Expression of SERPINA3s in cattle: focus on bovSERPINA3-7 reveals specific involvement in skeletal muscle

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α1-Antichymotrypsin is encoded by the unique SERPINA3 gene in humans, while it is encoded by a cluster of eight closely related genes in cattle. BovSERPINA3 proteins present a high degree of similarity and significant divergences in the reactive centre loop (RCL) domains which are responsible for the antiprotease activity. In this study, we analysed their expression patterns in a range of cattle tissues. Even if their expression is ubiquitous, we showed that the expression levels of each serpin vary in different tissues of 15-month-old Charolais bulls. Our results led us to focus on bovSERPINA3-7, one of the two most divergent members of the bovSERPINA3 family. Expression analyses showed that bovSERPINA3-7 protein presents different tissue-specific patterns with diverse degrees of N-glycosylation. Using a specific antibody raised against bovSERPINA3-7, Western blot analysis revealed a specific 96 kDa band in skeletal muscle. BovSERPINA3-7 immunoprecipitation and mass spectrometry revealed that this 96 kDa band corresponds to a complex of bovSERPINA3-7 and creatine kinase M-type. Finally, we reported that the bovSERPINA3-7 protein is present in slow-twitch skeletal myofibres. Precisely, bovSERPINA3-7 specifically colocalized with myomesin at the M-band region of sarcomeres where it could interact with other components such as creatine kinase M-type. This study opens new prospects on the bovSERPINA3-7 function in skeletal muscle and promotes opportunities for further understanding of the physiological role(s) of serpins.

1. Introduction

The serine protease inhibitors (serpins) constitute a large family of functionally diverse proteins that are found in all kingdoms including animals, plants, bacteria and some viruses [1–3]. Although their physiological functions are still not fully elucidated, most serpins are involved in numerous intracellular and extracellular processes such as blood coagulation, fibrinolysis, cell migration or tumour suppression [4,5]. Moreover, some studies have reported the association between serpins and numerous familial disorders or diseases known as serpinopathies [6,7]. In those cases, serpins act by forming large and stable multimers. Such conformational disorders are known for thrombosis induced by mutations in antithrombin [8], emphysema or liver cirrhosis induced by mutations in α1-antitrypsin [9,10], or dementia caused by polymerization and tissue deposition of the mutated neuroserpin [11]. In addition to the known protease inhibitory function of serpins [5,12], some members have evolved and acquired non-inhibitory roles in diverse events such as blood pressure regulation (angiotensinogen), chromatin condensation (protein MENT) or hormone transport (corticosteroid binding globulin, CBG and thyroxine binding globulin, TBG) [12,13]. In Drosophila, the serpin Spn27A is implicated in the formation of the dorsoventral axis during...
embryonic development [14]. In CBG and TBG cases, the decrease of hormone affinity results from the spontaneous insertion of reactive centre loop (RCL) domain into the β-sheet A upon cleavage. In the other cases, the RCL seems to be not functional, and it appears that one or more regions apart from the RCL are involved in biological activities of the serpins.

Despite their diversity of function(s), these proteins share a conserved 350-residue core globular domain consisting of three β-sheets and eight or nine α-helices [15]. They present a short flexible strand, the RCL domain, which contains the recognition region for the target protease. Many serpins inhibit their targets by forming stable complexes with the proteolytic enzymes. This interaction is RCL-dependent and induces conformational changes as in the mechanism of suicide substrate inhibitor [3,12,16].

Currently, the NCBI protein database contains at least 20 000 entries annotated as serpins. According to their gene structures, the exon–intron-based system includes six groups of vertebrate serpins [17]. Based on sequence similarities, the serpin family is divided into nine first clades (A to I) [5]. So far, the two largest groups are the antitrypsin-like and the ovalbumin-like serpins, named ‘serpin A’ and ‘serpin B’, respectively. The SERPINA3 genes typically consist of four exons with identical positioning and phasing of intron–exon boundaries. Chromosomal localization studies have highlighted the complexity of the family and revealed that serpin genes are often clustered and share similarities in structure, presumably as a result of gene duplication [17].

Among the known serpins, SERPINA3, also called 1-ACT, is one of the most studied and abundant serpins that appears to be physiologically essential. The main role of this protein is mediated by its inhibitory effect against cathepsin G and chymotrypsin-like enzymes [18,19]. Although its biological functions are not fully elucidated, 1-ACT seems to play an important role in immune response modulation. After tissue injury or infection, 1-ACT concentration increases leading to the inhibition of the natural cytotoxic activity of T-cell killer lymphocytes and the enhancement of antibody response [20,21]. Mutations in 1-ACT induce the formation of inactive polymers that cause the protein retention within hepatocytes and plasma deficiency [22]. More recently, some polymorphisms in the SERPINA3 gene have been associated with Alzheimer’s disease [23].

In contrast to humans, where α1-ACT is represented by the single SERPINA3 gene at position 14q32.1 [24], the SERPINA3 locus on other mammalian species is dramatically expanded. The mouse multi-genic locus, termed Spi-2, is a cluster of 14 members with 65–85% similarity and a markedly divergent RCL domain [25]. In pig, three α1-ACTs are detected at the protein level: P12 (SERPINA3-1), P13 and P14 (SERPINA3 paralogues); and an additional serpin, SERPINA3-2, is identified at the genomic level [26,27]. As observed in mice, pig P12 and SERPINA3-2 proteins show 76% amino acid identity; the main difference resides near the C-terminus region that includes the deduced RCL domain. More recently, we provided additional information about SERPINA3 status in the bovine genome. We characterized, a cluster of eight genes and one pseudo-gene at the 21q24 position [28]. Interestingly, this cluster contains an original subgroup of six members (bovSERPINA3-1 to bovSERPINA3-6) with an unexpectedly high degree of conservation (96%). This subgroup is not found in mouse and pig SERPINA3 clusters. The two remaining genes (bovSERPINA3-7 and bovSERPINA3-8) are more different (tables 1 and 2).

