Autolytic Activity in Aqueous Extracts of Chicken Skeletal Muscle

K. A. Caldwell

Autolytic changes in extracts of chicken skeletal muscle at 37° C have been investigated by measuring changes in acid soluble tyrosine equivalents. Autolysis is maximal at pH 3. This activity is enhanced by cysteine and by other thiols; it is inhibited substantially by 6-aminocaproic acid, iodoacetamide, cyanide, and mercuric ions; it is inhibited slightly by zinc ions and essentially unaffected by EDTA, ferrous, magnesium, or calcium ions. The inhibitory action of 6-aminocaproic acid is partially reversed by cysteine. The effects of protein concentration, length of assay, and temperature have also been determined. An activation energy of 7.9 kcal per mole was calculated. Data suggest that autolytic activity results from the actions of several cathepsins, especially cathepsins B and D.

Autolytic degradation of major tissue components has been attributed to the action of a group of particulate hydrolases with acid pH optima (de Duve, 1963); the cytoplasmic particles which envelop these enzymes are called lysosomes. The lysosomal enzyme complex includes several cathepsins as well as carbohydrases, lipases, nucleases, and more specialized enzymes. Present evidence indicates that these hydrolases function during both physiologic and pathologic autolysis, but the factors which initiate their action *in vivo* have not been clearly demonstrated. Although hydrolytic enzymes of organ tissue have been the subject of much research, acid hydrolases of skeletal muscle have received little attention. This result is probably owing to the relatively low concentration of lysosomes and/or of autolytic activity in muscle tissue. Even so, some investigators postulate a correlation between autolysis—especially proteolysis—and increased tenderness of muscle during post-mortem aging (Sharp, 1963; Zender *et al.*, 1958). Our laboratory has been investigating conditions which promote tenderization of poultry meat. In the present study, current knowledge of intracellular digestion has been applied to an investigation of some of the factors which influence the enzymatic hydrolysis of endogenous, sarcoplasmic proteins in aqueous extracts of poultry skeletal muscle.

METHODS AND MATERIALS

Preparation of Tissue Extracts. Commercial broilers ranging in weight from 1.4 to 2.6 kg. were slaughtered by exsanguination. Large breast muscles (*Pectoralis superficialis*) and mixed thigh-leg muscles were removed and chilled in ice for less than 45 minutes. Muscles were stripped of visible fat and connective tissue and a 25% homogenate in cold, deionized water was prepared in a laboratory blender; the period of homogenization was 1 minute. Homogenates were kept at 2° C. for about 24 hours. Lysosomal membranes should be completely disrupted by these treatments. Homogenates were then centrifuged at 10,400 G at 0° for 30 minutes and filtered. Extracts were stored at −18° C. in 20-ml. aliquots until used for autolysis experiments. Unused portions were refrozen.

Buffers. The following buffers were used: 0.2*M* glycine hydrochloride–glycine, pH 2 and 3; 0.2*M* acetic acid–sodium acetate, pH 4 to 5.8; 0.2*M* potassium dihydrogen phosphate–disodium hydrogen phosphate, pH 6 and 7.

Western Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, 800 Buchanan St., Albany, Calif. 94710

Assay of Autolytic Activity. Reaction systems usually contained 1 ml. of tissue extract and 1 ml. of buffer. However, when the effect of protein concentration was studied, a final volume of 2 ml. was attained by addition of the requisite amount of buffer, which varied from 0.67 to 1.5 ml. When the effect of an additive was studied, the additive was dissolved in buffer and the pH adjusted, when necessary. Incubations were carried out for 2 to 4 hours at 37° C. unless otherwise indicated. For each incubated sample, a corresponding control was kept at 0°. No measurable proteolysis occurred in control samples, since values for controls maintained at 0° for 1 to 5 hours were essentially equal. Reactions were terminated by addition of 2 ml. of 10% (v/v) perchloric acid. Reaction mixtures were then held at 0° for at least 15 minutes and precipitates removed by filtration through fluted filter papers.

