

Running Title: Effects of Soybean Peptide on Differentiation Markers

Effects of Low Molecular Weight Soybean Peptide on mRNA and Protein Expression
Levels of Differentiation Markers in Normal Human Epidermal Keratinocytes

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Abbreviations: K1, keratin 1; K5, keratin 5; K10, keratin 10; K14, keratin 14; LSP, low
molecular weight soybean peptide; NHEK, normal human epidermal keratinocytes;
NMF, natural moisturizing factor; SP, soybean peptide; TEWL, transepidermal water
loss; TGase, transglutaminase

Low molecular weight soybean peptide (LSP) was applied to normal human epidermal keratinocytes (NHEK), and the results showed a significant increase in the gene expression levels of involucrin, transglutaminase and profilaggrin. Filaggrin protein levels were also significantly higher. It is possible that LSP has an epidermal cell differentiation-promoting effect and may be able to regulate metabolism of the epidermis.

Key words: low molecular weight soybean peptide; keratinocyte; differentiation; skin; epidermis

The skin is composed of the epidermis and dermis, with epidermis being subdivided into the stratum corneum, the granular layer, the spinous layer and the basal layer, in order from the surface. Basal layer keratinocytes proliferate and differentiate by changing their morphology into cells that form each of these layers, finally becoming the enucleated corneocytes of the stratum corneum; the outermost layer of the stratum corneum then becomes dead skin and falls off. This manner of basal cell differentiation is how the skin is metabolized and renewed. The undifferentiated keratinocytes found in the basal layer produce keratin 5 (K5), which has a molecular weight of 58 kDa, and keratin 14 (K14). The stratum spinosum layer is composed of five to ten layers of spinous cells. Like the stratum basal layer, keratin filament expression can be observed, but with pairing of keratin 1 (K1), which has a molecular weight of 67 kDa, and keratin 10 (K10), which has a molecular weight of 56.5 kDa.¹⁾ In addition, there is expression of involucrin, an envelope protein rich in glutamine and lysine.^{2,3)} The stratum granular layer is composed of two or three layers of granule cells, and undergoes when flattening compared with spinous cells. Loricrin, which is produced by the granule cells,⁴⁻⁶⁾ is crosslinked with involucrin by enzymes such as transglutaminase to form the marginal zone of the stratum corneum.²⁾ Inside the granule cells, there are keratohyalin granules, which include large amounts of profilaggrin, a precursor of filaggrin.⁶⁻⁸⁾ Filaggrin is broken down in the stratum corneum into amino acids; it is also known as natural moisturizing factor (NMF), as it has a moisture retention function.⁹⁾ The stratum corneum is composed of about 15 layers of corneocytes, which are dead cells (corneocytes) that have been enucleated. The marginal zone described above is stable with respect to physical and chemical stimuli, and is largely responsible for the skin's barrier function.¹⁰⁻¹²⁾ Because of this release of specific proteins during keratinocyte differentiation, measuring these proteins makes it possible to estimate the extent of cell differentiation.

Soybean peptide (SP) is broken-down soybean protein. The average molecular weight of SP is about 3 to 10 kDa, and it is characterized as having a high glutamic acid

content. Reported actions of SP include a cholesterol inhibitory effect,¹³⁾ a weight loss effect,¹⁴⁾ an anti-fatigue effect¹⁵⁾ and an antihypertensive effect.¹⁶⁾ Low molecular weight SP (LSP), which is SP that has been further broken down, has an average molecular weight of about 500 to 1,000 Da; like SP, it has a high glutamic acid content.¹⁷⁾ About 60% of LSP is dipeptides and tripeptides, and it has been reported that di- and tripeptides are absorbed in the small intestine and elsewhere without being broken down.¹⁸⁾ In the cosmetics field, we have previously reported on LSP's collagen production-promoting effects in fibroblast cells¹⁷⁾ and its effect in improving stratum corneum moisture, transepidermal water loss (TEWL), and viscoelasticity in UVA-irradiated hairless mice.¹⁹⁾ In this manuscript, to establish how LSP affects keratinocyte differentiation, an important function in skin physiology, we applied LSP to keratinocytes and examined the gene expression levels of differentiation markers over time by quantitative real-time PCR, and examined protein levels by Western blotting.

