FEBS Letters 587 (2013) 1155–1163

FEBS Letters

journal homepage: www.FEBSLetters.org

Review

Commercial proteases: Present and future

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ARTICLE INFO

Article history:
Received 15 November 2012
Revised 19 December 2012
Accepted 20 December 2012
Available online 11 January 2013

Edited by Wilhelm Just

Keywords:
Commercial protease
Alkaline protease
Therapeutic protease
Protease engineering

ABSTRACT

This review presents a brief overview of the general categories of commercially used proteases, and critically surveys the successful strategies currently being used to improve the properties of proteases for various commercial purposes. We describe the broad application of proteases in laundry detergents, food processing, and the leather industry. The review also introduces the expanding development of proteases as a class of therapeutic agents, as well as highlighting recent progress in the field of protease engineering. The potential commercial applications of proteases are rapidly growing as recent technological advances are producing proteases with novel properties and substrate specificities.

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1. Introduction

It might come as a surprise to some that proteases, enzymes that cleave other proteins or even themselves in catalytic fashion, make up the largest single family of enzymes, constituting an estimated 2% of the human genome [1,2]. Proteases are classified into six groups: aspartate, cysteine, glutamate, metallo, serine, and threonine [3] based on characteristic mechanistic features consistent within each member of a group. Through structural and functional diversity, proteases carry out a vast array of critical functions ranging from intracellular protein recycling to nutrient digestion to immune system cascade

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biological roles of proteas structural scaffolds to converge with similar active site geometries with varied substrate recognition motifs. Despite their many different forms and functions, the underlying theme of peptide bond scission by all proteases is the same: polarization of the scissile amide C=O bond as well as activation of a nucleophilic group to attack the carbonyl carbon leading to hydrolysis.

There is already a large array of commercially used proteases ranging from detergent additives to effective therapeutics. The therapeutic proteases have recently been nicely reviewed [4]. The present review expands coverage to include the large variety of other commercial protease classes. In general, current protease products rely on naturally evolved cleavage specificities, although other properties such as solubility and overall stability have been effectively engineered. Nevertheless, there is an incredible landscape of potential uses for engineered next generation proteases once the power and specificity of their individual hydrolysis reactions can be tailored for specific needs (Fig. 1). Thus, we will also focus on recent advances in the engineering of new specificities into existing proteases. The key idea here is that the apparent plasticity of protease active sites has enabled them to evolve with varying degrees of substrate specificity and selectivity, thus making proteases a promising framework with which to engineer unique and useful new activities.

vated by changes in the environment to induce a conformational change or the binding of a small molecule/peptide to produce an active conformation. A common strategy for zymogen activation involves expression of the protease fused to an activation segment, ranging in size from 2 to 100 residues, which prevents proteolytic activity until it is cleaved. The most common activation segments are N-terminal sequences that sterically block the active site. Beyond the role of sterically hindering the active site, activation segments are often important for the folding, stability, and sorting of the precursor protease [5]. Some interesting recent work has focused on small molecule activators of several zymogens, such as procaspases and zymogens of the fibrinolytic and coagulation systems [6,7]. Zymogen pro-domains have also been engineered onto enzymes via alternate frame folding or circular permutation, such as the conversion of the cytotoxic ribonuclease barnase into an artificial zymogen activated by HIV-1 protease [8,9].

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http://dx.doi.org/10.1016/j.febslet.2012.12.019

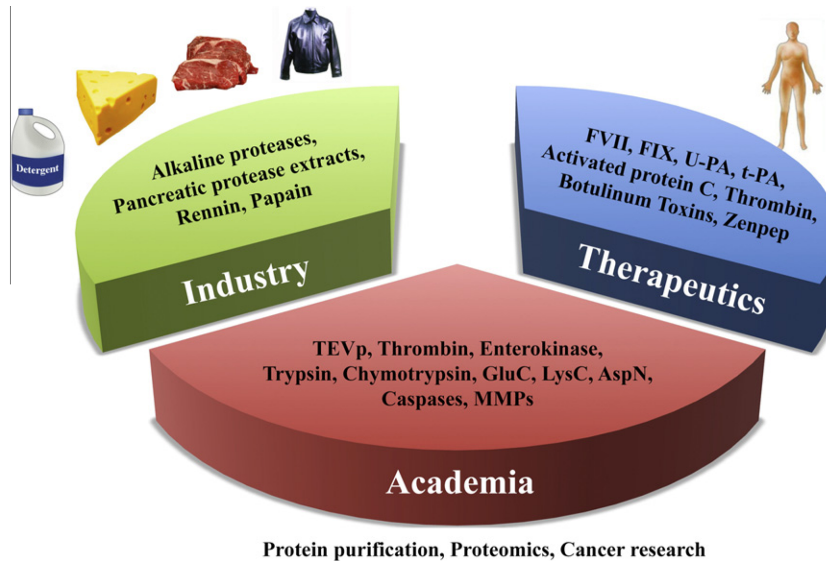

Fig. 1. An overview of protease applications.

