Absolute Quantification of Intact Proteins via 1,4,7,10-Tetraazacyclododecane-1,4,7-trisacetic acid−10-Maleimidoethylacetamide−Europium Labeling and HPLC Coupled with Species-Unspecific Isotope Dilution ICPMS

Xiaowen Yan,[†] Ming Xu,[†] Limin Yang,[†] and Qiuquan Wang*,[†,‡]

Department of Chemistry and the Key Laboratory of Analytical Sciences, College of Chemistry and Chemical Engineering, Xiamen University, Xiamen 361005, China, and State Key Laboratory of Marine Environmental Science, Xiamen University, Xiamen 361005, China

Quantitative proteomics requires novel analytical methodology to fill the gap related to absolute protein abundance in different physiological conditions. In this paper, we demonstrate a proof-of-concept study for absolute protein quantification. 1,4,7,10-Tetraazacyclododecane-1,4,7-trisacetic acid−10-maleimidoethylacetamide (MMA−DOTA) loaded with Eu was used to label lysozyme, insulin, and ribonuclease A, and they were subsequently quantified using HPLC coupled with ^{153}Eu species-unspecific isotope dilution inductively coupled plasma mass spectrometry (ICPMS). Labeling procedures were optimized using electrospray ionization mass spectrometry (ESI-MS) based on the labeling efficiency and specificity of the three intact proteins, which suggested that 10-fold or higher MMA−DOTA to cysteine sulphydryl rates at pH from 6.8 to 7.6 and 47 °C for 40 min were optimal conditions for the conjugation of the reduced-form proteins and that a 5-fold excess of Eu with respect to the DOTA present in the MMA−DOTA−conjugated proteins and pH 5.8 are optimal for Eu labeling. Subsequently, these three MMA−DOTA−Eu-labeled proteins were digested with trypsin, and the tryptic peptides were quantified via HPLC coupled with ^{153}Eu species-unspecific isotope dilution ICPMS. The results for the protein studied indicated that not only could 100% digestion efficiency not be achieved but also the resulting peptides needed a chromatographic separation at higher resolution. On the other hand, the labeled intact proteins were quantified without tryptic digestion. The average recovery was found to be 97.9% in six independent experiments, and the precision was evaluated to be 5.8% at the 10 pmol L^{-1} level. The detection limits (3σ) were determined to be 0.819, 1.638, and 0.819 fmol for lysozyme, the A chain of insulin, and ribonuclease A, respectively, using ICPMS with a normal concentric pneumatic nebulizer. These results demonstrated that high-quality absolute protein quantification could be achieved through labeling the intact proteins but not the tryptic peptides, implying that intact proteins may be more feasible and practical targets than tryptic peptides for ICPMS-based absolute protein quantification.

Quantitative proteomics is an important research field in modern life science. The main target of quantitative proteomics research is to identify and determine the diversity of protein expression in living systems during different physiological states. Accordingly, various types of tags have been developed to label proteins for detection and quantification. Fluorescent probes are widely used for protein labeling and imaging, but they suffer from spectral overlap, instability of the chromophores, photobleaching, and many other limitations when they are applied to protein quantification.[1] Molecular mass spectrometry has been the major technique for quantitative proteomics since the isotope-coded affinity tag was first introduced for relative protein quantification in 1999.[2] Various types of tags have been developed since then, such as ^{18}O labeling through enzyme digest in H$_2$^{18}O,[3] stable isotope labeling using amino acids in cell culture,[4] isobaric tags for relative and absolute quantitation,[5] and lanthanide-loaded chelating agents.[6−10] However, due to the inherent limitations

---

* To whom correspondence should be addressed. E-mail:qqwang@xmu.edu.cn.

† Department of Chemistry and the Key Laboratory of Analytical Sciences, College of Chemistry and Chemical Engineering.

‡ State Key Laboratory of Marine Environmental Science.

(1) Perfetto, S. P.; Chattopadhyay, P. K.; Roederer, M. *Nat. Rev. Immunol.* **2004**, *4*, 648–655.

(2) Gygi, S. P.; Rist, B.; Gerber, S. A.; Turecek, F.; Gelb, M. H.; Aebersold, R. *Nat. Biotechnol.* **1999**, *17*, 994–999.

(3) Schnölzer, M.; Jedrzejewski, P.; Lehmann, W. D. *Electrophoresis* **1996**, *17*, 945–953.

(4) Ong, S. E.; Blagoev, B.; Kratchmarova, I.; Kristensen, D. B.; Steen, H.; Pandey, A.; Mann, M. *Mol. Cell. Proteomics* **2002**, *1*, 376–388.

(5) Ross, P. L.; Huang, Y. N.; Marchese, J. N.; Williamson, B.; Parker, K.; Hattan, S.; Khainovski, N.; Pillai, S.; Dey, S.; Daniels, S.; Purkayastha, S.; Juhasz, P.; Martin, S.; Bartlet-Jones, M.; He, F.; Jacobson, A.; Pappin, D. J. *Mol. Cell. Proteomics* **2004**, *3*, 1154–1169.

(6) Whetstone, P. A.; Butlin, N. G.; Corneillie, T. M.; Meares, C. F. *Bioconjugate Chem.* **2004**, *15*, 3–6.

(7) Lee, S.; Young, N. L.; Whetstone, P. A.; Cheal, S. M.; Benner, W. H.; Lebrilla, C. B.; Meares, C. F. *J. Proteome Res.* **2006**, *5*, 539–547.

(8) Liu, H.; Zhang, Y.; Meng, L.; Qin, P.; Wei, J.; Jia, W.; Li, X.; Cai, Y.; Qian, X. *Anal. Chem.* **2007**, *79*, 7700–7707.

of molecular mass spectrometry, including a narrow dynamic range for calibration and the different signal response factors of different peptides in electrospray ionization mass spectrometry (ESI-MS) or MALDI-MS, especially; it is necessary to synthesize the individual isotope-labeled protein or peptide standard for absolute quantification of the corresponding protein or peptide.[11−14] Therefore, it is very expensive and difficult to apply routinely to the analysis of the thousands of proteins encountered in quantitative proteomics studies.

As an accurate and sensitive technique for detecting and quantifying elements, inductively coupled plasma mass spectrometry (ICPMS) has various unique features, including a broad dynamic range (9 orders of magnitude), a signal which is independent of the chemical species such as the kinds of peptide and/or protein, multielement capabilities, and excellent resolution between different element masses, making it an attractive detector for absolute protein quantification via the definite element stoichiometry in proteins. Thus, ICPMS-based absolute protein quantification methods have emerged in recent years.[15,16] For example, the naturally occurring heteroelements S,[17−19] P,[20,21] and Se,[22,23] which are covalently incorporated into proteins, were the first and are the most widely used for protein quantification by ICPMS. However, most of these heteroelements are not sensitive enough in ICPMS for the quantification of low-abundance proteins due to their low ionization efficiency and polyatomic spectral interference.[15] Recently, many more ICPMS-sensitive elements have been tagged to proteins as an alternative strategy for protein quantification.

