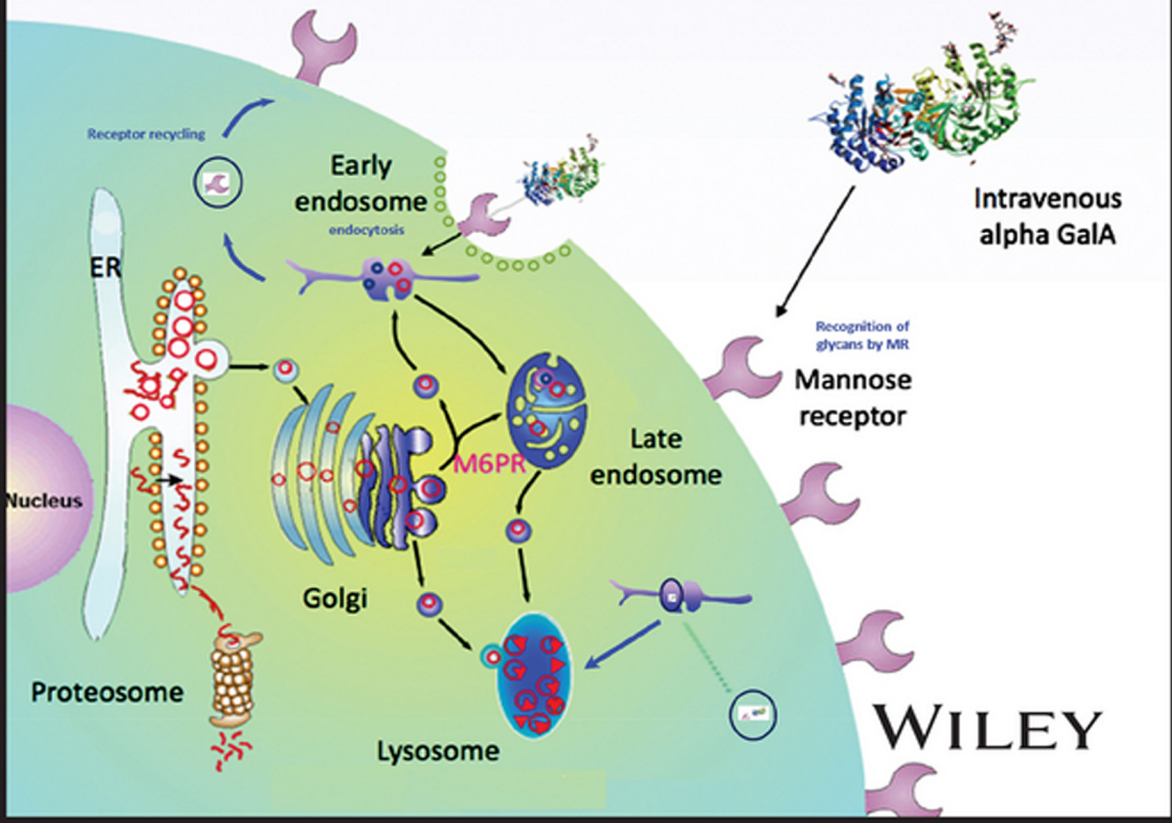
Chemical Biology of Enzymes for Biotechnology and Pharmaceutical Applications

Enzyme Technologies

Pluripotent Players in Discovering Therapeutic Agents

Edited by

Hsiu-Chiung Yang • Wu-Kuang Yeh • James R. McCarthy



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ENZYME TECHNOLOGIES

CHEMICAL BIOLOGY OF ENZYMES FOR BIOTECHNOLOGY AND PHARMACEUTICAL APPLICATIONS

(A Series Consisting of Three Volumes)

Volume I. *Enzyme Technologies: Metagenomics, Evolution, Biocatalysis, and Biosynthesis*
Editors: Wu-Kuang Yeh, Hsiu-Chiung Yang, and James R. McCarthy

Volume II. *Enzyme Technologies: Pluripotent Players in Discovering Therapeutic Agents*
Editors: Hsiu-Chiung Yang, Wu-Kuang Yeh, and James R. McCarthy

Volume III. *Enzyme Technologies: Two Facets of Modern Structure-Based Design*
Editors: James R. McCarthy, Hsiu-Chiung Yang, and Wu-Kuang Yeh

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PREFACE

The human genome was predicted to contain approximately 2742 genes that encode enzymes, which corresponds to 9.5% of the genome (HumanCyc version 7.5). These predicted enzymes can be subdivided into 1653 metabolic enzymes and 1089 nonmetabolic enzymes (including enzymes whose substrates are macromolecules, such as protein kinases and DNA polymerases). Enzymes play an important role in human physiology and the pathophysiology of disease. Understanding the function of an enzyme presents a significant opportunity for finding therapeutic agents. Therefore, a comprehensive understanding of all aspects of enzyme technology is critical in discovering therapeutic agents targeting enzymes, therapeutic enzymes, and enzyme-based applications, enabling research in drug discovery, chemistry, material science, and a vast number of other fields in science and technology.

This series on Chemical Biology of Enzymes for Biotechnology and Pharmaceutical Applications consists of three volumes. Volume I, *Enzyme Technologies: Metagenomics, Evolution, Biocatalysis, and Biosynthesis*, was published in 2010. Volume II, *Enzyme Technologies in Drug Discovery*, as listed in Volume I, has now been changed to *Enzyme Technologies: Pluripotent Players in Discovering Therapeutic Agents*. This book is intended both for biotech and pharmaceutical scientists in academic research institutes and industry as a comprehensive reference material for all common applications of enzyme technology in drug discovery. This book will be useful for biotechnology, biochemistry, molecular biology, and medicinal chemistry faculty members for teaching or conducting research in the field of enzyme technology. It is also a practical handbook for industrial scientists to study various aspects of enzyme technology and discover new treatments for unmet medical needs.

This book is divided into three parts: Part A: Enzymes – Essential Workhorses in Pharmaceutical Research; Part B: Enzymes – Indispensable Tools for Improving Druggability; and Part C: Enzymes – Powerful Weapons for Correcting Nature's Errors. Part A consists of four chapters. Chapter 1, by Dr. A. Roy et al., discusses the principles of assay development and cutting-edge technologies available for protease assays, using proteases as a prototype. Chapter 2, by Drs. Ohshiro and Tomoda, provides a case study on the design and development of selective enzyme inhibitors, using lipid metabolizing enzymes as a prototype. There is a belief that covalent enzyme inhibitors (also called "irreversible inhibitors") are not desirable for drug candidates. Chapter 3, by Dr. Mehdi, describes methods for characterizing covalent inhibitors and their therapeutic applications and explains how enzyme kinetics has been applied in drug discovery. Chapter 4, by Drs. Yeh and Peterson, provides a comprehensive coverage on various technologies that have been applied for *in vitro* enzymatic assays, as well as on common *in vivo* models to assess preclinical drug discovery for metabolic diseases. After going through this part, readers will have a better understanding as to how to select the best enzyme targets for drug discovery, the steps involved in designing enzyme inhibitors for therapeutic agents, and methods for evaluating selective enzyme inhibitors.

Part B consists of three chapters. It explains the principles of improving druggability and provides examples on how to utilize the properties of enzymes for designing therapeutic agents, specifically prodrugs. Chapter 5, by Dr. Hu et al., provides a comprehensive review of enzymes that are being, or can be, used to design prodrugs to improve druggability of existing drug molecules. Chapters 6 and 7 summarize case studies on the design of two successful prodrugs. Chapter 6, by Dr. McCarthy, provides a detailed approach and explains the hypothesis and rationale for the design and synthesis of a Gemcitabine prodrug. Chapter 7, by Drs. McKenna and Krylov, presents several examples of successful prodrug approaches that explicitly depend on enzyme-mediated activation. After going through this part, readers will have a complete understanding on how to select best target molecules for the prodrug approach to improve druggability and how to design successful prodrugs.

Part C consists of three chapters and provides a different viewpoint as to how enzymes can be used in pharmaceutical applications. There are many types of genetic in-born error disorders. Some of these disorders are involved in either deficiency or malfunctioning of specific enzymes. In such cases, functional enzymes can be reintroduced into patients. With recent advances in protein technology, there are several successful examples, such as the production of high-quality proteins and optimal methods for the delivery of these large molecules to the body. However, further improvements on either manufacturing or delivery of enzymes for therapeutics are still required. Chapter 8, by Dr. Beck, deals with Hunter's syndrome, while Chapter 9, by Dr. Lacbawan et al., discusses enzyme replacement therapy for Fabry disease. Chapter 10, by Dr. von Schassen et al., analyzes the level and activity of pancreatic enzymes as a means of diagnosing patients with pancreatic dysfunction, for example, Hunter's syndrome. After

going through this part, readers will have an insight into how enzymes can be applied as therapeutic agents or diagnostic tools. Furthermore, readers may be able to identify possible enzyme targets to treat genetic disorders that still do not have effective medication.

