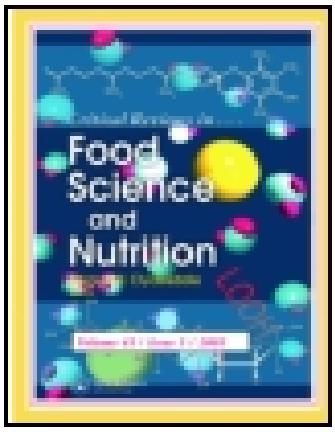

Critical Reviews in Food Science and Nutrition
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/bfsn20

Serine protease inhibitors as good predictors of meat tenderness: which are they and what are their functions?

Yasmine Boudida [a], Mohammed Gagaoua [a], Samira Becila [a], Brigitte Picard [b], Abdelghani Boudjellal [a], Carlos H. Herrera-Mendez [c], Miguel Sentandreu [d] & Ahmed Ouali [e]

[a] INATAA, University of Constantine, Aïn El Bey Road, 25000, Constantine, Algeria

[b] UMR1213 Herbivores, URH - AMUVI, INRA de Clermont Ferrand Theix, 63122, St Genès Champanelle, France

[c] Depart. Ingen. Agroind. Universidad De Guanajuato, 38900, Salvatierra, Guanajuato, Mexico

[d] Instituto de Agroquímica y Tecnología de Alimentos, CSIC, P.O. Box 73, 46100, Burjassot (Valencia), Spain

[e] UR370, QuaPA, INRA de Clermont Ferrand—Theix, 63122, St Genès Champanelle, France

Accepted author version posted online: 01 Aug 2014.

Serine protease inhibitors as good predictors of meat tenderness: which are they and what are their functions?

YASMINE BOUDIDA[1*], MOHAMMED GAGAOUA[1*], SAMIRA BECILA[1], BRIGITTE PICARD[2], ABDELGHANI BOUDJELLAL[1], CARLOS H. HERRERA-MENDEZ[3], MIGUEL SENTANDREU[4], AHMED OUALI[5§]

[1] INATAA, University of Constantine, Aïn El Bey Road, 25000 Constantine, Algeria

[2] UMR1213 Herbivores, URH - AMUVI, INRA de Clermont Ferrand Theix, 63122 St Genès Champanelle, France

[3] Depart. Ingen. Agroind. Universidad De Guanajuato, 38900 Salvatierra, Guanajuato, Mexico

[4] Instituto de Agroquímica y Tecnología de Alimentos, CSIC, P.O. Box 73, 46100 Burjassot (Valencia), Spain

[5] UR370, QuaPA, INRA de Clermont Ferrand – Theix, 63122 St Genès Champanelle, France

[*] These two authors contributed equally to the work and to this report.

§ **Corresponding author:** Ouali Ahmed mail: ouali@clermont.inra.fr

Address:

Dr Ouali Ahmed

INRA Clermont-Theix

63122 St Genes Champanelle

Phone: 33 4 73 62 41 63

Fax : 33 4 73 62 42 68

Mobile: 33 6 74 40 06 16

Mail: **ouali@clermont.inra.fr**

ABSTRACT

Since years, serine proteases and their inhibitors were an enigma to meat scientists. They were indeed considered to be extracellular and to play no role in postmortem muscle proteolysis. In the 1990's, we observed that protease inhibitors levels in muscles are a better predictor of meat tenderness than their target enzymes. From a practical point of view, we therefore choose to look for serine protease inhibitors rather than their target enzymes, i.e. serine proteases and the purpose of this report was to overview the findings obtained. Fractionation of a muscle crude extract by gel filtration revealed three major trypsin inhibitory fractions designed as F1 (Mr:50-70kDa), F2 (Mr:40-60kDa) and F3 (Mr:10-15kD) which were analyzed separately. Besides antithrombin III, an heparin dependent thrombin inhibitor, F1 and F2 comprised a large set of closely related trypsin inhibitors encoded by at least 8 genes bovSERPINA3-1 to A3-8 and able to inhibit also strongly initiator and effector caspases. They all belong to the serpin superfamily, known to form covalent complexes with their target enzymes, were located within muscle cells and found in all tissues and fluids examined irrespective of the animal species. Potential biological functions in living and postmortem muscle were proposed for all of them. In contrast to F1 and F2 which have been more extensively investigated only preliminary findings were provided for F3. Taken together, these results tend to ascertain the onset of apoptosis in postmortem muscle. However, the exact mechanisms driving the cell towards apoptosis and how apoptosis, an energy dependent process, can be completed *postmortem* remain still unclear.

Keywords: Serine Protease Inhibitors, Serpins, Caspases, Apoptosis, Cell differentiation, Bovine muscle

INTRODUCTION

In the 80's, 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 (For review see (Ouali, 1990). 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 (Pejler et al., 2007). Later on, several serine proteases were shown to be synthetized by muscle cells including plasmin, thrombin, kallikrein and others (reviewed in (Sentandreu et al., 2002).

It is well documented in the literature that, between the many quality attributes of meat, tenderness is considered as the most relevant by consumers. This attribute is going to strongly influence them in their decision to repurchase that meat (Jeremiah and Gibson, 2003). In relation to this, we have to stress forward that one of the main problems that nowadays the meat industry has to face is still the large variability of the final meat tenderness of carcasses and the impossibility to predict such variability. This situation is directly related to the complexity and biological diversity of skeletal muscle tissue. In addition to this, variability comes also from the complex biochemical reactions that take place during the conversion of muscle into meat just immediately after animal slaughter. Even if efforts have been made during the last decades in order to better understand these mechanisms, still many questions remain to be answered. What it is clear is that between these mechanisms, hydrolysis of the main structural proteins conforming myofibrils is one of the most relevant. These structural proteins would include specific myofibrillar and costamere proteins such as actin, myosin, titin, desmin and vinculin (Kemp et al., 2010). This 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 ageing. However, the relevance that the different groups of muscle peptidases can have in this process has been a matter of controversy for a long time. Over the last three decades, meat scientists have focused their efforts on the study of two main enzyme systems known as cathepsins, the first group of proteases to have been identified (De Duve et al., 1955) and calpains, a calcium dependent cysteine protease identified in the 1960's (Guroff, 1964). Unexpectedly, investigations carried out in the beginning of the 1990's demonstrate that the proteases inhibitor level in muscle is a better predictor of meat tenderness than that of their target enzyme (Ouali and Talmant, 1990; Shackelford et al., 1991). This explains our major interest in protease inhibitors in the following years (Ouali et al., 1995). Activity of these proteolytic systems is indeed finely regulated by specific inhibitors. Calpastatin specifically inhibits calpains, and is probably the most studied protease inhibitor in the meat science field. Activity of cathepsins is regulated by a family of cysteine peptidase inhibitors known as cystatins, but they have been much less studied in muscle.

In the scientific community of meat scientist, it is a general belief that the calpain-calpastatin system plays a major role in meat tenderization postmortem. However, the fact that other proteases also play an important role is gradually gaining attention. Several groups indeed think that postmortem proteolysis of meat proteins is a multienzymatic process involving a large set of proteases. In this context, knowledge of the specific biological functions of these proteases *in vivo* will be a prerequisite for the improvement of our understanding of the tenderizing mechanisms and for the search of biological markers of tenderness as well. To the previously mentioned proteases, we must add some others that still have not been so deeply studied in the

meat science field. This is the case of the proteasome and, more recently, the caspases, a particular group of cystein peptidases responsible for programmed cell death or apoptosis in cells, a phenomenon that has been proved to occur in postmortem muscle (Becila et al., 2010). In addition to this, as already pointed out, muscle tissue contains numerous other proteolytic enzyme groups that would have the potential to degrade a large set of muscle proteins. This is the case of muscle serine peptidases (Sentandreu et al., 2002).

Although the contribution of serine proteases to meat tenderization has never been clearly demonstrated, several studies identified muscle serine protease inhibitors' level as one of the best predictor of meat tenderness as compared to the concentration of calpains 1 and 2, calpastatin and cysteine protease inhibitors (Zamora et al., 2005; Zamora et al., 1996). The fact that proteases inhibitor level is a better predictor of meat tenderness than their target enzyme explained the aroused interest to identify them and their target proteases as well (Ouali and Talmant, 1990; Shackelford et al., 1991).

Based on these different observations and because of the probable importance of serine proteases in meat tenderization we decided to screen for serine proteases inhibitors in bovine muscle, to try to identify and characterize them and to identify their potential target intracellular proteases. For this purpose the bovine muscle selected was M. *Diaphragma pedialis*, a muscle containing, as all slow twitch muscles including heart, high amounts of proteases and inhibitors. The present work reviews all the work carried out by our group during more than one decade in relation to the characterization of serine peptidase inhibitors in muscle tissue, their potential target enzymes, their possible biological functions, and their potential role in postmortem muscle proteolysis and the development of meat tenderness.

MAJOR SERINE PROTEINASE FRACTIONS IN MUSCLE CRUDE EXTRACT

As previously mentioned, muscle crude extract prepared as described by (Bige et al., 1985) contained a large set of serine protease inhibitors exhibiting different Mr ranging from 10 to 85 kDa. For a purpose of clarity, analysis of the fractions obtained after gel filtration of the ammonium sulfate concentrated crude muscle extract on a Sephadex G100 superfine column (100 cm x 5 cm) were carried out separately. This step allowed the separation of three fractions called F1, F2 and F3 with Mr of about 85-60, 40-60 and 10-14 kDa respectively (Figure 1).

Each of the three fractions, F1, F2and F3, was analyzed separately for identification of their composition in serine protease inhibitors and their identification at the protein and gene level.

INHIBITORS PURIFIED FROM THE F1 FRACTION

Using different chromatography protocols, two trypsin/elastase inhibitors and a trypsin/thrombin inhibitor were purified to homogeneity and characterized (Tassy, 1998); (Herrera-Mendez et al., 2010; Herrera-Mendez et al., 2006b; Tassy et al., 2005). From the N-terminal partial sequences obtained by the EDMAN sequencing method, they were identified as:

- Trypsin/elastase inhibitors
  - Bov-Serpin A3.1previously designed Endopin 1A (Herrera-Mendez et al., 2010; Herrera-Mendez et al., 2006b; Tassy, 1998; Tassy et al., 2005)
  - Bov-Serpin A3.3 previously designed Endopin 1B (Herrera-Mendez et al., 2006b)
- Trypsin/Thrombin inhibitor
  - Antithrombin III (Herrera-Mendez et al., 2010)

From the F2 fraction, we failed to purify to homogeneity any serine proteinase inhibitor probably because all inhibitors of this fraction shared very close physicochemical properties.

All inhibitors from the F1 fraction are members of the serpin superfamily. Let's introduce now some basic concepts concerning this large and particular protein protease inhibitors family.

GENERAL PROPERTIES OF SERPINS

The serpin superfamily

Serpins are a group of proteins wth 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 serine proteases (**SER**ine **P**rotease **IN**hibitors). Over 3000 serpins have now been identified; these include 36 human proteins, as well as molecules in plants, fungi, bacteria, archaea and certain viruses (Law et al., 2006); (Olson and Gettins, 2011). Serpins are thus the largest and most diverse family of protease inhibitors.

While most serpins control proteolytic cascades, certain serpins do not inhibit enzymes, but instead perform diverse functions such as storage (ovalbumin), hormone carriage proteins (thyroxine-binding globulin) and tumor suppressor genes (maspin). The term SERPIN is used to describe these latter members as well, despite their non-inhibitory function. Inhibitory serpins were later shown to be cross-class inhibitors since they were shown to be able to inhibit other groups of proteinases especially cysteine peptidases (for review see (Silverman et al., 2001); (Gettins, 2002).

Inhibitory serpins are generally highly metastable proteins comprising several α-helices (eight or nine) and β-sheets together with an external reactive center loop (RCL) containing the active

site recognized by the target peptidase (**Fig 2a**). The sequence of the RCL defines the enzyme specificity pattern of each serpin. All inhibitory serpins are irreversible covalent 'suicide' protease inhibitors forming a highly stable covalent complex with their target enzyme, a complex detectable after gel electrophoresis in denaturing conditions (SDS-PAGE). (For more details see also the following reviews: (Olson and Gettins, 2011; Silverman et al., 2001; Silverman et al., 2010; Whisstock et al., 2010). A large set of information together with the serpin classification are also available at the following web site: http://en.wikipedia.org/wiki/Serpins.

