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ARTÍCULO DE REVISIÓN

Biomedical and Biological relevance of subtilases from the S8 family, diverse and widely distributed serine peptidases

Importancia biomédica y biológica de las subtilasas de la familia S8, peptidasas de tipo serino; diversas y ampliamente distribuidas

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ABSTRACT

Serine peptidases are enzymes widely distributed in all organisms. They are characterized for the presence of a serine residue at the active site, responsible of the nucleophilic attack during catalysis. Among serine peptidases, subtilases from the S8 family constitute the second more abundant family. They are divided in two subfamilies: subtilisin subfamily S8A and kexin subfamily S8B. Both subfamilies are widely distributed in all kingdoms, from bacteria to mammals, playing different functions. Prokaryotic subtilases are generally secreted outside the cell, and are mainly known to play a role in either nutrition or host invasion, acting as virulence factors. However, in eukaryotic cells, these enzymes are generally involved in the proteolytic processing of secreted proteins including cytokines, hormones, growth factors and receptors; and are involved in pathologies like cancer, diabetes or infectious diseases. All these elements, justify the great interest of the scientific community on this group of enzymes. This Rev. specifically highlights their Biol. roles as well as their biomedical relevance.

Keywords: Subtilases, biomedical relevance, virulence factors, diversity.

RESUMEN

Las peptidasas de tipo serino son enzimas ampliamente distribuidas en todos los organismos. Estas se caracterizan por la presencia de un residuo de serina en el sitio activo, responsable del ataque nucleofílico durante la catálisis. Entre las serino peptidasas, las subtilasas de la familia S8 constituyen la segunda familia más abundante de las proteasas de tipo serino. Estas enzimas están divididas en dos subfamilias: las subtilisinas de la subfamilia S8A y las kexinas de la S8B. Ambas subfamilias están ampliamente distribuidas en todos los reinos desempeñando

diversas funciones. Las subtilasas de origen procariótico son generalmente secretadas hacia el exterior celular, y comúnmente desempeñan funciones que pueden ir desde la nutrición hasta la invasión al huésped, actuando como factores de virulencia. Sin embargo, en las células eucariotas, estas enzimas están generalmente involucradas en el procesamiento proteolítico de proteínas secretadas incluyendo citoquinas, hormonas, factores de crecimiento y receptores; y están relacionadas con patologías como el cáncer, diabetes o enfermedades infecciosas. Todos estos elementos justifican el gran interés de la comunidad científica en este grupo de enzimas. Esta revisión resalta específicamente sus roles biológicos, así como su importancia biomédica.

Palabras clave: *subtilasas, relevancia biomédica, factores de virulencia, diversidad*

INTRODUCTION

Subtilases from the S8 family, are the second most abundant family of serine peptidases (Schaller *et al*., 2017). They can be divided in two subfamilies: subtilisin subfamily (S8A) and kexin subfamily (S8B) (Rawlings *et al*., 2018). Subtilisin subfamily members are widely distributed in all kingdoms, from bacteria like *Bacillus* spp. (Siezen & Leunissen, 1997) and *Dichelobacter* spp. (Park Han *et al*., 2018), to mammals (Sakai *et al*., 1998). They can also be found in plants, being involved not only in all aspects of the plant life cycle as well as in the response to biotic and abiotic stress (Figueiredo *et al*., 2018; Schaller *et al*., 2011). Likewise, kexin subfamily members are mainly distributed in eukaryote, like furin (*Homo sapiens*) which is involved in the proteolytic modification of many secreted proteins (Ven *et al*., 1990) and kexin (*Saccharomyces cerevisiae*), involved in alpha factor maturation on yeasts (Fuller *et al*., 1988).

Furthermore, these enzymes are key players on the life cycle of parasites like *Plasmodium falciparum* (Jean *et al*., 2005), *Toxoplasma gondii* (Kim, 2004), *Leishmania* spp. (Swenerton *et al*., 2010) and *Trypanosoma cruzi* (Lovato *et al*., 2011) and many viral pathogens including, coronavirus, flavivirus, pneumovirus, avian influenza, influenza A and HIV (Nemunaitis *et al*., 2020). Mammalian substrates of furin include cytokines, hormones, growth factors and receptors. Aberrant furin activity is associated with a variety of disorders including cancer, diabetes and neurological diseases (Braun & Sauter, 2019). Furin may not only promote disease upon aberrant expression, but also by activating a variety of pathogen-derived proteins like diphtheria toxin, *Pseudomonas* exotoxin A (Ogatas *et al*., 1992; Tsuneoka *et al*., 1993) and Shiga and Shiga -like toxins expressed by certain *Shigella* spp. Considering its biomedical and biochemical relevance, subtilases have promoted great interest on the scientific community and are the main subject of this revision.

General Biochemical features of subtilases

Serine peptidases are the enzymes with a serine residue in their active center responsible for the nucleophilic attack during catalysis. These enzymes are distributed in 14 clans according to the database MEROPS (Rawlings *et al*., 2018). Specifically, the SB clan is divided in two families: S8 (subtilases) and S53 (sedolisins) (Rawlings *et al*., 2018), being the subtilases (S8) the second more abundant family among serine peptidases (Schaller *et al*., 2017). Likewise, this family is divided in two subfamilies: subtilisins (S8A) and kexins (S8B) (Rawlings *et al*., 2018).