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PART A

ENZYMES – ESSENTIAL WORKHORSES IN PHARMACEUTICAL RESEARCH

1

ASSAY TECHNOLOGIES FOR PROTEASES

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I. INTRODUCTION

Proteases are ubiquitously expressed enzymes which catalyze hydrolysis of peptide bonds and work under a wide range of conditions using diverse catalytic mechanisms [1]. Proteases specifically cleave protein substrates either from the N- or C-termini (aminopeptidases and carboxypeptidases, respectively) or in the middle of the molecule (endopeptidases) [2]. Proteolytic enzymes modulate many physiological processes ranging from nonspecific hydrolysis of dietary proteins to highly specific and regulated proteolysis in cell cycle regulation, tissue remodeling,

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blood coagulation, blood pressure control, angiogenesis, apoptosis, inflammation, ovulation, fertilization, and embryonic development [3,4]. Over 500 proteases each from humans, rat, mouse, and chimpanzee have been annotated and compiled in the Degradome database (http://degradome.uniovi.es) [5,6]. Information on all known proteases and their substrates/inhibitors is listed in the MEROPS database [7]. Based on the amino acid or metal that catalyzes the nucleophilic attack on substrate peptide bonds, the proteases are classified into five major types: aspartic (Asp), metallo-, cysteine (Cys), serine (Ser), and threonine (Thr) proteases. Aspartic and metalloproteases use an activated water molecule as a nucleophile to attack the peptide bond of the substrate, whereas in Cys, Ser, and Thr proteases, a catalytic amino acid residue (Cys, Ser, or Thr, respectively) serves as a nucleophile (Fig. 1). As a result, acyl–enzyme intermediates are formed only in the reactions catalyzed by Ser/Thr and Cys peptidases. Within each class of protease type are several enzymes that may have overlapping or distinct substrate recognition sites. Rawlings and Barrett proposed a classification of proteases into families based on amino acid sequence similarity, and families with similar three-dimensional folding are assembled into clans, indicating common ancestry [7,8]. The focus of this article is mainly on mammalian proteases and retroviral proteases which are of significant therapeutic relevance.

While pepsin in gastric juices digests a variety of proteins with broad specificity, renin is an example of Asp protease that shows high substrate specificity. Most proteases bind their substrates in fairly similar manner, first elucidated for papain by Schechter and Berger [9–11]. The catalytic site is flanked on one or both sides by sites that confer specificity of substrate binding to the protease and accommodate a side chain of an amino acid residue of the substrate. The enzymatic binding sites toward the N-terminus of the substrate are the non-prime side designated as S1, S2, …, S*n* from the catalytic site, and the residues C-terminal to the cleavage site are the prime side designated as S1′, S2′, …, S*n*′ [8,12,13]. The amino acid residues in the protein substrate which correspond to their respective subsites are numbered P1, P2, …, P*n* and P1′, P2′, …, P*n*′ (Fig. 1). Only few of the substrate binding sites have stringent specificities. For instance, site S1 confers specificity for Ser proteases and caspases, whereas the site S2, a hydrophobic subsite, defines specificity for the papain family of Cys proteases. In addition to the sites close to the catalytic site of the enzyme, distant sites on the enzyme may also contribute to the binding of substrates to the protease [9]. The specificity and biological activity of caspases are also determined by S4, which is distant from the catalytic site [14]. Proteolytic processing is being recognized as a mechanism for regulation of enzymatic activities, localization, and fate of proteins that are activated by limited and specific hydrolysis of peptide bonds. Dysregulation of proteolytic activity, structure, or expression results in major pathologies in the areas of cardiovascular diseases, cancer, neurodegenerative disorders, osteoporosis, diabetes type II, pancreatitis, inflammation, arthritis, and infectious diseases [4]. A large number of marketed drugs target the proteolytic enzymes that are involved in pathogenesis of various diseases [15] (Table 1). Although only a relatively small number of proteases are currently targeted for

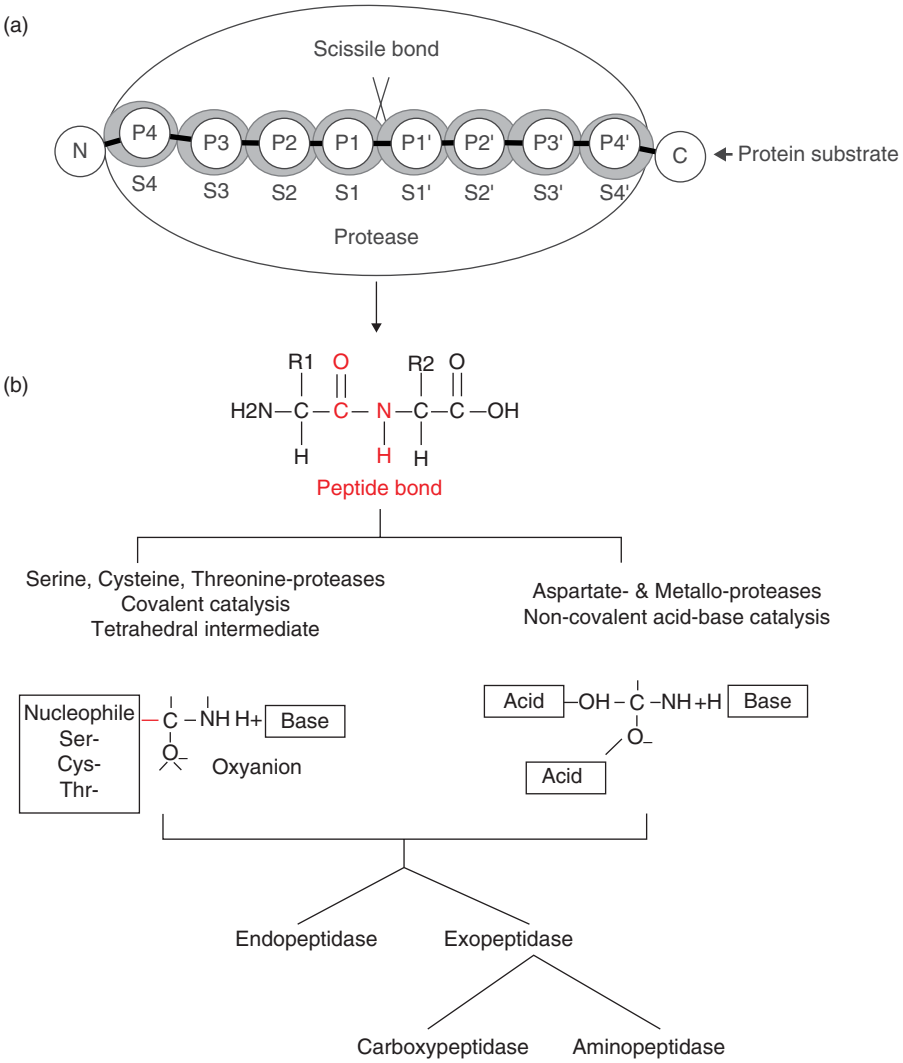

FIGURE 1 Schematic representation of binding of substrate to a protease site. (a) The binding sites of the protease are numbered on either side of the scissile bond, with the non-primed sites (S1, S2, …, S*n*) located toward the amino-terminus of the substrate and S1′ … S*n*′ or the primed subsites toward the carboxy-terminus. (b) Structure of the peptide bond which is hydrolyzed by proteases and the two basic catalytic mechanisms for all types of protease hydrolysis. In Ser, Cys, and Thr proteases, an amino acid at the active site serves as the nucleophile forming a transient covalent intermediate, whereas in metallo- and Asp proteases, an active water molecule functions as nucleophile (adapted from Reference [13]). The base in covalent catalysis is usually a His, and in non-covalent intermediate, Asp/Glu and zinc (metalloproteinases) serve as acids and bases. The proteases are also classified as endo- and/or exo-proteinases based on their ability to cleave within or at the amino-/carboxy-terminus of the peptide chain.

