Changes in Nonprotein Nitrogenous Constituents of Chicken Breast Muscle Stored at Below-Freezing Temperatures

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The nonprotein nitrogen fraction of chicken breast muscle, obtained by precipitating the protein in 5% trichloroacetic acid, increased noticeably during storage for 45 weeks at temperatures between −5° and −40° C. About 80% of the increase resulted from an increase in amino acids, mostly acidic, and aromatic and sulfur-containing amino acids. Guanidino compounds, mainly creatine and creatinine, increased slightly, while nucleic acid derivatives decreased. These changes increased with increasing storage temperature. Enzymes such as cathepsins were probably released as a result of damage during freezing and storage, causing limited proteolysis. Proteolysis may affect the solubility and ion-binding properties of the protein, and thus affect tenderness, loss of juiciness, and development of dryness in the meat.

In a previous paper concerned with the storage of chicken meat, the effect of frozen storage on the accumulation of protein-breakdown products was described (*15*). Ninhydrin-positive as well as Folin-Ciocalteu reagent-positive materials in the trichloroacetic acid-soluble fraction of the meat increased with increase in both temperature and time. The author thought that a more detailed study of the nonprotein nitrogen fraction in chicken breast muscle stored at different temperatures might show the nature of protein-breakdown materials formed during frozen storage and would also indicate the biochemical changes involved in quality deterioration. This paper describes results of an investigation made to determine the amino acid composition and to study accumulation of other major nitrogenous constituents, such as guanidino compounds and nucleic acid derivatives, and phenols and reducing substances, in the fraction obtained by precipitating protein in 5% trichloroacetic acid solution. Tests were made on muscle samples before freezing and after storage for 45 weeks at −40°, −20°, −10°, and −5° C.

Materials and Methods

Samples were obtained from eight 6-month-old chickens (male) from a single flock, processed under commercial conditions and aged in drained ice for 18 to 24 hours. To obviate bird-to-bird variability, comparisons were made between samples from left and right halves of the same bird. Breast meat from one half was stored at −40°C. (control sample) and from the other at −20°, −10°, or −5° C. or analyzed fresh (test sample). Breast meat to be stored was vacuum packaged in Cry-O-Vac bags and frozen in an air blast (300 to 500 feet per minute) at −30° C. before being placed in storage for 45 weeks at the desired temperature.

To prepare the nonprotein nitrogen fractions (also designated as TCA-filtrate), duplicate samples were ground with sand and potassium chloride–borate buffer (pH 7.5 and ionic strength 1.0, 1 ml. per gram of muscle), and made up to a known volume with trichloroacetic acid solution (sufficient to give 5% final concentration) and water. The suspensions were allowed to stand at room temperature for 2 hours and filtered to remove protein. To prevent loss of nitrogen in drippings, muscle samples were weighed while frozen and then ground in a mortar to include exudate.

The nonprotein nitrogen fraction was analyzed for total nitrogen (by standard micro-Kjeldahl procedure), for ninhydrin-positive nitrogen (*30*), and for amino acids. Ninhydrin-positive materials were determined both before and after hydrolysis of peptides and are expressed in terms of tyrosine equivalents. Hydrolysis was carried out by boiling TCA-filtrate in presence of 6*N* HCl for 20 hours under reflux. The hydrolysate was concentrated to dryness and the residue taken up in water to original volume. Ninhydrin-positive nitrogen estimated before hydrolysis is referred to as free amino nitrogen, and the increase in ninhydrin-positive nitrogen that occurred after hydrolysis is referred to as bound amino nitrogen. For amino acid analysis, a known volume of TCA-filtrate was hydrolyzed and evaporated to dryness, diluted to a known volume, and subjected to ion exchange chromatography using the "Technicon" automatic amino acid analyzer with operating conditions as described by Piez and Morris (*29*). Cystine and cysteine were determined chromatographically as cysteic acid on Dowex 1X2 columns using samples subjected to performic acid oxidation (*31*). Since methionine and tryptophan are destroyed by acid hydrolysis, methionine was estimated before hydrolysis by a colorimetric method (*23*), and tryptophan was estimated after alkaline hydrolysis by chromatography on starch columns (*8*, *25*). Hydroxyproline, which gives a weak color reaction with ninhydrin under standard conditions, was also determined separately using the procedure described by Troll (*36*).

