F:5

The Determination of the Myofibrillar and Connective Tissue Contents of Meats and Composite Meat Products

CONSTANTINOS G. ZARKADAS

Food Research Institute, Agriculture Canada, Ottawa, Ontario, K1A 0C6, and Department of Chemistry and Physics, McGill University, Macdonald Campus, Quebec, H9X 1C0, Canada (FRI Contribution No. 356).

INTRODUCTION

The precise assessment of the skeletal muscle, connective tissue and non-muscle protein content of composite meat products has always been a difficult analytical problem. Several of the microscopic, immunological and electrophoretic methods which have been described for their determination in such products (1-2) are limited mainly because of the extensive denaturation, structural changes and interactions which occur in these complex protein mixtures during processing. The more promising methods appear to be those which are based on the chromatographic separation and determination of the unique basic amino acid (3-6) and peptides (7) found in specific muscle and non-muscle proteins (5-10). However, until now, the multicolumn systems employed for these determinations have proven laborious, and the complete enzymatic hydrolysis of meat products and separation of these unusual amino acids and peptides has not been achieved. For these reasons, new chromatographic methods have been developed in this laboratory (11-15) for the complete separation and determination of all methylated basic amino acids, the diastereoisomers of 5-hydroxylysine [Lys(5OH)[1]], the stable crosslinks desmosine (Des) and isodesmosine (Ides) and related compounds in a single-column system, using the Durrum type DC-6A 11.0±1.0 µm or the Beckman type W-3 9.0±0.5 µm spherical resins. These sensitive methods are designed to be used with both conventional and fully automated amino acid analyzers, and have been successfully applied for the determination of the myofibrillar and connective tissue contents of meats and prepared composite meats.

This proposed chemical approach for evaluating the protein quality of composite meats is based on the direct determination of their myofilbrillar and connective tissue contents, since the contribution of these classes of proteins to the overall nutritive value of meats differs considerably. In this approach the myofibrillar myosin and actin contents of muscles and prepared composite meats can be determined from the amounts of N^{τ}-methylhistidine [His(τMe)] found in their acid hydrolysates. Collagen and collagen-like proteins (10, 16-18) can be calculated from the amounts of Lys(5OH) present and the elastin content from the amounts of Des and Ides (14-15). Therefore, when the sum of the myofibrillar and connective tissue nitrogen is subtracted from the total nitrogen of a composite meat hydrolysate sample, the difference is an accurate assessment of the non-muscle proteins present. This approach has the advantage over other methods that complete separation is possible in a single analysis in less than 7 h, and that the determination of the connective tissue and myofibrillar proteins can be carried out in both fresh muscles or processed meats as well as animal protein supplements (14-15).

[1]Abbreviations used: Des, desmosine, 4-(4-amino-4-carboxybutyl)-1-(5-amino-5-carboxypentyl)-3,5-bis(3-amino-3-carboxypropyl)pyridinium; Ides, isodesmosine, 2-(4-amino-4-carboxybutyl)-1-(5-amino-5-carboxypentyl)-3, 5-bis(3-amino-3-carboxypropyl)pyridinium; Lys(5OH), 5-hydroxylysine (*erythro*-5-hydroxy-L-lysine); aLys(5OH), allo-5-hydroxylysine (*threo*-5-hydroxyl-L-lysine); Lys(Me), N^6-methyl-L-lysine; Lys(Me_2), N^6-dimethyl-L-lysine; Lys(Me_3), N^6-trimethyl-L-lysine; GlcN, glucosamine; His(πMe), N^{π}-methylhistidine or *pros*methyl-L-histidine; His(τMe), N^{τ}-methylhistidine or *tele*methyl-L-histidine. The superscript denoted by *pros* (abbreviated by the symbol π) refers to the site of methylation of the nitrogen atom of the imidazole ring nearer the alanine residue of histidine, and the superscript *tele* (abbreviated τ) refers to the site of methylation of the imidazole N of histidine farther from the alanine side chain (see *Biochem. J.* 1972, 126, 773-780).

EXPERIMENTAL METHODS

Preparation of Composite Meat Products. Each of the commercially blended all-beef sausage (S) and wiener (W) emulsions with condiments (+c) or without, used in these studies, were obtained immediately after mixing from ordinary commercial sources. Composite meat blends (1000 g) were then prepared with the appropriate additions (w/wet w) of soya-bean (SB) concentrate (49%) to W emulsion samples shown in Table 1. The samples were homogenized in a Lourdes stainless steel blender (Lourdes Instr. Corp., Brooklyn, N.Y.) operated at top speed (3 min; 5°C), dried to constant weight in vacuo (95-100°C) or by lyophilizaton, pulverized in an electric driven end-runner mill, passed through a 152 µm mesh sieve, and their proximate composition was determined by standard methods (13-15), summarized in Table 1.

