Lysosomal Cathepsins of Chicken Skeletal Muscle: Distribution and Properties

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Activities of cathepsins A, B, C, and D in extracts of chicken skeletal muscle have been quantitated by using synthetic peptides and denatured hemoglobin as substrates. Experimental conditions have been established whereby most of the activity of each cathepsin is obtained in the latent or sedimentable form. The various cathepsins showed different degrees of latency. These activities are solubilized by Triton X-100 or by freezing and thawing; accordingly, muscle cathepsins appear to be associated with the lysosomes. The stability of lysosome-enriched fractions to osmotic and pH effects has been measured. The effects of pH, cysteine, and certain ions on the individual catheptic activities have been determined. Hydrolysis of hemoglobin by cathepsin D appears to be augmented by the supplementary actions of other cathepsins present in crude extracts.

Few efforts have been made to determine the subcellular localization of muscle cathepsins. Based on studies of organ tissues, lysosomes are described as subcellular reservoirs of acid hydrolases, which together have the capacity to degrade all biopolymers; lysosomal enzymes have been implicated in a number of catabolic processes both of physiologic and pathologic origin (de Duve, 1963). Membrane stability and impermeability have been suggested as control mechanisms for regulation of lysosome activity.

In earlier studies (Caldwell, 1970), the activation and inhibition of muscle autolysis by compounds which influence the reactivity of cathepsins B and C were observed. That study suggested the joint action of several muscle cathepsins on endogenous muscle proteins. Cathepsins B and D were especially implicated. Experiments reported here were undertaken both to characterize more definitely the catheptic activities present in skeletal muscle and to study their cellular localization.

METHODS AND MATERIALS

Preparation of Homogenates. Broilers, 1.5 to 2.6 kg, were obtained commercially and slaughtered by exsanguination. Large breast muscles (*Pectoralis superficialis*) were removed and chilled in cold sucrose solutions containing 1 mM ethylenediamine tetraacetate (EDTA); sucrose concentrations and solution pH varied as indicated in the text. Tissues were finely minced with scissors, suspended in nine parts of solution, and homogenized in a laboratory blender connected to a variable transformer set at 50% line voltage. Variations of the homogenization technique and differences attributable to such variations are described in the section on Results.

Crude homogenates were centrifuged at 0° C at 480 × g for 10 min; sediment was discarded. A portion of the postnuclear homogenate was centrifuged at 0° at 20,200 × g for 30 min to give a supernatant fraction which was used for determination of nonsedimentable catheptic activities; the pellet contains lysosomes and other subcellular organelles.

To disrupt lysosomal membranes, the remainder of the homogenate was either frozen at the temperature of acetone-dry ice and thawed three times or rehomogenized (six sweeps of a glass pestle) in presence of 0.2% Triton X-100. Such fractions were usually centrifuged at 20,200 × g to remove lysed membranes. Triton-treated or frozen-thawed samples served as enzyme source for determination of total activities. Extracts were stored at −18° C until assayed.

Osmotic and pH Activation. Aliquots of postnuclear homogenate were taken for preparation of nonsedimentable and total enzyme fractions, as described above. Bound or particulate activity was calculated as the difference between total and nonsedimentable activities. Additional aliquots were also centrifuged at 0° C at 20,200 × g for 30 min and the soluble phase discarded. Particulate fractions were carefully resuspended in the indicated media, maintained at 0° C for 30 min, and again centrifuged. Bound activity released from the particles into the soluble phase was measured. This procedure was used to obtain data of Table II.

Enzyme Assays. *N*-Carbobenzoxy-α-L-glutamyl-L-tyrosine and glycyl-L-phenylalanine amide were purchased from Mann Research Laboratories; benzoyl-L-arginine amide and bovine hemoglobin (hemiglobin) were purchased from Calbiochem.

All assays were carried out at 37° C with appropriate reagent and enzyme controls. Activities of cathepsins A, B, and C were assayed for 60 min; cathepsin D activity was measured for 120 min. Specific activity is expressed as nmoles product per mg protein per min.

