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Degradation of Myofibrillar Proteins by Cathepsins B and D

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1. The procedure of Barrett [(1973) *Biochem. J.* **131**, 809–822] for isolating cathepsins B and D from human liver was modified for use with rat liver and skeletal muscle. The purified enzymes appeared to be similar to those reported in other species. 2. Sephadex G-75 chromatography of concentrated muscle extract resolved two peaks of cathepsin B inhibitory activity, corresponding to molecular weights of 12500 and 62000. 3. The degradation of purified myofibrillar proteins by cathepsins B and D was clearly demonstrated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. After incubation with enzyme, the polypeptide bands representing the substrates decreased in intensity and lower molecular weight products appeared. 4. Cathepsins B and D, purified from either rat liver or skeletal muscle, were shown to degrade myosin, purified from either rabbit or rat muscle. Soluble denatured myosin was degraded more extensively than insoluble native myosin. Degradation by cathepsin B was inhibited by lack of reducing agent, or by myoglobin, iodoacetic acid and leupeptin, but not by pepstatin. The same potential modifiers were applied to cathepsin D, and only pepstatin produced inhibition. 5. Rat liver cathepsin B had a pH optimum of 5.2 on native rabbit myosin. The pH optimum of cathepsin D was 4.0, with a shoulder of activity about 1 pH unit above the optimum. 6. Rat liver cathepsins B and D were demonstrated to degrade rabbit F-actin at pH 5.0, and were inhibited by leupeptin and pepstatin, respectively. 7. The degradation of myosin and actin by cathepsin D was more extensive than that by cathepsin B.

Some 40% of total body protein is muscle protein and 60% of that is myofibrillar protein (Young, 1970). Under physiological conditions it is thought that myofibrillar proteins constitute a large and highly mobile protein reservoir. Myopathies and advanced starvation are typified by the disruption and disappearance of the orderly array of myofibrillar proteins. The enzyme system(s) responsible for protein degradation in skeletal muscle, however, has yet to be defined.

Few proteinases, other than those associated with lysosomes, have been identified in skeletal muscle. There is a postulated alkaline proteinase (Noguchi & Kandatsu, 1975) which has some of the same characteristics as alkaline cathepsin identified by Goldspink *et al.* (1970), an alkaline proteinase that appears to be bound to the myofibril (Mayer *et al.*, 1974), an enzyme that selectively degrades pyridoxine-dependent enzymes (Katunuma *et al.*, 1975), and a Ca^{2+}-activated neutral proteinase (Dayton *et al.*, 1976*a*), which has been reported to be the same proteinase described by Huston & Krebs (1968), Kohn (1969) and Busch *et al.* (1972) (see Dayton *et al.*, 1976*b*). Of these, only the Ca^{2+}-activated proteinase has been purified and shown to have proteolytic activity on isolated myofibrillar proteins. The Ca^{2+}-activated proteinase, a sarcoplasmic enzyme, removes Z-lines and partly degrades M-lines, and can degrade troponin, tropomyosin and C-protein (Dayton *et al.*, 1975). It cannot, however, degrade myosin or actin, and Dayton *et al.* (1976*b*) suggest that the enzyme may have a role in the disassembly of intact myofibrils during myofibrillar-protein turnover.

The present paper is part of a study concerned with the possible role of the lysosomal endopeptidases, cathepsins B and D, in myofibrillar protein turnover. Cathepsin B has been purified from several sources, including bovine tissues (Franklin & Metrione, 1972; Keilová & Tomásek, 1973; Etherington, 1974; Otto & Riesenkönig, 1975), sheep thyroid (Suominen & Hopsu-Havu, 1971), human liver (Barrett, 1973), human placenta (Swanson *et al.*, 1974), rabbit liver (Ogino & Nakashima, 1974) and rat skin (Järvinen & Hopsu-Havu, 1975). Cathepsin D has also been purified from several sources, and these include bovine tissues (Sapolsky & Woessner, 1972; Ferguson *et al.*, 1973; Smith & Turk, 1974), human and chicken liver (Barrett, 1970, 1973) and rat liver (Wiederanders *et al.*, 1974/76).

Cathepsins were purified from rat liver and skeletal muscle by the method of Barrett (1973) with modification, and their abilities to degrade myofibrillar protein *in vitro* were investigated. Purified myosin

and actin were used as substrates, since they represent 60% and 20% respectively of the myofibrillar protein of muscle (Ebashi & Nonomura, 1973), and consequently about 20% of the total body protein. An indigenous muscle proteinase has not previously been identified which can degrade myosin or actin.

Experimental

Materials

Male Sprague–Dawley rats (250–500g) were kindly given by Merck Chemical Division, Rahway, NJ, U.S.A., and also purchased from Sunrise Mousery, Whitehouse Station, NJ, U.S.A., as were New Zealand white rabbits (2–3kg).

Arquad 2C-50 [a 50% (w/v) solution of crude didodecyl ammonium chloride, also containing analogues of longer and shorter chain length, in aq. 50% (v/v) propan-2-ol], was kindly given by Dr. A. J. Barrett, Strangeways Research Laboratory, Cambridge, U.K. Leupeptin was a gift from Dr. A. Stracher, State University of New York, Downstate Medical Center, Brooklyn, NY, U.S.A.

Trizma base, dimethyl sulphoxide, cysteine (free base), β-mercaptoethanol, Bz-DL-Arg-$NHC_{10}H_7$ (α-*N*-benzoyl-DL-arginine 2-naphthylamide), Fast Garnet GBC (practical grade), 4-chloromercuribenzoic acid, 2-naphthylamine, bovine haemoglobin (type II), bovine serum albumin, ovalbumin (grade V), cytochrome *c* (horse heart, type III), myoglobin (horse skeletal muscle, type I), carbonic anhydrase (human erythrocyte), ascorbic acid, catalase (bovine liver), 5,5′-dithiobis-(2-nitrobenzoic acid) and α-chymotrypsinogen A (bovine pancreas, type II) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Phosphorylase *a* (rabbit muscle) and β-galactosidase (*Escherichia coli*) were purchased from Worthington Biochemical Corp., Freehold, NJ, U.S.A. 4-Aminophenylmercuric acetate was purchased from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Acrylamide, *NN′*-methylenebisacrylamide, ammonium persulphate, *NNN′N′*-tetramethylethylenediamine, Bromophenol Blue, glycine and β-alanine were purchased from Eastman Organic Chemicals, Rochester, NY, U.S.A. SDS* (sequanal grade) and Na_2EDTA were obtained from Matheson Coleman and Bell Manufacturing Chemists, Norwood, OH, U.S.A. Riboflavin was obtained from Nutritional Biochemicals Corp., Cleveland, OH, U.S.A., and CNBr was obtained from J. T. Baker Chemical Co., Phillipsburg, NJ, U.S.A. Sephadex G-75 (regular and superfine grades), DEAE-Sephadex (A-50) and Sepharose 4B were purchased from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A. CM-52 and DE-52 carboxymethyl- and DEAE-celluloses were obtained from H. Reeve Angel, Clifton, NJ, U.S.A., and Ampholine carrier ampholytes were obtained from LKB Instruments, Hicksville, NY, U.S.A. Pepstatin was purchased from Beckman Instruments, Palo Alto, CA, U.S.A. Coomassie Brilliant Blue R-250 was purchased from Colab Laboratories, Glenwood, IL, U.S.A. Sucrose and $(NH_4)_2SO_4$ (both special enzyme grade) were purchased from Schwarz/Mann, Orangeburg, NY, U.S.A., and Hyflo Super-Cel filter aid was a free sample from Johns Manville, Denver, CO, U.S.A. Ethylenediamine, Brij-35 and a variety of reagent-grade chemicals were purchased from Fisher Scientific Co., Pittsburgh, PA, U.S.A.

