Editors
Gianni Marone
Massimo Triggiani
Arturo Genovese

Translational Science: from Basic to Clinical Immunology and Allergy

Proceedings of the 28th Symposium of the *Collegium Internationale Allergologicum* (CIA)

PACINI
EDITORE
MEDICINA

ISBN 978-88-6315-466-5

Editing
PACINI EDITORE
Via A. Gherardesca
56121 Ospedaletto (Pisa)
www.pacinieditore.it
info@pacinieditore.it

Graphics and printing
Industrie Grafiche Pacini, Pisa, Italy

High-throughput NMR authentication of food allergens

K. Hoffmann-Sommergruber[1], S. Alessandri[2], A.I. Sancho[3], S. Vieths[4], P. Shewry[5], C. Mills[3]

[1] Department of Pathophysiology, Medical University of Vienna, Austria;
[2] Consorzio Interuniversitario Risonanze, Magnetiche di Metalloprotiene Paramagnetiche and Department of Agricultural Biotechnology, University of Florence, Italy;
[3] Institute of Food Research, Norwich, UK;
[4] Paul-Ehrlich-Institut, Langen, Germany; [5] Rothamsted Research, Harpenden, Hertfordshire

Summary

Background: Purified food allergens have to meet high quality criteria if they are to be used for *in vitro* diagnosis. Information on protein conformation from 1D-^{1}H-NMR analysis can make a significant contribution to the authentication of allergens. Furthermore, this method can be used to (i) compare the structures of allergens within a protein family, ii) determine the thermal stability of food allergens and iii) detect structural differences between recombinant and natural allergens.

Methods: The most important food allergens from cow's milk, hen's egg, fish, shrimp, peanut, hazelnut, celeriac, apple and peach were purified according to established purification protocols and their allergenic activity tested.

The presence and extent of protein tertiary structure was assessed by 1D-^{1}H-NMR experiments, using a 700 MHz field at 298K under variable buffer conditions. The impact of thermal treatment on the NMR spectra was also determined.

Results and conclusions: Analysis of the 1D-^{1}H-NMR spectra showed that some allergens had a rigid and extended tertiary structure while others showed only mobile flexible regions. A third group of allergens displayed both features with a rigid tertiary structure but also flexible mobile regions. In summary, 1D-^{1}H-NMR provides valuable information on the authenticity of allergen structure, requiring low protein concentrations and without the requirement for labelling with 15N and 13C.

Key words: food allergens, 1D-1H-NMR, circular dichroism, purified allergens

Introduction

Within the EC project EuroPrevall, a food allergen library was established based on coordinated and harmonised purification and characterisation. The most clinically relevant allergens of animal and plant food origin were selected and either produced as recombinant allergens or purified from natural sources (Tab. I) [1].

Since it is widely accepted that the structural integrity of allergens is relevant to their IgE binding capacity, the tertiary structure of the purified proteins was assessed by routinely used methods such as circular dichroism (CD) spectroscopy as well as by applying more specialised methods to determine structural features such as nuclear magnetic resonance spectrometry (NMR).

Address for correspondence:
Karin Hoffmann-Sommergruber
Assist. Prof, PhD, Department of Pathophysiology, Medical University Vienna, AKH-EBO 3Q, Waehringer Guertel 18-20, A-1090 Vienna, Austria.
Tel.: +43-1-40400-5104.
Fax: +43-1-404005130.
E-mail: Karin.Hoffmann@meduniwien.ac.at

Table I. Purified food allergens of plant and animal origin. After purification tertiary structure was assessed by 1D-NMR analysis.

Protein family	Source	Allergen	NMR analysis
nsLTP	Apple, peach, Hazelnut,	Mal d 3, Pru p 3, Cor a 8	Rigid tertiary structure
Profilin	Apple, Hazelnut, Celeriac	Mal d 4, Cor a 2, Api g 4	Rigid tertiary structure
Bet v 1 related	Apple, Peach, Hazelnut, Celeriac, Peanut	Mal d 1, Pru p 1, Cor a 1, Api g 1,Ara h 8	Rigid tertiary structure
2S Albumin	Peanut	Ara h 2, Ara h 6	Rigid tertiary structure
7/8 S Globulin	Hazelnut, Peanut	Cor a 11, Ara h 1	Rigid tertiary structure (flexible structural elements)
11 S Globulin	Hazelnut, Peanut	Cor a 9, Ara h 3/4	Flexible elements, no structure to be determined
Thaumatin-like protein	Apple	Mal d 2	Rigid tertiary structure
FAD-containing oxidase	Celeriac	Api g 5	Putatively folded
Casein	Cow's milk, Goat's milk	Bos d 8, goat's milk casein	No definite tertiary structure to be determined
Tropomyosin	Shrimp	Pen a 1	No definite tertiary structure to be determined
EF-hand domain (Parvalbumin)	Cod, Carp	Gad m 1, Cyp c 1	Rigid tertiary structure
Lipocalin (beta-lactoglobulin)	Cow's milk	Bos d 5	Rigid tertiary structure
C-type lysozyme (alpha-lactalbumin)	Cow's milk, hen's egg	Bos d 4, Gal d 4	Rigid tertiary structure
Kazal type protease Inhibitor	Hen's egg	Gal d 1	Rigid tertiary structure
Serpin	Hen's egg	Gal d 2	Rigid tertiary structure
Transferrin	Hen's egg	Gal d 3	Rigid tertiary structure
Serum Albumin	Hen's egg	Gal d 5	Rigid tertiary structure

