THE QUANTITATIVE DETERMINATION OF THE MYOFIBRILLAR AND CONNECTIVE TISSUE PROTEINS IN SKELETAL MUSCLES AND COMPOSITE MEATS

BY

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of Skeletal Muscle and Meat Products

Dedicated to my family and to Anthoula

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ABSTRACT

The myofibrillar and connective tissue protein contents of the costal bovine Diaphragm have been determined by use of analytical chromatographic methods developed to quantitate the unique amino acids that occur in these proteins. Myosin and actin were determined from the amounts of N^{τ}-methylhistidine found in the acid hydrolysates of the intracellular muscle protein fraction which was separated from the extracellular matrix of the Diaphragm in sodium dodecyl sulphate (2%) and $CaCl_2$ (0.05M). The costal Diaphragm contained 11.4% actin, and 23.6% myosin corresponding to 17.5 and 36.3% of the myofibrillar proteins. The content of collagen (2.61%) in the SDS-insoluble extracellular fraction was determined from the amounts of 5-hydroxylysine and the elastin content (0.19%) from the amounts of desmosine and isodesmosine present.

The same direct chromatographic approach was used to quantitate the myofibrillar and connective tissue protein contents of typical composite meat products. Mixed meat products varied considerably in their myosin (11.5 - 16.3%), actin (5.5 - 7.8%), collagen and collagen like proteins (7.3 - 16.3%), and non-meat protein additives (27.5 - 49.3%).

Linear regression equations have been developed to effectively predict the myofibrillar and connective tissue contents of composite meat blends from an amino acid analysis. Since a linear relationship was found to exist between the meat content and the levels of N^{τ}-methylhistidine, 5-hydroxylysine, and desmosine present in model meat mixtures (bovine-Diaphragm - soy protein concentrate), this chromatographic approach could be easily applied for enforcing meat regulations.

RESUME

Le contenu en protéine des tissus myofibrillaires et conjonctifs du Diaphramme costal bovin a été déterminé en utilisant des méthods analytiques développées pour exprimer en termes quantitatifs les acides aminés uniques composant ces protéins. Les taux de myosine et d'active furent determinés à partir des quantités de N^{τ}-methylhistidine des hydrolysates de l'acide de la fraction de la protéine musculaire intracellulaire. Cette fraction a été séparée de la matrice extracellulaire du Diaphramme avec du sulfate dodécyl de sodium (2%) et du $CaCl_2$ (0.05 M). Le Diaphramme costal contenait 11.4% d'actine et 23.6% de myosine correspondant respectivement a 17.5% et 36.3% de la protéine myofibrillaire. Le contenu en collagène (2.61%) de la fraction extracellulaire non-soluble de SDS peut être calculé a partir des quantités de 5-hydroxylysine et celui de l'élastine, de la quantité de desmosine présente.

On utilisa une approche similaire pour déterminer le contenu en protéine des tissus myofibrillaires et conjonctifs de viandes préparées. Les protéines de myosine (11.5 - 16.3%), d'actine (5.5 - 7.8%) et conjonctifs (7.3 - 16.3%) de ces produits ainsi que leur contenu en protéines végétales (27.5 - 49.3%) varient considérablement.

Des formules de régression furent développées pour être en mesure de prédire adéquatement le contenu de tissus myofibrillaires et conjonctifs de viandes préparées, a partir de l'analyse des acides aminés. Puisqu'il existe un rapport linéaire entre les contenus en viande et les quantités de N^{τ}-methylhistidine, 5-hydroxylysine et desmosine présentes, cette approche chimique pourrait être utilisée couramment dans le but de renforcer les reglements sur la commercialisation de la viande.

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LIST OF ABBREVIATIONS

The abbreviations, nomenclature and symbols used in the present study are those recommended by the Editorial Board of the Journal of Biological Chemistry (1985).

aLys(5-OH)	Allohydroxylysine
Anserine	β-alanyl-L-N^{π}-methylhistidine
ATP	Adenosine 5'triphosphates
ADP	Adenosine 5'diphosphates
Balenine	β-alanyl-L-N^{τ}-methylhistidine
Carnosine	β-alanyl-histidine
Des	Desmosine
DNA	Deoxyribonucleic acid
EAA	Essential amino acids
EDTA	Ethylenediaminetetraacetate
GlcN	Glucosamine
GalN	Galactosamine
His(π-Me)	N^{π}-methylhistidine
His(τ-Me)	N^{τ}-methylhistidine
HPLC	High pressure liquid chromatography
iDes	Isodesmosine
Lys(Me)	N^{6}-methyl-L-lysine
Lys(Me_2)	N^{6}-dimethyl-L-lysine

$Lys(Me_3)^+$	N^6-trimethyl-L-lysine
Lys(5-OH)	5-hydroxylysine
Mr	Relative molecular mass
Nle	Norleucine
Pro(4-OH)	4-hydroxyproline
SDS	Sodium dodecyl sulphate
$Tyr(NO_2)$	3-nitrotyrosine

SECTION I

GENERAL INTRODUCTION

The quantitative determination of the myofibrillar and connective tissue proteins in vertebrate skeletal muscle tissues or meats has always been a difficult analytical problem. However, in order to understand the molecular and cellular mechanisms involved during muscle development and growth, especially among young or adult animals from the major meat yielding species, it is necessary to quantitatively establish the levels of these structural muscle proteins in either skeletal muscle tissues or meats. In addition, there has been a continuing interest in the development of standards for labelling pre-packaged meats, and reliable analytical methods, useful for evaluating the protein quality and for the assessment of the skeletal muscle, connective tissue and non-muscle protein content of composite meat products [Pellet and Young, 1984; Benedict, 1987].

Past efforts to assess the protein quality of meats and meat products have been based on the microscopic examination, immunological and electrophoretic determination, of skeletal muscle and non-muscle protein additives in composite meats [reviewed by Pearson, 1975; Llewellyn, 1978; Olsman and Slump, 1981; Olsman et al., 1985; McNeal, 1986; Benedict, 1987]. These methods, however, have had limited success 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 specific peptides derived from actin [Anderson, 1981; Vandekerckhove et al., 1986] or from soybean proteins [Llewellyn et al., 1978; Hitchcock et al., 1981; Agater et al., 1986], which have been proposed as markers for the quantitative determination of muscle and non-muscle proteins respectively in composite meats. These methods, although promising, may not be readily adaptable to routine analysis of processed meats.

One major method presently in use for determining the myofibrillar myosin, actin, tropomyosin and troponin contents of muscle tissues involves the quantitative extraction, solubilization and separation of the contractile proteins by one- or two-dimensional electrophoresis on polyacrylamide gels [Potter, 1974; Yates and Greaser, 1983a; Murakami and Uchida, 1985], followed by densitometry. The results obtained by this method of quantitating contractile proteins in muscles showed considerable difference in their myosin and actin contents. Pepe and Drucker (1979) and Yates and Greaser (1983a; b) have discussed the problems associated with this method of quantitating contractile proteins, and pointed out that most of the variation was attributed to densitometric protein measurements of stained gels because more than one protein frequently comigrates in a single band. The quantitation of protein concentration in the stained proteins bands has also been carried out by eluting the separated myosin subunits and actin bands and determining their concentrations either from the extracted dye [Murakami and Uchida, 1985] or by amino acid analysis corrected for comigrating protein bands [Yates and Greaser, 1983a]. These measurements have shown that the molar ratio of myosin to actin in skeletal muscles is 1.0/6.0 [Potter, 1974; Murakami and Uchida, 1985]. Other methods used for determining the myosin

to actin mass ratios have shown similar variations [Hanson and Huxley, 1957], primarily due to differences in the myofibrillar volume and protein content of the myofibrils among muscle types, as determined by quantitative electron microscopy [Eisenberg and Kuda, 1976; Page and Surak-Droske, 1979].

The quantitation of N^{τ}-methylhistidine [His(τ-Me)], a unique basic amino acid found in both myosin and actin [Elzinga et al., 1973; Vandekerckhove and Weber, 1978a; 1979a; Elzinga and Collins, 1977], has been proposed as an index for determining these two principal myofibrillar proteins in tissues [Perry, 1970; Hibbert and Lawrie, 1972], and their turnover rates in skeletal muscle [Young and Munro, 1978; Harris and Milne, 1981; Bates and Millward, 1983]. This quantitation is based on the following findings: firstly, that actin and its isoforms contain one residue of His(τ-Me) at position 73 in their amino acid sequence [Elzinga et al., 1973; Vandekerckhove and Weber, 1978a; 1979a]; secondly, that mammalian myosin prepared from fast-twitch white muscle fibers contains one residue of His(τ-Me) at position 69 in each of the two heavy chains [Elzinga and Collins, 1977; Huszar, 1984]; thirdly, that avian myosin prepared from fast-twitch, slow red, and cardiac muscle fibers contains almost the same levels of His(τ-Me) [Masaki et al., 1986]; and fourthly, that His(τ-Me) is absent from all other proteins [reviewed by Huszar, 1984; Paik and Kim,1980].

Various measurements of the His(τ-Me) content of vertebrate skeletal muscle, which have been made using a variety of chromatographic methods, have yielded variable amounts of His(τ-Me) among the muscle tissues and meats studied. Although some of this variation was attributed to the presence of variable amounts of fast, slow, or mixed fast/slow classes of

myosin heavy chain (MHC) isoforms in different muscle types [Huszar, 1984], most of this variation was due to incomplete separation of small amounts of His(τ-Me) from other compounds present in the complex tissue hydrolysates by the multicolumn systems employed [Hancock and Harding, 1984]. Another complication has been the presence in skeletal muscles of variable amounts of balenine [Carnegie et al., 1982; 1983; 1984], a sarcoplasmic histidine dipeptide (β-alanyl-L-N^{τ}-methylhistidine) which yields β-alanine and His(τ-Me) in equimolar amounts upon acid hydrolysis [Harris and Milne, 1987], and must be extracted from muscle tissues or meats prior to acid hydrolysis.

The extracellular connective tissue proteins of skeletal muscle, i.e., collagen, elastin, muscle fiber 'ghost' proteins, fibronectin, laminin and glycoproteins [Cheah, 1985; Light and Champion, 1984; Loewy et al., 1983; Carrino and Caplan, 1986], which are involved in maintaining proper alignment of the muscle fibers, have also been implicated in a variety of morphogenetic and developmental processes [Wiens et al., 1984], and can influence either the synthesis of non-actin myofibrillar proteins or the assembly of cardiac contractile proteins into myofibrils [Wiens et al., 1984]. Thus the anatomical arrangement of the connective tissue in each level of muscle organization, i.e., epimysium, perimysium, and endomysium, can regulate and control the extent of contraction in young and adult normal and diseased muscles [Borg and Caulfield, 1980; Foidart et al., 1981; Kovanen et al., 1980; 1984; Rowe, 1981; 1986; Orcutt et al., 1986]. Low levels of 5-hydroxylysine [Lys(5-OH)] and structural collagen defects have, for example, been recently reported as prime factors in avian muscular dystrophy [DeMichele et al., 1985; 1986].

The connective tissue content of skeletal muscle is usually calculated from the amounts of 4-hydroxyproline [Pro(4-OH)] found in tissue hydrolysates [reviewed by Pearson, 1975 and Berg, 1982], or by quantitative extraction of the intracellular skeletal muscle proteins from the extracellular matrix and the estimation of the collagen and elastin contents [McCollester, 1962; 1963; Rosenthal et al., 1965; Comissioning and Hultin, 1976; Laurent et al., 1981; Stanley, 1983; Light and Champion, 1984]. The ratio of the polymorphic type I and III collagens in muscle tissues have been quantitated from the amounts of unique CNBr peptides separated by SDS-polyacrylamide gels [Laurent et al., 1981; Light et al., 1985; Light and Champion, 1984] or by HPLC [Van de Rest et al., 1980]. Measurements of elastin in muscle tissues is based on the determination of desmosine (Des) or isodesmosine (iDes) found in tissue hydrolysates [Faris et al., 1981; Soskel, 1987]. Other methods of elastin quantitation rely on the determination of the dipeptide, Val-Pro, in alkaline hydrolysates of elastin [Paz et al., 1982], or on the quantitation by HPLC of a unique pentapeptide, Val-Gly-Val-Pro-Gly, found only in thermolysin digests of elastin and tropoelastin [Sandberg et al., 1986]. Both methods, however, have yielded variable results.

To further study the possibility of using His(τ-Me) as a valid index for determining the myofibrillar myosin and actin contents of skeletal muscles and composite meats, it became apparent that an accurate determination of His(τMe) in vertebrate skeletal muscle, including those unique amino acids found in muscle connective tissue proteins, i.e., collagen and elastin, is essential. For these reasons, analytical chromatographic methods

have been developed in this laboratory to quantitate all methylated basic amino acids, the diastereoisomers of Lys(5-OH), the stable elastin crosslinks Des and iDes and related compounds in proteins and tissues [Zarkadas, 1979; Zarkadas et al., 1986b; 1987a].

The major objective of the present study was to provide statistically sound data on the occurrence and variation of these unique amino acids in Diaphragm muscle excised from young bovine animals and three major meat products, representing about 60 to 70% of the total volume of processed meats. Emphasis in this survey was given to the determination of the His(τ-Me), Lys(5-OH), Des and related basic amino acid contents of the isolated intracellular muscle protein and the extracellular matrix fractions, prepared and quantitated by the combined methods of McCollester (1962), Comissioning and Hultin, (1976), Stanley (1983), and Laurent et al. (1981) from the bovine Diaphragm muscle. The data so obtained in this study will be essential to establish the myofibrillar and connective tissue protein levels in skeletal muscle and composite meats such that precise regulatory standards can be established for meat products.

In this approach the assessment of the protein quality of composite meats is based on the direct determination of their myofibrillar and collagen contents from the amounts of His(τ-Me) and Lys(5-OH) found in their acid hydrolysates, respectively. Elastin can also be determined from the amounts of Des or iDes present. When the sum of the myofibrillar and connective tissue proteins is subtracted from the total protein of a composite meat hydrolysate sample, the difference is an accurate assessment of the non-muscle proteins present. These calculations are based on the total

protein content of these composite meat products determined by their detailed amino acid compositions which are also reported in the present study. This direct approach has the advantage over other methods in that the determination of all these classes of proteins can be carried out in both fresh muscles or processed meats, as well as animal protein supplements, and may prove especially valuable for industrial control and formulation, and could be easily applied on a routine basis for the proper enforcing of meat regulations.

Another objective of the present study was to show whether or not these unique amino acids, reported to occur exclusively in the myofibrillar myosin and actin, and connective tissue collagen and elastin, are in fact absent from plant-derived protein additives and ingredients most frequently used to formulate composite meat products. Typical non-meat protein additives and ingredients were analyzed for their His(τ-Me), Lys(5-OH) and related basic compound contents by the chromatographic methods developed to quantitate these unique amino acids in proteins and tissues.

The present study was also undertaken to determine whether a linear relationship exists between the myofibrillar protein and collagen contents of composite meats and the amounts of His(τ-Me) and Lys(5-OH) present in meat blends. Composite meat blends were prepared with the appropriate incremental addition of soybean protein concentrate to skeletal muscle, i.e., bovine Diaphragm, and analyzed by the present methods [Zarkadas et al., 1986b; 1987a]. The aim was to establish whether regression equations could be developed that would effectively predict the myofibrillar and

connective tissue contents of composite meat blends from an amino acid analysis.

SECTION II

LITERATURE REVIEW

Four muscle types are currently known in vertebrates: skeletal, cardiac, vascular smooth and enteric [Pollard and Cooper, 1986]. The following review will be devoted to the description of the vertebrate skeletal muscle.

2.1 Embryology and Development of the Vertebrate Skeletal Muscle

All vertebrate skeletal limb and trunk muscles originate from segmented paraxial mesoderm cells called somites which give rise to the presumptive myoblasts [Konigsberg, 1986]. The presumptive myoblasts contain nonmuscle contractile proteins and cannot fuse or synthesize muscle proteins [Fischman, 1986]. These cells are converted to myoblasts, which are bipolar in shape and are defined as "postmitotic," mononucleated cells containing many ribosomes. The myoblasts are capable of synthesizing some actin and myosin filaments, they are not organized into myofibrils, and permanently withdraw from the cell cycle [Allen et al., 1979]. These myoblasts are subsequently fused into cylindrical, multinucleated cells having central nucleation and named syncytial myotubes (8 - 15 μm in diameter) [Fischman, 1986]. The fusion of the myoblasts is an extremely cell-specific process which occurs after the mononucleated myoblasts are aligned in a direction parallel to their long axes, guided probably by fibronectin [Wakelam, 1985]. The multinucleated myotubes do not synthesize DNA or longer divide, but contain primitive muscle fibers in which longitudinal myofilaments (myosin, β- and γ-actin) are present at the cell periphery [Squire, 1981] along with

α-actinin, desmin, and troponin-T [Gomer and Lazarides, 1983a]. The sarcoplasmic reticulum and the T-tubules are formed on existing myofibrils [Squire, 1981].

The primary myotubes grow in length by incorporating other myoblasts, giving rise to secondary myotubes. As these myotubes mature, their interior is filled with myofibrils and become surrounded by their own basal lamina. The muscle fibers become thicker as the myofilaments multiply and differentiate into thin (actin) and thick (myosin) myofilaments arranged in overlapping bands [Allen et al., 1979].

At birth, muscle has grown in length and thickness, nuclei are located to the fiber periphery just under the sarcolemma, and the myofilaments are aggregated into bundles to form myofibrils. Although the number of muscle fibers does not increase substantially after birth, there is a large increase in total muscle DNA provided by the satellite cells [Allen et al., 1979]. Myofibrils can lengthen by an increase of their sarcomere numbers or by an increase of the sarcomeres length [Ishikawa, 1983].

The development of the early fibers can proceed without direct innervation but the transformation of myotubes into embryonic muscle fibers requires neural control [Kelly and Rubinstein, 1986].

2.2 Anatomy of the Vertebrate Skeletal Muscle

2.2.1 The Myofibril

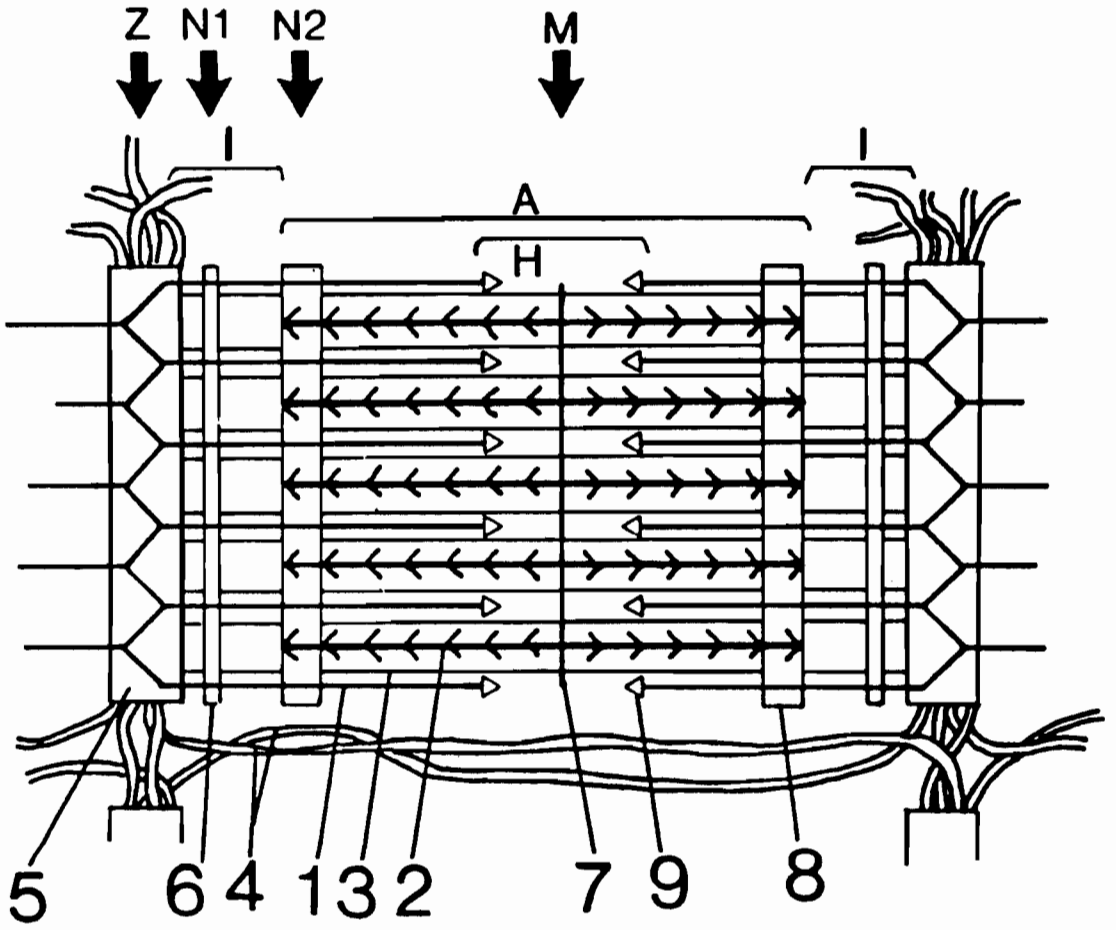
The skeletal muscle is composed of long, parallel, cylindrical muscle fibers, measuring 1 to 40 mm in length and 10 - 50 μm in width. Each muscle fiber (cell) is a multinucleated cell arranged in bundles called fasciculi

in which the fibers are longitudinally oriented and parallel to one another. Each muscle cell contains a large number of roughly cylindrical myofibrils (1 ≈ 2 μm in diameter) [Craig, 1986] composed of myosin and actin filaments. The myosin and actin filaments are parallel to the fiber axis [Lowey, 1986a], partly overlapping and highly organized in the basic unit of the myofibril contraction [Huxley and Brown, 1967] which is known as the sarcomere (2 ≈ 2.5 μm long). The sarcomere is the repeating unit giving the myofibril the characteristic striated appearance when observed under a light microscope. In the middle of the sarcomere is the A-band (Figure 1) (anisotropic band observed in the polarizing microscope) mainly composed of myosin filaments and C-protein. The region between two consecutive A-bands is called I-band (isotropic in polarized light) (Figure 1). Each half of the I-band is constructed by the F-actin filament (F for filamentous), which is in longitudinal register together with tropomyosin and troponin proteins. The actin filaments extend up to the H-band which resides in the middle region of the A-band (Figure 1) and therefore contains only thick filaments. The center region of the H-zone is called the M-band and is occupied with the rod-shaped light meromyosins tails of the myosin heavy chains [Squire, 1981]. The M-bridges and M-filaments are the structural elements of the M-line which probably stabilizes the longitudinal and transverse order of the thick filament [Craig, 1986] by connecting each thick filament with its five neighbours to make an hexagon. In the centre of the I band localizes a dense line known as the Z-line or Z-disc separating one sarcomere from the next and exactly aligned in all the myofibrils (Figure 1). The Z-line is composed of non-contractile proteins. Two faintly stained transverse

Figure 1

Diagram showing filament relationships in striated muscle sarcomere. The various structural components are as follows: (1) Thin filaments; (2) thick filaments; (3) connectin filaments; (4) intermediate filaments; (5) Z-lattice; (6) N1-line; (7) M-line; (8) N2-line; (9) end capping of thin filaments.

(From Sheterline, 1983)


Z
N1
N2
M
I
I
A
H
5
6
4
1
3
2
7
9
8

structures, the N1 and N2 lines, have been located within the I-band (Figure 1) but are not always observable in every striated muscle [Yarom and Meiri, 1971].

2.2.2 The Muscle Fiber

The basic structural unit of skeletal muscles are the muscle fibers arranged in bundles of fasciculi enveloped in a thin structure of connective tissue called the epimysium [Rowe, 1981]. The fasciculi (containing 30 ~ 40 muscle fibers) are separated from each other by a layer of connective tissue called the perimysium. The surface of each muscle fiber is surrounded by a lipid-protein membrane called the sarcolemma. The sarcolemma is composed of layers of plasma membrane which surrounds the muscle fiber, the glycocalyx and the basement membrane [Sanes, 1986]. The basement membrane is comprised of the basal and the reticular lamina. The basal lamina is formed by a network called lamina densa and a more dense area called lamina rara. The basal lamina provides external mechanical support for the fiber and facilitates muscle fiber regeneration; it contains type IV collagen, glycoproteins, glycolipids, aminoglycans and is associated with fibronectin [Sanes, 1986]. The reticular lamina contains collagen and other fibrillar proteins. On the basal lamina lies a network of connective tissue called endomysium composed of collagen and elastin [Rowe, 1986]. Endomysium and sarcolemma contribute to the elasticity of muscle and connect the muscle fiber to the tendinous part of the muscle [Sanes, 1986].

At the outer regions of the A-band, the myofibril is surrounded by a net of adjacent sheaths of anastomosing, flattened vesicles derived from the

endoplasmic reticulum called sarcoplasmic reticulum (SR) [Franzini-Armstrong, 1986]. The SR plays a very important role during the excitation-contraction coupling, by supplying calcium ions stored in its lumen interior compartment into the cytocol or by "pumping" Ca^{2+} back into the lumen of the SR, and thus is inhibiting contraction. The electrical signal for the Ca^{2+} release is carried by a series of membranous folds, the transverse tubules (T-tubules). This membrane system surrounds each myofibril at the level of the junction of the A and I bands [Ishikawa, 1983]. The SR is closely associated with the T-tubules forming complexes known as triads and feet or spanning protein constituted by calsequestrin which is a protein with a large capacity to bind Ca^{2+} [Franzini-Armstrong, 1986].

Inside the plasmalemma of the muscle cell there are also the basic common structures of mammalian cells: mitochondria, glycogen, lysosomes; golgi apparatus, and myosatellite cells which are closely associated with the skeletal muscle fiber and reside on its surface covered by the basal lamina [Ishikawa, 1983].

2.3 The Muscle Proteins

Muscle proteins within the muscle fiber can be classified in intracellular and extracellular proteins which reside inside and outside the sarcolemmal membrane of the muscle cell, respectively. The intracellular muscle proteins can be further classified into (1) the myofibrillar proteins, (2) the sarcoplasmic proteins, and (3) the intermediate filament or proteins of the cytoskeleton.

2.3.1 The Myofibrillar Proteins

The proteins that comprise the myofibrils within the muscle fiber are defined as myofibrillar proteins. They constitute 55 - 65% of the total muscle proteins or 10% of the weight of the vertebrate skeletal muscle [Yates and Greaser, 1983b]. Based on their location within the microfibril, myofibrillar proteins can be further classified into (1) the thick filament proteins and (2) the thin filament proteins.

2.3.1.1 The Thick Filament Proteins

Myosin

Myosin is the major protein of the thick filament located at the A-band of the sarcomere and is the principal protein involved in the contraction-relaxation cycle of the vertebrate muscle cell. It comprises 48 mg per g of fresh muscle or 430 mg/g of myofibril (range 380 - 520 mg) [Swynghedauw, 1986; Yates and Greaser, 1983b]. Each myosin molecule is about 160 nm long and 10 - 13 nm in diameter with a molecular weight of 500,000 [Lowey, 1986a]. There are about 300 myosin molecules per thick filament. The distance between adjacent levels of cross-bridges has been established to be 14.3 nm. Some researchers have suggested two [Lamvik, 1978], others have proposed three [Maw and Rowe, 1980], while still others consider four [Morimoto and Harrington, 1974; Pepe and Drucker, 1979] myosin bridges at each 14.3 axial repeat.

A myosin molecule is an assymetric hexamer, composed of two similar large polypeptide chains called "heavy chains" [Gazith et al., 1970], each having a Mr of about 200,000 [Lowey, 1986a] and three to four non-covalently associated small chains called "light chains" [Weeds and Lowey, 1971;

Stracher, 1969]. These myosin light chains are localized in the neck region of S-1 where the C-terminal heavy chain region is present. One of the light chains isolated with the sulphydryl reagent 5,5'dithiobis(2-nitrobenzoic acid) (DTNB) is called "DNTB light-chain" or RLC-2 (Mr-18,000) [Lowey, 1986a]. Another chain soluble in alkali is termed "alkali light-chain-2" or ALC-3 (Mr 16 - 16.5) and the one with a Mr of approximately 25,000 on SDS gels (21,000 by sequencing) [Lowey, 1986a] is designated "alkali light-chain 1" or ALC-1 [Frank and Weeds, 1974]. A probable role of light chains is to stabilize the myosin molecule against denaturation [Szent-Gyorgyi and Chantler, 1986]. The alkali light chains are essential to the ATPase activity of myosin, because they can be removed without destroying the ATPase activity of the heads. The alkali light chains have the ability to bind to the C-terminal region of actin [Trayer et al., 1987] or to control the lenght of synthetic thick filaments [Margossian et al., 1987]. The regulatory light chains (DTNB chains) are probably functioning in modulating the flexible region (between S1, S2 subfragments) of heavy chains while binding with actin during cross-bridge formation [Burker et al., 1983].

The COOH-terminal half of the heavy chain has an α-helix conformation and associates with a second heavy chain to form a double-stranded super-helix which is responsible for the assembly of myosin into thick filaments [Lowey, 1986a]. The N-terminal end of each heavy chain is folded separately to form a globular structure, associated with one essential and one regulatory light chain, which looks like an elongated pear-shaped lobe called head (19 nm long and 7 nm wide). Both heads of the myosin molecule

contain one ATP-binding site and have equivalent to each other's physiological function [Wagner and Weeds, 1977]. The heads of myosin are not necessarily identical [Craig, 1986]. The remaining α-helical region of the heavy chains is termed tail or rod; it is 150 nm long and extremely thin (2 nm) and closely packed in the filament backbone [Lowey, 1986a]. A region of the myosin tail lacks α-helical conformation and is susceptible to proteolytic enzyme action (e.g., trypsin, chymotrypsin and papain) [Lowey, 1986a]. Papain, for example, cleaves the myosin heavy chain into segments at a location 40 nm from the head/tail junction called "hinge domain". The cleaved globular head is known as heavy meromyosin (HMM; Mr of 350,000) and the remaining part is known as light meromyosin (LMM; Mr of 150,000). The HMM can be further cleaved by papain or trypsin to two subfragments called subfragments, S-1 and S-2 [Lowey, 1986a]. When the S-1 subfragment (95,000 Da) is subjected to limited tryptic digestion yields three major fragments of 25, 50 and 20 kDa [Harrington and Rodgers, 1984]. The nucleotide binding site is in the 25 kDa region and the actin-binding sites are found in both the 20 and 25 kDa peptides. In addition, the 20 kDa peptide contains two important thiol groups, SH1 and SH2, which are probably close to the ATP binding site [Harrington and Rodgers, 1984].

About 17% of the total amino acid residues found in myosin are basic, 18% are acidic and 38% have polar side groups [Kominz, et al., 1957]. Three unusual amino acids, namely N^{τ}-methylhistidine [His(τ-Me)], N^{6}-mono-methyl-lysine [Lys(Me)] and N-trimethyllysine [$Lys(Me_3)^+$] are present in the HMM region [Johnson and Perry, 1970; Kuehl and Adelstein, 1969; Hardy et al., 1970; Huszar, 1972]. Myosin from white fibers contains 2 His(τ-Me) residues

per myosin molecule [Huszar and Elzinga, 1971] at position 69 in each of the two heavy chains and 1 residue of Lys(Me), whereas myosin from red (slow) and cardiac muscle is devoid of them [Kuehl and Adelstein, 1970]. Fast muscles of unborn or newborn animals lack His(τ-Me) and Lys(Me). In contrast, 2 residues of $Lys(Me_3)^+$ per myosin molecule are found in all the mammalian, skeletal and cardiac muscles examined (Paik and Kim, 1980). The lack of histidine methylation in mammalian cardiac muscle is due to the absence of the histidine methylating enzyme from the cardiac muscle cells [Huszar, 1984]. In myosin prepared from white rat muscle the low levels of His(τ-Me) at birth increase and reach the adult levels about five weeks postnatal [Trayer et al., 1968; Kuehl and Adelstein, 1970; Huszar and Elzinga, 1972]. Some researchers have found the same age-dependent increase in the Lys(Me) and $Lys(Me_3)^+$ levels, whereas others reported that the levels of these two amino acids did not vary significantly during the life span [Paik and Kim, 1980]. Myosin prepared from avian fast-twitch muscle contains 1 residue of His(τ-Me) at position 754, 1 residue Lys(Me) at position 35 and 2 residues $Lys(Me_3)^+$ at positions 130 and 550 of the S1 heavy chain sequence [Maita et al., 1987]. Avian cardiac and red myosin contain the same levels of His(τ-Me) when compared to the levels of His(τ-Me) from the avian fast white myosin [Masaki et al., 1986). Other proteins such as histones, cytochromes, calmodulin, ribosomal and flagellar proteins contain methylated lysines but His(τ-Me) is constituent only of actin and myosin. Another methylated basic amino acid found in foetal but not in adult myosin is ω-N,ω'-N-dimethylarginine [ω'-$Arg(Me_2)$] [Reporter and Corbin, 1971].

The biological significance of methylated histidines remains to be elucidated. The ability of myosin to combine with actin does not seem to require the absence or presence of His(τ-Me) [Paik and Kim, 1980]. Tyihak et al. (1977) reported that methylation of the basic lysine and arginine may be significant in cell regulation proliferation. The basic amino acids are N-methylated while the polypeptide is still being synthesized on the ribosome. Additionally, N-methylation can occur on the nascent polypeptides in the absence of protein synthesis [Paik and Kim, 1980].

Non-Myosin Proteins of the Thick Filament

The vertebrate skeletal myofibril hosts a number of proteins other than myosin constituting up to 15% of the total proteins in the thick filament. The names of these proteins derive from their mobility on SDS polyacrylamide gels. Their presentation below is according to their location within the sarcomere. Their amounts in the myofibril and Mr are presented in Table 1.

A-band:

C-protein: It is an elongated molecule with no α-helical conformation, high Pro content that binds myosin at the light meromyosin and S-2 domains [Craig, 1986]. It also binds to F-actin, playing a regulatory role in the myosin and actin interactions [Yamamoto, 1986].

I-protein: It is found in the A-band except in the H-zone and has distinctly high Glu and Asp levels (36% of the total amino acid residues).

F-protein: It is considered to be the enzyme phosphofructokinase constituting 0.1% of the myofibrillar mass (Mr = 121,000) [Miyahara et al., 1980].

Table 1. Some Characteristics of Contractile Proteins of Vertebrate Skeletal Muscle[a].

	Amount in the Myofibril, %[b]	Mr	Function
A-band			
Myosin	43.0	521,000[b]	Cross-bridge and rigor bond formation; force generation.
C-protein	2.0	135,000 - 140,000	Maintain the thick filament integrity
I-protein	0.1[a]	50,000	Prevents unnecessary ATP hydrolysis during relaxation
F-, H-, X-proteins[c]	<1		Bind to myosin
M-line			
Creatine kinase (M-bridges)	<1	43,000	
Myomesin (M-filament)[d]	2.0	165,000	Binds to myosin
I-band			
Actin (G-form)	22.0	42,785	Involved in force generation
Tropomyosin	5.0	65,000 - 80,000	Assists in regulation of contraction
Troponin-C		18,000 - 21,000	Ca^{2+} regulation
Troponin-I	5.0	20,500 - 24,000	Inhibition of myosin-actin interaction
Troponin-T		30,500 - 37,000	Binds to tropomyosin
β-Actinin (free ends)	<1	62,000 - 71,000	Limits length and interaction between thin filaments
γ-Actinin	<1	70,800 - 80,000	Inhibits polymerization of G-actin to F-actin
Filamin[e] (also Z-line)	<1	230,000	Gelation of F-actin

Table 1 continued...

Table 1 continued

	Amount in the Myofibril, %[b]	Mr	Function
A-I Junction			
Titin[f]	10	1,200,000	Connects Z-lines in the same myofibril
N-line			
Nebulin[g]	5	550,000	
Z-line			
α-Actinin	2	190,000 - 210,000	Actin modification
Eu-Actinin[h]	<1	42,000	Binds to actin
Periphery of Z-line			
Desmin	<1	50,000	Links adjacent myofibrils
Synemin[e]	<1	220,000	Associates with desmin and vimentin
Vimentin[e]	<1	58,000	Forms transverse structures

[a]Summarized from Dutson and Calkins (1982).

[b]From Yates and Greaser (1983b).

[c]From Starr and Offer (1983).

[d]From Masaki and Takaiki (1977).

[e]From Granger and Lazarides (1979; 1980).

[f]From Wang et al. (1979).

[g]From Wang (1981).

[h]From Kuroda et al. (1981).

H-protein: It binds to myosin at a 1.46:1 (mol/mol) ratio, has a Mr = 74,000 and a Pro content similar to that of C-protein (Starr and Offer, 1983).

X-protein: It has a Mr of 152,000 and is found in slow and fast red fibers (Table 2) (Starr and Offer, 1983).

M-line:

M-protein: It is a single polypeptide chain with 13% α-helical and 35% β-pleated sheet structure which binds to the S-2 fragment of myosin at 0.8:1 mol ratio. M-protein has mostly been examined in chicken Pectoralis muscle. During enbryogenesis, M-protein is expressed in all fibers but during development its expression depends upon the muscle, species, fiber type and neural control. However, it is absent from cardiac and slow muscles [Grove et al., 1985]. In the M-band two more proteins are present: myomesin and creatine kinase (2 subunits) (Table 1).

2.3.1.2 Proteins of the Thin Filament

Actin

Actin is the major protein of the thin myofilaments (I band of the sarcomeres) and accounts for 25 mg/g of fresh tissue or 220 mg/g of myofibrillar protein (range 120 - 240 mg) [Swynghedauw, 1986; Yates and Greaser, 1983b]. The actin sequence is comprised of 374 residues with a Mr of 41,785 [Elzinga et al., 1973]. Actin binds 1 mol each of nucleotide (ADP or ATP) and Ca^{2+} or Mg^{2+} ions therefore, its Mr would account for 42,300 [Pollard and Cooper, 1986]. Actin exists in two forms: a globular (G-actin) and a filamentous (F-actin), depending upon the conditions. When

monovalent (KCl) or divalent ($MgCl_2$, $CaCl_2$) salts are added in solutions of G-actin, it polymerizes into F-actin in four steps [Pollard and Cooper, 1986]. The formed F-actin is 6 mm wide double-stranded, having a diameter between 95 and 100 Å, with right-handed helical structure whose half pitch it 37 nm, composed of 13.14 monomers [Craig, 1986]. The polypeptide chain of the F-actin is composed of β-pleated sheet surrounded by many α-helical chains, whereas G-actin contains approximately one quarter β-pleated sheet, one quarter α-helix and the rest random coil [Pollard and Cooper, 1986].

About 33% of the amino acid residues in actin have polar side chains, 13% of the residues are basic and about 2% are acidic [Kominz et al., 1957]. A phenylalanine residue is present at the COOH terminal end of the actin chain, whereas at the NH_2 terminal end an acetylated aspartic residue, added after synthesis, is present [Pollard and Cooper, 1986]. An unusual amino acid, His(τ-Me) has been located at position 73 in the amino acid sequence of actin [Elzinga et al., 1973; Vandekerckhove and Weber, 1978a; 1979a]. Smooth, cardiac, red skeletal, invertebrate and foetal rabbit skeletal actin contain the same levels of His(τ-Me) (1 residue per actin molecule) as adult rabbit skeletal actin [Paik and Kim, 1980]. The function of His(τ-Me) at position 73 is not known [Paik and Kim, 1980]. Solomon and Rubenstein (1986) reported that this methylated residue is not required for amino terminal post-translationsl processing or polymerization of actin <u>in vivo</u>. There are no methylated lysine or arginine residues in the actin sequence.

The sequence of actin is conservative during evolution; the chemical and physical structure of the molecule are very similar in <u>Physarum</u>, bovine cardiac and rabbit skeletal [Vandekerckhove and Weber, 1978a; 1978c; 1978d;

1979a; 1979b]. The actins from skeletal and cardiac muscles have been termed as α-actins, while those from non-muscle tissues (e.g., liver and brain) of higher vertebrates are designated β- and γ-isoactins; these isoactins have identical molecular weights but different isoelectric points. At least six different actin isoforms are found to be present in higher vertebrates [Vandekerckhove and Weber, 1978b]. One each in skeletal and cardiac muscles and two each in smooth and non-muscle tissues (such as brain and thymus) displaying more than 90% sequence homology. Both skeletal and cardiac actins are coexpressed to variable levels in striated muscles. In adult skeletal muscles, cardiac actin comprises less than 5% of the total actin, whereas in embryonic skeletal muscles is the major form (80%) [Vandekerckhove et al., 1986].

Actin and myosin are also present in the cytoplasm [Pollard, 1981]. In non-muscle cells actin is in excess of myosin e.g., in human platelet actin constitutes about 10% of the total protein whereas myosin only 1%, and in Acanthamoeba 14% and 0.3%, respectively. The actin to myosin ratio in skeletal muscle was found to be 6.0:1.0 [Murakami and Uchida, 1985], whereas in non-muscle cells this ratio was found to be too high (110/1) [Pollard, 1981]. In contrast to actin, the myosin of non-muscle cells is remarkably variable in size, shape, primary sequence and enzyme activity [Schliwa, 1986].

