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The Inactivation of Native Enzymes by a Neutral Proteinase from Rat Intestinal Muscle

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1. The solubilization and partial purification of a proteinase from the intestinal smooth muscle of rats fed on protein-free diets are described. 2. It has a mol.wt. of about 33000 and it is stable over a narrow pH range. 3. From its susceptibility to known modifiers of proteolytic enzymes, it appears to be a serine proteinase of a trypsin-like nature. Active-site titration with soya-bean trypsin inhibitor shows that the concentration of proteinase was about 3μg/g wet wt. of intestinal smooth muscle. However, the muscle proteinase demonstrates a marked ability for inactivating enzymes in their native conformation at neutral pH. It is about 100 times more efficient than pancreatic trypsin when the inactivating activities are compared on an approximately equimolar basis. 4. Inactivation of the substrate enzymes is accompanied by limited proteolysis, as demonstrated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. 5. An endogenous inhibitor was separated from the proteinase by fractionation with $(NH_4)_2SO_4$. 6. Contamination of the muscle tissue by lumen, mucosal or blood proteinases and inhibitors is shown to be unlikely. 7. A role for the neutral trypsin-like proteinase in initiating the degradation of intracellular enzymes 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 involves proteolysis at neutral pH and at a location distinct from the lysosomal matrix (Katunuma *et al.*, 1972). This could inactivate intracellular enzymes at different rates and might also 'prime' or initiate the subsequent non-specific digestion of the resultant products into amino acids, presumably in the lysosome (the organelle that is undoubtedly responsible for, at least, the terminal stages of protein breakdown). It was postulated that there might be group-specific proteinases, each responsible for initiating the catabolism of a susceptible form (e.g. the apoenzyme) of a certain type of enzyme. Several proteinases, supposedly active only towards pyridoxal phosphate-dependent enzymes, have been purified from a variety of rat tissues (Katunuma *et al.*, 1975). However, it is not obvious why the action of this type of group-specific proteinase should be limited to one class of substrate enzymes.

The present paper examines the susceptibility of several native enzymes to an inactivating activity in rat intestinal smooth muscle, a tissue that can be conveniently depleted of its blood content by perfusion so as to minimize the possibility of contamination from blood proteinases or inhibitors. Partial purification reveals that a trypsin-like serine proteinase is responsible for the inactivating activity. The problem of restricted specificity is examined by comparing the capacity of the muscle enzyme for inactivating native enzymes with that of pancreatic trypsin, an extracellular proteinase that preferentially attacks denatured polypeptide substrates.

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Materials and Methods

Di-isopropyl phosphorofluoridate was obtained from Calbiochem, Bishop's Stortford, Herts., U.K. Mitochondrial pig heart malate dehydrogenase [10mg/ml of solution in 50% (v/v) glycerol] and soya-bean trypsin inhibitor covalently coupled to agarose were gifts from Miles Research Laboratories, Stoke Poges, Slough, Bucks., U.K. Proflavin and pepstatin were gifts from Dr. R. McRorie, Department of Biochemistry, University of Georgia, U.S.A., and Professor H. Umezawa, Institute of Microbial Chemistry, Tokyo, Japan, respectively. Bovine liver glutamate dehydrogenase, pig heart isocitrate dehydrogenase, jack-bean urease and soya-bean trypsin inhibitor were from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. Rabbit muscle lactate dehydrogenase and yeast glucose 6-phosphate dehydrogenase were from Boehringer, Lewes, Sussex, U.K. Phosphorylase *b* was a gift from Dr. Philip Cohen. α-Trypsin was purified by the method of Schroeder & Shaw (1968). All other chemicals were

obtained from Sigma. Buffers were made from sodium phosphate. Soya-bean inhibitor concentrations were determined from $A^{1\%}_{280} = 9.1$ (Kay & Kassell, 1971).

Experimental animals

Male Wistar rats (150–250g) were maintained on the normal laboratory diet. They were then fed on a protein-free diet (obtained from Micro Bio Laboratories, Cambridge, U.K.) for 3 weeks. Food was removed from the animals 24h before they were killed by cervical dislocation. In some cases the animals were anaesthetized with ether before perfusion of the vascular bed of the gut with 0.9% NaCl.

Malate dehydrogenase assay

Malate dehydrogenase (L-malate–NAD^+ oxidoreductase, EC 1.1.1.37) was assayed by following the decrease in A_{340} in a Cecil Instruments CE 272 linear-readout spectrophotometer (thermostatically controlled) coupled to a Bryans 27000 chart recorder. The assay was performed at 25°C in 0.1M-sodium phosphate buffer, pH7.5, in the presence of 0.125mM-NADH and 0.3mM-oxaloacetate.

