Effects of soybean peptide and collagen peptide on collagen synthesis in normal human dermal fibroblasts

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Abstract

The collagen present in the dermis of the skin is a fibrous protein that fills the gaps between cells and helps to maintain tissue flexibility. Effectively increasing the collagen present in the skin is an important goal for cosmetic research. Recent research has shown that soybean peptide (SP) has anti-fatigue activity, antioxidant activity, and the ability to increase type I collagen, while collagen peptide (CP) has the ability to enhance corneal moisture content and viscoelasticity, as well as to increase levels of hyaluronic acid synthesizing enzymes in human skin. Little documented research, however, has been conducted on collagen formation in relation to these peptides. Therefore, this research applied SP and CP with molecular weights primarily around 500 and preparations containing both SP and CP to normal human dermal fibroblasts together with magnesium ascorbyl phosphate (VC-PMg), and used real-time PCR to determine the gene expression of type I collagen (COL1A1), which contributes to collagen synthesis, and Smad7, which contribute to collagen breakdown. In addition, ELISA was used to measure collagen content in the media. COL1A1 gene expression at 24 hours after sample addition was higher tendency in all samples and increased with time at 4, 8 and 24 hours after addition. Smad7 gene expression was not substantially different at 4 hours after addition.MMP-1 gene expression was higher following SP addition, but was lower after addition of CP and SP+CP. Medium collagen content was higher in all samples and increased with time at 8 hours after addition. Collagen levels were higher when SP and CP were added together.

Keywords: soybean peptide, collagen peptide, collagen, matrix metallo-proteinase

Introduction

The skin is a membrane that covers the surface of animals, and is responsible for retaining moisture, serving as a barrier, and regulating body temperature. The skin can be broadly classified as the epidermis and the dermis; keratinocytes and melanocytes make up the epidermis, while the dermis is composed of elastin, hyaluronic acid and other mucopolysaccharides, as well as collagen and other components of the extracellular matrix (ECM). These substances are produced by fibroblasts present in the dermis. Collagen acts to maintain, reinforce and bind skin structure, elastin confers elasticity to the skin, and hyaluronic acid helps the body retain moisture. The molecular weight of collagen is approximately 300,000, and the molecule is formed by three polypeptide chains with a right-handed helical structure (Veis and Brownell 1977).

Collagen synthesis begins when collagen genes are transcribed. Transcribed mRNA is translated to produce polypeptides inside the synthesizing cells, and prolyl hydroxylase converts the proline present in this polypeptide into hydroxyproline. Three of these polypeptides subsequently come together to form pro-collagen, which has a triple helical structure and is released outside the cell. Subsequently, pro-collagen is cleaved at the N and C termini, resulting in aggregation and cross-linking to form collagen fibers.

Collagenase is an enzyme that degrades collagen. The enzyme degrades collagen by cleaving its triple helical structure (Michaels et al. 1958). Collagenase is a matrix metallo-proteinase (MMP), a group of enzymes that break down the ECM (Vincenti et al. 1996), and the enzyme that breaks down type I collagen is known as MMP-1.

Vitamin C and transforming growth factor-β (TGF-β) are known promoters of collagen synthesis (Yamamoto et al. 1992). Vitamin C, a water-soluble vitamin, prevents DNA damage by free radicals (Fraga et al. 1991), alleviates endothelial cell dysfunction (Levine et al.

1996), reduces white blood cell adhesion caused by LDL (Lehr et al. 1995), and has a variety of other physiologic activities. TGF-β, a cytokine, contributes to cell growth (Massague et al. 2000) and synthesis of collagen and other components of the ECM (Ignotz and Massague 1986). A small-molecule signal known as Smad is used when transmitting TGF-β signals intracellularly, and Smad2 and 3 are activated by TGF-β to promote collagen synthesis (Nakao et al. 1997). The inhibitory Smad7 inhibits collagen synthesis (Nakao et al. 1997, Kopp et al. 2005).

Soybean peptide (SP) has been found to increase collagen content (Sekine et al. 2008), suppress cholesterol (Cho et al. 2007), enhance dieting attempts (Tamaru et al. 2007), alleviate fatigue (Yu et al. 2008) and reduce hypertension (Okamoto et al. 1995), while collagen peptide (CP) has been shown to suppress increases in blood pressure (Zhu et al. 2010), promote the synthesis of hyaluronic acid (Ohara et al. 2010) and increase moisture content in the human stratum corneum (Ohara et al. 2009). However, little research has been performed on the effects of these peptides on the skin or on the mechanism of collagen synthesis. Therefore, we applied, either alone or together, commonly used SP and CP rich in dipeptide and tripeptide forms having a molecular weight around 500 onto fibroblasts in order to investigate the effects on type I collagen synthesis, focusing on collagen synthesis and inhibition.

