Comparison of Enzyme and Waring Blendor Methods for Determination of Collagen in Beef

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Waring Blendor–centrifuge procedures often yield higher collagen nitrogen values in cooked than in raw beef. This may be due to inadequate blender dispersion of coagulated protein masses in cooked beef. The dispersive action of a protease inactive toward collagen was compared with that of the blender. Collagen values were lower in cooked than in raw meat by both methods, but differences between raw and cooked samples were generally greater by the enzyme method. The enzyme method yielded significantly lower collagen values for both raw and cooked beef, and these values showed better relationship to shear values and tenderness scores than those obtained by the blender method. The enzyme method offers promise for a procedure that is reproducible, rapid, and feasible for multiple sample operation.

PRESENT methods for chemical analysis of collagen in muscle tissue give inconsistent results and are time-consuming. The greatest difficulty lies in separating the soluble nitrogen-bearing components of muscle (protein and nonprotein) from connective tissue proteins. There have been many approaches to the problem, but the objectives of all methods were to facilitate the separation of muscle protein fractions and to minimize sampling variation. Miller and Kastelic (*7*) have reviewed chemical methods for determining collagen.

The Waring Blendor method of Hartley and Hall (*4*) used in this laboratory gave reproducible results for raw, but not for cooked beef. Moreover, it often indicated an apparent increase in collagen nitrogen during the cooking of meat. Hence, further work on this method seemed advisable.

The Waring Blendor method (for brevity, the blender method) consists of processing tissue and water in a Waring Blendor, adjusting to the apparent isoelectric point (*3*), pH 5.0, and washing with water by centrifugation. The high collagen values obtained by this method were attributed to inadequate dispersion of the coagulated protein masses.

Without extensive review of the literature, it is assumed that most meat researchers are aware of the controversial question of the importance of collagen to tenderness of meat. Such controversy may be expected because of the difficulty in defining the identity of collagen in tissue and estimating the amount therein. This investigation was aimed at developing some simple techniques that may help to overcome some of the difficulty mentioned above, and to permit a procedure for determining collagen that is reproducible, rapid, and feasible for multiple sample operation.

The procedures should be within the grasp of laboratory assistants without extensive and critical training and experience. Many meat research programs might be able to promote a project of this sort as one of several concurrent efforts involving the same carcasses or cuts of known history. This type of technology is not to be confused with the critically needed fundamental exploration typified by the paper of Loyd and Hiner (*6*). Their procedure would be outside the scope of many laboratories, both in the time involved and in qualified personnel. For these reasons, it was hoped to avoid the difficult-to-separate alkaline colloidal dispersions and critical hydroxyproline techniques involved in the method of Loyd and Hiner, and in the similar alkaline techniques of Lowry, Gilligan, and Katersky (*5*) and Miller and Kastelic (*7*).

In this investigation proteolytic enzymes, inactive toward collagen, were used to open up the complex connective tissue structure by hydrolysis of the simpler proteins, so that soluble nitrogenous material could be washed out by centrifugation. The objectives were to compare this procedure with the blender method, and to study the relationships among collagen nitrogen values, shear values, and tenderness scores.

Materials and Methods

Meat Used. Ten top rounds of beef, graded U. S. Good, and ripened for similar periods were purchased from a local wholesale meat company. Beginning at the proximal end, four 1.5-inch slices were cut from each top round, wrapped individually in aluminum foil, and stored at −20° F. until used.

Cooking Procedure and Shearing and Tenderness Tests. The experimental design provided for the slices from each round to be divided into two blocks. The first two slices from the proximal end of the round were in one block and the third and fourth slices in the other. One slice from each block was selected at random to be cooked under 10 pounds' pressure for 30 minutes and the other slice for 45 minutes.

A grid, prepared to conform to the shape of the semimembranosus muscle in the slices of top round, was divided into 1-inch squares. The squares were numbered, and a table of random numbers was used to select the numbers on the grid that represented the position in each slice to be used for shearing tests and chemical analyses and for organoleptic tenderness evaluation.

Prior to cooking, the slices were thawed 48 hours in a refrigerator (40° F.). Six 1-inch cores for determining raw shear values were removed from each slice. The slice was placed on a rack in an electric pressure cooker with $^1/_4$ cup of water and cooked under 10 pounds' pressure for either 30 or 45 minutes.