Cathepsin A was measured in 0.2 *M* acetate buffer, pH 5.0, containing 0.01 *M* *N*-carbobenzoxy-α-L-glutamyl-L-tyrosine and 0.15 ml of enzyme in a total volume of 1.5 ml. Reactions were terminated by addition of 1.5 ml of 10% trichloroacetic acid to 0.5-ml aliquots of assay mixture. After centrifugation, the supernatants were analyzed by the ninhydrin method of Moore and Stein (1954).

Cathepsin B activity was determined in 0.2 *M* acetate buffer, pH 5.0 containing 0.05 *M* benzoyl-L-arginine amide, 0.05 *M* cysteine, and 1 ml enzyme in a total volume of 2 ml. Aliquots (0.5 ml) were pipetted into 1 ml saturated sodium carbonate for liberation of ammonia, which was collected as ammonium sulfate by microdiffusion (Seligson and Seligson, 1951). Ammonium sulfate was eluted from the collection rods in 2 ml of water and measured by the Nesslerization technique described by Johnson (1941); samples were read at 420 mμ.

Cathepsin C activity was assayed in 0.2 *M* citrate buffer, pH 6.0 containing 0.05*M* glycyl-L-phenylalanine amide and 0.01 *M* cysteine hydrochloride. Liberated ammonia was determined as described for cathepsin B.

Cathepsin D activity was measured in 0.2 *M* acetate buffer, pH 4.0 in presence of 1% acid denatured hemoglobin, and 1 ml of enzyme in a total volume of 2 ml. Reactions were terminated with 1 ml of 10% trichloroacetic acid. Tyrosine equivalents in the supernatant were measured according to

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the Anson method as adapted by Gianetto and de Duve (1955).

Protein concentrations were determined by the Miller modification of the Lowry method (Lowry *et al.*, 1951; Miller, 1959).

RESULTS

Homogenate Preparation. Table I shows the influence of sucrose molarity, homogenization time, homogenization equipment, and pH on the extraction of total and nonsedimentable cathepsin D activities from chicken skeletal muscle. Varying the molarity of sucrose from 0.25 to 0.75 had marginal effects on the fraction of cathepsin D activity which was solubilized by the homogenization process, but the least disruption of subcellular structure was observed in 0.44*M* sucrose solutions.

When the homogenization time ranged from 30 to 60 sec, the percent nonsedimentable activity varied from 18 to 29. Several short bursts were more disruptive than a single but longer homogenization period: four 10- or three 15-sec periods gave 24 to 29% nonsedimentable activity, whereas a single 30- or 60-sec period gave 18 to 23% nonsedimentable activity. These differences are attributable to higher yields of total activity at longer homogenization periods without equivalent increases in the nonsedimentable activity.

Experiments summarized in Table I demonstrate the critical importance of homogenizer construction. Although several Waring Blendors—some of different sizes—were tested, the distribution of activity between supernatant and particulate fractions was not appreciably altered. Eighty-four percent of the activity was nonsedimentable. When an Omni-Mixer homogenizer fitted with a rotor-knife blade was used, 67% cathepsin D activity was solubilized. But when the Omni-Mixer was fitted with a specially constructed blade, 82% of the activity was associated with subcellular particles. The blade and shaft used in the latter experiments are shown in Figure 1. The blade was designed by R. N. Sayre of our laboratory and constructed in our mechanics shop; it has vertical slots in the stator to prevent vortex formation owing to rotor action.

The influence of solution pH on both total extractable activity and on percent nonsedimentable activity is shown in Table I. Compared with the yield of cathepsin D activity at pH 7.2, the relative extractable activity varied from 0.22 in deionized water to 1.18 in pH 4.0 buffer. Nonsedimentable activity varied from 14% at pH 7.2 to 67% in deionized water. Accordingly, a five-fold range was observed in yields of both total and bound activities.

Based on the above observations, optimal conditions for highest yields of both particulate and total cathepsin D activities include: 0.44 *M* sucrose, pH 7.2 potassium phosphate buffer as suspension medium; 1 min homogenization period; extrustion-type homogenizer blades. In a typical experiment, 2 g of tissue was homogenized in 18 ml of suspension medium.

