A THESIS FOR THE DEGREEOF MASTER OF SCIENCE

The Effects of The 57-kDa Protein Isolated from Korean Royal jelly on the Activity of Human-derived Osteoblast

국산 로얄젤리(royal jelly)에서 분리한 57-kDa 단백질의 인간유래조골세포 활성에 미치는 영향

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SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

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APPROVED AS A QUALIFIED THESIS OF SAEWOOM CHOI

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ABSTRACT

Royal jelly (RJ) is a secretion product of the cephalic glands of nurse bees that has been used for centuries for its extraordinary properties and health effects. Pharmacological actions of RJ such as antitumor, antiviral, and antioxidant activity have been well noted. RJ and its 57-kDa protein were also reported to enhance the bone-forming ability of osteoblasts and the production of albumin and to have protective effect on rat liver cells. However, the impact of RJ -derived materials, particularly 57-kDa protein, on Saos-2, a human osteoblast-like cell line, is not yet fully understood. Also, no information has been done to consider potential use of RJ -derived materials to manage ultra violet (UV) induced damages. In this study, the effects of RJ and its 57-kDa protein on Saos-2 and the cytoprotective activity of the materials toward Saos-2 cells damaged by UV were evaluated. The cytoprotective activity of the materials was

compared with that of *N*-acetyl-L-cysteine (NAC), a currently used antioxidant, for use as future commercial products with cytoprotective action using a MTS assay. Firstly, 57-kDa protein was purified from domestic RJ using fast protein liquid chromatography and gel filtration method. The 57-kDa protein at concentrations between 10 and 100 μg/mL exhibited a high correlation between protein concentration and cell viability (correlation coefficient (R^2) > 0.85). Pretreatment with 0.1 mg/mL 57-kDa protein and 2 mM NAC to Saos-2 cells resulted in 79 and 77% protection against UVB, respectively, whereas 1.5 mg/mL RJ showed no statistically significant protection from radiation induced cell death.

Osteoblast extracellular Ca^{2+}-sensing receptor regulates bone development, mineralization, and turnover (Dvorak-Ewell MM et al. 2011). The mitochondria are to generate the cell energy, ATP (i.e., phosphorylation of ADP), and Changes in alkaline phosphatase level and activity are involved in a variety of physiological and pathological events, such as bone development. The effects of 57-kDa protein on the calcium sensing receptor in live cells and mitochondria were compared with cell groups pretreated with UV using confocal microscopy. The microscopic observation revealed that the cells treated with 57-kDa protein showed normal cell shapes and organelles being maintained, similar to normal cells. Pretreatment of Saos-2 cells with 0.1 mg/mL 57-kDa protein significantly resulted in the increase in ALP activity from radiation induced cells. However, 1.5 mg/mL RJ and 2 mM NAC exhibited no statistically significant increase in ALP activity.

In conclusion, 57-kDa protein can be useful for the bone formation of osteoblasts and for protection from UV. For practical use of RJ and 57-kDa protein as novel anti- UV induced stress products to proceed, further research is needed to establish their human

safety and whether this activity could be exerted *in vivo* after consumption of the product by humans. In addition, their anti-UV induced stress modes of action need to be established and detailed tests are needed to understand how to improve anti-UV induced stress potency and stability for eventual commercial development.

Key word: Royal jelly, 57-kDa royal jelly protein, ultra violet irradiation, osteoblasts, protective effect, human osteoblast-like cell line, Saos-2 cell line

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INTRODUCTION

Bone mass consists of extracellular substances such as collagen and glycoprotein and numerous bone cell family such as osteoblasts, osteoclasts and osteopontin (Mikihiko et al., 2003). Osteoblasts are responsible for a matrix of osteoid which is composed mainly of Type I collagen and linked to mineralization of this matrix. This process needs some of the minerals such as zinc, copper, and sodium. Bone is a dynamic tissue that is being constantly reshaped by osteoblasts and osteoclasts. Osteoblasts are in charge of matrix and mineral production and osteoclasts break down the tissue. With ageing, the number of osteoblasts tends to decrease, affecting the balance of bone formation and absorption in the bone tissue (Salentijn, 2007) and potentially cause osteoporosis. For treatment of osteoporosis, some drugs such as vitamin D and calcium are commercially available and recommended although there however is an increased risk of myocardial infarctions (Bolland, 2010) and kidney stones (Virginia et al., 2013). Therefore, natural drugs with lesser side effects which can be an alternative of synthetic drugs have been substantially studied.

Exposure of human cells to UV leads to damage of cellular constituents and results in complicated cell responses, such as induction of genes and disturbance of various signaling pathways (Bender et al., 1997; Schwarz, 1998). The most serious effects of UVB are caused by the indirect DNA damage (free radicals and oxidative stress). In humans, prolonged exposure to solar UV radiation results in acute and chronic health effects on the skin, eye, and immune system (Lucas et al., 2006). Moreover, UVC can cause mutagenic or carcinogenic (Hogan, 2010).

Historically, RJ has long been used for conditions such as rheumatoid arthritis, hypertension, diabetes, chronic hepatitis, duodenal ulcer, menstrual problems, uterine bleeding and infertility, fatigue, weakness, poor nutrition, leucopenia, and cancer (Batchelder, 2002). No information has been done to consider potential use of RJ to manage UV induced damages, although excellent pharmacological actions of RJ have been well described by Crenguța I. P et al. (2011).

In this study, an assessment is made of the cytoprotective activity of the 57-kDa protein that comprises RJ toward Saos-2 cells damaged by UV using a MTS assay. The cytoprotective activity of the 57-kDa protein was compared with that of *N*-acetyl-L-cysteine (NAC), a currently used antioxidant, for use as future commercial products with cytoprotective action (Jun JH et al., 2008). Alkaline phosphatase (ALP) activity of 57-kDa protein-treated Saos-2 cells damaged by UV was examined using a *p*NPP ALP assay because changes in ALP level and activity are associated with various disease states in the liver and bone (Coates, 2013). In addition, physiological effects such as Ca^{2+} distribution and mitochondria activity were also investigated using confocal laser scanning microscopy because extracellular Ca^{2+} sensing receptor in osteoblast regulates bone development, mineralization, and turnover (Dvorak-Ewell et al., 2011). Mitochondria also are the key organelle that generate the cell energy and regulate cellular metabolism in live cells.

LITERATURE REVIEW

1. Royal jelly

Royal jelly (RJ) is secreted material of the bee for nursing bee larvae (Hoffmann, 1966). It is secreted from the glands in the mandibular or hypopharynx which is the head part of young worker bees (Graham, 1992). RJ is available to feeding all the larvae including the larvae group that will become workers. If honey bees need a queen bee, RJ is supplied bulk but only to the selected larvae during the first four days after birth. So after early intensive nutrition, the selected larvae which have mature ovaries enough to lay eggs and become a type of queen Bee. In fact, all the larvae eat RJ but imagos will not eat it except the queen. Only the queen bee feed RJ during her whole lifetime that is 40 times more than workers.

General composition of RJ is as follows: water (67%), proteins (13%), sugars (11%), fructose (6%), glucose (4%), and fatty acids (5%) which is shorter chains than usual for insects 8–10 carbons, minerals (Ca, Cu, Fe, Mg, Mn, Na, K, Zn, and Si) (1%), four phospholipids, and five glycolipids which supply energy (Graham, 1992). RJ also contains many vitamins (A, B, C, D, E, and K), minerals, sterols (methy-lenecholesterol (ca 0.3%), cholesterol, stigmastanol, stigmasterol, and testosterone), 7–9 different sterols (sitosterol, cortisol, and cholesterol), acetylcholine, amino acids (total 1.59%) (proline, lysine, glutamic acid, serine, alanine, arginine, aspartic acid, glycine, isoleucine, leucine, methionine, tyrosine, valine, glutamine, and taurine), and other nutrients. (Mohamed Fawzy R et al., 2012). Among amino acids, proline is the major amino acid (54–60%), followed by lysine and glutamic acid. Recently, the hydroxy fatty acids were reported. They include 10-hydroxy-*trans*-2-decenoic acid (10-HAD) (royal

jelly acid). 10-hydroxydecanoic acid (0.60–1.25%), 10-acetoxydecanoic acid, 11-*S*-hydroxydodecanoic acid, hydroxy-2*E*-decenoic acid 10-phosphate, and other acids (gluconic acid, sebacic acid, 2-decenedioic acid, *p*-hydroxybenzoic acid, 3-hydroxydecanoic, 8-hydroxydecanoic, and 3,10-dihydroxydecanoic acids) (Lavinia, 2011). The RJ proteins such as the major royal jelly protein family and some other previously identified proteins, peptides, glycoproteins, and biopterin are also included (Mohamed Fawzy R et al., 2011). Gamma-globulin, gelatin, adenosine monophosphate, and adenosine monophosphate N1-oxide were recently found from RJ (Mohamed Fawzy R et al., 2011). As the major active component of RJ, extensively recognized 10-HDA has been reported to be quite stable to heat, retaining chemically not damage in spite of deterioration of other components (Ramadana and Al-Ghamd, 2012). RJ itself is also very stable when refrigerated or in the frozen or dried state (Masaki K et al., 2001).

2. Biological activities of RJ

2.1. Cardioprotective action

The cardioprotective action is caused by RJ capacity to stimulate an increased secretion of adrenaline via acetylcholine (Vasily et al., 2006). RJ can reduce blood sugar level via insulin-like peptides and other compounds such as chromium, sulphur, and vitamins B3 and H. RJ is also capable to sustain the optimal blood level of sugars by taking part in the oxidation of glucose to obtain energy, through the insulinic effect of insulin-like peptides found in it (Batchelder, 2002).

2.2. Antioxidant and anti-aging actions

RJ has antioxidant and anti-aging abilities (Nagai T et al., 2006). Reportedly, RJ has significant effects on the hypothalamic-pituitary axis (Yukio et al., 2009). Hypothalamic-pituitary functioning generally decreases in aging. It can supply for age-associate reducing pituitary functions in rats (Yukio et al., 2009). It alleviates intra-cellular oxidation by acting as a scavenger of reactive oxygen species. RJ also has an effect on protein expression (Inoue et al., 2003). Peptides isolated from hydrolysates of water-soluble RJ proteins with protease P show significantly stronger hydroxyl radical-scavenging effects and antioxidant activity against lipid peroxidation. These results suggest that RJ peptides (RJPx) may inhibit LPO both *in vitro* and *in vivo* and helps prevent cell damage (Guo et al., 2003). Ames et al. (1993) studied the antioxidative and scavenging activities of enzymatic hydrolysates of RJ using three enzymes pepsin, trypsin, and papain. They reported that the antioxidative and scavenging activities of the enzymatic hydrolysates against active oxygen species such as superoxide anion radical and hydroxyl radical of each hydrolysate were strong in RJ.

Royal jelly has the basic components of collagen production against aging and affects the TGF-beta 1 (transforming growth factor-beta) which is an essential factor for collagen production (Koya-Miyata et al., 2004). A collagen production promoting factor from an extract of RJ and 10-hydroxy-2-decenoic acid isolated from RJ are identified as the factor which enhances a fibroblast cell line to produce TGF-beta 1 (Koya-Miyata et al., 2004). RJ treatment significantly promots transfer of fibroblasts in a dose-dependent manner at 8 hr. Among various lipid classes of fibroblasts, the levels of triglycerol and cholesterol are considerably decreased with 5 μg/mL RJ (Kim et al., 2010). The tympanic membranes of the guinea pigs showed marked fibroblastic orientation and

well-organized connective tissue after 3 months when royal jelly was supplied topically to the membranes after surgical operation (Kim et al., 2010). Application of RJ also promotes the success rate of closure compared to placebo (Calli et al., 2008).

2.3. Glucose metabolism

In humans, RJ ingestion is found to involve in the glucose metabolism. Twenty volunteers were tested the standardized oralglucose tolerance test (OGTT) and next, a second tested OGTT after ingestion of 20 g RJ. Serum glucose levels after 2 hrs and the area under the curve for glucose were considerably lower after RJ administration (Munstedt, 2009). Nomura et al. (2007) studied the effects of oral administration of royal jelly (10, 30, and 300 mg/kg, four weeks) on insulin resistance in 10-week-old rats, a type 2 diabetic model. They reported that RJ administration decreased systolic blood pressure and highly reduced serum levels of insulin and the HMA ratio, an index of insulin resistance. These results suggest that RJ could be an effective and functional food to prevent the development of insulin resistance as described previously by Zamami Y (2008).

2.4. Cholesterol management

The addition of RJ to the daily diet increases the HDL cholesterol, particularly in older patients (Münstedt, 2009). In humans, 50–100 milligrams dry weight of RJ per day reduced total serum cholesterol by 14% and lipids by 10%. The patients were treated with RJ by mouth and by injection but the results were quite similar for both delivery techniques (Vittek, 1995). The serum total cholesterol (TC) and serum low-

density lipoprotein (LDL) in the RJ group (n = 7, 6 g per day for 4 weeks) reduced considerably compared to other the control group ($P < 0.05$) (Guo, 2007).

2.5. Estrogenic effects

Fatty acids from RJ have many benefits toward autonomic imbalance, perimenopausal symptoms, osteoporosis, and other serious conditions. These abilities may attribute to the interaction of the fatty acids of RJ with an estrogen receptor in the human cells (Toshiaki et al., 2008). At concentrations of 10–100 µg/mL RJ exhibited a quiet low but significant estrogenic activity via estrogen receptor α (ERα), whereas it acted as a complete estrogen antagonist via estrogen receptor β (ERβ) at a concentration range between 50 and 200 µg/mL. These results suggest that RJ methanol extract may be a feasible natural modulator of estrogen signaling interaction with the ERα and ERβ subtypes (Tsiapara et al., 2009). It has been also reported that RJ competes with 17-β estradiol to bind to the human estrogen receptors such as α, β and offers that RJ has estrogenic activity through mediation with estrogen receptors by endogenous gene expressions (Mishima et al., 2005). Hale (1939) reported documented that RJ retained hormones that promote growth of ovarian activity in the white rat for a few days and an increase in fertility. Heyl (1939) injected RJ directly into female rat and found that it stimulated the ovarian growth in the same manner.

2.6. Bone formation

RJ as a whole or some of its properties enhances production of type I collagen and other activities for bone formation by action on osteoblasts through upregulation of procollagen I alpha1 gene expression (Narita, 2006).

2.7. Anticancer activity

Bisphenol A (BPA) is an environmental estrogen that stimulates proliferation of human breast cancer MCF-7 cells. RJ blocked the growth stimulating effect of BPA on MCF-7 cells (Nakaya, 2007). Fraction RJP30, gained by precipitation of the RJ with ammonium sulfate, declined by 2.5 fold the initial cell density of HeLa human cervicouterine carcinoma cells, after seven days of treatment (Salazar-Olivo, 2005). The 100 mg/kg of body weight doses of both RJ and green tea offered protection against cDDP (cisplatin)-induced nephrotoxicity, and both products enable as protector agents against cDDP-induced kidney damage in adult albino mice (Yapar et al., 2009).

2.8. Antibacterial immunologic activity

An antibacterial protein, royalisin, derived from RJ has potent antibacterial activity at low concentrations against Gram-positive bacteria, but not against Gram-negative bacteria (Fujiwara et al., 1990). Boukraa et al. (2008) studied the antibacterial activity of four varieties of honey and one variety of fresh RJ against *Staphylococcus aureus*. They reported a strong linear correlation amongst the minimum inhibitory concentration decrease of four varieties of honey and RJ (Boukraa et al., 2008).

2.9. Neurogenic effects of brain injury healing

Hattori (2007) studied the RJ and its unique fatty acid, 10-hydroxy-trans-2-decenoic acid, effects of AMP N1-oxide on the proliferation and differentiation of cultured neural stem/progenitor cells (NSCs) and reported that AMP N1-oxide is one of the components that facilitate astrogenesis by NSCs through activation of STAT3. AMP N1-oxide may

be a good elicitor for protection against and therapy for certain brain injuries, because astrocytes play important roles in brain development and the recovery from injury.

3. Ultra violet effect

A major part of the mutagenic and carcinogenic properties of sunlight has been attributed to UV rays. Ozone depletion results in an increased flux of biologically UV damaging radiations which directly reach the earth. UV induces harmful reactions for cells such as DNA damage, interfering with other components of receptor mediated signal transduction pathways and mediates immunosuppression by interfering with signal transduction (Bender et al., 1997). In particular, UV radiation in the middle-wavelength range between 290 and 320 nm (UVB) represents one of the most relevant environmental dangers because of its hazardous effects. Like alkylating chemicals and oxidants, exposure to UV light such as UVB can induce apoptosis in mammalian cells (Rehemtulla et al., 1997). Under UV irradiation, the stress-activated protein kinase pathway mediates cell death following injury induced by cis-platinum (Zanke et al., 1996). Undoubtedly, UVB-induced DNA damage is a crucial event in UVB-mediated apoptosis. On the other hand, UVB directly activates death receptors on the cell surface including CD95, implying that UVB-induced apoptosis can be initiated at the cell membrane through death receptor clustering (Kulms et al., 1999). In addition, epidemiological studies suggest that solar UV radiation is responsible for skin tumor development via gene mutations and immunosuppression, and possibly for photoaging (Ichihashi et al., 2003).

Moreover, the major concern is that UVB irradiation is known as a potent inducer of reactive oxygen species (ROS)-formation (Amaral et al., 2013). ROS such as

superoxides and hydrogen peroxide can cause severe damage to DNA, protein, and lipids. High levels of oxidant produced during normal cellular metabolism or from environmental stimuli such as UV radiation perturb the normal redox balance and shift cells into a state of oxidative stress (Amaral et al., 2013). Consequently, oxidative stress inhibits osteoblastic differentiation of bone cells by extracellular signal-regulated kinases and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Bai et al., 2004). ROS generation is a key modulator of damaged bone cell function. ROS promote bone loss by inhibiting osteoblast differentiation and enhancing osteoclastogenesis (Wauquie et al., 2009). Besides, the balance of bone formation and bone resorption tends to be negative with age, particularly in post-menopausal women, often leading to a loss of bone serious enough to cause fractures, which is called osteoporosis. Oxidative stress by itself and by influencing the regulatory cytokines such as tumor necrosis factor and interlukins are involved in osteoporosis (Abdollahi et al., 2005). Osteoblast extracellular Ca^{2+}-sensing receptor regulates bone development, mineralization, and turnover (Dvorak-Ewell MM et al. 2011). Physical stresses such as exposure to ultraviolet (UVB) may perturb the cell surface or alter receptor conformation (Caridad Rosette et al., 1996). Alkaline phosphatase (ALP) is one of the most frequently used biochemical markers of osteoblast activity. Oxidative stress such as exposure to ultraviolet (UVB) is one of the major causes of inhibition of ALP activity (Bai et al., 2004).

