Chapter 2

Identification, Purification, and Characterization of Staphylococcal Superantigens

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Abstract

Purifying natively produced staphylococcal superantigens is an important process in the study of these proteins, as many common methods of protein purification are affected by staphylococcal protein A contamination. Here, we describe a proven approach for identifying superantigens in vitro as well as for purifying novel superantigens both in His-tagged and native forms using modern genetic tools coupled with thin-layer isoelectric focusing.

Key words Staphylococcal superantigen, Purification, Identification, Characterization, Polyclonal antibody production

1 Introduction

Superantigens function as potent stimulators of the immune system and significantly contribute to disease progression in food poisoning [1], toxic shock syndrome [2], endocarditis [3], and pneumonia [4]. A new staphylococcal superantigen, staphylococcal enterotoxin-like X, has been described as recently as 2011 and was shown to contribute significantly to necrotizing pneumonia in the endemic methicillin-resistant *Staphylococcus aureus* (MRSA) strain USA300 [4]. This recent discovery, in conjunction with ~50 % of the *S. aureus* secretome being undefined [5], warrants a standard method in identifying, purifying, and characterizing new staphylococcal superantigens as they arise, as well as already known ones that are needed for pathogenesis studies.

Superantigens range in size from 19 to 30 kDa and lack post-translational modification. Their molecular weights can make them inherently difficult to identify by Western blot in a complex mixture of proteins due to the presence of protein A. Protein A is a staphylococcal protein that is both cell surface-associated and -secreted, having molecular weights of 15, 30, 45, and 60 kDa. Protein A is capable of binding the Fc portion of antibodies,

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making Western blot analysis difficult when desired superantigens fall in the same molecular weight as protein A. Our method described herein is capable of separating superantigens from the contaminating protein A based on their neutral to basic isoelectric properties.

Superantigen purification also benefits from the molecules' stabilities under many denaturing conditions such as boiling, pepsin/trypsin treatment, desiccation, and ethanol precipitation. Our protocol is able to harness the stability of superantigens during ethanol treatment to remove many small and large molecule contaminants. This precipitate can then be solubilized in water and proteins separated based on pH gradients set up by ampholytes in isoelectric focusing under continuous current. The purity of this natively expressed protein is typically ≥99 %, as determined by Coomassie blue R250 and silver staining.

As new superantigens arise, it is imperative to have a standard method to identify their production in culture fluids through stimulation of peripheral blood mononuclear cells (PBMCs) from humans, fever production in rabbits, and endotoxin enhancement in rabbits. It has been previously demonstrated that administering lipopolysaccharide (LPS), approximately 2–4 h after administration of a superantigen, synergizes to increase lethality by up to 10^6-fold [6]. These approaches have inherent difficulties with detecting superantigenicity because *S. aureus* is also capable of producing potent cytotoxins. Our protocols presented here have ways to avoid inadvertent proinflammatory responses from cytotoxins, allowing successful identification of superantigen-containing culture fluids.

Generation of antibodies to desired superantigens is important once purified proteins are isolated. IEF, as defined in this methodology chapter, or through use of His-tagged recombinant technology, as discussed in a previously published methodology [7], are both suitable sources of proteins for generating superantigen-specific polyclonal antibodies from rabbits. Previous work by our laboratory has demonstrated strong antibody responses develop to superantigens in sublethal-dose vaccination studies [8, 9], thus allowing us to use the same protein amounts to generate specific polyclonal antibodies, which in turn can be subsequently used for many immunological techniques.

2 Materials

2.1 Identification of New Staphylococcal Superantigens

All glassware and reagents *must be* maintained pyrogen-free to avoid LPS contamination.

1. 25 mL overnight culture grown in Todd Hewitt broth at 37 °C with aeration, of suspected superantigen-producing *S. aureus* strain.

