Quantitative evaluation of refolding conditions for a disulfide-bond-containing protein using a concise ^{18}O -labeling technique

Hiromasa Uchimura,¹ Yusam Kim,² Takaaki Mizuguchi,²
Yoshiaki Kiso,² and Kazuki Saito^{1,3*}

¹Laboratory of Proteomic Sciences, 21st Century COE Program, Kyoto Pharmaceutical University, Kyoto 607-8412, Japan

²Department of Medicinal Chemistry, Center for Frontier Research in Medicinal Science, Kyoto Pharmaceutical University, Kyoto 607-8412, Japan

³RIKEN Systems and Structural Biology Center, Yokohama 230-0045, Japan

Received 23 February 2011; Revised 28 March 2011; Accepted 29 March 2011

DOI: 10.1002/pro.637

Published online 15 April 2011 proteinscience.org

Abstract: A concise method was developed for quantifying native disulfide-bond formation in proteins using isotopically labeled internal standards, which were easily prepared with proteolytic ^{18}O -labeling. As the method has much higher throughput to estimate the amounts of fragments possessing native disulfide arrangements by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) than the conventional high performance liquid chromatography (HPLC) analyses, it allows many different experimental conditions to be assessed in a short time. The method was applied to refolding experiments of a recombinant neuregulin 1- β 1 EGF-like motif (NRG1- β 1), and the optimum conditions for preparing native NRG1- β 1 were obtained by quantitative comparisons. Protein disulfide isomerase (PDI) was most effective at the reduced/oxidized glutathione ratio of 2:1 for refolding the denatured sample NRG1- β 1 with the native disulfide bonds.

Keywords: high-throughput evaluation; isotopic labeling; MALDI-TOF-MS; neuregulin EGF-like motif; protein disulfide-bond formation; quantitative analysis

Abbreviation: NRG1- β 1, the EGF-like motif of neuregulin 1- β 1.

Grant sponsors: 21st Century COE Program and “Academic Frontier” Project for Private Universities: matching fund subsidy [Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan]; SUNBOR GRANT (Suntory Institute for Bioorganic Research).

*Correspondence to: Kazuki Saito, Laboratory of Proteomic Sciences, 21st Century COE Program, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607-8412, Japan. E-mail: saito@mb.kyoto-phu.ac.jp

Introduction

Most secretory proteins contain disulfide bridges (SS bonds),¹ which play very important roles in the acquisition and stabilization of their functional structures. However, the proteins are generally difficult to express in the native form with the correct disulfide-bond arrangements, using conventional *Escherichia coli* production systems.² Recombinant proteins with disulfide bridges often misfold and form incorrect SS bonds, consequently generating insoluble precipitates on expression.^{2,3} In such cases, the proteins must be converted into the native form by refolding procedures, in which

they are solubilized with denaturing and reducing agents, and then carefully refolded to form the correct disulfide bridges by removing or diluting the agents. Disulfide-containing proteins are sometimes expressed in a soluble form, but even in these rare cases, it is still not known if they have their native, correct disulfide-bond arrangements. The arrangements must be confirmed by analytical procedures.

Generally, to verify the arrangements, sample proteins are digested by a specific protease under nonreductive conditions, and the fragments possessing native disulfide bonds are identified and quantified by high performance liquid chromatography (HPLC).⁴ However, these procedures are often time-consuming and laborious, because every fragment peak in the chromatograms must be pooled and subjected to verification of native disulfide-bond formation, using amino-acid composition analyses, N-terminal amino-acid sequencing, MS measurements, and so on. On the other hand, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has recently been directly utilized with the digested mixture of proteins, without HPLC separation, as it can concisely identify the disulfide-bond-containing fragments in the mixture, and requires only a small amount of the protein sample. However, the peak intensities in the MALDI-MS spectra are much less quantitative, because the ionization efficiencies of sample compounds are affected by the coexisting salts and other sample molecules during the MALDI process.^{5,6} To estimate disulfide-bond formation accurately, the quantitative aspect of the MS analysis should be improved, while maintaining the advantage of its conciseness. Such improvement is required for the evaluation of disulfide-bond formation in parallel for many samples; for example, during the optimization of refolding conditions for recombinant disulfide proteins.