Guanidino compounds, essentially creatine and creatinine, were estimated by picric acid reagent after the conversion of creatine to creatinine by autoclaving the nonprotein nitrogen fraction at 117° to 120° C. for 20 minutes in the presence of 2*N* H_2SO_4 (*5*). Concentration changes in ribonucleic acid derivatives were determined by measuring ultraviolet absorption of the nonprotein nitrogen fraction at 260 mμ and by estimating total ribose content by orcinol reagent (*4*). Phenols and other reducing materials were estimated by Folin-Ciocalteu reagent (*9*), both before and after hydrolysis of TCA-filtrate with 6*N* HCl.

For paper chromatographic analysis of amino acids and Folin-Ciocalteu–positive materials, the nonprotein nitrogen fraction was evaporated to near dryness under vacuum, made to a known volume, and examined before and after (6*N* HCl) hydrolysis. Single-dimensional chromatography was carried out on buffer-impregnated papers using *n*-propanol–ethanol–pyrophosphate buffer, pH 8.9 (50–25–25, v./v.) as solvent system (*12*), and on Whatman No. 1 papers

Table I. Effect of Storage for 45 Weeks at Different Temperatures on Total and Ninhydrin-Positive Nitrogen Content of the Nonprotein Nitrogen Fraction in Chicken Breast Muscle

Storage Temp., ° C.	Nonprotein Nitrogen, Mg. per Gram of Muscle	Ninhydrin-Positive Nitrogen (expressed as tyrosine N)			
		Before hydrolysis		After hydrolysis	
		Mg. per gram of muscle	Nonprotein N, %	Mg. per gram of muscle	Nonprotein N, %
Fresh	5.14	0.35	7.0	0.69	13.5
−40°	4.97	0.39	7.8	0.73	14.6
−20°	5.54	0.58	10.6	1.16	21.1
−10°	5.72	0.77	13.5	1.35	23.7
−5°	6.13	0.93	15.2	1.62	26.3

Table III. Effect of Storage for 45 Weeks at Different Temperatures on Sulfur-Containing Amino Acids, Guanidino Compounds, and Nucleic Acid Derivatives Content of the Nonprotein Nitrogen Fraction in Chicken Breast Muscle

Storage Temp., ° C.	Mg. per Gram of Muscle				Ultraviolet Absorbance at 260 mμ
	Cysteic acid	Methionine	Creatine plus creatinine	Ribose	
Fresh	0.15	0.25	1.42	1.60	0.96
−40	0.17	0.25	1.40	1.42	0.92
−20	0.18	0.37	1.46	1.32	0.91
−10	0.32	0.56	1.55	0.95	0.88
−5	0.41	0.62	1.65	0.60	0.88

using *n*-butanol–acetic acid–water (60–15–25, v./v.) as solvent system (*21*). For two-dimensional paper chromatography, *n*-butanol–acetic acid–water (60–15–25, v./v.) was used as first solvent, and *n*-butanol–pyridine–water (6–6–6, v./v.) as second solvent. For color development, the papers were sprayed with ninhydrin reagent or with Folin-Ciocalteu reagent, followed by 30% aqueous sodium carbonate solution.

Results

Total nitrogen as well as ninhydrin-positive nitrogen content of the TCA-filtrate increased during storage, and the increase depended on storage temperature (Table I). Ninhydrin-positive nitrogen (found as free amino nitrogen and in the form of amino nitrogen–containing polymers) constituted about 13 to 15% of the nonprotein nitrogen in fresh muscle, and increased by 5 to 130% (as compared with fresh sample), depending on storage temperature.

