ELSEVIER

Journal of Chromatography B, 756 (2001) 71–84

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Review

Isolation of food allergens

Elide A. Pastorello*, Chiara Trambaioli

Allergy Centre, 3rd Division of General Medicine, Ospedale Maggiore IRCCS, Padiglione Granelli, Via Francesco Sforza 35, 20122 Milan, Italy

Received 24 February 2000; accepted 20 June 2000

Abstract

The identification of food allergens is a priority in the management of food allergy, because of the need to obtain standardized extracts and pure allergens for diagnosis and therapy. It is thus important to develop methods for purification of allergenic molecules in order to study their biological and immunological characteristics. Protocols for protein extraction from foods and for allergen purification are reviewed in this paper. We report published methods for extraction of allergens from either animal and vegetable foods and detailed purification methodologies including ion-exchange, gel filtration and reversed-phase chromatography of well known allergens.

Keywords: Reviews; Food allergy; Skin prick test; Radioallergosorbent assay

Contents

*Corresponding author. Fax: +39-2-5519-2842.
E-mail address: elidepa@box1.tin.it (E.A. Pastorello).

0378-4347/01/$ – see front matter
PII: S0378-4347(01)00072-X

1. Introduction

Treatment of food allergy is an area that today still presents unresolved problems. Firstly, skin prick test (SPT) and radioallergosorbent assay (RAST) diagnostic efficacy is low, thus it is necessary to base diagnosis on double-blind placebo-controlled food-challenge [1,2] and treatment involves eliminating the culprit food while no specific therapy is employed. This may be partially explained by the fact that there is scant knowledge about the majority of food allergens. Hence, diagnostic material is poorly standardized, and an attempt at specific immunotherapy is inconceivable.

Thus if food allergy management is to be refined, food allergen identification is of utmost priority. Once the allergens have been identified, the properties and biochemical and immunological activities of these can be investigated in detail, and there will be better understanding of sensitization mechanisms.

It is important to have the food allergens isolated as the protein shows a range of characteristics, which may be studied only under the natural form, among which the most relevant is the in vivo IgE binding activity.

The new techniques of molecular biology once again will provide for the availability of a larger amount of allergens in order to prepare commercial extracts for diagnosis and therapy. In some studies, however, the recombinant allergens did not show the same allergenic activity as the natural form as was for example the case for cherry thaumatin [3] and for Bet v 1 which presents 20 isoforms with different IgE binding activities [4,5].

The purification is not always an easy task; in many cases a very small amount of the protein is present in the alimentary source, or the food is full of different compounds such as fats, oil or phenols which make protein extraction difficult. In other cases the allergen is an enzyme, and it is able to inactivate its own allergenic activity.

Nonetheless, a lot of work has already been done in food allergen purification, and this will be summarized in the following paragraphs.

2. Protein extraction from food sources

Since a lot of different food sources are studied for their allergenic properties the first requirement for correct research procedure is to obtain good protein extracts, that is starting material with a sufficiently high protein concentration and suitably low lipid and sugar content so as to allow for protein separation. In the case of animal foods good extracts have often been achieved with simple incubation of the food in a buffer solution to extract proteins contained in the raw material, as reported for the extraction of egg allergens in Langeland study [6]. Hens' eggwhite antigen solution was prepared by stirring crude eggwhite with an equal volume of physiological saline for 4 h; after centrifugation, the supernatant was stored and used for clinical and immunological study. The method was substantially confirmed by the study of Bernhisel-Broadbent et al. [7], in which egg white was extracted by overnight incubation with PBS followed by centrifugation. A similar method was assessed [8] for preparation of extracts from different raw and cooked fish species (catfish, tuna, salmon, trout, codfish . . .) which were added separately to PBS in sterile centrifuge tubes. After overnight incubation the samples were centrifuged and the supernatants removed and lyophilized. This protocol was followed even for the preparation of allergenic extract from boiled Atlantic shrimp [9], while for oysters, crabs and mussels [10] the chosen protocol included extraction at room temperature in water maintained at pH 8 for 4 h, followed by dialysis and lyophilization of extracted proteins.

A more complex methodology is necessary for the isolation of milk allergens, because the separation of both casein and whey fractions is achieved only by acidic precipitation of proteins from skimmed milk. In the protocol set up by Bleumink and Young in

1968 [11] casein was separated from milk by precipitation at pH 4.65, obtained by milk titration with 1 *M* HCl. After sedimentation, the precipitated fraction was dissolved in water at pH 7 by slow addition of 1 *M* NH_4OH. This procedure was repeated twice and the solution subsequently dialyzed against distilled water and lyophilized. Whey allergenic proteins (β-lactoglobulin and α-lactalbumin) were isolated by acidic precipitation and recrystallization repeated three times [11].

In the case of vegetable foods, however, the procedure may be more complex as the presence of several non-proteinic compounds often causes problems because of their reactivity towards extracted proteins which may inactivate allergens.

The basic method for allergen extraction from vegetable food especially studied to reduce the amount of phenolic compounds was defined by Bjorksten et al. [12] in 1980. This has become the most widely used procedure for extracting proteins, especially from fruits, seeing as it was set up for studies on apple allergens. The protocol provides for homogenization of fruit pulp in 10 m*M* potassium phosphate buffer, pH 7.0, containing 2% suspended solid polyvinylpolypyrrolidone (PVPP), 2 m*M* ethylenediaminetetraacetic acid disodium salt (EDTA), 10 m*M* sodium diethyldithiocarbamate (DIECA) and 3 m*M* sodium azide (NaN_3). PVPP, EDTA and DIECA are added to inhibit reactions between proteins and phenolic compounds [13], while NaN_3 is an antimicrobial agent. The homogenate is then centrifuged at 40 000 *g* for 30 min and subsequently dialyzed for 20 h at 4°C against 10 m*M* potassium phosphate buffer, pH 7.0, containing 3 m*M* sodium azide. The resulting extract can be stored at −20°C if necessary without significant loss of allergenic activity.

This protocol can be used for protein extraction from a wide variety of foods, giving good results in almost all cases in which phenols elimination is necessary; the use of fruit pulp however leads to very low protein content, thus requiring concentration. The use of fruit peels instead of pulp can eliminate the need for time-consuming concentration steps and can also reduce the amount of sugar dissolved in protein preparations, making fruit extracts easier to manage.

The role of apple as an allergenic food has caused the development of several methods for protein extraction over the years, starting from the need to obtain reliable extracts for food allergy diagnosis and in vitro tests. Vieths et al. [14] prepared a specific apple extraction procedure by preparing apple extracts with a low-temperature 'acetone powder' method in which pieces of mature fresh apples were homogenized in acetone–dry ice at −60°C. The resulting precipitates were washed with acetone and with acetone–diethylether (1:1, v/v, −60°C), filtered and lyophilized. Protein extracts were obtained by incubating acetone dried powder with phosphate buffer, pH 7.4, 150 m*M* NaCl. This method can give very pure extracts with no contamination problems, but it is evident that the procedure is quite complex and thus cannot be easily followed by laboratories that do not have specific technical equipment and the required expertise.

A further method to enhance the protein content of fruit allergenic extracts and to lower carbohydrate content was developed in 1997 [15], and is based on ion-exchange column chromatography. This process, however, caused the loss of basic proteins (p*I*>8.8) which were eluted together with the sugars in the unretained fraction. The loss was low but the absence of basic proteins in processed food extracts can lead to severe underestimation of reactivity patterns, due to the importance of basic peptides (such as the lipid transfer protein family in vegetable food, or lysozyme in egg white) as major food allergens [16–18].

