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Using specificity to strategically target proteases

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Abstract—Proteases are a family of naturally occurring enzymes in the body whose dysregulation has been implicated in numerous diseases and cancers. Their ability to selectively and catalytically turnover substrate adds both signal amplification and functionality as parameters for the detection of disease. This review will focus on the development of activity-based methodologies to characterize proteases, and in particular, the use of positional scanning, synthetic combinatorial libraries (PS-SCL's), and substrate activity screening (SAS) assays. The use of these approaches to better understand a protease's natural substrate will be discussed as well as the technologies that emerged.

1. Introduction

Personalized medicine promises to improve the ability of a clinician to make an accurate diagnosis based on the detection of a molecular marker of disease. These biomarkers need to be well characterized, accessible, and in most applications able to be targeted by an agent linked to an imaging modality or diagnostic device. Proteases are a family of enzymes that account for 5–10% of the pharmaceutical targets in the current market.[1] Representing approximately 2% of all the proteins in an organism, proteases play important roles in bioregulation, matrix remodeling, digestion, and immune response.[2] The primary function of a protease is to discriminate among the many possible substrates available to it and to cleave a specific protein or peptide. The specificity of a protease is dictated by the subsites that assist in the selection and orientation of a given substrate.[3] These subsites are referred by their respective locations to the catalytic site with those located N-terminal of the catalytic site referred to as the non-prime side and those C-terminal referred to as the prime side. In addition, sites beyond the immediate active site can exist and are referred to as exosites. In terms of nomenclature, subsites are designated by the letter 'S', where the S1 and S1' locations are those immediately adjacent to the catalytic site. The corresponding substrate is designated by 'P', where a P1 amino acid is selected by the S1 subsite.[4] Proteases are divided into four major classes, each defined by the architecture of its active site and mechanism of hydrolysis. These are the aspartic proteases, the cysteine proteases, the serine proteases, and the metalloproteases, of which the serine proteases account for approximately one-third of the total.[2,5]

Due to their ubiquitous presence in virtually any biological process and their ability to catalytically turnover substrate, proteases are ideal biomarkers for the diagnosis and prognosis of disease. Several proteases have been implicated in the progression and initiation of certain diseases, in malaria, for example, falcipain 1 is a cysteine protease that helps in the invasion of the host cell by *Plasmodium falciparum*.[6–8] Other examples include HIV infection, where an aspartic protease (HIV-1 protease) is responsible for the maturation of the virus,[9–12] and prostate-specific antigen (PSA, human kallikrein 3) is a serine protease that is currently used in the clinic as a marker for prostate cancer.[13,14] Of Hanahan and Weinberg's six Hallmarks of Cancer, proteolysis can be found to participate in five of the six processes.[15]

The understanding of a protease's specificity and the identification of its substrates and hydrolytic products therefore becomes an important first step in the development of tools that can elucidate the mechanisms behind the cause and progression of disease, as well as present increasing opportunities for the development of drug

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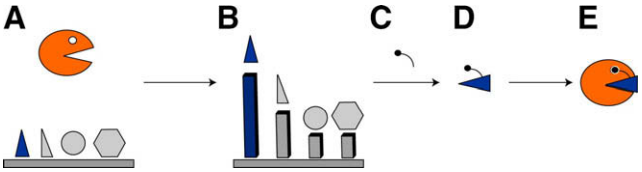


Figure 1. General illustration showing the use of a chemically diverse peptidic library to screen for specificity elements against a given protease (A). The identified specificity element (B) can then be derivatized with an irreversible inhibitor (C and D) to develop a potent activity-based probe (E).

candidates and their interventional use.[16–18] Such tools include targeted active-site inhibitors, also known as activity-based probes, which can be parameterized with imaging agents for the visualization of disease, used for drug delivery, or incorporated into devices capable of detecting the presence of a specific *active* protease and covalently binding to its active site.[19–23]

Since each subsite imparts its own specificity for an amino acid, one approach is to screen a protease against a synthetic combinatorial library composed of small molecules, in a format that systematically probes each subsite and collectively generates a positional profile. This information can be used to design a specificity element, or a compound with similar spatial and electronic properties as a protease's natural substrate. As illustrated in Figure 1, a general active-site inhibitor (also known as a mechanism-based irreversible inactivator) can be made more specific and potent against a protease of choice by tailoring a specificity element that has been designed with the information gained from a substrate profile.[24,25] There are several reviews that describe the use of positional profile information in combination with mechanism-based irreversible inactivators to design active-site protease inhibitors, some of which include disubstituted phosphonates against serine proteases,[26–30] and different small molecules against cysteine proteases[31–39] and metalloproteases.[39,40] Equipped with an imaging agent or therapeutic, these activity-based probes become effective delivery vehicles by selectively targeting and binding to the active form of a protease. This review discusses the practical applications of various peptidic and small molecule combinatorial library-based tools to not only elucidate a protease's active-site specificity, but to also design specificity elements that guide functionalized inhibitors to their protease target.