One intriguing question in the field is to address the biological significances of the eight closely related SERPINA3 proteins in cattle. BovSERPINA3-1 and bovSERPINA3-7 (often referred to as endopin 1 and endopin 2, respectively) have been studied in neurosecretory vesicles of chromaffin cells [29,30]. The authors suggested that bovSERPINA3-7 inhibits both serine and cysteine proteases of the regulated secretory pathway in chromaffin cells [30,31]. Also, our molecular and biochemical characterizations showed that bovSERPINA3-1 and bovSERPINA3-3 are expressed in skeletal muscles [32,33] and are able to strongly inhibit the initiator caspase 8 and the effector caspase 3 [34]. Although their biological roles are still uncertain, it could be assumed that both bovSERPINA3-1 and bovSERPINA3-3 are significantly relevant in situ as inhibitors of caspases and consequently programmed cell death [35].

To date, nothing is known about the expression of the other bovSERPINA3 members. In this study, we measured the expression levels of the eight bovSERPINA3 genes using a quantitative real-time PCR based on TaqMan® technology (custom

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**Table 1. Main characteristics of the members of the bovSERPINA3 family.**

| bovine serpines A3 | signal peptide | chain | length | mass (Da) whole/processed | UniProt | N-glycosylation sites |
|--------------------|----------------|-------|--------|---------------------------|---------|-----------------------|
| bovSERPINA3-1      | 1 – 24         | 387   | 411    | 46 240/43 650             | Q9TE1   | 100/180/230/264       |
| bovSERPINA3-2      | 1 – 24         | 387   | 411    | 46 240/43 650             | A217M9  | 100/180/230/264       |
| bovSERPINA3-3      | 1 – 24         | 387   | 411    | 46 330/43 780             | Q3ZEJ6  | 100/180/230/264/318   |
| bovSERPINA3-4      | 1 – 24         | 387   | 411    | 46 310/43 760             | A217N0  | 180/230/264/318       |
| bovSERPINA3-5      | 1 – 24         | 387   | 411    | 46 400/43 780             | A217N1  | 100/180/230/264/318   |
| bovSERPINA3-6      | 1 – 25         | 389   | 414    | 46 390/43 720             | A217N2  | 103/183/233/267/321   |
| bovSERPINA3-7      | 1 – 25         | 392   | 417    | 46 940/44 170             | A217N3  | 103/183/221/267       |
| bovSERPINA3-8      | 1 – 25         | 393   | 418    | 46 960/44 280             | A60Q2   | 103/183/233/268       |

| recombinant serpin A3 | N-His tag peptide | mass (Da) whole protein |
|-----------------------|-------------------|------------------------|
| recSERPINA3-3         | 1 – 23            | 387                    | 410                     | 46 540   |
| recSERPINA3-7         | 1 – 23            | 392                    | 415                     | 46 930   |
assays), and we analysed bovSERPINA3 protein patterns in different tissues of 15-month-old Charolais bulls. For the first time, we demonstrate that all the bovSERPINA3 family members are expressed at transcriptional and translational levels. As previously described [28], we have defined two subgroups. Many studies have been performed on bovSERPINA3-1 and A3-3, two members of the first subgroup [32–35]. In this report, we focus on one member of the second subgroup, bovSERPINA3-7 that shows more difference in the RCL domain. We characterized bovSERPINA3-7 expression, its tissue distribution and its glycosylation. By immunoprecipitation, we revealed that bovSERPINA3-7 and creatine kinase M-type interact in skeletal muscle. By immunostaining, we showed that bovSERPINA3-7 is preferentially localized in fast-type fibres, precisely in the M-band sarcomere. We propose that bovSERPINA3-7 and creatine kinase type M could interact within these cells. These investigations of the subcellular and tissue distributions of bovSERPINA3-7 contribute to knowledge of the biological roles of serpins, especially in skeletal muscle cells. The workflow of this study is shown in the electronic supplementary material, figure S1.

2. Results

2.1. Transcriptional expression pattern of bovSERPINA3 gene family

The expression analysis of bovSERPINA3 genes was carried out on eight different tissues from 15-month-old Charolais bulls (figure 1a). All bovSERPINA3 genes are expressed at different levels in the different tested tissues. BovSERPINA3 genes are highly expressed in the liver and weakly expressed in the lung, the testis and the thymus. In the other tissues (kidney, spleen, cerebellum and skeletal muscle), bovSERPINA3 genes are expressed at intermediate levels. We also evaluated the contribution of each serpin expression in those tissues (figure 1b; electronic supplementary material, table S3), and we observed that the most related bovSERPINA3 genes (bovSERPINA3-1 to A3-6) are more expressed than the two most divergent members of the serpin family (bovSERPINA3-7 and A3-8). At the transcriptional level, in each tested tissue except for liver and testis, bovSERPINA3-3/4 and bovSERPINA3-5 genes have nearly identical expression proportions. BovSERPINA3-1 has the highest proportion of expression in testis compared with lung, thymus or cerebellum, and it is weakly expressed comparatively to other bovSERPINA3 genes in liver, kidney, spleen and skeletal muscle. Surprisingly, bovSERPINA3-6, which belongs to the same subgroup, presents different expression proportions for each tissue except for kidney and spleen. BovSERPINA3-8 has a similar expression proportion in liver, spleen, cerebellum and skeletal muscle and a different expression proportion in other tested tissues. Finally, the bovSERPINA3-7 expression proportions are almost identical between testis and skeletal muscle and between thymus and cerebellum.

2.2. Expression patterns of bovSERPINA3 proteins in bovine tissues

Although it is known that both bovSERPINA3-3 and A3-7 are expressed in skeletal muscles [32,33] and in chromaffin cells [30,31], nothing is known about the presence of the
bovSERPINA3 family proteins in other tissues. To evaluate their distribution on diverse tissues of 15-month-old Charolais bulls, Western blot analyses were performed using a polyclonal antibody raised against purified bovSERPINA3-1. This antibody was evaluated for its capacity to recognize bovSERPINA3-3 and bovSERPINA3-7, two representative members of the bovSERPINA3 family. As previously described [36], on reducing SDS-PAGE, the antibody reveals the recombinant recSERPINA3-3 as a monomer form (50 kDa) and a dimeric form (100 kDa). This antibody also reveals the recombinant recSERPINA3-7 only under its monomeric form (47 kDa; figure 2a).

