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3.49 Proteases

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Glossary

aspartame The synthetic low-calorie sweetener produced from L-phenylalanine and L-aspartic acid, having the structure L-phe-L-asp-methyl ester.

BOTOX, BoNT, botulinum toxin The neurotoxin produced by *Clostridium botulinum*, responsible for food poisoning, which includes a zinc-dependent metalloprotease.

chymosin The predominant milk-clotting enzyme from the true stomach or abomasum of the suckling calf.

directed evolution Method used in protein engineering to harness the power of Darwinian selection to evolve proteins with desirable properties.

endoproteases Proteases that attack internal peptide bonds in the peptide chain remote from the C- or N-terminal.

GRAS list A list of microorganisms used in fermentation and food processes, which are 'generally regarded as safe' by the US Food and Drug Administration.

heterologous protein production Synthesis of foreign proteins in a host organism following transformation of that organism by a vector carrying genes from a different organism.

***k*-casein in cheese coagulation** *k*-Casein serves to maintain the micelles in milk in suspension. The cheese-making protease, rennet, hydrolyzes a single specific peptide bond in the *k*-casein fraction of milk resulting in destabilization of the milk micelles and coagulation.

protease engineering Techniques for creation of proteases with new or artificial amino acid sequences.

proteasome A large multicomponent barrel-like protease complex that digests a variety of proteins into short polypeptides and amino acids.

subtilisin A serine endopeptidase initially characterized from *B. subtilis*, but secreted in large amounts from many *Bacillus* species.

3.49.1 Introduction

Proteases represent a large and diverse group of hydrolytic enzymes that are classified by their site of action, enzyme active site structure, and specific reaction mechanisms. Proteases are ubiquitous in biology where they have a biochemical and/or physiological involvement in many aspects of cell and organism function, including nutrition, protein turnover, growth, adaptation, regulation, sporulation and germination, disease, and death. Proteases regulate much of what occurs in the human body including the essential cellular functions of differentiation, motility, division, and cell death. Proteases participate physiologically in intracellular protein-degradation processes using several systems, including lysosomes (membrane-bound organelles containing proteases) and the more recently characterized proteasome system, consisting of very large protein barrel-like stacked four-ringed complexes, the outer rings of which function as gates to proteins entering the central pore, while the interior surfaces of the inner

rings contain the proteolytic active sites [9]. Proteasome proteolytic mechanisms involve an active site threonine, and involve three different chymotrypsin-like, trypsin-like, and peptidyl–glutamyl peptide-hydrolyzing substrate specificities. Proteolysis is exploited physiologically for covalent activation, regulation, and inhibition of enzymes and other protein-based or linked effectors, for example, in the biochemical cascade leading to blood clotting and in production of insulin. Proteases remove signal sequences of peptides following their transport/secretion through membranes and they remove N-terminal methionine residues after translation.

Animal, plant, and, especially, microbial proteases represent the largest and most important segment of an industrial enzyme market where they are used in detergents, in food processing, in the leather industry, as biocatalysts in organic synthesis, and as therapeutics. Because proteases also function as causative agents of some diseases, protease inhibitors have also been developed as a class of therapeutics. Commercial fermentation processes for production of microbial proteases were developed from about the 1950s. Some of the workhorse protease-producing strains, including *Aspergillus, Rhizopus, and Bacillus* species, were the same species as were found in traditional fermented food fermentations where their proteases participated in the degradation of proteins and associated development of distinct flavors in solid mash substrates containing soy beans, cereal grains, and other plant-based food materials. The protease production fermentation processes and conditions were optimized through characterization and control of factors affecting microbial growth and enzyme production. Overproducing strains were isolated through extensive screening processes and were further improved through application of elaborate mutation/selection procedures. Developments in genetic engineering, including the ability to express recombinant proteins in different host organisms and the ability to manipulate and enhance transcription, translation, secretion, and other processes, have been exploited to enhance protease production and underlining the technoeconomics of their use. The ability to engineer proteins to modify properties, such as kinetics, specificity, and stability, has been applied to improve or expand on protease applications. Random and site-directed mutagenesis and other techniques have been particularly useful in designing, constructing, and characterizing the biocatalytic and stability properties of wholly new protease structures.

The estimated size of the industrial enzyme market is about $2.5–3 billion per year, which is made up of enzymes used in food processing (~$800 million), enzymes for animal feed (~$400 million), and nonfood/feed enzyme applications ($1.4–1.7 billion). Companies reported to have the largest share of the enzyme market are Novozymes (http://www.novozymes.com/en), Genencor International (http://www.genencor.com), and DSM (http://DSM.com/en_US/dnp/anh_enzymes.htm), with respective approximate market shares of 41–44%, 21%, and 8%. Some Japanese Companies including Shin Nihon continue to produce and commercialize enzymes using the solid culture (koji) process (http://www.aichi-brand.jp/corporate/type/chemical/shin-nihon-e.html). The Amano Enzyme Group produces a more specialized range of enzymes with major applications in biotransformations, diagnostics, and as dietary supplements (http://www.amano-enzyme.co.jp/aeu/product/presentation.html). The indicated internet sites and others provide valuable information on the principal applications of proteases as well as on new research, technology, and links to technical reports and publications. Other companies mainly based in Asia, Europe, and North America account for the residual approximately 27–30%. Proteases are thought to account for about 60% of the total enzyme market or $1.5–1.8 billion per year. Among protease applications, the most dominant use is in the detergents accounting for sales of alkaline protease of approximately $1 billion per year.

Evidence of the importance of proteases in industry may also be gleaned from a simple search of granted US patents (1976–July 2010) and patent applications (2001–July 2010) on the US Patent and Trademark Office (USPTO) site (http://patft.uspto.gov/netahtml/PTO/search-bool.html) as is indicated in **Table 1**. It should be noted that there is some overlap in the data since patents applied for and granted since 2001 will be counted under both headers. 'Protease' was used as the initial 'term' in the search, which was separately applied to the fields 'abstract' and 'claims'. In a second search, the search term 'protease inhibitor' was used. The presumption is that the difference between these two numbers, that is, proteases but not 'protease inhibitors' provides a good indication of numbers of granted patents and applications involving proteases. In the past 10 years, nearly 6000 patents have been filed with claims dealing with protease.

Examples of some selected recent patents (2008–10) related to proteases and protease inhibitors issued by the United States Patent Office are listed in **Tables 2** and **3**, respectively. Notable among the protease patents are strain- and detergent-related proteases, animal-feed applications, therapeutic applications, purification methods, diagnostic and detection assays, and applications of molecular methods including expression systems, immunogenic protease-mediated methods, and antibodies targeting proteases, engineered proteases, and protease activating factors. Notable among the protease inhibitor patents are inhibitors

Table 1 Search of the USPTO database for numbers of granted patents and patent applications related to proteases

	Search term	*In title*	*In abstract*	*In claims*
US patents granted(1976– 2010[a])	Protease	1671	2823	5121
	Protease inhibitor	102	276	756
	Protease, not protease inhibitor	1569	2547	4365
US patent applications(2001–10[a])	Protease	1335	2377	7702
	Protease inhibitor	102	256	1751
	Protease, not protease inhibitor	1467	2121	5951

[a]Search implemented on 19 July 2010.

