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Guanidination of Tryptic Peptides without Desalting for MALDI-TOF MS Analysis

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Abstract

Derivatizations that enhance mass spectral quality often require desalting which presents as a bottleneck in MALDI-TOF MS-proteomics. Guanidination, which converts lysine to homoarginine, an arginine analog, can increase detection of those peptides 5 – 15-fold. Our aim was to improve guanidination by using a novel reagent, *O*-methylisourea-freebase. In a simple reaction, interfering salts were removed prior to guanidination. Freebase preparation took about 30 min and could be applied to samples all at once as opposed to desalting samples one-by-one for 5 min each. For freebase guanidinated BSA tryptic peptides, more than 6-times the peptides were observed relative to tryptic peptides or those guanidinated with the conventional reagent, *O*-methylisourea hemisulfate. Peptide signals increased more than 10-fold relative to those from guanidination with the conventional reagent and were equivalent to those from conventional guanidination with desalting. In addition, freebase guanidination allowed for a lower limit of detection when combined with another derivatization, *N*-terminal sulfonation, as evidenced by MS/MS fragmentation analysis of in-gel digests of cytochrome *c*. Freebase guanidination of rat lung proteins after 2-D gel electrophoresis allowed for identification of all tested protein spots regardless of protein characteristics (MW or pI) or abundance. Co-derivatization with *N*-terminal sulfonation confirmed the identity of low abundance proteins in 2-D gel spots that contained more than one protein. The freebase guanidination reagent is simple to prepare and to implement. Desalting is not needed prior to MALDI-TOF MS. Freebase guanidination effectively increases the dynamic range of detection of lysine-containing peptides while decreasing the work needed for sample preparation.

Keywords

Guanidination; MALDI-TOF MS; *N*-terminal sulfonation; proteomics

INTRODUCTION

Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) is an important analytical tool for proteomics due to its high throughput capability and robustness. It is the method of choice for identifying proteins separated by 2-D gel electrophoresis (2-DGE), which in combination with MALDI-TOF MS is valuable for examining roles of proteins involved in biological processes¹⁻³ and for discovery of biomarkers.⁴⁻⁶

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Author Contributions

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2-DGE separates proteins into single protein spots that can be excised from the gel and digested with a protease, typically trypsin. Trypsin cleaves proteins at the *C*-terminus of arginine or lysine into peptides suitably sized for MS analysis (*i.e.*, 700 – 4000 Da). The basic functionality of the *C*-terminus of tryptic peptides stabilizes the positive charge necessary for MS.

In MALDI-TOF MS, a distinct bias is observed toward arginine-terminated peptides on the order of 5 – 15-fold intensity when compared to their lysine-terminated counter-parts.⁷ The guanidine side group of arginine (pKa 12.5) has a more favorable acid-base reaction with the matrix and ionizes more readily in the MALDI process than the amine side group of lysine (pKa 10.5).^{7,8} This so-called “arginine effect” is also observed during time of flight (*i.e.*, mass analysis), where peptides terminating in arginine are less prone to metastable fragmentation.⁹

Chemical derivatization is commonly employed to improve the quality of MALDI-TOF spectra.¹⁰ Guanidination is a derivatization that fully takes advantage of the “arginine effect” to increase the dynamic range of detection by converting lysine to homoarginine, an arginine analog. At an alkaline pH (pH > 10.5), *O*-methylisourea reacts with the α -amino group of lysine to form homoarginine in a selective manner.¹¹⁻¹³

Shifting the functionality of lysine to mirror that of arginine is beneficial for a number of reasons, the most apparent being an increase in detection of lysine-terminated peptides. Such an increase could improve proteome coverage in peptide mass fingerprinting (PMF)¹² studies and could allow for in-depth analysis of single proteins. For example, guanidination allowed for pinpointing of oxidation sites thought to be unique to Parkinson disease.⁵

