The Carboxyl-Terminal Penta-Peptide Repeats of Major Royal Jelly Protein 3 Enhance Cell Proliferation

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Royal jelly (RJ) is known as an important functional foodstuff that promotes several health benefits and contains various bioactive substances, including major royal jelly proteins (MRJPs). Among the MRJPs, MRJP3 possesses both cell proliferation and wound healing effects. As the carboxyl domain of MRJP3 contains tandem penta-peptide repeat (TPR) sequences unique to MRJP3 among the MRJPs, we purified the TPRs as glutathione-S-transferase (GST)-fusion proteins and demonstrated their dose-dependent effects on THP-1 and Vero cell proliferation. The GST-TPR protein with 19 repeats (GST-TPR19) showed cell proliferative activity equivalent to MRJP3 and higher than GST-TPR6. GST-TPR19 also exhibited wound healing activity at a level similar to MRJP3. Digestion of GST-TPR19 with trypsin had no effect on its cell proliferative activity, suggesting that the main digested products; i.e., penta-peptides (Q-N-x-N-[K/R]), maintain the cell proliferative ability of MRJP3. In conclusion, the TPRs of MRJP3 are critical to the beneficial effect(s) of RJ.

Key words royal jelly; major royal jelly protein 3 (MRJP3); penta-peptide repeat; cell proliferation; wound healing

INTRODUCTION

Royal jelly (RJ) is generally known as the exclusive food for the growth of queen honeybees (Apis mellifera) and contains many beneficial properties, such as antimicrobial, antioxidant, wound healing, anti-tumor, anti-aging, and anti-inflammatory effects.1–4) As these properties of RJ are widely considered to be useful for maintaining homeostasis and recovering from pathological conditions, RJ has been used in the form of cosmetics, health foods, or dietary supplements.5) Chemically, RJ is composed of water, carbohydrates, proteins, lipids, mineral salts and vitamins.6,7)

Major royal jelly proteins (MRJPs) are the most abundant components constituting more than 80% of the RJ proteins.8,9) The MRJP family has nine members (MRJP1–9) with composition ratios of MRJP1 (46%), MRJP2 (11%), MRJP3 (13%) and others.9) The amino acid sequences of these MRJPs have a homologous region at the major part of N-terminus, but those of the C-terminal region are unique to each molecule.10,11) MRJP3 has unique tandem penta-peptide repeat (TPRs) consisting of highly basic residues Q-N-[A/G/D]-[D/N/G]-[N/K/R] at its C-terminus.11) Consequently, these unique C-terminal TPRs in MRJP3 are considered to be responsible for the unique function(s) of MRJP3.

Each MRJP presents several biological activities such as the cell proliferative effects of MRJP1 on Jurkat and ICE-1 cells12) and the antimicrobial activities of MRJP1, MRJP2, and MRJP4,8,13,14) Kamakura and colleagues reported that the 57 kDa protein, most likely MRJP1, has cell proliferative effect on rat hepatocytes.15) MRJP1 induces mRNA expression of cytokines in cultured human keratinocytes.16) MRJP3 increased the cell proliferative and wound healing effects on HaCaT cells.17)

Regarding MRJP3, it has additional functions, including immune regulatory effects; i.e., suppression of both ovalbumin (OVA)/alum-induced allergic responses in mice18) and pro-inflammatory cytokine secretion from macrophages.19) Recently, it was reported that its C-terminus assembles with RNA to form extracellular ribonucleoprotein (RNP) and protect RNA from degradation.20)

In the present study, we elucidated the MRJP3-mediated cellular functions, including cell growth and wound healing, and found that the TPRs of MRJP3 induced THP-1 and Vero cell growth as well as the wound healing activity of Vero cells. Our study suggested that the C-terminus TPRs are critical for the application of RJ as functional foodstuffs and/or medical agents.

MATERIALS AND METHODS

Cells A human leukemic cell line, THP-1, was grown in RPMI medium (Sigma-Aldrich, St. Louis, MO, U.S.A.) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin (Thermo Fisher, Waltham, MA, U.S.A.). An African green monkey kidney epithelial cell line, Vero, was grown in Dulbecco’s modified essential medium (DMEM) supplemented as described above.

