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Peptide codes for multiple protease activity assay via high-resolution mass spectrometric quantitation

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RATIONALE: Proteases, which are involved in a number of biological processes, play important roles in human health. Alterations in protease activities have been associated with many diseases. Thus, effective methods for multiple protease assay are very essential and may help to discover more about their functions in different physiological processes. Here, we proposed a strategy of peptide codes for label-free analysis of multiple protease activities using high-resolution mass spectrometry (HRMS).
METHODS: The peptide codes were designed to contain a coding region and the substrate of the protease, respectively. Upon the cleavage of target proteases, the coding regions could be rapidly identified and directly quantitated by accurate *m/z* extractions without internal standard. Therefore, the coding region could be used as the unique "Protease ID" for the identification of the corresponding protease, and the amount of the cleavage product was used for protease activity analysis.
RESULTS: The method was validated by using trypsin and chymotrypsin as model proteases, with the designed "Trypsin ID" and "Chymotrypsin ID" occurring at *m/z* 761.270 and 711.243, respectively. The peak area of "Protease ID" was proportional to trypsin and chymotrypsin concentration in the range from 0.01 to 10 nM and 10 to 2000 nM, respectively. The proposed method could also be used for screening of protease inhibitors, which might be of great importance for drug discovery.
CONCLUSIONS: This method provided a label-free and accurate quantitative platform for convenient identification and activity analysis of multiple proteases, which could potentially be applied in clinical diagnosis.

Proteases, known as proteolytic enzymes, play fundamental roles in a series of biological processes, including protein metabolism, blood clotting, tissue remodeling, and immune defense.[1–5] Alterations in protease activities have been involved in many diseases, such as arthritis, inflammation, Alzheimer's disease, and cancer.[6–8] Therefore, effective methods for multiple protease activity assay are important and may help to uncover the roles of proteases in different physiological processes, as well as evaluate the drug candidates, which target the proteases.[9]

A traditional immunoassay method has been employed for the detection of protease concentrations, but this method is unsuitable for protease activity because the concentration is not necessarily proportional to the activity, and it often suffers from the risk of false-positives.[10–12] As an alternative, the substrate peptide cleavage technique has been applied for the multiple protease assay.[13–15] Upon cleavage by target proteases, the released or reserved signals can be detected with optical[13,14] or electrochemical[15] platforms. However, the labeling reagents might influence the intrinsic catalytic activity of proteases and thus limit the applications of these platforms.[16,17] Compared with these detection techniques, mass spectrometric (MS) analysis provides a label-free approach for the analysis of enzyme activities, which is significant to retain the biologically relevant protease activities and improve the assay accuracy by distinguishing the cleavage sites at different amino acid residues.[18]

As is well known, lanthanides have been adapted as elemental tags to multiple protease assays using inductively coupled plasma mass spectrometry (ICP-MS).[19,20] However, preparation and purification of the probes is still time-consuming. Our group proposed a peptide-encoded microplate for matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) analysis of trypsin and chymotrypsin, but internal standards were required for quantitation.[21] Considering the fact that high-resolution mass spectrometry (HRMS) can operate in the full-scan operation mode with the special advantages of high throughput[22] and can be used for accurate peptide quantitation without an internal standard, this work applied the peptide-encoding technique for HRMS analysis of protease activities.

The peptide codes were designed to contain a coding region as the "Protease ID" and the substrate for enzymatic cleavage respectively as previously reported (Fig. 1).[21] Upon cleavage by different target proteases, the formed "Protease ID" was identified and quantified using HRMS. As a proof-of-concept study, trypsin and chymotrypsin were selected as model proteases to achieve a duplex protease assay, with "Trypsin

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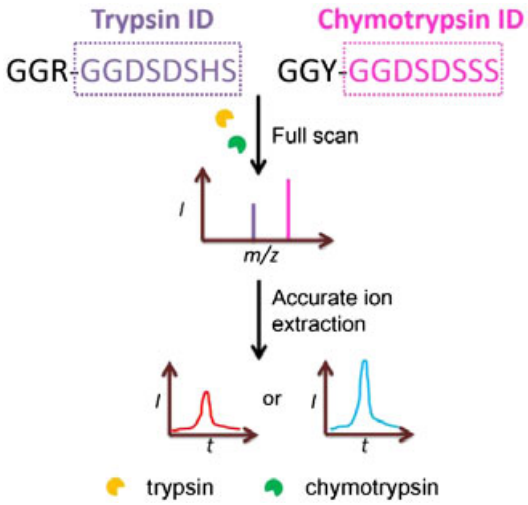

Figure 1. Schematic illustration of multiple protease activity assay using HRMS.

