



Proteomic allergen–peptide/protein interaction assay for the identification of human skin sensitizers

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ABSTRACT

Modification of proteins by skin sensitizers is a pivotal step in T cell mediated allergic contact dermatitis (ACD). In this process small reactive chemicals interact covalently or non-covalently with cellular or extracellular skin self-proteins or self-peptides to become recognized by the human immune system. Aiming to develop a novel non-animal in vitro test system for predicting sensitization potential of small reactive chemicals in human skin the allergen–peptide/protein interaction assay (APIA) has been developed. By applying modern proteomic technologies together with a target peptide containing all amino acids, the assay permits the profiling of all amino acid specific allergen–peptide interactions. Moreover, potentially crucial allergen-specific Cys-modifications are qualitatively monitored by mass spectrometry and confirmed by a dual peptide approach. Assay conditions chosen mimic the distinct human epidermal reactivity compartments of the skin surface (pH 5.5), stratum basale (pH 6.8), and typical physiological conditions (pH 7.4). An extreme as well as a moderate human contact sensitizer produced Cys-specific mass shifts, whereas a skin irritant did not. Our data indicate that MALDI–MS based and skin-related in vitro technology platforms – like the APIA – are promising tools in developing alternative non-animal allergen assays. This will assist in chemical classification and next generation risk assessment strategies, including REACH and experimental immunotoxicology.

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1. Introduction

Checkpoints of allergic contact dermatitis (ACD) induction comprise chemical skin penetration, interaction of small reactive chemicals with extracellular or cellular skin proteins or peptides, isochronic innate inflammatory cell responses, langerhans cell migration towards local lymph nodes, antigen processing and presentation, and the chemical-specific activation of human T cells followed by their recirculation into the epidermis (Karlberg et al., 2008; Kimber et al., 2011; Martin et al., 2010, 2006; Rustemeyer et al., 2001; Vocanson et al., 2009). Reducing, replacing, and refining the use of animal experimentation in risk assessment has been

an objective worldwide and specifically of the European Union for the last two decades. It has led to the 7th amendment to the EU Cosmetics Directive, prohibiting future animal use for cosmetic ingredient testing (EU, 2003). Thus, a new challenging research field has been created to address questions of how chemical-specific whole body immune reactions – as characteristic for the systemic local lymph node assay (LLNA) – may be potentially translated into novel in vitro assay systems. Consequently several new in vitro assays have been proposed or currently integrated into ECVAM's (European Centre for the Validation of Alternative Methods) validation strategy or are still under investigation (Adler et al., 2011; Aeby et al., 2010; Basketter and Kimber, 2010; Bauch et al., 2011; Galbiati et al., 2011; Hartung et al., 2011; Vandebriel and van Loveren, 2010). Representative examples are a peptide reactivity assay (Gerberick et al., 2008, 2004, 2007), a recently developed in vitro assay using the Nrf2-Keap1-ARE toxicity pathway as a cellular sensor for skin sensitizers (Emter et al., 2010; Natsch, 2010), or novel approaches concerning chemical-specific T cell reactivity (Dietz et al., 2010; Martin et al., 2010), and genomic biomarker signatures (Johansson et al., 2011; Lindstedt and Borrebaeck, 2011).

Abbreviations: ACD, allergic contact dermatitis; APIA, allergen–peptide/protein interaction assay; CA, cinnamic aldehyde; DNCB, 2,4-dinitrochlorobenzene; MS, mass spectrometry; SA, salicylic acid.

