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Synthesis and Characteristics of CM-1, 3-β-Glucan for Cosmetics

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Abstract

CM-1,3-β-Glucan was synthesis by introducing carboxy methyl group with molar ratio of monochloroacetic acid, and cytotoxicity, antioxidant, whitening effect, anti-inflammation, and anti-wrinkle characteristics were examined It was confirmed that carboxymethyl was quantitatively introduced to β-glucan. As a result of conducting test on cell stability of human keratinocyte cell lines (HaCaT), it did not present cytoxicity at both 0.1% and 0.5% concentration compared to control group and it was revealed to have no cytotoxicity at all. Presented high cell proliferation rate compared to control group at 0.1% concentration. Although anti-inflammation effect was confirmed with the reduction of COX-2 expression in CMB-1, CMB-5, CMB-6, CMBA-1, and CMBA-3 at 0.1% concentration as a result of conducting COX-2 expression influence test against animal cells, an anti-inflammatory test, it was revealed to have no effect in other substances. As a result of conducting MMP-1 expression influence test, an anti-wrinkle test, great anti-wrinkle effect was confirmed as MMI expression reduced in CMR-1, CMB-2, CMB-3, CMB-4, CMB-6, CMBA-1, and CMBA-3 at 0.1% concentration and CMB-1, CMB-2, CMB-3, CMB-4, and CMBA-1 at 0.5% concentration.

Keywords: *β-Glucan, CM-1,3-β-Glucan, cytotoxicity, whitening effect, anti-inflammation, anti-wrinkle*

1. Introduction

β-glucan is carbohydrate high molecular weight polysaccharide in which glucose molecules are connected together and it is known as substance with immunological enhancement and anticancer effect which exist in mushroom, yeast cell wall, grain, and others. It was first discovered as a substance that concerns with non-specific immonological reaction at yeast cell wall in 1940's. Krestin of bracket mushroom that belongs to basidiomycetes, lentinan of shiitake, shizophyllan of schizophyllum commune, and ploysaccharide contained

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in various edible mushrooms including ganoderma tsugae, sanghwang mushroom, honey mushroom, and others are actually used in clinical practice as medicine in Japan.

In regards to glucose, structure composed with backbone of β-1,3 bond is the most well-known and it possesses immunological function for overall human body by activating the secretion of various cytokine. β-glucan that is most widely produced nowadays is the extra-cellular polysaccharide produced by microorganism and it is mass produced using large fermentation tank. The produced β-glucan is high molecule composed of β-1,3-bond and there is a limitation in use as it is insoluble to water.

Therefore, in regards to domestic and foreign studies on the conversion into water soluble substance with bioactivity, although there has been studies to produce β-glucan in oligomer form by enzymatic treatment or convert it into low molecule by acid hydrolysis, manufacturing technology to produce it with consistent molecular weight has not been completed yet. Also, although methods to produce soluble β-glucan derivatives by carboxymethylation, sulfation, phosphorylation, tetrahydrofuranylation have been reported, not much is known about the manufacturing technology of these derivatives, their bioactivity, and their use.

In addition, due to increase in radiant heat and destruction of ozone layer followed by continuous increase in temperature caused by the change in earth environment, the frequency of exposure to ultraviolet rays has increased and skin aging is being activated with the acceleration of photoaging. Therefore, there is a demand for the development of materials for highly functiontional cosmetics that can defend and prevent it.

β-glucan is known to perform antioxidant reaction when exposed to the sunlight and it has effect of activating collagen production and fibroblast growth.(1, 2).

Therefore, method to acidize insoluble β-1,3-glucan, synthesize substance that prevents and defend skin aging through manufacturing water soluble carboxylation β-glucan by substituent synthesis, and cytotoxicity, anti-wrinkle activity, anti-inflammatory activity, and others regarding it were examined in this study.

