ROYAL JELLY PROTEINS AS A TOOL FOR DEVELOPMENT OF FUNCTIONAL INGREDIENTS FOR HEALTH

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Abstract

*The quality of honeybee (*Apis mellifera *L.) products has long been evaluated basing on static properties that can be defined by chemical, physical, and instrumental analyses. Nowadays, it is generally understood that quality of honeybee products should be defined in terms of a variety of dynamic functions of their individual components. Royal jelly (RJ) has been accepted and broadly used as a health promoting substance. There is a growing scientific evidence to support the concept that the most attractive bioactive compounds of honeybee products are the proteins of RJ. The recent discovery that RJ-proteins may have physiological functions as immunomodulators, suppressors of allergic reactions, their anti-hypertensive and proliferation stimulatory properties opened a new era in application of RJ and honey. Our systematic molecular-biological research of the individual proteins and peptides of RJ showed that their polyfunctional properties could be used as markers for standardization of dosages for application of RJ as a substance that can be consumed as a part of the daily diet, and which serves to regulate or otherwise affect a particular bodily process when ingested. Medical-pharmacological effects of honeybee products will be precisely evaluated and quantified on the basis of quantity of the individual proteins of RJ present in the used diet. The properties of individual RJ-proteins and their physiological functions during larval development and as ingredients of functional foods will be presented.*

Keywords: *honeybee royal jelly / protein / peptides / purification / physiological properties*

Introduction

The honeybee colony as a superorganism, composed of individual cells which are the honeybees themselves, is reflected also in mechanisms by which the honeybee foragers gathers, processes, and preserves nectar, honey, and pollen and ensures the nutrition of the brood. Proteins and peptides, synthesized by honeybee in cephalic glands, play a significant role in these processes as nutrition and protection of the honeybee brood against pathogens.

RJ is a secretion product of the cephalic glands of nurse bees and serves as a food for honeybee larvae and through prophylactic behavior is distributed between individuals of colony (CRAILSHEIM, 1992). RJ is a multiple-substance system containing proteins (12-15%), water (60-70%), total sugars (10-12%), lipids (3-7%), minerals, amino acids and vitamins (TAKENAKA, 1982; ŠIMÚTH, 2001). RJ is considered a unique nutrient developed in nature during evolution of animals.

Nursing and nourishing of the hatched honeybee larva is provided by young honeybees which, by secretions of their cephalic glands, provide the larva of the queen-to-be honeybee with royal jelly, the larva of the worker-to-be with worker jelly and the larva of the drone-to-be with the drone jelly. These jellies do not differ each from the other in chemical composition of the basic components, i.e. proteins, carbohydrates and lipids. The fundamental difference consists in that RJ contains, in contrast to worker jelly, a compound(s) which determines what will become of a genetically equal diploid egg: a queen honeybee or, in the absence of this compound(s), a worker honeybee. However, this compound has not been identified thus far. The key for defining this regulation mechanism is encoded in special genes of honeybee, which express in the initial stage of development of the queen-to-be larva and are repressed in the worker-to-be larva. It is the so-called differential expression of genes, often regulated hormonally and involved also in regulation of the expression of other genes responsible for various phenotypic signs distinguishing the queen honeybee from the worker one. Owing to this differentiation and to the fact that honeybees feed the queen with RJ for her whole life, the queen has a long-life span. The queen lives 4-5 years, while the worker only 3-4 weeks. This phenomenon made a basis for the opinion that RJ might be the means for long living of human beings. Though it is an experimentally not substantiated hypothesis, current scientific findings suggest that mainly the RJ proteins might act as a revitalization factor in the human nutrition. The proteins secreted by honeybee into its products have different functions in establishment of optimal development of honeybee colony. Hypopharyngeal, mandibular, and salivary glands are the sources of the most important honeybee proteins. In these glands synthesized are hundreds of various proteins and peptides which have an irreplaceable role in nutrition of the brood and its differentiation, in processing of flower pollen to pollen pellets and then to pollen bread as well as in unique technology of processing the nectar to honey. These proteins as *exogenous* secretion of honeybee make possible its direct contact with the source of nutrition (nectar - carbohydrates, pollen - proteins) and protection (larva-protective barrier of the honeybee colony against pathogens).