Structural features

As shown in **Fig 2b** for α_1-antitrypsin, upon cleavage of the RCL, the serpin adopt quickly a more stable conformation by insertion of the RCL into the β-strand series. This property is essential to the irreversible suicide substrate inhibitory mechanism of serpins. In the inhibitory pathway, the proteinase forms a non-covalent Michaelis-like complex (**Fig 2c**) through interactions with residues flanking the scissile bond (P1– P1**'**). Attack of the active site serine on the scissile bond leads to a covalent ester linkage between highly reactive Ser residue of the proteinase and the backbone carbonyl of the P1 residue and cleavage of the peptide bond. It is likely that only at this stage, with removal of the restraint, does the RCL start to insert into β-sheets and transport the covalently bound proteinase with it. Upon complete loop insertion, the proteinase is translocated to the distal side of the serpin (**Fig 2d**). This translocation induced an important distortion of the proteinase which became unable to complete the catalytic process. The energy needed to effect the distortion may come from the much greater stability of the cleaved loop-inserted conformation compared with the native-like conformation.

Kinetic analysis of protease–serpin interactions

Given the general mechanisms reported so far, kinetic values are determined under the assumption that inhibition is irreversible. In such case, the inhibition constant (Ki) is of no interest and will bring no information about the enzyme-serpin interaction. The major macroscopic parameters that define the effectiveness of serpin inhibition of a particular target protease are the stoichiometry of interaction (SI) and the apparent second order rate constant of inhibition (k_{ass}). Another parameter can be the stability of the covalent complex (vary from few seconds to several weeks (Bieth, 1980) during the inhibition process and often revealed by SDS-PAGE analysis, the complex being stable in the presence of SDS even after heating in boiling water.

SI was generally determined by titration of the enzyme with increasing amounts of pre-titrated inhibitor (illustrated in Figure 10 for the titration of trypsin and thrombin by purified antithombin III).

Practically, the Apparent Association rate constant (k_{app}) was measured according to the discontinuous method for enzymes interacting slowly with the serpins ($k_{ass} \leq 10^4$ $M^{-1}S^{-1}$) (Horvath et al., 2011) and the association rate constant then determined according to Equation.1:

$$k_{ass} = k_{app} * SI \qquad (1)$$

Otherwise, for rapid enzyme/serpin interaction, the continuous method was used to determine the Apparent Association rate constant (k_{app}) and the association rate constant (k_{ass}) was then obtained from Equation 2:

$$k_{ass} = k_{app}\,(1+[S]/Km) * SI \qquad (2)$$

where [S] is the substrate concentration used for activity measurement, Km the Michaelis constant characterizing the affinity of the protease towards the substrate and SI the stoichiometry of interaction.

CHARACTERIZATION OF Bov-SerpinA3-1 AND A3-3

Stoichiometry of interaction with inhibited proteases

The stoichiometry of interaction was determined by titration of the target enzymes (HLE, trypsin, caspases 3 and 8) with increasing amounts of either Bov-SerpinA3-1 (Swiss Prot ID: Q9TTE1) or A3-3 (Swiss Prot ID: Q3ZEJ6) as in (Schechter and Plotnick, 2004). As shown in **Table 1**, total inhibition of trypsin and human leukocyte elastase was achieved using equimolar concentrations of either serpins suggesting a 1:1 interaction ratio.

Regarding caspases, analysis of the findings of **Table 1** needs to remind that each molecule of caspases is a tetrameric structure comprising two associated moieties composed of a large and a small subunit, each moiety containing an active site, *i.e.* two active sites in each native caspase molecule. An equimolar interaction (1 mole of inhibitor per active site) was thus observed for the inhibition of caspase 3 by Bov-SerpinA3-1. Hence, for total inhibition of the caspase, one molecule of Bov-SerpinA3-1 must bind to each active site of caspase 3. In all other cases, namely inhibition of caspases 3 and 8 by Bov-SerpinA3-3 or inhibition of caspase 8 by Bov-SerpinA3-1, one mole of inhibitor inactivates simultaneously the two active sites (0.5 mole of inhibitor per active site), suggesting that association of the inhibitor to one of the two active sites induces a sufficient allosteric conformational change of the tetrameric native protease to make the second site unable to bind substrates.

Association rate constant 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. In this study, association rate constant towards a large set of serine and cysteine proteases was therefore determined for both serpins according to (Schechter and Plotnick, 2004) and (Horvath et al., 2011).

As depicted in Table 2, Bov-SerpinA3-1 and Bov-SerpinA3-3 showed a similar pattern towards the set of proteases tested. They were thus able to strongly inhibit elastase (k_{ass} = 1.3 x 10^6 $M^{-1}S^{-1}$) and trypsin (k_{ass} = 6.7 x 10^5 $M^{-1}S^{-1}$) whilst chymotrypsin and plasmin were only slightly inhibited and the k_{ass} values obtained for these two last enzymes are not of physiological significance (Herrera-Mendez et al., 2006b; Tassy et al., 2005). By contrast, no inhibition was detected against the other five serine proteases tested including cathepsin G, kallikrein, urokinase, plasminogen activator and thrombin.

As serpins are able to inhibit some cysteine proteases, Bov-SerpinA3-1 and A3-3 were tested against papain like enzymes including cathepsins B & L and papain itself and against calpains 1 and 2, two calcium dependent cysteine peptidases. Neither of these cysteine proteases was inhibited by the present serpins (Herrera-Mendez et al., 2006b; Tassy et al., 2005).

Bovine serpins were also tested against a group of cysteine peptidases called caspases. The first letter of the name « *C* » stands for the cysteine of the active site; « *asp* » defines the strict specificity of cleavage after an aspartic acid residue and *ase* is the su□x common to all enzymes. These are responsible of cell dismantling during apoptosis, a finely regulated cell death process and according to their function in the cell dying process; they were classified as initiator (caspases 8, 9 and 10) and effector (caspases 3, 6 and 7) caspases, initiator caspases being in charge of the limited proteolytic activation of effector enzymes. Caspase 8, an initiator caspase,

and caspase 3, an effector caspase, are strongly inhibited by both serpins (table 2) and k_{ass} values are in the range of 10^5 - 10^6 $M^{-1}S^{-1}$ indicating that this inhibition is of high physiological significance (Herrera-Mendez et al., 2009).

Interestingly, these were the first mammalian serpins identified as strong inhibitor of human initiator and effector caspases and able to form SDS-stable complexes with these (Herrera-Mendez et al., 2009). The only one other serpin found to inhibit caspases is crmA (Cytokine response modifier A) isolated from cowpox virus. In infected cells, crmA is suspected to block the apoptotic process during multiplication of the virus (Stennicke et al., 2002); (Dobo et al., 2006).

Ability of the serpins to form SDS-stable complexes with their target enzymes

Serpin inhibition of cysteine proteases proceeds according to the same trapping mechanism than for serine proteases (Swanson et al., 2007). Upon SDS-PAGE, we could therefore expect to identify the covalent complexes for all proteases shown to be strongly inhibited by Bov-SerpinA3 isoforms. As illustrated in Figure 3 for Bov-SerpinA3-1, bovine serpins (70 kDa) are thus able to form SDS-Stable complexes (C) with bovine pancreatic trypsin, human leukocyte elastase, human caspase 3 (apoptosis effector caspase) and human caspase 8 (apoptosis initiator caspase) (Herrera-Mendez et al., 2009; Herrera-Mendez et al., 2006b; Tassy et al., 2005).

Regarding caspases 3 and 8, preincubation of these enzymes with Bov-SerpinA3-1 leads to an SDS-stable complex of about 100–110 kDa. According to the Mr value, the complexes would comprise the cleaved inhibitor (70 kDa) and the covalently bound caspase (30 kDa) transported to the distal pole of the serpin. The covalently bound caspase is very likely an heterodimeric moiety (with or without the small subunit) of the tetrameric molecule, the other moiety being

very likely dissociated during heat denaturation of the sample in the presence of SDS. Further investigations are needed to clarify the exact mechanisms of the caspase/Bov-SerpinA3 complex formation and the behaviour of caspase subunits during complex formation (Herrera-Mendez et al., 2009). Note that in figure 3, the 140 kDa band observed for all peptidases corresponds to a dimer of the serpin as assessed by N-terminal sequence analysis (Tassy et al., 2005).

Cellular localization and tissue distribution

Immunolocalization of Bov-SerpinA3-1 was performed on transverse sections of freshly excised adult bovine *Longissimus* muscle using a specific polyclonal rabbit antiserum. As depicted in **Figure 4a**, the muscle serpin is highly concentrated between the plasma membrane and the myofibrils, whereas lower fluorescence intensity can be seen within the myofibrils, indicating that muscle serpin is exclusively intracellular with a preferential peripheral localization. No fluorescence was detected in the control sample for which the primary antibody was omitted. In primary myoblast in culture, the localization was essentially intracellular (see figure 4b).

Tissue distribution and content of Bov-SerpinA3-1 and other closely related serpins (see below) was assessed by ELISA in different bovine tissues and fluids including bovine plasma, liver, kidney and bovine *diaphragma* muscle. According to the results presented in Table 3, this serpin is very abundant in plasma (≈ 1 mg/ml) as compared to liver (14 µg/ g. wet tissue), kidney (2 µg / g. wet tissue) and muscle (1 µg/ g. wet tissue). These caspase inhibiting serpins showed a wide distribution in bovine tissues and fluids and were also detected in all tissues of other animal species analysed (rat, mouse, lamb, pork, …) including human.

Complexity of the bov-serpin A3 family

- ***Polymorphism of Bov-Serpin A3***

In the first step of the purification procedure developed for Bov-SerpinA3-1, the crude muscle extract was run on a SP-Sepharose column (5 cm x 10 cm) and proteins were eluted using a NaCl gradient (**fig 5a**). Western blot analysis of the fractions collected revealed a cross-immunoreactivity with the antibody raised against the purified serpin of most if not all fractions (**Fig 5b**). According to the result of **fig 5b**, the antibody recognized a series of proteins with different Mr suggesting that the Bov-SerpinA3 comprised different closely related members.

To confirm this assumption, pooled active fractions FI and FII obtained upon gel filtration of a muscle crude extract and identified in Figure 1 were analyzed by 2D gel electrophoresis and proteins revealed with the same anti-Bov-SerpinA3-1 antibody.

As assessed by western blot, 2D gel electrophoresis of the FI fraction revealed a complex protein pattern with pI ranging between about pH 4 and 6.8 (Figure 6a). It is impossible to determine the number of isoforms but the horizontal alignment of spots towards more acidic pH supports the presence of various degree of phosphorylation between these serpins (black line with close arrowhead). In addition some spots are distributed in a comma shape manner (arrows) suggesting that a large set of isoforms are glycosylated to various extent, a feature in good agreement with the overestimated Mr obtained by gel electrophoresis for both Bov-SerpinA3-1 and A3-3 (70 and 75 kDa versus 43-44 kDa for the Mr deduced from the protein sequences).

This was confirmed recently by PNGase F (progressive removal of all N-glycans) treatment of recombinant glycosylated Bov-SerpinA3-3 produced and purified from *S. cerevisiae* (Blanchet et al., 2012). Such treatment revealed six states of glycosylation corresponding to six different forms of different Mr separated by SDS-PAGE. A similar finding was obtained with purified Bov-SerpinA3-1 (not shown). These results are in good agreement with the five N-glycosylation sites identified in the sequence of this serpin (Herrera-Mendez et al., 2006b).

As compared with fraction F1, fraction F2 shows a wholly similar protein pattern with some additional spots of lower Mr (open arrows in Figure 6b). To conclude, all findings obtained so far emphasized the large molecular diversity of the bovine serpinA3 family which led us to identify the genes encoding these proteins in bovine genome.

- ***Genomic organization of the bovine serpinA3 genes***

Clustering of serpin genes frequently occurs in the genome of human and other animal species. In human, the chromosome 14q32 cluster comprised several serpins genes, encoding structurally related proteins with very diverse functions including SERPINA1 (α_1-antitrypsin), SERPINA3 (α_1-antichymotrypsin), SERPINA5 (PCI, protein C inhibitor), SERPINA9 (centerin), SERPINA10 (ZPI, protein Z-dependent protease inhibitor), SERPINA11 (not characterized yet) as well as SERPINA4 (kallistatin precursor) (Billingsley et al., 1993).

In mouse, 14 genes mapped on chromosome 12F1 cluster were identified and encoded for closely related serpins of the serpinA3 family (Forsyth et al., 2003). Similarly, 6 genes encoding serpinA3-like were mapped in rat on chromosome 6q32 cluster (Horvath et al., 2004)whereas in

pork, 6 similar genes were mapped on chromosome 7q23–q26 cluster (Musilova et al., 1995); (Archibald et al., 1996).