Assays for proteolytic activity

Activity was measured towards two types of substrate.

(*a*) *Assay depending on the inactivation of native enzymes, e.g. malate dehydrogenase.* Malate dehydrogenase (0.05mg) was incubated at 30°C with a suitable amount of muscle extract in a final volume of 0.25ml of 0.02M-sodium phosphate buffer, pH7.5. At suitable time intervals, samples were removed, diluted into cold 0.1M-sodium phosphate buffer, and assayed for residual malate dehydrogenase activity. One unit of activity is defined as that amount of enzyme causing an initial loss of malate dehydrogenase activity of 1%/min. The assay was initiated by addition of the dehydrogenase.

(*b*) *Assay with azocasein.* The digestion of azocasein was monitored by the release of dye-containing peptides soluble in 4% (w/v) trichloroacetic acid. Azocasein (11mg) was incubated with samples of the intestinal-muscle preparation from the various stages of purification (0.1ml) in a final volume of 1.1ml of 0.1M-sodium phosphate buffer, pH7.5. At suitable time intervals (usually 0.5, 1.0 and 1.5h) samples (0.25ml) were removed and added to 1ml of 4% trichloroacetic acid. After centrifugation at 800*g* for 5min, the A_{340} of each supernatant was determined. A linear rate of increase of A_{340} was observed provided that the final value did not exceed 0.3. The rate of increase of A_{340} was taken as a measure of proteolysis, 1 unit being defined as a rate of increase in A_{340} of 0.001/min.

A similar method was used for observing proteolysis of azoalbumin.

Preparation of the inactivating enzyme

Contamination of the muscle tissue by pancreatic proteinases which could have been present in the lumen of the small intestine was prevented by a rigorous washing procedure. Intestines were individually washed through the lumen with 9×25ml of 0.154M-KCl. No activity towards tosylarginine methyl ester, benzoyltyrosine ethyl ester (Hummel, 1959) or azocasein (see the above section) could be detected in the washings after the sixth wash. The limit of detection of pancreatic proteinases in all three of these assays is as little as 50ng/sample taken. The intestine was slit longitudinally, and the mucosal layer was removed by scraping the internal surface with a glass slide. Individual intestines were treated in this fashion and the muscle tissue thus obtained from four or five rats (about 2–5g per animal) was pooled and homogenized in 3vol. (w/v) of 0.02M-sodium phosphate buffer, pH7.5, with a Polytron PT2 homogenizer at half speed for 30s. This muscle homogenate contained less than 2% of the total intestinal maltase activity and less than 7% of the alkaline phosphatase activity that was present in whole, washed intestine (both of these enzymes are found in high concentrations in the mucosal tissue).

The homogenate was centrifuged at 100000*g* for 20min, to give a clear supernatant (S1) and a pellet that contained the proteolytic activity. The activity could be solubilized by incubation at 4°C with 0.02M- or 0.5M-phosphate buffer, pH7.5, or 0.5% (w/v) Triton X-100 for 20h. Centrifugation of the extracted material at 100000*g* for 1h gave a soluble form of the activity (S2), which was further purified by fractionation between 1.2M- and 2.55M-$(NH_4)_2SO_4$. This fraction was redissolved, dialysed against 0.02M-sodium phosphate buffer, pH7.5, and used in all of the studies reported in the present paper. It is referred to as the crude intestinal-muscle proteinase preparation.

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was carried out as described by Weber & Osborn (1969).

Studies on the nature of the inactivating enzyme

For determination of the molecular weight of the inactivating enzyme, a sample of the $(NH_4)_2SO_4$ extract was chromatographed on a column of Sephadex G-75 (1.5cm×20cm) equilibrated in 0.1M-sodium phosphate buffer, pH7.5. Glutamate

dehydrogenase (mol.wt. 330000), pepsinogen (mol.wt. 40000), soya-bean trypsin inhibitor (mol.wt. 22000), cytochrome *c* (mol.wt. 11700) and $K_2Cr_2O_7$ were used as molecular-weight markers to produce a linear calibration graph.