ID" and "Chymotrypsin ID" at m/z 761.270 and 711.243, respectively. Compared with previous schemes, the quantitation could be carried out directly and accurately by m/z extractions without use of an internal standard, which was of great convenience for the effective assay of protease activity.

EXPERIMENTAL

Chemicals and reagents

Peptides GGRGGDSDSHS (peptide code 1, M_w 1030.96) and GGYGGDSDSSS (peptide code 2, M_w 987.89) were synthesized and purified by Sangon Biotech (Shanghai, China) with purity greater than 95.0%. Trypsin (from bovine pancreas), α-chymotrypsin (from bovine pancreas, type VII), and 4-(2-aminomethyl)benzenesulfonyl fluoride hydrochloride (AEBSF, ≥97%) were purchased from Sigma-Aldrich (USA). All these reagents were used as received without further purification, and aqueous solutions were prepared using ultrapure water (18.2 MΩ, Milli-Q, Millipore).

Multiple protease assay

An aliquot of 10 μL peptide codes 1 and 2 (final concentration of 10 μM) was added to 80 μL reaction buffer (100 mM Tris–HCl buffer solution containing 10 mM $CaCl_2$, pH 7.8). Then 10 μL of trypsin (final concentration of 0.01 to 100 nM) and chymotrypsin (final concentration of 10 to 2000 nM) was added and the mixture was incubated at 37 °C for 1 h. After reaction, the resulting solution was diluted to 500 μL with 5 mM of NH_4OAc and submitted for HRMS analysis.

The lysates of HeLa, MCF-7, and PANC-1 cells were used as practical samples to verify the application of the assay, respectively. These cells were grown in cell culture media in a humidified atmosphere containing 5% CO_2 at 37 °C. After suspending with trypsin, they were washed thrice with phosphate-buffered saline (PBS, pH 7.4) containing 136.7 mM NaCl, 2.7 mM KCl, 8.72 mM Na_2HPO_4, and 1.41 mM KH_2PO_4 to remove the culture media and the used trypsin. The cell number was determined using a Petroff-Hausser cell counter (USA). These cells were dispersed in 100 mM Tris–HCl buffer solution containing 10 mM $CaCl_2$, sonicated for 15 min, and centrifuged to collect the supernatant. Subsequently, 10 μL of the 10× and 200× diluted supernatant was used to assay the corresponding protease activities of chymotrypsin and trypsin following the above protocol and submitted for HRMS analysis. The concentrations of the trypsin and chymotrypsin could be obtained by the peak areas of the "Protease ID" and the constructed calibration or working curves.

Inhibition assay

For trypsin inhibition assay, different concentrations of inhibitors AEBSF (0.01, 0.05, 0.1, 0.2, 0.5, and 1.0 mM) were pre-mixed with 100 nM trypsin for 20 min at room temperature, and 10 μL of the mixtures were added to peptide codes 1 and 2 in reaction buffer to incubate at 37 °C for 1 h. Afterward, 400 μL of 5 mM NH_4OAc aqueous solution was added to dilute the solution and submitted to HRMS analysis.

MS analysis

MS analysis was carried out on LTQ-Orbitrap XL mass spectrometer (ThermoFisher, Germany) coupled to a Thermo Surveyor LC system and a micro AS auto sampler (ThermoFisher, USA). The instrument was calibrated using the manufacturer's calibration solution (consisting of caffeine, the tetrapeptide MRFA, and ultramark). A guard column (Agilent, USA) was used as an online filter. The mobile phase was 50% acetonitrile aqoeous solution, and flow rate was 300 μL min^{-1}.

The mass spectrometer was operated in positive ion mode. Peptides were detected by full-scan mass analysis in the range of m/z 600–1200 at a resolving power of 60 000. Parameters of the ion source were as follows: capillary voltage 26 V, ion spray voltage 3.5 kV, tube lens 137 V, capillary temperature 350 °C, sheath gas flow 20 (arbitrary units), auxiliary gas flow 8 (arbitrary units), and sweep gas 0 (arbitrary units).

