Chapter 2

Allergen Extraction and Purification from Natural Products: Main Chromatographic Techniques

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Abstract

The development of techniques and methods for allergen purification is essential for diagnosis and the development of safe immunotherapeutic agents. The most common purification techniques include chromatographic methodologies. In this chapter, we review and describe the details of the methodologies of using ion-exchange, gel-filtration, and affinity chromatography to purify two well-known panallergens, profilin and parvalbumin.

Key words Allergy, Allergen extraction, Allergen purification, Chromatography, Chromatographic techniques, Profilin, Parvalbumin

1 Introduction

Allergy is becoming a serious health problem [1]. The improvement of allergy prevention and therapy depends mainly on the reliability of the diagnosis. The development of techniques and methods for allergen purification is an essential requisite for many of the advancements made in allergy diagnosis. For this reason, the extraction and purification of allergens for diagnostic and therapeutic purposes is an expanding field [2].

Allergen extracts are prepared from different source materials such as food, pollens, dander animal, arthropods, fungi, or dusts. The composition of allergen extracts can vary depending on the source, processing, and storage conditions. Allergen extracts are a heterogeneous mixture of proteins, glycoproteins, carbohydrates, nucleic acids, lipids, and other substances. From this complex sample, the allergens must be purified to be used for diagnostic purposes. Different methods have been described to achieve higher allergen yields and a removal of host contaminants, such as lipids, nucleic acids, polyphenols, and polysaccharides [3]. However, a standard protocol for allergen purification from any source is not available.

Jing Lin and Marcos Alcocer (eds.), *Food Allergens: Methods and Protocols*, Methods in Molecular Biology, vol. 1592,
DOI 10.1007/978-1-4939-6925-8_2,

The most common purification techniques undoubtedly include chromatographic techniques [4]. The main chromatographic techniques utilize the intrinsic properties of allergens to separate from other extract components. The different types of chromatography include [5]:

- **Ion-exchange chromatography**: based on the reversible adsorption of charged solute molecules to immobilized ion-exchange groups of opposite charge. Ion-exchange chromatography includes Anion-exchange chromatography and Cation-exchange chromatography.
- **Gel-filtration chromatography or size-exclusion chromatography**: 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.
- **Hydrophobic Interaction and Reversed-Phase Chromatography**: These techniques separate biomolecules according to differences in their hydrophobicity.
- **Affinity chromatography**: separates proteins on the basis of a reversible interaction between a protein (or group of proteins) and a specific ligand coupled to a chromatography matrix.

In this chapter, we review and describe the methodologies of using ion-exchange, gel-filtration, and affinity chromatography to purify profilin and parvalbumin, two well-known panallergens (proteins ubiquitous in nature, that share highly conserved sequence regions, structure, and function and responsible for many IgE cross-reactions between unrelated allergen sources) [6, 7].

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 MΩ cm at 25 °C). Store all reagents at 4 °C. To prevent clogging, always filter the sample through a 0.45 μm filter prior to loading the columns.

2.1 Chromatography Columns and Buffers

2.1.1 HiPrep Q XL 16/10 (GE Healthcare): Anionic-Exchange Column

1. Binding buffer: 50 mM Tris–HCl, pH 8.8. Weigh 6.05 g Tris-HCl and transfer to a 1 L cylinder. Add 900 mL of water, mix and adjust pH with HCl (6 N). Make up to 1 L with water. Store at 4 °C.
2. Elution buffer: Elution is achieved with 2 M NaCl. To prepare 1 M solution, weigh 116.88 g of NaCl in 1 L of water.

2.1.2 HiPrep DEAE FF 16/10 (GE Healthcare): Anionic-Exchange Column

1. Binding buffer: 50 mM Tris–HCl, pH 7.4. Weigh 6.05 g Tris and transfer to a 1 L cylinder. Add 900 mL of water, mix and adjust pH with HCl (6 N). Make up to 1 L with water. Store at 4 °C.

2. Elution buffer: Elution is achieved with a linear gradient from 0 to 1 M NaCl. To prepare 1 M solution, weigh 58.44 g of NaCl in 1 L of water.

