Published in final edited form as:

Curr Proteomics. 2010 July ; 7(2): 90–101.

Novel Sample Preparation for Mass Spectral Analysis of Complex Biological Samples

Eric A. Porsch¹, Cecelia A. Shertz¹, and Michael D. Boyle^{*,2}

¹ Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, NC 27707

² Department of Biology, Juniata College, 1700 Moore St. Huntingdon, PA 16652, USA

Abstract

The ability to combine a selective capture strategy with on chip MALDI-TOF analysis allows for rapid, sensitive analysis of a variety of different analytes. In this overview a series of applications of capture enhanced laser desorption ionization time of flight (CELDI-TOF) mass spectrometry are described. The key feature of the assay is an off-chip capture step that utilizes high affinity bacterial binding proteins to capture a selected ligand. This allows large volumes of sample to be used and provides for a concentration step prior to transfer to a gold chip for traditional mass spectral analysis. The approach can also be adapted to utilize specific antibody as the basis of the capture step. The direct and indirect CELDI-TOF assays are rapid, reproducible and can be a valuable proteomic tool for analysis of low abundance molecules present in complex mixtures like blood plasma.

Keywords

CELDI-TOF capture-enhanced laser desorption ionization time of flight mass spectroscopy; IdeS; Immunoglobulin G: albumin; cysteine proteases

1. INTRODUCTION

One of the major approaches to detailed proteomic analysis has been the development of protein chip technology [1–9]. This technique allows separation of proteins to be achieved on a surface that can then be directly transferred to a mass spectrometer for analysis. A variety of different surface chemistries including charge, hydrophobicity and reverse phase have been employed [1, 10–12]. In addition, specific antibody, sugar binding lectin or selective receptor surfaces have also been utilized [12]. More recently protein arrays and protein chips have been developed as an approach to analyze protein-protein interactions [13–18].

Despite the potential power of combining selective capture on a single surface prior to sample analysis, the protein chip system suffers from a number of significant technical limits based both on binding capacity that can be achieved on a protein chip [19, 20]. A variety of innovative nanotechnology solutions to enhance surface binding capacity have been developed [16–20], but the enhancement of surface capture capacity on a protein chip surface does not overcome the challenges associated with surface area and the ability to

effectively wash a chip to remove non-specifically bound or irrelevant proteins and avoiding cross contamination between spots on a protein chip has proved to be a major technical challenge.

A number of sample pre-treatment methods have been proposed to increase the specific activity of the analyte prior to applying the sample to a gold chip for mass spectral analysis for either selective concentration of the target [positive selection] or to deplete other abundant and irrelevant molecules in the sample [negative selection]. Inclusion of a selective capture step with antibody, an immobilized lectin or selective chemical binding agent can be used for certain applications [14, 21]. For biomarker studies using biological fluids, depletion of major plasma proteins like albumin and IgG has been suggested and many reagents are commercially available for this purpose (for review see [22–24]). In an attempt to circumvent some of these technical limitations our laboratory has utilized an off-chip selective capture approach [positive selection] to develop immunoproteomic protocols that can be coupled directly to a mass spectral platform for analysis [25–27].

In this report we describe an extension of this technique that uses selective surface bacterial binding proteins, rather than antibody, to provide analyte capture for subsequent analysis. It should be noted that the mass spectral instrument available for these studies (the Ciphergen PBSII instrument) has poor resolving properties and thus the peaks are not as well resolved as would be expected when using a higher quality mass spectrometer. The focus of the studies was to evaluate the modified capture enhanced laser desorption ionization time of flight (CELDI-TOF) mass spectrometry assay procedure and its application to analyze a variety of target ligands for which selective bacterial surface proteins have been identified. The key feature is that the immobilization of the analyte is achieved on a bacterial surface prior to transferring to a gold chip. This approach uses high affinity bacterial binding proteins that can be expressed at high density on the surface of certain Gram positive bacteria. These binding properties are retained after heat-killing and/or fixation with glutaraldehyde or other protein cross linking agents [23, 27–29]. The accuracy and precision of resolution of the captured analytes will be influenced by the capability of the mass spectrometer.

As shown in Fig. (1), there are a significant number of Gram positive bacterial isolates that express surface proteins with the ability to selectively interact with human plasma proteins including immunoglobulins, albumin and other physiologically relevant molecules [30–40]. The binding affinity of these bacterial proteins to target ligands often exceeds the affinity of specific antibodies to the same target by one or two orders of magnitude [30, 31]. In addition, selective receptors expressed by viruses for trace molecules such as cytokines [41] have the potential to be engineered into these surface structures [42, 43].

These natural capture supports have many advantages over synthetic alternatives such as affinity beads. The high capacity support can be generated in large volumes at low cost simply by growing bacteria. When grown under standardized conditions, the end product is a homogeneous size that can be readily harvested by centrifugation. The Gram positive cell wall is remarkably resistant to degradation by most enzymes and thus can be frozen and thawed or heat-treated with minimal loss of activity.

The basic strategy for the CELDI-TOF assay is shown in Fig. (2). Key to optimization of the signal to noise ratio in the assay is the selective capture and extensive washing that occurs in step 1. In step 2, further refinement in the selectivity of the assay can be achieved by optimizing the mass spectral analysis. There are two major components that impact the efficiency of the mass spectral analysis. The first variable is the laser energy. Establishing the minimal laser energy required to cause the targeted molecule to fly is important in

minimizing noise in the spectrum. The second major variable in achieving good sample analysis is the collection of data in a narrow molecular weight range. This allows non-specific noise to be excluded and the analysis to focus only on the molecular weight range of interest.

In addition to the direct CELDI-TOF assay described in Fig. (2), a modification of the technique can be employed using an antibody bound to bacteria expressing Fc-binding proteins (Fig. 6). In this immuno-CELDI-TOF variation, specificity for targeted antigens is provided by immobilized specific antibody bound to a bacterial surface Fc-binding protein (e.g. protein G). This approach has proved of particular value for analysis of post-translational modification processes in bacterial model systems [27, 44–46]. In this paper, examples of direct and indirect CELDI-TOF assays are provided and the use of this approach for different applications in proteomics discussed.

2. RESULTS AND DISCUSSION

2.1. Application of the Direct CELDI-TOF Procedure

The rationale for the development of CELDI-TOF applications described below was that Gram positive bacteria expressing high affinity binding proteins can capture a targeted ligand which can be retained on the bacterial surface while non-specific proteins are removed by extensive washing. The bacteria can be easily pelleted and resuspended in smaller volumes than the original sample to provide both selective enhancement and the ability to concentrate the targeted ligand in the sample. An aliquot of the bacterial capture reagent can be directly applied to a gold protein chip, treated with an energy absorbing matrix and transferred to a mass spectrometer for analysis. The general protocol is outlined in Fig. (2) and the bacterial capture reagent is selected based on the target ligand to be analyzed. A number of representative examples are provided below.