Six 1-inch cores were removed from the cooked slice for shear value determinations and six samples $^1/_6$ inch thick were scored for tenderness by a palatability panel. Tenderness scores were given within a range of 10 to 1; 10 represented extremely tender meat and 1, extremely tough meat. Both raw and cooked cores were sheared on a Warner-Bratzler (*9*) shearing apparatus. An average of the values obtained from 18 shears was the raw or cooked shear value for each slice. After shearing, the cores were ground

twice in a Universal No. 3 hand grinder with a fine blade, packed in 4-ounce jars with screw caps, and stored at −20° F. until analyzed for collagen nitrogen.

Chemical Analyses. The frozen, ground meat was thawed in a refrigerator. After being mixed thoroughly, duplicate 10-gram samples were analyzed for blender and enzyme collagen nitrogen.

BLENDER METHOD. In the Hartley and Hall (*4*) method, when the blender homogenate was washed with distilled water, turbidity appeared in the second or third washing. This condition was not suppressed by use of dilute calcium chloride, magnesium chloride, or sodium chloride wash solutions. The late appearance of the turbidity suggested that it might be caused by loss of some soluble muscle tissue constituent. Phosphate was suspected, and 0.06*M* phosphoric acid (approximately the concentration in muscle tissue) adjusted to pH 5.0 with sodium hydroxide was used as a wash solution. Turbidity was reduced greatly, and the solids packed firmly in centrifuging. Möhler and Kiermeier (*8*) observed that sedimentation of aqueous suspensions of finely minced meat was increased by addition of phosphate salts. Their interpretation was that an increase in cohesion occurred between protein particles.

Extraction with the phosphate wash removed about 30% of the total nitrogen from raw meat and about 10% from cooked meat. This is considerably less than the 80 to 90% removed from raw beef with 0.1*N* sodium hydroxide as reported by Miller and Kastelic (*7*). The phosphate, being essentially indifferent to muscle tissue in kind and concentration of solute, seems less likely to alter protein structure, but still capable of removing nitrogenous components soluble at the apparent isoelectric point of muscle tissue. The insoluble residue then may be autoclaved, and the soluble gelatin extracted from the residue without contamination by native soluble nitrogen-bearing components. After gelatin removal, the residue apparently contained the greater bulk of the muscle protein.

About one third of the nitrogenous material extracted from raw meat was heat-coagulable. When the coagulate from one sample was washed and autoclaved, the soluble hydrolyzate contained one twentieth as much nitrogen as the blender collagen found in the same sample. In cooking, this material is coagulated in varying degree before extraction, and in autoclaving it may be hydrolyzed partially and go into the gelatin fraction. Thus, it may help to cause apparently high values for blender collagen in cooked beef.

The turbidity that developed in washing the autoclaved residue with boiling water in the Hartley and Hall (*4*) method was avoided by use of boiling 0.2% sodium chloride solution. Rapid filtration and clear filtrates resulted.

ENZYME METHOD. Three enzymes (Protease 15, Rhozyme P-11, and Rhozyme A-4) were obtained from the Rohm & Haas Co. and were tried in the preliminary work to separate the soluble proteins from connective tissue in the muscle. The P-11 and A-4 gave inconsistent results and produced a colloidal dispersion during the washing with distilled water. However, the Protease-15 did not act in this manner and was used in the study. This enzyme is inactive toward native collagen (*10*).

Samples were weighed into 4-ounce screw-cap bottles and 40 ml. of water, 0.1 gram of enzyme, and enough 0.75*N* sodium hydroxide to bring the mixture to pH 7.35 were added. Each sample was mixed 1 minute with a motor stirrer and 1 ml. of toluene was added as an antiseptic.

The samples were placed in a shaker in a water bath maintained at a constant temperature of 40° C. for 16 hours, then cooled to 25° C. by placing the bottles in cold water. The samples were adjusted to pH 5.0 by the addition of 1*N* sulfuric acid. The procedure continued with the Hartley and Hall (*4*) method for washing and autoclaving.

