Over-All Assay and Partial Purification Procedures for Proteolytic Enzymes in Beef Muscle

R. A. SLIWINSKI, D. M. DOTY, and W. A. LANDMANN

American Meat Institute Foundation and Department of Biochemistry, University of Chicago, Chicago, Ill.

To clarify the nature of muscle proteases, an attempt was made to concentrate and purify the proteolytic enzymes in beef muscle. Purification of enzyme fractions was achieved by the preliminary use of ammonium sulfate, followed by dialysis and chromatography on diethylaminoethanol-modified cellulose. A purified enzyme preparation having an 18-fold increase in specific activity, based on nitrogen content, was obtained. An over-all assay procedure for screening fractions was developed, employing hemoglobin in 3M urea as a substrate. The assay reaction was carried out at pH 4.4 at 37° C. for 4 hours, and the extent of proteolytic action was determined from the amount of tyrosine produced in the protein-free supernatant.

THE PROTEOLYTIC ACTIVITY of muscle tissue, measured either by autolysis or hydrolysis of other protein substrates, is markedly lower than that of organ tissues such as liver, spleen, and kidney. However, it is of considerable importance, because of the suggested role of the proteolytic enzymes in meat tenderization, preservation, and flavor (*4*, *5*).

As the work of Balls (*2*) and Zender *et al.* (*12*) suggests that the specificity and concentration of muscle proteolytic enzymes are different from those of the cathepsins of liver, spleen, and kidney, the present study was undertaken to help clarify the nature of muscle proteinases. A sensitive assay procedure has been developed for determining the proteolytic activity of beef muscle, meat extracts, and purified enzyme fractions. Partial purification of the enzyme was achieved by fractionating meat extract with ammonium sulfate and diethylaminoethanol cellulose.

Materials and Procedures

Preliminary Extraction of Enzymes. Beef muscle tissue (usually round or chuck, U. S. Choice Grade, obtained from a local retail market) was freed from all separable fat and connective tissue and ground through a grinder with plates having holes $^1/_{16}$ inch in diameter. To 200 grams of the finely ground, well mixed muscle tissue, 400 grams of deionized water were added, and the slurry was homogenized in a Waring Blendor for 3 minutes. One milliliter of preservative (*7*) consisting of 1 part of fluorotoluene, 1 part of ethylene chloride, and 2 parts of butyl chloride, was added to the slurry, and the mixture was allowed to autolyze for 1 hour at room temperature with continuous stirring. The slurry was then centrifuged, the clear red supernatant was collected, and the residue was re-extracted with 200 grams of water, as described above. The combined supernatants subsequently used as the enzyme source were dialyzed against deionized water for 24 hours, and then adjusted with 1*N* hydrochloric acid to the desired pH of the assay mixture. Approximately 85% of the proteolytic activity of muscle tissue homogenate, as determined by the hemoglobin digestion procedure described subsequently, was extracted at pH 5.6, the original pH of meat.

Hemoglobin Substrate. Previous work by Doty and Wachter (*3*) employed a method of assay based on the liberation of tyrosine from casein as an index of activity. Casein, at the acid pH conditions of optimal activity of muscle proteinases, is relatively insoluble, so that for maximum activity, all test suspensions had to be stirred or shaken continuously during the 24-hour incubation period. In the work reported here, hemoglobin was chosen as the substrate, because of its complete solubility in acidic solutions, nonspecificity as a substrate for the assay of over-all proteolysis, and unlike casein or gelatin, hemoglobin is a reproducible substrate. A 10% stock hemoglobin solution in water was prepared from the readily soluble hemoglobin powder (Pentex Corp., Kankakee, Ill.). The stock hemoglobin solution was dialyzed overnight against running tap water followed by an 8-hour dialysis against deionized water. Portions of this hemoglobin solution, after treatment with the various denaturants were diluted so that the final concentration of each was 2% of hemoglobin in 0.2*M* sodium acetate–hydrochloric acid buffer at pH 4.4.

