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Peptides from *Bombyx* Fibroin Counter D-Galactose-induced Hair Aging in Mice

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Using proteases, we produced a peptide mixture from fibroin of the silkworm *Bombyx mori*. Mice received D-galactose by subcutaneous injection for 8 weeks to accelerate aging, and then received the peptide mixture orally for 7 weeks. In the mice aged with D-galactose, the coefficient of friction of hair increased significantly up to 1.6-fold, but in the mice subsequently given the peptide mixture, it was normal. Scanning electron microscopy showed improved hair cuticles in the latter mice too. These results indicate that oral administration with peptides from *Bombyx* fibroin counters the aging of hair cuticles.

Key words: *Bombyx mori*, Fibroin peptides, D-galactose, Friction coefficient, Hair cuticle

Introduction

Partial hydrolysis of *Bombyx mori* fibroin with calcium chloride or hydrochloric acid produced a powder originally tested as a commercial food (Chen *et al*., 1991, 1993). Since this finding, many peptide mixtures derived from silkworm fibroin have shown potential for biomedical applications.

Peptide mixtures derived from *Bombyx* fibroin have been shown to lower cholesterol (Chen *et al*., 1993; Hirao *et al*., 1999), treat diabetes (Hyun *et al*., 2004; Nahm *et al*., 1995), and lower blood pressure (Igarashi *et al*., 2006) in rats. Surprisingly, a peptide mixture produced from *Bombyx* pupae and cocoons (MW of 500-5000, called brain factor-7) improved learning and memory function in 25 normal elderly people (Kim *et al*., 2004). It protected neurons from amyloid beta toxicity and enhanced acetylcholine level in the brain in a rat model of memory impairment (Kim *et al*., 2005). It also improved attention and cognitive flexibility in children (Kim *et al*., 2009).

Although fibroin-derived peptide fragments enhance the proliferation of cultured human skin fibroblasts (Yamada *et al*., 2004) and are commercially available in cosmetics, there is little information on the effects of their oral administration. Accordingly, we evaluated the effect of a fibroin peptide mixture on hair quality in mice artificially aged by d-galactose. To our knowledge, this is the first report demonstrating that the oral administration of fibroin peptide mixture prevents the decline of hair quality in aged mice.

Materials and Methods

Preparation of fibroin peptide mixture, amino acid analysis, and electrophoresis

The fibroin peptide mixture was obtained through dissolution and proteolysis with proteases, followed by gel filtration chromatography (Hyun *et al*., 2004; Park *et al*., 2002; Yamada *et al*., 2004). Part of the preparation (1 mg) was hydrolyzed with 6 N HCl (1 ml) at 110°C for 24 h in an evacuated, sealed glass tube, and 50-µl aliquots were taken for the determination of amino acid composition with an AminoTac JLC-500/V amino acid analyzer (Japan Electron Optics Laboratories). Samples were separated by

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blue native polyacrylamide gel electrophoresis (PAGE; 4-16%) (Reisinger and Eichacker, 2006). The protein and peptide contents were obtained from the ratios of peptide or free amino acid weights to fibroin weight, as determined by precipitation in 10% trichloroacetic acid (Bensadoun and Weinstein, 1976) and by a BCA protein assay kit (Pierce).

Animals

Five-week old male C57BL/6J mice were purchased from SLC Japan and housed five per cage in an environmentally controlled room (23 ± 2°C; 12/12 h light/dark cycle; lights on 07:00-19:00). Tests were performed from 19:00 to 23:00. After an adaptation period of 10 days, all mice were fed a standard diet and their body weights were monitored during the experimental period. Food and water were given *ad libitum*, but their consumption was measured every other day. Mice within each cage were identified by ear clipping. This research was conducted in accordance with the Guidelines of the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals approved by the Animal Experiment Committee of Iwate University.