What about SERPINA3 like serpins'genes in bovine?

Based on the first sequence available for Bov-SerpinA3-1 and A3-3 (Herrera-Mendez et al., 2006b) several probes were designed and used to screen for similar genes in the bovine genome. A cluster of eight genes and one pseudogene sharing a high degree of identity and the same structural organization (5 exons and 4 introns) was characterized (Pelissier et al., 2008). Bovine SERPINA3 genes were localized by radiation hybrid mapping on 21q24 and only spanned over 235 Kilo bases. For all these genes, we proposed a new nomenclature from SERPINA3-1 to SERPINA3-8. They share approximately 70% of identity with the human SERPINA3 (α_1-antichymotrypsin) homologue. Preliminary expression analyses of these bovSERPINA3s showed different tissue-specific patterns and diverse states of glycosylation and phosphorylation a finding in good agreement with the tissue distribution and the polymorphism of the Bov-SerpinA3-like assessed at the protein level by different approaches. Hence we concluded that the bovine SERPINA3 family comprised at least eight different proteins members and probably more with regards to their variable degree of post-translational modifications (various degrees of glycosylation and of phosphorylation) (see fig 6).

- ***Comparative RCL sequences and potential enzyme targets***

As mentioned above, the sequence of the RCL defines the enzyme specificity pattern of each serpin. Comparison of the RCL sequences of the eight proteins encoded by the genes mapped on bovine chromosome 21 provides additional information about their structural and functional relationship. According to the RCL sequences depicted in figure 7, two subgroups can be

identified on the basis of their sequence homology. The first one would comprise Bov-SerpinA3-1 to A3-6 which contain an Arg (white on black bold letter R) residue at position 16 of the RCL and a Thr residues at position 17 (bold underlined T), two amino acid residues susceptible to explain their ability to inhibit strongly trypsin and elastase. The second group would comprise the two last serpins, namely Bov-SerpinA3-7 and A3-8, which have no Arg residues in similar position and would be unable to inhibit trypsin. By contrast, they both exhibit a Thr residue at position 16 and 17 and a Ser residue at position 17 and 18, respectively, suggesting that they will be able to inhibit elastase. Position of the P1 residues identified as preferential cleavage sites for trypsin and elastase agrees well with the supposed invariable RCL length value of about 17 amino acids.

As suggested in their name, caspases cleaved polypeptides essentially at the carboxyl side of Asp residues. No other potential P1 residues are actually known. On the other hand we found that purified Bov-SerpinA3-1 and A3-3 are strong inhibitors of caspases 3 and 8 ($k_{ass} > 10^5$ $M^{-1}S^{-1}$). The only one Asp residue in Bov-SerpinA3-1 to A3-6 RCL susceptible to be targeted by both caspases is Asp^{37} is far beyond the 17^{th} residue corresponding to the maximum length requested for serpin efficiency. Bov-SerpinA3-7 contains no Asp residue in its RCL while one Asp residue is observed at position 39 in its homologue Bov-SerpinA3-8. These observations strongly suggest that Bov-SerpinA3-1 to A3-6 would be able to inhibit caspases and to form SDS- stable complexes with them. Whether Bov-SerpinA3-7 will not be able to inhibit caspases since no Asp residue can be found in the RCL sequence, the ability of Bov-SerpinA3-8 to inhibit these cysteine tetrameric proteases would seem possible but this assumption needs to be tested experimentally. By contrast, these last serpins are very likely able to inhibit elastase for which

the P1 residue would be very likely Thr at position 16 for Bov-SerpinA3-7 and 17 for Bov-SerpinA3-8.

Regarding caspases, the present findings are therefore in total contradiction with the RCL length invariance of serpins established with various monomeric targeted serine proteases (trypsin, elastase…). Caspases are tetrameric cysteine proteases containing two active sites and whether the rule can be different for serpin interaction with these much larger proteases is questionable and calls for further clarification.

Possible biological functions of Bov-SERPINA3

Because of their ability to strongly inhibit caspases, two functions will be considered here because of the potential implications of cell death in these processes but this list is obviously not exhaustive and needs refinement. The first is of course apoptosis (see schematic diagram of the major regulation point in Figure 8) which is the primary process of concern with regards to the presently discovered function for these inhibitors 7 (Gagaoua et al., 2012). The second is the differentiation of muscle cells for which a resistance to cell death has often been reported in the first differentiation stages corresponding to the proliferation and confluence of myoblast. Cell survival is a prerequisite for differentiating primary myoblast (Lamkanfi et al., 2007), and the present caspases inhibiting serpins could be a potential candidate for such a function through total inhibition of caspases.

- ***Bov-SerpinA3: a new control point of apoptosis in mammals***

Since the 80's, it is well established that for most proteolytic systems, if not all, natural peptidase inhibitors constitute the major tool for controlling their biological activity. Regarding caspases, the first regulation level involves the conversion of zymogens to their active forms in response to inflammatory or apoptotic stimuli. This conversion is generally ensured by association of the peptidases with specified protein activator complexes. The second level of regulation involves the specific inhibition of active caspases by natural inhibitors. To date, members of three protein families have been found capable of ablating caspase activity *in vitro* and *in vivo* (Stennicke et al., 2002). One of these, the inhibitors of apoptosis protein (IAP) family regulates cellular apoptosis by direct caspase inhibition and is conserved from flies to humans (Deveraux and Reed, 1999). They are potent inhibitors of caspase 3, 7 and 9 (Dean et al., 2007), and they are the only one to be expressed in mammal cells and tissues. In addition to these endogenous regulators there are virally encoded inhibitors – cowpox virus crmA and baculovirus p35 – that are produced early in infection to suppress caspase-mediated host responses (Cassens et al., 2003). Protease inhibitors generally work by preventing hydrolysis of substrate by the enzyme, and almost all natural protease inhibitors achieve this by steric hindering access of substrates to the catalytic centre of the protease. Usually, inhibitors dock onto the same sites as substrates on the enzyme surface. By contrast, caspase inhibition by IAPs does not use these conventional mechanisms. To achieve their goal, IAPs block substrate access to the peptidase active site without directly docking into substrate pockets on the enzyme surface allowing hydrolysis of small molecules substrates after IAP binding (Stennicke et al., 2002); (Cassens et al., 2003).

It was therefore often thought that, for unknown reasons, cystatins or serpins do not seem to have been chosen for endogenous caspase regulation in eukaryotic cells. This gap now seems to be filled with the results reported here and invalidating this statement. Indeed, all findings obtained in the present work clearly demonstrate that eukaryotic cells have in fact developed very efficient pseudo-irreversible caspase inhibitors belonging to the serpin family, a family of inhibitors well known to form pseudo-irreversible complexes with their target peptidases. In contrast to the cowpox virus crmA, they form SDS-stable serpin/caspases complexes both *in vitro* and *in situ*. Such a more radical inhibition of the caspases than IAPs is indeed essential and constitutes probably an absolute prerequisite for a strict and efficient regulation of apoptosis. Supporting the *in vitro* findings, caspase-serpin complexes can be detected in postmortem muscle excised just after death by western blot using anti-caspase and anti-serpins antibodies (Figure 8B) stressing that caspase activation is completed in postmortem muscle in contrast to what has been suggested by some meat scientists. This probably explain why trypsin inhibitors quantified by titration in crude muscle extracts were found to be a better predictor of ultimate meat tenderness than calpain 1 & 2 levels, calpastatin concentration and level of cysteine proteases inhibitors assessed through inhibition of a pre-titrated papain preparation (Zamora et al., 2005; Zamora et al., 1996). These serine protease inhibitors are mainly composed of cross-class inhibitory serpins able to inhibit strongly and pseudo-irreversibly initiator and effector caspases as Bov-SerpinA3-1 to A3-6 and would be good candidates for accurate predictors of meat tenderness. Highest levels of serpins inhibiting caspases are thus observed in toughest meat (Zamora et al., 2005; Zamora et al., 1996).

- ***Possible role in 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 myoblast are protected against cell death by different still unclear mechanisms. Some authors suggested that this protection is mediated by thrombin (Chinni et al., 1999), a trypsin-like serine peptidase expressed in muscle cells (Citron et al., 1997) and decreasing significantly the number of apoptotic cells in culture performed in the presence of this peptidase (Chinni et al., 1999).

Because bovine SERPINA3s are strong inhibitors of initiator and executioner caspases, their expression in differentiating primary myoblasts was followed by immunohistochemistry using the anti-Bov-SerpinA3-1 antibody. As depicted in Figure 9, bovine SERPINA3s are expressed in proliferating (Figure 9a) and in confluent (Figure 9b) myoblasts but not in differentiated myotubes (Figure 9c). In figure 9c, we often observed a reminiscent fluorescence which can be very likely ascribed to myotubes which are still under differentiation. According to (Chinni et al., 1999), myoblasts exposed to thrombin, for a very short period, accumulated apoptosis inhibitory activity preventing cell death for the subsequent growth in the absence of thrombin suggesting that the protective action of thrombin could be effected through the synthesis of an unknown apoptosis inhibitory factor and more especially by serpins inhibiting caspases.

This observation therefore supports a real contribution of bovSERPINA3s to cell survival in differentiating primary myoblast, a result in good agreement with the expression pattern of these serpins during the different steps of the process.

CHARACTERIZATION OF ANTITHROMBIN III

Antithrombin III was purified from bovine skeletal muscle as previously described (Herrera-Mendez et al., 2010) and characterized. Identity of the purified serpin was first assessed using different technical approaches including N-terminal sequencing, immunoblotting using a polyclonal antibody raised against human antithrombin III and mass spectrometry peptide map. Primary sequence analysis using the Edman procedure provided the following sequence: H^{1}RSPVEDVCTAKPR^{14}. Similarity searches in databanks with this N-terminal sequence were carried out using BlastP2 (Bork Group, EMBL, Heidelberg). This sequence showed 100% similarity with the cDNA deduced sequence of the mature bovine AT-III (SwisProt accession number: P41361), a serpin inhibiting specifically thrombin. This identity was further confirmed by the strong labeling of the purified muscle AT-III by a polyclonal antibody raised against human AT-III and by mass spectrometry trypsin peptide map (Herrera-Mendez et al., 2010). Muscle AT-III was then characterized for its enzyme specificity pattern through association rate constant determination, its cellular localization and the expression of mRNA transcripts in both muscle tissue and differentiating primary myoblast in culture (paper in preparation).

Stoichiometry of interaction and SDS-stable complexes with the target enzymes

To assess the stoichiometry of interaction (SI), trypsin (7 nM) and thrombin (9 nM) were titrated with the purified AT-III in a total volume of 200 µL. As shown in Figure 10a, complete trypsin and thrombin inhibition was achieved for AT-III concentrations of 7.09 nM (mean ± SD for five independent determinations, 7.03 ± 0.04 nM) 0.5 nM), and 9.10 nM, (mean ± SD for five independent determinations, 9.05 ± 0.06 nM) respectively. These results stressed forward that

AT-III inhibition of both trypsin and thrombin occurs through a 1/1 enzyme/inhibitor ratio (SI = 1). Hence, concentration of AT-III was routinely determined by titration of trypsin solution of known concentration.

This finding was comforted by the formation of a 1/1 complex upon SDS-PAGE (Figure 10b). In contrast to Bov-SerpinA3-1 no dimer was obtained in such conditions, a result agreeing well with the absence of dimers for some isoform of Bov-SerpinA3 (Tassy et al., 2005) and other serpins (Gettins, 2002) including the cowpox virus crmA (Dobo et al., 2006).

Association rate constant towards different cysteine and serine proteases

Association rate constant towards a large set of cysteine and serine proteases was determined. According to the results depictured in **Table 4,** muscle AT-III inhibits strongly trypsin and thrombin and to a lesser extent plasmin and chymotrypsin. Note that no inhibition was observed against all cysteine proteases tested including papain, cathepsins B & L, calpains 1 & 2 and caspases 3 & 8 as well as against some other serine proteases (elastase, cathepsin G, Urokinase, plasminogen activator). Regarding thrombin addition of heparin caused a 100 fold increase in the association rate from 1.8×10^5 to 2.3×10^7 $M^{-1}S^{-1}$.

