A Low-Molecular-Weight Inhibitor of the Neutral Proteinase from Rat Intestinal Smooth Muscle

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1. Rat intestinal smooth muscle was shown to contain endogenous inhibitory activity towards the neutral trypsin-like muscle proteinase described previously [Beynon & Kay (1978) *Biochem. J.* 173, 291–298]. 2. Contamination of the muscle tissue by mucosal, blood and pancreatic inhibitors was shown to be unlikely. 3. The inhibitory activity was resolved into high- and low-molecular-weight components. 4. The low-molecular-weight component was purified to homogeneity. It had a molecular weight of approx. 9000 and was stable over the pH range 3–11. 5. It inhibited the muscle proteinase competitively ($K_i \approx 5 \mu\text{M}$), but had no effect on any of the other proteinases tested. 6. Leupeptin also inhibited the muscle proteinase competitively ($K_i \approx 0.3 \mu\text{M}$), whereas the low-molecular weight proteins gastrin, glucagon and insulin B-chain had very little effect. 7. A role for a weakly binding inhibitor in modulating the influence of the neutral proteinase on intracellular protein degradation is considered.

Intracellular proteins are degraded at markedly different rates within the cell, but the nature of the step that introduces the selectivity into the degradation process is not known. One possibility that has been suggested (for a review see Kay, 1978) involves proteolysis at neutral pH and at a location distinct from the lysosomal matrix. Indeed, it would appear that there may be two pathways for the general catabolism of intracellular proteins (Ballard, 1977): a lysosomal route that, in isolated hepatocytes, appears to account for about 70% of the total protein turnover (Seglen *et al.*, 1979), and a non-lysosomal pathway that accounts for the remainder and appears to be primarily responsible for the degradation of short-lived and abnormal proteins (Knowles & Ballard, 1976; Seglen *et al.*, 1979). The quantitative importance of the latter route in tissues such as muscle with a relatively low lysosomal content has yet to be evaluated.

In a previous report (Beynon & Kay, 1978) we described the presence in and partial purification from rat intestinal muscle of a trypsin-like serine proteinase. Although proteolysis of denatured proteins by this enzyme proceeded at rates similar to those observed with other neutral proteinases, the

muscle proteinase had a marked ability for attacking native protein structures at neutral pH. It was 100–300 times more efficient in inactivating enzymes in their native conformation than an equimolar amount of trypsin. Such an activity acting within the cell could degrade individual proteins at different rates at neutral pH and could have profound consequences for the metabolic status of that cell. The activity would have to be very carefully regulated, and it was shown that by altering the vulnerability of the substrate proteins, e.g. allosterically or as a result of changes in primary, secondary, tertiary or quaternary structure, it was possible to modulate the rate of proteolysis (Kay *et al.*, 1977; Carney *et al.*, 1978a). An alternative method of controlling such an active proteinase would be through the action of an endogenous inhibitor(s). Preliminary findings (Beynon & Kay, 1977; Carney *et al.*, 1978b) suggesting the existence of inhibitory activity have been reported. In the present paper, we describe the purification to homogeneity and characterization of a low-molecular-weight protein inhibitor from rat intestinal muscle.

Many previous papers have described proteinase inhibitors that are located extracellularly in diverse sources, e.g. in legume seeds (Kassell & Williams, 1976), in avian egg white (Kato *et al.*, 1978), in blood plasma (Heimbürger, 1974; Starkey & Barrett,

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1977) and nasobronchial fluids (Hochstrasser *et al.*, 1978). However, very little information has been reported for the complete characterization of an intracellular proteinase inhibitor.

Materials and Methods

Soya-bean trypsin inhibitor, azocasein, elastase, subtilisin and papain were obtained from Sigma (U.K.) Ltd., Poole, Dorset, U.K., and tosyl-Gly-Pro-Arg *p*-nitroanilide and the B-chain of oxidized insulin were purchased from Boehringer (U.K.), Lewes, Sussex, U.K. Agarose-trypsin, agarose-chymotrypsin, agarose-Pronase, trypsin, chymotrypsin, bovine serum albumin and pig heart mitochondrial malate dehydrogenase (in glycerol) were provided by Miles Laboratories, Stoke Poges, Slough, U.K. α -Trypsin was further purified by the method of Schroeder & Shaw (1968) and its concentration was determined by $A_{280}^{1\%} = 15.4$ (Kay & Kassell, 1971). Heparin and a set of molecular-weight standards (comprising CNBr fragments of myoglobin) for use in calibrating sodium dodecyl sulphate/urea/polyacrylamide gels were obtained from BDH Ltd., Poole, Dorset, U.K. Pepstatin was a gift from Professor H. Umezawa, Institute of Microbial Chemistry, Tokyo, Japan. Leupeptin and gastrin were gifts from Dr. A. J. Barrett, Strangeways Laboratory, Cambridge, and Professor R. A. Gregory, University of Liverpool, respectively. Glucagon and the metalloproteinase from *Armillaria mellea* were respectively gifts from Dr. A. Cryer and Dr. J. Basford of this Department. All other chemicals were obtained from Sigma. Buffers were made from sodium phosphate.