Exogenous proteins of honeybee

Classification of proteins secreted by honeybee into RJ and its products

A substantial part of RJ is made of proteins, which form about 50% of the dry mass of RJ (ŠIMÚTH, 2001). Major proteins accounting for 90% of total RJ proteins with molecular masses of 49-87 kDa assigned to one protein and gene family (HANES and ŠIMÚTH, 1992; SCHMITZOVÁ et al., 1998; MALECOVÁ et al., 2003). The minor part of RJ-proteins is composed of proteins and peptides with different functions including antimicrobial and antifungal properties (FUJIWARA et al., 1990; BÍLIKOVÁ et al., 2001; BÍLIKOVÁ et al., 2002; BACHANOVÁ et al., 2002).

Based on functions, exogenous proteins and peptides of honeybee could be classified as:

Technological enzymes - are involved in transformation of nectar to honey: α-glucosidase, glucose oxidase, catalase and amylase.

Nutritional proteins - are secreted into larval diet as the main source of protein nutrition of the honeybee larva.

Protective proteins and peptides - are secreted by honeybee into its products and protect mainly the developing brood against pathogens.

Physiologically active proteins and peptides - have various functions in the honeybee colony and influence processes in tissue cultures of animal cells *in vitro* conditions.

Structural properties of RJ

Generally RJ is defined as an emulsion. Scanning electronic microscopy (SEM) investigation of fresh RJ showed its unique structural features (ŠIMÚTH, 2001).

Examination by SEM showed that RJ in some areas of the RJ layer, contained relatively large globular spherical particles (Fig. 1). Their size ranged from 20 to 80 μm. These „globules" were connected with each other by a system of filament channels. The diameter of the filaments was about 2 μm and its length was variable. Higher power magnification of an individual globule showed filaments radiating from the shell-like surface of the globule. It was proposed that fine structure of RJ is generated in hypopharyngeal glands of honeybee.

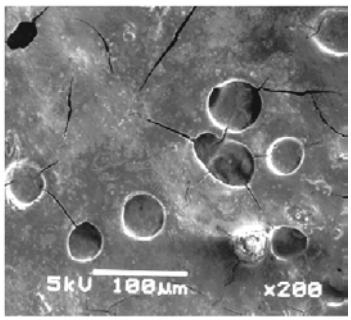

Figure 1 - SEM view of typical globular particles in natural RJ.
*An individual queen cell of 2-day-old larva (*Apis mellifera carnica *L.) from author's private apiary was cut out from the comb, the larva was removed and the cell containing RJ was frozen at -20 °C with the aim to inspect gross structural features of RJ by SEM. To prevent destruction of the original structure of RJ fixation procedures were omitted. The RJ samples were applied from the refrozen sample and allowed to develop at room temperature for 72 hours as a thin layer on aluminium discs with a diameter of 1.2 cm. The samples were then negatively stained with Cu in a vacuum chamber at 10^{-3} Pascal and examined using SEM (Jeol, model JSM-580, Japan).*

Preparation of RJ-proteins in natural form

In order to isolate the proteins in a possibly most natural state, we developed a method for fractionation of RJ by ultracentrifugation (ŠIMÚTH, 2001). Three physically distinct layers were obtained. The supernatant fraction was a green-yellowish fluid named plasma constituting 61 % (w/v) of native RJ. A yellowish-brown viscous fraction named jelly representing the mid-layer had a gelatinous consistence; this fraction constituted 32 % of RJ (w/v). The white sediment on the bottom (7 % w/v of RJ) appeared to be a nearly fixed substance. Significant amounts of fatty acids were concentrated in fractions with lower content of water. By ultracentrifugation was obtained a semi-solid golden-yellow, amber resembling gel. Biochemical analyses revealed that it is a major royal jelly protein (previously named as MRJP1) of the albumin type and therefore, it was named as *apalbumin-α.* It is reasonable to suggest that an interaction between apalbumin-*α* and fatty acids resulted in the formation of the water-insoluble protein fraction of RJ. It is interesting that other RJ proteins, which are located predominantly in supernatant fraction, i.e. apalbumin-β (previously named as MRJP2) and apalbumin-γ (previously named as MRJP3) do not have the ability to form gel, though they have high degree of homology with apalbumin-α (SCHMITZOVÁ et al., 1998).

