ICATION OF HIGH-MOLECULAR WEIGHT PROTEINASE INHIBITORS FROM BOVINE SKELETAL LE.

UCHON, M. ZABARI, M. BERRI, T. SAYD, J. SIBOULET AND A. OUALI
esearch Station, INRA Theix, 63122 Ceyrat, France

MARY :
olecular-weight proteinase inhibitors were purified from bovine *Diaphragma* muscle using selective extraction prior to conventional gel n and ion exchange chromatography techniques. Purification was concluded by using trypsin-agarose affinity and anti BSA-coupled se affinity. These inhibitors were discriminated on the basis of their efficiency to inactivate trypsin, papain or both. Proteinase rs with estimated molecular weight of 65 and 60 kDa were thus purified to homogeneity as assessed by SDS-polyacrylamide gel phoresis.

ODUCTION :
ent years a growing number of investigators have been interested in searching for novel inhibitors in biological fluids or tissues. The r of proteinase inhibitors isolated and identified so far is extremely large.
ajority of protein inhibitors known and characterised so far are directed towards serine proteinases. In a now classical review paper, OWSKI and KATO (1980) introduced for the first time a rational nomenclature by grouping these inhibitors into distinct families.This fication was based on sequence similarity, topological similarity, and mechanism of binding.
the last few years, a series of cysteine proteinases protein inhibitors have also been discovered and characterised (BARRETT et al., TURK and BODE, 1991). The cystatins are tight and reversibly binding inhibitors of the papain-like cysteine proteinases. They form a family of sequentially homologous proteins subdivided into three families, the stefins, the cystatins and the kininogens. The recently vered cathelin, a protein inhibitor of cathepsin L indicates that a new family of cysteine proteinase inhibitors may exist (RITONJA et al.,).

inhibitors might protect the cells from inappropriate endogenous or external proteolysis and could be involved in the control of the anisms responsible for intracellular protein breakdown. There is also growing information about the importance of these inhibitors in pathological events such as inflammation, muscle dystrophy, Alzeimer's disease, sclerosis and tumor malignancy.
ugh many studies have been carried out in order to identified proteinase inhibitors in different biological fluids and tissues, few is known proteinase inhibitors in muscle tissue. In the present study we describe the purification procedure, in bovine skeletal muscle, of three olecular-weight proteinase inhibitors.

ERIALS AND METHODS :

erials :
Sephadex G100 and G75 superfine , S-Sepharose, Q-Sepharose and CNBr Activated Sepharose 4B were purchased from Pharmacia Fine micals. Enzymes (trypsin, papain and chymotrypsin) and substrates (N-CBZ-Phe-Arg 7-amido 4 Methylcoumarin and N-Succinyl-Ala-Pro-Phe 7-amido 4 Methylcoumarin) were obtained from SIGMA Chemical CO. Trypsin affinity column: trypsin insoluble enzyme ked to cross-linked beaded agarose were from SIGMA Chemical CO.

uids :
mes and inhibitors activities : Trypsin and papain were assayed with N-CBZ-Phe-Arg 7-amido 4 Methylcoumarin as substrate by hethod of BARRETT (1980). Chymotrypsin was assayed by measuring N-Succinyl-Ala-Ala-Pro-Phe 7-amido 4 Methylcoumarin olysis (SAWADA, 1983). Inhibitors activities were measured by the decrease in proteolytic activities after preincubation with the inhibitors °C. Inhibition was expressed in per cent with respect to a control sample in which enzymes were incubated without inhibitor.
electrophoresis : Electrophoresis on 12,5 % polyacrylamide slab gel was performed according the procedure of LAEMMLI (1980) and ins were stained with Coomassie Brilliant Blue R-250. Molecular Weight was estimated by comparing mobilities of the bands with those e following marker proteins: phosphorylase-b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30

kDa), soybean trypsin inhibitor (20 kDa) and α lactalbumine (14 kDa).

Immunological analysis : Immunodiffusion based on the OUCHTERLONY method (1958) was performed using anti-bovine serum albumin to determine eventual inhibitor contamination by bovine serum albumin.

Preparation of anti bovine albumin affinity column : Anti-bovine serum albumin (developed in Rabbit) was coupled with CNBr activated Sepharose 4 B according to the manufacturer's instructions (Pharmacia). Approximately 5 mg antibodies were coupled with each ml of the Sepharose beads. The anti BSA-coupled Sepharose 4 B was packed in a column (1 X 5 cm).

Preparation of a crude inhibitor extract : A crude preparation was obtained by extraction from bovine *Diaphragma* muscle according to ZABARI et al (1991).