Because this antibody is able to recognize one member of each subfamily, it may recognize the other bovSERPINA3 family members. For all tested tissues, the antibody reveals several bands in the range 45–150 kDa that could correspond to several (or all) bovSERPINA3 proteins with different post-translational modifications (figure 3). Moreover, these band patterns are quite different in each tissue. Except in the serum and the liver, a 150 kDa form is observed and could correspond to dimers. Indeed, some serpins such as bovSERPINA3-3 are known to dimerize under denaturing conditions and these dimers disappear on native PAGE [36,37]. Surprisingly, an additional band of 96 kDa is specifically observed in the skeletal muscle. We proposed that this 96 kDa band could be related to a muscle-specific conformation, a complex, or post-translational modifications of one of the most divergent serpins. To address these hypotheses, we designed a specific anti-bovSERPINA3-7 antibody allowing us to analyse specifically this protein.

2.3. Expression patterns of bovSERPINA3-7 protein in bovine tissues

We have produced both SERPINA3-3 [36] and A3-7 in Escherichia coli. We first evaluated the specificity of anti-bovSERPINA3-7 antibody against these bacterial proteins by Western blot. As shown in figure 2b, the antibody reacts with purified recSERPINA3-7, but not with recSERPINA3-3.

Thus, we used this antibody to analyse the bovSERPINA3-7 patterns in several tissues of 15-month-old Charolais bulls by Western blot. For all samples, Western blot analysis reveals a protein at 65 kDa (figure 4a). This molecular weight is higher than that predicted from the amino acid sequence of bovSERPINA3-7 (44.17 kDa), i.e. without its signal peptide. Because four potential N-glycosylation sites are predicted in the protein sequence (UniProt data), the approximately 20 kDa difference in molecular weights between evaluated and apparent sizes of the protein could be explained by the presence of N-glycans at the four N-glycosylation sites. In addition to the 65 kDa band, we detected two other proteins of 59 and 75 kDa in the serum.

Figure 1. Tissue expression of bovSERPINA3s genes. (a) Relative amounts of transcripts (RQ ± S.D.) of bovSERPINA3s were determined by qPCR in 8 tissues. Each sample corresponds to 3 animals pooled cDNAs and was measured in triplicate. bovSERPINA3s expression levels were normalized by TFIIID, one stable housekeeping gene and calibrated by testis tissue. The data were plotted on a logarithmic scale to simplify comparison of expression profiles. (b) Proportions of transcripts of bovSERPINA3 genes for each tissue were calculated in per cent ± S.D.
and the liver; these bands are not equally represented in these tissues, supporting the idea that bovSERPINA3-7 could be differentially glycosylated according to the tissue. In the cerebellum and skeletal muscle, an additional band of 45 kDa is observed and could correspond to the bovSERPINA3-7 without any post-translational modifications.

Finally, as shown in figure 4a, a band of 96 kDa is exclusively detected in skeletal muscle. To determine if this band is expressed in all the three muscle types, we also performed Western blot analyses on total proteins extracted from bovine longissimus thoracis, cardiac and smooth muscles (figure 4b). Surprisingly, whereas the 65 kDa band is observed in the three muscle types, the 96 kDa band is specifically expressed in skeletal muscle.

2.4. Glycosylation analyses of bovSERPINA3-7 in skeletal muscle

UniProt data and our Western blot analyses suggested that bovSERPINA3-7 could be N-glycosylated at four putative sites. To confirm this hypothesis, we performed Western blot analyses...
analysis on total proteins extracted from longissimus thoracis of 15-month-old Charolais bulls which were treated by different combinations of glycosidases. To remove N-glycans, we used the endoglycosidase PNGase F [38]. As indicated in figure 5a, PNGase F treatment of bovSERPINA3-7 induces the disappearance of the 65 kDa protein in favour of the 45 kDa which corresponds to the theoretical molecular weight of the non-glycosylated bovSERPINA3-7. To determine whether the four putative sites are N-glycosylated, partial digestion with PNGase F was performed on the same sample. As shown in figure 5b, bovSERPINA3-7 immunoblot revealed five different bands corresponding to the fully glycosylated protein at 65 kDa, the partially glycosylated protein at 60.5, 57.5 and 52 kDa, and the non-N-glycosylated protein at 45 kDa.

We completed our glycosylation analysis by submitting the muscle proteins to treatments allowing the removal of mucin-type O-glycans that are frequently present on secreted proteins [39]. The enzyme combinations to remove N- and O-glycans do not generate an additional shift of the molecular weight compared with the one obtained with the PNGase F alone (figure 5c). This result suggests that bovSERPINA3-7 in skeletal muscle is an N-glycoprotein harbouring four N-glycans and no type-mucin O-glycans. However, the treatment with the sialidase enzyme induces a shift in the migration of the serpin (form about 60 kDa indicated by arrow in figure 5c, lane 4). This result indicated that the N-glycans of bovSERPINA3-7 present in skeletal muscle are mainly sialylated.

Surprisingly, after 16 or 24 h of treatment, the 96 kDa band was still observed (figure 5a,c). Thus, this band does not reflect a muscle-specific glycosylation of bovSERPINA3-7. Moreover, as shown in figure 2, recSERPINA3-7 is not able to form a homodimer as is observed for recSERPINA3-3 in denaturing conditions. Altogether, these results indicate that the 96 kDa band is not related to a skeletal muscle-specific glycosylation of bovSERPINA3-7 or to a homodimer of non-glycosylated SERPINA3-7. Thus, we propose that this band could correspond to a skeletal

![Figure 5. Glycosylation states of bovSERPINA3-7.](image-url)

(a) Bovine longissimus thoracis proteins (40 μg) were treated with PNGase F (2.5 units) for 16 h at 37°C. Proteins were revealed by Western blot using the polyclonal antibody raised against bovSERPINA3-7. (b) Kinetics of PNGase F digestion (2.5 units, 37°C) were performed on total proteins (40 μg) extracted from longissimus thoracis muscle. Five states of glycosylation (0–4) were detected by Western blot using the polyclonal antibody raised against bovSERPINA3-7. They correspond to fully, partially or non-glycosylated bovSERPINA3-7. (c) Deglycosylation assays of bovSERPINA3-7 were performed on proteins (50 μg) extracted from longissimus thoracis muscle. Lane 1: protein extract without treatment; lane 2: protein extract treated with PNGase F during 16 h; lane 3: protein extract treated with both PNGase F and a mixture of enzymes to remove O-glycans (sialidase A for cleavage of terminal sialic acid residues, O-glycosidase to remove the core Gal-β(1→3)-GalNAc, β(1→4)-galactosidase and β-N-acetylglucosaminidase to remove sugars associated with specific O-linked glycan structures) during 16 h; lane 4: protein extract treated with sialidase during 16 h.
muscle-specific complex of the non-glycosylated SERPINA3-7 with another/other protein partner(s).