Preparation of Tissue Hydrolysates. Triplicate samples (0.5 g) of all blends were hydrolyzed under vacuum (below 25 µm of Hg) with double glass-distilled constant-boiling HCl (6.0 M) containing 0.2% (w/v) phenol at 110±1.0°C for periods of 24, 48, 72 and 96 h. Foaming during evacuation or filtration was greatly suppressed by the addition of 5 µl octanol to each hydrolysate. After hydrolysis the tubes (Pyrex No. 9860) were scored and opened, the hydrolysates filtered through 0.22 µ type GS (24/25 mm) microfilters (Millipore Corp., Bedford, Mass.) into Pyrex tubes (18 x 150 mm) and dried under vacuum (below 750 µm of Hg) in a Rotary Evapo-Mix (Buchler Instr., Fort Lee, N.J.). The residue was taken up quantitatively in 5.0 ml pH 2.2 citrate buffer (0.2 M) and refiltered. Analyses of individual acid-hydrolysates were performed on the clear filtrate in duplicate by methods described previously (12-14).

Amino Acid Calibration Standards and Chemicals. The acquisition and preparation of all the unusual amino acid standards employed for peak identification and standardization of the instruments was as previously described (12-14). All other chemicals and reagents were of the highest purity commercially available and were used without further purification.

Procedures for Amino Acid Analyses. Amino acid analyses were carried out on either a Model 120C conventional (Method B) or a fully automated Beckman Spinco Model 121MB amino acid analyzer (Method C) using single-column methodology (12, 14) as follows:

Method B - Aliquots of 100 µl of the hydrolysate were applied to the special 60 x 0.9 cm column of Durrum type

-6A resin and initially developed with 0.35 M sodium citrate buffer, pH 5.700±0.002, on the Beckman Model
0 C analyzer operated at 27°C and 35 ml/h (0.92 cm min^{-1}). After the emergence of the neutral and acidic amino
ids (1 h), the regular ninhydrin reagent was pumped into the effluent stream at 17.5 ml/h (12). At 415 min
e buffer was changed to 0.35 M sodium citrate, pH 5.000±0.002, containing the same concentrations of octanoic
id (0.1 ml/ℓ) and phenol (1.0 g/ℓ).

thod C - In this system, aliquots of 20 µl of composite meat hydrolysate (0.1 g/ml) were applied to the
x 0.28 cm microcolumn of Beckman type W-3 (AA-10) resin and initially developed at 35°C (120 min) with sodium
trate buffer, pH 5.734±0.002, but at a flow rate of 5.35 ml/h (1.45 cm min^{-1}). After the emergence of the
tral and acidic amino acids (50 min), the dimethyl sulfoxide-ninhydrin reagent was pumped into the effluent
eam at 2.68 ml/h. At 125 min the temperature of the microcolumn was reduced to 28°C, and a second citrate
fer (0.35M; pH 5.000) containing the same concentrations of octanoic acid (0.1 ml/ℓ) and phenol (1.0 g/ℓ) was
roduced at 244 min so that both His(πMe) and His(τMe) emerged as discrete peaks following the ammonia peak
g. 1A).

RESULTS AND DISCUSSION

Previous work from this laboratory (12, 14) showed that all methylated basic amino acids, the diastereo-
mers of Lys(5OH), Ides, Des, GlcN and related compounds could be completely separated by a single-column
tem using Durrum type DC-6A or Beckman W-2(AA-20) spherical resins (11.0±1 µm). In the present study it was
nd that the smaller and more uniform spherical resin (9.0±0.5 µm) used permitted faster flow rates and re-
ted in a higher resolving power of all the unique basic amino acids most likely to be encountered in protein
tissue hydrolysates primarily because resin equilibrium was attained more rapidly. As may be seen in Fig. 1A,
selecting the optimum conditions of temperature, buffer and pH, and adjusting the flow rate to 1.45 cm min^{-1},
h of the basic components of the synthetic calibration mixture applied to the micro-column emerged as a dis-
te, well-separated peak in less than 7 h. Within the precision of the chromatographic procedure (±3.0%),
reproducibility of such determinations was perfect and recoveries were found to be quantitative.

