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Profiling of skin anti-aging related proteins in human dermal fibroblasts by decursin in *Angelica gigas* Nakai

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Abstract−The extract of *Angelica gigas* Nakai by supercritical CO_2 increased the expression of collagen synthesis-related proteins in human dermal fibroblast, including type 1(α-2) collagen chain precursor (pI 9.08, MW 129.7), procollagen C-endopeptidase (pI 7.40, MW 47.97), and prolyl 4-hydroxylase (pI 5.49, MW 60.90). It also increased the expression level of interaction-related proteins, α-actinin (pI 5.47, MW 105.5), integrin-β1 (pI 5.27, MW 88.46). The expression levels of these proteins by pure decursin were similar to those by supercritical fluid extract. By the dose concentration experiment, decursin in *A. gigas* was found to play the major role in expression level increases. Proteome analysis proved that decursin in *A. gigas* promoted synthesis of proteins related to skin anti-aging in human dermal fibroblasts.

Key words: Desursin, *Angelica gigas* Nakai, Skin Anti-aging, Collagen Synthesis

INTRODUCTION

Aging is sometimes defined as the accumulation of molecular damage over time [1]. 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 [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 chains assume 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.

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Skin aging is associated with the interaction between the matrix and the fibroblasts as well as reductions in 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].

Angelica gigas Nakai, which in Korea is referred to as 'Chamdanggui', is widely utilized as an important folk medicine, not only for the treatment of anemia but also as a sedative and anodyne agent. It has been reported to contain a variety of compounds, including coumarins, essential oils, and volatile flavors [6]. Decursin and decursin angelate, members of the coumarin family, can perform an important role in hydrogen peroxide scavenging [7], anti-tumor [8], and anti-platelet activities [9]. However, until now, no studies have been conducted on the skin whitening and skin anti-aging properties of decursin and decursinol angelate. In this study, we extracted decursin from *A. gigas* via three extraction methods. Among the various effects of decursin noted, an anti-aging effect was observed via proteome analysis. Proteome analysis based on 2-dimensional electrophoresis (2D-PAGE) was applied for the profiling of protein factors involved in the observed skin anti-aging effects.

MATERIALS AND METHODS

1. Materials

10 g of dried *A. gigas* was extracted with three extraction methods: hot water, 95% ethanol, and supercritical CO_2 fluid. Hot water extract was prepared for 24 hours with dH_2O at 100 °C. The ethanol extract was extracted for 24 hours with 95% ethanol at 60 °C. Supercritical CO_2 fluid extraction was conducted at a temperature of 40-60 °C and a pressure of 200-400 bar. Water and ethanol extracts

were lyophilized after the removal of the solvent with a rotary vacuum evaporator. For the dose experiments, the extract was dissolved in dimethyl sulfoxide (DMSO) and diluted with DMEM.

2. Analysis

Decursin was analyzed with a DS 6200 gas chromatograph (Donam Instruments INC., Korea) and separated on EC-5 (30 m×0.25 mm ID×0.25 μm, Altech INC., USA). The carrier gas used was nitrogen, at a flow rate of 5 ml/min. The injector and flame ionization detector (FID) were both set to a temperature of 270 ºC. The GC oven temperature was maintained at 250 ºC for 1 min and raised to 251 ºC at a rate of 0.2 ºC/min, and then maintained for 1 min at 251 ºC. The system was then programmed to increase the temperature to 271 ºC at a rate of 10 ºC/min, and then maintained at a constant temperature of 271 ºC for 5 min (total running time: 14 min). The split ratio was 1 : 10.

3. Cell Cultures and Viability Assay

Human dermal fibroblasts were purchased from the American Type Culture Collection (ATCC, CRL-1635). Fibroblasts were cultured in DMEM (WelGene lnc.) with 10% fatal bovine serum (Gibco), and 1% Antibiotic-antimycotic (Gibco). Cultures were prepared in a humidified CO_2-controlled (5%) incubator at 37 ºC. 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 3×10^3 cells/well and allowed 24 hours for surface adhesion. The culture medium was removed after 24 hours, then exchanged with a new medium containing *A. gigas* extracts at variable concentrations from 0-100 mg/L. The cultures were then maintained for three days at 37 ºC 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 ºC. 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].

