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# APOPTOSIS REGULATION IN *POSTMORTEM* MUSCLE: CROSS-CLASS INHIBITION OF CASPASES BY TWO BOVINE SERPINS

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**Abstract** –In living cells, protein inhibitors constitute a key control point of major biological processes. The serpins, acronym of serine peptidase inhibitors is the largest family identified to date. In mammalian tissues, the only known specific inhibitors of caspases, a cysteine protease family which play a major role in programmed cell death or apoptosis, are the inhibitor of apoptosis proteins or IAPs, a complex family of proteins targeting specifically executor caspases. In the present report, we provide strong evidence supporting the expression in mammalian tissues of a complex family of cross-class inhibitory serpins inhibiting strongly initiator and executor caspases. Two of these serpins (bovSERPINA3-1 and A3-3) were purified from bovine *Diaphragma* muscle and their kinetic of interaction with human recombinant caspases 3 and 8 characterized. Association rate constants obtained stressed forward that these serpins are very likely significant and relevant *in situ* inhibitors of caspases and consequently of apoptosis. Their functions as regulator of apoptosis and as biomarkers of meat tenderness are discussed.

**Key Words** – *Bovine* muscle, proteases, inhibitors, serpins, caspases

## I. INTRODUCTION

Muscles conversion into meat involved a number of various interacting endogenous and as yet poorly-understood biological factors. Recently, meat tenderization was reconsidered through the introduction of a new step known as apoptosis [1] taking place immediately after death and constituting therefore the first step of the conversion of muscle into meat. Key proteins of that process are members of the caspase

family, an acronym of Cysteine **AS**PartyI peptid**ASES**, a group of proteases orchestrating cell demolition. In postmortem muscle, caspases are probably the first proteolytic system to be implicated in *postmortem* proteolysis and meat tenderization [1-2]. Rate and extent of apoptosis is dependent on a delicate balance between the amounts of pro- and anti-apoptotic effectors. As members of the anti-apoptotic family of proteins, caspase inhibitors constitute the last point control of cell death. We previously reported the existence in bovine muscles of a complex family of serine proteases inhibitors belonging to the serpin superfamily, an acronym of **SER**ine **Peptidase Inhibitors** [3]. Most of them were shown to inhibit trypsin and human leukocyte elastase [4-5]. As serpins are also able to inhibit cysteine proteases through similar pseudo-irreversible suicide mechanisms [6-8], their activity was tested against, human initiator and effector caspases 8 and 3, respectively. We reported here results attesting the complexity of this family of serpins which appear to be strong inhibitors of caspases forming covalent stable complexes upon SDS-PAGE electrophoresis and susceptible to constitute the primary control point of apoptosis through a pseudo-irreversible inhibition of caspases. Their ability to be good predictors of meat tenderness is also discussed.

## II. MATERIALS AND METHODS

Purification of bovSERPINA3-1 and A3-3 inhibitors, enzyme and inhibitor titration was carried out as previously described [9]. Specific irreversible inhibitors Z-Asp-Glu-Val-Asp-FMC

and Z-Val-Glu-Leu-Asp-FMC were used for titration of commercial recombinant caspases 3 and 8 respectively. The activity was determined by using the fluorescent substrates N-Acetyl-Asp-Glu-Val-Asp-NHMec and Ac-Ile-Glu-Pro-Asp-NHMec, respectively.

The stoichiometry of interaction (SI) was determined by titration of defined concentrations of caspases 3 and 8 with increasing amounts of either bovSERPINA3-1 or A3-3. The association rate constants characterizing caspases 3 and 8 interaction with bovSERPINA3-1 and A3-3 were determined according to Horvath *et al.* [10] by the continuous method using the following equation:  $k_{ass} = k_{app} (1+[S]/K_m) \cdot SI$  where [S] is the substrate concentration used for activity measurement and  $K_m$  the Michaelis constant characterizing the affinity of the protease towards the substrate. SDS-PAGE performed to reveal the Enzyme-Inhibitor (E/I) complexes was done as in [11] on 12% polyacrylamide slab gels. Two-D gel electrophoresis was carried out as in [12]. Polyclonal rabbit antibody raised against bovSERPINA3-1 was used to reveal the serpins and their complex with proteases by western blot upon 1D and 2D gel electrophoresis.