Cellular localization and transcripts expression

As thrombin is expressed in muscle cells (Citron et al., 1997), the expression of its specific inhibitor antithrombin III (AT-III) could be expected an assumption supported by the immunohistolocalization of this serpin in all fibers of mouse skeletal muscle (Businaro et al., 1995). The work carried out in our group intended to verify this hypothesis.

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 immuno-histochemistry with the same antibody. As shown in Figure 11a, western blot analysis of crude muscle extracts subjected to SDS-PAGE always revealed one band with a Mr of about 58 kDa (Fig.11a, lane 1) and running as the purified AT-III (Fig. 11a, lane 2). 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 figure 11b 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. Within muscle fibers, AT-III labeling seemed to be often highly concentrated in the vicinity of the plasma membrane as compared to the inside cytosolic fibers area. When the primary AT-III antibody was omitted, no labeling was detected. These findings are wholly similar to those of (Businaro et al., 1995) who localized three different serum protease inhibitors in mouse skeletal muscle including AT-III. To attest this statement, we then looked for the expression of antithrombin in differentiating myoblast in culture and the expression of mRNA in muscle tissue and in differentiating primary myoblast.

In developing muscle, thrombin increase the number of cells during myoblast proliferation (Kelvin et al., 1989); (Suidan et al., 1996); (Guttridge et al., 1997); (Chinni et al., 1999) but delayed skeletal muscle myogenesis through inhibition of myoblast fusion (Suidan et al., 1996); (Chinni et al., 1999). To test this statement and to comfort our 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 using 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 experiments carried out, the cell nuclei were stained in red using the Hoechst method. As depicted in Figure 12a, 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 myoblastes (Fig 12b), fusing myoblastes and myotubes (Fig 12c). Despite the common properties shared by Protease nexin-1 (PN-1 or SERPINE2) and AT-III, PN-1 does not cross-react immunologically with AT-III (Farrell et al., 1986). Hence it can be ascertained that the labeled protein in the myoblast culture is AT-III and not PN-I.

As plasma antithrombin 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 Antithrombin III is expressed in bovine liver (www.ncbi.nhm.gov). cDNAs obtained by reverse transcription from bovine liver and diaphragm were probed by nested PCR using specific sets of primers designed from the bovine AT-III gene sequence (GeneBank, AC : 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 7 bases downstream and must amplified a 2463 bp fragment from genomic DNA and a 389 bp fragment from cDNA.

As indicated in Fig 12d, *lane m*, a nested fragment of approximately 400 bp was generated with liver and diaphragm cDNAs. The PCR conditions don't allow to amplify long fragments, superior to 2000 bp on genomic DNA. Upon direct sequencing, a complete identity between sequences of the amplified AT-III cDNA (NM_001034698) was found certifying that the bovine gene is effectively transcribed in this muscle.

As observed before on differentiating primary muscle cultured cells, AT-III is expressed when thrombin must be inactivated to allow the myoblast-myotube transition. The chronological expression of AT-III in muscle cells was confirmed by total RNA analysis, with the same probes than indicated above for skeletal muscle RNA, where mRNA transcripts are only found in the late stages of myoblast differentiation (confluence and fusion: fig. 12d, lane 2) and in myotubes (Fig. 12d, lane 3) but not in proliferating myoblastes (Fig. 12d, lane 1).

Taken together, these findings stressed the expression of AT-III in muscle cells and support that this serpin would be the primary regulator of thrombin activity in muscle, a function often wrongly ascribed to protease nexin-1 (Rosenberg and Damus, 1973); (Baker et al., 1980).

Biological functions in living and postmortem muscle

Besides the major role of thrombin in the vascular system reviewed by (Fenton, 1986) and (Mann, 1999), several extra-vascular functions have been reported since the 80's especially in the nervous system and in skeletal muscle. Regarding the nervous system, thrombin is assumed to modulate the shape of astrocytes (Cavanaugh et al., 1990); (Nelson and Siman, 1990) and to mediate neurite retraction in neuronal cells (Gurwitz and Cunningham, 1988); (Grand et al., 1989); (Suidan et al., 1992); (Jalink and Moolenaar, 1992). In muscle, thrombin is synthesized by muscle cells (Citron et al., 1997) and acts locally by contributing to synapse remodeling and elimination at the neuromuscular junction (Liu et al., 1994); (Zoubine et al., 1996). Its activation at the membrane level by the thrombin activator complex is strictly dependent on the translocation of phosphatidylserine phospholipids from the inner leaflet of the plasma membrane

to the outer leaflet (Majumder et al., 2005) through the action of diverse translocases (Bevers et al., 1996). In postmortem muscle, externalization of phosphatidylserine, an early hallmark of apoptosis, will trigger the activation of thrombin which will in turn rapidly disconnect nerve from muscle cells, a feature supported by the prompt *postmortem* decrease in the efficiency of low-voltage electrical stimulation which uses only the nervous system for the electrical field conduction (Ouali et al., 2006) (Herrera-Mendez et al., 2010).Therefore, thrombin might contributes efficiently to isolate dying cell from neighboring ones and from any other external signals, a major hallmark event of apoptotic cells.

In addition to its function in synapse plasticity, thrombin plays a major role in muscle cells differentiation. Thrombin thus increases the number of cells during myoblast proliferation but delayed skeletal muscle myogenesis through inhibition of myoblast fusion (Kelvin et al., 1989); (Suidan et al., 1996); (Guttridge et al., 1997); (Chinni et al., 1999). Thrombin has been even considered as a survival factor for proliferating myoblast through inhibition of apoptosis (Chinni et al., 1999). Our findings are in good agreement with the above two first functions of thrombin, namely myoblast proliferation enhancement and inhibition of myoblast fusion, thrombin/inhibitor ratio being high during proliferation (thrombin must be active) and low during the fusion phase (thrombin totally inhibited by AT-III) (See fig 12). Consideration of thrombin as a survival factor is however very doubtful since no direct evidence of any implication of this serine protease is actually available. Furthermore, survival of cells might be more probably ensured by inhibition of apoptosis through SERPINA3 like serpins which are able to inhibit pseudo-irreversibly caspases and in turn block the cell dying process (see above).

SERINE PROTEASE INHIBITORS FROM THE F3 FRACTION

Characterization of the serine protease inhibitors from this fraction is just starting and only preliminary results will be presented hereafter. From this fraction we previously purified and characterized a low Mr cysteine protease inhibitor binding tightly to cathepsin H ($k_{ass} \approx 3.7\ 10^4$ $M^{-1}S^{-1}$) and, though to a lower extent, cathepsin B (Bige et al., 1985).

Regarding serine proteinase inhibitors, two low Mr inhibitors designed spi-1 and spi-2were purified to homogeneity (fig 13a). Both are thermostable (stable between 40 and 90°C) and resistant to acidic and alkaline pH from 3 to 10. Spi-1 exhibits a Mr of 14.3 kDa and a pI value of 6.8 whereas spi-2 shows a Mr of 11.4 kDa and a pI value of 6.2 (fig 13b).

Both spi-1 and spi-2 are unable to inhibit all cysteine proteases tested including papain, cathepsins H, B and L as well as caspase 3. In contrast they can inhibit trypsin and, though to a lesser extent, chymotrypsin (Fig 14 a and b).

Much remains to be learned about these two low Mr spi and investigations are under progress to characterize their interaction with different serine proteases, to identify them at the protein level by mass spectrometry and to determine the association rate with potential target proteases.

DISCUSSION-CONCLUSION

Serine proteases were for a long time an enigma in meat science (for review, see (Ouali, 1990) and (Sentandreu et al., 2002). These included proteasome, first described by (Orlowski and Wilk, 1981) which also exhibits activities of the serine protease type. This proteolytic complex was shown to reproduce changes occurring in postmortem muscle and could contribute significantly

to intracellular protein degradation and, hence, to meat tenderization (Dutaud et al., 2006). Whether knowledge about the proteasome grew very fast in the next years, for other serine proteases, things were much less clear.

In the 1980's, serine proteases were first considered as only present in mast cells located in the extracellular matrix. 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 (Stauber et al., 1983a). Concomitantly these authors localized serine protease inhibitors within muscle cells (Stauber et al., 1983b). In 1986, we fractionated a series of inhibitors with a high affinity towards diverse serine proteases and located them within muscle cells (Ouali et al., 1986). 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, including kallikrein, thrombin, plasmin, protease nexin 1, plasminogen activator inhibitor or PAI, antithrombin III, kallistatin … (reviewed in (Sentandreu et al., 2002).

The present report provides some answers to the numerous questions about serine proteases and their inhibitors. The high Mr fractions (F1 and F2) 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 (Taylor et al., 2008). Taken together, these investigations suggest that there exists very likely a degree of compensation and redundancy within this serpin family wholly comparable to that reported for the set of caspases identified so far (Fuentes-Prior and Salvesen, 2004). The high Mr Fractions also contain antithrombin III, another serpin inhibiting strongly thrombin in an heparin

dependent manner and trypsin. All these protease have essential function in the apoptotic process, thrombin contributing to isolate the dying cells from the nervous system and caspases targeting several hundred proteins and primarily proteins from the cytoskeletal 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. Note that the high Mr fraction contains also a series of cysteine proteases inhibitors unable to inhibit serine proteases and not yet characterized (Ouali et al., 1995). Regarding the serine protease inhibitors purified from the F3 fraction, the reported results are preliminary findings and further investigations are needed in order to identify these protein inhibitors and their potential target proteases (Becila et al., 2010).

We recently hypothesized that after death, muscle will engage in the cell death program with apoptosis rather than necrosis being the probable candidate (Ouali et al., 2006); (Herrera-Mendez et al., 2006a). This hypothesis was confirmed more recently by (Ouali et al., 2007) and (Becila et al., 2010) who provide evidence of cell shrinkage starting few minutes after death and of the concomitant rapid actin degradation, a major protein of the transversal cytoskeletal filaments binding to the costameres and to the integrins, two findings supporting the onset of apoptosis immediately after animal death. In a recent review, (Kemp and Parr, 2012) emphasized this hypothesis and give a large set of evidence supporting it. The observation of a caspase 3/serpin complex in muscle extracts definitely ascertains that caspase 3 is totally active in postmortem muscle excised within one hour after death and, consequently, that muscle cells are already engaged in apoptosis.

However, despite the prominent progress in that domain, much remain to be done if we would expect to better understand the onset and progress of apoptosis in postmortem muscle and how the cell provide enough energy for the completion of that process. This will be a prerequisite to the identification of reliable biological markers of meat tenderness we are looking for since years.

ACKNOWLEDGEMENTS

This work would have not been possible without the Partenariat Hubert Curien (Franco-Algerian TASSILI project) which allowed regular visit to our laboratory of our colleagues from the University of Constantine (Algeria) during the four last years and funded the consumables. Thanks to the French and Algerian government.

REFERENCES

Archibald, A. L., Couperwhite, S., Mellink, C. H., Lahbib-Mansais, Y., and Gellin, J. (1996). Porcine alpha-1-antitrypsin (PI): cDNA sequence, polymorphism and assignment to chromosome 7q2.4- > q2.6. *Anim Genet.* **27**: 85-89.

Arrigo, A. P. (2005). Heat shock proteins as molecular chaperones. *M S-Medecine Sciences*. **21**: 619-625.

Baker, J. B., Low, D. A., Simmer, R. L., and Cunningham, D. D. (1980). Protease-nexin: a cellular component that links thrombin and plasminogen activator and mediates their binding to cells. *Cell*. **21**: 37-45.

Becila, S., Herrera-Mendez, C. H., Coulis, G., Labas, R., Astruc, T., Picard, B., Boudjellal, A., Pelissier, P., Bremaud, L., and Ouali, A. (2010). Postmortem muscle cells die through apoptosis. *European Food Research and Technology*. **231**: 485-493.

Beere, H. M. (2005). Death versus survival: functional interaction between the apoptotic and stress-inducible heat shock protein pathways. *Journal of Clinical Investigation*. **115**: 2633-2639.

Bevers, E. M., Comfurius, P., and Zwaal, R. F. (1996). Regulatory mechanisms in maintenance and modulation of transmembrane lipid asymmetry: pathophysiological implications. *Lupus*. **5**: 480-487.

Bieth, J. G. (1980). Pathophysiological interpretation of kinetic constants of protease inhibitors. *Bull Eur Physiopathol Respir*. **16**: 183-197.

Bige, L., Ouali, A., and Valin, C. (1985). Purification and characterization of a low molecular weight cysteine proteinase inhibitor from bovine muscle. *Biochim Biophys Acta*. **843**: 269-275.

