Biodegradation of exogenously applied silk peptides by stratum corneum cysteine proteases

Omoi, Miyuki[1]*; Arai, Yasuhiro[1]; Inamura, Kunihiko[1]; Yamamoto, Asuka[2]; Kawasaki, Tomomi[2]; Hirao, Tetsuji[2]

[1] R&D Center, IKEDA CORPORATION, Yokohama, Japan; [2] Graduate School of Pharmaceutical Sciences, Mukogawa Women's University, Nishinomiya, Japan

*Miyuki Omoi, IKEDA CORPORATION, 12F MM Park Building, 3-6-3 Minatomirai, Nishi-ku, Yokohama 220-0012, Japan, Tel.: +81-45-228-2880, Email: m.omoi@ikeda-corp.co.jp

ABSTRACT

Background

Various peptides are included in cosmetics. However, no study has reported the action of stratum corneum (SC) proteases on exogenous peptides applied to the SC. We hypothesized that exogenous peptides can be hydrolyzed into amino acids by SC proteases and that these amino acids may serve as natural moisturizing factors (NMFs). In this study, we aimed to clarify whether peptides can be hydrolyzed by SC proteases and determine the characteristics of these proteases involved in peptide degradation.

Methods

Samples of the outermost SC were collected non-invasively from healthy volunteers by stripping with adhesive tape, and were immersed in silk peptide solution at 37 ℃ for 48 h. Degradation of peptides was monitored by measuring the increase of amino groups quantified by the ninhydrin method.

Results

The incubation of peptides with tape-stripped SC resulted in an increase in the quantity of amino groups. The pH dependence and protease inhibitor profiles of the peptide degradation by SC proteases showed the involvement of cysteine proteases that function optimally at neutral pH. As we also detected the activity of aminopeptidase, it was suggested that bleomycin hydrolase (BH) is involved in the degradation of peptides by SC proteases. These results suggest that exogenously applied peptides are degraded by multiple SC proteases.

Conclusion

We found that exogenously applied silk peptides are degraded by cysteine proteases such as BH. This concept of peptide degradation upon application to the SC provides a novel approach to skin moisturization.

KEYWORDS
stratum corneum; cysteine protease; silk peptides; amino acids; biodegradation; bleomycin hydrolase

INTRODUCTION

The stratum corneum (SC), the outermost layer of the skin, is responsible for barrier and water-retaining functions, and determines skin appearance. The barrier function mainly controls the entry of foreign hazards from the outside and loss of water from the inside, protecting the body. In addition, the water-retaining function keeps the SC itself moisturized and soft. The SC consists of intercellular lipids and flat, dead corneocytes, which adhere to each other via desmosomes and intercellular lipids. Moreover, the corneocytes are surrounded by a structure known as the cornified envelope, and they are filled with keratin filaments and natural moisturizing factors (NMFs).

NMFs consist of water-soluble small molecules, including amino acids and their derivatives, organic acids, and mineral salts, which make a major contribution to moisturization of the SC. Amino acids, as major constituents of NMFs, are produced by the degradation of filaggrin, which is expressed as a precursor protein, profilaggrin, in the granular layer. Profilaggrin is degraded into filaggrin in the process of transition to SC. Furthermore, filaggrin is degraded into numerous amino acids by various SC proteases.

Previous studies revealed that filaggrin is degraded by proteases such as caspase 14, calpain I, and then finally by bleomycin hydrolase (BH), an aminopeptidase member of the cysteine protease family [1] that is involved in degrading filaggrin-derived peptides into free amino acids. Interestingly, it has been reported that BH expression is reduced in the epidermis of atopic dermatitis cases, suggesting that BH plays an important role in moisturizing the skin [2].

In addition, various proteases in the SC are also involved in homeostasis of the skin [3]. It is well known that corneodesmosomes are degraded by trypsin-like proteases such as kallikrein (KLK)5 and chymotrypsin-like proteases such as KLK7 in the exfoliation of the SC [4]. It has been reported that cathepsin D, an aspartic protease, is also involved in the degradation of corneodesmosomes [5]. Thus, although SC is a biologically dead tissue, various proteases in the SC are biologically active and maintain homeostasis of the healthy SC by tactically controlling their activities.