LSP was kindly provided by Fuji Oil Co., Ltd. (HI-NUTE AM; Osaka, Japan). The LSP used contains large amounts of aspartic acid and glutamic acid, and contained numerous substances with a molecular weight of about 500 to 1,000 Da.¹⁷⁾ Normal human epidermal keratinocytes (NHEK) were purchased from Biopredic (Rennes, France). NHEK were cultured in HuMedia-KG2 obtained from Kurabo (Osaka, Japan), and were seeded (2.0×10^5 cells/dish) into 60-mm dishes. After culture for 1 day, cells were treated with a final concentration of 0.1 $\mu\text{g/mL}$ LSP for 1, 3 or 9 days. mRNA expression was assayed by real-time PCR. Total RNA was isolated using RNAiso Plus (Takara Bio, Shiga, Japan) and purified with an PrimeScript® RT reagent Kit (Takara Bio). cDNA was generated using a SYBR® Premix Ex Taq™ (Takara Bio). Primer sequences were as follows: GAPDH forward, 5' -GAAGGTGAAGGTCGGAGT- 3', and reverse, 5' -GAAGATGGTGATGGGATTTC- 3'; K5 forward, 5' -GAGCTGAGAAACATGCAGGA- 3', and reverse, 5' -TCTCAGCAGTGGTACGCTTG- 3'; K10 forward, 5'

-CCATCGATGACCTTAAAAATCAG- 3', and reverse, 5'
 -GCAGAGCTACCTCATTCTCATACTT- 3'; involucrin forward, 5'
 -TGCCTGAGCAAGAATGTGAG- 3', and reverse, 5'
 -TTCCTCATGCTGTTCCCAGT- 3'; profilaggrin forward, 5'
 -CCATCATGGATCTGCGTGG- 3', and reverse, 5'
 -CACGAGAGGAAGTCTCTGCGT- 3'; TGase forward, 5'
 -TCTTCAAGAACCCCCTTCCC- 3', and reverse, 5'
 -TCTGTAACCCAGAGCCTTCGA- 3'. Real-time PCR was performed using the ABI

PRISM® 7500 Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA). Protein levels were assayed by Western blotting. NHEK were washed and subsequently dissolved in lysis buffer solution (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate). Samples were then run on 10% SDS-PAGE gels, blotted on polyvinylidene difluoride membranes, and immunodetected with antibodies against K10, involucrin, filaggrin and β -actin, and with secondary anti-rabbit IgG-HRP-labeled antibodies. Detection was performed using a Lumino image analyzer LAS-1000 plus (Fujifilm Corp., Tokyo, Japan). Analysis was carried out with a MultiGauge (Fujifilm Corp., Tokyo, Japan).

The effects of LSP on viable cell number count were assessed among keratinocytes. To keratinocytes, 0.1 μ g/mL LSP was added and viable cell number counts after 24 and 72 h of culture were obtained by trypan blue staining. The results showed that adding LSP did not change cell proliferation or viable cell number (data not shown). To keratinocytes, 0.1 μ g/mL LSP was added and gene expression levels for K5, K10, involucrin, profilaggrin and transglutaminase were measured by real-time PCR on the 1st, 3rd and 9th days. The data of gene expression levels were conducted using Tukey's multiple statistical tests. The results of relative quantification where gene expression levels with no SP of 1st day (1st day control) are "1" showed no changes in gene expression levels for K5 and K10 on the 1st, 3rd and 9th days after LSP was added (Fig. 1A and B). Gene expression levels for involucrin on the 9th day after LSP was

Fig.1

added, however, significant increased to 1.29-fold ($p<0.05$) that of the control on the 9th day (Fig.1C). Gene expression levels for profilaggrin and for transglutaminase showed significant increases at about 1.73-fold ($p<0.01$) and about 1.61-fold ($p<0.01$) that of the control on the 9th day, respectively (Fig. 1D and 1E). On the other hand, there were no changes in gene expression levels for involucrin, profilaggrin and transglutaminase on the 1st and 3rd days after addition of LSP. To keratinocytes, 0.1 $\mu\text{g/mL}$ LSP was added and protein levels for filaggrin after the 9th day were measured by Western blotting. The data of protein levels of filaggrin was conducted using Student's t-test. The results of relative quantification where gene expression levels with no SP were "1" showed that protein levels for filaggrin on the 9th day after LSP was added had increased by 2.3-fold ($p<0.01$).

Fig.2

This manuscript examined the effects of LSP on differentiation marker expression in epidermal cells. We examined the effects on cell differentiation among keratinocytes. LSP (0.1 $\mu\text{g/mL}$) was added to epidermal cells and gene expression levels for differentiation markers after the 1st, 3rd and 9th days of culture were measured. In this manuscript, we applied and examined 0.1 $\mu\text{g/mL}$ LCP, which increased a gene expression of COL1A1 for skin cells in a previous study.¹⁷⁾ The results showed that gene expression levels for all differentiation markers remained unchanged on the 1st and 3rd days after LSP was added (Fig. 1). Nine days after LSP was added, however, the gene expression levels for involucrin, a late differentiation marker, showed an increase (Fig. 1C), while gene expression levels for profilaggrin and TGase showed significant increases (Fig. 1D and 1E). Protein levels for filaggrin also increased significantly (Fig. 2). No changes in cell number caused by LSP were observed (data not shown), for which LSP is believed to have an epidermal differentiation-promoting effect. It is possible that LSP maintains the balance between ceramide, NMF and the like by activating turnover of the epidermis. As LSP did not have an effect on cell proliferation, it was assumed to only affect cell differentiation. Calcium, retinoic acid, vitamin D and other substances have been reported to promote differentiation, but LSP

contains none of these. LSP itself was therefore assumed to have induced differentiation.

