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귤피 펙틴 유래 효소적 가수분해물의 세포 보호 효과

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Cell Protective Effects of Enzymatic Hydrolysates of Citrus Peel Pectin

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요 약: Pectin은 식물 세포벽의 주요성분으로 과실이나 채소류의 세포막이나 세포막 사이의 엷은 층에 존재하며, 고점도의 수용성 다당체로 염과 pH에 의한 점도 변화가 심하며, 알코올류와 만나 gelation 되는 특징을 가지고 있다. 식품분야에서 펙틴은 점도의 증가 및 겔 형성제로 사용되어 왔으나, 화장품 분야에서는 그 사용이 극히 제한적이었다. 본 연구에서는 pectin 효소 분해물의 분해 정도 및 분자량 분포를 정확히 확인하기 위해, HPLC (GPC)를 이용한 분석 조건을 확립하였으며, 생물 전환 공정을 통해 저분자량 pectin oligomer가 형성되는 것을 확인하였다. 그리고, 2종의 효소에 대한 저분자량 pectin oligomer 생산 최적 조건 실험을 진행하여 최적 생산 조건을 확립하였으며, 이로 부터 제조한 pectin 효소분해물에서 저분자량 pectin oligomer를 선별적으로 분리하는 공정도 개발하였다. 이러한 공정을 통해 제조된 저분자량 pectin oligomer 소재 LMPH A 와 B는 약 200 ~ 2,700 Da 정도의 분자량 분포를 가지는 것으로 확인되었다. LMPH A와 B의 생리활성을 확인한 결과, 2종 모두 항산화 활성을 보였다. 게다가, 이들이 pectin 및 D-galacturonic acid 보다 상대적으로 우수하며, 농도의존적으로 증가함을 보였다. 또한 자외선(UVB)에 의한 피부세포의 광손상 및 이로 인한 apoptosis를 방어하는 효과를 나타내었다. 세포 활성화 효과 측정결과는 LMPH A, B 모두 0.025% 이상의 농도에서 세포 활성화 효과를 보였으며, 농도가 0.5%에 이를 때까지 농도 의존적으로 증가하는 것을 확인할 수 있었다. 특히, LMPH B의 경우, 0.5% 농도에서 약 30%, LMPH A도 약 22%의 매우 우수한 세포 활성화 효과를 가지는 것으로 확인되었다. 결론적으로, 본 연구를 통해 개발된 2종의 LMPH가 우수한 생리활성과 동시에 우수한 안전성을 보임으로써, 향후 화장품 소재로 응용 가능성이 매우 높을 것으로 기대된다.

Abstract: Pectin, a naturally occurring polysaccharide, has in recent years attracted considerable attention. Its benefits are increasingly appreciated by scientists and consumers due to its safety and usefulness. The chemistry and gel-forming characteristics of pectin have enabled to be used in pharmaceutical industry, health promotion and treatment. Yet, it has been rarely used in cosmetics because of its incompatibility with many cosmetic ingredients, including alcohols, and unstable viscosity of pectin gels under various pH and salt conditions. However, low-molecular-weight pectin oligomers have excellent biological activities, and depolymerization of pectin to produce cosmetic ingredients would be very useful. In this study, we attempted the development of cosmetic ingredients using pectin with an excellent effect on human skin. We developed a bio-conversion process that uses enzymatic hydrolysis to produce pectin hydrolysates containing mainly low-molecular-weight pectin oligomers. Gel permeation chromatography was used to determined the ratio of hydrolysis. The molecular weight of the pectin hydrolysates obtained varied between 200 and 2,700 Da. The two newly developed

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low-molecular-weight pectin hydrolysates, LMPH A and B, had higher anti-oxidative activities than pectin or D-galacturonic. Exposure to UVB radiation induces apoptotic cell death in epidermal cells. Annexin V binding and propidium iodide uptake were measured by flow cytometry to evaluate UVB-induced cell death in HaCaT cells. Both LMPH A and B reduced UVB-induced cell death and increased cell proliferation by 22% and 30% at 0.5% concentration respectively, while pectin had no significant activity. In conclusion, this study suggests that the newly developed low-molecular-weight pectin hydrolysates can be used as safe and biologically active cosmetic ingredients.

