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Guanidination of Tryptic Peptides without Desalting for Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight Mass Spectrometry Analysis

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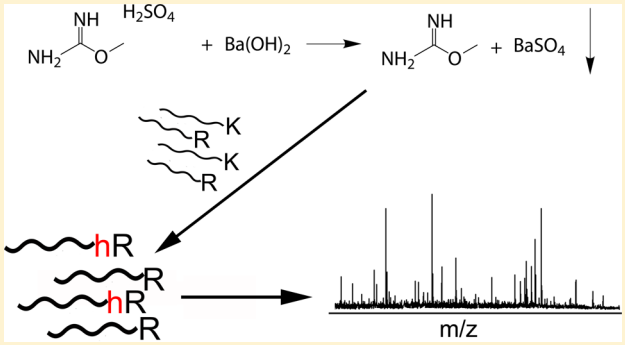
ABSTRACT: Derivatizations that enhance mass spectral quality often require desalting, which presents as a bottleneck in matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS)-proteomics. Guanidination, which converts lysine to homoarginine, an arginine analogue, can 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 tandem mass spectrometry (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 p*I*) 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.



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 processes[1−3] and for discovery of biomarkers.[4−6]

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 counterparts.[7] The guanidine side group of arginine (pK_a 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 (pK_a 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 analogue. At an alkaline pH (pH > 10.5), *O*-methylisourea reacts with the ε-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

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for pin-pointing of oxidation sites thought to be unique to Parkinson's 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 proteins[1] and in *de novo* sequencing of novel proteins.[15−19] In quantitative proteomics, guanidination was used directly as an isotopic tag[20] 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 (Δ 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). C_{18} NuTips were from PolyLC (Columbia, MD). Other reagents included *O*-methylisourea-hemisulfate (Sigma), barium hydroxide octahydrate (J.T. Baker Chemicals), ammonium hydroxide (NH_4OH) (Mallinckrodt, Inc.), and 2-sulfobenzoic acid cyclic anhydride (Acros Organics, New Jersey). Acetonitrile (LC−MS grade) and ammonium bicarbonate (NH_4HCO_3) 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 a 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 V h. The second dimension was run on a precast gel (Any kD Mini-Protean TGX, Bio-Rad) at a 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 pipet 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 Harbor[32] 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 of trypsin (1-D gel bands) in 50 mM NH_4HCO_3] at 37 °C for 16 h. Peptides were extracted by sonication for 5 min at ambient temperature in 25 mM NH_4HCO_3, 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 three aliquots for comparison of three 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 μL 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 μL 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 μL) was collected, avoiding the pellet. Next, 112 μL 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 three phases, one

