RIFICATION AND CHARACTERIZATION OF INTERMEDIATE Mr INHIBITOR OF CYSTEINE PROTEINASE IN VINE SKELETAL MUSCLE.

BERRI, M. ZABARI, P. ROUCHON, T. SAYD, J. SIBOULET and A. OUALI

sion de Recherche sur la Viande, Unité Biochimie des Proteines, INRA Theix, 63122 CEYRAT, France.

MMARY : Four papain fractions named FI, FII, FIII and FIV were fractionated from bovine crude extract muscle using a gel filtration matography on Sephadex G100. From the FIII fraction, a cysteine proteinase inhibitor was purified by two successive anionic exchange matography on a Q-Sepharose column and its properties investigated. This inhibitor protein shows a Mr of about 34.2 KDa, stable over a e pH game (5-8), thermostable (40-100°C), bind tightly to papain and cathepsin L, and, though to a lesser extent, to cathepsins B and H. is in good agreement with their corresponding Ki values : 70 nM for papain, 29 nM for cathepsin L, 600 nM for cathepsin B and 410 nM cathepsin H. The kinetic features of enzyme - inhibitor binding suggest a possible role for this inhibitor protein in controlling "in vivo" epsin L proteolytic activity.

TRODUCTION : Meat tenderness is probably the most important quality attribute for consumers. Management of this quality needs provement of our understanding of both the original causes and the nature of the mechanisms responsible for the postmortem improvement eat texture. Two sets of mechanisms are probably involved in this process. The first which is physicochemical in nature, mainly concerns crease in postmortem muscle osmotic pressure. The second is proteolysis of muscle proteins which is still considered as the primary hanism of meat tenderization (For review see OUALI, 1990). In this respect, two proteolytic systems have received much attention from d and non-meat scientists, since in live animals these were also involved in the degradative processes of proteins. These systems are the lysosomal proteinases (cathepsins B, H, L and D) and the calcium dependent neutral proteinases (calpains).

einase protein inhibitors naturally occurring within the cells largely contribute to the regulation of proteolysis in both living animals and mortem muscle. In regard to lysosomal proteinases, the existence of cathepsins inhibitors in muscle was reported for rabbit (TSUMOTO et al. 1983 ; MATSUISHI et al. 1988) and bovine (BIGE et al. 1985 ; OUALI et al. 1986). Recently ZABARI et al. (1991) dy show that the equipment of bovine muscle in cysteine proteinase inhibitors other than calpastatin is very complex. In fact four papain sing fractions FI, FII, FIII and FIV were fractionated from bovine crude extract muscle using gel filtration chromatography on Sephadex 0 column indicating the presence of four classes of inhibitors showing different Mr in the range of 12 to 70 KDa. While FI, FII and FIV sions inhibited trypsin and chymotrypsin together with papain, FIII fraction is only active against papain. The present work intended efore to purify and characterize the inhibitor from the FIII papain inhibiting fraction.

TERIALS AND METHODS :

ymes assays : Cathepsins B, H, and L were prepared from rat liver lysosomes according to OBLED et al. (1984). Cathepsins B, L, pain and trypsin activities were measured in accordance to BARRETT et al. (1980) using Z-Phe- Arg- NMec as substrate. Cathepsin H was ved with Arg-NMec as substrate (BARRETT et al; 1980) whereas chymotrypsin activity was tested according to SAWADA et al. (1983) g Suc-Ala-Ala-Pro-Phe-NMec as substrate. Activity of pepsin against haemoglobin was determined as previously reported by OUALI and AN (1981).

ume titration : titration of papain, cathepsins B, H and L was carried out essentially as described by BARRETT et al. (1982) using 4), a specific inhibitor of cysteine proteinases. As evaluated by this method, active proteinase concentration in the stock solution were 20 -250 nM, 60 nM and 350 nM for papain, cathepsins B, H, and L respectively.

steine proteinase inhibitor assays : Inhibition experiments were performed at constant enzyme concentration and variable centration of inhibitor. The mixture was preincubated at 37° for a previously defined fixed period, i.e 25 min for papain, 30 min for epsins L , 40 min for cathepsin H, 60 min for cathepsin B, 20 min for trypsin, chymotrypsin and pepsin. The reaction started by addition the substrate at final concentration of 10 µM. The progress of the reaction was followed fluorimetrically by monitoring the release of NMec (Exc. λ = 360nm, Emi. λ = 460 nm).

ification of muscle cysteine proteinase inhibitor : the details of the purification scheme were as previously described (ZABARI al 1991). Briefly the crude extract prepared from 350 g of *diaphagma* bovine muscle was loaded on a Sephadex G100 column (100x5 cm) ich separated four papain inhibiting fractions called FI, FII, FIII and FIV. The complete purification of the inhibitor from the FIII fraction

was achieved by using two successive exchange chromatography steps on a Q-Sepharose column (14 , 2.5 cm) run at different pH (8.15 and 7.6 respectively). The proteins were eluted by a 0 - 0.5 M NaCl gradient and 2 ml fractions were collected and tested for their inhibitory activity.

