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Methods for Mucin Analysis: A Comparative Study

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The aim was to compare five techniques commonly used to quantify mucin concentrations in ileal digesta collected from three growing pigs that had been fed a diet in which the sole protein was casein. Ileal mucin output was estimated by the periodic acid−Schiff, ethanol precipitation, and phenol−sulfuric acid methods as 25.1, 19.3, and 20.7 g kg^{-1} of dry matter intake (DMI), respectively. The mucin concentration estimated from sialic acid was only 5.9 g kg^{-1} of DMI. On the basis of the concentrations of the hexosamines *N*-acetylglucosamine and *N*-acetylgalactosamine, mucin output was estimated as 44.9 g kg^{-1} pf DMI. Of the five assays studied, the ethanol precipitation, periodic acid−Schiff, phenol−sulfuric acid, and sialic acid methods may considerably underestimate mucin in the digesta, which calls into question the accuracy of all of these approaches. In contrast, the gas chromatography method for the determination of hexosamines gave more information on the type and state of the mucin present.

KEYWORDS: Mucin; analysis; comparative study; hexosamines; *N*-acetylglucosamine; *N*-acetylgalactosamine; neutral sugar; polysaccharide; sialic acid; ethanol precipitation

INTRODUCTION

Given the nature of the alimentary canal, the gastrointestinal mucosal epithelia are exposed to the external environment. Bile salts, acids, digestive enzymes, and pathogenic bacteria all contribute to make the intestinal lumen a particularly noxious environment (*1*, *2*). To protect itself, the gastrointestinal mucosa is covered with a layer of mucus, characterized by aggregated glycoprotein molecules (mucins) secreted by specialist cells of the underlying mucosa. Mucins are a family of polydisperse molecules with high molecular mass and a high proportion of covalently bound oligosaccharide side chains (*3*), which afford high resistance to the effects of acid and digestive enzymes. They may be characterized as secreted or membrane-bound.

Secreted mucins, up to $(0.5-20) \times 10^6$ Da (*4*, *5*), contain a central polypeptide core, of 1500−4500 amino acids in length, with 100−200 oligosaccharide side chains that contain 1−20 or more monosaccharides; such oligosaccharides may account for 50−80% of the molecule's mass (*6*). In mammals, mucins typically contain fucose, galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine, and sialic acid, together with small amounts of sulfate and mannose. The carbohydrate chains are bound to the polypeptide by O-glycosidic linkages between *N*-acetylgalactosamine and the hydroxylated amino acids serine and threonine (*6*). Highly glycosylated regions of the polypeptide, rich in threonine, serine, and proline, may account for 70−80% of the molecule, in a structure reminiscent of a bottle brush. The poorly glycosylated regions of the mucin molecule contain less serine and threonine but are rich in cysteine, which allows the formation of disulfide bridges between mucin molecules to form very high molecular mass mucous polymers (*7*). The unique capacity of the secreted mucins to protect the delicate epithelial surfaces of the mucosa is primarily due to the polymerization of mucin monomers to form viscoelastic gels (*7*).

Membrane-bound mucins share many of the structural properties of secreted mucins but remain monomeric, do not form gels, and stretch out from the epithelial surface to form the cell-surface membrane glycocalyx. The mucin layer also contains a number of other compounds including bicarbonate ions, epidermal growth factor, trefoil peptides, bactericidal factors, protease inhibitors, and surface-active lipids (*7*). Such compounds, when incorporated into the mucus layer, guard against its degradation and protect the underlying mucosa from gastric acid and pancreatic enzymes.

Within the gastrointestinal (GI) tract, a dynamic equilibrium exists between the rate at which mucin is synthesized and secreted and the rate at which it is degraded, by both proteolysis and physical erosion. As a result of these degradative processes, mucins constitute a significant proportion of the endogenous protein that reaches the terminal ileum. It has been estimated that between 50 and 65% of the endogenous protein secreted into the gut is mucin (*5*). Lien et al. (*8*) estimated that mucin accounted for 5−11% of the endogenous protein leaving the ileum of pigs fed a protein-free diet (with amino acid supplementation), whereas, in the calf, protein derived from mucin was estimated to account for 19% of the total basal endogenous losses at the terminal ileum (*5*). This equates to a mass of mucin at the terminal ileum of preruminant calves and of pigs amounting to 7.5 and 3.9 g kg^{-1} of dry matter intake (DMI), respectively, with 25% of this originating from the upper GI tract (*8*, *9*).

