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Extracted tissue-specific atelocollagens have distinctive textural properties.

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Abstract

Food texture is a very important factor for elderly persons, children, and patients who have difficulty swallowing. Collagen and its hydrolysis product, gelatin, are used as ingredients in foods, dietary supplements, and medical materials. In this study, we extracted atelocollagen from nonedible porcine tissues, including ear, nose, and skin, and analyzed the biophysical properties of each tissue. Extracted whole auricle collagen (AEC) showed superior springiness, while only the skin region of auricle collagen (ASC) showed superior hardness, springiness and brittleness. Body skin collagen showed high hardness but low springiness. In a shear stress test, ASC gels showed high

shear strength, and their strains coincided with hardness in a textural examination, while nose and AEC showed low maximum strains. In viscosity, the auricular collagens showed higher viscosity regardless of the region of the ear. Fibril formation in collagen from each tissue and organ varied a great deal in width and morphology. We found that the same type of collagen had a unique texture and viscosity under physiological conditions depending on the tissue or organ of extraction. The results show that the collagen extracted from each organ has a unique texture and unique possibilities to serve as an ingredient in food or supplements.

Introduction

The danger of food aspiration in dysphagic patients and elderly people demands careful consideration of the physical properties of the foods they eat. Oral texture is represented in the brain areas that also represent taste, including the primary taste cortex, the orbitofrontal cortex, and the amygdala. Some neurons represent viscosity, and their responses correlate with the subjective thickness of a food and other neurons respond to other aspects of texture (Edmund, 2020; Igarashi, Arai, & Yamada, 2002).

Collagen and gelatin from the skin of fish and other animals are used as nutritional supplements due to the abundance of extracted volume and easy preparation (Gómez-Guillén, Giménez, López-Caballero, et al., 2011). Recently, it was reported that collagen peptide by oral administration was effective for promotion of wound healing in a rat model of pressure ulcers (Mistry, Steen, Clifford, et al., 2021). On the other hand, ear and nose tissues are not popular materials for these applications. Ear tissue, known for its elasticity, is eaten as a delicacy in some cultures and is also used in animal feed. Until now, neither ear nor nose tissue has been used in nutritional supplements because they provide less extractable collagen than body skin.

Cartilage of the nose and ears is a resilient and viscoelastic tissue composed of chondrocytes that cover and protect bones at the articular joints. Porcine auricular cartilage has significantly higher glycosaminoglycan and collagen contents compared to human, rat, and rabbit auricular cartilage (Chung, Erickson, Mauck, & Burdick, 2008; Derks, Sturm, Haverich, & Hilfiker 2013). The glycosaminoglycan and collagen contents of nasal tissue range from 40% to 60% of dry weight (Correro-Shahgaldian,

Introvigne, Ghayor, et al., 2016). The elasticity of organs such as nose and ear has been measured, but the physiological properties of the collagen extracted from various tissues and organs have not been revealed (Zopf, Flanagan, Nasser, et al, 2015; Chiu, Giardini-Rosa, et al., 2017). Previously, we showed that types I and III collagen extracted from the skin of pig auricle (ASC) had higher viscoelasticity and greater cell proliferative potential of fibroblast and keratinocyte compared to skin collagen ((Ishi, Hoshi, Takahashi, et al., 2019).

In this study, we extracted types I and III atelocollagen from porcine tissues of the auricle, nose, and skin, and measured the viscosity of solution, gel texture, and shear stress. We found that tissue-specific collagens have different biophysical properties in terms of texture and fibrillogenesis. In addition, we suggested that knowledge about collagen extracted from the ear and nose may be critically useful in dietary supplements for elderly persons and children.

Materials and Methods

Materials

Auricle, nose, and skin from approximately 3- to 7-month-old pigs were provided by A & V Project Co., Ltd. (Maebashi, Gunma, Japan). The pigs were a mixed breed derived from Landrace, Duroc, and Yorkshire breeds. All reagents in the extraction of collagen from porcine organs and other experimental reagents were of biochemical grade and were purchased from Wako Co. Ltd. (Tokyo, Japan). As a positive control, types I and

III collagen from porcine skin were purchased from Nippi-Collagen (Tokyo, Japan). Porcine skin collagen was purchased from Wako. Fish skin collagen was purchased from Taki Chemical Co., Ltd. (Hyogo, Japan).

