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A review on Royal Jelly proteins and peptides

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ABSTRACT

Royal Jelly (RJ) is a nutrient rich substance secreted by the hypopharyngeal glands of young worker bees. Owing to the exceptional biological properties, RJ is used in pharmaceutical, food and cosmetics industries. Several studies vouch that RJ has anti-ageing, antibacterial, anti-fatigue, anti-inflammatory, antioxidant, antitumor, anti-diabetic and antimutagenic potentials. These activities are mainly attributed to bioactive components it contains.

One of the principal bioactive components is Major Royal Jelly Proteins (MRJPs) which is considered to be a major factor in honey bee queen development. In this review, we explore large number of studies that have been undertaken to elucidate the functions and characterization of Royal Jelly proteins and peptides.

1. Introduction

For millennia, products from honey bees have been used as food and medicine because of their high nutritive value and nutraceutical properties. Honey bees are a source not just for honey but for several other valuable natural products with cosmetic and health-promoting compounds such as bees wax, pollen, and Royal Jelly (RJ). Demand for these ingredients far exceeds supply.

2. Royal Jelly

RJ is a thick milky-white fluid produced and secreted by nurse honey bees (young newly emerged workers of 5–15 days old) from their hypopharyngeal gland (Fig. 1). It is fed to all the bee larvae in the early stages of their life and to the queen bee until she dies. After hatching, the larvae destined to be workers are fed with a mixture of RJ, honey and pollen (Snodgrass, 1984). Key nutrients in RJ and the duration of nourishment with it determine whether the female larvae develop into short-living infertile workers or the long-living fertile queen (Knecht & Kaatz, 1990). The infertile worker bees have a life span of approximately six to eight weeks, while the queen which is continually fed on RJ lives a fertile life of up to four to five years. Even though the queen and the workers are genetically identical they vary extensively in their phenotypic, physiological and functional characteristics. It is thus evident that RJ has a strong epigenetic influence in the differentiation of the larvae into the sub-populations of workers and queens. This is thought to be achieved by epigenetic modification to DNA and regulation of gene expression through methylation of CpG islands (Kucharski, Maleszka, Foret, & Maleszka, 2008). How RJ achieves this biological outcome is an enigma that scientists have not been able to convincingly answer despite numerous investigations and tests. On the basis of these observations numerous experiments have been carried out to investigate whether the effect of RJ is similar in organisms other than bees with respect to their development, maturation and longevity.

3. The source of Royal Jelly – the hypopharyngeal gland and the Mandibular gland

The hypopharyngeal gland (hpg) and the Mandibular gland (mbg) are the organs involved in the production of RJ. Mbg is a pair of sac like glands which is found only in queen and worker bee. It is located on both sides of the head, directly above the mandibles (Örösi-Ṕ l, 1957). Similar to hpg, RJ secretion by mbg also changes with age of worker bees (Huo et al., 2016). Hpg is a paired long tuberous organ located within the frontal part of the worker bee's head (Fig. 2). It has many small sac like structures called acini composed of secretory cells (Huang & Otis, 1989). The genes in these secretory cells temporally express a variety of proteins that regulate age (time) dependent changes in honey bees. These proteins are regulated by phosphorylation to optimize their cellular activity (Qi et al., 2015). The size of the acini increases around the 6th day after hatching coinciding with the very high rate of expression of RJ but is significantly reduced after the 15th day with the

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Fig. 1. Image of honey comb cell filled with Royal Jelly.

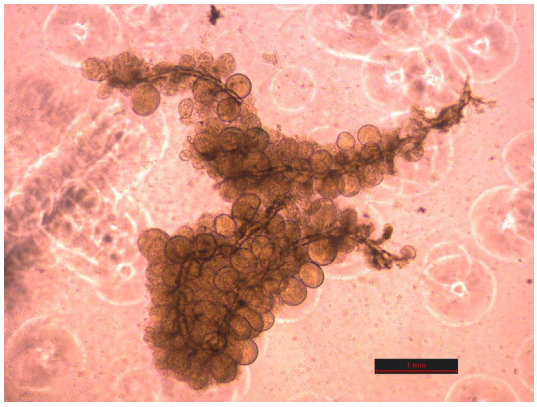

Fig. 2. Hypopharyngeal gland: Image from light microscopy.

colour changing from cream to pale yellow (Chanchao, Srimawong, & Wongsiri, 2006; Hrassnigg & Crailsheim, 1998; Huang & Otis, 1989; Liu, Wang, Zhou, & Zeng, 2015).

Both hpgs and mbgs produce RJ but only hpgs are involved in the production of proteins. Rate of protein synthesis in the hpg is thus highest in nurse bees and lowest in foragers (Knecht & Kaatz, 1990). This coincides with the synthesis and expression levels of protein rich RJ when the female worker bees are in the young nurse stage and the cessation of its expression when they start flying out as foragers.

When the bees become foragers, the glands shrink and switches to the expression of enzymes such as α-glucosidase, lucine arylamidase and invertase which are needed for the production of honey (Chanchao et al., 2006; Kubo et al., 1996; Simpson, Riedel, & Wilding, 1968; Suwannapong, Chaiwongwattanakul, & Benbow, 2010). Thus the foragers are no longer able to produce Royal Jelly.

For an apiculturist, on a bio-commercial perspective, a bio-technological intervention that succeeds in retaining a healthy honey bee colony will be advantageous in boosting the production of RJ.

4. Composition of Royal Jelly

RJ has a pH between 3.6 and 4.2. Water forms the major component at 60–70% (w/w) followed by proteins at 9–18% (w/w) and total sugars at 10–16% (w/w). It also contains small amounts of lipids, vitamins, salts and free amino acids (Bogdanov, 2011; Ramadan & Al-Ghamdi, 2012; Rembold & Dietz, 1966; Sabatini, Marcazzan, Caboni, Bogdanov, & Almeida-Muradian, 2009; Xue, Wu, & Wang, 2017).

