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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

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ABSTRACT

Thrombin/antithrombin III (AT-III) proteolytic system is well known for its function in blood coagulation. Thrombin is expressed in skeletal muscle but nothing is known about the presence of AT-III in the tissue. In postmortem muscle this system has been and is still totally ignored. We therefore successfully attempted to purify AT-III from bovine skeletal muscle and characterized the purified protein (identified as AT-III by N-ter sequencing and mass spectrometry finger print) for its physicochemical and inhibitory properties. As the human blood serpin, muscle AT-III is thermolabile and stable only at alkaline pH (pH 9–10). The muscle serpin inhibits strongly thrombin in a heparin dependent manner and trypsin. Phosphatidylserine (PS) externalization demonstrated in the present work suggested that prothrombin can be activated to thrombin through binding of the activator complex on the external PS groups. PS externalization is concomitant with shrinkage of muscle fibers indicating that muscle cells are engaged in the cell death program known as apoptosis few minutes after death. We then discussed the potential role of this proteolytic system in postmortem apoptosis development as well as in the control of low voltage electrical stimulation efficiency.

1. Introduction

Besides the major role of thrombin in the vascular system reviewed by Fenton (1986) and Mann (1999), several extravascular functions have been reported since the 1980s especially in muscle. In this tissue, thrombin is synthesized by muscle cells (Citron, Smirnova, Zoubine, & Festoff, 1997) and acts locally by contributing to synapse remodeling and elimination at the neuromuscular junction (Liu, Fields, Festoff, & Nelson, 1994; Zoubine, Ma, Smirnova, Citron, & Festoff, 1996) but plays also an essential role in muscle cell differentiation (Chinni et al., 1999; Suidan, Niclou, Dreessen, Beltraminelli, & Monard, 1996). A prerequisite to the activation of thrombin at the neuromuscular junction is the local externalization of phosphatidylserine phospholipids which contribute to the binding of thrombin activator complex to the membrane (Boon, Lambert, Sisson, Davis, & Smith, 2003).

By contrast, the regulation of thrombin activity by specific inhibitors is still debatable. Protease nexin I is the first thrombin inhibitor identified in muscle tissue and since then has been considered to be the natural inhibitor of thrombin (Baker, Low, Simmer, & Cunningham, 1980; Rosenberg & Damus, 1973). However, the exact origin of PN-I in muscle has never been clearly established. More importantly, the possible local expression of AT-III by muscle cells or other neighboring cell types has never been tested despite the fact that the presence of AT-III within muscle cells has been reported about 10 years ago (Businaro, Toesca, Ortolani, & Fumagalli, 1995). In agreement with the finding of these last authors, preliminary analysis of muscle tissue by either western blot or immunohistochemistry always detected the presence of significant amounts of antithrombin III in this tissue where it has been further located intracellularly (Ishida et al., 2003).

On the other hand, we recently provide evidence supporting that apoptosis sets up in postmortem muscle few minutes after animal bleeding (Ouali et al., 2007), a finding in good agreement with our earlier statements (Herrera-Mendez, Becila, Boudjellal, & Ouali, 2006). This supposed that translocation of phosphatidylserine, an early hallmark of apoptosis and a necessary event preceding thrombin activation, would be detectable soon after

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death. In this case, all required conditions for thrombin activation would be fulfilled in postmortem muscle and its activity will depend only on the amount of antithrombin III or on the antithrombin III/thrombin ratio at the time of death.

Actually, nothing is known about the status of thrombin and its specific inhibitor antithrombin III in postmortem muscle and about the contribution of this enzyme to postmortem proteolysis. Moreover, we previously emphasized that protease inhibitors are better markers of the extent of postmortem proteolysis than their target enzymes (Sentandreu, Coulis, & Ouali, 2002). Hence, to clarify the function of AT-III/thrombin couple in postmortem muscle, we undertook to purify antithrombin III from this tissue and to characterize its physicochemical and inhibitory properties with reference to the blood serpin. We also verified the externalization of PS which is a prerequisite for thrombin activation in postmortem muscle. Finally, the potential role of thrombin in postmortem muscle was shortly discussed with regards to its contribution to the conversion of muscle into meat and to some processing technologies applied postmortem such as low voltage electrical stimulation. It was concluded that antithrombin III is present in muscle cells and that thrombin might participate to apoptosis development through its contribution to isolate cells from neighboring ones and from the nerve network. Whether the enzyme contributes to the degradation of intracellular proteins remains unclear but the degradation of the nerve plate will be very likely responsible for the well known time dependent decreasing efficiency of low voltage electrical stimulation.

2. Materials and methods

2.1. Materials

Antithrombin III (AT-III) was purified from bovine *Diaphragma* muscle. Thrombin from bovine plasma (EC 3.4.21.5) was from Roche diagnostics (Meylan, France). Bovine pancreatic trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1) and plasmin (EC 3.4.221.7) were from bovine plasma, N-CBZ–Phe–Arg–NHMec, Boc–Val–Pro–Arg–NHMec, Suc–Ala–Phe–Lys–NHMec, Suc–Ala–Ala–Pro–Phe–NHMec (where: CBZ, benzyloxycarbonyl; NHMec, aminomethyl coumarylamide; Boc, *t*-butyloxycarbonyl; Suc, succinyl), heparin, N-*trans*-cinnamoyl-imidazole, 4-Nitrophenyl-p-guanidinobenzoate and the rabbit anti-antithrombin III polyclonal antibody were from Sigma Chemical Co. (St Quentin-Fallavier, France). Sephadex G-100, SP-Sepharose, Q-Sepharose, Ultrogel ACA44 were purchased from Amersham Pharmacia Biotech (Orsay, France). Fractogel EMD-DEAE-650 was from Merck (Norgent-sur-Marne, France). Goat anti-rabbit IgG conjugated with FITC was from Jackson Immunoresearch Laboratories (Baltimore, MD, USA).