There is a number of proteins, the so called actin-binding or actin-associated proteins [reviewed by Pollard and Cooper, 1986; Schliwa, 1986], that specifically interact with cytoplasmic actin. The capping proteins constitute a class of proteins that bind to the actin filament and stabilize

its length and inhibit loss or addition of subunits. Other actin associated classes of proteins are the crosslink proteins (α-actinin), the bundling proteins, and the membrane attachment proteins (vinculin, Mr = 130,000) [reviewed by Pollard and Cooper, 1986].

Regulatory Proteins

This class of proteins is not directly involved in cross-bridge formation but plays a role in the contraction-relaxation cycle [Craig, 1986]. This group includes the tropomyosin and troponin complex of proteins.

Tropomyosin

It comprises 5% (50 mg/g) of the myofibrillar mass [Yates and Greaser, 1983b] (Table 1) and is composed of an α-chain and a β-chain of 34 and 36 kdaltons, respectively with α-helical conformation [Craig, 1986; Perry, 1986]. Tropomyosin blocks actin-myosin interaction; it is probably associated with actin filaments at the Z-line and within the myofibril in a similar manner [Kamisova et al., 1986].

Troponins

This is a group of three non-covalently linked subunits, with a total molecule length of 26 nm. Troponin-T (TN-T) which binds to tropomyosin, Troponin-C (TN-C) which binds with Ca^{2+} ions and Troponin-I (TN-I) which inhibits actomyosin ATPase [Craig, 1986]. The molar ratio of TN-T:TN-C:TN-I is 1:1:2, respectively; the molar ratio of actin, TN-T, TN-C, TN-I and tropomyosin is 7:1:1:1:1. The troponins constitute 50 mg/g myofibrillar protein [Yates and Greaser, 1983a] (Table 2). Troponin-C consists of 159 amino acid residues and contains four Ca-Mg binding sites and forms complexes with TN-I and TN-T. Van Eyk et al. (1986) calculated that binding of

Mg^{2+} to these sites of TN-C inhibits the actomyosin ATPase and therefore promotes relaxation whereas Ca^{2+} binding promotes contraction.

Other I-band Proteins:

β-actinin: It is a heterodimer that inhibits the formation of F-actin in solution. It comprises 0.1% of the total myofibrillar protein mass [Pollard and Cooper, 1986].

γ-actinin: It is a homodimer rich in serine and glycine (about 25% of the total amino acid residues); it contains one Cys residue which seems to be important for the biological role of the molecule. γ-actinin prevents the polymerization of actin during the nucleation step [Pollard and Cooper, 1986].

Proteins of the Z-band:

α-actinin: It is a homodimer containing about 74% α-helix; it is 30 - 40 nm long, 2.4 nm in diameter and connects actin filaments at a regular interval of about 30 nm [Craig, 1986]. α-actinin accelerates polymerization of G-actin to F-actin when its molar ratio to eu-actinin is 1:2 and can bind 1 or 2 filaments of actin [Masaki and Takaiki, 1969]. The α-actinin to actin ratio in skeletal muscle has been calculated to be 0.197 ± 0.049. A positive significant correlation between α-actinin and Z-line width has also been reported [Schachat et al., 1985; Yamano et al., 1986].

Eu-actinin: It comprises 1 - 2% of the total myofibrillar mass [Kuroda et al., 1981] together with α-actinin; it is a dimer with a composition similar to that of actin except of its low proline content; it promotes

actin polymerization, though it has higher affinity for α-actinin than for actin [Kuroda et al., 1981].

Z-protein: It constitutes 0.3% of the total myofibrillar protein and has a Mr = 55,000. Ohashi and Maruyama (1979) isolated this protein after the removal of myosin from KI-insoluble residue of skeletal muscles. Z-protein does not interact with either actin or α-actinin.

Z-nin: It constitutes 0.4% - 0.8% of the total myofibrillar mass and has a Mr of 300,000 - 400,000 [Suzuki et al., 1983].

2.3.1.3 Muscle Contraction

Huxley and Niedergerke (1954) proposed that muscle sarcomeres shortened due to the filament sliding past one another, while the filaments themselves retain a constant length [Huxley and Brown, 1967] (e.g., the I-band that is the part of the actin microfilaments not covered by myosin decreases in length and the dense A-band remains unchanged). This proposed model is known as the sliding filament model of muscle contraction and is the most accepted mechanism of force production in eucaryotic cells [Cooke, 1986].

The hydrolysis of ATP to ADP and inorganic phosphate (Pi) provides the energy during the contraction of the muscle cell. The accepted model of the muscle contraction cycle is briefly the following: a heavy meromyosin head in the presence of ATP forms a charged intermediate form that binds to an actin filament. The complex of myosin-actin-ATP is hydrolyzed to ADP + Pi, but the hydrolysis products remain bound to the myosin head, which is energized and rotates at the flexible hinge region to a perpendicular to the

thin filament position (Pollard, 1987). A rise in intracellular Ca^{2+} concentration leads to the binding of the myosin head to an adjacent filament (Cooke, 1986). This myosin head undergoes local and global structural changes and it pulls by moving to a 45° angle with the actin filament [Lowey, 1986a]. The resulting "rigor complex" remains until a new ATP molecule binds to the myosin head detaching it from the thin filament. The bound ATP molecule is hydrolyzed to ADP and Pi and the myosin head returns to its original conformation and is ready for a second cycle (Pollard, 1987). In the intact muscle, in the absence of ATP, the overlap region of the sarcomere is strongly cross-linked, because the myosin heads are bound to the adjacent actin filaments and remain combined. This is the rigor state of the muscle and it is similar to rigor mortis that occurs after death because of the loss of ATP production [Cooke, 1986]. According to Huxley and Kress (1985), during contraction there is a 40% of weakly and 20% of strongly bound myosin heads to the actin filaments. The carboxy-terminal of the tail and the thick filament are not required for movement along actin [Hynes et al., 1987]. During force production of vertebrate skeletal muscle only myosin molecules with two native heads are involved, which do not cooperate in catalyzing ATP [Hynes et al., 1987]. There is no preferential binding of one of the two HMM heads to actin in the presence of ADP or at an equimolar actin to myosin heads ratio [Pliszka, 1987].

2.3.1.4 Muscle Fiber Types and Their Contractile Protein Isoform Spectrum

The overall properties and performance of a vertebrate skeletal muscle under various functional demands, primarily depend upon its fiber

composition. Muscle fibers have been classified on the basis of glycogen content, mitochondrial enzymic activity, fatigue resistance and type of contraction as presented in Table 2. The fast-twitch fiber subtypes IIA, IIB and the intermediate IIC (Table 2) are differentiated histochemically based on differences in the pH stability of their myofibrillar actomyosin ATPase activity [Gauthier, 1986; Staron and Pette, 1987a].

Each of the subunits of the myosin molecule exists in different isoforms or isoenzymes; these terms are used to describe proteins that arise from the same gene and have similar biological activity but different structures [Swynghedauw, 1986]. Based on biochemichal and immunochemical studies in skeletal muscles, the heavy chain has two isoforms, DTNB light chain has three and alkali light chain has four [d'Albis et al., 1979; Perry et al., 1984]. Among the various fiber types myosin exists as isoenzyme with similar or different ATPase activity [Young, 1982] and structures.Different heavy chain are linked with the same or different myosin light chains (MLC) [Gauthier and Lowey, 1977]. The MHC polymorphism has been demonstrated by means of biochemical analysis [Huszar,1972] and electrophoresis under non-denaturing conditions [d'Albis et al., 1979; Fitzsimons and Hoh, 1981]. In type I (slow-twitch) fibers, three slow isomyosins can result from the combination of distinct MHCs, alkali and regulatory light chains [Lowey, 1986b; Staron and Pette, 1987a;b]. Slow skeletal muscle myosin has a lower ATPase activity when compared to that of fast myosin. Adult slow myosin is not methylated [Kuehl and Adelstein, 1970].

Table 2. Vertebrate Adult Skeletal Muscle Fiber Types : Physiological, Structural, Histochemical and Biochemical Properties[a].

	White fast IIB	Intermediate IIC	Red fast IIA	Red slow I	Superfast IIM
Motor Unit Type	FF	F (int)	FR	S	-
Contraction Time	Fast	Fast	Fast	Slow	-
Fatigue Resistance	Low	Int.	High	High	-
Z-line	Narrow	Narrow	Wide	Wide	Narrow
Mitochondria (SDH)	Low	Int.	High	High	-
Glycolytic(G) or Oxidative(O)	G	O	O	O	G + O
Myosin Histochemistry					
ATPase (pH 10.8)	High	High	High	Low	High
ATPase (pH 4.6)	Moderate	Moderate	Low	High	Very high
ATPase (pH 4.4)	Low	Low	Low	High	Very high
Main Isoforms					
Myosin Heavy Chain	MHC-fB	f + s	MHC-fA	s(s1 + s2)	Superfast
Tropomyosin	αα			αβ	
Troponin	f			s	
Mammalian Synonyms					
Peter et al., 1972	FG	-	FOG	SO	-
Brooke and Kaiser, 1970	IIB	-	IIA	I	-
Ashmore and Doerr, 1971	α-W	-	α-R	β-R	-
Barnard et al., 1971	Fast-twitch white	-	Fast-twitch red	Slow-twitch intermediate	-

[a]Summarized from Gauthier (1986) and Swynghedauw (1986).

Type II (fast-twitch) fibers have stronger reaction for myofibrillar actomyosin ATPase activity than type I fibers in alkaline pH. The IIA fibers are represented by the MHC-fA and the IIB fibers by the MHC-fB type of heavy chain (Table 2). The myosin light chains of these fast-twitch fibers are identical, but different from those present in type I fibers [Staron and Pette, 1987a; b]. By using non-denaturing conditions on pyrophosphate gel [Hoh et al., 1976], myosin from fast skeletal muscles has been shown to exist as three isomyosins with the same MHC-f (fast) but with three light chain combinations. Staron and Pette (1987a;b) reported that a total of 54 - 60 theoretical isomyosins may exist in the fiber population of rabbit Soleus and Tibialis anterior mucles.

In addition to fast- and slow-twitch fibers, an intermediate type fiber (IIC) with coexisting slow and fast myosins in various proportions has been reported (Table 2) [Gauthier, 1986; Staron and Pette, 1987a;b]. The existence of IC fibers (between types I and IIC), IIAB fibers (between types IIA and IIB) [Staron and Pette, 1987a; b] and superfast fibers (IIM) (Table 3) [Swynghedauw, 1986] has also been reported.

The expression of myosin-heavy and -light chain isoforms is developmentally regulated in both skeletal and cardiac muscles [Lowey and Risby, 1971; Lowey, 1986b]. In rat slow skeletal muscle the established forms MHC-emb (embryonic) and MHC-neo (neonatal) disappear at four weeks after birth. Embryonic mammalian myosin is structurally similar to that of the slow myosin based on the lower ATPase activity, immunological tests and the lack

of His(τ-Me) [Trayer et al., 1968]. Huszar (1972) explained that the absence of His(τ-Me) in fast muscle from a three-week old animal is due to the fact that myosin is carried over from the foetal to the adult stage.

The MHC are encoded by a highly conserved multigene family [Buckingham et al., 1985] and can be divided into two groups: the sarcomeric MHC and the smooth muscle/non-muscle cell MHC. The sarcomeric MHC gene group is further composed of one group including MHC-emb, adult skeletal white and red (types IIB and IIA), MHC-neo and extra-ocular muscle myosin-heavy chain [Izumo et al., 1986], and another group including the α-MHC genes that code for MHC in ventricles (V1) and atria (A1) and the β-MHC gene coding for MHC in ventricles (V3) and for MHC in slow skeletal muscles [Izumo et al., 1986]. The nervous system and hormones are equally important for regulating the myosin gene expression during foetal life [Kelly and Rubinstein, 1986]. The expression of the MHC genes is variable and tissue-specific [Izumo et al., 1986].

Actin is present in identical forms in both fast and slow skeletal muscles [Perry, 1985]. Actin is not species-specific [Vandekerckhove and Weber, 1979a]. The actin gene family codes for a lower number of proteins than its encoded genes [Buckingham et al., 1985].

The regulatory proteins of the thin filament are similarly found in a number of isoforms. Avian and mammalian muscles express three troponin I isoforms, with highly homologous C-terminals, specific to cardiac, slow and fast skeletal muscles [Perry, 1986]. The slow forms of troponins I, C, T exist in slow muscles (type I fibers), whereas the fast troponins C, T and the slow troponin I are expressed in the fast muscles [Perry, 1985]. Tropomyosin exists in at least 10 isoforms that bind to actin with different

affinities [Perry, 1986]. The different composition of α- and β-chains gives rise to αα, αβ and ββ types of tropomyosin. Types I and II fibers contain the αβ and αα subunits (Table 2) of the tropomyosin molecule, respectively. The α:β ratio is muscle and species-specific [Perry, 1985; 1986].

From the rest of the muscle proteins the C-protein is found in different isoforms in slow and fast muscles, with different Mr and amino acid compositions [Obinata et al., 1986].

2.3.2 The Sarcoplasmic Proteins

This is a soluble class of proteins of the sarcoplasm located within the sarcolemma constituting about 30 - 45% of the total muscle protein or about 5% of the muscle weight [Szent-Gyorgyi, 1955; Hanson and Huxley, 1957). There are about 100 proteins belonging in this group of muscle proteins which can be separated by sucrose gradient technique and centrifugation into four subclasses [Asghar and Pearson, 1980]: (a) a nuclear fraction composed of nucleoproteins, RNA, DNA, lipoproteins; (b) a mitochondrial fraction consisting of mitochondria, enzymes of the TCA-cycle and the electron transport chain; (c) a microsomal fraction containing microsomes, sarcoplasmic reticulum, T-system and lysosomes and (d) a cytoplasmic fraction composed of the enzymes of glycolytic pathway and glucogenesis, myoglobin and the soluble proteins and enzymes of the cytochrome system. The sarcoplasmic proteins have an average Mr of 80,000, they are globular and soluble in H_2O or low concentrations of salt solutions [Asghar and Pearson, 1980].

2.3.3 The Intermediate Filaments and Proteins of Skeletal Muscle

This term is used to describe a class of highly insoluble filaments of the myofibrillar cytoskeleton with an intermediate diameter (10 nm) between that of actin (6 - 7 nm) and myosin (15 nm) [Lazarides, 1980]. Morphologically the intermediate filaments resemble collagen fibrils. It has been proposed that the intermediate filament cytoskeleton play an important role in mechanical continuity (titin or connectin) along the entire length of the myofibril and maintainance of the A-band at the centre of the sarcomere. In addition, the cytoskeletal proteins provide elasticity to the myofibril during the contraction-relaxation cycle [Lazarides, 1982]. In skeletal muscle intermediate filaments are present as: transverse filaments which connect Z to Z and M to M lines (interfibrillar bridges); longitudinal filaments connecting peripheries of successive Z-discs (intrafibrillar); doublet disc-like structure composed of desmin which "sandwiches" each Z-line; internal filaments in the gaps of doublet Z-structures [Wang and Ramirez-Mitchell, 1983], and interconnections joining adjacent myofibrils at the N_2-lines of sarcomeres [Lazarides, 1980]. The proteins included in this group are: connectin, titin, nebulin, desmin, vimentin, synemin and filamin (Table 1).

Connectin: It is located at A-I junction of the sarcomere, has a Mr ~ 1,000,000 and comprises 5% of the total protein. It is a fibrous protein, forms nets of about 2 nm and binds to myosin and actin. Maruyama et al. (1977) isolated connectin from the KI-insoluble muscle residues by solubilizing it with boiling for 5 minutes in 1% SDS at pH 7.0. The amino

acid composition of connectin closely resembles that of actin and eu-actinin except for its methionine and alanine contents [Maruyama et al., 1977].

Titin and Nebulin: These two proteins have been also isolated from KI-insoluble residues of skeletal muscle [Wang, 1981; Wang and Ramirez-Mitchell, 1983] (Table 1) and play an important role in the assembly of the myofibril [Kurpakus and Huiatt, 1986]. Titin has been located at the A-I junction, crossing through the A-band, and M-line, H-zone, N_2-line and at the ends of the thick filaments [Wang and Ramirez-Mitchell, 1983]. Nebulin has been located at the N_2-line where forms a nebulous transverse structure [Wang and Ramirez-Mitchell, 1983]. Nebulin protein has been recently reported as the canditate for the product of the Duchenne-muscular dystrophy gene (Robertson, 1987). Both titin and nebulin are extremely protease-sensitive and partially soluble in SDS and urea [Wang and Ramirez-Mitchell, 1983]. It is believed that titin is the high molecular weight component isolated by Wang (1981) from a group of proteins that Maruyama et al. (1977) had collectively called "connectin."

Desmin: This protein has been isolated from KI-insoluble residue of skeletal muscle with 1 M acetic acid [Lazarides, 1982]. It is located at the periphery of the Z-line and forms a 10 nm transverse network reaching the side of the muscle fiber and linking neighbouring Z-line [Lazarides, 1982]. It comprises about 5% of the total protein in gizzard muscle but only 0.18% of the skeletal muscle protein [Huiatt et al., 1980]. Lazarides (1980) and Bilak et al. (1987) consider desmin to be the major component of

the KI-insoluble residue of skeletal muscle. However, Wang and Ramirez-Mitchell (1983) have reported that desmin comprises only a minor component of the total KI-insoluble residue of the skeletal muscle.

Vimentin: It is localized at the periphery of Z-line forming transverse structures [Lazarides, 1982] (Table 1). In embryonic skeletal muscle the vimentin to desmin ratio is 75:25 whereas in the adult muscle fibers the ratio is 5:95 [Bilak et al., 1987].

Synemin: This protein has also been localized in the periphery of Z-disk and associates with desmin and vimentin [Lazarides, 1982].

Filamin: It is a dimer (Mr of 50,000) that helps in the gelation of F-actin [Weeds, 1982]. Filamin has been found to localize at the periphery of Z-disk of avian fast muscle and at the I-band of slow muscle [Gomer and Lazarides, 1983a], with the latter containing ten times more filamin (on the basis of actin) than in white muscle [Gomer and Lazarides, 1983b].

Spectrin ($\alpha\beta$-spectrin) and ankyrin proteins are also located at the Z-line domain around the myofiber and establish the continuity of the myofibers with the surrounding sarcolemma [Lazarides and Capetanaki, 1986].

Skelemins: The cytoskeleton of mammalian and smooth muscle cells contain two proteins of Mr ranging from 200,000 to 220,000, in a molar ratio of 1:1:20 with desmin [Price, 1987]. There are 54 µg skelemins/g of myofibrils. Skelemins form narrow rings at the periphery of the M-disks and probably serve to maintain lateral registration and prevent dissociation of the myofibrils during contraction.

2.3.4 The Cytoskeletal Breakdown of the Skeletal Muscle

The degree of the myofibrillar solubilization during their extraction from the muscle cell, as recommended by McCollester (1962; 1963) and Stanley (1983) and adopted in our study, seems to depend upon the nature of the interactions of the sarcoplasmic and myofibrillar proteins with the cytoskeletal proteins. It is established that the cytoskeleton of the muscle cell consists of connectin, titin, nebulin, and desmin. McCollester (1962) was the first to report that the "cytoskeleton" of the muscle cell has to be broken down before the contents of the muscle cell (myofibrillar proteins) are released, leaving behind the empty sarcolemmal sheath; this has been termed the "emptying phenomenon."

It has been reported that homogenization of the muscle tissue in 0.05 M $CaCl_2$ and subsequent incubation for half an hour in Tris-H_2O buffer is adequate for the successful emptying of the muscle cells [McCollester, 1962; 1963]. McCollester and Semente (1964) concluded that the successful emptying depends on both enzymatic and ionic effects. The ease emptying of aged muscle cells has been related [Hultin and Westort, 1969; Stanley and Hultin, 1968; Comissioning and Hultin, 1976] to the formation of actomyosin. In fresh muscle, ATP is the predominent nucleotide, whereas in aged muscle it disappears and only AMP is present [Comissioning and Hultin, 1976]. The actomyosin complex can be dissociated by ATP and ADP and therefore the emptying process is inhibited by the presence of these nucleotides. Actomyosin formation and removal of Z-line are necessary for the emptying phenomenon [Stanley, 1983].

The function of Ca^{2+} in fresh muscle is to stimulate the myosin ATPase activity which removes ATP, thereby forming actomyosin. McCollester (1962) emphasized that the presence of Ca^{2+} in the homogenizing medium plays an additional role apart from preventing marked contraction of the muscle cells during homogenization. If $CaCl_2$ is omitted, no emptying of fresh muscle will be observed [Hultin and Westort, 1969; Stanley, 1983]. Aged muscle (for 4 h at 4°C in H_2O or saline buffer) produces more extensive emptying than fresh muscle when $CaCl_2$ is not present in the homogenizing medium [Hultin and Westort, 1969; Stanley, 1983]. Calcium ions play a dual role: (1) they activate the intracellular Ca^{2+} proteases present in skeletal muscles [Dayton et al., 1976] and (2) they favor conformation destabilization and depolymerization of protein, involving subunit association and dissociation equilibria [Von Hippel et al., 1973] by decreasing the water holding capacity [Winegrad, 1965] and forming cross bridges between peptide chains and thereby condensing the muscle structure. Calcium chloride interacts strongly with amide groups of proteins by electrostatic ion-dipole interaction [Bello et al., 1966].

Two forms of calcium activated factors (CAF) have been identified in muscles [Busch et al., 1972; Etherington, 1984]. The first (CAF-I) requires 1 - 2 mM Ca^{2+} and the second (CAF-II) 50 - 100 μM Ca^{2+} for maximal activity [Etherington, 1984]. Koohmaraie et al. (1987) suggested that CAF-I plays the most important role in the postmortem fragmentation of myofibrils and therefore in the improvement of meat tenderness. The CAF has been reported to degrade Z-line releasing α-actinin, M-line, C-protein, Troponins-T,-I but does not affect myosin and actin [Koohmaraie et al., 1984; Etherington,

1984; Slinde and Kryvi, 1986]. In addition, CAF is capable of degrading desmin, vimentin [Slinde and Kryvi, 1986] and α-actinin [Elgasim et al., 1985]. Digestion of titin (connectin) and nebulin by CAF starts after the Z-disc has been damaged [Slinde and Kryvi, 1986]. Goodman, (1987) reported that an increase in free cytoplasmic Ca^{2+} in muscle was associated with the breakdown of non-myofibrillar, but not myofibrillar proteins. Crude preparation of CAF purified from bovine muscle acts at the periphery and mid-region of the Z-line which is removed completely [Slinde and Kryvi, 1986].

Cathepsins B and D are two groups of enzymes which are involved in muscle protein degradation during storage [Etherington, 1984]. These enzymes have an optimum pH of 5.5 and therefore seem unlike to be active at pH 7.4, which is the recommended by McCollester (1962) pH value for the extraction procedure.

2.4 The Extracellular Matrix of Vertebrate Skeletal Muscle

The muscle cells are surrounded by the extracellular matrix, which consists of collagen, elastin, fibronectin, laminin, chondroitin sulfate A,B and C, (Parthasarathy and Tanzer, 1987) dermatan sulfate, keratan sulfate, heparan sulfate, and proteoglycans [Carrino and Caplan, 1986]. The extracellular matrix plays an important role in the alignment of muscle fibers and blood vessels [Rowe, 1981], the maintainance of the structure of the neuromuscular junction and the organization of the nerve terminal, in the transmission and distribution of force from muscle to tendon, and in muscle regeneration [Sanes, 1986].

2.4.1 Collagen

The main component of the connective tissue is collagen which, is a glycoprotein. Collagen consists of three tropocollagen monomers, 300 nm long and 1.5 nm in diameter. Two of them are chains called α_1-chain while the third chain is named α_2-chain. Each α-chain has a Mr of 95,000, and consists of 1000 amino residues and coils in a left-handed minor α-helix with 3 amino acids per turn. The collagen molecule is formed from the three α-chains which coil with one another into a right-handed super helix [Fietzek and Kuhn, 1976]. The different types of collagen are composed of the following α-chains [Eyre, 1980]: α1(I)-chain (reads as alpha-one, type-one chain), α2(I)-chain, α1(II)-chain, α1(III)-chain, α1(IV)-chain and α1(V)-chain. The genetically distinct collagen α-chains may be as many as twenty [Boedtker and Aho, 1984].

Biosynthesis of Collagen

There are about twenty genes that code for the α-chains and at least 12 different vertebrate collagen types are expressed [Cheah, 1985; Martin et al., 1985]. The genes for the collagens are first transcribed into corresponding pre-mRNAs with copies of both exons and introns and cytoplasmic translatable mRNA is formed after about 50 excision and splicing events [Kuhn, 1984; Harwood, 1979]. The polypeptide chains of collagens are synthesized on membrane-bound ribosomes and pass into the lumen of the rough endoplasmic reticulum while being assembled [Harwood, 1979]. The collagen molecules are synthesized from procollagen chains which give rise to tropocollagens having their N- and C-terminal regions in non-helical conformation and are called "telopeptides". The tropocollagen molecules are packed together side by side, stabilized by chemical cross-links between the chains. A small gap separates the "head" of one tropocollagen from the "tail" of the next [Minor, 1980]. The central triple helical region is a polymer of the $[Gly\text{-}X\text{-}Y]_{340}$ structure; about one-third of the X and Y residues are proline, Pro(4-OH) or alanine.

Collagens have a unique amino acid composition. All types of collagen contain high levels of glycine (33%), proline (11.7%) and Pro(4-OH) (11.0%), whereas tryptophan is absent [Miller and Gay, 1982]. Types I, II and III contain more alanine compared to types IV and V. Cyst(e)ine is present only in types III, IV collagen. The α_1(IV) and α_2(IV)-chains contain high levels of Pro(4-OH) and Lys(5-OH). Minor (1980) reported that the non-polar amino acids comprise 60%, the hydroxy- 17%, the basic 11% and the acidic 9% of the total amino acid residues present in the collagen molecules [Minor, 1980].

Table 3. Amino Acid Composition of Collagen Types Present in Skeletal Muscles (Amino Acid Residues/1000 Residues)

	Collagen Type Standards[a]			
	I	III	IV	V
	Chain Association			
Amino Acids	$[\alpha1(I)]_2\alpha2(I)$	$[\alpha1(III)]_3$	$[\alpha1(IV)]_2\alpha2(IV)$	$[\alpha1(V)]_2\alpha2(V)$
Aspartic acid	42.7	42.0	46.3	49.3
Threonine	17.0	13.0	22.7	23.7
Serine	36.0	39.0	35.3	26.7
Glutamic acid	71.3	71.0	73.7	96.3
Proline	120.3	107.0	81.0	122.3
Glycine	334.7	350.0	330.7	331.7
Alanine	110.7	96.0	35.7	44.0
Cyst(e)ine	0	2.0	0.67	0
Valine	25.7	14.0	31.0	20.3
Methionine	6.3	8.0	14.7	9.67
Isoleucine	8.67	13.0	34.0	16.3
Leucine	22.7	22.0	53.3	36.3
Tyrosine	2.0	3.0	5.67	3.33
Phenylalanine	12.0	8.0	30.0	11.6
Histidine	6.0	6.0	6.0	7.33
Lysine	23.3	30.0	6.3	13.7
Arginine	50.0	46.0	28.7	42.7
4*Hydroxyproline	103.0	125.0	118.0	108.7
5*Hydroxylysine	10.0	5.0	45.3	31.7
3*Hydroxyproline	1.0	0	1.0	4.33

Table 3 continued ...

Table 3 continued

	Chain Association			
	$[\alpha1(I)]_2\alpha2(I)$	$[\alpha1(III)]_3$	$[\alpha1(IV)]_2\alpha2(IV)$	$[\alpha1(V)]_2\alpha2(V)$
WE[b] (μg/nmol)	0.091175	0.090897	0.095895	0.0950411
Distribution in Skeletal Muscle [c], %	61.5	33.5	5.0	d

[a] Average values for each collagen type were calculated from the data of Miller and Gay (1982).

[b] The residue weight (WE) for each collagen standard was calculated according to Horstmann (1979).

[c] From Light and Champion (1984) and Light et al. (1985).

[d] Type V collagen is present in skeletal muscle but not yet accurately quantitated (Light and Champion, 1984).

The amino acid composition [Miller and Gay, 1982] and the mean residue weight (WE) of types I, III, IV and V present in skeletal muscles are shown in Table 3.

The hydroxylation of peptidyl proline and lysine residues and glycocylations of peptidyl hydroxylysine residues is catalyzed by peptidyl proline 3-hydroxylase, proline 4-hydroxylase and peptidyl lysine hydroxylase, which reside in the endoplasmic reticulum. All three enzymes require ascorbate, molecular oxygen, ferrous ions and 2-oxoglutarate [Prockop et al., 1979; Eyre, 1980]. Peptidyl proline-4- and peptidyl lysine-hydroxylase are specific for the Y position of the Gly-X-Y sequence of the triple-helix.

The hydroxy- group of Pro(4-OH) when present at Y position stabilizes the collagen triple helix and enhances the stability of the assembly of collagen into fibrils [Nemeth and Scheraga, 1986]. The Pro(4-OH) possibly contributes to the higher thermal stability or to the resistant digestion of basement membrane collagens with mammalian collagenase [Minor, 1980]. The hydroxylated lysine act as the sites for carbohydrate attachment and stabilize the intermolecular collagen crosslinks within fibrils [Kivirikko and Myllyla, 1982; Prockop and Kivirikko, 1984]. The variability in the extent of lysine hydroxylation and proline-3 hydroxylation among genetically distinct collagen types, within the same collagen type from different tissues and state of development [Barnes et al., 1974; Eyre, 1980] can be explained by regulation of single peptidyl lysine hydroxylase and peptidyl proline 3-hydroxylase activities and not due to the presence of different isoenzymes

[Minor, 1980]. Proline in position Y in types I, II and III is not hydroxylated in only 2 or 3 positions but is probably completely hydroxylated in others to give an overall level of 90 - 95% hydroxylation of the proline residues present in these collagens [Kivirikko and Myllyla, 1982]. Marked differences have been found in the extent of hydroxylation of lysine, proline-3 and hydroxylysyl glycosylation whereas the hydroxylation of proline-4 shows smaller variation. Babel and Glanville (1984) reported that IV collagen is underhydroxylated considering proline hydroxylation but is completely hydroxylated and glycosylated with respect to lysine hydroxylation; this is the reverse of the extent of the hydroxylation in interstitial collagens (I, II, III). Type I collagens present the lowest degree of hydroxylation and type IV the highest [Kivirikko and Mylylla, 1982; Eyre et al., 1984]. In a specific collagen type the content of Pro(4-OH) shows smaller variation when compared to that of Pro(3-OH) or Lys(5-OH).

The crosslinks in collagen are formed from ε-NH_2 groups of lysine residues located in the nonhelical N- and C-extensions, which are oxidatively deaminated by a Cu^{2+}-dependent enzyme, known as lysyl oxidase [Kivirikko and Myllyla, 1982]. The resulting aldehyde is then linked with other ε-NH_2 groups of lysine and Lys(5-OH) residues of the helical and nonhelical regions of the macromolecule to yield intra- and intermolecular linkages [Kivirikko and Myllyla, 1982]. The acid labile crosslinks present in collagen are: α,β- unsaturated "aldol," lysinonorleucine (intramolecular band), N^{ε}-glucitol-lysine, hydroxylysinonorleucine, dihydroxylysinonorleucine (major intermolecular cross-link in young collagens), N^{ε}-mannitol-lysine, N^{ε}-hexosylhydroxylysine, hydroxymerodesmosine, aldol-histidine, and

histidino-hydroxy-merodesmosine [Tanzer, 1973]. Barnard et al. (1987b) identified an amino acid of high Mr deriving from lysine of insoluble mature collagen. This compound was named M amino acid and was proposed to be a major crosslink of mature collagens important for the stabilization of the molecule. Crosslinks increase in amount and strength within collagen fibers, with animal age [Schimokomaki et al., 1972]. Species, breed, sex and nutritional status of the animal influence the collagen's crosslink composition [Henrickson et al., 1984].

In addition to its presence in collagen, Pro(4-OH) has also been found in elastin [Bentley and Hanson, 1969], the Clq component of complement, in the collagenous tail of the enzyme acetylcholinesterase [Anglister et al., 1976] and in the primary cell wall plant glycoproteins i.e., extensins [McNeil et al., 1984]. The $(Gly\text{-}X\text{-}Y)_n$ sequence, characteristic of the collagen molecule has not been shown to have its analogous in the plant proteins [Adams and Frank, 1980]. The plant cell wall Pro(4-OH) is formed by the hydroxylation of protein-bound prolyl residues via an enzyme similar to that of the animal prolyl hydroxylase. The Lys(5-OH) has also been found in the Clq subcomponent of the complement system in the blood and the tail structure of acetylcholinesterase [Porter and Reid, 1978].

Localization, Morphology and Collagen Content of Skeletal Muscle

Skeletal muscle collagen is organized in the epimysium, perimysium and endomysium [Rowe, 1981]. The presence of types I, III and V collagen in skeletal muscles has been demonstrated by the use of SDS gel electrophoresis of pepsin-solubilized collagen [Bailey and Sims, 1977; Duance et al., 1977]

and immunofluorescent techniques. Type-specific anti-collagen and fluorescent antibodies methods revealed that, type IV collagen is localized exclusively in the lamina densa of the basal lamina, while type V is present in the outer layer of the basal lamina. Low levels of type V collagen is also found in the perimysium and endomysium. Type I collagen by contrast is present at high levels in the epimysium and at low levels in both the perimysium and endomysium. Type III collagen is mainly found in the perimysium forming a thin network of fine fibrils and at smaller amounts in the epimysium and endomysium [Bailey et al., 1979; Stephens et al. 1982a, b; Duance et al., 1977; Foidart et al., 1981]. Orcutt et al. (1986) reported that the collagen of the perimysium has a "laminar-like row" structure. Endomysial collagen appears to be in an ordered structure [Borg and Caulfield, 1980; Orcutt et al., 1986] with rows of reticular fiber arranged at an angle or perpendicular to the long axis of muscle fiber [Sanes, 1986]. The capillaries, the nerve filaments, the fibroblasts, the histocytes, and the mast cells are also present in this layer.

It has been estimated that the mean collagen content of skeletal muscle ranged from 4.02% of the total muscle protein (range 1.6% - 9.7%) on a dry, fat-free basis [Bendal, 1967] to 4.15% (range 2.21% - 5.59%) [Dransfield, 1977]. Light et al. (1985) estimated that the collagen content of the epimysium, perimysium and endomysium of bovine skeletal muscles was 6.2%, 4.7% and 0.3% respectively (expressed as percentage of the total muscle dry weight). Of the total collagen quantitated in bovine muscles, 61.5% is type I, 33.5% type III and 5% type IV [Light et al., 1985] (Table 3). Type IV collagen constitutes 30 ~40% of the basement membrane dry weight [Barnard et

al., 1987a]. Type V collagen is present in amounts less than 0.5% of the total collagen present in skeletal muscles [Light et al., 1985].

Although different muscles of various species are qualitatively similar, [Rowe, 1981] collagen content differs between muscles, locations within the same muscle [Rowe, 1981] and animal grade [Prost et al., 1975]. Collagen content is not affected by sex, diet or slaughter age [Dikeman et al., 1986], while others [Alnaqeeb et al., 1984] report that connective tissue of young muscles is poorly developed. The collagen content of muscles of the rat involved in the continuous support of posture (fast contracting muscles) is higher, than that of those muscles used mainly in voluntary movements [Kovanen et al., 1980]. This difference also applies at the level of the individual fibers [Kovanen et al., 1984]. The amount and distribution of collagen can regulate and control the extent of contraction in young and adult, normal and diseased muscles [Borg and Caulfield, 1980; Foidart et al., 1981; Alnaqeeb et al., 1984]. An increase in muscle collagen content has been observed in both myogenic and neurogenic muscle wasting diseases [Myllyla et al., 1982].

The extracellular matrix of skeletal muscles, in addition to collagen and elastin, contains other proteins such as fibronectin, laminin, and fibrillin [Hakomori et al., 1984]. Fibronectin (Mr of 220,000-240,000) has a role in binding collagen IV and glycosaminoglycans [Hay, 1981; Aumailley and Timpl, 1986]. It is present in the basal lamina, reticular lamina, epimysium and perimysium [Sanes, 1986].

Laminin is a large glycoprotein, composed of two polypeptide chains in the shape of an assymetrical cross. It is present in the lamina densa

region of the basal lamina and binds to the type IV collagen [Charonis et al., 1985]. Laminin is involved in cell adhesion, growth, morphology, differentiation, migration and matrix assembly [Kleinman et al., 1985].

Sakai et al. (1986) identified a new protein in the muscle connective tissue, the fibrillin. It has a Mr of 350,000 and is present in elastin-associated microfibrils. The proteoglycans of the muscle consist of four heparin sulphate chains attached to a small protein core. Parthasarathy and Tanzer (1987) consider chondroitin sulfate (Mr of 95,000) as the major proteoglycan of adult skeletal muscle.

2.4.2 Elastin

Elastin is the second major structural component of the extracellular matrix of the mammalian skeletal muscle. Three mRNAs isolated from different tissues encode for elastins a, b, c containing 747, 733 and 713 amino acid residues and having a molecular mass of 64.2, 62.7 and 60.9 kDa, respectively [Raju and Anwar, 1987].

Elastin contains high levels of glycine (1/3 of the total residues), proline (10-13%), valine, alanine, phenylalanine, leucine, isoleucine and no cyst(e)ine or Lys(5-OH); over 40% of the total amino acid residues have hydrophobic side chains. It also contains about 1% Pro(4-OH) [Bendall, 1967]. Elastin is distinguished into fibrillar and amorphous. The fibrous elastin appears first in the extracellular space and contains high alanine levels, 25% more than any other known protein [Urry, 1983]. The amorphous component of elastin consists of 95% polar amino acids and is deposited on the network of filamentous structures of the fibrous elastin [Urry, 1983].

Fibrous elastin is insoluble in most reagents [Franzblau and Faris, 1982] due to the presence in its molecule of the unique crosslink amino acids, Des and iDes [Thomas et al., 1963]. These crosslinks are formed by oxidative deamination of ε-NH_2 groups of specific Lys residues and is catalyzed by lysyl oxidase [Kagan et al., 1986]. Most of the lysine residues in elastin occur in pairs separated by 2 or 3 amino acid residues. A Des or iDes is formed when a pair of lysines followed by an alanine and another pair followed by a hydrophobic residue or when groups of three lysine residues and a lysine residue come into alignment. When two groups of three lysines followed by a hydrophobic residue come into alignment, a Des or iDes and lysinonorleucine is formed [Raju and Anwar, 1987]. Other crosslinks found in elastin are dehydrolysinonorleucine, allysine aldol and dehydromerodesmosine [Francis et al., 1973; Foster et al., 1974].

Muscle elastin constitutes a very small proportion of the total muscle protein. In 29 bovine muscles examined by Bendall, (1967), it was found that elastin ranged from 0.05% - 0.8% (% of dry and fat-free muscle protein) . In some muscles such as bovine Latissimus dorsi and Semitendinosus elastin accounted for 33% and 37% respectively of the total connective tissue [Bendall, 1967]. In muscle the network of elastin is qualitatively the same in muscles of high and low elastin content and is present as thin (1 - 2 μm) and thick (5 - 10 μm) fibers. The thick fibers are more concentrated in epimysium and perimysium, whereas the thin fibers are associated with the epimysial and perimysial collagen sheets [Rowe, 1986]. The thick elastin fibers restore energy when they are elongated,

whereas the thin fibers restore the rest length configuration of the perimysial connective tissue when the muscle is relaxed [Rowe, 1986].

Elastin content varies significantly between species within a phylum and considerably between phyla [Sage and Gray, 1979]. The amino acid composition of elastins purified from different tissues of each species are similar; differences occur between the neutral, aromatic amino acids [Starcher and Galione, 1976] and the Des, iDes levels. The levels of the unique Des and iDes are constant among various tissues of the same species, but vary considerably between species with the smaller species (i.e., avian) having usually lower levels of these amino acids [Starcher and Galione, 1976]. The amino acid sequence of bovine and porcine elastins are very well conserved but they are different from the chick elastin [Raju and Anwar, 1987]. Most of the reported variation of the crosslinks of the mature elastin can be attributed to different methods used for the extraction of this protein from different tissues and species, and to the different methods employed for the quantitation of these unique amino acids [Sandberg, 1976]. Alkali treatment, often used [Bendall, 1967] to isolate the extremely insoluble elastin from tissues, is destructive and reduces the percentage of Des, iDes and other intermediate crosslinks [Paz et al., 1982; Eyre et al., 1984].