2.5. Identification of a partner to bovSERPINA3-7 in skeletal muscle

To identify the protein partner associated with bovSERPINA3-7 in longissimus thoracis of 15-month-old Charolais bulls, we performed an immunoprecipitation of bovSERPINA3-7 in this tissue. Western blot and Coomassie blue staining analyses revealed the presence of a 96 kDa band in the eluted fraction after bovSERPINA3-7 immunoprecipitation (figure 6).

Two samples of this 96 kDa band were analysed by nanoLC-MS/MS. The combined analysis for the two samples leads to the identification of bovine creatine kinase M-type with a coverage of 61% and a log(E) value of −68.77. Creatine kinase is the only protein detected with a high confidence level that has a molecular weight in accordance with the formation of a complex of 96 kDa with the bovSERPINA3-7 protein. Details of the analysis are given in electronic supplementary material, table S4.

The cytosolic creatine kinase M-type is an important player of muscle metabolism as it regulates the optimal ATP/ADP ratio during muscle contraction. As the bovSERPINA3-7 protein is expressed in skeletal muscle and could interact with creatine kinase M-type, we analysed the bovSERPINA3-7 localization in skeletal muscle by immunostaining.

Transversal and longitudinal sections of longissimus thoracis (figure 7a and b, respectively) showed that bovSERPINA3-7 is distributed throughout the individual muscle fibres. The co-immunostaining with an anti-laminin antibody confirmed that bovSERPINA3-7 is localized within the muscle fibres. The immunostaining also revealed that all the fibres are not equally stained (figure 8a).

Muscle fibre types were then characterized according to their myosin isoforms. We observed that the bovSERPINA3-7 protein is less expressed in type I compared with type II fibres (figure 8c). According to these results, we concluded that the bovSERPINA3-7 protein is mainly expressed in fast-type (type II) fibres and partially in slow-type (type I) fibres.

Analysis by confocal microscopy, using the antibody raised against the bovSERPINA3 family, showed that all the fibres are stained (figure 9a), and the intensity of fluorescence is variable according to fibre type. Contrary to our observation for bovSERPINA3-7, the double staining SERPINA3s/type I myosin reveals that most of the bovSERPINA3 family proteins are preferentially expressed in type I fibres (figure 9c).
Therefore, in skeletal muscle, it seemed that the bovSERPINA3-7 protein is preferentially expressed in type II fibres, whereas the other members of the bovSERPINA3 family (or most of them) are expressed in type I fibres. Finally, the longitudinal sections of bovine longissimus thoracis muscle allowed us to localize bovSERPINA3-7 inside the sarcomeric structures including the Z-disk and M line identified by myotilin and myomesin stainings, respectively. As shown in figure 10, confocal imaging indicates clearly that bovSERPINA3-7 localizes into the sarcomeric M-band as established by its colocalization with myomesin (yellow in figure 10c). The RGB profile along a line (figure 10c) confirms the colocalization of these two proteins and clearly demonstrates that bovSERPINA3-7 is localized between two Z-discs stained by myotilin (figure 10b,d). Taken together, these data revealed that within skeletal muscle fibres, bovSERPINA3-7 is specifically localized in the sarcomeric M-band where it could interact with other specific M-band component(s).

3. Discussion

In contrast to human, where the serpin A3 is represented by a unique gene and a unique protein α1-ACT, the situation seems to be more complex in cattle and remains original in mammals. The family of bovine SERPINA3s contains a...
subgroup of six closely related proteins bovSERPINA3-1 to bovSERPINA3-6 and two other members, bovSERPINA3-7 and bovSERPINA3-8, that present differences, notably in the RCL [28]. This suggests that these proteins have different physiological roles.

To gain knowledge on the potential physiological role(s) of each serpin, it is essential to examine their expression in various tissues. For that purpose, studies at both transcriptional and translational levels were carried out on all bovSERPINA3s. In this study, we were able to quantify at the transcriptional level the expression of each gene. Our results confirmed that bovSERPINA3 family members are ubiquitously expressed in all Charolais-tested tissues. Except in the liver (major secretory organ of SERPINA3) which presents a high level of each transcript, their expressions are highly different for other tissues. Thus, for the same tissue, the mRNA expression level can be very different between bovSERPINA3 genes. In the same manner, for the same bovSERPINA3 gene, its expression level varies between tissues. Whereas bovSERPINA3 genes are located on the same locus and/or cluster, these results suggest different mechanisms of regulation for the expression of each member of the bovine family.

Because of the very high sequence similarity between the eight proteins, it is difficult to produce a specific antibody for each serpin. However, using a polyclonal antibody, we detected all these serpins in a panel of bovine tissues. Although bovSERPINA3 family members are ubiquitously expressed, their translational expression levels are highest in the liver and serum. This result is in agreement with the liver being the major secretory organ of several serpins, including α1-ACT and α1-antitrypsin (AAT) as described in humans [18]. Moreover, these serpins represent the most abundant serpins in human plasma. These potent plasma inhibitors are capable of inhibiting several serine proteases involved in inflammation [40] or leucocyte-derived proteases that are released at the site of skin injury [41].