2. Peptide-based libraries and inhibitors

Several different types of positional scanning, synthetic combinatorial libraries (PS-SCL's) have been developed to rapidly determine the substrate specificity of a protease. The majority of unbiased PS-SCL's are targeted against a protease's non-prime side subsites although there are very few reports of PS-SCL's that screen the prime side specificity of a protease.[32,41–43] Since a protease's target is a peptide or protein, peptide-based PS-SCL's are designed as a series of diverse sublibraries where one amino acid in a tetrapeptide is fixed and the

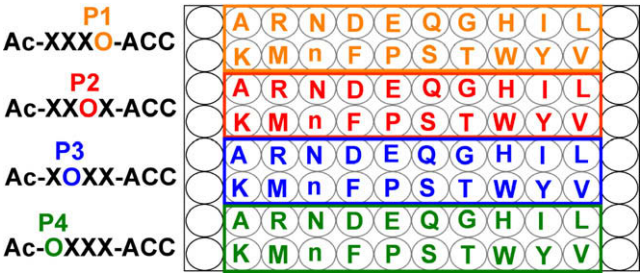
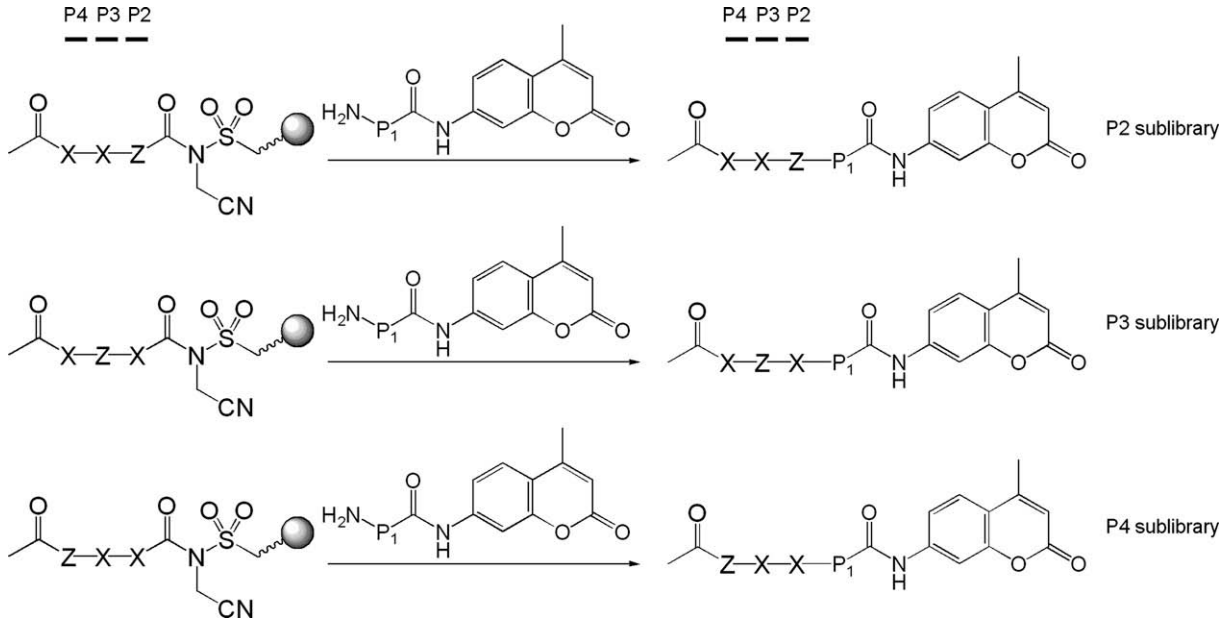


Figure 2. An illustration describing the experimental set-up of a peptide-based PS-SCL in a 96 well format which is arranged by the identity of the amino acid that is in fixed in the tetrapeptide substrate library. For example, the first two rows contain substrates where the P2–P4 amino acids are completely diverse, while the P1 position is fixed (designated by 'X' and 'O', respectively, in the tetrapeptide). n = norleucine, Ac = acetylated N-terminus, and ACC = 7-amino-4-carbamoylmethylcoumarin

remaining positions are a randomized mixture of amino acids (Fig. 2). By individually tracking the hydrolysis reaction at each subsite with a known fixed amino acid, these libraries allow for the simple and rapid determination of the P1–P4 preferences of a protease.[44]