Several studies have shown that the loss of intestinal mucin may be affected by certain dietary components such as fiber,

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Table 1. Ingredient Compositions of the Basal Diet

ingredient	g kg^{-1} of air-dry wt	ingredient	g kg^{-1} of air-dry wt
cooked wheat[a]	485.8	vitamin/mineral mix[c]	2.5
sucrose	168	sodium chloride	1.6
lactic casein[b]	159	synthetic methionine	0.7
skim milk powder	80	calcium carbonate	0.2
soybean oil	80	antioxidant[d]	0.2
dicalcium phosphate	22		

[a] Weet-bix, Sanitarium, Auckland, New Zealand. [b] NZMP, Palmerston North, New Zealand. [c] Vitalean, Vitec Nutrition Ltd., Auckland, New Zealand. Vitamins provided: (g kg^{-1} of diet) vitamin A, 3.0; (mg kg^{-1} of diet) cholecalciferol, 500.0; choline, 83.3; niacin, 12.5; panthotenic acid, 8.3; riboflavin, 2.1; vitamin B6, 1.7; vitamin E, 41.7; vitamin K, 1.7; (μg kg^{-1} of diet) biotin, 8.3; folic acid, 417.0; thiamin, 833.0; vitamin B12, 8.3. Minerals provided: (mg kg^{-1} of diet) Cu, 104.0; Fe, 83.0; Mn, 38.0; Zn, 100.0; (μg kg^{-1} of diet) I, 833.0; Co, 417.0; Se, 250.0. [d] Ethoxyquin, Kemin Industries Ltd., Auckland, New Zealand.

Table 2. Composition of the Experimental Diet[a]

ingredient	g kg^{-1} of air-dry wt	ingredient	g kg^{-1} of air-dry wt
maltodextrin	453	sodium hydrogen carbonate	18
sucrose	161	titanium dioxide	3
soybean oil	154	lactic casein	211
nitrogen content of the test diet	31.9		

[a] No vitamins, minerals, or fiber was added to this diet as the same diet was used in a separate acute feeding study with human subjects (*42*).

peptides, and antinutritional factors (*5*, *10*−*12*). As mucin is so important in the protection of the mucosa of the GI tract, any mechanism that alters this defensive barrier has important physiological implications, especially in the control and management of inflammatory diseases of the bowel (*13*). There are few studies that detail the quantification of gastrointestinal mucin because it is particularly difficult to assay (*14*).

Because ileal digesta are a complex mixture of endogenous and exogenous substances, the isolation and quantification of mucin in ileal digesta is often undertaken using carbohydrate markers specific to glycoproteins, such as amino sugars and sialic acid. The aim of this study was to compare some of the common techniques used to quantify mucin concentrations in mammalian ileal digesta.

MATERIALS AND METHODS

Animals and Diets. Digesta samples were collected from three Large White × Duroc pigs having a mean body weight of 79 (±4.8) kg (±SEM), housed individually in steel metabolism crates, in a room maintained at 24 ± 1 °C, at the Small Animal Production Unit, Massey University, Palmerston North, New Zealand. Approval for the study was granted by the Massey University Animal Ethics Committee (protocol 05/29). After 1 week's acclimation, each pig was fitted with a postvalve T cecum cannula, as described by van Leeuwen et al. (*15*). Following surgery, food was progressively reintroduced within 1 week up to a daily level of 0.08 metabolic body weight ($W^{0.75}$) kg day^{-1}, and this level of food intake was maintained for the remainder of the trial. Collection of digesta took place 8 weeks following surgery. The pigs were fed a lactic casein-based basal diet (**Table** 1) mixed with water (1:1, w/w), three times daily (8:00 a.m., 12:00 p.m., and 4:00 p.m.), in equal portions. Water was available ad libitum. On the day of the digesta collection at 8:00 a.m. the pigs were fed one-third of the daily intake of an experimental diet (**Table** 2). Digesta were then collected into polythene bags for a period of 10 h after the morning meal. Each specimen was weighed before sodium benzoate (10 g kg^{-1} of digesta), as a bactericide, and phenylmethanesulfonyl fluoride (0.37 g kg^{-1} of digesta), as an antiprotease, were added according to the protocol of Salgado et al. (*16*). The digesta samples were then pooled and frozen at −20 °C until chemical analysis. The pigs received no food during digesta collection.

Chemical Analysis. Dry matter was determined by drying the material to a constant mass in a forced-air oven at 95 °C. Total nitrogen was determined according to the Leco total combustion method (*17*), a variation of the Dumas method. Duplicate samples were combusted at 1050 °C in oxygen gas; the nitrogen was then reduced to N_2 by a copper catalyst at 750 °C, and this was measured by a thermal conductivity cell in a Leco FP2000 analyzer (Leco Corp., St. Joseph, MI). Titanium dioxide was determined according to the method of Short et al. (*18*). Amino acid concentrations were measured using a Waters ion exchange HPLC system (Waters, Millipore, Milford, MA) calibrated against a reference amino acid mixture. Norleucine and lysozyme were used as internal and external standards, respectively. Methionine and cysteine were measured as methionine sulfone and cysteic acid, respectively, after hydrolysis of samples that had been oxidized using performic acid. Tryptophan was not determined. Amino acid concentrations are given as a percentage of total determined amino acid concentration.

