Korean J Food Preserv
19(4), 463-469 (2012)

사단법인 한국식품저장유통학회
The Korean Society of Food Preservation

Anti-oxidant and Anti-skin-aging Effects of Abalone Viscera Extracts in Human Dermal Fibroblasts

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전복내장추출물의 항산화 및 human dermal fibroblasts에 대한 항피부노화 효과

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Abstract

In this study, the anti-oxidant and anti-elastase activities of four abalone viscera extracts were investigated to screen the most promising extract. This extract was further studied in terms of its anti-skin-aging properties. In the DPPH-scavenging assay, the Tris-HCl extract showed a 58.60±0.88% radical-scavenging activity, which was followed closely by the ethanol extract that had a 55.40±0.62% scavenging activity. In the anti-elastase assay, however, the ethanol extract showed the significantly highest elastase inhibition activity. Furthermore, none of the extracts had a harmful effect on the human dermal fibroblast, as revealed in the MTT assay. In the cell study, the effect of the ethanol extract at various concentrations on the human dermal fibroblast was investigated. At the 10 μg/mL concentration, the ethanol extract boosted the pro-collagen type I synthesis to 705.30±3.06 ng/mL and reduced the MMP-1 to 54.30±0.80 ng/mL, which was considered the optimum concentration. This is the first study that focused on the anti-oxidant and anti-skin-aging effects of abalone viscera extract. Its results may provide fundamental data for further study.

Key words : abalone, antioxidant, anti-skin-aging, MMP-1, Pro-collagen

Introduction

Aging is one of the inevitable procedures that all forms of life must endure. Along with the decay of vitality and the increase of weariness, all bio molecules, cells and organs in the human body are gradually damaged by this complex and irreversible process. And among all the organs, much more attention has been paid to the skin aging for its visual and social impact. Many research articles have been published about the two primary skin aging processes: intrinsic and extrinsic. Intrinsic skin aging is considered as the result of the elapsed time, and which is thought to be governed by the individual genetic background (1). Extrinsic skin aging is believed to be induced by various environmental factors include smoking, chronic exposure to the sun, excessive alcohol consumption and poor nutrition (2). Many studies also mentioned the important role played by reactive oxygen species (ROS) both in the intrinsic and extrinsic skin aging process (1,3-5). Therefore, the relationship between anti-oxidants and skin aging has been intensively studied, and anti-oxidants such as vitamins E and C, coenzyme Q10, glutathione, retinoid and others are considered to possess the ability to alleviate signs of aging (6-8).

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In recent years, the anti-skin-aging abilities of extracts and compounds obtained from plants or animals have been reported by many researchers. Green tea polyphenols have been demonstrated to prevent oxidative damage and inhibit matrix metalloproteinases expression in animal study (3). Gold kiwi fruit has been reported to reduce oxidative stress and inhibit glycation activity in the human skin cell study (9). And fucoidan is known for its inhibiting activity of the MMP-1 expression in the human skin cell (2).

Pacific abalone, *Haliotis Discus HannaiIno*, is important aquatic economic species widely cultured in East Asia. For thousands of years Korean and many other Asian peoples have used abalone as a traditional functional food. But during the process of abalone manufacture, abalone viscera, which accounts for nearly 20% of body weight, is generally discarded directly or sold at a very low price. This procedure not only causes environmental problems but also become a big economical waste. Therefore, effective utilization methods of abalone viscera are required to address this problem.

Recently, several studies have been published focusing on the nutrition and pharmacy values of abalone recently. González et al examined the nutritional value of abalone and the effects on serum cholesterol concentration in rats (10). Peng et al investigated the learning and memory improving ability of abalone extracts on mice (11). Lee et al studied the anti-tumor effect of abalone viscera extract (12). Li et al purified a glycosaminoglycan-like polysaccharide from abalone and the anticoagulant activity was investigated *in vitro* (13). However, to our best knowledge, no studies have been published on the anti-skin-aging ability of abalone viscera extract.

