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A simple method for assigning multiple immunogens to their protein on a two-dimensional blot and its application to asthma-causing allergens

A “one-step” procedure, that not only removes the color and blocking proteins used in the colorimetric immunodetection step but also stains the proteins originally on the blot, is presented. Following immunostaining and recording of immunoreactive spots, the blot was allowed to air-dry overnight (or longer) at room temperature and then counterstained with a colloidal gold solution. This “air-drying” process apparently altered the affinity of the blocking proteins (and possibly other proteins added subsequently to the blotting step) towards the nitrocellulose membrane causing them to be removed by the acidic colloidal gold solution while the “blotted” proteins were being stained. The sensitivity of this counterstained blot was comparable to that of the blot without going through the immunodetection process. Since both immunodetection and protein staining were carried out on the same blot, this allowed easy identification of many immunoreactive spots to their corresponding proteins when the two profiles were superimposed. Using this procedure, we have detected 25 immunoreactive spots (or allergens) from the whole body extract of the German cockroach (*Blattella germanica*) that contribute to asthma and assigned them to their corresponding proteins on a two-dimensional (2-D) protein map. The apparent M_r and pI for each of the allergens were determined. We have also located one of the major cockroach allergens, Bla g 5 (glutathione *S*-transferase). Two-dimensional zymography revealed the presence of ten gelatinase-type proteolytic enzymes. Only one of the ten proteases comigrated with the immunoreactive proteins indicating that unlike other allergen-producing systems, most of the cockroach allergens do not possess protease activity.

Keywords: Two-dimensional blots / *Blattella germanica* / Immunodetection

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Immunodetection of proteins on blot, first introduced by Towbin *et al.* [1], is the method of choice for identifying specific proteins after electrophoresis. Due to its high resolution, immunoprobings of 2-D gels has been extensively used as a means of localizing specific proteins among hundreds or even thousands of separated proteins [2]. Typically, a blot is subjected to a general protein stain such as Coomassie blue [3], India ink [4, 5] or colloidal gold [6, 7] and a separate blot is used for the detection of immunoreactive proteins. A “side by side” comparison of the two blots reveals the general location of the immunogens [8]. During the immunodetection step, blocking proteins are usually added and unlike the “blotted” proteins, the overlaid blocking proteins are only weakly bound to the surface of the membrane. This weak interac-

tion is designed to hinder nonspecific binding of primary or secondary antibodies to the membrane. It has been reported that the blocking proteins bind more tightly to some membranes (e.g., nitrocellulose) than to others such as PVDF [9]. To locate precisely the immunogens on a 2-D blot, however, both protein staining and immunodetection steps must be performed on the same blot. A method utilizing a colorimetric immunodetection followed by counterstaining the same blot with Coomassie blue was reported [9]. The presence of proteins added during the immunodetection step produced a high staining background, making it difficult to locate some of the immunogenic proteins [9]. Therefore, it is generally recommended that chemical staining of proteins on the blot be carried out before the immunodetection step [10]. Care must be taken that the introduced chemical dyes do not interfere with subsequent immunodetection using either chromogenic or enhanced chemiluminescent substrates. This restriction limits the use of protein staining dyes to those that are reversible (e.g., Ponceau S, Fast green or metal chelates) [10]. Because of the low sensitivity of these “reversible” dyes, they provide only “land-

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Abbreviation: TTBS, Tris-HCl/Tween 20/NaCl buffer

mark" spots for matching immunoreactivity patterns (obtained from the same blot) to a separate, highly sensitive silver-stained gel pattern [10]. Methods that combine either Indian ink [11] or colloidal gold [12] staining of proteins with enhanced chemiluminescent detection have been introduced. In the latter method, the nonspecific interaction between the secondary antibody or protein A with colloidal gold provides a background staining for proteins [12]. The detection of specific immunogenic protein spots therefore relies on the relative exposure time of the film [12]. Although this method represents an improvement in assigning proteins on a 2-D blot, it can only be used to detect highly immunogenic proteins before the background of nonspecific interaction of proteins with the colloidal gold masks the weaker immunogenic proteins. Thus, currently available 2-D immunodetection methods do not work well when dealing with a mixed population of strong and weak immunogens. Here, we report a simple method that not only removes the color produced by the immunodetection step but also retains high sensitivity of protein detection thereby allowing for easy assignment of many immunoreactive spots to their corresponding proteins on the same 2-D blot. To illustrate the usefulness of this method, we have identified at least 25 immunoglobulin E (IgE)-reactive immunoreactive spots (or allergens) produced by the German cockroach, *Blattella germanica*.

