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ANALYSIS OF ALLERGENIC PROTEINS BY MASS SPECTROMETRY

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ADDENDUM

Montalbán-López, M., Spolaore, B., Pinato, O., Martínez-Bueno, M., Valdivia, E., Maqueda, M., and Fontana, A. (2008) Characterization of linear forms of the circular enterocin AS-48 obtained by limited proteolysis. *FEBS Lett.* 582, 3237–324.

Pinato, O., Spolaore, B., Canton, M., Polverino de Laureto, P., and Fontana, A. The Interaction Of Apomyoglobin With Oleic Acid Leads To A Protein Complex That Displays Cellular Toxicity. *Manuscript in preparation.*

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ABBREVIATIONS

AcCN	acetonitrile
AEBSF	4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride
α-H	α-chain hemoglobin
β-H	β-chain hemoglobin
α-LA	bovine α-lactalbuimin
β-LG	β-lactoglobulin
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BSA	bovine serum albumin
CA	carbonic anhydrase
$CaCl_2$	calcium chloride
CAM	carbamidomethylation
CNBr	cyanogen bromide
DNPS-Cl	2,4-dinitrofenilsulfenyl chloride
ELISA	enzyme-linked immunosorbent assay
E:S	enzyme to substrate ratio
ESI	electrospray ionization
Gdn·HCl	guanidine hydrochloride
HIC	hydrophobic interaction chromatography
IAA	iodoacetamide
IgE	immunoglobulin type E
LC-MS/MS	liquid chromatography tandem mass spectrometry
LYS	lysozyme
MALDI	matrix-assisted laser desorption ionization
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MW	molecular weight
m/z	mass to charge ratio
NBT	nitroblue tetrazolio
NH_4HCO_3	ammonium bicarbonate
OA	oleic acid
OVA	ovalbumin
OVM	ovomucoid
PAGE	polyacrylamide gel electrophoresis
Q-Tof	quadrupole-time of flight
RP-HPLC	reverse phase high pressure liquid chromatography
SDS	sodium dodecyl sulphate
TCEP	Tris(2-carboxyethyl)fosfine
TEMED	tetra(methylethylene)diamine
TFA	trifluoroacetic acid
Tris·HCl	tris(hydroxymethyl)aminomethane
UV	ultraviolet

ABBREVIATION OF AMINO ACIDS

Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

SUMMARY

MS Analysis of Allergenic Proteins. Food allergy is a significant worldwide public health issue. Proteins from cow's milk, chicken eggs, soybean and peanuts are the most frequent allergens contained in the complex foods prepared by industrial processes. Allergenic proteins can induce allergic reaction in their native structural state or upon chemical or conformational changes induced by the industrial treatments. Nowadays, the identification of allergenic proteins in foods is conducted by using immunochemical methods such as ELISA tests, but these techniques suffer from several limitations due to cross-reactivity and false negative results. Indeed, alterations in the allergen's structure or chemical modifications can prevent the interaction with the antibody, thus causing misleading data.

Since allergens are toxic even in trace amounts, there is a need for reliable and sensitive analytical methods for allergenic proteins. The purpose of this PhD project was to develop procedures for the identification of these proteins in food samples by using mass spectrometry (MS), likely overcoming some limitations of the immunochemical assays. Indeed, the MS approach for identifying proteins makes use of data pertaining to the amino acid sequence of the protein, while immunochemical methods are linked to the integrity of the three-dimensional structure of proteins.

In order to test immunochemical approaches, polyclonal antibodies raised against the main allergenic proteins of milk (α-lactalbumin and β-lactoglobulin) and eggs (ovomucoid, ovalbumin and lysozyme) were purchased. Preliminary studies were performed in order to check the quality of the antibodies, in terms of specificity of recognition and cross-reactivity. Moreover, the responses of the antibodies using as antigens the purified commercial proteins and the same proteins contained in complex food matrices after thermal treatment were checked.

Since allergenic proteins usually are contained in complex mixtures of huge amounts of other proteins, the methodology nowadays named "targeted proteomics" was considered very appropriate. By this approach, a protein contained in a complex mixture can be identified by a MS analysis of a peptide fragment that is specific for the protein of interest and contained in the very complex mixture of a tryptic digest of a protein sample. The procedure involves specific labelling and isolation of the specific peptide, named

"proteotypic". To this aim, tryptophan (Trp) residues in proteins were modified by reaction with 2,4-dinitrophenyl-sulfenyl chloride (DNPS-Cl), that leads to a Trp-derivative with the DNPS label attached at 2-position of the indole nucleus. The selection of Trp(DNPS)-peptides from the complex mixture of a tryptic digest of a protein sample was achieved by exploiting the significant change in hydrophobicity and retention time of DNPS-modified peptides in a reverse-phase HPLC column. Moreover, DNPS-labelled Trp-peptides were isolated by hydrophobic interaction chromatography, as well as by immunoaffinity chromatography using a column prepared with anti-DNP antibodies. The "targeted proteomics" procedure was optimised using a mixture of model proteins and then applied to identify a protein allergen contained in a raw bakery product. Overall, it was demonstrated that the novel procedure of selective labelling and isolation of Trp-peptides allows a considerable simplification of the fingerprinting/MS approaches nowadays used for the identification of proteins in proteomics research.

Other Research Activities. During the PhD course I had the opportunity to collaborate with other members of the lab in a couple of additional projects, partly as a continuation of previous research conducted for the doctoral thesis. Documentation of this activity is herewith included as an Appendix at the end of this PhD Thesis.

The molecular properties of the complex formed by α-lactalbumin with oleic acid were investigated in detail. This complex appears to be very interesting, since it has been shown to display cellular toxicity specifically for cancer cells. It was shown that the protein in the complex is in an oligomeric state, at variance from previous statements that the protein was monomeric. Moreover, it was shown the oleic acid can interact also with other proteins, including apomyoglobin. The main conclusion of this work was that the protein moiety serves as a carrier of the otherwise poorly soluble fatty acid, thus leading to an enhancement of its water solubility and consequently of its intrinsic cytotoxic properties. A manuscript rescrubbing these results is in an advanced state of preparation.

Enterocin AS-48 is a 70-residue circular polypeptide produced by *Enterococcus faecalis* displaying a wide antibacterial activity. Limited proteolysis of AS-48 was used to prepare a linear form of this enterocin, as well as 38- and 55-residue fragments. Nicked AS-48 showed a lower helicity by far-ultraviolet circular dichroism and a reduced stability to thermal denaturation, but it was active against the sensitive bacteria assayed. The fragments also partly retained the biological activity of the intact protein. These results indicate that the circularization phenomenon is not required for the antibacterial activity,

but it is crucial for the stabilization of the native structural state. This research was published in *FEBS Lett.* (2008).

Summing up, the PhD Thesis is composed of a major part dealing with the development of MS methods for the identification of allergenic proteins in food matrices. The novelty of the MS method herewith investigated resides in the use of a selective labelling reaction for Trp-residues using DNPS-Cl, allowing an easy separation of Trp-peptides by reverse-phase HPLC, hydrophobic interaction chromatography and immunoaffinity chromatography using immobilised anti-DNP antibodies. The DNPS-labelled Trp-peptides are then analysed for their amino acid sequence by MS/MS and then related to the parent protein by interrogating protein databases. The method has been validated using a mixture of model proteins, as well as an extract of proteins from a raw bakery product. The research activity conducted in the ambit of the PhD allowed me to acquire significant expertise in modern methods of analysis of proteins, ranging from chromatographic techniques, biospectroscopy, bioinformatics and mass spectrometry.

RIASSUNTO

Analisi di proteine allergeniche mediante spettrometria di massa. Le allergie alimentari rappresentano ormai una problematica clinica di livello mondiale. Tra i prodotti alimentari considerati pericolosi per il loro elevato contenuto in proteine allergeniche troviamo il latte bovino, le uova, la soia e le arachidi. Le proteine allergeniche possono scatenare reazioni allergiche sia mantenendo la loro struttura nativa, sia in seguito a modifiche chimiche e conformazionali indotte dai processi industriali. I metodi d'elezione applicati per l'identificazione di proteine allergeniche negli alimenti sono rappresentati dai saggi immunochimici come i test ELISA. Tali metodi presentano però numerose limitazioni causate da fenomeni di cross reattività e da falsi positivi. Inoltre, alterazioni nella struttura delle proteine allergeniche o eventuali modifiche chimiche possono modificare l'interazione con gli anticorpi specifici, invalidando i risultati.

Dal momento che gli allergeni sono tossici anche in tracce, è necessario sviluppare dei metodi analitici efficaci e affidabili per la loro identificazione. Lo scopo di questo progetto di tesi è stato quello di sviluppare delle procedure per l'identificazione di proteine allergeniche mediante spettrometria di massa (MS) che possano superare i limiti metodici dei saggi immunologici. Oltretutto, l'identificazione delle proteine mediante MS si basa sull'analisi della sequenza amminoacidica di quest'ultime, mentre i saggi immunochimici sono strettamente dipendenti dall'integrità della struttura tridimensionale della proteina antigenica.

Al fine di testare la validità dell'approccio immnuchimico, sono stati testati alcuni anticorpi policlonali diretti contro le principali proteine allergeniche di latte (α-lattalbumina e β-lattoglobulina) e uova (ovomucoide, ovalbumina e lisozima). Sono stati condotti alcuni studi preliminari per validare la qualità di questi anticorpi, in termini di specificità di riconoscimento della proteina antigenica e della presenza di eventuali fenomeni di cross reattività. Inoltre, è stata valutata la risposta anticorpale usando come antigeni sia le proteine commerciali purificate, sia le stesse proteine contenute in prodotti alimentari prima e dopo trattamento termico.

Dato che le proteine allergeniche sono contenute in miscele complesse costituite da altre proteine, è stata considerata estremamente appropriata l'applicazione di una tecnica detta "*targeted chromatography*". Secondo questo strategia, è possibile identificare

mediante MS una proteina contenuta in una miscela complessa attraverso l'analisi di alcuni frammenti peptidici derivati dalla digestione triptica, che sono specifici della proteina stessa. Questa procedura prevede la modifica chimica e il successivo isolamento di specifici peptidi detti "prototipici". A tale scopo, i residui di triptofano contenuti nelle proteine sono stati chimicamente modificati mediante una reazione con il composto 2,4-dinitrofenilsulfenil cloruro (DNPS-Cl), che porta alla formazione di un derivato triptofanilico, con il DNPS legato in posizione 2 dell'anello indolico. La selezione dei peptidi modificati con il DNPS-Cl contenuti in una miscela triptica è stata effettuata sfruttando l'aumento di idrofobicità e del tempo di ritenzione di questi peptidi modificati in una colonna HPLC a fase inversa. Inoltre, gli stessi peptidi modificati con DNPS-Cl sono stati isolati mediante cromatografia per immunoaffinità utilizzando una resina derivatizzata con anticorpi monoclonali diretti contro il gruppo DNP. La strategia di "*targeted proteomics*" è stata ottimizzata utilizzando una miscela modello di sette proteine e successivamente è stata applicata per l'identificazione di una proteina allergenica contenuta in un prodotto dolciario. È stato inoltre dimostrato che queste nuove procedure di modifica selettiva e di selezione dei peptidi triptofanilici permette di semplificare considerevolmente l'analisi di fingerprinting/MS che è solitamente utilizzata per l'identificazione di proteine nei protocolli di proteomica.

Altre attività di ricerca. Durante il periodo di dottorato, ho avuto l'opportunità di collaborare con altri membri del laboratorio in due progetti addizionali come continuazione di un progetto di ricerca precedente. La documentazione relativa a queste attività è riportata in appendice alla tesi di dottorato.

Sono state investigate le proprietà molecolari del complesso formato da α-lattalbumina con l'acido oleico. Il complesso appare interessante poiché ha mostrato avere tossicità cellulare diretta selettivamente contro cellule tumorali. È stato dimostrato che la proteina nel complesso ha una struttura oligomerica, diversamente da quanto riportato nelle prime osservazioni, che ipotizzavano fosse in uno stato monometrico. Inoltre, è stato osservato che l'acido oleico interagisce anche con altre proteine, come l'apomioglobina. La principale conclusione di questo lavoro è stata che il motivo oligomerico della proteina veicola l'acido oleico, normalmente poco solubile, favorendo quindi la solubilizzazione dell'acido grasso e conseguentemente della sua proprietà citotossica. È in preparazione un articolo riguardante questi risultati.

L'enterocina AS-48, è un polipeptide circolare di 70 residui prodotto da *Enterococcus faecalis* che mostra a vere attività antibatterica. La proteolisi limitata è stata

usata per preparare una forma lineare e due frammenti di questa enterocina. Misure di dicroismo circolare nel lontano ultravioletto hanno dimostrato che la proteina ha una bassa ellitticità e una ridotta stabilità alla denaturazione termica, ma mantiene la sua attività antibatterica., mentre i frammenti presentano un'attività ridotta. Questi risultati indicano che la circolarizzazione è un fenomeno che non è richiesto per l'attività antibatterica, ma è cruciale per la stabilizzazione della struttura nativa. Questa ricerca è stata pubblicata in *FEBS Lett.* (2008).

Riassumendo, questa tesi di dottorato verte principalmente sullo sviluppo di metodologie di MS per l'identificazione di proteine allergeniche contenute in prodotti alimentari. La peculiarità e la novità del metodo MS qui investigato risiede nell'uso di una reazione di derivatizzazione dei residui di triptofano con DNPS-Cl, permettendo una separazione semplificata dei peptidi triptofanilici in HPLC su fase inversa, in cromatografia per interazione idrofobica e in cromatografia per immunoaffnità utilizzando una resina derivatizzata con anticorpi contro il gruppo DNP. La sequenza dei peptidi modificati con DNPS è stata poi analizzata mediante MS/MS e l'identità della proteina precursore è stata validata mediante ricerca in banca dati. Il metodo è stato validato su una miscela di sette proteine modello e sull'estratto proteico totale di un prodotto dolciario crudo. L'attività di ricerca condotta durante il periodo di dottorato mi ha permesso di acquisire delle competenze significative dei moderni metodi di analisi delle proteine, spaziando dalle tecniche cromatografiche, biospettroscopiche, bionfirmatiche e di spettrometria di massa.

I. INTRODUCTION

1. Food allergy

Allergy is a hypersensitivity reaction where symptoms appear rapidly following exposure to macromolecules (*1*). They are commonly mediated by specific antibodies belonging to the class of immunoglobulin E (IgE), which are normally generated as part of immune reactions to parasitic infections. For reasons that are only partly understood, allergy can also be generated upon exposure to environmental agents such as pollen, dusts and food. Adverse reactions to food or a food component, normally a protein, represent a prominent and actual problem all over the world (*2-3*). The food intolerance or allergy is a phenomenon that is increasing year by year, according to the significant changes in our feeding habits. Proteins are the most common food allergens and often they can induce an immediate allergic reaction that can have severe consequences in sensible subjects. The reasons for an individual to become intolerant towards a specific food protein are unclear. The amount of protein required to elicit an allergic response in a sensitized person varies considerably from patient to patient and protein to protein (Fig. 1).

IgE-mediated food allergies have two phases. The first involves sensitisation, where an IgE-response is generated towards a target macromolecule. The second phase occurs on re-exposure to an allergen when an allergic reaction takes place as pre-existing IgE binds the target macromolecules, causing the release of histamine and other inflammatory mediators. Food allergy symptoms vary from mild localized symptoms to severe anaphylaxis that may be even fatal (*4*). The spectrum of food allergy symptoms may include urticaria, angioedema, diarrhea, nausea, vomiting or hypotension. It is generally assumed that sensitisation to the classical food allergens, such as milk, egg, peanut and fish, occurs via the gastrointestinal tract, while other types of food allergy, particularly to fresh fruits and vegetables, occur as a consequence of prior sensitisation to inhaled allergens such as pollen.

Individuals who become sensitised to birch pollen develop IgE to the major birch allergen Bet v1, which also recognises homologous proteins present in fresh fruits and vegetables, giving rise to so-called cross-reactive allergy syndromes (*5-6*). It has been estimated that 1–2% of the population, up to 8% of children, suffer from some type of IgE-

mediated food allergy (*7*). Only few types of foods are responsible for causing the majority of food allergies, such as peanuts, tree nuts, wheat and soy, as well as allergens of animal origin including cow's milk, egg, fish and shellfish (*8*).

Since there are no medical treatments currently available for curing food allergies, the best way to prevent unintended exposure to a food allergen is the complete avoidance of the offending food. Correct food labelling in combination with good manufacturing practices can improve consumer safety and support food manufacturers, distributors and transporters to utilize food ingredients in an efficient and safe way. However, even if best intentions and practices are observed, the presence of trace contaminants of offending agents cannot be ruled out, unless accurate methods are available to detect their presence. For these reasons, reliable and specific detection methods capable of detecting even trace quantities of the food allergens must be developed.

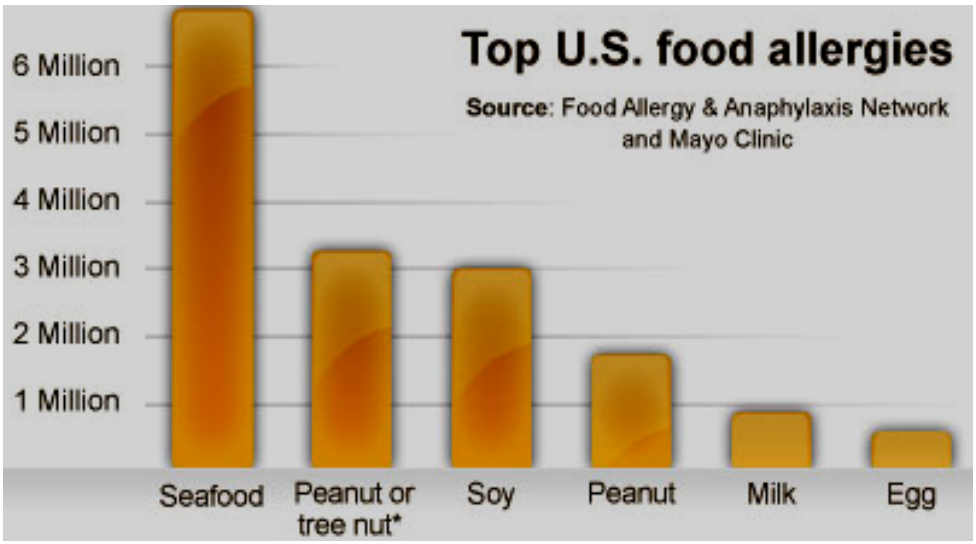

Figure 1. Histogram reporting the incidence of the main food allergies in the USA population.

Allergenic proteins and their structural features. Food allergens are almost always proteins, but not all food proteins are allergens. All proteins assume a specific three-dimensional structure that is of essential importance for their function (*9*).The primary structure is the amino acid sequence of the polypeptide chain, the secondary structure creates highly regular sub-structures by their patterns of hydrogen bonds between the main-chain peptide groups. The tertiary structure is the three-dimensional (3D) structure of a protein molecule, the spatial arrangement of the secondary structures. The quaternary structure is an oligomeric complex of several protein molecules or polypeptide chains.

Proteins are synthesized by ribosomes associated with the membranes of the endoplasmic reticulum and many of them undergo proteolytic cleavage following translation. A variety of proteins are synthesized as inactive precursors, which are activated under peculiar physiological conditions by limited proteolysis. Protein chains may undergo a variety of post-translational modifications by the action of specific enzymes. Protein glycosylation is a well known protein modification mediated by specific enzymes and the resulting glycoproteins consist of proteins covalently linked to carbohydrates. Phosphorylation is a most common protein modification occurring at the level of the side-chains of Ser, Thr and Asn. The majority of protein phosphorylation plays a role in the regulation of the biological activity of a protein and as such is transient.

Proteins which are allergens are often modified and food allergens have several biochemical characteristics in common(*10*). Often these include glycosylation patterns, stability to proteases, as well as to heat and protein denaturants. Their structure usually is globular and very compact and the most peculiar and significant characteristic of a protein allergen is stability to the proteolytic and acidic conditions of the digestive tract. This imparts to the protein an increased probability of reaching the intestinal mucosa, where absorption can occur. Indeed, several allergenic proteins have been tested for their stability to simulated gastric fluid and have been shown to resist for extended periods of time when compared with other non allergenic food proteins. Even though protein stability has been demonstrated for a variety of protein allergens, the molecular mechanism underlying this enhanced stability is not known (*11*). Studies have shown that higher order structures are largely responsible for their resistance to enzymatic digestion and therefore the persistence of their allergenicity (*12*).

Effects of food processing on allergen stability. The portion of a food protein that may cause an allergic reaction may be a simple stretch of a few amino acids along the primary structure or it may be a unique three-dimensional (3D) moiety of the protein structure, respectively referred to as linear and conformational epitopes. Since food ingredients are often subjected to a variety of processing treatments, alteration in epitopes may affect the allergenicity properties. Processing can destroy existing epitopes on a protein or can even generate new ones (neo-antigens), as a result of change in protein conformation. Neo-antigens are always formed when foods are cooked, even if conformational epitopes are usually removed at the same time. In some cases, the effect is to increase allergenicity, while in others heating may result in a decrease of allergenicity (*13-14*). This fact appears to explain why some people can tolerate unprocessed food, or a food ingredient, but not the corresponding processed counterpart. Often, processing treatments have been associated with decreased in allergenicity or with no significant effect.

Conformational epitopes are typically expected to be more susceptible to processing destruction than the linear epitopes on the same allergen. Linear epitopes are more likely to be altered if they are hydrolyzed. Processing may alter food in a way which could permit masking or unmasking of allergenic epitopes, thus reducing or enhancing allergen recognition. The effects of food processing on the structural stability of food allergens have been reviewed (*15*).

Detection methods for food allergens. Sensitized patients are dependent on reliable product labels and on the availability of foods not containing the respective allergens as contaminants (*16*). However, still a wide range of products is suspected or even reported to contain undeclared allergens and thus to cause adverse reactions (*17-19*) Consequently, the detection of hidden allergens in food products is an important aim both for the food industry and the food control authorities. There is an urgent need for analytical methods which should be highly specific, sensitive, rapid, cost-effective and thus capable of detecting even traces of allergens.

Immunoassays are presently the method of choice for detection and identification of a wide range of food components including food allergens (*20*). The most sensitive method used to screen the presence of hidden allergens in food matrices is the enzyme-linked immunosorbent assay (ELISA) (*21-22*). The technique called "sandwich" ELISA is used to improve the detection of an antigen. The antigen is attached to the plate with the specific capture antibody bound on the surface. Then, the primary antibodies, which bind specifically to the antigen, are applied. Subsequently, the enzyme-linked secondary antibody, which is specific to the primary antibodies, is used. Afterwards, the binding is detected, as evidenced by the color or fluorescent signal resulting from an enzymatic reaction of a substrate (Fig. 2). However, the presence of false-positive and false-negative results demands the availability of confirmatory methods. In addition, the ELISA quantitative data are strongly dependent on the matrix tested leading to lack of accuracy (*23-24*).

The gold standard of any diagnostic procedure in food allergy remains the positive outcome of a standardized food challenge. Of great help to an accurate diagnosis are skin prick tests as well as *in vitro* diagnostic tests. In the case of *in vitro* diagnostic tests, only a few commercially serum IgE testing methods are currently available. Further improvements of the *in vitro* diagnosis may include correlation with clinical diagnosis as well as definition of clinically relevant cross-reactivities. Finally, there is the possibility of using *in vitro* diagnosis in order to predict clinical sensitivity (*25-26*).

Given the limitations of the methods described above, it is clear that a confirmatory method is needed to provide an unambiguous identification of allergenic proteins. Analysis by mass spectrometry (MS) allows a combination of contemporaneous separation and identification of individual proteins and thus MS methods likely can provide suitable analytical approaches. Indeed, MS techniques have been employed for the identification of

allergens (*27*) and certainly hold potential to detect allergenic proteins in food products (*28-29*). No doubt that, despite the need for a costly and specialized equipment, MS methods can provide unambiguous identification of allergenic proteins in foods thus providing an alternative to the use of immunological techniques. Nowadays, MS has become an important method to biomarker discovery and disease diagnosis (*30-32*).

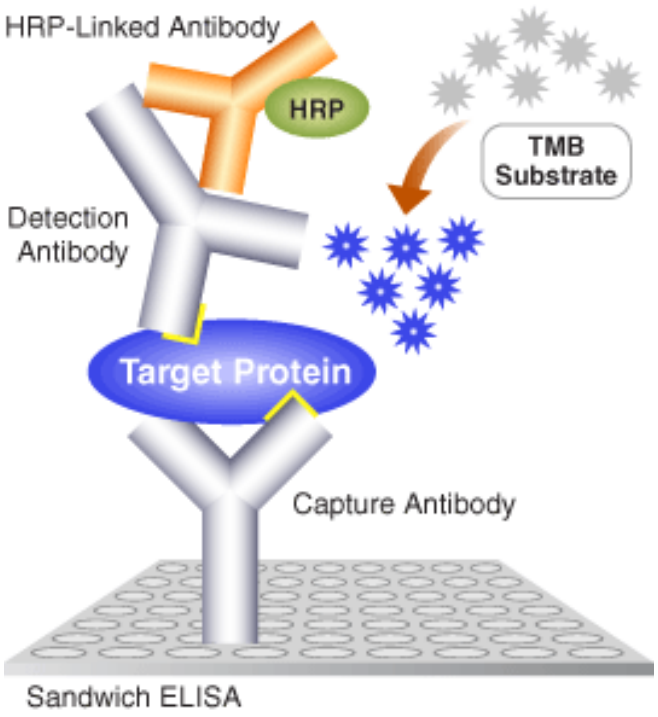

Figure 2. Scheme of a sandwich ELISA**.** A plate is coated with a capture antibody that immobilizes the antigen (Target Protein). A Detection Antibody is added that binds to the antigen. An enzyme-Linked secondary Antibody interacts with the Detection Antibody and the binding is measured by the enzyme-mediated conversion of a substrate into a detectable form.

Allergenic proteins from milk and eggs. Milk allergy is an adverse reaction to proteins that are present in milk from ruminants as cow and goat. The milk allergenic proteins are the same or very similar in terms of structural, functional and biological properties. From an immunological point of view, cow's milk allergy is a IgE-mediated reaction to milk proteins and may induce cutaneous (atopic dermatitis, urticaria, angioedema), respiratory (rhinitis, asthma, cough) and gastrointestinal (vomiting, diarrhea, gastro-esophageal reflux) reactions and in some extreme cases even systemic anaphylaxis. Different food products could be responsible for this type of allergy, since milk proteins are used as constituents of many industrial foods and, therefore, a large number of food products may contain residual amounts of milk proteins.

Milk contains several proteins that are considered antigenic and capable of inducing immune responses. Studies carried out on large populations of allergic patients have indicated that the most abundant proteins in milk such as β-lactoglobulin (β-LG), casein (CN) and α-lactalbumin (α-LA) are also the major allergens. However, also other proteins such as bovine serum albumin (BSA), lactoferrin (LF) and immunoglobulins (Ig) can have a role in developing milk allergy .

The estimated prevalence of egg allergy varies between 1.6 and 3.2% and thus makes it the second most common cause of food allergies in children . In fact, egg allergy has been reported as the most prevalent food hypersensitivity in the paediatric population, exceeding that of cow's milk allergy . The total absence of a specific allergen in food products is difficult to demonstrate, mainly because of food-manufacturing practices and difficulties in the quantification of egg contaminants in food matrices. Moreover, the immuno-reactivity of egg proteins can be altered during food processing. Denaturation or aggregation of food proteins during heat-mediated processing or matrix effects due to lipids or Maillard reactions may influence the detection tests(*33*).

It has been reported that the major egg allergens are, in decreasing order, lysozyme (LYS) > ovalbumin (OVA) > ovomucoid (OVM), based on skin tests, with ovomucoid being the dominant egg allergen . Frequency of recognition in egg allergic patients was reported to range from 6 to 67% for lysozyme and to reach up to 22% for ovotransferrin. The two major allergens OVM and OVA constitute about 11 and 54% of egg white proteins, respectively . Both proteins are glycosylated, with the polysaccharide moiety being as high as 25% of the mass of OVM.

2. Mass spectrometry in protein analysis

The main topic of the research conducted in the ambit of this PhD Thesis was the development of mass spectrometry (MS) methods for the identification of allergenic proteins in foods. A MS instrument connected on-line with a micro-column of a liquid reverse-phase liquid chromatograph (LC-MS/MS) was used. Approaches and methodologies are similar to those nowadays used in the general area of proteomics, considered the analysis of all proteins contained in a tissue or cellular extract (*34-38*). Indeed, proteomics refers to the analysis of all proteins in a living system, including the description of co- and post-translationally modified proteins and/or alternatively spliced variants. This includes their spatial and temporal distributions within cells and how all these are affected by changes in the extracellular and intracellular conditions.

A mass spectrometer consists of three basic components, i.e., an ion source, a mass analyser and an ion detector. MS measurements are carried out on ionised analytes in the gaseous phase, requiring a method to transfer protein molecules from a solution or solid phase into the gaseous state. The two most commonly used techniques are matrix-assisted laser-desorption ionisation (MALDI) and electrospray-ionisation (ESI) (Fig. 3). Both MALDI and ESI are soft ionisation techniques in which ions are produced with low internal energies and thus undergo little fragmentation. In MALDI, protein samples are co-crystallised with an organic matrix. A pulsed laser is used to excite the matrix, which causes rapid thermal heating of the molecules and the subsequently desorption of ions into the gas phase. MALDI produces packets of ions rather than a continuous beam. Therefore, it is often coupled to a mass analyser, which can measure either a complete mass spectrum without scanning a mass range, or trap all the ions for subsequent mass analysis.

ESI is based on spraying electrically-generated ions into the inlet of a mass spectrometer at atmospheric pressure. In ESI, a liquid is pushed though the capillary, with the analyte dissolved in a large amount of solvent, which is usually much more volatile than the sample (*39*). The analyte exists as an ion in the solution, either in its anion or cation form. Because of the charges repulsion, the liquid pushes itself out of the capillary and forms an aerosol. An uncharged carrier gas, such as nitrogen, is used to help nebulize the liquid and evaporate the neutral solvent in droplets. As the solvent evaporates, the analyte molecules are forced closer together, repel each other and break up the droplets. When the analyte is an ion free of solvent, it moves to the mass analyzer. After ionisation, the sample reaches the mass analyser, which separates ions by their mass-to-charge (m/z)

ratios. Ion motion in the mass analyser can be modulated by electric or magnetic fields to direct ions to a detector, which registers the numbers of ions at each individual m/z value.

Four basic mass analysers are nowadays used, i.e., the time-of-flight (Tof), ion trap, quadrupole and Fourier-transform ion cyclotron resonance (FT-ICR) analysers. All four differ considerably in sensitivity, resolution, mass accuracy and the possibility to fragment peptide ions. The combination of ion source, mass analyser and detector is usually determined by the specific application. ESI is most frequently coupled to ion traps and hybrid tandem mass spectrometers like quadrupole time-of-flight (Q-Tof) instruments.

For the experimental work of this PhD thesis, a Micro Q-Tof instrument (Micromass, Manchester) composed of an ESI source was used (Fig. 4). The Tof analyzer uses an electric field to accelerate the ions through the same potential and then measures the time they take to reach the detector. If all particles have the same charge, then their kinetic energies will be identical and their speed will depend only on their masses. Lighter ions will reach the detector first. The data produced are represented in a mass chromatogram of total ion current (TIC), measured in the ion source. The instrument acquires a mass spectrum of the peptides in terms of an intensity *vs*. m/z (mass-to-charge ratio) plot. Afterwards, the instrument can also determine the sequence of the peptides though a tandem mass spectrometry analysis (Fig. 5).

