# Investigation of water-soluble elastin as a multifunctional cosmetic material: Moisturizing and whitening effects

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#### Synopsis

Elastin and collagen are extracellular matrix proteins that are widely distributed in the body. Although elastin essentially functions as a skin moisturizer, there have been few reports on its other fundamental chemical and biological functions. In this study, we investigated the moisturizing and whitening (tyrosinase inhibition) effects of elastin to examine its usefulness as a cosmetic material. Water-soluble hot alkali pig aorta (HAPA)-elastin was prepared from pig aorta using the hot alkali method. HAPA-elastin showed a widely distributed molecular weight and had a coacervation property that mediated reversible self-assembly of its molecules with increasing temperature. Amino acid analysis of HAPA-elastin showed a high content (81.5%) of hydrophobic amino acids such as Gly, Ala, Val, and Pro. Des (desmosine) and Ide (isodesmosine), which are characteristic amino acids of elastin, accounted for more than 0.4% of the total amino acid content. HAPA-elastin showed a moisture-retaining property. The water content of skin samples treated with and without HAPA-elastin was 77.2%  $\pm$  7.8% and 49.4%  $\pm$  10.1%, respectively. HAPA-elastin also inhibited tyrosinase activity by 11.3%  $\pm$  3.9%. The results obtained indicate that elastin has a useful function as a cosmetic material.

#### INTRODUCTION

Elastin is an extracellular matrix protein, which imparts elasticity to connective tissues such as ligaments, arterial walls, lungs, and skin (1). The reversible self-assembly of elastin molecules, which is temperature dependent, is termed "coacervation." This characteristic is important for the formation of mature elastin and the expression of elasticity in elastic tissues. Elastin plays principal roles in achieving elasticity in the body. It is important to maintain the elastin content in human tissues; however, the elastin content decreases owing to various factors such as aging (2). Therefore, increasing and sustaining the content of elastin in tissues can contribute to the maintenance of health and beauty. It has been reported that ingesting water-soluble elastins prepared from several animal sources has a positive influence on the human health and beauty. For example, ingestion of water-soluble elastin improved the skin condition, such as improvement in texture and moisture of skin (3,4).

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Various compounds have been applied as cosmetic materials to improve the skin condition. Recently, some cosmetics containing amino acids such as Gly, Ala, and Pro, which are abundantly present in elastin, have been noted to preserve the skin moisture. These amino acids maintain a healthy skin condition and have moisturizing effects (5–7). Several elastins prepared from bovine ligamentum and porcine aorta show distinctive amino acid compositions and are rich in hydrophobic amino acids, Gly (30–36%), Ala (23–25%), Val (10–13%), and Pro (9–12%). These amino acid compositions are characteristic of the elastins and retain the skin moisture (8). Elastin is widely distributed in the body and is known to be almost nonantigenic (9). The abovementioned features of elastin make it a favorable cosmetic and medicinal material.

There are growing numbers of elastin-containing cosmetic products on the market, validating the moisturizing action of elastin. However, there are only a few reports explaining the fundamental chemistry of elastin as a useful cosmetic compound (3,4). Therefore, we investigated whether elastin has other desirable effects as a cosmetic material, such as whitening effect that has not been reported.

In this study, we used hot alkali pig aorta (HAPA)-elastin (water-soluble elastin), which was prepared from pig aorta and was a mixture of elastin, and its degradation products. This material is sold as an ingredient for functional foods. The molecular weight distribution and amino acid composition of the elastin fractions of HAPA-elastin were examined to identify their cosmetic characteristics. We focused on the moisturizing and whitening effects of HAPA-elastin. We also studied its moisture-retention effect using a three-dimensional cultured human epidermis, as well as its *in vitro* inhibitory effect on tyrosinase activity.

# MATERIALS AND METHODS

#### MATERIALS

Water-soluble HAPA-elastin (Farm elastin<sup>®</sup> for cosmetics, E&C HealthCare Ltd. Kagoshima, Japan) prepared from pig aorta by the hot alkali hydrolysis method was a kind gift from E&C HealthCare Ltd. We purchased both 3,4-dihydroxy-L-phenylalanine (DOPA) and "tyrosinase from mushroom" from Sigma-Aldrich (St. Louis, MO). All other chemicals were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

#### AMINO ACID ANALYSIS

HAPA-elastin was hydrolyzed using 6 M HCl for 48 h at 110°C *in vacuo*. After hydrolysis, the samples were dried and redissolved in 0.02 M HCl. Amino acid analysis was carried out with an amino acid analyzer (JLC-500/V, JEOL Ltd., Tokyo, Japan), using lithium buffers at an increasing pH. The analysis was performed at Yamaguchi Prefectural Industrial Technology Institute (Yamaguchi, Japan).

