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

Herrera M. Carlos[1], Gomez L. Blanca[1], Hernandez M. Alejandro[1],
Alejo L. Sergio[1], Arroyo F. Gabriela[1], Vargas R. Lorena[1] and Martinez F. Dora[2]

[1]Departamento de Ingenieria Agroindustrial, Campus Celaya-Salvatierra, Universidad de Guanajuato, Salvatierra, Gto. Mexico;
[2]División de Negocios, Universidad Anahuac, Mayab, Merida, Yucatan, Mexico.

Abstract – In the present study, Antithrombin III (AT-III) was purified from bovine muscle. Identity of the purified serpin was assessed using different approaches including N-terminal sequencing, Western-blot and Maldi-tof mass spectrometry peptide map. All results demonstrated that the purified muscle serpin is undoubtedly antithrombin III.

Key Words – Bovine, muscle, antithrombin III, serin proteinase, serpin.

I. INTRODUCTION

In blood, thrombin activity is mainly driven by antithrombin III (AT-III) [1]. AT-III was found inside all fibres of mouse skeletal muscles suggesting that, as thrombin, this protein is also expressed in muscle cells [2]. Despite the fact that AT-III must be very likely expressed in skeletal muscle, the protein has never been purified and characterized.

II. MATERIALS AND METHODS

Purification of AT-III was carried out from a crude extract prepared from bovine *Diaphragma* muscle within 1h post exanguination. The procedure comprise four to five chromatography steps. Monitoring of trypsin inhibitory activity in collected fractions, enzyme titration, stoechiometry of the enzyme/inhibitor interaction, measurement of the association rate constants, polyacrylamide gel electrophoresis were perfomed. ***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° 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. ***Immunolocalization*** of AT-III was perfomated on traverse sections of bovine *Diaphragma* muscle using the rabbit anti-human AT-III polyclonal antibody.

III. 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 (Fig. 1, lane b).

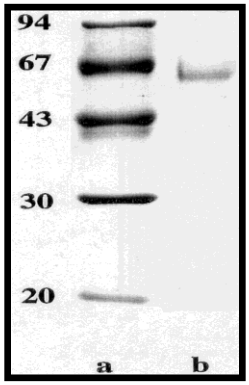

Figure 1. The inhibitor was run in denaturating conditions on a 12.5% polyacrylamide slab gel and revelated by silver staining. (a) Mr markers. (b) Purified AT-III.

The rabbit anti-human AT-III polyclonal antibody specificity was analysed using muscle crude extract and the purified protein. As shown in Fig 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 (Fig 2b lane 1) running as the purified AT-III (Fig 2b, lane 2). These findings suggest a high specificity of the antibody which will be used for the AT-III localisation in muscle tissue.

Primary sequence analysis using the Edman procedure provided the following sequence: H1RSPVEDVCTAKPR14. This sequence showed 100% similarity with the cDNA deduced sequence of the mature bovine AT-III (SwisProt accession number: P41361). The strong labelling of the purified muscle AT-III by a polyclonal antibody raised against human Antithrombin III comforted our findings. Finally, mass spectrometry trypsin peptide map identified this protein as bovine AT-III.

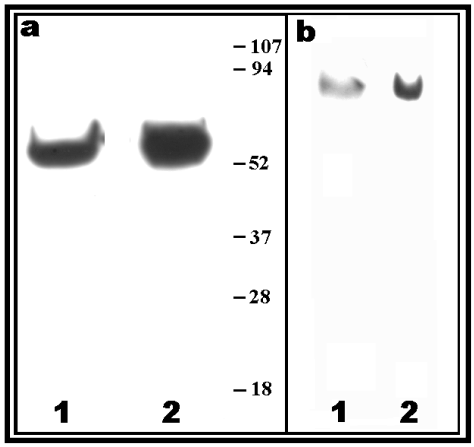

Figure 2. Analysis by immunoblotting using the rabbit anti-human AT-III polyclonal antibody. (2a, line 1): crude extract; (2a line 2): purified AT-III. In non denaturanting conditions (2b line 1): crude extract; (2b line 2): purified AT-III.

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 (Fig. 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.

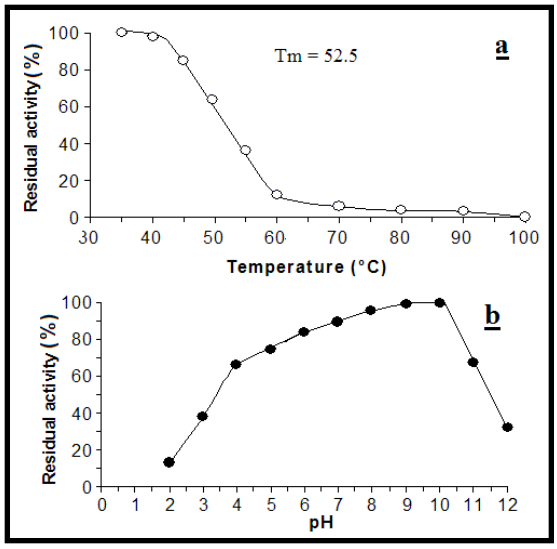

Figure 3. Residual activity of AT-III: (a) Heated 15 min, temperatures ranging 40° to 100°C. (b) Pre-incubated for 1h at different pH ranging from 2 to 12.

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 (Table 1) indicate that all peptidases are sensitive to the inhibitory action of AT-III. In the presence of heparin, the kass for thrombin increased about 100 fold. 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.

Table 1. Association rate constant (kass) for serine proteinases

Proteinase tested	kass (M^{-1}, s^{-1})
Trypsin	5×10^5
Chymotrypsin	6.8×10^4
Plasmin	1.7×10^4
Thrombin (- heparin) (+ heparin)	 1.8×10^5 2.3×10^7

IV. CONCLUSION

Antithrombin III has never been so far 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 be absolutely 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.

ACKNOWLEDGEMENTS

This work was supported by the CONACYT (Mexico) and French National Institute for Agricultural Research (INRA).

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