

Original Article

Non-invasive collection of stratum corneum samples by a tape-stripping techniqueYumi Kamitani¹⁾, Masayuki Yagi¹⁾, Keitaro Nomoto²⁾, Mio Hori²⁾, Junko Naito²⁾, Yoshikazu Yonei²⁾

1) Glycation Stress Research Center, Graduate School of Life and Medical Sciences, Doshisha University

2) Anti-Aging Medical Research Center, Graduate School of Life and Medical Sciences, Doshisha University

Abstract

Objective: An *in vivo* non-enzymatic reaction between proteins and reducing sugars results in the production of advanced glycation endproducts (AGEs). This reaction is referred to as glycation. Glycation in the skin is considered to contribute to skin aging. The accumulation of fluorescent AGEs in the skin can be quantitated by measuring autofluorescence (AF) intensity with AGE Reader. The objective of this study was to non-invasively collect stratum corneum samples by a tape-stripping technique and measure the content of *N*^ε-(carboxymethyl)lysine (CML), a non-fluorescent AGE, in the stratum corneum, and the area of stratum corneum to examine whether the degree of skin aging can be evaluated based on the degree of glycation in the skin.

Methods: Stratum corneum samples were collected by applying a commercially available adhesive sheet (Corneum Checker) at the same site of the medial aspect of an upper arm and then peeling it off the skin (= the tape-stripping method). This procedure was repeated 3 times sequentially to collect samples from the first to third layers of stratum corneum. The adhesive sheets used for stratum corneum sampling were subjected to protein extraction using xylene, toluene, ethanol or extraction buffer (50 mmol/L Tris-HCl buffer (pH 7.5), 120 mmol/L NaCl, 1 mmol/L Na₃VO₄, and 0.1% sodium dodecyl sulfate (SDS)) as solvent and a handy micro homogenizer, an ultrasound bath, a handy pestle, or by keeping them immersed for 10 minutes as the extraction method, and protein yields obtained by each combination of solvents and extraction method were compared. The amounts of stratum corneum proteins and CML in each extract were measured by DC Protein Assay (Lowry's method) and CML /*N*^ε-(carboxymethyl) Lysine ELISA Kit, respectively.

Results: Proteins were most efficiently extracted from the adhesive sheets used for stratum corneum sampling by the tape-stripping method using the extraction buffer and a handy micro homogenizer. Therefore, this study decided to use this solvent and extraction method for stratum corneum protein extraction in the following experiments. The amount of stratum corneum proteins extracted by the tape-stripping method was less variable between measurements in the second and third layers than in the first layer. The protein recovery rate by the current tape-stripping method was 99.1 ± 1.6% (mean ± standard deviation).

Discussion: This study suggested that the measurement of CML content in stratum corneum by a tape-stripping technique using a commercially available adhesive sheet. The use of the tape-stripping method enabled the extraction of stratum corneum proteins from the second and third layers with high reproducibility.

Conclusion: The measurement of CML content using stratum corneum samples non-invasively collected by the tape-stripping method may enable evaluation of skin aging based on the degree of glycation in the skin.

KEY WORDS: skin aging, advanced glycation end products, corneocytes, *N*^ε-(carboxymethyl)lysine (CML), stratum corneum proteins

Introduction

An *in vivo* non-enzymatic reaction between proteins and reducing sugars results in the generation of an irreversible substance called advanced glycation endproducts (AGEs). This reaction is referred to as glycation. These AGEs deposit in tissues and bind to receptors called RAGE (receptor for AGEs) to induce inflammatory changes in skin and other tissues. The biological stress caused by reducing sugars and aldehydes and a series of reactions that follow are referred to collectively as glycation stress¹⁾. The accumulation of AGEs increases with age and possibly acts as a risk factor for the development and progression of aging and aging-related disorders²⁾.

Glycation also occurs in the skin and the accumulation of AGEs in the skin is considered to contribute to skin aging by altering skin tone and elasticity¹⁾.

