APPROACHES TO ISOLATE MUCIN AND THREONINE UTILIZATION IN THE GUT

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APPROACHES TO ISOLATE MUCIN AND THREONINE UTILIZATION IN THE GUT

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Abstract

Intestinal mucin synthesis is sensitive to dietary threonine supply which suggests that the gut's requirement for threonine may comprise a significant proportion of the whole body requirement. We used a continuously perfused gut loop model and intraluminal flooding dose technique in six young pigs to study the acute effects of varying luminal availability of threonine on intestinal protein and mucin syntheses. A complete amino acid mixture containing 0, 75 or 200% of the whole body threonine requirement was continuously perfused in isolated loops for 120 min, including a 30 min ^{3}H-phenylalanine flooding dose. Fractional synthesis rates of total mucosal protein and mucin were measured by analyzing ^{3}H-phenylalanine incorporation. Fractional rates of total mucosal protein synthesis were significantly higher in loops perfused with solutions containing threonine at 200% (66 ± 4 %/d) compared to 0% (42 ± 9 %/d) and 75% (53 ± 6 %/d) ($P < 0.05$). For mucin, fractional rates of synthesis were significantly different between 0% (323 ± 72 %/d), 75% (347 ± 49 %/d) and 200% (414 ± 31 %/d) ($P < 0.05$).

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Abbreviations

GIT	Gastrointestinal Tract
BSA	bovine serum albumin
CsCl	cesium chloride
DTT	dithiothreitol
ELISA	enzyme linked immunosorbent assay
GuHCl	guanidium hydrochloride
LLO	listeriolysis O
PAS	Periodic acid/Schiff`s base
RER	rough endoplasmic reticulum
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate – polyacrylamide gel eletrophoresis
YE	Yersinia enterocolitica
TPN	Total Parenteral Nutrition
PDV	Portal Drained Viscera
IgA	Immunoglobulin A
TCA/PCA	Trichloroacetic acid/Perchloric Acid
^{3}H-Phe	Tritiated Phenylalanine
PBS	Phosphate Buffered saline
MWCO	Molecular Weight Cut Off
HCl	Hydrochloric Acid

Abs	Absorbance
ID	Inner Diameter
OD	Outer Diameter
NaCl	Sodium Chloride
H_2O	Water
NaOH	Sodium Hydroxide
TEA	Triethanolamine
PITC	Phenylisothiocyanate
PSR	Protein synthesis rate
ATP	Adenosine Triphosphate
TTBS	Combination of 20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween
AP	Aminopyridine

1.0 Review of Literature

1.1 Small intestine physiology

Absorption is the primary physiological role of the small intestine. The surface of the small intestine, or gut, is covered with millions of projections called villi which serve to increase the surface area of the small intestine. This increase in surface area allows for optimal absorptive capacity of the gut. At the base of the villus is the crypt where multipotent stem cells undergo mitosis. After proliferation, new crypt cells differentiate and begin to mature and migrate up to the villus tip. Most of these cells will mature into absorptive enterocytes while the remaining cells will become goblet cells **(Figure 1.1);** Paneth and enteroendocrine cells also derive from crypt stem cells.

A secondary role of the small intestine is protection. The gastrointestinal tract (GIT) is the first site of exposure to bacteria and toxins that may be ingested from the environment. The cells of the mucosal epithelium that are critical to this protection include goblet cells. These cells synthesize and secrete glycoproteins called mucins. These mucins are water-insoluble and are responsible for the gel-like properties of the mucus layer (Deplancke & Gaskins, 2001). The mucus gel layer that lines the lumen of the intestine is a mixture of glycoproteins, water, cellular macromolecules, electrolytes, microorganisms, and sloughed cells (Faure et al., 2002).

The mucus lining covers the epithelial surface from the mouth to the large intestine and varies in thickness with the thinnest portion lying in the small intestine (Deplancke & Gaskins, 2001). Atuma et al. (2001) used an in vivo model and a micropipette technique to observe the mucus layer. They observed that the mucus layer can actually be divided into two distinct layers, loose and firmly attached. They noted that the thickness of the mucus layer is actually thicker than some of the previous *in vitro* studies have noted. As well, in the small intestine, when the loose layer was suctioned off, a very thin discontinuous mucus layer remained. This did not occur in any other areas of the gastrointestinal tract.

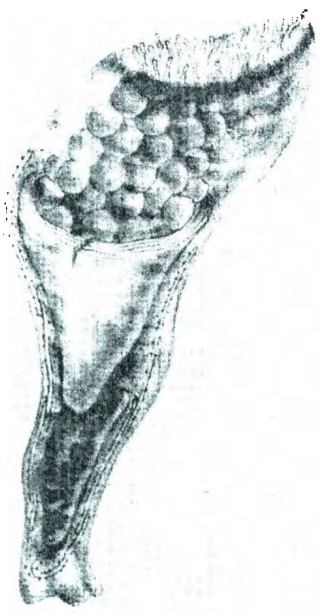

Figure 1.1. Mature colonic goblet cell. The mucin granules are located in the apical side of the goblet cell. (Adapted from Specian and Oliver, 1991).

1.2 Structure of mucin

Mucin is a glycoprotein which contains both protein and carbohydrate components in its structure. Mucin consists, by mass, of 10-20% protein and 80-90% carbohydrate. The protein backbone is primarily composed of threonine, serine and proline (**see Table 1.1**). These three amino acids make up more than 40% of the total amino acid composition (Allen et al., 1982). The five types of carbohydrates in the oligosaccharide side chains are N-acetylglucosamine, N-acetylgalactosamine, fucose, galactose and sialic acid. Depending on the carbohydrate composition, mucins are either neutral or acidic. The acidic mucins can be further categorized as sulfated (sulfomucin) or nonsulfated (sialomucin). The oligosaccharide side chains are often terminated by sialic acid or a sulfate group providing them with a negative charge (Allen et al., 1982). The immature goblet cells contain mostly neutral mucins; however, as goblet cell maturity occurs, the composition changes to a higher percentage of sialated mucins. The oligosaccharides prevent the formation of a helix and prevent bending of the molecule (Lamont, 1992).

Small intestinal mucin has a molecular weight of 2×10^6 daltons and consists of 8 subunits which are connected by disulfide bonds. There are 78 disulfide bridges per molecule (Starkley et al.,1974). These subunits have a molecular weight of 2.4×10^5 Da and become separated from each other when the glycoprotein has been exposed to proteolytic attack (Mantle et al., 1981).

Table 1.1 Amino acid composition of the small intestinal mucus glycoprotein
Adapted from Mantle & Allen (1981).

Amino Acid	(nmol/mg dry wt of glycoprotein)	(mol/100 mol of the protein)
Lys	39 ± 7	2.22
His	24 ± 2	1.37
Arg	39 ± 4	2.22
Asp	81 ± 10	4.63
Thr	464 ± 35	26.53
Ser	182 ± 10	10.40
Glu	74 ± 7	4.20
Pro	270 ±13	15.44
Gly	98 ± 4	5.60
Ala	66 ± 6	3.77
'Cys	75 ± 2	4.28
Val	125 ± 11	7.15
Met	22 ± 4	1.25
Ile	55 ± 6	3.14
Leu	75 ± 6	4.28
Tyr	26 ± 9	1.46
Phe	35 ± 9	2.00

Mucin has a linear peptide core with oligosaccharides radiating out from the central core. The structure of mucin is often referred to as a "bottle brush" structure with the bristles of the brush being the carbohydrate chains and the wire being the protein core (Allen et al., 1982) **(Figure 1.2)**. This structure allows for two distinct protein core regions: the glycosylated or native area, and the non-glycosylated or "naked" region. The carbohydrate portion of the glycoprotein is linked by O-glycosidic bonds to threonine or serine. These carbohydrates are so tightly packed together that they serve as an area of protection of the protein core from any enzymes present in the gut. It is the non-glycosylated areas which are subject to degradation (Allen et al., 1982). The non-glycosylated region is globular in shape and is rich in glutamic acid, aspartic acid and other acidic amino acids. As well, it is due to the high degree of glycosylation that mucin has a density of around 1.4 g/ml (Van Klinken et al., 1998). Purification techniques make use of the high degree of glycosylation of this glycoprotein.

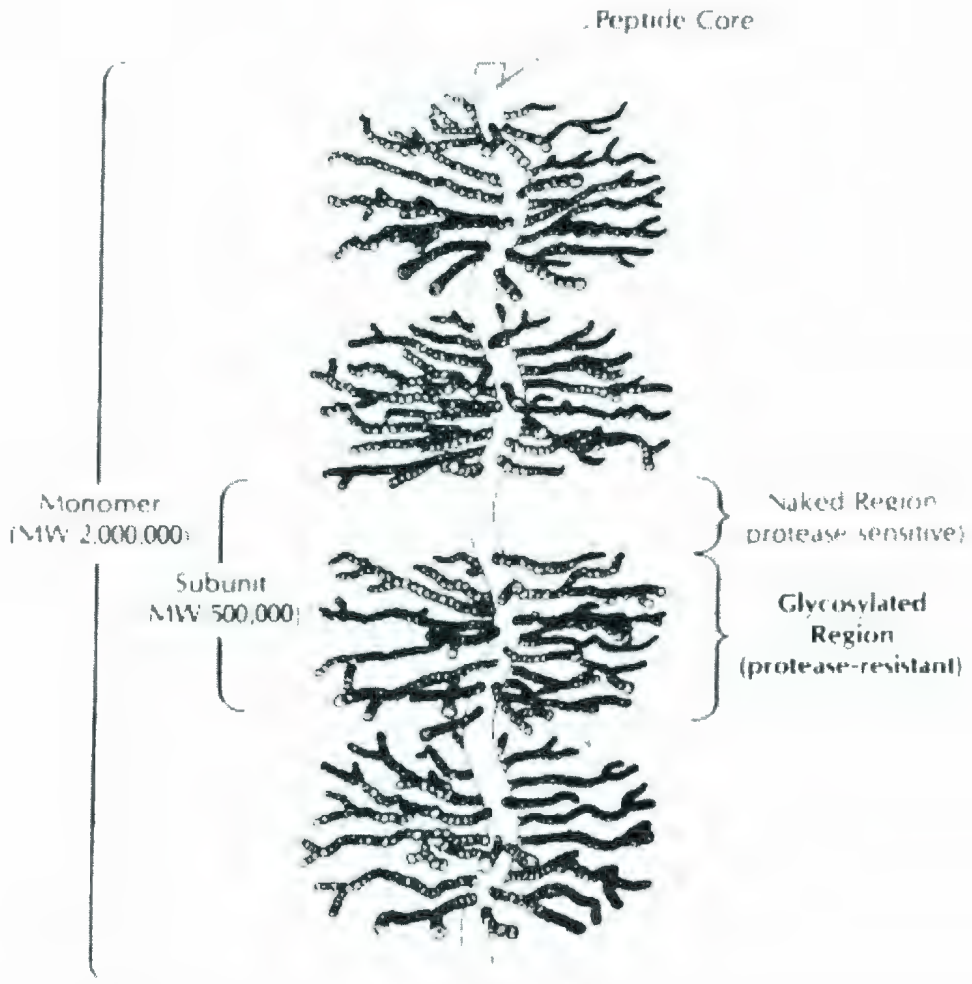

Figure 1.2. Non-glycosylated regions and "bottle brush" appearance of glycosylated regions and of the mucin monomer. (Adapted from Lamont, 1992)