In the present study, we first evaluated the anti-oxidant and anti-elastase activity of the four verities of abalone viscera extracts. And from four extracts, we selected one which showed better result and employed it in the cell tests. With all the data obtained in this study, we hope to demonstrate the anti-skin-aging properties of abalone viscera and contribute to further exploitation in this field.

Materials and methods

Abalone sample preparation and crude extract

Fresh abalone (*Haliotis Discus* HannaiIno) was bought from local aquatic market in Wando-gun, Jeonnam-do, which was harvested in the local mariculture farm in February 2011. After abalone was shucked and eviscerated, the viscera was gathered and homogenized and stored at -20℃ before frozen at -70℃ for 48 hr. The viscera was to a freeze dryer (IlShin BioBase, Korea) and dried under vacuum over 72 hr. The extracts of abalone viscera were carried out with 4 times (v/w) of methanol, ethyl alcohol, water and 0.2 M Tris-HCl buffer (pH 8.0) at 60℃ for 6 hr. The extraction procedures were conducted triplicate. After extraction, all extracts were filtrated and then concentrated under reduced pressure.

Proximate composition

Moisture, crude protein, crude fat, and ash were determined by the AOAC official methods. Crude protein was estimated from the total nitrogen by multiplying by 6.25.

Antioxidant assay

The antioxidant activity of the extracts was examined by the conventional DPPH scavenging assay. All the extracts were dissolved in MeOH and each sample solution was mixed with 0.2 mM DPPH solution and after 10 min reaction at room temperature, the optical density was measured at 517 nm on a spectrophotometer (UV-160, Shimadzu, Japan). BHT was used as reference solution in this experiment.

Elastase assay

The method employed in this study was described previously in the literature. Elastase from porcine pancreas (Sigma, St Louis, MO, USA) was dissolved in 0.2 mM Tris-HCl buffer (pH 8.0) to make 1 unit of stock solution. The substrate suc-$(ala)_3$-p-nitroanilide was dissolved in buffer at 0.8 mM. The extracts were incubated with the enzyme solution for 30 minutes before adding substrate to start the reaction. Absorbance values at 410 nm were measured immediately following addition of the substrate. Ursolic acid(31.0 μg/mL) was employed as reference solution in this assay.

Cell culture

The human dermal fibroblast, CCD-1064SK (ATCC CRL-2076), was obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in Iscove's Modified Dulbecco's Midium containing 10% fetal bovine serum (FBS). The cells were incubated in an atmosphere of 95% air and 5% CO_2 at 37℃ and split twice per week with alternate 1/3 and 1/4 dilutions. The cell line from passages 4 to 10 was used in this test.

MTT assay

The cytotoxicity of extracts was evaluatedwith 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazoli-um bromide (MTT) assay. The human dermal fibroblasts were trypsinized, centrifuged at 200×g for 5 min and resuspended in Iscove's Modified Dulbecco's Midium. The cells were placed in microtiter plates (96 wells) at a density of 2×10^4 cells per well and were allowed to be incubated for 24 hr with extracts at concentrations of 50, 100 and 200 μg/mL. One row contained medium only for background subtraction. The cells were washed with fresh medium and subjected to MTT test as described previously. And the absorption was determined in an ELISA reader at λ=540 nm after the background readings were automatically subtracted. The results were expressed as percentage of untreated control ones.