Various peptides are ingrediated in skincare and haircare cosmetics for the purposes of adhesion, repair, and moisturization of the skin and hair. It is supposed that these peptides may also be degraded by SC proteases, but no study has reported the changes of peptides

exogenously supplied (exogenous peptides) from outside of the skin. Therefore, we hypothesized that exogenous peptides would be hydrolyzed to amino acids by various proteases in the SC. We thus established the present study to reveal the degradation of exogenous peptides by SC proteases and to clarify the characteristics of such proteases involved in this degradation.

MATERIALS AND METHODS

Materials

The following materials were used in this study. Silk peptides (SILKPRO® F, Hydrolyzed Silk, average molecular weight 500 Da) and Hydrolyzed Soy Protein (hSP, TOFUPRO® U, average molecular weight 700 Da) were obtained from IKEDA CORPORATION, Japan. Hydrolyzed Conchiolin Protein (hCP) and Hydrolyzed Rice Protein (hRP) were obtained from Seiwa Kasei Co., Ltd., Japan. The amino acid composition and profile of these peptides are shown in Table 1. Protein Assay BCA kit and dithiothreitol (DTT) were purchased from Nacalai Tesque, Inc., Japan. Ninhydrin, iodoacetic acid, and disodium dihydrogen ethylenediaminetetraacetate dihydrate (EDTA) were purchased from Kanto Chemical Co., Inc., Japan. 1-(Transeposuccinyl-1-leucylamide)-4-guanidinobutane (E-64), leupeptin, pepstatin, L-arginine-4-methylcoumaryl-7-amide (Arg-MCA), and 7-amino-4-methylcoumarin (AMC) were purchased from Peptide Institute Inc., Japan. N-Ethylmaleimide (NEM) was purchased from Tokyo Chemical Industry Co., Ltd., Japan. 4-(2-Aminoethyl)benzenesulfonyl fluoride (AEBSF) was purchased from Funakoshi Co., Ltd., Japan. All reagents were of analytical grade unless otherwise stated.

Table 1 Amino acid composition and profile of peptides.

INCI name			Hydrolyzed Silk (silk peptides)	Hydrolyzed Soy Protein (hSP)	Hydrolyzed Conchiolin Protein (hCP)	Hydrolyzed Rice Protein (hRP)
Concentration (%)			10	20	3	25
Average molecular weight			500	700	600	400
Amino acid		Molar mass (g/mol)	Amino acid profile (mol%)			
Gly	Glycine	75.07	49.9	8.9	35.8	10.8
Ala	Alanine	89.09	33.8	5.8	13.4	10.3
Ser	Serine	105.09	6.6	5.4	5.7	6.2
Val	Valine	117.15	2.4	5.1	4.6	6.1
Glu	Glutamic acid	147.13	1.5	22.8	3.5	17.2
Asp	Aspartic acid	133.11	1.5	13.3	8.2	9.3
Phe	Phenylalanine	165.19	0.7	4.2	2.6	5.1
Thr	Threonine	119.12	0.6	1.3	1.6	2.1
Tyr	Tyrosine	181.19	0.5	1.3	2.3	2.9
Pro	Proline	115.13	0.5	5.9	4.1	6.8
Ile	Isoleucine	131.17	0.5	4.4	2.9	2.6
Leu	Leucine	131.17	0.4	8.4	7.1	8.7
His	Histidine	155.15	0.4	1.9	0.8	1.7
Arg	Arginine	174.20	0.4	3.6	3.2	2.3
Met	Methionine	149.21	0.1	0.9	1.4	2.8
Lys	Lysine	146.19	0.1	5.3	2.1	1.9
CySo3H	Cysteine acid	169.16	0.1	0.6	0.2	2.5
1/2Cys	1/2 Cystine		0.1	0.2	0.5	0.7

Collection of SC by tape-stripping

Healthy Japanese volunteers (4 females and 2 males) from whom we obtained informed consent were enrolled in this study. Samples of the outermost SC were collected non-invasively from the cheek by stripping with adhesive tape (2.5 × 2.5 cm; Kakushitsu Checker AST-01; Asch Japan Co., Ltd., Tokyo, Japan). The research protocol was approved by the ethics committee of Mukogawa Women's University.

