± 17 kDa (Fig. 1, purple line; Fig. S4). Thus, the complex eluted earlier than in 50 mM Tris/HCl, 150 mM NaCl, pH 7.5 and with an apparently higher molecular weight. The gradual addition of 10-HDA to MRJP1/apisimin/24-MC in 150 mM NaCl shifted the elution peak significantly closer to the void volume (Kruskal Wallis ANOVA, H = 18.229, n = 21, p = 0.0057) with the peak eluting at 8.2 ± 0.1 ml at the highest 10-HDA concentration (805.20 µM) (Table 1, Fig. 1).

DISCUSSION

In the pH adjusted buffer at pH 7.5, MRJP1/apisimin/24MC eluted with an apparent molecular weight of 283 kDa. This is in the same range as the expected theoretical molecular weight of 230.6 kDa for the $MRJP1_4$/$apisimin_4$/$24MC_8$ complex (4 × glycosylated MRJP1 monomer 51,320 Da (Buttstedt et al., 2016), 4 × apisimin 5,540.39 Da (ProtParam (Gasteiger et al., 2005)), 8 × 24-methylenecholesterol 398.7 g/mol (Da) (Kim et al., 2021)) and the with native mass spectrometry experimentally determined molecular weight of 231.9 kDa (Mandacaru et al., 2017). A with size exclusion determined molecular weight of 280 kDa for oligomeric MRJP1 has been reported earlier, which at that time was thought to be a MRJP1 pentamer (Tamura et al., 2009). The higher apparent molecular weight determined using size exclusion chromatography is expected as column calibration is typically performed with globular proteins not entirely reflecting the behaviour of nonglobular proteins such as $MRJP1_4$/$apisimin_4$/$24MC_8$, which has an H-shaped structure, with dimensions of 100 × 50 × 110 Å (height, width, length) (Tian et al., 2018). In the buffer at pH 4.0, MRJP1/apisimin/24MC elutes in the void

volume of the column, which is typical for the complex in the fibrillar state (Buttstedt et al., 2018; Tian et al., 2018).

In 150 mM NaCl, MRJP1/apisimin/24MC eluted earlier than in buffer at pH 7.5 and with a 1.7-fold higher molecular weight (485 ± 17 kDa). Thus, in the unbuffered solution two $MRJP1_4$/$apisimin_4$/$24MC_8$ complexes might have assembled and eluted together. Given that for fibril formation complexes assemble on top of each other (Mattei et al., 2020), two complexes together have dimensions of 100 × 100 × 110 Å (height, width, length), which indicates a rather globular state of two assembled complexes. This is confirmed by the experimentally determined molecular weight of 485 ± 17 kDa, which corresponds to the theoretically expected molecular weight of 461.2 kDa.

Adding low amounts of 10-HDA (26.84 and 134.20 µM) to MRJP1/apisimin/24MC without a buffering substance shifted the elution volume further towards lower values resulting in higher calculated molecular weights. Finally, the addition of 201.3 µM 10-HDA was enough to shift the elution volume of MRJP1/apisimin/24MC into the void volume of the column as is described for fibrillar MRJP1/apisimin/24MC (Buttstedt et al., 2018; Tian et al., 2018).

Fatty acids are due to their carboxyl group weak acids that dissociate in water into an carboxylate anion and aqueous proton (Nelson & Cox, 2004). This results in a decrease in solution pH down to the acid dissociation constant (pK_a) which is for saturated fatty acids with C10 to C18 chain lengths 4.01 ± 0.32 (Quast, 2016). When added to neutral aqueous solutions these fatty acids dissociate into anion and aqueous protons until a pH corresponding to their pK_a is reached, after which no further dissociation occurs. Thus, when 10-HDA is added to MRJP1/apisimin/24MC in 150 mM NaCl, aqueous protons are released lowering solution pH and inducing fibril formation. As MRJP1/apisimin/24MC is also known to fibrillate in buffers with pH 4.0 (Buttstedt et al., 2016, 2018; Tian et al., 2018) it might well be that any substance capable of adjusting solution pH to around 4.0 induces fibrillation. However, in RJ only organic acids, of which 10-HDA is by far the most abundant (50–70%) (Barker et al., 1959; Isidirov et al., 2012), are present as pH-lowering ingredients.