Inhibitors and activators were added in 0.02M-sodium phosphate buffer, pH7.5, to the intestinal-muscle preparation as purified to the $(NH_4)_2SO_4$-fractionation stage. After preincubation for 30min at 30°C, the residual inactivating activity towards malate dehydrogenase as substrate was measured, except with di-isopropyl phosphorofluoridate and Tos-Lys-CH_2Cl (7-amino-1-chloro-3-L-tosylamidoheptan-2-one). For these modifiers, it was possible to monitor the time-dependence of the loss of inactivating activity by removing samples at various time points and assaying the residual activity towards malate dehydrogenase or azocasein. With *p*-hydroxymercuribenzoate, the presence of the reagent in the digestion would itself have modified the malate dehydrogenase activity. Therefore, after preincubation with this modifier, the reaction mixture was dialysed against a 2000-volume excess of 0.02M-sodium phosphate buffer, pH7.5, before assay for residual inactivating activity. The activity remaining after these treatments was compared with a sample of the preparation treated in an identical fashion except for the omission of the inhibitor.

Assay of native enzymes

For the determination of the proteolytic activity towards various native enzymes, the enzymes were assayed under conditions where there was a linear relationship between velocity and concentration of the enzyme. The inactivation of these native enzymes by suitable amounts of the intestinal-muscle preparation or by α-trypsin was monitored under identical conditions (0.2mg of enzyme/ml at 30°C; 0.02M-sodium phosphate buffer, pH7.5) except for (*a*) glutamate dehydrogenase and (*b*) phosphorylase *b*: these were inactivated in 0.1M-sodium phosphate and 0.02M-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffers, both pH7.5, respectively because of instability at low ionic strength for the former enzyme and the necessity to assay phosphorylase by the release of phosphate from glucose 1-phosphate in the latter case.

Results

Purification of the inactivating enzyme

The possibility of contamination of the intestinal-muscle homogenates by luminal or mucosal activities was excluded on the basis of marker-enzyme measurements (see the Materials and Methods section). The initial steps of a representative purification of the inactivating activity are summarized in Table 1. Protein values are not included for the homogenate nor for resuspended pellet fractions, since it was difficult to obtain accurate measurements owing to problems of pipetting and light scattering, particularly for the insoluble fraction. It is likely that this fraction contained the contractile proteins from the muscle tissue, since the homogenization was carried out at low ionic strength.

The solubilized form of the activity was fractionated with $(NH_4)_2SO_4$. At concentrations lower than 1.2M, little of the activity was precipitated, whereas at concentrations up to 2.8M, marked increases in the total activity recovered were observed (Fig. 1). Consequently for routine preparations an initial fractionation at 1.2M-$(NH_4)_2SO_4$ was carried out and the unwanted protein was removed by centrifugation at 100000*g* for 20min. The supernatant was adjusted to 2.55M-$(NH_4)_2SO_4$ to recover the inactivating activity.

The rate of inactivation of malate dehydrogenase by the muscle preparation, as obtained after the $(NH_4)_2SO_4$ stage, showed a linear dependence on the amount of extract used over the range tested (0–10 units of activity contained in 0–85μg of protein in this particular preparation).

Nature of the inactivating enzyme

Characterization studies were performed on the inactivating activity as purified after the $(NH_4)_2SO_4$ stage. Chromatography of this material on a calibrated column of Sephadex G-75 gave a single peak of activity, which was eluted with a mol.wt. of 33000. The recovery of inactivating activity was 85%.

Several known modifiers of proteolytic enzymes were tested for their ability to affect the activity in the intestinal-muscle preparation (Table 2). Complete inhibition was obtained with di-isopropyl phosphoro-

Table 1. *Partial purification of the intestinal-muscle inactivating enzyme*

Intestinal muscle (16g from five rats) was homogenized in 50ml of 0.02M-sodium phosphate buffer, pH7.5. S1 is the high-speed supernatant. Overnight extraction of the pellet with 0.02M-phosphate buffer, pH7.5, followed by centrifugation gives a second supernatant (S2). One unit of proteolytic activity causes an initial loss of malate dehydrogenase activity of 1%/min.

Fraction	Protein (mg)	Activity (units)	Recovery (%)
Homogenate	—	1040	100
S1	480	0	0
Resuspended pellet	—	1140	109
S2	108	1500	144
$(NH_4)_2SO_4$ fraction (1.2–2.55M)	30	3500	336

