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Comparison of automated in-gel digest methods for femtomole level samples

A comparison of automated in-gel digestion methods for low picomolar to femtomolar levels of protein is presented. Gel spots with 4 pmol to 120 fmol of protein were stained with either Coomassie colloidal blue or SYPRO Ruby and digested using an automated platform. The sequence coverages and average peak intensities obtained from a matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) analysis are compared. Results show that methods using an acetonitrile extraction or digest times greater than the standard 4 h give no significant increase in peptide sequence coverage for automated digestion of low protein level samples. It is also shown that digests from SYPRO Ruby-stained gels show a greater improvement upon ZipTip cleanup than digests from Coomassie colloidal blue-stained gels. The digests from SYPRO Ruby-stained gels are also shown to give a higher average peptide intensity if a method with minimal gel plug washing is used.

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1 Introduction

A widely used method in proteome studies is the separation of mixtures of proteins by two-dimensional electrophoresis (2-DE) and the subsequent characterization and analysis of the isolated protein spots by mass spectrometry (MS) (reviewed in [1]). MS analysis can be used to obtain peptide mass fingerprints [2] or *de novo* sequences (*via* tandem MS). With the growing interest in proteomics and with improvements in the sensitivity of MS-based approaches, the number of 2-DE spots being studied is increasing and the quantity of protein present in targeted spots is decreasing. To address this problem, several laboratories and vendors have developed automated platforms to aid in the many post-2-DE, pre-MS steps including "spot picking", enzymatic in-gel digestion, sample concentration and cleanup, and spotting onto MALDI target plates [3]. While the use of such instruments has significantly improved the quality of life of laboratory personnel, these instruments do have some limitations including questions about protein adsorption to surfaces [3] and somewhat lower peptide recoveries when compared to manual methods.

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Abbreviations: ABC, ammonium bicarbonate; **AEI**, acetonitrile extraction method, initial digest; **AER**, acetonitrile extraction method, dried down, and reconstituted; **AES**, acetonitrile extraction method, dried down, reconstituted, and sonicated; **BLG**, β-lactoglobulin; **EXT**, extended digest method; **FTA**, fresh trypsin addition method; **HCT**, higher concentration trypsin method; **MWS**, minimal washing method; **MYO**, myoglobin; **STD**, standard digest method

One of the most important pre-MS sample preparation steps is the enzymatic digestion of protein with a protease such as trypsin. Because of the kinetics of enzymatic reactions, the amount of protein digested is not linearly dependent on the amount of protein present. The percentage of peptides recovered decreases with decreasing protein concentration [3]. Thus, the optimization of digest procedures for low protein concentration spots is particularly important. For low picomole to femtomole level samples, digestion of proteins in-gel provides better peptide recovery than digestion of proteins immobilized onto a membrane [4]. A variety of in-gel digest methods have been proposed and compared [5–14]. The majority of these methods are variations of the method described by Rosenfeld *et al.* [5] and most published comparisons of digest methods have focused on protein spots or SDS-PAGE bands that contain more than a picomole of protein – a relatively high concentration by current standards. To better understand the observed losses of protein during in-gel digestion, Speicher *et al.* [12] quantified the percent protein loss at each step. They found that the percentage of protein lost during certain steps of the digest procedure depended on the initial amount of protein, with lower level samples tending to lose a higher percentage.

Speicher *et al.* [12] also observed that losses during certain steps (such as destaining) depended on the type of stain used to visualize the spots on the electrophoresis

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gel. Other work by Lauber *et al.* [15] shows that not all protein stains are equally compatible with subsequent analysis by MS. This investigation compared both stain sensitivity and resulting peptide sequence coverage from MALDI-TOF-MS analysis, and concludes that the recently developed fluorescent stains (such as SYPRO Ruby) are more compatible with mass spectrometry than are the traditional stains (such as colloidal Coomassie blue).

Here, we compare staining and automated digest methods applied to samples containing low picomole to femtomole amounts of protein. Because results were expected to be protein dependent, three different proteins (bovine serum albumin, β-lactoglobulin, and myoglobin) were studied. We performed automated in-gel digestion of both colloidal Coomassie blue-stained and SYPRO Ruby-stained spots. We compared the sequence coverage and average intensity of matched peaks obtained when the digested samples were analyzed on a MALDI-TOF/TOF instrument (Applied Biosystems 4700) [16].