RESULTS AND DISCUSSION

Fractionation of the crude extract by gel filtration :

Gel filtration of the crude inhibitor extract on a Sephadex G100 column (5 x 100 cm) separated four papain inhibiting fractions referred to FI, FII, FIII, FIV (ZABARI et al., 1991). The first active fraction FI eluted near the void volume corresponded to protein with Mr ranging from 40 to 70 kDa.The second fraction FII included proteins with Mr ranging from 30 to 50 kD while FIII exhibited proteins with molecular weight between 20 and 30 kD. The last fraction FIV, eluted just after myoglobin corresponded to proteins with low molecular weight (≤ kDa). In addition to papain, FI, FII and FIV inhibited also trypsin and chymotrypsin while FIII show no activity against these serine proteinases. Only the first fraction FI containing inhibitors of highest molecular weight was investigated here.

Cation exchange chromatography on S-Sepharose :

Fraction FI dialysed against acetate sodium buffer pH 5.4 was first loaded on a S-Sepharose column (2.5 x 16 cm) equilibrated with the same buffer. Figure 1 shows that this step separated three inhibitory fractions: non adsorbed material (S_0) and two peaks (S_1, S_2) eluted with a 0.5 M linear NaCl gradient. S_0 (unretained fraction) and S_1 eluted from the column at about 0.08 M NaCl, inhibited papain and trypsin whereas S_2 eluted at about 0.15 M NaCl inhibited trypsin but not papain. All these fractions show a complex electrophoretic pattern.

Anion exchange chromatography on Q-Sepharose :

Each active fraction describe above was loaded on a Q-Sepharose column (2.5 x 16 cm).

-S_0 (unabsorbed material from S-Sepharose) was dialysed towards 0.05 M pipperazine-formic acid buffer, pH 4.0. The dialysed sample was applied to the column equilibrated in the same buffer. The inhibitory activity was recovered into two fractions : the unabsorbed proteins (S_0Q_0) and a peak (S_0Q_1) eluted within the linear gradient at 0.15 M NaCl (Figure 2). Both fractions showed inhibitory activity against papain, trypsin and chymotrypsin. On SDS-PAGE, S_0Q_0 showed several bands emerging at position between 40 and 70 kDa. Further studies of this fraction are under progress. S_0Q_1 analysed by SDS-PAGE showed one band with a molecular weight of about 65 kDa.

S_1 and S_2 were dialysed towards 0.03 M Tris-HCl buffer pH 8 and were separately loaded on Q-Sepharose equilibrated in this buffer.

-From S1 we obtained a single peak (S_1Q) inhibiting both papain and trypsin and eluted with 0.25 M NaCl (Figure 3). SDS-PAGE shows two bands with a Mr of 40 and 60 kDa, respectively.

-From S_2 we also obtained a single peak (S_2Q) inhibiting only trypsin and eluted with 0.2 M NaCl (Figure 4). SDS-PAGE show one band Mr 60 kDa and two bands around 25 kDa.

Gel filtration on Sephadex G75 superfine :

S_1Q and S_2Q were concentrated by chromatography on a FPLC mono Q HR5 column and then applied to a Sephadex G75 column (2.5 x cm) equilibrated with a solution of 0.03 M Tris-HCl buffer, pH 8, containing 0.4 M NaCl. For each fraction (S_1Q and S_2Q) only one inhibitory peak was obtained which was estimated in each case to have a molecular weight of 60 kDa by comparison with elution volume of standard proteins : bovine albumin (Mr 68 kDa), egg albumin (Mr 43 kDa), pepsin (Mr 35 kDa) and soybean trypsin inhibitor (Mr 20 kDa). The homogeneity of both inhibitors was examined by polyacrylamide gel electrophoresis. A single band with molecular weight of 60 kDa was obtained from each inhibitory fractions.

Affinity chromatography on trypsin agarose :

In order to determine whether both inhibitory activities (against papain and trypsin) associated with the S_1Q 60 kDa inhibitor are brought by only one protein, this Sephadex G75 fraction was loaded on a trypsin agarose affinity column (0.5 x 2 cm) equilibrated in Tris-HCl buffer pH

1 : **Chromatography of fraction FI on S-Sepharose.** applied on a S-Sepharose column (16 x 2.5) equilibrated in acetate sodium buffer pH 5.4. Proteins were eluted with a gradient of 0 - 0.5 M NaCl in the same buffer at a flow rate of 3 A 280 nm ——— papain inhibition % ⟿ hibition % ⟿