Percentages of various amino acids found in the hydrolysate of the nonprotein nitrogen fraction from breast muscle stored at −40° and −10° C. for 45 weeks are given in Tables II and III. In samples stored at −10° C., a substantial increase was noted in α-amino acids, acidic amino acids, aromatic amino acids, and in the sulfur-containing amino acids. Increases in the heterocyclic amino acids and in the basic amino acids were small, except for arginine which increased considerably. Since chicken meat is poor in tryptophan (*17*), the small increase of this amino acid in the nonprotein nitrogen fraction was not surprising. Paper chromatography gave similar results.

Changes in the guanidino compounds and nucleic acid derivatives were small (Table III). Guanidino compounds (mainly creatine and creatinine) increased slightly during storage and accounted for about 10% of the total increase in nonprotein nitrogen in samples stored at −20°, −10°, and −5° C. Nucleic acid derivatives showing ultraviolet absorption at 260 mμ decreased slightly with increase in storage temperature, but the decrease in the total ribose content occurred more rapidly than the loss of ultraviolet absorption. The rapid loss of ribose seems to have occurred as a result of interaction of amino acids and other metabolic products (*14*, *32*) with free ribose found in chicken muscle (*19*) and of ribose formed during breakdown of ribotides and ribosides to nucleic acid bases.

Folin-Ciocalteu reagent-positive materials increased during storage depending on storage temperature (Table IV). This increase occurred as a result of formation of free as well as polymer-containing Folin-Ciocalteu reagent-positive materials as shown by comparison of results before and after hydrolysis. Folin-Ciocalteu reagent has been used to measure primary cleavage

Table II. Relative Percentage of Various Amino Acids Found in the Hydrolysate of the Nonprotein Nitrogen Fraction in Chicken Breast Muscle Stored at −40° and −10° C. for 45 Weeks

Amino Acid[a]	Ninhydrin-Positive Nitrogen,[b] %	
	−40° C.	−10° C.
Hydroxyproline	Trace	Trace
Aspartic acid	1.7	2.4
Threonine	0.6	2.6
Serine	0.8	2.5
Glutamic acid	4.0	5.8
Proline	Trace	1.3
Glycine	1.6	8 1
Alanine	2.1	7.7
Valine	0.9	2.7
Methionine	Trace	0.9
Isoleucine	Trace	2.4
Leucine	0.5	5.2
Norleucine	...	2.4
Tyrosine	Trace	1.1
Phenylalanine	3.0	5.0
Ammonia	24.6	11.0
Lysine	35.3	17.3
Histidine	18.0	11.4
Arginine	Trace	3.6

[a] Listed in the order in which they were eluted from the column.

[b] Ninhydrin-positive nitrogen present in samples stored at −40° and −10° C., respectively, was 0.73 and 1.35 mg. per gram of muscle.

Table IV. Effect of Storage for 45 Weeks at Different Temperatures on Folin-Ciocalteu Reagent-Positive Materials in the Nonprotein Nitrogen Fraction in Chicken Breast Muscle

Storage Temp., ° C.	Mg. of Tyrosine per Gram of Muscle	
	Before hydrolysis	After hydrolysis
Fresh	0.25	0.52
−40	0.27	0.54
−20	0.29	0.64
−10	0.33	0.70
−5	0.40	0.79

in proteins due to proteolysis and to react with tyrosine (free and in peptide form), phenols, tryptophan, cysteine, sulfhydryl compounds including H_2S, and other reducing materials (*3*). Paper chromatography of the nonprotein nitrogen fraction showed the presence of three major Folin-Ciocalteu reagent-positive components, one of them having the R_F value of tyrosine, while the other two gave a negative reaction with ninhydrin. None of these components had R_F values corresponding to tryptophan, while cysteic acid, which is formed from cysteine during hydrolysis and chromatography, did not react with this reagent. No further attempt was made to identify the other two components.