Organomercurial-Sepharose was prepared as described by Barrett (1973). Saturated $(NH_4)_2SO_4$ was prepared by adding 2kg of $(NH_4)_2SO_4$ to 2.5 litres of water, heating into solution, and then storing at 4°C for at least 2 days before use.

* Abbreviation: SDS, sodium dodecyl sulphate.

Methods

Enzyme assays. Cathepsin B activities were measured by the method of Barrett (1972), in which the substrate was Bz-DL-Arg-$NHC_{10}H_7$. One unit of activity released, at pH6.0, 1μmol of 2-naphthylamine/min at 40°C. Cathepsin D activities were measured by the method of Anson (1938) as modified by Canonico & Bird (1970), in which bovine haemoglobin was the substrate. One unit of activity released, at pH3.8, 1μmol of tyrosine equivalents (soluble in trichloroacetic acid)/min at 40°C.

Determination of protein. Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard, and, during the course of enzyme purifications, by measurement of A_{280}, for which $A^{1\%}_{1cm}$ was assumed to be 10.0.

Assay of endogenous cathepsin B inhibitors. Purified rat liver cathepsin B (0.1ml, containing 0.01unit of activity) was mixed with 0.4ml of each column fraction, and then assayed by the standard procedure. Blanks contained 0.1ml of enzyme dilution solution [10mM-sodium acetate buffer, pH4.3, containing 1mM-Na_2EDTA and 0.1% (w/v) bovine serum albumin] instead of cathepsin B. Percentage inhibitions were determined by comparing the cathepsin B activity with that determined for 0.1ml of the enzyme diluted with 0.4ml of the column buffer, which represented 100% activity (0% inhibition).

Analytical isoelectric focusing. The method was that of Barrett (1970), and 1% (w/v) carrier Ampholines of pH ranges 5–7 and 3–10 were used for cathepsin D and cathepsin B respectively. Gels were stained for protein and cathepsin B activity as described by Barrett (1973). Some gels were bisected longitudinally, and one half was used to detect protein or enzyme activity and the other half was cut

into 5mm segments, which were each eluted overnight in 0.5ml of water for the measurement of pH.

Molecular-weight determinations by gel filtration. A column of Sephadex G-75 (2.5cm×36cm; 180ml), equilibrated at 4°C with 20mM-KH_2PO_4/Na_2HPO_4, pH6.5, with 0.10M-NaCl, was used at a flow rate of 3.0ml/h per cm^2. The column was calibrated with bovine serum albumin (68000mol.wt.), ovalbumin (45000mol.wt.), chymotrypsinogen A (25000mol.wt.), myoglobin (17200mol.wt.), and cytochrome *c* (13000mol.wt.).

Purification of cathepsins B and D from rat liver and skeletal muscle. The method of Barrett (1973), which allows the simultaneous isolation of cathepsins B and D from human liver, was modified for use with the rat tissues.

An extract, prepared from 1.5kg of well-homogenized liver, was left to autolyse overnight at pH4.5, after which a 47–64% (v/v) acetone fraction was prepared and chromatographed stepwise on CM-cellulose. This was carried out essentially as in the original procedure (Barrett, 1973), except that autolysis was at 25°C, since this gave better recoveries and purifications than 40°C. The pool from CM-cellulose was applied to a column (1.5cm×17cm; 30ml) of organomercurial-Sepharose and the adsorbed material, which included both cathepsins B and D, was eluted with 10mM-β-mercaptoethanol, which gave better recoveries than the recommended 10mM-cysteine. The cathepsins were then separated by chromatography on Sephadex G-75 (superfine grade). The concentrated sample volume (4.0–4.2ml) and column dimensions (1.6cm×105cm, 210ml) were designed to provide complete resolution.

The pool of cathepsin B activity from Sephadex G-75 was dialysed overnight against 2 litres of 20mM-sodium acetate buffer, pH5.0, and then applied to a column (1.6cm×8.5cm; 17ml) of CM-cellulose, equilibrated with the same buffer. Elution with 2 bed vol. of column buffer was followed by a linear gradient (120ml) of 0–0.20M-NaCl in the column buffer. One peak of cathepsin B activity, contaminated by an overlapping peak of inactive protein, was eluted between 0.10 and 0.15M-NaCl. Fractions with high specific activity were combined, dialysed overnight against 2 litres of column buffer, and rechromatographed on the same column of CM-cellulose after it had been washed with several bed volumes of 1.0M-NaCl in column buffer and re-equilibrated with the column buffer. The column was eluted with 1 bed vol. of 50mM-sodium acetate buffer, pH5.0, and then with a linear gradient (120ml) of 0–10M-NaCl in the 50mM-acetate buffer. Two peaks of protein were resolved, the second of which coincided with the single peak of cathepsin B activity. Fractions with high specific activity were pooled and stored as suggested by Barrett (1973).

The pool of cathepsin D activity from Sephadex G-75 was chromatographed on a column (1.6cm×6.5cm; 13ml) of DEAE-cellulose as described by Barrett (1973), except that the sample was applied rather than adsorbed batchwise. A single peak of cathepsin D activity appeared about midway through the gradient. This was further purified and resolved into isoenzyme forms by preparative isoelectric focusing in a sucrose gradient with pH5–7 carrier ampholytes (1%), by using an LKB Ampholine 8101 column.

Purification from rat skeletal muscle included additional modifications. A well-homogenized solution was initially prepared from 500g of hind-leg muscle (except soleus) in 2 litres of 0.15M-KCl/2% (v/v) butan-1-ol/1mM-Na_2EDTA. The autolysed extract was centrifuged at 13000*g* for 15 min and the supernatant fractionated with satd. $(NH_4)_2SO_4$ solution. Cathepsin B recovery and specific activity in the 45–65%-satd-$(NH_4)_2SO_4$ fraction were 3-fold those that could be obtained by acetone fractionation. This fraction was dialysed extensively against 20mM-sodium acetate buffer, pH5.0, containing 1mM-Na_2EDTA, and then applied to a column (2.5cm×16cm; 80ml) of CM-cellulose. The column was re-equilibrated with 2 bed vol. of the column buffer (50mM-sodium acetate buffer, pH5.0, containing 1mM-Na_2EDTA), and then eluted with 0.15M-NaCl in the same buffer. A peak of cathepsin B activity was resolved from a major slower-moving peak of inactive protein. Cathepsin D activity was eluted as a broad peak, and only that which co-eluted with the cathepsin B activity was kept for further purification. Owing to relatively low recoveries, the muscle cathepsins were not purified as extensively as those from liver after Sephadex chromatography. Cathepsin B was chromatographed on a column (0.8cm×4.6cm; 2.3ml) of CM-cellulose as for liver, except that the gradient was 30ml and all solutions contained 1mM-Na_2EDTA. A single peak of cathepsin B activity was eluted about midway through the gradient, and this was pooled and referred to as purified muscle cathepsin B. Cathepsin D was chromatographed on a column (0.8cm×5.6cm; 2.8ml) of DEAE-cellulose as described for liver. A single peak of cathepsin D activity with a slight shoulder was eluted about midway through the gradient, and this was pooled and referred to as purified muscle cathepsin D.