#### GEL FILTRATION CHROMATOGRAPHY

The gel filtration chromatography was performed on a 1220 Infinity LC System (Agilent Technologies, Santa Clara, CA) using a TSK gel G2000 SW<sub>XL</sub> column (7.8  $\times$  300 mm, 5  $\mu$ m; Tosoh Co., Tokyo, Japan). The mobile phase was 50 mM phosphate buffer containing

0.3 M NaCl (pH 6.9). The flow rate was set at 0.2 ml/min. Absorbance was measured at 220 nm. A lyophilized mixture of molecular weight markers ranging from 1,350 to 670,000 Da was used as the molecular mass standard (Bio-Rad Laboratories, Inc., Hercules, CA).

# MEASUREMENT OF THE TURBIDITY OF HAPA-ELASTIN

The coacervation potency of HAPA-elastin was evaluated by measuring its turbidity in distilled water (D.W.). The measurement was performed at 400 nm with a JASCO Ubest V-560 spectrophotometer (JASCO Co., Tokyo, Japan). HAPA-elastin was dissolved in D.W. at a concentration of 50 or 100 mg/ml at a low temperature ( $<4^{\circ}$ C). The measurements were taken during a progressive heating and cooling (0.5°C/min) cycle from 5°C to 65°C.

# SEPARATION OF ELASTIN BY THE COACERVATION TECHNIQUE

The coacervation technique was used to obtain two fractions from the HAPA-elastin solution. HAPA-elastin was dissolved in D.W. at a concentration 160 mg/ml, which is a sufficient concentration for the self-assembly of water-soluble elastin molecules. The solution was then heated to  $65-70^{\circ}$ C using a thermostatic bath and centrifuged at 2,350 g for 2 min at 40°C to obtain the two fractions by coacervation. The supernatant was collected as the low-molecular-weight fraction (fraction 1) and the precipitate was the high-molecular-weight fraction 2). The procedure is outlined in Figure 1.

# WATER CONTENT OF RECONSTRUCTED HUMAN EPIDERMIS

LabCyte EPI-MODEL 12 (LabCyte, Japan Tissue Engineering Co., Ltd. Gamagori, Japan) was cultured from human epidermal cells and stratified using polyethylene terephthalate membrane as a supporting layer. It morphologically resembles the human skin. LabCyte consists of a stratum corneum and a viable epidermis that is made up of a granular layer, stratum spinosum, and a basal layer (10). It is used in many skin irritation and corrosion studies as a substitute for laboratory animals (11–13). In this study, the water

# Solution of HAPA-elastin (160 mg/ml)



Figure 1. Separation of HAPA-elastin into its fractions by coacervation.

content of the skin was investigated using the three-dimensional cultured human epidermis of LabCyte.

We used a Sep-Pak<sup>®</sup> C18 cartridge (Waters Co., Milford, MA) to completely remove salts from HAPA-elastin and its fractions. This was important as residual salts may affect the results of moisture-retaining test. HAPA-elastin solutions were prepared in D.W. at the following concentrations: 0.02%, 0.1%, 1.0%, and 8.0% (w/w). The water content of LabCyte was determined by measuring its weight, which is a simple and easy method. Solutions of fractions (fractions 1 and 2) were also prepared in D.W. at the following concentrations: 0.02%, 0.1%, and 1.0% (w/w). A 200 µl aliquot of sample solution was applied on the surface of LabCyte and the mixture was incubated at 32°C. After 30 min, the elastin solution was removed and culture plates were further incubated at 32°C for 60 min to dry the LabCyte. These procedures were repeated three times. The reconstructed epidermal tissues were cut from the culture inserts using forceps and their wet weights were measured. The skin tissues were then dried at 60°C–80°C for 24 h. Within this temperature range, the samples did not show signs of thermolysis or combustion. Finally, the dry weights of the skin tissue samples were measured. Water content was determined using the following equation:

