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Effects of Guanidination with Trypsin, Lys-C, or Glu-C Digestion on Mass Spectrometric Signal Intensity and Protein Sequence Coverage

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The conventional peptide modification process of guanidination, in which the amino groups of lysine residues are converted to guanidino groups using *O*-methylisourea to create more basic homoarginine residues, is often used to improve the signal intensity of lysine-containing peptides in matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). Here, we used three different protease enzymes (trypsin, Lys-C, and Glu-C) to evaluate the effects of guanidination on the MS signals of two enzymatically digested proteins. Horse heart myoglobin and bovine serum albumin were guanidinated either before or after digestion with trypsin, Lys-C, or Glu-C. The resulting peptides were subjected to MALDI-MS, and signal intensities and sequence coverage were systematically evaluated for each digest. Guanidination prior to Glu-C digestion improved sequence coverage for both proteins. For myoglobin, guanidination before enzymatic digestion with trypsin or Lys-C also enhanced sequence coverage, but guanidination after enzymatic digestion enhanced sequence coverage only with Lys-C. For albumin, guanidination either before or after Glu-C digestion increased sequence coverage, whereas pre- or post-digestion guanidination decreased sequence coverage with trypsin and Lys-C. The amino acid composition of a protein appears to be the major factor determining whether guanidination will enhance its MALDI-MS sequence coverage.

Key Words: Guanidination, Homoarginine, *O*-methylisourea, MALDI, Proteins

Introduction

Generally, the matrix-assisted laser desorption/ionization-mass spectrometric (MALDI-MS) signal peaks from arginine-containing peptides are more intense than those of lysine-containing peptides because arginine residues are more basic than lysine residues.[1,2] To enhance the signal intensity of peptide fragments in MALDI-MS, researchers have used several different sample preparation processes involving the addition of basic moieties to the analyte. For example, acetoamidination of N-terminal and lysine side-chain amino groups using *S*-methylthioacetimidate significantly enhances the observed signals and has aided in the identification of proteins.[3] Also, a recent study introduced the picolinamidination of N-terminal and lysine residues using ethyl picolinimidate tetrafluoroborate synthesized from picolinamide and triethyloxonium tetrafluoroborate.[4] Finally, the recently established Lys Tag method involves the specific modification of lysine side-chain amino groups with an imidazole derivative,[5] increasing the basicity of lysine-containing peptides.

Protein guanidination is a well-known protein modification,[6] while peptide guanidination is used conventionally to improve the MALDI-MS signal intensity of lysine-containing tryptic peptides. In guanidination, the amino groups of lysine residues are converted to guanidino groups using *O*-methylisourea, thereby creating homoarginine residues.[7-12] Homoarginine, which is similar in structure to arginine, is more basic than lysine and enhances the ionization efficiency of MALDI-MS. In MALDI-MS, the observed mass of guanidinated peptides is increased by 42.0 Da ($C_1H_2N_2$) relative to that of unmodified peptides. Thus, tryptic peptides not detected in unmodified digests can be detected after guanidination with an increased mass of 42 Da.

Because protease enzymes other than trypsin, such as Lys-C and Glu-C, are also useful in protein analyses,[13,14] we evaluated the degree of MALDI-MS signal improvement following guanidination of lysine residues for peptides digested with trypsin, Lys-C, and Glu-C. Lysine residues of enzymatically produced peptides of horse heart myoglobin and bovine serum albumin were guanidinated; alternatively, each of the two proteins was guanidinated prior to enzymatic digestion to evaluate the importance of the process sequence. MALDI-MS mass spectra were obtained for unmodified proteins, guanidinated proteins, enzymatically produced peptides of unmodified proteins, guanidinated peptides of enzymatic digests, and enzymatically produced peptides of guanidinated proteins. These data were used to determine the degree of signal improvement and enhancement in sequence coverage resulting from guanidination.

Experimental Section

Materials. Anhydrous sodium carbonate (Na_2CO_3) was obtained from Sanchun (Seoul, Korea). Horse heart myoglobin, bovine serum albumin, iodoacetamide, DL-dithiothreitol, endoproteinase Lys-C from Lysobacter enzymogenes, trypsin, α-cyano-4-hydroxycinnamic acid (CHCA), sinapic acid, *O*-methylisourea hydrogen sulfate, and guanidine hydrochloride were from Sigma-Aldrich (St. Louis, MO, USA). Endoproteinase Glu-C was obtained from Roche (Basel, Switzerland). ZipTipC18 and Microcon (YM-3) filter columns for desalting of sample solutions were acquired from Millipore (Billerica, MA, USA), and μ-focusing MALDI plates were obtained from ASTA Inc. (Suwon, Korea).

MALDI-TOF MS analysis. Mass spectra were recorded on an AXIMA-CFR mass spectrometer (Kratos, Manchester, UK)

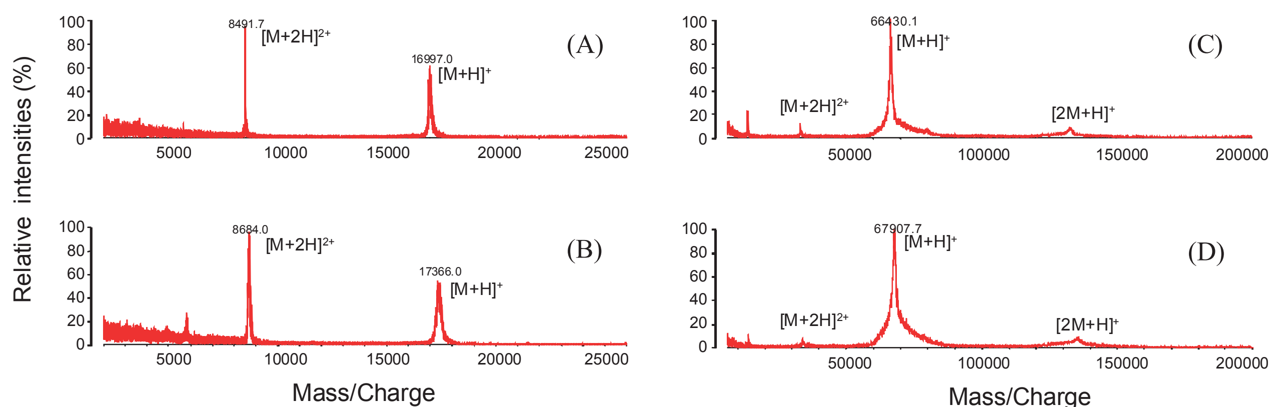

Figure 1. MALDI mass spectra of horse heart myoglobin (A, B) and bovine serum albumin (C, D) before (A, C) and after (B, D) guanidination. The mass spectra of myoglobin and albumin were obtained using 10- and 7.5-pmol samples, respectively.