Quantitative determination of collagen type I secretion

Quantitative determination of collagen type I secretion was indirectly detected using a procollagen type 1 C-peptide (PIP) *in vitro* enzyme immunoassay (EIA) kit (Takara Bio Inc., Wisconsin, USA). In brief, the human dermal fibroblasts were placed in microtiter plates (96 wells) at a density of 1×10^5 cells per well and incubate with the selected extract at the concentrations of 3.125, 6.25, 10, 12.5, 25, 50, 100 and 200 μg/mL for 24 hr. The incubation conditions were the same as the ones listed above. Retinoic acid (50 μg/mL) was employed as positive control. After the incubation, 100 μL of antibody-POD conjugate solution was added to each well and followed by the addition of 20 μL sample diluents or standard solution. After standing for 3 hr, the contents were removed by suction and all the wells were washed 4 times with 400 μL of PBS. And 100 μL of substrate solution was added into each well and incubate at room temperature for 15 min. The reaction was stopped with the adding of 100 μL stop solution to each well. The absorbance was measured at 450 nm using an ELISA reader (Labsystems, Helsinki, Finland).

MMP-1 inhibition assay

The MMP-1 activity was examined with the MMP-1 immunoassay kit (R&D Systems, Minneapolis, MN, USA). The human dermal fibroblasts were placed in microtiter plates (96 wells) at a density of 1×10^5 cells per well. The cells were pretreated with 2 ng/mL of TNF-α to stimulate the expression of MMP-1. Then all the cells were incubated with the selected extract at the concentrations of 3.125, 6.25, 10, 12.5, 25, 50, 100 and 200 μg/mL for 24 hr with the same conditions listed above. The activity of MMP-1 in the culture solution was determined by the method described in the kit.

Statistical analysis

To verify the statistical significance of the studied parameters, data are expressed as means and standard deviation (mean ± SD). Comparisons were made using the one-way analysis of variance (ANOVA). The p-values <0.05 was considered a statistically significant difference.

Results

Proximate composition

The general proximate composition of lyophilized abalone viscera was listed in the Table 1. From the table we learn that the crude protein is the largest part of the lyophilized abalone viscera, which is followed by crude lipid and total carbohydrate in succession. And the proportion of crude ash is 3.1%, a little higher than that of the moisture. The result obtained in this study was similar to the previous published results (14).

Table 1. Proximate composition of lyophilized abalone visceral

(%)

Moisture	Crude ash	Crude protein	Crude lipid	Total carbohydrate
2.6±0.12	3.1±0.04	53.3±0.36	20.7±0.36	20.3±0.35

Antioxidant assay

The DPPH radical scavenging activity of extracts from the abalone viscera was showed in Fig. 1. The antioxidant potential of Tris-HCl buffer extract was significantly higher than the other three extracts, and was lower than the BHT solution (60.0 μM dissolved in methanol) which was employed as a positive reference in this experiment. Closely following Tris-HCl buffer extract, the ethanol extract presented much higher scavenging activity than the other two extracts. The relation between oxidative stress and skin-aging has attracted researchers' attention for a long time. Free radicals, such as reactive oxygen species (ROS), are believed to induce the skin-aging process, as least partially. Various exogenous and endogenous factors, include UV exposure, life stress, smoking and normal metabolic processes, are engaged in the formation of free radicals (5,15). Further, many of the anti-oxidants obtained from natural resource have been

proved the anti-skin-aging activity. Ascorbic acid can eliminate most ROS and is a cofactor of prolylhydroxylase which prolylresedues in procollagen and in elastin in the skin (16). Vitamin E can reduce various oxidative stresses and suppress the activity of 12-O-Tetradecanoylphorbol-13-acetate, which is a well-known tumor promoter, induces oxidative stress (17). The anti-oxidative activity presented by the extracts from abalone viscera suggested the potential of their anti-skin-aging activity and the following testes were carried out to authenticate this hypothesis.

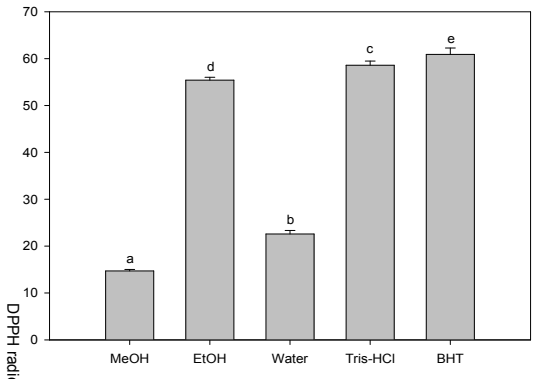

Fig. 1. DPPH radical scavenging activity of the extracts from the abalone visceral.

