British Journal of Nutrition (2012), **108**, 1726–1735
doi:10.1017/S0007114511007306
© The Authors 2012

Dietary silk protein, sericin, improves epidermal hydration with increased levels of filaggrins and free amino acids in NC/Nga mice

Hyunae Kim[1], Yu-ji Lim[2], Ji-Ho Park[2] and Yunhi Cho[1]*

[1]*Department of Medical Nutrition, Graduate School of East-West Medical Science, Kyung Hee University, Yongin-si, Gyeonggi-do 446-701, Republic of Korea*
[2]*Department of East-West Medical Science, Graduate School of East-West Medical Science, Kyung Hee University, Yongin-si, Gyeonggi-do 446-701, Republic of Korea*

(Submitted 20 May 2011 – Final revision received 26 October 2011 – Accepted 4 December 2011 – First published online 16 January 2012)

Abstract

Epidermal hydration is maintained primarily by natural moisturising factors (NMF), of which free amino acids (AA) are major constituents that are generated by filaggrin degradation. To identify dietary sources that may improve skin dryness of atopic dermatitis (AD), we investigated dietary effects of silk proteins, sericin and fibroin, on epidermal levels of hydration, filaggrins and free AA, as well as PPARγ, peptidylarginine deiminase-3 (PAD3) and caspase-14 proteins involved in filaggrin expression and degradation processes. NC/Nga mice, an animal model of AD, were fed a control diet (group CA: atopic control) or diets with 1 % sericin (group S) or fibroin (group F) for 10 weeks. In group S, epidermal levels of hydration, total filaggrins and total free AA, as well as PPARγ, PAD3 and caspase-14, which were reduced in group CA, were increased to higher or similar levels of a normal control group of BALB/c mice (group C). Furthermore, profilaggrin, a precursor with multiple filaggrin repeats, and three repeat intermediates were increased, while two repeat intermediates and filaggrin were decreased in parallel with increased levels of glutamate and serine, major AA of NMF in group S. Despite increased levels of total filaggrins, total free AA, PPARγ and PAD3, epidermal levels of hydration, glutamate, serine and caspase-14 were not increased, but other minor AA of NMF were highly detected in group F. Dietary sericin improves epidermal hydration in parallel with enhancing profilaggrin expression and degradation into free AA that is coupled with elevated levels of PPARγ, PAD3 and caspase-14 proteins.

Key words: Sericin: Epidermal hydration: Filaggrins: Free amino acids

Epidermal hydration (water content in the epidermis) is largely maintained by natural moisturising factors (NMF) and the epidermal barrier in the epidermis[1,2]. Compared with the epidermal barrier, in which the multilayered lamellar structure of ceramides, cholesterol and fatty acids provides a hydrophobic barrier against water loss in the epidermis[1], NMF consist primarily of amino acid (AA) and their derivatives together with lactate, sugars, urea and inorganic ions, which confer the hygroscopic ability of maintaining epidermal hydration as a complex mixture of water-soluble components[1,2]. AA, the major components of NMF, are formed by the degradation of filaggrin during epidermal differentiation. Filaggrin is initially synthesised as profilaggrin, which consists of multiple repeats of filaggrin[2,3]. Through the generation of two-repeat intermediate (2RI) or three-repeat intermediate (3RI) of filaggrin, profilaggrin is degraded to form monomeric filaggrin, and then undergoes subsequent degradation into free AA[3,4], which consists of multistep processes, in which various regulators and proteases are involved[5]. The similarities in AA profiles between filaggrin and NMF indicate that filaggrin is the sole source of AA in NMF, as glutamate, serine and glycine accounted for about >50 % of the total free AA in either filaggrin or NMF[2,4]. The reduced expression of filaggrin protein is paralleled by decreased amounts of free AA in dry skin diseases such as atopic dermatitis (AD) and ichthyosis[6–8].

The recognition of dry skin condition in various skin diseases[6–8], coupled with the inconvenience of applying moisturiser and emollients frequently, and undesirable side effects of some pharmaceutical agents, has led to the development of alternatives for dry skin protection from dietary and natural sources. Silk protein purified from the cocoons of silkworms (*Bombyx mori*) has been reported to have beneficial effects for dry skin protection[9,10]. Silk consists of two types of

Abbreviations: 2RI, two-repeat intermediate; 3RI, three-repeat intermediate; AA, amino acid; AU, arbitrary units; C, BALB/c mice fed a control diet; CA, NC/Nga mice fed a control diet; F, NC/Nga mice fed a diet supplemented with 1 % fibroin; NMF, natural moisturising factors; PAD3, peptidylarginine deiminase-3; S, NC/Nga mice fed a diet supplemented with 1 % sericin.

* **Corresponding author:** Professor Y. Cho, fax +82 31 201 3715, email choyunhi@khu.ac.kr

British Journal of Nutrition

https://doi.org/10.1017/S0007114511007306 Published online by Cambridge University Press

https://doi.org/10.1017/S0007114511007306 Published online by Cambridge University Press

British Journal of Nutrition

proteins, fibroin and sericin. In silk textile processing, sericin, which envelops fibroin with successive sticky layers, is mostly removed and fibroin, a fibrous protein, is purified. Although fibroin has been reported to be a useful biomaterial in the skin[11,12], sericin has been particularly reported to prevent dry skin condition[9,10]. Possibly due to its high serine content (30–33 % of total AA) and the AA profile similarity to NMF[13], human studies have indicated that topically applied sericin increases epidermal hydration[9,10]. Sericin may therefore be a valuable source of natural dry skin protection for cosmetic and food industries[13]. However, the dry skin protection activities of sericin have been suggested mostly by topical application (*in vitro* studies)[9,10], and little information is available on the dietary effects of sericin. In this regard, we previously reported that dietary sericin increases the total amount of ceramides[14], the major lipid maintaining the lamellar structure of the epidermal barrier[1], in NC/Nga mice, an animal model of AD[15,16]. To further investigate the dietary effect of silk protein on improving skin dryness, we compared the dietary effects of sericin and fibroin on epidermal hydration in NC/Nga mice, and delineated a possible mechanism of these effects in profilaggrin expression and its degradation into free AA in the present study. Specifically, altered levels of filaggrin species and free AA, as well as regulators and proteases involved in profilaggrin expression and degradation processing, were evaluated.

Experimental methods

Preparation of sericin and fibroin protein powders

Sericin and fibroin powders were provided by the Rural Development Administration, Republic of Korea[14,17]. In brief, sericin was extracted twice by boiling small pieces of silk cocoons of *B. mori* (100 g) with 3 litres of distilled water at 110°C for 5 h, and filtering through Whatman no. 2 paper[14,17]. Fibroin extracts were prepared by boiling small pieces of silk cocoons of *B. mori* (100 g) with 5 litres of 0·03 % sodium carbonate and 0·05 % marceillous soap for 30 min and washed twice with boiling water[14,17]. An electrodialysis system (Acilyzer-02; Astom) was used to remove salt residues from fibroin extracts[14]. All sericin and fibroin extracts were lyophilised to yield sericin and fibroin powders. The molecular weights of sericin and fibroin protein powders obtained were confirmed to be about 65 300 (sericin) and 101 700 (fibroin) by gel electrophoresis[14].