Preparation of muscle-protein substrates. Purified myosin was prepared from rabbit and rat skeletal muscle essentially as described by Offer *et al.* (1973) for rabbit muscle, except the procedure was scaled down to 10%. For the native substrate, myosin was resuspended with a minimum volume of 0.5M-NaCl and then diluted with the same before use. For denaturated myosin, resuspension was with minimum volumes of β-mercaptoethanol, 10% (v/v) SDS and 50mM-sodium phosphate buffer, pH7.0, to give a

final solution containing 4% β-mercaptoethanol, 4% SDS and 10mM-phosphate buffer. The solution was heated for 15min in a boiling-water bath, and then dialysed against 3×50vol. of 10mM-sodium phosphate buffer, pH7.0, over 36h at 20°C, followed by similar dialysis at 4°C. (The efficiency of removal of SDS was not determined.) The solution was clarified by centrifugation at 18000*g* for 10min and then diluted with the 10mM-phosphate buffer before use.

Rabbit actin was purified by the method of Seraydarian *et al.* (1967). The polymerized actin was dialysed overnight against 2 litres of 0.1M-NaCl, and then diluted with the same before use.

Demonstration of proteolytic activity on muscle proteins. (1) Assay procedure. The appropriate amount of enzyme was diluted to 25μl with water, mixed with 150μl of incubation buffer, and then preincubated for 5min at 37°C. For routine assays, the incubation buffer was 66.7mM-sodium acetate buffer of various pH values which also contained 2mM-dithiothreitol and 2mM-Na_2EDTA for assays with cathepsin B. Muscle protein substrate (4.0mg of native rabbit myosin or actin/ml, or 8.0mg of denatured rabbit myosin/ml), in a volume of 25μl, was added to the mixture with an automatic pipette, and then incubated at 37°C in a shaking water bath. At the end of the incubation period, 200μl of solubilization solution [0.5M-Tris/HCl, pH6.8, containing 4% (w/v) SDS, 10% (v/v) β-mercaptoethanol, 20% (v/v) glycerol and 0.002% (w/v) Bromophenol Blue] was added with agitation on a vortex mixer. The assay tube was quickly covered with aluminium foil and immediately transferred to a boiling-water bath for 5min. Reagent blanks were prepared by withholding the enzyme until just before heating or by substituting water for enzyme (both methods yielded the same results in SDS/polyacrylamide gels). Samples were applied to SDS/polyacrylamide slab gels within the next 48h.

To compensate for a polypeptide contaminant (133000mol.wt.) contained within the rat myosin substrate, the concentration of substrate protein added to reaction mixtures was twice that used for corresponding rabbit substrates. In a few cases (i.e. assay of rat muscle enzymes and substrates), larger assay volumes of enzyme and/or substrate were used to compensate for low activity and/or concentration respectively. The volumes and concentrations of the other components of the reaction mixtures were manipulated accordingly, and the standard final concentrations were maintained.

(2) SDS/polyacrylamide-gel electrophoresis. Disc electrophoresis in polyacrylamide slab gels containing SDS was carried out in the buffer system described by Laemmli & Favre (1973). Slab gels (8.2cm×8.2cm×0.27cm) were prepared in gel cassettes constructed with the glass plates, spacers and tape supplied in the Pharmacia gel-cassette kit. Each sample (10–100μl) was applied to a sample well in the stacking gel. Electrophoresis was performed at 60V for 15min, or until the samples entered the gels, and then at 120V until the tracking dye migrated to within 1cm from the bottom of the gel (about 1h). The gels were stained with Coomassie Brilliant Blue R250, and then destained in a Pharmacia GD-4 gel destainer with a Pharmacia DPS destainer power supply essentially as suggested by the manufacturer.

(3) Estimation of molecular weight of hydrolysis products. Molecular-weight estimates were made by comparing the mobilities of the polypeptide bands in SDS/polyacrylamide gels with the appropriate calibration curve. Gels polymerized with 7.5% (w/v) acrylamide were calibrated with myosin heavy chain (200000mol.wt.), β-galactosidase (130000mol.wt.), phosphorylase *a* (100000mol.wt.), bovine serum albumin (68000mol.wt.) and ovalbumin (43000mol.wt.); carbonic anhydrase (29000mol.wt.) migrated with the tracking dye, and served as a convenient marker. Gels polymerized with 12.5% (w/v) acrylamide were calibrated with bovine serum albumin, ovalbumin, carbonic anhydrase and cytochrome *c* (11700 mol.wt.). The molecular weights of the standard proteins are those suggested by Weber & Osborn (1969).

(4) Quantification. Some gels were analysed at 530nm in a Canalco K densitometer, connected to a Canalco 8G recorder. Relative amounts of polypeptides were determined by measuring the areas under the recorded peaks.

Results

Purification of cathepsins B and D

Rat liver cathepsins B and D were isolated in highly purified states (Table 1). Preparative isoelectric focusing of cathepsin D resolved three major isoenzymes with isoelectric points of 5.7 (α), 6.1 (β), and 6.8 (γ), and each gave a distinct band when focused in gels. Two minor forms with pI5.4 (α) and 6.8 (γ) were also resolved. The major isoenzymes behaved identically in either cationic (Reisfeld *et al.*, 1962) or anionic (Davis, 1964) gel electrophoresis, and each gave one band of protein with cathepsin D activity. A minor band of 53000mol.wt., representing less than 5% of the γ isoenzyme protein, was observed in SDS/polyacrylamide gels (Laemmli & Favre, 1973) if sufficient protein was electrophoresed. This increased in intensity with the decreasing specific activities of the other isoenzymes and therefore probably represented a minor inactive contaminant.

Cationic electrophoresis of cathepsin B revealed one diffuse band of protein with Bz-DL-Arg-$NHC_{10}H_7$-hydrolysing activity, whereas anionic electrophoresis revealed two closely migrating zones of protein and one very minor band of higher mobility. Owing to

Table 1. *Purification of rat liver cathepsins B and D*

The yields of protein and enzyme activities are those obtained from 1500g of tissue. Protein was determined by A_{280} as described in the text, except where distinguished by an asterisk (*). In these cases, proteins were measured by the method of Lowry *et al.* (1951).

		Cathepsin B			Cathepsin D		
	Protein (mg)	Activity (units)	Specific activity (units/mg of protein)	Recovery (%)	Activity (units)	Specific activity (units/mg of protein)	Recovery (%)
Homogenate	277000*	43	0.00015*	8	1722	0.0062*	100
Arquad supernatant	108000*	86	0.00080*	16	1148	0.011*	66
Autolysed extract	106000*	548	0.0052*	100	1050	0.010*	61
Acetone (47–64%) fraction	1840	396	0.215	72	439	0.239	25
CM-cellulose (stepwise elution)	546	240	0.439	44	295	0.540	17
Organomercurial-Sepharose	206	146	0.710	27	187	0.909	11
Ultrafiltration concentration	165	124	0.753	23	170	1.03	10
Sephadex G-75 superfine	53	78	1.48	14			
CM-cellulose (gradient elution)	18	59	3.28	11			
CM-cellulose (gradient elution), re-chromatography	6.0 3.8*	40	6.69 10.64*	7			
Sephadex G-75 superfine	35.0				155	4.46	9
DEAE-cellulose (gradient elution)	17.0				116	6.94	7
Isoelectric focusing	0.6; 0.2*				5	7.1; 18.4*	0.3
	1.3; 0.8*				15	11.7; 18.3*	0.9
	2.1; 1.3*				34	16.7; 25.7*	2.0
	2.8; 1.9*				63	22.1; 32.6*	3.7
	0.3; 0.1*				1	3.9; 9.1*	0.1

Table 2. *Purification of rat skeletal-muscle cathepsins B and D*

The yields of protein and enzyme activities are those obtained from 500g of tissue. Protein was determined by A_{280} as described in the text, except where distinguished by an asterisk (*). In these cases, protein was measured by the method of Lowry *et al.* (1951).