2. 25 mL defibrinated rabbit red blood cells.
3. 0.45 μm filters.
4. 0.8 % agarose in phosphate-buffered saline (PBS) kept at 50 °C.
5. 50 mL polypropylene conical tubes.
6. Glass microscope slides.
7. 100 mm × 15 mm Petri dish.
8. 5 mL serological pipette and dispenser.
9. 4 mm vacuum-assisted hole punch.
10. 6 New Zealand white rabbits (2 kg each).
11. 1 mL syringes.
12. 25-gauge needles.
13. Lipopolysaccharide (LPS): isolated from *Salmonella enterica* serovar Typhimurium, 1 mg/mL solubilized in PBS.
14. Fast reading, electronic rectal thermometer.

2.2 Superantigen Purification

All materials *must be* maintained pyrogen-free to avoid LPS contamination.

1. *Dialyzed Beef Heart (BH) growth medium:* Todd-Hewitt medium is useful in everyday studies of staphylococcal cultures however, for the purpose of superantigen purification it is optimal to use a dialyzed medium. Many medium proteins fall within a molecular weight of 20–50 kDa and superantigens are also within this range. Excessive medium protein can contaminate and cause false readings when determining protein purity by Coomassie blue R250 or silver stain.

   To bypass this problem we use a dialyzed beef heart (BH) medium, made directly from bovine hearts which is summarized below [10].

   (a) Add 10 lb of ground beef heart to 3 L of pyrogen-free H_2O and heat to 70 °C.

   (b) Stir tissue constantly, to avoid burning, for 30 min at 70 °C.

   (c) Cool the mixture to ~45 °C.

   (d) Add 16 g of porcine pancreas trypsin to proteolyze the proteins in the tissue.

   (e) A pH of 8 must be maintained via titration of 10 M NaOH, as needed, with vigorous stirring for 2 h.

   (f) After 2 h, bring the pH to 8.5, cover with foil and store at 4 °C for 18 h.

   (g) The next day, remove mixture from 4 °C and remove any large masses of fat floating on the top as this can affect the dialysis process.

(h) Be sure to adjust the pH to 8.0 if the pH has dropped below that point.

(i) Slowly add the trypsinized tissue mixture to 3 ft long segments of 45 mm, 12–14 k molecular weight (MW) cut-off dialysis tubing (*see* **Note 1**).

(j) After filling tubes, place in baked 20 L glass carboys with an equal number of dialysis tubing per carboy.

(k) Fill each carboy with 10 L of pyrogen-free water and dialyze for 4–5 days at 4 °C.

(l) After dialysis, the dialysate should be brown. This will contain small molecular weight BH proteins and other essential factors; the dialysate is used as the preferred microbiological medium.

(m) Combine the dialysates from each carboy and mix well.

(n) We find 1200 mL of BH media in 3 L Fehrenbach flasks provides the optimal growth conditions for aeration and superantigen production in ambient air. This volume will be used throughout the protocol.

2. All glassware is heated (baked) to 165 °C for a minimum of 3 h before use to avoid LPS contamination. Any items that cannot be heated are soaked in 10 % sodium dodecyl sulfate solution for 30 min and washed with pyrogen-free water.
3. Two to four 3 L Fehrenbach flasks containing 1200 ml of BH medium each (as prepared in Subheading 2.2, **item 1**).

(a) BH medium is supplemented 1:100 (v/v; 1 % final volume) with glucose-phosphate buffer.

(b) Glucose-phosphate buffer: 0.33 M dextrose, 0.5 M $NaHCO_3$, 0.68 M NaCl, 0.12 M $Na_2HPO_4 \cdot 7H_2O$, 0.027 M L-Glutamine.

4. Overnight culture of bacterial isolate grown in 25 ml Todd Hewitt broth.
5. Approximately 20 L of 200 proof ethanol for toxin precipitation.
6. Washed G-75 Sephadex: prepared by following steps. Washing is done to completely dehydrate and collapse the Sephadex beads. The collapsed Sephadex is used as a gel matrix for proteins to migrate through without being hindered by molecular weight.

(a) Swell Sephadex in a 4 L flask with 4 L of water overnight at 4 °C.

(b) Exhaustively dehydrate with ethanol the next day using a vacuum collection flask with Whatman paper in a funnel as a filter.

(c) Allow Sephadex to dry to a fine powder in a chemical safety hood before straining to remove large aggregates and returning to original container.