In this study, the MALDI-TOF-MS analysis was improved by the introduction of stable-isotope-labeling of digested peptides. On ionization, the isotopically labeled compounds behave in exactly the same manner as the nonlabeled ones, while they can be separately detected at different mass-to-charge (m/z) ratios in the MS spectra. Previously, using proteolytic ¹⁸O-labeling,^{7,8} we quantitatively revealed the disulfide-bond rearrangements in an epidermal growth factor (EGF)-like motif of neuregulin 1- β 1 (NRG1- β 1), a 71-residue polypeptide with three disulfide bonds.⁹ In the experiments, NRG1- β 1 was subjected to weakly basic conditions and digested with trypsin under nonreductive conditions in a buffer prepared with ¹⁸O-labeled water (H₂¹⁸O), whereas the untreated, native protein was digested in a buffer with natural ¹⁶O-water. After mixing equal amounts of both digestion mixtures, the rearrangement induced by the basic treatment was successfully evaluated by simple MALDI-TOF-MS measurements of the mixed sample. By introducing the isotope-labeling technique, the improved peak intensities

of the MALDI-TOF-MS spectra allowed a quantitative comparison of the digested fragments of the sample proteins, which were treated under different conditions. This result suggested that the ¹⁸O-labeled digested fragments could be utilized as internal standards for the quantitative estimation of native disulfide-bond formation. Therefore, in this study, using ¹⁸O-labeled digested fragments as the standards, a concise and systematic method was developed for optimizing the refolding conditions for recombinant disulfide proteins.

Results

First of all, to obtain the isotope-labeled standards with the proteolytic ¹⁸O-labeling, the authentic sample of NRG1- β 1, the model disulfide protein in this study, was digested under nonreductive conditions in a buffer prepared with ¹⁸O-water. Briefly, 50 μ g of commercially available NRG1- β 1 (R&D Systems, Minneapolis, MN) was digested with 2.5 μ g (1/20, by weight) of sequence grade modified trypsin (Promega Corporation, Madison, WI) at 37°C for 20 h in ammonium acetate buffer (50 mM, pH 6.9), which was prepared with ¹⁸O-labeled water ($\sim$ 97% atom ¹⁸O, Sigma-Aldrich, St. Louis, MO). On the digestion, two ¹⁸O-atoms from the solvent water molecules were incorporated into the carboxyl moieties of the C-terminal residues of the generated peptide fragments.^{7,8} As revealed in our previous study,⁹ because the disulfide-bond arrangements are affected by a weakly basic treatment, the tryptic digestion was performed in a neutral pH buffer (50 mM CH₃COONH₄, pH 6.9), although the optimum pH range of trypsin is more basic, pH 8–9. In fact, the MALDI-TOF-MS spectra revealed that ¹⁸O-fragments had the native SS bonds as expected, and neither cleaved nor rearranged SS bonds were observed in the digested fragments (data not shown). In addition, digested fragments possessing residual ¹⁶O-atoms were hardly detected, except for the most C-terminal fragment of the protein [9 in Fig. 1(A)], which still contained ¹⁶O-atoms in the carboxy group, as the group was not involved in the tryptic digestion in the ¹⁸O-labeled buffer.

Next, the sample recombinant disulfide protein, NRG1- β 1, was obtained with a conventional *E. coli* production system. The vector was designed for the expression of the protein (amino-acid residues 176–246 of the mature parent protein) with signal and His₆-tag sequences at the N- and C-termini, respectively. The signal sequence was included for the secretion of the expressed protein into the periplasmic space of the cells, while the His₆-tag was for subsequent purification by chromatography. As a result, the sample protein was produced with additional amino-acid residues, as compared with the authentic protein that was used for the ¹⁸O-fragment standards [Fig. 1(A)]. After the protein localized in the periplasm was extracted by osmotic shock,^{10,11} it was collected as an

Figure 1. (A) Tryptic fragments generated from two different NRG1- β 1 samples. One was commercially obtained and the other was recombinantly prepared in this study. Although the latter recombinant preparation had some additional residues, as compared with the former authentic one, fragments ②–⑨ were commonly generated by tryptic digestion. (B) Brief outline of the refolding experiments and the disulfide-bond verification for NRG1- β 1. After refolding was performed under various conditions, the samples were analyzed for native disulfide-bond formation by nonreductive tryptic digestion. To quantify the generated fragments with correct SS bonds, such as ⑦–⑧ and ②–④–⑥, the digested samples were measured by MALDI-TOF-MS, after mixing with the ^{18}O -labeled internal standards.