Other methods: Protein Mr were estimated by SDS-PAGE electrophoresis performed as described by LAEMMLI (1970) on 15% slab gels using the Pharmacia low-Mr calibration Kit. Proteins were stained with Coomassie Brillant Blue R-250. The pI was determined by isoelectric focusing on polyacrylamide slab gel. Protein concentration was measured according to LOWRY et al. (1951).

RESULTS :

General properties of the inhibitor : As summarised in table 1, a protein inhibitor was purified 1890 fold from the Sephadex G100 fraction. By SDS-PAGE, the final fraction shows only one band. Before that step, the inhibitory activity measured corresponds to a complex mixture of inhibitors and can not be taken into account. As regard its specificity towards different types of proteinases, this inhibitor was inactive against trypsin and chymotrypsin, two serine proteinases, as well as pepsin, a well known aspartyl proteinase. By contrast, cathepsins B, H, L and papain, four enzymes of the cysteine proteinase class were strongly inhibited suggesting that this inhibitor was specific of the cysteine proteinases group (Table 2).

Table - 1 : Purification of cysteine proteinase inhibitor from bovine skeletal muscle. One activity unit was defined as the concentration of inhibitor inactivating one unit of papain.

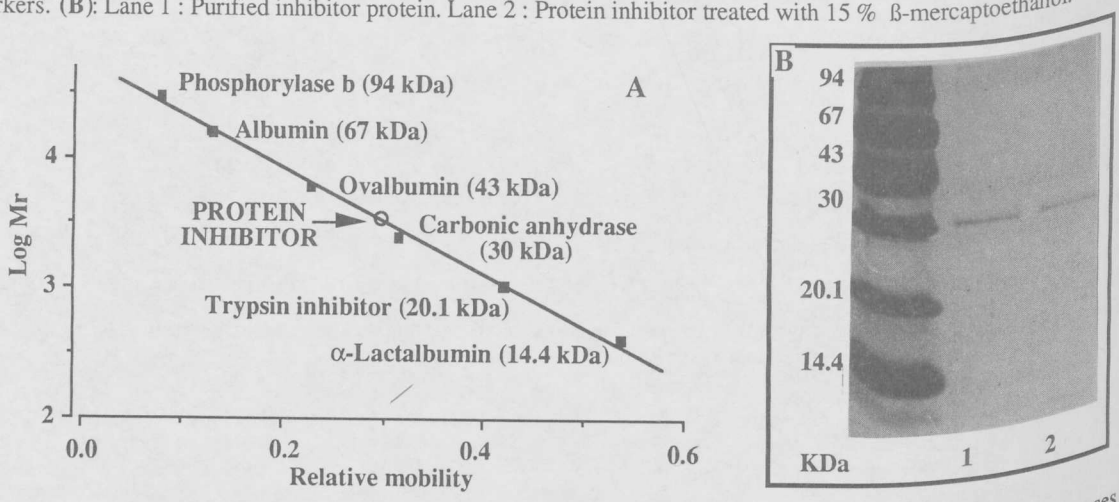
Purification Step	Total protein (mg)	Total activity (U)	Specific activity (U)	Purification factor
Gel filtration	91.0	107	1.26	1
Q-Sepharose - pH 8.15	6.4	180	28.1	22
- pH 7.60	0.105	250	2380	1890

Table - 2 : Inhibition spectrum of the 34.2 kDa inhibitor. Activity expressed as µg of inhibitor inactivating by 50% 1µg of enzyme. NI : not inhibited.

