A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Anti-wrinkle effect of fucoidan isolated from Miyeokgui (*Undaria pinnatifida* sporophyll) by enzyme-assistant extraction

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국문초록

최근에 국내 및 국제의 기능성 화장품 시장의 규모가 점점 커지고, 천연물로부터 화장품의 소재를 개발하는 연구가 활발히 진행 됨에 따라서 이 연구를 수행하였다. 연구의 소재로는 미역의 포자엽인 미역귀(*Undaria pinnatifida* sporophyll)를 선택하였다. 불과 얼마 전만 해도 미역귀는 미역 줄기를 사용하고 난 후에 버려지는 부위였지만, 몸에 이로운 작용을 하는 비타민, 알긴산, 미네랄등의 성분들 뿐만 아니라 생리활성이 뛰어난 fucoidan의 함량이 풍부함이 알려짐에 따라서 각광받기 시작하였다. Fucoidan은 단당류인 fucose에 sulfate가 결합한 구조를 가지고 있으며, 미역, 톳, 다시마, 모즈쿠 등 갈조류에 풍부하다고 알려져 있다. 미역귀로부터 분리한 fucoidan은 항산화, 항염증, 항비만, 항당뇨, 면역조절 등의 다양한 효과가 있다고 알려져 있으며, 특히 암세포의 apoptosis를 유도하는 효과가 뛰어나다고 알려져 있다.

Fucoidan의 apoptosis 유도 효과는 1996년 제 55회 일본암학회에서 처음 발표 되었다. 암에 대한 효과를 주목 받기 시작하고, 많은 수의 의학논문들이 발표 되었다. 우리 나라는 일본에 비해 뒤늦게 후코이단에 대한 연구 및 사업을 시작하였지만, 빠른 속도로 규모가 커지고 있다. 일본의 fucoidan의 주요 원료는 모즈쿠라는 해

조류인데, 본 연구에서 사용된 미역귀의 fucodian은 모즈쿠의 fucoidan보다 sulfate의 함량이 매우 높다. 기능성의 지표가 될 수 있는 sulfate는, 그 함량이 높을수록 효과가 좋다. 일본에 비해 높은 sulfate의 함량을 보유한 fucoidan을 보유한 미역귀를 소재로 하여 다양한 연구 및 사업이 진행되고 있다. 이러한 이로운 기능들과 개발 가능성을 근거로 하여 식품 및 의약품이 아닌 기능성 화장품 소재로서의 가능성을 확인하기 위하여 본 연구를 진행 하였다.

기능성 화장품은 산화 억제, 피부에 대한 염증보호, 미백, 자외선에 대한 보호, 주름개선, 보습효과 등의 기능을 필수적으로 포함한다. 그 중에서 주름에 대한 보호 및 개선 효과가 가장 중요하다고 생각되어 미역귀 유래 후코이단을 소재로 하여 연구를 진행하였다. 주름은 피부의 진피층의 기질 단백질이 분해 됨에 따라서 그 탄력성을 잃고 형성된다. 주름의 형성 원인은 자외선, 흡연, 내인적 스트레스 등 다양하다. 피부가 주름 형성 요인들에 의해서 자극을 받게 되면, reactive oxygen species (ROS가 생성되어진다. 생성된 ROS는 진피의 growth factor receptor를 자극하여 FOS와 JUN으로 구성되어 있는 activator protein-1 (AP-1)을 활성화 시킨다. 활성화된 AP-1은 기질 구조 단백질인 collagen의 전구체인 procollagen의 합성을 억제하고, collagen을 분해시키는 matrix-metallopreoteinases (MMPs)을 활성화 시킨다.

미역귀 유래 fucoidan은 human dermal fibroblast에서 무처리군과 비교하였을 때 최고 농도 200 μg/mL에서 132.2%의 증가량을 보였다. 또한 UVA로 인해 증가된 MMP-1의 발현량을 크게 감소 시켰다. 그러므로 미역귀 유래 fucoidan은 anti-wrinkle 효과를 가진 기능성 화장품의 원료로서 사용할 수 있다고 사료된다.

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ABSTRACT

Fucoidan isolated from the cultured Korean brown seaweed sporophyll (Miyeokgui) *Undaria pinnatifida* (Miyeok) is an interesting polysaccharide due to its various biological activities. This polysaccharide was isolated using five carbohydrate enzymes including AMG, Celluclast, Termamyl, Ultraflo, and Visoczyme. The five enzyme-assistant extracts were measured for their elastase and collagenase inhibition activity by chemical methods. Based on the results, we selected Celluclast enzymatic extract because of it's high level of biological activity. Then, the polysaccharides were separated from Miyeokgui cellucalst extract by mixing three volume of ethanol and storing in 4°C over night after proper mixing. Ethanolic supernatant was removed by centrifugation and the alginates were collected by $CaCl_2$ precipitation. The crude extract was further purified by DEAE–cellulose chromatography. Peak (carbohydrate contents) of fraction was showed on concentration of flow buffer 0 M, 0.2 M, 0.4 M, 0.6 M, 0.8 M, 1 M, and 1.2 M. The collected fractions were analysed for their sulfate content and monosaccharide composition. The F3 fraction was found to possess the highest sulfate and fucose contents. Therefore, further investigations were carried out using the F3 fraction.

Fucodian isolated from Miyeokgui by enzyme-assistant extracts were treated on human dermal fibroblast (HDF) cells for collagen synthesis activity and matrix-metalloproteinases inhibition activity. After 24 hours, culture media were collected for measured collagen synthesis activity and cells were lysed by lysis buffer for western blotting for measured matrix-metalloproteinases inhibition activity.

Fucoidan isolated from Miyeokgui by enzyme-assistant extracts increased the collagen synthesis with the increments of 69.3%, 111.8%, and 132.2%, at each concentration and

inhibited the matrix-metalloproteinases expression to 78.5%, at 200 μg/mL concentration. These results suggests that fucoidan isolated from Miyeokgui can be used as a potential ingredient in anti-wrinkle material in functional cosmeceutical industry.

1. INTRODUCTRION

Recently, functional cosmetics have become the focus of cosmeceutical industry with the increased interest about the appearance and harmful effects on skin. The major function of cosmetics is skincare that includes aspects like whitening effect, UV protective effects, anti-inflammation, anti-oxidant and anti-wrinkle activities. Most of the people, especially women are interested in having a beautiful skin. But nowadays no only the women also the men are interested in having a beautiful skin. Therefore the no of available skin care products in market is rapidly getting increased and many resources are invested for skincare research. One of the most important function of cosmetics is the prevention of wrinkling.

Wrinkling can be due to various factors such as UV, smoking, stress and physical shock. These factors induce generation of reactive oxygen species (ROS) that activate growth factor receptor. Activated growth factor receptors stimulate activation of activator protein-1 (AP-1) composed Fos and Jun protein. AP-1 induces expression of matrix-metalloproteinase (MMP) genes and inhibits expression of procollagen genes. MMPs are classified according to their biological function. For example, MMP-1 and MMP-12 are collagenase and elastase that disassemble collagen and elastin. Thus MMPs degrade extracellular matrix as the result of disassemble collagen and elastin in dermis which is the cause of wrinkle formation. Therefore, inhibitor or preventor of MMPs have used as functional cosmetic components. However, synthetic chemicals such as oxybenzone, benzophenone, butylated hydroxyanisole (BHA), have side effects to human body. Therefore, many researchers have found beneficial compounds from natural materials.

More than 70% of the earth surface is covered by the massive ocean and nearly 90% of organisms inhabit this environment. One of the that, Miyeokgui (Undaria tinnatifida

sporophyll) is sporophyll which situated at the root part of Miyeok (Undaria tinnatifida). A few years ago Miyeokgui did not received much attention, but recently it has become the focus of many researchers and companies with the invention of a variety of various beneficial substances including fucoidan.