4. Proteomic Analysis

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 4,000 ×g. The supernatant was collected and preserved at −20 ºC. 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 (13 cm, 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 performed for 60 min at 500 V, 60 min at 2,000 V, and then increased to 8,000 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 mM tris-HCl, 30% glycerol and 1% DTT), and then for an additional 15 min in the same solution containing 2.5% iodoacetamide rather than DTT [13]. After equilibration, the second dimension was run on 11.5% polyacrylamide homogeneous 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

1. Decursin Identification

The decursin content was assessed with different extraction sol-

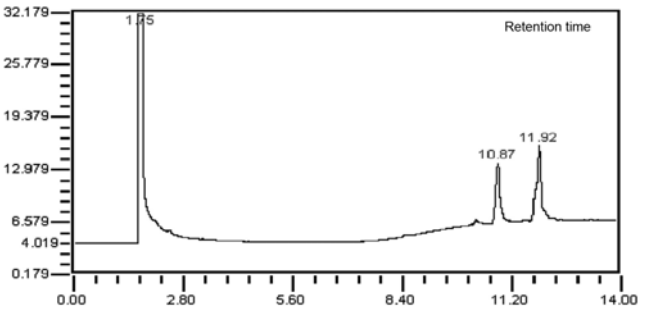

Fig. 1. GC chromatogram of decursin (RT 10.87 min) and decursinol angelate (RT 11.92 min) in *A. gigas* extract.

Table 1. Decursin contents in *Angelica gigas* Nakai extracts by various extraction methods

Extraction methods	Hot water	95% Ethanol	Supercritical CO_2 fluid					
			60 ºC			300 bar		
	100 ºC, 24 h	60 ºC, 24 h	200 bar	300 bar	400 bar	40 ºC	50 ºC	70 ºC
Decursin (%)	0	5.8	10.8	13.1	12.5	9.9	11.4	10.1

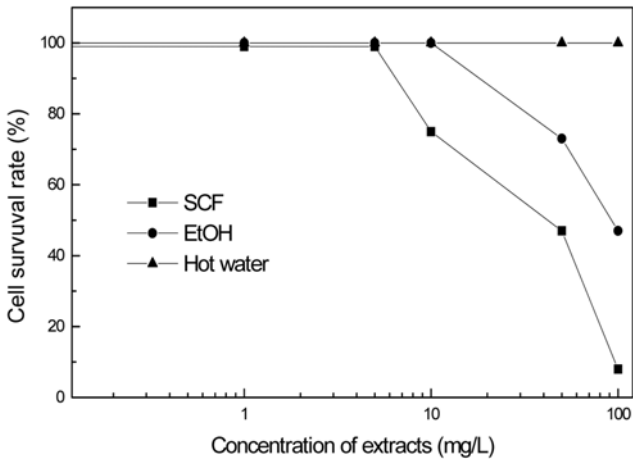

Fig. 2. Effects of *A. gigas* extracts on cell viability of human dermal fibroblast. Cell viabilities were observed after three days from dosing in various concentrations of *A. gigas* extracts by various extraction solvents, hot water, ethanol, and supercritical fluid (SCF).

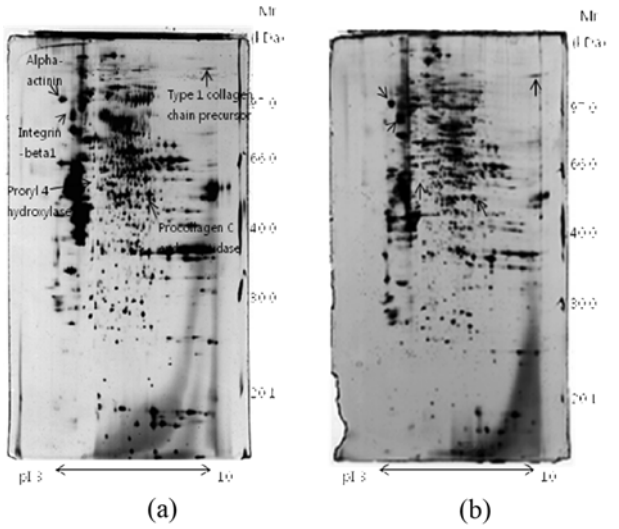

Fig. 4. 2D gel electrophoresis of total protein extracted from human dermal fibroblast. Cells treated with and without 650 μg/L decursin at 48 h; (a) control, (b) decursin.