Dialysis tubing was obtained from The Scientific Instrument Centre, London WC1X 9JA, U.K., and Spectrapor 6 dialysis tubing (which nominally retains molecules of mol.wt. greater than 3500) was purchased from Raven Scientific, Haverhill, Suffolk, U.K. The latter tubing was rinsed with running tap water for several hours at room temperature and then with several changes of distilled water to remove the glycerol included as humectant.

UM2 and PM10 ultrafiltration membranes (which nominally retain molecules of greater than 1000 and 10000 daltons respectively) were the products of Amicon Ltd., High Wycombe, Bucks., U.K.

Experimental animals

Adult male Wistar-strain rats (200–250 g) were maintained on the stock laboratory diet (Pilsburys, Edgbaston, Birmingham, U.K.) with a protein content of 17% (w/w). Food was removed from the animals 24 h before they were anaesthetized with ether. Heparin (0.2 mg/ml in 0.9% NaCl; 1 ml) was injected through the penial vein and, after exsanguination by cannulation of the dorsal

aorta, the vascular bed of the gut was washed out with a retrograde infusion of 0.9% NaCl through the hepatic portal vein to prevent any possibility of contamination by blood-plasma inhibitors. During the perfusion, the pancreas increased greatly in volume and was completely excised from the duodenal tissue. However, in order to be certain that the inhibitory activity was not being derived from the pancreas, the initial 15 cm length of duodenum was discarded.

The lumen of the remaining small intestine was subjected to a rigorous washing procedure with 0.9% NaCl to prevent contamination from residual pancreatic proteins or inhibitors, and the muscle layer was isolated and homogenized in 3 vol. of 0.02 M-sodium phosphate buffer, pH 7.5, as described by Beynon & Kay (1978). Centrifugation of homogenates at 100000 *g* for 20 min produced a supernatant (S1) that contained all of the inhibitory activity.

The intestinal-muscle proteinase was prepared from the muscle tissue of rats that had been fed on a protein-free diet (Micro-Bio Laboratories, Cambridge, U.K.) for 3 weeks before death, and its concentration was determined by active-site titration with soya-bean trypsin inhibitor, as described by Beynon & Kay (1978).

Assays for proteolytic activity

The activity of the intestinal muscle proteinase was measured at pH 7.5 by using two types of substrate:

(a) the inactivation of native enzymes, i.e. malate dehydrogenase, as described by Beynon & Kay (1978);

(b) the hydrolysis of a peptide substrate, tosyl-Gly-Pro-Arg *p*-nitroanilide. This was carried out at 30°C in 0.88 ml of 0.02 M-sodium phosphate buffer, pH 7.5. The peptide substrate (8 mg/ml in ethanol; 10 μ l) was added and the reaction was initiated by addition of the proteinase in a small volume (1–10 μ l). The rate of release of *p*-nitroaniline was monitored at 405 nm in a Cecil Instruments CE272 spectrophotometer fitted with a Bryans 27000 chart recorder. One unit of proteolytic activity is defined as that amount of proteinase causing an increase in A_{405} of 0.01/min (equivalent to approx. 50 ng of proteinase).

The activity of other neutral proteinases was measured at pH 7.5 with azocasein as the substrate (Beynon & Kay, 1978).

Assays for proteinase-inhibitory activity

Inhibitory activity was measured by preincubation of samples of putative inhibitor with the proteinase for 1 min at 30°C, followed by determination of the residual proteolytic activity either in the

malate dehydrogenase-inactivating assay or by the peptide-hydrolysis assay. One inhibitory unit is that which causes the loss of 1 unit of proteolytic activity. A constant amount of proteinase was always used.

When for an inhibitor $K_i \leq K_m$, high concentrations of inhibitor have to be included in the assay system in order to see an appreciable effect (under steady-state conditions). Alternatively, the concentration of substrate can be lowered, but this may lead (as with tosyl-Gly-Pro-Arg *p*-nitroanilide) to changes in absorption that are too small to be measured accurately.

Values for K_m and V_{max} can also be found from an analysis of the progress curve of an enzyme-catalysed reaction (Cornish-Bowden, 1976) by using the equation:

$$\frac{t}{\ln(S_0/S)} = \frac{1}{V_{max}} \cdot \frac{(S_0 - S)}{\ln(S_0/S)} + \frac{K_m}{V_{max}}$$

where S_0 is the initial substrate concentration and S is the concentration at time t . Thus, a plot of $t/\ln(S_0/S)$ versus $(S_0 - S)/\ln(S_0/S)$ is a straight line with slope $1/V_{max}$ and an intercept on the abscissa of $-K_m$. Diagnosis of the type of inhibition can also be made by using this plot. For example, if progress curves are obtained at several concentrations of a competitive inhibitor and plotted according to this equation, a series of lines parallel to that obtained with the substrate alone should be observed (Corney, 1979).