Because of the relevant role played by cereals in human feeding, and thus even in hypersensitivity reactions to foods, the methodology for the extraction of cereal proteins is of particular importance. Cereal seed storage proteins require complex methods for extraction and are characterized on the basis of their solubility in different solvents: the albumin and globulin fractions are soluble in neutral solutions [19] and are thus quite easy to extract, as reported by Sandiford et al. [20] who performed extraction from wheat grains and flour by incubation in 100 m*M* ammonium hydrogencarbonate by overnight shaking. The extracts were then dialysed and freeze-dried. Several variations of this method regard the incubation time and the extraction buffer; the outcome of these extractions is, however, quite similar, thus allowing researchers to choose better conditions for

their extract preparation according to the general design of the whole purification process. Other more complex protocols are necessary to obtain glutelins, which are soluble in diluted acid solutions [19], and prolamines [21]. Whereas the albumin and globulin storage proteins are widely distributed in flowering plants, the prolamins are restricted to the grasses family, which includes the major cereals. In these plants prolamins usually account for approximately half of the total grain nitrogen, and are characterized by two features: their extraction and solubility in alcohol–water mixtures, and their high content of proline and low levels of charged acidic and basic amino acids [22]. These molecules are known to be the responsible agent for coeliac disease; moreover they have been recently identified as the allergens involved in exercise-induced anaphylaxis after wheat ingestion [23].

To obtain a protein preparation completely free of sugars, lipids and phenolic compounds, which often bind to allergenic proteins and cause problems for purification procedures, the best choice is probably the precipitation of proteinaceous fraction from the whole food extract by organic solvents or by a high salt concentration. This method has been successfully followed by Desormeaux et al. [24] for the extraction of a wheat phospholipid transfer protein from both seeds and flour. Salting-out precipitation is preferable to the organic solvent method because its conditions cause less denaturing and its procedures are easier to handle, allowing for higher recovery of proteins even though a consistent loss of material is typical of this kind of extraction [14].

The presence of lipids in food, especially in seeds and nuts, is never desirable because of the problems it causes when the extracts are submitted to electrophoretic separations and to liquid chromatography, where they threaten both resolution between different proteins and column working capacity itself. Some methods were thus defined to remove lipids from foods without damaging their allergenic properties, so as to obtain lipid-free raw material to be used for extraction of proteins according to known protocols. The first protocols for defatting procedure were reported by Sun et al. [25] for Brazil nut kernels and by Yunginger and Jones for peanuts in 1987 [26] and required nuts to be ground into a fine paste and defatted by extraction with hexane. After three extractions with fresh hexane, the residue was recovered by filtration under reduced pressure and dried under vacuum overnight. The resulting material was then used for protein extraction with a suitable buffer.

A very similar protocol is that reported by Pastorello et al. [27], in which Brazil nut pulp, ground in a mixer, was defatted by several incubations in acetone followed by paper filtration and dried overnight to obtain lipid-free powder for extraction procedures.

A defatting step was necessary for peanut extraction too, as reported in the studies by Burks and co-workers [28,29] about major peanut allergen identification: defatting was performed by incubation of the roasted nuts with hexane, and the defatted powder was then used for protein extraction in 20 m*M* sodium phosphate buffer, pH 7, with 1 *M* NaCl and 8 *M* urea.

3. Electrophoretic techniques

The basic step required for purification is the identification of allergens in a defined allergenic source. Several techniques have been developed to monitor the allergenicity of both allergen source and individual allergens, all of which rely on detecting the binding of allergen specific IgE to the material under investigation. Before the discovery of IgE, the only methods available were biological, such as in vivo skin test reaction; in the following years different in vitro techniques were developed. However it is still considered necessary to confirm allergenic activity in at least one biological assay.

As for in vitro techniques, the first method used to test extract allergenicity was the RAST, still widely used for diagnostic purposes. For research studies another method was later developed by combining electrophoretic separation of allergens in the first dimension of gel with electrophoresis into antibody in second dimension; antigens precipitated with antiserum and were detected either by staining the plate (CIE) or by incubating it with allergenic serum and then by revealing allergen-bound IgE with radiolabelled anti-IgE and autoradiography (CRIE) [30,6]. This technique was employed for the identifi-

cation of crustacean allergens [31] from shrimp extract; eighteen precipitating antigens with anodic mobility were detected and were identified by CRIE analysis using sera from six subjects with elevated RAST values to shrimp extract. This assay identified seven molecules that bound patients' IgE antibodies and were subsequently studied in comparison with other crustacean extracts to establish cross-reactivity between food antigens [32].

3.1. Monodimensional and bidimensional gel electrophoresis

At present the most widely-used technique to identify allergen molecules in raw extracts is mono- and bidimensional electrophoretic separation in polyacrylamide gel followed by immunoblotting with allergenic serum; the IgE bound to individual allergens is then detected using an enzyme or radiolabelled anti-IgE reagent and the reactivity detected after appropriate treatment.

This method has been used for almost all studied allergenic proteins, and a lot of publications regard allergen identification and their definition as major or minor allergens on the basis of IgE reactivity of more or less than 50% of patients towards the specific molecule.

In some cases the allergenic proteins are well separated using SDS–PAGE, especially when allergens are the most relevant component of the studied extract; they can thus be directly eluted from the gel, so obtaining the purified molecules of interest with a one-step procedure. Simple preparative SDS–PAGE followed by electro-elution was used by Vieths et al. for the purification protocol for Mal d 1 [33], in parallel to the chromatographic method set up by Fahlbusch et al. [73]. The purity of the preparation was confirmed by sequencing and high biological activity was maintained although denaturing and reducing conditions were applied. The described method is one of the simplest procedures to purify an allergen, although it is only suitable when small amounts of protein are required.

The alternative in this field is two-dimensional electrophoretic separation, which combines separation based on molecular mass with that based on isoelectric points of the different molecular species. Two-dimensional PAGE with immobilized pH gradients (IPG–DALT) [34] has a unique capacity for the resolution of complex mixtures of proteins e.g. foods. Posch et al. [35] optimized IPG–DALT for the micropreparative isolation of high sample loads of wheat seed proteins to identify wheat grain allergens.

4. Chromatographic techniques

4.1. Ion-exchange chromatography

The technique of ion-exchange chromatography is based on the interaction between charged solute molecules and oppositely charged moieties covalently linked to a chromatography matrix. Today, ion-exchange is the most frequently used chromatographic technique for the separation and purification of proteins, polypeptides, nucleic acids, polynucleotides and other charged biomolecules [36]. The differences in charge properties of biological compounds are often considerable, and since ion-exchange chromatography is capable of separating species with very minor differences in properties it is a very powerful separation technique [37]. In principle, one could use either an anion- or a cation-exchanger to bind amphoteric samples by selecting the appropriate pH value. In practice however, the choice is based on which exchanger type and pH give the best separation of the molecules of interest, within the constraints of their pH stability.

The first protein to be purified in the history of allergen isolation was the major allergen of codfish, named Gad c 1, which was isolated by ion-exchange chromatography, gel filtration and isoelectrofocusing [38,39] and identified as a parvalbumin, a calcium binding protein typical of fish and amphibians muscle tissues.