After the first centrifugation, white floats of fat were observed on the filtrates from the samples treated with enzymes. These floats may have been clumped by the use of toluene, a surface-type antiseptic. As it was believed that some connective tissue might be occluded and lost in the washings, chloroform instead of toluene was used as a preservative. The resulting filtrates were clear with no floats; the fat apparently clumped with the chloroform in the bottom of the tube. However, after being autoclaved, the solutions were colloidal and difficult to filter. It was postulated that the fat was partially hydrolyzed and colloidally dispersed after removal of the chloroform in the autoclave, and that this condition caused the difficulty in filtering. An analysis of the filtrate containing the float indicated that only minimal quantities of collagen nitrogen were present, and not enough collagen was lost to affect the results. Therefore, the use of chloroform was discontinued, and toluene was used again. This observation is in contrast to Loyd and Hiner's (*6*) finding of an astonishing 71% of the longissimus dorsi hydroxyproline in the fat floats. The long period of alkaline digestion employed by them appears to be the principal difference in treatment of the tissue and may induce a difference in the adsorptive action of the fat.

Collagen nitrogen values were calculated as percentage of total nitrogen. Total nitrogen was determined by the AOAC method (*1*).

Table I. Average of Mean Collagen Nitrogen Values[a] Determined by Enzyme and Blender Methods

Round No.	Enzyme Method			Blender Method		
	Raw	Cooked, Min. 30	Cooked, Min. 45	Raw	Cooked, Min. 30	Cooked, Min. 45
I	7.6	7.2	6.7	9.9	9.5	9.2
II	8.5	6.1	5.9	10.2	9.5	8.5
III	8.9	6.5	6.3	10.6	9.7	8.6
IV	8.8	6.2	6.5	10.0	8.7	8.6
V	10.4	6.9	6.5	11.1	9.2	8.7
VI	9.2	6.5	6.7	9.8	9.3	9.3
VII	9.2	7.3	7.4	11.1	9.8	8.9
VIII	10.2	9.9	9.6	10.8	10.1	9.3
IX	11.1	10.0	9.9	11.1	9.6	9.6
X	11.8	10.3	9.9	10.4	9.9	9.1
Av.	9.6	7.7	7.5	10.5	9.5	9.0

[a] Per cent of total nitrogen.

Table II. Comparison, by *t* Test, of Collagen Nitrogen Values Obtained by Enzyme and Blender Methods

Measurements Compared	*t* Value	Conclusion
Enzyme *vs.* blender method		
Raw	4.50, 39 df[a,b]	Collagen nitrogen higher with blender method
Cooked 30 min.	5.20, 19 df[b]	
Cooked 45 min.	4.12, 19 df[b]	
30-minute *vs.* 45-minute		
Enzyme method	0.37, 38 df, ns[c]	No cooking time effect
Blender method	3.33, 38 df[b]	Collagen nitrogen greater for 30-min. cooking than for 45-min. (very low error variance)

[a] df degrees of freedom.
[b] Very highly significant ($P<0.001$).
[c] ns, nonsignificant.

Table III. Average of Mean Shear Values and Organoleptic Tenderness Scores[a]

Round No.	Shear Values, Lb.			Tenderness Scores	
	Raw	30 min.	45 min.	30 min.	45 min.
I	25.8	20.6	12.5	8.0	8.7
II	19.4	16.4	11.0	8.1	9.0
III	22.9	19.1	15.3	7.7	8.5
IV	20.5	11.3	10.9	6.4	7.8
V	27.5	11.6	14.4	7.5	8.2
VI	27.3	16.1	16.0	7.3	7.9
VII	27.5	16.4	11.9	7.6	7.8
VIII	24.6	21.3	17.0	6.6	8.4
IX	24.4	22.2	22.2	7.2	6.4
X	26.2	17.3	17.4	7.0	7.9
Av.	24.6	17.2	14.9	7.3	8.1

[a] 10, extremely tender.
1, extremely tough.