2.1.3 Superdex 75 10/300 (GE Healthcare): Size-Exclusion Column

1. Equilibration Buffer: Buffered-phosphate saline (PBS), pH 7.9. Weigh 2.39 g NaH_2PO_4 (20 mM), 2.84 g NaH_2PO_4 (20 mM) and 8.76 g NaCl (150 mM) and transfer to a 1 L cylinder, add 900 mL of water, mix and adjust to a pH required with a 6 N HCl solution. Make up to 1 L with water. Store at 4 °C.

2.1.4 Mono-Q 5/50 GL (GE Healthcare): Anionic-Exchange Column

For Profilin Purification

1. Binding buffer: 20 mM Tris–HCl, pH 7.9. Weigh 2.42 g Tris and transfer to a 1 L cylinder. Add 900 mL of water, mix and adjust pH with HCl (6 N). Make up to 1 L with water. Store at 4 °C.
2. Elution buffer: Elution is achieved with a linear gradient from 0 to 0.5 M NaCl. To prepare 0.5 M solution NaCl, weigh 29.22 g of NaCl in 1 L of water.

For Parvalbumin Purification

3. Binding buffer: 10 mM Tris–HCl, pH 7.5. Tris 10 mM, pH 7.5. Weigh 1.21 g Tris and transfer to a 1 L cylinder. Add 900 mL of water, mix and adjust pH with HCl (6 N). Make up to 1 L with water. Store at 4 °C.
4. Elusion buffer: Elution is achieved with a linear gradient from 0 to 1 M NaCl. To prepare 1 M solution, weigh 58.44 g of NaCl in 1 L of water.

2.1.5 Sepharose Poly-Proline: Affinity Chromatography Column

1. Binding buffer (Buffer A): 100 mM KCl, 100 mM Gly, 10 mM Tris–HCl, 0.5 mM dithiothreitol (DTT); pH 7.8. Weigh 7.45 g KCl, 7.5 g Gly, 1.21 g Tris, and 77 mg 0.5 DTT. Transfer to a 1 L cylinder, add 900 mL of water, and adjust pH with HCl (6 N). Make up to 1 L with water. Store at 4 °C.
2. Elution buffer (Buffer B): 100 mM KCl, 100 mM Gly, 10 mM Tris–HCl, 0.5 mM dithiothreitol (DTT), 3 M urea; pH 7.8. Weigh 7.45 g KCl, 7.5 g Gly, 1.21 g Tris, 77 mg 0.5 DTT, and 180.18 g urea. Transfer to a 1 L cylinder, add 900 mL of water, and adjust pH with HCl (6 N). Make up to 1 L with water. Store at 4 °C.
3. Elution buffer (Buffer C): 100 mM KCl, 100 mM Gly, 10 mM Tris–HCl, 0.5 mM dithiothreitol (DTT), 8 M urea; pH 7.8. Weigh 7.45 g KCl, 7.5 g Gly, 1.21 g Tris, 77 mg 0.5 DTT, and 480.48 g urea. Transfer to a 1 L cylinder, add 900 mL of water, and adjust pH with HCl (6 N). Make up to 1 L with water. Store at 4 °C.

2.2 Other Buffers and Components

1. 0.1 M ammonium bicarbonate buffer. Weigh 0.79 g ammonium bicarbonate and transfer to a 1 L cylinder, add make up to 1 L with water. Rinse the dialysis membrane (Spectra/Por 6 RC Dialysis Membranes, 32 mm wide, Spectrum Chemical Corp) into deionized water for 10 min. Store at 4 °C (*see* **Note 1**).
2. Affinity chromatography activation buffer: 0.1 M $NaHCO_3$, pH 8.3 buffer with 0.5 M NaCl. Weigh 0.84 g $NaHCO_3$, and transfer to a 1 L cylinder, add 900 mL of water and adjust pH with HCl (6 N). Make up to 1 L with water. Store at 4 °C. Affinity chromatography blocking buffer: 1 M Tris–HCl, pH 8. Weigh 121.14 g Tris, and transfer to a 1 L cylinder, add 900 mL of water and adjust pH with HCl (6 N). Make up to 1 L with water. Store at 4 °C.
3. Amicon stirred cell system (Millipore) (*see* **Note 2**).