Plasmids and Purification of Glutathione-S-transferase (GST)-Fusion Proteins Full length MRJP3 cDNA was synthesized as a DNA string codon-optimized for expression in Escherichia coli (E. coli) by GeneArt strings DNA fragments (Thermo Fisher). DNA fragments encoding amino-acid posi-
tions (aa 401–454 or 401–519 of MRJP3) were amplified by PCR using the primers listed in Supplementary Table 1, and cloned between the BamHI and Xhol sites of the pGEX-6P-1 plasmid (GE Healthcare, Chicago, IL, U.S.A.), resulting in pGEX-MRJP3-TPR6 and pGEX-MRJP3-TPR19, respectively. The integrity of the inserts was confirmed by DNA sequencing. E. coli strain BL21(DE3) (New England Biolabs, Ipswich, MA, U.S.A.) was transformed with the pGEX-derived plasmids and stimulated with isopropyl-β-d-thiogalactoside (IPTG). After expression of large quantities of the GST-fusion proteins, bacterial cells in ice-cold phosphate buffered saline (PBS) containing a protease inhibitor cocktail (Sigma-Aldrich) were lysed by brief sonication, and the GST-fusion proteins were purified with glutathione beads (GE Healthcare). The purified GST-fusion proteins were dialyzed against PBS, irradiated with UV and filtrated at a pore size of 0.2 µm (Merek Millipore, Burlington, MA, U.S.A.) to eliminate bacterial debris.

One milligram of the purified GST or GST-TPR19 proteins was digested with trypsin (0.05 mg/mL) at 37 °C for 20 min, followed by heating at 60 °C for 30 min to inactivate residual trypsin activity.

AlamarBlue Viability Assay and Trypan Blue Dye Exclusion Assay THP-1 cells suspended in RPMI medium without FBS at a density of 1 × 10^4 cells/well were plated in each well of 96-well plates in the presence or absence of GST-fusion proteins at various concentrations and then incubated at 37 °C. The amount of protein used in the experiments were calculated from the recommended daily intake of RJ. Vero cells cultured in DMEM-10% FBS at a density of 1 × 10^4 cells/well were plated in each well of 96-well plates. After overnight culture, the culture medium was replaced with DMEM-0% FBS containing the GST-fusion proteins at various concentrations and then the cells were incubated at 37 °C. After incubation for 48 h, 0.1 vol. of alammarBlue reagent (Thermo Fisher) was added and incubated for 4 h. Culture supernatants were transferred to the wells of a black plate (Corning Inc., Corning, NY, U.S.A), and fluorescence signals were counted with a GloMax Multi plate reader (Promega, Madison, WI, U.S.A.). Data were obtained from the triplicated independent experiments and each set of experimental results were obtained from 3 independent wells. The data for the cell proliferation assay with trypsinized protein products were analyzed with one-way ANOVA followed by Tukey’s multiple comparison test using GraphPad PRISM software ver.8.3 (GraphPad Software, San Diego, CA, U.S.A.). The data for the cell proliferation assay with trypsinized protein products were analyzed with the Wilcoxon rank sum test.

**RESULTS**

The Expression and Purification of GST-MRJP3 Fusion Proteins The C-terminal domain of MRJP3 is made of 19 tandem penta-peptide repeats (TPRs) (Figs. 1A–C). These penta-peptide repeats can be classified to three patterns based on the sequence position of the basic amino acid residues (N, K, or R). i.e., Q-N-A-x-N on the N-terminal side (hatched boxes), Q-N-x-N[K/R] in the central region (gray boxes), and R-N-G-N-R on the C-terminal side (dotted box) (Figs. 1A, B). The C-terminal sequences encoding 6 or 19 TPRs were cloned into the pGEX-6P-1 plasmid to express the GST-fusion proteins GST-TPR6 and -TPR19, respectively (Fig. 1A). The sixth TPR has an R residue at the C-terminus for trypsin digestion.

**Fig. 1. The C-Terminal Structure of MRJP3 and Expression of GST-TPR Proteins**

(A) MRJP3 possesses a signal peptide (SP) at the N-terminus (aa 1–16) and tandem penta-peptide repeats (TPRs) at aa 425–519, near the C-terminal end. The regions containing 6 or 19 TPRs were fused with GST, respectively, as indicated. The TPRs are indicated as hatched (Q-N-A-x-N), gray (Q-N-x-N[K/R]) and dot-