Keywords: *pectin, enzymatic hydrolysis, UVB, photo-damage*

1. Introduction

Skin aging can be categorized into chronological (intrinsic) aging and photo-aging (extrinsic). The term "intrinsic aging" describes irreversible physiological processes, while extrinsic aging is mainly a consequence of environmental damage[1], primarily caused by cumulative exposure to ultraviolet (UV) radiation from the sun, which generates reactive oxygens species (ROS)[2]. Solar UV radiation, particularly ultraviolet B (UVB) with a wavelength range between 290 and 320 nm, elicits many adverse effects in the skin, which include development of skin cancer[3], suppression of the immune system[4,5] and photo-aging[6]. ROS generated by UV radiation result in oxidative damage to cellular components such as mitochondrial and nuclear DNA which in turn accelerates aging and contributes to skin cancers[7].

The induction of programmed cell death, or apoptosis, by UV radiation is an important protective mechanism from neoplastic transformation for the skin. Apoptosis, a prevalent phenomenon in multi-cellular organisms, constitutes a basis for tissue homoeostasis and individual development and is responsible for the clearance of senescent cells, inhibition of cancerogenesis and immune response, the disturbance of which would almost inevitably lead to tumourigenesis, immune disorders or other major physiological disturbances in the body[8].

Programmed cell death can be induced by innumerable stimuli, including radiation, reactive oxygen species, heavy metals, aliphatic acids, virus infection, and anti-tumour drugs[9-13]. UV irradiation represents one of the most powerful natural apoptotic stimuli in diverse cell types within the skin, including keratinocytes, lymphocytes, and Langerhans cells[14,15].

Pectins are very complex and heterogeneous molecules, which are regular ingredients of all higher plants. Pectin is a complex polysaccharide, which consists of the α (1-4)-linked polygalacturonic acid backbone with intervening rhamnose residues, modified with neutral sugar side chains and non-sugar components such as acetyl, methyl, and ferulic acid groups. Galacturonic acid residues in pectin are partly present as methyl esters. The degree of esterification is defined as the percentage of carboxyl groups esterified. Pectin with a degree of esterification above 50% is named high-methyl-ester pectin or high-ester-pectin, whereas pectin with a degree of esterification below 50% is referred to as low-methyl-ester pectin or low-ester-pectin. Most pectin found in plant material, such as fruits, vegetables, and grass, is high-methyl-ester pectin.

Pectin galacturonic acid is present in three polymeric forms: homogalcturonan, a linear polymer of α (1-4)-linked galactuonic acid residues containing variable levels of methylesterification of the carboxyl group and acetylation on C-2; rhamnogalacturonan I, a repeating disaccharide of galacturonic acid and rhamnose; and rhamnogalacturnon II, a homogalacturonic backbone with numerous complex sidechains containing rhamnose and other neutral sugars. How these various polymers are attached to each other is still an open question[16].

Besides their functions in living tissues, pectins are also of commercial interest, as they are used as gelling agents in the manufacture of jams, jellies, marmalades and confectionery and for the stabilization of acidified dairy drinks[17]. Pectin also has several unique properties that have enabled it to be used as a matrix for the entrapment

and/or delivery of a variety of drugs, proteins, and cells.

Yet, pectin has been rarely used in cosmetics because of its incompatibility with many cosmetic ingredients including alcohols, and several inconveniences because it is negatively charged and viscosity of pectin gels is unstable.

The potential beneficial features of pectin are also limited by its molecular size. Pectin contains from a few hundred to about 1000 saccharide units in a chain-like configuration; this corresponds to average molecular weights from about 50,000 to 150,000 Da. Thus, its high molecular mass interferes with efficient skin penetration.

The current pectin production method employs high temperature (70 ~ 90 ℃) in combination with acidic hydrolysis using nitric, hydrochloric or sulphuric acid. The pH is between 1.5 and 2.5, and the reaction is continued for several hours. The main disadvantage of acid hydrolysis technology, and one which raises environmental concerns, is the generation of large volumes of acidic effluent that require further treatment before release. Moreover, harsh acidic treatment causes depolymerization and deesterification of the pectin chains. Enzymatic extraction of pectin seems more advantageous in terms of energy consumption and waste management. The enzymatic process is usually carried out at a pH between 3 and 5 and temperatures around 50 ℃ which is more beneficial in terms of economy and environmental impact. However, enzymatic extraction provides low pectin yields, low molecular weights of the product, and low galacturonan content[18].

In this study, we developed a bio-conversion process to produce low-molecular-weight pectin hydrolysates using chondroitinase and hyaluronidase to evaluate the ability of these hydrolysates to prevent the damage in UVB-irradiated HaCaT cells, a human keratinocyte line.