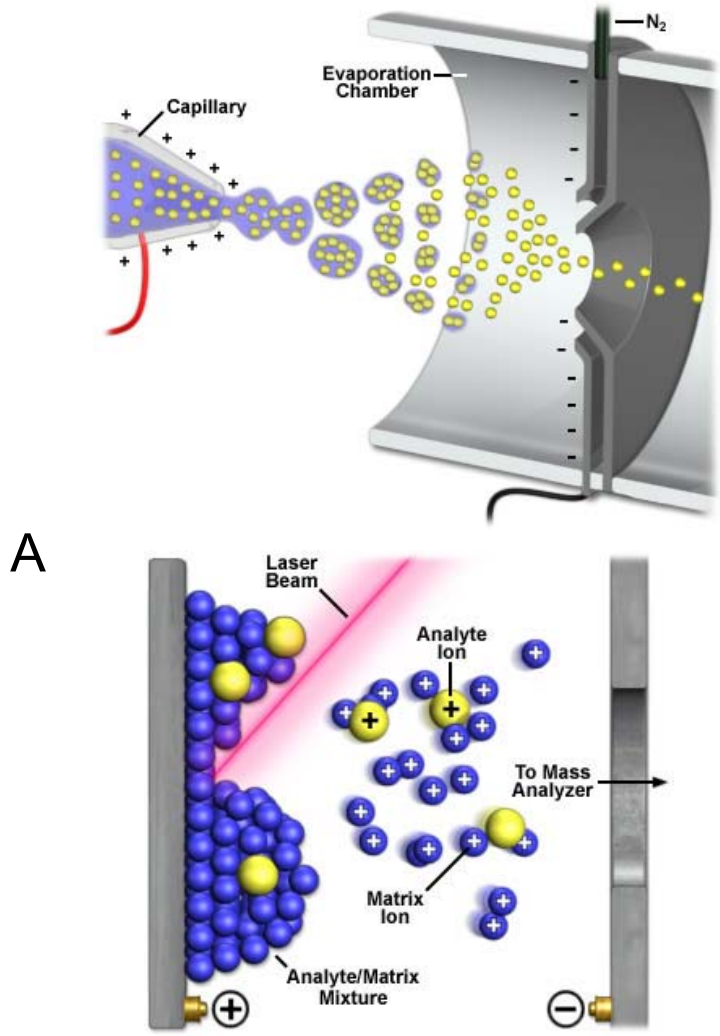

Figure 3. Schematic representations of the two main soft ionization techniques used in the mass spectrometric analysis of protein samples. (A) An ESI source operating in the positive ion mode is represented. A spray of fine droplets containing the charged analytes and solvent molecules is generated upon the application of a high electrical tension on a needle. The voltage polarity of the metal capillary is positive or negative for positive or negative ion generation, respectively. (B) In a MALDI source, the sample is mixed or dissolved with an excess amount of a matrix component having an absorption wavelength, which matches closely with the laser wavelength. Upon laser irradiation, a plume of neutral molecules and ions are desorbed. Ions are then guided to the mass analyzer and the detector by electrostatic lenses.

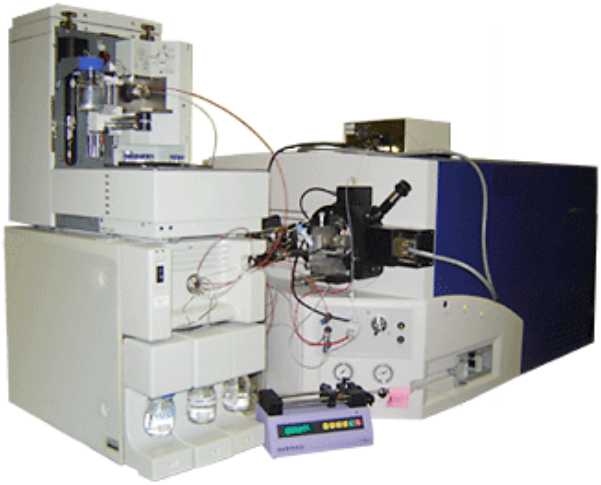

Figure 4. The Micromass CapLC unit (Waters) interfaced to a Micromass Q-Tof Micro mass spectrometer (Waters) equipped with a nanospray source.

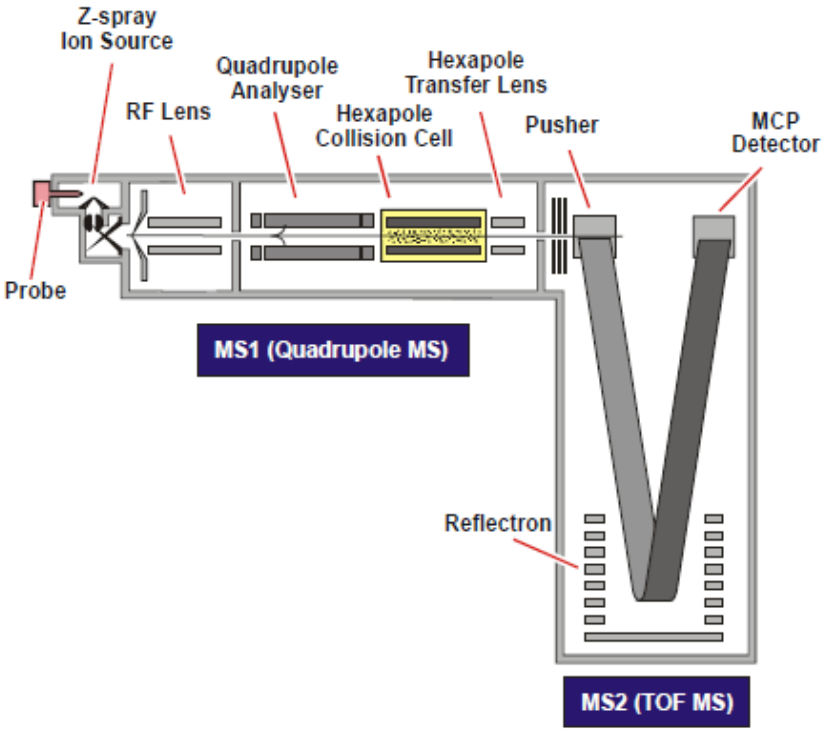

Figure 5. Schematic representation of an Hybrid Q-Tof instrument.

The tandem mass spectrometry MS/MS experiment involves multiple steps of mass selection or analysis, usually separated by some form of fragmentation. The first mass analyzer isolates one peptide from many entering a mass spectrometer. Then, a second one stabilizes peptide ions while they collide with a gas (argon, helium or nitrogen), causing them to fragment by collision-induced dissociation (CID). Finally, a third mass analyzer registers the fragments produced from the most abundant peptides. The ion of the initial peptide is called "precursor ion", while the ions produced in the MS/MS spectrum are called product ions. A typical fragmentation of a peptide is observed and a specific nomenclature of the product ions in a MS/MS experiment is used (Fig. 6).

A peptide sequence tag obtained by tandem mass spectrometry can be used to identify a peptide in a protein database. Peptide fragment ions are indicated by a, b, or c, if the charge is retained on the N-terminus, and by x, y or z if the charge is maintained on the C-terminus (*40-41*). Although peptide backbone cleavage is the most useful method for sequencing and peptide identification, other fragment ions may be observed under certain conditions. These include the side-chain loss ions and immonium ions. The amino acid sequence is determined in the MS/MS spectra by the difference from a fragment with the immediately subsequent one, which is the molecular weight of a specific amino acid.

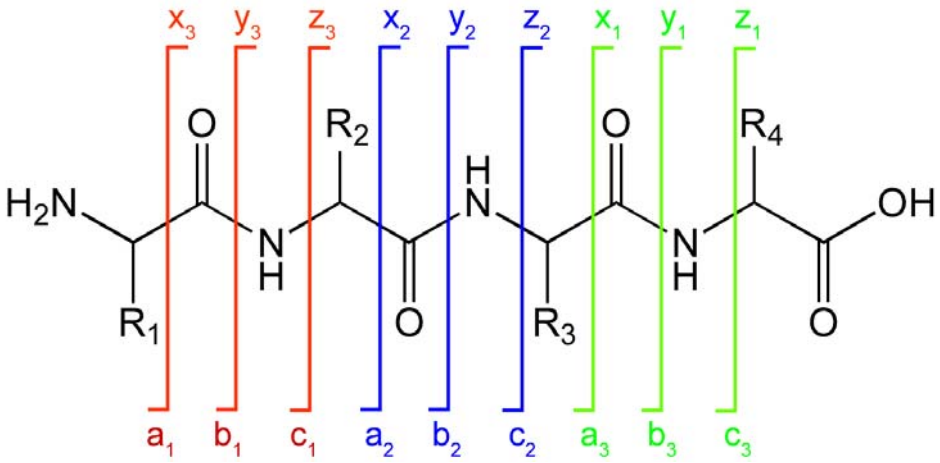

Figure 6. Nomenclature of peptide fragments produced in a tandem mass spectrometry experiment.

Proteomics experiments most often invoke the simultaneous analysis of several thousands of protein species from complex biological samples. The instrument is usually interfaced with a liquid chromatography system, where the protein samples, or the tryptic fragments, are loaded in a RP-HPLC column. The peptides are separated with a gradient of organic solvent, usually acetonitrile, following their hydrophobic scale. This system is miniaturized and specific to load minute amounts of sample. The separation of peptides and proteins is therefore a key element in proteomics analysis providing a method to simplify complex mixtures and deliver molecules to the ionisation source.

The following step of a proteomic experiment is to use the sequence data derived from product ion MS/MS spectra from one or more peptides for retrieve amino acid sequence data from protein databases. A number of search engines can correlate this information to protein sequence database, the most important sequence engines being SEQUEST and MASCOT. Sequence information is significantly more discriminating than molecular mass alone for identifying proteins, since even a single peptide of 10-15 residues is sufficient to identify a protein.

3. Chemical tagging in proteomics

The successful outcome of a proteomics analysis relies on the sample handling and pre-fractionation steps that come before MS protein identification (*42-43*). Even the most advanced MS instrumentation available today is not able to deal with very complex biological samples like cell extracts as a whole, without prior pre-fractionation. In the past, reduction of sample complexity has mostly depended on two-dimensional gel electrophoresis (2-DE) to efficiently separate protein mixtures. Drawbacks of the technique are the limitations in the pI and molecular weight of proteins, difficult automation and reproducibility problems.

While 2-DE still can be considered the most widely used separation technique prior to MS analysis, liquid chromatographic separations are increasingly used in so-called “gel-free approaches”. For this set-up, all proteins present in the sample are usually enzymatically cleaved into smaller peptides to obtain a very complex mixture that is then subjected to additional separation steps. Gel-free techniques overcome some of the limitations of gel-based techniques although they lack in resolution of individual sample

constituents, even when two-dimensional HPLC typically consisting of an ion exchange step in the first dimension and reversed-phase separation in the second one is performed.

For the analysis of proteins from complex biological mixtures, a suitable strategy involves the labelling of proteins or peptides for the purposes of an easier isolation and characterisation by MS. Chemical tagging implies the modification of functional groups of amino acid residues in proteins and peptides. By this strategy it is possible to enrich a subpopulation of peptides from a total protein digest, since it is possible to select only the labelled peptides by using chromatographic techniques (Fig. 7). If a rare amino acid, like cysteine or tryptophan, is chosen as a target for tagging, only a small fraction of peptides will carry this residue, resulting in a significant reduction of the sample complexity. Frequently, affinity tagging is also combined with stable-isotope labelling in order to conduct a quantitative MS analysis.

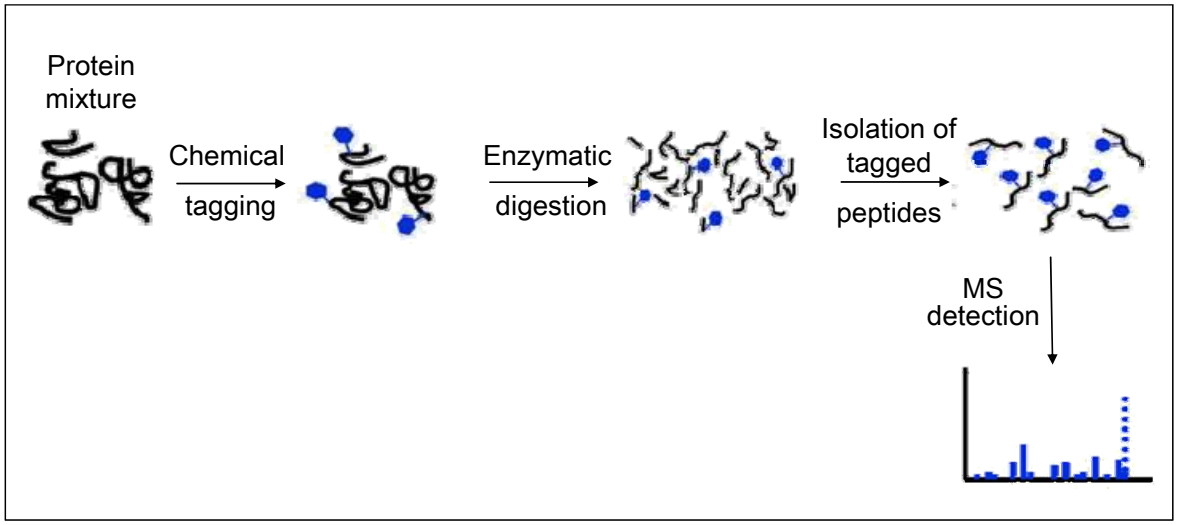

Figure 7. Scheme of sample fractionation using a chemical tagging strategy. A protein mixture is labelled with a chemical tag and then digested with an enzyme. The labelled peptides are subsequently selected by a chromatographic step, so that ideally only the tagged peptides are analysed by MS.

Chemical tagging of tryptophan in proteins with DNPS-Cl. Sulfenyl halides have been introduced in protein chemistry as a new class of reagent for the selective modification of tryptophan and cysteine residue in proteins. The sulfenyl halides react with tryptophan in aqueous organic solution at pH 8-9, giving the sulfenammide; but in acidic solution, in which protonation of the amino group occurs, the indole nucleus behaves as the nucleophilic reagent and is converted by reaction with the sulfenyl halide into a thioether in the 2-position. Quantitative modification of tryptophan residues in proteins is readily achieved by using a low molar ratio of reagent to protein. The acidic solvent conditions are necessary for the selectivity of the reaction, since protonation of amino groups inhibits sulfenamide formation.(*44-46*)

Recently, chemical labelling of tryptophan residues for proteomic applications has been reported (*47-48*). Arylsulfenyl halides are known for their selective reactivity towards the indole nucleus of tryptophan under acidic conditions (*49-50*). It has been found that 2-nitrobenezenesulfenyl chloride (NBS-Cl) can be successfully used for labelling tryptophan residues in protein and peptide mixtures (*47-48*). Moreover, NBS-Cl incorporating six ^{13}C or six ^{12}C in its benzene ring was employed in order to perform isotope-tagging of Trp-peptides for a quantitative MS analysis (*51-53*). The NBS-labelled Trp-peptides were enriched by exploiting the affinity of NBS-labelled peptides for Sephadex LH-20 or for a phenyl-resin matrix. The NBS-Cl modification has been already used in proteomics studies and has already raised a strong interest. For this PhD project, the 2,4-dinitrobenzenesulfenyl chloride reagent was employed (*54*). Previously, in the area of peptide chemistry, this reagent was named 2,4-dinitrophenylsulfenyl chloride and herewith it is preferred to use the classical abbreviation DNPS-Cl for the reagent, as used since 1965. (Fig. 8).

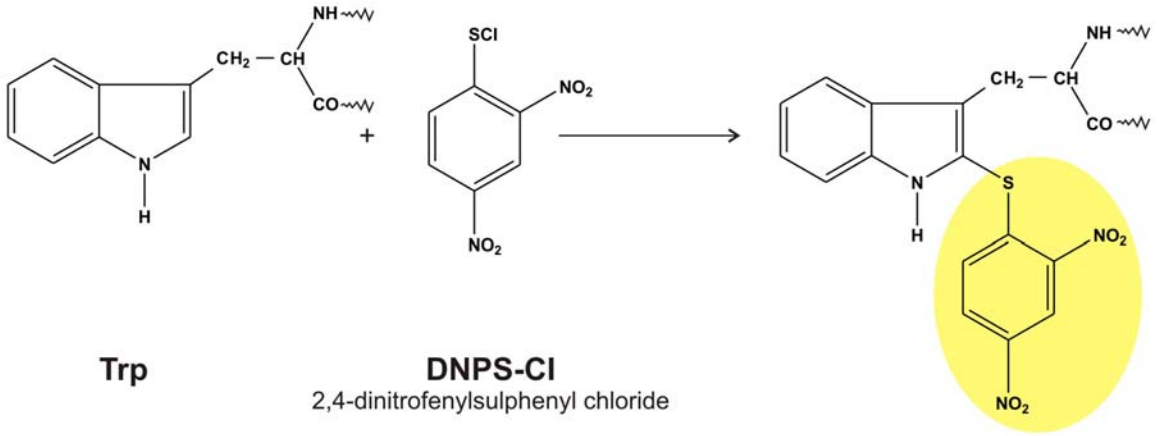

Figure 8. Chemical reaction of the tryptophan amino acid residue with DNPS-Cl. Under acidic conditions, the indole nucleus of Trp behaves as the nucleophilic reagent and is converted by reaction with the sulfenyl halide into a thioether in the 2-position.

4. Diagonal chromatography

The *diagonal* chromatography approach in RP-HPLC, combined with specific chemical modifications, can reveal a few modified peptides within an enzymatic mixture of thousands. Several reverse-phase LC techniques that sort specific classes of peptides have been developed (*55-59*). The central procedure is based on the technique of diagonal electrophoresis that was used for the isolation of cysteine peptides by diagonal paper electrophoresis(*60*).

Gevaert and co-workers have introduced reverse-phase liquid chromatography for isolating representative peptides in a system that closely resembles the original idea of diagonal chromatography. In this way, different sets of representative peptides can be isolated from complex peptide mixtures by chromatography without relying on specific affinity. A set of tryptic peptides from a protein digest is separated by RP-HPLC and all the fractions are collected, subjected to the labelling reaction and then reloaded on the same column and under the same conditions as the first separation. Those peptides that did not react with the derivatization reagent will be eluted with the same retention time in the pre- and post-reaction HPLC runs (Fig. 9). In a graph of retention time (1st dimension) *versus* retention time (2nd dimension), they will all lie on a straight diagonal line, hence the name of *diagonal*. However, those peptides that were modified by the reagent will have modified retention times, so will be shifted off the diagonal.

In order to reduce the number of repetitive chromatographic runs, several fractions from the primary run can be combined and subjected to the sorting reaction. For this reason, a version of diagonal chromatography combined fractional diagonal chromatography (COFRADIC) was developed. This technique was originally optimized for RP-HPLC by to the analysis of methionine-containing peptides in a gel-free protocol, allowing the identification of several hundred proteins in an *E. coli* extract. The total cell lysate was digested and processed by diagonal chromatography, using methionine oxidation as the differentiator and all diagonally shifted peptides were identified by mass spectrometry (*58*).

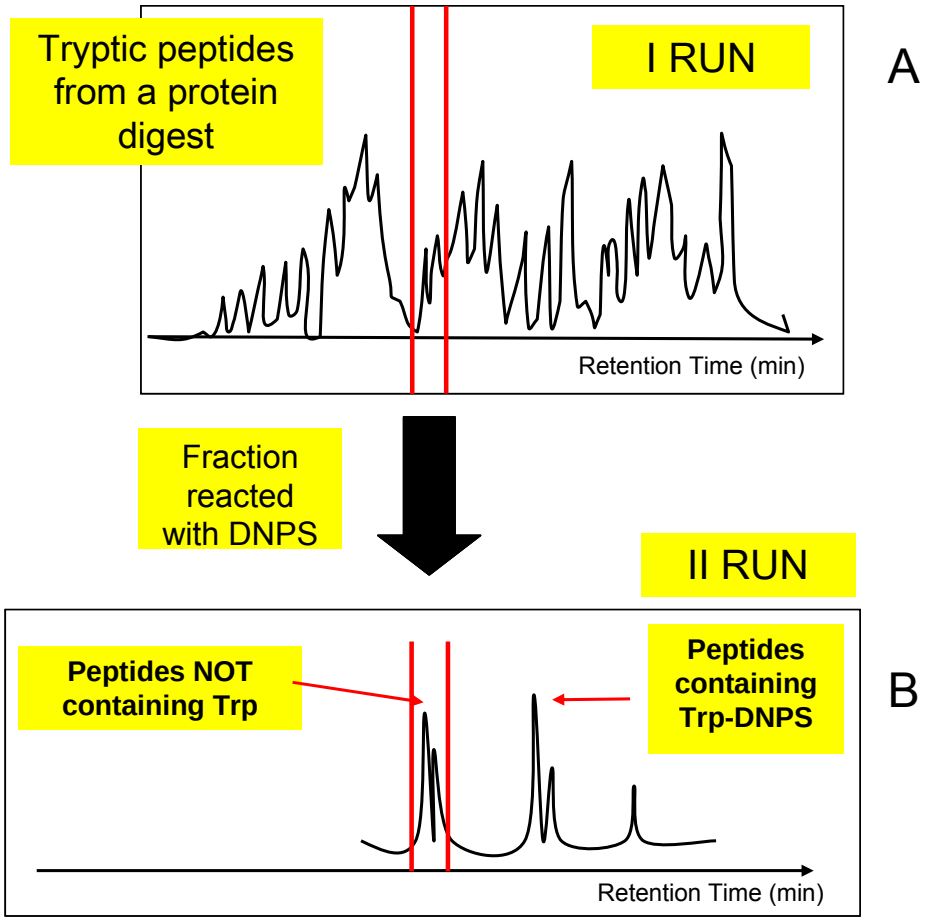

Figure 9. Scheme of a diagonal chromatography experiment. (A) RP-HPLC analysis of a complex tryptic peptide mixture. (B) RP-HPLC chromatogram of the peptide fraction which was collected from the first run (A) and then modified at Trp residues by DNPS-Cl. The second RP-HPLC separation is conducted using the same conditions of the first analysis. Since the chemical modification increase the hydrophobicity of the Trp-containing peptides, the modified peptides will be eluted whit a shift in retention time.

5. Immunoaffinity chromatography

Affinity chromatography separates proteins on the basis of a reversible interaction between a protein and a specific ligand coupled to a chromatography matrix. By affinity chromatography, it is possible to purified the protein of interest with a high selectivity and resolution an it can be collected in a purified, concentrated form (*61*). In a single step, affinity purification can offer vast time-saving over less selective multistep procedures (Fig. 10). Target molecules can be purified from complex biological mixtures, native forms can be separated from denatured forms of the same substance and small amounts of biological material can be purified from high levels of contaminating substances. Successful affinity purification requires a specific ligand that can be covalently attached to a chromatography matrix. The coupled ligand must retain its specific binding affinity for the target molecules and, after washing away unbound material, the binding between the ligand and target molecule must be reversible to allow the target molecules to be removed in an active form. Immunoaffinity chromatography utilizes antigens or antibodies as ligands to create highly selective media for affinity purification. Antibodies are extremely useful as ligands for antigen purification, especially when the substance to be purified has no other apparent complementary ligand.

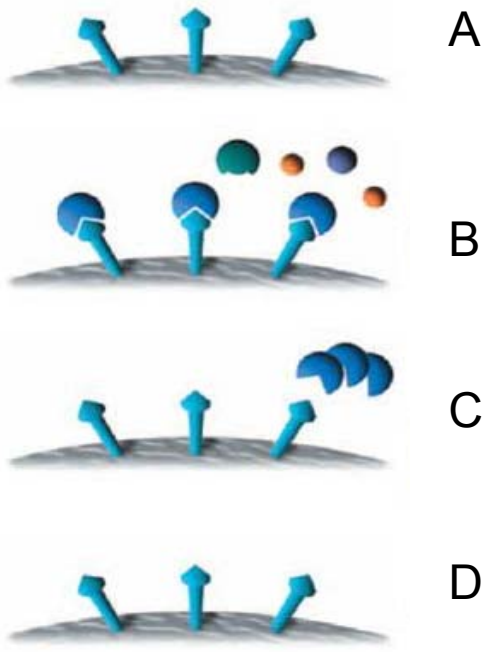

Figure 10. Affinity chromatography. (A) (A) The affinity medium is equilibrated in binding buffer.
(B) The sample is applied under conditions that favor specific binding of the target molecule to the ligand. The target molecule binds specifically, but reversibly, to the ligand whereas the unbound material washes through the column.
(C) The target molecule is recovered by applying conditions that favor elution, as for example by using a competitive ligand or by changing the pH, ionic strength or polarity of the solvent.
(D) The affinity medium is re-equilibrated with binding buffer.

Isolation of Trp-labeled peptides by affinity chromatography. A method was described for the specific selection of peptides containing modified residues from selectively modified proteins. The method is based on the affinity of specific antibodies to the group introduced by the modification (*62*). Antibody-Sepharose conjugates were thus used as a column to isolate specifically and in high yields the peptides which contained the modified residue. These peptides can be eluted from the affinity columns under conditions which dissociate antigen-antibody interaction. This method can be extended to other chemical modifications, if antibodies specific to the modified moiety are used. Specific antibodies with high affinity can be raised against almost any small molecule and they are therefore suitable regents for the isolation of modified peptides which have such small molecules attached to them. The high capacity of such an antibody column, the relatively high affinity of antibodies for a ligand and the fast rate of antigen-antibody interaction make this method a very powerful tool for the selection of labelled peptides from the whole peptide mixture of a protein digest (*63-64*).

6. Hydrophobic interaction chromatography

Hydrophobic interaction chromatography (HIC) is a technique that exploits the hydrophobic interaction of proteins with a hydrophobic matrix (*65*). Also a ligand characterized by a hydrophobic moiety and an ionisable group can be used. In this case, the HIC matrix can adsorb proteins at low-salt concentrations and then desorption of the bound proteins can be achieved by rendering the ligand hydrophilic due to its protonation (Fig. 11).

HIC has been exploited also by using a matrix bearing as hydrophobic ligand the 4-mercaptoethyl-pyridine moiety (Fig. 12), which in turn can be made hydrophilic by protonation. The high-ligand density of the MEP-HyperCel (70–125 μmol/mL) matrix enables an adsorption capacity much greater than other more traditional HIC matrices. This matrix, having a pKa of 4.8, can be easily used for protein separation by adsorption in its uncharged form at neutral pH and subsequently elution of adsorbed molecules being eluted at pH 4.0. MEP-HyperCel has been used previously for the purification of antibodies and other proteins. Binding is based on mild hydrophobic interactions and is achieved under near physiological conditions, without the addition of salts. The ability to bind proteins

under neutral conditions minimizes the risk of protein/peptide precipitation and/or aggregation and may contribute to better recovery.

In this PhD study, the MEP-HyperCel matrix has been used for the purification of DNPS-labelled tryptophan peptides contained in a protein digest. It was shown that the technique has several advantages and that indeed can be a very suitable technique for a mild and effective isolation of DNPS-labelled peptides.

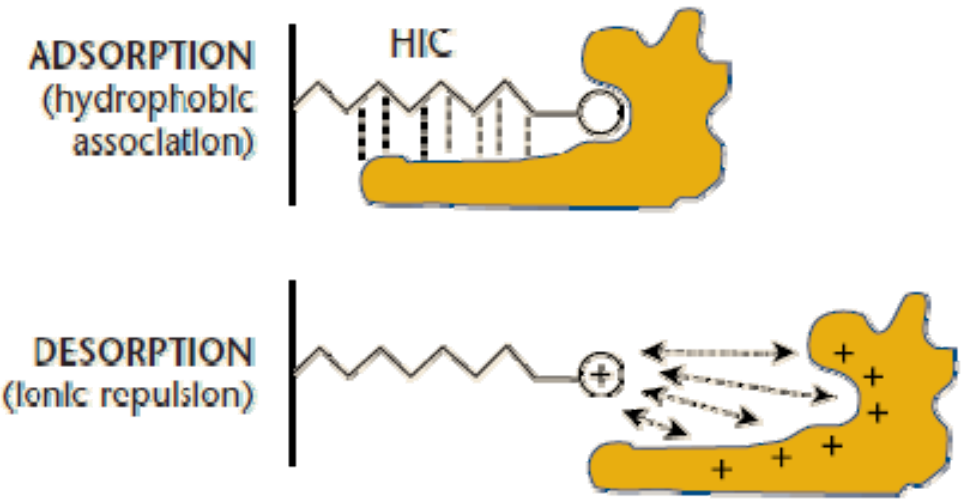

Figure 11. HIC performed using the MEP-HyperCel matrix. Peptides are adsorbed to the resin by hydrophobic interactions. Reduction of the pH in the elution solvent determines the protonation of the pyridine ring of the ligand and dissociation of the peptides from the matrix due to ionic repulsion.

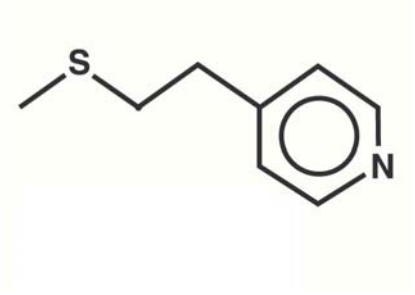

Figure 12. The heterocyclic ligand: 4-mercapto-ethyl-pyridine of the MEP-HyperCel matrix.

7. Main aims of the Thesis

The identification of protein allergens in foods is nowadays conducted by using immunochemical methods such as ELISA tests, but these techniques suffer from several limitations due to cross-reactivity and false negative results. Indeed, alterations in the allergen's structure or chemical modifications can prevent the interaction with the antibody, thus causing false negative results. Since allergens are toxic even in trace amounts, there is a need for reliable and sensitive analytical methods to detect allergenic proteins. The purpose of this PhD project was to develop procedures for the identification of these proteins in food samples by using mass spectrometry (MS), likely overcoming some limitations of the immunochemical assays. Studies will be conducted on allergenic proteins from eggs and milk.

Since allergenic proteins usually are contained in complex mixtures of huge amounts of other proteins, the methodology nowadays named "targeted proteomics" was considered very appropriate for the purposes of this PhD study. By this approach, a protein contained in a complex mixture can be identified by a MS analysis of a peptide fragment that is specific for the protein of interest and contained in the very complex peptide mixture of a tryptic digest of a protein sample. This procedure involves (1) chemical tagging or specific modification of an amino acid side chain with a specific reagent or reaction, (2) selective isolation of the tagged peptide by chromatography and (3) amino acid sequence analysis of the isolated tagged peptide by MS/MS.

It is aimed to modify tryptophan (Trp) residues in a complex mixture of peptides contained in a tryptic digest of a protein extract by reaction with 2,4-dinitrobenzene-sulfenyl chloride (DNPS-Cl), that leads to a Trp-derivative with the DNPS label attached at 2-position of the indole nucleus. The selection of Trp(DNPS)-peptide(s) from the complex mixture of a tryptic digest of a protein sample will be achieved by exploiting the significant change in hydrophobicity and retention time of DNPS-modified peptides in a reverse-phase HPLC column. Moreover, DNPS-labelled Trp-peptides will be isolated by hydrophobic interaction chromatography (HIC), as well as by immunoaffinity chromatography using a column prepared with anti-2,4-dinitrophenyl (DNP) antibodies.

The “targeted proteomics” procedure will be optimised using a mixture of model proteins and then applied to identify a protein allergen contained in a raw bakery product. Hopefully, it will be possible to demonstrate that the novel procedure of selective labelling and isolation of Trp-peptides will allow a considerable simplification of the fingerprinting/MS approaches nowadays used for the identification of proteins in proteomics research.