D-Galactose-induced aging model

D-Galactose injection is used to create aging models of the brain and in anti-aging pharmacology research. We devised an experimental schedule (Fig. 1) in accordance with models for the induction of senescence by D-galactose (Ji *et al*., 2009; Lu *et al*., 2006; Tsushima *et al*., 2010; Wei *et al*., 2005). Mice were randomly divided into four groups: normal control (NC), aging control (AC), and fibroin peptide mixture (FPM) at either 1% or 2% (w/w). The NC and AC groups were injected subcutaneously each day with 0.9% saline (NC) or D-galactose (Sigma-Aldrich, MO, USA) at 100 mg/kg (AC) for 8 weeks. The

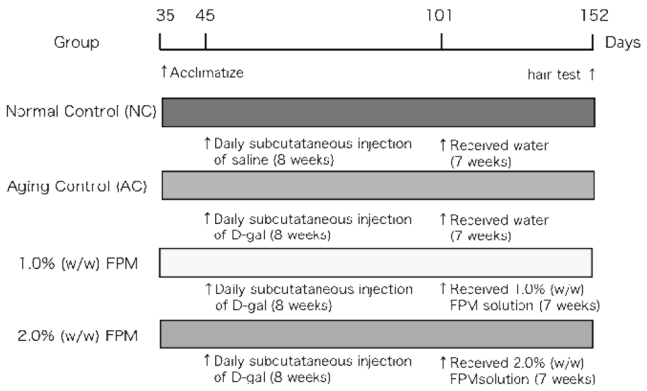

Fig. 1. Experimental schedule of fibroin peptide feeding and hair friction measurement in the mice aged with D-galactose.

two FPM groups received D-galactose as above, followed by fibroin peptide mixture in sterilized water for 7 weeks. We chose 7 weeks in consideration of the duration used in similar experiments: 2 weeks with silkworm fibroin (Kim *et al*., 2005), 3 weeks with *Vespa simillima* extract (Fujiwara *et al*., 2007), and 6 weeks with *Cordyceps sinensis* extract (Ji *et al*., 2009).

Gel filtration

The fibroin peptide mixture was dissolved in 0.1 M acetic acid and separated by fast protein liquid chromatography (FPLC; ÄKTA purifier, GE Healthcare) at 4°C with 0.1 M acetic acid at a flow rate 0.5 ml/min on a column packed with Superdex peptide 10/300 GL resin (GE Healthcare) and equilibrated with the same solution. The elution profile was monitored by UV absorbance at 215 nm.

ESI-LC/MS analysis

The mass spectrum of crude silk powder was determined by electrospray-ionization liquid chromatography / mass spectrometry (ESI-LC/MS) on a Hypersil Gold C18 column (2 mm i.d. × 50 mm; Thermo Scientific) at a flow rate of 200 µl/min. For each sample, 20 µl (1 µg/µl) was injected into the ESI-LC/MS system. The high-performance LC (HPLC) gradient was 5% B (0-2 min) – 40% B (2-40 min) – 100% B (40-45 min) – 5% B (45-60 min) (A: 100% H_2O, 0.1% formic acid; B: 20% H_2O/80% MeCN, 0.1% formic acid). The LC system was coupled to an LTQ Orbitrap XL (Thermo Scientific) MS operated in positive ion mode for data-dependent acquisition (full MS scan Orbitrap RP @ 30 000; top 1: MS/MS w/HCD 45% Orbitrap RP @ 7500; top 1: MS/MS w/HCD 35% Orbitrap RP @ 7500; top 2: MS/MS w/HCD 45% Orbitrap RP @ 7500; top 2: MS/MS w/HCD 35% Orbitrap RP @ 7500) of peptide fragmentation (MS/MS) spectra by collision-induced dissociation in helium gas. The spray voltage was 5 kV and the capillary temperature was 350°C.

Peptide sequence identification

All MS/MS fragmentation spectra were searched against the UniProt database by the Sequest database-searching tool in PEAKS Studio 4.5 proteomic MS software (Bioinformatics Solutions Inc.) with consideration of *B. mori* fibroin heavy chain (acc. no. P05740), fibroin light chain (acc. no. P21828), and fibrohexamerin (P25; acc. no. P04148).

