Freeze-Dried Royal Jelly Maintains Its Developmental and Physiological Bioactivity in Drosophila melanogaster

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Received June 27, 2012; Accepted August 10, 2012; Online Publication, November 7, 2012 [DOI: 10.1271/bbb.120496]

Royal jelly (RJ), a honeybee-derived product, has been found to possess developmental and physiological bioactivity in the fruit fly, Drosophila melanogaster, but little is known about the in vivo bioactivity of freeze-dried RJ (FDRJ) powder, which is another form of RJ processed for human use. To address this, we used Drosophila as a model animal to examine the effects of FDRJ in multicellular organisms. When flies were reared on food supplemented with FDRJ, the developmental time from larva to adult was shortened, the adult male lifespan was prolonged, and female fecundity was increased without any significant morphological alterations. Moreover, the expression of dilp5, an insulin-like peptide, its receptor InR, and the nutrient sensing molecule TOR, the target of rapamycin, was significantly increased in FDRJ-fed female flies as compared with ones reared on standard and on protein-enriched food. These findings suggest that like RJ, FDRJ maintains its bioactivity even after processing from RJ, what is expected to have bioactivity for multicellular organisms, including humans.

Key words: freeze-dried royal jelly; bioactive substance; insulin/insulin-like growth factor signaling; Drosophila melanogaster; functional food

Insect derivatives are currently the focus of new alternative medicines due to their functional properties in human health.1,2 Bioengineered natural insect products are also used since 1930, while insects themselves are used as models in studying essential mammalian processes such as immune responses to pathogens.3 Royal jelly (RJ), a well-known health-promoting compound, is one such remarkable product derived from insects. It is a viscous substance secreted from the hypopharyngeal and mandibular glands of honeybee (Apis mellifera) workers. It is an essential food for queen bee larvae and the queen herself.4,5 Many reports have documented the biological effects of RJ on cell proliferation,6 and antitumor,7 antioxidant,8,9 and anti-hypertensive10 activities. Moreover, diverse biological effects of RJ have been suggested, such as an effect on hypo-pigmentary mechanisms,11 as an agonist of transient receptor potential ankyrin 1 (TRPA1), and in Ca2+-permeable nonselective cation channels mediating thermogenesis and energy expenditure enhancement.12

RJ consists of 50–60% (w/w) water, 18% (w/w) proteins, 15% (w/w) carbohydrates, 3–6% (w/w) lipids, and 1.5% (w/w) other compounds such as minerals and vitamins.13,14 A search for the bioactive compound(s) in RJ recently resulted in the isolation of a 57-kDa protein designated royalactin, which is considered to be the major inducer of queen differentiation in honeybees.15 Royalactin was found to increase body size and ovary development while shortening developmental time, not only in honeybees, but also in the fruit fly Drosophila melanogaster. Researchers also examined the bioactivity of RJ at a concentration of 20% (w/v) in D. melanogaster using intact RJ stored at 40°C within 30 d of sampling from honeybees.15 The results suggest that fresh RJ can influence the physiology of other organisms include humans. However, both dose debulking and processing of RJ are required for use in humans to ensure biological efficacy and commercial feasibility.16 Although RJ is popular as a health-promoting agent,4 little is known as to whether processed RJ (freeze-dried RJ, FDRJ) maintains bioactivity like that of intact fresh RJ. In this study, we chose the fruit fly as a model animal to examine the bioactivity of FDRJ. We hypothesized that if developmental and physiological bioactivity is indeed maintained, FDRJ affects Drosophila development and physiology in a manner similar to RJ in the honeybee.

Materials and Methods

Freeze-dried fresh royal jelly (FDRJ). FDRJ was provided by Apis (Gifu, Japan). Samples were prepared from fresh RJ collected in China, processed, and stored at −18°C. Pooled frozen samples were thawed and filtrated twice for sterilization. After filtration, a vacuum dryer was used to convert liquid RJ into a powder. The dried RJ powder represented FDRJ. The resulting sample was stored at 4°C until use.

