ANTITHROMBIN III FROM BOVINE SKELETAL MUSCLE: PURIFICATION, CHARACTERISATION AND CELLULAR LOCATION

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Introduction

In blood, thrombin activity is mainly driven by antithrombin III (AT-III), a specific inhibitor. Extravascular thrombin functions must be regulated and AT-III is a potential candidate. The enzyme expressed by muscle cells is located in the vicinity of the external leaflet of the plasma membrane at the neuromuscular junction level. This feature is in good agreement with a thrombin regulatory function at the neuromuscular junction (Liu *et al.*, 1994). AT-III was found inside all fibres of mouse skeletal muscles suggesting that, as thrombin, this protein is also expressed in muscle cells (Businaro *et al.*, 1995). In cattle, the presence of a signal peptide is undoubted (Mejdoub *et al.*, 1991). Indeed, because thrombin is located extracellularly, the control of its activity by muscle AT-III means that serpin must be exported. Despite the fact that AT-III is most likely expressed in skeletal muscle, the protein has never been purified and characterized. The present work is therefore intended to purify and characterize AT-III from bovine skeletal muscle.

Materials and Methods

Purification of AT-III was carried out from a crude extract prepared as in Bige *et al.*, (1985) from bovine *diaphragma* muscle within 1h post exanguination. The procedure comprises of four to five chromatography steps. *Inhibitory activity* against trypsin was measured as described in Tassy *et al.*, 2005. *Titration* of bovine pancreatic trypsin, bovine plasmin and thrombin were carried out as in Chase and Shaw (1967). Chymotrypsine was titrated as described in Kezdy and Kaiser (1969). *Stoichiometry* of the enzyme–inibitor interaction was determined: predefined concentrations of enzyme were incubated with increasing amounts of muscle AT-III and the residual activity was measured. *Second order association rate constant* (K_{ass}) of the muscle AT-III for the peptidases tested: thrombin, trypsin, chymotrypsin and plasmin were determined as described in Schechter and Plotnick (2004). *SDS/PAGE* was perfomed as described previously (Laemmli, 1970). Molecular masses were estimated by using the Pharmacia low M_r calibration kit. *Immunoblots* were carried out as described previously (Sentandreu *et al.*, 2003). *pH stability* of the purified inhibitor was tested in the pH range 2 to 12. *Heat stability* of the AT-III was determined at different temperatures ranging from 40°C to 100°C. *Sequencing N-terminal* of muscle AT-III was perfomed using an Applied Biosystems 477A pulsed liquid sequencer. *MALDI-TOF mass spectrometry analysis*: peptide map of the trypsin digest was perfomed as previously described (Sentandreu *et al.*, 2003). *Immunolocalization* of AT-III was perfomated on traverse sections of bovine onglet muscle using the rabbit anti-human AT-III polyclonal antibody.

Results and Discussion

Identity of the purified serpin was assessed using different technical approaches. SDS-PAGE analysis of purified AT-III showed only one band with an estimated molecular mass of 58 kDa (Figure 1, lane b). The rabbit anti-human AT-III polyclonal antibody specificity was analysed using muscle crude extract and the purified protein. As shown in Figure 2a, after SDS-PAGE, the antibody labelled only one band in the crude extract (lane 1) running similarly to the purified AT-III (lane 2). In non denaturing conditions, this antibody recognized also one band in the crude extract (Figure 2b lane 1) running as the purified AT-III (Figure 2b, lane 2). These findings suggest a high specificity of the antibody which will be used for the AT-III localisation in muscle tissue.

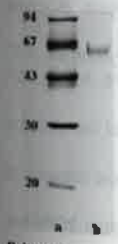

Figure 1: The inhibitor was run in denaturating conditions on a 12.5% polyacrylamide slab gel and revelated by silver staining. (a) Mr markers. (b) Purified AT-III.

