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REVIEW PAPER

Recent Development of Highly Sensitive Protease Assay Methods: Signal Amplification through Enzyme Cascades

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Abstract Proteases are involved in almost all biological processes, and therefore, aberrant activity of many of these enzymes is an important indicator of disease. Various methods have been developed to analyze protease activity, among which, protease assays based on resonance energy transfer are currently used most widely. However, quantitative methods with relatively higher sensitivity are needed, especially for disease diagnosis at early stages. One of the strategies to achieve higher sensitivity is to implement signal amplification of the protease activity. In this review, we briefly summarize the protease assay methods based on resonance energy transfer, and then elaborate the efforts to develop sensitive protease assays through signal amplification by using enzyme cascades.

Keywords: protease assay, enzyme cascade, signal amplification, resonance energy transfer

1. Introduction

Proteases (or proteolytic enzymes) hydrolyze the peptide bond of proteins by recognizing the side chains of specific amino acid sequences. Approximately 2% of human genes encode proteases or their homologs, and proteases are involved in various biological processes, such as development, immunity, blood clotting, and wound healing [1,2]. Therefore, aberrant protease activity is associated with various diseases, including cancer, cardiovascular disease, Alzheimer disease, inflammatory disease, and virus-related diseases [3-7]. To treat diseases resulting from hyperactivity of proteases, small molecule inhibitors have been developed. Inhibitors against matrix metalloproteinase (MMP) and cathepsin, which play an important role in cancer metastasis, have been actively investigated [4,8,9]. Protease inhibitors against the HIV protease are one of the most successful approaches to controlling the disease [7]. On the other hand, in diseases that are characterized by low or no protease activity, recombinant proteases are introduced into humans; for example, Factor IX for hemophilia B and tissue-plasminogen activator (tPA) for breakdown of blood clots [10]. Because of this relevance of proteases to disease states, the activity of specific proteases is an important indicator in the diagnosis of many diseases. For example, serine protease kallikrein 3, also known as prostate specific antigen, is a diagnostic marker for prostate cancer [11]. Cathepsin, urokinase, and MMPs are also known as markers of cancers [4,8,12,13]. In addition, high activity of calpain is reported to be associated with altered calcium homeostasis, resulting in various pathologies [14].

A number of methods have been developed to assay proteases. Immunoassays that rely on capturing proteases of interest by using specific antibodies have been developed to detect their abundance [15]. However, it should be noted that the protease activity, rather than its quantity, is indicative of disease states. Consequently, the immunological methods have limitation, and are rarely applied in screening protease inhibitors. Traditional biochemical methods, such as liquid chromatography and gel

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electrophoresis, have also been used to measure the activity of proteases [16]; however, most of them are time-consuming, and thus, cannot be easily adapted for high-throughput analysis of samples.

Recently, resonance energy transfer (RET)-based approaches have been actively investigated [17,18]. Two molecules, involved in RET, are linked by peptide substrates and cleavage of the substrate separates the two molecules and alters attributes of detectable signals. In the first part of this review, we summarize these methods briefly. Early diagnosis of disease is closely associated with the likelihood of success in treatment of many diseases, and methods with high sensitivity and low noise are needed to facilitate early diagnosis. In the later parts, we introduce one of the strategies for developing sensitive protease assays, in which the signals generated by proteases are amplified through a process of enzymatic cascade.

2. Protease Assays Based on RET

RET or fluorescence resonance energy transfer (FRET) are processes in which a donor chromophore, in its excited state, transfers energy to an acceptor chromophore (typically, at a distance closer than 10 nm to the donor) *via* non-radiative dipole-dipole coupling. In the protease assays based on FRET, the donor and acceptor molecules are linked together through short peptide substrates, and cleavage of the peptides results in reduction of the FRET efficiency (Fig. 1) [17,18]. Two types of systems have been developed. In the first system (Fig. 1A), the donor is a fluorophore, and the acceptor is a quencher. The emission spectrum of the donor overlaps with the absorption spectrum of the acceptor, and the quencher reduces the intensity of fluorescence from the fluorophore. Upon hydrolysis of the peptide substrate by protease, the donor and the acceptor move apart, and the emission of light from the fluorophore is restored. In the second configuration, a second fluorophore instead of the quencher can be linked to the donor fluorophore, (Fig. 1B), and the acceptor absorbs light from the donor and emits light of a different wavelength. Cleavage of the peptide linker between the two fluorophores results in an increase in the fluorescence from the donor, and in the second system, a reduction or elimination of the acceptor fluorescence.