2.1.1. Analysis of Post-Translationally Modified forms of Bovine Serum Albumin (BSA)—In previous studies from our laboratory, we had demonstrated the expression of a high affinity albumin-binding protein expressed by certain bovine group G streptococcal isolates that could selectively remove human serum albumin from plasma [23]. This stably expressed surface activity can also be used as a selective capture reagent for different sources of serum albumin, including bovine [23]. To evaluate the utility of the CELDI-TOF approach to monitor different post-translationally modified forms of BSA, we used as capture reagent a bovine group G streptococcal isolate, BG8 that we had previously characterized for expression of albumin-binding activity [23].

Isolate BG8 was selected for its high level of surface expression of a high affinity albumin-binding protein(s) [23]. BG8 was grown overnight in Todd-Hewitt broth and harvested by centrifugation. A 10% wet weight/volume suspension was prepared and heat killed by boiling at 100°C for 15 minutes. The washed pellet was collected and used as the capture reagent. In preliminary studies the bacterial reagent was shown to capture human, bovine, rabbit and sheep albumin but failed to bind ovalbumin. Optimal conditions for capturing albumin from 10–1,000 µg/mL samples were established for each species of albumin. No significant difference was noted in binding kinetics or efficiency among the species tested (data not shown).

Once capture conditions had been optimized empirically, a standard assay protocol was developed in which 10 µL of a 1 mg/mL (1 µg) albumin sample was added to 50 µL of a 10% wet weight/volume of heat-killed bacteria expressing surface albumin-binding proteins in a final volume of 1 mL 0.1 M Tris buffered saline (TBS). The reactants were incubated for 30 minutes at ambient temperature on a rocking platform. At the end of the incubation,

the bacterial pellet was recovered by centrifugation ($10,000 \times g$ for 2 minutes) and unbound fluid phase proteins were discarded. The resulting bacterial pellet was washed and transferred to a gold chip for mass spectral analysis as described in the Methods.

Albumin can be post-translationally modified *in vivo* as well as acquire many different types of molecules as cargo [47, 48]. In order to evaluate if CELDI-TOF approaches could be used to analyze variants of albumin, a series of experiments were conducted with native and a commercial preparation of chemically modified BSA preparation with 10–20 moles of glucosamide/mole of BSA (Sigma). This preparation demonstrates a major broad peak of protein in the mass spectrum at a $M_r \sim 75,000$ compared to the mass of the native protein $M_r \sim 66,000$ (Fig. 3).

An equimolar mixture of native and modified BSA was subjected to CELDI-TOF analysis using isolate BG8 as the capture reagent. As shown in Fig. (3) (panels A and B) the bacterial capture reagent could interact with either the native or glucosamide modified form of BSA. When the two variants were mixed at equal molar concentrations the CELDI-TOF assay was capable of capturing both the modified and unmodified forms that could then be resolved by mass spectral analysis, (see Fig. 3) panel C. This result suggests that different post-translational forms of albumin present in serum samples could be analyzed efficiently using this simple capture approach and the basic CELDI-TOF procedure described in Fig. (2). A more refined analysis could be achieved using a mass spectrometer with higher resolving power than the instrument used in this study.

To demonstrate the general application of CELDI-TOF to more complex mixtures of proteins, the next series of applications use a bacterial capture reagent for IgG Fc-fragments to measure the activity of a selective bacterial IgG protease.

2.1.2. Application of CELDI-TOF to Analysis of a Unique Bacterial

Endopeptidase—Bacterial cysteine proteases are virulence factors that have been studied extensively for their role in promoting the pathogenesis and pathophysiology of infections [49, 50]. In general, these enzymes have broad substrate specificity and, as a consequence, these gene products are usually translated as zymogen forms that become activated following secretion. Recently Bjorck and colleagues [51–55] have identified a novel cysteine protease, IdeS, expressed by certain *S. pyogenes* isolates. IdeS is expressed as a mature protein that does not require processing before becoming enzymatically active [51–55]. The enzyme also displays limited substrate specificity with certain species of IgG being the only substrate recognized thus far [51–55]. The enzymatic product generated by the action of IdeS on mammalian IgG is a $M_r \sim 25,300$ Fc fragment that retains the ability to bind streptococcal protein G. This provides the basis for a CELDI-TOF assay to monitor IdeS activity using bacteria expressing surface protein G as the capture reagent [56].

The initial characterization of IdeS enzymatic activity was monitored using purified IgG as substrate and an SDS-PAGE, HPLC or Biacore readout for detection of the Fc product. Our laboratory has adapted a protein G capture and CELDI-TOF procedure to monitor IdeS activity [59–61]. This approach has a number of technical advantages. For example, the capture and washing approaches accompanying the CELDI-TOF procedure enable the assay to be carried out with the IgG substrate present in complex mixtures like plasma [56]. In addition the relative technical simplicity of the assay allows for more efficient processing of samples and the ability to use lower concentrations of reagent.

To further demonstrate the utility of the CELDI-TOF approach to monitor IdeS activity and expand these observations, we have compared the ability of IdeS to cleave IgG present in plasma from different mammalian species. For these experiments 1% dilutions of human,

sheep, horse, goat, cow and rabbit plasma was incubated with recombinant IdeS (2 μ g) in a final volume of 70 μ l PBS for 30 min at ambient temperature. At that time, an aliquot of the reaction mixture was subjected to a capture step and subsequent mass spectral analysis. For this application the capture reagent used was G1400, which expresses high levels of the IgG-binding protein, protein G [56].

The results presented in Fig. (4) demonstrate the appearance of the characteristic Mr~25,300 Fc fragment in all of the plasma samples treated with IdeS. This peak was not observed in samples in which 20 mM iodoacetate, an IdeS inhibitor, was added prior to addition of the enzyme (data not shown). In the spectra from any sample containing a source of the mammalian plasma, a Mr~66,000 peak and a Mr~33,000 double charge peak representing contaminating albumin was also observed. These peaks do not overlap with the Fc fragment peak and by focusing on a narrow range of the spectra surrounding the Fc fragment peak the albumin contamination did not interfere with the ability to quantify the Fc peak.