Fucoidan is a polysaccharide composed of L-fucose as the main sugar unit and with attached sulfate groups. It is abundant in brown seaweeds such as *Ecklonia cava*, *Hizikia fusiformis*, *Undaria pinnatifida* and possesses a wide range of bioactivities including anti-oxidants, anti-inflmmatory, anti-diabet, immune-modulatory activities. Fucoidan isolated from Miyeokgui, is known to have various bioactivities, especially it has excellent anti-cancer activities. Due to these effects, Miyeokgui fucoidan is being sold as powder and as a drink.

Several extraction process are required to obtain fucoidans. The process is divided to 4 step as extraction of sample, precipitation of polysaccharides, precipitation of alginic acid, and purification of fucoidan. In the extraction step, hydrochloric acid (HCl) was used as a solvent. HCl is a strong acid which can cause serious harmful effects to human. Therefore, in this study, isolation of fucoidan from Miyeokgui was carried out using enzymes which do not have harmful effects on human being. Finally our results suggests the potential of Miyeokgui fucoidan as a functional cosmetic material in cosmeceutical preparations.

2. MATERIALS AND METHOHS

2.1. Chemicals and Reagents

The cells of a human dermal fibroblast (HDF) cell were provided from Dermapro Co. Five carbohydrate digestive enzymes that are Viscozyme L, Celluclast 1.5 L F G, AMG 300 L, Termamyl 120 L, and Ultraflo L were purchased from Novo Co. (Novozyme Nordisk, Bagsvaerd, Denmark). Procollagen Type I C-Peptide (PIP) EIA Kit was purchased from TaKaRa Korea Co. Antibody of MMPs including MMP-1 were purchased CST Co. (Cell Signaling Technology). Elastase from Porcine Pancreas Type I 4.0 Units/mg Protein, Collagenase from Clostridium histolyticum, DEAE-cellulose, N-Succinyl-Ala-Ala-Ala-p-nitroanilide, Collagenase Chromophore-Substrate, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St.Louis, MO). All other chemicals and reagents were analytical grade.

2.2. Plant material and extraction

Miyeokgui of cultured Korean Miyeok was used as the source of polysaccharides in this study. Samples were collected from southern coastal area of Wando, Korea. Sand and epiphytes were washed using tap water. After washing, Miyeokgui sample was soaked in fresh water for reducing alginic acid and salt. Then, Miyeokgui sample was freeze-dried and grounded in to a powder. The enzyme-assistant extraction was performed according to a previously reported method (Kang et al., 2014). In this study, the enzyme-assisted extracts of Miyeokgui were obtained by enzymatic hydrolysis using the five carbohydrases including

AMG, Celluclast, Termamyl, Ultraflo, and Viscozyme. A 10 g of Miyeokgui powder was homogenized with distilled water (D.W, 500 mL), and mixed with 250 μL of enzyme. After 24 hour, extract was filterd and then mixed with 3 volume of ethanol (EtOH) to obtain the crude polysaccharides (CPS) of Miyeokgui enzyme-assisted extracts. Then, mixture was centrifugated at 12000 rpm for 20 minute at 4°C and precipitate was collected and dissolved in D.W. Then, dissolved precipitate was mixed 4 M calcium chloride ($CaCl_2$) to obtain crude fucoidan (CF) from the alginic acid removed polysaccharides.

2.3. Anion-exchange chromatography

The crude fucoidan from Miyeokgui (200 mg) obtained by using the procedure described above was applied to a DEAE-cellulose column (45 cm x 2.5 cm). Elution was carried out at a flow rate 100 mL/h with a linear gradient of 0-1.2 M NaCl. Fractions of 3 mL were collected and measured for polysaccharide by phenol-H_2SO_4 assay. According to sulfate and composition of monosaccharide guided fractionation, elution was collected. Then, samples were desalted using membrane filtration.

2.4. Monosaccharide analysis

The extracts, CPS, CF, and the purified fucoidan was isolated from Miyeokui were hydrolyzed in a sealed glass tube with 4 M of trifluoroacetic acid for 4 h at 110°C to analyze the monosaccharide compositions. Then, the samples were digested using 6 N of HCl for 4 hours. Then the samples were separately applied to CarboPac PA1 cartridge (4.5 mm x 50 mm) column to analyze the neutral and amino sugars respectively. The column was eluted

using 16 mM of NaOH at 1.0 mL/min flow rate. Each sugar of the samples was detected by using ED50 Dionex electrochemical detector and data were analyzed by Peack Net on-line software.

2.5. Elastase inhibition activity

The elastase-catalyzed hydrolysis of substrate was measured in the presence of various concentrations of mistletoe extracts and its fractions. The measurement of enzyme activity was demonstrated by spectrophotometric monitoring of the release of *p*-nitroaniline at 410 nm from the enzymatic hydrolysis of the substrate: *N*-succinyl-trialanine-*p*-nitroanilide. The mixture of 20 μl of the sample and 340 μl of substrate (1.015 mM in Tris-HCl buffer) was preincubated at 25°C for 20 min and 20 μl of porcine pancreatic elastase were added and incubated at 25°C for indicated time period. The appearance of the substrate hydrolysis product (*p*-nitroaniline) was measured at 410 nm. The percentage of inhibition of enzyme activity was expressed as {1-(A-B)/C} x 100 (A; absorbance at 410 nm in the presence of test sample and enzyme, B; absorbance at 410 nm in the absence of test sample and enzyme, C; absorbance at 410 nm in the absence of test sample and in the presence of enzyme).

2.6. Collagenase inhibition activity

Collagenase inhibition activity was determined by the method of Wunsch and Heidrich (1963) with slight modification. For the reaction, 4 mM $CaCl_2$ was added to 0.1 M Tris-HCl buffer (pH 7.5) and 4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-Arg (0.3 mg/mL). Next, 100 μL of hydrolysate sample was mixed with 250 μL of the solvent described above. 150 μL

of collagenase (0.2 mg/mL) was added and incubated 20 minutes at room temperature. 500 μL of 6% citric acid was added to stop the reaction then 1500 μL ethyl acetate (EA) was added. The supernatant was removed and the O.D. was determined by UV/Vis spectrometer at an absorbance of 320 nm. Collagenase inhibition activity was determined using the following equation.

Collagenase inhibition activity
={1-(sample O.D.-blank O.D.)/con O.D.} x 100

2.7. Cell culture

The human dermal fibroblast (HDF) cells were maintained at 37°C in an incubated, under a humidified atmosphere containing 5% CO_2. The cells were cultured in DMEM containing 3:1 ratio of DMEM:F-12 medium, 10% heat-inactivated FBS, streptomycin (100 μg/mL), penicillin (100 unit mL^{-1}).

2.8. Collagen synthesis activity

The collagen synthesis activity was determined using Procollagen Type I C-Peptide (PIP) EIA Kit. 2 x 10^4 HDF cells were seeded into 6 well plate and incubated in 37°C for 24 hour. Then, the culture medium was removed and replaced by serum-free medium with different concentrations of samples. After 24 hours, the medium was collected and measured according using PIP kit. Absorbance was recorded with a Synerge HT microplate reader at 450 nm.

2.9. UVA irradiation and treatment

Prior to UV irradiation, cells were rinsed with HBSS and exposed to a radiation dose of 6 J/cm^2 of UVA light (UV Lamp, VL-6LM, Vilber Lourmat, France) using a thin layer of HBSS. After irradiation, the cells were rinsed with HBSS and replaced with different concentrations of samples in serum-free culture medium for 24 hours. Concomitantly, control cells (without irradiation) were treated in the same manner, although the wells were covered with aluminum foil to prevent irradiation.