2.5 The Cytoplasmic Histidine Dipeptides of the Vertebrate Skeletal Muscle

N^{τ}-methylhistidine occurs exclusively in myosin and actin sequences [Asatoor and Armstrong, 1967; Elzinga and Collins, 1977]. In muscle tissues, His(τ-Me) can also be found in the free form (0.3 - 17% of the

total non-protein bound) [Huszar et al., 1983; Harris and Milne, 1987], deriving from the breakdown of the muscle proteins or balenine and can be detected in blood and urine samples [Young et al., 1972]. In addition, 83 - 99.7% of the total non-protein bound His(τ-Me) has been identified in muscle extracts of mammalian and avian species as the sarcoplasmic dipeptide balenine (β-alanyl-L-N^{τ}-methylhistidine) [Rangeley and Lawrie, 1976; Carnegie et al., 1982; 1983; 1984; Harris and Milne, 1987]. Two other histidine dipeptides are normal constituents of skeletal muscles, namely carnosine (β-alanyl-L-histidine) and anserine (β-alanyl-L-N^{π}-methylhistidine) [Olsman and Slump, 1981]. The function of these dipeptides is not clearly known. They can act as buffers and retain constant pH because of changes due to lactic acid production during and after muscle function or may act as neurotransmitters in the muscle cell [Crush, 1970].

In spite of the large variation of the amounts of these histidine dipeptides between muscles and individual animals, their levels have been used as indicators for the presence of different meat species in composite meats [Olsman and Slump, 1981; Carnegie et al., 1984].

Carnosine, anserine and balenine, upon acid hydrolysis, release histidine, His(π-Me) and His(τ-Me), respectively, plus β-alanine. During amino acid analysis these released amino acids are co-chromatographed with the respective protein-bound compounds, leading to their overestimation. Therefore these sarcoplasmic dipeptides must be extracted from muscle tissues prior to acid hydrolysis. Successful removal of these dipeptides requires the disruption of the muscle fiber bundles into smaller fragmented myofibrils, the swelling of the myofibrils, and the cytoskeletal breakdown

of fresh or aged muscles [McCollester, 1962;1963; Stanley, 1983]. In meat products, sodium chloride and polyphosphates are mixed with meat during the comminution process in order to dissociate actomyosin from postrigor muscle and extract myosin. Extracted myosin is necessary for the protein-protein interactions and formation of a structural gel matrix within the composite meats [Voyle et al., 1984]. Therefore, it seems likely that the myofibrillar proteins present in processed meats would be readily extractable under even milder conditions [Rangeley and Lawrie, 1976; Bligh and Dyer, 1959] than those needed for their extraction from muscle tissues [McCollester, 1962; Laurent et al., 1981; Stanley, 1983].

2.6 The Determination of the Myofibrillar and Connective Tissue Proteins in Skeletal Muscles

Myofibrillar Proteins

One of the currently used methods for determining the myofibrillar myosin, actin, tropomyosin and troponin contents of muscle tissues involves the quantitative extraction (either from a pure preparation of myofibrils or from muscle homogenates) and separation of the contractile proteins by electrophoresis. The sarcoplasmic proteins are extracted from these preparations by repeated washings with a low ionic strength phosphate buffer (0.15 M K_2HPO_4, pH 5.8 - 7.0). The myofibrils are separated from nuclei and collagen fibrils with the use of Percoll gradients and centrifugation [Yates and Greaser, 1983b]. When a preparation of a whole muscle is used, the myofibrillar proteins are solubilized with 3% SDS in combination with urea

or thiourea. The myofibrillar proteins of the intact unwashed or extensively washed muscle are separated by one- or two-dimensional electrophoresis on polyacrylamide gels [Treager and Squire, 1973; Morimoto and Harrington, 1974; Potter, 1974; Pepe and Drucker, 1979; Yates and Greaser, 1983a; 1983b; Murakami and Uchida, 1985], and are quantitated by densitometry. The levels of myosin and actin found in vertebrate skeletal muscles have revealed significant variation. This variation has been attributed to the washing technique of the muscle (extensive or limited), and to the loss of myosin filaments during homogenization or to the incomplete extraction of the myofibrillar proteins [Yates and Greaser, 1983b]. The drawback of the SDS polyacrylamide electrophoresis, which leads to an overestimation of the muscle proteins, is the densitometric protein determination of the stained SDS gels, because more than one protein frequently comigrate in an electrophoretic band; for example, tropomyosin and eu-actinin have been reported to comigrate in the actin band [Pepe and Drucker, 1979; Cohen and Murphy, 1979]. The quantitation of the protein content in the stained protein bands on the SDS polyacrylamide gels has also been carried out by eluting the separated myosin and actin bands and determining their concentrations from the extracted dye [Murakami and Uchida, 1985]. Troponin-C and tropomyosin have different relative dye binding capacities from the other myofibrillar proteins, and Troponin-T comigrates with the sarcoplasmic proteins [Murakami and Uchida, 1985]. The protein content of the stained bands has also been calculated by amino acid analysis, corrected for comigrating protein bands, by means of multiple linear regression analysis [Yates and Greaser, 1983a]. The above methods

have shown that the molar ratio of myosin to actin in skeletal muscle is 1.0:6.0 [Potter, 1974; Murakami and Uchida, 1985] and that of actin to tropomyosin is 7.0:1.0 [Yates and Greaser, 1983a]. Results obtained showed that myosin contributes 43% and actin 22% of the myofibrillar protein mass [Yates and Greaser, 1983b].

Many researchers have used several buffer systems for the quantitative separation of the different protein fractions from fresh or glycerinated muscles under varying conditions of pH, ionic strength, temperature, buffer to sample ratio and extraction time [Szent-Gyorgyi et al., 1955; Hanson and Huxley, 1957]. Based on the above methods myosin has been estimated to account for 37.4 to 53.8% of the myofibrillar protein [Yates and Greaser, 1983b]. The variation of the results obtained by these methods has been attributed to the fact that the buffers used do not cleave selectively a particular type of bond and especially to differences in the myofibrillar volume among the various muscle types. This was indicated by the results of quantitative electron microscopy [Eisenberg and Kuda, 1976; Page and Surak-Droske, 1979]. Yates and Greaser (1983b) have summarized the published data on the myosin and actin contents of vertebrate skeletal muscle based on different methods. Actin has been found to range from 12 - 24.5% and myosin from 45 - 52% of the myofibrillar protein.

The quantitation of His(τ-Me), a unique basic amino acid found exclusively in both myosin and actin sequences [Elzinga et al., 1973; Vandekerckhove and Weber, 1978a; 1979a; Elzinga and Collins, 1977], has been proposed as an index for the estimation of these two myofibrillar proteins in tissues [Perry, 1970] and in meat products [Hibbert and Lawrie, 1972;

Zarkadas, 1981; McNeal, 1986; Ashworth, 1987]. This quantitation is based on the following findings: actin and its isoforms contain one residue of His(τ-Me) at position 73 in their amino acid sequence [Elzinga et al., 1973; Vandekerckhove and Weber, 1978a; 1979a], myosin prepared from mammalian and avian fast-twitch (IIA) fibers contains one residue His(τ-Me) in each heavy chain [Elzinga and Collins, 1977; Huszar, 1984; Maita et al., 1987], avian myosin prepared from fast-white, slow-red and cardiac muscles contains similar levels of His(τ-Me) (0.40 - 0.45 mol per 100,000 g of protein) [Masaki et al., 1986] and His(τ-Me) is absent from all other muscle or non-muscle proteins [Paik and Kim,1980; Huszar, 1984].

To validate the use of His(τ-Me) as an index for the determination of the myofibrillar proteins in meat products [Hibbert and Lawrie, 1972] the levels of His(τ-Me) should be established throughout the muscles of a given species and between the muscles of different species. Various measurements of the His(τ-Me) content of vertebrate skeletal muscle have been performed using a variety of chromatographic methods [Rangeley and Lawrie, 1976; 1977; Skurray and Lysaght, 1978; Jones et al., 1982]; these methods, however, have yielded variable amounts of His(τ-Me) among the muscle tissues or meats examined. Bovine muscles were found to contain 4.8 - 6.2 mg His(τ-Me)/g of non-collagen nitrogen (NCN) [Jones et al., 1982; Rangeley and Lawrie, 1976, 1977; Poulter and Lawrie, 1980a; Arneth and Herold, 1985]. In the case of pork muscles the levels cited varied from 4.2 - 8.6 mg His(τ-Me)/g of NCN [Jones et al., 1982; Poulter and Lawrie, 1980a]. The His(τ-Me) levels in avian muscles were also found to vary from 5 - 10.3 mg/g NCN [Poulter and Lawrie, 1980a; Rangeley and Lawrie, 1976; 1977; Jones et al., 1982; Arneth

and Herold, 1985]. The His(τ-Me) content of lamb muscles showed similar variation ranging from 4.7 mg/g NCN [Jones et al., 1982] to 6.1 - 6.2 mg/g of NCN [Rangeley and Lawrie, 1976, 1977]. Jones et al. (1985; 1987) reported an even greater variation of His(τ-Me) content in meat cuts of beef, pork and chicken. Organ meats (kidney, lung, etc.) often used in the manufacturing of meat products may contain His(τ-Me) levels anywhere from 0% to 65% of those found in skeletal muscles [Jones et al., 1985].

The variation in the His(τ-Me) levels has been attributed to the different levels of this unique amino acid in the myosins of fast, slow or mixed fast/slow muscle fibers, whereas the His(τ-Me) content of isoactins is found to be similar [White and Lawrie, 1985b; Johnson et al., 1986]. This variation has also been attributed to the different levels of connective tissue and sarcoplasmic proteins in the muscle tissues analyzed [Jones et al., 1985].

A plethora of chromatographic techniques concerning the determination of His(τ-Me) has been published because of the experimental importance of this unusual amino acid as index of the catabolic rates of the myofibrillar proteins and their levels in muscles and meat products. The major limitation of ion-exchange chromatographic procedures used to separate the His(τ-Me) from histidine or other basic ninhydrin-positive compounds present in meat, meat products or urine acid hydrolysates has been the lengthy analysis [Haverberg et al., 1975; Rangeley and Lawrie, 1976; 1977; DiFerrante et al., 1980] and preparation of the samples. Several other methods have been published concerning the quantitation of His(τ-Me) based on ion-exchange chromatography [reviewed by Paik and Kim, 1980 and Hancock

and Harding, 1984; Zarkadas, 1975; Nishizawa et al., 1978; Zunic et al., 1984; Fitch et al., 1986], and on the application of gas chromatography [Larsen and Thornton, 1980; Marcucci et al., 1984].

The methodology of His(τ-Me) determination has been improved with the introduction of the HPLC [Wassner et al., 1980; Hancock and Harding, 1984; Qureshi et al., 1986], combined with fluorometric determination of His(τ-Me) after a post-column reaction of the compound with O-phthaldehyde [Friedman et al., 1980]. Skurray and Lysaght (1978) quantitated the His(τ-Me) content of muscles on HPLC on silica after preliminary treatment of the meat hydrolysate with an ethanolic solution of 1-fluoro-2,4-dinitrobenzene (FDNB) and subsequent extraction to isolate the dinitrophenyl derivatives of the basic amino acids. Poulter and Lawrie (1980a) used a second derivatization step with methyl chloroformate. Jones et al. (1982) and White and Lawrie (1985a) described an HPLC method based on the derivatization of neutralized meat hydrolysates with fluorescamine reagent during which step only histidine and His(τ-Me) yielded acid-stable fluorescent derivatives [White and Lawrie, 1985a]. Although the methods mentioned above are sensitive and with the use of HPLC are rapid, they are suitable for the determination of only one posttranslationally modified amino acid residue found in muscles or meat product hydrolysates. Other researchers have reported methods that allow the quantitation of all the methylated amino acids present in the sequences of myosin and actin [reviewed by Hennecke and Plapp, 1984]. There are a few published methods concerning the quantitation of Lys(5-OH) and His(τ-Me) [DiFerrante et al., 1980] or Lys(5-OH), Lys(Me), and His(τ-Me) in the same analytical run [Villaneuva et al., 1987]. Zarkadas (1979) described an

ion-exchange chromatographic method where the crosslinks of elastin and collagen, and the methylated lysine and histidine were completely separated in a single run. Zarkadas et al. (1987a) improved the previous method and have achieved baseline resolution and complete separation from the parental amino acid lysine and histidine of all the basic unique compounds found in the four major classes of proteins present in muscles in 360 min. This was achieved on a microcolumn packed with DC-4A resin, using two sodium citrate buffers (0.35 M) pH 5.700 and 4.501 and two column temperatures (31.5° changes to 73°C at 295 min after injection) [Zarkadas et al., 1987a].

The traditional and official method for the determination of protein content in meats is the measurement of the total nitrogen by the Kjeldahl digestion, the protein content being calculated by means of a suitable factor [reviewed by Benedict, 1987]. This method is not accurate because it can not distinguish between the nitrogen originating from the contractile proteins and that derived from connective tissue proteins, vitamins, creatine, creatinine and nucleotides. The factor 6.25 which is used to calculate the protein content from the total nitrogen is not accurate and the protein content value thus calculated may be up to 10% higher than the actual protein content of a meat sample [Jones et al., 1982; Benedict, 1987]. Horstmann (1979) developed an accurate means of calculating the total protein content of a sample, which is based on the fact that the total amino acids found in a sample's hydrolysate (as determined by the ninhydrin reaction) reflect the total protein mass present. Since each protein has a different mean amino acid weight conversion factor, the relative amino acid content of each protein must be determined before the method can be used

[Horstmann, 1979; Benedict, 1987]. The method of protein determination proposed by Horstmann (1979) is used in the present investigation for determining the protein contents of muscles and meat products.

Other methods that can be used for protein measurement in meats and meat products include the biuret method for peptide bonds, the Lowry method for Tyr content and the dye binding method for charged groups [reviewed by Ellis, 1987 and Benedict, 1987].

Collagen

Direct biochemical estimation of the amounts of collagen present in tissues is difficult. This difficulty arises from the presence of intra- and inter-molecular crosslinks which make the collagens very insoluble. The collagen protein is usually quantitated from the amounts of the protein-bound Pro(4-OH) [reviewed by Jackson and Cleary, 1967; Pearson, 1975; Berg, 1982]. The determination of Pro(4-OH) has been based on its oxidation to a pyrrole-2-carboxylic acid or on its oxidation with p-toluenesulfonchloramide (Chloramine-T) in the presence of a known excess of alanine [reviewed by Jackson and Cleary, 1967]. These methods are sensitive to interference from other than the Pro(4-OH) compounds, such as salts, other amino acids, and have low reproducibility mainly due to differences between standards and unknown samples caused by the instability and colour variability of the formed chromogen compounds [reviewed by Jackson and Cleary, 1967]. Determination of Pro(4-OH) in a protein or a tissue hydrolysate can be achieved by ion exchange chromatography [Zarkadas et al., 1986b] or HPLC [Jones et al., 1986; Macek and Adam, 1986]. In a ring experiment in which

the chemical composition of lean beef was examined the results of the collagen content based on Pro(4-OH) turned out to be the least accurate of all the tests performed [Dransfield et al., 1983].

Besides collagen, Pro(4-OH) is also found in elastin [Bendall, 1967]. In skeletal muscle, elastin content is relatively low but in some muscles, such as in Semitendinosus, it is found in high levels [Bendall, 1967] and if not separated from collagen, gives an underestimation of the total collagen. Elastin is insoluble in hot alkali and this method or autoclaving are used to remove all the tissue collagen as soluble gelatin. The protein-bound Pro(4-OH) is then determined in the tissue residue obtained by any of the above methods and is transformed to its equivalent collagen after multiplied by a suitable factor [Etherington and Sims, 1981].

Light and Champion (1984) demonstrated the selective isolation and purification of muscle epimysium, perimysium and endomysium collagens by combining the methods of McCollester (1962; 1963) and Laurent et al. (1981). The phosphate buffers employed [Light and Champion, 1984] are very effective in increasing the water holding capacity of muscle and dissociating the actomyosin complex [Offer and Trinick, 1983]. The presence of NaCl (0.15 M) in phosphate buffers causes slight increase in pH but also lowers the isoelectric point of the proteins [Offer and Trinick, 1983]. The method proposed by Light and Champion, (1984) involves several fractionation steps and pepsin digestion that make the method time-consuming.

The method of McCollester (1962; 1963) has been used by many researchers to separate the intracellular contents of aged or fresh muscle cell from the sarcolemmal sheath [Hultin and Westort, 1969; Comissioning and Hultin,

1976; Stanley, 1983]. Laurent et al. (1981) used 2% SDS to remove all the non-collagen proteins from the collagenous proteins in lung tissue. The 2% SDS does not solubilize the bulk of collagen. Light and Champion, (1984) however, found that 0.7% of the total collagen content can be solubilized by SDS washing at room temperature for one hour, which was attributed to the pH (5.51) of the SDS solution. The combined methods of McCollester (1962; 1963), Laurent et al. (1981) and Stanley (1983) have been adopted in the present investigation to quantitatively isolate the myofibrillar and connective tissue proteins from skeletal muscles. The incubation of the muscle tissue in Tris-H_2O buffer as recommended by McCollester (1962; 1963), was ommited due to the uncontrolled nature of this step which probably involves extensive proteolysis and bacterial growth during the course of the incubation. Incorporation of enzyme inhibitors during the incubation step prevents the emptying phenomenon [McCollester and Semente, 1964].

The ratio of the main type I and III collagens found in skeletal muscles is estimated by purification of the muscle tissue (with one of the mentioned extraction methods) and then carboxymethyl cellulose chromatography and differential precipitation [with ethanol, NaCl or $(NH_4)_2SO_4$]. The disadvantage of this method is that salts have to be removed before further electrophoresis or chromatography [Miller, 1984]. Furthermore, the ratio of types I and III can be determined by molecular sieve chromatography, visualization of antibodies specific for types I and III, or quantitation of specific CNBr peptides derived from types I and III and separated either by SDS-polyacrylamide gels [Laurent et al., 1981; Light et al., 1985; Light and Champion, 1984], or by HPLC [Van de Rest et al., 1980]. Recently developed

spectrophotometric methods for the determination of collagen content involving the use of specific dyes can only be used in a very specific range (1-50 µg of protein) [Marotta and Martino, 1985] and need mathematic or spectrophotometric correction for the noncollagen proteins present. The enzyme linked immunoabsorbent methods (ELISA) [Rennard et al., 1980; Bellon, 1985], although sensitive at the level of ng of collagen protein, are not accurate and reproducible when applied to whole tissues because some collagens are more insoluble than others; another limiting factor is that collagens are poor immunogens and the ELISA methods may also measure cross-reactivity. Radioimmunoassays are prominent methods, but it is difficult to obtain highly labeled collagens which retain antigenicity. Zarkadas (1981) proposed that the collagen content of muscle tissues and meat products can be accurately quantitated from the levels of the protein-bound Lys(5-OH) found in their acid hydrolysates. Zarkadas et al. (1986b; 1987a) developed very sensitive ion-exchange chromatographic procedures where the unique stable crosslinks of collagen and elastin [Lys(5-OH) and Des, iDes] are completely resolved from other basic amino acids found in meat tissue hydrolysates.

Elastin

The determination of elastin in muscle tissues is based on the determination of Des and iDes found in tissue hydrolysates [Zarkadas et al., 1986b; 1987a] or in purified elastin by paper chromatography and subsequent ion exchange chromatography or HPLC [Soskel, 1987; Faris et al., 1981]. The enzyme linked immunoabsorbent assays [Gunja-Smith, 1985] recently developed

to determine elastin from Des levels are sensitive but show 20% cross-reactivity towards a nonreducible collagen crosslinking residue present in many tissues.

Due to the high insolubility of elastin to protein solvents and its resistance to hydrolysis, very strong methods have been used for extracting it from various tissues [reviewed by Jackson and Cleary, 1967; Cleary and Gibson, 1983]. The tissue is either autoclaved to remove the collagen or extracted in hot (98°C) 0.1 M NaOH for 45 - 50 min [Cross et al., 1973a; Bendall, 1967]. The prolonged autoclaving, although necessary for consistent protein yields and amino acid determinations results in losses of elastin and an increase in NH_2 terminal residues [Starcher and Galione, 1976]. Elastin extraction from muscle most often includes digestion of the tissue with trypsin and reaction with CNBr in formic acid assuming that no methionine residues are present in the elastin sequence. Collagenase and CNBr has been used for elastin extraction [Rasmussen et al., 1975], but is not accepted by others [Sage and Gray, 1979] because it leads to errors due to the present of methionine in some elastins [Starcher and Galione, 1976].

Other methods of elastin quantitation rely on determination of Val-Pro peptide [Hauschka and Gallop, 1979] found in alkaline hydrolysates of elastin; quantitation by HPLC of a pentapeptide Val-Gly-Val-Pro-Gly [Sandberg et al., 1986] found in thermolysin digests of either purified elastin or whole tissues. However the Val-Pro peptide is also found in high sulphur keratin, α-casein and acidic microfibrillar protein [Hauschka and Gallop, 1979]; the pentapeptide is found in the phosphofructokinase from Bacillus stearothermophillus [Haushka and Gallop, 1979]. The levels of

these peptides from elastin of different species vary [Raju and Anwar, 1987] and depend upon the different procedures used for the elastin preparation. The methods based on the specific peptides, though sensitive (2 ≈ 3 μg of elastin), have yielded variable results in the species examined because of incomplete cleavage or partial degradation of the pentapeptide during the thermolysine digestion of elastin [Sandberg et al., 1986].

SECTION III

THE QUANTITATION OF MYOFIBRILLAR AND CONNECTIVE TISSUE PROTEINS IN THE BOVINE Diaphragm MUSCLE

3.1 INTRODUCTION

There has been a continuing interest in the development of analytical methods for the quantitative determination of the myofibrillar and connective tissue proteins in vertebrate skeletal muscle tissues.

A thorough understanding of the structural organization of the muscle cell during development and growth of an animal requires the exact quantitation of the various muscle cell protein levels. The muscle mass of the Diaphragm has been reported to vary considerably among various nonthoracic diseases, i.e., chronic bronchitis, emphysema [Steele and Heard, 1973; Thurlbeck, 1978], in cardiac diseases, and in pleural or chronic obstructive lung disease [Ishikawa and Hayes, 1973; Scott and Hoy, 1976], and in Duchenne muscular dystrophy. In the latter, which is the most rapidly progressive of the muscular dystrophies, the muscle cells die and eventually the Diaphragm muscle is affected. Death of 90% of the patients by their twenties is directly related to chronic respiratory insufficiency [Rideau et al., 1983]. Since changes in diaphragmatic muscle mass appear to be clinically and physiologically important, normal levels of the myofibrillar and connective tissue proteins found in the mid-costal region of the young bovine Diaphragm can be useful as a reference standard.

One major method presently in use for estimating the myofibrillar protein contents of muscles involves the quantitative extraction, solubilization and separation of the contractile proteins by one- or two-dimensional electrophoresis on polyacrylamide gels [Potter, 1974; Yates and Greaser, 1983a; Murakami and Uchida, 1985], followed by densitometry. This method of quantitating contractile proteins in muscle tissues showed considerable variation in the myosin and actin contents, mainly due to comigration of different proteins on the gel.

Protein levels in the stained bands have also been estimated by eluting the separated myosin and actin bands; their protein concentrations were assessed either by amino acid analysis corrected for comigrating proteins [Yates and Greaser, 1983a;b] or by measuring the amount of extracted dye [Murakami and Uchida, 1985]. The molar ratio of myosin to actin in skeletal muscles thus determined is 1.0/6.0 [Potter, 1974; Murakami and Uchida, 1985]. The myosin to actin mass ratio, whose determination involves extraction of fresh or glycerinated muscle with various buffers, has also shown variation [Szent-Gyorgi et al., 1955; Hanson and Huxley, 1957]. This variation has been attributed to differences in both myofibrillar volume and protein content between fast and slow muscle types, as determined by quantitative electron microscopy [Eisenberg and Kuda, 1976; Page and Surak-Droske, 1979].

The quantitation of N^{τ}-methylhistidine [His(τ-Me)], a unique basic amino acid found in both myosin and actin, [Elzinga et al., 1973; Vandekerckhove and Weber, 1978a; 1979a; Elzinga and Collins, 1977] has been proposed as an index for determining the levels of these two principal

myofibrillar proteins in tissues [Perry, 1970; Hibbert and Lawrie, 1972] and their turnover rates in skeletal muscle [Young and Munro, 1978; Bates and Millward, 1983].

The chromatographic methods used to quantitate the His(τ-Me) content of skeletal muscles [Rangeley and Lawrie, 1976; Haverberg et al., 1974; Jones et al., 1982; White and Lawrie, 1985a] have yielded variable levels of this unique amino acid in different muscle tissues from various species. For example, bovine muscles were found to contain 4.79 - 6.2 mg His(τ-Me)/g of non-collagen nitrogen (NCN), porcine muscles 4.15 - 8.56 mg His(τ-Me)/g of NCN, and avian muscles were found to contain 5 - 10.3 mg His(τ-Me)/g of NCN [Poulter and Lawrie, 1980a; Rangeley and Lawrie, 1976; 1977; Jones et al., 1982; Arneth and Herold, 1985].

Although some of this variation was attributed to the presence of variable amounts of fast, slow or mixed fast/slow classes of myosin heavy chain isoforms in different muscle types [Huszar, 1984; Johnson et al., 1986], most of this variation was due to incomplete separation of the very small amounts of His(τ-Me) from other compounds present in such complex tissue hydrolysates by the multicolumn systems employed [Hancock and Harding, 1984]. Another complication has been the presence of variable amounts of balenine and free His(τ-Me) in skeletal muscles of all mammalian and avian species studied [Carnegie et al., 1982; 1983; 1984; Harris and Milne, 1987].

The extracellular matrix proteins of skeletal muscle appear to play an important role in the morphogenesis, development [Carrino and Caplan, 1986; Stephens et al., 1982a; 1982b; Lowey et al., 1983; Wiens et al., 1984], and

function of skeletal muscle [Borg and Caulfield, 1980; Rowe, 1981; 1986] in addition to their contribution to structure. Collagen structural defects have for example been reported as main factors in avian muscular dystrophy [DeMichele et al., 1985; 1986]. The connective tissue content of skeletal muscle is usually calculated from the amounts of Pro(4-OH) found in tissue hydrolysates [reviewed by Pearson, 1975 and Berg, 1982], or by quantitative separation of the intracellular skeletal muscle proteins from the extracellular matrix followed by the determination of the collagen and elastin content after CNBr cleavage [McCollester, 1962; 1963; Rosenthal et al., 1965; Stanley, 1983; Laurent et al., 1981; Light and Champion, 1984].

Measurement of elastin in muscle tissue is based on the determination of the unique crosslinks, desmosine (Des) and isodesmosine (iDes), found in tissue hydrolysates [Starcher, 1977; Faris et al., 1981; Soskel, 1987] or, alternatively on the determination of specific peptides [Hauschka and Gallop, 1979; Sandberg et al., 1986] found in alkaline hydrolysates and thermolysin digests of elastin. However, the peptides quantitated have been found in other proteins [Hauschka and Gallop, 1979] and variable levels of elastin have been determined in the species studied [Sandberg et al., 1986] based on the latter method.

Analytical ion-exchange single-column chromatographic methods have been developed [Zarkadas, 1979; Zarkadas et al., 1986b; 1987a] to validate the use of His(τ-Me) as an index of myosin and actin content in skeletal muscles, and Lys(5-OH) and desmosines can be used as quantitive markers of collagen and elastin, respectively.

The aim of the present study was, using these sensitive and high resolution single-column chromatographic methods, to quantitate the levels and variation of the unique and other amino acids of the intracellular and the extracellular fractions (F1 and F2, respectively) quantitatively isolated from young bovine Diaphragm muscle by the combined methods of McCollester (1962; 1963), Rosenthal et al. (1965), Comissioning and Hultin (1976), Stanley, (1983), and Laurent et al. (1981) . Calculations were based on the total protein of the intracellular (F1) and extracellular (F2) protein fractions of costal Diaphragm as determined by detailed amino acid analysis [Zarkadas et al., 1986b; 1987a]. The aim was to determine whether the levels of these unique amino acids in the isolated intracellular and extracellular muscle protein fractions could be used to quantitate the myofibrillar and connective tissue proteins present in the Diaphragm muscle.

3.2 MATERIALS AND METHODS

Chemicals and Resins

The different cation-exchange spherical resins used in the present study included types DC-5A (6.0 ± 0.5 µm; lot No. 746), DC-4A (9.0 ± 0.5 µm; lot No. 750) and DC-6A (11.0 ± 1.0 µm; lot No. 3280) obtained in the sodium form from Dionex Chemical Co., Sunnyvale, CA, type W-3 resin which was also in the sodium form obtained as 9.0 ± 0.5 µm particle size diameter from Beckman Instruments, Inc., Palo Alto, CA. Amino acid standard H and norleucine were obtained from Pierce Chemical Company (Rockford, IL) and type I standard amino acid calibration mixture was purchased from Beckman Instruments, Inc., Palo Alto, CA. L-Tryptophan and DL-ornithine (5-aminorvaline) were purchased from Schwarz/Mann, Orangeburg, NY.

The uncommon amino acid (uncommon amino acids are defined as the ones not incorporated into protein under mRNA direction) standards were obtained as follows: the diastereoisomer mixture of allo- and 5-hydroxy-DL-lysines, N^6-methyl-L-lysine, N^6-dimethyl-L- and N^6-trimethyl-L-lysines-bis(p-hydroxyazobenzene-p'-sulfonate) hydrates, N^τ-methyl-L-histidine, N^π-methyl-L-histidine hydrate and 4-hydroxyproline from Calbiochem-Behring, La Jolla, CA; 3-nitro-L-tyrosine from Aldrich Chemical, Milwaukee, WI. Isopropanol and methanol were products of Caledon LaboratorieS, Georgetown, ON. Hydrolysed potato starch was purchased from the Connaught Medical Research Laboratories of the University of Toronto, Toronto, ON. Phenol was a product of J.T. Baker Chemical Co., Phillipsburg, NJ. Octanoic acid, calcium

chloride, sodium chloride, and sodium phosphate (monobasic and dibasic) were obtained from Allied Fisher Scientific Company, Fairlawn, NJ. Sodium dodecyl sulphate was a product of Sigma Chemical Company, St. Louis, MO.

The buffers used for the high-sensitivity standard single-microcolumn analysis [Zarkadas et al., 1987a] were obtained as concentrates (B2: pH 4.25, 0.20 M; B3: pH 6.40, 1.0 M) and as ready to use solution (B1: pH 3.28, 0.20 M) from Beckman Instruments, Inc., Palo Alto, CA. The buffers for the other microcolumn analyses [Zarkadas et al., 1986b; 1987a] were prepared with sodium citrate.$2H_2O$ (crystals) obtained from J.T. Baker Chemical Co., Phillipsburg, NJ. Ninhydrin, stannous chloride.$2H_2O$, sodium acetate buffer and Piersolve (ethylene glycol monomethyl ether) were purchased from Pierce Chemical Co., Rockford, IL. Hydrochloric and trichloroacetic acids were products of Anachemia, Champlain, NY. Triple glass-distilled constant boiling HCl (5.7 M) was prepared according to the method described by the Association of Official Agricultural Chemists (1955), with the usual precautions described by Hamilton and Myoda, (1974).

All reagents and buffers used were prepared with high-purity laboratory water prepared by one of the procedures described by Ganzi (1984). Distilled water was passed through two mixed bed ion exchange resins and a charcoal filter (Continental Water System, El Paso, TX), redistilled in a glass distillation apparatus (Corning, AC-3, Corning Glass Works, Parkersburg, WV), transferred in acid washed Pyrex containers and passed through an RG501-X8 mixed bed resin (Biorad Lab., Richmond, CA) prior to use. All other chemicals and reagents used in this study were of the highest purity commercially available.

Sampling and Preparation of Muscle Tissue

Samples of bovine Diaphragm were excised as 5-cm-thick muscle sections from the left (or right) side of two commercial carcasses weighing approximately 110-130 kg each, which were randomly selected from young (12 - 14 months old) Angus steers (Canada Grade A) obtained from Abattoir Soulanges, Les Cedres, Quebec. Representative samples (250 g each) from the mid-costal region of the Diaphragm were trimmed of adhering fat and connective tissue, cut into small cubes, dried on Whatman No. 3 filter paper, frozen (-173 °C), and then pulverized in an electric driven end runner coffee mill (Moulinex Canada Ltd., Weston, Ontario). The bovine Diaphragm powders were then stored at -72 °C until needed.

Preparation of the Intracellualar SDS-Soluble (F1) and Extracellular SDS-Insoluble Protein (F2) Fractions from Bovine Costal Diaphragm

The selected specimens for this experiment were excised one-day postmortem from the costal region of the bovine Diaphragm which is attached to the lumbar vertebrae [Ashdown and Done, 1984] and consists primarily of striated skeletal muscle. The procedure employed for the extraction and preparation of a sodium dodecyl sulphate SDS-soluble intracellular muscle protein fraction (F1), and an SDS-insoluble extracellular matrix protein fraction, designated F2, combines the original method of McCollester (1962, 1963) and the procedures described by Stanley (1983) and Laurent et al. (1981) as follows: Ten g of frozen and well mixed sample were soaked in 75 mL of 0.05M $CaCl_2$ until it thawed (10 - 15 min) (Sanes and Hall, 1979). The sample was then homogenized in a VirTis Model 45 blade homogenizer

(VirTis Co. Inc., Gardiner, NY) for 10 s, (speed setting of 40/100) at 4 °C. The homogenizer was equipped with two blades which were reversed to increase shredding and reduce shearing of the muscle cells as recommended by Rosenthal et al. (1965). The homogenate was centrifuged at 4000 x g (Sorvall centrifuge model RC-2; rotor SS-34; 6,000 rpm) for 20 min at 4 °C, the supernatant was decanted through eight layers of cheese cloth to trap fat particles, and was retained at 4 °C so that muscle proteins could be precipitated with 5% trichloroacetic acid. The pellet was resuspended in 50 mL of ice-cold phosphate-buffered saline (PBS: 0.15 M NaCl/0.02 M sodium phosphate buffer, pH 7.4) and rehomogenized for 3 min at 4 °C. The homogenate was centrifuged at 4000 x g for 20 min and the supernatants combined. The sediment was resuspended in 50 mL 2% SDS (w/v) adjusted to pH 7.0 (25 °C), rehomogenized at the same speed (40/100) for 3 min at room temperature, recentrifuged and the supernatants combined. The wash procedure with SDS was repeated four more times as recommended for lung tissues by Laurent et al. (1981). The final pellet was then homogenized a further three times with 50 mL of PBS to remove the bulk of SDS. The supernatants were combined, and the intracellular SDS-soluble skeletal muscle protein fraction (F1) was recovered by precipitation with 5% trichloroacetic acid at 4 °C overnight and centrifugation at 14,000 rpm (Beckman model L5-75 ultracentrifuge; rotor #19) for 30 min at 4°C. The precipitated SDS-soluble muscle proteins (F1) were desalted by exchaustive dialysis at 4 °C against deionized water, 40% methanol (v/v) and again deionized water (Mr dialysis tube cut off 12,000), and finally freeze-dried, weighed, ground finely in

the end runner mill, and stored at -20 °C. The combined pellet containing the SDS-insoluble connective tissue proteins was rehomogenized in acetone for 3 min, centrifuged at 4,000 x g for 10 min at 4 °C and the supernatant discarded. This step was repeated twice and the pellet was dialyzed as before, dried by lyophilization, weighed and stored at -20 °C.

Procedures for Amino Acid Analyses

Preparation of Amino Acid Calibration Standards

The uncommon amino acid calibration standards used for the standardization of the amino acid analyzers and for peak identification were prepared as described by Zarkadas (1975; 1978; 1979). Desmosine and isodesmosine standard solutions were prepared in concentrations of 2.5 µmol/mL in 0.1 M HCl (triple glass-distilled, 5.7 M) from purified concentrated solutions [Zarkadas, 1979]. Norleucine and 3-nitrotyrosine were used as internal standards [Riordan and Giese, 1977]. Since the commercial 3-nitrotyrosine product it was found to contain impurities that coeluted with histidine, these were removed by gel chromatography on a Sephadex G-10 (100 x 1.5 cm) column equilibrated and eluted with 0.01 M hydrochloric acid according to Riordan and Giese (1977) and Zarkadas et al. (1986b). Stock solutions of norleucine and 3-nitrotyrosine (2.5 µmol/mL) in 0.2 M HCl were kept at -20°C untill needed. The diastereoisomers of 5-hydroxylysine were prepared after epimerization in 5.7 M triple glass-distilled HCl at 110° ± 1°C for 96 h as described previously [Zarkadas, 1975].

Regeneration and Equilibration of the Resins

To optimize separation on the microcolumns, and eliminate the gradual increase in back pressure associated with their continous use, the following special precautions were necessary for regenerating and equilibrating the resin, essentially as described previously [Zarkadas, 1979]. Before pouring the column or after prolonged use, the resin (10 g) was heated (75 °C) with agitation in 40 mL 2.0 M NaOH containing 0.25 g/L EDTA for 90 min. Deionized glass distilled water (20 mL) was added to the hot resin and the later was centrifuged for 10 min at 3,000 rpm in a Model IEL HN-SII swinging backet rotor (International Equipment Company, Damon/IEC Division, Needman Heights, MA). The washing with double deionized water was repeated six to seven times until the supernatant was neutral (Whatman BDH indicator paper). The resin was then heated (75-80 °C) with agitation in 5.7 M constant boiling glass-triply distilled HCl for 90 min, centrifuged and rinsed again in the above manner, slurried and deaerated in 20 mL 0.2 M NaOH containing 0.10 g/L EDTA. After packing, the microcolumns were regenerated and equilibrated before each analysis as described previously [Zarkadas et al., 1986b; 1987a].

Preparation of Buffers and Ninhydrin

The composition of the sodium citrate buffers employed for high-sensitivity single-microcolumn standard amino analyses by the methods described by Zarkadas et al. (1986b; 1987a), is given in Table 4. The preparation of the buffers used for the analyses of tryptophan, 4-hydroxyproline, on a 0.6 x 25 cm column was carried out as described

Table 4. Composition of Sodium Citrate Buffers Used for Amino Acid Analyses[a].

Parameters	Type of Analysis								
	Basic Amino Acids		Standard Amino Acids			Cysteic Acid, Methionine	S,S-dioxide	4-Hydroxy proline	Tryptophan
	A1	A2	B1[c]	B2[d]	B3[e]	A	B	C	D
Molarity (M)	0.35	0.35	0.20	0.20	1.0	0.20	0.20	0.20	0.21
Final pH[b]	5.700±0.002	4.501±0.002	3.280±0.002	4.250±0.002	6.400±0.002	3.280±0.002	4.100±0.002	2.910±0.002	4.775±0.002
Sodium Citrate (g)	137.26	137.26	-	-	-	78.43	78.43	78.43	82.35
HCl, 5.7 M (mL)[f]	26.0	80.0	-	1.5	-	85.0	50.0	90.0	38.4
Isopropanol	-	-	27.0	-	-	80.0	80.0	80.0	80.0
Octanoic Acid (mL)	1.0	1.0	-	-	-	0.4	0.4	0.4	0.4
Liquified Phenol (mL)	4.0	4.0	-	-	-	4.0	4.0	4.0	4.0
Thiodiglycol 25% (mL)	-	-	-	-	-	10.0	10.0	40.0	-
Final Volume (L)	4.0	4.0	.927	1.0	1.0	4.0	4.0	4.0	4.0

[a]Summarized from Zarkadas et al. (1986b, 1987a).

[b]pH measured at 25°C.

[c]Ready to use solution.

[d]Prepared from a 10 X concentrated buffer.

[e]Prepared from a 5 X concentrated buffer, containing 0.8 M sodium chloride.

[f]Triple glass distilled HCl.

previously [Zarkadas et al., 1986b] and that for cysteic acid and methionine S,S-dioxide on a 0.6 x 45 cm column as described by Fauconnet and Rochemont (1978) (Table 4).

The 0.35 M sodium citrate buffers (A1 and A2) used for the analysis of the methylated and crosslink basic amino acids (Table 4), were prepared in 4.0 L quantities, using acid washed (1:1 concentrated nitric and sulfuric acids) Pyrex reagent bottles, and after a filtration step (0.22 μm type GS, Millipore microfilters) were purified on a 45 x 0.9 cm Dionex DC-6A column (regenerated with 0.2 N NaOH containing 0.25 g/L EDTA, and equilibrated with 0.2 N HCl) as recommended by Zarkadas et al. (1987a). The column was operated at 73°C at a flow rate of 45 mL/h. All sodium citrate buffers were degassed before use under vaccum and sonication.

The ninhydrin reagent was prepared as described by Fauconnet and Rochemont (1978) in brown-glass bottles, acid washed to remove metal traces that would otherwise interfere with the ninhydrin-amino acid reaction [D'Aniello et al., 1985]. The ninhydrin reagent (72 g) was added and mixed to a degassed (for 15 min with pure N_2) solution of 2.70 L Piersolve and 0.90 L sodium acetate buffer (pH 5.51; 4.0 M). After the ninhydrin reagent was completely dissolved, 1.44 g of stannous chloride crystals were added and dissolved [James, 1984] with continuous bubbling of pure nitrogen. The 3.6 L ninhydrin reagent was kept under an atmosphere of nitrogen (purity 99.99%) at 2°C [Zarkadas et al., 1987a] and remained stable for two months.