		Cathepsin B			Cathepsin D		
	Protein (mg)	Activity (units)	Specific activity (unit/mg of protein)	Recovery (%)	Activity (units)	Specific activity (unit/mg of protein)	Recovery (%)
Homogenate	62100*	1.15	0.000018*	27.0	45.0	0.00072*	100.0
Arquad supernatant	14700*	0.73	0.000050*	17.0	20.9	0.0014*	46.0
Autolysis supernatant	3200*	4.24	0.0013*	100.0	17.8	0.0056*	40.0
$(NH_4)_2SO_4$(45–65%-satd.) fraction	369.0	2.58	0.0070	61.0	9.10	0.025	20.0
CM-cellulose (stepwise elution)	82.0	1.74	0.021	41.0	4.30	0.052	9.5
Organomercurial-Sepharose	9.0	1.05	0.117	25.0	3.47	0.385	7.7
Ultrafiltration concentration	8.5	0.81	0.095	19.0	1.45	0.170	3.2
Sephadex G-75 superfine	2.3	0.39	0.169	7.5			
CM-cellulose (gradient elution)	0.38	0.18	0.474	4.2			
Sephade G-75 superfine	1.7				0.79	0.468	1.8
DEAE-cellulose (gradient elution)	0.44				0.32	0.718	0.7

inactivation of cathepsin B at basic pH (Barrett, 1973), it was not possible to determine whether all of the bands were due to forms of cathepsin B. Gel isoelectric focusing revealed four major bands of protein, each corresponding to a band of Bz-DL-Arg-$NHC_{10}H_7$-hydrolysing activity. The isoelectric points were 5.3, 6.2, 6.7 and 6.9. A minor polypeptide band (21500mol.wt.), representing about 15% of the

Table 3. *Some characteristics of rat liver and skeletal-muscle cathepsins B and D*

The enzymes from skeletal muscle and liver, including each of the major cathepsin D isoenzymes, gave the same results. 'nmol of enzyme/unit of activity' for cathepsin D was based on the specific activity of the γ isoenzyme and the lower mol.wt. of 41000 (to balance out the slight contamination factor). The calculation for cathepsin B was based on 85% purity and an average mol.wt. of 26000.

	Cathepsin B	Cathepsin D
Assay substrate	Bz-DL-Arg-$NHC_{10}H_7$	Bovine haemoglobin
pH optimum	6.0	3.5
Requirements	Reducing agent	—
Inhibited by	Leupeptin, thiol inhibitors (i.e. iodoacetic acid, $HgCl_2$)	Pepstatin
Molecular weight		
Gel filtration	24000–27000	42000–45000
SDS/polyacrylamide-gel electrophoresis	27000	41000
nmol of enzyme/unit of activity	3.1	0.75

total protein, was resolved in SDS/polyacrylamide gels. The identity of the band was unclear from the data available.

The purification from rat skeletal muscle (Table 2), although less satisfactory than from liver, yielded enzyme samples of suitable purity for comparative studies. The enzymes from both tissues had the same characteristics (Table 3) and resembled those isolated from human liver (Barrett, 1973) and other mammalian tissues. Rat cathepsin D, however, was adsorbed to organomercurial-Sepharose, indicating that, unlike the human enzyme, it may contain a thiol group(s) free to react with the adsorbent.

Naturally occurring inhibitors of cathepsin B

The addition of rat muscle extract to purified cathepsin B has been demonstrated to inhibit the enzyme activity (Bird & Schwartz, 1977). When muscle extract was incubated overnight at pH4.5 and 25°C, the total cathepsin B activity increased gradually to about 6-fold the original activity (Fig. 1). This was apparently a result of inactivation of endogenous inhibitors, as may also occur with rat liver extract (Table 1). Chromatography of a concentrated muscle extract sample on Sephadex G-75 resolved two peaks of inhibitory activity, corresponding to mol.wts. 12500 and 62000. Järvinen & Hopsu-Havu (1975) have also identified cathepsin B inhibitors (12500 and 62000 mol.wts.) in rat skin. The significance of these indigenous muscle inhibitors is unclear, but the Bz-DL-Arg-$NHC_{10}H_7$-hydrolysing activity of muscle homogenates (and liver homogenates) appears not to be a valid indication of the amount of cathepsin B actually present.

Degradation of rabbit myosin by rat liver cathepsins B and D

Degradation of native and denatured myosin. Native myosin, at the approximate physiological ionic strength of the reaction mixture, was in filamentous form and insoluble, whereas denatured myosin remained soluble. (The solubility of the denatured myosin did tend to decrease below pH5.0, and was therefore not used at the lower pH values.) The consequent difference in effective substrate concentration precluded direct comparison of the rates of hydrolysis of the two substrates.

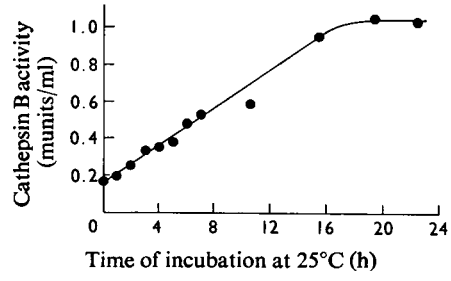
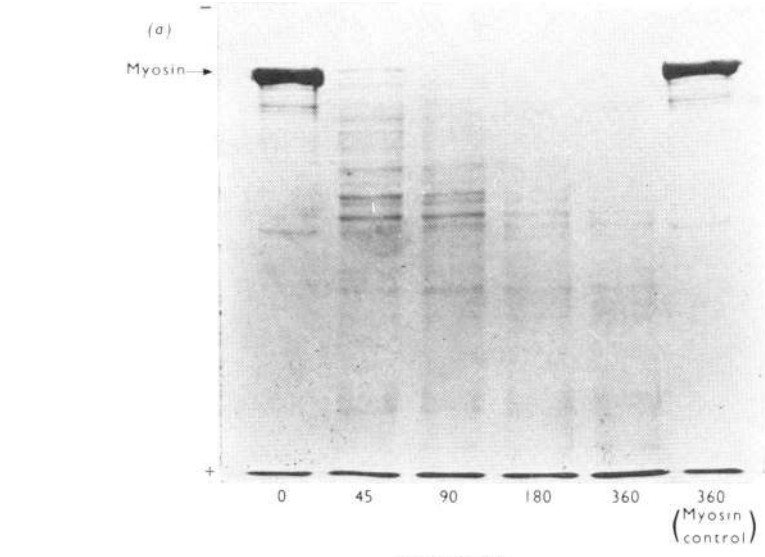
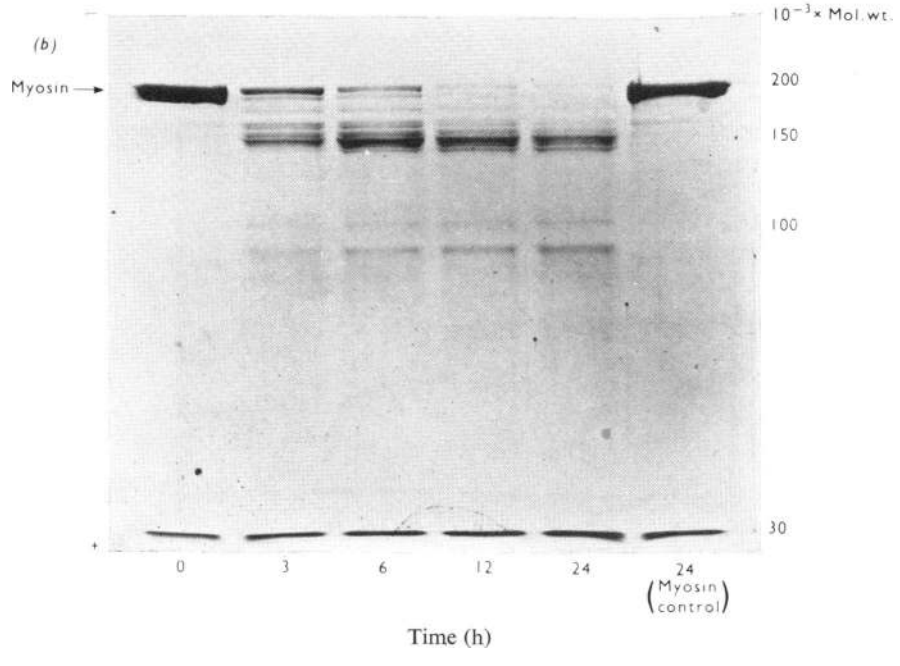