Animals and diets

Male BALB/c mice (5 weeks old, *n* 10) and male NC/Nga mice (5 weeks old, *n* 30) were purchased from SLC Japan (Shizuoka, Japan). NC/Nga mice, an animal model of AD, in which dry skin is raised by conventional environments[15,16] (not by genetic defects in *FLG*, profilaggrin gene)[18,19], were assigned to three groups of ten mice each: an atopic control group (group CA) fed a control diet, and groups S and F fed diets supplemented with 1·0 % powdered extracts of sericin and fibroin, respectively. Mice were fed the experimental diets for 10 weeks to compare the dietary effects of sericin and fibroin on epidermal hydration. Simultaneously, group C, a normal control group which consisted of BALB/c mice, was fed the control diet for 10 weeks. The compositions of the experimental diets are shown in Table 1, and the AA compositions of sericin and fibroin are shown in Table 2.

During the 10-week feeding period, all mice were maintained under conventional laboratory conditions without air filtration to induce AD as described previously[15,16]. Mice were housed under conditions of controlled temperature (22–24°C), humidity (55–60 %) and light (lights on from 07.00 to 19.00 hours). Food intakes and body weights of all groups were monitored weekly over the 10-week feeding period, and there were no significant differences between the groups. Animal care and handling conformed to the guidelines provided by the Animal Care and Use Review Committee of Kyung Hee University. At the end of week 10, all mice were killed by cervical dislocation.

Assessment of epidermal hydration

Under standardised conditions of 22–24°C and 55–60 % humidity, epidermal hydration was measured on the dorsal skin surface at the end of week 10 using a corneometer (model MPA-5; Courage + Khazaka Electronic GmbH). Data are expressed as means with their standard errors (capacitance in arbitrary units (AU)).

Table 1. Compositions of the experimental diets

	Experimental diets (g/kg dry diet)			
Composition	C	CA	S	F
Casein*	230	230	220	220
Sericin	–	–	10	–
Fibroin	–	–	–	10
L-Cystine	3	3	3	3
Maize oil	100	100	100	100
Cellulose	50	50	50	50
Vitamin mix†	10	10	10	10
Mineral mix‡	35	35	35	35
Sucrose	200	200	200	200
Maize starch	372	372	372	372

C, BALB/c mice fed a control diet; CA, NC/Nga mice fed a control diet; S, NC/Nga mice fed a diet supplemented with 1 % sericin; F, NC/Nga mice fed a diet supplemented with 1 % fibroin.

* Casein (nitrogen × 6·25), 870 g/kg.

† Vitamin mix composition, AIN-93 vitamin mix #310025 (Dytes, Inc.): niacin, 3 g/kg; calcium pantothenate, 1·6 g/kg; pyridoxine HCl, 0·06 g/kg; thiamin HCl, 0·6 g/kg; riboflavin, 0·6 g/kg; folic acid, 0·2 g/kg; biotin, 0·2 g/kg; vitamin E acetate (500 IU/g), 15 g/kg; vitamin B_{12} (0·1 %), 2·5 g/kg; vitamin A palmitate (500 000 U/g), 0·8 g/kg; vitamin D_3 (400 000 IU/g), 0·25 g/kg; vitamin K_1/dextrose mix (10 mg/g), 7·5 g/kg; sucrose, 967·23 g/kg.

‡ Salt mix composition, AIN-93G salt mix #210025 (Dytes, Inc.): calcium carbonate, 357 g/kg; potassium phosphate (monobasic), 196 g/kg; potassium citrate H_2O, 70·78 g/kg; NaCl, 74 g/kg; potassium sulphate, 46·6 g/kg; magnesium oxide, 24 g/kg; ferric citrate USP, 6·06 g/kg; zinc carbonate, 1·65 g/kg; manganous carbonate, 0·63 g/kg; cupric carbonate, 0·3 g/kg; potassium iodate, 0·01 g/kg; sodium selenate, 0·01025 g/kg; ammonium paramolybdate.$4H_2O$, 0·00795 g/kg; sodium metasilicate·$9H_2O$, 1·45 g/kg; chromium potassium sulphate·$12H_2O$, 0·275 g/kg; lithium chloride, 0·0714 g/kg; boric acid, 0·0815 g/kg; sodium fluoride, 0·0635 g/kg; nickel carbonate, 0·0318 g/kg; ammonium vanadate, 0·066 g/kg; sucrose finely powdered sucrose, 221·026 g/kg.

Table 2. Amino acid composition of casein and silk proteins

		Silk protein	
Amino acid	Casein	Sericin	Fibroin
EAA			
Arg	3·5	2·8	0·5
His	2·9	0·9	0·2
Ile	5·2	1·4	0·7
Leu	8·9	0·6	0·5
Lys	7·6	10·2	0·3
Met	2·8	<0·05	0·1
Phe	4·8	0·4	0·6
Thr	3·9	6·0	0·9
Trp	1·2	0·0	0·2
Val	6·4	2·6	2·2
NEAA			
Ala	2·8	4·6	29·3
Asp	6·7	19·1	1·3
Cys	0·5	<0·05	0·2
Glu	20·2	4·1	1·0
Gly	1·7	12·2	44·4
Pro	10·7	0·8	0·3
Ser	4·9	30·4	12·1
Tyr	5·3	3·8	5·2
Total (%)	100·0	100·0	100·0

EAA, essential amino acids; NEAA, non-essential amino acids.

Immunofluorescence analysis

Dissected dorsal skin was fixed in 4 % paraformaldehyde solution at 4°C for 24 h. Subsequently, skin was dehydrated and embedded in paraffin. Serial sections were obtained at a thickness of 5 μm. For antigen retrieval, sections were boiled in 0·05 M-citrate buffer for 10 min, and then completely cooled. After washing in 0·01 M-TBS, non-specific binding was blocked with 5 % goat serum and 0·1 % Triton-X in TBS for 1 h at room temperature. Skin sections were incubated at 4°C for 24 h with anti-rabbit antibody against filaggrin (ab81468; Abcam) diluted at 1:1000 in blocking buffer[20]. After washing again, the sections were incubated for 1 h in the dark at room temperature with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (Sigma) at a dilution of 1:200[20]. Slides were rinsed and mounted with Permafluor™ aqueous mounting medium (Thermo Scientific). The sections were photographed under light microscope (200 × magnifications) and fluorescence images were captured using a Zeiss microscope (Zeiss LSM 510; Carl Zeiss). The intensity of fluorescence was graded visually on a scale from 1 to 5 (1, low; 3, moderate; 5, high, 0·5 scale) by a pathologist who was unaware of the experimental diets and groups.

Western blot analysis

For the detection of profilaggrin (>220 kDa), degradative intermediates of profilaggrin including 3RI (90 kDa), 2RI (60kDa) and filaggrin (28 kDa)[21], the epidermis was isolated after 18 h incubation of the whole skin in PBS (pH 7·4) containing 10 mM-EDTA at 4°C, and immediately homogenised in 50 mM-Tris buffer containing 9 M-urea, 2 % SDS, 2 mM-EDTA and a protease inhibitor cocktail with polytron (P-8340; Sigma)[22]. For the detection of PPARγ, peptidylarginine deiminase-3 (PAD3) and caspase-14, a regulator and proteases involved in profilaggrin expression and its degradation processing into free AA[5], or detection of cytokeratin 5 (loading control), the epidermis was isolated after overnight incubation of the whole skin in an ice-cold 1:1 mixture of dispase II (2·4 units/ml; Roche) and Roswell Park Memorial Institute (RPMI) medium supplemented with 10 % fetal bovine serum at 4°C[23], and homogenised in 20 mM-Tris–HCl buffer (pH 7·5) containing 2 mM-EDTA, 0·5 mM-EGTA and 2 mM-phenylmethylsulfonyl fluoride with polytron[24]. Epidermal homogenates were centrifuged at 300 ***g*** for 5 min, and after the removal of debris, a portion of the homogenates was used to measure protein concentration by a modified Lowry method[25].

