Protein Quantification

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Europium-Labeled Activity-Based Probe through Click Chemistry: Absolute Serine Protease Quantification Using ^{153}Eu Isotope Dilution ICP/MS**

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The development of molecular mass spectrometry (MS), including various types of molecular MS with electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), as well as isotope-labeling strategies have greatly advanced relative protein quantification recently.[1] Among the limited number of methods reported using the molecular MS for absolute protein quantification, the results are primarily achieved using multiple reaction monitoring (MRM) of unique peptides representing target proteins on a triple-quadrupole molecular MS.[2] The MRM strategy has proven to be highly reproducible and accurate, but it calls for proper and unique peptide standards to generate high-quality MRM analysis. In some cases, the unique peptides from the target proteins have low ionization efficiencies and are difficult to analyze using the molecular MS. It is especially difficult to synthesize hundreds and thousands of peptide standards based on a whole proteome, but the standards are necessary, because different peptides have varying ionization efficiencies in the molecular MS. To overcome the intrinsic limitations of the molecular MS, the feasibility of using elemental mass spectrometry [especially inductively coupled plasma mass spectrometry (ICP/MS)] was recently explored for absolute protein quantification through the determination of either the naturally occurring heteroelements (S,[3] P,[4] Se[5]) of proteins and metals in the metalloproteins,[6] or the exogenous elements (I,[7] Hg,[8] Ln[9]) attached to the protein side chains. Besides extremely high sensitivity and selectivity as well as a broad dynamic range of up to nine orders of magnitude, a signal that is independent of chemical species (peptides and/or proteins) is another unique advantage of ICP/MS over the molecular MS when considering the absolute protein quantification. On the basis of this advantage, proteins can be quantified absolutely by coupling high-performance liquid chromatography (HPLC) with species-unspecific isotope dilution ICP/MS without the use of individual peptide or protein standards,[10] which are required in the molecular MS. It should, however be pointed out that a prerequisite for the success of the absolute protein quantification is the peak purity during HPLC. In other words, all the peptides and proteins in a complex proteome sample should be baseline separated. Actually, this is nearly impossible using the currently available separation techniques when the absolute protein quantification is applied to a real-world proteome sample. In contrast, recent targeted proteomics for obtaining the information for targeted proteins, rather all the proteins in a proteome, has developed rapidly.[2] Based on this concept of targeted proteomics, we considered using a biospecific labeling strategy, such as activity-based protein profiling (ABPP),[11] for a class of functional proteins to solve the problems mentioned above. In contrast to the immuno affinity assay, whose specificity comes from the noncovalent interaction between the antigen and antibody,[12] ABPP is based on the design and synthesis of an activity-based probe, which consists of two functional groups: 1) a group on an irreversible enzyme inhibitor that covalently binds to the active site of an active enzyme, and 2) a reporter group to readout the enzyme activity. By specifically labeling a class of active enzymes in a complex proteome sample, ABPP can decrease the sample complexity and reduce the separation requirements of current techniques, especially when using ICP/MS because only the labeled target enzymes are determined.

To the best of our knowledge, we report herein the first absolute protein quantification method using species-unspecific isotope dilution HPLC/ICP/MS together with an activity-based labeling strategy (see Figure S1 in the Supporting Information), which is expected to boost the development of the absolute protein quantification in the near future. As one of the largest and most widely distributed enzyme families in human cells, serine hydrolase comprises about 1% of the human proteome including about 176 serine proteases (SPs), which hydrolyze proteins.[13] SPs were used as examples to demonstrate the feasibility of this strategy.