II. MATERIALS AND METHODS

MATERIALS

Bovine α-lactalbumin and the other commercial proteins used in this thesis (β-lactoglobulin, lysozyme, ovomucoid, ovalbumin, bovine serum albumin, carbonic anhydrase, hemoglobin, myoglobin and aprotinin), AEBSF, the trypsin inhibitor, the CNBr-activated matrix, Sephadex G-25 SF resin and the reagents for dot and western blot assays were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Trypsin was obtained from Promega (Madison, WI, USA). MEP HyperCel resin was purchased from Biosepra (New York, NW, USA). 2-D Quant Kit was provided by GE Healthcare (Uppsala, Sweden). PepClean™ C-18 Spin Columns and dialysis cassettes were supplied by Pierce (Rockford, Il, USA). Antibody batches were provided by Chemicon (Rosemont, IL, USA,) Koma Biotech (Gayang, Seoul, Korea), Millipore and Sigma-Aldrich. 2,4-dinitrophenylsulfenyl chloride (DNPS-Cl),trifluoroacetic acid, and the other high purity salts used for spectroscopic analyses were obtained from Fluka (Buchs, Switzerland). The acetonitrile used for reverse phase HPLC chromatography is from Carlo Erba Reagenti (Italy). Reagents and solvents for electrophoresis were purchased from Bio-Rad (Richmond, IL, USA). All other chemicals were of analytical reagent grade and were obtained from Sigma or Fluka. The composition of the PBS buffer is 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl and 0.137 M NaCl, pH 7.4. The composition of TBS buffer is 1.37 M NaCl, 0.027 M KCl, 0.25 M Tris·HCl pH 7.4.

METHODS

1. Protein extraction from the bakery product

Bakery product was made by mixing 200 g of flour, 2 eggs, 200 g of sugar, 100 mL of milk, 50 g of butter and baking powder. The cake mixture was divided into two samples, a part was maintained in a raw state, and the other one was cooked for 40 min at 180 °C. Two aliquots of 10 g of raw and cooked cake were reduced into powder in liquid nitrogen and then suspended in 40 mL of 20 mM Tris·HCl pH 7.2. The samples were stirred for 4 hours at room temperature. Afterwards, the extracts were centrifuged at 10,000 g for 50 minutes at 4 °C in order to eliminate some precipitate and fatty acids. The two supernatants were filtered with a 1.2 μm cut-off cellulose filter. Then, the cake extracts were dialyzed with a 3000 Da cut-off membrane against 20 mM Tris·HCl pH 7.2 at 4 °C overnight. The protein concentration in the samples was calculated as indicated below and each sample was collected in aliquots and stored at -20 °C.

Protein quantification of the bakery product extract. A calibration curve with bovine serum albumin (BSA) was obtained in the range 0–50 μg protein. Six standard solutions were prepared (0, 10, 20, 30, 40 and 50 μg). For protein quantification the 2D quantification kit from GE Healthcare was used. The method is based on the binding of copper ions to proteins present in solution. Tubes containing 1–50 μL of the protein extracts to be assayed were prepared in duplicate. Precipitant and co-precipitant were added to each tube before mixing. The tubes were centrifuged at 16,000g for 5 min and the supernatant was completely removed. Copper solution and milliQ water were added and the samples were shaken briefly. 1 mL of working colour reagent was added to each tube. The samples were incubated at room temperature for 15–20 min and the absorbance of each sample and standard was read at 480 nm. Standard curves were generated by plotting the absorbance of the standards against the quantity of protein and used to determine the unknown protein concentration of the samples (Fig. 13).

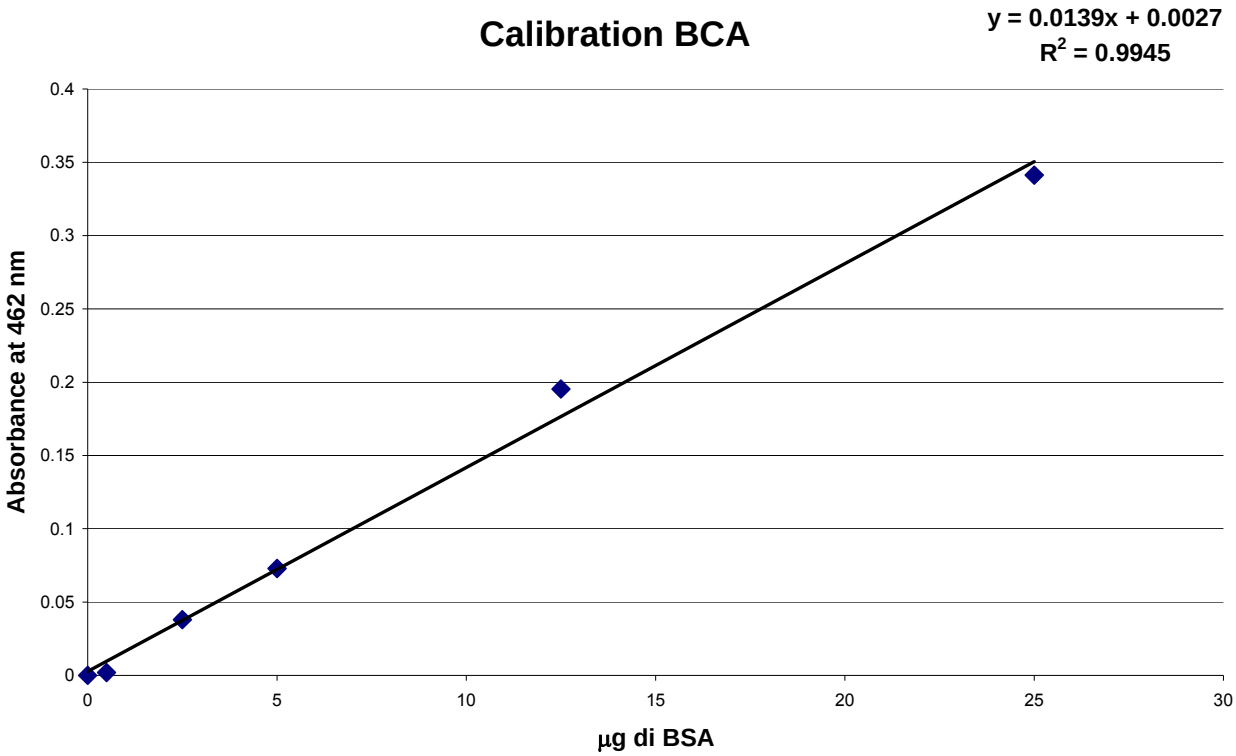

Fig. 13. Reference line obtained plotting the absorbance data at 642 nm as a function of the µg of protein.

2. SDS-PAGE electrophoresis

In SDS polyacrylamide gel electrophoresis (SDS-PAGE) charged proteins migrate in response to an electric field. Their rate of migration depends on the strength of the field and on the net charge, size and shape of the molecules. Sodium dodecyl sulphate (SDS) is an anionic detergent, which denatures proteins by "wrapping around" the polypeptide backbone. Electrophoresis experiments were conducted with the Mini-Protean II electrophoresis system purchased from BioRad using plates of 10.2 x 8.2 cm and spacer of 0.75 mm thickness. For the separation of proteins the gel was composed by 12% T (% total acrylamide and bis-acrylamide) and 0.4% C (% of bis-acrylamide). The running and stacking gel are composed of a solution of acrylamide bis-acrylamide, 30% T and 0.8% C in running and 5% T in stacking gel. The buffer was 1.5 M Tris·HCl, pH 8.5, with 0.3% SDS. The acrylamide polymerization was obtained by adding TEMED and a 10% solution of ammonium persulfate (APS). The electrophoresis buffer was 25 mM Tris·HCl, 250 mM glycine, 0.1% SDS, pH 8.5. Prior to apply to the gel, samples were dissolved in 0.3 M Tris·HCl, pH 6.8, with 0.1% SDS, 2.5% β-mercaptoethanol, 50% glycerol and traces of blue bromophenol and denaturated by heating at 100 °C for 5 min. The electrophoresis was performed at room temperature at 25 mA. Bands were revealed by silver staining.

3. Dot and Western blot

Dot Blot. Dot blot is a technique for detecting, analyzing, and identifying proteins, similar to the Western blot technique but differing from this in that protein samples are not separated by electrophoresis but are spotted through circular templates directly onto the membrane or paper substrate (Fig. 14).

Procedure:

1. A squared piece of the nitrocellulose membrane was cut and a grid with 1 cm x 1 cm dimensions was drown on the nitrocellulose paper.
2. The membrane was then placed face up in a Petri dish for dotting.
3. 2-5 μL of protein samples were spotted onto the nitrocellulose membrane in the middle of the grid. All the proteins were dissolved in PBS pH 7.4.
4. Non-specific sites on the nitrocellulose were blocked by soaking in 10% BSA in TBS-Tween for 1 h at room temperature.
5. The membrane was washed three times for 5 minutes with TBS-Tween. It was incubated with a primary antibody diluted to the appropriate concentration in TBS-Tween with 5% BSA. The binding was performed for 1 h at room temperature. For primary antibodies directly conjugated with the horseradish peroxidase (Rabbit anti-OVM antibody), only one incubation was required.
6. The membrane was washed three times for 5 minutes with TBS-Tween, and it was incubated with a secondary antibody conjugated with PA, diluted 1:10,000 in TBS-Tween with 5% BSA. Incubation was carried out for 1 h at room temperature.
7. The nitrocellulose was finally washed three times for 5 minutes with TBS-Tween and the washing solution was discharged.
8. Detection by horseradish peroxidase (HRP):

   · The working solution was prepared by mixing 1 part of chemiluminescent reagent with 1 part of the chemiluminescent reaction buffer, mixed and equilibrated at room temperature for 30 minutes before use.

   · The membrane was incubated with the working solution for 3-5 minutes at room temperature.

   · The chemiluminescent signal was acquired by Kodak Capture Twain.

9. Detection by alkaline phosphatase (AP):

· The membrane was incubated in the substrate solution BCIP/NBT for AP. Upon incubation, a dark blue-purple reaction product was developed on membrane sites where alkaline phosphatase was localized.

· The reaction was stopped by rinsing briefly with deionized water, and the image was acquired by an image scanner.

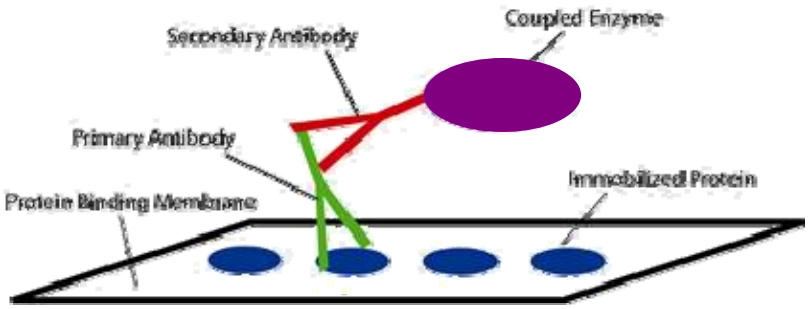

Figure 14. Dot blot scheme. The proteins are spotted on a nitrocellulose membrane. The membrane are incubated with a primary antibody specific for the protein target. A secondary antibody with a coupled enzyme is added. The membrane is incubated with the substrate solution. The reaction produces a coloured signal which can be acquired.

Western blot. Western blot analysis, also known as immunoblotting, is used to detect specific proteins from a heterogeneous sample. The protocol was first developed by Harry Towbin *et. al (66)* using a nitrocellulose membrane (Fig. 15).

The method is composed of four main steps:

- electrophoresis of the sample containing the target protein
- electrophoretic transfer to a nitrocellulose membrane
- labelling of target protein with specific primary and secondary antibodies
- detection and imaging of the target protein

Procedure:

1. SDS-PAGE analysis was performed as described in methods section 3.
2. The "western blot sandwich" was prepared by orienting cathode, filter paper, gel, nitrocellulose membrane, filter paper and anode. The protein transfer goes in the cathode to anode direction.
3. The transfer phase was performed for 90 minutes at 100V or overnight at 4 °C at 20V.
4. The membrane was incubated in the blocking solution for 1 hour at room temperature or at 4 °C overnight while shaking. The blocking solution was composed of 10% BSA in TBS-Tween.
5. The nitrocellulose was incubated with a primary antibody dissolved in TBS-Tween with 5% BSA, at room temperature for 1 hour.
6. The antibody solution was removed and the membrane was washed three times for 10 minutes in TBS-Tween.
7. The membrane was incubated with the secondary antibody dissolved in TBS Tween with 5% BSA, for 1 hour at room temperature.
8. The antibody solution was discharged and the membrane was washed three times for 10 minutes in TBS-Tween.
9. The detection steps were carried out in the same conditions as the dot blot protocol described above.

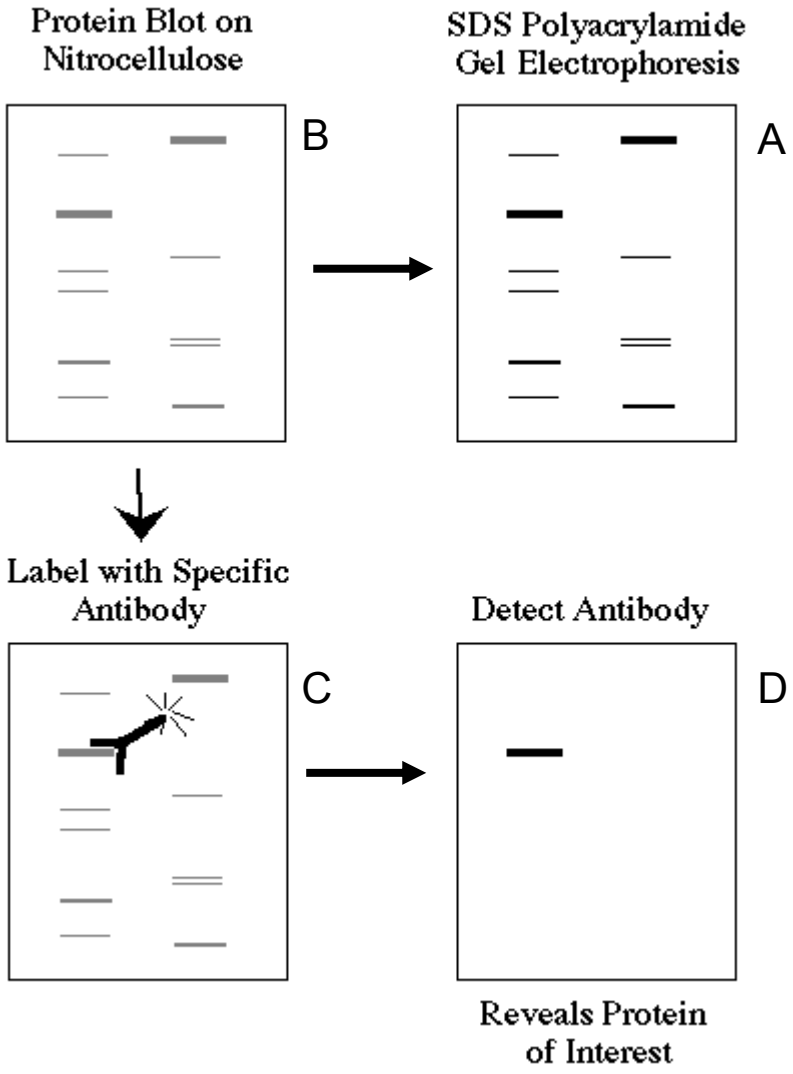

Figure 15. Western blot scheme. (A) The proteins are resolved by SDS-PAGE electrophoresis and then are transferred from the gel to the nitrocellulose membrane (B). (C) Labelling step with the specific antibody. (D) Revealing step of the protein target.

4. Reaction of α-LA and of a model proteins mixture by DNPS-Cl

The conditions for the derivatization of Trp residues with DNPS-Cl were optimized on a model protein, α-LA. After reduction and alkylation, chemical derivatization of 100 μg of α-LA 5 mg/mL was performed by adding a 30-fold molar excess of 2-4,dinitrobenzene-sulfenyl chloride in 35 μL of glacial acetic acid for 1 hour at room temperature. Upon addition of water, the unreacted DNPS-Cl decomposed and formed mostly an insoluble nitro-aryl-disulfide, which was separated by centrifugation. For diagonal chromatography experiment, the samples isolated according to the chromatographic peaks derived from the RP-HPLC were dried in a vacuum centrifuge and labelled by subsequently dissolving them in 15 μL of a 1 mg/mL solution of DNPS-Cl in glacial acetic acid.

Concerning the model mixture, 100 μL of the carbamidomethylated proteins mixture (lysozyme, apomioglobin, hemoglobin, carbonic anhydrase, albumin, α-lactalbumin and aprotinin) were desalted by Sephadex G-25 SF column equilibrated in 5% acetic acid. The eluted fraction, which contains the protein mixture, was dried in a vacuum concentrator and then dissolved in 120 μL of 5% acetic acid. 450 μL of DNPS-Cl in 100% acetic acid were added to the dried proteins mixture. The molar ratio between protein:DNPS-Cl was about 1:20. The reactions were performed at room temperature in the dark for 4 hours upon stirring. The reaction was quenched by dilution with water. 50 μL of the protein mixture were loaded on a RP-HPLC and the eluted material was analyzed by MS in order to confirm the modification of the proteins by DNPS-Cl. The mixture was then dissolved in 100 μL of 6 M Gdn·HCl and reloaded in a Sephadex G-25 SF column eluted with 50 mM Tris·HCl, 0.5 M Gdn·HCl, pH 8.5. Finally, the protein mixture was digested by trypsin.

5. RP-HPLC and the "diagonal" chromatography approach

Reversed phase HPLC (RP-HPLC) consists of a non-polar stationary phase and an aqueous, moderately polar, mobile phase, in a high pressure system. The RP-HPLC analysis was performed with a liquid chromatograph instrument at high pressure, purchased from Agilent Technologies, model 1100 (Waldbronn, Germany). The column used was a Jupiter C_{18} (Phenomenex, Torrance, CA, USA), whose dimensions are 4.6 x 250 mm with a diameter particles of 5 μm. Chromatographic separations were performed at flow rate of 0.8 mL/min by a linear water/acetonitrile gradient, containing 0.1% and 0.085% respectively of trifluoroacetic acid (TFA), from 10 to 80% in 40 minutes. For diagonal chromatography experiment on seven model protein mixtures the gradient of AcCN was from 10 to 80% in 37 minutes and the second one. The absorbance of the effluent from the RP column was monitored at 226 nm.

Diagonal chromatography is characterized by two chromatographic steps occurring in the same condition in terms of AcCN gradient and RP-column type. The whole tryptic digest of a protein mixture is resolved on a RP-HPLC run. The fractions of interest are then modified by addition of the chemical tag DNPS-Cl and reloaded in RP-HPLC in the same conditions as the first RP-separation. Peptides that did not react with DNPS-Cl are eluted with the same retention time of the first run, while the DNPS-Cl modified peptides show a shift in the retention time according to the increase in hydrophobicity induced by DNPS-Cl. In the experiments on the seven model proteins, the whole tryptic digest was loaded on the RP-HPLC column according to the previously described conditions. RP-fractions were collected at a constant time interval of 1 minute, for 30 minutes and they were dried in a vacuum concentrator. The 30 fractions were incubated with DNPS-Cl. The fractions with a 15 minutes shift in the retention time of the first run were loaded together in the second RP-HPLC run. The peaks with a shift to higher retention time were collected and dried. Four fractions were reloaded individually because the potential peaks were overlapping. For the "targeted" diagonal chromatography approach, the whole tryptic digest of the raw cake was resolved on a RP-HPLC run using an organic solvent gradient from 10 to 80% of AcCN in 40 minutes. RP-fractions were collected at a constant time interval of 1 minute, for 50 minutes. On the basis of retention times of Trp-containing peptides of the standard α-LA, four main eluted fractions were dried and reacted by DNPS-Cl. The modified fractions were individually loaded in RP-HPLC and all the eluted peaks were recovered and dried for further MS analyses.

6. Affinity column prepared with anti-DNP antibodies

In order to select the α-LA Trp-labelled peptides by affinity enrichment, the CNBr-activated Sepharose (Fig. 16) was derivatized with the monoclonal antibody anti-dinitrophenyl group clone SPE-7, purchased from Sigma-Aldrich. The IgE concentration at 280 nm was 1 mg/mL in PBS, pH 7.4 in the presence of 15 mM sodium azide.

Coupling reaction of Antibodies anti- and Sepharose CNBr-activated medium:

1. 200 μg of anti-dinitrophenyl monoclonal antibody was provided in phosphate buffer pH 7.4 containing 15 mM sodium azide as a preservative. For the coupling reaction, the antibody should be in 0.1 M $NaHCO_3$ 0,5 M NaCl buffer pH 8.5. The antibody solution was dialyzed against the coupling $NaHCO_3$/NaCl buffer at 4 °C overnight, using the dialysis cassettes, with a cut-off of 10,000 Da.
2. 100 μg of dry Sepharose CNBr-activated resin were washed with 10-15 volumes of cold 1 mM HCl. The supernatant, which contains lactose, was removed between successive additions.
3. The resin was washed with 5-10 column volumes of distilled water, then the medium was conditioned with the $NaHCO_3$/NaCl coupling buffer and immediately transferred to the antibody solution in coupling buffer.
4. The antibody was mixed with the medium overnight at 4 °C. The coupling rate was checked by measuring the UV absorbance at 280 nm of the supernatant after coupling reaction .
5. The unreacted ligand was washed away using $NaHCO_3$/NaCl coupling buffer
6. The unreacted groups were blocked with 0.1 M Tris·HCl pH 8.0 for 2 hours at room temperature.
7. The blocking solution was removed firstly with basic coupling buffer at pH 8.5, then with acetate buffer 0.1 M, pH 4 containing 0.5 M NaCl.
8. The washing step (see point 7) was completed after 4-5 repetitions.
9. Finally 250 μL of drained coupled-gel was obtained.

Elution of Sepharose-conjugated DNP antibody. Sepharose-conjugated DNP antibody was packed in a micro-spin column (2 x 0.6 cm), moved to a receiver tube, washed and conditioned with 0.1 M NH_4HCO_3 pH 8.0. To avoid unspecific cleavage of the antibody coupled to the medium, the trypsin inhibitor AEBSF in aqueous solution was added to a final concentration of 0.1 mM before starting the affinity selection. Then the α-LA digest (2 μg) modified by DNPS-Cl was diluted to a final volume of 30 μL with 0.1 M NH_4HCO_3 and loaded on the resin bed. The top and the bottom caps of the column were inserted and the system was mixed with an end-over-end mixer for 30 minutes to optimize the interaction between the antibodies and the DNP moiety of the peptides. To ensure complete binding, the medium was centrifuged and the flow-through was recovered and reloaded onto the column. The second flow-through was retained to confirm the sample binding. The unabsorbed non-tryptophan peptides were washed out with 0.1 M NH_4HCO_3 pH 8.0 in 5 washing steps. The resin was rinsed 4-5 times with milliQ water. The matrix-adsorbed tryptophan-DNP peptides were eluted with 10% formic acid in 5 steps. Finally the medium was washed with 6 M Gdn·HCl and stored in 20% ethanol. All the fractions recovered from each washing and elution steps (150 μL) were collected and gently dried in a vacuum concentrator for following MS analysis.

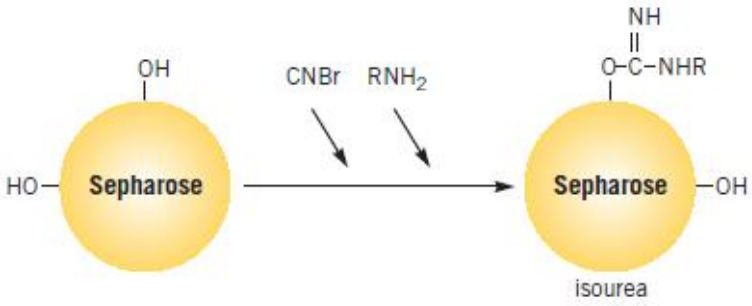

Figure 16. Activation by cyanogen bromide and coupling to the activated matrix.

7. Hydrophobic interaction chromatography (HIC)

In order to isolate Trp-containing peptides from a whole tryptic digest, hydrophobic interaction chromatography (HIC) was carried out. In HIC the peptides are adsorbed by hydrophobic interaction followed by a pH reduction that positively charges the pyridine ring of the ligand, thus causing ionic repulsion and the subsequent elution of the peptides.

500 μL of MEP HyperCel matrix was packed in a micro spin column (2 x 0.6 cm), moved to a receiver tube, washed and conditioned with 0.1 M NH_4HCO_3 pH 8.0. Then, 50 μg of α-LA digest (carbamidomethylated and DNPS-labelled α-LA) was diluted to a final volume of 400 μL with 0.1 M NH_4HCO_3 and applied on the top of the resin bed. The sample was eluted from the column by gravity. To ensure complete binding, the medium was centrifuged and the flow-through was recovered and reloaded into the column. The second flow-through was retained to confirm the sample binding. The unabsorbed non-tryptophan peptides were washed out using 0.1 M NH_4HCO_3 pH 8.0 in four washing steps. The resin was washed 2 times with milliQ water until the pH dropped. The adsorbed tryptophan peptides were eluted with 50 mM sodium citrate or using 0.1% formic acid in four steps. Finally the medium was washed with 6 M Guanidine hydrochloride and stored in 20% ethanol. All the fractions recovered from each washing-elution step (400 μL) were collected and gently dried in a vacuum concentrator for subsequent MS analysis.

8. Fingerprinting analysis in water/acetonitrile

The “in-solution digestion” was performed according to the procedure described by Delahunty *et al*.(*67*). Fingerprinting with trypsin is the most used method to analyze a protein. The procedure implies first the reduction of disulfide bridges of the protein to free thiols and then the subsequent S-alkylation of the free thiols. Trypsin reaction with the S-alkylated protein produces a tryptic digest and thus to the generation of peptide fragments whose identity is determined by mass spectrometry.

For fingerprinting analysis in solution, the protein sample was dissolved in a small volume of 100 mM Tris·HCl, 8M Urea pH 8.5. To reduce the disulfide bridges Tris(2-carboxyethyl)fosfine (TCEP) was added to a final concentration of 5 mM and it was incubated at room temperature for 15 minutes. After reduction, the cysteine residues were alkylated with Iodoacetamide (IAA). The IAA reaction was carried out in the same buffer and it was added to a final concentration of 25 mM at room temperature, in the dark for 30 minutes. Before adding trypsin, the protein solution was diluted to 2M Urea with 50 M ammonium bicarbonate pH 8.0, and 1 mM $CaCl_2$ was added. Finally, the reaction with trypsin was performed at a ratio of 1:50 (enzyme:substrate), and the solution was incubated at 37 °C for 18 hours.

An organic solvent-assisted tryptic digestion procedure was optimized during the affinity chromatography selection of Trp-labelled peptides of α-LA (*68-70*). The protein sample was dissolved in a small volume of 100 mM Tris·HCl, 8M Urea pH 8.5 and the protein was reduced and alkylated as described above. The carbamidomethylated α-LA was labelled by DNPS-Cl and the excess of salts and reagent were removed by desalting in C-18 spin column. The protein was then eluted in 50 mM Tris·HCl, 80% AcCN pH 7.6. The fingerprinting reaction was performed in the organic solvent mixture at a ratio of 1:50 (enzyme:substrate), and the solution was incubated at 37 °C for 18 hours.

9. Mass spectrometry ESI Q-Tof and LC-MS/MS

A mass spectrometer determines the mass of a molecule by measuring the mass-to-charge ratio (m/z) of its ion. Ions are generated by inducing either the loss or gain of a charge from a neutral species. Once formed, ions are electrostatically directed into a mass analyzer, where they are separated according to m/z and finally detected. The result of the molecular ionization, ion separation and ion detection is a spectrum that can provide an estimate of molecular.

Mass spectrometry analysis were performed with a Micromass mass spectrometer Q-Tof micro (Manchester, UK), connected with a micro-HPLC CapLC (Waters, Milford, MA, USA). Samples were dissolved in 0.1% formic acid and separated on a column Atlantis C_{18} nano-ESI, 75 μm x 150 mm (Waters). Elution from the column was performed with milliQ water containing 5% acetonitrile and 0.1% formic acid (solvent A) and acetonitrile containing 5% water and 0.1% formic acid (solvent B). The linear gradient of solvent B was from 5 to 70% in 40 min at a flow rate of 200 nL/min.

10. Database research

The MS/MS spectra were analyzed with the MASCOT software (http://www.matrixscience.com) for the database research, selecting trypsin as proteolytic enzyme and S-carbamidomethylation (S-CH_2CONH_2) as modification occurring at cysteine residues. Possible modifications of the amino acid side chains, such as oxidation of Met and phosphorylation of Ser, Thr and Tyr, were considered during the query.

III. RESULTS

1. Immunochemical approaches for the identification of allergenic proteins in foods

The standard analytical procedure usually applied for the identification of allergenic proteins in food matrices is represented by the immunological assays. These methods are based on the selective interaction and recognition of an antigenic protein by the specific antibodies. It has been demonstrated that this approach suffers from several limitations due to the low specificity of antibody recognition and to cross-reactivity reactions. Indeed, allergenic proteins can induce allergic reactions in their native structural state or after chemical or conformational changes induced by industrial treatments. These alterations in the allergen's structure or chemical modifications can prevent the interaction with the antibody and this is the cause of many false negative results.

In order to test the immunochemical approach, different polyclonal antibodies raised against the main allergenic proteins of milk (α-lactalbumin and β-lactoglobulin) and eggs (ovomucoid, ovalbumin and lysozyme) were purchased. Preliminary studies were conducted in order to check the quality of the antibodies in terms of specifity of recognition and cross-reactivity. Moreover, the responses of the antibodies were checked using as antigens the purified commercial proteins and the same proteins contained in complex food matrices before and after thermal treatment.

In Fig. 17, dot blot tests are reported which were performed using the commercial polyclonal antibodies raised against α-lactalbumin (α-LA), β-lactoglobulin (β-LG) and lysozyme (LYS). It is interesting to report that a first batch of antibodies against LYS was substituted by the company because it lacked specificity both in dot and western blot tests. Indeed, it recognized all the negative controls that were tested (data not shown). Each antibody was tested in dot blot assays using several negative control proteins and the total protein extracts of a raw and a cooked bakery products, as it is indicated in block scheme (Fig 17 A). It was observed that the antibodies against α-LA (panel B) are specific for the protein which is recognized in the spot of the raw cake protein extract. In panels C and D, the dot blot assays are shown that were conducted with the antibodies against β-LG and LYS respectively. Both the antibodies detect the proper allergenic protein and they gave positive results also with the protein extracts of a raw and a cooked bakery products. The

application of several negative controls during these tests allows to confirm the specifity of the antibodies and to exclude cross-reactions with other antigenic proteins as α-LA and LYS.

