Proteolytic Activity of Rat Skeletal Muscle

I. EVIDENCE FOR THE EXISTENCE OF AN ENZYME ACTIVE OPTIMALLY AT pH 8.5 to 9.0*

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A considerable number of intracellular animal proteases have been described (1, 2). Many of the enzymes studied exhibit pH optima in the acid range, whereas some show optimal activity at or near neutrality. Relatively few of the cathepsins described to date show maximal activity in the alkaline pH range. One such protease, found in aqueous extracts of whole rat lung, exhibits a pH optimum at 8.4 for the hydrolysis of urea-denatured hemoglobin (3). Another enzyme, found in erythrocytes, attacks hemoglobin optimally at pH 8 (4).

Smith (5) has reported that aqueous extracts of rat skeletal muscle show little or no proteolytic activity against the endogenous muscle proteins of the extract in a pH range of 4.0 to 8.0. In the studies reported in this paper, it will be shown that rat skeletal muscle homogenates do, in fact, contain a proteolytic enzyme which readily attacks the endogenous muscle proteins at an optimal pH of 8.5 to 9.0. A preliminary account of this work has already been published (6).

EXPERIMENTAL

Preparation of Homogenates—Adult Sprague-Dawley rats were killed by exsanguination. A homogenate of skeletal muscle from the hind limbs was prepared by the method of Snoke and Neuring (7). After being kept at 4° for about 18 hours, the muscle homogenate was filtered through two layers of surgical gauze to remove connective tissue. This preparation was used in all experiments.

Assay of Autolytic Activity—One volume of muscle homogenate was mixed in an ice bath with 2 volumes of either dilute hydrochloric acid or potassium hydroxide of appropriate strength to achieve the pH desired in a given experiment.

The reaction mixtures were preincubated at 37° for 5 minutes to attain constant temperature. Then 2-ml aliquots, taken at zero time and after 1 hour of incubation, were pipetted into centrifuge tubes containing 5 ml of 5% trichloroacetic acid. The pH of the test systems, determined electrometrically before and after incubation, was found to vary less than 0.2 unit. After standing for 1 hour at room temperature, the trichloroacetic acid-treated mixtures were centrifuged and the clear supernatant solutions decanted and saved for analysis. The optical density of the supernatant solutions was determined in a Beckman DU spectrophotometer at 280 mp. Alternatively, acid-soluble peptides were measured after reaction of the supernatant solutions with the Folin-Ciocalteu reagent (8).

Autolytic activity was expressed as the optical density of the complete system incubated for 1 hour minus that of the corresponding control at time zero. The analyses were always performed in duplicate.

Assay of Proteolytic Activity with Hemoglobin—A urea-denatured hemoglobin (Fisher Scientific Company) solution, used as substrate for the assay of proteolytic activity, was diluted to 2.5%, as estimated by dry weight, and frozen until use.

The procedure used for the determination of proteolytic activity was essentially that of Kunitz (9). To 5 ml of a 2.5% aqueous solution of urea-denatured hemoglobin, previously adjusted to pH 9.0 with concentrated KOH, were added 5 ml of muscle homogenate and enough 0.1 M KOH to make the pH of the mixture 9.0. The final incubation mixture was diluted to 15 ml with H2O. Appropriate control incubation systems were prepared in the same manner except that water was substituted for hemoglobin. Other control incubation systems contained 1 volume each of hemoglobin, dilute base, and distilled water in place of the muscle homogenate. The reaction mixtures were preincubated at 37° for 5 minutes to attain constant temperature. Then 2-ml aliquots withdrawn from the reaction mixtures at the start and also after 1 hour of incubation, were pipetted into each of two test tubes containing 5.0 ml of 5% trichloroacetic acid. The final steps of the assay were carried out as described previously for the determination of autolytic activity.

Determination of a-Amino Nitrogen Released during Incubation of Muscle Homogenate—Skeletal muscle homogenate, 200 ml, was dialyzed with continuous agitation against two changes of 4 liters of 2% KCl at 4° for a total of 18 hours. The volume of the muscle homogenate did not change measurably during the course of the dialysis procedure. The dialyzed rat muscle homogenates, which were used in all subsequent experiments, contained 20 to 30 mg of protein per ml as determined by the method of Lowry et al. (10).