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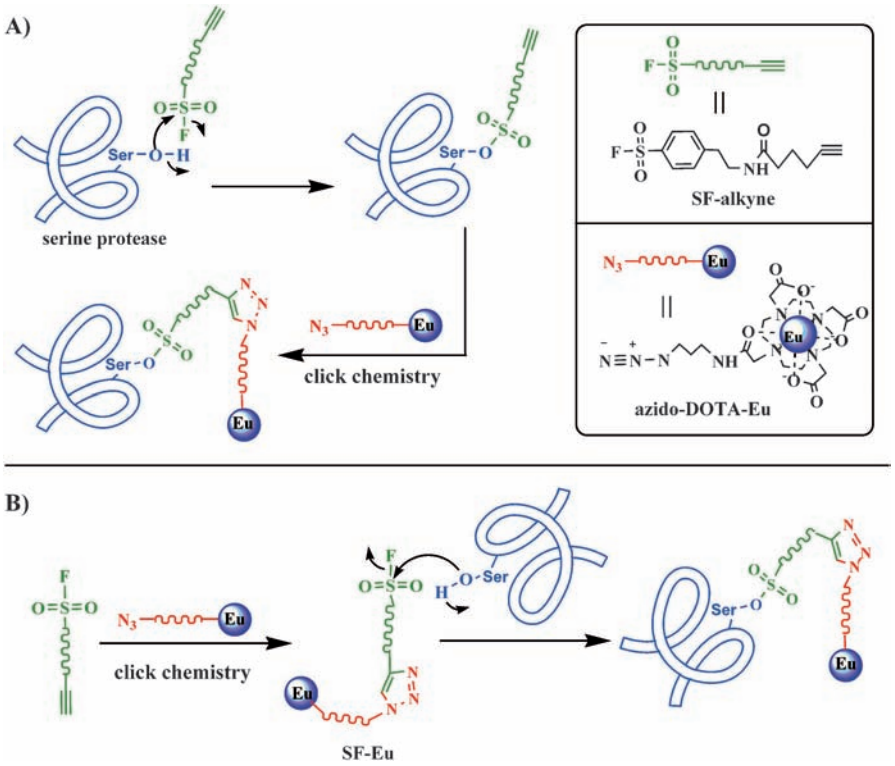
To design and synthesize an activity-based SP probe containing a lanthanide (which is a very sensitive element in ICP/MS and has a low background noise in biological systems), we chose a widely used irreversible SP inhibitor, 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF).[14] The sulfonyl fluoride (SF) group in AEBSF can bind covalently and specifically to the hydroxy group of the serine residue in the active site of SPs, thus resulting in a sulfonyl–SP derivative which is stable for long periods of time.[15] On the other side of the AEBSF, the amine group is conjugated with 6-heptynoic acid to produce an SF–alkyne unit (see Figure S2 in the Supporting Information), which can then undergo click chemistry with a europium-loaded azido-monoamide-DOTA (azido-DOTA-Eu; Scheme 1). The click chemistry is an [3+2] cycloaddition reaction between a terminal alkyne and azide using Cu^{I} as a catalyst.[16] In this proof-of-concept study, we tried to attach azido-DOTA-Eu to SPs in two different ways: 1) SPs were first conjugated with SF–alkyne and subsequently labeled with azido-DOTA-Eu through a click reaction (Scheme 1 A); 2) The azido-DOTA-Eu unit was conjugated to SF–alkyne to give SF–alkyne/azido-DOTA-Eu (SF-Eu), which was then used to label the SPs (Scheme 1 B). In both cases, the SF–alkyne unit needs to be synthesized first. To minimize the hydrolysis of the SF group in alkaline aqueous solution, the amidation reaction between ASBSF and 6-heptynoic acid was carried out in DMF, and a carboxyl activating agent, O-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU), and an organic base, *N*,*N*-diisopropylethylamine (DIPEA), were used to complete the reaction. The SF–alkyne unit was synthesized and purified using HPLC, and the structure was confirmed using ESI/MS (see Figure S3 in the Supporting Information), and ^{1}H and ^{13}C NMR spectroscopy (Figures S4 and S5).

To investigate the efficiency of labeling SPs with the purified SF–alkyne, we used chymotrypsin and elastase as model SPs. After incubating 1 mM SF–alkyne with freshly prepared chymotrypsin and elastase in 100 mM Tris-HCl (pH 7.5), the model SPs were completely labeled with one SF–alkyne unit within 1 hour (even though the numbers of the serine residues of chymotrypsin and elastase were 26 and 22, respectively), as confirmed by using ESI/MS (Figure 1). The deconvolution molecular weights (DMs) of intact chymotrypsin and elastase were 25443 Da and 25896 Da, respectively; and the DMs of these two SPs increased to 25736 Da and 26186 Da, respectively after they were labeled with one SF–alkyne unit (MW = 311). Thus, the DMs match the theoretical molecular weights (TMs) of 25734 Da and 26187 Da, respectively. Differences of 2 and 1 Da between the DM and TM values are due to the relatively low-mass resolution of the ion trap MS employed. These results demonstrated that SF–alkyne was quite efficient and specific for labeling the hydroxy group of the serine residue in the active site of the SPs, and is consistent with other studies on the labeling of chymotrypsin[17] and elastase[18] with SF derivatives.



Scheme 1. Schematic illustration of the strategies for labeling serine protease using a Eu-labeled activity-based probe. A) First, the hydroxy group of the serine residue in the active site of serine protease was labeled with SF-alkyne, and subsequently labeled with azido-DOTA-Eu by click chemistry. B) SF-alkyne-azido-DOTA-Eu probe (SF-Eu) was synthesized first by conjugating SF-alkyne and azido-DOTA-Eu by click chemistry, and then used to label SP directly.