General methods

The protein concentration of samples was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Molecular-weight determination on small proteins was carried out by electrophoresis in sodium dodecyl sulphate/urea/polyacrylamide gels as described by Swank & Munkres (1971). This utilizes 10% acrylamide gels with an acrylamide/bisacrylamide ratio of 10:1 (w/w). Samples containing approx. 50 µg of protein were dissolved in a loading solution containing 1% (w/v) sodium dodecyl sulphate, 8 M-urea, 1% (w/v) mercaptoethanol, 0.01 M- H_3PO_4 adjusted to pH 6.8 with solid Tris, and 0.0025% (w/v) Bromophenol Blue. The solutions were incubated at 60°C for 10 min, cooled and stored overnight at room temperature before electrophoresis. Gels were stained with Coomassie Blue and destained as described by Weber & Osborn (1969).

Samples for amino acid analysis were hydrolysed in 6 M-HCl *in vacuo* at 105°C for 24, 48, 72 and 96 h. The hydrolysates were evaporated to dryness, redissolved in 0.2 M-sodium citrate buffer, pH 2.2, and loaded into a Locarte amino acid analyser fitted with a six-channel automatic loader.

Results

Nature of the inhibitory activity

The muscle layer of the small intestine from rats that had been fed on a normal balanced diet was homogenized in 0.02 M-sodium phosphate buffer, pH 7.5 (see the Materials and Methods section), and centrifuged at 100 000 *g* for 20 min. Samples of the supernatant were incubated with the intestinal-muscle proteinase (Fig. 1), and all of the 'inhibitory' activity present originally in the homogenate (Table 1) was recovered in this supernatant (S1).

A peptide factor has been observed in liver which stabilizes phosphofructokinase (Dunaway & Segal, 1976) and ATP citrate lyase (Osterlund & Bridger, 1977) against proteolytic and thermal inactivation. The possibility that the S1-fraction 'inhibitory' activity might be acting in a similar manner by protecting malate dehydrogenase directly, i.e. without interacting with the muscle proteinase, was tested by making use of the fact that, in order to obtain equivalent rates of inactivation of malate dehydrogenase by the trypsin-like muscle proteinase and by trypsin, it is necessary to add about 170 times more trypsin (Beynon & Kay, 1978). If the S1-fraction 'inhibitory' activity was acting by stabilizing malate dehydrogenase, it should also protect the substrate against tryptic digestion, whereas it would not be able to inhibit such a large amount of trypsin to any significant extent. Supernatant S1 had no effect on the tryptic digestion of malate dehydrogenase (Fig. 1), so that it would appear that the inhibitory activity

Fig. 1. Effect of unfractionated S1 supernatant on the inactivation of malate dehydrogenase by the intestinal-muscle proteinase and by trypsin

Malate dehydrogenase (0.05 mg) was incubated at 30°C in a final volume of 0.25 ml of 0.02 M-sodium phosphate buffer, pH 7.5, with the intestinal-muscle proteinase (0.2 µg; ●) or trypsin (30 µg; ■) alone or in the presence of 50 µl of the initial S1 supernatant (○ and □ respectively). At appropriate times, samples were removed and assayed for the dehydrogenase activity remaining.

is not due to a stabilizing factor analogous to the peptide found by Dunaway & Segal (1976).

The pH-stability of the inhibitory activity was investigated as a method for further fractionation. Samples of the S1 fraction (containing 1300 units of inhibitory activity) were adjusted to pH 3.0 or to pH 11.0 and incubated at 37°C. Samples were removed after 1 and 2 h and adjusted back to pH 7.5. After removal of insoluble material by centrifugation (5000g for 10 min), the residual inhibitory activities were measured and compared with that of a sample maintained at pH 7.5. The inhibitory activity

was completely stable at both pH values (1300 units were recovered in every case). Consequently, for routine purification purposes, the S1 supernatant was incubated at pH 3 for 1 h at 37°C and then re-adjusted to pH 7.5 (Table 1).

Overnight dialysis of a sample of S1 supernatant (containing 1300 units) in conventional dialysis tubing resulted in a loss of approx. 50% of the inhibitory activity (600 units recovered). Prolonging the period of dialysis (together with changes of buffer) did not decrease the recovery of inhibitory activity further (600 units). By contrast, dialysis of super-

Table 1. *Purification of the low-molecular-weight proteinase inhibitor from rat intestinal muscle*

Intestinal muscle (12g) was homogenized in 36 ml of 0.02M-sodium phosphate buffer, pH 7.5. The homogenate was centrifuged at 100000g for 20 min to produce the supernatant, S1. The values for total inhibitory activity are the measured values in each case. The values of low-molecular-weight inhibitory (LMWI) activity for the homogenate, S1 and pH 3-treated supernatants were back-calculated as explained in the text.