Guanidination can also prevent unwanted side-reactions to lysine when the *N*-terminus is to be derivatized. *N*-Terminal sulfonation, which promotes uniform fragmentation in MALDI-TOF MS/MS, is arguably the most common derivatization to combine with guanidination.¹⁴ Such a combination was used to increase coverage of 2-D gel excised proteins¹ and in *de novo* sequencing of novel proteins.¹⁵⁻¹⁹ In quantitative proteomics, guanidination was used directly as an isotopic tag²⁰ or as a protecting group prior to labeling the *N*-terminus with a tag.^{21,22} Another advantage of guanidination is the mass shift of 42 Da which helps distinguish lysine from glutamine (0.04 Da).¹¹

Salts from guanidination interfere with MALDI-TOF MS and desalting is required.²³ Desalting is time consuming, labor intensive, and costly. Consequently, guanidination is often performed as a second pass on protein spots that did not result in a statistically significant identification.^{1,24} Relative quantitation using isotope labeled guanidination reagents could be automated except for the desalting step.²⁰

Alternative guanidination methods do not require desalting, however, there are disadvantages. Desalting is optional for a commercial guanidination kit that uses a low concentration of *O*-methylisourea-salt.²⁵ However, potential problems include incomplete derivatization, which complicates spectra and may interfere with quantitation. Although in-gel guanidination of proteins prior to digestion was assumed to be complete, it is incompatible with in-solution digests.^{26,27} Sergeant et al.²⁶ reported that trypsin has lower digest efficiency toward homoarginine resulting in longer peptides which tend to be more difficult to analyze.

The objective of this study was to develop freebase guanidination, which is a simple and robust alternative to the conventional guanidination procedure. Use of *O*-methylisourea-freebase increases sensitivity and high throughput capability of the MALDI-proteomics workflow. In a simple reaction, *O*-methylisourea-salt is reacted with barium hydroxide, the

products of which separate easily—*O*-methylisourea-freebase goes to the aqueous phase and unwanted salts precipitate.²⁸ The freebase guanidination reagent gives complete guanidination and does not leave behind interfering salts. This is especially useful for applications, as demonstrated, for which additional purification steps are not desirable.

EXPERIMENTAL SECTION

Materials and Reagents

Trypsin Gold, MS grade was purchased from Promega (Madison, WI). Standard proteins: BSA, ovalbumin (chicken), myoglobin (horse), and cytochrome *c* (horse) (Sigma, St. Louis, MO) were used. The MALDI matrix α -cyano-4-hydroxycinnamic acid (HCCA) and Peptide Calibration Standard II were from Bruker (Billerica, MA). C₁₈ NuTips™ were from PolyLC (Columbia, MD). Other reagents included *O*-methylisourea-hemisulfate (Sigma), barium hydroxide octahydrate (J.T. Baker Chemicals), ammonium hydroxide (NH₄OH) (Mallinckrodt, Inc.) and 2-sulfobenzoic acid cyclic anhydride (Acros Organics, New Jersey). Acetonitrile (LC-MS grade) and ammonium bicarbonate (NH₄CO₃) were from Fisher Scientific. Water was purified on a Milli-Q™ Advantage A10™ system (Millipore, Billerica, MA). Laemmli sample buffer, Bio-Safe Coomassie Stain, and other gel electrophoresis supplies were purchased from Bio-Rad (Hercules, CA). Rat lung tissues from 8 week-old Sprague Dawley Crj:CD 1GS male rats were provided by the Korean Rural Development Administration (Gyeonggi-do, Korea).

One-Dimensional Gel Electrophoresis (1-DGE)

Mixtures of standard proteins were separated *via* 1-DGE in triplicate.²⁹ The mixtures consisted of 12.5, 25, or 50 pmol each of BSA, ovalbumin, myoglobin, and cytochrome *c*. Proteins were separated on a 12% polyacrylamide gel with a MiniProtean apparatus (Bio-Rad) at constant 15 mA for 2 h. The gel was stained with Bio-Safe Coomassie Stain according to the Bio-Rad protocol.

