**In vitro activities of the components from scallop shells**

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**ABSTRACT:** In the present study, in order to enable effective utilization of scallop shells, the *in vitro* activities of components that were extracted from scallop shells were investigated. In particular, the scallop shell's useful ability to protect skin was evaluated. The following were found: (i) scallop shell extract inhibited generation of the superoxide anion, which was generated by xanthine and xanthine oxidase; (ii) when the scallop shell extract was supplied to culture medium for skin fibroblast cells, the cell growth rate was increased; and (iii) the scallop shell extract showed strong inhibitory activities for elastase. In the present paper, we describe the possibility of the effective utilization of scallop shells as cosmetics.

**KEY WORDS:** biological activities, cosmetic, scallop shells, utilization.

**INTRODUCTION**

Molluscan shells are mineralized by composites of CaCO$_3$ and organic proteins, which exhibit nanoscale regularity and strength. Although the organic proteins typically constitute only 1–5% of the weight of biomineralized composite material, they are responsible for its organization and the resulting strength enhancement. In recent years, there have been several reports on the cloning of the shell organic matrix proteins.1-3

The shells are composed of two kinds of CaCO$_3$ polymorphs of the prismatic layer and the nacreous layer. The nacreous layer (pearl) has been used in Chinese medicine since ancient times for keeping the skin moist and maintaining a stable emotional state. The nacreous layer is also used as a cosmetic in Japan. However, only few studies on the bioactive substances in the shell have been reported.4-6

Scallop is one of the major marine products in Hokkaido, Japan. As a result, about 300,000 tons per year of scallop shells are generated as industrial waste. Although scallop shells have been used efficiently as a source of CaCO$_3$, calcium and material for desulfurization, further uses are currently desired. First, we investigated the *in vitro* activities of components from scallop shells. In particular, we considered the possibility of using the scallop shells as a cosmetic, because the nacreous layer (pearl) has been used for keeping skin moist in Chinese medicine. In the present study, we describe the ability of components from scallop shells to protect skin.

**MATERIALS AND METHODS**

**Reagents**

Elastase (porcine pancreas), trypsin (porcine pancreas) and α-chymotrypsin (bovine pancreas) were purchased from Sigma Chemical Co., St Louis, MO, USA, as were α-N-benzoyl-DL-arginine-β-nitroanilide, N-succinyl-Ala-Ala-Pro-Phe-β-nitroanilide and N-succinyl-Ala-Ala-Pro-Phe-β-nitroanilide.

**Extraction of scallop shell components**

Scallop shells were isolated from *Pinctada yessoensis* and brushed to remove any adhered material and then crushed to a powder. The powdered shells were dialyzed against 1 L of 5% acetic acid in order to decalcify completely. This was followed by exhaustive dialysis against 1 L deionized water to remove the acetic acid. After dialysis, the decalcified solution was centrifuged at 16,000×g for 20 min at room temperature. The supernatant and the pellet were employed as the water-soluble fraction and the water-insoluble fraction, respectively. The water-soluble fraction was concentrated and loaded onto a Sephacryl S-200 gel filtration column (0.5×35 cm; Amersham Pharmacia, Little...
Chalfont, Buckinghamshire, UK) previously equilibrated with deionized water, and fractions of 1 mL were collected. Each fraction was employed as the scallop shell extract for the protease inhibitory assay and the free radical scavenging assay. The water-insoluble fraction was extracted with 70% methanol again. The methanol-soluble fraction was concentrated and dissolved at a concentration of 153 mg/mL (wet weight/vol.) in 10% dimethyl sulfoxide. The methanol-soluble fraction was also employed as scallop shell extract for the growth assay of skin fibroblast cells.

**Measurement of protease activities**

Elastase activity was measured by the method of Ito* et al.* The reaction mixture was 50 mM HEPES-NaOH (pH 7.5) and 0.3 mM butoxycarbonyl-alanine-4-nitrophenol as substrate in the presence and the absence of the scallop shell extract. After 1 μg pancreas elastase was added to the reaction mixture the color development was measured at 405 nm.

Trypsin activity was measured in a solution containing 12.5 mM Tris-HCl (pH 8.0) and 0.0625 mg/mL α-N-benzoyl-DL-arginine-p-nitroanilide in the absence and the presence of the scallop shell extract. The reaction was started by adding trypsin at a concentration of 0.5 mg/mL, and the optical density at 405 nm was then measured.

In the α-chymotrypsin inhibitory assay, 0.2 mM N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide was dissolved in 10 mM HEPES-NaOH (pH 7.5) in the absence and the presence of scallop shell extract. The reaction was started by adding α-chymotrypsin at a concentration of 0.0013 mg/mL and the optical density at 595 nm was then measured.

