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A rapid and efficient two-step gel electrophoresis method for the purification of major rye grass pollen allergens

Purified proteins are mandatory for molecular, immunological and cellular studies. However, purification of proteins from complex mixtures requires specialised chromatography methods (*i.e.*, gel filtration, ion exchange, *etc.*) using fast protein liquid chromatography (FPLC) or high-performance liquid chromatography (HPLC) systems. Such systems are expensive and certain proteins require two or more different steps for sufficient purity and generally result in low recovery. The aim of this study was to develop a rapid, inexpensive and efficient gel-electrophoresis-based protein purification method using basic and readily available laboratory equipment. We have used crude rye grass pollen extract to purify the major allergens Lol p 1 and Lol p 5 as the model protein candidates. Total proteins were resolved on large primary gel and Coomassie Brilliant Blue (CBB)-stained Lol p 1/5 allergens were excised and purified on a secondary "mini"-gel. Purified proteins were extracted from unstained separating gels and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analyses. Silver-stained SDS-PAGE gels resolved pure proteins (*i.e.*, 875 µg of Lol p 1 recovered from a 8 mg crude starting material) while immunoblot analysis confirmed immunological reactivity of the purified proteins. Such a purification method is rapid, inexpensive, and efficient in generating proteins of sufficient purity for use in monoclonal antibody (mAb) production, protein sequencing and general molecular, immunological, and cellular studies.

Keywords: Protein / Purification / Grass / Pollen / Allergen

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1 Introduction

Grass pollen is a major outdoor cause of hayfever and allergic asthma in about 30% of the population in temperate climates of the world [1]. Allergy is triggered as a result of mediator release, including histamine and leukotrienes, from basophils and mast cells when certain pollen proteins bind and cross-link cell-bound human immunoglobulin E (IgE) antibodies [2]. Due to the high clinical significance of grass pollen allergens, a great deal of research effort has been invested in allergen characterisation, purification, and molecular cloning [3]. It is anticipated that the results of such work and the availability of highly purified natural and recombinant allergens will be pivotal for the production of standardised reagents for diagnosis and treatment of grass pollen allergy.

In Australia, the major atmospheric grass pollen is from rye grass (*Lolium perenne*), as up to half a tonne of pollen can be produced per hectare [1, 4]. For the last decade, we have been interested in the immunological characterisation and molecular cloning of major allergens from rye grass pollen [3]. The major allergens of rye grass pollen are designated Lol p 1 and Lol p 5 under the International Union of Immunological Societies (IUIS) nomenclature system [5] and their cross-reactive homologues occur in other grass species highlighting their clinical importance [6]. Up to 90% of individuals allergic to rye grass pollen demonstrate IgE reactivity to both of these allergens which are therefore termed major allergens. Although some isoforms of Lol p 1 and Lol p 5 have been cloned, sequenced, and expressed in bacteria and yeast [3, 7], the need for natural allergens is of great importance for immunological, cellular and clinical studies.

A number of studies have already employed a range of chromatography methods including gel-filtration, ion-exchange, and affinity for allergen purification [8–12]. However, such methods employ expensive and highly specialised chromatography columns and equipment such as fast protein liquid chromatography (FPLC) and HPLC. These are very tedious and time-consuming, often

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Abbreviations: FPLC, fast protein liquid chromatography; IgE, immunoglobulin G; mAb, monoclonal antibody

requiring multistep purification protocols and generating semipurified samples with low recovery. The difficulty is particularly enhanced when dealing with complex mixtures of proteins, like rye grass pollen extract, from which highly purified protein samples need to be derived for analysis. Therefore, an efficient and inexpensive protein purification system would be an invaluable tool for any researcher. Here, for the first time, we describe a highly efficient, rapid, and inexpensive two-step gel electrophoresis protein purification method. The major rye grass pollen allergens Lol p 1 and Lol p 5 were used as the model protein candidates. Moreover, the method employs readily available gel electrophoresis equipment and buffers avoiding unnecessary, expensive establishment costs.

