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Effects of Myrrh Extracts on Collagenase Activity and Procollagen Synthesis in Hs68 Human Fibroblasts and Tyrosinase Activity

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Abstract

This study was designed to investigate the collagen metabolism and tyrosinase activity of Myrrh Extracts (ME). The currently known effects of ME are to move blood, calm pain, solve edema, and promote tissue regeneration. The effect of ME on type I procollagen production and collagenase (matrix metalloproteinase-1, henceforth referred as MMP-1) activity in human normal fibroblasts Hs68 after ultraviolet B (UVB, 312 nm) irradiation was measured using the ELISA method. The tyrosinase activity after treatment with ME was measured as well. There was no cytotoxicity at all tested concentrations. ME significantly inhibited the increased collagenase activity after UVB damage, whereas it did not recover the reduced type I procollagen production in UVB damaged Hs68 cells. It did not reduce the L-DOPA oxidation. However, it significantly reduced the tyrosinase activity. In conclusion, ME showed the anti-wrinkle effects via the collagenase inhibitory mechanism and whitening effects via the tyrosinase inhibitory mechanism. Although further research is needed to validate its efficacy, these results suggest that ME may have potential as an anti-aging ingredient in cosmetic herb markets.

Keywords: *Myrrh, type I procollagen, collagenase, tyrosinase*

1. Introduction

Myrrh is a resin obtained from *Commiphora myrrha* Engl. Its effects are to activate blood circulation and relieve pain. It reduces swelling and promotes generation of flesh in the cases of skin lesions and ulcerations. It has an excellent ability to reduce swelling, relieve pain, promote generation of flesh, and enhance healing of skin lesions and ulcerations. Heat-clearing and blood-cooling herbs are usually added to reduce inflammation. It has been applied with Ru Xiang (olibanum) as powder topically for the treatment of skin lesions and ulcerations The latest trend today is to look younger than one's age. In these days, aging seems to be treated not as an inescapable destiny to accept but as a disease or a disorder to overcome. There are two major theories of aging: the programmatic theory that aging is an inherent genetic process, and the stochastic theory that aging represents random environmental damage. Processes that are associated with cellular damage and aging are the production of free radicals (a process much enhanced after ultraviolet irradiation) and an increasing number of errors during DNA replication. Cellular manifestations of intrinsic aging include decreased life span of cells, decreased responsiveness of cells to growth signals, which may reflect loss of cellular receptors for growth factors, and increased responsiveness to growth inhibitors. All these findings are more pronounced in cells derived from photodamaged skin [3].

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It has been shown that UV irradiation leads to the formation of reactive oxygen species (ROS) that activate the mitogen-activated protein (MAP) kinase pathway, which subsequently induces the expression and activation of matrix metalloproteinases (MMPs) in human skin in vivo [4-5]. MMPs including collagenase are considered key factors in the photoaging process.

In the present study, we investigated the effect of Myrrh Extracts (ME) on type I procollagen production and collagenase activity in human normal fibroblasts Hs68 after UVB (312 nm) irradiation. The tyrosinase activity after treatment with ME was measured as well.

2. Methods

2.1. Sample Preparation

Myrrh was purchased from Omniherb (Korea). ME was prepared as follows. 100 g of Myrrh in 2,000 ml distilled water was heated in a heating extractor for 3 hours. The extract was filtered and concentrated using the rotary evaporator. The extract was lyophilized by using a freeze dryer (6.2 g). The extract was dissolved in water and filtered three times through micro-filter paper and syringe filter (Whatman #2, 0.45 μm to 0.2 μm). Filtered material was placed in the disinfected vial and was sealed for further study.

2.2. Reagents

All reagents were purchased from Sigma-Aldrich except those mentioned below (St. Louis, MO, USA).

2.3. Cell culture

Hs68 human fibroblasts (Health Protection Agency Culture Collections, UK) were cultured in Dulbecco's Modified Eagle's medium (Gibco, USA) containing 10% fetal bovine serum, 1% antibiotics at 37°C in a humidified atmosphere of 5% CO_2. When cells reached above confluency, subculture was conducted at a split ratio of 1:3.

2.4. UVB Irradiation

A UVB lamp (Vilber Lourmat, France) was used as a UVB source. In brief, Hs68 cells were rinsed twice with phosphate-buffered saline (PBS), and all irradiations were performed under a thin layer of PBS (200 μl/well). Immediately after irradiation, fresh serum-free medium was added to the cells. Responses were measured after a 24-hour incubation period. Mock-irradiated blanks followed the same schedule of medium changes without UVB irradiation.

