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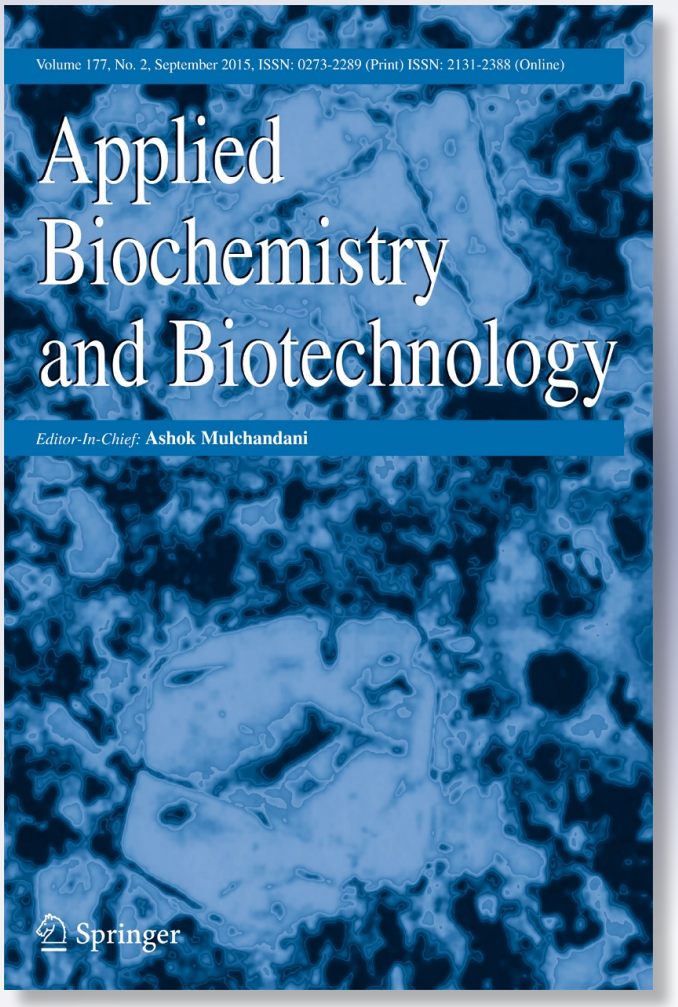

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Caspases and Thrombin Activity Regulation by Specific Serpin Inhibitors in Bovine Skeletal Muscle

Mohammed Gagaoua[1,3] · Kahina Hafid[1] · Yasmine Boudida[1] · Samira Becila[1] · Ahmed Ouali[2] · Brigitte Picard[3,4] · Abdelghani Boudjellal[1] · Miguel Angel Sentandreu[5]

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Abstract In living cells, after activation, protein inhibitors constitute the last step of proteases activity regulation. This review intends to provide original information about a group of bovine muscle serine proteases inhibitors belonging to the Serpin superfamily and characterized at the gene and protein level. This report is the only one and the first to provide much information on this group of proteases inhibitors of the serpin type and their potential biological functions. Amongst the eight genes identified in bovine, three serpins were purified from the muscle tissue and characterized. These are two members of the bovSERPINA3 family, i.e., bovSERPINA3-1 and A3-3, and the last one is antithrombin III (AT-III or BovSERPINC1). BovSERPINA3 family comprises at least eight protein members encoded by different genes mapped on chromosome 7q23–q26 cluster. BovSERPINA3-1 and A3-3 were shown to locate within muscle cells and are cross-class inhibitors strongly active against trypsin as well as against human initiator and effector caspases 8 and 3. They constitute a key apoptosis control in mammals. They were thus expressed in proliferating and confluent myoblasts phases where cells must be alive but not in myotubes. Antithrombin III inhibits

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trypsin and, in a heparin dependent manner, thrombin. AT-III and its mRNA were expressed in muscle cells and in differentiating primary myoblasts in culture.

Keywords Bovine skeletal muscle · Serine proteases inhibitors · Serpins · Apoptosis · Myoblast differentiation · Antithrombin

Introduction

Serine proteases are found ubiquitously in every type of living organisms as well as in many viral genomes. In the 1980s, the presence of serine proteases within muscle cells was doubtful and a large set of investigations suggested that some of them if not all originated from mast cells [63]. The major serine proteases identified in mast cells that are in charge of the secretion of essential molecules including heparin, serotonin, and else are known as chymases and tryptases [70]. Later on, several serine proteases were shown to be synthesized by muscle cells including plasmin, thrombin, kallikrein, and others [80]. Understanding the proteolytic machinery in muscle cells, the regulation of their activity, and their biological importance represents a major challenge.

All surveys carried out over the last four decades on meat quality, emphasized that tenderness is the most important sensory trait of meat in comparison to the other attributes. Many studies indicated that consumers are willing to pay a premium for beef meat as long as it is guaranteed tender [57]. As a result, the major problem that the meat industry has to solve is the management of the large variability in the ultimate tenderness of meat cuts and the absence of accurate predictors of this quality attribute. To date, numerous strategies have been assayed to try to discover those mechanisms that would allow us to understand the main causes of the variability in meat quality [29, 65, 72].

This situation is directly related to the complexity and biological diversity of the skeletal muscle tissue. Unsuccessful management of this quality also results from our lack of knowledge about the complex biochemical mechanisms taking place in post-mortem muscle immediately after animal slaughter. Despite the large efforts developed over the last decades to better understand these mechanisms, many questions remain unsolved. Amongst them, proteolytic processes responsible for hydrolysis of the main structural proteins conforming myofibrils is one of the most relevant with respect to the post-mortem meat tenderness improvement.

Post-mortem protein breakdown is assumed to be caused by the proteolytic action of different endogenous muscle enzyme groups during carcass storage, a period known as meat aging. However, the relevance that the different groups of muscle peptidases can have in this process has been a matter of controversy since years. For a long time, the calpain system was considered to be the primary system responsible for meat tenderization until the discovery that the first event affecting muscle cells is the onset of apoptosis, a complex process governing the cell death process and the concomitant degradation of structural proteins [4, 36, 66].

Efficiency of the endogenous proteolytic system is mainly determined by the level of their specific inhibitors [69, 81]. This statement was confirmed in earlier studies by Zamora et al. [99, 100] who showed that after animal bleeding, serine protease inhibitors levels are the best meat tenderness predictors amongst about 30 quantitative variables measured including

calpains 1 and 2, calpastatin, cysteine protease inhibitors, …etc. Interestingly, most of them belong to the serpins (serine protease inhibitors) superfamily and some were further shown to be strong inhibitors of caspases [28, 35], a proteolytic group of cysteine proteases considered as the main driver of apoptosis [14, 53].

Because of their major role in post-mortem proteolysis regulation, muscle serine protease inhibitors were extensively investigated in our group over the last decades and characterized. Protease inhibitors are ubiquitous in the animal kingdom but relatively little is known about their biological functions in the bovine skeletal muscle. The present paper reviews the main outcomes regarding their identity, their target proteases, their biological functions, and their implication in post-mortem improvement in meat tenderness. Before that, it would be interesting to briefly present the serpin superfamily and their properties.

General Properties of Serpins

The Serpin Superfamily

Serpins are a group of proteins with similar structures that were first identified as a set of proteins able to inhibit proteases. The acronym serpin was originally coined because many serpins inhibit chymotrypsin-like serine proteases (serine protease inhibitors). Over 3000 serpins have now been identified, which are subdivided into 16 subgroups (A–P) according to their common ancestry. They were found abundantly in eukaryotes and even in some bacteria and archea and in some viruses [44, 60]. Serpins are thus the largest and most diverse family of protease inhibitors [73].

