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Original article

Reduction effect of oxidized protein hydrolase (OPH) on advanced glycation end products and OPH-like activity in human stratum corneum.

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Abstract

***Purpose*:** Accumulation of advanced glycation end products (AGEs) *in vivo*, which is caused by glycative stress, induces the browning reaction of proteins and hardening of proteins by crosslinking, which consequently affects the deterioration of biological functions. Accumulation of AGEs in skin is involved in the decline of elasticity and texture of skin. Thus, this could be a trigger factor for visual impressions of the aging process. This study focused on oxidized protein hydrolase, OPH. OPH, which is one kind of serine protease, exists in biological tissues of a variety of animals. OPH is recognized as acylamino acid releasing enzyme (AARE), which isolates *N*-terminal acylamino-acid of proteins. OPH degrades oxidized and glycated proteins and is involved in the proteolysis of blood carbonyl modifying protein in diabetic model rats. Furthermore, OPH contributes to the proteolysis and reduction of aged proteins in cooperation with proteasome. However, OPH effects on AGEs have not been verified. This study explored the existence and non-existence of activity of OPH in stratum corneum and to verify the effects of OPH on AGEs, focusing on age-related glycation of skin tissue and defensive mechanisms towards glycative stress.

***Methods and results*:** OPH-like activity was recognized in stratum corneum extract of cheek and inside upper arm. As a result of gel filtration chromatography analysis, where an extracted solution of cheek stratum corneum and OPH were fractionated, OPH-like activity was recognized in the same elution fraction. When Advanced Glycation End Product-modified Human Serum Albumin (AGE-HSA) was used as substrate and OPH was reacted, OPH decreased the amount of fluorescent AGEs in AGE-HSA and the amount of CML (N^{ε}-(carboxymethyl) lysine) ($p < 0.01$). As a result of reaction between extracted solution of cheek corneum and CML, the amount of CML in the reaction mixture was decreased ($p < 0.01$). Measuring corneum OPH-like activity, amount of corneum CML and accumulation amount of skin AGEs, which were provided by 52 healthy females at age range from 21 to 75, negative correlation was shown between age and OPH-like activity ($y = -2.9662x + 322.02$, $R^2 = 0.119$, $p < 0.05$). Positive correlation was shown between age and the accumulation of skin AGEs ($y = 0.0137x + 1.3534$, $R^2 = 0.225$, $p < 0.01$) and between age and the amount of corneum CML ($y = 0.0012x + 0.0378$, $R^2 = 0.079$, $p < 0.05$). Negative correlation was shown between corneum OPH-like activity and the amount of corneum CML ($y = -547.39x + 227.16$, $R^2 = 0.077$, $p < 0.05$) and between corneum OPH-like activity and the accumulation of skin AGEs ($y = -82.107x + 340.26$, $R^2 = 0.076$, $p < 0.05$).

***Conclusion*:** It may be possible that OPH-like activity, as existence was recognized in stratum corneum, was involved in proteolysis of stratum corneum CML and skin AGEs. It was indicated that the decline of stratum corneum OPH-like activity could be involved in an increase in the amount of stratum corneum CML and the accumulation of skin AGEs.

***KEY WORDS*:** oxidized protein hydrolase, corneum, degradation of advanced glycation end products

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Introduction

Accumulation of advanced glycation end products (AGEs) *in vivo*, which is caused by glycative stress, induces browning reaction of proteins and hardening of proteins by crosslinking, which involves inflammation induced by the receptor for AGEs (RAGE) and affects the deterioration of biological functions [1]. Accumulation of AGEs *in vivo* caused by glycative stress acceleration is one of trigger factors of diabetic complication, Alzheimer's disease, osteoporosis, arteriosclerosis and other diseases. Moreover, glycative stress acceleration, including chronic high fasting blood sugar levels and an increase of AGEs in skin, is involved in the development of the visual impression of aging [2, 3]. As aging in human skin, pentosidine and CML (N^{ε}-(carboxymethyl) lysine), which are categorized as AGEs, are accumulated in collagen fiber and elastin fiber of dermis [4, 5]. Accumulation of CML is recognized in keratin of epidermis [6]. It is indicated that accumulation of AGEs in skin causes the decline of elasticity and the resilience of skin [7] and the loss of skin texture [8]. Thus, the accumulation AGEs in skin could be a trigger factor for symptoms of aging. Furthermore, AGEs, having photosensitizer characteristics, which forms active oxygen due to ultraviolet light irradiation [9]. Thus, effects on skin cells have been a subject of discussion [10, 11]. Therefore, it is expected to be an effective anti-aging countermeasure to inhibit, degrade and eliminate skin AGEs for reduction of glycative stress of skin, including photo-aging.