Scawen & Allen (1976) studied the structure of mucin and its subunits when subjected to proteolytic attack. They noted that when observed alone, the mucin subunits contain the same amount of carbohydrate as the original polymer. However, the difference lies in the amino acid content. Threonine, serine and proline constitute 18.6 – 25.6% of the undigested glycoprotein which is increased to 61-67% in the cleaved glycoprotein. The digested area found in the non-glycosylated regions of the glycoprotein is rich in the amino acid cysteine which allows for the formation of disulfide bridges (Allen, 1981). The non-glycosylated region makes up 25% of the protein portion of glycoprotein (Clamp et al., 1978).

Bell et al. (1985) studied pig gastric and duodenal mucus and discovered that the mechanical properties of these two mucus layers are similar to those found in human mucus layers of the same organ. This indicates that pig mucus is a good model for human mucus layers. Most mucins found in mammals have similar structures (Lamont, 1992). The neonatal piglet is an effective model for studying gut metabolism since it is very similar physiologically and metabolically to the human infant (Mantle et al., 1981).

1.3 Role of mucus in the gastrointestinal tract

The mucus layer serves many physiological functions. As mentioned previously (section 1.1), the mucus gel layer protects the underlying epithelium from various luminal factors. For example, it serves as a physical barrier since it is the first site of exposure of the

intestinal epithelium to gut bacteria (Deplancke & Gaskins, 2001). As well, the mucus layer acts as a lubricant by coating various particles, non-digested food and sloughed cells in order to facilitate their clearance through the intestine (Faure et al., 2002). It also aids in transport between the lumen and the epithelial lining in addition to regulating hydration of the epithelium (Deplancke & Gaskins, 2001). The mucus gel layer also protects the epithelium of the intestine from mechanical damage that may occur during digestion and through the corrosive action of acidic gastric juice and proteolysis by digestive enzymes (Montagne et al., 2004). The mucus layer of the intestine is also a free radical scavenger (Cross et al., 1984) and a selective barrier (Allen et al., 1985). Overall, the mucus layer plays an important role in the overall nutrition and health of the animal due to its role in protection and maintenance of adequate functionality.

The carbohydrate structure of the mucin glycoprotein provides a function in the lumen of the gastrointestinal tract as well. The oligosaccharide chains can immobilize enzymes which prevent them from being removed by peristalsis thereby improving their utilization efficiency (Montagne et al., 2004). The carbohydrate chains also provide attachment sites for immunoglobulins and lysozymes to ensure that they are maximally utilized in the lumen. If this binding did not occur, these useful proteins would be washed away and degraded by bacteria present in the lumen (Lamont, 1992). The side chains also play an important role in protection from pathogens and toxins by serving as an area of attachment for various bacteria thereby preventing bacterial access to the epithelial cells and allowing easy removal of any invading organisms (Lamont, 1992).

The non-glycosylated protein core is hydrophobic and may be involved in binding other molecules such as lipids and other proteins (Lamont, 1992), while the oligosaccharide chains are hydrophilic and are therefore involved in binding water. These properties account for the gel-forming properties of mucin (Lamont, 1992).

1.4 Synthesis of mucin and regulation of secretion

Mucin is synthesized in intestinal goblet cells. Since the life span of mucin is approximately 3 days from production in the goblet cell to being secreted into the lumen, the regulation of its release is an area of interest. The process of mucin synthesis originally occurs in the rough endoplasmic reticulum (RER) where it is then passed to the Golgi apparatus. Here it is glycosylated and packaged into a membrane-bound mucin granule. The addition of carbohydrates to mucin is facilitated by membrane-bound glycosyltransferases which transfer monosaccharides from nucleotide sugar donors in the Golgi apparatus. The addition of sulfate groups for the termination of the oligosaccharides is accomplished by transfer from 3'-phosphoadenosine-5'-phosphate by Golgi sulfotransferases (Deplancke & Gaskins, 2001).

Upon development, the immature goblet cells, which contain only a few mucin granules and are found only in the crypts, begin producing mucin granules immediately. The immature goblet cells are large and have the shape of a pyramid and contain organelles which are located between the mucin granules. As the cell moves towards the villus tip, it decreases in volume through the loss of cytoplasm and some of the organelles. As the

movement continues and the volume decreases, the mature goblet cell takes a cup-like shape with the apical portion filled with mucin granules and the narrow basal side containing the remaining organelles and the nucleus. The mature goblet cells are located in the intestinal glands and the villi and contain many mucus globules. When the goblet cells release a mucus granule, the mucus is released and covers the epithelial surface.

The mucus layer is not static in thickness due to removal by luminal contents. Therefore, in healthy animals, there is a continuous balance being achieved between the production of mucin on the mucosal side and removal on the luminal side (Lamont, 1992). The integrity of the mucus layer is maintained by the process of basal or continuous slow exocytosis (Oliver & Specian, 1995). Basal secretion is used for proper maintenance of the mucus layer which would be needed to replenish mucin lost due to erosion and digestive processes. This process occurs by releasing a single mucin granule. When goblet cells are exposed to various stimuli or secretagogues **(Section 1.5)**, an increase in the release of mucin is observed due to acceleration of secretion. During this process many granules move to the plasma membrane of the goblet cell and fuse. At the same time, all of these granules are released expelling their contents (Lamont, 1992). The factors that stimulate the process of mucin secretion are not fully understood. The goblet cell, after expelling its contents, is able to restore its mucin content within 1-2 hours and can participate in baseline or accelerated secretion again (Lamont, 1992). The process of mucin secretion can be accelerated in the presence of a variety of materials known as mucin secretagogues.

1.5 Mucin secretagogues

The regulation of the release of mucin plays a part in the protective role of the mucus layer. The mucus layer has been shown to interact with various luminal factors which alter the secretory activity of the goblet cells. These factors are known as secretagogues. This is an interesting area of research, since an increased release of mucin may possibly alter the amount of threonine required to maintain the production of mucin, and in turn the protective capacity of mucus. Studies have been conducted on the effects of secretagogues, such as fiber, protein, bacteria, drugs and hormones, antinutritional factors and short chain fatty acids, on mucin secretion. These studies are summarized below.

1.5.1 Fiber

There have been numerous studies performed using fiber as a secretagogue (Lien et al., 1996, Satchithanandam et al., 1996). Barcelo et al. (2000) observed the effect of various food components using the isolated perfused rat colon. They looked at the effect of algal polysaccharide alginate since this is a component of gelling agents, thickeners, stabilizers or emulsifiers used in food. They found that alginate increased mucin secretion. Furthermore, they found that luminal administration of ulvan (a fiber extracted from green seaweed) and glucuronic acid (a component of ulvan) also caused an increase in mucin secretion indicating a role of glucuronic acid in the secretion process.

In another study, a wheat-based diet was supplemented with pea fiber and an increase in the ileal output of mucin was observed (Lien et al., 2001). An increase in ileal output of mucin in pigs fed a diet containing fiber from wheat straw, corn cob and wood cellulose has also been observed (Mariscal-Landin et al., 1995). When fed a diet containing bran, the goblet cell numbers increased when compared to a fiber-free control group (Dunsford et al., 1991). As well, with the use of labeled glucose and sulfate, it was observed that there was an increase in incorporation of these molecules into mucin when rats were fed a diet containing wheat bran and cellulose as compared to the control (Schmidt-Wittig et al., 1996). When exposed to citrus fiber, the stomach and small intestine of the rat increased mucin secretion by 390% in the stomach and 210% in the small intestine (Satchithanandam et al., 1990). Lundin et al. (1993) also showed that when oat bran, rye bran and soybean hull were supplemented into a low-fiber diet, there was an increase in the volume and number of goblet cells in the jejunum and ileum of hamsters.

As indicated, fiber and mucin secretion has been studied extensively over the years. There is a well-defined link between fiber and an increase in mucin secretion. Allen (1981) determined that fiber's role in mucin secretion is due to its effect on erosion of the mucus layer of the epithelium and the effect it has on enzymatic proteolysis. Insoluble fiber abrades the mucus layer causing mucin to be sloughed off in larger quantities than normal. Chemically, fiber alters enzymatic activity and the distribution of enzymes in the lumen which plays a role in mucin secretion (Schneeman et al., 1982). Fiber and its role on mucin secretion has proven to be very interesting. However, it is just one of many nutrients that cause an increase in mucin secretion.