[a-e]superscript letters indicate significant difference at p<0.05 as determined by one-way analysis of variance.

Elastase assay

Elastase belongs to the endopeptidasefamily and is capable of degrading a wide range of extracellular matrix proteins, especially collagens and elastin (18,19). Its activity increases with age which results in reduced skin elasticity (20,21). So that to inhibit the elastase activity is considered as an effective method for protection against skin aging. And many studies on the anti-elastase and anti-skin-aging activity of extracts or compounds obtained from natural sources have been published (22,23). The elastase inhibition activity of abalone viscera extracts is presented in Fig. 2. As we can see, ursolic acid which was employed as positive control showed the highest anti-elastase activity (77.7%). All the four abalone viscera extracts revealed the anti-elastase potential except for the water extract which had only 1% inhibition activity. The ethyl alcohol extract showed significantly higher activity than Tris-HCl extract and followed by methanol in succession. So unlike its slightly lower anti-oxidant activity compared with Tris-HCl extract, the ethyl alcohol extract showed significantly higher elastase inhibition activity than the other extracts. And the difference between anti-oxidant activity and anti-elastase activity is not uncommon, as is revealed in previous study (24,25). While according to the results listed above, we selected ethyl alcohol extract as the more promising candidate for the future anti-skin-aging study.

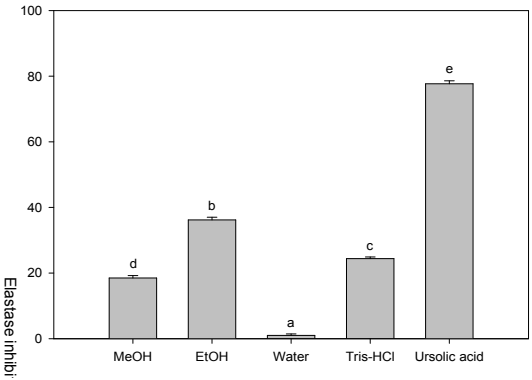

Fig. 2. Elastase inhibition activity of extracts from abalone visceral.

[a-e]superscript letters indicate significant difference at p<0.05 as determined by one-way analysis of variance.

The MTT assay

The cytotoxicity of the four abalone viscera extracts was evaluated by the MTT assay. The viability of human dermal fibroblast which was treated with various abalone viscera extracts was presented in the Table. 2. The control group was considered as 100% while the viability of other groups was obtained by dividing the viability of the control group. The retinoic acid was used as the reference with the same concentration range from 50 to 200 μg/mL. Many studies have proven the stimulation affect of retinoic acid on the proliferation of human dermal fibroblast (26,27). This observation was affirmed in this study as the retinoic acid treated groups showed higher cell viability than the control group. Moreover, all the abalone viscera extracts treated

Table 2. The cytotoxicity of extracts from abalone visceral

Material	Concentration (μg/mL)	Cell viability (%)
Control		100.0
MeOH	50	100.0±0.54
	100	125.3±2.63
	200	131.9±1.20
EtOH	50	137.7±1.47
	100	134.3±0.54
	200	146.3±2.03
Water	50	134.7±1.00
	100	131.1±0.97
	200	139.7±1.05
Tris-HCl	50	139.4±1.33
	100	142.8±2.19
	200	145.6±2.83
Retinoic acid	50	117.5±1.53
	100	129.5±1.05
	200	125.3±1.32

groups manifested significantly higher cell viability than the control one. But the difference between the four extracts was not significant except the MeOH extract at 50 μg/mL which showed nearly the same cell viability compared with the control group. The study suggested that all the abalone extracts had no harmful effect on the human dermal fibroblast in the present experimental conditions. Since human dermal fibroblast is the main producer of collagen, increased dermal fibroblast may alleviate the skin aging.