4. Osteoblast

Bone is a dynamic tissue that is constantly being reshaped by osteoblasts, which are in charge of production of matrix and mineral, and osteoclasts, which break down the

tissue. The number of osteoblasts tends to decrease with age, affecting the balance of formation and desorption in the bone tissue (Pittenger et al., 1999). Osteoblasts produce a matrix of osteoid, which is composed mainly of Type I collagen (Reddi et al., 1977). Osteoblasts are also responsible for mineralization of this matrix. Zinc, copper, and sodium are some of the minerals required in this process (Murshed et al., 2005).

5. 57-kDa Protein of royal jelly

The 57-kDa protein which is monomeric and molecular weight 57 in RJ is specifically degraded in proportion to both storage temperature and storage period compare to other components such as 10-HAD, vitamins, amino acids, and proteins. It has been suggested that 57-kDa protein could be useful as a marker of freshness of RJ (Kamakura et al., 2000). In addition, it enhances proliferation of primary cultured rat hepatocytes and increases albumin production in the absence of serum. The 57-kDa protein stimulates hepatocyte DNA synthesis and prolongs the proliferation of hepatocytes, as well as increases albumin production (Kamakura et al., 2001). Moreover, a 57-kDa protein may be an essential growth factor for regulating cast differentiation of honey bee larvae into queen by increasing body size, ovary development and shortened developmental time. It also exhibits similar effects on the *D. melanogaster* (Kamakura et al., 2001).

MATERIALS AND METHODS

1. Royal jelly

Fresh RJ (50 g) was obtained from Korea Honey Farm and stored at –70℃ until use. A voucher specimen (RJ-K01) was deposited in the Research Institute for Agriculture and Life Science, College of Agriculture and Life Sciences, Seoul National University.

2. Materials

(3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetra-zolium) inner salt (MTS) was purchased from Promega (Madison, WI, USA). Coomassie Brilliant Blue R-250 was by Bio-Rad Life Sciences (Hercules, CA, USA). Rosewell Park Memorial Institute (RPMI) 1640 and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). *p*NPP (*p*-Nitrophenyl Phosphate) Alkaline Phosphatase Assay Kit were purchased from AnaSpec (Fremont, CA, USA) DEAE FF 16/10 column, Mono Q column, Superdex 75 gel filteration column were supplied by GE Healthcare Life Sciences (Pittsburgh, PA, USA). Collagen IV-coated plates (96-well plates) were purchased from BD Biosciences (Bedford, MA, USA). CellTiter 96® AQueous One Solution Cell Proliferation assay kit was purchased from Promega (Madison, WI, USA). MitoTracker® Red FM and Fluo-4/AM were purchased from Invitrogen Molecular Probes (San Diego, CA, USA). The Precision Plus Protein all blue standards were provided by Bio-Rad Life Sciences (Hercules, CA, USA). All of the other chemicals and reagents used in this study were of analytical grade quality and available commercially.

3. Cell line and culture conditions

Saos-2 cell line has the characteristics of osteoblasts such as the formation of bone proteins and the activity of ALP and is widely used for the experimental model. Saos-2 (KCLB 30085), a human osteoblast-like cell line, was purchased from the Korean Cell Line Bank (Seoul, ROK). The cell line was maintained in RPMI 1640 supplemented with 10% charcoal dextran-treated FBS and 1% antibiotics (penicillin + streptomycin) in a humidified incubator at 37℃ and 5% CO_2 for cell proliferation assays. The cell line was stored at –70℃ until use.

4. Protein purification

The method of protein purification was followed by Kamakura et al., (2001) with minor modification.

4.1. Ultrafiltration procedure

RJ (30 g) in 1L of 20 mM Tris-HCl buffer (pH 7.0) was centrifuged at 1 × 5000 *g* for 10 min at 4℃ using Supra 25K ultra vacuum speed centrifuge (Hanil Science, Incheon, ROK) and filtered using an Advantec 240 mm filter paper (Advantec MFS, Dublin, CA, USA). The combined supernatant (60 mL) was concentrated to 20 mL with 20 mM Tris-HCL buffer (pH 7.0) using QuixStand Benchtop System (GE Healthcare Life Sciences, Buckinghamshire, UK) incorporated with a 10,000 nominal molecular weight cutoff (NMWL) polysulfone Hollow Fiber Cartridge (Xampler Ultrafiltration Cartridges (UFP-1-C-4X2M), 1400 cm^2 [1.5 ft^2], from (140) 0.5 mm ID fibers). The concentrate was added to 80 mL of 20 mM Tris-HCL buffer (pH 7.0). These procedures were repeated five times. The final filtrated samples were transferred to Amicon Ultra-15mL

centrifuge tubes (EMD Millipore, Darmstadt, Germany), and the tubes were centrifuged at 3000 *g* for 30 min at 4℃.

4.2. Fast protein liquid chromatography

4.2.1. DEAE FF 16/10 column chromatography

The supernatant obtained from ultrafiltration stated previously was applied to DEAE FF 16/10 column equilibrated with 20 mM Tris-HCl buffer (pH 7.0) using AKTA Fast protein liquid chromatography (FPLC) System (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) with a linear gradient of 0–1.0 M NaCl in a total volume of 10 mL at a flow rate of 5 mL/min. The samples were centrifuged at 3000 *g* for 30 min at 4℃ as stated in Section 4.1. Fractions (5 mL) were collected and analyzed by 15% sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE).

4.2.2. Mono Q column chromatography

The fraction (5 mL) obtained from DEAE FF 16/10 column was applied to a 5 × 50 mm Mono Q column equilibrated with 20 mM Tris-HCl buffer (pH 7.0). The column was eluted with a linear gradient of 0–1.0 M NaCl in a total volume of 5 mL at a flow rate of 5 mL/min. The samples (5 mL) were centrifuged at 3000 *g* for 30 min at 4℃ as stated in Section 4.1 and then electrophoresed on 15% SDS-PAGE.

4.2.3. Superdex 75 gel filteration column chromatography

The fraction (1 mL) obtained from Mono Q column was applied to Superdex 75 column equilibrated with 0.15 M NaCl in 50 mM phosphate buffer (pH 7.0). Elution was carried out with the same buffer at a flow rate of 1.0 mL/min. The samples were

centrifuged at 3000 *g* for 30 min at 4℃ as stated in Section 4.1. Fractions (1 mL) were collected and analyzed by 15 % SDS-PAGE.

5. Polyacrylamide gel electrophoresis and Protein concentration determination

SDS-PAGE was performed according to the method of Laemmli (1970). Protein samples were loaded onto a 15 % polyacrylamide gel. Protein bands were observed after using Coomassie Brilliant Blue R-250 stain. The protein concentration was determined by a protein assay kit with BSA as a standard as described previously by Bradford et al. (1976). The protein was identified by Hybrid LC/MS/MS System (Applied Biosystems, Foster, CA, USA) as it is 94 % the same between purified and 57-kDa protein (RJ protein).

6. Measurement of Saos-2 cells protective effect and viability

UVB irradiation on Saos-2 cells was performed using UVM-225D Mineralight (UVP, Phoenix, AZ, USA) emitting wavelength of 302 nm. UV strength was measured using a HD2102-2 UV meter (Delta OHM, Padova, Italy). The cells were treated with 50 mJ/cm^2 of UVB for the *in vitro* experiments. Saos-2 cells were seeded onto a 96-well microplate at a concentration of 1×10^4 cell/well with 200 μL of fresh RPMI 1640 containing 10 % FBS and incubated for 24 h. Based on the preliminary test results, cells pretreated with 1.5 mg/mL RJ and 0.1 mg/mL 57-P were cultured for 24 h, followed by UVB irradiation. The cell viability of the test materials was determined based on the reduction of MTS to formazan according to the manufacturer's instruction. After removing the medium, 200 μL RPMI containing MTS was added to each well and incubated at 37℃ for 1 h. NAC (2 mM) served as a positive control. Absorbance was

read at 490 nm by using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA). The resulting sample was assayed at 490 nm by using a microplate fluorometer (Molecular Devices, Sunnyvale, CA, USA). All bioassays were repeated three times in duplicates.

7. Confocal microscopy

Saos-2 cells were seeded in confocal dishes (35 × 10mm) at a concentration of 1 × 10^5 cell/dish for 24 h. The culture medium was then removed, and the plates were washed three times with phosphate-buffered saline (PBS) (pH 7.4) and replaced with new serum free media containing 1.5 mg/mL RJ, 0.1 mg/mL 57-kDa, and 2 mM NAC. After 24 h, the medium was removed and washed with PBS three times. Cells were incubated for 45 min with 10 μM Mitotracker Red (excitation/emission spectra for Mitotracker Red is 578/598 nm) to detect mitochondria. The Ca^{2+} indicator dye Fluo-4/AM (excitation/emission maxima of 488/520 nm) was used to assess the cytoplasmic calcium concentration. Cells were stained by Fluo-4 AM and incubated at 37℃ for 45 min. They were then rinsed by PBS three times to remove any free dye to allow complete deesterification of AM ester. Ca^{2+} and mitocondria levels were measured from fluorescent living cell images using a Fluoview FV10i Confocal Laser Scanning Microscope (Olympus, Tokyo, Japan).

8. Alkaline phosphatase (ALP) activity test

The ALP activity of the test materials was evaluated as described previously by the manufacturer's instruction. Saos-2 cell was seeded onto a 96-well culture plate at a concentration of 1 × 10^4 cell/mL at 37 ℃ with 5 % CO_2 for 24 h. The medium was

removed and the plates were replaced with serum-free media containing 1.5 mg/mL RJ, 0.1 mg/mL 57-kDa, and 2 mM NAC and the cells were further incubated for 24 h. The culture medium was removed and washed with PBS three times. After 24 h incubation, ALP activity was measured using *p*NPP ALP assay kit at 405 nm.

9. Data analysis

Statistical analysis was carried out using GraphPad Prism 5 software (La Jolla, CA, USA). Data were expressed as mean ± standard mean error (SEM) from at least 3 independent experiments. Data from two groups were analyzed by a Student's *t*-test, and multiple groups were analyzed by a one-way analysis of variance and Bonferroni multiple comparison test.

RESULTS

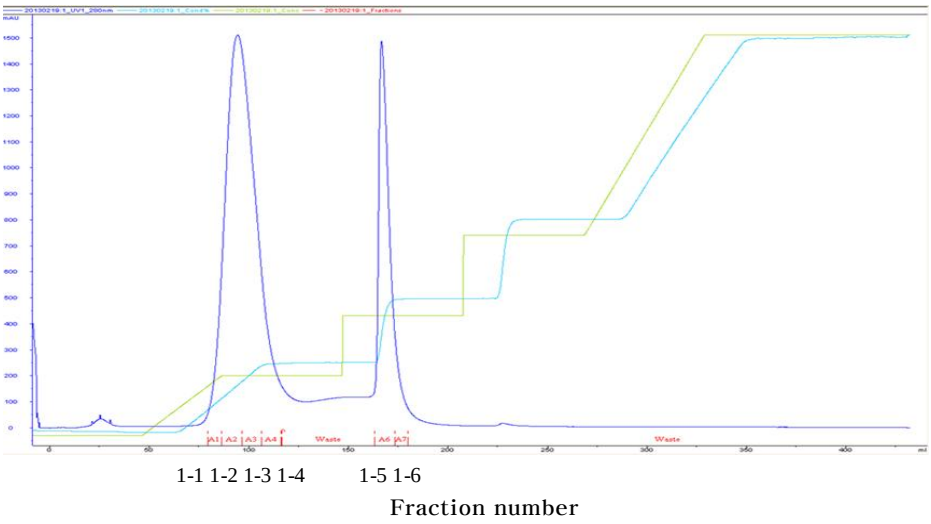
1. Purification of 57-kDa protein

As illustrated in Fig. 1A, the supernatant of the RJ extract was divided into six fractions by ion-exchange DEAE 16/10 column chromatography. These fractions were analyzed by SDS-PAGE, and 57-kDa protein band were found only in fractions 1-4, 1-5, and 1-6 (Fig. 1B).

Fractions 1-4 and 1-5 from DEAE 16/10 column chromatography was subjected to AKTA FPLC MONO Q ionic exchange as illustrated in Fig. 2A. Several protein peaks were eluted out. The SDS-PAGE analysis revealed that 57-kDa protein bands were found in fraction 2-2 (Fig. 2B).

Fraction 2-2 from AKTA FPLC MONO Q ionic exchange was subjected to Superdex 75 gel filtration (Fig. 3A). Fraction 3-3 was subjected to SDS-PAGE analysis and the peaks 3-3-1 and 3-3-2 were found to be homogeneous proteins with a molecular mass around 57-kDa as illustrated in Fig. 3B. It matched 94% sequence I.D between purified protein and original protein in Protein Bank by LC/MS/MS system (Fig. 4).

(A)



(B)

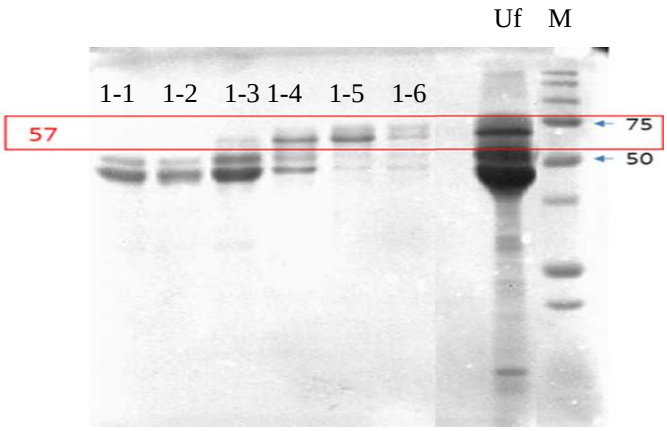
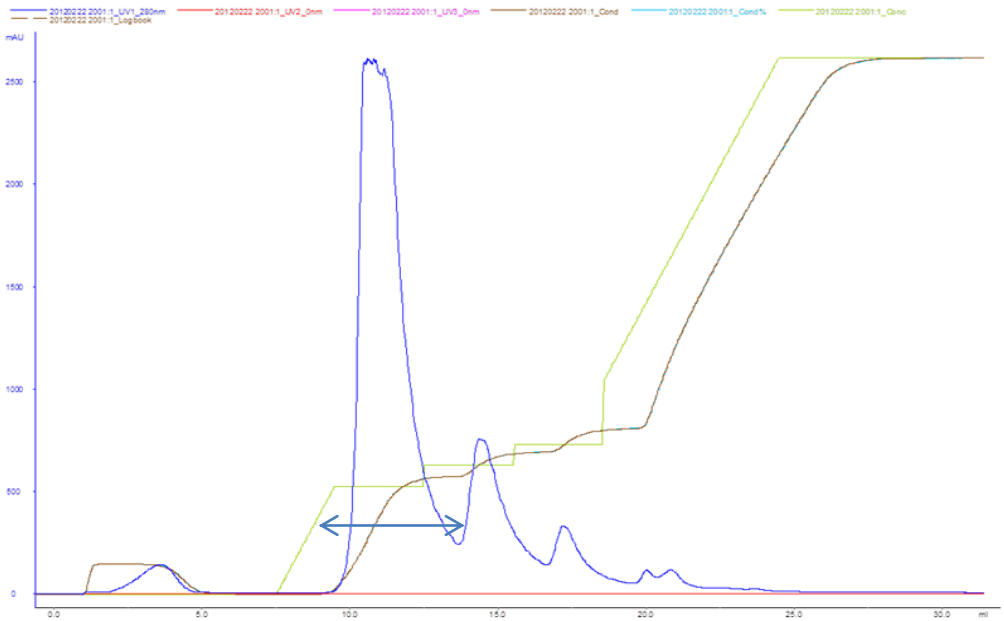


Fig. 1. Elution profile of 57-kDa protein on ion-exchange DEAE FF 16/10 column chromatography. (A) Each fraction obtained from ultrafiltration was subjected to DEAE FF 16/10 column. Six fractions (1–6) were collected, and absorbance was read at 280 nm. (B) SDS-PAGE profile. Lane U, fraction from ultrafiltration; Lanes 1-1–1-6 represent the fraction obtained from DEAE FF 16/10 column. The position of 57-kDa protein is indicated by red box.