80%-saturated ammonium sulfate precipitate. For the refolding experiments, the protein precipitate was solubilized in denaturation buffer (10 mM NH_4HCO_3 , 5 M guanidine hydrochloride, 1 mM dithiothreitol (DTT), 20 mM imidazole, and 0.5 M NaCl, pH 8.3), and was crudely purified by chromatography on a Ni-chelating affinity column, eluted with a linear gradient of imidazole (from 20 to 500 mM). The protein thus obtained was passed through a desalting column, to remove the imidazole and the reductant DTT from the buffer. (For further experimental details, see the section “Materials and Methods.”)

A brief outline of the refolding experiments is illustrated in Figure 1(B). To start the refolding procedure, immediately after passage through the desalting column, the solution containing the denatured recombinant protein was diluted with a buffer containing several redox agents. After an incubation at 25°C for 30 min, the refolding products were digested with trypsin under nonreductive conditions, in a neutral pH buffer (50 mM Tris-HCl, pH 7.0) prepared with natural ^{16}O -water. To quantitatively evaluate the native disulfide-bond formation in the products, each sample solution was mixed with an equal amount of the ^{18}O -standards, and concentrated and desalted with a ZipTip_{C18} (Millipore Corporation, Billerica, MA), according to the manufacturer’s instructions. The bond formation was estimated from the peak intensity ratios in the MALDI-TOF-MS spectra, between ^{16}O - and ^{18}O -fragments possessing the native disulfide bonds.¹²

Before the refolding experiments were performed, according to the outline shown in Figure 1(B), various tryptic digestion conditions were considered for sufficient cleavage of the sample pro-

teins, because the protease is less active in a neutral pH buffer, but complete digestion of the sample protein is very important in our quantitative evaluation method for the disulfide-bond formation. To adjust the digestion conditions, solutions of the denatured recombinant NRG1- β 1 (3 μg) were acidified to pH $\sim$ 3, and then 0.03, 0.06, 0.15, or 0.6 μg of trypsin (1/100, 1/50, 1/20, or 1/5, by weight, respectively) was added. The digestion reactions were initiated by the addition of a small aliquot of concentrated Tris-HCl buffer (1 M, pH 7.0, final dilution to 50 mM, pH 7.0), and the solutions were incubated at 37°C for 15 h. At this point, one-half of each sample solution was mixed with 4% (v/v) trifluoroacetic acid (TFA) to terminate the digestion reaction, whereas the other half of the solution was further incubated with additional trypsin (0.6 μg , 1/5, by weight) for 12 h. The additional digestion of the latter samples was also terminated with the aqueous TFA solution (4%, v/v). All the digested samples were mixed with equal amounts of the ^{18}O -fragment standards, and were concentrated and desalted with a ZipTip_{C18}. The samples eluted from the ZipTip_{C18} were measured by MALDI-TOF-MS, using α -cyano-4-hydroxycinnamic acid (CHCA) as the matrix. (For details of the MS sample preparation procedure, refer to the Supporting Information of our previous paper.⁹)

The digestion efficiencies with different amounts of trypsin were evaluated by comparing the amounts of fragment ⑤ (residues 201–207) generated, as the fragment has no cysteine residues in its sequence, and would be generated by the tryptic digestion, regardless of the SS bond formation in NRG1- β 1. The amounts of the ^{16}O -fragment ⑤ in the digestion mixtures are plotted as the ratios against the ^{18}O -