1.5.2 Dietary protein sources

Although the effects of dietary protein have been studied to a lesser degree than fiber, protein has been shown to cause an increase in mucin secretion. Claustre and colleagues (2002) determined that a 5% (wt/vol) lactalbumin (a component of casein) hydrolysate caused an increase in mucin secretion in the jejunum by 335% when compared to that of the control group. Chicken egg albumin and meat hydrolysates did not have the same effect on mucin secretion as the other two protein hydrolysates, indicating that mucin discharge is sensitive to the protein source.

In the same study, Claustre found that when casein hydrolysate or β-casomorphin-7 (an opioid peptide derived from bovine β-casein) was exposed to the lumen of the vascularly perfused rat jejunum, there was a strong increase in mucus secretion. Trompette and colleagues' (2003) findings suggest that opioid peptides may activate μ-receptors, which may be involved with gastrointestinal protection through an increase in mucin secretion. This is an important observation since it has been found that after consumption of a bovine milk diet, β-casomorphins have been found in the human small intestine and in the plasma of calves (Umbach et al., 1985). Claustre et al. (2002) also determined that neither native casein nor an amino acid mixture of glutamine and glutamic acid had an effect on mucin secretion. Overall, these finding are important to the nutritional community since milk is the only protein source for the suckling neonate. In addition, this could be very interesting when considering the consumption of infant formulas based on hydrolyzed casein, such as Carnation GoodStart®. This concept would be of

particular interest when considering the link between mucin secretion and amino acid requirements, particularly in the neonate.

1.5.3 Bacteria

In addition to nutritional components, bacteria have been repeatedly demonstrated to be a mucin secretagogue. One of the most studied bacteria with regards to mucin secretion is Cholera and its toxin. When the small intestine is exposed to cholera toxin, mucin synthesis increases (Leitch, 1988, Moore et al., 1993 , Roomi et al., 1984, Yardley et al., 1972, Forstner, et al., 1982, Forstner et al., 1981, George et al., 1983, Njoku et al., 1983, Sherr et al., 1979, Chadee et al., 1991, Epple et al., 1997, Elliott et al., 1970, Moore et al., 1993) and the goblet cells of the intestine are depleted of mucin (Yardley et al., 1972). The process of mucin secretion into the lumen of the intestine responds rapidly to the presence of cholera toxin (George and Leitch, 1983, Leitch, 1988, Njoku et al., 1982). It is thought that the mechanism of action in the intestine via cholera toxin since *Vibrio cholerae* actually colonizes the intestine and releases cholera toxin. The small intestine responds by secreting large amounts of water and salt and mucin to aid in bowel movement and excretion of the toxin (Flach et al., 2004). Moore et al. (1993) used an *in vitro* open loop model to observe mucin secretion stimulated by cholera toxin. They observed a 4-fold increase in luminal mucin content when the proximal and distal portions of the rat small intestine were exposed to the cholera toxin.

In one study reported by Steinberg et al. (1975), proximal jejunal loops were isolated and cannulated with all blood supply left intact. This model was then used to observe the effects of shigella toxin on mucin secretion and how it compares to the response observed with cholera toxin. Both toxins induced mucin discharge, however, the onset of secretion of shigella toxin was 105 minutes whereas cholera was much quicker with an onset of 15 to 30 minutes. Despite this difference, identical rates of mucin secretion were observed.

Another bacterial toxin that causes mucin secretion is purified listeriolysis O (LLO) (an exotoxin of *L. monocytogenes*). This bacterium has been found to apically increase mucin exocytosis in cultured human HT29-MTX cells (Coconnier et al., 1998). *L. monocytogenes* is a gram-positive pathogen found in the food of humans and animals. In addition, similar results have been observed with the use of Entamoeba histolytica (Chadee et al., 1991) using a colonic loop model as well as Yersinia enterocolitica (YE) (Mantle et al., 1989) in the mid and distal rabbit small intestine.

The study of bacteria and mucin secretion is of particular interest since bacteria in our food supply present a chronic exposure and challenge to the GIT. This chronic exposure is even more of a concern in the typical environment of agricultural animals. Moreover, the bacterial toxin's role in mucin secretion provides a good model to examine the extreme circumstances of the mucosal defense process which includes mucin secretion.

1.5.4 Antinutritional factors

Lectins have been shown to bind glycoproteins and therefore lead to decreased mucin hydrolysis through the digestive process in the small intestine. This in turn causes an increase in mucin fermentation in the colon and cecum. As well, lectins have been shown to increase the release of histamine which has been shown to be another mucin secretagogue (Haas et al., 1999).

Antinutritional factors and their interactions with mucin are important in agricultural feeds. Threonine is the second limiting amino acid in most feedstuffs in Canada. These feedstuffs also contain other antinutritional factors such as tannins, lignins and other fiber components. This abundance of mucin secretagogues in feed may have significant implications on feed amino acid supplementation.

1.5.5 Hormones and drugs

Various drugs have been shown to be mucin secretagogues as well. Carbachol, when exposed to the rat small intestine, depleted the mucin secretory granules found in goblet cells in as little as 5 minutes (Phillips, 1992). Similar results were found in the isolated vascularly perfused rat colon when exposed to neurotransmitter and hormones such as bethanechol, bombesin and vasoactive intestinal peptide. As well, serotonin, peptide YY,

bromolasalocid, interleukin-1β, sodium nitroprusside and dimethyl-PGE_2 caused an increase in mucin discharge in the rat colon (Plaisancié et al., 1998).

1.5.6 Short chain fatty acids

A mixture of short chain fatty acids or individual short chain fatty acids, with the exception of succinate and lactate, have been shown to cause an increase in mucin secretion in the rat colon. Shimotoyodome et al. (2000) tested the short chain fatty acids, acetate, propionate, and butyrate, and observed an increase in mucus secretion.

Overall, the area of nutritional, bacterial and pharmacological effects on mucin secretion has been widely studied and has proven to be very interesting. However, the overall role that this process plays in the organism from a nutritional cost point of view has not been investigated.

1.6 Degradation of mucin

The glycoproteins found in the mucus layer of the intestine are gradually degraded as they move through the intestine. The protein core is partially degraded in the upper gastrointestinal tract; however, the enzymes that are necessary for the breakdown of the carbohydrate portion of the glycoprotein are present only in the large intestine. It has been proposed that the broken down protein core serves as a food source for anaerobic

bacteria located in the colon. These bacteria possess the glycosidase enzymes necessary to degrade the carbohydrates (Allen et al., 1981). As well, there is an enzyme in the intestine called mucinase that is responsible for mucin degradation and research has been done to investigate how this enzyme is regulated. Some dietary factors such as guar gum, pectin and carrageenan have been found to reduce mucinase activity whereas cellulose-free and fiber-free diets have been shown to increase mucinase activity (Shiau & Chang, 1983).

Degradation of mucin is difficult due to the high amount of oligosaccharides structurally present. Therefore mucin could be a significant endogenous protein loss (Montagne et al., 2004, Reeds et al., 1999). This is important nutritionally since the protein and carbohydrate that is broken down from the mucin structure is not reabsorbed by the large intestine. Particularly, if the threonine from the protein core is excreted, this would affect the threonine requirement and protein needs of the animal.

1.7 Mucins and disease

Adequate mucus gel layer integrity is required for proper functioning of the small intestine. Inadequate mucin secretion can lead to various disease states. Changes in the mucus gel layer are observed in a variety of intestinal and nutritional disorders such as enteric infections, inflammatory bowel disease, colon cancer, diseases which require total parenteral nutrition (TPN), Helicobacter infection, ulcerative colitis, Crohn's disease and

gastritis. This demonstrates the importance of the development and maintenance of mucin production (Deplancke & Gaskins, 2001, Corfield et al., 2000).

Infection by *Helicobacter pylori* can lead to gastritis and ulcers. During *H. pylori* infection, the glycosylated portion of mucin becomes altered in an irreversible manner. This alteration of the carbohydrate area allows for favorable attachment of the bacteria (Ota et al., 1998) and results in ulcer formation.

In ulcerative colitis, a reduction in the amount of goblet cells has been observed leading to a decrease in the amount of mucin produced which consequently reduces the thickness of the mucus layer. (McCormick et al., 1990, Pullan et al., 1994). Mucus thickness is often altered in different disease states which could possibly be an indication of a decrease in the rate of mucin synthesis (Faure et al., 2002). When the composition of mucus is examined in patients with ulcerative colitis, it has been noted that there is an increased secretion of a mucin species that is usually retained within the goblet cell granules (Smith and Podolsky, 1987).

Probiotics, when combined with a protein hydrolysate, can reduce the intestinal inflammation and mucosal barrier permeability (Majamaa et al., 1997). This could be an indication of how bacteria interact with the mucus layer and a possible alteration in mucin synthesis. The presence of probiotics may impede any invading organisms from entering the mucus layer by attaching to the carbohydrate side chains. Therefore, the

preferred binding sites of the invading bacteria are already occupied leaving excretion as the fate for the intruder.

Mucin genetics also play a role in various disease states. In numerous studies, the MUC genes have been studied extensively (Buisine et al., 1998, Gendler and Spicer, 1995, Gum et al., 1991, Tytgat et al., 1995, Tytgat et al., 1994) and it has been determined that of the thirteen MUC genes found in the body (Corfield et al., 2001), MUC 2, MUC 3, MUC 4 and MUC 6 are present in the small intestine (Gender & Spicer, 1995). The study of the regulation and expression of these genes provide insight into how mucins play a role in diseases such as cancer, cystic fibrosis, asthma and other respiratory illnesses (Gender & Spicer, 1995). It has been found that there is a correlation between *H. pylori* infections, which can lead to mucosal inflammation of the stomach and intestine, and decreased rates of mucin synthesis (Ece et al, 2004).

1.8 Dietary effects on mucins

The presence of nutrients in the intestinal lumen has a major influence on the structure and quantity of mucin. The dietary effects on mucin are not only important because of the chemical changes that may occur in its structure, such as the acidic or neutral population of mucins in goblet cells, but also because of the impact it has on the synthesis and release of mucin from goblet cells.