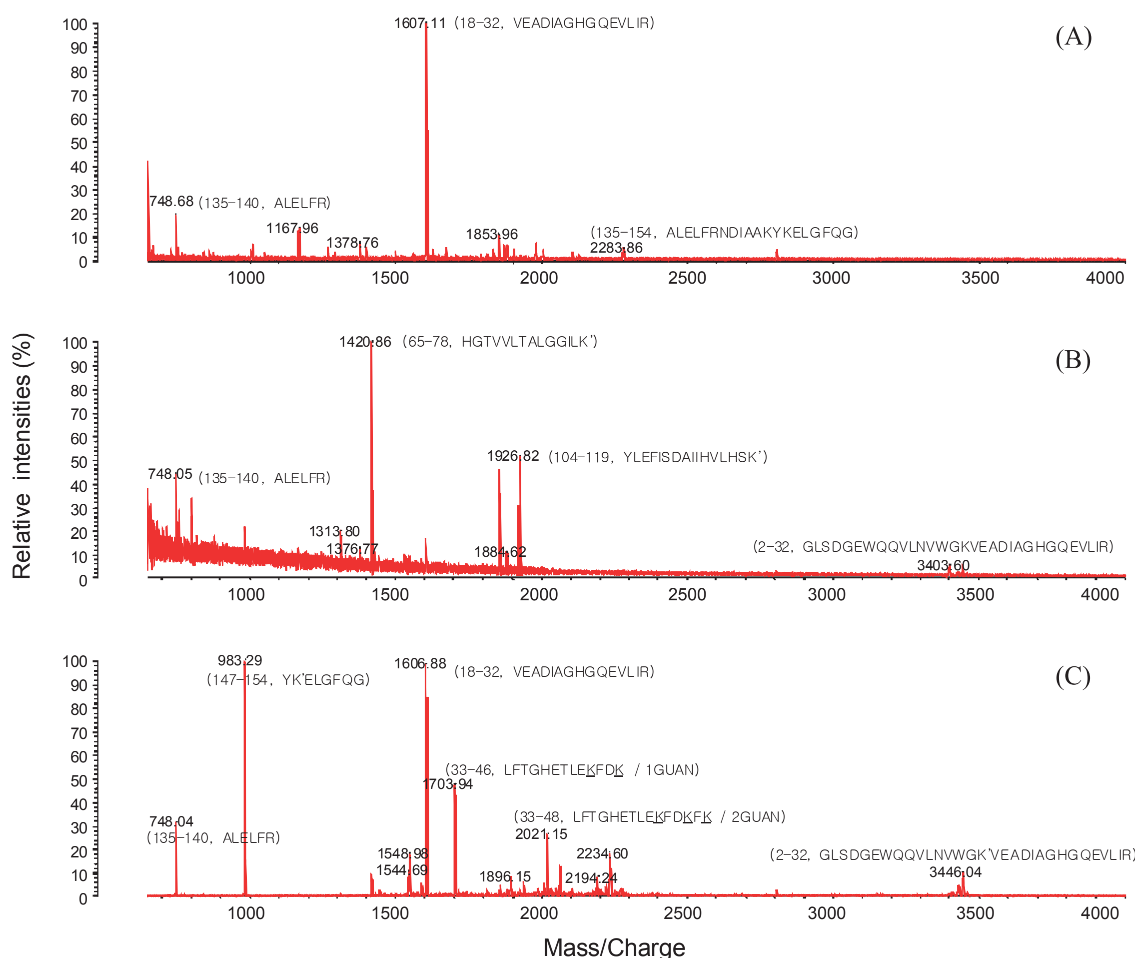

Figure 2. MALDI mass spectra of trypsin-related myoglobin peptides: (A) tryptic peptides of myoglobin; (B) guanidinated tryptic peptides of myoglobin; (C) tryptic peptides of guanidinated myoglobin. Several of the peaks are annotated with the sequence and amino acid position of the corresponding peptide. K', guanidinated lysine; K̲, partially guanidinated lysine; GUAN, number of guanidinated lysine residues.

in positive-ion mode. Protein spectra were taken in linear mode from *m*/*z* 1,000 to 40,000 using a sinapic acid matrix solution. Peptide spectra were observed in reflectron mode from *m*/*z* 500 to 6,000 using a CHCA matrix solution. Matrix solutions contained 7.5 mg CHCA or 10 mg sinapic acid in 1 mL of 50% acetonitrile/H_2O in 0.1% trifluoroacetic acid (v/v). To prepare MALDI spots, 0.6 μL of matrix solution was deposited on the plate, allowed to dry, and then covered with a 1 μL aliquot of sample-matrix mixture. The mass spectrometer was calibrated using angiotensin I, bradykinin, and adrenocorticotropic hormone immediately prior to measurement.