The composition of RJ varies with seasons and ecological conditions around the location where the bees inhabit and forage. It also differs according to the race (Sano et al., 2004) and caste of the honey bee (Brouwers, Ebert, & Beetsma, 1987), physiological and metabolic differences between the nurse bees (Brouwers et al., 1987; Lercker, Caboni, Vecchi, Sabatini, & Nanetti, 1993), and the time of harvest of RJ (Scarselli et al., 2005; Zheng, Hu, & Dietemann, 2010).

Among the 9–18% proteins, majority of them are categorised as Major Royal Jelly Proteins (MRJPs), of which Major Royal Jelly Protein 1 (MRJP1) accounts for more than 45% (Furusawa et al., 2008). This is the most studied protein and considered to be the key factor that directs the development of the honey bee queen (Kamakura, 2011).

5. Proteins from Royal Jelly

Analyses of the Royal Jelly proteins show that 82–90% (w/w) is constituted by MRJPs (Drapeau, Albert, Kucharski, Prusko, & Maleszka, 2006; Santos et al., 2005; Schmitzova et al., 1998). Santos et al. (2005) and Shinkhede and Tembhare (2009) used evidence obtained through histological and transmission electron microscope studies to show that MRJPs are produced in the hpg of nurse bees. Apart from hpg, Buttstedt, Moritz, and Erler (2013a) also examined the expression of mrjps in various body parts (head, thorax and abdomen) of worker bees (nurse and foragers), queen bee (mated and unmated) and drones.

MRJPs are named by the order of the molecular weight or simply numbered according to the order in which they were discovered resulting in different names for the same proteins. MRJPs are named by the order of the molecular weight or simply numbered according to the order in which they were discovered resulting in different names for the same proteins. Table 1 shows the different names by which the major royal jelly proteins are known. Within the RJ protein family, nine members have been identified viz. MRJP1, MRJP2, MRJP3, MRJP4, MRJP5, MRJP6, MRJP7, MRJP8 and MRJP9 which are encoded by nine different genes. Fig. 3, briefly shows the classification and functions of MRJPs.

These complex proteins of MRJP family contain high amounts of amino acids necessary for nourishing both the queen bee and larvae. Arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine are the ten essential amino acids most commonly seen in MRJPs; with MRJP1 having 48%, MRJP2 having 47%, MRJP3 having 39.3%, MRJP4 having 44.5%, MRJP5 having 51.4%, MRJP6 having 42%, MRJP7 having 48.3%, MRJP8 having 49.5% and MRJP9 having 47.3% amino acid content. MRJP5 is rich in arginine and methionine while the predominant amino acids in MRJP1, MRJP2 and MRJP4 are leucine and valine. The major amino

Table 1
Names and molecular weights for MRJP1-MRJP9.

Proteins	Amino-acid content	Alternative name	Molecular weight (kDa)	References
MRJP1	48%		56	Kubo et al. (1996) and Ohashi et al. (1997, 2000)
			57	Kamakura, Fukuda, et al. (2001), Kamakura, Suenobu, et al. (2001)
		Apalbumin 1	NA[a]	Bilikova et al. (2002, 2009), Biilikova and Simuth (2010) (2010), Majtán et al. (2006), Scarselli et al. (2005), Šimúth (2001), Šimúth et al. (2004), Tao et al. (2008), Fontana et al. (2004), Rosmilah et al. (2008), Šimúth et al. (2004), Shen et al. (2015) and Xia et al. (2006)
		Apalbumin α	NA[a]	Schmitzova et al. (1998) and Šimuth, Bíliková, and Kovácová (2003)
		D III protein	NA[a]	Moriyama, Ito, Omote, Miura, and Tsumoto (2015) and Watanabe et al. (1996,1998)
		P56kP-4	NA[a]	Kamakura and Sakaki (2006), Ohashi et al. (1997) and Schmitzova et al. (1998)
		RJP-1	NA[a]	Kamakura, Fukuda, et al. (2001)
		RJP-3	NA[a]	Kucharski et al. (1998)
		RJPX	NA[a]	Klaudiny, Kulifajová, et al. (1994)
		Royalactin	55	Kamakura (2011)
		MRJP1 oligomer	280	Kamakura (2011) and Ramadan and Al-Ghamdi (2012)
		MRJP1 oligomer	350	Bilikova et al. (2002), Kimura et al. (2003) and Šimúth (2001)
		MRJP1 oligomer	420	Tamura, Amano, et al. (2009)
MRJP2	47%		50	Kubo et al. (1996), Ohashi et al. (2000)
		Royal jelly glycol-protein (RJGP)	55	Kimura et al. (1996)
		Apalbumin 2	NA[a]	Scarselli et al. (2005) and Šimúth et al. (2004)
		Apalbumin β	NA[a]	Schmitzova et al. (1998) and Šimuth et al. (2003)
			52	Nozaki et al. (2012)
			49	Schmitzova et al. (1998)
MRJP3	39.3%		64	Kubo et al. (1996) and Ohashi et al. (1997, 2000)
		Apalbumin 3	NA[a]	Rosmilah et al. (2008) and Scarselli et al. (2005)
		Apalbumin γ	NA[a]	Schmitzova et al. (1998) and Šimuth et al. (2003) Schmitzova et al.(1998)
		RJP-1	NA[a]	Kucharski et al. (1998)
		RJP57-1	NA[a]	Beye et al. (1998), Klaudiny, Hanes, et al. (1994), Klaudiny, Kulifajová, et al. (1994), Scarselli et al. (2005)
MRJP4	44.5%		60	Li et al. (2007) and Schmitzova et al. (1998)
		RJP-2	NA[a]	Kucharski et al. (1998)
		RJP57-2	NA[a]	Klaudiny, Hanes, et al. (1994), Klaudiny, Kulifajová, et al. (1994)
MRJP 5	51.4%		77	Schmitzova et al. (1998)
			80	Li et al. (2007) and Schmitzova et al. (1998)
			87	Schmitzova et al. (1998)
MRJP 6	42%		47.5	Buttstedt, Moritz, and Erler (2013b)
MRJP 7	48.3%		48.6	Buttstedt et al. (2013b)
MRJP 8	49.5%		45.06	Buttstedt et al. (2013b)
MRJP 9	47.3%		46.27	Buttstedt et al. (2013b)

[a] NA: Not Available.

acids in MRJP3 are arginine and lysine (Schmitzova et al., 1998). Leucine is the major amino acid in MRJP6, 7 and 8 and isoleucine is the predominant one in MRJP9. The amino acid content of MRJPs is included in Table 1.

