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Original article

Estimation of ileal output of gastro-intestinal glycoprotein in weaned piglets using three different methods

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Abstract – Mucin is the main constituent of gastrointestinal mucus and is responsible for its physico-chemical and physiological properties. Previous studies have suggested that this glycoprotein represents a major component of undigested endogenous protein at the ileum. The aim of the study was to estimate the ileal output of this glycoprotein using three methods: direct ELISA, hexosamine-based method and ethanol precipitation. For setting up the ELISA assay, the glycoprotein was isolated from intestinal mucus scraping by cesium chloride density gradient ultracentrifugation and a rabbit hyperimmune plasma was raised against the purified glycoprotein. Ileal outputs of hexosamine and glycoprotein were measured in weaned piglets fed a control diet (C) based on casein or diets which contained 50% crude protein supplied by white (WCP) or black (BCP) chickpea. The hexosamine output was higher ($P < 0.05$) with the WCP diet (2.3 and 1.5 g·kg^{-1} of dry matter intake for glucosamine and galactosamine, respectively) than with diet C (1.1 and 0.7 g·kg^{-1} of DMI). The hexosamine-based and ethanol precipitation methods, but not the ELISA, showed significant differences between the diet treatments ($P < 0.05$). Although hexosamine-based and ethanol precipitation methods for the estimation of ileal glycoprotein appeared to be more satisfactory than the developed ELISA to display diet effects in this study, it remains to be determined whether the higher glycoprotein data variability observed with ELISA reflects the actual biological variability of the phenomenon or not.

small intestine / methods / glycoprotein / piglet

1. INTRODUCTION

The mucus layer is part of the various protection systems developed at the intestinal epithelial cell level. In the gastrointestinal tract, in particular, the main functional properties of the mucus are protection against microorganisms and physical and chemical attacks, lubrication of the gut and action as a diffusion barrier [1]. Mucin is the major glycoprotein of the mucus gel. Each mucin subunit (molecular weight > 10^6) is constituted of a protein backbone rich in serine and threonine and by many carbohydrate chains, accounting for more than 50% by weight of the glycoprotein, and protecting

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the protein core against proteolysis [2]. The mucus layer is in a dynamic balance between mucin synthesis and secretion from goblet cells of the underlying epithelium, and erosion on the lumenal side releasing mucin into the gut lumen. An intact mucus layer is required at the surface of the epithelium for optimal protection, depending on both quantitative (thickness) and qualitative (sugar composition of carbohydrate chains) aspects [3, 4]. Because this glycoprotein is poorly digested before reaching the large intestine, where it is fermented by enteric bacteria [5], it represents one of the most significant contributions to endogenous protein flowing out of the small intestine [6]. There is now some evidence that intestinal glycoprotein flow is modulated by dietary components including fiber, protein and antinutritional factors [7–9]. So, measuring the glycoprotein flow in the gut lumen could provide insights into the effect of the diet or dietary components on gut physiology. However, despite this interest, information on glycoprotein recovery in the digesta is scarce, probably because it is difficult to assay.

The aim of the present study was to quantify glycoprotein output in the ileal digesta of weaned piglets using three methods: (1) an enzyme-linked immunosorbent assay (ELISA) that we developed; (2) hexosamine-derived calculations [10]; and (3) glycoprotein precipitation by ethanol [11]. The piglets were fed either a control diet based on casein as the source of protein or a diet containing white or black chickpeas.

2. MATERIALS AND METHODS

2.1. Purification of gastro-intestinal glycoprotein and preparation of a hyperimmune plasma

2.1.1. Glycoprotein purification

Pig intestinal glycoprotein was purified by a modification of the method of Mantle and Allen [12]. Briefly, mucus was collected by gently scraping the small intestinal mucosa from ten freshly slaughtered pigs (approximately 25 kg of body weight). Mucus scrapings (300 g) were solubilized in 3 L of Tris buffer (50 mM, pH 7.5) containing 6 M guanidine hydrochloride, for 1 h at +4 °C. Then, the glycoprotein was chemically reduced by adding 0.1 M dithiothreitol (+4 °C, 24 h) and sulfhydryl groups were carboxymethylated by adding 0.25 M iodoacetamide (+4 °C, 24 h, dark). The solubilized crude mucus solution was adjusted to a density of 1.49 $g \cdot mL^{-1}$ by adding 55 g of solid cesium chloride per 100 mL. It was then, ultracentrifuged (model L8-70M, Beckman Instruments, Fullerton, CA, USA) twice at 203 000 × *g* for 48 h at +12 °C. After each run, the eight density fractions obtained were dialyzed (cut off level 12 000 to 16 000 from Viskase, Poly Labo, Strasbourg, France) and analyzed as described below.

2.1.2. Analysis of density fractions and purified glycoprotein

Aliquots of density fractions collected after ultracentrifugation (density from 1.29 to 1.59 $g \cdot mL^{-1}$) were evaporated (Speed-Vac SC 110, Savant Instruments, New York, USA), dissolved in Tris buffer (10 mM, pH 7.5) containing 35 mM sodium dodecyl sulfate (SDS) and analyzed for total protein content using the Lowry method with serum albumin as a standard [13] and for total glycoprotein using the orcinol-sulfuric acid procedure, with galactose as the standard [14]. Nucleic acids were monitored at an optical density of 260 nm (U Vikon 922, Kontron Instruments, UVK-Lab, Trappes, France). The purity of the density fractions for protein was analyzed by SDS-PAGE electrophoresis on mini-gels (80 × 90 mm) (system model 6450, Touzard & Matignon, Vitry-sur-Seine, France) using 7.5 and 4% polyacrylamide gels for migrating and stacking gels, respectively [15]. Migration was conducted at room temperature, in a Tris-glycine buffer (25 mM Tris, 0.192 M glycine, pH 8.3) containing 3.5 mM SDS,

under a constant current of 20 mA per slab. The proteins were stained with Coomassie brilliant blue (Amersham Pharmacia Biotech, Uppsala, Sweden) (0.25% in methanol/acetic acid/water 5/1/4). The glycoproteins were also stained by the periodic acid-Schiff (PAS) technique [16]. This SDS-PAGE technique and associated PAS or Coomassie blue stainings were also used to characterize other samples including a commercial porcine gastric mucin sample (ref M2378, Sigma, La Verpillière, France), porcine gastric, intestinal and colonic crude mucus scrapings, ileal digesta and feed extracts. Western blotting was also conducted using a semi-dry trans-blot apparatus (Pharmacia, Uppsala, Sweden) for transferring proteins separated by SDS-PAGE as described earlier by Salgado et al. [17]. Carbohydrate and amino acid compositions of purified glycoprotein were determined by ionic chromatography (CarboPac PA1, Dionex) using the method of Quemener et al. [18], and by ion-exchange chromatography (Pharmacia LKB Alpha Plus, Uppsala, Sweden) using the method of Moore and Stein [19], respectively. Tryptophan, which is destroyed by acid hydrolysis, was not analyzed. Amino acid composition of the purified glycoprotein was compared to published amino acid compositions of mucin from different animal species by the χ^2 distance as previously described by Guilloteau et al. [20]. The lower the χ^2 distance, the higher the similarity between the compared proteins.