2. Experiment

2.1. Synthesis of CM-1,3-β-Glucan

After dispersing β-Glucan to water and producing alkali β-Glucan at low temperature by adding NaOH, monochloroacetic acid(MCA) was added to molar ratio for 24 hour reaction at room temperature and then non-reactant was removed by cleaning it with water. It was synthesized according to rate of moles.

Also, synthesized CM-1,3-β-Glucan was converted into low molecule by acidolysis. (CMBA-1, 2, 3)

2.2. IR Measurement

In order to examine the molecular structure of carboxymethyl β-glucan derivatives, chemical structure was examined through KBr disc method with FT-IR (Japan Spectrum, FT-3000).

2.3. Measurement of Molecular Weight

Molecular weight is measured with GPC method. Molecular weight was measured by connecting 2 ultrahydrogel linear columns and 120 ultrahydrogel column, using 0.1 M NaCl solution as mobile phase at the velocity of 0.8 ㎖/min. Pluran (MW: 5.9kDa, 11.2kDa, 45kDA, 115kDa, 446kDa, 780kDa) was used as reference material.

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2.4 Exploration on Functionality

1. Skin Cell Safety *in vitro* test(MTT)

1.1. Cell cultivation and test method

① **Cell cultivation**

Human skin cell line (keratinocyte cell line HaCaT) was cultivated under the condition of 37℃, 5% CO_2 in 100 mm/60.1 cm² culture dish together with Dulbecco's Modified Eagle's Medium(DMEM), 10% fetal bovine serum(FBS), antibiotic-antimycotic (GIBCO, Cat No. 15240-062). Subculture was conducted when the confluence of over 80% occurred for this cell.

② **Test Method**

The cell is cultivated until it reaches the confluence of 50~60% under the cell cultivation condition after dividing it as 1×10^4 cell/well in 24 well. Conduct additional cultivation of 48 hours after throwing away the culture media and processing the test substance at certain concentration after putting new culture media that does not contain 10% FBS. Additional cultivation of 3 hours is conducted after putting 50ul of MTT solution (6.6mg/ml, 3-(4,5-dimethyl thiazol-2yl)-2,5 diphenyl-2H-tetrazolium bromide solution) to each well. After removing the culture solution, put 200ul of dimethylsulfoxide(DMSO, Amresco, 0231-500ML) and shake it for 10 minutes. Then, take 100µl in 96 well and measure absorbance at 540nm with ELISA (Enzyme-Linked Immunosorbent Assay) reader. The degree of cytotoxicity was expressed in percentage with the absorbance of control group using pure water as its standard.

Cell Stability(%) = (Absorbance of Test Group/ Absorbance of Control Group)x100

2. Antioxidant *in vitro* test

2.1. Material & Test Method

① **Antioxidant test through DPPH free radical scavenging activi**ty

This test method can grasp the degree of direct reaction of antioxidant effect by testing scavenging activity against free radicals generated by 1,1-Diphenyl-2-picryhydrazyl(DPPH, Sigma D9132-1G) on ethanol. Compound 1,1-Diphenyl-2-picryhydrazyl(DPPH, Sigma D9132-1G) generates free radicals within ethanol. By mixing it with CM-β-glucan of 0.1%, 0.5% concentration, the degree of reduction in amount of free radicals was examined. To be specific, DPPH solution 0.5ml of 0.1mM and low molecule hyaluronic acid 0.1ml diluted to the concentration of 0.1% and 0.5% was added to ethanol 0.4ml. After strongly vortexing it for 10 seconds, react it for 30 minutes in cool and dark place. The absorbance was measured at 517nm using ELISA and the degree of antioxidant capacity was expressed in percentage with absorbance of control group which used ethanol as its standard.

Free radical activation inhibition rate(%) = 100-{(active absorbance of each sample/ active absorbance of blank)X100}

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3. Anti-inflammatory Activation Test

3.1. Cell Cultivation & Test Method

① Cell Cultivation

Human skin cell line (keratinocyte cell line HaCaT) was cultivated under the condition of 37℃, 5% CO_2 in 100 mm/60.1 cm² culture dish together with Dulbecco's Modified Eagle's Medium(DMEM), 10% fetal bovine serum(FBS), antibiotic-antimycotic (GIBCO, Cat No. 15240-062). Subculture was conducted when the confluence of over 80% was occurred for this cell.