Subtilases are proteins with an average Mol. weight between 26 and 29 kDa and a general folding consisting on seven beta sheets flanked by two alpha helix layers (Rawlings *et al*., 2018) (Fig. 1). These enzymes are mostly monomeric proteins, usually they have only one catalytic domain (Rawlings *et al*., 2018) and are encoded as preproproteins that once synthetized are modified in both extremes of polypeptide chain (van der Hoorn, 2008). Most members of this family are active at neutral-mildly alkali pH. Casein is often used as a protein substrate and a typical synthetic substrate is Suc-Ala-Ala-Pro-Phe-NHPhNO_2 (Rawlings *et al*., 2018).

Typical catalytic triad of serine peptidases consist on Asp, His and Ser (Rawlings et al., 2018). The order of these residues in the sequence variates between families like S1 (His-Asp-Ser), S9 (Ser-Asp-His), S10 (Ser-Asp-His) and S8(Asp-His-Ser) (Rawlings et al., 2018). In subfamily S8A, the active site residues frequently occur in the motifs Asp-Thr/Ser-Gly (which is similar to the sequence motif in families of aspartic endopeptidases in clan AA (AA)), His-Gly-Thr-His and Gly-Thr-Ser-Met-Ala-Xaa-Pro (Fig. 1A). In subfamily S8B, the catalytic residues frequently occur in the motifs Asp-Asp-Gly, His-Gly-Thr-Arg and Gly-Thr-Ser-Ala/Val-Ala/Ser-Pro (Rawlings et al., 2018) (Fig. 1B).