Experimental animals, food, and maintenance. Standard culture medium (standard food, SF) consisted of 10% (w/v) glucose, 7% (w/v) corn meal, 4% (w/v) yeast extract, and 0.55% (w/v) agar medium containing 0.3% (v/v) propionic acid and 0.35% (v/v) butyl p-hydroxybenzoate as antifungal agents. Three forms of RJ-supplemented...
media were prepared, as follows: RJ, SF plus 2% (w/v) intact fresh RJ; bRJ, SF plus 2% (w/v) RJ boiled at 100 °C for 5 min; and FDRJ, SF plus 2% (w/v) FDRJ. Each of the various forms was added to SF by mixing at 40 °C and then dispensed into glass vials and allowed to solidify. As an experimental control, whey-supplemented medium (Whey: SF plus 2% whey powder, Healthy Best, Osaka, Japan) was prepared in the same manner. The total calories of RJ and whey were adjusted to similar levels: 81.6 and 75.0 kcal/20 g respectively. Only the intact RJ diet failed to result in the development of Drosophila (data not shown), consistently with the results of a previous study.15 Oregon-R, a laboratory wild-type strain of D. melanogaster, was used in all experiments. The flies were reared under a 12 h light–dark cycle at 50% humidity at 23 °C (in all other experiments).

Observation of adult morphology and measurement of developmental time. Approximately 50 pairs of adult flies were placed in a single vial containing SF, Whey, RJ, bRJ, or FDRJ medium. They were transferred to a fresh vial every 2–3 d. Hatched larvae were maintained at least 120 per vial to prevent developmental delay as a result of a density effect. The vials were placed in a room at 23 °C, and the number of eclosed flies was recorded twice a day to determine developmental time and the eclosion rate, which was calculated using the eclosed adults/hatched larval ratio. The body weight and wing size of each eclosed fly were also measured.

Adult longevity assay. A longevity assay was performed as described previously,17 with some modifications. Briefly, in each lifespan experiment, fully developed adult flies reared on SF medium were collected, anesthetized with diethyl ether, and separated into virgin males and females. Flies (>20) were placed in a single vial containing SF, SF + 0.1% FDRJ, SF + 0.5% FDRJ, or SF + 1% FDRJ and transferred every 3–4 d to a fresh vial. The number of dead flies was recorded until no living flies remained. RJ concentrations were determined by the methods of previous in vitro experiments.18,19

Female fecundity assay. Eclosed virgin adult females were transferred to a vial (3–5 animals/vial) containing SF (33 vials, n = 161), FDRJ (32 vials, n = 150), or Whey (31 vials, n = 153) with an equal number of males for 2 d. Then only the females were transferred to new vials containing SF, and the time-dependent change in the number of eggs in each vial was recorded.

RNA preparation and quantitative RT-PCR. Total RNA was isolated from 10 adults and purified using RNAzol RT (Molecular Research Center, Cincinnati, OH). Adults were grouped according to whether they were fed FDRJ or Whey, as well as according to strain, sex, and the occurrence of eclosion (5-d-old adults). First-strand cDNA was synthesized using ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan) with 2 μg of total RNA from each strain as template and an oligo (dT) 20mer primer (Toyobo). All procedures were conducted following the manufacturer’s instructions. RT-PCR was carried out to confirm the first cDNA products using first strand cDNA as template and specific sets of primers for the InR (5′-GATGGACAGTCTGAG-GTTTG-3′, 5′-AACCGACGCAATCACTACAG-3′), InsR (5′-TGTCG-TGGAACGCAAACTC-3′, 5′-GAGATGCAGCTCTTATCC-3′), and TGR (5′-TGATGACGTCAAAAGTCTTG-3′, 5′-GGCCCTGCTATT-GGAAGTAACT-3′) genes. A single band was detected under normal PCR cycling conditions. Quantitative RT-PCR was performed on a ROTOR-GENE 6000 (Qiagen, Hilden, Germany) following the manufacturer’s instructions with SYBR green-based detection of PCR products. Relative quantification of target gene expression was done using β-tubulin and rp49, as previously described.20 The threshold cycle and the standard curve method were used to calculate the relative amount of target mRNA using ROTER-GENE 6000 software v1.7. Melting curves were examined after amplification to confirm single-product measurements.

Statistical analysis. Statistical analysis was performed using one-way ANOVA (Dunnnett’s correction) and Microsoft Excel 7.0 software, with significance set at p < 0.05.