At present, glutamine receptors, which are transporters of the amino acids glutamine and glutamic acid, are understood to affect differentiation.²⁰⁾ One of the characteristics of LSP is its high glutamic acid content. It is thus possible that the glutamic acid present in LSP affects differentiation. In the reports given above, however, differentiation was inhibited by glutamic acid, in contrast to our results here. It is not known whether LSP acts as an amino acid or as a peptide. In addition, the mechanisms by which epidermal cell differentiation are promoted must be elucidated, and because 0.1 µg/mL LSP was added in this experiment, which has previously exhibited a type-I collagen production-promoting effect,¹⁷⁾ it is possible this effect was not observed. In the future, its usefulness as a cosmetic ingredient should be elucidated by examining additive concentrations or examining the use of amino acid mixtures formulated with high molecular weight SP or LSP amino acid compositions.

In conclusion, LSP has been shown to have an epidermal cell differentiation-promoting effect. Together with the promotion of type-I collagen production and the improvement of skin in UVA-irradiated hairless mice, which we have reported previously, LSP has the potential to regulate skin metabolism, and is thus a material of great interest as a cosmetic additive.

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Figure legends

Fig. 1. Effects of LSP on mRNA Expression Level of Differentiating Gene in Normal Human Keratinocytes.

NHEK cells were incubated with 0.1 µg/mL of LSP or medium (vehicle) and gene expression levels for K5 (A), K10 (B), Involucrin (C), Profilaggrin (D) and TGase (E) genes expression were measured by real-time PCR on the 1st, 3rd and 9th days. The results of relative quantification where gene expression levels with no SP of 1st day (control on the 1st day) are “1”. The mRNA levels were normalized by dividing by the quantity of GAPDH in each sample. Values are means± SD of three experiments. *: $p < 0.05$, **: $p < 0.01$, : compared to the control on the 9th day group. The statistical analysis was conducted using Tukey’s multiple statistical tests.

Fig. 2. Effects of LSP on Filaggrin Protein Expression Level in Normal Human Keratinocytes.

NHEK cells were incubated with 0.1 µg/mL or medium (vehicle) and gene expression levels for filaggrin protein expression was measured by Western blotting on the 9th days. The results of relative quantification where protein expression levels with no SP of 9th day (control on the 9th day) are “1”. Values are means± SD of three experiments. *: $p < 0.05$: compared to the control on the 9th day, Student’s t-test.