An important component of these assays is the readout. The most commonly used assays utilize a simple-to-interpret photochemically based signal as substrate is consumed, such as a change in absorbance or fluorescence properties. There are several examples of FRET (Förster Resonance Energy Transfer) based assays that generate an 'activated' fluorescence signal upon proteolysis.[45–49] The general application of these assays is limited to those proteases whose active site is not affected by the sterics of the large quencher-fluorophore pairs. In addition, hydrolysis at any of the peptide bonds would illicit a response without designating which bond was cleaved, complicating the interpretation of the readout. Another approach is to use a simpler and smaller fluorophore that generates a traceable signal when a known bond is cleaved. Coumarin has been the fluorophore of choice due to its small size and its photochemical sensitivity to the nature of appended functional groups.[50,51]

A widely used coumarin derivative in PS-SCL technology is the 7-amino-4-methyl coumarin (AMC), which has photochemical properties that are dependent on the substitution at the nitrogen in the 7- position. When AMC is coupled to an amino acid it has an emission at 395 nm (λ_{ex} = 325 nm) and exhibits a red-shifted emission at 440 nm (λ_{ex} = 345 nm) when the anilide bond between the AMC and the P1 amino acid is hydrolyzed.[52] Thus, monitoring at the latter wavelength and tracking the appearance of the fluorescence signal is a convenient method to follow the progress of hydrolysis. Exploiting this, Thornberry and co-workers developed a PS-SCL that used sublibraries composed of an ACC fluorophore coupled to a tetrapeptide, with an aspartic acid in the P1 position and randomized P2–P4 positions.[53,54] This library was built stepwise by first appending an aspartic acid derivatized AMC directly to a solid-state resin via the carboxylic acid on the Asp side chain. The remaining P2–P4 positions were then added sequentially following



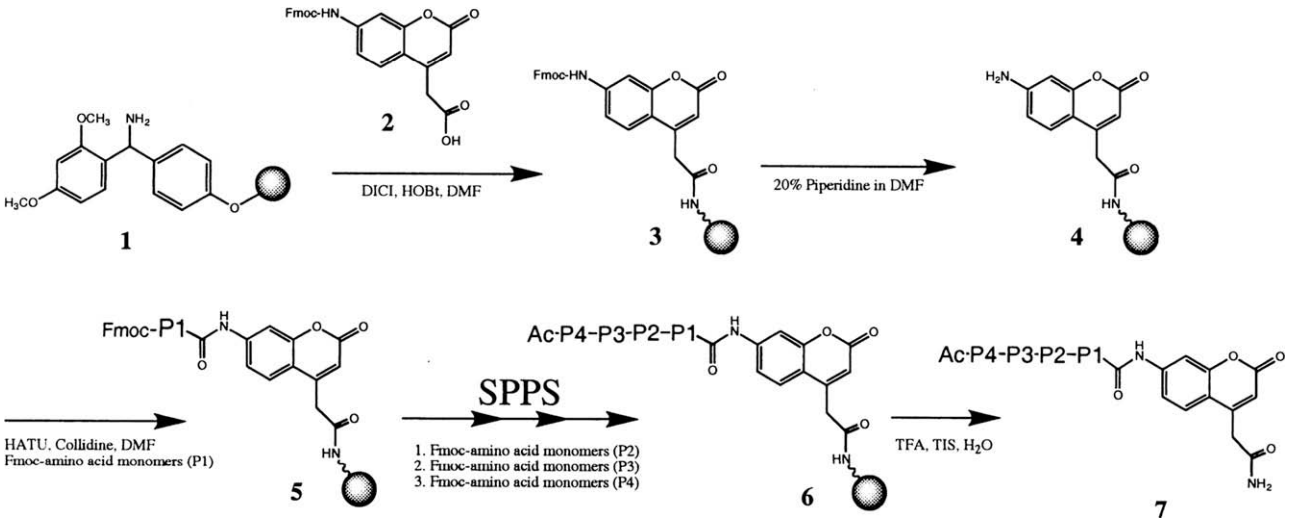
Scheme 1. Synthesis of the tetrapeptide–AMC library by combining two separately synthesized components onto a cyano-methane sulfonamide linker. The first is an AMC with a fixed amino acid in the P1 position and the second is the resin bound, N-acetylated peptide with a fixed amino acid in the 'Z' position and completely randomized amino acids in the 'X' positions. Reproduced with permission from Ref. 55.