Osmotic and pH Activation of Particulate Fraction. The data shown in Table II demonstrate that, in absence of adequate osmotic protection, 20 to 48% particulate cathepsin D was released into the incubation media. In presence of 0.44 *M* sucrose, only 1 to 4% of the enzyme was redistributed. Therefore, adequate osmotic protection appears to be more necessary for particle stability than regulation of media pH. In both the presence and absence of sucrose, particles were most stable at pH 5 and 6 and least stable at more acid and more alkaline pH values or in deionized water. The greater stability of particles at pH 5 and 6 (Table II) contrasts with the lower yield of activity in this pH range (Table I).

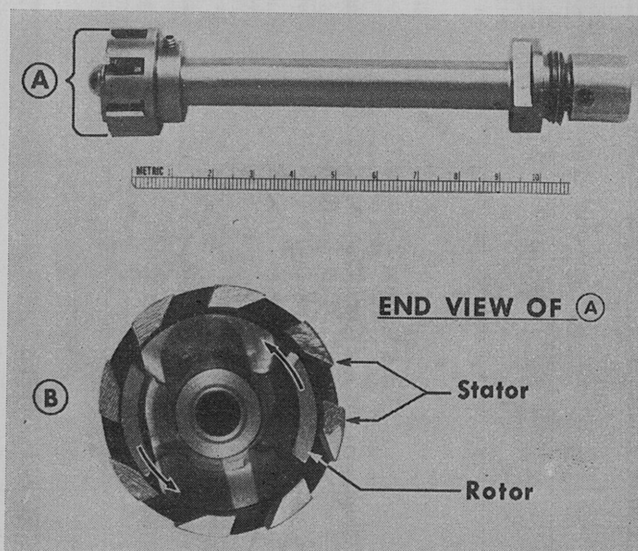

Figure 1. Extrusion-blade assembly for Omni-Mixer homogenizer

Table I. Influence of Various Experimental Parameters on Distribution and Total Activity of Cathepsin D[a]

Sucrose Molarity, pH 7.2[b,c]		% Nonsedimentable
0.25		27
0.44		24
0.50		25
0.75		27
Homogenization Time, Sec[c]		
60		18
30		23
10 + 10 + 10 + 10		29
15 + 15 + 15		24
Homogenizer Construction[c]		
Waring Blendor		84
Omni-Mixer		
Rotor-knife blade assembly		67
Extrusion blade assembly		18
Homogenization Medium[d]	**Relative Extractable Activity[e]**	
pH 7.2, phosphate	1.00	14
Deionized water	0.22	67
pH 6.0, phosphate	0.63	26
pH 5.0, acetate	0.33	64
pH 4.0, acetate	1.18	35

[a] 10% Homogenates (w/v) in 0.44 *M* sucrose, pH 7.2 potassium phosphate buffer were prepared by homogenizing for 60 sec in the Omni-Mixer with extrusion blade assembly, except as indicated. [b] Homogenization time = 3 × 15 sec. [c] Total activities measured on frozen-thawed samples. [d] Total activities measured on Triton X-100 treated samples. [e] Specific activity = 6.10.

Table II. Osmotic and pH Activation of Particulate Fractions[a]

Medium	Percent Nonsedimentable Cathepsin D Activity: No Sucrose	Percent Nonsedimentable Cathepsin D Activity: 0.44 *M* Sucrose
Deionized water	46	3
pH 7.2, Phosphate	44	3
pH 6.0, Phosphate	27	1
pH 5.0, Acetate	20	1
pH 4.0, Acetate	48	4

[a] Experimental details given in text.