Dialyzed muscle homogenate, 25 ml, was adjusted to pH 9.0 with 0.1 M KOH and diluted to 50 ml with H2O. A second 25-ml portion of the homogenate was adjusted to pH 12.3 with 1 M KOH and allowed to stand at room temperature for 15 minutes; this aliquot was then adjusted to pH 9.3 with 1 M HCl and diluted to 50 ml with H2O.

Immediately thereafter, both test systems were incubated at
Paper Chromatography of Alcohol-soluble Products of Autolysis—For the paper chromatographic studies, 1-ml aliquots of the previously described incubation mixtures were withdrawn at 0, 15, 30, 45, 60, and 90 minutes and mixed with 8 ml of 80% ethanol. The precipitate was removed by centrifugation, and 0.2-ml aliquots of the alcoholic supernatant solutions were spotted on Whatman No. 1 paper (12 × 44 cm). The papers were chromatographed in an n-butanol-acetic acid-water (4:1:1, by volume) solvent system for 18 hours. After drying, the papers were sprayed with 0.5% anilinonaphthol solution of ninhydrin and allowed to develop at room temperature.

Temperature Coefficient of Reaction—A dialyzed muscle homogenate, 60 ml, was carefully adjusted to pH 9.0 with 0.1 M KOH. Then 50 ml of this preparation were mixed with an equal volume of 0.05 M borate buffer, pH 9.0. Four aliquots were incubated at 22°, 37°, 42°, and 47°, respectively. The pH of the incubation mixtures did not fall more than 0.1 unit after 40 minutes’ incubation. After 0, 10, 20, 30, and 40 minutes of incubation, 2-ml aliquots were removed from the test systems and mixed with 5 ml of 5% trichloroacetic acid solution. After standing at room temperature for 1 hour, the precipitates were removed by centrifugation and the optical density of the supernatant solutions was determined at 280 mμ.

RESULTS AND DISCUSSION

Effect of pH of Incubation Mixture on Release of Substances Absorbing at 280 mμ and at 700 mμ after Reaction with Folin-Ciocalteu Reagent—Fig. 1 shows the effect of pH on the autolysis of a crude skeletal muscle homogenate. The ordinate represents the increase in optical density at 280 mμ or 700 mμ for an incubation time of 1 hour. It is clear that the muscle preparation exhibited maximal autolysis at pH 3.5 to 4.0 and at pH 8.5 to 9.0. The small amount of activity observed at pH 9.0 in the alkaline range was quite unexpected in view of the results of Smith (5). The finding that the optical density increments measured at 280 and 700 mμ increased in parallel in the alkaline range is evidence that acid-soluble substances containing tyrosine and tryptophane are liberated during the incubation of skeletal muscle homogenates. Presumably the release of these substances at pH 9.0 was due to the action of proteases on the endogenous proteins of the skeletal muscle homogenate. It is unlikely that the release of the chromogenic substances was a result of non-enzymatic chemical hydrolysis of proteins by alkali, since the reaction exhibited a true pH optimum and was almost completely and irreversibly inhibited in either strongly acidic or basic solution.

In another experiment, a muscle homogenate was extensively dialyzed against 2% KCl in order to remove small molecular weight compounds, i.e., peptides containing aromatic amino acids which might serve as substrates for peptidases or other muscle enzymes. The pH-dependence curve for the autolysis of the dialyzed muscle homogenate was similar to that for the undialyzed homogenate. Furthermore, the “autolytic activity” of a dialyzed muscle preparation incubated at pH 8.7 was, by ultraviolet method, 92% of, or, by Folin-Ciocalteu method, equal to

1 T. R. Koszalka, unpublished observations.
that of an undialyzed preparation. These findings are compatible with the hypothesis that high molecular weight nondialyzable substances serve as substrates for the observed reaction. Heating the dialyzed muscle homogenate at 70° for 10 minutes or treating it with strong acid or base destroyed nearly all of the autolytic activity at pH 9.0 as measured by both methods of analysis, an observation which lends support to the view that the reaction is enzymatic in nature.

Nature of 280 mp Absorbing Materials—The optical density increment has been plotted as a function of wavelength (Fig. 2) for a test system containing undialyzed muscle homogenate which has been incubated at pH 8.6 for 1 hour. The curve exhibits an absorption maximum at 275 mp. The optical density increment at 260 mp is less than one-half of the value found at 275 mp or at 280 mp. Since acid-soluble nucleic acid derivatives generally show absorption maxima near 260 mp (12), these findings indicate that such purine- or pyrimidine-containing substances were not responsible for the “activity” as measured by the ultraviolet method of analysis. On the other hand, the data support the view that aromatic amino acids or their derivatives are liberated from muscle homogenates incubated at pH 8.5 to 9.0. It seems highly unlikely that the enzymatic hydrolysis of endogenous nucleic acids could contribute significantly to the apparent proteolytic activity, since rat skeletal muscle contains only 25 µg of RNA-phosphorus and 5 µg of DNA-phosphorus per 100 mg of fresh tissue (13).