Materials and methods

1. Cells and cell culture

Normal human dermal fibroblasts (NHDFs) were purchased from Kurabo (Osaka, Japan). NHDFs were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2.

2. Samples and reagents

SP was supplied by Fuji Oil Co., Ltd. (HI-NUTE AM, Osaka, Japan), and CP was extracted from fish (SCP-AS-L; Nippi Inc., Tokyo, Japan). DMEM purchased from Wako Pure Industries Inc. (Osaka, Japan). FBS was obtained from Nichirei BioSciences (Tokyo, Japan). RNAiso Plus, PrimeScript® RT reagent Kit and SYBR® *Premix Ex Taq*™ were purchased from Takara Bio Inc. (Shiga, Japan). Human collagen type 1 ELISA kit was obtained from Applied Cell Biotechnologies Inc. (Kanagawa, Japan). Magnesium ascorbyl phosphate (VC-PMg) was supplied by Nikko Chemicals Co. Ltd. (Tokyo, Japan).

3. Amino acids compositions of SP and CP

The amino acid compositions of SP and CP were analyzed using a JLC500/V amino acid analyzer (JEOL, Tokyo, Japan) after hydrolysis in 6 N HCl, performic acid oxidation for methionine and cysteine analysis, at 110°C for 24 h *in vacuo*. To analyze for tryptophan, hydrolysis was carried out with barium hydroxide.

4. HPLC conditions

The HPLC system was a series 1100 HP consisting of a G1322A quaternary pump, a G1322A degasser, a G1330A thermostatic control, a G1316A column heater, a G1329A autosampler and a G1314A ultraviolet detector (Agilent Technologies, Palo Alto, CA). Columns were supplied by TOSOH (Tokyo, Japan). Separation was performed on TSK gel G3000 SWXL+ TSK gel G2000 SWXL at room temperature. Detection wavelength was set at 220 nm. Mobile phase was a mixture of 50 mM phosphate buffer, 1% sodium dodecyl sulfate and 1.17% NaCl at a flow rate of 0.4 mL/min.

5. Sample addition

Samples were prepared using medium containing 0.5% FBS. Medium alone was added to the normal sample, and VC-PMg, a vitamin C derivative, was added to the control sample to a final concentration of 10 μg/mL. SP was added to final concentrations of 10^{-6} to 10^{-3}% and CP was added to a final concentration of 10^{-5}%. The SP+CP sample contained both peptides at a concentration ratio of 1:1 and a final concentration of 10^{-5}%. VC-PMg was added to all samples at a concentration of 10 μg/mL.

6. RNA extraction

Medium was removed from the wells of a 96-well plate and 200 μL of RNAiso Plus was added to each well to dissolve the cells. Next, 1 mL of this cellular solution was collected and agitated in a tube containing 200 μL of chloroform and centrifuged at 4°C and 12,000 × g for 15 min. The aqueous layer was collected and agitated in a new micro-tube containing 200 μL of chloroform and again centrifuged at 4°C and 12,000 × g for 15 min. The aqueous layer was collected and gently agitated in a new micro-tube containing 500 μL of chilled isopropyl alcohol, and then placed on ice. The tube was subsequently centrifuged for 10 min at 4°C and

12,000 × g. Then, 500 μL of chilled 70% ethanol was added to the resulting RNA precipitate, which was centrifuged at 4°C and 12,000 × g for 15 min, followed by washing of the RNA. The resulting precipitate was dissolved in 7 μL of RNase-free water and allowed to stand on ice for 15 min. Next, 1 μL of RNA solution was collected and diluted by a factor of 100 in RNase-free water. Absorption was measured at 260 nm and 280 nm using a microplate reader. Total RNA content was then calculated. RNA purity was determined at $OD_{260/280}$. The Tabletop Micro-centrifuge 3500 (Kubota Corporation, Tokyo, Japan) was used as the centrifuge, and the SpectraMax M2^{e} (Molecular Devices, Inc., Sunnyvale, CA, USA) was used as the microplate reader.

7. Quantitative real-time PCR

Extracted total RNA was reverse transcribed using the PrimeScript® RT reagent Kit. For each reaction, 2 μL of 5×PrimeScript® Buffer, 0.5 μL of PrimeScript® RT Enzyme Mix, 0.5 μL of Oligo dT Primer, and 0.5 μL of Random 6-mers were mixed, and 3.5 μL of this mixture was added to each tube of an 8-tube strip. Then, 0.5 μg of total DNA samples dissolved in RNase-free water were added to each tube, and RNase-free water was added to bring the final volume to 6.5 μL. Reverse transcription was performed with a thermal cycler (Veriti, Applied Biosystems, Foster City, CA, USA) to prepare cDNA .