**Free radical scavenging activity**

Superoxide anion was generated by xanthine and xanthine oxidase. Xanthine oxidase activity was measured according to the method of Wede* et al.* The reaction mixture was 340 μM xanthine and 20 mM potassium phosphate buffer (pH 6.5) in the presence and the absence of the scallop shell extract. After the addition of 0.05 U/mL xanthine oxidase, absorbance at 290 nm was measured.

Production of superoxide radical was measured by the reduction of nitroblue tetrazolium. The reaction mixture contained 170 μM xanthine, 320 μM nitroblue tetrazolium and 20 mM potassium phosphate (pH 6.5). Various concentrations of the scallop shell extracts were added to the reaction mixture. The reaction was started by adding 0.05 U/mL xanthine oxidase and the reduction of nitroblue tetrazolium was detected by measuring absorbance at 560 nm.

**Protein concentrations**

Protein concentrations were measured according to the Lowry method.

**Quantification of saccharides**

Each fraction (10 μL) after Sephacryl S-200 gel filtration was mixed with orcinol-sulfuric acid reagent. After 20 min, the absorbance at 425 nm was measured.

**Sodium dodecylsulfate–polyacrylamide gel electrophoresis**

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli.

**Cell culture**

Human skin fibroblast cells (TIG-101) were purchased from the Japanese Collection of Research Bioresources (JCRB; Osaka, Japan) cell bank. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum (FCS) under a gas mixture of 95% air/5% CO₂.

**Growth assay of skin fibroblast cells**

Skin fibroblast cells were seeded at a density of 4 × 10⁴ cells in a volume of 50 μL per well. After 24 h, the scallop shell extract (methanol-soluble fraction) was added to the culture medium at various concentrations. The cells were treated for 24 h with the scallop shell extract and then the cell numbers were quantified by the 3-(4,5-dimethyl) ethizazole (MTT) assay.

**RESULTS AND DISCUSSION**

After decalcifying the scallop shells using 5% acetic acid, the water-soluble and -insoluble fractions were pooled as described above. SDS-PAGE revealed that the water-soluble fraction contained at least three kinds of proteins with molecular masses of 90 kDa, 20 kDa and 17 kDa (Fig. 1). In con-
contrast, the water-insoluble fraction did not show any distinct bands (Fig. 1). The water-soluble fraction was separated by a Sephacryl S-200 gel filtration column (Fig. 2a). Although the 20 kDa and 17 kDa proteins eluted in fractions 14–20 (Fig. 2b), the 90 kDa protein was not detected in any fractions by staining with Coomassie Brilliant Blue (CBB) after SDS-PAGE. Silver staining of proteins after SDS-PAGE showed that the 90 kDa protein eluted in fractions 21–23. The delay of the elution of the 90 kDa protein may be due to the interaction with the Sephacryl S-200 gel. The small amount of 90 kDa protein did not often stain with CBB, as found in a variety of other highly anionic matrix proteins. However, the 20 kDa and 17 kDa proteins did not often stain with silver but did stain with CBB.

Next, saccharides, polysaccharides or glycoproteins in each fraction were measured by the orcinol method, as it is known that the shell organic matrix contains saccharides and glycoproteins. When 10 μL of each fraction was mixed with orcinol-sulfuric acid reagent, color development was detected in many fractions (Fig. 2c), suggesting

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**Fig. 1** Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the (S) water-soluble and (I) water-insoluble fractions. The gel was stained with Coomassie Brilliant Blue (CBB).

**Fig. 2** Sephacryl S-200 gel filtration column chromatography of the scallop shell extract. (a) The water-soluble fraction from the scallop shells was separated by a Sephacryl S-200 gel filtration column equilibrated with deionized water at a flow rate of 5 mL/h. After absorbance at 280 nm, each fraction was measured and employed for protease inhibitory assay and free radical scavenging assay. (b) Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of fractions employed in the present study. The fraction number obtained by gel filtration is indicated. (S) indicates the water-soluble fraction. The gel was stained with Coomassie Brilliant Blue (CBB) except that fraction 21 was stained with silver. (c) Polysaccharide, saccharide or glycoprotein in each fraction was examined by the orcinol method as described in Materials and Methods.
that many fractions, except proteins, contain saccharides, polysaccharides or glycoproteins. In the present study, the fractions obtained after gel filtration and the water-insoluble fraction were employed as the scallop shell extract for the following assays.