2.3 Components for Watermelon Extract Preparation

1. Extraction buffer: 10 mM K_2HPO_4 pH 7, 2% (p/v) PVPP (Polyvinyl poly-pyrrolidone) and 2 mM EDTA (Ethylene diamine tetra acetic acid) buffer. Weigh 1.74 g K_2HPO_4 and transfer to a 1 L cylinder, add 500 mL of water weigh 20 g PVPP and 5.84 g EDTA and adjust pH with HCl (6 N). Make up to 1 L with water (*see* **Note 3**).
2. Cellulose acetate 0.22 μm pore filter (Corning, New York).

2.4 Components for Fish Extract Preparation

1. Extraction buffer: Phosphate-buffered saline (PBS), pH 7.2 with 1 mM PMSF (phenylmethylsulfonyl fluoride). To prepare 100 mM of PMSF weigh 17.4 mg of PMSF per milliliter of isopropanol and store at −20 °C. Add 10% volume of 100 mM PMSF to PBS pH 7.2 buffer.
2. Delipidation buffer: Diethyl ether 98% (Sigma) (*see* **Note 4**).

3 Methods

3.1 Production of the Watermelon Extracts

1. Homogenize watermelon pulp in extraction buffer 1/20 (w/v).
2. Extract proteins overnight (12 h), at 4 °C under magnetic stirring.
3. Centrifuge at 15,000 × *g* at 4 °C for 30 min.
4. Discard the pellet, consisting of non-soluble material, and clarify supernatant by filtration through a cellulose acetate 0.22 μm pore filter.

3.2 Pre-purification

In order to enrich the protein content in the extract, carbohydrates are removed using an anionic-exchange chromatography column HiPrep Q XL 16/10 (GE Healthcare):

1. Dialyze the watermelon extract against Tris–HCl 50 mM, pH 8.8 buffer.
2. Load the column with 200 mL of watermelon extract.
3. Wash the column with three volumes of binding buffer.
4. Elute with 2 M NaCl in binding buffer in ten column volumes (200 mL).
5. Restore column conditions following the instructions provided by the manufacturer.
6. Concentrate the eluted fraction using the Amicon system (Millipore) and cellulose membranes.
7. Dialyze against 0.1 M ammonium bicarbonate, adjust protein concentration to 1 mg/mL and freeze dry for storage.

3.3 Isolation of Native Profilin

We present two chromatographic methods to isolate watermelon profilin as described before [8].

After each chromatographic step, eluted fractions should be dialyzed against 0.1 M ammonium bicarbonate and freeze dried for storage if necessary.

3.3.1 Conventional Chromatography-Based Method

This method is based on conventional chromatography using a FPLC AKTA Purifier (GE Healthcare).

HiPrep DEAE FF 16/10

1. Dialyze the watermelon extract, previously prepurified by the HiPrep Q XL 16/10 (GE Healthcare), against 50 mM Tris–HCl 50 mM, pH 7 (binding buffer).
2. Load 25 mL of watermelon extract in the chromatography column.
3. Wash the column with three column volumes of binding buffer (60 mL).
4. Elute with a linear gradient from 0 to 1 M NaCl in binding buffer in 15 column volumes (300 mL). Fraction with profilin, together with other proteins, should elute in the range of 40–170 mM NaCl.
5. Dialyze the fraction (or fractions) of interest against ammonium bicarbonate and freeze dry.
6. Restore column conditions following the instructions provided by the manufacturer.

Superdex 75 10/300

1. Resuspend the sample in 100 μL of PBS and filter through a 0.22 μm filter.
2. Inject the sample onto the Superdex 75 10/300 chromatography column.

3. Wash with PBS and collect the fraction containing the profilin. This should appear approximately after 12–14 mL.
4. Dialyze the fractions of interest against 0.1 M ammonium bicarbonate.
5. Restore column conditions following the instructions provided by the manufacturer.

Mono-Q 5/50 GL

1. Dialyze the fractions collected in the previous step against 20 mM Tris–HCl pH 7.9.
2. Inject the sample onto the Mono-Q 5/50 GL chromatography column (*see* **Note 5**).
3. Wash the column with five volumes of binding buffer (5 mL) at a flow rate of 2 mL/min.
4. Elute with a linear gradient from 0 to 0.5 M NaCl in binding buffer in 15 column volumes (15 mL). Fraction containing purified profilin should be eluted approximately at 60 mM NaCl.
5. Dialyze against 0.1 M ammonium bicarbonate and freeze dry.
6. Restore column conditions following the instructions provided by the manufacturer.