2.1.3. Production and characterization of a hyperimmune plasma to pig glycoprotein

Rabbit hyperimmune plasma was raised against the purified gut porcine glycoprotein as previously described [21]. The specificity of the anti-glycoprotein plasma was tested by ELISA and western blotting against glycoprotein from the different sites of the pig gut and crude mucus or digesta from various animal species (see below).

2.2. Measurement of ileal glycoprotein in the ileal digesta of piglets

2.2.1. ELISA for piglet glycoprotein

A direct ELISA was set up for intestinal piglet glycoprotein according to a procedure developed in preruminant calves by Montagne et al. [21]. Freeze–dried digesta were homogenized, reduced and alkylated as described above for glycoprotein purification. This preparation was then centrifuged (30 000 × *g* for 15 min, +4 °C), and the supernatant corresponding to crude soluble mucus was dialyzed 48 h against sodium azide (50 mM). An aliquot was evaporated (Speed-Vac SC 110) and then dissolved in carbonate buffer (15 mM Na_2CO_3 and 35 mM $NaHCO_3$, pH 9.6) containing 2 M guanidine hydrochloride. This preparation was assayed for protein by the method of Bradford [22] using the Bio-Rad protein assay reagent (Bio-Rad, Hemel Hempstead, UK).The samples were then diluted to different concentrations (200 to 350 ng·mL^{-1} of soluble protein) in carbonate buffer. Microtiter plates (Nunc 969914, Roskilde, Denmark) were coated in triplicate with diluted samples (200 µL per well) and left to incubate overnight at +4 °C. All the solutions used subsequently were made in PBS (pH 7.4). After saturation of the binding sites with 0.1% skim milk (2 h at +4 °C), our rabbit anti-glycoprotein plasma (dilution 1:2500) was added and the plates were incubated for 1 h 30 at +37 °C. After washing, an anti-rabbit peroxidase conjugate (dilution 1:2000) was added and incubated for 1 h 30 at +37 °C. Peroxidase activity was revealed by incubating 200 µL of substrate [2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) 0.01% and H_2O_2 0.01%; Sigma] for 30 min at room temperature in the dark. The reaction was stopped by adding 50 µL of HCl 6 M. The optical density was measured at 495 nm against a blank prepared without coated glycoprotein (plate reader Argus 300 from Packard Instruments Co., Meriden, CT).

Purified glycoprotein solutions ranging from 0 to 1000 ng of glycoprotein protein per mL were used in triplicate to construct standard curves. The linear portion of response curves was determined after a logarithmic transformation of glycoprotein concentration. The concentration of glycoprotein protein in the assayed samples was determined from standard curves by linear regression. The data were expressed per milliliter and per g of protein in the coating solution and then in g per 100 g of dry matter (DM) in the ileal digesta to have the glycoprotein concentration. Since the Bio-Rad protein assay underestimated the glycoprotein concentration, probably due to a high degree of mucin glycosylation, the glycoprotein content was estimated to be 1.6 fold the Bio-Rad protein value on the basis of the sum of amino acids determined in the purified glycoprotein.

2.2.2. Determination of glycoprotein using hexosamine assays

Glucosamine and galactosamine were assayed by the method of Combe et al. [23]. Ten to 30 mg of freeze-dried ileal digesta samples were hydrolyzed in 2 mL of HCl, 5 M for 2 h at +110 °C. The concentration of hexosamine was then determined by ion exchange chromatography (Pharmacia LKB Alpha Plus). Pure glucosamine and galactosamine were used as standards (Sigma).

The concentration of native glycoprotein (no proteolytic digestion) was calculated from the determination of galactosamine output as reported by Lien et al. [10] as follows:

glycoprotein output = galactosamine output / % galactosamine

with galactosamine output in $g \cdot kg^{-1}$ of DMI.

The percentage of galactosamine in a mixture of glycoproteins was estimated from the observed glucosamine to galactosamine ratio (x) according to the following empirical regression equation:

% galactosamine = $32.30 - 22.74x + 8.83x^2 - 1.37x^3$ [10].

2.2.3. Determination of glycoprotein by ethanol precipitation

A small fraction of ileal digesta was used to estimate glycoprotein concentration by a rather non specific method based on ethanol precipitation. The precipitate is composed mainly of raw mucus glycoprotein [11] and is considered to be representative of the glycoprotein fraction in the digesta [24]. Three grams of freeze-dried ileal digesta were suspended in 25 mL of a 0.15 M sodium chloride solution and mixed by vortex. After centrifugation (12 000 × *g*, 30 min, +4 °C), 15 mL of the supernatant was added to 22 mL ethanol (0 °C), kept for one night at –20 °C and centrifuged (1400 × *g*, 10 min, +4 °C). The precipitate was recovered in 15 mL of the sodium chloride solution, precipitated again with ethanol (same conditions), freeze-dried and weighed.

2.3. Digestion experiment with piglets

In order to compare the three methods described above (ELISA, hexosamine-based calculation and ethanol precipitation method), glycoprotein was measured in the ileal digesta quantitatively collected in a previous experiment [25].

2.3.1. Diets

Seeds from two varieties (white and black) of chickpea (*Cicer arietinum* L.) were used in the previously reported experiment. A basal diet containing casein (C) as the only source of crude protein and two diets in which 50% of crude protein was supplied by white (WCP) or black (BCP) chickpeas were compared. Data on diet composition are given in Table I.

