Short Technical Report

Application of Immuno-Mass Spectrometry to Analysis of a Bacterial Virulence Factor

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ABSTRACT

*Here we describe a novel antibody-based assay that combines specificity of antibody with precision of mass spectral analysis. The assay is carried out in three steps using a single antigen capture and transfer reagent. The first step of the assay involves antibody immobilization. The second step is antigen capture and washing to remove unbound proteins. The third step involves the analysis of the captured antigens by surface enhanced laser desorption ionization time-of-flight mass spectrometry. The assay is facilitated by the ability of a single nonviable bacterial preparation expressing immunoglobulin-binding proteins that enable antibody immobilization, specific capture of fluid-phase antigen, and direct sample transfer to a protein chip for mass spectral analysis. Proof-of-concept studies using a model *Streptococcus pyogenes* virulence factor, the secreted cysteine protease SpeB, are presented.*

INTRODUCTION

Analysis of virulence factors and coordinately regulated gene expression in bacteria has been studied extensively using 2-D gel electrophoresis methods (1,2,19,25,26). These studies represent the foundation of the current concept of

proteomics that seeks to identify key differences between two variants of a single organism associated with mutation, selection, or environmental conditions (19,25). Although there are a number of technical limitations associated with 2-D gel analysis, this technology has proved to be very useful for identifying virulence markers on bacteria and, when combined with mass spectrometry, has even identified protein products not predicted from genomic analysis (15). These approaches are being complemented with immunological assays that involve sera from convalescent patients to be used in an immunoblotting format, following 2-D gel separation of extracts of pathogens (14) or in immunoscreening assays of bacterial libraries to identify bacterial antigens that are only expressed during infection (IVIAT technology) (12). The ability to identify unique proteins associated with a virulent form of a microorganism offers great potential for identification of vaccine candidates and as a potential target for diagnostic assays.

Once a key virulence factor has been identified, it will no longer be necessary to follow changes in the entire proteome; rather, a target focus on the virulence factor itself will be warranted. This could be achieved using quantitative real-time PCR methods or immunoassays. However, there is considerable experimental evidence from microbial systems that message levels do not always predict the phenotype (20). Furthermore, the post-translational modification of gene products may significantly influence its biological properties (e.g., phosphorylation of signaling molecules).

In light of these observations, it is important not only to monitor the production of a specific virulence molecule but also to assess if it is present in its native or post-translationally modified form. These issues are particularly important for understanding the role of the secreted cysteine protease of *Streptococcus pyogenes*, SpeB, in streptococcal infections. SpeB is first secreted as a zymogen and then must undergo autocatalytic activation before it is believed to have a significant effect on bacterial virulence (22,23). To help elucidate the role of SpeB in group A streptococcal infection, we have developed a novel immuno-mass spectrometric assay to monitor different molecular mass forms of the SpeB protein. This assay combines the specificity of an antibody capture step with the rapid resolving power of mass spectrometry.

A number of earlier antibody-based mass spectral methods have been reported. These involve the direct immobilization of antibody on a chip surface that can be used to capture antigen for subsequent mass spectral analysis (5,7,28). These approaches are technically limited by the concentration of antibody that can be immobilized and the volume of antigen-containing sample that can be analyzed. Furthermore, attempts to analyze multiple samples on a single chip result in significant cross-contamination between sample spots, even if a bioprocessing device is used.

To overcome these significant technical difficulties, we have developed a system that enables the antigen capture and washing steps to be separated from the mass spectral analysis step (Figure

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Figure 1. Schematic representation of immuno-mass spectrometry assay involving antibody (Ab) immobilization (step 1), antigen (Ag) capture (step 2), and mass spectral analysis of bound antigen (step 3). For precise experimental details, see the Materials and Methods section.

1). This is facilitated by the ability of a single reagent to act to immobilize antibodies, to capture antigen, and then be directly transferred to a protein chip for mass spectral analysis.

MATERIALS AND METHODS

Bacteria and Antiserum

S. pyogenes isolate 1881 (serotype M1, SpeB positive, opacity factor negative) was obtained from the Centers for Disease Control and Prevention and has been characterized fully elsewhere (21,24). The cysteine protease, SpeB,

was purified from culture supernatant of *S. pyogenes* isolate 1881 according to the protocol of Berge and Bjorck (4). Rabbit antisera specific for streptococcal SpeB was purchased from Toxin Technology (Sarasota, FL, USA).

Immuno-Mass Spectrometry Assay

The immuno-mass spectrometry assay described in this study involves three sequential steps, as summarized in Figure 1. The first step involves the immobilization of the specific antibody, the second step involves the capture of antigen, and the third step involves the mass spectral analysis of the captured

antigen. This process utilizes a single immobilized reagent support (proACTR) that enables the transition from step to step to be accomplished seamlessly.