While most serpins control proteolytic cascades, certain serpins do not inhibit enzymes but instead perform diverse functions such as storage (ovalbumin, in egg white), hormone carriage proteins (thyroxine-binding globulin, cortisol-binding globulin), and molecular chaperones (HSP47). The term serpin is used to describe these latter members as well, despite their non-inhibitory function [83]. Early studies on serpins revealed that the mechanism by which these proteins inhibit target proteases appeared to be distinct from the lock-and-key-type mechanism used by small protease inhibitors such as the Kunitz-type inhibitors (e.g., basic pancreatic trypsin inhibitor). Indeed, serpins form covalent complexes with their target proteases by trapping the enzyme and transporting it to the distal end of the core of the serpin. Hence, the conformational structural change induced to the enzyme makes it no more able to complete the hydrolytic reaction [30, 42].

Serpins are metastable proteins comprising the core of the protein with several strands and an external loop at the C-terminal end called reactive center loop (RCL). This loop contains the target scissile bond for proteases, and its sequence defines the specificity of serpins (Fig. 1a). In the presence of the target enzyme (e.g., trypsin), a Michaelis complex (Fig. 1b) is formed as for all enzyme-substrate interaction and as soon as the acyl-enzyme complex is obtained, the RCL inserts into the protein core transporting with it the enzyme to the opposite side. This induces a large distortion of the active site responsible for the slowing and subsequent abolishment of the catalyzed hydrolysis of the acyl intermediate (Fig. 1c).

Furthermore, it is worth noting that some serpins are able to inhibit cysteine proteases (e.g., caspases and cathepsins) through the same trapping mechanism, by

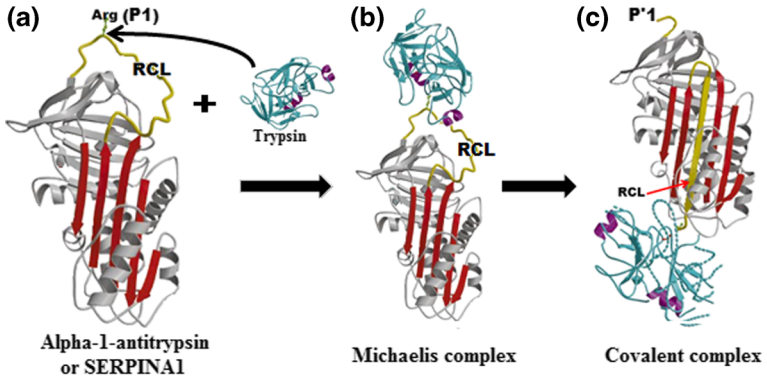

Fig. 1 Serpin structures and conformation. **a** Native α_1-antitrypsin (PDB entry 1QLP) [19]; **b** Michaelis complex between SERPINA1 (Alaserpin from *Manducasexta*) and trypsin (PDB entry 1K9O) [98]; **c** Covalent complex between α_1-antityprsin and trypsin (PDB entry 1EZX). The enzyme is transported to the distal part of the serpin and undergoes an irreversible deformation responsible of the enzyme inactivation via the reactive center loop (RCL) [43]

alteration of the trapped acyl intermediate from an oxyacyl species to a thioacyl intermediate, leading to a covalent complex with the targeted protease and its concomitant transfer to the distal end of the serpin [17, 35, 79, 90].

Kinetic Analysis of Protease-Serpin Interactions

Stoichiometry of Interaction: Signification and Mode of Determination

The stoichiometry of interaction (SI) reflects the efficiency of the serpin as a proteinase inhibitor (Fig. 2a). SI is the number of moles of serpin needed to generate one mole of kinetically trapped covalent serpin-proteinase complex (E-I). As soon as the acyl intermediate (EI*) has occurred between the proteinase and the exposed RCL, there is a competition between inhibitory (k_4) and substrate (k_3) pathways, the last one regenerating the active enzyme and the cleaved serpin. The relationship of SI to the rate constants is therefore $SI=(k_3+k_4)/k_4$. Efficient inhibitors are therefore those with SI values close to 1 ($k_4 \gg k_3$), whereas predominantly substrate reactions have large SI ($k_3 \gg k_4$). For accurate comparison between different enzyme/serpin couples, the apparent second-order rate constants (k_{ass}) must be corrected by being multiplied by SI ($k_{ass}=k_{app}*SI$) [77]. Finally, the trapped enzyme/inhibitor complex can dissociate by hydrolysis of the acyl bond, but this occurs at a very slow rate and generally concerns a small amount of the total complex pool [31].

The stoichiometry of interaction is generally measured by titration of a defined concentration of the target enzymes (in the nanomolar range) by addition of increasing amounts of serpins (Fig. 2b). In the example provided, total inhibition of trypsin by bovSERPINA3-1 is achieved in the presence of an equimolecular concentration of serpin ([I]/[E]=1). Hence, this serpin must be considered as a very efficient inhibitor of trypsin, but the present observation does not mean that trypsin is the in vivo target enzyme.

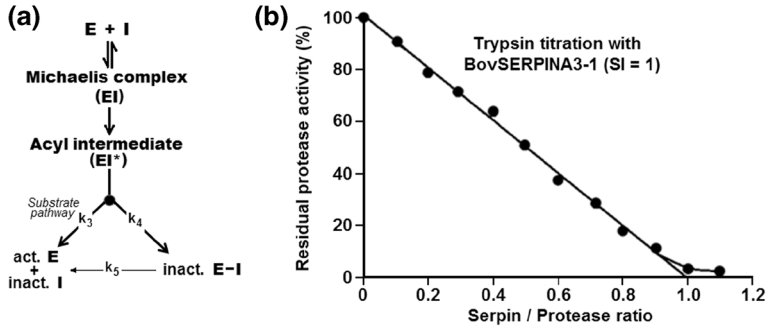

Fig. 2 Serpin pathway mechanism with two potential outcomes (adapted from [83]) (**a**) and determination of the stoichiometry of interaction (SI) of trypsin with bovSERPINA3-1 (**b**). **a** Schematic major reactions sequence for interaction of serpins (*I*) with proteinases (*E*) with well-defined intermediates, reactants, and products. After cleavage of the scissile bond and formation of the acyl intermediate (*EI**), insertion of the RCL into the core of the serpin can occur and two outcomes are then possible for this intermediate: (1) successful completion of the substrate cleavage pathway with release of active protease (*act. E*) and cleaved serpin (*Inact. I* or truncated I) (rate constant k_3); (2) translocation of the protease to the distal end of the serpin and kinetic trapping of the EI* complex through distortion of the protease (rate constant k_4). The final enzyme/serpin complex (*E-I*) can be hydrolyzed very slowly (k_5) releasing, as in the substrate pathway, truncated serpin I (*intact. I*) and active enzyme (*act. E*). **b** Stoichiometry of interaction of trypsin (5 nM) with bovSERPINA3-1 giving a SI value of 1 indicative a very good efficiency of the serpin to inhibit trypsin

Association Rate Constant k_{ass}: Methods of Determination

The rate of protease inhibition by serpins is generally determined by using either the discontinuous or the continuous/progress curve method. The discontinuous method is generally used for low association rate constants of inhibition $<10^4$ M^{-1} s^{-1}. Faster association rate constants (k_{ass} $\geq 10^6$ M^{-1} s^{-1}) are usually determined using the progress curves method [41]. With the previous methods, the serpin concentration is higher than that of the enzyme level ([Io]>[Eo]). However, under low reactant concentrations (in the nanomolar range) and under second-order conditions ([Eo]=[Io]), k_{ass} can be determined according using an appropriate method [21].

Major Serine Proteinase Fractions in Bovine Muscle Crude Extract

The bovine muscle mostly used for this purpose was M. diaphragma pedialis, a muscle containing, as all slow twitch muscles including heart, high amounts of proteases, and inhibitors. Muscles contained a large set of serine protease inhibitors with Mr ranging from 10 to 85 kDa [5, 6, 68, 80]. Gel filtration of a muscle crude extract fractionated three major fractions F1, F2, and F3 [80] with Mr of about 60–85, 40–60, and 10–14 kDa, respectively.