Preparation of Tissue Hydrolysates

Acid Hydrolysis

Complete amino acid analyses were carried out on each of the two fractions, F1 and F2, prepared from the bovine Diaphragm of two animals. Ten mg of the F1 fraction and 5 mg of the F2 fraction were hydrolysed in Pyrex test tubes (18 x 150 mm) under vacuum (below 10μm of mercury) with 7.0 mL of triple glass-distilled constant boiling HCl (5.7 M) at 110 °C in duplicate for 24, 48, 72 and 96 h respectively with the usual precautions described by Moore and Stein (1963). Foaming of the hydrolysates was supressed during evacuation by the addition of 5-10 μL of octanoic acid. Liquified phenol (5-10μL) was also added to each hydrolysate [Blackburn, 1978]. The appropriate volume of 2.5 μmol/mL Nle, used as internal standard, was incorporated before hydrolysis in all the hydrolysates, as recommended by Gardner (1985). Additionally, 3-nitrotyrosine internal standard, was incorporated in the 96 h hydrolysates [Zarkadas et al., 1987a]. The small amounts of insoluble materials formed during acid hydrolysis were removed by filtration (0.22 μm type GS, Millipore microfilters). Exhaustive washing was essential in order to eliminate losses due to adsorption of amino acids onto the inside of the hydrolysis tubes [Robel, 1973]. The clear filtrates were then evaporated, dried under vacuum (below 750 μm of mercury) in a Rotary EvapoMix (Buchler Instruments, Fort Lee, NJ) at 42°C, and transferred to 1 or 5 mL volumetric flasks, with sodium citrate sample-dilution buffer pH 2.20, 0.2 M Na^+ (19.61 g/L sodium citrate, 32 mL/L 6 M HCl, 20.0 mL/L

Table 5. Summary of the Operational Parameters for Amino Acid Analyses[a].

Parameters	Type of Analysis				
	Basic Amino Acids	Standard Amino Acids	Cysteic Acid, Methionine S-S,dioxide	4-Hydroxy-proline	Tryptophan
Col.Dimension (cm)	50.0 X 0.28	23.5 X 0.28	37.0 X 0.6	21.0 X 0.6	21.0 X 0.6
Resin Type	DC-4A	DC-5A	W-3	DC-6A	DC-6A
Resin Size (μm)	9.0 ± 0.5	6.0 ± 0.5	9.0 ± 0.5	11.0 ± 1.0	11.0 ± 1.0
Buffer change (min)[b]					
ΔA → B	215.7	27.1	45.0	-	-
ΔB → C	-	52.6	-	-	-
Initial Temperature (°C) (T1)	31.5	42.5	43.0	45.0	52.0
Final Temperature (°C) (T2)	73.0	73.0	73.0	-	65.0
Temperature Change (min)[b]					
ΔT1 → T2	294.7	12.7	20.0	-	0
Flow Rate (mL/h)					
Buffer	5.65	5.75	30.0	30.0	30.0
Ninhydrin	5.60	5.60	15.0	15.0	15.0
Regeneration time (min)[c]	8.0	4.0	30.0	20.0	20.0
Equilibration time (min)[d]	30.0	20.0	50.0	40.0	40.0
Sample Volume (μL)	100	100	250	250	500
Reaction Temperature (°C)	126	126	100	100	100

[a]Summarized from Zarkadas et al. (1986b, 1987a).

[b]Refers to time after injection.

[c]Regeneration with 0.2 M NaOH containing 0.25 g/L EDTA.

[d]Equilibration with the first eluting buffer.

thiodiglycol, 1.0 mL/L liquified phenol) by repeated washings and sonication, made up to volume with the dilution buffer and stored in Pyrex screw cap tubes (No. 9826) at 4°C. An aliquot of this dilution was transferred to a 1.5 mL micro-centrifuge tube and centrifuged in a Beckman Microfuge-B (Beckman Instruments, Palo Alto, CA) for 5 min at 11,600 rpm before its application to the amino acid analyzer or any subsequent dilutions.

Alkaline Hydrolysis

Tryptophan was determined separately after alkaline hydrolysis [Hugli and Moore, 1972] in the bovine costal Diaphragm isolated fractions (150-200 µg of protein per injection) by an improved chromatographic procedure using 3-nitrotyrosine as an internal standard [Zarkadas et al., 1986b] (Table 5).

Performic Acid Oxidation Procedure

Cyst(e)ine and methionine were determined in separate samples (80-120 µg protein/250 µL) by the performic acid method of Moore (1963), as described by Zarkadas et al. (1986b) (Table 5). Norleucine was added in the hydrolysates (before the oxidation procedure) as an internal standard [Gardner, 1985], and the recoveries of cyst(e)ine as cysteic acid and methionine as the dioxide were calculated in proportion to the yields obtained by the performic acid treatment of standard solutions of these amino acids and relative to alanine and leucine present in each sample.

Instrumentation

Analyses of cysteic acid, methionine S,S-dioxide, tryptophan and 4-hydroxyproline were performed on a conventional Beckman amino acid analyzer Model 120C [Zarkadas et al., 1986b]. The common and the unique methylated and crosslink amino acids were determined on a fully automated Beckman Model

120C amino acid analyzer (equivalent to Beckman Model 121MP) as described by Zarkadas et al. (1987a).

Methods for Amino Acid Analyses

All samples were analyzed by the standard procedure described by Zarkadas et al. (1987a) (Table 5). Norleucine was used as internal standard. A full loop (100 µL containing 0.6 - 1.5 µg of protein) was used for all injections. The data reported for threonine, serine, tyrosine and ammonia represent the average of values extrapolated to zero time of hydrolysis assuming first order kinetics [Blackburn, 1978]. The values for valine, isoleucine, leucine and phenylalanine are averages of data from 48, 72 and 96 h of hydrolysis. All other values of amino acids are reported as the average value from 24, 48, 72 and 96 h of hydrolysis. 4-Hydroxyproline was determined separately from a concentated 24 h hydrolysate (equivalent to 200-250 µg of protein per analysis) using a single column (21 x 0.6 cm) packed with Dionex type DC-6A resin [Zarkadas et al., 1986b] (Table 5). Recoveries of Pro(4-OH) were calculated relative to alanine. The determination of the methylated basic amino acids, the diastereoisomers of Lys(5-OH) and related compounds were carried out with concentrated 96 h acid hydrolysates (equivalent to 180-220 µg of protein per analysis) by the single-microcolumn (50 x 0.28 cm) system using Dionex DC-4A resin [Zarkadas et al., 1987a] (Table 5). This amount of protein injected yielded peaks equivalent to 1-2 nmol His(τ-Me) (in the analyzed F1 fraction) and 10-14 nmol Lys(5-OH), 0.25 - 0.40 nmol iDes, Des (in the F2 fraction). The unusual amino acid calibration standards (2 nmol per injection for each unique

basic amino acid with the exception of iDes=0.9610 nmol and Des=1.0378 nmol) employed for peak identification and computation of the relative response factors were prepared as described previously, using 3-nitrotyrosine as the internal standard. Recoveries of these unique basic amino acids were calculated based on the protein content of individual hydrolysates determined by the procedure described by Horstmann (1979).

Protein Determination

The protein content of the isolated muscle fractions was determined in individual hydrolysates as described by Horstmann (1979) and Nguyen et al. (1986). According to these procedures, the precise protein content of a tissue hydrolysate can be calculated by multiplying the sum of the amino acids (in nmol), determined in the acid hydrolysates of the particular tissue by detailed amino acid analysis, by its weight equivalent (WE in µg/nmol). The weight equivalent (WE) or average residue weight is characteristic of each protein or protein mixture and is calculated from the molar concentration (a_i) of a particular amino acid i (determined in the analyzed acid hydrolysate) multiplied by the anhydrous molecular weight b_i (in µg) of the i amino acid according to the formula:

$$WE = \sum_{i=1}^{20} (a_i b_i) \qquad (1)$$

Tryptophan and cyst(e)ine are destroyed during acid hydrolysis, therefore a conversion factor (F in µg/nmol) is essential, in order to determine

the protein mass found in acid hydrolysates and is calculated according to the formula:

$$F = \frac{\sum_{i=1}^{18} (a_i b_i)}{1 - (a_{Trp} + a_{Cys})} \qquad (2)$$

where a_i is the mol fraction of a particular amino acid $\underline{i}$ per mol of total amino acid composition [Nguyen et al., 1986].

A factor F' was also calculated in order to determine the protein content in a particular acid hydrolysate in the absence of tryptophan, cyst(e)ine, proline and Pro(4-OH) according to the formula:

$$F' = \frac{\sum_{i=1}^{16} (a_i b_i)}{1 - [a_{Trp} + a_{Cys} + a_{Pro} + a_{Pro(4\text{-}OH)}]} \qquad (3)$$

The F' conversion constant is unique for the isolated muscle fractions (F1 and F2) or meat products and can be used in any latter protein quantitation [Nguyen et al., 1986], in hydrolysates of the same tissues as follows: the total concentration (c) of amino acids (in nmol) determined in an acid hydrolysate of the particular tissue is multiplied by the F' factor. The protein mass can then be computed according to the formula:

$$P_{conc} = F' . c \quad (\text{in } \mu g) \qquad (4)$$

The Quantitation of Collagen, Elastin and Connective Tissue Proteins in Skeletal Muscles

The collagen content of skeletal muscles can be calculated from the amount of the unique amino acid Lys(5-OH) found in their acid hydrolysates, by the modified formula of Jackson and Cleary (1967).

$$C_{COL} = \frac{C_{Lys(5\text{-}OH)} \cdot 1000 \cdot WE_{COL}}{n \cdot Mr_{Lys(5\text{-}OH)}} \qquad (5)$$

where C_{COL} is the concentration of collagen expressed in g/kg of total protein, $C_{Lys(5\text{-}OH)}$ is the concentration of Lys(5-OH) found in the acid hydrolysates of muscle tissues (in g/kg of total protein), WE_{COL} is the average mean residue weight of skeletal muscle collagen, n is the average number of Lys(5-OH) residues per thousand amino acid residues in skeletal muscle collagen, and $Mr_{Lys(5\text{-}OH)}$ is the anhydrous molecular weight of Lys(5-OH).

Types I, III and IV collagen account for an estimated 61.5%, 33.5% and 5% respectively, expressed as percentage of the total collagen quantified in five bovine skeletal muscles [Light and Champion, 1984; Light et al., 1985]. Since the average Lys(5-OH) content of skeletal muscle collagen was calculated to be 10.09 residues per 1000 amino acid residues [Miller and Gay, 1982; Tables 3 and 8); the average mean residue weight of muscle collagen is WE_{COL} = 0.091318 µg/nmol (Table 8), calculated according to the distribution of types I, III, IV collagen in muscle and their amino acid composition

[Miller and Gay, 1982; Table 3), $Mr_{Lys(5*OH)}$ is 0.14851 then the amount of collagen present in the bovine Diaphragm can be calculated according to the formula:

$$C_{COL} \text{ (g/kg of total protein)} = C_{Lys(5*OH)} \times 62.34 \quad (5a)$$

The elastin content of skeletal muscle can also be calculated from the levels of Des and iDes determined in the acid hydrolysates of muscle tissues. The mean composition of elastin purified from bovine tissues [Starcher and Galione, 1976] contains 4.1 residues of total desmosines (Des + iDes) per 1000 amino acid residues and has a mean residue weight WE_{EL} = 0.08504 µg/nmol. The anhydrous Mr of Des or iDes is 0.4545 µg. Therefore, the amount of elastin present in these muscle tissue fractions can be computed by the following equation:

$$C_{EL} \text{ (g/kg of total protein)} = C_{(Des + iDes)} \times 45.63 \quad (5b)$$

The total connective tissue content of skeletal muscle can be computed from equation (5) as follows:

$$C_{CON} = \frac{C_{Pro(4*OH)} \cdot 1000 \cdot WE_{CON}}{n \cdot Mr_{Pro(4*OH)}} \quad (6)$$

where C_{CON} is the concentration of connective tissue proteins in g/kg of total protein, $C_{Pro(4*OH)}$ is the concentration of Pro(4*OH) in g/kg total

protein found in the acid hydrolysates of the muscle tissue, WE_{CON} is the average mean residue weight of connective tissue proteins and is equal to 0.09088 (Table 8), and n is the mean Pro(4-OH) content of connective tissue proteins equal to 104.3 residues per 1000 total amino acids (Table 8). These calculations were based on the data reported for 33 bovine skeletal muscles [Bendall, 1967] which have been found to contain a mean collagen content of 93.05% and a mean elastin content of 6.95% (expressed as a percentage of the total connective tissue proteins). The Pro(4-OH) content of collagen and elastin is 111.12 and 13.0 residues per 1000 residues, respectively (Table 8).

Therefore, the total connective tissue in g/kg total protein in skeletal muscles can also be calculated as shown below:

$$C_{CON}\ (g/kg\ \text{total protein}) = C_{Pro(4\text{-}OH)} \times 7.7027 \qquad (6b)$$

Determination of Total Myofibrillar Proteins

Since the amount of His(τ-Me) in muscle tissues determined by the chromatographic method described by Zarkadas et al. (1987a) represents the sum of the distribution of His(τ-Me) in myosin and actin present in the Diaphragm according to the expression:

$$C_{His(\tau\text{-}Me)} = C_A + C_M \qquad (7)$$

where $C_{His(\tau\text{-}Me)}$ is the total protein-bound His(τ-Me) in myosin and actin, C_A is the amount (in g) of His(τ-Me) in actin, and C_M is the amount (in g) of His(τ-Me) in myosin in one kg of total protein.

The relative amounts of actin [Mr_A = 41,782; Elzinga et al., 1973] and

myosin [Mr_M = 521,000; Yates and Greaser, 1983b] per unit of tissue, expressed as mol/kg of total protein are related to the distribution of His(τ-Me) in myosin and actin of the Diaphragm, and can be calculated by the following equation:

$$\sum_{j}^{2} [P_A + P_M] = \left[\frac{A}{A + 2M} \cdot \frac{41,782}{151.2} + \frac{M}{A + 2M} \cdot \frac{521,000}{151.2}\right] \cdot C_{His(\tau\text{-}Me)} \quad (8a)$$

Substituting the molar ratio of actin to myosin reported by Murakami and Uchida (1985) [A/M = 6] in eqn (8a), the sum of actin and myosin in the Diaphragm can be calculated as follows:

$$\sum_{j}^{2} [P_A + P_M] = [207.25 + 430.72] \cdot C_{His(\tau\text{-}Me)} \quad (8b)$$

or

$$\sum_{j}^{2} [P_A + P_M] = 637.97 \cdot C_{His(\tau\text{-}Me)} \quad (8c)$$

Since the sum of myosin and actin in the myofibril accounts for 65% of the total myofibrillar protein by weight [Yates and Greaser, 1983b], the total myofibrillar protein in g/kg total protein in the Diaphragm can also be calculated as shown below:

$$\text{amt myofibrillar protein} = 981.49 \cdot C_{His(\tau\text{-}Me)} \quad (8d)$$

Statistical Analysis

Processing and linear regression of the amino acid analysis data were carried out by a Fortran computer program especially developed for this purpose. Analysis of variance on the amino acid data of the F1, F2 fractions isolated from the young bovine Diaphragm was carried out by the Statistical Analysis System (SAS) (1982), using the computing centre (VAX) at Datacrown, Inc., IBM, Toronto, Ontario. A complete randomized design with two animals was used [Steel and Torrie, 1980].

3.3 RESULTS AND DISCUSSION

The present studies investigate the possible use of four unique basic amino acids as markers for determining the specific myofibrillar and connective tissue proteins in vertebrate skeletal muscle. The young bovine Diaphragm chosen as an example of a typical skeletal muscle for these studies is a dome-shaped sheet of skeletal muscle tissue, separating the thoracic and abdominal cavities. The muscle fibers are large and project radially from its central dome near the sternum to the periphery and the two crura of the diaphragm, thus giving at least three distinct tissue sites [George and Susheela, 1961; Ashdown and Done, 1984]: the vertebral (dorsal), the costal (lateral) and sternal (ventral) regions and a largely tendinous central dome, which can be compared in each animal species for their myofibrillar and connective tissue contents. Although the presence of all three muscle cell types with fast, mixed fast/slow, and slow properties have been indicated [Green et al., 1984], the percentage of red fibers (slow, I) in the mammalian Diaphragm is related to the breathing frequency and metabolic rate [Gauthier and Padykula, 1966]; therefore, Diaphragm of larger mammals contains a greater proportion of slow twitch (type I) fibers, i.e., bovine Diaphragm (> 70%), whereas smaller animals have higher proportions of type IIA and IIB fibers [Davies and Gunn, 1972; Green et al., 1984]. In six bovine Diaphragms (costal region) examined by Young (1982), only 5% of the fibers were found to be fast and the rest of the fibers were found to be two variants of slow fiber type in approximately equal amounts. In addition these Diaphragms were found to have the slowest ATPase activity

after Masseter muscle (100% slow fibers) among 18 bovine muscles studied [Young, 1982]. To minimize the risk of variation in fiber types among the major diaphragmatic sites [Sieck et al., 1983], the mid-costal region of the left hemidiaphragm was sampled in the present study.

Accurate and detailed determination of the unique and other amino acid content of the intracellular and extracellular muscle protein fractions (F1 and F2) separated from the bovine Diaphragm was carried out at the picomole range by the single-column methodology described by Zarkadas et al. (1987a). A preliminary note illustrating the analytical capability of the present methodology and its application to complex tissue hydrolysates, i.e., the central dome region of the bovine Diaphragm, has been presented previously [Zarkadas et al., 1987a]. The procedure employed for the separation of the F1 and F2 fractions from the costal Diaphragm, as free as possible from all soluble amino acids and peptides, combines the original method of McCollester (1962; 1963), using aged for one day muscle [Stanley, 1983], and the SDS washing procedure described by Laurent et al. (1981). The results presented in Table 6 show that, even after exhaustive dialysis [Light and Champion, 1984], the separated SDS-soluble muscle protein fraction (F1) contained 54.78% protein compared to the extracellular SDS-insoluble connective tissue residue (F2) which contained only 31.25% protein. The total quantity of the intracellular muscle protein fraction F1 found in the bovine costal Diaphragm is on the average 95.27% of the total protein while the average quantity of SDS-insoluble extracellular matrix represents 4.73% of the total muscle protein. Since the protein concentrations in each fraction was determined from their amino acid composition [Horstmann, 1979], also

reported in Table 6, it is unlikely to cause large errors in the relative protein values reported for these fractions. Deviations from these averages may reflect variations in the thickness of the tendinous septa surrounding the myofibrils. The overall amino acid composition of the intracellular and extracellular muscle protein fractions (F1 and F2) from the young bovine Diaphragm, and levels of statistical significance obtained from analysis of variance as presented in Table 6, represent the average values of two replicates and duplicate determinations obtained from duplicate 24, 48, 72 and 96 h hydrolysates. The values in Table 6 show deviations of less than ± 2.0% from the average values obtained between two animals within the same treatment. The least variability in tissue amino acid content was found when the results were expressed as g of amino acid per kg of anhydrous, fat- and ash- free tissue protein, since the influence of both fat and moisture is eliminated [Tristram and Smith, 1963; Zarkadas et al., 1987b]. The protein concentration of individual hydrolysate samples was determined by the procedure described by Horstmann (1979). The average weight equivalents (WE in µg/nmol) and F, F' conversion factors obtained for both fractions F1 and F2 are given in Table 6, so that they can be used in all subsequent quantitations of protein in these muscle fractions following standard procedures [Horstmann, 1979].

The values obtained for protein-bound His(τ-Me) in the intracellular muscle protein fraction (F1) isolated from the costal region of the bovine Diaphragm show high reproducibility, low coefficient of variation, and within the precision of the chromatographic procedure (100 ± 2.5%) (Table 6). As shown in Figure 2A, the analysis of the intracellular muscle protein

Figure 2

Separation of the methylated basic, crosslink amino acids and related compounds present in the myofibrillar and connective tissue proteins of the costal region of the young bovine Diaphragm on an analytical 50 x 0.28 cm microcolumn of Dionex DC#4A resin. The arrows indicate the buffer and temperature changes. The curves show absorbance at 570 nm.

(A) Separation of methylated lysines and histidines in the intracellular muscle protein fraction (F1) (96 h hydrolysate) (219.0 µg protein/injection);

(B) Analysis of a 96 h acid hydrolysate of the extracellular matrix, (F2) fraction (65 µg protein/injection).

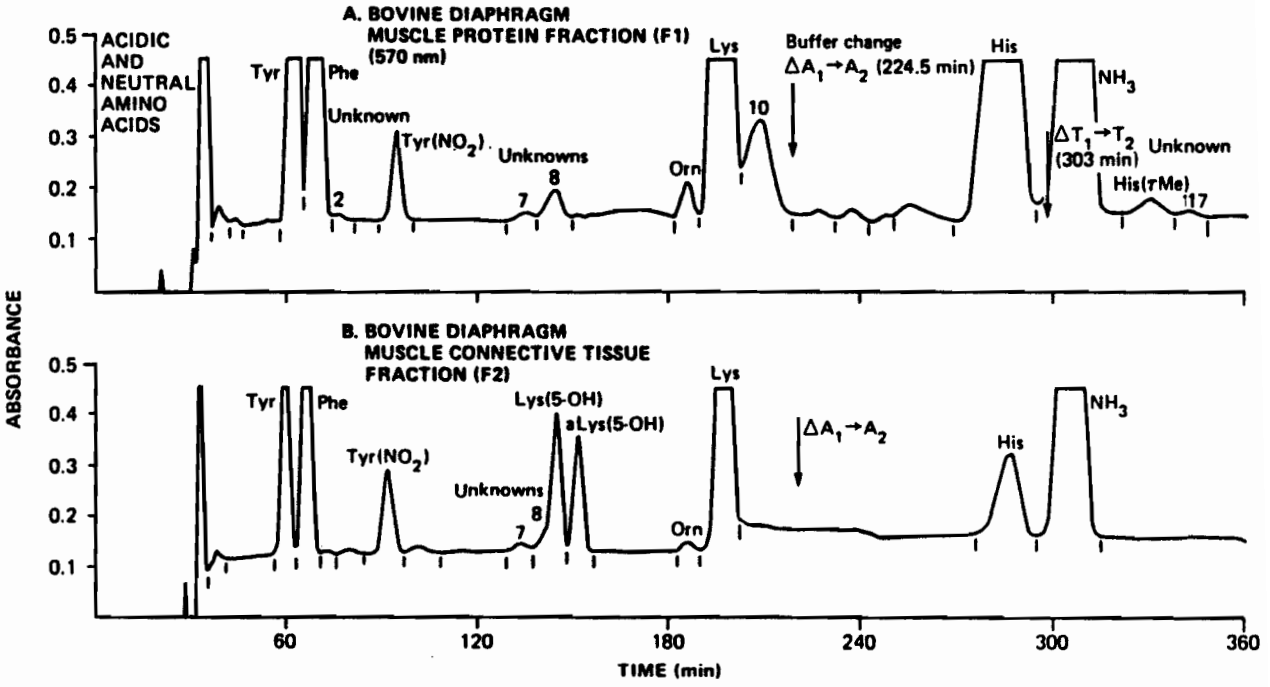

A. BOVINE DIAPHRAGM
MUSCLE PROTEIN FRACTION (F1)
(570 nm)
ACIDIC
AND
NEUTRAL
AMINO
ACIDS
Tyr
Phe
Unknown
Tyr(NO2)
Unknowns
Orn
Lys
Buffer change
ΔA1→A2 (224.5 min)
His
NH3
ΔT1→T2
(303 min)
Unknown
His(τMe)
B. BOVINE DIAPHRAGM
MUSCLE CONNECTIVE TISSUE
FRACTION (F2)
Lys(5-OH)
aLys(5-OH)
ABSORBANCE
0.5
0.4
0.3
0.2
0.1
60
120
180
240
300
360
TIME (min)

TABLE 6. Amino Acid Composition of the Intracellular (F1) and Extracellular (F2) Muscle Protein Fractions Isolated From the Mid-Costal Region of Young Bovine Diaphragm Muscle.

(g Amino Acid/kg of Protein)

Amino Acid	F1, SDS-Soluble Muscle Proteins[a]			F2, SDS-Insoluble Muscle Proteins[a]		
	Mean ± SEM[b]	CV[b]	F[b]	Mean ± SEM[b]	CV[b]	F[b]
Aspartic acid	99.89 ± 0.47	1.33	0.01^{ns}	68.96 ± 0.16	0.56	0.03^{ns}
Threonine	48.75 ± 0.66	2.71	0.86^{ns}	24.77 ± 0.43	2.71	2.72^{ns}
Serine	41.64 ± 0.84	2.38	6.34^{ns}	40.20 ± 0.18	0.75	1.97^{ns}
Glutamic acid	166.36 ± 0.54	0.48	3.26^{ns}	116.04 ± 0.27	0.40	1.86^{ns}
Proline	31.43 ± 0.33	2.35	0.30^{ns}	108.79 ± 2.19	4.19	0.70^{ns}
Glycine	30.14 ± 0.07	0.54	0.07^{ns}	180.02 ± 0.52	0.70	0.06^{ns}
Alanine	53.14 ± 0.18	0.68	0.93^{ns}	81.24 ± 0.37	0.41	12.92^{ns}
Cysteine	8.59 ± 0.09	0.76	19.27^{*}	8.13 ± 0.27	0.25	2112.6^{**}
Valine	55.22 ± 0.36	1.14	1.67^{ns}	39.30 ± 0.51	2.09	2.49^{ns}
Methionine	27.22 ± 0.05	0.44	0.05^{ns}	11.07 ± 0.41	3.32	12.81^{ns}
Isoleucine	52.17 ± 0.12	0.39	2.32^{ns}	23.79 ± 0.31	2.77	0.69^{ns}
Leucine	91.24 ± 0.18	0.34	1.84^{ns}	42.51 ± 0.28	0.84	5.24^{ns}
Tyrosine	40.24 ± 0.36	2.18	0.08^{ns}	16.55 ± 0.65	3.08	17.11^{**}
Phenylalanine	45.34 ± 0.22	1.16	0.11^{ns}	25.84 ± 0.35	1.05	17.98^{*}
Histidine	28.20 ± 0.11	0.87	0.15^{ns}	10.89 ± 0.41	8.94	0.10^{ns}
Lysine	95.16 ± 0.27	0.37	5.12^{ns}	42.07 ± 0.32	0.08	1075.1^{**}
Arginine	67.64 ± 0.08	0.27	0.06^{ns}	80.29 ± 0.39	1.06	0.56^{ns}
Tryptophan	16.79 ± 0.14	0.79	10.05^{ns}	-		
N^{ϵ}-methyllysine	0.121 ± 0.008	14.31	0.82^{ns}	-		
N^{τ}-methylhistidine	0.548 ± 0.008	3.41	0.27^{ns}	-		
4-Hydroxyproline	-			70.23 ± 0.16	0.29	5.50^{ns}

Table 6 continued...

TABLE 6 (continued)

Amino Acid	(g Amino Acid/kg of Protein) F1, SDS-Soluble Muscle Proteins[a] Mean ± SEM[b]	CV[b]	F[b]	F2, SDS-Insoluble Muscle Proteins[a] Mean ± SEM[b]	CV[b]	F[b]
5-Hydroxylysine	*			8.85 ± 0.08	1.88	0.80[ns]
Isodesmosine	*			0.43 ± 0.01	1.47	2.32[ns]
Desmosine	*			0.44 ± 0.01	2.77	5.03[ns]
Ornithine	0.273 ± 0.008	4.49	4.55[ns]	0.32 ± 0.01	3.75	2.89[ns]
Ammonia	12.37 ± 2.80	46.38	0.05[ns]	14.89 ± 0.18	68.67	1.37[ns]
Total AA-N[c]	170.17			190.43		
Total recovery (g protein/kg dry weight)	547.80 ± 0.02			312.50 ± 1.02		
% Yield (g protein /100g total protein)	95.27			4.73		
WE (µg/nmol)[d]	0.11284			0.093944		
F (µg/nmol)[d]	0.115093			0.094645		
F' (µg/nmol)[d]	0.119547			0.113246		

[a]Isolated by the methods of McCollester (1962;1963), Laurent et al. (1981) and Stanley (1983).

[b]Mean values and standard error of measurements (SEM) for 2 replicates and 16 determinations. F, values from analysis of variance; **, P<0.01; *, P<0.05; ns, not significant; CV, coefficient of variation.

[c]Calculated according to Heidelbaugh et al. (1975).

[d]The WE constant and F,F'conversion factors were calculated according to Horstmann (1979), using eqns (1) (2) and (3) respectively.

fraction (F1) isolated from the costal region of the bovine Diaphragm (100 µL equivalent to 219.0 µg protein analysis) by the present method revealed the complete separation, at pH 4.501 and 71°C, of His(τ-Me) from an unknown ninhydrin-positive peak, designated No. 17. In addition, a number of as yet unidentified minor and major ninhydrin-positive peaks, designated 2, 7, 8 and 10 were apparent (Figure 2A), and the results calculated relative to the internal standard [Tyr(NO_2)] are presented in Table 7. Two of these major unknown compounds (Nos. 8 and 10) found in relatively large amounts in most skeletal muscle tissues examined. Similarly the unknown compound No. 17 has also been found in variable amounts in other proteins and tissues. As may be seen from Figure 2A, the intracellular F1 fraction is devoid of amino acids unique to collagen or elastin thus demonstrating the successful separation of the intracellular proteins fraction from the extracellular matrix connective tissue proteins fraction. By the chromatographic method employed as little as 10 pmol of Lys(5-OH) can be quantitated, which is equivalent to 90.50 ng of collagen [assuming 10.09 pmol of Lys(5-OH) per 91.32 ng of collagen; Table 10]. In addition, Pro(4-OH) was absent from hydrolysates (24 h) of the F1 fraction, analyzed by the chromatographic method of [Zarkadas et al., 1986b] having a detection limit of 1 nmol.

The data for protein-bound His(τ-Me) (0.548 g/kg protein or 3.63 µmol/g protein; Table 6) in the costal bovine Diaphragm agrees with those reported by Haverberg et al. (1975) for rat Diaphragm (0.395 g/kg mixed protein) and by Elia et al. (1979) for human Diaphragm (3.00 µmol/g fat and moisture free weight; range 2.44-3.61) and is higher than the figure reported by Mussini et al. (1984) for normal rat Diaphragm (0.221 g/kg of total noncollagen

Table 7. Elution Times and Contents of Unknown Ninhydrin-Positive Compounds Determined in the Mid-Costal Region of the Young Bovine Diaphragm[a].

Unknown[b] Peak	Elution Time[b] (min)	Mean ± SEM[c]	C.V.[d]	F[d]
F1, Intracellular, SDS-Soluble Muscle Proteins (nmol/mg of Protein)				
No. 2	(77)	1.06 ± 0.23	17.88	15.40[ns]
No. 7	(137)	0.46 ± 0.06	22.62	2.19[ns]
No. 8	(149)	3.60 ± 0.10	2.51	12.19[ns]
No. 10	(211)	21.34 ± 0.98	5.36	6.87[ns]
No. 11	(238)	0.82 ± 0.09	12.24	7.22[ns]
No. 12	(255)	0.74 ± 0.07	2.30	213.53**
No. 13	(261)	4.91 ± 0.24	11.02	0.27[ns]
No. 17	(365)	0.89 ± 0.18	5.49	13.95[ns]
F2, Extracellular, SDS-Insoluble Muscle Proteins (nmol/mg of Protein)				
No. 2	(77)	1.98 ± 0.21	3.36	119.05**
No. 7	(137)	3.31 ± 0.02	1.34	0.21[ns]

[a]Analyzed by the method of Zarkadas et al. (1987a); Figure 2.

[b]Unknown compounds present in 96 h hydrolysates of the F1, F2 fractions containing 219 and 65 µg of protein respectively.

[c]Mean values and standard error of measurements (SEM) for 2 replicates and 12 determinations. The relative concentrations of the unknown peaks were calculated by assuming a relative response factor (RRF) equivalent to that of the internal standard Tyr(NO_2) (RRF = 1.000).

[d]CV, coefficient of variation; F, values from analysis of variance; **,$P<0.01$; ns, not significant.

proteins). In addition the levels of His(τ-Me) in the bovine Diaphragm agree with those reported by Nishizawa et al. (1979) for skeletal muscle of cattle (0.594 g/kg total protein), and by Huszar et al. (1983) for bovine skeletal muscle (3.80 μmol/g muscle dry weight) and are in between the levels reported for four bovine skeletal muscles [0.643 g/kg protein; range 0.598 - 0.696 g/kg protein as recalculated from the original data on the assumption that skeletal muscle contains 20% protein] and those found in bovine Masseter muscle (0.415 g/kg protein) [White and Lawrie, 1985b]. The reported variation of His(τ-Me) levels, above,may be attributed to different methods used and to technical difficulties to quantitate this marker amino acid in the presence of considerable amounts of histidine or ammonia, changes in the contents of myofibrillar, connective tissue and sarcoplasmic proteins or due to the different extent of histidine methylation [Kuehl and Adelstein, 1970]. Furthermore various myosin isoforms are expressed differentially in different species and muscles [Izumo et al., 1986].

As may be seen in Figure 2B the analysis (100 μL equivalent to 65 μg of fraction F2) of the SDS-insoluble extracellular matrix muscle protein fraction (F2) isolated from the young costal Diaphragm revealed the complete separation of the diastereoisomers of Lys(5-OH) along with two as yet unidentified stable components, designated 7 and 8. It was found that unknown peak No. 8 partially coeluted with one of the diastereoisomers of Lys(5-OH), and that another unknown ninhydrin-positive compound also present in the connective tissue fraction (F2) appeared to coelute with the same diastereoisomers of Lys(5-OH), even after epimerization, thus interfering with its quantitation (Figure 2B). Because of this, the determination of

the aLys(5-OH) diastereoisomer is being routinely used for quantitating the total levels of Lys(5-OH) after epimerization (96 h). Unlike the tendinous dome portion of the adult bovine Diaphragm [Zarkadas et al., 1987a] the young costal Diaphragm contains very small amounts of the elastin crosslinks iDes and Des separating as minor peaks before and after the internal standard peak, Tyr(NO_2) (Figure 2B), and the results obtained are summarized in Table 6. As may seen from the pattern of Figure 2B, His(τ-Me) was absent from the extracellular matrix (F2) fraction. The detection limit of the chromatographic method employed [Zarkadas et al., 1987a] is 5-10 pmol His(τ-Me), therefore the major myofibrillar proteins (myosin and actin) were not present in amounts more than 1.24 μg [based on the data reported by Yates and Greaser (1983b) and on the finding that myosin and actin contain 2 and 1 His(τ-Me) residues per molecule of protein, respectively].

The amino acid profile of the extracellular matrix fraction F2 as presented in Tables 6 and 8, is highly characteristic of connective tissue proteins, i.e., collagen, elastin, etc., and completely different from that of any other group of muscle proteins. Glycine is the most abundant and accounts for almost 30% of the total amino acid residues. Alanine accounts for 10.6% of all residues. Proline and Pro(4-OH) taken together account for a further 16.1% of the total residues. Thus these four amino acids account for 56.7% of the total amino acid residues. The frequency of the acidic amino acids is approximately 14%, while the total basic amino acids including arginine, lysine, histidine and Lys(5-OH) account for almost 9.1% of the total residues. The high content of Pro(4-OH), together with serine and threonine and the small amounts of tyrosine and Lys(5-OH), bring the

Table 8. Comparison of the Amino Acid Composition of the Extracellular Matrix Protein Fraction (F2) Isolated from the Bovine Costal Diaphragm With Muscle Collagen, Lung Collagens and Bovine Elastins.

	Number of Amino Acid Residues per 1000 Amino Acid Residues				
Amino acid	Bovine Costal Diaphragm F2 fraction[a]	Weighted Average of Muscle Collagen[b]	Lung Collagen[c]	Bovine Semi-tendinosus Elastin[d]	Purified Bovine Elastins[e]
Aspartic Acid	55.3	42.63	53	7.4	9
Threonine	22.6	15.94	24	8.2	11
Serine	42.6	36.97	40	8.3	11
Glutamic Acid	83.0	71.34	79	17.1	19
Proline	103.5	113.90	89	110.9	114
Glycine	291.1	339.61	306	325.9	324
Alanine	105.5	102.01	103	223.1	223
Cysteine	6.5	0.70	7	-	-
Valine	36.6	22.03	28	145.8	134
Methionine	7.8	7.31	1.8	-	-
Isoleucine	19.4	11.39	15	26.5	27
Leucine	34.7	23.98	32	61.4	61
Tyrosine	9.4	2.52	4.2	8.4	8
Phenylalanine	16.2	11.56	18	29.8	30
Histidine	7.3	6.0	8	0.6	0.4
Lysine	30.3	24.71	23	5.3	5
Arginine	47.5	47.59	48	5.7	7
3-Hydroxyproline	nd	0.67	nd	-	-
4-Hydroxyproline	57.3	111.12	101	12.4	13
5-Hydroxylysine	5.8	10.09	13	-	-

Table 8 continued...

Table 8 continued

Amino acid	Bovine Costal Diaphragm F2 fraction[a]	Weighted Average of Muscle Collagen[b]	Lung Collagen[c]	Bovine Semitendinosus Elastin[d]	Purified Bovine Elastins[e]
	Number of Amino Acid Residues per 1000 Amino Acid Residues				
Isodesmosine	0.087	-	-	1.2	1.7
Desmosine	0.089	-	-	2.1	2.4
$\frac{Pro(4\text{-}OH)}{Pro+Pro(4\text{-}OH)}$	0.356	0.494	0.532	0.093	0.093
$\frac{Lys(5\text{-}OH)}{Lys+Lys(5\text{-}OH)}$	0.161	0.290	0.361	-	-
WE (µg/nmol)[f]		0.09132	0.09288	0.08506	0.085034

[a] Average of 16 determinations (young calves), present study; key: nd=not determined

[b] Average values calculated from the data presented in Table 3 and according to the formula : ${}_{A}R_{i} = (0.615 \times {}_{1}R_{i} + 0.335 \times {}_{2}R_{i} + 0.05 \times {}_{3}R_{i})$ where ${}_{n}R_{i}$ = amino acid residue/1000 residues for column n (1,2,3) and row i of the Table 3; type V collagen was not included .

[c] CNBr-soluble material in SDS-extracted residue from rabbit lung (Laurent et al.,1981).

[d] Data from bovine Semitendinosus muscle elastin (Bendall, 1967).

[e] Average amino acid composition of elastin purified from five bovine tissues (Starcher and Galione, 1976).

[f] Calculated according to Horstmann (1979);

The average residue weight for muscle collagen is:

$$\sum_{i=1}^{3} WE_{col} = [(0.615 \times 0.091175) + (0.335 \times 0.09897) + (0.05 \times 0.095895)] = 0.09132 \text{ (µg/nmol)}$$

The average residue weight of muscle connective tissue is:

$$\sum_{i=1}^{4} WE_{con} = [(0.9305 \times 0.091318) + (0.0695 \times 0.085034)] = 0.09088 \text{ (µg/nmol).}$$

total content of hydroxylated residues to 12.8% compared to 12.4% for the total hydrophobic amino acids. The amino acid composition of the isolated (F2) fraction of the bovine Diaphragm, with the exception of Pro(4-OH) and Lys(5-OH), closely resembled that of rabbit lung and skeletal muscle collagens (Table 9). The lower levels of these two amino acids in bovine Diaphragm could be attributed to the fact that young animals were examined in the present study [Schimokomaki et al., 1972]. Kruggel and Field, (1974) reported that the intramuscular collagen of bovine foreshank muscle group (active) contained lower levels of intramuscular cross-linkages as compared to Longissimus muscle collagen, supporting the view that active muscles contain less collagen crosslinks than quiescent muscles. It is possible that a muscle used in locomotion is more highly cross-linked than a muscle like the Diaphragm that contracts moderately and at regular intervals throughout the life of an animal. Tables 6 and 8 show that the small amounts of Des and iDes found in the extracellular matrix (F2) of the Diaphragm may have derived from the network of capillaries often separated with the SDS-insoluble membrane residue (F2). Although they may account for up to half the dry weight of the preparation [McCollester, 1962], methods to remove these capillaries by chemical means during the numerous washes are not available at present [Bendall, 1967]. Rowe (1986) has recently shown that elastin associated with capillaries in muscles of high and low elastin content constitutes only a very small portion of the total elastin present in these muscles.

The Actin and Myosin Components of the Diaphragm

The results presented in Table 9 indicate that the quantitation of

Table 9. The Myofibrillar and Connective Tissue Protein Contents in Vertebrate Skeletal Muscles

	Mid-Costal Bovine Diaphragm		Rabbit Psoas major		
Skeletal Muscle Proteins	F1, Intracellular, SDS-Soluble Proteins g/kg Total Protein[a]	% of the Total Muscle Protein			
Myofibrillar[b]	537.86±7.85	51.24	57.71[e]	62.00[f]	40 - 48[g]
Actin[c]	113.57±1.66	10.82	12.69	12.00	-
Myosin[d]	236.04±3.45	22.49	24.82	34.00	25 - 30
Actomyosin	349.61±5.10	33.31	37.51	46.00	-
Soluble Proteins	451.50	43.00	-	34.00	41.60
	F2, Extracellular, SDS-Insoluble Proteins g/kg Total Protein[a]	% of the Total Proteins			
Collagen[h]	551.70	2.61			
Elastin[j]	39.70	0.19			
Total Connective[k] Tissue	591.40	2.80			
	540.96	2.56			

[a]Isolated by the combined procedures of McCollester (1962;1963), Laurent et al. (1981) and Stanley (1983). Distribution of the isolated fractions in the costal Diaphragm: intracellular proteins = 95.27%; extracellular matrix proteins=4.73%

[b, c, d]Calculated from eqns (8b) and (8d).

