Chapter 13

Honeybee Venom Detected by Enzyme Immunoassay

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Introduction

The following lab exercise began as a student-initiated project in my immunochemistry class at the University of Alaska Anchorage. The student was a beekeeper who had become allergic to honeybee venom, and was curious as to whether she had antibodies in her blood serum against protein components in the bee venom. She developed a Western blotting procedure where she separated bee venom proteins by SDS-polyacrylamide gel electrophoresis, electroblotted the separated proteins from the gel to nitrocellulose membrane, then immunodetected the proteins using commercially prepared antivenom as a positive control. She was able to demonstrate antibodies in her own serum to several of the bee venom components as well.

By bypassing the complicated electrophoresis and blotting steps, it has been possible to adapt this lab exercise for a single lab period, where the main emphasis is immunodetection. In the lab exercise presented here, students perform a simple immunoassay where they immunodetect different concentrations of honeybee proteins spotted directly onto nitrocellulose membranes. The students learn how to make dilutions and have an opportunity to practice micropipetting very small volumes. Immunodetection is carried out using primary antibodies to bee venom proteins, followed by secondary antibodies conjugated with an enzyme which will convert a colorless substrate to a colored product. The intensity of the colored product on the nitrocellulose is thus proportional to the concentration of bee venom spotted onto the nitrocellulose. The exercise provides an opportunity to discuss the sensitivity of immunodetection methods and to talk about the medical uses of enzyme immunoassays such as the Western blot and enzyme linked immunosorbent assay (ELISA).

While students perform this simple exercise, I demonstrate the normal Western blotting technique using gel electrophoresis and electrophoretic transfer of the separated proteins onto nitrocellulose.

The exercise can be adapted to any protein for which antibodies are available, either commercially or from an investigator at your institution. In some years, because of my interest in fish immunology, I have the students spot rainbow trout serum onto nitrocellulose and detect the fish proteins using an antiserum that I use in my research. Alternatively, one could purchase antibodies directed against plant or animal proteins found in foods, as for example, to detect contamination of supposed 100% beef with turkey meat.

Notes for the Instructor

This exercise is readily adaptable to both beginning biology students and advanced students, depending on how much background information you are ready to give your students and their familiarity with laboratory techniques such as pipetting. I find it best to do this lab exercise after students have had at least an overview of the immune system, though the concept of specific binding is an intuitive one, and knowledge of allergies is widespread among young adults.

Although it is possible to perform this lab exercise in one 3-hour period if the students are well organized, the exercise is best performed over two 3-hour lab periods. Sufficient time is then

available to spend on immunologic and biochemical concepts, on practicing micropipetting, and on discussing results expected at the detection step. The time intervals noted in the Student Outline are applicable if one must complete the exercise in one 3-hour lab period. The best place to interrupt the exercise for use in two lab periods is after the blocking step in non-fat milk (step 5). After blocking in milk, the membrane should be rinsed briefly in 5 ml TTBS, inserted into a sealable plastic bag, and stored in the refrigerator until the next lab period. During the second lab period, incubation times in both the primary and the secondary antibodies can be increased to 60 minutes each for more sensitivity.

If there is a student in the class who is allergic to bee venom and if your institution permits the use of human serum in the laboratory, an interesting variation on this experiment can be performed. An individual allergic to bee venom would be expected to have IgG and/or IgE antibodies in their serum. In a separate petri dish, instead of using the commercially-prepared rabbit anti-venom antibodies in the assay, the student's serum can be used as the source of primary antibodies. The serum should be diluted 1:10 in the 1% gelatin-TTBS buffer (see below). The secondary antibody-enzyme conjugate must then be one which is specific for human immunoglobulins, that is, goat anti-human IgG-alkaline phosphatase. A positive result indicates that the student has IgG antibodies to at least one of the components of the bee venom. The method is not sufficiently sensitive for the detection of IgE antibodies.

A "real" Western blot can be shown as a demonstration during the lab. In the Western blot, individual venom components are separated by SDS-polyacrylamide gel electrophoresis and electroblotted to nitrocellulose. I use the Mini-Protean apparatus and Mini-TransBlot Cell from BioRad, but there are other setups available. Immunodetection is performed as described in the student outline, resulting in purple bands on the nitrocellulose at positions corresponding to the distance the individual proteins migrated in the gel during electrophoresis. If prestained molecular weight markers are run on the gel, they will transfer to the nitrocellulose, and the students can calculate the molecular weights of the venom components. In addition, if one has serum from an allergic student, one can determine to which proteins in the venom the student has antibodies!