Using different chromatography protocols, two trypsin/elastase inhibitors and a trypsin/thrombin inhibitor were purified to homogeneity from the F1 fraction and characterized [37, 38, 92, 93]. From the N-terminal partial sequences obtained by the Edman sequencing method, they were identified as the following:

– Trypsin/elastase inhibitors

  BovSERPINA3-1 previously designed Endopin 1A [37, 38, 92, 93]

BovSERPINA3-3 previously designed Endopin 1B [38]

– Trypsin/thrombin inhibitor

Antithrombin III [37]

From the F2 fraction, we failed to purify to homogeneity any serine proteinase inhibitor probably because (1) most inhibitors of this fraction first shared very close structural and physicochemical properties with the F1 entities and (2) most of them are apparently common to F1 and F2 fractions according to their cross immuno-reactivity towards the antibody raised against bovSERPINA3-1 [27].

The BovSERPINA3 Family

Genomic Organization of the Bovine SERPINA3 Genes

Clustering of serpin genes frequently occurs in mammals genomes as illustrated for human [7], mouse [24], rat [40], and pork [2, 58]. Regarding bovine SERPINA3, a number of eight genes encoding these proteins were sequenced and appeared to be encompassed in the same cluster. They were thus mapped on chromosome 7q23–q26 cluster and spanned over 235 kb [71]. The large number of genes encoding these serpins suggests a probable great complexity of the bovSERPINA3 subfamily of serpins. This complexity was supported by the two-dimensional gel electrophoresis of fractions F1 and F2 who showed multiple spots of glycosylated and phosphorylated proteins when revealed with a polyclonal antibody raised against purified bovSERPINA3-1 [27]. The high sequence homology between the identified members of the bovSERPINA3 family (>75 %) suggests that it exists a degree of compensation and redundancy between them comparable to the redundancy noted for caspases [25]. This redundancy may be concern their respective function in living cells, the multiplicity of their potential target proteases, their mode of action, …etc.

Stoichiometry of Interaction with Inhibited Proteases

The stoichiometry of interaction was determined by titration of the target enzymes (human leukocyte elastase (HLE), trypsin, caspases 3 and 8) with increasing amounts of either bovSERPINA3-1 (Swiss Prot ID: Q9TTE1) or A3-3 (Swiss Prot ID: Q3ZEJ6). As shown in Table 1a, total inhibition of trypsin and human leukocyte elastase was achieved using equimolar concentrations of either serpins suggesting a 1:1 interaction ratio.

Most serpins so far reported inhibited enzymes with only one active site like trypsin, chymotrypsin, …etc., and the concentration of enzymes equals the concentration of active sites. Active enzyme concentration is therefore the most accurate value for SI determination ([E]=[active site]). By contrast, titration of caspases with specific inhibitors as Z-VAD-FMK provides the concentration of active sites and not the concentration of active enzymes. As native active caspases contain two active sites, the concentration of native tetrameric caspases equals to half the concentration of active sites ([E]=0.5*[active Sites]). For caspases, the concentration of active sites is the accurate value to be considered for the determination of the SI values.

Table 1 Kinetic analysis of protease-serpin interactions. a) Stoichiometry of interaction of bov-serpins with the targeted serine and cysteine proteases (adapted from [35] and [27]). b) Inhibitory pattern of bov-serpinA3-1 and A3-3 and association rate constant (k_{ass} in M^{-1} s^{-1}) against different serine and cysteine proteases [38, 93]. The proteases strongly inhibited by the serpins are in italic whereas those for which the inhibition is of no physiological significance are in normal style. Proteases tested are bovine pancreatic trypsin and chymotrypsin, bovine serum plasmin, human leukocyte elastase (HLE), and human recombinant caspases 3 and 8

a)	Stoichiometry of interaction (SI)	
	SerpinA3-1	SerpinA3-3
Trypsin	1.01±0.03	0.98±0.01
HLE	1.04±0.02	1.01±0.03
Caspase 3[a]	1.01±0.07	0.55±0.07
Caspase 8[a]	0.49±0.09	0.51±0.06
b)	Association rate constant (k_{ass}) (M^{-1} s^{-1})	
Proteases	BovSERPINA3-1	BovSERPINA3-3
Trypsin	3.9×10^6	6.7×10^5
HLE	2.4×10^7	1.3×10^6
Chymotrypsin	$<10^3$	$<10^3$
Plasmin	$<10^3$	$<10^3$
Caspase 3[a]	4.2×10^5	1.5×10^5
Caspase 8[a]	1.4×10^6	2.7×10^6

[a]SI=[I]/[caspase active sites]

According to the results in Table 1a, a 1:1 interaction of bovSERPINA3-1 with active sites was needed to fully inactivate caspase 3, i.e., two moles of inhibitor for one mole of active caspases. In other words, binding of one mole of inhibitor to each active site and two serpins molecules for each native tetrameric caspases are required for total inhibition of caspase 3. With respect to all other enzyme/inhibition interactions, i.e., caspase 3/bovSERPINA3-3, caspase 8/bovSERPINA3-1, and caspase 8/bovSERPINA3-3, binding of one mole of inhibitor inactivates two actives sites, meaning that binding of one serpin molecule induces a sufficient allosteric conformational change of the tetrameric native caspase to make the second site unable to bind substrates. Another reason could be the dissociation of the tetrameric caspase structure due to the important constraints induced to the multimeric protease as observed for the inhibition of caspases by cytokine response modifier A [17]. Such dissociation will indeed lead to the disappearance of the second active site in the native enzyme which is no more tetrameric.

Association Rate Constant (k_{ass}) Towards Different Cysteine and Serine Proteases

Regarding this constant, we must keep in mind that k_{ass} values $\leq 10^3$ M^{-1} s^{-1} are of no physiological significance. Association rate constant towards a set of serine and cysteine proteases was therefore determined for both serpins according to Schechter and Plotnick [77] and Horvath et al. [41]. As previously reported [27], both bovSERPINA3-1 and A3-3 do not inhibit the following serine proteases: cathepsin G, elastase kallikrein, urokinase, plasminogen activator, and thrombin. No inhibition was observed against the most common cysteine proteases including papain, cathepsins B, H, and L, and calpains 1 and 2. By contrast, they both strongly inhibit

trypsin and elastase, two serine proteases, together with caspases 3 and 8. Both caspases 3 and 8 were further shown to form SDS-stable complexes with these serpins [27, 28, 35].

Three essential particular features of bovSERPINA3/caspases interactions must be emphasized: (a) formation of SDS-stable complexes with targeted caspases, (b) nature and localization of the target scissile bond, (c) in situ caspases/bovSERPINA3 complexes detection in post-mortem muscle and consequences.

Formation of SDS-Stable Complexes with Target Proteases

One hallmark of serpins is their ability to form SDS-stable complexes with their target proteases. Serpins are restricted to inhibiting proteinases of only the serine mechanistic class. However, at least two serpins are now known to demonstrate cross-class inhibition of several different types of cysteine proteinases: the viral serpin cytokine response modifier A (*Crm*A) inhibits caspase-1 [51], a member of the caspases family, a group of cysteine proteases distinct from the papain-like cysteine proteases [95, 97], and the human serpin squamous cell carcinoma antigen 1 (SCCA1) inhibits the papain-like cysteine proteinases cathepsins S, K, and L [78, 79, 91]. For both serpins, it was demonstrated that they use very likely a common reactive center loop (RCL)-dependent mechanism to inhibit serine and cysteine proteases [17, 78]. We previously reported that bovSERPINA3-1 and A3-3 are strong inhibitors of initiator and effector caspases [27, 28, 35] and form stable complexes with their target cysteine proteases, a feature supported by the half-life times of the different caspase/bovSERPINA3 complexes ranging between 7 min and 16 days [39]. These are therefore new mammalian serpins capable to inhibit cysteine proteases especially human caspases 8 and 3 but not cysteine proteases of the papain family. In contrast to the viral CrmA, both serpins form an SDS-stable complex with either caspases. Interestingly, all complexes exhibit a similar Mr of about 112–113 kDa as assessed by SDS-PAGE and comprise very likely the serpin covalently bound to half of the tetrameric native caspases, i.e., a heterodimeric moiety. As for CrmA, trapping of the caspases by bovSERPINA3s and its distortion to the core of the serpin induces very likely a rapid dismantling of the initial native tetrameric caspase structure into their heterodimeric moiety which would explain that only one mole of serpin inhibited two active sites. Although the mechanisms of action of bovSERPINA3-1 and A3-3 on caspases inhibition have not been investigated in detail, one could think that they use the same trapping mechanism than with serine proteases, an assumption agreeing well with the earlier studies by Gettins group [90].