AGEs include fluorescent and non-fluorescent forms. The accumulation of fluorescent AGEs in skin can be quantitated non-invasively as autofluorescence (AF) intensity using AGE Reader (DiagnOptics Technologies BV, Groningen, The Netherlands)^{3,4)}. *N*^ε-(carboxymethyl)lysine (CML), a typical non-fluorescent AGE that accumulates in skin⁵⁾, can be quantitated by assays such as enzyme linked immunosorbent assay (ELISA). CML has also been detected in the epidermal layer, a tissue that undergoes rapid turnover⁶⁾.

The area of a corneocyte increases with age. The size

of corneocytes is closely correlated with the turnover rate of stratum corneum; the cells become larger as turnover slows down. The turnover rate of stratum corneum reflects cell growth rate in the basal layer, allowing for the determination of skin condition (degree of skin metabolism) by measuring stratum corneum turnover rate^{7,8)}.

In this study, we non-invasively collected stratum corneum samples by a tape-stripping technique using a commercially available adhesive sheet to determine the optimal condition for extracting stratum corneum proteins from the adhesive sheet and examine whether CML content in the stratum corneum protein extract and the area of stratum corneum can be measured.

Methods

Extraction of stratum corneum proteins by a tape-stripping technique

The current method for extracting stratum corneum proteins from skin by a tape-stripping technique was developed based on the methods described by Suzuki *et al.*⁹⁾ and in the Patent Gazette¹⁰⁻¹²⁾.

Subjects washed their upper arms with soap, rinsed thoroughly with running water and wiped water thoroughly off the skin surface. A stratum corneum sample was collected by pressing a commercially available adhesive sheet (Corneum Checker: AST-01, Asahi Biomed, Yokohama, Kanagawa, Japan) on the medial aspect of the right upper arm with a finger for 5 seconds and then peeling it off the skin. The peeled adhesive sheet was cut into a 2.5 × 2.5 cm piece and placed into a 2-mL tube with the adhesive side facing inside. Protein extraction was performed using 600 µL of a mixture of xylene, toluene, ethanol and extraction buffer (50 nmol/L Tris-HCL buffer (pH 7.5), 120 mmol/L NaCl, 1 mmol/L Na₃VO₄, and 0.1% sodium dodecyl sulfate (SDS))⁹⁾ using the following 4 different methods (N = 2):

- 1) Homogenize at 9,000 rpm for 2 minutes with a handy micro homogenizer (NS-360D, MICROTEC, Funabashi, Chiba, Japan)
- 2) Treat in an ultrasound bath (5510 J-MT, Yamato Scientific, Chuo-ku, Tokyo, Japan) for 10 minutes
- 3) Homogenize with a handy pestle (Mimi Cordless Grinder, Funakoshi, Bunkyo-ku, Tokyo, Japan) for 2 minutes
- 4) Keep immersed for 10 minutes

After each treatment, the adhesive tape was removed from the tube. The fraction extracted by xylene, toluene and ethanol was centrifuged at 3,000 rpm for 5 minutes. The supernatant was collected and evaporated to dryness under reduced pressure. The dried sample was then dissolved in 100 µL of 100 mmol/L phosphate buffer (pH 7.4) containing 0.1% of SDS and used as the stratum corneum protein extract. The fraction extracted by the extraction buffer was used as a stratum corneum protein extract without further processing.

Quantitation of stratum corneum proteins

The protein content in the stratum corneum protein extract was measured using a DC Protein Assay kit of micro-assay method (Bio-Rad Laboratories, CA, USA). Specifically, 40 µL

of the stratum corneum protein extract was mixed with 20 µL of DC Reagent A' (added 20 µL of DC Reagent S to each 1 mL of DC Reagent A) and 160 µL of DC Reagent B provided with the kit, the mixture was left to stand at room temperature for 30 minutes, and subjected to absorbance measurements at 750 nm on a microplate reader (N = 2). A calibration curve for protein quantitation (0.625-125 µg/mL) was created using human serum albumin (HSA, Sigma-Aldrich, St. Louis, MO).