Real-time PCR was conducted with SYBR® *Premix Ex Taq*™. Prepared cDNA was diluted by a factor of 2 with sterile distilled water. Reagents and primers (Table 1) were prepared and added at 18 μL to each tube of an 8-tube strip. Next, 2 μL of sterile distilled water (negative control) or prepared cDNA was added to bring the total volume to 20 μL. Amplified cDNA was detected and analyzed in real time using the ABI PRISM® 7500 Real-Time PCR system (Applied Biosystems) and intercalation by SYBR® Green . Relative

data were evaluated using a calibration curve. cDNA was prepared at concentrations of 0.16, 0.8, 4 and 20 relative to a concentration of 100 for undiluted cDNA in order to prepare the calibration curve.

Table 1

8. ELISA

Analysis of collagen content in the cultured medium was subsequently conducted according to the instructions of the ELISA kit.

Results

1. Characterization of SP and CP

Amino acid analysis of SP and CP revealed that SP was rich in aspartic acid and glutamic acid, and that CP was rich in glycine, proline and hydroxyproline (Table 2). Molecular weights were estimated using a molecular sieve. Calibration curves are prepared by using molecular weight markers with known weights to find the retention time and plotting the logarithm of the molecular weight (Figure 2). Measurement of the estimated molecular weights of SP and CP based on the relationship between molecular weights and retention times of marker molecules revealed that, although content differences were present, both SP and CP contained numerous substances with a molecular weight of around 500.

Figure 1

Figure 2

Table 2

2. Investigation of the effects of magnesium ascorbyl phosphate (VC-PMg) on COL1A1, Smad7 or MMP-1 gene expression

The collagen promoting effects of VC-PMg have been investigated. Addition of a VC-PMg to fibroblasts increased tendency COL1A1 gene expression, but did not alter the expression of Smad7 or MMP-1. This indicates that the collagen synthesis promoting effects of VC-PMg only affect COL1A1 gene expression, not the expression of Smad7 or MMP-1 (data not shown).

3. Investigation of the effects of SP concentration on COL1A1 gene expression

SP was added to fibroblasts to a final concentration of 10^{-6} to 10^{-3}%. Following 24 hours of incubation, COL1A1 gene expression was determined using real-time PCR. The results are shown in Figure 3.

Relative expression was determined against the sample with no SP added (0%), which was taken to be 1. The highest level of COL1A1 gene expression, 1.24, was increased tendency in the sample with 10^{-5}% SP (not significantly). Expression was not elevated at other concentrations. Subsequent experiments were therefore conducted at a concentration of 10^{-5}%.

Figure 3

4. Changes over time in COL1A1 gene expression caused by SP, CP and SP+CP

Real-time PCR was used to measure changes in COL1A1 gene expression in fibroblasts following addition of SP, CP and SP+CP. The results are shown in Figure 4. Peptides were applied at a concentration of 10^{-5}% for 4, 8 or 24 hours. The concentration used to investigate the combined effects of SP and CP was 5×10^{-6}%.

With relative expression in the control taken to be 1, COL1A1 gene expression was no different after 4 hours in the SP group, but had increased to 1.2 and 1.1, respectively, in the CP and SP+CP groups, and was same level in the SP group. After 8 hours, expression in the SP, CP and combined groups amounted to 1.1, 1.3 and 1.3, respectively. After 24 hours, expression in the SP, CP and combined groups was higher at 1.3, 1.5, and 1.4, respectively. Especially, COLA1A gene expression of CP and CP+SP administrated groups was significantly higher than VC-PMg group.

Figure 4

5. Changes over time in MMP-1 and Smad7 gene expression caused by SP, CP and SP+CP

Real-time PCR was used to measure changes in MMP-1 and Smad7 gene expression in fibroblasts following the addition of SP, CP and SP+CP. With relative expression in the control taken to be 1, MMP-1 gene expression was elevated at 1.5 after 4 hours in the SP group. Expression was lower in the CP and SP+CP groups at 0.6 and 0.8, respectively (Figure 5). Although significant increased in Smad7 mRNA expression was not observed (data not shown).

Figure 5

6. Changes over time in collagen content caused by SP, CP and SP+CP

ELISA was used to measure changes in collagen content in fibroblast culture medium following addition of SP, CP and SP+CP. The concentration in the control was 72.9 μg/mL. Collagen content after 8 hours was increase tendency at 82.5, 108.9 and 120.2 μg/mL, respectively, for SP, CP and combined addition (Figure 6A). Only SP+CP group was significantly higher than control group ($p<0.05$, Tukey's multiple comparison test). The concentration in the control group was 285.1 μg/mL after 24 hours, and were 393.9, 415.0 and 431.5 μg/mL, respectively, for SP, CP and combined addition (Figure 6B).