Table 2 Examples of protease patents issued by the US Patent Office during 2008–10

US patent no	*Protease patent title*
7,763,430	Diagnostic assay for anti-von Willebrand Factor cleaving protease (ADAMTS13) antibodies
7,759,103	Extracellular serine protease
7,750,134	Nucleic acids encoding microbial SUMO protease homologs
7,745,395	Proaerolysin containing protease activation sequences and methods of use for treatment of prostate cancer
7,737,263	Modified Cry35 proteins having altered protease stability
7,718,766	Fret protease assays for botulinum serotype A/E toxins
7,718,763	Substrate polyeptides for von Willebrand factor cleaving protease ADAMTS-13
7,709,621	Vectors with modified protease-dependent tropism
7,709,608	Substrates useful for FRET protease assays for botulinum serotype A/E toxins
7,696,309	Protease resistant mutants of stromal cell derived factor-1 in the repair of tissue damage
7,691,597	Method for measuring protease activity of transglutaminase and transglutaminase composition
7,678,550	FRET protease assays for botulinum serotype A/E toxins
7,670,821	Method for the purification of microbial protease
7,662,860	3D-structure model of SARS coronavirus 3CL protease and anti-SARS drugs
7,659,380	Method of isolation and purification of trypsin from pronase protease and use thereof
7,658,965	Use of acid stable protease in animal feed
7,638,277	Transmembrane serine protease overexpressed in ovarian carcinoma and uses thereof
7,635,679	Protease resistant mutant of human HMGB1 high affinity binding domain Box-A (HMGB1 Box-A)
7,622,558	Antibodies which are directed against the Marburg I polymorphism of factor VII-activating protease (FSAP), and their preparation and use
7,622,292	Regulation of human transmembrane serine protease
7,618,801	Streptomyces protease
7,611,700	Protease resistant modified interferon alpha polypeptides
7,608,444	Use of acid stable protease in animal feed
7,601,807	Protease, DNA encoding the same, and method for manufacturing protease
7,595,183	Cathepsins L-like cysteine protease derived from northern shrimp (Pandalus eous)
7,588,927	Composition and methods relating to SENP1– a sentrin-specific protease
7,569,226	Alkaline protease from Bacillus sp. (DSM 14392) and washing and cleaning products comprising said alkaline protease
7,563,872	Protease, a gene therefor and the use thereof
7,563,607	Acid fungal protease in fermentation of insoluble starch substrates
7,541,156	Method of identifying antinociceptive compounds using protease activated receptor-2
7,538,205	Regulation of human prostasin-like serine protease
7,537,901	Extracellular serine protease
7,531,317	Fluorescence polarization assay to detect protease cleavage
7,521,209	Insertion of furin protease cleavage sites in membrane proteins and uses thereof
7,501,117	Composition exhibiting a von Willebrand factor (vWF) protease activity comprising a polypeptide chain with the amino acid sequence AAGGILHLELLV
7,494,768	Mutational profiles in HIV-1 protease and reverse transcriptase correlated with phenotypic drug resistance
7,494,660	HCV NS3-NS4A protease resistance mutants
7,482,147	Ubiquitin protease
7,473,821	Nucleic acids encoding Cry8Bb1 endotoxins engineered to have insect-specific protease recognition sequences
7,473,524	Mutational profiles in HIV-1 protease correlated with phenotypic drug resistance
7,462,760	Genes encoding plant protease-resistant pesticidal proteins and method of their use
7,455,992	Lactobacillus acidophilus nucleic acid sequences encoding protease homologues and uses therefore
7,452,708	Human PRSS11-like S2 serine protease and uses thereof
7,452,690	Protease EFC cell surface fusion protein assay
7,449,187	Alkaline protease from Bacillus gibsonii (DSM 14391) and washing and cleaning products comprising said alkaline protease
7,442,514	Mutants of the factor VII-activating protease and detection methods using specific antibodies
7,439,240	Purine-or pyrrolol[2,3-d]pyrimidine-2-carbonitiles for treating diseases associated with cysteine protease activity
7,432,093	Soluble, functional apoptotic protease-activating factor 1 fragments
7,427,666	Antibody directed against a ubiquitin-specific protease occurring in the brain
7,425,323	Treatment and composition for achieving skin anti-aging benefits by corneum protease activation
7,416,855	Immunoassay methods for detecting interleukin-1 beta converting enzyme like apoptosis protease-3
7,410,769	Peptide biosensors for anthrax protease
7,410,765	System for protease mediated protein expression
7,402,427	Vectors with modified protease-dependent tropism
7,384,773	Crystal of HIV protease-cleaved human beta secretase and method for crystallization thereof
7,384,758	Bioluminescent protease assay with modified aminoluciferin or derivatives thereof
7,374,896	GFP-SNAP25 fluorescence release assay for botulinum neurotoxin protease activity

(*Continued*)

Table 2 (*Continued*)

US patent no	*Protease patent title*
7,361,748	von Willebrand factor (vWF)–cleaving protease
7,355,015	Matriptase, a serine protease and its applications
7,344,856	Method of controlling cleavage by OmpT protease
7,339,092	Plant activation of Cry8Bb1 by insertion of a plant protease-sensitive site
7,332,567	Fret protease assays for clostridial toxins
7,332,320	Protease producing an altered immunogenic response and methods of making and using the same
7,329,506	Apparatuses and methods for determining protease activity
7,323,558	Nucleic acids encoding dendritic cell transmembrane serine protease
7,320,887	Alkaline protease variants
7,320,878	Protease assay for therapeutic drug monitoring
7,314,744	Hyperthermostable protease gene
7,314,730	Regulation of human transmembrane serine protease

directed to target protease active site categories in particular against aspartic, serine, and cysteine proteases or against pathogenic proteases including hepatitis, human immunodeficiency virus (HIV), severe acute respiratory syndrome (SARS), or malaria and also against diseases including certain diabetic states. In addition, many filings are directed to specific chemical groups or analogs acting as inhibitors including heterocycles, sulfonamides, pyrimidines, quinolines, ketoamides, aminoalkanes, and substituted peptides. These tables illustrate the broad scope of current research and technological developments in this field, in the context of intellectual property protection.

Some prior reviews on proteases, having a strong biotechnology emphasis are: Kumar and Takagi [6], Rao *et al*. [12], Ward *et al*. [20], and Sumantha *et al*. [14]. The reader is also referred to the proteolysis map that integrates five databases related to proteases and proteolysis (http://www.proteolysis.org/proteases).