Billingsley, G. D., Walter, M. A., Hammond, G. L., and Cox, D. W. (1993). Physical mapping of four serpin genes: alpha 1-antitrypsin, alpha 1-antichymotrypsin, corticosteroid-binding globulin, and protein C inhibitor, within a 280-kb region on chromosome I4q32.1. *Am J Hum Genet*. **52**: 343-353.

Blanchet, X., Pere-Brissaud, A., Duprat, N., Pinault, E., Delourme, D., Ouali, A., Combet, C., Maftah, A., Pelissier, P., and Bremaud, L. (2012). Mutagenesis of the bovSERPINA3-3 demonstrates the requirement of aspartate-371 for intermolecular interaction and formation of dimers. *Protein Sci*. **21**: 977-986.

Businaro, R., Nori, S. L., Toesca, A., De Renzis, G., Ortolani, F., De Santis, E., and Fumagalli, L. (1995). Immunohistochemical detection of three serum protease inhibitors in mouse skeletal muscle by confocal laser scanning microscopy. *Ital J Anat Embryol*. **100 Suppl 1**: 123-130.

Cassens, U., Lewinski, G., Samraj, A. K., von Bernuth, H., Baust, H., Khazaie, K., and Los, M. (2003). Viral modulation of cell death by inhibition of caspases. *Arch Immunol Ther Exp (Warsz)*. **51**: 19-27.

Cavanaugh, K. P., Gurwitz, D., Cunningham, D. D., and Bradshaw, R. A. (1990). Reciprocal modulation of astrocyte stellation by thrombin and protease nexin-1. *J Neurochem*. **54**: 1735-1743.

Chinni, C., de Niese, M. R., Tew, D. J., Jenkins, A. L., Bottomley, S. P., and Mackie, E. J. (1999). Thrombin, a survival factor for cultured myoblasts. *J Biol Chem*. **274**: 9169-9174.

Citron, B. A., Smirnova, I. V., Zoubine, M. N., and Festoff, B. W. (1997). Quantitative PCR analysis reveals novel expression of prothrombin mRNA and regulation of its levels in developing mouse muscle. *Thromb Res*. **87**: 303-313.

De Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R., and Appelmans, F. (1955). Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. *Biochem J*. **60**: 604-617.

Dean, E. J., Ranson, M., Blackhall, F., Holt, S. V., and Dive, C. (2007). Novel therapeutic targets in lung cancer: Inhibitor of apoptosis proteins from laboratory to clinic. *Cancer Treat Rev*. **33**: 203-212.

Deveraux, Q. L., and Reed, T. C. (1999). IAP family proteins - suppressors of apoptosis. *Genes & Development*. **13**: 239-252.

Dobo, J., Swanson, R., Salvesen, G. S., Olson, S. T., and Gettins, P. G. (2006). Cytokine response modifier a inhibition of initiator caspases results in covalent complex formation and dissociation of the caspase tetramer. *J Biol Chem*. **281**: 38781-38790.

Dutaud, D., Aubry, L., Sentandreu, M. A., and Ouali, A. (2006). Bovine Muscle 20s Proteasome: I. Simple Purification Procedure and Enzymatic Characterization in Relation With Postmortem Conditions. *Meat Science*. **74**: 337-344.

Elliott, P. R., Pei, X. Y., Dafforn, T. R., and Lomas, D. A. (2000). Topography of a 2.0 A structure of alpha1-antitrypsin reveals targets for rational drug design to prevent conformational disease. *Protein Sci*. **9**: 1274-1281.

Engh, R., Lobermann, H., Schneider, M., Wiegand, G., Huber, R., and Laurell, C. B. (1989). The S variant of human alpha 1-antitrypsin, structure and implications for function and metabolism. *Protein Eng*. **2**: 407-415.

Farrell, D. H., Van Nostrand, W. E., and Cunningham, D. D. (1986). A simple two-step purification of protease nexin. *Biochem J*. **237**: 907-912.

Fenton, J. W. (1986). Thrombin. *Annals of the New York Academy of Sciences*. **485**: 5-15.

Forsyth, S., Horvath, A., and Coughlin, P. (2003). A review and comparison of the murine alpha1-antitrypsin and alpha1-antichymotrypsin multigene clusters with the human clade A serpins. *Genomics*. **81**: 336-345.

Fuentes-Prior, P., and Salvesen, G. S. (2004). The protein structures that shape caspase activity, specificity, activation and inhibition. *Biochem J*. **384**: 201-232.

Gagaoua, M., Boudida, Y., Becila, S., Picard, B., Boudjellal, A., Sentandreu, M. A., and Ouali, A. (2012). New Caspases' inhibitors belonging to the serpin superfamily: a novel key control point of apoptosis in mammalian tissues. *Adv Biosci Biotechnol***. in press**

Gettins, P. G. (2002). Serpin structure, mechanism, and function. *Chem Rev*. **102**: 4751-4804.

Grand, R. J., Grabham, P. W., Gallimore, M. J., and Gallimore, P. H. (1989). Modulation of morphological differentiation of human neuroepithelial cells by serine proteases: independence from blood coagulation. *Embo J*. **8**: 2209-2215.

Guroff, G. (1964). A Neutral, Calcium-activated Proteinase from the Soluble Fraction of Rat Brain. *Journal of Biological Chemistry*. **239**: 149-155.

Gurwitz, D., and Cunningham, D. D. (1988). Thrombin modulates and reverses neuroblastoma neurite outgrowth. *Proc Natl Acad Sci U S A*. **85**: 3440-3444.

Guttridge, D. C., Lau, A., Tran, L., and Cunningham, D. D. (1997). Thrombin causes a marked delay in skeletal myogenesis that correlates with the delayed expression of myogenin and p21CIP1/WAF1. *J Biol Chem*. **272**: 24117-24120.

Herrera-Mendez, C. H., Becila, S., Blanchet, X., Pelissier, P., Delourme, D., Coulis, G., Sentandreu, M. A., Boudjellal, A., Bremaud, L., and Ouali, A. (2009). Inhibition of human initiator caspase 8 and effector caspase 3 by cross-class inhibitory bovSERPINA3-1 and A3-3. *FEBS Lett*. **583**: 2743-2748.

Herrera-Mendez, C. H., Becila, S., Boudjellal, A., and Ouali, A. (2006a). Meat ageing : Reconsideration of the current concept. *Trends in Food Science and Technology*. **17**: 394-405.

Herrera-Mendez, C. H., Becila, S., Coulis, G., Sentandreu, M. A., Aubry, L., and Ouali, A. (2010). Purification and partial characterization of antithrombin III from bovine skeletal muscle and possible role of thrombin in postmortem apoptosis development and in efficiency of low voltage electrical stimulation. *Food Research International*. **43**: 356-363.

Herrera-Mendez, C. H., Bremaud, L., Coulis, G., Pelissier, P., Sentandreu, M. A., Aubry, L., Delourme, D., Chambon, C., Maftah, A., Leveziel, H., and Ouali, A. (2006b). Purification of the skeletal muscle protein Endopin 1B and characterization of the genes encoding Endopin 1A and 1B isoforms. *FEBS Lett*. **580**: 3477-3484.

Horvath, A. J., Forsyth, S. L., and Coughlin, P. B. (2004). Expression patterns of murine antichymotrypsin-like genes reflect evolutionary divergence at the serpina3 locus. *J Mol Evol*. **59**: 488-497.

Horvath, A. J., Lu, B. G., Pike, R. N., and Bottomley, S. P. (2011). Methods to measure the kinetics of protease inhibition by serpins. *Methods Enzymol*. **501**: 223-235.

Huntington, J. A., Read, R. J., and Carrell, R. W. (2000). Structure of a serpin-protease complex shows inhibition by deformation. *Nature*. **407**: 923-926.

Jalink, K., and Moolenaar, W. H. (1992). Thrombin receptor activation causes rapid neural cell rounding and neurite retraction independent of classic second messengers. *J Cell Biol*. **118**: 411-419.

Jeremiah, L. E., and Gibson, L. L. (2003). The effects of postmortem product handling and aging time on beef palatability. *Food Research International*. **36**: 929-941.

Kelvin, D. J., Simard, G., Sue, A. Q. A., and Connolly, J. A. (1989). Growth factors, signaling pathways, and the regulation of proliferation and differentiation in BC3H1 muscle cells. II. Two signaling pathways distinguished by pertussis toxin and a potential role for the ras oncogene. *J Cell Biol*. **108**: 169-176.

Kemp, C. M., and Parr, T. (2012). Advances in apoptotic mediated proteolysis in meat tenderisation. *Meat Sci*. **92**: 252-259.

Kemp, C. M., Sensky, P. L., Bardsley, R. G., Buttery, P. J., and Parr, T. (2010). Tenderness--an enzymatic view. *Meat Sci*. **84**: 248-256.

Lamkanfi, M., Festjens, N., Declercq, W., Vanden Berghe, T., and Vandenabeele, P. (2007). Caspases in cell survival, proliferation and differentiation. *Cell Death Differ*. **14**: 44-55.

Law, R. H., Zhang, Q., McGowan, S., Buckle, A. M., Silverman, G. A., Wong, W., Rosado, C. J., Langendorf, C. G., Pike, R. N., Bird, P. I., and Whisstock, J. C. (2006). An overview of the serpin superfamily. *Genome Biol*. **7**: 216.

Liu, Y., Fields, R. D., Festoff, B. W., and Nelson, P. G. (1994). Proteolytic Action of Thrombin Is Required for Electrical Activity-Dependent Synapse Reduction. *Proc Natl Acad Sci U S A*. **91**: 10300-10304.

Majumder, R., Weinreb, G., and Lentz, B. R. (2005). Efficient thrombin generation requires molecular phosphatidylserine, not a membrane surface. *Biochemistry*. **44**: 16998-17006.

Mann, K. G. (1999). Biochemistry and physiology of blood coagulation. *Thromb Haemost*. **82**: 165-174.

Musilova, P., Lahbib-Mansais, Y., Yerle, M., Cepica, S., Stratil, A., Coppieters, W., and Rubes, J. (1995). Assignment of pig alpha 1-antichymotrypsin (AACT or PI2) gene to chromosome region 7q23-q26. *Mamm Genome*. **6**: 445.

Nelson, R. B., and Siman, R. (1990). Thrombin and its inhibitors regulate morphological and biochemical differentiation of astrocytes in vitro. *Brain Res Dev Brain Res*. **54**: 93-104.

O'Riordan, M. X., Bauler, L. D., Scott, F. L., and Duckett, C. S. (2008). Inhibitor of apoptosis proteins in eukaryotic evolution and development: a model of thematic conservation. *Dev Cell*. **15**: 497-508.

Olson, S. T., and Gettins, P. G. (2011). Regulation of proteases by protein inhibitors of the serpin superfamily. *Prog Mol Biol Transl Sci*. **99**: 185-240.

Orlowski, M., and Wilk, S. (1981). A multicatalytic protease complex from pituitary that forms enkephalin and enkephalin containing peptides. *Biochem Biophys Res Commun*. **101**: 814-822.

Ouali, A. (1990). Meat Tenderization: Possible Causes and Mechanisms. A Review. *Journal of Muscle Foods*. **1**: 129-165.

Ouali, A., Bige, L., Obled, A., Lacourt, A., and Valin, C. (1986). Small and high molecular weigth protease inhibitors from bovine skeletal muscle.in Cysteine Proteinases and their Inhibitors. *Walter de Gruyter and Co., Berlin (Turk, V., ed.),*: 545-554.

Ouali, A., Herrera-Mendez, C. H., Coulis, G., Becila, S., Boudjellal, A., Aubry, L., and Sentandreu, M. A. (2006). Revisiting the conversion of muscle into meat and the underlying mechanisms. *Meat Science*. **74**: 44-58.

Ouali, A., Herrera-Mendez, C. H., Coulis, G., Samira, B., Boudjellal, A., Harhoura, K., Aubry, L., and Sentandreu, M. A. (2007). Meat tenderisation and muscle cell death, two highly related events. *Tehnologija Mesa*. **48**: 1-15.

Ouali, A., Rouchon, P., Zabari, M., Berri, M., Zamora, F., and Tassy, C. (1995). Natural serine and cysteine proteinase inhibitors in bovine skeletal muscle. *In Expression of tissue proteinases and regulation of protein degradation as related to meat quality Edited by: Ouali A, Demeyer D, Smulders FJM. Utrecht (NL): ECCEAMST,*: 173 - 198.