Figure 6(A) and 6(B)

Discussion

This research investigated the effects of SP and CP, both alone and in combination, on collagen synthesis in fibroblasts. The amino acid compositions and molecular weight distributions of the peptides used were also determined. Analysis revealed that the SP was rich in aspartic acid and glutamic acid and that the CP was rich in glycine, proline and hydroxyproline. An investigation of molecular weight distributions using high-performance liquid chromatography with a gel filtration column showed that the peptides used were rich in molecules with a molecular weight of around 500.

Expecting to observe the promotion of collagen synthesis, we added SP and CP both alone and in combination in order to investigate their effects on collagen synthesis. COL1A1 gene expression and collagen content increased over time in each group. Addition of SP, CP and SP+CP resulted in increased COL1A1 gene expression and the promotion of collagen synthesis. The final concentration of peptides when added in combination was set at 10^{-5}%, i.e., SP and CP were each applied at a concentration of 5×10^{-6}%; thus, the effects of this combination were likely substantial.

Increases in collagen content are dependent on collagen synthesis and collagen metabolism. This means that collagen synthesis must be promoted and collagen metabolism must be suppressed in order to effectively increase collagen content. We therefore decided to investigate the effects of adding SP, CP and SP+CP on the expression of Smad7 and MMP-1, which contribute to collagen metabolism. Smad7 gene expression was unaltered by any of the compounds investigated. MMP-1 gene expression was elevated at 4 hours after application in the SP group. In contrast, expression decreased a short time after CP and SP+CP application. Possible explanations for these differences include differences in amino acid sequences and mean molecular weights, and differences in the rates of cellular uptake due to receptors for

binding and endocytosis. We plan to investigate these differences in detail. It is considered that SP and/ or CP have produced on promoter of the COL1A1 gene. In addition, we think this effect is not direct but indirectly. It is not understood whether influence is a growth factor or cytokine. We are considering future study.

Conclusions

Collagen levels were highest when soybean peptide and collagen peptide were added to fibroblasts together. Our data suggest that their application regulated collagen synthesis by altering both COL1A1 gene expression and MMP-1 gene expression. This collagen synthesis promoting effect of SP, CP and SP+CP suggests the potential benefits of these peptides in improving skin condition when applied in food products and cosmetics.

Acknowledgements

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Table 1. Primer sequences

Primer	Direction	Sequence
GAPDH	Forward	5’ -GAAGGTGAAGGTCGGAGT- 3’
	Reverse	5’ -GAAGATGGTGATGGGATTTC- 3’
COL1A1	Forward	5’ -CCGCCGCTTCACCTACAGC- 3’
	Reverse	5’ -TTTTGTATTCAATCACTGTCTTGCC- 3’
Smad7	Forward	5’ -CCTTAGCCGACTCTGCGAACTA- 3’
	Reverse	5’ -CCAGATAATTCGTTCCCCCTGT- 3’
MMP-1	Forward	5’ -AAGCGTGTGACAGTAAGCTA- 3’
	Reverse	5’ -AACCGGACTTCATCTCTG- 3’

Table 2. Amino acid compositions of soybean peptide and collagen peptide

Amino Acids	SP	CP
Asp	12.6	4.6
Thr	3.8	2.7
Ser	5.5	3.7
Glu	22.1	7.7
Gly	4.2	23.9
Ala	3.9	10.6
Cys	1.3	0.0
Val	4.0	1.9
Met	1.1	0.4
Ile	3.8	1.2
Leu	6.7	2.7
Tyr	3.4	0.1
Phe	4.6	1.8
Hylys	ND	0.4
Lys	6.6	3.7
His	2.7	0.7
Arg	8.1	8.9
Pro	5.6	13.8
Hypro	ND	11.2
Total	100.0	100.0

ND : not detected

Figure Legends

Fig. 1. Molecular weight distribution of SP and CP by size exclusion liquid chromatography. Solid line: soybean peptide; dotted line: collagen peptide.

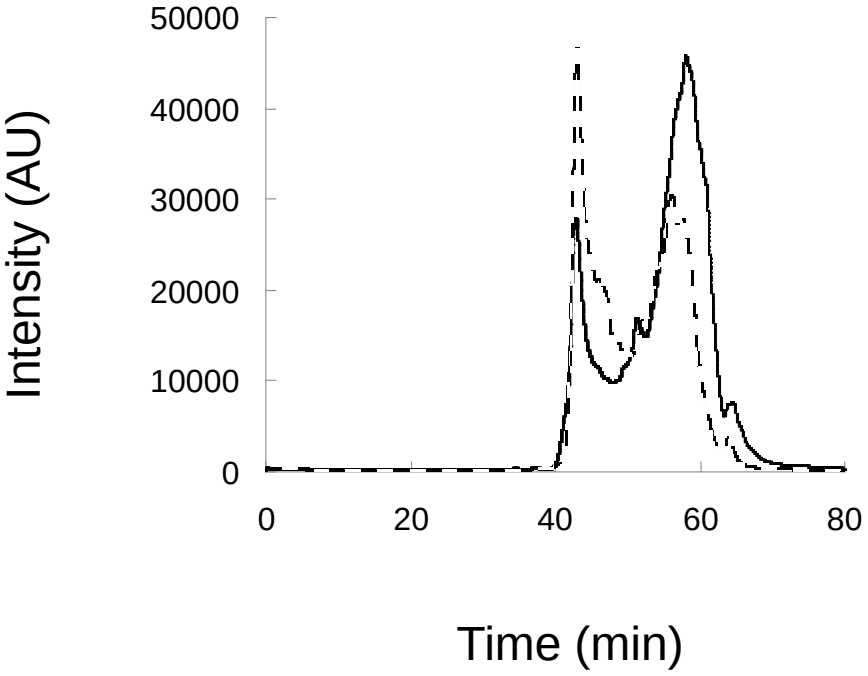
Fig. 2. Calibration curve of the molecular weight and retention time by known molecular weight compounds.