These are therefore some examples that lead us to ask questions about the assertion of Gettins [31]. In his review, the author pointed out that it is doubtful to think that "cysteine proteinases might be inhibited by the same conformation change-based mechanism as serine proteinases arguing that no high molecular weight SDS-stable band has been demonstrated on PAGE for a serpin-cysteine proteinase complex that would correspond to a covalent thioacyl intermediate between serpin and proteinase". The author concluded "that it is unlikely that a thioester present in the intact cysteine proteinase-serpin complex would resist to the analytical procedure", i.e., the usual preliminary heating at 95 °C for 5 min in the presence of 2–4 % SDS for SDS-PAGE analysis. The only one exception to the basic rule of SDS-stable complex formation between serpin and cysteine protease is CrmA for which such complex has never been detected after using the normal denaturing conditions. Such complex has been only detected after mild denaturing conditions or after electrophoresis in non-denaturing conditions [17].

Nature and Localization of the Target Scissile Bond Within the RCL

It is well established that from relations between the known target proteinases of certain serpins and the P1 residue of the serpin RCL that the primary determinant of specificity is the P1 residue. For example, the viral serpin CrmA has a P1 aspartate and inhibits aspartate-specific proteinases of the caspase family. On the other hand, mechanism of kinetic trapping of proteinases by serpins requires at least a distortion of the proteinase active site to compromise or abrogate its catalytic competence. Achievement of this goal needs (i) an over-tight apposition of the two proteins in the covalent complex after insertion of the cleaved RCL into β-sheet A, (ii) the concomitant translocation of the proteinase to the distal end of the serpin, and (iii) its following compression to the core of the serpin and subsequent distortion. Full insertion of the RCL within the core of the serpin implies a similar length of the β-sheet and the inserted arm of the RCL. Statistically, the length of this arm (RCL length up to the scissile bond) is almost always 17 residues which reduced extensively the potential P1 residue identity for inhibition of target proteases. The invariance of the RCL length is an absolute necessity to allow a full translocation of the proteinase to the distal pole of the serpin and its increase seems to induce a predominance of the substrate pathway (k_3 in Fig. 2a), with a release of active enzyme and truncated serpin, at the expense of the formation of a stable complex ((k_4 in Fig. 2a).

The respective RCL sequences of bovSERPINA3-1 and A3-3 are depictured in Fig. 3. As previously reported, and according to the cleaving specificities of both proteases, the P1 residue for trypsin is Arg^{16} (R) whereas that of elastase is Thr^{17} (T) [38, 93]. For both serine proteases, the bovSERPINA3's RCL length up to the scissile bonds agrees well with the established rule of a maximum of 17 residues (16 residues for trypsin and 17 residues for elastase). As caspases are aspartate-specific proteases, we have to look for an Asp residue in these sequences. The only one Asp (D) residue is at position 37 in the RCL sequence of both bovine serpins far away from the common 17th residue we generally refer to. To confirm this feature, Asp^{37} (Asp^{371} in the native protein) residue of recombinant bovSERPINA3-3 was substituted to Ala^{37} by site-directed mutagenesis and the activity of both mutated (M-D37/A37) and wild-type (WT) recombinant proteins was tested against both trypsin and caspase 3. Trypsin is similarly inhibited by the recombinant WT and the M-D^{37}/A^{37} bovSERPINA3-3, and both mutated and WT serpins formed a SDS-stable complex with the serine protease. By contrast, caspase 3 is no more inhibited with the mutant M-D^{37}/A^{37} bovSERPINA3-3 as compared to the wild-type recombinant protein and, as expected, SDS-stable complex was obtained for the WT but not for the mutated serpin [8, 35]. This allows us to conclude that the P1 residue for caspases is very likely Asp^{37} or Asp^{371} in the native protein.

The major differences between serine proteases and caspases is that the latter contains two active sites and are two- to threefold larger than trypsin and elastase, the two serine proteases

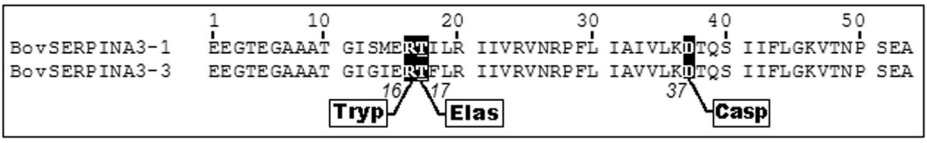

Fig. 3 RCL sequence of bovSERPINA3-1 and A3-3 (adapted from [35]). *White letters* on black background indicate the P1 residues for the different inhibited enzymes including trypsin (*Tryp*), elastase (*Elas*), and caspases (*Casp*)

strongly inhibited by both bovSERPINA3-1 and A3-3. Whether such long RCL needed to trap the high Mr caspases remains an open question further studies of the time dependent behavior of the caspase/serpin complexes will be necessary. Another point needing additional investigations is providing more insights to the question on whether the substrate pathway (k_3) of Fig. 2a will predominate over the caspase/serpin stable complex pathway (k_4) as soon as trapping of caspases has been completed as suggested for CrmA/complexes [17].

In Situ Caspases/SERPINA3 Complexes Detection in Post-mortem Muscle

BovSERPINA3 strongly inhibit caspases 3 and 8 and are able to form covalent complex with their target cysteine proteases. Therefore, we recently sought to verify whether such complexes can be detected in post-mortem muscles. This was done in different bovine muscles including the diaphragma pedialis (DP), rectus abdominis (RA), and semitendinosus (ST) excised 1 to 1.5 h post-mortem. Unexpectedly, a large amount of SDS-stable complexes (Fig. 4b) with a similar Mr (≈113 kDa) than those obtained in vitro after SDS-PAGE analysis of caspase/bovine serpin mixtures (Fig. 4a) were obtained. In post-mortem muscle (Fig. 4b), the amount of free bovSERPINA3 varies from one muscle to another. The highest amounts of free bovSERPINA3 were found in DP muscle where the dimer of about 140 kDa is still discernible. Otherwise, no 70 kDa (bovSERPINA3) band and only a few amount of the latter are detectable in RA and ST muscles, respectively. The dimer is no more detectable in both RA and ST muscles [39]. Although such complexes are not often observed in post-mortem muscles, this finding suggest that, in situ, caspases are probably the target enzyme of members of the bovSERPINA3's family capable of strong inhibition of these aspartate-specific cysteine proteases. Furthermore, in agreement to an earlier report, large amounts of such complexes will slow down the apoptotic process and probably favors greater ultimate toughness of the meat cuts [99].

