Advances in protease engineering for laundry detergents

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Proteases are essential ingredients in modern laundry detergents. Over the past 30 years, subtilisin proteases employed in the laundry detergent industry have been engineered by directed evolution and rational design to tailor their properties towards industrial demands. This comprehensive review discusses recent success stories in subtilisin protease engineering. Advances in protease engineering for laundry detergents comprise simultaneous improvement of thermal resistance and activity at low temperatures, a rational strategy to modulate pH profiles, and a general hypothesis for how to increase promiscuous activity towards the production of peroxycarboxylic acids as mild bleaching agents. The three protease engineering campaigns presented provide in-depth analysis of protease properties and have identified principles that can be applied to improve or generate enzyme variants for industrial applications beyond laundry detergents.

Introduction

Q2 Proteases (proteinases or peptidases) are enzymes that catalyze the hydrolysis of peptide bonds. They are found in all organisms, where they play an essential role in metabolic and physiological processes. Substrate unspecific proteases participate in protein recycling and digestion, whereas sequence specific proteases are essential for zymogen activation, catalytic cascades, and other physiological processes related to cell survival or death [1]. Proteases are classified by their catalytic mechanism into aspartic, glutamic, serine, cysteine or metalloproteases, by their ability to cleave terminal amino acids as exo- or endopeptidases, or by pH conditions for optimal activity (acid, neutral or alkaline proteases) [2].

Serine proteases are the most abundant type of protease containing a serine as essential catalytic amino acid residue. Serine initiates a nucleophilic attack on the peptide bond in an electronic environment provided by a neighboring histidine and aspartic acid [3]. Further classification of serine proteases is dependent on substrate specificity and structural homology to well established proteases [4]. The main subclasses of serine proteases are subtilisin-like, chymotrypsin-like, wheat serine carboxypeptidase II-like, prolyligopeptidase-like, myxobacter α-lytic and *staphylococcal* proteases [2].

In addition to their physiological importance, proteases are of great use in industrial enzymatic applications such as laundry detergents, automatic dishwashing, feed additives, food preparation, leather, diagnostics, therapeutics and pharmaceutical industries [2,5]. The major use of proteases in the food industry is the enhancement of flavor in dairy, meat, and fish products. In the leather and wool industry, proteases find their application for soaking, de-hairing, and hydrolysis of overlapping scales on wool fibers [6]. In different medical treatments, proteases are used as active agents (treatment of osteoarthritis, removal of dead tissue, wound healing) [7].

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Subtilisin-like proteases are serine proteases produced as extracellular enzymes with a molecular weight ranging from 18 to 90 kDa and are mainly employed in industry due to their outstanding properties such as high stability and broad substrate specificity [5].

Proteases as additives in laundry detergent applications

Proteases in the form of trypsin and chymotrypsin were introduced for the first time as an active ingredient in laundry detergents for degradation of proteinaceous stains in 1913 by the German chemist Otto Röhm [8]. The first commercial detergent containing bacterial proteases was produced by Gebrüder Schnyder in 1959. By 1985, approximately 70% of the heavy-duty laundry detergents in Europe contained enzymes. In the last 50 years, proteases and other enzymes in laundry detergents switched from being minor additives to key ingredients. The selection and evaluation of proteases to be used in detergents is based on important parameters defined by laundry detergent manufacturers. A detergent protease needs to have efficient washing performance at broad alkaline pH and over a wide range of temperatures (from low temperatures for synthetic fibers, to high temperatures for cotton). The performance of a good detergent protease is defined by multiple parameters such as proteinaceous stain degradation, compatibility with other detergent components (e.g. nonionic and anionic surfactants, complexing agents, perfumes, and other enzymes), stability in the presence of oxidizing agents as bleach, and shelf life in detergent formulations. The leading enzyme suppliers and detergent manufacturers are actively pursuing the development of new enzyme activities that address consumer needs for improved cleaning, fabric care and antimicrobial properties. Hence, research on proteases has focused on discovery and engineering enzymes that are more robust with respect to pH, temperature, stability and substrate specificity by using techniques of protein engineering and rational design.

In this review we will focus on three important properties for the application of subtilisin proteases in the laundry detergent industry that have been tackled by protein engineering; activity and thermal resistance of *Bacillus gibsonii* alkaline protease (BgAP) was simultaneously increased [9], promiscuous activity of subtilisin Carlsberg was increased towards peroxycarboxylic acids production [10], and the pH activity profile of BgAP was shifted towards higher activity at lower pH (pH range 8.5–10) [11].