2.3.2. Pig feeding and digesta collection

Eighteen crossbred (Duroc × Landrace) male piglets from six litters were weaned at 28 days of age (8.23 ± 0.26 kg body weight). They were randomly assigned to the experimental treatments in a complete block design

Table I. Ingredients and chemical composition of the experimental diets.

Diet	C[1]	WCP[2]	BCP[3]
Ingredients (g·kg^{-1} air-dried diet)			
Maize starch	451	119	129
Casein	250	127	127
White chickpea	–	491	–
Black chickpea	–	–	478
Sucrose	100	100	100
Straw	60	30	30
Soybean	70	70	70
Calcium carbonate	4	5	4
Dicalcium monophosphate	47	41	44
Sodium chloride	5	5	5
L-Lysine	–	–	1
DL-Methionine	3	2	2
L-Tryptophan	–	1	1
Vitamin-trace-mineral[4]	10	10	10
Nutrients analysis or specified (g·kg^{-1} air-dried diet)			
Dry matter (g·kg^{-1})	918	917	914
Ash	69	72	76
Crude protein	233	221	241
Crude fat	76	99	97
Starch	491	314	278
Non-starch carbohydrates[5]	21	172	190
Neutral detergent fiber	55	68	89
Gross energy (MJ·kg^{-1} dry matter)	19	20	20

[1] Control diet.
[2] White chickpea diet.
[3] Black chickpea diet.
[4] Mineral and vitamin mixture supplied per kg of diet: 12000 IU Vitamin A, 2000 IU Vitamin D_3, 20.0 mg Vitamin E, 1.0 mg Vitamin B_1, 4.0 mg Vitamin B_2, 1.5 mg Vitamin B_6, 0.2 mg Vitamin B_{12}, 2.0 mg Vitamin K_3, 0.1 mg Vitamin H_2, 25.0 mg nicotinic acid, 0.5 mg folic acid, 15.0 mg pantothenic acid, 400.0 mg choline chloride, 0.6 mg IO_3, 40.0 mg MnO_2, 125.0 mg $FeSO_4.7H_2$, 100.0 mg ZnO_2, 160.0 mg $CuSO_4.5H_2O$, 0.15 mg Na_2SeO_3, 0.4 mg $CoSO$, 100.0 mg *Bacillus Toyi,* 50.0 mg carbadox, 500.0 mg flavoring, 60.0 mg antioxydant (butylated hydroxytoluene), 1050 mg phosphorique acid, 150.0 mg citric acid.
[5] Non-starch carbohydrates = organic matter – (crude protein + crude fat + starch + sucrose + α-galactosides).

on the basis of their litter origin and average body weight at weaning. They were surgically prepared with ileo-rectal anastomosis allowing quantitative collection of ileal digesta over a period of seven days. Digesta were collected at 4 h intervals using weighed plastic trays located under the cages, and to which sodium benzoate (10 g·kg^{-1} of digesta) and phenylmethylsulfonyl fluoride (0.37 g·kg^{-1} digesta) were added as preservatives. The digesta were freeze-dried until analysis (for more details see [25]).

2.3.3. Feed and digesta analysis

DM and nitrogen contents were determined in feed and ileal digesta by oven-drying at +105 °C and according to the Kjeldahl method, respectively. Glycoprotein content was determined by our ELISA technique, and the hexosamine and the precipitation methods described above. The concentration of glycoprotein in the digesta was used to calculate mucin output at the terminal ileum.

2.3.4. Mucin output calculation and statistical analysis

The outputs of glycoprotein and hexosamines at the ileum are expressed in g·kg^{-1} of DM intake (DMI). Ileal output of hexosamines was not corrected for those of dietary origin, following the published ways of calculations [23, 26]. The variance homogeneity of the data was assessed using the Hartley test [27]. Data had heterogeneous variances between diets, and so non parametric tests were performed. Data for hexosamine ileal concentrations and outputs measured in three different groups of piglets (each group testing a different diet) were analyzed for the diet effect using the Kruskal-Wallis one-way analysis of variance [28]. Data for glycoprotein concentration and outputs in ileal digesta estimated with three different methods were analyzed for diet and method effects simultaneously using the Friedman two-ways analysis of variance. The correlations between the methods were evaluated by the non-parametric

test of Spearman. All statistical analyses were performed using StatView (Abacus Concepts, Inc., version 4.55, Berkeley, CA, USA). Differences were considered significant at the probability level of 0.05.

3. RESULTS

3.1. Isolation and characterization of small intestinal porcine glycoprotein

The analytical results of the glycoprotein fractions obtained from the second ultracentrifugation are presented in Figure 1. They show that fractions F3, F4 and F5 had the lowest nucleic acid content while fraction F5 had the highest glycoprotein content. The eight fractions collected after the second ultracentrigugation were also analyzed for purity using SDS-PAGE followed by PAS and Coomassie brilliant blue stainings (Fig. 2). The PAS coloration revealed that the glycoprotein was mostly present in the two bands of high molecular weight (> 97 kDa) (Fig. 2a). The heaviest one was located on the top of the separating gels and corresponded probably to non-fully reduced glycoprotein polymers which did not enter the separating gels. The second band migrated a little in the separating gel but still had a molecular weight higher than the heaviest molecular weight marker (97 kDa). These fractions did not apparently contain contaminating proteins as revealed by the Coomassie brilliant blue staining (Fig. 2b). Thus fraction F5 which had a density of 1.49 $g \cdot mL^{-1}$ was considered as the purest and kept for raising a hyperimmune plasma and as the glycoprotein standard in our ELISA (see the following sections).

The protein content of purified glycoprotein was 22.3% by weight (Tab. II). The major amino acids ($g \cdot 100\ g^{-1}$ of total assayed amino acids) were threonine (15.4), proline (11.5), glutamic acid + glutamine (11.3), aspartic acid + asparagine (9.2) and serine (8.8). The sialic acids corresponded to 3.1% by weight of the purified glycoprotein whereas galactosamine and glucosamine represented 3.4 and 3.7%, respectively.

