A New Marker for Evaluation of Royal Jelly

*Kikuji Yamaguchi¹, ², Toru Kono¹, Takanori Moriyama³

Department of Surgery, Asahikawa Medical College¹, Japan Royal Jelly Co., Ltd², Laboratory Science, Faculty of Health Sciences, Hokkaido University³

We focused attention on rapid growth of bee larvae (BL) in royal cells promoted by royal jelly (RJ). The substance which promotes the growth of BL has not been clarified, however high quality RJ should abundantly contain this growth-promoting substance. Firstly, we performed fundamental study to disclose the content ratio of soluble proteins in RJ samples using HPLC. Soluble RJ proteins were universally separated into five peaks. Among these peaks, the peaks of 280 kDa and 72 kDa were major and others were minor components. These proteins were extracted and identified by 2-dimensional electrophoresis and MALDI TOF/TOF MS, respectively. The main 280 kDa and 72 kDa peaks on HPLC were Major RJ Protein 1 (MRJP1) and MRJP2, respectively. It has been reported that MRJP1 shows maintenance of cell activities and cell growth activity in vitro. Consequently, MRJP1 is the first candidate of the growth-promoting substance. To test this hypothesis, we performed an experimental apiculture. RJ was collected sequentially (every 4 hours from 24 to 72 hours after the transferring BL) from artificial royal cells and the total amount of protein (TP), trans-10-hydroxy-2-decenoic acid (DA) and MRJP1 were determined. TP and MRJP1 decreased with growth of BL but DA did not show any significant changes. The promotion effect of MRJP1 on the growth of BL was examined in vivo. BL were treated with RJ containing various concentrations of MRJP1. BL growth was promoted by MRJP1 treatment in a dose-dependent manner. From these results, it is strongly demonstrated that MRJP1 is a key protein of BL growth. The amounts of MRJP1 in commercially available RJ samples were determined using HPLC. The amount of MRJP1 was remarkably varied ranged from 30 mAU to 2000 mAU, peak value of HPLC among RJ samples. The study is of novel significance as it demonstrates for the first time that MRJP1 is a key in the growth of BL, and we strongly propose use of MRJP1 to evaluate the quality of RJ.

Key words: royal jelly, major royal jelly protein 1, bee larvae, protein, quality, decenoic acid
A New Marker for Evaluation of Royal Jelly

Royal Jelly (RJ) is the primary food that is secreted from the hypopharyngeal and mandibular grand of nurse honeybee. And it plays the specific and important role in queen honeybee development, however it has not been cleared that what kinds of ingredients of RJ were consumed by larva to grow into a queen. The queen honeybee is fed RJ through the period in bee larva (BL), while nurse honey bee is fed RJ for only 3 days (Srisuparbh et al. 2003; Simuth 2001). Comparison the size of BL after the extraction between 48 and 72 hrs showed that 72 hrs BL is much bigger than 48 hrs. (Figure 1)

![Figure 1](image1.png)

A bee larva (BL) after extraction of 48hrs

A BL after extraction of 72hrs

RJ is contained various components: 60-70 % of moisture, 12-15 % of protein, 10-16 % of total sugar, lipids, vitamin, salt, and free amino acid (Simuth 2001; Howe et al. 1985; Chen C.S and S.Y. Chen 1995). The substance which encourages such as rapid growth is the major active component of RJ, and the primary candidate is the protein component. However, this protein component has not been identified so far. Therefore, it is considered that identifying and quantitative analysis of this protein component will be the basic study of RJ.

First of all, we examined that whether RJ protein is consumed by BL in company with growing. The results showed that content rate of protein component has been decreased over 4% during 48 hrs from 24 hrs to 72 hrs compared to 24 hrs since transferred. (Figure 2)

![Figure 2](image2.png)

Figure 2
In contrast, decenoic acid (DA) which has been established as quality criterion of RJ has hardly changed as little as 0.5%. Thus, decrease of protein component with BL growth in royal cell is unlikely to be explained by DA. (Figure 2) Therefore, it is assumed that it would be another protein component besides DA. The more than 80% of soluble RJ protein is major royal jelly proteins (MRJPs) (Simuth 2001; Schmitzova et al. 1998). It has considered that MRJPs were main factor of RJ physiological specific role for queen honeybee development, because MRJPs were composed a lot of essential amino acids, compatible ovalbumin or casein (Schmitzova et al. 1998).