② Test Method

- COX-2 Expression Influence Test against Animal Cell

This test is a method to examine the degree of COX-2 expression, an inflammation inducing enzyme in keratinocyte cell lines (HaCaT), at mRNA level. In specific, keratinocyte cell line (HaCaT) was cultivated under the condition of 37℃, 5% CO_2 in 100 mm/60.1 cm² culture dish together with Dulbecco's Modified Eagle's Medium(DMEM), 10% fatal bovine serum(FBS), 1% antibiotic-antimycotic (GIBCO, Cat No. 15240-062). When the confluence of over 80% was occurred for this keratinocyte cell lines, it was divided as 1×10^4 cells/well in 96 well plate and cultivated until it reached the confluence of over 80% under the cell cultivation condition. After removing the culture media, examining the UV of 50m.J, exchanging it with new culture media containing CMB-1, CMB-2, CMB-3, CMB-4, CMB-5, CMB-6, CMBA-1, CMBA-2, CMBA-3, and B.G.O at 0.1% and 0.5% concentration, and conducting additional cultivation for 24 hours under the cell cultivation condition, the influence of each substance on the expression of COX-2 that is the inflammation related gene induced by UV test was examined. Real-time PCR was conducted for the test and the sequence of the test is as following.

i. Synthesis of RNA Isolation & cDNA

RNA and cDNA were separated and synthesized with the use of Fastline Cell RT-PCR kit(QIAGEN). Cell removed of culture media was cleaned with FCW(cell wash buffer), an component of Kit, and it was processed for 5 minutes at room temperature by adding Cell processing mixture (mixture in which gDAN Wipeout buffer is added to Buffer FCPL added Buffer FCPW) of 50ul. Then, it was incubated for 5 minutes at 75℃ after moving to new E-tube.

ii. Real-time PCR

In order to analyze each gene, Real-time PCR was conducted with synthesized cDNA. After mixing cDNA with probe(GAPDH, COX-2) and component (2x QuantiTect SYBR Green RT-PCT Master mix, QuantiTect RT mix, RNase- free water) of QuantiTect® SYBR® Green RT-PCR kit(Qiagen), it was conducted with the use of Rotor Gene Q Real-time PCR(Qiagen). In regards to all genes, it was conducted once for 30 minutes at 50℃, once for 15 minutes at 95℃, and 40 times for 15 seconds at 94℃, 30 seconds at 60℃ and 72℃ respectively. For the primer, QuantiTect® primer assays of Giagen was used in the test. The expression rate was presented by adjusting its own GAPDH.

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4. Anti-wrinkle Activation Test

4.1. Cell Cultivation & Test Method

① **Cell Cultivation**

Human dermal fibroblast was cultivated under the condition of 37℃, 5% CO_2 in 100 mm/60.1 cm² culture dish together with Dulbecco's Modified Eagle's Medium(DMEM), 10% fetal bovine serum(FBS), antibiotic-antimycotic (GIBCO, Cat No. 15240-062). Subculture was conducted when the confluence of over 80% was occurred for this cell.

② **Test Method**

- Collagenase MMP-1 Activation Inhibition Test

As a method to examine the degree of MMP-1 expression at mRNA level in human dermal fibroblast, specific method of cultivating human dermal fibroblast under the condition of 37℃, 5% CO_2 in 100 mm/60.1 cm² culture dish together with Dulbecco's Modified Eagle's Medium(DMEM), 10% fetal bovine serum(FBS), 1% antibiotic-antimycotic (GIBCO, Cat No. 15240-062) was used in this study. When the keratinocyte cell is confluenced over 80%, it was cultivated until it reached the confluence of over 80% under the cell cultivation condition after dividing it as 1×10^4 cells/well in 96 well plate. In order to induce the wrinkle caused by photoaging after removing the culture media, the test was conducted with 50mJ of UV, exchanging it with new culture media containing CMB-1, CMB-2, CMB-3, CMB-4, CMB-5, CMB-6, CMBA-1, CMBA-2, CMBA-3, and B.G.O at 0.1% and 0.5% concentration, conducting additional cultivation under the 24 hour cell cultivation condition, the influence of each substance on the expression of collagenase MMP-1, the lyase of collagen, induced by the UV test, was examined. In regards to the test method, real-time PCR was used and the sequence of test is as following.