2 Materials and methods

2.1 Electrophoresis and staining

Three standard proteins with a range of molecular weights were chosen: bovine serum albumin (#A-7638; Sigma, St. Louis, MO, USA), β-lactoglobulin A (#L-7880; Sigma), and myoglobin (#100862; horse skeletal muscle; ICN Biomedicals, Aurora, OH, USA). Protein solutions were made by dissolving each protein in deionized water. These solutions were used to create three protein mixtures: low, medium, and high concentration. The concentrations of these mixtures were such that 0.5 μg of each protein will be loaded onto a 1-D SDS-PAGE gel for the high concentration solution, 0.1 μg of each protein for the medium concentration solution, and 0.05 μg of each protein for the low concentration solution. Prior to electrophoresis, the mixtures were reduced with DTT reducing agent (New England Biolabs, Beverly, MA, USA) according to the manufacturer's directions. Each protein mixture was loaded onto the first nine wells of two precast 12% Tris-HCl Ready Gels (Bio-Rad Laboratories, Hercules, CA, USA; #161–1156) and 5 μL of Broad Range Protein Marker (New England Biolabs) was loaded into the last well. For gels loaded with the low protein concentration mixture, a more concentrated protein mixture was run on the last well in place of the protein marker. This procedure ensured that even if the low protein concentration could not be detected by one of the staining methods, the location of the protein bands could be determined for subsequent spot picking. Two gels (one pair for each level of protein loading) were run simultaneously at a constant voltage of 150 V for approximately 50 min in a Mini Protean 3 Cell (Bio-Rad Laboratories) until the dye front reached the bottom of the gel. The gels were removed from the glass backing and rinsed for 5 min in deionized water. The two identical gels were then stained separately. One gel was stained overnight with colloidal Coomassie blue using the Colloidal Blue Stain kit (Invitrogen, San Diego, CA, USA). Staining was performed following the manufacturer's instruction. The other gel was cut in half and each half was stained overnight in 15 mL of SYPRO Ruby Protein Gel Stain (Molecular Probes, Eugene, OR, USA) in a covered plastic petri dish. Both gels were then destained overnight at room temperature on a shaker: the colloidal Coomassie blue gel in deionized water and the SYPRO Ruby gel in 10% ACS-grade methanol (Mallinckrodt Baker, Paris, KY, USA)/7% ACS-grade acetic acid (Fisher, Fairlawn, NJ, USA). The colloidal Coomassie blue gel image was obtained by scanning it on a desktop scanner. The SYPRO Ruby gel image was obtained by scanning it on a FLA-3000 Fluorescent Image Analyzer (Fuji Photo Film Company, Tokyo, Japan).

2.2 Spot excision

To visualize protein spots for manual excision, colloidal Coomassie blue gels were placed on a lightbox and SYPRO Ruby gels on a Dark Reader transilluminator (Clare Chemical Research, Dolores, CO, USA). Four spots were manually cut from each protein band using a One-Touch manual excision tool with a 1.5 mm cutting diameter (The Gel Company, San Francisco, CA, USA). Care was taken to excise only spots that were completely within the stained band. The spots were placed directly into digest plates (one gel plug per well) for the Investigator Progest (Genomic Solutions, Chemsford, MA, USA). The spot picker was rinsed in ethanol (Pharmco, Brookfield, CT, USA) and dried with a kimwipe between cuts. The cut gel plugs were washed with 20 μL of 50% HPLC-grade ACN (Mallinckrodt Baker) for 5 min. After removal of the 50% ACN, the digest plates were covered and stored at −70°C until digestion.

2.3 In-gel digestion

All gel plugs were digested in the Investigator Progest (Genomic Solutions). All digest procedures were variations of the standard digest method as described below. The method variations chosen were those that could potentially improve sequence coverage for a wide range of proteins, not only those proteins that share a specific characteristic (*e.g.*, hydrophobic proteins).

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2.3.1 Standard digest method (STD)

Gel plugs were destained twice by alternating between a solution of 25 mM ammonium bicarbonate (ABC) (Fisher) and 100% ACN. Plugs were then incubated with a solution of 10 mM DTT (Bio-Rad Laboratories) in 25 mM ABC for 30 min. The temperature was set to 56°C for the first 10 min and then allowed to cool to room temperature during the last 20 min. The reducing solution was then replaced with 100 mM iodoacetamide (Fluka, Buchs, Switzerland) in 25 mM ABC and incubated for 45 min. The gel plugs were then washed twice with alternating solutions of 25 mM ABC and 100% ACN. The gel plugs were dried by two additions of 100% ACN. 10 μL of modified sequencing-grade trypsin (Promega, Madison, WI, USA) at a concentration of 10 μg/mL was then added. The entire vial of trypsin (20 μg) was initially dissolved in 200 μL of 1% formic acid (Genomic Solutions), and 1.8 mL of 25 mM ABC was added just before the trypsin was aliquoted to each well. The gel plugs incubated in the trypsin solution for 5 min and then 15 μL of 25 mM ABC were added to each well. The gel plugs were digested for 4 h at 37°C and the reaction was stopped by adding 7 μL of 3% formic acid to each well. After a 10 min delay, the supernatant was removed. During digestion in this instrument, the wells are not completely sealed. Because of this, we have observed differences in the final volume depending on the humidity and temperature of the lab (especially during longer digests). To prevent variation caused by environmental fluctuations, the digest plate was sealed during the trypsin digest step by covering the top of the digest microtiter plate with Thermowell Sealing Tape (Corning Inc., Corning, NY, USA), placing the digest microtiter plate into the final sample microtiter plate, and covering the sides with Parafilm. The assembly was then placed back into the Progest heat block and was disassembled after the digest at 37°C and before the 3% formic acid was added.

