Vol. 120, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 16, 1984 Pages 96-102

IDENTIFICATION OF FOUR DISTINCT SERINE PROTEINASE INHIBITORS IN RAT SKELETAL MUSCLE

Lothar Kuehn, Burkhardt Dahlmann and Hans Reinauer

Biochemische Abteilung, Diabetes-Forschungsinstitut,
Auf'm Hennekamp 65, D-4000 Düsseldorf, Germany

Received February 28, 1984

SUMMARY: The serine proteinase inhibitory capacity in the cytosolic fraction of rat skeletal muscle tissue is accounted for by several discrete inhibitory activities. Three of these activities are identical with the proteinase inhibitors α_1-proteinase inhibitor, rat proteinase inhibitor I and rat proteinase inhibitor II respectively, which have been recently characterized as major serine proteinase inhibitors in rat serum (Kuehn, L., Rutschmann, M., Dahlmann, B. and Reinauer, H. (1984) Biochem. J. 218, in the press). The other inhibitor molecule, having an M_r of about 15 000, appears to be an endogeneous inhibitor.

During recent years, increasing attention has focused on the physiological role of proteinase inhibitors (1). Detailed studies have been conducted on the properties and function of plasma proteinase inhibitors in man (2) and in other mammals such as the rat (3). On the other hand, while a potential role for proteinase inhibitors as modulators of intracellular proteolysis has been recognized, the molecules involved are not well defined. Since tissue proteins are subject to continuous turnover, a control mechanism must exist to ensure protein homoeostasis and this control could also be performed by proteinase inhibitors.

In several conditions showing an accelerated protein breakdown in skeletal muscle, an increase in alkaline serine proteinase activity has been observed (4). Specifically, we have found that, during insulin deficiency in the streptozotocin-diabetic rat, the capacity of a serine proteinase (chymase) is increased and that this activity can be normalized by administration of insulin (5,6). Such adaptive behaviour has suggested that chymase may be involved in the overall protein breakdown, at least in pathological situations. Although the enzyme originates in intramuscular mast cells, it is equally present in certain muscle cells (7), possibly by a mechanism of 'transgranulation' (8), and the enzyme degrades both myofibrillar (9) and muscle cell cytosolic proteins (10).

ABBREVIATIONS: α_1-PI, α_1-proteinase inhibitor; RPI I and RPI II, rat proteinase inhibitors I and II respectively; Bistris, 2-[-bis-(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; Bistrispropane, 1,3-bis-[(1,1-dihydroxy-2-hydroxymethyl)isopropylaminopropane]; BSA, bovine serum albumin; Bz-, benzoyl; -NPhNO$_2$, 4-nitroanilide; SDS, sodium dodecyl sulphate.

0006-291X/84 $1.50

This, together with the finding that muscle tissue extracts contain inhibitory activity against alkaline serine proteinase (4,11-13), has prompted us to examine whether these inhibitors might be involved in the modulation of proteinase activity. As a first step, we have analyzed the inhibitory spectrum in cytosol from rat skeletal muscle, and we report here on the identification of the individual components.

MATERIALS AND METHODS

Fed, male Wistar rats (200-230 g) were used throughout and the animals were divided into two groups: one group of rats was killed by exsanguination under anaesthesia and the blood was drained prior to removal of the gastrocnemius muscles; the other animals (control group), were anaesthetized and the blood was removed from muscle capillaries by non-recirculating hind-limb perfusion (14) with Krebs-Ringer-bicarbonate buffer/5 mM glucose, pH 7.4 (perfusion rate: 10 ml/min, total volume: 100 ml). Gastrocnemius muscles were quickly excised and frozen at -20°C.

For isolation of serine proteinase inhibitors, gastrocnemius muscles were dissected and homogenized in a fivefold volume (v/w) of icecold, 25 mM Bistris-propane/HCl buffer, pH 6.7 (Sigma, München) in an MSE homogenizer for 1 min. at 12 000 g (4°C) and the resulting supernatant was used as the source of serine proteinase inhibitory activity. This cytosolic fraction contained no alkaline serine proteinase activity as assessed enzymatically (15) or with immunological methods, using a monospecific antiserum (16). Anion-exchange chromatography on Mono Q™ HR 5/5 (Pharmacia, Uppsala) in conjunction with the Pharmacia FPLC (fast protein liquid chromatography) system and chromatography on Affi-gel Blue (Bio-Rad, München) was performed as in (17). Gel filtration on Sephadex G-75 superfine grade (Pharmacia) was done as in (18), but using 25 mM Bistrispro-pane/HCl, pH 6.7.