To assess the accuracy of the proposed method, the activities of trypsin and chymotrypsin were also detected with BAEE (*N*-α-benzoyl-L-arginine-ethyl ester at 253 nm) and BTEE (*N*-α-benzoyl-L-tyrosine-ethyl ester at 256 nm) methods, respectively. Briefly, the assay was carried out in 100 mM Tris–HCl buffer (pH 8.0) containing 6 mM $CaCl_2$, 10 μM substrate (BAEE or BTEE), and trypsin or chymotrypsin at different concentrations for constructing the working curves or the same 10× diluted cell lysates as samples. The absorbance was measured at 25 °C on a UV-3600 spectrophotometer (Shimadzu, Japan).

RESULTS AND DISCUSSION

Feasibility of multiple protease assay

Trypsin can specifically cleave peptides at the C-terminal of the Arg/Lys residue, while chymotrypsin cleaves the peptides at the C-terminal of the Tyr/Phe/Trp residue. To demonstrate the feasibility of the proposed assay for distinguishing these proteases, peptide code 1 GGRGGDSDSHS and peptide code 2 GGYGGDSDSSS were mixed with the two target proteases for enzymatic reactions and subsequently submitted for HRMS analysis. The MS spectrum showed that typical peaks of "Protease ID" in the presence of trypsin and chymotrypsin occured at m/z 761.270 and 711.243, respectively (Fig. 2A).