Biodegradation of silk peptides by SC proteases

Degradation of silk peptides by SC proteases was monitored by measuring the increase of amino groups in silk peptide solution upon incubation with the tape-stripped SC. Briefly, adhesive tape with SC on the surface (tape-stripped SC) was immersed in the reaction solution consisting of 50 mmol/L sodium phosphate buffer (pH 7.0) with silk peptides and

left at 37 ℃ for 48 h. After incubation, the amino groups contained in the reaction mixture were quantified by the ninhydrin method, in accordance with the protocol reported by Abernathy et al. [6]. Briefly, 20 μL of each sample was added to 400 μL of ninhydrin reagent, consisting of 2% ninhydrin–0.025% $SnCl_2$–75% ethylene glycol in 1 mol/L Na-acetate buffer (pH 5.5), followed by incubation at 104 ℃ for 10 min. Absorbance at 570 nm was then measured with a microplate reader (MTP-900 Lab; Corona Electric, Japan) to quantify the amino groups, with calibration being performed using glycine as a standard. The increase of amino groups upon incubation with SC was considered to reflect degradation by SC proteases, which was corrected by the quantified protein content of the tape-stripped SC as described in the following section.

Determination of the SC protein content by bicinchoninic acid method

SC protein was solubilized by incubation with 0.1 mol/L NaOH–1% SDS solution at 60 ℃ for 2 h, followed by neutralization with 2 mol/L HCl [7]. Protein content in the sample was determined using the protein assay BCA kit with bovine serum albumin (Sigma-Aldrich) as a standard protein.

Determination of optimum pH

The optimum pH for SC protease activity was determined using silk peptides as a substrate in buffer solutions at various pH: 50 mmol/L sodium acetate buffer (pH 4.0–6.5), 50 mmol/L sodium phosphate buffer (pH 5.5–8.5), and 50 mmol/L sodium borate buffer (pH 8.0–9.0).

Detection of aminopeptidase activity in the SC

The tape-stripped SC (1.25 × 1.25 cm) was immersed in 1000 μL of the reaction mixture consisting of 0.4 mmol/L Arg-MCA–50 mmol/L sodium phosphate buffer (pH 7.0)–10 mM DTT, and incubated at 37 ℃ for 24 h. Then, fluorescence of the reaction mixture was measured with a microplate reader at Ex 340/70 nm and Em 450/12 nm to quantify the amount of AMC liberated by the degradation of Arg-MCA.

RESULTS

Degradation of exogenous peptides by SC proteases

The degradation of exogenously applied silk peptides by SC proteases was examined. The number of amino groups was successfully increased upon the incubation of silk peptide solution with a tape-stripped SC in a manner dependent on the incubation time (Fig. 1). This increase was dependent on the substrate (silk peptides) concentration (Fig. 2) and also on the SC dose (the area of tape-stripped SC) (Fig. 3).

The number of amino groups was not increased in the case of incubation with heat-treated (100 ℃ for 10 min) SC (Fig. 4), suggesting that the proteases had been inactivated. These results clearly showed that the incubation of exogenously applied silk peptides with SC resulted in enzymatic degradation by SC proteases.

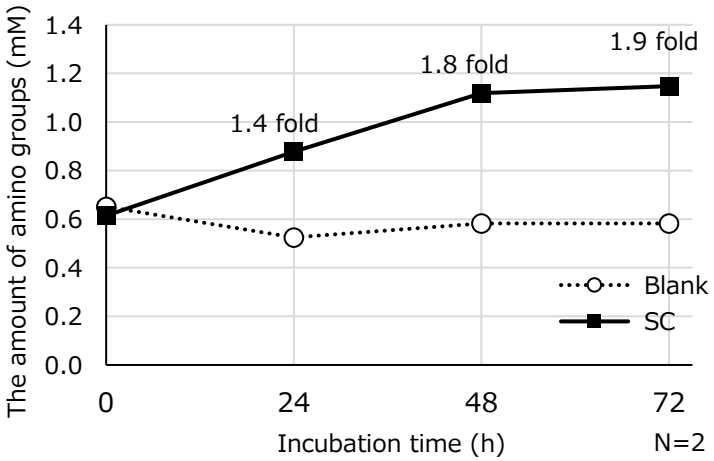

Fig. 1 Time-dependent increase of amino groups upon incubation of silk peptides with SC.
Silk peptides (0.03%) were incubated with tape-stripped SC at 37 ℃ for different times. The level of amino groups in the reaction mixture was determined by the ninhydrin method. Data are expressed as the mean of duplicate experiments. The fold increases of amino groups at each period against that at 0 h are shown.