2. Materials and Methods

2.1. Materials

Annexin V-Alexa Fluor 488 were obtained from Invitrogen/ Molecular Probes. Pectin from citrus peel (galacturonic acid ≥ 74.0%), pectinase from *Rhizopus* sp. (Macerozyme R-10), pectinase from *Aspergillus aculeatus* (Pectinex® Ultra SPL), pectinase from *Aspergillus niger* (Pectinex 3XL®), pectolyase from *Aspergillus japonicus* and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich. Other commercially available reagents and solvents were used as received.

2.2. DPPH Radical Scavenging Activity

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity was evaluated according to the method of Blois, et al.[19] with minor modifications. DPPH solution (0.1 mM in methanol) was added to an equal volume of sample solution and allowed to react for 10 min at room temperature, after which the optical density was measured at 565 nm using a microplate autoreader (ELX800, Bio-Tek, USA).

2.3. Superoxide Radical-Scavenging Activity

ROS-scavenging activity was measured by monitoring the reduction of nitroblue tetrazolium. Briefly, the sample, Na_2CO_3 buffer (50 mM, pH 10.2), xanthine (3 mM in Na_2CO_3 buffer), ethylene diamine tetraacetic acid (3 mM), nitroblue tetrazolium (0.75 mM), and bovine serum albumin solution were mixed, and the mixture was incubated at 25 ℃ for 10 min. Xanthine oxidase (0.25 units/mL) solution was then added and the sample was further incubated at 25 ℃ for 25 min. The reaction was quenched with $CuCl_2$ (6 mM). Scavenging activity was calculated by comparing the optical density at 565 nm of the control with that of the sample using a microplate autoreader.

2.4. Gel Permeation Chromatography

Gel permeation chromatography was carried out using a Waters Alliance2695 separation module (Waters, USA), with a high-pressure gradient pump, an Alliance autosampler, an evaporative light scattering (ELSD) detector (ELSD 2000, Alltech Associates, Inc., Deerfield, IL, USA), and with a Shodex OHpak KB-804 column (300 × 0.8 mm, Showa Denko K.K., Tokyo, Japan), which was eluted in isocratic mode with acetic acid buffer (pH 4.75) at 50 ℃ and a flow

rate of 0.6 mL/min. Peaks were detected by using the ELSD detector. The molecular weight of the products of pectin hydrolysis was estimated from a calibration curve.

2.5. Preparation of Pectin Hydrolysates

Varying amounts of each enzyme were added to pectin suspensions (5%, w/v, 2 mL) in 50 mM acetate buffer (pH 4.0) or 50 mM citrate buffer (pH 5.0). The suspensions were agitated for 18 h on a reciprocating shaker, and an equal volume of ethyl alcohol was added to remove high-molecular-weight unhydrolyzed pectin and the enzymes. The suspensions were kept at 4 ℃ for 4 h followed by removal of the precipitate by centrifugation at 15,000 rpm. The supernatants were evaporated in a rotary vacuum evaporator until complete removal of ethyl alcohol, lyophilized, and stored at -70 ℃.

2.6. Cells

HaCaT cells were cultured in DMEM with 10% fetal bovine serum and 100 units/mL penicillin/streptomycin at 37 ℃ in a 5% CO_2 atmosphere. Cells were cultured to 90% ~ 95% confluence and then maintained in serum-free DMEM for 24 h before UVB exposure.

2.7. Cell Viability Assay

The number of viable cells was determined from the ability of mitochondria to convert 3(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) to formazan dye. All procedures were performed at 37 ℃ in a humidified 5% CO_2 atmosphere. HaCaT cells were cultured overnight in a 96-well plate, at a density 2×10^4 cells/well. The cells were then treated with pectin hydrolysates, according to the experimental design. After 24 h, the medium was removed and 10 μL of MTT (10 mg/mL) was added to each well, and the cells were incubated for a further 4 h. The absorbance was then measured at 570 nm using a microplate reader (Sunrise Basic, Austria).

2.8. UVB Irradiation of HaCaT Cells

HaCaT cells maintained in serum-free DMEM for 24 h were incubated with pectin or its hydrolysate for 1 h before UVB irradiation. After the incubation, the cells were washed once in PBS, which was then removed. Cells were irradiated with a dose of 100 mJ/cm^2 using a bank of two UVB lamps (peak emission of 310 ~ 315 nm). Following irradiation, HaCaT cells were again washed with PBS and returned to serum-free DMEM.