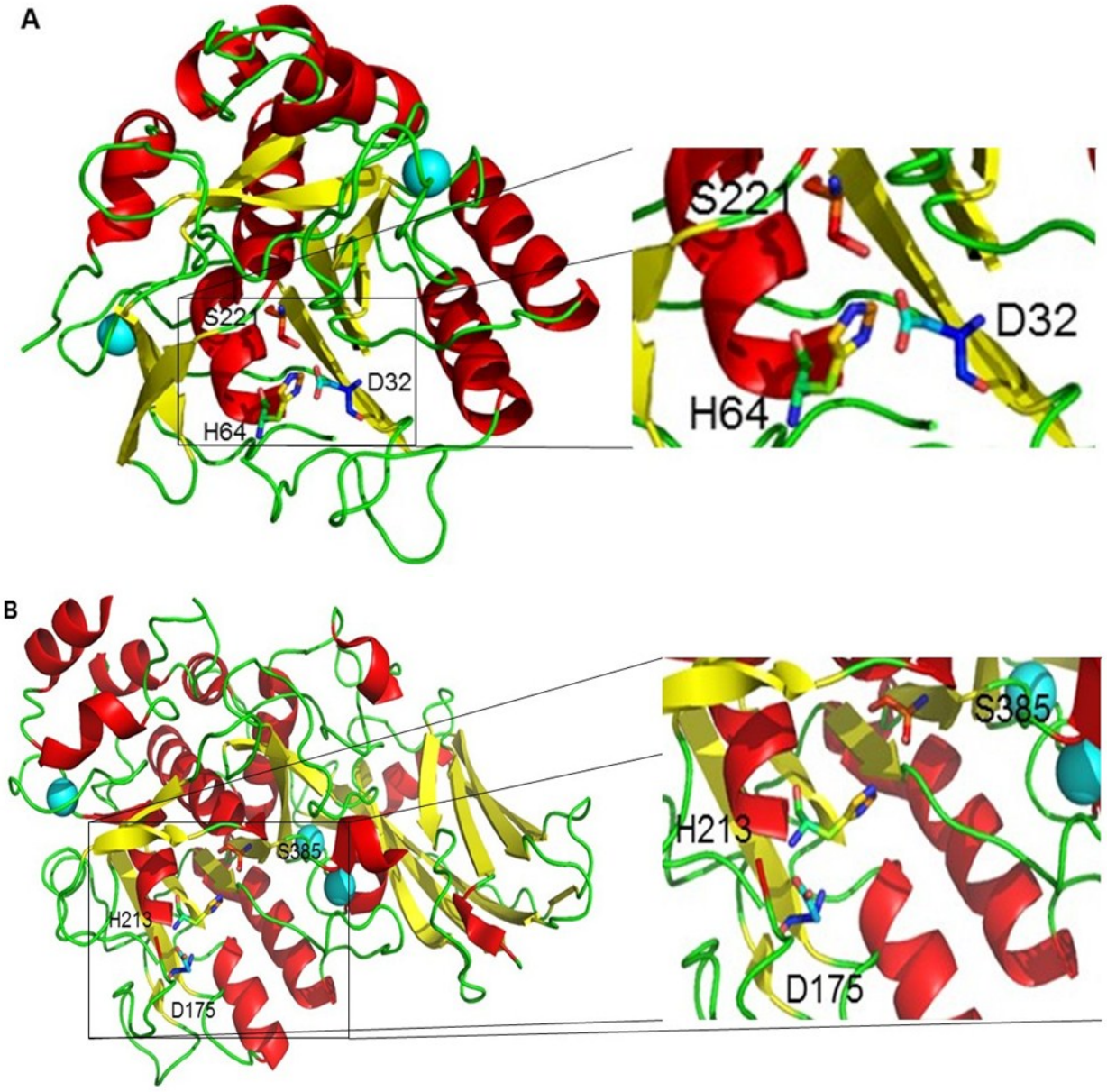

Figure 1. Typical 3D structure of S8A and S8B subfamilies. **A**, subtilisin Carlsberg 3D structure (*B. licheniformis*). **B**, kexin 2 3D structure (*S. cerevisiae*). Images in cartoon representation were obtained using the software Pymol 1.7.2.1 (http:www.pymol.com) and PDB files: 1CSE-A (A) y 1OT5-A (B). Catalytic residues are shown in sticks representation and labeled with their position in the sequence. Calcium atoms are shown in cyan, α helix in red, β sheets in yellow and loops in green. At the right side of the images there are zooms of the active center.

Figura 1. *Study Estructuras 3D típicas de las subfamilias S8A y S8B. A, estructura 3D de la subtilisina Carlsberg (*B. licheniformis*). B, estructura 3D de la kexina 2 (*S. cerevisiae*). Las estructuras se representan en cintas y se obtuvieron usando el programa Pymol 1.7.2.1 (http:www.pymol.com) y los archivos PDB: 1CSE-A (A) y 1OT5-A (B). Los residuos catalíticos se muestran en representación de varillas y se etiquetaron con su posición en la secuencia. Los átomos de calcio están mostrados en cian, las hélices alfa en rojo, las hojas beta en amarillo y los lazos en verde. A la derecha de las imágenes se encuentra un aumento del centro activo.*

In the S8 family, a channel able to accommodate at least six amino acids (P4-P2´) of a polypeptide substrate forms the active center. Generally, subtilases specificity seems to be determinated by the interaction of P4-P1 residues of substrate with S4-S1 pockets of enzyme (Groen *et al*., 1992). These sites have several common features (Enzyme numbering is from subtilisin BPN sequence) (Siezen & Leunissen, 1997) (Fig. 2):

S2´: hydrophobic pocket of variable size, which depends on the lateral chain orientation of the aromatic residue conserved in the position 189.

S1: long cleft flanked by 125 to 128 residues and from 152 to 155.

S2: small cleft flanked in one side by 100 residue and histidine 64 of active center. At the bottom it is limited by the hydrophobic residue 96 and the aspartic 32 of the active center.

S3: It is not relevant because P3 residue it is generally oriented to the solvent.

S4: a pocket formed by residues from 101 to 104 and from 126 to 130, which seem to have two subsites with different characteristics.

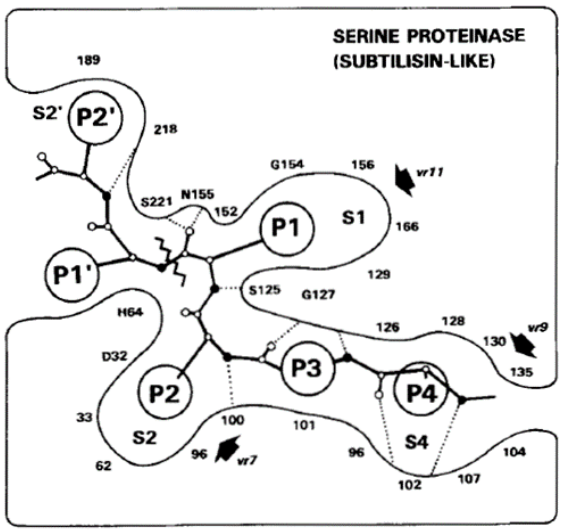

Figure 2. Schematic representation of substrate/inhibitor (bold lines) binding to the active center of a subtilisin-like serine peptidase (smooth surfaces) Side chains of the **P4-P2'** residues are shown as large circles; positions of the enzyme residues that may interact with these **P4-P2'** side chains are shown surrounding the binding sites **(SI, S2,** etc). Image was taken from Siezen and Leunissen, 1997 (Siezen & Leunissen, 1997).

Figura 2. *Representación esquemática de la unión del sustrato/inhibidor (líneas gruesas) al centro activo de una serino peptidasa tipo subtilisina (superficies suaves). Las cadenas laterales de los residuos de P4-P2´ están mostrados como círculos, las posiciones de los residuos de la enzima que pueden interactuar con esas cadenas laterales de P4-P2´ están mostrados alrededor de los sitios de unión (S1, S2, etc). La imagen fue tomada de Siezen y Leunissen, 1997 (Siezen & Leunissen, 1997).*

On the other hand, most of subtilases present autolysis and heat denaturation in concert to limit the stability of subtilisins (Ottesen *et al*., 1970). However, it has been observed in most of them, that the binding of calcium to gain protection diminish the thermal denaturation and proteolytic degradation (Frommel & Hohne, 1981; Voordouw *et al*., 1976).