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Using an in vitro approach with three different synthetic peptides – lysine containing Ac-RFAAKAACOOH peptide, cysteine containing Ac-RFAACAA-COOH, and histidine carrying Ac-RFAA-HAA-COOH – Gerberick et al. established a promising direct peptide reactivity assay (DPRA) for screening of chemical allergens (Gerberick et al., 2004). In this useful assay, chemical peptide reactivity is quantitatively examined as percent peptide depletion, which is measured as the difference of the peptide concentration in the chemical peptide samples relative to the controls. Information on qualitative chemical–peptide interaction is not given. In contrast to this HPLC based peptide reactivity approach Aleksic et al. have investigated the intrinsic ability of selected sensitizers or non-sensitizers to modify specific amino acid residues of human serum albumin and skin-related human model proteins (Aleksic et al., 2007, 2008). By using modern proteomics technologies, like matrix assisted laser desorption/ionization mass spectrometry (MALDI–MS) and LC–MS/MS, their data reveal DNCB-specific modifications of Lys, Cys and Tyr residues on human serum albumin (HSA) as well as on keratinocyte-related keratin 14 and cofilin protein. Results reported by both studies converge in demonstrating evidence that small reactive chemical dependent cysteine modifications seem to play a significant role in peptide/protein haptentation, being indicative for skin sensitizers but not for skin irritants.

To combine both, Cys-specific peptide depletion and qualitative information on chemical induced amino acid residue modification the allergen–peptide/protein interaction assay, APIA, has been developed. The APIA as presented in its initial form is a dual peptide approach focussing on sensitizer induced Cys modifications being monitored by modern mass spectrometry. Similar to Aleksic's work with HSA (Aleksic et al., 2007), skin-related in vitro conditions were chosen to mimic the physiological skin reactivity compartment. Assay limitations as well as future improvements are also considered.

Standard operation procedure of the allergen–peptide/protein interaction assay for identifying potentially allergenic small reactive chemicals.

2. Purpose

The haptentation process is regarded as a central indicative element of a consecutive allergic or non-allergic reaction. The allergen–peptide/protein interaction assay (APIA) therefore focuses on the initial haptentation processes in human skin and evaluates interaction reactions between small reactive chemicals – the potential allergens – and (skin-related) proteins or peptides (Bauer et al., 2011). One known useful skin-related model protein is human serum albumin, but to simplify the mass spectrometric test in its initial phase naturally occurring or synthetic target peptides are utilized. The current test system uses two synthetic model peptides: the all amino acids containing peptide-21 (plus Cys) and the peptide-20 (minus Cys). Both, Cys-specific adduct formation (yes/no) and the concomitant chemical-specific peptide mass shifts and peptide depletions are detected. In vitro conditions chosen reflect partially the skin reactivity in vivo compartments (pH5.5, pH6.8, pH7.4) but are not optimized to non-physiological chemical-specific or more reaction type specific conditions, e.g. with no skin-related higher/lower pH values (electrophils/nucleophils; e.g. Lys residues are typically protonated at pH 7.4, and therefore not nucleophilic at this pH). The mass spectrometric based, allergen–peptide/protein interaction assay is also applicable to higher throughput screening.

3. Limitations

- assay focus lies on allergen-/chemical-specific Cys-peptide modification (dual peptide approach), but other amino acid modifications may occur; however, they are co-analyzed by both peptide modifications (peptide-20 and peptide-21);

- skin-related pH values do not completely reflect in situ micro-environments, and peptides actually used are not yet skin-related;
- allergen–protein interactions are more complex, but can also be evaluated if in-depth MS analyses follow;
- non-covalent small chemical reactions may not be detected or valued as unspecific reactions; respiratory allergens may partially be recognized;
- reactivity or chemical–peptide adduct formation is influenced by reaction time, incubation temperature, and allergen or chemical to peptide ratios;
- expected chemical-specific adduct formation has to be calculated beforehand;
- immunological and immunotoxicological issue: chemical-specific modifications may be unexpectedly protective/simple detoxifying/tolerance inducing;
- a complementary software data analysis tool – related to the LLNA data – would be extremely helpful;
- similar to previous observations (Aleksic et al., 2007, 2008), relative signal intensities may vary depending on the position of the MALDI–MS laser beam and laser intensity at the time of analysis, but may be compensated by repeated runs and additional qualitative and quantitative calibrations.

4. Method outline

Chemical reactivity is tested by incubation of small reactive chemicals with two similar peptides, peptide-21 (plus Cys) and peptide-20 (minus Cys). As end points of this dual peptide approach chemical-specific peptide shifts (m/z), peptide depletion, and Cys reactivity are measured. Three different skin-related in vitro conditions (varying pH) are analyzed.

