
Preface

Superantigen Protocols assembles experimental protocols that have proved useful for the study of superantigens. These techniques will allow researchers from various areas of cell biology, microbiology, immunology, biochemistry, and molecular biology to assess the physical characteristics and biological effects of well-known superantigens as well as of putative substances that might have superantigenic activities, and to explore therapies for superantigen-induced effects.

Microbial exotoxins have been studied for decades as virulence factors because of their pathogenic effects. The term “superantigen” was coined a decade ago for some of these molecules because of their potent T-cell stimulatory activities. In recent years, advances in molecular biology provide recombinant as well as natural superantigens in highly purified form for physical characterization. Superantigens are now used extensively as tools to study interactions between receptors on cells of the immune system as they bind to major histocompatibility complex class II molecules on antigen-presenting cells and V_{β} regions of T-cell receptors. The biological effects that result from these interactions are studied both *in vitro* and *in vivo*. The intent of this book is, therefore, to bring together up-to-date techniques developed by experts in the field of biochemistry, immunology, and molecular biology for the study of superantigens.

Superantigen Protocols begins with an overview of the field to provide background information on the various classes of superantigens and their structure. This summary is followed by a group of chapters on methods to define binding and other physical interactions of superantigens with their cellular receptors. Finally, techniques used in the study of the biological effects of superantigens are covered to guide researchers in the assessment of cellular activities and molecules induced by superantigens. In addition, approaches to the study and the development of therapeutics in suppressing the superantigen-induced effects are presented.

Teresa Krakauer, PhD

Expression, Purification, and Detection of Novel Streptococcal Superantigens

John K. McCormick and Patrick M. Schlievert

1. Introduction

Superantigens (SAGs) are a class of bacterial or viral proteins that aberrantly alter immune system function through simultaneous interaction with lateral surfaces of major histocompatibility (MHC) class II molecules on antigen presenting cells, and to particular variable regions of the T-cell antigen receptor (TCR) β -chain. Among the secreted virulence factors from group A streptococci are the streptococcal pyrogenic exotoxins (SPEs), also commonly known as erythrogenic toxins or scarlet fever toxins. Together with the enterotoxins and toxic shock syndrome toxin-1 (TSST-1) from *Staphylococcus aureus*, the SPEs belong to a larger family of related exotoxins collectively known as the “pyrogenic toxin” class of superantigens, which share functional activities, similar amino acid sequences, and conserved three-dimensional structures (1,2). Due to their notable association with scarlet fever and streptococcal toxic shock syndrome (TSS), SPE A (3,4) and SPE C (5) are generally considered to be the prototypical streptococcal superantigens.

Genome sequencing projects have recently revealed that group A streptococci possess genes encoding multiple superantigen sero-

types (6). From an early search of the incomplete *S. pyogenes* SF370 genome, Proft and colleagues characterized two novel streptococcal superantigens, termed SPE G and SPE H, and identified a portion of the *SPE J* gene (7). Both SPE I (8) and SPE J (8,9) have been characterized since then, and including the streptococcal superantigen (SSA) (10) and the multiple streptococcal mitogenic exotoxin Z (SMEZ) serotypes (11,12), there are eight described streptococcal superantigens. For discussion purposes, SPE A and SPE C will be referred to as the prototypical streptococcal superantigens while SPE serotypes G, H, I, J, SSA, and SMEZ will be referred to as novel streptococcal superantigens. Although SPE B and SPE F share the “SPE” nomenclature, these proteins have enzymatic activity (protease and DNase, respectively) and neither shares significant amino acid homology to the other streptococcal superantigens. Superantigen activity of both SPE B and SPE F remains debated in the literature.

The potency of bacterial superantigens borders on the absurd where significant T cell proliferation can be detected below the picogram (10^{-12} g)/mL range. Owing to this extreme potency, many debates exist in the literature regarding the ability of a novel protein to function as a superantigen that stem from the possibility that the protein in question was contaminated with trace amounts of genuine superantigen. For this reason, and the apparent ability of streptococci to produce multiple superantigens, it is prudent to obtain recombinant superantigen proteins expressed from *Escherichia coli* for characterization of biological activities. In this chapter, we will focus on two methods used in our laboratory to purify novel streptococcal superantigens for further functional, biochemical, and immunological characterization, and will also discuss various standard methods used for their detection.