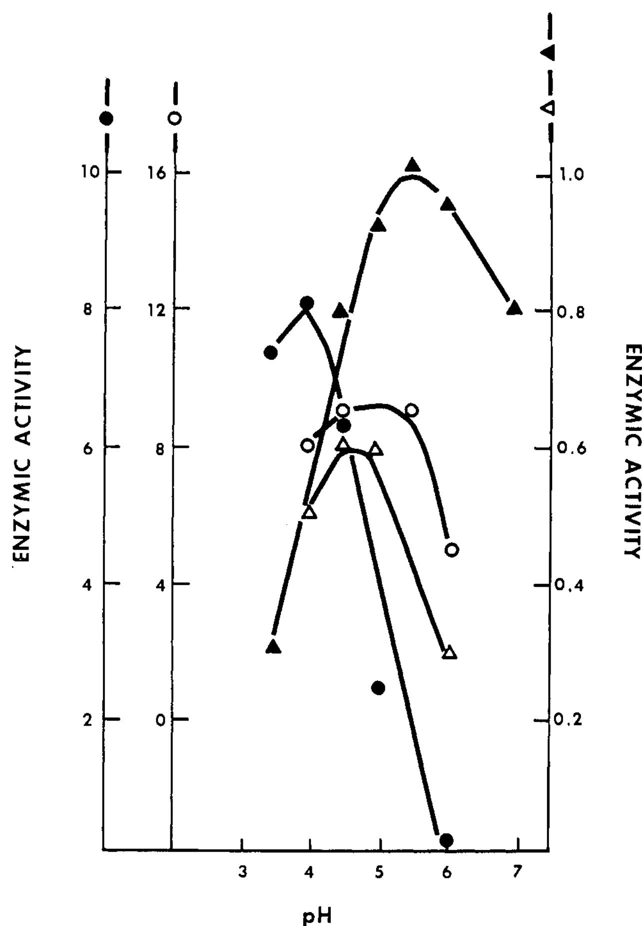

Figure 2. Influence of pH on catheptic activities. Assays are described in Methods and Materials except pH varied; enzyme source was Triton X-100 treated postnuclear homogenates. Results are expressed as nmoles product per mg protein per min. Cathepsin: A(○); B(△); C(▲); D(●)

Table III. Catheptic Activities of Chicken Breast Muscle[a]

Cathepsin Measured	Specific Activity	Percent Nonsedimentable
A	88	26
B	0.59	29
C	0.95	41
D	8.10	14

[a] Experimental details given in text.

Catheptic Activities of Muscle. Results obtained from assay of cathepsins A, B, C, and D are shown in Table III. In order of decreasing specific activity, the enzymes are cathepsins A, D, C, and B. The latter two activities are of the same order of magnitude; cathepsin A activity was approximately 100 times greater than those of cathepsins B and C, and 10 times greater than that of cathepsin D. Although some variation among the enzymes was noted, each was distributed in both the supernatant and particulate fractions; the greater proportion of each activity was in the particulate phase.

Influence of pH on Muscle Cathepsins. Figure 2 summarizes the activity of these enzymes as a function of assay pH: optima of 5.0, 4.8, 5.5, and 4.0 were observed for cathepsins A, B, C, and D, respectively.

Table IV. Influence of Cysteine on Catheptic Activities[a]

Cysteine Molarity	Relative Specific Activity[c] Cathepsin B	C[b]	D
0	1.0	1.0	1.0
0.01	...	2.2	...
0.05	2.4	1.9	1.4
0.10	...	1.0	...
0.25	2.3	...	...
0.50	1.6	...	...

[a] Experimental details given in text. [b] Cysteine added as cysteine hydrochloride. [c] Specific activities: 0.20, 0.43, and 8.10 for cathepsin B, C, and D, respectively.

Table V. Influence of Several Compounds on Catheptic Activities[a]

Additive	Relative Specific Activity[b] Cathepsin B	C	D
None	1.0	1.0	1.0
Potassium cyanide	1.4	1.4	0.8
Iodoacetamide	0.5	0.5	0.5
Mercuric chloride	0.8	0.9	0.7

[a] Conc. cyanide and iodoacetamide = 25 mM, conc. mercuric chloride = 2.5 mM. Other details given in text. [b] Specific activities: 0.58, 0.96, and 8.10 for cathepsin B, C, and D, respectively.

Influence of Cysteine on Activities. Increased activities of 2 to 2.5-fold were observed for cathepsins B and C in presence of 0.01 *M* to 0.05 *M* cysteine (Table IV). At higher concentrations, cysteine was less effective. Although purified preparations of cathepsin D reportedly are not activated by thiols, in experiments summarized here, hemoglobin hydrolysis at pH 4.0 increased 40% in presence of 0.05 *M* cysteine.