Another set of SDS-PAGE gels followed by PAS and Coomassie brilliant blue stainings was also conducted with various purified and crude glycoprotein samples, ileal digesta and experimental feed (Fig. 3). Again, the PAS staining revealed two bands of high molecular weight glycoproteins, as described above, in all the samples, except the feed extracts (Fig. 3a). In addition a third stained band appeared at the bottom of the gel at the level of the solvent migration front for most samples, except our purified intestinal glycoprotein and the commercial sample of porcine gastric mucin. This staining probably corresponded to heavily hydrolyzed glycoproteins with very low molecular weights (<< 14.1 kDa). Coloration with the Coomassie brilliant blue (Fig. 3b) confirmed that our purified glycoprotein fraction was apparently not contaminated with proteins. This was also true for the commercial sample of porcine gastric mucin. In contrast, and as expected, the other samples displayed various patterns of proteins (although, for unexpected reasons, this staining was weak for the digesta samples otherwise well stained by PAS).

3.2. Characteristics and specificity of ELISA assay for porcine gastro-intestinal glycoprotein

The linear range of this ELISA assay was 4 to 150 $ng \cdot mL^{-1}$ of glycoprotein protein (Fig. 4). Intra-plate and inter-plate variations (n = 6) were 4.2 and 12.9%, respectively. Prior to the ELISA assay, the glycoproteins must be extracted from freeze-dried samples by denaturation with guanidine hydrochloride, reduction, and alkylation, to enhance glycoprotein solubility. The coefficient of variation of the resulting amount of collected protein varied between 11 and 20% whereas the final coefficient of variation of glycoprotein ileal ELISA data was 52 to 99% depending on the diet.

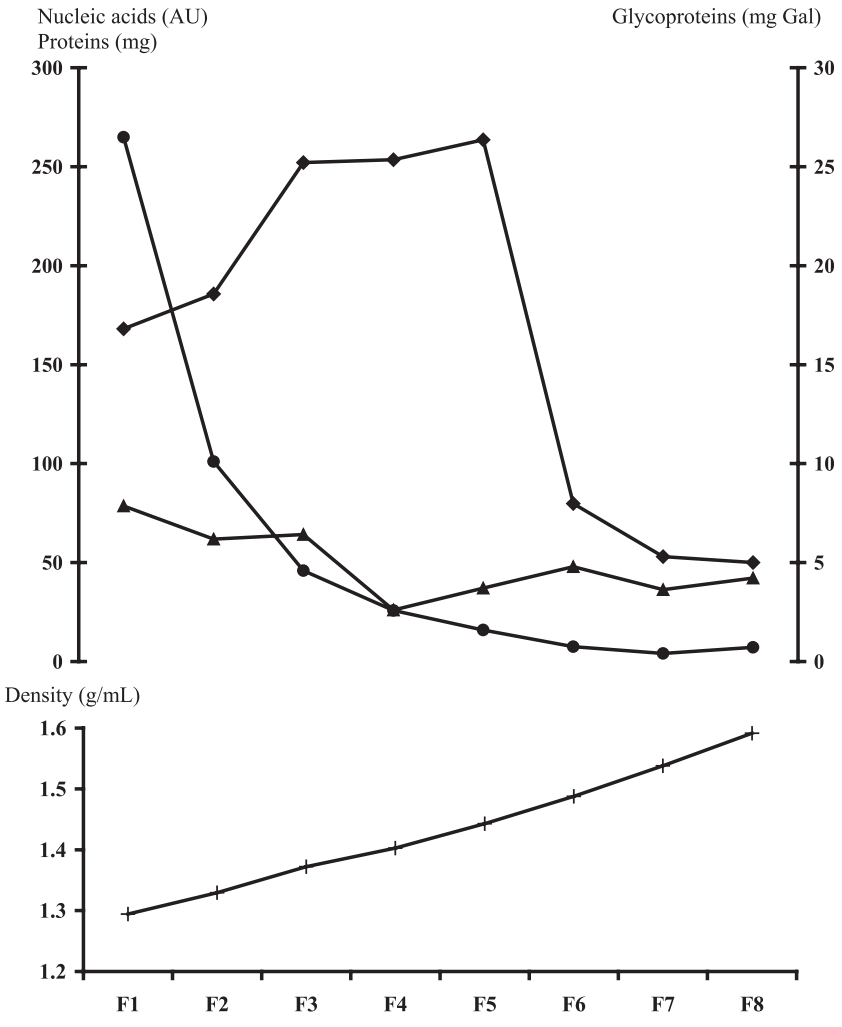

Figure 1. Cesium chloride density gradient profile after the second ultracentrifugation. Glycoprotein-containing fractions obtained after the first ultracentrifugation were pooled and CsCl was added to an initial density of 1.49 g·mL^{-1}. After ultracentrifugation (203 000 × *g*, 48 h, +12 °C), fractions were collected and analyzed for density (+), protein (●), glycoprotein (♦) and nucleic acids (▲). The F5 fraction is the one containing the purest porcine small intestinal glycoprotein. Therefore F5 was used to raise an hyperimmune plasma and served as the glycoprotein standard in the ELISA test set up. AU: arbitrary units. Gal: galactose equivalent.

The hyperimmune plasma raised against porcine gastro-intestinal glycoprotein did not recognize bovine casein or plant proteins including those from maize, black and white chickpeas used in our experimental diets for piglets. Surface glycoprotein recovered by gentle mucosal scraping from the small intestine of piglets displayed an average glycoprotein concentration of 40 μg·mL^{-1}. The limited number of individual or pooled samples assayed by ELISA suggested a high cross-recognition with porcine colonic and

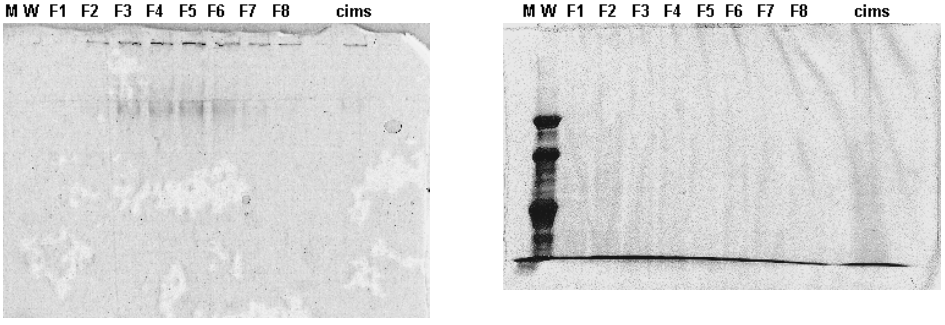

Figure 2. SDS-PAGE analysis of the glycoprotein fractions obtained after the second ultracentrifugation and of crude intestinal mucus scraping followed by PAS (a, left panel) and Coomassie brilliant blue (b, right panel) stainings. MW: molecular weight markers (kDa), F1 to F8: ultracentrifugation fractions, cims: crude intestinal mucus scraping. Amounts of protein deposited: 10 µg per lane.