Figure 2. Quantitative optimization of neutral pH tryptic digestion, by comparison with the generated amounts of NRG1-β1 fragment ⑤. (A) The recombinant samples were digested with 1/100, 1/50, 1/20, and 1/5 (w/w) of trypsin in Tris-HCl buffer (50 mM, pH 7.0) at 37°C for 15 h. (B) After the digestions performed in A, they were continued with additional 1/5 (w/w) of trypsin in the same neutral pH buffer at 37°C for 12 h. The generated amounts of fragment ⑤ are plotted as the abundance ratios between ¹⁶O-peptide from the samples and ¹⁸O-peptide from the standards. Data are shown as averages with standard deviations, which were obtained for three spots of the same MALDI-TOF-MS samples.

labeled fragment of the internal standards in Figure 2. When the denatured sample protein was digested for the first 15 h, more fragment ⑤ was generated as more trypsin was added to the reaction solutions [Fig. 2(A)]. Larger amounts of the protease greatly promoted the tryptic digestion in the neutral pH buffer. On the other hand, after the additional digestion with a large amount of trypsin (1/5 of the substrate, by weight), similar amounts of fragment ⑤ were finally generated in every digestion solution [Fig. 2(B)], indicating that the additional digestion cleaved most of the residual substrate protein in the solutions. As 1/5 weight of trypsin in the first 15-h

digestion generated the largest amount of fragment ⑤, which was closest to those after the additional digestion, this amount of protease (1/5, by weight) may be sufficient for the digestion step in the refolding experiments.

The ¹⁸O-standards were then reprepared, by digestion with 1/5 weight of trypsin, because the previous standards were obtained with a smaller amount (1/20) of the protease, which may not have generated maximum digests from the authentic NRG1-β1. In fact, when the previous ¹⁸O-standards were merged with the tryptic fragments generated by the longer digestion, every ratio between the ¹⁶O- and ¹⁸O-peptides was somewhat larger than 1.0 [Fig. 2(B)]. The previous standards contained a slightly smaller amount of fragment ⑤ than that generated by the exhaustive digestions of the recombinant protein. After the treatment with 1/5 weight of trypsin, the digestion mixture of the authentic protein hardly showed any peaks from incompletely digested fragments with missed cleavages in its MALDI-TOF-MS spectra (data not shown), indicating that the new digestion conditions achieved complete cleavage of the authentic NRG1-β1, although some lysine and arginine residues are adjacent to the bridged cysteines. As the protease fully digested the sample disulfide protein with the native fold, which generally has a compact structure, the conditions must be applicable to the digestion step for the refolding experiments. The ¹⁸O-standards, newly prepared with a sufficient amount of trypsin, were used throughout the following experiments.

Using the optimized digestion step, the denatured recombinant NRG1-β1 samples were subjected to the refolding experiments, as described in Figure 1(B). After passage through the desalting column, the solutions of the sample protein (3 μg) were diluted with Tris-HCl buffers (10 mM, pH 8.5), containing reduced and oxidized glutathione (GSH and GSSG, respectively) at different ratios. In addition, protein disulfide isomerase (PDI) (Takara Bio, Shiga, Japan) was examined for its ability to accelerate disulfide-bond formation. After an incubation at 25°C for 30 min, the disulfide-bond formation was terminated by the addition of 4% (v/v) TFA. The refolding products were digested with the optimized amount of trypsin (0.6 μg, 1/5, by weight) in the neutral pH buffer (50 mM Tris-HCl, pH 7.0) under nonreductive conditions at 37°C for 15 h. The digested samples were combined with equal amounts of the new ¹⁸O-standards, and were concentrated and desalted with a ZipTip_{C18}.

Native disulfide-bond formation in the refolding products was estimated by quantification of the generated fragments with correct SS bonds, such as ⑦⑧ and ②④⑥ in Figure 1(A). The amounts of the generated fragments are plotted in Figure 3, as the abundance ratios between ¹⁶O-peptides from the

Figure 3. Quantitative evaluation of native disulfide-bond formation in fragments 7-8 (A) and 2-4-6 (B) of the NRG1- β 1 refolding products. The denatured sample protein solutions were diluted with different redox buffers (containing GSH:GSSG at ratios of 1:10, 1:2, 1:1, 2:1, 10:1, and 200:1) in the absence (open bars) or presence (closed bars) of protein disulfide isomerase (PDI), and were incubated at 25°C for 30 min. After the refolding products were digested with 1/5 (w/w) of trypsin in a neutral pH buffer at 37°C for 15 h, the generated amounts of the fragments were estimated in the same manner as described in the legend of Figure 2.

refolding samples and ^{18}O -peptides from the internal standards. In these experiments, the sample protein was completely digested under all the examined conditions, because the $^{16}\text{O}/^{18}\text{O}$ ratios of fragment 5 were always around 1.0 (data not shown).

