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WATER ORGANISMS AS A SOURCE OF PROTEASES AND ITS INHIBITORS. CALCIUM-DEPENDENT PROTEASES (CALPAINS)

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Proteases are enzymes that are essential to all life. They are biology's version of Swiss army knives (Seife, 1997) that cut up biological polymers. Proteases regulate the fate, localization, and activity of many proteins, modulate protein-protein interactions, create new bioactive molecules, contribute to the processing of cellular information, and generate, transduce, and amplify molecular signals. As a direct result of these multiple actions, proteases influence DNA replication and transcription, cell proliferation and differentiation, tissue morphogenesis and remodeling, heat shock and unfolded protein responses, angiogenesis, neurogenesis, ovulation, fertilization, wound repair, stem cell mobilization, hemostasis, blood coagulation, inflammation, immunity, autophagy, senescence, necrosis, and apoptosis (López-Otín and Bond, 2008). Proteases are also essential in viruses, bacteria and parasites for their replication and the spread of infectious diseases, as well as for effective transmission of disease, and in animal hosts for the mediation and sustenance of diseases (Turk, 2006).

The recent availability of the genome sequence of different organisms has allowed the identification of their entire protease repertoire (termed degradome) (Quesada et al., 2009) (table). Thus, the human degradome contains over 500 human proteases (561 known proteases, 175 putative proteases and pseudogenes, 400 inactive homologues), accounting for 2% of structural genes in humans.

Table. Complexity of degradomes (data from *MEROPS* and *Degradome* Databases)

Organisms	Representative species	Number of proteases
Mammals	Homo sapiens	561
	Rattus norvegicus	646
	Mus musculus	656
	Ornithorhynchus anatinus (platypus)	>500
Birds	Gallus gallus	382
Fish	Danio rerio	503
Amphibia	Xenopus tropicalis	278
Insects	Drosophila melanogaster	558
Nematodes	Caenorhabditis elegans	403
Plants	Arabidopsis thaliana	723
	Populus trichocarpa	955

Many families of human proteases are also clearly recognizable in the genomes of *D. melanogaster*, *C. elegans* and *A. thaliana*. This indicates the existence of universal proteolytic routines in these organisms, although they are frequently expanded in vertebrates. It has become evident that, in addition to highly conserved proteolytic routines, there are also specific roles played by unique proteases in different species. These comparative genomic studies have also provided valuable insights into the conservation, evolution, and functional relevance of this group of enzymes.

Proteases likely arose at the earliest stages of protein evolution as simple destructive enzymes necessary for protein catabolism and the generation of amino acids in primitive organisms. Through evolution, proteases have adapted to the wide range of conditions found in complex organisms

(variations in pH, reductive environment and so on). Despite proteases share a common biochemical function, their catalytic domains exhibit high sequence diversity. Thus catalytic core of proteases characterizing mechanisms of action classifies them as either serine, cysteine or threonine proteases (*N*-terminal nucleophile hydrolases), or as aspartic, metallo and glutamic proteases (with glutamic proteases being found only in fungi) (Rawlings et al., 2010). Protease diversity is further increased by the frequent attachment of ancilliary, non-proteolytic domains to the catalytic moieties (López-Otín and Overall, 2002). A variety of specialized functional modules that provide substrate specificity, guide their cellular localization, modify their kinetic properties, and change their sensitivity to endogenous inhibitors. These non-catalytic domains include archetypal sorting signals that direct these enzymes to their proper location, autoinhibitory prodomains that prevent premature activation, and ancillary domains that facilitate homotypic interactions or heterotypic contacts with other proteins, substrates, receptors, or inhibitors. It is very likely that the substantial combinatorial activity observed in protease genes has been a driving force in the protease transition from nonspecific primitive enzymes to highly selective catalysts responsible for subtle proteolytic events that are at the heart of multiple biological processes.

