Biotechnological Aspects and Pharmaceutical Applications of Bacterial Proteases

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ABSTRACT

Proteases enzymes are capable of hydrolyzing peptide bonds in proteins. They can be found in all living organisms. Bacterial proteases enzymes have great pharmaceutical importance due to their key role in biological processes and in the life-cycle of many pathogens. New technologies for rationally protein engineering proteases, as well as improved delivery options, will expand greatly the potential pharmaceutical applications of enzymes. Proteases are extensively applied agents in several sectors of pharmaceutical industry. Furthermore, numerous research applications predominant use of proteases has been in killing tumor cells, they are also emerging as useful agents in

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the treatment of digestive disorders, inflammation, and other diseases. The aim of this paper is to review the biotechnological aspects of proteases enzymes and summarize their pharmaceutical applications in the life sciences.

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INTRODUCTION

Proteases (EC 3.4) are group of enzymes which have the ability to hydrolyze peptide bonds [1]. This enzyme category is subdivided into two major groups as exopeptidases and endopeptidases, based on the position of the peptide bond cleavage. The further classification of proteases can be categorized into six types. These include, aspartic proteases (EC 2.4.23), cysteine proteases (EC 2.4.22), metalloproteases (EC 2.4.17), glutamic (3.4.23.32), serine proteases (EC 2.4.16) and threonine proteases (3.4.25.1), depending on the nature of the active site [2-4]. They are also classified as acidic (pH 2.0 to 6.0), neutral (pH 6.0 to 8.0) or alkaline proteases (pH 8.0 to 13.0) based on their pH optima [5-8]. Based on the sequence and structural similarities, all the known proteases are classified into clans and families and are available in the MEROPS database [9-11] (Table 1).

Table-1: classification of proteases with EC numbers depends on MEROPs database (Alan Barrett and his colleagues in Cambridge, U.K., have developed a more detailed classification system for proteases) [10,11]

Class	Descriptions	Examples	EC groups	
Endopeptidases	Hydrolyse internal, alpha-peptide bonds, tending to act away from the N or C terminus.	Chymotrypsin; Pepsin; Papain.	Serine	3.4.21
			Cysteine	3.4.22
			Aspartic	3.4.23
			Metallo-	3.4.24
			Threonine	3.4.25
Omega-peptidases	Hydrolyse internal peptide bonds, but tend to work closer to the termini with no requirement for a free terminus	Ubiquitinyl hydrolase-L3; pyroglutamyl peptidase; gamma-glutamyl hydrolases.	3.4.19	
Aminopeptidases (Exopeptidase)	Requires a free N terminus, releases a single amino acid.	Aminopeptidase N; Aminopeptidase C.	3.4.11	

Carboxypeptidases (Exopeptidase)	Requires a free C terminus, releases a single amino acid.	Carboxypeptidase A1; CarboxypeptidaseY.	3.4.16 3.4.17 3.4.18
Dipeptidyl-peptidases (Exopeptidase)	Hydrolyses a dipeptide off the N terminal of the protein.	Dipeptidyl peptidase I; Dipeptidyl peptidase III	3.4.14 (shared with tripeptidyl peptidases)
Tripeptidyl-peptidases (Exopeptidase)	Hydrolyses a tripeptide off the N terminal of the protein.	Tripeptidyl peptidase I; Tripeptidyl peptidase II.	3.4.14 (shared with dipeptidyl peptidases)
Peptidyl-dipeptidase (Exopeptidase)	Hydrolyses a dipeptide off the C terminal of the protein.	Peptidyl-dipeptidase A.	3.4.15
Dipeptidase (Exopeptidase)	Hydrolyses a dipeptide off the C terminal OR the N terminal of the protein. Usually requires both to be free.	Dipeptidase A; Membrane dipeptidase.	3.4.13

It was estimated previously that about 2% of the human genes encode proteolytic enzymes (peptidases, proteases, or proteinases) and due to their necessity in many biological processes proteases have become important therapeutic targets [12]. They are intensively studied to explore Protein engineering to improve their thermos stability, efficiency and to change their specificity for industrial or therapeutic purposes [13]. Studying proteases enzymes is highly justified by their key role in several fields of industry [14,15].

