

Application of Proteomics for the Characterization of Biological Allergen Extracts

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1. Introduction

Quality control for the release of allergenic extracts used for desensitization relies mainly on methods focusing on allergens. The latter include ELISA-inhibition for the immunological activity, as well as specific antibody-based assays for the quantification of major allergens.

Yet, allergenic extracts are complex molecular mixtures in which allergens account only for a fraction of the overall composition. In this context, exploratory methods aimed at characterizing non-allergenic molecules, most particularly proteins, allow to further document the robustness of a given manufacturing process of allergenic products, as well as the comparability between distinct manufacturing processes. To this end, it is now possible to harness the power of proteomics. The latter includes methods allowing to analyze accurately protein profiles of complex biological extracts, such as:

- 2D-PAGE (two-dimensional polyacrylamide gel electrophoresis) and data searching against expressed sequence tags (EST) [1],
- 2D-DIGE (two-dimensional differential gel electrophoresis) [2–3],
- SELDI-TOF MS (surface enhanced laser desorption/ionization time-of-flight mass spectrometry) [4].

Proteomic techniques remain complex and cumbersome to implement. As such, they cannot be used for routine batch release but should rather be used for exploratory purposes, such as molecular characterization and support to industrial process development.

Herein, we document the application of 2D-PAGE and data analysis against EST,

2D-DIGE and SELDI-TOF MS to extracts (e.g. grass pollen or mite) for three different purposes, namely:

- characterization of such extracts,
- demonstration of the robustness of a manufacturing process,
- comparability of distinct manufacturing processes.

2. Methods

2.1. 2D-PAGE and data searching against EST

Proteins from a *D. pteronyssinus* extract are first separated using 2D-PAGE. Then, protein spots are excised from the Coomassie stained gels, washed, dried and analyzed by mass spectrometry after trypsin digestion. Identification of the proteins is performed using data searches against the in-house dust mite EST databases produced at the National University of Singapore [1].

2.2. 2D-DIGE

Proteins from two batches of five grass pollen extracts (namely ORALAIR® Grasses drug substance) are labeled with distinct fluorescent markers (cyanines) that are excitable at two different wavelengths (Fig. 1A). After labeling, the two batches are mixed and their proteins are co-separated by two-dimensional gel electrophoresis (Fig. 1B). Using the appropriate excitation wavelength, the image related to each batch is obtained (Fig. 1C). The two images are then compared quantitatively over

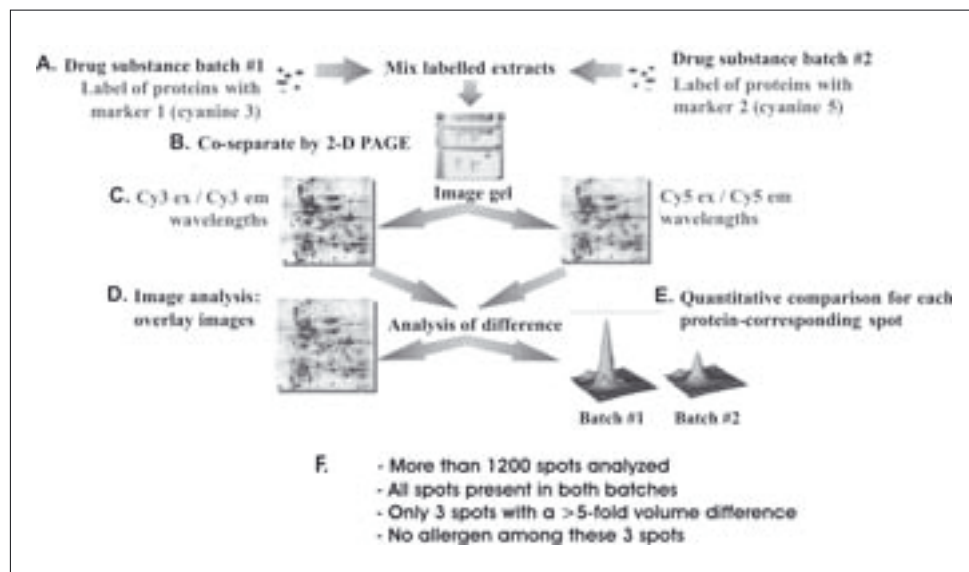


Fig. 1. Principle of 2D-DIGE (A-E) and results obtained for two batches of ORALAIR® Grasses drug substance (F).

the whole set of spots to determine whether each protein, corresponding to one spot, is more or less represented in one batch relative to the other one (Fig. 1D and E).