Oxidized protein hydrolase, OPH, which is serine protease, exists in diversified biological tissues of animals such as pig liver, rat brain and human blood [12]. OPH is recognized to be the same enzyme as acylpeptide hydrolase (APEH) and acylamino acid releasing enzyme (AARE, EC 3.4.19.1) [13-15], which isolates *N*-terminal acylamino-acid of proteins. Some recent studies reported that OPH had the ability to degrade oxidized and glycated proteins in proiority [12]. In research of rats with diabetes mellitus induced by streptozotocin, serum OPH activity increased and blood carbonyl modifying protein decreased, as blood glucose level increased [16]. Therefore, it would be possible that OPH is related to the inhibition of development and progression of diabetes mellitus and aging-related symptoms. Moreover, OPH contributes to the breakdown of dysproteinemia in cooperation with proteasome [17]. However, OPH effects on accumulated AGEs *in vivo* have not been verified.

This study explored, focusing on aging-related glycation of skin tissue and defensive mechanisms towards glycative stress, to examine the existence and non-existence of OPH-like activity in stratum corneum and to verify that OPH would have the reduction effects of AGEs.

Methods

OPH and enzyme substrate

As an OPH, acylamino-acid releasing enzyme (AARE) was used, which is commercially available (Takara Bio Inc., Otsu, Shiga, Japan). As an enzyme substrate for OPH, *N*-acetyl-L-alanine *p*-nitroanilide (AAPA; Bachem AG, Bubendorf, Switzerland) and/or Advanced Glycation End Product-modified Human Serum Albumin (AGE-HSA) (human serum albumin; HSA, Sigma-Aldrich, St. Louis, MO, USA) were used. AGE-HSA was prepared, adjusting to mix 40 mg/mL HSA solution, 100 mmol/L sodium phosphate buffer solution (pH 7.4), 2.0 mol/L glucose aqueous solution and distilled water in a ratio of 2 : 5 : 1 : 2 and then reacting at 60°C for 40 hours.

Measurement of OPH-like activity in extraction of stratum corneum

Stratum corneum was exfoliated and sampled from skin of the cheek and inside upper arm provided by 5 healthy males at an age range from 20 to 69. Extraction of stratum corneum was prepared in the following manner:

Transparent adhesive tape (CELLOTAPE™ CT-24, Nichiban Co., Ltd. Bunkyo-ku, Tokyo, Japan) was stuck on the skin. The adhesive tape used for sampling was cut into 12 mm wide and was immersed in 2mL of 200 mmol/L Tris-HCl buffer solution (pH 7.4), with ice cold, which was containing 140 mmol/L NaCl and 0.1% Triton X-100. Then, the samples were processed four times by ultrasonication for twenty seconds.

In measurement of OPH-like activity, the amount of *p*-nitroaniline, which was isolated using AAPA as substrate, was measured by absorbance change (405 nm), after mixing stratum corneum extract and 50 mmol/L AAPA in the ratio of 9 : 1 and reacting at 37°C for 1 hour. The amount of protein in the stratum corneum extract was measured using a Protein Assay Bradford Reagent (Wako Pure Chemical Industries, Ltd., Chuo-ku, Osaka, Japan). The specific activity of OPH was calculated, as one unit was determined to be the amount of *p*-nitroaniline that was formed as substrate of AAPA for 1 hour, using calculation per protein amount. Moreover, OPH-like activity was measured in 2 other samples; adding, instead of corneum extract, trypsin from Porcine Pancreas (Wako Pure Chemical Industries) and Trypsin from Bovine Pancreas (Wako Pure Chemical Industries, Ltd. Chuo-ku, Osaka, Japan), which were serine protease and recognized to exist in corneum.

OPH-like activity in fractionation of corneum extraction by gel filtration chromatography

For fractionation of corneum extract by gel filtration chromatography analysis, stratum corneum was exfoliated and sampled from the skin of the right cheek of healthy females at ages from 20 to 29, after they washed their faces. The adhesive tape used for sampling was processed in the same manner above-mentioned to obtain extraction of corneum.

Extraction of right cheek corneum was fractionated by gel filtration high performance liquid chromatography (HPLC). Fractionation conditions of HPLC were the following:

Equipment: Prominence HPLC system (Shimadzu Corporation, Nakagyo-ku, Kyoto, Japan)

Analysis condition: Column YMC-Pack Diol-200, 500×8.0 mm I.D. (YMC Co. LTD, Shimogyo-ku, Kyoto, Japan), Eluent: 100 mmol/L of potassium phosphate buffer solution (pH 7.4) containing 200 mmol/L NaCl Flow rate: 0.5 mL/min, Detection: UV 230 nm, Injection amount: 50-130 µL, Fractionation: Fraction 1 (6.00-6.99 min)-49 (54.00-54.99 min). Meanwhile, OPH was compared by fractionation of gel filtration chromatography. The molecular weight of each elution fraction was estimated by the elution time in MW Marker (HPLC) (Oriental Yeast Co., Ltd., Itabashi-ku, Tokyo, Japan). OPH-like activity in fractionation was measured by absorbance with 405nm, after AAPA was

added to extraction of corneum and/or elution fraction of fractionated OPH and reacted at 37°C for 24 hours.