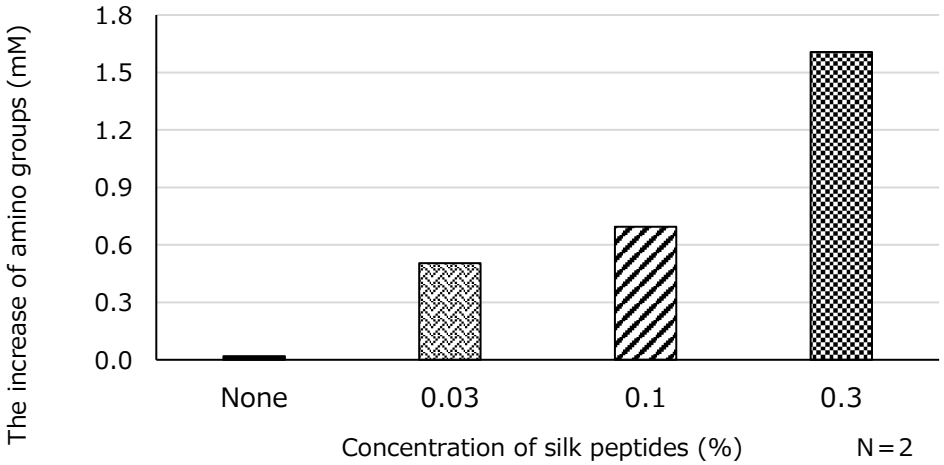

Fig. 2 Effect of silk peptide concentration on degradation by SC.
Various concentrations of silk peptides were incubated with tape-stripped SC at 37 ℃ for 48 h. The level of amino groups in the reaction mixture was determined by the ninhydrin method. The increase of amino groups (mM) upon incubation is shown as the mean of duplicate experiments.

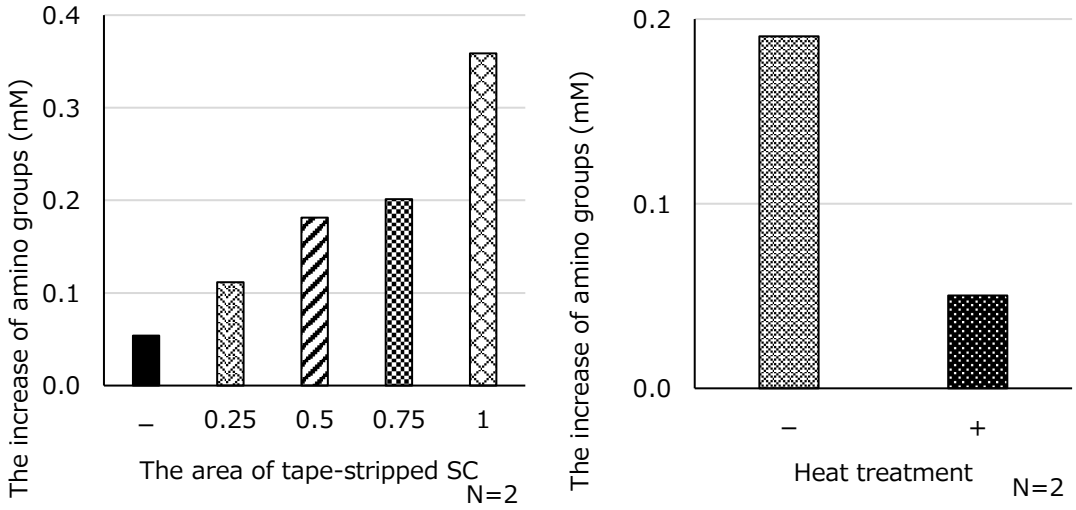

Fig. 3 Effect of the SC area on silk peptide degradation.
Silk peptides (0.03%) were incubated with various sizes of tape-stripped SC at 37 ℃ for 48 h. The level of amino groups in the reaction mixture was determined by the ninhydrin method. The increase of amino groups (mM) upon incubation is shown as the mean of duplicate experiments. The area of "1" corresponds to 6.25 cm^2.

Fig. 4 Effect of heat treatment of the SC on silk peptide degradation.
Tape-stripped SC was treated at 100 ℃ for 10 min, followed by incubation with silk peptides (0.03%) at 37 ℃ for 48 h. The level of amino groups in the reaction mixture was determined by the ninhydrin method. The increase of amino groups (mM) upon incubation is shown as the mean of duplicate experiments.

Determination of optimum pH

To clarify the effect of pH on the degradation of silk peptides by SC proteases, tape-stripped SC was incubated with silk peptide solutions in a buffer with various pH. Fig. 5A shows that the degradation of silk peptides by SC proteases was effectively promoted at pH 7–8, while SC proteases also functioned at other pH between 4 and 9. We confirmed that silk peptides are preferentially degraded by SC proteases at pH 7.0, compared with degradation at pH 5.5, using SCs collected from several donors (Fig. 5B).