Fraction	Protein (mg)	Total inhibitory activity (units)	Inhibitory activity from LMWI (units)	Specific activity of LMWI (units/mg)	Purification (fold)	Recovery (%)
Homogenate	1032	10300	6000	5.8	1	100
S1	258	10300	6000	23	4	100
Supernatant obtained after pH 3 treatment	146	10300	6000	41	7	100
Material retained by PM10 ultrafilter	84	3500	0	—	—	34 (of total)
Material passing through PM10 ultrafilter	56	4900	4900	87	15	81
Material retained by UM2 ultrafilter	48	4700	4700	98	17	78
Pooled fractions from Sephadex G-25	12	2600	2600	217	37	42

Fig. 2. *Chromatography of the inhibitory activity on Sephadex G-75*

A sample of the S1 supernatant was incubated at pH 3 for 1 h, re-neutralized to pH 7.5 and, after removal of the insoluble material, concentrated by ultrafiltration over a UM2 membrane. The concentrated material was applied to a column (2 cm × 60 cm) of Sephadex G-75 equilibrated in 0.02M-sodium phosphate buffer, pH 7.5. Fractions were measured for A_{280} (O) and for inhibitory activity (●) by using the malate dehydrogenase assay.

nant S1 in Spectrapor 6 tubing resulted in a recovery of inhibitory activity of 1100 units = 85%. This indicated the possibility of more than one proteinase inhibitor being present in the supernatant fraction.

A sample of the material obtained after the pH 3-treatment step was applied to a column of Sephadex G-75 (Fig. 2). The inhibitory activity was resolved into a high-molecular-weight component (peak A) and a peak of lower molecular weight (peak B), with approximately equal amounts of inhibitory activity in each peak. This resolution could be explained by:

(a) the existence of two (or more) discrete proteinase inhibitors;

(b) an association-dissociation system of peak-B inhibitor in equilibrium with an oligomeric form of the inhibitor, giving rise to peak A;

(c) proteolysis of the high-molecular-weight component *in vivo* or during the purification procedure, e.g. by lysosomal enzymes, which would be active during the pH 3 incubation.

The last possibility was tested by inclusion of pepstatin (an inhibitor of carboxyl proteinases such as cathepsin D; Barrett, 1977) and leupeptin [an inhibitor of thiol proteinases such as cathepsins B, L and H (Kirschke *et al.*, 1977), as well as the intestinal-muscle proteinase; see below] both at a final concentration of 0.1 mM in the initial homogenization buffer.

The homogenate was centrifuged (100000g for 20 min) and the supernatant was acidified, re-neutralized and chromatographed on Sephadex G-75 as before. An elution pattern identical with that in Fig. 2 was obtained, except that a third peak of

inhibitory activity was eluted with the total column volume (corresponding to the leupeptin added).

Thus it would appear unlikely that the two peaks of inhibitory activity resulted from proteolysis during the isolation procedure. Inhibitory activity was also lowered on dialysis of the whole S1 fraction (i.e. before the pH 3 step), so that the low-molecular-weight component must have been present in the S1 fraction. On repeated dialysis, however, only a portion of the total inhibitory activity was lost, so that it is improbable that the two peaks of inhibitory activity were involved in a monomer-oligomer equilibrium system. It would thus appear that the two pools of inhibitory activity were due to the existence of (at least) two discrete proteinase inhibitors. Further details of the high-molecular-weight component have been described elsewhere (Carney, 1979).

Purification of the low-molecular-weight inhibitor

Since the peak-B inhibitory activity appeared to be of low molecular weight, much of the unwanted protein was conveniently removed by passage of the acid-treated supernatant through an ultrafilter fitted with a PM10 membrane. Table 1 shows that approx. 50% of the total initial inhibitory activity passed through the PM10 membrane, but was retained in a subsequent ultrafiltration in a second ultrafilter fitted with a UM2 membrane (nominal molecular-weight cut-off 1000).

The material thus concentrated by using the latter membrane was applied to a column of Sephadex G-25 (Fig. 3). The inhibitory activity was eluted as a single peak. Fractions were pooled as indicated by

Fig. 3. Chromatography of the low-molecular-weight proteinase inhibitor from rat intestinal muscle on Sephadex G-25. The material concentrated by ultrafiltration over a UM2 membrane was applied to a column (2 cm $\times$ 60 cm) of Sephadex G-25, equilibrated in 0.02 M-sodium phosphate buffer, pH 7.5. Fractions (5 ml) were collected and assayed for A_{280} (○) and for inhibitory activity (●) by using the malate dehydrogenase assay.