standard solid-phase peptide synthesis (SPPS) to form a completely diverse library composed of approximately 8000 peptides. In a collaboration between the Ellman and Craik laboratories, the versatility of these libraries was expanded beyond P1-Asp specific peptides. A novel synthetic strategy was employed that can be used for incorporating any of 19 amino acids (almost all natural amino acids, including norleucine and excluding cysteine and methionine) in the P1 position to generate a library composed of approximately 160,000 peptides.[55] This approach involves the separate synthesis of two components, which are later combined on a solid-state resin (Scheme 1). The first section is a tri-peptide sublibrary that is bound to the resin at the C-terminus via a 'safety-catch' alkane sulfonamide linker. One of the amino acids in the tri-peptide is fixed and the remaining positions are a completely diverse mixture of amino acids. This resin-bound sulfonamide linker, developed by Ellman and Backes,[56] allows for the side-on nucleophilic addition of the second component, an AMC-fluorophore functionalized with a P1 amino acid. The complete AMC-coupled tetrapeptide is then cleaved off the resin following a standard protocol. This strategy improves the previous approach developed by Thornberry by removing the limitation of having only P1 amino acids with side chains capable of binding to a resin. Among the over 150 proteases that have been profiled, AMC-based libraries have been used to screen against the cysteine protease families of caspases,[53,57] the malaria associated falcipains,[58] as well as several other serine proteases.[54,55,59–61]

The strategy described above for the AMC-based PS-SCL was still complicated by the requisite sulfonamide linker and the combination of two separately synthesized components. With the goal of creating a SPPS compatible library that can be synthesized in one sequential step, the Ellman and Craik laboratories substituted AMC with a similar coumarin derivative known as 7-amino-4-carbamoylmethylcoumarin (ACC). This bifunctional fluorophore has a carboxylic acid handle that can be used to load ACC directly to the solid-state resin, and an accessible primary amine to conjugate a peptide.[62,63] The peptide is constructed directly onto the resin-bound fluorophore following standard solid-phase peptide synthesis protocols, and the tetrapeptide–ACC is simply cleaved from the resin (Scheme 2). This strategy has also been used to directly synthesize individual ACC-labeled substrates for the quantitative measurement of substrate turnover. Similar to AMC, the photochemical properties of ACC are also sensitive to the substitution at the nitrogen, a P1-bound ACC has an emission at 400 nm (λ_{ex} = 325 nm) and following hydrolysis of the anilide bond, its emission maximum red-shifts to 460 nm (λ_{ex} = 380 nm). It is also slightly more sensitive with the intensity of its emission being relatively stronger (2.8×) compared to AMC. Included among the over 200 proteases profiled using the ACC-based PS-SCL are a large series of cysteine proteases,[37] the 3Clpro protease in SARS coronovirus,[64] the lymphoma-associated Granzyme M,[65] Factor VIIa which is a serine protease responsible for blood coagulation,[66] and the prostate-specific serine protease KLK4.[61] The Ellman laboratory has further expanded the versatility of the ACC PS-SCL by translating the library into a microarray format, minimizing sample and enzyme usage.[67–71]

2.1. Translating the information from a PS-SCL to design a peptide-based inhibitor

The power of the PS-SCL libraries lies in the ability to quickly generate substrate profile data which can be used to design a potent and specific inhibitor against



Scheme 2. Synthetic strategy of the tetrapeptide–ACC substrate which allows for the sequential addition of the ACC fluorophore and peptide on the resin, where the peptidyl portion following P1 uses standard solid-phase peptide synthesis (SPPS) methods. Reproduced from Ref. 62.