5. Definitions/abbreviations

CA	cinnamic aldehyde
Cys	cysteine
DMSO	dimethyl sulfoxide
DNCB	2,4-dinitrochlorobenzene
HCCA	α -cyano-4-hydroxycinnamic acid
HSA	human serum albumin
LC–MS	LIQUID chromatography mass spectrometry
MALDI–MS	matrix-assisted laser desorption/ionization mass spectrometry
MS	mass spectrometry
Peptide-20	synthetic peptide, minus Cys
Peptide-21	synthetic all amino acids carrying peptide, plus Cys
SA	salicylic acid
TFA	trifluoroacetic acid
TOF	time of flight

6. Materials

6.1. Peptides

- Peptide-21 (LHKSMGRTWQFDYNPEACVIK) and
- peptide-20 (LHKSMGRTWQFDYNPEAVIK) were synthesized according to the method of Dormeyer et al. (Dormeyer et al., 2005), and are commercially available from PSL Laboratories (Heidelberg, Germany).

6.2. Technical equipment

- Eppendorf Thermomixer® R Dry Block Heating and Cooling Shaker (Eppendorf AG, Hamburg, Germany).

- Bruker Daltonics ultraflex MALDI TOF/TOF Mass Spectrometer (Bruker Daltonik, Bremen, Germany).
- MTP AnchorChip™ 384 T F; MALDI target for scout MTP ion sources with 384 anchors (approx. 800 µm diameter) for sample preparation plus 96 calib-anchors (additional anchor for external calibration); dedicated for autoflex and ultraflex series mass spectrometers and compatible with Dual ESI/MALDI Source (identical with former MTP AnchorChip™ 800/384 T F; Bruker Daltonik, Bremen, Germany).
- C18 ZipTip pipette tips; ZipTip is a 10 µL pipette tip with a 0.6 or 0.2 µL bed of chromatography media, and it is used for concentrating and purifying samples (Millipore, Schwalbach, Germany).

6.3. Chemicals, Chemical Solutions, MALDI–MS Calibration Kit, Reagent Tubes

– Chemicals

Cinnamic aldehyde (CA), 2,4-dinitrochlorobenzene (DNCB), salicylic acid (SA), and further chemicals of highest purity available were used, like DMSO, HCCA and TFA, all supplied by Sigma–Aldrich (Munich, Germany).

– Chemical Solutions

Example: Stock solutions were made in 100% dimethyl sulfoxide to final concentrations of 2.5 M 2,4-dinitrochlorobenzene (DNCB), 1 M cinnamic aldehyde (CA) and 1 M salicylic acid (SA). CA was diluted in 50 mM ammonium acetate buffer to final working solutions of 10 mM and 100 mM. All working solutions were freshly prepared and diluted to 1 mM, 2.5 mM or 5 mM final concentration. DNCB was diluted in 50 mM ammonium acetate buffer to final working solutions of 10 mM and 100 mM. All working solutions were freshly prepared and diluted to 1 mM, 2.5 mM or 5 mM final concentration. SA was diluted in 50 mM ammonium acetate buffer to final working solutions of 10 mM and 100 mM. All working solutions were freshly prepared and diluted to 1 mM, 2.5 mM or 5 mM final concentration.

– MALDI–MS Calibration Kit:

- ProteoMass™ Peptide MALDI–MS Calibration Kit MSCAL2 was from Sigma–Aldrich (Munich, Germany).
- Reagent Tubes:
- 1.5 ml safe-lock microcentrifuge tube, e.g. Eppendorf, 0030 120.086,
- 15 ml tubes, e.g. BD Falcon, 352096,
- 50 ml tubes, e.g. BD Falcon, 352070.