2.3.2 Extended digest method (EXT)

The time allowed for trypsin digestion was only 4 h in the standard digest protocol, however, most manual digest methods perform this step overnight. Therefore, an automated method was tested with an increased digest time of 12 h. Other conditions were kept the same as in the STD method.

2.3.3 Acetonitrile extraction method (AEI, AER, AES)

ACN extraction has been commonly used in published proteome studies, but some researchers have questioned the method's applicability to low-protein-level samples [12]. After the digest was finished, the initial supernatant was transferred to the final sample plate. Next, 30 μL of 100% ACN was added to the gel plug and incubated for 10 min at room temperature. The ACN was then removed and added to the initial supernatant in the final sample plate. The mixture was immediately spotted onto the MALDI target plate (AEI method). The remaining digest was transferred to an Eppendorf microcentrifuge tube that was previously deplasticized with 100% methanol. The samples were then completely dried down in a Speedvac (Savant Instruments, Holbrook, NY, USA). The dried digests were reconstituted with 10 μL of 0.1% HPLC-grade trifluoroacetic acid (Baker, Phillipsburg, NJ, USA) and spotted onto the MALDI target plate (AER method). The unused digest was ultrasonicated (VWR Aquasonic Model 75T) for 5 min and then spotted onto the MALDI target plate (AES method).

2.3.4 Higher concentration trypsin method (HCT)

Because of enzyme kinetics, more enzyme is needed for low-concentration samples to achieve the same yield as a high-concentration sample in the same amount of time. However, our experience had shown us that increasing the trypsin concentration for an in-gel digest can lead to trypsin autolysis peaks large enough to obscure smaller sample peptide peaks (data not shown). We expect that only small amounts of protein will diffuse out of the gel plug during the digest and thus digestion will occur primarily by enzyme absorbed into the dried gel plug at the beginning of the digest. Therefore, we anticipated that the addition of a high concentration of trypsin followed by the removal of excess trypsin solution not absorbed by the gel may increase the amount of trypsin available for digestion without leading to large autolysis peaks. In this method, trypsin at a concentration of 20 μg/mL in 25 mM ABC was added to the instrument immediately before the instrument aliquoted 10 μL of the enzyme to each well. After a 10 min incubation with the gel plugs, the excess trypsin solution that had not been absorbed was expelled. The 25 mM ABC digest buffer was then added as in the standard digest method.

2.3.5 Fresh trypsin addition method (FTA)

In the standard digest method, the trypsin, dissolved in 1% formic acid, was added to the machine at the beginning of the run. This solution sat in the instrument at room temperature for several hours before mixing with 25 mM ABC (to increase the pH) and then being added to the gel plugs. We believe that some trypsin autolysis may occur during the initial waiting period, so a method where tryp-

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sin was rehydrated (20 μg rehydrated in 2 mL of 25 mM ABC) and added to the instrument immediately before being aliquoted to the sample plate wells is tested.

2.3.6 Minimal washing method (MWS)

In the STD method, the two washing stages (destaining at the beginning and washing after alkylation with iodoacetamide) were done by adding 25 mM ABC and then 100% ACN to the gel plugs, repeating both steps once. In the MWS method, this was changed to a single wash of 25 mM ABC followed by 50% ACN. This shortened approach was attempted because it has been suggested that washing stages result in a higher protein loss in SYPRO Ruby-stained samples [12]. Some samples from this digest were also desalted and concentrated to 4 μL using ZipTips (Millipore, Bedford, MA, USA). The ZipTip cleanup procedure used was that recommended by the manufacturer.

2.4 MALDI-MS analysis

Digests were immediately spotted onto a 192-well stainless steel target plate for the 4700 Proteomics Analyzer. Spotting was achieved by first pipetting 0.5 μL of the sample onto the MALDI target plate and then adding 0.5 μL of 10 mg/mL recrystalized α-cyano-4-hydroxycinnamic acid (Sigma) in 50% acetonitrile/0.1% trifluoroacetic acid. 4700 Proteomics Analyzer Calibration Mixture (Applied Biosystems) was spotted down for external calibration. All samples were allowed to air-dry at room temperature. Using the CTS version of the 4700 instrument, peptide mass fingerprint sequence coverage data were collected in positive MS reflector mode from 1000 laser shots for each sample. For MS/MS analysis on this instrument, the ion of interest is isolated using a timed ion selector located after the first TOF mass analyzer [16]. The timed ion selector is composed of two tandem deflection gates. The isolated ions enter a collision chamber filled with atmospheric gas at a pressure of 1E-6 torr. The fragments resulting from collisions between peptides and air are then reaccelerated and analyzed by a second TOF mass analyzer [16]. The isolation of the peak of interest was done at a relative resolution of 50 (full width at half maximum), and data from 2000 laser shots were collected.