2 Materials and methods

The schematic diagram in Fig. 1 outlines the steps involved in the gel-electrophoresis purification protocol. These steps are explained in detail below.

2.1 Primary gel

Rye grass pollen was obtained from Greer Laboratories (Lenoir, NC, USA) and used to extract total soluble proteins following established protocols [13]. Briefly, 100 mg of pollen was shaken in 1 mL of phosphate-buffered saline (PBS) at 4°C for 3 h. The sample was then clarified by centrifugation at 6000 rpm for 15 min at 4°C and the supernatant removed promptly and respun to remove any debris. Protein concentration was determined as described elsewhere [13]. Total rye grass pollen proteins (8 mg) were mixed with equal volumes of sample buffer (125 mM Tris buffer (pH 6.8), 0.4% SDS, 0.008% dithiothreitol (DTT), 2% glycerol), boiled for 5 min, loaded into a single well and separated on a 1 mm thick “maxi” (20 × 20 cm) 10% homogeneous SDS-polyacrylamide gel (SDS-PAGE) using the BioRad Protean II gel apparatus (BioRad, Hercules, CA, USA). Gels were electrophoresed in tank buffer (25 mM Tris (pH 8.3), 190 mM glycine SDS) at 32 mA until the dye front reached the stacking gel/separating gel interface, then run at 50 mA until the dye front ran through. At the completion of electrophoresis, gels were stained in Coomassie Brilliant Blue (CBB) stain for 1–2 h and destained (3% v/v glycerol, 7% v/v acetic acid, 20% v/v methanol) overnight until the background was removed.

2.2 Excision

The stained gel was washed in three changes of Milli-Q water for 5 min each and protein bands identified as the major rye grass pollen allergens Lol p 1 and Lol p 5 [3, 14]

were excised with a scalpel blade over a light box. The gel slices were equilibrated in tank buffer for 5 min before resolving in a secondary gel as described below.

2.3 Secondary gel

Equilibrated gels slices were loaded directly and layered into a single well of a 14% homogeneous 1.5 mm thick secondary “mini”-SDS-PAGE gel (10 × 7.5 cm) using a Xcell II Mini-Cell (Novex, San Diego, CA, USA). Gels were electrophoresed in gel tank buffer at 125 V until the dye front ran through. The secondary gel comprised 1/3 14% separating gel and 1/3 4% stacking gel allowing the remaining 1/3 space as a well to layer primary gel slices (see Fig. 1).

2.4 Extraction and collection

After electrophoresis, the separating gel component of each gel was excised, sealed in a separate plastic bag with 2.5 mL of extraction buffer (0.1 M Tris, pH 9.5) and incubated on a rotating wheel at 37°C for 2 h. To increase or assist elution of proteins, the gel slices can be cut into smaller pieces to increase surface area. After incubation, the eluted protein sample was collected and a further 2.5 mL extraction buffer added for a second followed by third extraction of the same amount. The protein content of the eluted samples were determined [13] and subjected to immunoblot analysis as described below.

2.5 Immunological assay

Eluted protein samples, along with total rye grass pollen proteins, were resolved on 14% homogeneous “mini”-SDS polyacrylamide gels as described above. Following electrophoresis, the gels were either silver stained (Novex) or transferred onto nitrocellulose (NC) for immunoblot analysis using our established protocols [15]. Briefly, after transfer of proteins, the free protein binding sites of NC were blocked with 10% w/v skim milk powder in PBS, washed and probed separately with Lol p 1-specific (FMC A1) and Lol p 5-specific (FMC A7) monoclonal antibodies (mAb). Monoclonal antibody binding was detected with horse radish peroxidase (HRP)-conjugated anti-mouse Ig and developed with the substrate 4-chloro-1-naphthol [15]. NC blots containing the same proteins were also incubated with only the HRP-conjugated anti-mouse secondary antibody as negative control.