Table II. Carbohydrate and amino acid composition of purified pig small intestinal glycoprotein.

Assayed carbohydrates (g·100 g^{-1} DM)[2]		Assayed amino acids (g·100g^{-1} AA)[1]			
		Indispensable		Dispensable	
N-acetylgalactosamine	3.4	Thr	15.4	Asx	9.2
N-acetylglucosamine	3.7	Val	5.3	Ser	8.8
Sialic acids	3.1	Ile	4.1	Glx	11.3
		Leu	6.2	Pro	11.5
		Phe	3.2	Gly	5.1
		Lys	4.8	Ala	5.8
		His	2.3	Tyr	2.6
		Arg	3.4	Cys	0.2
		Met	0.8		
		Protein (g·100g^{-1} of glycoprotein)			22.3

[1] Trypotophan not assayed. [2] Fucose, galactose, mannose, etc., not assayed.

gastric glycoprotein (Tab. III). Mucosal scrapings from the small intestine of calves contained 28 µg·mL^{-1} of piglet glycoprotein equivalent, also suggesting an important cross-recognition. By contrast, the limited numbers of scrapings or digesta samples collected in rabbits, chickens, rats and trouts suggested a lower cross-recognition with piglet gastro-intestinal glycoprotein.

The hyperimmune plasma raised against our porcine intestinal glycoprotein was also tested by western blotting on various samples (Fig. 5). Although this part was not completely optimized, this plasma clearly stained our purified glycoprotein. However, this staining appeared not to be restricted to the two bands revealed by the PAS staining (Figs. 2 and 3) but rather to cover a wide range of molecular weights. By contrast, the commercial porcine gastric mucin sample did not react at all despite its glycoprotein nature as revealed by PAS (Fig. 3). Stained smears, although less intense, appeared for piglet gastro-intestinal crude mucus samples and ileal digesta. A weak staining was evidenced with WCP and BCP feed extracts,

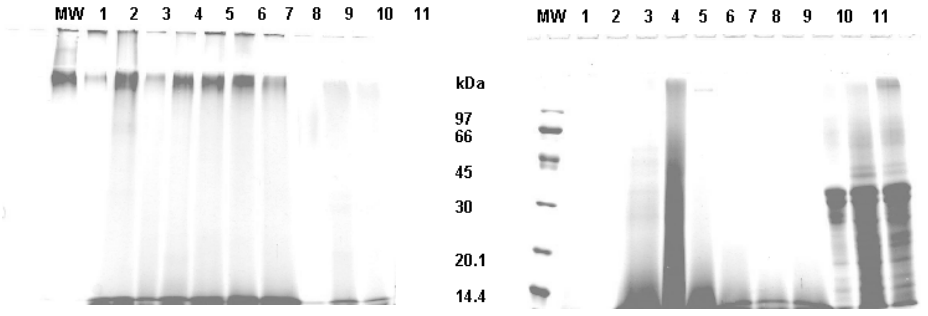

Figure 3. SDS-PAGE analysis of various samples followed by PAS (a, left panel) and Coomassie brilliant blue (b, right panel) stainings. MW: molecular weight markers (kDa), lane 1: fraction F5, lane 2: commercial porcine gastric mucus, lanes 3, 4 and 5: porcine crude gastric, small intestinal and colonic mucus scrapings, lanes 6, 7 and 8: ileal digesta of piglets fed the control, WCP and BCP diets, respectively, lanes 9, 10 and 11: extracts from the control, WCP and BCP diets, respectively. Amounts of protein deposited: lanes 1 and 2: 20 µg, lanes 3 to 8: 200 µg, lanes 9 to 11: 100 µg.

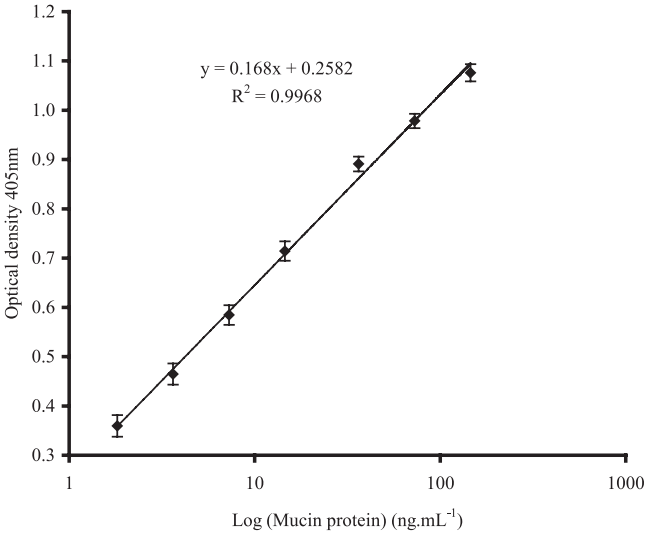

Figure 4. Standard curve of the ELISA assay (mean ± sem for 6 assays).

thus contrasting with the lack of recognition of these samples by ELISA.

3.3. Concentration and output of hexosamines and glycoprotein in the ileal digesta of piglets

Hexosamines were assayed in diets and in ileal digesta of the three groups of piglets (Tab. IV). Limited amounts of glucosamine and no galactosamine were detected in the diets based on chickpeas. In the ileal digesta, the concentration of glucosamine was significantly higher with WCP compared to the BCP diet ($P < 0.05$), the control diet giving an intermediate value. However, there was a tendency ($P < 0.10$) to get more galactosamine with BCP than with the control diet.

Table III. ELISA response for glycoprotein from different sites of the digestive tract of piglets and from different animal species.