2. Materials

1. Bacterial strains: We typically do our cloning in *E. coli* XL1-blue (Stratagene, Cedar Creek, TX) but other commonly used cloning strains are acceptable. We do our recombinant protein expression in

- E. coli* BL21 (DE3) (Novagen, Madison, WI). This strain harbors the DE3 lysogen, which encodes the T7 RNA polymerase under inducible control by isopropyl β -D-galactopyranoside (IPTG). Thus, strains that contain the gene to be expressed under control of the T7 promoter can be induced by the addition of IPTG.
2. Plasmids: For all of our streptococcal superantigen expression work, we have used the pET28 plasmid series (Novagen).
 3. Media: We routinely grow *E. coli* in Luria-Bertani (LB) media (**I3**) and although for protein expression we use a dialyzed beef heart medium (**I4**), growth in LB or other standard *E. coli* medium is acceptable.
 4. Water: We use either double-distilled or MilliQ filtered H₂O for all solutions and procedures after growth of the bacteria.
 5. Chemicals: We obtain NaCl, Tris-HCl, NiSO₄, imidazole, and urea from Sigma Chemical Co. (St. Louis, MO).
 6. Antibiotics: For cloning and plasmid maintenance of pET28, we use kanamycin (Sigma) at 50 μ g/mL. Stocks are made at 50 mg/mL in MilliQ water, filter sterilized (0.22 μ m) and stored at -20° C.
 7. IPTG: For induction of gene expression in *E. coli* BL21 (DE3) we use IPTG (Sigma) at 0.2 mM. Stocks are made at 200 mg/mL in MilliQ water, filter-sterilized (0.22 μ m) and stored at -20° C.
 8. Ethanol: We use 200 proof ethanol that does not contain other additives.
 9. Dialysis tubing: We use both 6000–8000 (23 \times 14.6 mm) and 12,000–14,000 (45 \times 29 mm) molecular-weight cut-off dialysis tubing (Spectrum Laboratories Inc., Rancho Dominguez, CA).
 10. Preparative Isoelectric focusing (IEF) apparatus: We use a LKB Multiphor 2117 electrophoresis module (Amersham Pharmacia Biotech, Piscataway, NJ). Other commercial available preparative isoelectric focussing equipment should also be acceptable (e.g., Biorad) (**I5**).
 11. Sephadex: For preparative IEF, we use Sephadex G-75 (Sigma) that has been swelled in water, exhaustively washed with absolute ethanol, and dried.
 12. Ampholytes: We use 3.5–10 pH gradient and 6.0–8.0 pH gradient ampholytes (Amersham Pharmacia Biotech AB, Sweden).
 13. Nickel column: We use the His-Bind Resin and buffer kit (Novagen) for purification of 6 \times His-tagged proteins.
 14. Charge buffer: 50 mM NiSO₄.

15. Binding buffer: 500 mM NaCl, 20 mM Tris-HCl, pH 7.9.
16. Phosphate-buffered saline (PBS): 5 mM Na₂HPO₄/NaH₂PO₄, 150 mM NaCl, pH 7.2.

3. Methods

This section describes two methods used in our laboratory to purify novel streptococcal superantigens, and discusses standard methods used for their detection. The two purification methods differ in respect to the presence or absence of an affinity tag engineered onto the N-terminus of the protein to aid in purification. Despite the methods discussed here, novel streptococcal superantigens have also been purified from culture supernatants using affinity chromatography with anti-superantigen IgG conjugated Sepharose (e.g., SSA) (*10*) or ammonium sulfate precipitation, chromatofocusing, and hydrophobic interaction (e.g., SMEZ) (*11*). Furthermore, novel streptococcal superantigens have been expressed as glutathione-S-transferase (GST) fusion proteins in *E. coli* and purified using glutathione (GSH) agarose followed by cation exchange chromatography (e.g., SPEs G, H, I, J) (*7,8,12,16*).