Protein extracts were electrophoretically separated on either 4–12 % Bis-Tris gel for various filaggrin species[21,22] or 10 % SDS-PAGE gel for other proteins (NuPage Invitrogen)[24]. Gels were blotted onto nitrocellulose membranes and incubated with primary antibodies against either filaggrin (ab81468), PPARγ (sc-6285), caspase-14 (sc-56 041), PAD3 (ab50246) or keratin-5 (ab53121) (filaggrin, PAD3 and keratin-5 antibodies: Abcam; PPARγ and caspase-14 antibodies: Santa Cruz Biotechnology, Inc.) followed by incubation with IgG-horseradish peroxidase (HRP) secondary antibodies (Cell Signaling Technology, Inc.)[24]. Immunoreactive proteins were visualised with an enhanced chemiluminescence (ECL) detection system (GE Healthcare), and band intensity was quantified by densitometry.

Free amino acid analysis

Epidermis isolated with an ice-cold 1:1 mixture of dispase II (2·4 units/ml; Roche) and RPMI supplemented with 10 % fetal bovine serum at 4°C[23] was homogenised in 15 % TCA with polytron and placed at 4°C for 16 h followed by centrifugation at 12 000 ***g*** for 10 min to remove the acid-precipitated protein. After filtration (0·2 μm; Sartorius), the supernatant was subjected to free AA analysis by ion-exchange chromatography using an automatic AA analyser (Model LC-5A; Shimadzu). Free AA were eluted from a lithium high-performance column by sequential usage of lithium citrate buffers with increasing concentrations and pH (0·20 M, pH 2·80; 0·30 M, pH 3·00; 0·50 M, pH 3·15; 0·9 M, pH 3·50; 1·65 M, pH 3·55) in the mobile phase[26,27]. The column temperature was maintained at 47°C and the flow rate was 0·45 ml/min. Post-column derivatisation with ninhydrin was followed by spectrophotometric detection at 440 and 570 nm. The CV of multiple analyses was within 5 %. The internal standard method eliminated the error caused by the loss associated with the analytical technique. With measuring protein concentration of epidermal homogenates[25], free AA contents were reported as nmol/mg epidermal protein.

Statistical analysis

Data are expressed as means with their standard errors. All data were analysed by one-way ANOVA using SPSS software (SPSS version 13.0; SPSS, Inc.)[28], and differences among the groups (C, CA, S and F) were determined by Duncan's multiple comparison test. $P<0·05$ was considered significant. Data for epidermal hydration (Fig. 1) were further analysed by the unpaired Student's *t* test at $P<0·05$ or $P<0·01$.

British Journal of Nutrition

https://doi.org/10.1017/S0007114511007306 Published online by Cambridge University Press

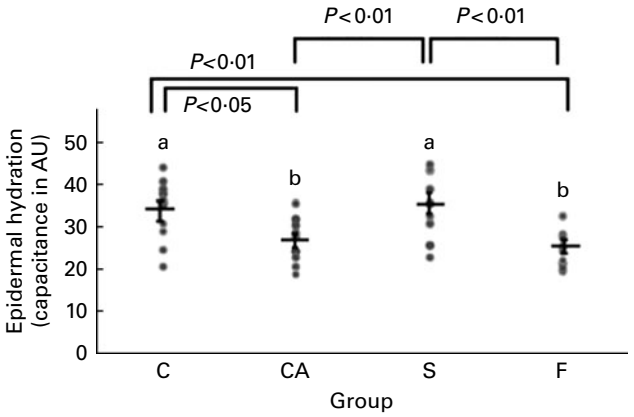

Fig. 1. Dot plot illustrating the epidermal hydration in BALB/c mice fed a control diet (group C) and NC/Nga mice fed a control diet (group CA) or diets supplemented with 1 % sericin (group S) or fibroin (group F) for 10 weeks. Each point in the dot plot represents one mouse, but occasionally overlapping data points obscure the sample size of ten in each group. The central line within the column of points represents the mean values (n 10), with standard errors represented by vertical bars. [a,b] Groups not sharing a common letter are significantly different ($P<0·05$) using one-way ANOVA and Duncan's multiple comparison test. $P<0·05$ or $P<0·01$, between groups as indicated by the unpaired Student's t test. AU, arbitrary unit.

Results

Assessment of epidermal hydration

Decreased epidermal hydration has been reported in NC/Nga mice with the induction of AD-like skin lesions[15,16]. When the distribution of the assessed level of epidermal hydration was illustrated as the dot plot, the dots in each group indicated normally distributed variances (Fig. 1). The epidermal hydration in group CA (27·2 (SEM 1·67) AU) was significantly lower than in group C (34·1 (SEM 2·32) AU), the normal control group. In group S, epidermal hydration (36·0 (SEM 2·41) AU) was higher than that in group CA and was similar to that of group C. However, the epidermal hydration of group F (25·7 (SEM 1·52) AU) was similar to that of group CA, indicating that dietary supplementation with sericin only improves epidermal hydration to similar levels of the normal control group and that dietary fibroin has no beneficial effects on epidermal hydration.

Expression of profilaggrin, degradative intermediates and filaggrin proteins

In immunofluorescence analyses (Fig. 2(A) and (B), the filaggrin proteins were highly expressed in the upper regions (i.e. stratum granulosum and stratum corneum) of the epidermis, in which terminal differentiation is processed, as reported previously[3], in the skin sections of all groups. The protein expressions of filaggrins in group CA became faint, when compared with those of group C. After dietary supplementation with sericin or fibroin, the protein expressions of filaggrins in groups S and F were higher than those of group CA. On a quantitative basis, the fluorescence intensity score of group CA was lower than those of other groups in relation to the level of epidermal hydration (Fig. 1). Although the fluorescence intensity scores of filaggrins in both groups S and F were higher than those in group CA, group F had lower scores than group S. The fluorescence intensity scores in group S were higher than those of group C.

Further studies to characterise the altered filaggrin species demonstrated that the protein expressions of total filaggrins including profilaggrin, 3RI, 2RI and monomeric filaggrin in group CA were significantly lower than those in group C (Fig. 3), reflecting the faint fluorescence intensity of filaggrins shown in Fig. 2. The protein expressions of total filaggrins in both groups S and F were higher than those in group CA, and were similar to group C. However, the protein expressions of