NMR spectroscopy allows macromolecules of biological interest to be studied under physiological conditions at atomic resolution, providing detailed information on the structural, chemical and dynamic properties of proteins. While 2D NMR techniques are usually applied for detailed structural analysis, little work has been carried out to evaluate the use of 1D-^{1}H-NMR as a high throughput first step to assess the structural authenticity of purified proteins [2]. We therefore applied 1D ^{1}H-NMR to the range of purified protein allergens from the Europrevall allergen library to provide information on their tertiary structures.

Materials and Methods

The food allergens listed in Table I were purified as previously described [3-12]. For NMR analysis, purified allergens (0.06-0,39 mmol) in aqueous buffers (either 20-50 mM sodium phosphate buffer, pH 7.4 with and without 150 mM NaCl, or MOPS 20 mM, 0.5M NaCl, pH 7.4, 11% D_2O) analysed with an Avance 700 spectrometer (Bruker Com. Madison, WI, USA) at 700MHz. Two sets of experiments (minimum of 256 scans each) were performed at 25°C, one with the water signal being minimised and the other using water selective pulses. The consist-

ency of the spectra with information available on the structures of the allergens was also compared. For selected allergens the thermal stability was assessed by stepwise heating of the protein up to 85°C followed by cooling down to 25°C. The control and heated/cooled samples were then subjected to 1D-^{1}H-NMR analysis on a 600MHz spectrometer (Bruker Com. Madison, WI, USA) and the spectra compared.

Results

One dimensional NMR provides information on the structural properties of a folded protein at the atomic level under physiological conditions. A typical spectrum of a protein with a rigid tertiary structure, rPru p 1 which is the Bet v 1 homologue from peach, is presented in Figure 1A. A series of well resolved peaks above 9 ppm can be assigned to the backbone NH while the region below 8 ppm provides signals from the aromatic ring protons and side chain NH_2. Between 5.8 and 3.5 ppm the backbone alpha proton signals are seen, followed by the aliphatic region (3.5-0 ppm). Side chain methyl signals are found between 1 and -1 ppm. Using the 1D ^{1}H-NMR analysis structural changes due to physicochemical manipulations such as heat treatment can be identified. The Pru p 1 preparation was therefore heated stepwise to 80°C and the spectrum determined after cooling to 25°C. This heating and cooling did not affect the structure of Pru p 1 with well-resolved patterns of signals from 8.5 down to 0 ppm being virtually identical in the heated and unheated control samples (Fig. 1B). However, when Pru p 1 was heated to 85°C, changes in the overall structure were evident. In this case the well-resolved peaks are replaced by unresolved broad peaks, especially in the area of the side chain HN and the alpha protons (6-4 ppm; Fig. 1B). These data are consistent with previously reported CD data for Pru p 1 which also show irreversible structural changes on thermal treatment at temperatures above 80°C [8].

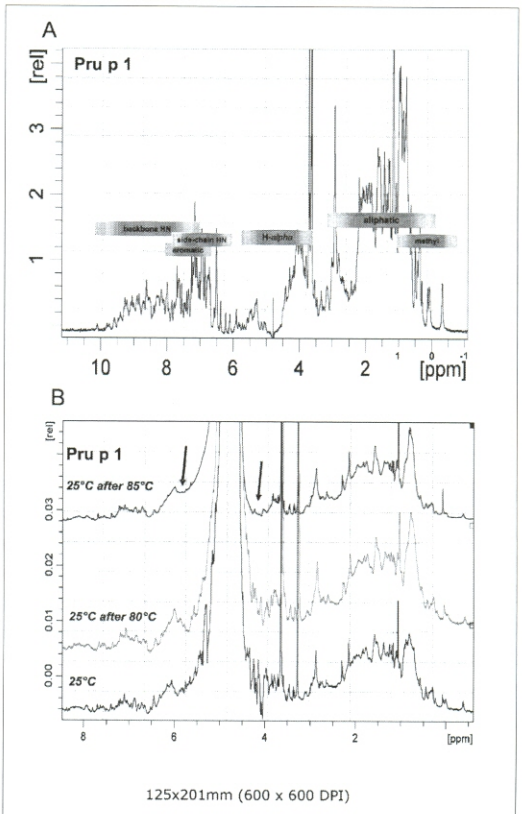

Figure 1. A: NMR 1D-^{1}H Spectra (600 MHz) of purified recombinant Pru p 1 (Bet v 1 homologue from peach). B: After stepwise heating followed by cooling down to 25°C NMR 1D-^{1}H spectra were obtained. Some changes in tertiary structure of Pru p 1 due to thermal treatment are marked by arrows.