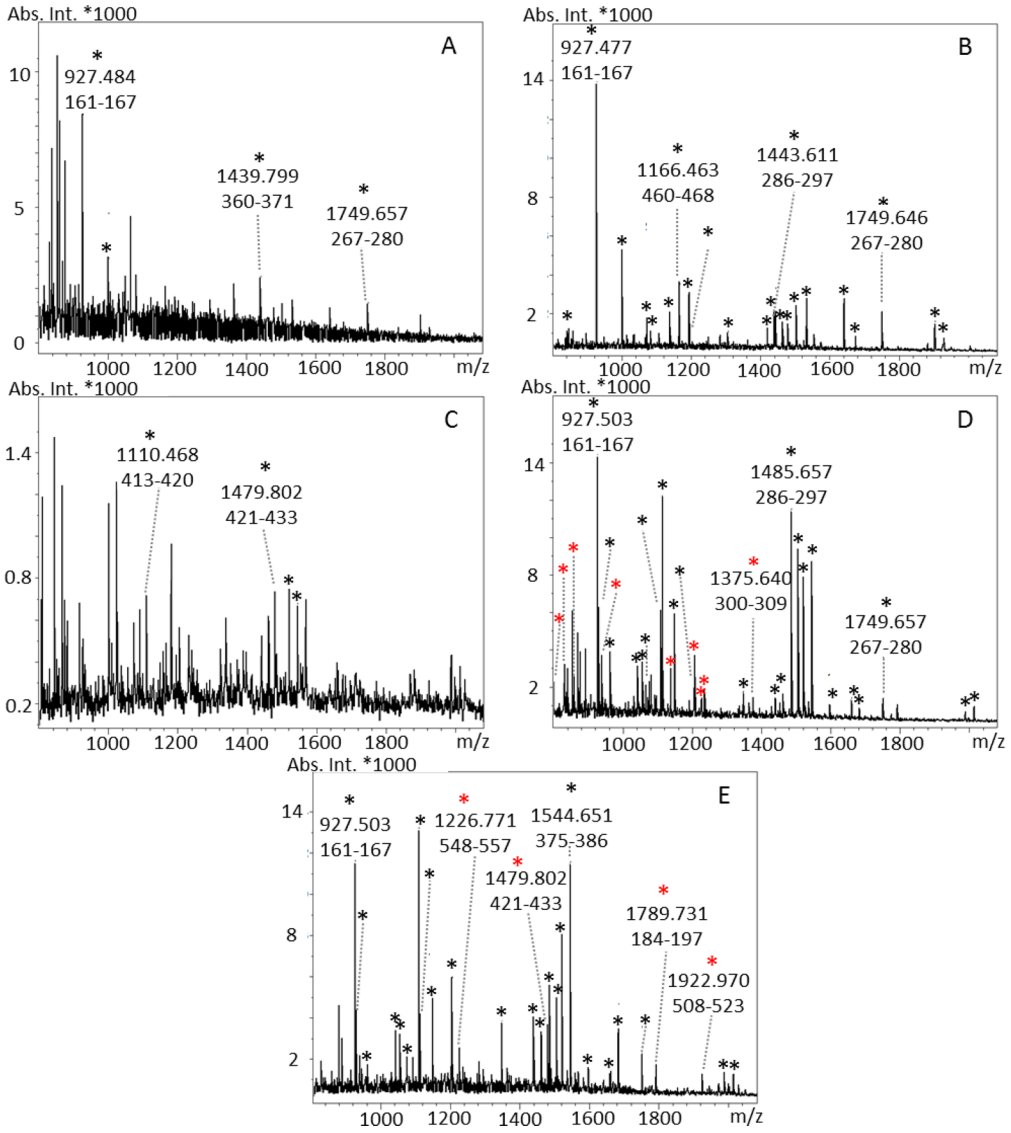

Figure 1. MALDI-TOF spectra of BSA (25 fmol) tryptic peptides before (A) and after desalting (B), after conventional guanidination without desalting (C) and with desalting (D), and after freebase guanidination (E). For reference, the *m*/*z* and peptide range (BSA; UniProt accession P02769) are given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

solid precipitate and two liquid phases. An aliquot of 100 μL (2.11 M *O*-methylisourea-freebase in 74% acetonitrile, pH 9–10) was carefully collected from the top liquid layer and mixed with 25 μL of 14.8 N NH_4OH, 35 μL of acetonitrile, and 51 μL of water to prepare a 1.0 M *O*-methylisourea-freebase solution in 50% acetonitrile containing 1.75 N NH_4OH (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 μL of water, 163.2 μL of acetonitrile, and 96.5 μL of 14.8 N NH_4OH, making a solution of 1.0 M *O*-methylisourea, 40% acetonitrile, 3.5 M NH_4OH (pH 11).

Guanidination. Dried peptides were dissolved in 5 μL 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 μL of 25 mM triethylammonium bicarbonate, 50% acetonitrile. Next, 2.5 μL 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 μL of 0.25% TFA, 50% acetonitrile (pH 2). Conventionally guanidinated peptides (with or without sulfonation) were optionally desalted with C_{18} NuTips as per the manufacturer's instructions. After desalting, the sample was eluted with 5 μL 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 μL was deposited on the AnchorChip target plate (Bruker) and allowed to air-dry. Alternatively, 0.5 μL of

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the sample was deposited on a standard steel target followed by 0.5 μL 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 search[35] 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, *O*-methylisourea-freebase separated into the aqueous phase while salts precipitated. The salts are aqueous soluble to an extent (barium sulfate [2.5 μg/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 cocrystallization with HCCA. Addition of 5 μL of that solution (which is equivalent to 1 reaction volume) had little or no effect on cocrystallization.

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 (Figure 1A). The four peptides used for identification resulted in 7% sequence coverage. Desalting and sample concentration with C_{18} NuTips improved sequence coverage by more than 5-fold (39% sequence coverage; Figure 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% sequence coverage; Figure 1C). Desalting of conventionally guanidinated peptides removed interfering salts, increasing signal and lowering background noise (50% sequence coverage; Figure 1D). With freebase guanidination, interfering salts are removed prior to guanidination. After freebase guanidination, there was minimal background noise and the signal intensity was equivalent to that of desalted samples (Figure 1B,D, and E). Both freebase guanidination and the conventional procedure are alleviated from the "arginine effect," whereas for tryptic peptides, one peak dominated the spectrum (Figure 1B; *m/z* 927; YLYEIAR). Sequence coverage after freebase guanidination was comparable to the conventional method with desalting (46% sequence coverage). Differences in peptide ions contributing to sequence coverage were noted (red asterisks; Figure 1D,E). 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} microcolumn leads to the loss of more hydrophobic peptides.[23,36] No underivatized lysines were detected after freebase guanidination, indicating a quantitative reaction.