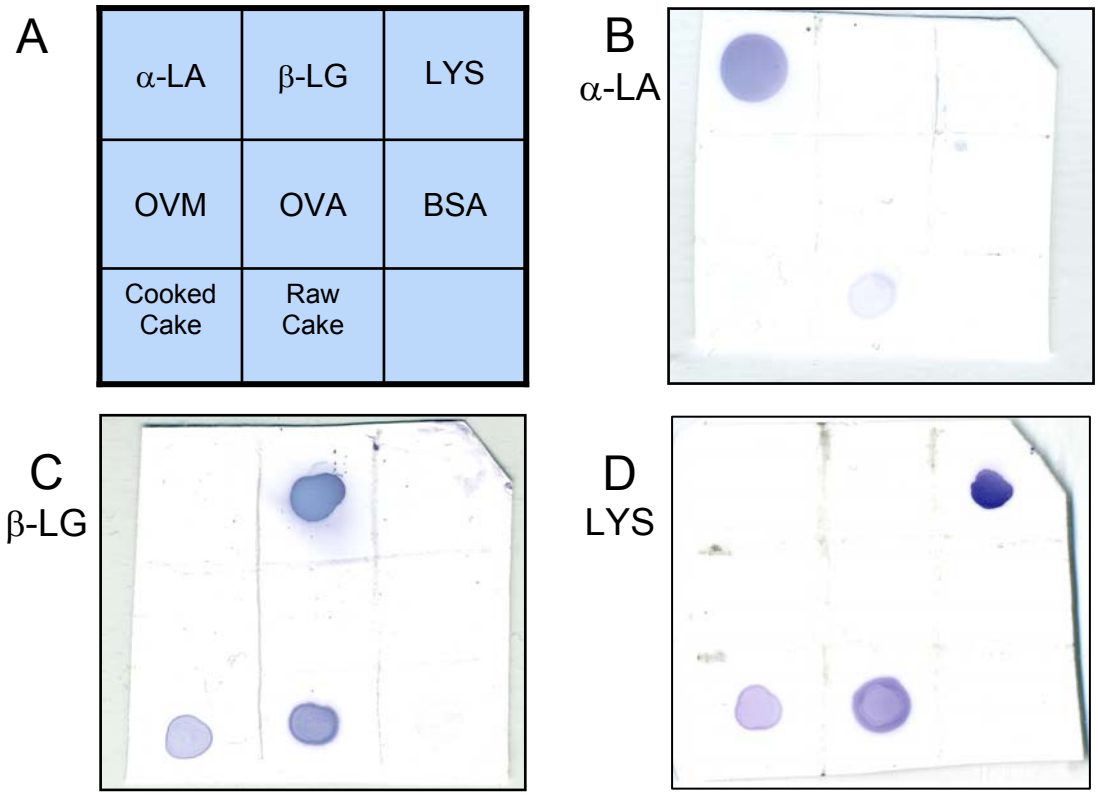

Figure 17. Dot blot assays of the main allergenic proteins of milk and eggs contained in commercial batches and in a cooked and raw bakery products. The tests were conducted on a nitrocellulose membrane. (A) In the blue square, the loading scheme of the blot membranes is reported. The protein samples were dissolved in PBS pH 7.4 and 5 μg (corresponding to 2-4 μL) were blotted on the nitrocellulose support. (B) Dot blot test conducted with a primary antibodies against α-LA diluted at a ratio of 1:5,000. (C) Dot blot test conducted with a primary antibodies against β-LG dilute at a ratio of 1:5,000. (D) Dot blot test conducted with a primary antibodies against LYS diluted at a ratio of 1:5,000. The membranes were incubated with a secondary antibody conjugated to alkaline phosphatase (AP) diluted 1:10,000. The detection was performed using a BCIP/NBT solution.

In Fig. 18, dot blot assays are shown that evaluate the specificity of antibodies raised against OVM and OVA. The assays were performed using the same negative controls of the previous experiments and on the total protein extracts of a raw and a cooked cakes. Interestingly, both antibodies recognize OVM and OVA. Since the observed lack of specificity can be caused by the cross-contamination of the two proteins in the two commercial samples, the purity of the two batches of OVA and OVM were checked by RP-HPLC and the chromatograms are shown in Fig. 19. The RP-HPLC separations demonstrate that the commercial sample of OVM is pure (Fig. 19A) whereas that of OVA is contaminated by traces of OVM, as evidenced by the presence of a small peak with the retention time of OVM (Fig. 19B). The results of the RP-HPLC analyses indicate that the antibodies against OVM recognized the spot of the commercial batch of OVA due to a contamination of this commercial sample. On the other hand, they suggest that the recognition of the antibodies anti-OVA of the commercial sample of OVM is not due to cross contamination but it is caused by a lack of specificity of the antibody. In order to further test the specificity of the antibodies against OVA and OVM, the eggs allergenic protein LYS, OVM and OVA were purified by RP-HPLC and analyzed by dot blot as shown in Fig 20. The antibodies anti-OVM recognize only the spot of the purified protein, whereas interestingly, the antibodies anti-OVA recognize selectively the spot of purified OVM, thus clearly confirming the unspecific recognition of this antibody preparation.

In Fig 21, two western blot assays are reported that were performed with the antibodies against OVM and OVA used in the previous dot blot tests. The blots were loaded with the commercial allergenic proteins α-LA, β-LG, LYS, OVM and OVA and with protein extracts from a raw and a cooked cake. In these tests, the antibodies anti-OVA recognize all the proteins that were blotted whereas the antibodies against OVM confirm their specificity both in dot and western blot assays and even on a protein extract from a cooked cake. It can be concluded that the antibodies anti-OVA show also a difference in specificity between dot and western blot assays. Antibody recognition is extremely sensitive to the three-dimensional structure of a protein and as a consequence to conformational changes of the antigenic protein during the immunochemical assay. On this basis, it is probable that in dot blot analyses the anti-OVA antibodies show a higher specificity because the proteins maintain a native conformation whereas in western blot assays the antigens are denatured by the SDS-PAGE separations.

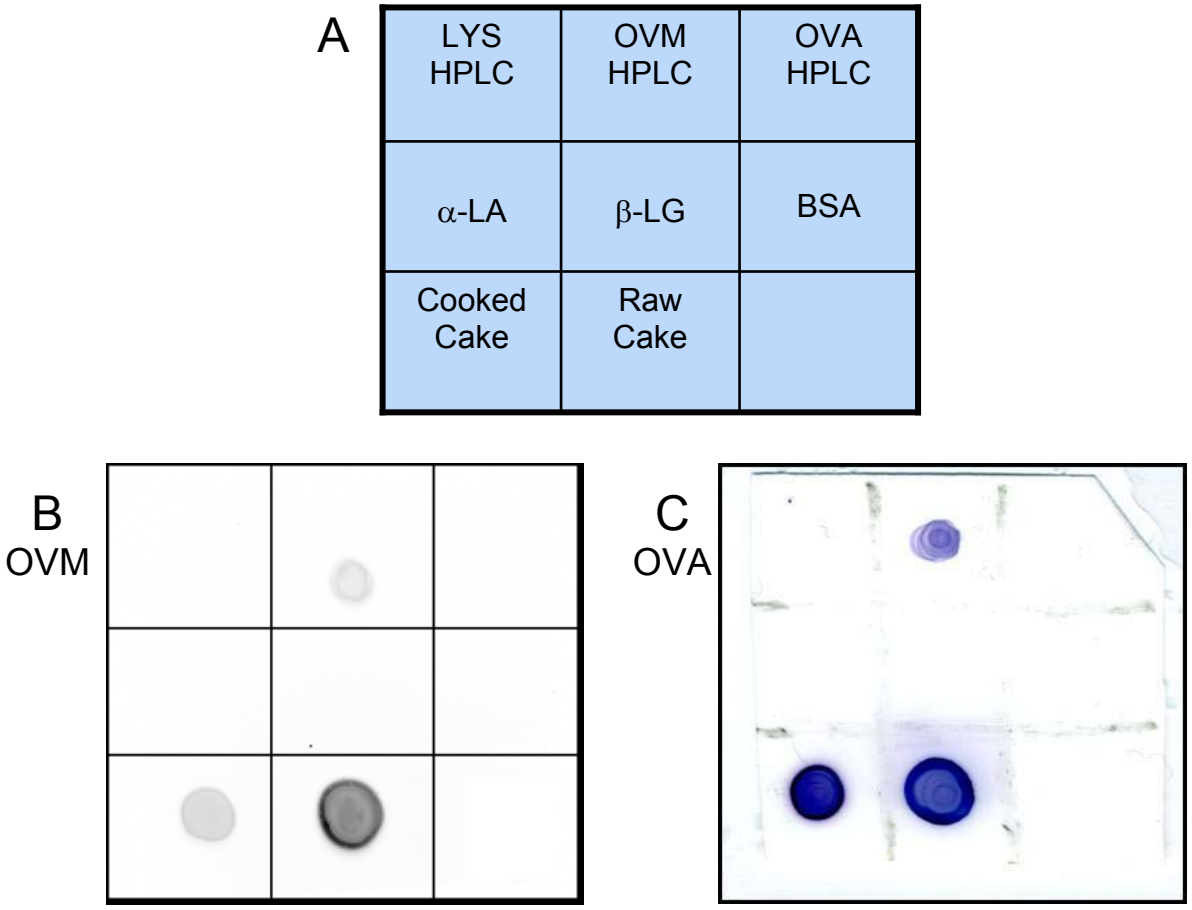

Figure 18. Dot blot assays of the main allergenic proteins of milk and eggs contained in the commercial batches and the protein extract of a cooked and raw bakery products. The protein samples of LYS, OVM and OVA were previously purified by RP-HPLC. The tests were conducted on a nitrocellulose membrane. (A) In the blue square, the loading scheme of the blot membranes is reported. The protein samples were dissolved in PBS pH 7.4 and 5 μg (corresponding to 2-4 μL) were blotted on the nitrocellulose support. (B) Dot blot test conducted with a primary antibodies against OVA diluted at a ratio of 1:2,000. The membrane was incubated with a secondary antibody conjugated with AP diluted 1:10,000. The detection was performed with a BCIP/NBT solution. (C) Dot blot test conducted with a primary antibodies against OVM diluted at a ratio of 1:10,000 which is directly conjugated with the horseradish peroxidase. The detection was performed using a chemiluminescent reagent and the signal was acquired with Kodak Capture Twain.

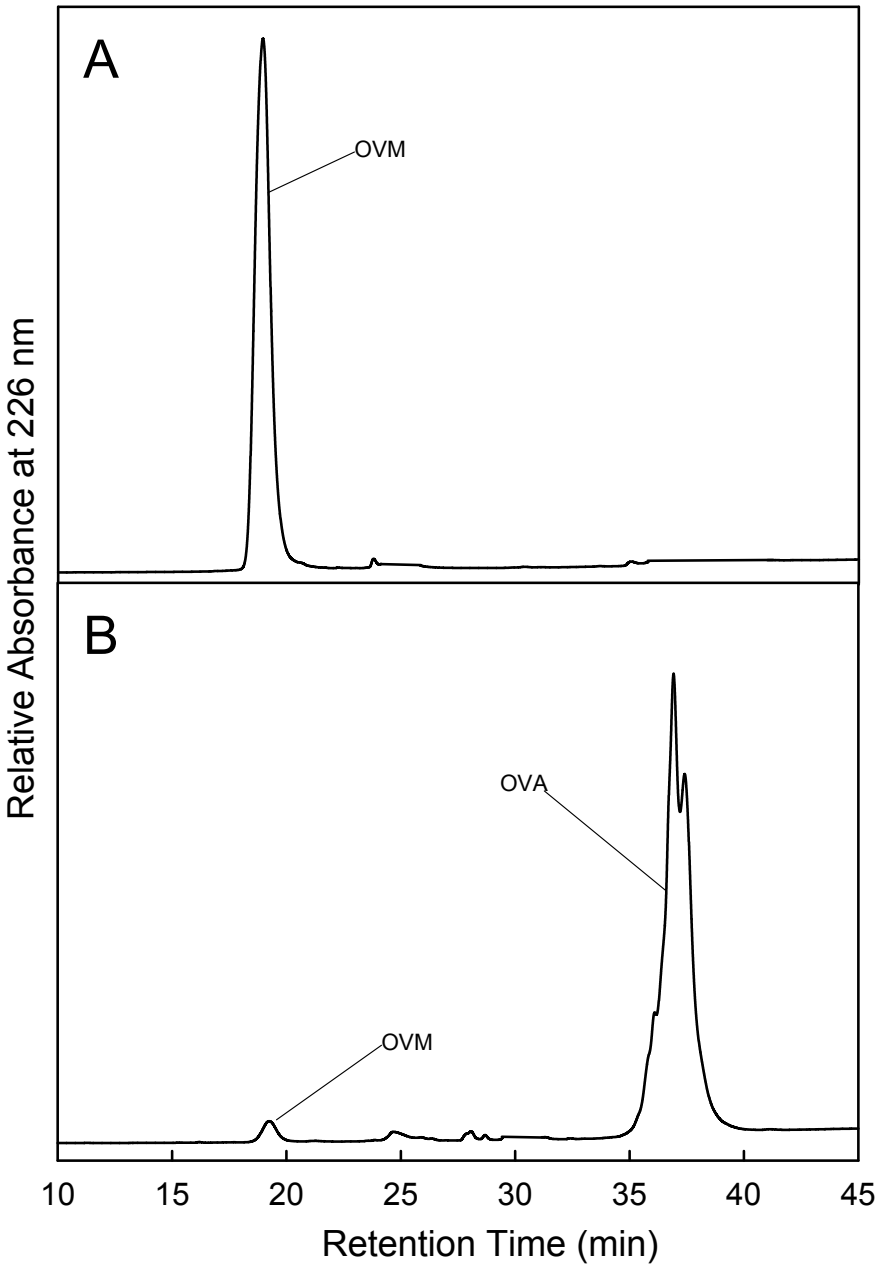

Figure 19. RP-HPLC chromatograms of the commercial batches of ovomucoid (A) and ovalbumin (B) in the dot and western blot assays. The proteins were loaded on a Vydac C_4 column (250 x 4.60 mm) eluted at a flow rate of 0.8 mL/min with a linear gradient of acetonitrile containing 0.1% TFA, from 5 to 60% in 40 min. The absorbance of the effluent from the column was monitored at 226 nm and identities of the proteins were confirmed by MS analysis.

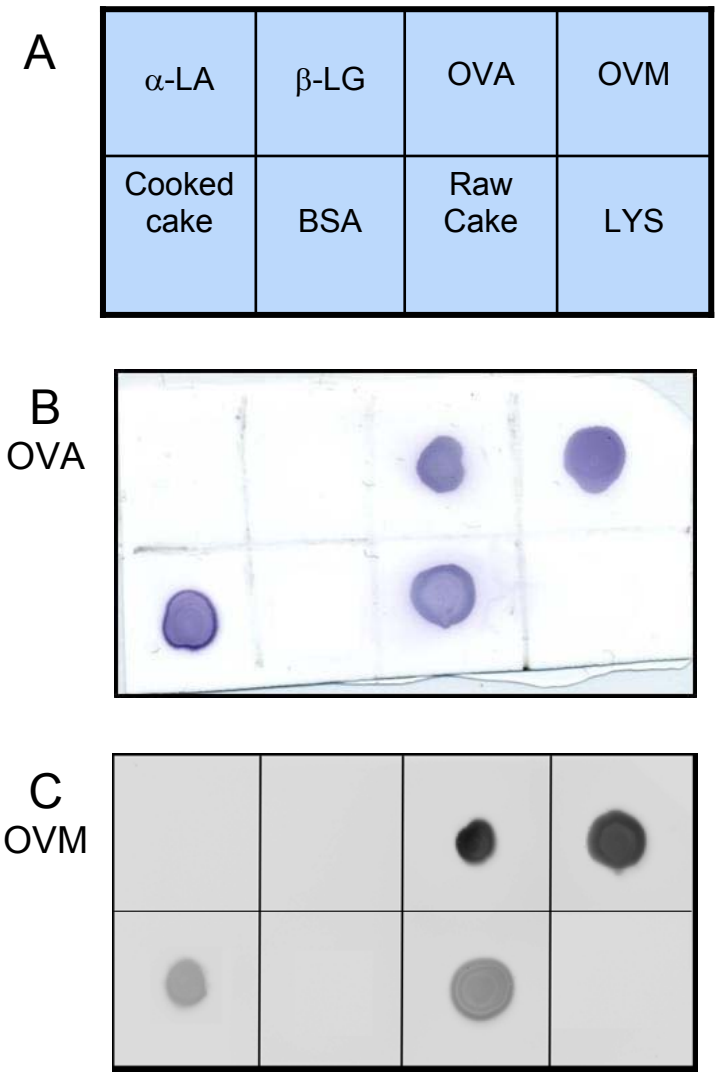

Figure 20. Dot blot assays of the main allergenic proteins of milk and eggs contained in the commercial batches and in a cooked and raw bakery products. The tests were conducted on a nitrocellulose membrane. (A) In the blue square, the loading scheme of the blot membranes is reported. The protein samples were dissolved in PBS pH 7.4 and 5 μg (corresponding to 2-4 μL) were blotted on the nitrocellulose support. (B) Dot blot test conducted with a primary antibodies against OVA diluted at a ratio of 1:2,000. The membrane was incubated with a secondary antibody conjugated with AP diluted 1:10,000. The detection was performed whit a BCIP/NBT solution. (C) Dot blot test conducted with a primary antibodies against OVM diluted at a ratio of 1:10,000 which is directly conjugated with the horseradish peroxidase. The detection was performed using a chemiluminescent reagent and the chemiluminescent signal was acquired with Kodak Capture Twain.

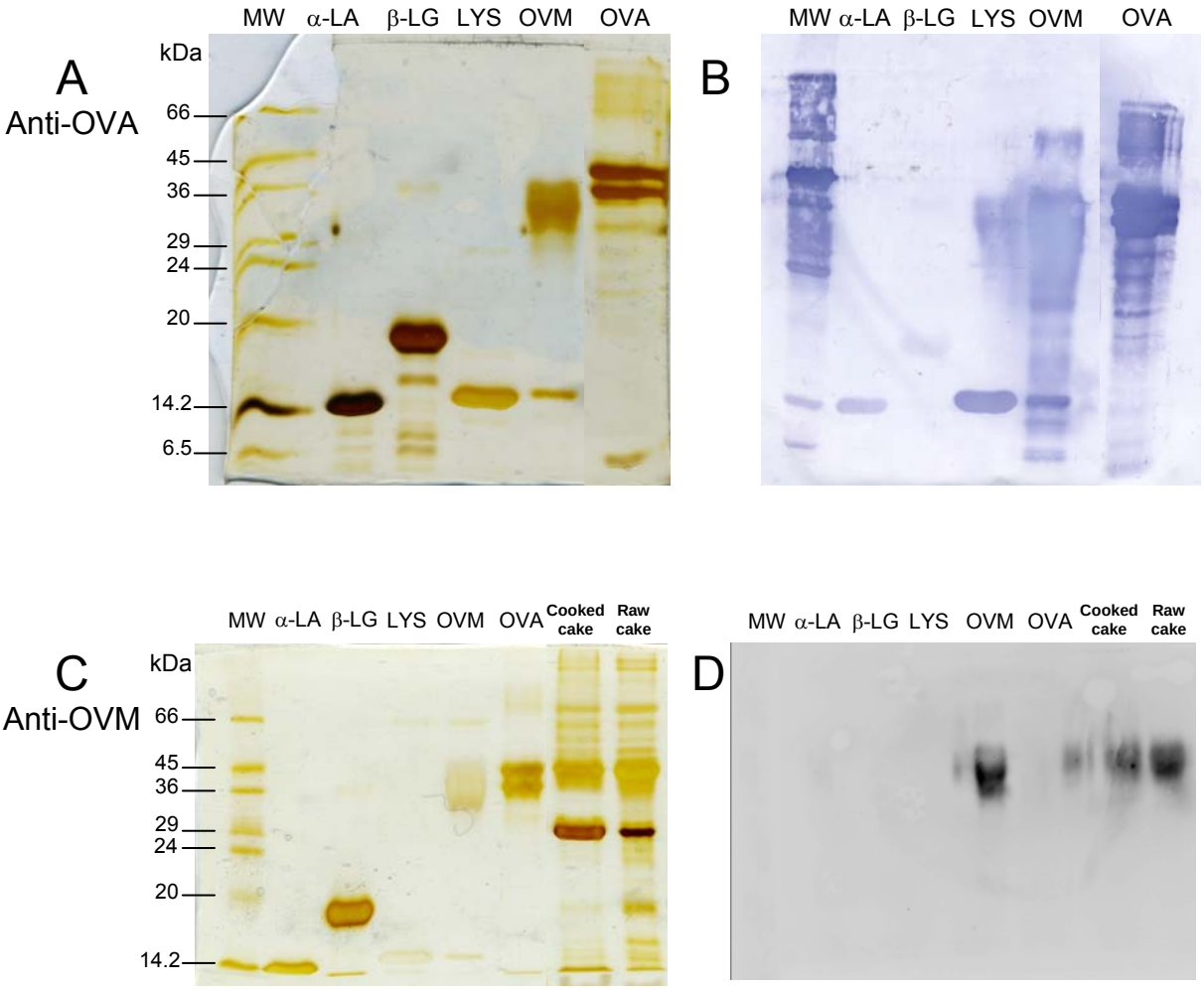

Figure 21. SDS-PAGE and Western blot assays of the main allergenic proteins of milk and eggs, contained in the commercial batch and in a raw and cooked bakery product. SDS-PAGE 15% gel electrophoresis analyses of the commercial proteins (A) and of the total protein extracts form a raw and cooked cake (C). In the first lane (MW) the standard molecular weights are indicated. Western blot assays of the corresponding SDS-PAGE gels (B,D). The tests were conducted on a nitrocellulose membranes. (B) Western blot test was conducted with primary antibodies against OVA dilute at a ratio of 1:2000. The membrane was incubates with a secondary antibody conjugated with AP diluted 1:10,000. The detection was performed with a BCIP/NBT solution. (D) Western blot test conducted with primary antibodies against OVM dilute at a ratio of 1:10,000 which is directly conjugated with the horseradish peroxidase. The detection was performed using a chemiluminescent reagent and the signal was acquired with Kodak Capture Twain.

Summing up, the immunological tests performed in the present study suggest that the specificity of the antibodies used for the detection of protein allergens in food matrices has always to be carefully checked. In general, the observed lack of specificity of the anti-OVA antibodies can be due to the method of preparation of the antibodies, and in particular to the characteristics of the sample of antigenic proteins used to induce the formation of the antibodies and to the method of isolation of the specific polyclonal antibodies from the whole animal serum. We also observed a dependence of the specificity of the antibodies on the type of immunological test used for the analysis, thus suggesting that also the conformational features of the antigenic proteins are an important aspect of immunological assays. Conformational changes can thus be considered one of the cause of the false negative and false positive results which give an ambiguous indication of the presence of the allergenic proteins in food matrices.

2. Diagonal chromatography approach for the selection of Trp-containing peptides

2.1. RP-HPLC analysis of standard α-lactalbumin reacted with DNPS-Cl

The aim of this experiment is to demonstrate that the chemical tagging of a protein by DNPS-Cl determines an increase of hydrophobicity and as a consequence a shift in retention time in a RP-HPLC analysis. Bovine α-LA was selected as model protein because it contains four tryptophan residues in position 26, 60, 104 and 118 of the polypeptide chain and it is one on the major allergenic proteins of bovine milk. α-LA was labelled with the chemical tag DNPS-Cl. Best conditions for the chemical labelling reaction were found the addition of 30-fold molar excess of DNPS-Cl dissolved in glacial acetic acid to an aqueous solution of carbamidomethylated α-LA. The reaction mixture was incubated for 1 hour in the dark and then it was quenched by dilution with water. This solution was analyzed by RP-HPLC (Fig. 22) and the eluted material was collected, dried in a vacuum concentrator and analyzed by MS (Tab. 1).

The RP-HPLC chromatograms of native α-LA, of carbamidomethylated α-LA and of this last form of the protein after derivatization with DNPS-Cl are compared in Fig. 22. From the figure, it is evident the shift of the peak of α-LA towards higher retention times when the protein is reduced and alkylated and after reaction with DNPS-Cl. In the chromatogram of DNPS modified α-LA, another species with a lower retention time in respect to the conjugated protein was also observed which corresponds to labelled α-LA oxidized at the level of a Met residue, as confirmed by MS analysis. The RP-HPLC analyses clearly demonstrate that the DNPS moiety increases the hydrophobicity of the Trp residues since it causes a shift in the retention time of labelled α-LA. Moreover, the reaction takes place with a high yield since the chromatographic peak of the unmodified protein is no more detectable. On the basis of the mass spectrometry data, the reaction is strictly selective to the four Trp residues since the molecular mass of the product corresponds to the expected mass for α-LA conjugated to four DNPS moieties (Table 1). Peptide mass fingerprinting data confirm this result (see below).

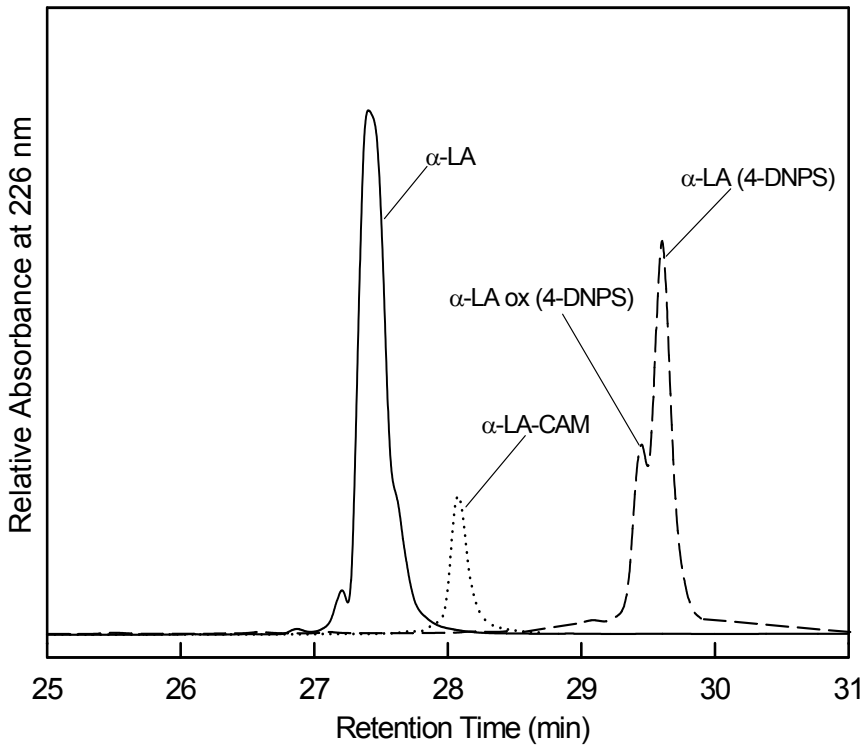

Figure 22. RP-HPLC chromatograms of native α-LA (*solid line*), of the protein after reduction and carbamidomethylation (*dotted line*) and after reaction with DNPS-Cl (*dashed line*). The figure was obtained by overlaying three distinct RP-HPLC runs. The protein was loaded on a Phenomenex C_{18} column (250 x 4.60 mm) eluted at a flow rate of 0.8 mL/min with a linear gradient of acetonitrile containing 0.1% TFA from 10 to 80% in 40 min. The absorbance of the effluent from the column was monitored at 226 nm and identities of the proteins were confirmed by MS analysis.

Table 1. Molecular masses of native α-LA and of the protein after reduction and carbamidomethylation and after reaction with DNPS-Cl.

Protein	*Calculated Mass*[a]	*Found Mass*[b]
α-LA	14178.12	14178.08± 0.13
α-LA-CAM[c]	14642.54	14642.13± 0.17
α -LA-DNPS[d]	15435.18	15435.56± 0.23

[a]Molecular masses calculated from the amino acid sequence of α-LA, α-LA-CAM and α-LA-DNPS.
[b]Experimental molecular masses determined by ESI-MS.
[c] α-LA-CAM is commercial α-LA which was reduced by TCEP and alkylated by IAA.
[d]α -LA-DNPS is α-LA-CAM which was modified with DNPS-Cl.

2.2. Fingerprinting analysis of α-lactalbumin before and after reaction by DNPS-Cl

During this part of the project, the peptide fingerprinting of α-LA was performed to characterize the commercial protein and the products of the reaction with DNPS. The fingerprint technique consists on the digestion of proteins with a protease, generally trypsin and the subsequent analysis by MS of the peptide fragments produced in the reaction. Standard α-LA was reduced and carbamidomethylated and then digested with trypsin. Proteolysis was conducted for 18 hours at 37° C in 100 mM NH_4HCO_3 pH 7.8, with an enzyme to substrate ratio of 1:50 by weight. After incubation, the reaction was quenched upon addition of 2% TFA. The digestion mixture was analyzed by RP-HPLC (Fig. 23) and the identity of the eluted species was confirmed by MS analysis using a ESI Q-Tof instrument (Tab. 2). The sequence coverage of the protein calculated on the basis of the identified tryptic peptides was of 96 %.

In order to confirm the chemical tagging by DNPS-Cl at the level of the Trp residues of α-LA, the labelled protein was also digested with trypsin. The RP-HPLC chromatograms of the fingerprinting of α-LA before and after reaction by DNPS-Cl are compared in Fig. 24. The identities of the tryptic peptides were determined by MS analysis and the measured massed are reported in Table 2. In Fig. 24A, five Trp-containing peptides are indicated, that correspond to the most intense peaks in the RP-HPLC chromatogram of the digestion mixture of the unmodified protein. After the chemical tagging reaction, these peptides elute at higher retention times with shifts ranging from 2.5 to 7.1 minutes (Fig. 24B, Table 3-4).

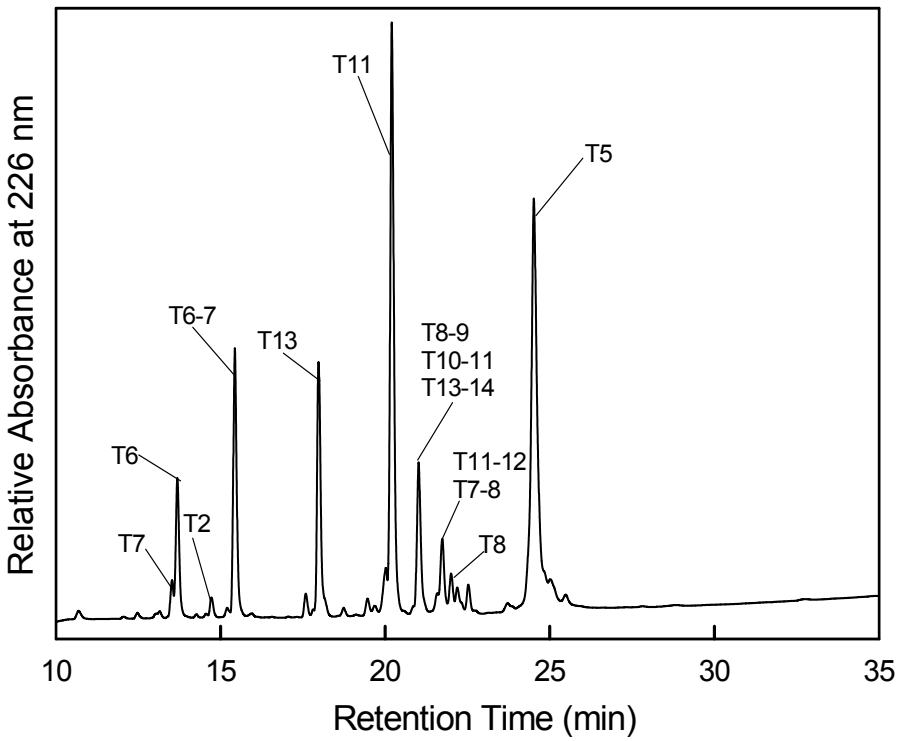

Figure 23. RP-HPLC chromatogram of the tryptic digest of α-LA. Proteolysis by trypsin was conducted for 18 h at 37 °C in 2M Urea, 0.1 M NH_4HCO_3 pH 7.8, with an E/S ratio of 1:50. The reaction was stopped upon addition of 2% TFA and loaded on a Phenomenex C_{18} column (250 x 4.60 mm) eluted at a flow rate of 0.8 mL/min with a linear gradient of acetonitrile containing 0.1% TFA from 10 to 80% in 40 min. The absorbance of the effluent from the column was monitored at 226 nm and identities of the fragment species were confirmed by MS analysis.