Fig. 3. Effects of SP concentration on COL1A1 mRNA expression in fibroblasts at 24 h after application. Vitamin C magnecium phosphate (10 μg/mL) is added to all samples. Each data point represents the mean and standard deviation of at least three independent experiments.

Fig. 4. Effects of SP, CP and SP+CP on COL1A1 mRNA expression in fibroblasts at 4 h, 8 h and 24 h after application. White bar: 4 h; gray bar: 8 h; black bar: 24 h. Each data point represents the mean and standard deviation of at least three independent experiments. Vitamin C magnecium phosphate (10 μg/mL) is added to all samples. Each data point represents the mean and standard deviation of at least three independent experiments. * $p < 0.05$, ** $p < 0.01$, as compared to control group (Tukey's multiple comparison test).

Fig. 5. Effects of SP, CP and SP+CP on MMP-I mRNA expression in fibroblasts at 4 h after application. Vitamin C magnecium phosphate (10 μg/mL) is added to all samples. Each data point represents mean and standard deviation of at least three independent experiments. * $p < 0.05$, as compared to control group (Tukey's multiple comparison test).

Fig. 6. Effects of SP, CP and SP+CP on collagen concentration in fibroblasts at 8 h (A) and 24 h (b) after application. Vitamin C magnecium phosphate (10 μg/mL) is added to all samples. Each data point represents mean and standard deviation of at least three independent experiments. * $p < 0.05$, as compared to control group (Tukey's multiple comparison test).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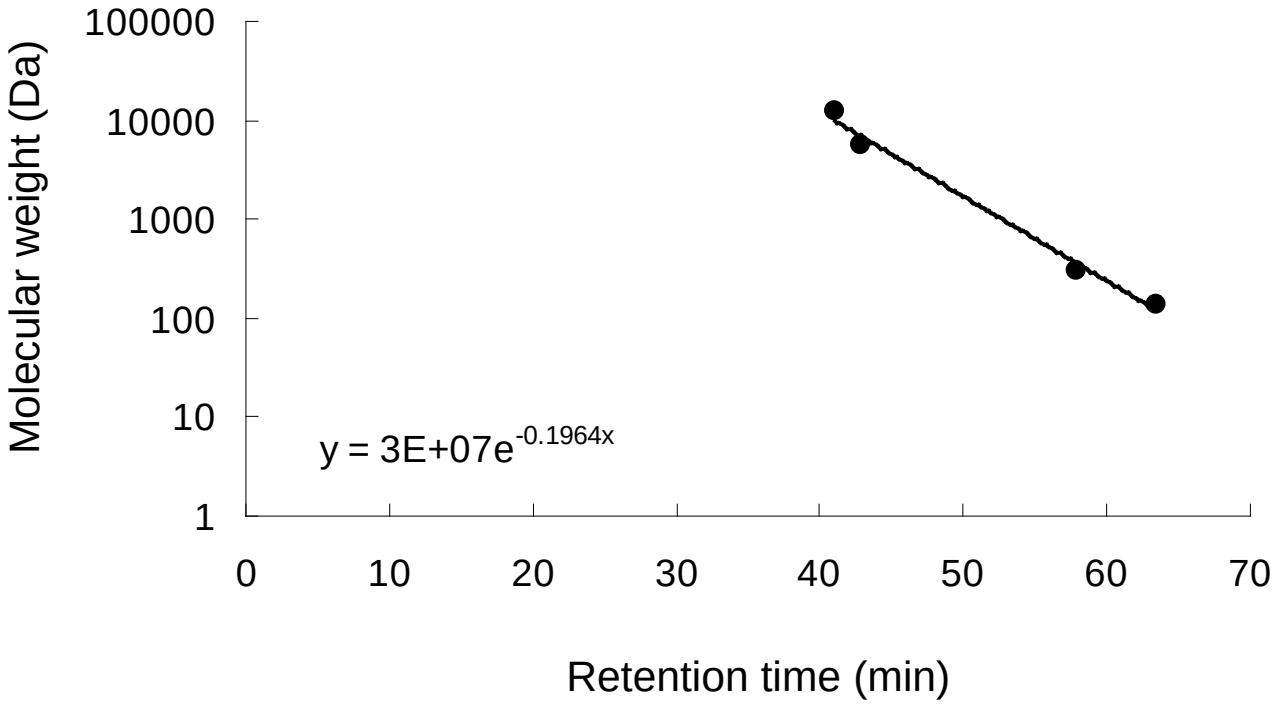
Time (min)