Once the procedure was set up, a large number of applications soon followed and opened the field of allergen purification. In principle, the acidic proteins can be isolated by interaction with anion exchangers, while basic proteins are purified with cation exchangers because of their positive charge. On this basis, a suitable chromatography can be set up for each known allergenic molecule.

4.1.1. Anion-exchange chromatography

The first and most important allergens to be purified by the anion-exchange technique were the peanut allergens, whose 'celebrity' is due to the fact that peanuts are among the most common causes of immediate hypersensitivity reactions to foods. The identification and purification of the major peanut allergen Ara h 1 dates to 1991 with the study of Burks et al. [28], who were the first to develop an effective methodology for protein isolation, whereas the previous studies [40–43] reported only immunological analysis and revealed a lot of allergenic components whose nature had not yet been assessed. The purification of the allergen was achieved by anion-exchange chromatography performed with a Mono Q column (Pharmacia, Uppsala, Sweden). The raw peanut extract was dialyzed against 20 m*M* Tris-bis-propane (pH 7.2) and 8 *M* urea, and then loaded onto the Mono Q column; a stepwise salt gradient of 0 to 1.5 *M* NaCl was applied and fractions collected and lyophilized. Dot blotting and SDS–PAGE/immunoblotting analysis led to the identification of fraction 3 as a 63 500 protein with p*I* 4.55 and positive PAS staining, defined as a major peanut allergen and named Ara h 1. Further studies identified Ara h 1 as a seed storage protein belonging to the vicilin family. Biochemical and structural analysis of epitopes was later carried out and led to the determination of the peptides and amino acids essential to IgE binding [44].

A subsequent study by the same authors [29] led to the identification and purification of a second major allergen of peanut by a procedure very similar to that used for Ara h 1. The method was based on anion-exchange chromatography of the raw extract in 20 m*M* Tris-*bis*-propane (pH 7.2) without urea on a PL-SAX column (Polymer Laboratories, Amherst, MA, USA) developed with a stepwise salt gradient from 0 to 1.5 *M* NaCl. The fraction 4 contained the protein of interest which has two closely migrating components whose mean molecular mass was 17 000 and mean p*I* was 5.2; the allergen was named Ara h 2 and its epitope specificity was later studied by Burks et al. [45].

Another important family of proteins with high allergenic activity studied with ion-exchange chromatography was that of egg-white proteins. Egg-white is a complex mixture of more than 20 proteins, among which the four proteins ovomucoid, ovalbumin, ovotransferrin and lysozyme have been claimed to be the main allergens [46,47,6,48], respectively named Gal d 1, Gal d 2, Gal d 3 and Gal d 4. As the commercial preparations have been demonstrated to contain large amounts of cross-contaminating proteins, a protocol for their purification was established by Ebbehoj et al. [49] in order to obtain pure allergens for further immunological studies. Commercial preparations of the proteins ovomucoid, ovotransferrin and ovalbumin were subjected to a two-step purification. Anion-exchange chromatography was done on a Q-Sepharose FF column (Pharmacia) equilibrated with 20 m*M* triethanolamine, pH 7.3, and eluted by a salt gradient from 0 to 1 *M* NaCl. Beside the expected peaks for each one of the analyzed proteins, a few additional peaks were found in all the preparations. In all the cases the gel filtration contributed to the purification of the proteins, in addition to serving as desalting steps. A very similar protocol for these proteins was assessed by Bernhisel et al. [7] and consisted in a one-step purification by anion-exchange chromatography on DEAE Sephacel column with a 0–0.2 *M* NaCl gradient with 10 m*M* phosphate buffer, pH 6.8.

Even the major allergen of rice Ory s 1 was purified with anion-exchange chomatography [50] from ammonium sulfate precipitated extract applied on a DEAE cellulose column, equilibrated with 20 m*M* Tris–HCl buffer, pH 8.6, and eluted with salt gradient 0–0.1 *M* NaCl. The IgE reactive fractions were collected and then fractionated on a Sephadex G-50 column to obtain a single band fraction in SDS–PAGE analysis. The allergenic protein was then cloned [51], and the deduced sequence showed considerable similarity to wheat and barley α-amylase/trypsin inhibitors.

Another allergen studied with this powerful technique was the major allergen of kiwi fruit identified [52] and purified by Pastorello et al. in 1997 [53]. The protein of interest was separated from the crude extract with an anion-exchange chromatography on a ResourceQ (Pharmacia Biotech) column equilibrated in 20 m*M* Tris–HCl, pH 7.5, and eluted with salt gradient from 0 to 0.5 *M* NaCl. The fourth fraction obtained from the chromatography contained the purified major allergen with molecular mass of 30 000; isoelectrofocusing and amino-acid sequenc-

ing led to the identification of the protein as actinidin, a proteolytic enzyme belonging to the class of thiol-proteases. The allergen was named Act c 1 and its important role in hypersensitivity to kiwi fruit was confirmed also by Fahlbusch et al. [54], who purified the allergen according to the reported method [53].

4.1.2. Cation-exchange chromatography

Cation-exchange chromatography is the other side of ion-exchange technique, and uses negatively charged groups linked to the column matrix; they are typically sulphonic residues which belong to strong exchangers and bind molecules with positive net charge.

A large family of allergens, the lipid transfer protein group, was isolated from different foods [16–18] using this powerful technique and subsequently indicated as a new class of panallergens [55] within vegetables.

The first allergen to be identified as such a molecule and then purified was the major allergen of peach, Pru p 3 [16]. The protein was isolated from the peach extract by cation-exchange chromatography on a ResourceS (Pharmacia Biotech) column, equilibrated with 50 m*M* sodium acetate buffer, pH 5, and developed with salt gradient from 0 to 1 *M* NaCl. The peak eluted with the gradient contained the 9000 allergen together with other contaminants and was thus passed on a gel filtration Superdex75 column to achieve final purification. Isoelectric point (p*I*>9) and amino terminal sequence of the pure protein proved its high homology with the lipid transfer protein of almond. This opened a wide field of study because of the cross-reactivity between different vegetable foods, which to further investigation proved to be due to common low molecular mass allergens of the LTP family [56].

In addition to the the allergen of peach, other LTPs identified as major allergen in apricot (Pru ar 3) [17] and minor allergen in apple (Mal d 3) [18] were also purified by cation-exchange chromatography.

As regards the study of eggwhite allergens, we reviewed the purification schemes for ovomucoid, ovotransferrin and ovalbumin with anion-exchange chromatography in the previous paragraph. Here we report the methodology for the isolation of the fourth allergen of eggwhite Gal d 4, currently called lysozyme, from the study of Ebbehoj et al. [49]. Lysozyme was purified by cation-exchange chromatography on a MonoS HR 5/5 (Pharmacia) column with 20 m*M* Bicine–HCl buffer, pH 7.8, as eluent. Desorption was carried out by a salt gradient from 0 to 1 *M* NaCl in the same buffer and lysozyme was eluted at 0.16 *M* NaCl. Pooled fractions from several runs were freeze-dried, dissolved in 50 m*M* NH_4HCO_3, pH 7.8, and purified by gel filtration in the same buffer on a Sephacryl S-200 HR column (Pharmacia) to obtain pure allergen.