Resolution of protein profile of RJ by size exclusion HPLC gave five peaks of 40 kDa, 280 kDa, 100 kDa, 72 kDa and 4.5 kDa. Among these peaks, the 280 kDa and 72 kDa components were identified as MRJP1 and MRJP2 respectively (Imjongjirak, Klinbunga, & Sittipraneed, 2005; Tamura, Kono, Harada, Yamaguchi, & Moriyama, 2009). On the basis of N-terminal sequencing, cDNA sequencing, SDS-PAGE and two dimensional electrophoresis the molecular weight of the subunits of MRJP1, MRJP2, MRJP3, MRJP4 and MRJP5 have been determined to be 55 kDa, 49 kDa, 60–70 kDa, 60 kDa and 80 kDa respectively (Albert, Bhattacharya, Klaudiny, Schmitzová, & Simúth, 1999; Klaudiny, Hanes, Kulifajova, Albert, & Simuth, 1994; Nozaki et al., 2012; Sano et al., 2004; Schmitzova et al., 1998; Su, Albert, Chen, & Zhong, 2005; Tamura, Kono, et al., 2009).

MRJP1 is primarily studied for its role as a 'queen maker' (Kamakura, 2011), but the other MRJPs also play important roles in determining the fate of the developing larvae (Schmitzova et al., 1998). Xin et al. (2016) working on *Drosophila melanogaster*, recently reported that not only MRJP1 but all the MRJPs together influence and enhance the longevity of *D. melanogaster*. In addition to extending the life span, RJ enhanced feeding rate and fecundity in both sexes of *D. melanogaster*. The major factor attributed to the extension of life span was the antioxidant property of the MRJPs. They observed that the levels of Super Oxide Dismutase the super-oxide scavenging enzyme were up-regulated when *D. melanogaster* was fed on RJ. MRJPs in the diet also up-regulate S6K, MAPK and Egfr in the Egfr mediated signalling pathway to extend the life span of *D. melanogaster*. This work was supported by Detienne, De Haes, Ernst, Schoofs, and Temmerman (2014) in the nematode, *Caenorhabditis elegans* where MRJP1 was shown to act on Egfr and its receptor thereby modulating the EGFR signalling system to extend the life span on *C. elegans*.

Buttstedt, Ihling, Pietzsch, and Moritz (2016) refuted the observations of Kamakura, observing that there is no single factor for queen differentiation like MRJP1. They suggested that the growth is driven by the amount of foods ingested while MRJP1 provides essential amount of nutrients for the queen larvae.

5.1. MRJP1

The most abundant and the first protein to be identified was MRJP1 (Hanes & Šimuth, 1992). It is also known in different names like apalbumin (Šimúth, 2001) and royalactin (Kamakura, 2011). Several researchers also report that in addition to its presence in the hypopharyngeal gland, MRJP1 is detectable in the cytoplasm of brain cells of the antennal lobe, optical lobe and mushroom bodies both in nurse and