TABLE 1 FDA-Approved Drugs for Select Proteases

Protease	Class	Compound	Company	Indication
HIV-1 protease	Asp	Atazanavir	Bristol-Myers Squibb	AIDS
		Darunavir	Prezista	
		Fosamprenavir	GlaxoSmithKline	
		Indinavir	Merck	
		Lopinavir	Abbott	
		Nelfinavir mesylate	Pfizer	
		Ritonavir	Abbott	
		Saquinavir	Hoffmann-La Roche	
		Tipranavir	Boehringer Mannheim	
Renin	Asp	Aliskiren (Tekturna)	Novartis	Hypertension
ACE	Metallo	Captopril	Bristol-Myers Squibb	Hypertension
		Enalapril	Merck	
		Lisinopril	AstraZeneca	
Carboxypeptidase A	Metallo	Penicillamine		Hypertension
MMP-1 and MMP-2 collagenases	Metallo	Periostat	Galderma Labs	Periodontitis
Enkephalinase	Metallo	Racecadotril		
Thrombin	Ser	Ximelagatran	AstraZeneca	Thrombosis
		Argatroban	Mitsubishi Pharma	
		Lepirudin	Aventis	
		Desirudin	Novartis	
Human FXa	Ser	Fondaparinux	Sanofi-Synthélabo	Thrombosis
Human neutrophil elastase	Ser	Sivelestat	Ono	Respiratory disease
Trypsin-like protease	Ser	Camostat mesilate	Ono	Pancreatitis I
Broad-spectrum protease	Ser	Nafamostat mesilate	Japan Tobacco	Pancreatitis inflammation
Plasminogen activator	Ser	Streptokinase		
Proteasome	Thr	Bortezomib (Velcade)	Millennium	Multiple myeloma
DPPIV	Ser	Pioglitazone	Takeda	Diabetes mellitus type II
		Saxagliptin	Bristol-Myers Squibb	
		Linagliptin	Boehringer Ingelheim	
Cathepsin K	Cys	Odanacatib	Merck	Osteoporosis/ bone cancer
		ONO5334	Ono	Osteoporosis

drug development, the commercial success of angiotensin-converting enzyme (ACE) inhibitors and human immunodeficiency virus (HIV) protease inhibitors makes the protease family a valuable target for disease treatment [16]. We have worked on a variety of protease assay formats using proprietary substrates for HTS and will present an overview of common protease assay technologies. We will end the review by discussing computational approaches to designing substrates for protease binding sites.

II. PROTEASE ACTIVITY ASSAYS

Identification of appropriate substrate is the first major step toward characterizing a protease and developing an assay to monitor its activity. A protein containing a short recognition sequence for a protease may work well in an *in vitro* assay, but may not be a physiologically relevant substrate for the proteolytic enzyme. In cases where the peptide sequences are unknown or ill defined, positional scanning experiments or phage display methods are used for screening combinatorial libraries of peptides for specificity determination. Bioinformatics-based prediction of cleavage sites and determination of protease preferences on synthetic substrates are important techniques in predicting natural protease substrates. Figure 2 summarizes the techniques employed in mapping substrates, a rapidly evolving field which is beyond the scope of this article and has been extensively reviewed [17–20]. The design of sensitive and selective synthetic peptide-substrate cleavage assays and the comprehensive mapping of active site specificity determinants are crucial for developing protease inhibitor drugs. The assays for proteolytic enzymes are generally continuous, homogeneous assays that can be performed in medium- to high-throughput formats. The assays are more relevant when the enzyme activity is measured with their specific native substrates, but assays with long native proteins have low hydrolytic rates and are time-consuming and costly. The substrate peptide sequences for most of the common proteases are very well established, and the fact that most proteases catalyze hydrolysis of small peptides has led to the development of technically simple and sensitive assays using fluorometric, colorimetric, and bioluminescent methods in which a single specific peptide bond is cleaved and the cleavage is monitored spectrometrically. A large number of kits are available from various vendors that serve to assay protease families using generic substrate peptides. In general, the minimal defined peptide substrate (average three to six amino acid residues) is synthesized based on the information on the binding fragments of natural substrates or inhibitors of proteases. In the case of many proteases like caspases, matrix metalloproteinases (MMPs), cathepsins, or HIV-1 proteases, the same substrate peptide or a minor variant of a substrate sequence is conjugated with either fluorophores or chromogenic groups or tagged with aminoluciferin to allow development of fluorescence-, absorbance-, or luminescence-based assay formats. Both cell-based and rapid mix-and-read biochemical methods have been developed for assaying the protease activity. The biochemical assays based on purified recombinant enzymes being

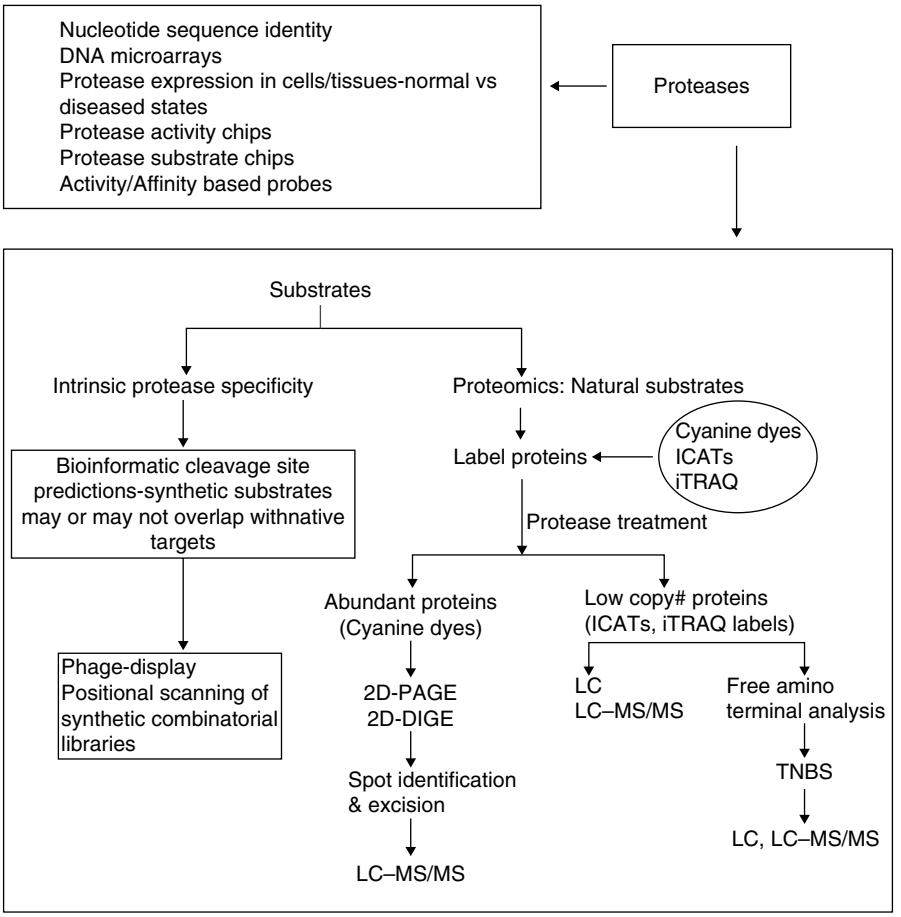

FIGURE 2 Overview of substrate and protease characterization. Protease identification/characterization may initiate from sequence identity/homology, from expression and activity chips, and from loss- or gain-of-function studies in cells or mouse models to establish their role in disease. *In vitro* biochemical hydrolysis and binding experiments based on short synthetic sequences of amino acids are often used to determine protease site binding and hydrolysis. Bioinformatics is useful for building theoretical predictions of substrate sequences, but may differ from real *in vivo* biological targets. Proteomics tools help in the identification of native substrates in cell environment. The protease-treated or protease-untreated cells are labeled with different cyanine dyes (Cy3, Cy5), and the proteins are resolved on two-dimensional (2D) gels (difference gel electrophoresis (DIGE) or 2D DIGE). The substrate and cleavage products are obtained from superimposition of the images from protease treated and controls. The proteins of interest are excised from gels and analyzed by liquid chromatography and mass spectrometry (LC–MS, multidimensional liquid chromatography, MuDPIT). Low-copy-number proteins can be labeled with more sensitive isotope mass tags (isotope-coded affinity tags (ICAT)), or label all primary amines in a trypsinized proteome (iTRAQ), followed by LC and LC–MS/MS. A comprehensive overview of protease substrate identification is available in Overall et al. [1].

more specific are useful for studying kinetics and for the screening of small-molecule libraries or peptidomimetic modulators. Figure 3 shows general aspects of activity assay optimization like defining optimal buffer components, pH, and stability; temperature determination for various substrates of interest; determining the concentration range for the purified protease; and determining the linearity of the reaction. The continuous assays are well suited for miniaturization and adaptation for HTS for identification of protease activity modulators. The modulators identified from the *in vitro* or cell-based assays are generally tested in orthogonal assays to ascertain specificity and selectivity (Fig. 3). The detailed guidelines for development and optimization of any enzyme including proteases for HTS are given in NIH assay guideline manual (http://assay.nih.gov/assay/), which, among others, is a useful reference manual. PubChem (http://pubchem.ncbi.nlm.nih.gov/assay) database lists over 3000 protease activity assays, which were used to identify modulators of protease activity. A few representative assays for proteases compiled from PubChem database are listed in Table 2. The following three major assay formats are widely employed for assaying the activity of various members of protease family:

A. Colorimetric Assays

The colorimetric assays, though less sensitive, have traditionally found more utility in active site titrations for estimating active protease concentrations or for detection of activity in biological fluids. Colorimetric assays based on substrates like amino acid–ethyl, amino acid–methyl, or amino acid–alpha-naphthyl esters were used earlier for quantification of activity of proteases such as trypsin, thrombin, chymotrypsin, pronase, plasmin, and urokinase [21]. These reactions could be used for kinetic analysis and for zymogram studies, but many of these substrates were unstable. The use of tripeptide or other polypeptide substrates afforded higher specificity and stability. In majority of direct colorimetric assays, a substrate peptide (three to five residues) is linked to a chromophore, usually *para*-nitroanilide (*p*-NA). Enzymatic hydrolysis of the substrate releases free *p*-NA, a yellow-colored compound which has high absorbance at 405 nm and is detected spectrophotometrically (Fig. 4a). In the presence of excess substrate concentration, an increase in the rate of absorbance of chromophore released is linearly related to enzyme concentration. A large number of chromogenic substrates are available from Spectrozyme and American Diagnostica for assaying general Ser proteases as well as coagulation factors and kallikreins. For example, ACTICHROME® factor X (fX) is a chromogenic assay for the measurement of fX activity in human plasma. The assay involves activation of fX in the plasma to factor Xa (FXa) by Russell's viper venom. Activated FXa hydrolyzes the Spectrozyme® chromogenic substrate and releases the chromophore, *p*-NA. The color of the reaction solution is read spectrophotometrically at 405 nm. In a large number of colorimetric caspase activity assays, short four-residue peptides linked to *p*-NA serve as substrates. For instance, the DEVD-*p*-NA chromogenic substrate is used to assay caspase-3 and caspase-8 activities in cell lysates. The colorimetric QuantiCleave Protease Assay

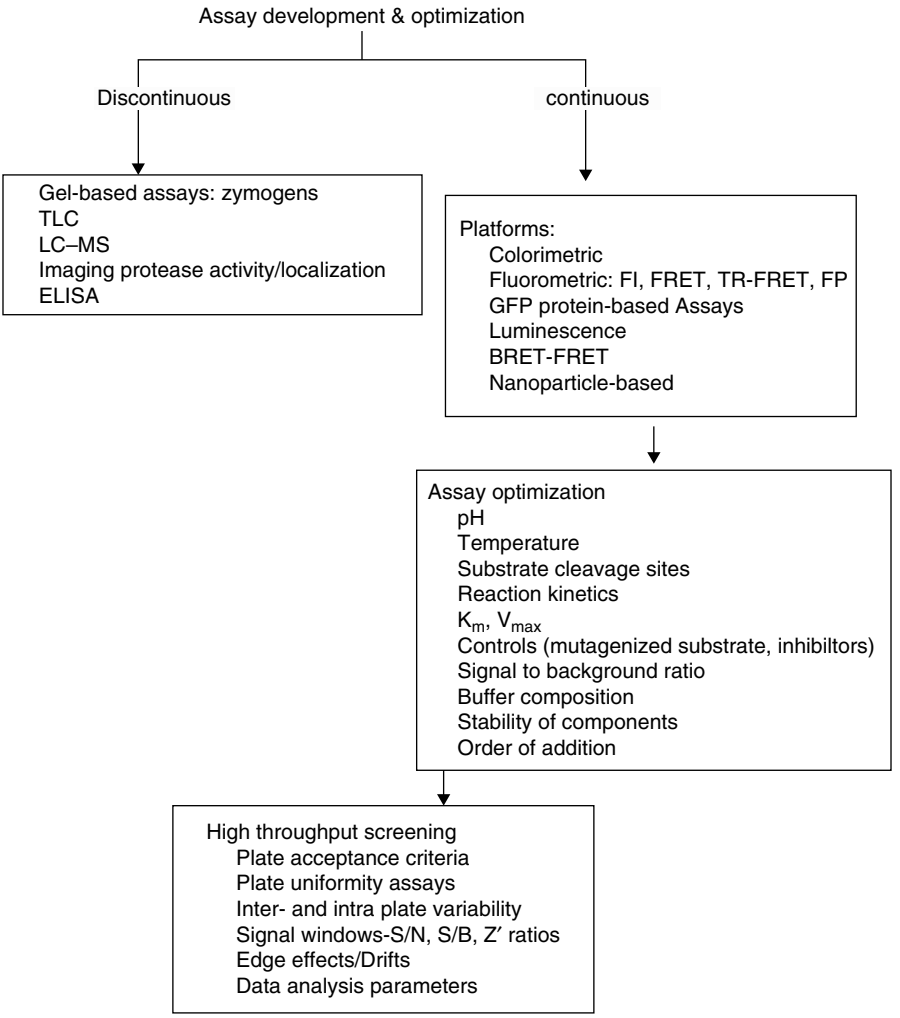

FIGURE 3 Protease activity assay development and optimization. Protease assays can be discontinuous or homogeneous. Examples of discontinuous, low throughput assays include protease activity determination in zymogens, analysis of peptide fragments by TLC or via LC–MS, and imaging of proteases in cell environment by using tagged peptides and ABPs. Biochemical or cell-based assays are continuous assays which are based on colorimetric, fluorometric, or luminescence platforms. The three basic formats can be used in conjunction with nanoparticles or used as such with substrates labeled with appropriate tags. The continuous assays can be further miniaturized and adapted for HTS by further optimizations to conform to stringent statistical acceptance criterion of HTS assays.

TABLE 2 Representative Protease Screens Compiled from PubChem Database

Protease	Assay format	Disease/significance
Cathepsin L	Fluorescence increase by hydrolysis of Z-Phe-Arg-AMC	Proteolysis by cathepsin L required for entry and replication of SARS and Ebola virus
High-temperature-responsive antigen (HTRA-1) Ser protease	FP with FP-TAM probe	Osteoarthritis, AD, and age-related macular degeneration
Insulin-degrading enzyme (IDE), an Abeta-degrading zinc metalloprotease	Fluorescence polarization: fluorescein-Abeta-(1–40)-Lys-biotin (FAbetaB) peptide incubated with IDE dissociates biotin moiety from fluorescein. Addition of avidin to the reaction increases the mass of intact substrate, slowing their rotation rate and decreasing depolarization of plane-polarized light. The low-molecular-weight cleaved substrate rotates rapidly and causes strong depolarization. Ratios determine the relative amounts of cleaved and intact forms of the FAbetaB substrate	AD is characterized by accumulation of amyloid beta-protein
Proteasome, ATP-dependent protease	Flow cytometry: FLAG-tagged, fluorescent proteasomes were captured on anti-FLAG beads, and disassembly was monitored by loss of bead fluorescence in the presence of ATP. The beads were sorted via high-throughput flow cytometer	Inhibitors of proteasome assembly and activity
Sentrin-specific protease-8 (SENP-8)	Luminescence: SENP8-dependent RLRGG-aminoluciferin peptide-substrate hydrolysis – releases aminoluciferin, measurable in a coupled luminescence detection assay	Involved in maturation of SUMO precursors (endopeptidase cleavage) and deconjugation of the targets (isopeptidase cleavage)
SARS coronavirus 3C-like protease	FRET assay: HiLyte Fluor TM 488 is attached at the N-terminus of a 3CLpro peptide substrate (HiLyte Fluor 488-ESATLQSGLRKAK (QXL520)-NH2 (AnaSpec) and is quenched by a QXLTM520 moiety at the C-terminus. Following cleavage, separation of the fluorescent compound and quencher leads to an increase in fluorescence	Cold, lower respiratory tract infections, and diarrhea

(*Continued*)

TABLE 2 (*Continued*)

Protease	Assay format	Disease/significance
Ub-specific peptidase 2 isoform a	Ub-IsoPro1 kit (Progenera, LifeSensors, Inc.): a Ub or ubiquitin-like (UBL) protein conjugated to a reporter enzyme, phospholipase A2 (PLA2), which has an absolute requirement for a free amino-terminus. Fusion of a UBL to the N-terminus of PLA2 inactivates PLA2. When the UBL–PLA2 reporter enzyme is cleaved by USP2, the activated reporter can subsequently act on its substrate, available NBD C6-HPC (Invitrogen), with a fluorescent readout	Ub homeostasis

Source: http://pubchem.ncbi.nlm.nih.gov/assay.

Kit (Thermo Scientific) is based on the usage of modified casein as substrate, in which all surface primary amines have been succinylated. When succinylated casein is cleaved at peptide bonds by proteases, free alpha-amino-terminal groups of peptide fragments are released. The primary amines react with trinitrobenzene sulfonic acid (TNBSA) to form a yellow-colored TNB–peptide adduct [22] which is measured spectrophotometrically at 450 nm (Fig. 4b). The non-succinylated casein serves as a control substrate in these assays. Replacement of the peptide cleavage bond with a thioester bond results in creation of free sulfhydryl in an assay reported for MMPs [23,24]. The free sulfhydryl group reacts with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent) to form 5-nitro-5-thiobenzoate, a colored compound with absorbance at 412 nm. A thiopeptide (Ac-PLG-[2-mercapto-4-methyl-pentanoyl]-LG-OC2H5)-based assay kit for MMP-9 is available from Enzo Life Sciences. Most colorimetric assays have been replaced by more sensitive fluorometric- or luminescence-based formats.