Application of this accelerated method to composite meats was then tested with acid hydrolysates of com-
cially prepared all-beef wiener emulsion, sausage and hamburger. The chromatographic separations shown in
. 1B-C are typical of those obtained when the acid hydrolysates of two composite meat products were analyzed
this method. As shown in Fig. 1B-C the analysis of all-beef wiener with condiments (W+c) samples and a com-
ite wiener (W3) product containing 17.3% SB revealed the complete separation of the diastereoisomers of Lys(5OH)
His(τMe) along with eighteen as yet unidentified compounds that are stable to acid hydrolysis. Each of the
hteen unknown peaks was assigned an Arabic number from 1 to 18 to indicate its relative order of elution from
microcolumn. Although the identity of all these unknown compounds remains to be confirmed, the two major
hydrin-positive components, designated as peaks 17 and 18 (Fig. 1B-C), that eluted from the column after
(τMe) warrant further close investigation as possible markers for identifying specific proteins present in
h mixtures. These results clearly demonstrate the high resolving power of this simple microcolumn chromato-
phic system which can be conveniently used as a standard analytical method for the determination of all these
ual basic amino acids in protein or composite meat hydrolysates.

le 1. Proximate composition of commerically prepared all-beef wiener (W) and sausage (S) emulsions
with condiments (+c) or without, composite meat blends (1 to 7) and hamburger (H).

ple	Protein source in blend (g/kg; w/w) from		Dry matter (DM; g/kg)	Total N	Crude protein (N x 6.25)	Total lipid
	W	Soybean		(g/kg DM)		
	1000	-	412.8 ± 0.5†	39.77 ± 0.83	248.6 ± 5.2	688.3 ± 2.5
	958.3	7.2	348.4 ± 1.2	47.05 ± 0.90	294.1 ± 5.6	593.2 ± 2.7
	875.0	21.4	337.4 ± 4.7	49.30 ± 0.76	308.1 ± 4.8	606.7 ± 2.4
	833.0	28.6	339.7 ± 3.6	46.58 ± 1.36	291.1 ± 8.5	591.1 ± 4.3
	750.0	42.8	330.9 ± 2.1	50.17 ± 1.01	313.6 ± 6.3	569.9 ± 3.1
	675.0	64.3	299.0 ± 1.7	60.23 ± 2.97	376.4 ± 18	470.2 ± 2.9
	500.0	85.7	249.1 ± 5.8	66.63 ± 2.14	416.4 ± 13	368.7 ± 2.3
	250.0	128.6	220.9 ± 0.9	71.85 ± 1.25	440.1 ± 7.8	297.2 ± 3.8
	-	-	403.0 ± 0.5	45.32 ± 0.62	283.3 ± 3.9	555.2 ± 5.8
	-	-	408.5 ± 4.8	37.40 ± 1.70	233.8±10.1	705.9 ± 5.8
	-	-	436.1 ± 3.4	39.23 ± 1.02	245.2 ± 6.3	615.2 ± 4.2
	-	-	344.5 ± 4.5	78.82 ± 0.86	492.6 ± 5.4	453.0 ± 7.5

n values with standard deviations for six determinations.

The results obtained for the His(τMe) and Lys(5OH) content of composite wiener blends (W1 to 7) are pre-
ted in Table 2, and represent the average values from 24, 72 and 96 h hydrolyses. Since complete separation
ween His(τMe), His(πMe) and the major unknown peak No. 17 (Fig. 1) was achieved, and the reproducibility of
h determinations was excellent, the recoveries of His(τMe) were found to be quantitative. Similarly the
unts of Lys(5OH) present in such products can be quantitated accurately from the sum of the values obtained
the diastereoisomers after epimerization (12). From the results presented in Table 2, it is evident that a
ear relationship exists between the amounts of His(τMe) and Lys(5OH) found in the composite meat blends
to 7) analyzed and the percentage of meat emulsion (W) in the mixture. The results obtained from analyses
both high and low concentrations of these unusual amino acids in mixtures (W) clearly demonstrates the high
sitivity and resolving power of this method which has the added advantage that nanogram amounts of these
que compounds can be determined with an accuracy of less than ±3.0%. For comparison, Table 2 lists the

Table 2. The N^{τ}-methylhistidine and 5-hydroxylysine contents of commercially blended all-beef wiener (W) and sausage (S) emulsions with condiments (+c) or without, composite meat blends (1 to 7), and hamburger (H).