3 Results and discussion

Purified proteins are important in a range of *in vitro* and *in vivo* experiments and assays. In grass pollen allergy, purified allergens are mandatory for use in immunological and cellular assays to study their molecular interaction

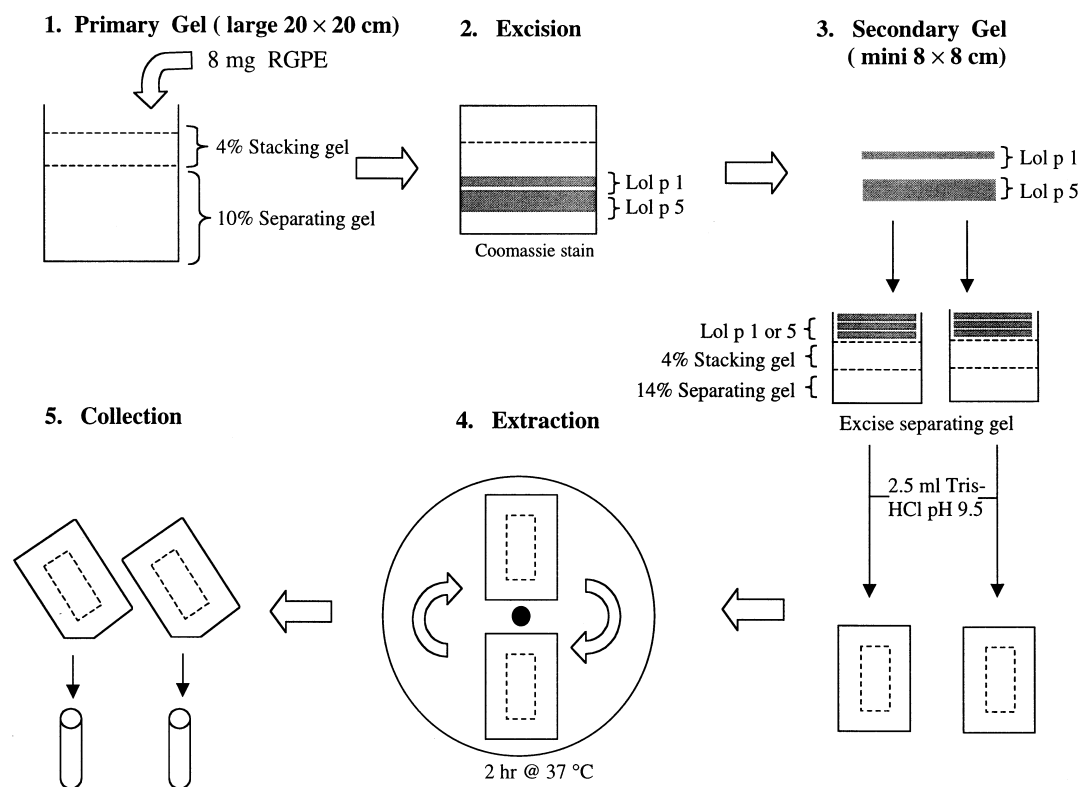


Figure 1. Schematic diagram of the two-step gel electrophoresis purification protocol. Rye grass pollen extract is denoted by RGPE.

with the human immune system in order to produce standardised allergen preparations for use in allergy diagnosis and treatment. However, total grass pollen extracts are a complex mixture of proteins and purification of certain allergens has been difficult and laborious [3, 12]. Here, we have used grass pollen as a model to describe a rapid and efficient two-step gel electrophoresis purification method that generates purified proteins which can be used directly or as an effective starting material for other purification methods.

Our purification method employs two major steps; namely, primary and secondary gel electrophoresis (Fig. 1). The primary gel electrophoresis employs a 1 mm thick “maxi”-gel format to allow ample sample loading and enhanced protein separation. The secondary gel electrophoresis employs a 1.5 mm thick “mini”-gel format allowing easy loading, protein concentration and electroelution of the CBB stain from the primary gel slices. This gel is optimised enabling ample primary gel slices to be loaded and allowed to stack so that the protein of interest is predominantly in the separating gel when the dye front runs out. Indeed, staining of the primary gel slices and stacking gel did not reveal any residual proteins while proteins were only visualised in the separating gel (data not

shown). However, to cater for proteins of different molecular mass, the percentage of the separating gel can be optimised to allow maximal resolution.