Studies have shown that diet may affect the types of mucin and the number of goblet cells present as well as the rate of mucin secretion from these goblet cells (Deplancke & Gaskins, 2001). In one study, it was found that the total number of goblet cells was higher in the jejunum and ileum of a TPN-fed neonatal piglet as opposed to an enterally fed piglet (Deplancke & Gaskins, 2001). Kleesen et al. (2003) found that the thickness of the epithelial mucus layer and the number of goblet cells present were altered in rats fed a diet containing a fructan called oligofructose-long chain inulin. In addition, when piglets were fed a diet of carboxymethylcellulose (a highly viscous nonfermentable soluble polysaccharide), Piel et al. (2005) observed that there was an increase in the number of total ileal goblet cells per villus and in ileal flow of mucin when compared to the control group.

Another indication of the effect of diet on mucin is found through the weaning process. Early weaning causes various health issues and it is thought that these problems are related to intestinal goblet cell function and numbers. This is an important issue in the agricultural industry since animals are being weaned earlier in life to increase productivity. Studies have shown that there is a decrease in acidic and neutral carbohydrate-containing goblet cells after weaning (Dunsford et al., 1991), which is thought to increase the exposure of the epithelium to the luminal contents. As well, the decrease in the goblet cell population may be due to a change in the rates of maturation and proliferation. Further study needs to be done in this area to fully understand the effects of weaning on the mucus layer.

1.9 Threonine utilization in the gut

Protein synthesis rates in the small intestine are very high, accounting for 9-12% of daily whole body protein synthesis. Mucosal digestive enzymes, mucins, trefoil particles and hormones are just a few of the proteins that are synthesized in the small intestinal tissue (Baracos et al., 2000).

When compared to the rest of the body, the gut requires a different amino acid profile in order to maintain adequate growth and maintenance. This difference in amino acid requirement is due to the gut's role in protection, digestion and absorption. Therefore, determining this requirement for specific amino acids is important for the understanding of the growth and development of the gut in animals. There are numerous methods for determining whole body amino acid requirements which include growth assays, nitrogen balance and amino acid oxidation techniques. With adaptations of these techniques, it has been determined that of all of the amino acids, the threonine requirement of the gut is proportionately the greatest of the amino acids and it is believed that this is primarily due to mucin synthesis.

Bertolo and co-workers (1998) showed that when a piglet was fed via total parenteral nutrition (TPN, bypassing the gut) versus enteral feeding, the threonine requirement was decreased by 55%. These findings were supported by Stoll et al. (1998) who showed in pigs that 61% of the dietary threonine was extracted by the portal drained viscera (PDV), which is dominated by gut metabolism, whereas other indispensable amino acids were

extracted at only 14-33%. They also showed that 90% of the threonine that was metabolized by the gut was either secreted as mucosal proteins or catabolized. As well, it has been shown that 84% of the dietary threonine is retained by the gut in pigs fed a high protein diet and 100% is retained when fed a low protein diet (van Goudoever et al., 2000). This high demand for threonine is thought to be the result of mucin synthesis that occurs in the goblet cells of the small intestine. The mucus layer is continuously being removed. Therefore, mucin synthesis must occur at a rate which maintains the layer's integrity. When feeding is occurring via TPN, mucin synthesis is reduced, which in turn reduces the threonine requirement of the gut. Law et al. (2007) showed that there was reduced goblet cell number and gut growth in general when piglets were fed a threonine-deficient diet gastrically.

These studies suggest that threonine is important in small intestinal metabolism. However, the metabolic fate of threonine that is used by the gut is unknown. It is possible that dietary threonine is incorporated into mucosal proteins. Dietary threonine could also be oxidized; however, Schaart et al. (2005) determined that the PDV utilized a large amount of threonine for intestinal mucosal protein synthesis and secretory protein synthesis in pigs since their study showed that only 2-9% of dietary threonine was oxidized. Mucosal protein synthesis can utilize amino acids from both the luminal and arterial sources. Zhao et al. (1986) discovered, among his research in threonine balance, that there was a loss of threonine through a non-oxidative pathway and he suggested that this loss was through mucin secretion; 71% of the threonine available was incorporated into small intestinal proteins. As well, Schaart et al. (2005) discovered that when pigs

were fed a high protein diet, most of the dietary threonine used by the small intestine is incorporated from the luminal threonine supply. However, when a low protein diet is consumed, systemic threonine is extracted for the metabolic needs of the small intestine.

1.10 Mucin and Threonine requirements

Mucin is particularly rich in the essential amino acid threonine. Mucin accounts for 19% of the protein in calf ileal digesta (Montagne et al., 1999) and 5-11% of protein losses in pig ileal digesta, with threonine constituting 28-35% of the total amino acids (Lien et al., 1997). Threonine that is lost from the ileum enters the colon where microbes digest mucin, but the threonine released is not reabsorbed and is in turn lost from the body. Therefore, the threonine requirement of the small intestine is of particular interest due to the high amount of threonine lost in the gut. Nutritionally, threonine requirements and utilization in the gut are very important since individuals with impaired gut function or intestinal disease may require increased amounts of threonine to meet the requirements for proper gut integrity. Adequate amounts of threonine are required to maintain the mucus gel layer of the intestine. The maintenance of this layer is important since it serves as a protective barrier for the gut. If threonine that is utilized during the first-pass metabolism for mucin synthesis is increased, then the availability of threonine in portal circulation will decrease and in turn decrease threonine that is available for peripheral tissues (Reeds et al., 1999). Not only is the basal threonine requirement of the gut of

interest, but so is the requirement under a variety of nutritional and pathological conditions which compromise functionality of the gut.

Various studies have demonstrated the critical role threonine plays in mucin synthesis. In one of these studies, Faure et al. (2005) found that when feeding rats for 14 days diets with various threonine levels, mucosal protein synthesis rates did not differ between groups. However, mucin synthesis rates were sensitive to dietary threonine levels and there was a significant difference observed in rats fed 30% and 100% of the theoretical threonine requirement for rats. This difference was observed throughout the entire small intestine and colon.

1.11 Experimental Rational and Objectives

Mucin is a glycoprotein that is rich in the amino acids proline, serine and threonine. It serves as a protective barrier for the epithelium of the small intestine from luminal factors such as bacteria, enzymes and digestive material. This protective layer is maintained by a continuous process of mucin synthesis and secretion. Since mucin is rich in threonine, the availability of this essential amino acid is crucial to the production of mucin and in turn, to the integrity of the small intestine.

Therefore, the objective of our study was to determine if the synthesis of small intestinal mucosal and mucin protein is sensitive to varying luminal threonine levels. To fulfill this objective, we had to:

1. Confirm the effectiveness of mucin purification techniques and establish these techniques in our laboratory.
2. To develop the gut loop model as a novel technique to study the interaction between luminal nutrients and intestinal protein synthesis.
3. To determine if small intestinal protein and mucin synthesis is sensitive to luminal threonine supply

We hypothesize that:

1. The gut loop model developed by Adegoke et al. (1999a) will prove to be an effective model for monitoring the interaction between luminal nutrients and intestinal protein synthesis.
2. The mucosal protein and mucin synthesis rates will be limited by a low luminal threonine supply and will increase with increasing threonine availability.

2. Materials and Methods

2.1 Mucin Isolation Techniques

The classical technique used to quantitatively isolate mucin involves density gradient centrifugation with subsequent assays to assess purity (Faure et al., 2002). A recent alternative approach (Faure et al., 2002) was developed using digestive proteases to exploit mucin's resistance to these enzymes. We decided to isolate mucin using both techniques to validate the newer method in our laboratory.

2.1.1 Animal Procedure

A recently weaned, 5-week-old, 11 kg Yorkshire pig was anaesthetized using an injection of ketamine hydrochloride (20 mg/kg) and xylazine (2 mg/kg), and was maintained with 2% halothane gas mixed with oxygen. A midline incision was made and a 30 cm section of jejunum was excised, emptied and rinsed with phosphate buffered saline (PBS) to remove any chyme. The section of gut was cut lengthwise to expose the lumen. On a glass plate imbedded in ice, crude mucus was collected by 'lightly' scraping along the length of the section with a glass microscope slide. In another section of gut, mucosa was collected by 'firmly' scraping along the section recovering the entire mucosal tissue leaving only muscularis.

2.1.2 Isopycnic Density Gradient Centrifugation

With the crude mucus obtained **(see section 2.11),** a cesium chloride density gradient was performed and the sample was analyzed as per **Figure 1.3.** This technique makes use of the high degree of glycosylation of mucins and its impact on the density of the glycoprotein. With this method, the unmodified protein was separated because of its lower density than mucins. The nucleic acids were also separated because their density is greater than that of both glycoproteins and protein (Allen et al., 1981).

2.1.3 Mucin Purification using Protease Digestion and Gravity Gel Filtration Chromatography

Faure et al. (2002) also recently developed a quick and convenient way to purify glycoproteins **(Figure 1.4 for Summary of Method)**. Their technique made use of mucin's resistance to proteases and its high molecular weight. Scraped mucosa was homogenized in PBS and exposed to protease digestion using the commercial product Flavourzyme, a fungal complex of exopeptidases and endoproteases (Novozymes, Bagsvaed, Denmark). Since mucin's structure contains protease-resistant areas, most of the contaminating protein was completely digested leaving mucin relatively intact. By using gel filtration chromatography with Sephacryl S-300 High Resolution, purified mucin was isolated from the small amount of remaining contaminating protein. The mucin-containing fractions were then identified by sodium dodecyl sulphate –

polyacrylamide gel electrophoresis (SDS-PAGE) with periodic acid/Schiff's base (PAS) and Coomassie Brilliant Blue staining.

2.1.4 SDS-PAGE

Samples for SDS-PAGE were prepared by taking 20 μL of the experimental sample and mixing it with 3 μL of 80% glycerol, 2 μL of 1 M DTT and 5 μL of 35 mM SDS in an Eppendorf tube. The solution was then boiled in water for 3 minutes.