Recovery rate for stratum corneum proteins

To determine the recovery rate for stratum corneum proteins, 10-30 µL of 1 mg/mL aqueous HSA solution was applied onto the adhesive side of the adhesive sheet cut into a 2.5 × 2.5 cm piece. The sheet was then placed in an airtight container containing a desiccating agent (silica gel) and was left to stand for about 30 minutes until completely dried. Then, protein was extracted from the sheet in the extraction buffer with a handy micro homogenizer and quantitated using a DC Protein Assay kit of micro-assay method (Bio-Rad).

Measurement of protein content in different layers of stratum corneum

Stratum corneum samples were collected by applying 3 adhesive sheets sequentially (the first to third layers) at the same site of the medial aspect of the right upper arm of 22 year-old female and 23 year-old male. Then, stratum corneum protein was extracted from each sheet in 600 µL of extraction buffer with a handy micro homogenizer and quantitated using a DC Protein Assay kit of micro-assay method (Bio-Rad).

Measurement of CML content in skin stratum corneum

Stratum corneum samples were collected by applying 2 adhesive sheets sequentially (the first and second layers) at the same site of the medial aspect of the right upper arm of 5 healthy women aged between 21 and 43 years with no skin problems. The stratum corneum protein extract was obtained from the adhesive sheet for the second layer. Protein purification from the protein extract was performed as follows. To 400 µL of the protein extract, 80 µL of 14% perchloric acid (PCA; Wako Pure Chemical Industries, Osaka, Japan) was added, mixed, left to stand for 20 minutes, and centrifuged at 10,000 rpm for 15 minutes. The precipitate was dissolved in 400 µL of 50 mmol/L Tris-HCl buffer (pH 8.0).

CML content was measured by ELISA using a commercially available assay kit of CML/N^ε-(carboxymethyl) Lysine ELISA Kit (CycLex, Ina, Japan)¹³⁾. 180µL of each reacted sample or CML standard mixed 180µL of anti-CML adduct monoclonal antibody (clone name: MK-5A10) solution was added to each diluted sample, stirred, and 100µL of each mixture was dispensed into a well of an antigen-coated microplate (N = 3). The plates were incubated for one hour at room temperature; washed with 0.2% Tween-20; 100µL of horse radish peroxidase conjugated anti-mouse IgG polyclonal antibody solution was dispensed into each well, and further incubated for one hour; washed with washing buffer; 100µL of tetra-methyl-benzidine solution was added to each well, and the plate was wrapped in aluminum foil and incubated at room temperature for 10 minutes; 100µL of stop solution was

added and the absorbance was measured at dual wavelengths of 450nm/540nm using a spectrophotometric microplate reader (SPECTRA MAX 190, Molecular Devices, Chuo-ku, Tokyo, Japan) within 30 minutes. A calibration curve for CML quantitation (0.08-1.25 $\mu\text{g}/\text{mL}$) was created using CML-HSA standards (CycLex, Ina, Nagano, Japan).

Ethical considerations

A study explanatory session was held prior to skin stratum corneum sampling by the tape-stripping method, according to the ethical guidelines for epidemiological studies. Subjects were required to fully understand the content of the study and provide written consent before voluntarily participating in the study.

Results

Measurement of CML content in stratum corneum by the tape-stripping method

Determination of the optimal solvent and method for extracting stratum corneum proteins

Four solvents, xylene, toluene, ethanol and extraction buffer, were used in determining the optimal method for extracting stratum corneum proteins. The use of the extraction buffer and a handy micro homogenizer resulted in protein yields of 7.85 and 9.05 $\mu\text{g}/\text{cm}^2$ skin from the first and second layers, respectively, with a small variation between measurements (Fig. 1). When xylene or toluene was used, protein yields were less than a quarter of those obtained with the extraction buffer. Treatment with an ultrasound bath resulted in very low protein yields with any of the 4 solvents tested (0.03-0.49 $\mu\text{g}/\text{cm}^2$ skin). The use of a handy pestle resulted in high protein yields of 1.58 and 3.37 $\mu\text{g}/\text{cm}^2$ skin, but with a substantial variation between measurements (about 2-fold difference). The 10-minute immersion method resulted in very low protein yields with any of the 4 solvents tested (0.14-1.40 $\mu\text{g}/\text{cm}^2$ skin).