Table 3 Examples of protease inhibitor patents issued by the US Patent Office during 2008–10

US patent no	*Protease inhibitor patent title*
7,763,733	Ritonavir analogous compound useful as retroviral protease inhibitor, preparation of the ritonavir analogous compound and pharmaceutical composition for the ritonavir analogous compound
7,763,641	Broadspectrum heterocyclic substituted phenyl containing sulfonamide HIV protease inhibitors
7,763,609	Heterocyclic aspartyl protease inhibitors
7,759,499	Compounds as inhibitors of hepatitis C virus NS3 serine protease
7,759,354	Bicyclic guanidine derivatives as asparyl protease inhibitors, compositions, and uses thereof
7,759,353	Substituted spiro iminopyrimidinones as aspartyl protease inhibitors, compositions, and methods of treatment
7,754,737	Diaminoalkane aspartic protease inhibitors
7,745,444	Inhibitors of serine proteases, particularly HCV NS3-NS4A protease
7,741,345	AZA-peptide protease inhibitors
7,737,300	Processes and intermediates preparing cysteine protease inhibitors
7,728,148	Acyclic oximyl hepatitis C protease inhibitors
7,723,380	Antiviral protease inhibitors
7,718,769	Tri-peptide hepatitis C serine protease inhibitors
7,718,691	Compounds as inhibitors of hepatitis C virus NS3 serine protease
7,718,633	Prodrugs of HIV protease inhibitors
7,718,612	Pyridazinonyl macrocyclic hepatitis C serine protease inhibitors
7,709,646	Tetrahydroquinoline derivatives useful as serine protease inhibitors
7,709,446	Multifunctional protease inhibitors and their use in treatment of disease
7,704,958	Methods and compositions for inhibiting apoptosis using serine protease inhibitors
7,700,645	Pseudopolymorphic forms of a HIV protease inhibitor
7,700,605	2-Cyano-pyrimidines and -triazines as cysteine protease inhibitors
7,696,250	Alpha ketoamide compounds as cysteine protease inhibitors
7,696,226	HIV protease inhibiting compounds
7,696,173	Compositions comprising bowman-birk protease inhibitors and variants thereof
7,691,967	Smart pro-drugs of serine protease inhibitors
7,687,459	Arylalkoxyl hepatitis C virus protease inhibitors
7,671,057	Therapeutic agent for type II diabetes comprising protease-inhibiting compound
7,671,032	HCV NS-3 serine protease inhibitors

(*Continued*)

Table 3 (*Continued*)

US patent no	*Protease inhibitor patent title*
7,662,849	Amidino compounds as cysteine protease inhibitors
7,662,779	Triazolyl macrocyclic hepatitis C serine protease inhibitors
7,659,404	Broad spectrum 2-(substituted-amino)-benzothiazole sulfonamide HIV protease inhibitors
7,649,015	Cellular accumulation of phosphonate analogs of HIV protease inhibitor compounds
7,645,425	Protease inhibitor sample collection system
7,642,049	Method for identifying HIV-1 protease inhibitors with reduced metabolic affects through detection of human resistin polymorphisms
7,635,694	Cyclobutenedione-containing compounds as inhibitors of hepatitis C virus NS3 serine protease
7,632,635	Method for measuring resistance of a patient HIV-2 to protease inhibitors
7,622,490	Broadspecturm 2-amino-benzoxazole sulfonamide HIV protease inhibitors
7,619,094	Ketoamides with cyclic P4's as inhibitors of NS3 serine protease of hepatitis C virus
7,608,632	Sulfonamide inhibitors of aspartyl protease
7,605,159	Cathepsin cysteine protease inhibitors and their use
7,605,126	Acylaminoheteroaryl hepatitis C virus protease inhibitors
7,601,709	Macrocyclic hepatitis C serine protease inhibitors
7,598,029	Method for identifying HIV-1 protease inhibitors with reduced metabolic affects
7,595,299	Peptides as NS3-serine protease inhibitors of hepatitis C virus
7,592,420	Expression in filamentous fungi of protease inhibitors and variants thereof
7,592,368	Sulphonamide derivatives as prodrugs of aspartyl protease inhibitors
7,582,605	Phosphorus-containing hepatitis C serine protease inhibitors
7,575,888	Method of determining chymase activity with secretory leukocyte protease inhibitor
7,569,678	Protease inhibitor conjugates and antibodies useful in immunoassay
7,566,719	Quinoxalinyl macrocyclic hepatitis C serine protease inhibitors
7,553,844	Methods for treatment of HIV or malaria using combinations of chloroquine and protease inhibitors
7,553,618	Method for determining human immunodeficiciency virus type 1 (HIV-1) hypersusceptibility to the protease inhibitor amprenavir
7,550,559	Acylsulfonamide compounds as inhibitors of hepatitis C virus NS3 serine protease
7,550,474	Substituted phenyl acetamides and their use as protease inhibitors
7,550,427	Poly-pegylated protease inhibitors
7,547,701	Haloalkyl containing compounds as cysteine protease inhibitors
7,531,538	.alpha.- and .beta.-Amino acid hydroxyethylamino sulfonamides useful as retroviral protease inhibitors
7,514,557	Process for preparing acyclic HCV protease inhibitors
7,507,763	Resistance-repellent retroviral protease inhibitors
7,504,382	Protease inhibitors for coronaviruses and SARS-CoV and the use thereof
7,501,398	Inhibitor of the folding of the HIV-1-protease as antiviral agent
7,488,848	Alpha ketoamide compounds as cysteine protease inhibitors
7,462,760	Genes encoding plant protease-resistant pesticidal proteins and method of their use
7,453,002	Five-membered heterocycles useful as serine protease inhibitors
7,449,447	Peptidomimetic NS3-serine protease inhibitors of hepatitis C virus
7,442,695	Depeptidized inhibitors of hepatitis C virus NS3 protease
7,429,604	Six-membered heterocycles useful as serine protease inhibitors
7,417,063	Bicyclic heterocycles useful as serine protease inhibitors
7,413,877	Bacterial expression of bowman-birk protease inhibitors and variants thereof
7,402,586	Heteroaryl aminoguanidines and alkoxyguanidines and their use as protease inhibitors
7,399,749	Substituted prolines as inhibitors of hepatitis C virus NS3 serine protease
7,385,085	Oxime derivative substituted hydroxyethylamine aspartyl protease inhibitors
7,384,734	Compositions and methods for determining the susceptibility of a pathogenic virus to protease inhibitors
7,375,218	Process for preparing macrocyclic HCV protease inhibitors
7,371,747	Cyanoalkylamino derivatives as protease inhibitors
7,365,092	Inhibitors of serine proteases, particularly HCV NS3-NS4A protease
7,342,041	3,4-(Cyclopentyl)-fused proline compounds as inhibitors of hepatitis C virus NS3 serine protease
7,339,078	Bis-amino acid hydroxyethylamino sulfonamide retroviral protease inhibitors

3.49.2 Protease Types

The different protease types are briefly described here with emphasis where possible on commercially important examples of each type. For a more detailed discussion of reaction mechanisms the reader is also referred to Rao *et al.* [12], Ward *et al.* [20], and Fujinami and Fujisawa [3]. The principal enzyme types are exoproteases, which act at or near the ends of the peptide chains, delineated as aminopeptidases and carboxypeptidases to indicate their action is at the N- or C-terminals of the peptide substrates. These enzymes may be further differentiated depending on the size of the moiety that is cleaved off, be it, for example, an amino acid, a dipeptide, or a tripeptide. Industrial organisms known to produce aminopeptidases include *Aspergillus oryzae*, *Bacillus licheniformis*, *B. stearothermophilus*, *and Escherichia coli*. Carboxypeptidases, which are produced by species such as *Aspergillus*,

Penicillium, and *Saccharomyces* species, are differentiated further into three groups based on the presence of certain amino acid substituents at their active sites, namely the serine carboxyproteases, the metallocarboxyproteases, and the cysteine carboxyproteases.