Analytical procedures: Sources of pancreatic trypsin, chymotrypsin and elastase as well as determination of proteinase inhibitory activity were as detailed in (17) except that Bz-Val-Gly-Arg-NPhNO$_2$ (Boehringer, Mannheim) was used for measuring trypsin-activity. One mUnit of inhibitory activity was defined as that amount of a preparation which completely inhibited 1 pmol of active proteinase.

Protein was determined as in (19) with Lab-Trol (Dade, Miami) as the standard.

Ouchterlony double immunodiffusion analysis was performed as described in (20).

The statistical significance between the mean values of the data obtained for the non-perfused and the perfused muscles was assessed by Student's t-test.

RESULTS

As rat serum contains a number of serine proteinase inhibitors (21), it was important to exclude that the proteinase inhibitory activity measured in the muscle cytosolic fraction was due to a contamination of the tissue with blood. Therefore, trypsin inhibitor levels were measured in muscles from exsanguinated animals and were compared with the levels in hind-limb perfused (control) muscles. As shown in Table 1, however, no statistically different values were found for the two preparations. During all subsequent experiments, muscles from exsanguinated animals were used.

To further characterize the inhibitory activity, the cytosolic fraction was subjected to anion-exchange chromatography. As shown in Fig. 1, the activity is resolved into three distinct peaks. Thus, peak 1 is inhibitory towards trypsin

TABLE 1. Effects of hind-limb perfusion and exsanguination of rats on muscle weight, on protein content and on trypsin-inhibitory activity in cytosolic fractions of rat gastrocnemius muscle. For full experimental details, see text.

Experiment	Muscle weight (g)	Cytosolic protein (mg)	Antitrypsin activity (mUnits)	Specific activity (mUnits/mg)
Perfusion	1.7 ±0.1	77±7.6	1185±117	14±1.1
Exsanguination	1.54±0.11 (n.s.)	75±2.2 (n.s.)	1217±114 (n.s.)	16±1.4 (n.s.)

Values given are means ± standard error of the means from 6 separate experiments; n.s., not significant ($P>0.05$)

only, peak 2 is active against all three serine proteinases tested, while peak 3 is inhibitory towards trypsin and chymotrypsin, but not elastase. Recovery of inhibitory activity during this step was quantitative (96±8%, means ± SEM for 6 independent experiments), suggesting that these activities represented the spectrum of inhibitors, in the muscle cytosol fraction, towards pancreatic serine proteinases. Since ion-exchange chromatography was analogous to the method described for fractionation of inhibitors from rat serum (17), peak 2 and peak 3 inhibitory activities were possibly due to the presence, in muscle cytosol, of the serum proteinase inhibitors α_1-PI and RPI I (together with RPI II). This assumption is based on the finding that i.) the two activities were eluted at the same salt concentrations as had been the case for the inhibitors from rat serum and that ii.) they showed the same activity pattern towards the three pancreatic serine proteinases tested. In contrast, the trypsin-inhibitory activity eluted with 50-70 mM NaCl (peak 1) had not been previously identified in

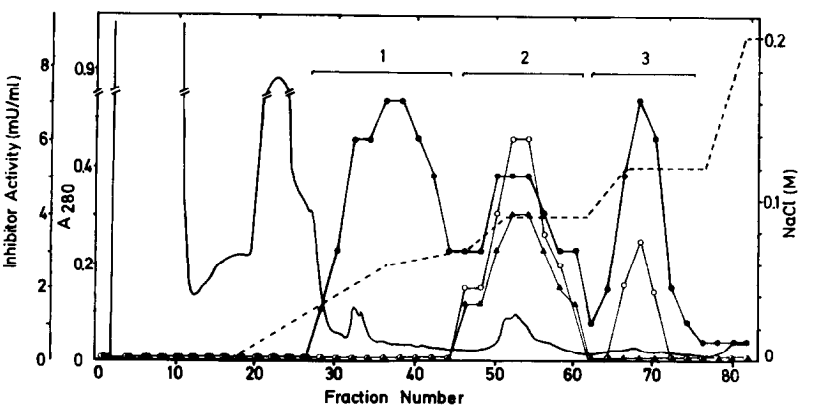

Fig. 1. Anion-exchange chromatography of rat skeletal muscle cytosol on Mono Q. 12 ml of muscle cytosol, containing 22 mg protein was applied to the column (0.5 x 10 cm) equilibrated with 20 mM Bistris/HCl buffer, pH 6.0. Starting at fraction 16, the column was eluted with a linear/isocratic gradient of 0-0.2 M NaCl in the buffer. The flow rate was 1 ml/min., and 1 ml fractions were collected. ----, concn. of NaCl; ——, A_{280}; ●—●, antitrypsin activity; ○—○, antichymotrypsin activity; ▲—▲, anti-elastase activity.