(A)



Fraction number 2-1 2-2 2-3 2-4 2-5

(B)

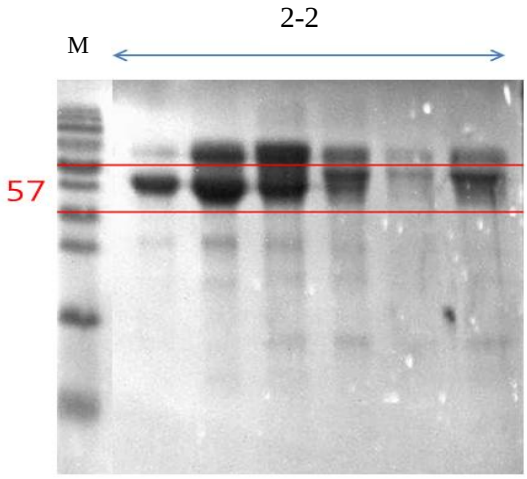
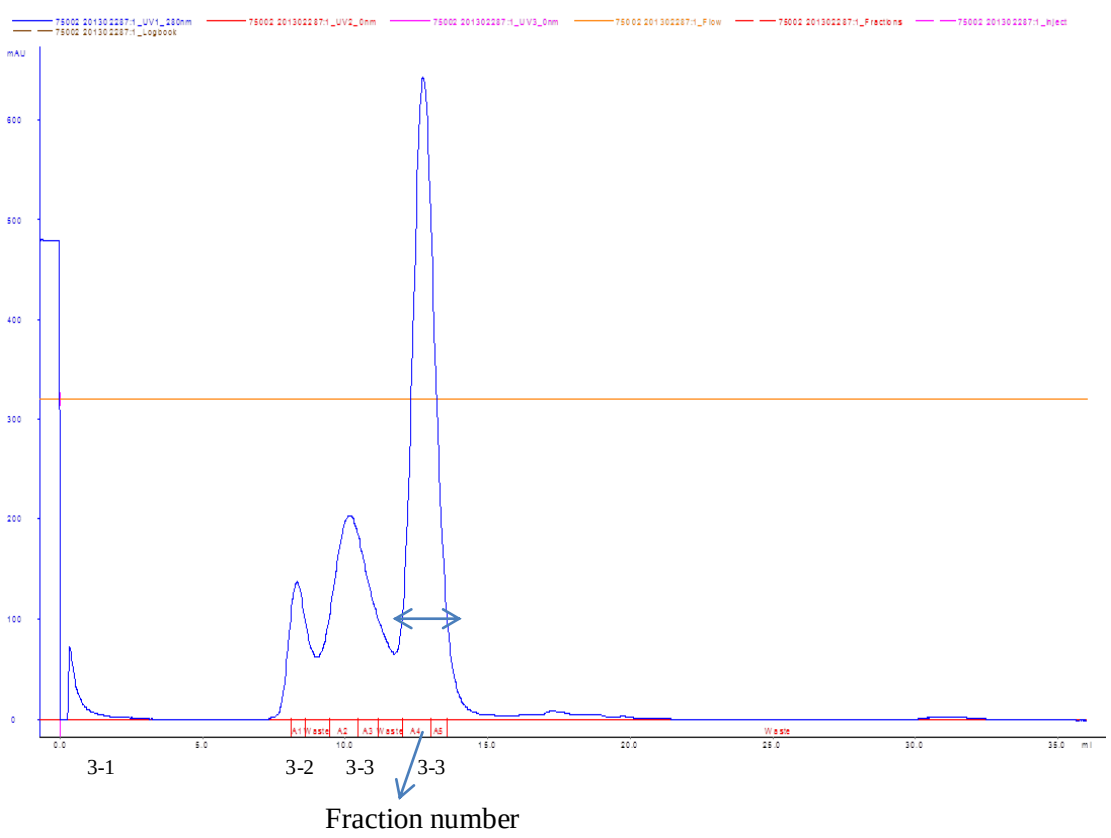


Fig. 2. Elution profile of 57-kDa protein on ion-exchange chromatography Mono Q. (A) Each fraction obtained from DEAE FF 16/10 column was subjected to Mono Q column. The fraction 2-2 was collected and absorbance was read at 280nm. (B) SDS PAGE profile of the fraction 2-2. The position of 57-kDa protein is indicated by red box.

(A)



(B)

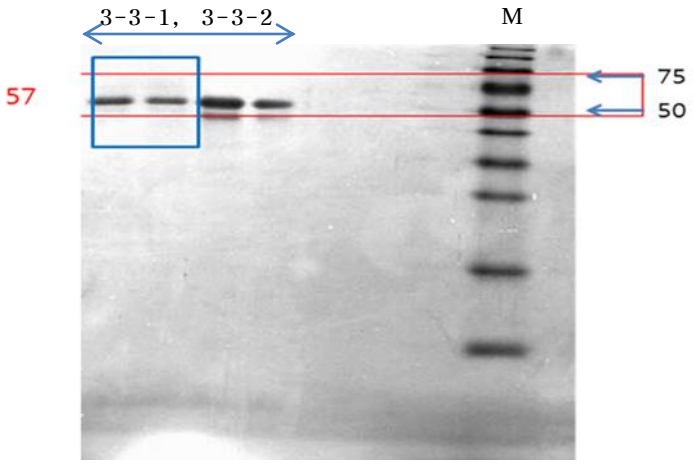


Fig. 3. Elution profile of 57-kDa protein on Superdex 75 gel filtration. (A) The fraction from Mono Q column was subjected to Superdex 75 gel filtration. Four fractions were collected and absorbance was read at 280 nm. (B) SDS-PAGE profile of each fraction. The position of 57-kDa protein is indicated by red box.

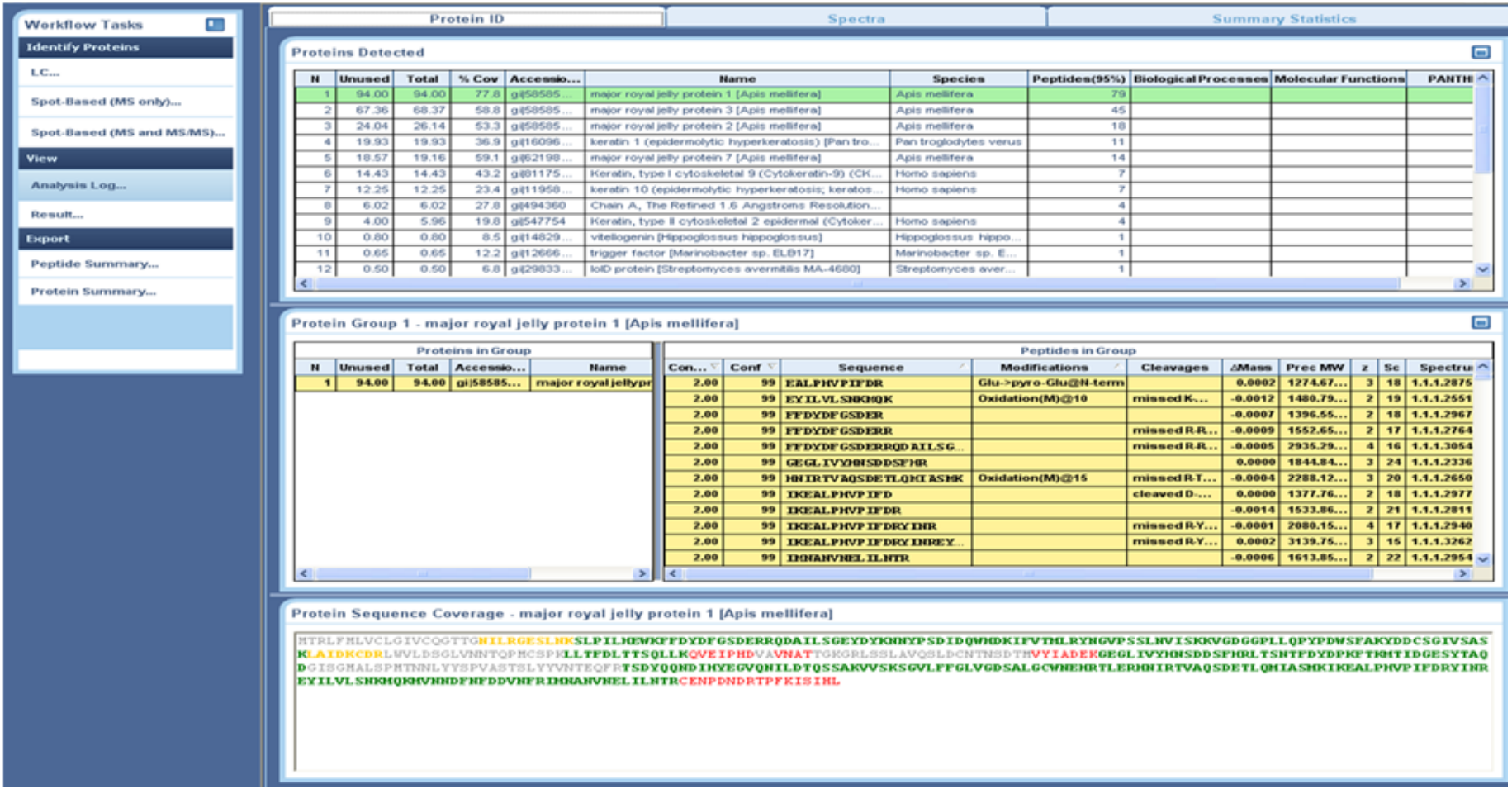


Fig.4. Protein identification by hybrid LC/MS/MS system. Protein from Superdex 75 was identified by hybrid LC/MS/MS system. The results indicates that 94% similarity was seen to purified and 57-kDa protein (RJ protein).

2. Effect of 57-kDa protein from royal jelly on Saos-2 cell viability

The concentration-dependent effects of purified 57-kDa protein derived from RJ on Saos-2 cell viability were evaluated using a MTS assay (Fig. 5). The 57-kDa protein at concentrations between 10 and 100 μg/mL exhibited a high correlation between protein concentration and cell viability (correlation coefficient (R^2) > 0.85).

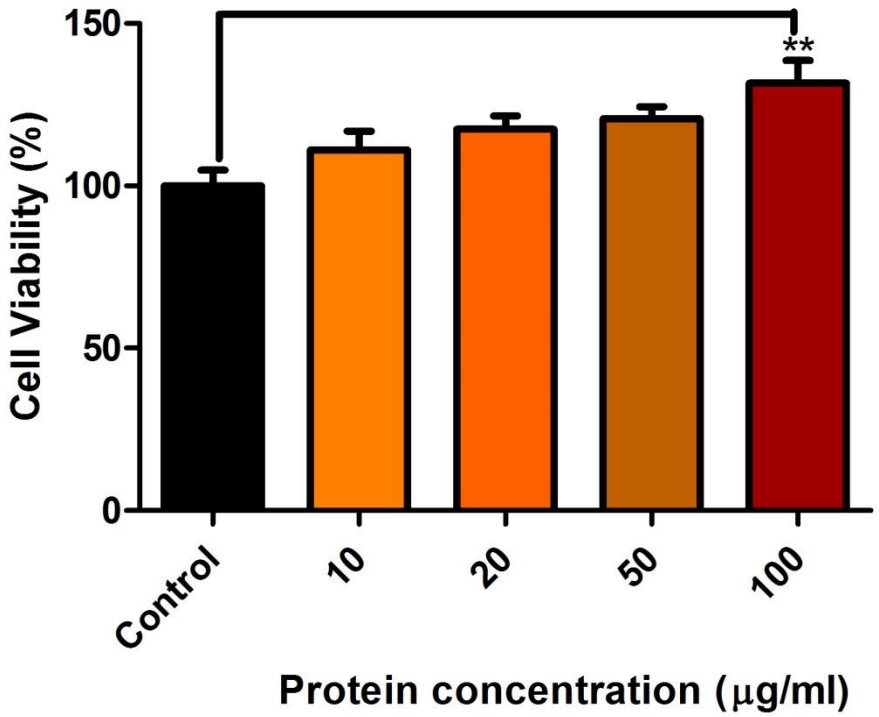


Fig. 5. Concentration-dependent effect of 57-kDa protein on Saos-2 cell viability. The 57-kDa proteins were added to culture medium at various concentrations (10, 20, 50, and 100 μg/mL) and cell viability was measured at 490 nm using a MTS assay. The data are expressed as a percentage of control. Each bar represents the mean ± SEM of triplicate samples of three independent experiments. ** Significant at $P < 0.01$, according to a Student's *t*-test.

3. Effect on viability of UVB irradiated Saos-2 cells

Cell viability of Saos-2 cells following UVB irradiation either with or without the test material was likewise examined (Fig. 6). When pretreated with 0.1 mg/mL 57-kDa protein and 2 mM NAC 24 h prior irradiation, Saos-2 cells were protected 79 and 77% by the protein and NAC treatments, respectively, whereas 1.5 mg/mL RJ showed no statistically significant protection from radiation induced cell death.

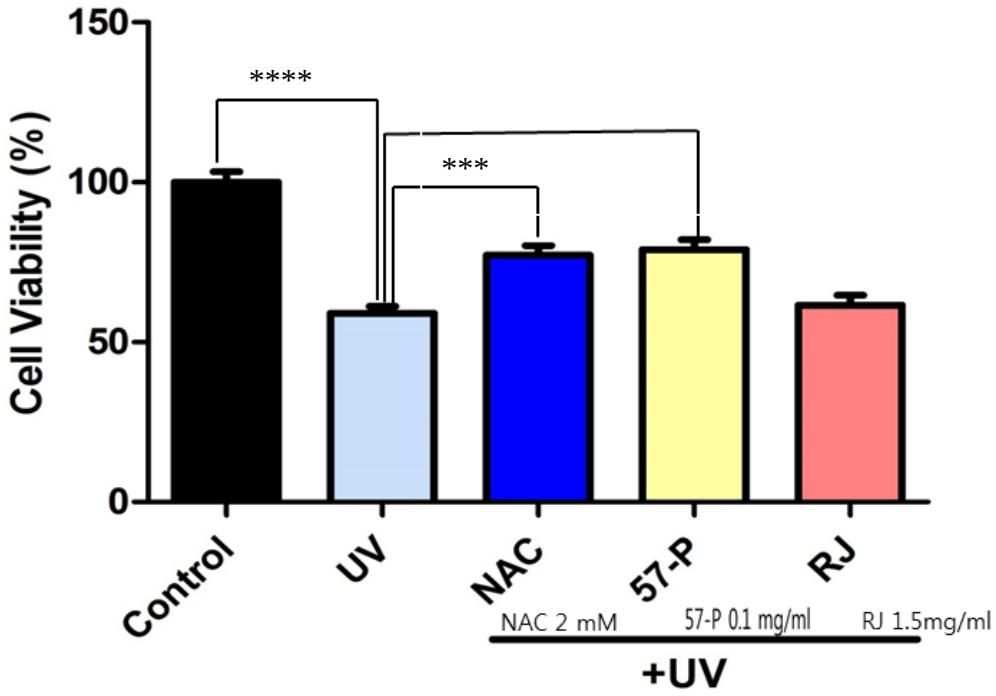


Fig. 6. Cell viability of Saos-2 cells following UVB irradiation either with or without the test materials. Saos-2 cells were cultured at 1×10^4 cells/well. Plates were irradiated with UVB 50 mJ/cm^2, the Saos-2 was killed at level of 40.91%. The 1.5 mg/mL RJ, 0.1 mg/mL 57-kDa protein, and 2 mM NAC were added to each well, and their viability was measured by MTS assay at 24h. Each bar represents the mean ± SEM of duplicate samples of three independent experiments. *** Significant at $P < 0.001$, according to a Student's *t*-test.

4. Cytoprotective effect on UVB irradiated Saos-2 cells

The cytoprotective effect of 57-kDa protein on Ca^{2+} and mitochondria was assessed using confocal microscopy. The confocal microscopic image showed that the control group had the highest density and strong red florescence and distributed green florescence in 10× magnification (Fig. 7). However, 0.1 mg/mL 57-kDa protein, 1.5 mg/mL RJ, and 2 mM NAC treated groups showed more proliferation and Ca^{2+} distribution and mitochondria activity than only UVB treated group even if they had lower density and florescence than control. The 60× magnification images demonstrated that treated groups still maintained their cell morphology compared to untreated group (Fig. 8).

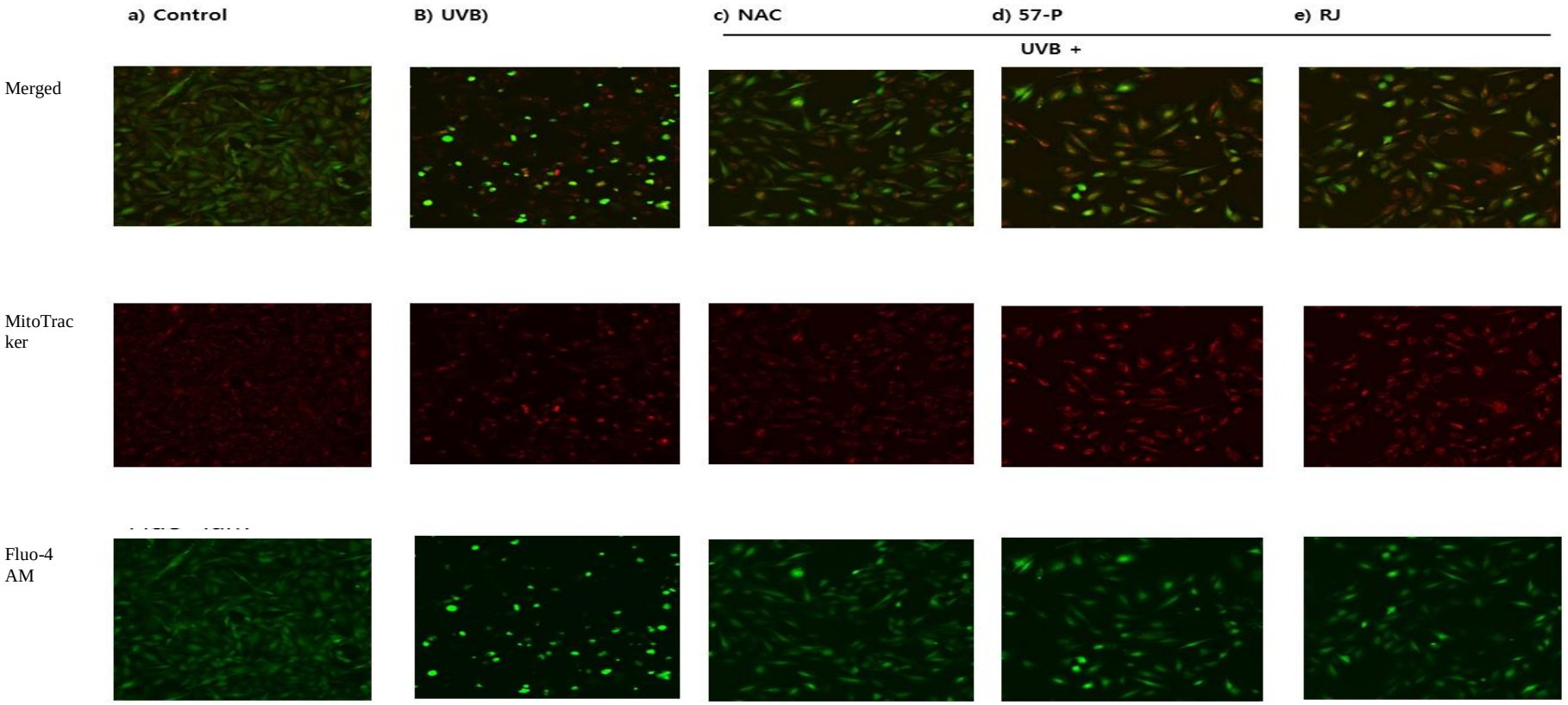


Fig. 7. Cytoprotective effect of 57-kDa protein on UVB stress using two different dyes (10×). Cells were seeded 1×10^5 in confocal dishes for 24 h and then washed with phosphate-buffered saline (PBS) three times for treating with 57-k Da protein, royal jelly (RJ) and NAC in no supplemented media. After another 24 h, cells were stained by Fluo-4 AM (Ca^{2+}sensitive dye) and MitoTracker (excitation/emission spectra for Mitotracker Red is 578/598 nm) and incubated about 45 min at 37℃. They were then rinsed by PBS three times to remove any free dye to allow complete deesterification of AM ester (excitation, 485 nm; emission, 512 nm). Ca^{2+} and mitocondria levels on cell damage induced by UVB measured from fluorescence live cell images using a Fluoview FV10i Confocal Microscope. The confocal microscopic image showed that the control group had the highest density and strong red florescence and distributed green florescence.