Element-tagged immunoassay and element-tagged affinity labeling are the two main strategies developed for protein detection and quantification using ICPMS. The element-tagged immunoassay, which combines the sensitivity of ICPMS and the specificity of an antibody, has made notable progress in recent years. Metal nanoparticles or metal chelating polymers are mostly utilized to label antibodies for signal amplification and multiplexed assays.[24−32] Due to the specificity of the antibody, this strategy does not need chromatographic separation, but much care must be taken to prevent reducing or removing antibody activities when antibodies are labeled with tags.[32] For the element-tagged affinity labeling technique, organic mercurial,[33−37] iodine,[38,39] and lanthanide-loaded chelating agents (diethylenetriamine pentaacetic acid (DTPA)[40,41] and 1,4,7,10-tetraazacyclododecane-1,4,7-trisacetic acid (DOTA)[42−44]) are used to introduce ICPMS-sensitive elements.

The chelating agent DOTA was first introduced as an element-coded affinity tag for relative protein quantification in 2004,[6] but it was not combined with ICPMS for absolute protein quantification until a metal-coded affinity tag technique (MeCAT) was developed; this MeCAT approach together with flow injection analysis ICPMS for eye lens proteomics quantification was applied in 2007.[42] The same chelating agent was also used for protein quantification with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and HPLC.[43,44] More recently, peptides were quantified absolutely using Lu-DTPA labeling combined with nano-HPLC/ICPMS and postcolumn isotope dilution analysis, demonstrating that this technique is highly sensitive, accurate, and precise.[41] However, until now, to the best of our knowledge, most of the published literature using ICPMS with a chelating agent labeling strategy were for small peptides, and only a few studies demonstrated labeling of intact proteins with metal tags, for example, Ahrends et al. demonstrated the labeling of α-lactalbumin, bovine serum albumin, and *Sus scrofa* eye len proteins by lanthanide chelates. The labeled proteins were separated with gel electrophoresis and then determined using offline flow

(9) Ahrends, R.; Pieper, S.; Neumann, B.; Scheler, C.; Linscheid, M. W. *Anal. Chem.* **2009**, *81*, 2176–2184.
(10) Pieper, S.; Beck, S.; Ahrends, R.; Scheler, C.; Linscheid, M. W. *Rapid Commun. Mass Spectrom.* **2009**, *23*, 2045–2052.
(11) Gerber, S. A.; Rush, J.; Stemman, O.; Kirschner, M. W.; Gygy, S. P. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 6940–6945.
(12) Pan, S.; Zhang, H.; Rush, J.; Eng, J.; Zhang, N.; Patteron, D.; Comb, M. J.; Aebersold, R. *Mol. Cell. Proteomics* **2005**, *4*, 182–190.
(13) Beynon, R. J.; Doherty, M. K.; Pratt, J. M.; Gaskell, S. J. *Nat. Methods* **2005**, *2*, 587–589.
(14) Mallick, P.; Schirle, M.; Chen, S. S.; Flory, M. R.; Lee, H.; Martin, D.; Ranish, J.; Raught, B.; Schmitt, R.; Werner, T.; Kuster, B.; Aebersold, R. *Nat. Biotechnol.* **2007**, *25*, 125–131.
(15) Prange, A.; Pröfrock, D. *J. Anal. At. Spectrom.* **2008**, *23*, 432–459.
(16) Sanz-Medel, A.; Montes-Bayon, M.; De La Campa, M. D. R. F.; Encinar, J. R.; Bettmer, J. *Anal. Bioanal. Chem.* **2008**, *390*, 3–16.
(17) Wind, M.; Wegener, A.; Eisenmenger, A.; Kellner, R.; Lehmann, W. D. *Angew. Chem., Int. Ed.* **2003**, *42*, 3425–3427.
(18) Schaumloffel, D.; Giusti, P. H.; Preud'Homme, H.; Szpunar, J.; Lobinski, R. *Anal. Chem.* **2007**, *79*, 2859–2868.
(19) Wang, M.; Feng, W.; Lu, W.; Li, B.; Wang, B.; Zhu, M.; Wang, Y.; Yuan, H.; Zhao, Y.; Chai, Z. *Anal. Chem.* **2007**, *79*, 9128–9134.
(20) Navaza, A. P.; Encinar, J. R.; Sanz-Medel, A. *J. Anal. At. Spectrom.* **2007**, *22*, 1223–1237.
(21) Navaza, A. P.; Encinar, J. R.; Sanz-Medel, A. *Angew. Chem., Int. Ed.* **2007**, *46*, 569–571.
(22) Giusti, P.; Schaumlöffel, D.; Encinar, J. R.; Szpunar, J. *J. Anal. At. Spectrom.* **2005**, *20*, 1101–1107.
(23) Xu, M.; Yang, L.; Wang, Q. *J. Anal. At. Spectrom.* **2008**, *23*, 1545–1549.
(24) Zhang, C.; Wu, F. B.; Zhang, Y. Y.; Wang, X.; Zhang, X. R. *J. Anal. At. Spectrom.* **2001**, *16*, 1393–1396.
(25) Zhang, C.; Zhang, Z.; Yu, B.; Shi, J.; Zhang, X. *Anal. Chem.* **2002**, *74*, 96–99.
(26) Baranov, V. I.; Quinn, Z.; Bandura, D. R.; Tanner, S. D. *Anal. Chem.* **2002**, *74*, 1629–1636.
(27) Baranov, V. I.; Quinn, Z. A.; Bandura, D. R.; Tanner, S. D. *J. Anal. At. Spectrom.* **2002**, *17*, 1148–52.
(28) Quinn, Z. A.; Baranov, V. I.; Tanner, S. D.; Wrana, J. L. *J. Anal. At. Spectrom.* **2002**, *17*, 892–96.
(29) Ornatsky, O.; Baranov, V.; Bandura, D. R.; Tanner, S. D.; Dick, J. *J. Immunol. Methods* **2006**, *308*, 68–76.
(30) Hu, S. H.; Zhang, S. C.; Hu, Z. C.; Xing, Z.; Zhang, X. R. *Anal. Chem.* **2007**, *79*, 923–929.
(31) Lou, X.; Zhang, G.; Herrera, I.; Kinach, R.; Ornatsky, O.; Baranov, V.; Nitz, M.; Winnik, M. A. *Angew. Chem., Int. Ed.* **2007**, *46*, 6111–6114.
(32) Ornatsky, O. I.; Kinach, R.; Bandura, D. R.; Lou, X.; Tanner, S. D.; Baranov, V. I.; Nitzb, M.; Winnik, M. A. *J. Anal. At. Spectrom.* **2008**, *23*, 463–469.
(33) Takatera, K.; Watanabe, T. *Anal. Sci.* **1992**, *8*, 469–474.
(34) Guo, Y.; Chen, L.; Yang, L.; Wang, Q. *J. Am. Soc. Mass Spectrom.* **2008**, *19*, 1108–1113.
(35) Kutscher, D. J.; Busto, M. E. C.; Zinn, N.; Sanz-Medel, A.; Bettmer, J. *J. Anal. At. Spectrom.* **2008**, *23*, 1359–1364.
(36) Guo, Y.; Xu, M.; Yang, L.; Wang, Q. *J. Anal. At. Spectrom.* **2009**, *24*, 1184–1187.
(37) Kutscher, D. J.; Bettmer, J. *Anal. Chem.* **2009**, *81*, 9172–9177.
(38) Jakubowski, N.; Messerschmidt, J.; Añorbe, M. G.; Waentig, L.; Haye, H.; Roos, P. H. *J. Anal. At. Spectrom.* **2008**, *23*, 1487–1496.
(39) Navaza, A. P.; Encinar, J. R.; Ballesteros, A.; González, J.; Sanz-Medel, A. *Anal. Chem.* **2009**, *81* (13), 5390–5399.
(40) Patel, P.; Jones, P.; Handy, R.; Harrington, C.; Marshall, P.; Evans, E. H. *Anal. Bioanal. Chem.* **2008**, *390*, 61–65.
(41) Rappel, C.; Schaumlöffel, D. *Anal. Chem.* **2009**, *81*, 385–393.
(42) Ahrends, R.; Pieper, S.; Kühn, A.; Weisshoff, H.; Hamester, M.; Lindemann, T.; Scheler, C.; Lehmann, K.; Taubner, K.; Linscheid, M. W. *Mol. Cell. Proteomics* **2007**, *6*, 1907–1916.
(43) Jakubowski, N.; Waentig, L.; Hayen, H.; Venkatachalam, A.; Bohlen, A.; Roos, P. H.; Manza, A. *J. Anal. At. Spectrom.* **2008**, *23*, 1497–1507.
(44) Koellensperger, G.; Groeger, M.; Zinkl, D.; Petzelbauer, P.; Hann, S. *J. Anal. At. Spectrom.* **2009**, *24*, 97–102.