Results

FDRJ affected the developmental time of Drosophila without affecting morphology

RJ-fed Apis mellifera queens are known to have a body size twice as large as that of workers, but with a shorter developmental time.21 To determine whether this phenomenon also occurs with FDRJ and whether it is common in multicellular organisms, we reared Drosophila larvae on intact and modified RJ-containing media or standard medium alone, and compared developmental times from larva to adult. To determine whether the developmental time was dependent on nutrition, especially protein supplementation, whey-containing medium was also prepared as a nutritional control. The mean developmental time of the intact RJ-fed individuals was 15.9 d, 0.3 d (1.9%) faster than that of the individuals reared on SF (16.2 d) (Table 1). Although slight, this difference is significant (p = 0.034). Similar growth stimulation was seen in both bRJ (15.8 d) and FDRJ (15.8 d) (0.4 d fasterers, 2.5%). In contrast, the developmental time of the individuals reared on Whey was slower (17.6 d) than those reared on SF. This suggests that the RJ-induced reduction in developmental time is not the result of simple protein supplementation. The eclosion rates during development from larvae to adults for the SF-, FDRJ-, and Whey-reared flies were 73.7%, 75%, and 68.8% respectively, with no significant differences among the media (data not shown).

The developmental time was assessed for rearing at under 23 °C, which is lower than the optimum rearing temperature of 25 °C. The developmental time from larva to adult depends on temperature,22,23 and differences were deemed easier to detect at this lower temperature. Similar results were obtained for rearing at under 25 °C (Table 1). In addition, no significant morphological changes in body weight or wing size were detected in the FDRJ-supplemented flies as compared with the SF- and Whey-supplemented individuals (Supplemental Fig. 1; see Biosc. Biotechnol. Biochem. Web site). The findings thus indicate show that FDRJ also has developmental and physiological bioactivity, which decreases the larva-to-adult development time in Drosophila without affecting adult morphology.

Table 1. Effects of Different Types of RJ on Drosophila Development

<table>
<thead>
<tr>
<th>Food</th>
<th>Rearing temperature (°C)</th>
<th>Development time (days, mean ± SE)</th>
<th>n</th>
<th>p value (vs. SF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF</td>
<td>23</td>
<td>16.2 ± 0.11</td>
<td>294</td>
<td>—</td>
</tr>
<tr>
<td>RJ</td>
<td>23</td>
<td>15.9 ± 0.09</td>
<td>390</td>
<td>*</td>
</tr>
<tr>
<td>FDRJ</td>
<td>23</td>
<td>15.8 ± 0.07</td>
<td>546</td>
<td>***</td>
</tr>
<tr>
<td>bRJ</td>
<td>23</td>
<td>15.8 ± 0.09</td>
<td>352</td>
<td>**</td>
</tr>
<tr>
<td>Whey</td>
<td>23</td>
<td>17.6 ± 0.14</td>
<td>127</td>
<td>***</td>
</tr>
<tr>
<td>SF</td>
<td>25</td>
<td>11.3 ± 0.06</td>
<td>163</td>
<td>—</td>
</tr>
<tr>
<td>FDRJ</td>
<td>25</td>
<td>10.7 ± 0.04</td>
<td>165</td>
<td>***</td>
</tr>
<tr>
<td>Whey</td>
<td>25</td>
<td>11.8 ± 0.07</td>
<td>145</td>
<td>***</td>
</tr>
</tbody>
</table>

SF, standard food; RJ, SF containing 2% intact RJ; FDRJ, SF containing 2% freeze-dried RJ; bRJ, SF containing 2% boiled RJ; Whey, SF containing 2% whey. Significant differences are denoted as *p < 0.05, **p < 0.01, ***p < 0.001.
Freeze-Dried Royal Jelly Bioactivity in Drosophila

Prolongation of adult male longevity with 1% FDRJ intake

In honeybees, workers and queens have the same genotype, yet they exhibit a 10-fold difference in lifespan.21,22,23) To determine whether FDRJ maintains its bioactivity in other multicellular organisms, adult virgin Drosophila were exposed to SF containing 0.1%, 0.5%, or 1% (w/v) FDRJ, and their lifespans were determined. Although no significant differences were seen in adult female lifespans, the mean lifespan of adult males reared on 1% FDRJ was significantly prolonged by 3.6 d (8.5%), as compared with those reared without FDRJ (Fig. 1, Table 2). This suggests that FDRJ extends the adult lifespan, at least in males.