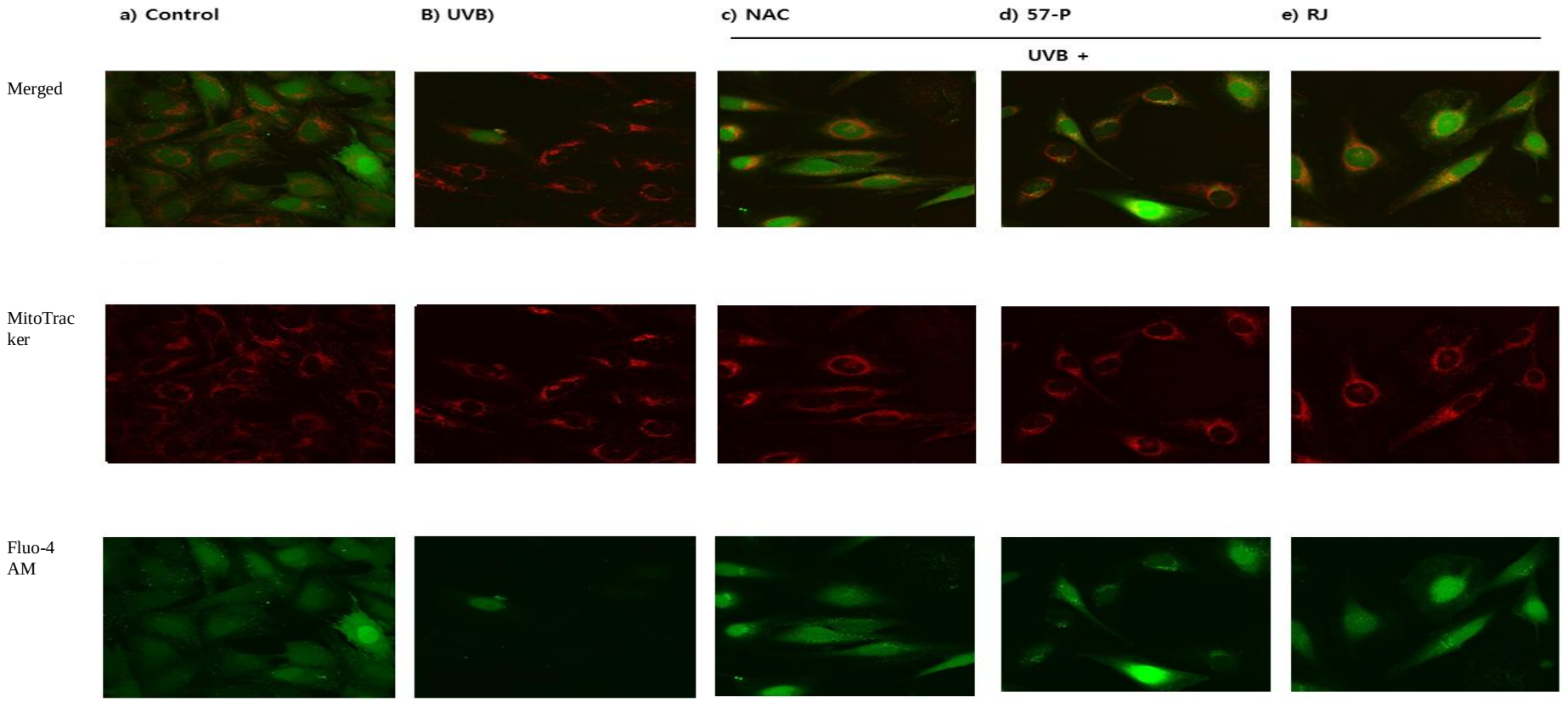


Fig. 8. Cytoprotective effect of 57-kDa on UVB stress using two different dyes (60×). Cells were seeded 1×10^5 in confocal dishes for 24 h and then washed with phosphate-buffered saline (PBS) three times for treating with 57-kDa protein, royal jelly (RJ) and NAC in no supplemented media. After another 24 h, cells were stained by Fluo-4 AM (Ca^{2+}sensitive dye) and MitoTracker (excitation/emission spectra for Mitotracker Red is 578/598 nm) and incubated about 45 min at 37℃. They were then rinsed by PBS three times to remove any free dye to allow complete deesterification of AM ester (excitation, 485 nm; emission, 512 nm). Ca^{2+} and mitocondria levels on cell damage induced by UVB measured from fluorescent live cell images using a Fluoview FV10i Confocal Microscope. These images demonstrated that treated groups still maintained their cell morphology compared to untreated group.

5. Effect on alkaline phosphatase activity of UVB irradiated Saos-2 cells

The ALP activity of Saos-2 cells following UVB irradiation either with or without the test material was examined using a MTS assay (Fig. 6). Pretreatment of Saos-2 cells with 0.1 mg/mL 57-kDa protein 24 h prior irradiation significantly attenuated the increase in ALP activity from radiation induced cells. However, 1.5 mg/mL RJ and 2 mM NAC exhibited no statistically significant increase in ALP activity.

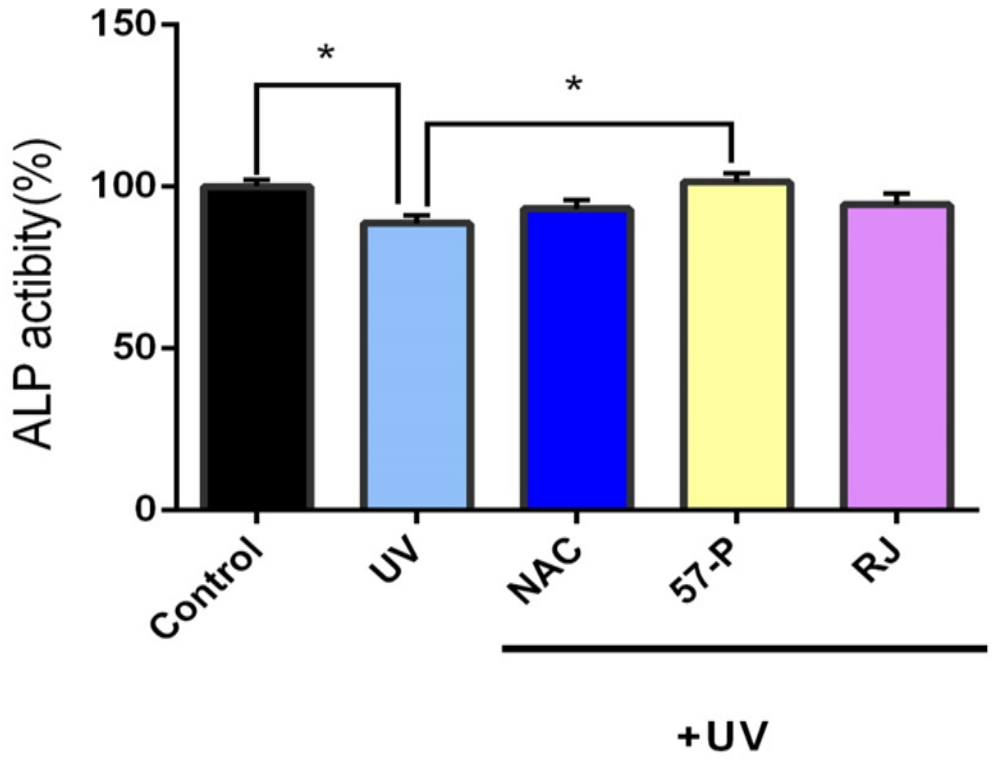


Fig. 9. Alkaline phosphatase (ALP) activity of Saos-2 cells following UVB irradiation either with or without the test materials. Saos-2 cells were cultured at 1×10^4 cells/well. Plates were irradiated with UVB 50 mJ/cm^2. The 1.5 mg/mL RJ, 0.1 mg/mL 57-kDa protein, and 2 mM NAC were added to each well, and their ALP activity was measured using *p*NPP ALP assay kit at 405 nm at 24h. Pretreatment of Saos-2 cells with 0.1 mg/mL 57-kDa protein significantly attenuated the increase in ALP activity from radiation induced cells. Each bar represents the mean ± SEM of duplicate samples of three independent experiments. * Significant at $P < 0.05$, according to a Student's *t*-test.

DISCUSSION

RJ is secreted material from mandibular and the hypopharyngeal glands on the head of worker bees and is food for all bee larvae. It is a famous nutritional material for queen differentiation of honey bee larvae as well as human health. RJ possesses metabolic activity, antioxidant, anti-aging properties, estrogenic effects, anti-cancer and antibacterial activity (Crenguța I. P et al., 2011). Recently, it has been reported that a certain component of the RJ causes to fully develop a bee into a queen. It appears a single monomeric protein that has been called the 57-kDa protein because of its molecular weight or it is named royalactin, on the basis that it is driven from RJ and activates not only rat hepatocytes but also is important for becoming a queen honey bee (Masaki K et al., 2001).

Osteoblasts are cells which originate in the bone marrow and contribute to the production of new bone. These cells build up the matrix of the bone structure and also play a role in the mineralization of the bone matrix. Bone is constantly being built up and broken down by the body, making osteoblasts rather critical. The counterpart to the osteoblast is the osteoclast, a cell which is responsible for breaking down bone. As people get older, their production of osteoblasts decreases. Bone health can also be influenced by the amount of available calcium in the diet, as osteoblasts need calcium to work with in the process of building up bone (Osteoblast, wikipedia). Nevertheless, the 57-kDa protein effects are still unknown on human cells. In this study, treatment with the 57-kDa protein at 100 µg/mL resulted in significant cell viability than control group. The 57-kDa protein does not have any toxicity against Saos-2 human osteoblast cell line.

Ultraviolet B (UVB) radiation acts as a strong apoptotic trigger in many cell types, in tumor and normal cells. Several studies have demonstrated that UVB-induced cell death occurs through the generation of reactive oxygen species (Sara Salucci et al., 2013).The consequent oxidative stress includes the impairment of cellular antioxidants, the induction of DNA damage and the occurrence of apoptosis. (Sara Salucci et al., 2013) Therefore, UVB irradiation is known as a potent inducer of ROS formation as a tool to assess ROS-induced damage (Amaral et al., 2013). Ca^{2+} ions are important molecules and play physiological roles for calcium signaling range widely. Calcium ions enter the cytoplasm and exert allosteric regulatory effects on many enzymes and proteins. In addition, calcium ions can play a predominant role as an activator of signal transduction in ion channels or as a second messenger caused by indirect signal transduction pathways such as G protein-coupled receptors. These include muscle contraction, neuronal transmission as in an excitatory synapse, cellular motility (including the movement of flagella and cilia), fertilization, cell growth or proliferation, learning and memory as with synaptic plasticity, and secretion of saliva (Berridge et al., 2000). Other biochemical roles of calcium include regulating enzyme activity, permeability of ion channels, activity of ion pumps, and components of the cytoskeleton (Koolman et al., 2005). Osteoblast extracellular Ca^{2+}-sensing receptor regulates bone development, mineralization, and turnover (Dvorak-Ewell et al., 2011,). The most important roles of mitochondria are to generate the cell energy, ATP (i.e., phosphorylation of ADP), through respiration, and to regulate cellular metabolism (Ivannikov et al., 2013). In addition to supplying cellular energy, mitochondria are involved in other tasks such as signaling, cellular differentiation, cell death, as well as the control of the cell cycle and cell growth (McBride HM et al., 2006). UVB occur oxidative damage to the cell.

Consequently, Normal oxidative damage to mitochondrial and nuclear DNA is extensive (Richter C et al., 1988).

In this study, the cytoprotective effects of pretreatment with RJ, 57-kDa protein, and NAC on Saos-2 cells damaged by UVB were evaluated using a MTS assay and confocal microscopy. The 57-kDa protein significantly enhanced cell viability by increasing ALP activity and prolonged cell survival in UVB irradiated condition (57-kDa : 79 and NAC : 77% protection).. In addition, the living cell observation revealed that the cells treated with 57-kDa protein showed normal cell shapes and organelles being maintained, similar to normal cells. These results indicate that the 57-kDa protein may affect the extracellular Ca^{2+}-sensing receptors and mitochondria activity in living osteoblast. Therefore, the 57-kDa protein might improve cell protective ability and viability or specific functions in osteoblasts. This original finding indicates that RJ and 57-kDa protein can hold promise as novel and effective anti-UV products.

In conclusion, 57-kDa protein can be useful for the bone formation of osteoblasts and for protection from UV induced stress. For practical use of RJ -derived materials as novel anti-UV induced stress products to proceed, further research is needed to establish their human safety and whether this activity could be exerted *in vivo* after consumption of the product by humans. In addition, their exact action mechanisms related to bone formation and anti-UV induced stress of action need to be established and detailed tests are needed to understand how to improve UV induced stress and stability for eventual commercial development.

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국산 로얄젤리(royal jelly)에서 분리한 57-kDa 단백질의 인간유래조골세포 활성에 미치는 영향

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최 새움

초 록

로얄젤리는 꿀벌 성충이 꽃가루와 꿀을 흡수시켜서 머리의 인두선에서 분비하는 물질로서, 꿀벌 유충을 여왕벌로 분화시키는 모든 핵심요소들을 포함하고 있다. 로얄젤리의 57-kDa 단백질이 여왕벌 분화의 핵심요소이며 이는 곤충세포의 상피세포 수용체를 자극하여 여왕벌의 몸을 크게 하고 생식기관을 발달시킨다. 그러나, 항암, 항바이러스, 항산화 같은 로얄젤리 효능에 대한 연구는 매우 광범위하고 로얄젤리의 생리적으로 뛰어난 효과는 이미 알려져 있지만 57-kDa 단백질이 여왕벌의 분화에 핵심역할을 하는 물질임이 알려졌음에도 아직 이 단백질이 인체 유래 세포에 미치는 영향에 대해서는 아직 많이 알려져 있지 않다.

본 연구는 로얄젤리가 쥐 유래 조골세포의 뼈 형성능력을 증가시키며, 57-kDa 단백질이 쥐 유래 간세포의 활성에 도움을 준다는 연구결과를 바탕으로, 57-kDa 단백질이 인간 유래 조골세포 (Saos-2 Cell line)에 미치는 영향을 조사하였다. 국산 로얄젤리에서 크로마토그라피 및 겔 여과법을 이용하여 57-kDa 단백질만을 분리

정제한 다음 이 단백질이 조골세포의 활성에 영향을 미치는 적정 농도를 실험한 결과, 100 μg/mL 농도에서 대조군에 비해 통계적으로 유의한 세포활성을 나타내었다. 또한, 57-kDa 단백질이 쥐 간세포의 수명과 세포활성, 알부민의 생산을 촉진시킨다는 연구결과를 바탕으로 이 단백질이 조골세포를 보호하는 효과를 조사하였다. 57-kDa 단백질을 처리한 세포들을 자외선으로 손상시킨 뒤, 자외선 조사로 촉발된 산화제를 소거하는 역할로 잘 알려진 화합물 NAC 와 비교하였을 때, 통계적으로 유의한 세포 보호 효과를 보였다 (79%) (UVB; 59.09%, NAC 2 mM; 77.16%, RJ 1.5 mg/ml; 61.57%). 그리고, 살아있는 세포의 칼슘 sensing 리셉터와 미토콘드리아를 형광 염색하여 오로지 자외선만 조사한 세포 그룹과 비교하였을 경우, 57-kDa 단백질을 처리한 세포들은, 염색된 세포 소기관이나 세포 형태가 정상세포와 비슷하게 유지되고 있음을 관찰하였다. 또한, 뼈 분화에 중요한 염기성 인산분해효소도 0.1 mg/mL 57-kDa 단백질 처리 그룹이 다른 그룹보다 현저하게 증가하였다 (RJ 1.5 mg/ml; 94.44%, NAC 2 mM; 93.18%, UVB; 88.74%).

이상의 결과를 바탕으로, 본 논문은 인체 유래한 세포에 대해 잘 알려지지 않은 57-kDa 단백질의 활성을 연구했다는 것에 그 의의가 있고, 57-kDa 단백질에 대한 분리 정제와 그 분리 정제된 단백질이 인간유래 조골세포에 작용하는 세포 보호 효과를 밝힘으로서 이 단백질의 활용 가능성을 보여주었다. 그러나 57-kDa 단백질의 자극으로 인해 세포 안에서 일어나는 여러 작용 메카니즘에 대한 분자생물학적인 보다 상세한 측정과 동물모델을 이용한 생물검정이 요구된다.

검색어 : 로얄 젤리, 조골세포, 57-kDa 단백질.