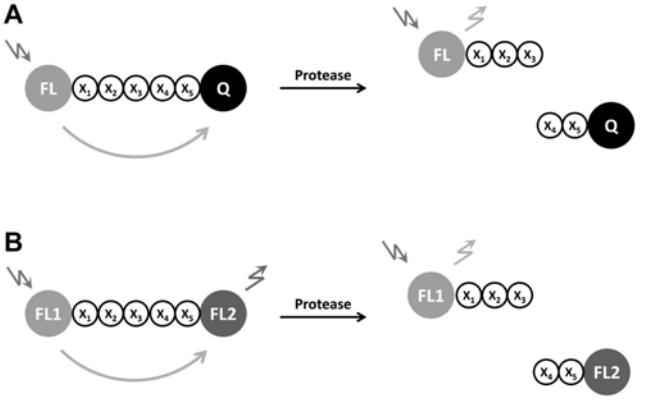

Fig. 1. Protease assay methods based on fluorescence energy transfer (FRET). The donor chromophore (green ball) is linked *via* protease substrates (X_1–X_5) to the quencher (black ball) (A) or acceptor chromophore (red ball) (B). Cleavage of the linker restores the fluorescence from the donor, and in the second method, reduces the acceptor fluorescence.

FRET methods have been widely used to assay protease activities because of its advantages, especially coming from the fact the signal is based on fluorescence [17,18]. Compared to the traditional biochemical methods, the signal-to-noise ratio is high, the signal can be measured without any additional purification step after reaction, and the assay cost is generally low. In particular, the platform can be easily developed into a high-throughput assay method, which makes it possible to analyze the samples in parallel for determining the substrate specificity of a protease or for finding protease inhibitors [19-22]. The chemical compounds inhibiting proteases can then be developed further into therapeutics for diseases resulting from hyperactivity of proteases. In addition, these assays can also be used to reverse-engineer desired substrate specificity for a given protease. For instance, a high-throughput FRET-based method for assaying OmpT endopeptidase activity was used for engineering the substrate specificity of the enzyme [23,24].

Various organic dyes have been used as donors and acceptors [18,25]. In particular, with the development of new chromophores with different absorption and emission wavelengths, various combinations are now possible, which enables multiplexed analyses of samples. Despite advantages with using synthetic organic chromophores, their applications are limited because of some unfavorable inherent photophysical properties [17]. Recently, much attention has been focused on synthesis of nanoparticles with enhanced properties, such as photostability, absorption capacity, quantum yield, and fluorescence lifetime, compared with those of organic dyes [17,18].

With the discovery and engineering of new fluorescent proteins, protein chromophores have emerged as alternatives to synthetic probes [26]. Fluorescent proteins are linked to synthetic compounds, such as organic dyes or nanoparticles, or two fluorescent proteins are connected to each other through a peptide linker. In the latter case, the entire protease sensor system can be expressed in a recombinant form in engineered cells, and the protein FRET pairs can be used for monitoring protease activity inside the cells. However,

synthetic chemical compounds generally have difficulty in penetrating the cellular membrane, thus, limiting their application *in vivo*.

One limitation of FRET-based methods that employ fluorescent chromophores as donors is the requirement for external luminescence to initiate fluorescence transfer. In addition, nanoparticles are not usually used as acceptors because they have broad absorption spectra, and thus, can be excited by external light that is used for exciting donor probes. In order to overcome these drawbacks, the luciferase enzyme has been investigated as a source for photon emission, in a process referred to as bioluminescence resonance energy transfer (BRET) [17]. Luciferase oxidizes luciferin into oxyluciferin, which relaxes back to the ground state by emitting a yellow-green light. Because an external light source is not needed for excitation, BRET methods have a lower background signal and usually have higher sensitivity than FRET-based methods.

