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## Asian Pacific Journal of Tropical Medicine

journal homepage: <http://ees.elsevier.com/apjtm>Original research <http://dx.doi.org/10.1016/j.apjtm.2016.08.001>

## Skin whitening and anti-corrugation activities of glycoprotein fractions from liquid extracts of boiled sea cucumber

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## ARTICLE INFO

## Article history:

Received 10 Apr 2016

Received in revised form 3 Jun 2016

Accepted 15 Jun 2016

Available online 20 Aug 2016

## Keywords:

Sea cucumber

Glycoprotein

Cytotoxicity

Tyrosinase

Elastase

## ABSTRACT

**Objective:** To determine skin whitening and wrinkle improvement efficacy, glycoprotein fractions were extracted from liquid extracts of boiled sea cucumber and their effects on tyrosine and elastase inhibitory activities were assayed.

**Methods:** Fractions above and below 50 kDa (>50 kDa and <50 kDa) were extracted via a series of steps involving: boiling, filtering, desalting and freeze drying. Cytotoxicity, skin whitening and wrinkle-removing effects of boiled liquid were determined.

**Results:** Our MTT data showed that neither glycoprotein fraction of boiled liquid induces cellular cytotoxicity up to a concentration of 10 mg/mL treatment of the mouse melanoma cell line, B16F10, with 10 mg/mL >50 kDa enhanced tyrosinase and elastase inhibitory activities by 50.84% and 28.78%, respectively. Correlations of the >50 kDa concentration with tyrosinase inhibitory ( $R^2 = 0.968$ ) and elastase inhibitory ( $R^2 = 0.983$ ) efficacy were significant.

**Conclusions:** >50 kDa glycoprotein fraction isolated from liquid extracts of boiled sea cucumber, which can serve as a functional cosmetic ingredient for whitening and wrinkle improvement of skin.

## 1. Introduction

Sea cucumbers are marine animals from the class Holothuroidea. About 1 200 known species of sea cucumber exist in worldwide, with using in food or folk remedies [1]. Sea cucumbers have traditionally been employed as medicines owing to their therapeutic efficacy in various diseases such as hypertension, asthma, rheumatism, incisions, burns, erectile dysfunction, and constipation. Over 300 compounds, including saponins, cerebrosides, polysaccharides, and peptides, have been identified in sea cucumber extract [2–5], with anti-angiogenic, anti-coagulation, anti-bacterial, anti-oxidation, and anti-tumor activities [6–10]. In terms of food science, sea cucumbers contain 90.5% water, 3.2% protein, 0.2% fat, and 3.5% minerals [11,12].

In addition, these animals are known as “ginseng of the sea” due to their high saponin content known for its efficacious ginseng ingredient [6,13]. The key components of dried sea cucumber are glycoprotein and chondroitin. Earlier research has focused on mucous-like polysaccharides and sulfate chondroitin structure [14]. In particular, glycoprotein, derived from marine organisms, has been shown to enhance anticancer activities and immune functions [15,16]. However, the mechanisms underlying the effects of these compounds on diseases at the molecular level remain to be elucidated.

Liquid extracts of boiled sea cucumber are byproducts of the dried cucumber production process. In general, the majority of this liquid is discarded, and results in large economic losses and raising concerns regarding environmental pollution. In addition, despite the high content of functional ingredients (for instance, polyphenol, protein, and glycogen), most studies on sea cucumbers to date have been related to food product development [17] and limited research has focused on extracting functional ingredients from the aspect of sea cucumber cooked liquid or developing functional products that based on the constituents.

In this study, liquid extracts of boiled sea cucumber were extracted from Korean sea cucumbers and glycoproteins that

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Peer review under responsibility of Hainan Medical University.

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separated by purifying the fractionated liquid based on molecular size. We evaluated the skin whitening and anti-corrugation activities of each glycoprotein fraction, with a view to determining the feasibility of using functional ingredients from sea cucumber cooked liquid for preparation of cosmetics.