A range of allergenic proteins of both plant and animal origin was subjected to 1D-^{1}H-NMR to assess their structural integrity after purification, as summarised in Table I. The majority of the proteins, including members of the Bet v 1 family, profilins, 2S albumins, and thaumatin-like proteins from plants, displayed a rigid tertiary structure. Allergens with similar defined structures from animal origin were parvalbumins, beta-lactoglobulin, and members of the C-type lysozyme, kazal-type protease inhibitor and serpin, transferrin and serum albumin families of proteins. By contrast, no tertiary structure was detected in the

case of caseins from cows' and goats' milk. These data are consistent with the accepted features of caseins which display a flexible, non-compact structure. The absence of a rigid tertiary structure characterises also the 1D-^{1}H-NMR spectrum of tropomyosin from shrimp. Similarly, no defined tertiary structure could be determined for 11S globulins from hazelnut and peanut. The spectra of several other proteins, the FAD- containing oxidase, Api g 5 from celeriac and Ara h 1, the 7S globulin from peanut, showed regions of both rigid and flexible/mobile structure.

Conclusions

A total of 31 purified allergens of diverse molecular masses (9 kDa-78 kDa) were analysed by 1D-^{1}H-NMR to determine their structural integrity. 1D ^{1}H NMR allows identification of the structural and dynamic properties of proteins at the atomic level under physiologic conditions. The method is less time consuming compared to 2D-NMR and provides data on the presence or absence of a rigid tertiary structure. In addition, structural changes due to thermal or enzymatic treatment can be readily identified by this method. However, 1D-^{1}H-NMR is currently limited to the analysis of proteins of molecular masses up to approximately 70 kDa. In summary, one dimensional 1H-NMR proved a highly useful method to determine the structural authenticity of proteins, even at low protein concentrations and without 15N and 13C labelling.

References

1 Hoffmann-Sommergruber K, Mills EN, Vieths S. *Coordinated and standardized production, purification and characterization of natural and recombinant food allergens to establish a food allergen library*. Mol Nutr Food Res 2008;52(Suppl.2):159-65.

2 Scheich C, Leitner D, Sievert V, et al. *Fast identification of folded human protein domains expressed in E. coli suitable for structural analysis*. BMC Struct Biol 2004;4:4.

3 Blanc F, Bernard H, Alessandri S, et al. *Update on optimized purification and characterization of natural milk allergens*. Mol Nutr Food Res 2008;52(Suppl.2):166-75.

4 Jacobsen B, Hoffmann-Sommergruber K, Have TT, et al. *The panel of egg allergens, Gal d 1-Gal d 5: Their improved purification and characterization*. Mol Nutr Food Res 2008;52(Suppl.2):176-85.

5 Albrecht M, Alessandri S, Conti A, et al. *High level expression, purification and physico- and immunochemical characterisation of recombinant Pen a 1: a major allergen of shrimp*. Mol Nutr Food Res 2008;52(Suppl.2):186-95.

6 Ma Y, Griesmeier U, Susani M, et al. *Comparison of natural and recombinant forms of the major fish allergen parvalbumin from cod and carp*. Mol Nutr Food Res 2008;52(Suppl.2):196-207.

7 Oberhuber C, Ma Y, Marsh J, et al. *Purification and characterisation of relevant natural and recombinant apple allergens*. Mol Nutr Food Res 2008;52(Suppl.2):208-19.

8 Gaier S, Marsh J, Oberhuber C, et al. *Purification and structural stability of the peach allergens Pru p 1 and Pru p 3*. Mol Nutr Food Res 2008;52(Suppl.2):220-9.

9 Bublin M, Lauer I, Oberhuber C, et al. *Production and characterization of an allergen panel for component-resolved diagnosis of celery allergy*. Mol Nutr Food Res 2008;52(Suppl.2):S241-50.

10 Rigby NM, Marsh J, Sancho AI, et al. *The purification and characterisation of allergenic hazelnut seed proteins*. Mol Nutr Food Res 2008;52(Suppl.2):251-61.

11 Lauer I, Alessandri S, Pokoj S, et al. *Expression and characterization of three important panallergens from hazelnut*. Mol Nutr Food Res 2008;52(Suppl.2):262-71.

12 Marsh J, Rigby N, Wellner K, et al. *Purification and characterisation of a panel of peanut allergens suitable for use in allergy diagnosis*. Mol Nutr Food Res 2008;52(Suppl.2):272-85.

Acknowledgements

This work was carried out within the EuroPrevall project, funded by the EU (contract number FOOD-CT-2005-514000). Rothamsted Research and IFR receive grant-aided support from the Biotechnology and Biological Sciences Research Council (BBSRC) of the UK.