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Characterization and purification of a Ca^{2+} ion-activated neutral proteinase inhibitor in rabbit skeletal muscle

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Summary. This paper describes the isolation, purification and properties of a specific inhibitor of calcium-activated neutral proteinase (CaANP) in rabbit skeletal muscle.

The inhibitor was a thermo-acid-stable protein degraded by trypsin and chymotrypsin and seemed to contain two polypeptide chains with molecular weights of 70 000 and 13 000 daltons. Maximal inhibitory activity was obtained at neutral pH. High salt concentrations were needed to suppressinhibition.

Inhibitor concentration had no effect on the optimal Ca^{++} ion levels for CaANP. These experiments also show that enzyme inhibitor association was instantaneous and did not need any incubation.

Introduction.

A Ca^{2+}-activated sarcoplasmic (CAS) factor disrupting myofibrils by removing the Z-disc was described by Busch *et al.* (1972). Subsequently, during CAS factor degradation of these Z-discs, α-actinin release (Suzuki, Nonami and Goll, 1975) and a decrease in ATPase activity (Suzuki and Goll, 1974) were reported. This proteinase might also account for certain *post-mortem* changes in myofibrillar structures (Penny, 1974 ; Penny, Voyle and Dransfields, 1974 ; Olson, Parrish and Stromer, 1976 ; Olson *et al.*, 1977). Reddy *et al.* (1975), Dayton *et al.* (1976), Ishiura *et al.* (1978) and Azanza *et al.* (1979) tried to purify this enzyme.

In attempting to purify the enzyme from rabbit skeletal muscle, no calcium-stimulated proteolytic activity on casein was detected in the crude extracts but after ion-exchange chromatography, this activity was easily measured.

Up to now, many proteinase inhibitors in various organs or tissues have been presumed (Noguchi *et al.*, 1974 ; Drummond and Duncan, 1966), or partially purified (Waxman and Krebs, 1978 ; Kopitar *et al.*, 1978 ; Nishiura *et al.*, 1978 ; Nishiura, Tanaka and Mukachi, 1979) but inhibition studies have never been carried out on pure electrophoretic species.

This paper describes the isolation and purification of a specific inhibitor of Ca^{2+} ion-activated neutral proteinase (CaANP) in rabbit skeletal muscle. The purification protocol led to a new molecule containing two polypeptide chains with molecular weights of 70 000 and 13 000 daltons in SDS polyacrylamide gel electrophoresis. Analysis of their physico-chemical properties may be considered to be a fundamental step in interaction studies on the enzyme inhibitor complex.

If highly specific proteinase inhibitors participate in proteolytic enzyme regulation processes, new perspectives may be opened concerning the *in vivo* regulation of CaANP and its potential functions in the turnover of myofibrillar proteins.

Material and methods.

1) *Purification procedure.* — The same homogenate of fresh rabbit skeletal muscle was used as starting material for preparing the CaANP and its inhibitor. Immediately after the rabbit had been killed, the skeletal muscles were quickly removed and homogenized with 2.5 volumes of 5 mM Tris-HCl buffer (pH = 7.40) containing 4 mM EDTA, 50 mM NaCl and 2 mM 2-mercaptoethanol in a Waring blender. The homogenate was centrifuged at 27 000 × g for 40 min and the soluble fraction filtered through cheesecloth. The pH was then adjusted to 7.00 and the new precipitate removed by centrifugation at 3 000 × g for 10 min.

Chromatography on Sephacel DEAE : the supernatant was run on a column of Sephacel DEAE (Pharmacia, France) (2.5 × 60 cm) equilibrated in 5 mM Tris/HCl buffer (pH = 7.40) containing 0.1 mM NaCl, 2 mM 2-mercaptoethanol. The protein was eluted from the column by a linear gradient of KCl (10 to 500 mM) in the same buffer. Enzyme purification was carried out by the usual methods (Azanza *et al.*, 1979). Only the inhibitor underwent the next steps.

Gel filtration on Sephacryl S-200 : the DEAE pool inhibitor fraction was concentrated tenfold by ultrafiltration on an Amicon XM50 membrane and applied to a column (2.5 × 100 cm) of Sephacryl S-200 (Pharmacia, France) equilibrated with 5 mM Tris/HCl buffer (pH = 7.40) containing 1 mM EDTA, 10 mM NaCl, 2 mM 2-mercaptoethanol.