Ouali, A., and Talmant, A. (1990). Calpains and calpastatin distribution in bovine, porcine and ovine skeletal muscles. *Meat Sci*. **28**: 331-348.

Pejler, G., Abrink, M., Ringvall, M., and Wernersson, S. (2007). Mast cell proteases. *Adv Immunol*. **95**: 167-255.

Pelissier, P., Delourme, D., Germot, A., Blanchet, X., Becila, S., Maftah, A., Leveziel, H., Ouali, A., and Bremaud, L. (2008). An original SERPINA3 gene cluster: elucidation of genomic organization and gene expression in the Bos taurus 21q24 region. *BMC Genomics*. **9**: 151.

Philchenkov, A. (2004). Caspases: potential targets for regulating cell death. *J Cell Mol Med*. **8**: 432-444.

Rosenberg, R. D., and Damus, P. S. (1973). The purification and mechanism of action of human antithrombin-heparin cofactor. *J Biol Chem*. **248**: 6490-6505.

Schechter, N. M., and Plotnick, M. I. (2004). Measurement of the kinetic parameters mediating protease-serpin inhibition. *Methods*. **32**: 159-168.

Sentandreu, M. A., Coulis, G., and Ouali, A. (2002). Role of muscle endopeptidases and their inhibitors in meat tenderness. *Trends in Food Science & Technology*. **13**: 400-421.

Shackelford, S. D., Koohmaraie, M., Miller, M. F., Crouse, J. D., and Reagan, J. O. (1991). An evaluation of tenderness of the longissimus muscle of Angus by Hereford versus Brahman crossbred heifers. *J Anim Sci*. **69**: 171-177.

Silverman, G. A., Bird, P. I., Carrell, R. W., Church, F. C., Coughlin, P. B., Gettins, P. G., Irving, J. A., Lomas, D. A., Luke, C. J., Moyer, R. W., Pemberton, P. A., Remold-O'Donnell, E., Salvesen, G. S., Travis, J., and Whisstock, J. C. (2001). The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. Evolution, mechanism of inhibition, novel functions, and a revised nomenclature. *J Biol Chem*. **276**: 33293-33296.

Silverman, G. A., Whisstock, J. C., Bottomley, S. P., Huntington, J. A., Kaiserman, D., Luke, C. J., Pak, S. C., Reichhart, J. M., and Bird, P. I. (2010). Serpins flex their muscle: I. Putting the clamps on proteolysis in diverse biological systems. *J Biol Chem*. **285**: 24299-24305.

Stauber, W. T., Fritz, V., Dahlmann, B., and Reinauer, H. (1983a). Immunohistochemical localization of two proteinases in skeletal muscle. *J Histochem Cytochem*. **31**: 827-830.

Stauber, W. T., Ong, S. H., Fritz, V. K., Esnard, F., and Gauthier, F. (1983b). Protease inhibitor localization in control and streptozotocin-diabetic skeletal muscles. *Histochem J*. **15**: 1079-1086.

Stennicke, H. R., Ryan, C. A., and Salvesen, G. S. (2002). Reprieval from execution: the molecular basis of caspase inhibition. *Trends Biochem Sci*. **27**: 94-101.

Suidan, H. S., Niclou, S. P., Dreessen, J., Beltraminelli, N., and Monard, D. (1996). The thrombin receptor is present in myoblasts and its expression is repressed upon fusion. *Journal of Biological Chemistry*. **271**: 29162-29169.

Suidan, H. S., Stone, S. R., Hemmings, B. A., and Monard, D. (1992). Thrombin Causes Neurite Retraction in Neuronal Cells through Activation of Cell-Surface Receptors. *Neuron*. **8**: 363-375.

Swanson, R., Raghavendra, M. P., Zhang, W. Q., Froelich, C., Gettins, P. G. W., and Olson, S. T. (2007). Serine and cysteine proteases are translocated to similar extents upon formation of covalent complexes with serpins - Fluorescence perturbation and fluorescence resonance energy transfer mapping of the protease binding site in CrmA complexes with granzyme B and caspase-1. *Journal of Biological Chemistry*. **282**: 2305-2313.

Tassy, C. (1998). Purification et caractérisation d'un inhibiteur de sérine protéinases à partir de muscle squelettique de bovin. *PhD Thesis, EPHE, Paris.*

Tassy, C., Herrera-Mendez, C. H., Sentandreu, M. A., Aubry, L., Bremaud, L., Pelissier, P., Delourme, D., Brillard, M., Gauthier, F., Leveziel, H., and Ouali, A. (2005). Muscle endopin 1, a muscle intracellular serpin which strongly inhibits elastase: purification, characterization, cellular localization and tissue distribution. *Biochem J*. **388**: 273-280.

Taylor, R. C., Cullen, S. P., and Martin, S. J. (2008). Apoptosis: controlled demolition at the cellular level. *Nature Reviews Molecular Cell Biology*. **9**: 231-241.

Whisstock, J. C., Silverman, G. A., Bird, P. I., Bottomley, S. P., Kaiserman, D., Luke, C. J., Pak, S. C., Reichhart, J. M., and Huntington, J. A. (2010). Serpins flex their muscle: II. Structural insights into target peptidase recognition, polymerization, and transport functions. *J Biol Chem*. **285**: 24307-24312.

Ye, S., Cech, A. L., Belmares, R., Bergstrom, R. C., Tong, Y., Corey, D. R., Kanost, M. R., and Goldsmith, E. J. (2001). The structure of a Michaelis serpin-protease complex. *Nat Struct Biol*. **8**: 979-983.

Zamora, F., Aubry, L., Sayd, T., Lepetit, J., Lebert, A., Sentandreu, M. A., and Ouali, A. (2005). Serine peptidase inhibitors, the best predictor of beef ageing amongst a large set of quantitative variables. *Meat Sci*. **71**: 730-742.

Zamora, F., Debiton, E., Lepetit, J., Lebert, A., Dransfield, E., and Ouali, A. (1996). Predicting variability of ageing and toughness in beef M. Longissimus lumborum et thoracis. *Meat Sci*. **43**: 321-333.

Zoubine, M. N., Ma, J. Y., Smirnova, I. V., Citron, B. A., and Festoff, B. W. (1996). A molecular mechanism for synapse elimination: novel inhibition of locally generated thrombin delays synapse loss in neonatal mouse muscle. *Dev Biol*. **179**: 447-457.

TABLES

Table 1. Stoichiometry of interaction of Bov-serpins with the target enzymes: Bovine pancreatic trypsin, Human Leukocyte Elastase (HLE), human recombinant caspases 3 and 8.

	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[(1)]	1.01 ± 0.07	0.55 ± 0.07
Caspase 8[(1)]	0.49 ± 0.09	0.51 ± 0.06
[(1)] SI= [I]/[Caspase active sites]		

Table 2. Inhibitory pattern of bovSERPINA3-1 and A3-3 and association rate constant (k_{ass} in $M^{-1}s^{-1}$) against a large set of cysteine and serine proteases tested. (NI: no inhibition detected). In bold style are the proteases strongly inhibited by the serpins; in normal style are the proteases for which the inhibition is of no physiological significance; in italic style are the non-inhibited proteases.

PROTEASES		Association rate constant (k_{ass}) ($M^{-1}s^{-1}$)	
		bovSERPINA A3-1	bovSERPINA A3-3
Serine Proteases	**Trypsin**	**3.9×10^6**	**6.7×10^5**
	Chymotrypsin	1.0×10^2	9.0×10^2
	Plasmin	1.8×10^3	2.7×10^3
	Elastase	**2.4×10^7**	**1.3×10^6**
	Cathepsin G	*NI*	*NI*
	Kallikrein	*NI*	*NI*
	Urokinase	*NI*	*NI*
	Plasminogen activator	*NI*	*NI*
	Thrombin	*NI*	*NI*
Cysteine Proteases	*Papain*	*NI*	*NI*
	Cathepsin B	*NI*	*NI*
	Cathepsin L	*NI*	*NI*
	Calpains 1 & 2	*NI*	*NI*
	Caspase 3	**4.2×10^5**	**1.5×10^5**
	Caspase 8	**1.4×10^6**	**2.7×10^6**

Table 3. ELISA (Enzyme Linked ImmunoSorbant Assay) quantification of Bov-SerpinA3-1 in different bovine fluids and tissues. **"**Present NQ", means that the serpin was detected by other methods but not quantified by ELISA (Tassy et al., 2005).

ELISA Quantification of Bov-	
Fluids/Tissues	[Bov-SerpinA3-1]
Plasma	1,0 mg/ml
Liver	14 µg/g wet tissue
Kidney	2,0 µg/g wet
Muscle	1,0 µg/g wet
Thymus	Present NQ
Spleen	Present NQ

Table 4. Inhibitory pattern of bovine ATIII and association rate constant (k_{ass} in $M^{-1}s^{-1}$) against a large set of cysteine and serine proteases tested. In italic style are the non-inhibited proteases (NI).

	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 (5µM)	**2.3×10^7**
Elastase	none	NI
Cathepsin G	none	NI
Kallikrein	none	NI
Urokinase	none	NI
Plasminogen activator	none	NI

FIGURE LEGENDS

Figure 1: Elution profile of a muscle crude extract from a Sephadex G100 superfine column (5x100 cm) and profile of antitrypsin-activity (Herrera-Mendez et al., 2006b).

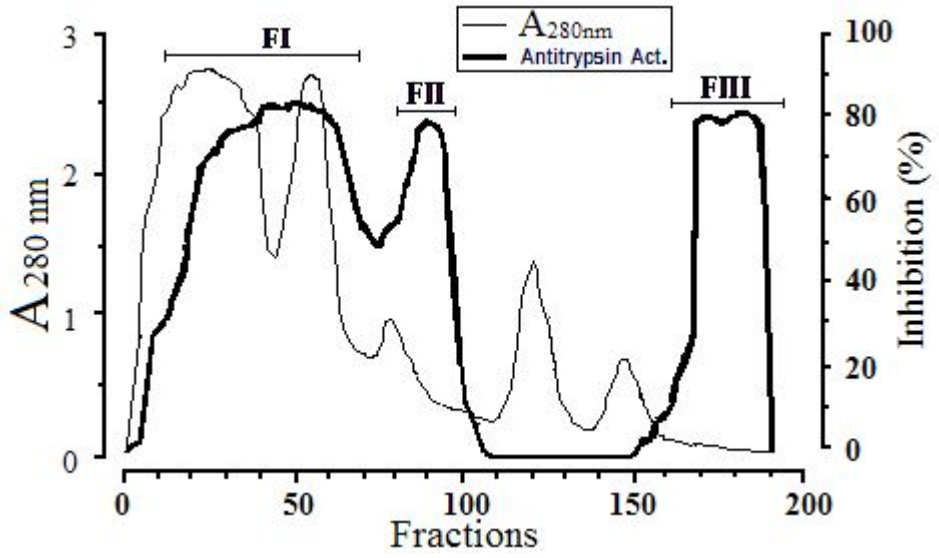

Figure 2: Serpin structures and conformation. (a), native α_1-AT (Protein Data Bank (PDB) entry 1QLP) (Elliott et al., 2000); (b), cleaved α_1-AT (PDB entry 7API) Upon RCL cleavage, the loop inserts into the serpin core constituting an additional strand (Engh et al., 1989); (c), Michaelis complex between Serpin A1 (Alaserpin from *Manduca sexta*) and trypsin (PDB entry 1I99 (Ye et al., 2001); (d), covalent complex between α_1-AT 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 (Huntington et al., 2000). In (b) and (d) the inserted RCL is in purple. AT, antitrypsin or SERPINA1.