2.5. Cell Viability

General viability of cultured cells was determined by reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. The Hs68 cells were seeded in 24-well plates at a density of 2×10^5/ml per well and cultured at 37°C in 5% CO_2. Cells were pretreated with the sample at a concentration of 10, 30, and 100 μg/ml for 24 hours prior to UVB irradiation. After UVB irradiation, cells were retreated with the sample and incubated for additional 24 hours, before being treated with 0.05 mg/ml (final concentration) of MTT. The blank and control groups were cultivated without sample treatment. The cells were then incubated at 37°C for an additional 4h. The medium

containing MTT was discarded, and the MTT formazan that had been produced was extracted with 200 μl of DMSO. The absorbance was read at 595 nm with a reference wavelength of 690 nm. The cell viability being calculated as follows:

Cell viability (%)

= [(OD595 of sample) / (OD595 of control)] × 100

2.6. Assays of Collagen Type I Synthesis and Collagenase Inhibition

Hs68 human fibroblasts were inoculated into 24-well plates (2×10^5 cells/well) and cultured at 37°C in 5% CO_2. Cells were pretreated with the sample at a concentration of 10, 30, and 100 μg/ml for 24 hours prior to UVB irradiation. After UVB irradiation, cells were retreated with the sample and incubated for additional 24 hours. The blank and control groups were cultivated without sample treatment. After culturing, the supernatant was collected from each well, and the amount of pro-collagen type I was measured with a procollagen type I C-peptide assay kit (Takara Bio, Japan). The activity of collagenase was measured with a matrix metalloproteinase-1 (MMP-1) human biotrak ELISA system (Amersham life science, USA).

2.7. Tyrosinase Inhibition Assay

Tyrosinase activity was determined essentially as previously described [6]. The reaction mixtures were prepared by adding 40U of mushroom tyrosinase to 20 μl of ME dissolved in distilled water (0.1, 1, and 10 mg/ml), and then adding 40 μl of 1.5 mM L-tyrosine and 220 μl of 0.1 M sodium phosphate buffer (pH 6.5). The resulting mixture (300 μl) was incubated for 10 min at 37°C, and then absorbance at 490 nm was measured. The same mixture, but without ME, was used as a control.

2.8. Inhibition of L-DOPA Oxidation

The inhibitory effect of ME on L-DOPA oxidation was determined according to the method of Joshi with a slight modification [7]. 50 μl of ME dissolved in 0.1 M sodium phosphate buffer (0.1, 1, and 10 mg/ml) was added to 40 U of mushroom tyrosinase in 900 μl of 0.1 M sodium phosphate buffer (pH 6.5). After 6 min of incubation at 37°C, 3 mM of L-DOPA was added. Then the mixture was incubated at 37°C for 15 min. Activities were quantified by measuring absorbance at 475 nm. The same mixture, but without ME, was used as a control.

2.9. Statistical Analysis

The results were expressed as means ± standard error of the mean (SEM). Significances of changes were evaluated using the one-way ANOVA with Dunnett's post-hoc test. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Cytotoxicity on Hs68 Human Fibroblasts

In order to evaluate the cytotoxicity of ME, samples were prepared at various concentrations and used to treat human fibroblasts (Hs68). The results of this evaluation are shown in Figure 1 at concentrations of 10, 30, 100 μg/ml. The cell viability was recalculated into 100% of control group. The cell viabilities of ME 10 μg/ml treated, ME

30 μg/ml treated, ME 100 μg/ml treated are 100.5 ± 0.9%, 97.3 ± 0.3%, and 99.6 ± 0.0%, respectively. ME showed no cytotoxicity up to the effective concentration for anti-wrinkle activity (less than 100 μg/ml).