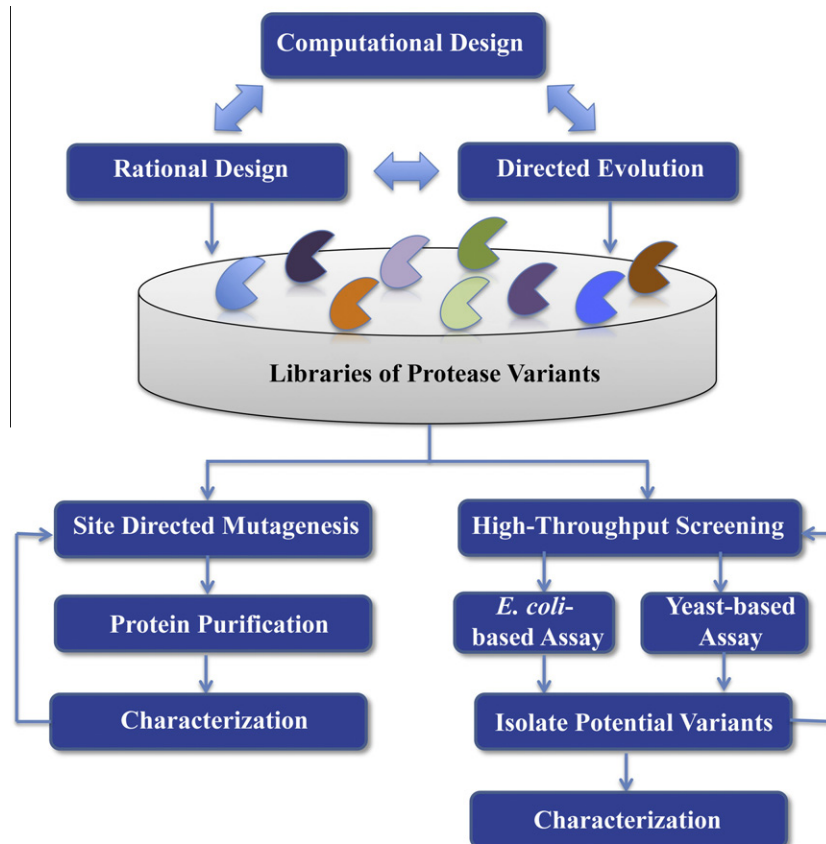

Fig. 2. Schematic diagram outlining the general approaches of protease engineering.

Proteases act within a context of complex networks comprised of small molecule activators and inhibitors, peptides, receptors, substrates, and binding domains, which also influence the spatial and temporal localization of their activity. The human genome contains over 550 presumed protease genes, the most abundant of which are metallo, serine and cysteine proteases represented by 191, 178 and 161 genes, respectively [10]. Threonine and aspartic acid proteases are of a relatively low abundance with only 27 and 21 genes, respectively [10]. Recent advances in proteomics have allowed researchers to gain a more comprehensive understanding of protease expression, regulation and activity that is now referred to as the degradome [11].

The goal of this review is to discuss the general categories of commercial proteases then survey the successful strategies (Fig. 2) now being used to improve protease properties, including the engineering of entirely new substrate selectivities, for industrial and therapeutic applications. The market for engineered proteases is already large, but with the advent of a more widespread ability to tailor stability, specificity and selectivity, the commercial future of proteases appears very bright indeed.

2. Protease engineering in industrial applications

Proteases were first applied in the detergent, leather and food industries several hundred years ago. For instance, alkaline proteases have been used to remove hair from hides, pancreatic protease extracts for use in detergents and leather processing, proteases in rennet from unweaned calves to coagulate milk for cheese production, and papain from papayas to tenderize meat [12]. The unique catalytic activities of proteases make them an inexpensive choice for hydrolyzing peptide bonds for industrial uses. However, the application of proteases for commercial use often requires them to maintain high activity in non-physiological conditions such as high temperatures and pH, intensive calcium chelating agents, and detergents. The absence of such selection pressures during the natural evolution of proteases means that most of them are unstable or inactive in these non-physiological conditions. Protease engineering by site-directed mutagenesis or directed-evolution can generate proteases with improved functions to meet the requirements of commercial applications. The proteolytic enzyme subtilisin [13], an important biocatalyst used in many detergent formulations, will be mainly discussed in detail as a model case study for the improvement of protease functions by protein engineering.