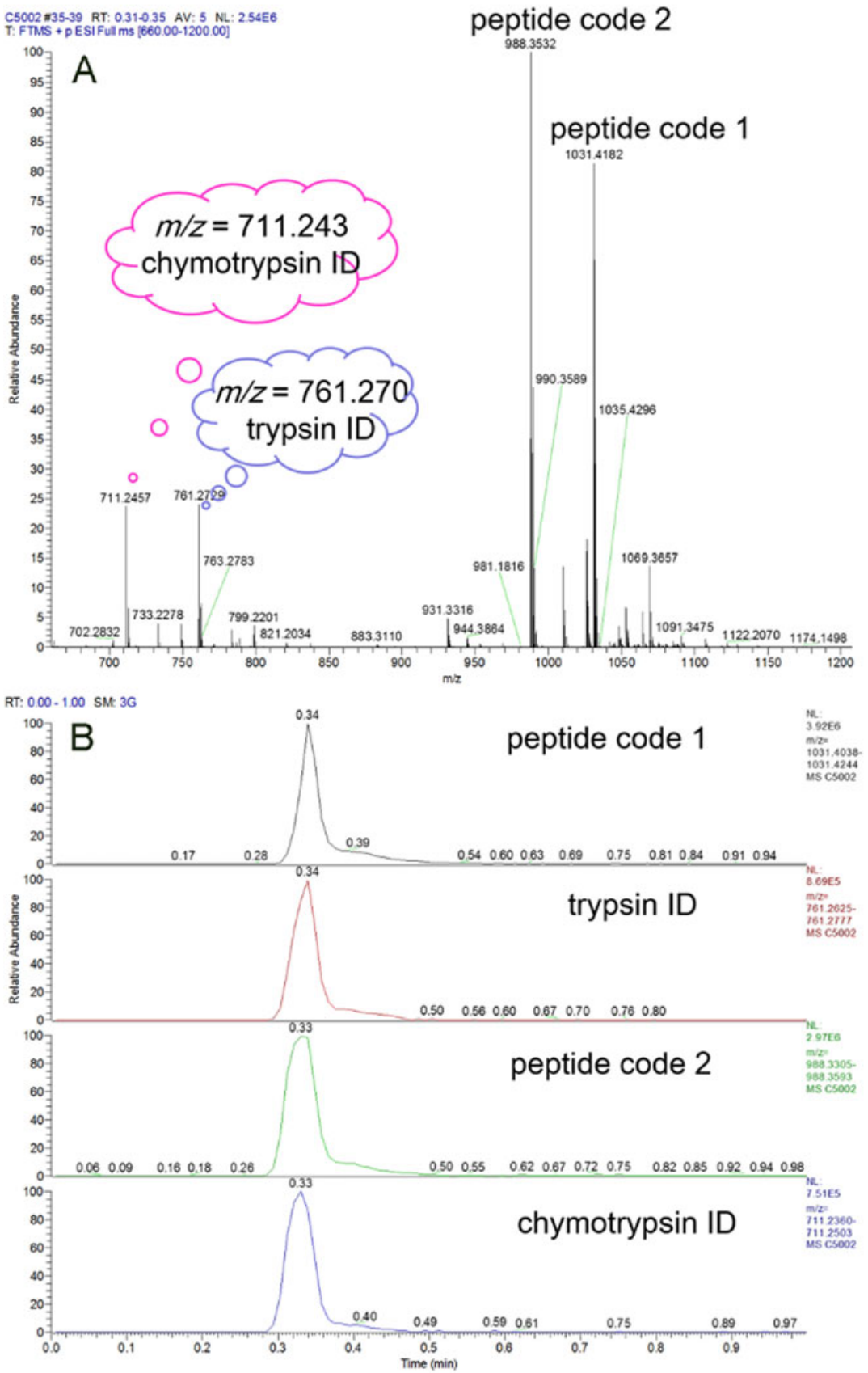

Figure 2. (A) Mass spectrum of the solutions after reaction with 5 nM of trypsin and 500 nM of chymotrypsin at 37 °C for 1 h and (B) extracted ion chromatograms from (A) using a mass extraction window of ±10 ppm.

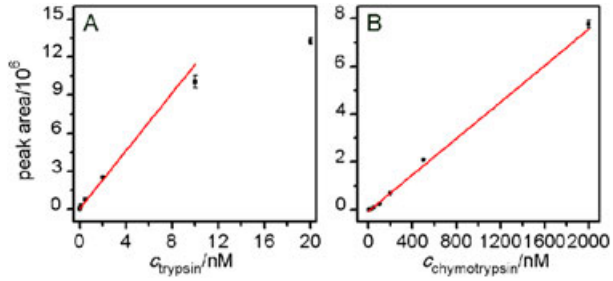

Figure 3. Plots of the peak areas of (A) trypsin ID and (B) chymotrypsin ID vs trypsin and chymotrypsin concentrations, respectively. All experiments were carried out in triplicate.

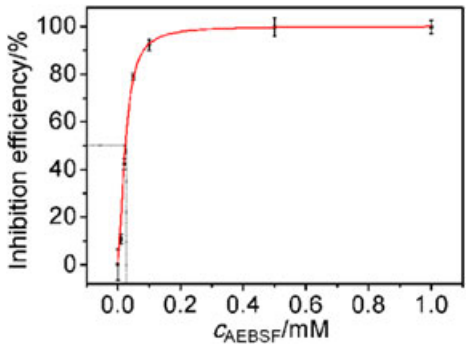

Figure 4. Plots of inhibition efficiency of AEBSF to 20 nM trypsin vs its concentrations. All experiments were carried out in triplicate.

These phenomena indicated that both trypsin and chymotrypsin cleaved their corresponding peptide codes at predicted sites. The cleavage products were just the coding regions GGDSDSHS (m/z = 761.270) and GGDSDSSS (m/z = 711.243), which were further verified with a MS/MS method (data not shown). Therefore, this platform could be used for identification of the types of proteases with the unique "Protease ID" peaks.

Quantitative detection of protease activities

For quantitative assay of the protease activity, accurate ions for "Protease ID" with the m/z tolerence of 10 ppm were extracted from the MS spectrum as shown in Fig. 2(B). At various concentrations of target proteases, the MS spectra of reaction solutions showed the increasing peak areas of "Trypsin ID" at m/z 761.262–761.278 and "Chymotrypsin ID" at m/z 711.236–711.250 with the increasing concentration of trypsin and chymotrypsin. The linear ranges for trypsin and chymotrypsin were 0.01–10 nM and 10–2000 nM, respectively (Fig. 3). Their detection limits (3σ) were 3.8 pM and 2.2 nM, respectively, which were lower than those of 2.3 and 5.2 nM for the previous MALDI-TOFMS analysis[21] and comparable with some fluoresence, phosphorescence, or label-free colormetric assays.[23–25] All these results implied the effectiveness of the proposed method for multiple protease assay. The proposed method coded the information whether targets existed into unique numbers, which served as the "Protease ID", rather than the relatively complicated optical or electronic signals. Furthermore, this platform could offer directly quantitative data without the need for internal standards, thus simplifying the detection process. Besides, a more masterly design of peptide codes could benefit more specific proteases by employing the proposed method.