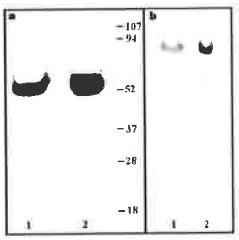

Figure 2: Analysis by immunoblotting using the rabbit anti-human AT-III polyclonal antibody. (2a, line 1): crude extract; (2a line 2): purified AT-III. In non denaturanting conditions (2b line 1): crude extract; (2b line 2): purified AT-III.

Primary sequence analysis using the Edman procedure provided the following sequence: H^{1}RSPVEDVCTAKPR^{14}. 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 (Mejdoub *et al.*, 1991). The strong labelling of the purified muscle AT-III by a polyclonal antibody raised against human antithrombin III supported our findings. Finally, mass spectrometry trypsin peptide map identified this protein as bovine AT-III, the peptide finger print covering about 60 to

70% of the bovine protein sequence (not shown). Several sets of evidence therefore stressed that the purified serpin is undoubtedly bovine AT-III.

Treatment of AT-III for 15 min at different temperatures ranging from 35 to 100°C confirmed the low thermal stability of normal bovine AT-III (Figure 3a). A decrease of about 2 to 5% in the activity was already observed after 15 min at 40°C. At 45 °C an activity loss of 15% is generally achieved. Analysis of the data using a sigmoid curve fitting programme gave a temperature of transition Tm of 52.7 °C. Bovine AT-III is relatively stable only at pH 10. Above this value the activity falls sharply to about 30% of its maximal activity at pH 12.

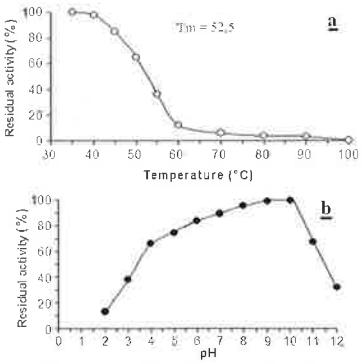

Figure 3: (a) Residual activity (percentage of the control) of AT-III: (a) Heated 15 min at different temperatures ranging 40° to 100°C. (b) Pre-incubated for 1h at different pH ranging from 2 to 12.

Table 1: Association rate constants for inhibition of serine proteinases.

	Cofactor	k_{ass} ($M^{-1}s^{-1}$)
Trypsin	-	5×10^5
Chymotrypsin	-	$6,8 \times 10^3$
Plasmin	-	$1,7 \times 10^4$
Thrombin	- heparine -	$1,8 \times 10^5$
	+ heparine (5 µM)	$2,3 \times 10^7$

Stoichiometry of inhibition (SI): These results stressed forward that AT-III inhibition of both trypsin and thrombin occurs through a 1/1 enzyme/inhibitor ratio (SI=1). Inhibitory pattern of bovine muscle AT-III and association rate constant: the results depicted in Table 1 indicate that, though to various extent, all peptidases are sensitive to the inhibitory action of AT-III. In the presence of heparin, the k_{ass} for thrombin increased about 100 fold whereas other peptidases did not show any sensitivity to this cofactor (not shown). Several other serine peptidases were also tested including Human leukocyte elastase, cathepsin G Human kidney urokinase, porcine pancreatic kallikrein and tissue-plasminogen activator and none of them seemed to be inhibited by bovine muscle AT-III. The muslce AT-III is highly concentrated between the plasma membrane and the myofibrils, indicating that muscle AT-III is exclusively intracellular with a preferential peripheral localization.

Conclusion

Antithrombin III has so far never been purified from muscle tissue and this is the first time that a purification procedure was proposed. The present work further stressed that AT-III must definitely be included as a new partner in the studies on the role of thrombin in muscle development. Using intact skeletal muscle and primary muscle cells, examination, at the gene and protein levels, of the AT-III functions in muscle cell differentiation and muscle development will be therefore the next step in our view of a better understanding of the role of thrombin/antithrombin in these complex processes.

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