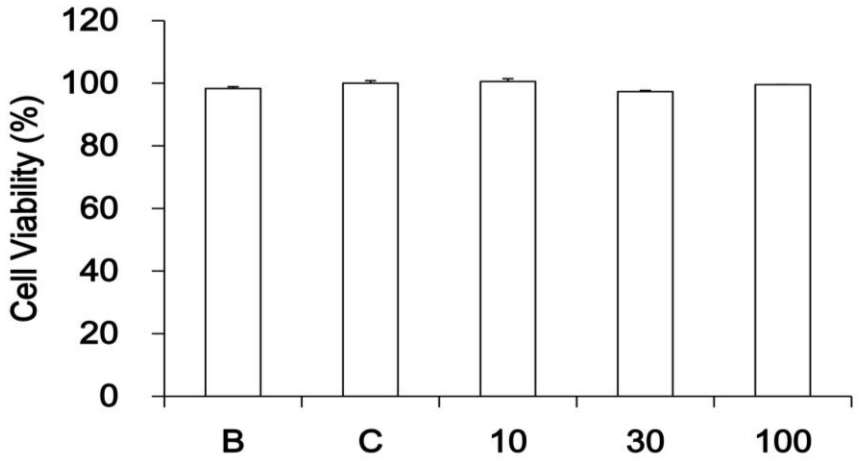

Figure 1. Cell viability of ME on Hs68 Human Fibroblasts. B: Blank, Distilled Water Treated Group without UVB Irradiation. C: Control, Distilled Water Treated Group with UVB Irradiation. 10, 30, and 100: Myrrh Extracts (ME 10, 30, and 100 μg/ml) Treated Group. Data are Expressed as the Mean ± SEM of Three Experiments

3.2. Assay of Collagen Type I Synthesis

To evaluate the amount of collagen type I synthesis that occurred upon exposure to the sample, collagen type I was quantitatively detected by using the procollagen type I C-peptide assay kit previously described in the methods section. Collagens are synthesized as precursor molecules, called procollagens. These molecules contain additional peptide sequences, usually referred to as 'propeptides', at both the amino-terminal end and the carboxy-terminal end. These propeptides are cleaved from the collagen triple-helix molecule during its secretion, after which the triple-helix collagens are polymerized into extracellular collagen fibrils. Thus, the amount of free propeptides stoichiometrically reflects the amount of collagen molecules synthesized [8]. The amounts of type I collagen synthesis of ME were shown in Figure 2. ME did not increase the expression of type I collagen at all concentrations of 10, 30, and 100 μg/ml (7.9 ± 1.3 ng/ml, 5.4 ± 2.0 ng/ml, and 5.4 ± 2.1 ng/ml) compared with control group (14.0 ± 0.5 ng/ml, Figure 2).

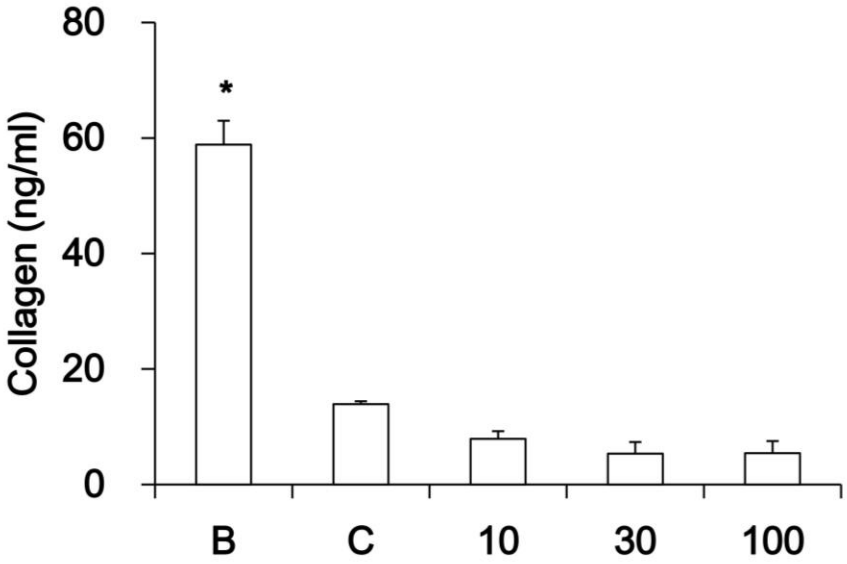

Figure 2. Effect of ME on Collagen Type I Synthesis in Human Fibroblast Cells. B: Blank, Distilled Water Treated Group without UVB Irradiation. C: Control, Distilled Water Treated Group with UVB Irradiation. 10, 30, and 100: Myrrh Extracts (ME 10, 30, and 100 μg/ml) Treated Group. Data are Expressed as the Mean ± SEM of Three Experiments. *: Significantly Different from the Control, $p < 0.05$

3.3. Assay of Collagenase Activity

To evaluate the collagenase activity, MMP-1 activity was quantitatively measured by using the previously described matrix metalloproteinase-1 assay kit. The activities of MMP-1 of ME treatment were recalculated into 100% of control group (Figure 3). ME reduced the MMP-1 activity at concentrations of 10 μg/ml, 30 μg/ml, and 100 μg/ml in a dose dependent manner (32.4 ± 8.4%, 20.7 ± 5.8%, and 13.0 ± 3.4%). All ME concentrations showed the statistical significances ($p < 0.05$).