Screening of protease inhibitor

The peptide-encoding technique was also tested against AEBSF, a small molecular inhibitor. The inhibition efficiency (%) was calculated with $(1 - A_{761.270}/A_0) \times 100\%$, where A_0 and $A_{761.270}$ are the peak areas in the absence and presence of inhibitor, respectively. Figure 4 shows a plot of the inhibition efficiency toward trypsin versus the concentration of AEBSF. The addition of the inhibitor reduced the activity of trypsin and thus reduced the cleavage of peptide code 1. The IC_{50} (defined as 50% inhibition efficiency) for AEBSF was estimated to be 0.028 mM. The results demonstrated the application of the coding assay in the screening of a protease inhibitor, which might be of great importance for drug discovery.[26–28]

Protease activity assay in cell lysates

Although trypsin and chymotrypsin are normally secreted by pancreatic acinar cells for food digestion, elevated levels of these two proteases in body fluids are reported to be associated with pancreatic disease and cancer.[29,30] Therefore, to demonstrate the feasibility of the proposed method in biological samples, it was applied to assay trypsin and chymotrypsin in the cell-related samples including two non-pancreatic cells (HeLa and MCF-7) as negative control and one sample of pancreatic carcinoma cells (PANC-1). The detection results are listed in Table 1, which indicated that the proposed method could differentiate non-pancreatic and pancreatic cells. The "Protease ID" was further verified by a MS/MS method (Fig. 5), which demonstrated the specificity of the proposed method in the analysis of complex samples and indicated that interference from co-eluted ions with quantitation results was negligible. The measurement accuracy for trypsin and chymotrypsin in the same samples

Table 1. Detection results of cell lysates using the proposed and reference methods.

	Trypsin/μM			Chymotrypsin/nM		
Sample	proposed method	reference method	relative error/%	proposed method	reference method	relative error/%
HeLa	ND	ND		ND	ND	
MCF-7	ND	ND		ND	ND	
PANC-1	1.29 ± 0.07	1.34	−3.7	106.44 ± 5.23	112.33	−5.2

Cell concentration: HeLa, 7.5×10^5 cell mL^{-1}; MCF-7, 5.0×10^5 cell mL^{-1}; PANC-1, 4.5×10^5 cell mL^{-1}.

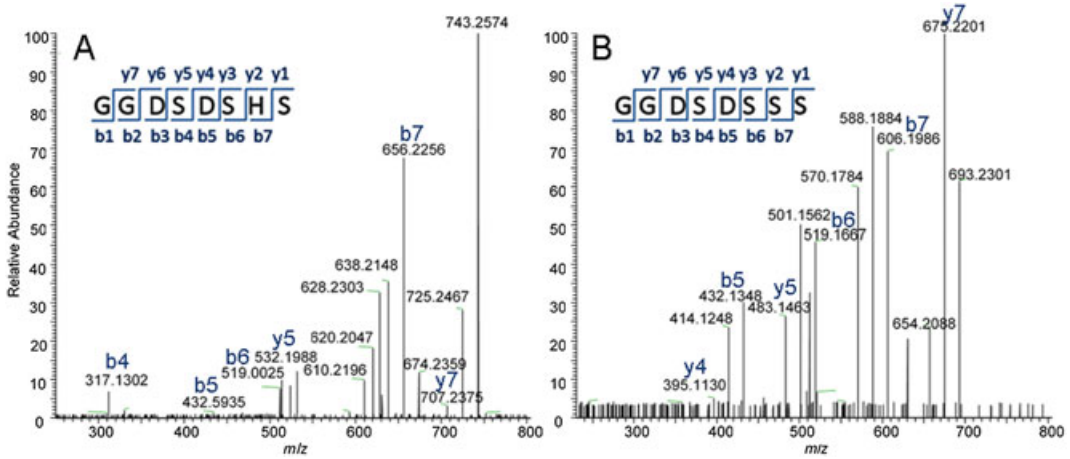

Figure 5. MS/MS spectra of (A) trypsin ID at m/z 761.262–761.278 and (B) chymotrypsin ID at m/z 711.236–711.250.

was also examined by comparing the results with those obtained from BAEE and BTEE methods, which were also reliant on the substrate cleavage to assay protease activities[31–33] and showed relative errors less than 5.2%. Thus, the proposed peptide-encoding strategy could be potentially applicated in clinical diagnosis and expanded to protease activity assays in blood samples, while necessary dilution and desalting protocols might be needed to obtain sacrifatory results.