Table IV. Correlation Coefficients for Collagen Nitrogen Values with Shear Values and Collagen Nitrogen Values with Tenderness Scores

Measurements Correlated	Correlation Coefficient	Conclusion
Collagen nitrogen *vs.* shear values		
Raw		
Enzyme	0.10 ns[a]	No relationship
Blender	0.22 ns	No relationship
Cooked		
Enzyme 30 min.	0.39 near[b]	May be a weak relationship
Enzyme 45 min.	0.68[c]	Collagen nitrogen values related to shear values
Blender 30 min.	0.21 ns	No relationship
Blender 45 min.	0.28	No relationship
Collagen nitrogen *vs.* tenderness scores		
Enzyme 30 min.	−0.44[b]	Slight relationship
Enzyme 45 min.	−0.51[b]	Slight relationship
Blender 30 min.	0.04 ns	No relationship
Blender 45 min.	−0.28 ns	No relationship

[a] Nonsignificant.
[b] Significant ($P<0.05$).
[c] Very highly significant ($P<0.001$).

Statistical Analyses. The t test was used to compare collagen nitrogen values by the enzyme method (enzyme collagen nitrogen) *vs.* collagen nitrogen values by the blender method (blender collagen nitrogen), and collagen nitrogen values for samples cooked 30 minutes *vs.* the values for samples cooked 45 minutes for both the enzyme and blender methods of determining collagen. Correlation coefficients for raw meat and for meat cooked 30 and 45 minutes were obtained for enzyme collagen nitrogen values with shear values, blender collagen nitrogen values with shear values, enzyme collagen nitrogen values with tenderness scores, and blender collagen nitrogen values with tenderness scores.

Results and Discussion

Averages of mean values for collagen nitrogen in raw and cooked semimembranosus muscle from 10 top rounds, graded U.S. Good, as determined by the blender and enzyme methods are presented in Table I.

Although results in this investigation indicated lower blender collagen values in cooked than in raw beef, experience in this and other laboratories (*2*) has shown that this relation is often reversed. This reversal has been observed in samples in frozen storage more than a year in this laboratory (unpublished data). In the study reported here, samples were processed promptly after preparation. The possibility is suggested that collagen may be degraded by enzyme action in frozen, stored raw meat, whereas enzymes are inactivated by cooking the meat.

The t test showed that blender collagen nitrogen values were significantly ($P<0.001$) greater than enzyme collagen nitrogen values for both raw and cooked meat (Table II). Blender collagen nitrogen values were significantly ($P<0.001$) greater for samples cooked 30 minutes than for those cooked 45 minutes. The latter result may be explained by the greater opportunity for hydrolysis of collagen under moist heat when the meat was cooked for 45 minutes than when it was cooked 30 minutes. This is further reflected in the average shear values and tenderness scores given in Table III. Shear values generally were lower in the longer-cooked samples, and tenderness scores were slightly higher, with the exception of those for round IX. The enzyme method failed to show a significant difference in collagen nitrogen attributable to cooking time (Table II), but it indicated greater differences in collagen between raw and cooked samples in 7 of the 10 rounds for both cooking periods (Table I).

Of greater importance, enzyme collagen values (Table IV) gave a very highly significant ($P<0.001$) correlation ($r = 0.68$) with shear values for samples cooked 45 minutes, and a nearly significant correlation ($r = 0.39$) for those cooked 30 minutes. Also, enzyme collagen values were correlated significantly ($P<0.05$) with tenderness scores for samples both 30 ($r = -0.44$) and 45 ($r = -0.51$) minutes (Table IV). Obviously enzyme collagen values were correlated better with shear values and tenderness scores for the samples cooked 45 minutes than for those cooked 30 minutes. For the raw meat there was no apparent relationship between shear values and collagen nitrogen as determined by either method.

For these reasons it is believed that the use of proteolytic enzymes for collagen separation and estimation offers some promise. Since this investigation was made, the enzyme method has been improved by the use of 5 mg. of aureomycin hydrochloride in the incubation mixture (without toluene or chloroform) to suppress the activity of contaminating microorganisms. No putrefactive odor develops after the addition of aureomycin hydrochloride. Valenzuela (*11*) found that aureomycin hydrochloride (0.1 mg. per ml.) inhibited bacterial growth without interfering with growth of tissue in artificial media.

Also, the phosphate wash used in the blender method has been substituted for distilled water to remove water-soluble protein following incubation. The incubation temperature has been increased to 50° C., where Protease-15 appears more effective.