In fragment 7-8, the generated amounts of the peptide were quite small in the refolding products without PDI at any GSH:GSSG ratios [open bars in Fig. 3(A)]. However, this isotope-assisted method allows even such small amounts of the peptide to be quantitatively compared with each other, as supported by their small statistical deviations. Under more reductive conditions with higher GSH:GSSG ratios, more fragment 7-8 was generated, although it reached only 0.24-fold of the standard under the most reductive conditions examined in this study (GSH:GSSG = 200:1). By contrast, in the presence of PDI, the generation of the fragment was dramati-

cally promoted [closed bars in Fig. 3(A)]. At every GSH:GSSG ratio, the yield of the fragment was much greater than that obtained in the absence of PDI. Especially, at a 2:1 ratio of the redox agents, the amount of fragment 7-8 generated was as much as that in the ^{18}O -standards prepared by the sufficient digestion, as the abundance ratio between the ^{16}O - and ^{18}O -peptides became ~ 1.0 . This result suggests that the native disulfide bond between 7 and 8 was fully reconstructed at the 2:1 glutathione ratio, during the refolding process with PDI. The enzyme obviously promoted the native disulfide-bond formation most efficiently at the 2:1 ratio, whereas the bond was readily generated at 200:1 in the absence of PDI. Intrinsically, PDI catalyzes disulfide-bond formation in the endoplasmic reticulum (ER) of eukaryotic cells.^{13,14} As the GSH:GSSG ratio in the ER is known to be within the range of 1:1–3:1,¹⁵ the results obtained in this study are consistent with the intrinsic properties of PDI.

In fragment 2-4-6, the other fragment possessing native disulfide bonds, the generation of the fragment was also strongly promoted by the addition of PDI [Fig. 3(B)]. The yield of the fragment was the greatest at a 2:1 glutathione ratio, as similarly observed for fragment 7-8 in the presence of PDI. On the other hand, without PDI, the generation of fragment 2-4-6 was somewhat different from that of 7-8. The yield of fragment 2-4-6 was maximal around a 2:1 ratio of the redox agents, whereas the yield of 7-8 was better under more reductive conditions. Considering the results for both fragments 7-8 and 2-4-6, native NRG1- β 1 was most efficiently generated at a GSH:GSSG ratio of 2:1 in the presence of PDI, and thus these experimental conditions should be used for the large-scale preparation of natively refolded NRG1- β 1, as the first choice.

Discussion

The usefulness of the ^{18}O -assisted quantification method was verified by application to refolding experiments of a disulfide protein, NRG1- β 1. By the use of the ^{18}O -labeled standards, the formation of native disulfide bonds can be easily estimated with conventional MALDI-TOF-MS measurements. Therefore, many products generated under different conditions can be compared, without any complicated procedures using HPLC or other separation techniques, for seeking the best refolding conditions of disulfide proteins.

When a tryptic fragment has multiple disulfide bonds, such as 2-4-6, its isomers with different internal disulfide configurations cannot be discriminated by their m/z values in the MS spectra. In this case, secondary digestion of the fragment with another protease is often useful, as shown in the Supporting Information of our previous paper.⁹ However, such additional procedures reduce the

throughput of the experiments, and are not always necessary in the optimization of refolding conditions for a disulfide protein. The generated amounts of ② ④ ⑥ can be used as one of the primary indexes in the optimization experiments, even without the elucidation of its internal disulfide configuration, as the lower amounts of the fragment still obviously indicate the lack of native disulfide bridges in the refolding products of the recombinant NRG1-β1.