A variation of this CELDI-TOF capture assay has also been used to monitor the production of IdeS in culture media of *S. pyogenes* during growth in a complex nutrient broth (Todd Hewitt broth containing beef heart extract). All *S. pyogenes* isolates contain the gene for SpeB, another cysteine protease with broad substrate specificity [51]. IdeS activity has only been detected in the culture media of group A streptococci that fail to secrete the potent cysteine protease SpeB. (Shertz and Boyle, unpublished data). Since SpeB is known to degrade surface and secreted proteins [57, 58], it is not clear whether the failure to detect IdeS in culture media of SpeB- secreting bacteria is due to failure to express the *ides* gene product or post-translational destruction of the secreted protein.

IdeS and SpeB differ in their ability to be inhibited by the cysteine protease inhibitor E64 [53]. IdeS is not inhibited by E64, while SpeB can be effectively inhibited at doses that do not affect the ability of *S. pyogenes* organisms to divide [58]. By growing bacteria in the presence of E64 and measuring IdeS activity in supernatant fluids using the CELDI-TOF assay it is practical to determine if SpeB is degrading IdeS or if the active enzyme is not present.

To distinguish among the possible explanations for the absence of IdeS activity in culture media of SpeB secreting isolates, a mouse skin selected variant 2109SR, that fails to secrete SpeB and the parent SpeB secreting wild type parent isolate 2109 were grown in culture with or without E64 (1 μ M). Bacteria-free culture media was analyzed for IdeS activity using a modification of the CELDI-TOF assay in which differing volumes of culture media were added as the source of enzyme. As shown in Fig. (5), IdeS activity was present in supernatants from isolate 2109SR grown either in the presence or absence of E64. IdeS activity was not detected even in the presence of E64 in culture media from the SpeB producing isolate 2109. These results suggest that IdeS and SpeB gene expression may be oppositely regulated in *S. pyogenes*.

In summary, the direct CELDI-TOF procedure can be used when a suitable capture reagent is available. Examples have been provided using well-characterized bacterial binding proteins for albumin or IgG Fc regions. As noted in Fig. (1), there are many additional binding activities that could be used for the capture step in the basic CELDI-TOF procedure summarized in Fig. (2). For example, bacteria expressing IgA Fc binding proteins [39, 40] could be used as the basis of an assay for IgA proteases [59] or a modification of the procedure using a plasminogen-binding protein could be developed to monitor different forms of human plasminogen [35, 36]. Plasminogen-binding proteins on streptococci have been shown to differentiate among different forms of Glu- and Lys-plasminogen as well as binding predictably to different plasminogen fragments [36].

A modification of the approach has also been used to monitor binding of different fibrinogen fragments to group A streptococci [37]. This allows structure-function analysis of the domains on a ligand that binds to a bacterial-binding protein as well as provides an efficient screening strategy to identify additional naturally occurring bacteria or engineered variants that could be used as capture reagents in CELDI-TOF assays.

The potential to engineer functional binding domains from non-bacterial sources, for example, viral cytokine-binding proteins [41] into the genes for surface expressed proteins on Gram positive organisms is practical. Pozzi and colleagues [43] have developed a system in which a significant portion (~100 amino acids) of the surface-exposed domain of the M protein from *S. pyogenes* can be replaced with a desired sequence. This construct can be successfully expressed on the surface of *Streptococcus gordonii* [42, 43]. A similar strategy could be used to engineer any identified protein binding domain into a CELDI-TOF capture reagent. This would provide an additional source of reagents for use in a direct CELDI-TOF format.

2.2. Indirect Immuno-CELDI-TOF Assays

In order to increase the versatility of the capture step in the CELDI-TOF assay, a versatile way to capture selective target molecules for analysis is desirable. This approach takes advantage of the specificity of antibodies provided by immunoglobulin Fab2 regions and the ability to anchor the IgG molecule to a bacterial support via the Fc region. This allows a virtually unlimited range of targeted antigens that can be analyzed by combining bacteria expressing high affinity Fc receptors with available sources of monoclonal or polyclonal antibodies.

The basic immunoproteomic variation of the CELDI-TOF procedure is summarized in Fig. (6) and is carried out in three stages. The first stage involves immobilization of a specific antibody, the second step involves the capture of antigen and the third step involves the mass spectral analysis of the captured antigen. This bacterial reagent serves to immobilize the specific antibody and acts as the antigen capture and transfer reagent (ACTR). To generate a specific capture reagent either purified antibody or an appropriate antiserum is incubated with the protein G expressing bacteria as described in the Methods.

In the second stage, the immobilized antibody is incubated in a 1 mL volume of sample containing the targeted antigen as described in the Methods. During the procedure antigen-antibody complexes are formed and unbound antigen is lost during the washing steps. After the final wash, the bacterial pellet is resuspended in 10 μ L TBS. This represents a concentration step in which the original soluble antigen sample (1 mL) is captured in an immobilized form in 1% of the original sample volume. The CELDI-TOF mass spectrometry analysis step is carried out as described in the methods. For each antigen-antibody combination the intensity of the laser energy needs to be determined from preliminary empiric experiments to establish the minimal laser energy required to fly the targeted antigen without releasing antibody or non-specific proteins associated with the ACTR complex.

The initial applications of this immunoproteomic strategy have been used to monitor a variety of post-translational modification events in bacterial systems. These included the zymogen activation reaction of the streptococcal cysteine protease SpeB as well as assays to monitor SpeB processing of surface M protein [44–46]. The value of this approach is that it allows the specificity of the antibody to enhance the selectivity of the capture reaction and to analyze related events occurring in complex mixtures and dynamic biological systems.

The results in Fig. (7) demonstrate the versatility of this technique to monitor the association between the presence of an active cysteine protease in the culture media of an M1 streptococcal isolate and the form of M1 protein expressed on the cell surface. For these studies isolate WT1881 (SpeB⁺) was inoculated into Todd-Hewitt Broth and grown at 37°C for varying times. Aliquots were harvested at late logarithmic, early and late stationary phase. Bacteria were pelleted by centrifugation and surface M1 protein was by treatment with CNBr as described previously [59, 61]. Culture supernatants were collected on ice and the cysteine pro-tease inhibitor E64 added to a final concentration of 10 µM to prevent the autoactivation of any zymogen SpeB that may be present in the sample [58].

Culture supernatants were analyzed for the presence and molecular form of SpeB at different phases of bacterial growth using the previously described immuno-CELDI-TOF assay with a specific rabbit anti-SpeB antiserum (Toxin Technologies, Tampa, FL) providing the specificity [45, 46]. In this system the capture reagent is an immobilized rabbit anti-SpeB antibody or as a control, an equivalent concentration of normal rabbit serum. The specific and control capture reagent were incubated with 1 mL of culture media harvested at different times. After the antigen capture step and washing, the bacterial capture reagent was transferred to a protein chip for mass spectral analysis.