i. Synthesis of RNA Isolation & cDNA

RNA and cDNA was separated and synthesized with the use of Fastline Cell RT-PCR kit(QIAGEN). Cell removed of culture media was cleaned with the component of kit FCW(cell wash buffer) and cell processing mixture (mixture of Buffer FCPL added Buffer FCPW and gDAN Wipeout buffer) of 50 ul was added for 5 minute processing at room temperature. Then it was moved to new E-tube and incubated for 5 minutes at 75℃.

ii. Real-time PCR

In order to analyze each gene, Real-time PCR was conducted with synthesized cDNA. After mixing cDNA with probe(GAPDH, COX-2) and components (2x QuantiTect SYBR Green RT-PCT Master mix, QuantiTect RT mix, RNase- free water) of QuantiTect® SYBR® Green RT-PCR kit(Qiagen), it was conducted with the use of Rotor Gene Q Real-time PCR(Qiagen). In regards to all genes, it was conducted once for 30 minutes at 50℃, once for 15 minutes at 95℃, and 40 times for 15 seconds at 94℃, 30 seconds at 60℃ and 72℃ respectively. For the primer, QuantiTect® primer assays of Giagen was used in the test. The expression rate was presented by adjusting its own GAPDH.

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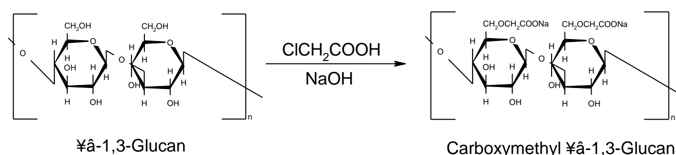
3. Results and Discursion

2.1. Synthesis of CM-1,3-β-Glucan

CM-1,3-β-Glucan was synthesized as Scheme 1 after dispersing β-Glucan to water, producing alkali β-Glucan at low temperature by adding NaOH, adding monochloroacetic acid to molar ratio for 24 hour reaction at room temperature, and then cleaning it with water. The result is as Table 1.



Scheme 1. Schematic diagram of CM-1,3-β-Glucan

Also, synthesized CM-1,3-β-Glucan was converted into low molecule by acidolysis (CMBA-1, 2, 3).

Table 1. Synthesis of CM-1,3-β-Glucan

Sample	β-Glucan (g/unit mole)	MCA (g/mole)	Yield (%)	Mw
CMB-1	50/0.31	22	96.26	145,000
CMB-2	50/0.31	28	102.74	145,000
CMB-3	50/0.31	42	107.0	145,000
CMB-4	50/0.31	56	112.0	145,000
CMB-5	50/0.31	70	106.76	145,000
CMB-6	50/0.31	84	142.0	145,000
CMBA-1	50/0.31	28	-	35,000
CMBA-2	50/0.31	28	-	11,000
β-glucan Oligosaccharide	-	-	-	1250

As a result of measuring FT-IR to examine molecular structure of carboxymethyl β-glucan derivative, it was revealed that CM-1,3-β-Glucan has been synthesized as the absorption band by -OH of broad β-Glucan appeared at around 3500cm^{-1}, absorption band by carbonyl at 1698cm-1, and absorption band by -O- of carbonyl at around 1100 as seen in Figure 1.