[e, f, g]From Yates and Greaser (1983b), Hanson and Huxley (1957), Szent-Gyorgi et al. (1955), respectively.

[h, j, k]Calcualted from eqns (5a), (5b) and (6b).

protein-bound His(τ-Me), known to occur exclusively in myosin and actin [Elzinga et al., 1973; Elzinga and Collins, 1977; Huszar, 1984], can be used as an index for determining these two principal myofibrillar proteins in the costal region of the bovine Diaphragm using eqn (8d). The results, expressed as g of protein per kg of anhydrous, ash-free total muscle protein, are presented in Table 9. The data show deviations of less than ± 3% from the average values. These values also show that in the costal Diaphragm, myosin accounts for 22.49% of the total muscle protein corresponding to 43.04% of the myofibrillar proteins, and actin accounts for an estimated 10.82% of the total muscle mass or about 20.71% of the myofibrillar proteins. These results are in accord with those reported by Yates and Greaser (1983b) who have shown that the rabbit Psoas major muscle, which is composed of at least 90% fast-twitch (IIB) fibers [Schachat et al., 1985], contains 57.71% myofibrillar protein of the total muscle mass, and that the myofibrils contain 22% actin and 43% myosin by weight. Similar values, corrected on the basis that muscle contains 20% protein and 12% myofibrils [Yates and Greaser, 1983b], have been reported by other authors using different methods for determining the myofibrillar protein content of skeletal muscles [Hanson and Huxley, 1957; Szent-Gyorgi et al., 1955], and their results are included in Table 9 for comparison. For example Hanson and Huxley (1957) found by quantitative extraction that the rabbit Psoas major myofibrils contained 21% of actin and 43% of myosin by weight, compared to the myosin value reported by Szent-Gyorgi et al. (1955) which accounted for 38% of the myofibrillar proteins.

These results (Table 9) provide additional evidence to indicate that the relative amounts of actin and myosin per unit of tissue, as related to the distribution of His(τ-Me) in these two muscle proteins, remains constant. Deviations from the average figure of 57.71% reported by Yates and Greaser (1983b) for the myofibrillar protein content of Psoas major muscle may reflect variations in the amount of these muscle proteins present in this anatomical region of the costal Diaphragm and not in its distinct actin to myosin distribution. The possibility remains, of course, that there exist minor differences in the amounts of slow (type I) and fast (types IIA and IIB) classes of myosin heavy chain isoforms present in the Diaphragm [Young, 1982; Green et al., 1984; Izumo et al., 1986; Lowey, 1986b] which do not contain methylated histidine residues in their amino acid sequence [Kuehl and Adelstein, 1970]. The contribution of myosin and actin originating from nonmuscle cells of the Diaphragm would increase the actin to myosin ratio; however, this seems unlikely since the figure of 33.3% (percentage of actomyosin of the muscle weight) obtained in the present study agrees with the value of 37.5 % reported by Yates and Greaser (1983b).

Soluble Proteins in the Bovine Diaphragm

An SDS-soluble fraction (other than the myofibrillar proteins) in the bovine Diaphragm was estimated to account for 43.0% of the total muscle proteins (Table 9). This value agrees with the figure of 41.6% soluble proteins found by Szent-Gyorgi et al. (1955) in fresh muscle (rabbit Psoas major) but is significantly higher than the amount of 28% and 34% sarcoplasmic proteins found in glycerinated and fresh muscle respectively by Hanson

and Huxley, (1957). The differences between the sarcoplasmic development of red fibers (bovine Diaphragm) and white fibers (Psoas major) [Seideman et al., 1984] could not account for the 14% more soluble protein extracted from the former muscle. Increased extractability of sarcoplasmic proteins has been found after 16 h postmortem [Chaudhry et al., 1969]. It is possible that the 2% SDS solution employed during the isolation procedure [Laurent et al., 1981] solubilized cell membranes, organelles and proteins of the Z-band. It is evident that there is an increased extractability of myofibrillar and soluble proteins due to the disintegration of the insoluble Z-line in aged muscles [Davey and Gilbert, 1967] probably accounting for the greater amount of the extracted soluble fraction in the bovine Diaphragm.

Determination of the Connective Tissue Proteins

The connective tissue content of the extracellular matrix proteins (F2) determined in the bovine costal Diaphragm is also presented in Table 9. The extracellular matrix proteins (F2) accounted for an average of 4.73% of the total muscle protein. The collagen and elastin contents of the young bovine costal Diaphragm were estimated to be 551.70 g/kg and 39.70 g/kg of isolated intramuscular connective tissue proteins, respectively (Table 9). Thus using equations (5a and 5b), it was found that in young, costal bovine Diaphragm, collagen acounts for an estimated 2.62% and elastin for 0.19% of the total muscle proteins. Of the total connective tissue proteins, collagen accounted for an estimated 93.24% and elastin for 6.76%. The value of 551.70 g collagen/kg protein is remarkably similar to the figure of 0.55 mg total collagen/mg of isolated intramuscular collagen found by Burson et al.

(1986c) in bovine Longissimus dorsi muscle. These authors suggested that their isolated intramuscular collagen contained other muscle proteins resistant to the isolation procedure [Burson and Hunt, 1986b]. As in our study, their method involved a 2% SDS solution.

These results (2.61% collagen of the total muscle proteins present in the Diaphragm) are in agreement with those reported by McClain et al. (1971) for young bovine Semimembranosus muscle (3% of its total muscle mass), Seideman, (1986) for five young bovine muscles (average 2.60%; range 1.51 - 3.47%), and Burson and Hunt (1986a) for four bovine muscles (mean 3.18%; range 1.97 - 4.44%). Furthermore these figures are within the range of the collagen values reported by: Mc Keith et al. (1985) for 13 adult bovine muscles (range 1.48% - 9.04%; on a dry weight and fat-free basis), Bendall (1967) for 32 adult bovine skeletal muscles (mean 4.02%; range 1.6 - 9.7%), Dransfield (1977) for 18 young (18 months old steers) bovine muscles (average 4.15%; range 2.21 - 5.59%), and Cross et al. (1973b) for five bovine muscles from three age groups (mean 3.38%; range 2.50 - 4.13%).

The value obtained for elastin in the costal region of the Diaphragm 0.19% (6.76% of the total connective tissue proteins) is within the range of elastin content reported by Bendall (1967) for 29 bovine muscles, ranging from 0.05% (Triceps brachii) to 0.8% (Trapezius thoracis) or 0.61 - 16.7% as percentage of the total connective tissue. Three of the muscles examined in that study [Bendall, 1967], namely Latissimus dorsi, Semitendinosus and Panniculus muscles, contained 2%, 1.82%, and 1.13% elastin (or 33%, 37%, and 10.4% as percentage of the total connective tissue) and were not included in the above range of elastin content for the 29 muscles. The level of elastin

in the Diaphragm, (as percentage of the total connective tissue) are also, within the range of elastin content reported by Vognarova et al. (1968) for seven veal cuts (4.65 ⇔ 9.61%) and seven beef cuts (5.44 ⇔ 15.24%). The anatomical location and function of a muscle correlates directly to the connective tissue content [Kovanen et al., 1980]. The Diaphragm muscle contracts moderately at regular intervals throughout the life of an animal [Rochester, 1985]. The variation in the duration and intensity of work performed by Diaphragm is reflected in the anatomical and histological architecture of the muscle.

As may be seen from Table 9 the total connective tissue proteins (assessed from the amounts of collagen and elastin) of young bovine Diaphragm account for 2.80% of the total muscle proteins whereas the extracellular matrix (F2) fraction accounts for 4.73% of the total muscle protein (Table 6). The difference of 1.93% muscle proteins seems to belong to the transcellular Diaphragm matrix. Therefore the (F2) fraction seems to contain collagen, elastin and other proteins, such as structural and microfibrillar proteins [Sakai et al., 1986], fibronectin, dermatan sulphate, laminin etc., resistant to the 2% SDS washings [Burson and Hunt, 1986b] and having an overall amino acid composition similar to that of the (F2) fraction (Table 8). Lowey et al. (1983) have shown the presence of "ghost" proteins belonging to the transcellular muscle matrix. These authors found that exhaustive extraction of skeletal muscle with 6.0 M guanidinum HCl and thiol leaves a residue of 1% which, when treated with collagenase, produces a residue accounting for only 0.2% of the total original muscle protein.

The connective tissue content was also calculated by multiplying the factor 7.7027 of equation (6b) by the levels of Pro(4*OH) found in costal bovine Diaphragm (Table 6). This factor was determined from the estimated weight equivalent (WE=0.09088 µg/nmol) and the Pro(4*OH) content (104.3 residues per 1000 total amino acid residues) of the connective tissue proteins in bovine skeletal muscles (Table 8). These calculations were based on the data reported by Bendall (1967) for 33 bovine skeletal muscles, which were found to contain a mean collagen content of 93.05% and a mean elastin content of 6.95% (expressed as a percentage of the total connective tissue proteins). The Peronaeus tertius muscle which contains exceptionally high collagen levels was not included in these calculations. The WE=0.091318 µg/nmol of bovine muscle collagen and its Pro(4*OH) content (111.12 residues per 1000 total residues) were calculated from the data of Miller and Gay, (1982) and Light and Champion (1984) as presented in Table 8. The corresponding values for elastin, extracted from five bovine tissues [Starcher and Galione, 1976], were 0.08504 µg/nmol and 13.0 residues per 1000 residues, respectively (Table 8). The so calculated connective tissue accounted for an estimated 2.56% of the total muscle proteins (Table 9).

3.4 CONCLUSION

The myofibrillar and connective tissue protein contents of the young bovine mid-costal Diaphragm were determined. The bovine Diaphragm, a slow skeletal muscle, was subjected to a fractionation procedure including 0.05 M $CaCl_2$, PBS (0.17 M Na^+, pH = 7.4) and 2% SDS; by this method the Diaphragm muscle was separated in an intracellular fraction (F1) containing all the soluble in PBS and 2% SDS myofibrillar proteins, organelles, Z-line proteins, etc., and an extracellular matrix fraction (F2) composed of the insoluble, in 2% SDS, proteins. The myosin and actin contents of the costal Diaphragm, were determined from the levels of His(τ-Me) found in the F1 fraction. Collagen was determined from the levels of Lys(5-OH) found in the F2 fraction, which was found to have an amino acid composition similar to other connective tissues. The determination of elastin and total connective tissue proteins was based on the quantitation of Des, iDes and the Pro(4-OH) levels found in the F2 fraction. All of the above calculations were based on the total protein content of the F1 and F2 isolated fractions, determined by their detailed amino acid composition.

The above method of quantitating myofibrillar and connective tissue proteins could be useful in determining their levels in both normal and diseased muscles.

SECTION IV

THE DETERMINATION OF MYOFIBRILLAR AND CONNECTIVE TISSUE PROTEINS IN COMPOSITE MEAT PRODUCTS.

4.1 INTRODUCTION

Meats and processed meat products constitute the most important protein source in meeting human dietary requirements. However, the protein quality and nutritive value of processed meats and poultry products vary considerably depending upon the type and amount of specific meat cuts and non-meat ingredients used to formulate them. Such composite meat products are prepared from cheaper meat cuts, which are frequently high in connective tissue, from the major meat yielding species [Young and Pellet, 1984; Porteous, 1981]. Formulations usually include a number of other non-muscle animal and plant protein additives including milk protein hydrolysate, non fat dry milk, egg powders, gelatin, soy and other types of oil seed products, cereal additives, vegetable starches, spices, etc., [Ellis, 1987] prepared by various separation and extraction processes. The actual levels of protein used in such processed meats vary and are tailored to meet the cost and nature of such ingredients available to the processor. These mixtures are then ground, chopped or comminuted, encased to retain defined shapes or forms, and then processed into a variety of meat products [Terrell, 1982; Rust, 1982].

There has been a continuing interest in the development of standards for labelling prepackaged meats, and reliable analytical methodology useful

for the precise assessment of the skeletal muscle, connective tissue and non-muscle protein content of composite meat products [Pearson, 1975; Olsman and Slump, 1981; McNeal, 1986; Benedict, 1987; Ellis, 1987]

The use of His(τ-Me) as a quantitative marker for the estimation of the myofibrillar content in muscles and composite meat products has been proposed [Perry, 1970; Hibbert and Lawrie, 1972; Zarkadas, 1981; Olsman and Slump, 1981; McNeal, 1986]. The reported levels of His(τ-Me) in various muscles of the meat yielding species differ significantly [White and Lawrie, 1985b; Jones et al., 1985; Johnson et al., 1986]. This variability may be attributed to the difficulty of the chromatographic systems employed to completely separate the small levels of His(τ-Me) from other ninhydrin-positive compounds present in the complex meat hydrolysates analyzed [Jones et al., 1982; Hancock and Harding, 1984] and to the various derivatization treatments of the meat hydrolysates prior to analysis [Skurray and Lysaght, 1978; Ward, 1978; Nakamura and Pisano, 1979; Poulter and Lawrie, 1980a; b; Jones et al., 1982]. An excellent baseline separation of all the unique protein bound compounds found in myosin, actin, collagen, and elastin was achieved by Zarkadas (1979) and Zarkadas et al. (1987a). Free His(τ-Me), and the variable amounts of the naturally occurring sarcoplasmic dipeptide balenine [Harris and Milne, 1987] may contribute to the reported variability of the His(τ-Me) levels found in meats and meat products.

An accurate, reliable and sensitive method was essential to examine the validity of the use of His(τ-Me) as index of the myofibrillar proteins (myosin and actin) and the use of Lys(5-OH) as valid quantitative index of collagen content in composite meats.

The major objective of the present study was to provide experimentally sound data on the levels of these unique, stable amino acids in three major meat products, representing 60 - 70% of the total market volume of composite meats, and to analyze typical non-meat protein additives, ingredients and protein supplements commonly used to formulate composite meats for their His(τ-Me) and Lys(5-OH) contents. The categorical data obtained in this survey will be essential in establishing the myofibrillar and connective tissue protein levels in skeletal muscle and composite meats so that valid and precise regulatory standards can be established for meat products.

In this approach the assessment of the protein quality of composite meats is based on the determination of their myofibrillar and collagen contents from the amounts of His(τ-Me) and Lys(5-OH) respectively found in hydrolysates of these products. These unique compounds are quantitated by the chromatographic procedures described by Zarkadas et al (1986b; 1987a). When the sum of the myofibrillar and connective tissue proteins is subtracted from the total protein of a composite meat hydrolysate, the difference is an accurate assessment of non-muscle proteins present. These calculations are based on the total protein contents of these meat products as determined by their detailed amino acid composition [Horstmann, 1979].

This direct approach has the advantage over other methods in the sense that the determination of all these classes of proteins can be carried out on fresh or processed meats as well as on animal protein supplements. It may prove especially valuable for industrial quality control programs and formulations.

4.2 MATERIALS AND METHODS

Sampling and Preparation of Commercially Processed Meats for Analysis.

The three major meat products examined in the present study including mixed meat sausages, bologna and frankfurters and four of the ingredients used in their preparation, were obtained from ordinary commercial sources (Hygrade-LaBelle Fermiere, Montreal, Quebec). Three representative samples of these typical mixed-meat products originating from different batches were selected at random in 1.0 kg quantities. For amino acid analysis, approximately 200 g were sampled from each of these products, and after the casings were removed the samples were cut into small cubes and ground separately, then frozen (-173 °C) and lyophilized. The freeze dried samples were then pulverized in a standard electrically driven end runner mill as before and then stored at -20 °C untill needed.

Proximate and Elemental Analysis

Total nitrogen content of the freeze-dried meat products was determined by the official MicroKjeldahl method [Association of Official Agricultural Chemists, 1980; Section 2.057] using the automated Technicon II system [Technicon Instruments Co., Tarrytown, NY] for the analysis of the digests [Association of Official Agricultural Chemists, 1980; Section 24.028]. Moisture was determined by the procedure described in Sections 7.003 and 24.002 from the Association of Official Agricultural Chemists (1980). The total ash was determined as described in Association of Official Agricultural Chemists (1980) Sections 24.009 and 31.012. The total lipids

were determined by the Petroleum-ether Goldfish method [Association of Official Agricultural Chemists, 1980; Sections 10.132 and 24.005].

The freeze-dried samples were prepared for elemental analysis by the wet digestion procedure using a mixture of 15 mL of concentrated nitric acid and 8 mL of perchloric acid in a 100 mL Kjeldahl flask as described by Parks and Dunn (1963). The molybdovanadate method [Parks and Dunn, 1963] was used for the determination of phosphates. Analyses for calcium, magnesium, potassium, zinc and iron were performed in separate freeze-dried samples by the official lanthanum oxide method [Association of Official Agricultural Chemists, 1980; Section 2.109] using a fully automated atomic absorption Spectrophotometer (Varian Model AA-975) equipped with a programmable sample changer (Varian Model 55) and printer-plotter (Hewlett Packard Model HP92805A).

Chlorine was determined separately by the modified Volhard method [Caldwell and Moyer, 1955] using an automatic chloride titrator (Amino-Cotlore American Instrument Co., Silver Spring, MD) equipped with a silver generator electrode and a silver anode.

Procedures for the Extraction of Soluble Dipeptides Present in Meat Products

To effectively remove the soluble histidine dipeptides including balenine from the lyophilized meat samples [Carnegie et al., 1984], the following three different extraction solvents were used: (a) a mixture of 75% in 0.1 M HCl [Rangeley and Lawrie, 1976] (b) a mixture of methanol-chloroform as described by Bligh and Dyer (1959), and (c) distilled water.

The lyophilized mixed-meat sausage powdered sample was thoroughly mixed and divided into four portions of approximately 10.0 g each. One of the samples was used as a control while the other three samples were treated as follows:

(a) Hydrochloric acid-Ethyl alcohol extraction, [Rangeley and Lawrie, 1976]

The appropriate volume of distilled water (10 mL) was added to 10 g of freeze-dried tissue (mixed meat sauasage) in order to duplicate the moisture content of the fresh product, and then homogenized in a VirTis homogenizer with 3 volumes of 0.1 M HCl in 75% ethanol at a speed setting of 40/100 for 10 min. The homogenate was then centrifuged in a Sorvall RC-2B centrifuge (using rotor SS-34) at 14,000 x g for 15 min at 4°C. The supernatant was collected while the residue was resuspended in 3 volumes of 0.1 M HCl in 75% ethanol and homogenized for 10 min and recentrifuged as before. The residue was extracted three more times and the pellet was suspended in 5 volumes of acetone, homogenized for 15 min in the VirTis homogenizer and centrifuged as before. The acetone wash step was repeated three more times. The residue was dried under vacuum in a desiccator over potassium hydroxide (crystals) and stored at -20 °C until needed.

(b) Methanol:Chloroform extraction [Bligh and Dyer, 1959]

An appropriate volume of distilled water (40 mL) was added to 10 g of lyophilized tissue to restore a moisture level of 80 ± 1.0% [Bligh and Dyer, 1959]. After the addition of 50 mL of chloroform and 100 mL of methanol, the sample was homogenized at room temperature in a 250 mL flask in a VirTis homogenizer at a speed setting of 40/100 for 2 min as described by Bligh and

Dyer, (1959). Chloroform (50 mL) was added and after 30 s of further homogenization at the same speed, 50 mL of distilled H_2O were added, and rehomogenized for 1 min. The homogenate was then filtered with suction through a Whatman No. 1 filter, and the dried residue was extracted a further two times. The volumes of water, chloroform, and methanol, before and after dilution, were kept in the specified proportions 0.8:1:2 and 1.8:2:2, respectively. The final residue was homogenized in 100 mL chloroform for 5 min, filtered through the same filter, dried overnight in an evacuated desiccator at room temperature, ground to pass through a 40mm screen and stored at -20 °C untill needed.

(c) Water extraction procedure:

The freeze-dried tissue (10 g), was reconstituted to the moisture content of the fresh product (mixed meat sausage) with the addition of deionized water (10 mL), was homogenized in 20 volumes of water in a VirTis model 45 homogenizer at high speed for 10 min. The homogenate was filtered with suction through a Whatman No. 1 filter. The dried residue was homogenized in 100 mL of acetone for 10 min using a magnetic stirrer. The homogenate was then filtered through the same filter paper and the filtrates were combined. The homogenization step with 50 mL of acetone was repeated twice. The residue was dried overnight over potassium hydroxide crystals in an evacuated desiccator and stored at -20 °C.

Amino Acid Analysis

Standard amino acid analyses of lyophilized samples (0.1 g) were carried out essentially as described by Zarkadas et al. (1986b; 1987a) and

in Section III (Materials and Methods) of the present study. Tryptophan determination in each of the meat products (0.05 g) was carried out by the Hugli and Moore (1972) method as described by Zarkadas et al. (1986b).

Protein Determination

The protein content of each meat product hydrolysate was determined by the methodology described by Horstmann (1979) and Nguyen et al. (1986) using eqns 1, 2, and 3 (Section III).

The Determination of Myosin, Actin , and Connective Tissue Proteins in Meat Products

The amount of actin and myosin in meat products can be calculated from the amounts of His(τ-Me) and the molar ratio of actin and myosin in skeletal muscles according to the following eqn 8a (Section III):

$$\sum_{j}^{z} [P_A + P_M] = \left[\frac{A}{A + 2M} \cdot \frac{41,782}{151.2} + \frac{M}{A + 2M} \cdot \frac{521,000}{151.2}\right] \cdot C_{His(\tau\text{-}Me)}$$

Therefore, myosin, actin, actomyosin and myofibrillar proteins can be determined by using eqns 8b, 8c, and 8d (Section III).

The mean value of other SDS-soluble muscle proteins found in the costal bovine Diaphragm, a typical mammalian skeletal muscle, accounted for 43.0% of the total muscle protein (Section III), therefore this soluble muscle protein fraction in meat products can be computed as follows:

$$\text{amount of muscle soluble proteins} = \frac{\sum_{j}^{2} [P_A + P_M]}{\frac{44.0}{43.0}} \cdot C_{His(\tau\text{-}Me)} = 623.47 \cdot C_{His(\tau\text{-}Me)} \quad (8e)$$

The sum of the myofibrillar and other soluble muscle proteins calculated from eqns (8d) and (8e) represents the total intracellular muscle proteins found in composite meat products.

The average collagen content in 32 bovine skeletal muscles was found to account for 4.15% of the total muscle proteins [Bendall, 1967; Dransfield, 1977; Light and Champion, 1984; Light et al., 1985], then the amount of muscle collagen in composite meats can be calculated as follows:

$$\text{amount of muscle collagen} = \frac{\sum_{j}^{2} [P_A + P_M]}{\frac{44}{4.15}} \cdot C_{His(\tau\text{-}Me)} = 60.17 \cdot C_{His(\tau\text{-}Me)} \quad (8f)$$

If the amount of muscle collagen is subtracted from the total collagen of a composite meat product, the difference is an accurate estimate of the non-muscle collagen added to this product.

Assuming that the transcellular matrix in skeletal muscle accounts for an estimated 1.92% of the total muscle proteins [Loewy et al., 1983; bovine Diaphragm; Section III], then the extracellular matrix (in g/kg of total protein) of composite meats can similarly be calculated as shown below:

$$\text{amount of extracellular matrix} = \frac{\sum_{j}^{2} [P_A + P_M]}{\frac{44}{(4.15+1.92)}} \cdot C_{His(\tau\text{-}Me)} = 88.01 \cdot C_{His(\tau\text{-}Me)} \quad (8g)$$

Therefore, when the sum of the intracellular and extracellular muscle proteins is subtracted from the total protein of a composite meat product, which was quantitated by amino acid analysis, then the difference is an accurate assessment of the non-muscle protein additives and ingredients present.

Statistical Analysis

Data processing and linear regression analyses were performed with a Fortran program written for this purpose. Statistical analysis of the results and the effect of the extraction procedure was performed with a Statistical Analysis System package (1982) by using the computing centre (VAX) at Datacrown, Inc., IBM, Toronto, Ontario. A complete randomized design[Steel and Torrie, 1980] was used. Two factors were taken into account in this experiment: the meat product with three levels (sausages, frankfurters, bologna) and the treatment (untreated or solvent treated meat product) to which each product was subjected. Each treatment combination (3 x 2) was duplicated. Additionally, in order to increase the accuracy of the data each experimental unit was repeated three times (e.g., three bologna sampled at different production times).

4.3 RESULTS AND DISCUSSION

Proximate and Mineral Composition of Meat Products

The data on moisture, nitrogen, fat and ash contents of mixed meat sausages, frankfurters and bologna, as presented in Table 10, represent the means of 12 determinations (3 experimental units x 4 determinations per unit). Significant variations in moisture content were found in the mixed meat sausage samples ($P<0.05$) among the products evaluated. Frankfurters had the highest (61%) and mixed meat sausages the lowest (50.5%) moisture content. Differences in total nitrogen content were noted among the meat products examined. Mean values (4.08g/100 g dry weight basis) ranged from 2.85 in mixed meat cured sausages to 4.93 and 4.45 in frankfurters and bologna, respectively. The total lipid content among these products varied significantly. Table 10 shows that the amount of petroleum extractable fat was highest in sausages (70.9 g/100 g dry weight) and lowest in frankfurters (41.14 g/100 g dry weight). There was an inverse relationship between the fat and moisture, and nitrogen and moisture contents in the processed meat products studied. Total ash was lowest in mixed meat sausages and highest in bologna product (Table 10).

Meat products appear to be high in Na and Cl (Table 10). Mean values for Na ranged from 4.83 (frankfurters) to 5.48 (sausages) g/100 g dry weight and fat-free basis, or from 0.79 (mixed meat sausages) to 1.18 (bologna) g/100 g of fresh meat product. These values are in close agreement with the respective values reported by Pennington et al. (1986) for similar products. Mixed meat sausages contained 56.9 mg Ca/100 g of dry weight, on a fat free basis, whereas in frankfurters and bologna, Ca ranged from 256.2 to 504.7

Table 10. Proximate and Mineral Composition of Selected Composite Meat Products.

Nutrient	Composite Meat Products								
	Mixed Meat Sausage			Bologna			Frankfurters		
	Mean ± SEM	C.V.	F	Mean ± SEM	C.V.	F	Mean ± SEM	C.V.	F
	g / 100 g dry weight basis (N = 3)								
Moisture	50.54 ± 0.29	2.03	7.60*	56.89 ± 0.12	0.71	3.57^{ns}	60.95 ± 0.19	0.68	3.07^{ns}
Total Nitrogen (Kjeldahl-N)	2.85 ± 0.03	3.57	14.80**	4.45 ± 0.02	1.65	0.61^{ns}	4.93 ± 0.03	2.40	2.79^{ns}
Total Lipid	70.92 ± 0.47	2.30	0.18^{ns}	43.76 ± 0.32	2.49	0.08^{ns}	41.14 ± 0.37	2.92	3.61^{ns}
Total Ash	3.99 ± 0.04	3.63	4.35*	8.02 ± 0.01	0.59	2.60^{ns}	7.79 ± 0.04	1.61	0.26^{ns}
Minerals	mg / 100 g dry weight, fat-free basis (N = 3)								
Calcium	56.93 ± 3.00	15.81	2.44^{ns}	504.69 ± 5.92	3.32	1.96^{ns}	256.22 ± 8.31	9.73	8.34*
Phosphorus	586.08 ± 19.90	10.19	129.00^{ns}	717.78 ± 12.23	4.82	1.07^{ns}	617.47 ± 18.11	8.80	1.04^{ns}
Magnesium	68.78 ± 4.61	20.09	22.49**	86.72 ± 1.37	4.47	1.70^{ns}	64.32 ± 2.78	12.95	6.79*
Potassium	926.25 ± 24.04	7.79	3.33*	635.40 ± 15.82	7.04	13.91**	686.99 ± 11.51	5.03	4.42^{ns}
Sodium	5477.69 ±136.80	7.50	0.30^{ns}	4852.70 ± 68.86	4.00	4.85^{ns}	4832.73 ± 41.54	2.58	2.84^{ns}
Chlorine	7248.36 ±142.29	2.78	-	6157.74 ± 64.70	2.35	-	6056.96 ± 87.86	3.55	5.85^{ns}
Zinc	8.02 ± 0.24	9.05	0.09^{ns}	6.53 ± 0.16	5.01	1.03^{ns}	7.71 ± 0.07	2.62	23.12**
Iron	6.95 ± 0.26	11.22	6.63*	10.14 ± 0.25	6.57	1.26^{ns}	8.49 ± 0.14	4.99	0.74^{ns}

Significance denoted by F test:

* = $P<0.05$; ** = $P<0.01$; ns = not significant; C.V. = coefficient of variation; S.E.M. = standard error of means for 12 determinations.

mg/100 g, respectively (Table 10) which are in general higher than the respective values reported by Pennington et al. (1986). Bologna contained twice as much P as the mixed meat sausages. Frankfurters contained slightly lower levels of P than those of the bologna sample (Table 10). Composite meats appeared to be a poor source of Fe and Zn, containing less than 2.5 mg/100 g fresh product (Table 10). The bologna product had a much higher Fe and Mg levels but lower Zn content than either frankfurters or sausages (Table 10). These results are in agreement with the values of these elements reported for similar products by Pennington et al. (1986).

Amino Acid Composition

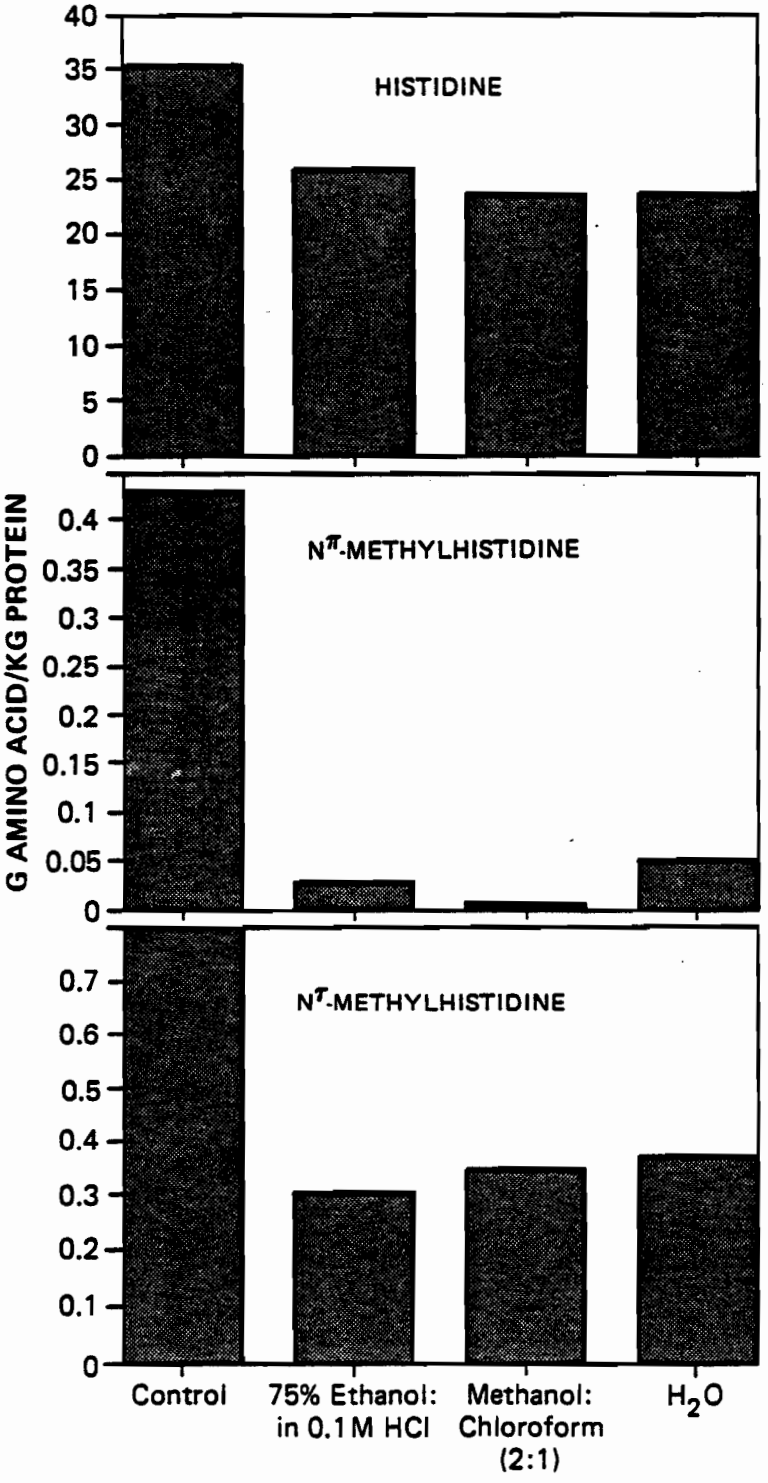
Mixed meat sausages, frankfurters and bologna products were examined in the present study. Meat products have been reported to contain variable amounts of soluble histidine dipeptides, including carnosine, anserine and balenine [Carnegie et al., 1982; 1983; 1984; Harris and Milne, 1987] which upon acid hydrolysis yield β-alanine, histidine, His(π-Me) and His(τ-Me). To quantitatively establish the levels of protein-bound His(τ-Me) in processed meats, the first objective was to find which of the existing solvent extraction procedures would quantitatively extract all soluble compounds including the histidine dipeptides from the lyophilized meat samples prior to acid hydrolysis. Three solvent systems were compared, a mixture of 75% ethanol in 0.1 M HCl (Rangeley and Lawrie, 1976), a mixture of methanol:chloroform (Bligh and Dyer, 1959), and distilled water. Other extraction procedures, i.e., 0.6 M perchloric acid (Happich et al., 1984) and 0.9% saline in 8% 5-sulphosalicylic acid (Carnegie et al., 1984), have

also been reported to be effective in extracting these histidine dipeptides, but have the limitation that the solvents must be removed prior to subjecting the tissue to amino acid analysis.

As may be seen in the histogram of Figure 3 both the Bligh and Dyer (1959) and Rangeley and Lawrie (1976) solvent extraction procedures were equally effective in extracting soluble amino acids peptides including histidine dipeptides from processed meats at ambient temperature, while distilled water was the least efficient. The results presented in Figure 3 are expressed as g amino acid residue per kg total protein. The main advantage of this unit of expressing the composition of a meat mixture, i.e., moisture, fat and ash-free basis, is that the usual practise of subtracting the percentage of connective tissue present in such complex protein mixtures (Olsman and Slump, 1981) is no longer required. The constants, weight equivalent (WE in µg/nmol) and conversion factors (F and F' in µg/nmol), for each of the meat products investigated have been determined (Tables 11 to 13), and can be used in all subsequent quantitations of these products following standard procedures [Horstmann, 1979]. The protein content of the meat products examined was found to be most accurate when calculated according to Horstmann (1979) (Tables 11, 12 and 13). The actual protein of these products was overestimated up to 27.9% when the Kjeldahl nitrogen (Table 10) and the traditional factor 6.25 were used for protein determination. The protein content of these products determined according to the above methods and according to Kjeldahl nitrogen by using the factors calculated from amino acid analysis (Heidelbaugh et al., 1975) is shown in Tables 11 to 13.

Figure 3

Histogram representing the effect of three different extraction solvents, prior to amino acid analysis, on the soluble histidine dipeptides, including carnosine [β-alanyl-L-histidine], anserine [β-alanyl-L-N^{π}-methylhistidine] and balenine [β-alanyl-L-N^{τ}-methylhistidine] present in a mixed meat sausage sample : (A) a mixture of 75% ethanol in 0.1 M HCl [Rangeley and Lawrie, 1977]; (B) a mixture of methanol-chloroform [Bligh and Dyer, 1959]; and (C) distilled water.


HISTIDINE
N$^{\pi}$-METHYLHISTIDINE
N$^{\tau}$-METHYLHISTIDINE
G AMINO ACID/KG PROTEIN
40
35
30
25
20
15
10
5
0
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
75% Ethanol: in 0.1M HCl
Methanol: Chloroform (2:1)
H_2O

The amino acid composition of the three major meat products and ingredients analyzed by the new chromatographic methods [Zarkadas et al., 1986b; 1987a] and levels of statistical significance obtained from analysis of variance are summarized in Tables 11 to 15 and 17 and compared with those of previous investigators in Tables 11 to 13. The composition of lyophilized sausage samples before and after solvent extraction, given in Table 11, shows that only 1.77% (amount of protein determined in the filtrate) of the total amino acid residues was extracted by this method [Rangeley and Lawrie, 1976] prior to acid hydrolysis. A sizeable proportion of the total amino nitrogen, extracted from the processed meats was free ammonia, which accounts for 8.14%, 10.5% and 9.02% of the total nitrogen in the solvent treated sausages, bologna and frankfurters, respectively (Tables 11 to 13). Practically all of the His(π-Me) (97.5 - 99.5%) found in composite meats was extracted at ambient temperature by this procedure (Figure 3). It was found that as much as 33.7% of the total histidine present in untreated sausages (Table 11) could thus be extracted from the lyophilized mixture. Similarly the soluble histidine found in bologna accounts for 14.3% compared to 19.7% extracted from frankfurters (Tables 12 and 13). Similarly, it was found that 60.1%, 20.1%, and 10.4% of the total His(τ-Me) was removed from sausage, frankfurter and bologna products respectively during the extraction procedure. Carnegie et al. (1984) and Olsman and Slump (1981) have reported that the ratio of carnosine to anserine or vice versa could be used as an index for the identification of the meat yielding species in processed meats. Assuming that all of the extracted histidine, His(π-Me) and His(τ-Me) were originally bound to β-alanyl and according to the experimental

Table 11. The Amino Acid Composition of Lyophilized Mixed Meat Sausage Before and After Extraction With 0.1 M HCl in 75% Ethyl Alcohol (g of Amino Acids / kg of Protein)

Amino Acid	Mixed Meat Sausages				F^a	USDA[b]
	Control		Extracted		Between	Handbook
	Mean ± SEM[a]	CV[a]	Mean ± SEM[a]	CV[a]	Treatments	No. 8-7
Aspartic acid	77.29 ± 0.81	2.56	95.23 ± 0.56	1.43	109.53**	84.78
Threonine	44.92 ± 0.83	4.50	41.04 ± 0.37	2.22	34.60*	34.70
Serine	41.23 ± 0.86	5.10	36.73 ± 0.55	3.65	45.17*	38.06
Glutamic acid	137.02 ± 1.26	2.25	148.17 ± 0.90	1.49	34.59*	148.81
Proline	59.50 ± 1.49	6.10	62.02 ± 0.99	3.90	6.98^{ns}	50.22
Glycine	70.45 ± 2.33	8.08	69.65 ± 1.84	6.47	0.37^{ns}	63.96
Alanine	56.08 ± 0.64	2.82	59.15 ± 0.34	1.38	24.62*	57.76
Cysteine	8.25 ± 0.49	14.32	6.92 ± 0.21	7.14	10.39^{ns}	7.84
Valine	52.00 ± 0.54	2.54	51.20 ± 0.35	1.64	16.68^{ns}	34.63
Methionine	23.82 ± 0.54	5.58	24.37 ± 0.32	3.14	0.59^{ns}	27.16
Isoleucine	48.84 ± 0.57	2.85	46.03 ± 0.36	1.92	20.48*	36.19
Leucine	75.21 ± 0.83	2.69	77.07 ± 0.40	1.27	0.23^{ns}	60.45
Tyrosine	41.18 ± 0.65	3.86	35.61 ± 0.46	3.13	109.51**	28.81
Phenylalanine	40.52 ± 0.79	4.76	40.09 ± 0.19	1.25	1.11^{ns}	30.22
Histidine	36.58 ± 0.45	3.03	24.62 ± 0.42	4.18	1120.11**	29.48
Lysine	73.36 ± 0.68	2.26	68.05 ± 0.51	1.85	166.70**	67.39
Arginine	68.62 ± 0.35	1.26	73.95 ± 0.32	1.05	118.43**	57.09

Table 11 continued...