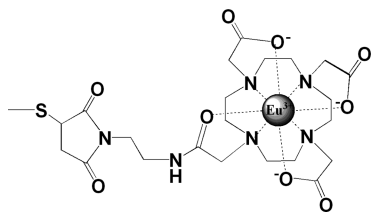**Figure 1.** Structure of MMA−DOTA−Eu labeled−SH.

injection ICPMS.[42] In his PhD thesis (Humboldt University Berlin, 2009), the labeling of transferrin and the proteome of *E. coli* by the same method was also described. In the most recently published paper, Kutscher and Bettmer demonstrated the complete labeling of insulin with *p*-hydroxymercuribenzoic acid.[37] More effort is still needed to develop sophisticated strategies for labeling relatively large intact proteins to achieve directly their absolute quantification using ICPMS.

In this proof-of-concept study, a commercial available bifunctional chelating agent, 1,4,7,10-tetraazacyclododecane-1,4,7-trisacetic acid−10-maleimidoethylacetamide (maleimido−mono−amide−DOTA or MMA−DOTA), was used for protein labeling with Eu (Figure 1). The metal chelating moiety DOTA forms an extremely stable complex with lanthanide ions,[45−47] while the functional maleimide group binds covalently to the sulphydryl (−SH) in the proteins with high specificity and efficiency under mild conditions.[48] The labeling procedure was optimized for pH range, reaction time, and temperature, as well as the ratios of MMA−DOTA to the −SH group. Under optimized conditions, all −SH groups exposed to lysozyme, ribonuclease A, and insulin were completely labeled with MMA−DOTA−Eu after suitable reduction of the proteins. Subsequently, isotope dilution analysis combined with ICPMS was carried out for absolute protein quantification. Eu was selected since it contains only two stable isotopes and high-purity [153]Eu-enriched Eu_2O_3 was available. The sensitivity, accuracy, and precision of this method for intact proteins and tryptic peptides were also evaluated and compared.

EXPERIMENTAL SECTION

Chemicals and Materials. Tris(2-carboxyethyl)phosphine (TCEP, ≥98.0%), insulin (from bovine pancreas, ≥27 units mg^{-1}), ribonuclease A (from bovine pancreas, ≥90%), lysozyme (from chicken egg white, 95%), and tosylphenylalanine chloromethyl ketone (TPCK)-treated trypsin (from porcine pancreas, proteomic grade, ≥10 000 units mg^{-1}) were all purchased from Sigma-Aldrich (St. Louis, MO). MMA−DOTA was purchased from Macrocyclics (Dallas, TX). [153]Eu-enriched Eu_2O_3 (99.8%) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). Concentrated HNO_3 (GR grade) and HPLC grade acetonitrile (ACN) were obtained from Merck KGaA (Darmstadt, Germany). All other chemicals and reagents were at least of analytical grade and were also purchased from Sigma-Aldrich. Ultrapure water (18.2 MΩ. cm) was prepared in a

Milli-Q system (Millipore Filter Co., Bedford, MA) and used throughout this study.

Natural isotopic abundance Eu solution for protein labeling was prepared by dissolving the solid Eu_2O_3 (from Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, purity greater than 99.9999%) in concentrated HNO_3 at room temperature, and then, this was evaporated gradually to dryness on an electric stove; the residue finally dissolved to a concentration of 500 mmol L^{-1} using 2% HNO_3. The [153]Eu isotope enriched Eu stock solution used for species-unspecific isotope dilution analysis was prepared from solid [153]Eu_2O_3 in the same way, and the final concentration of [153]Eu was determined via inverse isotope dilution analysis to be 61.9 nmol L^{-1}.

Protein stock solutions of ribonuclease A and lysozyme were prepared by dissolving an appropriate amount of ribonuclease A or lysozyme in H_2O to obtain a concentration of 100 nmol mL^{-1}. These stock solutions were further diluted in the corresponding buffers to a final concentration of 10 nmol mL^{-1}. Insulin stock solution was prepared in dilute hydrochloric acid at pH 2.5. For further experiments, insulin samples were diluted with different buffers so as to give a concentration of 15 nmol mL^{-1}.

Apparatus. *HPLC/ESI-MS.* Chromatographic separations were carried out on an Agilent 1100 series chromatographic system (Agilent Technologies, Palo Alto, CA) using a Zorbax 300SB C18 column (1.0 I.D. × 150 mm length; particle size, 3.5 *μ*m). The gradient elution program was as follows: The 95% mobile phase A (0.05% TFA in ultrapure water) was maintained for 4 min for desalting, and then mobile phase B (0.05% TFA in ACN) was increased from 20 to 70% in 13 min with a flow rate of 0.05 mL min^{-1}. The HPLC was directly coupled to an Esquire-LC ESI ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). All ESI-MS experiments were performed in the positive mode in the range of 300−2000 *m/z*. The operational parameters were as follows: nebulizer, 75 psi; dry gas, 8 L min^{-1}; dry temperature, 300 °C; capillary voltage, −3500 V; end plate offset, −500 V.