1-D PAGE has been used by many investigators to separate the complex IgE-reactive German cockroach allergens [13, 14]. (We used the term "allergens" here in accordance with the published literature even though we have not ruled out the possibility that some of these allergens may be related, *i.e.*, degradation products or even isoforms). Due to the large number of allergens present, it became clear that this system was inadequate in resolving them. A 2-D PAGE method was therefore used to determine the total number of allergens. Whole body extract was prepared by homogenizing German cockroaches in a phosphate-buffered saline solution (PBS) at pH 7.4 followed by centrifugation at $13000 \times g$ for 5 min at 4°C. The supernatant was centrifuged two more times to obtain a clarified extract which was dialyzed overnight against the same buffer at 4°C. This was followed by further dialysis of the extract against water to remove the bulk of ions that might interfere with the isoelectric focusing step. The first-dimensional IEF gels were prepared according to the procedure described by O'Farrell [15]. The gel mixture contained 9.2 M urea, 2% CHAPS, 4% acrylamide/N, N'-methylenebisacrylamide and 2% carrier ampholytes (0.4% pH 3–10 and 1.6% pH 5–7). Whole body extract was mixed with the above gel mixture (0.4 mg total protein/mL gel mixture). The mixture was loaded into the gel tube (200 µL/gel tube) and electrophoresed initially at 500 V for 10 min and then at 850 V for 5 h. The IEF gels were then equilibrated with SDS equilibration buffer (0.0625 M Tris-

HCl, pH 8.8, 2.3% SDS, 5.0% 2-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue) before transferring onto the second-dimensional gel. The second-dimensional gel electrophoresis was carried out using a Mini-Protein II 2-D cell (Bio-Rad, Hercules, CA, USA). Proteins from the 2-D gels were blotted onto a nitrocellulose membrane (0.2 µm from Bio-Rad) at room temperature using a semidry electrophoretic transfer cell (Bio-Rad) in Towbin's buffer [1] at 15 V for 1 h. The blot was then immersed in a blocking solution containing 5% non-fat dry milk in Tris-buffered saline/Tween 20 (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.05% Tween 20; (TTBS) for 2 h. The blot was washed with TTBS and immunoprobed with a primary antibody solution at 1:500 dilution for either 2 h or overnight. This primary antibody was obtained from pooled sera collected from individuals that were known to have asthmatic symptoms caused by German cockroaches. The blot was washed and then incubated for 2 h with a biotin-coupled secondary antibody solution (anti-human IgE from Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) at 1:1000 dilution. Following incubation with the streptavidin-biotinylated alkaline phosphatase complex [16, 17] for 2 h, the allergens were detected by staining with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) (Sigma Chemical Co., St. Louis, MO, USA).

Figure 1 (top panel) shows a typical 2-D protein profile from the whole body extract of *B. germanica* stained with a colloidal gold solution [18]. A separate 2-D blot generated under identical conditions was immunoprobed using the amplified alkaline phosphatase system described above, the allergen profile is also presented in Fig. 1 (middle panel). A control allergen profile using pooled sera from individuals that were known to have no asthmatic symptoms was used to eliminate several minor spots present in Fig. 1 (results not shown). Additionally, a second control employing blank incubation with the secondary (but not the primary) antibody was also used to eliminate nonspecific spots. Because the protein and allergen profiles were performed with different blots (from the same experiment), it was difficult to correlate them. With more than 20 allergen proteins and many of them apparently expressed at low levels (*i.e.*, proteins that were below the threshold of detection even with the sensitive colloidal gold stain [7]), it was necessary to use the same blot for precise assignment of allergens to their protein spots.

A popular method for stripping the proteins introduced during the immunodetection step was to use a buffer containing 2% SDS and 0.1 M 2-mercaptoethanol at either 50°C [19] or at 70°C [10, 20]. This stripping procedure was originally designed for immunodetection using enhanced chemiluminescence so that the stripped blot can be reprobed with a different antibody. For this procedure

