Fractionation and characterization of proteinase inhibitors from bovine skeletal muscle.

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SUMMARY : In this work we attempted to fractionate proteinase inhibitors from bovine *Diaphragma* muscle using two chromatography steps. The crude extract was first run on a Sephadex G100 column (100x5 cm) which fractionated four papain inhibiting fractions F-I, F-II, F-III and F-IV with Mr in the range of 40-70 kD for F-I and F-II, 30 kD for F-III and 12-14 kD for F-IV. While F-I, F-II and F-IV fractions inhibited also trypsin, activity against both trypsin and chymotrypsin was detected for F-I and F-II fractions. Composition of each G100 fractions was further analysed by chromatography on a Q-Sepharose column (15x2.4 cm) equilibrated in Tris-HCl buffer of adapted pH. Two of them, namely F-III and F-IV were purified to homogeneity.

Each active fraction was tested for their activity against papain, trypsin and chymotrypsin and further analyzed by SDS-PAGE.

INTRODUCTION : The cystatins are a group of proteins that ase described as tight - binding inhibitors of cysteine proteinases of the papain family. Similarities in their amino-acid sequence and other properties show that the cystatins comprise a single evolutionary superfamily (BARRETT et al. 1986) comprising:

- The Stefin family consisting of low molecular-weight (10 to 14 kD) inhibitors, mainly intracellular. The molecular chains are formed by about 110 amino-acid residues with no disulfide bonds.
- The Cystatin family consisting of low molecular-weight (13 kD) proteins, essentially found in body fluids. On average, they contain about 115 residues, with two disulfide bonds near the COOH terminus.
- The Kininogen family consisting of high molecular -weight inhibitors which contain divergent copies of the cystatin family sequence but with nine disulfide bonds.

Inhibitors of cysteine proteinases are present in tissues and body fluids of mammalian species (BARRETT et al. 1985). They could be involved in the control mechanisms responsible for intracellular or extracellular protein breakdown.

Although many studies have been carried out on the cysteine proteinase inhibitors, only few informations have been brought about the cysteine proteinase inhibitors in muscle tissue. As to the skeletal muscle, the presence of such inhibitors in rat (SCHWARTZ and BIRD 1977) and rabbit (MATSUMOTO et al. 1983, MATSUISHI et al. 1988) and bovine skeletal muscle (BIGE et al. 1985, OUALI et al. 1986) was reported, and only one inhibitor with a low molecular-weight of 14 kDa has been purified from bovine skeletal muscle (BIGE et al. 1985) by using gel filtration on Sephadex G75 and affinity chromatography on carboxymethyl-papain Sepharose. Bovine muscle was further shown to contain at least three high-molecular inhibitory fractions (Mr>60 kD) inhibiting both the cysteine and serine proteinases (OUALI et al. 1986). Therefore, the present work was an attempt to clarify the composition of muscle cell in proteinase inhibitors.

MATERIALS AND METHODS : Extraction of the crude extract from minced muscle was performed at 4°C according to BIGE et al. (1985). Briefly, about 400 g of bovine diaphragm muscle were minced in a Moulinex mincer and homogenized for 30 s with an Ultra-Turax in 3 vol. of 0.05 M Tris- HCl buffer (pH 7.6) containing 4 mM EDTA and 0.15 M NaCl.The homogenate was then centrufuged at 1000xg for 5 mn and at 2000 xg for 30 mn. The 20 000xg (30 mn) supernatant was adjusted to pH 10 with 5 M NaOH and incubated at 37°C for 30 mn. After adjusting the pH to 6.0 with 5 M HCl, the suspension was centrifuged at 20 000xg and adjusted to pH 7.6 before salting out between 40 and 70% ammonium sulfate saturation. Precipitated protein were dissolved in a minimum volume of 0.03 Tris-HCl buffer (pH 7.6) containing 0.15 M NaCl, dialyzed overnight against this buffer and loaded on Sepadex G100 superfine column (5x100cm) equilibrated in the same buffer. The flow rate was 21 ml/h and fractions of 10 ml were collected.

Activities against papain and trypsin were assayed as reported by BARRETT (1980),whereas chymotrypsin activity was tested according to SAWADA et al. (1983).