Fig. 1. *Increase in cathepsin B activity of muscle extract during incubation at pH4.5 and 25°C*

Equal volumes of fresh muscle extract were adjusted to pH4.5 with 5M-sodium formate buffer, pH2.8, and then incubated for different periods of time at 25°C. Cathepsin B activities correspond to the activities of 0.5ml samples of the autolysis supernatants (i.e. after centrifugation) as determined by the cathepsin B assay.

Cathepsin B, at pH5.2, degraded the 200000-mol.wt. heavy chains of denatured myosin into progressively smaller fragments, ultimately less than 30000 mol.wt., since they migrated with the tracking dye (Plate 1*a*). Degradation of native myosin, however, released a heterogeneous group of polypeptides (10000–50000 mol.wt.) and yielded major products of about 150000 mol.wt. which were essentially resistant to additional proteolysis (Plate 1*b*). The activity of cathepsin D on denatured myosin (Plate 2*a*)

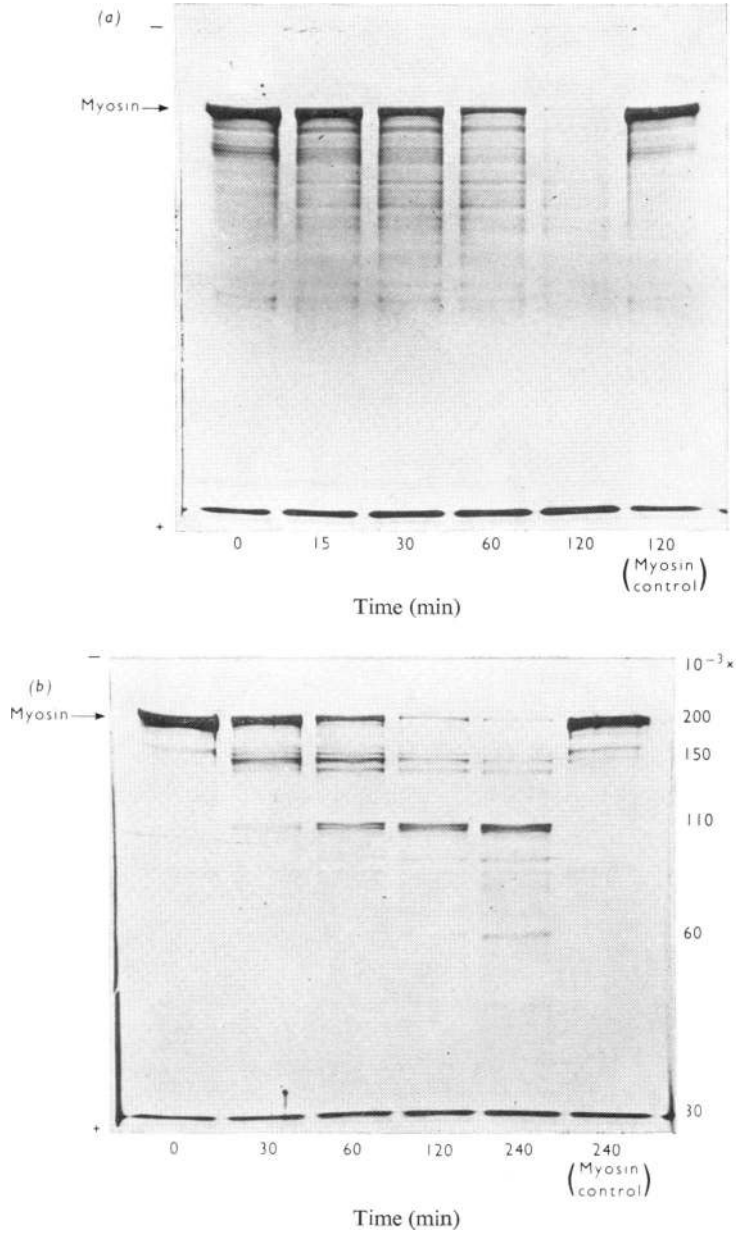




EXPLANATION OF PLATE 1

Degradation of denatured and native rabbit myosin by rat liver cathepsin B

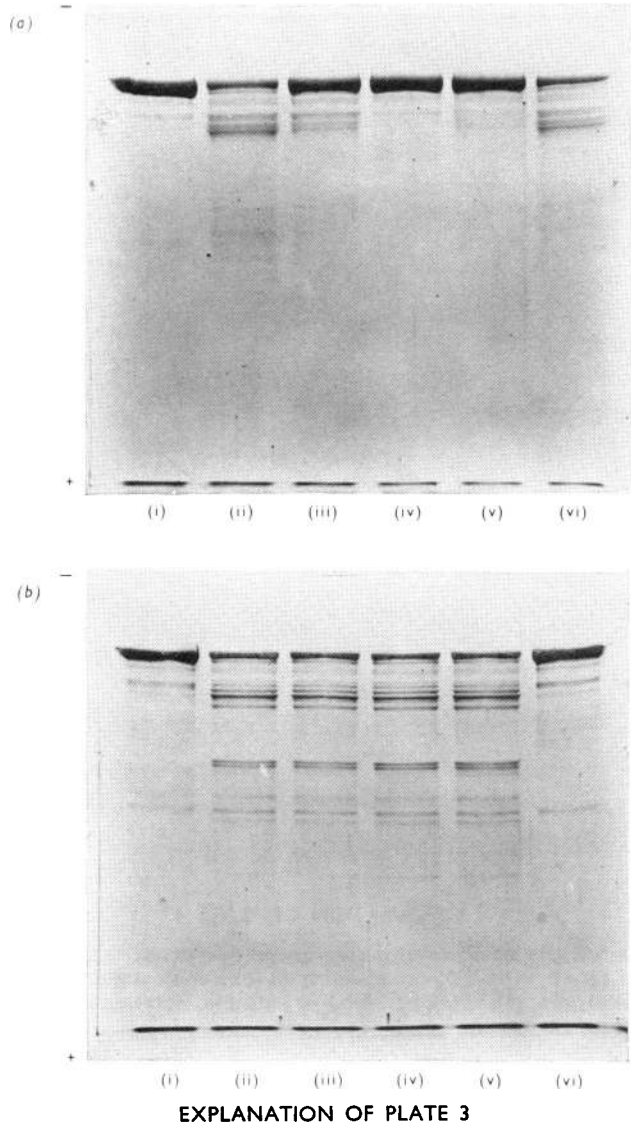
(*a*) Denatured myosin (1.0mg/ml) was incubated with cathepsin B (0.14unit/ml) at 37°C in 50mM-sodium acetate buffer, pH5.2, containing 1.5mM-Na_2EDTA and 1.5mM-dithiothreitol. The myosin control was incubated with water instead of enzyme. Assays were terminated at the designated times by treatment with solubilization solution, as described under 'Methods'. Samples (15μl), equivalent to 7.5μg of substrate protein, were subsequently applied to the 7.5% gel (with 4% stacking gel) and electrophoresis in the presence of SDS was performed as described under 'Methods'. The gel was stained with Coomassie Brilliant Blue R-250 after the stacking gel had been removed. (*b*) The substrate was native myosin (0.5mg/ml) and reaction mixtures also contained 62.5mM-NaCl. Samples (30μl) were required for application of the 7.5μg of substrate protein to the gel. All other conditions were as described for (*a*).