2.9. Annexin V/propidium Iodide Labeling of Cells

DMEM containing floating cells was collected 12 h after UVB irradiation. Attached cells were then washed once with PBS and detached with trypsin supplemented with EDTA, and trypsin was inactivated with DMEM containing 10% fetal bovine serum. The detached cells were then pooled with floating cells, the entire sample was pelleted by centrifugation, and DMEM was removed. The cells were resuspended in annexin-binding buffer [10 mM HEPES (pH 7.4), 140 mM NaCl, and 2.5 mM $CaCl_2$] at a concentration of 1×10^6 cells/mL and transferred to 1.5 mL microcentrifuge tubes. Cells were incubated for 30 min with Annexin V-Alexa Fluor 488 and propidium iodide according to the manufacturer's instructions. Cells were then diluted to a total volume of 0.5 mL in annexin binding buffer and analysed by two-colour flow cytometry using a total of 10,000 events. The emission fluorescence of the Annexin V conjugate was detected and recorded through a 530 / 30 band-pass filter in the FL1 detector. Propidium iodide was detected in the FL2 detector through a 585 / 42 band-pass filter. List mode data files gated on forward scatter versus side scatter were acquired and analysed. Appropriate electronic compensation was adjusted by acquiring cell populations stained with each dye or fluorophore individually, as well as an unstained control. Apoptotic cells were only those which stained positive for Annexin V and negative for propidium iodide, located in the bottom right quadrant.

2.10. Statistical Analysis

All experiments were performed in triplicate. Data are presented as mean ± standard error (SE). Statistical comparison was conducted using Student's *t*-test after ANOVA.