Freebase versus Conventional Guanidination of Standard Proteins Digested In-Gel. Guanidination is pH sensitive. The reaction relies on the ε-amino group of lysine being deprotonated. The optimal pH is 11.3[13] 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 four standard proteins was separated by 1-DGE (Figure 2). At 12.5 pmol of each protein,

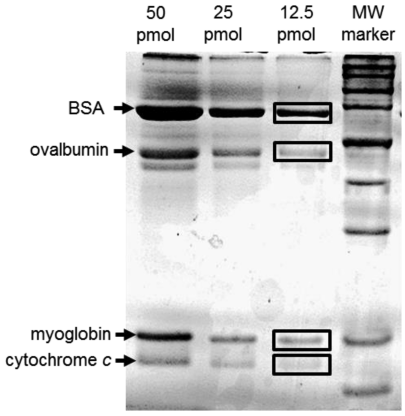

Figure 2. 1-DGE of mixtures of standard proteins (50, 25, or 12.5 pmol). Boxes indicate bands excised for further experiments.

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

Table 1. Comparison of Freebase Guanidination to Conventional Guanidination with and without Desalting[a]

			freebase		conventional without desalting		conventional with desalting	
protein[b]	MW (kDa)	p*I*	score[c]	sequence coverage (%)	score	sequence coverage (%)	score	sequence coverage (%)
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 *c*	12	9.6	160	56	56[a]	19[a]	145	53
repeatability[d] (%)			100		48		100	

[a]Results are for samples from 1-DGE in Figure 2 and are representative of all replicates except cytochrome *c* from conventional guanidination without desalting, which had only 1 replicate with interpretable results. [b]75 fmol per spot. [c]Mascot PMF score. [d]The % 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 *c*	
	score[b]	peptides	score	peptides	score	peptides	score	peptides
freebase	406	14	342	7	180	6	158	3
conventional without desalting	190	6	178	6				
conventional with desalting	393	16	437	9	137	7		

[a]Results for samples from 1-DGE (Figure 2). [b]Summed ions scores from Mascot MS/MS search.

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 (Figure 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 four 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 the 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 electrospray (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 coderivatization 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 three 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 (Figure 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 two additional peptides were identified by MS/MS fragmentation analysis leading to a higher score. Freebase guanidination well identified four out of four proteins, whereas the conventional method with desalting identified three out of four. This and previous studies showed that desalting is needed for the conventional guanidination method,[23] and sample losses are an unavoidable concern when desalting with C_{18} microcolumns.[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 development[2] 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

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identifications regardless of protein characteristics or abundance.

Whole protein extracts from rat lung were separated via 2-DGE. A total of 20 spots, which captured a wide range of molecular weight and isoelectric points with detection by Coomassie staining, were chosen from the gel (Figure 3).

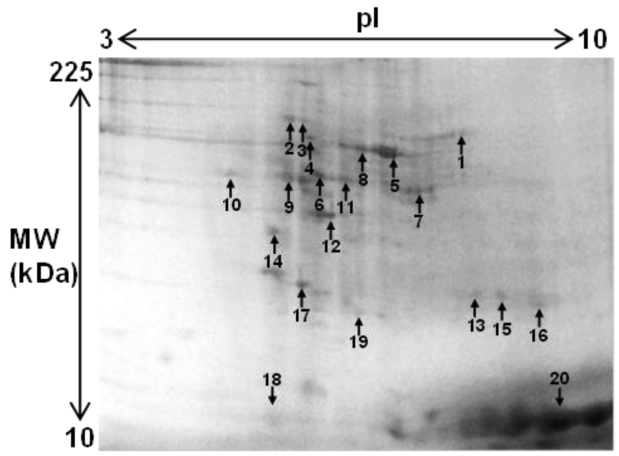

Figure 3. 2-DGE of rat lung tissues. Numbers indicate protein spots excised from the gel and used for protein identification (Table 3).

Identification was achieved through PMF of freebase guanidinated samples (Table 3). Further confirmation was achieved by coderivatization 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 p*I*) or abundance. Fragmentation analysis after coderivatization 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 refs 31 and 34). For a 97.2 kDa protein, phosphorylase b, 50−100 fmol was required for MS/MS analysis[34] and the lower LOD for Coomassie staining was 8−28 ng of protein,[31] 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 (Figure 4A). An initial search in the Swiss-Prot database[41] returned no significant hits. A Mascot search using a larger database, NCBI nr, resulted in a match to the predicted protein collagen α-1 (VI) for which there is currently no protein level data available (UniProt Knowledgebase[42] entry last updated May 29, 2013 for accession number D3ZUL3_RAT). A total of 20 peaks, within the mass range of m/z 995−3257, were used in the identification (Figure 4A). Of these, five 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, eight sulfonic acid derivatives were detected (Figure 4B). A red dashed line indicates a shift of 184 Da for a peptide (m/z 1187, Figure 4A) to its sulfonic acid derivative (m/z 1371, Figure 4B), which was selected for MS/MS fragmentation (Figure 4C). In the fragmentation spectrum, the highest peak was from a loss of the aromatic sulfonic acid group and ammonia (Δ 201, $HO_3S-C_6H_4-CO-NH_2$, Figure 4C). This and the observed