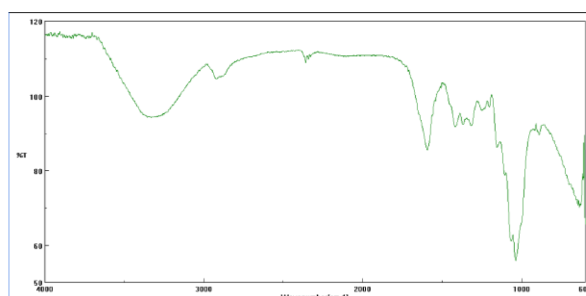


Figure 1. IR spectra of CM-1,3-β-Glucan

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Cell Stability of Keratinocyte Cell Lines (HaCaT)

Cell stability of CMB-1, CMB-2, CMB-3, CMB-4, CMB-5, CMB-6, CMBA-1, CMBA-2, CMBA-3, and B.G.O in keratinocyte cell lines HaCaT was evaluated. As a result, the cytotoxicity wasn't presented at concentration 0.1% and 0.5% compared to control group and it was revealed that there is no cytotoxicity.

(Standard: cell viability of over 80% is considered as the range without cytotoxicity)

Cell Proliferation Capacity

CMBA-1(about 42%) presented high cell proliferation rate compared to control group at 0.1% concentration.

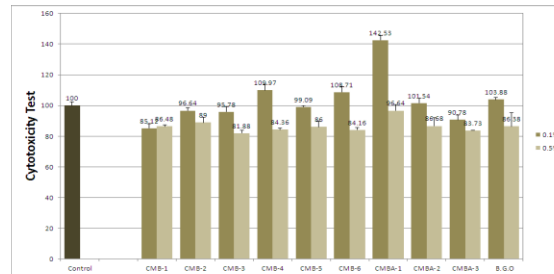

Figure 2. Cytotoxicity of CM-1,3-β-Glucan

Antioxidant Test

DPPH Free Radical Scavenging Ability

In order to examine the antioxidant capacity of CM-β-glucan in vitro, free radical activation inhibition rate was examined at 0.1% and 0.5% concentration in regards to CMB-1, CMB-2, CMB-3, CMB-4, CMB-5, CMB-6, CMBA-1, CMBA-2, CMBA-3, and B.G.O. As a result, Vitamin C 0.1% used as positive control group presented free radical activation inhibition rate of 61%. Although it is not as high activation inhibition rate as Vitamin C, CBB-1, CMB-2, CMB-4, CMB-5, CMB-6, and CMBA-3 sample also presented free radical activation inhibition rate of 25.75, 28.4%, 5.6%, 21.2%, 10.22%, and 16.6% respectively at 0.5% concentration.

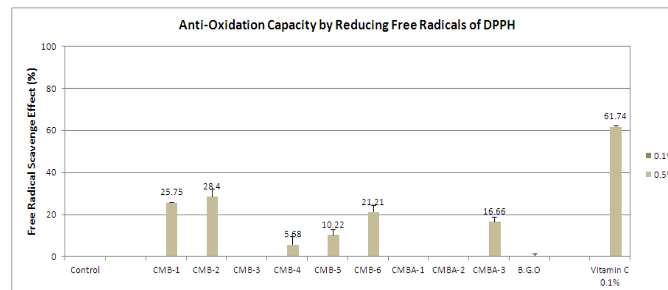

Figure 3. Anti-oxidation capacity by reducing free radicals of DPPH for CM-1,3-β-Glucan

Anti-inflammation Test Result

COX-2 Expression Influence Test against Animal Cells

In order to examine the influence of 0.1% and 0.5% concentration processed CMB-1, CMB-2, CMB-3, CMB-4, CMB-5, CMB-6, CMBA-1, CMBA-2, CMBA-3, and B.G.O on COX-2 expression in keratinocyte cell lines HaCaT, Real-time PCR was conducted. As a result, in comparison with positive control group which induced inflammation through UV test, the COX-2 expression at 0.1% concentration was reduced in CMB-1, CMB-5, CMB-6, CMBA-1, and CMBA-3 thus anti-inflammation effect was examined but it was revealed that there is no effect in other items.