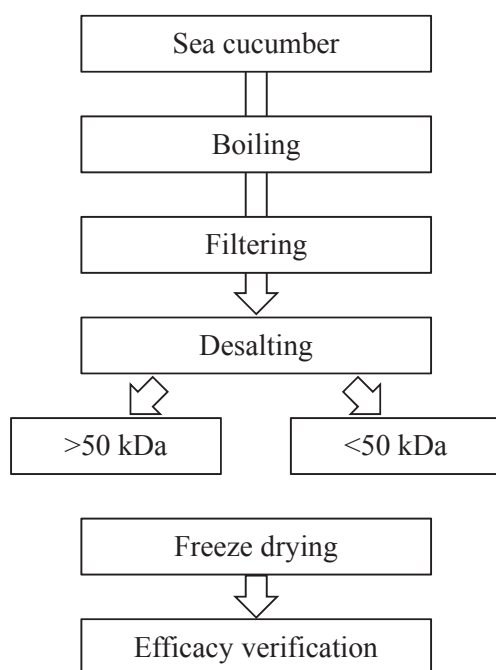
## 2. Materials and methods

### 2.1. Production of liquid extracts

We used approximately 200 g of Korean sea cucumbers (*Stichopus japonicus*, Selenka) harvested on the day of testing. Animals were eviscerated via a 3 cm incision from the anus to the center of the back and cleaned using fresh water for removal of salt and impurities. About 50 kg of eviscerated sea cucumbers were placed in boiling water and the mixture continuously stirred, and causes leakage of liquid extracts. Heating temperature was maintained at 80–90 °C. After removal of solid materials, the collected liquid extracts were stored at –20 °C until separation of glycoprotein fractions.

### 2.2. Separation and purification of glycoprotein fractions

After defrosting of the liquid extracts at 4–6 °C, the first decompression and filtration steps were conducted using a SUPRAcap depth filter (K500 P grade), followed by second depression and filtration to remove impurities using a SUPRAcap depth filter 10 inch capsule. Glycoprotein fractions with molecular weights of above and below 50 kDa (>50 kDa and <50 kDa) were separated using by an ultrafiltration system (BTR), lyophilized, and used for bioactivity assay. The separation and purification steps of glycoprotein fractions are shown in Figure 1.



**Figure 1.** Schematic diagram of isolation of glycoprotein fractions from sea cucumber.

### 2.3. Separation and purification of glycoprotein fractions

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### 2.4. Cytotoxicity assay of glycoprotein fractions

Mouse melanoma cell line suspensions (100 µL; 5 000 cells/well) (B16-F10, ATCC LOT. 60508145) were dispensed into a 96-well plate and preincubated at 37 °C in 5% CO<sub>2</sub> for 24 h for cell adherence to the well plate. The control contained 100 µL Dulbecco's Modified Eagle Medium (ATCC) without cells. A 10 µL aliquot of fractions of >50 kDa and <50 kDa of liquid extracts was added to each well for each concentration (0.1, 1, 2, and 5 mg/mL). For the negative control, 10 µL medium was added and the mixtures reacted at 37 °C in 5% CO<sub>2</sub> for 24 h. After incubation of each well with 10 µL CCK-8 solution for 1 h, absorbance was measured at 450 nm.

$$\text{Survival rate (\%)} = (A_{\text{sample}} - A_{\text{b}}) / (A_{\text{c}} - A_{\text{b}}) \times 100$$

$A_{\text{sample}}$ : sample absorbance,  $A_{\text{b}}$ : blank absorbance (value obtained with sample containing no cells),  $A_{\text{c}}$ : negative control absorbance (value obtained with distilled water containing cells). Experiments were performed in triplicate, and the results recorded as means ± SD.