Sample	No. of samples	Glycoprotein concentration (µg/mL)[1]	ELISA response (%)[2]
From different sites of piglet digestive tract			
Small intestine[3]	5	40 ± 13	100
Colon[3]	2 pools (n = 5)	32 ± 12	80
Stomach[3]	pool (n = 10)	63	156
From different animal species			
Calf[3]	7	28 ± 17	70
Rabbit[4]	3	10 ± 7	25
Chicken[4]	pool (n = 3)	15	40
Rat[4]	pool (n = 3)	12	24
Trout[3]	pool (n = 10)	2	5

[1] Means ± SD.
[2] Values are expressed as a percentage of the response measured with our purified pig gastro-intestinal glycoprotein.
[3] Glycoprotein in mucosal scraping.
[4] Glycoprotein in intestinal digesta.

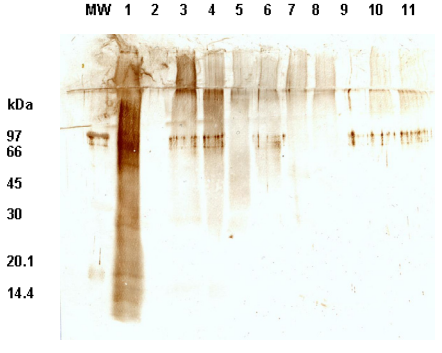

Figure 5. Western blot analysis of various samples. MW: molecular weight markers (kDa), lane 1: fraction F5, lane 2: commercial porcine gastric mucus, lanes 3, 4 and 5: porcine crude gastric, small intestinal and colonic mucus scrapings, lanes 6, 7 and 8: ileal digesta of piglets fed the control, WCP and BCP diets, respectively, lanes 9, 10 and 11: extracts from the control, WCP and BCP diets, respectively. The amounts of protein deposited per lane are the same as those mentioned in the legend of Figure 3. Antibody dilutions: anti-porcine glycoprotein hyperimmune plasma: 1:312, conjugated rabbit antibody: 1:600.

The glucosamine and galactosamine ileal outputs were higher ($P < 0.05$) with WCP than with the control. The BCP diet gave an intermediate value without any significant difference from the WCP and control diet.

Glycoprotein concentrations and outputs in the ileal digesta of piglets as determined by the three tested methods, are compared in Table V. The ELISA test did not provide evidence of significant differences in glycoprotein concentration or output between the diet treatments. By contrast, the two other methods revealed a significant diet effect ($P < 0.05$) for both glycoprotein ileal concentration and output.

3.4. Comparison between the three glycoprotein assay methods

Significant differences between the methods were evidenced for both glycoprotein ileal concentration and output in the case of the control and BCP diets ($P < 0.05$). By contrast, such differences did not reach the significance level in the case of the WCP

Table IV. Hexosamine concentration in ileal digesta and output at the ileum of piglets.

Hexosamines (in % of DM)	Diet[1]			*P* diet[2]
	Control	WCP	BCP	
Glucosamine	0	0.35	0.23	
Galactosamine	ND	ND	ND	
Ileal concentration (% of DM)				
Glucosamine	0.8^{ab}	1.2^{a}	0.7^{b}	0.01
	(0.6–0.9)	(0.8–1.3)	(0.7–0.9)	
Galactosamine	0.5	0.73	0.6	0.09
	(0.4–0.7)	(0.5–1.1)	(0.5–0.7)	
Ileal output ($g \cdot kg^{-1}$ of DMI)				
Glucosamine	1.1^{b}	2.3^{a}	1.7^{ab}	0.01
	(0.9–1.7)	(1.4–2.9)	(1.4–2.2)	
Galactosamine	0.7^{b}	1.5^{a}	1.4^{ab}	0.02
	(0.6–1.3)	(0.9–2.4)	(1.2–1.7)	

Values in ileal digesta are medians with minimum and maximum values below.
[a,b] Medians with different superscript letters in a row are different ($P < 0.01$).
[1] Diets supplemented with white (WCP) or black (BCP) chickpea.
[2] Probability of a diet effect tested with the Kruskal-Wallis rank test.
ND: not detected.

Table V. Mucin concentration in ileal digesta and output at the ileum of piglets estimated by different methods.

Hexosamines (in % of DM)	Diet[1]			*P* diet[2]
	Control	WCP	BCP	
Ileal mucin concentration (% of DM)				
Hexosamine method	3.8^{x}	5.7	3.8^{x}	0.074
	(3.0–4.5)	(3.6–6.6)	(3.5–4.7)	
ELISA	1.3^{y}	1.7	1.9^{y}	0.819
	(0.9–3.4)	(0.8–8.1)	(0.9–3.7)	
Precipitation	$2.6^{ab, y}$	3.9^{a}	$2.9^{b, y}$	0.022
	(2.2–3.6)	(3.7–5.3)	(2.5–3.2)	
P method[2]	0.041	0.311	0.006	
Ileal mucin output ($g \cdot kg^{-1}$ of DMI)				
Hexosamine method	5.0^{x}	11.4	8.6^{x}	0.091
	(4.2–8.8)	(6.9–14.8)	(7.6–11.0)	
ELISA	1.6^{y}	3.2	4.2^{y}	0.549
	(1.3–6.7)	(1.4–18.3)	(5.8–7.7)	
Precipitation	3.9^{b}	7.6^{a}	6.7^{ab}	0.015
	(2.9–5.2)	(6.8–11.6)	(5.8–7.7)	
P method[2]	0.041	0.311	0.006	

Values in ileal digesta are medians with minimum and maximum values below.
[1] Diets supplemented with white (WCP) or black (BCP) chickpea. Medians in the same raw with different letters (a,b) differ between diets. Medians in the same column with different letters (x,y) differ between methods.
[2] Probabilities of diet and method effects tested using the Friedman rank test.

diet. There were significant correlations between the precipitation and hexosamine methods (ρ Spearman = 0.73, $P < 0.01$) and the hexosamine and ELISA assays (ρ = 0.71, $P < 0.01$). By contrast, a lower correlation was obtained between the ELISA and the ethanol precipitation method (ρ = 0.43, $P < 0.10$).

4. DISCUSSION

4.1. Isolation and characterization of small intestinal porcine glycoprotein

The porcine intestinal glycoprotein purified here presented a high molecular weight (> 97 kDa). Its PAS staining pattern was very similar to those published for bovine [21] and rat [29] intestinal glycoproteins. Since the work in rats [29] was carried out with a proteolysis step in order to keep the protease-resistant glycoprotein as the material of interest, it is probable that our present purified glycoprotein corresponded to this protease-resistant part. The apparent molecular weight of bovine intestinal glycoproteins was estimated to be between 250 and 300 kDa [21]. Here, we did not attempt to determine more precisely the molecular weight of our purified glycoprotein. Silver staining which is more sensitive than Coomassie brilliant blue did not reveal contaminating proteins in the purified bovine glycoprotein [21]. Based on this observation from our laboratory, we did not carry out silver staining of SDS-PAGE gels because purification procedures for bovine and porcine intestinal glycoprotein were essentially similar.