2.3. SELDI-TOF MS

Proteins from an extract are captured on solid-phase chip surfaces coated with various chemistries (e.g. negatively charged carboxylate chemistry that acts as a weak cation exchanger). After co-crystallization with a matrix, proteins are desorbed and ionized using a laser beam. The ionized molecules are accelerated under vacuum in an electric field towards an ion detector. The mass/charge (m/z) ratio of each protein species is recorded on the basis of the time required for the ions to reach the detector (the so-called “time-of-flight” or “TOF”). The output generated from the TOF MS analysis is a trace or a map showing the relative abundance versus the molecular masses of the detected proteins.

3. Results and discussion

3.1. Characterization of *D. pteronyssinus*

Table 1 displays examples of proteins from *D. pteronyssinus* that were identified using 2D-PAGE and data searching against EST after mass spectrometric analysis. This identification was performed for the 50 most abundant proteins of *D. pteronyssinus*, leading to the detection of four main categories of proteins, including allergens, within the proteome of this mite species.

Protein category	Examples
Cuticle proteins	adult specific rigid cuticular-like protein, cuticle-like protein 2
Cytoskeleton proteins	actin, tubulin
Enzymes and related proteins	acetylcholinesterase, protease inhibitor (ovalbumin type)
Allergens	Group 1, 2, 7, 10, 13, 20

Table 3. Some characteristics of methods to elucidate the quaternary structure of a protein

3.2. Robustness of a manufacturing process for allergenic extracts

In order to confirm the robustness of the manufacturing process of ORALAIR® Grasses drug substance, two batches were compared using 2D-DIGE. More than 1200 spots were analyzed, which were all present in the two batches. Moreover, only three spots (i.e. less than 0.3% of the spots) displayed a quantitative difference that was above of a 5-fold difference. When compared to 2D-PAGE followed by Western blotting, none of those three spots corresponded to an allergen.

Thus, 2D-DIGE confirmed the robustness of the manufacturing process of ORALAIR® Grasses drug substance.

3.3. Comparability of distinct manufacturing processes

In order to establish the equivalence between concurrent extraction of five grass pollens (*Dactylis glomerata*, *Phleum pratense*, *Lolium perenne*, *Anthoxanthum odoratum* and *Poa pratensis*) and mixture of five individual extracts of those grass pollens, extracts obtained under those two conditions were compared using SELDI-TOF MS. The patterns of protein masses obtained for the two kinds of extracts are fully comparable (Fig. 2), thus establishing the equivalence between the corresponding processes. It is worth noting that, due to its intrinsic variability, this method is not suitable for comparing the relative abundance of each protein.

SELDI-TOF was also used for comparing two scales of production of the ORALAIR® Grasses drug substance, namely 60-liter and 300-liter scales. A total of 21 peaks representative of the whole spectrum of masses of detected proteins were selected for statistical analysis (see Fig. 3 as an example). According to the latter, there was no statistical difference between the protein profiles obtained for the 60-L and the 300-L extracts. This established the comparability between the two scales of production, which was further confirmed using a 2D-DIGE analysis (data not shown).

4. Conclusion

Proteomic methods allow a better characterization of allergenic extracts by extending the data obtained for allergens. Such methods are most particularly suitable for documenting the robustness, as well as the comparability of manufacturing processes.

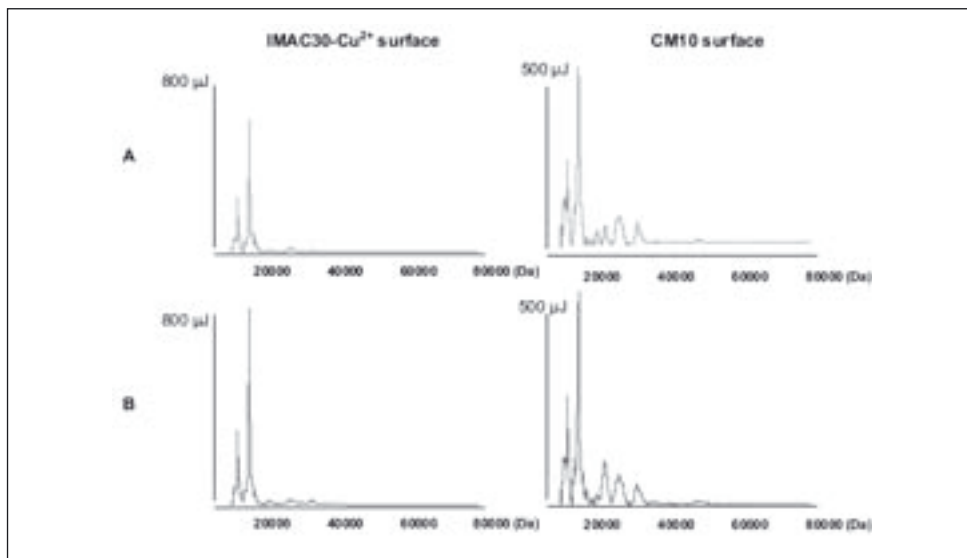


Fig. 2. Protein profiles of (A) a drug substance obtained after concurrent extraction of five grass pollens and (B) a mixture of drug substances obtained after individual extraction of the same five grass pollens, obtained by SELDI-TOF MS using two different surface chemistries, namely IMAC30-Cu²⁺ (i.e. Cu²⁺ coupled to nitroloacetic acid) and CM10 (i.e. negatively charged carboxylate that acts as a weak cation exchanger).