Proteinases	µg inhibitor
Papain	4.46
Cathepsin L	0.46
Cathepsin B	19.4
Cathepsin H	40.4
Trypsin	NI
Chymotrypsin	NI
Pepsin	NI

Physicochemical properties of this inhibitor : The apparent Mr of this inhibitor estimated by SDS-PAGE was 34.2 KDa (Fig. 1A). Whether the electrophoretic analysis was performed in the absence or in the presence of thiol compounds, only one band was observed suggesting that this inhibitor is a monomeric protein (Fig. 1B). This protein subjected to isoelectric focusing on polyacrylamide gel displayed a pI of 6.7 (data no shown). The temperature stability was tested over a large temperature range (40-100°C) by incubating the pure fraction

Figure 1 : Electrophoretis pattern of the purified inhibitor fraction : (**A**) Calibration curve established using the Pharmacia low Mr markers. (**B**): Lane 1 : Purified inhibitor protein. Lane 2 : Protein inhibitor treated with 15 % ß-mercaptoethanol.



during 30 min at different temperatures. The papain inhibiting activity was unchanged between 40 and 80°C whereas it decreases by about 25% 30% between 80 and 100°C. The pH stability was performed by incubating the inhibitor at various pH ranging from 5 to 8 in the absence of the enzyme during 3 h. This protein seemed to be highly stable in this pH range since no activity loss was noted.

Kinetic properties : the availability of highly purified inhibitor preparations enables determination of the molar concentration of the inhibitor using a Mr of 34.2 KDa and a protein content of 50 µg/ml. Assuming that all inhibitor molecules were active, the molar concentration was estimated to be 1.1 µM.

Activity against cathepsins and papain : for all proteinases , the extent of inhibition by the muscle inhibitor is time dependent. Enzymes were preincubated for various time with equimolar levels of inhibitor until constant residual activity was obtained (BIETH, 1980). Whereas maximum inhibition of papain and cathepsin L was achieved after 25 min and 30 min respectively, a 60 min and 40 min preincubation was needed for cathepsins B and H respectively (Fig. 2).

igure 2: Time course decrease in the fractional free
nzyme concentration when preincubated with equimolar
vels of inhibitor.

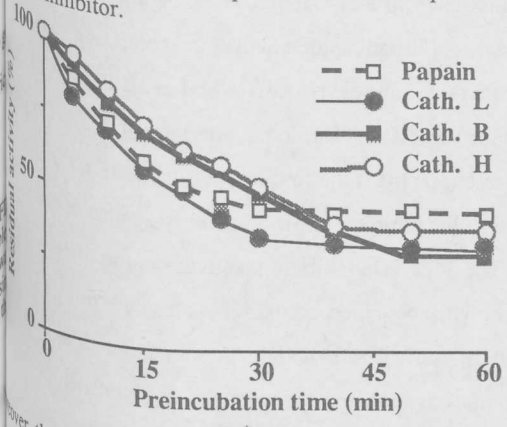
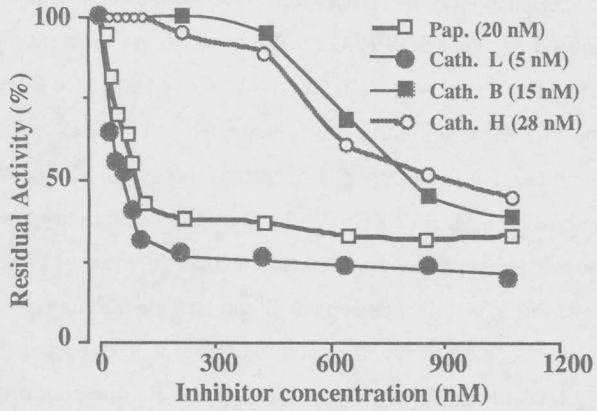


Figure 3: Changes in the fractional free enzyme concentration upon treatment by increasing levels of inhibitor