(B) The amino acid sequence of the MRJP3 C-terminal region. TPRs consist of Q-N-A-x-N, Q-N-x-N[K/R] and R-N-G-N-R (underlined). Numbers show amino acid positions in MRJP3. (C) The penta-peptide species and repeat numbers of MRJP3, GST-TPR6, and GST-TPR19. Region (aa) indicates the amino-acid positions of MRJP3. (D) Purified GST, GST-TPR6 (TPR6), GST-TPR19 (TPR19) and MRJP3 proteins were separated in a SDS-PAGE gel followed by CBB staining.
The GST-fusion proteins, which were purified as described in Materials and Methods, and the purified MRJP3 protein (kindly provided by Dr. Yonekura) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie Brilliant Blue (CBB) staining (Fig. 1D). The molecular weights of GST-TPR6 and -TPR19 were consistent with those calculated based on the sequences, approximately 33 kDa and 40 kDa, respectively. The purified MRJP3 was revealed to be approximately 70 kDa by the SDS-PAGE, which is consistent with previous reports.9,18)

The Proliferative Activities of MRJP3 and GST-TPR Proteins To evaluate the effects of MRJP3-TRRs on cell proliferation, the purified GST-fusion proteins were added at the indicated final concentrations to the THP-1 and Vero cell cultures, and the growth of cells was analyzed using an alamarBlue assay (Figs. 2A, B). No detectable changes in cell morphology were observed by the addition of GST-fusion proteins and MRJP3 (data not shown). The purified MRJP3 was revealed to be approximately 70 kDa by the SDS-PAGE, which is consistent with previous reports.9,18)

The Proliferative Activities of MRJP3 and GST-TPR Proteins To evaluate the effects of MRJP3-TRRs on cell proliferation, the purified GST-fusion proteins were added at the indicated final concentrations to the THP-1 and Vero cell cultures, and the growth of cells was analyzed using an alamarBlue assay (Figs. 2A, B). No detectable changes in cell morphology were observed by the addition of GST-fusion proteins and MRJP3 (data not shown). The proliferative activities of THP-1 cells and Vero cells were increased in the presence of 1 µM protein concentration (Figs. 2C, D). In both THP-1 and Vero cells, cell viabilities, measured as fluorescence units, were increased by the addition of GST-TPR6, GST-TPR19, and MRJP3 in a dose-dependent manner. In addition, the trypan blue dye exclusion assay revealed a significant increase in viable THP-1 cells in the presence of 250 µg/mL of GST-TPR6, GST-TPR19, and MRJP3 (Fig. 3A). The cell-doubling time ($T_d$) of THP-1 cells grown in the presence of 250 µg/mL of GST, GST-TPR6, GST-TPR19, and MRJP3 was 54.6, 49.0, 40.1, and 36.6 h, respectively. Furthermore, cell viabilities were increased in the presence of TPRs (Fig. 3B). The results show that the proliferative activity was increased as the number of TPRs. In all experiments, the proliferative activity of GST-TPR19 was similar to that of MRJP3 and higher than that of GST-TPR6. These results indicate that the C-terminal TPRs are the major contributors to the cell proliferative activity of MRJP3.

Wound Healing Effects of the C-Terminal Repeat Fusion Proteins Wound healing effects of the GST-TPR proteins were evaluated in the in vitro scratch-wound healing model using Vero cells as described in Materials and Methods. Cell migration activities were measured as the percentage of gap coverage over a period of 24 h. As shown in Fig. 4, the gap was clearly and quickly filled by the addition of MRJP3 or GST-TPRs, indicating that the C-terminal TPRs of MRJP3 are responsible for the cell migratory activity. Furthermore, longer TPRs showed higher cell migratory activity.
The Cell Proliferative Activity of Trypsin-Treated GST-TPR19

In order to determine the minimal essential structure of TPRs for cell proliferative activity, GST and GST-TPR19 digested with trypsin were added to Vero cells and analyzed by the alamarBlue viability assay. Relative numbers of the cells treated with the indicated protein in triplicated wells were obtained, with that of the cells treated with GST used as a 100% control. The mean ± S.E.M. of relative cell numbers for three independent experiments are shown. The p-values were determined by one-way ANOVA followed by Tukey's multiple comparison test. *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001.