EXPLANATION OF PLATE 2

Degradation of denatured and native rabbit myosin by rat liver cathepsin D

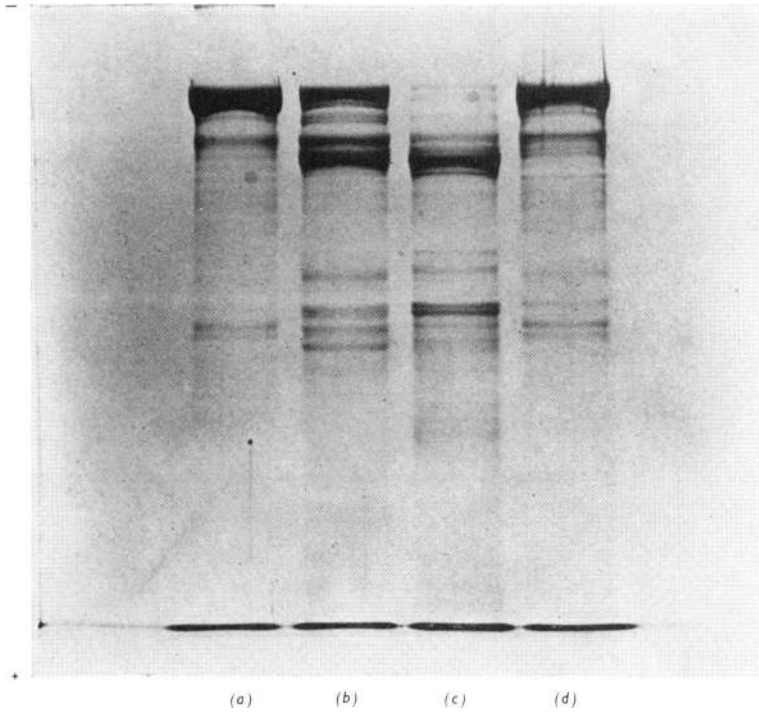
(*a*) Incubation was with cathepsin D γ-isoenzyme (0.10unit/ml) at pH5.0, and Na_2EDTA and dithiothreitol were not included in the reaction mixtures. All other conditions were as described in Plate 1(*a*). (*b*) Incubation was with cathepsin D γ-isoenzyme (0.10unit/ml) at pH4.0, and Na_2EDTA and dithiothreitol were not included in the reaction mixtures. All other conditions were as described in Plate 1(*b*).



EXPLANATION OF PLATE 3

Effects of modifiers of enyzme activity on the degradation of rabbit native myosin by rat liver cathepsins B and D

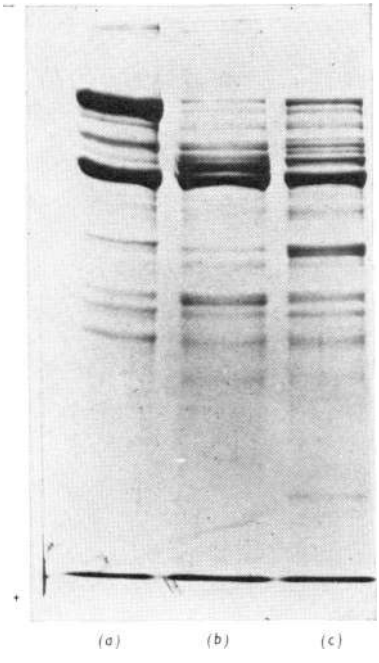
Potential modifiers were introduced as part of the incubation buffers. (*a*) Incubations were with cathepsin B (0.14 unit/ml) for 2 h at pH 5.0. All other conditions were as described for Plate 1(*b*). (i) Myosin; (ii), as (i), but with enzyme; (iii), as (ii), but without dithiothreitol; (iv), as (ii), but with 10 mM-iodoacetic acid; (v), as (ii), but with 10 μM-leupeptin; (vi), as (ii), but with 2 μM-pepstatin. (*b*) Incubations were with cathepsin D γ-isoenzyme (0.10 unit/ml) for 40 min at pH 4.0. All other conditions were as described in Plate 2(*b*). (i), (ii), (iv), (v) and (vi) were the same as described above for (*a*); (iii) contained 1.5 mM-Na_2EDTA and 1.5 mM-dithiothreitol.



EXPLANATION OF PLATE 4

Comparison of rabbit native myosin degradation by liver and muscle cathepsin B

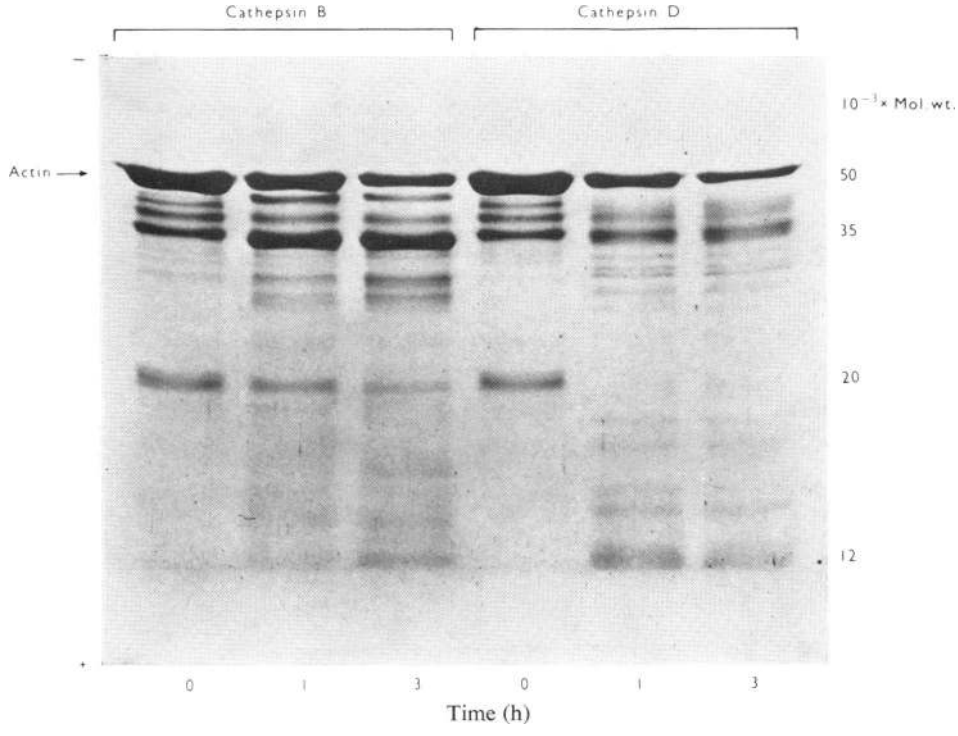
Incubations were at pH5.2 for 4h, with 0.035unit of cathepsin from either source/ml. All other conditions were as described for Plate 1(*b*). (*a*) Myosin; (*b*), as (*a*), but with liver cathepsin B; (*c*), as (*a*), but with muscle cathepsin B; (*d*), as (*c*) but with 10μM-leupeptin.