Protein concentrations were determined from the absorbance at 280 nm using $E^{1\%}$ =0.1 (BRADFORD, 1976). Molecular-weight of the inhibitor was estimed by gel filtration on Sepadex G100 and by SDS-PAGE using the corresponding calibration Kits. SDS polyacrylamide gel electrophoresis (PAGE) was carried out on 15% and 12% polyacrylamide slab gels and proteins stained with Coomassie Brillant Blue (LAEMMLI, 1970).

RESULTS

1 - Fractionation of the Crude Extract by Gel filtration:

The crude extract was run on a gel filtration column as soon as possible after its preparation since the proteins were denatured and the inhibiting activity was partly lost when this operation was delayed. As shown in Fig-1, gel filtration on Sephadex G100 of the crude extract separated four papain inhibiting fractions **(F-I, F-II, F-III, F-IV),** indicating the presence of four kinds of inhibitors with different molecular-weight ranging from 14 kD to 70 kD.

The two first active fractions (F-I and F-II) eluted near the void volume correspond to protein with molecular weight ranging from 40 to 70 kD. The second fraction eluted just after F-II exibited a Mr of 30 kD. The last one eluted just after myoglobin and shows a Mr corresponding to protein with very low Mr (≤ 14 kD).

All fractions were also tested for their inhibiting activity against trypsin and chymotrypsin.
F-I and F-II inhibited either trypsin and chymotrypsin, but F-IV inhibited only trypsin. F-III showed no activity against both enzymes.
On SDS-PAGE, all fractions showed a complex banding pattern.

Each of them was further run on anionic exchange chromatography Q-Sepharose column (15x2.4) equilibrated with Tris-HCl buffer of adapted pH and protein were eluted by a NaCl gradient (0-0.5 M). All fractions collected were similary tested for their inhibitory activity against papain, trypsin and chymotrypsin.

2 - Composition of each G 100 Fractions as assessed by Anionic Exchange Chromatography:

- Composition of F-I and F-II

All active fractions collected were pooled and dialysed overnight against Tris-HCl buffer pH 8.0, then loaded on a Q-Sepharose column equilibrated with the same buffer.
As shown in Fig 2, anionic exchange chromatography separated two fractions eluted in the gradient between 0.2 and 0.3 M NaCl. F-I a inhibited papain, trypsin and chymotrypsin while F-I b inactivated essentially papain and trypsin.
This Sephadex G100 fraction showed only one active peak eluted at 0.2 M NaCl and inhibiting only papain. On SDS-PAGE, Q-Sepharose fractions obtained from F-I and F-II showed a complex pattern comprising bands emerging at position between 40 kD and 70 kD. Further studies are now being conducted to purify and characterize these inhibitors.

- Composition of F-III

Complete purification of the inhibitor present in the F-III G 100 fraction was achieved by using two anionic exchange chromatography steps. After overnight dialysis of the G 100 fraction against Tris-HCl buffer, pH 8.15, the sample was loaded on a Q-Sepharose column previously equilibrated in the same buffer and protein eluted with a 0 - 0.5 M Na Cl gradient. Eluted papain inhibiting fractions were then pooled, dialyzed overnight against Tris-HCl buffer, pH 7.60 and loaded on a Q-Sepharose equilibrated in the pH 7.60 buffer. One papain inhibiting peak was eluted in the gradient between 0.2 and 0.25 M NaCl (Fig 4b). Neither trypsin nor chymotrypsin inhibiting activity were observed. Analysis of these fractions by SDS-PAGE showed only one band of Mr 30 kD (Fig 4a).

Under reducing conditions (15% 2- mercaptoethanol), all active fractions analysed by SDS-PAGE showed one band of similar Mr (data not shown). This result demonstrated that this protein was not converted to proteins of lower molecular-weight in the presence of a thiol reducing agent.

The pH stability was tested over a large pH range (5.0-8.0) by incubating the pure fractions at different pH and at 37° C for either 1.5 h or 3h. By comparing their activity to that of unincubated fractions, activity loss appeared not significant and did never exceed 5% suggesting that this inhibitor is very stable over this pH range.