B. Fluorescence Assays

In recent years, homogeneous fluorescence- and luminescence-based assay formats have gained wider acceptance due to higher sensitivity, larger signal-to-noise windows, high adaptability to enzymes from various sources, and requirement for very low volumes of reagents. Fluorescence assays are most commonly employed for developing assay platforms for protease activity [25], and most of the assays are based on conjugating extrinsic fluorophores like fluorescein, rhodamine, and BODIPY dyes to a substrate of interest [26]. The fluorophores with distinct excitation (Ex) or emission (Em) properties can be multiplexed within the same reaction, enabling simultaneous measurements of multiple endpoints. Sensitivity of fluorescence-based assays is dependent on accumulation of cleaved product and may be limited by

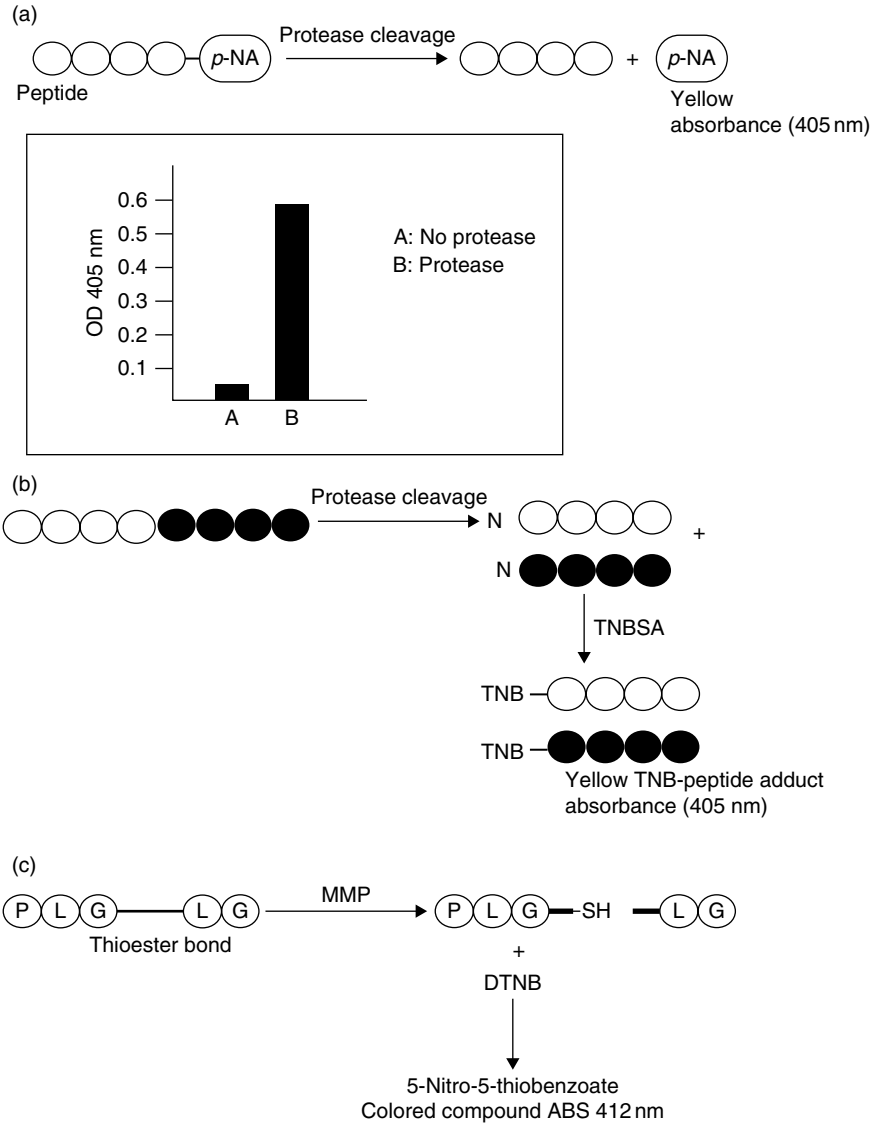

FIGURE 4 Colorimetric assays for proteases. Examples of some colorimetric methods in which protease cleavage is followed by increased absorbance in direct or in coupled assays. (a) Short peptides are conjugated with *p*-NA, which is released following hydrolysis of amino acid–*p*-NA bond, resulting in increased absorbance at 405 nm. (b) TNBSA reacts with the amino-termini of the protease hydrolyzed peptides to increase absorbance at 405 nm. (c) Thioester bond at the peptide cleavage site releases a peptide fragment with reduced sulfhydryl group which reacts with DTNB to form a colored compound with absorbance at 412 nm.

residual fluorescence of peptide-conjugated fluorophores or spectral overlap of cleaved fluorescent products. Fluorescence assays may involve either direct fluorescence intensity measurements [27], Förster resonance energy transfer (FRET), fluorescence polarization (FP) [28,29], or time-resolved measurements (homogeneous time-resolved fluorescence (HTRF), time-resolved Förster resonance energy transfer (TR-FRET)). The measurement method is dependent upon the basic design of the synthesized peptide substrate. In addition, FRET measurements are also performed using fluorescent protein-based substrates. In the simplest substrate type, a short peptide substrate is linked to a fluorophore, which is quenched when bound to the peptide, but fluorescence is detected upon peptide cleavage by a protease (Fig. 5). Many Ser and Cys protease assays are based on simple peptide-conjugated fluorophores. In these proteolytic reactions, the substrate specificity is defined by S subsites, and an aminoacyl intermediate is formed during proteolysis. Fluorogenic substrates for these proteases contain aromatic amines like 7-amino-4-methylcoumarin (AMC) or 4-methyl-7-coumarylaminde (NH-Mec), which are conjugated to short peptides that confers specificity. The cleavage of amide bond by the proteases increases fluorescence. The fluorescent group occupies the S1′ pocket of the proteases and undergoes a change in its fluorescence characteristics on acylation of the amino acid. Many of the caspase activity assays are based on such substrates in which a tetrapeptide is linked to the fluorophore like 7-amino-4-trimethylcoumarin. Cleavage of these substrates by purified caspases or apoptosis-induced cell lysates results in an increase in fluorescence. One of the disadvantages of such substrates is an associated high background signal and proper storage and intactness of the peptide substrate. This format may greatly reduce the sensitivity range for an assay. In other cases, the peptide substrates are synthesized such that the fluorophore Em is quenched by proximity of the second group, the quencher. The peptidase activity physically separates the quencher from the fluorophore and helps restore detectable Em of fluorescence. In FRET, FP, and HTRF/TR-FRET, the peptide substrate is designed to possess two fluorophores, one of which is the donor, which transfers energy to the second fluorophore, the acceptor. The Em spectrum of the donor overlaps with the absorption spectrum of the acceptor (Fig. 5). The acceptor either can emit energy (fluorescence increase) or may serve as a quencher. The same peptide substrate is utilized for FRET and FP assays, and many such peptides are commercially available for generic protease activities. In FRET, direct fluorescence intensity is measured at defined Ex and Em wavelengths and is sensitive to absorptive interference from colored compounds. This spectral interference can be bypassed using FP assay, in which the ratio of orthogonal fluorescence intensities is measured. An interesting FP assay was developed for assaying human cytomegalovirus protease activity using a peptide substrate which was biotinylated at amino-terminus and was conjugated to a fluorophore at the C-terminus [28,29]. After incubation of the substrate with recombinant cytomegalovirus protease, avidin was added. Binding of avidin to the biotin end of the probe produced a polarization signal which was a function of relative amounts of cleaved and uncleaved substrate. The higher-molecular-weight uncleaved substrate bound avidin to generate a high polarization value, whereas the cleaved, low-molecular-weight peptide produced a low polarization value.