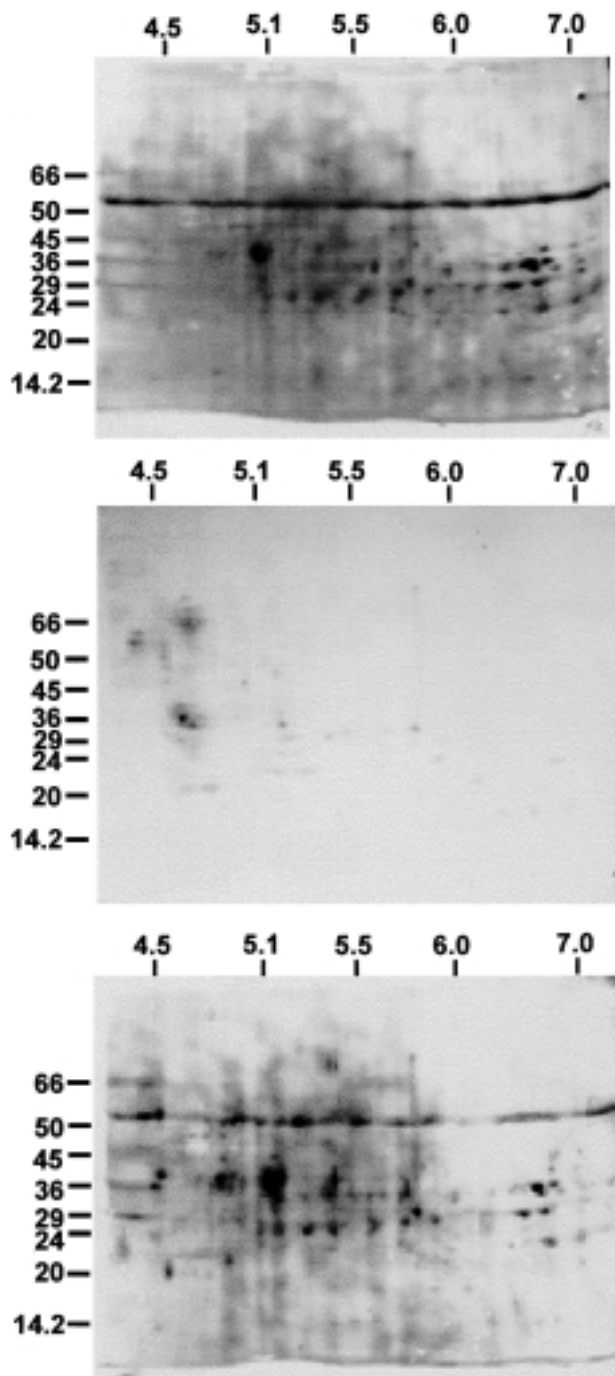


Figure 1. 2-D protein and allergen profiles from the whole body extract of *B. germanica*. Top panel: proteins from *B. germanica* were subjected to 2-D PAGE, blotted onto nitrocellulose membrane and stained with a colloidal gold solution [18]. Middle panel: immunoprobings of 2-D blot with the amplified alkaline phosphatase system. Bottom panel: immunoprobed 2-D blot was allowed to air-dry overnight at room temperature followed by washing with TTBS solution three times (20 min each time). The blot was then rinsed with water three times (2 min each time) and counterstained with the colloidal gold solution [18].

to work well, the blot must be stored wet at 4°C after each immunodetection step [19]. Initially, we used the stripping method of Magi *et al.* [10] in the hope of removing the antibodies and their associated color from the 2-D blot. The immunostained blot was incubated in 2% SDS, 0.1 M 2-mercaptoethanol and 62.5 mM Tris-HCl, pH 6.7, for 45 min at 70°C. The blot was then washed twice with 10 mM Tris-HCl, pH 7.4. We found, however, that the color on the blot still remained, indicating that this method cannot be applied for stripping the antibodies used in the colorimetric immunostaining. Unexpectedly, we discovered that when the immunoblot was allowed to air-dry overnight or longer at room temperature (rather than kept wet at 4°C as suggested), the affinity of the proteins introduced during the immunodetection step toward the membrane was apparently altered. When the blot was subsequently stained with the acidic colloidal gold solution, not only the color (representing the alkaline phosphatase attached to the secondary antibody) disappeared but it also produced a rather clear background indicating the removal of blocking proteins as well (Fig. 1, bottom panel). Furthermore, this air-drying step produced a protein profile comparable in sensitivity to that of the nonimmunoprobed blot (compare Fig. 1, top and bottom panels). Thus, this step represents a significant improvement in the immunodetection and makes it rather easy to assign many immunoreactive spots to their corresponding proteins on the same 2-D blot.

This discovery led us to assign the location of the 25 German cockroach allergens on the 2-D blot (Fig. 2). The apparent M_r and pI for each of the allergens were tabulated in Table 1. We found that there was a cluster of three allergens (spots 11–13) having pI approximately 4.6 and M_r of 33 000 to 38 000 with spot 12 (M_r 36 000) being the most active allergen. This allergen is most likely the Bla g 2 protein reported by Chapman and his co-workers [21]. One of the major German cockroach allergens is Bla g 5 which was reported to possess the glutathione S-transferase (GST) activity [22]. Since GST is an important detoxification enzyme, we decided to purify the enzyme *via* affinity chromatography using a glutathione-agarose column (Sigma Chemical Co.) 0.5 mL of the whole body extract from *B. germanica* was loaded onto a 1.0 mL glutathione-agarose column. The column was incubated for 30 min at room temperature. The unbound proteins were washed with 50 mM PBS (pH 7.4) and the bound proteins were eluted with 10 mM reduced glutathione in 50 mM Tris (pH 8.5). Two GST bands with M_r of approximately 23 000 and 22 000 were detected by 1-D SDS-PAGE (results not shown). The 23 kDa protein band reacted strongly with the antibody whereas the 22 kDa protein band exhibited only weak immunogenicity (results not shown). Location of these two immunoreactive allergens on the 2-D blot