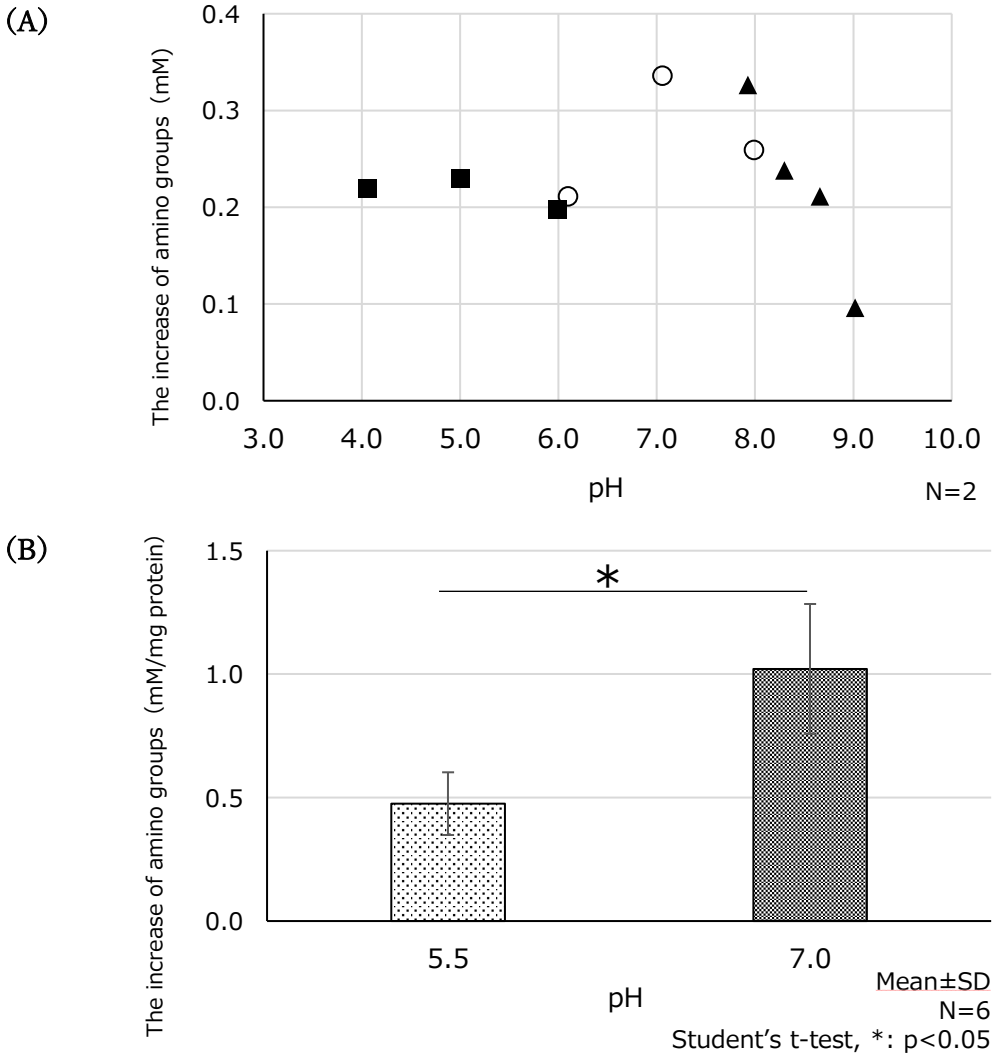

Fig. 5 The pH dependence of silk peptide degradation by SC.
(A) Silk peptides (0.03%) were incubated with tape-stripped SC at 37 ℃ for 48 h with Na-acetate (■), Na-phosphate (○), and Na-borate (▲) buffers at various pH. The level of amino groups in the reaction mixture was determined by the ninhydrin method. The increase of amino groups (mM) upon incubation is shown as the mean of duplicate experiments.
(B) Silk peptides (0.03%) were incubated with tape-stripped SCs from various donors at 37 ℃ for 48 h at pH 5.5 or 7.0. Data are expressed as the mean ± SD (N=6). Higher SC protease activity was observed at pH 7.0 than at pH 5.5.