### III. RESULTS AND DISCUSSION

In living cells, after activation through limited proteolysis or not, protein inhibitors constitute the primary and last step of proteases activity regulation. Regarding serine proteases, the largest family of inhibitors is serpins, a group of protein inhibiting both serine and cysteine proteases in a pseudo-irreversible suicide mechanisms [6-8]. Bovine serpins identified constitute a complex family of polymorphic proteins encoded by at least 8 different genes [3]. Analysis by 2D-gel electrophoresis of a muscle crude extract concentrated by ammonium sulfate precipitation emphasized this complexity (Figure 1). The fraction revealed a complex protein pattern with pI ranging between from pH 4.5 and 6.8. 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 of these ser-pins (black line with close arrowhead). In addition some spots are distributed in a

comma shape manner (black arrow) indicative of various protein glycosylation state.

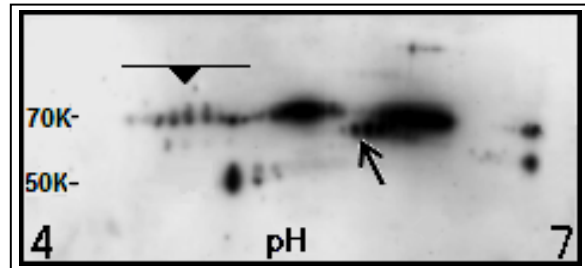


Figure 1: Polymorphism of the Bov-SERPINA3 family as assessed by 2D gel electrophoresis of a crude muscle extract after ammonium sulfate concentration. Western blots were revealed using the rabbit polyclonal antibody raised against Bov-Serpina3-1. *Black arrow*: comma shape alignment of spots indicative of glycosylation; *Black line with arrowhead*: linear alignment of spots with various extent of phosphorylation.

Two of these serpins, bovSERPINA3-1 (Swiss Prot ID: [Q9TTE1](#)), a 70 kDa protein, and A3-3 (Swiss Prot ID: [Q3ZEJ6](#)), a 75 kDa were purified and their protease specificity pattern was analyzed through determination of SI (Stoichiometry of Interaction) and  $k_{ass}$  (association rate constant) which characterized serpins/proteases interactions. As depicted in Table 1 for trypsin and elastase, two proteases containing only one active site/mole, the stoichiometry is 1 mole inhibitor/1 mole enzyme and the association rate ranged from  $10^5$  to  $10^6$   $M^{-1} \cdot s^{-1}$ , a series of  $k_{ass}$  values physiologically significant. Regarding caspases, each mole contains two active sites. According to the findings of Table 1, only caspase 3/SERPINA3-1

Table 1: Stoichiometry of interaction (SI) and association rate constants for the inhibition of recombinant caspases 3 and 8, human leukocyte elastase and trypsin by bov-SERPINA3-1 and A3-3 [4-5].

Enzymes	Constant	SERPINA3-1	SERPINA3-3
<b>Caspase 3</b>	<sup>a</sup> SI	1.01	0.55
	<sup>b</sup> $k_{ass}$	$4.2 \times 10^5$	$1.5 \times 10^5$
<b>Caspase 8</b>	SI	0.49	0.51
	$k_{ass}$	$1.4 \times 10^6$	$2.7 \times 10^6$
<b>Elastase</b>	SI	1.04	1.01
	$k_{ass}$	$2.4 \times 10^7$	$1.3 \times 10^6$
<b>Trypsin</b>	SI	1.01	0.98
	$k_{ass}$	$3.9 \times 10^6$	$6.7 \times 10^5$

<sup>(a)</sup> SI =  $[I]/[Caspase \text{ active sites}]$ ; <sup>(b)</sup>  $k_{ass}$  in  $M^{-1} \cdot s^{-1}$  ( $k_{ass}$  values greater than  $10^4$  are physiologically significant)

showed a 1/1 ratio suggesting that total protease inhibition can be achieved only upon binding of 1 mole of inhibitor to each active site (2 moles of inhibitor/active caspase 3). In other cases the SI was 0.5 indicating that one mole of inhibitor is sufficient to inhibit both caspase active sites probably through a significant conformational modification of the protease. All  $k_{ass}$  values indicate that these serpins are strong inhibitors of caspases 3 and 8 and that their interactions are of physiological significance. Note that all inhibited enzymes including caspases formed SDS-stable complexes (Figure 2).