Samples of digesta were fractionated by differential centrifugation using the method of Metges et al. (*19*), first at 250*g* for 15 min at 4 °C to separate food particles and porcine cells and then at 14500*g* for 30 min at 4 °C to separate microbial cells and porcine cellular detritus. The soluble mucins were assumed to be quantitatively recovered in the supernatant, together with proteins, peptides, free amino acids, neutral sugars, urea, creatinine, and ammonia. Amino acid concentrations were determined as described by Hodgkinson and Moughan (*20*).

Determination of Mucin and Mucin Markers. An attempt was made to prepare a standard mucin solution by redissolving pure mucin obtained from Sigma (Sigma-Aldrich Corp., St. Louis, MO). This proved to be difficult, however, particularly because many surfactants interfered with the colorimetric methods. To overcome this, for the colorimetric assays, a standard solution was obtained using mucin precipitated from porcine ileal digesta by ethanol. The standard curve obtained correlated with that of Mantle and Allen (*21*) ($\rho = 0.99$; $P < 0.001$).

Quantification of Mucin by Ethanol Precipitation. Following the method of Piel et al. (*14*), the digesta were treated in the following manner: Three milliliters of each fractionated digesta was added to 25 mL of a 0.15 M sodium chloride solution and mixed by vortex. Each of the diluted digesta samples was centrifuged at 12000*g* for 30 min at 4 °C; 15 mL of each supernatant was added to 22 mL of ethanol at 0 °C, mixed, and then kept overnight at −20 °C. Each supernatant/precipitate mixture was then centrifuged at 1400*g* for 10 min at 4 °C. The precipitate from each mixture was recovered and redissolved in 15 mL of 0.15 M sodium chloride solution before being precipitated again using the same procedure. Each precipitate was then resolubilized in 10 mL of distilled water, frozen, freeze-dried, and weighed.

Colorimetric Analysis of Mucin Based on the Periodic Acid−Schiff (PAS) Histological Stain Method. The determination of digesta mucin using PAS method followed a method taken from Mantle and Allen (*21*). Both the Schiff reagent and 50% periodic acid solution were purchased from BDH Chemicals (Merck, Darmstadt, Germany). A periodic acid solution was prepared by adding 10 μL of periodic acid (50% solution from BDH Chemicals) to 10 mL of a 7% ethanoic acid solution. Samples and standards, containing between 5 and 100 μg of mucin and a blank dissolved in 2 mL of water, were incubated for 2 h at 37 °C with 200 μL of freshly made periodic acid solution. After periodic acid oxidation, 200 μL of the Schiff solution was added to the glycoprotein solutions. The resultant solutions were then incubated for 30 min at room temperature for maximum color development. Absorbances were read at 555 nm.

Colorimetric Analysis of Mucin by the Phenol−Sulfuric Acid Assay for Neutral Sugars. The method used for the detection of neutral sugars using phenol−sulfuric acid was adapted from that of Beeley (*22*), a scaled down version of that used by Dubois et al. (*23*). Concentrated sulfuric acid (specific gravity = 1.84), D-mannose, and phenol were purchased from BDH Chemicals (Merck, Darmstadt, Germany). A standard aqueous solution of D-mannose (40 μg/ml) was preprepared and stored at −18 °C. In test tubes (14 mm i.d.), 0.5 mL of an aqueous test, blank, or standard solution was added to 300 μL of a phenol reagent (5%

w/v aqueous solution) and then mixed. Two milliliters of concentrated sulfuric acid was then added rapidly from a fast flowing pipet and mixed immediately. A blank (water) and standards of 5−20 μg of mannose were included in each assay. The test, blank, and standard solutions were left for 30 min for maximum color development and to allow the tubes to cool before the absorbance was read at 484 nm.

Total, Bound, and Free Sialic Acids. Sialic acids were determined following the method of Beeley (*22*), who adapted a periodate−thiobarbituric acid assay developed by Aminoff (*24*). The following reagents were preprepared several hours before they were required: periodate, 0.025 M periodic acid in 0.0625 M sulfuric acid, pH 1.2; sodium arsenite, 2 wt %/v sodium arsenite in 0.5 M HCl; 2-thiobarbituric acid, 0.1 M 2-thiobarbituric acid in water adjusted to pH 9.0 by adding NaOH; acid butan-1-ol, butan-1-ol containing 5% (v/v) of 12 M HCl; an aqueous standard of *N*-acetylneuraminic acid, in a range of 10−80 μg/mL. All chemicals were purchased from BDH Chemicals (Merck, Darmstadt, Germany). The procedure for the analysis of free sialic acid was as follows: Five hundred microliters of sample, blank, or standard was added to 0.25 mL of the periodic acid reagent and then mixed by vortex. The tubes were incubated for 30 min at 37 °C before 1 mL of the sodium arsenite was added to reduce the excess periodic acid and vortexed. The tubes were left for 1−2 min for the yellow color of the liberated iodine to disappear. Two milliliters of the thiobarbituric acid reagent were added before another vortexing. The samples were then heated in a boiling water bath for 7.5 min before being cooled on ice. Once cool, 5 mL of the acid-butanol was added and then mixed once more by vortex. The tubes were centrifuged briefly to separate the two phases before the butanol layer was collected and the absorbance measured at 549 nm.