Gel filtration on Sephadex G-100 : after heat treatment (70 °C for 5 min) and centrifugation at 5 000 × g for 10 min, the concentrated supernatant was separated by gel filtration on a column of Sephadex G-100 (Pharmacia, France) eluted by 0.1 mM Tris/HCl buffer (pH = 7.40) containing 0.1 mM EDTA, 50 mM NaCl and 2 mM 2-mercaptoethanol.

Gel filtration on Sephacryl S-200 in 6M guanidinium chloride : the inhibitor fractions concentrated with Amicon XM50 were put into a Sephacryl S-200 column equilibrated in 10 mM Tris/HCl buffer (pH 7.40) containing 6M guanidinium chloride.

2) *Assays of proteolytic activity* :

— on casein : casein was used as a substrate to quantitatively measure proteolytic activity according to Azanza *et al.* (1979) ;

— on myofibrils : myofibrils, prepared as described by Etlinger, Zak and Fischman (1976), were used as a substrate to quantitatively measure the effect of proteinase on α-actinin release (Azanza *et al.*, 1979).

Inhibition tests on Ca^{2+} ion-activated neutral proteinase : appropriate amounts of inhibitor and enzyme were both preincubated at 30 °C for 1 min before the reaction was started by adding casein substrate (1.5 ml). The reaction was subsequently stopped by adding 5 p. 100 TCA (v/v) and centrifuging the mixture. With each reaction, we ran control samples, adding the TCA immediately after the casein.

3) *Protein concentration.* — Protein concentration was determined by modifying the methods of Lowry *et al.* (1951), Azanza *et al.* (1979) and Bradford (1976) using bovine serum albumin as a standard.

4) *Sodium dodecyl sulphate polyacrylamide gel electrophoresis.* — SDS gel electrophoresis was performed according to Azanza *et al.* (1979).

5) *Molecular weight determination.* — The molecular weight of the pure inhibitor was determined as follows :

— by Sephadex G-100 filtration on a column calibrated with Dextran blue, α-actinin, bovine serum albumin, avalbumin, α-chymotrypsin and cytochrome-C ;

— by SDS/polyacrylamide gel electrophoresis using bovine serum albumin, ovalbumin, α-chymotrypsin and cytochrome-C as standards.

Results and discussion.

Inhibitor purification.

As shown in figure 1, the CaANP and its inhibitor contained in the supernatant were dissociated by Sephacel DEAE chromatography. These results confirm our hypothesis explaining the absence of Ca^{2+}-dependent neutral proteolytic activity owing to the presence of an inhibitor in the crude muscular extract.

After chromatography, the CaANP was purified (Azanza *et al.*, 1979) and the Inhibitory peak P_1 (fig. 1) was first filtered on Sephacryl S-200. The P_2 pool (fig. 2) was heated at 70 °C for 5 min and centrifuged. This treatment was the most important step of our purification procedure since it eliminated a protein contaminant and maintained 100 p. 100 of the inhibitory activity (table 1).

After filtration through a G-100 column, SDS/polyacrylamide gel electrophoresis showed that the inhibitory peak (pool P_3) (fig. 3) contained two distinct fractions having 70 000 and 13 000 daltons molecular weight (fig. 4). In order to determine if these two fractions were necessary to inhibitory activity, pool P_3 was concentrated and filtered through a S-200 column in guanidinium chloride.

After dialysis of the P_4 and P_5 pools (fig. 5), inhibitory activity was measured and compared to the control fractions. Inhibitory strength was found to be partially contained in peak P_4. However, our first results seem to indicate that when casein is used as a substrate, mixing P_4 and P_5 increases inhibitory activity. This suggests that the peptide with MW = 13 000 may have interfered with the inhibition procedure.

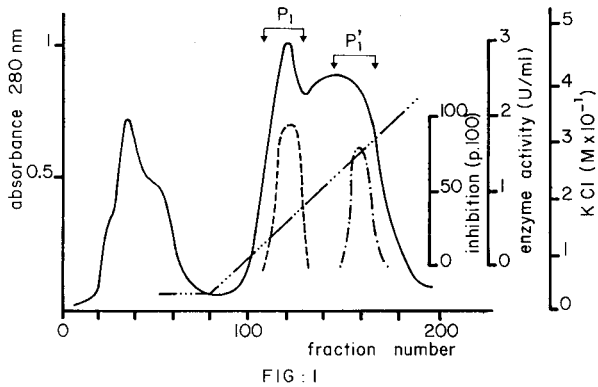

FIG : 1

FIG. 1. — *Fractioning of proteolytic and inhibitory activities of skeletal muscle on Sephacel DEAE* (——) absorbance at 280 nm ; (—·—) activity on casein ; (- - -) inhibition toward caseinolytic activity of CaANP : (—··—··-) KCl molarity.