7. Autoclaved, nano-purified deionized pyrogen-free water contained in baked glassware.
8. Spectra/Por dialysis membrane, 45 mm, 12–14 k MW cutoff and 23 mm, 6–8 k MW cutoff (Spectrum Laboratories Inc., Rancho Dominguez, CA)
9. Pharmacia-LKB Biotechnology Multiphor 2117 thin-layer isoelectric focusing apparatus able to accommodate a plate size of 26 × 12.5 cm (Amersham Pharmacia Biotech, Piscataway, NJ). Plate needs to be maintained on a glass continuous water flow chamber at 2 °C. A constant power source capable of maintaining 1000 V, 20 mAmps, and 8 W for 24 h is required to run this machine.
10. A 26 × 12.5 cm glass bottom thin-layer isoelectric focusing plate with rubber boarders.
11. Isoelectric focusing (IEF) electrodes (GE Healthcare, Uppsala, Sweden).
12. IEF electrode strips.
13. Solutions of 50 mL 1 M NaOH and 50 mL of 1 M H_3PO_4 to set up the IEF electrodes.
14. Ampholytes with pH ranges 3–10, 6–8, and 7–9 (Sigma-Aldrich, St. Louis, MO).
15. 3MM Whatman paper.
16. 10 % trichloroacetic acid.
17. Coomasie R250 protein stain and destain.
18. Antibodies against your superantigen for Western blot analysis (if available).
19. Pyrex™ glass wool.
20. 0.45 μm syringe filter.

2.3 Mitogenicity Assay

Use only plastic materials when working with lymphocytes.

1. 50 mL human blood.
2. 50 mL polypropylene conical tubes.
3. 15 mL polypropylene tubes.
4. 200 proof ethanol.
5. Pyrogen-free H_2O.
6. 100 mL Histopaque-1077® solution (Sigma Aldrich, St. Louis, MO).
7. 500 mL Roswell Park Memorial Institute medium (RPMI)-1640.
8. 20 mL Complete RPMI: supplemented with 1 % Penicillin-Streptomycin (10,000 U/mL), 2 % Fetal Calf Serum, 20 μM glutamine.
9. Hemocytometer.

10. 96-well tissue culture plate.
11. 100 scintillation vials.
12. 300 mL Cytoscint™-ESLiquid Scintillation Cocktail (MP Biomedicals, LLC, Solon, OH).
13. Titertek® Plus Cell Harvester.
14. Pressed fiber-glass paper.
15. Scintillation counter capable of making counts per minute (cpm) readings.

2.4 Generating Polyclonal Antibodies in Rabbits

1. New Zealand White rabbit (under 6 months of age).
2. Freund's incomplete adjuvant.
3. Three aliquots 10–20 μg of superantigen in PBS at final volumes of 500 μl.
4. 3 ml sterile syringe.
5. 5 ml sterile glass culture tubes.
6. 18- and 20-gauge syringe needles.
7. 1 and 60 mL sterile syringes.
8. 50 ml conical centrifuge tube.
9. Sterile wooden applicator sticks.
10. Sterile microfuge tube.
11. Glass microscope slide.
12. 5 mL serological pipette and dispenser.
13. 4 mm vacuum-assisted hole punch.
14. 0.8 % agarose in PBS (kept at 50 °C until dispensed).
15. Non-specific protein of choice to test antibody specificity.
16. Ketamine 100 mg/ml.
17. Xylazine 100 mg/ml.
18. Ethasol® (Active ingredients 390 mg/ml pentobarbital sodium and 50 mg/ml phentoin sodium) (Vibrac AH, Inc., Fort Worth, Texas).

3 Methods

3.1 Identification of New Staphylococcal Superantigens

1. Centrifuge overnight culture of *S. aureus* strain (25 ml) at 4000 × *g* for 10 min.
2. Remove bacterial supernate and filter-sterilize through a 0.45 μm filter.
3. Add 1:1 defibrinated rabbit red blood cells to bacterial supernate and rock gently at 37 °C for 1 h.
4. Centrifuge blood/supernate mixture at 4000 × *g* for 10 min.
5. Remove supernate and filter-sterilize through a 0.45 μm filter.