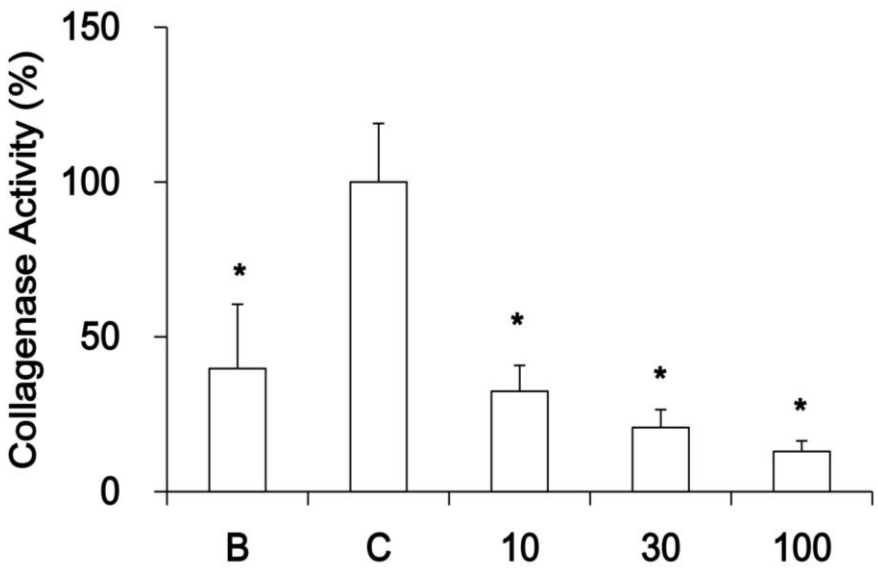

Figure 3. Effect of ME on Collagenase Activity in Human Fibroblast Cells. B: Blank, Distilled Water Treated Group without UVB Irradiation. C: Control, Distilled Water Treated Group with UVB Irradiation. 10, 30, and 100: Myrrh Extracts (ME 10, 30, and 100 μg/ml) Treated Group. Data are Expressed as the Mean ± SEM of Three Experiments. *: Significantly Different from the Control, $p < 0.05$

3.4. Inhibitory Effects on Tyrosinase Activity

The activities of ME on tyrosinase activity were recalculated into 100% of control group (Figure 4). The tyrosinase activity of ME 0.1, 1, and 10 mg/ml treated groups showed the dose-dependant reductions (85.7 ± 3.2%, 76.2 ± 5.8%, and 10.3 ± 4.7%). 10 mg/ml treated group showed the statistical difference ($p < 0.05$).

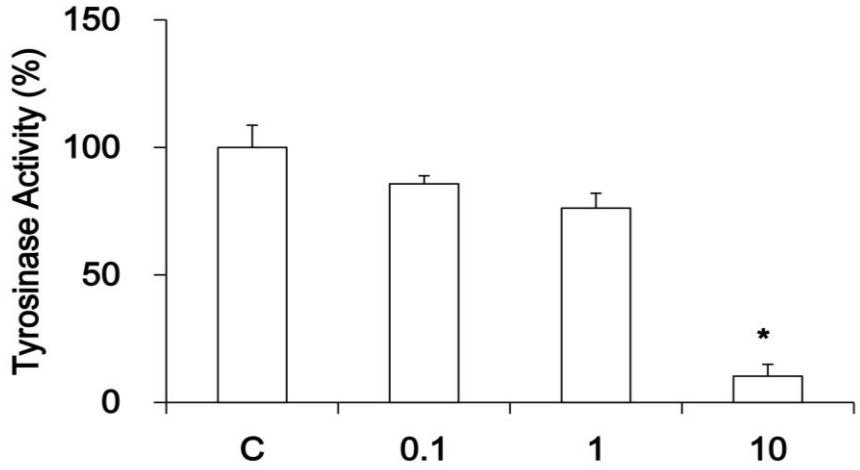

Figure 4. Effect of ME on Tyrosinase Activity. C: Control, Distilled Water Treated Group. 0.1, 1, and 10: Myrrh Extracts (ME 0.1, 1, and 10 mg/ml) Treated Group. Data are Expressed as the Mean ± SEM of Three Experiments. . *: Significantly Different from the Control, p < 0.05