2.1. Thermostability enhancement

High thermostability is a critical property required of proteases for applications in laundry detergent or leather processing. Increasing temperatures can disrupt the non-covalent interactions within a folded protein, leading to protein unfolding [14]. During the unfolding process, the exposure of buried hydrophobic residues can potentially induce protein aggregation. Moreover, proteins can also be inactivated through some covalent mechanisms, such as deamidation of the Asn and Gln residues, hydrolysis of peptide bonds, beta-elimination of disulfide bonds, and oxidation of cysteine and methionine residues. Protein engineering has provided means to modulate protease stability to fit desired requirements. In general, a first step when engineering proteins for enhanced thermostability is to search for any naturally evolved stabilizing residues by comparing the sequences of the corresponding homologous proteins from mesophiles and thermophiles. Additionally, several other strategies of protein engineering have been extensively explored.

2.1.1. Disulfide bonds

Introducing non-natural intramolecular disulfide bonds to stabilize protein structure is one of the well-known strategies to increase the stability of proteins. The additional intramolecular disulfide bonds can lower the entropy of the unfolded polypeptide chain, thus stabilizing the folded state. Based on structural comparison with a thermophilic subtilisin-type protease, aqualysin I, an unnatural disulfide bridge of Cys-61/Cys-98 was introduced in subtilisin E, resulting in a significant enhancement of the thermal stability [15]. A disulfide bond between Cys-22 and Cys-87 was also reported to improve the stability of subtilisin BPN′, with a more significant effect in the absence of calcium [16]. In some other cases, the introduction of disulfide bonds at incorrect positions may undermine the stability of proteases [17].

2.1.2. Amino acid modification

Methionine, cysteine, or tryptophan residues close to or within the active site of enzymes are normally involved in the oxidative inactivation of the enzyme. Subtilisin was well known to be inactivated by the oxidation of Met222, a catalytically important residue, to its sulfoxide form. Estell et al. investigated the functional consequences of replacing Met222 with the other 19 natural amino acids [18]. Under non-oxidative conditions, the sulfur-containing amino acids, Cys and Met exhibited the most specific activities towards the substrate, sAAPF-pNA, followed by the small amino acids and amino acids with amides and aliphatic side chains. Interestingly, under oxidizing conditions of 0.1 to 1.0 M H_2O_2, replacement of Met 222 to non-oxidizable amino acids under these conditions, Ala, Ser or Leu, improved the oxidative tolerance of subtilisin. Based on the fact that the high specific activity of subtilisin is linked to the presence of a sulfur atom at position 222, Breddam and co-workers chemically modified an M222C mutant by thiomethylating the cysteine with the thioalkylating reagent, methyl methane thiosulfonate (Me-MTS) [19]. The engineered thiomethylated subtilisin derivative exhibited an extraordinarily enhanced stability and catalysis under oxidative conditions compared to both wild-type and the unmodified M222C variant during treatment with hydrogen peroxide. Thus, both mutagenesis and chemical modification are feasible strategies to engineer oxidation-labile residues in proteases for improved oxidation-resistant properties.

Other amino acid modifications, such as deamination of asparagine, which is prone to occur via cyclization reactions with a C-terminal glycine, also play important roles in protein inactivation [20]. Narhi and colleagues described studies on the effect of point mutations on the stability of aprA-subtilisin, which contains Asn-Gly pairs at positions 109–110 and 218–219 [21]. The substitution of N109S showed a 3 °C increase in transition temperature, while the substitution of N219S led to a 4 °C increase in transition temperature. Combination of these two mutations (N109S, N219S) exhibited stability gains that had a cumulative beneficial effect on subtilisin stability, with a 7 °C higher transition temperature.

2.1.3. Metal binding

Metal binding sites can function to both stabilize and activate enzymes. For instance, a highly thermostable protease from *Bacillus* sp. AK.1 in the subtilisin superfamily, is strongly stabilized by binding three Ca^{2+} ions to specific sites in its tertiary structure [22], preventing autolysis and thermal unfolding. Pantoliano et al. explored the engineering of binding affinity at metal ion binding sites for the stabilization of subtilisin [23]. Introducing negatively charged Asp residues into the low-affinity calcium-binding site via substitutions at Gly131 and Pro172 strengthened the electrostatic interaction between subtilisin and calcium ions, leading to a significant improvement in stability. On the other hand, engineered subtilisin variants exhibiting high stability and catalytic activity in the absence of calcium are required for use in laundry detergents due to the high concentration of chelating agents present in most brands. To solve this problem, an evolved subtilisin variant, Sbt88, was isolated [24]. The calcium binding potential was initially abolished by deleting the calcium-binding loop, followed by improvement of the stability via directed evolution. Sbt88 contained a 1000-fold greater stability in strong chelating conditions with native-like catalytic activity [24]. Moreover, by sequentially randomizing 12 amino acid positions in Sbt88, a highly stable subtilisin variant, Sbt140, was obtained with a 15 000-fold enhancement in its calcium-free half-life at elevated temperatures [25].