2-DGE

Proteins were extracted from homogenized rat lung tissues in rehydration buffer (Bio-Rad) and the concentrations were determined with a Bradford assay.³⁰ Lung proteins were separated *via* 2-DGE according to the Bio-Rad protocol.³¹ Whole protein extract (200 µg) was applied to an immobilized pH gradient strip (7 cm, pH 3-10, linear) using active rehydration at 50 V for 16 h at 20 °C on a Protean i12 isoelectric focusing (IEF) cell (Bio-Rad). IEF consisted of rapid ramping to 250 V (15 min), gradual ramping to 4,000 V (1 h), and then rapid ramping at 4,000 V for 15,000 Vh. The second dimension was run on a pre-cast gel (Any kD™ Mini-Protean TGX™, Bio-Rad) at constant 200 V for 0.5 h. The gel was stained with Bio-Safe Coomassie Stain as described for 1-DGE. Spots were manually picked with a pipette tip, modified by cutting off the end of the tip.

In-Gel Trypsin Digest

In-gel trypsin digestion was conducted according to a protocol from Cold Spring Harbor³² with only slight modification to peptide extraction. Following reduction and alkylation, proteins were digested with trypsin [12.5 (2-D gel spots) or 25 ng trypsin (1-D gel bands) in 50 mM NH₄CO₃] at 37 °C for 16 h. Peptides were extracted by sonication for 5 min at ambient temperature in 25 mM NH₄CO₃, followed by addition of acetonitrile and repeated sonication. After transferring the supernatant into fresh vials, peptides were extracted in 0.05% trifluoroacetic acid (TFA) and then 0.05% TFA, 50% acetonitrile. 1-D gel extracts were divided into 3 aliquots for comparison of 3 guanidination methods (freebase, conventional, and conventional with desalting) and then were dried *via* SpeedVac.

In-Solution Trypsin Digest

In-solution trypsin digestion of BSA (200 pmol) was conducted in triplicate according to Russell et al.³³ which was completed in 1 h by incorporating 80% acetonitrile into the digest solution. Reduction and alkylation were also in 80% acetonitrile solution. Peptides were dried *via* SpeedVac and resuspended in 100 μ L of 5% acetonitrile, 0.3% formic acid and stored at -20 °C.

Guanidination Reagent Preparation

The freebase guanidination reagent was prepared with significant modification to Imbeah et al.²⁸ in order to make the final product suitable for downstream MS application. To prepare *O*-methylisourea-freebase, 50 mg of *O*-methylisourea hemisulfate was dissolved in 51 μ L of water and then added to 64 mg of barium hydroxide octahydrate to yield an 8 M *O*-methylisourea and 4 M barium hydroxide solution. It is important to note that barium hydroxide should be in a 1:1 (mol/mol) ratio with sulfate. The freebase reaction was mixed by pulse vortexing (10 min) and then centrifuged (20,800 *g*) at 4 °C for 5 min. The clear supernatant (40 μ L) was collected, avoiding the pellet. Next, 112 μ L of acetonitrile was added to bring it to ~74% acetonitrile. The solution was vortexed for 15 s and a precipitate formed. The precipitate was pelleted as in the previous step. The mixture separated into 3 phases- 1 solid precipitate and 2 liquid phases. An aliquot of 100 μ L (2.11 M *O*-methylisourea-freebase in 74% acetonitrile, pH 9 – 10) was carefully collected from the top liquid layer and mixed with 25 μ L of 14.8 N NH₄OH, 35 μ L of acetonitrile, and 51 μ L of water to prepare a 1.0 M *O*-methylisourea-freebase solution in 50% acetonitrile containing 1.75 N NH₄OH (pH 11) as the working solution. The conventional guanidination reagent was prepared with slight modification to Beardsley and Reily.²³ *O*-methylisourea hemisulfate (50 mg) was dissolved in 119.3 μ L of water, 163.2 μ L of acetonitrile, and 96.5 μ L of 14.8 N NH₄OH, making a solution of 1.0 M *O*-methylisourea, 40% acetonitrile, 3.5 M NH₄OH (pH 11).