The complexity of proteases is further increased through post-transcriptional events such as alternative splicing and differential polyadenylation of genes encoding proteases (Freije et al., 1994; Mitsui et al., 2008), by the occurrence of gene copy number variations or polymorphic variants that may contribute to the modification of protease functions or alter their regulatory mechanisms (Masson et al., 2008), or by post-translational modifications. Finally, proteases act in the context of complex cascades, pathways, circuits, and networks, comprising many protein partners that dynamically interact to form the so-called protease web (auf dem Keller et al., 2007).

All known endogenous protease inhibitors are proteins, although some microorganisms produce small non-protein inhibitors. To date, the number of identified endogenous inhibitors is considerably lower than that of proteases. As an illustrative example, a total of 105 genes encoding protease inhibitors have been annotated in the human genome, and there are only 1–2 inhibitors per prokaryote genome. Nature inhibitor specificity varies from one target protease (such as calpain for calpastatin) to numerous proteases of several catalytic types (such as plasma α_2-macroglobulin).

Consistent with the essential roles of proteases in cell behavior, survival and death, alterations in spatiotemporal patterns of expression of proteases or abnormal levels of natural inhibitors/activators underlie multiple pathological conditions such as cancer, neurodegenerative disorders, and inflammatory and cardiovascular diseases. Furthermore, mutations in protease genes result in over 80 hereditary diseases in human (Puente et al., 2003). The key role of proteases and protease inhibitors in many physiological and pathophysiological processes makes them attractive targets for pharmaceutical industry as potential drug targets or as diagnostic and prognostic biomarkers (Leung et al., 2000; Turk, 2006). Their best-known representatives include angiotensin-converting enzyme (ACE) inhibitors and HIV protease inhibitors. Finally, proteases are also important tools of the biotechnological industry because of their usefulness as biochemical reagents or in the manufacture of numerous products (Saeki et al., 2007).

Calpains (EC 3.4.22.17) or Ca^{2+}-dependent cysteine neutral proteases along with cathepsins and proteasome are one of the main proteolytic systems present in any cell of various organisms – from protozoan to humans. Total protein degradation in cells is probably the result of the synergistic proteolytic action of proteases indicated above (Ouali et al., 1992; Goll et al., 2003), even if only calpains are sometimes described to mediate the proteolysis or the early stage of this process (Ladrat et al., 2000). Calpains are constitutive enzymes, but as noted by Cottin et al. (1994), they should not be considered as housekeeping enzymes because the transcription of the calpain gene could be regulated. Although definitive physiological roles are not yet clearly identified, calpains, having regulatory or signaling function in cells rather than a digestive function such as the lysosomal proteases or the proteasome (Goll et al., 2003), are believed to participate in numerous cellular and physiological processes: proliferation, differentiation, cell migration, signal transduction, skeletal muscle growth, metabolic disorders or degenerative diseases, and cell death (via necrosis or apoptosis) (Goll et al., 2003; Tidball and Spencer, 2000; Wang, 2000).

Calpains constitute divergent protease family C2 (cysteine protease clan CA) (*MEROPS* database) consisting of 643 nucleotide sequences (14 in human), 26 identifiers, including 3 proteins of known tertiary structures. Typical members are composed of four domains, including prodomain, catalytic domain of

cysteine protease (catalytic triad $Cys^{105}/His^{262}/Asn^{286}$), C2-like domain, and calmoduline-like domain with five EF-hand Ca^{2+}-binding motifs. Catalytic core are highly conservative among family. Atypical calpains contain ancillary functional domains instead of EF-hand-containing domain IV. Despite the absence of Ca^{2+}-binding EF-hand motifs all known calpains are Ca^{2+}-dependent (Croall and Ersfeld, 2007). Ubiquitously expressed calpains 1 and 2 form heterodimers with regulatory small subunit, other calpains are monomeric proteins expressed mainly in tissue-specific manner (Goll et al., 2003). Some calpain-dependent human diseases, so-called calpainopathies, are described. Some of them are hereditary, such as limb-girdle muscular dystrophy type 2A associated with single-nucleotide mutation in calpain 3 gene, whereas other depends on regulatory defects such as calcium inbalance.