vents. The results demonstrated that the concentration of decursin as a main compound of *A. gigas* was increased in the supercritical CO_2 fluid extract as compared to the ethanol extract. However, it was not detected in the hot water extract. The decursin peak was detected at 10.87 min and identified via GC-MS (decursin MW. 328) (Fig. 1). The decursin content was 5.8% in the ethanol extract. The supercritical CO_2 fluid extracts contained high levels of decursin, 9.8-13.1%, according to the temperature and pressure conditions utilized (Table 1). This result was thought to be attributable to the special chemical characteristics of decursin. Supercritical fluid extraction, usually by supercritical carbon dioxide, can be used to extract oil compounds from natural products, and does not produce substantial thermal degradation neither solvent pollution nor alteration of the extracts. We believe that the use of supercritical fluid as a decursin extraction solvent may prove to be a very effective extraction method.

2. Cytotoxic Effects of *A. gigas* Extracts on Human Dermal Fibroblasts

The cytotoxic effects of *A. gigas* extracts differed with different extraction methods; hot water, 95% ethanol, and supercritical CO_2 fluid (300 bar, 60 °C) extractions were investigated against human skin fibroblasts. An MTT assay was conducted with *A. gigas* extracts at various concentrations, in a dose range of 0.001-100 mg/L. According to the results of the MTT assay, hot water extract evidenced no cytotoxic activities on fibroblasts at concentrations up to 100 mg/L, whereas supercritical fluid and ethanol extracts evidenced cytotoxic activity at concentrations over 10 mg/L and 50 mg/L, respectively (Fig. 2). The cytotoxic effects differed with different extracts, because the decursin contents in the *A. gigas* differed with

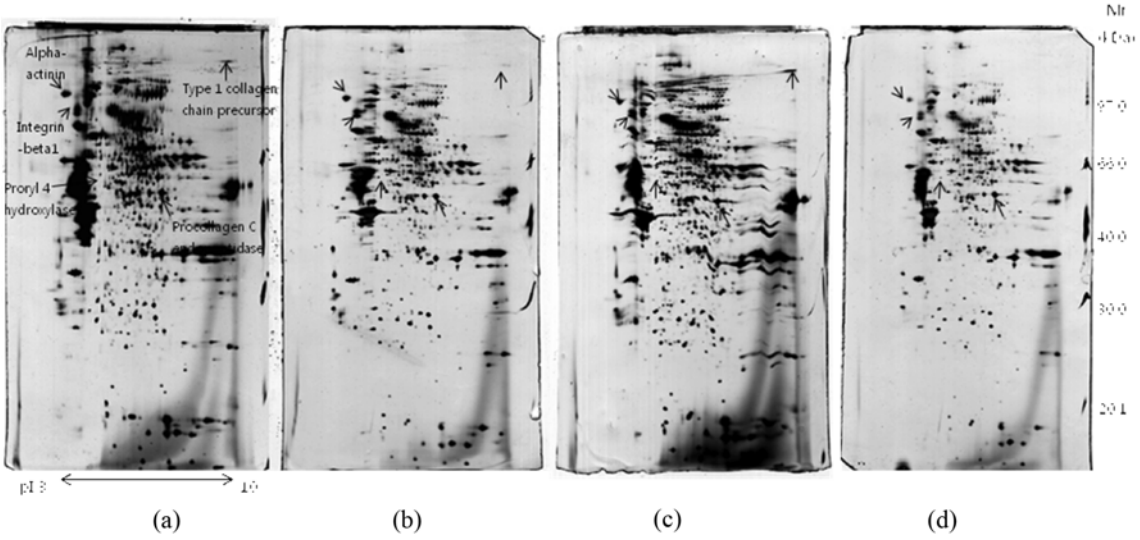

Fig. 3. 2D gel electrophoresis of total protein extracted from human dermal fibroblast. Cells treated with and without 5 mg/L *A. gigas* extracts at 48 h; (a) control, (b) ethanol extract, (c) supercritical fluid (SCF) extract, (d) hot water extract.

different extraction methods.