In the next step, we optimized the click reaction between azido-DOTA-Eu and SF–alkyne (see Figure S6a in the Supporting Information), with respect to the reducing reagents, tris(benzyltriazolylmethyl) amine (TBTA; Cu^{I}-stabilizing ligand),[19] the reaction time, and the kinds of buffer used. We found that sodium ascorbate was a much more efficient reducing reagent than tris(2-carboxyethyl)phosphine in our reaction system (see Figures S6b,c). When three times the amount of azido-DOTA-Eu was reacted with SF–alkyne in a mixture containing 5 mM sodium ascorbate, 1 mM $CuSO_4$, 0.1 mM TBTA, 100 mM Tris-HCl (pH 7.5), and 20% *tert*-butyl alcohol for 1 hour, more than 99% of the SF–alkyne was labeled with azido-DOTA-Eu to produce SF-Eu (see Figures S6c,d). The object of using 20% *tert*-butyl alcohol was to prevent precipitation of TBTA in pure aqueous solution. When we attempted to conjugate the SPs to SF–alkyne to obtain alkyne-SF-SP, which was then reacted with azido-DOTA-Eu through the click chemistry (Scheme 1 A), the SPs unfortunately

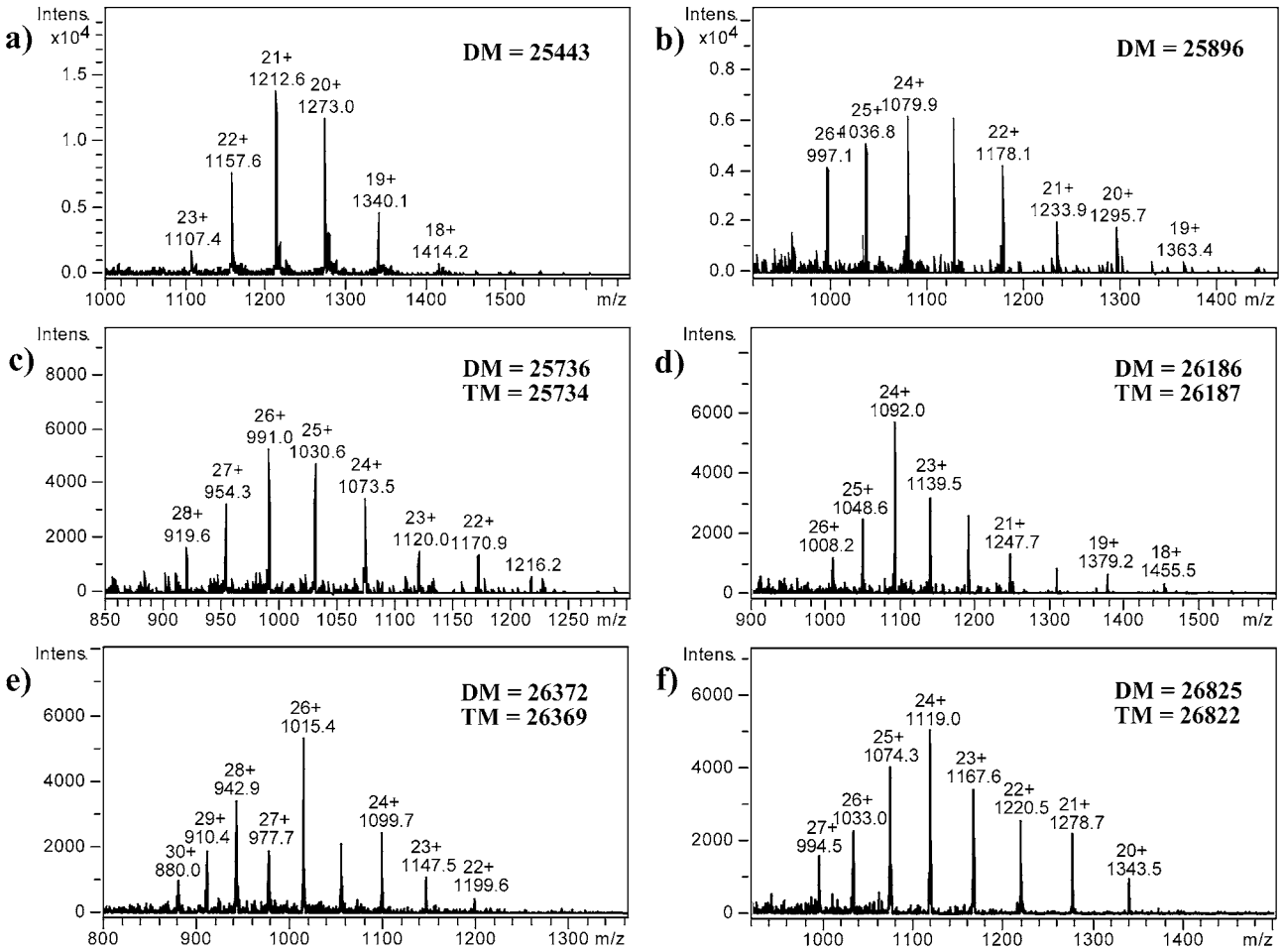


Figure 1. ESI/IT/MS spectra of a) intact chymotrypsin, b) intact elastase, c) SF-alkyne-labeled chymotrypsin, d) SF-alkyne-labeled elastase, e) SF-Eu-labeled chymotrypsin, and f) SF-Eu-labeled elastase. DM and TM in the MS spectra denote deconvolution and theoretical molecular weights, respectively.

