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.[3] 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.[3] 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 ($S^{[3]}$, $P^{[3]}$, $Se^{[5]}$) of proteins and metals in the metalloproteins, [6] or the exogenous elements ($I^{[7]}$, $Hg^{[8]}$, $La^{[9]}$) 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-specific 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). 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 a 3+2 cycloaddition reaction between a terminal alkyne and azide using CuI 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 1A); 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 1B). 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 the resulting SF–alkyne was labeled with azido-DOTA-Eu through the click reaction (Scheme 1A); 3) The azido-DOTA-Eu unit was synthesized first by conjugating SF–alkyne to give SF–alkyne/azido-DOTA-Eu (SF-Eu), which was then used to label the SPs 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(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 CuSO4, 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 alkylne-SF-SP, which was then reacted with azido-DOTA-Eu through the click chemistry (Scheme 1A), the SPs unfortunately...
precipitated out. The speculated reason for this phenomenon was the use of TBTA, according to a previous report. However, TBTA is a very important reagent for stabilizing CuI, and plays a catalytic role in the click reaction. No SF-Eu was found after 1 hour in the case where neither CuI nor TBTA were used. We therefore changed our strategy (Scheme 1B) 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 1e,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 2a, 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 153Eu standard [153Eu-
(NO₃)₃ through a three-way connector, and the signals of ¹⁵³Eu and ¹⁵¹Eu 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 chymotrypsin and elastase in the sample. Different from specific-unspecific 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 ¹⁵³Eu-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-¹⁵³Eu-labeled chymotrypsin and elastase from the crude protein samples using HPLC, and the purified SF-¹⁵³Eu-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 ¹⁵³Eu-labeled chymotrypsin and elastase were spiked into the natural Eu-labeled sample for ¹⁵³Eu species-specific isotope dilution HPLC/ICP/MS analysis, the ratio detected \( R_{\text{sample}} \) of ¹⁵³Eu-labeled chymotrypsin and elastase versus natural Eu-labeled chymotrypsin and elastase matched the theoretical \( R_{\text{theory}} \) very well (see Figure S7 in the Supporting Information), and indicated the accuracy of ¹⁵³Eu 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 \((17.8 \pm 0.5) \times 10^{-3} \text{ mol L}^{-1}\) and \((9.1 \pm 0.3) \times 10^{-3} \text{ mol L}^{-1}\), respectively, and corresponds 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 (3 σ criterion) of Eu in the mass-flow chromatogram with a RSD lower than 3% \((n = 5\) at pmol level). These quantitative results demonstrate that the sensitivity and precision of ¹⁵³Eu 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 isotopically-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 ¹⁵³Eu 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 ¹⁵³Eu-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-¹⁵³Eu-labeled chymotrypsin and elastase from the crude protein samples using HPLC, and the purified SF-¹⁵³Eu-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 ¹⁵³Eu-labeled chymotrypsin and elastase were spiked into the natural Eu-labeled sample for ¹⁵³Eu species-specific isotope dilution HPLC/ICP/MS analysis, the ratio detected \( R_{\text{sample}} \) of ¹⁵³Eu-labeled chymotrypsin and elastase versus natural Eu-labeled chymotrypsin and elastase matched the theoretical \( R_{\text{theory}} \) very well (see Figure S7 in the Supporting Information), and indicated the accuracy of ¹⁵³Eu 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 \( (0.45 \pm 0.02) \times 10^{-3} \text{ mol L}^{-1} \) and \( (0.23 \pm 0.01) \times 10^{-3} \text{ mol L}^{-1} \) corresponding to \( (95.2 \pm 3.3)\% \) and \( (50.3 \pm 1.8)\% \) in the crude solid protease powders. Provided that these results could be considered “true” values, those obtained using ¹⁵³Eu 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 ¹⁵³Eu species-unspecific isotope dilution HPLC/ICP/MS for the absolute quantification of the SPs in biological samples, different 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 \( (17.8 \pm 0.5) \times 10^{-3} \text{ mol L}^{-1} \) and \( (9.1 \pm 0.3) \times 10^{-3} \text{ mol L}^{-1} \), 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 (3 σ 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 ¹⁵³Eu 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 isotopically-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 ¹⁵³Eu 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 ¹⁵³Eu-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-¹⁵³Eu-labeled chymotrypsin and elastase from the crude protein samples using HPLC, and the purified SF-¹⁵³Eu-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 ¹⁵³Eu-labeled chymotrypsin and elastase were spiked into the natural Eu-labeled sample for ¹⁵³Eu species-specific isotope dilution HPLC/ICP/MS analysis, the ratio detected \( R_{\text{sample}} \) of ¹⁵³Eu-labeled chymotrypsin and elastase versus natural Eu-labeled chymotrypsin and elastase matched the theoretical \( R_{\text{theory}} \) very well (see Figure S7 in the Supporting Information), and indicated the accuracy of ¹⁵³Eu 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 \( (0.45 \pm 0.02) \times 10^{-3} \text{ mol L}^{-1} \) and \( (0.23 \pm 0.01) \times 10^{-3} \text{ mol L}^{-1} \) corresponding to \( (95.2 \pm 3.3)\% \) and \( (50.3 \pm 1.8)\% \) in the crude solid protease powders. Provided that these results could be considered “true” values, those obtained using ¹⁵³Eu 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 ¹⁵³Eu 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 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% in brain and pancreas), and we found that different kinds of the active SPs in Tris-HCl method to investigating the level of SPs inhibitors and their degree of inhibition effect. The active 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% in brain and pancreas, and we found that different kinds of the active 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% in brain and pancreas.