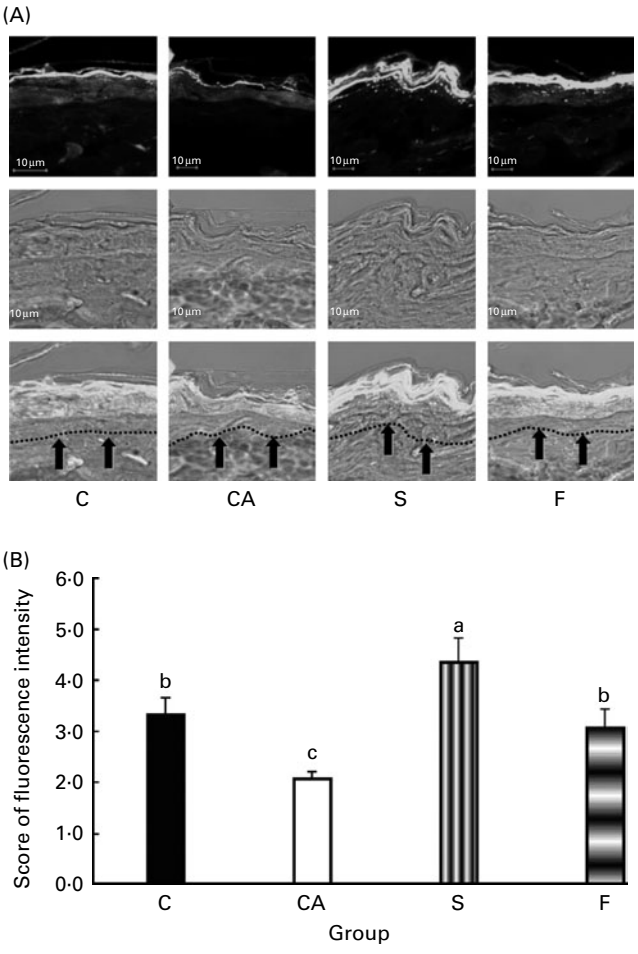

Fig. 2. Indirect immunofluorescence of filaggrins in the epidermis of BALB/c mice fed a control diet (group C) and NC/Nga mice fed a control diet (group CA) or diets supplemented with 1 % sericin (group S) or fibroin (group F) for 10 weeks. Skin specimens of the dorsal trunk were harvested for immunofluorescence staining with a primary antibody of filaggrin, which recognises an epitope within the filaggrin repeat domain and therefore detects profilaggrin, three-repeat or two-repeat intermediates and filaggrin, and using a fluorescein isothiocyanate (FITC)-conjugated secondary antibody. (A) Images in fluorescence microscopy (top) and differential interference contrast light microscopy (middle); co-captured images in fluorescence microscopy and differential interference contrast light microscopy (bottom). Arrows and dotted lines indicate the bottom layer of the epidermis. A representative section is shown from each group. Scale bar: 10 μm. (B) Score of fluorescence intensity. Values are means (n 10), with standard errors represented by vertical bars. [a,b,c] Mean values with unlike letters were significantly different ($P<0·05$; one-way ANOVA and Duncan's multiple comparison test).

British Journal of Nutrition

https://doi.org/10.1017/S0007114511007306 Published online by Cambridge University Press

total filaggrins in group S were significantly higher than those in group F, and profilaggrin and 3RI were specifically expressed at higher levels than those in group C. In contrast, the protein expressions of 2RI and filaggrin in group S were lower than those in group C. In group F, the protein expressions of filaggrin species were similar or modestly lower than those in group C.

Expression of PPARγ, peptidylarginine deiminase-3 and caspase-14 proteins

Although multistep processes of profilaggrin expression and degradation into free AA are poorly understood, several regulators including activator protein-1 (AP-1) (Jun and/or Fos)[29] and PPARγ[30] have been reported to enhance the profilaggrin expression during terminal differentiation of the epidermis[5]. Once synthesised, >220 kDa profilaggrin is initially dephosphorylated by phosphatase type 2A and rapidly cleaved to filaggrin (28 kDa) by a variety of proteases including matriptase, protasin and probably kallikrein-5, which then binds to and condenses the keratin cytoskeleton with transglutaminases and PAD, specifically PAD3[5,31]. Ultimately, filaggrin monomers undergo degradation by a variety of proteases including caspase-14, into free AA, the major constituents of NMF[5,31].

Of the various regulators and proteases involved in profilaggrin expression and degradation processes[5,29–31], the protein expressions of PPARγ, PAD3 and caspase-14 are shown in

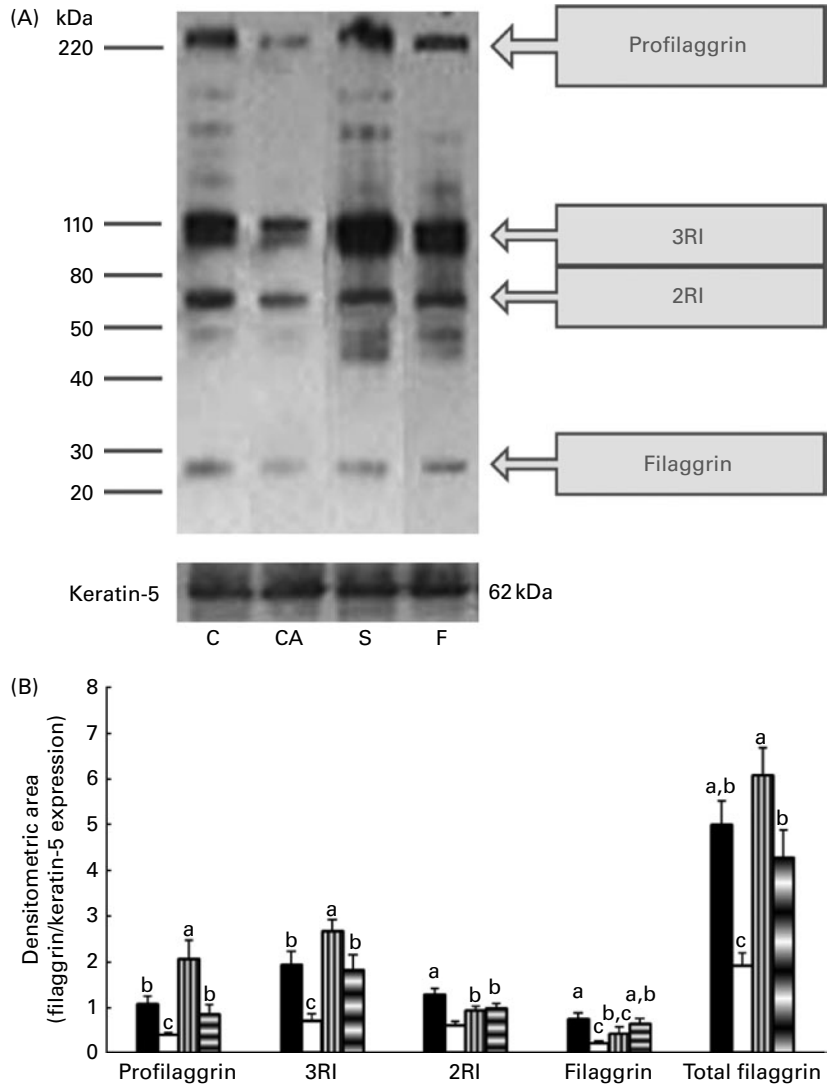

Fig. 3. Altered protein expression of profilaggrin, three-repeat (3RI) or two-repeat intermediates (2RI), and filaggrin in the epidermis of BALB/c mice fed a control diet (group C, ■) and NC/Nga mice fed a control diet (group CA, □) or diets supplemented with 1 % sericin (group S, ◧) or fibroin (group F, ▩) for 10 weeks. Protein extracts (5 μg each) from epidermal homogenates in 50 mM-Tris buffer containing 9 M-urea, 2 % SDS, 2 mM-EDTA and a protease inhibitor cocktail were electrophoretically separated on 4–12 % Bis-Tris gel and immunoblotted with anti-rabbit antibodies against filaggrins, which recognises an epitope within the filaggrin repeat domain and therefore detects profilaggrin, 3RI, 2RI and filaggrin. (A) Representative expressions of profilaggrin, 3RI, 2RI and filaggrin in the epidermis of mice. (B) The signal intensities from multiple experiments of (A) were quantified and the integrated areas were normalised to the corresponding value of keratin-5 (loading control). Values are means (n 10), with standard errors represented by vertical bars. ^{a,b,c} Mean values with unlike letters in each protein expression were significantly different ($P<0{\cdot}05$; one-way ANOVA and Duncan's multiple comparison test).