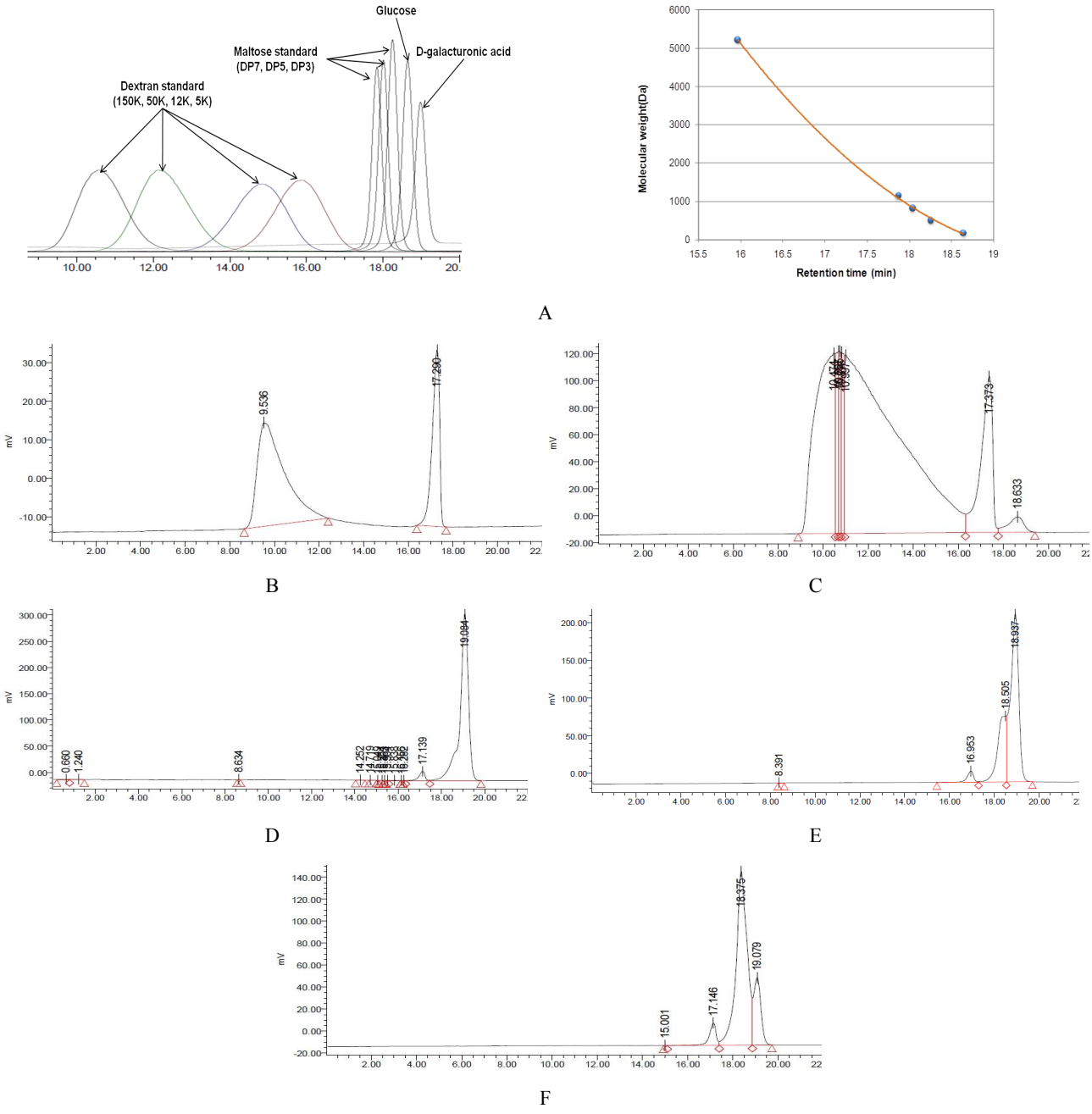


Figure 1. Molecular weight distribution of low-molecular-weight fractions of pectin hydrolysates. (A) A GPC calibration curve of maltose and dextran standards. (B) A gel permeation chromatogram of untreated pectin showing the main high-molecular-weight peak with an elution time of approximately 9.5 min and a low-molecular-weight sub-peak with an elution time approximately 17 min. (C) Treatment with Macerozyme R-10 shifted the main peak to 10.7 min, but this enzyme could not effectively hydrolyse high-molecular-weight pectin to low-molecular-weight oligomers. (D) Pectin was hydrolysed to very low-molecular-weight monomers or dimers with a retention time of 19 min by Pectinex® Ultra SPL. (E), (F) Both Pectinex 3XL® (E) and pectolyase (F) effectively produced low-molecular-weight oligomeric pectin hydrolysates, resulting in peaks with an elution time of 18 ~ 19 min.

The results were considered to be significant at $p < 0.001$.

3. Results and Discussion

We produced low-molecular-weight fractions of citrus peel pectin hydrolysates using the pectolytic enzymes Macerozyme R-10, Pectinex® Ultra SPL, Pectinex 3XL®, and pectolyase, and examined the molecular weight distribution of the hydrolysates by gel permeation chromatography (Figure 1).

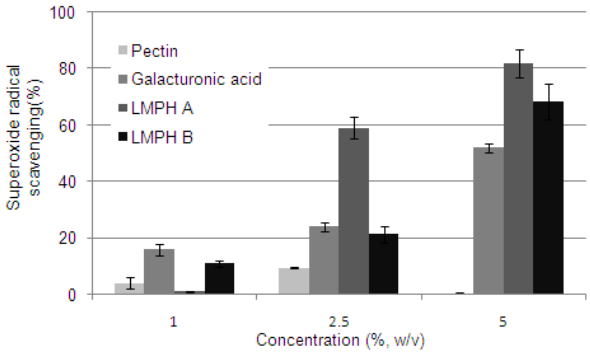
3.1. Gel Permeation Chromatography Analysis of Ppectin Hydrolysates

The peak of untreated high-molecular-weight pectin was eluted at approximately 9.5 min, with a low-molecular-weight sub-peak with elution time of approximately 17 min. We estimated the molecular weights of these two peaks as approximately 300 kDa and 2 kDa, respectively, using a GPC calibration curve of dextran standards (Figure 1A). Incubation with Macerozyme R-10, an enzyme derived from *Rhizopus* sp., shifted the main pectin peak to 10.7 min but was unable to effectively hydrolyse high-molecular-weight pectin to low-molecular-weight oligomers (Figure 1C). Pectinex® Ultra SPL (derived from *A. aculeatus*), hydrolysed pectin to very low-molecular-weight monomers or dimers, which eluted after 19 min (Figure 1D). Pectinase from *A. niger* (Pectinex 3XL®) and pectolyase from *A. japonicus* effectively produced low-molecular-weight oligomeric pectin hydrolysate with the molecular weight between 200 to 2700 Da (Figure 1E, F). Thus, we defined the hydrolysates produced by Pectinex 3XL® and pectolyase as low-molecular-weight pectin hydrolysates (LMPH) A and B, respectively.