Discussion

The nonprotein nitrogen fraction in fresh chicken breast muscle obtained by

precipitating the protein in 5% trichloroacetic acid solution increased by about 1 to 2% during frozen storage in 45 weeks and accounted for about 14 to 15% of the total nitrogen in the muscle. About 80% of the increase in the nonprotein nitrogen occurred as a result of accumulation of amino acids. Although muscle proteins appear to be the source of these amino acids, the possibility of a source other than protein splitting was considered. Release of amino acids from peptides, such as glutathione, carnosine, and glutamine, or from ribonucleic acids involved in protein synthesis (*13*) would account neither for the increase in amino acid content reported here nor for the accumulation of amino nitrogen containing polymers in the nonprotein nitrogen fraction. The release of tyrosine, phenylalanine, lysine, arginine, and leucine during storage suggests the activity of cathepsins (*34*). Isolation and purification of cathepsins from beef muscle active at low temperatures has already been reported by Ball (*1*). Cathepsins have been shown to be bound, along with other hydrolytic enzymes, in lysosomes (*6*) and to have very low activity when the granules are intact and increased activity as a result of damaging treatment such as homogenization or incubation at 37° C. Therefore, it appears that the freezing and storage conditions used here caused damage liberating these enzymes. Absence of hydroxyproline in the nonprotein nitrogen fraction may indicate that proteolysis did not involve connective-tissue proteins (*27*). These findings support previous results that the stroma fraction is not affected during frozen storage (*15*).

Since the results indicated that proteolysis of muscle proteins occurred during frozen storage, the relationship of proteolysis to changes in protein and to changes in quality were considered. During frozen storage, myofibrillar proteins of chicken breast muscle are denatured so that actomyosin becomes insoluble at its isoelectric point and also loses its myosin-adenosinetriphosphatase activity (*15*). In view of the accumulated data on the action of proteolytic enzymes on muscle proteins (*10, 24, 38*), and in view of the results of experiments made earlier in studying changes in chicken muscle proteins during aseptic storage at refrigeration temperatures (*16*), neither the loss of protein solubility nor the loss of myosin-adenosinetriphosphatase activity appears to be the result of proteolysis in myofibrillar proteins, but the possibility of "limited proteolysis" cannot be ruled out. Alanine, aspartic acid, glutamic acid, serine, and threonine, which accumulated in nonprotein nitrogen fraction during storage, are the abundant end-groups in muscle proteins (*20*). Since all properties of proteins depend on structure and interaction of elements in this structure with environment, any alteration in the arrangement of their constituent amino acid residues will affect the acid-base dissociation of the proteins and may lead to a change in solubility at a particular isoelectric point, as well as a change in ion-binding properties (*18*). Since concentration of absorbed anions and cations influence the water retention of meat (*33*) and affect its tenderness (*11*), proteolysis may affect all three of these properties of muscle. Loss of juiciness and development of dryness in frozen poultry (*28, 35*) may be related to changes in water-holding capacity of meat and to changes caused by "limited proteolysis" in myofibrillar proteins.

Results presented in this paper and those of other published work indicate that loss of flavor and development of off-odor in frozen stored meat is related to proteolysis. In beef, decrease in consumer-type taste panel preference based on flavor has been correlated to the action of endocellular tissue proteolytic enzymes (*7*). Also, formation of H_2S and mercaptan from sulfur-containing compounds has been shown to be involved in the development of off-odor in irradiated meat (*22*). Amino acid mixture obtained by proteolysis in fish has been shown to have a bad taste (*26*), and interactions between amino acids and sugars and their breakdown products are known to produce mercaptals, many of which have disagreeable odors (*2, 32*). A previous study (*37*) has shown a concomitant increase in protein-breakdown products as estimated by ninhydrin and Folin-Ciocalteu reagents and a decrease in odor and flavor ratings of chicken meat held at refrigeration temperatures. A similar relationship may be true for meat stored at below-freezing temperatures.

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