Measurement of degradation activity on AGEs by OPH

For the measurement of OPH degradation activity on AGEs, reaction solution was prepared; a mixture of 200 mmol/L Tris-HCl buffer solution (pH 7.4), 0.5 unit/mL OPH, and AGE-HSA in the ratio of 15:1:18 was incubated at 37°C for eighteen hours. The amount of fluorescent AGEs (excitation wavelength: 370 nm, detection wavelength: 440 nm) was measured. The CML amount was measured using CircuLex CML/Nε-(carboxymethyl) lysine ELISA Kit (Circulex, Ina, Nagano, Japan).

Measurement of degradation activity on corneum CML extract

Extraction of corneum, which was obtained from the right cheek of males at an age range from forty to forty nine was prepared in a cup method (diameter 25 mm)[18, 19] where 100 mol/L phosphate buffered saline (PBS(-)) was the extraction liquid. The protein amount in the corneum extract was measured, using Protein Assay Bradford Reagent and was adjusted to 16.8 μg/100 μg of protein. A mixture of corneum extract and 1 mg/mL CML (Lonza AG, Braine-l'Alleud, Belgium) at a ratio of 1:1 was reacted at 37°C for 120 min. The CML amount in the reaction solution was measured using CircuLex CML/Nε-(carboxymethyl) lysine ELISA Kit.

Measurement of OPH-like activity and skin AGEs in corneum

The subjects were 52 healthy females at an age range from 21 to 75. After the right cheek of subjects was cleansed by commercially available cleansing sheet, corneum was exfoliated and collected three times per site using a commercially available sheet for peeling corneum (Corneum Checker AST-01; Asahi Biomed, Chiyoda-ku, Tokyo, Japan).

The amount of CML in corneum was measured in the same manner as the previous report[20] using a tape stripping technique. After the protein was extracted from the second and the third layer of the sheet for peeling corneum, the amount of CML was measured. For the measurement of OPH activity, half of the second layer of the corneum-peeling sheet (1.25 cm × 2.50 cm) was inserted into a 2.0 mL screw cap tube. One stainless bead (diameter 4 mm) (Taitech Co., Ltd. Koshigaya, Saitama, Japan) and ice cold 300 μL of PBS(-) containing 0.025% Triton X-100 were added and processed at 3,000 rpm in a bead pulverizing machine for 5 min (Taitech Co., Ltd.). The obtained extract was processed by centrifugal separation (10,000 rpm, 4°C, 5 min) and supernatant liquid was used as corneum extract. After 100 μL of corneum extract and 50 μL of one mmol/L AAPA were mixed and reacted at 37°C for 120 min, OPH-like activity was measured by absorbance with 405 nm. The quantity of protein was determined, using the Bradford protein assay. OPH specific activity was calculated in the same manner mentioned above. AGE accumulation in skin was measured with autofluorescence (AF) of inside right upper arm, using an AGE Reader (DiagnOptics, Groningen, Netherlands).

Statistical analysis

The analysis was performed using IBM SPSS Statistics 24 (IBM Japan, Ltd., Chuo-ku, Tokyo, Japan). As for the assay method for measurement items, an unpaired t-test was employed for comparison between two groups and Pearson product-moment correlation coefficient was employed for the correlation test. The significance level was under 5% in a two-sided test.

Ethical standard

The examinations of this study were conducted in compliance with the ethical principles of the Declaration of Helsinki. Subjects were fully informed of the contents of the examination, about sampling of corneum and measurement of AF by AGE Reader, and voluntarily participated in the examination. This study was performed with written consent for participation in this research.

Results

OPH activity in stratum corneum

As an index, the effect that OPH isolated *p*-nitroaniline with AAPA used as substrate, existence and non-existence of OPH-like activity in corneum extract was verified. As a result, existence of OPH-like activity was recognized in the skin corneum of cheek and the inside of the upper arm. However, it was indicated that OPH-like activity was different according to individual subjects. When OPH-like activity was examined for each subject, in the case of subject A, C, and E, corneum from the cheek was significantly stronger than that of inside the upper arm ($p < 0.05$, ***Fig. 1***). On the other hand, OPH-like activity was not recognized in trypsin and α-chymotrypsin (data not shown).