Effect of inhibitors and reducing agent DTT

Table 2 summarizes the effects of various inhibitors on SC protease activity. E-64 and leupeptin strongly inhibited SC protease activity at pH 7.0, while iodoacetic acid inhibited SC protease activity at pH 5.5, but NEM, pepstatin, AEBSF, and EDTA had little or no effect.

Additionally, the effect of DTT on the degradation of silk peptides by SC proteases was investigated, as DTT is known as a reducing agent for a thiol residue in the active site of cysteine proteases [8]. As a consequence, the addition of DTT to the reaction solution of silk peptides and SC proteases promoted the degradation of silk peptides (Table 2), suggesting that DTT leads to the activation of cysteine proteases involved in degrading silk peptides.

Table 2　Effect of protease inhibitors and reducing agent.

	Substance	Concentration	Relative activity (%)	
			pH 5.5	pH 7.0
Inhibitor	None		100	100
	E-64	160 μM	40	23
	Leupeptin	120 μM	55	20
	Pepstatin	120 μM	97	81
	AEBSF	1 mM	112	132
	NEM	1 mM	75	72
	Iodoacetic acid	1 mM	16	50
	EDTA	1 mM	105	120
Reducing agent	DTT	10 mM	255	159

Silk peptides (0.03%) were incubated with tape-stripped SC at 37 ℃ for 48 h in 50 mM Na-acetate buffer (pH 5.5) or Na-phosphate buffer (pH 7.0) in the presence of inhibitors or a reducing agent. The level of amino groups in the reaction mixture was determined by the ninhydrin method. The increase of amino groups (mM/mg protein) upon incubation was calculated. Data are expressed as activity relative to the group without inhibitors.

Detection of aminopeptidase activity in the SC

To investigate proteases involved in the degradation of silk peptides, aminopeptidase activity in the SC was examined using a fluorogenic substrate, Arg-MCA. Tape-stripped SC exhibited aminopeptidase activity against Arg-MCA, observed as the release of free AMC, while no degradation was observed for the adhesive tape without SC. This aminopeptidase activity in the SC was most active at pH 7.0 (Fig. 6). The release of free AMC by SC aminopeptidase activity was observed in both incubation time-dependent and SC area-dependent manners (data not shown). The linear Lineweaver–Burk plot of this reaction confirmed the presence of an aminopeptidase in the SC (data not shown).

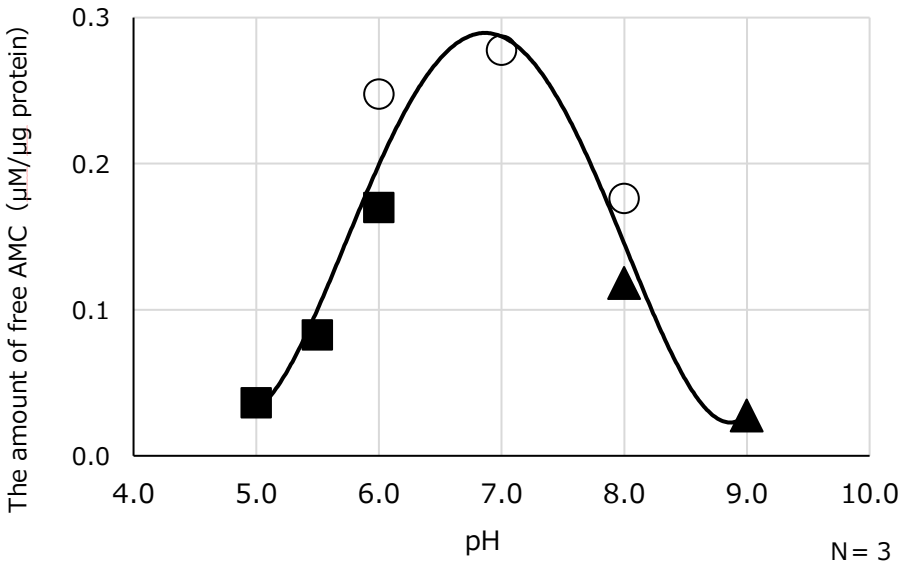

Fig. 6 The pH dependence of SC aminopeptidase activity.
Tape-stripped SC was incubated with 0.4 mM Arg-MCA in 50 mM Na-acetate buffer (■) , Na-phosphate buffer (○), or Na-borate buffer (▲) at 37 ℃ for 6 h. The amount of free AMC (μM/μg protein) was determined and expressed as the mean of triplicate experiments.