Pro-Collagen type I synthesis

The effect of abalone viscera ethanol extract on the pro-collagen type I synthesis ability of human dermal fibroblast was examined in this study. Human dermal fibroblast was treated with distilled water and retinoic acid (at the concentration of 0.02 μg/mL) which was considered as control and reference respectively. The experimental group was treated with abalone viscera ethanol at concentrations of 3.125, 8.250, 10.0, 12.5, 25.0, 50.0, 100.0, 200.0 μg/mL. The result showed in Fig. 3 revealed that while retinoic acid stimulated the pro-collagen type I synthesis which confirmed the previous study (28,29), all the abalone extract treated cells except the 200.0 μg/mL treated one secrete significantly more collagen than the control and the retinoic acid treated one. Furthermore, the cells treated with 10.0 and 12.5 μg/mL extract synthesized the highest pro-collagen type I level and the collagen synthesis decreased along with the extracts concentrations range from 25.0 to 200.0 μg/mL. On the other hand, when treated at 200.0 μg/mL the human dermal fibroblast synthesized even lower collagen than the control one which may be considered as inhibited the collagen synthesis. So, as a conclusion, abalone viscera ethanol extract could stimulate the synthesis activity of human dermal fibroblast in the concentration range from 3.125 to 100.0 μg/mL. And at 10.0 and 12.5 μg/mL, human dermal fibroblast showed the highest collagen level. But at 200.0 μg/mL, abalone viscera extract would inhibit collagen synthesis.

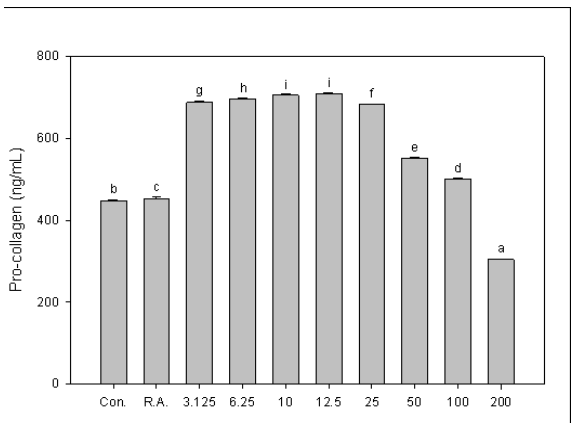

Fig. 3. Type I Pro-collagen synthesis of control and abalone viscera ethanol extract treated group.

Distilled water and retinoic acid was considered as control and reference and abalone viscera ethanol extracts treated to human dermal fibroblast at concentrations of 3.125 to 200.0 μg/mL.

[a-i)]Superscript letters indicate significant difference at $p<0.05$ as determined by one-way analysis of variance.

MMP-1 inhibition assay

Matrix metalloproteinases (MMPs) belong to a family of secreted or membrane-associated zinc endopeptidases capable of digesting extracellular matrix components (30,31). The correlation of MMP-1 and the skin aging has been reported in many studies (32,33). The MMP-1 activity of human dermal fibroblast treated with abalone viscera ethanol extract at various concentrations was presented in Fig.4. In this test, TNF-α at the concentration of 2 ng/mL was used to increase the activity of MMP-1. After treated by TNF-α, the cells were incubated with abalone viscera ethanol extract at the concentrations of 3.125, 8.25, 10.0, 12.5, 25.0, 50.0, 100.0, 200.0 μg/mL respectively. From the result we can see that all the ethanol extract treated groups presented lower MMP-1 than the control one. Moreover, the 10.0 μg/mL treated group had significant lower MMP-1 activity than other ethanol extract treated groups. Together with the result listed above, it revealed that human dermal fibroblast treated at 10.0 μg/mL had higher pro-collagen I level and lower MMP-1 activity.