7. Methods

7.1. Allergen/chemical–peptide interaction

Described chemical working solutions were added to 0.05 mM final concentration of peptide-21 or of peptide-20. Solutions were prepared at pH values of pH 5.5, pH 6.8 and pH 7.4, starting with molar chemical:peptide ratios of 20:1, 50:1 or 100:1. Ratios were adapted from chemical:protein ratios as applied in the qualitative mass spectrometric study of Aleksic et al. (2007). One identical, and additional lower ratios were chosen, since under in vivo conditions higher and lower chemical:peptide or chemical:protein ratios have to be expected in human skin. Negative controls were examined with peptide-21 or peptide-20 using the same conditions. Each single incubation mixture was gently shaken for 24 h at 37 °C (Eppendorf Thermomixer®). The reaction was stopped by freezing the samples at –20 °C. Experiments were performed in

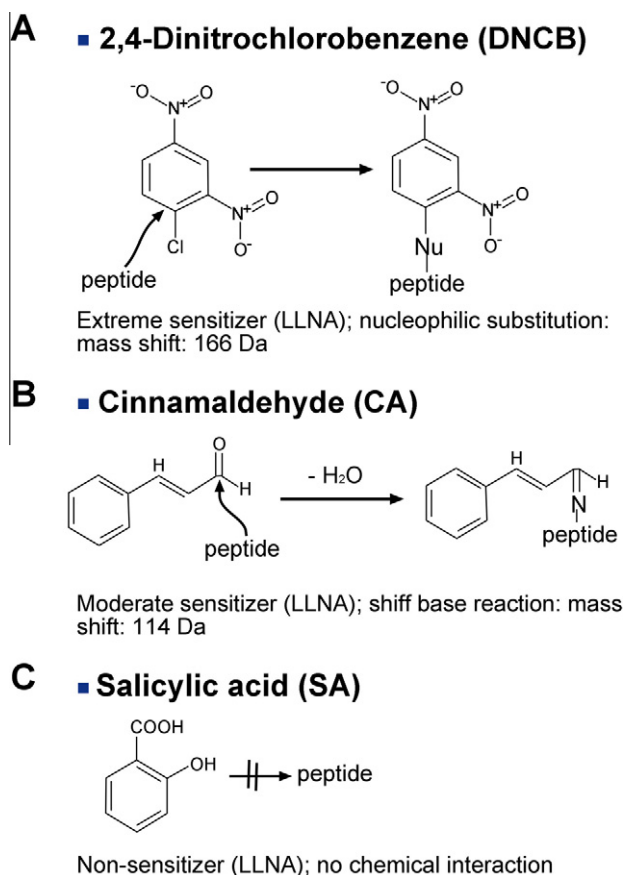


Fig. 1. Chemical reactions of two skin sensitizers, 2,4-dinitrochlorobenzene (extreme sensitizer, LLNA) and cinnamaldehyde (moderate sensitizer, LLNA), and a non-sensitizer, salicylic acid.

quadruplicates and expected peptide modifications are shown in Fig. 1.

7.2. MALDI-TOF readout analyses

Each sample was diluted 1:20 in 0.1% TFA to reduce the DMSO content to a maximum of 0.05%. Samples were concentrated using 10 µL C18 ZIPTIP pipette tips according to the manufacturer's instructions. Subsequently samples were eluted with 1 µL HCCA solution (saturated HCCA was diluted 1:7 (v/v) in 1:1 acetonitrile:0.1% TFA (v/v)) and spotted on a MALDI 800–384 AnchorChip target plate according to the manufacturer's instructions. Analysis was performed on an Ultraflex I MALDI TOF instrument using Bruker Flex-control, Flex-analysis, and Biotoools software.

Calibration was performed externally using SIGMA ProteoMass™ Peptide MALDI–MS Calibration Kit. MALDI mass spectra were recorded within an m/z range of 700–3500 in the positive ion reflector mode. Mass spectra were obtained by averaging up to 300 individual laser shots. Each analysis was performed in triplicate.

7.3. Endpoints and data analysis

Endpoints of chemical–peptide adduct formation are assessed based on chemical-specific calculated mass shifts by MALDI-TOF mass spectrometry (m/z). Peptide depletion is regarded as a secondary readout criterion, and cysteine specificity is confirmed by dual peptide approach of peptide-21 and peptide-20. Since the chemical to peptide ratio of 20:1 was most effective, this ratio should be used as standard.

8. Health safety and environment

According to material safety data sheets (MSDS), available from the specific suppliers, e.g. Sigma (www.sigmaaldrich.com/catalog/DisplayMSDSContent.do).

