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Method for detecting the reactivity of chemicals towards peptides as an alternative test method for assessing skin sensitization potential

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HIGHLIGHTS

- The skin sensitization potential evaluated using spectrophotometric-monitoring methods reactivity of chemicals toward peptides.
- The combination results of cysteine and lysine peptide depletion showed good positive correlations.
- New in vitro model is a good alternative evaluation model for the prediction of the skin sensitization potential.

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ABSTRACT

Cosmetics are normally composed of various ingredients. Some cosmetic ingredients can act as chemical haptens reacting toward proteins or peptides of human skin and they can provoke an immunologic reaction, called as skin sensitization. This haptenation process is very important step of inducing skin sensitization and evaluating the sensitizing potentials of cosmetic ingredients is very important for consumer safety. Therefore, animal alternative methods focusing on monitoring haptenation potential are undergoing vigorous research. To examine the further usefulness of spectrophotometric methods to monitor reactivity of chemicals toward peptides for cosmetic ingredients. Forty chemicals (25 sensitizers and 15 non-sensitizers) were reacted with 2 synthetic peptides, e.g., the cysteine peptides (Ac-RFAACAA-COOH) with free thiol group and the lysine peptides (Ac-RFAAKAA-COOH) with free amine group. Unreacted peptides can be detected after incubating with 5,5′-dithiobis-2-nitrobenzoic acid or fluorescamine™ as detection reagents for free thiol and amine group, respectively. Chemicals were categorized as sensitizers when they induced more than 10% depletion of cysteine peptides or more than 30% depletion of lysine peptides. The sensitivity, specificity, and accuracy were 80.0%, 86.7% and 82.5%, respectively. These results demonstrate that spectrophotometric methods can be an easy, fast, and high-throughput screening tools predicting the skin sensitization potential of chemical including cosmetic ingredient.

1. Introduction

Some chemicals can elicit allergic contact dermatitis (ACD) via skin sensitization. Estimation of the skin sensitization potential for new ingredients is an important part of the safety assessment in cosmetics and topical drugs. For many decades, identification of potential skin sensitization hazards for cosmetic ingredients have evaluated by guinea-pig maximization test (GPMT) (Magnusson and Kligman, 1969). However, GPMT determined the skin sensitization potential by evaluating eczema and edema in elicitation phase so cannot provide quantitative data (Nukada et al., 2012). Moreover, concerning the animal welfare, the animal experiment is restricted for cosmetics and cosmetic ingredient since 2009 (EC, 2003). For these reason, the murine local lymph node assay (LLNA) was developed and adapted by OCED Test Guideline 429 (OECD, 2002). In comparison with the guinea pig methods, LLNA can provide quantitative data of skin sensitization potential by the measurement of lymphocyte proliferation in draining lymph nodes (EC3: Estimated concentration that produces a stimulation index of 3 in the murine local lymph node assay) (Kimber and Basketter, 1992). And LLNA showed a similar degree of sensitivity and specificity in terms of hazard identification compare to guinea pig methods (Dean et al.,

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2001) by use of reduced number of animals and shorter test period. However, this method still requires animals for testing and cannot satisfy the demand for full replacement of animals.

In the cosmetic industry, alternative methods to animal experiments are undergoing a vigorous progress. Cosmetics Europe (Cosmetics Europe – The Personal Care Association) is involved in researching alternative studies and risk assessment methodologies, and the following 3 test methods are in the ECVAM (European Centre for the Validation of Alternative Methods) pre-validation stage: human cell line activation test (h-CLAT), myeloid U937 skin sensitization test (MUSST), and direct peptide reactivity assay (DPRA) (Adler et al., 2011).

Skin sensitizers itself cannot directly induce skin sensitization. In skin sensitization process, the formation of adducts between chemical and endogenous protein and/or peptides in skin is essential (Dupuis et al., 1980; Landsteiner and Jacobs, 1935; Patlewicz et al., 2001). In skin proteins, the side chains of many amino acids contain electron-rich groups capable of reacting with nucleophilic allergens. Lysine and cysteine are those most often cited as electron-rich amino acids that can react with electrophiles strongly. Haptens (generally small molecules with a molecular weight less than 1000 Da) can interact with biological macromolecules in skin by formation of bonds of various strengths and these reactions called hapten–protein conjugation in mechanism of skin sensitization. The strength and bond stability of interaction between chemical and protein can critical point of hapten–protein conjugation that is first step of skin sensitization (Gerberick et al., 2008). Almost chemicals can form a hapten–protein conjugate and can induce a skin sensitization, thus the evaluation of the reactivity between chemicals and proteins and/or peptides can serve as an animal alternative for skin sensitization (Divkovic et al., 2005; Gerberick et al., 2008). In previous studies, the measurement of the reactivity between chemicals with protein and/or peptides has been formed by two methods. One is by monitoring the depletion of a selected peptide in the presence of chemicals using liquid chromatography with ultraviolet (UV) detection (Gerberick et al., 2004, 2007a), the other is by monitoring adduct formation between the peptide and the chemical using liquid chromatography mass spectrometry (LC/MS) or nuclear magnetic resonance (NMR) (Ahlfors et al., 2005; Aleksic et al., 2007, 2008; Alvarez-Sanchez et al., 2004a,b). The former chromatography-based methods have an advantage, that a very small quantity of sample is needed for the analysis (Alvarez-Sanchez et al., 2004a,b; Gerberick et al., 2007a; Natsch et al., 2007). The analysis using LC/MS- and NMR provide more detailed information than methods based on liquid chromatography. Although HPLC-, LC/MS-, and NMR-based analysis methods have the abovementioned advantages, these methods are rather expensive, time-consuming, and require skilled operators. In contrast, spectrophotometry is a conventional method used to detect changes in coloured samples and can be used even when very small quantities are available for analysis. Previously, a rapid and inexpensive spectrophotometric assay was developed for determining the reactivity of chemicals towards glutathione (GSH) (Schultz et al., 2005). The free thiol group of GSH was monitored by derivatization of GSH with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) and subsequent spectrophotometric detection and used to determine the reactivity of unsaturated carbonyl-containing compounds towards GSH.