3.5. Inhibitory Effects on L-DOPA Oxidation

The activities of ME on L-DOPA oxidation were recalculated into 100% of control group (Figure 5). ME 0.1, 1 and 10 mg/ml treated groups did not show any activity (112.9 ± 6.4%, 86.8 ± 3.5%, and 86.3 ± 8.5% respectively).

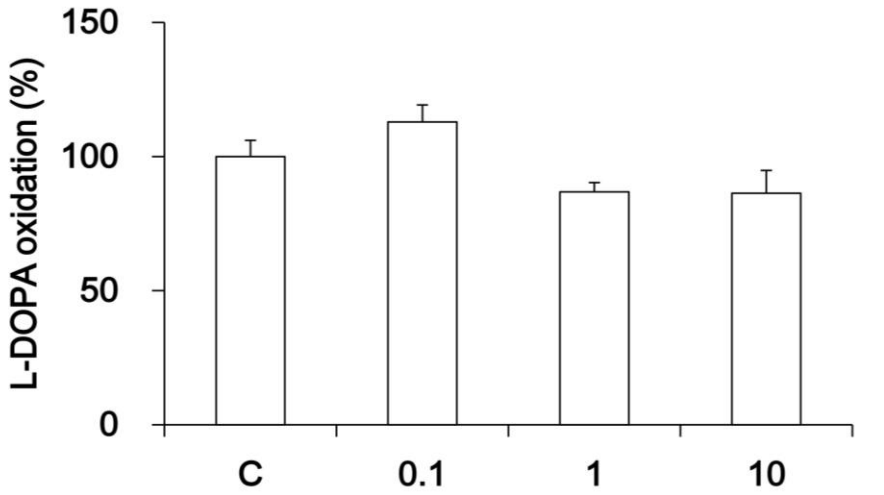

Figure 5. Effect of ME on L-DOPA Oxidation. C: Control, Distilled Water Treated Group. 0.1, 1, and 10: Myrrh Extracts (ME 0.1, 1, and 10 mg/ml) Treated Group. Data are Expressed as the Mean ± SEM of Three Experiments

4. Discussion

The ME has been used for the relief of pain symptoms caused by the stagnation of qi and blood circulation. Clinical manifestations are dysmenorrhea, amenorrhea, epigastric pain, and traumatic injuries. It may be applied with olibanum for skin lesions and ulcerations. Their topical application one to two times daily for 3 to 5 days shows satisfactory results in patients suffering from acute sprain of the lower back and legs as well. [1-2].

Its chemical compositions are heerabomyrrholic acid, commiphoric acid, commiphorinic acid, heerabomyrrhol, heeraborescene, commiferin, ergenol, m-cresol, and cumin aldehyde [2].

Skin aging is one of the most obvious evidence of aging. The skin is increasingly exposed to ambient UV-irradiation, thus increasing risks for photooxidative damage with long-term detrimental effects like photoaging, which is characterized by wrinkles, and loss of skin tone and resilience. Photoaged skin displays alterations in the cellular component and extracellular matrix with accumulation of disorganized elastin and its microfibrillar component fibrilin in the deep dermis and a severe loss of interstitial collagens, which are the major structural proteins of the dermal connective tissue. It has been shown that UV irradiation leads to the formation of reactive oxygen species (ROS) that activate the mitogen-activated protein (MAP) kinase pathway, which subsequently induces the expression and activation of matrix metalloproteinases (MMPs) in human skin in vivo [4-5]. MMPs are known to be over-expressed in human fibroblasts within hours after exposure to UV irradiation. Therefore, MMPs are considered key regulators in the photoaging process. Inhibiting the major collagen-degrading enzymes like MMPs would be useful agents for anti-aging.

In order to evaluate the cytotoxicity of ME, samples were prepared at various concentrations and used to treat human fibroblasts (Hs68). There was no cytotoxicity in all treated concentrations.