Hair friction measurement

To evaluate the effects of the treatments on hair condition of the mice, we measured the coefficient of friction (COF) of the mouse hair with a portable tribometer developed by

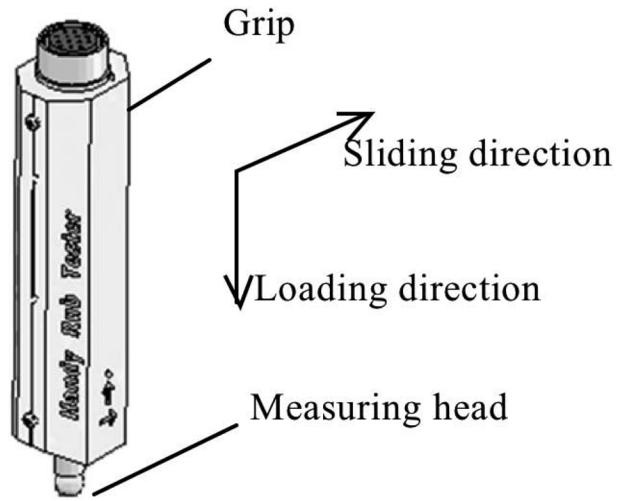

Fig. 2. Schematic drawing of the portable tribometer developed by Uchidate and Iwabuchi (2008).

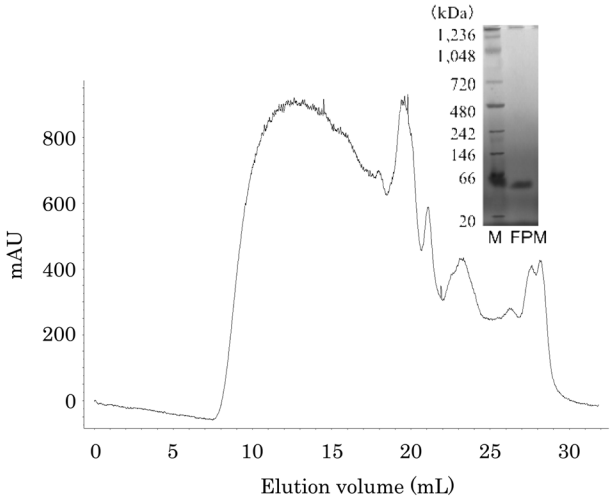

Fig. 3. Fast protein liquid chromatography and blue native PAGE (inset) of the fibroin peptide mixture.

Uchidate and Iwabuchi (2008) of our group (Fig. 2). The measuring head of the tribometer was covered with an artificial skin made of a polymer and was slid against the back of the mice at a load of approximately 0.3 N. Mice were anesthetized with diethyl ether during the measurements. COF was obtained as the ratio of the frictional force to the applied load.

Observation by scanning electron microscope

Hairs were observed by SEM (JOEL, JSM-6510 LA). Skin tissue was biopsied from the mid-back region of anesthetized mice and placed in a fixative solution of glutaraldehyde (2.5%) in phosphate buffer (0.1 M; pH 7.4) for 24 h. The specimens were sectioned into 0.5-mm × 10-mm slabs and washed in Milli Q water. The slabs were sliced longitudinally and attached to conductive carbon tape on the specimen mounting stage. They were coated with palladium–platinum in an ion spatter coater (Hitachi, E-102) and examined under the SEM.

Histological analysis

Mice were deeply anesthetized with diethyl ether and decapitated. The sebaceous glands in the skin were dissected out and washed in cold 50 mM phosphate buffer (0.1 M; pH 7.4). They were fixed in paraformaldehyde (4.0%) in phosphate buffer (0.1 M; pH 7.4) at 4°C overnight. Samples were embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin (HE).

Statistical analysis

All statistical analyses were performed in JMP 8 software (SAS Institute Inc., USA). One-way ANOVA was carried out with the Tukey–Kramer HSD *post hoc* test for multiple comparisons. Data are expressed as means ± SEM. Statistical signicance was set at $P < 0.05$.