2.10. Cell viability and LDH release

Cell viability was determined using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide]. Briefly, 2 x 10^4 cells were seeded in a 24 well plate. The cells were then exposed to a series of sample concentrations for 24 hours. After 24 hours, MTT reagent was added and incubated for another 4 hours. MTT reagent was removed and replaced with 300 μL of dimethyl sulfoxide (DMSO) for dissolving formazan. Absorbance was red with a Synerge HT microplate reader at 540 nm. LDH release was determined using cytotoxicity assay kit (Cyto Tox 96® Non-Radio., Promega). Cell culture supernatant was transfered into a 96 well plate and mixed with assay buffer of the cytotoxicity kit. Then, it was incubated 30 minutes in dark room and mixed with stop solution. Absorbance was read with a Synerge HT microplate reader at 490 nm.

2.11. Intracellular reactive oxygen species (ROS) measurement

Intracellular ROS was measured using increasing fluorescent by DCFH-DA. DCF-DA diffuses into cells, where as it is hydrolyzed by intracellular esterase to polar 2',7'-dichlorodihydrofluorescein. This non-fluorescent fluorescin analog gets trapped inside the cells, and is oxidized by intracellular oxidants to a highly fluorescent, 2',7'-dichlorofluorescein. The 2 x 10^4 HDF cells were seeded in 48 well plates. 24 hours after plating, the cells were treated with samples and incubated for 24 hours. Then, DCFH-DA (25 μg/mL) was treated to the cells, and irradiated with 6 J/cm^2 of UVA radiation. Fluorescence was detected at an excitation wavelength of 485 nm and an emission wavelength of 535 nm, using a Synergy HT spectrometer.

2.12. Western blot analysis

Western blotting was performed for the detection of MMP-1as described previously (Kim et al., 2013). The cells were collected and washed twice with cold PBS. The cells were lysed in a lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 25 μg/mL aprotinin, and 25 μg/mL leupeptin] and maintained on ice for 30 minutes. The cell lysates were collected via centrifugation, and the protein concentrations were determined using a BCA™ protein assay kit. Aliquots of the lysates (40 μg of protein) were separated on 12% SDS-polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane (BIO-RAD) with a glysine transfer buffer [192 mM glycine, 25 mM Tris-HCl (pH8.8), 20% methanol (v/v)]. After blocking the nonspecific sites with 1% bovine

serum albumin (BSA), the membranes were incubated overnight with specific primary antibody at 4°C. The membranes were then incubated for an additional 60 minutes with a peroxidase-conjugated secondary antibody (1:3000, Vector Laboratories, Burlingame, USA) at room temperature. The immunoactive proteins were detected using an enhanced chemiluminescence (ECL) Western blotting detection kit.

2.13. Statistical analysis

All data are expressed as means ± S.D. Significant differences among the groups were determined using the unpaired Student's *t*-test. A value of $*P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Yields, carbohydrate contents, and sulfate contents of Miyeokgui extracts

Miyeokgui was extracted with five carbohydrase and HCl. Extraction yields of AMG, Celluclst, Termamyl, Ultraflo, Viscozyme, and HCl were 40.2%, 37.6%, 40.3%, 39%, 38%, and 35.4%, respectively. Carbohydrate and Sulfate contents of extractions described above were 26.1%, 27.8%, 25.4%, 30.3%, 26.4%, and 22.1% and 7.8%, 8.3%, 7.5%, 7.9%, 7.7%, and 6.9%, respectively (Table. 1.). Elastase and collagenase inhibition experiments were performed on all extracts due to the fact that they indicated similar efficacy of extraction.

Table 1. Yields, carbohydrate contents and sulfate contents of Miyeokgui extracts.

	Assistant	Yields (%)	Carbohydrate contents (%)	Sulfate contents (%)
Extracts	A	40.2±1.2	26.1±1.7	7.8±0.14
	C	37.6±1.5	27.8±1.7	8.3±1.5
	T	40.3±1.1	25.4±0.3	7.5±0.1
	U	39±3.0	30.3±0.7	7.9±0.4
	V	38±1.7	26.4±0.3	7.7±1.1
	H	35.4±1.4	22.1±1.1	6.9±1.2

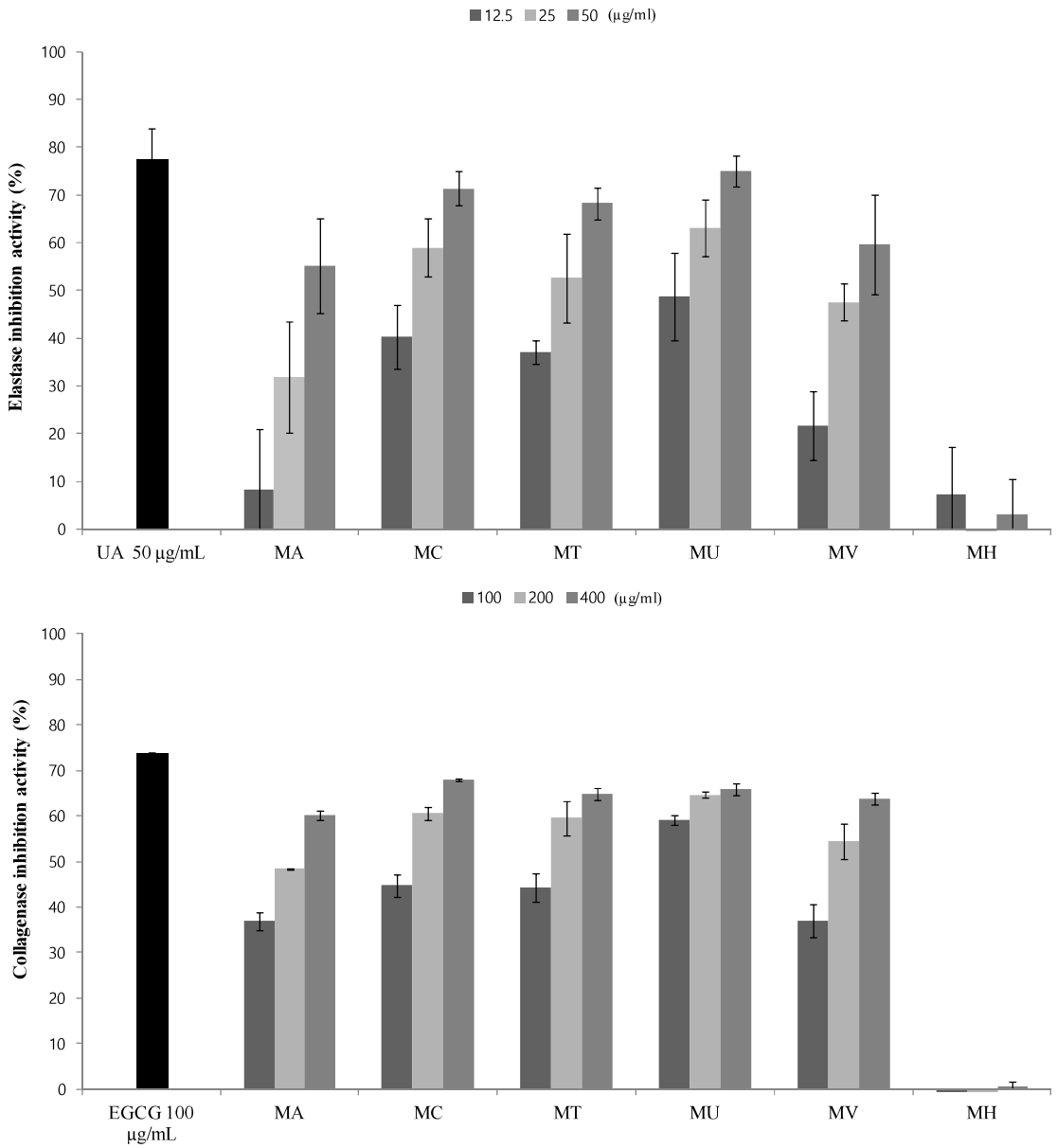
A : AMG C : Celluclast T : Termamyl U : Ultraflo V : Viscozyme H : HCl