For this original bovine family, it was essential to determine more specifically which SERPINA3s are present in these bovine-tested tissues. The present evidence that mRNAs and proteins of some of these serpins are expressed in different tested tissues clearly indicates a complexity of the potential physiological role(s) of these inhibitors. Using the antibody which recognized all members of bovSERPINA3 family, the range of molecular weight detected by Western blot results from the expression of several members of this family in the same tissue and/or the presence of more than one glycoform of each serpin. Indeed, in addition to the bands of about 45 kDa (which correspond to the non-glycosylated forms of bovSERPINA3s) observed in the liver, kidney and skeletal muscle, several other bands corresponding to different glycosylated states were also detected in all tested tissues. This particular status has been described for the protein C inhibitor (PCI or SERPINA5) which is a serpin type of serine protease inhibitor. The N-glycosylation of the seminal plasma PCI differs strikingly compared with those of both blood-derived and urinary PCI. In addition, the PCI glycoforms displayed different activities. The authors concluded that the N-glycans of PCI are tissue-specific and could be responsible for PCI conformational changes [42]. Similarly, glycosylation variants of human corticosteroid-binding globulin (CBG or SERPINA6) have been characterized. Importantly, some of them have been shown to affect the affinity of CBG for its receptors [43–45].

One intriguing question in the field remains regarding the biological significance of the eight closely related SERPINA3 proteins in cattle. In this study, we focused on the characterization of bovSERPINA3-7, one of the two most different members of the bovSERPINA3 family. We took advantage of a sequence upstream of the bovSERPINA3-7 RCL, which
differs from the one found in the other serpins, to design a specific anti-bovSERPINA3-7 antibody. The expression pattern of this protein illustrates perfectly the complexity previously described. In all tested tissues, bovSERPINA3-7 is expressed as an N-glycosylated protein of 65 kDa. The 59 kDa band, observed in both serum and liver, corresponds to bovSERPINA3-7 with an intermediate glycosylated state, as it disappears after PNGase F treatment (data not shown). A form without post-translational modification (45 kDa) is also detected in cerebellum and muscle. For the glycosylated forms, total and partial deglycosylation experiments proved that bovSERPINA3-7 is a glycoprotein with sialylated N-glycans. To complete this study and that of Hwang et al. [31], we suggest that the four sites of bovSERPINA3-7 are N-glycosylated. Surprisingly, both in liver and serum, the 75 kDa band revealed by the polyclonal antibody specific for bovSERPINA3-7 is not affected by PNGase F treatment (data not shown) and remains to be identified. Furthermore, a 96 kDa band, resistant to PNGase, is detected only in skeletal muscles of 15-month-old Charolais bulls with both antibodies raised against bovSERPINA3-7 and all bovSERPINA3s. Our results indicate that bovSERPINA3-7 does not exist in a homodimeric form unlike bovSERPINA3-3 (see §2.2). In previous data [34], we have shown that bovSERPINA3-3 expressed in E. coli (without N-glycans) or purified from muscle (N-glycosylated) remains active and displays comparable association rate constants when tested against trypsin and caspase 3. To assert that the recombinant protein bovSERPINA3-7 corresponds to an active serpin, the in vitro inhibitory activity was tested against elastase. We found that recSERPINA3-7 inhibits this serine protease with an association rate (k_{ass}) of 1.7 × 10^{5} M^{-1} s^{-1}, a similar value to that measured by Hwang et al. [31]. This suggests that non-glycosylated bovSERPINA3-7 is functional in vivo. Therefore, we can assume that the 96 kDa band correspond to a stable homodimer of non-glycosylated bovSERPINA3-7 (45 kDa) and a protein partner (about 50 kDa).

NanoLC–MS/MS analysis of the 96 kDa-specific skeletal muscle band reveals the presence of bovine creatine kinase M-type. Muscle cytosolic creatine kinase isoform plays an important role in muscle energetic metabolism and is a very efficient system of maintaining optimal ATP/ADP ratio during muscle contraction. The active form of this enzyme is a homodimer of 80–86 kDa [46]. As reported, creatine kinase migrates at 43 kDa under denaturing conditions [47]. The association between this monomeric form and a nonglycosylated form of bovSERPINA3-7 can achieve a complex of 96 kDa, as observed. In addition, it is well documented that creatine kinase M-type is located in the M-band of the sarcomere [48] where it interacts with myomesin and M protein [49], especially in fast-twitch skeletal muscles [50]. More recently, computational simulations to predict creatine kinase-associated factors [51] have shown that creatine kinase M-type can potentially interact with 85 significant partners. Among these proteins, there are M-band components such as myomesin, M-band protein, titin and myosin. The function of most of the other candidate proteins has not been reported yet and remains unknown in skeletal muscles. Among them, we note the presence of SERPINF1, a strong inhibitor of angiogenesis [52]. SERPINF1 presents a full-length significant homology of about 50% with bovSERPINA3-7. Using immunostaining, we showed in this work that bovSERPINA3-7 is preferentially present within the sarcomeric M-band of type II (fast-type) muscle fibres and co-localizes with myomesin, supporting the hypothesis of an interaction between bovSERPINA3-7 and creatine kinase M-type in skeletal muscle. While most of the other bovSERPINA3s are detected in type I (slow-type) fibres, A3-7 is preferentially observed in type II fibres, suggesting that bovSERPINA3-7 plays a specific role in skeletal muscle. The determination of partners can further promote our understanding of the physiological role(s) of bovSERPINA3-7.

4. Material and methods

4.1. Samples

For quantitative real-time PCR and Western blot analyses, tissue samples were obtained from three 15-month-old Charolais bulls. All tissue samples were collected from young piglets. The tissues collected included liver, spleen, thymus, cerebellum, kidney, lung, testis, serum, skeletal muscles (longissimus thoracis and rectus abdominis), aorta and heart. All these bovine tissues were obtained from the INRA experimental slaughterhouse (Theix, France). The specimens used for all experiments were snap frozen upon extraction and stored at −80°C until use.

4.2. Frozen sections

Skeletal muscle longissimus thoracis from 15-month-old Charolais bulls was dissected, snap frozen in isopentane, cooled by liquid nitrogen and stored at −80°C until sectioning. Cryosections of samples were cut using a Reichert Frcoguc 2800 (Leica, Heidelberg, Germany) with a cutting temperature of about −20°C. Thick sections of 10 μm were made across or longitudinally to the muscle fibres. Cryosections from each tissue sample were collected on Teflon printed diagnostic (Immuno-Cell, Mechelen, Belgium) and dried for 1 h at room temperature before treatments.