BIOLOGICAL ROLES OF SUBTILASES

S8A subfamily

Subtilisins (S8A subfamily) are widely distributed from bacteria (Han *et al*., 2012) to mammals (Rockwell *et al*., 2002), and play several essential roles (Table 1).

In some bacteria, fungi and archaea these enzymes have a nutritive function. Therefore, most of them are secreted to extracellular space to digest other proteins and peptides before it can be internalized and used as a carbon source (Siezen & Leunissen, 1997).

Archaea

Staphylothermus marinus, is an archaeal organism strictly anaerobic, sulphur dependent hyper thermophile. This microorganism was isolated from deep sea

fumaroles growing within a temperature range of 65–98 °C and its main energy source is the fermentation of peptides (Mayr *et al*., 1996). STABLE, is a subtilisin subfamily member isolated from *S. marinus*. This enzyme possesses an unusual resistance to denaturation by heat, pH or chemical agents, guarantying the nutrition process even at these extreme temperatures (Mayr *et al*., 1996). Pyrolysin; is an extracellular extremely thermostable protease from *Pyrococcus furiosus*. *P. furiosus* is an hyperthermophilic archaebacterium, obligate heterotroph and uses peptidases like pyrolysin to grow up on polymeric substrates such as protein up to temperatures of 103 °C (Eggen *et al*., 1990). As pyrolysin and STABLE, several highly thermostable peptidases have been isolated and characterized from hyperthermofilic archaeas like *Desulfurococcus* spp. (Cowan *et al*., 1987), *Sulfolobus solfataricus* (Burlini *et al*., 1992) and *Thermococcus stetteri* (Klingeberg *et al*., 1995)*;* playing nutrition roles*.*

Bacteria

There are other subtilisins involved on precursors processing or maturation reactions of bacteriocins (lantibiotics), extracellular adhesins and enzymes of spore germination in bacteria like *Clostridium* spp. (Adams *et al*., 2013; Siezen & Leunissen, 1997). Spores are the major transmissivity form of the nosocomial pathogen *Clostridium difficile*, a leading cause of health care associated diarrhea worldwide. Successful transmission of *C. difficile* requires that its hardy, resistant spores germinate into vegetative cells in the gastrointestinal tract. A critical step during this process is the degradation of the spore cortex, a thick layer of peptidoglycan surrounding the spore core. In *Clostridium* sp., cortex degradation depends on the proteolytic activation of the cortex hydrolase, SleC. CspB is a subtilisin-like serine protease essential for efficient SleC cleavage and *C. difficile* spore germination (Adams *et al*., 2013).

Fungi

Fungal subtilisins from S8A subfamily are mostly extracellular and are involved in proteolytic processing of proteins related with several roles.

Burton and coworkers found in 1993 a subtilisin like protease, similar to proteinase K; involved in senescence mechanisms of the mushroom *Agaricus bisporus* (Burton *et al*., 1993). *A. bisporus* is the most extensively cultivated mushroom species in the world.

After harvest, the sporophore continues to develop by expansion of the cap and stipe, and production of spores. Harvest changes protein composition, mainly peptidase activity (Burton *et al*., 1993). The function of the post-harvest peptidases may be to release amino acids for the nutritionally starved, excised sporophore following the depletion of carbohydrate reserves, and/or to activate enzymes.

White rot basidiomycetes have received extensive attention because of their lignin-degrading activity. *Pleurotus ostreatus* is a white rot basidiomycete, which belongs to the subclass of ligninolytic microorganisms that produce laccases, manganese peroxidases, and veratryl alcohol oxidases but no lignin peroxidase. PoSl is an extracellular subtilase involved in the ligninolytic activity of *P. ostreatus* by degrading POXA1b (phenol oxidase A1b) and activation of another laccase isoenzyme (Palmieri *et al*., 2001); playing a regulatory role in laccase activity in *P. ostreatus*.

Plant

Recently have been described subtilisins involved on several cellular process in plants, like development regulation in several stages of life cycle, cell wall modification, development of seeds and fruits, processing of peptide growth factors and epidermal development (Figueiredo *et al*., 2018).

Nonetheless, they have gained more prominence for its potential agriculture relevance. Granell and coworkers in 1987 reported the first evidence of subtilisins involvement in plant-pathogen interactions in tomato plants. These authors identified the accumulation of the subtilase P69 in tomato leaves after infection with citrus exocortis viroid (Granell *et al*., 1987).

After the discovery of the P69 involvement in tomato resistance against the citrus exocortis viroid (CEV) and *Pseudomonas syringae*, the protein isoform P69B was also associated to the defense response against *Phytophthora infestans* (Tian *et al*., 2005).

Furthermore, several subtilisins have been found related in plant pathogen interaction. In grapevine, subtilases were identified in response to two biotrophic pathogens, *Plasmopara viticola* and *Erysiphe necator*, the causing agents of downy and powdery mildews respectively. In the *Vitis vinifera-Plasmopara viticola* pathosystem, several studies have highlighted the involvement of subtilases in the establishment of a successful resistance response (Figueiredo *et al*., 2018).