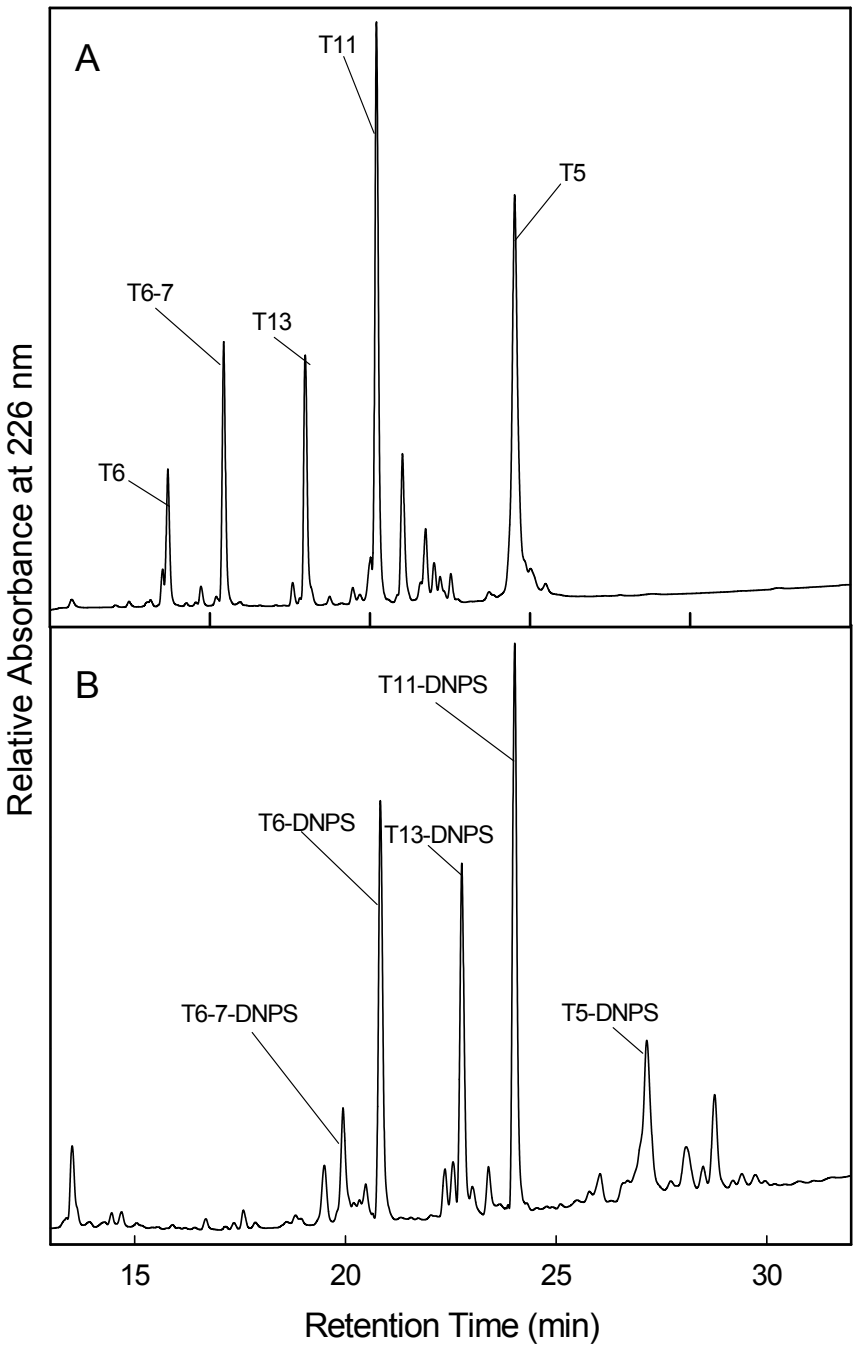

Figure 24. RP-HPLC chromatograms of the tryptic digest of α-LA-CAM (A) and of DNPS-labelled α-LA (B). Proteolysis with trypsin was conducted as described in fig. 23. The reaction was stopped upon addition of 2% TFA and loaded on a Phenomenex C_{18} column (250 x 4.60 mm) eluted at a flow rate of 0.8 mL/min with a linear gradient of acetonitrile containing 0.1% TFA, from 10 to 80% in 40 min. The absorbance of the effluent from the column was monitored at 226 nm and identities of the fragment species were confirmed by MS analysis. Arrows indicate the α-LA fragments which contain Trp residues (A) and the same residues modified by DNPS-Cl (B).

Table 2. Molecular masses of the tryptic peptides of α-LA produced in the fingerprinting reaction. The identity of the peptides were confirmed by MS analysis using an ESI Q-Tof instrument.

Protein	*Tryptic Fragment*		*Sequence*	*Calc. Mass*[a]	*Found. Mass*[b]
α-LA	T10	95-98	ILDK	487.30	487.31
	T6	59-62	IWBK	605.30	605.33
	T9-10	94-98	KILDK	615.40	615.78
	T12	109-114	ALBSEK	706.33	706.32
	T2	6-10	BEVFR	709.32	709.56
	T3-4	11-16	ELKDLK	744.44	744.44
	T13	115-122	LDQWLBEK	1090.51	1090.63
	T11	99-108	VGINYWLAHK	1199.65	1199.81
	T13-14	115-123	LDQWLBEKL	1203.60	1203.81
	T10-11	95-108	ILDKVGINYWLAHK	1668.94	1668.68
	T8	80-93	FLDDDLTDDIMBVK	1698.75	1698.76
	T8-9	80-94	FLDDDLTDDIMBVKK	1826.84	1826.99
	T11-12	99-114	VGINYWLAHKALBSEK	1887.97	1887.65
	T7	63-79	DDQNPHSSNIBNISBDK	2002.81	2002.61
	T6-7	59-79	IWBKDDQNPHSSNIBNISBDK	2590.10	2590.25
	T7-8	63-93	DDQNPHSSNIBNISBDKFLDDDLTDDIMBVK	3683.55	3683.87
	T5	17-58	GYGGVSLPEWVBTTFHTSGYD TQAIVQNNDSTEYGLFQINNK	4713.09	4713.45

[a]Molecular masses calculated from the amino acid sequence of α-LA-CAM.
[b]Experimental molecular masses determined by ESI-MS.

Table 3. Molecular masses of α-LA Trp-containing peptides before and after the reaction with DNPS-Cl.

Trp Peptides	*Sequence*	*Calc. Mass*[a]	*Found Mass*[b]	*Calc. Mass*[a] *+DNPS*	*Found Mass*[b] *+DNPS*
T 6	IWBK	605.30	605.33	803.27	803.30
T 6-7	IWBKDDQNPH SSNIBNISBDK	2590.10	2590.24	2788.07	2788.20
T 13	LDQWLBEK	1090.51	1090.63	1288.49	1288.62
T 11	VGINYWLAHK	1199.65	1199.81	1397.62	1397.83
T 5	GYGGVSLPEWVBTTFHT SGYDTQAIVQNNDSTEY GLFQINNK	4713.09	4713.45	4911.25	4911.51

[a]Molecular masses calculated from the amino acid sequence of native and of DNPS-labelled α-LA.
[e]Experimental molecular masses determined by ESI-MS.

Table 4. Retention times (RT) in the RP-HPLC analyses of the Trp-containing tryptic peptides of α-LA and the shift in RT determined by the derivatization with DNPS-Cl.

Tryptic peptides of α-LA	*RT (min)*	*ΔRT (min)*
T6	13.8	7.1
T6-7	15.6	4.5
T13	18.1	4.7
T11	20.3	3.8
T5	24.7	2.5

2.3. Chemical tagging by DNPS-Cl and fingerprinting analysis of a mixture of proteins

In order to set up the diagonal chromatography approach for the selection of Trp-containing peptides from a complex mixture of tryptic peptides, the method was tested on the tryptic digest of a model mixture of seven proteins (lysozyme, apomyoglobin, hemoglobin, carbonic anhydrase, bovine serum albumin, α-lactalbumin and aprotinin). All these proteins contain Trp residues except aprotinin that is considered as the negative control of the method. Moreover, these proteins have different molecular weights and are commercially available. The selection of only the Trp-labelled peptides of the proteins contained in the mixture can considerably reduce the number of the peptides to be analyzed by MS. Table 5 reports the calculated number of tryptic peptides and of Trp-containing peptides produced by trypsin hydrolysis of the seven model proteins. Digestion with trypsin can produce at minimum 200 tryptic peptides, but only 20 of these peptides contain Trp-residues. MS analysis of only the Trp-containing peptides can thus reduce the complexity of the digest and greatly simplify protein identification.

Table 5. Tryptic peptides and Trp-containing tryptic peptides produced by trypsin digestion of the seven model proteins.

Protein	*Calc. Mass*[a]	*N° Trp*	*Tryptic peptides*	*Trp-containing Tryptic peptides*
Lysozyme	14305.14	6	18	4
α-Lactalbumin	14186.12	4	14	4
Albumin	66433.22	2	78	2
Carbonic anhydrase	29023.67	7	25	6
Apomyoglobin	16951.50	2	23	1
α-Hemoglobin	15053.19	1	14	1
β-Hemoglobin	15954.42	2	18	2
Aprotinin	6517.58	0	10	0
Total peptides number			200	20

[a]Molecular masses calculated from the amino acid sequence of α-LA, LYS, BSA, CA, apoMb, aprotinin, α-H and β-H.

The mixture of the seven model proteins was reduced, carboxamidomethylated and digested with trypsin according to the protocol reported in Material and Methods (*67*). The digest was analyzed by RP-HPLC and fractions were collected at time intervals of 1 minute as indicated in Fig. 25A by vertical lines. Each fraction was dried and modified by DNPS-Cl using the reaction conditions optimized for α-LA. The derivatized fractions were then analyzed by RP-HPLC under the same chromatographic conditions of the first run in terms of gradient of organic solvent and RP-column. The peaks which eluted with a shift in the retention time were collected and analyzed by MS (Table 6). As an example, Fig. 25B reports the RP-HPLC chromatogram of the fraction collected at 16 minutes after derivatization with DNPS-Cl. The peptide material that still elutes at 16 minutes is represented by peptides without Trp residues. On the contrary, by MS it was confirmed that the peptides that show a shift towards higher retention times contain Trp residues that are modified by DNPS-Cl.

In the RP-HPLC separation of the tryptic digest a considerable number of fractions was collected which means that the RP-HPLC analysis of each DNPS-derivatized fraction would be quite time consuming. In order to reduce the number of these RP-HPLC analyses, the DNPS-derivatized fractions were loaded in the number of two for each chromatographic analysis. The paired fractions were chosen so that they differ of 15 minutes in retention time because this interval between the first and the second fraction was estimated to be was sufficient to resolve the Trp-labelled peptides contained in the first fraction. The modified peptides that eluted with a shift in retention time were collected and analyzed by MS and their identities were determined on the basis of the measured molecular masses. Table 6 lists the calculated and found masses of the Trp-tryptic peptides which were identified by MS. The Trp-peptides of all the proteins contained in the model mixture were identified, except for aprotinin which does not contains Trp residues.

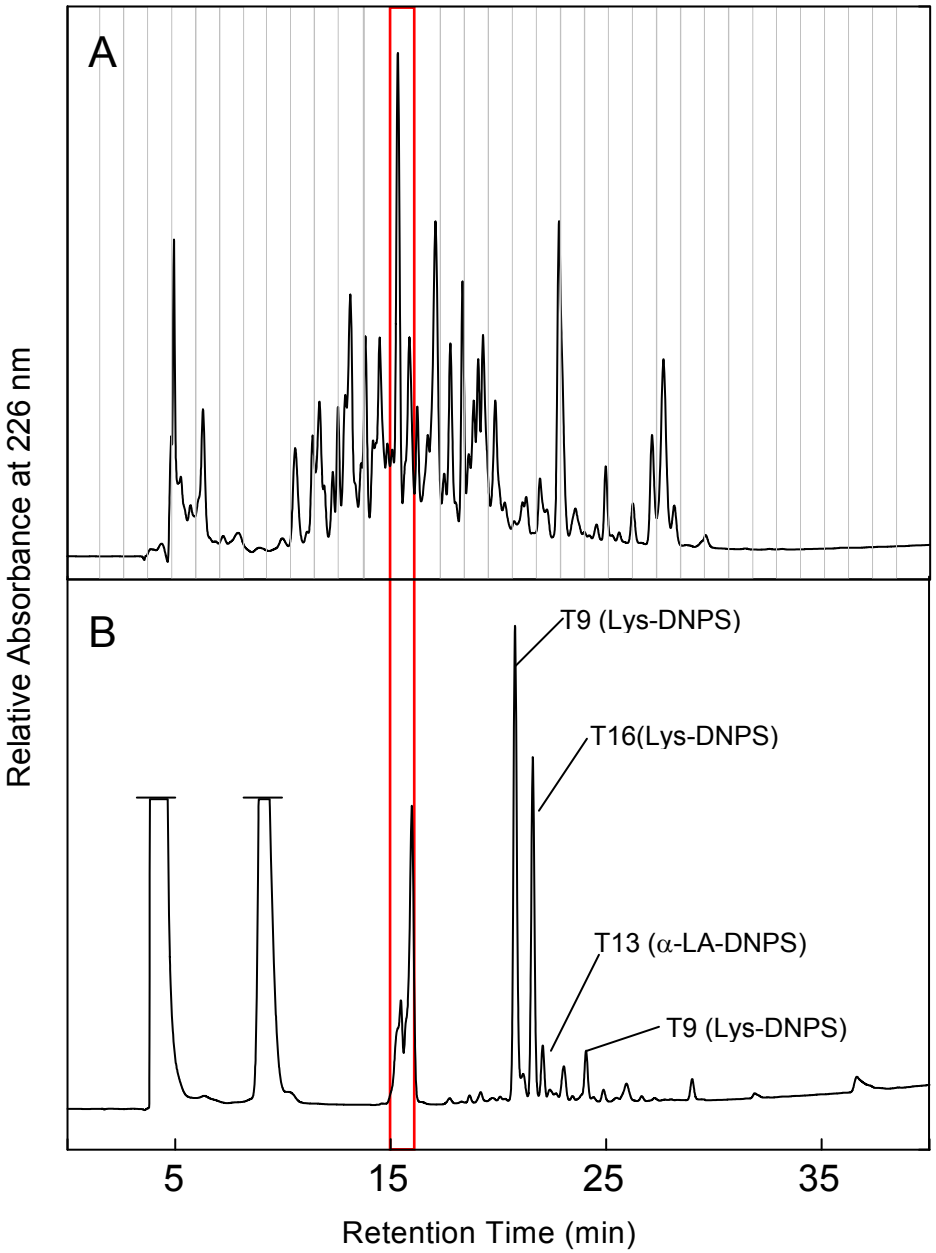

Figure 25. Analyses by RP-HPLC of the total raw cake digest mixture (A) and of the fraction 15-16 min after reaction by DNPS-Cl (B). Red lines indicate the fraction collected and derivatized with DNPS-Cl. Proteolysis with trypsin was conducted for 18 hours at 37 °C in 0.1 M NH_4HCO_3 pH 7.8, with an E/S ratio of 1:50. The reaction was stopped upon addition of 2% TFA and loaded on a Phenomenex C_{18} column (250x4.60 mm) eluted at a flow rate of 0.8 mL/min with a linear gradient of acetonitrile containing 0.1% TFA, from 10 to 80% in 40 min. The absorbance of the effluent from the column was monitored at 226 nm and identities of the fragment species were confirmed by MS analysis.

Table 6. Molecular masses of the Trp-containing tryptic peptides of the seven model proteins isolated using the diagonal chromatography approach.

Protein	*Tryptic peptide*		*Sequence*	*Mass + DNPS-Cl*	
				Found[b]	*Calculated*[a]
α-Lactalbumin (α-LA)	T5	(17-26)	GYGGVSLPEWVBTTFHTSGYD TQAIVQNNDSTEYGLFQINNK	4911.25	4911.51
	T6	(59-62)	IWBK	803.27	803.30
	T11	(99-104)	VGINYWLAHK	1397.62	1397.83
	T13	(115-122)	LDQWLBEK	1288.49	1288.62
Lysozyme (Lys)	T6	(22-33)	GYSLGNWVCAAK	1522.73	1522.60
	T9	(62-68)	WWUNDGR	1190.36	1190.37 (1 DNPS)
				1388.47	1388.34 (2 DNPS)
	T13	(98-112)	IVSDGNGMNAWVAWR	2070.75	2070.74 (1 DNPS)
	T16	(117-125)	GTDVQAWIR	1242.56	1242.51 (1 DNPS)
Albumin (BSA)	T16-17	(132-136)	KFWGK	862.43	862.34
	T31	(212-217)	AWSVAR	886.39	886.34
Aprotinin	None	None	None	0	0
Carbonic Anhydrase (CA)	T1	(1-8)	SHHWGYGK	1210.49	1210.42
	T2	(9-17)	HBGPZHWHK	1338.54	1338.48
	T10	(89-110)	LVQFHFHWGSSBBQGSEHTVDR		2781.13
	T13	(113-125)	YAAELHLVHWNTK	1778.92	1778.70
	T19	(171-211)	STDFPNFDPGSLLPNVLDYWT YPGSLTTPPLLESVTWIVLK		4991.54
	T22	(226-250)	TLNFNAEGEPELLMLANWRPAQPLK		3051.45
Apomyoglobin (apoMb)	T1	(1-11)	GLSDGEWQQVL	1428.56	1428.56
	T1	(12-16)	NVWGK	800.32	800.39
α-Hemoglobin (α-H)	T3	(12-16)	AAWGK	729.27	729.28
β-Hemoglobin (β-H)	T2	(8-16)	AAVTAFWGKLLVVYPWTQR	1147.69	1147.50
	T5	(30-39)	LLVVYPWTQR	1471.69	1471.72

[a]Molecular masses calculated from the amino acid sequence of α-LA, Lys, BSA, Aprotinin, CA, apoMb, α-H and β-H, which were labelled by DNPS-Cl.
[b]Experimental molecular masses determined by ESI-MS

2.4. MS/MS analysis of Trp-labelled peptides in the tryptic digest of the protein mixture

MS/MS measurements were conducted on the Trp-labelled peptides isolated by the diagonal chromatography approach in order to confirm the peptide identity and to study the effect of the DNPS modification on the pattern of fragmentation. The MS/MS spectra of the tryptic peptide T6 of lysozyme and of the tryptic peptide T2 of the β-chain of hemoglobin are reported in Fig. 26 and the N-terminal and C-terminal product fragments are indicated as b and y fragments, respectively. These two series of product ions are the most common fragments observed in the MS/MS spectra acquired with the Q-Tof mass spectrometry instrument, with the series of the b fragments usually less represented than the y series (*36*). By MS/MS analysis of the labelled peptides, the sequence coverage of 7 amino acid residues out of 12 was obtained for the tryptic peptide T6 of lysozyme and of 7 amino acids residues out of 9 for the tryptic fragment T2 of β-chain of hemoglobin. The MS/MS measurements demonstrated the specificity of the reaction with DNPS-Cl at the level of Trp residues and its compatibility with the acquisition and interpretation of MS/MS spectra. A noteworthy aspect is the presence in the MS spectra of the labelled peptides of a signal at 357.1 m/z that corresponds to the immonium ion of DNPS-tryptophan. This immonium ion is formed during the peptide fragmentation and it is present only in the MS/MS spectra of the DNPS-labelled peptides. From this observation it is possible to consider this species as a specific marker in the MS/MS spectrum of the presence in the sequence of the peptide of a modified Trp residue.

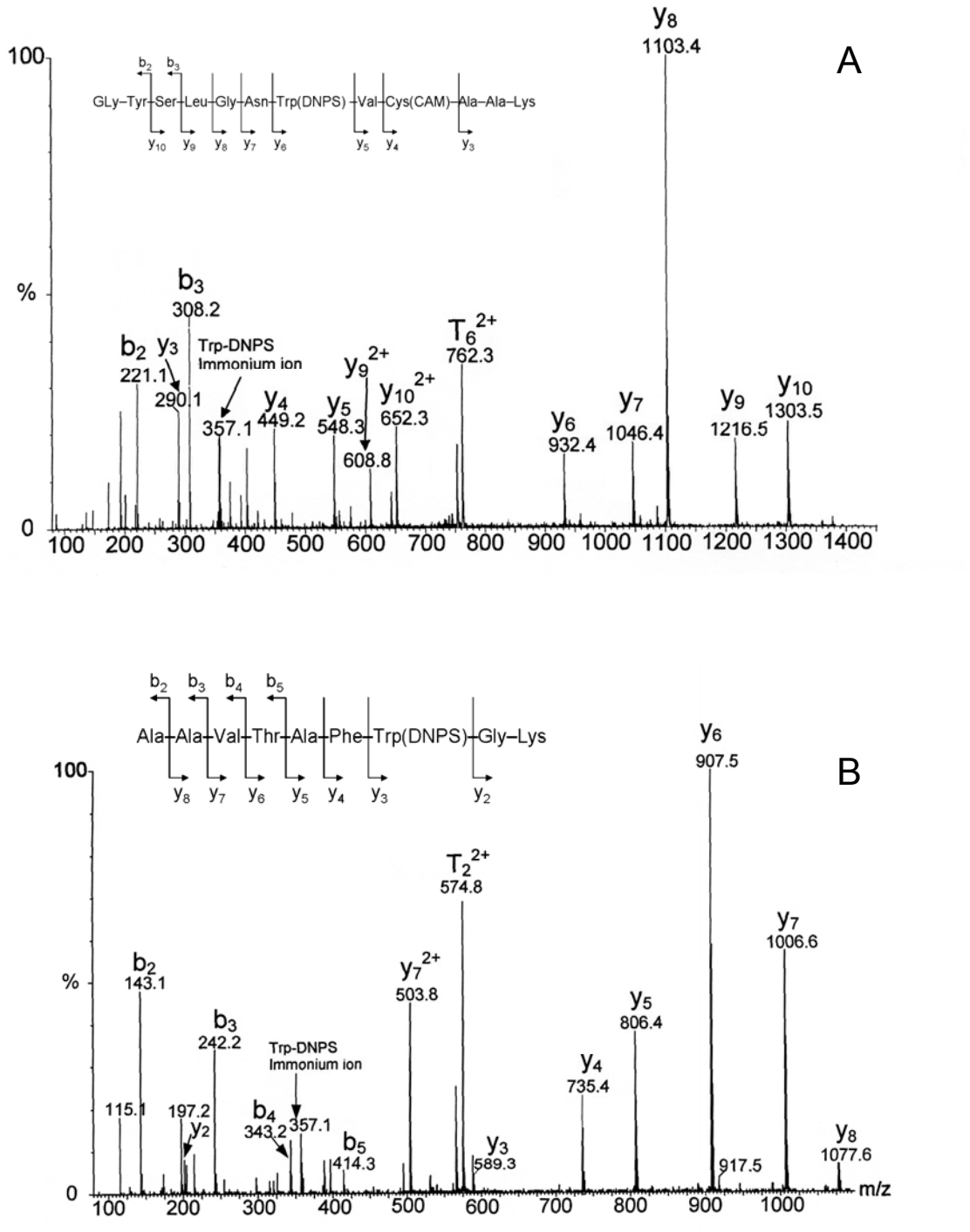

Figure 26. MS/MS spectra of the ion $[M+H]^{2+}$ of the tryptic peptide T6 of Lysozyme (A) and of the ion $[M+H]^{2+}$ of the tryptic peptide T2 of β-chain of Hemoglobin (B). In the spectrum, the **y** and **b** ions are indicated. The location of the product fragments in the peptide sequence is also shown.

2.5. Chemical tagging with DNPS-Cl and fingerprinting analysis of a raw bakery product

In order to optimize the diagonal chromatography approach for the identification of allergenic proteins in complex food matrices, this method was applied to a raw bakery product made of several ingredients such as bovine milk, chicken eggs, flour, butter, sugar and baking powder. The analysis was limited to the selection and identification of the Trp-containing peptides of α-LA which is one of the major allergenic proteins of milk. To this aim, only the RP-HPLC fractions which were supposed to contain the Trp-peptides of α-LA were modified by DNPS-Cl and analyzed by RP-HPLC.

Proteins contained in the raw cake were extracted using the protocol described in the methods section 1. The total protein concentration estimated by the 2D quantification kit (GE Healthcare) was of 2.5 mg/mL. In order to perform the tryptic digestion, a dried aliquot of 0.5 mg of raw cake extract was dissolved in 0.1 M NH_4HCO_3 pH 7.8. Proteolysis by trypsin was conducted for 18 hours at 37 °C with an enzyme to substrate ratio of 1:50 by weight. After incubation, the reaction was quenched upon addition 2% TFA. The whole tryptic digest was analyzed by RP-HPLC and fractions were collected at a time interval of one minute. The chromatogram is reported in Fig. 27A. The fractions where the Trp-containing peptides of α-LA elute were collected, reacted with DNPS-Cl and loaded on the RP-HPLC column under the same conditions of the first run. In Fig. 27B the RP-HPLC separation of the fraction collected at 21 minute is reported. This fraction was supposed to contain the tryptic peptide T11 of α-LA. The peptides which do not contains Trp residues were eluted at the same retention time as the first RP-HPLC run. On the contrary, the peptides which contain labelled Trp residues were eluted with a shift in the retention time according with the increase of hydrophobicity determined by the DNPS moiety. The peaks that displayed a higher retention time were collected and analyzed by MS and MS/MS to determine their identities. In Table 7, the Trp-labelled peptides of α-LA are reported that were selected by the diagonal chromatography approach and identified by MS and MS/MS analysis. Among these peptides, we identified the peptide T11 of α-LA modified by DNPS (Fig. 27B, Fig 28). The MS/MS spectrum of the DNPS-labelled tryptic peptide T11 allowed the identification of the peptide since 9 amino acid residues out of 11 were sequenced. Moreover, it was possible to confirm the localization of the modification at the level of the Trp residue. In the MS/MS spectrum, the signal of the immonium ion of

Trp-DNPS is also detectable as a further indication of the presence of this chemical modification.

Table 7. Molecular masses of the Trp-peptides of α-LA isolated using the diagonal chromatography approach from the protein extract of a raw cake.

α-LA -DNPS peptides	*Calculated Mass*[a]	*Found Mass*[b]
T6	803.27	803.30
T13	1288.49	1288.62
T11	1397.62	1397.83
T5	4911.25	4911.51

[a]Molecular masses calculated from the amino acid sequence of DNPS-labelled α-LA.
[b]Experimental molecular masses determined by ESI-MS.

These results demonstrate the usefulness of the diagonal chromatography approach for the identification of a specific protein from a complex peptide mixture. Indeed, by MS/MS sequence analysis of few selected labelled peptides it is possible to confirm the identity and consequently the presence of the parent protein. We demonstrated the feasibility of this approach with the detection of the allergenic protein α-LA in a complex peptide mixture obtained after digestion of a raw cake protein extract. We define this specific application of the diagonal chromatography approach as "targeted". Indeed, estimating peptide populations of thousand of species in the digest of complex protein mixtures, by the targeted diagonal chromatography approach it is possible to focus the MS analysis only towards the peptides of a specific allergenic protein present in the mixture. By reducing sample complexity, this approach simplifies the MS analysis in terms of time spent for data acquisition and volume of MS data to be interpreted.

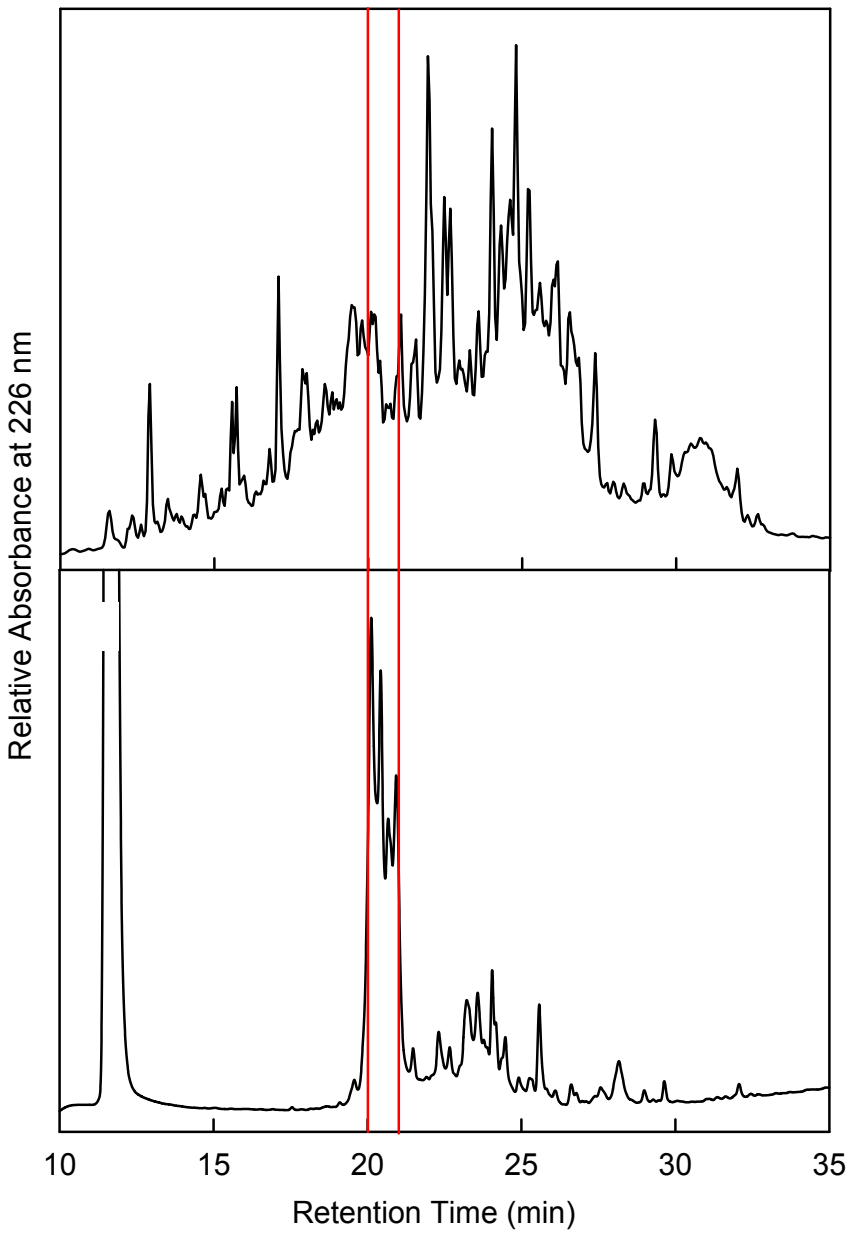

Figure 27. Analyses by RP-HPLC of the total raw cake digest mixture (A) and of the fraction 20-21 min after reaction with DNPS-Cl (B). Red lines indicate the fraction collected and derivatized with DNPS-Cl. Proteolysis by trypsin was conducted for 18 hours at 37 °C in 0.1 M NH_4HCO_3 pH 7.8, with an E/S ratio of 1:50. The reaction was stopped upon addition of 2% TFA and loaded on a Phenomenex C_{18} column (250x4.60 mm) eluted at a flow rate of 0.8 mL/min with a linear gradient of acetonitrile, containing 0.1% TFA, from 10 to 80% in 40 min. The absorbance of the effluent from the column was monitored at 226 nm and identities of the fragment species were confirmed by MS analysis.