The most critical point of this methodology is the availability of the authentic proteins possessing the native disulfide-bond arrangement. In recent years, a variety of systems for producing difficult proteins have become available, such as those using mammalian or insect cultured cells, yeast, and so on,^{16,17} but the protein yields of these systems are still lower, and the production costs are much more expensive than those of the conventional systems using *E. coli*.¹⁸ If even a small amount of the authentic sample protein can be obtained from such advanced systems, or from commercial sources as in this study, then the isotope-assisted method can be used to optimize the refolding conditions of a disulfide protein, using the small amount of the authentic sample, and will greatly facilitate the large-scale preparation at a reasonable cost.

In addition, as this concise method with ¹⁸O-standards can estimate the local disulfide-bond formation separately, as observed in fragments ⑦ ⑧ and ② ④ ⑥ of NRG1-β1, it may also be applied to experiments to reveal the dynamic processes of protein folding, by quantitatively tracing the formation of individual disulfide bonds.

Materials and Methods

Protein expression and extraction of the sample recombinant protein from *E. coli* periplasm

The EGF-like motif of neuregulin 1-β1 (NRG1-β1) was used as the model disulfide protein in this study. NRG1-β1 (amino-acid residues 176–246 of the mature parent protein) was expressed in *E. coli* strain BL21(DE3), using the pET-26b(+) vector (Novagen®, Merck KGaA, Darmstadt, Germany) with signal and His₆-tag sequences at the N- and C-termini, respectively. The signal sequence was for the secretion of the expressed protein into the periplasmic space of the cells, whereas the His₆-tag was for the subsequent purification of the protein. Both sequences were derived from the pET-26b(+) vector. The transformed *E. coli* cells were cultured in 9 mL of Luria-Bertani (LB) medium, containing 25 µg/mL kanamycin, at 37°C for 4.3 h, and then they were directly transferred to 900 mL of LB medium, containing 25 µg/mL kanamycin and 0.75% glucose. The culture was incubated at 30°C until the optical density at 600 nm (OD₆₀₀) reached ~0.5, and then protein expression was induced with 0.5 mM isopro-

pyl-β-D-1-thiogalactopyranoside (IPTG), and the culture was further incubated for 15 h.

The recombinant protein expressed in the periplasm was extracted by the osmotic shock procedure, as follows: 1 g (wet weight) of the cells, collected by centrifugation (3170g, 4°C, 10 min), was suspended in 80 mL of 30 mM Tris-HCl, pH 8.0, containing 20% sucrose. The suspension was stirred in the buffer with 1 mM ethylenediaminetetraacetate (EDTA) at room temperature for 10 min. After centrifugation (14,500g) at 4°C for 10 min, the resulting pellet was immediately resuspended in cold distilled water (an equal volume to the original suspension) containing 1 mM EDTA and 0.4 mM phenylmethylsulfonyl fluoride (PMSF), and was gently stirred in an ice bath for 40 min. After centrifugation (14,500g) at 4°C for 10 min, the periplasmic proteins obtained in the supernatant were precipitated with 80%-saturated ammonium sulfate. The precipitate was collected by centrifugation (14,500g, 4°C, 10 min), and stored at –80°C until use.

Preparation of the denatured sample protein for refolding experiments

For the refolding experiments, the precipitated sample protein (~1.4 g, extracted from 3 L of the cell culture) was solubilized in 160 mL of denaturation buffer (10 mM NH₄HCO₃, 5 M guanidine hydrochloride, 1 mM DTT, 20 mM imidazole, and 0.5 M NaCl, pH 8.3), and was applied to a Ni-chelating affinity column (HisTrap FF column, GE Healthcare Bio-Sciences AB, Uppsala, Sweden), equilibrated with denaturation buffer. The protein was eluted from the column with a linear gradient of imidazole (from 20 to 500 mM) in 10 mM NH₄HCO₃, 5 M guanidine hydrochloride, and 1 mM DTT, pH 9.2. Fractions containing the protein were collected and lyophilized.

The lyophilized sample (~0.1 g) was suspended in distilled water (~100 µL) and centrifuged (2300g) at room temperature for 2 min. The resulting supernatant was passed through a PD-10 desalting column (GE Healthcare Bio-Sciences AB) (10 mM Tris-HCl and 5 M guanidine hydrochloride, pH 8.5) to remove the imidazole and DTT from the sample solution. Fractions containing the sample protein were collected, and the protein concentration was measured with a BCATM protein assay kit (Pierce, Rockford, IL), according to the manufacturer's instructions. As a result, ~63 µg of the sample protein was obtained from 3 L of the cell culture. This yield was rather low, as compared with those of other recombinant proteins, as NRG1-β1 is a difficult disulfide protein to prepare, and it readily precipitates on expression. Refolding and disulfide-bond verification experiments were performed as described in the section "Results," using 3 µg of the prepared sample protein for each refolding condition.