Denaturation of Hemoglobin Substrate. During the development of the assay procedure, optimal enzyme activity was not obtained when using the hemoglobin substrate as described by Anson (*1*). Various methods for denaturing hemoglobin were investigated, because there is evidence that all proteins are digested by proteolytic enzymes via their denatured form. Urea was chosen as a chemical denaturant, and ultrasonic vibrations and gamma radiation were used as physical means for denaturation.

Hemoglobin denatured with 6*M* urea, prior to adjusting the pH to 4.4, was tried, but the proteolytic activity of a muscle extract was much lower than that obtained when employing Anson's hemoglobin substrate which did not contain urea. Obviously, the urea was denaturing the enzyme as well as the hemoglobin. Studies using a series of urea concentrations showed that it was possible to establish conditions at which the proteases appeared to suffer little or no denaturation, while the substrate behaved as though it were maximally denatured. For these studies, portions of the stock hemoglobin solution were denatured by bringing the urea concentration to 6.0, 3.0, or 1.5*M*. The solutions were stirred in the presence of the sodium acetate buffer for 1 hour at room temperature, after which the pH was adjusted to 4.4 with dilute hydrochloric acid.

A portion of the stock solution was also subjected to ultrasonic vibrations using a 10-kc. instrument for 2 hours. The buffer was then added and the pH was

adjusted to 4.4 with dilute hydrochloric acid.

For studies involving hemoglobin denatured by irradiation, a 10-gram sample of dry hemoglobin powder was subjected to gamma irradiation from a cobalt-60 source until it received a dosage of 10 megarads. The irradiated material was then dissolved in the proper amount of water to give the same concentration as used in the other solutions, and buffered as indicated previously.

For the comparison of these substrates, 2 ml. of a water extract of beef muscle (enzyme source) were added to 2 ml. of each of the various hemoglobin substrates. The preservative (0.1 ml.) previously described was used to prevent microbial growth in all test mixtures during incubation. The reaction mixtures were incubated for 4.0 hours at 37° C. After incubation, 4 ml. of 10% trichloroacetic acid were added to precipitate the undigested protein and stop the enzyme reaction. The precipitates formed were centrifuged after standing overnight. The concentration of the unprecipitated protein fragments, taken as a measure of the proteolytic activity, was determined as tyrosine by measuring the absorbance of the solutions at 276 mμ using a Beckman Model DU spectrophotometer with a photomultiplier attachment.

The results of these studies (Table I) indicate that the maximum proteolytic enzyme response was obtained if the hemoglobin substrate was denatured with 3*M* urea. Hemoglobin denatured by physical means, such as ultrasonic vibrations and gamma irradiation, showed no increase in its ability to be digested by the muscle cathepsins. Wu (*11*) advanced the theory that denaturation consists of an alteration of the specific tertiary structure of the protein molecule, wherein the closely folded peptide chains unfold. The type of alteration caused by urea or acid would hardly be the same as that produced by ultrasonic vibrations or gamma irradiation. The data in Table I support this view. They also indicate that urea is capable of changing the hemoglobin molecule so that it is more susceptible to proteolytic digestion, and that by controlling the concentration of urea the enzyme itself is not inactivated.

Absorption Spectra of Tyrosine and Acid-Soluble Fragments of Enzymatic Reaction. Holiday (*6*) reported the maximum absorption spectrum of tyrosine and tryptophan at 2800 A. Recently Wetlaufer, Edsall, and Hollingworth (*10*) showed that the maximum absorption of tyrosine at pH 6 is 2745 A. The absorption spectra of tyrosine—122 γ per ml.—and of the clear supernatant liquid from a sample of the reaction mixture were determined against a 5% trichloroacetic acid blank using a Beckman recording spectrophotometer Model DK 2 with a 10-mm. quartz cell. Figure 1 shows the relationship between the absorbance of the solutions and wave length and indicates the maximum absorption at 2760 A. of both pure tyrosine and the acid-soluble products of the enzyme digest, in 5% trichloroacetic acid. A standard curve was prepared by plotting the tyrosine concentration *vs.* the absorbance at 2760 A. The relationship was linear over a range of 40 to 1120 γ of tyrosine.