Guanidination

Dried peptides were dissolved in 5 μ L of *O*-methylisourea-freebase or *O*-methylisourea hemisulfate solution by sonication for 5 min. After guanidination at 65 °C for 20 min, the solution was dried *via* SpeedVac for approximately 5 min.

Sulfonation

Sulfonation was conducted with modification to Keough et al.¹⁴ Sulfonation solutions were prepared daily by dissolving 20 mg of 2-sulfobenzoic acid cyclic anhydride in 1 mL of dry tetrahydrofuran. Dried guanidinated peptides were dissolved in 2.5 μ L of 25 mM triethylammonium bicarbonate, 50% acetonitrile. Next, 2.5 μ L of the sulfonation solution was added to bring it to pH 8 – 9, followed by sonication for 5 min at ambient temperature. After the reaction, the solution was evaporated *via* SpeedVac.

Sample Preparation for Mass Spectrometry

Freebase guanidinated peptides (with or without sulfonation) were dissolved in 5 μ L of 0.25% TFA, 50% acetonitrile (pH 2). Conventionally guanidinated peptides (with or without sulfonation) were optionally desalted with C₁₈ NuTips™ as per the manufacturer's instructions. After desalting, the sample was eluted with 5 μ L of 0.05% TFA, 60% acetonitrile into a clean tube.

Samples were mixed 1:1 with the matrix (0.7 mg/mL HCCA in 85% acetonitrile, 0.1% TFA, 1 mM ammonium phosphate, monobasic) and 1 μ L was deposited on the AnchorChip™ target plate (Bruker) and allowed to air dry. Alternatively, 0.5 μ L of the sample was

deposited on a standard steel target followed by 0.5 μ L of the matrix (20 mg/mL HCCA in 70:30 [v/v] acetonitrile: 5% formic acid) and allowed to air dry. Peptide II calibration standard, in the same matrix, was spotted adjacent to the samples.

Mass Spectrometry

MALDI-TOF MS/(MS) was conducted with an Ultra Flex III (Bruker) using Flex Control v. 3.4. All spectra were acquired in positive reflectron mode and were externally calibrated. For MS mode, 1,000 laser shots were summed. For automated acquisition, 10 attempts were made before aborting acquisition. Manual data acquisition was then attempted for aborted acquisitions. Parent ions were fragmented in LIFT mode.³⁴ Data were sent to Flex Analysis v. 3.4 for spectral processing. Peak picking and residue assignments were manually verified.

Protein Identification

Spectral data were subjected to a Mascot search³⁵ on a local Mascot server v. 2.2.7 (Matrix Science, Boston, MA) *via* ProteinScape v. 3 (Bruker). For PMF, the mass tolerance was 50 ppm and carbamidomethyl cysteine was a global modification. Upon verification that no underivatized lysines were detected, homoarginine was set as a global modification. Fragmentation spectra were submitted to a Mascot MS/MS search with a mass tolerance of 50 ppm and MS/MS tolerance of 0.3 Da. Carbamidomethyl cysteine and homoarginine were global modifications and *N*-terminal sulfonation, listed as 3Sulfo, was a variable modification. Identifications were based on significant matches at $p < 0.05$.

RESULTS AND DISCUSSION

O-Methylisourea-Freebase Preparation and Use

The improved guanidination reagent, *O*-methylisourea-freebase, was prepared in less than 30 min while waiting for gel peptide extracts to dry. In a simple reaction between *O*-methylisourea hemisulfate salt and barium hydroxide (1:1 mol/mol), *O*-methylisourea-freebase separated into the aqueous phase while salts precipitated. The salts are aqueous soluble to an extent (barium sulfate [2.5 μ g/mL]; unreacted *O*-methylisourea hemisulfate [100 mg/mL]; unreacted barium hydroxide octahydrate [38.9 mg/mL]), but are not soluble in organic solvents. Upon agitation in 74% acetonitrile, residual salts precipitated. The final freebase guanidination solution includes: 1.0 M *O*-methylisourea-freebase, which was efficient for complete guanidination of tryptic peptides,²³ 50% acetonitrile which aided in resolubilizing dried peptides, and 1.75 N NH_4OH which maintained the reaction pH (pH 11). Drying the peptides after the 20 min reaction effectively removed NH_4OH . Dried freebase guanidinated peptides were dissolved in 0.25% TFA, 50% acetonitrile prior to co-crystallization with HCCA. Addition of 5 μ L of that solution (which is equivalent to 1 reaction volume) had little or no effect on co-crystallization.