In coffee leaves, a proteomic analysis of apoplastic fluid in incompatible (resistant) and compatible (susceptible) *Coffea arabica-Hemileia vastatrix* interactions, has also revealed an increase of subtilase proteins at all time-points in the resistant genotype (Guerra-guimarães *et al*., 2015).

Another interesting fact found in plant subtilases is their role in the response to abiotic environment stimulus. Subtilases were reported to be involved in drought and salt resistance mechanisms (Budic *et al*., 2013). A well characterized example is the *Arabidopsis thaliana* subtilase AtSBT6.1 associated to the unfolded protein response upon salt stress, through the cleavage of an ER-resident type II membrane protein (bZIP28). The cleavage of the bZIP28 protein is essential for the activation of genes associated to salt stress response (Figueiredo *et al*., 2018).

Animals

Subtilisins from this family (S8A) are also present in mammals. One example is Site-1 protease (S1P), involved in lipid composition regulation of animal cells (Sakai *et al*., 1998). Cholesterol and fatty acids are the hydrophobic building blocks of cell membranes. Their synthesis and uptake must be coordinated to supply sufficient amounts for new membrane synthesis while avoiding over accumulation. Coordination is achieved by a family of transcription factors designated as sterol regulatory element binding proteins (SREBPs) that are bound to membranes of the endoplasmic reticulum (ER) and nuclear envelope (Brown & Goldstein, 1997). A crucial component in this regulatory pathway is the Site-1 protease, which makes the first cut in the SREBPs, thereby initiating their release (Duncan *et al*., 1997; Sakai *et al*., 1996).

S8B family

Kexin family members (S8B family) are less distributed among organisms. They can be found mainly in eukarya, like kexin from yeast (*Saccharomyces cerevisiae*)(Fuller *et al*., 1988), or furin from mammals (*Homo sapiens*) (Rockwell *et al*., 2002). Site-specific proteolysis is crucial in regulating many fundamental Biol. pathways, including the activation of many proteins (Jin *et al*., 2005). Peptidases involved in this process are called proproteins convertases (PC) and belong to the S8B family of serine peptidases (Rawlings *et al*., 2018).

Fungi

The first proprotein processing peptidase to be discovered was Kex2 peptidase (kexin) from *S. cerevisiae*. The *KEX2* gene was initially identified as a genetic locus required for killer toxin *ex*pression (Wickner & Leibowitz, 1976). It was subsequently shown to be required for production of α-factor, the mating pheromone secreted by *MAT*α haploid cells (Leibowitz & Wickner, 1976). The enzyme has a single transmembrane domain followed by a cytosolic tail, which is responsible for Kex2 localization to late compartments of the yeast secretory pathway.

Animals

In mammalian cells were identified three Kex2 homologues. Two of these, PC1/3 and PC2, were the prohormone *c*onvertases (or proprotein convertases) responsible for the processing of proinsulin and other prohormones and neuropeptide precursors (Rockwell *et al*., 2002; Seidah *et al*., 1990, 1991; Smeekens *et al*., 1991). These cytosolic enzymes, are expressed in neuroendocrine cells localized in the regulated secretory pathway (Rockwell *et al*., 2002). The third homologue, furin, works in a more analogous manner to Kex2, cycling among late compartments of the constitutive secretory pathway (Rockwell *et al*., 2002).

Furin, was the first proprotein convertase to be identified in humans in 1986 and is the product of the *FUR* gene (Ven *et al*., 1990). Furin is a type I transmembrane protein that is ubiquitously expressed in vertebrates and invertebrates (Mbikay *et al*., 1997). It is localized to the Golgi and *trans*-Golgi network where it cleaves multiple proteins (Nemunaitis *et al*., 2020). Due to furin's ubiquitous expression and localization it is able to process a large amount and variety of proteins including growth factors, cytokines, hormones, adhesion proteins, collagens, membrane proteins, receptors as well as other classes (Tian *et al*., 2011).

BIOMEDICAL RELEVANCE OF SUBTILASES

Subtilases have great scientific interest due its biomedical relevance: as virulence factors during tissue invasion by bacterial pathogens (DuMont & Cianciotto, 2017; Windhorst *et al*., 2002), essential processing in parasites (Alam, 2014; Kim, 2004; Swenerton *et al*., 2010) and processing key enzymes for virus entry/ egress on host cells (Braun & Sauter, 2019).