Influence of Time, Temperature, and pH on Proteinase Activity. Studies were undertaken to obtain information regarding the optimal conditions of reaction of muscle proteolytic enzymes with respect to temperature, pH, reaction time, etc. These studies were carried out by using 3*M* urea denatured hemoglobin as the substrate and the water extract of muscle tissue, as previously described, as the enzyme source. By using a 1 to 1 mixture of enzymes and substrate the final concentration of hemoglobin in the reaction mixture was 1% in 0.1*M* acetate–hydrochloric acid buffer.

A series of enzyme activity tests was made employing three different dilutions of the enzyme solution 1 to 0, 2 to 1, and 1 to 2 with water (Figure 2). Each reaction was run at pH's varying from 3.5 to 5.5, at temperatures of 25°, 37°, 45°, and 53° C., for periods of incubation ranging from 30 minutes to 24 hours. Tests made at the extremes of these experimental conditions with respect to temperature, pH, and reaction times, showed decidedly low activity and therefore are not reported here.

Studies at 37° C. indicated that as the reaction time increased the optimal pH also increased (Figure 3). Concentration, reaction rate, or stability differences of the enzymes may contribute to the optimal pH shift with increased reaction time. Several proteolytic enzymes may be present, each having a different pH optimum. If the more heat-labile enzymes have a lower pH optimum, then the longer the incubation period, the more inactivation there is of the heat-labile enzymes. With these enzymes no longer functioning, the increased pH optimum of the heat-stable enzymes becomes apparent as a shift in the curve.

Table I. Effect of Substrate Denaturation on Enzyme Activity

(1% hemoglobin, 4 hours at 37° C., pH 4.4)

Hemoglobin Substrate Treatment	Activity of Tyrosine Liberated, γ
Untreated	208
6*M* urea	64
3*M* urea	520
1.5*M* urea	304
Ultrasonic vibrations	216
Gamma irradiation	184

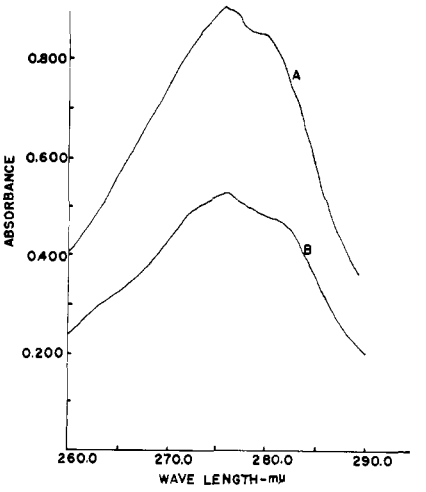

Figure 1. Absorption spectra of crystalline tyrosine and acid-soluble tyrosine products of enzyme digest

A. Recrystallized tyrosine, 122 γ/ml. in 0.3*M* trichloroacetic acid
B. Acid-soluble products in 0.3*M* trichloroacetic acid from enzyme digest

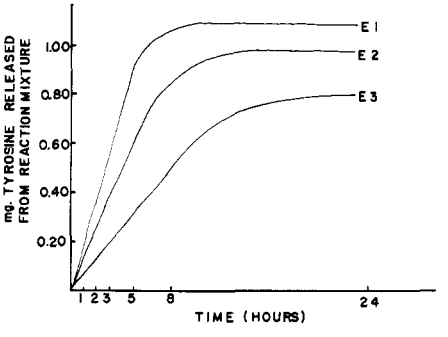

Figure 2. Relationship of proteolytic activity *vs.* time at pH 4.4, and 37° C. for three different dilutions

E-1. Original extract
E-2. Original extract diluted 2 to 1 with water
E-3. Original extract diluted 1 to 2 with water