Endoproteases, which attack internal peptide bonds in the peptide chain remote from the C- or N-terminal, are further differentiated into the following subgroups based on their specific mechanism of action:

1. Serine endoproteases, having a serine residue at their active sites which participates in the catalytic reaction, have broad specificities and indeed these enzymes catalyze hydrolytic reactions involving esters and amides as well as peptides. Important well-known enzymes of this subgroup include the chymotrypsins and the subtilisins. Many serine proteases have high pH optima in the range 7–12, with those in the pH range 9–10 being known as serine alkaline proteases or the first generation of detergent proteases and those with pH optima of 11–12 being known as the high alkaline proteases, the second generation of detergent proteases. The subtilisins are an important family of serine proteases produced by *Bacillus* species. These enzymes were also the starting material for development of a third generation of detergent enzymes, resistant to oxidation by bleach and related detergent oxidants, which involved substitution of an oxidation-sensitive amino acid near the enzyme's active site with an oxidation-resistant amino acid. These enzymes are further discussed under commercial *Bacillus* proteases below.
2. Aspartic endoproteases, have low pH optima (3–4) and contain a pair of aspartic acid residues at their active sites, and are sorted into two groups: pepsins and pepsin-like enzymes (e.g., produced by *Aspergillus*, *Penicillium*, and *Rhizopus* species) and rennet and rennet-like enzymes (e.g., produced by *Mucor pusillus*, *M. Miehei*, and *Endothia* species).
3. Cysteine/thiol endoproteases contain a cysteine–histidine dyad at their catalytic sites and generally require reducing agents for retention of catalytic activity and are denatured or inhibited by sulfhydryl reagents. Subgroups include papain and papain-like enzymes (including clostripain and streptopain from *Clostridium histolyticum* and *Streptomyces* species, respectively).
4. Metalloendoproteases. Biocatalysis requires the presence of a bound divalent cation. These enzymes are inhibited by ethylenediaminetetraacetic acid but not by sulfhydryl reagents or diisopropylfluorophosphate. The highly thermostable neutral zinc protease produced by *B. stearothermophilus*, thermolysin, is a well-known member of this subgroup. Protein stability is enhanced by four calcium atoms. Other group members include the metalloprotease collagenases (microbial example from *C.histolyticum*), the neutral metalloprotease elastase (microbial example from *P. aeruginosa*), and the alkaline cell wall lytic protease I from *Myxobacter* sp.
5. Glutamic acid and threonine endoproteases represent recently characterized new enzyme families having reaction mechanisms that involve specific participation of active-site glutamic acid and threonine, respectively.

An important link dealing with protease classification and mechanisms of protease action includes MEROPS: the peptidase database (http://merops.sanger.ac.uk/).

3.49.3 Principal Industrial Sources/Production Processes

3.49.3.1 Production of Animal and Plant Enzymes

Enzymes have been extracted from animal sources since ancient times where milk being carried in calf stomachs was observed to clot (due to the activity of calf rennet or chymosin). The best source of the enzyme is the fourth stomach of unweaved calves, where it is present together with pepsin, but the ratio of pepsin to rennet is low. In Denmark, Christian Hansen started commercial production of chymosin by extracting calf stomach with saline. Similar approaches were used to produce bovine pepsin. Typically, the enzyme-containing tissues are preserved at the slaughterhouse by salting, freezing, or drying. Salted stomachs may be washed and dried prior to rennet extraction. The dried calf stomach may be sliced, blended or ground, and extracted with water containing 5–10% NaCl to produce what is known as single-strength rennet. Concentration of the single-strength liquid product using ultrafiltration is used to produce double- or triple-strength rennet.

Plant proteolytic enzymes, such as papain from *Carica papaya* and bromelain from the pineapple family *Bromeliaceae*, have also been produced commercially. In the case of bromelain, the stem component of the pineapple plant after harvesting of the fruit is peeled, crushed, and pressed to recover a juice extract containing the soluble bromelain enzyme. Papain is obtained from the green fruit. The latex is released by making incisions in the fruit, which is laden with liquid in the early morning. The latex tends to congeal on the surface of the fruit from which it may be collected. The collected gel-like latex may be dried on trays at temperatures up to 55 °C using forced air or alternatively it may be liquefied, filtered, and spray dried to produce the crude enzyme. Further purification may involve water extraction and solvent precipitation. Carbohydrate/plant gum-depolymerizing enzymes may be added to remove cloud and viscous plant polysaccharides from crude aqueous extracts. During processing, or when the final product is in the form of a liquid, the labile active site sulfhydryl groups are protected by addition of sodium bisulfite.

3.49.3.2 Production of Microbial Proteases

Initial commercial production of enzymes by fermentation evolved from traditional koji solid substrate oriental food fermentations, which utilized media containing starchy-protein constituents such as wheat, bran, soy beans, and others. These methods were

used up to the mid-1950s in the United States and are still applied in Japan. Following the development of the penicillin-submerged culture fermentation at the US Northern Regional Research Laboratories, submerged fermentations, including the production of proteases, were developed for enzyme production, initially in the United States and Europe, initially for applications in food and beverage processing. These deep-stirred and aerated production fermentors typically have working volumes of 50–200 m^3 and are designed to withstand 'autoclaving' conditions, namely >15 psi and >121 °C for sterilization. It is worth mentioning in passing that it is well established that the overall mix of enzymes produced in solid substrate fermentations tends to be more complex than the corresponding enzyme profile produced by the same strain in submerged culture, likely reflecting the more complex nature of the substrate components used in solid substrate fermentations. Interestingly, when chymosin yields produced by a recombinant *Aspergillus* strain in solid substrate and submerged cultures were compared, much higher yields were observed in the solid-state culture.