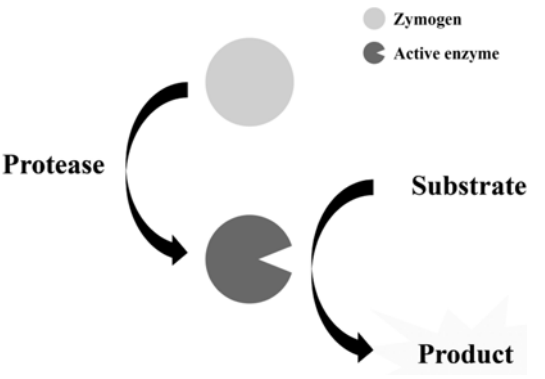

Fig. 2. Signal amplification strategy using enzyme cascades. A protease activates a zymogen into an active one, which then generates a detectable signal. The activated enzyme can convert more than one substrate; thus, the protease activity signal is amplified, and the readout signal is theoretically higher than that generated directly by the protease activity, such as the signal generated in Fig. 1.

3. Amplification of Protease Activity Signal through Enzyme Cascades

Detection of small amounts of marker proteins enables disease diagnosis at early stages, which often correlates with success in the treatment of these diseases. Quantitative methods with high sensitivity and low noise are crucial for developing these diagnostic methods. Even though FRET-based protease assay methods have significantly improved with advancements in technologies for synthesizing fluorescent molecules and for light-detection instruments, there is still a need to develop new assay methods with higher sensitivity, while maintaining at least the same level of noise. One strategy for improving the assay sensitivity is to take advantage of a signal amplification processes *via* enzyme cascades (Fig. 2 and Table 1). In these methods, a protease of interest activates another enzyme by removing the restrictions on the enzyme *via* a cleavage event, and the activated enzyme then generates detectable signals. These methods include signal amplification steps by reporter enzymes, and the degree of amplification is dependent on the catalytic activity of the activated enzymes. In the remaining part of this review, we will summarize recent research focused on developing methods of measuring protease activity utilizing the enzyme cascades.

3.1. Pro-protease protease sensors

A recent report described an assay method for enteropeptidase, which employs a naturally occurring enzyme-

Table 1. Protease assay methods developed using enzyme cascades

Reporter system			Protease	Reference
Zymogen	Substrate	Signal		
Trypsinogen	FRET peptide	Fluorescence	Enteropeptidase	[27]
Pro-urokinase	Chromogenic peptide	Absorbance	MMPs	[28]
ER-luciferase fusion protein	Luciferin	Luminescence	Caspase 3	[30]
Circularly permutated luciferase	Luciferin	Luminescence	Caspase 3/7 Caspase 8 Enteropeptidase TEV Presission PSA SARS 1	[29]
Autoinhibited split luciferase	Luciferin	Luminescence	TEV Caspases	[40, 41]
Autoinhibited split lactamase	Fluorocillin	Fluorescence	TEV	[40]
Phospholipase A_2	NBD C_6-HPC	Fluorescence	SUMO protease	[34]

cascade reaction. Enteropeptidase is a serine protease and converts trypsinogen into active trypsin by cleaving the N-terminal peptide (DDDDK) of the zymogen [27]. In this assay, trypsinogen serves as the substrate for enteropeptidase, and the activated trypsin generated by the action of enteropeptidase cleaves a FRET peptide substrate. The signal generated by enteropeptidase is amplified by the catalytic activity of trypsin. To develop the assay method, the authors first optimized FRET peptide substrates of trypsin based on the FRET efficiency and its cleavage efficiency/specificity, and further tested a range of enteropeptidase concentrations, using the optimized substrates and trypsinogen. The limit of detection (LOD) was around 200 fM enteropeptidase in a complex biological mixture of *Escherichia coli* lysate, as well as in a buffered solution. This is probably the lowest LOD reported for any enteropeptidase assay, and the improved sensitivity is probably attributable to the signal amplification by the enzymatic cascade. An enteropeptidase assay is, perhaps, of limited clinical significance. However, the authors mentioned that the system can be adapted for the detection of any other protease. To apply this strategy for other proteases, the N-terminal sequence of trypsinogen should be engineered in such that the protease of interest cleaves the modified sites; while the engineered trypsinogen remains inactive. One, and seemingly the only, approach is to insert a peptide sequence that the protease of interest recognizes and cleaves between the N-terminal peptide and trypsin. However, small changes in protein sequences can alter the function/structure of proteins, and additional studies, especially using proteases that are disease markers, are necessary to generalize the strategy employed in this study.