9. Additional information

Website: www.sens-it-iv.eu and Sens-it-iv Newsletters (see homepage).

10. Results

Interaction of small reactive chemicals with self-proteins or -peptides is a key step in human skin sensitization, and essential to induce chemical-specific, disease triggering human T cell responses. Hence, to mimic physiological pH values found in different layers of human skin corresponding assay conditions were chosen, pH 5.5 – for skin surface, pH 6.8 – for stratum basale conditions, and pH 7.4 as typical physiological pH. To examine proposed assay system three distinct model chemicals were selected from the chemical list established by EU project Sens-it-iv (www.sens-it-iv.eu): extreme sensitizer 2,4-dinitrochlorobenzene, moderate sensitizer cinnamic aldehyde, and salicylic acid as non-sensitizer. Each compound was incubated in quadruplicates with peptide-21 or peptide-20 for 24 h, and chemical reaction was stopped by freezing. Then, after peptides have been concentrated by C18 ZIPTIPs, mass spectrometric analyses were performed. As demonstrated by Fig. 2A treatment with strong sensitizer DNCB resulted in new MS-signals corresponding to the 166 *m/z* mass shift of peptide-21 at all skin relevant pH conditions tested (pH 5.5, pH 6.8, pH 7.4). DNCB modified peptide-21 showed the highest adduct formation rates at pH 6.8 and pH 7.4. Moreover, no peptide modification was observed with Cys-free variant of peptide-21 (peptide-20), indicating an amino acid specific reaction preference for DNCB under skin-related pH conditions chosen (Fig. 2B). Treatment with moderate sensitizer cinnamic aldehyde also resulted in new adduct formation, here with chemical-specific corresponding 114 *m/z* mass shifts of peptide-21, at all skin relevant pH conditions applied (pH 5.5, pH 6.8, pH 7.4). Similar results were obtained with cinnamic aldehyde. CA modified peptide-21 showed the highest adduct formation rates at pH 6.8 and pH 7.4, and no cinnamic aldehyde dependent peptide modification was observed with the Cys-free peptide-20 (Fig. 2B). However, an additional oxygen dependent MS-peak – plus 16 *m/z* – was measured after incubation of peptide-21 with cinnamic aldehyde suggesting selective oxidative product reactivity (Fig. 2A).

Next, potential salicylic acid dependent peptide modifications were studied. Remarkably, mass spectra of both target peptides revealed neither any modification of peptide-21 nor of peptide-20 by SA under skin-related in vitro conditions (Fig. 2A and B). In summary, data clearly reveal pH dependent differential allergen DNCB- and CA-specific peptide interaction and adduct formation, with concomitant partial peptide depletion, and selective modification of Cys-residues as demonstrated by peptide-21. Non-allergenic compound SA did not interfere with Cys containing peptide-21 or Cys-free peptide-20.

11. Discussion

Many factors contribute to the manifestation of human ACD, such as agent bioavailability in human skin, chemical structure and properties, peptide/protein interaction reactivity and specificity, cellular migration and differentiation of human antigen presenting cells, clonal activation of effector/regulatory T cells in the

draining local lymph nodes, memory cell generation and cellular recirculation to human epidermis. Furthermore, concomitant inflammatory innate immune responses such as those from keratinocytes and fibroblasts also influence ACD (Cavani, 2008; Karlberg et al., 2008; Martin et al., 2011; Smith Pease, 2003; Toebak et al., 2009). Thus, trying to replace immunologically very complex animal models, like the LLNA for skin sensitization, by just one single in vitro assay seems to be almost impossible. However, collective observations from a battery of new different types of predictive in vitro assays may give sufficient information to replace such whole body models.

Chemical-peptide/protein interaction either with extracellular skin proteins or with cell-associated proteins or peptides is one essential checkpoint in ACD. Remarkably, most related studies focusing into the chemical-peptide or chemical-protein interactions used either quantitative depletion readout or analyzed qualitative adduct formation, but did not combine quantitative and qualitative data simultaneously (Gerberick et al., 2008). To overcome this limitation we established a MALDI-MS based allergen-peptide/protein interaction assay, APIA. The APIA, as introduced in 2008, at the Sens-it-iv meeting in Estoril, Portugal, provides relative quantitative information on peptide depletion and additional qualitative mass spectrometric data on the modified amino acid target. However, relative quantitative analyses are technically restricted to laser beam and peptide flight quality as well as to peptide distribution on the anchor chip utilized, thus appropriate quality control experiments are necessary.