### 2.5. Measurement of tyrosinase inhibitory activity

Tyrosinase inhibitory activity was assessed according to the method of Kim *et al.* [18]. A 500 µL cell suspension (50 000 cells/well) was dispensed into a 24-well plate (B16-F10, ATCC LOT. 60508145), and cells adhered by preincubating at 37 °C in 5% CO<sub>2</sub> for 24 h. Fractions >50 kDa and <50 kDa of liquid extracts and vitamin C were added to each well for all the concentrations tested and incubated at 37 °C in 5% CO<sub>2</sub> for 24 h. The pellet obtained from trypsin treatment was dissolved in 1% Triton X-100 PBS (0.5 mL), mixed with 0.2% L-DOPA (0.1 M) and 0.5 mL sodium phosphate buffer, and incubated at 37 °C for 2 h. Absorbance was measured at 490 nm.

$$\text{Inhibition (\%)} = (1 - A_1 / A_0) \times 100$$

( $A_0$  = control group absorbance,  $A_1$  = experimental group absorbance). Experiments were performed in triplicate, and the results recorded as means ± SD.

### 2.6. Elastase inhibitory activity assay

The elastase inhibitory effect was measured using the method of Kim *et al.* [19]. Fractions >50 kDa and <50 kDa of liquid extracts and vitamin C were prepared, and an aliquot of 10 µL pipetted into a test tube and mixed with 50 µL porcine

pancreas elastase (10 µg/mL) solution dissolved in 50 mM Tris–HCl buffer (pH 8.6). Next, 100 µL N-succinyl-(L-Ala)<sup>3</sup>-p-nitroanilide (0.5 mg/mL) dissolved in 50 mM Tris–HCl buffer (pH 8.6) was added as a substrate and reacted for 20 min. Elastase inhibitory activity was determined as the reduction in absorbance between groups with and without sample solutions.

$$\text{Inhibition(\%)} = (1 - A_1/A_0) \times 100$$

( $A_0$  = control group absorbance,  $A_1$  = experimental group absorbance). Experiments were performed in triplicate, and the results recorded as means  $\pm$  SD.

### 2.7. Statistical analysis

Statistical analysis was performed using SPSS statistical software. Normality and homogeneity of data were verified using analysis of variance (ANOVA), and the differences among experimental groups evaluated using one-way ANOVA and Duncan's multiple range tests.

## 3. Results

### 3.1. Cytotoxicity of glycoprotein fractions

Cytotoxicity of sea cucumber liquid extracts was analyzed using the MTT assay (Figure 2). Cells were treated with fractions above and below 50 kDa (>50 kDa and <50 kDa) derived from liquid extracts at concentrations of 0.1, 1, 2, and 5 mg/mL, and viability measured. Cell viability remained >90% with all treatment concentrations, suggesting no significant cytotoxicity of sea cucumber glycoprotein fractions at the cellular level. In contrast, positive controls (treated with 100% ethanol) showed <10% cell viability.

### 3.2. Measurement of tyrosinase inhibitory activity

Upon treatment of cells with >50 kDa and <50 kDa fractions at concentrations of 0.1, 1, 5, and 10 mg/mL, the inhibitory activity of the >50 kDa fraction was 20.54%–50.84% higher, whereas that of the <50 kDa fraction was not significant (Figure 3). In particular, tyrosinase inhibitory activity (50.84%) of the >50 kDa fraction (10 mg/mL) was high accounting for

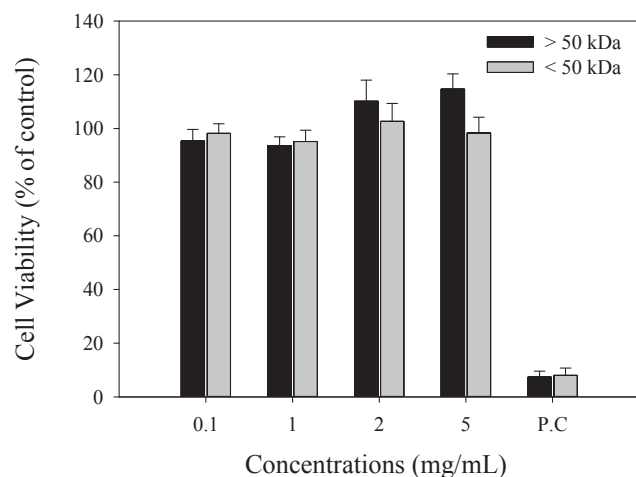


Figure 2. Cytotoxicity assay of fractions isolated from sea cucumber.