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ACIDS OF TOMATOES

The Separation of Organic and Inorganic Acid Anions in Filtered Tomato Purée by Partition Chromatography

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A method, employing silicic acid partition chromatographic techniques, is described for the separation of those organic and inorganic anions which contribute to the acidity of tomatoes. Acids were converted to the hydrogen form by passing filtered purée through the cation exchange resin, Dowex 50. Titratable and total acidity were determined by titrating an aliquot of filtered tomato purée before and after resin treatment, respectively, with 0.1*N* sodium hydroxide to a phenol red end point. Separation of acids was quantitative and total recovery was approximately 94% of the total acidity. Ten acids (acetic, lactic, fumaric, malic, pyrrolidone carboxylic, citric, phosphoric, hydrochloric, sulfuric, and galacturonic) were found to be present in the filtered tomato purée. The phosphate, chloride, sulfate, and galacturonate ions had not previously been separated from tomato purée by partition chromatography or reported as constituents of the total acidity.

Procedures reported for the separation of the organic acids of tomato fruits (*2*, *3*, *7*) were adaptations of the methods outlined by Isherwood (*4*) and Marvel and Rands (*6*). The Isherwood method, as employed by Bulen, Varner, and Burell (*2*) and Rice and Pederson (*7*), to isolate the organic acids in tomatoes required the use of sulfuric acid in the initial extraction of the organic acids as well as in the preparation of the silicic acid partition column. The author found in preliminary studies that the sulfuric acid was eluted in sufficiently high concentrations to mask the presence of acids eluted after citric acid.

The purpose of this paper is to report analytical procedures found adaptable for the separation and identification of organic and inorganic acids present in tomatoes. The data as presented can be used as a general guide in determining the content of individual acids in other fruits. The ratio of chloroform to 1-butanol as well as the quantity of solvent may have to be modified to meet the specific requirement of the fruit.

Experimental

Preparation and Preservation of Tomato Purée Samples. Twenty-five pounds of freshly picked tomatoes were used for each sample. The fruits were washed, trimmed, macerated, and heated to 155° F. in a steam-jacketed kettle and held at this temperature for 4 minutes with constant stirring. This material was then passed through a Langsenkamp laboratory pulper operating at approximately 1600 r.p.m. and equipped with a 0.027-inch finishing screen. This operation removed skins and seeds and reduced the pulp to a purée. The resultant purée was heated to 200° F., placed in cans, sealed, processed in boiling water for 10 minutes, and then cooled immediately with water.

Removal of Cations by Ion Exchange. Approximately 150 ml. of canned purée were centrifuged to separate the red pulp from the clear amber liquid. The supernatant liquid was filtered to remove traces of pulp. Fifty milliliters of the filtrate were passed through a 30-ml. volume of the cation exchange resin, Dowex 50 in the hydrogen ion cycle, in a 17 × 0.75-inch glass tube. The eluent containing acids, sugars, and neutral material not attracted to cation exchange resin was collected in a 100-ml. volumetric flask at the bottom of the ion exchange tube. Five- to 10-ml. increments of distilled water were added to the top of the column to ensure a quantitative removal of the acids. Sugars present after cation exchange did not hinder the separation of acids on the silicic acid partition column.

The resin was regenerated by treating with four bed volumes of 2.5*N* hydrochloric acid and washing with distilled water (with occasional back flushing) until the eluent was free of chloride (silver nitrate test).

Separation of Acids by Partition Chromatography. The partition column was set up in a manner similar to that reported by Marvel and Rands (*6*). Only the coarser fraction of the silicic acid was used for the partition column. Six samples were run at one time using a battery of six ion exchange tubes and six partition chromatographic tubes.

Resin-treated filtrate, containing approximately 1 meq. of total acid (determined by titrating to phenol red end point) was pipetted into a 50-ml. beaker and concentrated on a water bath to 1.5 to 2 ml. at temperatures below 40° C. Two grams of oven-dried silicic acid were mixed thoroughly with the sample in a manner similar to that reported by Wise (*9*) until a free-flowing powder was obtained. The mixture was placed on top of the column in a dry form. Five milliliters of chloroform were used to remove any residue in the beaker and to suspend the sample at the top of the tube. A wad of cotton was placed on top of the sample to prevent disturbance of the sample or partition column as solvents were added.

The nine solvents used to elute the acids were composed of different concentrations of 1-butanol and chloroform