The procedure for analysis of total sialic acid was as follows: One hundred microliters of 0.5 M HCl was added to 0.4 mL of digesta, and the tubes were incubated at 80 °C for 1 h. After the hydrolysates had cooled, the procedure for analysis of free sialic acid as described above was repeated.

Hexosamines by Gas−Liquid Chromatography (GLC). The hexosamines, *N*-acetylglucosamine (GlcNAc) and *N*-acetylgalactosamine (GalNAc), used as mucin markers, were estimated as the alditol acetates by the GLC method described by Lien et al. (*8*) from the procedures of Blakeney et al. (*25*) and Kraus et al. (*26*), using a Shimadzu 2010 (Shimadzu Scientific Instruments Inc., Columbia, MD) chromatograph with a DB-17 fused silica capillary column (J&W Scientific, Folsom, CA; 0.25 mm internal diameter × 30 m) and using helium (1.5 mL/min) as the carrier. For the extraction, hydrolyzation, and acetylation of hexosamine sugars, all chemicals were purchased from BDH Chemicals (Merck, Darmstadt, Germany) and the method was as follows: To approximately 50 mg of freeze-dried digesta was added 1.5 mL of 12 M sulfuric acid, and the mixture was left for 1 h at room temperature. The solutions were then diluted to 3 M by adding 4.5 mL of water before being hydrolyzed at 110 °C for a further hour. Following hydrolysis, 200 μL of an internal standard, *N*-methylglucamine, was added (10 mg/mL in distilled water). One milliliter aliquots of the acid hydrolysates were then cooled on ice before being made basic by adding 0.7 mL of concentrated ammonium hydroxide. One hundred microliters of this basic solution was reduced by adding 1 mL of sodium borohydride (30 mg/mL in anhydrous dimethyl sulfoxide) and incubating at 40 °C for 90 min. Excess sodium borohydride was then decomposed by adding 200 μL of glacial acetic acid. Acetylation was achieved by the addition of 0.2 mL of 1-methylimidazole and 2 mL of acetic anhydride and then leaving the mixture for 10−15 min at room temperature. Following acetylation, the acetic anhydride was decomposed by adding 5 mL of water and allowing the solutions to cool to room temperature. The alditol acetates were extracted into 4 mL of dichloromethane by vortexing. Following a brief centrifugation the aqueous layer was discarded. The dichloromethane solutions were then washed twice with another 4 mL of water before the final dichloromethane layer was evaporated to dryness under a stream of nitrogen. Before analysis by GLC, the alditol residues were redissolved in 1 mL of dichloromethane.

Five microliters of the redissolved alditol acetate solutions was injected onto a DB-17 fused silica capillary column (J&W Scientific, Folsom, CA; 0.25 mm internal diameter × 30 m). The gas chromatograph, a Shimadzu 2010, was set up using the following conditions: carrier gas, helium, at a rate of 1.5 mL/min; injector temperature, 270 °C. The oven temperature was raised from 50 to 190 °C at 30 °C min^{-1} and maintained for 3 min, then increased by 5 °C min^{-1} to 270 °C and maintained for 10 min. The detector temperature was set at 270 °C. Peak area integration was analyzed using the Shimadzu GC solutions V2-30su6 data system.

Table 3. Amino Acid Composition of the Terminal Ileal Digesta

	amino acid concn[a]	SEM[b]		amino acid concn[a]	SEM[b]
Indispensable Amino Acids					
arginine	2.6	0.23	lysine	3.4	0.42
cysteine	2.2	0.40	methionine	1.1	0.09
histidine	2.5	0.23	phenylalanine	2.7	0.36
isoleucine	3.8	0.48	threonine[c]	9.4	1.67
leucine	5.8	0.68	valine	6.5	0.86
Dispensable Amino Acids					
alanine	7.7	0.96	proline[c]	8.8	0.73
aspartic acid	9.3	0.65	serine[c]	7.8	0.78
glutamic acid	14.8	0.90	tyrosine	2.0	0.24
glycine	9.6	1.34			

[a] Amino acid concentration as a percentage of total determined amino acid concentration. The absolute flows of digesta amino acids have been reported previously (*30*). Tryptophan was not assayed. [b] SEM, standard error of mean. [c] Predominant amino acids in mucin.