CONCLUSIONS

In this work, the peptide-encoding technique is applied for quantitative analysis of protease activity using HRMS. The peptide codes contain a coding region and a cleavage site for different proteases, respectively. Thus, multiple proteases can be simply identified with the specific m/z of the cleaved encoding regions on MS spectra. Meanwhile, the quantitations can be directly carried out by extracting accurate ions from the spectra with m/z tolerence of 10 ppm without the aid of an internal standard. This proposed strategy shows good analytical performance with convenience, high specificity as well as acceptable precision. It is anticipated that the peptide-encoding technique, combined with HRMS, will provide a convenient and powerful tool for multiple sensing, as well as for the investigation of numerous protease-related processes.

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REFERENCES

[1] B. Turk. Targeting proteases: successes, failures and future prospects. *Nat. Rev. Drug Discov.* **2006**, *5*, 785.
[2] C. López-Otín, L. M. Matrisian. Emerging roles of proteases in tumour suppression. *Nat. Rev. Cancer* **2007**, *7*, 800.
[3] N. A. Thornberry, Y. Lazebnik. Caspases: enemies within. *Science* **1998**, *281*, 1312.
[4] X. S. Puente, L. M. Sánchez, C. M. Overall, C. López-Otín. Human and mouse proteases: a comparative genomic approach. *Nat. Rev. Genet.* **2003**, *4*, 544.
[5] D. Leung, G. Abbenante, D. P. Fairlie. Protease inhibitors: current status and future prospects. *J. Med. Chem.* **2000**, *43*, 305.
[6] P. Friedl, K. Wolf. Tube travel: the role of proteases in individual and collective cancer cell invasion. *Cancer Res.* **2008**, *68*, 7247.
[7] R. Q. Yan, M. J. Bienkowski, M. E. Shuck, H. Y. Miao, M. C. Tory, A. M. Pauley, J. R. Brashler, N. C. Stratman, W. R. Mathews, A. E. Buhl, D. B. Cater, A. G. Tomasselli, L. A. Parodi, R. L. Heinrikson, M. E. Gurney. Membrane-anchored aspartyl protease with Alzheimer's disease β-secretase activity. *Nature* **1999**, *402*, 533.
[8] G. Blum. Use of fluorescent imaging to investigate pathological protease activity. *Curr. Opin. Drug Discov. Dev.* **2008**, *11*, 708.
[9] A. Dufour, C. M. Overall. Missing the target: matrix metalloproteinase antitargets in inflammation and cancer. *Trends Pharmacol. Sci.* **2013**, *34*, 233.
[10] J. Wu, Z. F. Fu, F. Yan, H. X. Ju. Biomedical and clinical applications of immunoassays and immunosensors for tumor markers. *TrAC, Trends Anal. Chem.* **2007**, *7*, 679.
[11] B. F. Cravatt, A. T. Wright, J. W. Kozarich. Activity-based protein profiling: from enzyme chemistry to proteomic chemistry. *Annu. Rev. Biochem.* **2008**, *77*, 383.
[12] M. Wu, E. Petryayeva, W. R. Algar. Quantum dot-based concentric FRET configuration for the parallel detection of protease activity and concentration. *Anal. Chem.* **2014**, *86*, 11181.
[13] H. B. Wang, Q. Zhang, X. Chu, T. T. Chen, J. Ge, R. Q. Yu. Graphene oxide–peptide conjugate as an intracellular protease sensor for caspase-3 activation imaging in live cells. *Angew. Chem. Int. Ed.* **2011**, *50*, 7065–7069; *Angew. Chem.* **2011**, *123*, 7203.
[14] Y. Huang, M. Shi, K. Hu, S. L. Zhao, X. Lu, Z. F. Chen, J. Chen, H. Liang. Carbon nanotube-based multicolor fluorescent peptide probes for highly sensitive multiplex detection of cancer-related proteases. *J. Mater. Chem. B* **2013**, *1*, 3470.
[15] R. P. Liang, X. C. Tian, P. Qiu, J. D. Qiu. Multiplexed electrochemical detection of trypsin and chymotrypsin based on distinguishable signal nanoprobes. *Anal. Chem.* **2014**, *86*, 9256.
[16] C. I. Soderstrom, F. P. Spriggs, W. Song, S. Burrell. Comparison of four distinct detection platforms using