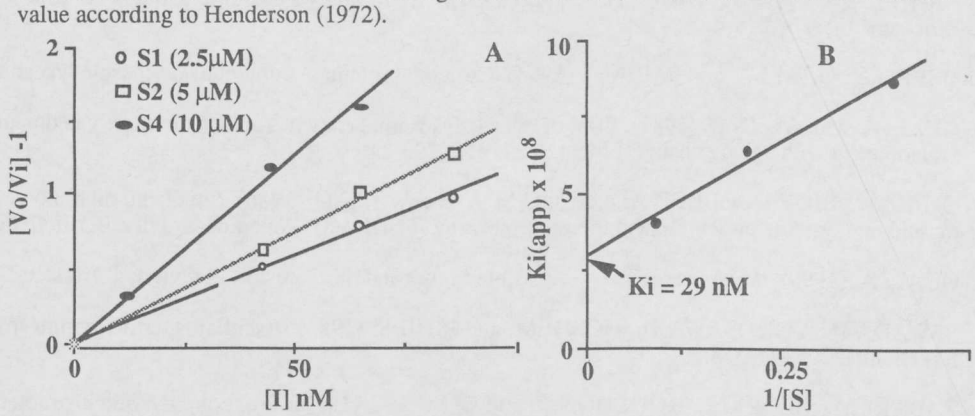


wever, the efficiency of the inhibitor to inactivate cysteine proteinases is highly variable. As shown in Fig. 3, papain and cathepsin L were much more sensitive than cathepsins B and H for which a decrease in the residual activity occurred at higher inhibitor concentration (> 430

lue for papain and cathepsins B and H : for these proteinases, the equilibrium constant (Ki) was determined according to BIETH (1980) ing the non reversibility of the proteinase-inhibitor association, a condition achieved by working at low enzyme and inhibitor ntrations in the assays which led to a non linearity of the titration curve. Hence, equimolar inhibitor and enzyme levels ranging from 10^{-7} $^{-8}$ M were preincubated in a minimum final volume. In such experimental conditions, Ki(app) can be determined by the EASSON-DMAN method (HENDERSON, 1972). This method is based on the following equation : **Io/1-a = Ki/a + Eo (1)** where a is the nal free enzyme level ([EI]/[Eo] or Vi/Vo), Io the initial free inhibitor level and Eo the initial free enzyme level. The plot of [Io/1-a] versus yields a straight-line whose slope is Ki(app). As the Km values of the enzymes for their respective specific substrates was higher than the substrate concentration (Km > So) , the Ki values can be deduced from the following equation : **Ki(app) = Ki (1+So/Km)** (2). the Km values reported by BIGE (1985), Ki values calculated for papain, cathepsin B and cathepsin H according to equation 2 were 70 600 nM and 410 nM respectively (Table 3).

lue for cathepsin L : optimisation of the inhibitor-cathepsin L interaction was achieved by working with a very low proteinase-inhibitor [E]/[I]<0,01). Under such conditions the EASSON-STEDMAN plot cannot be used for the Ki determination, but as described by NICKLIN al. (1984), when [I]>[E], it's easily shown that : **1/a = 1 + I/Ki(app).** On plotting **[1/a-1]** against **[I]**, a straight-line plot with a gradient /Ki(app) was obtained (Fig. 4A). The experimental Ki(app) values obtained in the presence of 2.5 , 5 , 10 µM of Z-Phe-Arg-NMec were 6 and 39 nM respectively. Ki(app) thus decreased as a function of substrate concentration which is typical of uncompetitive mechanism ENDERSON, 1972). According to this author, a further replot of Ki(app) versus 1/S was linear and the extrapolated intercept on the ase yielded a true Ki value of 29 nM (Fig. 4B).

Figure 4 : Determination of the equilibrium constant Ki characterising the interaction of the inhibitor with cathepsin L ([Eo] = 17 nM). **(A) :** Estimation of the apparent Ki using three different substrate concentrations according to Nicklin et al. (1984). **(B) :** Estimation of the Ki value according to Henderson (1972).



le 3 : Ki values for binding of the hibitor with cathepsins B, H, L and Papain

Proteinases	Ki (nM)
Papain	70
Cathepsin L	29
Cathepsin B	600
Cathepsin H	410

CUSSION AND CONCLUSION : Bovine skeletal muscle contained several cysteine proteinase inhibitor fractions referred to as : FII, FIII and FIV according their increasing elution volume from a Sephadex G100 column (ZABARI et al. 1991). From the FIII fraction,