Freebase *versus* Conventional Guanidination of In-Solution BSA Digests

Tryptic peptides from in-solution digestion of BSA were used to optimize freebase guanidination conditions and to examine the benefits of freebase guanidination compared to conventional guanidination, which uses *O*-methylisourea hemisulfate, with and without desalting. In the present study, 25 fmol of BSA was the lowest amount for which a positive identification could be obtained for the tryptic peptides *via* a database search (Fig. 1A). The four peptides used for identification resulted in 7% sequence coverage. Desalting and sample concentration with C_{18} NuTipsTM improved sequence coverage by more than 5-fold (39% Seq. Cov.; Fig. 1B). Conventional guanidination without desalting resulted in a marked reduction in ion intensity and a noisy background which prevented detection of all but 4 peptide ions (7% Seq. Cov.; Fig. 1C). Desalting of conventionally guanidinated peptides removed interfering salts, increasing signal and lowering background noise (50%

Seq. Cov.; Fig. 1D). With freebase guanidination, interfering salts are removed prior to guanidination. After freebase guanidination, there was minimal background noise and signal intensity was equivalent to that of desalted samples (Fig. 1B and 1E). Both freebase guanidination and the conventional procedure are alleviated from the “arginine effect,” whereas for tryptic peptides, one peak dominated the spectrum (Fig. 1B; m/z 927; YLYEIAR). Sequence coverage after freebase guanidination was comparable to the conventional method with desalting (46% Seq. Cov.). Differences in peptide ions contributing to sequence coverage were noted (red asterisks; Fig. 1D and 1E). Peptide ions exclusive to conventional guanidination with desalting were found in the lower end of the mass range, whereas those exclusive to freebase guanidination were larger. This is in line with the observations that desalting with a C_{18} micro-column leads to the loss of more hydrophobic peptides.^{23,36} No underivatized lysines were detected after freebase guanidination, indicating quantitative reaction.

Freebase versus Conventional Guanidination of Standard Proteins Digested In-Gel

Guanidination is pH sensitive. The reaction relies on the α -amino group of lysine being deprotonated. The optimal pH is 11.3¹³ with a minimum of pH 10.²³ The freebase guanidination solution was adapted to ensure that it is compatible with standard in-gel digest procedures, which use acidic conditions to extract peptides. A mixture of varying amounts of the 4 standard proteins was separated by 1-DGE (Fig. 2). At 12.5 pmol of each protein, cytochrome *c* was barely detectable, so this amount was used in the remainder of the study. The higher acid content of gel extracts required 10-times more NH_4OH than in-solution digested peptides to achieve the optimal reaction pH. When a lesser amount of base was used, guanidination was incomplete.

High throughput capability with MALDI-TOF MS relies on efficient automated data acquisition. Samples were spotted on an AnchorChip™ target and data were collected in automated mode. Sequence coverage and the Mascot score were about the same for freebase guanidination and conventional guanidination with desalting (Table 1). The sequence coverage for these was about 10 percentage points higher than conventional guanidination without desalting for all proteins except cytochrome *c*, which was more than 30 percentage points lower. Only one replicate from conventional guanidination without desalting was interpretable for cytochrome *c*, which was near the limit of detection (LOD) for Coomassie staining (Fig. 2). These data indicate major advantages of freebase guanidination over conventional guanidination with or without desalting at low protein concentration conditions for cytochrome *c*, potentially for other proteins as well. Freebase guanidination is therefore effective in increasing the dynamic range of detection while decreasing the work needed in sample preparation.