Verheijen *et al.* took a similar approach to develop an assay method for MMPs, which are known to be involved in cancer metastasis [28]. Pro-urokinase (pro-uPA) is activated to urokinase (uPA) through proteolytic cleavage by plasmin, and the activity of uPA can be detected by using a chromogenic substrate (pyro-Glu-Gly-Arg-p-nitroanilide). To engineer a form of pro-uPA that can be activated by MMPs instead of by plasmin, the authors replaced the sequence of amino acids in pro-uPA that is recognized by plasmin (PRFL ↓ IIGG, where the arrow indicates the cleavage site) with one that can be hydrolyzed by many MMPs (RPLG ↓ IIGG). Using this system, the authors observed an increased MMP activity in synovial tissue extracts from patients with rheumatoid arthritis, compared to the activity of extracts from patients with osteorarthritis. The samples tested in this study might not have detectable uPA activity, enabling detection of increased activity. However, blood and urine, which are most widely used for disease diagnosis, have significant activity of the reporter enzyme, uPA, which definitely imposes a limitation on the clinical application of this assay method.

3.2. Engineered firefly luciferase protease sensors

Firefly luciferase catalyzes the oxidation of firefly luciferin in the presence of Mg·ATP and oxygen into oxyluciferin, which emits a yellow-green light upon relaxation. Luciferase provides high sensitivity and a wide dynamic range, and it has been widely used as both an *in vivo* and an *in vitro* bioluminescence reporter. In an attempt to exploit the beneficial properties of luciferase, Fan *et al.* engineered luciferase asprotease sensors [29]. Firefly luciferase has 2 domains, a large N-terminal domain and a small C-terminal domain; both domains are connected through a hinge-like linker. The C-terminal domain rotates and translocates to the N-terminal domain upon substrate binding (Fig. 3A). Based on these structural features, the authors engineered a circularly permuted construct: the N- and C-termini were connected through a linker that included sequence cleavable by a protease, and new C- and N-termini were created at the 233 and 234 positions, respectively (Fig. 3B). The linker restricts the conformational change induced by substrate binding, and the circularly permuted luciferase exhibits very little luminescence, several 1,000-fold lower than the wild-type enzyme. However, protease treatment increases the luminescence, in the range of 100 ~ 1,000-fold, depending on the specific protease and cleavable sequence used.

Laxmane *et al*. took another approach to engineer luciferase enzymes with attenuated activity, which can be activated by the action of caspase-3 [30]. The authors constructed caspase-3 reporters by fusing the estrogen receptor regulatory (ER) domain to the C-terminus alone, or to both the N- and C-termini of firefly luciferase, with a caspase-3 cleavable sequence (DEVD) in the linker region between ER and luciferase (Fig. 3C). The fusion of the ER domain silences the activity of the reporter, and cleavage of the linkers by caspase-3 restores the luciferase activity. The mechanism by which the ER domain inhibits luciferase has not been elucidated; however, the association of the ER domain with heat shock proteins seems to play a role in the attenuation of luciferase activity [30]. In the case that both N- and C-termini of luciferase are fused to the ER domain, the cleavage of the linkers resulted in approximately 10-fold increase in luminescence. In the study, the caspase-3 activity induced by tumor necrosis factor α-related apoptosis-inducing ligand (TRAIL) was monitored real-time in tumors implanted in mice.

Despite the successful applications of the engineered luciferases, the systems have an important limitation. The enzymes are unstable in their purified forms, and the applications are restricted to *in vivo* bioluminescence or to *in vitro* analyses in an unpurified state, such as in cell lysate