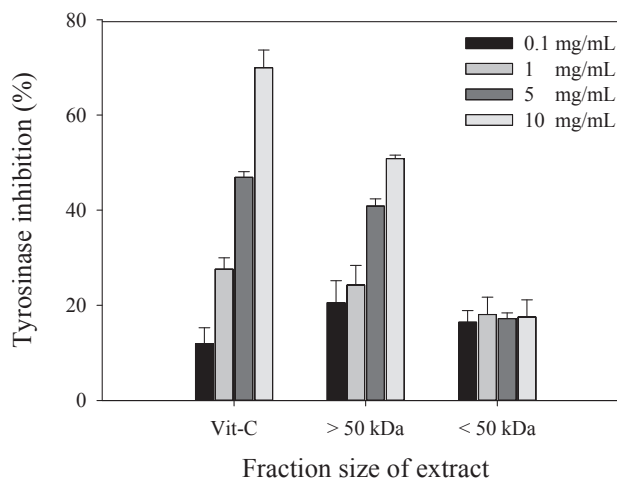


Figure 3. Tyrosinase inhibitory activities of fractions isolated from sea cucumber.

73% activity (69.95%;  $P < 0.05$ ) of ascorbic acid used as the control.

### 3.3. Analysis of elastase inhibitory activity

Upon treatment of cells with fractions >50 kDa and <50 kDa fractions at concentrations of 0.1, 1.0, 5.0, and 10.0 mg/mL, the elastase inhibitory activity of the >50 kDa fraction increased significantly to 5.75%–28.78% higher, and that of the <50 kDa fraction to 3.49%–10.40% (Figure 4). In particular, the elastase inhibitory activity of the >50 kDa fraction (28.78%) was over 2.8 times that of the <50 kDa fraction (10.40%) ( $P < 0.05$ ).

### 3.4. Correlation analysis

Correlation analyses of tyrosinase and elastase inhibitory activities with concentrations of >50 kDa glycoprotein fraction were conducted. High correlation was observed between >50 kDa levels and tyrosinase inhibitory activity ( $R^2 = 0.968$ ;  $Y = 3.096X + 21.652$ ) as well as elastase inhibitory activity ( $R^2 = 0.983$ ,  $Y = 2.468X + 4.731$ ), as shown in Figures 5 and 6.