Thereupon, we analysis the soluble protein component of RJ in royal cell by size-exclusion HPLC method. Soluble RJ proteins were extracted by dialysis followed by several centrifugation techniques. Soluble RJ proteins were universally separated into five peaks, 640 kDa, 280 kDa, 100 kDa, 72 kDa, and 4.5 kDa by size-exclusion HPLC on Superose 12 column. (Figure 3)

The heights indicate the contents (mAU) of proteins figured out by HPLC. We hypothesized, if the protein which was consumed by larva exclusively, including in these 5 peaks, the numerical value of peaks should be decreased in time course. So we examined the RJ in royal cell with HPLC method (n=113). As a result, only Peak 2 decreased remarkably in time course. (Figure 4) Suggest Peak 2 is the active component of RJ.
Continuation we verified the Peak 2 with electrophoresis, 2D electrophoresis and MALDI-TOF/TOF MS (matrix assisted laser desorption ionization time of flight/time of flight mass spectrometry) to clear the ingredients. Below show the results of electrophoresis and 2D electrophoresis analysis.

**SDS-PAGE Patterns of Soluble Royal Jelly Proteins**

HPLC refined sample analyzed by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), the Peak 2 band is detected at 55,000, and anion exchanged sample is detected at the same band. (Figure 5) Refer to the reported RJ protein component corresponds to this molecular weights; we found that MRJP1 is a substance which was conform to this protein. Furthermore we show the results of 2D electrophoresis analysis in the Figure 6.
2-D Electrophoresis Patterns of Soluble Royal Jelly Proteins

![Image of electrophoresis patterns](image)

Crude Dialyzed Sample  Main Peak Fraction of Mini Q HPLC  Figure 6

Left patterns are the crude samples post dialysis, and the right one is the electrophoresis result of anion exchanged HPLC main peak. Red circled area spots assumed MRJP1 are observed as molecular weight of 55,000 spots which are distributed at pI 4.2-6.5. (Figure 6) Also similar spot has been observed as the most dominant spot among crude soluble protein sample.

Next, fractionalized red circle spot into three and examined mass analysis by MALDI TOF/TOF MS. We performed proteome analysis by 2-DE and MALDI TOF/TOF MS in order to identify the 55 kDa protein. By 2-DE, crude soluble RJ proteins were separated a number of spots ranged from pH 3.8 to 10.7 and from 5kDa to 100 kDa. After reductive alkylation, trypsinization, and dechlorination by common procedure for fractionalized gel, each sample is applied to target. Result of mass analysis shown below.

Match to: gi|58585098  
Score: 126  Expect: 1.1e-006  
**major royal jelly protein 1** [Apis mellifera]

As a result of mass analysis by MALDI TOF/TOF MS, indicated this spot is identified as MRJP1 (so-called Apisin) at identical score 126 from search by MASCOT program. (Figure 7)

Thus, filtrated Peak 2 by HPLC analysis is defined as MRJP1, the height of Peak 2 obtained by HPLC analysis is the amount of MRJP1 in RJ, and it is verified that amount
of MRJP1 can be quantifiable by HPLC analysis.

It has been reported that MRJP1 have plenty biological functions, such as maintenance cell life and encourage cell proliferation in vitro (Kamakura et al. 2001a, 2002a; Watanabe et al. 1996, 1998; Narita et al. 2006 ; Mishima et al. 2005 ; Simuth et al. 2004). Thus, we compared the difference of BL growth in depending on MRJP1 to examine whether MRJP1 is involved to BL growth in royal cell or not in vivo. According to result, the growth of BL which has been given RJ contained 500mAU MRJP1 is inhibited obviously compare to BL which has been given RJ contained 1500mAU MRJP1; suggest that MRJP1 directly influence to BL growth. (Figure 8)

Therefore, it is proved first time that MRJP1 is important factor of BL rapid growth. Thus, RJ with high MRJP1 content equal high quality. Now, we propose MRJP1 content as a novel assessment method (marker) for RJ.

Using this new marker, compare the different lot of the undiluted solution RJ (n=70) from the same harvested-local area, MRJP1 content are varied between 1500mAU ~ 1900mAU. (Figure 9)
Next, we compared the differences among the vintage (2005 to 2007). Each bar expressed the mean of MRJP1 in the same harvested-local area (n=30). Apparently, harvested vintage (i.e. local weather) has big influence on the MRJP1 content in RJ. (Figure 10)

Then, we compared the commercial undiluted solution RJ which contains over 1.8% of DA value (n=7). The result shows there is a huge difference between them such as 32mAU to 1840mAU. (Figure 11)
Analyzed MRJP1 amount using HPLC as a marker greatly contributes for evaluation of RJ quality. Next step our study is verifying the appropriateness to use MRJP1 content as a marker. For that, large scale verification will be needed internationally; therefore we must correct and analyze RJ samples from the different countries in the world. Secondly, it is noted that expensive measuring instruments such as size-exclusion HPLC and more over advanced special knowledge and techniques are required. To be solved this, versatile simple measuring maneuver such as ELISA is needed to be developed, and we have already success to develop the anti-MRJP1 monoclonal antibody.

In conclusion, the study is of novel significance as it demonstrates for the first time that MRJP1 is a key in the growth of BL and amount of MRJP1 content has highly possibility to be used as new quality assessment method of RJ.
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