2.2. Stability and activity enhancement in organic solvent

Industrial applications of enzymes can be greatly expanded if an enzyme can perform in the presence of organic solvents rather than just an entirely aqueous environment [26]. Proteases including subtilisin have demonstrated their potential applications in organic synthesis [27], peptide synthesis [28], and enzymatic esterification of oligosaccharides [29] in anhydrous dimethylformamide (DMF). Principally, a layer of solvent water bound to the

folded protein allows the protein to maintain the correct and functional conformation when transferred from water to an organic solvent [30]. However, water-miscible organic solvents, such as DMF, can also strip away the layer of water associated with the folded protein and destabilize the native protein [30]. Moreover, organic solvents may also destabilize a reaction transition state, thus decreasing the catalytic activity of the enzyme. As a powerful strategy, protein engineering by directed evolution provides a promising approach to recover the lost stability and catalytic activity of enzymes in organic solvent. Chen and Arnold generated a randomly mutated library of subtilisin E by error prone PCR, and screened the variant possessing enhanced catalytic activity of hydrolyzing sAAPF-pNA in the presence of DMF [31]. A triple mutant, D60N/Q103R/N218S, was identified with 38-fold improvement in catalytic activity compared to wide-type subtilisin E in 85% DMF. To further tune the activity of subtilisin E, a variant containing four effective substitutions (D60N/D97G/Q103R/N218S) underwent sequential rounds of random mutagenesis and screening. Mutations with six additional amino acid substitutions (G131D/E156G/N181S/S182G/S188P/T255A) were obtained, yielding a 256-fold higher activity than wild-type subtilisin E in 60% DMF [32].

3. Protease engineering in therapeutic applications

The concept of using engineered proteases for therapeutic applications has long been a goal in the pharmaceutical industry. The catalytic activity of proteases makes them a potentially unique therapeutic, allowing for smaller dosages, the potential to inactivate numerous target proteins and higher efficacy. In combination, these effects can lead to lower and less frequent dosages and more importantly, lower costs. Various protease therapies are currently under clinical investigation, a number of which are expected to reach the market over the next few years. The FDA has already approved a variety of natural proteases for use in treatments such as wound debridement (collagenase), thrombolysis (urokinase), haemophilia (factor VIIa), sepsis (activated protein C) and muscle spasms (botulinum toxin A and B) [4,33]. Of the therapeutic proteases currently on the market, most are serine proteases, and only two have been engineered for enhanced properties such as serum half-life or stability [4]. To date, none have been engineered for enhanced selectivity or specificity. Tailoring protease specificity is an important yet lofty goal in advancing the use of engineered proteases as therapeutics.

3.1. Protein engineering to improve the efficacy of therapeutic proteases

Improving the efficacy of therapeutic proteins is one of the major objectives in protein engineering for therapeutic applications. Notable successes in enhancing the efficacy of therapeutic proteases have so far only been achieved by rational design. For instance, Novo Nordisk redesigned the residues in the activation pocket of factor VIIa (FVIIa) by comparing the crystal structures of selected trypsin family members, identifying five important positions for the allosteric regulation of FVIIa activity [34]. One of the engineered FVIIa variants (V158D/E296V/M298Q/L305V/K337A) displayed 100-fold faster activity for TF-independent factor X activation than the wild type protease, thus enhancing the procoagulant and anti-fibrinolytic activities [34]. Another impressive example of improved protease efficacy was achieved by increasing localization affinity [35]. Interaction between the Gla domain of factor VII and phospholipid membranes is essential for effective hemostasis. Based on structural information of a vitamin K-dependent protein with high membrane affinity, Harvey et al. generated a FVII variant (Y4/P10Q/K32E/D33F/A34E) exhibiting a 150-fold higher membrane binding affinity and 40-fold enhanced factor X activation by introducing more Gla domains at selected positions in the membrane contact site. Multiple Gla domains enhanced the interaction of factor VII with membranes containing acidic phospholipids [35].

3.2. Protein engineering to improve the specificity of therapeutic proteases

The potential of engineering proteases with narrowed specificity to only one or a few target sequences should decrease side effects, while maintaining desired therapeutic benefits. Bifunctional thrombin presents a good example. It not only plays an important role in activating multiple pro-coagulant substrates in the blood-clotting pathway, but also acts as an anticoagulant agent to attenuate clot formation by activating protein C when bound to thrombomodulin on the vascular endothelium [36,37]. Thrombin has been engineered to enhance its specificity for its anticoagulant functionality by restricting the interaction with pro-coagulant substrates. Alanine scanning mutagenesis of the solvent exposed charged and polar amino acids revealed that a single substitution (E229A) could alter thrombin's specificity to favor the anticoagulant substrate, protein C [38]. In the cynomolgus monkey model, the E229A mutant, acting as a selective endogenous protein C activator, exhibited the function of reversible anticoagulation without any procoagulant side effects. A thrombin double mutant (W215A/E217A) was engineered to compromise its procoagulant activities, exhibiting a 20000-fold slower k_{cat}/K_M for cleaving fibrinogen compared to that of wild-type thrombin, with comparable specificity for activating protein C in the presence of thrombomodulin [39]. X-ray structural studies indicated that the W215A/E217A mutations stabilized the E* form, in which the E*–E equilibrium could only be substantially shifted by the binding of protein C and thrombomodulin [40]. The treatment of platelet-dependent thrombosis in a baboon model with the W215A/E217A mutant exhibited safe and potent anticoagulant and antithrombotic effects without any detectable hemorrhage or organ failure [41].