Collagen is a group of naturally occurring proteins. In nature, it is found exclusively in animals, especially in the flesh and connective tissues of mammals [9]. It is the main component of the connective tissues, and is the most abundant protein in mammals, making up about 25% to 35% of the whole-body protein content [10].

Collagen, in the form of elongated fibrils, is mostly found in fibrous tissues such as the tendon, ligament and skin, and is also abundant in the cornea, cartilage, bone, blood vessels, the gut, and intervertebral disc. In muscle tissue, it serves as a major component of endomysium. Collagen constitutes 1% to 2% of muscle tissue, and accounts for 6% of the weight of strong, tendinous muscles [11]. Collagen occurs in many places throughout the body. So far, only 29 types of collagen have been identified and described. Over 90% of the collagen in the body, however, is of type I, II, III, and IV. Among them, collagen type I is placed at skin, tendon, vascular, ligature, organs, and bone (main component of bone). Collagen-related diseases most commonly arise from genetic defects or nutritional deficiencies that affect the biosynthesis, assembly, postranslational modification, secretion, or other processes involved in normal collagen production [11].

In the present study, the amount of collagen type I did not increase. However, ME significantly reduced the collagenase activity (MMP-1 activity). The collagen metabolism is regulated by modulating collagen synthesis and collagenase activity. It also reduced the tyrosinase activity. Consequently, ME would be thought to increase the collagen in tissue by the collagenase-inhibitory mechanism.

5. Conclusions

In conclusion, ME showed inhibitory activity on the collagenase (MMP-1) and tyrosinase. The reduced collagenase activity might delay the process of skin-aging. Accordingly, these results suggest that ME may have potential as an anti-aging ingredient in cosmetic herbal drugs. It is to be hoped that further studies will be needed to unravel the underlying molecular mechanisms.

Acknowledgments

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References

[1] I. R. Kim, H. C. Kim, Y. B. Kuk, S. J. Park, Y. K. Park, J. H. Park, B. I. Seo, Y. B. Seo, H. J. Song, M. K. Shin, Y. J. Lee, Y. C. Lee, J. H. Lee, K. H. Leem, S. I. Cho, J. K. Chung, Y. S. Joo and H. Y. Choi, Boncho-Hak, Young-Lim Press, Seoul, **(1999)**.
[2] J. K. Chen and T. T. Chen, "Chinese Medical Herbology and Pharmacology", Art of Medicine Press, CA, **(2004)**.
[3] M. Yaar, B. A. Gilchrest and J. Dermatol, Surg. Oncol, vol. 16, no. 10, **(1990)**, pp. 915.
[4] G. J. Fisher, S. C. Datta, H. S. Talwar, Z. Q. Wang, J. Varani, S. Kang and J. J. Voorhees, "Nature", vol. 379, no. 6563, **(1996)**, pp. 335.
[5] J. Y. Shin, W. Hur, J. S. Wang, J. W. Jang, C. W. Kim, S. H. Bae, S. K. Jang, S. H. Yang, Y. C. Sung, O. J. Kwon and S. K. Yoon, "E+xp. Mol. Med.", vol. 37, no. 2, **(2005)**, pp. 138.
[6] A. Vanni, D. Gastaldi and G. Giunata, "Ann. Chim.", vol. 80, no. 35, **(1990)**.
[7] P. C. Joshi, C. Carraro and M. A. Pathak, "Biochem. Biophys. Res. Commun.", vol. 142, no. 1, **(1987)**, pp. 265.
[8] Y. H. Kim, C. B. Chung, J. G. Kim, K. I. Ko, S. H. Park, J. H. Kim, S. Y. Eom, Y. S. Kim, Y. I. Hwang and K. H. Kim, "Biosci. Biotechnol. Biochem", vol. 72, no. 2, **(2008)**, pp. 303.
[9] W. E. G. Müller, "Integr. Comp. Biol.", vol. 43, no. 1, **(2003)**, pp. 3.
[10] G. A. Di Lullo, S. M. Sweeney, J. Korkko, L. Ala-Kokko and J. D. San Antonio, Journal of Biol. Chem.", vol. 277, no. 6, **(2002)**, pp. 4223.
[11] Z. E. Sikorski, "Chemical and Functional Properties of Food Proteins", CRC Press, Boca Raton, **(2001)**.