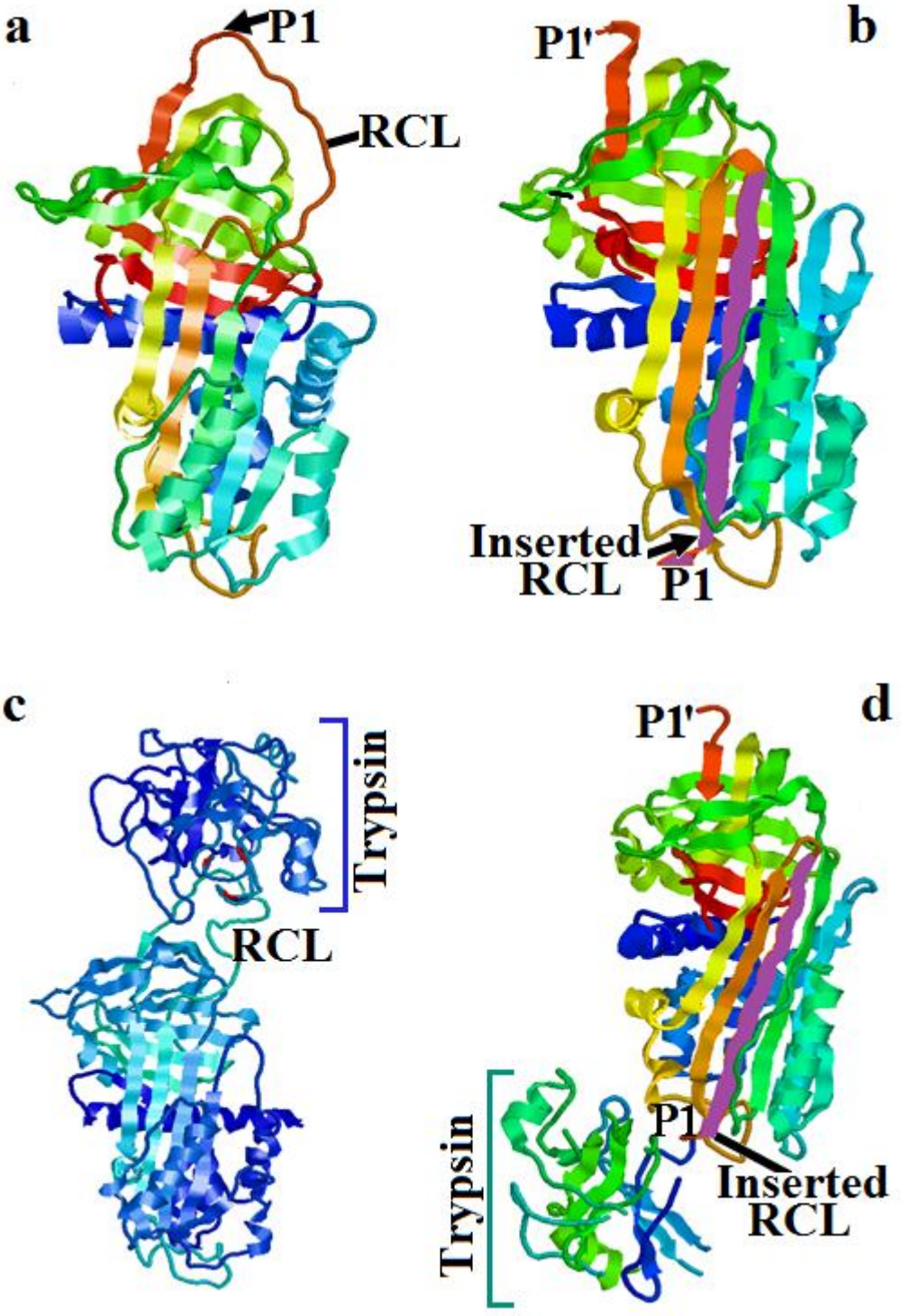

Figure 3. Covalent complexes upon SDS-PAGE between Bov-SerpinA3-1 and proteases as revealed by western blot, using the rabbit polyclonal antibody raised against this serpin (Herrera-Mendez et al., 2009). Similar findings were obtained with Bov-Serpin A3-3 (not shown). Note that the 140 kDa band is a dimer of the 70 kDa serpin (Tassy et al., 2005). C, band corresponding to the complex formed with the different target enzymes: trypsin, Human Leukocyte Elastase (HLE) and human recombinant caspases 3 and 8. (-) inhibitor alone; (+) inhibitor incubated with the target enzyme (Enz).

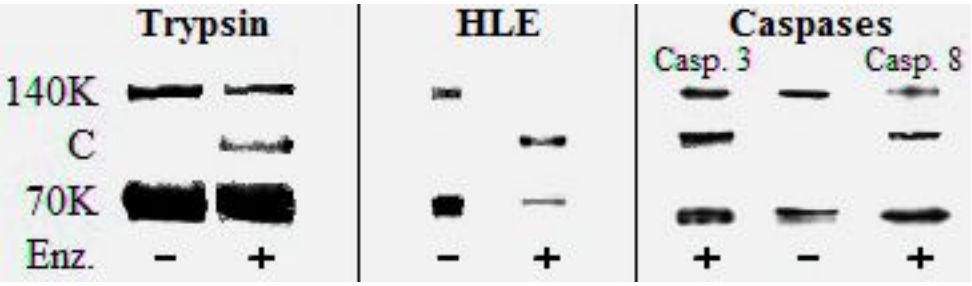

Figure 4. Cellular localization of Bov-SerpinA3-1 in transversal cuts of bovine skeletal muscle (a) and in primary bovine myoblast culture (b) using the rabbit polyclonal antibody raised against the purified serpin. Inserts of Figure4a and 4b are control samples where the primary antibody has been omitted.

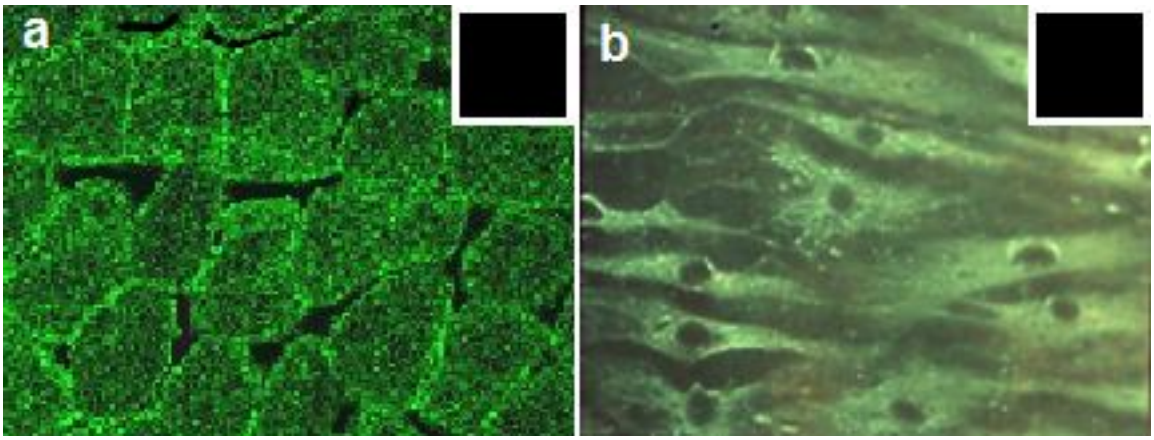

Figure 5: Polymorphism of the Bov-Serpin family as assessed by western blot: (a) Elution profile of a muscle crude extract from a SP-Sepharose column [18]. (b) Western blot analysis of the fractions eluted throughout the NaCl gradient (fraction number under each lane).Proteins were revealed using the rabbit polyclonal antibody raised against Bov-serpinA3-1. As a control the purified Bov-SerpinA3-1 (70 kDa) was loaded in the first well (A3-1).

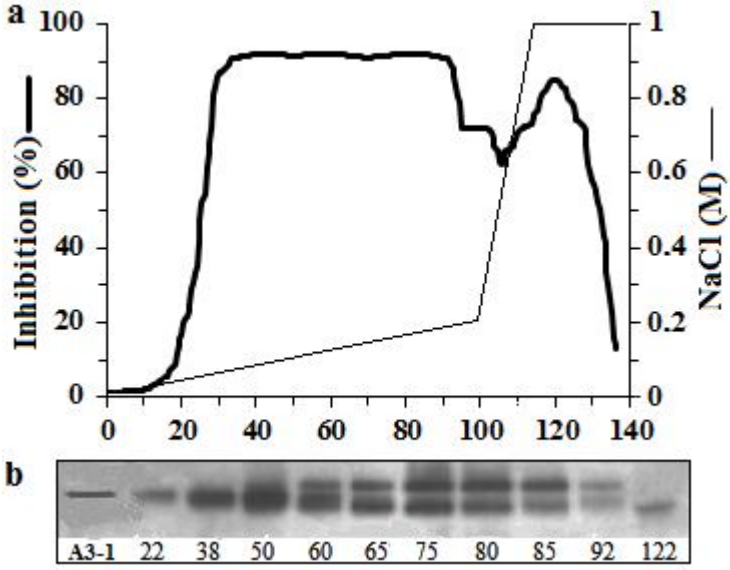

Figure 6: Polymorphism of the Bov-SERPINA3 family as assessed by 2D gel electrophoresis of fractions F1 and F2 identified in Figure 1. (a) 2D Gel analysis of the F1 fraction. (b) 2D gel analysis of fraction F2. Proteins were revealed by western blot using the rabbit polyclonal antibody raised against Bov-SerpinA3-1. In (a) and (b), the line with a down arrowhead indicates aligned spots corresponding to various degree of phosphorylation. Black arrows indicate a comma shape alignment of spots corresponding to different degree of glycosylation. In (b), open arrows designed new spots of slightly lower Mr not observed in the F1fraction or more abundant in F2.

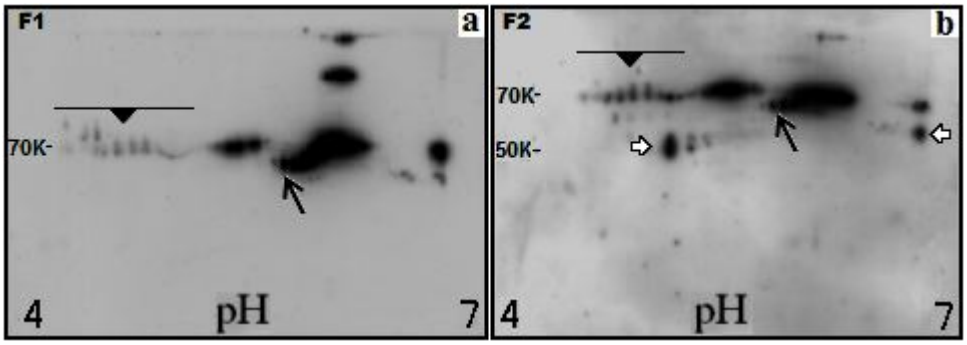

Figure 7: Alignment of RCL sequences of bovSERPINA3-1 to bovSERPINA3-8 obtained from coding gene sequences (Pelissier et al., 2008). (a) Reactive Centre Loop (RCL) sequences of bovSERPINA3-1 and A3-3 used throughout this work. (b) RCL sequence with highest similarities with bovSERPINA3-1. (c) RCL sequence of the most divergent bovSERPINA3-7 and A3-8. Residues in white bold type indicate P1 residues for trypsin (Arg^{16}) and putative P1 residue for caspases 3 and 8 (Asp^{37} for A3-1 to A3-6; Glu^{39} and Asp^{39} for A3-7 and A3-8).

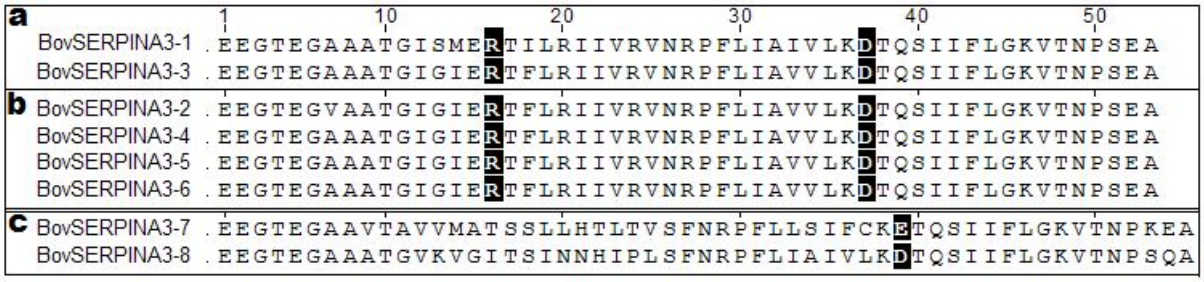

Figure 8. Schematic diagram of apoptosis regulation and complex detection in crude muscle extracts.

(A) Schematic and simplified diagram summarizing the major regulation points of the apoptotic process (adapted from (Philchenkov, 2004). With regards to the extrinsic pathway (**Path 1**), stimuli will bind to the death receptor inducing the activation of caspases 8 and 10 by association with their activator complexes (DISC or death-inducing signaling complex and CARD or caspase-recruitment domain). Besides the activation of effector caspases, caspase 8 will cleave Bid, a pro-apoptotic member of the Bcl2 protein family, which is at the cross between the intrinsic and extrinsic pathways. Cleaved Bid (tBid) will then initiate the contribution of mitochondria to the cell death process. Activation of caspase 8 by the activator complex can be lowered down by FLIP (FLICE inhibitory protein) a protein competing for binding to the activator complex. Activated caspase 8 will then in turn activate executioner caspases 3 and 7. The apoptotic status of the cell will be comforted by the release from mitochondria of diverse pro-apoptotic components including cytochrome c, a necessary member of the apoptosome complex responsible of the procaspase 9 activation. For more detailed explanations, see the review of (Ouali et al., 2006).