In agreement with earlier studies [62], SpeB is only secreted by *S. pyogenes* in late logarithmic or early stationary phase (see Fig. 7). Initially the enzyme is secreted as the zymogen form of the protein (Mr~40,300) but autoactivates over time. By late stationary phase, the predominant form of SpeB is the fully activated Mr~28,000 variant (see Fig. 7).

Analysis of the M1 protein present in CNBr extracts of the cells collected at the corresponding time points is also shown in Fig. (7). In this experiment the internal ~41,000 CNBr fragment of the M1 protein is used to normalize the data. This is necessary since expression of the M1 protein is growth phase regulated. At the earliest time point studied, all of the M1 protein was in the native ~45,500 Daltons form (Fig. 7). At later times, the M1 protein was present in both its native (~45,500) and post-translationally modified forms (~43,000) (see Fig. 7).

In a parallel series of studies in which the bacteria were grown in 10% human plasma, it was not possible to detect the production of SpeB due to the overwhelming signal due to contaminating proteins in the mass spectra. In this assay, the selectivity of the capture step was not sufficient to allow for a SpeB peak to be detected in the presence of a double charged human serum albumin peak that gave a strong signal with a broad peak around Mr ~33,000.

It was, however, possible to analyze the surface M1 protein since the CNBr extraction method also fragmented albumin and the fragments did not interfere with the ability to detect M protein in the assay. The M1 protein extracted from bacteria grown in the presence of 30% human plasma was unprocessed by SpeB (see Fig. 8). This indicated that either SpeB was not produced in the presence of plasma, failed to activate or that there were competitive substrates in human plasma that prevented the efficient modification of the surface bacterial proteins. In a series of related studies of message levels for the *speB* gene, we found that in the presence of human serum the bacteria did not transcribe message for SpeB (Rezcallah and Boyle, unpublished data). These studies demonstrate the value of being able to combine transcriptional analysis with proteomic data to determine whether phenotypic differences occur at the transcriptional, translational or post-translational levels.

3. EXPERIMENTAL SECTION

3.1. Materials

3.1.1. Chemicals, Biochemicals and General Laboratory Reagents—All general laboratory chemicals were obtained from Sigma Chemical Company, St. Louis, MO. All chemical and reagents were highest grade available. Bovine serum albumin and a chemically modified glucosamide derivative were obtained from Sigma and human IgG was obtained from Athens Research & Technology, Athens, GA.

3.1.2. Bacterial Strains—*Streptococcus pyogenes* isolate 2109 and a spleen-recovered variant (2109SR) obtained following injection into a skin air sac and recovery from the spleen have been described fully in [63]. The two genetically paired isolates differ in their ability to secrete the streptococcal pyrogenic exotoxin B (SpeB) [63]. SpeB is a cysteine protease that is secreted as a zymogen capable of undergoing autoactivation when the pH of the media decreases [44]. The active enzyme can modify a number of surface and secreted streptococcal proteins [57, 58].

For studies of the M1 protein and its modification by SpeB, isolate 1881 was used. This isolate was originally obtained from the Centers for Disease Control in Atlanta and its phenotypic characteristics have been detailed in [60, 61]. The surface M1 protein was extracted using CNBr extraction techniques as described in [61].

3.1.3. Preparation of Recombinant IdeS and Analysis of Enzyme Activity—Immunoglobulin-degrading enzyme of streptococci (IdeS) was cloned from *S. pyogenes* isolate AP1 as described in [56]. The cloned gene was expressed in pBAD (Invitrogen) as a fusion protein containing a poly-His tail to facilitate efficient affinity purification using metal chelate chromatography [56]. The direct CELDI-TOF assay for IdeS activity is based on the ability to quantify a Mr~25,300 Fc fragment following capture by G1400 [56]. The use of this assay to measure IdeS activity using either purified IgG or IgG in mammalian plasma has been described previously [56]. A modification of this assay approach to monitor IdeS secretion by bacteria grown in culture has also been described [56]. Examples of these assays are provided in the results section.

Essentially, the assay for IdeS activity uses human immunoglobulin G (IgG) as substrate and is performed in a final volume of 70 μ L 100 mM Tris buffered saline (TBS), pH 7.4. The ratio of IdeS to IgG and the time and temperature of incubation depend on the nature of the experimental study being conducted. At the end of the appropriate incubation time, 20 mM iodoacetate is added to the reaction mixture to prevent further enzymatic action. In the direct CELDI stage of the assay, generation of Fc fragments by the action of IdeS on IgG is determined by mixing the enzyme reaction mixture with 50 μ L of a 10% wet weight/volume heat-killed suspension of protein G-expressing G1400. The mixture was rocked at ambient temperature for 30 minutes. After incubation, the bacterial suspension was centrifuged at $10,000 \times g$ for 2 minutes. The resulting pellet was washed three times with 500 μ L of phosphate buffered saline pH 7.4 (PBS) and then resuspended in 50 μ L of PBS. One microliter of the suspension was spotted directly onto a gold CIPHERGEN[®] protein chip and analyzed with CIPHERGEN[®] Protein Chip Software, Version 3.0 as described above.

3.2. Methods

3.2.1. General Protocol for Direct and Indirect CELDI Assay—The selectivity of the CELDI-TOF assay is determined by the capture reagent. In preliminary experiments optimal conditions for each capture reagent need to be determined empirically, which involves establishing the relationship between analyte concentration, peak height, and area

under the peak for a given laser energy setting. The general protocol for the direct CELDI-TOF assay is summarized in Fig. (2). Following the capture and washing step, the mass spectral analysis is carried out by applying 1 μ L of the concentrated heat-killed bacterial suspension directly to a spot on a gold Ciphergen[®] chip. After drying, each spot is coated with an energy-absorbing matrix consisting of saturated 4-hydroxy-3,5-dimethoxycinnamic acid in 50% acetonitrile and 0.05% trifluoroacetic acid and transferred to the Ciphergen[®] PBSII protein chip mass spectrometer for analysis. The data is collected averaging 65 shots from a N₂ 320 nm-UV-laser on different positions of each protein chip spot and analyzed using Ciphergen[®] Protein Chip Software, Version 3.0, which identifies protein peaks, assigns masses, and calculates intensities. Details for analysis of particular analytes are provided in the Results section.

The indirect CELDI-TOF assays follow an identical protocol to that described for the direct assay with the addition of an initial step to generate the antibody coated capture reagent (see step 1 of Fig. 6). In these assays the immobilized support for the specific polyclonal or monoclonal antibody is a heat killed group G streptococcus, G1400, that stably over-expresses protein G, a high affinity Fc binding protein for mammalian IgG [27].