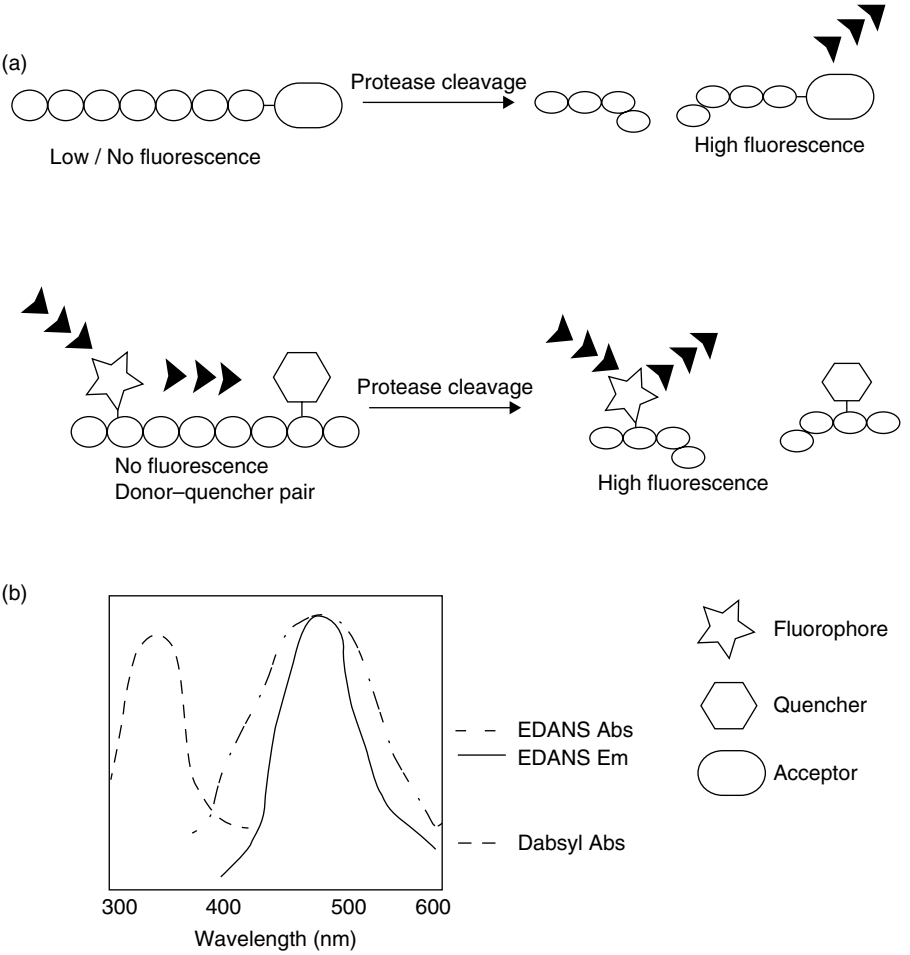

FIGURE 5 Fluorometric assays for proteases. Fluorescent groups are added to peptide substrates to generate substrates for fluorescence-based assays. The simplest substrate is synthesized by adding a coumarin class of fluorophores to linear peptide sequence. An increase in signal is registered with the release of fluorophore post-protease-mediated hydrolysis. (a) Donor–quencher combination in peptide substrate ensures higher signal above background. (b) The overlap of Em spectra of EDANS and Ex spectra of DABSYL characterizes a suitable FRET pair useful for many fluorescence-based platforms.

For the FRET/FP assays, the labeling moiety does not need to be placed at the catalytic site and permits design of substrates with optimal residues at the active site. Since FRET is based on electromagnetic energy transfer between the donor and acceptor pairs of fluorophores separated by very short distance of 10–100 Å, the substrates are designed such that fluorescence increases on cleavage of peptide as a result of loss of intramolecular fluorescence quenching. The selection of donor–acceptor fluorophores requires a high degree of overlap between the Em spectrum of

the donor and absorption spectrum of the acceptor, preferably in the long wavelength region; the high quantum yield of the donor; and an effective quenching of donor fluorescence [27]. Several donor–acceptor pairs have been developed like EDANS/DABSYL (4-(4′-dimethylaminophenylazo) benzoic acid, Dansyl/Trp, and naphthalene/anthracene (5 (2′aminoethylamino) naphthalenesulfonic acid). In EDANS/DABSYL pair, quenching of EDNAS fluorescence in substrates with amino-terminal DABSL group is very efficient because of almost complete overlap between the excited state of EDANS and the DABSYL absorbance and the high molar extinction coefficient of DABSYL (Fig. 5). Many aspartyl proteases and metalloproteases require amino acids P′ to the cleavage site for enzyme recognition, and their peptide substrates are designed such that the fluorophore and quencher are separated by less than three or four residues, allowing short-range intramolecular interactions. Many proteasome-mediated events like conformational changes in protein or protein degradation, which change the distance between the acceptor and donor pairs, are efficiently assayed using FRET-based techniques.

The small stokes shifts and near-UV absorption/Em spectra of donor–acceptor pairs in FRET assays have significant drawbacks in HTS of compound libraries since FRET reactions are influenced by high backgrounds from compounds, microplates, reagents, low signal-to-background ratios, and short fluorescence lifetimes. Using a combination of fluorophores with widely separated wavelengths, for example, a protease assay using dual substrates labeled with fluorescent probes with nonoverlapping Ex/Em wavelengths like rhodamine 110- and coumarin-based fluorophores, in the same well allowed for identification of true inhibitors which were active at both fluorescence settings [30]. These problems of FRET assays are circumvented by TR-FRET assays or HTRF assays. The TR-FRET/HTRF assays, introduced by *Cisbio* International, are based on using rare earth lanthanides like dysprosium (Dy), samarium (Sm), terbium (Tb), and europium (Eu). The lanthanides have poor intrinsic fluorescence but can be complexed to rare earth chelates and cryptates to enhance their fluorescence and prevent decay (cisbio.com). The complexed lanthanides have large stokes shifts and long Em half-lives of 100–1000 μs and help avoid signal contamination by shorter-lived signals contributed by other components of the assay. The HTRF assays generally use complexed cryptates like EuK as an energy donor and XL665 protein as an acceptor (Em 665 nm) [31]. The Em spectra of donor and the Ex spectra of the acceptor overlap and the donor–acceptor pairs are placed at 10 nm or less from one another. A highly sensitive time-resolved fluorescence quench assay (TR-FQA) was developed for caspase-3 [32] using a hexapeptide substrate labeled with fluorescent Eu chelate at one end and a Dabacyl quencher at the other end. Cleavage with caspase-3 allowed measurement of time-resolved Eu signal to the acceptor allophycocyanin. Time resolution allowed the separation of the fluorescence signal by spectral and temporal filtering and resulted in very high signal-to-background ratios. In addition to the extrinsic fluorophores, many protease assays are based on the naturally fluorescent proteins, like the green fluorescent protein (GFP), which can be used for both *in vitro* and *in vivo* assay formats.

Protein-Based Fluorescence Reporter Substrates The reporter constructs in which the protease substrate sites are introduced between the coding sequences of the fluorescent proteins are used in many cell-based protease assays. The cell-based

protease assays require transient or stable expression of the GFP which absorbs blue light and emits green light, in the absence of any cofactor requirement. The GFP chromophore arises from posttranslational modifications and comprises of *p*-hydroxybenzylideneimidazolinone formed by cyclization of Ser65, Tyr66, and Gly67 and 1,2-dehydrogenation of the tyrosine (Tyr) [33].The wild-type GFP has been extensively mutagenized (Fig. 6a, [33,34]) to improve spectral characteristics and quantum yields and generate more useful variants like eGFP, blue fluorescent protein (BFP), cyan fluorescent protein (CFP), and yellow fluorescent protein (YFP) [33,34]. Many protease assays use FRET-based reporters for monitoring protease activity in cellular environment in which a protease cleavage site is placed between two fluorescent variants of GFP or between a GFP and a fluorophore, with overlapping donor Em and acceptor Ex spectra [35]. Induction of protease activity in cells expressing the fusion protein results in peptide cleavage followed with the loss of the FRET signal between the donor and acceptor, when excited at the wavelength of donor absorbance. In general, both live cells expressing FRET constructs as well as cell lysates can be used for studying protease activity. Onuki et al. [36] reported a caspase-8 assay, in which its native substrate, BID, a BH3-interacting domain death agonist peptide, was flanked by CFP, the cyan fluorescent variant of GFP, and YFP, the yellow fluorescent variant. The absorbance of light at 433 nm by CFP results in energy transfer to YFP, which emits fluorescence at 527 nm (Fig. 6b). The hydrolysis of the protease site by caspase-8 results in loss of FRET Em at 527 nm, with a gain of Em at 475 nm from CFP [36]. The ratio of light Em at the two wavelengths is used for FRET quantification and is proportional to protease activity. Most assays are based on energy transfers between CFP/BFP and GFP or YFP separated by specific protease cleavage sites. Since most cells express a wide variety of proteases which have redundant activities, a direct correlation between the protease type and measured activity is obtained only when cells are transfected with highly specific peptidase sites in the FRET expression constructs. The FRET-based reporters can be used for imaging cell systems with detectable GFP expression. In addition to the reporter assays expressing fluorescent proteins, short fluorescence-based cell-permeable probes have been used for studying specific protease activities and their localization in live or fixed cells and in the small animal models.