OPH-like activity in fractionation of corneum extraction by gel filtration chromatography

OPH-like activity was recognized in a fraction of corneum extract and OPH by gel chromatography (***Fig. 2***). OPH-like activity was recognized in 19 fraction (elution time: 24.00-24.99 min) of corneum extract and OPH. Molecular weight in 19 fraction was estimated as 218-288 kDa.

Degradation activity of AGEs due to OPH

The degradation activity due to OPH was verified for fluorescent AGEs and CML in AGE-HSA. The result of this study showed that OPH decreased fluorescent AGEs and CML in AGE-HSA significantly ($p < 0.01$, ***Fig. 3***). The rate of decrease was 4% in fluorescent AGEs and 47% in CML; the rate of decreasing for CML was higher.

Degradation activity of CML due to stratum corneum extract

Corneum extract obtained from the right cheek decreased the amount of CML ($p < 0.01$, ***Fig. 4***). The rate of decrease was 7% in CML.

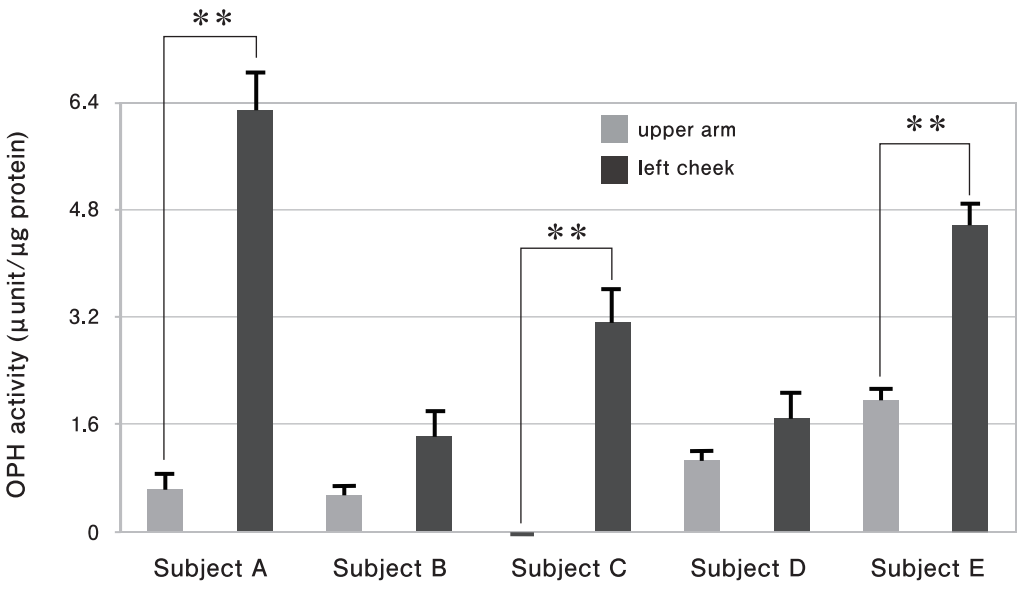

Fig. 1. **Comparison of OPH activity in corneum of inside upper arm and cheek.**

Results are expressed as mean ± SD, n = 3, ** $p < 0.01$. Reactive condition: AAPA (37.5mmol/L) + corneum extract, 37°C, 60 min. Detection: 405 nm. OPH, oxidized protein hydrolase; AAPA, *N*-acetyl-L-alanine *p*-nitroanilide; SD, standard deviation.