Species-Unspecific Isotope Dilution HPLC/ICPMS. Prior to HPLC/ICPMS, separation of the labeled protein was carried out on an LC-20AD LC system (Shimadzu, Kyoto, Japan) with the same C18 column used in HPLC/ESI-MS. The gradient elution program was as follows: The 95% mobile phase A (0.05% TFA in ultrapure water) was maintained for 5 min, and then mobile phase B (0.05% TFA in ACN) was increased from 20 to 60% in 30 min (or from 5 to 60% in 55 min for tryptic peptides) with a flow rate of 0.05 mL min^{-1}. The effluent from the column was mixed with the enriched [153]Eu spike solution and continuously pumped by a syringe pump (Cole-Parmer, East Bunker Court Vernon Hills, IL) through a four-way connector, while ultrapure water (0.9 mL min^{-1}) was introduced continuously to mitigate the effect of increasing the ACN in the mobile phase on the plasma stability and ionization efficiency. The online isotope ratio of [153]Eu to [151]Eu was monitored using an ELAN DRC II ICPMS (PerkinElmer, SCIEX, Canada) equipped with a concentric pneumatic nebulizer and a cyclonic spray chamber. The ICPMS operational parameters were as follows: nebulizer gas, 0.88 L min^{-1}; auxiliary gas, 1.0 L min^{-1}; plasma gas, 15 L min^{-1}; RF power, 1200 W; dwell time, 100 ms; lens voltage, 7.2 V.

(45) White, D. H.; Delearie, L. A.; Moore, D. A.; Wallace, R. A.; Dunn, T. J.; Cacheris, W. P.; Imura, H.; Choppin, G. R. *Invest. Radiol.* **1991**, *26*, S226–S228.
(46) Bianchi, A.; Calabi, L.; Corana, F.; Fontana, S.; Losi, P.; Maiocchi, A.; Paleari, L.; Valtancoli, B. *Coord. Chem. Rev.* **2000**, *204*, 309–93.
(47) Bunzli, J. C. *Acc. Chem. Res.* **2006**, *39*, 53–61.
(48) Gregory, J. D. *J. Am. Chem. Soc.* **1955**, *77*, 3922–3923.

Parameters such as nebulizer gas flow and lens voltage were optimized daily to obtain the best sensitivity.

Procedures. *Labeling of Proteins.* Before conjugating, the disulfide bonds in lysozyme (10 μL, 10 nmol mL^{-1}) were reduced with TCEP (5× in excess compared to the disulfide bonds) at 37 °C for 30 min in 60 μL of 8 mol L^{-1} urea and 100 mmol L^{-1} NH$_4$Ac (pH 6.8). Subsequently, MMA−DOTA was added to conjugate the nascent free −SH groups directly.

To optimize the mole ratio of MMA−DOTA to −SH groups, different amounts (0.5×, 1×, 5×, 10×, 15×, 20×, 30×, and 50× in excess compared to −SH groups) of MMA−DOTA were added to conjugate free −SH groups in 8 mol L^{-1} urea and 100 mmol L^{-1} NH$_4$Ac (pH 6.8) at 37 °C for 2 h,; while to find the optimal pH range, conjugating reactions were carried out in 8 mol L^{-1} urea and 100 mmol L^{-1} NH$_4$Ac at different pHs (4.5, 5.1, 5.6, 6.1, 6.8, 7.6, 8.1, and 8.6) at 37 °C for 2 h. Under the optimized reaction conditions of pH and mole ratio of MMA−DOTA to −SH groups, reaction temperature and time were further optimized.

In the chelation step, to avoid precipitation of Eu at high pH, the reaction had to be performed at pH ≤ 5.8. Therefore, the protein−MMA−DOTA solution was buffered with 500 mmol L^{-1} NH$_4$Ac (pH 5.8) and a 5-fold molar excess of Eu with respect to DOTA was added to load the Eu for 1 h at 37 °C. Then EDTA solution was added to integrate any free Eu^{3+} which existed. The Eu−EDTA was eluted within the dead volume in each chromatographic run, so as not to interfere with the determination of labeled proteins.

For HPLC/ESI-MS, after the salts were eluted at the dead volume, the effluent was directly injected into the ESI-MS, while for HPLC/ICPMS analysis, the sample was first desalted on a Zorbax 300SB C18 column (1.0 I.D. × 50 mm length; particle size, 3.5 μm) with an Agilent 1100 series chromatographic system, and then, the protein fraction was collected, blow dried with N$_2$, and reconstituted in 500 mmol L^{-1} NH$_4$Ac (pH 5.8) before injection into the HPLC system.

Tryptic Digestion of the Eu-Labeled Proteins. The proteins were first denatured and reduced, and then, the exposed −SH groups were labeled with MMA−DOTA−Eu. The obtained Eu-labeled proteins were subjected to digestion with TPCK-treated trypsin directly at 37 °C overnight. The weight ratio of protein to trypsin was 1:20. Before digestion, the Eu-labeled proteins were desalted and reconstituted in 100 mmol L^{-1} NH$_4$HCO$_3$ (pH 7.8).

RESULTS AND DISCUSSION

Efficiency and Specificity of Protein Labeling. The efficiency and specificity of the labeling procedure were monitored using ESI-MS to evaluate the reaction conditions of pH, mole ratio of MMA−DOTA to protein, time, and temperature. Lysozyme was chosen as the model protein to optimize the labeling conditions.

The reduction condition using TCEP was chosen based on previous studies in our laboratory,[34] and the reduction was approved to be complete and efficient in the experiments which followed. TCEP contains no −SH groups which react with maleimido groups in MMA−DOTA. However, according to the report by Getz et al., TCEP decreases the labeling yield of −SH
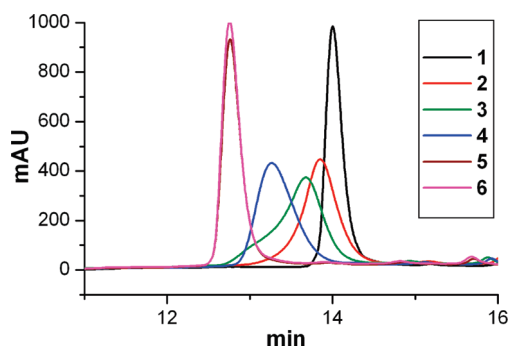

Figure 2. HPLC/UV (214 nm) chromatogram showing the optimization of n (MMA−DOTA): n (−SH) for lysozyme labeling in 8 mol L^{-1} urea, 100 mmol L^{-1} NH$_4$Ac (pH = 6.8) at 37 °C for 2 h. Peaks 1, 2, 3, 4, 5, and 6 refer to a 0-, 0.5-, 1-, 5-, 10-, and 50-fold excess of MMA−DOTA in relation to −SH groups.

groups with maleimide.[49] In this study, the possible decrease of labeling efficiency of MMA−DOTA toward the sulfhydryls in the proteins studied caused by TCEP was overcome by the use of excessive amounts of MMA−DOTA. Urea (8 mol L^{-1}) was used to denature the proteins to make the reduction and labeling efficient and complete. Figure 2 shows the HPLC/UV chromatogram when different amounts of MMA−DOTA were used to conjugate the lysozyme. Since there are eight cysteine −SH groups in the reduced form of lysozyme, when conjugation of the MMA−DOTA to the −SH groups was not complete under the conditions of 1−5-fold excess of MMA−DOTA, then mixed MMA−DOTA−conjugated lysozyme adducts coexisted in the solution, leading to broad absorption peaks as shown in Figure 2, peaks 2−4, and further confirmed by ESI-MS (Figure 3b). Along with the increase in the amount of MMD−DOTA added, the retention time of the MMA−DOTA−conjugated lysozyme peak decreased because more hydrophilic MMA−DOTA was conjugated into lysozyme. Once a 10-fold or 50-fold molar excess of MMA−DOTA (4-fold or 20-fold excess with respect to the amount of TCEP used) was added, the chromatogram peaks (Figure 2, peak 5 and 6) were as sharp and symmetrical as those of the unconjugated lysozyme (Figure 2, peak 1), which corresponded to a fully MMA−DOTA−conjugated lysozyme (MMA−DOTA−lysozyme = 8:1) as confirmed by ESI-MS (Figure 3c). The mass spectra were clean, and the deconvolution molecular weight (MW) was 18520.2, corresponding to the MW of 8 MMA−DOTA−labeled lysozyme (526.54 × 8 + 14303.9 = 18516.2). The mass difference of 4.0 between the measured and theoretical MW was due to the low mass accuracy of the ion trap mass spectrometer employed.