3.2. Elastase and collagenase inhibition activity of enzyme-assistant extracts and HCl extracts

Elastase and collagenase inhibition activities were measured by chemical experiment in order to, select the carbohydrase enzyme with the most efficacy. Elastase inhibition activity was analysed for the enzyme-assistad extracts for concentrations of 12.5, 25 and 50 μg/mL and of ursolic acid (UA) (50 μg/mL) which was used as the positive control (Fig 1-A). Collagenase inhibition activity was determined for the enzyme-assistant extracts for sample concentrations of 100, 200, and 400 μg/mL, and 100 μg/mL of epigallocatechin gallate (EGCG) was used as the positive control (Fig 1-B).

Elastase inhibition activity of UA was found to be 77.4 % and the enzyme-assistad extracts showed dose-dependant increase of elastase inhibition activity. Among the six enzyme-assistad extracts, Cellucalst and Ultraflo extracts showed the highest levels of elastase inhibition activity (71.4% and 75%) at 50 μg/mL. These elastase inhibition activities were closed to UA.

Collagenase inhibition activity of EGCG was found to be 73.8% and the enzyme-assistad extracts showed dose-dependant increase of collagenase inhibition activity. In this experiment, Celluclast and Ultraflo extracts showed the highest levels of collagenase inhibition activity (67.9% and 65.8%) at 400 μg/mL.



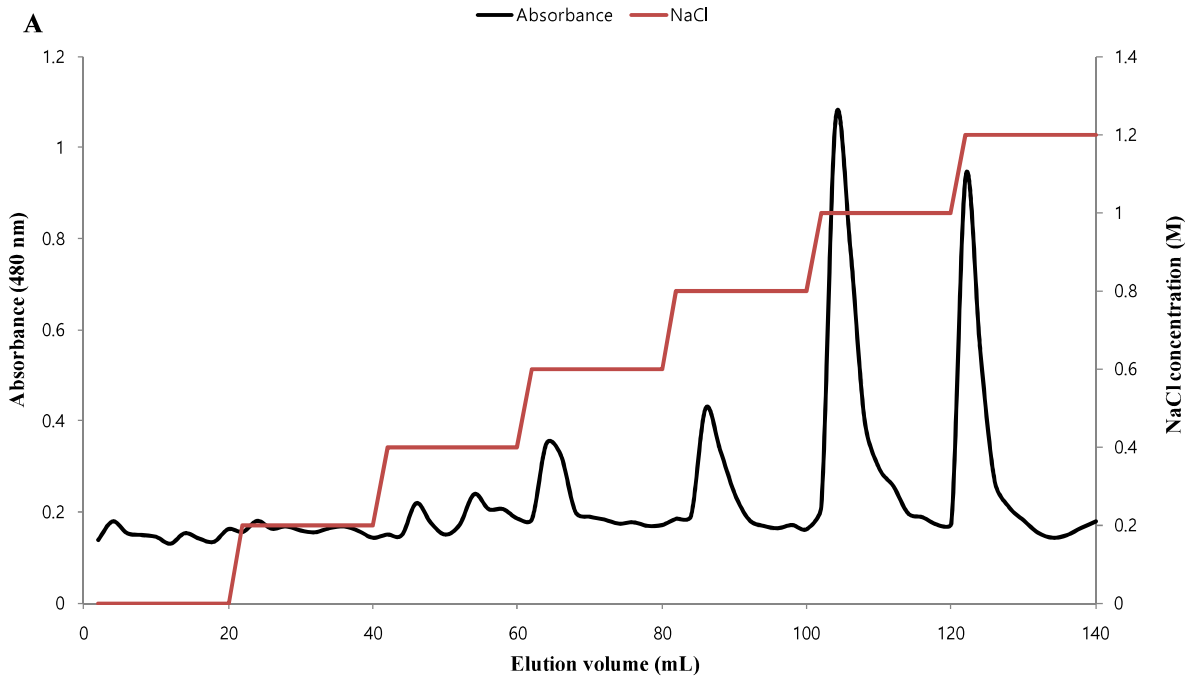
UA : Ursolic acid EGCG : Epigallocatechin gallate MA : Miyeokgui AMG extract MC : Miyeokgui celluclast extract
MT : Miyeokgui termamyl extract MU : Miyeokgui ultraflo extract MV : Miyeokgui viscozyme extract MH : Miyeokgui HCL extract

Fig 1. Elastase and collagenase inhibition activity of enzyme-assistant and HCl extracts isolated from Miyeokgui.

3.3. Purification of a polysaccharide with a higher sulfate contents

Crude polysaccharides of Miyeokgui Celluclast extracts were isolated by three volume of EtOH precipitation followed by the separation of alginic acid from the crude polysaccharides with the addition of $CaCl_2$. The alginic acid-removed crude polysaccharides were purified by DEAE-cellulose column chromatography with a NaCl buffer gradient (0-1.2 M) according to sulfate content and monosaccharide composition. A sample weight of 200 mg of alginic acid-removed Miyeokgui celluclast crude polysaccharides were loaded in to the column and 200 mL of NaCl buffer per concentration was eluted out and collected from the column. Then, the carbohydrate content of each fractions were measured and the separated fractionations of the chromatogram were labeled as 4 peaks, F1, F2, F3, and F4 (Fig 2-A). Among the fractions, F3 showed the highest contents of sulfate (Fig 2-B). Therefore, F3 was considered as the fucoidan rich fraction purified from Miyeokgui.