3. Proteome Analysis

Human skin fibroblasts dosed with 5 mg/L of *A. gigas* extracts were 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 the extracts. In the control gel, which was not treated with *A. gigas* extract, 485 spots were identified and 447 spots were counted in the gel with *A. gigas* extracted by ethanol. Among the spots matched, 172 spots were up-regulated and 94 spots were down-regulated by ethanol extract of *A. gigas*. In the gel used with the supercritical fluid extract, 444 spots were identified. When compared with the control gel, 253 spots were found to match. 137 of these spots were up-regulated and 77 of the spots were down-regulated. In the gel used with the hot water extract, 256 spots were identified. As the result of matching with the control gel, 55 spots were up-regulated and 127 spots were down-regulated (Fig. 3).

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, Score 58), procollagen C-endopeptidase (pI 7.40, MW 47.97, Score 53), 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, Score 59), integrin-β1 (pI 5.27, MW 88.46, Score 67).

The anti-aging effect of *A. gigas* extract has been generally attrib-

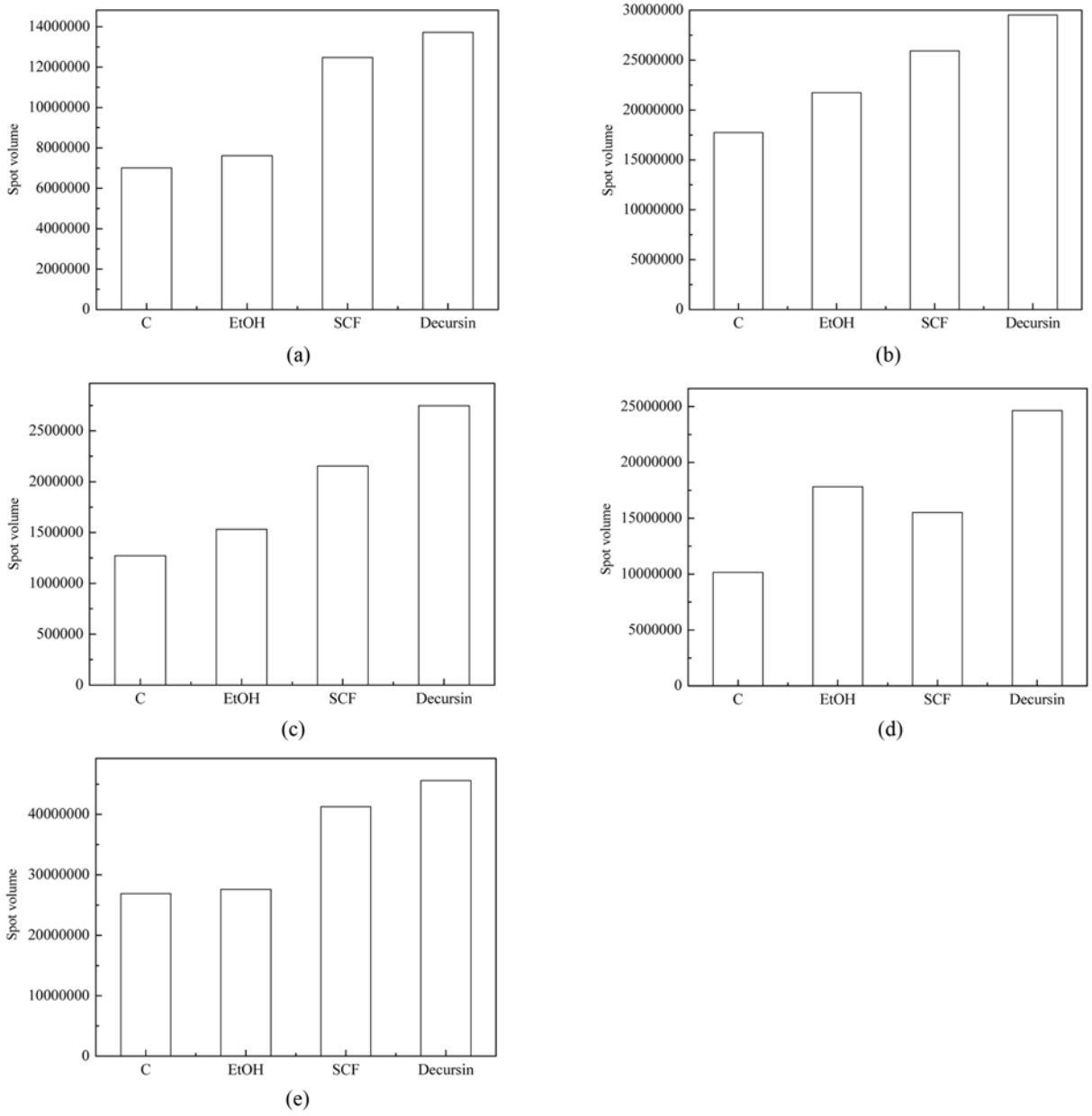

Fig. 5. Spot volume of expressed proteins related to skin anti-aging by *A. gigas* extracts (5 mg/L) and decursin (650 μg/L)-stimulated human dermal fibroblast; (a) type 1(α-2) collagen chain precursor, (b) procollagen C endopeptidase, (c) proryl 4-hydroxylase, (d) α-actinin, (e) integrin-β1.