2.5 Data processing

The spectra were analyzed using GPS Explorer (Version 1.1, Applied Biosystems) which acts as an interface between the Oracle database containing raw spectra and a local copy of the MASCOT search engine (Version 1.8, [17]). Peptide peaks with a signal/noise ratio greater than 5 and a mass between 1100 and 4000 *m/z* were searched against the NCBInr database [18]. Up to one missed trypsin cut was allowed and the data were searched using oxidation of methionine and carbamidomethylation of cysteine as variable modifications. If the correct protein match was found, the number of matched peptides, sequence coverage, and intensities of matched peaks were taken directly from the MASCOT output. If no correct match was found, the list of peptide masses with signal/noise ratio greater than 5 was manually compared to a list of predicted peaks generated by ExPASy PeptideMass [19]. If no matching peaks were found, little or no trypsin autolysis peaks were present, and the other digest replicates gave reasonable data, it was assumed that the spotting onto the MALDI target plate was done poorly and the data was not included in this study (2 out of 600 spotted digests met this criteria). For MS/MS analysis, all peaks with a signal-to-noise ratio greater than 5 were searched against the NCBI vertebrate database using the same modifications as the MS database searches.

3 Results and discussion

3.1 Quantification of gel spot protein content

The quantity of protein present in each gel plug was estimated by measuring the ratio of the area of the cut spot to the entire monomer band, and multiplying the ratio by the amount of protein loaded. For bovine serum albumin (BSA), a dark band appeared above the monomer BSA band on the 1-D gel. Image analysis (Multi-Analyst, Bio-Rad Laboratories) determined that 15% of the loaded protein was in this heavier band. For the high protein level gels the amount of protein in each excised spot was approximately 1 pmol BSA, 4 pmol β-lactoglobulin (BLG), and 4 pmol myoglobin (MYO). For the medium level gels, the amount per spot was 170 fmol BSA, 700 fmol BLG, and 700 fmol MYO. For the low level gels, the amount per spot was 120 fmol BSA, 400 fmol BLG, and 400 fmol MYO.

3.2 Gel stain comparison

The resulting peptide mass fingerprint sequence coverages are summarized in Fig. 1. The data represent the average value for the digests from the four gel spots taken from each SDS-PAGE band. The sequence coverage gives a measure of the number of matching peptide

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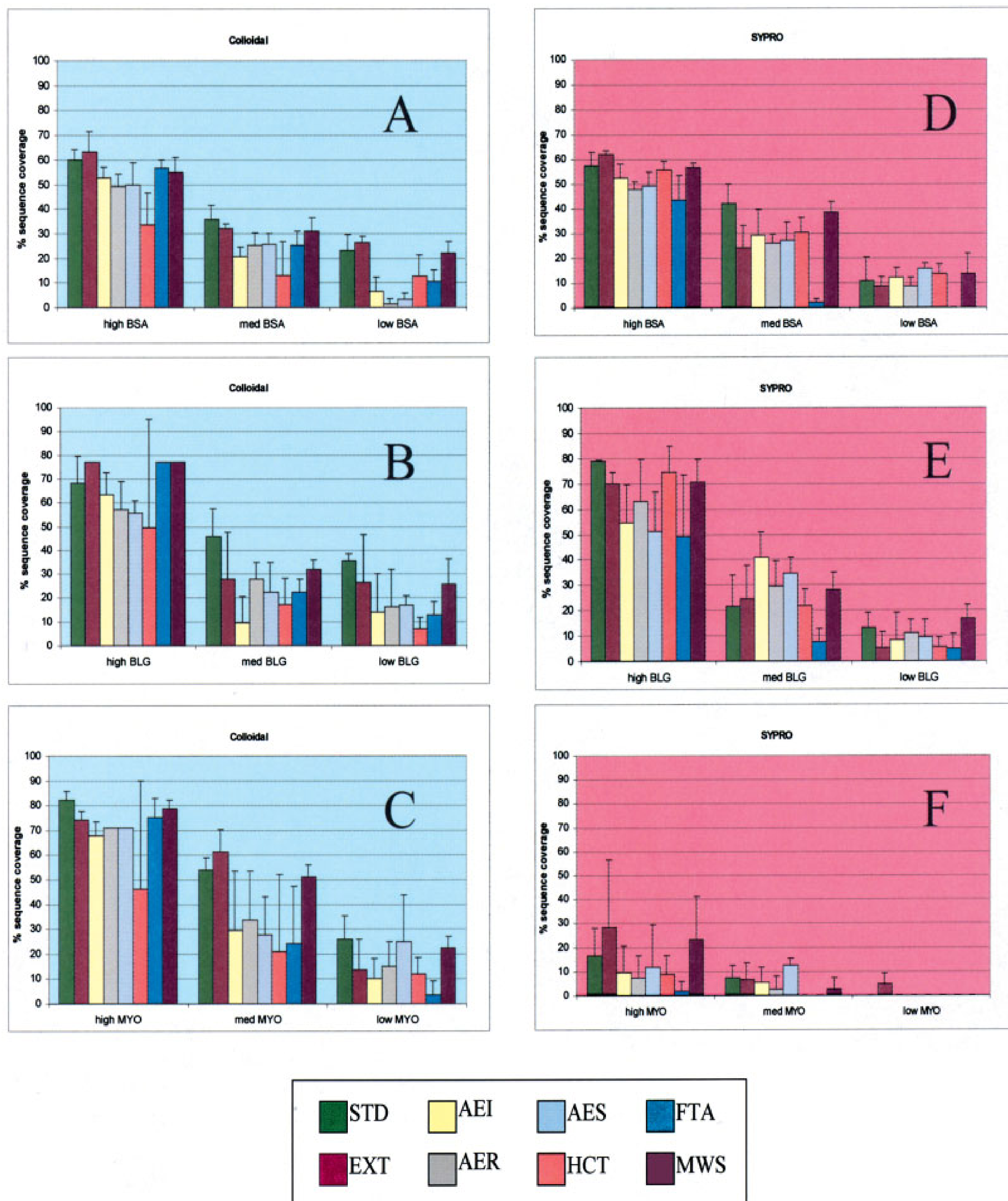