The specific capture reagent can be generated using either purified antibody or an appropriate antiserum by incubation with the protein G-expressing bacteria. Antibodies are selectively bound to the bacteria via their Fc regions and unbound proteins are removed by washing. This procedure results in both the capture of antibody and the orientation of the Fab2 regions away from the immobilization reagent to maximize interaction with fluid phase antigen in the next stage of the assay.

When an antiserum is used as the source of antibody, this step is essentially an affinity purification procedure for the antibody since non-IgG serum proteins are removed by washing. As with all forms of immunoassay, it is necessary to include a control preparation of affinity capture reagent with normal IgG or an isotype matched antibody of irrelevant specificity to ensure immunological specificity in the assay. Once the specific capture reagent is generated the assay follows an identical series of steps to the direct CELDI-TOF procedure described above.

4. FUTURE DIRECTIONS AND CONCLUSIONS

In this paper we describe direct and indirect CELDI-TOF procedures that can be used to monitor a variety of post-translational modification events associated with dynamic biological systems. There are a number of key elements that make this approach of practical value for multiple applications. The advantage of the off-chip capture approach is that it allows large sample volumes to be analyzed and enables efficient washing to remove unbound or irrelevant molecules from the system prior to mass spectral analysis. In addition, by resuspending the capture reagent-analyte complex in a significantly smaller volume than the original sample, a concentration step is included. When coupled to the selective nature of the capture reagent, the signal to noise ratio of the assay is greatly enhanced. This approach overcomes many of the technical limitations associated with direct coupling of capture reagents to the limited surface area on a protein chip and the problems of cross-spot contamination that can occur when attempting to wash a multi-spot chip.

The availability of many natural bacterial binding proteins with high affinity surface binding structures for an array of mammalian plasma proteins (see Fig. 1) offers the potential for positively selecting certain physiologically important molecules for analysis. Many of these target molecules are known to exist in a variety of post-translational forms. The availability of multiple different natural binding proteins (e.g. IgA, or plasminogen-binding proteins)

also offers the potential to extend this basic simple direct CELDI approach to multiple applications.

The bacteria used in the capture step perhaps represent the ultimate in nanotechnology products. Not only do they express very high densities of a high affinity binding activity on a particle that can be harvested by centrifugation but these bioparticles are easy to manufacture by virtue of their ability to self replicate in inexpensive bacterial culture media. Furthermore the ability to engineer binding peptides into bacterial surface proteins [42, 43] would suggest that the potential to generate new bacterial capture reagents with a desired selectivity for use in direct assays would be practical.

The indirect capture approach offers a wide range of analyzable targets not only using existing monoclonal and polyclonal antibodies but the technology lends itself to exploiting Fc fusion proteins. Many receptor activities have already been engineered with an Fc fragment [64, 65]. These fusion proteins could be used with existing capture reagents expressing high affinity Fc-binding proteins in indirect CELDI-TOF assays. Thus with existing technology the capture reagent is unlikely to limit the range of target molecules that can be analyzed using direct or indirect CELDI-TOF approaches.

The sensitivity of the technique and the ability to resolve subtle differences in modified forms of a given analyte will be in large part a function of the mass spectrometer used for the analytic assay. By combining capture technology with optimization of laser energy and focusing on a limited mass region of the spectra containing the target analyte, it is possible to maximize the signal to noise ratio and obtain quantitative data. Currently semi-quantitative results have been obtained using the area under the peak for the analyte [25].

Developing more rigorous quantitative methods will require the use of better instrumentation and development of appropriate assay protocols. Quantification can be improved by inclusion of standards of the targeted analyte selectively modified to migrate at a distinct molecular mass in each sample prior to analysis (e.g. a biotinylated derivative) [66]. Obtaining a linear relationship between the area under the peak in the mass spectra and the concentration of the targeted molecule being analyzed will require careful experimentation to establish the linear range for the assay. In the indirect CELDI approaches, a standard curve would need to be included in each assay if absolute quantification is required.

The simplicity of the off-chip capture assay with the ability to use large volumes of sample, efficient washing and a concentration step just prior to analysis make this approach attractive for studies designed to analyze post-translational modification events in complex biological systems. Furthermore, by combining the capture aspect of direct and indirect CELDI assays with selective enzymatic treatment of bound analyte (e.g. with specific glycosidases or phosphatases), with modification specific antibodies (e.g. phosphoserine antibodies) and with enzymatic fragmentation analysis (e.g. tryptic digestion), the nature of the post-translational event can be confirmed and/or more precisely characterized. To measure subtle post-translational events e.g. a single phosphorylation modification, it may be necessary to include phosphospecific antibodies and/or protease fragmentation to be able to effectively identify subtle post-translational modification events.

As detailed molecular analysis of complex biological systems continues to advance there will be an increased need to develop simple measures of post-translational processing in complex mixtures. CELDI-TOF offers a simple technique to monitor subtle changes in specific gene products despite the presence of large molar excess of irrelevant proteins and other molecules. The use of capture reagents with higher affinity than conventional antibodies has a number of additional advantages over current immunoassays. In particular,

it will permit further sample manipulation after selective capture to increase the ability to precisely map post-translational modification events. The advantages of the off-chip capture approach suggest that CELDI approaches are likely to be valuable in studies of complex and dynamic biological systems under more realistic physiological conditions.

Acknowledgments

This work was supported in part by the William J von Liebig Foundation and grant AI 072722 from the National Institutes of Health.