Preparation and characterization of auricular collagen molecules

Atelocollagen from porcine auricle, nose, and skin was prepared by acetic acid extraction and pepsin digestion (Ishi, Hoshi, Takahashi, et al., 2019; Miller, & Rhodes, 1982; Miles, 1967). Prior to collagen extraction, all of these organs were shaved to remove all hair and the top layer of skin, cut with scissors, and blended in a blender. Crude extracted collagen was purified using 0.7 M NaCl solution. Purified collagen was centrifuged at 10,000 ×*g* for 1 h, and the pellet was dissolved in 0.1 M of acetic acid and dialyzed at 4°C against 0.1 M of acetic acid solution for 5 days. The solution was lyophilized and weighted. The extracted collagens were characterized by SDS-PAGE with a 7.5% polyacrylamide gel with 4 M urea and stained with coomassie brilliant blue (Sykes, Puddle, Francis, & Smith, 1976).

Shear force and texture measurements of collagen gels

The shear force and texture of collagen gel derived from each tissue were measured by a creep meter (RE-33005, Yamaden, Tokyo, Japan). Collagen gel samples prepared with a concentration of 2.7 mg/mL and incubated at 37°C for 3 h. The samples were 15 mm in diameter and 10 mm in length and were set on tissue culture plates. Shear force was measured using a 1.0 mm^2 cuneate probe. moving into the gel at a speed of 0.5 mm/sec,

with a load cell of 0-20 N. The breaking point of a gel specimen was defined as the first peak top or plateau point of the stress strain curve (Nomura, Toki, Ishii, & Shirai, 2000). Textural measurements were carried out using 11-mm-diameter columnar probe. The probe was moved into the gel at a speed of 0.5 mm/sec, with a compression ratio of 40%; trigger force of 20 N. The Szczesniak method was used to assess five textural parameters: hardness, brittleness, springiness, cohesiveness, and adhesiveness (Zheng, Zhou, Wang, et al., 2021). Shear force test was repeated twelve times in triplicate and more samples. Texture measurement was repeated nine times in triplicate and more samples.

Measurement of viscosity of extracted collagen

The viscosity of each collagen solution was measured using tuning fork vibro viscometers SV-10 (A&D Company, Ltd, Tokyo, Japan). The viscosity of the atelocollagen solution from each tissue was measured at a concentration of 3.0 mg/mL every 10 min. Each experiment was repeated at least three times in triplicate.

In vitro fibril formation of collagen

Fibril formation of each collagen was measured by monitoring the increases in the absorbance of the collagen solution under physiological condition at 400 nm (Kadler, Holmes, Trotter, & Chapman, 1996). For the assay, 0.1 w/v % collagen solution was prepared with 0.15 mM of Tris-HCl at pH 7.4 and centrifuged at 3,000 ×*g* for 5 min at 4°C for deaeration. These samples were transferred to a cuvette and incubated in a

37°C water bath. Optical density at 400 nm was monitored every 5 min with a photometer (Shimadzu, Kyoto, Japan) for 90 min. Each experiment was repeated at least five times in triplicate.

Transmission electron microscopy

Either 0.1 or 0.05 w/v % of each collagen sample was incubated in pH 7.5 Tris-HCl at 37°C for 24 hr, and the gel sample was loosened vigorously. For transmission electron microscopy (TEM), the samples were transferred to formvar-coated copper grids using a micropipette and allowed to settle. The samples were stained with freshly prepared 0.25％ ammonium molybdate solution for 10 min. Excess buffer was then gently removed using a filter paper (Fera, Dosemeci, Sousa, et al., 2012; Fera, Farrington, Zimmerberg, et al., 2012). The grids on the samples were measured using an electron transmission microscope (JEM-1400; JEOL, Tokyo, Japan). Each experiment was repeated at least three times in triplicate. The widths of the collagen fibrils by TEM imaging were measured using Image J over at least 20 collagen fibrils.