4.3. RNA extraction and first strand cDNA synthesis

Total RNA was isolated from tissue samples using the RNasy Midi kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The digestion with DNase I (Qiagen) was performed on the spin column. Concentration of the isolated RNA and the 260/280 nm absorbance ratio were measured with the NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). RNA integrity was additionally assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). All samples had RIN ≥ 8. RNA was stored at −80°C when the reverse transcription step was not immediate. Two micrograms of total RNA were reverse transcribed in a total volume of 20 μl using 250 ng of random hexamers primers (Invitrogen, Carlsbad, CA) and 200 units of Superscript™ III Reverse Transcriptase (Invitrogen) according to the manufacturer’s guidelines. The reaction was incubated for 5 min at 25°C,
Table 3. Primers sequences for qPCR and accession numbers of genes.

| primer and probe names | nucleotide sequences | transcript targets | accession numbers |
|------------------------|----------------------|--------------------|------------------|
| SA3-1F                 | 5'-CGACAGGCGCTCTGCTATC-3' | bovSERPINA3-1       | GenBank: AY911536 |
| SA3-1R                 | 5'-GAGACTACAGGTTCCGCGG-3' |                    |                  |
| TMSA3-1                | 5'-AGCCTCGGGACTGGTCGGCG-3' |                    |                  |
| SA3-3/4F               | 5'-GATGAGGCGAGAGAGATTCGACC-3' | bovSERPINA3-3      | GenBank: AY911537 |
| SA3-3/4R               | 5'-CTGGTCTGGCGGCTTTCCAAC-3' | and A3-4           | GenBank: EF153627 |
| TMSA3-3/4              | 5'-CCCTCTGGCCTGGGCTCTC-3' |                    |                  |
| SA3-5F                 | 5'-CTCCGGCAACATCTCGGG-3' | bovSERPINA3-5       | GenBank: EF153628 |
| SA3-5R                 | 5'-GTACATCAGGGAGCCTCCAC-3' |                    |                  |
| TMSA3-5                | 5'-TGCTGAGACCCAGAGGAGG-3' |                    |                  |
| SA3-6F                 | 5'-ACCCCTTACCTCCGGAGC-3' | bovSERPINA3-6       | GenBank: EF153629 |
| SA3-6R                 | 5'-ACAGAGCTCCTTCCGGTC-3' |                    |                  |
| TMSA3-6                | 5'-CACGTGCTTCTGCTGGCAGG-3' |                    |                  |
| SA3-7F                 | 5'-AGGGCTTCCTCCGCTCC-3' | bovSERPINA3-7       | GenBank: EF153630 |
| SA3-7R                 | 5'-TCCTCCTGCTGCTCCCTC-3' |                    |                  |
| TMSA3-7                | 5'-ACCAAGCTGCTGAGGTCCTCCAG-3' |                    |                  |
| SA3-8F                 | 5'-CTGGGCTCTGCTGCTGCTG-3' | bovSERPINA3-8       | GenBank: EF153631 |
| SA3-8R                 | 5'-GGAAAGCCAAAGGCTTGGAGG-3' |                    |                  |
| TMSA3-8                | 5'-GAAGAAGAAGGAGGCTGAGGCCC-3' |                    |                  |
| TFIID-F                | 5'-CGTGGGCAAAATGTTGGAAT-3' | TFIID              | GenBank: NM_001075742 |
| TFIID-R                | 5'-TTCACCTTTGGCTCTGTTGGA-3' |                    |                  |
| TMTFIID                | 5'-TAAGAGAGGCCGCCAGGACTGCA-3' |                    |                  |

60 min at 50°C and enzyme was inactivated for 15 min at 70°C. cDNAs were stored at −20°C until use.

4.4. Primers and probes design

Each set of primers and the corresponding probe were chosen to be as specific as possible for the different transcripts. Primers and TaqMan® probes were designed using the primer analysis software PRIMER EXPRESS v. 2.0 (Applied Biosystems, Foster City, CA). Primers were synthesized by MWG Biotech (Courtaboeuf, France) and TaqMan® FAM dye-labelled probes by Applied Biosystems.

4.5. Quantitative real-time PCR

The sets of primers (table 3) were selected for their reaction efficiencies (close to 100%) determined from calibration curves and melting curve analysis. These primers were validated with cDNA dilution series (10⁻¹, 10⁻², 10⁻³, 10⁻⁴ and 10⁻⁵) from adult liver cDNA. For each set of primers, melting curve analyses were done and PCR products were purified and sequenced for confirmation. Each assay was performed with three pooled cDNA samples and was made in triplicate. No acceptable suitable set of specific primers was found for bovSERPINA3-2. Only a common set of primers was designed for bovSERPINA3-3 and bovSERPINA3-4. Transcription factor IID (TFIID) was used as internal RNA control to normalize samples for expression.

Using optimized qPCR conditions, all targeted mRNAs were detected using TaqMan® technology in all examined bovine tissues. For each set of experiments, the results were calibrated using the value of the testis expressed genes. The cycle threshold (Ct) values varied (electronic supplementary material, table S1) indicating that transcript abundance is gene and tissue-related.

Quantitative real-time PCR was carried out in triplicate using the ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems) in 96 well microtitration plates. Each qPCR was performed in a final volume of 20 μl containing 8 μl of appropriate dilution cDNA template (three pooled equivalent samples), 10 μl of MasterMix (2 × TaqManTM Universal PCR MasterMix, no amperase UNG-Applied Biosystems), 200 nM of labelled probe and 300 nM of forward and reverse primers. The qPCR protocol was as following: denaturation by a hot start at 95°C for 10 min followed by 40 cycles of a two-step programme (denaturation at 95°C during 15 s and annealing/extension at 60°C for 1 min). qPCR data were analysed using the appropriate threshold set-up as recommended by Applied Biosystems (SDS 2.3) before being transferred to Microsoft EXCEL. The slope of the calibration curve was calculated from the plot of log₁₀ of initial target copy number versus the corresponding Ct. The PCR efficiency (E) was determined from the slope of the curve obtained with serially diluted samples, as $E = 10^{-1/\text{slope}}$. In a sample, the expression levels of target genes are normalized to the endogenous control (average of 2). This is given by $ΔCt$, were $ΔCt$ is determined by subtracting the average endogenous gene Ct value from the average target gene Ct value $[Ct \text{ target gene} - Ct \text{ average (endogenous gene)}]$. The calculation of $ΔΔCt$ involves subtraction of the $ΔCt$ value for the controls
from the $\Delta C_i$ value for the cases $[\Delta C_i \text{ target gene}_{(\text{case})} - \Delta C_i \text{ target gene}_{(\text{control})}]$. The $RQ$ value ($RQ = 2^{-\Delta C_i}$) is the relative expression of the target gene compared with the control (electronic supplementary material, table S2). Proportions of transcripts of bovSERPINA3 genes are calculated by subtraction of $C_i \text{ target gene}_{(\text{case})}$ from the number of cycles divided by the addition of $[C_i \text{ target gene}_{(\text{case})} - \text{number of cycles}]$ and are expressed as percentages (electronic supplementary material, table S3). Data were transferred to Microsoft Excel, and proportions of transcripts of bovSERPINA3 genes were statistically evaluated through numerous pairwise comparisons using Student's $t$-test. Differences were considered significant when $p < 0.05$.