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A THESIS FOR THE DEGREEOF MASTER OF SCIENCE

The Effects of The 57-kDa Protein Isolated from Korean Royal jelly on the Activity of Human-derived Osteoblast

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APPROVED AS A QUALIFIED THESIS OF SAEWOOM CHOI

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The Effects of The 57-kDa Protein Isolated from Korean Royal jelly on the Activity of Human-derived Osteoblast

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ABSTRACT

Royal jelly (RJ) is a secretion product of the cephalic glands of nurse bees that has been used for centuries for its extraordinary properties and health effects. Pharmacological actions of RJ such as antitumor, antiviral, and antioxidant activity have been well noted. RJ and its 57-kDa protein were also reported to enhance the bone-forming ability of osteoblasts and the production of albumin and to have protective effect on rat liver cells. However, the impact of RJ -derived materials, particularly 57-kDa protein, on Saos-2, a human osteoblast-like cell line, is not yet fully understood. Also, no information has been done to consider potential use of RJ -derived materials to manage ultra violet (UV) induced damages. In this study, the effects of RJ and its 57-kDa protein on Saos-2 and the cytoprotective activity of the materials toward Saos-2 cells damaged by UV were evaluated. The cytoprotective activity of the materials was

compared with that of *N*-acetyl-L-cysteine (NAC), a currently used antioxidant, for use as future commercial products with cytoprotective action using a MTS assay. Firstly, 57-kDa protein was purified from domestic RJ using fast protein liquid chromatography and gel filtration method. The 57-kDa protein at concentrations between 10 and 100 μg/mL exhibited a high correlation between protein concentration and cell viability (correlation coefficient (R^2) > 0.85). Pretreatment with 0.1 mg/mL 57-kDa protein and 2 mM NAC to Saos-2 cells resulted in 79 and 77% protection against UVB, respectively, whereas 1.5 mg/mL RJ showed no statistically significant protection from radiation induced cell death.

Osteoblast extracellular Ca^{2+}-sensing receptor regulates bone development, mineralization, and turnover (Dvorak-Ewell MM et al. 2011). The mitochondria are to generate the cell energy, ATP (i.e., phosphorylation of ADP), and Changes in alkaline phosphatase level and activity are involved in a variety of physiological and pathological events, such as bone development. The effects of 57-kDa protein on the calcium sensing receptor in live cells and mitochondria were compared with cell groups pretreated with UV using confocal microscopy. The microscopic observation revealed that the cells treated with 57-kDa protein showed normal cell shapes and organelles being maintained, similar to normal cells. Pretreatment of Saos-2 cells with 0.1 mg/mL 57-kDa protein significantly resulted in the increase in ALP activity from radiation induced cells. However, 1.5 mg/mL RJ and 2 mM NAC exhibited no statistically significant increase in ALP activity.

In conclusion, 57-kDa protein can be useful for the bone formation of osteoblasts and for protection from UV. For practical use of RJ and 57-kDa protein as novel anti- UV induced stress products to proceed, further research is needed to establish their human

safety and whether this activity could be exerted *in vivo* after consumption of the product by humans. In addition, their anti-UV induced stress modes of action need to be established and detailed tests are needed to understand how to improve anti-UV induced stress potency and stability for eventual commercial development.

Key word: Royal jelly, 57-kDa royal jelly protein, ultra violet irradiation, osteoblasts, protective effect, human osteoblast-like cell line, Saos-2 cell line

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INTRODUCTION

Bone mass consists of extracellular substances such as collagen and glycoprotein and numerous bone cell family such as osteoblasts, osteoclasts and osteopontin (Mikihiko et al., 2003). Osteoblasts are responsible for a matrix of osteoid which is composed mainly of Type I collagen and linked to mineralization of this matrix. This process needs some of the minerals such as zinc, copper, and sodium. Bone is a dynamic tissue that is being constantly reshaped by osteoblasts and osteoclasts. Osteoblasts are in charge of matrix and mineral production and osteoclasts break down the tissue. With ageing, the number of osteoblasts tends to decrease, affecting the balance of bone formation and absorption in the bone tissue (Salentijn, 2007) and potentially cause osteoporosis. For treatment of osteoporosis, some drugs such as vitamin D and calcium are commercially available and recommended although there however is an increased risk of myocardial infarctions (Bolland, 2010) and kidney stones (Virginia et al., 2013). Therefore, natural drugs with lesser side effects which can be an alternative of synthetic drugs have been substantially studied.

Exposure of human cells to UV leads to damage of cellular constituents and results in complicated cell responses, such as induction of genes and disturbance of various signaling pathways (Bender et al., 1997; Schwarz, 1998). The most serious effects of UVB are caused by the indirect DNA damage (free radicals and oxidative stress). In humans, prolonged exposure to solar UV radiation results in acute and chronic health effects on the skin, eye, and immune system (Lucas et al., 2006). Moreover, UVC can cause mutagenic or carcinogenic (Hogan, 2010).

Historically, RJ has long been used for conditions such as rheumatoid arthritis, hypertension, diabetes, chronic hepatitis, duodenal ulcer, menstrual problems, uterine bleeding and infertility, fatigue, weakness, poor nutrition, leucopenia, and cancer (Batchelder, 2002). No information has been done to consider potential use of RJ to manage UV induced damages, although excellent pharmacological actions of RJ have been well described by Crenguța I. P et al. (2011).

In this study, an assessment is made of the cytoprotective activity of the 57-kDa protein that comprises RJ toward Saos-2 cells damaged by UV using a MTS assay. The cytoprotective activity of the 57-kDa protein was compared with that of *N*-acetyl-L-cysteine (NAC), a currently used antioxidant, for use as future commercial products with cytoprotective action (Jun JH et al., 2008). Alkaline phosphatase (ALP) activity of 57-kDa protein-treated Saos-2 cells damaged by UV was examined using a *p*NPP ALP assay because changes in ALP level and activity are associated with various disease states in the liver and bone (Coates, 2013). In addition, physiological effects such as Ca^{2+} distribution and mitochondria activity were also investigated using confocal laser scanning microscopy because extracellular Ca^{2+} sensing receptor in osteoblast regulates bone development, mineralization, and turnover (Dvorak-Ewell et al., 2011). Mitochondria also are the key organelle that generate the cell energy and regulate cellular metabolism in live cells.

LITERATURE REVIEW

1. Royal jelly

Royal jelly (RJ) is secreted material of the bee for nursing bee larvae (Hoffmann, 1966). It is secreted from the glands in the mandibular or hypopharynx which is the head part of young worker bees (Graham, 1992). RJ is available to feeding all the larvae including the larvae group that will become workers. If honey bees need a queen bee, RJ is supplied bulk but only to the selected larvae during the first four days after birth. So after early intensive nutrition, the selected larvae which have mature ovaries enough to lay eggs and become a type of queen Bee. In fact, all the larvae eat RJ but imagos will not eat it except the queen. Only the queen bee feed RJ during her whole lifetime that is 40 times more than workers.

General composition of RJ is as follows: water (67%), proteins (13%), sugars (11%), fructose (6%), glucose (4%), and fatty acids (5%) which is shorter chains than usual for insects 8–10 carbons, minerals (Ca, Cu, Fe, Mg, Mn, Na, K, Zn, and Si) (1%), four phospholipids, and five glycolipids which supply energy (Graham, 1992). RJ also contains many vitamins (A, B, C, D, E, and K), minerals, sterols (methy-lenecholesterol (ca 0.3%), cholesterol, stigmastanol, stigmasterol, and testosterone), 7–9 different sterols (sitosterol, cortisol, and cholesterol), acetylcholine, amino acids (total 1.59%) (proline, lysine, glutamic acid, serine, alanine, arginine, aspartic acid, glycine, isoleucine, leucine, methionine, tyrosine, valine, glutamine, and taurine), and other nutrients. (Mohamed Fawzy R et al., 2012). Among amino acids, proline is the major amino acid (54–60%), followed by lysine and glutamic acid. Recently, the hydroxy fatty acids were reported. They include 10-hydroxy-*trans*-2-decenoic acid (10-HAD) (royal

jelly acid). 10-hydroxydecanoic acid (0.60–1.25%), 10-acetoxydecanoic acid, 11-*S*-hydroxydodecanoic acid, hydroxy-2*E*-decenoic acid 10-phosphate, and other acids (gluconic acid, sebacic acid, 2-decenedioic acid, *p*-hydroxybenzoic acid, 3-hydroxydecanoic, 8-hydroxydecanoic, and 3,10-dihydroxydecanoic acids) (Lavinia, 2011). The RJ proteins such as the major royal jelly protein family and some other previously identified proteins, peptides, glycoproteins, and biopterin are also included (Mohamed Fawzy R et al., 2011). Gamma-globulin, gelatin, adenosine monophosphate, and adenosine monophosphate N1-oxide were recently found from RJ (Mohamed Fawzy R et al., 2011). As the major active component of RJ, extensively recognized 10-HDA has been reported to be quite stable to heat, retaining chemically not damage in spite of deterioration of other components (Ramadana and Al-Ghamd, 2012). RJ itself is also very stable when refrigerated or in the frozen or dried state (Masaki K et al., 2001).

2. Biological activities of RJ

2.1. Cardioprotective action

The cardioprotective action is caused by RJ capacity to stimulate an increased secretion of adrenaline via acetylcholine (Vasily et al., 2006). RJ can reduce blood sugar level via insulin-like peptides and other compounds such as chromium, sulphur, and vitamins B3 and H. RJ is also capable to sustain the optimal blood level of sugars by taking part in the oxidation of glucose to obtain energy, through the insulinic effect of insulin-like peptides found in it (Batchelder, 2002).

2.2. Antioxidant and anti-aging actions

RJ has antioxidant and anti-aging abilities (Nagai T et al., 2006). Reportedly, RJ has significant effects on the hypothalamic-pituitary axis (Yukio et al., 2009). Hypothalamic-pituitary functioning generally decreases in aging. It can supply for age-associate reducing pituitary functions in rats (Yukio et al., 2009). It alleviates intra-cellular oxidation by acting as a scavenger of reactive oxygen species. RJ also has an effect on protein expression (Inoue et al., 2003). Peptides isolated from hydrolysates of water-soluble RJ proteins with protease P show significantly stronger hydroxyl radical-scavenging effects and antioxidant activity against lipid peroxidation. These results suggest that RJ peptides (RJPx) may inhibit LPO both *in vitro* and *in vivo* and helps prevent cell damage (Guo et al., 2003). Ames et al. (1993) studied the antioxidative and scavenging activities of enzymatic hydrolysates of RJ using three enzymes pepsin, trypsin, and papain. They reported that the antioxidative and scavenging activities of the enzymatic hydrolysates against active oxygen species such as superoxide anion radical and hydroxyl radical of each hydrolysate were strong in RJ.

Royal jelly has the basic components of collagen production against aging and affects the TGF-beta 1 (transforming growth factor-beta) which is an essential factor for collagen production (Koya-Miyata et al., 2004). A collagen production promoting factor from an extract of RJ and 10-hydroxy-2-decenoic acid isolated from RJ are identified as the factor which enhances a fibroblast cell line to produce TGF-beta 1 (Koya-Miyata et al., 2004). RJ treatment significantly promots transfer of fibroblasts in a dose-dependent manner at 8 hr. Among various lipid classes of fibroblasts, the levels of triglycerol and cholesterol are considerably decreased with 5 μg/mL RJ (Kim et al., 2010). The tympanic membranes of the guinea pigs showed marked fibroblastic orientation and

well-organized connective tissue after 3 months when royal jelly was supplied topically to the membranes after surgical operation (Kim et al., 2010). Application of RJ also promotes the success rate of closure compared to placebo (Calli et al., 2008).

2.3. Glucose metabolism

In humans, RJ ingestion is found to involve in the glucose metabolism. Twenty volunteers were tested the standardized oralglucose tolerance test (OGTT) and next, a second tested OGTT after ingestion of 20 g RJ. Serum glucose levels after 2 hrs and the area under the curve for glucose were considerably lower after RJ administration (Munstedt, 2009). Nomura et al. (2007) studied the effects of oral administration of royal jelly (10, 30, and 300 mg/kg, four weeks) on insulin resistance in 10-week-old rats, a type 2 diabetic model. They reported that RJ administration decreased systolic blood pressure and highly reduced serum levels of insulin and the HMA ratio, an index of insulin resistance. These results suggest that RJ could be an effective and functional food to prevent the development of insulin resistance as described previously by Zamami Y (2008).

2.4. Cholesterol management

The addition of RJ to the daily diet increases the HDL cholesterol, particularly in older patients (Münstedt, 2009). In humans, 50–100 milligrams dry weight of RJ per day reduced total serum cholesterol by 14% and lipids by 10%. The patients were treated with RJ by mouth and by injection but the results were quite similar for both delivery techniques (Vittek, 1995). The serum total cholesterol (TC) and serum low-

density lipoprotein (LDL) in the RJ group (n = 7, 6 g per day for 4 weeks) reduced considerably compared to other the control group ($P < 0.05$) (Guo, 2007).

2.5. Estrogenic effects

Fatty acids from RJ have many benefits toward autonomic imbalance, perimenopausal symptoms, osteoporosis, and other serious conditions. These abilities may attribute to the interaction of the fatty acids of RJ with an estrogen receptor in the human cells (Toshiaki et al., 2008). At concentrations of 10–100 µg/mL RJ exhibited a quiet low but significant estrogenic activity via estrogen receptor α (ERα), whereas it acted as a complete estrogen antagonist via estrogen receptor β (ERβ) at a concentration range between 50 and 200 µg/mL. These results suggest that RJ methanol extract may be a feasible natural modulator of estrogen signaling interaction with the ERα and ERβ subtypes (Tsiapara et al., 2009). It has been also reported that RJ competes with 17-β estradiol to bind to the human estrogen receptors such as α, β and offers that RJ has estrogenic activity through mediation with estrogen receptors by endogenous gene expressions (Mishima et al., 2005). Hale (1939) reported documented that RJ retained hormones that promote growth of ovarian activity in the white rat for a few days and an increase in fertility. Heyl (1939) injected RJ directly into female rat and found that it stimulated the ovarian growth in the same manner.

2.6. Bone formation

RJ as a whole or some of its properties enhances production of type I collagen and other activities for bone formation by action on osteoblasts through upregulation of procollagen I alpha1 gene expression (Narita, 2006).

2.7. Anticancer activity

Bisphenol A (BPA) is an environmental estrogen that stimulates proliferation of human breast cancer MCF-7 cells. RJ blocked the growth stimulating effect of BPA on MCF-7 cells (Nakaya, 2007). Fraction RJP30, gained by precipitation of the RJ with ammonium sulfate, declined by 2.5 fold the initial cell density of HeLa human cervicouterine carcinoma cells, after seven days of treatment (Salazar-Olivo, 2005). The 100 mg/kg of body weight doses of both RJ and green tea offered protection against cDDP (cisplatin)-induced nephrotoxicity, and both products enable as protector agents against cDDP-induced kidney damage in adult albino mice (Yapar et al., 2009).

2.8. Antibacterial immunologic activity

An antibacterial protein, royalisin, derived from RJ has potent antibacterial activity at low concentrations against Gram-positive bacteria, but not against Gram-negative bacteria (Fujiwara et al., 1990). Boukraa et al. (2008) studied the antibacterial activity of four varieties of honey and one variety of fresh RJ against *Staphylococcus aureus*. They reported a strong linear correlation amongst the minimum inhibitory concentration decrease of four varieties of honey and RJ (Boukraa et al., 2008).

2.9. Neurogenic effects of brain injury healing

Hattori (2007) studied the RJ and its unique fatty acid, 10-hydroxy-trans-2-decenoic acid, effects of AMP N1-oxide on the proliferation and differentiation of cultured neural stem/progenitor cells (NSCs) and reported that AMP N1-oxide is one of the components that facilitate astrogenesis by NSCs through activation of STAT3. AMP N1-oxide may

be a good elicitor for protection against and therapy for certain brain injuries, because astrocytes play important roles in brain development and the recovery from injury.

3. Ultra violet effect

A major part of the mutagenic and carcinogenic properties of sunlight has been attributed to UV rays. Ozone depletion results in an increased flux of biologically UV damaging radiations which directly reach the earth. UV induces harmful reactions for cells such as DNA damage, interfering with other components of receptor mediated signal transduction pathways and mediates immunosuppression by interfering with signal transduction (Bender et al., 1997). In particular, UV radiation in the middle-wavelength range between 290 and 320 nm (UVB) represents one of the most relevant environmental dangers because of its hazardous effects. Like alkylating chemicals and oxidants, exposure to UV light such as UVB can induce apoptosis in mammalian cells (Rehemtulla et al., 1997). Under UV irradiation, the stress-activated protein kinase pathway mediates cell death following injury induced by cis-platinum (Zanke et al., 1996). Undoubtedly, UVB-induced DNA damage is a crucial event in UVB-mediated apoptosis. On the other hand, UVB directly activates death receptors on the cell surface including CD95, implying that UVB-induced apoptosis can be initiated at the cell membrane through death receptor clustering (Kulms et al., 1999). In addition, epidemiological studies suggest that solar UV radiation is responsible for skin tumor development via gene mutations and immunosuppression, and possibly for photoaging (Ichihashi et al., 2003).

Moreover, the major concern is that UVB irradiation is known as a potent inducer of reactive oxygen species (ROS)-formation (Amaral et al., 2013). ROS such as

superoxides and hydrogen peroxide can cause severe damage to DNA, protein, and lipids. High levels of oxidant produced during normal cellular metabolism or from environmental stimuli such as UV radiation perturb the normal redox balance and shift cells into a state of oxidative stress (Amaral et al., 2013). Consequently, oxidative stress inhibits osteoblastic differentiation of bone cells by extracellular signal-regulated kinases and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Bai et al., 2004). ROS generation is a key modulator of damaged bone cell function. ROS promote bone loss by inhibiting osteoblast differentiation and enhancing osteoclastogenesis (Wauquie et al., 2009). Besides, the balance of bone formation and bone resorption tends to be negative with age, particularly in post-menopausal women, often leading to a loss of bone serious enough to cause fractures, which is called osteoporosis. Oxidative stress by itself and by influencing the regulatory cytokines such as tumor necrosis factor and interlukins are involved in osteoporosis (Abdollahi et al., 2005). Osteoblast extracellular Ca^{2+}-sensing receptor regulates bone development, mineralization, and turnover (Dvorak-Ewell MM et al. 2011). Physical stresses such as exposure to ultraviolet (UVB) may perturb the cell surface or alter receptor conformation (Caridad Rosette et al., 1996). Alkaline phosphatase (ALP) is one of the most frequently used biochemical markers of osteoblast activity. Oxidative stress such as exposure to ultraviolet (UVB) is one of the major causes of inhibition of ALP activity (Bai et al., 2004).