Figure 1


100000
10000
1000
100
10
1
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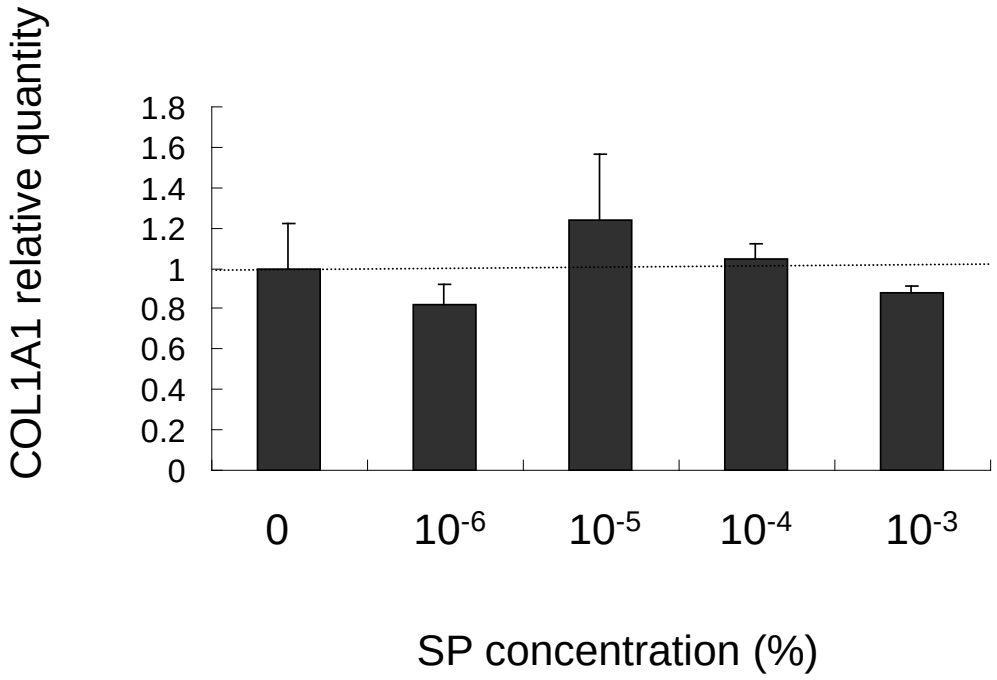
20
30
40
50
60
70
Retention time (min)

Figure 2


COL1A1 relative quantity
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1.6
1.4
1.2
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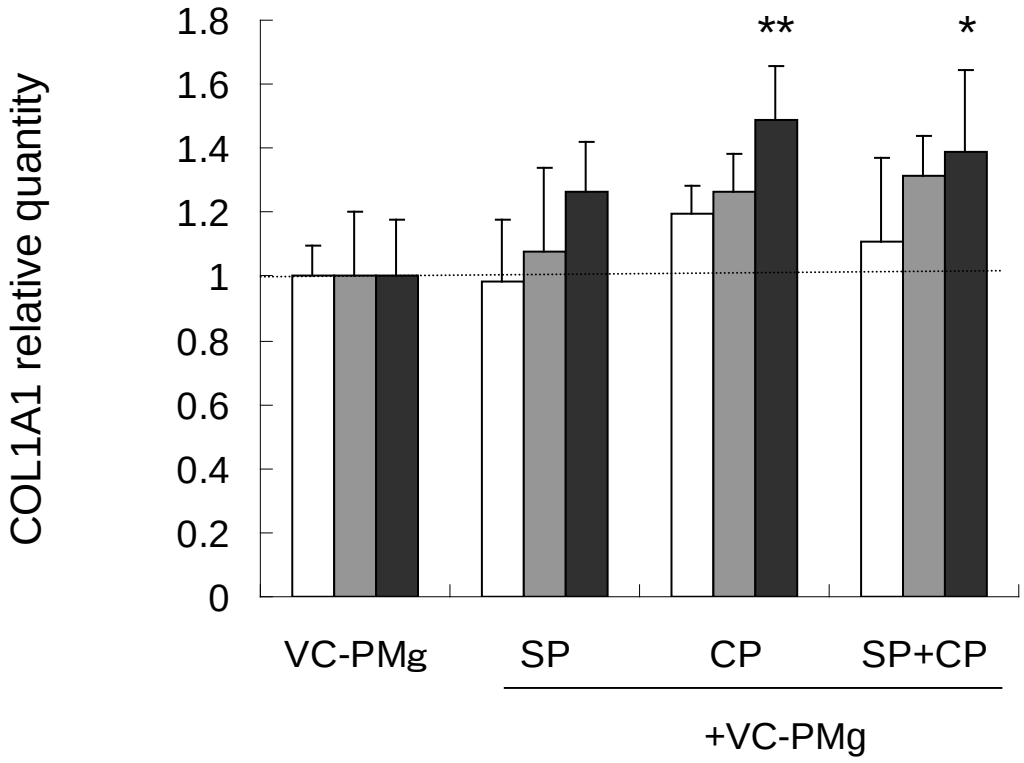
0.4
0.2
0
0
10-6
10-5
10-4
10-3
SP concentration (%)