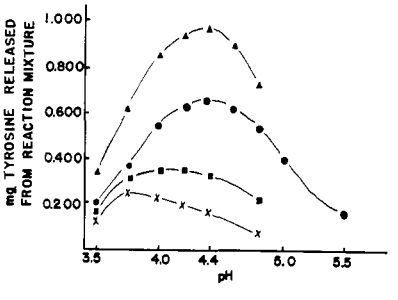

Figure 3. The effect of pH on proteolytic activity at 37° C. for various periods of incubation

▲ 24 hours
● 4 hours
■ 2.5 hours
X 1 hour

Table II. Effect of Dialysis upon Enzyme Activity of Extracts

Extracting pH	Treatment	Absorbance of Enzyme Digest Supernatant			Activity Tyrosine Liberated, γ
		Blanks	Exptl.	Difference	
4.1[a]	Undialyzed	0.845	1.190	0.345	376
	Dialyzed	0.290	0.770	0.480	520
5.6	Undialyzed	1.055	1.490	0.435	472
	Dialyzed	0.300	0.890	0.590	640
7.1	Undialyzed	1.070	1.520	0.450	488
	Dialyzed	0.320	0.920	0.600	648
8.0[a]	Undialyzed	1.030	1.410	0.380	408
	Dialyzed	0.315	0.815	0.500	540

[a] The meat slurry gelled slightly at these extracting pH's, so that the separation of the aqueous phase from the residue, either by filtration or centrifugation, was hindered somewhat, hence the extractions may not have been efficient.

Table III. Activity of Proteolytic Fractions Obtained during Purification

	Tyrosine Liberated, γ	Specific Activity[a], γ	Purification Factor
Water extract of beef muscle	268	2.67	
Ammonium sulfate fraction	440	10.00	3.7
Active fraction from DEAE cellulose column	640	48.75	18.2

[a] γ of tyrosine liberated per mg. of protein N_2 per 4 hours.

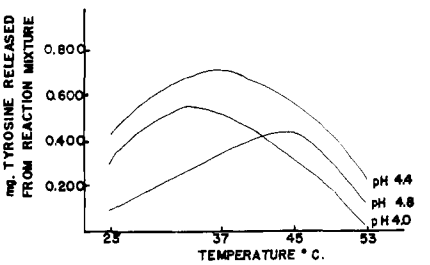

Figure 4. Effect of temperature on proteolytic activity at various pH's during a 4-hour incubation

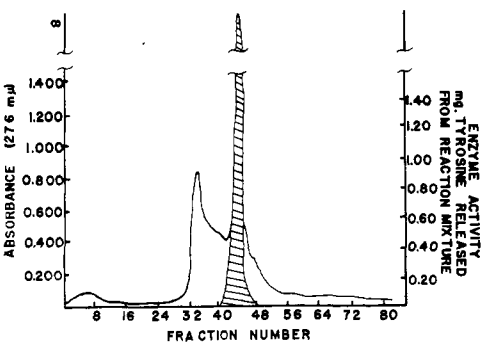

Figure 5. Effluent diagram of ammonium sulfate fractionated beef muscle proteins on DEAE cellulose anion exchanger.

Shaded area represents portion of proteolytic activity in protein fractions. Fraction volume, 4 ml.

Optimal temperature differences at various pH's also indicate that more than one enzyme is involved in the over-all proteolysis scheme. The optimal temperature increases as the reaction mixture becomes less acid (Figure 4). Again, this phenomenon may be attributed to one or more of the factors mentioned above.

Final Assay Procedure. As graphical representation of the data showed that optimal enzyme response was obtained under rather limited experimental conditions, a set of conditions was chosen which would reflect the over-all optimal proteolytic activity of the proteolytic enzymes of beef muscle tissue. The standardized procedure adopted for this work employed as a substrate a hemoglobin solution consisting of 2% hemoglobin, 3*M* urea, 0.2*M* sodium acetate, with enough 1*N* hydrochloric acid to bring the pH to 4.4. An equal volume (2 ml.) of the enzyme solution was added and the mixture was incubated for 4 hours at 37° C. The reaction was stopped and the protein precipitated by the addition of 10% trichloroacetic acid (4.0 ml.). After standing overnight, the solutions were centrifuged and the clear supernatant liquids were used for absorbance determinations. Readings were taken on a Beckman Model DU spectrophotometer at 276 mμ, and converted to micrograms of tyrosine by referring to a previously prepared standard curve.