Another important molecule purified by cation-exchange chromatography was the major allergen of shrimp Pen a 1, which was isolated from boiled shrimp extract with a complex procedure [57] including ammonium sulfate precipitation, chromatography on DEAE–Sephacel column with Tris–HCl buffer, pH 8, and two additional passages on BioGel P-200 and Sepharose 4B to reach complete purification. The IgE binding molecule of interest was then identified as tropomyosin on the basis of sequential homology with the protein of *Drosophila melanogaster* and of electrophoretic behaviour in the presence of 6 *M* urea [58,59]. A protein later studied for its allergenic properties in brown shrimp, *Metapenaeus ensis*, was also identified as tropomyosin and cloned for further characterization [60].

4.2. Gel-filtration chromatography

Gel-filtration chromatography, also called size-exclusion chromatography, is one of the most widely known and used separation techniques for the analysis of protein mixtures. It is based on the possibility of parting molecules in solution on the basis of their size as they pass through a column packed with a gel [61].

The volume of the sample influences the size of column which is needed, while viscosity places an upper limit on the sample concentration which is permissible. Ionic strength, pH and composition are not significant as long as they do not affect the sizes or stability of the molecules to be separated and are not outside the stability range of the gel-filtration medium [62]. These features make gel filtration the technique of choice for the separation of well distinguished molecular mass proteins in a solution

with few different molecules, and this is the reason for the wide use of size-exclusion chromatography for the last purification step of allergens, which have been previously isolated from whole food extracts by other chromatograpic techniques [16–18].

However, in case of high amount of proteins in a given food source, as happens for seed storage proteins, it was possible to set up a one-step purification like that reported [25,27] for the isolation of the major allergen from Brazil nut, Ber e 1, which was purified by a size-exclusion chromatography on a Superdex75 column (Pharmacia) with high salt concentration in sodium phosphate buffer, pH 7.5, giving a single-protein fraction, corresponding to the 2S albumin of the seed storage proteins of the nut.

Similar purification protocols were established even for the isolation of the major allergens of yellow mustard Sin a 1 [63], purified with a single gel filtration on Sephadex G-75 with 0.15 *M* ammonium bicarbonate, pH 8, and of oriental mustard seeds Bra j 1 [64], whose fraction from gel filtration displayed the presence of several isoallergenic forms in an analysis following cationic-exchange chromatography.

Other important allergens isolated by gel filtration were those of wheat and of cow's milk. The major allergen of wheat was purified from ammonium sulfate precipitated extract loaded onto a AcA 44 size-exclusion column, equilibrated and eluted with PBS, pH 7.3. The purified 15 000 component was then N-terminal sequenced and identified as corresponding to the published sequence of α-amylase inhibitor [65,66]. To this family belong the major allergens of rice [50] and of barley [67], whose isolation protocols are reported in other sections of this review [68,69]. Casein was identified as the major allergen of cow's milk [69] and its isolation was carried out by size-exclusion chromatography on a Sephacryl S-300 column, eluted with phosphate buffer, pH 8.4, which caused the isolation of high-molecular-mass aggregates then disrupted by treatment with 5% 2-mercaptoethanol and 3 *M* urea to obtain monomeric form [70].

The other typical use of gel-filtration chromatography is the final polishing of semipurified fractions from other separation techniques, as reported in purification protocols for eggs white allergens [49] and for LTPs [16–18] that we reviewed in the paragraphs about ion-exchange chromatography.

In summary, the advantages of gel-filtration technique are that the behaviour of a known protein can be generally anticipated on the basis of its molecular mass and structure, the technique can be adapted to almost any different purification protocol because of the possibility to use any needed ionic strength, pH and composition for the elution buffer and the method is very useful for the removal of small quantities of contaminants in the last step of purification procedures.

4.3. Reversed-phase chromatography

In reversed-phase chromatography the binding of mobile phase solute to an immobilised *n*-alkyl hydrocarbon or aromatic ligand occurs via hydrophobic interaction [71]. Molecules that possess some degree of hydrophobic character, such as proteins, peptides and nucleic acids, can be separated by reversed-phase chromatography with excellent recovery and resolution. In addition, the use of ion-pairing modifiers, such as trifluoroacetic acid, in the mobile phase allows reversed-phase chromatography even of charged solutes such as hydrophilic peptides. Preparative reversed-phase chromatography has found applications ranging from micropurification of protein fragments for sequencing to process scale purification of recombinant protein products [72].

Initially, experimental conditions are designed to favour adsorption of the solute from the mobile phase to the stationary phase. Bound solute molecules will then sequentially desorb and elute from the column, according to their individual hydrophobicities, by decreasing the polarity of the mobile phase; this is usually achieved by increasing the percentage of organic modifier (usually acetonitrile) in the mobile phase itself.

In the development of a purification scheme, consisting of various steps with increasing specificity for the desired biomolecule and decreasing capacity, the reversed-phase chromatography is not a suitable method for the first step of separations from raw extracts because of the presence of lipids and other highly hydrophobic contaminants which bind strongly and can be difficult to remove from the column. The method is thus usually employed as a second step following the first capture stage, as reported in the study by Fahlbusch et al. [73] for Mal d 1 purification and by Sanchez-Monge et al. [74] for

isolation of wheat flour peroxidase. To be employed as the first isolation stage, reversed-phase chromatography requires an extensive processing of the raw extract in order to lower lipid and contaminant content; this approach has been used in studies by Inschlag et al. [3] to purify Pru a 2 allergen from lyophilized cherry extract and by Sanchez-Monge et al. [55] to isolate LTP from lyophilized defatted fruit extracts. In the intermediate stages of purifications, however, where the critical requirements are recovery and resolution, reversed-phase chromatography is a suitable technique because of the high resolution that can be achieved while removing undesired contaminants. Polishing is the final step in the preparation of a pure product, and at this stage typical contaminants may include conformational and structural variants of the target molecule. Polishing can be performed using size exclusion, as already reported in this review, especially when dimers and aggregates must be removed. However, when dealing with slight structural variants and microheterogeneities, reversed-phase chromatography with its excellent resolving power is the method of choice. It has been, therefore, extensively employed for allergen purification. Here we report the methodologies used for the isolation of some allergens from fruits and wheat flour, as they correspond to the aim of this review about food allergens. The first procedure including reversed-phase chromatography was reported in the study of Fahlbusch et al. in 1995 [73], who isolated the major allergen of apple, later named Mal d 1. The purification protocol included a first capture step performed with anion-exchange chromatography of the lyophilized apple extract on a ResourceQ column in 20 m*M* Tris–HCl buffer, pH 8. The fraction of interest among those eluted with salt gradient was then processed to be loaded onto a RP-C4 column (Vydac, 214TP54) equilibrated in water with 0.1% TFA. The proteins were eluted with a linear gradient of 0–50% acetonitrile in 0.1% TFA for 30 min and then with 50–80% acetonitrile for 5 min. Eluted proteins were then analyzed by SDS–PAGE and immunoblotting, to confirm the allergenicity of the purified 18 000 component. Further studies showed high homology of this protein with the major allergen of birch pollen, Bet v 1, thus giving a confirmation on molecular basis to the clinical cross-reactivity between birch pollen and apple [75].