Peptides were identified using Swiss-Prot tools (http://expasy.org/tools/findpept.html) with guanidination specified with an atomic composition of C:1, H:2, and N:2 at K positions. The

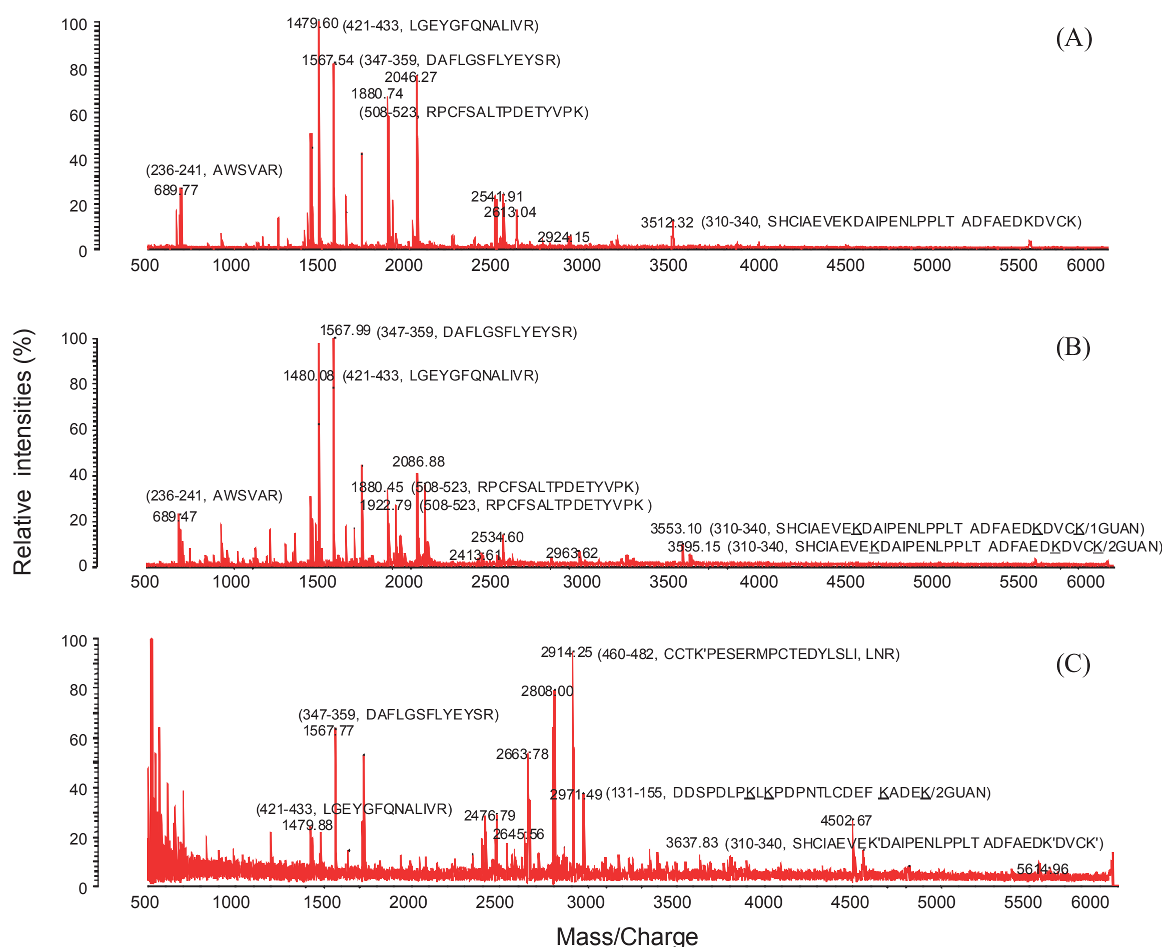

Figure 3. MALDI mass spectra of trypsin-related albumin peptides: (A) tryptic peptides of albumin; (B) guanidinated tryptic peptides of albumin; (C) tryptic peptides of guanidinated albumin. Several of the peaks are annotated with the sequence and amino acid position of the corresponding peptide. K', guanidinated lysine; K, partially guanidinated lysine; GUAN, number of guanidinated lysine residues.

Swiss-Prot accession number/entry names used for horse heart myoglobin and bovine serum albumin were P68082/MYG_HORSE and P02769/ALBU_BOVIN, respectively.

Protein digestion. Peptide solutions were prepared from 10 mg/mL protein stock solutions. For myoglobin, 170 μg of protein was digested with trypsin, Lys-C, or Glu-C at a protein: enzyme ratio (w/w) of 50:1 in a volume of 100 μL overnight at 37 °C, resulting in a myoglobin peptide solution of approximately 100 pmol/μL. Albumin was denatured before digestion by the addition of 6 M guanidine hydrochloride to 100 μg of protein in a total volume of 100 μL. The albumin disulfide bonds were then reduced by incubation with 5 μL of 10 mM DL-dithiothreitol for 30 min at 37 °C and alkylated by incubation with 20 μL of 40 mM iodoacetic acid for 30 min at room temperature in the dark. The reduced, alkylated albumin solution was desalted and concentrated into 100 μL of 50 mM NH_4HCO_3 using a Microcon filter (YM-10). It was then incubated with trypsin, Lys-C, or Glu-C at a protein:enzyme ratio (w/w) of 50:1 overnight at 37 °C, resulting in an albumin peptide stock solution of approximately 15 pmol/μL.

The 100 pmol/μL myoglobin digestion solution was diluted 1:10 into CHCA solution to create a 10 pmol/μL digestion mixture that was used as the MALDI myoglobin peptide sample. Alternatively, the 15 pmol/μL albumin digestion solution was diluted 1:2 into CHCA solution to yield a 7.5 pmol/μL digestion mixture that was used as the MALDI albumin peptide sample.

Guanidination of enzymatically digested peptides. Twenty μL of each enzymatically digested protein solution was mixed with an equal volume of 0.6 M *O*-methylisourea, and the pH of these mixtures was adjusted to approximately 10.5 by the addition of 1.6 M Na_2CO_3 in distilled water. The total volumes were brought to 100 μL with 50 mM NH_4HCO_3, a pH of 10.5 was confirmed with pH paper, and the mixtures immediately incubated for 2 h at 37 °C. The guanidinated peptide mixtures were then desalted using ZipTipC18 columns according to the manufacturer's protocol.