The worldwide market for proteases enzymes was estimated to reach (USD 4) billion dollars' value in (2015) and is expected to witness significant growth over the next eight years on account of its increasing application in detergents, pharmaceuticals, and food & beverages [14,16]. The pharmasutecal protease share was about comprising around 1.5%. The major contributor is the North America market, which accounts for 40% of the global protease sales (Figure 1) [17]. Importantly, the proportion of peptides in pharma is anticipated to increase, since it is estimated to grow faster (9.4% annual growth in 2012–2015) than the global industry (3-6% annual growth in 2012–2015) [18].

Figure-1: The global market of protease [17].
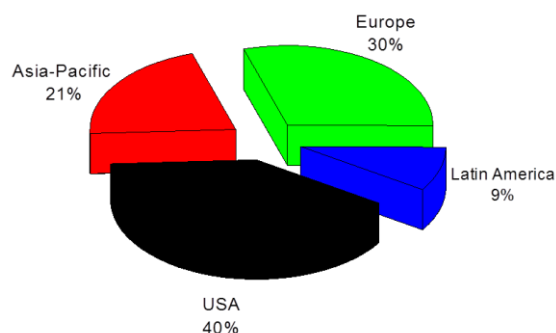
Proteases are found in a wide diversity of sources such as plants, animals, and microorganisms. Increased interest in microbial (bacteria and fungus) proteases resulted from the inability of plant and animal proteases to meet current world demand; they are preferred to other sources because they possess almost all characteristics desired for biotechnological applications [8]. The wide diversity and specificity of proteases have been used to great advantage in developing effective healing agents [19]. Thus, depleting the body's enzyme capacity is the cause of all the pains, sprains, injuries, inflammation, indigestion, immune deficiency and degenerative diseases like cancer, cardiovascular disease, and infection [20]. Enzymes do not give temporary relief from problems but they address the dominant underlying causes of many health problems [21]. Scientists are working to explore pharmaceutical applications of bacterial enzymes as the information is scarce. Following biotechnological aspects and pharmaceutical applications of bacterial proteases have been reviewed in this article.

Bacterial proteases

Most commercial proteases (Figure 2a), mainly neutral and alkaline, are produced by organisms belonging to the genus *Bacillus*. Bacterial neutral proteases are active in a narrow pH range from 5 to 8 and have relatively low thermotolerance. Due to their intermediate rate of reaction, neutral proteases generate less bitterness in hydrolyzed proteins than do the animal proteinases and hence are valuable for use in the pharmaceutical industry [22,23].

BPN or protease Carlsberg was 86% or 72%, respectively; and sequences were conserved around three essential amino acids (Ser221, His64, Asp32) in the catalytic center [35]. This sequence around the catalytic triad of these

three amino acids is highly conserved among alkaline proteases and it has been suggested that the gene for both the intracellular and extracellular proteases evolved from a common ancestor by divergent evolution [36].

Wells et al. was the first report on DNA sequence determination was on protease BPN from Bacillus amyloliquefaciens [24]. Since then, nucleotide sequences of the alkaline proteases, such as protease E from Bacillus subtilis [25], protease Carlsberg from Bacillus licheniformis [26], protease amylosacchariticus from Bacillus subtilis var. amylosacchariticus [27], protease NAT from Bacillus subtilis natto [28,29] and alkaline proteases from Bacillus alcalophilus PB92 [30] and Bacillus sp. 221 [31], have been determined. These studies on DNA and protein sequence similarity are necessary for a variety of purposes and have therefore become routine in computational molecular biology [32]. Knowledge of full nucleotide sequences of the enzyme genes has facilitated the deduction of the primary structure of the encoded enzymes and in many cases identification of various functional regions. These sequences also serve as the basis for phylogenetic analysis of proteins and assist in predicting the secondary structure of proteins, thereby helping in the study of structure-function relationships of the enzyme [33].

