# Production of an egg yolk antibody against *Parietaria judaica* 2 allergen

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**ABSTRACT** Specific antibodies are essential tools for studying proteins as well as for diagnostic research in biomedicine. The egg yolk of immunized chicken is an inexpensive source of high-quality polyclonal antibodies. The 12-kDa *Parietaria judaica* 2 allergen was expressed as a fusion protein and was used to immunize Leghorn chickens. In this paper, we show, using 2-dimensional gel electrophoresis and immunoblotting, that chicken antibodies raised against a recombinant allergen can be used to recognize similar proteins from a pollen raw extract. Allergen identity was confirmed by nanoLC-nanospray-tandem mass spectrometry analysis. Our data demonstrate for the first time that a synergistic combination of molecular biology, 2-dimensional PAGE, and use of nonmammalian antibodies represents a powerful tool for reliable identification of allergens.

**Key words:** antibody, allergy, *Parietaria judaica*, pollen

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## INTRODUCTION

A rapid and cost-effective production of antibodies against specific antigens is necessary in several fields of biomedical and veterinary research as well as in various clinical applications for diagnosis and therapy. Antibodies can be used in a vast array of methodological applications, from Western blot to ELISA assays, immunohistochemistry, and cell sorting.

Conventional methods to produce commercially available immunoglobulins, in either polyclonal or monoclonal forms, rely on animals such as rabbits, goats, and rodents. Alternatively, it is possible to use chickens as originally described by Polson et al. (1980). The immunization of chickens for antibody production is an attractive approach for several reasons (Asaoka et al., 1992; Zhang, 2003): chicken housing is inexpensive, egg collection is noninvasive, IgY antibodies are concentrated in egg yolks and isolation is fast and simple, and chicken immunoglobulins do not react with mammalian IgG or IgM and do not activate mammalian complement factors. This last property helps to reduce the false positive due to the interference of complement factors in mammalian serum samples (Vikinge et al., 1998). Furthermore, the amount of antibodies recovered from an egg is equivalent to that from around 200 mL of mammalian blood, thus lowering the cost for micrograms of antibody produced.

Allergic airway disease attributable to inhalant allergens such as pollen has been increasing steadily, with a recent population-based survey in Europe reporting a prevalence of 23%, with values as high as 40% in some countries (D'Amato et al., 2007). Pollen is a major natural cause of allergy induced by inhalation. *Parietaria judaica* belongs to the Urticaceae family, is commonly found in urban and rural areas, and its pollen is one of the main causes of allergy in the Mediterranean area, with a 60 to 80% prevalence in Italy and Greece and 25 to 40% prevalence in Spain and southern France (Masullo et al., 1996).

*Parietaria judaica* pollen allergens have been identified and characterized by immunochemical and molecular-biological techniques (Amoresano et al., 2003; Stumvoll et al., 2003); Parj1 and Parj2 represent 2 major allergens belonging to a family of glycoproteins known as nonspecific lipid transfer proteins (Duro et al., 1997; Salcedo et al., 2004). However, other proteins, specific isoforms, or posttranslational modifications of known allergens can contribute to hypersensitivity reactions in allergic individuals (Westphal et al., 2003). The accurate diagnosis of hypersensitive patients as well as the in-depth knowledge of the mixtures for an effective immunotherapy depend on the molecular characterization of all allergenic components from a biological source. Antibodies are a crucial tool to meet this need. The goal of the research reported herein was to produce and


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characterize polyclonal antibodies to Parj2, one of the main allergens contained in *P. judaica* pollen.

## MATERIALS AND METHODS

### Antigen Preparation

Antigen preparation was performed as described previously (Duro et al., 1996). In brief, the PCR-amplified fragment corresponding to the full length coding region of Parj2 was overexpressed as a fusion protein with the histidine tag (Parj2-His). After purification by a $Ni^{2+}$ affinity column, the Parj2-His was lyophilized, resuspended in water, aliquoted, and stored at −80°C until used.