A 1 mm thick polyacrylamide gel (4% acrylamide stacking/7.5% acrylamide separating) at 20 mA was used. Mucins can be distinguished by their low mobility and their ability to stain well with PAS stain for glycoproteins. To test for the presence of any contaminating proteins, Coomassie Brilliant Blue (G-250) stain (Sigma) was used. Molecular weight markers of 30,000 – 200,000 kDa (Sigma, Catalogue # SDS6H2) were used. This method was adapted from that of Laemmli (1970).

2.1.5 PAS Staining of SDS-PAGE Gels

To perform PAS staining of a polyacrylamide gel, the gel was placed in a plastic container and was submerged in 5% v/v phosphotungstic acid (Sigma) in 2 N HCl. The gel was left to rock gently for 90 minutes at room temperature. The 5% phosphotungstic acid solution was then drained and the gel was immersed in 500 mL of a 7% methanol-14% acetic acid solution (v/v) for 1 hour to allow for proper leaching of the SDS. This

solution was then poured off and the gel was placed in a 1% periodic acid-7% trichloroacetic acid solution (v/v) for another 60 minutes to allow for oxidization of the oligosaccharides. To remove excess periodic acid, 250 mL of 0.5% sodium metabisulfite in 0.1 N HCl was added. The gel was left for 1 hour at room temperature and then drained. The gel was then stained with Schiff's reagent (Fuchsin-sulfite reagent, Sigma) in the dark on ice and was left overnight.

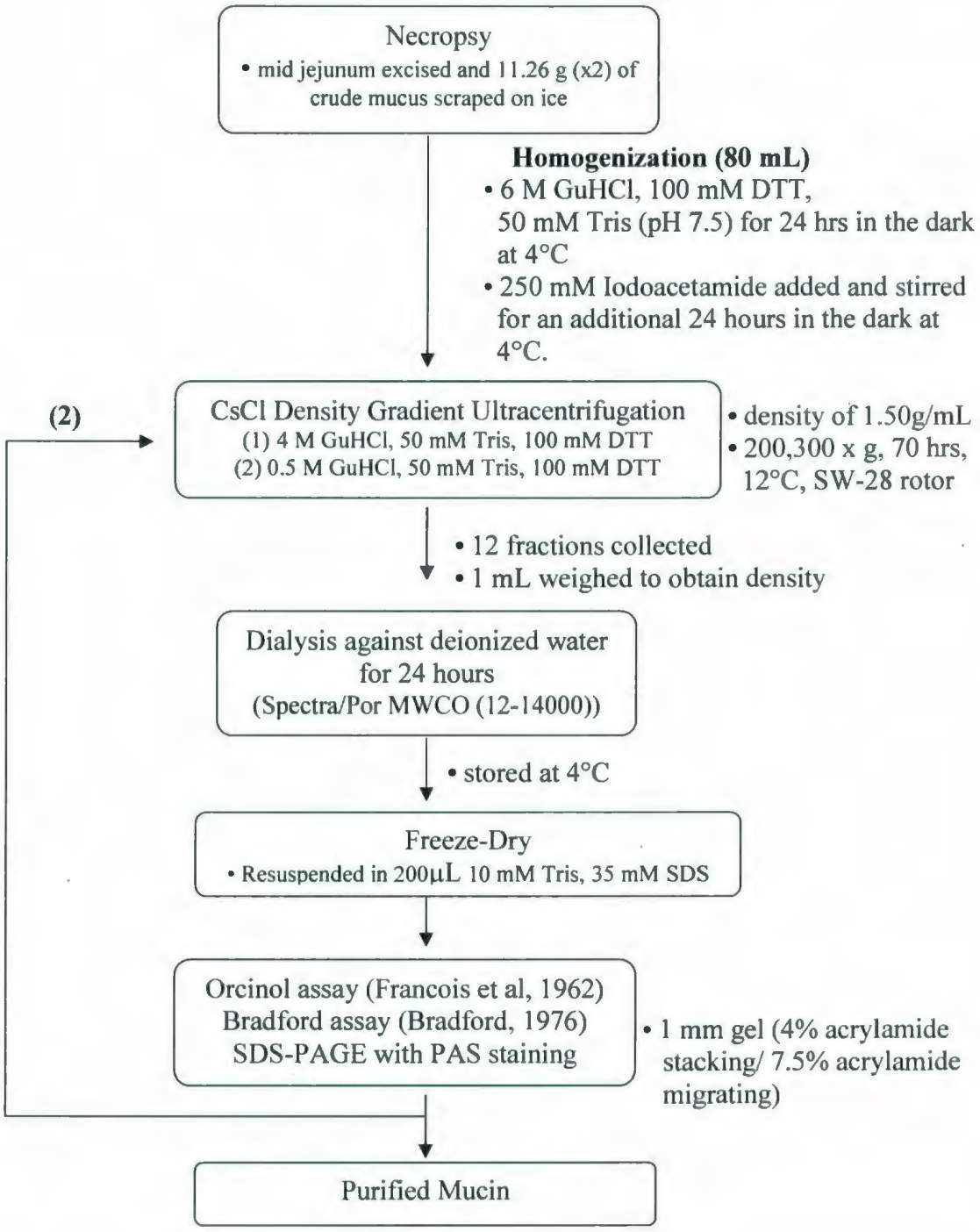

Figure 1.3 Summary of mucin isolation procedures using isopycnic density gradient ultracentrifugation

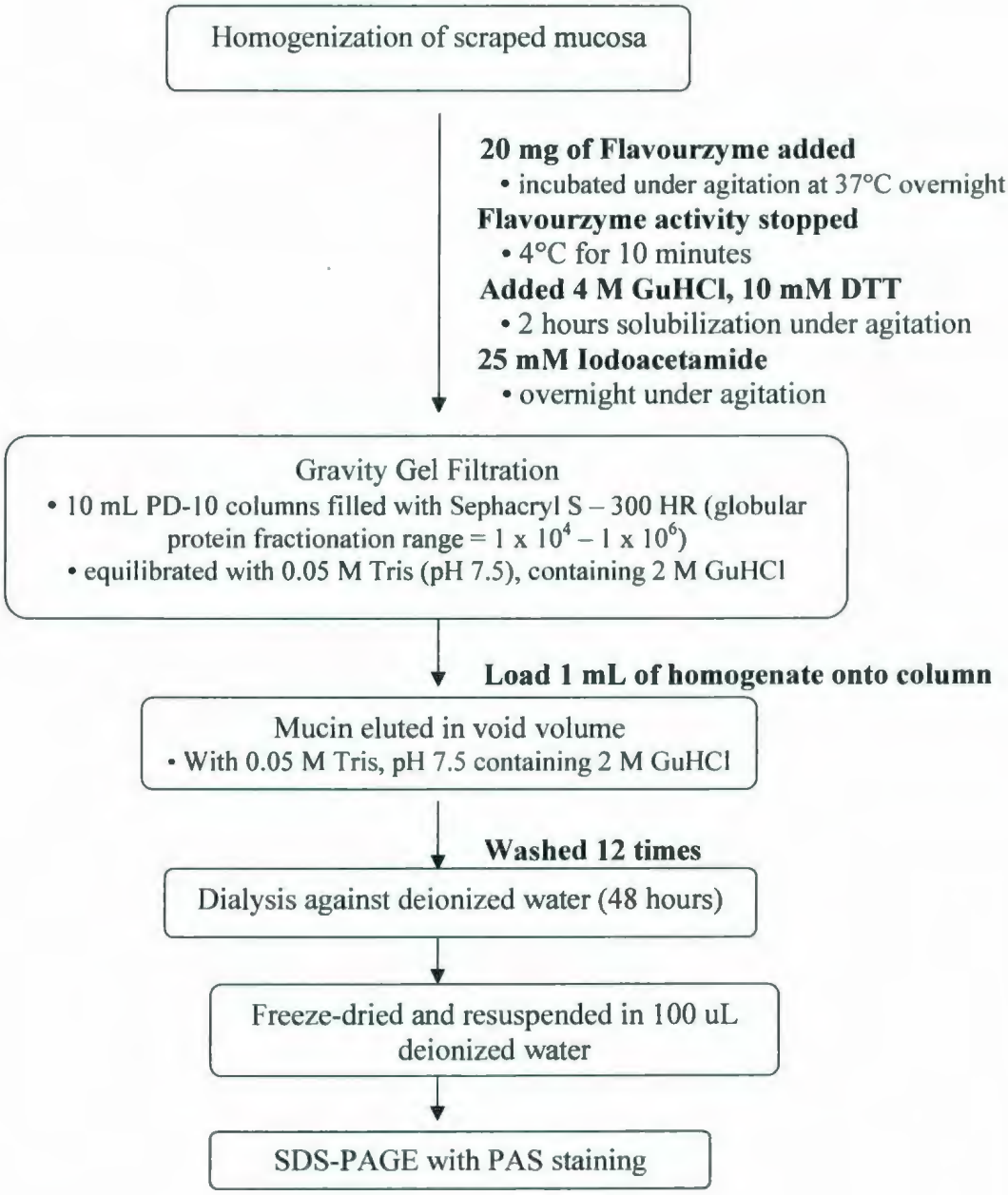

Figure 1.4 Summary of protocol for isolating mucin via gel filtration chromatography.

2.2 Gut Loop Model

2.2.1 Animal Preparation

Seven fasted, ~24-kg domestic pigs were anaesthetized using an initial flow of 4% halothane mixed with oxygen and maintained with 2% halothane and oxygen. The pig was then cleaned thoroughly with soap and periodine and a midline incision was made to expose the small intestine which was laid gently outside of the abdominal cavity. The ligament of Treitz (end of duodenum) was identified and 15 cm distal of the ligament, an inlet cannula (ID, 1/16 in.; OD, 1/8 in., Watson Marlow, Cornwall, England) was inserted into a hole made with an 18 gauge needle through the intestinal wall into the lumen. The tubing was secured by tying a suture around the intestine and tubing and closing off that end of the intestine. A 10-cm section of gut was then measured and the same procedure was performed at the opposite end providing an outlet cannula (ID, 3/32 in.; OD, 5/16 in., Watson Marlow, Cornwall, England) for the loop. A total of four loops were prepared similarly with 50 cm of intestine between loops. The loops were flushed with PBS (144.6 mM NaCl, 15.9 mM Na_2HPO_4, 1.2 mM $NaH_2PO_4 \cdot H_2O$, pH 7.4, 37°C) to remove any chyme that may be present. The intestines were then rinsed with warm 0.9% saline and placed gently back into the abdominal cavity for the duration of the experiment. To prevent adhesion between the handled intestines, adequate moisture levels were maintained by spraying the intestine with warm 0.9% saline solution and by covering the gut with plastic wrap. The pig's heart rate, respiration, body temperature and oxygen saturation were monitored throughout the experiment.