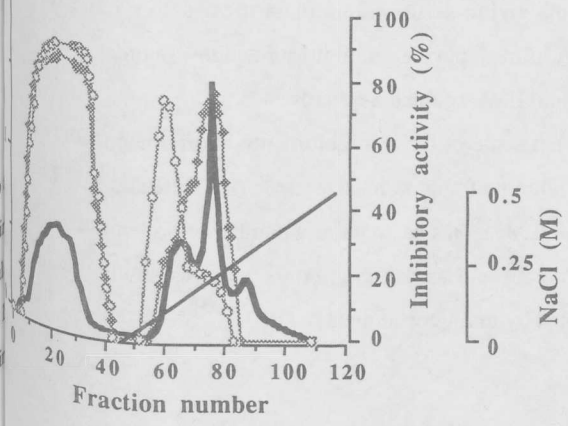
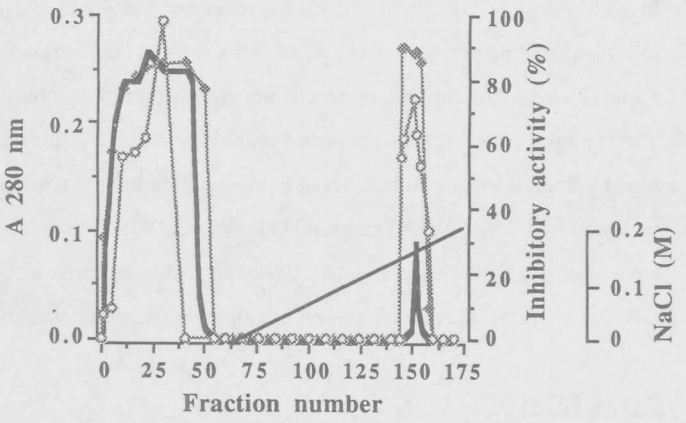


Figure 2 : Chromatography on Q-Sepharose of fraction S1 Fraction S2 was applied on a Q-Sepharose column equilibrated in 0.05 M pipperazine formic acid buffer, pH 4. Proteins were eluted with a linear gradient of NaCl (0 - 0.2 M) in the same buffer at a flow rate of 3 ml/min. A 280 nm ——— trypsin inhibition % ⟿ papain inhibition % ⟿



3 : **Chromatography of fraction S1 on Q-Sepharose.** ple was applied to a Q-Sepharose column (16 x 2.5 cm) ed in 0.03 M Tris-HCl buffer, pH 8.0. Proteins were eluted Cl gradient at a flow rate of 3 ml/min and 4.5 ml fractions ected. A 280 nm ———; trypsin inhibition % ⟿; hibition % ⟿

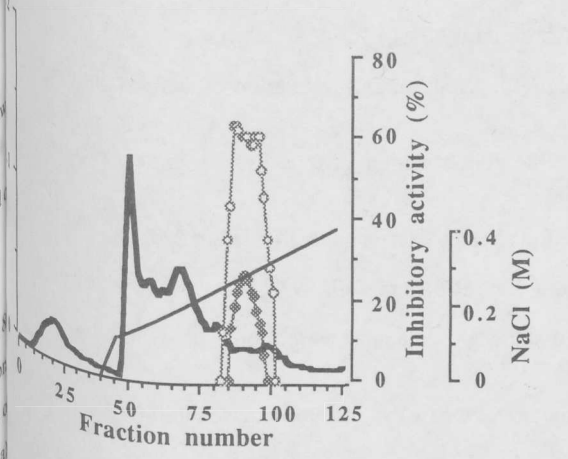
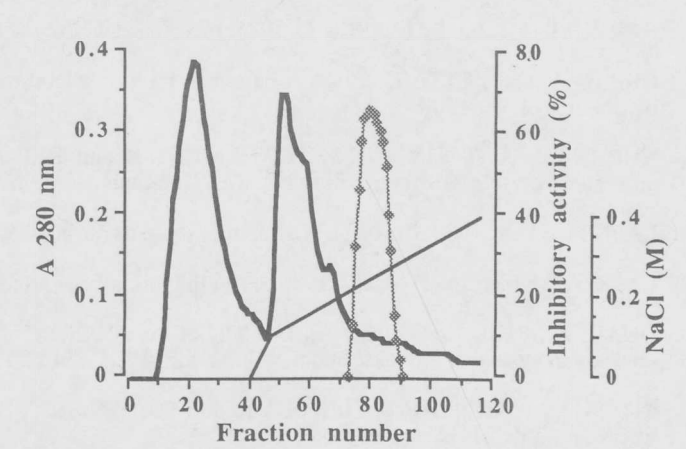


Figure 4 : Chromatography of fraction S2 on Q-Sepharose. S2 was applied to a Q-Sepharose column equilibrated in 0.03 M Tris-HCl buffer, pH 8.0. Proteins were eluted with a NaCl gradient in the same buffer. Other conditions were as given in the legend to fig. 3. A 280 nm ———; trypsin inhibition % ⟿



8 and unbound proteins were eluted with the same buffer. In the unbound fraction, only the papain inhibiting activity was recovereds, a findi which clearly desmonstrate that papain and trypsin inhibitory activities were brought by different proteins. However, we did not succeed our attempt to eluate the bound trypsin inhibitory activity even after an extensive washing of the column with 25 mM HCl. Two reasons mi account for this failure : (1) the inhibitor was unstable at acidic pH and could not be detected or (2) because the protein inhibitor binds tightl the column, itt was not release in such conditions. These two assumptions have not yet been tested.