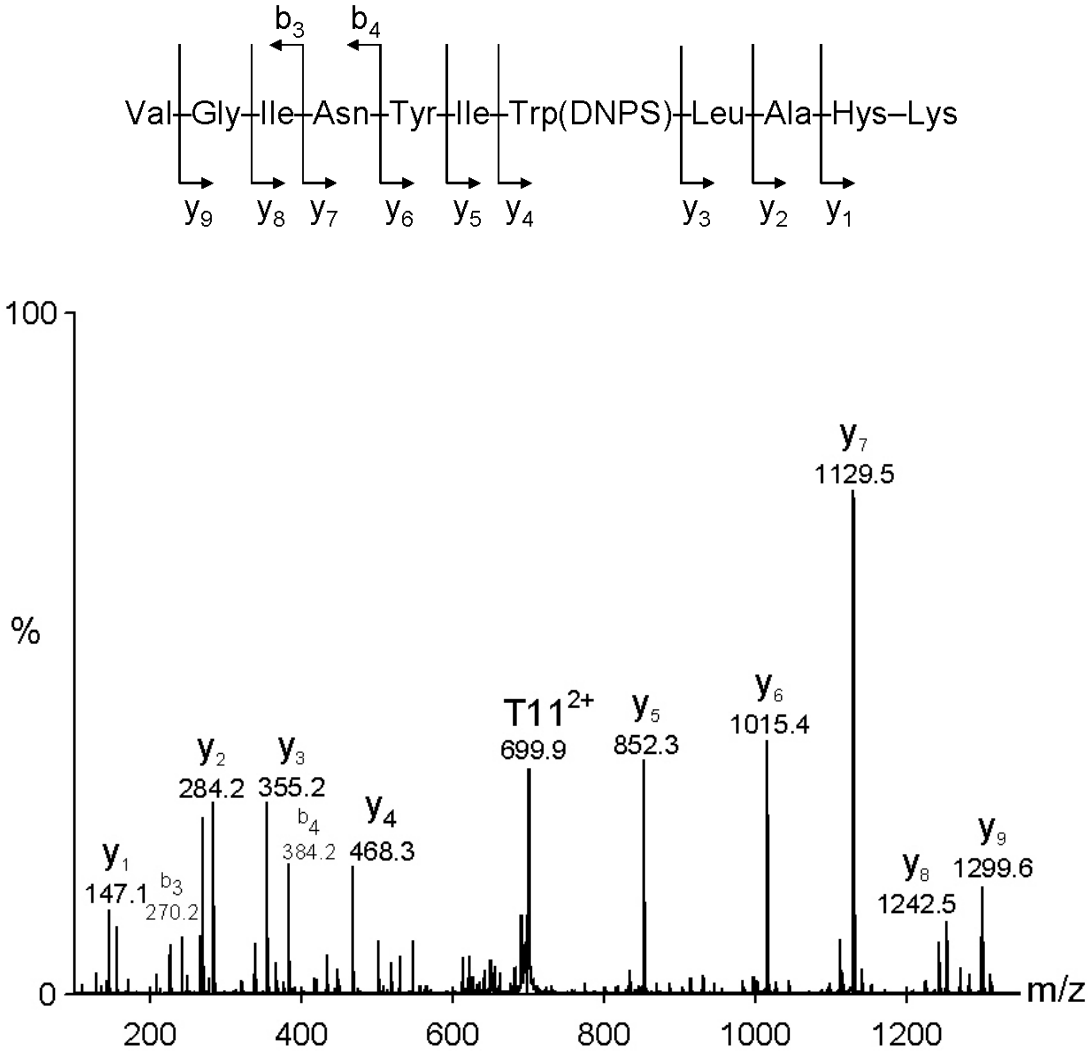

Figure 28. MS/MS spectrum of the ion $[M+H]^{2+}$ of the α-LA tryptic peptide T11 witch contain Trp residue modified by DNPS-Cl. In the spectrum, the **y** and **b** ions are indicated as well as their location in the peptide sequence.

3. Selection of Trp-containing peptides by affinity chromatography

In order to improve methods for the selection and the enrichment of Trp-labelled peptides from a whole tryptic digest, an immunoaffinity chromatography approach was developed using labelled α-LA as model protein. To this aim, a Sepharose CNBr-activated medium was covalently modified with monoclonal antibodies raised against the dinitrophenyl group as described in the section 5 of Methods and schematically shown in Fig 29. After drying, the fractions of the flow-through of the column and of the elution step with 10% formic acid were dissolved in 0.1% formic acid and analyzed by LC-MS/MS using a Micromass CapLC unit (Waters) interfaced to a Micromass Q-Tof Micro mass spectrometer (Waters) equipped with a nanospray source.

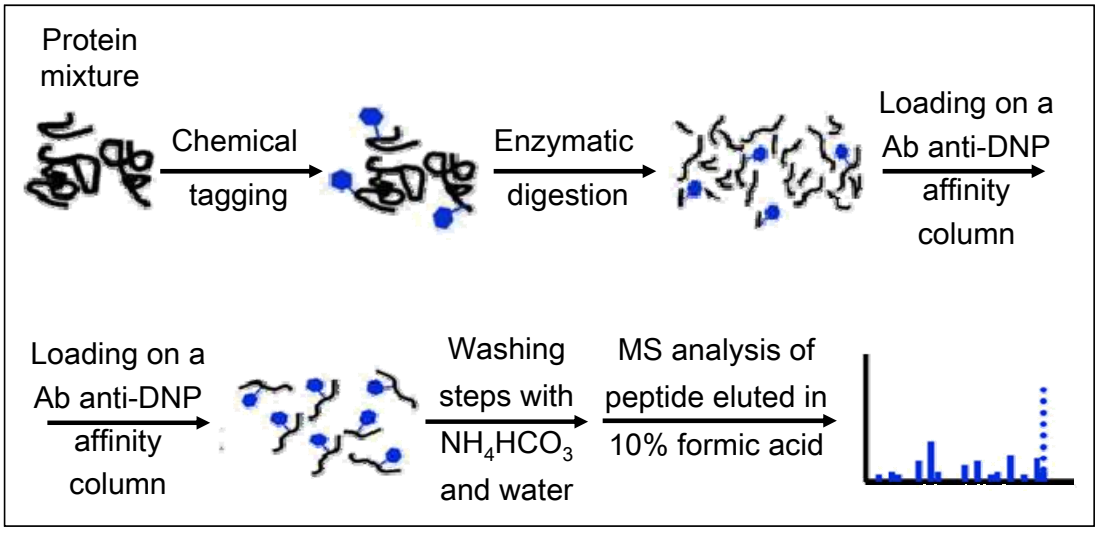

Figure 29. Workflow of the immunoaffinity chromatography approach used for the enrichment of Trp-containing tryptic peptides of DNPS-labelled α-LA.

By MS measurements, we could demonstrate that the tryptic peptides of α-LA are not retained by the immunoaffinity column and indeed we observed their m/z signals in the LC-MS/MS analysis of the flow-through. On the other end, DNPS-labelled peptides are retained by the column and their m/z signals are detected only in the MS spectra of fraction eluted with formic acid.

In Fig. 30. the MS spectra of the different affinity enrichment steps are shown. The region of the mass spectrum containing the signal of an unlabelled peptide of α-LA (peptide T7, $[M+H]^{3+}$ 668.4 m/z; Fig. 30A) and a labelled peptide (peptide T6, $[M+H]^{2+}$ 402.6 m/z; Fig. 30B) are compared. The α-LA tryptic peptide T7 that does not contains tryptophan residues, is not retained by the immunoaffinity matrix and it elutes in the flow-through (Fig. 30A). On the contrary, the α-LA tryptic peptide T6-DNPS which contains tryptophan residues, is retained by the affinity media due to the interaction of DNPS groups with the anti-DNP antibodies and it elutes only with formic acid. In Fig. 31, the MS/MS spectrum of the Trp-labelled peptide T6 of α-LA is reported. Interpretation of the pattern of fragmentation confirms the identity of the peptide since the sequence of 3 amino acid residues out of 4 was obtained. Of interest, the signal of the immonium ion of Trp-DNPS is quite intense in the spectrum confirming that it can be used as a probe of the modification with DNPS of the peptide.

Using the immunoaffinity approach, all the four DNPS-modified tryptic peptides of α-LA were selected and their identities were confirmed by MS and MS/MS analysis. Table 8. lists the masses of the Trp-containing peptides detected in the LC-MS/MS analysis of the fractions eluted with formic acid, whereas as a further example of the results of the analysis, in Fig. 32 the MS/MS spectrum of the tryptic labelled peptide T13 of α-LA is reported. The MS/MS analysis allowed to confirm the sequence of 7 amino acid residues out of 8 of the peptide and also the site of the modification.

Table. 8. Molecular masses of the Trp-labeled peptides of α-LA selected by immunoaffinity chromatography.

α-LA -DNPS peptides	*Calculated Mass*[a]	*Observed Mass*[b]
T6	803.27	803.32
T13	1288.49	1288.54
T11	1397.62	1397.76
T5	4911.25	4911.55

[a]Molecular masses calculated from the amino acid sequence of DNPS-labeled α-LA.
[b]Experimental molecular masses determined by ESI-MS.

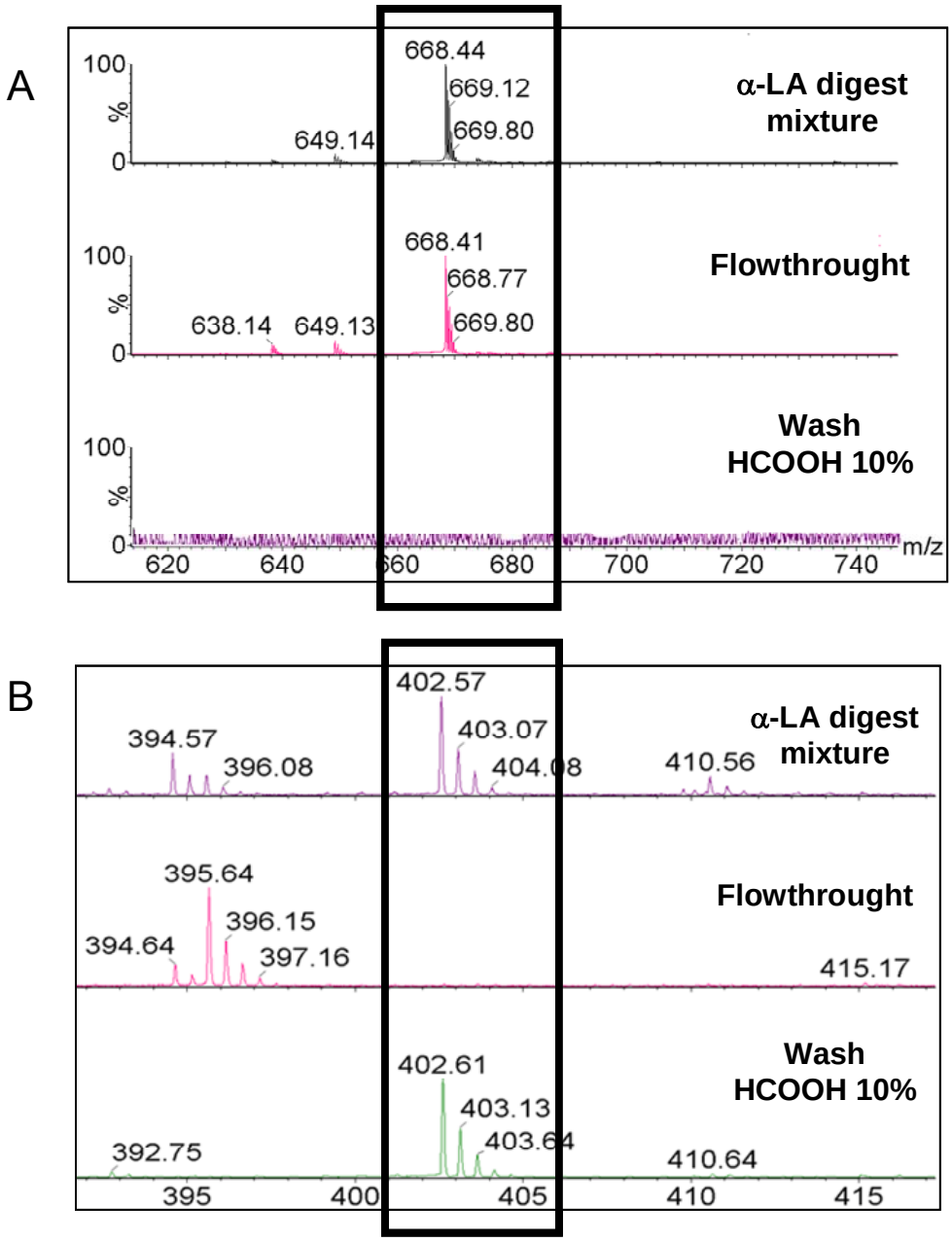

Figure 30. Mass spectra of the fractions collected at each elution steps from the immunoaffinity chromatography column are shown. (A) Mass spectra of the ion $[M+H]^{3+}$ of the α-LA tryptic peptide T7 witch does not contains Trp residues. This peptide is not retained by the affinity column and it elutes in the flow-through. (B) Mass spectra of the ion $[M+2H]^{2+}$ of the α-LA tryptic peptide T6-DNPS. The DNPS-labelled peptide T6 is retained by the affinity media due to the interaction of DNPS groups with anti-DNP antibodies and it was eluted only with formic acid. For the RP-HPLC analyses, a column Atlantis C18 nano-ESI was employed and the elution of the column was performed with milliQ water containing 5% acetonitrile and 0.1% formic acid (solvent A) and acetonitrile containing 5% water and 0.1% formic acid (solvent B). The linear gradient of solvent B was from 5 to 70% in 40 min at a flow rate of 200 nL/min.

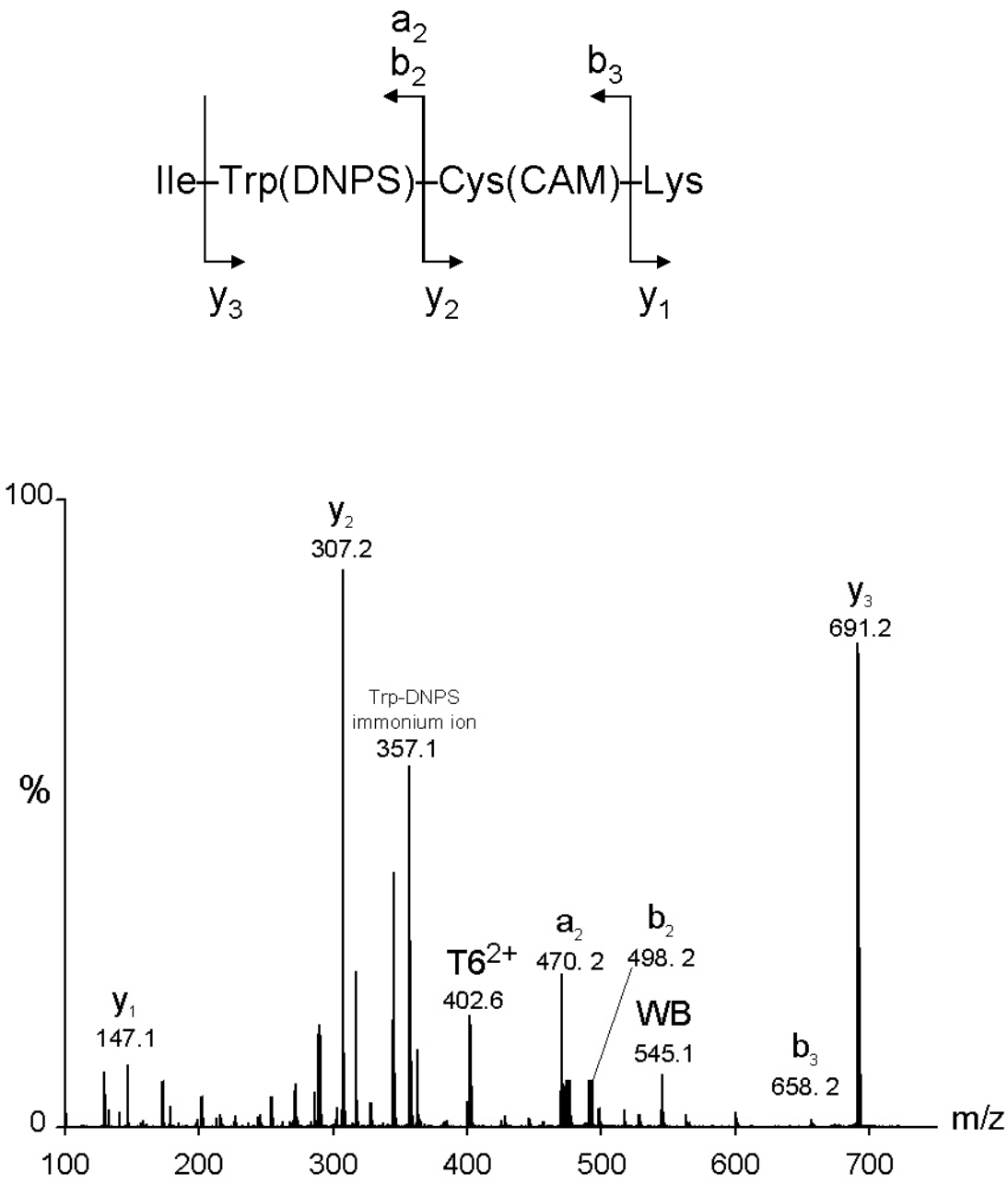

Figure 31. MS/MS spectrum of the ion $[M+H]^{2+}$ of the α -LA tryptic peptide T6 witch contains one Trp residue modified by DNPS-Cl. In the spectrum, the **y** and **a** ions are indicated as well as their location in the peptide sequence.

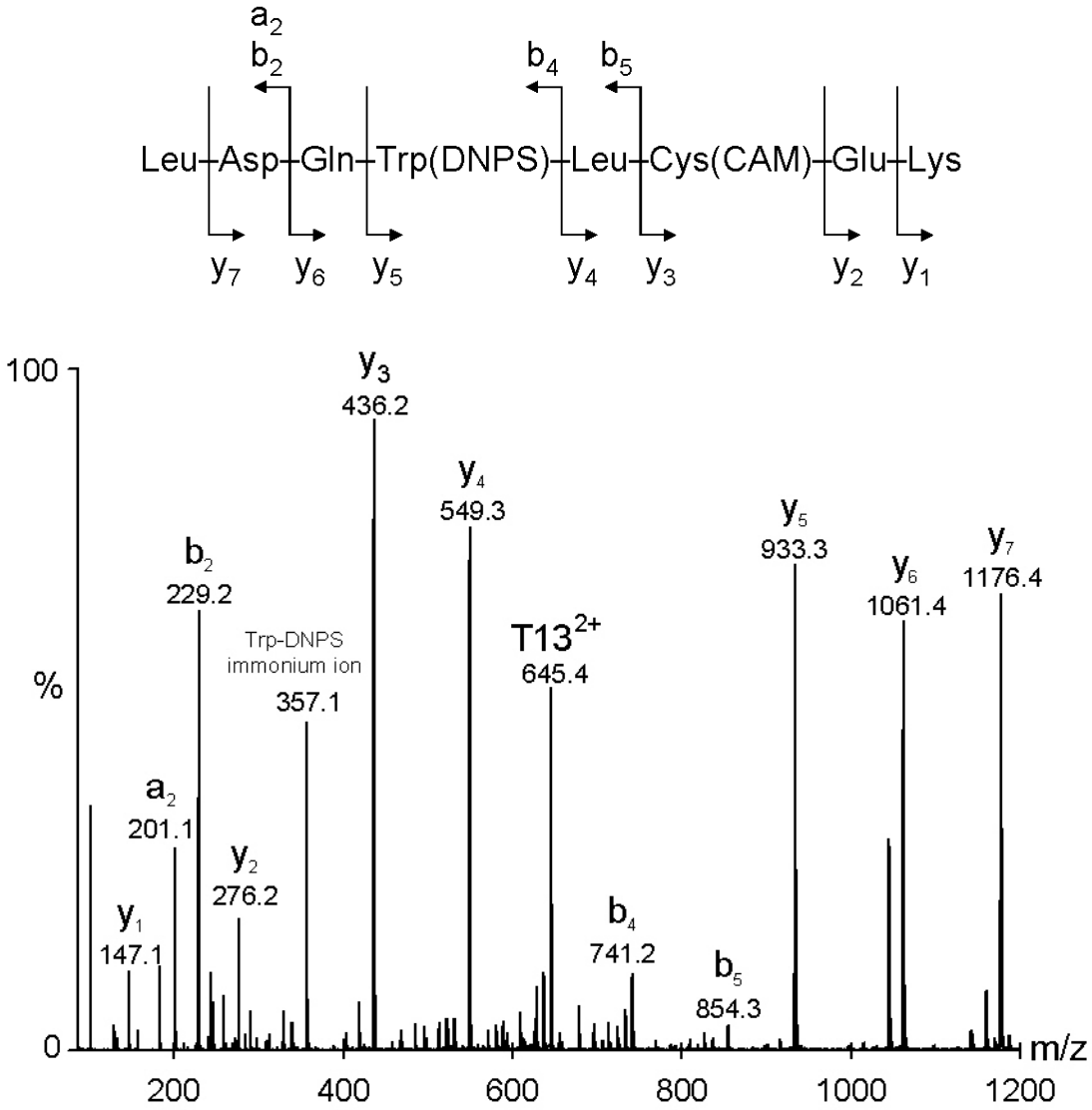

Figure 32. MS/MS spectrum of the ion $[M+H]^{2+}$ of the α -LA tryptic peptide T13 witch contains one Trp residue modified by DNPS-Cl. In the spectrum, the **y, b** and **a** ions are indicated as well as their location in the peptide sequence.

4. Fingerprinting analysis of labelled α-LA in water/acetonitrile.

In order to optimize the protocol of the affinity chromatography selection approach, conditions for the tryptic digestion in the presence of organic solvents were developed. This strategy can reduce sample loss and the time necessary for the preparation of the samples to be loaded on the columns. Indeed, the reaction in organic solvents has the advantage of being faster than in aqueous buffer, thus decreasing the duration of the proteolysis reaction. In the literature, several organic solvent mixtures are proposed for fingerprinting analyses [72-73]. For the organic solvent assisted fingerprinting of α-LA here reported, a solution of 50 mM Tris·HCl, 10 mM $CaCl_2$, 80% acetonitrile (AcCN) pH 7.6 was chosen and the results of this reaction were compared with the standard digestion protocol in aqueous buffer. The proteolytic reaction was conducted on carbamidomethylated α-LA and the DNPS-labelled protein. The digestion mixtures were analyzed by RP-HPLC and the identities of the peptides were confirmed by MS analysis.

In fig 33, the RP-HPLC chromatograms of the trypsin digests of carbamidomethylated α-LA in 80% AcCN after one and 18 hours of reaction at 37 °C are reported. After one hour, the tryptic peptide T6 and T13 are not present in the whole digest and the most abundant species are represented by tryptic fragments with one missed cleavage in the sequences. On the contrary, after 18 hours of incubation most of these peptides containing missed cleavages are further digested indicating that the reaction has gone to completion. Indeed, after one hour the peptides T11-12 and T 10-11 are two of the main species in the digestion mixture, whereas after 18 hours their intensities decrease for the formation of peptide T11. The situation is similar for the tryptic peptide T13-14 which contains one missed cleavage. The intensity of the peak of peptide T13-14 decreases with time because its hydrolysis produces fragment T13. The RP-HPLC chromatograms of digests of carbamidomethylated α-LA in 80% AcCN and in aqueous buffer are compared in Fig. 34. Both reactions were incubated at 37 °C for 18 hours. The species T6-7 and T7-8 are preferably produced in aqueous buffer than in the presence of organic solvent whereas peptide T5-6 is observed only in the reaction conducted in the presence of AcCN. A part from these differences, the overall pattern of hydrolysis is similar in the two conditions indicating that AcCN does not inhibit the hydrolysis of the protein by trypsin.

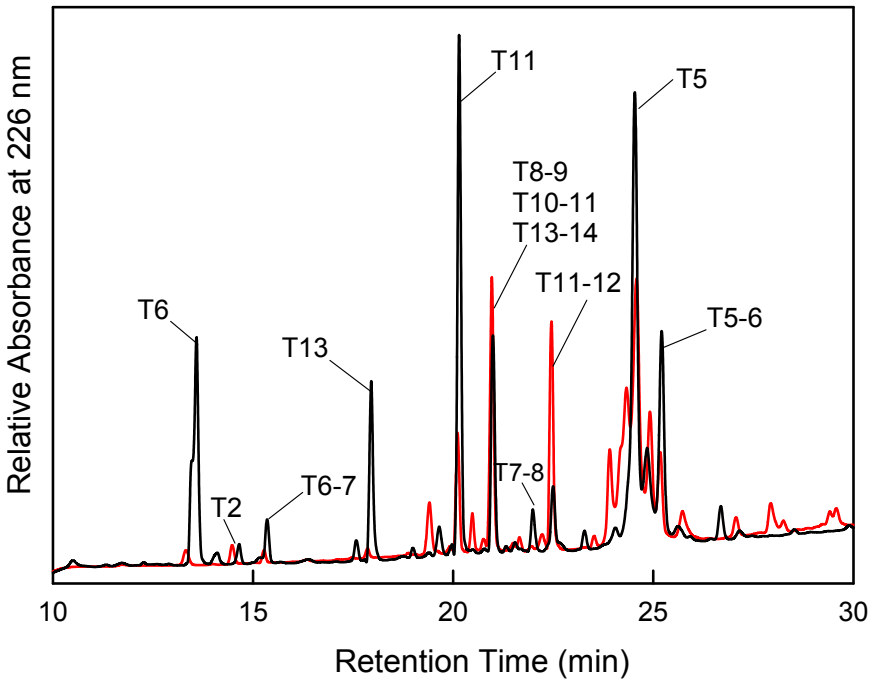

Figure 33. RP-HPLC chromatograms of the proteolysis mixture of the α-LA-CAM digested with trypsin after one (red line) and 18 hours (black line) of incubation. Proteolysis with trypsin was conducted at 37 °C in 50 mM Tris·HCl, 80% AcCN pH 7.6 with an E/S ratio of 1:50. After incubation, the reaction was stopped upon addition of 2% TFA and loaded on a Phenomenex C_{18} column (250 x 4.60 mm) eluted at a flow rate of 0.8 mL/min with a linear gradient of acetonitrile containing 0.1% TFA, from 10 to 80% in 40 min. The absorbance of the effluent from the column was monitored at 226 nm and identities of the fragments were determined by MS.

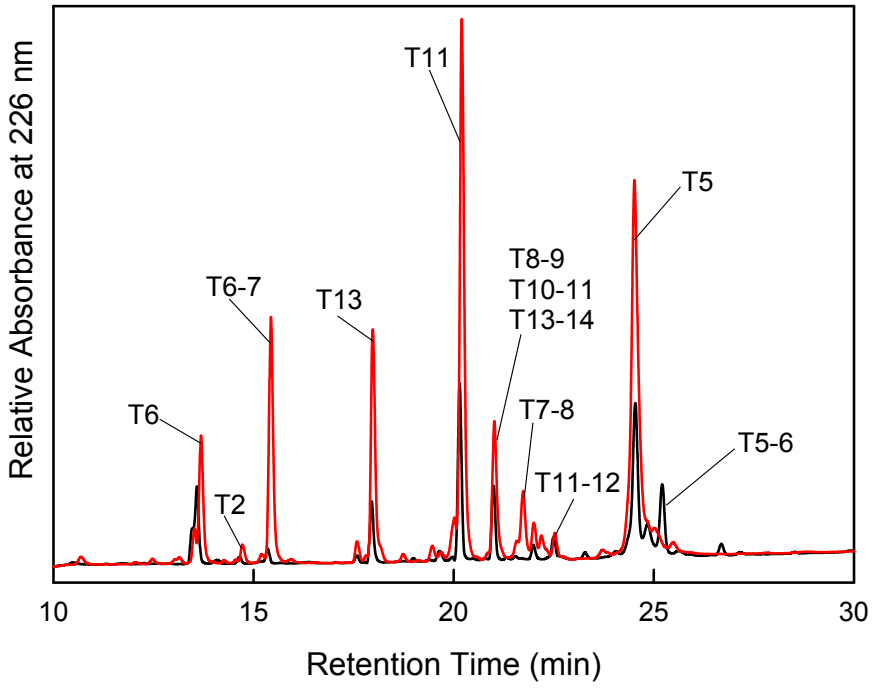

Figure 34. RP-HPLC chromatograms of the proteolysis mixture of the carbamidomethylated α-LA digested with trypsin. Proteolysis with trypsin was conducted for 18 hours at 37 °C in 0.1 M NH_4HCO_3 pH 7.8 (red line) and in 50 mM Tris·HCl, 80% AcCN pH 7.6 (black line) with an E/S ratio of 1:50. After incubation, the reaction was stopped upon addition of 2% TFA and loaded on a Phenomenex C_{18} column (250 x 4.60 mm) eluted at a flow rate of 0.8 mL/min with a linear gradient of acetonitrile containing 0.1% TFA, from 10 to 80% in 40 min. The absorbance of the effluent from the column was monitored at 226 nm and identities of the fragment were determined by MS.

The conditions of proteolysis in 80% AcCN were also applied for the fingerprinting reaction of DNPS-labelled α-LA. The RP-HPLC chromatograms after one and 18 hours of reaction at 37 °C are reported in Fig. 35. The proteolytic pattern is similar between the two times of reaction, the main difference being some tryptic species carrying one missed cleavage that are further digested after 18 hours of reaction. The RP-HPLC chromatograms of the tryptic digests of DNPS-labeled α-LA in 80% AcCN and in aqueous buffer after 18 hours of proteolytic reaction at 37 °C are also compared in Fig. 36. The overlay of the two chromatographic profiles indicates that the production of peptide species with one or more missed cleavages is promoted in the presence of organic solvent, thus resulting in a different pattern of proteolysis. Indeed, the tryptic peptide T6 is produced only in the reaction conducted in aqueous reaction whereas in organic solvent fragment T5-6 is very stable. Moreover, fragments T6, T11 and T13 are the most abundant peptides in the reaction conducted in aqueous buffer while peptides T11-12, T13-14, T12-13, T10-11 and T8-9 containing missed cleavages are detected only in the organic solvent assisted reaction. We repeatedly observed also the formation of a semitryptic peptide encompassing amino acid 17 to 31 of α-LA (T5*). This peptide is produced only in the digest of DNPS-labelled α-LA obtained in aqueous buffer and not in the same reaction conducted on carbamidomethylated α-LA. In general, we observed that the pattern of the fingerprinting reaction is different not only upon changing the conditions of the reaction (aqueous buffer or organic solvent) but also under the same digestion conditions, if the substrate of the reaction is the protein modified with DNPS-Cl or carbamidomethylated α-LA.

In conclusion, trypsin digestion conducted in organic solvents can be useful for the fingerprinting analysis of a protein or a protein mixture. This strategy has the advantage to reduce the reaction time and to avoid drying steps before the MS measurements, which can lead to peptide precipitation and loss of sample. The reactions conducted on carbamidomethylated α-LA and on the DNPS-labelled protein indicated that in general the pattern of proteolysis is similar for the reactions conducted in aqueous buffer and in organic solvents, with the exception of peptides that contain missed cleavages that are more abundant in the presence of AcCN. This phenomenon has already been reported in the literature for reactions conducted in organic solvents but the cause is not clearly understood. One reason that has been proposed is a reduced solvation at the level of the hydrophilic site of hydrolysis in the presence of organic solvents [72-73].