Repeatability measures how reliable a method is. A few samples from freebase guanidination and conventional guanidination with desalting failed during automated acquisition, whereas most from conventional guanidination without desalting failed. The missing data were collected manually. For the 4 standard proteins and each of the replicates, a significant identification could be obtained 100% of the time when the freebase method or the conventional method with desalting was used. Less than half of those examined after conventional guanidination without desalting were successfully identified (Table 1; Repeatability). AnchorChip™ targets are most convenient for automated data acquisition.³⁷ These targets are also known for increasing sensitivity by concentrating the sample into a smaller area which comes with the consequence of other components concentrating as well.³⁷ As a result, an enhancement was not observed for freebase guanidinated samples and the data quality for conventional guanidination without desalting was critically reduced. Also, sample drying time was increased with AnchorChip™ use. The best compatibility was with the standard target plate, which can be used for automated data acquisition.

Freebase versus Conventional Guanidination when Combined with *N*-Terminal Sulfonation

In MALDI-TOF MS/MS, fragmentation occurs through a charge-remote mechanism, so spectra are not as readily interpretable as those from ESI-MS/MS.³⁸ *N*-Terminal sulfonation facilitates unambiguous interpretation of MALDI-TOF MS/MS fragmentation spectra wherein *y*-ions are almost exclusively detected.¹⁴ A major advantage of guanidination is that its co-derivatization with *N*-terminal sulfonation gives no side reactions.¹³

N-Terminal sulfonation with 2-sulfobenzoic acid could be conducted without sample cleanup.²⁶ Therefore, the 3 guanidination methods were compared after *N*-terminal sulfonation of the standard protein digests with this chemical (Table 2). The 20 most intense peaks were subjected to MS/MS fragmentation and scores of significant peptide identifications were summed. For cytochrome *c*, which stained very faintly after 1-DGE (Fig. 2), a positive identification from the fragmentation spectra could only be obtained with freebase guanidinated samples. Compared to conventional guanidination without desalting, freebase guanidination had more peptides identified and higher scores from the Mascot MS/MS search. For ovalbumin, conventional guanidination with desalting is the preferred method since 2 additional peptides were identified by MS/MS fragmentation analysis leading to a higher score. Freebase guanidination well identified 4 out of 4 proteins, whereas the conventional method with desalting identified 3 out of 4. This and previous studies showed that desalting is needed for the conventional guanidination method²³ and sample losses are an unavoidable concern when desalting with C₁₈ micro-columns.^{23,36} Freebase guanidination did not require desalting which improved sensitivity of detection (Table 2). This was particularly important since *N*-terminal sulfonation decreases sensitivity.²⁶ A more thorough investigation demonstrating applicability to a wider range of proteins is given in the following section.

Application of Freebase Guanidination and its Co-Derivatization with *N*-Terminal Sulfonation to Analysis of Rat Lung Proteins from 2-DGE

Lung diseases continue to be challenging to diagnose and treat.^{39,40} Insights into the biology of lung cancer,^{4,6} as well as an understanding of pulmonary development² have been gained with the use of proteomics. A major objective of the present study was to implement freebase guanidination in the analysis of lung proteins separated by 2-DGE. Successful implementation was assessed by the capacity to yield statistically significant protein identifications regardless of protein characteristics or abundance.

Whole protein extracts from rat lung were separated *via* 2-DGE. Twenty spots which captured a wide range of molecular weight and isoelectric point and of detection by Coomassie staining were chosen from the gel (Fig. 3). Identification was achieved through PMF of freebase guanidinated samples (Table 3). Further confirmation was achieved by co-derivatization with *N*-terminal sulfonation and MS/MS fragmentation of at least one peptide. For each of the 20 proteins, a significant PMF identification was obtained regardless of protein characteristics (MW or pI) or abundance. Fragmentation analysis after co-derivatization with *N*-terminal sulfonation allowed for successful identification of 18 out of 20 spots. The two that were not identified (spots 10 and 19) exhibited very low staining. The LOD by Coomassie staining coincides with that of MALDI-TOF MS/MS (estimated according to^{31,34}). For a 97.2 kDa protein, phosphorylase b, 50 – 100 fmol was required for MS/MS analysis³⁴ and the lower LOD for Coomassie staining was 8 – 28 ng of protein³¹ which is equivalent to 46 – 160 fmol of phosphorylase b.