3.1. Purification of Native (Untagged) Streptococcal Superantigens

This section describes the methods we have used to purify novel streptococcal superantigens without the aid of an affinity tag. In our experience, inclusion of DNA encoding the superantigen signal peptide in the expression plasmid results in very poor yields of protein. Thus, we clone all of our genes lacking the signal peptide. Briefly, superantigen genes are cloned by polymerase chain reaction (PCR) into the pET28 plasmid. The forward primer incorporates a *NcoI* restriction site onto the PCR product while the reverse primer incorporates a *BamHI* restriction site following the stop codon of the gene. The *NcoI* site is engineered to allow for an in frame translational fusion with the ATG start codon encoded within the restriction site (CCATGG). Clones are verified by restriction digests and

DNA sequencing. Alternatively, if the gene contains an internal *NcoI* site, *NdeI* can be used with this vector. Other than this, cloning procedures are beyond the scope of this chapter. Once the proper clone is obtained and transformed into *E. coli* BL21 (DE3), proceed as follows:

1. Grow *E. coli* BL21 (DE3) containing the appropriate plasmid overnight at 37°C with shaking in 50 mL of LB broth containing 50 µg/mL kanamycin.
2. The following morning, subculture the culture at 1% into fresh prewarmed (37°C) beef heart medium or other suitable medium containing 50 µg/mL kanamycin. We typically grow 1200 mL of culture but the method can be scaled up or down as desired. A typical yield following the protocol is about 5 mg of pure superantigen per liter of culture.
3. Monitor growth until the absorbance at 600 nm is approx 0.5. Induce superantigen gene expression with the addition of IPTG (0.2 mM). Continue to grow 3–4 h.
4. Precipitate the cultures with 4 volumes of absolute ethanol (80% final concentration). This step causes lysis and dehydration of the *E. coli* cells releasing intracellular protein. This concentration of ethanol typically results in the differential precipitation of proteins greater than ~10,000 Da. Proteins are generally precipitated at 4°C for 2 or more days.
5. The ethanol is poured or siphoned off and the cell debris is concentrated by centrifugation.
6. Concentrated crude protein and cell debris is dried and resuspended in water; the precipitated superantigen protein will resolubilize. Culture supernatants (up to approx 10 L) are concentrated to 50 mL.
7. Centrifuge the cell debris at 10,000g, retain the supernatant, and dialyze (12,000–14,000 molecular weight cut off) the supernatant against 2 L of water overnight at 4°C.
8. We run two consecutive separations via flatbed isoelectric focussing (pH 3.0–10.0 ampholytes and pH 6.0–8.0 ampholytes) with a LKB Multiphor 2117 electrophoresis apparatus using Sephadex G-75. Ampholytes are added at 5% to a total sample volume of 50–75 mL. Sephadex is added to achieve a semi-solid support, which is poured onto the IEF plates to achieve a smooth, flat layer. The IEF gradient is run overnight (≥18 h) at 1000V, 8W, and 20mA. The initial

- 3.5–10.0 pH gradient removes a substantial amount of cell debris and contaminants (brown color at the acidic end of the gradient).
9. The visible band (due to a change in refractive index of the focused toxin in high concentration) is harvested. If an band is not visible, the entire basic end of the IEF (except for the brown acidic end) is harvested.
 10. The fraction is usually refocused in a pH 6.0–8.0 gradient under the same conditions used for the 3.5–10.0 pH gradient. Residual 3.5–10.0 pH ampholytes are not removed. For some superantigens with a lower pI (e.g., SPE A or SSA) we use pH 4.0–6.0 ampholytes for the second gradient.
 11. The visible band is harvested, Sephadex is removed by filtering through a syringe containing glass wool, and ampholytes are removed by extensive dialysis against water (4 d, 4°C) with 6,000–8,000 molecular-weight cut-off dialysis tubing (*see Note 1*). If a visible band is not seen, the Sephadex gel will require fractionating and the various fractions can be screened by antibody or sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).
 12. To assess purity, we run 10 µL of each sample on a 15% SDS-PAGE. **Figure 1** shows an example of this purification procedure for recombinant SPE J (**9**).