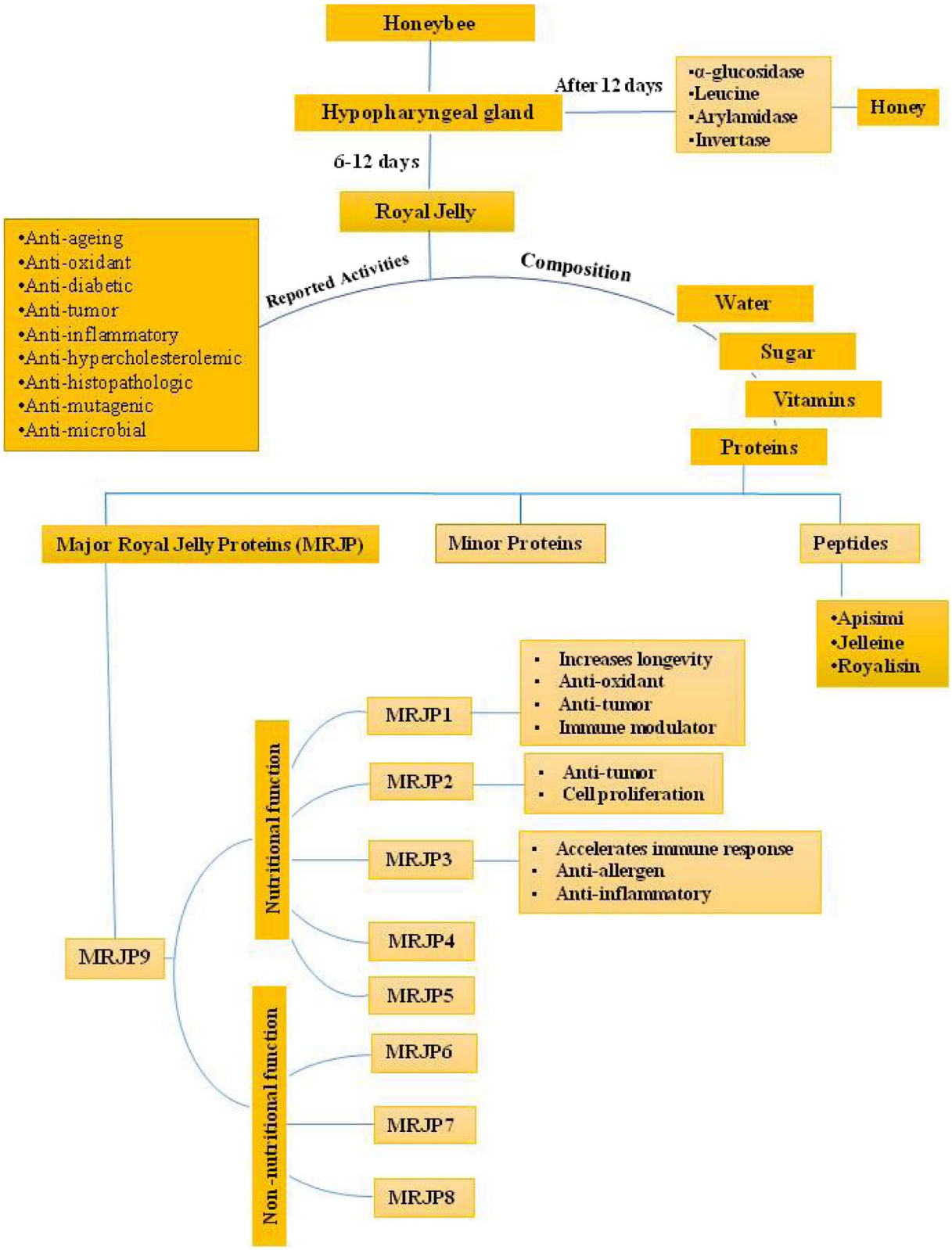

Fig. 3. Flow chart shows the proteins of Royal Jelly and their functions.

worker bees (Garcia et al., 2009; Kucharski, Maleszka, Hayward, & Ball, 1998; Peixoto et al., 2009). MRJP1 has also been detected in low quantities in honey, honey bee pollen pellet and pollen bread (Malecová et al., 2003).

MRJP1 is found in different forms such as monomers, oligomers, and water soluble forms. The monomeric form of MRJP1, royalactin functions as the queen determiner (Foret et al., 2012; Kamakura, 2011). It induces the physiological changes that results in the differentiation of larvae into queen bees, shortens the developmental time and increases both body and ovary size. The oligomeric forms of MRJP1 show self-assembling ability in aqueous solutions probably by associating with fatty acids (Šimúth, 2001).

Using size exclusion HPLC, different studies estimated the molecular size of MRJP1 oligomer to be 280 kDa (Kamakura, 2011; Ramadan & Al-Ghamdi, 2012), 350 (Šimúth, 2001) or 420 kDa (Tamura, Amano, et al., 2009). The profile of the HPLC peaks also showed that the quantity of the 280 kDa protein varies depending on the areas of apiculture (Imjongjirak et al., 2005; Tamura, Kono, et al., 2009). Native PAGE analysis showed that the MRJPI oligomer is a 290 kDa protein which is cleaved into two smaller proteins, 55 kDa (MRJP1 monomer) and 5 kDa (apisimin). The 5 kDa protein apisimin acts as a subunit-joining-protein with MRJP1 monomer to form MRJP1 oligomer by non-covalent bonds (Shen et al., 2010; Tamura, Amano, et al. (2009)). Four molecules of MRJP1 and four molecules of apisimin forms MRJP1 oligomer complex with a molecular weight of 232 kDa (Mandacaru et al., 2017). Šimúth (2001) recognised the 420 kDa protein as oligomeric apalbumin. In another study Bilikova et al. (2002) reported that apalbumin is a 420 kDa protein that combines with an oligomer of apisimin (5.5 kDa) to form the stable MRJP1 protein complex of approximately 450 kDa.

The consensus 350 kDa protein (later named as apisin) is a glycoprotein with six subunits; each with a molecular mass of approximately 58 kDa (Furusawa, Arai, Kato, & Ichihara, 2016; Kamakura, Fukuda, Fukushima, & Yonekura, 2001; Kimura et al., 2003). It is a heterocomplex consisting of two distinct subunits: one with the N-terminal sequence of MRJP1 (Asn-Ile-Leu-Arg-Gly similar to) and the other one being apisimin with the N-terminal sequence (Lys-Thr-Ser-Ile-Ser). Consolidating all these studies, Tamura, Amano, et al. (2009) confirmed that MRJP1 oligomer is a heterocomplex consisting of MRJP1 monomer and apisimin. The factors which influence the oligomerisation of MRJP1 have not yet been well defined. One of the reasons might be that the MRJP1 differs in glycosylation in different honey bee species (Majtan, Kumar, Majtan, Walls, & Klaudiny, 2009). Tamura, Amano, et al. (2009) suggested that post-translational-modifications by glycosylation and phoshporylation create a wide disparity in isoelectric points in MRJPs. But Zhang et al. (2012) argue that phosphorylation could not be a major reason for polymorphisms of MRJPs because of the low number of phosphorylation sites detected in MRJP1. According to them, phoshporylation was characterized in MRJP1 and 2. MRJPs derived from different species of honey bees show heterogeneities in terms of molecular weights and isoelectric points (Ohashi, Natori, & Kubo, 1997; Schmitzova et al., 1998). MRJP1 shows eight isoforms with similar isoelectric points ranging from 4.5 to 5.0 in *Apis mellifera* (Hanes & Šimuth, 1992). Sano et al. (2004) identified two isoforms of MRJP1 in African strain honey bees and one form in European honey bees. In another study, Santos et al. (2005) reported that MRJP1 has six different forms with a molecular weight ranging from 48,810 to 59,995 Da and pI values ranging from 4.23 to 5.50 in African nurse bee. Studies by Tamura, Amano, et al. (2009) revealed that MRJP1 has a pI ranging from 4.9 to 6.3. Cruz et al. (2011) found nine different isoforms of MRJP1 with pI from 4.7 to 5.2 by 2D electrophoresis. The authors conclude that these multiplicities may be due to glycosylation or the genetic variability among the bees in the hive (Ohashi et al., 1997; Schmitzova et al., 1998).