6. Using 0.8 % agarose in PBS, add rabbit red blood cells to a final concentration of 3 % red blood cells.
7. Quickly dispense 4 ml of agarose/blood mixture onto microscope slides.
8. Allow agarose/blood mixture to solidify at room temperature. Keep solidified agarose slide in 100 mm × 15 mm Petri dish on top of a moist paper towel to provide a humidified environment.
9. Using a vacuum-assisted hole punch, punch a 4 mm hole into the agarose, remove the plug, and add 20 μl of supernate from **step 5**.
10. Incubate at 37 °C for 4 h.
11. If major cytolysins have been removed, no zone of clearing should be seen surrounding the supernate.
12. If zones of clearing are present, repeat **steps 1–5** as needed to remove residual cytolysins.
13. Once cytolysins have been removed, make 1:10 and 1:100 serial dilutions from the original supernate.
14. Inject, intravenously, two rabbits per group with 2 mL of each dilution (undiluted, 1/10, and 1/100), as well as two PBS-only controls (*see* **Note 2**).
15. Measure fever responses hourly over 4 h, noting that superantigens typically cause linear rises in body temperatures, peaking at 4 h (*see* **Note 3**).
16. After 4 h, intravenously inject each rabbit, excluding PBS control, with 5 μg of LPS (1/100 of LD_{50} dose) (*see* **Note 4**).
    (a) LPS is given at this time point because LPS and superantigen synergism is optimal at this point and will cause rapid lethality if superantigen is present.
17. Assess lethality over a 48-h period (*see* **Note 5**).
18. If fever and enhancement of LPS lethality are seen, proceed to purification steps.

3.2 Purification of Staphylococcal Superantigens

1. Inoculate 2–4 × 1200 mL flasks of BH plus 1 % glucose-phosphate buffer with overnight culture of desired superantigen-producing *S. aureus*.
2. Incubate cultures for 48 h at 37 °C at 220 × g (*see* **Note 6**).
3. After 48 h incubation, pour cultures into 6 L Erlenmeyer flasks already containing 4800 mL of 100 % ethanol, and let cultures precipitate for 2–5 days at room temperature or 4 °C, until liquid in flasks is transparent and mats of precipitate are at the bottoms (*see* **Note 7**).
4. Slowly pour off and discard the majority of clear supernate without agitating the precipitate into solution (*see* **Note 8**).

5. Collect precipitate in 500 mL plastic centrifuge bottles and centrifuge for 10 min at 4000 × *g* at 4 °C (*see* **Note 9**).
6. Allow pellets to air-dry for 2–3 h to remove ethanol. Pellet is considered free of ethanol when the pellet surface looks matte but not completely dry.
7. Add 75 mL of pyrogen-free H_2O and stir pellet with a magnetic stir bar and plate at 1000 × *g* for a minimum of 4 h at room temperature.
8. Remove stir bar and centrifuge at 8000 × *g* for 30 min at 4 °C; save the supernate and discard pellet.
9. Dialyze supernate in 45 mm, 12,000–14,000 MW cutoff dialysis tubing in 4 L pyrogen-free water overnight at 4 °C with gentle stirring (*see* **Note 1**).
10. Dry dialyzed sample (contents of the dialysis bag) to ~75–90 mL (*see* **Note 10**).
11. Empty sample into a 150 mL beaker and add 2.5 mL of pH 3–10 ampholytes.
12. Add approximately 4.5 g washed G-75 Sephadex to sample, stirring slowly to avoid air bubbles (*see* **Note 11**).
13. Cut 3 IEF electrode strips for each end of IEF plate, each a little smaller than the plate to allow for expansion after wetting.
14. Wet electrode strips with ~2.5 ml pyrogen-free water.
15. Pour Sephadex-containing sample into IEF plate and spread/smooth with a flat-edged spatula and light tapping plate on bench top to make a completely smooth surface.
16. Dry plate until the surface no longer looks glossy (should have a matte appearance). (If cracks occur *see* **Note 12.**)
17. After drying, cut two more wicks (slightly longer than the prior three wicks and slightly shorter than plate) for dipping in either 1 M NaOH or 1 M H_3PO_4 for placement on ends of the IEF plate; these will be the anionic and cationic ends, respectively.
18. Place IEF plate on thin-layer IEF water coolant plate.
19. Set the maximum settings: Volts → 1000; Watts → 8; mAmps → 20.
20. Let IEF run for 24 h.
21. Remove plate from apparatus, and with a flat-edged spatula, scrape non-cloudy portion of gel (NaOH end) into a new 150 ml beaker (*see* **Note 13**).
22. Bring non-cloudy fraction to 100 mL using sterile pyrogen-free H_2O and repeat **steps 11–20**, except this time use ampholytes with a pH range of 6–8 or 7–9, depending on isoelectric point of the superantigen you are trying to purify.