The new assay uses a dual peptide approach to focus into amino acid and specifically into crucial cysteine modifications (peptide-21/-20, $\pm$ Cys; Fig. 3). Interaction of the model peptide-21, composed of all amino acids, with sensitizer DNCB and/or sensitizer CA demonstrated exclusive thiol-dependent Cys modification at all pH values tested, corresponding to the skin surface (pH 5.5), stratum basale in the epidermis (pH 6.8), and physiological pH (pH 7.4) (Ohman and Vahlquist, 1994). In contrast, non-sensitizer SA did not chemically interfere with any amino acid offered by peptide-21 or peptide-20. Thus, usage of synthetic peptide-21 and peptide-20 gives an initial reactivity profile of each chemical examined, allowing to determine chemical specific modifications of distinct amino acids, e.g. cysteine, lysine, or tyrosine by mass spectrometry. Moreover, such reactivity profiles generated under skin-related in vitro conditions may not only help to classify chemicals, and to distinguish between sensitizers and non-sensitizers, but may also help to explain chemical-specific T-cell responses or unresponsiveness. Several studies supporting this view have already shown, that sensitizer-specific modified self-proteins like albumin – and perhaps also peptides – are capable to induce sensitizer-specific human T cell responses. All of them, human albumin conjugated to penicillin G (Brander et al., 1995) or bound to common contact allergen nickel (Thierse et al., 2004, 2005) or conjugated to sensitizer PPD (Jenkinson et al., 2010) were capable to induce allergen-specific human T cell responses.

Evidences for a dominant role of Cys but not Lys involvement during ACD are given by several other observations. By using a synthetic model carrier peptide with all nucleophilic amino acids, H-Pro-His-Cys-Lys-Arg-Met-OH Ahlfors et al. showed significant peptide interaction of two electrophilic haptens to Cys but not to Lys residue. Moreover, they found – like in our study – in contrast to the immunologic hapten-lysine theory, that the only site of modification on the model peptide was on the thiol group of cysteine and not on Lys (Ahlfors et al., 2003). Further evidence of thiol group participation during sensitization is given by the observation that sensitizer induced tyrosine phosphorylation by contact sensitizers can be blocked by compounds with free sulfhydryl groups (Bruchhausen et al., 2003); and that thiol-groups seem to play an important role in activation of THP1 and dendritic cells by small