Cellular Localization, Tissue Distribution, and Polymorphism

Immunolocalization of bovSERPINA3-1 carried out on transverse sections of freshly excised adult bovine longissimus muscle using a specific polyclonal rabbit antiserum revealed that muscle serpins are highly concentrated between the plasma membrane and myofibrils, whereas

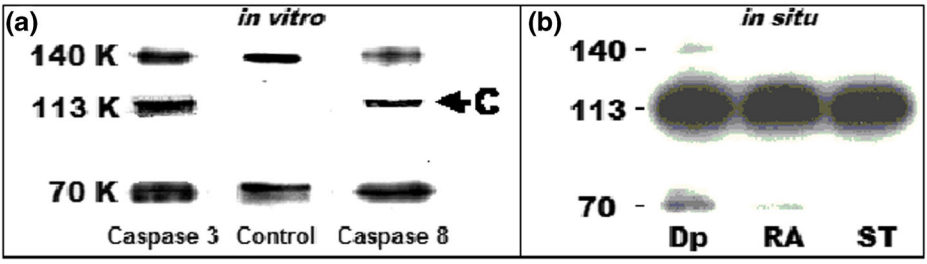

Fig. 4 SDS-stable complexes of bovSERPINA3 with target enzymes as revealed **a** in in vitro analysis of caspase/serpin mixtures (from [35, 65]) and **b** in post-mortem muscle extracts from three different bovine muscles: *Dp* M. diaphragma pedialis, *RA* rectus abdominis, and *ST* semitendinosus (unpublished data). Proteins were labeled with a rabbit polyclonal antibody raised against purified bovSERPINA3-1

lower fluorescence intensity can be seen within the myofibrils, indicating that muscle serpins are exclusively intracellular with a preferential peripheral localization [93].

BovSERPINA3-1, A3-3, and other closely related serpins seem to be ubiquitous [27, 93] and present in most bovine tissues and fluids. Highest concentrations were found in bovine plasma (≈1 mg/ml) followed by liver (14 μg/g wet tissue) and kidney (2 μg/g wet tissue) [27]. The lowest levels were observed in striated muscles especially muscle diaphragma with about 1 μg/g wet tissue. Using the same anti-bovSERPINA3-1 polyclonal antibody, closely related serpins were found in all tissues of other species tested including rat, mouse, lamb, and pork and in human tissues and plasma as well.

At least eight genes encoding this family of homologous proteins were identified suggesting that a minimum of eight different proteins would be expected. In fact, as they were glycosylated [8] and phosphorylated to various extent, the number of isoforms is much larger. This assumption was confirmed by either one- or two-dimensional gel electrophoresis of fractionated muscle extracts [27, 28]. BovSERPINA3 therefore constitutes a highly polymorphic group of serpins, most of them being able to strongly inhibit initiator and effector caspases [27, 28, 35, 38].

Potential Biological Functions

Based on their ability to inhibit initiator and effector caspases, two major functions have been identified. The first one concerns the regulation of apoptosis through direct pseudo-irreversible inhibition of caspases. The second is a consequence of the first one since they probably contribute to the survival of muscle cells in the two first phases of differentiation, i.e., proliferation and confluent phases. But before that, let us come back briefly on apoptosis events and their regulation.

Major Apoptosis Events and Regulation

Apoptosis is a programmed cell death that involves the controlled dismantling of intracellular components while avoiding inflammation and damage to surrounding cells. The mechanisms of apoptosis are highly complex and sophisticated, involving an energy-dependent cascade of molecular events [15, 20, 25]. To date, research indicates that there are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. Whatever the nature of the initiator stimuli, the apoptotic process is essentially driven by caspases, a group of cysteine-ASPartyl proteASES specifically cleaving polypeptide chains after an aspartyl residue and ends up with the activation of executioner caspases that subsequently coordinate their activities to demolish key structural proteins and activate other enzymes [20, 25].

The extrinsic signaling pathways that initiate apoptosis involve transmembrane receptor-mediated interactions. Upon ligand binding to the death receptors, cytoplasmic adapter proteins (fas-associated death domain (FADD), TNF receptor-associated death domain (TRADD)) which will in turn associates with initiator caspases, catalyzes the auto-catalytic activation of procaspases (caspases 8 or 10). Death receptor-mediated apoptosis can be inhibited by a protein called FLICE-inhibitory protein (c-FLIP) which will bind to FADD and caspase 8, rendering them ineffective [47, 76]. Downstream, apoptosis can be inhibited by inhibitor of apoptosis proteins (IAPs) a group of caspases' inhibitors. Once initiator caspases

are activated, the execution phase of apoptosis is triggered through proteolytic activation of the effector caspases [50].

The intrinsic signaling pathways involved a series of diverse mitochondrial-initiated events. The stimuli that initiate the intrinsic pathway produce intracellular signals that may act in either a positive or negative fashion. Negative signals involve the absence of certain growth factors, hormones, and cytokines that can lead to failure of suppression of death programs. Positive signals include, but are not limited to, radiation, toxins, hypoxia, hyperthermia, viral infections, and free radicals. In post-mortem muscle, hypoxia is very likely the main signal triggering apoptosis. All of these stimuli cause changes in the mitochondrial membrane that results in the release of two main groups of normally sequestered pro-apoptotic proteins from the inter-membrane space into the cytosol especially cytochrome c but not only [65, 75]. The control and regulation of these apoptotic mitochondrial events occur through members of the Bcl-2 family of proteins [13]. The Bcl-2 family of proteins governs mitochondrial membrane permeability and can be either pro-apoptotic (Bax, Bak, Bid, Bad, Bim/Bad, Noxa…) or anti-apoptotic (Bcl-2, Bcl-XL, Mcl-1…) [34, 96]. These proteins have a great significance since they can determine if the cell commits to apoptosis or aborts the process. It is thought that the main mechanism of action of the Bcl-2 family of proteins is the regulation of cytochrome c release from the mitochondria via alteration of mitochondrial membrane permeability [46, 82].

The extrinsic and intrinsic pathways both end at the point of the execution phase, considered as the final pathway of apoptosis (Fig. 5). Once activated, effector caspases (caspase-3, -6, and -7) activate in turn a cytoplasmic endonuclease, which degrades nuclear material, and degrade the nuclear and cytoskeletal proteins including cytoskeletal and filamentous gelsolin [52], a protein acting as a nucleus of actin polymerization and many others [86].

BovSERPINA3: an Additional Ultimate Control Point of Apoptosis

It is well established that pathological conditions featuring excessive apoptosis may benefit from inhibiting apoptosis. In this respect, the identification and exploitation of new targets remain a considerable focus of attention [59] including inhibition of Bcl-2 proteins, stimulation of the IAP (inhibitors of apoptosis proteins) family, the only one caspase inhibitor known to be expressed in mammalian cells [26], …etc. BovSERPINA3 subfamily comprises natural caspases inhibitors and might constitute an interesting new target for apoptosis inhibition in vivo and post-mortem muscle [27]. It is probably even truer that these serpins are much more effective inhibitors of caspases than IAPs.

This latter assumption is supported by the fact that trypsin inhibitors, most of which are also caspases inhibitors, quantified by titration in crude muscle extracts were found to be a better predictor of ultimate meat tenderness than calpain 1 and 2 levels, calpastatin concentration, and level of cysteine proteases inhibitors assessed through inhibition of a pre-titrated papain preparations. These serine protease inhibitors are mainly composed of cross-class inhibitory serpins able to inhibit strongly and pseudo-irreversibly initiator and effector caspases as bovSERPINA3-1 to A3-6 and would be good candidates for accurate biomarkers of meat tenderness. Highest levels of serpins inhibiting caspases are thus observed in toughest meat [99, 100]. As depicted in the schematic representation of apoptotic events (Fig. 5), bovSERPINA3 may constitute a serious pathway of apoptosis inhibition by acting against initiator (caspase 8 or caspase 9) and effector caspases (caspases 3, 6, and 7). Recent findings indeed emphasized the ability of bovSERPINA3