Statistical analysis

Statistical analysis was performed using Student's *t*-test or one-way ANOVA followed by the Tukey-Kramer method for multiple comparisons and Student’s t-test. Significant differences were defined as $p \leq 0.05$ or 0.01.

Results and Discussion

Identification of type of collagen molecules from auricle, nose, and skin tissues

To examine the physiological characteristics of collagens from auricle, skin, and nose, we extracted collagen with acetic acid and pepsin and purified it with 0.7 M NaCl. Collagen was extracted from auricular tissue in either of two ways. The auricular elastic region of collagen (AEC) was prepared by mincing whole auricle, including the chondrocyte region, and the auricular skin region collagen (ASC) was prepared by extracting small fragments from the auricle skin. Extracted collagen molecules were purified with 0.7 M NaCl solution and molecular patterns of the types of collagen were analyzed using SDS-PAGE. That analysis revealed that collagen molecules extracted from auricle, skin, and nose showed molecular patterns consistent with types I and III collagen (Figure 1). In a previous report, we showed that the molecular composition of ASC was 90% type I collagen and about 10% type III collagen molecules (Ishi, Hoshi, Takahashi, et al., 2019). In the present study, the extracted atelocollagens with acid and pepsin showed no differences among tissues (lane2-5). Future research will include an analysis of amino acid composition in relation to auricular type I collagen's biophysiological properties.

Texture analysis of tissue-specific collagen

The physiological properties of the collagen extracted from various tissues and organs have not been revealed. We measured textures of collagen gels extracted from auricle, skin, and nose under physiological conditions. These obtained data were used to calculate five textural parameters hardness, brittleness, springiness, cohesiveness, and adhesiveness by the Szczesniak method. For the hardness, ASC gel showed a value of 23 KPa, significantly higher than other tissues (Figure 2A). Skin collagen was over 17 KPa, and nose collagen was nearly 15 KPa. On the other hand, AEC showed significantly lower hardness than the other tissues, at 7 KPa. The brittleness is the force at which a material is liable to fracture when subjected to stress (Figure 2B). ASC was irrefrangible, with a value of 700 Pa among other tissues, while skin and nose collagen gels showed 400 Pa and 500 Pa, respectively. AEC showed significant brittleness, 150 Pa. Springiness was defined as the rate at which an area compressed by a force returns to its position after the force is removed. AEC showed significantly greater springiness than other tissues (Figure 2C). ASC had the next best high springiness but lower than AEC. Skin collagen gels were the least springy tissue among other tissues about 23% that of AEC. Nose collagen gels showed almost the same springiness as ASC. The adhesiveness of AEC and nose collagen were higher than that of skin (Figure 2D), but did not show significant differences compared to other tissues in experiments. As for the cohesiveness, AEC and nose collagen gels had high values, while skin collagen gel had a lower value, but the differences among tissues were not significant (Figure 2E).

Each organ and tissue had a different texture. Especially, ASC and AEC were extracted from auricle tissue but showed different textures. AEC collagen had the lowest hardness

but superior springiness compared to other tissues and showed relatively high adhesiveness and cohesiveness. On the other hand, ASC collagen gel showed high hardness and brittleness but lower springiness than AEC. Skin collagen, the most predominant use of collagen as a food or other material, showed relatively high hardness but the lowest springiness, adhesiveness, and cohesiveness. Nose collagen gel was middle texture among other tissue and was relatively high adhesiveness and cohesiveness. Dentin and bone derive their mechanical properties from a complex arrangement of collagen type I fibrils reinforced with nanocrystalline apatite mineral in extra- and intrafibrillar compartments (Silver, Freeman, Horvath, & Landis, 2001).

These results suggested that the extracted tissue-specific collagen gels had unique textual properties.