Figure 1. Comparison of the measured sequence coverage resulting from different in-gel digestion methods. The label on the horizontal axis indicates the protein (BSA, BLG or MYO) and the concentration of protein in the gel plug (high, medium, low). (A) BSA gel plugs stained with colloidal Coomassie blue. (B) BLG gel plugs stained with colloidal Coomassie blue. (C) MYO gel plugs stained with colloidal Coomassie blue. (D) BSA gel plugs stained with SYPRO Ruby. (E) BLG gel plugs stained with SYPRO Ruby.(F) MYO gel plugs stained with SYPRO Ruby.

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masses (and thus the quality of the peptide mass fingerprint) and an estimate of the maximum peptide sequence that could be derived from MS/MS experiments. The error bars representing the standard deviation of the sequence coverage are relatively large because, for the peptide mass range studied here, each matched peptide contains around 10–20 amino acids. Therefore, even if the number of matched peptides from two digests only differs by one, the sequence coverage will reflect a change of at least ten amino acids. For the smaller MYO and BLG proteins, ten amino acids is about 6% of the sequence coverage. Based on the results in Fig. 1, there is no significant difference in sequence coverage between spots stained with colloidal Coomassie blue and SYPRO Ruby at higher concentrations. Colloidal blue staining did give slightly better sequence coverage for spots at low protein levels. MYO was not stained well by SYPRO Ruby at any of the protein concentrations tested. This posed a technical challenge to excise the spots from the correct location, especially at the medium and low concentration levels. The sequence coverage was very low for the SYPRO Ruby-stained MYO digests (Fig. 1F), but it was not possible to determine whether this result was caused by the stain's impact on digestion, or by incorrect spot excision.

It has been shown that sample cleanup and concentration using ZipTips improves sequence coverage and signal intensity [20]. Anecdotal evidence suggests that digests from SYPRO Ruby-stained gels may benefit more from ZipTip treatment than digests from colloidal Coomassie blue-stained gels. To test this, digests from the low protein level gels, digested using the minimal washing method, were cleaned and concentrated to 4 μL with ZipTips and then spotted on the MALDI target plate. The sequence coverage before and after sample cleanup

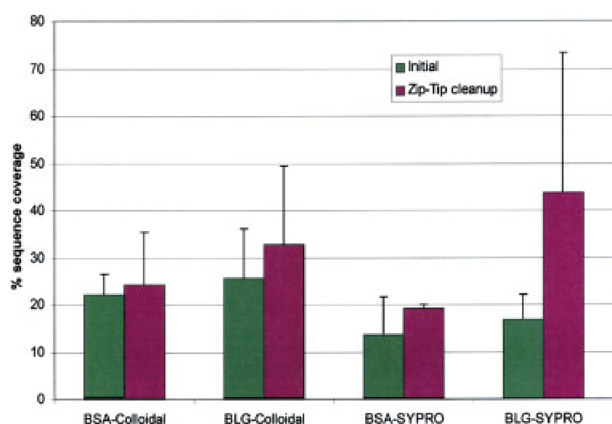

Figure 2. Comparison of measured sequence coverage before and after ZipTip cleanup. These gel plugs were from a low concentration gel and were digested using the minimal washing method.