When I demonstrate the Western blot, I run the electrophoresis and blotting during one lab period, then block the nitrocellulose, rinse, and store the blot in a sealable plastic bag in the refrigerator until the next lab period as noted above. Alternatively, the electrophoresis and electroblotting can be done just before class, and the immunodetection steps performed along with the students in a single lab period. The incubation periods lend themselves well to student questions and to further discussion of the assay techniques.

Safety Issues: Any student who is allergic to bees, wasps, yellow jackets, or other stinging insects should wear gloves, a lab coat, and safety glasses when pipetting and diluting the bee venom. Once the bee venom has been immobilized on the nitrocellulose, the risk of exposure to the allergenic components in the venom becomes virtually zero, as long as the membrane is handled with forceps.

Materials

For a class of 20 students working individually (or 40 students working in pairs):

Nitrocellulose paper, 4 × 5 inch sheets, Schleicher and Schuell, 0.2 µm (2, each cut into 10 2.5 cm × 4 cm pieces)
Plastic petri dishes, 60 mm (40)
Tris buffered saline (TBS) in capped tubes (25 ml per student, but you should make up enough to make 3 liters of TTBS as well. I make up 4 liters of TBS at a time, but you may need to make

up smaller amounts, depending on the size of flask or beaker you have available). TBS: 20 mM Tris, 500 mM NaCl, pH 7.5. To make 1 liter: Add 2.42 g Tris to 29.24 g NaCl and bring to 900 ml with distilled or distilled deionized water. Adjust to pH 7.5 with HCl, then bring the final volume to 1 liter with distilled water..

Tris buffered saline containing 0.05% Tween-20 (TTBS) in 125 ml glass bottles (100 ml per student is a generous amount). Add 0.5 ml Tween-20 to 1 liter of TBS.

1% gelatin-TTBS (2.5 g blotting grade gelatin in 250 ml TTBS, for making up antibody solutions). Store in the refrigerator, and liquefy in a 37°C water bath or incubator just before use.

Bee venom from *Apis mellifera*, lyophilized, Sigma #V-3375, stock reconstituted in distilled water at 25 mg/ml. One vial of bee venom will last many years. Store stock at −20°C.

Anti-venom, to *Apis mellifera* made in rabbit, lyophilized, Sigma #A-5026, reconstituted in 2 ml distilled water, and diluted 1:100 in 1% gelatin-TTBS. A class of 20 requires 100 ml, but extra should be provided. Store stock at −20°C.

Goat anti-rabbit IgG-alkaline phosphatase conjugated secondary antibody and BCIP/NBT substrate, BioRad kit #170-6460. Antibody solution is made up in 1% gelatin-TTBS. Recommended dilutions vary and are supplied with the kit instructions. A dilution of 1:3000 to 1:5000 is customary. The BCIP/NBT substrate solution is simple to make up in the kit buffer just before use. A class of 20 requires 100 ml, but extra should be provided. The antibody conjugate is also available separately.

Instant non-fat dried milk, 5% in TTBS, 125 ml (5 ml per student, plus extra)

Normal rabbit serum, Sigma #R9133, 1 µl per student. (This is a positive control. The secondary goat-anti-rabbit antibodies will bind to the rabbit antibodies in the rabbit serum and allow the colorimetric reaction to proceed on the nitrocellulose.)

Bovine serum albumin (BSA), 1 mg/ml, 1 µl per student. (This is a negative control. None of the antibodies should be capable of binding BSA spotted onto the nitrocellulose, so there will be no color change on the membrane.)

Micropipettors (Eppendorf Ultramicropipettors are great for students because they can be autoclaved) to deliver 1–10 µl, 1 per two students. Capillary devices or Hamilton syringes will also work.

Micropipettor tips to fit the micropipettors (several per student in a petri dish)

Pipets for buffer transfers (transfer pipets are economical and work well), 5 ml (several per student)

Forceps for handling of nitrocellulose (20)

Pencils and rulers (for dividing nitrocellulose into quadrants)

Variable speed rocker or shaker to hold 20 60-mm petri dishes (1), or students can rock their plates by hand

Container (100 ml) for primary antibody solution, which can be re-used

Animal waste container for secondary antibody solution

Blow dryer

Preparation Before Class

1. Cut nitrocellulose membrane into 2.5 cm × 4 cm pieces (one per student or student pair) and hold them in a petri dish until class. Leave the protective blue papers on both sides of the nitrocellulose for protection.