Viscosity and shear stress tissue-specific type I and III collagen molecules

Fat and oil viscosity and texture are activated in the human brain and mouth (Edmund 2019). Some neurons represent viscosity, and their responses correlate with the subjective thickness of a food, while other neurons respond to other aspects of texture (Edmund, 2020; Igarashi, Arai, & Yamada, 2002). We measured viscosity of extracted collagen solution and shear stress of collagen gel under physiological conditions and from tissues. Auricular collagen, either ASC or AEC, showed high viscosity of about 90 or 80 mPa per second at 4 ℃, respectively (Figure 3). The viscosity of auricular collagen was 1.5 times high that of skin and nose collagen..

The viscosity of macromolecules depends significantly on molecular structure. For

example, xanthan gum, a thickener, is useful for increasing the viscosity of a liquid without substantially changing its other properties (Araie, Ono-Minagi, Usami, et al., 2020). Structure of xanthan gum is a double-helical conformation, and its properties include high viscosity due to the entanglement of high molecular structure. Structure of collagen molecules forms rigid fibrils composed of a triple-helix molecular structure (Holmes, Kirk, Tronci, et al., 2017). We measured molecular structures of the extracted AEC, ASC, nose, and skin collagen molecules using circular dichroism (CD) spectra. ASC showed a highly rigid α-helical molecular formation compared to other tissues (data not shown). This result suggested a correlation between the conformation and viscosity of collagen.

Next, we examined shear stress tests of the reconstituted collagen gels from the four tissues and organs. As shown Figure 4A, the ASC gel showed superior hardness, followed by the skin and nose gels. On the other hand, AEC maintained its structure up to 100% strain but showed small shear stress and less hardness than the other tissues. These results suggested that ASC and AEC were of the same organ but showed different physiological characteristics against shear force. For nose collagen, the maximum shear force reached 75% of strain. In strain forces of 20％ and 60％, nose collagen showed the highest stress among the tissues, and these results were associated with brittleness in the texture analysis. The nose gel had a character soft and fragile by even a small strain. In maximum shear stress, ASC showed the highest shear force among the specimens, at 230 KPa, followed by skin, at 170 KPa (Figure 4B). On the other hand, the maximum shear force of AEC and of nose were only about one-third that of ASC. The maximum shear force of tissues

were associated with hardness in the textural analysis.

Fibrillogenesis and morphology of porcine collagen fibrils

An important factor in the mechanical strength of biomacromolecules is fibrillogenesis by self-association under physiological conditions (Harris, Soliakov, & Lewis, 2013). We evaluated biophysiological properties of fibrillogenesis collagen of each tissue. We used optical to examine the fibrillogenesis of the collagen. As shown in Figure 5A, AEC had the highest turbidity among the tissues, and absorbance value was increased to about 1.4 for 90 min. ASC showed high fibrillogenesis similar to that of AEC.

Turbidity reflects the extent to which collagen molecules self-assembled fibrils (Shayegan, & Forde, 2013; Williams, Gelman, Poppke, et al., 1978). AEC fibrillogenesis on the log phase showed superior among all tissues and the variation in the turbidity of it increased with time, reaching a near plateau after 10 min (Figure 5B). Fibrillogenesis of body skin collagen was lower than that of ASC, and nose collagen had the lowest fibrillogenesis on the log phase. Results of fibrillogenesis of collagen molecules on log phase were associated with results of springiness in texture analysis. Shark skin collagen showed the characteristics of well-developed fibrils compared to those of pig skin collagen, and a shark skin concentration of 0.2% gel had over 2.0 MPa mechanical strength, which was 5 times that of pig skin gel (Notbohm, Nokelainen, Myllyharju, et al., 1999). This was attributed to the fact that shark collagen gel is composed of more homogeneous fibrils and a network having more points of fibril-to-fibril interaction than pig collagen gel.