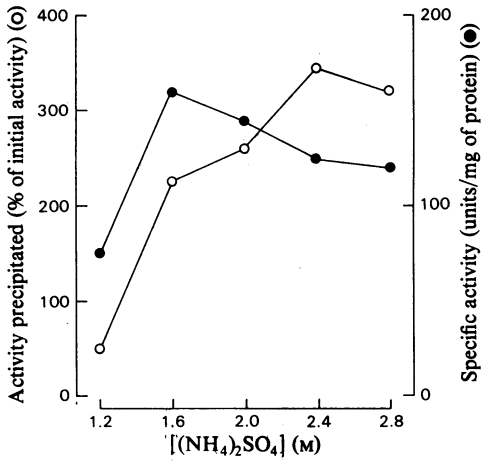

Fig. 1. *Recovery of inactivating activity on $(NH_4)_2SO_4$ fractionation*

Solid $(NH_4)_2SO_4$ was added to equal amounts of fraction S2 to give the indicated concentration. The precipitate was obtained by centrifugation at 100000*g* for 20min, and after dialysis of the redissolved pellet against 0.02M-phosphate buffer, pH7.5, proteolytic activity towards malate dehydrogenase, together with protein concentration, was determined. Total activity (○) is expressed as a percentage of the original value for the unfractionated material.

Table 2. *Effect of modifiers on the inactivating activity of the muscle preparation*

Samples of the intestinal-muscle preparation (10 units) were preincubated with the indicated concentrations of modifiers for 30min at 30°C before determination of the residual inactivating activity towards malate dehydrogenase. Results are expressed as a percentage of the activity obtained in the absence of modifiers. Abbreviation: Tos-Phe-CH_2Cl, 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one.

Modifier	Concn.	Activity (%)
None	—	100
Soya-bean trypsin inhibitor (mol.wt. 22000)	5μM	0
Lima-bean trypsin inhibitor (mol.wt. 9000)	10μM	0
Whole-rat serum	4% (v/v)	0
Di-isopropyl phosphorofluoridate	150μM	0
Tos-Lys-CH_2Cl	37μM	0
Benzamidine	1.0mM	12
Proflavin	0.5mM	22
α-*N*-Benzoylarginine	1.0mM	54
Tos-Phe-CH_2Cl	0.1mM	100
D-Tryptophan *O*-methyl ester	1.0mM	100
Pepstatin	0.4mM	100
EDTA	10.0mM	100
$CaCl_2$	1.0mM	100
2-Mercaptoethanol	15.0mM	100
p-Hydroxymercuribenzoate	0.1mM	100

fluoridate and soya-bean and lima-bean inhibitors, suggesting that the inactivating activity was due to a serine proteinase. The inhibition by Tos-Lys-CH_2Cl and benzamidine further indicates that this proteinase is trypsin-like in nature. Time courses were determined for the abolition of the activity by di-isopropyl phosphorofluoridate and Tos-Lys-CH_2Cl (Fig. 2) by removing samples from the incubation mixtures at various times and measuring the residual activity towards malate dehydrogenase. None of the ligands tested had any direct effect on the activity of malate dehydrogenase.

An attempt was made to determine the concentration of the proteinase [as obtained after the $(NH_4)_2SO_4$ stage] by titration with the potent inhibitor of trypsin-like proteinases, soya-bean inhibitor (Fig. 3). The activity towards malate dehydrogenase was lost in parallel with the azocasein-hydrolysing activity of the preparation, suggesting that the activity towards the two substrates was derived from the same enzyme. By extrapolation, complete inhibition of the inactivating and digesting activities was attained with 3.33pmol of soya-bean trypsin inhibitor. Assuming a 1:1 stoicheiometry of binding of inhibitor to proteinase (see the Discussion section), this gives a concentration of proteinase of 3.33pmol/3μl of extract. Taking a mol.wt. of approx. 33000 for the inactivating enzyme, then the concentration of proteinase was approx. 37μg/ml. The protein concentration of this particular $(NH_4)_2SO_4$ extract was 4.5mg/ml, so that the amount

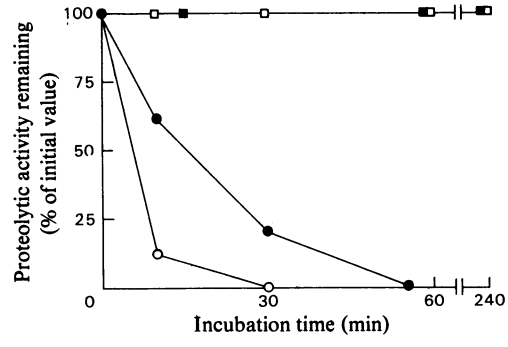

Fig. 2. *Effect of various inhibitors on the inactivating activity*

Samples (containing 70 units of activity) of the intestinal-muscle preparation were incubated at 30°C in 0.14ml of 0.02M-sodium phosphate buffer, pH7.5, with 50μM-di-isopropyl phosphorofluoridate (●), 100μM-Tos-Phe-CH_2Cl (■) or 37μM-Tos-Lys-CH_2Cl (○). At various times, samples (20μl) were removed, added to 0.23ml of 0.02M-phosphate buffer, pH7.5, and the inactivating activity towards malate dehydrogenase was measured. Control samples (□) showed no loss in activity.