Table 1. Biol. roles of subtilases from the S8 family
Tabla 1*: Funciones biológicas de subtilasas de la familia S8*

ORGANISM	ENZYME	BIOL. ROLE	REFERENCE
ARCHAEA			
Staphylothermus marinus	STABLE	Nutrition	(Mayr *et al*., 1996)
Pyrococcus furiosus	Pyrolysin	Nutrition	(Eggen *et al*., 1990)
BACTERIA			
Cochliobolus carbonum	Alp2	Nutrition	(Murphy & Walton, 1996)
Dichelobacter nodosus	AprV5	Virulence factor	(Han *et al*., 2012)
Clostridium difficile	CspB	Spore germination	(Adams *et al*., 2013)
Streptococcus pneumoniae	HtrA	Stress response and protein quality control	(Stoppelaar *et al*., 2013)
FUNGI			
Saccharomyces cerevisiae	Kexin	α factor production (mating pheromone)	(Wickner and Leibowitz, 1976)
Agaricus bisporus	*Agaricus bisporus proteinase*	Senescence	(Burton *et al*., 1993)
Pleurotus ostreatus	PoSl	Proteolytic processing at extra-cellular space	(Palmieri *et al*., 2001)
PROTIST			
Cryptosporidium parvum	CpSUB1	Proteolytic processing of surface proteins	(Wanyiri *et al*., 2009)
Plasmodium falciparum	PfSUB	Erythrocyte invasion and egress	(Alam, 2014)
Toxoplasma gondii	TgSUB2	Rhoptry protein maturation	(Kim, 2004)
Neospora caninum	NC-p65	Host cell invasion and egress	(Louie & Conrad, 1999)
Babesia divergens	BdSUB-1	Erythrocyte invasion	(Montero et al., 2006)
PLANT			
Lycopersicum esculentum	P69	Developmental processes and defense	(Granell *et al*., 1987)
Arabidopsis thaliana	AtSBT6.1	Salt stress response	(Figueiredo *et al*., 2018)
Verticillium dahliae	GbSBT1	Defense mechanisms against pathogens	(Duan *et al*., 2016)
ANIMALS			
Homo sapiens	Furin	Proprotein processing	(Ven *et al*., 1990)
Homo sapiens	Site 1 protease*	Lipid composition regulation	(Sakai *et al*., 1998)

*This enzyme is present in all animal cells.

Bacterial diseases

The major secretory protease from the nosocomial pathogen *Stenotrophomonas maltophilia* (StrmP1) is a subtilase that induces matrilysis and anoikis in human lung epithelial cells, as well as inflammation (DuMont & Cianciotto, 2017; Windhorst *et al*., 2002). Surveillance studies have shown that *S. maltophilia* is the 6th most common cause of worldwide respiratory tract infections (Chang *et al*., 2015), causing severe disease in immune-compromised individuals (Paez *et al*., 2008). According to Dumont and Cianciotto (2017) the enzyme StrmP1 induces rounding and detachment of the A549 cells, and the observed effects of degradation of fibronectin, fibrinogen and IL-8 was similar when StrmP1 was compared with the well-known subtilisin Carlsberg from *Bacillus licheniformis* (model of the S8 family) (DuMont & Cianciotto, 2017).

Further examples of subtilases playing key role in inflammation processes are the EpiP peptidases from *Staphylococcus epidermidis* (Geibler *et al*., 1996) and *Staphylococcus aureus* (Kuhn *et al*., 2014), mainly known to process the epidermis leader peptide, but also capable to degrade collagen. EpiP from *S. aureus* is a protective antigen against lethal infection in mice, which might be associated with EpiP antibodies blocking the function of the protein. Given that EpiP peptidases are released to the extracellular medium, there is a possible role of these enzymes in invasive infection, as has been shown for some of its homologs, as the SpyCEP from *Staphylococcus pyogenes* (Kuhn *et al*., 2014).

Subtilases are also known as virulence factors in bacterial pathogens as *Dichelobacter nodosus* (Han *et al*., 2012), the urinary tract pathogen *Proteus mirabilis* (Alamuri *et al*., 2009) and the zoonotic bacteria *Streptococcus suis* (Bonifait *et al*., 2010).

Parasitic diseases

Malaria, caused by protozoan parasites of *Plasmodium* genus, is a major global parasitic disease (Breman, 2001). Malaria in humans is caused by five *Plasmodium* species, namely, *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*. Of these, *P. falciparum* is the causative agent of severe malaria and the major cause of malaria-related fatality (Alam, 2014).

In *P. falciparum* genome there are three genes known encoding for subtilisin-like peptidases, these enzymes are PfSUB1, 2, and 3 (Alam, 2014). All of them are highly expressed at late asexual blood stages (Le *et al*., 2014). Of these, PfSUB1 and 2 have been extensively characterized and implicated in egress and invasion during asexual blood stage life cycle of the parasite (Arastu-kapur *et al*., 2008; Harris *et al*., 2005; Yeoh *et al*., 2007). The resurgence of malaria burden due to resistance to chloroquine and other therapeutic strategies like sulfadoxine-pyrimethamine (Zucker *et al*., 2003) and artemisinin-based combination therapies (Anderson *et al*., 2010; White, 2010) increases the need to focus on different drug targets to develop novel drugs. PfSUB1 and PfSUB2 qualify as attractive antimalarial drug targets given its essentiality for parasite blood and liver stages, proteolytic activity on multiple parasite proteins, and its role in egress and invasion (Alam, 2014).