The components of extracellular matrix possess different structural formations as well as different mechanical properties such as viscoelasticity depending on the tissue (Zopf, Flanagan, Nasser, et al., 2015; Chiu, Giardini-Rosa, Weber, et al. 2017; Ishi, Hoshi, Takahashi, et al., 2019; Wood, & Keech, 1960; Parry, 1988). We observed structural differences among tissues of collagen molecules using TEM. The TEM findings revealed that the collagen fibrils had different structures depending on each tissues (Figure 6). ASC and AEC collagen fibrils were extracted from the same organ yet had quite different shapes. ASC fibrils were thinner but significantly longer (Figure 6A, 6E), while AEC collagen fibrils were thicker than those of ASC (Figure 6B, 6F). The difference in fibril structures between ASC and AEC may suggest differences in physiological characteristics as well as in hardness, brittleness, and shear stress. The bold fibrils of AEC may be formed by ASC conformation and proceed to fibrillogenesis. Skin type I collagen formed networks of flat fibrils (Figure 6C, 6G). On the other hand, collagen fibrils extracted from nose tissue showed a fine and waggly structure (Figure 6D, 6H). Nose collagen was not hard, but its adhesiveness and easy deformability from shear stress. Collagen fibrillogenesis may involve a correlation between fibril textural properties and the formation of each organ and tissue.

Conclusion

In this study, we extracted atelocollagen with acid and pepsin, and purified collagen samples from porcine organs or tissues, including the ear, skin, and nose. The molecular patterns of extracts revealed same types I and III atelocollagen in all of tissues and organs

by SDS-PAGE. Extracted collagen solution and gel specimens were evaluated for texture, shear stress, and fibril formation by each tissue. Another possible cause of the different properties among each tissue collagens is that the minor type of collagen may be slightly present below the detection level in this study.

Interestingly, ASC and AEC were both extracted from the auricle but their fibrils had different physiological characteristics. The texture analysis showed that ASC was the hardest and also springy, while AEC had superior springiness but was easy to fracture. Skin collagen had superior hardness but showed very little springiness, adhesiveness, or cohesiveness among other tissues. The physiological characteristics of nose collagen were not hardness and easy deformability, but relatively high adhesiveness. Fibrils from collagen extracted from nose showed a fine and waggly structure compared to the other collagen fibrils.

Extracted types I and III collagen from skin and ASC were derived from both skin regions, which showed the same hardness, while springiness and fibril figures showed noticeably differences between skin and ASC.

We prepared gelatin samples which a concentration of 6.5 mg /ml of auricular collagen (ASC) and skin collagen following the methods of JIS by hydrolyzation with 0.1M HCl at 60 ℃ for 24 hours. Gelatin derived from auricle (1.75 ± 0.3 mPa･s) showed higher viscosity than gelatin of skin (1.49 ± 0.16 mPa･s). When collagen molecules were degraded, regardless of whether skin and auricle tissue significantly decreased viscosity. These results suggested that tissue-specific collagen gels textures may contribute to fibril formation self-associated of molecules.

Generally, collagen and gelatin used in protein supplements and most other applications are extracted from skin, bone, and tendon. The differences in texture among the source organs and tissues make certain collagens more suitable than others for different applications. A hard oral texture is important for stimulating the brain in elderly persons and children, while brittleness is necessary to prevent elderly persons from accidentally swallowing and thus contracting pneumonia.

The results of this study suggested that knowledge about extracted collagen from auricle and nose may be critically useful in dietary supplements for dysphasic patients, elderly persons and children. We anticipate formulating new collagen supplements derived from various organs to stimulate the brain. Additionally, in this study we analyzed only nonedible tissues, including those of the auricle, nose, and skin, and obtained knowledge about their textures. Our research may promote the more effective use of resources obtained domestic animals.

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Conflicts of Interest:

Authors do not have any conflicts of interest.

Ethical Statements

This study does not involve any human or animal testing.

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Figures

Figure. 1.