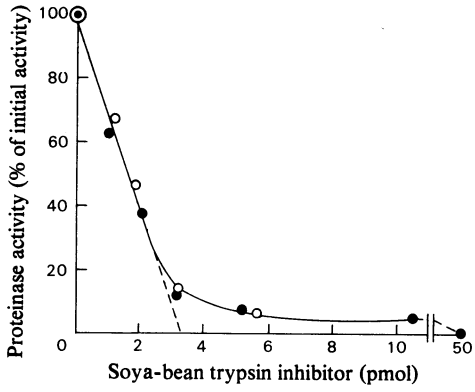

Fig. 3. *Titration of the activity of the intestinal-muscle proteinase with soya-bean trypsin inhibitor*

Samples (3 μl, containing 10 units of activity towards malate dehydrogenase) of the muscle proteinase preparation were preincubated at 30°C in 0.25 ml of 0.02 M-sodium phosphate buffer, pH 7.5, with various amounts of soya-bean trypsin inhibitor (5 μg/ml) before determination of the residual inactivating activity towards malate dehydrogenase (●). This was repeated (with 30 units of activity), except that the residual activity was measured towards azocasein (○).

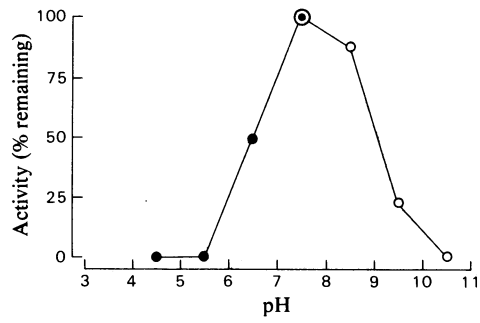

Fig. 4. *Effect of pH on the stability of the proteinase*

Samples of the muscle proteinase preparation (10 units) were preincubated at 30°C for 60 min in 0.1 ml of 0.01 M-sodium succinate (●) or 0.01 M-sodium glycinate (○) buffers at the indicated pH values, before addition of 0.15 ml of 0.1 M-sodium phosphate buffer to return the pH to 7.5. The residual activities towards malate dehydrogenase were measured and values are expressed as a percentage of those of control samples kept at pH 7.5 by dilution first into the phosphate buffer and then adding 0.1 ml of the succinate or glycine buffers.

of proteinase present was 8.2 μg/mg of protein. This value varied from one preparation to the next.

No attempt was made to determine the pH optimum of the inactivating activity, since malate dehydrogenase as the substrate is sensitive to pH alterations. However, the effect of pH on the stability of the proteinase was tested by preincubating samples of the $(NH_4)_2SO_4$ extract for 60 min at various pH values before re-adjusting the pH to 7.5 and measuring the inactivating activity remaining towards malate dehydrogenase (Fig. 4). The activity shows a narrow pH range within which it is stable, and the instability at lower pH values suggests a non-lysosomal origin, although the behaviour of the solubilized enzyme *in vitro* may not be reflected in the situation *in vivo*.

Specificity of the inactivating activity

The $(NH_4)_2SO_4$ extract of intestinal muscle contained only low amounts of proteinase in terms of protein. The level of activity isolated from intestines that had been perfused before homogenization of the muscle layer (3.4 μg of proteinase/g of muscle) was no different from that from unperfused tissue (3.2 μg/g of muscle). However, the activity of this small amount of proteinase towards malate dehydrogenase was very high. This prompted us to investigate the ability of the proteinase to inactivate other native enzymes and to digest general proteolytic substrates, by using the material as purified to the $(NH_4)_2SO_4$ stage rather than to attempt further to purify the rather small amounts of protein to homogeneity before carrying out these studies. An attempt was also made to assess the vulnerability of each enzyme to inactivation by including a parallel series with highly purified trypsin. An approximate concentration for the proteinase was calculated in the preceding section, and Table 3 shows that on an equimolar basis, the ability of the intestinal-muscle enzyme to digest general protein substrates, e.g. azocasein and azoalbumin, is very similar to that of trypsin. However, in all cases, when the ability of the intestinal-muscle proteinase for inactivating native enzymes is compared with that of trypsin, the intestinal proteinase is more active by one to two orders of magnitude. In each case, the inactivation could be prevented by means of a short preincubation with very small amounts of soya-bean or lima-bean trypsin inhibitors. Similarly it was always possible to arrest inactivation at any time by addition of one of these inhibitors to the digestion mixture.