Degradation of other peptides by SC proteases

Degradation of other peptides than silk peptides by SC proteases was also examined in the same way by measuring the increase of amino groups upon incubation with the tape-stripped SC. As shown in Fig. 7, hSP, hCP, and hRP were degraded by SC proteases. Notably, hCP was degraded by SC proteases at pH 5.5, but hardly degraded at pH 7.0.

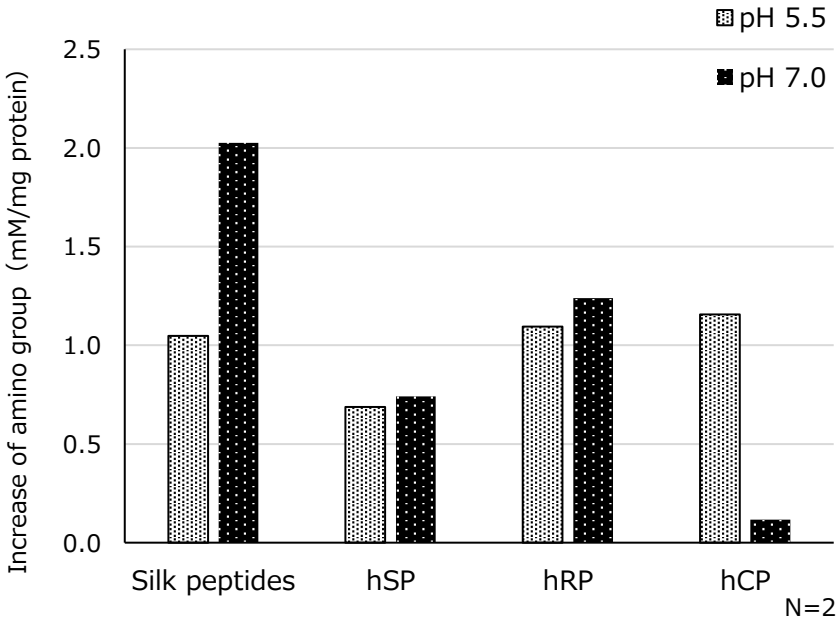

Fig. 7 Degradation of various peptide ingredients by SC proteases.
Each cosmetic ingredient was adjusted to approximately 0.03% and incubated with tape-stripped SC at 37 ℃ for 48 h. The level of amino groups in the reaction mixture was determined by the ninhydrin method. The increase of amino groups (mM/mg protein) upon incubation is shown as the mean of duplicate experiments.

DISCUSSION

In the present study, we elucidated that exogenous peptides applied cosmetically onto the skin can be degraded by SC proteases, including cysteine proteases such as BH, using non-invasively collected SC. The results suggested that SC proteases degrade not only endogenous proteins but also exogenous peptides into amino acids, which can function as moisturizing factors supporting NMFs (Fig. 8).

Biochemical characterization of silk peptide degradation by SC, including a time-course experiment (Fig. 1), dependence on substrate dose (Fig. 2), dependence on SC dose (Fig. 3),

and heat inactivation (Fig. 4), clearly showed the involvement of certain proteases in the degradation. Further experiments on pH dependence (Fig. 5A) and the inhibitor profile (Table 2) strongly suggested the involvement of BH in the SC. Actually, we confirmed the presence of aminopeptidase activity in the SC using a fluorogenic substrate, Arg-MCA (Fig. 6).