A



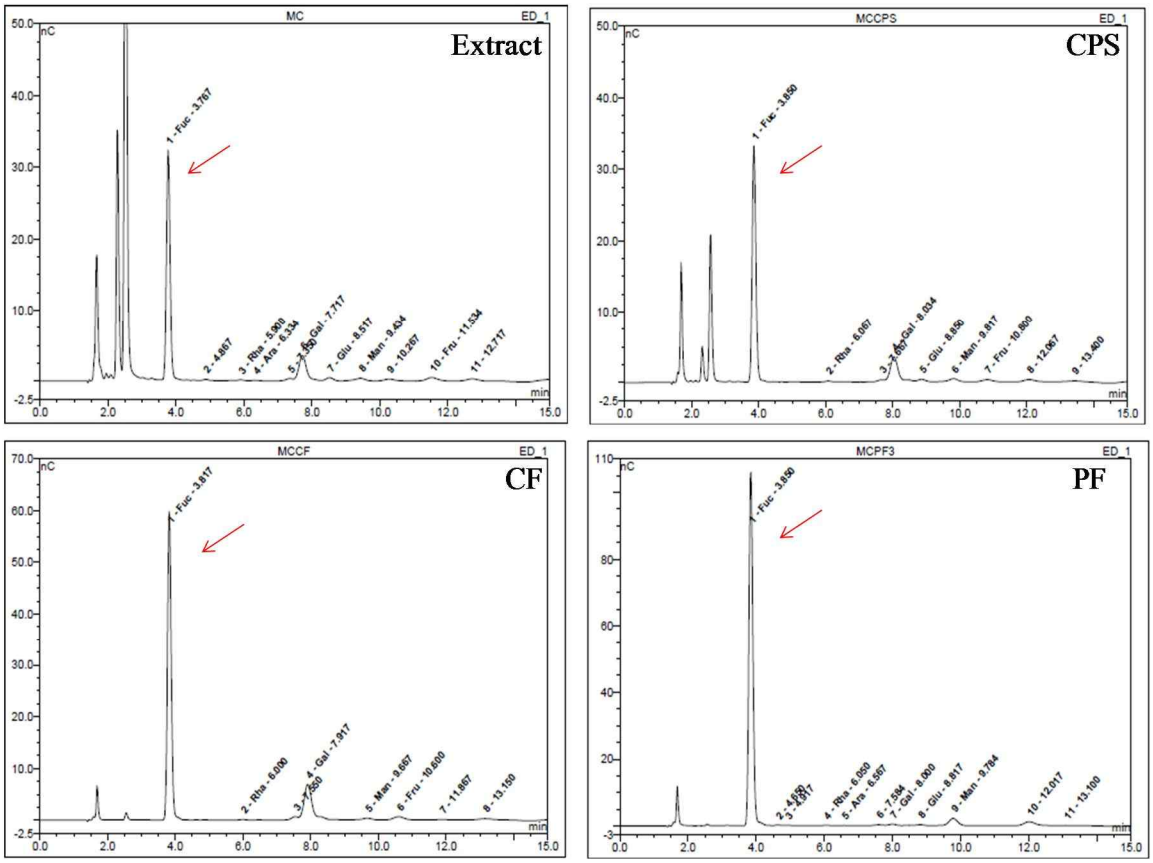
B

Sulfate content (%)			
F1	F2	F3	F4
8.9±1.1	9.4±0.4	27.8±0.9	15.1±3.4

Fig 2. DEAE-cellulose chromatogram of the polysaccharide without alginic acid isolated from Celluclast extract of Miyeokgui (A) and sulfate contents of F1, F2, F3, and F4 (B).

3.4. Monosaccharide compositions of extract, CPS, CF, and purified fucoidan isolated form Miyeokgui

The extracts, CPS, CF, and the purified fucoidan isolated from Miyeokui were hydrolyzed with 4 M of trifluoroacetic acid for 4 h at 110°C to analyze the monosaccharide composition. Then, the samples were digested using 6 N of HCl for 4 hours. From the Peack Net on-line software analysis, the monosaccharides of the samples were found to be fucose, galactose, glucose, and mannose. The major monosaccharide of the extract was fucose (63.1%) and other sugars, galactose (15.53%), glucose (1.62%), and mannose (2.19%) as minor components. Also the major monosaccharide of CPS, CF and purified fucoidan was fucose (67.68%, 73.29% and 89.2%), respectively and other sugars, galactose (13.07%, 18.06% and 0.64%), glucose (0.99%, 1.06% and 0.32%), and mannose (2.42%, 22.08% and 4.03%) were present as minor constituents respectively (Fig. 3).



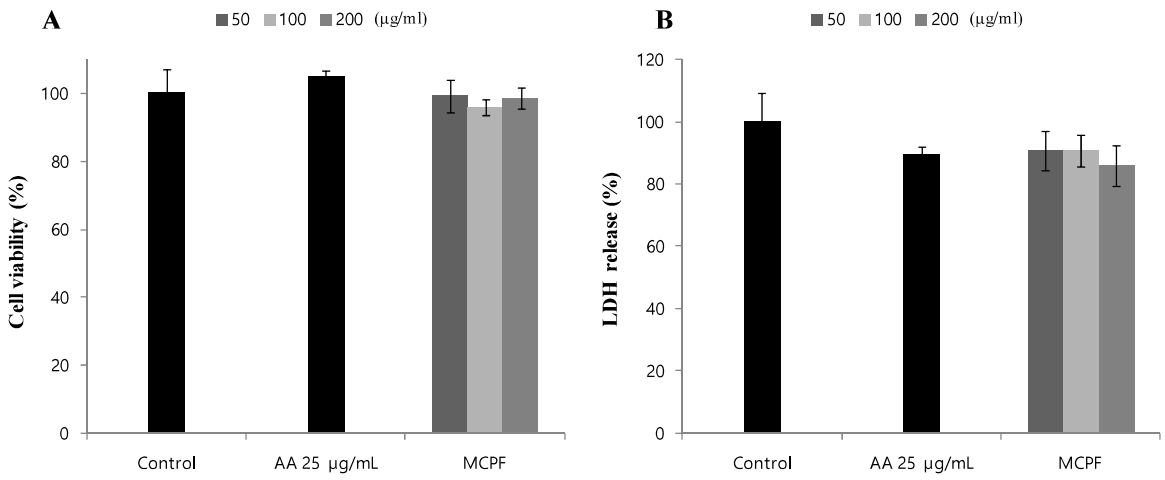
	Assistance	Sample	Fucose	Galactose	Glucose	Mannose
Composition (%)	Celluclast	Ext	63.10	15.53	1.62	2.19
		CPS	67.68	13.07	0.99	2.42
		CF	73.29	18.06	1.06	2.08
		PF	89.20	0.64	0.32	4.03

Ext : Extract CPS : Crude polysaccharides CF : Crude fucoidan PF : Purified fucoidan

Fig 3. The monosaccharide compositions of extract, CPS, CF, and purified fucoidan isolated from Miyeokgui

3.5. Cytotoxicity of fucoidan

The cell viability and LDH release in HDF cells were evaluated in order to determine the toxicity of the isolated fucoidan. The cells were treated with 50, 100, and 200 μg/mL of fucoidan and incubated for 24 hour in serum-free medium. And then, 50 μg/mL of the culture medium was transferred into 96 well plate for the measurement of LDH release. A volume of 100 μg/mL of MTT (2 mg/mL) reagent was treated in to each well for the MTT assay. Level of cell viability and LDH release were measured by colorimetric methods. Cell viability of Miyeokgui Celluclast purified fucoidan was 99.6%, 95.7%, 98.6%, at each concentration (Fig. 4-A). LDH release of Miyeokgui Celluclast purified fucoidan was 90.6%, 90.6%, 85.9 at each concentration (Fig. 4-B). Therefore, Miyeokgui Celluclast purified fucoidan can be considered to have no cytotoxic effect up to the concentration of 200 μg/mL. Based on these results 50, 100, and 200 μg/mL concentrations were found to be effective and were used for further study.