Among the major IgE-binding proteins identified in wheat flour, an uncharacterized protein band of approximately 35 000 has been frequently reported [76]; to characterize this allergen, a purification protocol was defined by Sanchez-Monge et al. in 1997 [74]. It involved a first passage of the wheat flour salt extract on Sephadex G-100 to fractionate it by gel filtration, using 0.1 *M* ammonium acetate, pH 6.8, as elution buffer. Fractions showing the highest level of allergen by immunodetection were subjected to reversed-phase chromatography on a preparative Vydac-C4 column, eluting with a three-step linear gradient of acetonitrile in 0.1% TFA: 10–20% in 30 min, 20–50% in 160 min, 50–85% in 60 min. The peak corresponding to the allergen was then identified as a glycosylated protein of 36 600 with amino-acid sequence corresponding to a seed specific peroxidase.

Other wheat allergens recently isolated by reversed-phase chromatography belong to the gliadin family [23] and were extracted from seeds with acetonitrile and TFA incubation. Acetonitrile-soluble wheat proteins were purified on a Resource RPC (Pharmacia) column with a linear gradient of acetonitrile in 0.1% TFA giving the purification of the 65 000 γ-like gliadin. Reversed phase chromatography on ProRPC HR 5/10 of a previously gel filtrated water soluble fraction gave instead a purified 40 000 protein whose sequence proved to be identical to α-gliadin.

To the characterized allergens of cereal family belongs even the major allergen of barley, Hor v 1, which was purified by reversed-phase chromatography on a Vydac C4 column of the 50% ammonium saturated extract eluted with a linear gradient of acetonitrile in 0.1% TFA [68]. The purified IgE binding 15 000 protein was then sequenced and identified as a member of the α-amylase/trypsin inhibitor. Its monomeric glycosylated form was a second time isolated with a two-step procedure including crude extract fractionation on Sephadex G-100 and subsequent preparative reversed-phase HPLC on an Ultrapore 300-5 C3 column [69].

Another important molecule isolated using reversed-phase chromatography was a major allergen of cherry, named Pru a 2 [3] and identified on the basis of N-terminal sequence and corresponding cDNA nucleotide sequence as a thaumatin-like protein belonging to the group 5 of pathogenesis-related

proteins [77]. Cherry protein extract was purified by reversed-phase HPLC employing a linear gradient of 2-propanol in 60 min at room temperature on a C_8 Hypersyl WP300 column. Mobile phase A was 0.1% TFA in water, and gradient was developed from 0 to 80% mobile phase B, containing 90% 2-propanol and 0.1% TFA. The purified cherry protein fraction with apparent molecular mass of 30 000 eluted in two distinct peaks from the Hypersyl column. The two fractions were tested in IgE immunoblots, but only one of them showed IgE reactivity with sera from cherry-allergic patients. Both fractions were then subjected to amino-acid sequencing, and the analyzed 22 N-terminal amino-acid residues revealed 100% identity to the mature thaumatin-like protein from cherry. The mass analysis carried out on both samples gave a value of 23 350, according to the calculated value of 23 330 for the thaumatin-like protein in cherry. These results led to the conclusion that one of the purified fractions contained an IgE non-reactive form of the molecule, probably due to structural characteristics, such as incorrect three-dimensional folding.

Peach and apple LTPs were also purified by reversed-phase chromatography, besides the cation-exchange chromatography method assessed by Pastorello and co-workers [16–18], as reported by Sanchez-Monge et al. [55] in which defatted fruit extracts were fractionated on a preparative Vydac-C4 column.

4.4. Hydrophobic interaction chromatography

Hydrophobic interaction chromatography (HIC) is an alternative way of exploiting the hydrophobic properties of proteins, working in a more polar and less denaturing environment compared with RPC. The polarity of the complete system of HIC is increased by adding salt to the mobile phase, which promotes ligand–protein interactions [78–80]. Most of the bound proteins are then effectively desorbed by simply washing the HIC adsorbent with water or dilute buffer solutions at near neutral pH. Used in the first step of a purification protocol HIC can serve as an effective means of concentrating a dilute sample. Other typical points in a purification scheme where HIC fits in naturally are after an ammonium sulphate precipitation and after an ion-exchange step where the sample is eluted with a rather high ionic strength.

From this short paragraph it is easy to see the effectiveness of this chromatographic technique in protein separation; unfortunately, to our knowledge up to now no protocols for allergen isolation include HIC, so that only general indications can be given on this subject.

4.5. Affinity chromatography

Affinity chromatography can be considered the ideal type of chromatographic technique because of its absolute specificity for the protein to be isolated as the matrix is coupled with a chemical compound closely interacting with the peptide of interest on the basis of biochemical affinity. Ligands can thus be substrates or their analogues, activators or reversible inhibitors of the enzymatic activity of the proteins, allosteric effectors, molecules with chemical affinity due to hydrophobic interactions with protein surface or whole receptor proteins which in vivo closely interact with target molecules. Affinity chromatography requires detailed knowledge of structure and specific characteristics of the molecule to be purified, in order to carefully set up the separation conditions which ensure the highest yield of purified product. It is thus a powerful technique, but not easy to use until the allergenic molecule of interest is fully identified and studied, unless an isolation protocol set up for different purposes can be exploited for allergen separation.

The most famous allergen purified by affinity chromatography is surely profilin, a panallergen isolated from several different sources by chromatography on poly(L-proline) column. Identification of profilin as a novel pollen allergen was made in 1991 by Valenta et al. [81] by cloning and sequencing cDNAs encoding IgE-binding birch pollen proteins distinct from Bet v 1. One of these allergens was identified as birch profilin by sequence homology and affinity to poly-L-proline. This characteristic was exploited for profilin purification since 1988 [82] with setting up of chromatography on poly-L-proline linked to activated Sepharose 4B and equilibrated with 0.1 *M* KCl, 0.1 *M* glycine, 10 m*M* Tris–HCl, pH 7.8, plus 0.5 m*M* dithiothreitol. Profilin and profilin-actin complexes together with small amounts

of contaminating proteins were eluted with the same buffer containing 30% dimethylsulphoxide but without dithiothreitol. Profilin was then identified as a common allergen in different sources, such as celery [83], peach and apple [84], latex [85] and vegetable foods in general [86,87], and was purified with several little variations of the reported protocol.

5. Immunochemical techniques

5.1. Immunoprecipitation

Most of immunochemical techniques are based upon the binding of the antigen to its specific antibody to form the antigen–antibody complex. This interaction can be used not only for analytical purposes, but also as a preparative technique to isolate a specific antigen from an heterogeneous mixture, due to the possibility of removing the precipitated antigen–antibody complex from the solution by centrifugation. The immunoprecipitation technique has the advantage of requiring a lower amount of specific serum with respect to immunoaffinity chromatography, and moreover it often gives higher recovery of the molecule of interest [88]; it was not however reported as a means for allergen purification.

5.2. Immunoaffinity chromatography

The ability to generate antibodies to a large array of chemically distinct target molecules, coupled with the technology of monoclonal antibody production, has made immunoaffinity purification a powerful and routine laboratory technique [89,90].

The antigenic and allergenic structure of Ara h 1, a major allergen of peanut, was investigated with the use of seven monoclonal antibodies obtained from BALB/c mice immunized with purified Ara h 1 [91]. Monoclonal antibody 8D9 was then used to prepare an immunoaffinity specific column to purify the allergenic protein, by coupling the Mab to activated Sepharose. The affinity column was equilibrated with 20 m*M* NaH_2PO_4, 1 *M* NaCl and loaded with crude peanut extract. Ara h 1 was eluted with 100 m*M* triethylamine, pH 11.5, into test tubes containing 0.1 ml of 1 *M* NaH_2PO_4 buffer to neutralize the eluate. Ara h 1 eluted in this way from multiple runs was pooled, dialysed against 100 m*M* ammonium bicarbonate buffer, lyophilized and subsequently analyzed for binding capacity to human IgE from patients with peanut hypersensitivity.

