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Proteome analysis for skin anti-agingactivities of *pinuskoraiensis*in human dermal fibroblasts

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ABSTRACT

The*Pinuskoraiensis* seed oil extracted by supercritical CO_2showed anti-oxidation activity. It also had reducing power caused by unsaturated fatty acids. Proteome analysis proved that *Pinuskoraiensis* seed oil promoted proteome synthesis related to skin anti-aging in human dermal fibroblasts. Itincreased the expression of collagen synthesis-related proteins in human dermal fibroblast. It also increased the expression level of anti-oxidant enzyme catalase 3.2 times. © 2016 Trade Science Inc. - INDIA

KEYWORDS

Pinuskoraiensis;
upercritical CO_2;
Skin anti-aging;
Collagen synthesis.

INTRODUCTION

The skin-aging process can be attributed both to intrinsic-aging and photo-aging. Intrinsic-aging damage is attributable to the passage of time, and photo-aging is the result of repeated exposure to ultraviolet radiation. Intrinsic-aged skin is smooth, pale, and finely wrinkled; photo-aged skin is coarsely wrinkled. In the dermis, the regulation of fibroblast proliferation, differentiation, migration, and apoptosis is dependent on the integrity of the extra-cellular matrix (ECM), and age-associated matrix changes are probably determinants of alterations in dermal functions. The dermis layer is composed of connective tissue and blood vessels. Dermal connective tissue contains collagen and elastin. Collagen fibers account largely for the volume of the skin and the bulk of its tensile strength, whereas elastic fibers are associated with skin's elasticity and resilience[1,2].

Collagen alterations are the most relevant changes occurring within the dermis in intrinsic and photo-aged skin. During the skin-aging process, collagen synthesis and enzymes involved in the post-translational processing of collagen are reduced[3]. Thus,reduced collagen contents have been suggested as a cause of the skin wrinkling observed in aged skin[4]. Procollagens, a precursor form of collagen,are synthesized within the endoplasmic reticulum (ER). Newly synthesized three-procollagen chainsassume a triple-helix form as the result of proryl 4-hydroxylase. Properly folded procollagens are secreted from the cell and help in the formation of collagen,whereas improperly folded abnormal procollagens are retained within the ER.

Skin aging is associated with the interaction between the matrix and the fibroblasts as well as reductionsin collagen content, and the formation of

a matrix structure within the dermal layer. In the skin's extracellular matrix, integrin and actin are believed to play a pivotal role in intracellular adhesion and cell protein adhesion, and thus also in the organization and assembly of the extracellular matrix,upon which skin firmness depends[5].

Pinuskoraiensis,which in Korea is referred to as pine nut, is widelyutilized as an important folk medicine. It has been reported that the Pinus plats have effects on anti-aging and anti-inflammation. It contains a variety of compounds, including phenolics and flavonoids, essential oils, and volatile flavors[6,7]. In seed of *Pinuskoraiensis*, various essential amino acid components were found. In seed oil, remarkable anti-oxidation activity was reported[8,9]. In this study, we extracted seed oil from *Pinuskoraiensis*via supercritical fluid extraction. Among the various effects of seed oil, anti-agingeffects were observed via proteome analysis. Proteome analysis based on 2-dimensional electrophoresis was applied for the profiling of protein factors involved in the observed skin anti-aging effects.

MATERIALS AND METHODS

Materials

Pinuskoraiensis seed oil was extracted with supercritical CO_2 fluid. Supercritical CO_2fluid extraction was conducted at a temperature of 40-60! and a pressure of 200-400 bar. For the dose experiments, the extract was dissolved in dimethyl sulfoxide (DMSO) and diluted with DMEM.

Cell cultures and viability assay

Human dermal fibroblastswere purchased from the American Type Culture Collection (ATCC, CRL-1635). Fibroblasts were cultured in DMEM (WelGenelnc.) with 10% fatal bovine serum (Gibco), and 1% Antibiotic-antimycotic (Gibco). Cultures were prepared in a humidified CO_2-controlled (5%) incubator at 37!. The cells were sub-cultured every seven days. An MTT assay was utilized to determine the ability of viable cells that convert a soluble tetrazolium salt, 3-(4, 5-dimethyl-2-tetrazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT), into an insoluble formazan precipitate. In brief, the fibroblasts were seeded into 96-well plates at $3x10^3$ cells/well and allowed 24 hours for surface adhesion. The culture medium was removed after 24 hours, then exchanged with a new medium containing *Pinuskoraiensis*extracts at variable concentrations from0~100 mg/L. The cultures were then maintained for three days at 37! in a CO_2 incubator. After 3 days of cultivation, the medium was removed. The cells were washed twice in PBS and incubated for 5 hours in MTT solution at 37!. After incubation, the MTT solution was removed. The formazan crystals formed were dissolved with DMSO for 15 min. Cell viability was determined by measuring the optical density of the formazan solution at 540 nm[10].