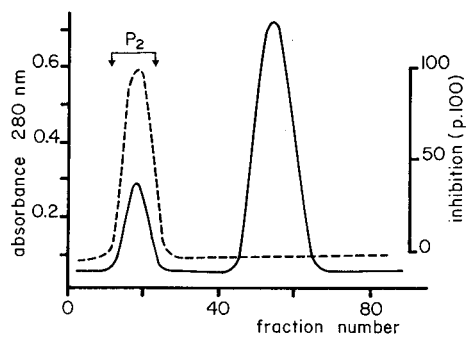

FIG : 2

FIG. 2. — *Gel filtration profile of the P_1 fraction inhibitor on Sephacryl S-200.* (——) absorbance at 280 nm ; (- - -) inhibition toward caseinolytic activity of CaANP.

TABLE 1

Some properties of the Ca^{2+} ion-activated neutral proteinase inhibitor from rabbit skeletal muscle.

Treatment of inhibitor sample		Inhibitory activity remaining (p. 100)
Control (without inhibitor)		80
+ RNAse		78
+ DNAse		79,5
+ trypsin		0
+ α-chymotrypsin		2
Heat treatment	50 °C for 20 min	78
	60 °C for 20 min	78
	70 °C for 20 min	80
	90 °C for 20 min	80
Acid treatment	pH = 1	78
	pH = 2	78
	pH = 3	80

Aliquots (3 ml, 0.2 mg/ml) from purified inhibitor were dialysed against 10 mM Tris-HCl (pH = 7.40) containing 50 mM NaCl, 10 mM $MgCl_2$, 3 mM 2-mercaptoethanol and treated under various conditions. Assays with proteases :

— *DNAse and RNAse* : 250 μg of inhibitor samples were preincubated with 100 μg of each enzyme at 30 °C for 1 hr ; assay was carried out directly on CaANP activity as previously described.

— *Trypsin and α-chymotrypsin* : 250 μg of inhibitor samples were preincubated with 100 μg of each enzyme at 37 °C for 1 hr.

pH : 8.00 and 7.60. The reactions were stopped by heating at 80 °C for 30 min and inhibition of CaANP activity was tested as previously described.

— *Heat treatment* : inhibitor samples were preincubated at 50, 60, 70 and 90 °C for 20 min and tested. on CaANP activity.

— *Acid treatment* : pH changes were obtained with HCl (N) and NaOH (N).

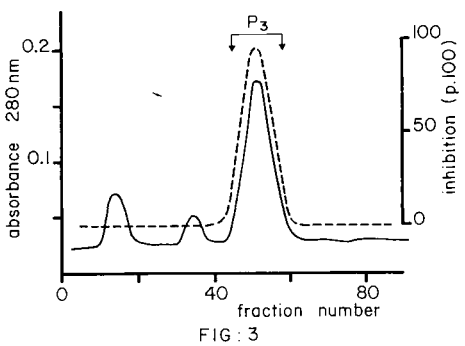

FIG : 3

FIG. 3. — *Sephadex G-100 gel chromatography of the heated P_2 inhibitor fraction.*
(——) absorbance at 280 nm ; (- - -) inhibition toward caseinolytic activity of CaANP.

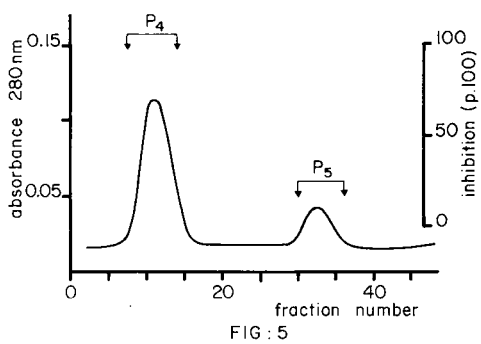

FIG : 5

FIG. 5. — *Sephacryl S-200 gel chromatography of the P_3 fraction inhibitor in 6M guanidium chloride.*
(——) absorbance at 280 nm ; (- - -) inhibition toward caseinolytic activity of CaANP.