As the proteins in the secondary gel are no longer stained or fixed, their elution becomes more efficient. Moreover, since the secondary gels can be loaded with several layers of gel slices, high protein concentration and ample recovery can be achieved. A general Tris elution buffer has been used in our method. To enhance elution, separating gels can be cut into smaller pieces or homogenised to increase surface area. In our studies, elution of proteins from intact separating gel slices was satisfactory obtaining as much as 175 µg/mL of Lol p 1 and Lol p 5 after one round of extraction, that is, 437.5 µg of each protein from a 8 mg starting material (Fig. 2). Further extractions can be performed to elute more protein from the same separating gel slice. We have performed three extractions and depending on the protein and its concentration in the separation gel, the second extraction generated the same amount of protein as the first one (Fig. 2A) while amounts significantly declined by the third extraction (Fig. 2B). Therefore, two extractions are adequate for ample protein recovery. Again, in this report we describe the purification method and the extraction step itself

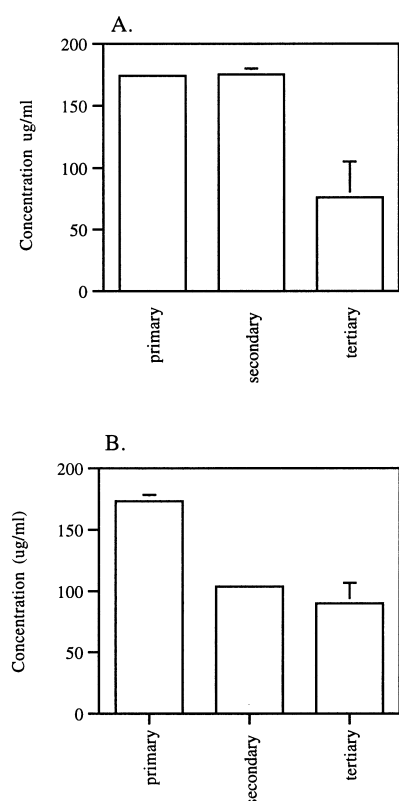


Figure 2. Multiple extraction profiles of the purified proteins. Protein estimations of Lol p 1 (A) and Lol p 5 (B) extractions.

should be further optimised for different proteins of interest. For example, temperature should be optimised according to the temperature stability of the protein under study or a different extraction buffer may be needed. Additionally, protease inhibitors or other preservatives may be added to the extraction buffer.

In order to check protein purity and conservation of immunological reactivity, we performed SDS-PAGE and immunoblot analyses (Fig. 3). Silver staining of the eluted proteins revealed the expected molecular mass corresponding to the single Lol p 1 allergen and the doublet Lol p 5 allergen and corresponded to the native proteins in the total rye grass pollen extract (Fig. 3A). When the NC blots of the same gel were probed with Lol p 1-specific mAb FMC A1 and Lol p 5-specific mAb FMC A7, specific binding was obtained to Lol p 1 (Fig. 3B) and Lol p 5 (Fig. 3C), respectively. Incubation of an identical NC blot with only the secondary anti-mouse antibody did not show any nonspecific binding (data not shown) highlighting the specificity of the mAbs in the observed results (Fig. 3).

It may be argued that other non-Lol p 1 and non-Lol p 5 proteins may occupy the same molecular mass of these allergens and thus be contaminating our excised protein

bands. From our previous findings of two-dimensional PAGE analysis using silver staining of gels, and probing NC transfers of the same gels with Lol p 1- and Lol p 5-specific mAbs and human serum IgE, the only other proteins that share the same molecular mass but possess different isoelectric points were isoforms of Lol p 1 and Lol p 5 (7, 16). However, if multiple proteins occupy the same molecular mass and single species of the protein are required, one can either use the partially purified sample generated by our two-step method reported here in a follow-up ion-exchange (or similar) chromatography or use 2D gel spots as the primary gel (*i.e.*, step 1) in our method. Therefore, the lower intensity of mAb binding to Lol p 5 (Fig. 3C) from that of silver-stained Lol p 5 (Fig. 3A) may not be attributed to a contamination of non-Lol p 5 proteins but is as a result of mAb dilution that has been optimised to give adequate binding to visualise the two Lol p 5 bands discretely, since high mAb binding will generate a single broad protein reactivity. An interesting observation was made with both allergens, although to a greater extent with Lol p 1, with the appearance of a protein band that was double the molecular mass of the original allergen (Fig. 3, arrows). We have previously observed a similar phenomenon with both of these allergens and hypothesise that these are dimers of the allergens that have not been completely reduced by SDS-PAGE [3, 7].