Attempts to measure proteolysis as a function of change in absorbancy at 280 mμ were unsuccessful because of small and erratic changes in optical density. Therefore, analyses of tyrosine equivalents in the filtrates were carried out on 0.2-ml. aliquots according to the method of Lowry *et al.* (1951); nonlinearity between tyrosine equivalents and sample size was observed when aliquots greater than 0.4 ml. were analyzed. The difference in acid-soluble tyrosine equivalents between an incubated sample and its control was taken as the tyrosine equivalents released autolytically. At pH 3, in absence of additives, this difference was approximately 50% greater than control values. Protein concentrations were determined by the method of Lowry *et al.* (1951) as modified by Miller (1959). Specific acitivity is expressed as μmoles tyrosine equivalents released per mg. of protein per minute. In the region of pH 6 to 7 in absence of activators and at other pH values when certain inhibitors are present, the change in optical density is sometimes slightly negative, *e.g.*, −0.005. While these values probably reflect the absence of measurable enzyme activity and are within the experimental error, they are reported here as negative specific activities.

RESULTS

Effect of pH. Figure 1 shows the pH activity curves for autolytic activity in breast and thigh extracts. Maximal autolysis was observed at pH 3.0 for both tissues. As the pH is increased from 3, the reaction rate decreases, such that little or no autolysis is evident at pH 6 or 7. The pH profiles of breast and thigh extracts are qualitatively similar. However, specific activities of thigh extracts were about twice those of breast. Differences in specific activity are attributed to differences in extractable protein. This is probably owing

Table I. Effect of Various Thiols on Muscle Autolysis[a]

Thiol	Relative Specific Activity
Control[b]	100
β-Mercaptoethylamine	224
Cysteine	383
Glutathione	415
2,3-Dimercaptopropanol	527
Dithiothreitol	623

[a] All assays were carried out in 0.2*M* glycine hydrochloride buffer, pH 3.0 in presence of 25m*M* thiol. [b] Specific activity = 1.68.

Table II. Effect of Various Compounds on Muscle Autolysis[a]

Compound	Relative Specific Activity
Control[b]	100
Ferrous ammonium sulfate	103
EDTA	101
Magnesium chloride	94
Calcium chloride	90
Zinc acetate	77
Potassium cyanide	64
Mercuric chloride	24

[a] All reactions were carried out in 0.2*M* glycine hydrochloride buffer, pH 3.0 in presence of 25m*M* additive, except that the concentration of mercuric chloride was 2.5m*M*. [b] Specific activity = 1.77.

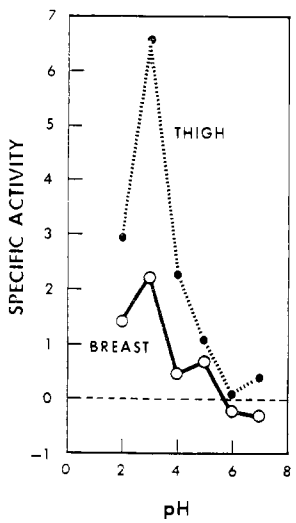

Figure 1. Influence of pH on autolytic activity

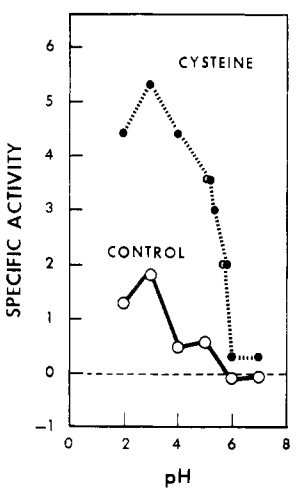

Figure 2. Effect of cysteine on autolytic activity. Concn. CySH, 40m*M*

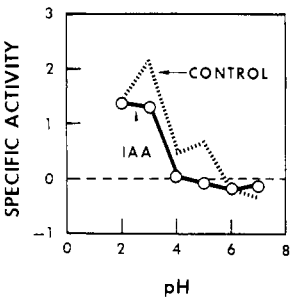

Figure 3. Effect of iodoacetamide on autolytic activity. Concn. IAA, 25m*M*

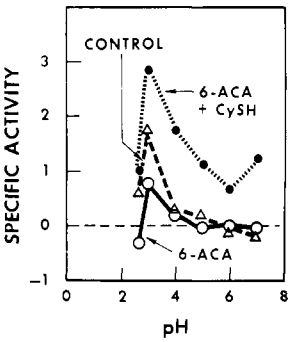

Figure 4. Effect of 6-aminocaproic acid on autolytic activity. Concn. 6-ACA, 0.4*M*; concn. CySH, 0.02*M*