The market for industrial enzymes expanded substantially during the 1960s when alkaline proteases were first commercialized for use in detergents. The most important microbial strains used in protease production are *Bacillus* species, used to produce the alkaline serine proteases with applications in detergents and in food processing, and the neutral proteases that are used in brewing and related cereal-mashing applications as well as in general food processing. Fungal proteases are also produced commercially for food applications, with *Aspergillus* species as dominant producers. *Bacillus* and *Aspergillus* species are attractive hosts for production of food proteases as many species of these genera have long been known to be participating in traditional food fermentations and some of the strains (*B. subtilis*, *B. licheniformis*, *A. niger*, and *A. oryzae*) are on the GRAS list of the US Food and Drug Administration (FDA; http://www.accessdata.fda.gov/scripts/fcn/fcnNavigation.cfm?rpt=grasListing), meaning these organisms and their products are 'generally regarded as safe'.

All of the predominant industrial microbial proteases are secreted as extracellular enzymes into the culture medium. *Bacillus* and *Aspergillus* species exhibit high growth rates, and this contributes to short fermentation durations. Biotechnology aspects of these species have been discussed by Schallmey *et al*. [13] and Ward *et al*. [19]. The bacterial and fungal enzyme final production fermentation processes have typical durations of 2–4 and 3–5 days, respectively. Extracellular enzyme concentrations of up to 30 $g\,l^{-1}$ may be achieved. Following the fermentation, the biomass is separated by centrifugation (for bacteria) or filtration (filamentous fungi) and the clarified supernatant/filtrate is usually concentrated as a liquid to the desired final product strength for commercial use. Where alkaline proteases are to be added to detergent powders, the enzymes in liquid concentrates are dried into powder form and subsequently prepared as the characteristic wax-containing granules present in enzyme detergents, which protects the user from undesirable inhalation of protease dusts.

'Wild-type' *Bacillus* alkaline proteases have been characterized into two generations of enzymes based on their source organisms and pH optima. A third generation of alkaline proteases enzymes has been artificially engineered by amino acid substitutions to improve their properties. This is discussed in more detail in the detergent enzyme section. Commercial neutral proteases produced by *Bacillus* species are typically zinc metalloproteases with pH optima at or near 7.0, with applications in brewing for production of amino nitrogen to support yeast growth, for optimization of cereal extract recovery during mashing, for chill-proof haze removal from beverages such as beer, and for modification of soy, milk, or other commercial protein preparations.

One of the most important industrial proteases produced by *Aspergillus* species is the pepsin-like acid protease, produced by *A. saitoi*, which is widely used for hydrolysis of soybean protein in the manufacture of soy sauce. The principal commercial acid protease produced by *A. oryzae* is an endoacting enzyme, which consequently only produced a small amount of free amino acids, with a pH optimum of 4–4.5. This enzyme needs to be combined with a protease having high carboxypeptidase activity, for use in soy sauce manufacture, as it is the high concentration of free amino acids in soy sauce that contribute to the characteristic flavor. Various *Aspergillus* species produce Aspergillopeptidase A, which has a low pH optimum, which renders it suitable for use as a human digestive aid.

3.49.3.3 Impact of Recombinant Technology on Microbial Protease Production

Among the Gram-positive bacteria, priority was given to characterizing the genome of *B. subtilis* and its complete sequence was published in Kunst *et al*. [7]. The identification of genes associated with the major secretory pathway in *Bacillus* species, encoding five type I and a type II signal peptidase were perceived as important to the capacity of these species to secrete large amounts of extracellular enzymes. The whole genome of *B. halodurans* [15] has also been sequenced which is especially important given this strain's capacity to produce extracellular enzymes including protease having high alkaline activity and stability. Early recombinant research strategies with *B. subtilis*, involving introduction of engineered plasmids were problematical because recombination processes over many generations caused plasmid sequence deletions, a problem which was somewhat resolved by development of mutant strains lacking certain recombinant enzymes. A few instances have been cited regarding the development of high-level expression of *B. subtilis* genes, which involved the use of native alpha-amylase native high enzyme-producing promoter. More stable clones were achieved by strategies that involved direct insertion of recombinant DNA into the chromosome and strategies for amplification of gene copy number were also successful.

Molecular and genetic studies were concentrated on a number of industrially important *Aspergillus* species, including *A. nidulans*, and the naturally occurring enzyme production hosts, *A. niger* and *A. oryzae*. Genomes of these organisms have been sequenced. Websites providing molecular and genetic information on these *Aspergillus* species are summarized in Ward *et al*. [19]. In addition, the latter review also discusses causes of low levels of production of heterologous proteins by *Aspergillus* species, including

limitations at the sites of transcriptional, translational, and posttranslational levels and strategies to improve heterologous gene expression through gene fusion, protease-deficient mutants and other approaches.

The first recombinant heterologous product to gain approval from the US FDA was calf chymosin, for use in cheese making, produced by an *Aspergillus* species. The precursor, preprochymosin is first synthesized after which the 16-amino-acid presequence is cleaved off during the secretion process. A 42-amino-acid prosequence is subsequently removed during further processing of the protein at a low pH. Improved yields of chymosin were achieved using a combination of strategies including gene fusion, classical mutation/selection, and through use of aspergillopepsin-minus mutants. On the other hand, *Kluyveromyces lactis* is the microbial host used by DSM for production of their recombinant chymosin product, Maxiren.

Rao *et al*. [12] have provided extensive tables on the cloning, sequencing, and expression of protease genes from microbial sources, including bacteria (including *Bacillus*, *Lactococcus*, *Streptomyces*, *Serratia*, *Pseudomonas*, *Aeromonas*, *Vibrio* species, and *E. coli*), fungi (including *Aspergillus*, *Fusarium*, *Rhizopus*, and *Mucor* species), yeasts (including *Saccharomyces*, *Candida*, and *Kluyveromyces* species), animal viruses (including herpesviruses, adenoviruses, retroviruses, and picornaviruses), and plant mosaic viruses.

3.49.4 Principal Applications of Proteases

3.49.4.1 Detergents

The most characterized subtilisins are subtilisin Carlsberg (produced by *B. licheniformis*, *B. pumilus*, and *B. subtilis*), and subtilisin BPN (produced by *B. amyloliquefaciens*, *B. stearothermophilus*, and *B.subtilis*). Subtilisin Carlsberg, with pH optimum values of pH 9–10, produced by neutralophilic *Bacillus* species, represents the first generation of alkaline proteases commercialized for use in detergents (e.g., Alcalase (Novozymes) and Nagarase (Nagase, www.nagase.com/technology/enzymes.asp)).

A second generation of detergent proteases, the high-alkaline proteases, produced in alkalophilic *Bacillus* species, such as *B. clausii* and *B. halodurans*, exhibited higher optimal pH values (pH 11–12) and greater thermostability (up to 60 °C, without calcium) are also serine proteases, albeit with some unique amino acid residue features thought to contribute to alkaline- and thermostability, which have also been observed in alkaline cellulase [3]. Examples of commercial high alkaline detergent proteases are M-protease (Kao Corporation: www.kao.com/jp/en/corp_rd/development_02_01.html) and Savinase (Novozymes). For example, the amino acids Arg19, Glu271, Thr274, and Arg275 of the commercialized second-generation detergent proteases, M-protease, appear to form a pattern of stabilizing hydrogen bond networks, which also link both C- and N-terminals. These patterns appear to be conserved among second-generation proteases, whereas they are not observed in the subtilisin Carlsberg-type alkaline proteases.