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]

			guanidination only		guanidination + sulfonation	
spot	accession[b]	protein	protein score[c]	sequence coverage (%)	ions score[d]	peptides
1	P12346	serotransferrin	233	31	49	1
2	D3ZUL3	predicted protein collagen α-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 β-5 chain	114	44	39	1
9	P17475	α-1-antiproteinase	146	42	35	1
10	P18418	calreticulin	149	26		
11	P85125	polymerase I and 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 β 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 α-1/2	62	37	47	1
19	P42930	heat shock protein β-1[g]	79	32		
20	P02091	hemoglobin subunit β-1[g]	209	95	92	2

[a]2-DGE spot (Figure 3). [b]UniProt Knowledgebase accession number. [c]Mascot PMF score. [d]Summed ions scores from MS/MS Mascot search. [e]Background proteins are serum albumin. [f]Background proteins are hemoglobin subunit β-1. [g]Background proteins are hemoglobin subunit α-1/2.

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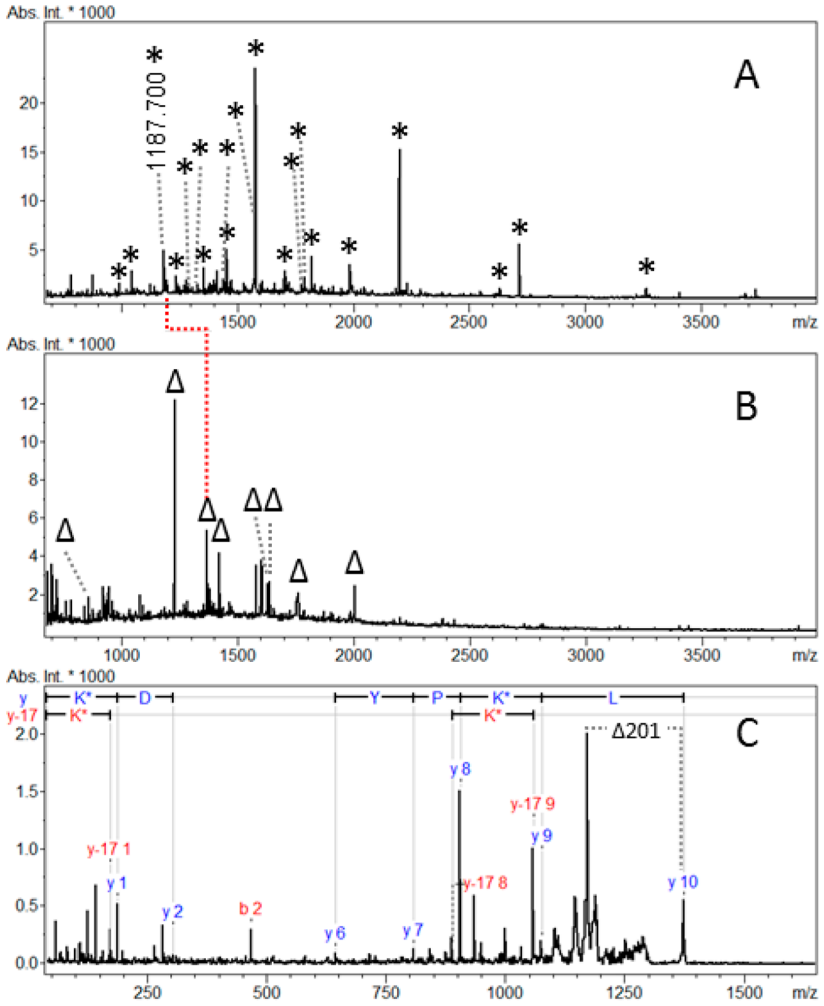

Figure 4. Identification of collagen α-1 (VI) from 2-DGE spot 2 with MALDI-TOF spectra of tryptic peptides after freebase guanidination (A) and coderivatization with *N*-terminal sulfonation (B). A red dashed line indicates the sulfonic acid derivative of m/z 1187 which was used for MS/MS fragmentation analysis (C). Interpretation of the y-, y-17-, and b-ions led to identification of a collagen α-1 (VI) peptide (LKPYGALVDK). Asterisks indicate peaks contributing to protein identification, and triangles indicate N-terminally sulfonated tryptic peptides.

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; Figure 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 co-derivatization with N-terminal sulfonation, which is known to negatively impact sensitivity. In the 2-DGE workflow, sample preparation was streamlined by avoiding time-consuming cleanup steps and PMF identification was obtained regardless of protein abundance.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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