Figure 3


COL1A1 relative quantity
1.8
1.6
1.4
1.2
1
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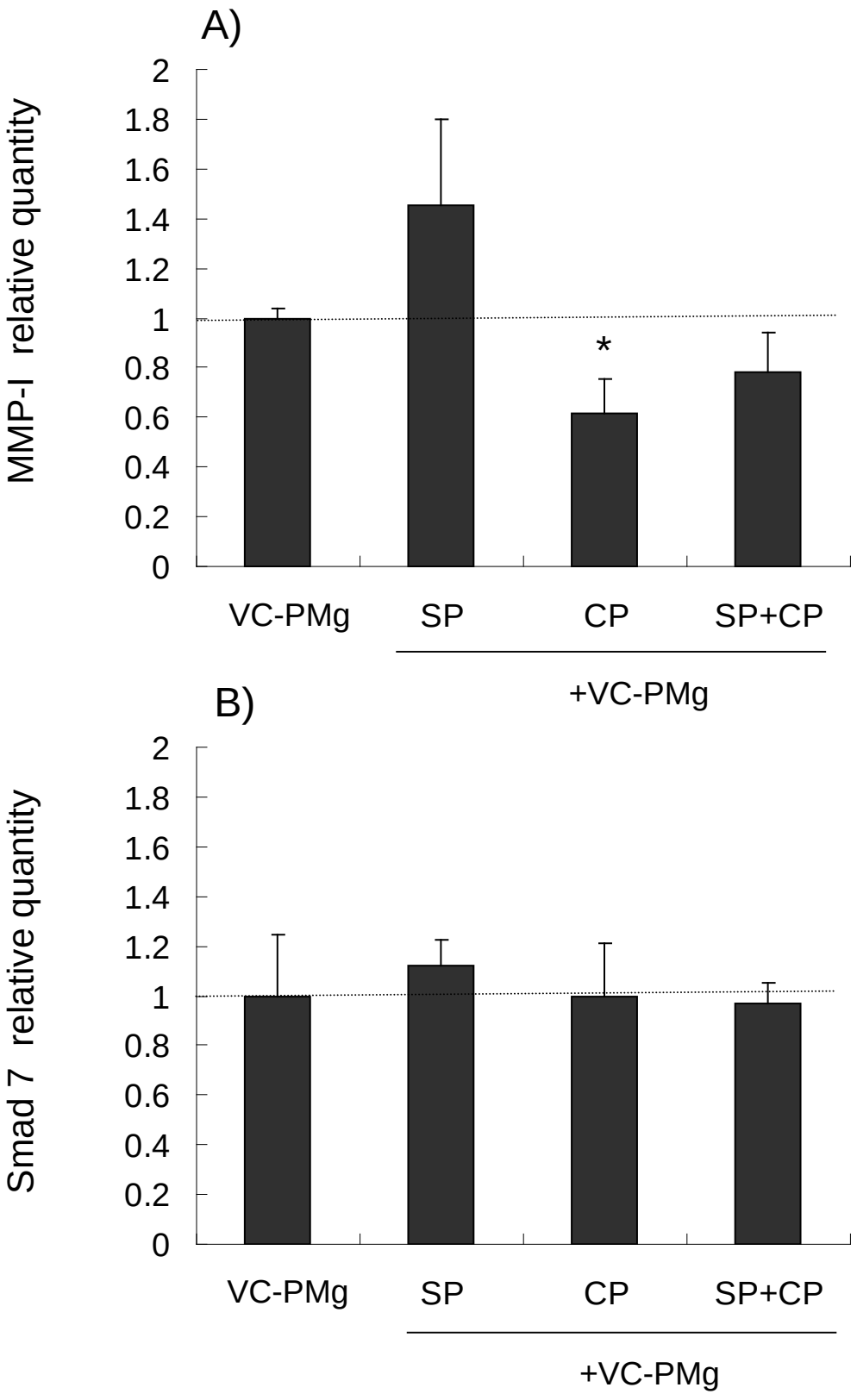
**
*
VC-PMg
SP
CP
SP+CP
+VC-PMg