and concentration is shown in Fig. 2. We observed the following increases in percent sequence coverage for digests from colloidal Coomassie blue-stained gels. BSA was at 22% sequence coverage initially and went to 24% sequence coverage after ZipTip cleanup. BLG went from 26% to 33% sequence coverages. The change in sequence coverage for digests from SYPRO Ruby-stained gels were larger. BSA went from 14% to 19% sequence coverage and BLG went from 17% to 44% sequence coverage. The end result was protein-dependent, with BLG showing a higher sequence coverage from digests of SYPRO Ruby-stained gels and BSA showing a higher sequence coverage from digests of colloidal Coomassie blue-stained gels. However, there was variability in the observed sequence coverage for both proteins.

3.3 Sequence coverage comparison of digest methods

Using the STD method, 20–30% sequence coverage was achieved even with only a few hundred femtomoles of protein in the gel plug. The EXT method does not appear to be an improvement over the STD method, despite the extended time available for enzymatic digestion. Interestingly, for some gel spot sets, the average sequence coverage was lower than that for the STD method. The increase in peptides due to the longer digestion time [14] is balanced by the amount of trypsin autolysis products (which can suppress the peptide signal) which also increases with time. Although it is possible that a smaller increase in the digestion time may increase sequence coverage, these results indicate that an overnight digestion is not necessary.

In Fig. 1, only one gel plug set (medium BLG-SYPRO) shows the initial sample from the ACN extraction method (AEI) as having a greater sequence coverage than that achieved with the STD method; the other gel plug sets show the AEI method yielding less sequence coverage. This decrease may be caused by the low concentration of proteins used in the study. The small increase in peptide concentration due to the second extraction with ACN may have been negated by the increase in sample volume. To increase the peptide concentration (and remove the ACN so ZipTip cleanup can be performed), the initial sample was completely dried and reconstituted in a nonorganic, low-volume solution (AER method). However, drying the samples may lead to sample loss by adsorption to surfaces. If so, this process explains why the reconstituted samples did not show an overall increase in sequence coverage, even though a greater percentage of the sample was loaded onto the MALDI plate. The reconstituted digests were sonicated in an attempt to

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dissolve some of the adsorbed protein (AES method). Only the sequence coverage of the low protein level spots seemed to improve with sonication.

The next two digest methods, HCT and FTA, did not provide as high a sequence coverage as the STD method. The results of the higher concentration trypsin method appear to be inconsistent. Some gel plug sets (such as medium BLG-SYPRO) showed the HCT method producing an average sequence coverage similar to the STD method. Others (such as medium BSA-colloidal Coomassie blue) showed an average sequence coverage far below that of the STD method. Furthermore, the standard deviations of the sequence coverage for the HCT method were large when compared to those of other methods. In this method, the only trypsin available for digesting the protein was that which entered the gel plug when the plug was being rehydrated in the trypsin buffer (after rehydration the excess buffer was removed). These observations are consistent with variable amounts of trypsin entering the gel plug. This may be because, unlike in the manual digest where the gel plugs are often dried down in a Speedvac prior to rehydration, in the automated mode they are dried down with ACN only. Differences in dryness between gel plugs would lead to variations in the amount of the trypsin solution that was absorbed.

The FTA method gave percent sequence coverages similar to the STD method for gel spots from the high protein level colloidal Coomassie blue gels. However, coverage was lower than with the STD method for all other gel spot sets. It is not clear why this method did not work as well as the STD method. The trypsin was rehydrated within minutes of being added to the gel plugs, so it cannot be due to extensive autolysis.

The final digest method tested was the minimal washing (MWS) method. This method gave similar sequence coverage results to the STD method, though it often gave a slightly lower average sequence coverage for digests from colloidal Coomassie blue-stained spots. It has been shown that complete destaining is important for digestion of colloidal Coomassie blue-stained gels [21]. The minimal washing method may not have been effective in destaining some of the colloidal Coomassie blue gel plugs.