Guanidination and mass spectra of proteins. Myoglobin samples (170 μg) and albumin samples (500 μg) were guanidinated using the procedure outlined above in "Guanidination of enzymatically digested peptides" in total volumes of 100 μL to yield solutions of 100 pmol/μL and 75 pmol/μL, respectively. After guanidination, the solutions were passed through Microcon YM-3 filters, according to the manufacturer's protocol. The guanidinated protein solutions were diluted 1:10, and mass spectra were obtained using 1 μL of the resulting 10 pmol guanidinated myoglobin solution or 7.5 pmol guanidinated albumin

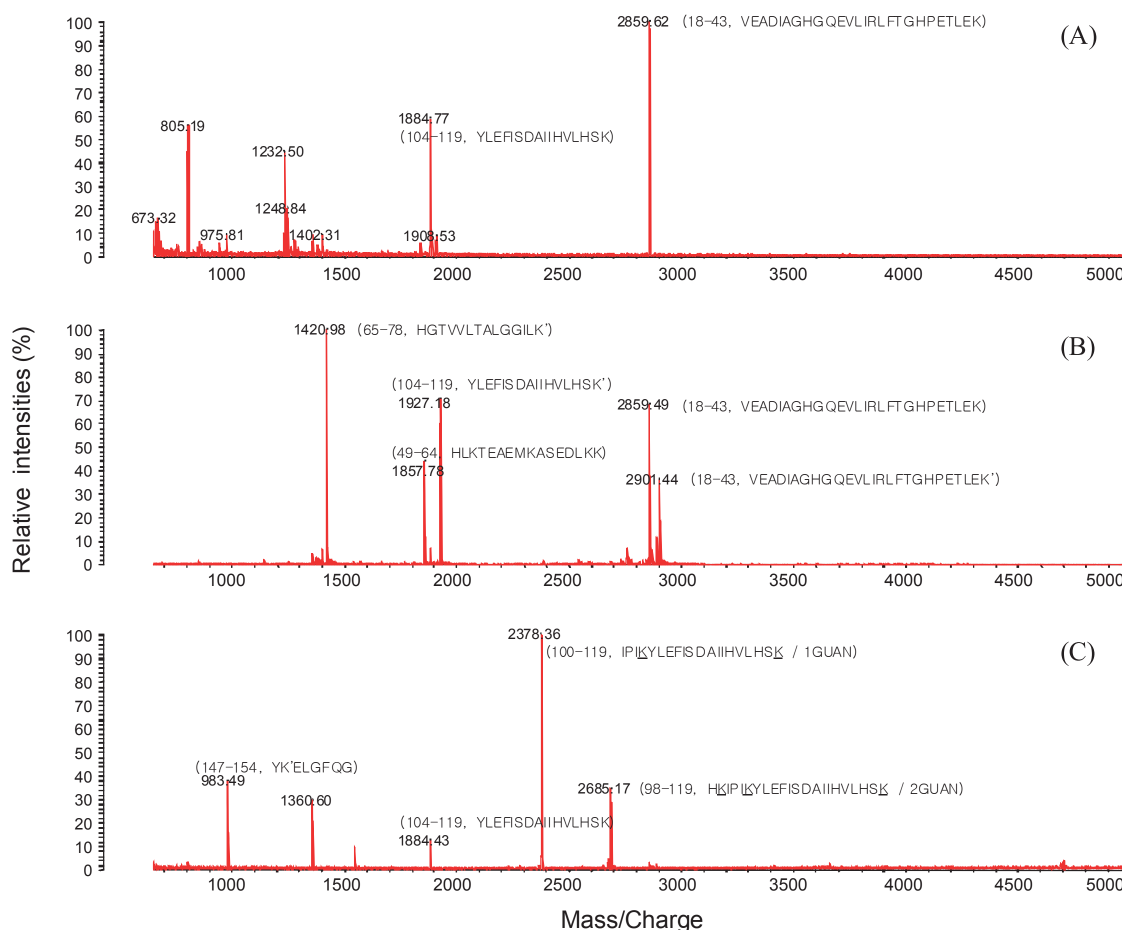

Figure 4. MALDI mass spectra of Lys-C-related myoglobin peptides: (A) Lys-C-digested myoglobin; (B) guanidinated Lys-C-digested myoglobin; (C) Lys-C-digested guanidinated myoglobin. Several of the peaks are annotated with the sequence and amino acid position of the corresponding peptide. K', guanidinated lysine; K, partially guanidinated lysine; GUAN, number of guanidinated lysine residues.

solution. Mass spectra of the non-guanidinated proteins were also obtained using 10 pmol of myoglobin or 7.5 pmol of albumin.

Digestion of guanidinated proteins. Guanidinated proteins were incubated with digestion enzyme (trypsin, Lys-C, or Glu-C) at 37 °C overnight according to the method described above in "Protein digestion" ZipTipC18 columns were used to completely remove salts from the samples prior to deposition on the MALDI plate.

Results and Discussion

Guanidination conditions. The most important factors affecting guanidination efficiency are *O*-methylisourea concentration, temperature, and the pH of the reaction mixture.[9] In this study, 20 μL of 0.6 M *O*-methylisourea was used for guanidination.[15] This amount (12 μmol) was believed to be sufficient to convert all of the lysine residues in both the protein and peptide mixtures to homoarginine. [In 20 μL of 100 pmol/μL myoglobin or 15 pmol/μL albumin solution, there are 38 nmol (20 μL × 100 pmol/μL × 19 lysine residues/protein) or 17.7 nmol (20 μL × 15 pmol/μL × 59 lysine residues/protein) of lysine residues, respectively]. The reaction time for guanidination depends on temperature. Based on published examples,[7,16] the guanidination reaction was carried out for 2 h at 37 °C to modify all of the lysine residues.

Both the N-terminal and lysine side-chain amino groups of a peptide can react with *O*-methylisourea. The pK_a of the N-terminus is less than 10, and a lysine side chain has a pK_a of approximately 10.30.[17] The conversion of a lysine residue to a homoarginine residue requires an adjustment to the pH of the reaction mixture. At pH 10.5, both amino groups can theoretically be guanidinated, but guanidination is observed primarily at lysine side chains, which are less sterically hindered than N-terminal amino groups. Guanidination of N-terminal glycine residues has, however, been reported to occur because of reduced steric hindrance, and other amino acids at the N-terminus have been guanidinated with increased reaction time.[9] In the current experiment, guanidination was observed only at lysine side chains.