### Immunization of Hens and Isolation of IgY from Egg Yolks

Approximately 0.2 mg of Parj2 resuspended in water was emulsified with an equal volume of complete Freund's adjuvant and the mixture was injected into the pectoral muscle of hens. Further injections of the protein, emulsified as described above with the incomplete adjuvant, were given, alternating the side of the chicken breast, 14 and 28 d later. From the 30th day, the eggs were collected daily, marked, and stored at 4°C until use. The antibodies were purified using the IgY Eggspress Kit (Gallus Immunotech Inc., Fergus, Canada; Weiler et al., 2007).

### Pollen Collection and Protein Extraction

Pollen from *P. judaica* was purchased from Allergon (Ängelholm, Sweden). The pollen grains were defatted with diethyl ether and extracted by magnetic stirring in PBS for 20 h at 4°C. The extract was clarified by centrifugation at 5,600 × *g* for 30 min at 4°C and the supernatant was dialyzed against ultrapure distilled water, lyophilized, and stored at −80°C until use.

### Western Blot Analysis and Competition Assays

The titer and specificity of the antibodies purified from the eggs were analyzed by SDS-PAGE followed by Western blotting as described previously (Taverna et al., 2008). Briefly, different amounts of recombinant Parj2 (200 ng to 1 μg) or *P. judaica* pollen extracts (1 to 15 μg) were separated on 12% SDS-PAGE gel. The proteins were transferred to a nitrocellulose membrane (Hybond-ECL, Amersham Biosciences, Little Chalfont, UK) and the membrane was incubated in blocking solution (5% nonfat dry milk, 20 m*M* Tris, 140 m*M* NaCl, 0.1% Tween-20) for 1 h at room temperature. After several washings in the buffer, the filters were incubated overnight at room temperature with the chicken anti-Parj2 antibody (1:10,000 dilution). The filters were then incubated for 1 h at room temperature with a 1:10,000 dilution of horseradish peroxidase-conjugated rabbit anti-chicken IgY (Abcam, Cambridge, UK). Immunocomplexes were detected by the enhanced chemiluminescence detection system (Super Signal, Pierce, Rockford, IL). For competition assay, before the addition to the membrane, the IgY anti-Parj2 were preincubated for 3 h at room temperature with increasing amounts of recombinant allergen (10, 50 μg/mL) and then immunocomplexes were detected as described.

### 2-Dimensional Gel Electrophoresis and Immunoblotting

A 2-dimensional gel electrophoresis (**2DE**) PAGE was performed as described previously (Fontana et al., 2008). Briefly, proteins were solubilized in a buffer containing 8 *M* urea, 4% wt/vol 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 40 m*M* Tris, and 65 m*M* dithioerythritol (**DTE**), and protein concentrations were determined using the Coomassie Protein Assay (Pierce). The first dimension was performed by Ettan IPGphor Isoelectric Focusing System with Immobiline DryStrip pH 3–10 Non Linear or Immobiline DryStrip pH 6–9, 18 cm long (Amersham Biosciences, Uppsala, Sweden). Aliquots of total proteins, 2 mg for preparative gels and for 2DE Western blot, were included in a buffer containing 8 *M* urea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 1% DTE, 0.5%, IPG buffer (Amersham Biosciences, Uppsala, Sweden), and a trace amount of bromophenol blue. Samples were applied to the gel strip and run for 1 h at 20°C and then for 10 h at 30 V. Focusing was performed by linearly increasing the voltage from 200 to 3,500 V during the first 4.5 h and then keeping the voltage constant at 8,000 V for 10 h.

After first dimension, strips were equilibrated for 30 min in 6 *M* urea, 2% SDS, 0.05 *M* Tris-HCl pH 6.8, 30% glycerol, and 2% DTE, then for 20 min in the same buffer replacing DTE with 2.5% iodoacetamide and adding a trace amount of bromophenol blue. The second dimension was run on Ettan Dalt*6* (GE Healthcare, Buckinghamshire, UK) with vertical slab gels with 9 to 16% polyacrylamide linear gradient (SDS-PAGE). Gels were stained with ammoniacal silver nitrate for analytical gels (Hochstrasser et al., 1988) or Coomassie Brilliant Blue R-250 for preparative gels. For immunoblots, the gel was transferred to nitrocellulose membrane (Hybond-ECL, Amersham Biosciences, Little Chalfont, UK). Immunodetection of chicken anti-Parj2-binding proteins or IgY-binding proteins was performed as described earlier.