Activated protein C (APC) is another example of a bi-functional protease in the blood coagulation system. Its dual mode of action as an anticoagulant and anti-inflammatory agent benefits septic patients, and led to the development of Xigris, which is the first FDA-approved drug for severe sepsis. On the other hand, the bifunctionality of Xigris complicates its clinical use. The anticoagulant activity of the protease increases the risk of severe bleeding events and patient death [42]. In order to minimize the undesired side effects, considerable effort has been devoted to APC for selective alteration of the multiple activities by reducing its anticoagulant properties while retaining its anti-inflammatory activity. It has been shown that five basic residues in loop 37 and the Ca^{2+}-binding loop of APC bind to factor Va, initiating cleavage at Arg-506 of factor Va, generating its anticoagulant activity. However, these five basic residues are not required for its cytoprotective effects. Mosnier et al. engineered an APC variant (RR229/230AA, KKK191-193AAA) with smaller, neutral amino acid side chains on the APC-factor Va contact surface, minimizing anticoagulant activity, yet maintaining normal cytoprotective activities [43]. Moreover, Bae et al. demonstrated that stabilizing the Ca^{2+}-binding loop of APC by an engineered disulfide bond (Cys67–Cys82) dramatically abolished the anticoagulant activity of APC with little impact on its cytoprotective and anti-inflammatory properties [44]. Another successful strategy to eliminate the anticoagulant activity of APC is via mutations within the Gla domain to disrupt the binding interactions between APC and protein S cofactor. Harmon et al. showed that a single mutation (L138D) in the Gla domain of APC almost entirely abated its anticoagulant activity [45]. Such APC

variants with narrowed specificity may have enhanced performance in severe sepsis therapy and are currently in preclinical development.

3.3. Protein engineering to extend the serum half-life of therapeutic proteases

Development of next generation therapeutic proteases with prolonged half-lives will benefit patients via reduced dosing frequency. In general, protease inactivation by endogenous inhibition has been implicated as a major cause of the short serum half-lives of active therapeutic proteases. Understanding protease-inhibitor interactions is especially significant for therapeutic protease engineering. Serpins use suicide-inhibition to inhibit proteases by generating a covalent complex with the protease. Disruption of serpin–protease interactions can be applied to create an inhibitor-resistant protease. Using tissue plasminogen activator (t-PA) as an example, inhibitors of t-PA prevented systemic activation of plasminogen and also attenuated the beneficial therapeutic property of t-PA as a thrombolytic agent [46]. Based on the known structural model of trypsin, Madison et al. predicted that the residues of t-PA that are in contact with plasminogen activator inhibitor-1 (PAI-1), are not involved in the catalytic interactions with the substrate, plasminogen [47]. Wild type t-PA was completely inactivated by PAI-1, while the mutant t-PA with a seven amino-acid (296–302) deletion was capable of retaining ~95% of its enzymatic activity under the same conditions. In addition, heavy glycosylation of active snake venom proteases appears to provide a steric barrier to inactivation by inhibitors, leading to significantly enhanced serum half-lives [48]. By analogy, selective glycosylation of the residues around a protease's active site may provide an option for engineering therapeutic proteases with prolonged half-lives.

Another well-established technology for improving serum half-lives of therapeutic proteins is the attachment of polyethylene glycol (PEG) chains that increase the hydrodynamic volume of proteins, thus reducing the kidney clearance rate [49]. Other benefits from PEGylation include lowering immunogenicity, reducing protein aggregation, and improving protein thermal stability. In contrast to the random coupling of PEG groups to amino groups on side chains, site-selective PEGylation, such as glycoPEGylation, can generate homogeneous PEGylated proteins while preserving bioactivity. Recombinant FVIIa and factor IX (FIX) have been engineered via glycoPEGylation and are currently in Phase I clinical trials for the treatment of hemophilia [50]. In a recent report, the half-life of a glycoPEGylated FIX has been increased from 18 to 93 h in humans while maintaining the bioactivity of native FIX [51]. Other general strategies to extend serum half-lives of therapeutic proteins include genetic fusion to a carrier protein with a naturally long half-life, such as albumin or antibody-constant domain (Fc). CSL Behring applied the recombinant albumin fusion technology to FVIIa to generate a fusion protein with a short and flexible glycine/serine linker, resulting in an extended half-life of 4.4 h in rat, which is 7-fold greater than that of wild-type FVIIa [52]. A similar strategy was also used to engineer an FIX-albumin fusion protein. In this case a cleavable linker was incorporated between the FIX and albumin moieties to avoid the interference of FIX's biological activity by the bulky albumin portion at the active site. The FIX-albumin fusion, currently in a phase I/II clinical trial, exhibited a 5-fold improved half-life over that of wild type FIX in animal models, while providing sustained hemostatic efficacy [52].