Table 2. *Amino acid composition of the low-molecular-weight inhibitor*

Samples were hydrolysed for 24, 48, 72 or 96 h *in vacuo* in 6 M-HCl at 105°C. The number of residues of each amino acid was calculated on the basis of 10 residues of glutamic acid/molecule.

Amino acid	Amino acid composition (mol of residue/mol) after hydrolysis for				Best integer
	24 h	48 h	72 h	96 h	
Lys	8.44	8.59	8.62	8.21	9
His	2.00	2.00	2.00	2.02	2
Arg	5.01	5.01	5.32	4.64	5
Asp	8.37	9.10	8.83	8.20	9
Thr	4.73	4.34	4.29	4.25	5
Ser	5.73	5.58	4.79	4.51	6
Glu	10.00	10.00	10.00	10.00	10
Pro	6.97	6.90	6.76	6.36	7
Gly	7.59	7.90	7.68	7.98	8
Ala	6.83	7.11	6.70	6.94	7
Val	5.16	5.61	5.68	6.02	6
Met	0.94	0.86	0.52	1.32	1
Ile	3.23	3.83	4.13	3.88	4
Leu	4.87	5.18	5.35	5.22	5
Tyr	1.15	1.19	1.07	1.14	1
Phe	1.53	1.66	1.61	1.18	2
Cys		Not determined			
Trp		Not determined			
Total number of residues					87

the bar and the purified material was concentrated finally by ultrafiltration using a UM2 membrane.

A summary of the purification is given in Table 1, where the values for the purification and recovery of the inhibitor are calculated from the total activity at the start of the procedure and the contribution of the low-molecular-weight material to this activity. The latter value was calculated by assuming that the proportion of inhibitory activity that passed through the PM10 membrane [$4900/(3500 + 4900) = 58\%$] represents the fraction of the total activity in the homogenate that was due to the low-molecular-weight inhibitor. In addition, the value for the final protein concentration as determined by the Lowry *et al.* (1951) method (with bovine serum albumin as the reference protein) was 1.5 times that of the true protein concentration as determined by amino acid analysis (Table 2). Thus the final specific activity of the purified inhibitor was 325 units/mg, which represents a purification of approx. 60-fold.

A sample (containing 50 µg) of the concentrated inhibitor obtained after chromatography on Sephadex G-25 was subjected to sodium dodecyl sulphate/urea/polyacrylamide-gel electrophoresis. The inhibitor migrated as a single band (Fig. 4), with a mobility of 0.33 compared with that of Bromophenol Blue. A molecular weight of 7400 was calculated for the inhibitor by comparison with the mobilities of proteins of known molecular weight from a linear calibration graph.

Fig. 4. *Sodium dodecyl sulphate/urea/polyacrylamide-gel electrophoresis of the low-molecular-weight rat inhibitor*

Fig. 5. Effect of substrate (malate dehydrogenase) concentration on the initial rates of inactivation by the intestinal-muscle proteinase and by trypsin

Malate dehydrogenase was incubated at various concentrations at 30°C in 0.02 M-sodium phosphate buffer, pH 7.5, with the intestinal-muscle proteinase (●) and with trypsin (○). The unit of v is nmol of substrate protein inactivated by the proteinase in unit time.

The amino acid composition of the inhibitor is given in Table 2. The molecular weight calculated from this composition was 8900, in reasonable agreement, for such a small protein, with that determined by gel electrophoresis.

Comparison of the activities of the intestinal-muscle proteinase and trypsin towards a native protein substrate and a peptide substrate

Accurate determination of kinetic parameters with a protein such as malate dehydrogenase as the substrate is difficult, since the number of peptide bonds split in the inactivation step is not known. The values obtained (Fig. 5) for inactivation of malate dehydrogenase by the two proteinases show that K_m was 32 μM for the muscle proteinase and 96 μM for trypsin, and k_{cat} ($V_{\text{max}}/[\text{proteinase}]$) was 8.4×10^4 and $1.5 \times 10^3 \text{ min}^{-1}$ respectively. Thus k_{cat}/K_m for the muscle proteinase is 2625, approx. 175 times the ratio (15) for trypsin. At fixed concentrations of proteinase and substrate, then, malate dehydrogenase will be inactivated 175 times faster by the muscle proteinase than by trypsin, as observed previously (Beynon & Kay, 1978).

The peptide tosyl-Gly-Pro-Arg *p*-nitroanilide would appear to have greater potential as a convenient substrate for investigation of the mode of action of inhibitors. The initial rates of hydrolysis at various concentrations of this peptide by the intestinal muscle proteinase and by trypsin were measured (Fig. 6).

K_m values of 6.6 and 2.1 μM were obtained for the two proteinases respectively, and the respective k_{cat} values were 2.7×10^3 and $0.54 \times 10^3 \text{ min}^{-1}$. k_{cat}/K_m is therefore 4.1×10^2 for the muscle proteinase and 2.6×10^2 for trypsin, so that the two enzymes display similar activities towards this peptide substrate.