PMF results warranted MS/MS fragmentation analysis for two main reasons: 1) the protein was not represented in the Swiss-Prot database,⁴¹ which is manually annotated and reviewed; or 2) the spot contained a mixture of proteins. The mass spectrum of spot 2 after

trypsin digestion and freebase guanidination is shown (Fig. 4A). An initial search in the Swiss-Prot database⁴¹ returned no significant hits. A Mascot search using a larger database, NCBI nr, resulted in a match to the predicted protein collagen -1 (VI) for which there is currently no protein level data available (UniProt Knowledgebase⁴² entry last updated May 29, 2013 for accession number: D3ZUL3_RAT). Twenty peaks, within the mass range of m/z 995 – 3257, were used in the identification (Fig. 4A). Of these, 5 were found to contain hydroxyproline (m/z 995, 1356, 1572, 1708, and 1724). Sulfonation with sulfobenzoic acid cyclic anhydride results in a mass shift of 184 Da ($O_3S-C_6H_4-CO$). After *N*-terminal sulfonation, 8 sulfonic acid derivatives were detected (Fig. 4B). A red dashed line indicates a shift of 184 Da for a peptide (m/z 1187; Fig. 4A) to its sulfonic acid derivative (m/z 1371; Fig. 4B), which was selected for MS/MS fragmentation (Fig. 4C). In the fragmentation spectrum, the highest peak was from a loss of the aromatic sulfonic acid group and ammonia (-201 ; $HO_3S-C_6H_4-CO-NH_2$; Fig. 4C). This and the observed y_{17} -ions are expected for sulfonic acid derivatives of peptides containing an internal arginine or homoarginine.^{14,26}

Differential post-translational modification and proteolytic cleavage of a highly abundant protein can result in occurrence of that protein in more than one spot.³⁹ This phenomenon may obscure detection of low abundance and potentially relevant proteins. Several such high abundance proteins were detected in the present study, including serum albumin and hemoglobin. Fragmentation spectra were instrumental in verifying low abundance components in various spots (3, 4, 7, 8, 13, 15, 16, 19 and 20; Fig. 3 and Table 3). For example, PMF of spot 8 revealed that serum albumin peaks accounted for 75% of the ion intensity, whereas those of hemopexin accounted for only 11%. Analysis of MS/MS fragmentation spectra confirmed the presence of hemopexin in the mixture.

CONCLUSIONS

Problems associated with guanidination using *O*-methylisourea hemisulfate appeared to stem exclusively from the salts, making use of *O*-methylisourea-freebase ideal. In the present study, no underivatized lysines were detected, suggesting that freebase guanidination is quantitative. This contributes to better sensitivity and reproducibility. The benefits of freebase guanidination were most apparent under demanding sample conditions as was seen after coderivatization with *N*-terminal sulfonation, which is known to negatively impact sensitivity. In the 2-DGE workflow, sample preparation was streamlined by avoiding time-consuming clean-up steps and PMF identification was obtained regardless of protein abundance.

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Figure 1. MALDI-TOF spectra of a BSA (25 fmol) tryptic peptides before (A) and after desalting (B), after conventional guanidination without desalting (C) and with desalting (D), and after freebase guanidination (E). For reference the m/z and peptide range (BSA; UniProt accession: P02769) are given for some peaks. Asterisks indicate peaks contributing to protein identification and red asterisks highlight the peaks that differ between D and E.

Figure 2.
1-DGE of mixtures of standard proteins (50, 25, or 12.5 pmol). Boxes indicate bands excised for further experiments.

Figure 3.
2-DGE of rat lung tissues. Numbers indicate protein spots excised from the gel and used for protein identification (Table 3).

Figure 4.