The ratio of *N*-acetylglucosamine (GlcNAc) to *N*-acetylgalactosamine (GalNAc) in mucins varies according to the origins of the mucin molecules (*8*). Utilizing this difference, Lien et al. (*8*) estimated mucin output using regression equations derived from the ratios of these hexosamines in purified gastric and intestinal mucins. These equations have been used in this study and are as follows:

$$\text{mucin output (g day}^{-1}) = \text{GalNAc (g day}^{-1})/\%\ \text{GalNAc}$$

$$\%\text{GalNAc} = 32.30 - 22.74x + 8.83x^2 - 1.37x^3 \quad (1)$$

(x = GlcNAc:GalNAc ratio).

The contribution (%) of gastric mucin was determined using the following equation:

$$\%\text{gastric mucin} = -80.23 + 183.26x - 71.19x^2 + 11.05x^3 \quad (2)$$

The recovery of an indigestible marker (TiO_2) was used to correct the concentrations of mucin and mucin markers for each of the methods using the equation

$$\text{corrected concn of mucin or mucin marker [g kg}^{-1}\text{ of dry matter intake (DMI)]} = [\text{concn of TiO}_2\text{ in the diet (g kg}^{-1}\text{ of DMI)} \times \text{concn of TiO}_2\text{ in the diet (g kg}^{-1}\text{ of DMI)}]/[\text{concn of TiO}_2\text{ in the ileal digesta (g kg}^{-1}\text{ of DMI)}]$$

Having obtained values for the concentrations of mucin markers by the PAS, phenol−sulfuric acid, and sialic acid assays, the final concentration of mucin was calculated using multiples derived from the composition data published by Mantle and Allen (*27*).

RESULTS

The amino acid composition of the total ileal digesta is presented in **Table** 3. In diminishing order of concentration the amino acids glutamic acid, glycine, threonine, aspartic acid, proline, and serine were predominant. Glutamic acid was particularly high, being nearly 15% of the total amino acid content of the digesta. The concentration of tryptophan was not determined in this study.

Table 4 gives the concentrations of the mucin markers in the ileal digesta of pigs fed a casein-based diet determined using the various methods studied. It may be noted that the concentration of sialic acid found in the digesta is low in comparison to the other

Table 4. Mean (± SEM) Concentrations of Mucin Markers in the Ileal Effluent of Pigs Given a Casein-Based Diet

method	ref	mucin marker	mucin marker concn (g kg^{-1} of DMI)	SEM
periodic acid−Schiff	*21*	polysaccharide	20.7	0.77
phenol−sulfuric acid	*23*	neutral sugars	17.1	1.43
Aminoff	*24*	sialic acid	1. total, 0.17	6.1×10^{-3}
			2. free, 0.12	4.5×10^{-3}
			3. bound, 0.05	1.7×10^{-3}
hexosamines by GLC	*8*	GalNAc [a]	6.5	0.33
		GluNAc [a]	8.6	0.28

[a] GalNAc, *N*-acetylgalactosamine; GluNAc, *N*-acetylglucosamine.

Table 5. Mean (± SEM) Concentrations of Mucin [1] in the Ileal Effluent of Pigs Given a Casein-Based Diet

method	ref	mucin concn (g kg^{-1} of DMI)	SEM
periodic acid−Schiff	*21*	25.1[a]	0.95
phenol−sulfuric acid	*23*	20.7[a]	1.76
Aminoff	*24*	5.9[a]	0.13
ethanol precipitation	*14*	19.3	1.50
hexosamines by GLC	*8*	44.9[b]	1.48

[a] Calculated using data from Mantle and Allen (*27*). [b] Calculated using regression equations from Lien et al. (*8*).

Table 6. Pearson Correlation Coefficients for Relationships between Chosen Mucin Analysis Methodsa

mucin marker	hexosamine	periodic acid−Schiff	phenol−sulfuric acid	ethanol precipitation
periodic acid−Schiff	0.687			
phenol−sulfuric acid	0.623	0.949		
ethanol precipitation	0.634	0.953	0.944	
sialic acid	0.777	0.761	0.781	0.770

[a] All correlations were significant at $p < 0.001$.

mucin markers. Nearly 74% of the total sialic acid is unbound, with only 26% remaining bound to mucin subunits. These data are only indirectly representative of the mucin present in the ileal digesta.

The concentrations of mucin predicted on the basis of the markers are presented in **Table** 5. These concentrations were calculated using compositional data from the study of Mantle and Allen (*27*). The mucin concentration determined by the hexosamine method was 44.9 g kg^{-1} and considerably higher than that found with the other methods. In comparison, the PAS, PSA, and ethanol precipitation methods were 56, 46, and 43% of the concentration on mucin determined by the hexosamine method. The mucin concentration determined by using the Aminoff method was only 5.9 g kg^{-1}, a value only 13% of that determined using the hexosamine method.