Protein guanidination. The mass spectra of horse heart myoglobin (10 pmol) and bovine serum albumin (7.5 pmol) are shown in Figure 1A and C, respectively. The spectra were obtained in positive-ion linear mode and exhibit $[M+2H]^{2+}$ and $[M+H]^{+}$ peaks. The corresponding mass spectra of guanidinated myoglobin and guanidinated albumin are shown in Figure 1B and D, respectively. Since myoglobin contains 19 lysine resi-

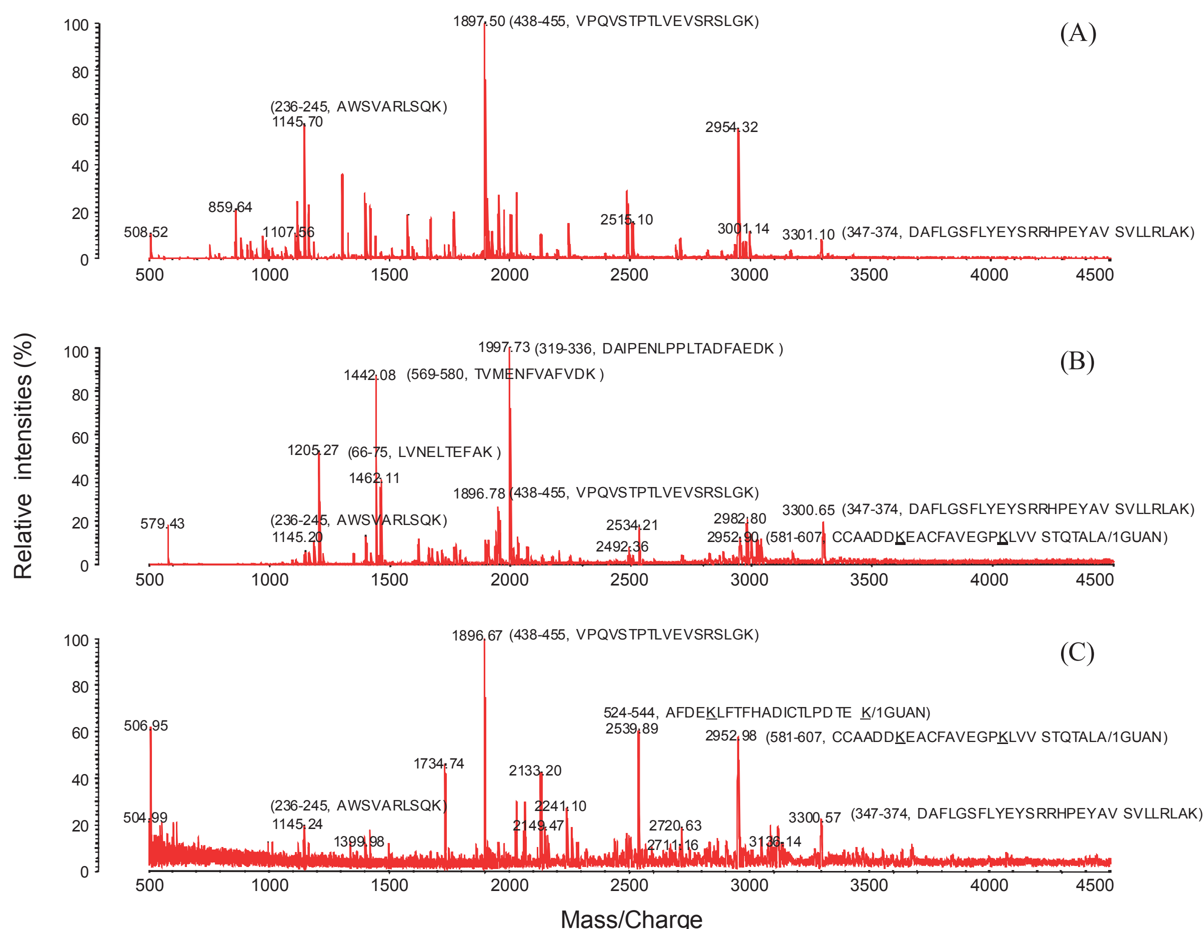

Figure 5. MALDI mass spectra of Lys-C-related albumin peptides: (A) Lys-C-digested albumin; (B) guanidinated Lys-C-digested albumin; (C) Lys-C-digested guanidinated albumin. Several of the peaks are annotated with the sequence and amino acid position of the corresponding peptide. K', guanidinated lysine; K, partially guanidinated lysine; GUAN, number of guanidinated lysine residues.

dues, complete guanidination would increase its mass by 798 Da (19 lysine residues ×42 Da). The observed mass increase was 370 Da, indicating that approximately half of the lysine residues were guanidinated. Since albumin contains 59 lysine residues, complete guanidination would increase its mass by 2,478 Da (59 lysine residues ×42 Da). The observed mass increase of 1,477 Da indicates that approximately 35 lysine residues were guanidinated. Partial protein guanidination was already reported where guanidination of native form of kappa-elastin converted about 50% lysine residues to homoarginine.[18] The "buried" unmodified lysine residues were detected after partial acid hydrolysis followed by exhaustive guanidination,[18] or dansylated after drastic reduction followed by dansylation.[19]

Trypsin-related peptides. The trypsin-related mass spectra of myoglobin and albumin are shown in Figures 2 and 3, respectively. The mass spectra in Figure 2A and 3A are from unmodified tryptic peptides of myoglobin and albumin, respectively. In these spectra, the peaks of arginine-containing peptides were more intense than those of peptides lacking arginine residues. The most intense peaks of myoglobin and albumin were VEADIAGHGQEVLIR (residues 18 - 32 of myoglobin) at *m*/*z* 1607.11 (Fig. 2A) and LGEYGFQNALIVR (residues 421 - 433 of albumin) at *m*/*z* 1479.60 (Fig. 3A), respectively.