Conventional protein purification methods include gel filtration, ion exchange and affinity chromatography to name just a few. However, one needs the expensive and dedicated hardware (*i.e.*, FPLC and HPLC) to perform such purifications. Moreover, such purification strategies are limited by the amount of protein that can be loaded on the column and consequently this is reflected in the low amounts and diluted output of the purified product. We are not claiming that our method described here is superior to or replaces such sophisticated and essential chromatography methods but is a novel, rapid and simple method that uses inexpensive equipment already established in most protein chemistry laboratories. Therefore, even though the protein sample generated by our method may not be 100% pure due to presence of isoforms at the same molecular mass, it would provide a concentrated sample for use in, for example, ion-exchange chromatography. Indeed, for some proteins, FPLC and HPLC may be the only way for successful purification, but we strongly feel that our method offers unique advantages that will suggest it as the first method of choice for trial protein purification. Indeed, we routinely use this method to purify proteins for immunological and cellular assays and for preparation of antigens for murine injections to produce specific polyclonal and monoclonal antibodies.

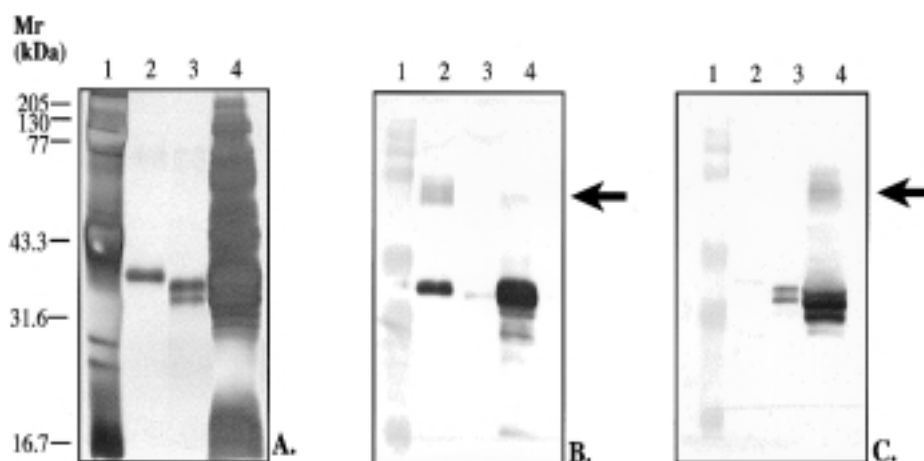


Figure 3. SDS-PAGE and immunoblot analyses of the purified proteins. Samples were run on 14% SDS-PAGE gels and either (A) silver stained or (B) transferred onto NC for immunoblot analysis using Lol p 1-specific mAb FMC A1 and (C) Lol p 5-specific mAb FMC A7. Gels contained pre-stained molecular mass markers (lane 1), purified Lol p 1 (lane 2), purified Lol p 5 (lane 3) and crude rye grass pollen extract (lane 4). Arrows indicate possible dimerisation of Lol p 1 and Lol p 5 proteins.

Although some of the steps in our purification method presented here are not novel on their own, it is the overall arrangement of these steps that makes our method novel. For example, primary gel involves routinely used SDS-PAGE and Coomassie staining of the gel for protein identification, which are not novel. However, our method becomes novel after step 2 which involves the SDS-PAGE of the excised protein-containing gel slices. The secondary gel has been optimised to contain adequate space for sample loading, stacking and separating which we consider novel. Finally, the secondary gel is isolated for protein extraction and collection, which are not new techniques on their own but novel for this application. We strongly believe that our SDS-PAGE purification system has not been previously reported and is thus novel.