Hot and cold, the temperature challenge of modern detergent proteases

Nowadays trends in energy efficiency raise the awareness in society for washing at low temperature. Designing sustainable laundry detergents with high performance at low temperatures requires the development of enzymes with high efficiency at broad temperature range especially at temperatures <20°C [12]. Proteases adapted to low temperatures can be isolated from naturally occurring psychrophilic microorganisms, displaying high proteolytic activity (15°C) [13–15]. Unfortunately, such enzymes generally do not meet industrial requirements due to inherent low stability at temperatures above 20°C and low product yields in large scale production [5,12,16]. On the other hand, subtilisins isolated from mesophilic organisms exhibit at the same time higher catalytic efficiency and temperature stability at temperatures from 30°C to 45°C. In order to adapt mesophilic subtilisins to the current trend of washing at low temperature (20°C) directed evolution and rational design approaches have been employed. One directed evolution campaign was performed with *Bacillus sphaericus* subtilisin (SSII) using random mutagenesis followed by recombination of improved variants. A remarkable increase in the turnover number (k_{cat} at 10°C increased 6.6-fold) and increased catalytic efficiency (9.6-fold higher than wild type) was achieved [17]. In another approach a chimeric enzyme was generated by replacing the highly flexible 12 amino acid region (MSLGSSGESSLI) in the psychrophilic Antarctic *Bacillus* TA39 protease (S39) with the corresponding amino acid sequence (LSLGSPSPSATL) of the mesophilic subtilisin Savinase® with a temperature optimum at 55°C. The resulting hybrid enzyme showed a higher specific activity for synthetic substrates and a broadened substrate profile at room temperature [18].

Stability of subtilisins is a property which has been studied extensively by random mutagenesis and rational approaches. Bryan *et al*. summarized, in an intensive analysis, amino acid substitutions and their effects in over 50% of the 275 amino acids of subtilisin [19]. Research on subtilisin stabilization focused on calcium dependent and independent stability, as well as stabilization by the introduction of disulfide bonds [19–25]. However, a general mechanism describing the stability of proteases has to be elucidated. In most proteins, intramolecular interactions such as salt bridges are essential for thermal stability. It has been shown that thermal stability was dramatically reduced by removal of salt bridge-networks in aqualisin I, a thermostable subtilisin protease [26,27]. In another approach, the apparently opposite properties of high thermal stability in combination with increased activity at low temperatures were investigated in *B. gibsonii* alkaline protease (BgAP) [9]. BgAP was of special interest for laundry detergent applications as it exhibits superior activity and stability in comparison to other subtilisins [28]. The mesophilic protease BgAP with a temperature optimum of 45–50°C shows a rapid decrease in activity at temperatures lower than 45°C, as well as at temperatures higher than 50°C. In order to broaden the temperature range a directed evolution campaign using three iterative rounds of Sequence Saturation Mutagenesis (SeSaM) was performed [29]. In the first round of SeSaM, five variants were identified with increased activity at 15°C for the artificial substrate succinyl-Ala-Ala-Pro-Phe-*para*-nitroanilide (suc-AAPF-*p*NA) accompanied by decreased thermal resistance at 58°C. The amino acid substitutions identified in these five variants were independently saturated to elucidate the effect of each position on the increased activity at 15°C. Upon sequence analysis, key mutations Ile21Val, Met122Leu and Asn253Asp were identified as essential for increased activity at 15°C. In parallel, screening for increased thermal resistance at 58°C resulted in a variant harboring amino acid substitutions Ser39Glu, Asn74Asp and Asp87Glu. Both sets of amino acid substitutions were combined in one hybrid variant MF1 (Ile21Val, Ser39Glu, Asn74Asp, Asp87Glu, Met122Leu, and Asn253Asp) in order to recover thermal resistance (see Fig. 1).

MF1 showed 1.5-fold improved k_{cat} (35.3 s^{-1}) and a 100-fold increased half-life at 60°C (224 min) in comparison to BgAP wild type (k_{cat} of 23.2 s^{-1} and half-life at 60°C of 2 min). In order to gain deeper insights into the location of the amino acid substitutions, a

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