Water content 
$$(\%) = \frac{\text{wet weight} - \text{dry weight}}{\text{wet weight}} \times 100$$
 (1)

TYROSINASE INHIBITION ASSAY

Tyrosinase inhibition was measured by the colorimetric method, using a 96-well plate (14). A 20-µl sample of elastin solution (1.0 or 10 mg/ml), 40 µl of tyrosinase (40 U/ml), and 100 µl of phosphate buffer solution (pH 6.8, 1/15 M) were mixed and added to each well in the plate and incubated at 25°C for 3 min. Subsequently, 50 µl of DOPA (2.5 mM) was added to each well and absorbance was recorded after 10 min using a microplate reader at 490 nm (Immuno-Mini NJ-2300, Nalge Nunc International Co., Rochester, NY). The amount of dopachrome produced during the reaction was determined using the following equation:

Tyrosinase inhibition (%) = 
$$(1 - A_{\text{sample}} / A_{\text{control}}) \times 100$$
 (2)

Where " $A_{\text{sample}}$ " represents the absorbance of the sample containing elastin, and " $A_{\text{control}}$ " represents the absorbance of control.

# STATISTICAL ANALYSIS

The data have been presented as the mean of triplicate measurements for each experiment. The data were analyzed using the Dunnett's test and statistical significance, was considered at p < 0.05.

#### RESULTS

#### AMINO ACID COMPOSITION

The amino acid composition of HAPA-elastin was determined by partial hydrolysis of pig aorta and purification of elastin peptide mixtures, as previously reported (15,16). The data are summarized in Table I. For all three elastin samples studied, the total content of Gly, Pro, Ala, and Val was in the range of 80.1–81.5%. Conversely, the contents of acidic amino acid residues, Asp and Glu, and basic amino acid residues, Lys, His, and Arg, were relatively lower and were in the ranges of 2.5-3.6% and 1.3-1.7%, respectively. It is well known that elastin contains two characteristic amino acids, Des and Ide. Together, Des and Ide residues formed 0.2-0.4% of the total amino acids in the HAPA-elastin. Our results are in good agreement with the amino acid composition of pig elastin obtained through gene sequencing, as reported in the National Center for Biotechnology Information database (NCBI, GenBank: BAP76077.1). Three main amino acid components of HAPAelastin (Gly, Ala, and Pro) are also present in high amounts in the human epidermis. It has therefore been suggested that this water-soluble elastin is a useful cosmetic material, which can supply the epidermis with the abovementioned beneficial amino acids (5-7,17).

#### COACERVATION MEASUREMENTS

HAPA-elastin showed an apparent coacervation property at a protein concentration of 100 mg/ml (Figure 2). The HAPA-elastin solution was initially clear but became turbid

Amino acid composition of HAPA-elastin and elastin peptides from porcine aorta.			
Amino acid	Residues / 1,000 total residues		
	HAPA-elastin	Elastin peptides from porcine (15)	Elastin peptides from porcine (16)
Нур	9	6	11
Asp	9	3	6
Thr	4	21	14
Ser	6	11	11
Glu	27	23	19
Pro	114	107	117
Gly	336	325	330
Ala	234	242	234
Val	131	128	120
Met	1	0	0
Ile	16	16	18
Leu	53	53	54
Tyr	11	21	16
Phe	26	29	33
Des + Ide*	4	2	3
His	7	0	1
Lys	8	8	6
Arg	2	7	6

Table I

\*Des. desmosine: Ide. isodesmosine.



Figure 2. Coacervation property of HAPA-elastin. The concentration of HAPA-elastin was either 50 or 100 mg/ml.

at 37°C, as the temperature of the solution was increased from 5°C to 65°C. Maximum turbidity was observed at 45°C. When the temperature of the solution was decreased from 65°C to 5°C, turbidity decreased in a temperature-dependent manner, and the solution became clear again at 5°C. Consequently, the profiles for change in turbidity with temperature were almost identical for both the heating and cooling cycles. This indicates that the coacervation of HAPA-elastin may be reversible; however, the reversibility is temperature dependent. As shown in Figure 2, HAPA-elastin did not show a complete coacervation property at 50 mg/ml, indicating that the coacervation of HAPA-elastin is concentration dependent.