Table 11 (continued)

Amino Acid	Mixed Meat Sausages				F^a	USDA[b]
	Control		Extracted		Between	Handbook
	Mean ± SEM[a]	CV[a]	Mean ± SEM[a]	CV[a]	Treatments	No.8-7
Tryptophan	12.64 ± 0.48	9.18	10.43 ± 0.40	9.39	5.34^{ns}	7.99
4-Hydroxyproline	29.21 ± 1.39	11.64	26.95 ± 0.89	8.06	6.92^{ns}	"
N^{π}-Methylhistidine	0.57 ± 0.09	38.01	0.003 ± 0.001	143.94	117.85**	"
Ammonia	25.84 ± 1.27	12.05	17.69 ± 0.89	6.50	13.85^{ns}	"
Total AA-N[c]	187.28		178.88			
Protein[e](g/100g D.M)	12.856 ± 3.11		47.042 ± 13.95			
Crude protein						
(Kjeldahl-N X 6.25)[f]	17.81 ± 0.19		nd			
(Kjeldahl-N X 5.34)[c]	15.22 ± 0.16		nd			
Total EAA(mg/g N)[d]	2613.04		nd			
EAA Index[d]	71.83		nd			
Protein score[d]	60.91		nd			
WE[e] (µg/nmol)	0.107278		0.107101			
F[e] (µg/nmol)	0.109007		0.108533			
F'[e] (µg/nmol)	0.120440		0.199948			

[a]Mean values and standard error of measurements (SEM) for 3 replicates and 48 determinations **, P<0.01; *, P<0.05; key: ns, not significant; nd, not determined; CV, coefficient of variation.

[b]Values converted from Richardson's et al. (1980) USDA Handbook No.8-7 (07075).

[c]Calculated according to Heidelbaugh et al. (1975); Nitrogen factor 100/18.73= 5.34.

[d]From Oser (1951) and Block and Mitchell (1946).

[e]The WE constant and F, F' conversion factors were calculated from the amino acid composition of each hydrolysate according to Horstmann (1979) using eqns 1, 2 and 3.

[f]Kjeldahl nitrogen value was taken from Table 10.

curves reported by Carnegie et al. (1984) the sausage sample contained mostly pork meat, whereas frankfurter and bologna contained pork and chicken meats in the proportions 0.3:0.7 and 0.2:0.8, respectively.

The data presented in Tables 11 to 15 indicates that each of these meat products has a characteristic amino acid profile depending upon the amounts of specific meats or meat cuts and non-muscle ingredients used to formulate each product. The following features between values for individual amino acids, however, seem to be common to all products. Glutamic acid, for example, is the most abundant amino acid in all untreated products and accounts for almost 14-18% of all amino acid residues. Aspartic acid, proline, glycine, leucine, lysine and arginine, which are the next most abundant amino acids in processed meats, when taken together account for a further 40 to 43%. Thus seven amino acids account for approximately 54-61%, so that only one-third of the positions in the polypeptide chains of processed meats are available for the remaining forty amino acids and ninhydrin-positive compounds which have been determined in this study (Table 11 to 15). Serine and threonine account for 8.0% to 8.6% and tyrosine for another 4.0% of the total amino acid residues. Pro(4-OH) together with small amounts of Lys(5-OH), bring the total composition of residues with hydroxyl groups to nearly 16.0%, which is relatively frequent. The amino acids with hydrophobic side chains [Barrantes, 1973] ranged between 27-29%. Leucine accounts for about 7.5-8.0%, valine for a little more than 5%, tryptophan for only 1.25% and tyrosine, isoleucine and phenylalanine for approximately 13.0%. Methionine accounts for only 2 to 2.8%. This sulfur amino acid has been reported to become oxidized into methionine S-oxide

Table 12. The Amino Acid Composition of Lyophilized Mixed Meat Frankfurters Before and After Extraction With 0.1 M HCl in 75% Ethyl Alcohol

(g of Amino Acids / kg of Protein)

Amino Acid	Mixed Meat Frankfurters				F[a] Between Treatments	USDA[b] Handbook No. 8-7
	Control		Extracted			
	Mean ± SEM[a]	CV[a]	Mean ± SEM[a]	CV[a]		
Aspartic acid	81.78 ± 0.19	0.56	96.66 ± 0.67	1.69	345.25**	98.85
Threonine	41.23 ± 0.30	1.78	40.90 ± 0.55	3.27	24.31*	35.99
Serine	38.56 ± 0.93	5.58	36.15 ± 1.07	7.26	16.91[ns]	40.96
Glutamic acid	156.22 ± 1.03	1.61	163.66 ± 1.13	1.68	11.97[ns]	164.18
Proline	61.35 ± 0.45	1.80	56.16 ± 0.52	2.27	53.91*	48.05
Glycine	61.49 ± 0.68	2.73	57.68 ± 1.00	4.22	5.55[ns]	73.05
Alanine	55.55 ± 0.42	1.83	55.21 ± 0.31	1.37	1.96[ns]	68.77
Cysteine	10.00 ± 0.46	14.32	8.12 ± 0.19	5.70	27.94*	11.52
Valine	49.96 ± 0.56	2.77	53.72 ± 0.20	0.90	16.92[ns]	41.76
Methionine	24.67 ± 0.88	8.73	24.25 ± 0.23	2.24	2.85[ns]	20.21
Isoleucine	50.87 ± 1.15	5.50	48.34 ± 0.41	2.10	8.54[ns]	43.00
Leucine	81.10 ± 0.81	2.46	82.53 ± 0.47	1.37	0.01[ns]	72.67
Tyrosine	40.98 ± 0.66	3.93	38.18 ± 0.61	3.91	50.99**	27.84
Phenylalanine	43.93 ± 0.32	1.81	43.54 ± 0.22	1.24	31.04*	31.83
Histidine	32.33 ± 0.19	0.21	25.95 ± 0.15	1.41	13576.8**	31.03
Lysine	71.73 ± 0.93	3.15	71.64 ± 0.27	0.92	1.16[ns]	80.14
Arginine	66.01 ± 0.56	2.07	69.84 ± 0.35	1.22	4.78[ns]	75.27
Tryptophan	11.24 ± 0.56	30.19	11.31 ± 0.36	7.85	0.01[ns]	7.27

Table 12 continued...

Table 12 (continued)

Amino Acid	Mixed Meat Frankfurters: Control Mean ± SEM[a]	Control CV[a]	Mixed Meat Frankfurters: Extracted Mean ± SEM[a]	Extracted CV[a]	F[a] Between Treatments	USDA[b] Handbook No. 8-7
4-Hydroxyproline	16.79 ± 2.07	2.77	14.12 ± 0.08	1.50	1.65[ns]	-
N^{π}-Methylhistidine	2.02 ± 0.08	9.91	0.05 ± 0.005	21.97	345.01**	-
Ammonia	24.92 ± 0.68	6.76	19.48 ± 0.72	8.98	82.91*	-
Total AA-N[c]	183.42		177.68			
Protein[e] (g/100g D.M)	22.924 ± 1.45		49.497 ± 8.95			
Crude protein						
(Kjeldahl-N X 6.25)[f]	30.81 ± 0.19		nd			
(Kjeldahl-N X 5.44)[c]	26.81 ± 0.16		nd			
Total EAA (mg/gN)[d]	2680.89		nd			
EAA Index[d]	70.60		nd			
Protein score[d]	65.55		nd			
WE[e] (µg/nmol)	0.108461		0.108955			
F[e] (µg/nmol)	0.110343		0.110636			
F'[e] (µg/nmol)	0.120735		0.119968			

[a]Mean values and standard error of measurements (SEM) for 3 replicates and 48 determinations. **, P<0.01; *, P<0.05; key: ns, not significant nd, not determined; CV, coefficient of variation.

[b]Values converted from Richardson's et al. (1980) USDA Handbook No. 8-7 (07023).

[c]Calculated according to Heidelbaugh et al. (1975); Nitrogen factor 100/18.34 = 5.44.

[d]From Oser (1951) and Block and Mitchell (1946).

[e]The WE constant and F, F' conversion factors were calculated from the amino acid composition of each hydrolysate according to Horstman (1979) and by using eqns 1, 2 and 3 respectively

[f]Kjeldahl nitrogen was taken from Table 10.

during processing [Spindler et al., 1984] or in the presence of carbohydrates [Rayner, 1985] and was determined both as methionine S,S-dioxide [Moore, 1963] and in regular acid hydrolysates as methionine [Zarkadas et al., 1987a]. Both methods yielded identical results. Thus the frequency of hydrophobic groups in processed meats greatly exceeds that of hydroxyl groups.

The following differences between values for individual amino acids were noted among the extracted and untreated composite meats evaluated. The aspartic acid values of the extracted sausages, bologna and frankfurter samples were some 12.0% to 18.8% higher than the corresponding untreated samples (Tables 11 to 13). USDA Handbook No.8 (Richardson et al., 1980) recalculated values for aspartic acid are 84.78, 98.85 and 87.94 g/kg protein for mixed meat sausages, frankfurters and bologna, respectively. These values are considerably higher than the respective values found in the untreated samples examined in this study, but lower than the corresponding present in the solvent treated samples. The mean values obtained for arginine in the solvent treated composite meats ranged from 63.34 to 73.95 g/kg protein (Tables 11 to 13), with the sausages being much higher in arginine (73.95g/kg) compared to 68.62 g/kg protein found in the untreated sample. Richardson et al., (1980) reported arginine values of 57.09 and 59.79 g/kg protein for sausages and bologna (Tables 11 and 13), respectively, but higher values for frankfurters (75.25 g/kg protein; Table 12). The glutamic acid values for the extracted products exceed those of the untreated samples, but are in reasonable agreement with Richardson's et al. (1980) recalculated data. It is possible that the values obtained for

Table 13. The Amino Acid Composition of Lyophilized Mixed Meat Bologna Before and After Extraction With 0.1 M HCl in 75% Ethyl Alcohol

(g of Amino Acids / kg Protein)

	Mixed Meat Bologna				F[a]	USDA[b]
	Control		Extracted		Between	Handbook
Amino Acid	Mean ± SEM	CV[a]	mean ± SEM[a]	CV[a]	Treatments	No.8-7
Aspartic acid	74.41 ± 1.17	3.88	84.75 ± 0.33	0.94	37.42*	87.94
Threonine	40.22 ± 0.78	4.73	38.31 ± 0.22	1.44	8.55[ns]	43.37
Serine	43.37 ± 1.34	7.54	37.06 ± 0.42	2.81	32.67*	43.54
Glutamic acid	178.18 ± 1.77	2.44	196.27 ± 0.61	0.79	32.47*	160.31
Proline	67.09 ± 0.30	1.10	65.18 ± 0.23	1.47	1876.65**	63.99
Glycine	54.41 ± 0.38	1.69	47.95 ± 0.29	1.47	1621.94**	73.91
Alanine	50.66 ± 0.21	0.99	48.59 ± 0.30	1.52	104.22**	62.36
Cysteine	9.79 ± 1.28	6.55	9.65 ± 0.20	4.90	34.63*	11.63
Valine	50.47 ± 0.59	0.99	53.15 ± 0.06	0.25	3.61[ns]	53.12
Methionine	28.24 ± 0.92	8.00	23.73 ± 2.77	2.97	24.88*	23.70
Isoleucine	49.67 ± 1.09	5.39	49.10 ± 0.31	1.53	370.00[ns]	43.37
Leucine	80.77 ± 0.28	0.85	82.93 ± 0.37	1.07	0.76[ns]	76.82
Tyrosine	40.23 ± 1.64	10.02	41.92 ± 1.54	9.00	0.19[ns]	30.72
Phenylalanine	46.16 ± 0.20	1.06	45.81 ± 0.09	0.50	695.29**	39.52
Histidine	29.51 ± 0.15	1.29	25.30 ± 0.03	0.25	587.11**	27.20
Lysine	63.41 ± 1.28	2.02	57.13 ± 0.19	0.79	129.76**	75.54
Arginine	60.33 ± 0.33	1.37	63.34 ± 0.14	0.53	4.70[ns]	59.79
Tryptophan	13.87 ± 0.20	3.48	12.62 ± 0.14	2.66	27.44*	8.98
4-Hydroxyproline	15.65 ± 0.14	2.16	15.83 ± 0.25	3.89	0.77[ns]	-

Table 13 continued.....

Table 13 (continued)

Amino Acid	Mixed Meat Bologna				F^a	USDA[b]
	Control		Extracted		Between	Handbook
	Mean ± SEM[a]	CV[a]	Mean ± SEM[a]	CV[a]	Treatments	No. 8-7
N^{π}-Methylhistidine	2.06 ± 0.07	8.84	0.05 ± 0.004	23.86	543.80**	-
Ammonia	28.35 ± 0.89	7.63	22.49 ± 0.22	2.44	317.14**	-
Total AA-N[c]	182.15		174.88			
Protein[e] (g/100g D.M)	21.824 ± 0.29		51.083 ± 5.12			
(Kjeldahl-N X 6.25)[f]	27.81 ± 0.13		nd			
(Kjeldahl-N X 5.34)[c]	24.43 ± 0.11		nd			
Total EAA (mg/g N)[d]	2652.52		nd			
EAA[d]	72.78		nd			
Protein score[d]	73.15		nd			
WE[e] (µg/nmol)	0.109379		0.110458			
F[e] (µg/nmol)	0.111444		0.112462			
F'[e] (µg/nmol)	0.122787		0.123751			

[a]Mean values and standard error of measurements (SEM) for 3 replicates and 48 determinations. **, P<0.01; *, P<0.05; ns, not significant; nd, not determined; CV, coefficient of variation.

[b]Converted from Richardson's et al. (1980) USDA Handbook No. 8-7 (07008).

[c]Calculated according to Heidelbaugh et al. (1975); Nitrogen factor 100/18.22 = 5.49.

[d]From Oser (1951) and Block and Mitchel (1946).

[e]The WE constant and F, F' conversion factors were calculated from the amino acid composition of each hydrolysate according to Horstmann (1979) by using eqns 1, 2 and 3 respectively.

[f]Kjeldahl value was taken from Table 10.

aspartic acid, glutamic acid and arginine in the extracted samples are slightly augmented by the presence of unknown compounds in the region of these peaks, but this would probably be insufficient to account for the difference, because of the low coefficient of variation and high resolution achieved between these peaks by the chromatographic method employed [Zarkadas et al., 1987a]. Abnormalities of the aspartic and glutamic acids chromatography after treatment of the tissues to be analyzed has been reported [Joy et al., 1980; Happich et al., 1984]. The lower levels of Pro(4-OH) and Lys(5-OH) found in the solvent treated bologna and frankfurters as compared to those values in the control samples agree closely with the data of Happich et al. (1984) for freeze-dried beef extracted with three solvents. Herring et al. (1967) reported an increase in Pro(4-OH) extractability after storage of muscle at 4 °C for 10 days. Finally, the mean values obtained for the remaining amino acids in the extracted samples are slightly lower than the corresponding untreated samples, except as mentioned earlier for histidine. The weighted mean values obtained for the amino acid composition of all products investigated are consistenly higher compared to those recalculated from the USDA Handbook No. 8-7 [Richardson et al., 1980].

Processed meats contained significant amount of all amino acids commonly found in proteins with the exception of cyst(e)ine and methionine and possibly valine, tryptophan, phenylalanine and isoleucine. A comparison between the EAA profile of mixed meat sausages, bologna and frankfurters (Tables 11 to 13) with the total EAA (mg/g N) of reference proteins showed that all of these products were much lower than cow's milk (3200 mg/g N) or

hen's whole egg (3215 mg/g N) protein [FAO/WHO, 1965]. Similar results were obtained from EAA indices and protein scores calculated from the amino composition of these products (Tables 11 to 13) by the methods of Oser (1951) and Block and Mitchell (1946). Although these predictive tests are based on the known amino acid composition of these selected processed meats which afford a valuable guide in compounding the protein mixtures or meat products, they fail to take into account differences in the digestibility and availability of individual amino acids and the quality of the various proteins present [Pellet and Young, 1984]. Because of this Sarwar et al.(1985), Happich et al. (1984) and McLaughlan et al. (1980) developed reliable and rapid methods of assessing the nutritive value and protein quality of foods including available amino acid score, net protein ratio (NPR) and relative net protein ratio (RNPR). These methods, however, do not provide any information about the identity of the limiting or the levels of other amino acids present in the protein or protein mixture being evaluated. For these reasons, Pellet and Young, (1984) have found that the best estimate of the protein quality of a food is its amino acid composition, and recommended that whenever the overall protein quality of individual foods is required, the amino acid composition rather than the standard rat bioassay procedures be used.

To validate the use of His(τMe) as an index for assessing the myofibrillar protein content of processed meats, accurate and detailed determination of His(τMe) in three selected composite meat products was carried out. The major objective of this survey was to provide statistically sound data on the occurence and variation of His(τMe) and other

unique basic amino acids found in these major meat products. All determinations were carried out by new single-column chromatographic methods [Zarkadas et al., 1987a]. The chromatograms illustrated in Figures 4,5 and 6 are typical of the separations obtained by one of these methods [Zarkadas et al., 1987a]. Good separation from a standard containing all the unique basic amino acids likely to be encountered in a biological system was obtained (Figures 4A to 6A). The unusual basic amino acid components of processed meats were determined from concentrated 96 h hydrolysate samples (220-270 µg/100 µL in the case of the untreated products and 550-650 µg/100 µL in the case of the solvent treated products) so that reasonable sized peaks (50 to 250 pmol/100 µg protein) for these unique components could be obtained. As may be seen in Figures 4B and 4C, the analysis of sausage hydrolysates (96 h) before and after solvent extraction by this method enabled the complete separation of all methylated basic amino acids including the diastereoisomers of Lys(5-OH) and revealed four major peaks (1, 5, 13, and 17) and thirteen minor as yet unidentified ninhydrin-positive components. The elution times and relative concentrations of the four major unknown peaks are presented in Table 14, while the retention times of the minor components shown in Figures 4B and 4C are as follows: peak 2, 44.0; 3, 47.0; 4, 55.0; 6, 105.0; 7, 124,0; 8, 130.0; 9, 135.0; 10, 142.0; 11, 179.0; 12, 183.2; 14, 258.0; 15, 267.0; and 16, 270.0 min. Similar separations of all these unknown peaks were obtained with both bologna and frankfurter composite meat samples investigated and the chromatographic runs are presented in Figures 5 and 6. The relative concentrations of the four major unknown peaks found in extracted sausage, bologna and frankfurters samples

(Figures 4, 5 and 6) are presented in Table 14. Their concentrations were calculated by assuming a relative response factor (RRF) equal to that of the internal standard, 3-nitrotyrosine (RRF=1.0000). It was rather surprising that the stable crosslinks of elastin, Des and iDes, were not present in the chromatographic patterns of Figures 4, 5, and 6, since it has been reported that connective tissue rich in elastin is added in comminuted meat products [Young and Pellet, 1984]. Considering that the detection limit of the chromatographic procedure employed [Zarkadas et al., 1987a] was 20 pmol for either Des or iDes, and that 4.1 μmol of desmosines (Des + iDes) are present in 85.03 mg of elastin (Table 8), as little as 0.42 μg of elastin would be quantitable, if present in the meat products examined.

The elution profiles obtained in the analysis of acid hydrolysates of extracted and untreated sausage samples shows that both His(π-Me) and unknown No.13 have been completely extracted with the 75% ethanol:0.1 M HCl mixture, prior to hydrolysis (Figure 4C), compared to the untreated sample (Figure 4B) while unknown No.6 increased considerably. Since the positions of both His(π-Me) and His(τ-Me) in the chromatogram are very sensitive to pH, rigid control of the pH of the second eluting buffer (pH 4.501 ± 0.002) is also necessary to obtain good resolution [Zarkadas et al., 1987a]. Thus, as shown in Figure 4A, by introducing the second buffer (pH 4.501) just before the elution of Lys(Me), and by increasing the temperature to 73 °C, both His(π-Me) and His(τ-Me) were completely separated following the ammonia peak. It should also be noted (Figures 4 to 6) that at pH 4.501, His(τ-Me) is completely separated from an unknown compound (No. 17) which appeared to occur in variable amounts (Table 14) in most meat and plant tissue

Figure 4

Chromatographic separation of methylated lysines and histidines, the diastereoisomers of 5-hydroxylysine and related compounds in mixed meat sausages: (A) separation of a synthetic amino acid calibration mixture; (B) typical separation of an untreated 96 h hydrolysate of sausage; and (C) analysis of an extracted sausage sample (96 h hydrolysate). The curve shows absorbance at 570 nm.

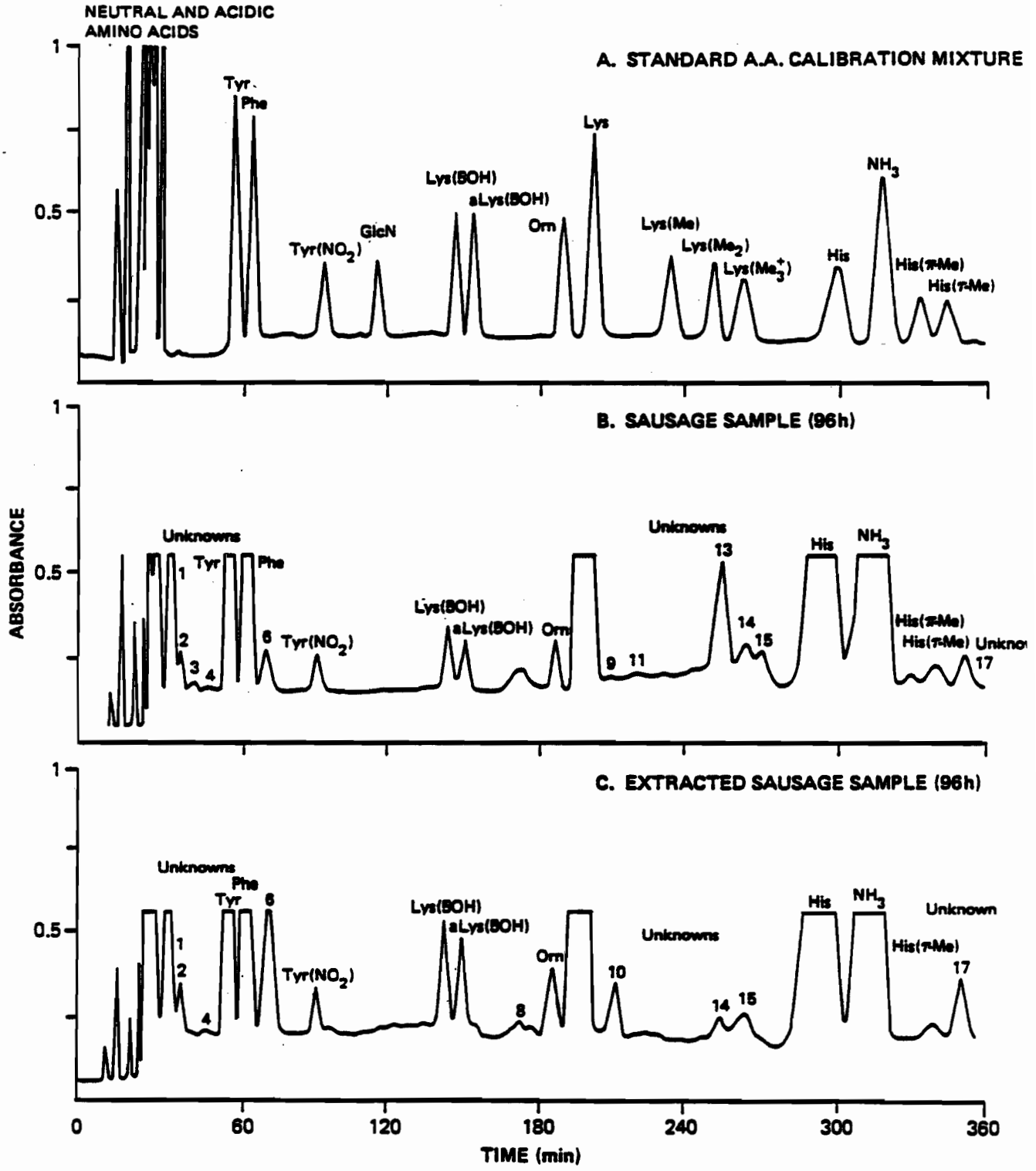

NEUTRAL AND ACIDIC AMINO ACIDS
A. STANDARD A.A. CALIBRATION MIXTURE
Tyr
Phe
Tyr(NO2)
GlcN
Lys(BOH)
aLys(BOH)
Orn
Lys
Lys(Me)
Lys(Me2)
Lys(Me3+)
His
NH3
His(π-Me)
His(τ-Me)
B. SAUSAGE SAMPLE (96h)
Unknowns
Tyr
Phe
Tyr(NO2)
Lys(BOH)
aLys(BOH)
Orn
Unknowns
His
NH3
His(π-Me)
His(τ-Me)
C. EXTRACTED SAUSAGE SAMPLE (96h)
Unknowns
Phe
Tyr
Tyr(NO2)
Lys(BOH)
aLys(BOH)
Orn
Unknowns
His
NH3
Unknown
His(τ-Me)
ABSORBANCE
1
0.5
0
60
120
180
240
300
360
TIME (min)

Figure 5

Typical elution patterns of the methylated basic amino acids and related compounds in bologna sample. (A) Separation of a synthetic mixture of 15 unique basic amino acids on an analytical 50 x 0.28 cm microcolumn of Dionex DC-4A resin; (B) elution profile of an extracted bologna (96 h hydrolysate) sample; (C) chromatographic separation of an untreated bologna (96 h hydrolysate) sample. The curves denote absorbance at 570 nm.

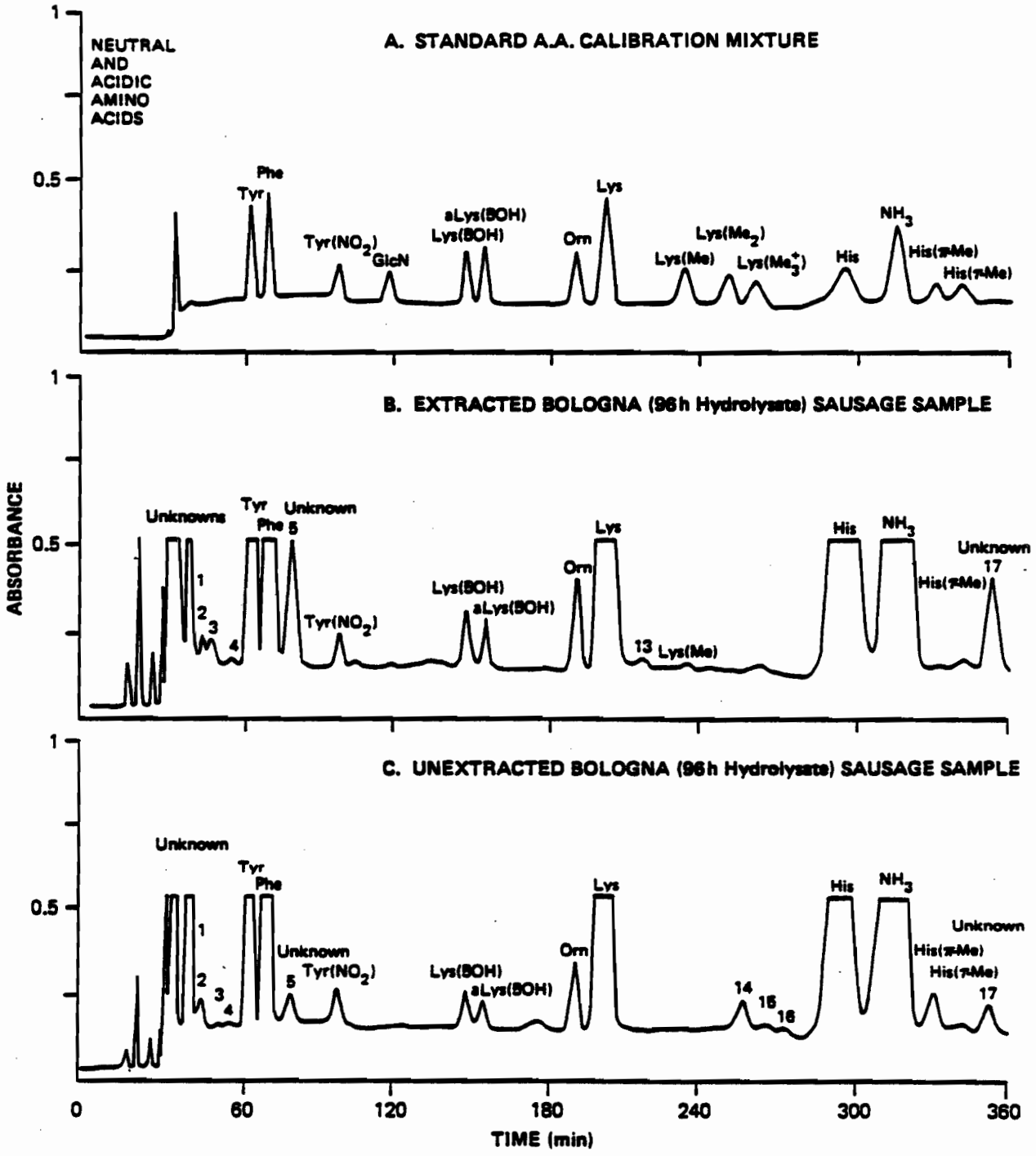

A. STANDARD A.A. CALIBRATION MIXTURE
NEUTRAL AND ACIDIC AMINO ACIDS
Phe
Tyr
Tyr(NO2)
GlcN
aLys(BOH)
Lys(BOH)
Orn
Lys
Lys(Me)
Lys(Me2)
Lys(Me3+)
His
NH3
His(π-Me)
His(τ-Me)
B. EXTRACTED BOLOGNA (96h Hydrolysate) SAUSAGE SAMPLE
Unknowns
Tyr
Phe
Unknown 5
Tyr(NO2)
Lys(BOH)
aLys(BOH)
Orn
Lys
13 Lys(Me)
His
NH3
His(τ-Me)
Unknown 17
C. UNEXTRACTED BOLOGNA (96h Hydrolysate) SAUSAGE SAMPLE
Unknown
Tyr
Phe
Unknown
Tyr(NO2)
5
Lys(BOH)
aLys(BOH)
Orn
Lys
14
15
16
His
NH3
Unknown
His(π-Me)
His(τ-Me)
17
ABSORBANCE
1
0.5
0
60
120
180
240
300
360
TIME (min)

Figure 6

Separations of all methylated lysines and histidines, the diastereoisomers of 5-hydroxylysine, and related compounds in frankfurter samples. (A) typical elution profile of a synthetic mixture of 15 unique basic amino acids on a 50 x 0.28 cm microcolumn of Dionex DC-4A resin; (B) analysis of a frankfurter (96 h hydrolysate) sample; (C) separation of a 96 h hydrolysate of an extracted frankfurter sample. The curves represent absorbance at 570 nm.

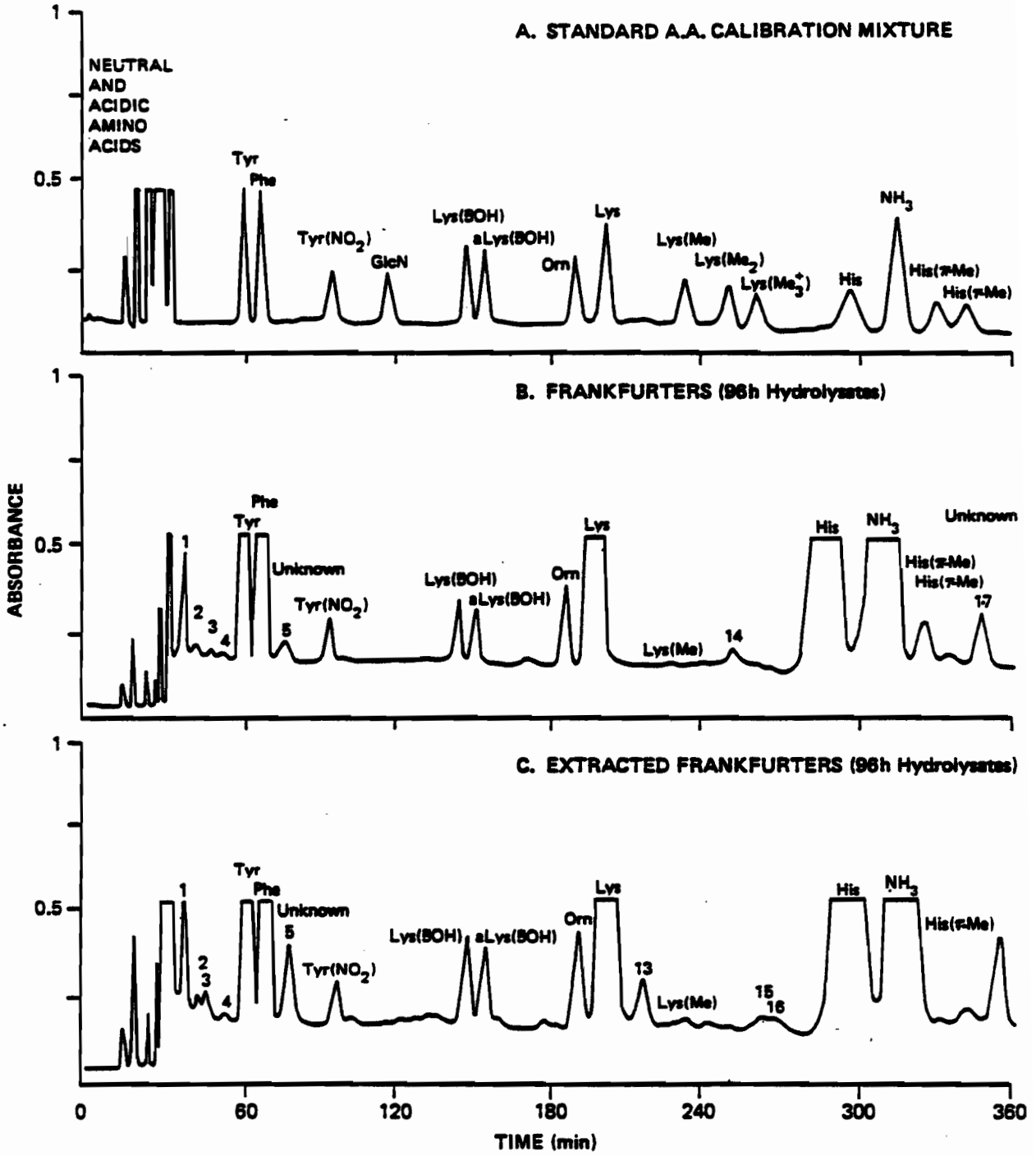

A. STANDARD A.A. CALIBRATION MIXTURE
NEUTRAL AND ACIDIC AMINO ACIDS
Tyr
Phe
Tyr(NO2)
GlcN
Lys(BOH)
aLys(BOH)
Orn
Lys
Lys(Me)
Lys(Me2)
Lys(Me3+)
His
NH3
His(π-Me)
His(τ-Me)
B. FRANKFURTERS (96h Hydrolysates)
Phe
Tyr
Unknown
Tyr(NO2)
Lys(BOH)
aLys(BOH)
Orn
Lys
Lys(Me)
His
NH3
Unknown
His(π-Me)
His(τ-Me)
1-7
C. EXTRACTED FRANKFURTERS (96h Hydrolysates)
Tyr
Phe
Unknown
Tyr(NO2)
Lys(BOH)
aLys(BOH)
Orn
Lys
Lys(Me)
His
NH3
His(π-Me)
ABSORBANCE
1
0.5
0
60
120
180
240
300
360
TIME (min)

Table 14. Elution Times and Contents of Major Unknown Ninhydrin-Positive Peaks Present in Hydrolysates of Commercially Prepared Comminuted and Emulsified Type Meat Products Extracted With 0.1 M HCl in 75% Ethanol [a]

Compound[b]	Elution Time (min)	(nmol / mg of Protein) Meat Products: Sausages Mean ± SEM[c]	Sausages CV[c]	Bologna Mean ± SEM[c]	Bologna CV[c]	Frankfurter Mean ± SEM[c]	Frankfurter CV[c]
Unknown 1	38.0	nd		nd		nd	
5	78.0	15.698 ± 0.619	9.66	17.513 ± 0.226	3.16	8.902 ± 0.302	8.30
13	216.0	5.826 ± 0.713	27.37	2.186 ± 0.908	101.75	4.222 ± 0.241	13.97
17	354.0	7.133 ± 0.384	13.18	13.927 ± 0.775	12.45	7.822 ± 0.358	11.21
Ornithine	190.0	5.757 ± 0.349	14.85	8.267 ± 0.359	10.65	7.809 ± 1.002	31.44

[a] Separated according to the method described by Zarkadas et al. (1987a). (Figures 4, 5 and 6).

[b] Each of the unknown peaks is assigned an arabic number to indicate its relative order of elution from the microcolumn.

[c] Mean values and standard error of measurements (SEM) of 12 determinations; CV, coefficient of variation; nd, not determined. The relative concentrations of the various unknown peaks were calculated by assuming a relative response factor equal to that of the internal standard, 3-nitrotyrosine (RRF=1.0000).

hydrolysates investigated. Rangeley and Lawrie (1977) and Jones et al. (1982) have reported the presence of a peak, in hydrolysates of vegetable proteins, eluting at the same position or after the elution of His(τ-Me) in the chromatographic systems they employed. Figures 4, 5, and 6 also show that an accurate determination of the Lys(5-OH) content of composite meats was performed from the values obtained for one of its diastereoisomers, aLys(5-OH), after epimerization. For the above reasons, caution must be exercised in interpreting the data available in the literature concerning the contents of His(τ-Me) and Lys(5-OH) of various muscles from different meat yielding species, and processed meats.

Table 15 compares the His(τ-Me) and Lys(5-OH) contents of the three commercially prepared mixed meat products before and after solvent extraction with 75% ethanol:0.1 M HCl mixture and all-beef hamburger [Karatzas and Zarkadas, 1987]. The range and levels of statistical significance obtained from the analysis of variance of the His(τ-Me) and Lys(5-OH) contents of the these processed meats are also presented in Table 15. The values obtained for His(τ-Me) and Lys(5-OH) of all composite meat products show high reproducibilty. The data presented in Table 15 shows that each of these meat products has a typical His(τ-Me) and Lys(5-OH) profile depending upon the amounts of specific meats or meat cuts and non-muscle ingredients used to formulate each product. A comparison, for example, between different types of meat sausages, bologna and frankfurters (Table 15), indicates that significant differences exist among these products with respect to their His(τ-Me) and Lys(5-OH) contents. The mixed meat sausages had high levels of Lys(5-OH) and appear to contain a higher proportion of connective tissue

Table 15. The N^{τ}-Methylhistidine and 5-Hydroxylysine Contents of Commercially Prepared Comminuted and Emulsified Type Meat Products and, Hamburger Before (un) and After (ex) Solvent Extraction With 0.1 M Hcl in 75% Ethyl Alcohol.

(g of Amino Acids/kg of Protein)

Meat Product	N^{τ}-Methylhistidine Mean ± SEM[a]	Range Min.-Max.	CV[a]	F[a] Between Treatments	5-Hydroxylysine Mean ± SEM[a]	Range Min.-Max.	CV[a]	F[a] Between Treatments
Mixed Meat								
Sausages								
Protein Bound	0.303 ± 0.013[c]	0.268 0.351	10.85		2.628 ± 0.221	1.927 3.269	20.56	
Total (un)	0.759 ± 0.010[b]	0.715 0.789	3.26	429.43**	2.237 ± 0.093	1.839-2.485	10.17	3.06[ns]
Bologna								
Protein Bound	0.266 ± 0.020	0.219 0.335	18.45		1.171 ± 0.053[c]	1.017-1.332	11.01	
Total (un)	0.297 ± 0.014	0.261-0.356	11.32	0.78[ns]	1.594 ± 0.060[b]	1.414-1.759	9.21	36.78*
Frankfurters								
Protein Bound	0.378 ± 0.013	0.341-0.419	8.34		1.737 ± 0.078[c]	1.586-2.081	11.02	
Total (un)	0.473 ± 0.017	0.417-0.525	8.90	17.95[ns]	2.067 ± 0.045[b]	1.960-2.235	5.32	87.15*
Hamburger (ex)[d]								
Protein Bound	0.533 ± 0.027	0.480-0.572	10.17		1.755 ± 0.035	1.632-1.794	3.97	

[a]Mean values and standard error of measurements (SEM) for 3 replicates and 12 determinations; **, P<0.01; *, P<0.05; ns, not significant; CV, coefficient of variation.

[b,c]Means within a column with different superscripts are significantly different.

[d]From Karatzas and Zarkadas (1987).

compared to bologna and frankfurters. The values of Table 15 show that the frankfurter meat samples contained the highest levels of His(τ-Me), indicating that this product had the highest levels of myofibrillar protein contents. The protein-bound His(τ-Me) levels determined in the sausage, frankfurter and bologna products (Table 15) correspond to 2.4227, 2.6755, and 1.7700 mg His(τ-Me)/g nitrogen (fat and collagen free), respectively. These results are in reasonable agreement with those reported by other authors [Rangeley and Lawrie, 1977; Poulter and Lawrie, 1980b; Olsman and Slump, 1981]. Some differences noted, however [Jones et al., 1985], 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.

The Myofibrillar and Connective Tissue Proteins of the Meat Products

In this study, an attempt was made to relate the protein quality of three typical meat products and hamburger to their amino acid composition for the direct determination of their myofibrillar and connective tissue contents using the very sensitive and high resolving power chromatographic method which has been developed [Zarkadas et al., 1987a].

From the results presented in Table 15 on the His(τ-Me) and Lys(5-OH) contents of three composite meat products and hamburger samples, it has been possible to determine their myofibrillar and connective tissue protein contents and the results are summarized in Table 16. In this chemical approach the actin, myosin, actomyosin and total myofibrillar protein contents of prepared composite meats and hamburger can be determined from the amounts of His(τ-Me) found in their acid hydrolysates using eqns 8b, 8c, and 8d.