Another application of immunoaffinity interaction was set up by Eigenmann et al. [92] for the identification of unique peanut and soy allergens by adsorption of cross-reacting sera on affinity columns coated with either peanut or soy extracts, in order to remove cross-reacting antibodies and to have an IgE response specific towards a unique food. These sera were then used for the study of specific soy and peanut allergens, revealing IgE binding specific for soy 46 000 and 21 000 proteins, whose clinical role was still to be elucidated, and six unique allergenic fractions of peanut at 13 000, 17 000, 25 000, 34 000, 55 000 and 63 500, which included the major allergens Ara h 1(63 500) and Ara h 2 (17 000) and other fractions still to be studied.

6. Conclusions and perspectives

As we extensively reported in this review, much work has been done regarding protocols for allergen purification from natural sources. Nonetheless, there is still a lot to be achieved before newly characterized molecules for further in-depth studies are obtained. However, for the preparation of large amounts of allergenic proteins to set up reliable diagnostics and therapeutics the most suitable method is based upon molecular biology techniques. cDNA clones have been isolated for a number of allergens in recent years, not only for inhalant allergens (the most famous are Bet v 1 and Bet v 2 [93]) but also for food allergens. The relatively easy production of these molecules has given a new impulse to allergological studies, which now are often directed towards the elucidation of molecular mechanisms involved in hypersensitivity reactions [94]. To this end, particular care was recently taken in the study of peanut allergens Ara h 1 [95], Ara h 2 [96] and Ara h 3 [97], which were cloned and subjected to site-directed mutagenesis to modify the IgE-binding epitopes; these molecular modifications led, in most cases, to a significant reduction or loss of IgE binding, opening new possibilities to obtain

improved diagnostic and therapeutic agents for the treatment of peanut allergy. The literature has reported many cases of other cloned food allergens which have been subjected to molecular characterization, such as, for example, the major allergens of celery (Api g 1) [98], of cherry (Pru a 1 [99] and Pru a 2 [3]), of apple (Mal d 1) [75], of mustard (Sin a 1) [100] and of English walnut (Jug r 1) [101]. The basis of this approach is the precise knowledge of physical and biochemical characteristics of natural allergenic molecules, and this can only be done with allergens purified from natural sources, i.e. foods. With regard to this particular feature, it should be noted that for the expression in *E. coli* of Sin a 1 [100] the authors gave detailed information about the remarkable difficulties of expressing a soluble, immunologically active recombinant allergen, since natural Sin a 1 is a cysteine-enriched protein composed of two polypeptides linked together by disulfide bridges, and these biochemical properties cause a lot of problems in molecular cloning. The expression of the recombinant allergen was successfully achieved thanks to some devices based on the knowledge of the natural molecule, and this strongly confirms the necessity of basic physical and biochemical studies on natural allergens prior to shifting to recombinant DNA technologies and first of all the confirmation of its allergenic activity in vivo.

All the cloned allergens, once expressed in the heterologous host chosen for the production and purified from the culture lysates, were carefully evaluated for their reactivity with IgE antibodies present in sera from intolerant patients to check that the whole expression process had been successfully completed. This required the availability of the natural allergen to have a definite term for comparison, and that is the other basic reason for natural allergen purification. Researchers need to compare biochemical and immunological characteristics of recombinant molecules with those of native ones, to verify that molecular expression in heterologous systems does not significantly modify the IgE response pattern, which would make recombinant proteins useless for diagnostic purposes. The loss of allergenicity was in fact reported for Pru a 2 molecule, probably due to incorrect folding of the polypeptide chain [3], and even though it seems an uncommon case, the occurrence of such an event in forthcoming studies cannot be excluded.

These are only few examples concerning the use and the problems proposed by allergens obtained by recombinant methodology, which however seems undoubtedly the future of the allergologic scientific development. Difficulties with cloned allergens are summarized and discussed in the review about recombinant food allergens written by Lorenz et al. and reported in this issue [102].

7. Nomenclature

SPT	skin prick test
RAST	radioallergosorbent test
CIE	crossed immunoelectrophoresis
CRIE	crossed radioimmunoelectrophoresis
SDS–PAGE	sodium dodecylsulphate–polyacrylamide gel electrophoresis
p*I*	isoelectric point

References

[1] H.A. Sampson, R. Albergo, J. Allergy Clin. Immunol. 74 (1984) 26.
[2] C. Ortolani, M. Ispano, E.A. Pastorello, R. Ansaloni, A. Magri, J. Allergy Clin. Immunol. 83 (1989) 683.
[3] C. Inschlag, K. Hoffmann-Sommergruber, G. O'Riordain, H. Ahorn, C. Ebner, O. Scheiner, H. Breiteneder, Int. Arch. Allergy Immunol. 116 (1998) 22.
[4] M.D. Spangfort, H. Ipsen, S.H. Sparholt, S. Aasmul-Olsen, P. Osmark, F.M. Poulsen et al., Adv. Exp. Med. Biol. 409 (1996) 251.
[5] M. Breitenbach, F. Ferreira, A. Jilek, I. Swoboda, C. Ebner, K. Hoffmann-Sommergruber et al., Adv. Exp. Med. Biol. 409 (1996) 117.
[6] T. Langeland, Allergy 37 (1982) 521.
[7] J. Bernhisel-Broadbent, H.M. Dintzis, R.Z. Dintzis, H.A. Sampson, J. Allergy Clin. Immunol. 93 (1994) 1047.
[8] J. Bernhisel-Broadbent, S.M. Scanlon, H.A. Sampson, J. Allergy Clin. Immunol. 89 (1992) 730.
[9] J.F. Crespo, C. Pascual, R. Helm, S. Sanchez-Pastor, I. Ojeda, L. Romualdo, M. martin-Esteban, J.A. Ojeda, Allergy 50 (1995) 918.
[10] A.M. Witteman, J.A. Akkerdaas, J. van Leeuwen, J.S. van der Zee, R.C. Aalbersee, Int. Arch. Allergy Appl. Immunol. 105 (1994) 56.
[11] E. Bleumink, E. Young, Int. Arch. Allergy 34 (1968) 521.
[12] F. Björksten, L. Halmepuro, M. Hannuksela, A. Lahti, Allergy 35 (1980) 671.
[13] W.D. Loomis, Methods Enzymol. 31 (1974) 528.
[14] S. Vieths, B. Schoning, A. Petersen, Int. Arch. Allergy Immunol. 104 (1994) 399.