The proteinase was totally inactive towards the trypsin substrates tosylarginine methyl ester and benzoylarginine ethyl ester, the chymotrypsin substrate *N*-acetyltyrosine ethyl ester and the elastase substrate *N*-t-butoxycarbonylalanine *p*-nitrophenyl ester.

The inactivation of the substrate enzymes was shown to be due to limited proteolytic cleavage by removing samples at various time points during the inactivation of malate dehydrogenase and glutamate dehydrogenase (Fig. 5) and analysing them by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

In both cases, the band corresponding to the original subunit diminished in staining intensity, accompanied by the appearance of only one digestion product of lower molecular weight.

Discussion

The release of an inactivating enzyme from intestinal smooth muscle into a soluble form was achieved by overnight extraction of the insoluble pellet fraction with solutions of low ionic strength. This suggests that the enzyme is probably not an intrinsic membrane protein, but either it may be

Table 3. *Inactivation of native enzymes by the intestinal-muscle proteinase and by trypsin*

Each enzyme (0.2mg/ml) was incubated at pH7.5, 30°C, with the proteinase preparation (0.15–1.5 μg of proteinase) or with α-trypsin (16–160 μg). Samples were removed at various times for determination of the residual activity of the substrate enzyme. Initial rates of inactivation were calculated from tangents to the initial slopes of these inactivation plots. Hydrolysis of azocasein and azoalbumin by both proteinases was measured by the increase in A_{340} resulting from solubilization of dye-containing peptides.

Substrate	Initial rate of inactivation (% of initial activity lost/min per pmol) by: Trypsin	Intestinal-muscle proteinase
Isocitrate dehydrogenase	0.055	4.80
Phosphorylase *b*	0.028	4.14
Malate dehydrogenase	0.008	2.40
Glucose 6-phosphate dehydrogenase	0.004	0.65
Glutamate dehydrogenase	0.004	0.12
Lactate dehydrogenase	0.0002	0.11
Urease	0	0
	Initial rate of hydrolysis (ΔA_{340}/min per nmol)	
	Trypsin	Intestinal-muscle proteinase
Azocasein	0.14	0.18
Azoalbumin	0.017	0.020

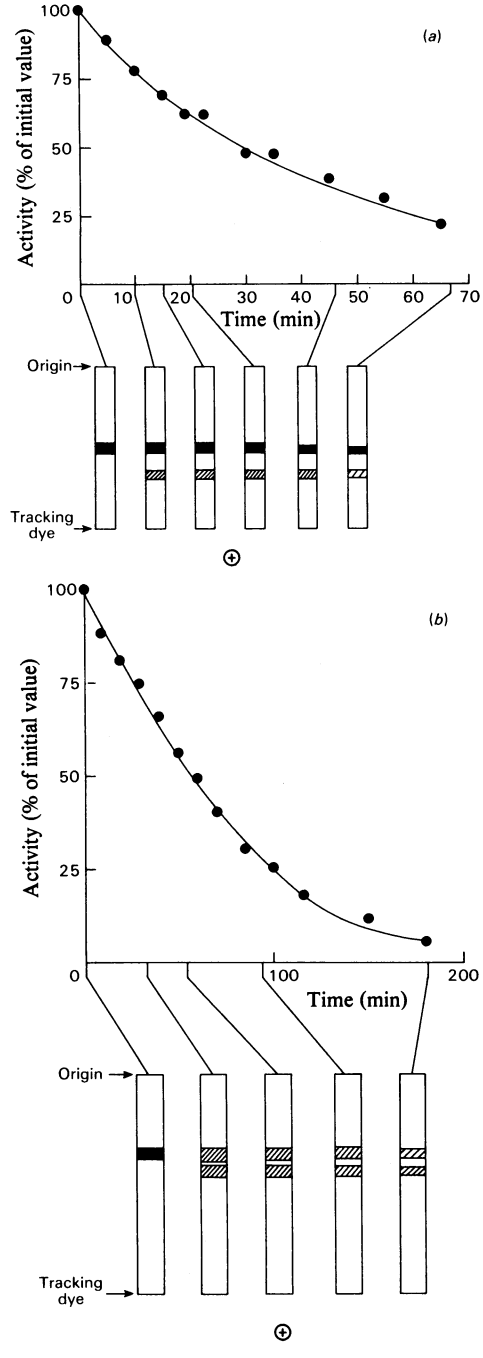

Fig. 5. *Time course and extent of digestion of malate dehydrogenase and glutamate dehydrogenase by the intestinal-muscle proteinase*