Identification of collagen -1 (VI) from 2-DGE spot 2 with MALDI-TOF spectra of tryptic peptides after freebase guanidination (A) and co-derivatization with *N*-terminal sulfonation (B). A red dashed line indicates the sulfonic acid derivative of m/z 1187 which was used for MS/MS fragmentation analysis (C). Interpretation of the *y*-, *y*₋₁₇- and *b*-ions led to identification of a collagen -1 (VI) peptide (LKPYGALVDK). Asterisks indicate peaks contributing to protein identification and triangles indicate *N*-terminally sulfonated tryptic peptides.

Table 1Comparison of freebase guanidination to conventional guanidination with and without desalting.^a

protein ^b	MW (kDa)	pI	freebase		conv. without desalting		conv. with desalting	
			score ^c	s.c. (%)	score	s.c. (%)	score	s.c. (%)
BSA	66	5.6	227	45	226	35	267	45
ovalbumin	42	5.2	158	57	131	41	133	55
myoglobin	17	7.4	173	75	159	65	170	74
cytochrome <i>c</i>	12	9.6	160	56	56 ^a	19 ^a	145	53
repeatability ^d (%)			100		48		100	

^aResults are for samples from 1-DGE in Fig. 2 and are representative of all replicates except cytochrome *c* from conventional guanidination without desalting which had only 1 replicate with interpretable results.

^b75 fmol per spot.

^cMascot PMF score.

^dThe % identified out of 48 (4 proteins × 3 replicates × 4 technical replicates).

Table 2

Comparison of freebase guanidination to conventional guanidination with and without desalting when combined with *N*-terminal sulfonation.^a

	BSA		ovalbumin		myoglobin		cytochrome <i>c</i>	
	score ^b	peptides	score	peptides	score	peptides	score	peptides
freebase	406	14	342	7	180	6	158	3
conv. without desalting	190	6	178	6	-	-	-	-
conv. with desalting	393	16	437	9	137	7	-	-

^aResults for samples from 1-DGE (Fig. 2).

^bSummed ions scores from Mascot MS/MS search.

Table 3

List of proteins from 2-DGE of rat lung tissues identified with freebase guanidination and confirmed by co-derivatization with *N*-terminal sulfonation.^a

spot	accession ^b	protein	guanidination only		guanidination + sulfonation	
			protein score ^c	s.c. (%)	ions score ^d	peptides
1	P12346	Serotransferrin	233	31	49	1
2	D3ZUL3	Predicted protein collagen -1 (IV)	149	24	70	2
3	P06761	78 kDa glucose-regulated protein ^e	248	41	47	2
4	P63018	Heat shock cognate 71 kDa protein ^e	125	23	47	1
5	P02770	Serum albumin	356	62	238	8
6	P31000	Vimentin	265	67	72	2
7	Q8VIF7	Selenium-binding protein 1 ^e	117	29	32	2
8	P20059	Hemopexin ^e	121	31	54	1
9	P69897	Tubulin beta-5 chain	114	44	39	1
9	P17475	Alpha-1-antiproteinase	146	42	35	1
10	P18418	Calreticulin	149	26	-	-
11	P85125	Polymerase I & transcript release factor	58	12	61	2
12	P60711	Actin, cytoplasmic 1	171	59	75	1
13	P25113	Phosphoglycerate mutase 1 ^f	99	51	19	1
14	P58775	Tropomyosin beta chain	91	37	53	1
15	B0BNN3	Carbonic anhydrase 1 ^{fg}	100	56	60	2
16	P08010	Glutathione S-transferase Mu 2 ^{fg}	126	51	23	1
17	O35244	Peroxiredoxin-6	244	61	23	1
18	P01946	Hemoglobin subunit alpha-1/2	62	37	47	1
19	P42930	Heat shock protein beta-1 ^g	79	32	-	-
20	P02091	Hemoglobin subunit beta-1 ^g	209	95	92	2

^a2-DGE spot (Fig. 3).

^bUniProt Knowledgebase accession number.

^cMascot PMF score.

^dSummed ions scores from MS/MS Mascot search. Background proteins are

^eserum albumin

^fhemoglobin subunit beta-1

^ghemoglobin subunit alpha-1/2.