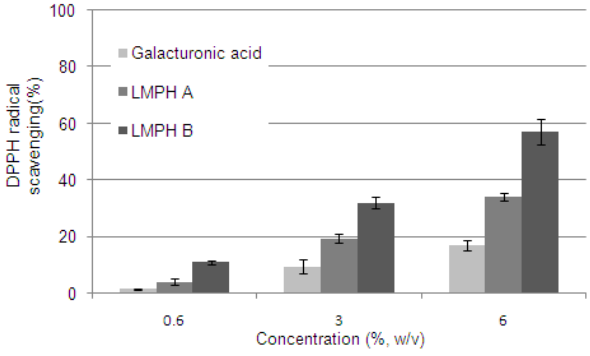
Next, we evaluated the biological activities of LMPH A and B.

3.2. Anti-oxidative Activity of Low-molecular-weight Pectin Hydrolysate

Figure 2 presents the results of evaluation of the scavenging activities of LMPH A and B against DPPH radical, a stable free radical, and superoxide anion, generated



A



B

Figure 2. Anti-oxidative activities of low molecular weight pectin hydrolysates. (A) LMPH A (produced by using Pectinex 3XL®; 2.5%, w/v) removed 59% of superoxide anion; LMPH B (produced by using pectolyase; 2.5%, w/v) removed 21.3% of superoxide anion. (B) LMPH A (3.0%, w/v) scavenged 19.4% of DPPH radical; LMPH B (3.0%, w/v) scavenged 34.2% of DPPH radical.

by the xathine/xanthine oxidase system. LMPH A and B had higher anti-oxidative activities than pectin and D-galacturonic acid (the predominant sugar residue of pectin). LMPH A removed 59% of superoxide anion at a concentration of 2.5% (w/v) and scavenged 19.4% of DPPH radical at a concentration of 3.0% (w/v). Similarly, LMPH B removed 21.3% of superoxide anion and scavenged 34.2% of DPPH radical at the same concentrations. Untreated pectin and D-galacturonic acid used in the range of concentrations from 1% to 5% did not show scavenging activities above 16% with DPPH radical or superoxide anion. Therefore, we assume that bio-conversion of pectin

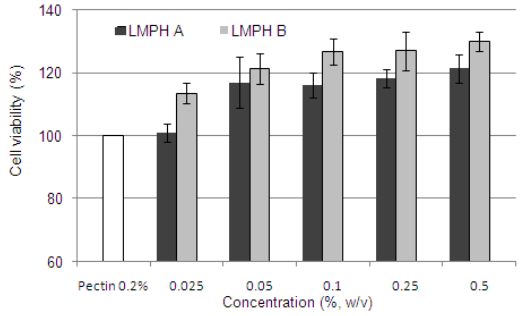


Figure 3. Effect of low-molecular-weight pectin hydrolysates on cell proliferation. Both hydrolysates dose-dependently induced cell proliferation (LMPH A, to approximately 1.2-fold; LMPH B, to approximately 1.3-fold) in the range of concentrations from 0.025% to 0.5% in comparison with the control (0.2% untreated pectin), whereas untreated pectin showed no significant activity (data not shown).

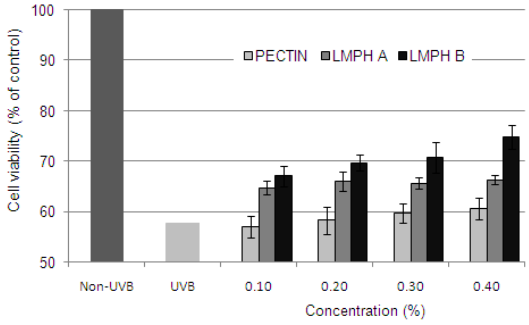


Figure 4. Cell protective by low-molecular-weight pectin hydrolysates after UVB radiation. Viability of UVB-irradiated HaCaT cells was 58% of that of non-irradiated cells. Viability of cells pre-treated with LMPH A (0.1%) before UVB exposure increased to 64%. Viability of cells pre-treated with LMPH B before UVB exposure increased dose-dependently (up to 75% at 0.4% LMPH B). Untreated pectin showed no significant activity.

molecules contributed to the anti-oxidative activities to LMPH A and B.