Results

Peptides of fibroin peptide mixture

The four main amino acids present were glycine (42.7%), alanine (28.6%), serine (9.8%), and tyrosine (4.5%) (data not shown). This composition is consistent with that of *Bombyx* fibroin reported by Shimura *et al*. (1976).

Blue native PAGE revealed a band of 60 kDa with potentially many peptides in it (Fig. 3). Thirty-seven peptide fragments were sequenced, 25 singly charged and 12 divalently charged, most originating from the heavy or light chain of fibroin (Table 1 and 2). These results suggest that the profile of peptide fragments is attributable to enzymatic digestion (Jeong and Hur, 2010; Yamada *et al*., 2004). Our preparation (the 60-kDa protein comprised around 2% of total weight) was composed of 15 fragments from fibroin light chain, 13 from fibroin heavy chain, 2 from P25 protein, and 7 unknown. The 28 fibroin peptide fragments ranged in length from 3 to 10 amino acids and accounted for 52% of the total weight (the remainder may be free amino acids).

Effect of fibroin peptide mixture on hair quality of aging mice

The mean COF was significantly higher ($P < 0.001$) in AC than in NC (a 1.6-fold difference), yet returned to NC level (~0.23) in the FPM groups (Fig. 4). These results indicate that FPM feeding improved COF in mice artificially aged by D-galactose.

Table 1. Fibroin-peptide mixture sequences of singly charged ion identified by LC/MS/MS and their occurrences in the known sequence of fibroin

RTc	Precursor m/z	Charge state	Peptide MW	Sequence	Original Protein	Peak Intensity
4.1	367.1614	1	366.1542	SGFG	FIBH_BOMMO	1.00E+06
11.7	374.2286	1	373.2213	EII or EIL	FIBL_BOMMO	1.10E+06
10.2	459.1875	1	458.1802	GYGY	NA	1.00E+06
6.6	459.2349	1	458.2277	FGHV	FIBL_BOMMO	2.90E+06
4.7	581.2567	1	580.2495	VADGGY	NA	6.00E+06
7.1	509.2352	1	508.228	VNGGY	FIBH_BOMMO	3.70E+05
10.9	596.2929	1	595.2857	VLDSY	NA	2.00E+06
25.4	617.3299	1	616.3226	WDAIL	FIBL_BOMMO	7.30E+05
17.3	714.31	1	713.3027	SSGFGPY	FIBH_BOMMO	3.90E+06
3.5	423.1876	1	422.1804	SGPY	FIBH_BOMMO	2.30E+06
19.9	538.3601	1	537.3528	LLPPV	FIBL_BOMMO	5.40E+06
12.7	551.2826	1	550.2754	GVGVGY	FIBH_BOMMO	1.30E+06
17.2	737.4566	1	736.4494	LLPPVAQ	FIBL_BOMMO	1.30E+06
6.7	395.1927	1	394.1853	GVGY	FIBH_BOMMO	1.90E+07
17.7	627.2778	1	626.2705	SGFGPY	FIBL_BOMMO	7.90E+06
17.7	929.4011	1	928.3939	GTGSSGFGPY	FIBH_BOMMO	5.50E+06
8.6	638.2787	1	637.2715	SQSGPY	FIBH_BOMMO	2.00E+06
5.6	583.2358	1	582.2286	SDFGTG	FIBH_BOMMO	3.90E+06
10	373.2447	1	372.2375	AGII	FIBL_BOMMO	2.80E+06
15.4	483.2235	1	482.2162	FGPY	FIBH_BOMMO	1.10E+06
17.2	771.3314	1	770.3242	GSSGFGPY	FIBH_BOMMO	6.00E+06
17.3	540.2453	1	539.238	GFGPY	FIBH_BOMMO	4.60E+06
24.4	604.3346	1	603.3273	EIPVF	SI25_BOMMO	1.90E+06
5.6	670.2681	1	669.2609	SDFGTGS	FIBH_BOMMO	1.00E+06
28.9	603.3504	1	602.3432	GGLPIF	SI25_BOMMO	9.50E+05

Table 2. Fibroin-peptide mixture sequences of divalently charged ion identified by LC/MS/MS and their occurrences in the known sequence of fibroin.