References

1. Davies HA. The ProteinChip® System from CIPHERGEN: A new technique for rapid, micro-scale protein biology. *J Mol Med.* 2000; 78:B29. [PubMed: 11043389]
2. von Eggeling F, Davies H, Lomas L, Fieldler W. Tissue-specific microdissection coupled with Protein Chip Array® technologies: Applications in cancer research. *Biotechniques.* 2000; 29:1066–1070. [PubMed: 11084869]
3. Wright GL Jr, Cazares LH, Leung SM, Nasim S, Adam B, Yip TT, Schellhammer PF, Gong L, Vlahou A. Prote-inChip®-surface enhanced laser desorption/ionization (SELDI) mass spectrometry: A novel protein biochip technology for detection of prostate cancer biomarkers in complex protein mixtures. *Pros Can Pros Dis.* 2000; 2:264–276.
4. Merchant M, Weinberger SR. Review: Recent advancements in surface-enhanced laser desorption/ionization-time of flight-mass spectrometry. *Electrophoresis.* 2000; 21:1164–1167. [PubMed: 10786889]
5. Weinberger SR, Morris TS, Pawlak M. Recent trends in protein biochip technology. *Pharmacogenomics.* 2000; 1:395. [PubMed: 11257925]
6. Kiehnopf M, Siegmund R, Deufel T. Use of SELDI-TOF mass spectrometry for identification of new biomarkers: potential and limitations. *Clin Chem Lab Med.* 2007; 45:1435–1449. [PubMed: 17970700]
7. Hodgetts A, Levin M, Kroll JS, Langford PR. Biomarker discovery in infectious diseases using SELDI. *Future Microbiol.* 2007; 2:35–49. [PubMed: 17661674]
8. Engwegen JY, Gast MC, Schellens JH, Beijnen JH. Clinical proteomics: searching for better tumour markers with SELDI-TOF mass spectrometry. *Trends Pharmacol Sci.* 2006; 27:251–259. [PubMed: 16600386]
9. Poon TC. Opportunities and limitations of SELDI-TOF-MS in biomedical research: practical advices. *Expert Rev Proteomics.* 2006; 4:51–65. [PubMed: 17288515]
10. Camarero JA. Recent developments in the site-specific immobilization of proteins onto solid supports. *Biopolymers.* 2008; 90(3):450–458. [PubMed: 17618518]
11. Rusmini F, Zhong Z, Feijen J. Protein immobilization strategies for protein biochips. *Biomacromolecules.* 2007; 8:1775–1789. [PubMed: 17444679]
12. Tang N, Tornatore P, Weinberger SR. Current developments in SELDI affinity technology. *Mass Spectrom Rev.* 2004; 23:34–44. [PubMed: 14625891]
13. Seibert V, Wiesner A, Buschmann T, Meuer J. Surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI TOF-MS) and Protein Chip technology in proteomics research. *Pathol Res Pract.* 2004; 200:8–94.
14. Camarero JA. Recent developments in the site-specific immobilization of proteins onto solid supports. *Biopolymers.* 2008; 90:450–458. [PubMed: 17618518]
15. Blättler T, Huwiler C, Ochsner M, Städler B, Solak H, Vörös J, Grandin HM. Nanopatterns with biological functions. *J Nanosci Nanotechnol.* 2006; 6:2237–2264. [PubMed: 17037832]
16. Uttamchandani M, Wang J, Yao SQ. Protein and small molecule microarrays: powerful tools for high-throughput proteomics. *Mol Biosyst.* 2006; 2:58–68. [PubMed: 16880923]
17. Ivanov YD, Govorun VM, Bykov VA, Archakov AI. Nanotechnologies in proteomics. *Proteomics.* 2006; 6:1399–1414. [PubMed: 16447155]

18. Tomizaki KY, Usui K, Mihara H. Protein-detecting microarrays: current accomplishments and requirements. *Chembiochem*. 2005; 6:782–799. [PubMed: 15791688]
19. Poon TC. Opportunities and limitations of SELDI-TOF-MS in biomedical research: practical advices. *Expert Rev Proteomics*. 2007; 4:51–65. [PubMed: 17288515]
20. Gast MC, Engwegen JY, Schellens JH, Beijnen JH. Comparing the old and new generation SELDI-TOF MS: implications for serum protein profiling. *BMC Med Genomics*. 2008; 31:1–4.
21. Liu T, Qian WJ, Mottaz HM, Gritsenko MA, Norbeck AD, Moore RJ, Purvine SO, Camp DG 2nd, Smith RD. Evaluation of multiprotein immunoaffinity subtraction for plasma proteomics and candidate biomarker discovery using mass spectrometry. *Mol Cell Proteomics*. 2006; 5:2167–2174. [PubMed: 16854842]
22. Cho SY, Lee EY, Lee JS, Kim HY, Park JM, Kwon MS, Park YK, Lee HJ, Kang MJ, Kim JY, Yoo JS, Park SJ, Cho JW, Kim HS, Paik YK. Efficient prefractionation of low-abundance proteins in human plasma and construction of a two-dimensional map. *Proteomics*. 2005; 5:3386–3396. [PubMed: 16047310]
23. Coyle EM, Blazer LL, White AA, Hess JL, Boyle MD. Practical applications of high-affinity, albumin-binding proteins from a group G streptococcal isolate. *Appl Microbiol Biotechnol*. 2006; 71:39–45. [PubMed: 16317541]
24. Huang L, Fang X. Immunoaffinity fractionation of plasma proteins by chicken IgY antibodies. *Methods Mol Biol*. 2008; 425:41–51. [PubMed: 18369885]
25. Hess JL, Blazer L, Romer T, Faber L, Buller RM, Boyle MD. Immunoproteomics. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2005; 815:65–75.
26. Boyle MD, Hess JL, Nuara AA, Buller RM. Application of immunoproteomics to rapid cytokine detection. *Methods*. 2006; 38:342–350. [PubMed: 16487723]
27. Saouda M, Romer T, Boyle MD. Application of immuno-mass spectrometry to analysis of a bacterial virulence factor. *Biotechniques*. 2002; 32:916–923. [PubMed: 11962613]
28. Frohman MA, Frohman LA, Goldman MB, Goldman JN. Use of protein A--containing *Staphylococcus aureus* as an immunoabsorbent in radioimmunoassays to separate antibody-bound from free antigen. *J Lab Clin Med*. 1979; 93:614–621. [PubMed: 429860]
29. Boyle, MDP.; Faulmann, EL. Application of bacteria expressing immunoglobulin binding proteins to immunoprecipitation reactions. In: Boyle, MDP., editor. *Bacterial Immunoglobulin Binding Proteins, Volume II*. Academic Press; San Diego, CA: 1990. p. 273-289.
30. Boyle, MDP. Bacterial IgG-binding proteins. In: Delves, PJ.; Roitt, IM., editors. *Encyclopedia of Immunology*. 2. Academic Press; San Diego, CA: 1998. p. 323-327.
31. Boyle, MDP. Immunoglobulin-binding proteins expressed by Gram-positive bacteria. In: Cunningham, MW.; Fujinami, RJ., editors. *Effects of Microbes on the Immune System*. Lippincott Williams & Wilkins; Philadelphia, PA: 2000. p. 195-217.
32. de Château M, Björck L. Identification of interdomain sequences promoting the intronless evolution of a bacterial protein family. *Proc Natl Acad Sci USA*. 1996; 93:8490–8495. [PubMed: 8710897]
33. Talay SR, Grammel MP, Chhatwal GS. Structure of a group C streptococcal protein that binds to fibrinogen, albumin and immunoglobulin G *via* overlapping modules. *Biochem J*. 1996; 315:577–582. [PubMed: 8615832]
34. Jaffe J, Natanson-Yaron S, Caparon MG, Hanski E. Protein F2, a novel fibronectin-binding protein from *Streptococcus pyogenes*, possesses two binding domains. *Mol Microbiol*. 1996; 21:373–384. [PubMed: 8858591]
35. Berge A, Sjöbring U. PAM, a novel plasminogen-binding protein from *Streptococcus pyogenes*. *J Biol Chem*. 1993; 268:25417–25424. [PubMed: 8244975]
36. Broder CC, Lottenberg R, Boyle MD. Mapping of the human plasmin domain recognized by the unique plasmin receptor of group A streptococci. *Infect Immun*. 1989; 57:2597–2605. [PubMed: 2547717]
37. Hess JL, Boyle MD. Fibrinogen fragment D is necessary and sufficient to anchor a surface plasminogen-activating complex in *Streptococcus pyogenes*. *Proteomics*. 2006; 6:375–378. [PubMed: 16287173]