The occurrence of several other post-translational modification processes such as methylation and de-amidation have been investigated in MRJPs in which methylation has been shown to play a central role in the polymorphism of MRJP 1–5. De-amidation of MRJPs enhances shelf-life and thus enables the long-term storage of RJ by protecting the degradation of the component proteins from non-enzymatic reactions (Zhang et al., 2012).

Structural analysis of MRJP1 show that there are three glycolysation sites (Ohashi et al., 1997). Based on the amino acid sequence, Šimúth (2001) predict the secondary structure of MRJP1 which is about 20% α-helix and 17.6% β-sheets. Cruz et al. (2011) proposed a secondary structure consisting of 9.6% α-helix and 38.3% β-sheets and 20% beta turns based on data from Circular Dichroism analysis. Their results emphasize that it is a highly stable protein. Studies of Calabria, Hernandez, Teixeira, de Sousa, and Espindola (2008) identified MRJP1 as a calmodulin-binding protein (CaMBP). Calmodulin interacts with target proteins under regulation by calcium ions. Consequently presence of calcium ions (2mM) causes conformational changes on MRJP1 and that makes it susceptible to temperature (ΔG^{25} over 62 kJ mol^{-1}) and pH (6 and 7) (Cruz et al., 2011).

Like proteins, peptides from RJ impart multifunctional properties to it. Resolution of RJ by reverse phase HPLC and Q-Tof-MS/MS identified a series of short peptides named Jelleines-1, Jelleines-II, Jelleines-III, and Jelleines-IV. Most of the Jelleines, are antibacterial peptides derived from the C-terminus of MRJP1. Fontana et al. (2004) demonstrated that Jelleines-I-III are effective against Gram-positive, Gram-negative bacteria and yeast. Jelleines IV have no anti-microbial activity. Jelleines are composed of 8–9 amino acid residues with an overall charge of 1+ or 2+. Even though Jelleines are shorter than most anti-microbial peptides, they have the basic structural properties of anti-microbial peptides similar to those identified from ancient bacteria such as gramicidin from *Bacillus brevis* and subtilisin from *Bacillus subtilis*. Cationic anti-microbial peptides are generally present from 12 to 50 amino acid residues with a net positive charge of 2+ or 7+ (Fontana et al., 2004).

Royalisin, another anti-bacterial protein protects RJ from getting contaminated with Gram-positive bacteria. It is an amphipathic protein consisting of 51 amino acid residues with the C-terminal half of the molecule being rich in charged amino acids with three disulfide intramolecular linkages (Fujiwara et al., 1990). The presence of this protein in the RJ prevents infections against Gram-positive bacteria (Fontana et al., 2004; Fujiwara et al., 1990). Royalisin is a defensin like polypeptide with specific anti-bacterial activity against the honey bee pathogen *Paenibacillus larvae larvae* which causes American Foul Brood disease the most destructive honey bee brood disease. It also acts against other Gram-positive bacteria such as *Bacillus subtilis* and *Sarcina lutea*. It shows anti-fungal activity against *Botrytis cinerea* (Bărnutiu et al., 2011; Bilikova, Wu, & Simuth, 2001; Fujiwara et al., 1990; Scarselli et al., 2005). Worker bees secrete both Royalisin and Jelleines into RJ to provide a broad spectrum protection against microbial infections (Fontana et al., 2004).

MRJPs have multiple biological functions in addition to their role in honey bee larval development. MRJPs and royalactin are the main factors which provide the antibacterial activity to RJ especially against Gram positive bacteria (Fratini, Cilia, Mancini, & Felicioli, 2016). In investigations using rat hepatocytes as the model, inclusion of MRJP1 in the culture media where observed to stimulate cell proliferation and induce the production of albumin even in the absence of foetal bovine serum, possibly by acting as bio-similars or substitutes for growth factors (Kamakura, Suenobu, & Fukushima, 2001). For this reason, it can be included in human and animal cell culture media and medications when cell proliferation is the desired outcome such as in tissue regeneration in burn victims and in wound-healing processes (Kamakura, Suenobu, et al., 2001; Majtán, Kováčová, Bíliková, & Šimúth, 2006; Shen et al., 2010).

5.2. MRJP2

MRJP2 is a basic protein with an N-terminal sequence A-I-V-R-E-N-S and only one N-linked sugar chain which has a structure close to that of mannose. Based on superpose 12 HPLC analysis, the molecular weight of MRJP2 has been determined to be 72 kDa (Imjongjirak et al., 2005; Schmitzova et al., 1998).

Su et al. (2005) reported that MRJP2 protein is not as polymorphic in *Apis mellifera* but Albert, Klaudiny, and Simúth (1999) contradicts that it is polymorphic in *Apis cerana*. In 2008, Qu et al. reported that the MRJP2 and MRJP3 proteins produced by Chinese honey bees are less polymorphic when compared to those produced by the European and African strains. These variations in the MRJP proteins have been attributed to genetic differences as well as post-translational

modifications (Qu et al., 2008; Sano et al., 2004).

Studies show that in MRJP2-MRJP5, post-translational modification results in expanding the pI range from 6.3 to 8.3 (Li, Wang, Zhang, & Pan, 2007; Santos et al., 2005; Scarselli et al., 2005; Schönleben, Sickmann, Mueller, & Reinders, 2007; Tamura, Kono, et al., 2009). In 2002, Bilikova et al. observed that eight proteins were present with molecular weights approximating 49 kDa and isoelectric point ranging from 7.5 to 8.5. In 2004, Sano et al. observed 12 and 15 isoforms in European and African honey bees respectively while in 2005 Santos et al. identified eight different forms of MRJP2 in European honey bees with molecular weights ranging from 50,673 to 59,995 Da and isoelectric point ranging from 4.92 to 7.02. However Santos et al. (2005) attributes this high number of isoforms to glycosylation/de glycosylation and proteolysis that occur during the storage of RJ.