Sample	% N in blend	% Protein contribution to blend from W	Amino acid (g/16gN)	
			His(τMe)	Lys(5OH)
W	3.98	100	0.037 ± 0.0010†	0.266 ± 0.0002
W1	4.71	95.7	0.035 ± 0.0010	0.238 ± 0.0020
W2	4.93	87.1	0.033 ± 0.0010	0.209 ± 0.0010
W3	4.66	82.7	0.027 ± 0.0010	0.161 ± 0.0020
W4	5.02	74.3	0.025 ± 0.0010	0.129 ± 0.0030
W5	6.02	63.3	0.022 ± 0.0005	0.095 ± 0.0001
W6	6.66	54.0	0.016 ± 0.0010	0.082 ± 0.0001
W7	7.19	44.3	0.012 ± 0.0004	0.031 ± 0.0002
W+c	4.53		0.037 ± 0.0010	0.217 ± 0.0020
S	3.74		0.062 ± 0.0007	0.302 ± 0.0020
S+c	3.92		0.050 ± 0.0004	0.210 ± 0.0250
H	7.88		0.058 ± 0.0008	0.194 ± 0.0030

†Mean values with standard deviations for nine determinations.

His(τMe) and Lys(5OH) contents of commercially prepared sausages and hamburger samples. Both of these meat products were found to contain considerably higher levels of His(τMe) compared to wiener blends (W1 to 7) but lower Lys(5OH). Although the data reported in Table 2 are in reasonable agreement with those reported by other authors (8-9, 22), some differences have been noted. These may arise from the fact that other methods were employed for these determinations or because of variations in the levels of muscle or non-muscle proteins used in the preparation of such products. Until now 4-hydroxyproline [Pro(4OH)] was thought to be confined almost exclusively to the connective tissue fibrous proteins, collagens and elastin (19-20, 23), and has been used as an indicator of both the presence of collagen and elastin in tissues and the assessment of the connective tissue content of meats (1). However, the discovery in the present studies of small amounts of Pro(4OH) in both potatoes (0.46 g/kg d.wt.) and oat groats (0.22 g/kg d. wt.), and by others in higher plants, algae (21), and animal proteins including acetyl cholinesterase, Clq etc., (17-18), suggest that Pro(4OH) should no longer be used as an accurate index for determining the connective tissue content of either muscles or composite meats. From the foregoing results, it is evident that the mycofibrillar and connective tissue contents of prepared meats can be determined from the amounts of His(τMe) and Lys(5OH) present in their acid hydrolysates, respectively, and that this direct chemical approach could be easily applied in a routine basis for enforcing meat regulations. However, since the levels of His(τMe) and Lys(5OH) in various muscles and composite meats have not yet been established, a complete survey to determine the levels of these unique amino acids in skeletal, cardiac and smooth muscle proteins of the major meat yielding species and meat products would be required so that precise regulatory standards for these components can be established.

The author wishes to acknowledge the excellent assistance of Dr. Susan Loeb, Miss Carol Beddard and Mr. John P. Emery.

REFERENCES

1. Pearson, D. (1975) Analyst 100: 73-81.
2. Llewellyn, J.W. (1979) Intl. Flav. Fd Add. 10: 115-118 and 128.
3. Paik, W.K. and Kim, S. (1980) In Protein Methylation (Meister, A., ed.) Vol. 1; pp. 53-68, John Wiley & Sons, New York.
4. Horakova, M. and Deyl, Z. (1978) J. Chromat. 159: 227-314.
5. Perry. S.V. (1970) In The Physiology and Biochemistry of Muscle as a Food (Briskey, E.J., Cassens, R.G. and March, B.B., eds.), Vol. 2, pp. 537-553 and 588, Univ. of Wisconsin Press, Madison, Milwaukee.
6. Vickery, H.B. (1972) In Advances in Protein Chemistry (Anfinsen, C.B., Edsall, J.T. and Richards, F.M., eds.), Vol. 26, pp. 81-171, Academic Press, New York.
7. Llewellyn, J.W., Dean. A.C., Sawer, R., Bailey, F.J. and Hitchcock, C.H.S. (1978) J. Fd. Technol. 13: 249-252.
8. Rangeley, W.R.D. and Lawrie, R.A. (1976) J. Fd. Technol. 11: 143-159.
9. Skurray, G.R. and Lysaght, Y.A. (1978) Fd. Chem. 3: 111-114.
10. Bailey, A.J. (1978) J. Clin. Path. 31: Suppl. (Roy. Coll. Path.), 12: 49-58.
11. Zarkadas, C.G. (1974) Proc. Can. Fed. Biol. Soc. 17: 255.
12. Zarkadas, C.G. (1975) Can. J. Biochem. 53: 96-101.
13. Zarkadas, C.G. (1978) Can. J. Biochem. 56: 952-957.
14. Zarkadas, C.G. (1979) J. Appl. Biochem. 1: 148-163.
15. Hulan, H.W., Proudfoot, F.G. and Zarkadas, C.G. (1979). Br. J. Nutr. 41: 163-173.