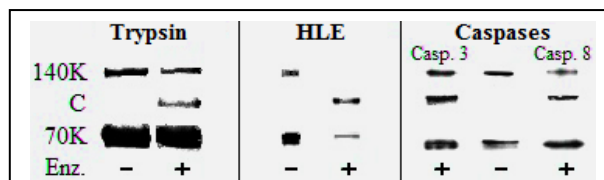


Figure 2: Covalent complexes upon SDS-PAGE between bovSERPINA3-1 and proteases as revealed by western blot, using the rabbit polyclonal antibody raised against this serpin. Similar findings were obtained with bovSERPINA3-3 (not shown). Note that the 140 kDa band is a dimer of the 70 kDa serpin. *Abbreviations:* C, band corresponding to the complex with the target enzymes: Trypsin, Human Leukocyte Elastase (HLE) and human caspases 3 and 8. (-) inhibitor alone; (+) inhibitor incubated with the enzyme (Enz) [4]

Apoptosis is a complex finely regulated process and multiple factors contribute at different levels to this regulation. Besides the currently mentioned stimuli activating/inhibiting this cell death process and because of their high concentration in muscle cells together with their ability to inhibit pseudo-irreversibly initiator and effector caspases, these serpins constitute an important control point of apoptosis. In recent *in vitro* studies, inactivation of the corresponding human serpins in growing human prostate cancer cells in culture was shown to reverse the cell proliferation/cell death ratio comforting the potential role of these serpin family members in apoptosis regulation (unpublished data). This feature is supported by Huang *et al.* [13] who reported a decrease of protein degradation in muscle injected with specific inhibitors of caspase 3.

This is the first time that serpin able to inhibit caspases have been identified in mammalian tissues where they showed a wide distribution. The only one serpin reported as a potent caspase inhibitor is the cowpox virus CrmA (Cytokine response modifier A). However, in contrast to the bovine serpins, CrmA is unable to form SDS stable complexes with any of the target caspases tested, the E/I complex being detected only upon electrophoresis in non-denaturing conditions suggesting a non-covalent association [14]. The other known inhibitor of caspases are IAPs (Inhibitor of Apoptosis Proteins) which differ from all other protease inhibitors in their mode of action as they just ensure an hindering of the active site to limit its accessibility to substrate but not to small peptides. There two binding sites are located outside but close to the active site on the surface of the protease.

The bovine serpins inactivate strongly trypsin with which they formed a SDS-stable complex. According to their abundance in skeletal muscle (Figure 1), these serpins represent very likely a major part of the trypsin inhibitors quantified by titration with this enzyme in the studies of Zamora *et al.* [15-16] who identified at death serine protease inhibitors concentration as the best marker of meat ultimate tenderness amongst about 30 quantitative variables measured including calpains 1 and 2, cysteine protease inhibitors and calpastatin. In the context of biomarkers of meat tenderness, quantification of these serpins must be reconsidered using more specific immunological methods. For more details about serine protease inhibitors in muscle cells and their functions see Boudida, Gagaoua *et al.* [5] which will appear soon online.

#### IV. CONCLUSION

The present short report demonstrates the expression in bovine muscle cells of a widely distributed complex family of cross-class inhibitory serpins able to bind very tightly to initiator and executor caspases, with physiologically significant association rate constants. Their efficiency to inhibit caspases suggests that they might play a major role in apoptosis regulation and thus the implication of this key control point together with their

mechanisms of action on caspases call for further detailed investigations. Finally, screening and characterization of proteases inhibitors in muscle which are better predictors of meat tenderness as compared to their target enzymes are far from being completed and much work remains to be done in this context.

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