to the larger content of lipids and connective tissue in thigh muscle, which appears to decrease the total extractable protein from thigh muscle without appreciably influencing the extraction of proteolytic activity. This conclusion is based on observations that the rate of release of tyrosine equivalents from the two extracts was essentially equal but protein concentrations of breast extracts were about twice those of thigh. In all other respects, responses of breast and thigh enzymes were similar; literature reports more frequently concern enzymic activities and other properties of breast muscle rather than those of thigh. Accordingly, subsequent data describe results obtained with breast extracts only.

Either physiologic or pathologic autolysis would be expected to occur in the range pH 5 to 7; therefore, the influence of various activators or inhibitors on reaction rates was investigated. This was done with the view both to determine the nature of enzymes involved and to assess the possible significance of such activity in the region of physiological pH.

Effect of Cysteine and Other Thiols. Cathepsins A, B, and C have pH optima in the range pH 5 to 7 (Fruton, 1960). The latter two enzymes also require sulfhydryl activators for maximum activity. Accordingly, the effect of cysteine on reaction rates was tested. Data show (Figure 2) that in presence of 40m*M* cysteine, reaction rates are increased throughout the range pH 2 to 7. Largest relative increases were observed above pH 4, where the original activity was either low or negligible: a three- and a sixfold increase in activity were observed at pH 3 and pH 5, respectively. At pH 5.8 the increase in relative activity was infinite, owing to the lack of measurable activity in absence of cysteine.

All tested thiols enhanced autolytic activity, but the increase varied from two- to sixfold (Table I). The effect of cysteine, which was used in many of these experiments, was intermediate to that of the most active and the least active of the tested thiols.

Effect of Iodoacetamide. Iodoacetamide is reported to be a potent inhibitor of both cathepsins B and C during the hydrolysis of synthetic substrates (Fruton, 1960). In presence of 25m*M* iodoacetamide, autolysis at pH 3 was reduced to 60% control values and autolysis at pH 4 and 5 was completely abolished (Figure 3).

Effect of 6-Aminocaproic Acid. Cathepsin B acts selectively on α-*N*-acylated esters or amides of L-arginine or L-lysine. It is inhibited by 6-aminocaproic acid, which is a structural analog of arginine (Ali *et al.*, 1967). Data of Figure 4 show that 6-aminocaproic acid inhibits autolysis throughout the acid pH range. At pH 3, activity is reduced to about 45% control values. If the reaction is repeated in presence of 20-m*M* cysteine, inhibition by 6-aminocaproic acid is at least partially counteracted. At pH 3, for example, the cysteine-activated enzyme retains at least 80% its original activity in presence of 6-aminocaproic acid.

Effect of Various Compounds. Many compounds are reported to inhibit or activate the protease or peptidase action of various cathepsins. The effects of some of these chemicals on muscle autolysis are summarized in Table II. EDTA, ferrous, magnesium, or calcium ions had little or no effect on the reaction rate, but zinc ions reduced the activity to 77% control values. The most potent inhibitors of this group are potassium cyanide and mercuric chloride, which decreased autolysis to 64 and 24% of control values, respectively.

Effect of Time, Temperature, and Protein Concentration. The enzymatic nature of these reactions was demonstrated by

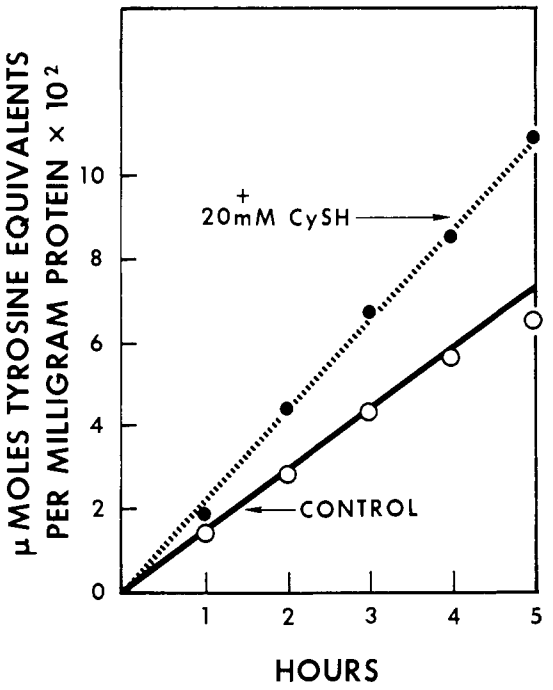

Figure 5. Effect of protein concentration on autolytic activity. All assays carried out at pH 3 in presence of 20 m*M* CySH