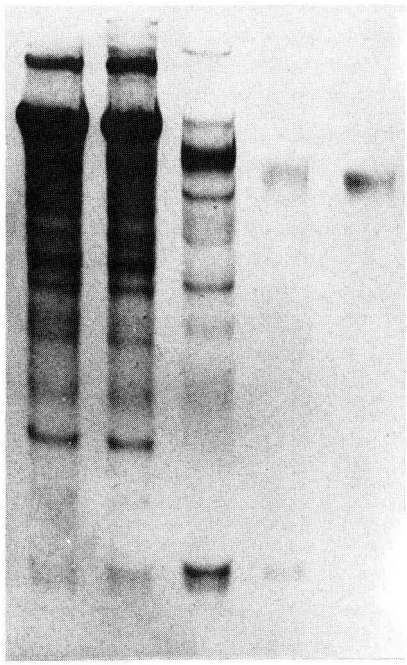

FIG. 4. — *Analytical SDS/polyacrylamide gel electrophoresis at various stages in the purification of rabbit skeletal-muscle inhibitor.*
(*a*) after Sephacel DEAE chromatography, (*b*) after Sephacryl S-200 gel filtration, (*c*) after heating at 70 °C for 5 min, (*d*) after Sephadex G-100 gel filtration, (*e*) after Sephacryl S-200 gel filtration in 6M guanidium chloride, 20 μg (*a*) (*b*), 30 μg (*c*) (*d*), 40 μg (*e*) of protein were added.

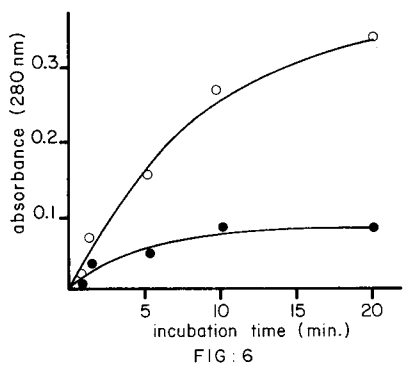

FIG : 6

FIG. 6. — *Kinetic inhibition of α-actinin release.*
2 ml of CaANP (150 μg/ml) were preincubated for 10 min at 30 °C with 4.5 ml of 10 mM Tris-(HCl) (pH = 7.40) containing 2 mM 2-mercaptoethanol or 4.5 ml of the same buffer containing (0.2 mg/ml) the CaANP inhibitor and then incubated with 20 ml of myofibril suspension (6 mg/ml) at 30 °C. At specified times, samples were withdrawn and the reaction stopped with EDTA (240 mV v/v). After centrifugation (42 000 × g for 10 min)), activity was evaluated by supernatant absorbance at 280 nm. ●——● Kinetics with inhibitor ; o——o Kinetics without inhibitor.

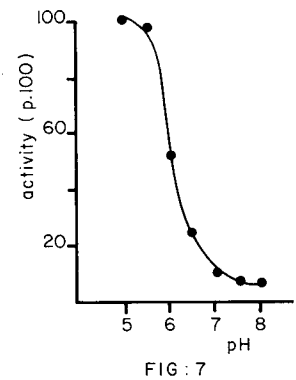

FIG : 7

FIG. 7. — *Effect of pH on the stability of the enzyme-inhibitor complex.*
70 μl of CaANP (150 μg/ml) were preincubated with 250 μl of inhibitor (0.4 mg/ml). Kinetic reaction was started by adding a casein solution (1.5 ml) in 200 mM Tris maleate, pH 5.5 to 7.5.

Inhibitor properties.

Table 1 shows that the inhibitor is a thermo-acid-stable protein degraded by trypsin and chymotrypsin. Waxman and Krebs (1978) and Nishiura, Tanaka and Murachi (1979) also reported the thermo-acid stability of a CaANP inhibitor extracted from liver and heart tissues and showing molecular weights of 270 000 and 300 000 daltons, respectively. However, considering the purification procedure and the lack of purification test results, we cannot say whether or not these molecules were similar. Busch *et al.* (1972) and Azanza *et al.* (1979) showed that CaANP induced α-actinin release during myofibril incubation with this enzyme. When the inhibitor was added to the incubation medium. CaANP enzymatic activity stopped (fig. 6).