Two-dimensional gel electrophoresis

Cells washed in PBS were isolated via centrifugation. Proteins were extracted by cell lysis. The lysis buffer solution was prepared with 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 2% Carrier ampholyte, 4% PIC, and 0.002% BPB. Cells in lysis buffer were sonicated for 1 min and maintained for 30 min at room temperature. The solution was centrifuged for 5 min at 4000×g. The supernatant was collected and preserved at -20°C. The amount of total protein extracted was measured via the modified Bradford method[11]. Bovine γ-globulin was utilized as the standard protein.

Immobiline Dry strips (13cm, pI 3-10L, Amersham Biosciences, Sweden) were used with an IPGphore fixed-length strip holder. The strip was rehydrated for 12 h with rehydration solution and the sample proteome was injected simultaneously. The rehydration solution was prepared with 7 M urea, 2 M Thiourea, 2% CHAPS, 1% DTT, 2% Carrier ampholyte, 10% glycerol, 0.002% BPB. Isoelectric focusing was conducted in IPGphore (Amersham Bioscience, Sweden). After 8 hours of rehydration, stepwise focusing was performedfor 60 min at 500 V, 60 min at 2000 V, and then increased to 8000 V and maintained until no current change was observed[12]. Focused IPG strips were equilibrated for 15 min in a solution (7 M urea, 2 M Thiourea, 2% SDS, 50 mMTris-HCl, 30% glycerol and 1% DTT), and then for an additional 15 min in the same solution containing 2.5% iodoacetamiderather

than DTT[13]. After equilibration, the second dimension was run on 11.5% polyacrylamide homogenous gels (T-13%, C-2.5%, 18×24 cm)[14]. The gels were stained with silver nitrate[15]. The stained gel was scanned and the protein spot images were analyzed using 2D Elite (Amersham Biosciences, Sweden) image analysis software.

Differently expressed protein spots between the groups were manually excised from the stained gels, and protein digestion was performed according to the methods of Yoo *et al*.[15]. The samples were then desalted using C18 ZipTip (Millpore) and the digested proteins were dissolved in buffer solution prepared with 50% acetonitrile and 0.5% trifluoroacetic acid. The matrix solution was prepared with saturated α-cyano-4-hydroxy-trans-cinnamic acid in acetonitrile and 0.1% trifluoroacetic acid (1:1, v/v). The sample solution was mixed with the matrix solution at the same volume ratio. The mixed solution was loaded into an Ettan MALDI-TOF Pro system (Amersham Bioscience, Sweden). Peptide mass fingerprinting data were analyzed using MASCOT (http://www.matrixscience.com/search_form_select.html) with a mass tolerance of 0.1 Da and 1 missed cleavage allowance.

RESULTS AND DISCUSSION

Seed oil composition

The seed oil of *Pinuskoraiensis*was extracted with supercritical CO_2. The composition of seed oil in fatty acid composition was analyzed with GC after the oil was hydrolyzed with methylation. The most abundant fatty acid was linoleic acid. 46% of all fatty acids was linoleic acid. The next abundant fatty acid was oleic acid as 28%. Palmitic acid and stearic acid were 5 and 3%. The abundant composition of unsaturated fatty acids explains the pine nut seed oil have high reducing power.

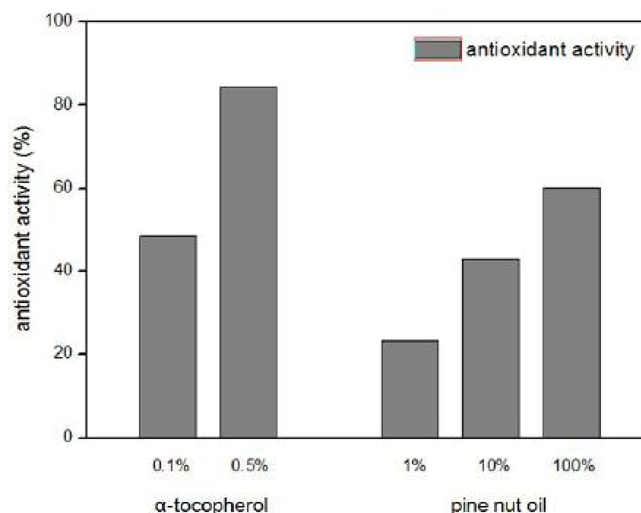

Figure 1 : Anti-oxidation activities of pine nut oil measured by ß-carotene bleaching method