3.4 Peak intensity comparison of digestion methods

One concern for the analysis of gel spots with low levels of protein is that there may not be enough peptide masses measured with MS to identify an unknown protein solely by its peptide mass fingerprint. In these cases, matching sequence ions from tandem MS becomes essential for protein characterization. However, not all peptide fragments can be successfully identified using MS/MS. One important factor in determining whether MS/MS will be successful using a MALDI platform is the intensity of the parent peak. Figure 3A shows a typical MS spectrum from a high-protein-level BSA spot digested with the minimal wash method. Figure 3B shows a typical MS spectrum from a medium-protein-level BSA spot from the same digest. MS/MS data were acquired on 1439.8 *m/z* from both of the samples. The resulting spectra are shown in Figs. 3C and D. A database search of the spectra in Fig. 3C (whose parent peak from 3A had an intensity of 9000) was positively identified as BSA with a MOWSE score of 39 and a confidence interval (calculated by GPS Explorer) of 98%. A score over 34 for MS/MS data indicates extensive homology or identity. The confidence interval is a statistical calculation that can be used to compare results between different database searches. The closer the confidence interval is to 100%, the more likely the identification is correct. The spectra in Fig. 3D (whose parent peak from 3B had an intensity of 1700) had a correct BSA match for the top-scoring protein, but the MOWSE score was only 16 and the confidence interval was 0%.

To estimate how well different digest methods would work for MALDI-TOF/TOF-MS/MS analysis, the average intensity per matched peak was determined. The intensity per matched peak was found by dividing the total intensity of all matched peaks by the total number of matched peaks. This number was then averaged over the four gel plugs from that protein band. The average intensity per matched peak was then scaled by dividing it by the average intensity of the 2211.1 *m/z* trypsin autolysis peak for that gel spot set. Note that for the acetonitrile extraction method series (AEI, AER, AES) all of the intensities per matched peak were scaled using the intensity of the 2211.1 *m/z* peak of the initial sample (AEI). Only the methods whose variation from the STD method should not change the degree of trypsin autolysis are included in this comparison: STD, AEI, AER, AES, and MWS. This data is shown in Fig. 4 for BSA and BLG. Two observations can be drawn from this data. First, the subsequent steps in the acetonitrile extraction methods (drying down and sonicating) did increase the average intensity of the peptide peaks present. Second, and perhaps more important, the minimal wash method gave a higher intensity than the STD method in all cases except the high-protein-level BSA-colloidal Coomassie blue gel plug set. These results can be explained by the findings mentioned earlier that suggest that SYPRO Ruby-stained gels may lose more protein during washing steps than some other stains [12], and that stain removal is very important for digestion of colloidal Coomassie blue-stained gels [21].

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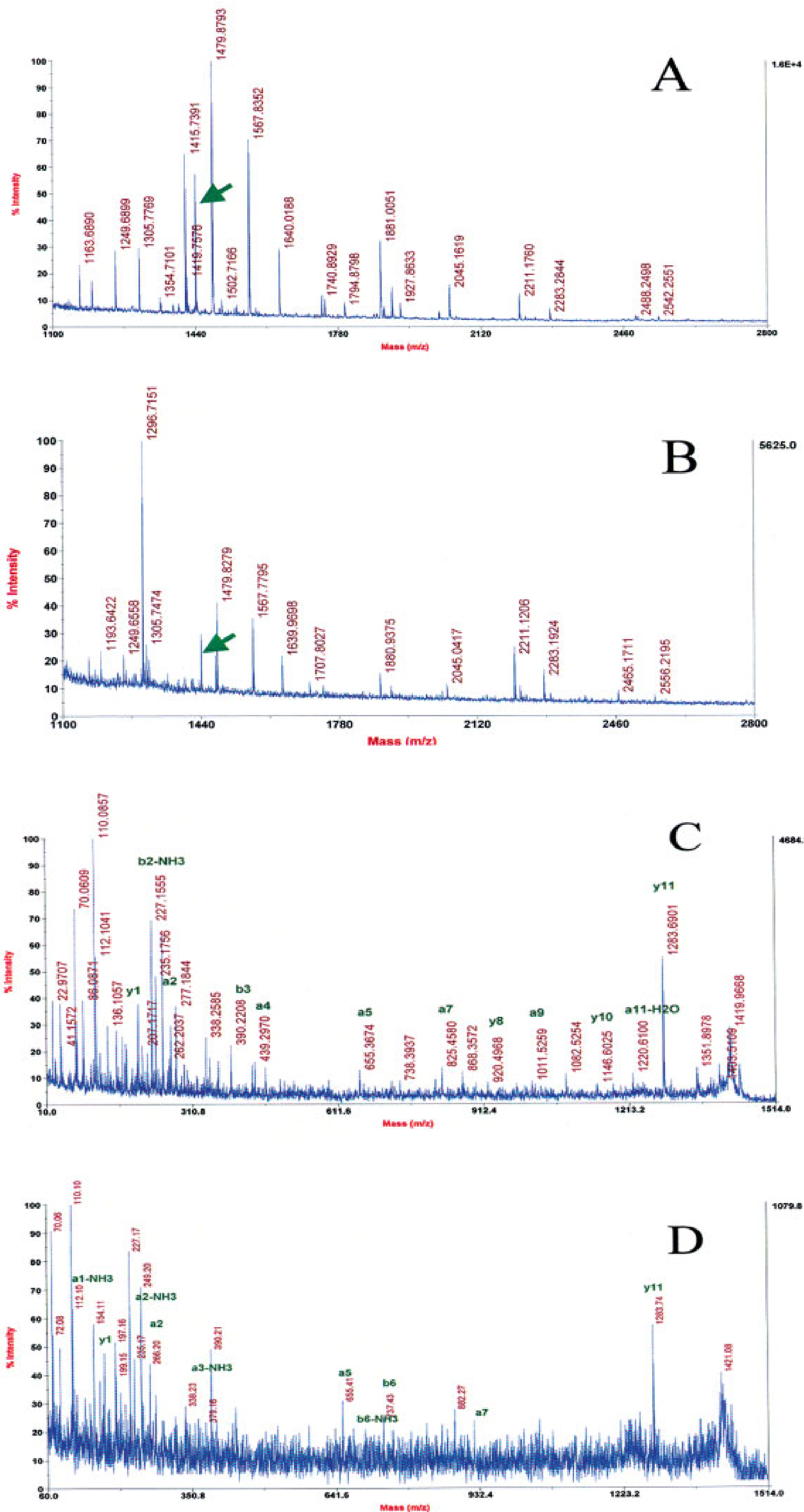