### Protein Identification by Mass Spectrometry

***In-Gel Protein Digestion.*** All chemical products used for mass spectrometry (**MS**) analysis were of analytical or HPLC-grade quality. Sequence-grade trypsin was purchased from Promega (Madison, WI) and α-cyano

4-hydroxycinnamic acid from LaserBioLab (Sophia-Antipolis, France).

Coomassie Blue-stained protein spots were excised from the preparative gels and cut into 1-mm pieces. In-gel digestion was performed as described by Shevchenko et al. (1996) with minor modifications. Destaining was obtained by successive washes with 20 m*M* $NH_4HCO_3$ buffer and $H_2O$:$CH_3CN$ (50:50) mixture. For proteolytic digestion, the gel was treated by 5 to 20 μL of a trypsin solution (20 ng/uL in 50 m*M* $NH_4HCO_3$) for 5 h at 37°C. The resulting tryptic peptides were extracted from the gel by centrifugation and the supernatant fraction was recovered. A second extraction step was performed using 10 to 15 μL of $H_2O$:$CH_3CN$:HCOOH (60:36:4; vol/vol/vol) mixture for 30 min. An aliquot of the extract was dried in a vacuum concentrator and resuspended in 0.1% trifluoroacetic acid solution and used for nanoLC-nanospray-tandem MS (**LC-ESI-MS/MS**) experiments.

***LC-ESI-MS/MS.*** Experiments were performed on a Q-STAR XL instrument (Applied Biosystems) equipped with a nanospray source using a distal-coated silica-tip emitter (FS 150-20-10-D-20, New Objective, Woburn, MA) set at 2,300 V. Independent-data acquisition mode allowed peptide ions within a mass:charge ratio (***m/z***) 400 to 2,000 survey scan mass range to be analyzed for subsequent fragmentation. The tandem MS spectra were acquired in the *m/z* 65 to 2,000 range for +2 to +4 charged ions. The collision energy was automatically set by the software (Analyst 1.1) and was related to the charge of the precursor ion. The MS and tandem MS data were recalibrated using internal reference ions from a trypsin autolysis peptide at *m/z* 842.510 $[M + H]^+$ and *m/z* 421.759 $[M + 2H]^{2+}$.

Tryptic peptides were separated using an UltimatenanoLC (Dionex, Voisins Le Bretonneux, France) with a $C_{18}$ PepMap microprecolumn (5 μm; 100 Å; 300 μm × 5 mm ; Dionex) and a $C_{18}$ PepMap nanocolumn (3 μm; 100 Å; 75 μm × 150 mm ; Dionex). The chromatographic separation was developed using a linear 60-min gradient from 0 to 50% B, where solvent A was 0.1% HCOOH in $H_2O$:$CH_3CN$ (95:5) and solvent B was 0.08% HCOOH in $H_2O$:$CH_3CN$ (20:80) at approximately 200 nL/min flow rate.

Protein identification was achieved by the ProID database-searching software (Analyst version 1.1, Applied Biosystems) using a peptide and a fragment mass tolerance of ±0.15 and ±0.1 Da, respectively. A ProtScore above 2 defined a percentage of confidence better than 99%.

## RESULTS AND DISCUSSION

### Characterization of Anti-Parj2 IgY Antibody

Egg yolk antibodies from hens immunized with Parj2-His fusion protein were extracted and purified from individual eggs as described in the Material and Methods. Immunoblot analyses using the purified antigen (Figure 1a) or pollen extract (Figure 1b) detected a single specific band of the correct molecular weight of 12 kDa. The quality of the chicken antibodies was also suggested by the recognition of as little as 200 ng of the recombinant purified antigen. Figure 2a shows the result of a competition assay, representative of an experiment done in triplicate, in which preincubation of the antibody with increasing concentration of recombinant Parj2 diminished specific IgY binding to the allergen. Figure 2b is the densitometric analysis of the blot showing that 50 μg of antigen caused approximately a 50% inhibition of antibody binding.