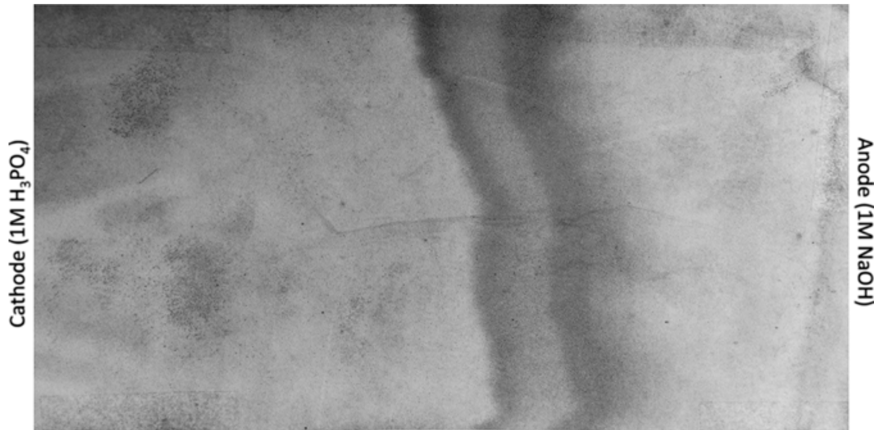

Fig. 1 Zymogram print of isoelectric focusing of TSST-1 during purification process. This is the secondary separation at pH 6–8. The two bands are TSST-1 with a difference in pH of 0.04, showing the high resolving power of this technique

23. Typically, after 18–24 h, you will notice a clear band in the second gel due to the light diffraction properties caused by the focusing of the superantigen. This band can be carefully scraped into a 50 mL conical tube, suspended in 10 mL pyrogen-free water, and then you can skip to **step 26**. If not proceed to next step.
24. The highest concentration of protein can be detected at this point through the use of a zymogram print on 3 MM Whatman paper. Briefly, over-lay plate with Whatman paper, wash for ~30 s with 10 % trichloroacetic acid and stain with Coomassie R250 and destain yielding a pattern seen in Fig. 1. This was a secondary separation at pH 6–8. The two bands are TSST-1 with a difference in pH of 0.04, showing the high resolving power of this technique.
25. Grid the plate into roughly 15 equal-sized fractions (from anode to cathode), and using a flat-edged spatula, scrape each into 50 mL conical tubes, rinsing spatula between fractions.
26. Suspend each fraction in 5 mL of sterile pyrogen-free H_2O, invert 5–10 times, and let settle at 4 °C overnight.
27. Carefully pipette ~10 μl of supernate out of each tube, avoiding Sephadex (*see* **Notes 14** and **15**), and run on SDS-PAGE to examine protein content. At the same time, the supernate can be placed in a pH electrode for isoelectric point determination.
28. Identify fractions of desired protein in highest concentrations through Coomassie blue R250 and Western blot (if antibodies for the superantigen you are purifying are available) and then pool those fractions.

29. Pack 1 cm of glass wool into the bottom of a 60 mL syringe. Tape needle-tip end down to the top of a 50 mL conical tube and pour desired fractions in to allow gel filtration to occur. When most of the liquid has run through, plunge Sephadex/protein mix through the glass wool to remove the majority of the remaining liquid. This step will remove most of the Sephadex from the purified superantigen (superantigen remains in liquid portion).
30. Filter liquid containing your superantigen through a 0.45 μm pore size syringe filter.
31. Dialyze the purified superantigen in 6–8 k MW cutoff tubing for 4 days at 4 °C in 1 L pyrogen-free water with slow continuous stirring (*see* **Note 16**).
32. Quantify desired protein using Bradford reagent with a standard curve generated using a previously described superantigen for best accuracy.
33. An additional SDS-PAGE gel with subsequent Coomassie blue R250 staining should reveal a protein purity of ≥99 %.