To optimize the pH range, conjugating reactions were investigated at different pH values. The result at pH 6.1 is shown in Figure 3d. The mass spectrum was complex, and 7 (MMA−DOTA)−conjugated lysozyme and 8 (MMA−DOTA)−conjugated lysozyme are seen in Figure 3e (an enlarged spectrum of Figure 3d from 900 to 1050 *m/z*). In contrast, when reactions took place under a pH ranging from 6.8 to 7.6, the mass spectra were as clean as those in Figure 3c, indicating that lysozyme was completely conjugated by the 8 (MMA−DOTA).

Reaction temperature and time were further optimized using ESI-MS (data not shown), suggesting that 47 °C and 40 min were

(49) Getz, E. B.; Xiao, M.; Chakrabarty, T.; Cooke, R.; Selvin, P. R. *Anal. Biochem.* **1999**, *273*, 73–80.
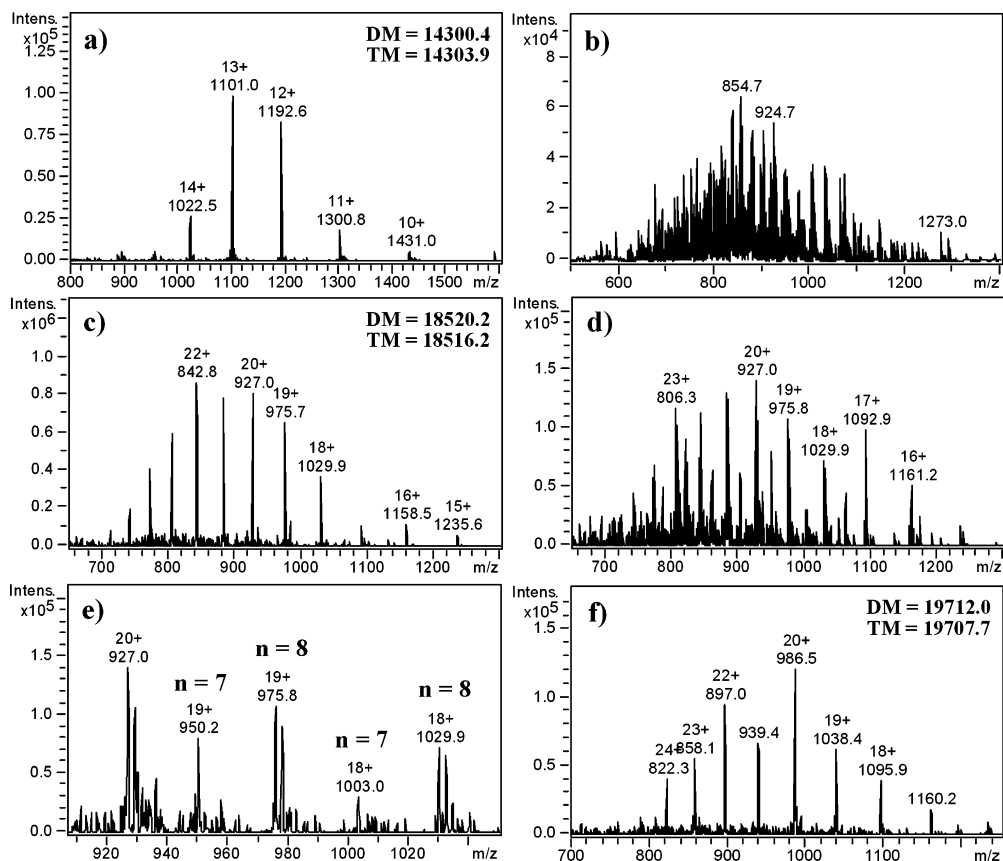
Figure 3. ESI-MS spectra of (a) lysozyme, (b) mixed labeled lysozyme, and (c) completely labeled lysozyme under 0-, 5- and 10-fold excess of MMA−DOTA to −SH groups in 8 mol L^{-1} urea, 100 mmol L^{-1} NH$_4$Ac (pH = 6.8) at 37 °C for 2 h, and (d) the mixed 7 (MMA−DOTA)-labeled and 8 (MMA−DOTA)-labeled lysozyme at pH 6.1 under 10-fold of MMA−DOTA to −SH groups. (e) Shows an enlarged ESI-MS spectrum from 900 to 1050 *m/z* of (d) where *n* represents the number of MMA−DOTA groups conjugated per lysozyme molecule. (f) ESI-MS spectrum of MMA−DOTA−Eu-labeled lysozyme. DM and TM in the MS spectra denote deconvolution and theoretical molecular weights, respectively.

optimal. The overall optimized MMA−DOTA−conjugating conditions were, thus, as follows: 47 °C, pH 6.8−7.6, 40 min, and 10-fold or higher MMA−DOTA to the total −SH groups exposed. Finally, Eu was loaded through chelation by DOTA at pH 5.8 to avoid possible hydrolytic precipitation of Eu under high pH. When a 5-fold molar excess of Eu with respect to DOTA was present in the MMA−DOTA−conjugated lysozyme, the lysozyme−MMA−DOTA−Eu complex was confirmed using HPLC/ESI-MS to have a deconvolution of MW 19712.0 (Figure 3f), while mass signals from unlabeled lysozyme−MMA−DOTA were not observed. Due to the extreme stability of the DOTA−Eu complex (log K = 23.5),[42] no loss of Eu was observed during HPLC separation, when 0.05% TFA was added to the ACN/H$_2$O mobile phase.

Under these optimized reaction conditions, ribonuclease A and insulin were also labeled with MMA−DOTA−Eu. Since insulin consists of two chains (A and B) with four cysteine residues in the A chain and two cysteine residues in the B chain, when insulin was reduced and labeled, two polypeptides appeared because disulfide bonds between the two chains were broken. As shown in Figure 4, and as expected, the measured MW matched the corresponding theoretical MW well. It should be noted that all the reactions were carried out within one centrifuge tube, which would greatly reduce protein losses during sample preparation.