Purified native or recombinant allergens are recommended for the diagnosis and therapy of allergic disorders (Valenta and Kraft, 2002). The identification of relevant allergens (i.e., specific proteins using appropriate methodologies) is therefore required for this purpose. We show evidence that chicken antibodies have reliable immunological properties, at least comparable to mammalian antibodies, that should give them an advantage as a convenient, inexpensive alternative for allergy research.

### 2DE Immunoblotting of P. judaica Pollen

*Parietaria judaica* is a wind-pollinated dicotyledonous weed and represents the main cause of allergy in the Mediterranean area, where up to 80% of pollinosis patients are sensitized against the proteins composing

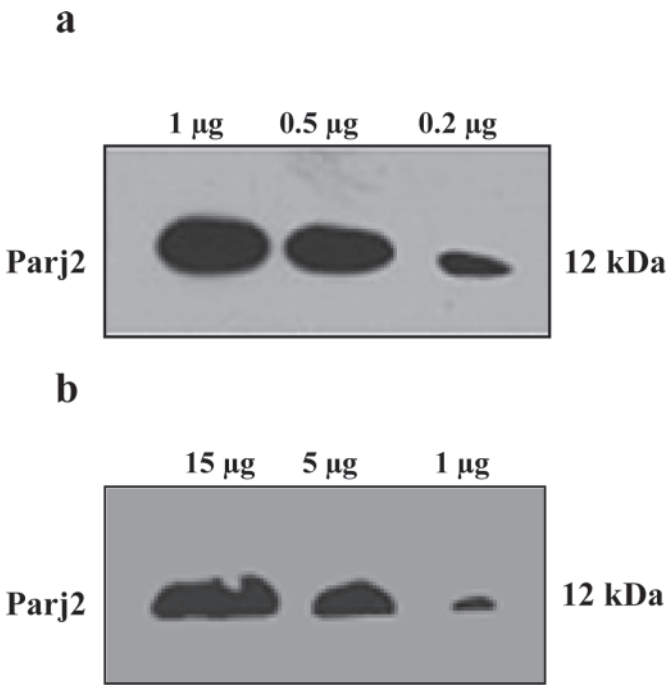


**Figure 1.** Western blot analysis of recombinant Parj2-His allergen (a) or *Parietaria judaica* extract (b). Different concentrations of purified allergen (1 μg to 200 ng) or whole pollen extract (15 to 1 μg) were loaded on a 12% SDS-PAGE gel, transferred on nitrocellulose membrane, and then probed with a 1:10,000 dilution of chicken anti-Parj2 antibody. A single band was observed in all conditions. Up to 200 ng of purified antigen is recognized by specific IgY.

the *P. judaica* pollen. Although molecular biology and immunological methodologies have allowed the identification of Parj1 and Parj2 as the major pollen components responsible for allergic reaction (Asturias et al., 2004), a comprehensive and detailed analysis of pollen allergens has never been achieved by using sophisticated analysis such as the 2DE, immunoblot, and after MS. In our study, we used approximately 1.5 g of mature pollen for protein extraction. Aliquots of the protein samples were subjected to 2-DE analysis and representative silver nitrate-stained 2DE gel is shown in Figure 3. Preliminary identifications of protein spots by MS have recognized Parj allergens in the right bottom part of the gel (bordered area). Because these basic proteins are not well separated with this type of Immobiline DryStrip pH 3–10 Non Linear, the samples were run on strips pH 6 to 9, which allow a better discrimination of the polypeptides with an isoelectric point higher than 7. Figure 4 shows Coomassie-stained 2DE gels and a specific immunoblot with IgY from Parj2 immunized hens. As shown in Figure 4b, 4 spots corresponding to a protein of about 12 kDa as recognized by chicken polyclonal antibodies. Preabsorption of IgY antibodies with recombinant allergen induced a complete inhibition of IgY binding to the membrane (Figure 4c), thus confirming the specificity of the antibody. To better characterize the biochemical identities of immunoreactive proteins, the spots of interests (Figure 4a, no. 1 to 7) were excised from Coomassie-stained 2-DE gel (Figure 4a) and subjected to LC-ESI-MS/MS. Table 1 shows the results of protein spot identifications. Protein spots 1 and 2 are isoforms of Parj1 allergen, protein spots 3 to 5 are isoforms of Parj2 P8, whereas protein spots 6 and 7 are isoforms of Parj2 P2 allergen. Sequence comparison between Parj1 and Parj2 peptides reveals a 55% homology that explains the crossreactivity of our antibody.