3.3 Lymphocyte Mitogenicity Assay

The following procedure should be carried out in biological safety cabinet.

1. Draw 50 mL of human blood into 5 mL heparin to prevent clotting.
2. Dilute blood 1:1 with RPMI-1640 medium at room temperature into two 50 mL conical centrifuge tubes and mix gently by inversion.
3. At room temperature, pour 18 mL of Histopaque® into 4 × 50 mL tubes and carefully layer 24 mL of diluted blood on top (*see* **Note 17**).
4. Centrifuge at 400 × *g* for 30 min
5. Collect 5–6 mL of the buffy coat (central interface) by pipetting slowly, and combine in 50 mL centrifuge tube. Discard the remaining blood components.
6. Dilute buffy coat with RPMI-1640 to 50 mL and centrifuge at 400 × *g* for 10 min.
7. Discard supernate.
8. Resuspend pellet (lymphocytes) by gently pipetting 10 mL RPMI-1640 into the tube and gently pipetting up and down against the pellet.
9. Pipette resuspended cells into a 15 mL centrifuge tube and add RPMI-1640 to 15 mL total.
10. Centrifuge at 400 × *g* for 10 min.
11. Decant supernate and resuspend pellet in 15 mL RPMI-1640.

12. Centrifuge at $400 \times g$ for 10 min.
13. Decant supernate and resuspend pellet in 5 mL of *complete* RPMI.
14. Count the lymphocytes in a hemocytometer; adjust cells to a concentration of 2×10^5 cells/200 μL.
15. Add 200 μL of lymphocytes to 96-well, flat-bottom microtiter plates (quantity of wells will depend on total tests being carried out).
16. Serial dilute superantigen-containing fractions tenfold (final dilution 10^{-5}) from purification process to be added in 20 μl volumes per well of the microtiter plates (test samples in triplicate or quadruplicate) (*see* **Note 18**).
17. Serial dilute 1 mg/ml staphylococcal enterotoxin B (SEB) as a positive control (you want 1 μg/well–10^{-6} μg/well) and diluent from fractions (usually pyrogen-free water) as a negative control.
18. Each well will contain the following:
    - 20 μl of toxin dilution or control to appropriate well
    - 200 μl of diluted lymphocytes to each well
19. Incubate in 5 % CO_2 at 37 °C for 3 days.
20. On day 3, add 1 μCi tritiated thymidine to each well in a 20 μl volume.
21. Incubate in 5 % CO_2 at 37 °C for 24 h.
22. Titertek® Plus Cell Harvester is used to harvest lymphocyte population. First load empty scintillation vials into a vial holder to correspond with plate design.
23. Turn on vacuum attached to cell harvester, then turn the harvester on.
24. Fill two metal dishes, one with distilled H_2O and one with absolute ethanol.
25. Place white filter paper (fiberglass paper) on machine in designated places, and clamp it down to hold in place.
26. Use the vacuum to draw (~24 psi) H_2O onto the filter paper to wet it.
27. Use the vacuum to draw lymphocytes out of corresponding wells onto the filter papers, and wash with H_2O for seven fills of the wells.
28. Draw up ethanol onto the filter papers and pull vacuum for 1–2 min to help dry the filter paper.
29. Switch harvester to pull filter disks to the apparatus.
30. Turn off harvest switch, raise cut filter pieces, and place filter disks in corresponding tubes.

31. Repeat process for all wells
32. Let filter paper disks dry in tubes for at least 1 h
33. Add 3 mL of Cytoscint™-ESLiquid to each vial and cap.
34. Count on scintillation counter in cpm (or dmp) units (*see* **Note 19**).