British Journal of Nutrition

https://doi.org/10.1017/S0007114511007306 Published online by Cambridge University Press

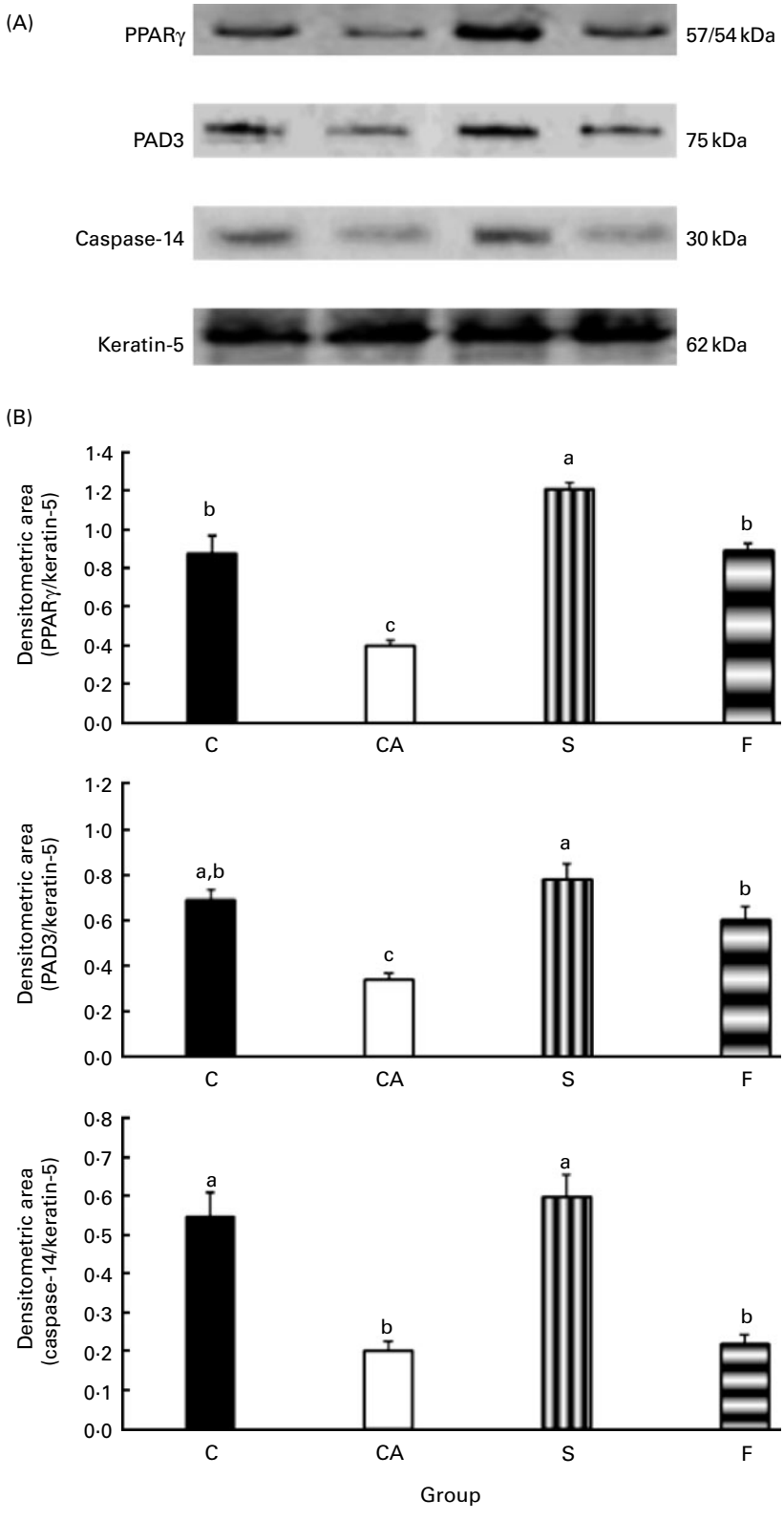

Fig. 4. Altered protein expression of PPARγ, peptidylarginine deiminase 3 (PAD3) and caspase-14 in the epidermis of BALB/c mice fed a control diet (group C) and NC/Nga mice fed a control diet (group CA) or diets supplemented with 1 % sericin (group S) or fibroin (group F) for 10 weeks. Protein extracts were electrophoretically separated on 10 % SDS-PAGE gel and immunoblotted with primary antibodies against PPARγ (57/54 kDa), PAD3 (75 kDa) or caspase-14 (30 kDa). (A) Representative expressions of PPARγ, PAD3 and caspase-14 in the epidermis of mice. (B) The signal intensities from multiple experiments of (A) were quantified and the integrated areas were normalised to the corresponding value of keratin-5 (loading control). Values are means (*n* 8), with standard errors represented by vertical bars. [a,b,c] Mean values with unlike letters in each protein expression were significantly different ($P<0{\cdot}05$; one-way ANOVA and Duncan's multiple comparison test).

British Journal of Nutrition

https://doi.org/10.1017/S0007114511007306 Published online by Cambridge University Press

Fig. 4. The protein expressions of PPARγ, PAD3 and caspase-14 in group CA were significantly less than those in group C. In group S, the expressions of these proteins were higher than those in group CA, and were similar (PAD3 and caspase-14) to or even higher (PPARγ) than those in group C. Although the protein expressions of PPARγ and PAD3 were similar to those in group C, the protein expression of caspase-14 in group F was less than that in group C. Although further studies are required to investigate the dietary modulation of profilaggrin expression and degradation processes with sericin supplements in depth, these results indicate that the increase in PPARγ, PAD3 and caspase-14 may, at least in part, contribute to an initial increase in profilaggrin expressions, and further to the enhanced degradation of the intermediates of filaggrins and filaggrin itself into free AA, ultimately recovering dry skin conditions that were onset with AD induction.

Free amino acid analysis

Comparison of free AA contents in the epidermis of each group is shown in Table 3 and Fig. 5. The total amounts of AA in groups S and F were higher than those in group CA, and were similar to those in group C (Table 3). Further analysis of the individual AA content in group C of BALB/c mice demonstrated that glutamate and serine were most abundant in the epidermis, followed by lysine, glycine, histidine, aspartate and alanine, revealing that glutamate and serine are the most prominent free AA, comprising almost 62·0 and 28·8% of total free AA, respectively, in the epidermis (Table 3 and Fig. 5). Arginine, phenylalanine, valine, cysteine and tyrosine were barely detected, and the combined contents of these free AA were only 0·7% of total free AA in the epidermis. Isoleucine, leucine, methionine, threonine, tryptophan and proline were not detected in the epidermis of group C, similar to the AA profile of filaggrin in BALB/c mice as well as the AA profile of the human epidermis, in which glutamate, serine and glycine are the major AA[4].