The data presented in Table 16 indicate that the myofibrillar protein content of all-beef hamburger accounts for an estimated 52.3% of the total muscle proteins. Actin accounts for 11.05% while myosin for another 23.0% of the total muscle protein in hamburger sample. These results are in accord with those reported for skeletal muscle by Hanson and Huxley (1957) and Yates and Greaser (1983b) who have shown that the rabbit Psoas major contains 57.71% myofibrillar proteins, and that actin accounts for an estimated 12.69% while myosin accounts for 24.82% of the total muscle proteins. In contrast, the content of the myofibrillar proteins in mixed meat sausages and bologna ranged from 29.7 to 26.1% compared to 37.1% found in frankfurters (Table 16). These results indicate that the actual levels

Table 16. The Myofibrillar and Connective Tissue Contents of Selected Composite Meat Products and All-Beef Hamburger.

Protein Class	Composite Meat Products			All-Beef[f]
	Sausages	Frankfurters	Bologna	Hamburger
	Skeletal Muscle Proteins (g of Protein / kg of Total Protein)			
i. Intracellular (ia + ib)[b]	485.30	606.67	426.92	855.44
ia. Myofibrillar[b]	297.39 ± 12.41	371.00 ± 12.41	261.08 ± 19.63	523.13 ± 14.12
Actin[b]	62.80 ± 2.69	78.34 ± 2.69	55.13 ± 4.15	110.46 ± 5.59
Myosin[b]	130.51 ± 5.37	162.81 ± 5.60	114.57 ± 8.61	229.57 ± 11.61
Actomyosin[b]	193.31 ± 8.07	241.15 ± 8.29	169.70 ± 12.76	340.03 ± 17.10
ib. Other soluble proteins[b]	188.91 ± 7.32	235.67 ± 8.01	165.84 ± 12.32	332.31 ± 8.87
ii. Extracellular matrix[c]	26.67 ± 1.13	33.27 ± 1.13	23.41 ± 1.74	46.91 ± 1.25
iia. Collagen[c]	18.23 ± 0.90	22.74 ± 0.78	16.00 ± 1.21	32.07 ± 0.88
Total (i + ii)	511.97	639.94	450.33	902.35
	Non-muscle ingredients and additives			
iii. Connective tissue[d]	203.83 ± 3.03	106.79 ± 0.61	119.72 ± 1.89	83.72 ± 0.08
iv. Total collagen and collagen-like proteins[e]	163.83 ± 13.78	108.28 ± 4.86	73.00 ± 3.30	109.41 ± 2.18
v. Added collagen (iv − iia)	145.60	85.54	57.00	77.34
Total (i + ii + v)	657.57	725.48	507.33	979.69
Added non-muscle proteins	342.43	274.52	492.67	20.31

[a]Samples extracted with a mixture of 75% ethyl alcohol in 0.1 M HCl [Rangeley and Lawrie, 1977].

[b]Calculated from eqns (8b, 8c, 8d, and 8e) and His(τ-Me) levels (Table 15).

[c]Calculated from eqns (8f and 8g) and His(π-Me) levels (Table 15).

[d]Calculated from the Pro(4-OH) data (Tables 11 to 13) using eqn (6b, Section III).

[e]Calculated from the Lys(5-OH) levels (Table 15) using eqn (5a, Section III).

[f]From Karatzas and Zarkadas (1987).

of meat cuts used to formulate each of the composite meats evaluated varied significantly, and that certain of these products contained substantial amounts of non-meat protein ingredients and additives. These results also show that the direct approach used in this study for evaluating protein quality in composite meats has the advantage over other methods in that it is based on the determination of at least two classes of high quality muscle proteins: the myofibrillar myosin and actin, and other intracellular soluble muscle proteins (Table 16), which can be compared in each of the meat products evaluated.

Since the mean value of the other SDS-soluble muscle proteins reported for the costal region of the bovine diaphragm account for 43.0% of the total muscle proteins (Table 9), this muscle protein fraction in composite meat products could also be calculated by eqn 8e, and the results are summarized in Table 16. This intracellular muscle protein fraction includes sarcoplasmic proteins, organelles, Z-band and other membrane proteins, etc., which are soluble in 2% SDS as described by McCollester (1962) and Laurent et al. (1981).

The weighted mean collagen and connective tissue contents of commercially prepared composite meats and hamburger are presented in Table 16. In this approach the content of total collagen in meat products was determined from the amounts of Lys(5-OH) found in their acid hydrolysates and the content of total connective tissue proteins from the amounts of Pro(4-OH) present using equns 5a and 6b (Section III). The accuracy of such calculations, however, will depend on the purity of the collagen or elastin on which their Lys(5-OH) and Pro(4-OH) contents are based (Table 8). Although

Pro(4-OH) was once thought to be unique to collagen [Eastoe, 1967] except for the comparatively small amounts (1.3 to 1.6%) found in elastin (Bendall, 1967), and this amino acid can be used as an index for determining the connective tissue content of various muscle tissues or meats, its application to composite meats is limited for the following reason. It is evident that Pro(4-OH) is a constituent of certain seed proteins including extensin and other 4-hydroxyproline-rich glycoproteins of plant cell walls [McNeil et al., 1984; Adams and Frank, 1980]. Therefore the values reported for the connective tissue contents of processed meats in Table 16, which usually include plant protein additives such as soy bean and other types of oil seed proteins (Table 17), could be an overestimate of the actual levels of connective tissue proteins found in composite meats.

For these reasons, the determination of Lys(5-OH) is now being routinely used in this laboratory as an index for quantitating the collagen content of various muscles and composite meats, since this amino acid remains relatively constant among the various genetic forms of collagen [Miller and Gay, 1982; Cheah, 1985], and because the relative distribution of the various collagen isoforms among normal skeletal muscles also appear to be constant [Light and Champion, 1984; Light et al., 1985]. The average collagen content of 32 adult and 18 young bovine skeletal muscle tissues was found to account for 4.02% and 4.15% respectively of the total muscle proteins on a dry, fat free basis [Bendall, 1967; Dransfield, 1977] and the transcellular insoluble matrix in skeletal muscle accounts for 1.92% of the total muscle proteins [Lowey et al., 1983]. Thus the higher collagen value (10.94%) determined in the hamburger samples (Table 16), compared to 4.1%

collagen calculated for skeletal muscles, may be attributed to cheaper meat cuts being used in this product. These data correspond closely to those reported by Terrell (1982) for beef plate and cow meat and by Porteus (1981) for beef anf hog meats used in sausage formulations.

From the data presented in Table 16 it is apparent that differences in the collagen composition exist among the meat products analyzed. Mean values for total collagen ranged from 7.3% to 16.4% in meat products. If the amount of collagen normally associated with muscle is subtracted from the total collagen found in composite meats, the difference is an accurate assessment of the non-muscle collagen being added to these products. For example, mixed meat sausages contained 16.4% total collagen (Table 16), 14.6% of which was added as non-muscle collagen to this product. Values for collagen being added to bologna and frankfurters were also high, and ranged from 5.70 to 8.55 g/kg total protein, respectively. The bologna sample contained the lowest levels of collagen of all the products studied and the highest amounts (49.3%) of added non-muscle proteins (Table 16).

Four additives (H-82, H-93, H-190 and H-64) used in the manufacture of the commercially prepared meats examined in the present study and a number of plant and animal ingredients were analyzed for their His(τ-Me), Lys(5-OH), related ninhydrin-positive compounds and Pro(4-OH) contents using the chromatographic procedures described by Zarkadas et al. (1986b; 1987a). The results of these analyses are summarized in Table 17. In the ingredients analyzed His(τ-Me), Lys(5-OH) and desmosines were absent, whereas Lys(Me) and Lys(Me_3)+ were present in all but the Hygrade additives. An unknown as yet unidentified compound No. 17 was found in all the ingredients in levels

Table 17. The Unique Basic Amino Acid, Unknown Compound No. 17 and Pro(4-OH) Contents Found in Hydrolysates (96 h) of Non-meat Protein Additives and Ingredients Used in the Formulations of Meat Products[a].

Non-Meat Protein	nmol/mg Protein[b]						
	Orn	Lys(Me)	$Lys(Me_2)$	$Lys(Me_3)^+$	Unknown No.17	His(τ-Me)	Pro(4-OH)
Additives							
Oilseed Derived							
Mustard seed full-fat flour	2.78 ± 0.63	-	1.57 ± 0.04	7.37 ± 0.61	43.52 ± 2.31	-	87.35 ± 0.16
Textured soybean flour, Promate	3.43 ± 0.09	-	1.15 ± 0.01	1.11 ± 0.03	25.92 ± 1.11	-	nd
Soybean protein isolate	1.66 ± 0.03	-	1.05 ± 0.03	1.54 ± 0.01	6.52 ± 0.33	-	nd
Cereal Grain Derived							
Biscrum flour	trace	-	trace	-	-	-	nd
Vital wheat gluten	1.81 ± 0.02	trace	1.75 ± 0.04	-	9.12 ± 0.30	-	trace
Hygrade flour, H-82	8.03 ± 0.94	-	-	-	22.09 ± 1.47	-	1.80 ± 0.01
Hygrade flour, H-93	5.35 ± 0.77	-	-	-	15.26 ± 0.81	-	0.86 ± 0.01
Animal Derived							
Milk solid non-fat powder	5.32 ± 0.49	-	trace	-	trace	-	nd
Egg white solids	2.01 ± 0.09	-	-	-	4.59 ± 0.63	-	nd
Sensory Enhancers							
Mixed: non-meat binders and Flavourings (H-190)	7.80 ± 1.00	-	-	-	18.16 ± 0.68	-	6.97 ± 0.03
Flavourings: spices and spice extractives (H-64)	9.88 ± 1.07	-	-	-	35.45 ± 1.80	-	1.60 ± 0.05
Protein Supplements							
Gluten bran	6.07 ± 1.01	0.19 ± 0.01	0.91 ± 0.81	2.55 ± 0.21	16.30 ± 0.48	-	trace
Gluten feed	8.04 ± 0.31	0.35 ± 0.01	-	0.87 ± 0.01	0.43 ± 0.01	-	trace
Potato protein	7.63 ± 0.74	-	0.56 ± 0.01	0.80 ± 0.11	10.32 ± 0.11	-	95.24 ± 0.78
Alfalfa meal	5.63 ± 1.21	0.18 ± 0.02	-	0.65 ± 0.12	7.63 ± 0.54	-	273.96 ± 1.97

[a]Determined by the methods described by Zarkadas et al. (1986b; 1987a).

[b]Means and standard error of measurements for four determinations; nd=not determined.

ranging from 0.43 to 43.52 nmol/mg of protein (Table 17). All the ingredients examined contained appreciable amounts of Pro(4-OH), ranging from 0.86 to 273.96 nmol/mg of total protein. This finding substantiates the reports of others that Pro(4-OH) is a constituent of cell wall proteins of the plants [Adams and Frank, 1980; McNeil et al., 1984].

4.4 CONCLUSION

From the foregoing results, it is evident that this proposed direct approach for evaluating the protein quality of composite meats is based on the determination of their myofibrillar and connective tissue protein contents, since the contribution of these classes of proteins to the overall nutritive value of meats differs considerably. In this chemical approach the myofibrillar myosin and actin contents of muscles and prepared composite meats marketed today can be determined from the amounts of His(τ-Me) found in their acid hydrolysates, and collagen and collagen-like proteins can be calculated from the amounts of Lys(5-OH) present. The presence of Pro(4-OH) in a number of ingredients studied excludes its use as valid index of collagen content in meat products. When the sum of the intracellular and extracellular matrix skeletal muscle proteins is subtracted from the total protein of a composite meat hydrolysate sample, the difference is an accurate assessment of the non-muscle proteins present (Table 19). This direct approach has the advantage over other methods that complete separation of these unique basic amino acids is possible in a single analysis in less than 5.7h, and that the determination of all these classes of proteins can be carried out in both fresh muscles or processed meats, as well as animal protein supplements [Zarkadas et al., 1986b; Nguyen et al., 1986). In addition, this method may prove especially valuable for industrial control and formulation and could be easily applied for enforcing meat regulations.

SECTION V

THE RELATIONSHIP BETWEEN N^{τ}-METHYLHISTIDINE, 5-HYDROXYLYSINE, DESMOSINE, 4-HYDROXYPROLINE LEVELS AND SOY PROTEIN CONCENTRATE AMOUNTS PRESENT IN BOVINE DIAPHRAGM-SOY PROTEIN CONCENTRATE MODEL MIXTURES.

5.1 INTRODUCTION

The nutritional evaluation of processed meats and poultry products requires an accurate determination of the protein content present in such products. Since this protein originates from cheaper meat cuts, which are frequently high in connective tissue [Porteus, 1981] and from a number of other non-muscle animal and plant protein additives including milk and egg powders, soy and other types of oil seed products, gelatin, etc., [Ellis, 1987] a reproducible and accurate methodology for the quantitative analysis of vegetable or meat proteins in composite meats is essential.

Most of the microscopic, electrophoretic and immunological methods which have been described and recently reviewed by Olsman and Slump (1981), Olsman et al. (1985), Hitchcock and Crimes (1985) and Berkowitz and Webert (1987) for the determination of specific muscle and/or non-muscle proteins in meat products containing different kind of proteins, are limited mainly because of the extensive denaturation, structural changes and interactions which occur in these complex protein mixtures during thermal processing. Similar limitations were encountered in the determination of specific tryptic peptides derived from actin [Anderson, 1981; Vandekerckhove et al., 1986] and soy bean proteins [Llewellyn et al., 1978; Agater et al., 1986]

for assessing the levels of muscle and non-muscle proteins in composite meats, since complete solubilization and enzymatic hydrolysis of processed meat proteins has not been achieved [reviewed by Ellis, 1987 and Berkowitz and Webert, 1987].

Quantitation of protein-bound His(τ-Me), a unique basic amino acid which occurs exclusively in myosin and actin [Johnson and Perry, 1970; Asatoor and Armstrong, 1967] has been proposed as a marker for determining these two myofibrillar proteins in tissues and meat products [Perry, 1970; Hibbert and Lawrie, 1972; Olsman and Slump, 1981; Zarkadas, 1981; Rangeley and Lawrie, 1976; 1977; McNeal, 1986; Benedict, 1987]. Hibbert and Lawrie (1972) have indicated that a linear relationship exists between the myofibrillar contents and the amounts of His(τ-Me) present in various meat blends examined, but their results were not statistically analyzed. These findings led to the development of several ion-exchange and HPLC methods for the separation of His(τ-Me) [Rangeley and Lawrie, 1976; Jones et al., 1982; White and Lawrie, 1985a] and numerous studies have reported the distribution and occurence of His(τ-Me) in muscle tissues and a variety of processed meats [Rangeley and Lawrie, 1977; Jones et al., 1982; 1985; 1987; White and Lawrie, 1985b; Johnson et al., 1986]. These studies however have yielded variable amounts of His(τ-Me) among the muscle tissues and processed meats investigated [Rangeley and Lawrie, 1977; Jones et al., 1985; 1987;], mostly due to incomplete separation of His(τ-Me) from other ninhydrin positive compounds by the multicolumn systems employed [Hancock and Harding, 1984].

Until now Pro(4-OH), which was thought to be confined exclusively to the connective tissue fibrous proteins, collagen and elastin [Pearson, 1975]

has been used as the basis for determining the connective tissue content of meats [reviewed by Jackson and Cleary, 1967]. However, the recent findings that this amino acid is also present in large amounts in the Pro(4-OH)-rich glycoproteins, i.e., extensins. etc., of the primary cell walls of all dicotyledonous plants [McNeil et al., 1984], suggests that Pro(4-OH) should no longer be used as an index for determining the connective tissue content of composite meats containing plant protein additives.

To validate the possible use of His(τ-Me) and Lys(5-OH) as markers for determining the myofibrillar and connective tissue proteins of composite meats, it became apparent that an accurate determination of these unique amino acids in meat mixtures was essential.

The present study was undertaken to investigate whether regression equations could be developed that would effectively predict the myofibrillar and connective tissue protein contents of composite meat model mixtures from an amino acid analysis. Model meat mixtures were prepared with the appropriate incremental addition of soy protein concentrate to bovine Diaphragm, a typical skeletal muscle used in processed meats. Analyses were carried out by the sensitive chromatographic procedures described by Zarkadas et al. (1986b; 1987a) and the His(τ-Me), Lys(5-OH), Des and Pro(4-OH) contents of these mixtures were accurately determined. The total protein content of these mixtures was determined based on their detailed amino acid composition [Horstmann, 1979].

5.2 MATERIALS AND METHODS

Materials and Resins

The resins, amino acid calibration standards, buffers and reagents used in the present study are essentially the same as those described in Section III of the present study.

Sampling of Muscle Tissues

The bovine Diaphragm muscles used in the present study were obtained from Abattoir Soulanges, Les Cedres, Quebec. The thoracic and abdominal regions of the Diaphragm muscle were excised [Ashdown and Done, 1984] from three mature animals (Canada, Grade C1, Holstein-Friesian cows) two days postmortem. To avoid variation in the relative frequency of fiber types [Sieck et al., 1983] only the left mid-costal region was sampled [Ashdown and Done, 1984]. The muscle specimens were placed on ice and transported to the laboratory. They were cleaned of visible fat and connective tissue, and approximately 500 g of each muscle were cut in small cubes and ground frozen (-173°C) to a fine powder in a pre-cooled (electrically driven) end runner coffee mill (Moulinex Canada Ltd., Western Ontario). The frozen powder was then lyophilized and pulverized as before to pass through a No. 40 mesh sieve [opening = 0.425 μm; Newark Wire Cloth Co., Newark, NJ]. The soy protein concentrate (SC; Gl 301) commercial sample was kindly provided by Griffith's Laboratories Ltd., Scarborough, Ontario.

Extraction Procedure for the Histidine Dipeptides and Free Amino Acids

The lyophilized Diaphragm and SC samples (20 g) were extracted with a mixture of chloroform, methanol and distilled water essentially as described by Bligh and Dyer (1959). The only modification introduced was the use of a VirTis model 45 homogenizer (VirTis, Gardiner, New York) instead of the recommended Waring blender. Since the moisture of the lyophilized samples was low it was adjusted to a final level of 80 ± 1% by the addition of distilled H_2O. The volumes of chloroform, methanol, and H_2O before and after dilution, were kept in the specified proportions 1:2:0.8 and 2:2:1.8, respectively. The delipidated proteins in the methanol layer were recovered by filtration. The extraction of the insoluble protein fraction was repeated twice and the final residue was homogenized further with 150 mL chloroform. The protein residue was dried overnight under vacuum (over KOH pellets) in a desiccator, ground in coffee mill (Moulinex Canada, Ltd., Weston ON) to pass through a No. 40 mesh screen (Newark Wire Cloth Co., Newark, NJ) and stored at -20 °C until needed (see also Materials and Methods of Section III of the present study).

Preparation of Diaphragm - Soy Protein Concentrate Mixtures

Meat mixtures (10.0 g) were then prepared (in triplicate) with the appropriate incremental additions (w/w; dry weight basis) of SC to each of the three bovine Diaphragm samples as shown in Table 18. The samples were homogenized in an electric driven coffee mill as before and their protein

Table 18. Amounts of Soy Protein Concentrate Added in Extracted Lyophilized Diaphragm Muscle

Sample description, % soy concentrate added to the mixture	g ingredients in mixture (dry weight basis)	
	Soy Concentrate	Diaphragm
0%	0	10.0
0.5%	0.05	9.95
1.0%	0.10	9.90
1.5%	0.15	9.85
3.0%	0.30	9.70
6.0%	0.60	9.40
9.0%	1.00	9.00
12.0%	1.20	8.80
15.0%	1.50	8.50
18.0%	1.80	8.20
21.0%	2.10	7.90
100.0%	10.00	0

content and amino acid composition were determined by standard methods [Horstmann, 1979; Nguyen et al., 1986; Zarkadas et al., 1986b; 1987a].

Preparation of Hydrolysates & Amino Acid Analyses

Duplicate samples (0.1 g) from the extracted SC, the Diaphragm muscles (three animals) and their mixtures were hydrolyzed for 24 and 96 h at 110°C under vacuum (5 - 20 μm of Hg) in 10 mL of triple-glass distilled constant boiling HCl (5.7 M) as described previously [Nguyen et al., 1986]. Duplicate samples (0.1 g) of the Diaphragm (Animal 1) - SC mixtures were hydrolyzed for 48 and 72 h. Methionine and cyst(e)ine of these (Animal 1 - SC) mixtures were determined separately after performic acid oxidation [Moore, 1963] and analyzed according to the procedures described by Zarkadas et al. (1987a).Tryptophan in mixtures (0.05 g) was determined separately after alkaline hydrolysis for a period of 24 h as described by Hughli and Moore (1972). Tryptophan analyses were performed on a Beckman 120C amino acid analyzer as described by Zarkadas et al. (1986b). 4-Hydroxyproline analysis was carried out in 24 h hydrolysates on a conventional Beckman Model 120C amino acid analyzer [Zarkadas et al., 1986b]. The basic methylated, the diastereoisomers of Lys(5-OH) and other compounds present in 96 h concentrated hydrolysates were analyzed on an updated fully automated amino acid analyzer according to the methods described by Zarkadas et al. (1987a). Recoveries were calculated relative to the protein content of each 96 h hydrolysate, determined from their detailed amino acid composition as reported by Horstmann (1979) and Nguyen et al. (1986) (see also Materials and Methods, Section III).

Statistical Analysis

Data processing and linear regression analysis of the amino acid data were carried out by a Fortran computer program developed for this purpose. Statistical analysis were carried out using GLM (general linear model) procedure of Statistiacl Analysis System (SAS) (1982) using the computing centre (VAX) at Datacrown, Inc., IBM, Toronto, Ontario. A complete randomized design was set up using three animals as replicates.

5.3 RESULTS AND DICSUSSION

In the previous section of the present study (section III) it has been shown that the determination of His(τ-Me), Lys(5-OH) and Des can be used as quantitative indices for calculating the myofibrillar and connective tissue protein contents of a typical skeletal muscle, the bovine Diaphragm. To further validate the possible use of these unique amino acids as markers for determining the amounts of muscle proteins present in composite meat products, regression equations were developed between the levels of His(τ-Me), Lys(5-OH), Des and the amounts of SC present in solvent treated bovine Diaphragm - SC model mixtures (0 - 21% added SC).

The results of the amino acid composition of bovine Diaphragm, SC and ten represenative meat mixtures prepared with the appropriate addition (w/w) of SC to Diaphragm, often used for meat processing, are presented in Tables 19 to 21. A comparison of different methods of expressing results [Eastoe, 1967] indicated that, within any given meat mixture the least variability occured when the data is expressed on a moisture-, fat- and ash-free basis. Results have therefore been calculated as g of amino acid residues (anhydro amino acid) per kg of total protein. Values for all determinations show a reproducibility of 100 ± 3% for all amino acids and better than 100 ± 2% for amino acids present in amounts greater than 3% in a given sample. The accuracy and reproducibility (precision) of the analytical method used in this study for the determination of the standard amino acids [Zarkadas et al., 1987a] was also tested by forty eight non-consecutive analyses of a standard calibration amino acid mixture. Precision was estimated as the

Table 19. Amino Acid Composition of Soy Protein Concentrate and Lyophilized Bovine Diaphragm, Extracted With a Methanol:Chloroform (2:1) Mixture.

Amino Acid	Soy Concentrate Mean ± SEM[a]	CV[a]	Diaphragm Mean ± SEM[a]	CV[a]
Aspartic acid	113.14 ± 0.63	0.63	95.26 ± 0.01	0.01
Threonine[b]	39.67 ± 0.15	0.15	45.21 ± 1.18	3.70
Serine[b]	52.13 ± 1.84	1.88	39.45 ± 0.84	3.00
Glutamic acid	186.43 ± 0.37	0.38	158.31 ± 1.38	1.23
Proline	50.48 ± 2.70	2.76	39.94 ± 0.38	1.35
Glycine	36.49 ± 0.06	0.23	37.62 ± 0.93	3.48
Alanine	41.17 ± 0.20	0.67	54.39 ± 0.89	2.31
Cysteine	11.86 ± 0.01	0.08	8.52 ± 0.03	0.34
Valine	52.74 ± 0.42	1.13	53.38 ± 0.05	0.12
Methionine	12.15 ± 0.24	2.41	29.11 ± 0.44	1.84
Isoleucine	50.68 ± 1.16	1.16	50.97 ± 0.33	0.93
Leucine	79.75 ± 0.55	0.98	87.12 ± 0.80	1.30
Tyrosine[b]	41.72 ± 0.50	1.71	40.95 ± 0.38	1.31
Phenylalanine	53.82 ± 0.28	0.74	45.74 ± 0.06	0.19
Histidine	26.45 ± 0.10	0.52	30.34 ± 0.61	2.84
Lysine	64.34 ± 0.14	0.30	93.05 ± 0.46	0.70
Arginine	72.60 ± 0.11	0.21	66.22 ± 0.74	0.61
Tryptophan	14.04 ± 0.09	1.00	14.62 ± 0.80	7.71
Ammonia[b]	16.84 ± 2.71	22.76	12.95 ± 0.43	4.61

Table 19 continued.....

Table 19 (continued)

Amino Acid	Soy Concentrate Mean ± SEM[a]	CV[a]	Diaphragm Mean ± SEM[a]	CV[a]
N^6-methyllysine	-		0.0867 ± 0.015	30.30
N^6-trimethyllysine	-		0.3377 ± 0.050	27.43
Ornithine	0.5419 ± 0.009	2.75	0.2562 ± 0.03	20.59
Unknown No. 17[f]	14.36 ± 0.8	7.88	0.52 ± 0.035	6.79
Total AA-N[c]	171.3433		171.5311	
Total EAA[d] (mg/g N)	2871.94		3118.13	
EAA Index[d]	75.44		83.80	
Protein Score[d]	44.85		65.38	
Protein Content[e] (g/kg DM)	573.20 ± 7.67		884.38 ± 4.78	
WE[e] (µg/nmol)	0.112231		0.111784	
F[e] (µg/nmol)	0.114578		0.113834	
F'[e] (µg/nmol)	0.121978		0.120446	

[a]Mean values and standard error of measurements (SEM) of 8 determinations; CV, coefficient of variation.

[b]Mean values and standard error of estimates.

[c]Calculated according to Heidelbaugh et al. (1975).

[d]Calculated according to Block and Mitchell (1946) and Oser (1951). Protein score reported is based on Cys, Met.

[e]The protein content, and WE, F, F' constants were calculated from the amino acid composition of each hydrolysate according to Horstmann (1979), and by using eqns 1, 2, and 3 respectively.

[f]Expressed in nmol/mg of protein.

standard deviation (SD) of the forty eight determinations, expressed as the percentage error by the following expression:

$$\% \text{ Error} = \frac{SD}{\bar{x}}$$

where $\bar{x}$ is the mean of forty eight determinations. The data obtained showed that the reproducibility for all amino acids ranged from 0.1 - 2.1% (n = 48) except for proline which showed reproducibility of 3.9% (n = 48). The accuracy of this method was also tested for all amino acids by means of the one-sample t test as follows:

$$t = \frac{(\bar{x} - \zeta)}{SD}$$

where ζ is the known concentration for each amino acid in the standard calibration amino acid mixture (ζ = 1 nmol). The accuracy for this chromatographic method was calculated to range from 0.5 - 4.1% (n = 48) for all the amino acids except for that of proline which was calculated to be 5.4% (n = 48). The precision (reproducibility) and accuracy of the method [Zarkadas et al., 1987a] used for the determination of methylated basic and crosslink amino acids was similarly computed to be: 2.3% and 3.1% for His(τ-Me) (n = 28), 2.6% and 4.1% for Lys(5-OH) (n = 28), 1,9% and 2.2% for aLys(5-OH) (n = 28) and 4.3% and 3.9% for Des (n = 6), respectively.

The amino acid composition of Diaphragm muscle as presented in Table.19 is typical of a skeletal muscle tissue [Young and Pellet, 1984]. The amino acid profile of SC sample appeared to be high in acidic amino acids which when taken together account for almost 30% of all residues, while the total basic amino acids including lysine, arginine, histidine account for about 16% of the total amino acid residues (Table 19). The total content of

hydroxylated amino acids account for almost 14% compared to 22% for the total hydrophobic amino acids. Agreement between the mean values obtained in the present study with those values reported by Wolf (1982) is good both in the amino acid composition as a whole and in many of the individual values. The SC sample contained higher aspartic, serine, glutamic, proline, cyst(e)ine and phenylalanine levels and lower threonine, alanine, methionine, leucine and lysine levels as compared to those found in the Diaphragm muscle (Table 19). Soy concentrate sample contained 1.10 g Pro(4-OH) per kg of protein, compared to Diaphragm which contained 8.01 g Pro(4-OH)/kg of total protein (Table 21). These results are in accord with those reported by McNeil et al. (1984), Adams and Frank, (1980).

Bovine Diaphragm and SC samples contained significant amounts of all amino acids commonly found in proteins with the exception of cyst(e)ine and methionine and possibly valine, isoleucine, threonine, and tryptophan. A comparison between the EAA profiles of SC and bovine Diaphragm, used as ingredients (Table 19), with the total EAA (mg/g N) of references proteins showed that all of these products were much lower than hen's whole-egg (3215 mg/g N) protein or cow's milk (3200 mg/g N) (FAO/WHO, 1965). Similar differences were noted from EAA indices and protein scores calculated from the amino acid composition of these mixtures (Table 20) by the methods of Oser (1951) and Block and Mitchell (1946). These results, however, are in close agreement with those reported by Noda et al. (1977) when soy is replacing meat in composite mixtures. It should be noted that the above presented data is based on the extracted with methanol:chloroform samples and do not absolutely resemble the nutritional value of the raw samples. Happich et

al. (1984) however have reported that the nutritional quality of beef as measured by the protein efficiency ratio (PER) is not affected by the extraction solvent system employed to remove the fat present in the beef tissue.

The nitrogen and protein contents of SC, Diaphragm and each of the composite meat blends were calculated as described by Heidelbaugh et al. (1975) and Horstmann (1979) and the results obtained are summarized in Tables 19 and 20. These authors have recommended that whenever accurate data on the protein content of individual foods is required, the actual amino acid nitrogen content should be used. The calculated nitrogen conversion factors (Kjeldahl) for SC and bovine Diaphragm samples were found to be 5.84 and 5.83 respectively. About 35% of the total nitrogen present in the SC sample was contributed by glutamic, arginine and ammonia, compared to 40% found for the Diaphragm. The protein content of the extracted SC sample was found to be 573.20 g/kg of dry matter, which is well below the reported 70% protein content of such products based on the Kjeldahl nitrogen determination [Rakosky, 1974; Mattil, 1974]. This difference is due to the fact that Kjeldahl nitrogen does not distinguish between the protein-bound amino acid nitrogen and the non-protein nitrogen in the form of free amino acids, dipeptides, purines, pyrimidines, etc., [Benedict, 1987]. The weight equivalent (WE in µg/nmol) and conversion factors (F and F' in µg/nmol) for each of the SC - Diaphragm mixtures have been determined (Table 20), and can be used in all subsequent quantitations of these mixtures following standard procedures as described by Horstmann (1979) and Nguyen et al. (1986).

Table 20. Amino Acid Composition of Soy Protein Concentrate-Bovine Diaphragm Mixtures Extracted With a Methanol:Chloroform (2:1) Mixture.

	Soy Protein Concentrate Added[a]									
	0.5%		1.0%		1.5%		3.0%		6.0%	
	(g of amino acids / kg of protein)									
Amino Acids	Mean ± SEM[b]	CV[b]	Mean ± SEM[b]	CV[b]	Mean ± SEM[b]	CV[b]	Mean ± SEM[b]	CV[b]	Mean ± SEM[b]	CV[b]
Aspartic acid	95.43 ± 0.30	0.037	96.43 ± 0.34	0.05	94.64 ± 0.34	0.52	95.98 ± 0.09	0.15	97.13 ± 0.04	0.06
Threonine[c]	46.21 ± 0.49	1.51	45.95 ± 0.25	0.78	45.92 ± 0.19	0.59	45.30 ± 0.90	2.78	46.18 ± 0.66	2.05
Serine[c]	40.06 ± 1.56	5.50	41.90 ± 0.04	0.14	41.33 ± 0.16	0.55	39.37 ± 1.90	6.85	40.53 ± 1.01	3.53
Glutamic acid	160.58 ± 0.26	0.22	162.16 ± 0.46	0.40	161.13 ± 1.14	1.01	162.86 ± 0.17	0.15	164.19 ± 0.27	0.23
Proline	41.84 ± 0.66	2.23	40.61 ± 1.44	5.03	45.85 ± 1.40	4.31	41.28 ± 0.03	0.10	41.49 ± 0.64	2.21
Glycine	37.68 ± 0.03	0.12	36.60 ± 0.11	0.40	36.92 ± 0.15	0.57	37.37 ± 0.15	0.58	37.55 ± 0.03	0.12
Alanine	54.38 ± 0.18	0.46	53.56 ± 1.44	1.44	52.68 ± 0.23	0.61	53.17 ± 0.15	0.38	53.47 ± 0.30	0.87
Cysteine	9.06 ± 0.01	0.03	8.77 ± 0.19	2.96	10.05 ± 0.70	9.86	10.03 ± 0.03	0.23	10.63 ± 0.06	0.74
Valine	53.10 ± 0.37	0.98	52.56 ± 0.09	0.23	51.23 ± 0.36	1.02	52.23 ± 0.05	0.13	52.38 ± 0.33	0.39
Methionine	28.97 ± 0.16	0.80	28.07 ± 0.03	0.16	27.85 ± 0.21	1.05	27.75 ± 0.05	0.27	27.46 ± 0.15	0.39
Isoleucine	51.10 ± 0.22	0.60	50.61 ± 0.28	1.32	49.68 ± 0.23	0.65	50.21 ± 0.31	0.87	49.89 ± 0.15	0.41
Leucine	87.76 ± 0.42	0.70	88.70 ± 0.54	0.86	88.28 ± 0.08	0.13	87.72 ± 0.09	0.14	86.95 ± 0.19	0.31
Tyrosine[c]	40.85 ± 0.66	2.28	40.49 ± 0.58	2.00	40.77 ± 0.86	2.98	42.24 ± 1.58	5.27	38.16 ± 0.92	3.43
Phenylalanine	44.68 ± 0.06	0.19	46.71 ± 0.05	0.14	44.04 ± 0.96	3.06	45.36 ± 0.19	0.60	44.45 ± 0.11	0.35
Histidine	29.62 ± 0.01	0.02	29.20 ± 0.21	1.05	29.22 ± 0.04	0.21	29.34 ± 0.17	0.80	29.02 ± 0.03	0.15
Lysine	93.52 ± 0.21	0.31	94.25 ± 0.64	0.97	94.03 ± 0.04	0.05	93.17 ± 0.01	0.01	92.90 ± 0.10	0.15
Arginine	67.78 ± 0.05	0.10	67.70 ± 0.71	1.48	67.66 ± 0.35	0.71	67.36 ± 0.42	0.86	67.39 ± 0.04	0.08
Tryptophan	10.04 ± 0.11	1.51	10.00 ± 0.17	2.29	12.15 ± 0.03	0.30	13.33 ± 1.01	10.54	13.06 ± 0.52	5.60
Ammonia[c]	10.24 ± 0.97	0.97	12.96 ± 0.69	7.53	12.53 ± 0.52	5.92	22.46 ± 2.54	16.01	17.13 ± 1.46	12.05
N^{ϵ}-methyllysine	0.0694 ± 0.01	15.60	0.0718 ± 0.01	2.19	0.0668 ± 0.01	7.36	0.0572 ± 0.01	7.87	0.0502 ± 0.01	13.45

Table 20 continued...

Table 20 (continued)

Amino Acids	Soy Protein Concentrate Added[a] 0.5% Mean ± SEM[b]	CV[b]	1.0% Mean ± SEM[b]	CV[b]	1.5% Mean ± SEM[b]	CV[b]	3.0% Mean ± SEM[b]	CV[b]	6.0% Mean ± SEM[b]	C V [b]
	(g of amino acids / kg of protein)									
N$^{\epsilon}$-trimethyllysine	0.2071 ± 0.02	17.94	0.1941 ± 0.01	11.37	0.1822 ± .01	10.21	0.1713 ± 0.02	17.46	0.1665 ± 0.01	16.22
Ornithine	0.1970 ± 0.01	6.23	0.2540 ± 0.02	13.28	0.1930 ± 0.02	14.18	0.204 ± 0.04	36.84	0.231 ± 0.02	13.72
Unknown No. 17[f]	0.59 ± 0.07	20.53	0.713 ± 0.02	4.05	0.95 ± 0.16	28.80	1.11 ± 0.18	27.90	1.86 ± 0.12	10.90
Total AA-N[d]	169.55		171.50		171.30		179.22		175.02	
Total EAA[e] (mg/g N)	3144.01		3106.59		3103.57		2998.52		3025.08	
EAA Index[e]	81.80		80.58		81.77		79.09		80.68	
Protein Score[e]	66.31		64.26		66.25		65.66		66.85	
Protein Content[g] (g/kg DM)	882.45 ± 3.67		879.24 ± 8.80		880.23 ± 6.78		870.45 ± 9.88		868.34 ± 14.45	
WE[g] (µg/nmol)	0.111521		0.111700		0.111689		0.111888		0.111621	
F[g] (µg/nmol)	0.113313		0.113459		0.113756		0.114043		0.113822	
F'[g] (µg/nmol)	0.119847		0.119776		0.120877		0.120437		0.120378	

Table 20 continued ...

Table 20 (continued) Amino Acid Composition of Soy Protein Concentrate – Bovine Diaphragm Blends Extracted With a Methanol:Chloroform (2:1) Mixture

	Soy Protein Concentrate Added[a]									
	9.0%		12.0%		15.0%		18.0%		21.0%	
	(g of amino acids / kg of protein)									
Amino Acids	Mean ± SEM[b]	CV[b]	Mean ± SEM[b]	CV[b]	Mean ± SEM[b]	CV[b]	Mean ± SEM[b]	CV[b]	Mean ± SEM[b]	CV[b]
Aspartic acid	100.87 ± 0.42	0.59	100.61 ± 0.76	1.07	101.23 ± 0.21	0.29	102.18 ± 0.29	0.40	102.52 ± 0.04	0.05
Threonine[c]	44.55 ± 0.66	2.10	44.15 ± 0.28	0.91	44.98 ± 0.31	0.98	45.19 ± 0.27	0.83	44.71 ± 0.68	2.16
Serine[c]	36.83 ± 1.72	6.62	39.13 ± 1.01	3.64	41.01 ± 0.35	1.18	41.42 ± 0.04	0.14	40.55 ± 1.22	4.25
Glutamic acid	163.56 ± 3.54	3.07	168.14 ± 0.68	0.58	169.49 ± 0.56	0.46	171.08 ± 0.08	0.06	171.04 ± 0.45	0.37
Proline	39.97 ± 0.33	1.19	39.02 ± 0.95	3.46	40.22 ± 0.31	1.10	37.56 ± 0.22	0.82	38.61 ± 1.66	6.08
Glycine	39.10 ± 1.58	5.71	37.06 ± 0.09	0.37	37.01 ± 0.25	1.00	36.76 ± 0.03	0.10	36.95 ± 0.02	0.06
Alanine	55.53 ± 0.02	0.05	53.57 ± 0.01	0.01	52.54 ± 0.23	0.61	52.75 ± 0.11	0.29	51.84 ± 0.03	0.09
Cysteine	10.36 ± 0.63	8.64	9.95 ± 0.28	3.98	7.94 ± 0.72	12.77	9.72 ± 0.21	3.04	10.01 ± 1.22	17.24
Valine	53.34 ± 0.38	1.00	53.55 ± 0.26	0.69	53.28 ± 0.12	0.33	53.40 ± 0.12	0.32	53.40 ± 0.01	0.04
Methionine	26.68 ± 0.28	1.48	25.74 ± 0.02	0.09	25.16 ± 0.21	1.15	25.02 ± 0.04	0.32	24.01 ± 0.38	2.26
Isoleucine	50.79 ± 0.62	1.74	50.54 ± 0.07	0.18	51.05 ± 0.24	0.66	51.18 ± 0.08	0.22	51.06 ± 0.05	0.14
Leucine	86.45 ± 0.87	1.43	85.66 ± 0.14	0.23	86.32 ± 0.40	0.66	86.14 ± 0.08	0.13	86.23 ± 0.20	0.33
Tyrosine[c]	39.23 ± 1.11	4.00	39.88 ± 0.72	2.55	38.92 ± 0.83	3.03	38.43 ± 0.23	0.85	41.29 ± 1.0	3.26
Phenylalanine	46.24 ± 0.16	0.49	45.24 ± 0.06	0.19	45.57 ± 0.33	1.01	46.60 ± 1.26	3.85	46.45 ± 0.03	0.10
Histidine	30.26 ± 0.32	1.50	28.42 ± 0.07	0.36	28.87 ± 0.15	0.71	28.53 ± 0.06	0.27	28.32 ± 0.06	0.29
Lysine	89.96 ± 0.85	1.34	90.29 ± 0.23	0.36	89.61 ± 0.06	0.10	88.57 ± 0.05	0.08	87.66 ± 0.27	0.43
Arginine	67.51 ± 0.04	0.09	66.94 ± 0.04	0.84	67.32 ± 0.01	0.03	67.16 ± 0.05	0.11	68.24 ± 0.91	1.89
Tryptophan	12.59 ± 0.23	2.56	16.38 ± 1.30	11.21	13.47 ± 1.05	11.06	12.08 ± 0.01	0.14	10.38 ± 0.21	2.82
Ammonia[c]	19.10 ± 0.08	0.51	13.74 ± 0.85	8.77	15.81 ± 2.01	17.96	12.79 ± 1.14	12.55	16.36 ± 1.26	2.23

Table 20 continued....