3.4 Generating Superantigen-Specific Polyclonal Antibodies

1. Take ~1 mL of blood from a rabbit's marginal ear vein, to be used as a non-immune serum control.
2. Dispense 0.5 mL of Freund's incomplete adjuvant into glass culture tube.
3. Using 3 mL syringe and 18-gauge needle, add 0.5 mL of superantigen preparation to the incomplete adjuvant, and continuously pull up and push down (*see* **Note 20**).
4. Once mixture has become opaque and highly viscous, inject mixture in bolus doses, subcutaneously, into the nape of the neck of the rabbit (multiple sites).
5. **Steps 1**–**3** are repeated two more times on days 7 and 14 post primary immunization, for a total of three immunizations.
6. Seven days after final immunization, the rabbit is anesthetized using 0.8 ml Ketamine (100 mg/ml) and 0.4 ml Xylazine (100 mg/ml).
7. Direct cardiac puncture through the ribcage is performed using a 60 ml syringe and 18-gauge needle.
8. Exsanguinate the animal through this cardiac puncture. Roughly 80–100 mL of blood can be obtained from a 4 to 5 kg rabbit.
9. Our laboratory uses a 1 mL dose of Euthasol® (390 mg pentobarbital sodium and 50 mg phenytoin sodium) for premature euthanasia and then subsequent opening of the thoracic cavity to ensure animal expiration.
10. Blood should be distributed evenly across 50 ml conical tubes and stored at room temperature.
11. After 1 h at room temperature, ring around the blood clot using a sterile wooden applicator.
12. Tubes should then be stored at 4 °C for 18 h.
13. After 18 h, centrifuge tubes at 4000 × *g* for 30 min.
14. ***Carefully remove serum using a serological pipette and transfer to another conical tube.
15. Filter-sterilize serum using a 0.45 μm syringe filter.
16. Set up a double immunodiffusion assay by pipetting 4 mL molten 0.8 % agarose in PBS onto a microscope slide and allow it to harden at room temperature.
17. Carefully punch three 4 mm holes, 4 mm apart, into the agarose.

18. Add 20 μl of immunized serum to one end well and 20 μl of non-immunized serum on the other side.
19. In the center well, add 20 μl of a 100 μg/ml preparation of the purified toxin used for immunization.
20. Incubate slide in humidified chamber (Petri dish with moistened paper towel at the bottom) at 37 °C for 4-8 h.
21. Immunized serum should react with purified protein within 4-8 h, as depicted by a white immunoprecipitate between the serum and protein well.
22. Serum containing polyclonal antibody against superantigen should be aliquotted in 1 ml amounts and stored at -20 °C until needed.

4 Notes

1. All dialysis tubing should be thoroughly hydrated in water before adding any BH or protein sample. This can be done by submerging membranes in a beaker of water for ~1 min. Open the tubing by rubbing between your gloved fingers. Knot one end and fill with water to ensure no leaks are in the tubing before adding your sample.
2. LPS-only control rabbits can also be used.
3. In contrast, if there is contaminating LPS, the fever responses will peak first at 1 h and then at 3 h post-injection.
4. Alternatively, at the 4 h time point, the PBS rabbits may be injected with LPS to verify lack of enhanced lethality.
5. Synergistic effects of superantigen and LPS are potent. If a superantigen is present, it is likely all rabbits will die or be severely ill by 2-4 h, but we keep the animals for 48 h.
6. Secreted virulence factors, i.e. superantigens, are typically made in early stationary phase. Therefore, 48 h ensures they are produced to maximum amount and maximally released from the cell wall.
7. A 1 (culture):4 (ethanol) dilution is critical for ethanol precipitation to ensure small molecules <10 kDa remain in the supernate, while heavier molecules precipitate. This removes the majority of the medium proteins.
8. Precipitate will slide and collect on one side of the flask. This is expected but caution should be taken to avoid pouring out too much of the precipitate as this contains the desired superantigen.
9. This step will have to be repeated multiple times until all flasks are empty. Simply pour off supernate after each centrifugation, and add more precipitate.