precipitated out. The speculated reason for this phenomenon was the use of TBTA, according to a previous report.[20] However, TBTA is a very important reagent for stabilizing Cu^{I}, and plays a catalytic role in the click reaction.[19] No SF-Eu was found after 1 hour in the case where neither Cu^{I} nor TBTA were used. We therefore changed our strategy (Scheme 1 B) such that the SF-Eu probe was synthesized first and then used to label the SPs directly. The purified SF-Eu was used to label chymotrypsin and elastase in 100 mM Tris-HCl (pH 7.5), and the ESI/MS spectra showed that both of the SPs were completely labeled with one SF-Eu unit (MW = 946) within 1 hour and without any precipitate (Figures 1 e,f). Although SF-Eu is larger than SF–alkyne by one DOTA-Eu group, it could still label the model SPs efficiently. The linker between the SF and DOTA-Eu groups could place the DOTA-Eu group far enough away from SF to avoid a steric effects.

A sample containing three peptides (vasopressin, oxytocin, and somatostatin) and five proteins [ribonuclease A (RNase A), cytochrome C (Cyt C), lysozyme, bovine serum albumin (BSA), and carbonic anhydrase], together with the two SPs was chosen to investigate the specificity of SF-Eu to SPs (Table 1). The results obtained (Figure 2) suggested that only chymotrypsin and elastase reacted with SF-Eu under the labeling conditions. As shown in Figure 2 a, SF-Eu and SF-Eu-labeled peptides or proteins in the sample were baseline separated on a C18 column using HPLC with the optimized gradient elution program and UV (214 nm) detection (see the Supporting Information). The effluent from the HPLC was subsequently mixed with the enriched ^{153}Eu standard ^{153}Eu-

Table 1: Ten model peptides and proteins used.

No.	Name[a]	Number of serine residues	Molecular weight [kDa]
1	vasopressin	0	1.0
2	oxytocin	0	1.1
3	RNase A	15	13.7
4	somatostatin	1	1.6
5	Cyt C	0	12.3
6	lysozyme	10	14.3
7	BSA	28	66.4
8	chymotrypsin	26	25.4
9	elastase	22	25.9
10	carbonic anhydrase	30	29.0

[a] The SWISS-PROT numbers for the proteins are given in the Supporting Information.

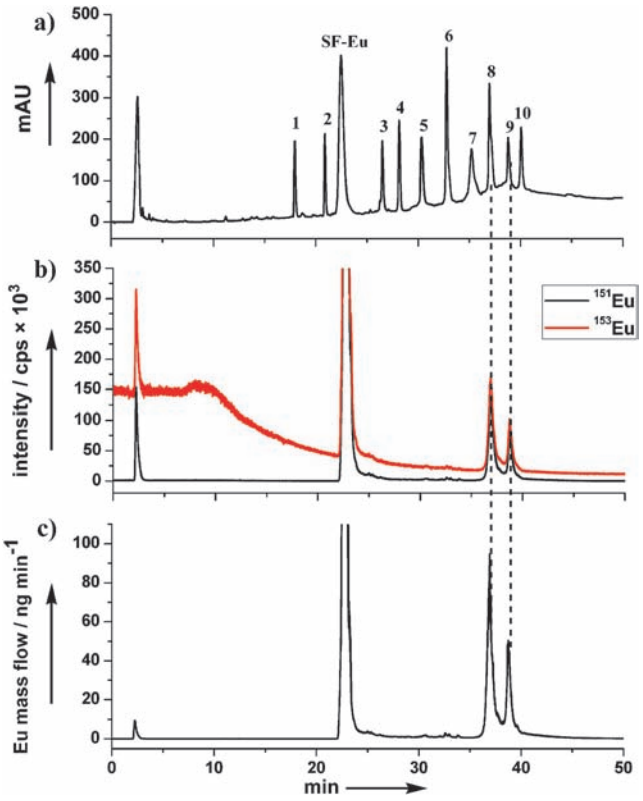


Figure 2. Typical chromatograms of the peptide/protein sample using a) HPLC/UV (214 nm), b) HPLC/ICP-MS for monitoring ^{153}Eu and ^{151}Eu isotopes with species-unspecific isotope dilution, c) the Eu mass-flow chromatogram of the SF-Eu-labeled peptide/protein transformed from (b) according to the on-line isotope dilution equation (Equation S1). Peak Nos. in (a) denote the corresponding peptide/protein listed in Table 1.