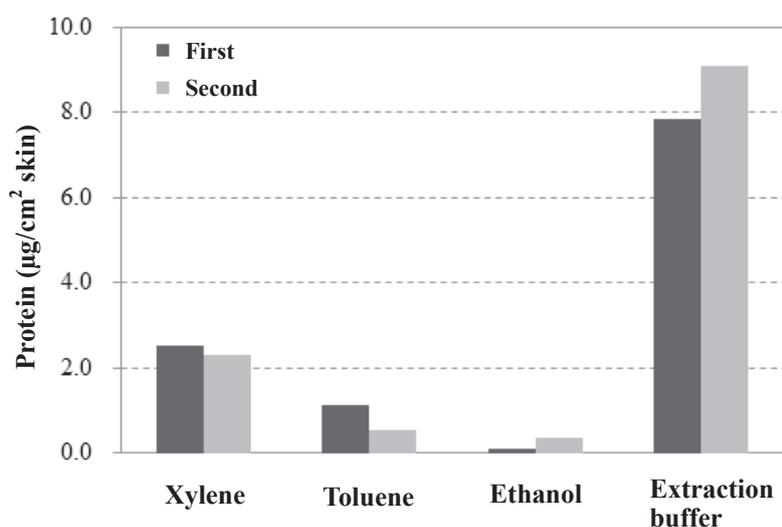


Fig. 1. Amount of extracted protein treated by handy microhomogenizer. (N=2)

Given these results, the use of the extraction buffer and a handy micro homogenizer was considered the optimal method for extracting stratum corneum proteins by the tape-stripping method using an adhesive sheet.

Recovery rate for stratum corneum proteins

When proteins were extracted from adhesive sheets onto which 0 (only water) to 30 μg (A-D) of HSA had been applied and dried, using the extraction buffer and a handy micro homogenizer, the recovery rate for HSA was $99.1 \pm 1.6\%$ (mean \pm standard deviation) (Fig. 2).

Protein content in different layers of stratum corneum

When 3 adhesive sheets were sequentially attached to the same site of the medial aspect of the upper arm and the protein content was measured in the first to third layers of stratum corneum, the protein yield from the first layer was greater than those from the second and third layers. The second and third layers showed a small variation in protein content between measurements (Fig. 3).

Measurement of CML content in skin stratum corneum

The mean (\pm standard deviation) CML content in the second-layer stratum corneum extract from 5 healthy women with no skin problems (age ranged 21-43 years) was 1.27 ± 0.60 ng/ μg protein.

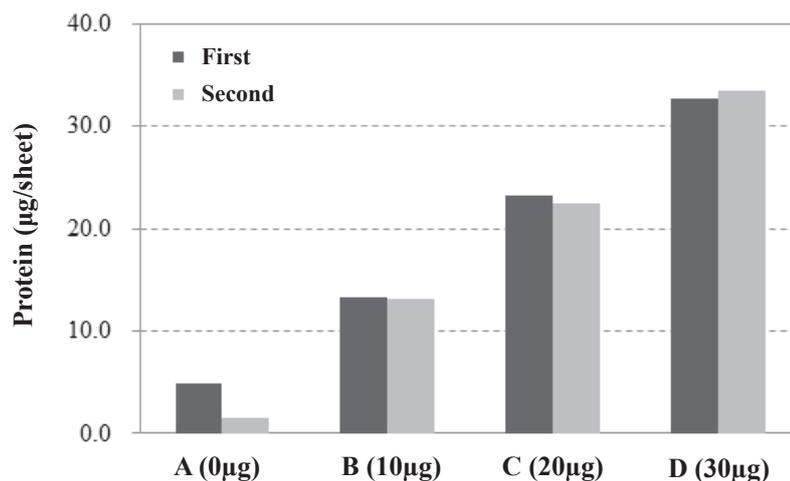


Fig. 2. Amount of recovered protein. Human serum albumin (HSA) was put on and extracted from adhesive sheets. Parenthesis indicate dose of human serum albumin. (N = 2)