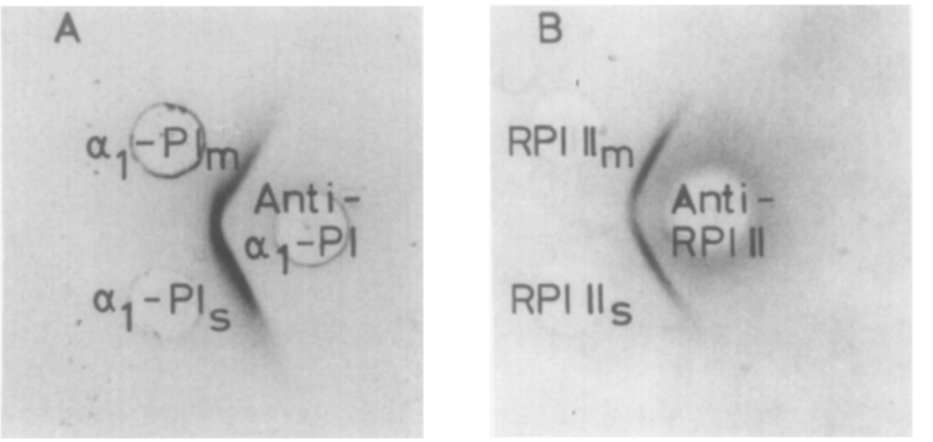

Fig. 2. Double immunodiffusion of (A) anti-α_1-PI antiserum (10 μl) against α_1-PI from rat serum (α_1-PI$_s$, 1 μg) and from gastrocnemius muscle (α_1-PI$_m$, 5 μg) and of (B), anti-RPI II antiserum (10 μl) against RPI II from rat serum (RPI II$_s$, 1 μg) and from gastrocnemius muscle (RPI II$_m$, 6 μg).

rat serum. As specific antisera against α_1-PI and RPI II from rat serum were available, we tested the above assumption by immunological methods. Double immunodiffusion analysis showed that the inhibitors from muscle were precipitated by the respective antisera, forming single precipitin lines and these precipitin lines completey fused with those formed between serum α_1-PI and RPI II, respectively (Fig. 2). This result clearly demonstrated the identity of two inhibitors isolated from the different sources.

The suggestion that peak 3 inhibitory activity in Fig. 1 is the composite of RPI II and RPI I could be confirmed by chromatography, of this fraction, on Affi-gel Blue , a step which allows the separation of RPI I from RPI II, in rat serum (17). As shown in Fig. 3, peak 3 material from the previous step is elu-

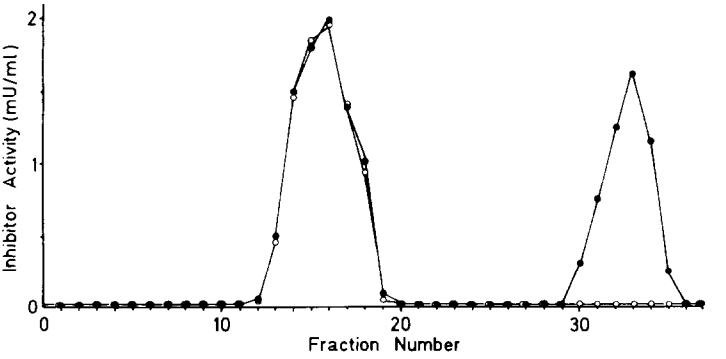

Fig. 3. Separation of RPI I from RPI II by Affi-gel Blue chromatography. The concentrated, dialyzed peak 3 fraction from Mono Q chromatography (2 ml) was applied to an Affi-gel Blue column (1 x 15 cm), equilibrated with 25 mM Bistris-propane/HCl buffer, pH 6.7. After sample application, the column was eluted with equilibration buffer. The flow rate was 13 ml/cm^2 per h, and 1 ml fractions were collected. ●—●, trypsin-inhibitory activity; ○—○, chymotrypsin-inhibitory activity.