Physical-chemical properties of apalbumin-α

It was showed that apalbumin-α formed a subunit structure (ŠIMÚTH, 2001). The basic subunit of about 420 kDa is built up from the basic 55 kDa monomer. Microscopic observations showed that apalbumin-α in aqueous solutions forms structures similar to those occurring also in RJ. In dependence on the concentration of apalbumin-α, various regular repeating structures are generated (Fig. 2). It is a self-assembling structure of the protein as a result of oligomerization of its subunits. It is interesting that other RJ proteins do not have the ability of oligomerization, though they have high degree of sequential homology with apalbumin-α.

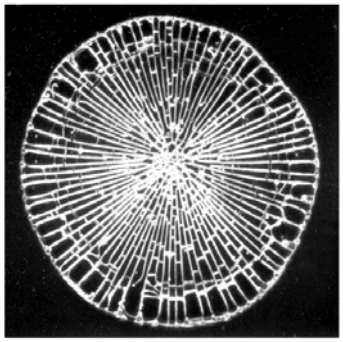

Figure 2 - The self-assembling of regular filamentous structures of apalbumin-α. The light microscopy view was performed 20 min after applying a drop (3 μl) of apalbumin-α (80 mg per 1 ml water) on cover slip.

What is the function of the self-forming structures of apalbumin-α in honeybee colony? Many proteins are much more stable in super-molecular structure than in monomeric forms. When considering the high content of proteins in RJ, then from theoretical point of view it cannot be assumed that all nourishing RJ proteins are immediately metabolized. High concentration of amino acids would bring about disproportionately high osmotic pressure in the digestive tract of larva. We have found further that apalbumin-α occurs also in honey and pollen kit. Honeybee can to utilize the structural properties of apalbumin-α in processing of the flower pollen into pollen kit. It looks like the honeybee wraps the pollen into apalbumin as its bread. Apalbumin-α is a texture-forming factor in RJ and in cosmetics products containing RJ. It seems that the apalbumin-α has more functions than its nutritional role in the larval development.

Physiological properties of RJ-proteins

Few generalizations can be made about physiological properties of major royal jelly proteins (MRJPs). The behavioral evidence, though suggestive, still does not tell us directly whether MRJPs do serve the similar function in early as well as in later larval development. The study on break down of RJ protein in the midgut of the larvae (TSAO and SHUEL, 1968) showed that some RJ proteins may have passed through the gut epithelium unchanged. High content of essential amino acids predestines their assumed role in the honeybee colony as nutritional.

The protein fraction of honeybee RJ contains many valuable components and biologically active substances. Besides the MRJPs, low amounts of several minor proteins including antibiotics peptides (FUJIWARA et al., 1990; BÍLIKOVÁ et al., 2001; BÍLIKOVÁ et al., 2002) are present in RJ.

Of particular interest are bioactive proteins and peptides that are present in the amino acid sequence of food proteins. These peptides, inactive within the sequence of the parent protein, can be released by enzymatic proteolysis for example during gastrointestinal digestion or during food processing. Once they are liberated in the body, bioactive peptides may act as regulatory compounds with hormone-like activity. The structures of biologically active sequences were not yet obtained and further research directed towards *in vitro* enzymatic and/or *in vivo* gastrointestinal digests of the appropriate proteins is necessary.