Release of α-Amino Nitrogen during Incubation of Dialyzed Muscle Homogenate at pH 9.0—If the reaction observed upon incubating a muscle homogenate at pH 9.0 is proteolytic in nature, then it should produce an increase in the number of titratable carboxyl and α-amino groups as a result of the splitting of peptide bonds. Consequently the α-amino nitrogen level of dialyzed muscle homogenates incubated at pH 9.0 was studied as a function of time of incubation. In addition, unidimensional paper chromatography was used to identify the ninhydrin-reactive products of the reaction.

The results given in Fig. 3 show that incubation of a dialyzed muscle homogenate at pH 9.0 produced an increase in nonprotein α-amino nitrogen proportional to the time of incubation. In contrast, the alkali-“denatured” dialyzed muscle homogenate incubated at pH 9.0 showed no increase in α-amino nitrogen with time. These results are compatible with and lend support to the view that muscle homogenates undergo proteolysis when incubated at pH 9.0 at 37°. The finding that the exposure of the muscle homogenate to strong alkali completely inhibited the increase of α-amino nitrogen upon incubation at pH 9.0 is further evidence that the reaction is of an enzymatic nature.

The results of the paper chromatography experiments (Fig. 4) also attest to the proteolytic nature of the reaction. Examina-

Fig. 3. The production of ninhydrin reactive substances by incubation of a dialyzed muscle homogenate at 37°, pH 9.0. Untreated system, O; homogenate adjusted to pH 12.3 with 1 M KOH and then readjusted to pH 9.3 with 1 M HCl, X.

Fig. 4. Chromatogram of amino acids and peptides detected in the alcoholic supernatant obtained after treating with ethanol a dialyzed muscle homogenate incubated at 37°, pH 9.0. Incubations were carried out for 0, 15, 30, 45, 60, and 90 minutes. 200-µl aliquots of an ethanolic supernatant from the reaction mixture were spotted on paper.

Assuming that the average molecular weight of proteins in muscle is 5 X 10^4, a calculation from the data in Fig. 3 reveals that approximately three peptide bonds are cleaved per hour per molecule of protein. This is a minimum estimate which would be increased by any factor restricting the proteolytic action to a particular protein fraction.
Use of Urea-denatured Hemoglobin as Substrate for Assay of Muscle Proteolytic Activity—The data in Table I show that although there was no measurable nonenzymatic hydrolysis at pH 9.0 in the control containing hemoglobin alone, there was a large significant increase in the optical density of trichloroacetic acid supernatant solution of the control containing only muscle homogenate. In fact, the optical density increment of the complete test system was equal to that of the second control. The indications are, therefore, that the addition of hemoglobin resulted in no further net increase in proteolysis, and that the endogenous muscle protein affords enough substrate to saturate the enzyme in the absence of hemoglobin. The finding that the autolytic reaction follows apparent zero order kinetics for at least 30 minutes at 37°C is in accord with the latter assumption.

SUMMARY

Rat skeletal muscle homogenates prepared in 2% KCl and incubated at 37°C exhibited minimal autolysis at pH 3.5 to 4.0. Maximal autolysis was observed at pH 8.5 to 9.0 as measured by the release of trichloroacetic acid-soluble substances which absorb light at 280 μm.

Several lines of evidence supported the view that the observed reaction at pH 8.5 to 9.0 is enzymatic and proteolytic in nature. This evidence includes (a) the ease of inactivation by heat, acid, and alkali; (b) a temperature coefficient of 1.85 to 2.0 for the reaction; (c) the spectral studies of the trichloroacetic acid-soluble substances released during the reaction and the effect of pH on the reaction; (d) the release of ninhydrin-reactive nitrogen during the reaction at pH 9.0; and (e) the failure of dialysis of muscle preparation to affect autolysis.

The addition of denatured hemoglobin to the muscle preparation did not enhance the activity observed at pH 9.0. This finding indicates that the native muscle protein affords enough substrate to saturate the enzyme when no hemoglobin is added.

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