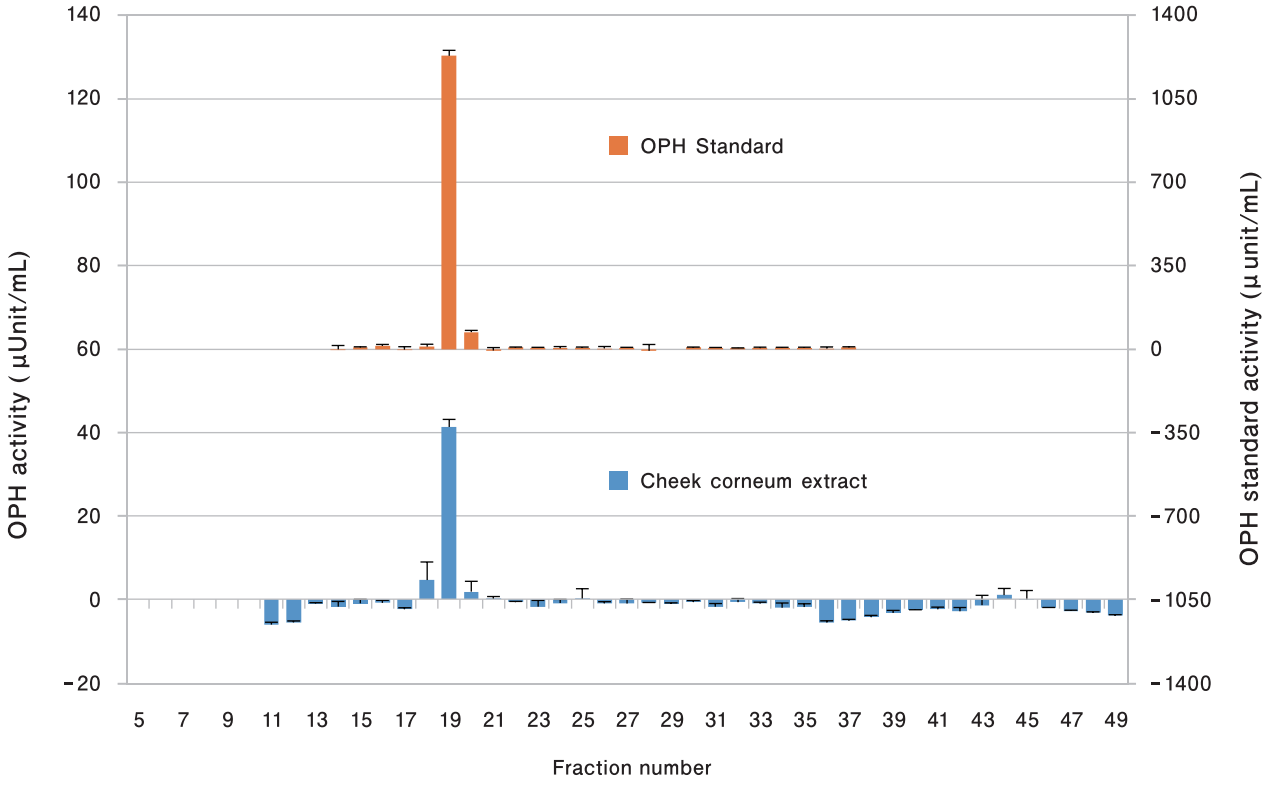

Fig. 2. **Fraction of gel filtration chromatography of cheek corneum extract.**

Results are expressed as mean ± SD, n = 3. Column: YMC-Pack Diol-200 (500×8.0 mm I.D.), Eluent: 100 mmol/L phosphate buffer (pH 7.4) + 0.2 mol/L NaCl, Flow rate: 0.5 mL/min, Injection volume: 50 μL (OPH standard) or 130 μL (Cheek corneum extract), Fraction number: 1 (6.00～6.99 min) ～ 49 (54.00～54.99 min), Reactive condition: AAPA (37.5 mmol/L) + gelfiltratin fraction, 37°C, 4 hr (OPH standard) or 24 hr (Cheek corneum extract), Detection: 405 nm. OPH, oxidized protein hydrolase; AAPA, *N*-acetyl-L-alanine *p*-nitroanilide; SD, standard deviation.