multiple ligand binding assay formats. *J. Immunol. Methods* **2011**, *371*, 106.
[17] Z. Y. Xia, Y. Xing, M. K. So, A. L. Koh, R. Sinclair, J. H. Rao. Multiplex detection of protease activity with quantum dot nanosensors prepared by intein-mediated specific bioconjugation. *Anal. Chem.* **2008**, *80*, 8649.
[18] A. Liesener, U. Karst. Monitoring enzymatic conversions by mass spectrometry: a critical review. *Anal. Bioanal. Chem.* **2005**, *382*, 1451.
[19] X. W. Yan, L. M. Yang, Q. Q. Wang. Lanthanide-coded protease-specific peptide–nanoparticle probes for a label-free multiplex protease assay using element mass spectrometry: a proof-of-concept study. *Angew. Chem. Int. Ed.* **2011**, *50*, 5130; *Angew. Chem.* **2011**, *123*, 5236.
[20] U. S. Lathia, O. Ornatsky, V. Baranov, M. Nitz. Multiplexed protease assays using element-tagged substrates. *Anal. Biochem.* **2011**, *408*, 157.
[21] J. J. Hu, F. Liu, H. X. Ju. Peptide code-on-a-microplate for protease activity analysis via MALDI-TOF mass spectrometric quantitation. *Anal. Chem.* **2015**, *87*, 4409.
[22] M. M. Gómez-Ramos, C. Ferre, O. Malato, A. Agüera, A. R. Fernández-Alba. Liquid chromatography-high-resolution mass spectrometry for pesticide residue analysis in fruit and vegetables: screening and quantitative studies. *J. Chromatogr. A* **2013**, *1287*, 24.
[23] X. G. Gu, G. Yang, G. X. Zhang, D. Q. Zhang, D. B. Zhu. A new fluorescence turn-on assay for trypsin and inhibitor screening based on graphene oxide. *ACS Appl. Mater. Interfaces* **2011**, *3*, 1175.
[24] P. Miao, T. Liu, X. X. Li, L. M. Ning, J. Yin, K. Han. Highly sensitive, label-free colorimetric assay of trypsin using silver nanoparticles. *Biosens. Bioelectron.* **2013**, *49*, 20.
[25] P. Wu, T. Zhao, J. Y. Zhang, L. Wu, X. D. Hou. Analyte-activable probe for protease based on cytochrome C-capped Mn:ZnS quantum dots. *Anal. Chem.* **2014**, *86*, 10078.
[26] M. A. Hanson, T. K. Oost, C. Sukonpan, D. H. Rich, R. C. Stevens. Structural basis for BABIM inhibition of botulinum neurotoxin type B protease. *J. Am. Chem. Soc.* **2000**, *122*, 11268.
[27] Y. Q. Chen, R. A. McClure, Y. P. Zheng, R. J. Thomson, N. L. Kelleher. Proteomics guided discovery of flavopeptins: anti-proliferative aldehydes synthesized by a reductase domain-containing nonribosomal peptide synthetase. *J. Am. Chem. Soc.* **2013**, *135*, 10449.
[28] A. F. Kisselev, W. A. van der Linden, H. S. Overkleeft. Proteasome inhibitors: an expanding army attacking a unique target. *Chem. Biol.* **2012**, *19*, 99.
[29] A. Paju, U. H. Stenman. Biochemistry and clinical role of trypsinogens and pancreatic secretory trypsin inhibitor. *Crit. Rev. Clin. Lab. Sci.* **2006**, *43*, 103.
[30] D. M. Goldberg. Proteases in the evaluation of pancreatic function and pancreatic disease. *Clin. Chim. Acta* **2000**, *291*, 201.
[31] A. E. Altshuler, A. H. Penn, J. A. Yang, G. R. Kim, G. W. Schmid-Schönbein. Protease activity increases in plasma, peritoneal fluid, and vital organs after hemorrhagic shock in rats. *PLoS One* **2012**, *7*, e32672.
[32] I. Németh, S. Juhász. A trypsin and chymotrypsin inhibitor from the metacestodes of *Taenia pisiformis*. *Parasitology* **1980**, *80*, 433.
[33] S. T. Jiang, J. W. Wu, M. J. Su, S. S. Tzeng. Purification and characterization of calpastatin from grass prawn muscle (*Penaeus monodon*). *J. Agric. Food Chem.* **2000**, *48*, 3851.