4.6. Expression and purification of recombinant bovSERPINA3-7

Expression plasmid encoding mature bovSERPINA3-7, excluding the putative signal peptide, was constructed in pET19b plasmid vector (Novagen, Madison, WI) as previously described for bovSERPINA3-3 [36]. The bovSERPINA3-7 protein was expressed in Escherichia coli BL21-RP CodonPlus (DE3)-RP strain (Stratagene, La Jolla, CA). Protein was expressed with an NH2- His tag to the N-terminus, which allows affinity purification on a Ni2+- column. Purification was performed using a Ni-NTA Fast Start column (Qiagen) according to the manufacturer’s instructions, and subsequent dialysis.

4.7. Protein extraction and SDS-PAGE analyses

One hundred milligrams of tissue pieces and 800 $\mu$L of extraction buffer (Tris-HCl 50 mM pH 7.5, KCl 150 mM, EDTA 4 mM) supplemented with protease inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany) and 200 mM $\beta$-mercaptoethanol were added to a 2 ml lysis Matrix E tube (MP Biomedicals, Santa Ana, CA). The tube was processed in the FastPrep FP120 instrument (Thermo Savant, Holbrook, NY) for three periods of 20 s at a setting of 6. After homogenization, the tube was centrifuged at 10 000g for 1 min at 4°C and supernatant was collected. Concentrations of the proteins extracted from bovine tissues were measured as described in [53]. SDS-PAGE was performed as described previously [54], under reducing conditions on 10% or 12% acrylamide separating gels. Proteins (40 $\mu$g) were solubilized with 2 $\times$ reducing loading buffer (2% SDS, 20% glycerol, 100 mM Tris-HCl, pH 6.8, 0.1% bromophenol blue, 5% $\beta$-mercaptoethanol). Molecular masses were estimated using the Precision Plus Protein Standards calibration kit (BioRad, Hercules, CA). Proteins were revealed with 0.25% Coomassie brilliant R-250 solution.

4.8. Antibodies

Six primary antibodies were used in this study. Polyclonal anti-bovine SERPINA3s antibody was raised against purified bovSERPINA3-1 [55]. It cross-reacted with all of the bovine SERPINA3 proteins. Rabbit antisera specific for bovSERPINA3-7 (Agro-Bio, La Ferté St. Aubin, France) was raised in rabbit against a synthetic peptide FFKAQWKTTPFNPNHLYES found only in the amino acid sequence of bovSERPINA3-7 and in no other animal or bacterial protein. Immunoglobulins (IgGs) from antisera were purified by protein A sepharose according to the manufacturer’s protocol (GE Healthcare, Chalfont St Giles, UK) and used for immunoblotting at a dilution of 1:1500.

Four commercial antibodies were also used in this study: IgG1 mouse monoclonal anti-myotilin (A. Menarini Diagnostics France Bond & Novoceastra Reagents, Rungis, France), goat polyclonal anti-myomesin-1 (Santa Cruz Biotechnology, Information Systems, Santa Cruz, CA), IgG1 mouse monoclonal anti-slow skeletal myosin heavy chain antibody (Abcam, Paris, France) and rat monoclonal anti-laminin 2 alpha (Abcam).

For Western blot analyses, a second antibody, swine anti-rabbit IgG conjugated to horseradish peroxidase (DAKO, Glostrup, Denmark), was used (dilution 1:1000). For microscopy analyses, several second antibodies were used and are described in §4.16.

4.9. Western blot analyses

Separated proteins were then transferred onto a PVDF Western blotting membrane (Roche Diagnostics) and electroblotted for 40 min at 200 mA. After overnight saturation at 4°C, the membrane was first incubated under agitation with a primary antibody for 1 h at 20°C, and then with a second antibody conjugated to horseradish peroxidase. The immunoblot was processed by chemiluminescence detection (Chemiluminescence Blotting Substrate (POD), Roche Molecular Biochemicals, Mannheim, Germany).

4.10. Deglycosylation

For deglycosylation assays, protein extracts were treated with different combinations of enzymes from an enzymatic protein deglycosylation kit (Sigma-Aldrich, St Louis, MO) according to the manufacturer’s instructions. O-Deglycosylation assays required the use of sialidase A for cleavage of terminal sialic acid residues, O-glycosidase to remove the core Galβ(1→3)-GalNAc, β(1→4)-galactosidase and β-N-acetylglucosaminidase to remove sugars associated with specific O-linked glycan structures. To remove all N-linked glycans, we used the PNGase F.