Precursor m/z	Charge state	Peptide MW	Sequence	Original Protein	Peak Area (Auto detection)
354.1956	2	706.3766	GFRQSL	FIBL_BOMMO	6.20E+05
365.7265	2	729.4386	ITDLLR	FIBL_BOMMO	7.00E+05
366.2134	2	730.4124	VISRAW	FIBL_BOMMO	2.30E+06
369.2314	2	736.4484	LLPPVAQ	FIBL_BOMMO	3.40E+06
402.7216	2	803.4288	RQSLGPF	FIBL_BOMMO	5.20E+06
409.7295	2	817.4445	SVISRAW	FIBL_BOMMO	7.30E+05
415.7034	2	829.3924	SDNEIPR	FIBL_BOMMO	4.70E+06
482.2347	2	962.455	VDDTDKSIA	FIBL_BOMMO	2.40E+06
416.1956	2	830.3766	SDDELPR	NA	7.50E+06
479.7328	2	957.451	SDNELPRQ	NA	2.10E+06
562.7824	2	1123.5503	VLDSYTDGVR	NA	2.10E+06
641.3818	2	1280.749	APPAPAPVAPLLPA	NA	3.40E+06

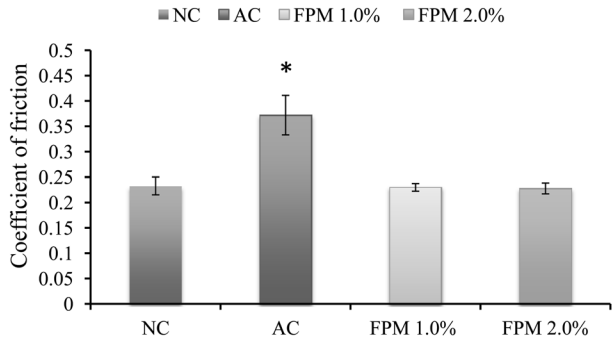

Fig. 4. Effect of fibroin peptide mixture (FPM) on coefficient of friction of mice hair. NC, normal control; AC, aging control. Results are mean ± SEM (n = 8). *P < 0.0001 by Tukey–Kramer HSD test.

SEM of hair wear and histology of sebaceous glands

SEM showed that 2% FPM restored the hair cuticle to the quality of NC, but AC caused rough wear (Fig. 5). This result corresponds to the COF result.

On the other hand, cross-sections of sebaceous glands did not show any remarkable differences in histology (Fig. 6). These results suggest that FPM feeding is more crucial to hair nutrition than to sebaceous glands in aged mice.

Discussion

Fibroin preparations derived from the cocoons of *B. mori* are used in the food industry, cosmetics, and other applications. Most cosmetic products are supplied as emulsions, lotions, gels, or powders for topical application (Secchi, 2008). Hair cosmetics containing a fibroin peptide mixture of MW 300 to 500 restored hair damaged by chemical treatment (Hyun *et al*., 2008). Our data show that oral administration with fibroin peptide mixture counters the aging of mouse hair cuticle.

Whether the fibroin peptide mixture or a component peptide is responsible for the anti-aging activity has not yet been elucidated. The anti-aging function remains to be analyzed, but oral administration may provide a practical use of silk fibroin in cosmetic products.