In group CA of NC/Nga mice, glutamate was not detected and the serine content was significantly lower than that in group C, which was only at about 25·8% of that in group C (Table 3 and Fig. 5). In contrast, methionine, which was not detected in group C, was abundant and alanine was modestly, but significantly increased in group CA. After dietary supplementation of sericin in NC/Nga mice, serine as well as glycine, arginine and valine in group S were significantly higher than those in group CA. In addition, glutamate, which was not detected in group CA, was modestly increased at about 26·7% of that in group C. Furthermore, methionine, which was highly detected in group CA, was decreased, and alanine was not detected in group S. In group F, serine and glutamate were detected at very low levels, and glycine, arginine and valine were either barely or not detected. Although methionine was not detected, tyrosine, cysteine, phenylalanine and isoleucine, minor free AA in NMF[4], which were either barely or not detected in other groups, were highly detected in group F. These data demonstrated that recovery of serine and glutamate, the major AA in NMF[4], coupled with decreases in methionine, normalised the free AA contents of group S, in parallel with enhancing the epidermal hydration (Fig. 1). Despite no detection of methionine, dietary

Table 3. Free amino acid (AA) contents in the epidermis of mice
(Mean values with their standard errors, *n* 10)

	Experimental groups							
	C		CA		S		F	
AA	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
EAA (nmol/mg epidermal protein)								
Arg	5·6^{b}	1·4	6·8^{b}	2·9	92·4^{a}	20·1	12·0^{b}	5·9
His	22·5^{a}	6·5	33·2^{a}	4·4	49·9^{a}	17·7	20·4^{a}	5·0
Ile	ND		ND		ND		32·2	6·8
Leu	ND		ND		ND		ND	
Lys	57·7^{a}	15·9	37·9^{a}	15·5	43·9^{a}	9·7	33·5^{a}	4·2
Met	ND		230·0^{a}	30·5	89·1^{b}	40·6	ND	
Phe	0·6^{b}	0·2	0·6^{b}	0·1	1·2^{b}	0·3	35·4^{a}	4·4
Thr	ND		ND		ND		ND	
Trp	ND		ND		ND		ND	
Val	1·1^{b}	0·9	2·3^{b}	0·6	19·1^{a}	6·2	ND	
NEAA								
Ala	11·9^{b}	3·3	32·1^{a}	5·9	ND		ND	
Asp	20·1^{a}	4·1	25·8^{a}	6·7	24·7^{a}	3·8	ND	
Cys	1·2^{b}	0·6	ND		0·3^{b}	0·2	682·4^{a}	103·6
Glu	1024·7^{a}	259·8	ND		274·1^{b}	11·0	16·3	6·1^{c}
Gly	27·2^{b}	9·1	30·9^{b}	4·7	85·7^{a}	29·7	ND	
Pro	ND		ND		ND		ND	
Ser	476·2^{b}	150·4	122·8	28·5^{c}	1092·9^{a}	222·8	28·7	4·6^{d}
Tyr	3·3^{b}	1·0	4·7^{b}	0·6	6·6^{b}	1·4	718·8^{a}	88·3
Total AA	1652·1^{a}	453·3	527·1^{b}	100·4	1779·9^{a}	363·6	1579·7^{a}	229·1

C, BALB/c mice fed a control diet; CA, NC/Nga mice fed a control diet; S, NC/Nga mice fed a diet supplemented with 1% of sericin; F, NC/Nga mice fed a diet supplemented with 1% fibroin; EAA, essential AA; NEAA, non-essential AA; ND, not detected.
^{a,b,c} Mean values within a row with unlike superscript letters were significantly different ($P<0·05$; one-way ANOVA and Duncan's multiple comparison test).

British Journal of Nutrition

https://doi.org/10.1017/S0007114511007306 Published online by Cambridge University Press

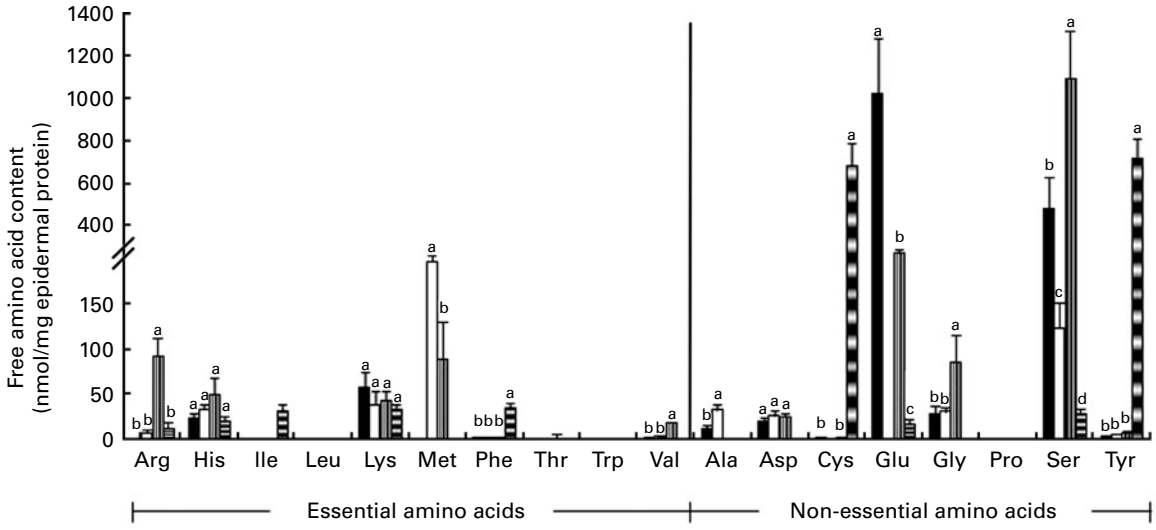

Fig. 5. Comparison of free amino acid contents in the epidermis of BALB/c mice fed a control diet (group C, ■) and NC/Nga mice fed a control diet (group CA, □) or diets supplemented with 1 % sericin (group S, ▥) or fibroin (group F, ▤) for 10 weeks. [a,b,c] Mean values with unlike letters for each amino acid were significantly different ($P < 0{\cdot}05$; one-way ANOVA and Duncan's multiple range comparison test).

supplementation of fibroin did not normalise the free AA contents in the epidermis without the recovery of glutamate and serine, or with abnormal increases of minor free AA in NMF.

Discussion

Although filaggrin is initially synthesised as profilaggrin and degraded into free AA, the major components of NMF[2,3], little is known about the dietary modulation of profilaggrin and filaggrin levels or of free AA levels related to improving the dry skin condition such as AD. The present study indicates that decreased levels of total filaggrins including profilaggrin, 3RI or 2RI of filaggrins, and filaggrin are paralleled by the reduction of epidermal hydration in group CA. The low protein expressions of filaggrins in group CA could be due to either the decreased synthesis of filaggrins or their enhanced degradation into free AA in dry skin conditions. However, although filaggrin degradation is further stimulated in the dry condition (stable in the hydrated condition)[32], the total free AA in group CA were significantly lower than those in group C, which is consistent with the results of previous studies, in which reduced amounts of total free AA are paralleled with the decreased immunostaining intensity or protein expression of filaggrins in the dry epidermis of either AD or ichthyosis patients[6–8]. Therefore, the reduction of epidermal hydration in group CA is more probably due to the decreased expressions of filaggrins.

In the same manner, the recovery of epidermal hydration in group S to a level similar to that of group C could be explained by the increased expressions of filaggrins. In fact, the protein expressions of total filaggrins in group S were significantly higher than those in group CA and even higher (in immunofluorescence analysis) than those in group C. However, further analysis of filaggrin species in group S reveals that despite higher expressions of profilaggrin and 3RI proteins than those in group C, the protein expression of 2RI was lower than that in group C, and the protein expression of filaggrin was further decreased similar to that in group CA. In addition, total amounts of free AA in group S were significantly higher than those in group CA, and were increased to a similar level to that in group C, indicating that dietary sericin initially increases the protein expression of profilaggrins and enhances their degradation into free AA.