$(NO_3)_3$ through a three-way connector, and the signals of ^{153}Eu and ^{151}Eu isotopes in the mixed effluent were monitored using ICP/MS (see Figure S1). The data in Figure 2b indicates that almost only chymotrypsin and elastase were labeled with SF-Eu and detected using ICP/MS, whereas the ICP/MS signals of the other peptides and proteins such as Cyt C and lysozyme were below 1.0% compared with the signal of chymotrypsin, thus demonstrating the specificity of SF-Eu for the SPs. Moreover, it should be pointed out that even though there were other peptides/proteins of the same retention time as those of the labeled SPs on the HPLC, only the labeled SPs would be determined when using ICP/MS.

The chromatograms for the ^{153}Eu and ^{151}Eu isotopes (Figure 2b) show that the signal intensity of Eu decreased when the acetonitrile content of the effluent for the C18 column was changed in accordance to the gradient elution program (see the Supporting Information). To calibrate this effect, as both isotopes are affected to the same extent and the isotope ratio remains stable, the chromatograms for ^{151}Eu and ^{153}Eu isotopes were transformed into a Eu mass-flow chromatogram (Figure 2c) using the on-line isotope dilution equation given in Equation S1 in the Supporting Information. By integrating the peak areas corresponding to chymotrypsin and elastase in the Eu mass-flow chromatogram, the concentrations of chymotrypsin and elastase in the mixed protein solution, prepared from the crude chymotrypsin and elastase solid powder obtained from Sigma–Aldrich, were determined to be $(17.8 \pm 0.5)\ \mu mol\,L^{-1}$ and $(9.1 \pm 0.3)\ \mu mol\,L^{-1}$, respectively, and corresponded to $(90.3 \pm 2.5)\%$ and $(46.9 \pm 1.3)\%$ within the purity values provided by the producer. The detection limits of this method for the SPs were calculated to be 0.2 fmol based on the signal-to-noise ratio (3σ criterion) of Eu in the mass-flow chromatogram with a RSD lower than 3% ($n = 5$ at pmol level). These quantitative results demonstrated that the sensitivity and precision of ^{153}Eu species-unspecific isotope dilution HPLC/ICP/MS on the absolute quantification of the SPs were very high and accurate. It should be mentioned here that fluorophore[21] and isotope-coded tags[22] based on ABPP have been used to monitor the activities of the SPs, whereas the absolute quantification of the SPs remains unreported, because of the limitations of the detection techniques used. Obviously, the Eu-labeled activity-based probe together with species-unspecific isotope dilution ICP/MS overcame the limitations and realized the absolute quantification of the SPs.

To validate the accuracy of the ^{153}Eu species-unspecific isotope dilution HPLC/ICP/MS with the Eu-labeled activity-based probe, we further applied species-specific isotope-dilution HPLC/ICP/MS to measure the contents of chymotrypsin and elastase in the sample. Different from specific-unspecific isotope dilution, species-specific isotope dilution method could account for any peptide/protein loss or contamination during the processes from sample preparation and HPLC separation through detection, which was carried out by spiking the ^{153}Eu-labeled SP standards into the natural Eu-labeled sample and analyzed using the HPLC/ICP-MS (Figure S1). To obtain the SP standards, we purified SF-^{153}Eu-labeled chymotrypsin and elastase from the crude protein samples using HPLC, and the purified SF-^{153}Eu-labeled chymotrypsin and elastase were further quantified using the standard Bradford assay and ICP/MS (data were not shown here). When a known amount of ^{153}Eu-labeled chymotrypsin and elastase were spiked into the natural Eu-labeled sample for ^{153}Eu species-specific isotope dilution HPLC/ICP/MS analysis, the ratio detected ($R_{detected}$) of ^{153}Eu-labeled chymotrypsin and elastase versus natural Eu-labeled chymotrypsin and elastase matched the theoretical R_{theory} very well (see Figure S7 in the Supporting Information), and indicated the accuracy of ^{153}Eu species-specific isotope dilution HPLC/ICP/MS. The concentrations of chymotrypsin and elastase in the mixed protein solution prepared from the crude chymotrypsin and elastase solid powder obtained from Sigma–Aldrich were determined to be $(0.45 \pm 0.02)\ \mu mol\,L^{-1}$ and $(0.23 \pm 0.01)\ \mu mol\,L^{-1}$ corresponding to $(95.2 \pm 3.3)\%$ and $(50.3 \pm 1.8)\%$ in the crude solid proteases powders. Provided that these results could be considered "true" values, those obtained using ^{153}Eu species-unspecific isotope dilution HPLC/ICP/MS were in good agreement with the true values, and the recovery of chymotrypsin and elastase were within $(94.8 \pm 2.7)\%$ and $(93.1 \pm 2.6)\%$ ($n = 5$), respectively.