ProACTR is a selected type III IgG-binding protein (protein G) expressing nonviable bacterial preparation, which was obtained from ReceptorPro (Ann Arbor, MI, USA). The immuno-mass spectrometry method is carried out as follows.

Step 1. Antibody Immobilization

In the initial step, specific or control anti-serum or antibody is immobilized on the proACTR reagent by incubation of a dilution of serum (or antibody) with 50 μ L reagent for 30 min at room temperature. Unbound proteins are removed by washing the bacterial reagent three times with 0.1 M Tris-buffered saline (TBS), pH 7.4. The resulting antigen capture and transfer reagents (ACTRs) are then used for the antigen capture step (Step 2).

It should be noted that the specific capture reagent can be generated using either antiserum or purified antibody. The immobilized protein G on proACTR enables both immobilization and selective purification of IgG from serum. Furthermore, the antibody is immobilized via the Fc region. This orients the F(ab)₂ regions away from the support to maximize interaction with the fluid-phase antigen.

The antibody-coated ACTR binds directly and evenly to an H4 protein chip (CIPHERGEN, Palo Alto, CA, USA) containing a long-chain aliphatic surface capable of binding proteins by reverse-phase interaction. Other sources of

Figure 2. Capture and detection of enzymatically active SpeB present in Todd-Hewitt broth collected from *S. pyogenes* isolate 1881 grown overnight at 37°C. One milliliter of a 1:2 dilution of culture media was incubated with 50 μ L ACTR pre-coated with 1:1000 dilution of rabbit monospecific polyclonal antiserum to SpeB (upper panel) or normal rabbit sera (lower panel). Three microliters of the ACTR coated with antibody and captured antigen were applied to an H4 protein chip and analyzed by SELDI-TOF mass spectrometry as described in the Materials and Methods section.

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immobilized IgG-binding proteins (e.g., protein A or protein G Sepharose) were not suitable for this purpose because of their poor centrifugation properties and their failure to bind uniformly to a protein chip surface (data not shown).

Step 2. Selective Capture of Antigen

Specific anti-SpeB coated ACTR (50 μ L) or the normal rabbit control ACTR were incubated with 1 mL sample containing the target antigen for 30 min at room temperature (Figure 1, step 2) in a 1.5-mL Eppendorf[®] centrifuge tube. Unbound antigen was removed by centrifugation at 10 000 $\times$ *g* for 2 min, followed by two washing steps with 1 mL TBS. After the final wash, the specific and control ACTR was resuspended in 10 μ L TBS. This provides a concentration step in which the original soluble antigen sample (1 mL) is recovered in 1% of the original volume.

Step 3. Analysis by SELDI-TOF

The surface enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectrometry analysis step is carried out using the ProteinChip[®] system (Ciphergen). The ACTR suspension (3 μ L), with its associated antibody-antigen complex, was applied directly to individual spots on an H4 protein chip and allowed to dry at room temperature. To the dry spot, 0.5 μ L energy-absorbing molecules, EAM [3,5-dimethoxy-4-hydroxycinnamic or sinapinic acid in 50% acetonitrile, and 0.5% trifluoroacetic acid (all from Sigma, St. Louis, MO, USA)] was applied. Once dry, a second application of 0.5 μ L EAM was applied and allowed to dry. The chip was transferred to the SELDI reader (Ciphergen), and samples were analyzed following desorption of bound proteins by short, intense pulses from an N₂ 320-nm UV laser and detected by time-of-flight in a mass spectrometer.

The intensity of the laser energy was determined from preliminary empirical experiments to efficiently desorb bound antigen without releasing antibody or nonspecific proteins associated with the ACTR complex. This result was anticipated based on the higher affinity binding of protein G for rabbit IgG Fc (approximately 10⁻⁹ M)

compared with the affinity of the interaction between specific F(ab)₂ and antigen (10⁻⁷–10⁻⁸ M) (17).

RESULTS AND DISCUSSION

The immuno-mass spectrometry assay outlined in Figure 1 was applied to capture and analysis of a key virulence factor of *S. pyogenes*, SpeB. SpeB is a cysteine protease secreted by *S. pyogenes* in the zymogen form (approximately 40 kDa) that undergoes autocatalytic activation to yield an active enzyme (approximately 28 kDa) (6,18). The molecular form of the molecule is critical to understanding the contribution of this molecule to virulence in *S.*

pyogenes infection (22,23).

In the initial experiment, the ability to capture active SpeB from an overnight culture of *S. pyogenes* grown in Todd-Hewitt broth containing beef extract was tested. Under these growth conditions, the fully activated (approximately 28 kDa) form of SpeB is present (6). The results presented in Figure 2 demonstrate that the expected SpeB protein peak at approximately 28.3 kDa was only identified in the sample using proACTR pre-incubated with anti-SpeB antiserum. No protein peak was observed with the proACTR reagent pre-incubated with normal rabbit serum (Figure 2).