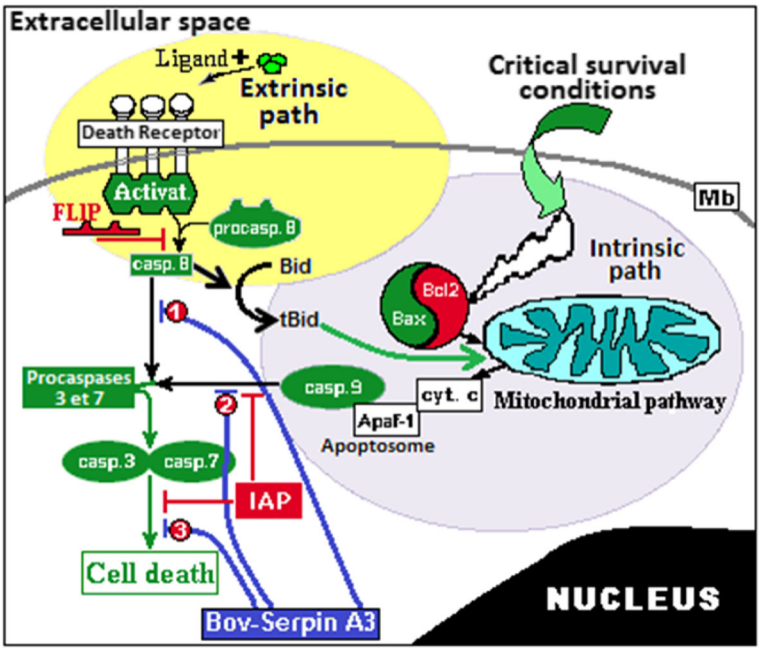

Fig. 5 Schematic representation of the two major pathways of apoptosis and the main regulatory points (adapted from [27]. These are the extrinsic pathway initiated by extracellular signals binding to the death receptor and the intrinsic pathway or mitochondrial pathway activated as soon as cells are in critical survival conditions (radiation, toxins, hypoxia, hyperthermia, viral infections, …etc.). The pathway is controlled at the level of the caspase 8' activator complex by FLIP (FLICE-inhibitory protein) acting as a competitive ligand to the activator and, downstream in the process, by IAPs, a group of proteins inhibiting caspase 9 but not caspase 8 and effector caspases. The intrinsic pathway is mainly regulated by the ratio of pro- and anti-apoptotic members of the Bcl-2 family. The main mechanism of action of the Bcl-2 family of proteins is the regulation of cytochrome c release from the mitochondria via alteration of mitochondrial membrane permeability. Released cytochrome c then binds to Apaf-1 to form the apoptosome complex in charge of caspase 9 activation. BovSERPINA3 family members are able to inhibit apoptosis through initiator (caspases 8, 9, and 10) and effector (caspases 3, 6, and 7) caspases inactivation

proteins to inhibit efficiently caspases 6, 7, 9, and 10 in addition to caspases 3 and 8 (unpublished data), covering therefore all inhibition steps of the apoptotic' phases.

BovSERPINA3: Potential Control of Muscle Cells Differentiation

During the process of muscle development, myoblasts proliferate and then undergo differentiation, fusing to form multinucleated myotubes. During the proliferating phase, it was suggested that myoblasts are protected against cell death by different still unclear mechanisms. Some authors even suggested that this protection is mediated by thrombin [11], a trypsin-like serine peptidase expressed by muscle cells [12].

Because of their function in apoptosis regulation, bovSERPINA3 members could be potential candidates for myoblast survival through inhibition or control of apoptosis. It was therefore decided to follow the expression of these serpins in bovine primary myoblast culture at the different phases of cell differentiation including proliferation, confluence, and myotubes.

In primary myoblast in culture, serpin localization was essentially intracellular in both proliferating (Fig. 6a) and confluent steps (Fig. 6b). When the primary antibody was omitted, no fluorescence was detected (inserts Fig. 6a, b). By contrast, no fluorescence was obtained in differentiated myotubes (Fig. 6c) suggesting that these serpins are no more expressed after fusion into myotubes. These findings support a probable primary role of these serpins in proliferating and confluent phases by prevention of myoblasts from cell death through caspases and, hence, apoptosis inhibition.

Muscle Antithrombin III (AT-III) or BovSERPINC1

Besides the major role of thrombin in the vascular system [23, 55], several extravascular functions have been reported since the 1980s especially in muscle. In this tissue, thrombin is synthesized by muscle cells [12] and acts locally by contributing to synapse remodeling and elimination at the neuromuscular junction [54, 101] but plays also an essential role in muscle cell differentiation [11, 89]. A prerequisite for the activation of thrombin at the neuromuscular junction is the local externalization of phosphatidylserine phospholipids, which contribute to the binding of thrombin activator complex to the membrane [9].

By contrast, the regulation of thrombin activity by specific inhibitors is still debatable. Protease nexin I (PN-I) is the first thrombin inhibitor identified in muscle tissue and since then has been considered to be the natural inhibitor of thrombin [3, 74]. However, the exact origin of PN-I in muscle has never been clearly established. More importantly, the possible local expression of antithrombin III (AT-III), a serpin protein spelled SERPINC1, by muscle cells or other neighboring cell types has never been tested despite the fact that the presence of AT-III within muscle cells has been reported about 10 years ago [10]. In agreement with the finding of these last authors, preliminary analysis of muscle tissue by either western blot or immunohistochemistry always detected the presence of significant amounts of AT-III in this tissue where it has been further intracellularly located [45].

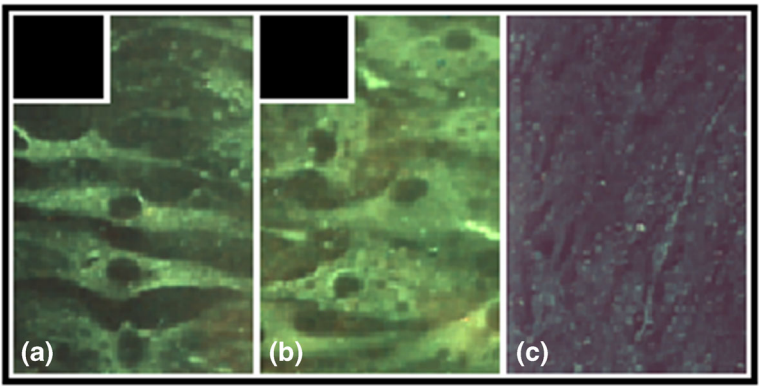

Fig. 6 Cellular expression of bovSERPINA3-1 in primary bovine myoblast culture using the rabbit polyclonal antibody raised against the purified serpin (adapted from [27]. **a** Proliferating myoblasts, **b** confluent myoblasts, **c** growing myotubes. Inserts of Fig. 6a, b are controls for which the primary antibody was omitted

General Outlines of AT-III Biochemical Properties and Cell Localization

The protein purified from bovine muscle with a Mr of 58 kDa has been identified as antithrombin III (BovSERPINC1) using different approaches including western blot carried out with a specific AT-III polyclonal antibody, N-terminal sequencing and MALDI-TOF mass spectrometry analysis [37].

Stoichiometry of interaction (SI) with trypsin (7 nM) and thrombin (9 nM), the most strongly inhibited serine proteases, was performed by adding increasing amounts of this serpin. As depicted in Fig. 7a, for both proteases, equimolar concentrations of AT-III are needed for total inhibition suggesting a 1:1 molar interaction ratio and, hence, a SI of 1. Association rate constants were then determined using trypsin, chymotrypsin, plasmin, and thrombin as target enzymes.

As depicted in Table 2, trypsin and thrombin were inhibited with similar efficiency with k_{ass} of 5.0×10^5 and 1.8×10^5 M^{-1} s^{-1}, respectively. Chymotrypsin and plasmin are less sensitive to this serpin with k_{ass} one magnitude lower and close to 10^4 M^{-1} s^{-1}. Importantly, addition of heparin does not affect the interaction of AT-III with trypsin, chymotrypsin, or plasmin. By contrast, in the presence of 5 μM of heparin, the association rate constant with thrombin is two magnitudes higher than in the absence of heparin shifting from 1.8×10^5 to 2.3×10^7 M^{-1} s^{-1}. Note that all other cysteine (papain, cathepsins B, H, and L, calpains 1 and 2 and caspases 3 and 8) and serine (Elastase, cathepsin G, kallikrein, urokinase, and plasminogen activator) proteases tested were not inhibited by purified muscle AT-III. Serpins are known, as discussed above, to form SDS-stable covalent complexes readily detected as "band shifts" after gel electrophoresis in denaturing conditions with their target enzymes and AT-III did not escape to that rule at least with thrombin as the target enzyme (Fig. 7b).