Pollen extracts contain several proteins that behave as allergens (Rossignol et al., 2006). In *P. judaica*, 3 proteins causing an allergic response have been identified, Parj1, Parj2, and Parj3 (Duro et al., 1997; Costa et al., 2000; Asturias et al., 2004); these allergens belong to the nonspecific lipid transfer protein family and are characterized by a high resistance to both heat treatment and proteolytic digestions. In our map, 3 spots (3 to 5) correspond to Parj2 P8 protein, 2 spots (6 and 7) correspond to Parj2 P2 protein, whereas 2 spots (1 and 2) correspond to Parj1 P1 protein.

A search of Parj1 and 2 protein sequence, using specific software supported by ExPaSy Proteomics Server evidenced both *O*-glycosylation sites (http://www.cbs.dtu.dk/services/NetOGlyc) and phosphorylation sites (http://www.cbs.dtu.dk/services/NetPhos).

In particular, Parj1 P1 protein has 4 glycosylation sites and 7 phosphorylation sites, Parj2 P8 protein has 1 glycosylation site and 5 phosphorylation sites, and Parj2 P2 protein has 1 glycosylation site and 4 phosphorylation sites. These posttranslational modifications do not significantly change the molecular weight of a protein, but they may be responsible for the shifts in isoelectric points. At the same time, posttranslational

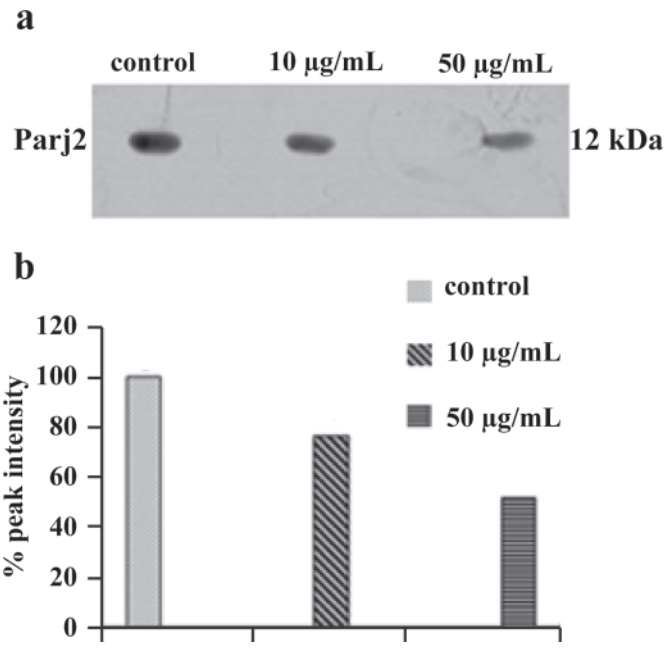


**Figure 2.** Western blot inhibition assay. The recombinant allergen Parj2 was run on a 12% SDS-PAGE gel and transferred on a nitrocellulose membrane as described in Materials and Methods. Immunoglobin Y anti-Parj2 were preincubated for 3 h at room temperature with increasing amounts of recombinant allergen (10, 50 µg/mL) and then immunocomplexes were detected by incubation with horseradish peroxidase-conjugated rabbit anti-chicken IgY (a). Panel b shows a densitometric analysis of the competition assay revealing a 50% inhibition of IgY binding the recombinant Parj2 when 50 µg of the peptide was used.