To demonstrate the feasibility of ^{153}Eu species-unspecific isotope dilution HPLC/ICP/MS for the absolute quantification of the SPs in biological samples, different concentrations

of chymotrypsin and elastase were spiked into human serum samples collected from Xiamen University Affiliated Zhongshan Hospital. We found the SPs could not be labeled and detected when the concentration of the spiked chymotrypsin and elastase was below 5 and 2.5 μM, respectively, mainly because of the high level of SPs inhibitors such as alpha-1 proteinase inhibitor (1–2 mg mL^{-1}) in human serum.[23] Within the ranges of 10–50 and 2.5–25 μM, the recoveries of chymotrypsin and elastase, respectively, were found to be from 34.0 to 51.0% and 18.4 to 56.1%, respectively (see Figure S8 in the Supporting Information), which meant that about 66.0 to 49.0% and 81.6 to 43.9% of these two SPs were inhibited by the inhibitors in the human serum samples. This phenomenon implied that one can additionally apply this method to investigating the level of SPs inhibitors and their degree of inhibition effect in a real biological sample, besides the absolute quantification of the active SPs. We further applied this strategy to quantify the active SPs in Tris-HCl extractable fraction of rat tissues (kidney, heart, liver, lung, brain and pancreas), and we found that different kinds of the SPs were expressed in these tissues and they ranged from 0.4 to 7.6 nmol g^{-1} (see Figure S9). When 36 pmol chymotrypsin and 12 pmol elastase were spiked, we found that the recoveries of these two SPs in the tissues except pancreas were above 90% (see Figure S10), which meant that the contents of SPs inhibitors in the rat tissues were relatively low. It should be noted that the recovery of chymotrypsin in pancreas was only 38.4% while that of elastase was 96.7%, thus suggesting that chymotrypsin-preferable inhibitors existing in the pancreas significantly inactivated chymotrypsin.[24] All these results indicate the feasibility of this method for analyzing the active SPs in real biological samples.

In conclusion, we developed a highly sensitive, precise, and practical method for the absolute quantification of active SPs. To the best of our knowledge, this is the first report of using this newly designed and synthesized Eu-labeled activity-based SP probe together with isotope dilution ICP/MS for active SP specific labeling and absolute quantification. Further application of this strategy is ongoing in our laboratory to quantify other SPs. Not limited to the SPs shown in this report, we believe that this method can be easily extended to other classes of enzymes, such as cysteine proteases,[11a,25] metalloproteases,[26] oxidoreductases,[27] and cytochrome P450[28] by developing the corresponding lanthanide-labeled activity-based probes.

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[1] a) S. P. Gygi, B. Rist, S. A. Gerber, F. Turecek, M. H. Gelb, R. Aebersold, *Nat. Biotechnol.* **1999**, *17*, 994–999; b) S. E. Ong, B. Blagoev, I. Kratchmarova, D. B. Kristensen, H. Steen, A. Pandey, M. Mann, *Mol. Cell. Proteomics* **2002**, *1*, 376–386; c) P. L. Ross, Y. N. Huang, J. N. Marchese, B. Williamson, K. Parker, S. Hattan, N. Khainovski, S. Pillai, S. Dey, S. Daniels, S. Purkayastha, P. Juhasz, S. Martin, M. Bartlet-Jones, F. He, A. Jacobson, D. J. Pappin, *Mol. Cell. Proteomics* **2004**, *3*, 1154–1169.

[2] a) S. A. Gerber, J. Rush, O. Stemman, M. W. Kirschner, S. P. Gygi, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 6940–6945; b) R. J. Beynon, M. K. Doherty, J. M. Pratt, S. J. Gaskell, *Nat. Methods* **2005**, *2*, 587–589; c) L. Anderson, C. L. Hunter, *Mol. Cell. Proteomics* **2006**, *5*, 573–588; d) V. Brun, A. Dupuis, A. Adrait, M. Marcellin, D. Thomas, M. Court, F. Vandenesch, J. Garin, *Mol. Cell. Proteomics* **2007**, *6*, 2139–2149.

[3] a) M. Wind, A. Wegener, A. Eisenmenger, R. Kellner, W. D. Lehmann, *Angew. Chem.* **2003**, *115*, 3547–3549; *Angew. Chem. Int. Ed.* **2003**, *42*, 3425–3427; b) D. Schaumlöffel, P. Giusti, H. Preud'Homme, J. Szpunar, R. Łobiński, *Anal. Chem.* **2007**, *79*, 2859–2868; c) M. Wang, W. Y. Feng, W. W. Lu, B. Li, B. Wang, M. T. Zhu, Y. Wang, H. Yuan, Y. L. Zhao, Z. F. Chai, *Anal. Chem.* **2007**, *79*, 9128–9134.

[4] A. Pereira Navaza, J. Ruiz Encinar, A. Sanz-Medel, *Angew. Chem.* **2007**, *119*, 575–577; *Angew. Chem. Int. Ed.* **2007**, *46*, 569–571.