Figure-2: Ribbon diagram of the crystal structure of native (a) serine proteases and (b) protease BPN from coordinates obtained from [37, 22].
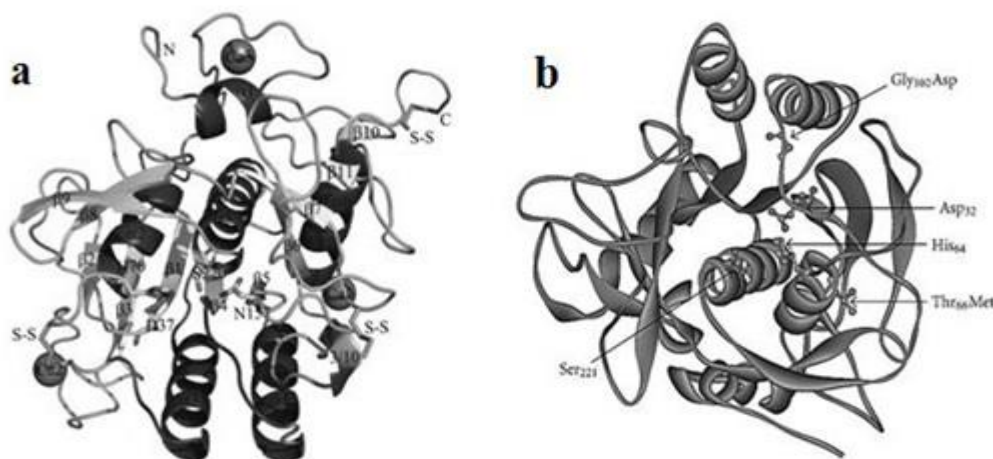
80% similarity was found between the DNA sequence of the protease structural genes from Bacillus subtilis and Bacillus amyloliquefaciens; and the translated mature coding sequence was 85% identical to the protein sequence of protease subtilisin BPN (subtilopeptidase) [34] (Figure 2b). The nucleotide sequence of the protease NAT gene from Bacillus subtilis natto was nearly identical to that of protease E and protease amylosacchariticus, with discrepancies

only at 13 and 27 nucleotides, respectively, of the 1,473 nucleotides sequenced [30]. The primary structure of the mature region of the NAT gene has 99.5% and 99.3% similarity with that of the primary structure of mature protease E and protease amylosacchariticus. The homology of the total amino acid residues between protease NAT and protease. They have drawn using the program Discovery Studio [38]. Relative locations of the catalytic residues and mutations are indicated [39].

Protein engineering

Many pharmaceutical industrial applications of proteases require enzymes with properties that are nonphysiologically. Protein engineering allows the introduction of predesigned changes into the gene for the synthesis of a protein with an altered function that is desired for the application. Recent advances in recombinant DNA technology and the ability to selectively exchange amino acids by site-directed mutagenesis (SDM) have been responsible for the rapid progress of protein engineering [40]. Identification of the gene and knowledge of the three-dimensional structure of the protein in question are the two main prerequisites for protein engineering. The X-ray crystallographic structures of several proteases have been determined [41,42]. Proteases from bacteria have been engineered to improve their properties to suit their applications.

Proteases from many bacteria have been chosen as a model system for protein engineering since a lot of basic information about this commercially important enzyme is available [43]. A protein engineering study was undertaken to determine the functions of one of the largest loop insertions (residues 205 to 219), predicted to be spatially close to the substrate binding region of the SK11 protease from *Lactobacillus delbrueckii* and susceptible to autoproteolysis [44]. Deletion or modification of the loop of the protease was shown to affect the activity and auto processing. It have been shown that random mutagenesis of the substrate binding site of a lytic protease, a serine protease secreted by the soil bacterium *Lysobacter* enzymogenes, generated enzymes with increased activities and altered primarily specifies. Substitution of His120 by Ala in the LasA protease of *Pseudomonas aeruginosa* yielded an enzyme devoid of staphylolytic activity. Thus, His120 was shown to be essential for LasA activity [45,46].

Pharmaceutical applications

For many years, different enzymes such as penicillin acylase, amylases and many others have been applied in pharmaceutical industries for either direct application or preparation of bioactive molecules [47-53]. Proteases are an expanding class of drugs that hold great promise. The U.S. Food and Drug Administration (FDA) have approved

many protease therapies, and a number of next generation or completely new proteases are in clinical development. Although they are a well-recognized class of targets for inhibitors, proteases themselves have not typically been considered as a drug class despite their application in the clinic over the last several decades; initially as plasma fractions and later as purified products [54-56]. Although the predominant use of proteases has been applied as the killer of tumor cells, they are also emerging as useful agents in the treatment of skin problems, digestive disorders, inflammation, and other diseases [57].

Skin treatment

Alkaline proteases are used for developing products of medical importance [58]. Kudrya and Simonenko exploited the elastolytic activity of *B. subtilis* 316M for the preparation of elastoterase, which was applied for the treatment of burns, purulent wounds, carbuncles, furuncles, and deep abscesses [59]. It has been reported the use of alkaline protease from *Bacillus* sp. strain CK 11-4 as a thrombolytic agent having fibrinolytic activity [60].