[15] A. Martinez, M. Fernandez-Rivas, J. Martinez, R. Palacios, Allergy 52 (1997) 155.
[16] E.A. Pastorello, L. Farioli, V. Pravettoni, C. Ortolani, M. Ispano, M. Monza, C. Baroglio, E. Scibola, R. Ansaloni, C. Incorvaia, A. Conti, J. Allergy Clin. Immunol. 103 (1999) 520.
[17] E.A. Pastorello, F. Purello D'Ambrosio, V. Pravettoni, L. Farioli, G. Giuffrida, M. Monza, R. Ansaloni, D. Fortunato, E. Scibola, F. Rivolta, C. Incorvaia, A. Bengtsson, A. Conti, C. Ortolani, J. Allergy Clin. Immunol. 105 (2000) 371.
[18] E.A. Pastorello, V. Pravettoni, L. Farioli, M. Ispano, D. Fortunato, M. Monza, M.G. Giuffrida, F. Rivolta, E. Scibola, R. Ansaloni, C. Incorvaia, A. Conti, C. Ortolani, J. Allergy Clin. Immunol. 104 (1999) 1099.
[19] P.R. Shewry, J.A. Napier, A.S. Tatham, Plant Cell 7 (1995) 945.
[20] C.P. Sandiford, R.D. Tee, A.J. Newman Taylor, Clin. Exp. Allergy 24 (1994) 549.
[21] Publication No. 84 T.B. Osborne, The Proteins of Wheat Cereal, Carnegie Institute of Washington, 1907.
[22] P.R. Shewry, Biol. Rev. 70 (1995) 375.
[23] K. Palosuo, H. Alenius, E. Varjonen, M. Koivuluhta, J. Mikkola, H. Keskinen, N. Kalkkinen, T. Reunala, J. Allergy Clin. Immunol. 103 (1999) 912.
[24] A. Desormeaux, J.E. Blochet, M. Pezolet, D. Marion, Biochim. Biophys. Acta 1121 (1992) 137.
[25] S.M. Sun, F.W. Leung, J.C. Tomic, J. Agric. Food Chem. 35 (1987) 232.
[26] J.W. Yunginger, R.T. Jones, in: Proceedings of the Fourth International Paul Ehrlich Seminar on the Regulatory Control and Standardization of Allergenic Extracts, Gustav Fischer Verlag, Stuttgart, 1987, p. 251.
[27] E.A. Pastorello, L. Farioli, V. Pravettoni, M. Ispano, A. Conti, R. Ansaloni, F. Rotondo, C. Incorvaia, A. Bengtsson, F. Rivolta, C. Trambaioli, M. Previdi, C. Ortolani, J. Allergy Clin. Immunol. 102 (1998) 1021.
[28] A.W. Burks, L.W. Williams, R.M. Helm, C. Connaughton, G. Cockrell, T. O'Brien, J. Allergy Clin. Immunol. 88 (1991) 172.
[29] A.W. Burks, L.W. Williams, C. Connaughton, G. Cockrell, T. O'Brien, R.M. Helm, J. Allergy Clin. Immunol. 90 (1992) 962.
[30] S.T. Holgate, M.K. Church, Allergy, Gower Medical Publishing, London, 1993, 1.6.
[31] S.B. Lehrer, M.L. McCants, J.E. Salvaggio, Int. Arch. Allergy Appl. Immunol. 77 (1985) 192.
[32] S.B. Lehrer, Ann. Allergy 57 (1986) 267.
[33] S. Vieths, K. Janek, H. Aulepp, A. Petersen, Allergy 50 (1995) 421.
[34] A. Görg, W. Postel, S. Gunther, Electrophoresis 9 (1988) 531.
[35] A. Posch, W. Weiss, C. Wheeler, M.J. Dunn, A. Görg, Electrophoresis 16 (1995) 1115.
[36] J. Bonnerjera, S. Oh, M. Hoare, P. Dunhill, Bio/Technology 4 (1986) 954.
[37] S.R. Himmelhoch, Methods Enzymol. 22 (1971) 273.
[38] K. Aas, J.W. Jebsen, Int. Arch. Allergy Appl. Immunol. 32 (1967) 1.
[39] S. Elsayed, K. Aas, Int. Arch. Allergy Appl. Immunol. 40 (1971) 428.
[40] D. Barnett, B.A. Baldo, M.E.H. Howden, J. Allergy Clin. Immunol. 72 (1983) 61.
[41] S. Meier-Davis, S.L. Taylor, J. Nordlee, R. Bush, J. Allergy Clin. Immunol. 79 (1987) 218.
[42] D. Barnett, M.E.H. Howden, B. Bonham, R.W. Burley, Proc. Sydney Allergy Group 4 (1985) 104.
[43] S.L. Taylor, J.A. Nordlee, J.W. Yunginger, R.T. Jones, M.I. Sach, R.K. Bush, J. Allergy Clin. Immunol. 69 (1982) 128.
[44] D.S. Shin, C.M. Compadre, S.J. Maleki, R.A. Kopper, H. Sampson, S.K. Huang, A.W. Burks, G.A. Bannon, J. Biol. Chem. 273 (1998) 13753.
[45] A.W. Burks, G. Cockrell, C. Connaughton, A. Karpas, R.M. Helm, J. Allergy Clin. Immunol. 95 (1995) 607.
[46] J. Anet, J.F. Back, R.S. Baker, D. Barnett, R.W. Burley, M.E.H. Howden, Int. Arch. Allergy Appl. Immunol. 77 (1985) 364.
[47] D.R. Hoffman, J. Allergy Clin. Immunol. 71 (1983) 481.
[48] T. Langeland, O. Harbitz, Allergy 38 (1983) 131.
[49] K. Ebbehøj, A.M. Dahl, H. Fræhør, A. Nørgaard, L.K. Poulsen, V. Barkholt, Allergy 50 (1995) 133.
[50] A. Urisu, K. Yamada, S. Masuda, H. Komada, E. Wada, Y. Kondo, F. Horiba, M. Tsuruta, T. Yasaki, M. Yamada, S. Torii, R. Nakamura, Int. Arch. Allergy Appl. Immunol. 96 (1991) 244.
[51] R. Nakamura, T. Matsuda, Biosci. Biotech. Biochem. 60 (1996) 1215.
[52] E.A. Pastorello, V. Pravettoni, M. Ispano, L. Farioli, R. Ansaloni, F. Rotondo, C. Incorvaia, I. Asman, A. Bengtsson, C. Ortolani, J. Allergy Clin. Immunol. 98 (1996) 601.
[53] E.A. Pastorello, A. Conti, V. Pravettoni, L. Farioli, F. Rivolta, R. Ansaloni, M. Ispano, C. Incorvaia, M.G. Giuffrida, C. Ortolani, J. Allergy Clin. Immunol. 101 (1998) 531.
[54] B. Fahlbusch, O. Rudeschko, C. Schumann, F. Steurich, M. Henzgen, G. Schlenvoigt, L. Jager, J. Invest. Allergol. Clin. Immunol. 8 (1998) 325.
[55] R. Sanchez-Monge, M. Lombardero, F.J. Garcia-Selles, D. Barber, G. Salcedo, J. Allergy Clin. Immunol. 103 (1999) 514.
[56] E.A. Pastorello, C. Ortolani, L. Farioli, V. Pravettoni, M. Ispano, A. Borga, A. Bengtsson, C. Incorvaia, C. Berti, C. Zanussi, J. Allergy Clin. Immunol. 94 (1994) 699.
[57] S. Nagpal, L. Rajappa, D.D. Metcalfe, P.V. Subba Rao, J. Allergy Clin. Immunol. 83 (1989) 26.
[58] K.N. Shanti, B.M. Martin, S. Nagpal, D.D. Metcalfe, P.V. Subba Rao, J. Immunol. 151 (1993) 5354.
[59] C.B. Daul, M. Slattery, G. Reese, S.B. Lehrer, Int. Arch. Allergy Immunol. 105 (1994) 49.
[60] P.S. Leung, K.H. Chu, W.K. Chow, A. Ansari, C.I. Bandea, H.S. Kwan, S.M. Nagy, M.E. Gershwin, J. Allergy Clin. Immunol. 94 (1994) 882.
[61] P. Andrews, Biochem. J. 96 (1965) 595.
[62] T.C. Laurent, J. Killander, J. Chromatogr. 14 (1964) 317.
[63] L. Menendez-Arias, I. Moneo, J. Dominguez, R. Rodriguez, Eur. J. Biochem. 177 (1988) 159.
[64] M.A. Gonzalez de la Pena, L. Menendez-Arias, R.I. Monsalve, R. Rodriguez, Int. Arch. Allergy Appl. Immunol. 96 (1991) 263.