Fig. 6. Effect of substrate (tosyl-Gly-Pro-Arg *p*-nitroanilide) concentration on the initial rates of hydrolysis by the intestinal-muscle proteinase and by trypsin: inhibition by leupeptin

Tosyl-Gly-Pro-Arg *p*-nitroanilide was incubated at various concentrations at 30°C in 0.02 M-sodium phosphate buffer, pH 7.5, with the intestinal-muscle proteinase (●) and with trypsin (○). Inhibition of the muscle proteinase by leupeptin was examined by inclusion of this compound at concentrations of 1.3 (□), 1.9 (■) and 3.2 μM (▲). The unit of v is μmol of substrate peptide hydrolysed by the proteinase in unit time.

Fig. 7. Effects of leupeptin (●) and the low-molecular-weight muscle inhibitor (○) on the apparent value of K_m (K_m^{app}) for the hydrolysis of tosyl-Gly-Pro-Arg *p*-nitroanilide by the intestinal-muscle proteinase

Effect of a known proteinase inhibitor, leupeptin

Leupeptin is an *N*-acylated tripeptide that inhibits trypsin-like serine proteinases and thiol proteinases (Aoyagi & Umezawa, 1975; Kirschke *et al.*, 1977). It inhibits the amidase activity of trypsin (as measured towards benzoylarginine *p*-nitroanilide) non-competitively: $K_i \approx 0.4 \mu\text{M}$ (Aoyagi & Umezawa, 1975). The initial rates of hydrolysis of the peptide tosyl-Gly-Pro-Arg *p*-nitroanilide by the intestinal-muscle proteinase were measured at various concentrations of the substrate in the absence and presence of several concentrations of leupeptin (Fig. 6). It appears that leupeptin is a competitive inhibitor of the hydrolysis of this amide substrate by the trypsin-like muscle proteinase. The values of K_m^{app} obtained were plotted against inhibitor concentration (Fig. 7), and K_i was found to be 0.3 μM .

Kinetics of the inhibition of the muscle proteinase by the purified low-molecular-weight rat protein

Since this protein proved to have a K_i value similar in magnitude to that of K_m for tosyl-Gly-Pro-Arg *p*-nitroanilide and only limited amounts of material were available, it was necessary to derive kinetic parameters from progress-curve analysis (see the Materials and Methods section). Progress curves for the hydrolysis of the peptide substrate by the muscle proteinase were obtained at several concentrations of the low-molecular-weight rat inhibitor. The results (not shown) were expressed as plots of $t/\ln(S_0/S)$ versus $(S_0 - S)/\ln(S_0/S)$; this resembles a plot of S_0/v against S_0 derived for the steady-state case. A series of lines was obtained parallel to that for the substrate alone, so that the low-molecular-weight protein would appear to be a competitive inhibitor. The values of $K_m^{app.}$ obtained were plotted against inhibitor concentration (Fig. 7) and K_i was determined to be $5 \mu\text{M}$.

From this series of experiments, K_m for tosyl-Gly-Pro-Arg *p*-nitroanilide was found to be $6 \mu\text{M}$, and $k_{cat.}$ was $2.5 \times 10^3 \text{ min}^{-1}$, in close agreement with the values derived previously from the steady-state plot (Fig. 6). Such agreement also indicates that product inhibition was insignificant in the analysis of the progress curves, which were always followed to completion. An approximate value for K_i for inhibition of the intestinal-muscle-proteinase-catalysed inactivation of malate dehydrogenase by the low-molecular-weight protein was calculated by substituting the values obtained from Fig. 5 into the equation

$$v = \frac{V_{max.} [S]}{[S] + K_m \left(1 + \frac{[I]}{K_i}\right)}$$

Inclusion of the purified inhibitor ($20 \mu\text{M}$) resulted in a decrease in the initial rate of inactivation of malate dehydrogenase from 96 to 32 nmol/min. Substitution in the above equation produced a K_i value of $4 \mu\text{M}$, which is in reasonable agreement with the more accurate value derived from the progress-curve analysis. This K_i value is higher than that of leupep-

tin and several orders of magnitude higher than those normally associated with extracellular protein proteinase inhibitors. Consequently, several other low-molecular-weight proteins that have definite functions other than as proteinase inhibitors were tested for their ability to inhibit the muscle proteinase.

Effects of low-molecular-weight proteins on the hydrolysis of tosyl-Gly-Pro-Arg p-nitroanilide by the muscle proteinase

Progress curves were obtained for the hydrolysis of tosyl-Gly-Pro-Arg *p*-nitroanilide in the presence of each protein at the indicated concentrations (Table 3). Values for K_m and $V_{max.}$ were then obtained by progress-curve analysis as described above.