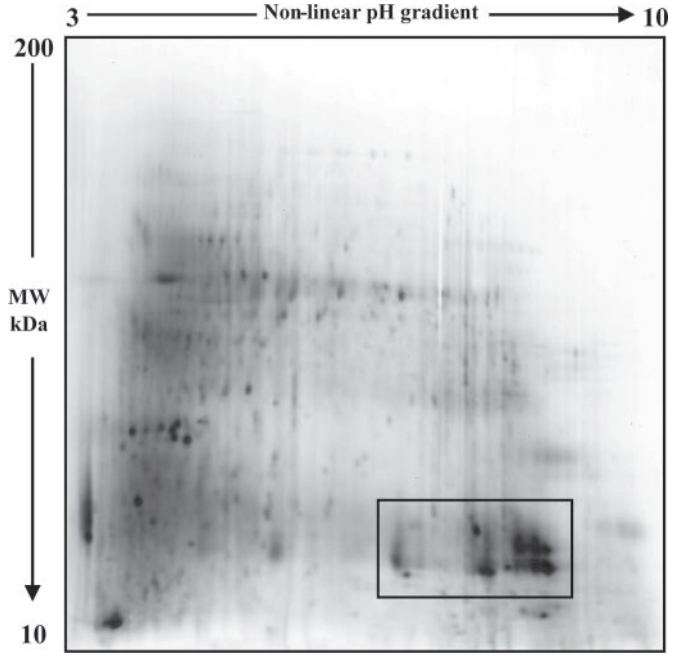


**Figure 3.** Representative proteomic map of *Parietaria judaica* pollen. In the bordered area are located most of the protein spots corresponding to Parj allergens. MW = molecular weight.

modifications might have an influence on the Ig binding; therefore, knowledge of the structure of allergens is important to improve diagnosis and therapy of allergies (Ferreira et al., 2006). A large number of allergens and allergen isoforms have been described in recent years and most of them have not been confirmed at the protein level often due to the lack of good quality antibodies (Pauli, 2000). The data presented in our study show for the first time the valuable use of chicken antibodies in the identification of pollen allergens through 2DE Western blot, thus representing satisfactory tools for immunological characterization and classification of the proteins from a raw plant extract.

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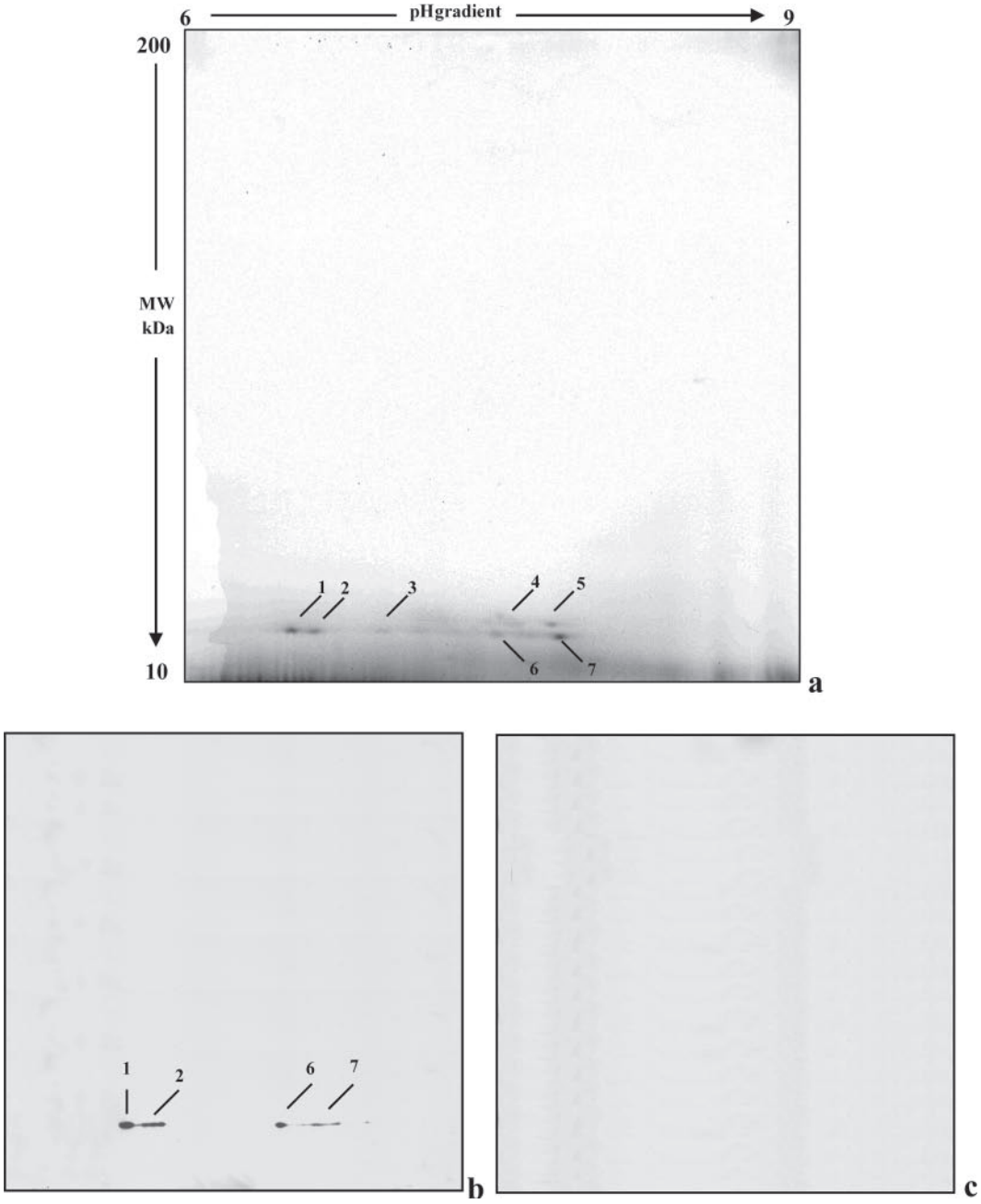