3.4. Protein engineering to reduce the immunogenicity of therapeutic proteases

Although protein-based therapeutics hold great promise for the treatment of human diseases and have expanded dramatically in the last two decades, immunogenicity remains a problem. As a category of protein-based therapeutics, proteases also face the same clinical concerns of immunogenicity. The development of neutralizing antibodies to therapeutic proteases might attenuate the clinical effect of the treatment, and may also result in serious life-threatening adverse reactions. For instance, antibody induced therapy failure (ABTF) is a big concern for botulinum toxins (BoNTs) [53], which are FDA-approved therapeutics for the treatment of various neurological disorders. Several strategies are being explored to reduce the risk factors for the immunogenicity of BoNTs, including limiting the BoNT drug's protein load, improving drug formulation to remove the complexing proteins, development of high affinity BoNT, and shielding antigenic BoNT epitopes [54]. In addition, other general strategies for reducing immunogenicity by protein engineering take advantage of modifying proteins by glycosylation or PEGylation, fusing therapeutic protein to a human Ig Fc fragment, and minimizing T cell epitopes by mutagenesis [55–58].

4. Practical considerations of protease engineering

4.1. Protease engineering through structure-guided mutagenesis

Since 1982, when site-directed mutagenesis was first used to engineer enzymes with known structure [59], extraordinary achievements have been accomplished for the enhancement of the innate properties of proteases such as catalytic activity and stability (Fig. 2) [34,47,60,61]. Although the achievements obtained with subtilisin and human coagulation factors, as mentioned above, have had a measurable positive impact, it can still be argued that the wider application of commercial proteases has been limited in part because little progress has been made toward altering protease substrate specificity.

The first landmark progress on altering substrate specificity was achieved with trypsin twenty years ago [62]. Based on the similarity in tertiary structures of trypsin and chymotrypsin, trypsin was engineered to be a chymotrypsin-like protease via key residue replacements in the S1 binding pocket as well as a critical surface loop. This trypsin variant presented chymotrypsin-like substrate binding patterns, differentiating substrates in the acylation step during the catalysis. In addition, computer graphic analysis was also used to guide the engineering of trypsin to increase its specificity to arginine and lysine substrate [63]. Further research has focused on the surface loops [64] and S1′ subsite [65] broadening the application of the engineered trypsin.

Subtilisin is another excellent example, which has been successfully engineered for various purposes. After altering the substrate specificity to dibasic residues [66], subtilisin BPN′ was further engineered to resemble furin, called furilisin, that could cleave tribasic substrates after site-mutagenesis in the residues that consisted of the S4 subsites in subtilisin [67]. 3-D structure modeling using the published structures of different proteases revealed that two crucial residues, Gly124 and Gly152, which are located on both sides of the waist of the S1 pocket, were involved in the substrate specificity alteration of subtilisin YaB [68]. Mutations of these two residues significantly enhanced protease activity. Other examples of successful subtilisin engineering include substitution of its catalytic histidine with alanine to increase the protease specificity against histidine-containing substrates [69], and the alteration of substrate specificity at the P4 subsite [70,71].

Extending beyond the ground-breaking accomplishments of the engineered trypsin and subtilisin variants, other proteases have been successfully engineered based on making changes inspired by structural and sequence comparisons. Examples include swapping substrate specificities of the neuropeptidase, neurolysin, and thimet oligopeptidase [72], enhancing the P1 substrate specificity

of serine carboxypeptidases [73], and altering the S1 substrate specificity of porcine pepsin to resemble fungal aspartic endopetidases [74]. The S2 subsite specificity of several other proteases were also altered in this way, such as the engineering of papain [75], human cathepsin S [76], human cathepsin D [77] and aqualysin I [78]. Although these and similar efforts pioneered the field of protease engineering, generating protease variants with truly novel substrate specificities not found in homologs has remained more elusive.

4.2. Protease engineering via directed evolution

An approach to the isolation of engineered proteases with entirely new properties, such as substrate specificities not found in homologs, involves the use of directed evolution (Fig. 2). In the directed evolution approach, proteases with desirable properties are derived from libraries of protease variants having various levels of sequence randomization. Two significant challenges are often encountered when trying to engineer new sequence specificity into a protease. First, multiple simultaneous mutations can be required to alter substrate specificity, necessitating the use of relatively large libraries of protease variants to insure that rare mutation combinations are present. Second, the directed evolution of enzyme function commonly leads to enzyme variants showing promiscuous rather than specifically altered substrate specificity. To overcome these two obstacles, various high-throughput screening methods have been developed in *Escherichia coli* and yeast platforms over the last ten years (Table 1).