a protease of choice. In one example, the Kaposi's sarcoma-associated herpesvirus protease (KSHV protease) was chosen as a potential therapeutic target.[72,73] KSHV protease is a serine protease that is inactive as a monomer and is activated upon the formation of an immature viral capsid. The protease then cleaves at two sites, which leads to the eventual formation of a mature herpesvirion. In this report, KSHV protease was screened against an ACC-based PS-SCL and found to be highly specific for P1-Ala, and moderately specific for P2-Nle (norleucine), P3-Val. Surprisingly, the enzyme showed a strong preference for aromatic residues at the P4 position. Using this information, a specificity element was designed with slight modifications to the substrate profiling data to improve solubility and with additional information from KSHV protease's natural substrate to expand it to P5 and P6. This hexapeptide specificity element was then converted to an activity-based probe by appending it onto a diphenylphosphonate affinity label, a serine protease inhibitor that can conform to a structure similar to the transition state of the catalytic mechanism and is capable of covalently modifying the active-site serine via the formation of a phosphonate-ester bond.[27] The specificity element-bound activity-based probe was observed to be more potent than the general serine protease inhibitor, diisopropyl fluorophosphate (DFP), which is unable to completely inhibit KSHV. By using the probe against a series of mutated KSHV proteases, it was discovered that catalysis occurs only in the dimeric form which reduces the activation energy required to reach the transition state by placing the active amino acids of the protease in proximity to each other. This report documented both a new avenue for designing drug candidates against KSHV and established methodology that is translatable to other herpesviruses.[74] In addition, this was also the first direct evidence that supported the role of dimerization as a mechanism for activation in herpesvirus proteases. Similar methodology was also used to build activity-based probes that can differentiate and target proteases in the Granzyme family, which are serine proteases that participate in the innate immune response.[75]

A PS-SCL designed specificity element can also be incorporated into a device for the real-time, in-situ detection of active proteases. Ellman and co-workers used a specificity element-nanocrescent particle based probe to detect PSA in patient samples, using surface enhanced Raman scattering with sample volumes in the femtoliter range.[76]

2.2. Non-peptidic libraries: substrate activity screening

Ellman and co-workers have recently developed a technique known as substrate activity screening (SAS) that utilizes a library-based approach to quickly discover and optimize potent and low molecular weight, non-peptidic protease inhibitors.[77] As illustrated in Figure 3, this assay involves the initial screening of the protease against a fluorogenic library composed of a diverse set of substrates that are individually appended to *N*-acyl aminocoumarin. Similar in mechanism with the PS-SCL in the presence of active protease, hydroly-

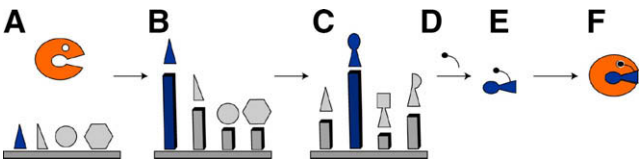


Figure 3. An illustration describing the use of substrate activity screening to discover a non-peptidic substrate of a protease. Using a similar approach as the PS-SCL described in Figure 1, a protease is screened against a library containing different small molecules (A). After its identification (B), the substrate is then derivatized spatially and/or electronically as another library, which is screened against the protease (C). The optimal substrate is then appended with an active-site inhibitor (D) to create a non-peptidic activity-based probe (E) capable of inhibiting a protease of choice (F).

sis of the amide bond results in the release of the fluorophore generating in a traceable readout. The catalytic turnover of the substrate amplifies the fluorescent signal, allowing weakly binding substrates to be also identified. The product of this first step is a substrate with a known structure that serves as a scaffold to systematically design similar substrates with varying electronic and steric properties. These derivatized compounds can then be appended to *N*-acyl aminocoumarin and screened against the target protease to determine the optimal substrate, which is then used as a specificity element. This specificity element can then be translated into an activity-based probe by appending it to the appropriate mechanism-based irreversible inactivator. The SAS approach has been used to develop potent and non-peptidic inhibitors of the cysteine protease cathepsin S[77–79] and the serine protease chymotrypsin.[1] This technology is not limited to proteases and it has also been used to identify and build selective inhibitors of phosphatases, enzymes that remove phosphate from their specific substrate and can be inhibited by a variety of different phosphate isosteres.[80]

3. Conclusion

The proteomic strategy for advancing personalized medicine has focused on the presence or absence of specific proteins as evidence of disease. This approach is not without its flaws, as exemplified by the false-negatives and false-positives associated with the use of ELISA to detect PSA for the diagnosis of prostate cancer.[81,82] One possible solution is to look at protein *activity* as an indicator of disease, and proteases may be the appropriate targets as these add both functionality and signal amplification parameters to these devices. For example, it has been recommended that newer prostate cancer diagnostics should determine the ratio of free PSA versus PSA bound to natural serum protease inhibitors, where the latter form of the protease requires that it is active.[83] Proteases also show promise for advancing the field of theragnostics, where continuous monitoring of disease-associated protease activity can be used to determine the efficacy of a therapeutic. New discoveries continue to associate their enzymatic activity in the initiation and progression of various diseases. Coupled with the wealth of information generated from a substrate profile, these findings can be used to fuel the emergence of novel, activity-based technologies.

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