#### SEPARATION BY COACERVATION

HAPA-elastin is prepared from pig aorta and contains a mixture of elastin degradation products. To understand in detail the characteristics of each component of the elastin degradation mixture, HAPA-elastin was further separated into its individual components by using a phase separation method and gel filtration chromatography. The solution was separated into two layers via coacervate formation. Starting with 24 g of HAPA-elastin, we obtained 20 g of fraction 1 (supernatant) and 2.4 g of fraction 2 (precipitate), which translates to 89.3% and 10.7%, respectively, of each fraction.

The molecular weight of each fraction was determined by gel filtration chromatography (Figure 3). The molecular weights of the fractions ranged from 1,350 to 670,000 Da, with those of fractions 1 and 2 ranging from 1,000 to 44,000 Da and 2,000 to 670,000



**Figure 3.** Chromatograms for HAPA-elastin and its fractions derived from gel filtration chromatography. Chromatograms for (a) HAPA-elastin, (b) fraction 1, and (c) fraction 2. Elution times of the molecular mass standards: (i) thyroglobulin (670,000 Da), (ii)  $\gamma$ -globulin (158,000 Da), (iii) ovalbumin (44,000 Da), (iv) myoglobin (17,000 Da), and (v) vitamin B<sub>12</sub> (1,350 Da) are indicated with arrows.

Da, respectively. As shown in Figure 3, the distinctive peak observed in the chromatograms for HAPA-elastin and fraction 1 represents a peptide with a molecular weight of 7,000 Da. The intensity of this peak decreased in the chromatogram for fraction 2. It confirmed that the molecular weight distribution in fraction 2 was different from that in HAPA-elastin and fraction 1. In addition, only fraction 2 showed a broad peak representing a high-molecular-weight peptide.

#### MOISTURE-RETAINING PROPERTY

The water content of LabCyte treated with HAPA-elastin, fraction 1, and fraction 2 is shown in Figure 4. The water content of LabCyte was measured to demonstrate the use-fulness of elastin in maintaining the skin moisture. The water content of the control LabCyte was  $49.4\% \pm 10.1\%$ . Treatment of LabCyte surface with HAPA-elastin at elastin concentrations of 0.1% (w/w) and 1.0% (w/w) increased the water content significantly to  $58.4\% \pm 5.7\%$  and  $65.7\% \pm 8.9\%$ , respectively. However, increasing the HAPA-elastin concentration to 8.0% resulted in a LabCyte water content of only  $52.4\% \pm 4.9\%$ . In fact, the highest concentration of HAPA-elastin that induced the highest water content in LabCyte was 1.0% (w/w).

Similar results were observed with fractions 1 and 2. The water content of LabCyte was  $59.5\% \pm 4.9\%$ ,  $65.4\% \pm 5.7\%$ , and  $77.2\% \pm 7.8\%$  when fraction 1 was applied at concentrations of 0.02% (w/w), 0.1% (w/w), and 1.0% (w/w), respectively. There were significant differences in the water contents between the control and fraction 1-treated samples at all the concentration levels studied. The water content of LabCyte was  $52.5\% \pm 8.2\%$ ,  $55.9\% \pm 6.8\%$ , and  $66.6\% \pm 8.1\%$  at fraction 2 concentrations of 0.02%, 0.1%, and 1.0% (w/w), respectively. A statistically significant difference was detected in the water content between the control LabCyte and that treated with 1.0% fraction 2. The highest water content in LabCyte in the study was 77.2%, which was approximately 1.5-fold higher than that of the control, and was obtained using fraction 1 at a concentration of 1.0% (w/w).

#### INHIBITION OF TYROSINASE ACTIVITY

The inhibitory effects of HAPA-elastin and fractions 1 and 2 on the activity of tyrosinase were examined in an *in vitro* assay using mushroom tyrosinase (Figure 5). Vitamin C (1.0 mg/ml) was used as a positive control and showed  $99\% \pm 0.86\%$  inhibition. HAPA-elastin showed a weak inhibitory effect on tyrosinase activity. At concentrations of 1.0 and 10 mg/ml, HAPA-elastin inhibited the activity of tyrosinase by about 4% and 12%, respectively. These results indicate the concentration-dependent inhibitory effects of HAPA-elastin on tyrosinase activity. At 10 mg/ml, fraction 1 showed a relatively weaker inhibitory effect than HAPA-elastin on tyrosinase activity. Conversely, fraction 2 inhibited tyrosinase to about the same extent as HAPA-elastin did. These results clearly indicate that the high-molecular-weight fractions in HAPA-elastin have an inhibitory effect on tyrosinase, demonstrating their potential as skin-lightening materials in cosmetics provided the activity can be further enhanced.