Figure 3. (A) MALDI-TOF-MS spectrum of a digest from a BSA gel plug with high protein concentration, digested with minimal washing method. (B) MALDI-TOF-MS spectrum of a digest from a BSA plug with medium protein concentration, digested with minimal washing method. (C) MALDI-TOF/TOF-MS/MS spectrum of 1439.8 *m/z* peak from spectrum A (shown with green arrow). The precursor ion had an intensity of 9000, and the MS/MS spectrum was correctly identified with a MOWSE score of 39. (D) MS/MS spectrum of 1439.8 peak *m/z* from spectrum B (shown with green arrow). The precursor ion had an intensity of 1700 and, although the top MASCOT match was BSA, it only received a MOWSE score of only 16.

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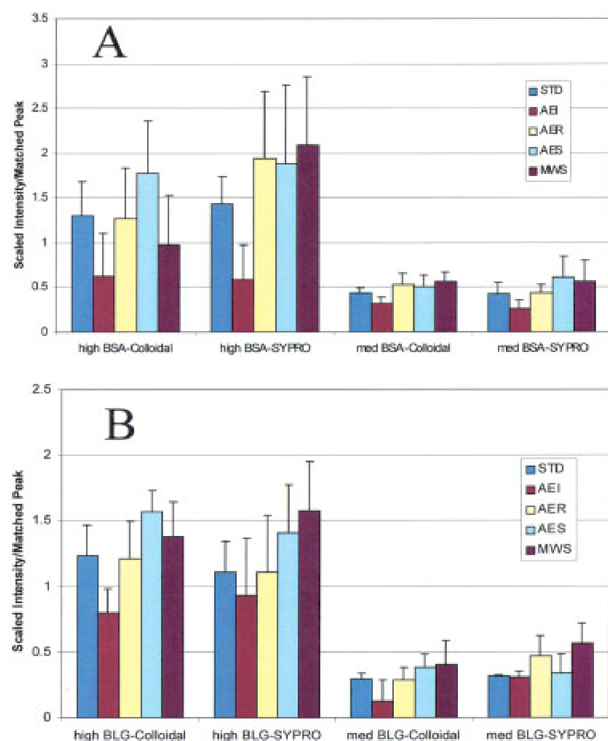

Figure 4. Comparison of average scaled intensity per matched peak for digests of (A) BSA and (B) BLG analyzed with MALDI-TOF-MS. The average intensity per matched peak was scaled by the average intensity of the 2211.1 *m/z* trypsin autolysis peak for that digest method.

4 Concluding remarks

This is the first method comparison focusing on the automated in-gel digestion of femtomole level proteins. We have found that, although widely used in published proteome studies, the ACN extraction method is not necessary, and may even lower sequence coverage, for automatic digestion of low-protein-level gel spots. The sequence coverages after drying, reconstituting, and sonicating the ACN extracts were similar to those observed with the standard method. However, the post-digestion steps associated with the AES method greatly increased sample preparation time and could not be easily automated. We also showed that although manual methods often use an overnight digestion, the automatic digestion of low-protein-level gel spots did not show a significant improvement in sequence coverage when the digestion time was increased to from 4 to 12 h. When comparing digests from differently stained gel plugs, we observed that digests from SYPRO Ruby-stained gels showed a greater increase in sequence coverage after ZipTip cleanup and concentration than digests from colloidal Coomassie blue-stained gels. We also found that the average intensity of matched peaks in digests from SYPRO Ruby-stained gels increased when a method that minimized gel plug washing was used.

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