uted to an induced increase in collagen synthesis-related proteins. (The data from the hot water extract was not shown because the expression of anti-aging related proteins was not increased as compared with the controls). We assumed that the anti-aging effect of *A. gigas* extract was due to decursin, the major compound in the extract. To prove the presumption, we performed proteome analysis to evaluate the anti-aging effect of pure decursin. Decursin (650 μg/L) at the same concentration as the decursin contained in *A. gigas* supercritical CO_2 fluid extract was applied to human dermal fibroblasts. In the results of 2D-analysis (Fig. 4), expression levels of the type 1(α-2) collagen chain precursor and procollagen C-endopeptidase by pure decursin were similar to those by the supercritical fluid extract. The expression levels of these proteins directly relevant to anti-aging were increased by more than 2-fold as compared with the controls (Fig. 5).

We also evaluated the effect of decursin concentration on the expression levels of collagen synthesis-related proteins. Various concentrations of decursin in the range of 0-650 μg/L were dosed and the expression levels were observed by 2D-analysis. Fig. 6 shows that the expressions of collagen synthesis-related proteins were increased as the decursin concentration increased. This is true to type 1(α-2) collagen chain precursor, procollagen C-endopeptidase, and prolyl 4-hydroxylase. This is another proof that anti-aging effect of *A. gigas* extract was due to decursin. The expression levels of col-