EXPLANATION OF PLATE 5

Degradation of rat native myosin by rat skeletal-muscle cathepsins B and D

Rat native myosin (1.0mg/ml) was incubated with cathepsin B at pH5.2 or cathepsin D at pH4.0 for 3h at 37°C (myosin control was at pH4.0). Sample volumes (30μl) applied to the gel contained 15.0μg of substrate protein. All other conditions were as described in Plate 1(*b*) for cathepsin B and Plate 2(*b*) for cathepsin D. (*a*) Myosin; (*b*), as (*a*), but with cathepsin B (0.035unit/ml); (*c*) as (*a*), but with cathepsin D (0.026unit/ml).



EXPLANATION OF PLATE 6

Degradation of rabbit F-actin by rat liver cathepsins B and D

F-actin (0.5mg/ml) was treated with cathepsin B (0.13unit/ml) or cathepsin D γ-isoenzyme (0.47unit/ml) at 37°C in 50mM-sodium acetate buffer, pH5.0, containing 12.5mM-NaCl. Reaction mixtures with cathepsin B also contained 1.5mM-Na_2 EDTA and 1.5mM-dithiothreitol. Samples (45μl) of terminated reaction mixtures, each containing 11.2μg of substrate protein, were applied to the 12.5% gel (with 4% stacking gel). All other conditions were as described in Plate 1(*b*).

was similar to that of cathepsin B, but native myosin was degraded more extensively (Plate 2*b*). Initially, proteolytic products of 150000–175000mol.wt. appeared and were further degraded. Two major (110000 and 107500mol.wt.) and several minor products were formed, which were particularly resistant to additional proteolysis. Each of the three major cathepsin D isoenzymes gave the same degradation products, and the activities appeared to be proportional to their haemoglobin-degrading activities in the standard assay.

Incubation of both cathepsins B and D with native myosin revealed no synergism, and the hydrolysis products resembled those of cathepsin D alone. Each enzyme was incubated with the hydrolysis products of the other, and cathepsin D degraded the 150000-mol.wt. products of cathepsin B, but cathepsin B had no additional activity on the products of cathepsin D.

Effect of pH. Only degradation of the 200000-mol.wt. myosin band was measured, since activities on secondary substrates (i.e. hydrolysis products) appeared not to affect the results significantly. The assays were initially demonstrated to be linear for the incubation times used. The pH optimum for the degradation of native myosin by cathepsin B was 5.2 (Fig. 2). A similar pH curve was obtained when purified rat muscle cathepsin B was incubated with denatured rabbit myosin, and proteolysis measured colorimetrically by an increase in ninhydrin-reactive substances (Schwartz & Bird, 1976). The pH optimum of cathepsin D was about pH4.0, with a shoulder of activity about 1 pH unit above the optimum (Fig. 3). A similar pH curve, although shifted about 1 pH unit to the left, has been described for the activities of human and chicken liver cathepsin D on haemoglobin (Barrett, 1970).

Effects of various modifiers of enzyme activities. Degradation of native or denatured myosin by cathepsin B (Plate 3*a*) was unaffected by pepstatin, but was greatly decreased by the absence of dithiothreitol and was abolished by iodoacetate or leupeptin, or by myoglobin at 0.5mg/ml. This concentration caused only 50% inhibition of cathepsin B with Bz-DL-Arg-$NHC_{10}H_7$ as substrate (Bird & Schwartz, 1977). Only pepstatin inhibited the degradation catalysed by cathepsin D (Plate 3*b*).

Comparison of rabbit myosin degradation by cathepsins B and D from rat liver and muscle

The purified muscle cathepsin B preparation had at least 2-fold the myosin-degradative activity of the liver enzyme on the basis of its naphthylamidase activity, and was also inhibited by leupeptin (Plate 4). The degradation products, however, appeared to be the same for both enzyme samples. The myosin-degradative activities observed with equal units of liver and muscle cathepsin D were the same, as were the degradation products, and both were inhibited by pepstatin (2μM).

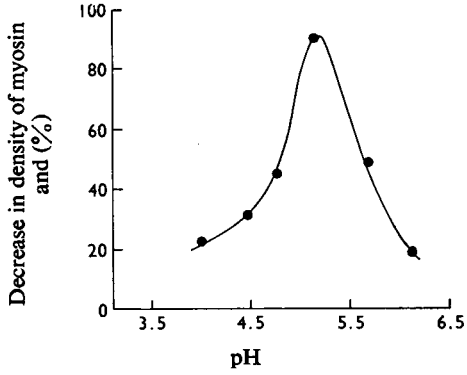


Fig. 2. *Degradation of native rabbit myosin by rat liver cathepsin B as a function of pH*

Incubation with cathepsin B (0.14unit/ml) was for 40min. The buffers used were 50mM-sodium formate (pH3.5), 50mM-sodium acetate (pH4.0–5.5) and 50mM-sodium phosphate (pH6.0). All other conditions were as described for Plate 1(*b*), except that gels were scanned at 530nm. The percentage decrease in density of the myosin band was calculated with respect to the reagent blank incubated at the respective pH. Points on the graph correspond to pH values measured in reaction mixtures at room temperature.

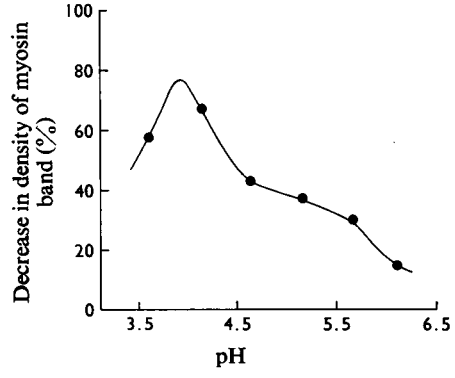


Fig. 3. *Degradation of native rabbit myosin by rat liver cathepsin D as a function of pH*

Incubations with a mixture of the three major cathepsin D isoenzymes (0.10unit/ml) were for 30min. All other conditions were as described for Fig. 2, except that Na_2EDTA and dithiothreitol were not included in the reaction mixtures.

Degradation of rat myosin by cathepsins B and D from rat liver and muscle

Myosin prepared from rat muscle contained additional polypeptide contaminants, but degradation of the 200000-mol.wt. myosin heavy chain by cathepsins B and D, also purified from rat skeletal muscle, was clearly demonstrated (Plate 5). The pattern of degradation was as demonstrated above on rabbit myosin. The same results were obtained with the liver enzymes.