The mass spectra of the guanidinated tryptic peptides of myoglobin and albumin are shown in Figures 2B and 3B, respectively, with each lysine guanidination resulting in a mass increase of 42.0 Da. The mass spectrum in Figure 2B shows the signal enhancement resulting from guanidination of peptides with C-terminal lysines. The peptide HGTVVLTALGGILK (residues 65 - 78 of myoglobin; one guanidination) at *m*/*z* 1420.86 yielded the most intense peak. However, the improvement from guanidination was insignificant for the albumin tryptic digest (Fig. 3B); the most intense peak in the mass spectrum was from the arginine-containing peptide DAFLGSFLYEYSR (residues 347 - 359).

The mass spectra of tryptic peptides of guanidinated myoglobin and albumin are shown in Figures 2C and 3C, respectively. The signals for the peptides with a C-terminal arginine (VEADIAGHGQEVLIR, *m*/*z* 1606.88) (Fig. 2C), guanidinated lysine (YKELGFQG, *m*/*z* 983.29; one guanidination) (Fig. 2C), or both (CCTKPESERMPCTEDYLSLILNR, *m*/*z* 2914.25; one guanidination) (Fig. 3C) became dominant. Unmodified proteins are presumed to differ from guanidinated proteins in trypsin accessibility. Guanidination of undigested proteins resulted in partial guanidination of lysine residues, as shown in Figures 2C and 3C. The observed trypsin-related myoglobin

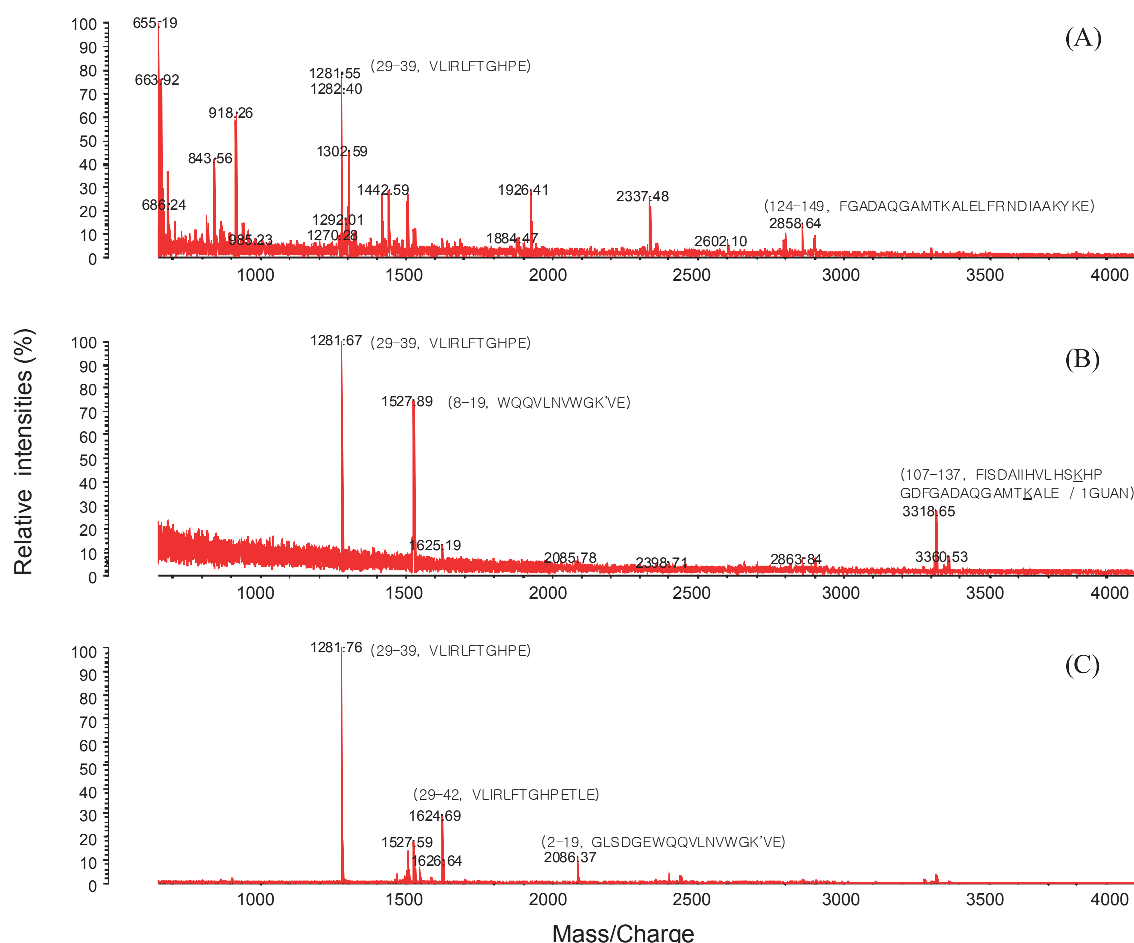

Figure 6. MALDI mass spectra of Glu-C-related myoglobin peptides: (A) Glu-C-digested myoglobin; (B) guanidinated Glu-C-digested myoglobin; (C) Glu-C-digested guanidinated myoglobin. Several of the peaks are annotated with the sequence and amino acid position of the corresponding peptide. K', guanidinated lysine; K̲, partially guanidinated lysine; GUAN, number of guanidinated lysine residues.

and albumin peptides with fully or partially guanidinated lysine residues are summarized in Tables S1 and S2, respectively.

Lys-C-related peptides. Lys-C, which cleaves C-terminal to lysine residues, is sometimes used in place of trypsin to increase sequence coverage and signal intensity.[13] The Lys-C-related mass spectra from myoglobin and albumin are shown in Figures 4 and 5, respectively. The Lys-C-related peptides produced results similar to those of the trypsin-related myoglobin peptides; peptides that had been undetectable in the mass spectra of Lys-C-digested peptides (Figs. 4A and 5A) produced observable peaks when guanidinated (Figs. 4B and 5B), and Lys-C-digested peptides of guanidinated proteins provided different mass spectral peak patterns (Figs. 4C and 5C). In particular, peaks corresponding to guanidinated Lys-C-digested peptides were not comparable to those of Lys-C-digested guanidinated peptides. In other words, the MS peak patterns in the two spectra were completely different.