The only one inhibitor of caspase and hence of apoptosis so far identified in mammals are IAPs (Inhibitor of apoptosis proteins) which targeted initiator caspase 9 and effector caspase 3 and 7 (O'Riordan et al., 2008). If these steps failed, the process will continue to its end and lead to cell death and dismantling. The new control pathway reported here, namely the pseudo-irreversible inhibition of caspase by dedicated serpins, affect different step of the apoptotic process. The first control point (1) is the inhibition of initiator caspases, i.e. caspase 8 and probably also caspase

10. The second (2) is the probable inhibition of caspase 9, another initiator caspase. The third and last point is the inhibition of caspase 3 and, according to recent findings, caspase 7.

In the present diagram, we introduce a third path to emphasize the major role of Heat Shock Proteins (HSPs). These proteins are generally expressed as soon as a cell undergoes a stress to preserve all cell functions by protecting their targets proteins from any structural and/or activity loss. HSPs may have therefore diverse anti-apoptotic actions by protecting the target protein substrates from hydrolysis by caspases and other any proteolytic system of concern and by forming complexes with diverse caspases at different stage of the process thus hindering their function (Arrigo, 2005; Beere, 2005).

(B), Western blot analysis of at death muscle extracts using a rabbit anti-Bov-SerpinA3-1 (lane 1) and anti-caspase 3 polyclonal antibody (lane 2). The complex is revealed with both antibodies emphzising the caspase activation in postmortem muscle and the caspase-serpin complex formation.

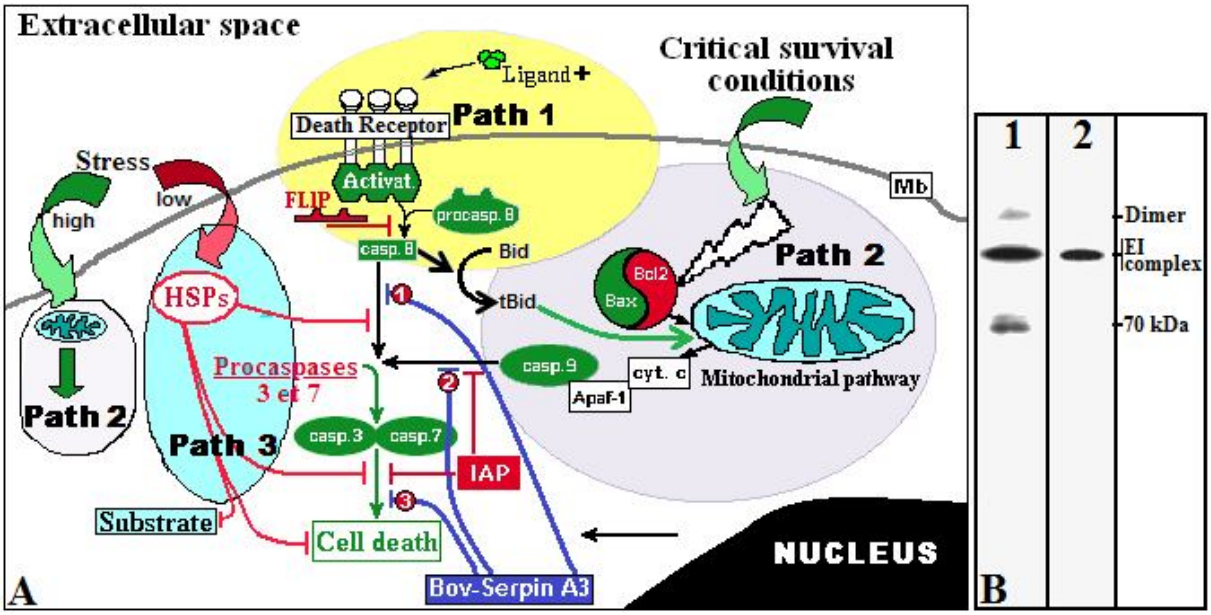

Figure 9. Expression of Bov-SerpinA3 during primary myoblast differentiation. (a) proliferating myoblasts; (b) confluent myoblasts; (c) differentiated myoblasts (myotube); (d) control with omitted primary antibody. Expression of these serpins was revealed by immunohistochemistry using the polyclonal rabbit antibody raised against Bov-SerpinA3-1 as the primary antibody and a FITC labelled anti-rabbit IgG as the secondary antibody.

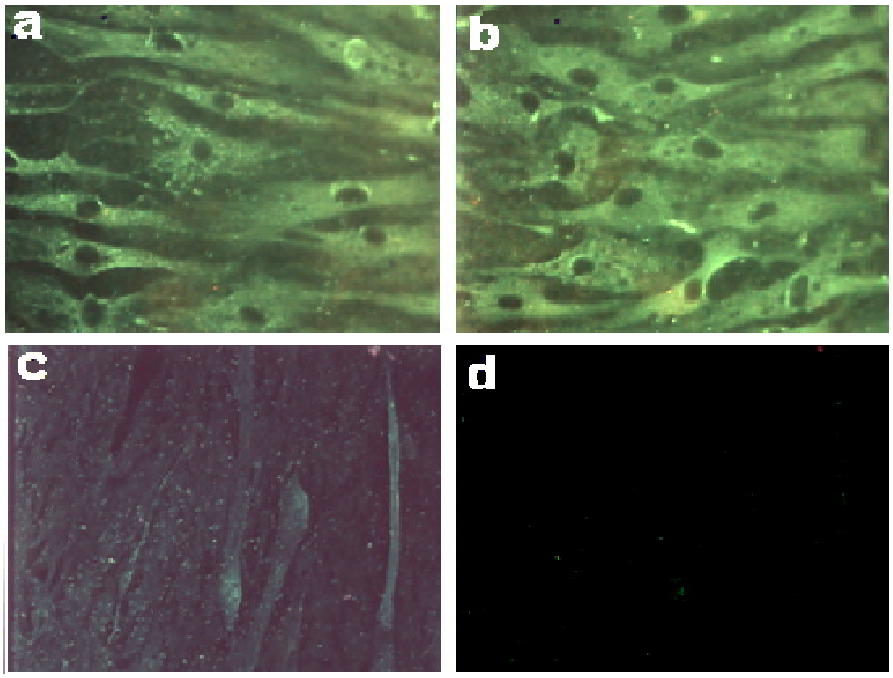

Figure 10: Stoichiometry of interaction of bovine ATIII with trypsin and thrombin (a) and thrombin/serpin complex formation upon SDS-PAGE analysis of the enzyme/inhibitor mixture.

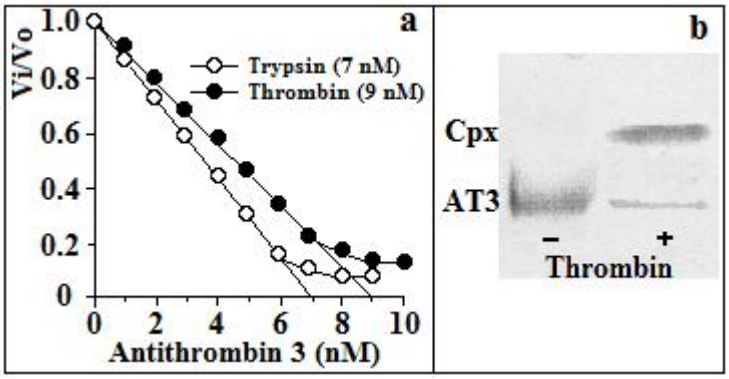

Figure 11: Expression of antithrombin III in muscle crude extracts and in muscle cells as assessed by western blot and immunohistochemistry. (a) Western blot analysis. of a crude muscle extract (lane 1) and of purified AT-III (lane 2) using a rabbit polyclonal anti-AT3. (b) Immunolocalization of the bovine muscle AT III in bovine Diaphragma pedialis muscle using the same antibody (1/50) as the primary antibody and the FITC-labelled goat anti-rabbit IgG as the secondary antibody (1/500). (c) Imunolocalization of ATII in differentiating primary bovine myoblast in culture in similar conditions than in (a). Cell nuclei are stained in red with the Hoechst method. Inserts in (b) and (c) are control samples in which the primary antibody was omitted.

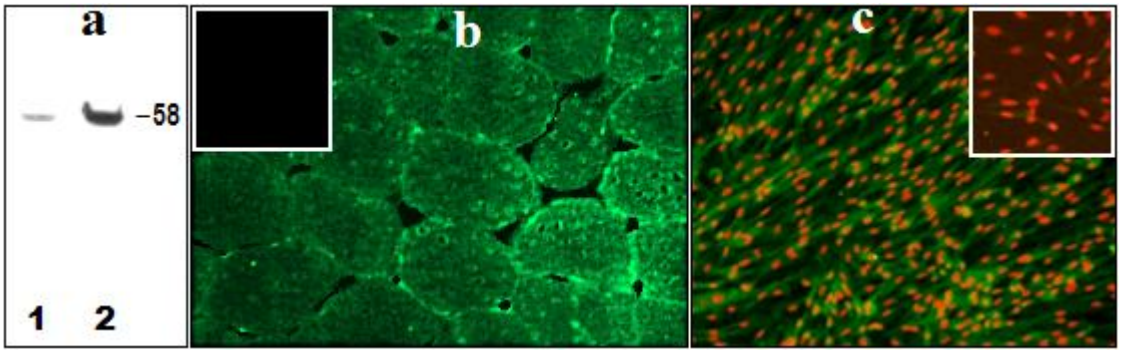

Figure 12: Expression of AT-III in differentiating primary bovine myoblasts and RT-PCR analysis of bovine AT-III mRNAs . (a) absence of expression of ATIII in proliferating primary bovine myoblast in culture (no fluorescence was observed). (b) expression of ATIII in confluent primary bovine myoblast in culture. (c) expression of AT-III. (c) expression of ATIII in growing myotubes. Note that cell nuclei were coloured in red using the Hoechst staining method. The antibody used was a rabbit polyclonal anti-AT3 diluted to 1/50. (d) RT-PCR analysis of total cells mRNAs prepared from proliferating myoblast (lane 1), confluent and fusing myoblast (lane 2) and growing myotubes (lane 3). Lane m showed the RT-PCR analysis of total mRNAs prepared from bovine muscle.

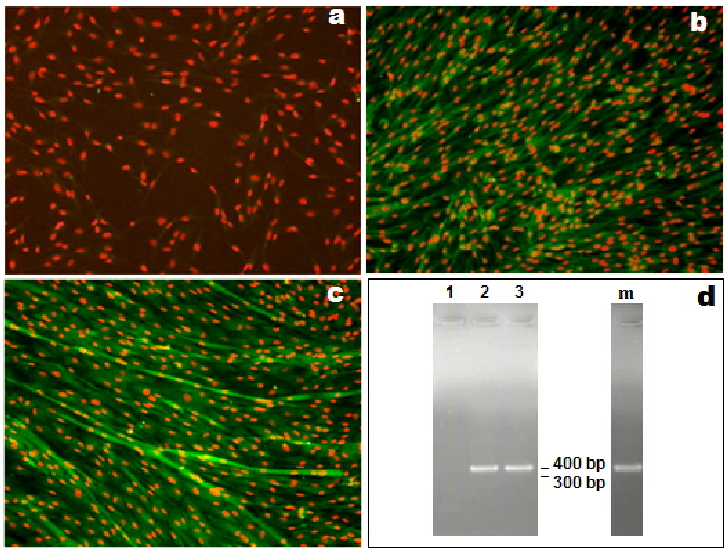

Figure 13: (a) Analysis by SDS-PAGE of total fraction FIII (see Figure 1) and of purified serine protease inhibitors 1 (spi-1) and 2 (spi-2). Proteins were revealed by silver staining. (b) Molecular mass (Mr) and isoelectrical point (pI) of spi-1 and spi-2.

b

	spi-1	spi-2
Mr (kDa)	14.3	11.4
pI	6.8	6.2

Figure 14: Inhibition profile of trypsin (≈20 nM) and chymotrypsin (≈20 nM) by spi-1 and spi-2.