For several years, protease-activated receptors are targets of science regarding various diseases and platelet aggregation [61]. Wound healing is an important step after skin injury and is connected with the involvement of protease-activated receptors and inflammation. An important point in skin inflammation is the coagulation-dependent skin inflammation [62]. Protease-activated receptors are a special kind of receptors, being activated by proteases cleavage or chemical agonists. They may play an important role in various physiological processes. It is shown that the proteases are involved in many diseases, for example, Parkinson's disease and Alzheimer's disease. The fact, that proteases regulate the coagulation, and are involved in interleukin and cytokine release leads to the conclusion that they are involved in inflammation processes [62].

Digestive aids

The wide diversity and specificity of proteases are used to great advantage in developing effective treatment agents. Oral administration of proteases from *Aspergillus oryzae* has been used as a digestive aid to correct certain lytic enzyme deficiency syndromes [63]. Virtually all patients with cystic fibrosis suffer from severe intestinal malabsorption that is due mainly to a deficiency in pancreatic enzymes [64]. Abnormal levels of bile salts, bicarbonate deficiency, and other factors contribute to the problem. Effective treatments, therefore, should allow a normal to high-fat diet, control symptoms such as a pancreatic lesion and achieve normal nutrition [57]. Pancreatic enzyme replacement therapy involving a defined mixture of proteases, lipases, and amylases can be used to achieve

normal or near-normal absorption in most people with cystic fibrosis [57]. Zenpep® (Eurand), or pancrelipase, is a porcine-derived pancreatic enzyme product that was recently approved for cystic fibrosis patients and that has been shown to improve both fat and nitrogen uptake in patients [65]. It has been reported, it is one of many such digestive protease preparations that have been approved and marketed [66].

Anti-inflammatory

Secretory leukocyte protease and Serine protease enzyme derived from bacteria as an alternative to serrapeptase in dietary supplements for cardiovascular, anti-inflammatory against pulmonary infection and mouse colitis subsequently [67-69]. Serrapeptase showed both the ability to improve the viscoelasticity and clearance of sputum. Several in vitro and in vivo studies have indicated that serrapeptase may have the potential to increase incidence and severity of certain viral infections, including influenza and pneumonia; this did not happen with serrazime [20].

Malignant tumors

The ability to deliver proteases continuously and intracellularly would expand greatly the potential pharmaceutical applications of numerous proteases [70]. In addition, Gene therapy for protease replacement has additional potential applications for indications that are less well recognized for the key contributions made by proteases enzymes [71]. Proteases may be promised to act therapeutically in the treatment of cancer. Several groups are exploring the applications of proteases involved in apoptosis delivered using gene therapy to selectively kill cancer cells [72]. Activation of certain caspases, which are cysteine proteases, is one such approach. Using an expression system for caspase 8, in a model system containing the oncogenic human telomerase reverse transcriptase gene, the inhibition of tumors in mice has been observed [73]. Later experiments indicated that caspase 6, a downstream protease of caspase 8, was superior in its ability to induce apoptosis in two different malignant glioma cell lines. The addition of caspase combinations to cancer cell lines may illicit synergistic results [74]. Recently, small molecule approaches to caspase activation have been demonstrated [75]. In Kurschus and Jenne, work, a high-throughput screening was used to identify agents capable of activating directly procaspase 3 and 6, and that were subsequently capable of promoting cell death [76]. Another apoptotic machinery has also been investigated for use as selective killing agents. For example, granzyme B, a serine protease typically found in the secretory vesicles of natural killer cells, is suggested as an effector domain for immunotoxins that has been actively pursued. Such strategies may become more

robust following additional advances in our ability to selectively deliver molecules into the cytosol of target cells [77].

CONCLUSION

The applications of proteases in pharmaceutical have grown rapidly. Novel protein engineering strategies and techniques will continue to expand the commercial protease markets. Encouragingly, the recent success of apoptotic caspase activation with engineered proteases [78]. It represents a new way to specifically control protease activity for pharmaceutical applications. In addition, taking advantage of the proteases activities in diseased tissues may also offer a new strategy for site-specific drug targeting [79] and tumor imaging [80]. 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

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