The observed Lys-C-related peptides from myoglobin and albumin are listed in Tables S3 and S4, respectively. Almost none of the guanidinated peptides found in the guanidinated Lys-C-digested protein sample were also found in the Lys-C-digested guanidinated protein sample, and *vice versa*. Peaks corresponding to unmodified peptides were common to both samples. In the identified peptides from Lys-C-digested guanidinated protein (column GUAN-Lys-C in Tables S3 and S4), one unmodified lysine residue was always present, with the exception of the C-terminal peptides (YKELGFQG and EACFAVEGPKLVVSTQTALA for myoglobin and albumin, respectively). Furthermore, peptides containing guanidinated lysine residues always contained missed cleavages. That is, homoarginine-containing peptides were observed only when the peptide included a missed cleavage. These observations strongly indicate that Lys-C is inactive toward guanidinated lysine residues, thereby resulting in completely different MS patterns for peptides guanidinated before *vs*. after Lys-C digestion.

Glu-C-related myoglobin peptides. The Glu-C-related mass spectra from myoglobin and albumin are shown in Figures 6 and 7, respectively. C-terminal arginine and lysine residues are not relevant to Glu-C-digestion because Glu-C exclusively cleaves at the C-terminus of glutamic or aspartic acid.[14] However, arginine-containing peptides, such as VLIRLFTGHPE (*m*/*z* 1281.55) and DYLSLILNRLCVLHE (*m*/*z* 1857.64), yielded the most intense peaks in the mass spectra of Glu-C-digested peptides (Figs. 6A and 7A). Although guanidination significantly improved the peak intensity of trypsin- and Lys-C-related peptides containing lysine, the peak intensity for Glu-C-related

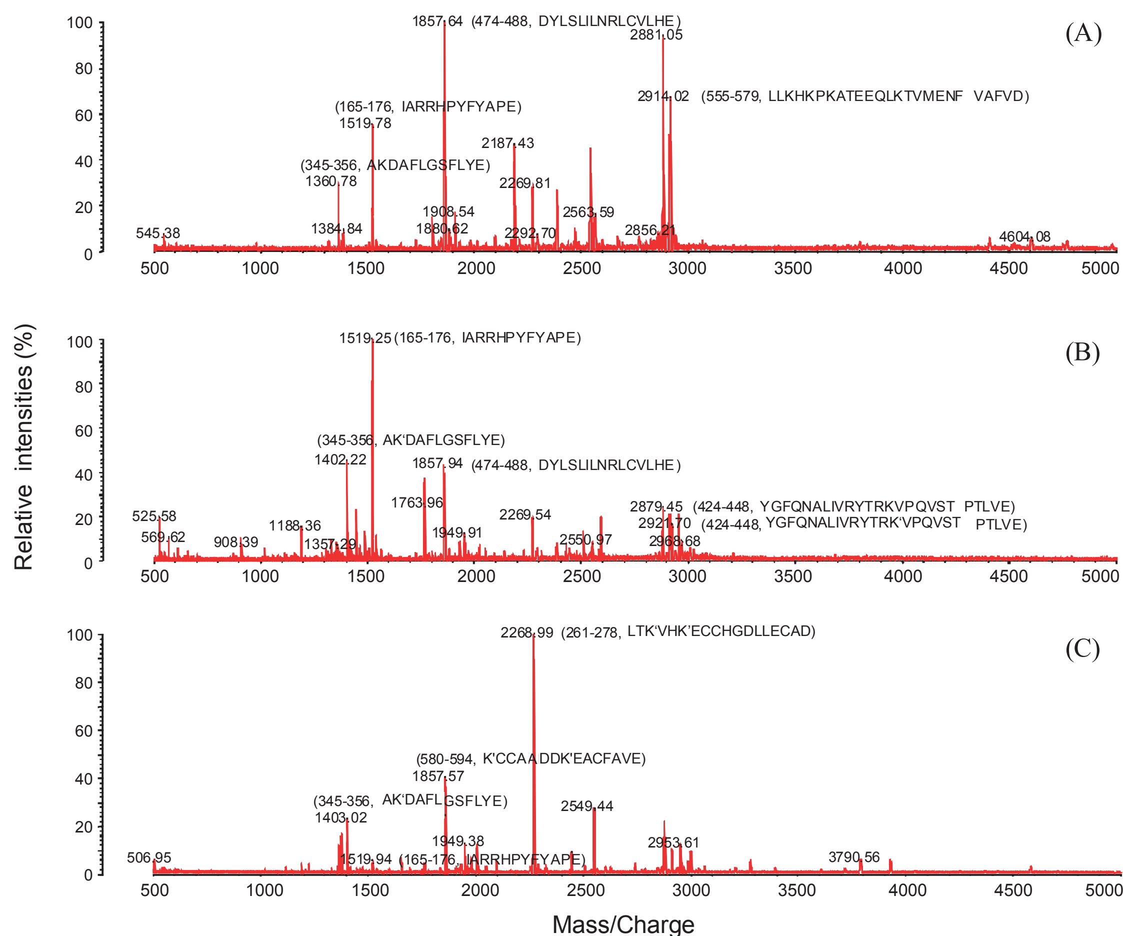

Figure 7. MALDI mass spectra of Glu-C-related albumin peptides: (A) Glu-C-digested albumin; (B) guanidinated Glu-C-digested albumin; (C) Glu-C-digested guanidinated albumin. Several of the peaks are annotated with the sequence and amino acid position of the corresponding peptide. K', guanidinated lysine; K, partially guanidinated lysine; GUAN, number of guanidinated lysine residues.

peptides was not considerably affected by guanidination. The same two peptide peaks remained intense in the mass spectra of guanidinated tryptic peptides (Figs. 6B and 7B) and of tryptic peptides of guanidinated proteins (Figs. 6C and 7C). Some lysine-containing peptides [residues 8 - 19 (m/z 1527.89) and 107 - 137 (m/z 3318.65) of myoglobin (Fig. 6B) and residues 261 - 278 (m/z 2268.99) of albumin (Fig. 7C)] exhibited increased intensity from guanidination. The identified Glu-C-related myoglobin and albumin peptides are summarized in Tables S5 and S6, respectively.