Figure 4


A)
MMP-I relative quantity
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1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
VC-PMg
SP
CP
SP+CP
+VC-PMg
B)
Smad 7 relative quantity
2
1.8
1.6
1.4
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VC-PMg
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CP
SP+CP
+VC-PMg

Figure 5

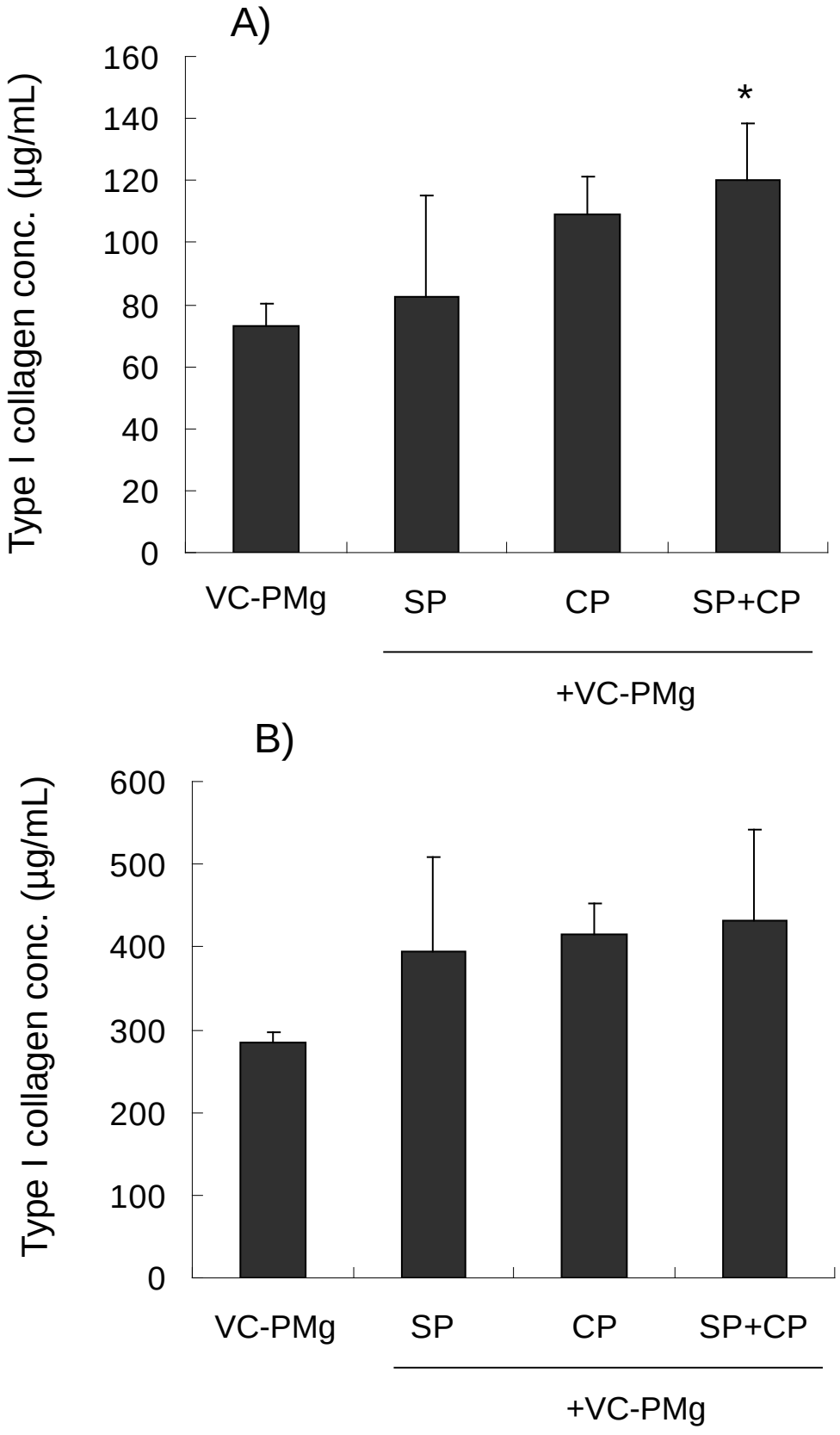
A)
Type I collagen conc. (μg/mL)
160
140
120
100
80
60
40
20
0
*
VC-PMg
SP
CP
SP+CP
+VC-PMg
B)
Type I collagen conc. (μg/mL)
600
500
400
300
200
100
0
VC-PMg
SP
CP
SP+CP
+VC-PMg

Figure 6