Pearson correlation coefficients describing the degree of relationship between the respective mucin analysis methods are presented in **Table** 6. The relationship between the ethanol precipitation analysis and the two colorimetric methods of PAS and PSA gave particularly high Pearson correlation coefficients of 0.953 and 0.944. The correlation between the two acid colorimetric methods was also high (0.949). The correlations between the hexosamine method and the other methods were considerably lower, only 0.687, 0.623, 0.634, and 0.777 for the PAS, PSA, ethanol precipitation, and Aminoff methods, respectively. Interestingly the correlations between the Aminoff method and the other methods remained fairly constant, being 0.777, 0.761, 0.781, and 0.770 for the hexosamine, PAS, PSA, and ethanol precipitation methods, respectively.

Table 7. Composition of Mucus Glycoproteins from the Mammalian Gastrointestinal Tract [after Mantle and Allen (*27*)]

mucin	percentage by weight: sulfate	carbohydrate[a]	protein
salivary mucins			
pig	0.0	66	34.0
gastric mucins			
human	7.0	76	17.0
pig	3.1	83.9	13.0
small intestinal mucins			
human	1.6	80.4	18.0
pig	2.6	79.4	18.0
colonic mucins			
human	2.0	66.9	31.1
pig	3.0	83.7	13.3

[a] Data from Mantle and Allen (*27*). The percentage of carbohydrate was calculated as the difference between the percentages given for sulfate and protein.

DISCUSSION

The proportion of protein in purified adult porcine small intestinal glycoprotein has been reported as 23% (*14*, *28*) and the sum of serine, proline, and threonine in the protein core of the glycoprotein being as much as 52% (by weight) according to Mantle and Allen (*27*, *29*). In the present study these three amino acids represented only 23% (by weight) of the total amino acids present in the digesta, a result that reflects the presence of other sources of protein in the digesta. In earlier research (*30*) it was demonstrated that only 14% of the total protein present in the ileal digesta originated from mucin. Although the six most abundant amino acids in the digesta are those associated with mucin, they are diluted by the high proportion of amino acids originating from bacterial sources, nearly 61% of the total protein found in the ileal digesta of animals fed a casein-based diet (*30*).

Ileal digesta may contain several different mucin types, originating from a range of secretory organs in different parts of the GI tract. The chemical composition of these mucins is known to vary with both their origin and animal species (**Table** 7) (*3*, *27*). However, it would be expected that, in ileal digesta, mucins originating from the stomach and the small intestine would predominate.

The value of GalNAc as a mucin marker lies in its limited occurrence in dietary constituents (*8*). GluNAc is less specific as it is found in some dietary proteoglycans (*31*). However, no GalNAc or GluNAc was found in the dietary material in this study. The GluNAc/GalNAc ratio differs considerably between gastric and intestinal mucin (*8*, *29*, *32*), as gastric mucin contains approximately 30% GlcNAc and 13% GalNAc, whereas intestinal mucin contains approximately 20 and 40%, respectively. Given this difference in the concentrations of these two amino sugars, the relative proportions of gastric and intestinal mucins in the ileal digesta can be estimated.

The concentrations of GalNAc and GluNAc in the ileal digesta were estimated to be 6.5 and 8.6 g kg^{-1} of DMI (**Table** 4), respectively. Using regression equations derived by Lien et al. (*8*) the mucin output was then calculated to be 44.9 g kg^{-1} of DMI (**Table** 5), a value much higher than the estimates reported by Lien et al. (*8*) for pigs fed a protein-free diet. This discrepancy may be due to the influence of dietary protein per se (*12*).

GluNAc/GalNAc ratios in purified pig gastric and intestinal mucins of 2.8 and 0.6, respectively, were reported by Mantle and Allen (*27*). The ratio observed in this study for the mixed proteins in the ileal digesta was 1.36, which suggests that there was proportionately more gastric mucin than intestinal mucin in the ileal effluent. This is corroborated by the ratio of threonine/serine. Ratios for threonine/serine of 1.15 and 2.55 in purified pig gastric and intestinal mucins, respectively, were reported by Mantle and Allen (*27*). In the present study the threonine/serine ratio was 1.32, similar to a mean value of 1.29 reported by Lien et al. (*8*) for adult pigs given a protein-free diet. Correlation analysis of the concentrations of the two hexosamines over the three pigs yielded a Pearson correlation coefficient (ρ) of 0.995 ($P < 0.001$), indicating a constancy in the ratio of gastric and small intestinal mucins between animals given the casein-based diet.

Degradation of the oligosaccharide side chains may reduce the concentration of GluNAc and GalNAc found in the ileal digesta; however, as every oligosaccharide side chain begins with one GalNAc residue, these would be conserved more than any other. As GluNAc is found along the length of the oligosaccharide side chains, the ileal concentration of this hexosamine may be subject to greater variation than that of GalNAc when the side chains are degraded. The ratio of GluNAc to GalNAc appears to be relatively constant (as evident from the high correlation coefficient), suggesting that GalNAc and GluNAc are reliable mucin markers.