Gastrin at concentrations up to 2 mM had no effect (Table 3). The influence of glucagon was unusual, causing an increase in both $K_m^{app.}$ and $V_{max.}^{app.}$ in a manner reminiscent of the hyperbolic inhibition discussed by Cornish-Bowden (1976). Insulin B-chain acted as a simple competitive inhibitor. In an attempt to compare directly the inhibitory efficiencies of these proteins, the degree of inhibition per μmol of protein is given in Table 3. On this basis, it appears that the rat muscle protein is about 50–100 times more efficient as an inhibitor of the muscle proteinase than the other non-specific proteins chosen at random. The inhibition produced by the purified muscle protein seems to be the result of an inherent inhibitory activity rather than the non-specific action of a low-molecular-weight protein component of the cell acting as an alternative substrate for the proteinase. There was no indication of the observed degree of inhibition decreasing during incubation of the low-molecular-weight inhibitor with the proteinase.

Effect of the low-molecular-weight inhibitor on other purified proteinases

The ability of the muscle inhibitor to interact with other proteinases was measured by using azocasein as a general proteinase substrate (Table 4). Only the

Table 3. *Effect of various proteins on the hydrolysis of tosyl-Gly-Pro-Arg p-nitroanilide by the intestinal-muscle proteinase*

Progress curves were observed at 405 nm for the hydrolysis of tosyl-Gly-Pro-Arg *p*-nitroanilide at 30°C in 0.02 M-sodium phosphate buffer, pH 7.5, by the intestinal-muscle proteinase in the presence of each protein at the indicated concentration.

Protein added	$K_m^{app.}$	$V_{max.}^{app.}$	Inhibitory equivalents (units/ μmol of protein)
None	6.6	9.8	—
Gastrin (2 mM)	6.6	9.8	0
Glucagon (0.1 mM)	151	21	1.3
Glucagon (0.5 mM)	79	16	1.4
Insulin B-chain (20 mM)	74	9.4	2.0
Rat low-molecular-weight inhibitor (20 μM)	29	9.8	85

Table 4. *Effect of the low-molecular-weight protein from rat intestinal muscle on the hydrolysis of azocasein by various proteolytic enzymes*

Each proteinase (0.25 μ g in 0.1 ml of 0.1 M-sodium phosphate buffer, pH 7.5) was incubated with 1.0 ml of azocasein (11 mg/ml of buffer) at 30°C in the absence or presence of the low-molecular-weight inhibitor (70 units). Samples were removed into 4% (w/v) trichloroacetic acid for deproteinization at various times and the initial rates of hydrolysis were calculated from the A_{340} values measured for the resultant supernatants.

Proteinase	$10^3 \times \text{Initial rate}$ ($\Delta A_{340}/\text{min}$)	
	–Inhibitor	+Inhibitor
Intestinal-muscle proteinase	1.82	1.42
Trypsin	1.41	1.41
Chymotrypsin	1.49	1.49
Elastase	0.41	0.41
Subtilisin	1.40	1.41
Papain*	1.01	1.01
<i>Armillaria mellea</i> proteinase	1.50	1.52

* The digestions involving papain were carried out in the presence of 5 mM-cysteine and 2 mM-EDTA.

Table 5. *Interaction of the low-molecular-weight inhibitor with immobilized proteolytic enzymes*

The inhibitor (90 units) was incubated in 0.1 ml of 0.02 M-sodium phosphate buffer, pH 7.5, for 15 min at 30°C with 250 μ g of each of the proteinases insolubilized on agarose. After centrifugation for 5 min at 800 g, the inhibitory activity in the supernatants was measured against the intestinal-muscle proteinase.

Gel added	Inhibitory activity recovered in supernatant (% of initial activity)
Agarose–trypsin	97
Agarose–chymotrypsin	102
Agarose–Pronase	98
Agarose	100

intestinal-muscle proteinase appeared to be inhibited. However, azocasein is used at a concentration of 10 mg/ml in this assay, so that if the inhibitor had only a very weak affinity for the other proteinases, any effect would have been overwhelmed by the excess of substrate.

Consequently, the inhibitor was incubated in the absence of substrate with several proteolytic enzymes immobilized through attachment to agarose (each insolubilized proteinase had been assayed against azocasein immediately beforehand, and had the activity specified by the manufacturer). After preincubation at a molar ratio of active proteinase:inhibitor of approx. 2:1 in each case, the mixtures were centrifuged (5000 g for 10 min) to remove

any insoluble-enzyme–inhibitor complexes that had formed. The residual inhibitory activity remaining in each supernatant was then measured towards the intestinal-muscle proteinase by using the malate dehydrogenase assay system. Table 5 shows that the low-molecular-weight inhibitor did not bind significantly to any of the immobilized enzymes tested.