4. Osteoblast

Bone is a dynamic tissue that is constantly being reshaped by osteoblasts, which are in charge of production of matrix and mineral, and osteoclasts, which break down the

tissue. The number of osteoblasts tends to decrease with age, affecting the balance of formation and desorption in the bone tissue (Pittenger et al., 1999). Osteoblasts produce a matrix of osteoid, which is composed mainly of Type I collagen (Reddi et al., 1977). Osteoblasts are also responsible for mineralization of this matrix. Zinc, copper, and sodium are some of the minerals required in this process (Murshed et al., 2005).

5. 57-kDa Protein of royal jelly

The 57-kDa protein which is monomeric and molecular weight 57 in RJ is specifically degraded in proportion to both storage temperature and storage period compare to other components such as 10-HAD, vitamins, amino acids, and proteins. It has been suggested that 57-kDa protein could be useful as a marker of freshness of RJ (Kamakura et al., 2000). In addition, it enhances proliferation of primary cultured rat hepatocytes and increases albumin production in the absence of serum. The 57-kDa protein stimulates hepatocyte DNA synthesis and prolongs the proliferation of hepatocytes, as well as increases albumin production (Kamakura et al., 2001). Moreover, a 57-kDa protein may be an essential growth factor for regulating cast differentiation of honey bee larvae into queen by increasing body size, ovary development and shortened developmental time. It also exhibits similar effects on the *D. melanogaster* (Kamakura et al., 2001).

MATERIALS AND METHODS

1. Royal jelly

Fresh RJ (50 g) was obtained from Korea Honey Farm and stored at –70℃ until use. A voucher specimen (RJ-K01) was deposited in the Research Institute for Agriculture and Life Science, College of Agriculture and Life Sciences, Seoul National University.

2. Materials

(3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetra-zolium) inner salt (MTS) was purchased from Promega (Madison, WI, USA). Coomassie Brilliant Blue R-250 was by Bio-Rad Life Sciences (Hercules, CA, USA). Rosewell Park Memorial Institute (RPMI) 1640 and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). *p*NPP (*p*-Nitrophenyl Phosphate) Alkaline Phosphatase Assay Kit were purchased from AnaSpec (Fremont, CA, USA) DEAE FF 16/10 column, Mono Q column, Superdex 75 gel filteration column were supplied by GE Healthcare Life Sciences (Pittsburgh, PA, USA). Collagen IV-coated plates (96-well plates) were purchased from BD Biosciences (Bedford, MA, USA). CellTiter 96® AQueous One Solution Cell Proliferation assay kit was purchased from Promega (Madison, WI, USA). MitoTracker® Red FM and Fluo-4/AM were purchased from Invitrogen Molecular Probes (San Diego, CA, USA). The Precision Plus Protein all blue standards were provided by Bio-Rad Life Sciences (Hercules, CA, USA). All of the other chemicals and reagents used in this study were of analytical grade quality and available commercially.

3. Cell line and culture conditions

Saos-2 cell line has the characteristics of osteoblasts such as the formation of bone proteins and the activity of ALP and is widely used for the experimental model. Saos-2 (KCLB 30085), a human osteoblast-like cell line, was purchased from the Korean Cell Line Bank (Seoul, ROK). The cell line was maintained in RPMI 1640 supplemented with 10% charcoal dextran-treated FBS and 1% antibiotics (penicillin + streptomycin) in a humidified incubator at 37℃ and 5% CO_2 for cell proliferation assays. The cell line was stored at –70℃ until use.

4. Protein purification

The method of protein purification was followed by Kamakura et al., (2001) with minor modification.

4.1. Ultrafiltration procedure

RJ (30 g) in 1L of 20 mM Tris-HCl buffer (pH 7.0) was centrifuged at 1 × 5000 *g* for 10 min at 4℃ using Supra 25K ultra vacuum speed centrifuge (Hanil Science, Incheon, ROK) and filtered using an Advantec 240 mm filter paper (Advantec MFS, Dublin, CA, USA). The combined supernatant (60 mL) was concentrated to 20 mL with 20 mM Tris-HCL buffer (pH 7.0) using QuixStand Benchtop System (GE Healthcare Life Sciences, Buckinghamshire, UK) incorporated with a 10,000 nominal molecular weight cutoff (NMWL) polysulfone Hollow Fiber Cartridge (Xampler Ultrafiltration Cartridges (UFP-1-C-4X2M), 1400 cm^2 [1.5 ft^2], from (140) 0.5 mm ID fibers). The concentrate was added to 80 mL of 20 mM Tris-HCL buffer (pH 7.0). These procedures were repeated five times. The final filtrated samples were transferred to Amicon Ultra-15mL

centrifuge tubes (EMD Millipore, Darmstadt, Germany), and the tubes were centrifuged at 3000 *g* for 30 min at 4℃.

4.2. Fast protein liquid chromatography

4.2.1. DEAE FF 16/10 column chromatography

The supernatant obtained from ultrafiltration stated previously was applied to DEAE FF 16/10 column equilibrated with 20 mM Tris-HCl buffer (pH 7.0) using AKTA Fast protein liquid chromatography (FPLC) System (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) with a linear gradient of 0–1.0 M NaCl in a total volume of 10 mL at a flow rate of 5 mL/min. The samples were centrifuged at 3000 *g* for 30 min at 4℃ as stated in Section 4.1. Fractions (5 mL) were collected and analyzed by 15% sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE).

4.2.2. Mono Q column chromatography

The fraction (5 mL) obtained from DEAE FF 16/10 column was applied to a 5 × 50 mm Mono Q column equilibrated with 20 mM Tris-HCl buffer (pH 7.0). The column was eluted with a linear gradient of 0–1.0 M NaCl in a total volume of 5 mL at a flow rate of 5 mL/min. The samples (5 mL) were centrifuged at 3000 *g* for 30 min at 4℃ as stated in Section 4.1 and then electrophoresed on 15% SDS-PAGE.

4.2.3. Superdex 75 gel filteration column chromatography

The fraction (1 mL) obtained from Mono Q column was applied to Superdex 75 column equilibrated with 0.15 M NaCl in 50 mM phosphate buffer (pH 7.0). Elution was carried out with the same buffer at a flow rate of 1.0 mL/min. The samples were

centrifuged at 3000 *g* for 30 min at 4℃ as stated in Section 4.1. Fractions (1 mL) were collected and analyzed by 15 % SDS-PAGE.

5. Polyacrylamide gel electrophoresis and Protein concentration determination

SDS-PAGE was performed according to the method of Laemmli (1970). Protein samples were loaded onto a 15 % polyacrylamide gel. Protein bands were observed after using Coomassie Brilliant Blue R-250 stain. The protein concentration was determined by a protein assay kit with BSA as a standard as described previously by Bradford et al. (1976). The protein was identified by Hybrid LC/MS/MS System (Applied Biosystems, Foster, CA, USA) as it is 94 % the same between purified and 57-kDa protein (RJ protein).

6. Measurement of Saos-2 cells protective effect and viability

UVB irradiation on Saos-2 cells was performed using UVM-225D Mineralight (UVP, Phoenix, AZ, USA) emitting wavelength of 302 nm. UV strength was measured using a HD2102-2 UV meter (Delta OHM, Padova, Italy). The cells were treated with 50 mJ/cm^2 of UVB for the *in vitro* experiments. Saos-2 cells were seeded onto a 96-well microplate at a concentration of 1×10^4 cell/well with 200 μL of fresh RPMI 1640 containing 10 % FBS and incubated for 24 h. Based on the preliminary test results, cells pretreated with 1.5 mg/mL RJ and 0.1 mg/mL 57-P were cultured for 24 h, followed by UVB irradiation. The cell viability of the test materials was determined based on the reduction of MTS to formazan according to the manufacturer's instruction. After removing the medium, 200 μL RPMI containing MTS was added to each well and incubated at 37℃ for 1 h. NAC (2 mM) served as a positive control. Absorbance was

read at 490 nm by using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA). The resulting sample was assayed at 490 nm by using a microplate fluorometer (Molecular Devices, Sunnyvale, CA, USA). All bioassays were repeated three times in duplicates.

7. Confocal microscopy

Saos-2 cells were seeded in confocal dishes (35 × 10mm) at a concentration of 1 × 10^5 cell/dish for 24 h. The culture medium was then removed, and the plates were washed three times with phosphate-buffered saline (PBS) (pH 7.4) and replaced with new serum free media containing 1.5 mg/mL RJ, 0.1 mg/mL 57-kDa, and 2 mM NAC. After 24 h, the medium was removed and washed with PBS three times. Cells were incubated for 45 min with 10 μM Mitotracker Red (excitation/emission spectra for Mitotracker Red is 578/598 nm) to detect mitochondria. The Ca^{2+} indicator dye Fluo-4/AM (excitation/emission maxima of 488/520 nm) was used to assess the cytoplasmic calcium concentration. Cells were stained by Fluo-4 AM and incubated at 37℃ for 45 min. They were then rinsed by PBS three times to remove any free dye to allow complete deesterification of AM ester. Ca^{2+} and mitocondria levels were measured from fluorescent living cell images using a Fluoview FV10i Confocal Laser Scanning Microscope (Olympus, Tokyo, Japan).

8. Alkaline phosphatase (ALP) activity test

The ALP activity of the test materials was evaluated as described previously by the manufacturer's instruction. Saos-2 cell was seeded onto a 96-well culture plate at a concentration of 1 × 10^4 cell/mL at 37 ℃ with 5 % CO_2 for 24 h. The medium was

removed and the plates were replaced with serum-free media containing 1.5 mg/mL RJ, 0.1 mg/mL 57-kDa, and 2 mM NAC and the cells were further incubated for 24 h. The culture medium was removed and washed with PBS three times. After 24 h incubation, ALP activity was measured using *p*NPP ALP assay kit at 405 nm.

9. Data analysis

Statistical analysis was carried out using GraphPad Prism 5 software (La Jolla, CA, USA). Data were expressed as mean ± standard mean error (SEM) from at least 3 independent experiments. Data from two groups were analyzed by a Student's *t*-test, and multiple groups were analyzed by a one-way analysis of variance and Bonferroni multiple comparison test.

RESULTS

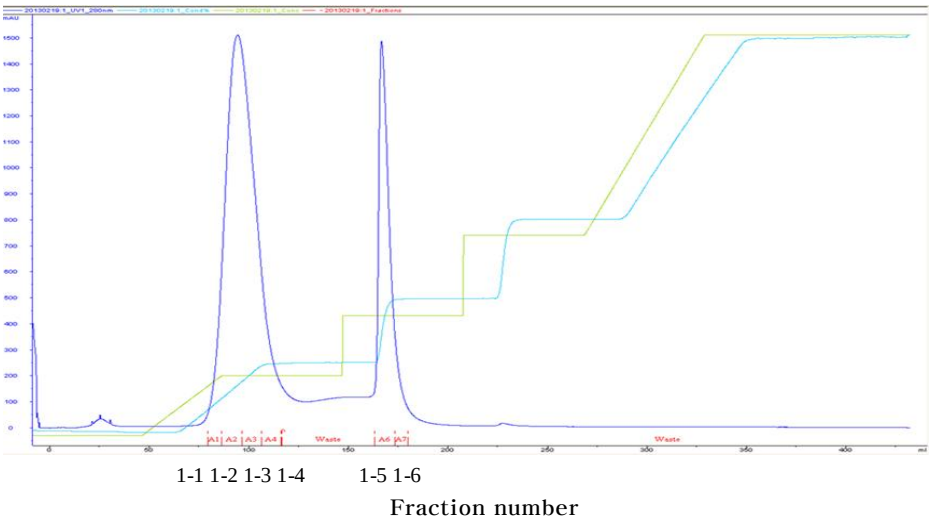
1. Purification of 57-kDa protein

As illustrated in Fig. 1A, the supernatant of the RJ extract was divided into six fractions by ion-exchange DEAE 16/10 column chromatography. These fractions were analyzed by SDS-PAGE, and 57-kDa protein band were found only in fractions 1-4, 1-5, and 1-6 (Fig. 1B).

Fractions 1-4 and 1-5 from DEAE 16/10 column chromatography was subjected to AKTA FPLC MONO Q ionic exchange as illustrated in Fig. 2A. Several protein peaks were eluted out. The SDS-PAGE analysis revealed that 57-kDa protein bands were found in fraction 2-2 (Fig. 2B).

Fraction 2-2 from AKTA FPLC MONO Q ionic exchange was subjected to Superdex 75 gel filtration (Fig. 3A). Fraction 3-3 was subjected to SDS-PAGE analysis and the peaks 3-3-1 and 3-3-2 were found to be homogeneous proteins with a molecular mass around 57-kDa as illustrated in Fig. 3B. It matched 94% sequence I.D between purified protein and original protein in Protein Bank by LC/MS/MS system (Fig. 4).

(A)



(B)

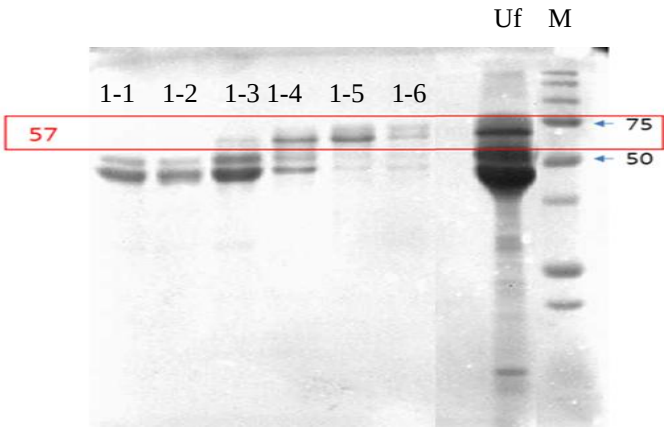
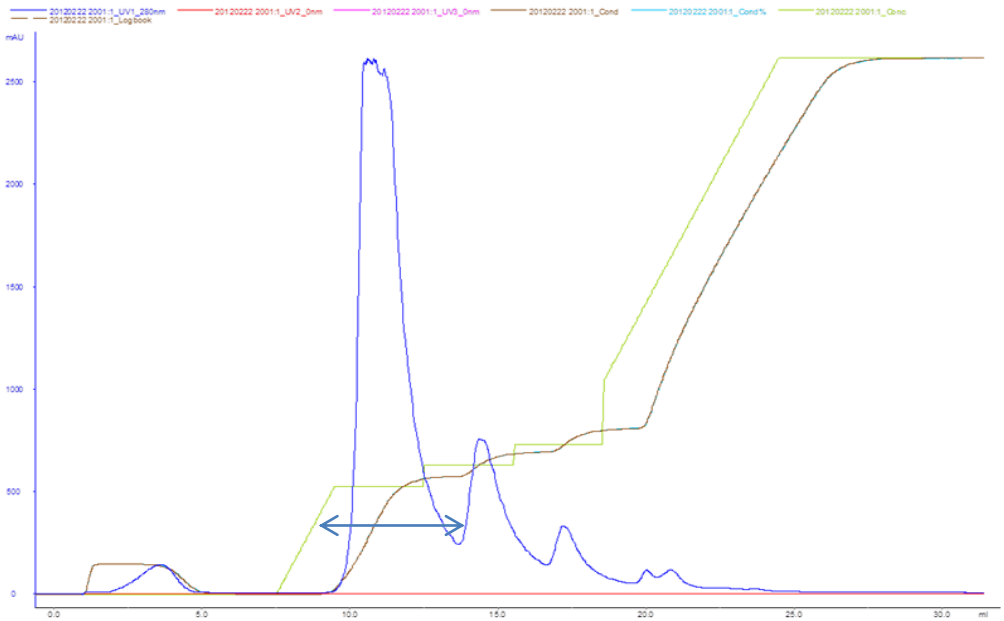


Fig. 1. Elution profile of 57-kDa protein on ion-exchange DEAE FF 16/10 column chromatography. (A) Each fraction obtained from ultrafiltration was subjected to DEAE FF 16/10 column. Six fractions (1–6) were collected, and absorbance was read at 280 nm. (B) SDS-PAGE profile. Lane U, fraction from ultrafiltration; Lanes 1-1–1-6 represent the fraction obtained from DEAE FF 16/10 column. The position of 57-kDa protein is indicated by red box.