As thrombin is expressed in muscle cells [12], the expression of its specific inhibitor AT-III could then be expected, an assumption supported by the immuno-histolocalization of this serpin in all fibers of mouse skeletal muscle [10].

The presence of AT-III in bovine muscle was first assessed by analysis of crude muscle extracts by western blot using a polyclonal antibody raised against human AT-III and then by

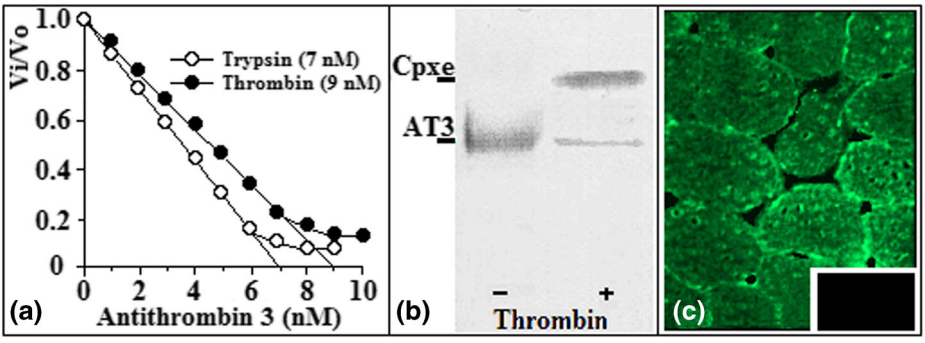

Fig. 7 Inhibitory properties of antithrombin III and cell localization (adapted from [37]). **a** Stoichiometry of interaction with trypsin and thrombin (SI=1). **b** Thrombin/serpin complex formation upon SDS-PAGE analysis of the enzyme/inhibitor mixture. **c** Immunolocalization of AT-III in bovine diaphragma pedialis muscle using a rabbit polyclonal AT-III (1/50) as the primary antibody and the FITC-labeled goat anti-rabbit IgG as the secondary antibody (1/500) (unpublished data)

Table 2 Association rate constants of AT-III for a set of inhibited proteases in the absence or the presence of heparin [37]

Proteases	Cofactor	k_{ass} (M^{-1} s^{-1})
Trypsin	None	5.0×10^{5}
Chymotrypsin	None	6.8×10^{4}
Plasmin	None	1.7×10^{4}
Thrombin	None	1.8×10^{5}
	Heparin (5 μM)	2.3×10^{7}

immunohistochemistry with the same antibody. All western blots performed on muscle crude extracts revealed detectable amounts of AT-III with a Mr of about 58 kDa. Immunohistochemical localization of AT-III was then carried out on transverse section of bovine Diaphragma pedialis muscle to confirm the presence of this protein within muscle fibers. As shown in Fig. 7c, no fluorescence was detected in the extracellular space suggesting that AT-III is a constitutive intracellular protein of muscle cells and is very likely genetically expressed in these cells, an assumption supported by the presence of AT-III's mRNA in the total pool of bovine muscle mRNA. Within muscle fibers, AT-III labeling seemed to be often highly concentrated in the vicinity of the plasma membrane as compared to the inner cytosolic fibers area. When the primary AT-III antibody was omitted, no labeling was detected (insert Fig. 7c). These findings are wholly similar to those of earlier studies [10], which localized three different serum protease inhibitors in mouse skeletal muscle including AT-III.

Potential Biological Functions of Muscle Antithrombin III

Major Functions Ascribed to Thrombin in Skeletal Muscle

Thrombin is mainly known for its pro-coagulant action in blood [23, 55]. However, thrombin has also additional function in other tissues such as brain, vascular endothelium, and skeletal muscle. For more details about thrombin functions in skeletal muscle, the reader must refer to previous reports [37, 66].

Two major functions have been indeed ascribed to thrombin in skeletal muscle cells, which are known to express this protein [32]. The first one is its implication in synapse remodeling and elimination occurring at the neuromuscular junction [101] where the enzyme has been located [1, 56]. The second is the important regulation function in muscle development and muscle cell differentiation [32]. Thrombin was found to cause an increase in the number of cultured myoblasts, possibly through an increase in proliferation [89]. Besides the increase in myoblast number, thrombin further inhibits both myoblast fusion and apoptosis suggesting that thrombin functions as a survival factor for myoblasts [16].

In both functions, thrombin activity is probably regulated by specific inhibitors. As AT-III is expressed by muscle cells, this serpin could be the primary candidate for the control of thrombin activity in muscle tissue. But despite its intracellular localization, we still do not know if this protein is expressed by muscle cells. To address this issue, the transcriptional expression of AT-III in muscle and differentiating myoblasts using liver as a control was investigated.

Transcriptional Expression of AT-III in Liver, Muscle, and Differentiating Myoblasts

The intracellular origin of AT-III was verified by partial cDNA identification in bovine diaphragm muscle. Total RNA was isolated from bovine diaphragm muscle and liver, and reverse transcription was performed. As plasma AT-III is synthesized in liver and then exported, liver PCR was used as positive control. Expression profile suggested by analyses of EST counts indicated that AT-III is expressed in bovine liver (www.ncbi.nhm.gov). cDNAs obtained by reverse transcription from bovine diaphragm muscle and liver were probed by nested PCR using specific sets of primers designed from the bovine *AT-III* gene sequence (GeneBank: NC_007319). The first set of primers corresponds to positions 4097–4116 (in exon 4) and 6553–6571 (in exon 5). The second set of primers were overlapping and designed seven bases downstream and amplified a 2463-bp fragment from genomic DNA and a 389-bp fragment from cDNA.

As indicated in Fig. 8, lanes lv and m, a nested fragment of approximately 400 bp was generated with liver and diaphragm cDNAs. The PCR conditions do not allow to amplify long fragments, superior to 2000 bp on genomic DNA.

Direct sequencing revealed a complete identity between sequences of the amplified DNAs and AT-III cDNA (NM_001034698) thus certifying that the bovine *AT-III* gene is effectively transcribed in this muscle.

To comfort these findings by using a similar approach, the transcriptional expression of AT-III in differentiating myoblasts was investigated. As depicted in Fig. 8, lane 1, no AT-III cDNA was detected in proliferating myoblast, a result in good agreement with previous reports suggesting an improvement of cell proliferation in the presence of active thrombin [11, 33, 48, 89]. By contrast, AT-III cDNA is detected in confluent myoblasts (Fig. 8, lane 2) and in growing myotubes (Fig. 8, lane 3) agreeing well with the inhibition of fusion by active thrombin which must be totally inhibited at these stages. Hence, in differentiating myoblast,

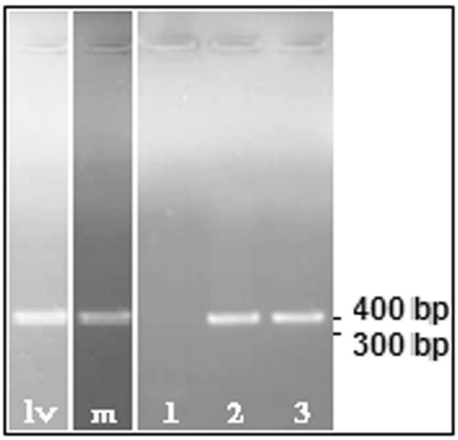

Fig. 8 Transcriptional expression AT-III in muscle and differentiating myoblasts [37]. RT-PCR analysis of total cells mRNAs prepared from liver (*lane lv*), bovine skeletal muscle (*lane m*), proliferating myoblasts (*lane 1*), confluent and fusing myoblast (*lane 2*), and growing myotubes (*lane 3*). A similar nested fragment of approximately 400 bp was generated in the pool of cDNAs isolated from liver, bovine diaphragma pedialis muscle, confluent myoblasts, and myotubes. The PCR conditions do not allow amplifying longer fragments on genomic DNA

expression of AT-III is observed only in confluent myoblasts and in myotubes, two phases where thrombin activity is unsuitable and must be totally inhibited [11, 16, 89].