16. Bornstein, P. and Sage, H. (1980) Ann. Rev. Biochem. 49: 957-1003.
17. Porter, R.R. and Reid, K.B.M. (1978) Nature 275: 699-704.
18. Adams, E. and Frank, L. (1980) Ann. Rev. Biochem. 49: 1005-1061.
19. Fischer, E. (1902) Chem. Ber. 35: 2660-2665.
20. Bentley, J.P. and Hanson, A.N. (1969) Biochim. Biophys. Acta 175: 339-344.
21. Lamport, D.T.A. (1977) Recent Adv. Phytochem. 11: 79-115.
22. Poulter, N.H. and Lawrie, R.A. (1980) Meat Science 4: 15-31.
23. Man, M. and Adams, E. (1975) Biochem. Biophys. Res. Commun. 66: 9-16.

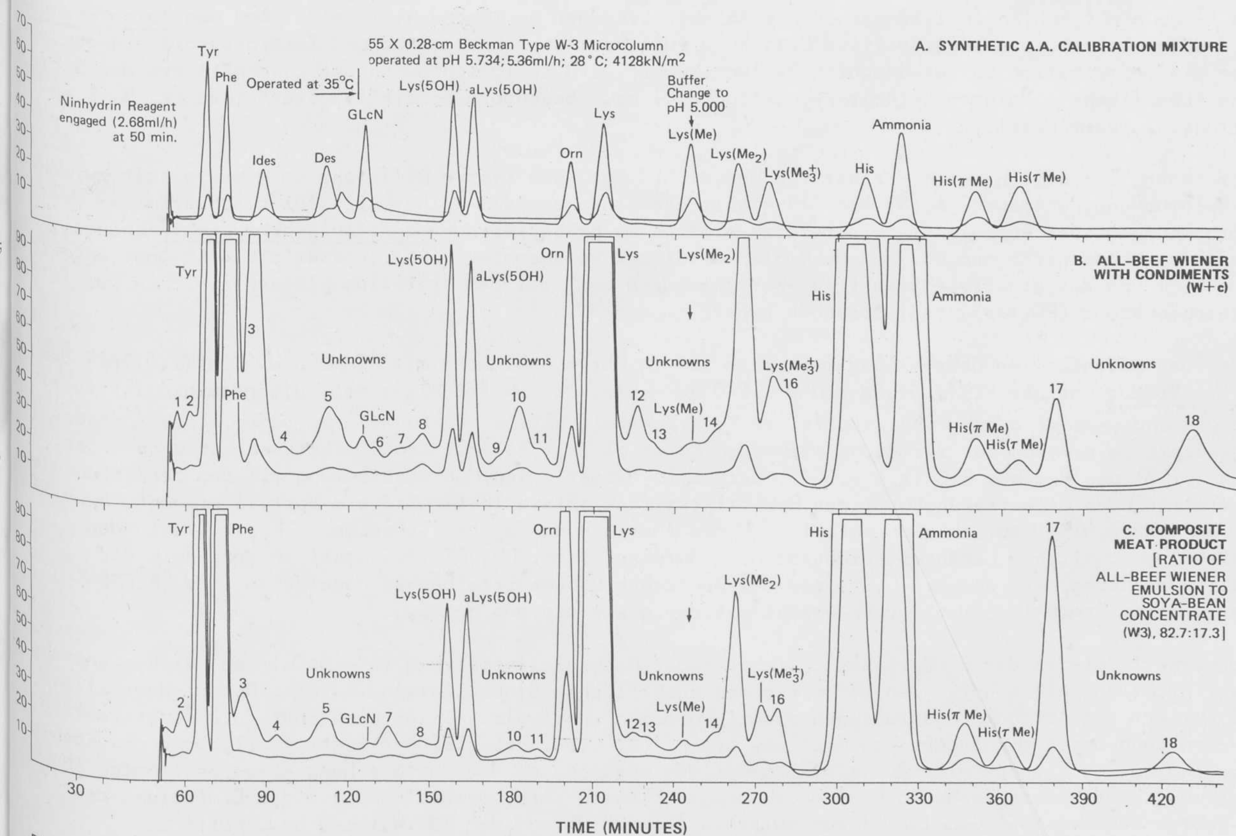

FIG. 1 Chromatographic separation of all methylated basic amino acids, the diastereoisomers of 5-hydroxylysine, stable crosslinking amino acids, and related compounds in typical 96h composite meat hydrolysates by Method C. The upper curve shows absorbance at 570nm and the lower curve the absorbance at 440nm.