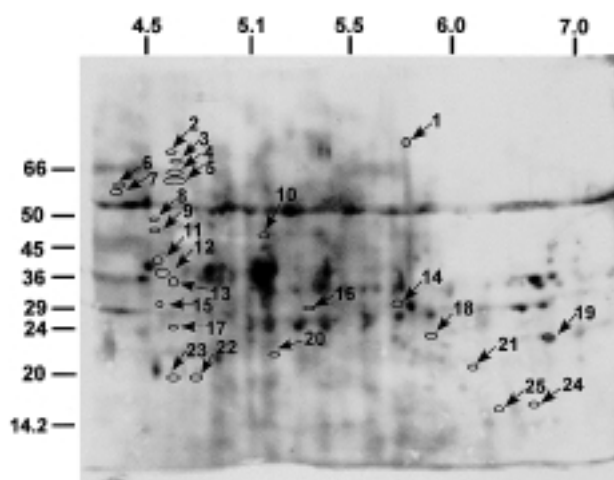


Figure 2. Location of the immunoreactive spots from the whole body extract of *B. germanica* on the 2-D blot. Immunostained blot (Fig. 1, middle panel) and restained "air-dried" blot (Fig. 1, bottom panel) were superimposed to locate the position of the 25 allergens on the 2-D blot.

Table 1. Apparent M_r and pI of the 25 immunoreactive spots from whole body extract protein of *B. germanica*

Immunoreactive spots	Approximate M_r	Approximate pI
1	85 000	5.7
2	74 000	4.6
3	70 000	4.6
4	64 000	4.6
5	60 000	4.6
6	59 000	4.3
7	56 000	4.3
8	48 000	4.5
9	46 000	4.5
10	43 000	5.1
11	38 000	4.6
12	36 000	4.6
13	33 000	4.6
14	30 000	5.7
15	30 000	5.3
16	29 000	4.6
17	24 000	4.6
18	23 000	5.9
19	22 000	6.8
20	22 000	5.5
21	20 000	6.1
22	19 000	4.7
23	19 000	4.6
24	16 000	6.7
25	15 000	6.5

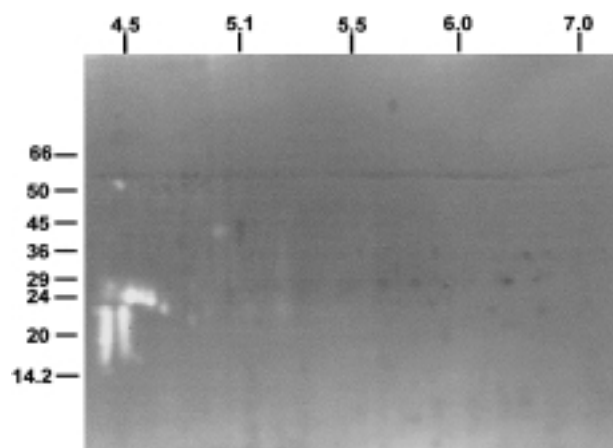


Figure 3. 2-D zymogram from whole body extract of *B. germanica*. IEF gel was transferred onto the top of a 12% polyacrylamide slab gel containing 0.05% gelatin and electrophoresed at a constant voltage of 200 V at room temperature for 35 min in SDS. After washing the gel with 2.5% Triton X-100 solution for 30 min with gentle agitation, it was incubated with 50 mM sodium phosphate buffer, pH 6.5 for 30 min. The gel was subsequently fixed and stained overnight with Coomassie blue.

was determined with spot 18 ($M_r = 23\ 000$ and $pI\ 5.8$) as the major allergen and spot 19 ($M_r = 22\ 000$ and $pI\ 6.8$) as the minor allergen (Fig. 2). Our affinity-purified fraction also contained three additional glutathione binding proteins as revealed by 2-D PAGE (results not shown).

The majority of allergens in other systems (e.g., dust mites) were shown to possess 25 protease activity [23]. In an effort to determine whether some of the allergens from *B. germanica* contain protease activity, a 2-D zymography procedure was used [24]. Figure 3 shows that 10 proteases were detected and of these only one comigrated with the allergen (spot 17 in Fig. 2). Thus, we conclude that, unlike the house dust mite and other systems, most of the cockroach allergens do not possess gelatinase-type protease activity.

Our simple procedure for removing the color produced by the immunodetection and staining of the originally blotted proteins in one step provides a new tool for identification and assignment of many immunostained antigens to their corresponding proteins on the 2-D blot. Preliminary results indicated that some (if not most) of the antibodies introduced during the immunodetection step were also removed in the "air-drying" step. The identification of cockroach allergens from this study should also facilitate their functional studies in the future.

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