2. Reconstitute bee venom according to manufacturer's instructions and dilute some of it to 1.0 mg/ml. Store 10 µl aliquots in microfuge tubes in the freezer, thaw just before class, and hold on ice. A vial of bee venom will last many years. Venom should not undergo repeated thawing and freezing cycles.

3. BSA and normal rabbit serum should be aliquoted into microfuge tubes as well, stored in the freezer, and thawed just before class. Hold on ice.

4. Make up 4 liters of Tris buffered saline (TBS), some of which will be used for TTBS. Aliquot out 25-ml portions for each student. Store in the refrigerator until class.

5. Using the remaining TBS above, make up 3 liters of TTBS, Tris buffered saline containing 0.05% Tween 20, and dispense 100 ml into each of 20 glass bottles for the students. Store in the refrigerator until class. The remainder will be used for the milk and antibody solutions, and any leftover should be stored in the refrigerator.

6. Make up 125 ml of 5% blocking solution by adding 6.25 g instant non-fat dried milk to 125 ml TTBS. This should be made just before class and can sit at room temperature.

7. Make up 125 ml of antivenom: Reconstitute antivenom in distilled water as directed on the vial, and then dilute 1.25 ml in 125 ml of 1% gelatin-TTBS. Store in the refrigerator, then place in a 37°C water bath for a few minutes just before use. Reconstituted stock antivenom should be aliquoted and stored in the freezer at –20°C if possible.

8. I put at each station one of each of the following: bee venom aliquot, BSA aliquot, and NRS aliquot all on ice; 25 ml TBS, 100 ml TTBS. Blocking solution, antibodies, and enzyme substrate are all held at the front of the classroom and are used communally.

During Lab And Just Before Use

1. Make up 125 ml of alkaline phosphatase conjugate: Dilute stock goat anti-rabbit IgG-alkaline phosphatase antibody 1:5000 in 1% gelatin-TTBS (25 µl antibody in 125 ml 1% gelatin-TTBS).

2. Make up BCIP/NBT substrate: 1.0 ml Solution A, 1.0 ml Solution B, and 100 ml Kit Buffer. Store stock substrate components as suggested by the supplier. Alternatively, purchase 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) separately and make up solutions as follows: 15 mg BCIP in 1 ml N,N-dimethylformamide (DMF) and 30 mg NBT in 0.7 ml DMF plus 0.3 ml water. Add 1 ml BCIP and NBT to 100 ml carbonate buffer (0.1 M $NaHCO_3$, 1.0 mM $MgCl_2$, pH 9.8) just before use. The DMF should be opened and used under a chemical hood.

Student Outline

Objectives

1. To learn the principle of the enzyme immunoassay detection method.
2. To appreciate the immunologic basis of allergic reactions.
3. To learn how to pipet small volumes.
4. To learn about the specificity of the antigen-antibody reaction.
5. To gain an understanding of the concept of sensitivity and detection limits.

Introduction

The venom of the honeybee, *Apis mellifera*, contains numerous enzymes, volatiles and other interesting substances. Many individuals are allergic to the protein components of bee venom, or become allergic after working with bees. The growing problem with Africanized bees in the United States has revitalized interest in the properties of venom and in the immune response to the venom components. Allergic individuals usually develop a localized skin lesion called a hive at the site of a bee sting, but more serious systemic reactions also occur, including anaphylactic shock and death. Both local and systemic manifestations of bee venom allergy are due to immunoglobulin E antibodies to antigenic components in *Apis mellifera* venom. Bee venom antigens can easily be detected in the laboratory using an enzyme immunoassay (EIA) technique. Anti-venom antibodies can be detected by EIA as well, though clinical allergy testing is based on radioisotopic techniques.

Enzyme immunoassay is one of the most frequently used detection methods in the clinical laboratory today and is rapidly supplanting assays based on radioisotopes. Enzyme immunoassay is sensitive, safe, and highly specific. Two examples of EIA methods in common use are the enzyme-linked immunosorbent assay (ELISA), and the Western blot, both used in testing for exposure to the Human Immunodeficiency Virus.