3.2. Purification of 6× His-Tagged Streptococcal Superantigens

This procedure generally follows the His-Bind kit protocol by Novagen (www.novagen.com) with small modifications.

1. Repeat **steps 1–3** from **Subheading 3.1**.
2. Pellet the cells by centrifugation and wash once with binding buffer. Although the manufacturer's protocols suggest using 40 mM imidazole in the binding buffer, we omit imidazole from the binding buffer.
3. Resuspend the cells at 50× concentration in binding buffer.
4. Lyse the cells with 4 rounds of sonication on ice, or alternatively using a French Press.
5. Spin down cell debris and filter (0.45 µm) the supernatant. We generally do not experience inclusion body formation with the streptococcal superantigens from this procedure, but if soluble protein yields are poor, the bacterial pellet should be solubilized in 6 M urea and checked for protein of the expected size (*see Note 2*).

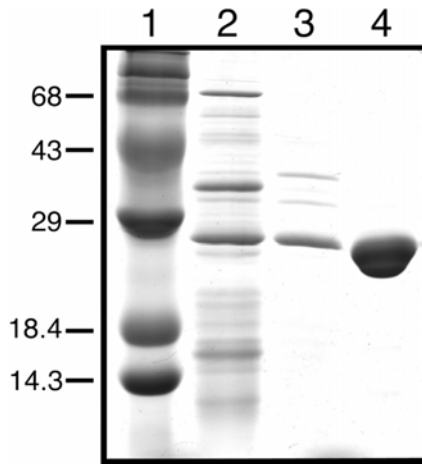


Fig. 1. Purification of streptococcal pyrogenic exotoxin J by ethanol precipitation and preparative isoelectric focusing. Shown is a 15% SDS-polyacrylamide gel stained with Coomassie brilliant blue. Lane 1, molecular-weight marker; lane 2, ethanol precipitated *E. coli* cell lysate; lane 3, protein preparation after pH 3.5–10.0 isoelectric focusing gradient; lane 4, protein preparation after pH 6.0–8.0 isoelectric focusing gradient.

6. Prepare the nickel column by gently resuspending the resin and pipeting the matrix into a standard column. The binding capacity of this column is approx 8 mg of recombinant protein per mL of resin and we generally use 1 mL of matrix for every 100 mL of *E. coli* culture. The resin can be reused for multiple purification runs of the same superantigen.
7. For 1 mL of resin we wash with 3 mL of H₂O, 5 mL of charge buffer, and 5 mL of binding buffer (*see Note 3*).
8. Because the loading volume for the nickel column is not an issue, dilute the sample 10× in binding buffer to dilute any remaining salts in the supernatant. Load 20 mL of diluted sample per 1 mL of column matrix. Collect the run through fraction.
9. Wash the column with 5 mL each of:
 - a. Binding buffer;
 - b. Binding buffer containing 30 mM imidazole;
 - c. Binding buffer containing 60 mM imidazole; and
 - d. Binding buffer containing 200 mM imidazole.Collect all fractions. The manufacturer's protocols recommend