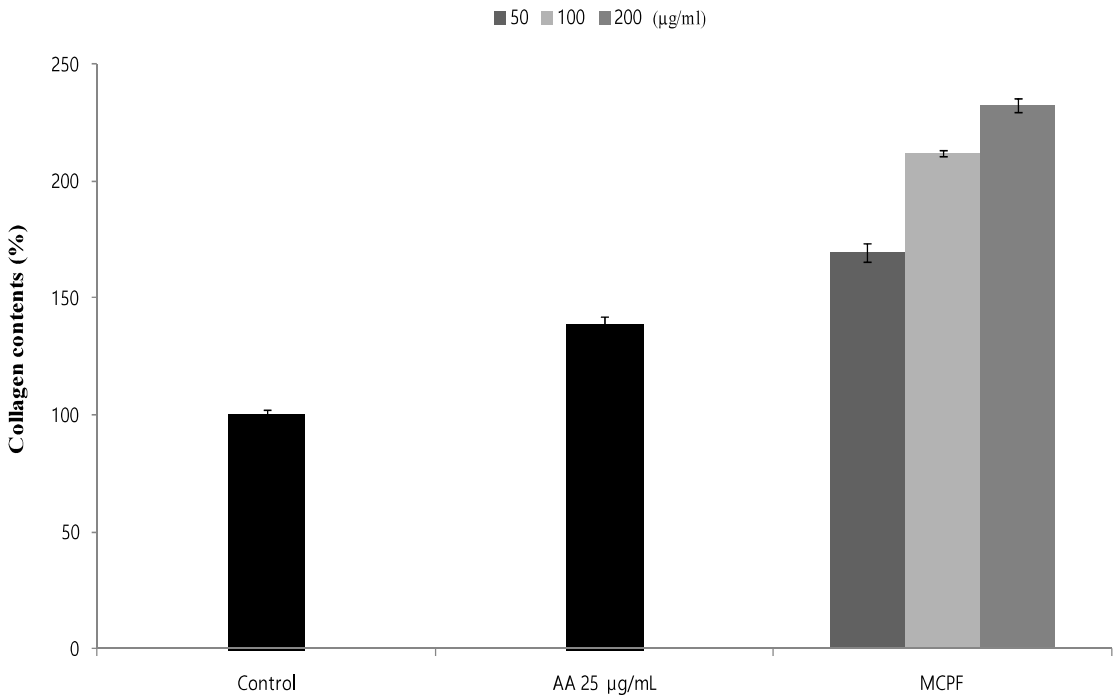


AA : Ascorbic acid MCPF : Miyeokgui celluclast purified fucoidan

Fig 4. Cytotoxicity of purified fucoidan by MTT assay (A) and LDH assay (B) on human dermal fibroblast.

3.6. Collagen synthesis activity of fucoidan

Collagen synthesis in Miyeokgui Celluclast purified fucoidan (MCPF)-treated group increased dose dependently with increasing concentrations of MCPF compared with that of the control group. The increasing rate of collagen content with MCPF was found to be 69.3%, 111.8%, and 132.2%, at each concentration (Fig. 4.). Miyeokgui Celluclast purified fucoidan showed a higher collagen synthesis activity, indicating a higher effect more than that of 25 μg/mL of ascorbic acid as reported in a previously study. Therefore, we suggests that Miyeokgui can be utilized as a functional cosmetic material because of the increase of collagen produced by its fucoidan may prevent wrinkles by strengthening the extracellular matrix.

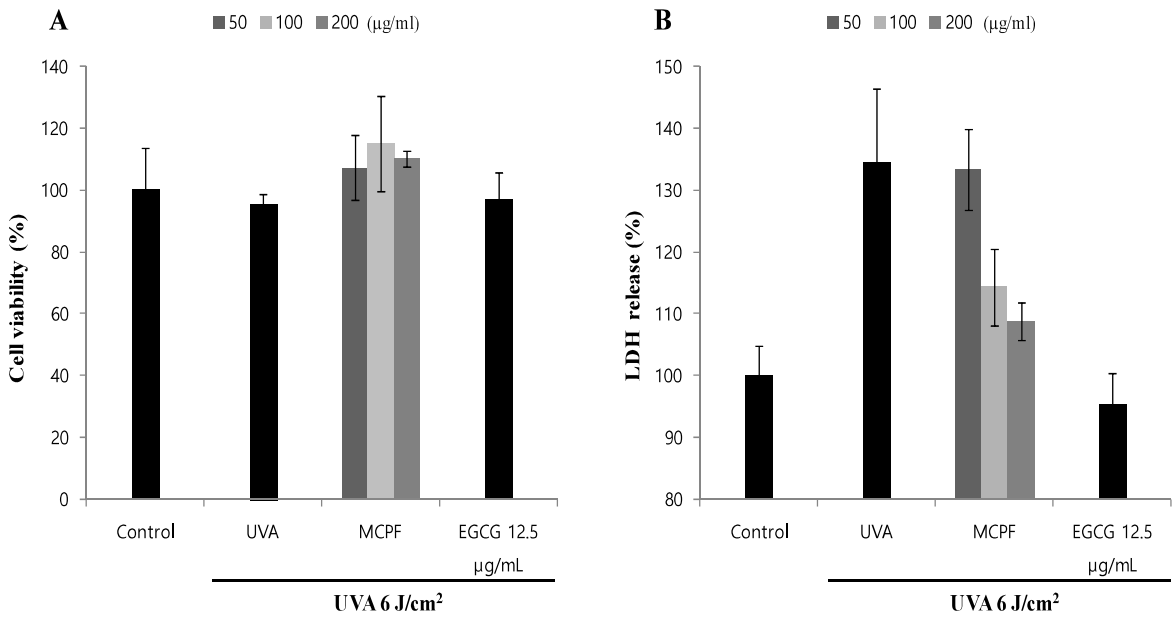


AA : Ascorbic acid MCPF : Miyeokgui celluclast purified fucoidan

Fig 5. Collagen synthesis activity of purified fucoidan by an ELISA kit on human dermal fibroblast.

3.7. UVA-induced cell damage

MTT assay and LDH assay was performed in order to evaluate the UVA-induced cell damage and LDH release. Exposed to UVA, was found to induce the production of reactive oxygen species (ROS) which leads to cell damage. ROS can cause a decrease of cell viability and increase the LDH release. Therefore, at first we optimized the methods for achieving appropriate energy for UVA irradiation that can cause cell damage without a significant effect on cell viability to be 6 J/cm^2 of UVA. It maintained a significant level of cell viability with a increased LDH release. The cells irradiated with UVA without the application of any reagent showed similar level as the control (95.4% of cell viability). Also, Miyeokgui Celluclast purified fucoidan treated cells showed similar levels with their respective controls (Fig. 5-A). However, LDH release of UVA-irradiated cells increased to 34.4% compared with that of the control. Miyeokgui Celluclast purified fucoidan showed to decrease the LDH release to 1.1%, 20% and 25.6%, at each sample concentration (Fig. 5-B).



EGCG : Epigallocatechin gallate MCPF : Miyeokgui celluclast purified fucoidan

Fig 6. Measurement of UVA-induced cell damage by MTT assay (A) and LDH assay (B) on human dermal fibroblast.

3.8. Measurement of intracellular ROS induced UVA irradiation

The HDF cells were exposed 6 J/cm^2 of UVA for the measurement of intracellular ROS production. Miyeokgui Celluclast purified fucoidans were treated to HDF cells at different concentrations and 12.5 μg/mL of EGCG was treated as the positive control. After 24 hours, 25 μg/mL of DCFH-DA reagent was treated into each well and incubated for 30 minutes. Then, the culture medium was removed by suction pump and each well was washed twice using HBSS. For irradiation of UVA, each well was replaced with a thin layer of HBSS. After irradiation, HBSS was removed and replaced with serum-free medium containing different concentration of Miyeokgui Celluclast purified fucoidan. Then, the cells were incubated for 1 hour and fluorescence value was measured by Synergy HT microplate reader. The cells exposed to UVA without any treatment indicated 102.2% of fluorescence value compare with the control. Cells treated with Miyeokgui Celluclast purified fucoidan showed a gradually decreasing levels of fluorescence 108.2%, 87.1%, 61.8%, respectively at each concentration (Fig. 6.). Miyeokgui Celluclast purified fucoidan reduced the UVA-induced ROS production that triggers the initiation of wrinkle mechanism. Therefore they can protect UVA-induced wrinkle formation as well as other skin diseases. In conclution Miyeokgui Celluclast purified fucoidan have the ability to inhibit the mechanism of wrinkle formation.