The major advantages of our method over other methods are cost-effectiveness, efficiency, and ease of use. On the other hand, our method is limited to abundant proteins that can only be detected on a gel by Coomassie stain. Therefore, in regard to minimum protein quantities, any protein of sufficient amount that can be detected by Coomassie staining (*i.e.*, 0.5 µg/cm²) would be suitable for our method. It should be also stressed here that the protein recoveries from gel slices are excellent (Fig. 2) and combined with sample overloading of the second gel, ample protein can be purified with ease. Finally, our gel-electrophoresis purification method can be optimised to include purification of proteins under native conditions (*i.e.*, native gels) and according to both their molecular mass and isoelectric point (*i.e.*, 2-D gels). Moreover, the method may be optimised to include copper- or silver-stained gels. Therefore, our method is applicable to any protein that can be resolved by SDS-PAGE, native- PAGE and 2-D PAGE.

4 Concluding remarks

We have developed a rapid, simple, efficient, and inexpensive two-step gel electrophoresis protein purification method using major rye grass pollen allergens as the model candidates. This method generated ample protein with exceptional purity and immunological reactivity. We strongly feel that this method will be very useful and popular not only among allergy researchers purifying allergens but also others who have limited or no access to FPLC or HPLC chromatography methods and equipment.

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5 References

- [1] Knox, R. B., Suphioglu, C., *Trends Plant Sci.* 1996, 1, 156–164.
- [2] Roitt, I. M., Brostoff, J., Male, D. K., *Immunology*, Mosby, London 1998, pp. 22.2–22.4.
- [3] Suphioglu, C., *Clin. Exp. Allergy* 2000, 30, 1335–1341.
- [4] Smart, I. J., Tuddenham, W. G., Knox, R. B., *Aust. J. Bot.* 1979, 27, 333–342.
- [5] King, T. P., Hoffman, D., Lowenstein, H., Marsh, D. G., Platts-Mills, T. A., Thomas, W., *Int. Arch. Allergy Immunol.* 1994, 105, 224–233.
- [6] Suphioglu, C., Singh, M. B., Knox, R. B., *Int. Arch. Allergy Immunol.* 1993, 102, 144–151.
- [7] Suphioglu, C., Mawdsley, D., Schäppi, G., Gruehn, S., de Leon, M., Rolland, J. M., O'Hehir, R. E., *FEBS Lett.* 1999, 462, 435–441.
- [8] Verma, J., Singh, B. P., Gangal, S. V., Arora, N., Sridhara, S., *Int. Arch. Allergy Immunol.* 2000, 122, 251–256.

- [9] Yokoyama, M., Miyahara, M., Shimizu, K., Kino, K., Tsunoo, H., *Biochem. Biophys. Res. Commun.* 2000, *275*, 195–202.
- [10] Suck, R., Weber, B., Kahlert, H., Hagen, S., Cromwell, O., Fiebig, H., *Int. Arch. Allergy Immunol.* 2000, *121*, 284–291.
- [11] Tsai, J. J., Shen, H. D., Chua, K. Y., *Int. Arch. Allergy Immunol.* 2000, *121*, 205–210.
- [12] Pacakova, V., Stulik, K., Ticha, M., *J. Chromatogr. B* 1997, *699*, 403–418.
- [13] Suphioglu, C., Singh, M. B., Simpson, R. J., Ward, L. D., Knox, R. B., *Allergy* 1993, *48*, 273–281.
- [14] Suphioglu, C., Smith, P. M., Ong, E. K., Knox, R. B., Singh, M. B., *Adv. Exp. Med. Biol.* 1996, *409*, 147–155.
- [15] Sutherland, M. F., O’Hehir, R. E., Czarny, D., Suphioglu, C., *J. Allergy Clin. Immunol.* 1999, *104*, 889–890.
- [16] Smith, P. M., Ong, E. K., Singh, M. B., Knox, R. B., *Mol. Immunol.* 1994, *31*, 491–498.