Tryptic Digestion of MMA−DOTA−Eu-labeled Proteins and Quantification of Tryptic Peptides Using HPLC Coupled with Species-Unspecific Isotope Dilution ICPMS. Proteolytic peptides and intact proteins are two main targets for protein quantification. However, for molecular mass spectrometry-based quantitative proteomics, proteins are identified and quantified mostly from their proteolytic peptides, which is called "bottom-up" proteomics. The reason for this situation is that high-quality protein identification information can be obtained much easier from MS/MS data of proteolytic peptides than from MS/MS data of intact proteins, and signals of "light" and "heavy" stable isotope tag labeled peptides can be transferred into relative quantitative information directly. Therefore, proteolytic digestion is demanded for most proteomic research using molecular mass spectrometry. On the other hand, for absolute quantification of proteins using ICPMS, the absolute amount of the labeled proteins is obtained by translation of the amount of the element naturally incorporated or labeled to the proteins, and so, it is crucial to find out the stoichiometry of the protein−tag conjugate. In other words, we should first know how many binding sites the target proteins have. Therefore, protein digestion and identification by molecular mass spectrometry are essential for ICPMS-based absolute protein quantification, and the relationship between ICPMS and molecular mass spectrometry in life sciences should be recognized as
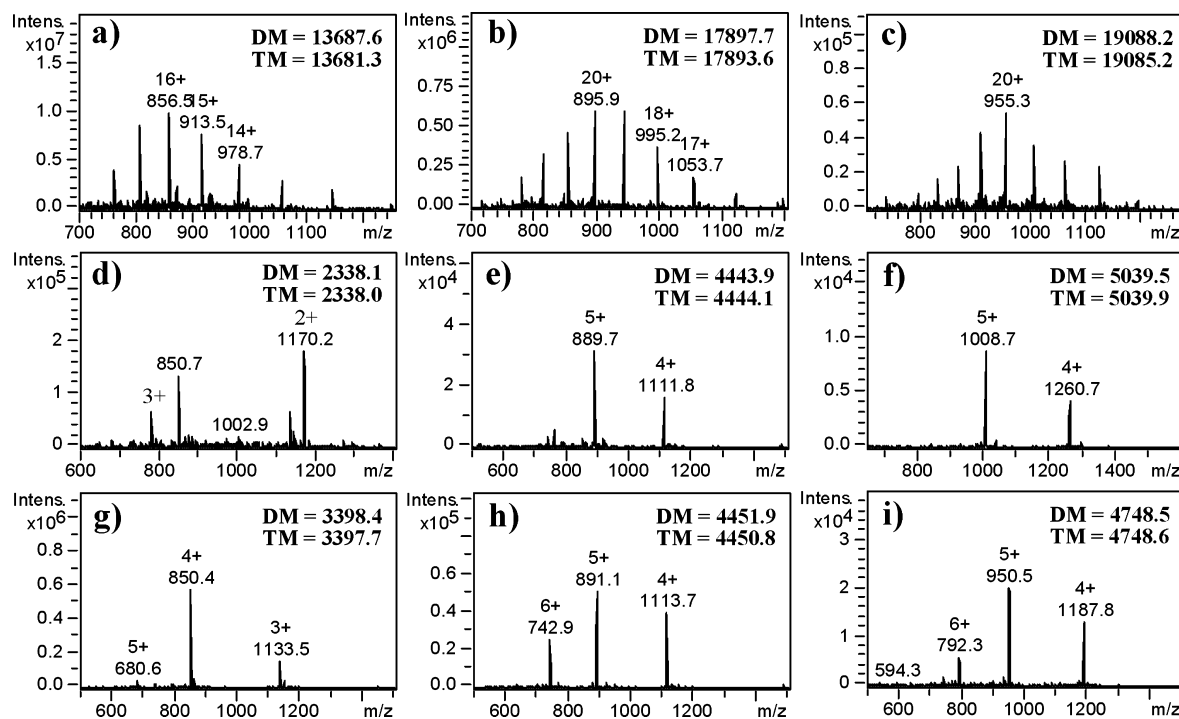
Figure 4. ESI-MS spectra of (a) intact ribonuclease A, (b) MMA−DOTA−labeled ribonuclease A, (c) MMA−DOTA−Eu-labeled ribonuclease A, (d) insulin A chain, (e) MMA−DOTA-labeled A chain, (f) MMA−DOTA−Eu-labeled A chain, (g) insulin B chain, (h) MMA−DOTA-labeled B chain, and (i) MMA−DOTA−Eu-labeled B chain. DM and TM in the MS spectra denote deconvolution and theoretical molecular weights, respectively.

"synergy".[50] Thus, in the following experiments, we investigated the tryptic behavior of MMA−DOTA−Eu-labeled proteins using both ICPMS and ESI-MS, and we tried to quantify these proteins from the corresponding tryptic peptides.

Species-unspecific isotope dilution analysis was performed by continuously mixing the [153]Eu-enriched spike solution with the HPLC effluent, and both the [151]Eu and [153]Eu isotopes were monitored using ICPMS. The online measured [151]Eu/[153]Eu isotope ratio chromatogram was transformed into a Eu mass flow chromatogram as described in ref 41. The three MMA−DOTA−Eu-labeled proteins insulin, lysozyme, and ribonuclease A were digested with TPCK-treated trypsin which cleaves proteins at the C-terminal sites of lysine and arginine residues. For insulin, there was no cleavage site in the A chain, and so, it remained in entirety after tryptic digestion. The B chain possessed two cleavage sites; therefore, three peptides resulted as shown in Figure 5a: One peptide had 22 amino acids (FVNQHLCGSHLVEALYLVCGER) (Figure 5a, peak 3) which was confirmed by ESI-MS (Figure 5d), one had 7 amino acids (GFFYTPK) (Figure 5a, peak 2, and Figure 5c), and the third was a single alanine (not preserved on the column). Of these four tryptic peptides (including the insulin A chain), only FVNQHL-CGSHLVEALYLVCGER and the A chain contained cysteine residues, which could be labeled with MMA−DOTA−Eu, and so, it was detected by HPLC coupled with species-unspecific isotope dilution ICPMS as shown in Figure 5b. The contents of insulin calculated from the A chain and FVNQHLCGSHLVEALYLVCGER determined were 13.6 ± 0.8 and 9.3 ± 1.2 nmol mL^{-1}, respectively, suggesting that the recoveries of insulin are, respectively, 90.7%

and 62% because 9.3% of the A chain and 38.0% of the B chain were lost during the process of tryptic digestion or chromatographic separation. A similar phenomenon was observed in a previous study,[41] indicating that even for insulin, which was considered to be the smallest protein, quantification from its tryptic peptides was still difficult. Therefore, if one wants to quantify insulin from its tryptic peptides, calibration factors of 1.1 and 1.6 should be introduced for the A chain and B chain, respectively.