3.3. Effect of Low-molecular-weight Pectin Hydrolysates on Cell Proliferation

The effects of LMPH A and B treatment on cell proliferation were evaluated for the production of a potent low molecular weight pectin oligomer. Dermal fibroblasts are one of the essential cell types in the dermis. They play an important role in repairing skin wounds by providing growth factors and extracellular matrix proteins that attract new cells, which eventually restore the physiological continuity of the skin. LMPH A and B (0.5%) significantly induced proliferation of human dermal fibroblasts (by about 1.3-fold; Figure 3). These results show that bio-conversion of pectin molecules contributes to the stimulation of cell proliferation by LMPHs.

3.4. Cell-protective Activity of Low-molecular-weight Pectin Hydrolysates after UVB Radiation

To clarify the ability of low-molecular-weight pectin hydrolysates to protect cells from UVB radiation, HaCaT keratinocytes were pre-treated with different concentrations of LMPH A and B and exposed to UVB. The UVB dose used in this study (100 mJ/cm^2) was chosen because it caused an about 50% decrease in cell viability, as determined by the MTT assay. After further incubation for 24 h in the presence or absence of low-molecular-weight pectin hydrolysates, cell viability was measured by this assay. We found that both LMPH A and B dose-dependently reversed UVB-induced cell death (Figure 4). Irradiated cells without LMPH showed about 58% survival rate in comparison with that of non-irradiated cells, while the viability of cells pre-treated with LMPH A (0.1%) before UVB exposure increased to 64%. Pre-treatment with LMPH B before UVB exposure also dose-dependently increased cell viability (to 75% at 0.4% LMPH). Untreated pectin showed no significant activity. These results show that LMPHs protect cells from photo-damage induced by UVB radiation.

3.5. Effects of Low-molecular-weight Pectin Hydrolysates on Apoptosis of UVB-irradiated HaCaTs Cell

To quantify the apoptotic response of HaCaT cells to LMPH A or B treatment, Annexin V staining was performed to identify changes in the apoptotic cell population. Both pectin hydrolysates effectively reduced apoptosis of UVB-irradiated HaCaT cells. The mean percentage of Annexin V-positive / propidium iodide-negative cells among untreated cells was 6.3% (Figure 5A, E), and this population was increased significantly to