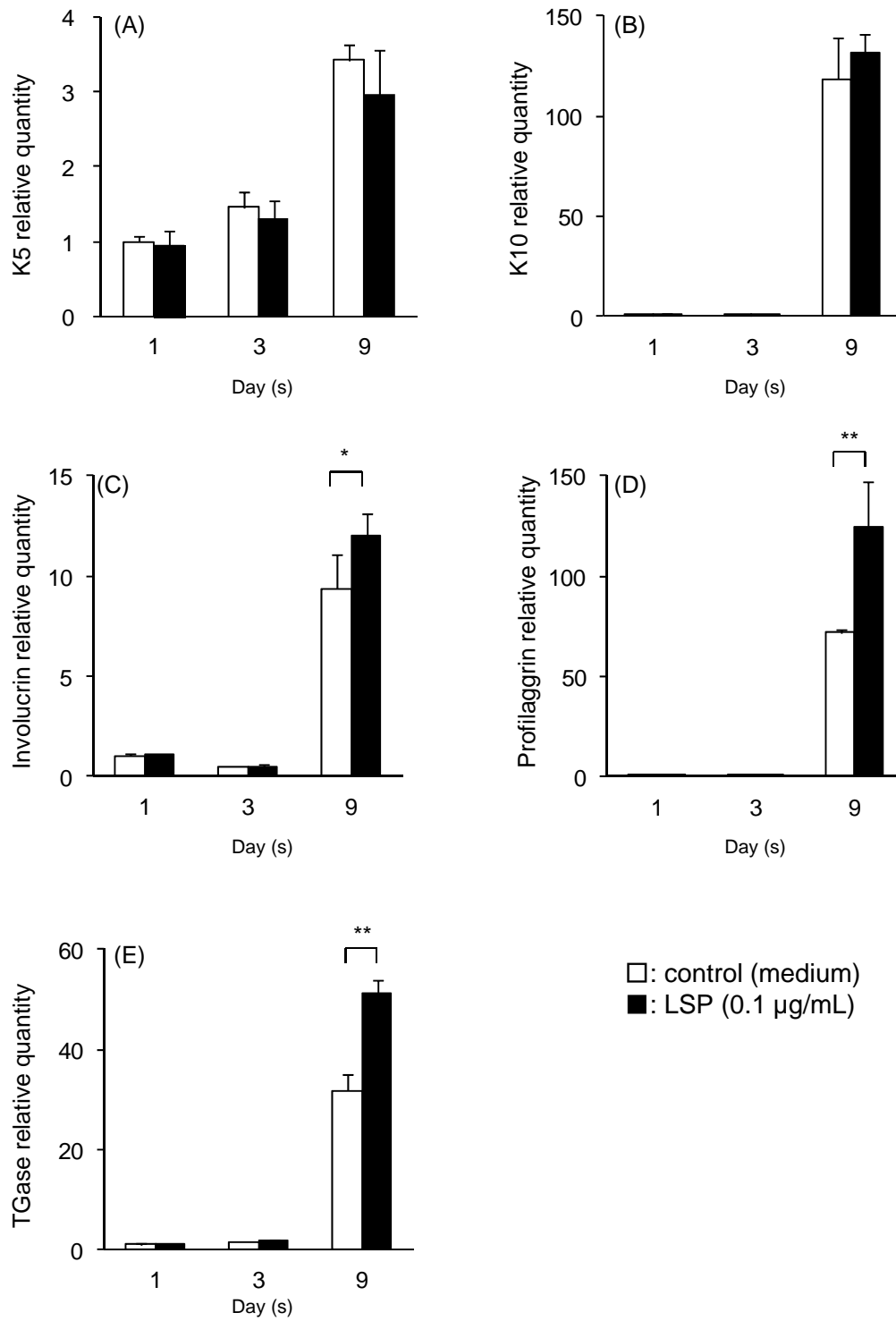


Fig. 1

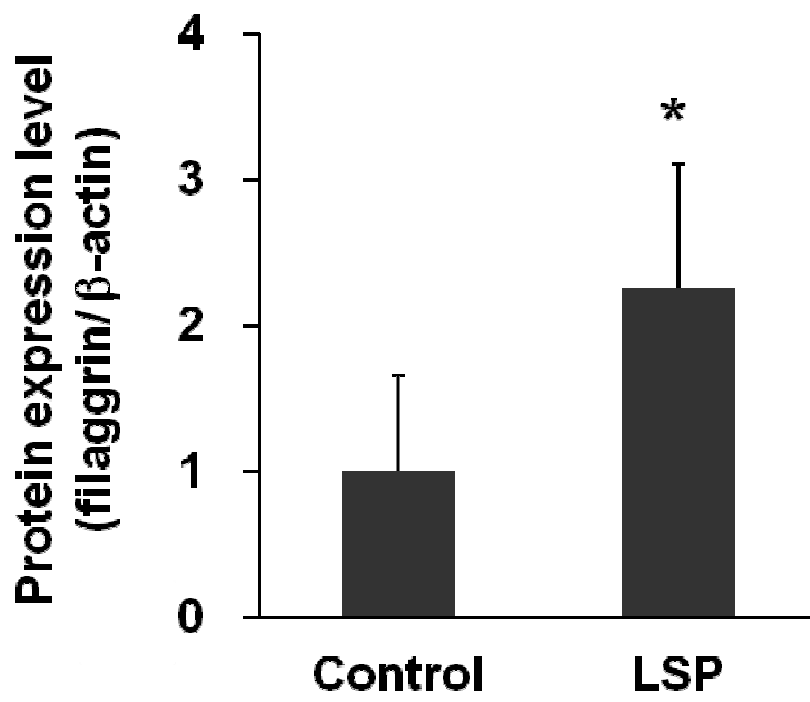
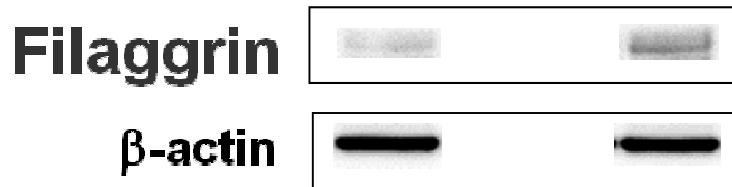


Fig. 2