In the purified piglet glycoprotein, the protein fraction represented 22.3% of the whole molecule, a value similar to that found in the adult porcine mucin (21%) [12], but higher than that found in rat (12–16%) or human mucin (12%) and lower than that found in bovine mucin (53%) [21, 30]. The total protein content of mucin seems to be greater in young animals compared to adults [31], suggesting increased glycosylations over lifetime. Our piglet glycoprotein had a high content of threonine, serine and proline residues which are characteristic of mucus glycoprotein. We found that our piglet glycoprotein samples had an AA composition relatively close to that of humans ($\chi^2 = 15$) and rats ($\chi^2 = 60$) but substantially different from that of adult pig ($\chi^2 = 210$) and calf ($\chi^2 = 230$) glycoprotein. In comparison to glycoprotein from the adult pig, our purified fraction contained more aspartic acid + asparagine, glutamic acid + glutamine and alanine but less arginine and cysteine. This difference may be explained by a modification of glycoprotein composition with animal age, as reported between newborn and adult rats [31]. It is also possible that the considerable polymorphism demonstrated for mucin alleles [32] and the size heterogeneity of biochemically purified mucin [1] were implicated. The low amount of cysteine in our purified mucin sample could be explained by the fact that this AA is particularly well represented at the terminal parts of the glycoprotein, which are poorly glycosylated and thus very sensitive to protease attacks [1]. As mentioned above, our purified fraction was probably the protease-resistant part of the glycoprotein. In addition, we cannot rule out inter-laboratory assay differences.

The proportion of galactosamine, glucosamine and sialic acid (3.4, 3.7 and 3.1% by weight, respectively) are lower than those measured in adult pig intestinal mucin [12]. In rats, a lower proportion of hexosamines in young animals compared to adults has also been observed [31, 33]. The glycosylation level of mucin depends on the AA composition and sugar chain elongation [1]. The expression of glycosyltransferases which control the quality of glycan chains is regulated during postnatal development [34].

The sugar and assayed AA of our purified glycoprotein sample revealed a yield much less than 100% (32.5%). This difference may be accounted for by non analyzed AA (tryptophan), sugars (fucose, galactose, mannose), sulfate and other compounds [12].

Unfortunately, due to the very low amounts of highly purified glycoprotein produced here, we were unable to carry out all these extra analyses.

4.2. Hyperimmune plasma characterization and ELISA assay set up

Our purified glycoprotein was used to produce a specific hyperimmune plasma for setting up an ELISA. Our limited investigations using Western blotting showed this plasma to bind our purified fraction as well as porcine gastro-intestinal crude mucus and ileal digesta. However, this staining pattern was rather broad even in the case of our purified material. This is in contrast with our previous observations with purified bovine glycoprotein [21]. It suggests a degradation of our porcine glycoprotein with time since Western blot was conducted approximately two years after having purified the porcine glycoprotein. However, corresponding SDS-PAGE gels stained PAS conducted either at the time of purification (Fig. 2) or very recently (Fig. 3) displayed essentially the same pattern of glycoprotein staining. This was also true for crude gastro-intestinal glycoprotein preparations and ileal digesta. Thus it is possible that limited glycoprotein degradation, although not revealed by either PAS or Coomassie brilliant blue, might have been sufficient to be detected by our hyperimmune plasma.

In our procedure, the glycoprotein to be assayed was directly coated on the ELISA plate by non-covalent binding. The direct ELISA, as compared with competitive or sandwich designs, often leads to decreased detectability and increased variability, as a consequence of antigen desorption at each step. Moreover, adsorption of the antigen on the plate could mask some epitopes and diminish the response. However, in the case of gastro-intestinal glycoproteins, these phenomena were probably limited because of its chemical characteristics, particularly its high molecular weight (>> 97 kDa). A major reason for developing a direct ELISA assay was the difficulty to solubilize the intestinal glycoprotein. This setup allowed removal of the excess of guanidine hydrochloride, necessary for solubilization of mucin, by extensive washing before immunological reaction. Similar direct ELISA assays for intestinal glycoproteins of calves and rats have been successfully developed in our laboratory [21] and elsewhere [35], respectively.

Our ELISA assay had a linear range of 4 to 150 $ng \cdot mL^{-1}$ of glycoprotein protein. This range was intermediate between other direct ELISA assays for human (2 to 15 $ng \cdot mL^{-1}$) [36], rat (0 to 20 $ng \cdot mL^{-1}$) [35] or preruminant calf (20 to 640 $ng \cdot mL^{-1}$) [21] small intestinal glycoprotein. The ELISA intra- and inter-plate variabilities were very similar between porcine (present work) and bovine glycoprotein (previous work, [21]).

The hyperimmune plasma cross-reacted with crude mucus from various regions of the pig gastrointestinal tract including the stomach, small intestine and colon. This was also described in the literature for bovine [21], human [36] and rabbit [37] gastro-intestinal glycoprotein. This suggests the presence of common antigenic determinants among these glycoproteins both within and between animal species. From the intestinal digestion perspective, this also implies that the glycoprotein concentrations and outputs we determined using the present ELISA included both gastric and intestinal components. The relative proportion of gastric and intestinal glycoprotein contained in the ileal digesta cannot be determined by our approach. By contrast, in some studies, regression equations derived from hexosamine ratios in purified gastric glycoprotein were used to calculate its contribution to ileal digesta glycoprotein [10].