(A)



Fraction number 2-1 2-2 2-3 2-4 2-5

(B)

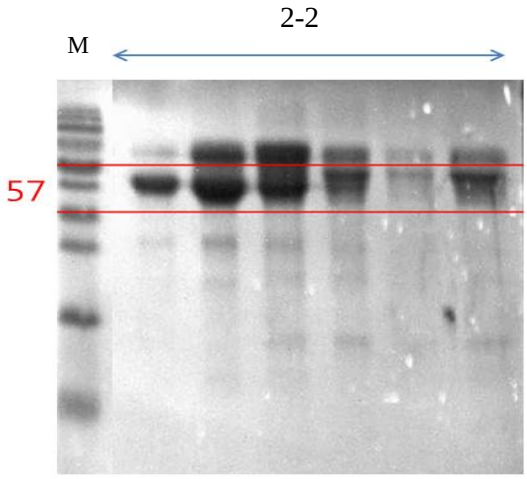
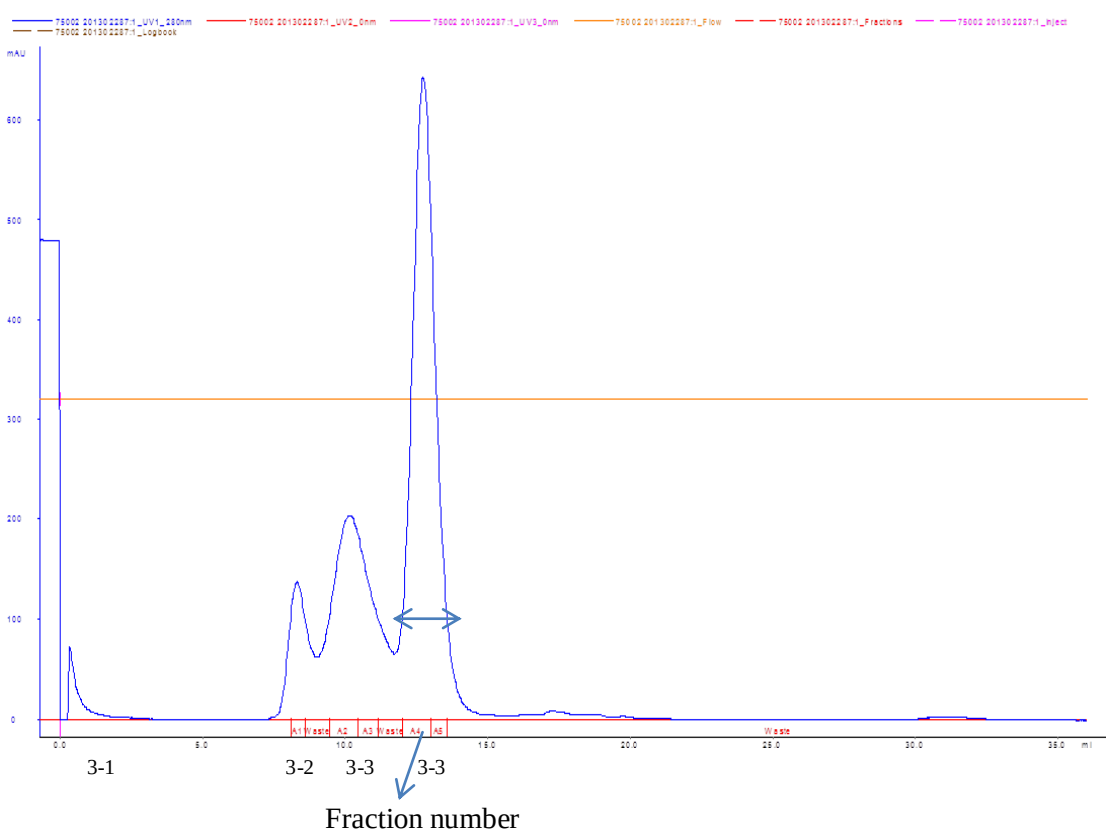


Fig. 2. Elution profile of 57-kDa protein on ion-exchange chromatography Mono Q. (A) Each fraction obtained from DEAE FF 16/10 column was subjected to Mono Q column. The fraction 2-2 was collected and absorbance was read at 280nm. (B) SDS PAGE profile of the fraction 2-2. The position of 57-kDa protein is indicated by red box.

(A)



(B)

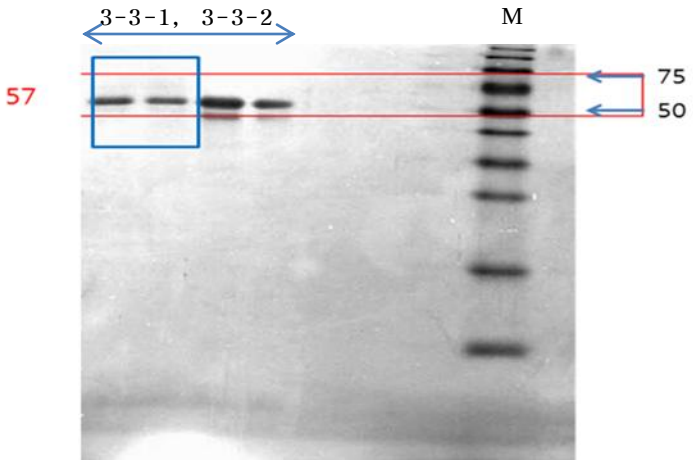


Fig. 3. Elution profile of 57-kDa protein on Superdex 75 gel filtration. (A) The fraction from Mono Q column was subjected to Superdex 75 gel filtration. Four fractions were collected and absorbance was read at 280 nm. (B) SDS-PAGE profile of each fraction. The position of 57-kDa protein is indicated by red box.

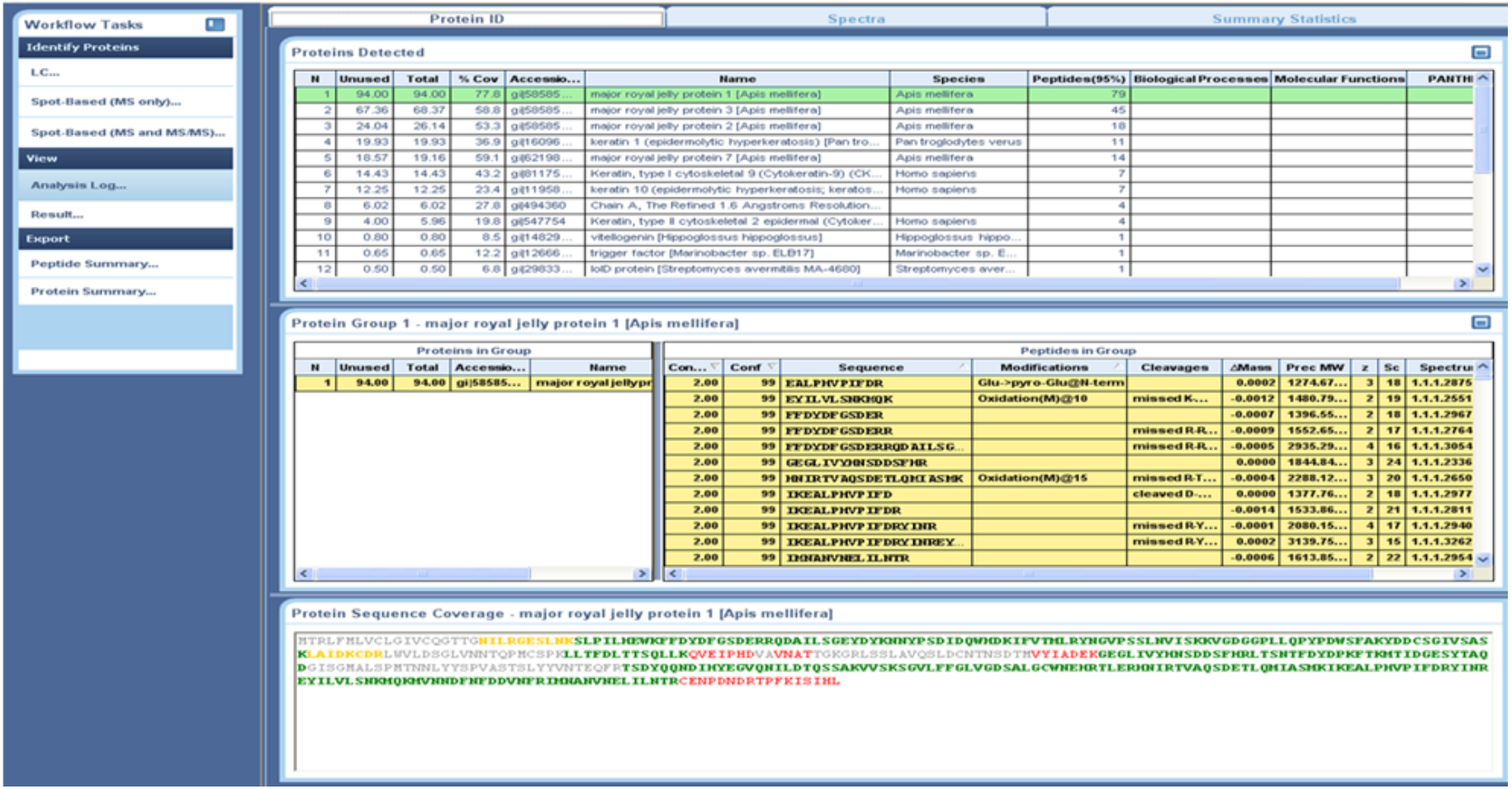


Fig.4. Protein identification by hybrid LC/MS/MS system. Protein from Superdex 75 was identified by hybrid LC/MS/MS system. The results indicates that 94% similarity was seen to purified and 57-kDa protein (RJ protein).

2. Effect of 57-kDa protein from royal jelly on Saos-2 cell viability

The concentration-dependent effects of purified 57-kDa protein derived from RJ on Saos-2 cell viability were evaluated using a MTS assay (Fig. 5). The 57-kDa protein at concentrations between 10 and 100 μg/mL exhibited a high correlation between protein concentration and cell viability (correlation coefficient (R^2) > 0.85).

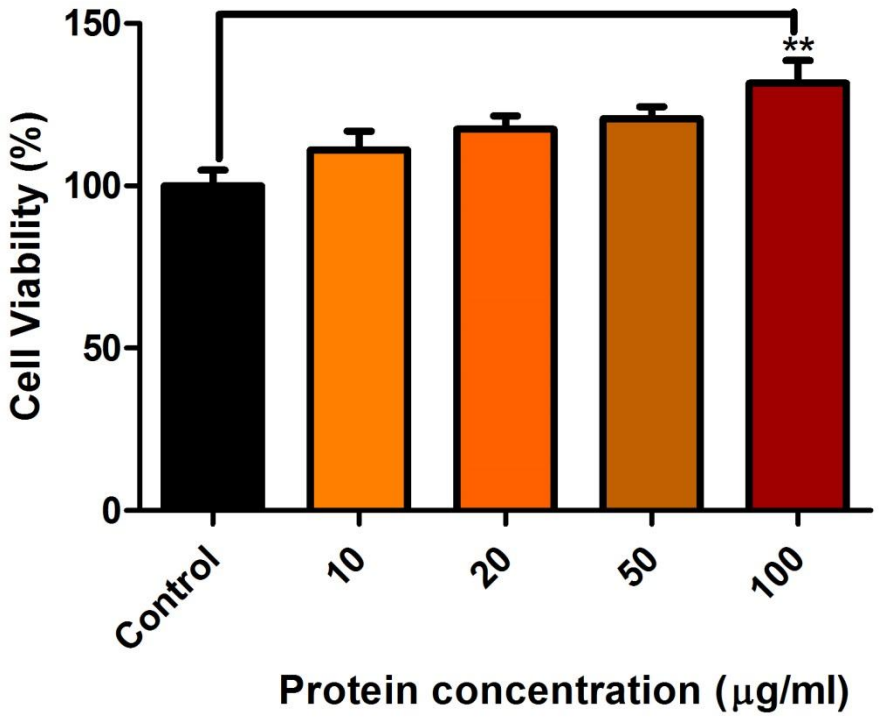


Fig. 5. Concentration-dependent effect of 57-kDa protein on Saos-2 cell viability. The 57-kDa proteins were added to culture medium at various concentrations (10, 20, 50, and 100 μg/mL) and cell viability was measured at 490 nm using a MTS assay. The data are expressed as a percentage of control. Each bar represents the mean ± SEM of triplicate samples of three independent experiments. ** Significant at $P < 0.01$, according to a Student's *t*-test.

3. Effect on viability of UVB irradiated Saos-2 cells

Cell viability of Saos-2 cells following UVB irradiation either with or without the test material was likewise examined (Fig. 6). When pretreated with 0.1 mg/mL 57-kDa protein and 2 mM NAC 24 h prior irradiation, Saos-2 cells were protected 79 and 77% by the protein and NAC treatments, respectively, whereas 1.5 mg/mL RJ showed no statistically significant protection from radiation induced cell death.

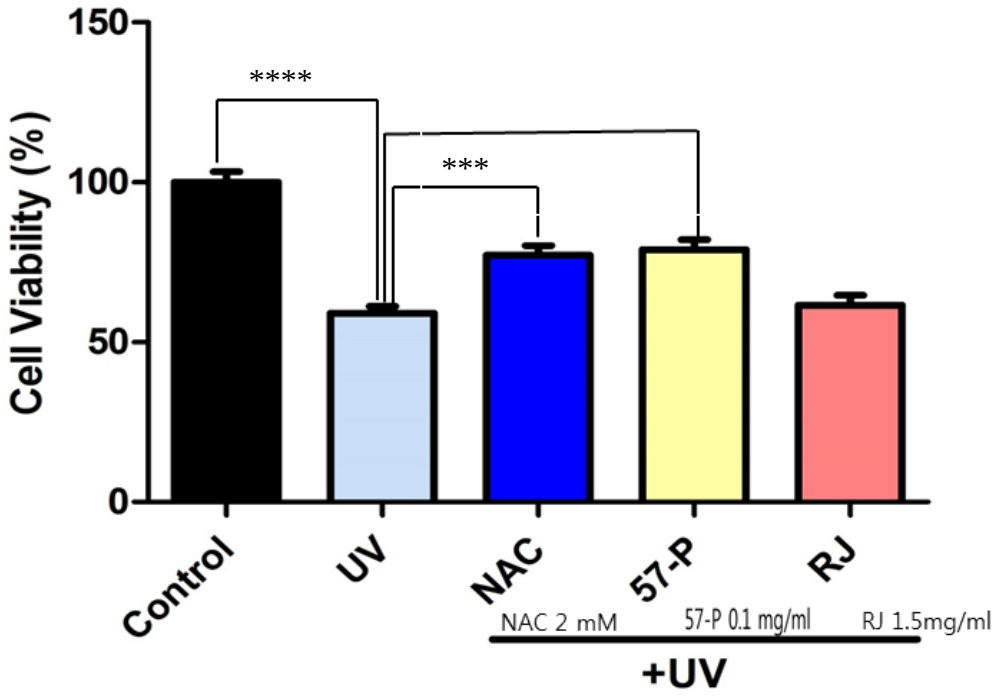


Fig. 6. Cell viability of Saos-2 cells following UVB irradiation either with or without the test materials. Saos-2 cells were cultured at 1×10^4 cells/well. Plates were irradiated with UVB 50 mJ/cm^2, the Saos-2 was killed at level of 40.91%. The 1.5 mg/mL RJ, 0.1 mg/mL 57-kDa protein, and 2 mM NAC were added to each well, and their viability was measured by MTS assay at 24h. Each bar represents the mean ± SEM of duplicate samples of three independent experiments. *** Significant at $P < 0.001$, according to a Student's *t*-test.

4. Cytoprotective effect on UVB irradiated Saos-2 cells

The cytoprotective effect of 57-kDa protein on Ca^{2+} and mitochondria was assessed using confocal microscopy. The confocal microscopic image showed that the control group had the highest density and strong red florescence and distributed green florescence in 10× magnification (Fig. 7). However, 0.1 mg/mL 57-kDa protein, 1.5 mg/mL RJ, and 2 mM NAC treated groups showed more proliferation and Ca^{2+} distribution and mitochondria activity than only UVB treated group even if they had lower density and florescence than control. The 60× magnification images demonstrated that treated groups still maintained their cell morphology compared to untreated group (Fig. 8).

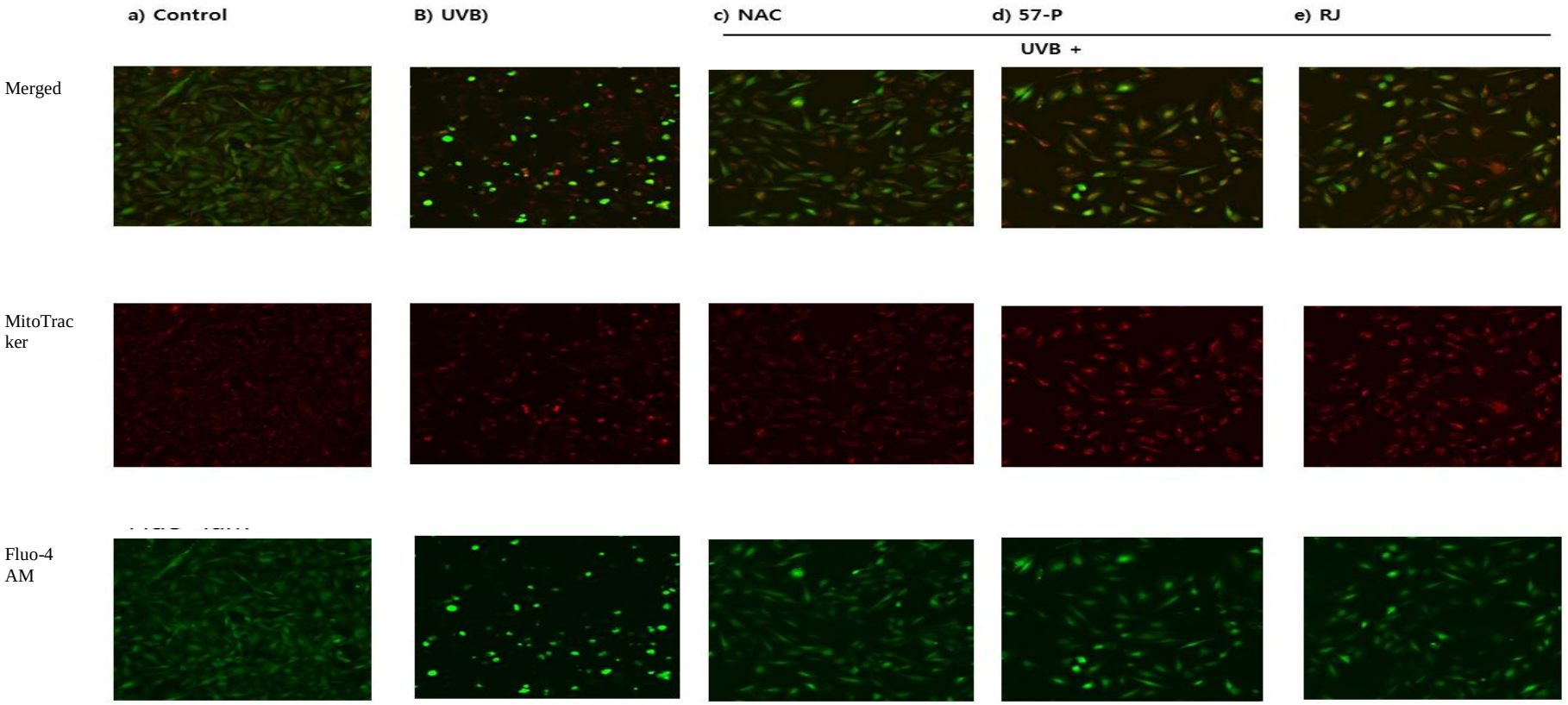


Fig. 7. Cytoprotective effect of 57-kDa protein on UVB stress using two different dyes (10×). Cells were seeded 1×10^5 in confocal dishes for 24 h and then washed with phosphate-buffered saline (PBS) three times for treating with 57-k Da protein, royal jelly (RJ) and NAC in no supplemented media. After another 24 h, cells were stained by Fluo-4 AM (Ca^{2+}sensitive dye) and MitoTracker (excitation/emission spectra for Mitotracker Red is 578/598 nm) and incubated about 45 min at 37℃. They were then rinsed by PBS three times to remove any free dye to allow complete deesterification of AM ester (excitation, 485 nm; emission, 512 nm). Ca^{2+} and mitocondria levels on cell damage induced by UVB measured from fluorescence live cell images using a Fluoview FV10i Confocal Microscope. The confocal microscopic image showed that the control group had the highest density and strong red florescence and distributed green florescence.