- Composition of F-IV

As F-I, F-II, and F-III, the G 100 F-IV fraction was run on a Q-Sepharose column in Tris-HCl, pH 7.2 in order to eliminate pigment, which was the main contaminant. Anionic exchange chromatography separated three fractions referred to as F-IVa, F-IVb and F-IVc and eluted in the gradient between 0 and 0.3 M NaCl (Fig 5A):

- F-IV a: was eluted at 0.10 M NaCl and strongly inhibited papain whereas no activity against trypsin and chymotrypsin was detected.
- F-IV b: eluted at 0.15 M NaCl, inhibited papain and moderately trypsin. SDS-PAGE pattern of fractions F-IVa and F-IVb showed only one band of Mr 14 kD (Fig 5B).
- F-IV c: was eluted at 0.2 M NaCl and inhibited only papain. On SDS-PAGE F-IVc showed two bands of Mr 14 and 12 kD, respectively.

Chromatofocusing on a Mono-P column using FPLC system of the F-IVc fraction separated three major papain inhibiting protein fractions eluted respectively at pH 6.2, 5.8 and 5.5. As shown by SDS-PAGE, the first active fraction eluted at pH 6.2 corresponded to the 14 kD protein and those eluted at pH 5.8 and 5.5 corresponded to two isoforms of the 12 kD inhibitor.

With regard to its heat stability, the low-Mr cysteine proteinase inhibiting fractions from G100 appeared completely stable at high temperature over a range 50 to 100°C.

DISCUSSION - CONCLUSION

Our study demonstrates the presence in bovine skeletal muscle of several cysteine protease inhibitors with Mr ranging from 12 kD to 65-70 kD. All these inhibitors show activity against papain (cysteine proteinase), some of them inhibited also trypsin and others are active towards both trypsin and chymotrypsin, the two serine proteinases tested.

About inhibitors isolated from fraction F-I, obtained after G100 chromatography, ours results agree with previous works conducted in this laboratory (OUALI et al. 1986) in the course of which three inhibitors with a Mr of about 62kD and inactiving both cysteine and serine proteinases were isolated and partially characterized.On the other hand the inhibitor obtained from the fraction F-II inhibited papain, trypsin and chymotrypsin and have a Mr of about 47 kD.This 47 kD protein inhibitor might be compared to the papain inhibiting fraction detected by MATSUMOTO et al. (1983) and MATSUISHI et al. (1988) in rabbit muscle and showing with a Mr of 50 kD. Complete purification and characterization of these inhibitors are in progress.

According to MATSUICHI et al. (1988) who isolated an inhibitory fraction from rabbit skeletal muscle with a Mr value of 30 kD, we purify in three steps an inhibitor showing an identical Mr. This inhibitor is stable over a large range of pH and temperature. Conversely to

the papain inhibiting fraction described by the previous authors, the present 30 kD inhibitor appears to be not sensitive to reducing agents. Indeed, wether SDS - PAGE was performed in the absence or in presence of mercaptoethanol at a concentration as high as 15%, only one band was observed on the gel which migrates as a protein with a Mr of 30 kD. Moreover, as this inhibitor was distributed in at least five to six different fractions when eluted from the second Q-Sepharose, it could by stressed that this 30 kD protein inhibitor is composed of different isoforms, a point which is under investigation.

Concerning the low molecular weight inhibitors Q-Sepharose chromatography fractionated the F-IV G100 fraction into three active peaks. The first one (F-IVa) contains an inhibitor with a Mr value of 14 kD inactiving only papain. This might be compared to the one observed by SCHWARTZ and BIRD (1977) in rat skeletal muscles and to the other one purified by BIGE et al. (1985) from bovine skeletal muscle, respectively. Fraction (F-IVc) is similar to that reported by ZEECE et al. (1991) which separated from bovine cardiac muscle one fraction active against cysteine proteinases. In contrast to the previous ones, the last fraction (FIVb) appeared to be a strong inhibitor of both papain and trypsin. However, at the present time no definite conclusion can be drawn on whether the activity of this fraction against trypsin and papain might be ascribed to one or two different proteins or protein isoforms of similar Mr.

The present investigations clearly shows that the equipment of muscle cells in proteinase inhibitors other than calpastatin, a specific inhibitor of calcium dependent neutral proteinases, is very complex. Hence, much works remain to be done for clarifying their physiological function together with their possible role in meat tenderization.

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