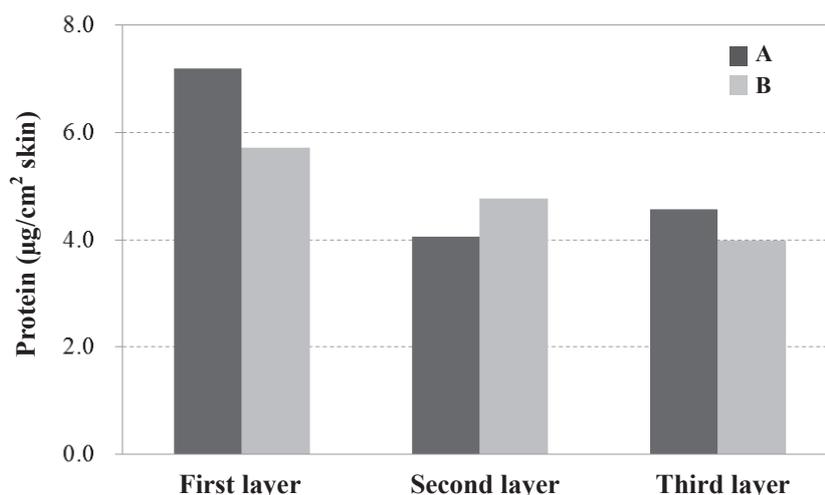


Fig. 3. Amount of extracted protein from the first, second and third layers of skin stratum corneum. A; 22 year-old female, and B; 23 year-old male. (N = 2)

Discussion

In this study, we found that the use of a commercially available adhesive sheet (Corneum Checker), extraction buffer consisting of 50 mmol/L Tris-HCl, 120 mmol/L NaCl, 1 mmol/L Na_3VO_4 and 0.1% SDS, and a handy micro homogenizer enables the optimal extraction of stratum corneum proteins, with a protein recovery rate of $99.1 \pm 1.6\%$.

Suzuki *et al.*⁹⁾ compared 3 different types of adhesive tapes and 16 different types of organic solvents in extracting stratum corneum proteins by the tape-stripping method and found that proteins were efficiently extracted by xylene, while

almost no protein was extracted by chloroform or hexane due to interactions between adhesives and stratum corneum proteins. They also demonstrated that the extraction efficiency for stratum corneum proteins varied depending on the type of adhesive tape. The use of buffers containing surfactants for extracting stratum corneum proteins from adhesive sheets has also been described; the addition of Triton X-100¹⁴⁾ and SDS¹⁰⁾ has been shown to improve the extraction efficiency for these proteins. Sodium orthovanadate (Na_3VO_4) can also be added to the extraction solvent to inhibit the dephosphorylation of proteins. The use of scrapers, ultrasonic devices, pestles and other tools to scrape the adhesive surface has also been

described⁹⁾. It has been suggested that the suitability of the extraction method is determined by the combination of the adhesive sheet, adhesive agent and solvent.

Proteins were most efficiently extracted from the first layer and relatively consistently extracted from the second and third layers of the stratum corneum. The stratum corneum is composed of 15-16 layers in most parts of the body, with exceptions including the palms, soles of the feet and other exceptional areas. The moisture content of the stratum corneum varies with depth. The results may be related to the fact that the first layer is exposed to the outside environment. Results obtained by measuring CML content in the stratum corneum protein extracted from 5 healthy women aged 21 to 43 years showed a substantial individual difference in CML content, suggesting that CML content in stratum corneum should be determined for each subject when using the parameter to assess the status of skin glycation.

Conclusion

The present tape-stripping method using a commercially available adhesive sheet enabled the extraction of skin stratum corneum in a non-invasive manner and the measurement of CML content in stratum corneum as well as corneocyte area. These observations suggest that the measurement of CML content in stratum corneum and corneocyte area by the tape-stripping method may be applied to the evaluation of skin aging and the effectiveness of functional materials.

Conflicts of Interest

The authors have no conflicts of interest in this study.

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