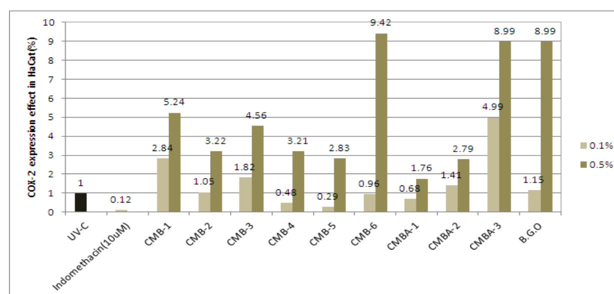

Figure 4. COX-2 expression effect in HaCat for CM-1,3-β-Glucan

Anti-wrinkle Test Results

MMP-1 Expression Influence Test Results

In order to examine the influence of 0.1% and 0.5% concentration processed CMB-1, CMB-2, CMB-3, CMB-4, CMB-5, CMB-6, CMBA-1, CMBA-2, CMBA-3, and B.G.O on the MMP1 expression in human dermal fibroblast, real-time PCR was conducted. As a result, it was found to have anti-wrinkle effect as MMI expression was reduced in CMR-1, CMB-2, CMB-3, CMB-4, CMB-6, CMBA-1, and CMBA-3 at 0.1% concentration and CMB-1, CMB-2, CMB-3, CMB-4, and CMBA-1 at 0.5% concentration compared to negative control group that induced the wrinkle caused by photoaging through UV test.

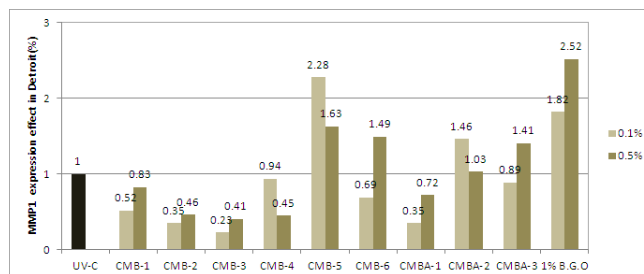

Figure 5. MMP1 expression effect in detroit for CM-1,3-β-Glucan

4. Conclusion

By introducing carboxymethyl to water insoluble β-glucan and synthesizing CM-1,3-β-Glucan with molar ratio, cytotoxicity, antioxidant, whitening, anti-inflammation, and anti-wrinkle characteristics were examined and the result is as following.

1. It was confirmed that carboxymethyl was quantitatively introduced to β-glucan.

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2. As a result of conducting test on cell stability of human keratinocyte cell lines (HaCaT), it did not present cytoxicity at both 0.1% and 0.5% concentration compared to control group and it was revealed to have no cytotoxicity at all. Particularly, low molecular weight substance CMBA-1 (about 42%) presented high cell proliferation rate compared to control group at 0.1% concentration.

3. In regards to antioxidant test DPPH free radical scavenging activity, CBB-1, CMB-2, CMB-4, CMB-5, CMB-6, and CMBA-3 presented free radical activation inhibition rate of 25.75, 28.4%, 5.6%, 21.2%, 10.22%, and 16.6% respectively at 0.5% concentration although it is not as high activation inhibition rate as Vitamin C.

4. Although anti-inflammation effect was confirmed with the reduction of COX-2 expression in CMB-1, CMB-5, CMB-6, CMBA-1, and CMBA-3 at 0.1% concentration as a result of conducting COX-2 expression influence test against animal cells, an anti-inflammatory test, it was revealed to have no effect in other substances.

5. As a result of conducting MMP-1 expression influence test, an anti-wrinkle test, great anti-wrinkle effect was confirmed as MMI expression reduced in CMR-1, CMB-2, CMB-3, CMB-4, CMB-6, CMBA-1, and CMBA-3 at 0.1% concentration and CMB-1, CMB-2, CMB-3, CMB-4, and CMBA-1 at 0.5% concentration.

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