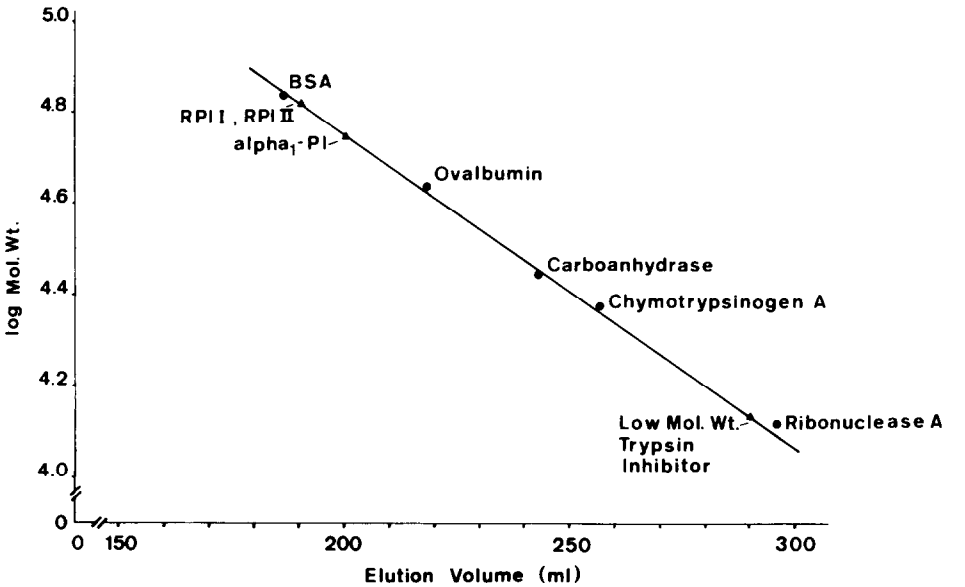

Fig. 4. M_r determination of serine proteinase inhibitors isolated from rat skeletal muscle cytosol, by gel-filtration on Sephadex G-75 superfine grade. The following proteins were used for calibration of the column : BSA (M_r 68 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 30 000), chymotrypsinogen A (M_r 25 000), and ribonuclease A (M_r 13 800). For experimental details, see the text.

ted from the Affi-gel Blue column as two distinct peaks, fractions 12-19 being active against trypsin and chymotrypsin (RPI I) and fractions 30-35 being active towards trypsin but not chymotrypsin (RPI II). The order of elution of the two activities was found to be identical with that of RPI I and RPI II from rat serum (17).

Finally, evidence for the identity of three of the inhibitors from muscle cytosol and α_1-PI, RPI I and RPI II from serum was obtained by gel-filtration experiments (Fig. 4). Thus, the inhibitors having the inhibitory specificities of α_1-PI, RPI I and RPI II, respectively are eluted as molecules with apparent M_r values of about 55 000, 65 000 and 65 000, values which are the same as those found for the respective inhibitor proteins from rat serum, by analysis in SDS polyacrylamide gels (17).

During gel filtration the other, trypsin-inhibitory component of muscle cytosol (peak 1, Fig.1) was eluted at a position corresponding to about M_r 15 000. There appears to be no counterpart of this molecule in rat serum.

DISCUSSION

Several studies have reported the presence of serine proteinase inhibitory activity in rat skeletal muscle tissue and have suggested a potential role of these activities in the control of intracellular proteolytic activity (4,11,13). Furthermore, by use of immuno-histochemical methods, discrete inhibitory spe-

cies such as the exogeneous proteinase inhibitors α_1-PI and α_1-inhibitor 3 have been demonstrated inside rat skeletal muscle cells (22). The present study confirms these authors' finding by showing that α_1-PI is a component of the serine proteinase inhibitory system of rat skeletal muscle tissue. In addition, the exogeneous inhibitors RPI I and RPI II as previously identified in rat serum (17) were found to be two further components of this system. The failure to have detected, in the present study, α_1-inhibitor 3 is probably due to the low levels of this inhibitor in rat serum (23) and this is most likely true in the muscle tissue as well. The low molecular weight inhibitor which accounts for about 40% of the trypsin inhibitory activity measured in muscle cytosol, appears to be an endogeneous inhibitor. The apparent M_r value and the lack of inhibitory actvity against glandular kallikrein (L. Kuehn, unpublished observation) argues against this protein to be related to aprotinin, localized in mast cells (24). The inhibitor appears also to be different from the Kunitz-type inhibitor (M_r 14 000) a product that is released from α_1-inhibitor 3 by tryptic digestion (25) and which shows a broader spectrum of proteinase inhibition than does the inhibitor reported here. Furthermore, its molecular size suggest it to be different from the inhibitor of neutral proteinase with an M_r of 7 500-9 000 (26). Finally, since the inhibitor is inactive towards cysteine proteinases (L.Kuehn, unpublished work) it is different from sericystatin, an endogeneous inhibitor with presumed activity towards both serine and cysteine proteinases (27).