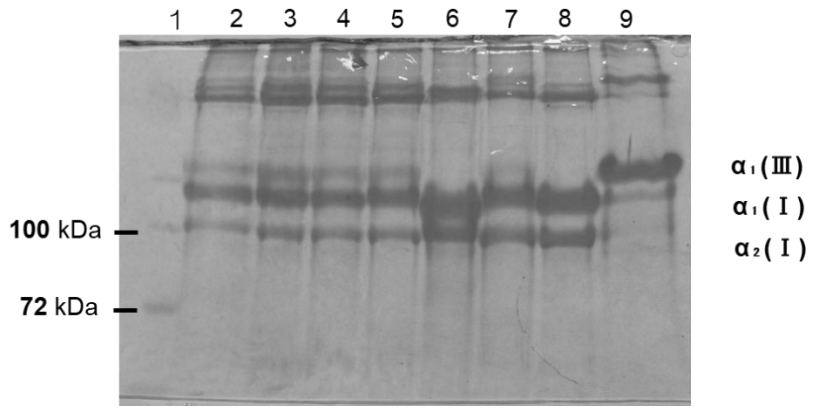

Figure .2.

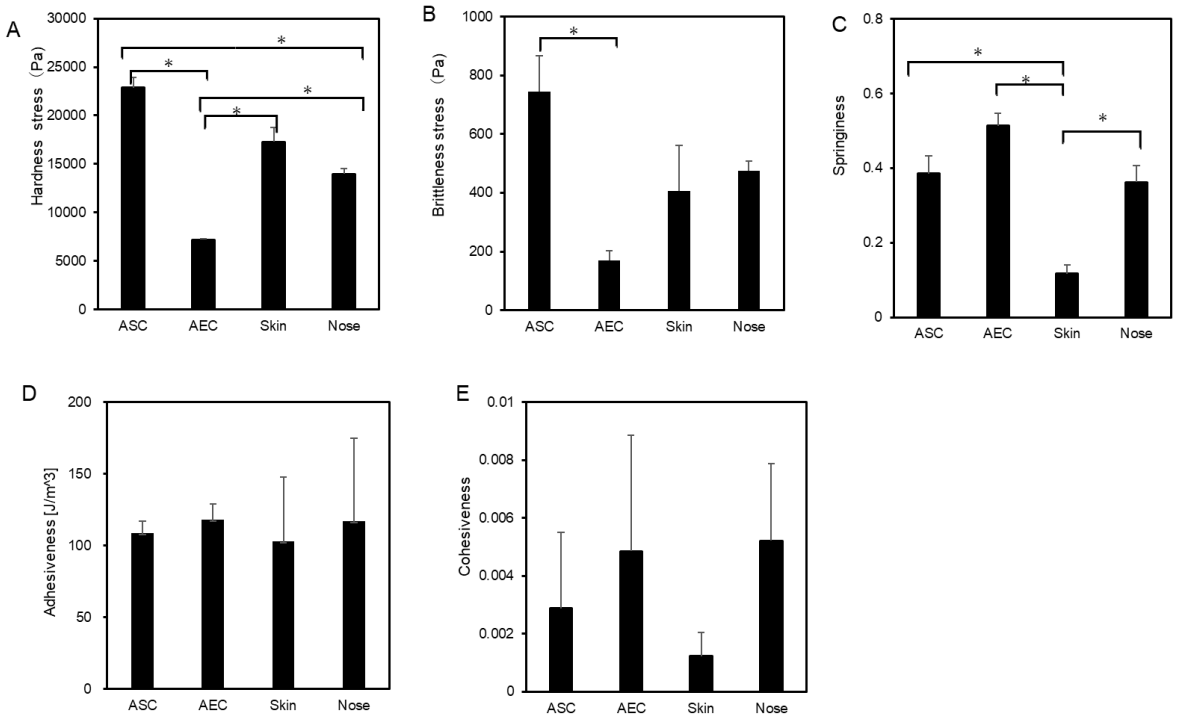

Figure. 3.