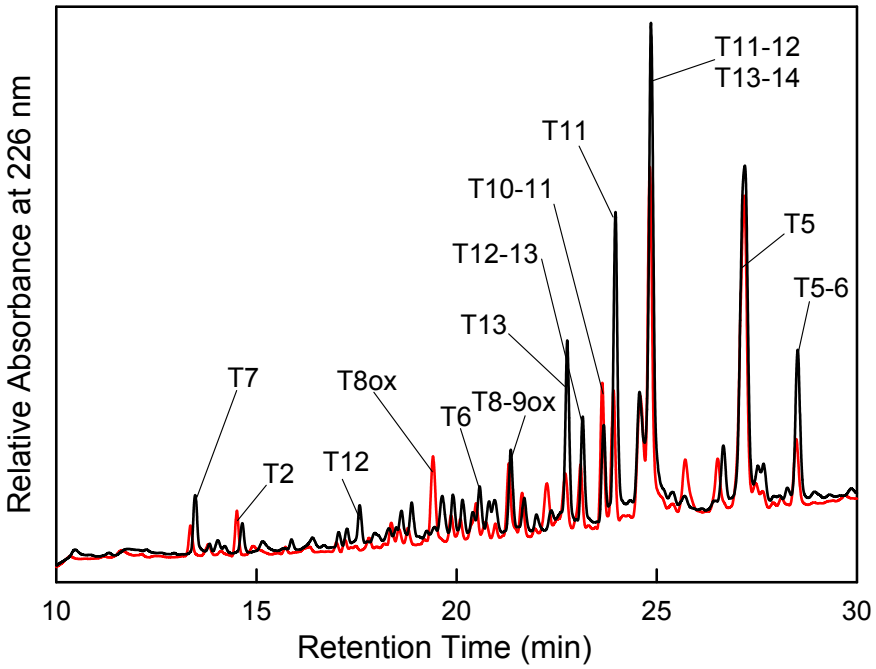

Figure 35. RP-HPLC chromatograms of the proteolysis mixture of the DNPS-labelled α-LA digested with trypsin after one (red line) and 18 hours (black line) of incubation. Proteolysis with trypsin was conducted at 37 °C in 50 mM Tris·HCl, 80% AcCN pH 7.6 with an E/S ratio of 1:50. After incubation the reaction was stopped upon addition of 2% TFA and loaded on a Phenomenex C_{18} column (250 x 4.60 mm) eluted at a flow rate of 0.8 mL/min with a linear gradient of acetonitrile containing 0.1% TFA, from 10 to 80% in 40 min. The absorbance of the effluent from the column was monitored at 226 nm and identities of the fragment were determined by MS. Fragments T8-9 and T8 are present in an oxidized form because the tryptic digestion was conducted on a sample of DNPS-labelled α-LA that was partly oxidized.

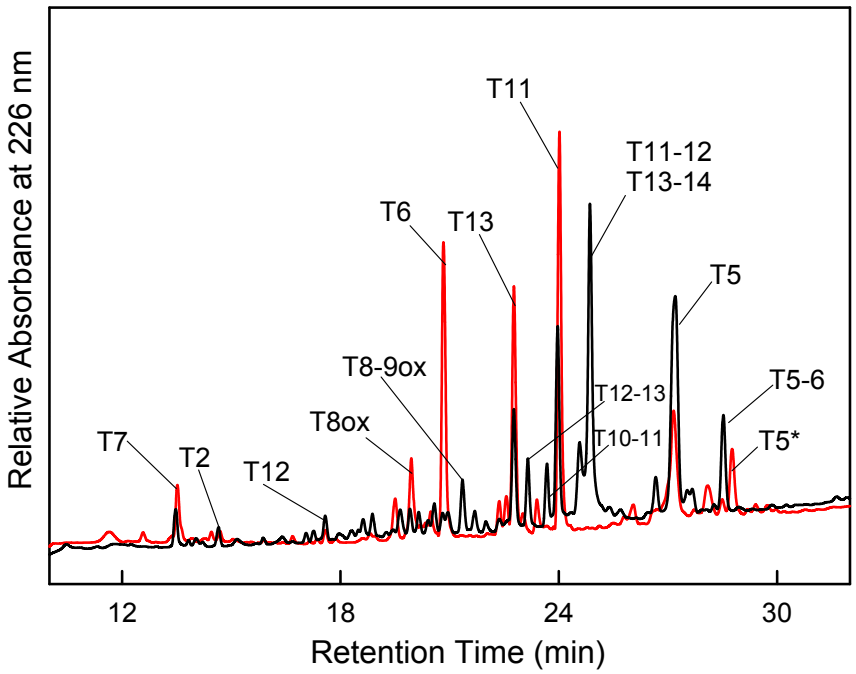

Figure 36. RP-HPLC chromatograms of the proteolysis mixture of the DNPS-labelled α-LA digested with trypsin. Proteolysis with trypsin was conducted for 18 hours at 37° C in 0.1 M NH_4HCO_3 pH 7.8 (red line) and in 50 mM Tris·HCl, 80% AcCN pH 7.6 (black line) with an E/S ratio of 1:50. After incubation, the reaction was stopped upon addition of 2% TFA and loaded on a Phenomenex C_{18} column (250 x 4.60 mm) eluted at a flow rate of 0.8 mL/min with a linear gradient of acetonitrile, containing 0.1% TFA, from 10 to 80% in 40 min. The absorbance of the effluent from the column was monitored at 226 nm and identities of the fragment were determined by MS. T8-9 and T8 are present in an oxidized form because the tryptic digestion was conducted on a sample of DNPS-labelled α-LA that was partly oxidized. T5* is a semitryptic peptide derived from peptide T5 (see text).

5. Selection of DNPS-labelled Trp-containing peptides by Hydrophobic Interaction Chromatography (HIC).

A few studies in the literature have reported the enrichment of 2-nitrobenzenesulfenyl (NBS)-labelled peptides by hydrophobic chromatographic matrices, in particular LH-20 and Phenyl Sepharose matrices (*47-48*). The NBS group is used to modify Trp residues in proteins and it differs from the DNPS moiety by the absence of the nitro group in *para* of the benzene group. The increase in hydrophobicity conferred by the NBS group to the peptide is less than that obtained with the conjugation by DNPS-Cl but it is sufficient to allow the enrichment of the NBS-modified peptides by the above mentioned hydrophobic matrices. In order to develop a method for the selection of the DNPS-labelled Trp-containing peptides from a whole digest mixture, we decided to test the Hydrophobic Interaction Chromatography (HIC) using a MEP HyperCel matrix provided by BioSepra. The MEP HyperCel sorbent is composed of a cellulose matrix to which 4-Mercapto-Ethyl-Pyridine (4-MEP) is linked. In HIC, the peptides are adsorbed by hydrophobic interactions to the pyridine ring of the matrix and then eluted upon pH reduction, that determines the protonation of pyridine and the dissociation of the peptides due to ionic repulsion. The workflow of the technique for DNPS-labelled α-LA is shown in Fig. 37.

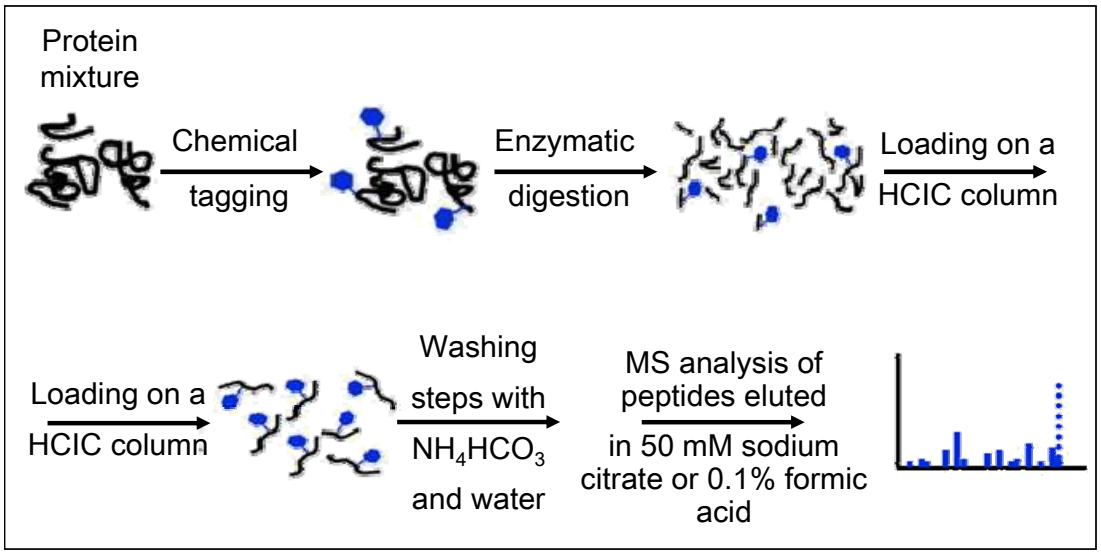

Figure 37. Workflow of the HIC chromatographic approach used for the enrichment of Trp-containing tryptic peptides of DNPS-labelled α-LA.

In order to optimize the method, the HIC chromatographic technique was initially tested using the tryptic digest of carbamidomethylated α-LA. The absence of the modification by DNPS of the Trp residues of the protein allows to verify the presence of specific interactions between the tryptic peptides and the matrix. The fractions collected from the HIC column after loading the digest (flow-through), after washing with 0.1 M NH_4HCO_3 pH 7.8 and elution with 50 mM sodium citrate pH 4.0 were analysed by RP-HPLC and the chromatograms are reported in Fig. 38. From the comparison of the RP-HPLC analyses of the digest and of the flow-through (Fig. 38A), it is evident that the two Trp-containing peptides T11 and T5 are retained by the resin because the corresponding chromatographic peaks are not detected in the flow-through fraction. The peptide T11 was selectively eluted in the washing step with 0.1 M NH_4HCO_3 pH 7.8 (Fig. 38B), whereas peptide T5 dissociated from the matrix after elution with 50 mM sodium citrate pH 4.0 (Fig. 38C). Since both peptides contain Trp residues, the results of this experiment suggest that the HIC matrix has an intrinsic affinity for Trp-containing peptides. Moreover, the order elution of the two Trp-containing peptides reflects differences in hydrophobicity since T5 is longer in sequence and more hydrophobic than T11 and thus it needs an acidic pH to be eluted.

The same experiment was conducted on the tryptic digest of DNPS-labelled α-LA conducted in organic solvent and the RP-HPLC chromatograms of the fractions collected from the HIC matrix are shown in Fig. 39. In Fig. 39A, it can be observed that five tryptic peptides were retained by the HIC matrix and MS analyses confirmed that all of these peptides contain DNPS-labelled Trp residues. Besides to the tryptic peptides T11 and T5 which strongly interact with the matrix even in the absence of the DNPS modification, the Trp-labeled peptides T6, T13 and T17-31 are also retained after conjugation to DNPS. The T6 and T13 fragments eluted in the washing step with 0.1 M NH_4HCO_3 pH 7.8, as it is shown Fig. 39B. After derivatization with DNPS, the peptide T11 elutes in 50 mM sodium citrate pH 4.0 at difference from the previous experiment where it eluted in the washing step (Fig. 39C). A similar phenomenon is observed for peptide T5 which is strongly retained by the HIC matrix and it dissociates only after washing with 6M Gdn·HCl (Fig. 39D). As expected, the observed higher affinity of the HIC matrix for Trp-containing peptides is caused by the increase in hydrophobicity induced by the chemical tagging with DNPS-Cl. Since the peptides adsorb to the HIC resin by hydrophobic interactions, an increase in hydrophobicity favors the retention of the peptides on the matrix thus requiring stronger elution conditions.

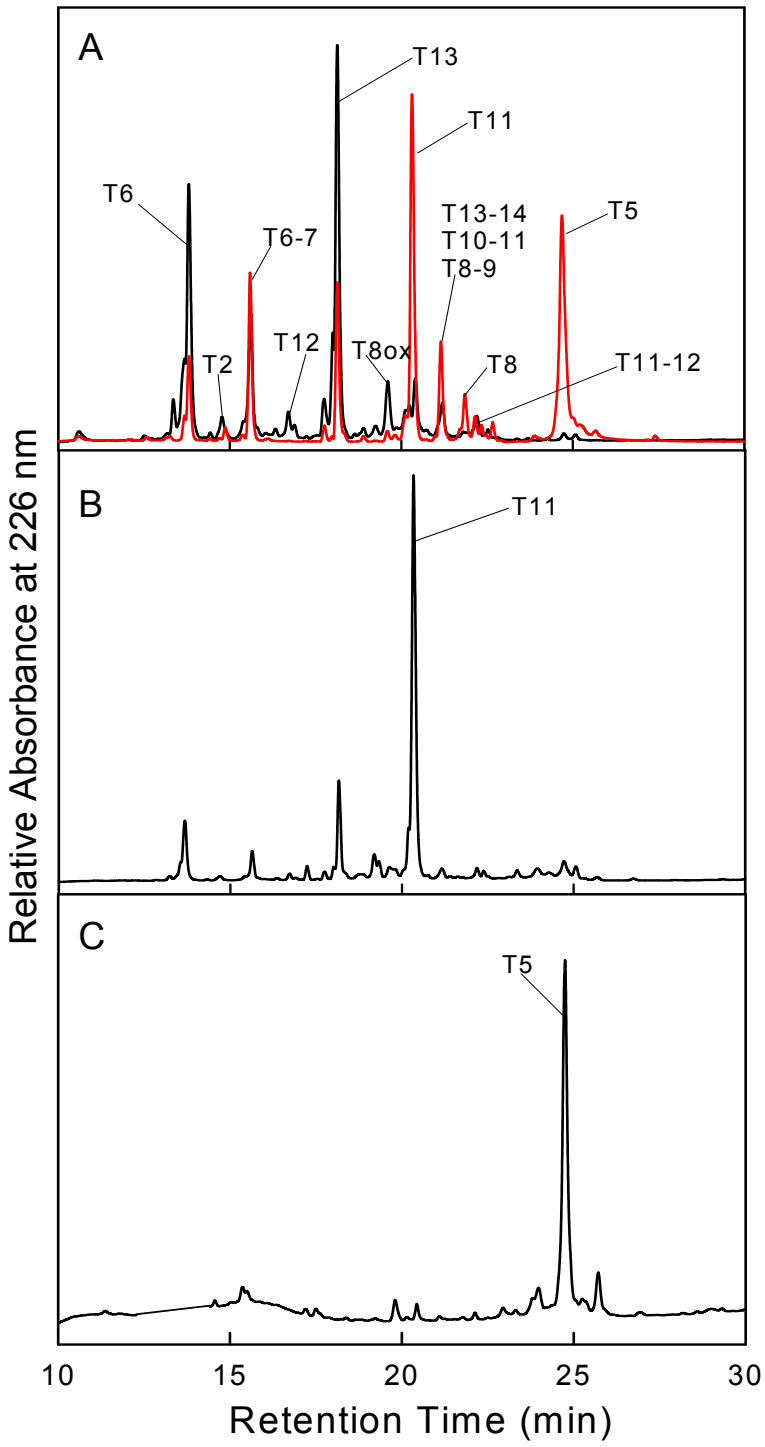

Figure 38. RP-HPLC chromatograms of the elution steps of the HIC chromatography approach, tested on a tryptic digest of carbamidomethylated α-LA. (A) RP-HPLC chromatograms of the fingerprinting reaction mixture of α-LA loaded on the HIC (red line) and of the flow-through fraction from the HIC medium (black line). Proteolysis with trypsin was conducted for 18 hours at 37 °C in 0.1 M NH_4HCO_3 pH 7.8, with an E/S ratio of 1:50. (B) RP-HPLC chromatogram of the washing step with 0.1 M NH_4HCO_3 pH 7.8. (C) RP-HPLC chromatogram of the elution step with 50 mM sodium citrate pH 4.0. The eluted fractions from HIC matrix were loaded on a Phenomenex C_{18} column (250 x 4.60 mm) eluted at a flow rate of 0.8 mL/min with a linear gradient of acetonitrile containing 0.1% TFA, from 10 to 80% in 40 min. The absorbance of the effluent from the column was monitored at 226 nm and the entities of the peptides were confirmed by MS analysis.

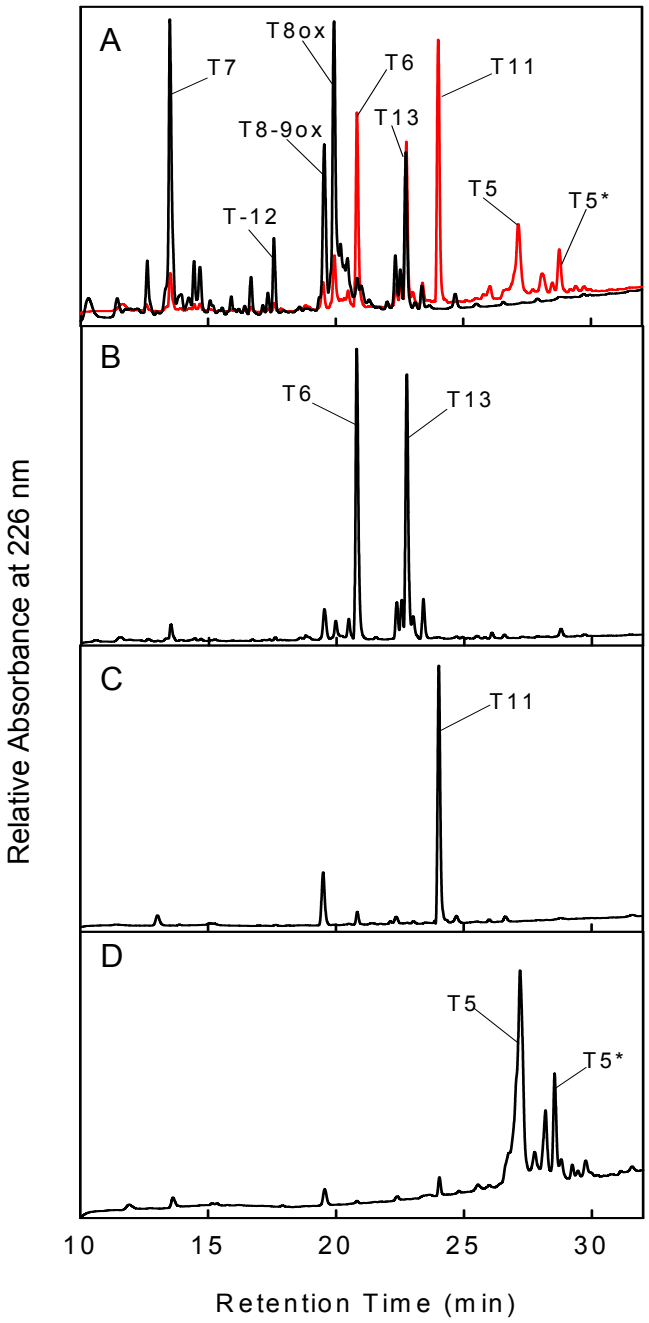

Figure 39. RP-HPLC chromatograms of the elution steps of the HIC chromatography approach, tested on a tryptic digest of DNPS-labelled α-LA. (A) RP-HPLC chromatograms of the fingerprinting reaction mixture of labelled α-LA loaded on the HIC (red line) and of the flow-through fraction from the HIC resin (black line). (B) RP-HPLC chromatogram of the washing step with 0.1 M NH_4HCO_3 pH 7.8. (C) RP-HPLC chromatogram of the elution step with 50 mM sodium citrate pH 4.0. (D) RP-HPLC chromatogram of the elution step with 6 M Gdn·HCl. Proteolysis with trypsin was conducted for 18 hours at 37 °C in 0.1 M NH_4HCO_3 pH 7.8, with an E/S ratio of 1:50. The eluted fractions from the HIC matrix were loaded on a Phenomenex C_{18} column (250 x 4.60 mm) eluted at a flow rate of 0.8 mL/min with a linear gradient of acetonitrile, containing 0.1% TFA, from 10 to 80% in 40 min. The absorbance of the effluent from the column was monitored at 226 nm and the identities of the peptides were confirmed by MS analysis.

The elution conditions used in these experiments were those suggested in the datasheet of the resin and they were developed for the purification of antibodies. In order to optimize the separation protocol and allow the direct MS analysis of the fractions eluted under acidic conditions, an elution step in formic acid was tested. The experiment was conducted on the whole tryptic digest of carbamidomethylated α-LA. The RP-HPLC analyses of the elution pattern from the HIC matrix of the tryptic digest are reported in Fig. 40. The loading and washing steps were conducted using the same conditions of the experiment shown in Fig. 38 (Fig. 38A and 38B), whereas the elution step was performed with 0.1% formic acid, pH 2.5 in order to collect the peptides in a solvent suitable for the MS analysis. Interestingly, during the different chromatographic steps the behaviour of the tryptic peptides were the same observed in the first experiment since the peptide T11 eluted in the washing step with NH_4HCO_3, whereas fragment T5 in formic acid (previously it eluted in 50 mM sodium citrate pH 4.0). These results suggest that formic acid can substitutes the elution step in 50 mM sodium citrate, thus allowing the direct analysis of the eluted peptides by MS or LC-MS/MS.

Overall, the experiments performed using HIC suggest that this chromatographic technique can be useful for the enrichment of Trp-containing peptides. Indeed, the analysis of the digest of α-LA indicated that only Trp-containing peptides are retained by the HIC matrix. Moreover, chemical tagging by DNPS can be used to increase the affinity of the Trp-modified peptides for the resin thus allowing a fast and selective enrichment of these peptides.

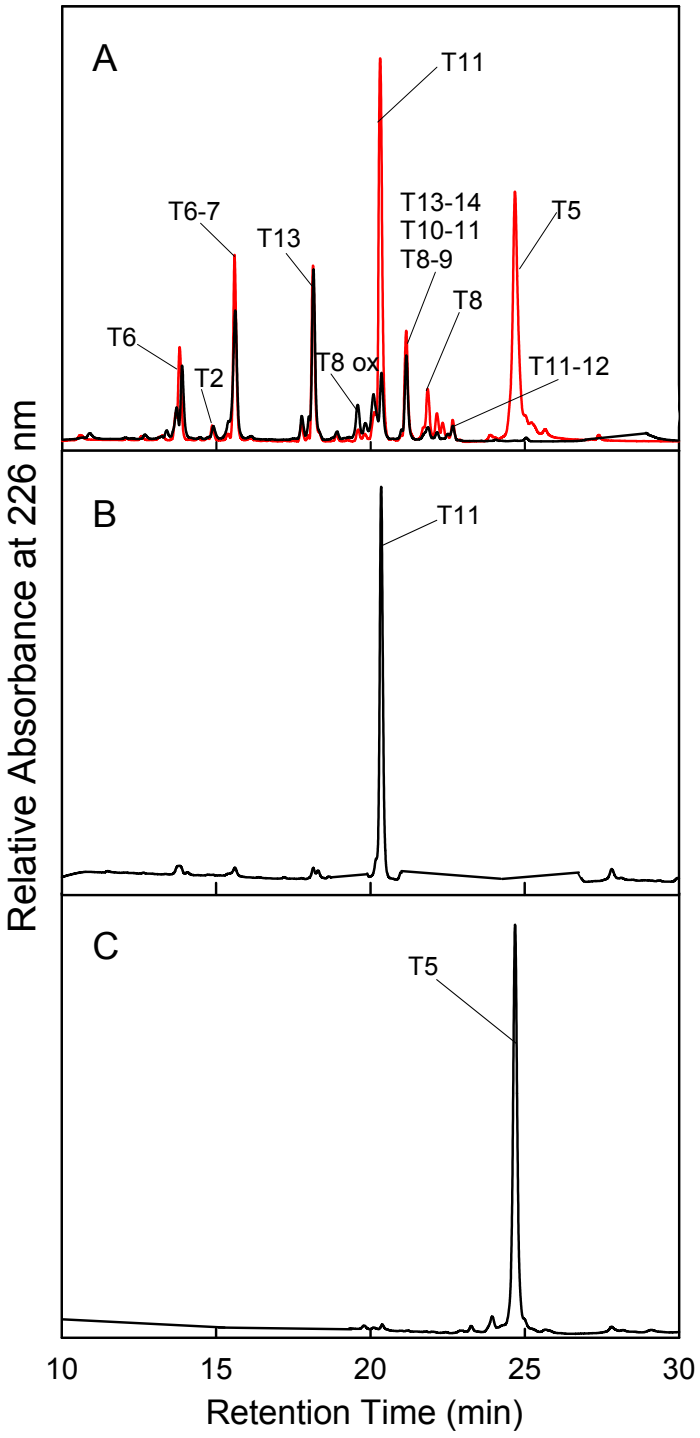

Figure 40. RP-HPLC chromatograms of the elution steps of the HIC chromatography approach, tested on a tryptic digest of carbamidomethylated α-LA. (A) RP-HPLC chromatograms of the fingerprinting reaction mixture of α-LA loaded on the HIC (red line) and of the flow-through fraction from the HIC resin (black line). (B) RP-HPLC chromatogram of the washing step with 0.1 M NH_4HCO_3 pH 7.8. (C) RP-HPLC chromatogram of the elution step by 0.1% formic acid pH 2.5. The eluted fractions from HIC matrix were applied to a Phenomenex C_{18} column (250 x 4.60 mm) eluted at a flow rate of 0.8 mL/min with a linear gradient of acetonitrile containing 0.1% TFA, from 10 to 80% in 40 min. The absorbance of the effluent from the column was monitored at 226 nm and identities of the peptides were confirmed by MS analysis.

IV. DISCUSSION

Nowadays we are eating sophisticated foods in terms of ingredients and industrial treatments, causing an increase of the number of allergic people and new allergies. The main problem for the consumers is represented by the hidden allergens in foods. Often allergens can be introduced in foods as preservatives, additives or as contaminants during the industrial preparation, but the problem is that often these substances are not indicated in the food labels. Moreover, the legislation may permit a food manufacturer not to list an ingredient if it is present in traces, thus increasing the risk for the consumers.

The most used methods to identify allergenic proteins in food matrices are immunochemical assays, but several limitation of these methods can produce misleading results. An important issue is the selection of the antibodies used for the immunological tests, since antibodies should be highly specific for the target protein in order reach an acceptable level of specificity and sensitivity. The antibody specificity strictly depends upon the purity of the protein antigen, so that usually a purified protein is required for developing an immunoassay. For this reason, the source and purity of the protein allergen, its structural integrity and the matrix effects on the protein are crucial aspects for the validation of an immunochemical assay.

The ELISA kits commercial available for the identification of allergenic proteins in foods are mostly based on the use of polyclonal antibodies. Immunochemical tests are easy to perform and rather cheap, but sometimes lead to false negative results. On the other hand, the MS approach requires costly instrumentation and specialized technicians in order to conduct successfully the entire analysis process. The most striking advantage of using MS resides in the fact that the identification of a protein is based upon the amino acid sequence of the protein and not the 3D structure of the protein (*71*).

Protein identification in a complex sample can be reached by the fingerprinting technique, by which (i) the protein sample is digested by trypsin, (ii) all tryptic peptides thus produced are separated by RP-HPLC, (iii) their sequence determined by MS/MS analysis and (iii) the parent protein identified after database research. However, this approach is made difficult by the complexity of the sample to be analyzed, considering the enormous amounts of different proteins eventually present in the sample.

Recently, an approach was developed by which only some specific peptides from the whole tryptic digest are chemically modified at the level of a relatively rare amino acid

residue. This procedure, named chemical tagging, allows to specifically separate the labeled peptides by chromatography and to prepare analytical samples enriched by a subpopulation of peptides from a very complex mixture, thus greatly simplifying the classical fingerprinting approach. In this PhD thesis, tryptophan was chosen as the amino acid target, because it is the least abundant amino acid in proteins and the selection of tryptophan-labelled peptides can reduces the number of analytes and the complexity of the entire analysis (Tab. 9). Since most proteins (>90%) in *Homo sapiens* contain at least one tryptophan residue, labelling of Trp residues appears to be much suitable for the *diagonal* analytical approach (*72-73*).

Table 9. Per cent of proteins lacking a specific amino acids in different species (Expasy).

Amino acid	*E. coli*	*Human*	*Yeast*
Met	0.33	2.02	0.9
Cys	14.56	4.42	8.69
His	4.12	3.8	2.55
Trp	11.34	8.74	10.32

In the ambit of this PhD Thesis the chemical labeling strategy was used in order to simplify the analysis of allergenic proteins contained in model protein mixtures, as well as food matrices. The selective reaction of DNPS-Cl with Trp residues, leading to the covalent binding of a DNPS-moiety at the 2-position of the indole nucleus of Trp, was exploited in order to achieve a selective labeling, as well as an easier purification of DNPS-labeled Trp-peptides. The strategy was tested on a mixture of seven model proteins and then on a total protein extract of a raw cake product. In the case of the sample containing the model proteins, it has been estimated that the total number of the tryptic peptides was 200 tryptic peptides, while several thousands of tryptic peptides were those originating from a cake.

The DNPS-labelled Trp-peptides can be easily purified from other peptides by RP-HPLC, due to their enhanced hydrophobicity. The results obtained indicate that the *diagonal* chromatography can be very reproducible and selective in enriching for Trp-

peptides a sample of tryptic peptides. Therefore, the "targeted" approach can reduce significantly the time required for a successful MS analysis and allows one to focus on the analysis of a single allergenic protein contained in a food matrix.

The selection of DNPS-labelled Trp-containing peptides was achieved by hydrophobic interaction chromatography (HIC), exploiting the enhanced hydrophobicity of the labelled peptides, since these are lately eluted form a hydrophobic chromatographic matrix. The use of HIC seems very appropriate, since it requires a relatively cheap matrix and thus seems to be suitable for possible industrial applications. The method has been validated by using a tryptic digest of α-LA labelled with DNPS-Cl. As expected, it has been observed that the labelled Trp-peptides are retained stronger than the unlabelled Trp-peptides by the hydrophobic matrix Sephadex LH-20 (*51-53*).

Immunoaffinity chromatography was also used as a technique for the enrichment in Trp-peptides an analytical sample derived from a complex mixture of tryptic peptides. For this strategy, we made use of an affinity column prepared with monoclonal antibodies raised against the DNP group. It has been demonstrated that immunoaffinity chromatography allows an efficient and selective isolation of DNPS-tagged Trp-peptides. A drawback of the procedure likely resides in the costly monoclonal antibody and, therefore, for a practical point of view the alternative use of (HIC) should be considered.

The sensitivity of the overall methodology of *diagonal* chromatography and MS analysis has to be evaluated and must await the outcome of additional studies. Of course, this is a relevant analytical problem, considering that protein allergens can be contained in traces in foods. Nevertheless, the experiments were conducted using only 2 μg of a sample of α-LA tryptic peptides and the labelled Trp-peptides were isolated in a single chromatographic run. Therefore, the tagging procedure, followed by RP-HPLC separation and MS/MS analysis, appears to be useful for the analysis also of low abundant protein allergens.

Summing up, in this PhD Thesis the possibility to analyse proteins displaying allergenic properties and contained in food matrices by the use of MS techniques has been demonstrated. Nowadays immunochemical techniques such as ELISA are the most use analytical procedures for allergenic proteins, but these techniques suffer from the disadvantage of false negative results, since allergenic proteins can de significantly changed in their 3D structural and thus immunogenic properties by food processing treatments. Instead, MS methods can identify the specific peptides originating from a target

protein, irrespective of its 3D structure. Therefore, MS techniques appear to offer at least a complementary technique to ELISA assays in current use.