Some uncertainty remains relative to the effect of thiols on cathepsin A. Iodice *et al.* (1966) observed no cysteine-activation of chicken skeletal muscle cathepsin A; Greenbaum and Sherman (1962) observed an absolute thiol requirement for cathepsin A of beef spleen. In present studies, cysteine-containing samples assayed after 0 and 60 min at 37° C showed an increase in ninhydrin-positive reactants during incubation. However, such increases were nonenzymic in nature, since equivalent changes occurred with enzyme blanks.

Influence of Several Reagents on Enzyme Activities. The effects of potassium cyanide, iodoacetamide, and mercuric chloride on cathepsins B, C, and D are shown in Table V. In general, cathepsins B and C were similarly affected by these reagents: both were activated by cyanide and inhibited by iodoacetamide and mercuric chloride. Cathepsin D activity was inhibited by each of the three reagents.

DISCUSSION

Skeletal muscle has been neglected as a source of lysosomes and lysosomal hydrolases because skeletal muscle is resistant to mechanical disruption, while preparation of lysosome-enriched particulates requires the maintenance of intact subcellular membranes. Skeletal muscle is also poor in lysosomes and perhaps, as in other tissues, a portion of those particles originally present agglutinate with or are adsorbed to the nuclear fraction (Bowers and de Duve, 1967; Pollack and Bird, 1968). Much activity which could be lysosome-bound *in vivo* may either be solubilized by mechanical disruption or sedimented with nuclear proteins during centrifugation. Per-

haps owing to such difficulties, several papers report negligible or low latency of acid hydrolyase activity in skeletal muscle (Buchanan and Schwartz, 1967; Shibko *et al.*, 1963). The strong dependence of extractable activity upon media pH (Table I) is of interest, since earlier reports of low catheptic activity in skeletal muscle may actually reflect incomplete separation or solubilization of catheptic enzymes. Studies reported here demonstrate that the chief difficulties can be obviated by proper selection of homogenization technique and of suspension medium. Postnuclear homogenates, rich in total yield and in subcellular particles, can be obtained. Where other experimental conditions are the same, it seems unlikely that the homogenization medium could greatly influence the number of cells which escape disruption; so greater yields of particulate and total activities at pH 7.2 must reflect a minimized tendency of muscle lysosomes to agglutinate and sediment with nuclear proteins.

Observed pH optima (Figure 2) are in good agreement with literature values for catheptic enzymes (Fruton, 1960; Iodice *et al.*, 1966). Based on acid pH optima and the activation of each cathepsin by Triton X-100 or by freezing and thawing, the particulate fractions obtained were rich in intact lysosomes.

Differences in latency among the four cathepsins (Table III) may reflect either real variations in the ratio of bound to free enzyme *in vivo* or heterogeneity of lysosome populations in skeletal tissue. Bowers and de Duve (1967) report at least two distinct lysosome populations in lymphoid tissue, one of which is rich in cathepsin D but apparently devoid of cathepsins B and C. Stagni and de Bernard (1968) observed different proportions of sedimentable activity for several lysosomal hydrolases of beef skeletal muscle, and individual hydrolases were not activated to the same degree by a given concentration of Triton X-100. Should chicken skeletal muscle contain more than one lysosome population, higher yields of particulate cathepsins A, B, and C may require slight modifications of the techniques employed for maximum yields of bound cathepsin D.

Of the enzymes tested, proteolysis of hemoglobin and other protein substrates by tissue extracts is usually attributed to the action of cathepsin D; several workers also report the hydrolysis of protein substrates by cathepsin B (Ali, 1964; Ali *et al.*, 1967; Greenbaum *et al.*, 1959). Cathepsins A and C are generally said to be peptidases devoid of proteolytic activity (Iodice *et al.*, 1966; Planta *et al.*, 1964); however, McDonald *et al.* (1969) indicate that cathepsin C hydrolyzes protein in presence of chloride ion. Earlier work from this laboratory revealed thiol-activation of autolysis in aqueous extracts of chicken skeletal muscle (Caldwell, 1970); potassium cyanide, mercuric chloride, and iodoacetamide inhibited the reaction. The work suggested the combined action of muscle cathepsins during tissue autolysis. It attributed special importance to the roles of cathepsins B and D, and suggested that cathepsins A, B, and C might function by hydrolyzing further the autolytic products of cathepsin D action. Present studies with synthetic substrates and denatured hemoglobin show the presence of at least four cathepsins in chicken skeletal muscle. The influence of cysteine on hemoglobin hydrolysis (Table IV) also suggests that the thiol-dependent cathepsins B and C augment the hydrolytic action of cathepsin D.