10. Drying of dialysis tubing can be significantly expedited if done on a vent in a biological safety cabinet. Rotate tube frequently to avoid uneven drying and cracking.
11. Sample plus Sephadex should be cake batter consistency to allow easy spreading and smoothing of the surface.
12. Again, drying process can be expedited by drying on a vent in biological safety cabinet. If cracks occur, scrape out sample and rehydrate to prior consistency before re-plating. If any cracks are present, the electrical circuit cannot be completed.
13. Cloudy portion (H_3PO_4 end) is largely contaminating cell debris and can be discarded. At the anionic end (NaOH end), a thin white band will appear. This should be carefully discarded as well as it disrupts the pH gradient.
14. After allowing the fractions to settle at 4 °C in 5 mL of water, a small band of separation can be seen above the settled Sephadex. This is the liquid you want to use in your SDS-PAGE.
15. If a specific superantigen has not been identified, proceed to superantigenic fraction identification as determined by lymphocyte mitogenicity assay upon exposure to each fraction from the IEF plate.
16. Do not allow dialysis to proceed longer than 4 days. Protein will irreversibly fall out of solution.
17. Layer carefully by placing pipette tip on side and slowly allow the blood to run down the side of the tube, ideally having ~10 mL clear layer on the bottom.
18. The lymphocyte mitogenicity assay can be used on known superantigens in order to check functionality of the purified protein. Alternatively, you can perform a mitogenicity assay on unknown, potential superantigens, in which you would test the individual fractions from the IEF purification process and look for tritiated thymidine uptake into DNA by PBMCs as an indication of superantigen-stimulated lymphocyte division.
19. Fractions with lymphocyte mitogenic activity will appear as higher readings on the scintillation counter. As many dilutions are used per fraction, it should be relatively clear what fraction the superantigen is located in, as more activity at more dilute levels of supernate indicate higher concentrations of superantigen.
20. As you plunge the fluid in and out of the syringe, the protein and emulsifying agent will begin to turn opaque and become difficult to draw into the syringe. It is important to keep the mixture moving, regardless of difficulty until it is injected into the rabbit.

References

1. Argudin MA, Mendoza MC, Rodicio MR (2010) Food poisoning and *Staphylococcus aureus* enterotoxins. Toxins (Basel) 2:1751–1773
2. Altemeier WA, Lewis S, Schlievert PM, Bjornson HS (1981) Studies of the staphylococcal causation of toxic shock syndrome. Surg Gynecol Obstet 153:481–485
3. Salgado-Pabon W, Breshears L, Spaulding AR, Merriman JA, Stach CS, Horswill AR, Peterson ML, Schlievert PM (2013) Superantigens are critical for *Staphylococcus aureus* infective endocarditis, sepsis, and acute kidney injury. mBio 4:e00494–13
4. Wilson GJ, Seo KS, Cartwright RA, Connelley T, Chuang-Smith ON, Merriman JA, Guinane CM, Park JY, Bohach GA, Schlievert PM, Morrison WI, Fitzgerald JR (2011) A novel core genome-encoded superantigen contributes to lethality of community-associated MRSA necrotizing pneumonia. PLoS Pathog 7:e1002271
5. Kusch H, Engelmann S (2014) Secrets of the secretome in *Staphylococcus aureus*. Int J Med Microbiol 304:133–141
6. Schlievert PM (1982) Enhancement of host susceptibility to lethal endotoxin shock by staphylococcal pyrogenic exotoxin type C. Infect Immun 36:123–128
7. McCormick JK, Schlievert PM (2003) Expression, purification, and detection of novel streptococcal superantigens. Methods Mol Biol 214:33–43
8. Spaulding AR, Lin YC, Merriman JA, Brosnahan AJ, Peterson ML, Schlievert PM (2012) Immunity to *Staphylococcus aureus* secreted proteins protects rabbits from serious illnesses. Vaccine 30:5099–5109
9. Spaulding AR, Salgado-Pabon W, Merriman JA, Stach CS, Ji Y, Gillman AN, Peterson ML, Schlievert PM (2014) Vaccination against *Staphylococcus aureus* pneumonia. J Infect Dis 209:1955–1962
10. Schlievert PM, Shands KN, Dan BB, Schmid GP, Nishimura RD (1981) Identification and characterization of an exotoxin from *Staphylococcus aureus* associated with toxic-shock syndrome. J Infect Dis 143:509–516