2.2. Purification of muscle AT-III

The purification procedure included five chromatography steps and starts from a crude extract prepared as in Bige, Ouali, and Valin (1985), from bovine *Diaphragma pedialis* muscle obtained within 1 h after killing. Briefly, a 40–70% $(NH_4)_2SO_4$ precipitate was dissolved in 40 ml of 20 mM NaCl, 30 mM Tris/HCl buffer, pH 7.6, dialyzed overnight against the same buffer and then clarified by centrifugation at 20,000*g* for 15 min. The dialyzed extract was fractioned on a Sephadex G-100 column (5 cm × 100 cm) previously equilibrated with the same buffer. Elution was carried out at a flow rate of 24 ml/h and 10 ml fractions were collected. Absorbance at 280 nm was determined for all fractions using a Beckman DU-70 spectrophotometer (Beckman Coulter France, Villepinte). Inhibitory activity against trypsin was measured in all fractions as described below. The first eluted active peak containing proteins of highest Mr was dialyzed against 50 mM sodium acetate buffer pH 5.2 (buffer A). This dialyzed fraction was then applied to a SP-Sepharose column (5 cm × 10 cm) equilibrated with buffer A and bound proteins eluted at 3 ml/min with a 0–0.2 M linear NaCl gradient. Fractions of 4.5 ml were collected. Fractions from the last active peak eluted at the end of the gradient was dialyzed against 30 mM Tris/HCl buffer pH 8.0 (buffer B) and loaded on a Q-Sepharose column (2.5 cm × 10 cm) previously equilibrated with buffer B. Bound proteins were eluted at 3 ml/min with a linear 0–0.25 M NaCl gradient and fractions of 4.5 ml collected. Fractions from the only peak active against trypsin were collected, dialyzed against buffer C (30 mM Bis/Tris buffer pH 6) and applied to a Fractogel EMD-DEAE-650 column (1 cm × 8 cm) equilibrated with buffer C. Proteins were eluted at 1 ml/min with a linear 0–0.25 M NaCl gradient and 2.0 ml fractions collected. Active fractions were pooled and low Mr contaminants were eliminated by gel filtration on an Ultrogel ACA44 column (1.7 × 77 cm). Pooled eluted fractions were concentrated on an Amicon cell (YM-3 membrane) and stored at 0–4 °C until subsequent use.

2.3. Monitoring of the trypsin inhibitory activity in collected fractions

Aliquots of each fraction (30 μl) were mixed with 750 μl of a trypsin solution (40 μg/ml) in 50 mM Tris/HCl buffer, pH 8.0, containing 10 mM $CaCl_2$, and then incubated at 37 °C for 20 min before addition of 250 μl of a 40 μM substrate stock solution (N–CBZ–Phe–Arg–NHMec). After 20 min, the reaction was stopped by adding 3 ml of a stopping solution containing 100 mM chloroacetate, 70 mM acetic acid and 30 mM sodium acetate, pH 4.3. Fluorescence was then measured using a PerkinElmer LS 50B spectrofluorimeter (λ excitation at 360 nm – λ emission at 440 nm). Unless otherwise indicated, all activity measurements were performed at 37 °C.

2.4. Enzyme titration

Titration of bovine pancreatic trypsin, bovine plasmin and thrombin were carried out as in Chase and Shaw (1970) using 4-nitrophenyl p-guanidinobenzoate. Chymotrypsin was titrated as described in Kezdy and Kaiser (1969) using N-*trans*-cinnamoyl-imidazole.

2.5. Stoichiometry of the enzyme–inhibitor interaction

Predefined concentrations of trypsin and thrombin were incubated with increasing amounts of muscle AT-III and the residual activity was measured as described above.

2.6. Measurement of the association rate constants

Second-order association rate constant (k_{ass}) of the muscle AT-III for the target peptidases tested, *i.e.* thrombin, trypsin, chymotrypsin and plasmin, were determined as described in Schechter and Plotnick (2004). The purified inhibitor (5 nM) was pre-incubated with equimolar amounts (5 nM) of trypsin in 50 mM Tris/HCl buffer, pH 8.0, containing 10 mM $CaCl_2$ for given periods of time. 100 μl of 100 μM substrate stock solution (N-CBZ–Phe–Arg–NHMec for trypsin) were then added and the mixture incubated for 20 min before measuring residual activity. The association rate with plasmin and chymotrypsin was determined by pre-incubating the enzymes (15 nM) with a 10-fold molar excess of inhibitor in 50 mM Tris/HCl buffer containing 100 mM NaCl, pH 7.5, in a total volume of 200 μl. After the substrates (Suc–Ala–Phe–Lys–NHMec and Suc–Ala–Ala–Pro–Phe–NHMec for plasmin and chymotrypsin respectively) were added at a final concentration of 100 μM and incubated for 20 min. The reaction was