Like MRJP1, MRJP2 stimulated the release of antitumor substance TNF-α in mouse macrophages (Šimúth, Bíliková, Kováčová, Kuzmová, & Schroder, 2004; Tamura, Amano, et al., 2009). As with MRJP1, supplementing serum free medium with MRJP2 helped in the regeneration of human cell lines (Simuth, 1998).

5.3. MRJP3

The polymorphisms in MRJP3 was analysed by immunoblot assays of the expressed protein and PCR analyses of genomic DNA. Polymorphism is more evident in the MRJP3 produced by *Apis mellifera carnica* colonies and in *Apis mellifera* sub-species (Albert, Klaudiny, et al., 1999). This variability is derived from the variable number of repeats at the genomic level within the honey bees in the colony. Hence, MRJP3 and their repetitive regions can be used as markers for genetic variations and evolutionary trends of honey bee colonies (Albert, Klaudiny, et al., 1999; Schmitzova et al., 1998).

MRJP3 exists as eight different alleles encoding 14–30 pentapeptide repeats. Eight protein isoforms of MRJP3 were observed by Beye et al. (1998) and Albert, Klaudiny, et al. (1999). But according to Santos et al. (2005) MRJP3 occur only as five isoforms with molecular weights ranging from 80,590 Da to 87,000 Da and isolectric points ranging from 7.05 to 8.04. However Sano et al. (2004) reported twenty-four and ten different isoforms from the RJ of European and African honey bees respectively. The authors hypothesize that the presence of a larger number of isoforms is the result of degradation of MRJP3 during storage which starts within the hive itself.

In studies that looked into the health aspects of MRJP3, by Kohno et al. (2004), Okamoto et al. (2003) and Tamura, Kono, et al. (2009) it has been found that MRJP3 influences immune responses of T-cells by down-regulating the production of IL-4, IL-2 and IFN-γ. They also report that MRJP3 subdues the production of IgE and IgG1, effectively functioning as an anti-allergic agent. Kohno et al. (2004) also reported that MRJP3 functions as an anti-inflammatory agent both *in vitro* and *in vivo* in activated mouse macrophages by inhibiting the production of pro inflammatory cytokines such as TNF-, IL-6 and IL-1. This was later confirmed by Qu et al. (2008).

5.4. MRJP4

Presence of MRJP4 in the hypopharyngeal gland was confirmed by Schmitzova et al. in 1998 who demonstrated the presence of its mRNA. It provides nutritive components such as essential amino acids to RJ (Scarselli et al., 2005; Schmitzova et al., 1998). However studies show that the level of *mrjp*4 expression in the hypopharyngeal gland is very low compared to the expression of the other MRJPs (Klaudiny, Kulifajová, Crailsheim, & Simuth, 1994; Kubo et al., 1996; Malecová et al., 2003; Ohashi et al., 1997).

The average molecular weight of MRJP4 is estimated to be approximately 60 kDa (Sano et al., 2004). This is higher than the molecular weight predicted from the cDNA sequences synthesised from *Apis mellifera* (Schmitzova et al., 1998). Like-wise, the molecular weight of MRJP4 inferred from the European honey bee genome is different from that inferred from the cDNA sequence of the Indian honey bee (Qu et al., 2008). These discrepancies in molecular weights seem to arise out of eight possible N-terminal glycosylation sites (Schmitzova et al., 1998), different degrees of glycosylation and honey bee species diversity (Qu et al., 2008). Liu et al. (2014) observed that MRJP4 is continuously expressed at different levels in all stages of *Apis mellifera*. No isoforms of MRJP4 were reported by Santos et al. (2005) from hpg of African nurse bees but Sano et al. in 2004 observed two and five different isoforms from European and African bees RJ respectively.

5.5. MRJP5

The most fascinating feature of the MRJP5 protein is a wide repeat region located between amino acids residues 367 and 540 (Qu et al., 2008). It is composed of a 58-fold repeated tri-peptide motif with a dominance of Dinucloetide Repeat Motif (DRM) sequence located further upstream than that of MRJP3 (Schmitzova et al., 1998). The overall length of the repeat region in MRJP5 is 174 amino acids which is longer than MRJP3 by 100 amino acids and the repeat units within this region are less conserved than in MRJP3. Both repeat regions consist of positively charged arginine/lysine residues as well as negatively charged aspartic residues (Albert, Bhattacharya, et al., 1999).

Like MRJP4, MRJP5 is also continuously expressed at different levels in all stages of *Apis mellifera* (Liu et al., 2014). It too provides nutritive components such as essential amino acids to RJ (Scarselli et al., 2005; Schmitzova et al., 1998).

MRJP5 exists in three different forms from the hpg of nurse honey bees, with molecular weight ranging from 79,075 to 79,471 Da and pI values ranging from 6.34 to 6.80 (Santos et al., 2005). The protein profile of the MRJP5 of European and African honey bees showed four and seven different isoforms (Sano et al., 2004). However, the authors do not rule out the possibility that they might be products of proteolysis or of glycosylation/de glycosylation during storage.

5.6. MRJP6, 7, 8, 9 and 10

MRJP6, MRJP7 and MRJP8 seem to have no nutritional function in *Apis cerana*. MRJP8 and MRJP9 are considered as the ancient and ancestral members of the MRJP family. Proteomic analysis of RJ showed single forms in MRJP6, MRJP7, and MRJP8 (Santos et al., 2005). MRJP6 and MRJP7 are constantly expressed in the hypopharyngeal gland in both worker and foragers. MRJP7 has also been detected in the brains of nurse bees in addition to the hpg. Unlike MRJP6 and MRJP7, MRJP8 is expressed only in forager stages in *Apis cerana* (Liu et al., 2014). Santos et al. (2005) reported the presence of MRJP8 in RJ of African honey bee. MRJP8 occurs in lesser quantity in RJ compared to other MRJPs (Buttstedt et al., 2013b). All the MRJPs except MRJP8 occur in RJ of *Apis mellifera* (Buttstedt et al., 2013b). Apart from RJ, the presence of MRJP8 and MRJP9 could be detected in bee venom too (Blank, Bantleon, McIntyre, Ollert, & Spillner, 2012; Peiren et al., 2005, 2008).