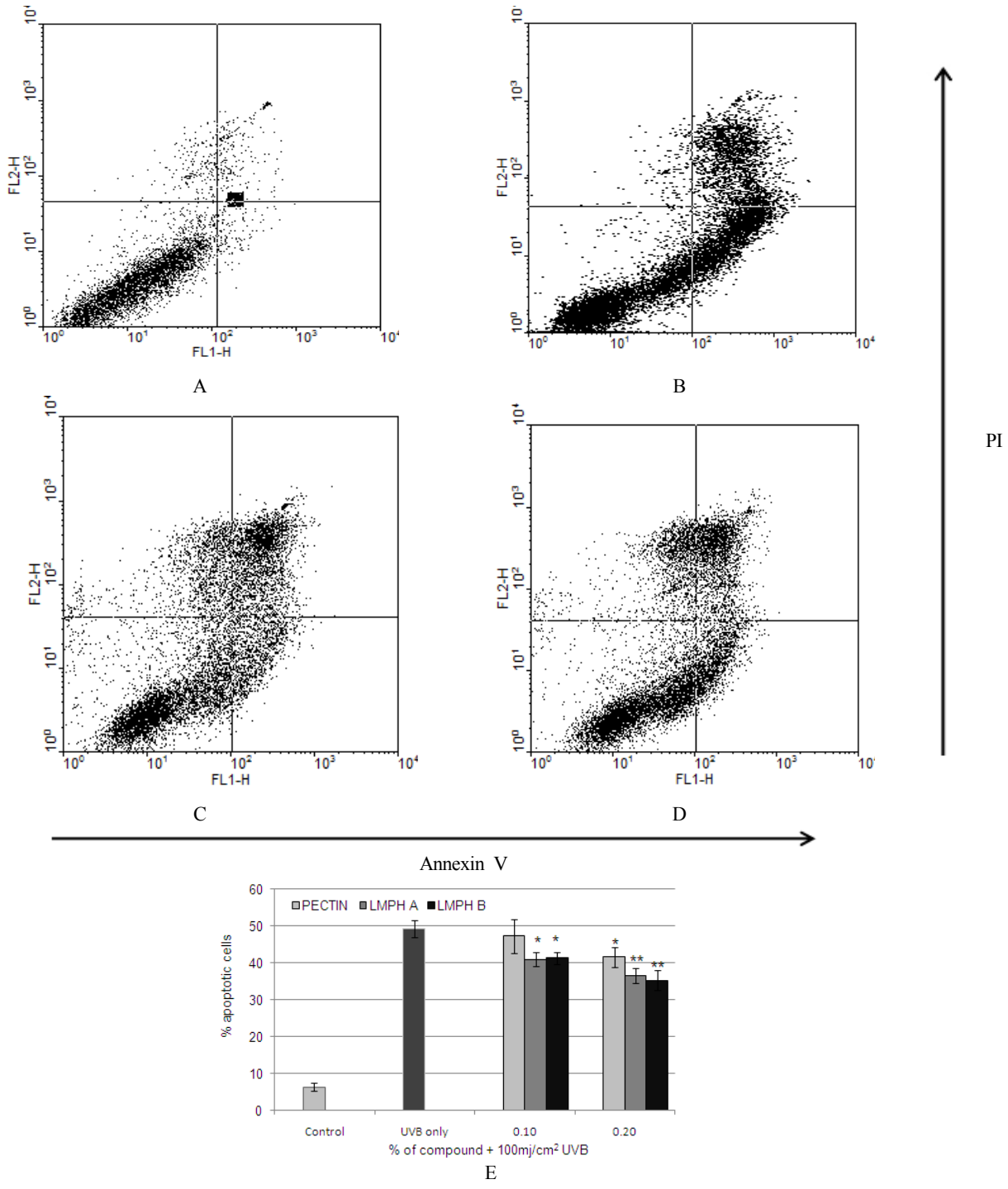


Figure 5. Effects of low-molecular-weight pectin hydrolysate on apoptosis of UVB-irradiated HaCaT cell. The percentage of Annexin V-positive/propidium iodide-negative cells among untreated HaCaTs was 6.3% (A and E), and this population was increased significantly (to 49.4%) by UVB irradiation (B and E). Treatment with LMPH A decreased the Annexin V-positive cell population (to 36.6% at 0.2% LMPH A; C and E). Treatment with LMPH B also decreased the Annexin V-positive cell population (to 35.3% at 0.2% LMPH B; D and E). One tailed Student's *t*-test: * $p < 0.05$, ** $p < 0.001$.

49.4% by UVB exposure (Figure 5B, E). Treatment with LMPH A decreased the Annexin V-positive cell population (to 36.6% at 0.2% LMPH A; Figure 5C, E). Treatment with LMPH B also decreased the Annexin V-positive cell population (to 35.3% at 0.2% LMPH B; Figure 5D, E). These results show that LMPHs protect HaCaT cells from UVB-induced apoptosis.

4. Conclusion

In this study, we developed a bio-conversion process to produce pectin hydrolysates obtained by enzymatic hydrolysis and containing mainly low-molecular-weight pectin oligomers. Gel permeation chromatography was used to determine the ratio of hydrolysis. The molecular weight of the pectin hydrolysates obtained varied between 200 and 2700 Da. The newly developed low-molecular-weight pectin hydrolysates, LMPH A and B, had higher anti-oxidative activities than pectin or d-galacturonic acid. The effects of LMPH A and B treatment on cell proliferation were evaluated for the production of a potent low-molecular-weight pectin oligomer. LMPH A and B significantly induced proliferation of human dermal fibroblasts (approximately 1.3-fold after treatment with 0.5% LMPH B), while untreated pectin had not significant activity. Therefore, we assume that bio-conversion of pectin molecules contributes to the anti-oxidative properties of LMPH A and B and to their ability to stimulate cell proliferation.

To examine the protective effect of low-molecular-weight pectin hydrolysates against UVB radiation, HaCaT keratinocytes were pre-treated with different concentrations of LMPH A and B and exposed to UVB. We found that LMPH A and B dose-dependently reversed UVB-induced cell death. Annexin V binding and propidium iodide uptake were measured by flow cytometry to evaluate UVB-induced cell death in HaCaT cells. We found that LMPH A and B reduced UVB-induced cell death. These results show that the use of LMPHs in cell cultures can protect them from photo-damage induced by UV-B radiation. Overall, this study suggests that the newly developed low-molecular-weight pectin hydrolysates can be used as safe and biologically active cosmetic ingredients.

Acknowledgements

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