Under the conditions of the assay, the activity of the water-extractable enzymes is proportional to the time of incubation up to a reaction time of 6 hours (Figure 2). Thus, the 4-hour digestion period is well within the range of the linear portion of the curve, where the enzyme reaction is not limited by inhibitors or insufficient substrate.

Extraction of Proteolytic Activity from Meat and Effect of Dialysis on Activity of Extracts. Certain extraneous chromogenic materials absorbing ultraviolet radiation in the region of tyrosine absorbance were being extracted by water from the muscle tissue along with the enzymes, and were interfering with the assay. After the water extracts of the muscle tissue were prepared, an 18-hour dialysis of the extracts against running deionized water prior to the assay, almost completely removed the extraneous chromogenic substances with a significant increase in the over-all proteolytic activity (Table II). As the apparent activity of the extracts was increased by dialysis, some inhibitory compound(s) were obviously present in the undialyzed extracts. The nature of these interfering substances is unknown.

Table II also shows the effect of the extraction pH on the enzyme activity of the water extracts. Fresh meat was ground and homogenized with water as previously described. The pH of the slurry was adjusted to 4.6 with 1*N* hydrochloric acid or to 7.1 and 8.0 with 1*N* sodium hydroxide. The extraction at pH 5.6 was carried out without any addition of acid or base, because this was the initial pH of the meat. Higher pH extractions were attempted, but the meat slurry gelled to such a degree that the extract could not be separated from the residue. Even at an extraction pH of 8.0, the slurry was slightly gelled and the results were uncertain. During the extraction procedure, final volumes of the slurry were kept constant to avoid differences due to dilution. The results from these experiments indicate that extractions were best performed at the initial pH of meat (5.6) or at neutrality. Because the results were not significantly better at a pH of 7.1, subsequent extractions were performed at the initial pH of excised muscle tissue (approximately 5.6).

Purification of Proteinases of Beef Muscle Extracts by Fractionation

The fractionation of water extracts of beef muscle was attempted with the use of ammonium sulfate at saturation levels of 10, 20, 50, 60, 70, and 85%. Aterf dialysis to remove the salt, the proteolytic activity was found mainly in those fractions which were precipitated by 50 to 60% ammonium sulfate. Modified cellulose anion exchangers were employed in the fractionation of the active ammonium sulfate fractions. Diethylaminoethanol (DEAE)-modified cellulose was prepared according to the method of Peterson and Sober (*9*). The material precipitated by 50 to 60% ammonium sulfate was dialyzed against carbon dioxide–free water and placed on a diethylaminoethanol anion exchanger column in ion-free water. The column was chromatographed with carbon dioxide–saturated water as suggested by

Mitz and Yanari (*8*). The column and collection tubes were cooled with circulating ice water at 3° to 4° C. Fractions of 5 ml. each were collected at a flow rate of 15 ml. per hour. Protein fractions were determined by the absorbances of the fractions at 2760 A. and were plotted against fraction number.

Two major protein peaks were obtained after carbon dioxide elution (Figure 5). Each fraction was assayed for proteolytic activity by the hemoglobin digestion method described. The activity is indicated by the shaded area of the graph. The proteolytic fractions contained activity in an amount equivalent to the total placed on the column with an 18-fold increase in specific activity (Table III) based on the nitrogen content determined by the micro-Kjeldahl method.

While these results seem to indicate that a single proteolytic enzyme has been isolated from meat, more than one enzyme will probably be found upon further fractionation of this material. The kinetic data, such as the relationship of activity to time, temperature, and pH, support the theory that a number of proteolytic enzymes are active in the meat extracts. Thus the partially purified protease preparations should be further purified and separated to characterize more completely the specific enzymes present in the proteolytic system of beef muscle.