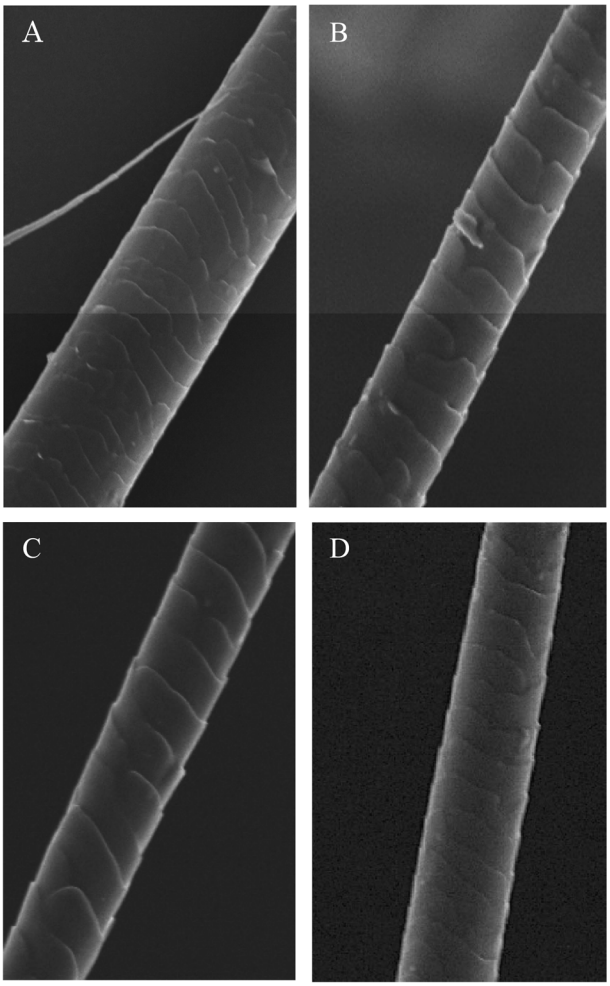

Fig. 5. SEM photographs of hair cuticles of mice. A, normal control (NC); B, aging control (AC); C, FPM 1.0%; D, FPM 2.0%.

Soluble fibroin extracts enhanced learning and memory in normal and demented persons (Chae *et al*., 2004; Kim *et al*., 2005) and cognition and attention in normal chil-

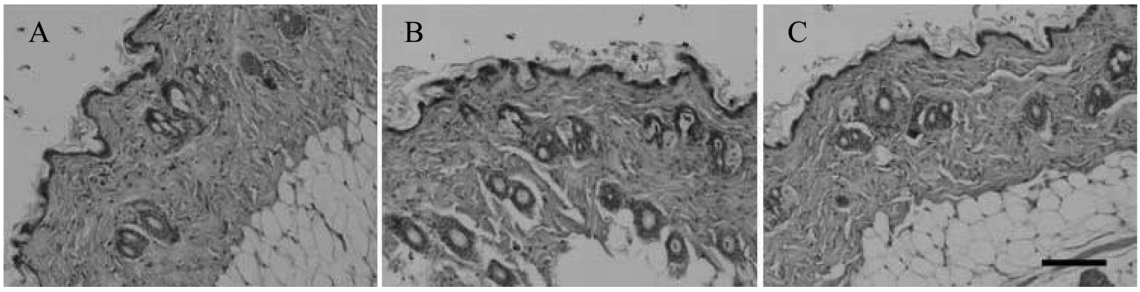

Fig. 6. HE-stained sebaceous glands of mice. A, normal control (NC); B, aging control (AC); C, FPM 2.0%. Scale bar, 100 μm.

dren (Kim *et al*., 2009). The components of a fibroin peptide mixture called brain factor-7 are assumed to protect neurons and maintain acetylcholine concentrations (Kim *et al*., 2005). Besides silk fibroin, extracts prepared from adult workers of *Vespa simillima* (Fujiwara *et al*., 2007) and *Paecilomyces tenuipes* cultured on silkworm pupae (Tsushima *et al*., 2010) have improved cerebral function in mammals.

The complex composition of the silk fibroin peptide mixture allows for the discovery of novel therapeutic agents. In particular, oral administration can both enhance brain function and retard the aging process. An increase in the coefficient of friction of human hair is attributable to damage (Bhushan *et al*., 2005). So our finding may facilitate the development of oral therapies to improve age-related hair nutrition and the elucidation of bioactive compounds from the fibroin peptide mixture.

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