It is proposed that fibril formation of $MRJP1_4$/$apisimin_4$/$24MC_8$ is dependent on the protonation state of specific glutamic acid residues in MRJP1 (Mattei et al., 2020). Whereas at pH 7.0 glutamic acid is deprotonated and thus negatively charged, at pH 4.0 the carboxyl group is mostly protonated (Grimsley at al., 2009). In the pH 4 buffer, the loss of the electrostatic repulsion between two complexes enables acidic residues in MRJP1, including Glu48 and Asp396, to come into close contact facilitating complex assembly and fibril formation via hydrophobic contacts (Mattei et al., 2020). As a result, the contact areas of the four MRJP1 molecules between the complexes are negatively charged at pH 7.0, but predominantly hydrophobic at pH 4.0 (Mattei et al., 2020). This loss of electrostatic repulsion is achieved by protonation of the carboxyl groups, which as shown here, can also be accomplished by the addition of 10-HDA.

The core of the $MRJP1_4$/$apisimin_4$/$24MC_8$ complex is highly hydrophobic and during structural elucidation elongated densities are recorded at the core, which are consistent with fatty acids (Mattei et al., 2020). Thus, in addition to lowering the pH of the solution, 10-HDA might be in very close proximity to the complex creating a microenvironment with a higher 10-HDA concentration than in the overall solution.

In RJ, 10-HDA concentration averages 21 mg/g RJ (Ferioli et al., 2007) corresponding to 124 mM (calculated with RJ density = 1.1 g/ml (Sabatini et al., 2009) and molecular weight 10-HDA = 186.29 g/mol). $MRJP1_4$/$apisimin_4$/$24MC_8$ concentration averages 41.5 mg/g in RJ (Yamaguchi et al., 2013) corresponding to 0.22 mM (calculated with RJ density = 1.1 g/ml (Sabatini et al., 2009) and molecular weight $MRJP1_4$/$apisimin_4$/$24MC_8$ complex = 209,477 g/mol). Thus, RJ contains per molecule of complex approximately 550 molecules of 10-HDA. During size exclusion chromatography shown here, about 85 molecules of 10-HDA per molecule of complex (201.30 µM 10-HDA and 2.39 µM $MRJP_4$/$apisimin_4$/24-MC_8) were sufficient to shift the elution volume of MRJP1/apisimin/24MC into the void volume. This is about 6.5-times lower than the ratio in RJ. Thus, either the worker bees add a high surplus of 10-HDA to RJ to ensure that the $MRJP1_4$/$apisimin_4$/$24MC_8$ complex fibrillates, or when 10-HDA is added to the purified complex in solution, it might become enriched at the core of the complex due to hydrophobic interactions leading to an increased local 10-HDA concentration even when overall 10-HDA concentration in the solution is rather low. Of course, a combination of both is also possible.

10-HDA has a multiplicity of functions in RJ. It has antibiotic activity against a variety of bacteria and fungi (Blum et al., 1959; Yousefi et al., 2012), it influences larval development and might partially regulate honey bee queen development (Kinoshita & Shuel, 1975; Spannhoff et al., 2011) and is known as worker substance during the analysis of female honey bee MDG signals. Whereas in queenright colonies, workers predominantly produce 10-HDA and 10-hydroxydecanoic acid (10-HDAA) in their MDGs, the major compounds of queen MDGs are (*E*)-9-oxodec-2-enoic acid (9-ODA) and (*E*)-9-hydroxydec-2-enoic acid (9-HDA), which function as a queen pheromone (Crewe & Velthuis, 1980; Plettner et al., 1996; Mumoki & Crewe, 2021). Here, another function for 10-HDA was identified: it protonates negatively charged amino acids in the $MRJP1_4$/$apisimin_4$/$24MC_8$ complex, thereby inducing oligomerization and fibril formation leading to an increased viscosity of RJ.

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