Anti-oxidation activity

The anti-oxidation activity of *Pinus koraiens is* seed oil was measured. The ß-carotene bleaching method is an appropriate method to test the anti-oxidation power of lipid and oil. Figure 1 shows that the pine nut oil in 100% had equivalent anti-oxidation power to 0.1% ±-tocopherol. The anti-oxida-

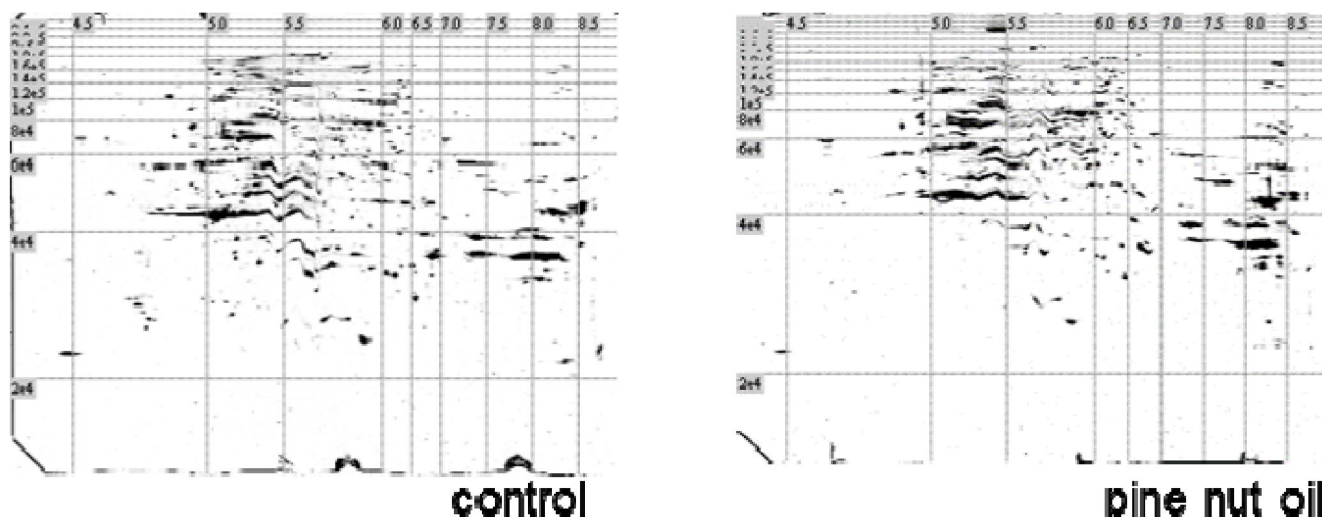

Figure 2 : Profiling of proteomes expressed in human dermal fibroblast

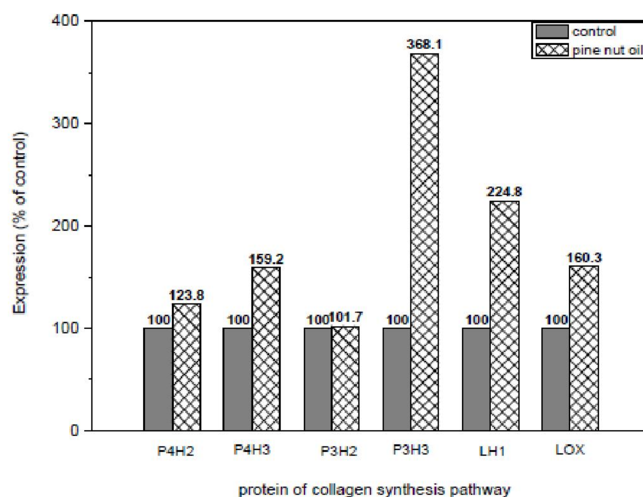

Figure 3 : Expression of enzymes related to collagen synthesis in human dermal fibroblast