The protozoan parasite *Toxoplasma gondii* commonly infects humans and other mammals, causing toxoplasmosis in immunocompromised individuals (Kim, 2004). *T. gondii* is an obligate intracellular pathogen, replicating only within a specialized parasitophorous vacuole formed in the cytoplasm of nearly all cell types. The sequence of events that occur during host cell invasion by Apicomplexa is generally conserved across the phylum (Kim, 2004). A variety of data has been published suggesting that peptidases are important in Apicomplexa invasion processes. Serine protease inhibitor studies have suggested that serine protease activity is necessary for successful invasion of host cells (Blackman, 2000; Conseil *et al*., 1999).

Furthermore, Miller *et al*. identified two subtilisin-like serine peptidases (TgSUB1 and TgSUB2) in *T. gondii* (Miller *et al*., 2001, 2003). TgSUB1 is homologous to PfSUB1 (Blackman, 2008) and is recognized by PfSUB1 antisera. TgSUB1 is a microneme protein and like many microneme proteins is secreted from the parasite in a calcium-dependent fashion (Miller *et al*., 2001). TgSUB1 contains a consensus C-terminus glycolipid anchor addition site and Biochem. data suggest that TgSUB1 is GPI-anchored (Kim, 2004). TgSUB2 amino acid sequence is highly homologous to other known apicomplexan subtilisins, particularly *P. falciparum* PfSUB2. Current evidence suggests that TgSUB2 plays a role in rhoptry protein maturation. TgSUB2 may activate proteins involved in rhoptry targeting and development (Kim, 2004).

Moreover, protozoan parasites of the genus *Leishmania* cause cutaneous, mucocutaneous, and visceral leishmaniosis in humans. Due to the lack of safe and effective treatments for leishmaniosis, the World

Health Organization classified this disease by as a Tropical Disease Res. Category I disease, an emerging or uncontrolled disease (WHO, 2004). In *Leishmania* a subtilisin-like serine protease, similar to eukaryotic Site-1 peptidase (Swenerton *et al*., 2010), was found to process the terminal peroxidases of the trypanothione reductase system. This system plays an important role in *Leishmania* survival within host macrophages and is being intensely studied as a target for antiparasitic drug development (Krauth-siegel & Comini, 2008).

Viral infections

Many viruses contain surface glycoproteins which when are cleaved by furin or other proprotein convertases are activated and viral propagation is achieved (e.g.: avian influenza, HIV, Ebola, Marburg and measles viruses) (Hallenberger *et al*., 1992; Thomas, 2002; Volchkov *et al*., 2000).

Furin is an enzyme localized to the Golgi and *trans*-Golgi network where it cleaves multiple proteins. Many pathogens like SARS-CoV-2 virus use it to cleave glycoproteins, a step essential for entry into host cells (Coutard *et al*., 2020; Thomas, 2002). The spike (S) protein of coronaviruses facilitates viral entry into target cells. Entry depends on binding of the surface unit, S1, of the S protein to a cellular receptor (ACE2), which facilitates viral attachment to the surface of target cells. SARS-CoV-2 relies on S1/S2 cleavage of protein S at viral entry like SARS-CoV (Coutard *et al*., 2020; Walls *et al*., 2020). This cleavage produces a conformational change on receptor-binding domain (RBD) (Fig. 2), allowing its exposition and posterior binding to ACE2 receptor. Following the attachment of the receptor-binding domain to the ACE2-binding cellular site, the affinity of which is 10- to 20-fold higher than SARS-CoV (Walls *et al*., 2020), the S1 subunit is shed resulting in a stable and accessible fusion domain (S2) subunit (Wrapp *et al*., 2020).

SARS-CoV-2 protein S, is cleaved at two sites, S1/S2 furin cleavage site (P**R**RA**R**↓S**V**) and a furin-like S2_cleavage site (**K**↓**SF**) (Nemunaitis *et al*., 2020). The presence of a unique furin cleavage site (RRAR) at the S1/S2 boundary in the SARS-CoV2 protein may provide