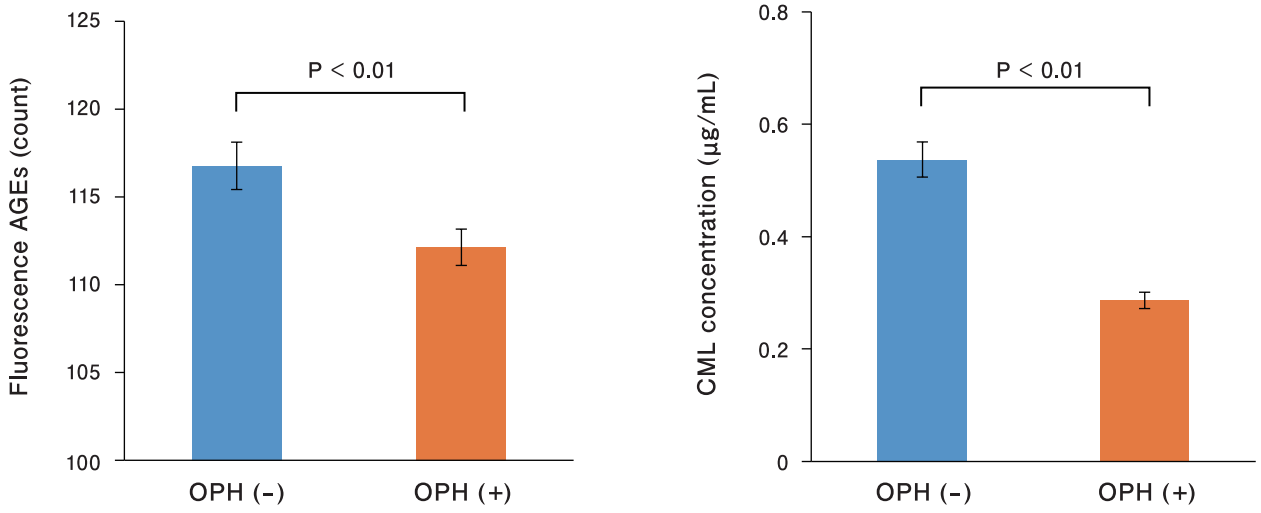

Fig. 3. **Degradation effect of AGEs by OPH.**

Results are expressed as mean ± SD, n = 3. Reactive condition: AGE-HSA (2.7 mg/mL) + OPH (0.02 unit/mL) + 125 mmol/L Tris-HCl (pH7.4), 37°C, 18 hr. AGEs measured fluorescence (ex 370 nm/em 440 nm) method, CML measured by ELISA. AGEs, advanced glycation end products; CML, N^{ε}-(carboxymethyl)lysine; HSA, human serum albumin; OPH, oxidized protein hydrolase; SD, standard deviation.

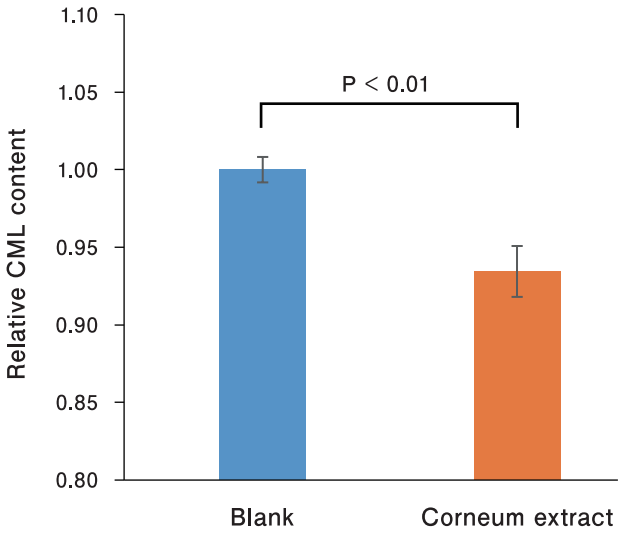

Fig. 4. **Degradation effect of CML by cheek corneum extract.**

Results are expressed as mean ± SD, n = 3. Corneum extract: collected cup method from right cheek. Reactive condition: CML (0.5 mg/mL) + corneum extract (8.4 μg protein), 37°C, 120 min. CML measured by ELISA. CML, N^{ε}-(carboxymethyl) lysine; SD, standard deviation.

Measurement of OPH-like activity and skin AGEs in corneum

Fig. 5 shows the measurement results of OPH-like activity of the right cheek corneum, corneum CML amount of right cheek and the accumulation of AGEs in the upper arm corneum; samples were collected from healthy females at an age range from 21 to 75. Negative correlation was shown between subject age and OPH-like activity in corneum ($y = -2.9662x + 322.02$, $R^2 = 0.119$, $p < 0.05$). Positive correlation was shown between age and skin AGEs accumulation ($y = 0.0137x + 1.3534$, $R^2 = 0.225$, $p < 0.01$) and between age and corneum CML amounts ($y = 0.0012x + 0.0378$, $R^2 = 0.079$, $p < 0.05$).

Furthermore, negative correlation was shown between corneum OPH-like activity and CML amount ($y = -547.39x + 227.16$, $R^2 = 0.077$, $p < 0.05$) and between corneum OPH-like activity and accumulation of skin AGEs ($y = -82.107x + 340.26$, $R^2 = 0.076$, $p < 0.05$, ***Fig. 6***).

Discussion

OPH-like activity in stratum corneum

The AGEs accumulation in skin is involved in the decline of elasticity and resilience of skin and the loss of skin texture [7, 8]. Therefore, the inhibition of AGE accumulation and the degradation of AGEs are important countermeasures of anti-aging skin [21, 22].

This study confirmed that human corneum had the same effect as OPHs which exist in pig livers, rat brains and human blood from biological tissue. Corneum OPH-like activity tended to be stronger in the cheek than in the inside upper arm. Therefore, there was a possibility that ultraviolet to skin, one of external factors, could affect OPH-like activity.