Sericin has low digestibility with pepsin and pancreatin due to its protease-resistant property[33]. Undigested sericin functions as a dietary fibre in the intestine, thereby improving constipation[33] or increasing the faecal excretion of cholesterol and TAG, ultimately altering systemic lipid profiles for the anti-hyperlipidaemic effect[34]. In a similar manner, undigested sericin in group S could alter epidermal barrier lipids in which ceramides are composed of a multilayered lamellar structure together with cholesterol and fatty acids[1]. In our previous study[14], the epidermal level of total ceramides, which was significantly decreased in group CA, was significantly increased in group S (1 % sericin and 22 % casein in the diet of group S). However, addition of a low percentage of sericin in casein does not inhibit pepsin and pancreatin digestion[33]. Furthermore, when either peptide (prepared by proteases treatment *in vitro*: molecular weight confirmed to be about 5410[14]) or AA types of sericin are supplemented in the diet as the same percentage of the protein type of sericin (group S), the epidermal level of total ceramides is far less than that in group CA[14]. These data, coupled with previous reports, in which although serum lipid levels were decreased, faecal excretion and digestibility of lipids with 4 % dietary sericin did not differ from that of the control group[35], suggest that the increased levels of ceramides[14] and filaggrins, and/or the enhanced filaggrin degradation into free AA in group S could not be explained solely by the altered systemic lipid profile with the low digestibility of sericin. Alternatively, dietary sericin increases the serum level of adiponectin[35], which indirectly mediates a decrease in hepatic lipogenesis[36], and

British Journal of Nutrition
https://doi.org/10.1017/S0007114511007306 Published online by Cambridge University Press

specifically increases the secretion of IgA from the colon without affecting the caecal profile of the microflora[37]. In our previous study[1,38], dietary sericin inhibited the protein expression of ceramidase, the degradative enzyme of ceramides, which was significantly increased in group CA[14]. Furthermore, in the present study[5], dietary sericin increased the protein expressions of PPARγ, PAD3 and caspase-14, a regulator or proteases involved in filaggrin metabolism, suggesting that ingested sericin or partially digested sericin peptide fragments, of which the AA sequences might be distinct from those prepared by the proteases treatment *in vitro*[14], could indirectly promote systemic responses to alter the protein expression and/or the activity of target enzymes or regulators in filaggrin or ceramide metabolism. The systemic responses of dietary sericin to filaggrin and/or ceramide metabolism could be due to complex mechanisms; these remain to be elucidated in future studies.

In contrast to the beneficial effects of sericin, fibroin, another silk protein examined in the present study, did not recover dry skin conditions despite increased levels of filaggrins and total free AA to levels similar to those found in group C. Compared with high serine content (30·4 % of the total AA) and the resemblance of the AA profiles of sericin to those of NMF[13], glycine (44·4 %) and alanine (29·3 %) are the most prominent AA and serine accounts for only 12·1 % of the total AA in fibroin. Glycine and serine can be converted to each other by a reversible reaction in mammal tissue[39]; dietary supplementation of the silk protein fibroin could possibly provide large quantities of serine, therefore increasing the synthesis of filaggrins and ceramides, the major lipid of the epidermal barrier which is synthesised *de novo* with the enzymatic condensation of serine and palmitoyl-CoA by serine palmitoyl transferase[1,38], to the similar level of group C. However, epidermal levels of total filaggrins as well as total ceramides in group F[14] were lower than those in group S, suggesting that serine supplied by glycine conversion in group F was less efficient for the synthesis of either filaggrin or ceramides in the epidermis than serine directly supplied in group S. Although epidermal levels of total free AA as well as of PPARγ and PAD3 proteins were increased to the similar level of group C and methionine, which is highly detected in group CA, was not detected, epidermal levels of the major AA of NMF such as glutamate and serine were not increased in group F. Notably, tyrosine, phenylalanine, cysteine and isoleucine, minor AA of NMF[4], were highly detected and the protein expression of caspase-14, a protease of filaggrin degradation into free AA, was less than that in group C. These data, coupled with the identification of possible substrates other than filaggrins for transglutaminases, PAD or protease[40,41], suggest that dietary fibroin did not enhance filaggrin degradation into free AA, and/or possible substrates other than filaggrins could become proteolysed by these enzymes, resulting in the abnormal free AA contents and therefore not enough for recovering the dry skin condition in group F despite the increase in total filaggrins and free AA to the similar level of group C.

Although the systemic responses of dietary sericin to filaggrin metabolism, specifically modulation of the activities or expressions of regulators and proteases, require further elucidation in depth, the present *in vivo* study demonstrated a novel observation that dietary supplementation of the silk protein sericin improved epidermal hydration in NC/Nga mice, in parallel with the profilaggrin accumulation and its enhanced degradation into free AA that is coupled with elevated protein expressions of PPARγ, PAD3 and caspase-14, a regulator or proteases in filaggrin metabolism. Sericin may be a potential alternative therapeutic agent, or may serve as an adjunct to conventional therapies for dry skin conditions such as AD.

Acknowledgements

We wish to thank Dr Peter M. Elias in the Dermatology Service and Research Unit, Department of Veterans Affairs Medical Center, University of California-San Francisco, CA, USA for his personal communication and precious advice on epidermal hydration and filaggrin degradation. This study was supported by a grant of the National Research Foundation (2010-0016554), Republic of Korea. H. K. was responsible for the development of the study design, evaluation and the interpretation of the data and statistics, and was involved in the drafting and writing of the manuscript. Y.-J. L. and J.-H. P. were responsible for the immunofluorescence analysis. Y. C. was responsible for the supervision of the analytical work, writing and final approval of the manuscript. All authors read and approved the final manuscript. None of the authors has any conflicts of interest.

References

1. Rawlings AV & Harding CR (2004) Moisturization and skin barrier function. *Dermatol Ther* **17**, Suppl. 1, 43–48.
2. Harding CR & Scott IR (2002) Stratum corneum moisturizing factors. In *Skin Moisturization*, pp. 61–80 [JJ Leyden and AV Rawlings, editors]. New York: Marcel Dekker.
3. Harding CR & Scott IR (1983) Histidine-rich proteins (filaggrins): structural and functional heterogeneity during epidermal differentiation. *J Mol Biol* **170**, 651–673.
4. Scott IR, Harding CR & Barrett JG (1982) Histidine-rich protein of the keratohyalin granules. Source of the free amino acids, urocanic acid and pyrrolidone carboxylic acid in the stratum corneum. *Biochim Biophys Acta* **719**, 110–117.
5. Sandilands A, Sutherland C, Irvine AD, *et al.* (2009) Filaggrin in the frontline: role in skin barrier function and disease. *J Cell Sci* **122**, 1285–1294.
6. Howell MD, Kim BE, Gao P, *et al.* (2007) Cytokine modulation of atopic dermatitis filaggrin skin expression. *J Allergy Clin Immunol* **120**, 150–155.
7. Takahashi M & Ikezawa Z (2005) Dry skin in AD and patients on hemodialysis. In *Dry Skin and Moisturizers: Chemistry and Function*, pp. 95–106 [M Lodén and HI Maiback, editors]. Boca Raton, FL: CRC Press.
8. Seguchi T, Cui CY, Kusuda S, *et al.* (1996) Decreased expression of filaggrin in atopic skin. *Arch Dermatol Res* **288**, 442–446.
9. Padamwar MN, Pawar AP, Daithankar AV, *et al.* (2005) Silk sericin as a moisturizer: an *in vivo* study. *J Cosmet Dermatol* **4**, 250–257.
10. Voegeli R, Meier J & Blust R (1993) Sericin silk protein: unique structure and properties. *Cosmet Toilet* **108**, 101–108.