[5] a) L. Hinojosa Reyes, J. M. Marchante-Gayón, J. I. García Alonso, A. Sanz-Medel, *J. Anal. At. Spectrom.* **2003**, *18*, 1210–1216; b) G. Ballihaut, F. Claverie, C. Pécheyran, S. Mounicou, R. Grimaud, R. Łobiński, *Anal. Chem.* **2007**, *79*, 6874–6880; c) M. Xu, L. Yang, Q. Wang, *J. Anal. At. Spectrom.* **2008**, *23*, 1545–1549.

[6] a) C. Sariego Muñiz, J. M. Marchante Gayón, J. I. G. Alonso, A. Sanz-Medel, *J. Anal. At. Spectrom.* **2001**, *16*, 587–592; b) Y. Nuevo Ordoñez, M. Montes-Bayón, E. Blanco-González, A. Sanz-Medel, *Anal. Chem.* **2010**, *82*, 2387–2394.

[7] a) N. Jakubowski, J. Messerschmidt, M. G. Añorbe, L. Waentig, H. Haye, P. H. Roos, *J. Anal. At. Spectrom.* **2008**, *23*, 1487–1496; b) A. Pereira Navaza, J. Ruiz Encinar, A. Ballesteros, J. M. González, A. Sanz-Medel, *Anal. Chem.* **2009**, *81*, 5390–5399.

[8] a) D. J. Kutscher, M. E. C. Busto, N. Zinn, A. Sanz-Medel, J. Bettmer, *J. Anal. At. Spectrom.* **2008**, *23*, 1359–1364; b) Y. F. Guo, M. Xu, L. M. Yang, Q. Q. Wang, *J. Anal. At. Spectrom.* **2009**, *24*, 1184–1187; c) M. Xu, X. Yan, Q. Xie, L. Yang, Q. Wang, *Anal. Chem.* **2010**, *82*, 1616–1620.

[9] a) R. Ahrends, S. Pieper, A. Kühn, H. Weisshoff, M. Hamester, T. Lindemann, C. Scheler, K. Lehmann, K. Taubner, M. W. Linscheid, *Mol. Cell. Proteomics* **2007**, *6*, 1907–1916; b) C. Rappel, D. Schaumlöffel, *Anal. Chem.* **2009**, *81*, 385–393; c) M. Terenghi, L. Elviri, M. Careri, A. Mangia, R. Łobiński, *Anal. Chem.* **2009**, *81*, 9440–9448; d) X. Yan, M. Xu, L. Yang, Q. Wang, *Anal. Chem.* **2010**, *82*, 1261–1269; e) X. Yan, L. Yang, Q. Wang, *Angew. Chem.* **2011**, *123*, 5236–5239; *Angew. Chem. Int. Ed.* **2011**, *50*, 5130–5133; f) Z. Zhang, X. Yan, M. Xu, L. Yang, Q. Wang, *J. Anal. At. Spectrom.* **2011**, *26*, 1175–1177.

[10] a) L. Rottmann, K. G. Heumann, *Fresenius J. Anal. Chem.* **1994**, *350*, 221–227; b) L. Rottmann, K. G. Heumann, *Anal. Chem.* **1994**, *66*, 3709–3715; c) J. Vogl, K. G. Heumann, *Anal. Chem.* **1998**, *70*, 2038–2043.

[11] a) Y. Liu, M. P. Patricelli, B. F. Cravatt, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 14694–14699; b) D. Greenbaum, K. F. Medzihradszky, A. Burlingame, M. Bogyo, *Chem. Biol.* **2000**, *7*, 569–581.

[12] a) C. Zhang, F. B. Wu, Y. Y. Zhang, X. Wang, X. R. Zhang, *J. Anal. At. Spectrom.* **2001**, *16*, 1393–1396; b) C. Zhang, Z. Y. Zhang, B. B. Yu, X. R. Zhang, *Anal. Chem.* **2002**, *74*, 96–99; c) V. I. Baranov, Z. Quinn, D. R. Bandura, S. D. Tanner, *Anal. Chem.* **2002**, *74*, 1629–1636; d) X. Lou, G. Zhang, I. Herrera, R. Kinach, O. Ornatsky, V. Baranov, M. Nitz, M. A. Winnik, *Angew. Chem.* **2007**, *119*, 6223–6226; *Angew. Chem. Int. Ed.* **2007**, *46*, 6111–6114; e) S. C. Bendall, E. F. Simonds, P. Qiu, E. D. Amir, P. O. Krutzik, R. Finck, R. V. Bruggner, R. Melamed, A. Trejo,