2.2.2 Perfusion

The perfusion solutions were continuously warmed in a water bath at 37°C. The inlet and outlet cannulae were attached to a multi-channel peristaltic pump (Watson Marlow, Cornwall, England) which pumped respective amino acid solutions through the loops **(Section 2.2.3)**; in one loop, PBS solution was used as a control. The various solutions and loops were randomly allocated. After a 90 minute perfusion was completed, the amino acid solution was replaced with an equivalent amino acid solution containing 100 µL of 0.5 mCi/mL ^{3}H-phenylalanine (Amersham Biosciences, Piscataway, NJ) in a 2 mM phenylalanine solution; this flooding dose of phenylalanine was infused for an additional 30 minutes . A schematic of the set up is shown in **Figure 1.5**.

After the flooding dose perfusion was completed, the loop was excised using cautery to prevent bleeding, flushed with cold 0.9% saline, cut lengthwise and scraped with a microscope slide on ice to remove the mucosa. The tissue was immediately frozen in liquid nitrogen and stored at -70°C until further analysis. The precise time of the removal of the loops was recorded.

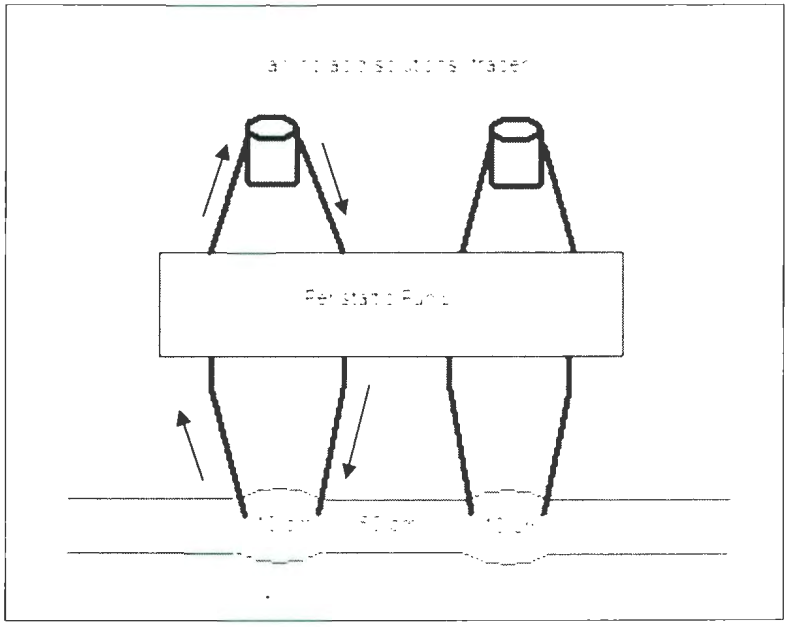

Figure 1.5 Schematic of gut loop model and amino acid perfusion set up

2.2.3 Amino Acid Mixture

The amino acid profile was based on that from analyzed sow's milk protein (Davis et al., 1994) with some minor modifications; the total amino acid concentration in each perfusion solution was based on that used by Adegoke et al. (2003). Amino acids (Sigma, St. Louis, MO) were weighed out individually as per **Table 1.2.**

Table 1.2. Basal amino acid profile of perfused nutrient solution (500 mL)

Amino Acid	Weight of Amino Acid (mg)
Alanine	63.4
Arginine	77.5
Aspartic acid	137.3
Cysteine	28.2
Glutamic acid	204.2
Glycine	56.3
Histidine	42.3
Isoleucine	70.4
Leucine	156.7
Lysine	114.4
Methionine	38.7
Proline	206.0
Taurine	8.8
Tryptophan	35.2
Tyrosine	68.7
Valine	81.0
Glutamine	140.9

The crystalline amino acids were weighed and mixed to make a homogeneous mixture which was added in small aliquots to 500 mL of deionized H_2O which was heated to 55-65°C. The temperature was maintained to dissolve the amino acids and the solution was exposed to a continuous flow of nitrogen gas to prevent oxidation. The temperature was monitored throughout to ensure it did not exceed 75°C.

Once all of the amino acids were dissolved, the volume was divided in half (250 mL). To one half, 37.85 mg of phenylalanine was added to make the basal 90 minute perfusion solutions. To the other half, 82.6 mg of phenylalanine was added to be used as the flooding dose solution (Adegoke et al., 1999a). The two mixtures were further divided into three 50 mL aliquots each (three for the 90 minute perfusion and three for the flooding dose solution).

The test amino acids, threonine and serine, were then added according to **Table 1.3**; serine was used to maintain isonitrogenicity between solutions. Bertolo et al. (1998) have previously estimated the requirement of threonine in orally-fed, 4-kg piglets as 28 mg/g amino acids. Three levels of threonine were tested: 0%, 75% and 200% of this estimated requirement. The test amino acids were added to each 50 mL solution. There were two 50 mL solutions mixed for each threonine treatment (one for the 90 minute perfusion and one for the flooding dose infusion).

Table 1.3 Threonine and Serine amounts (mg) for each 50 mL amino acid mixture containing varying levels of threonine

Percent of Requirement	Threonine (mg/50 mL)	Serine (mg/50 mL)
0%	0.0	14.7
75%	3.5	11.6
200%	9.4	6.4

To the flooding dose solution of each threonine treatment, 100 µL of 0.5 mCi/mL ^{3}H-phenylalanine was also added.

2.2.4 Measuring Tissue Free and Tissue Bound Amino Acids

The methods for the measurement of tissue free and tissue bound amino acids were adapted from those of Adegoke et al. (1999). Approximately 300 mg of mucosa was homogenized for 45 seconds in 3 mL of ice cold 2% (w/v) perchloric acid using a Polytron homogenizer set at 50% of maximum speed. The homogenized samples were then centrifuged at 2800 x g for 15 minutes and the resulting supernatant was decanted into a 5 mL vial. Standards (40 µL norleucine (25 µmol/mL) and 30 µL of ^{14}C-leucine (0.5 µCi/mL; 1,110,000 dpm/mL)) were added to the supernatant and the sample was frozen and stored until further analysis of tissue free amino acids.

The supernatant was neutralized with a half-volume of saturated potassium citrate (1 g in 0.65 mL deionized water) (Sigma, St. Louis, MO). The samples were left to sit on ice for 10 minutes and were then filtered using a 0.45 µm filter. The samples were frozen and stored until further analysis.

The pellet containing the tissue bound amino acids was washed with 4 mL of ice-cold 2% (w/v) perchloric acid by disrupting the pellet with a plastic disposable pipet. This disruption was followed by centrifugation at 2800 x g for 15 minutes. The supernatant was discarded and the rinsing procedure was repeated 4 times.

The remaining pellet was resuspended in 1.3 mL of 1 M NaOH by disrupting the pellet with a Teflon pestle. NaOH was added slowly to a total of 2.4 mL. The samples were then left to sit in a 37°C water bath for ~1.5 hours, or until they appeared clear, to solubilize the protein; 0.05 mL of the solution was kept for total protein analysis.

To the remaining tissue bound amino acid mixture, 1.2 mL of cold 20% (w/v) perchloric acid was added and the mixture was allowed to stand in an ice bath for 20 minutes to re-precipitate the protein. The samples were then centrifuged at 3000 x g for 15 minutes and the resulting supernatant was discarded. Standards (240 µL norleucine (25 µmol/mL) and 120 µL of ^{14}C-leucine (0.5 µCi/mL; 1,110,000 dpm/mL)) were then added to the pellet and 1.2 mL of 6 N HCl was added and the pellet was disrupted with a glass rod. This acid mixture was then transferred to a 25 mL pyrex digestion tube with a screw cap. 1.2 mL of additional HCl was added to rinse the plastic test tube and then transferred to the pyrex tube. A final rinse with 0.6 mL of 6 N HCl was added to the pyrex tube to make a final total of 5 mL of 6N HCl. The samples were placed in a drying oven set at 110°C for 24 hours for hydrolysis of the protein to occur.

After 24 hours, the tubes were placed in a fume hood to cool. Once cooled, the hydrolysates were transferred to an Erlenmeyer flask. The pyrex tubes were rinsed twice with deionized water, then the final volume was brought up to 25 mL. The contents were mixed thoroughly and then filtered with a 0.45 µm filter, into a labeled scintillation vial and stored until further analysis.

2.2.5 Amino acid derivatization steps

When ready for derivatization, 1 mL of each of the tissue free and tissue bound amino acids were frozen with liquid nitrogen and freeze dried overnight. The next day, a fresh 20:20:60 TEA:methanol:water mixture was made and 100 µL was added to each sample. The samples were freeze dried for approximately one hour. To each sample, 50 µL of a 10:10:70:10 water:TEA:methanol:PITC solution was added, vortexed, and left to sit for 35 minutes for the PITC (Pierce, Rockford, IL) to react to the amino acids. The samples were then freeze dried overnight and resuspended in 300 µL of sample diluent.

2.2.6 Mucin Hydrolysis

Mucin purification was performed using the Faure method as described in **Section 2.1.3**. The mucin-containing fractions were then combined and transferred to a pyrex tube. 5 mL of 6 N HCl, 10 µL of norleucine (2.5 µmol/mL) and 40 µL of ^{13}C-Leucine (0.5 µCi/mL; 1,110,000 dpm/mL) were added and the mixture was hydrolyzed at 110°C for 24 hours. After cooling, samples were then diluted with deionized water to 6 mL. 3 mL of the solution was placed in a plastic test tube and freeze dried (approximately 2 days). The samples were derivatized as described in **Section 2.2.5**.