Table 20 (continued)

Amino Acids	Soy Protein Concentrate Added[a] 9.0% Mean ± SEM[b]	CV[b]	12.0% Mean ± SEM[b]	CV[b]	15.0% Mean ± SEM[b]	CV[b]	18.0% Mean ± SEM[b]	CV[b]	21.0% Mean ± SEM[b]	CV[b]
	(g of amino acids / kg of protein)									
N^{ϵ}-methyllysine	0.0603 ± 0.01	22.23	0.0526 ± 0.01	7.92	0.0512 ± 0.01	20.32	0.0546 ± 0.01	16.09	0.0512 ± 0.01	12.88
N^{ϵ}-trimethyllysine	0.1644 ± 0.01	13.24	0.1553 ± 0.01	6.39	0.1563 ± 0.01	2.62	0.1588 ± 0.01	1.34	0.1514 ± 0.02	2.08
Ornithine	0.166 ± 0.02	20.27	0.186 ± 0.03	25.23	0.177 ± 0.02	17.23	0.176 ± 0.01	13.34	0.182 ± 0.03	27.74
Unknown No. 17[d]	2.34 ± 0.09	6.64	2.74 ± 0.06	3.91	3.77 ± 0.30	13.77	4.58 ± 0.23	8.70	5.10 ± 0.31	10.43
Total AA-N[e]	176.73		171.53		173.25		170.41		173.34	
Total EAA[f] (mg/g N)	2986.05		3080.03		3022.24		3072.04		3017.70	
EAA Index[f]	79.75		83.13		80.53		81.41		78.47	
Protein Score[f]	65.25		62.78		58.74		61.69		60.45	
Protein Content[g] (g/kg DM)	856.03 ± 12.34		846.70 ± 23.56		837.38 ± 6.67		828.06 ± 15.67		818.73 ± 2.78	
WE[g] (µg/nmol)	0.111515		0.111970		0.111844		0.111854		0.111892	
F[g] (µg/nmol)	0.113645		0.114333		0.113744		0.113881		0.113840	
F'[g] (µg/nmol)	0.119836		0.120409		0.119970		0.119836		0.119988	

[a]Dry weight to dry weight ratio.

[b]Mean values and standard error of measurements (SEM) of 8 determinations; CV, coefficient of variation.

[c]Mean values and standard error of estimates.

[d]Expressed in nmol/mg of protein.

[e]Calculated according to Heidelbaugh et al. (1975).

[f]According to Block and Mitchell (1946) and Oser (1951). Protein scores reported were based on Cys, Met levels.

[g]The protein content and WE, F factors were calculated from the amino acid composition of each hydrolysate according to Horstmann (1979). The factor F' was calculated according to eqn (3) for determining protein mass in the absence of Trp, Cys, Pro and Pro(4-OH).

Table 21. The N^{τ}-Methylhistidine, 5-Hydroxylysine, 4-Hydroxyproline, and Desmosine Contents of Solvent Treated (Methanol:Chloroform; 2:1) Soy Protein Concentrate - Bovine Diaphragm Model Meat Mixtures.

Mixture[a], %	His(τ-Me)[b]		Lys(5-OH)[b]		Pro(4-OH)[c]		Des[b]	
	Mean ± SEM[d]	CV[d]	Mean ± SEM[d]	CV[d]	Mean ± SEM[d]	CV[d]	Mean ± SEM[d]	CV[d]
0	0.4170±0.021	0.9	0.5484±0.003	0.81	8.01±0.87	18.90	0.1490±0.020	21.01
0.5	0.4120±0.002	0.62	0.5419±0.002	0.66	5.69±0.31	9.42	0.0214±0.002	12.25
1.0	0.4066±0.030	11.53	0.5348±0.004	1.38	5.19±0.52	17.38	0.0254±0.005	31.14
1.5	0.4046±0.003	1.39	0.5377±0.004	1.41	5.16±0.31	10.56	0.0222±0.003	22.59
3.0	0.4031±0.003	1.23	0.5330±0.001	0.21	5.47±0.04	1.48	0.0165±0.002	20.91
6.0	0.3907±0.001	0.64	0.5166±0.001	0.49	5.79±0.23	6.76	0.0232±0.001	4.96
9.0	0.3835±0.003	1.21	0.5070±0.002	0.57	4.86±0.18	6.54	0.0177±0.001	12.07
12.0	0.3641±0.004	2.09	0.4832±0.003	1.00	4.47±0.31	11.90	0.0179±0.001	13.86
15.0	0.3514±0.003	1.53	0.4661±0.005	1.97	4.77±0.38	13.64	0.0177±0.002	20.91
18.0	0.3406±0.006	2.93	0.4502±0.010	5.51	5.60±0.47	14.43	0.0191±0.002	21.23
21.0	0.3286±0.010	5.25	0.4344±0.008	3.19	5.94±0.03	0.92	0.0203±0.004	32.95
100.0	-		-		1.10±0.04	5.83	-	

[a]Weight/weight (dry basis ratio); soy protein concentrate added.

[b]Determined in 96 h concentrated hydrolysates, according to Zarkadas et al. (1987a).

[c]Determined in 24 h concentrated hydrolysates, according to Zarkadas et al. (1986b).

[d]Mean values and standard error of measurements (SEM) of 12 determinations; CV, coefficient of variation.

Analysis of 96 h hydrolysate of the solvent treated Diaphragm by the chromatographic procedure described by Zarkadas et al. (1987a) revealed the complete separation of Des, methylated lysines (Table 20), Des, aLys(5-OH), ornithine, lysine and His(τ-Me) (Table 21) from other ninhydrin positive compounds present in these hydrolysates (Figure 7). As shown in Figure 7A His(τ-Me) is completely separated from an unknown compound designated as Unknown No. 17. This separation was achieved with the introduction of a second citrate buffer (pH 4.501, 0.35 M) just before the elution of Lys(Me) (224.5 min) at high temperature 72°C [Zarkadas et al., 1987a]. Soy protein concentrate contained high levels of Unknown No. 17 (Figure 7B) compared to those found in the Diaphragm muscle (Table 19; Figure 7A). As may be seen in Figure 7A another unknown compound (No. 8), coeluted with Lys(5-OH). For this reason the levels of aLys(5-OH) were used to determine the total Lys(5-OH) content in both the Diaphragm muscle and in the meat model mixtures. For purposes of comparison, Figure 8 illustrates the separation of all these compounds present in acid hydrolysates (96 h) of SC - Diaphragm mixtures in which SC is present in amounts of 0.5%, 21%, and 60.5% respectively (w/w; dry weight basis).

The results presented in Table 21 show low coefficient of variation for His(τ-Me) and Lys(5-OH). These results demonstrate the accuracy and high reliability of the method even when very small amounts of SC have replaced meat protein in the model mixtures. Higher coefficient of variations, however were found for Des among the mixtures evaluated. This is probably due to the fact that quantification of this amino acid was performed close to the detection limit of the chromatographic method employed

Figure 7

Typical chromatographic separation of methylated basic and crosslink amino acids and related compounds present in solvent treated (methanol:chloroform) (A) bovine Diaphragm and (B) soy protein concentrate samples (96 h hydrolysate) on an analytical 50 x 0.28 cm microcolumn of Dionex DC-4A resin. The curves denote absorbance at 570 nm.

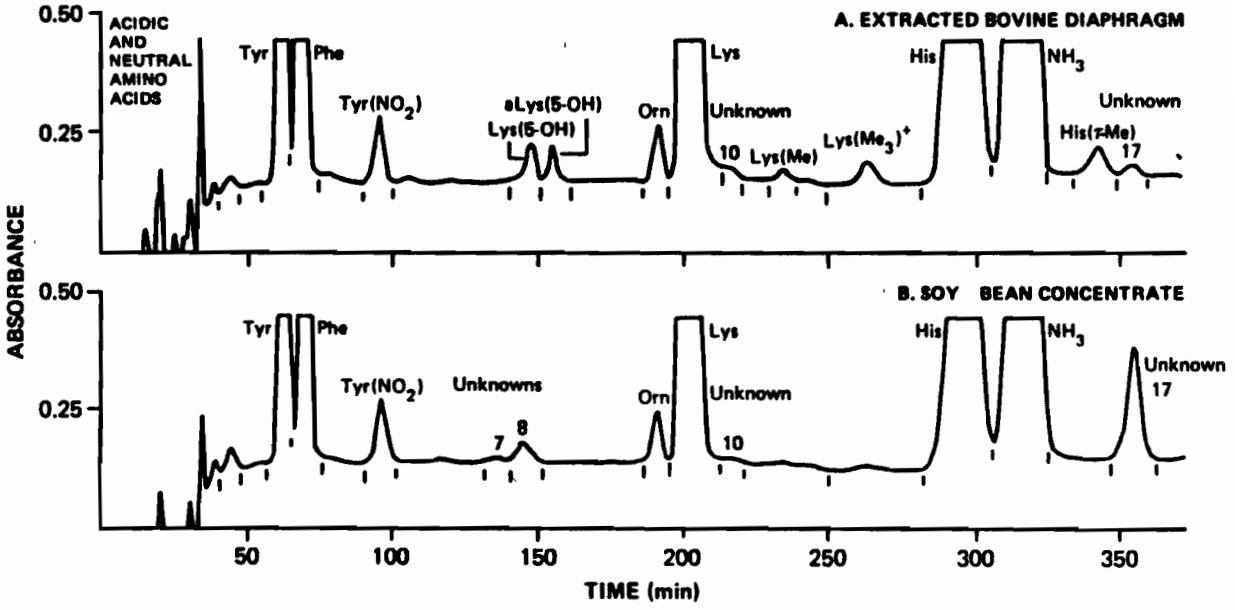
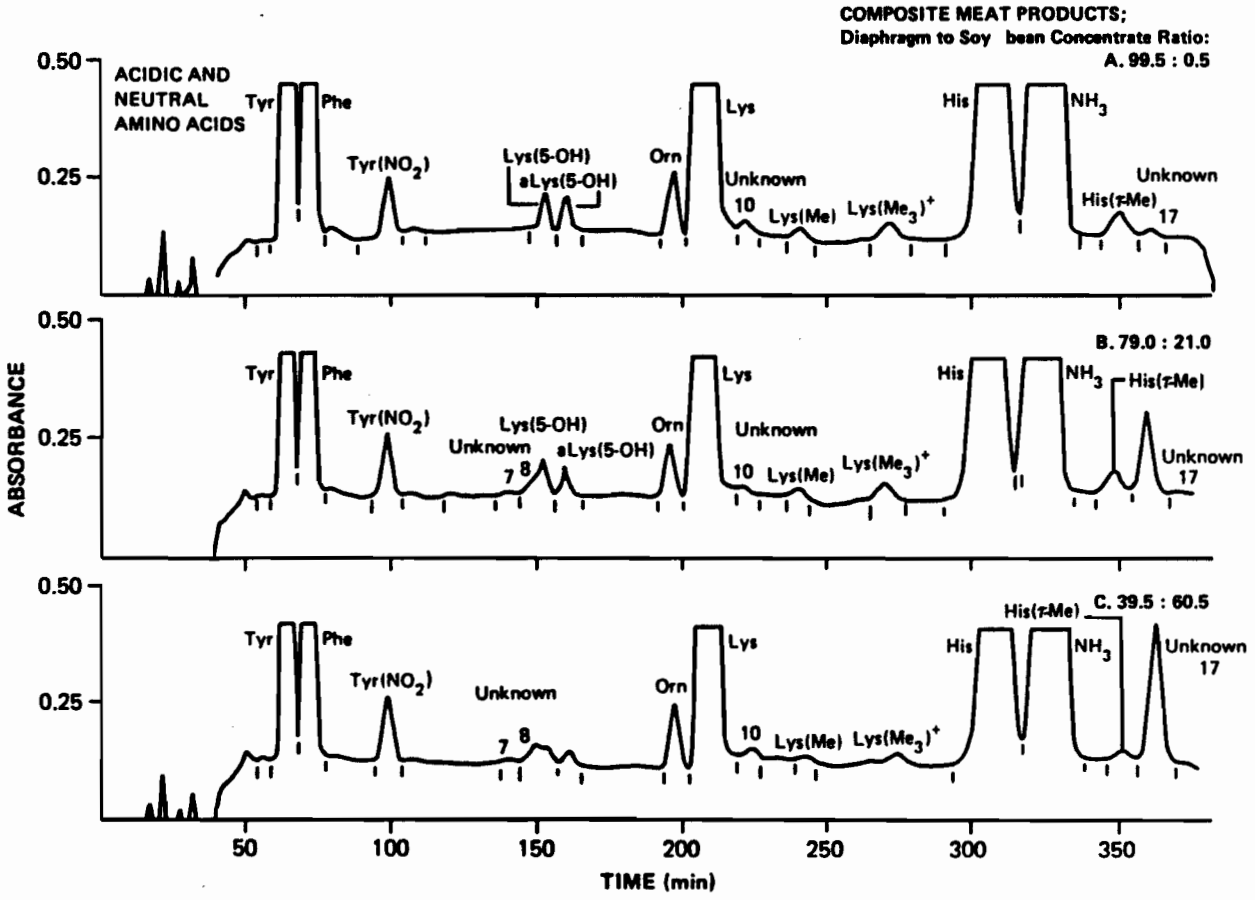

0.50
0.25
ABSORBANCE
ACIDIC AND NEUTRAL AMINO ACIDS
A. EXTRACTED BOVINE DIAPHRAGM
Tyr
Phe
Tyr(NO2)
aLys(5-OH)
Lys(5-OH)
Orn
Lys
Unknown
10
Lys(Me)
Lys(Me3)+
His
NH3
Unknown
His(τ-Me)
17
B. SOY BEAN CONCENTRATE
Tyr
Phe
Tyr(NO2)
Unknowns
7
8
Orn
Lys
Unknown
10
His
NH3
Unknown
17
50
100
150
200
250
300
350
TIME (min)

Figure 8

Separations of all methylated lysines and histidines, the diastereoisomers of 5-hydroxylysine, and related compounds present in solvent treated soy protein concentrate-bovine Diaphragm model mixtures (96 h hydrolysate). (A), (B), (C) Analysis of meat mixtures containing 0.5%, 21.0% and 60.5% (w/w; dry weight) of soy protein concentrate, respectively. The curves represent absorbance at 570 nm.


COMPOSITE MEAT PRODUCTS;
Diaphragm to Soy bean Concentrate Ratio:
A. 99.5 : 0.5
B. 79.0 : 21.0
C. 39.5 : 60.5
ACIDIC AND NEUTRAL AMINO ACIDS
Tyr
Phe
Tyr(NO2)
Lys(5-OH)
aLys(5-OH)
Orn
Lys
Unknown
10
Lys(Me)
Lys(Me3)+
His
NH3
His(τ-Me)
Unknown
17
7
8
0.50
0.25
ABSORBANCE
50
100
150
200
250
300
350
TIME (min)

(10 ≈ 20 pmol). The data reported in Tables 20 and 21 were evaluated by polynomial regression analysis as follows:

$$Y = \beta_0 \pm \beta_1 \cdot X \pm \beta_2 \cdot X^2 \pm \beta_3 \cdot X^3 \quad \text{where}$$

Y = is the content of a given amino acid found in the model mixtures X = is the predicted soy protein concentrate content of a given sample (expressed as percentage of dry weight), and β_1, β_2, β_3 are the coefficients of the linear, quadratic and cubic term of X of the above equation, respectively. These parameters were evaluated by minimizing the squared differences between experimental data points, and the results obtained are summarized in Table 22. It should be noted that a linear relationship exists between the percentage SC added to the model mixtures and the amounts of His(τ-Me), Lys(5-OH), Unknown No.17, Des, methionine, threonine, glycine, proline, leucine, phenylalanine, histidine, lysine, ornithine found in their acid hydrolysates. These results confirm the findings of Hibbert and Lawrie (1972) for His(τ-Me) while providing additional linear relationships [Lys(5-OH), Des] (Table 22) and correlations (Table 24) that exist in the examined meat mixtures. The data in Table 22 indicate that the relationship between the Pro(4-OH), Lys(Me), Lys(Me_3)$^+$ contents in model mixtures and the percentage SC added to these composite meat mixtures was found to be quadratic. In the case of cyst(e)ine and isoleucine however, this relationship is cubic (Table 22). The levels of significance, standard error of estimates and R^2 values of these relationships are also in Table 22. The amino acids aspartic, serine, glycine, alanine, valine, tyrosine, arginine, tryptophan and

Table 22. Polynomial Regressions for the Amino Acids Present in Solvent Treated Soy Protein Concentrate-Bovine Diaphragm Model Mixtures (0% - 21% Added Soy in Lyophilized Muscle)[a].

$$Y = \beta_0 \pm \beta_1 X \pm \beta_2 X^2 \pm \beta_3 X^3$$

Amino Acids[c]	β_0	β_1[b]	β_2[b]	β_3[b]	$R^2 100$[d]
Threonine	45.78 ± 0.25	-0.06 ± 0.02*	-	-	40.1
Glycine	160.40 ± 0.54	0.56 ± 0.05***	-	-	93.3
Proline	41.12 ± 0.76	-0.19 ± 0.07*	-	-	44.9
Cysteine	8.52 ± 0.37	0.8490 ± 0.2209**	-0.1000 ± 0.0268**	0.0030 ± 0.0008**	67.9
Methionine	28.65 ± 0.15	-0.22 ± 0.01***	-	-	96.3
Isoleucine	50.93 ± 0.25	+0.3903± 0.1481*	0.0478 ± 0.0180*	-0.0014 ± 0.0006*	62.5
Leucine	87.91 ± 0.27	-0.106 ± 0.025	-	-	65.7
Phenylalanine	44.96 ± 0.31	0.066 ± 0.029	-	-	37.2
Histidine	29.69 ± 0.23	-0.062 ± 0.022*	-	-	46.8
Lysine	93.98 ± 0.29	-0.303 ± 0.027***	-	-	93.5
4-Hydroxyproline	6.23 ± 0.30	-0.300 ± 0.089**	0.0140 ± 0.0044**	-	27.8
5-Hydroxylysine	0.547 ± 0.002	-0.005 ± 0.0002***	-	-	95.0
Desmosine	0.052 ± 0.02	-0.002 ± 0.001*	-	-	14.5
Ornithine	0.224 ± 0.011	-0.003 ± 0.001*	-	-	19.4
N^{ϵ}-methyllysine	0.075 ± 0.004	-0.003 ± 0.001**	0.0001 ± 0.00005*	-	42.8
N^{ϵ}-trimethyllysine	0.2343 ± 0.014	-0.013 ± 0.004**	0.0005 ± 0.0002*	-	38.8
N^{τ}-methylhistidine	0.4100 ± 0.004	-0.004 ± 0.0004**	-	-	77.0
Unknown17	0.679 ± 0.123	0.204 ± 0.011***	-	-	91.1

[a]Weight to weight (dry basis ratio); soy protein concentrate added.

[b]β_1, β_2, β_3 are the coefficients of the linear, quadratic and cubic term of X respectively and are reported with their corresponding standard error of estimates.

[c]Aspartic acid, serine, glycine, alanine, valine, tyrosine, arginine, tryptophan and ammonia were not found to have any significant correlation and are not presented in this table.

[d]$R^2 100$ is the coefficient of determination of multiple regression. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

ammonia (not presented in Table 22) did not show any significant relationship with the incremental addition of SC to the bovine Diaphragm.

The analysis of variance and the t-test of linear regression of His(τ-Me), Lys(5-OH), Des and Unknown No. 17 are summarized in Table 23. Figures 9 and 10 illustrate the linear relationship that exists between the myofibrillar and connective tissue protein contents and the amounts of His(τ-Me) and Lys(5-OH) present. The multiple correlation coefficient, R = 0.97, indicated that the model developed for His(τ-Me) was highly significant ($P < 0.001$; Table 22). Thus, the R^2 (coefficient of determination of multiple regression) statistic suggests that the model accounts for 77% of the total variation. The model developed for His(τ-Me) is reliable (R^2 = 0.77) for the practical prediction of soy protein concentration content in such model mixtures. Similarly, the models developed for Lys(5-OH) (R^2 = 95.0) and for Unknown No. 17 (R^2 = 91.1) are highly reliable. The model developed for Des, however, showed that it accounts for only 15% of the total variation (Table 22). The reason for the low R^2 of the Des model lies in the fact that the mean Des level of the Diaphragm muscle are very different from the Des levels found in all other mixtures (Table 21). The very small levels of Des found in the Diaphragm and the SC-Diaphragm mixtures could probably account for the high coefficient of variations and low R^2 values (Tables 21 and 22).

It may be concluded that the His(τ-Me), Lys(5-OH), lysine and methionine levels decrease with the addition of SC in the model meat mixtures, whereas glutamic and Unknown No. 17 increase respectively

TABLE 23. Analysis of Variance and t-Tests of Linear Regression Equations of N^{τ}-Methylhistidine, 5-Hydroxylysine, Desmosine and Unknown No.17.

His(τ-Me) Y = 0.410 + 0.004 . X

Source	SS	df	MS	F	Prob>F	R^2
Regression	0.0245033	1	0.0245033	103.69	0.0001	0.7699
Residual	0.0073254	31	0.0002363			

Parameter	Value	SD	t	Prob>t
Intercept	0.4096	0.0040	103.29	0.0001
Slope	0.0038	0.0004	10.18	0.0001

Lys(5-OH) Y = 0.547 + 0.005 . X

Source	SS	df	MS	F	Prob>F	R^2
Regression	0.0478895	1	0.0478895	586.44	0.0001	0.9498
Residual	0.0025315	31	0.0008166			

Parameter	Value	SD	t	Prob>t
Intercept	0.5465	0.0023	234.50	0.0001
Slope	-0.0053	0.0002	-24.22	0.0001

Des Y = 0.052 + 0.002 . X

Source	SS	df	MS	F	Prob>F	R^2
Regression	0.0080408	1	0.0080408	4.75	0.038	0.1449
Residual	0.0474439	28	0.0016944			

Parameter	Value	SD	t	Prob>t
Intercept	0.0517	0.0111	4.64	0.0001
Slope	-0.0022	0.0010	-2.18	0.0380

Un17 Y = 0.679 + 0.204 . X

Source	SS	df	MS	F	Prob>F	R^2
Regression	71.955049	1	71.955049	318.68	0.0001	0.9113
Residual	6.999573	31	0.225793			

Parameter	Value	SD	t	Prob>t
Intercept	0.6875	0.1226	5.54	0.0001
Slope	0.2042	0.0114	17.85	0.0001

Figure 9

The relationship between percentage soy protein concentrate (SC) and His(τ-Me) content present in solvent treated meat mixtures (Diaphragm:SC).

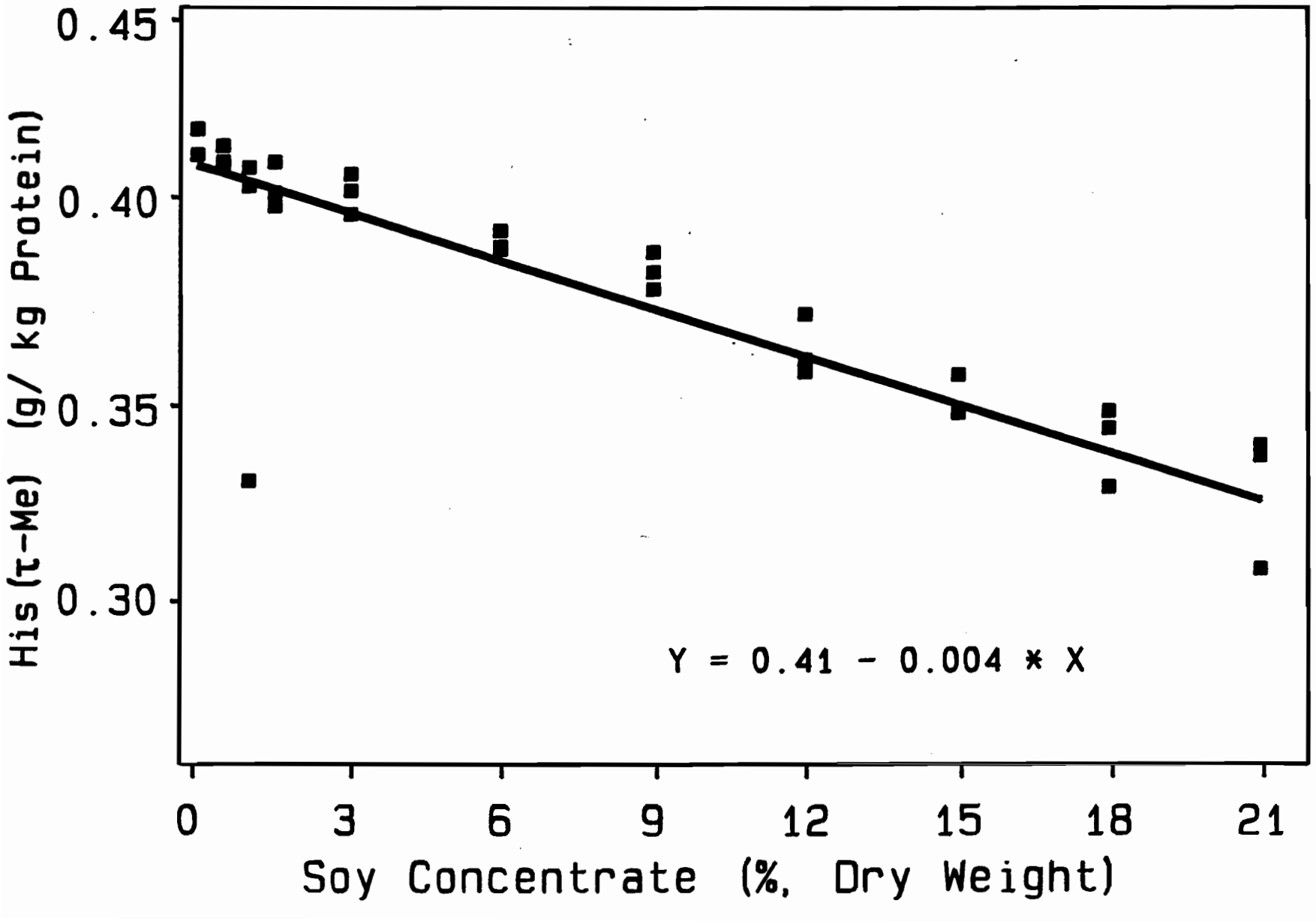
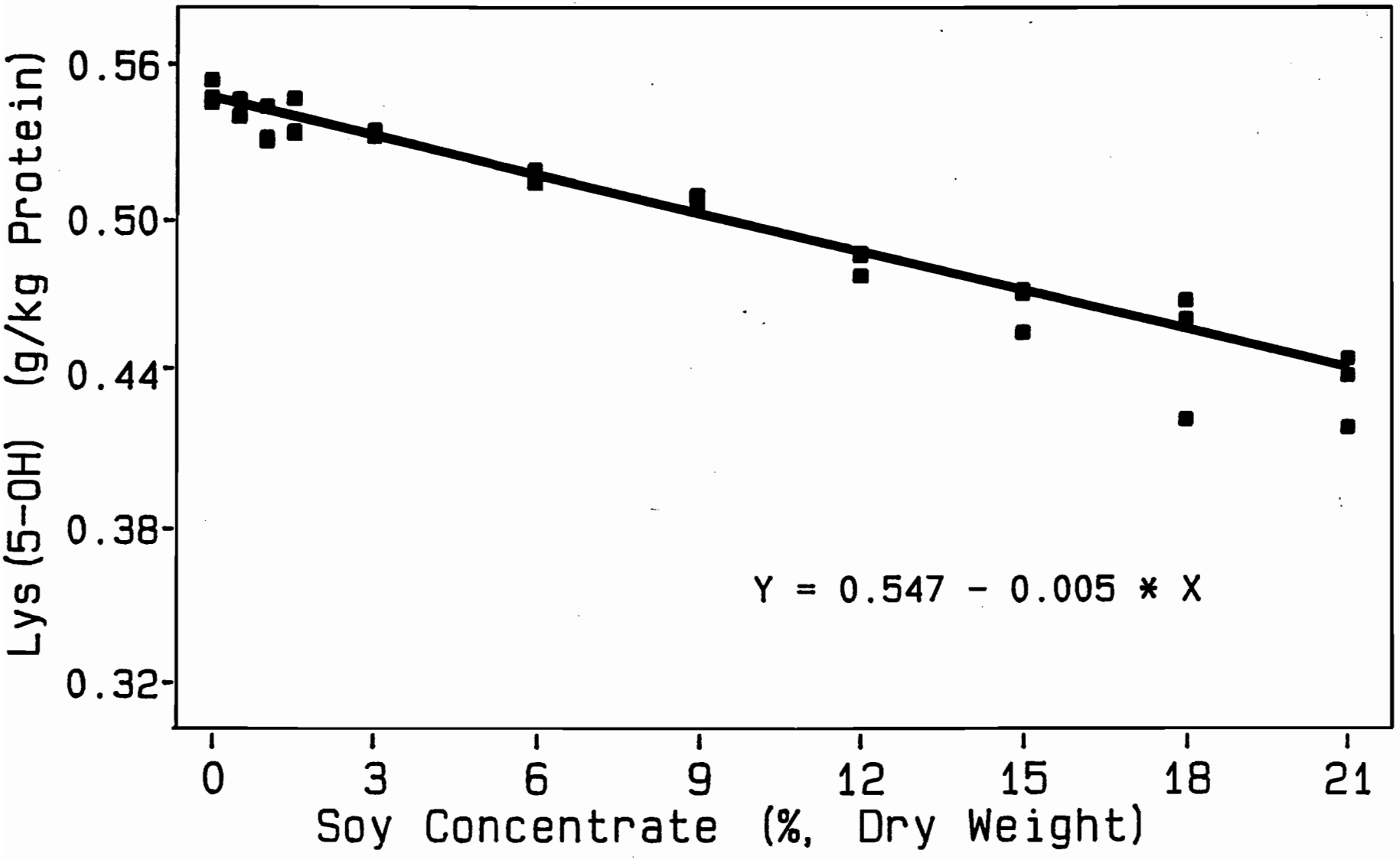

0.45
0.40
0.35
0.30
His(τ-Me) (g/ kg Protein)
Y = 0.41 - 0.004 * X
0
3
6
9
12
15
18
21
Soy Concentrate (%, Dry Weight)

Figure 10

The relationship between percentage soy protein concentrate (SC) and Lys(5-OH) content present in solvent treated meat mixtures (Diaphragm:SC).


Lys (5-OH) (g/kg Protein)
0.56
0.50
0.44
0.38
0.32
Y = 0.547 - 0.005 * X
0
3
6
9
12
15
18
21
Soy Concentrate (%, Dry Weight)

(Table 22). Moreover threonine, proline, leucine, histidine, Des and ornithine have a high negative correlation with the SC content of the mixtures.

A summary of the simple correlation coefficients obtained for each of the amino acids determined in all the SC-Diaphragm muscle model mixtures (0% - 21% added SC) analyzed is presented in Table 24. The His(τ-Me) content of each of these meat mixtures was positively correlated with the amounts of Lys(5-OH), methionine, lysine, leucine found in these mixtures. On the other hand, the His(τ-Me) content of these mixtures is negatively correlated with the amounts of glutamic and Unknown No. 17. The concentration of Lys(5-OH), increased as the concentration of methionine, leucine and lysine increased, and it decreased as the concentration of glutamic increased. The Des levels were positively correlated with Pro(4-OH) and negatively with arginine. There was a positive correlation between Pro(4-OH) and threonine or alanine or methionine. The Unknown No. 17 content of mixed meats was highly correlated with most of the amino acids except for proline, Des, Lys(Me) and Lys(Me_3)$^+$ (Table 24). It should also be noted that while there is positive correlation between Lys(5-OH) and Des, and between Des and Pro(4-OH), there was no significant correlation between Lys(5-OH) and Pro(4-OH). It was also found that the concentration of each amino acid was correlated with at least eight other amino acids except for aspartic, glycine, cyst(e)ine, valine, isoleucine, tyrosine (not presented in Table 24).

Table 24. Simple Correlation Coefficients Between Amino Acids Found in Model Blends[a] of Soy Protein Concentrate * Bovine Diaphragm Extracted with a Methanol : Chloroform Mixture (2:1)

	Thr	Ser	Glu	Pro	Ala	Met	Leu	Phe	His	Lys	Arg	Pro (4-OH)	Lys (5-OH)	Des	Orn	Lys (Me)	Lys (Me_3)+	His (τ-Me)
Ser	-.79**																	
Glu	-.88**	.81**																
Pro	-.62*	.79**	.46															
Ala	.89**	-.97**	-.90**	-.76**														
Met	.95**	-.88**	-.97**	-.64*	.96**													
Leu	.97**	-.80**	-.93**	-.57	.89**	.96**												
Phe	-.95**	.87**	.88**	.62*	-.92**	-.95**	-.93**											
His	.77**	-.84**	-.93**	-.52	.89**	.89**	.81**	-.75**										
Lys	.97**	-.88**	-.93**	-.66*	.95**	.99**	.98**	-.97**	.83**									
Arg	-.86**	.91**	.83**	.81**	-.94**	-.92**	-.84**	.90**	-.81**	-.93**								
Pro(4-OH)	.79**	-.76**	-.74**	-.69*	.78**	.79**	.72**	-.74**	.73**	.77**	-.80**							
Lys(5-OH)	.60*	-.14	-.98**	.66*	.63*	.98**	.76**	-.60	.73**	.94**	-.26	.16						
Des	.01	-.18	-.52	-.07	.37	.48	.08	.10	.62*	.26	-.73*	.80**	.39*					
Orn	-.79**	.91**	.63*	.84**	-.87**	-.78**	-.76	.86**	-.61*	-.83**	.86**	-.15	.39*	.59**				
Lys(Me)	.42	.24	-.60*	.11	.25	.66*	.46	-.08	.48	.52	-.46	.47	.60**	.55**	.43*			
Lys(Me_3)+	.49	-.06	-.87**	.33	.60	.89**	.58	-.28	.76**	.71*	-.41	.70**	.52**	.83**	.55**	.81**		
His(τ-Me)	.61	-.06	-.97**	.66*	.56	.98**	.80**	-.60	.67*	.96**	-.24	.18	.88**	.40*	.28	.52**	.52**	
Un17	-.93**	.85**	.97**	.53	-.93**	-.98**	-.96**	.94**	-.86**	-.98**	.87**	-.46**	-.91**	-.20	.49**	-.37*	-.32	-.81**

[a]Weight to weight (dry) ratio.

[b]Asp, Gly, Cys, Val, Ile, Tyr, Trp, NH_3 not presented in the table were not correlated with any of the other amino acids.

*, P<0.05; **, P<0.01; number of observations, n=33 for the correlations of the uncommon amino acids and n=11 for the correlations of the rest of the amino acids (from Ser through Arg).

5.4 CONCLUSION

From the foregoing results, it is evident that a linear relationship exists between the myofibrillar and connective tissue contents and the respective amounts of His(τ-Me) and Lys(5-OH) present in the meat blends analyzed, and that regression equations could be developed that would effectively predict these two classes of proteins in composite meats. The limit of detection was as low as 0.5% in SC content. Other linear relationships also exist between the SC content and the levels of methionine, lysine, glutamic, and Unknown No. 17 found in composite meats, and in conjunction with the levels of His(τ-Me) and Lys(5-OH) present, this direct approach could be easily applied on a routine basis for determining muscle and non-muscle proteins in composite meats. However, since a quadratic relationship was found to exist between the percentage of soy in these mixtures and the levels of Pro(4-OH) present, it is suggested that Pro(4-OH) should no longer be used as an index for determining the collagen content of composite meats.

For the equations developed to be feasible for routine analysis it would be necessary that the levels and variation of these unique amino acids, found to have a linear relationship with the SC or muscle contents of the meat mixtures examined, would be established and show a reasonable consistency between the major meat yielding species.

SECTION VI

SUMMARY

The levels of myosin, actin, collagen and elastin were quantitatively established in the costal region of the young bovine Diaphragm. This skeletal muscle was separated into an intracellular and an extracellular fraction after was subjected to an extraction procedure including $CaCl_2$ (0.05 M), phosphate buffer saline and sodium dodecyl sulphate (2%). The quantitation of myosin (22.5%) and actin (10.8%) as percentage of the total muscle proteins) was based on the direct chromatographic determination of His(τ-Me) levels found in the acid hydrolysates of the intracellular muscle protein fraction. The SDS-soluble proteins in the Diaphragm accounted for 43% of the total muscle proteins. The extracellular matrix fraction (SDS-insoluble) accounted for 4.73% and had an amino acid composition very similar to that of other connective tissues. The collagen content (2.61%) was determined from the amounts of Lys(5-OH) found and elastin (0.19%) from the levels of Des, iDes present in the SDS-insoluble fraction isolated from the bovine Diaphragm. Connective tissue proteins were found to account for 2.80% of the total muscle proteins, when calculated from the amounts of Pro(4-OH) present in the SDS-insoluble fraction. A small portion (1.92%) of the total muscle proteins was assumed to belong to the trancellular muscle matrix [Lowey, et al., 1983]. All of the above calculations were based on the detailed amino acid composition of the SDS-soluble and -insoluble fractions.

The protein quality of three typical commercial meat products (sausages, bologna, frankfurters) was evaluated based on the same direct quantitation of their myofibrillar and connective tissue protein contents. Treatment of the meat products with 0.1 M HCl in 75% ethyl alcohol was found to be effective in removing most of the free His(τ-Me) and balenine present in these products. The amounts of myosin and actin present were determined from the levels of the protein bound His(τ-Me) (solvent treated samples), and the collagen content from the amounts of Lys(5-OH) present in the acid hydrolysates of these products. The composite meat products contained 26.1-37.1% myofibrillar protein, 7.3-16.4% collagen, 5.7-14.6% added collagen and 27.5-49.3% added non-muscle proteins. The frankfurter sample contained the highest amount of myofibrillar proteins while the bologna sample the lowest. The protein content of these products was most accurately calculated from their detailed amino acid composition. Solvent treated sausages, frankfurters and bologna contained 47.1, 49.5 and 51.5g of protein/100g dry matter, respectively. The products examined had low chemical scores (range: 61 - 73% calculated based on the sulfuric amino acids), and EAA (70.6 - 72.8) indices when compared to reference proteins. The mean residue weights (WE) of sausages, bologna and frankfurters were 0.1073, 0.1094, and 0.1085 µg/nmol, respectively. These factors, after they are corrected for tryptophan, cysteine, proline, and Pro(4-OH), can be used for any subsequent protein determination in acid hydrolysates of the same products. The three meat products had variable fat (41.1 - 70.9%) and moisture (50.5 - 61%) contents. Their mineral content was similar with the exception of the high levels of Ca (on a fat free basis) found in bologna and frankfurters when

compared to those values found in mixed meat sausages. In addition mixed meat sausages had the highest Na content of the three products examined.

The unique amino acids His(τ-Me) and Lys(5-OH) were absent from fifteen non-meat protein additives commonly used in the manufacturing of meat products. These ingredients were analyzed by the same chromatographic techniques employed to quantitate these unique amino acids in acid hydrolysates of meat products. Furthermore, the additives examined did not exhibit any peaks which interfered with the accurate detrmination of the unique amino acids found in the muscle proteins. However, all the ingredients contained Pro(4-OH) levels which ranged from traces up to 2.74% (alfalfa meal protein supplement) and high levels of the unknown No. 17.

Model meat mixtures prepared with the incremental addition of soy protein concentrate to bovine Diaphragm (0-21% soy added, weight to weight ratio), were subjected to the chromatographic procedures described by Zarkadas et al. (1986b; 1987a). Linear regression relationships were found between His(τ-Me), Lys(5-OH) and Des contents and the soy protein concentrate present in these mixtures. A quadratic relationship was found to exist between Pro(4-OH) and soy protein concentrate present in the meat mixtures. The protein content of the solvent treated Diaphragm and soy concentrate as determined from their detailed amino acid composition was found to be 884.38 and 573.20 g/kg of dry weight, respectively. The mixture containing 21% soy protein concentrate contained 818.73 g protein/kg dry weight. It was found that the addition of soy concentrate up to 21% lowered the EAA index and the chemical scores of the model meat mixtures from 83.5

to 78.5 and from 65.5 to 60.5%, respectively. Highly significant correlations ($P<0.001$) were found to exist between His(τ-Me), Lys(5-OH), methionine and Unknown No.17.

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