Discussion

The presence of (at least) two proteinase-inhibitory components in the supernatant obtained from rat muscle is indicated by several lines of evidence: (1) approx. 50% of the inhibitory activity is lost on dialysis in conventional tubing; (2) approx. 50% passes through a PM10 ultrafiltration membrane; and (3) the activities can be resolved on Sephadex G-75 even in the presence of added proteinase inhibitors. Kinetic analysis of the type of inhibition observed with the material resolved into peaks A and B on Sephadex G-75 (Fig. 2) indicated that, although the peak-A material inhibited non-competitively, peak B contained a competitive inhibitor (Carney, 1979). Thus it appears that the low-molecular-weight component (peak B) resolved by chromatography on Sephadex G-75 was the same as the protein isolated by the slight modification of this procedure that was adopted as the routine method for purification to homogeneity.

Contamination of the preparations of the low-molecular-weight protein by inhibitors derived from blood plasma was excluded firstly by removal of blood from the tissue by prior perfusion with iso-osmotic saline and secondly by incubation of the tissue extracts at pH 3 for 1 h at 37°C. The major proteinase inhibitors of blood, α_1 -proteinase inhibitor and α_2 -macroglobulin, are rapidly inactivated at pH 3 (Küppers, 1974; Kress & Laskowski, 1974; Laurell & Jepson, 1975). Failure to observe such precautions has often led, in the past, to misassignment of the true source of proteinases and inhibitors, e.g. the elastase and cathepsin G of spleen are really derived from the neutrophil leucocytes in spleen (Starkey, 1977), and the various tissue proteinases described by Katunuma (1975) in reality have now been shown to be derived from mast cells (Woodbury *et al.*, 1978a,b).

It seems unlikely that the low-molecular-weight inhibitor described here was derived from the pancreas, since (1) the pancreas was easily and completely dissected away, (2) the initial 15 cm of the small intestine, with which the pancreas is associated, was discarded, (3) the K_i value found for the rat protein was considerably different from the K_i values reported for pancreatic inhibitors, and (4) the amino acid composition was totally different from those of, for example, the bovine pancreatic basic (Kunitz) and secretory (Kazal type) trypsin inhibi-

tors (Kassell & Laskowski, 1964; Greene *et al.*, 1966).

Both of these inhibitors of extracellular proteinases are also low-molecular-weight proteins, but they inhibit trypsin with $K_i \approx 10^{-11} \text{ M}$ (Putter, 1967). Similarly, other extracellular inhibitors, e.g. soya-bean and lima-bean trypsin inhibitors, α_1 -proteinase inhibitor, ovomucoid etc., also interact with proteinases, essentially irreversibly (Kassell & Williams, 1976). By contrast, the rat intracellular proteinase inhibitor has a K_i several orders of magnitude higher, in the 10^{-6} M range. This would appear to be logical, since in the extracellular situation an inhibitor has only a transient opportunity in dilute solution to combine to inactivate its target proteinase, so that reaction has to be rapid and essentially complete. On the other hand, it would appear unnecessary and wasteful in energetic terms to inactivate an intracellular proteinase irreversibly. It would seem much more useful to be able to modulate the activity reversibly so that the proteinase could be switched on and off in response to cellular metabolic demands.

The low-molecular-weight inhibitor was apparently homogeneous after 60-fold purification and so it appears to be a major protein component of the muscle tissue, contributing about 1% of the homogenate protein. Since 6000 inhibitory units were obtained from 12 g of tissue, an approximate tissue concentration of low-molecular-weight inhibitory activity of 500 units/g can be calculated. We had previously estimated that the proteinase concentration in muscle was approx. 250 units/g of tissue (Beynon & Kay, 1978). Thus a small excess of inhibitory activity over proteinase exists in the tissue, and relatively small changes in the amount of low-molecular-weight inhibitor could lead to dramatic changes in the expression of the proteinase activity, assuming that the two proteins have ready access to each other. Studies are required to extend our preliminary investigations (Beynon & Kay, 1977) into the balance between proteinase and inhibitor in the muscle tissue and alterations induced by nutritional and hormonal perturbations.

Modulation of the activity of this active proteinase in such a manner appears to provide a useful mechanism for the regulation of the net rate of degradation of intracellular proteins. The trypsin-like serine proteinase from muscle, like others in this category, was inhibited by leupeptin and rather more effectively than by the endogenous muscle inhibitor. However, leupeptin inhibited the muscle proteinase competitively, in contrast with its non-competitive effect on the amidase activity of trypsin (Aoyagi & Umezawa, 1975). Administration of leupeptin to perfused liver (Dunn & Aronson, 1977) and to various isolated cells such as hepatocytes (Bohley *et al.*, 1977; Hopgood *et al.*, 1977) and macrophages (Dean, 1979) results in inhibition of the lysosomal

route of intracellular protein degradation. It also appears, however, to inhibit the non-lysosomal degradation pathway (Seglen *et al.*, 1979). Indirectly, this lends substantiation to the involvement of a trypsin-like serine proteinase (such as the muscle proteinase) in intracellular protein degradation.

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