The precipitation of glycoproteins using high concentrations of ethanol is considered by many researchers to be nonspecific [e.g., Piel et al. (*14*)]. This is further illustrated by the variety of different compounds ethanol has been used to precipitate [e.g., α-galactosidase from a fungus (*33*); human-immunodeficiency-virus-inhibitory glycoprotein from aqueous extracts of a Caribbean sponge (*34*); and the two gonadotropins, a follicle-stimulating hormone and a luteinizing hormone, from the pituitary gland of halibut (*35*)]. Non-covalently bound proteins associated with mucin precipitated by ethanol have been noted by Leterme et al. (*36*) and are associated with the polymerization of mucin subunits. Although the validity of this assay for the determination of mucin in human gastric juices has been accepted by some workers [e.g., Azuumi et al. (*37*)], its effectiveness has been questioned by others (*14*, *34*). In the present study both the precipitate and the ethanolic supernatant were assayed for carbohydrates using the phenol−sulfuric acid technique, with mixed results. The final concentration of ethanol in the precipitation of glycoprotein from the digesta seems to be critical, as once the resulting precipitate was redissolved, its carbohydrate content varied from almost 0 to 124 μg mL^{-1} of ileal digesta. When the protein content of each fraction was determined, using Bradford reagent, there was often twice as much protein in the supernatant as there was in the precipitate. When Piel et al. (*14*) reported that their ELISA showed no immunoreactivity in the precipitates, they concluded that this may have been because high concentrations of ethanol are known to denature human gastric mucin (*38*). However, the unreliability of the assay found during this study suggests that ineffective precipitation may also be a factor and one that requires further investigation.

The estimate of intestinal mucin output obtained by ethanol precipitation was less than half that estimated by the hexosamine assay (**Table** 5). A similar discrepancy was reported by Piel et al. (*14*). In their assay the ethanol precipitate value was 78% of that determined by the hexosamine assay. Björling (*39*) reported that highly glycosylated glycoproteins remain soluble in relatively high concentrations of ethanol. Ineffective precipitation may well be the reason for such inconsistent results. In light of the results from this study and of others, the reliability of ethanol precipitation for quantitative and specific estimation of digesta glycoproteins must be queried. From our own observations and supported by other published results (*14*, *34*, *39*), it would appear that the ethanol precipitation method considerably underestimates mucin in digesta. Moreover, given the sialic acid, periodic acid−Schiff (PAS), and phenol−sulfuric acid (PSA) methods gave similar or lower values for mucin compared to ethanol precipitation (**Table** 4), this calls into question the accuracy of all of these approaches.

Table 8. Carbohydrate Concentration of Mucin Expressed as a Molar Ratio to GalNAc, after Mantle and Allen (*27*)

	carbohydrate composition[a]				
mucin	GalNAc[b]	GlcNAc[c]	galactose	fucose	sialic acid
salivary mucins					
pig	1	0.0	0.5	0.4	0.4
gastric mucins					
human	1	1.8	2.3	1.6	0.9
pig	1	2.8	2.9	1.9	0.2
small intestinal mucins					
human	1	1.1	1.0	1.0	0.4
pig	1	0.6	0.6	0.3	0.6
colonic mucins					
human	1	0.9	1.2	0.3	0.2
pig	1	2.9	2.5	1.5	0.2

[a] Carbohydrate composition expressed as the molar ratio to GalNAc (*27*). [b] GalNAc, *N*-acetylgalactosamine. [c] GluNAc, *N*-acetylglucosamine.

Although the PAS staining of electrophoresis gels or histological specimens containing glycoproteins is a sensitive and widely used procedure, its use for the analysis of mucins in biological fluids does not appear to be common. Developed by Mantle and Allen (*21*), the assay is quick and sensitive for the analysis of polysaccharides that are oxidized by periodate. However, there is considerable variation in the oligosaccharide side chains of the various mucins found within the gastrointestinal tract and between different species (**Table** 8). Also, pig gastric mucin contains many sugar residues that are 1−3-linked and therefore are not oxidized by periodic acid (*40*). Such differences in composition affect the sensitivity of the PAS assay such that 150 μg of pig intestinal mucin gives an absorbance at 555 nm 35% greater than that of a similar mass of pig gastric mucin (*21*). With the mixtures of different mucins that are present in ileal effluent, it is not possible to obtain absolute values for the mass of oxidizable sugar residues (*22*), and the aforementioned different monosaccharide composition may account for the apparent underestimation of mucin using this method (**Table** 5).