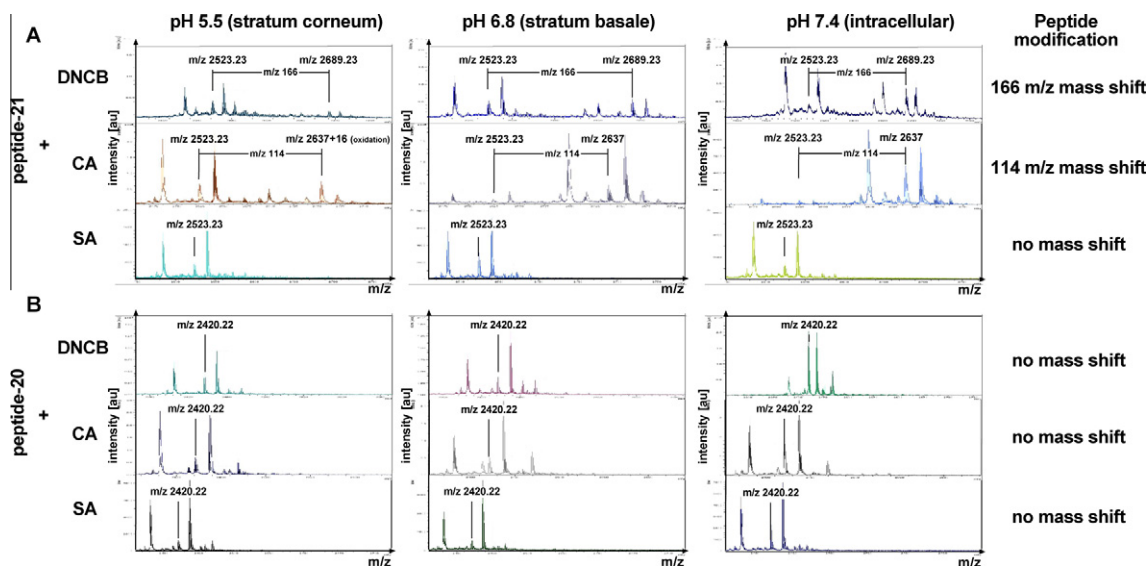


Fig. 2. Mass spectrometric identification of allergen-peptide/protein interactions are influenced by skin-related in vitro conditions (pH 5.5–pH 6.8–pH 7.4). (A) MALDI-TOF mass spectrometric analysis of DNCB- and CA-induced adduct formation with peptide-21 (Cys+) revealed new DNP-specific peptide signals (peptide-21-DNP) at skin-related pH conditions: pH 5.5 (stratum corneum), pH 6.8 (stratum basale), pH 7.4 (physiological). (B) No peptide modifications and mass shifts were detected after incubation of peptide-20 (Cys-) with either DNCB and CA, or SA, regardless of pH condition. Data are representative of 3 independent experiments and MALDI-MS signal intensity is given in arbitrary units (a.u.).

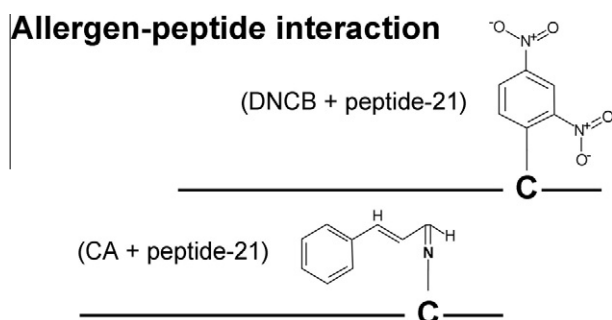


Fig. 3. Dual peptide approach: peptide-21 (Cys+) reacts with DNCB and CA, whereas peptide-20 (Cys-) does not react. The dual peptide analysis allows direct confirmation of amino acid specific modification by small reactive chemicals. Non-Cys peptide modifications are expected to generate additional adduct MS-signals with both peptide-21 (Cys+) and peptide-20 (Cys-).

reactive sensitizers (Hirota et al., 2009; Kagatani et al., 2010; Suzuki et al., 2009). In addition, by using thiol-reactive model haptens, skin-related data of Simonsson et al., indicated that Cys 54 modification of keratin K5 and keratin K14 may have an important role in the induction of ACD (Lonsdorf and Enk, 2011a,b; Simonsson et al., 2011). Focusing on Cys reactive chemicals seems to be one indicative step during human ACD, thus supporting the implementation of the APIA assay with Cys-reactive peptide-21.

Furthermore, with the APIA we introduce skin-related reactivity conditions, partially reflecting physiological parameters of human skin (Ohman and Vahlquist, 1994). We are thereby trying to detect most relevant amino acid residue modifications triggering the adaptive immune response. The current assay protocol offers a robust proteomic in vitro analysis platform, which is also adaptable to high throughput screening (Bruker Daltonics). In addition, we are currently working on implementing naturally occurring human skin target peptides and proteins to the APIA discovery platform, which will be followed by a more comprehensive predictive chemical testing phase.

Complementary to recently developed LC-MS approaches (Aleksic et al., 2009; Natsch and Gfeller, 2008; Troutman et al.,

2011) MALDI-MS based APIA offers, qualitatively and quantitatively an additional, promising tool not only for predictive sensitization in vitro assay management, but also for REACH and general hazard risk assessment. However, when related to high throughput LC-MS data of Natsch and Gfeller, and to reach higher APIA assay robustness, further sensitizers and non-sensitizers remain to be assessed.

Conflict of interest statement

The author(s) declare that there are no conflicts of interest.

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