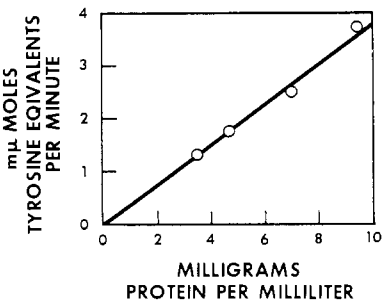

Figure 6. Time course of liberation of acid soluble tyrosine equivalents. Concn. CySH, 20m*M*

several experiments. pH-Dependence has already been shown in Figure 1. Figures 5 and 6 show that production of acid soluble tyrosine equivalents is linear with respect to assay time and that the specific activity is independent of protein concentration. Since muscle extracts provided both enzymatic and substrate protein, the difficulty of testing a wide range of protein concentrations is evident. Linearity with respect to time was observed both in presence and absence of cysteine.

The temperature-dependence of these reactions was demonstrated in samples which were incubated for two hours. Under these conditions, the cysteine-activated enzymes reveal a temperature optimum of 45° C. Autolytic enzymes were inactivated above 45° C. Temperature effects described here were shown to obey the Arrhenius relationship, from which an activation energy of 7.9 kcal per mole was calculated (Figure 7).

DISCUSSION

Comparison of the properties of the five known cathepsins with observations reported here implicates several of these enzymes in muscle autolysis. The cathepsins are usually isolated from organ tissue and have been characterized primarily as to pH optimum, action on synthetic substrates, and response to specific activators or inhibitors. Cathepsins A, B, and C all have pH optima in the range pH 5 to 7, but differ in their actions on synthetic peptides and in their response to specific activators and inhibitors (Fruton, 1960). Cathepsin D is optimally active at pH 3 to 4 against hemoglobin or serum albumin, but does not hydrolyze the synthetic peptides used to characterize cathepsins A, B, or C (Press *et al.*, 1960). Cathepsin E is similar to cathepsin D, but is maximally active against serum albumin at pH 2.5 (Lapresle and Webb, 1962).

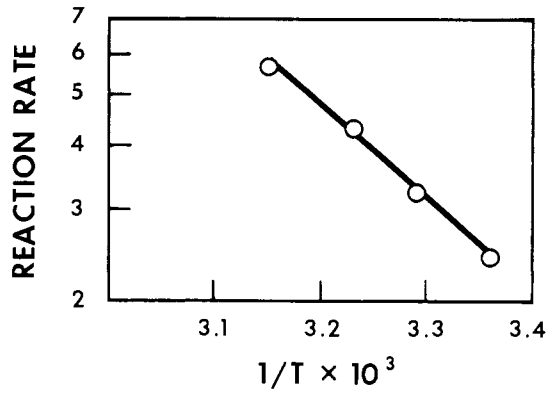

Figure 7. Arrhenius plot of autolytic activity between 25° and 45° C

Iodice *et al.* (1966b) report maximum autolysis of endogenous rabbit or chicken muscle proteins at pH 4.1 to 4.3. Moreover, addition of purified preparations of cathepsin D to autolyzing systems increased the reaction rate. Additionally, these workers observed negligible hydrolysis of denatured hemoglobin by a purified preparation of cathepsin A in absence of cathepsin D; but the combined action of cathepsins A and D was more than additive (Iodice *et al.*, 1966a). The restricted hydrolytic action by cathepsin A was attributed to its requirement for small proteins or peptides as substrates, which were provided by the initial action of cathepsin D. Similarly, Kozalka and Miller (1960) report a rat skeletal muscle enzyme which degrades endogenous muscle proteins maximally at pH 8.5 to 9.0 and less effectively at pH 3.5 to 4.0. Parrish and Bailey (1966, 1967) have shown that both bovine and porcine muscle cathepsins hydrolyze denatured hemoglobin optimally at pH 4 and at pH 8 to 10, with maximal activity at pH 4.