#### DISCUSSION

In this study, we investigated HAPA-elastin (water-soluble elastin) as a cosmetic material. The molecular weight of HAPA-elastin ranged from 1,350 to 670,000 Da (Figure 3).



**Figure 4.** Water contents of cells treated with HAPA-elastin, and fractions 1 and 2. Water content was  $49.4\% \pm 10.1\%$  without HAPA-elastin (i.e., only D.W.), which was used as the reference (unshaded bar). Water contents of cells treated with the respective concentrations of (a) HAPA-elastin, (b) fraction 1, and (c) fraction 2. Data are presented as mean  $\pm$  standard error of the mean. \*p < 0.05.



Figure 5. Rate of tyrosinase inhibition by HAPA-elastin and fractions 1 and 2. Tyrosinase activity was 100% when D.W. was added to the cells as the control. Data are presented as mean  $\pm$  standard error of the mean. \*p < 0.05.

Characteristically, the ratio of Des and Ide residues to total residues in HAPA-elastin is 4:1,000 (Table I). As a guideline for evaluating elastin, the Japan Health and Nutrition Food Association has stated that, the standard value of the total content of Des and Ide in pure elastin should exceed 0.2% per molar ratio (18). In this study, the total content of Des and Ide was 0.4% per molar ratio. It was within the accepted range and confirmed that HAPA-elastin was of high purity, and was efficiently prepared from the pig aorta.

Although several elastins sold on the market usually show little coacervation property, this study confirmed the coacervation ability of HAPA-elastin. In addition, HAPA-elastin has a wide molecular weight range, consisting of large-molecular-weight polypeptides and small fragments. It is considered that coacervation results from the intramolecular and intermolecular hydrophobic interactions within a sample (19,20). Amino acid analysis showed that HAPA-elastin contained many hydrophobic amino acids such as Gly, Ala, Val, and Pro. These hydrophobic amino acids accounted for more than 81.5% of the total amino acid content of HAPA-elastin. During the separation process, the elastin solution separates into two layers. One is the supernatant, which is a solution of soluble peptides (low-molecular-weight elastin). The other is the precipitate, which comprises coacervate aggregates of elastin (high-molecular-weight elastin) produced during the heating process (21,22). Fraction 1 accounted for 89.3% of the coacervate yield from HAPA-elastin and showed an apparent difference in molecular weight (Figure 3b) as compared to HAPA-elastin. Fraction 1 lacked high-molecular-weight proteins, whereas fraction 2, which accounted for 10.7% of the yield, contained a relatively higher amount of highmolecular-weight proteins (44,000-67,000 Da) (Figure 3c). HAPA-elastin is a useful

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material because it contains a high amount of hydrophobic amino acids and after its coacervation, high-molecular-weight polypeptides are produced.

In this study, we focused on the moisturizing and whitening effects of HAPA-elastin. As shown in Figure 4, the application of HAPA-elastin to LabCyte resulted in a high water retention in the epidermis. The water content of the cells increased in a concentration-dependent manner until a concentration of 8.0% was reached, following which, the water content decreased (Figure 4c). These results indicate that, at an optimum HAPA-elastin concentration, moisture can be sufficiently retained in the skin. Fractions 1 and 2 exhibited the same dose-dependent effects as HAPA-elastin. In particular, fraction 1 showed a significant moisturizing effect, producing the highest water content of 77.2% at a 1.0% elastin concentration. Some reports have claimed that oral ingestion of elastin can suppress water loss from the surface of the skin (3,4). These reports together with our results indicate that HAPA-elastin possesses a moisturizing effect, which is good for maintaining a healthy skin.