The principle of EIA detection is the same for all methods. Its specificity depends on the binding of antibodies to protein antigens immobilized on a surface, such as nitrocellulose paper or plastic. Its sensitivity depends on the level of enzymatic activity retained in the reaction mix capable of converting a colorless substrate to a colored one. In the Western blot, the colored substrate is deposited onto a paper surface, and the location of substrate deposition is highly informative. ELISA substrates, on the other hand, remain in solution and can be measured in a spectrophotometer. The ELISA test is highly sensitive and can be designed to be quantitative as well. Several enzyme-substrate systems in common use are listed in Table 13.1.

Table 13.1. Enzyme-substrate systems for immunodetection.

Enzyme	Substrate	Color	Use
alkaline phosphatase	p-nitrophenyl phosphate	yellow	ELISA
alkaline phosphatase	BCIP/NBT*	purple	blotting and histochemistry
horseradish peroxidase	ABTS **	green	ELISA
horseradish peroxidase	3-amino-9-ethylcarbazole	blue	blotting and histochemistry

* 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium
** 2,2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid

In this lab we will perform immunodetection of dilutions of honeybee proteins spotted onto nitrocellulose membranes. The procedure requires a series of incubation steps, each of which is followed by extensive washing of unbound proteins.

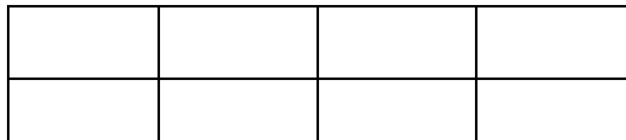
We will first pipet dilutions of bee venom proteins directly onto nitrocellulose membranes. The unoccupied portions of the membranes will then be blocked using a suspension of non-fat dried

milk. A commercially prepared anti-venom prepared in rabbits (rabbit anti-*Apis mellifera* venom antibodies) will supply the primary antibodies for binding to the immobilized bee venom proteins. The secondary antibody preparation is one prepared in mice which binds specifically to rabbit antibodies and at the same time is conjugated with the enzyme alkaline phosphatase (mouse anti-rabbit IgG-AP). When the substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) comes into contact with the immobilized enzyme, it is converted to its colored form and is deposited as a purple product on the nitrocellulose paper. The intensity of the localized color reaction is directly proportional to the amount of bee venom spotted onto the nitrocellulose in the first step.

The result is a thick open-faced sandwich, where the nitrocellulose is the "bread," the bee venom is the "butter," the primary antibody is the "lettuce," the secondary antibody-enzyme conjugate is the "salami," and the substrate is the "cheese," melting down and sticking to all of the sandwich components.

Procedure

Obtain a piece of nitrocellulose (NC) from the instructor. Handle NC with clean gloves or forceps, in order to avoid transferring skin proteins to the paper. Using a pencil and straight-edge, and with the protective blue papers still in place, draw lines on the paper, dividing it into eight sections, as shown. Press hard enough so that depressions are made in the NC. Mark one corner for orientation.
(5 minutes)



2. Pipet 5 ml Tris buffered saline (TBS) into a petri dish using a transfer pipet. Holding the NC with forceps, lower it slowly into the liquid, wetting the paper thoroughly. Wetting should occur by capillary action, not by dunking. Set the paper out on a paper towel to dry, or dry with a blow dryer. Discard the TBS, but save the petri dish for further incubations. (5 minutes)

3. *Note: A student who is allergic to bee venom should wear gloves and safety glasses when pipetting the venom in this step to avoid any possible contact with the allergen.* Dilute the bee venom using the 1 mg/ml stock. Make dilutions containing 0.5 mg/ml, 0.1 mg/ml, and 0.01 mg/ml of bee venom as follows:

   Step 1: Pipet 3 drops of distilled water onto parafilm: 5 µl, 8 µl, and 9 µl.

   Step 2: Pipet 5 µl of the 1 mg/ml bee venom stock directly into the 5 µl water drop and mix by pipetting carefully a few times. The drop will slide around on the parafilm a bit because of the hydrophobic nature of the parafilm and the high surface tension of the water. You have made a 1:1 dilution and the bee venom concentration in this drop should now be 0.5 mg/ml. This is sometimes called a 1 in 2 dilution.