In order to determine enzyme-inhibitor intereactions, we studied inhibitory strength after varying the pH, Ca^{2+} and salt concentration. Maximal inhibitory acti-

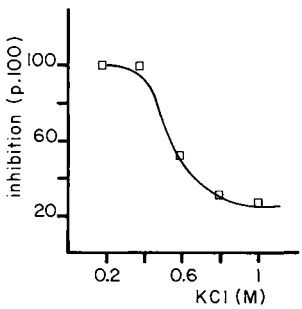

FIG. 8. — *Effect of salt concentration on the stability of the enzyme-inhibitor complex.*, 70 µl of CaANP (150 µg/ml and 250 µl of inhibitor 0.4 mg/ml) were both preincubated and then assayed on casein-substrate at various ionic strengths.

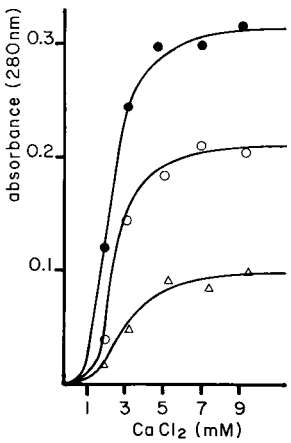

FIG. 9. — *Inhibition of CaANP by increasing amounts of CaANP inhibitor and Ca^{2+} concentration.* Increasing amounts of the inhibitor were preincubated with CaANP at pH = 7.5 for 1 min at 30 °C and then assayed on a casein solution containing different $CaCl_2$ concentrations. 50 µl of CaANP solution (150 µg/ml) were successively preincubated with : ●——● 250 µl of buffer (100 mM Tris-HCl, pH = 7.40, 2 mM 2-mercaptoethanol ; o——o 250 µl of inhibitor (0.28 mg/ml) ; △——△ 250 µl of inhibitor (0.4 mg/ml).

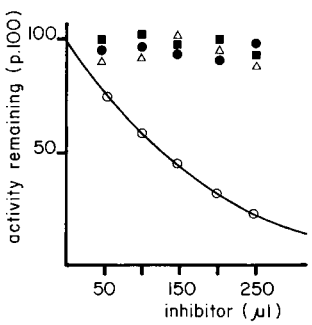

FIG. 10. — *Effect of the CaANP inhibitor on various neutral proteinases.* Increasing amounts of the inhibitor were preincubated with the tested enzyme at pH = 7.5 for 2 min at 30 °C and then the remaining caseinolytic activity was determined for 10 min at 30 °C. Tested enzymes : o——o 70 µl of CaANP (150 µg/ml) ; ●——● 70 µl of trypsin (150 µg/ml) ; ■——■ 70 µl of α-chymotrypsin (150 µg/ml); △——△ 70 µl of papaïne (150 µg/ml). The activity recorded with each tested enzyme in the absence of inhibitor was considered to be 100 p. 100.

vity (fig. 7) appeared at neutral pH ; pH decrease induced partial dissociation of the enzyme inhibitor complex. High salt concentrations were needed to suppress inhibition (fig. 8). Figure 9 shows that optimal Ca^{2+} levels were unaffected by inhibitor concentration, indicating that there was no competition between the Ca^{2+} and the inhibitor.

Experiments have shown that the enzyme-inhibitor complex does not need preincubation. When the enzyme and the inhibitor were mixed simultaneously with the casein substrate, inhibition strength did not decrease.

The calcium-activated neutral proteinase inhibitor was highly specific and had no effect on any other tested proteases, including trypsin, α-chymotrypsin and papaine (fig. 10).

These results suggest that in experimental conditions similar to those *in vitro*, inhibition is constant and does not depend on physiological Ca^{2+} content. The presence of the inhibitor in the crude extract is thus a problem as far as the cellular role of the enzyme is concerned. Its activity must imply a different subcellular localization or the existence of a Ca^{2+}-protein binding modulator system which might be involved in suppressing the inhibition.

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Résumé. Cette recherche a conduit à la purification d'un inhibiteur spécifique du « calcium activated Neutral proteinase » (CaANP) à partir de muscles de lapin et à l'étude de ses propriétés.

Cet inhibiteur de nature protéique est composé de deux fractions de poids moléculaire 70 000 et 13 000 daltons. Il est thermostable et résistant aux pH acides. Son activité inhibitrice est maximale à pH 7 et diminue pour des concentrations en KCl supérieures à 0,3 M.

Les exigences en calcium du CaANP sont identiques quelle que soit la concentration de l'inhibiteur. La formation du complexe enzyme-inhibiteur est instantanée et ne nécessite pas de préincubation.

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