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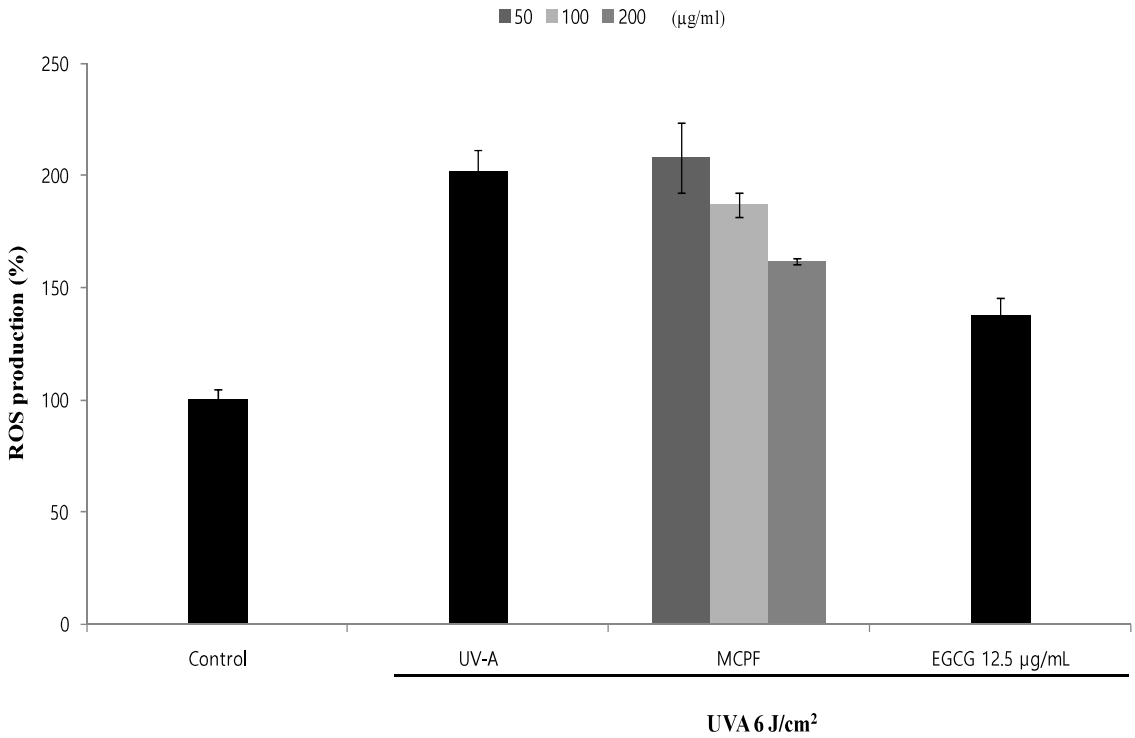
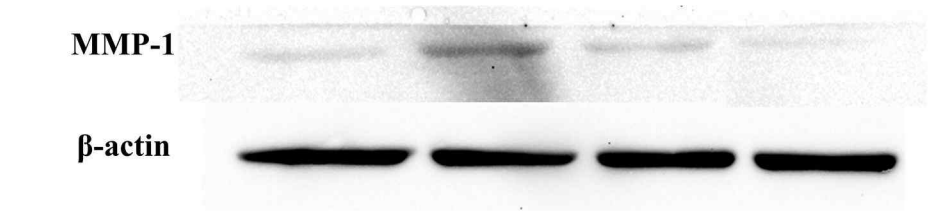


EGCG : Epigallocatechin gallate MCPF : Miyeokgui celluclast purified fucoidan

Fig 7. UVA-induced ROS inhibition activity of purified fucoidan by a fluorescence die method on human dermal fibroblast.

3.9. MMP-1 expression and inhibition activity of fucoidan

Western blot analysis was performed for the measurement of MMPs expression. The HDF cells were initially seeded in 60 π dish as 2 x 10^4 number of cells and incubated for 24 hours. Then, the cells were treated with Miyeokgui Celluclast purified fucoidan at concentration of 50, 100, and 200 μg/mL. After 24 hours, cells were washed twice with HBSS and exposed UVA-irradiation in thin layer of HBSS. Then, the cells were exposed Miyeokgui Celluclast purified fucoidan at same concentration as before in a serum-free medium. After incubating for 24 hours, we removed the medium and washed it twice with cold PBS. Then the cells were lysed with lysis buffer as described in section 2. 12. for 1 hour and separated the lysate and using centrifugation at 14000 rpm, 4°C, 20 minutes. Electrophoresis, was carried out using a SDS-PAGE and the separated proteins were transferd to a membrane, after blocking with blocking buffer, membranes were exposed to primary and secondary antibodies respectively. MMP-1 was found to be expressed in 52 kDa of molecular weight fraction and β-actin was expressed in 43 kDa of molecular weight fraction.



UVA (6 J/cm$^{2)}$	-	+	+	+
EGCG (50 μg/mL)	-	-	-	+
MCPF (200 μg/mL)	-	-	+	-

EGCG : Epigallocatechin gallate MCPF : Miyeokgui celluclast purified fucoidan

Fig 7. Western blot analysis of the inhibitory effects of purified fucoidan against UVA-induced expression of MMP-1 on human dermal fibroblast.

4. Discussion

Miyeokgui extracts by enzyme-assistant and HCl showed a similar level of yield, carbohydrate content, and sulfate content. Therefore, elastase and collagenase inhibition experiments were performed using all the extracts using the carbohydrase enzymes which has the most efficiency and potential activity. Elastase inhibition activities were evaluated for the concentrations of 12.5, 25, and 50 μg/mL enzyme-assistant extracts. Elastase inhibition activity of UA was 77.4 % and the enzyme-assistant extracts showed a dose-dependant inhibition of elastase activity. Among the six enzyme assisted extracts, Cellucalst and Ultraflo extracts showed the highest levels of elastase inhibition activity (71.4% and 75%) at 50 μg/mL. These elastase inhibition activities were closed to that of UA. Collagenase inhibition activity was determined for the 100, 200, and 400 μg/mL concentrations of the enzyme-assistaed extracts. Collagenase inhibition activity of EGCG was 73.8% and the enzyme-assisted extracts indicated a dose-dependent inhibition of collagenase activity. Additionally, the Celluclast and Ultraflo extracts also showed high levels of collagenase inhibition activity (67.9% and 65.8%) at 400 μg/mL. However, HCl extracts didn't indicated elastase and collagenase inhibition activities. The reason for this might be the molecular weight of the extracts. The function of the five enzymes are focused on the hydrolysis of the carbohydrates at specific sites. But hydrolysis through HCl proceed without any specificity. Therefore, we assume that activities of enzyme-assisted extracts and HCl-assisted extracts are different from each other. These results suggests that polysaccharides isolated from Miyeokgui by enzymes contain substances with anti-wrinkle activity. Therefore, elastase inhibition activity, collagenase inhibition activity, and commercial efficacy of enzymes were consideredfor the further experiments using Celluclast for the isolation of fucoidan and for *in vitro* experiments.

The isolation of CPS, CF, and purified fucoidan from Miyeokgui Celluclast extract (PF)was

performed using a DEAE-cellulose column. Based on the column seperation the eluates were divided in to four fractions as F1, F2, F3, and F4. And then, the sulfate content and the monosaccharide compositions of fractions were measured. Among the fractions F3 showed the highest sulfate contents and fucose composition. Therefore, F3 was determined as the purified fucoidan isolated from Miyeokgui. The F3 fraction was used for further experiments.

In vitro experiments including cytotoxicity, collagen synthesis, protection against UVA, inhibition of ROS, and inhibition of MMP-1 were performed for the identification of anti-wrinkle activity of PF.

Cell viability and LDH release in HDF cells were analyzed in order to determine the toxicity of the fucoidans. Cell viability of PF showed as 99.6%, 95.7%, 98.6%, at each concentration. LDH release of PF showed as 90.6%, 90.6%, 85.9 at each concentration. Therefore, PF was considered not to have any cytotoxic effect up to the concentration of 200 μg/mL and within the concentrations of 50, 100, and 200 μg/mL. Aforementioned concentrations were used continuously in the further studies.