O. I. Ornatsky, R. S. Balderas, S. K. Plevritis, K. Sachs, D. Péer, S. D. Tanner, G. P. Nolan, *Science* **2011**, *332*, 687 – 696.
[13] a) C. López-Otín, L. M. Matrisian, *Nat. Rev. Cancer* **2007**, *7*, 800 – 808; b) G. M. Simon, B. F. Cravatt, *J. Biol. Chem.* **2010**, *285*, 11051 – 11055.
[14] J. C. Powers, J. L. Asgian, O. D. Ekici, K. E. James, *Chem. Rev.* **2002**, *102*, 4639 – 4750.
[15] a) A. M. Gold, D. Fahrney, *Biochemistry* **1964**, *3*, 783 – 791; b) A. M. Gold, *Biochemistry* **1965**, *4*, 897 – 901.
[16] H. C. Kolb, M. G. Finn, K. B. Sharpless, *Angew. Chem.* **2001**, *113*, 2056 – 2075; *Angew. Chem. Int. Ed.* **2001**, *40*, 2004 – 2021.
[17] a) P. B. Sigler, H. C. W. Skinner, C. L. Coulter, J. Kallos, H. Braxton, D. R. Davies, *Proc. Natl. Acad. Sci. USA* **1964**, *51*, 1146 – 1151; b) A. Fenselau, D. Koenig, D. E. Koshland, Jr., H. G. Latham, Jr., H. Weiner, *Proc. Natl. Acad. Sci. USA* **1967**, *57*, 1670 – 1675; c) J. T. Gerig, D. C. Roe, *J. Am. Chem. Soc.* **1974**, *96*, 233 – 238.
[18] a) H. C. Watson, D. M. Shotton, J. M. Cox, H. Muirhead, *Nature* **1970**, *225*, 806 – 811; b) D. M. Shotton, H. C. Watson, *Nature* **1970**, *225*, 811 – 816.
[19] T. R. Chan, R. Hilgraf, K. B. Sharpless, V. V. Fokin, *Org. Lett.* **2004**, *6*, 2853 – 2855.
[20] A. E. Speers, G. C. Adam, B. F. Cravatt, *J. Am. Chem. Soc.* **2003**, *125*, 4686 – 4687.
[21] a) G. Blum, S. R. Mullins, K. Keren, M. Fonovic, C. Jedeszko, M. J. Rice, B. F. Sloane, M. Bogyo, *Nat. Chem. Biol.* **2005**, *1*, 203 – 209; b) L. E. Edgington, A. B. Berger, G. Blum, V. E. Albrow, M. G. Paulick, N. Lineberry, M. Bogyo, *Nat. Med.* **2009**, *15*, 967 – 973.
[22] a) A. E. Speers, B. F. Cravatt, *J. Am. Chem. Soc.* **2005**, *127*, 10018 – 10019; b) S. H. Verhelst, M. Fonović, M. Bogyo, *Angew. Chem.* **2007**, *119*, 1306 – 1308; *Angew. Chem. Int. Ed.* **2007**, *46*, 1284 – 1286; c) P. P. Geurink, B. I. Florea, N. Li, M. D. Witte, J. Verasdonck, C. L. Kuo, G. A. van der Marel, H. S. Overkleeft, *Angew. Chem.* **2010**, *122*, 6954 – 6957; *Angew. Chem. Int. Ed.* **2010**, *49*, 6802 – 6805.
[23] P. G. Gettins, *Chem. Rev.* **2002**, *102*, 4751 – 4804.
[24] a) M. Kunitz, J. H. Morthrop, *J. Gen. Physiol.* **1936**, *19*, 991 – 1007; b) Y. T. Wachtfogel, U. Kucich, C. E. Hack, P. Gluszko, S. Niewiarowski, R. W. Colman, L. H. Edmunds Jr., *J. Thorac. Cardiovasc. Surg.* **1993**, *106*, 1 – 9.
[25] a) D. Kato, K. M. Boatright, A. B. Berger, T. Nazif, G. Blum, C. Ryan, K. A. Chehade, G. S. Salvesen, M. Bogyo, *Nat. Chem. Biol.* **2005**, *1*, 33 – 38; b) F. Yuan, S. H. Verhelst, G. Blum, L. M. Coussens, M. Bogyo, *J. Am. Chem. Soc.* **2006**, *128*, 5616 – 5617.
[26] A. Saghatelian, N. Jessani, A. Joseph, M. Humphrey, B. F. Cravatt, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 10000 – 10005.
[27] a) G. C. Adam, S. E. J. Orensen, B. F. Cravatt, *Nat. Biotechnol.* **2002**, *20*, 805 – 809; b) G. C. Adam, J. Burbaum, J. W. Kozarich, M. P. Patricelli, B. F. Cravatt, *J. Am. Chem. Soc.* **2004**, *126*, 1363 – 1368.
[28] A. T. Wright, J. D. Song, B. F. Cravatt, *J. Am. Chem. Soc.* **2009**, *131*, 10692 – 10700.