   Step 3: Pipet 2 µl from the 0.5 mg/ml drop into the 8 µl drop of water and mix. This is a 1:4 or a 1 in 5 dilution and the bee venom concentration in this drop should now be 0.1 mg/ml.

   Step 4: Pipet 1 µl from the 0.1 mg/ml drop into the 9 µl drop of water and mix. This is a 1:9 or a 1 in 10 dilution and the bee venom concentration in this drop should now be 0.01 mg/ml.

4. Set the micropipettor at 1 µl and attach a white pipet tip. Spot 1 µl samples of the dilutions of bee venom in the four upper quadrants of the NC (left to right): 0.01 mg/ml, 0.1 mg/ml. 0.5 mg/ml, and 1.0 mg/ml. If you pipet the samples in this order, you will need only one pipet tip. Discard the tip into a tip disposal container. Using a fresh tip for each of the controls, spot 1 µl of normal rabbit serum (NRS) in one of the lower quadrants as a positive control, and pipet 1 µl of bovine serum albumin (BSA) into a second quadrant as a negative control. Allow the proteins to dry, or dry with a blow dryer. (10 minutes)

5. Re-wet the nitrocellulose as done previously by lowering it into 5 ml of a suspension of 5% non-fat dry milk in Tris buffered saline (TBS) containing 0.05% Tween-20 (TTBS). The proteins in the milk will bind to the sites on the paper which are not occupied by bee venom or serum, and will prevent non-specific binding by antibodies in subsequent steps. Incubate 15 minutes at room temperature on the shaker. The liquid should wash slowly back and forth across the membrane during this step. (15 minutes)

6. Pour off the milk into the sink and wash the NC three times in 5 ml of TTBS for 5 minutes each, on the shaker, or by hand. Tween-20 is a detergent present in the wash buffer to aid in releasing non-adherent proteins from the nitrocellulose without denaturing the proteins of interest. (15 minutes)

7. Pour off the last wash and pipet 5 ml of the primary antibody solution into the dish. The rabbit anti-*Apis mellifera* antibody has been diluted 1:100 in 1% gelatin-TTBS. Incubate the NC on the shaker for 45 minutes. During this incubation, we will discuss the experiment together as a class. (45 minutes)

8. Pour off the primary antibody into the designated container and wash the NC three times in TTBS as done previously. The primary antibody solution may be stored in the refrigerator up to 1 month and reused one or two times, as long as it does not become contaminated. (15 minutes)

9. Pour off the last wash and pipet 5 ml of the secondary antibody into the dish. The goat anti-rabbit IgG-alkaline phosphatase conjugate has been diluted 1:5000 in 1% gelatin-TTBS. Incubate the NC on the shaker for 30 minutes. (30 minutes)

10. Pour off the secondary antibody into the animal waste container and wash the NC two times in TTBS and then once in TBS. The secondary antibody solution cannot be reused. (15 minutes)

11. Pour off the last wash and pipet 5 ml of BCIP/NBT substrate into the dish. In the presence of alkaline phosphatase, the yellowish BCIP/NBT will turn purple and precipitate out onto the nitrocellulose paper wherever the enzyme has been localized. Incubate the NC on the shaker until spots are clearly visible and the background has not begun to darken. You should see spots of increasing intensity of color from left to right, and the positive serum control should be stained as well. The BSA spot should not be stained. (5–10 minutes)

12. Pour off the BCIP/NBT and wash your NC in several changes of distilled water over 10 minutes. Dry the NC between paper towels away from light. Store it in transparent wrap in your lab notebook.

Thought/Study Questions

1. Why must you not touch the NC directly?

2. What is the chemical basis for the binding of protein to NC?
3. Why is normal rabbit serum used as a control? What does it control for? Does this assay appear to be specific?
4. What do phosphatases do? How does alkaline phosphatase produce a colored product in this reaction?
5. What is Tween 20 and why is it included in the washes? Why is it not included in the last wash before addition of BCIP/NBT?
6. What is the function of gelatin in the antibody buffers? What is gelatin?
7. What is in bee venom, and how could you see if antibodies to specific components of venom are present in the antiserum?
8. In individuals allergic to bee venom, what antibody class predominates? How could you detect it using this type of system?
9. How much bee venom was in each spot? Remember, 1 mg/ml = 1 μg/μl and 1 μg = 1000 ng.
10. According to your results, how sensitive is this type of immunodetection assay? Do you know which substances in human blood are present in this approximate concentration?