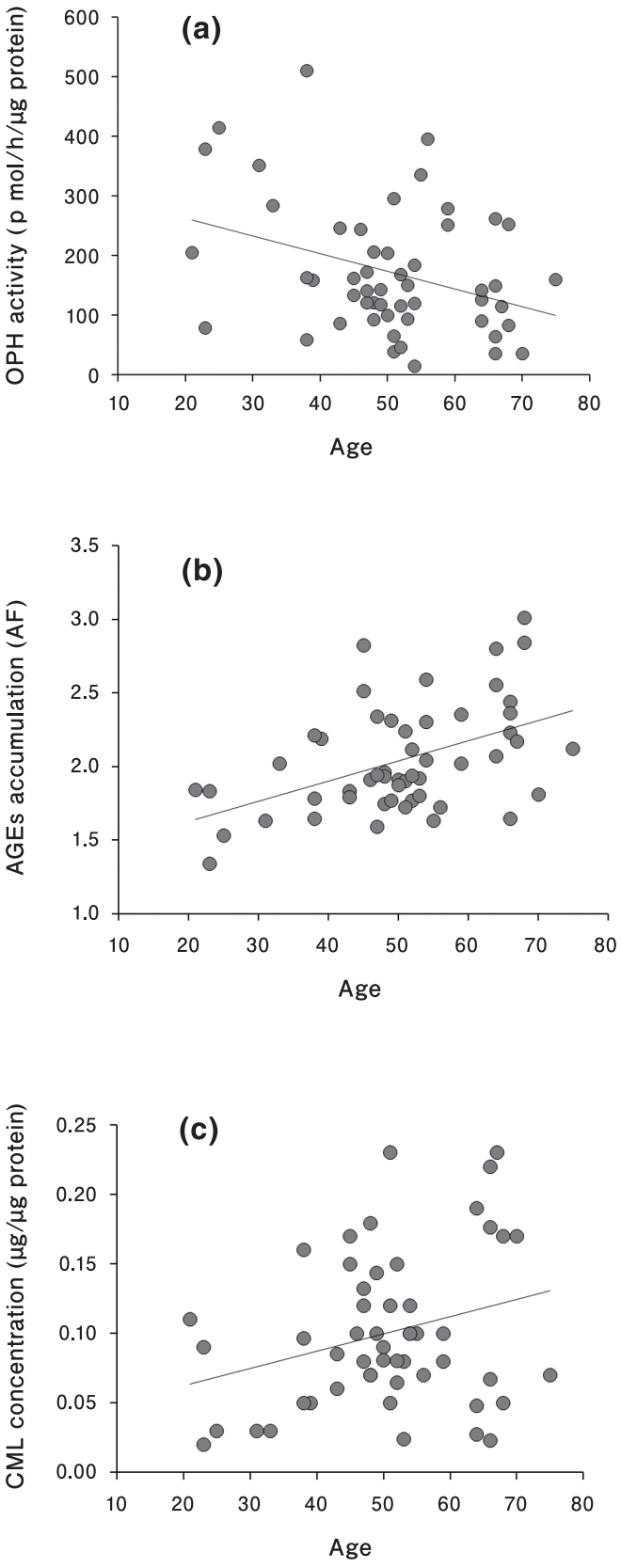

Fig. 5. **Relationship of OPH activity in the corneum and age.**

(a) OPH vs age: y = –2.9662x + 322.02, $R^2 = 0.119$, $p < 0.05$, **(b)** Skin AGEs accumulation vs age: y = 0.0137x + 1.3534, $R^2 = 0.225$, $p < 0.01$, **(c)** Cornem CML vs age: y = 0.0012x + 0.0378, $R^2 = 0.079$, $p < 0.05$. Subject: n = 52. AF: Autofluorescence reader value by the AGE Reader. OPH, oxidized protein hydrolase; AGEs, advanced glycation end products; CML, N^{ε}-(carboxymethyl)lysine.