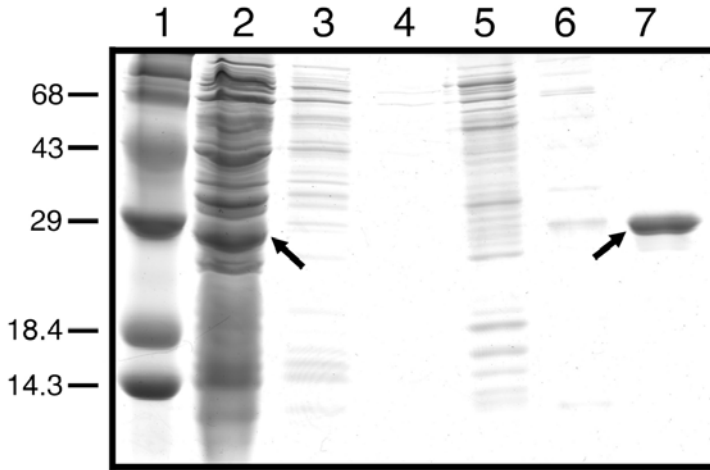


Fig. 2. Purification of 6 \times His-tagged streptococcal pyrogenic exotoxin G by nickel chelation chromatography. Shown is a 15% SDS-polyacrylamide gel stained with Coomassie brilliant blue. Lane 1, molecular-weight marker; lane 2, *E. coli* cell lysate; lane 3, fall through fraction after loading onto nickel column; lane 4, binding buffer wash fraction (0 mM imidazole), lane 5, 30 mM imidazole wash fraction; lane 6, 60 mM imidazole wash fraction; lane 7, 200 mM imidazole elution fraction. Arrows indicate SPE G in the crude cell lysate and purified SPE G in the 200 mM imidazole fraction.

elution of the protein with 1 M imidazole but 200 mM is typically sufficient.

10. Run 10 μ L of each sample on a 15% SDS-PAGE to assess purity. **Figure 2** shows an example of this purification procedure for recombinant 6 \times His-tagged SPE G. If cleavage of the 6 \times His-tag is desired, we suggest the Thrombin Cleavage Capture Kit (Novagen). Proteins are dialyzed against PBS before use in biological assays.

3.3. Detection of Novel Streptococcal Superantigens

We generally use a few standard techniques for the detection of the novel streptococcal superantigens during purification but these techniques can also be used for superantigen detection from clinical strains of group A streptococci. For the rapid detection of the presence of a streptococcal superantigen in high concentration

(>10 $\mu\text{g/mL}$) we use the Ouchterlony (double-immune diffusion) technique (17). Gels for double immunodiffusion are prepared with 0.75% agarose in PBS. Melted agarose is poured onto glass slides (4.5 mL/slide), solidified and 4 mm diameter wells are punched 4 mm apart in a hexagonal pattern. Twenty μL of each sample is placed in the outside wells and superantigen specific antisera is added to the center well. After approx 4 h incubation at 37°C, precipitin areas are indicative of superantigen reactive with the antiserum. For the detection of small amounts of a streptococcal superantigen we use Western-blot analysis, which is a standard procedure and is beyond the scope of this chapter.

The recombinant superantigens should be of sufficient purity (*see Figs. 1 and 2*) to perform most experiments including biological assays and immunizations. However, our structural biology collaborators many add a final “polishing” step prior to setting up crystallization plates (18).

4. Notes

1. Following purification of superantigen by preparative IEF, we remove ampholytes by dialysis. We typically do not extend the dialysis period past 4 d because in our experience the protein may precipitate as an insoluble form that we have not been able to satisfactorily solubilize.
2. During expression of recombinant protein from BL21 (DE3), occasionally the clone will no longer produce recombinant protein for reasons that are not readily clear. If this occurs, we generally retransform new BL21 (DE3) cells with the original plasmid and repeat the expression. Alternatively, if soluble protein levels of the superantigen in the crude extract are low, the pellet should be checked for inclusion body formation by solubilization in 8 M Urea. Because the affects of superantigen denaturation and subsequent refolding on superantigen function is unknown, we suggest growing bacteria at 30°C or using less IPTG for induction if inclusion body formation occurs.
3. It is important to avoid β -Mercaptoethanol, dithiothreitol (DTT), and EDTA in the binding buffer for use with His Bind resins because the reducing agents will precipitate the Ni^{2+} and EDTA will chelate the

Ni²⁺ from the column. Due to the extreme potency of these toxins, we do not use the same resin for different superantigen purifications.

Acknowledgments

This work was supported by USPHS grant HL36611 from NIH to Patrick M. Schlievert.

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