Both mercuric ions and iodoacetamide inhibit cathepsins A, B, or C, but not cathepsin D (Ali *et al.*, 1967; Ali and Evans, 1969; Fruton, 1960; Parrish and Bailey, 1966, 1967). Cyanide is reported to inhibit cathepsin B but to activate cathepsin C (Fruton, 1960; Greenbaum and Fruton, 1957); based on that report, it was hoped to distinguish the contributions of these two enzymes during hemoglobin hydrolysis. On the contrary, cyanide has been observed to activate slightly both cathepsins B and C; so comparisons of their roles could not be made. Inhibition of cathepsin D-catalyzed hemoglobin hydrolysis by ions which inhibit cathepsins A, B, or C implicates them together with cathepsin D during hydrolysis of proteins by crude tissue extracts (Table V).

These studies, together with an earlier paper (Caldwell, 1970), show that tissue cathepsins act jointly during degradation of either exogenous or endogenous protein substrates. Additional efforts are required to purify, characterize, and to define the roles and interrelationships of these enzymes when tissues undergo autolysis.

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LITERATURE CITED

Ali, S. Y., *Biochem. J.* **93,** 611 (1964).
Ali, S. Y., Evans, L., *Biochem. J.* **112,** 427 (1969).
Ali, S. Y., Evans, L., Stainthorpe, E., Lack, C. H., *Biochem. J.* **105,** 549 (1967).
Bowers, W. E., de Duve, C., *J. Cell. Biol.* **32,** 339 (1967).
Buchanan, W. E., Schwartz, T. B., *Amer. J. Physiol.* **212,** 732 (1967).
Caldwell, K. A., J. Agr. Food Chem. **18,** 276 (1970).
de Duve, C., "Ciba Foundation Symposium: Lysosomes," A. V. S. de Reuck and M. P. Cameron, Eds., pp. 1–35, Little, Brown, and Co., Boston, Mass., 1963.
Fruton, J. S., "The Enzymes," Vol. 4, part A, P. D. Boyer, Henry Lardy, and Karl Myrback, Eds., Chapter 11, p. 233, Academic Press, New York, 1960.
Gianetto, R., de Duve, C., *Biochem. J.* **59,** 433 (1955).
Greenbaum, L. M., Fruton, J. S., *J. Biol. Chem.* **226,** 173 (1957).
Greenbaum, L. M., Hirshkowitz, A., Shoichet, I., *J. Biol. Chem.* **234,** 2885 (1959).
Greenbaum, L. M., Sherman, B., *J. Biol. Chem.* **237,** 1082 (1962).
Iodice, A. A., Leong, V., Weinstock, I. M., *Arch. Biochem. Biophys.* **117,** 477 (1966).
Johnson, M. J., *J. Biol. Chem.* **137,** 575 (1941).
Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., *J. Biol. Chem.* **193,** 265 (1951).
McDonald, J. K., Zeitman, B. B., Reilly, T. J., Ellis, S., *J. Biol. Chem.* **244,** 2693 (1969).
Miller, G. L., *Anal. Chem.* **31,** 964 (1959).
Moore, S., Stein, W. H., *J. Biol. Chem.* **211,** 907 (1954).
Parrish, F. C., Jr., Bailey, M. E., J. Agr. Food Chem. **14,** 232 (1966).
Parrish, F. C., Jr., Bailey, M. E., J. Agr. Food Chem. **15,** 88 (1967).
Planta, R. J., Gorter, J., Gruber, M., *Biochim. Biophys. Acta* **89,** 511 (1964).
Pollack, M. S., Bird, J. W. C., *Amer. J. Physiol.* **215,** 716 (1968).
Seligson, D., Seligson, H., *J. Lab. Clin. Med.* **38,** 324 (1951).
Shibko, S., Caldwell, K. A., Sawant, P. L., Tappel, A. L., *J. Cellular Comp. Physiol.* **61,** 85 (1963).
Stagni, N., de Bernard, B., *Biochim. Biophys. Acta* **170,** 129 (1968).

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