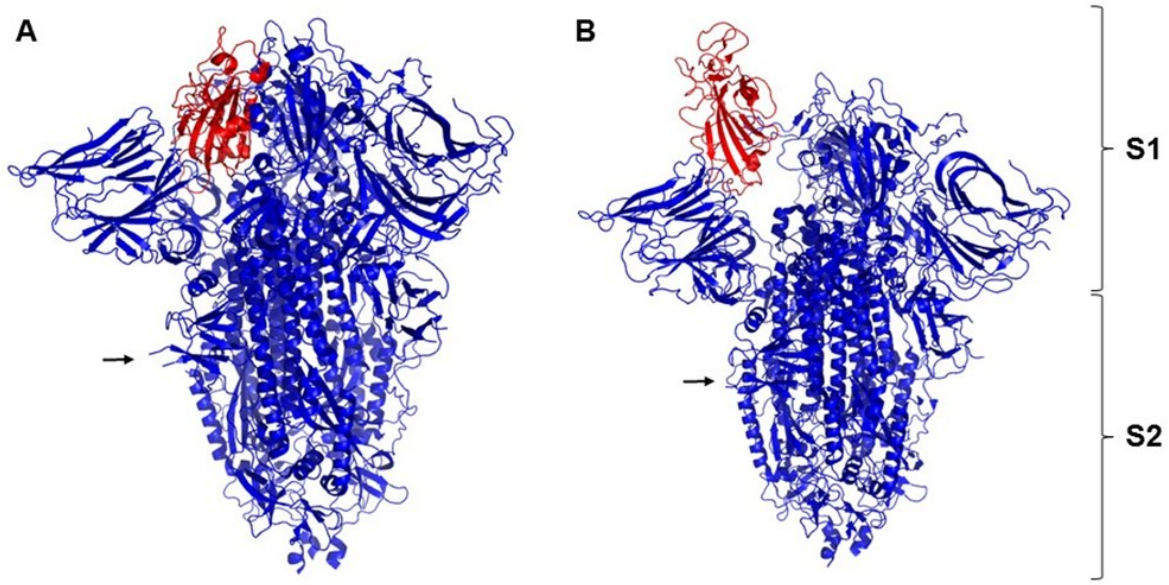

Figure 3. 3D structures of furin cleaved spike protein of Sars-CoV-2 virus in different conformations. A, closed conformation. B, opened conformation with one RBD exposed. Arrows indicate one S1/S2 furin cleavage site. Images were prepared using software Pymol 1.7.2.1 (http: www.pymol.org) and PDB files: 6ZGI and 6ZGG, respectively. RBD domain is in red and the rest of the structure is in dark blue).

Figura 3. *Estructuras 3D de la proteína de la espiga del virus Sars-CoV-2 en diferentes conformaciones después del corte con furina. A, conformación cerrada. B, conformación abierta con un RBD expuesto. Las flechas indican el sitio de corte entre S1 y S2. Las imágenes se prepararon usando el programa Pymol 1.7.2.1 (http: www.pymol.org) y los archivos PDB: 6ZGI y 6ZGG, respectivamente. El dominio RBD se muestra en rojo y el resto de la estructura en azul oscuro.*

a gain-of-function over SARS-CoV allowing cleavage during viral egress thereby directly or indirectly contributing to increased replication rate, transmission and disease severity (Coutard *et al*., 2020).

Furin-induced disorders

As it was described above, furin is an enzyme that belongs functionally to the proprotein convertases family. Their similarity with bacterial subtilisin and yeast kexin peptidases has coined the abbreviation PCSK (proprotein convertase subtilisin/kexin type). Humans encode nine members of this protease family (PCSK1–9), with PCSK3 representing furin (Braun & Sauter, 2019). To date; more than 200 cellular substrates of PCSKs have been described, including hormones, receptors, growth factors and adhesion molecules. Thus, it is not surprising that aberrant furin activity is associated with a variety of disorders including cancer, diabetes and neurological diseases (Braun & Sauter, 2019).

The canonical furin cleavage site is frequently described as R-X-K/R-R↓ (Nemunaitis *et al*., 2020). Bioinformatics analyses and functional studies uncovered more than 100 furin cleavage sites in mammalian proteins. These comprise growth factors and cytokines (e.g. IGF1, IGF2, TGFb, PDGFa, PDGFb, VEGF-C, NGF, CXCL10), hormones (e.g. PTH, TRH, GHRH), adhesion molecules (e.g. integrins, vitronectin), collagens, metalloproteinases, coagulation factors, receptors, membrane channels and albumin (S. Tian *et al*., 2011). The Physiol. importance of furin is reflected by furin knock-out mice, which die at embryonic day 11 because of cardial ventral closure defects and hemodynamic insufficiency (Bassi *et al*., 2005; Klein-szanto & Bassi, 2017; Roebroek *et al*., 1998). The furin aberrant expression or activation promotes the formation and progression of various malignancies including colon carcinoma, rhabdomyosarcoma, head and neck cancers, lung, skin and brain tumors (Jaaks & Bernasconi, 2017). In some cases, furin levels positively correlate with aggressiveness, and increased furin expression has been proposed as prognostic marker for advanced cancers. (Jaaks & Bernasconi, 2017). Aberrant furin activation promotes several steps of cancer development, including cell proliferation, vascularization, metastasis and antitumor immunity (Braun & Sauter, 2019). However, the relative contribution of individual furin substrates to tumor progression and the role of other proprotein convertases remain largely unclear.

Subtilases are distributed among several relevant pathogenic organisms playing diverse functions, mainly as virulent factors; involved in mechanisms like protein maturation, host invasion and protein damage; inducing disorders or infections. These pathogenic roles have encouraged to the scientific community the design and isolation of protease inhibitors in order to develop new drugs and treat several diseases involving external pathogens or uncontrolled and harmful processes within the host.

CONCLUSIONS

Subtilases from the S8 family are a large and diverse group of serine peptidases with a high importance for biomedicine and bioChem.. These enzymes play important roles as virulence factors and proteolytic processing of proteins related with development, nutrition or, at key steps during pathogenesis in several infectious diseases and Physiol. disorders in mammals as wells. All these elements confirm the relevance of the study of this diverse and widely distributed group of enzymes and enhance the importance of the search of new inhibitors as a way to treat these diseases.

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