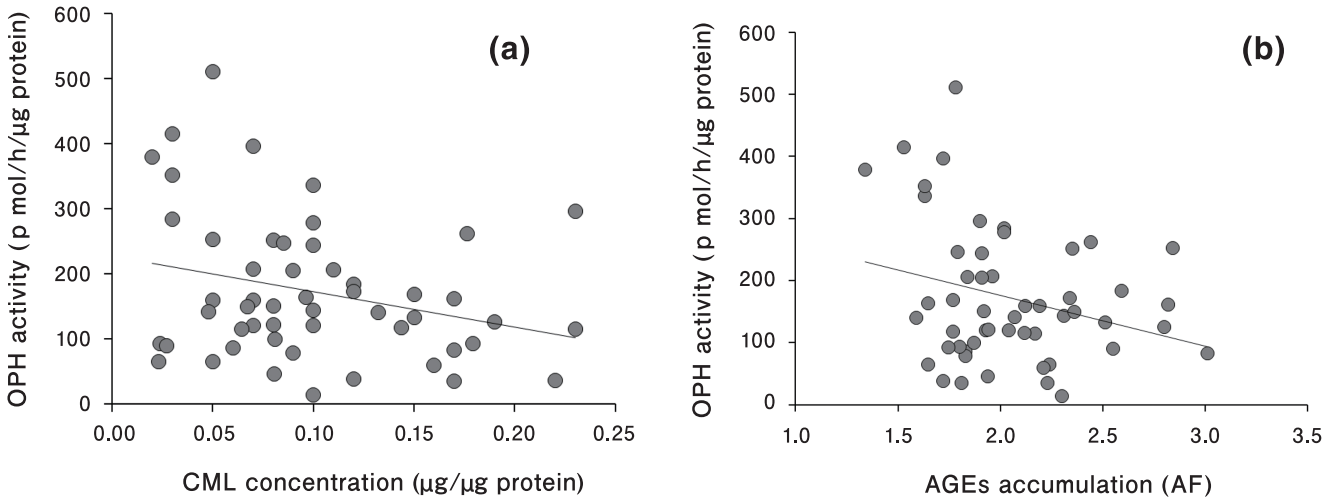

Fig. 6. **Relationship of OPH activity between corneum AGEs.**

(a) OPH vs corneum CML: $y = -547.39x + 227.16$, $R^2 = 0.077$, $p < 0.05$, **(b)** OPH vs skin AGEs accumulation: $y = -82.107x + 340.26$, $R^2 = 0.076$, $p < 0.05$. Subject: n = 52, AF: Autofluorescence reader value by the AGE Reader. OPH, oxidized protein hydrolase; AGEs, advanced glycation end products; CML, N^{ε}-(carboxymethyl)lysine.

OPH activity in fractionation of corneum extract by gel filtration chromatography

When corneum extract and OPH were eluted and fractionated by gel filtration chromatography, the result indicated that the fraction of OPH-like activity and the fraction of OPH activity were matched. Molecular weight of OPH-like activity elution fraction was estimated as 218-288 kDa. Molecular weight of OPH was 360 kDa by gel filtration chromatography, and 80 kDa by SDS-PAGE (sodium dodecyl sulfate - polyacrylamide gel electrophoresis)[12]. This molecular weight measurement showed a smaller estimation. The difference in the estimated molecular weight was thought to be influenced by a difference in the protein, which was used in the column by gel filtration chromatography and by molecular weight marker.

Degradation effect of AGEs due to OPH

The OPH which was studied decreased the fluorescence AGE amount and CML amount in AGE-HSA. However, the amount of decrease in the fluorescence AGEs was smaller than the amount of decrease in the CML. There are many types of fluorescence AGEs such as crossline, argpyrimidine and pyrropyridine. Therefore, it was possible that effects on AGEs of OPH could have substrate specificity; corneum extract also decreased CML amount. It has already been indicated that OPH degrades oxidized and glycated proteins[12]. CML is formed by oxidation and glycation of proteins[23]. Therefore, CML could have a property that would contribute to degradation by OPH.

Correlation between OPH activity in stratum corneum and skin AGEs

Corneum CML amount and AGEs accumulation in the right cheek corneum extracts, which were sampled from 52 healthy female subjects, showed a positive correlation with age and an increase according to aging. It has already been recognized that the pentosidine amount, CML amount[4] and AGE accumulation[24], in dermis collagen fiber, increases according to aging. Accumulation of CML in the epidermal layer is also known[6]. This study recognized that corneum CML increased due to aging. Meanwhile, a negative correlation was shown between age and OPH-like activity, and OPH-like activity decreased according to age. Moreover, negative correlation was recognized between the corneum OPH-like activity and both of the corneum CML and skin AGEs. Therefore, it was possible that a change of corneum OPH-like activity affects the accumulation of corneum CML and skin AGEs.

Skin immediately contacts the external environment and tends to receive external stress factors such as ultraviolet rays and substances in the air. Corneum OPH-like activity could protect the skin from glycative stress and oxidative stress and affect the development of aging. Therefore, OPH-like activity could play an important role in maintaining functions of the epidermis layer of the skin. Furthermore, it was indicated that maintenance of corneum OPH-like activity would inhibit skin AGEs accumulation and contribute to anti-aging skin, as corneum CML amount and skin AGEs accumulation increases according to age.

Conclusion

It was confirmed that OPH would have an effect to decrease the amount of AGEs and CML. OPH-like activity was recognized in stratum corneum and could be involved in degradation of corneum CML. A change in corneum OPH-like activity due to aging would affect the change in the amount of coreum CML and skin AGE accumulation.

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Conflict of interest statement

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