Based on these and similar reports, the optimum observed here at pH 3 results primarily from the action of cathepsin D. However, neither cathepsin D nor cathepsin A is influenced by sulfhydryl compounds. Accordingly, enhancement of autolytic reactions by thiols probably reflects activities of cathepsins B and C. Greenbaum *et al.* (1959) have shown that partially purified preparations of cathepsin B hydrolyze proteins, whereas other workers (Planta *et al.*, 1964) report cathepsin C to be devoid of proteolytic activity. Moreover, Ali (1964, 1967) and Ali *et al.* (1967) have implicated cathepsin B in the autolytic degradation of cartilage matrix. They report the release of a chondromucoprotein during cartilage autolysis. They suggest the presence of sufficient reducing substances in cartilage matrix to maintain cathepsin B in its reduced or fully active state, whereas additional reducing agents are required if experiments are carried out in dilute aqueous media (Ali *et al.*, 1967).

Both cathepsin B and C are activated by thiols and inhibited by iodoacetamide and mercuric ions; but whereas cathespin C is activated by cyanide, cathepsin B is inhibited by this ion

(Fruton, 1960). Further, 6-aminocaproic acid is a structurally specific inhibitor of cathepsin B (Ali *et al.*, 1967). Results of these experiments suggest that much of the observed autolytic activity can be attributed to cathepsin B. Data presented here do not preclude participation of any known cathepsin in muscle autolysis. Rather, the combined action of several cathepsins, some of which are thiol dependent, is indicated. Cathepsins B and D are especially active. The significance of this work lies in the demonstration of considerable proteolytic activity against endogenous muscle proteins in the range of physiologic pH in presence of sulfhydryl protectors. Like the cathepsins of cartilage matrix, the activity of muscle cathepsins may be higher than previously reported, provided that adequate concentrations of co-factors are maintained. The maintenance of these critical sulfhydryl groups in the reduced state would likely be true for normal muscle *in vivo*. Additional studies are required to define more clearly the role of individual acid cathepsins in the autolytic degradation of muscle proteins and to identify their endogenous substrates.

ACKNOWLEDGMENT

I acknowledge with thanks the technical assistance of I. V. Streeter and O. K. Grosjean.

LITERATURE CITED

Ali, S. Y., *Biochem. J.* **93,** 611 (1964).
Ali, S. Y., *Biochem. J.* **102,** 10c (1967).
Ali, S. Y., Evans, L., Stainthorpe, E., Lack, C. H., *Biochem. J.* **105,** 549 (1967).
de Duve, C., "Lysosomes," A. V. S. de Reuck and M. P. Cameron, Eds., pp. 1–35, Little, Brown, Boston, 1963.
Fruton, J. S., "The Enzymes," P. D. Boyer, H. Lardy, and K. Myrback, Eds., Vol. 4, part A, Chapter 11, p. 233, Academic Press, New York, 1960.
Greenbaum, L. M., Hirshkowitz, A., Shoichet, I., *J. Biol. Chem.* **234,** 2885 (1959).
Iodice, A. A., Leong, V., Weinstock, I. M., *Arch. Biochem. Biophys.* **117,** 477 (1966a).
Iodice, A. A., Leong, V., Weinstock, I. M., *Enzymol. Biol. Clin.* **6,** 269 (1966b).
Kozalka, T. R., Miller, L. L., *J. Biol. Chem.* **235,** 665 (1960).
Lapresle, C., Webb, T., *Biochem. J.* **84,** 455 (1962).
Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., *J. Biol. Chem.* **193,** 265 (1951).
Miller, G. L., *Anal. Chem.* **31,** 964 (1959).
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).
Press, E. M., Proter, R. R., Cebra, J., *Biochem. J.* **74,** 501 (1960).
Sharp, J. G., *J. Sci. Food Agr.* **14,** 468 (1963).
Zender, R., Lataste-Dorolle, C., Collet, R. A., Rowinski, P., Moulton, R. F., *J. Food Sci.* **23,** 305 (1958).

Received for review August 18, 1969. Accepted December 3, 1969.