3.3.2 Affinity Chromatography-Based Method

This method is based on affinity chromatography using a non-commercial Poly-L-Proline coupled to a Sepharose 4B (GE Healthcare) column.

Column Preparation

Use a glass column of 1 cm diameter and 25 cm height (column volume: 19.7 mL) to package the slurry (*see* **Note 6**).

1. Weigh 6 g Sepharose 4B (GE Healthcare).
2. To activate the Sepharose 4B (GE Healthcare), wash with 1 mM HCl (1/200 w/v) using a glass funnel fitted with Whatman filter.
3. Add 0.5% Poly-proline ligand in activation buffer: weigh 100 mg Poly-proline and resuspend in 20 mL of activation buffer.
4. Incubate the activated Sepharose 4B with the Poly-proline in activation buffer overnight at 4 °C under magnetic stirring conditions.
5. Centrifuge 5 min at 14,000 × *g* and discard supernatant containing the excess of Poly-Proline.
6. Wash with three column volumes (60 mL) of blocking buffer to avoid unspecific binding to the reactive groups, 2 h at 4 °C under magnetic stirring conditions.
7. Centrifuge 1 min at 14,000 × *g* and discard supernatant.

8. Perform three consecutive washes of 30 min each with five column volumes of alternative pH buffer, using 0.1 M acetic acid pH 4 and the binding buffer (*see* **Note 7**).
9. Mount the packaging reservoir with end piece and rinse with water.
10. Mount the column in the packaging reservoir vertically and pour the slurry taking care of no air bubbles are trapped and close with a top closing piece connected to a peristaltic pump.
11. After slurry packaging, load 500 μL of human serum, wash with five column volumes of buffer A (100 mL) and elute with buffer B, followed by buffer C (five column volumes each).
12. Equilibrate column in buffer A by washing with five column volumes (100 mL) of buffer A.

Profilin Purification

1. Dialyze the watermelon extract against buffer A.
2. Load 25 mL of the extract into the column.
3. Wash with three column volumes of buffer A (60 mL) to wash off the excess of proteins that do not bind to the poly-proline residues.
4. Elute with three column volumes (60 mL) of buffer B to elute the actin-profilin complexes.
5. Elute with three column volumes (60 mL) of buffer C to elute purified profilins.
6. Dialyze the fractions of interest against 0.1 M ammonium bicarbonate.

3.4 Preparation of Fish Extracts

1. 30 g of cooked muscle filets (100 °C for 30 min) is extracted in 10% (w/v) of PBS, pH 7.2, with 1mM PMSF at 4 °C undergoing constant stirring for 24 h (*see* **Note 3**).
2. After centrifugation at 12,000 × *g* for 30 min at 4 °C, lipids from supernatants are extracted using diethyl ether 98% (delipidation buffer) (*see* **Note 4**).
3. The delipidated extract is dialyzed against 0.1 M ammonium bicarbonate.
4. Protein extracts are lyophilized and stored at 4 °C.

3.5 Purification of Parvalbumins

Purification protocol is based on the protocol described by Bugajska-Schretter et al. [9] with some modifications [10].

1. The parvalbumin-enriched fraction was redissolved in Tris 10 mM, pH 7.5.
2. Inject the sample onto the Mono-Q 5/50 GL chromatography column (*see* **Note 5**).
3. Wash the column with five volumes of binding buffer (5 mL) at a flow rate of 2 mL/min.

