From gel to MS: factors influencing peptide recovery from gels

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Introduction

A typical gel based approach to proteomics involves the staining of a 1-D or 2-D polyacrylamide gel with a protein stain, excising the band(s) of interest, in-gel digestion and extraction of the resulting peptides for analysis by mass spectrometry. A number of factors influence the success of the technique and little effort has so far been devoted to determining the relative impact of these factors.

To assess the effects of different staining procedures, gels were stained with Coomassie blue, silver or zinc-imidazole (Zn-ImH) "reverse" staining. The effect of these stains was assessed using a number of standard proteins with and without destaining prior to digestion. Furthermore, the effect of the choice on recovery of peptides and subsequent protein identification from gels stored for longer periods was also assessed.

To demonstrate the compatibility of the Zn-ImH staining with mass spectrometry, we have applied our findings to endo- and exo- proteomic profiling of *Aspergillus* sp.

Materials and methods

Gel separation of proteins:
Bovine serum albumin (BSA), Enolase, Alcohol dehydrogenase (ADH) and α-casein, were separated on a 10% SDS-PAGE gel as per Laemmli [1].
Aspergillus nidulans cell extracts were prepared by detergent treatment before being analysed by 2-DE.
Growth medium from *Aspergillus niger* was concentrated by acetone precipitation prior to 1-DE.

Gel staining methods:
Described in Table 1

Digestion and peptide recovery:
In-gel digestion was carried out as described [3]. The resulting peptides were extracted by the addition of 2 volumes of ACN and dried prior to analysis.

MALDI-ToF Mass spectrometry:
Samples were desalted using ZipTips (Millipore). 1µl of sample was mixed with 1µl of matrix (CHCA in 50% ACN, 0.1% TFA) and spotted onto a stainless steel target. Samples were analysed on a Voyager STR (Applied Biosystems) instrument in reflectron mode.
Proteins were identified by comparison with theoretical peptide mass fingerprint (for standards). For *Aspergillus* protein identification, MALDI data was searched against an in-house MASCOT *Aspergillus* protein database.

LC-MS/MS:
Proteolytic digests were subjected to LC-MS/MS using a QTof1 upgraded to QTof2 specification (Waters) coupled to an UltiMate nanoflow HPLC (LC Packings). Following data acquisition using automatic function switching, generated data were subjected to peak picking and submitted for MASCOT searching using the MSDB database.

Gel staining comparison

Coomassie or silver staining techniques are commonly used to visualise gel separated proteins prior to processing and analysis. We have assessed the compatibility of Zn-ImH staining as an alternative to classical silver and Coomassie staining techniques. This stain offers similar limits of detection (Figure 1) and is relatively quick and simple to perform (Table 1).

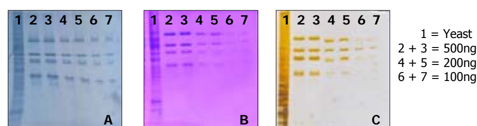


Figure 1: Comparison of Zinc (A), Coomassie (B) and silver (C) staining.

The advantages of destaining silver stained gel bands prior to processing have been well documented [4]. We have found that destaining of Coomassie and Zn-ImH stained bands also improves the quality of the data obtained. In the example below (Figure 2) the sequence coverage of this Coomassie stained enolase gel band was increased from 26% to 38% when bands were completely destained prior to digestion. Similar results were observed for other proteins and stains (data not shown).

Panel A shows MALDI spectrum for 400ng in gel digest of Coomassie stained enolase gel band processed using standard procedures, the sequence coverage was 26% (A). When stained bands were destained to completion prior to processing an improved sequence coverage of 38% was observed (B).

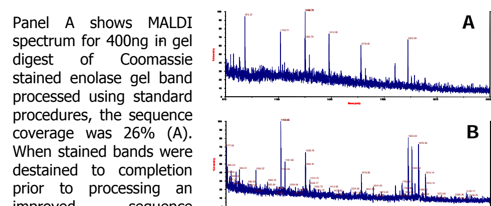
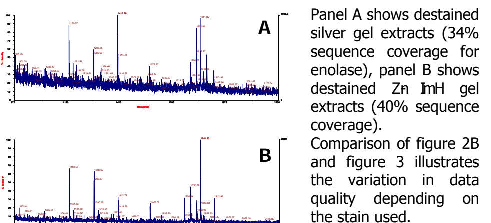


Figure 2: Enhanced peptide detection with gel destaining prior to processing



Panel A shows destained silver gel extracts (34% sequence coverage for enolase), panel B shows destained Zn ImH gel extracts (40% sequence coverage).
Comparison of figure 2B and figure 3 illustrates the variation in data quality depending on the stain used.