**DISCUSSION**

In this report, we found that the C-terminal TPRs of MRJP3 showed cell proliferative activities in THP-1 and Vero cells, which is essentially consistent with a previous report that demonstrated that MRJP3 induced cell proliferation and migration of HaCaT cells. It is consistent with the prediction that the C-terminal domain is the responsible region of proliferation activities, which supported by the properties that the C-terminal TPRs are unique for MRJP3. Although it has been reported that purified MRJP3 inhibits Jurkat cell proliferation, this discrepancy in results might be dependent on the purification method or origin of the RJ used. Our results indicated that the MRJP-TPRs themselves contributed to the cell proliferative activity, as recombinant proteins purified from bacteria were used in this report.

The number of TPRs is likely to have a significant impact on cell proliferation, as the result of 1 µM protein concentration (Figs. 2C, D). As trypsin-digested GST-TPR19 demonstrated significant cell proliferative activity, the C-terminal penta-peptide sequence Q-N-x-N-[K/R] appears to be the unit responsible for cell proliferation (Fig. 5B). In addition to this penta-peptide, trypsin digestion of GST-TPR19 is expected to generate a peptide of 33 aa in length derived from aa 422-454 of MRJP3 (Fig. 1C), which contains 5 copies of Q-N-A-[G/D]-N and one copy of Q-N-x-N-[K/R]. As shown in Fig. 2, GST-TPR6, which contains the 33 aa sequence, increased cell proliferative activity, Q-N-A-[D/G]-N penta-peptide may also possess cell proliferative activity. Our results indicated that GST-TPR19 demonstrated higher cell proliferative activities than did GST-TPR6. Therefore, the amino acid sequence derived from aa 455–519 is thought to possess more potent cell proliferative activity. Alternatively, it is possible that the number of repeats affects the proliferative activity of GST-TPRs.
The obtained results indicate that the Q-N-x-N-[K/R] peptide could be the responsible unit for cell proliferative activity, since cell proliferative activity was increased by the number of TPRs and was not decreased by the trypsin treatment. However, it is important to directly demonstrate whether the Q-N-A-x-N peptide possesses cell proliferative activity.

The residues at the positions 3, 4, and 5 of the penta-peptide show a good deal of diversity (Fig. 1C); therefore, the alterations in and optimization of amino acid residues are important to our understanding of the functional mechanism of MRJP3 TPRs. Although the precise mechanisms underlying the cell proliferative activity of MRJP3 TPRs remains to be elucidated, Lin et al. demonstrated by Cluster of Orthologous Groups (COG) analysis that a fraction containing MRJP2, 3, and 7 is associated with carbohydrate transport and metabolism.17) The C-terminal basic residues of the Q-N-x-N-[K/R] peptides change the charge as well as the α-amidation of the peptide. C-Terminal amidation is found in many physiologically active peptides, such as peptide hormones and antimicrobial peptides, and is known to enhance stability and receptor binding.21–23) The basic amino acid residues on the C-terminal side of the Q-N-x-N-[K/R] peptides may affect the activity of the penta-peptide in the same way as peptide hormones to lead to the higher cell proliferative activity of GST-TPR19. Peptide hormones, such as gastrin and cholecystokinin, induce cell proliferative activity through specific receptors24,25); thus, identification of the cellular receptor for MRJP3 and the penta-peptide would be important to our understanding of the mechanisms underlying the proliferative activity.

Alternative, TPRs might internalized directly as a cell penetrating peptide, since they are rich in R and K residues so that they may behave similarly to Tat peptide of human immunodeficiency virus (HIV) and penetratin.26)

RJ possesses various biological properties1–4): the intake of RJ significantly prolongs the mean lifespan of C3H/HeJ mice,27) and RJ and protease-treated RJ (pRJ) increase the cell proliferation rate and attenuate the decrease in muscle weight in aged mice and humans.28,29) Protease treatment of RJ generates many peptides derived from MRJPs30); thus, our results showing that the penta-peptide derived from MRJP3 possesses cell proliferative activity is consistent with those reports and warrant further studies on RJ-derived short peptides generated by digestive enzymes in the gut, which may provide further understanding of the biological functions of RJ.

RJ has many beneficial effects on human health and is widely used as a functional foodstuff. As our results indicate that the C-terminal TPRs of MRJP3 possess proliferative activity and it is likely that the penta-peptide is responsible for this activity.

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Conflict of Interest NI received a collaborative research fund from API Co., Ltd., a company that manufactures and sells bee products. However, the company had no involvement in the collection, management, analysis, or interpretation of the data, or in any processes related to manuscript preparation. All other authors declare no conflict of interest.

Supplementary Material The online version of this article contains supplementary materials.

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