A potential physiological role of two of the inhibitors described herein is suggested by the following observations: i.) α_1-PI and RPI I are potent inhibitors of chymase (12); ii.) during situations of enhanced muscle protein breakdown like diabetes the proteolytic capacity of this enzyme is increased several fold (5) while, inversely, levels of inhibitors like α_1-PI are significantly reduced (22) and iii.) levels of both the enzyme and the inhibitor are restored to normal by administration of insulin to the diabetic animals (5, 22).

Work is in progress to study this proposal in detail.

ACKNOWLEDGEMENT

This work was supported by the Ministerium für Wissenschaft und Forschung des Landes Nordrhein-Westfalen, Düsseldorf, by the Bundesministerium für Jugend, Familie und Gesundheit, Bonn and by a grant (SFB 113, Projekt G4) from the Deutsche Forschungsgemeinschaft, Bonn, Germany. We thank Mrs. M. Koenen for expert technical assistance and Dr. P. Schadewaldt for his help with setting up the hindquarter perfusion system.

REFERENCES

1. Laskowski, M.,Jr., and Kato, I. (1980) Ann. Rev. Biochem. 49, 593-626
2. Travis, J., and Salvesen, G.S. (1983) Ann. Rev. Biochem. 52, 655-709
3. Weström, B.R., and Carlsson, L.C.T. (1976) Int. J. Biochem. 7, 41-47

4. Dahlmann, B., Kuehn, L., Rutschmann, M., and Reinauer, H. (1981) Acta biol. med. germ. 40, 1349-1355
5. Röthig, H.J., Stiller, N., Dahlmann, B., and Reinauer, H. (1978) Horm. Metab. Res. 10, 101-104
6. Dahlmann, B., Schroeter, C., Herbertz, L., and Reinauer, H. (1979) Biochem. Med. 21, 33-39
7. Stauber, W.T., Fritz, V., Dahlmann, B., and Reinauer, H. (1983) J. Histochem. Cytochem. 31, 827-830
8. Greenberg, G., and Burnstock, G. (1983) Exptl. Cell Res. 147, 1-13
9. Reinauer, H., and Dahlmann, B. (1979) in Biological Functions of Proteinases (Holzer, H., and Tschesche, H., eds.) pp.94-101, Springer, Berlin
10. Dahlmann, B., Kuehn, L., and Reinauer, H. (1983) Biochim. Biophys. Acta 761, 23-33
11. Noguchi, T., and Kandatsu, M. (1969) Agr. Biol. Chem. 33, 1226-1228
12. Kuehn, L., Dahlmann, B., and Reinauer, H. (1983) Hoppe Seyler's Z. Physiol. Chem. 364, 1168 (Abstract)
13. Dudai, M., Mayer, M., and Kidron, M. (1982) Hoppe Seyler's Z. Physiol. Chem. 363, 651-654
14. Ruderman, N.B., Houghton, C.R.S., and Hems, R. (1971) Biochem. J. 124, 639-651
15. Dahlmann, B., and Reinauer, H. (1978) Biochem. J. 171, 803-810
16. Dahlmann, B., Block, I., Kuehn, L., Rutschmann, M., and Reinauer, H. (1982) FEBS Lett. 138, 88-90
17. Kuehn, L., Rutschmann, M., Dahlmann, B., and Reinauer, H. (1984) Biochem. J. 218, in the press
18. Rutschmann, M., Kuehn, L., Dahlmann, B., and Reinauer, H. (1982) Anal. Biochem. 124, 134-138
19. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254
20. Ouchterlony, O. (1964) in Immunological Methods (Ackroyd, J.F., ed.) pp. 55-78, Blackwell Scientific Publications, Oxford
21. Gauthier, F., Genell, S., Mouray, H., and Ohlsson, K. (1978) Biochim. Biophys. Acta 526, 218-226
22. Stauber, W.T., Ong, S.H., Fritz, V., Esnard, F., and Gauthier, F. (1983) Histochem. J. 15, 1079-1086
23. Esnard, F., and Gauthier, F. (1980) Biochim Biophys. Acta, 614, 553-563
24. Fritz, H., Kruck, J., Rüsse, I., and Liebich, H.G. (1979) Hoppe Seyler's Z. Physiol. Chem. 360, 437-444
25. Albrecht, G.J., Hochstrasser, K., and Schönberger, Ö.L. (1983) Hoppe Seyler's Z. Physiol. Chem. 364, 1697-1702
26. Carney, I.T., Curtis, C.G., Kay, J., and Birket, N. (1980) Biochem. J. 185, 423-433
27. Kopitar, M., Brzin, J., Locnikar, P., and Turk, V. (1981) Hoppe Seyler's Z. Physiol. Chem. 362, 1411-1414