2.2.7 Amino Acid Analysis using High Performance Liquid Chromatography (HPLC) and Fraction Collection

100 μL of mucosal protein samples and 300 μL of mucin samples were injected for amino acid separation using a Pico-Tag reverse-phase column (Waters, Mississauga, Ontario, Canada). The run time was 90 minutes. The fractions corresponding to the leucine and phenylalanine peaks were collected over a time window starting at least 2 minutes before and after the elution time of each amino acid using a Waters fraction collector (Waters, Milford, MA). The amount of radioactivity in each fraction was determined using a liquid scintillation counter (Wallac Fisher 1209 RackBeta LSC; counting for 15 minutes per sample with window open from 5-650) after adding 10 mL of Biodegradable Counting Scintillant (Amersham Biosciences, Buckinghamshire, England).

2.2.8 Calculations and Statistical Analyses

Protein synthesis rates (PSR) were calculated using the formula developed by Garlick et al. (1983) using the following equation:

$$K_s = \frac{SRA_h \times 100}{SRA_f \times t}$$

Where K_s is the fractional rate of protein synthesis in percentage of the tissue protein pool synthesized per day; SRA_h is the specific radioactivity of bound phenylalanine in the protein hydrolysate from the tissue; SRA_f is the specific radioactivity of phenylalanine in

the precursor pool; and t is the time allowed for incorporation of the radioactive phenylalanine into protein in days.

Data were analyzed using a one-way ANOVA with repeated measures (to account for blocking within pigs) and Student Newman Keuls multiple comparisons using Graph Pad Prism 4. Means were declared significantly different at a probability level of $P < 0.05$.

3.0 Results

3.1 Mucin Isolation Using Isopycnic Density Gradient Ultracentrifugation

Due to the high degree of extensive glycosylation, mucin is traditionally separated by CsCl density gradients. The buoyant density of mucin is around 1.40 g/mL which is higher than proteins and fats (<1.0 g/mL) and lower than nucleic acids (~1.7 g/mL) (Van Klinken et al., 1998). Therefore, after isopycnic density gradient ultracentrifugation, fractions with a density between 1.39 g/mL and 1.41 g/mL were expected to be mucin-containing **(see Figure 1.6)**.

Comparing our results to those of Piel et al. (2004), our results were similar in pattern across fractions. Our protein analysis pattern using the Bradford assay (Bradford, 1976) was similar (graph not shown). Traditionally, the orcinol assay is used to analyze carbohydrate content. However our attempts to develop this assay resulted in data that had high variability and did not resemble those established previously (Piel et al, 2004). Therefore, our numerous attempts led to the abandonment of the orcinol technique.

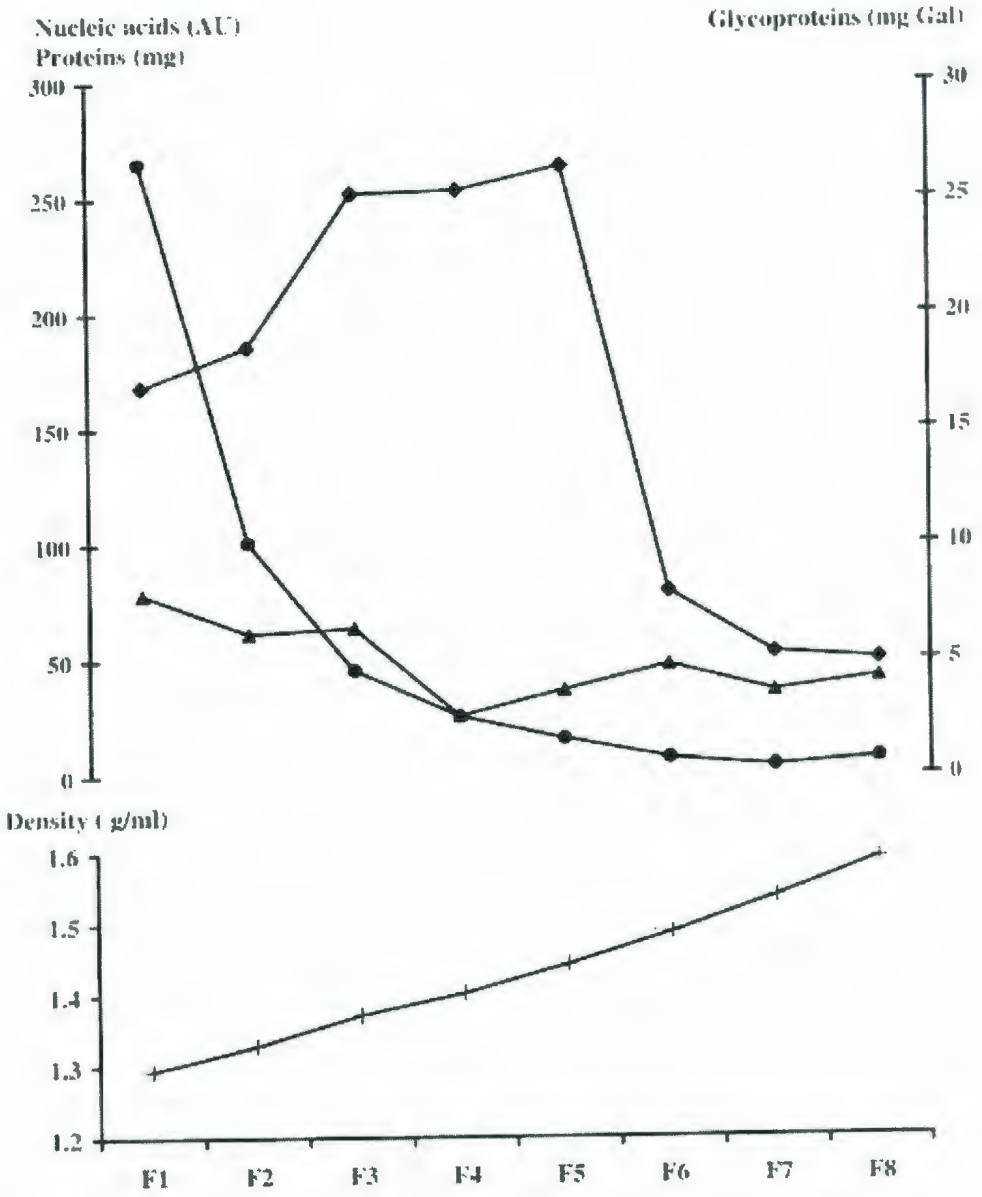

Figure 1.6. CsCl density gradient profile after second ultracentrifugation by Piel et al. (2004). Analysis of density (+), protein (●) by Bradford assay, glycoprotein (♦) by orcinol reaction, and nucleic acids (▲). (Adapted from Piel et al., 2004)

To replace the orcinol assay, we then employed an alternative method to detect glycoproteins,used by Mantle & Allen (1978). They observed that the PAS analysis of glycoproteins is 20 times more sensitive than the orcinol-sulfuric acid assay. Therefore, this technique can be utilized when a minimum quantity of mucin is available. The mode of action for this procedure, according to Mantle & Allen (1978), is that periodic acid cleaves the C:C bond in 1:2 glycols of monosaccharides. This process converts the glycols present to dialdehydes which are not further oxidized. Instead, the dialdehydes are localized by Schiff's reagent (Walsh & Jass, 2000). The intensity of the pink colour development is an indication of the number of reactive glycol structures.

Our results, consistent with those of Faure et al. (2002), indicated that mucin glycoprotein, which has a high molecular weight, only slightly migrated into the separating gel **(Figures 1.7 and 1.8)**. As can be seen in **Figure 1.7** from Faure et al. (2002), mucin was eluted primarily in fractions 6 and 7 which corresponded to fraction densities measured from the CsCl ultracentrifugation of 1.40 g/mL (fraction 6) and 1.44g/mL (fraction7) (densities obtained from weighing one mL of fraction). These densities also corresponded to the results obtained by Piel et al. (2004) **(see Figure 1.6)**.

The mucin-containing fractions should also have low, or no, contaminating protein. This can be indicated by staining a gel with Coomassie Brilliant Blue. As shown in **Figure 1.9**, the glycoprotein-containing fractions contained no contaminating protein. These observations show that SDS-PAGE and PAS staining, instead of the more traditional orcinol assay, is an effective means of verifying that mucin is isolated.

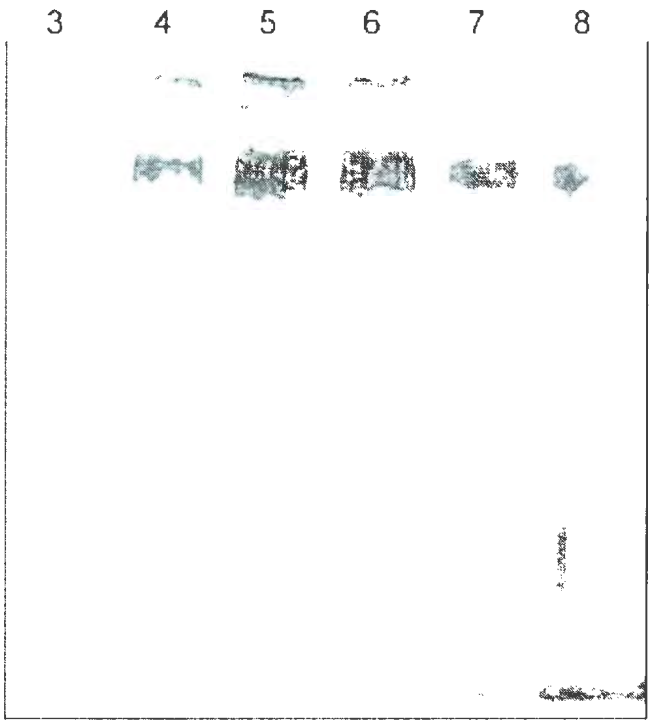

Figure 1.7 SDS-PAGE analysis of fractions 3-8 eluted from the Sepharose CL-4B column. Fractions (30 µL/lane) were loaded on a 3% stacking – 5% separating gel. The gel was stained with PAS. Taken from Faure et al. (2002).