4.11. Enzyme assay

Porcine pancreatic elastase (Calbiochem, La Jolla, CA) was titrated using human plasmatic α1-antitrypsin (Calbiochem). The activity was determined by using the fluorogenic substrate MeOSuc-AAPV-AMC (Calbiochem). Equimolar amounts of active enzyme and bovSERPINA3-7 (10 nM) were used for kinetic studies to determine the association rate constant ($k_{assoc}$). The serpin was pre-incubated with elastase in 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM CaCl2 at room temperature (25°C) for given periods of time. Substrate (100 $\mu$M) was then added, and residual enzyme activity was measured at timed intervals. The second-order rate constant $k_{assoc}$ was computed as the slope of the plot of the reciprocal of free enzyme [I]/[E] over time of incubation with inhibitor (t), based on the kinetic equation as 1/[E] = $k_{assoc}$ (t + 1/[E]0), where [E]0 = initial enzyme concentration and [E] = residual enzyme concentration [56].
4.12. Immunoprecipitation

Anti-bovSERPINA3-7 polyclonal antibody was used for immunoprecipitation experiments. In first step, IgG antibodies were purified from rabbit serum on a Hitrap protein AHP column packed with 1 ml of protein A sepharose (GE Healthcare Bio-Science, Uppsala, Sweden) according to the manufacturer’s instructions. Purified immunoglobulins were dialysed and then chemically cross-linked with the protein AG, a genetically engineered protein which combines the IgG binding domains of both proteins A and G, coated on the surface of the magnetic beads. Dissected muscle from *longissimus thoracis* (20 mg) was homogenized and solubilized in extraction buffer (Tris-HCl 50 mM, pH 7.5, KCl 150 mM, EDTA 4 mM, pH 8.0). After centrifugation at 10 000g for 20 min at 4°C, the resulting supernatant was incubated with 20 μl of beads for 30 min at room temperature under agitation. After four washing steps with an excess of neutral extraction buffer, immunocomplex was collected with 50 μl of acid elution buffer (glycine 0.1 M, SDS 0.1%, pH 2.5) and immediately neutralized with 3 μl Tris-HCl 1 M, pH 9.0. Aliquots were analysed by Western blot using the specific antibody relative to the component that we intended to detect.

4.13. In-gel protein digestion

All chemical products for mass spectrometry analysis were purchased from Sigma (St Louis, MO). Sequencing grade-modified trypsin used for protein digestion was purchased from Promega (Charbonnieres, France). Bands of interest were excised from the gel and in-gel digestion was performed as described [57] with minor modifications. Briefly, bands were destained and vacuum dried. After washes (acetonitrile 50% (twofold)), NH₄HCO₃ 25 mM, acetonitrile 50%, acetonitrile 100%), proteins were reduced with 25 mM DTT in 100 mM NH₄HCO₃ for 60 min at 56°C, and alkylated with 25 mM iodoacetamide in 100 mM NH₄HCO₃ for 60 min at room temperature in the dark. Trypsin digestion was performed overnight at 37°C (20 ng μl⁻¹ in 25 mM NH₄HCO₃). The resulting peptides were extracted successively by acetonitrile 50%–formic acid 0.25% (twofold), acetonitrile 100%. Extracted fractions were pooled and vacuum dried overnight and resuspended in 25 μl of acetonitrile 2%–trifluoroacetic acid 0.08% for mass spectrometry analysis.

4.14. LTQ-Orbitrap analysis and database searching

Four microlitres of the tryptic digest was analysed by nanoLC-MS/MS using LTQ-Orbitrap Discovery mass spectrometer (Thermo Scientific) interfaced with an Ultimate™ 3000 RSLCnano System (Thermo Scientific). Peptides were separated on a 2 μm C₁₈ PepMap column (Thermo Scientific). Mass data collected during analysis were processed by the open source Xtandem pipeline parser 3.3 (The Global Proteome Machine, http://www.thegpm.org) and the MS/MS data were used to query databases Uniprot_Bos_taurus_18072012.fasta, \ contaminants_standards.fasta and *Oryctolagus cuniculus*.fasta with the following criteria: 0.5 Da for peptide and fragment mass tolerances, one missed trypsin cleavage site allowed, carbamidomethylation of cysteine residues (from iodoacetamide exposure) and methionine oxidation as variable modifications. The protein identification, filtered for bovine and rabbit species, was established for protein score: logEvalue prot. < −2.6, peptide score: Evalue pep. <0.05 at least two peptides, and automatic elimination of the result set on the contaminating proteins.

4.15. Immunostaining

Cryosections were first blocked in phosphate-buffered saline (PBS) with 10% goat serum for 1 h at room temperature. After two washing steps of 3 min with PBS, primary antibodies were added (range of dilution: 1/400–1/200 according to the used antibody) and incubated for 1 h at room temperature. After two other washing steps of 3 min, secondary antibodies were added (range of dilution: 1/400–1/200 according to the used antibody) for 50 min at room temperature. Samples were washed again two times in PBS for 3 min before adding DAPI (dilution: 1/1000; Invitrogen-Life Technologies, Saint Aubin, France). The specimens were finally washed for a last time for 3 min and mounted on glass slides with Fluoromount™ Aqueous Mounting Medium (Sigma-Aldrich, Saint-Quentin Fallavier, France).

Controls for immunofluorescence staining were treated as above under the same conditions, except that the primary antibody was replaced by pre-immune serum or the same class of immunoglobulin (for an isotypic control). A final control of the specificity of the immunochromic procedure was checked by incubation of cryosections with the secondary antibodies without primary antibodies.

All incubations were carried out in a humid atmosphere.

4.16. Immunofluorescence light microscopy

Immunoreactions were detected with indirect fluorescence using F(ab”)₂ goat IgG anti-mouse Alexa Fluor® 488 (Invitrogen-Life Technologies) or F(ab”)₂ donkey IgG anti-goat Dylight® 488 (Abcam) or goat IgG anti-rat Alexa Fluor® 488 (Invitrogen-Life Technologies) for green fluorescence and F(ab”)₂ goat IgG anti-rabbit Alexa Fluor® 546 (Invitrogen-Life Technologies) or F(ab”)₂ donkey IgG anti-rabbit Dylight® 594 (Abcam) for red fluorescence.

Double labelling was performed simultaneously using one monoclonal and one polyclonal antibody followed by two secondary antibodies coupled to fluorochromes of different wavelengths.

Microscopy analyses were carried out using an epifluorescence Leica Digital Microscope inverted DMI 6000B (Leica Microsystems SAS All Microscopy and Histology, Nanterre, France). Image processing was done using the image processing software MetaMorph. Confocal laser microscopy analyses were performed using a laser scanning microscope Zeiss LSM 510 META (Carl Zeiss Jena GmbH, Jena, Germany). Images were recorded and processed using VOLUMETRY® three-dimensional image analysis software (PerkinElmer, San Jose, CA).

Competing interests. The authors declare that they have no conflict of interest.

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