38. Pleass RJ, Areschoug T, Lindahl G, Woof JM. Streptococcal IgA-binding proteins bind in the C alpha 2-C alpha 3 inter-domain region and inhibit binding of IgA to human CD89. *J Biol Chem.* 2001; 276:8197–8204. [PubMed: 11096107]
39. Faulmann EL, Duvall JL, Boyle MD. Protein B: a versatile bacterial Fc-binding protein selective for human IgA. *Biotechniques.* 1991; 10:748–755. [PubMed: 1878208]
40. Russell-Jones GJ, Gotschlich EC, Blake MS. A surface receptor specific for human IgA on group B streptococci possessing the Ibc protein antigen. *J Exp Med.* 1984; 160:1467–1475. [PubMed: 6387034]
41. Bai H, Buller RM, Chen N, Boyle MD. Viral binding proteins as antibody surrogates in immunoassays of cytokines. *Biotechniques.* 2002; 32:166–171.
42. Medaglini D, Pozzi G, King TP, Fischetti VA. Mucosal and systemic immune responses to a recombinant protein expressed on the surface of the oral commensal bacterium *Streptococcus gordonii* after oral colonization. *Proc Natl Acad Sci USA.* 1995; 92:6868–6872. [PubMed: 7624334]
43. Pozzi G, Contorni M, Oggioni MR, Manganelli R, Tommasino M, Cavalieri F, Fischetti VA. Delivery and expression of a heterologous antigen on the surface of streptococci. *Infect Immun.* 1992; 60:1902–1907. [PubMed: 1563781]
44. Boyle MD, Romer TG, Meeker AK, Sledjeski DD. Use of surface enhanced laser desorption ionization protein chip system to analyze streptococcal exotoxin B activity secreted by *Streptococcus pyogenes*. *J Microbiol Methods.* 2001; 46:87–97. [PubMed: 11412919]
45. Romer T, Rescellah M, Boyle MDP. Use of surface enhanced laser desorption ionization time-of-flight mass spectrometry to analyze post-translational modification of bacterial virulence factors. *Res Signpost.* 2002; 37:1–18.
46. Romer T, Boyle MDP. Application of immunoproteomics to analysis of post-translational processing of the anti-phagocytic M protein of *Streptococcus pyogenes*. *Proteomics.* 2003; 3:29–35. [PubMed: 12548631]
47. Gekle M. Renal tubule albumin transport. *Annu Rev Physiol.* 2005; 67:573–594. [PubMed: 15709971]
48. Odhiambo A, Perlman DH, Huang H, Costello CE, Farber HW, Steinberg MH, McComb ME, Klings ES. Identification of oxidative post-translational modification of serum albumin in patients with idiopathic pulmonary arterial hypertension and pulmonary hypertension of sickle cell anemia. *Rapid Commun Mass Spectrom.* 2007; 21:2195–2203. [PubMed: 17569101]
49. Lasakosvitsch F, Gentil LG, dos Santos MR, da Silveira JF, Barbieri CL. Cloning and characterization of a cysteine proteinase gene expressed in amastigotes of *Leishmania (L.) amazonensis*. *Int J Parasitol.* 2003; 33:445–454. [PubMed: 12705937]
50. Saouda M, Wu W, Conran P, Boyle MDP. Streptococcal pyrogenic exotoxin B (SpeB) enhances tissue damage initiated by other *Streptococcus pyogenes* products. *J Infect Dis.* 2001; 184:723–731. [PubMed: 11517433]
51. von Pawel-Rammingen U, Bjorck L. IdeS and SpeB: immunoglobulin-degrading cysteine proteinases of *Streptococcus pyogenes*. *Curr Opin Microbiol.* 2003; 1:50–55. [PubMed: 12615219]
52. von Pawel-Rammingen U, Johansson BP, Bjorck L. IdeS, a novel streptococcal cysteine proteinase with unique specificity for immunoglobulin G. *EMBO J.* 2002; 21:1607–1615. [PubMed: 11927545]
53. Vincents B, von Pawel-Rammingen U, Bjorck L, Abrahamson M. Enzymatic characterization of the streptococcal endopeptidase, IdeS, reveals that it is a cysteine protease with strict specificity for IgG cleavage due to exosite binding. *Biochemistry.* 2004; 43:15540–15549. [PubMed: 15581366]
54. Wenig K, Chatwell L, von Pawel-Rammingen U, Bjorck L, Huber R, Sondermann P. Structure of the streptococcal endopeptidase IdeS, a cysteine proteinase with strict specificity for IgG. *Proc Natl Acad Sci USA.* 2004; 101:17371–17376. [PubMed: 15574492]
55. Agniswamy J, Lei B, Musser JM, Sun PD. Insight of host immune evasion mediated by two variants of group A *Streptococcus* Mac protein. *J Biol Chem.* 2004; 279:52789–52796. [PubMed: 15466462]