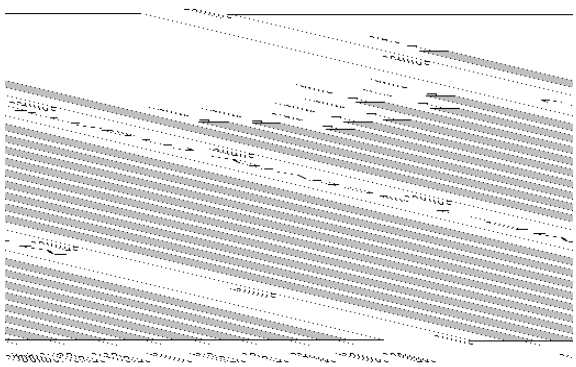

Fig. 4. MMP-1 activities of control group and ethanol extract treatment group in human dermal fibroblast.

Abalone viscera ethanol extracts treated to human dermal fibroblast at concentrations of 3.125 to 200.0 μg/mL.

[a-e)]Superscript letters indicate significant difference at $p<0.05$ as determined by one-way analysis of variance.

Conclusion

The anti-oxidant activity of four extracts obtained from abalone viscera was examined in this study. Among the four extracts, the Tris-HCl extract presented significant higher

DPPH radical scavenging activity than other extracts. And in the anti-elastase activity test, the ethanol extract showed much higher activity. The cytotoxicity test revealed that all the four extracts have no harmful effect to the human dermal fibroblast and they all promoted the cell viability. We selected ethanol extract to be employed in the further cell tests. Human dermal fibroblast was treated with various concentrations of ethanol extract and the Pro-Collagen type I synthesis and MMP-1 activity were analyzed. The result showed at 10.000 μg/mL the ethanol extract could significantly increase the pro-collagen type I synthesis and inhibit the MMP-1 activity. As a conclusion, abalone viscera extracts showed anti-oxidant and anti-elastase ability. Moreover, the ethanol extract stimulated the Pro-Collagen type I synthesis and inhibited MMP-1 activity in the human dermal fibroblast. This study may provide fundamental data about the anti-oxidant and anti-skin-aging ability of abalone.

요 약

전복가공부산물인 내장의 이용가치를 향상시키고자 열수추출, 에탄올추출 등의 방법으로 유효성분을 추출하고 항산화 및 항피부노화 활성을 측정하였다. DPPH 라디칼소거능 분석결과 Tris-HCl 추출물은 58.60±0.88%으로 높은 라디칼소거능을 보였으며, 다음으로 에탄올추출물은 55.40±0.62%의 라디칼소거능을 보였다. Anti-elastase활성을 분석한 결과 에탄올추출물이 다른 시험구에 비하여 현저하게 높은 활성을 보였다. 전복내장추출물의 세포독성을 확인하기 위하여 human dermal fibroblast 세포에 대하여 MTT 시험결과 세포독성은 전혀 나타나지 않았다. 에탄올추출물의 농도를 달리하여 human dermal fibroblast 세포에 대해pro-collagen type I 생합성능시험결과 10.0 μg/mL에서 705.30±3.06 ng/mL로 retinoic acid 453.60±2.82 ng/mL보다 우수한 결과를 보였다. MMP-1 활성은 10 μg/mL에서 45.30±0.80 ng/mL를 보여 control의 62.37±0.56 ng/mL 보다 감소정도가 낮았다. 본 연구는 전복내장추출물의 항산화와 항노화활성에 관하여 최초로 시도되었으며 향후 전복내장을 이용한 다양한 연구의 기초자료로서 중요한 의미가 있다.

Acknowledgement

This work was supported by a grant of Marine bio-industry development project from Jeonnam provincial government.

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(접수 2012년 4월 17일 수정 2012년 5월 18일 채택 2012년 7월 27일)