In the next series of experiments, the sensitivity of the immuno-mass spec-

Figure 3. Evaluation of the sensitivity of the immuno-mass spectrometry assay to the detection of SpeB in complex bacterial culture media. ACTR pre-coated with 1:1000 dilution of rabbit monospecific polyclonal antiserum to SpeB was incubated for 30 min at room temperature with 1 mL different concentrations of purified enzymatically active SpeB (188–1500 pg) diluted in Todd-Hewitt broth, washed twice, and then resuspended in 10 μ L 100 mM TBS, pH 7.4. Three microliters of the ACTR coated with antibody and captured antigen were applied to an H4 protein chip and analyzed by SELDI-TOF mass spectrometry as described in the Materials and Methods section. No detectable peaks were observed for any of these samples incubated with ACTR coated with normal rabbit serum (data not shown).

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trometry assay for the detection of SpeB was evaluated. Different concentrations of SpeB were added to Todd-Hewitt broth and incubated for 30 min at room temperature with the immobilized specific antibody reagent or a normal antibody control. Following the immunocapture and washing steps, 3 μ L bacteria-antibody-antigen mixture was applied to an H4 protein chip and analyzed by SELDI-TOF mass spectrometry as described in the Materials and Methods section.

The results presented in Figure 3 show that the optimized anti-SpeB ACTR could capture the antigen from the bacterial culture media. The peak size corresponding to the model antigen increased as the concentration of SpeB added to bacterial media increased (Figure 3). A parallel series of samples analyzed following immunocapture with pro-ACTR coated with normal rabbit serum contained no detectable SpeB protein when analyzed under identical experimental conditions (data not shown). These results would sug-

gest that the immuno-mass spectrometry technique, like other immunoassay formats, could provide semi-quantitative data provided appropriate standard antigen samples are included in the same assay and analyzed under identical experimental conditions.

SpeB is secreted as a zymogen protein that can autoactivate in a sequential manner to generate a fully active cysteine protease (6,8). The immuno-mass spectrometry assay can be applied to capture and detect the intermediates generated during the autoactivation process. The zymogen form of the protein can be maintained by adding the selective cysteine protease inhibitor E64 (3). Culture supernatant containing zymogen SpeB and E64 was diluted 1:10 to facilitate autoactivation. Samples were collected at various times, and the molecular mass of antigenic variants of SpeB was analyzed using the protocol outlined in Figure 1.

The results of these studies indicate that in addition to the zymogen and fully active form of SpeB three distinct mol-

Figure 4. Use of immuno-mass spectrometry to analyze different molecular weight forms of SpeB from culture media. ACTR pre-coated with 1:1000 dilution of rabbit monospecific polyclonal anti-serum to SpeB was incubated with culture supernatant of *S. pyogenes* isolate 1881 grown overnight in the presence of E64 to maintain SpeB in the zymogen form (upper panel) and analyzed by SELDI-TOF mass spectrometry. After E64 removal, the assay was repeated following 1 min (middle panel) or 5 min (lower panel) incubation at room temperature. Note the autoactivation intermediates present in the middle panel. No detectable peaks were observed for any of these samples incubated with ACTR coated with normal rabbit serum (data not shown).

ecular mass intermediates could be identified (Figure 4). These intermediates corresponded to those previously identified by conventional electrospray ionization using highly purified SpeB protein fragments (8).

The ability to use a single reagent to immobilize antibody, capture antigen, and transfer the bound antigen into the mass spectrometer allows the strategy outlined in Figure 1 to be applied to the analysis of any antigen/antibody pair. Based on the molecular mass of the antigen and the sensitivity of the SELDI-TOF instrument being used, post-translational modification events that involve ≥ 200 Da change in molecular mass can be reliably distinguished. This resolution would not be sufficiently sensitive to monitor a single phosphorylation event and thus may not be directly applicable to studies of signal transduction in eukaryotic systems. However, if specific antibodies can be generated that recognize phosphorylation of specific amino acid sequences, then the immuno-mass spectral approach may be applied (16). Alternatively, tryptic fragmentation of captured native and modified proteins could be used to generate peptides in a mass range in which the presence of a single phosphorylated residue could be resolved by an appropriate mass spectrometer. The sensitivity, rapidity, and economy of reagents and the more precise molecular mass profiling facilitated by mass spectrometry suggest that immuno-mass spectrometry assays may be a viable alternative to current Western immunoblotting techniques. The next developments in immuno-based mass spectral analysis are likely to incorporate advances in antibody engineering (5,9,13), the development of quantitative mass spectral-based assays (10,11), and technological improvements in protein chip-based proteomics (27).

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