The colorimetric assay for the quantitative analysis of neutral sugars, described by Dubois et al. (*23*), is undertaken in strongly acidic conditions and can be applied to free monosaccharides or to unhydrolyzed samples of glycoprotein (*22*). Although this assay is quick and simple, like the PAS reaction, it too lacks specificity as it cannot distinguish between monosaccharides such as galactose, fucose, and glucose. The extinction coefficients of these sugars also differ. Using correlation analysis of the concentrations of mucin for the three pigs as determined by the PAS and phenol−sulfuric acid assays, a Pearson correlation coefficient of 0.95 ($p < 0.001$) (**Table** 8) was obtained, a value that is not surprising as the two assays measure similar monosaccharides. There was also good agreement between the ethanol precipitation method and the two colorimetric assays ($\rho = 0.95$ and 0.94, respectively). If it is accepted that the ethanol precipitation method underestimates the mucin concentration because of incomplete precipitation (*14*, *39*), it is also not surprising that these three methods indicate such proportionality.

The concentrations of galactose and fucose also differ greatly between the different types of gastrointestinal mucin (**Table** 8). Pig gastric mucin has >5 times the amount of galactose and fucose than pig intestinal mucin. As the type of mucin found in digesta from the terminal ileum varies, the different concentrations of galactose and fucose cannot be relied upon for the determination of absolute concentrations of mucin. Similarly, the periodic acid−Schiff reaction also seems to be more suitable for comparative analysis and cannot be relied upon for determining absolute concentrations of mucin. Therefore, an apparent underestimation of mucin using this method (**Table** 5) may be attributable to the variance in monosaccharide composition, between the types of mucin present in the digesta, and their differential reactions to the chromophore.

Sialic acid is a collective term for the acylated derivatives of neuraminic acid. In mammalian glycoproteins sialic acids occur at the terminal end of oligosaccharide side chains of the glycoconjugate and give the mucin molecules an electronegative charge. Due to the weakness of its glycosidic linkage to the carbohydrate side chains, sialic acid units are easily cleaved from such side chains with only mild hydrolysis. Thus, the concentration of free sialic acid may be used as a measure of desialylation of the glycoconjugates (*41*), and the quantification of free sialic acid in the ileal effluent can indicate the degree of degradation of glycoproteins.

The mucin concentration estimated using the sialic acid assay was 5.9 g kg^{-1} of DMI, a value >7 times lower than the value obtained using the GC hexosamine assay (**Table** 5). The reason for such a large difference is not known; however, the degradation of sialic acid along the gastrointestinal tract and incomplete reaction of the breakdown products with the chromophore may account for the low values observed.

The concentration of free sialic acid (0.12 g kg^{-1} of DMI; **Table** 4) was nearly 2.4 times that of the bound fraction (0.05 g kg^{-1} of DMI), suggesting a substantial degree of mucin degradation. It is, therefore, not surprising that the sialic acid assay yielded lower, although still statistically significant, interassay correlations compared to the other colorimetric and ethanol precipitation assays.

With the ethanol precipitation method there may have been differential precipitation of highly glycosylated glycoproteins and of other non-mucin components. There seems to be no reason why this gravimetric assay produces results 2.3 times lower than the hexosamine assay, other than that it ineffectively precipitates degraded forms of mucin as indicated by the free sialic acid concentrations present in the digesta. Although the two colorimetric assays for neutral sugars are both straightforward and relatively inexpensive, they can be justifiably used only for comparative studies. Given that the ethanol precipitation method clearly gave low recoveries of mucin (based upon chemical analysis of the supernatant and precipitate), this also calls into question the accuracy of the absolute values for mucin excretion determined by the periodic acid−Schiff, phenol−sulfuric acid, and sialic acid methods, all of which gave values similar to or lower than the ethanol precipitation method. One reason for this may be that the mucins present have been degraded to such an extent that the other mucin markers present in the digesta may not be conserved as much as the two hexosamines. It would therefore be useful to determine the nature of the ethanol precipitate and the extent of its degradation.

One notable omission from the range of methods investigated here is the ELISA assay used by Piel et al. (*14*). In their hands, mucin levels in digesta estimated by this means were only one-third those derived from the hexosamine assay. They attribute this discrepancy to the hexosamine and ethanol precipitation assays being less specific. They also found a 4-fold greater individual variability with the ELISA technique than with the other two assays. They suggested that this may also be due to a lack of specificity. However, it seems to us that a more likely reason for these results stems from the extensive degradation of mucins during their transit of the GI tract, which is likely to diminish progressively the specificity of an antibody-based method.

Each of the five methods studied has advantages and disadvantages from a technical perspective. The present results highlight that both the hexosamine assay, using a gas−liquid chromatography method, and the quantification of free and bound sialic acid give information that is useful in describing the nature of the mucins present at the terminal ileum. The hexosamine assay gives a measure of the ratio of gastric to intestinal mucin, and the sialic acid assay gives a measure of the extent of digestive degradation, both useful in describing the composition of ileal effluent. If the quantities of gastric and small intestinal mucin are to be determined, it would appear that the hexosamine assay would be the current method of choice.

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