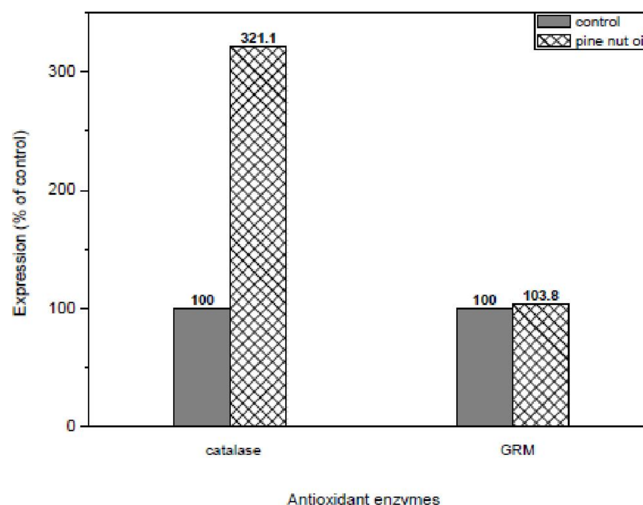

Figure 4 : Expression of anti-oxidant enzymes in human dermal fibroblast

tion power was assumed to come from the reducing power of pine seed oil. In addition, various bioactive compounds extracted by supercritical CO_2 could be other important factors for the anti-oxidation power.

Anti-aging activities with proteome analysis

Human skin fibroblasts dosed with 5 mg/L of *Pinuskoraiensis*seed oilwere harvested after 48 hours. 2D-PAGE was made with protein samples extracted from the harvested cells. Protein profiling was conducted to compare the proteins expressed with and without extract. In the control gel, which was not treated with *Pinuskoraiensis*seed oil, 1353 spots were identified and 1191 spots were counted in the gel with *Pinuskoraiensis*seed oil extracted with the supercritical fluid extraction. When compared with the control gel, 413 spots were found to match. 230 of these spots were up-regulated and 183 of the spots were down-regulated (Figure 2).

Among the regulated proteins, proteins related to anti-aging were identified via 2D-image analysis and MALDI-TOF. The collagen synthesis-related proteins were identified as follows: type 1(α-2) collagen chain precursor (pI 9.08, MW 129.7), procollagen C-endopeptidase (pI 7.40, MW 47.97), prolyl 4-hydroxylase (pI 5.49, MW 60.90), protein disulfide isomerase (pI 4.80, MW 57.50), interaction with ECM α-actinin (pI 5.47, MW 105.5), integrin-β1 (pI 5.27, MW 88.46). A representative MALDI-TOF database query result for type 1(α-2) collagen chain precursor. Database query result and scores, protein sequence and sequence coverage, and matched peptide fragment and error between experimental mass and theoretical mass proved the spot protein as the type 1(α-2) collagen chain precursor.

The anti-aging effect of *Pinuskoraiensis*seed oil has been generally attributed to an induced increase in collagen synthesis-related proteins. (Figure 3) Besides the role of collagen synthesis-related proteins, many other mechanisms are involved for skin anti-aging. The interaction between extra-cellular matrix such as collagen and cell substance is as important as collagen synthesis. The interaction of fibroblasts is mediated by specific receptors on their surfaces. α-actinin is a member of a family of actin filament crosslinking and bundling proteins. A family of membrane receptors referred to as the integrins are heterodimeric glycoproteins, which participate in intercellular adhesion and cell-protein adhesion within the extra-cellular matrix[5,16,17]. According to published results, the expression level of integrin β1 was reduced in the age-induced fibroblasts via UV irradiation as compared to fibroblasts without UV irradiation[5].

In addition to increases of enzymes related to collagen synthesis, increase of anti-oxidant enzymes in fibroblast was also observed. Figure 4 shows that *Pinuskoraiensis*seed oil increased expressions of anti-oxidant enzymes. Among them the expression of catalase was 3.2 times higher than the control.

This result has been well correlated to previous data of anti-oxidation activity of *Pinuskoraiensis*seed oil. The anti-oxidation power of *Pinuskoraiensis*seed oil could be from various bio-active compounds extracted by supercritical CO_2 as well as reducing power of unsaturated fatty acids.

Based on the results of this study, *Pinuskoraiensis*seed oil extracted with supercritical $CO_2$was found to inhibit skin-aging with different mechanisms, collagen synthesis and increased anti-oxidant enzymes. Proteome analysis proved that *Pinuskoraiensis*seed oil promoted proteome synthesis related to skin anti-aging in human dermal fibroblasts.

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