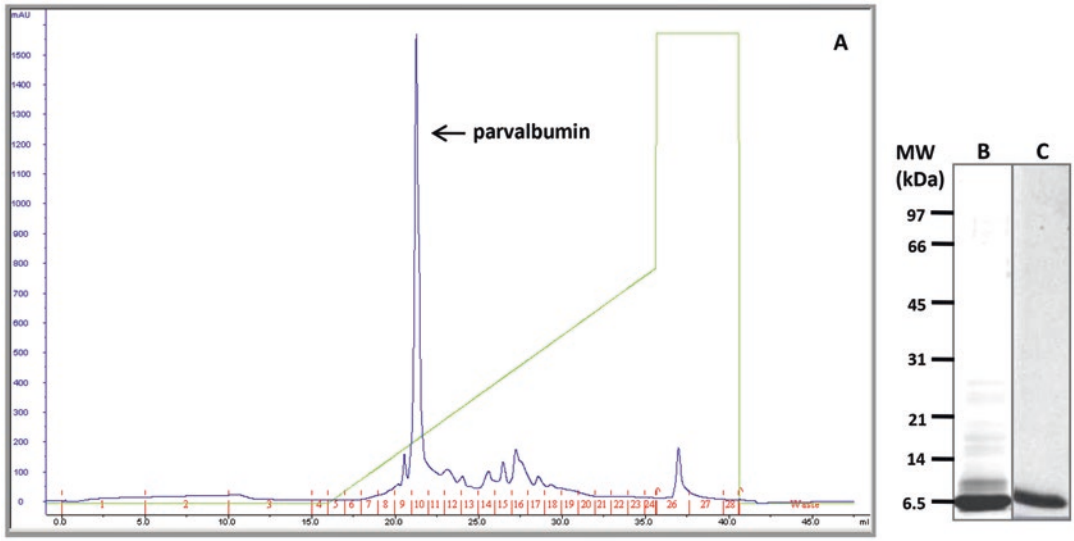

Fig. 1 Parvalbumin purification from whiff complete fish extract. (**a**) MonoQ 5/50 GL chromatogram showing A_{280nm} (*dark blue*) and elution buffer (*green*). (**b**) SDS-PAGE (14%) of eluted fraction. (**c**) Immunodetection obtained by incubation with sera from fish allergic patients

4. Elute with a linear salt gradient from 0 to 1 M NaCl in binding buffer in 15 column volumes (15 mL). Fraction containing purified parvalbumin should be eluted approximately at 140 mM NaCl (*see* Fig. 1).
5. Dialyze against 0.1 M ammonium bicarbonate and freeze dry.
6. Restore column conditions following the instructions provided by the manufacturer.

3.6 Purity and Reactivity Confirmation Tests

To confirm purity, fractions obtained after chromatography will be run in 14% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) as described elsewhere [13] and analyzed by mass spectrometry (MS). To confirm allergenic potential, immunodetection analysis must be performed using allergic serum pool [10].

Fractions containing more than 90% of purified protein can be used for further experiments. An example of these studies performed with purified parvalbumin is given in Fig. 1.

4 Notes

1. Attach one end of the membrane with the tubing closure provided by the kit and add the extract with a pipette. Attach the other end and put the membrane into the cylinder with a magnetic stir bar. Keep it O/N in constant stirring at 4 °C. Change the dialysis buffer twice for three days.

2. This system allows rapid concentration without the need of purification by applying nitrogen gas pressure (not exceeding a pressure limit of 75 psi). The cut-off will determine the solutes to be retained in the cell or be passing through the pore of the membrane. In this case a 3 kDa cellulose membrane was selected, to eliminate salts and water. Fill the cell with the extract to concentrate, after pressurization, put the system in a magnetic stirring table and concentrate up to the desirable volume.
3. The use of the water-insoluble PVPP is for the removal of phenolic compounds present in vegetable tissues that could interact with proteins [10, 12]. EDTA is a chelating agent frequently used as a protease inhibitor in protein extraction buffers [11].
4. Delipidation technique consists of forming a biphasic system by adding diethyl ether to the extracted fish extracts. 100 mL of extract is shaken vigorously with 50 mL of diethyl ether. After 5 min the lower layer is removed and mixed with another 50 mL of diethyl ether per each 100 mL. This cycle is repeated two times more.
5. Manufacturer recommends loading up to 45 mg.
6. To determine the volume of a packaging reservoir apply the formula:

$$\text{Volume} = \pi \times \text{reservoir height} \times \text{radius}^2$$

7. To change from one buffer to the other, centrifuge a few second at 15,000 × *g* and discard supernatant. Then add the new buffer.

Acknowledgement

This work was supported by grants from the Instituto de Salud Carlos III (PI 13/00477, PI 13/00928), co-supported by FONDOS FEDER and the Institute of Applied Molecular Medicine of CEU San Pablo University of Madrid.

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http://www.springer.com/978-1-4939-6923-4

Food Allergens
Methods and Protocols
Lin, J.; Alcocer, M. (Eds.)
2017, X, 299 p. 44 illus., 20 illus. in color., Hardcover
ISBN: 978-1-4939-6923-4
A product of Humana Press