we purified to homogeneity a 34.2 KDa inhibitor protein using two step anionic exchange chromatography on a Q-Sepharose column run at p
8.15 and 7.6 respectively. This inhibitor is thermostable and stable over a large pH range from 5 to 8. It appears to be not sensitive to reduc
agents. Indeed, whether SDS-PAGE was performed in the absence and in the presence of ß-mercaptoethanol at concentration as high as 15
only one band was observed. A fraction (Mr = 29 KDa) called I-T was isolated from rabbit muscle (MATSUISHI et al. 1988). Conversely
the bovine 34.2 KDa inhibitor, I-T was converted to a low molecular weight inhibitor I-M (10.7 KDa) in the presence of thiol reducing agen
suggesting that I-T was a trimer of I-M formed through dissulfide bonding. The present report is the first to describe a skeletal muscle papa
inhibitor showing a Mr 34.2 KDa. With regard to its specificity towards proteinases, this inhibitor show no activity against serine or aspar
proteinase but, although to various extent, inhibited all cysteine proteinases tested. The 34.2 KDa inhibitor inactivated strongly both papain an
cathepsin L and moderately cathepsins B and H. This finding is in good agreement with the experimental Ki values determined for the thr
enzymes. For all enzymes tested, the inhibitory activity was dependent on the inhibitor-enzyme ratio and the preincubation time. Moreover,
efficiency was shown to vary between enzymes a finding comforted by the Ki values determined according to HENDERSON (1972) an
NICKLIN and BARRETT (1984). Ki was estimated to be 70 nM for papain, 600 nM for cathepsin B, 410 nM for cathepsin H and 29 nM f
cathepsin L. The most sensitive enzyme was cathepsin L for which the mechanism was shown to be uncompetitive. Regarding this proteina
and using the approach of BIETH (1980), the low Ki value and the high I/Ki ratio >10 agree with a potential significant physiological role
assumption supported by the likely very high "in vivo" I/E ratio and the relatively high I/Ki ratio previously determined. However, othe
kinetic parameters might be determined (Kass, Kdiss, delay time and stability time) to confirmed this physiological role.

REFERENCES

BAICI A. and GYGER-MARAZZI M. (1982). The slow, tight-binding inhibition of cathepsin B by leupeptin. A hysteretic effect. Eur
Biochem. 129, 33-41.

BARRETT A.J. (1980). Fluorimetric assays for cathepsin B and cathepsin H with methylcoumarylamide substrates. Biochem. J. 187: 90
912.

BIETH J.G. (1980). Pathological interpretation of kinetic constants of protease inhibitors. Bull. Europ. Physiopa. Resp. 16, 183-195.

BIETH J.G. (1984). *In vivo* signification of kinetic constants of protein proteinase inhibitors. Biochemical medecine, 32, 387-397.

BIGE L., OUALI A. and VALIN C. (1985). Purification and characterization of a low molecular cysteine proteinase inhibitor from bov
muscle. Biochemica and Biophysica Acta. 843: 269-275.

BIGE L. (1985). Purification et caracterisation des inhibiteurs musculaires des cysteine proteinases lysosomiales. These de Docteur d'Un
Univ. de CLERMONT-FERRAND II.

HENDERSON P. J. F. (1972). A linear equation that describes the stady-states kinetics of enzymes and subcellular particles interacting wi
tightly bound inhibitors. Biochem. J., 127, 321-333.

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

LOWRY O. H., ROSEBROUGH N. J., FARA A.L. and RANDALL R.J. (1951). Protein measurement with the FOLLIN phenol reagen
Biol. Chem., 193, 256-268.

MATSUISHI M., OKITANI A., HAYAKAWA Y., and KATO H. (1988). Cysteine proteinase inhibitors from rabbit skeletal muscle. I
Biochem. 20: 259-264.

MATSUMOTO T., OKITANI A., KITAMURA Y. and KATO H. (1983). Some properties and postmortem changes of cysteines protei
inhibitors from rabbit skeletal muscle. Agri. Biol. Chem. 47: 2365-2371.

NICKLIN M. J. H. and BARRETT A. J. (1984). Inhibition of cysteine proteinases and dipeptidyl peptidase I by egg-white cystat
Biochem. J., 223, 245-253.

OBLED A., OUALI A. and VALIN C. (1984). Cysteine proteinase content of rat muscle lysosomes. Biochimie, 66, 609-616.

OUALI A. and VALIN C. (1981). Effet of muscle lysosomal enzymes and calcium activated neutral proteinase on myofibrillar ATPase activ
: relationship with ageing changes; Meat Science, 5, 233-245.

OUALI A., BIGE L., OBLED A., LACOURT A. and VALIN C. (1986). Small and high molecular weight proteinase inhibitors from bov
muscle.In: Cysteine proteinase and their inhibitors.(V. TURK ed). Walter de Gruyter. Berlin. New York, PP.545-554.

OUALI A. (1990). Meat tenderization : possible causes and mechanisms. A review. J. Muscle Foods. 1, 129-165.

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

ZABARI M., BERRI M., ROUCHON P. and OUALI A. (1991). Fractionation and characterization of proteinases inhibitors from bovine
skeletal muscle.37th Intern. Cong. of Meat Scien. and techn., 1, 452-456.