Free radical scavenging assay

Skin, which has a highly differentiated and complex structure, is vulnerable to free radical damage because of its contact with oxygen. The free radical damage of skin causes alteration in the extracellular matrix of connective tissue, such as collagen and elastin, resulting in loss of skin tone and wrinkles. First, the ability of scallop shell extract to scavenge superoxide anion generated by xanthine and xanthine oxidase was investigated. The activity of xanthine oxidase was measured by the production of uric acid from xanthine, and the generation of superoxide anion was measured by formazan production from nitroblue tetrazolium. The scallop shell extract (fraction number 16) inhibited generation of superoxide anion in a dose-dependent manner (Fig. 3a). The inhibition was about 67% at a protein concentration of 0.06 mg/mL. In contrast, the scallop shell extract did not inhibit the xanthine oxidase activity (Fig. 3b) even at the concentration (0.06 mg/mL), which inhibits the generation of superoxide anion, suggesting that the scallop shells contain free radical scavenging substances.

Cell growth assay

We investigated the effect of the scallop shell extract on the growth rate of human skin fibroblast cells as described above (Fig. 4). Skin fibroblast cells secrete matrix proteins such as collagen and elastin, which are necessary for the protection of skin. When the water-soluble fraction from the scallop shells was supplied to culture medium for skin fibroblast cells, the cell growth rate did not change significantly (Fig. 4a). However, the fraction that was extracted by 70% methanol significantly enhanced the growth rate of skin fibroblast cells (Fig. 4b). The number of cells was raised to approximately 135% at doses of 1.36 and 1.53 mg/mL (wet weight/vol.) when compared to the control culture. When the cell growth assay was performed in serum-free medium to exclude an influence of FCS, a similar result was found (data not shown). These results suggest that the substance in the scallop shell extract (methanol-soluble fraction) may act as growth factor for skin fibroblast cells.

It is generally believed that increasing collagen synthesis in skin reverses skin damage, such as photoaging and keeps skin moist. The increase of collagen synthesis may be achieved by functional activation or proliferation of skin fibroblast cells.
Elastase inhibitory assay

The increase of elastase activity in skin destroys elastic fibers, resulting in reduced skin elasticity. Therefore, the elastase inhibitor has been added to some cosmetics.

When the scallop shell extract after gel filtration was examined in an elastase inhibitory assay, two fractions (fraction numbers 18 and 19) inhibited elastase activity in a dose-dependent manner (Fig. 5b). The activity was inhibited to about 80% at a protein concentration of 0.06 mg/mL, suggesting that scallop shell extract contains the elastase inhibitor. When the elastase activities were measured in solutions containing various concentrations of substrates in the presence and the absence of the scallop shell extract, double reciprocal plots of elastase activities indicated that the scallop shell extract behaved as a non-competitive inhibitor (Fig. 5b).

Thus, free radical scavenging activity, growth-promoting activity for skin fibroblast cells, and elastase inhibitory activity by components from the scallop shell extract may raise the possibility of the utilization of the scallop shells as a cosmetic for protecting skin.

Protease inhibitory assay

To examine whether the scallop shell extract inhibits other serine protease activity, trypsin and α-chymotrypsin inhibitory assays were performed as described above. In the trypsin inhibitory assay, the activity was detected in several fractions (fraction numbers 15–20). The inhibition was about 70% at a protein concentration of 0.087 mg/mL (fraction number 18) (Fig. 6a). However, no fractions exhibited inhibitory activity for α-chymotrypsin (data not shown). In contrast, several fractions (fraction numbers 25–30)
enhanced the activity for α-chymotrypsin (Fig. 6b), but not for elastase and trypsin. When the fractions were incubated with substrate in the absence of α-chymotrypsin, no α-chymotrypsin-like activity was found (Fig. 6c). These results show that the scallop shell extract contains the inhibitor for trypsin and elastase activity and the activator for α-chymotrypsin activity.

The shell organic matrix constitutes proteins, polysaccharides, lipids etc. Analysis of the water-soluble fraction by SDS-PAGE revealed at least three kinds of proteins with molecular masses of
90 kDa, 20 kDa and 17 kDa (Fig. 1). SDS-PAGE analysis of the fractions (fractions 16–18), which inhibit elastase and trypsin activities and contain free radical scavenging substances, showed bands with molecular weights of 20 kDa and 17 kDa (Fig. 2b). However, we could not conclude that the inhibitory substance and the free radical scavenging substance are identical to the proteins, because this fraction may contain saccharides or polysaccharides (Fig. 2c). The fraction (fraction number 27) that activates \( \alpha \)-chymotrypsin activity and the methanol-soluble fraction did not show a distinct band in SDS-PAGE analysis (Fig. 2b), suggesting that \( \alpha \)-chymotrypsin-activating substance and growth-promoting substance for skin fibroblast cells may not be proteins. Now, it is unclear whether these substances are proteins, glycoproteins, saccharides or other components.

The results presented here show that scallop shell extract has a useful ability in protecting skin. The utilization of scallop shells as cosmetics may be expected after further investigations.

REFERENCES