References

1. Fahey RC, Hunt JS, Windham GC (1977) On the cysteine and cystine content of proteins. Differences between intracellular and extracellular proteins. *J Mol Evol* 10:155–160.
2. Marston FAO (1986) The purification of eukaryotic polypeptides synthesized in *Escherichia coli*. *Biochem J* 240:1–12.
3. Rudolph R, Lilie H (1996) In vitro folding of inclusion body proteins. *FASEB J* 10:49–56.
4. Chang J-Y, Li L, Lai P-H (2001) A major kinetic trap for the oxidative folding of human epidermal growth factor. *J Biol Chem* 276:4845–4852.
5. Cohen SL, Chait BT (1996) Influence of matrix solution conditions on the MALDI-MS analysis of peptide and proteins. *Anal Chem* 68:31–37.
6. Kussmann M, Nordhoff E, Rahbek-Nielsen H, Haebel S, Rossel-Larsen M, Jakobsen L, Gobom J, Mirgorodskaya E, Kroll-Kristensen A, Palm L, Roepstorff P (1997) Matrix-assisted laser desorption/ionization mass spectrometry sample preparation techniques designed for various peptide and protein analytes. *J Mass Spectrom* 32:593–601.
7. Mirgorodskaya OA, Kozmin YP, Titov MI, Körner R, Sönksen CP, Roepstorff P (2000) Quantitation of peptides and proteins by matrix-assisted laser desorption/ionization mass spectrometry using ^{18}O -labeled internal standards. *Rapid Commun Mass Spectrom* 14:1226–1232.
8. Yao X, Freas A, Ramirez J, Demirev PA, Fenselau C (2001) Proteolytic ^{18}O labeling for comparative proteomics: model studies with two serotypes of adenovirus. *Anal Chem* 73:2836–2842.
9. Saito K, Yasuo I, Uchimura H, Koide-Yoshida S, Mizuguchi T, Kiso Y (2010) Verification of protein disulfide bond arrangement by in-gel tryptic digestion under entirely neutral pH conditions. *Proteomics* 10:1505–1509.
10. Neu HC, Heppel LA (1965) The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of spheroplasts. *J Biol Chem* 240:3685–3692.
11. Oka T, Sakamoto S, Miyoshi K, Fuwa T, Yoda K, Yamasaki M, Tamura G, Miyake T (1985) Synthesis and secretion of human epidermal growth factor by *Escherichia coli*. *Proc Natl Acad Sci USA* 82:7212–7216.
12. Johnson KL, Muddiman DC (2004) A method for calculating $^{16}\text{O}/^{18}\text{O}$ peptide ion ratios for the relative quantification of proteomes. *J Am Soc Mass Spectrom* 15:437–445.
13. Gilbert HF (1998) Protein disulfide isomerase. *Methods Enzymol* 290:26–50.
14. Wilkinson B, Gilbert HF (2004) Protein disulfide isomerase. *Biochim Biophys Acta* 1699:35–44.
15. Hwang C, Sinskey AJ, Lodish HF (1992) Oxidized redox state of glutathione in the endoplasmic reticulum. *Science* 257:1496–1502.
16. Brondyk WH (2009) Selecting an appropriate method for expressing a recombinant protein. *Methods Enzymol* 463:131–147.
17. Clare JJ, Romanos MA, Rayment FB, Rowedder JE, Smith MA, Payne MM, Sreekrishna K, Henwood CA (1991) Production of mouse epidermal growth factor in yeast: high-level secretion using *Pichia pastoris* strains containing multiple gene copies. *Gene* 105:205–212.
18. Dyck MK, Lacroix D, Pothier F, Sirard M-A (2003) Making recombinant proteins in animals—different systems, different applications. *Trends Biotechnol* 21:394–399.