FDRJ-induced enhancement of female egg laying

Queen bees produce numerous offspring throughout their lifetime.21) To determine whether FDRJ has female-specific activity, egg production was examined in adult female Drosophila reared on FDRJ. The fecundity within 72 h of the females reared on FDRJ was significantly higher (2.9-fold) than those reared on SF (Fig. 2). Whey-fed females also laid more eggs than SF-fed individuals (2.1-fold higher), but not as many as FDRJ-fed females (1.4-fold higher than the whey individuals). This suggests that FDRJ influences the fecundity of adult female Drosophila.

Table 2. Longevity Assay of Adults Fed FDRJ

<table>
<thead>
<tr>
<th>Food</th>
<th>Sex</th>
<th>Mean lifespan (days, mean ± SE)</th>
<th>Maximum lifespan (days)</th>
<th>n</th>
<th>p value (v.s. SF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF</td>
<td>M</td>
<td>42.4 ± 1.15</td>
<td>75</td>
<td>209</td>
<td>—</td>
</tr>
<tr>
<td>SF + 0.1%FDRJ</td>
<td>M</td>
<td>41.5 ± 1.22</td>
<td>75</td>
<td>211</td>
<td>0.57</td>
</tr>
<tr>
<td>SF + 0.5%FDRJ</td>
<td>M</td>
<td>42.4 ± 1.16</td>
<td>75</td>
<td>212</td>
<td>0.90</td>
</tr>
<tr>
<td>SF + 1%FDRJ</td>
<td>M</td>
<td><strong>46.0 ± 1.15</strong></td>
<td>72</td>
<td>212</td>
<td><strong>0.03</strong></td>
</tr>
<tr>
<td>SF</td>
<td>F</td>
<td>38.2 ± 1.67</td>
<td>74</td>
<td>83</td>
<td>—</td>
</tr>
<tr>
<td>SF + 0.1%FDRJ</td>
<td>F</td>
<td>38.0 ± 1.94</td>
<td>76</td>
<td>84</td>
<td>0.93</td>
</tr>
<tr>
<td>SF + 0.5%FDRJ</td>
<td>F</td>
<td>37.6 ± 1.84</td>
<td>73</td>
<td>84</td>
<td>0.80</td>
</tr>
<tr>
<td>SF + 1%FDRJ</td>
<td>F</td>
<td>37.2 ± 2.16</td>
<td>76</td>
<td>84</td>
<td>0.70</td>
</tr>
</tbody>
</table>

SF, standard food; FDRJ, freeze-dried RJ; M, male; F, female.

Fig. 1. Lifespans of Adult Drosophila Reared on Freeze-Dried Royal Jelly (FDRJ).
Survivorship curves of males (A) and females (B) are shown for each medium. SF: standard food, n = 209 male, 83 female; SF + 0.1% FDRJ, n = 211 male, 84 female; SF + 0.5% FDRJ, n = 212 male, 84 female; SF + 1% FDRJ, n = 212 male, 84 female.

Fig. 2. Effect of FDRJ on Female Fecundity.
Eclosed virgin adult females previously housed with equivalent numbers of males for 2d were scored for egg production over 3 d. Data are expressed as mean ± SE values. Significant differences are denoted as *p < 0.05, **p < 0.005, ***p < 0.0005. SF, standard food, n = 161; Whey, SF containing 2% (w/v) whey, n = 153; FDRJ, SF containing 2% (w/v) FDRJ, n = 160.