For lysozyme, six tryptic peptides which contained cysteine residues were theoretically expected to form. Two of them (CK and GCR) were too hydrophilic to be preserved on the column under the HPLC conditions, and so, the remaining four peptides would appear in the HPLC/ICPMS chromatogram. As shown in Figure 6a, there were four main peaks in the HPLC/ICPMS chromatogram as expected. However, the peak area ratio of L.1 to L.2 to L.3 was calculated as 1:4.7:1.6, which did not match the theoretical ratio of 1:1:1 according to the number of cysteine residue(s) in the corresponding peptides. A similar result was also obtained for ribonuclease A (Figure 6b). More than six theoretically expected peaks were observed in the HPLC/ICPMS chromatogram, but baseline separation could not be achieved for some peptides as the peak areas could not be integrated accurately. Thus, the peak area ratio obtained was far beyond the theoretical one. These peptides might possibly be baseline separated after optimizing the chromatographic conditions or using other separation methods, but the requirements not only for more resolving power of the separation techniques for tryptic peptides than that for intact proteins but also for higher tryptic efficiency and specificity would not change. Therefore, satisfactory quantitative

(50) Becker, J. S.; Jakubowski, N. *Chem. Soc. Rev.* **2009**, *38*, 1969−1983.
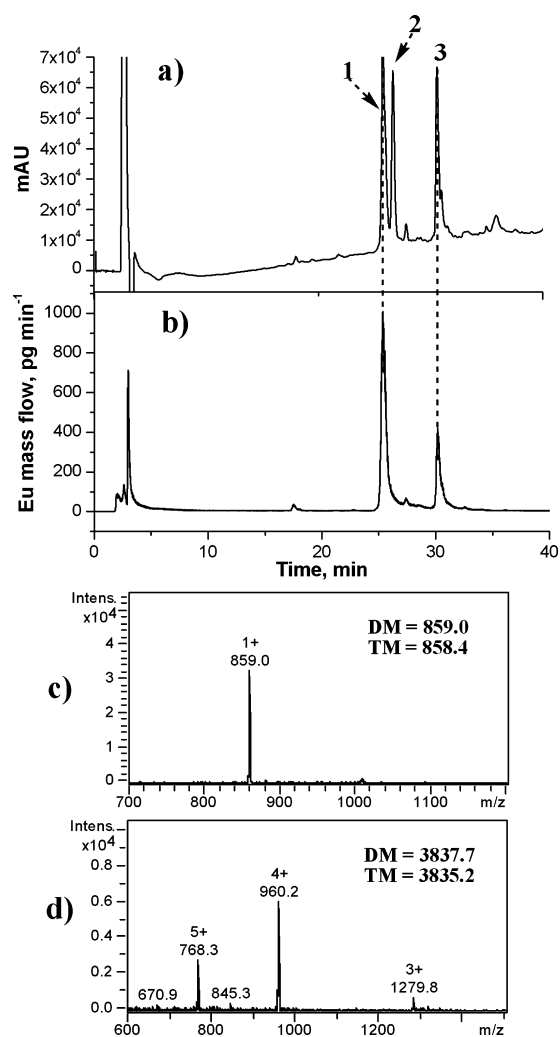
Figure 5. (a) HPLC/UV (214 nm) chromatogram of tryptic peptides of insulin, where peaks 1, 2, and 3 correspond to the MMA−DOTA−Eu-labeled A chain, GFFYTPK, and MMA−DOTA−Eu labeled FVNQHL-CGSHLVEALYLVCGER, respectively. (b) HPLC/ICPMS chromatogram for MMA−DOTA−Eu-labeled tryptic peptides of insulin. (c) ESI-MS spectrum of peak 2. (d) ESI-MS spectrum of peak 3. DM and TM in the MS spectra denote deconvolution and theoretical molecular weights, respectively.

results could not be obtained for lysozyme or ribonuclease A from their tryptic peptides under optimized conditions.

Compared with ICPMS, ESI-MS provides another dimension of separation for peptides and, thus, more tryptic peptides were expected to be detected. However, due to the different signal intensities of the different peptides, some low signal response peptides were missed in ESI-MS and only three of four and three of six expected MMA−DOTA−Eu-labeled peptides were detected from lysozyme and ribonuclease A, respectively (Table 1). A missed cleavage peptide (L.7 ^{66}CKPVNTFVHESLADVQAVC-SQK^{87}) was also detected which provided ESI-MS evidence showing the difficulty of complete digestion of ribonuclease A. All these results indicated that proteolytic digestion and chromatographic separation were the bottlenecks of ICPMS-based protein quantification via proteolytic peptides. If one wants to quantify proteins from their proteolytic peptides, complete proteolytic digestion and baseline separation of all the proteolytic peptides must first be fulfilled. From this point
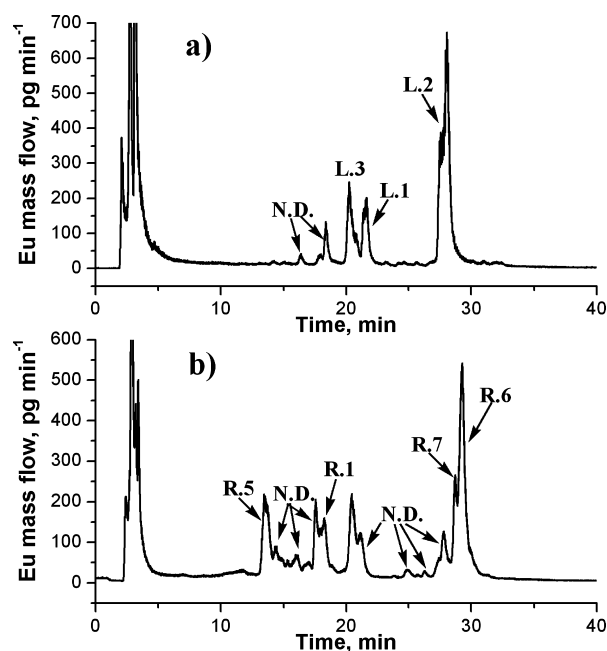

Figure 6. HPLC/ICPMS chromatograms of (a) tryptic digested lysozyme, where peaks L.1, L.2, and L.3 correspond to the tryptic peptides of lysozyme listed in Table 1 and (b) ribonuclease A, where peaks R.1, R.5, R.6, and R.7 correspond to the tryptic peptides of ribonuclease A listed in Table 1. N.D. represents peptides not detected by ESI-MS.

Table 1. Theoretical and Measured Molecular Weights (MWs) of MMA−DOTA−Eu-labeled Tryptic Peptides of Lysozyme (L) and Ribonuclease A (R) Using ESI-MS

MMA−DOTA−Eu-labeled tryptic peptide	theoretical MW	measured MW
L.1 ^{24}CELAAAMK^{31}	1511.9	1513.0
L.2 ^{40}GYSLGNWVCAAK^{51} + 1 Na	1964.1	1963.0
L.3 ^{80}WWCNDGR^{86} + 1 Na	1631.9	1630.6
L.4 ^{92}NLCNIPCSALLSSDITASVNCAK^{114}	4359.6	nd^b
R.1 ^{37}QHMDSSTSAASSSNYCNQMMK^{57}	2981.4	2983.4
R.2 ^{68}PVNTFVHESLADVQAVCSQK^{87}	2845.6	nd
R.3 ^{87}NVACK^{92}	1207.8	nd
R.4 ^{93}NGQTNCYQSYSTMSITDCR^{111}	3519.9	nd
R.5 ^{118}YPNCAYK^{124} + 1 Na	1553.9	1552.8
R.6 ^{131}HIIVACEGNPYVPVHFDASV^{150} + 1 Na	2862.6	2861.1
R.7 ^{66}C^{a}KPVNTFVHESLADVQAVCSQK^{87} + 1 Na	3776.2	3771.8

^{a} One missed cleavage site at lysine residue. ^{b} nd denotes not detected.

of view, intact proteins may be more feasible and practical targets for ICPMS-based protein quantification.