British Journal of Nutrition

https://doi.org/10.1017/S0007114511007306 Published online by Cambridge University Press

11. Inouye K, Kurokawa M, Nishikawa S, *et al.* (1998) Use of *Bombyx mori* silk fibroin as a substratum for cultivation of animal cells. *J Biochem Biophys Methods* **37**, 159–164.
12. Mason R (2008) Fabrics for atopic dermatitis. *J Fam Health Care* **18**, 63–65.
13. Kato N, Sato S, Yamanaka A, *et al.* (1998) Silk protein, sericin, inhibits lipid peroxidation and tyrosinase activity. *Biosci Biotechnol Biochem* **62**, 145–147.
14. Park KH, Choi YS, Kim HA, *et al.* (2007) Dietary effect of silk protein on ceramide synthesis and the expression of ceramide metabolic enzymes in the epidermis of NC/Nga mice. *J Korean Soc Food Sci Nutr* **36**, 554–562.
15. Aioi A, Tonogaito H, Suto H, *et al.* (1993) Impairment of skin barrier function in NC/Nga Tnd mice as a possible model for atopic dermatitis. *Br J Dermatol* **144**, 12–18.
16. Suto H, Matsuda H, Mitsuishi K, *et al.* (1999) NC/Nga mice: a mouse model for atopic dermatitis. *Int Arch Allergy Immunol* **120**, Suppl. 1, 70–75.
17. Madyarov S, Lee KG, Yeo JH, *et al.* (1999) Improved method for the preparation of silk fibroin hydrolysates. *Korean J Seric Sci* **41**, 108–115.
18. Heish M, Imai Y, Katayama H, *et al.* (2003) Gene expression analysis of atopic dermatitis-like skin lesions induced in NC/Nga mice by mite antigen stimulation under specific pathogen-free conditions. *Int Arch Allergy Immunol* **132**, 355–363.
19. Presland RB, Boggess D, Lewis SP, *et al.* (2000) Loss of normal profilaggrin and filaggrin in flaky tail (ft/ft) mice: an animal model for the filaggrin-deficient skin disease ichthyosis vulgaris. *J Invest Dermatol* **115**, 1072–1081.
20. Kang TH, Park HM, Kim YB, *et al.* (2009) Effects of red ginseng extract on UVB irradiation-induced skin aging in hairless mice. *J Ethnopharmacol* **123**, 446–451.
21. Kam E, Resing KA, Lim SK, *et al.* (1993) Identification of rat epidermal profilaggrin phosphatase as a member of the protein phosphatase 2A family. *J Cell Sci* **106**, 219–226.
22. Ginger RS, Blachford S, Rowland J, *et al.* (2005) Filaggrin repeat number polymorphism is associated with a dry skin phenotype. *Arch Dermatol Res* **297**, 235–241.
23. Maciejewski-Lenoir D, Richman JG, Hakak Y, *et al.* (2006) Langerhans cells release prostaglandin D_2 in response to nicotinic acid. *J Invest Dermatol* **126**, 2637–2646.
24. Kim HA, Oh I, Park KH, *et al.* (2009) Stimulatory effect of dietary red ginseng on epidermal hydration and ceramide levels in ultraviolet-irradiated hairless mice. *J Med Food* **12**, 746–754.
25. Lowry OH, Rosebrough NJ, Farr AL, *et al.* (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**, 265–275.
26. Park TS, Chang JS & Sung MK (1998) Plasma aminogram and urinary excretion of free amino acids in adult vegetarians compared with age-matched omnivores in Korea. *J Food Sci Nutr* **3**, 368–373.
27. Moore S & Stein WH (1963) Chromatographic determination of amino acids by the use of automatic recording equipment. In *Methods in Enzymology*, vol. 6, p. 819 [SP Colowick and NO Kaplan, editors]. New York: Academic Press.
28. Norusis MJ (2004) ANOVA method. In *SPSS 13.0 Procedures Companion*, pp. 179–181. New Jersey: Prentice Hall, Inc.
29. Jang SI, Steinert PM & Markova NG (1996) Activation protein 1 activity is involved in the regulation of the cell type-specific expression from the proximal promoter of the human profilaggrin gene. *J Biol Chem* **271**, 24105–24114.
30. Mao-Qiang M, Fowler AJ, Schmuth M, *et al.* (2004) Peroxisome-proliferator-activated receptor (PPAR)-gamma activation stimulates keratinocyte differentiation. *J Invest Dermatol* **123**, 305–312.
31. Prealand RB, Joseph A & Lawrence OT (2006) Profilaggrin and the fused S100 family of calcium-binding proteins. In *Skin Barrier*, pp. 111–140 [PM Elias and KR Feingold, editors]. New York: Taylor & Francis Group.
32. Scott IR & Harding CR (1986) Filaggrin breakdown to water binding compounds during development of the rat stratum corneum is controlled by the water activity of the environment. *Dev Biol* **115**, 84–92.
33. Sasaki M, Yamada H & Kato N (2000) A resistant protein, sericin improves atropine-induced constipation in rats. *Food Sci Technol Res* **6**, 280–283.
34. Seo CW, Um IC, Rico CW, *et al.* (2011) Antihyperlipidemic and body fat-lowering effects of silk proteins with different fibroin/sericin compositions in mice fed with high fat diet. *J Agric Food Chem* **59**, 4192–4197.
35. Okazaki Y, Kakehi S, Xu Y, *et al.* (2010) Consumption of sericin reduces serum lipids, ameliorates glucose tolerance and elevates serum adiponectin in rats fed a high-fat diet. *Biosci Biotechnol Biochem* **74**, 1534–1538.
36. Awazawa M, Ueki K, Inabe K, *et al.* (2009) Adiponectin suppresses hepatic SREBP1c expression in an AdipoR1/LBKB1/AMPK dependent pathway. *Biochem Biophys Res Commun* **382**, 51–56.
37. Okazaki Y, Tomotake H, Tsujimoto K, *et al.* (2011) Consumption of a resistant protein, sericin, elevates fecal immunoglobulin A, mucins and cecal organic acids in rats fed a high-fat diet. *J Nutr* **141**, 1975–1981.
38. Holleran WM, Takagi Y & Uchida Y (2006) Epidermal sphingolipid: metabolism, function, and roles in skin disorders. *FEBS Lett* **580**, 5456–5466.
39. Fallon HJ, Davis JL & Goyer RA (1968) Effect of protein intake on tissue amino acid levels and the enzymes of serine biosynthesis in the rat. *J Nutr* **96**, 220–226.
40. Tarcsa E, Marekov LN, Mei G, *et al.* (1996) Protein unfolding by peptidylarginine deiminase. Substrate specificity and structural relationships of the natural substrates trichohyalin and filaggrin. *J Biol Chem* **271**, 30709–30716.
41. Fischer H, Stichenwirth M, Dockal M, *et al.* (2004) Stratum corneum-derived caspase-14 is catalytically active. *FEBS Lett* **577**, 446–450.

British Journal of Nutrition

https://doi.org/10.1017/S0007114511007306 Published online by Cambridge University Press