4.3. Comparison between the three glycoprotein assay methods

The principles of the three methods compared are very different, thus probably limiting the comparisons, but contributing certainly to explain why correlations among

them were neither systematic nor strong. Glycoprotein ileal average/median concentration and output data usually ranged in the following order: hexosamines > ethanol precipitation > ELISA, the latter method providing data two- to three-folds lower that the other methods. It is probably hazardous to compare the absolute values because none of them can be considered as the reference. It is commonly accepted that galactosamine is particularly well suited for estimating glycoprotein on the basis of hexosamines because of its limited occurrence in diets and other endogenous sources, including non-mucin glycoconjugates such as glycolipids and proteoglycans from bacteria or epithelial cells [23]. However, this method bears the problem related to the presence of hexosamines (essentially glucosamine here) in the diet. It is hardly possible to envisage any correction since, to the best of our knowledge, literature is lacking on the degradation and absorption of hexosamines in the small intestine. Removing 100% of the dietary glucosamine contribution from the ileal output led to negative net values for the WCP and BCP diets. Thus we decided not to make any correction thus following calculations reported in previous studies [23, 26]. The ELISA method, although considered as the most specific for intestinal glycoprotein, suffers from the lack of demonstrated absolute specificity. In addition, a factor (1.6) was needed for correcting the protein assay values using the Bio-Rad method for taking into account the amount of amino acids assayed (although not all determined) in the purified sample. Having retained the correction factor determined for crude intestinal mucus scrapings (3.1) would have brought ELISA values closer to those obtained by the other methods. However, we did not use this ratio because it was the purified, and not the crude, glycoprotein which we used as the standard in our ELISA. On the contrary, it seems reasonable to consider having lower glycoprotein values with ELISA because the other methods are probably less specific. The third method using, ethanol precipitation, is rather non-specific as other precipitation methods. It has been shown to denature human gastric mucin structure, probably through conformational alterations of both the glycosylated and protein components of the molecule [38]. The fact that our ELISA was unable to reveal any immunoreactivity of the ethanol precipitates in the present study suggests such a structural denaturation for porcine intestinal glycoprotein. Although authors having used this precipitation method stated that the precipitate was composed mainly of raw mucus with limited contamination with covalently bound proteins [11], to our knowledge, this precipitation has not been explored further. Analysing glycoprotein ethanol precipitates using SDS-PAGE followed by PAS and Coomassie brilliant blue (or silver) stainings might provide some insights into what is precipited. The fact that comparing these methods is not straightforward is also suggested by the moderate to low correlations among these methods.

The ileal glycoprotein output with the control diet was 5.0, 1.6 and 3.9 $g \cdot kg^{-1}$ of DMI measured by the hexosamine method, ELISA and ethanol precipitation, respectively. The values obtained by the two latter methods are comparable to those obtained with the hexosamine method by Lien et al. [10] in 50 kg body weight pigs fed a nitrogen-free diet (3.8 $g \cdot kg^{-1}$of DMI), and that measured by ELISA in calves fed a milk replacer based on skim milk powder (3.4 $g \cdot kg^{-1}$ of DMI) [21].

One important point which comes out of these methodology comparisons for intestinal glycoprotein evaluation is the higher overall variability of concentration data with ELISA (52 to 99%, depending on the diet) as compared to hexosamine (12 to 18%) and ethanol precipitation (10 to 20%) methods. Individual variabilities in glycoprotein ileal concentrations determined by ELISA in the bovine study [21] amounted to 26 to 30%. Thus, ELISA variability in the present study was twice to three-folds higher than in our work with bovine glycoprotein. Since the ELISA intra- and inter-plate variabilities

were close between these two studies, it seems that the present ELISA was more variable, possibly due to the higher complexity of the piglet, as compared to the preruminant calf ileal digesta, but also to larger animal variations. By contrast, the two other methods provided more limited individual variability, twice lower as that for bovine glycoprotein ELISA [20] but four-folds lower than the present ELISA. Whether this can be related to the fact they are also less specific than ELISA, and therefore contribute to level individual variability, raises the question of the actual biological variability of glycoprotein ileal concentration and outputs.

4.4. Ileal glycoprotein output and differences between diets

A significant increase in the ileal glycoprotein output was observed when feeding the WCP diet as compared to the casein based diet as the control. This increase was significant with the hexosamine and ethanol precipitation methods but not with the direct ELISA. Chickpea seeds are rich in anti-nutritional factors such as α-galactosides, trypsin inhibitors, tannins and saponins [39, 40]. These constituents are known to alter the intestinal brush border and/or the digestion process [41]. So, the enrichment of the digesta in glycoprotein could be a defence mechanism to protect gastrointestinal mucosa.

There are few data on the effects of individual dietary constituents on gastro-intestinal glycoprotein secretion. The existing studies have mainly examined the effect of carbohydrates including dietary fiber. In the pig, addition of maize starch increased the galactosamine output from 0.64 to 1.38 $g \cdot kg^{-1}$ of DMI [42] and increased fiber intake enhanced hexosamine excretion [26, 43]. When increasing amounts of pea fiber were added to the pig diets, there was a trend towards a linear increase in the ileal glycoprotein output [44]. Additionally, the concentrations of lumenal immunoreactive glycoprotein were higher in the stomach (+350%) and small intestine (+200%) of rats fed a diet containing 5% citrus fiber, compared to those fed a fiber-free diet [35]. In the present study, differences in fiber contents between the experimental diets could partly explain those in the ileal glycoprotein output observed.

The differences between diets might also be an effect of the protein source. Indeed, in calves fed milk replacer diets based on plant protein, the output of glycoprotein protein increased by 70% at the duodenum and jejunum when skim milk powder was partially replaced by either a soybean protein concentrate or partially hydrolyzed isolate [21]. Glycoprotein output was shown to increase at the duodenum (+24%) and ileum (+52%) with potato protein concentrate [21]. In an in vitro model of the rat jejunum, two enzymatically hydrolyzed milk protein sources, namely casein and lactalbumin, induced mucin release in the lumen [7]. Thus, direct or indirect specific interactions could exist between dietary proteins and/or peptides resulting from their hydrolysis and goblet cells for releasing mucin. This hypothesis is supported in rats fed with a diet supplemented with purified phaseolin, peptides derived from its digestion being strongly fixed to the intestinal mucosa and probably acting as glycoprotein secretagogues [45].

To conclude, the ELISA assay is probably the most specific method but glycoprotein ileal concentrations and outputs appeared to be more variable than those obtained with the hexosamine and ethanol precipitation methods. The hexosamine calculation and the ethanol precipitation methods appeared to be interesting alternatives to ELISA but they are probably less specific and the precipitation method needs much larger amounts of samples (3 g DM). These methods thus await further work. Investigations are in progress to determine the impact of dietary constituents on the glycoprotein output at the end of the small intestine in weaned piglets.

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