Figure 3: Comparison of MALDI spectra obtained from destained silver and Zn-ImH stained gel bands

Whilst enough peptides for identification were detected by MS following application of all three staining techniques, both Coomassie and Zn-ImH staining gave superior quality results with both giving better sequence coverage and clearer, more intense, spectra with reduced background.

Application of Zn-ImH staining

We have applied our knowledge of factors influencing peptide recovery, and Zn-ImH staining, to our characterisation of the endo- and exo- proteomes of *aspergillus niger* and *nidulans*.

Detergent extracted proteins from *Aspergillus nidulans* were analysed by 2-DE. A number of protein spots were selected for gel processing and the peptides analysed by MALDI-ToF. Figure 4 shows one of the proteins identified.

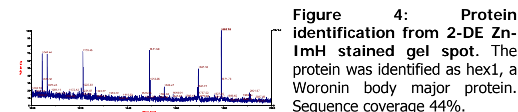
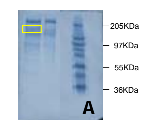
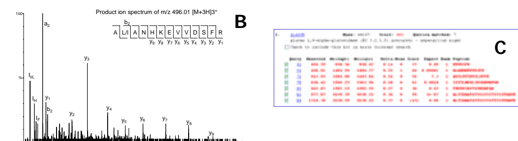


Figure 4: Protein identification from 2-DE Zn-ImH stained gel spot. The protein was identified as hex1, a Woronin body major protein. Sequence coverage 44%.

In another study, the proteins secreted into growth medium (the exo-proteome) of *Aspergillus niger* were separated by 1-DE, visualised using Zn-ImH staining and analysed by LC-MS/MS. This combines the sensitivity of the reverse staining technique and automation of LC-MS techniques to provide a high throughput method of protein secretion by *Aspergilli*. Figure 5 shows a typical protein identification.

Figure 5: Identification of media secreted proteins. A band (A) was excised, digested and analysed by LC-MS/MS (B). The protein was identified by MASCOT searching as α-glucosidase (C).





Zinc-Imidazole (Zn-ImH):
Staining and destaining were carried out as described [2].
Gels were washed briefly in water before incubation for 15 min in 0.2M imidazole, 0.1%SDS. Gel development was performed with 0.2M zinc sulfate.
Gel pieces were destained by treatment with 25mM Tris, 0.3M glycine pH8.0 or 1% acetic acid.

Coomassie brilliant blue (CBB):
Gels were incubated in staining solution (Sigma) for 30 min. The gel was then destained in 50% methanol, 5% acetic acid for 30 min until bands appeared.
Additional destaining prior to in-gel digestion was performed by incubation of the gel pieces at 37°C for 20 min in 50% 25mM ammonium bicarbonate in acetonitrile (ACN) as required.

Silver staining:
Silver staining was carried out as described previously [3]. Gel pieces were destained using 30mM potassium ferricyanate and 100mM sodium thiosulfate [4].

Table 1: Staining methods used in this study

Gel age affects peptide recovery

Superior results were obtained with gel bands processed immediately after staining when compared with those processed after 7 days storage in water (at 4°C). This was observed for all protein stains tested.

Figure 6 below illustrates this effect. Stained BSA gel bands processed immediately after staining resulted in a sequence coverage of 15-30% depending on the staining method used. In contrast, gel pieces processed after 7 days storage in water at 4°C resulted in sequence coverages of less than 10%, illustrating that all stains have a detrimental effect over time.

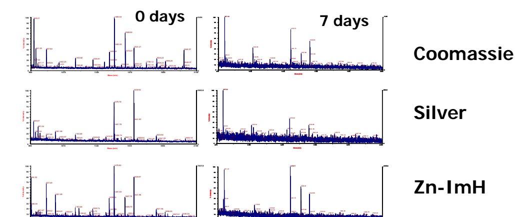


Figure 6: Gel age affects peptide yield. BSA bands were fully destained and digested immediately after staining or after 7 days storage. All bands analysed after 7 days storage resulted in a sequence coverage of 9%.

Summary

The success of an in-gel based approach is influenced by a number of factors. We have demonstrated that the choice of stain, destaining prior to digestion and the time of gel processing can affect the quality and reliability of the data obtained.

Although Zn-ImH staining is not quantitative, for many applications it may provide a viable alternative to classical silver staining. This stain is faster to visualise, and remove, offers similar limits of detection and gives superior MS results when compared to silver staining.

We have applied our knowledge of factors influencing peptide recovery from gels to our research into the exo- and endo- proteomes of *Aspergillus* species.

References

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