[65] J. Fränken, U. Stephan, H.E. Meyer, W. König, Int. Arch. Allergy Immunol. 104 (1994) 171.
[66] J.M. James, J.P. Sixbey, R.M. Helm, G.A. Bannon, A.W. Burks, J. Allergy Clin. Immunol. 99 (1997) 239.
[67] A. Armentia, R. Sanchez-Monge, L. Gomez, D. Barber, G. Salcedo, Clin. Exp. Allergy 23 (1993) 410.
[68] D. Barber, R. Sanchez-Monge, L. Gomez, J. Carpizo, A. Armentia, C. Lopez-Otin, F. Juan, G. Salcedo, FEBS Lett. 248 (1989) 119.
[69] M. Mena, R. Sanchez-Monge, L. Gomez, G. Salcedo, P. Carbonero, Plant Mol. Biol. 20 (1992) 451.
[70] G.H. Docena, R. Fernandez, F.G. Chirdo, C.A. Fossati, Allergy 51 (1996) 412.
[71] M.T.W. Hearn, HPLC of Proteins Peptides and Polynucleotides, VCH, New York, 1991.
[72] C.V. Olson, D.H. Reifsnyder, E. Canonva-Davis, V.T. Ling, S.E. Builder, J. Chromatogr. A 675 (1994) 101.
[73] B. Fahlbusch, O. Rudeschko, W.D. Muller, G. Schlenvoigt, S. Vettermann, L. Jager, Int. Arch. Allergy Immunol. 108 (1995) 119.
[74] R. Sanchez-Monge, G. Garcia-Casado, C. Lopez-Otin, A. Armentia, G. Salcedo, Clin. Exp. Allergy 27 (1997) 1130.
[75] M. Vanek-Krebitz, K. Hoffmann-Sommergruber, M. Laimer da Camara Machado, M. Susani, C. Ebner, D. Kraft, O. Scheiner, H. Breiteneder, Biochem. Biophys. Res. Commun. 214 (1995) 538.
[76] C.P. Sandiford, R.D. Tee, A.J. Newman Taylor, Clin. Exp. Allergy 25 (1995) 340.
[77] E.A. Pastorello, L. Farioli, V. Pravettoni, C. Incorvaia, E. Scibola, M. Ispano, R. Ansaloni, M. Monza, F. Rivolta, C. Ortolani, Giorn. It. Allergol. Immunol. Clin. 9 (1998) 24.
[78] J. Porath, L. Sundberg, N. Fornstedt, I. Olson, Nature 245 (1973) 465.
[79] S. Pahlman, J. Rosengren, S. Hjerten, J. Chromatogr. 131 (1977) 99.
[80] L.O. Narhi, Y. Kita, T. Arakawa, Anal. Biochem. 182 (1989) 266.
[81] R. Valenta, M. Duchene, K. Pettenburger, C. Sillaber, P. Valent, P. Bettelheim, M. Breitenbach, H. Rumpold, D. Kraft, O. Scheiner, Science 253 (1991) 557.
[82] U. Lindberg, C.E. Schutt, E. Hellsten, A.C. Tjader, T. Hult, Biochim. Biophys. Acta 967 (1988) 391.
[83] P. Vallier, C. Dechamp, R. Valenta, O. Vial, P. Deviller, Clin. Exp. Allergy 22 (1992) 774.
[84] R. van Ree, M. Fernandez-Rivas, M. Cuevas, M. van Wijngaarden, R.C. Aalberse, J. Allergy Clin. Immunol. 95 (1995) 726.
[85] P. Vallier, S. Balland, R. Harf, R. Valenta, P. Deviller, Clin. Exp. Allergy 25 (1995) 332.
[86] R. van Ree, V. Voitenko, W.A. van Leeuwen, R.C. Aalberse, Int. Arch. Allergy Immunol. 98 (1992) 97.
[87] C. Ebner, R. Hirschwehr, L. Bauer, H. Breiteneder, R. Valenta, H. Ebner, D. Kraft, O. Scheiner, J. Allergy Clin. Immunol. 95 (1995) 962.
[88] K. Wilson, K.H. Goulding, A Biologist's Guide To Principles and Techniques of Practical Biochemistry, Edward Arnold Publishers, London, 1986.
[89] G. Kohler, C. Milstein, Nature 25 (1975) 495.
[90] M. Wilchek, T. Miron, J. Kohn, Methods Enzymol. 104 (1984) 3.
[91] A.W. Burks, G. Cockrell, C. Connaughton, R.M. Helm, J. Allergy Clin. Immunol. 93 (1994) 743.
[92] P.A. Eigenmann, A.W. Burks, G.A. Bannon, H.A. Sampson, J. Allergy Clin. Immunol. 98 (1996) 969.
[93] R. Valenta, M. Duchene, S. Vrtala, T. Birkner, C. Ebner, R. Hirschwehr, M. Breitenbach, H. Rumpold, O. Scheiner, D. Kraft, J. Allergy Clin. Immunol. 88 (1991) 889.
[94] R. Valenta, S. Vrtala, S. Laffer, S. Spitzauer, D. Kraft, Allergy 53 (1998) 552.
[95] D.S. Shin, C.M. Compadre, S.J. Maleki, R.A. Kopper, H. Sampson, S.K. Huang, A.W. Burks, G.A. Bannon, J. Biol. Chem. 273 (1998) 13753.
[96] J.S. Stanley, N. King, A.W. Burks, S.K. Huang, H. Sampson, G. Cockrell, R.H. Helm, C.M. West, G.A. bannon, Arch. Biochem. Biophys. 342 (1997) 244.
[97] P. Rabjohn, R.M. Helm, J.S. Stanley, C.M. West, H.A. Sampson, A.W. Burks, G.A. Bannon, J. Clin. Invest. 103 (1999) 535.
[98] H. Breiteneder, K. Hoffmann-Sommergruber, G. O'Riordain, M. Susani, H. Ahorn, C. Ebner, D. Kraft, O. Scheiner, Eur. J. Biochem. 233 (1995) 484.
[99] S. Scheurer, K. Metzner, D. Haustein, S. Vieths, Mol. Immunol. 34 (1997) 619.
[100] M.A. Gonzalez de la Pena, R.I. Monsalve, E. Batanero, M. Villalba, R. Rodriguez, Eur. J. Biochem. 237 (1996) 827.
[101] S.S. Teuber, A.M. Dandekar, W.R. Peterson, C.L. Sellers, J. Allergy Clin. Immunol. 101 (1998) 807.
[102] A.R. Lorenz, S. Scheurer, D. Haustein, S. Vieths, J. Chromatogr. B 756 (2001) 255.