Although the above first and second generations of proteases originate from wild-type organisms, genetic and protein engineering methods have been used to artificially enhance proteases with respect to different desirable catalytic and/or stability properties. Subtilisin was used as the starting enzyme for development of a third generation of detergent proteases, whereby a methionine residue near the serine residue at the active site, which is undesirably oxidized by detergent additives such as bleach, is replaced with a nonoxidizable amino acid moiety (serine, alanine, or leucine) through site-directed mutagenesis to produce oxidant-resistant proteases. This kind of strategy has been used in oxidant-resistant commercial third-generation proteases such as Purafect OxP (Genencor) and Durazyme (Novozymes). In another example, a number of detergent proteases were engineered which exhibit relatively high activity at low temperatures, which are effective in cold-water washes at 10–20 °C. Novozymes' detergent alkaline protease, Kannase, is an example of such an enzyme.

3.49.4.2 Food Enzymes

3.49.4.2.1 Brewing and cereal processing

Proteases are used in the mashing stage of brewing and general cereal processing where it functions to increase the volume of filterable extract and enhances the amount of alpha-amino nitrogen in wort. The generally accepted amount of alpha-amino nitrogen requirement for yeast growth in a normal fermentation is 140–180 mg l^{-1}. In an all malt mash, the malt proteases typically produce the requisite amount of amino nitrogen from the principal proteins of barley, hordein and glutelin, both during the malting process and during mashing. As the proportion of malt is decreased or eliminated through use of unmalted cereals there is a requirement to replace the malt protease with industrial proteases, especially neutral proteases from *Bacillus* and also from *Aspergillus* species. *Bacillus* alkaline protease is not effective for this application. These enzymes may also be used for production of cereal extract concentrates, where filterable extract yield is the priority rather than amino nitrogen and, in this case, papain may also be used to promote proteolysis. It should also be noted that the temperature–time holds during mashing are designed to meet the activity requirements of the mashing enzymes including protease, amylases, and glucanases. In mashing with malt enzymes, the lowest temperature hold is around 50 °C, called the 'proteolytic' stand as malt proteases have temperature optima around this setting and quickly get denatured as the temperature is increased. Some of the microbial proteases and papain have higher temperature optima at 55–65 °C, enabling the mashing process to be implemented at high temperatures that are more optimal for starch gelatinization and hydrolysis of starch and betaglucans.

Proteases have a second application in brewing for removal of chill haze. After final beer filtration when beer is stored at low temperatures there is a tendency for a haze to develop due to low solubility of residual proteoglycans in finished beer. Papain is particularly effective at hydrolyzing these polymers, thereby removing the haze. These applications are further discussed in Ward [17, 18].

3.49.4.2.2 Cheese making

Acidic aspartic proteases are used as milk-clotting enzymes in cheese making because of their characteristic ability to coagulate milk proteins to form curds with associated release of whey. There are now three types of commercial milk-coagulating enzymes: animal rennets, microbial rennets, and genetically engineered calf rennet (chymosin), the latter two being developed due to a world shortage of calf rennet as a result of the substantial increases over the years in cheese production. The uniqueness of rennet as a protease is its extremely high substrate specificity, in that it hydrolyzes a single-specific peptide bond (Phe105–Met106) in the k-casein fraction of milk, to produce para-k-casein and a macropeptide, without attacking other caseins. As k-casein serves to maintain the milk micelles in suspension, whereas neither the para-k-casein nor a macropeptide serves this function, cleavage of this bond by rennet results in destabilization of the milk micelles and cheese coagulation. The initial stages of the cheese-making process typically involve the use of lactic acid producing starter cultures to reduce the pH of the milk (or cream) to about pH 4.6, where the predominant casein proteins approach their isoelectric pHs, rendering them more amenable to coagulation. At this point, rennet is added to effect coagulation. Thus, a unique property of rennet is its very high ratio of milk clotting to general proteolytic activity and the challenge in searching for microbial rennets was to find enzymes that approached this characteristic activity ratio. Many of the candidates tended to have higher proteolytic activity that manifested itself in the production of weaker curds or in softening of the cheese over time in storage. The most important native microbial cheese-making enzyme commercialized as a result of these searches was the enzyme produced by *Rhizomucor miehei*. Recombinant chymosin was introduced in the late 1980s. Whey, the byproduct of cheese making is rich in proteins, some of which may be rendered insoluble by heat denaturation. Both the insoluble and soluble proteins of whey may be hydrolyzed into peptides and amino acids, mediated by trypsin and microbial proteases with pH optima reflecting the pH properties of the whey.

3.49.4.2.3 Baking

Proteases are used in baking to modify gluten, a protein in wheat with viscoelastic properties that has the ability to expand as bread dough rises during the baking process. The dough-making process can be accelerated by addition of proteases to partially hydrolyze the gluten. For example, weak gluten flour is needed for biscuit making but is difficult to source. This problem may be resolved by protease-catalyzed degradation of gluten in more readily obtainable high gluten-containing flour. Typically, heat-labile fungal proteases are used for gluten hydrolysis in baking, such that the enzyme denatures as temperature rises in the early stages of baking. A second application of proteases in baking relates to the modifications linked to flavor and nutritional development. Bacterial neutral proteases may be used for this purpose, for example, in production of biscuits, cookies, and crackers.

3.49.4.2.4 Production of protein hydrolysates

Protein hydrolysates are widely used as food and feed additives, where they have a variety of protein property altering characteristics. Many types of proteins, including soy protein, gelatin, caseins, and whey proteins may be modified using proteases. Fish and meat hydrolysates are prepared using proteases, and proteases may also be used for meat tenderization. Indeed, natural meat tenderization mediated by endogenous proteases in the muscle after animal slaughter is known to occur. Proteases may be used to recover proteins from bones during animal rendering, which in turn may be incorporated into canned soups, sauces, and meats, especially cured meats and sausages. Microbial proteases, especially the *Bacillus* alkaline serine proteases have long been used for preparation of protein hydrolysates. However, neutral proteases, for example, from *B. licheniformis* are also used for this application. Where protein hydrolysis is implemented under more acidic conditions, proteases from fungi such as *A. oryzae* and *Rhizopus niveus*, and related strains are more frequently applied. Protein hydrolysates, after enzyme hydrolysis, may be used in human food formulations, including in infant formula and in animal feeds, or the protease may be added to the protein-containing food or feed, enabling proteolysis to take place during further processing of the food or feed. Protein hydrolysates have useful functional properties for fortifying fruit juices and other beverages. Protein hydrolysates are known to have important blood-pressure-regulating properties and may be used as therapeutic dietary agents.