In previous our study, we established spectrophotometric assay methods to determine the reactivity of chemicals towards 2 chemical groups, i.e., the thiol group of a cysteine-containing peptide (cysteine peptide) and the amino group of a lysine-containing peptide (lysine peptide). We used DTNB as the detection reagent for the free thiol group and fluorescamine as the detection reagent for the free amine group. We also investigated the possibility of using this method as an in vitro sensitization test. By combination of the 2 methods using each type of peptide, our new method achieved a high degree of sensitivity, specificity, and accuracy (Jeong et al., 2013).

In this study, we reconfirm the estimation for skin sensitization using previous our established spectrophotometric assay methods. In addition, we ensure the possibility of application for cosmetic ingredients by use of our previous in vitro alternative for skin sensitization.

2. Material and methods

2.1. Peptides and test chemicals

We used 2 model peptides, a cysteine peptide (Ac-RFAACAA-COOH) and a lysine peptide (Ac-RFAAKAA-COOH), which have already been used in a previous study (Gerberick et al., 2004). Two synthetic model peptides were purchased from Peptron Co. (Daejeon, Korea) with >95% purity. The chemical name, class, chemical abstract system (CAS) number, and LLNA data of 40 test materials are presented in Table 1. We selected 30 chemicals that have been well categorized by their sensitization potential obtained from LLNA results and clinical data. Another 10 chemicals were selected from common ingredients, which are used in cosmetics. The test chemicals included 25 known sensitizers (S) and 15 non-sensitizers (NS). The peptide-to-chemical ratios used were 1:5 for cysteine and 1:10 for lysine.

2.2. Measurement of cysteine peptide depletion

The cysteine peptide reaction solution was prepared by diluting the cysteine peptide stock solution to 400 μM with 100 mM sodium phosphate buffer (pH 8.0, 1 mM EDTA) before the experiment. Chemical reaction solutions were prepared by diluting chemical stock solutions to 2 mM with 100 mM sodium phosphate buffer (pH 8.0, 1 mM EDTA). Then, 90 μL of peptide reaction solution and 90 μL of chemical reaction solution were reacted in the 96-well ELISA plate for 24 h at room temperature. After 24 h, 20 μL of a 10 mM DTNB solution solubilized in sodium phosphate buffer (pH 8.0) was added to each well of the plate. The mixture was incubated for 3 min to achieve complete reaction between unreacted peptide and DTNB. Both, before the addition of the DTNB solution and after the reaction between unreacted peptide and DTNB, the optical density (OD) was measured using an UV–vis spectrophotometer (SpectraMAX 190™, Molecular Devices, CA, USA; absorption wavelength: 412 nm).

2.3. Measurement of lysine peptide depletion

The lysine peptide reaction solution was prepared by diluting the lysine peptide stock solution to 200 μM with 100 mM sodium phosphate buffer (pH 10.0, 1 mM EDTA) before the experiment and chemical reaction solutions were prepared by diluting chemical stock solutions to 2 mM with isopropanol. Then, 100 μL of peptide reaction solution and 100 μL of chemical reaction solution were reacted in the 96-well ELISA plate for 24 h at room temperature. After incubation, 180 μL of the reaction mixtures were transferred to a light-proof black clear-bottom 96-well plate (Greiner Bio-One, Frickenhausen, Germany). Next, 20 μL of a 20 mM fluorescamine solution (prepared in DMSO) was added to each well of the plate and incubated for 3 min to achieve complete reaction between unreacted peptide and fluorescamine. Fluorescence intensity was measured using a fluorometer before the addition of fluorescamine solution and after the reaction between unreacted peptide and fluorescamine (Flexstation 3, Molecular Devices; excitation: 390 nm, emission: 465 nm, 475 nm).