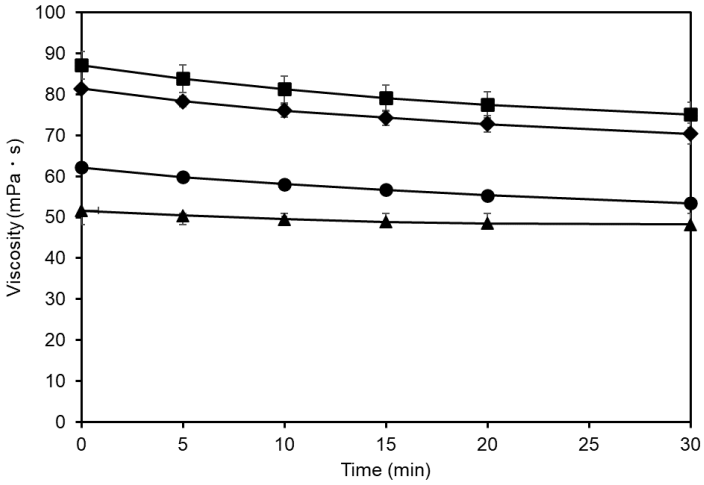

Figure. 4.

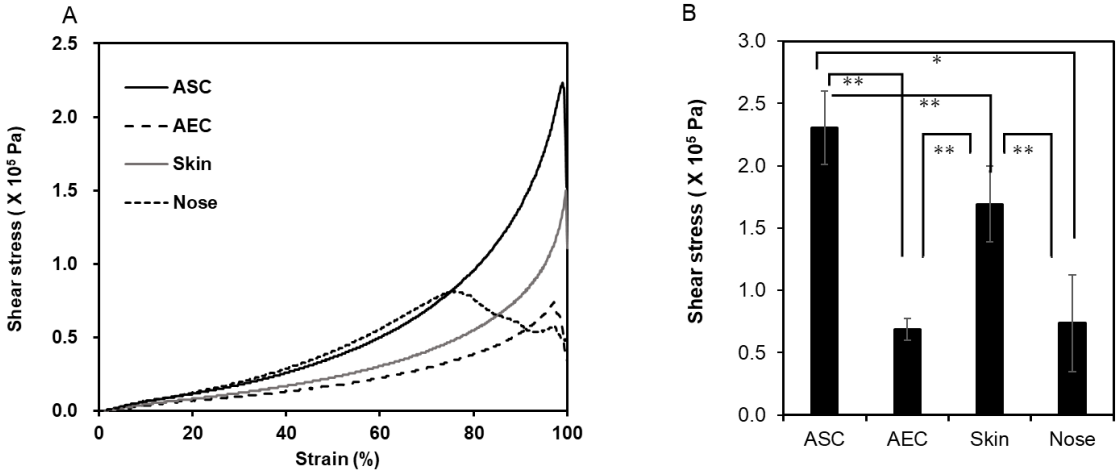

Figure. 5.

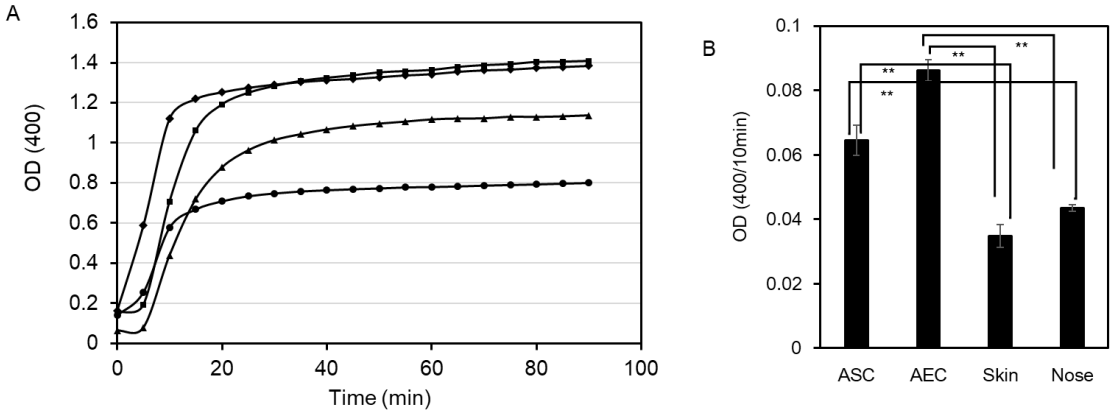

Figure. 6.