Malate dehydrogenase (*a*) and glutamate dehydrogenase (*b*), each at a concentration of 0.2mg/ml, were incubated with muscle proteinase in 0.02M- and 0.1M-sodium phosphate buffer, pH7.5, respectively at 30°C. The loss of the dehydrogenase activities was followed and samples (0.1ml) were removed at the indicated times and added to 0.1ml of insolubilized soya-bean trypsin inhibitor (sufficient to inactivate completely all of the proteolytic activity). After centrifugation, the supernatants were freeze-dried before analysis by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

loosely bound to the membrane or the soluble enzyme may be contained within membranes. It seems improbable that the activity is tightly associated with the contractile proteins of the muscle tissue, since it could also be solubilized by extraction of the resuspended pellet fraction with detergents. Further investigation is necessary to elucidate this and to establish the subcellular location of the activity, so that at present the inactivating activity can probably best be described as membrane-'limited'.

The solubilized form of the enzyme could be readily determined in a very sensitive assay by measuring its capacity for inactivating native enzymes. Malate dehydrogenase was used routinely for convenience and the initial rate of loss of its activity could be measured accurately by removing samples at appropriate time points. Similarly, with the other native enzymes tested, it was always possible to have actual measurements of the initial rate of loss of enzyme activity by appropriate sampling.

From the sodium dodecyl sulphate/polyacrylamide-gel patterns obtained on inactivation of the enzymes and from the studies with known modifiers of proteolytic enzymes, it was apparent that the inactivating activity was due to a serine proteinase of a trypsin-like nature in that it was inhibited by di-isopropyl phosphorofluoridate, proflavin, benzamidine, Tos-Lys-CH_2Cl, benzoylarginine and the bean trypsin inhibitors. No inhibition was observed by the chymotrypsin inhibitors Tos-Phe-CH_2Cl and D-tryptophan methyl ester, nor with modifiers of thiol, carboxy- or metallo-proteinases. This suggests that this proteinase is distinct from the other proteolytic enzymes that have been described in muscle tissue, such as the insulin-degrading proteinase (Duckworth *et al.*, 1974) and the pyridoxal phosphate-active group-specific proteinase purified from several different muscles (Katunuma *et al.*, 1975). The latter are chymotrypsin-like, with esterolytic activity towards acetyltyrosine ethyl ester. The myofibril-degrading proteinase from skeletal muscle (Dayton *et al.*, 1976) is activated by Ca^{2+}, but is not trypsin-like in nature. Intracellular proteinases from other tissues seem to be predominantly chymotrypsin-like, e.g. the histone-degrading proteinase from rat liver (Jusic *et al.*, 1976), the mast-cell proteinase (Kawiak *et al.*, 1971) and the neutral cathepsin G of leucocytes (Starkey, 1977). However, reports have appeared suggesting trypsin-like activities in rat uterus, another smooth muscle (Katz *et al.*, 1976; Woessner, 1977).

The inhibitors from soya bean and lima bean combine avidly with trypsin- or chymotrypsin-like enzymes to form 1:1 aggregates, and the soya-bean inhibitor, in particular, binds very tightly to trypsin (K_i approx. 0.1nM; Kassell & Williams, 1976). Consequently it seems reasonable to assume that it would bind equally avidly to the trypsin-like proteinase from intestinal muscle. By assuming a 1:1 stoicheiometry of binding between proteinase and inhibitor, a maximum concentration for the number of active sites was calculated. If more than 1 mol of inihibitor should bind per mol of proteinase, or if the effective concentration of the inhibitor should be decreased by non-specific binding to other proteins present in the intestinal-muscle proteinase preparation, then the active-site concentration for the proteinase would be appropriately decreased from this maximum value. The inactivating activity measured per mol of proteinase would then be correspondingly even higher. Thus allowing for errors in the calculation of the proteinase concentration, it is still apparent that on an approximate equimolar basis the proteinase has much the same ability as pancreatic trypsin for hydrolysing 'general' protein substrates, but it is much more efficient for inactivating native enzymes. Since all the proteolytic inactivations could be instantly and completely arrested at any stage by the addition of small amounts of soya-bean inhibitor, it seems unlikely that the $(NH_4)_2SO_4$ fraction contained more than one type of proteolytic activity.