The bioactive components of RJ are not fully evaluated, however, recent *in vitro* studies demonstrate that some MRJPs affect very important physiological processes. Bioactive peptide originating from RJ and MRJPs should be taken into account as potential modulators of various regulatory processes in the body. The structure-activity relationship and the mechanism by which MRJPs exert their immunomodulatory effects are not yet defined. However, the results obtained with 350 kDa and 55 kDa RJPs suggest that MRJPs may affect important cellular processes. The 350 kDa proteins with N-terminal amino acid sequence as apalbumin-α (SCHMITZOVÁ et al., 1998) stimulate the proliferation of human monocytes (U 937 cell line) and human-human hybridomas (HB4C5 cell line) (KIMURA et al., 1995). The cited authors have found that the structures of N-linked sugar chains of 350 kDa protein are the typical high mannose type structure (Man9-GlcNAc2) commonly found in animal, plant, and insect. The 55 kDa protein of RJ having N-terminal

amino acid sequence identical with the second most abundant protein of RJ i.e. apalbumin-β (SCHMITZOVÁ et al., 1998; BÍLIKOVÁ et al., 1999), maintains the high viability of rat primary cultured cells but not stimulate the proliferation of human monocytes (KIMURA et al., 1996). Estimation of chemical structures of bioactive MRJPs is the first step toward discovering the molecular mechanism of their physiological activities in synergy with other bioactive components of RJ.

From the point of view of physiological activity the dominant position have the most abundant albunoid's proteins of RJ: acidic apalbumin-α (ŠIMÚTH, 2001) and basic apalbumin-β (BÍLIKOVÁ et al., 1999) that are expressed also in the honeybee brain (KIMURA et al., 1995; KUCHARSKI and MALESZKA, 2002). These findings as well as the similarities between the immune systems of insects and mammals (DUSHAY et al., 1966; IMLER and HOFFMANN, 2001; BAUD and KARIN, 2001) showed that RJ-proteins could be a factor, which is involved in the regulation of important physiological processes. This was indirectly confirmed by founding that the 1% solution of native honey induced of TNFα release from unprimed monomac 6 cells, while artificial honey did not (TONKS et al., 2001).

Data on physiological properties of RJ proteins, such as stimulation of human monocytes proliferation (KIMURA et al., 1995), or on immunomodulatory properties of RJ (ŠVER et al., 1996), suppression of allergic reactions by RJ (OKA et al., 2001), or antihypertensive activity of bioactive RJ peptides (MATSUI et al., 2002) broaden their potential application in pharmacy and indicate their natural function in honeybee evolution, where they could play the role of inductor (trigger) of defense mechanisms during larval development. If this assumption is correct, then RJ proteins could induce production of bioregulators of cytokine type that have an important function in immune system, inflammatory processes, as well as play certain part in the control of cell proliferation, differentiation, and apoptosis. This assumption was corroborated by our preliminary experiments, in which we have detected induction of cytokines in murine macrophages by RJ proteins. Our observations indicate that RJ proteins are responsible for stimulation of production of TNF-α in human monocytes in vitro by 1% aqueous solution of honey (TONKS et al., 2001). This also indicates that action of honeybee proteins can stimulate a specific system (cytokine network) that activates genes responsible for production of defensive substances prior to occurrence of bacterial infection.

Honeybee, similarly to other insects, responses to bacterial infection by inducing higher expression of the genes coding for antimicrobial peptides, which are subsequently secreted into haemolymph. Such „immune response" is very fast (occurs in a few hours) but is often non-specific (CASTEELS, 1997; ZASLOFF, 2002). Certain honeybee antimicrobial peptides (apidaecin, abaecin, and hymenoptaecin) are induced specifically and are released into haemolymph only after bacterial infection, whereas honeybee defensin – royalisin (FUJIWARA et al., 1990) and apisimin (BÍLIKOVÁ et al., 2002) discovered in RJ are probably synthesized throughout whole honeybee's lifetime. Proteins and peptides from RJ can participate in defense mechanisms of honeybee against microbial pathogens by means of a direct inactivation of microorganisms occurring in honeybee products, as well as through induction of cytokines participating in regulation of transcription of defensive proteins and peptides.

The obtained data on biochemical and biological properties of RJ proteins will serve as a basis for functional genomics of the honeybee defensive system against diseases as well as for better understanding of physiological properties of honeybee products as the components of functional foods. It is still difficult to assess the clinical efficacy of RJ, but there is a high expectancy from people having RJ to evaluate the conditions of RJ activity, the mechanism of action or the suitable dose and its application period. The presented view on experimental study of proteins at molecular level secreted by honeybee into its products is an approach to define the bioactivity of protein and peptides of RJ as important nutraceutical.

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