Degradation of actin by cathepsins B and D

Native rabbit actin, in filamentous form under the assay conditions, was resolved as G-actin monomer (50000mol.wt.) in the SDS/polyacrylamide gels (Plate 6). Minor bands were also resolved and may have represented contaminating tropomyosin and troponin and/or proteolytic fragments produced during the purification procedure. Cathepsin B degraded actin at pH5, yielding a major polypeptide product of about 35000mol.wt. (Plate 6). Degradation by an approximately equimolar amount of cathepsin D was more extensive (Plate 6), and cathepsin D was also capable of degrading the 35000-mol.wt. product of cathepsin B. Actin degradation by cathepsins B and D was inhibited by leupeptin and pepstatin respectively. The activity of cathepsin D at pH4 was approximately twice that at pH5, whereas cathepsin B had less activity at the lower pH. The activities of both enzymes at pH6 were very low.

Discussion

The degradation *in vitro* of both the non-globular myosin heavy chain and actin was clearly demonstrated with SDS/polyacrylamide-gel electrophoresis. [The four globular myosin light chains (about 20000mol.wt.) migrated with the tracking dye in the SDS gels and were therefore not resolved.] After incubation of substrate with enzyme, the reaction mixture was heated at 100°C and then analysed in SDS/polyacrylamide gels. Proper controls showed that protein digestion was completely stopped by heating.

These results are in contrast with the conclusions of several previous studies cited by other investigators (Busch *et al.*, 1972; Dayton *et al.*, 1976*a*; Kar & Pearson, 1976). Those studies appear to have suffered from limitations that were circumvented in the present study. In the biochemical analyses of Park & Pennington (1967) and Bodwell & Pearson (1964) only proteolysis products soluble in trichloroacetic acid were measured, which would not include relatively large polypeptide fragments detectable in SDS/polyacrylamide gels. The techniques used by Sharpe (1963) and Friedman *et al.* (1969) did not provide the resolution necessary to detect changes in myofibrillar structure. Martins & Whitaker (1968) and Fukazawa & Yasui (1967) used incubation temperatures (4 and 0°C respectively) at which the cathepsins have little or no activity. In addition, all of these studies used substrates and/or enzyme preparations that were heterogeneous and not as well-defined as those described in the present paper. They also did not consider the possibility of endogenous inhibition.

Degradation of denatured myosin was more extensive than that of native myosin, suggesting that many bonds that are susceptible to hydrolysis are not accessible to the enzymes in the native molecule. This could be due to the native conformation of the myosin molecule and/or the myosin filament. The muscle cathepsin B preparation had greater myosin-degrading activity than did that from liver. The activities of both preparations were eluted within the same molecular-weight range by gel filtration, and were thiol-dependent and inhibited by leupeptin. Since different 'cathepsin B-like' lysosomal enzymes have been reported to occur in the rat (Kirschke *et al.*, 1974/76, 1976; Davidson & Poole, 1975; Towatari *et al.*, 1976), a likely explanation for these results is that the enzyme preparations contained different proportions of 'cathepsin B-like' enzymes. The significance of the relative activities in the purified preparations is questionable, however, since these may not proportionally reflect the ratios in the tissues.

The pH optimum for degradation of native myosin by cathepsin D was 4.0, but 50% of the activity remained at pH5.2 (Fig. 3), the pH optimum of cathepsin B. From the semi-quantitative results of gel scanning (Figs. 2 and 3) and the molar activities of the enzymes (Table 3), the activity of liver cathepsin B at pH5.2 was approx. 25% that of cathepsin D on a molar basis. Cathepsin B purified from muscle, having greater proteolytic activity than that from liver, was equivalent to 50% or more of the same cathepsin D activity. Treatment with cathepsin D, however, also resulted in smaller hydrolysis products, and reasonable activity was expressed over a much wider pH range. The cathepsins degraded actin less readily than myosin. Similar results have been reported for exogenous proteinases, such as trypsin (Ebashi & Nonomura, 1973). Degradation of F-actin by cathepsin D was of the order of 10% of its activity on myosin, whereas that by cathepsin B was about 20%. Actin, however, represents only one-third the amount of protein contributed by myosin in muscle. These experiments were with F-actin and do not account for higher degradation rates which might occur after depolymerization.

Considerations of actual concentrations *in vivo* gave a clearer estimate of the proteolytic potential of

the lysosomal endopeptidases. Thus 500g of muscle (wet wt.) contain 62.1g of protein and 45 units (34nmol) of cathepsin D (Table 2): 60% of the protein is myofibrillar protein, and 60% (22.4g) of this is myosin (Ebashi & Nonomura, 1973). Since 0.02 unit of cathepsin D can degrade, at pH4.0, 150μg of native myosin in 1h (Fig. 3), then 45 units of cathepsin D are capable of degrading 0.34g of native myosin in 1h, or 22.4g in 133h. At pH5, degradation would require twice this amount of time, or 266h (i.e. 11 days). The actual amount of cathepsin B present in the muscle is not known, but the amount free from inhibition in the autolysate is 4.24 units from 500g of muscle (Table 2). Assuming that this is equal to about half that present in the original homogenate, 500g of muscle contains 8.5 units, or 26nmol, which is 75% of the amount of cathepsin D. Since the proteolytic activity of cathepsin D appears to be 1–4-fold that of cathepsin B at pH5, the time required for total degradation of native myosin would be 14–60 days at this pH. The sum of activities of both cathepsins B and D at pH5 give a value of 6–9 days.

Although the described degradation is not complete, neither the contribution of the lysosomal exopeptidases nor the enhancing effect of possible denaturation were considered. We are well aware of the broad assumptions made on the basis of this isolated system *in vitro*. However, the above extrapolations do help illustrate the significance of the activities of the lysosomal proteinases present in muscle. Jensen & Bainton (1973) have measured intralysosomal pH values as low as 4. Millward & Garlick (1974/76) have estimated the half-life of actomyosin in the quadriceps and gastrocnemius muscles of the rat to be 26 and 36 days respectively. The myosin-degradative activities of cathepsin B and D are sufficient to be of physiological importance.

Recent experiments in our laboratory have demonstrated that myosin and actin can also be degraded in intact myofibrils or myofilaments by lysosomal extracts from normal muscle at pH5 (Spanier *et al.*, 1977). The effect was greater with treatment by lysosomal extracts from dystrophic muscle, in which catheptic activity and acidic autolysis are known to increase. These activities were completely blocked by the cathepsin B and D inhibitors leupeptin and pepstatin respectively. McGowan *et al.* (1976) have reported that these inhibitors inhibit the degeneration of muscle cells in culture, and noted significant differences in the number of myofibrils present. The effect was more marked in dystrophic muscle fibres, but similar differences were also noted in normal muscle fibres.

It is likely that intralysosomal digestion under physiological conditions would require initial disruption of the myofibrils by a neutral proteinase (Dayton *et al.*, 1976*b*; Bird & Schwartz, 1977), mechanical forces (Goldspink, 1971; Millward & Garlick, 1974/76), and/or other factors. The myofilaments could then be sequestered and digested within sarcoplasmic-reticulum components, which appear to be part of the lysosomal apparatus in skeletal muscle (Bird, 1975). In pathological states such as dystrophy (Weinstock & Iodice, 1969) and ischemia (Hoffstein *et al.*, 1975), the stimulation of the lysosomal system and/or membrane labilization and enzyme release which may occur could result in extensive damage by the lysosomal proteinases.

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