Wright (1977) has inferred that trypsin and chymotrypsin may have, in addition to their traditional specificities for certain amino acids, a specificity that recognizes certain secondary conformations in proteins. Thus, if it is possible for neutral, intracellular proteinases to be involved in the initiation of catabolism of native enzymes, then it seems reasonable to suggest that they might exhibit a different conformational specificity from those shown by trypsin and chymotrypsin, in accordance with their different functions. In this respect, it seems justifiable to use native enzymes as proteolytic substrates for measuring intracellular proteinases, since low-molecular-weight and denatured polypeptide substrates are 'optimized' for use in general proteolysis, but, as observed in the present paper, may not always be particularly susceptible to a proteinase with some degree of conformation selectivity.

However, the concept of proteinases having 'group-specificity', such as that suggested for the apoenzyme forms of pyridoxal-dependent enzymes (Katunuma *et al.*, 1972), is by no means certain, since Table 3 shows that if a comparison is made of the susceptibility of several enzymes to proteolysis by the intestinal-muscle proteinase or by trypsin, then the order of vulnerability to both proteinases is exactly the same, although the muscle proteinase, in quantitative terms, is about two orders of magnitude more efficient in its proteolytic capacity. A similar order can be obtained with inactivation catalysed by chymotrypsin.

Thus, it would seem that it is the susceptibility of the enzyme that determines the ease of proteolysis,

and that this would be a much better criterion to use as a basis for 'specificity'.

The activity of the intestinal-muscle proteinase at physiological pH values and the marked ability to inactivate native enzymes suggests that such a proteinase, acting within the cell, could have profound consequences on the metabolic status of that cell. It might be expected that such an activity would have to be regulated, perhaps through the action of endogenous inhibitors. During fractionation with $(NH_4)_2SO_4$ (Fig. 1), considerable increases in total activity were obtained and the 2.55M-$(NH_4)_2SO_4$ supernatant has been found to contain an inhibitor of the proteolytic activity (R. J. Beynon, unpublished work). The nature of this inhibitor and other possible methods for regulating the activity of the proteinase, such as alteration in the vulnerability of the enzyme via ligand-induced conformational changes, require further investigation.

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References

Dayton, W. R., Goll, D. E., Zeece, M. G., Robson, R. M. & Reville, W. J. (1976) *Biochemistry* **15**, 2150–2158

Duckworth, W. C., Heinemann, M. & Kitabchi, A. E. (1974) *Biochim. Biophys. Acta* **377**, 421–430

Hummel, B. C. W. (1959) *Can. J. Biochem. Physiol.* **37**, 1393–1398

Jusic, M., Seifert, S., Weiss, E., Haas, R. & Heinrich, P. C. (1976) *Arch. Biochem. Biophys.* **177**, 355–363

Kassell, B. & Williams, M. J. (1976) in *Handbook of Biochemistry and Molecular Biology of Proteins* (Fasman, G. D., ed.), vol. 2, 3rd edn., pp. 583–668, Chemical Rubber Co. Press, Cleveland, Ohio

Katunuma, N., Kominami, E., Kominami, S., Kito, K. & Matsuzawa, T. (1972) in *Metabolic Interconversions of Enzymes* (Wieland, O., Helmrich, E. & Holzer, H., eds.), pp. 159–174, Springer-Verlag, Berlin

Katunuma, N., Kominami, E., Kobayashi, K., Banno, Y., Suzuki, K., Chichibu, K., Hamaguchi, Y. & Katsunuma, T. (1975) *Eur. J. Biochem.* **52**, 37–50

Katz, J., Troll, W., Levy, M., Filkins, K., Russo, J. & Levitz, M. (1976) *Arch. Biochem. Biophys.* **173**, 347–354

Kawiak, J., Vensel, W. H., Komender, J. & Barnard, E. A. (1971) *Biochim. Biophys. Acta* **235**, 172–187

Kay, J. & Kassell, B. (1971) *J. Biol. Chem.* **246**, 6661–6665

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Schroeder, D. D. & Shaw, E. (1968) *J. Biol. Chem.* **243**, 2943–2949

Starkey, P. M. (1977) in *Proteinases in Mammalian Cells and Tissues* (Barrett, A. J., ed.), pp. 57–89, North-Holland, Amsterdam

Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412

Woessner, J. F. (1977) *Biochem. J.* **161**, 535–542

Wright, T. (1977) *Eur. J. Biochem.* **73**, 567–578