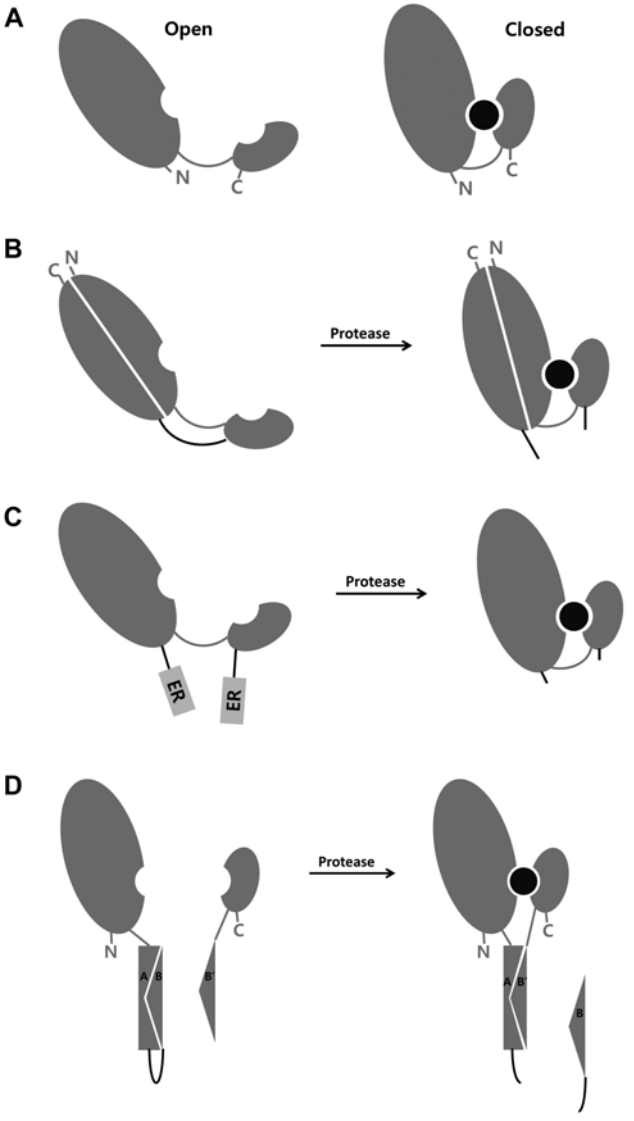

Fig. 3. Engineered luciferase zymogens. The action of a protease activates the luciferase zymogens into active luciferases, generating bioluminescence signals. (A) Models of firefly luciferase in the open conformation (without bound substance) or in the closed conformation (with the luciferyl-adenylate reaction intermediate shown as black circles) (adapted from reference [29]). (B) Circularly permuted luciferase. Linking the N- and C-terminal domains with a protease-cleavable peptide (black line) restricts the conformational change induced by substrate binding (adapted from reference [29]). (C) ER-luciferase fusion protein. Fusion of the estrogen receptor regulatory (ER) domain to the N- and C-termini of firefly luciferase *via* a linker, composed of a protease-cleavable peptide (black line), silences the activity of the reporter [30]. (D) Autoinhibited split luciferase. The N-terminal domain of firefly luciferase is linked to a heterodimeric coiled-coil (A-B), in which the two coils are connected *via* a protease-cleavable peptide (black line), and the C-terminal domain is linked to another coil (B'). The coiled-coil (A-B) is autoinhibited, and complementation of the two split fragments is thus prevented [40].

and in cell-free translation reactions. However, a system with a defined composition with purified proteins is needed in many cases to develop standardized diagnostic methods for disease detection. There are more stable luciferase enzymes from other organisms [31], but they have to be engineered to have the desired properties, which can sometimes be extremely difficult.

3.3. SUMO-PLA_2 fusion-protein protease sensors

Sumolyation, wherein a small ubiquitin-like modifier (SUMO) is covalently attached to the ε-NH_2 group of a lysine residue in proteins, through a series of enzymes (E1 activating enzyme, E2 conjugating enzyme, and E3 ligase), is a posttranslational modification and is known to be involved in many cellular processes, such as transport, transcription, apoptosis, and protein stability [32]. Sentrin-specific proteases (SENPs) are isopeptidases that remove SUMO moieties from proteins [32]. Sumolyation is a dynamic process, and thus, the activity of SUMO proteases is important in understanding SUMO-related biological processes. Phospholipase A_2 (PLA_2) is an enzyme that releases fatty acids from the second carbon of glycerol and hydrolyzes fluorogenic substrates, such as 2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (NBD C_6-HPC). The enzyme requires a free amino terminus for catalytic activity [33]. By using these features of PLA_2, the researchers at Progenra developed an assay platform for SENPs by constructing a fusion protein, SUMO-PLA_2, which itself is inactive [34]. SENPs remove SUMO from the fusion protein, and the activated PLA_2 hydrolyses NBD C_6-HPC and generates a fluorescence signal. Leach *et al*. used this assay system to determine Km values and to characterize inhibitors of SUMO proteases [34].