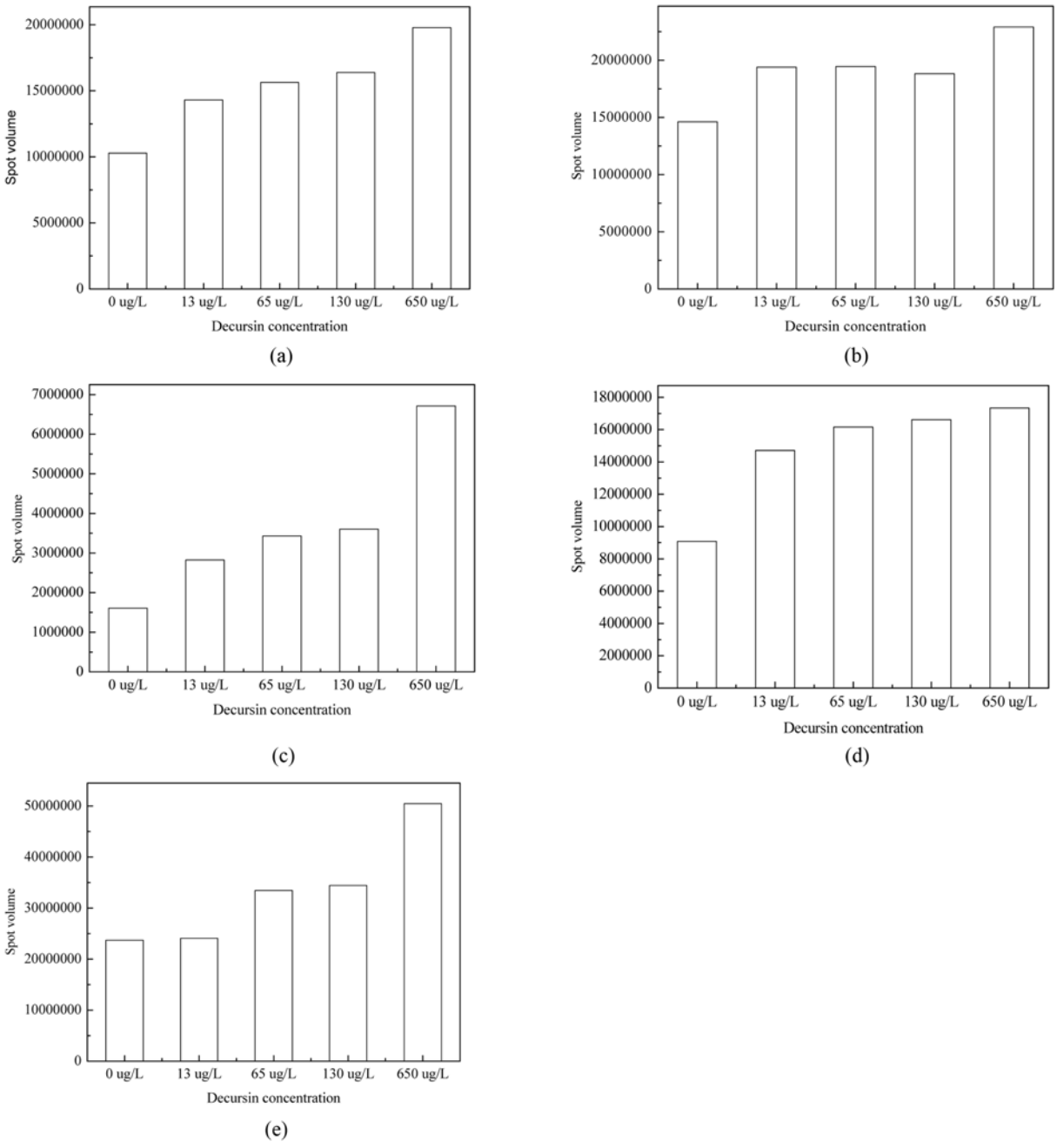

Fig. 6. Spot volume of expressed proteins related to skin anti-aging in decursin-stimulated human dermal fibroblast; (a) type 1(α-2) collagen chain precursor, (b) procollagen C endopeptidase, (c) proryl 4-hydroxylase, (d) α-actinin, (E) integrin-β1.

lagen synthesis-related proteins directly relevant to anti-aging were near proportionally controlled by decursin concentration in the range of 0-650 μg/L.

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 the 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].

As shown in Fig. 5, *A. gigas* extract, especially supercritical fluid extract, induced increase in interaction-related proteins. Proteome analysis was also carry out to evaluate the effect of pure decursin on interaction-related proteins. In the results of 2D-analysis (Fig. 4), expression levels of the α-actinin and integrin-β1 by pure decursin (650 μg/L) were similar to those by the supercritical fluid extract. The expression levels of these proteins were increased by around 2-fold as compared with the controls (Fig. 6). The effect of decursin concentration on the expression levels of interaction-related proteins was also evaluated. Various concentrations of decursin in the range of 0-650 μg/L were dosed and the expression levels were observed by 2D-analysis. Fig. 6 shows that the expressions of α-actinin and integrin-β1 were increased as the decursin concentration increased. The expression levels of these proteins directly relevant to anti-aging were controlled by decursin concentration in the range of 0-650 μg/L. The anti-aging effect of *A. gigas* extract was proved again with interaction-related proteins due to decursin.

Based on the results of this study, *A. gigas* extracted with supercritical CO_2 fluid was found to inhibit skin-aging with different mechanisms, collagen synthesis and elasticity by interaction increase. Decursin in *A. gigas* extract is the major effective compound of skin anti-aging. Proteome analysis proved that decursin in *A. gigas* promoted proteome synthesis related to skin anti-aging in human dermal fibroblasts.

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