Many applications of proteases in food processing and protein hydrolysates relate to flavor production. Manipulation of hydrolytic conditions for hydrolysis of soy protein by *Bacillus* alkaline protease can result in the development of contrasting functional and flavor properties. A well-recognized problem in the protease-mediated production of protein hydrolysates is the associated development of a characteristic bitter taste, which is attributed to the presence of hydrophobic amino acids as well as proline within the peptides generated by proteolysis. The bitterness is often attributed to terminal hydrophobic amino acids of the peptides produced, but nonterminal hydrophobic amino acids in small to medium-sized oligopeptides may also exhibit a bitter taste, whereas larger peptides appear nonbitter. Bitter tastes have been observed in some cheese-making processes and were attributed to a protease derived from *Pseudomonas R098*. Hence, the importance of controlling the hydrolysis process to minimize these bitter tastes.

3.49.4.3 Proteases in Leather and Fabric Processing

There are four principal steps in leather processing: soaking, dehairing, bating, and tanning. Soaking generally involves addition of alkali solutions that are applied to swell the hide. Application of high alkali concentrations combined with a hydrogen sulfide

treatment step for protein hair root solubilization contributes to conventional chemical removal of hair and/or wool from hides. As occurs in other industries, there is a strong move away from use of harsh chemicals in processing because of the associated safety risks to workers handling these materials. Also, these methods add significant high costs to effluent treatment and waste disposal and increase risks of spills and pollution. Therefore, more environmentally friendly approaches are desirable and proteases can fill this niche. Protease application in leather processing, thus, reduces the safety, higher handling, and disposal costs associated with chemical treatments. Furthermore, the potential to apply proteases to selectively attack noncollagenous protein constituents offers the opportunity to enhance leather quality over and above what is achievable through chemical treatment.

Proteases may be applied to processes for silk manufacture. The silk protein sericin imparts a rough texture to raw silk fibers and expensive processes involving starch application in shrink proofing and twist setting have been used to remove sericin from the core silk fiber proteins. A number of processes that use proteases for silk degumming prior to implementation of key dyeing processes have been described in the patent literature.

3.49.4.4 Other Applications

Proteases have also found more specialized applications in processes for purification of nonprotein products from animal or plant extracts including extraction of carbohydrate gums and mucopolysaccharides. Proteases may be used for solubilization of keratin materials to convert waste materials such as feathers to protein concentrates for use as animal feeds. An alkaline protease from *Streptomyces* species also has strong keratinolytic activity. The plant proteases, papain and bromelain are effective as meat tenderization enzymes, as is the *B. subtilis* neutral protease. Other industrial applications of proteases include their use in silver recovery from conventional gelatin-containing photographic film including X-ray film, and in the liquefaction of industrial and household organic waste. Proteases may also be consumed by humans and animals as digestive aids.

3.49.4.5 Proteases in Organic Synthesis

Although traditionally proteases have been applied for hydrolysis of peptide bonds, it has been known for a long time that proteases can catalyze the reverse reaction, namely enabling synthesis of peptide bonds, where the reaction mixture contains high concentrations of amino acids or peptides that drive the equilibrium of the reaction toward synthesis. More recently, hydrolytic enzymes have been applied for catalysis of reactions involving water as reactant or product, whereby the water concentration in the reaction medium is partially or wholly replaced by another solvent, thereby shifting the reaction equilibrium in favor of synthesis. As water molecules may also participate in maintaining the requisite conformational structure of proteins for biocatalysis, much research has been directed to understanding the impact of water, and indeed water concentration on enzyme activity and stability and the associated impact of implementing enzyme reactions in nonconventional organic media or biphasic aqueous–organic systems. In addition, enzymes have been identified or engineered to be more stable in organic solvents. For example, a microbial protease from *Thermus* strain Rt4A2 is very stable in organic solvents. Subtilisin has been rendered more stable in organic solvents by covalent modification of the enzyme using selected polymers or by application of covalent or noncovalent methods to bind the enzyme to supports. The research of Chen and Arnold (reviewed by Kuchner and Arnold [5]) applied the tools and concepts of directed evolution to developing subtilisin proteases that were active in the presence of organic solvents and, hence, applicable to using proteases catalyze synthesis of peptides and related bonds. Protease PST-01 from *Pseudomonas aeruginosa*, which exhibits a high level of homology to thermolysin, also contains a disulfide bond (one of two), which contributes substantially to its solvent stability. Another approach to replacing water in the reaction medium is to use supercritical fluids as the reaction solvent. These topics are discussed in detail in two excellent texts edited by Patel [10, 11]. The following are some examples of the applications of proteases in bioorganic synthesis:

1. The capacity of proteases to synthesize di- and oligopeptides by coupling of peptide esters to N-protected amino acids was exploited by Toya Soda Company Japan for aspartame (L-phe-L-asp-methyl ester) synthesis, mediated by thermolysin from *B. thermoproteolyticus*. The immobilized thermolysin catalyzes formation of aspartame through reaction of L-aspartyl-methyl ester with L-phenylalanine containing an amino group blocking agent, after which the blocking agent may be removed chemically.
2. Prolyl-endopeptidase from *Flavobacterium meningoseptum*, a thermostable protease from *C. Thermohydrosulfuricum*, and pronase from *Streptomyces griseus* have been used for synthesis of various peptides.
3. Proteases, such as subtilisin and clostripain have been used in glycoconjugate synthesis. Strategies included participation of the enzyme directly in acylation of the carbohydrate or in formation of a peptide bond between a glycopeptide and a peptide.
4. The ability of proteases to mediate a variety of other reactions in addition to peptide bond cleavage, namely reactions involving esters and amides of carboxylic acids, renders them interesting and effective tools for resolution of pairs of enantiomers in racemic mixtures through enantioselective bond cleavage or formation. Example enzymes that have been applied for this purpose include subtilisin, *A. oryzae* protease, and serine alkaline protease from *Thermoactinomyces vulgaris*. The leucine aminopeptidase from *P. putida* ATCC 12633 has been cloned into *E. coli* K-12 and has been commercialized for production of several optically pure L- and D-amino acids using resolution strategies. Subtilisin, thermolysin, chymotrypsin, and papain have been applied in preparative synthesis of Leu- and Met-enkephalin.

5. Subtilisin was effective in the regioselective deacylation of peracylated nucleosides. Subtilisin also mediated acylation reactions of dialkyl and diallyl carbonates in the resolution of racemic amines. Subtilisin efficiently resolved recemic amine mixtures, for example, in preparation of a precursor of the synthesis of rasagiline, useful in treatment of depression and dimentia as well as in synthesis of oral calcimimetic drugs. Subtilisin–ruthenium combinations were effective in dynamic kinetic resolution of secondary alcohols.