Effects of FDRJ on insulin/insulin-like growth factor signaling (IIS) and TOR network-related gene expression

The data related above indicate that FDRJ influences adult physiology through increases in developmental time, longevity, and female fecundity. Hence we investigated the gene expression of IIS/TOR (the target of rapamycin) signaling pathway-related genes, since this pathway is closely related to development, longevity, and female fecundity.26,27) Gene expression of Drosophila insulin dilp5, its receptor InR, and TOR was evaluated to determine the effects of FDRJ on IIS/TOR signaling. Quantitative RT-PCR revealed increases in dilp5, InR, and TOR expression in FDRJ-fed females, but no significant differences in males (Fig. 3). The expression of dilp5, InR, and TOR in the FDRJ-fed females was 4.3-, 5.3-, and 4.7-fold higher than in the SF-fed ones, respectively. These results suggest that FDRJ facilitates the IIS/TOR signaling pathway in adult females.
Discussion

Our findings suggest that in *Drosophila*, FDRJ affects developmental time, male longevity, and female fecundity, and induces the expression of IIS/TOR signaling pathway-related genes in females. A previous study found that *Drosophila* reared on a medium containing 20% RJ, 8% yeast, and 10% D-glucose had increased body size (body weight and length) and fecundity, as well as an extended lifespan and shortened developmental time, as compared with flies reared on a control medium or a casein medium, as seen for flies fed the 57-kDa protein royalactin. Although not all the developmental and physiological changes seen in the individuals reared on fresh 20% RJ or royalactin were observed for FDRJ, this study indicates that FDRJ bioactivity is almost identical to fresh RJ and royalactin. Although the dose of FDRJ used here differed from that of RJ/royalactin used previously, the present findings indicate that the endogenous bioactivity of royalactin in *Drosophila* is partially maintained in FDRJ. Despite these similarities, it is of course possible that there are other factors that differ between FDRJ and royalactin that influence development and physiology in *Drosophila*.

Several environmental factors are known to control growth rates in insects, including temperature, nutrition, and population density. Of *Drosophila*, it has been frequently reported that mutant alleles involved in the IIS/TOR pathway, such as *dilp (s)*, *InR*, and *TOR*, also cause developmental defects and delays. In addition, TOR activity is required by the prothoracic gland (PG), which secretes the molting hormone ecdysone, while activation of TOR solely in PG cells reverses the developmental delay caused by nutritional restriction.

Moreover, IIS/TOR pathway-related genes are known to play crucial roles in aging, but Kamakura concluded that royalactin drives queen development through an Egfr-mediated signaling pathway. Since the Egfr-mediated signaling and IIS pathways are exclusive parallel pathways, it is reasonable to assume that induction of *dilp5*, *InR*, and *TOR* gene expression does not occur in long-living males fed 1% FDRJ. The fact that FDRJ resulted in increased gene expression of *dilp5*, *InR*, and *TOR* in adult females, as well as higher fecundity, might reflect the increase observed in the number of cells expressing relatively high levels of *InR* and *TOR* (e.g., germ cells and adult fat cells) and the subsequent expression of *dilp5*. The honeybee TOR pathway plays a key role in the development of queen bees, the sole fertile individuals in the bee caste. Moreover, it has been reported that TOR is required for the proliferation, growth, and survival of differentiating germ cells, while germline stem cells and follicle stem cells in *Drosophila* ovaries have been found to respond to diet via IIS. Taken together, these findings suggest that Egfr-mediated signaling and the subsequent control of fecundity through ovary maturation is mediated not only by RJ and royalactin, but also by FDRJ.

In conclusion, this study indicates for the first time that FDRJ, a physically processed form of RJ, maintains certain bioactivities in multicellular organisms other than the honeybee. The present findings provide a first step in analyzing the molecular mechanism of FDRJ bioactivity using *Drosophila* mutants and in the development of disease models of life style- and age-related diseases. Before successful industrial application of RJ, further *in vivo* analysis of FDRJ bioactivity in mammals as well as clinical studies are required.
Acknowledgments

We thank the members of our laboratory for fruitful discussions during the course of this study. We also thank Dr. Toshiro Ohta for helpful comments that enabled us to improve the manuscript. This work was supported in part by the Japan Royal Jelly Fair Trade Council, a Grant-in-Aid for Young Scientist (B) (24700837) (to Y.K.), and by the Global Center of Excellence (COE) Program of the Ministry of Education, Culture, Sports, Science, and Technology of Japan. The funders had no role in study design, data collection and analysis, the decision to publish, or the preparation of the manuscript.

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