Acknowledgment

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Characterization of linear forms of the circular enterocin AS-48 obtained by limited proteolysis

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Abstract AS-48 is a 70-residue circular peptide from *Enterococcus faecalis* with a broad antibacterial activity. Here, we produced by limited proteolysis a protein species carrying a single nicking and fragments of 55 and 38 residues. Nicked AS-48 showed a lower helicity by far-ultraviolet circular dichroism and a reduced stability to thermal denaturation, but it was active against the sensitive bacteria assayed. The fragments also partly retained the biological activity of the intact protein. These results indicate that circularization is not required for the bactericidal activity, but it is important to stabilize the native structure. Moreover, it is possible to reduce the sequence to a minimal AS-48 domain without causing inactivation of this bacteriocin.

Keywords: Enterocin AS-48; Bacteriocin; Circular protein; Limited proteolysis; Protein fragment; Antimicrobial activity

1. Introduction

Circularization of polypeptide chains is a modification that stabilizes the native conformation of a protein and increases the resistance to proteolysis. There are many examples of natural circular proteins in different organisms. AS-48 is a 70-residue circular peptide from *Enterococcus faecalis* that belongs to an heterogeneous group of bacterial antimicrobial polypeptides, also called bacteriocins [1–5]. This protein shows a broad antimicrobial spectrum against both Gram-positive and Gram-negative bacteria [6,7]. The target of AS-48 is the bacterial cytoplasmic membrane in which it inserts in a voltage-independent manner forming pores and leading to the dissipation of the proton motive force [2]. The complete NMR assignment indicated that the AS-48 fold is characterised by five helices (α_1 to α_5) arranged to form a globular structure (Fig. 1) [8]. This bacteriocin is a strongly basic molecule (p$I \sim 10.5$) that contains a high proportion (49%) of hydrophobic and uncharged hydrophilic amino acid residues [1,9]. Basic residues are asymmetrically distributed at the level of the helices, being clustered in helices α_4 and α_5, whereas a hydrophobic surface is located in helices α_1, α_2 and α_3. The backbone circularization of the pro-AS-48 occurs in the middle of α_5-helix between Met1 and Trp70 and it confers an extremely high stability to the protein [10,11]. Because of its stability and broad antimicrobial spectrum, AS-48 is a good candidate as a natural food preservative [12–14].

Previous attempts to obtain a nicked AS-48 by chemical hydrolysis with BrCN were not successful [10]. The synthesis of a fragment encompassing the positive charged region also did not produce an active species [15]. Here, we have used limited proteolysis to produce linear forms of AS-48, considering that this technique often allows to obtain nicked forms and fragments of a protein able to maintain a native-like conformation and even biological activity [16–18]. We report the production of a nicked AS-48 form and fragments species of 55 and 38 residues that retain an α-helical secondary structure and partly the biological activity of the intact protein.

2. Materials and methods

2.1. Materials

Thermolysin was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All chemicals of analytical reagent grade were purchased from Sigma or Fluka (Basel, Switzerland). AS-48 was purified to homogeneity from the supernatant of the producer *Enterococcus faecalis* JH2-2(pAM401-81) strain in high cell density batch using the purification protocol earlier developed [19].

2.2. Limited proteolysis experiments

Purified samples of AS-48 were prepared upon dissolution of the lyophilised protein in 6 M Gdn-HCl followed by desalting using a Sephadex G-25 column equilibrated in 50 mM Tris–HCl, pH 7.5. Proteolysis reactions were conducted in 50 mM Tris–HCl, 0.1% sodium dodecyl sulfate (SDS), pH 7.5, upon addition of a stock solution of 10% SDS in 50 mM Tris–HCl, pH 7.5, to the protein sample collected from gel filtration. Proteolysis in 15% trifluoroethanol (TFE) were carried out upon dilution of the same sample with TFE. Experiments were performed at a protein concentration of 1.0 mg/ml using thermolysin at an enzyme to substrate (*E*/*S*) ratio of 1/50 (by weight) at room temperature for the proteolysis in 0.1% SDS, and at an *E*/*S* ratio of 1/20 at

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Abbreviations: CD, circular dichroism; *E*/*S*, enzyme to substrate ratio; ESI-MS, electrospray ionization-mass spectrometry; RP, reverse phase; HPLC, high-performance liquid chromatography; [θ], mean residue ellipticity; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; UV, ultraviolet; TFE, trifluoroethanol; Gdn-HCl, guanidine hydrochloride; MIC, minimum inhibitory concentration; $AS_{10/11}$, AS-48 nicked at the level of residues Ala10 and Val11; AS_{43-27}, fragment 43–27 of AS-48; $AS_{42,43-10}$, fragments 42–10 and 43–10 of AS-48

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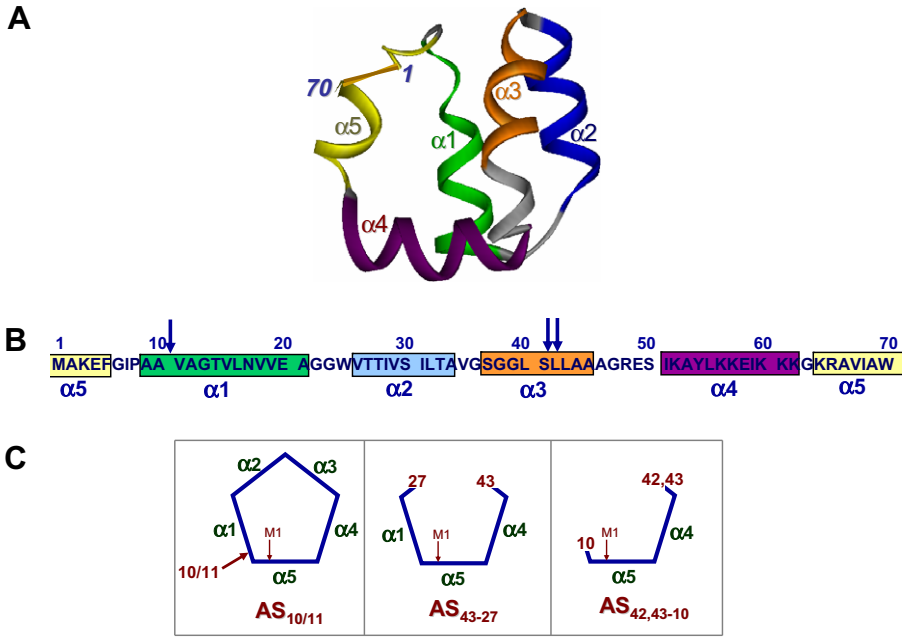

Fig. 1. Schematic three-dimensional structure and amino acid sequence of AS-48. (A) Three-dimensional structure of AS-48 derived from the NMR structure of the protein (file 1E68 taken from the Brookhaven Protein Data Bank) using the program WebLab Viewer Pro 4.0 (Molecular Simulations Inc., San Diego, CA, USA). Amino acid residues M1 and W70 appear separated in the model, but in the protein they are connected by a peptide bond. The five major helical regions of the protein spanning residues 9–21 (α1), 25–34 (α2), 37–45 (α3), 51–62 (α4) and 64–5 (α5) are indicated and showed with different colours (see B). (B) Amino acid sequence of AS-48. The regions of α-helical structure are indicated with coloured boxes and arrows indicate the sites of initial proteolytic cleavage by thermolysin (Th) in 0.1% SDS and 15% TFE. (C) Schematic representation of $AS_{10/11}$ and of fragments AS_{43-27} and $AS_{42,43-10}$ produced by limited proteolysis of AS-48.

37 °C for the hydrolysis in 15% TFE. The reactions were stopped by acidification with trifluoroacetic acid (TFA) in water. Stock solutions of thermolysin were prepared in 50 mM Tris–HCl, 5 mM $CaCl_2$, pH 7.5, for the reaction in 0.1% SDS and in 50 mM Tris–HCl, 1 mM $CaCl_2$, pH 7.5, for the reaction in 15% TFE, and stored at −20 °C. For the limited proteolysis reaction in 15% TFE a lower concentration of $CaCl_2$ was preferred, since it resulted in an increase of the yield of fragment AS_{43-27}. The proteolysis mixtures were analyzed by RP-HPLC using a Jupiter C_4 column (4.6 mm × 250 mm; Phenomenex, Torrance, CA, USA) connected to an anion exchange guard column and eluted with a gradient of acetonitrile/0.085% TFA vs water/0.1% TFA from 5% to 65% in 10 min, from 65% to 68% in 15 min and from 68 to 95% in 1 min. The effluent was monitored by recording the absorbance at 226 nm. The collected fractions were freeze-dried using a Speed-Vac system (Savant). $AS_{10/11}$ still eluted from the RP-HPLC separation with bound some SDS molecules that were eliminated upon precipitation with 6 M Gdn-HCl.

The sites of proteolytic cleavage of AS-48 were identified upon analysis by mass spectrometry of the protein fragments purified by RP-HPLC. Mass determinations were obtained with an electrospray ionization (ESI) mass spectrometer Q-Tof Micro from Micromass (Manchester, UK). The measurements were conducted at a capillary voltage 3 kV and a cone voltage 30–40 V. The molecular masses of protein samples were estimated using the MassLynx software 4.0 (Micromass) and compared with the sequence of AS-48 using the MassXpert 1.0 software. In order to determine the sites of nicking of AS-48, N-terminal sequencing was performed using an Applied Biosystems (Foster City, CA, USA) protein sequencer (model Procise HT491).

2.3. Circular dichroism

Protein concentration was determined by UV spectroscopy using extinction coefficient values calculated according to Gill and von Hippel [20]. Circular dichroism spectra were recorded at room temperature on a Jasco J-710 spectropolarimeter (Tokyo, Japan) equipped with a thermostatically controlled cell holder [18]. Thermal denaturation of $AS_{10/11}$ was performed by measuring the temperature dependence of the dichroic signal at 222 nm of the protein dissolved in 10 mM phosphate buffer, pH 2.5, at a concentration of 0.015 mg/ml and using a cuvette with a 1.0 cm path length. The circular dichroism (CD) signal was continuously recorded while heating the cuvette at a constant rate.

2.4. Activity assays

Purified AS-48, $AS_{10/11}$ and fragments AS_{43-27} and $AS_{42,43-10}$ dissolved in water:acetonitrile (1:1) plus 0.05% TFA were tested for their inhibitory activity against *Enterococcus faecalis* JH2-2 and *Listeria monocytogenes* CECT 4032 strains using the spot-assay method [21]. Spots with known protein concentration were deposited onto plates of buffered Mueller Hinton Agar (MHA-T, Scharlab), previously overlaid with 6 ml of BHA soft-agar inoculated with the indicator strain. Peptide activity was assessed by observing an inhibition zone after incubation overnight at 37 °C. To determine the minimum inhibitory concentration of the nicked form and fragments of AS-48, samples were diluted and tested against the indicator strains under identical conditions.

3. Results

3.1. Limited proteolysis of AS-48

Proteolysis experiments were carried out at neutral pH and in the presence of TFE or SDS and the proteolysis mixtures were analysed by RP-HPLC (Fig. 2). The sites of hydrolysis were determined upon analysis of the proteolytic products eluted in the chromatographic fractions by mass spectrometry (Table 1) and N-terminal sequencing. When limited proteolysis was conducted in 50 mM Tris–HCl, pH 7.5, we observed the production only of small peptides (Fig. 2A) which were not of interest for the purposes of this study. Addition of 15% TFE instead determined a slower degradation of AS-48 and it allowed the isolation of fragment 43–27 (AS_{43-27}) with a good yield (Fig. 2B and 1C). Upon increasing the TFE concen-

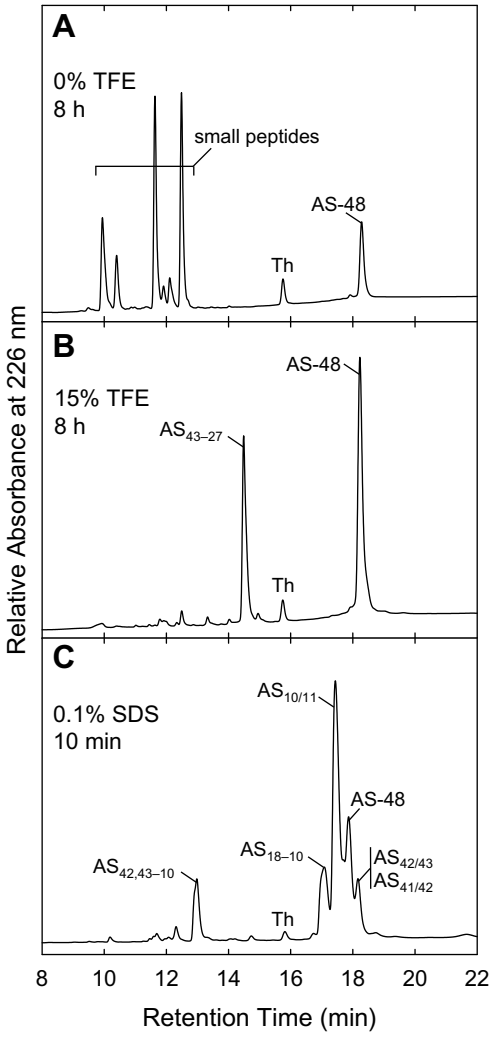

Fig. 2. Limited proteolysis of AS-48 by thermolysin. RP-HPLC analyses of the proteolytic mixtures of AS-48 reacted with thermolysin in 50 mM Tris–HCl, pH 7.5 (A), with the addition of 15% TFE (B) or in the presence of 0.1% SDS (C). Aliquots of the reaction mixtures were analysed after 8 h (A and B) and 10 min (C) of incubation.

Table 1
Molecular masses of fragments of AS-48 obtained by proteolysis with thermolysin in 0.1% SDS or 15% TFE

Protein species[a]	Molecular mass (Da)	
	Calculated[b]	Measured[c]
AS-48	7149.50	7149.42
$AS_{43–27}$	5798.93	5798.83
$AS_{10/11}$	7167.57	7167.25
$AS_{43–10}$	4141.41	4140.44
$AS_{42–10}$	4254.49	4253.52
$AS_{18–10}$	6512.80	6512.88

[a]Fragments obtained by proteolysis of AS-48 with thermolysin in 0.1% SDS or 15% TFE (Fig. 2).
[b]Molecular masses calculated from the amino acid sequence of AS-48. Mass values above 4500 Da are given as average molecular masses.
[c]Experimental molecular masses determined by ESI-MS.

tration (30% and 40%), the reaction became much slower and even if we isolated a nicked form of AS-48 opened between residues Leu42 and Leu43, the yield of this species was too low to perform additional studies (data not shown).

Limited proteolysis was also conducted at neutral pH in the presence of 0.1% SDS. Using these conditions, we obtained three forms nicked at the level of residues Ala10 and Val11 ($AS_{10/11}$), Leu42 and Leu43 ($AS_{42/43}$), Ser41 and Leu42 ($AS_{41/42}$), as well as fragments 18–10, 43–10 and 42–10 (Fig. 2C and 1C). The molecular masses of these species were determined by electrospray ionization-mass spectrometry (ESI-MS) and they are shown in Table 1. We isolated $AS_{10/11}$, fragments 43–10 and 42–10 in sufficient amount and homogeneity to perform further structural analyses and activity tests. Since the two fragments elute together from the RP-HPLC column, they were studied in mixture and we will refer to them as $AS_{42,43–10}$.

3.2. CD spectroscopy

Far-UV CD spectra were acquired on solutions of $AS_{10/11}$ and of fragments $AS_{43–27}$ and $AS_{42,43–10}$ in 50 mM Tris–HCl, pH 7.5 (Fig. 3). $AS_{10/11}$ and AS-48 have CD spectra typical of an α-helical conformation, but the intensity of the CD signal of the nicked species is reduced. We estimated a loss of ~35% of α-helical content in respect to that of the intact protein, being the percentage of α-helix calculated according to Scholtz et al. [22] 45% and 80% for $AS_{10/11}$ and AS-48, respectively (Fig. 3A). Conversely, far-UV CD spectra of $AS_{10/11}$ and AS-48 acquired in the presence of 0.1% SDS evidenced a much lower difference in helicity between the nicked and intact forms, being ~7% less for $AS_{10/11}$ in respect to that of intact AS-48. The surfactant appears to stabilise the helical conformation of $AS_{10/11}$, whereas it slightly denatures intact AS-48.

The far-UV CD spectra of fragments $AS_{43–27}$ and $AS_{42,43–10}$ are instead quite different (Fig. 3B). Indeed, the spectrum of fragment $AS_{43–27}$ displays the features of α-helical polypeptides, since minima of negative ellipticity occur near 208 and 222 nm. Interestingly, the helical content of this fragment species is substantial, since it shows a helicity of 71%, ~9% less than that of intact AS-48, thus suggesting that it retains a native-like conformation. Fragments $AS_{42,43–10}$ instead show a much lower helical content and an increase in the intensity of the minimum at 205 nm, which is indicative of a more unfolded conformation.

In order to determine the effect of nicking on the thermal stability of AS-48, we subjected $AS_{10/11}$ to thermal denaturation at pH 2.5 (Fig. 4). It has been reported that the thermal unfolding of AS-48 at neutral or alkaline pH leads to irreversible protein aggregation, in line with the fact that the protein at acidic pH is monomeric and forms a dimer at neutral pH [8,23]. At pH 2.5, $AS_{10/11}$ has a far-UV CD spectrum that has the features of an α-helical polypeptide, but it shows a much lower ellipticity than AS-48 (data not shown). On the basis of the thermal denaturation profile monitored by the temperature-dependence of the CD signal at 222 nm (Fig. 4A), $AS_{10/11}$ shows a low cooperativity of unfolding and a reduced stability if compared to native AS-48, which was shown to unfold at 102 °C [10,11]. However, even in the case of nicked AS-48, thermal unfolding is reversible at pH 2.5, as indicated from the nearly coincidence of the far-UV CD spectra recorded at 9.7 °C before and after heating (Fig. 4B). Interestingly, the CD melting curve of nicked AS-48 shows also some initial cold-induced unfolding below 20 °C, a phenomenon already

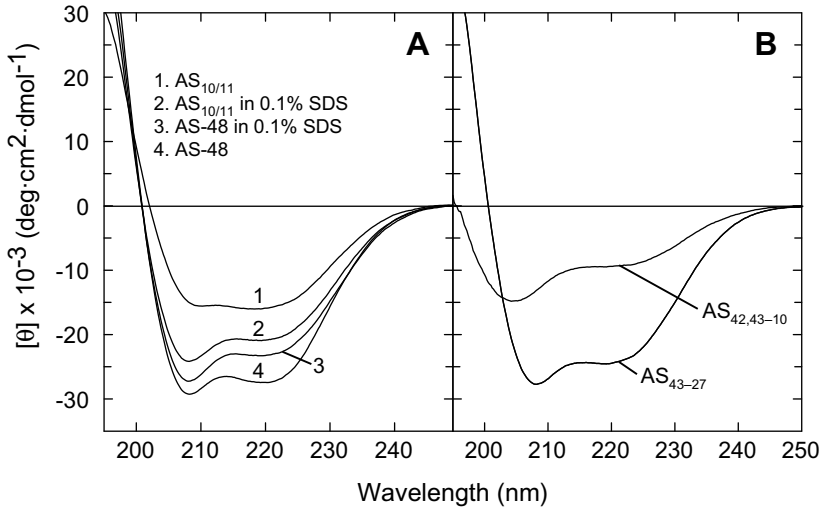

Fig. 3. Spectroscopic characterization by circular dichroism of $AS_{10/11}$ nicked form and $AS_{43–27}$ and $AS_{43,42–10}$ fragments. (A) Far-UV CD spectra of AS-48 and $AS_{10/11}$, in the presence and in the absence of 0.1% SDS. (B) Far-UV CD spectra of fragments $AS_{43–27}$ and $AS_{42,43–10}$. Spectra were recorded at room temperature in 50 mM Tris–HCl, pH 7.5.

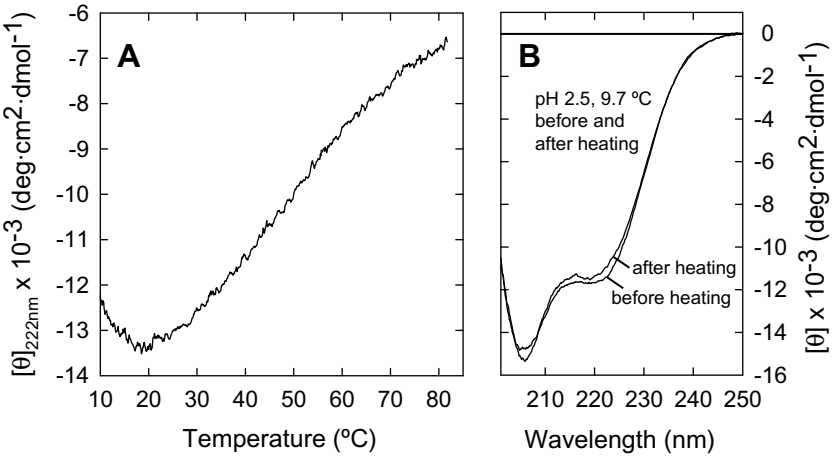

Fig. 4. Thermal denaturation of $AS_{10/11}$. (A) Melting profile of $AS_{10/11}$ in 10 mM phosphate buffer, pH 2.5. The temperature dependence of the dichroic signal at 222 nm was recorded. (B) Far-UV CD spectra of $AS_{10/11}$ at 9.7 °C recorded before and after the thermal denaturation.

described for other proteins [24]. A cold denaturation effect has been observed also for intact AS-48, but only under denaturing conditions, thus confirming that nicking of the protein reduces its stability [10].

3.3. Activity tests

The biological activity of the nicked form and fragments of AS-48 were evaluated by inhibition of the growth of *L. monocytogenes* CECT 4032 and *E. faecalis* JH2-2, both of them being sensitive bacteria against AS-48 wild-type (Fig. 5). $AS_{42,43–10}$, $AS_{43–27}$ and $AS_{10/11}$ samples (5 μl) were active against both the indicator strains when assayed at 0.3 mg/ml, demonstrating that all these linear AS-48 forms retain the inhibitory activity, *L. monocytogenes* being more sensitive. However, the stability of the samples in solution was in all cases less than that of the native AS-48, since the activity was lost during freezing and thawing processes. Finally, comparing the minimum inhibitory concentration (MIC) against *L. monocytogenes*, the nicked form was at least 300 times less

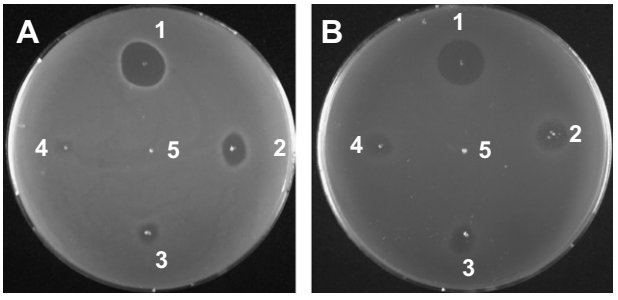

Fig. 5. Inhibition halos of AS-48 and its derivatives against *E. faecalis* (A) and *L. monocytogenes* (B) after 18 h of incubation at 37 °C. (1) Native AS-48 (control), (2) $AS_{10/11}$, (3) $AS_{43–27}$, (4) $AS_{42,43–10}$, (5) 50% acetonitrile containing 0.05%TFA (solvent control). Samples (5 μl) contained a protein or fragment concentration of 0.3 mg/ml.

effective than the native form and fragments AS_{43-27} and $AS_{42,43-10}$ were 500 and 1000 times less active, respectively.

4. Discussion

Proteolysis experiments were conducted using thermolysin on AS-48 dissolved in the presence of SDS and TFE, since under these solvent conditions the protein is hydrolysed at few peptide bonds, as often observed with folded protein species [25]. Several studies have demonstrated that SDS and TFE have a peculiar denaturing effect towards the native conformation of proteins, since they lead to partly folded protein conformations that appear to mimic those formed by proteins at the level of membranes [25,26]. Indeed, proteolysis experiments conducted in the presence of TFE and SDS lead to the hydrolysis of the protein at few peptide bonds encompassed by helices α_1, α_2 and α_3 (residues 9–45), suggesting that this region becomes more flexible and thus susceptible to attack by the protease (Fig. 1) [18].

Nicking of the peptide bond Ala10-Val11 at the N-terminus of helix α_1 of AS-48 leads to a consistent destabilization of the structure of the protein. Analysis by far-UV CD of the nicked $AS_{10/11}$ form shows a reduction in ellipticity in respect to the native protein which can be interpreted considering that hydrolysis alters the secondary structure of AS-48. Indeed, it is probable that the open form is less compact and rigid in respect to the intact protein, in agreement with the observation that structural fluctuations and reduction in helix length can lower the measured helicity [27]. The secondary structure of $AS_{10/11}$ is also less stable to thermal denaturation in respect to native AS-48, as expected for the favourable entropic contribution introduced by circularization [10,11]. In spite of the differences in helical content and stability, biological assays confirmed that the nicked form retains its functional properties even if it shows a 300 times lower inhibitory activity. These results suggest that circularization is not essential for the bactericidal activity, but that it can stabilise the three-dimensional structure of the protein. Interestingly, upon addition of 0.1% SDS the helical contents of native and nicked AS-48 became almost identical, demonstrating that $AS_{10/11}$ can acquire a conformation similar to that of intact AS-48 in the presence of a membrane-like environment. This observation suggests that nicking is compatible with the insertion into the bilayer lipid membrane that is required for the activity [2].

Fragment AS_{43-27} shows a high α-helix content close to that calculated from the NMR structure of AS-48 (71% and 78%, respectively), whereas the $AS_{42,43-10}$ species displays a far-UV CD spectrum characteristic of a more random conformation. Activity tests conducted on AS_{43-27} and $AS_{42,43-10}$ indicated that the first species is more active, but not as $AS_{10/11}$. All these fragments retain the positive charged region that is important for the interaction with bacterial membranes. The higher activity of AS_{43-27} could be explained by the presence in this last species of the hydrophobic α_1 helix and by the acquisition of a more structured conformation that can maintain the cluster of positive charges in a native-like arrangement. Indeed, the helical content has been demonstrated to be important for the activity of anti-bacterial peptides [28]. Interestingly, the previously described synthetic 21-residue peptide comprising residues 49–69 of AS-48 was fully devoid of biological activity [15]. The synthetic mini-bacteriocin differs from $AS_{42,43-10}$ by the absence of the last 11 residues 70–10 (WMAKEFGIPAA) of α_5 helix and by the substitution of residues Ile59 and Val67 with cysteinyl residues linked by a disulfide bond, that was introduced into the peptide in order to maintain the relative orientation of the helices (Fig. 1). We suggest that the presence of the Trp70 residue in $AS_{42,43-10}$ can be important for the biological activity of AS-48. Indeed, tryptophan residues are known to display a high affinity for the polar-apolar interface of the lipid bilayer and they are involved in the interaction with membranes, as demonstrated by mutational analysis in pediocin-like bacteriocins [29]. $AS_{42,43-10}$ thus appears to be the minimal sequence of AS-48 that still shows activity, analogous to the anti-mycobacterial domain identified in NK-lysin and granulysin [30].

Summing up, the results here presented confirm that it is possible to produce linear forms of AS-48 that can still retain biological activity. However, a proper distribution of electrostatic and hydrophobic surfaces in the protein species appears to be required for developing a full antibiotic activity and thus an efficient insertion into the membranes. Indeed, the biological activity of AS-48 requires not only the positively charged region of the protein, but also part of the hydrophobic region.

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Manuscript in preparation

α-Lactalbumin Forms with Oleic Acid a High Molecular Weight Complex Displaying Cytotoxic Activity

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Running Title: Oleic Acid Complex of Oligomeric α-Lactalbumin with Apoptotic Activity

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Abstract

α-Lactalbumin (LA) forms with oleic acid (OA) a complex, named HAMLET (human LA) or BAMLET (bovine LA), which induces the selective death of tumor cells by an apoptosis-like mechanism. Even if the use of the LA/OA complex in cancer therapy appears to be quite promising, molecular aspects of the interaction between the protein and the fatty acid are still not understood. In particular, initially it was reported that the active species is an aggregated form of LA, whereas subsequently it was asserted to be monomeric. Here, it is demonstrated that the toxicity of the LA/OA complex towards Jurkat cells is exerted by a high molecular weight protein/fatty acid complex. The aggregation state of OA under physiological conditions is changed by the addition of the protein and the fatty acid becomes more water soluble. The active LA/OA complex was isolated by gel filtration chromatography and it was shown to have a molecular mass of about 110 kDa and to be composed of an oligomeric form of the protein bound to the fatty acid at a molar ratio of protein over OA of about 1/17. Crosslinking experiments using glutaraldehyde provided evidence that the complex is mostly composed by 4-5 protein molecules. Circular dichroism measurements indicated that the protein in the complex adopts a partly folded conformational state, similar to the well known molten globule state of LA at low pH. Limited proteolysis experiments allowed us to infer molecular features of the LA/OA complex and, in particular, indicated that the α-helical domain of the protein is mostly involved in the interaction with the fatty acid, while the β-domain encompassing the central region of the 123-residue chain of the protein appeared to be loosely bound to OA and flexible and thus easily attacked by proteases. Overall, our data allow us to propose that the interaction of OA with a protein leads to a better solubility of the otherwise rather insoluble fatty acid and thus to an enhancement of its intrinsic apoptotic activity. Moreover, since the protein component in the LA/OA complex is both oligomeric and partly folded or misfolded, the cellular toxicity of the complex appears similar to that exerted by the earlier formed protein oligomers of amyloidogenic proteins. Therefore, both the oligomeric protein and the fatty acid can contribute in a synergic way to the toxicity of HAMLET-like complexes.

Manuscript in preparation

The Interaction Of Apomyoglobin With Oleic Acid Leads To A Protein Complex That Displays Cellular Toxicity

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Running Title: Oleic Acid Complex of Oligomeric Apomyoglobin with Apoptotic Activity

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This work was presented at the 53rd National Meeting of the Italian Society of Biochemistry and Molecular Biology (SIB) and National Meeting of Chemistry of Biological Systems, Italian Chemical Society (SCI-Section CSB) (September 23–26 2008), Commun. 15.15.

Abstract

Several studies have shown that fatty acids can induce the formation of toxic aggregates in some amyloidogenic proteins involved in neurodegenerative diseases. In particular, it has been demonstrated that oleic acid (OA) can induce the formation of oligomers in proteins such as tau, superoxide dismutase and amyloid β-peptide. Here, we have studied the interaction of OA with horse heart apomyoglobin (apoMb), which is not related to any pathology. This single chain and disulfide-free 153-residue protein is extensively used since decades as a model for studies of protein structure, folding, misfolding and aggregation. The interaction of OA with apoMb (1:10 molar ratio) leads to the formation of an apoMb/OA complex given by protein oligomers, as evidenced by protein cross-linking experiments with glutaraldehyde followed by SDS-PAGE. Indeed, cross-linked oligomers of apoMb are formed in the presence of OA, while apoMb alone does not form oligomers. Far-UV circular dichroism spectroscopy measurements indicated that apoMb maintains a highly helical conformation in the presence of OA. We tested the toxicity of the apoMb/OA complex on Jurkat cells and we have found that the protein complex causes cell death by an apoptosis-like mechanism. Conversely, the protein is not active when tested alone and OA shows a much reduced toxicity. Of interest, it has been demonstrated previously that OA can induce cellular toxicity by an apoptosis-like mechanism. Our results suggest that the interaction of OA with apoMb strongly enhance the water solubility of the otherwise insoluble fatty acid, thus leading to a significant enhancement of its intrinsic apoptotic activity. We suggest that the apoMb/OA complex acts as a delivery system of the toxic fatty acid to the cell. This mechanism could explain also the previously reported toxicity of OA complexes with other proteins, including those with human and bovine α-lactalbumin (LA). We conclude that the toxic effect for tumor cells of the OA/LA complex previously reported6 is not protein-specific and we suggest that other proteins can display similar toxic effects if combined in an OA complex.