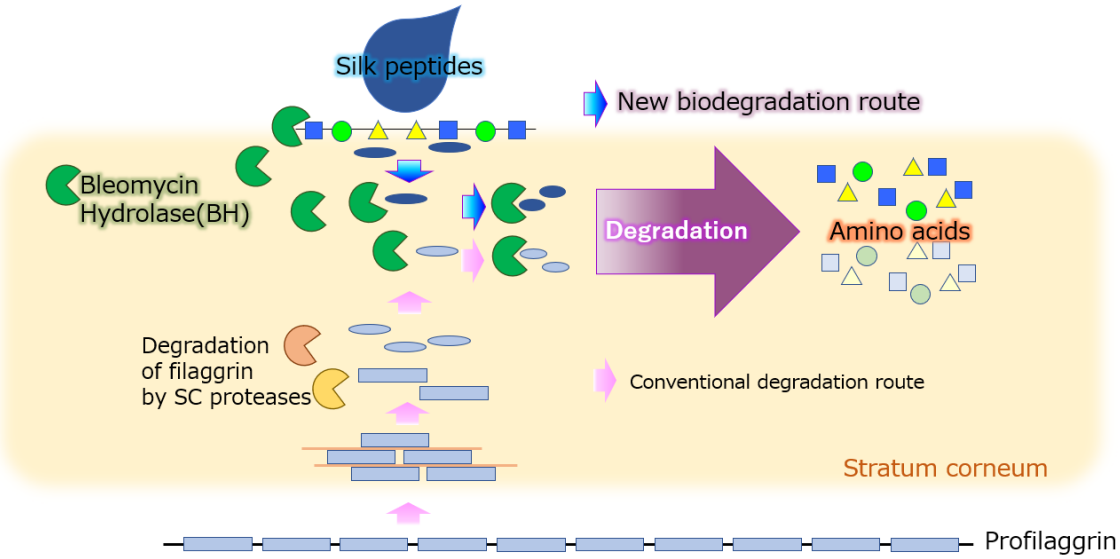

Fig. 8 Biodegradation of exogenously applied silk peptides.

Takeda et al. [9] reported the purification and characterization of BH from rat skin. The results of pH dependence and the protease inhibitor profile in this study are almost match those obtained in their study, although there are some discrepancies. The first discrepancy involves pH dependence. BH from rat skin exhibits maximum activity at pH 7–8, but no activity at pH under 6 [9]. However, silk peptide degradation preferentially occurred at pH 7–8, while the degradation was observed at pH 4–6 (Fig. 5A), suggesting the involvement of protease(s) other than BH in the silk peptide degradation by the SC. The second discrepancy involves the protease inhibitor profile. Among the inhibitors tested, E-64, leupeptin, and iodoacetic acid efficiently inhibited silk peptide degradation (Table 2). This profile was in agreement with that of BH activity [9]. However, NEM exhibited less inhibitory activity against silk peptide degradation (Table 2) compared with inhibition against BH activity [9]. This discrepancy might have arisen from a difference in the purity of the enzyme. Takeda et al. [9] carried out biochemical characterization using BH purified from rat skin, while we performed experiments using intact SC collected from human skin, which included a massive

amount of keratin fibers containing thiol groups, so the NEM did not effectively inhibit silk peptide degradation. As mentioned above, we can conclude that BH is involved, at least in part, in the silk peptide degradation by the SC. Interestingly, silk peptide degradation was accelerated by the addition of a reducing agent, DTT (Table 2), because BH is a member of the thiol proteases. This led us to assume that oxidative stress on the skin could be a factor inhibiting BH activity to generate NMFs. Therefore, oxidative stress might be a factor suppressing silk peptide degradation by the SC. Further studies are necessary to clarify this.

As shown in Table 1, silk peptide consists of high levels (over 80%) of Gly and Ala residues, but very few basic and aromatic amino acid residues, which are recognized by a trypsin-like enzyme, KLK5, and a chymotrypsin-like enzyme, KLK7, respectively. Therefore, it was suggested that neither KLK5 nor KLK7 is a major candidate for a silk peptide-degrading protease. Meanwhile, various peptides other than silk peptides could also be degraded by SC proteases (Fig. 7), although the pH preference varied among the peptides. In such cases, various proteases including KLKs and cathepsin D could be involved in degradation of the peptides, depending on their amino acid sequence as well as the molecular size of peptides. Although further studies are necessary to clarify the class of proteases engaged in peptide degradation, it is clear that exogenously applied peptides can be degraded by SC proteases.

Adhesive tape-stripped SC samples collected non-invasively provide valuable information reflecting the condition of the skin, and are useful for understanding events occurring at the skin surface. Using this technique, we successfully revealed that exogenous peptides can be degraded by SC proteases including BH. However, it is necessary to accumulate data from a number of individuals for more reliable evidence. Additionally, because this experimental model is a simple *ex vivo* system using a tape-stripped SC, it is also necessary to investigate the efficacy of cosmetic ingredients, including silk peptides, by *in vivo* clinical tests. Nevertheless, we hope that SC proteases will be helpful for developing cosmetics that mitigate dry skin by the degradation of exogenously applied peptides.

CONCLUSION

We found that exogenously applied silk peptides are degraded by cysteine proteases such as BH, which is known to degrade filaggrin peptides into amino acids. This suggests that amino acids of NMFs can be supplemented not only endogenously within the skin but also exogenously. This concept of peptide degradation upon application to the SC provides a novel approach to skin moisturization.

CONFLICT OF INTEREST STATEMENT

NONE.

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