Imaging of Protease Activities Fluorescence-based probes have been used in many *in vitro* and *in vivo* imaging techniques for monitoring and detecting active proteases within live cells and small animal models. For instance, the degradation of extracellular matrix (ECM) by collagenases, which are required for migration of cancer cells, was studied using BT549 breast cancer cells grown in the presence of fluorescent ECM protein substrate, FITC-labeled collagen IV. The areas containing cell clusters degraded the matrix and, after fixing, were imaged as nonfluorescent, cleared areas, showing that the ECM substrates have relevance in the cell migration through the tumor matrix. However, since high background fluorescence and cell fixation preclude the ability for real-time data imaging, the technical problems were circumvented by using the dye-quenched fluorescent protein substrates. The dye-quenched substrates contain high density of FITC molecules which are quenched due to high molecular proximity [37]. Protease-mediated cleavage of the substrate releases fluorescent degradation products. BioMol has developed cell-based assays which

(a)

Fluorescent Protein (FP)	Mutations	Excitation Max (nm)	Emission Max (nm)
Wt GFP	None	395, 495	511
Green (eGFP)	S65T, F64L	489	509
Blue (BFP)	Y66H, Y145F, F64L	385	445
Cyan (CFP)	Y66W, S65A, S72A, M153T, V163A	434	477
Yellow (YFP)	T203Y, S65G, S72A, V68L	514	527
T-Sapphire	T203I, Q69M, C70P, V163A, S17G	399	511

(b)

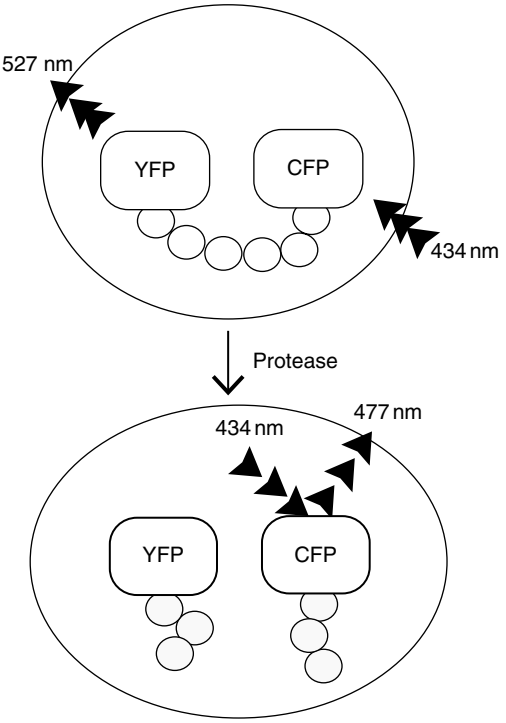

FIGURE 6 Fluorescent protein-based FRET assays. (a) Green fluorescent protein, an intrinsically fluorescent protein, and its variants derived from extensive mutagenesis of the wild-type GFP. (b) A schematic showing a FRET assay using YFP and CFP as acceptor/donor in a cell-based reporter assay. The peptide sequence between the two fluorescent proteins could represent any sequence specific for caspases or cathepsins or metalloproteinases.

utilize cell-permeable fluorescent substrate probes for *in situ* localization of active proteases. The cells with active Ser proteases stain green (FFCK-FAM) and cells with active caspases stain red (SR-VAD-FMK). Cells displaying either or both activities are easily detected using fluorescence microscopy and FACS. A number of

polymer-based near-infrared fluorescence (NIRF) probes have been introduced for imaging protease activity [38] in whole organism models. The NIRF probes consist of a high-molecular-weight carrier like polyethylene glycol (PEG), a cleavable peptide spacer, and a near-infrared fluorophore, indocyanine green, which is an FDA-approved tricarbocyanine dye. Many such probes have been used to monitor activity of caspases [39], metalloproteases, cathepsin, and coagulation enzymes [40]. In one such example, an NIRF peptide substrate was developed to monitor general cathepsin activity *in vivo* [38]. Since most lysosomal cathepsins cleave the polylysine peptides, fluorophores were coupled at high density to an inert copolymer composed of polylysine backbone stabilized with PEG side chains, and the NIRF peptide was injected along with a dye in mice. The high density of fluorophores quenched fluorescence Em, but *in vivo* cathepsin-mediated proteolysis of the peptide decreased the fluorophore density and increased the Em signal in the near-infrared range, the long wavelength which was not subject to scattering or absorption by tissues [38].

FRET-based probes are not specific and can generally be hydrolyzed by many cellular proteases. The intracellular responses to a variety of physiological and pathological conditions can be monitored with the help of more specific and stable, fluorescently labeled activity-based probes (ABPs) [41]. Activity-based probes form covalent complex with the protease active site since they are often based on specific protease inhibitors that occupy protease active sites. The highly hydrophobic ABPs are membrane permeable and can detect specific protease activity in a cellular microenvironment. All ABPs consist of three parts: reactive group, linker, and tag. The reactive functional group, the warhead, is an electrophilic group, which covalently binds to the catalytic nucleophiles (Ser, Cys, or Thr in Ser, Cys, or Thr proteases, respectively) located at the active site of the enzymes [41,42]. The steric hindrance between the warheads from the tag is reduced by a variable linker, which may be a nonspecific spacer of alkyl or PEG groups or may be designed after a substrate. All ABPs contain a tag to enable detection and/or purification of labeled proteins. The tags may be radiolabels (I-125, H-3), fluorescent groups (cyanins, BODIPY, rhodamines, TAMRA), or biotin, which have been used for activity profiling of Cys proteases like cathepsins [43], caspases, and proteasomes. The design of specific ABPs requires that the target proteases have well-defined catalytic mechanisms and known inhibitors for which structural and kinetic data are available [41]. The ABPs have been used extensively for Cys and Ser proteases. DCG-04 was designed based on E-64, a natural product inhibitor of papain family of Cys proteases, and was used to determine the role of papain-like proteases in tumor progression, parasitic invasion, and cell cycle regulation [44]. The caspase-1 of Cys protease family was identified using ABPs containing a biotin-tagged specific peptide sequence coupled to acyloxymethyl ketone (AOMK) and aldehyde reactive groups [45]. The ABP based on specific Ser protease inhibitor, diisopropyl fluorophosphonate (DFP), was used to profile Ser hydrolases for biomarkers for cancer, and activities of at least seven Ser hydrolases were found to be differentially expressed in MDA-MB-231 breast cancer cells in cell culture and in xenograft model [46]. The metalloproteases, with their zinc-containing active sites, hydrolyze the substrate peptide bond without forming acyl–enzyme intermediates. An ABP against metalloproteinase, based on its inhibitor peptide hydoxamate [47], a zinc chelator, and a photocrosslinking group were used to profile MMP activity in melanoma cell lines. The study

uncovered the role of neprilysin, a membrane protein, in progression of melanoma. The ABPs have proven useful in unraveling both previously unknown drug targets and novel players in various pathophysiological processes. In summary, fluorescence-based formats have extensive applications in development of many *in vitro* and *in vivo* imaging assays. At the same time there are several drawbacks in fluorescence-based approaches for biochemical and cell-based assays. Although fluorescence assays are very sensitive, they are susceptible to interference from inherent absorption characteristics of chemical compounds in the *in vitro* assays. In cell-based systems, the quantum yields of many GFP derivative proteins are weak, and many such fluorescent protein-based constructs have limited sensitivity, and the large size of fluorescent proteins limit the spatial resolution of the constructs. Many FRET-independent photochemical changes can result from external light source to initiate fluorescence transfer causing background noise or photobleaching [48,49]. In addition, the use of ultraviolet light for Ex of BFP in cell-based reporter assays has cytotoxic effects. The problems with fluorescent assays can often be circumvented using luminescence assay formats, which are also not affected by compound interference, photobleaching, and phototoxic effects.

C. Bioluminescence Assays

Bioluminescent assays are based on naturally occurring bioluminescence reactions catalyzed by the enzyme luciferase derived from *Photinus pyralis*. Bioluminescent assays are rapid, are sensitive, and are based on the reaction catalyzed by luciferase enzyme, which acts on its substrate luciferin or aminoluciferin in the presence of ATP and generates energy-rich peroxide intermediates, which spontaneously decompose and reach the ground state accompanied with Em of photons at 550–605 nm. Any modification of the amino or carboxy group of aminoluciferin makes the resultant aminoluciferin derivative insensitive to luciferase reactions [50]. Protease substrates are covalently conjugated via a peptide bond to the amino group or to the modified carboxy-terminal derivative of aminoluciferin. The protease-mediated cleavage of the peptide bond linking the substrate and aminoluciferin generates free aminoluciferin, which is available as a viable substrate for luciferase enzyme [51]. These assays are nonhomogeneous reactions that require compatibility between the protease assay conditions and the luciferase assay buffer especially with respect to pH requirements (Fig. 7a). The amount of aminoluciferin released is a direct measure of protease activity. A detailed comparison of the bioluminescent assays with corresponding fluorescent assays for caspase-3/7, caspase-8/9, DPPIV, and calpain showed that the luminescence assays were more sensitive (100–1000-fold) [50]. Ser and Cys proteases which do not require P′ sites for cleavage work well with aminoluciferin–peptides. A single-step homogeneous assay was reported for caspase-3, in which the aminoluciferin-tagged peptide substrate was premixed with luciferase enzyme before adding to wells containing caspase-3 enzyme [52]. Many FRET-based approaches have been replaced with BRET (bioluminescence resonance energy transfer) in which the fluorescent donor is replaced by a bioluminescent luciferase. The luciferase produces an initial photon Em, which is transferred to an acceptor (a fluorophore) that absorbs the donor energy and emits light at a longer