6. Human insulin may be synthesized from porcine insulin by protease-mediated exchange of amino acid No. 30 of the B insulin chain from alanine to threonine. Amino acid No. 29 of the B insulin chain is lysine and, hence, a lysine-specific protease clips off the terminal alanine. The des-B30-insulin product is then reacted with threoninyl-*tert*-butyl ester in an organic solvent medium, mediated by a lysine-specific protease from *Achromobacter* sp, after which the *tert*-butyl ester-blocking agent is removed to produce human insulin.

3.49.4.6 Examples of Nutritional and Therapeutic Applications of Proteases

At least three applications of proteases may be identified in health-care sector, in thrombosis, in specific cancer treatments, and as digestive aids:

1. The extracellular protease, streptokinase, produced by the hemolytic *Streptococcus* species, which has the ability to rapidly dissolve clots, is used to treat acute blocking of arteries, deep vein thrombosis, and pulmonary embolism and is a widely used therapy for treatment of coronary thrombosis caused by myocardial infarction. A contraindication is that it also degrades other blood protein factors (including factors V and VIII and plasmin), which can result in hemorrhaging. An alternative approach being developed relates to synthesis of the human thrombolytic enzyme, tissue plasminogen activator (t-PA), using Chinese hamster ovary cells. This recombinant product, produced by Genentech Corporation (www.gene.com/gene/products/information/…/activase/) has been approved by the FDA.

2. It has been observed that certain proteases can negatively impact certain tumor and may thus have therapeutic value. Tumor necrosis and solubilization has been observed to be induced by microinjection of solid tumors with proteases from *Serratia marcescens*. Use of carboxypeptidase G_1 from *Pseudomonas* sp. to cleave off the terminal glutamic acid from folic acid can induce a state of deficiency of folic acid, which can result in 'starvation' of certain tumor cells. The latter enzyme may also be exploited to produce a tissue-specific antiproliferative brain tumor agent, methothrexate, from a folic acid analog.

3. *Botulinum toxin*. Botulinum toxin (BOTOX, BoNT) is a neurotoxin produced by *C. botulinum*, is the causative agent of food poisoning (www.allergan.com). Over the years (1989 to date), BoNT has been approved by the FDA for various therapeutic treatments of involuntary muscle disorders and for cosmetic uses. BoNT contains a heavy and light chain and has the ability to target colinergic nerve endings and bind with high affinity to the presynaptic motor nerve endings. The light chain consists of a zinc-dependent metalloprotease that attacks a group of proteins (the SNAREs) which mediate neurotransmitter release from the motor nerve endings. Hence, the proteolytic action of BoNT results in blocking acetylcholine release and has applications in treatment of conditions resulting from hyperactivity at cholinergic nerve endings (spasms, excessive blinking, and migraine) and in blocking pain-sensitization processes. Examples of other applications are in wound healing and in cosmetic injection procedures to prevent formation of wrinkles. The structures and modes of action of different botulinum toxins have been reviewed by Dolly and Aoki [2].

4. Proteases are present in animal digestive fluids and the complex digestive fluids of venomous snakes are known to contain proteases that are known to have a variety of toxic physiological effects, including hemorrhagic and cyto-, myo- and hemo-toxic effects.

3.49.5 Protease Inhibitors

As proteases participate so widely in physiological functioning, it is to be expected that hyperproteolytic endogenous activity may contribute in some cases to abnormal physiological functioning and disease states. In addition, in humans and animals, proteases play significant roles in a variety of microbial and viral pathophysiological conditions, including acquired immune deficiency syndrome (AIDS), staphylococcal infections, candidiasis, and malaria as well as in certain cancers, which has given momentum to the identification and development of protease inhibitors as therapeutic agents against disease-causative proteases. It appears that aspartic-, cysteine- and metalloproteases are the predominant enzymes involved in human disease conditions such that inhibitors that modulate the mechanisms of these enzymes are target therapeutic candidates. An extensive list of protease inhibitors in provided on the Science Gateway Link (http://www.sciencegateway.org/resources/protease.htm).

Many microbial pathologic diseases are linked to infection of organs in one way or another exposed to the exterior environment including skin, mouth, lungs, eyes, ears, nose, other cavities, intestine, and soft tissues. Given that collagen accounts for around 30% of total mammalian protein, it is not surprising that proteases attacking collagen (collagenases) are associated with many

pathological disease conditions. Microbial collagenases, involving a host of clinical organisms, have been implicated in certain mycetomas, abscesses, ulcers, septicemia, emphysemia, ectyma gangrenosum, pregnancy complications, periodontal diseases, dental caries, and necrotizing diseases. Therefore, it is clear that inhibitors to collagenases have enormous potential in disease therapy. This is further discussed in Harrington [4].

Proteases from blood-feeding invertebrates, for example, the medical leech, *Hirudo medicinalis*, produce serine protease peptide inhibitors that interact with trypsin-like proteases to block blood coagulation in their hosts [21].

Proteasome inhibitors have been developed as chemotherapeutic agents with antitumor activity though induction of apoptosis [1]. Bortezomib is effective in the treatment of multiple myeloma, where increased levels of proteosomes are observed in blood serum. Other potential applications of bortezomid are in treatment of B-cell and pancreatic cancers. The HIV protease inhibitor therapeutic, ritonavir, also inhibits the trypsin-like activity of proteosomes and may have inhibitory activity against glioma cells. Proteasome inhibitors are also potential therapeutics for autoimmune diseases.

Although thrombolytic activity of the serine protease, t-PA, appears beneficial for acute stroke treatment, t-PA also may determine the extent of neuronal damage after injury of the central nervous system. Serine proteases are present in neurons and glial cells together with endogenous serine protein inhibitors that play a neuroprotective role [16]. Clearly, modulating the proper balancing of the interactions of the proteases and their inhibitors will be important in strategic treatment of brain injury and in using t-PA as a therapeutic agent.

Protease inhibitors have been developed against proteases that viruses use to cleave nascent proteins for assembly of new virions, for example, in treatment or prevention of viral infection by HIV or hepatitis C viruses. Protease inhibitors developed against HIV, sequinavir, ritonavir, and lipinavir have also exhibited antiprotozoal activity, for example, against malaria and Giardia infection (http://en.wikipedia.org/wiki/Proteasome).

Neutral endopeptidase (NEP) inhibitor, omapatrilat, acts to inhibit metalloproteases including the angiotensin-converting enzyme in treatment of hypertension and in clinical management of congestive heart failure. Omapatrilat also beneficially inhibits NEP that degrades atrial natriuretic peptide, secreted by the heart.

Resistance may develop to protease inhibitor treatment of viral diseases, especially when the inhibitor drug is used at suboptimal concentrations/dose rates. This is perceived as a serious problem, for example, in HIV treatment, where at least 10% of the amino acids of the HIV protease may be substituted through mutation while still retaining a viable virus [8] (http://www.aids.org).

References to some of the most cited papers on classification of protease inhibitors are summarized in the BioinfoBank Library (http://lib.bioinfo.pl/meid:190879).

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