3.4. Autoinhibited split-protein protease sensors

Split protein (or protein fragment) complement methods have been widely used to study biomolecular interactions [35,37]. A protein is split into two fragments, and each fragment is fused to proteins of interest. When the two proteins interact with each other directly, or *via* another molecule, the split fragments are assembled into an active protein. Autoinhibition is a naturally occurring mechanism for regulating protein activity: the protein has a polypeptide pseudosubstrate that blocks access to its active site, and the inhibitory region can be displaced through a conformational change or removed by a cleavage [38,39]. Shekhawat *et al*. employed these two mechanisms for regulation of protein activity, split protein complementation, and autoinhibition, to engineer protein reporters that can be activated by the action of protease (Fig. 3D) [40]. They used previously developed split protein complement systems: firefly luciferase and β-lactamase. One half of the reporter is linked to an antiparallel heterodimeric coiled coil (A-B), in which the

two coils are linked through a protease-cleavable linker, and the other half is linked to a coil (B'), which can dimerize with A. The heterodimeric coiled coil (A-B), which is autoinhibited, has a very low binding affinity to B', and the complementation of the 2 fragments is, therefore, unlikely. However, when a protease cleaves the linker connecting A and B, the interaction between A and B' can occur, and the split halves then assemble into an active reporter, such as luciferase or β-lactamase. Using these systems, the authors developed and optimized protease sensors for the TEV protease and caspase-3. The best luciferase sensor for TEV provided a 1,000-fold increase of signal after protease treatment. In addition, logic gates were constructed by fusing autoinhibited heterodimer coiled-coils to both split halves.

In a later study, the same group reported a panel of caspase sensors, which were used to investigate the substrate specificities of caspases and caspase activation pathways [41]. The protein sensors in this study were expressed using cell-free translation systems and then used to assess caspase activity in mammalian cytosolic extracts. However, it is plausible that the commercially available kits for *in vitro* translation would include proteases that activate the sensors. Actually, when the caspase sensors were expressed using a rabbit reticulocyte lysate (RRL), significant luminescence signals were observed without the addition of a caspase, which was attributed to endogenous caspase activity in RRL. In addition, as mentioned above, the sensors must be expressed immediately before analysis, which can be an obstacle in developing standardized methods for analysis or diagnosis.

3.5. Signal amplification *via* cycling probe technology

Cycling probe technology (CPT) is a technique originally developed for detecting specific DNA sequences by using the unique property of RNase H, which hydrolyzes the 3'-O-P bond of RNA in a DNA/RNA duplex [42]. Kim and Chung exploited the advantages of CPT to amplify the signal of a protease activity assay. This strategy differed from the ones that use the enzyme cascades that have been described so far, but the signal amplification method is worth introducing in this review [43]. The system used in this study included two kinds of gold nanoparticles (GNPs), GNPa, in which the GNP is conjugated to a peptide-DNA complex, and GNPb, in which the GNP is conjugated to a RNA-fluorescein isothiocyanate (FITC) complex. GNPb particles were also pegylated to minimize heteroduplex formation between the GNPa DNA and GNPb RNA. Thus, RNase H was prevented from cleaving the single-stranded RNA of GNPb, and the fluorescence of FITC was quenched by GNPs. When a protease of interest cleaves the peptide sequence linking the DNA and GNPa, the DNA oligomers are released from the nanoparticles and diffuse, eventually forming DNA/RNA duplexes with the RNA in GNPb. Duplex formation allows RNase H to hydrolyze the RNA linked to GNPb, and the quenched fluorescence of FITC is thus recovered. DNA oligomers, which are a product of the protease activity, are not consumed by the RNase H reaction, the cycle of duplex formation and RNA hydrolysis can be continued, serving as the signal amplification step. The authors applied this system to the MMP2 protease, and the sensitivity of the protease was improved, to the detection of MMP2 at levels as low as 10 pM.

4. Conclusion

Proteases play important roles in diverse biological processes and are important markers of a number of diseases. Various methods have been developed to analyze their activities, and the protease assay methods based on RET are, currently, the most common approaches. However, new tools with relatively higher sensitivity and at least the same level of noise, if not a further reduction in noise, are still needed. One solution to resolve this problem are the approaches that rely on amplifying the signal of protease activity by using enzyme cascades, and several specific strategies have been attempted so far. Improved sensitivity has been demonstrated for these methods, but the optimization of these methods or development of new ones is required for application to diverse purposes.

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