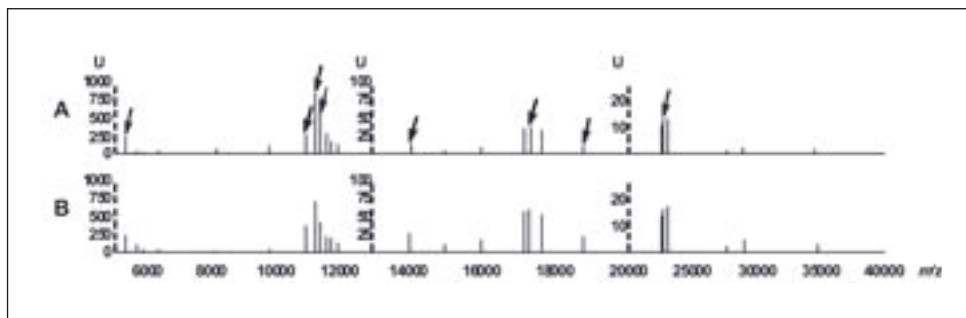


Fig. 3. Patterns of m/z values for ORALAIR® Grasses drug substance manufactured at the 60-liter (A) and the 300-liter (B) scales, obtained by SELDI-TOF MS using an IMAC- Cu^{2+} surface (i.e. Cu^{2+} coupled to nitroloacetic acid). Arrows point to peaks selected for statistical analysis.

Yet, they ideally have to be associated with functional assays (such as a basophil activation assay), as well as methods to analyze small molecules, such as the Th2-polarizing phytoprostanes found in pollen extracts [6].

5. References

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Discussion

Pauli: When you show the profiles of two different batches, can you not also show the different molecules in grass pollen, the important major allergens like *Phl p 1* and *Phl p 5*?

Batard: Actually, when we determined the profiles in SELDI-TOF and 2D-DIGE, e.g., we did not identify the proteins, this is a further step. We wanted to see the proteins as a whole and were not specially focusing on the allergens. Allergens are determined with other techniques, actually we especially used assays based on

monoclonal antibodies for the allergens. Of course it would be very nice to see the allergen profile – actually we did the 2D experiments with immunoprints and we were able to see the group 5a and group 1 isoforms. This way we were able to say that when we had three spots which were rather different between two batches that they were not allergens at all. But I could not show everything because that is too many data.

Fernandez-Caldas: It was a very nice talk. I wanted to ask you whether you used batches of grass pollens collected in different years for defining the robustness or comparability. Because we know they change in the composition of major and minor allergens. I see this as an exercise to look for consistency in the production or reproducibility of the extracts.

Batard: I do not have the details of the origin of these batches, but the batches are big batches and it is more than likely that all the batches that were used were from different years and also from different producers. And then they were mixed, yes.

Kober: I think these studies making use of proteomics to look at the allergen extracts are very interesting. I wonder whether you tried to correlate the results with immunoblotting or immunospot data or something else to assess the actual allergenic activities of these batches. As Bob Esch pointed out, the patients are very hard to standardize and you also have to consider the variability of the different patients. I mean it would be wonderful if we could use these nice proteomic measurements to define and to look at robustness. But how do they correlate with the actual clinical and biological activity of the extracts?

Batard: I would say we make it the other way round. We develop a product which is validated in clinical studies and during this procedure we characterize this extract. So we determine the allergen composition using methods based on monoclonal antibodies. Those extracts are manufactured reproducibly and we know they work, and that is it.

van Ree: Thierry, you stress so much that this is only exploratory, I do not understand why. When I see your 2D gel experiments this looks very nice. And even if you think that in a company that would be too much to ask, you could outsource it to a lab that does this very often. I think this type of techniques would be very nice as a batch-release test, but that is my personal opinion.

Batard: For documenting the dossier it is good, but as a batch-release test it still is really impossible. It is so cumbersome – for instance, it takes days for making 2D-DIGEs.

Vieths: I think one aspect maybe the cost because the staining technique is very expensive. So, if you do this for batch release, you pay a lot of money for your releasing procedure. The second comment I have is: I understand that you are afraid that we apply the 50–200 per-cent rule to 1200 spots on a gel. That is something I acknowledge.