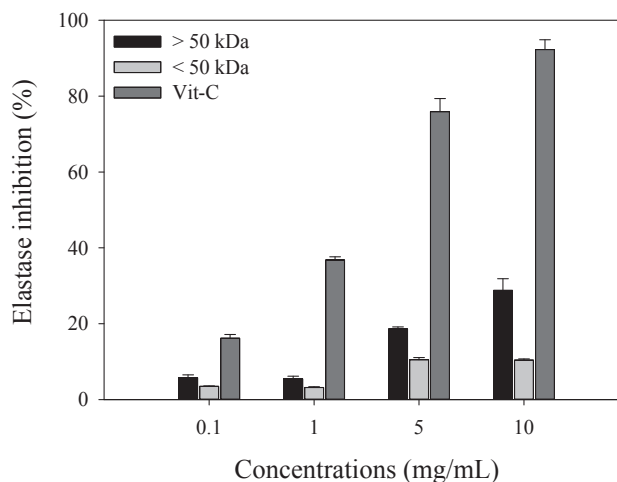
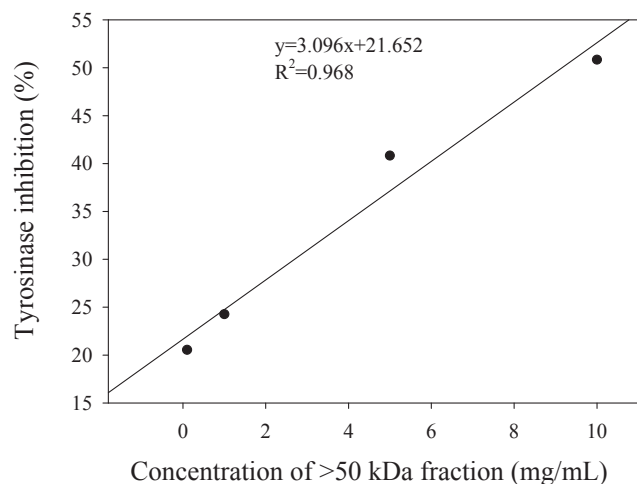
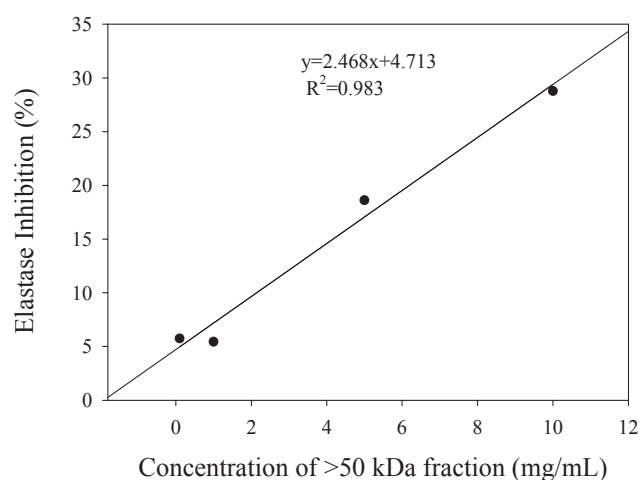


Figure 4. Elastase inhibitory activities of fractions isolated from sea cucumber.



**Figure 5.** Correlation between the >50 kDa concentration and tyrosinase inhibitory activity.



**Figure 6.** Correlation between the >50 kDa concentration and elastase inhibitory activity.

#### 4. Discussion

In this study glycoproteins from sea cucumber cooked liquid were separated according to molecular size and their cytotoxicity, whitening and anti-corrugation activities determined via MTT, tyrosinase inhibitory and elastase inhibitory assays, respectively. MTT assay is a cytotoxicity analysis technique that measures the amount of MTT-formazan, which generated from MTT by dehydrogenase present in mitochondria [20]. The whitening activity of the liquid extract obtained from boiling sea cucumber was determined by measuring dopachrome formed from L-tyrosine or L-DOPA at 490 nm [21]. Tyrosinase inhibition activity, which is effectively associated with suppression of melanin synthesis in the skin, and is useful for the development of skin whitening products [22]. Glycoprotein fractions of boiled sea cucumber (>50 kDa and <50 kDa) at concentrations of 0.1, 1.0, 2.0, and 5.0 mg/mL induced no cytotoxicity and tyrosinase inhibitory activity of the >50 kDa fraction was higher than that of the <50 kDa fraction (Figure 3), suggesting a significant whitening effect.

The connective tissues and elastic fibers of tendons mainly consist of elastin, and the elastase is key enzyme hydrolyzing the protein of key connective tissue substrate. Inhibition of elastase

activity prevents damage to elastic fibers due to UV or free radicals by protecting the skin from aging [23]. In our experiments, elastase inhibitory activity of the >50 kDa fraction was higher with increasing concentrations (Figure 4), clearly indicative of anti-corrugation activity. The anti-corrugation activity of glycoprotein, derived from sea cucumber steaming liquid, is proposed to be attributable to its anti-oxidant activity [24]. However, further studies are warranted to confirm this theory. Our findings collectively support the utility of sea cucumber extracts >50 kDa of as a valuable natural marine resource with potential functional application as a whitening and anti-corrugation ingredients in cosmetics.

#### Conflict of interest statement

The authors disclose no conflicts.

#### Acknowledgements

This research was supported by the Public Welfare & Safety Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (NRF-2013M3A2A1067529).

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