**Figure 4.** Coomassie-stained 2-dimensional gel of *Parietaria judaica* pollen extract (a), immunoblot of the same gel using anti-Parj2 IgY (b), or control Western blot using recombinant Parj2 peptide to compete for specific IgY (c). Proteins were separated in the first dimension by isoelectric focusing on gel strips with a narrow isoelectric point (pI) range of 6 to 9 and then in the second dimension using 9 to 16% SDS-PAGE. Distinct protein spots (n = 4) with specific IgY immunoreactivity as seen in corresponding immunoblots were subjected to tryptic digestion followed by nanoLC-nanospray-tandem mass spectrometry analysis for their identification (arrows). MW = molecular weight.

**Table 1.** Proteins identified by mass spectrometry analysis

| Spot no.[1] | Abbreviated name | Accession no.[2] | Protein name | Theoretical pI, MW (Da)[3] | ProtScore[4] | Sequence coverage (%)[5] | Matched species |
|---|---|---|---|---|---|---|---|
| 1 | NLT13 | Q40905 | Probable nonspecific lipid-transfer protein 1 (protein P1) | 7.03, 11,000 | 19.7 | 50 | *Parietaria judaica* |
| 2 | NLT13 | Q40905 | Probable nonspecific lipid-transfer protein 1 (protein P1) | 7.03, 11,000 | 13.52 | 68.1 | *P. judaica* |
| 3 | NLT22 | O04403 | Probable nonspecific lipid-transfer protein 2 (protein P8) | 8.63, 11,110 | 9.15 | 83.5 | *P. judaica* |
| 4 | NLT22 | O04403 | Probable nonspecific lipid-transfer protein 2 (protein P8) | 8.63, 11,110 | 15.2 | 48.1 | *P. judaica* |
| 5 | NLT22 | O04403 | Probable nonspecific lipid-transfer protein 2 (protein P8) | 8.63, 11,110 | 25.52 | 92.5 | *P. judaica* |
| 6 | NLT21 | P55958 | Probable nonspecific lipid-transfer protein 2 (protein P2) | 8.16, 11,345 | 10 | 36.8 | *P. judaica* |
| 7 | NLT21 | P55958 | Probable nonspecific lipid-transfer protein 2 (protein P2) | 8.16, 11,345 | 23.68 | 54.9 | *P. judaica* |

[1]Spot number reported on the 2-dimensional gel electrophoresis maps in Figure 4.

[2]Accession number used in the SWISS-PROT database.

[3]Theoretical isoelectric point (pI) and molecular weight (MW) values reported in the SWISS-PROT database.

[4]ProtScore from tandem mass spectrometry data (Analyst 1.1, Applied Biosystems, Courtaboeuf, France).

[5]Sequence coverage: data from nanoLC-nanospray-tandem mass spectrometry.

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