Sequence coverage. Comparisons of myoglobin and albumin sequence coverage for each enzyme are shown in Figures 8A and 8B, respectively. For myoglobin, only a slight improvement in sequence coverage was observed from guanidination before or after tryptic digestion, whereas significant improvements were obtained from guanidination before or after Lys-C digestion; this result explains the effective contribution of guanidination to the MS signal enhancement of C-terminal lysine peptides of myoglobin, which contains 19 lysine residues and only 2 arginine residues.

For albumin, however, the degree of sequence coverage decreased with guanidination before or after trypsin or Lys-C digestion. The decrease was larger when guanidination was performed prior to enzymatic digestion. Albumin contains 59 lysine residues and 23 arginine residues and thus has a relatively low lysine-to-arginine ratio (59:23) compared with that of myoglobin (19:2); this difference is believed to explain the decreased sequence coverage from guanidination. We assume that the signal enhancement from guanidination was considerably smaller for albumin than for myoglobin because the ratio of tryptic or Lys-C-produced peptides containing C-terminal lysines to those containing C-terminal arginines was significantly smaller for albumin than for myoglobin. Furthermore, the sample loss that occurs from the use of ZipTipC18 or Microcon (YM-3) desalting columns after guanidination of peptides or proteins, respectively, probably contributed to the decreased sequence coverage of myoglobin and albumin.

When guanidination was performed prior to Glu-C digestion, protein sequence coverage (69.4% and 42% coverage for myoglobin and albumin, respectively) increased relative to that of unmodified Glu-C-digested peptides (53.7% and 24.9%, respectively). The degree of sequence coverage from guanidination of Glu-C-digested peptides decreased slightly for myoglobin (49.1% coverage) and increased for albumin (39.1% coverage).

Conclusively, guanidination-induced improvement of sequence coverage was observed to depend on the protein se-

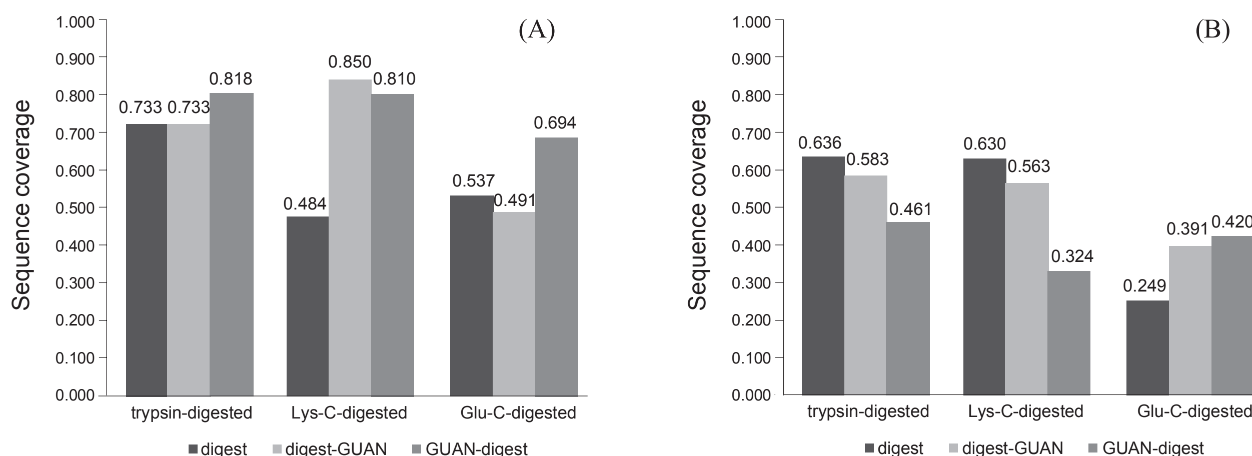

Figure 8. Sequence coverage comparisons of enzymatic digests of horse heart myoglobin (A) and bovine serum albumin (B). Digestions were performed with trypsin, Lys-C, or Glu-C. Digest, enzymatic digestion without guanidination; digest-GUAN, enzymatic digestion followed by guanidination; GUAN-digest, guanidination of myoglobin followed by enzymatic digestion. The first methionine of myoglobin and amino acids 1 - 24 of albumin in the Swiss-Prot entries were not included in sequence coverage calculations.

quence. For horse heart myoglobin, guanidination provided an improved or similar degree of sequence coverage relative to that without guanidination with all three enzymes (trypsin, Lys-C, and Glu-C). For bovine serum albumin, however, guanidination generally provided an increased degree of sequence coverage only with Glu-C digestion.

Conclusions

Lysine side chains in horse heart myoglobin and bovine serum albumin were guanidinated either before or after enzymatic digestion with trypsin, Lys-C, or Glu-C. The resulting improvement in MS signal intensity and sequence coverage was dependent on the protein sequence. The signal enhancement resulting from guanidination of lysine-containing myoglobin peptides was most significant with trypsin, followed by Lys-C and then Glu-C. For albumin, on the other hand, the enhancement was most significant with Lys-C, followed by Glu-C and then trypsin. Enzymatic digestion prior to guanidination increased sequence coverage only in the case of Lys-C-digested myoglobin and Glu-C-digested albumin. Guanidination of myoglobin or its peptides provided an improved or similar degree of sequence coverage with all three enzymes, whereas guanidination of albumin or its digests provided an increased degree of sequence coverage only with Glu-C.

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Supporting Information. Additional supporting information (Tables S1-S6) is available in the online version of this article.

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