Literature Cited

(1) Anson, M. L., *J. Gen. Physiol.* **22,** 79 (1938).
(2) Balls, A. K., *Ice and Cold Storage* **41,** No. 485, 143 (1938).
(3) Doty, D. M., Wachter, J. P., J. AGR. FOOD CHEM. **3,** 61 (1955).
(4) Hankins, O. G., Hiner, R. L., *Food Inds.* **12,** 49 (1940).
(5) Hiner, R. L., Madsen, L. L., Hankins, O. G., *Food Research* **10,** 312 (1945).
(6) Holiday, E. R., *Biochem. J.* **30,** 1795 (1936).
(7) Hutner, S. H., Bjerknes, C. A., *Proc. Soc. Exptl. Biol. Med.* **67,** 393 (1948).
(8) Mitz, M. A., Yanari, S. S., *J. Am. Chem. Soc.* **78,** 2649 (1956).
(9) Peterson, E. A., Sober, H. A., *Ibid.*, **78,** 751 (1956).
(10) Wetlaufer, D. B., Edsall, J. T., Hollingworth, B. R., *J. Biol. Chem.* **233,** 1421 (1958).
(11) Wu, H., *Chinese J. Physiol.* **5,** 321 (1931).
(12) Zender, R., Lataste-Dorolle, C. Collet, R. A., Rowinski, P., Mouton, R. F., *Food Research* **23,** 305 (1958).

Received for review May 13, 1959. Accepted July 30, 1959. Division of Agricultural and Food Chemistry, 134th Meeting, ACS, Chicago, Ill., September 1958. Journal paper No. 177 American Meat Institute Foundation. Work supported by Quartermaster Food and Container Institute for the Armed Forces, Quartermaster Research and Engineering Command, U. S. Army, approved as No. 971 in the series of papers for publication. The views and conclusions expressed herein are those of the authors.

RUMEN BIOCHEMISTRY

Physiological Activities of Rumen Mixed Cell Suspensions

H. A. BLADEN[1] and R. N. DOETSCH

Departments of Microbiology and Dairy Science, University of Maryland, College Park, Md.

An attempt was made to determine some of the factors that affect the production of volatile fatty acids and lactic acid by washed cell suspensions of rumen bacteria. Refrigeration or freezing of washed cell suspensions yielded preparations that did not give results comparable to those obtained from untreated washed suspensions. Time of sampling after feeding as well as variations of buffer and/or substrate concentration affects the amounts and ratios of volatile and lactic acids produced. The length of the incubation period influences the quantity of acids present, as does the presence of rumen fluid; the latter seems to have an inhibitory effect on the production of volatile acids. Reactions that require long-term incubations yield results of a variable nature, and hence are much more difficult to interpret in terms of in vivo events.

SEVERAL TECHNIQUES have been employed to study the specialized microbial flora of the rumen with the hope of defining the over-all contribution of the mixed bacterial population (*2, 3*). Among these is the washed cell suspension technique, in which the mixed bacterial (but not protozoan) flora is obtained by differential centrifugation of fresh rumen liquor and then subjected to various physiological experiments. Washed suspensions of rumen bacteria were studied by Johns (*10*), and Elsden and Sijpesteijn (*6*). Subsequently various modifications (*1, 4, 7, 8, 11, 13–15, 19, 20*) were introduced.

Limitations of the washed cell suspension technique have been noted (*3*), but efforts to avoid some of the deficiencies of pure culture experiments in studying one aspect of microbial ecology (*22*) should be encouraged Lewis (*15*) noted that certain treatments of washed cell suspensions would alter the production of various end products. It has been shown also (*18*) that the speed of centrifugation governs to some extent the production of lactic acid.

The purpose of the experiments reported here was to determine the parameters affecting the formation of certain chief end products of carbohydrate metabolism by rumen washed cell suspensions—namely, volatile fatty

[1] Present address, United States Department of Agriculture, Animal Husbandry Research Division, Beltsville, Md.