Based on the present study, there is a possibility that fraction 1, owing to its high content of low-molecular-weight peptides, can penetrate the epidermis. This could possibly explain the relatively high water content observed in the LabCyte cells after treatment with fraction 1. Generally, low-molecular-weight substances (<500 Da) can penetrate the skin (23); however, hyaluronic acid has a molecular weight of 400,000 Da, but it has been reported to penetrate both the epidermis and the dermis (24). It was reported that the water content of the skin increased by approximately 10% after 3 weeks of hyaluronic acid ingestion (25). Similarly, HAPA-elastin may be expected to exhibit moisture-retaining effects. Fraction 2 showed similar moisturizing effects that were comparable with HAPA-elastin, as shown in Figure 4. Since it is difficult for high-molecular-weight proteins to penetrate the epidermis, fraction 2 may produce a skin-covering effect and result in water retention, thereby moisturizing the skin. It can be suggested that the low-molecular-weight elastin fragments penetrate the skin to maintain its water content, whereas the high-molecularweight fragments may remain on the skin and act as a barrier against water evaporation. However, there is a need to further explore this effect.

The whitening effect of cosmetics has been widely studied in terms of effective inhibition of melanin production (26). Melanin is the main pigment present in the surface structures of vertebrates and is widely distributed in animals and plants. It is the principal factor responsible for the skin color and pigmentation. The oxidation of tyrosine by enzyme tyrosinase, which involves the hydroxylation of tyrosine to L-DOPA and the oxidation of L-DOPA to dopaquinone, is the critical step in melanin biosynthesis. The melanin biosynthesis, which consists of rate-limiting steps, is regulated by tyrosinase at an initial stage (27). Thus, inhibition of tyrosinase activity inhibits the melanin synthesis. HAPA-elastin inhibited the synthesis of melanin (Figure 5). Kojic acid and polyphenols such as flavonols are wellknown tyrosinase inhibitors (28). Furthermore, some studies have reported that several proteins and peptides derived from animals and plants inhibit tyrosinase activity (29,30). This inhibition of tyrosinase activity is mainly caused by hydrophobic and aliphatic amino acids such as Val, Ala, Leu, and Met (29). HAPA-elastin contains a large amount of all the hydrophobic and aliphatic amino acids listed earlier except Met (Table I). HAPA-elastin inhibited the tyrosinase activity by 2.0-11.3% in this study. This can be attributed to the rich hydrophobic and aliphatic amino acid content of HAPA-elastin. However, the HAPAelastin-mediated tyrosinase inhibition was lower than that mediated by other compounds such as a collagen (31). This gentle effect can be beneficial in the development of useful biomaterials without any side effects. Surprisingly, a significant difference in tyrosinase inhibition was observed between fractions 1 and 2 (Figure 5). The average molecular weights of HAPA-elastin, fraction 1, and fraction 2, determined using gel filtration chromatography (Figure 3), were 4,700, 3,900, and 10,000 Da, respectively. The molecular weights were used to calculate the molarities of each sample. The molarities for a 10 mg/ml solution of HAPA-elastin, fraction 1, and fraction 2 were 2.1, 2.6, and 1.0 mM, respectively. Although the molarities of HAPA-elastin and fraction 1 at a concentration of 10 mg/ml were almost similar, HAPA-elastin showed relatively higher tyrosinase inhibition than fraction 1 did. Interestingly, fraction 2 showed relatively higher tyrosinase inhibition than HAPAelastin and fraction 1 did, despite the low molarity of fraction 2 compared with either of them. It suggested that tyrosinase inhibition activity of elastin and elastin derivatives was not simply proportional to their molecular weight. It can be presumed that the observed difference in tyrosinase inhibition was due to the different sequences of amino acids in the various peptides and not merely due to the presence of different amino acids. It has been reported that no amino acid can, by itself, completely inhibit the activity of tyrosinase (32). Melanocytes have binding sites for the elastin-derived Val-Gly-Val-Ala-Pro-Gly (VGVAPG) hexapeptide. This hexapeptide is involved in bioactivities such as cell migration and proliferation (33). This may be the mechanism by which HAPA-elastin inhibited tyrosinase activity, which may subsequently promote skin cell turnover and result in whitening effects.

In conclusion, HAPA-elastin is useful not only as a functional food but also as a cosmetic material, because it maintains the water content of the skin and inhibits tyrosinase activity. The findings from this study may contribute to the development of better cosmetics with milder and more moisturizing effects on the skin.

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