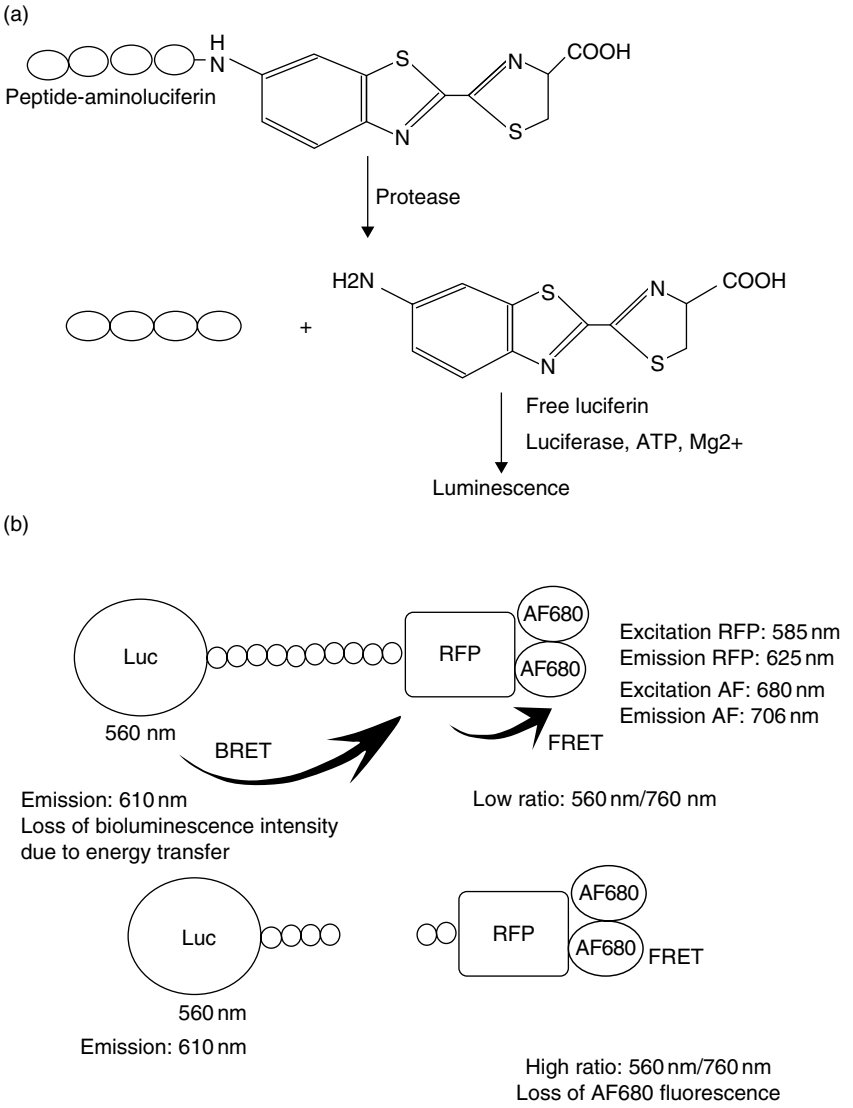

FIGURE 7 Luminescence-based protease assays. (a) Basic construction of the luciferase substrate conjugated to a protease-specific peptide sequence. The peptide–aminoluciferin has no detectable activity. Hydrolysis of the aminoluciferin–peptide bond by protease releases free aminoluciferin which is a usable substrate for luciferase in the presence of ATP and magnesium. (b) A luciferase-based bioluminescence transfer assay (BRET) in which luminescence is detected only after proteolysis.

wavelength. In one example of BRET format, the protease substrate decapeptides containing FXa, thrombin, or caspase-3 recognition sequences were flanked by the bioluminescent luciferase protein (Em 610 nm) and a red fluorescent protein (Ex 585 nm/Em 625 nm) covalently labeled with an NIRF dye, AF680 (Ex 680 nm/Em 705 nm). The energy transfers were possible due to overlap between the red bioluminescence Em at 617 nm of the Luc variant and the AF680 absorbance at 680 nm (Fig. 7b). The ratios of 560 to 760 nm, determined following protease cleavage of the protein substrates, were monitored by recording Em spectra and plotting the change in peak ratios over time [53]. The bioluminescent reporters have high sensitivity and have very high signal windows due to minimal autoluminescence of cells and tissues. The three basic assay formats described earlier are also being developed in conjunction with new technologies like nanoparticle-based assay systems.

D. Nanoparticle-Based Protease Activity Assays

Nanoparticles are nanometer-sized particles that have found wide utility in biology and other fields of study. Since nanoparticles are similar to subcellular components like proteins, they provide an interface to study events at nanoscale [54]. The nanoparticles are tagged with antibodies, fluorescent or chromogenic tags that enable detection and quantification of an enzyme activity, and in many cases the material composition of nanoparticles itself contributes to optical detection properties of the assays. Nanoparticle-based assays are very sensitive and can be used to monitor protease activity in real-time both *in vitro* and *in vivo*. The protease substrates immobilized onto a nanoparticle surface are acted upon by active enzymes resulting in a change of nanoparticle environment, which is measured via fluorescence, absorbance, and imaging or by biophysical methods. Gold nanoparticles (AuNP) exhibit characteristic size- and shape-dependent electronic and spectral properties, which have been exploited to develop protease activity assays. Colloidal AuNP tethered to peptides containing two Cys residues flanking protease cleavage site acquires blue color due to aggregation [55,56]. Protease-mediated cleavage of the activity on the immobilized substrate disperses the aggregates, changing the color to red [57]. In addition to colorimetric assays, AuNP and other metallic nanoparticles are known to quench fluorescence of transferred excited electrons [58]. The electrons transferred from fluorophore-containing peptides are quenched when attached to AuNP. After the cleavage of peptide by protease, the physical separation from the AuNP restores the previously quenched fluorescence [59]. An MMP-2 assay was established using Cy5.5-labeled substrate in which association with AuNP quenched the signals. With MMP-2 activity, the fluorescence signal recovered both *in vitro* and in mice experiments [60]. A new method to assay MMPs expressed in tumors was reported recently using composite gold–iron oxide (Au–Fe_3O_4) nanoparticles. An optical probe containing Cy5.5-GPLGVRG-TDOPA was immobilized on the iron oxide surface and SH-PEG (5000) for *in vivo* imaging on the gold surface. The methodology exploited the quenching properties of AuNP and stable surface chemistry of iron oxide nanoparticles. The MMP activity was assayed by fluorescence imaging of both *in vitro* and *in vivo* mouse tumor models [61]. In addition to AuNPs, magnetic [62,63] and

polymeric- and silica-based nanoparticles [64,65] are also used in protease sensing assays. In addition to the fluorescence quenching properties, the iron oxide-based magnetic nanoparticles [64] are useful for tracing distribution of injected samples by magnetic resonance technology. The nanoparticle-based protease assay systems are quantitative, specific, and sensitive and will soon find wide applications in drug screening campaigns and in routine assay development.

III. ASSAYS FOR SOME CLINICALLY SIGNIFICANT PROTEASES

Because of their therapeutic relevance, members from each of the protease families have been targeted as significant targets for drug discovery. A successful identification of protease activity in disease and its development as a therapeutic target requires an understanding of the *in vitro* chemical mechanism and kinetics of proteolytic activity as well as the complexity of *in vivo* function and biological processes regulating proteolysis. Some of these proteases and the assays used for activity determination and for identification of small-molecule/peptidomimetic modulators are discussed briefly in the following sections.

A. Aspartic Proteases

Aspartyl proteases play an important role in several aspects of our overall health and physiology, including blood pressure (renin), digestion (pepsin and chymosin), and in the maturation of the HIV-1 protease [66]. Human Asp proteases are a small class of proteases with only 15 members, most of which are significant drug targets [67]. The aspartate proteases are monomeric enzymes, except for the HIV protease, which is a homodimer [68]. Most aspartyl proteases can accommodate up to 9-amino acid-long peptides into the active site binding cleft and specifically cleave dipeptide bonds that have hydrophobic residues [67]. The aspartate proteases consist of two domains arising from gene duplication, and the two halves of the enzyme are independent but similarly folded units that move relative to each other [67]. In contrast, the HIV protease consists of two identical subunits which are interconnected with six-stranded antiparallel β-sheets. Most of the amino acid sequences of aspartate proteases are divergent except for the highly conserved catalytic site motif comprising of Asp-Thr-Gly. The amino- and carboxy-terminal domains each contribute one catalytic Asp acid residue to the active site, each of which has different pK value. Peptide bond cleavage occurs by a general acid–base catalytic mechanism. One of the two catalytic Asp residues acts as a general acid and is protonated in the enzyme–substrate complex. The other Asp residue acts as a general base activating a water molecule which then attacks the carbonyl carbon of the amide bond, resulting in the formation of a tetrahedral diol intermediate. Subsequent deprotonation of the hydroxyl group by one of the catalytic aspartates and simultaneous activation of the leaving amine by the other protonated Asp residue ultimately lead to peptide bond cleavage. At least three types of Asp proteases are discussed in the succeeding text.