PF showed an increasing rate of collagen synthesis activity (132.2%) at the highest concentration. This value is similar compared with the ascorbic acid a known compound possessing collagen synthesis activity. As these results suggests PF can be used to strengthen the extracellular matrix due to increased collagen contents.

And PF also indicated protective activity against UVA-induced cell damage. The energy of 6 J/cm2 of UVA didn't affect the cell viability, but increased the LDH release and ROS production. The PF decreased the LDH release and ROS production on a dose-dependent manor. These results suggested that the PF could protect dermal layer of human skin against UVA.

Finally, we measured MMP-1 inhibition activity of PF as an indicator of wrinkle formation.

The PF indicated a profound MMP-1 inhibition activity. Based on our data we suggests that PF decreases ROS, formation mechanisms that mediate wrinkle formation. However, further studies are in need for identifying the effects on the expression of upstream factors that regulate the expression of MMP-1.

5. CONCLUSION

In this study, we investigated anti-wrinkle activity of fucoidan isolated from Miyeokgui. Our results demonstrated a profound anti-wrinkle effect of fucoidan on collagen synthesis and MMP-1 inhibition in human dermal fibroblasts. Fucoidan isolated from Celluclast extract showed a dramatic increase in collagen synthesis and inhibition of MMPs. Therefore, we suggest that the enzyme-assisted isolation method of fucoidan is more efficient than HCl isolation method. Further, the fucoidan isolated from Miyeokgui showed higher levels of collagen synthesis and MMPs inhibition activities. This suggests the possibility of fucoidan isolated from Miyeokgui to be utilized as a potential material of functional cosmetics.

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학부 4학년에 연구실에 들어와 다른 선배님들의 연구와 실험을 돕고 배우면서, 연구자의 꿈을 키우게 되었고, 저 또한 좋은 주제로 연구를 하여 멋있게 졸업을 해서 석사 학위를 받고 싶다는 생각을 품게 되었는데 어느새 시간이 흘러 학위논문을 작성하게 되었습니다.

처음 연구실에 들어가겠다는 뜻을 밝혔을 때 학부생활을 하던 저의 모습을 보시고 반대하셨지만, 앞으로 변한 모습을 보여드리고 끝까지 노력하겠다는 저의 말을 믿고 받아주시고, 부족한 모습을 보여도 올바른 연구의 길로 인도해주신 전유진 교수님께 가장 먼저 감사의 말씀을 드리고 싶습니다. 실망을 안겨드린 적도 많았지만 변함없는 신뢰와 기회를 주셔서, 저 또한 더욱 힘을 내어서 무사히 석사과정을 마칠 수 있었습니다. 이 후에도 끝없이 새로운 것에 대한 탐구와 도전을 하시는 교수님의 모습을 본받아 더욱더 발전하여 교수님의 제자로 부끄럽지 않도록 하겠습니다.

엄격해 보이지만 다정하고 유쾌한 모습도 보여주시며, 승선실습으로 해양에 대한 관심을 더욱 높여주신 송춘복 교수님, 직접 실험을 하시며 연구자의 모습을 보여주시는 이제희 교수님, 멋진 외모와 목소리로 해양 미생물에 대해서 가르쳐

주신 허문수 교수님, 어려운 내용의 수업이지만 재미있는 말로 즐거운 수업을 만들어주시고, 아쿠아리움 견학 등 다양한 경험의 기회를 만들어 주신 여인규 교수님, 웃는 모습이 매력적이시고 항상 학생들에게 친근하게 다가와 주시는 정준범 교수님, 때로는 친한 친구처럼, 때로는 엄격한 지도자처럼 조언을 아끼지 않으신 이승헌 교수님, 처음으로 발표수업을 진행하시고 발표하는 방법과 중요성에 대해 가르쳐 주신 이경준 교수님, 학부가 달라서 아쉽게 수업을 듣지는 못했지만 인사를 드릴 때 격려와 조언을 아끼지 않아주신 최광식 교수님, 김기영 교수님, 박상률 교수님, 정석근 교수님, 교수님들께서 가르쳐 주신 지식과 건내주신 조언과 격려가 제가 석사 학위를 무사히 마치는 데 큰 힘이 되었습니다.

연구실에서 같이 생활 하지 않았지만 항상 후배들을 위해 멀리서도 힘써주신 수진이형, 친근하게 웃어주시며 다가와 주시는 길남이형, 실험실 생활 뿐만 아니라 사회생활을 하는 법을 가르쳐 주신 승홍이형, 몇 번 보지 못했지만 잘 챙겨 주시던 선희누나, 처음 실험에 대한 공부를 할 때 기초를 잡아 주신 석천이형, 짧은 시간밖에 못 봤지만 리더쉽이 뛰어난 성명이형, 넓은 분야에 대한 지식의 뛰어나 연구하는 법에 대해 조언을 아끼지 않으신 지혁이형, 항상 걱정해주고 챙겨주시는 긴내누나, 친근감 있게 많은 이야기를 해주신 원우형, 실험과 과제에 대해 물

어보면 적극적으로 돠주신 주영누나, 유쾌한 모습으로 기운이 나게 해주시는 아름다슬누나, 처음 연구실에 들어왔을 때 자리만 지키고 있던 저를 챙겨주시던 민철이형, 함께 살면서 즐거운 시간을 보내고 의지 할 수 있는 버팀목이 되어주던 준성이형, 무심한 듯 관심을 주시는 재영이형, 연구실에 들어오라고 적극 추천해 준 현수 형, 항상 웃는 모습으로 반겨주시는 은아누나, 같이 실험을 하며 많은 것을 가르쳐 주고, 항상 노력하는 모습을 보여주는 나래누나, 선뜻 다가가기 힘들면서도 더 가깝게 다가갈 수 있게끔 해주시는 선배님들 덕분에 오늘의 제가 있을 수 있어 감사드립니다. 때로는 친구처럼, 때로는 선배처럼 저에게 많은 도움을 준 서영이, 묵묵히 자신의 역할을 다하는 형호, 정확히 일처리를 하려고 노력하는 바로, 학부 때부터 봐오며 미운 정, 고운 정이 들고 많은 부분에서 도움을 준 동기 혜원이, 모든 연구실 멤버들에게 감사의 말씀 드리고 선배님들께는 부끄럽지 않은 후배가, 후배들에겐 자랑스런 선배가 될 수 있도록 노력하겠습니다.

제가 뜻을 펼 칠 수 있도록 전폭 지원 해 주신 우리 부모님과 두 동생에게 무한한 고마움을 느끼며 감사의 말을 드립니다. 가정을 위해 매일매일 노력하시는 우리 아버지, 짜증만 부리는 아들을 항상 걱정하고 돌봐주신 우리 어머니, 제가 미처 신경쓰지 못하는 것을 챙겨주고 연구실 생활에 집중 할 수 있게 도와준 완택

이와 민정이 너무너무 고맙고 자랑스런 형, 오빠가 될 수 있게 노력 하겠습니다.

마지막으로, 학부 때 만나서 대학원까지 같이 보낸 사랑하는 현경이, 힘든 일이 있을 때 서로 버팀목이 되어 주고 격려와 위로를 나누어서 큰 힘이 되었습니다. 혼자만의 고민으로 괴로울 때도 항상 옆에서 힘을 실어주고, 다른 연인들이 해주는 만큼 못 해줄 때도 이해를 하며 기다려준 현경이에게 너무나 고맙습니다.

제가 석사 학위를 마칠 수 있게 도와주신 모든 분들께 다시 한번 감사의 말씀 드리며, 이 분들의 도움이 가치 있는 것이 되게끔 더욱더 노력하고 발전하는 모습을 보여 드리겠다는 다짐을 하며, 최선을 다해 생활하겠습니다.