4.2.1. Directed evolution and high-throughput screening of proteases in E. coli-based systems

As a directed evolution platform, *E. coli* has several technical advantages including high transformation efficiencies and a fast growth rate. Several *E. coli* based methods have been developed that successfully alter protease activity (Table 1). For example, a method based on β-galactosidase inactivation relied on the observations of different X-gal degradation rates to isolate evolved proteases with desired substrate specificities [79]. In this approach, the designed substrate sequence was inserted into an accessible surface loop of β-galactosidase. Cleavage of the substrate sequence leads to a decrease in β-galactosidase activity. An HIV-1 protease (HIV PR) variant, which displayed a 2-fold increase in preference for a novel substrate with low cellular toxicity, was isolated via this approach. Another *E. coli* method utilized a genetic selection system (GSS) to engineer variants of the tobacco etch virus protease (TEV-P) with specificities favoring nominally poor substrates of wild-type TEV-P [80]. The desired new substrate sequence was inserted between two DNA-binding domain (DBD) moieties. Cleavage by a TEV-P variant resulted in the enhanced expression of reporter genes, such as HIS3, kanamycin resistance gene, and LacZ. These genes facilitated the host's growth rate and an increase in β-galactosidase activity. A TEV-P variant was evolved using this method with an altered P1′ specificity. Note that both of these methods required isolation of individual colonies on plates, limiting throughput. In addition, no ability to include a simultaneous counter-selection substrate screen into the assay was reported, making it impossible to exclude the more common promiscuous enzymes from the pool of those isolated. Finally, any protease activity that interfered with *E. coli* would likely be excluded from those isolated.

Directed evolution based upon *E. coli* in combination with fluorescence activated cell sorting (FACS) has proven to be the most prolific directed evolution-based protease engineering approach yet reported. It has been used to engineer a variety of new substrate selectivities into OmpT, an *E. coli* outer membrane protease, in a surprising general way, and without sacrificing overall catalytic activity. In the *E. coli*-FACS system, the negative charge of the *E. coli* outer surface is exploited to capture positively-charged and fluorescently-labeled cleavage products. Fluorescence intensity measured by FACS is proportional to catalytic turnover, providing a way to quantify and isolate the fastest protease variants in high throughput fashion from libraries exceeding 10^8 members. Multiple substrates, each containing a different cleavage sequence as well as a differently colored fluorophore, can be used with multi-color FACS to screen directly for highly specific variants that cleave only desired substrate sequences. Thus, the *E. coli* based FACS system can address both of the critical issues related to protease engineering through directed evolution described at the beginning of this section; namely, the ability to screen relatively large libraries as well as the ability to incorporate simultaneous counter selection substrates along with the selection substrate, insuring that relatively sequence-selective protease variants are isolated [81,82].

The *E. coli*-FACS system was used to isolate a number of OmpT variants that specifically prefer substrates with novel P1 and/or P1′ residues. Importantly, a relatively high level of catalytic activity along with the new selectivity was observed with all of these variants, demonstrating the plasticity of the OmpT active site [81–85]. Perhaps surprisingly, OmpT variants preferring neutral and even acidic residues at P1 and P1′ were isolated. Wild-type OmpT prefers dibasic residues, especially Arg, at these critical substrate positions. Thus, relatively dramatic alteration of substrate specificity, including the reversal of substrate charge at the site of cleavage, has been achieved with the same protease. Further, OmpT variants were engineered to specifically cleave substrates containing post-translationally modified tyrosine residues at P1, namely sulfotyrosine [84] and nitrotyrosine [85]. A subtilisin variant engineered to cleave specifically after phosphotyrosine has also been reported [86]. In each case, the engineered protease variants preferred the modified substrates over unmodified tyrosine. Since no protease with a naturally evolved specificity for post-translationally modified amino acids has been reported, the engineering of such novel and highly specialized variants predicts a bright future for those attempting to reprogram the substrate selectivities of proteases.

A different *E. coli* based system has been reported that exploits the sequence dependent ClpXP-mediated degradation pathway [87]. In particular, appending the so-called ssrA sequence to a protein targets that protein for rapid degradation. To generate a

Table 1
Pioneer work of the next generation protease engineering.