A lone study (Albert & Klaudiny, 2007) claims that MRJP9 present in RJ of African honey bees has no role in nutrition but is an immunosensitizing agent. Recently Helbing, Lattorff, Moritz, and Buttstedt (2017) added a new member to the MRJP family, *mrjp10* from the phylogenetically oldest honey bee species *A. florae*.

6. Geneology and genomic organization of Major Royal Jelly Proteins (MRJPs)

Genes coding for MRJPs occurs in the honey bee genome in high copy numbers (Drapeau et al., 2006). They seem to have evolved from a single progenitor gene that encodes a member of the yellow protein family. Yellow proteins were originally identified in *D. melanogaster* where they are required for development, locomotion, immunity,

sexual behaviour and cuticle pigmentation (Drapeau, 2003; Kucharski et al., 1998; Nash, 1976). They have also been detected in the genome of the parasitoid wasp *Nasonia vitripennis*, leafcutter bee *Megachile rotundata*, paper wasp *Polistes canadensis*, and in the leaf cutter ant *Atta cephalotes* in different copy numbers (Buttstedt et al., 2013a).

Albert and Klaudiny (2007) traced the phylogeny of all *mrjp*s and suggested that the ancestor of the *mrjp* family is *mrjp*9 as it does not contain any repetitive regions seen in the other *mrjp* members. The high copy number of the *mrjp* genes has been traced to the duplication of this ancient gene (Buttstedt et al., 2013a). A unique clade of this gene seems to be involved in caste specification in *Apis mellifera* (Ferguson, Green, Surridge, & Jiggins, 2011).

Genes that code for MRJPs have been identified in chromosome 11 in the honey bee genome (Buttstedt et al., 2013a). Nine genes arranged in a 65-kb tandem array have been recognised by the Honey bee Genome Sequencing Consortium (2006) (http://www.nature.com/nature/journal/v443/n7114/full/nature05260.html). In *Apis mellifera*, genes for MRJPs 1–5 are present as single copy genes per haploid honey bee genome (Malecová et al., 2003).

Upstream of all *mrjp* genes 1–5 is a putative promoter. These promoters have binding sites for the Ultraspiracle transcription factor (USP) and a cluster of dead ringer (Dri) genes in the 5′ non-coding region. Dri and USP have been shown to be involved in the expression of *mrjp* genes (Malecová et al., 2003). Juvenile hormones (JH) that regulate larval development in the honey bee binds to USP and thus functions as a regulator of USP's engagement with the promoter region. JH has also been reported to be involved in reprogramming and switching the function of the hypopharyngeal gland from expressing MRJP in nurses to expressing sugar processing enzymes in foragers (Ohashi et al., 1997). In all probability, this reprogramming may be involved in temporal activation leading to the developmental changes from nurses to foragers too.

7. Recombinant MRJP1

A colony of honey bee generally produces ~ 0.5–1 kg of RJ each year depending on the honey bee species. The production cost of RJ is high because of these low yields which is insufficient to meet the current commercial demand (Ramadan and Al-Ghamdi, 2012). Several studies have been carried out to address this problem, most of which are aimed at the expression of recombinant proteins to raise commercial production with the intention of meeting market demands. With this in view, Judova, Klaudiny, and Simuth (1998) demonstrated that *Escherichia coli* could be genetically modified to express recombinant MRJP1 from *Apis mellifera*. They cloned cDNA encoding MRJP1 into pQE32 expression vector. The expressed recombinant His-tagged MRJP1 (rMRJP1) was purified by affinity chromatography on Ni-NTA resin and separated by electroelution from the polyacrylamide gel. Even though only 0.6 mg of rMRJP1 could be obtained after 1 h from 1 L culture, the experiment demonstrated the scope of a biotechnological proposition for its synthesis. In another study on *Apis cerana*, Imjongjirak et al. (2005) isolated MRJP1 cDNAs (AcMRJP1 and AcMRJP2) and cloned it into pET17b system and expressed the protein in *E. coli*. The recombinant AcMRJPs (rAcMRJP1 and AcMRJP2) thus obtained was expressed in insoluble form from which they were able to purify them and get the final yield of 20 and 8 mg per litre of flask culture respectively. In another demonstration of the technique, Zhang, Ding, Zhang, Jin, & Shen (2010) expressed the MRJP1 (AccMRJP1) of *Apis cerana cerana* in recombinant *E. coli*. However, the recombinant proteins they obtained had a molecular weight of 48 kDa instead of the expected 57 kDa because bacteria were not able to perform the required post translational modifications. The lower molecular weight of the recombinant protein thus obtained can be traced to the lack of glycosylation of the protein as discovered by Ohashi et al. (1997) who reported that deglycosylation of the 57 kDa MRJP1 protein results in the 48 kDa protein.

Most of the recombinant proteins expressed in the prokaryotic system were not just unglycosylated, their expression levels too were very low and they lacked biological activities. Experiments to overcome this were conducted by Shen et al. (2010) and Zhang et al. (2010) in yeast expression systems. They succeeded in obtaining recombinant proteins which were glycosylated and the expression levels were comparatively higher than was obtained from bacteria. Shen et al. (2010) used the eukaryote *Pichia pastoris*, a methylotrophic yeast for producing recombinant MRJP1. *Pichia pastoris* has the capability to perform many types of post-translational modifications, build disulfide bonds, proteolytic process the protein and secrete it in its native, biologically functional form (Cereghino and Cregg, 2000). In addition, yeast and higher eukaryotes share aspargine linked glycosylation pathway. The recombinant AccMRJP1 thus obtained was a glycosylated protein (57 kDa), similar to the naturally occurring AmMRJP1. They were thus able to prove that yeast is a better organism to express the glycosylated recombinant protein to get a protein of improved biological activity (Kukuruzinska, Bergh, & Jackson, 1987; Zhang et al., 2010) than the prokaryote systems.