Chromatography on anti BSA-coupled Sepharose 4 B :

In the first steps of the purification procedure we noted the presence of bovine serum albumin in most active inhibitory fractio Immunodiffusion performed using anti-BSA revealed contamination by BSA of most purified inhibitory fractions. In order to eliminate BS samples where therefore run on an affinity chromatography column made of anti BSA-coupled Sepharose 4 B.

The presence of BSA, a major serum protein, in these fractions suggested that some of these inhibitors might originate from contamina blood a finding supported by a set of evidence showing the plasma origin of albumin found in muscle tissue (HEILIG and PETTE, 1988). reviewed by LASKOWSKI and KATO (1980) and TRAVIS and SALVESEN (1983), plasma contains a number of proteinase inhibitors wh have been extensively investigated. Hence, it would be important to focuse the present work on inhibitors which exist within muscle cells. this respect, tissue specificity as well as cellular localisation will be investigated by immunochemical methods.

CONCLUSION

Endogenous cysteine and serine proteinases are probably the most active muscle proteinases responsible for intracellular protein degradati The present study report the purification of four distinct inhibitors of these proteinases. The 65 kDa protein inhibitor inactivates both cyste and serine proteinases. Among the three other muscle inhibitor (each with a Mr of 60 kDa), two of them inhibit only trypsin and the last o inactivate only papain. The purified inhibitors can be compared with muscle inhibitors of serine type (WAXMAN and KREBS, 1978; KHU et al.,1984); cysteine type (SCHWARTZ and BIRD, 1977) or both serine and cysteine type (OUALI et al., 1986).

This work confirm the great complexity of the muscle equipment in proteinase inhibitors already suggested in earlier studies conducted in th laboratory (OUALI et al.,1986, ZABARI et al., 1991). Further investigations are needed in order to improve our knowledge about th possible function, their target enzymes as well as their relationship with the protein inhibitors isolated from various tissues and fluids.

REFERENCES

BARRETT A.J. 1980. Fluorimetric assay for cathepsin B and cathepsin H. Biochem. J. 187, 909 - 912.

BARRETT A.J. and SALVASEN G. 1986. Proteinase inhibitors. Elsevier, Amsterdam.

HEILIG A. and PETTE D. 1988. Albumin in rabbit skeletal muscle : origin, distribution and regulation by contractile activity. Eur. Biochem. 171, 503-508.

KUEHN L., RUTSCHMANN M., DAHLMANN B. and REINAUER H. 1984. Identification of four distinct serine proteinase inhibitor rat skeletal muscle. Biochem.. Biophys. Res. Commun . 120, 96-102.

LAEMMLI U.K. 1970. Cleavage of structural proteins during the assembly of the head bacteriophage T4. Nature 227, 680-685.

LASKOWSKI M. and KATO I. 1980 . Protein inhibitors of proteinases. Annu. Rev. Biochem.. 49, 593-626.

OUALI A., BIGE L., OBLED A., LACOURT A., VALIN C. 1986. Small and high molecular weight proteinases inhibitors from bovi muscle - In cysteine proteinase and their inhibitors p.545-554 (V.Turk.eds.).

RITONJA A., KOPITAR M., JERALA R. and TURK V. 1989. Primary structure of a new cysteine proteinase inhibitor from pig leucocyt FEBS lett. 255, 211-214.

SAWADA H., H. YOKOSAWA, M. HOSHI and S. ISHII. 1983. Ascidian sperm chymotrypsin-like enzyme; participation in fertilisatio Experientia. 39, 377-378.

TRAVIS J. AND SALVESEN G.S. 1983. Human plasma proteinase inhibitors. Ann. Rev. Biochem. 52, 655-709.

TURK V. and W. BODE .1991. The cystatins : protein inhibitors of cysteine proteinases. FEBS lett. 285, 213-214.

WAXMAN L. and KREBS E. 1978. Identification of two protease inhibitors from bovine cardiac muscle. J. Biol. Chem. 253, 5888-5891.

ZABARI M. BERRI M., ROUCHON P. and OUALI A. 1991. Fractionation and characterisation of proteinase inhibitors from bovine skelet muscle. 37 th international congress of meat science and technology, 1, 452-456.