These findings provide therefore a direct evidence for the transcriptional expression of AT-III in bovine skeletal muscle, a finding consistent with the presence of the protein within muscle cells. As expected, transcriptional expression of AT-III in differentiating primary culture of bovine myoblasts is only observed in the late phases (confluent myoblasts and growing myotubes) when thrombin must be fully inhibited to avoid any inhibition of the fusion process. To confirm these findings, expression of AT-III at the protein level was followed in differentiating primary culture of bovine myoblasts.

Expression of AT-III in Differentiating Primary Culture of Bovine Myoblasts

In developing muscle, thrombin increases the number of cells during myoblast proliferation but delayed skeletal muscle myogenesis through inhibition of myoblast fusion. To test this statement and to comfort the previous findings supporting the presence of AT-III in muscle cells, the expression of this serpin was followed by immunohistochemistry in differentiating primary bovine myoblast cultures. For that, we used a rabbit polyclonal antibody raised against human AT-III and revealed with a FITC conjugate goat anti-rabbit IgG as the secondary antibody. In all the experiments carried out, the cell nuclei were stained in red using the Hoechst method [62]. Hoechst dye, which binds to the minor goove of DNA, has been extensively used by many laboratories for studying nuclear changes occurring during apoptosis [85]. As depicted in Fig. 9a, proliferating myoblast showed no labeling indicative of the absence of this protein within muscle cells. By contrast, an intense green staining was observed in confluent myoblasts (Fig. 9b), fusing myoblasts and myotubes (Fig. 9c). In all cases, in the absence of the primary antibody, no fluorescence was detected (inserts Fig. 9b, c). Antithrombin III or SERPINC1 is therefore expressed only when thrombin must be fully inactivated to allow the myoblast-myotube transition. Despite the common properties shared by protease nexin I (PN-I) and AT-III, the former does not cross-react immunologically with the latter [22].

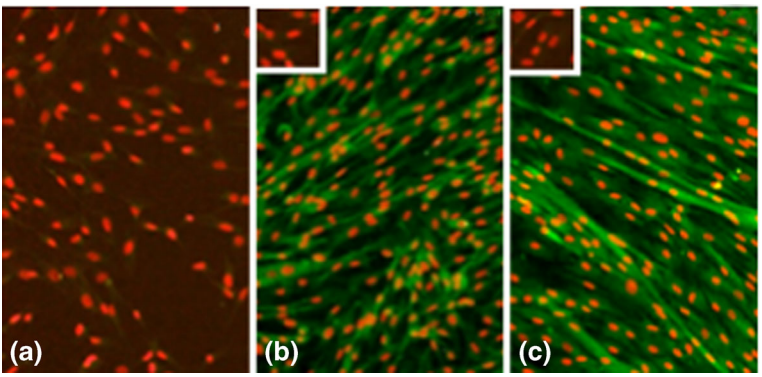

Fig. 9 Immunolocalization of AT-III in differentiating primary bovine myoblast in culture. **a** Proliferating myoblasts, **b** confluent myoblasts, **c** growing myotubes. Cell nuclei are stained in *red* with the Hoechst method [62]. Inserts in **b** and **c** are control samples in which the primary antibody was omitted. Immunolocalization was performed using a rabbit polyclonal AT-III (1/50) as the primary antibody and the FITC-labeled goat anti-rabbit IgG as the secondary antibody (1/500)

Hence, it can be ascertained that the labeled protein in the myoblast culture is AT-III and not PN-I.

Evidence of Post-mortem Synapse Degradation

Synapse remodeling and dismantling are the first thrombin functions identified. Post-mortem, it is well known that low voltage electrical stimulation can be applied only during the survival of the nervous system which is used for current propagation. In cattle, this is generally applied just after bleeding on undressed carcasses between 10 and 60 min after slaughter [84]. Even during this early post-mortem period, the efficiency of this technology in terms of extent of pH drop decreases sharply [37, 66]. One possible reason could be the alteration of the nerve plate by thrombin which is probably activated as soon as phosphatidylserine groups are externalized in the few minutes following animal death [4, 67].

Discussion—Conclusion—Future Prospects

Serine proteases were for a long time an enigma in meat science [63, 80]. These included proteasome [61], which also exhibits activities of the serine protease type. This proteolytic complex was shown to reproduce changes occurring in post-mortem muscle and could contribute significantly to intracellular protein degradation and, hence, to meat tenderization [18]. Whether knowledge about the proteasome grew very fast in the next years, for other serine proteases, many things were much less clear.

In the 1980s, serine proteases were first considered as only located in the extracellular matrix within the mast cells. As a result, it was suggested that they have no function in the meat tenderizing process. Later on, several reports suggested that some of them are located within muscle cells [87]. Concomitantly, Stauber and co-workers localized serine proteases inhibitors within muscle cells [88]. In 1986, we fractionated a series of inhibitors with a high affinity towards diverse serine proteases and located them within muscle cells [64]. It was then clear that muscle cells contain serine proteases together with their inhibitors and this was strengthened by the subsequent identification of some of them at the gene, transcripts, or protein levels [80].

The present review provides some answers to the numerous questions about serine proteases and their inhibitors. The high Mr fractions thus comprised predominantly cross-class inhibitory serpins able to inhibit serine and cysteine proteases and more specially caspases, a group of cysteine proteases orchestrating the demolition phase of apoptosis [94]. Taken together, these investigations suggest that, very likely, it exists a degree of compensation and redundancy within this serpin superfamily wholly comparable to that reported for the set of caspases identified so far [25]. The high Mr Fractions also contain AT-III, another serpin strongly inhibiting thrombin in a heparin dependent manner and trypsin. All these proteases have essential functions in the apoptotic process, thrombin contributing to isolate the dying cells from the nervous system, and caspases targeting several hundreds of proteins and primarily proteins from the cytoskeleton including actin of the transversal filaments, integrin to which transversal filaments of the M line bind dystrophin, a protein of the sarcoglycan complex designed costamere to which transversal filaments of the Z-line are bound, laminins, a group of proteins ensuring the connection of costameres and integrins to the extracellular matrix, …and others.

Otherwise, we recently hypothesized that after death, muscle cells will engage in the cell death program, with apoptosis rather than necrosis being the most probable process [36, 66]. This hypothesis was confirmed more recently by numerous studies [4, 49, 67], providing evidence of cell shrinkage starting few minutes after animal death. Also they revealed concomitant rapid actin degradation, a major protein of the transversal cytoskeletal filaments binding to the costameres and to the integrins. These two findings both support the onset of apoptosis immediately after animal death. The observation of a caspase 3/serpin complex in muscle extracts definitely ascertains that caspase 3 is totally active in post-mortem muscle excised within 1 h after death and, consequently, those muscle cells are already engaged in apoptosis. We also must keep in mind that apoptotic cell dismantling implicates not only caspases but a large set of other proteases including cathepsins (autophagic process), proteasomes, and others.

However, despite the prominent progress in that domain, much remains to be done if we expect to better understand the onset and progress of apoptosis in post-mortem muscle and how the cell provide enough energy for the completion of that process. This will be a prerequisite for the identification of reliable biomarkers of meat tenderness we are looking for since years. On the other hand, protease inhibitors are of essential help for any progress in this field as they provide valuable information about the proteolytic enzymes of concern and their potential role in post-mortem cell dismantling. Total screening for proteases inhibitors in muscle cells will be therefore of major importance for future developments in our understandings of post-mortem muscle proteolysis.

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Conflict of Interest The authors declare no conflict of interest.

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