However the major hurdle in producing recombinant proteins using the yeast expression system is that it adds impurities to the culture supernatant and to the recombinant protein itself, making its isolation and purification extremely difficult. In order to overcome these difficulties, Ibarra-Herrera, Torres-Acosta, Mendoza-Ochoa, Aguilar-Yañez, and Rito-Palomares (2014) developed an aqueous two-phase system for the partial purification of the recombinant protein from *P. pastoris* with limited success.

In yet another attempt, Judova et al. (2004) transferred the AmMRJP1 cDNA into tobacco plant using pBin19 as vector and expressed it in tobacco leaves. For expression of MRJP1 the plasmid was introduced into tobacco (*Nicotiana tabacum*) leaf using *Agrobacterium tumefaciens* mediated transformation protocol which carry the expression cassette composed of CaMV 35S RNA promoter, cDNA encoding MRJP1 with its native signal peptide and nos3′ as transcription terminator in binary vector pBin19 were used. Transgenic plants of F1and F2 generation were obtained from the seeds of primary transgenic plants and the leaf extracts showed expression of MRJP1 after immunoblot analysis. The yield obtained was 0.3 μg recombinant protein from 1 g of tobacco plant.

Expression of AccMRJP1 gene has also been demonstrated in larvae of the silkworm, *Bombyx mori* (Shen et al., 2010; Tao et al., 2008). They used a *Bombyx mori* nuclear polyhedrosis virus (BmNPV) Bacmid (a baculovirus shuttle vector) including a BmNPV bacmid and its *E. coli* DH10Bac/BmNPV baculovirus expression system. This eukaryotic expression system has advantages such as glycosylation, phosphorylation and post-translational modification thereby solving the problems encountered in the expression of MRJP1 in transgenic tobacco and in *E. coli*. The yield which was 486 μg per larva was higher than that achieved in *E. coli*.

Ma et al. (2012) constructed an MRJP1 mammary gland specific expression vector and expressed the recombinant MRJP1 in cow's milk. Along with vector pBC1-MRJP1-NEOr, the expression cassette consisted of goat β-casein promoter, cDNA encoding MRJP1 and a Neor selection marker. They suggested that the creation of transgenic mammary gland bioreactors to produce recombinant MRJP1 in milk could improve human health.

8. MRJPs used as freshness markers

Fresh RJ of good quality is whitish in colour, nutty flavoured, low in fermentation products, free of microorganisms and their by-products and contains no trace of rancidified fatty acids.

Acclaimed for its ability to facilitate and maintain a healthy life, RJ is a widely accepted food supplement with a high market demand. Consequently, it needs to be stored and transported from the places of its production (which are mostly apiaries in rural areas) until marketed

and used. Unlike honey, its quality degrades progressively with the duration and temperature at which it is stored and transported. Storage at room temperature changes its organoleptic properties like colour, viscosity, pH, enzymatic activity, amino acid content, etc. Improperly stored RJ thus loses its utility and effectiveness (Kamakura, Fukuda, et al., 2001; Shen et al., 2015).

MRJP1 and its degradation products could be useful as markers for assessing the freshness of RJ. The MRJP1 in RJ gradually and progressively degrades at storage temperatures above 4 °C because of the activity of trypsin like proteinase (Funakoshi, Shimada, & Kojima, 1993; Kamakura, Fukuda, et al., 2001). Recently, Shen et al. (2015) measured the drop in quantity of MRJP1 in RJ during storage using Enzyme Linked Immunosorbent Assay (ELISA) and confirmed that MRJP1 can be used as the marker for assessing the quality and freshness of RJ.

9. Some MRJPs act as allergens

Among the MRJPs, MRJP1 and MRJP2 act as allergens in some people. The risk of having allergy is higher in individuals who already have other allergies (Leung, Thien, Baldo, & Czarny, 1997) or allergies to other bee products (Pavel et al., 2011; Thien et al., 1996; Yonei et al., 1997). MRJP1 and 2 have been identified as the proteins that are recognised by Immunoglobulin E which is involved in triggering allergic reactions (Leung et al., 1997; Peacock, Murray, & Turton, 1995; Rosmilah et al., 2008; Takahashi, Matsuo, & Ohkido, 1983). Persons allergic to RJ may end up with acute asthma, contact dermatitis, anaphylaxis and in extreme cases even death triggered by IgE response (Rosmilah et al., 2008).

10. Conclusion

RJ is one of the most attractive commercial products in the food, medicine, (neutraceutical) and cosmetic industries. In addition to being nutritive, its natural functions in honey bees include regulating physiological and temporal mechanisms, development and reproduction, caste differentiation, providing antibiotic protection, and in extending the longevity of the queen bee. The properties of RJ are multi-factorial because of the complex matrix of bioactive compounds it contains and the variety of physiological processes it controls. Various studies have demonstrated that MRJPs are the dominant proteinaceous components in RJ. This review article aims to draw the attention of the scientific community to the cluster of current knowledge on an extremely interesting natural product that has been in use and in great demand from the start of civilisation. Natural products sourced from honey bees are among the few that have no alternatives – neither natural nor synthetic. With the advances made in biotechnology and health sciences, there is a large potential for applications for this product in neutraceutical and food sciences. The review highlights the need for more research into royal jelly and its components so that the mechanism by which it influences physiological pathways in bees as well as in consumers is unravelled.

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