56. Hess JL, Porsch EA, Shertz CA, Boyle MD. Immunoglobulin cleavage by the streptococcal cysteine protease IdeS can be detected using protein G capture and mass spectrometry. *J Microbiol Methods*. 2007; 70:284–291. [PubMed: 17543400]
57. Berge A, Björck L. Streptococcal cysteine proteinase releases biologically active fragments of streptococcal surface proteins. *J Biol Chem*. 1995; 270:9862–9867. [PubMed: 7730368]
58. Raeder R, Woischnik M, Podbielski A, Boyle MDP. A secreted streptococcal cysteine protease can cleave a surface expressed M1 protein and alter the immunoglobulin-binding properties. *Res Microbiol*. 1998; 149:539–548. [PubMed: 9795991]
59. Lamm ME, Emancipator SN, Robinson JK, Yamashita M, Fujioka H, Qiu J, Plaut AG. Microbial IgA protease removes IgA immune complexes from mouse glomeruli *in vivo*: potential therapy for IgA nephropathy. *Am J Pathol*. 2008; 172:31–36. [PubMed: 18165266]
60. Raeder R, Otten RA, Chamberlin L, Boyle MD. Functional and serological analysis of type II immunoglobulin G-binding proteins expressed by pathogenic group A streptococci. *J Clin Microbiol*. 1992; 30:3074–3081. [PubMed: 1452687]
61. Raeder R, Boyle MDP. Association between expression of immunoglobulin G binding proteins by group A streptococci and virulence in a mouse skin infection model. *Infect Immun*. 1993; 61:1378–1384. [PubMed: 8454339]
62. Chaussee MS, Ajdic D, Ferretti JJ. The *rgg* gene of *Streptococcus pyogenes* NZ131 positively influences extracellular SPE B production. *Infect Immun*. 1999; 67:1715–1722. [PubMed: 10085009]
63. Smith T, Sledjeski DD, Boyle MDP. *Streptococcus pyogenes* infection in mouse skin leads to a time-dependent up-regulation of protein H expression. *Infect Immun*. 2003; 71:6079–6082. [PubMed: 14500534]
64. Nicoletti F, Di Marco R, Papaccio G, Conget I, Gomis R, Bernardini R, Sims JE, Shoenfeld Y, Bendtzen K. Essential pathogenic role of endogenous IL-18 in murine diabetes induced by multiple low doses of streptozotocin. Prevention of hyper-glycemia and insulinitis by a recombinant IL-18-binding protein: Fc construct. *Eur J Immunol*. 2003; 33:2278–2286. [PubMed: 12884303]
65. Lira CB, Chu K, Lee YC, Hu MC, Lin SH. Expression of the extracellular domain of OB-cadherin as an Fc fusion protein using bicistronic retroviral expression vector. *Protein Expr Purif*. 2008; 61:220–226. [PubMed: 18620062]
66. Blazer LL, Boyle MD. Use of protein chip mass spectrometry to monitor biotinylation reactions. *Appl Microbiol Biotechnol*. 2007; 74:717–722. [PubMed: 17123078]

Bacterial Products	Predicted Reactive Domains
Emm 64/14	IgG ₁ IgG ₂ IgG ₄
Enn 64/14	IgG ₃
Mrp 64/14	IgG ₁ IgG ₂ IgG ₄ Fibrinogen
Enn 49	IgA
Emm 51	Plasminogen
Emm 2	IgG ₁ IgG ₂ IgG ₄ IgG
Arp 4	IgG IgA
Prt H	IgG ₁ IgG ₂ IgG ₄ IgG Albumin
BG8	Albumin
SIR 28	IgG ₁ IgG ₂ IgG ₄ IgG IgA
Emm 3	Albumin Fibrinogen Fibronectin
Emm 9	IgG ₁ IgG ₂ IgG ₄ IgG Albumin Fibrinogen

Fig. 1.

Representative functional domains present in surface binding proteins of selective Gram positive bacteria. Each of the binding proteins represented has been cloned sequenced and the binding properties confirmed. For detailed review see [31].

Fig. 2.
Schematic representation of the direct CELDI-TOF assay procedure.

Fig. 3. Application of the direct CELDI-TOF procedure to analysis of post-translationally modified BSA. One microgram of bovine serum albumin (BSA) (panel A), BSA-glucosamide (panel B), or a mixture of 1 μ g BSA and 1 μ g BSA-glucosamide (panel C) was captured with heat-killed BG8 and analyzed using the CELDI-TOF procedure detailed in the Methods.

Fig. 4.

Application of the direct CELDI-TOF procedure to measurement of IdeS activity in plasma. A 1% dilution of human, horse, cow, sheep, goat or rabbit plasma was incubated with 2 μ g of recombinant IdeS as described in the methods. An aliquot of this reaction was captured with heat-killed G1400 and analyzed for IgG digestion using the CELDI-TOF procedure detailed in the Methods.

Fig. 5.

Application of the direct CELDI-TOF procedure to measurement of IdeS activity in bacterial culture media. A control supernatant from culture lacking bacteria and supernatant from strains 2109 (SpeB⁺), 2109 SR (SpeB⁻) were incubated with 40 µg human IgG with or without the SpeB inhibitor E64 (1 µM). The reaction product was captured with heat-killed G1400 and analyzed using the CELDI-TOF procedure detailed in the Methods for IdeS activity.

Fig. 6.
Schematic representation of the indirect immune-CELDI-TOF assay procedure.

Fig. 7.

Analysis of SpeB production and M1 protein profiles for isolate 1881 in culture. WT1881 was grown in Todd-Hewitt broth at 37°C. At late log, early and late stationary phase, samples of culture supernatant and bacteria were harvested. The culture supernatants were analyzed for SpeB production and the zymogen form of the enzyme (Mr~40,350) was present in late log phase and the fully active form (Mr~28,650) was predominant in late stationary phase culture media. In the early stationary phase of growth zymogen and active SpeB as well as a number of activation intermediates could be identified. These profiles were associated with SpeB-related proteins since no peaks were observed using the immobilized normal rabbit serum control (data not shown). Bacteria harvested at each time point were extracted with CNBr. The results indicate the M1 protein in late log phase contains the native M1 protein (panel D) and the internal CNBr fragment while in the early log (panel E) and late stationary phases (panel F) an additional peak corresponding to a processed form of the M1 protein is apparent. Note: for this analysis, all M1 extracts were normalized against the internal (Mr~41 000) CNBr fragment to allow direct qualitative comparison of extracts containing different quantities of M1 protein. This figure was reproduced from with permission from Romer, T. and Boyle, M.D.P. (2003) Application of immunoproteomics to analysis of post-translational processing of the anti-phagocytic M protein of *Streptococcus pyogenes*. *Proteomics*. 3, 29–35. Copyright Wiley-VCH Verlag GmbH & Co. KGaA.

Fig. 8.

Analysis of M1 protein expressed on the surface of SpeB+ isolate 1881 grown in the presence of 30% human plasma. Panel A, the profile of CNBr extracted M1 protein for bacteria grown in media alone; panel B, the profile of CNBr extracted M1 protein for bacteria grown in media containing the cysteine protease inhibitor E64; panel C, profile of CNBr extracted M1 protein for bacteria grown in the presence of 30% human plasma. The analysis conditions were as described in figure 7. Note the additional $M_r \sim 35,000$ peaks in the lower panel represent CNBr fragments of human serum albumin.