The calpain proteolytic system in vertebrates consists of at least three components: 1) the form of the protease that is fully active at micromolar concentration of calcium (μ-calpain), 2) the form of the proteinase that is fully active at millimolar concentration of calcium (m-calpain), and calpastatin, which inhibits the activity of both μ- and m-calpains at their respective calcium requirement. However the structural and biochemical features of invertebrate calpains and contribution of calpain-mediated proteolysis in metabolic response reactions due to variable factors have not defined satisfactory yet (Mykles, 1998).

We studied calpain system in numerous freshwater and marine invertebrates and fish: Annelida (*Stylodrilus heringianus*, *Herpobdella octoculata*), Crustacea (*Asellus aquaticus*, *Gammarus* spp., *Polyphemus pediculus, Daphnia pulex*), Mollusca (*Limnaea intermedia, L. polustris, Viviparius viviparius, Planorbis planorbis, Dreissena polymorpha, D. bugensis*, *Mytilus edulis* L., *Unio longirostris*), Insecta (larvae of *Erythromma najas, Limnephilus stigma*, *Siphlonurus linneanus*, *Acilius* spp., *Chaoborus* spp.), Esocidae (pike *Esox lucius* L.), Cyprinidae (roach *Rutilus rutilus* L., crucian carp *Carassius carassius* L.), Percidae (perch *Perca fluviatilis* L.), Coregonidae (whitefish *Coregonus lavaretus*, *C. albula*), Gadidae (navaga *Eleginus navaga* Pall.), Salmonidae (Atlantic salmon *Salmo salar* L., rainbow trout *Salmo trutta* L.). Calpain-like activity was detected in all studied organisms have atypical calpains. The highest level of calpain activity was observed in the most primitive organisms (annelid worms) (Kantserova et al., 2010). Marine invertebrates (mussels and crustacean) are also a good source of calpains due to high expression and more simple purification procedure in the absence of endogenous inhibitors. It was confirmed that genes of calpastatine *CAST* and regulatory small subunit *CSS* are found only in vertebrates (fish). So invertebrates contain only monomeric calpains which are inherently not susceptible to calpastatin regulation. Fish contain both typical and atypical calpains as well as non-proteolytic homologues. Analysis of structural composition of fish and invertebrate calpains has shown that all known ancillary domains (T, PalB, Zn-finger, SOL, additional C2-like) are presented (Goll et al., 2003; Bondareva et al., 2008). Besides invertebrates calpains are known to have wider substrate specificity than vertebrate calpains. Thus myofibrillar proteins actin and myosin are substrates for crustacean calpains (Mykles, 1998) but not for vertebrate calpains. Due to easier degradome composition, protease abundance, lack of some regulatory mechanisms and wider substrate specificity invertebrates (especially marine invertebrates – crustacean and mussels) are a rich source for calpains. Possessing all human calpain homologs fish and invertebrates are a good model to study basic calpain-mediated processes such as proliferation, cell cycle, biogenesis of organelles, autophagy as well as inducible calpain-dependent disorders: muscular dystrophy, cataractogenesis, necrosis, etc.

Despite indisputable academic interest there are some practical aspects of calpain-calpastatine study. Pharmaceutical industry needs: (1) drug design on the basis of calpastatin inhibitory sequences, (2) recombinant tissue-specific calpains to treat hereditary deficit. Consider role of calpain-calpastatine system in the postmortem tenderization process (Koohmaraie, 1992) the genetic modification of cattle and fish breeds is a tool to enhance quality of meat and fish fillet. There are some successful examples such as cattle and fish breeds characterized by μ-calpain overexpression, specific SNPs in μ-calpain gene (*CAPN1*) or reduced calpastatine expression (Chéret et al., 2007; Salem et al., 2007). Furthermore, a net increase in the calpain/CAST mRNA ratio with a corresponding increase in calpain catalytic activity induced by starvation was shown in rainbow trout (Salem et al., 2005).

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