System	Host	Reaction Location	Concept	Evolved Protease	Alteration of the Substrate Specificity	Ref.
β-gal inactivation	*E. coli*	Cytoplasm	X-gal selection/ cell growth	HIV-PR	∼2-fold change	[79]
GSS	*E. coli*	Cytoplasm	Cell growth rate/ report gene	TEV-P	P1′ specificity, ∼100-fold change	[80]
ssrA	*E. coli*	Cytoplasm	GFP degradation /FACS	TEV-P	NA	[87]
OmpT	*E. coli*	Cell Surface	Electrostatic cells surface/FACS	OmpT	10^5–10^7-fold change	[82]
YBGA	*Yeast*	Cytoplasm	Cell growth rate	TEV-P	NA	[83]
GASP	*Yeast*	Cytoplasm	Transcription enhancer/X-gal	HAV 3CP	P2 specificity, ∼160-fold change	[90]

GSS: genetic selection system; YBGA: yeast-based growth assay; GASP: genetic assay for site-specific proteolysis.

selection method useful for protease engineering, a putative protease substrate sequence is inserted between the ssrA sequence and green fluorescent protein (GFP). If there is no cleavage by an added protease or protease variant, the ssrA tag leads to rapid degradation of GFP in the cytoplasm, preventing the folding of GFP and its transport to the periplasm. Cleavage by the protease removes the ssrA tag, so the GFP is successfully transported to the periplasm and the cells become fluorescent, enabling high throughput isolation by FACS. This approach is still relatively new and no comprehensive protease engineered using it has been reported. However, the substrate profiling of the wild-type TEV-P was performed [87].

4.2.2. Directed evolution and high-throughput screening of proteases in yeast-based systems

The OmpT system is a powerful tool and platform for protease engineering, but it is not without drawbacks. This strategy is limited to enzymes that can be displayed in an active form on the surface of *E. coli*, an important constraint in the engineering of most proteases, especially eukaryotic proteases [88,89]. Because the protein folding and secretory pathways of yeast more closely approximate those of mammalian cells compared to *E. coli*, such as promoting efficient oxidative protein folding and N-linked glycosylation, methods were developed in yeast with the purpose of engineering eukaryotic proteases. As of yet, only two methods have been reported on the engineering of proteases in a yeast-based platform (Table 1), more than likely due to the complexity of manipulation in yeast.

The yeast-based growth assay (YBGA) was the first high-throughput screening method reported in yeast [83]. In YBGA, a temperature sensitive *Saccharomyces cerevisiae* strain, CDC25-2, was used as the host to isolate the evolved proteases. The CDC25-2 cells can only grow at the non-permissive temperature of 36 °C in the presence of an active protease variant against the designed substrate in the cells. The concept of the YBGA was well demonstrated with wild-type TEV-P, however, no engineered TEV-P variants with novel specificities were reported using this method.

The yeast GASP (genetic assay for site-specific proteolysis) system is another reported method for protease engineering in yeast [90,91]. In brief, the LexA-b42 moiety, which is a plasma membrane-anchored triple fusion protein (STE2-substrate-LexA-b42), can be released to activate the expression of the LexA operon regulated reporter genes, such as Leu2 and LacZ, in the presence of an active protease variant. Using the yeast GASP approach, Sellamuthu and his colleagues successfully altered the substrate specificity for the P2 position of HAV 3CP [90], and further evolved the HAV 3CP to recognize the Q8 substrate (QQQQQ/QQQ) [91].

Although novel high-throughput screening methods significantly expedite the development of protease engineering, several challenges still exist. The size of the mutant library is currently limited to less than 10^{10} in most of the methods, which can only cover the saturated mutagenesis of 7 residues. However, multiple mutations are often required to alter substrate specificity. In the case of engineered OmpT, changing the P1 specificity required as many as nine mutations around the substrate-binding pocket [85]. The limited mutant library size significantly lowers the odds of isolating a desired variant. In order to cover as many residues, yet maintain a similar sized library, a computationally designed "smart library", based on the known three-dimensional structure, should be applied in combination with the high-throughput screening methods. In addition, the *E. coli*- and yeast-based platforms have their disadvantages of being unfavorable for the expression, folding, and post-translational modifications of human proteins. Because of the potential of therapeutic applications of human proteases, a more powerful high-throughput screening method, which could be generally applied to all proteases, especially human proteases, is still needed.

5. Future directions

The applications of proteases in industry and therapeutics have grown rapidly in the last two decades. Novel protein engineering strategies and techniques will continue to expand the commercial protease markets. Encouragingly, the recent success of apoptotic caspase activation with engineered small-molecule-activated proteases [92] represents a new way to specifically control human protease activity for clinical applications. In addition, taking advantage of the proteolytic activities of proteases in diseased tissues may also offer a new strategy for site-specific drug targeting [93] and tumor imaging [94]. With the development of synthetic biology, computational design, crystallography, and screening technologies, we can anticipate that the future of protease engineering will be a multi-disciplinary task with many dramatic successes to come.

Acknowledgements

This research was conducted in whole or in part by Clayton Foundation for Research. We are thankful to Drs. Mark Gebhard and Moses Donkor for comments on the manuscript.

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