Absolute Quantification of Intact Proteins Using HPLC Coupled with Species-Unspecific Isotope Dilution ICPMS. Due to the poor protein quantification results obtained from tryptic peptides, intact proteins were quantified directly without tryptic digestion. Since the proteins were all completely labeled with MMA−DOTA−Eu (as discussed before), quantification results from intact proteins should be more precise and accurate. To support this expectation, MMA−DOTA−Eu-labeled lysozyme was quantified first to validate the proposed strategy using HPLC coupled with species-unspecific isotope dilution ICPMS. The average recovery was found to be 97.9% in six independent experiments, and the precision was evaluated to be 5.8% at the 10 pmol level, demonstrating that high quality absolute protein quantification was achieved for intact

Table 2. Overview of Different Element Tags Used for Peptide/Protein Labeling and Quantification by ICPMS

year	sample type	labeling tag	separation technique	detector	detection limit (fmol)	ref
2009	insulin	pHMB	RP-uHPLC	ICPMS with a DS-5 nebulizer, MALDI-TOFMS, ESI-MS	subfmol	37
2009	bovine pancreatic ribonuclease A, lysozyme and, insulin	CH_3Hg^+	RP-HPLC	ICPMS with a direct injection high efficiency nebulizer, ESI-ITMS	400, for insulin	36
2008	ovalbumin	pHMB	RP-capHPLC	ICPMS with a DS-5 nebulizer, MALDI/ESI-TOF-MS	1	35
2009	tyrosine, three peptides from reference material (NIST 8327), tryptic digests of β-casein	iodine	RP-capHPLC	ICPMS and ESI-QTOFMS	0.48	39
2008	bovine serum albumin, lysozyme, pepsin	iodine	1 D SDS-PAGE and membrane blotting	ICPMS and laser ablation, nanoESI-FTICR-MS	150	38
2009	kemptide, adrenocorticotropic hormone fragment 1−10, neuropeptide Y fragment 1−24, and Met-Leu-Phetripeptide	Lu-DTPA	RP-nanoHPLC	ICPMS with postcolumn IDA, ESI-QTOFMS	0.179	41
2007	bradykinin, substance P	Eu-DTPA	RP-HPLC	ICPMS and ESI-MS	1.0×10^7	40
2008	$B\beta_{15-42}$	In-DOTA	RP-HPLC	ICPMS with a PFA-nebulizer, ESI-TOFMS	1.7	44
2008	bovine serum albumin and lysozyme	lanthanides-DOTA	1 D SDS-PAGE and membrane blotting	ICPMS and laser ablation, nanoESI-FTICR-MS	15	43
2007	HIV integrase inhibitor peptide, bovine serum albumin, α-lactalbumin, and Sus scrofa eye lens proteins	lanthanides-DOTA	1 and 2 D SDS-PAGE	flow injection ICPMS, MALDI/ESI-TOF-MS	0.11, for BSA	42
this study	insulin, lysozyme, and ribonuclease A	Eu-DOTA	RP-HPLC	species-unspecific isotope dilution ICPMS with a concentric pneumatic nebulizer, ESI-ITMS	0.819, for lysozyme	

lysozyme via HPLC coupled with species-unspecific isotope dilution ICPMS. On the basis of the detection limit (DL) for Eu, 6.55 fmol based on the signal-to-noise ratio (3σ criterion) in the mass flow chromatogram, the corresponding DLs were estimated to be 0.819, 1.638, and 0.819 fmol for lysozyme (eight −SH groups), the A chain of insulin (four −SH groups), and ribonuclease A (eight −SH groups), respectively. The DLs obtained in this study were comparable to those previously reported (Table 2). Because of the low sample introduction efficiency of the concentric pneumatic nebulizer used in this study and the 18-fold dilution of the HPLC effluents due to the makeup of ultrapure water flow (0.9 mL min^{-1}), a lower DL is expected to be achieved via a total consumption nebulizer in the near future.

Ribonuclease A, lysozyme, and insulin in a protein sample were labeled, baseline separated, and absolutely quantified with this method. As shown in Figure 7, the ^{151}Eu and ^{153}Eu isotope chromatogram was transformed into a Eu mass flow chromatogram, and the concentrations of ribonuclease A, lysozyme, and the A and B chains of insulin were calculated to be 10.4, 9.8, 14.7, and 15.2 nmol mL^{-1}, respectively, which matched the theoretical contents of 10, 10, 15, and 15 nmol mL^{-1} well.

CONCLUSIONS

In this proof-of-concept study, a complete, specific, and efficient method was developed to label intact proteins with MMA−DOTA−Eu. Subsequently, three MMA−DOTA−Eu-labeled proteins were tryptic digested and quantified from the corresponding tryptic peptides via HPLC coupled with species-unspecific isotope dilution ICPMS. Quantitative results from the tryptic peptides indicated that

more accurate tryptic digestion and efficient chromatographic separation were required. Furthermore, intact proteins were quantified directly without tryptic digestion, and the results demonstrated this strategy was much more precise, accurate, and sensitive. On the basis of all these results, we concluded that intact protein might provide a more feasible and practical target for ICPMS-based protein quanti-
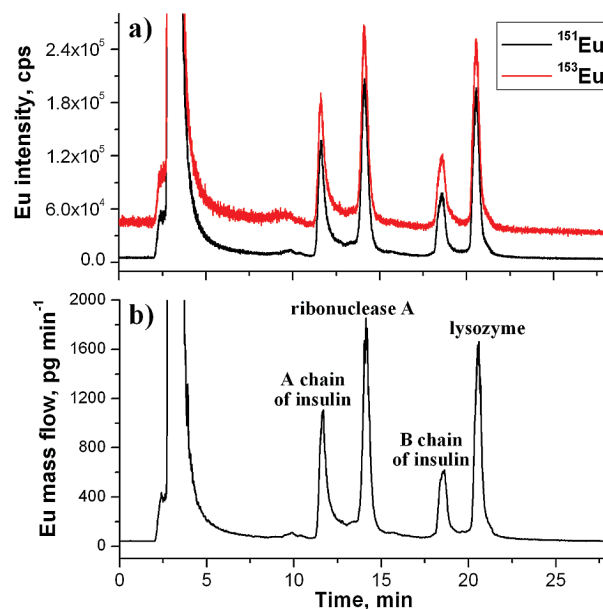

Figure 7. (a) ^{151}Eu and ^{153}Eu isotope chromatograms of the A chain of insulin, ribonuclease A, B chain of insulin, and lysozyme (from left to right). (b) Eu mass flow chromatogram translated from (a).

fication, owing to the easier quantitative chromatographic separation and not requiring complete and exact proteolytic digestion. Although ICPMS-based protein quantification with the element labeling affinity tag approach is still in its nascent period, potential quantitative capability has been proven in a few recently published papers (Table 2). More sophisticated labeling strategies and multidimensional separation techniques are urgently needed to extend the application of species-unspecific isotope dilution ICPMS.