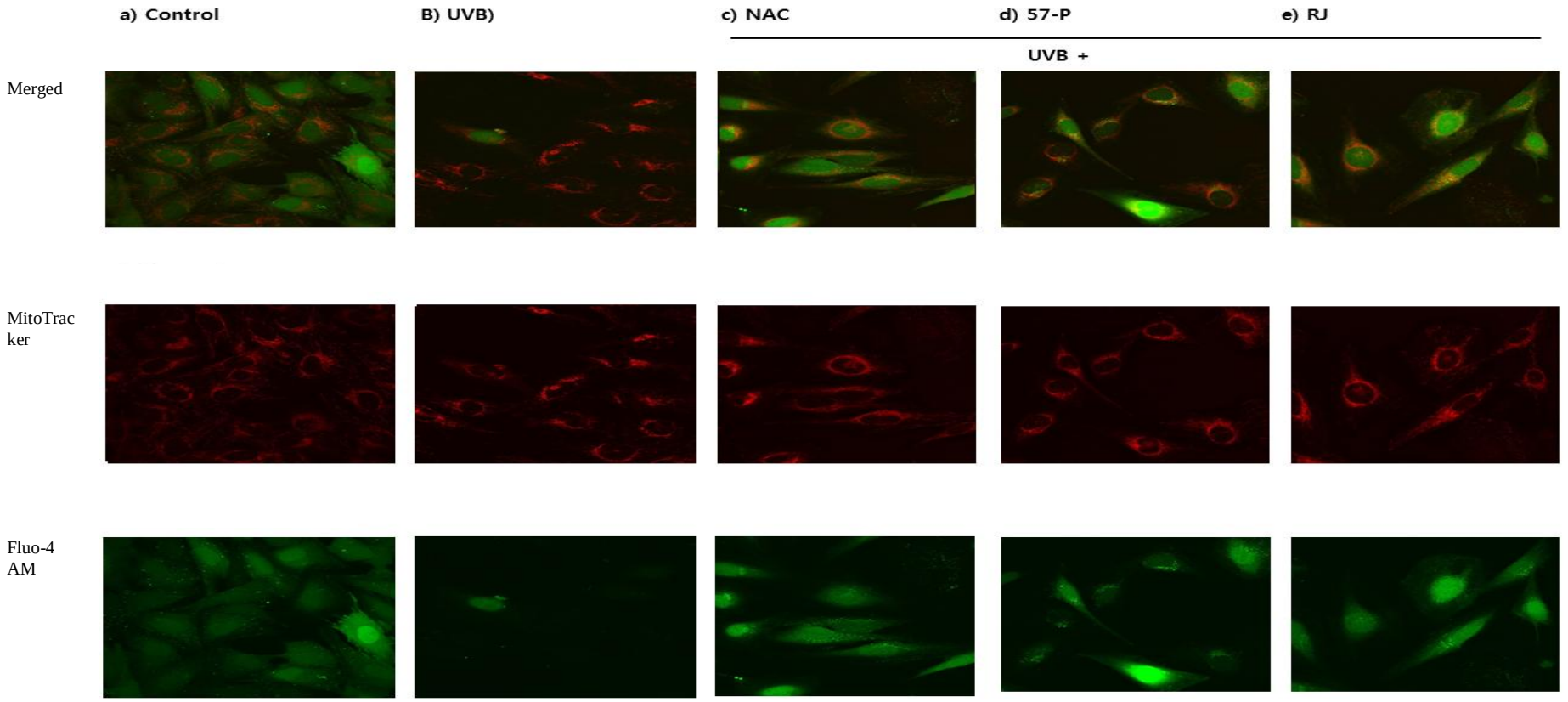


Fig. 8. Cytoprotective effect of 57-kDa on UVB stress using two different dyes (60×). Cells were seeded 1×10^5 in confocal dishes for 24 h and then washed with phosphate-buffered saline (PBS) three times for treating with 57-kDa protein, royal jelly (RJ) and NAC in no supplemented media. After another 24 h, cells were stained by Fluo-4 AM (Ca^{2+}sensitive dye) and MitoTracker (excitation/emission spectra for Mitotracker Red is 578/598 nm) and incubated about 45 min at 37℃. They were then rinsed by PBS three times to remove any free dye to allow complete deesterification of AM ester (excitation, 485 nm; emission, 512 nm). Ca^{2+} and mitocondria levels on cell damage induced by UVB measured from fluorescent live cell images using a Fluoview FV10i Confocal Microscope. These images demonstrated that treated groups still maintained their cell morphology compared to untreated group.

5. Effect on alkaline phosphatase activity of UVB irradiated Saos-2 cells

The ALP activity of Saos-2 cells following UVB irradiation either with or without the test material was examined using a MTS assay (Fig. 6). Pretreatment of Saos-2 cells with 0.1 mg/mL 57-kDa protein 24 h prior irradiation significantly attenuated the increase in ALP activity from radiation induced cells. However, 1.5 mg/mL RJ and 2 mM NAC exhibited no statistically significant increase in ALP activity.

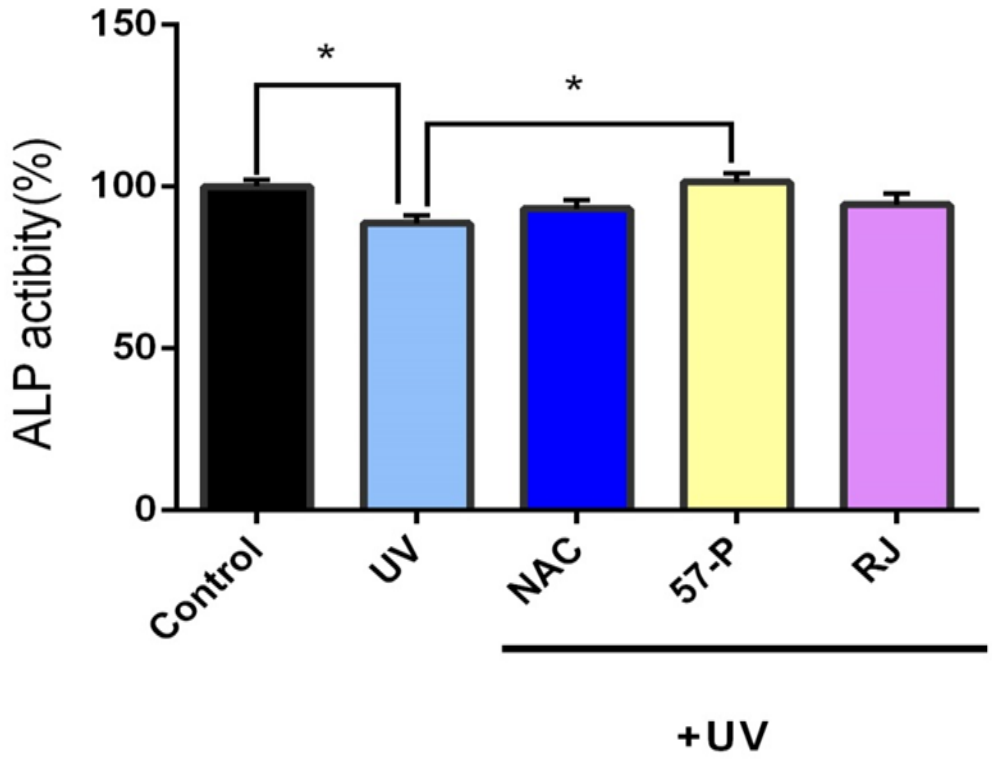


Fig. 9. Alkaline phosphatase (ALP) activity of Saos-2 cells following UVB irradiation either with or without the test materials. Saos-2 cells were cultured at 1×10^4 cells/well. Plates were irradiated with UVB 50 mJ/cm^2. The 1.5 mg/mL RJ, 0.1 mg/mL 57-kDa protein, and 2 mM NAC were added to each well, and their ALP activity was measured using *p*NPP ALP assay kit at 405 nm at 24h. Pretreatment of Saos-2 cells with 0.1 mg/mL 57-kDa protein significantly attenuated the increase in ALP activity from radiation induced cells. Each bar represents the mean ± SEM of duplicate samples of three independent experiments. * Significant at $P < 0.05$, according to a Student's *t*-test.

DISCUSSION

RJ is secreted material from mandibular and the hypopharyngeal glands on the head of worker bees and is food for all bee larvae. It is a famous nutritional material for queen differentiation of honey bee larvae as well as human health. RJ possesses metabolic activity, antioxidant, anti-aging properties, estrogenic effects, anti-cancer and antibacterial activity (Crenguța I. P et al., 2011). Recently, it has been reported that a certain component of the RJ causes to fully develop a bee into a queen. It appears a single monomeric protein that has been called the 57-kDa protein because of its molecular weight or it is named royalactin, on the basis that it is driven from RJ and activates not only rat hepatocytes but also is important for becoming a queen honey bee (Masaki K et al., 2001).

Osteoblasts are cells which originate in the bone marrow and contribute to the production of new bone. These cells build up the matrix of the bone structure and also play a role in the mineralization of the bone matrix. Bone is constantly being built up and broken down by the body, making osteoblasts rather critical. The counterpart to the osteoblast is the osteoclast, a cell which is responsible for breaking down bone. As people get older, their production of osteoblasts decreases. Bone health can also be influenced by the amount of available calcium in the diet, as osteoblasts need calcium to work with in the process of building up bone (Osteoblast, wikipedia). Nevertheless, the 57-kDa protein effects are still unknown on human cells. In this study, treatment with the 57-kDa protein at 100 µg/mL resulted in significant cell viability than control group. The 57-kDa protein does not have any toxicity against Saos-2 human osteoblast cell line.

Ultraviolet B (UVB) radiation acts as a strong apoptotic trigger in many cell types, in tumor and normal cells. Several studies have demonstrated that UVB-induced cell death occurs through the generation of reactive oxygen species (Sara Salucci et al., 2013).The consequent oxidative stress includes the impairment of cellular antioxidants, the induction of DNA damage and the occurrence of apoptosis. (Sara Salucci et al., 2013) Therefore, UVB irradiation is known as a potent inducer of ROS formation as a tool to assess ROS-induced damage (Amaral et al., 2013). Ca^{2+} ions are important molecules and play physiological roles for calcium signaling range widely. Calcium ions enter the cytoplasm and exert allosteric regulatory effects on many enzymes and proteins. In addition, calcium ions can play a predominant role as an activator of signal transduction in ion channels or as a second messenger caused by indirect signal transduction pathways such as G protein-coupled receptors. These include muscle contraction, neuronal transmission as in an excitatory synapse, cellular motility (including the movement of flagella and cilia), fertilization, cell growth or proliferation, learning and memory as with synaptic plasticity, and secretion of saliva (Berridge et al., 2000). Other biochemical roles of calcium include regulating enzyme activity, permeability of ion channels, activity of ion pumps, and components of the cytoskeleton (Koolman et al., 2005). Osteoblast extracellular Ca^{2+}-sensing receptor regulates bone development, mineralization, and turnover (Dvorak-Ewell et al., 2011,). The most important roles of mitochondria are to generate the cell energy, ATP (i.e., phosphorylation of ADP), through respiration, and to regulate cellular metabolism (Ivannikov et al., 2013). In addition to supplying cellular energy, mitochondria are involved in other tasks such as signaling, cellular differentiation, cell death, as well as the control of the cell cycle and cell growth (McBride HM et al., 2006). UVB occur oxidative damage to the cell.

Consequently, Normal oxidative damage to mitochondrial and nuclear DNA is extensive (Richter C et al., 1988).

In this study, the cytoprotective effects of pretreatment with RJ, 57-kDa protein, and NAC on Saos-2 cells damaged by UVB were evaluated using a MTS assay and confocal microscopy. The 57-kDa protein significantly enhanced cell viability by increasing ALP activity and prolonged cell survival in UVB irradiated condition (57-kDa : 79 and NAC : 77% protection).. In addition, the living cell observation revealed that the cells treated with 57-kDa protein showed normal cell shapes and organelles being maintained, similar to normal cells. These results indicate that the 57-kDa protein may affect the extracellular Ca^{2+}-sensing receptors and mitochondria activity in living osteoblast. Therefore, the 57-kDa protein might improve cell protective ability and viability or specific functions in osteoblasts. This original finding indicates that RJ and 57-kDa protein can hold promise as novel and effective anti-UV products.

In conclusion, 57-kDa protein can be useful for the bone formation of osteoblasts and for protection from UV induced stress. For practical use of RJ -derived materials as novel anti-UV induced stress products to proceed, further research is needed to establish their human safety and whether this activity could be exerted *in vivo* after consumption of the product by humans. In addition, their exact action mechanisms related to bone formation and anti-UV induced stress of action need to be established and detailed tests are needed to understand how to improve UV induced stress and stability for eventual commercial development.

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국산 로얄젤리(royal jelly)에서 분리한 57-kDa 단백질의 인간유래조골세포 활성에 미치는 영향

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초 록

로얄젤리는 꿀벌 성충이 꽃가루와 꿀을 흡수시켜서 머리의 인두선에서 분비하는 물질로서, 꿀벌 유충을 여왕벌로 분화시키는 모든 핵심요소들을 포함하고 있다. 로얄젤리의 57-kDa 단백질이 여왕벌 분화의 핵심요소이며 이는 곤충세포의 상피세포 수용체를 자극하여 여왕벌의 몸을 크게 하고 생식기관을 발달시킨다. 그러나, 항암, 항바이러스, 항산화 같은 로얄젤리 효능에 대한 연구는 매우 광범위하고 로얄젤리의 생리적으로 뛰어난 효과는 이미 알려져 있지만 57-kDa 단백질이 여왕벌의 분화에 핵심역할을 하는 물질임이 알려졌음에도 아직 이 단백질이 인체 유래 세포에 미치는 영향에 대해서는 아직 많이 알려져 있지 않다.

본 연구는 로얄젤리가 쥐 유래 조골세포의 뼈 형성능력을 증가시키며, 57-kDa 단백질이 쥐 유래 간세포의 활성에 도움을 준다는 연구결과를 바탕으로, 57-kDa 단백질이 인간 유래 조골세포 (Saos-2 Cell line)에 미치는 영향을 조사하였다. 국산 로얄젤리에서 크로마토그라피 및 겔 여과법을 이용하여 57-kDa 단백질만을 분리

정제한 다음 이 단백질이 조골세포의 활성에 영향을 미치는 적정 농도를 실험한 결과, 100 μg/mL 농도에서 대조군에 비해 통계적으로 유의한 세포활성을 나타내었다. 또한, 57-kDa 단백질이 쥐 간세포의 수명과 세포활성, 알부민의 생산을 촉진시킨다는 연구결과를 바탕으로 이 단백질이 조골세포를 보호하는 효과를 조사하였다. 57-kDa 단백질을 처리한 세포들을 자외선으로 손상시킨 뒤, 자외선 조사로 촉발된 산화제를 소거하는 역할로 잘 알려진 화합물 NAC 와 비교하였을 때, 통계적으로 유의한 세포 보호 효과를 보였다 (79%) (UVB; 59.09%, NAC 2 mM; 77.16%, RJ 1.5 mg/ml; 61.57%). 그리고, 살아있는 세포의 칼슘 sensing 리셉터와 미토콘드리아를 형광 염색하여 오로지 자외선만 조사한 세포 그룹과 비교하였을 경우, 57-kDa 단백질을 처리한 세포들은, 염색된 세포 소기관이나 세포 형태가 정상세포와 비슷하게 유지되고 있음을 관찰하였다. 또한, 뼈 분화에 중요한 염기성 인산분해효소도 0.1 mg/mL 57-kDa 단백질 처리 그룹이 다른 그룹보다 현저하게 증가하였다 (RJ 1.5 mg/ml; 94.44%, NAC 2 mM; 93.18%, UVB; 88.74%).

이상의 결과를 바탕으로, 본 논문은 인체 유래한 세포에 대해 잘 알려지지 않은 57-kDa 단백질의 활성을 연구했다는 것에 그 의의가 있고, 57-kDa 단백질에 대한 분리 정제와 그 분리 정제된 단백질이 인간유래 조골세포에 작용하는 세포 보호 효과를 밝힘으로서 이 단백질의 활용 가능성을 보여주었다. 그러나 57-kDa 단백질의 자극으로 인해 세포 안에서 일어나는 여러 작용 메카니즘에 대한 분자생물학적인 보다 상세한 측정과 동물모델을 이용한 생물검정이 요구된다.

검색어 : 로얄 젤리, 조골세포, 57-kDa 단백질.

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