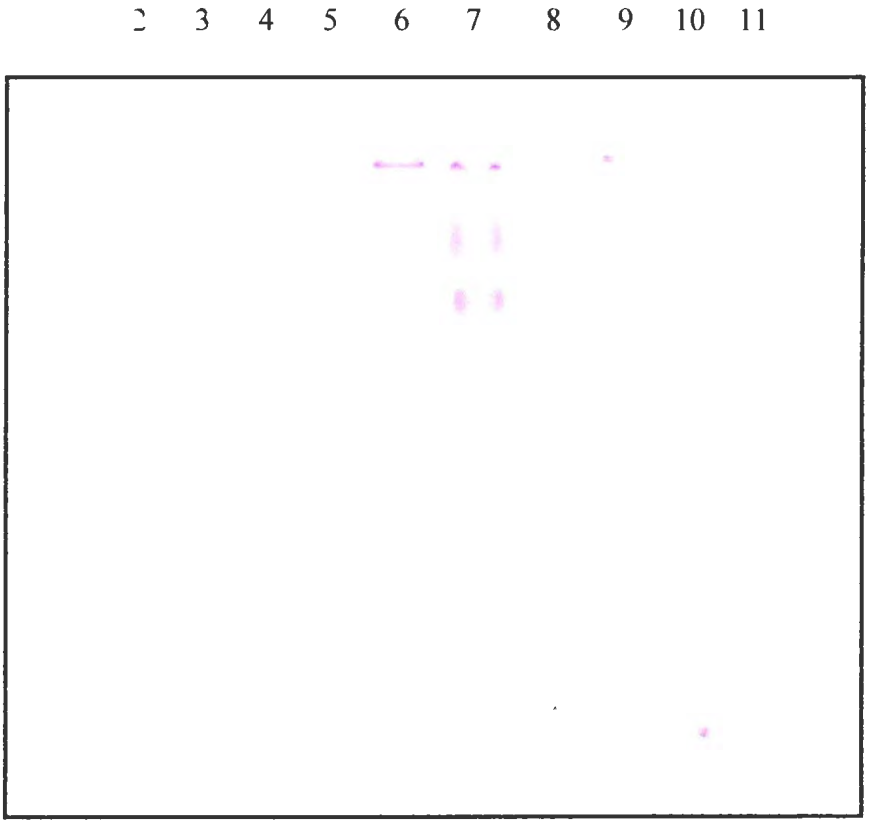

Figure 1.8. SDS-PAGE analysis of fractions 2-11 obtained from CsCl density gradient. Fractions (20µL/lane) were loaded on a 4 % stacking/7.5% separating gel. The gel was stained with PAS. The bands are most intense in fractions 6 and 7, which correspond to fractions predicted using densities measured from the CsCl ultracentrifugation (Fraction 6: 1.40 g/mL; Fraction 7: 1.44g/mL).

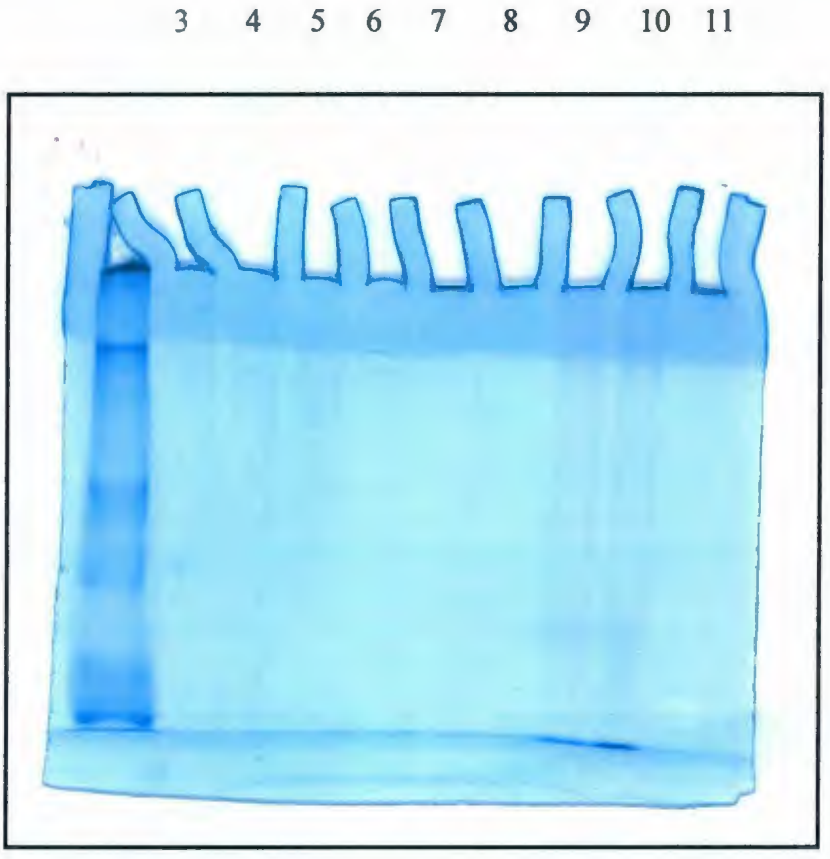

Figure 1.9. SDS-PAGE analysis of fractions 3 to 11 obtained from CsCl density gradient. Fractions (20μL/lane) were loaded on a 4 % stacking/7.5% separating gel. The gel was stained with Coomassie Brilliant Blue G-250. No contaminating protein is visible in mucin-containing fractions 6 and 7 **(see Figure 1.8)**. Molecular weight marker 30,000 – 200,000 kDa (Sigma).

3.2 Mucin Isolation using Protease Digestion and Gravity Gel Filtration

Faure et al. (2002) developed a more convenient procedure for mucin isolation. They made use of mucin's resistance to protease activity due to the high degree of glycosylation and their high molecular mass. Due to this glycosylation, mucin is protected from proteolytic attack. However, any other proteins present are not. Thus, any unprotected proteins in a mucosal homogenate will be digested by Flavourzyme and then will be easily separated from the mucin glycoprotein by gravity gel filtration chromatography. The verification steps are similar to those after isolation by density gradient ultracentrifugation. Mucin-containing fractions that are released in the void volume of the column are then dialyzed against deionized water and analyzed by SDS-PAGE.

The results from our initial gel filtration **(Figure 2.0)** showed strong glycoprotein bands in washes 2, 3, 4, 5 and 6 of the column. However, **Figure 2.1** indicates the presence of protein bands in these fractions when stained with Coomassie Brilliant Blue. These results indicated inadequate proteolysis by Flavourzyme. Therefore, we increased the amount of Flavourzyme used from 10 mg to 20 mg and increased the incubation period from 2 hours to overnight (approximately 17 hours).

After increasing the Flavourzyme concentration and incubation period, glycoprotein bands were located in a similar wash pattern **(see Figure 2.2)**. However, no

contaminating protein bands were observed indicating the complete proteolysis of contaminating protein and in turn, the purification of mucin **(see Figure 2.3)**.

The isolation of mucin glycoprotein is a challenging procedure due to their large size and heterogeneous structure (Davies & Carlstedt, 2000). The more traditional technique of cesium chloride density gradients has been developed extensively over the years (Claustre et al., 2002, Starkey et al., 1974, Mantle et al., 1981, Dekker et al. 1989). The technique produced by the Faure group (2002) is a more recent development and was limited to the isolation of intestinal mucin from one species (rats) and only by their laboratory. Given this, we decided to develop both techniques in our laboratory in order to validate results using Faure's new method against the results from the more established density gradient approach. Each of these techniques utilize different fundamental approaches to isolation but used the same verification assays, namely SDS-PAGE with PAS staining. Because the PAS-staining bands from the cesium chloride density gradient technique showed a similar pattern across fractions to that seen in the gravity filtration technique developed by Faure et al. (2002), we concluded that the rapid and convenient method produced by Faure et al. (2002) gave satisfactory results compared to the traditional, more established method. Since our laboratory had not established either method, this approach of using two independent methods to isolate mucin helped confirm our results and we therefore decided to use the protease digestion technique for future experiments.

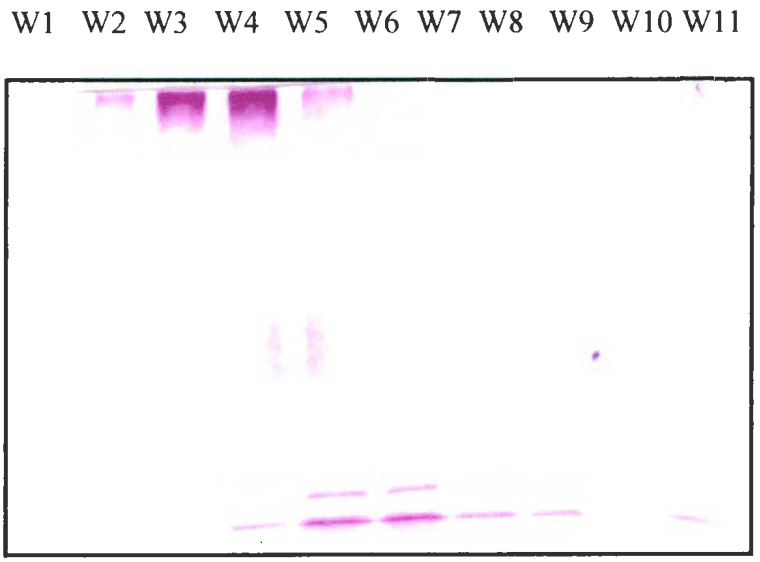

Figure 2.0. SDS-PAGE analysis of washes 1 to 11 eluted from the Sephacryl S-300 HR column after 2 hours of Flavourzyme protease (10 mg) digestion. Fractions (20µL/lane) were loaded on a 4 % stacking/7.5% separating gel. The gel was stained with PAS.

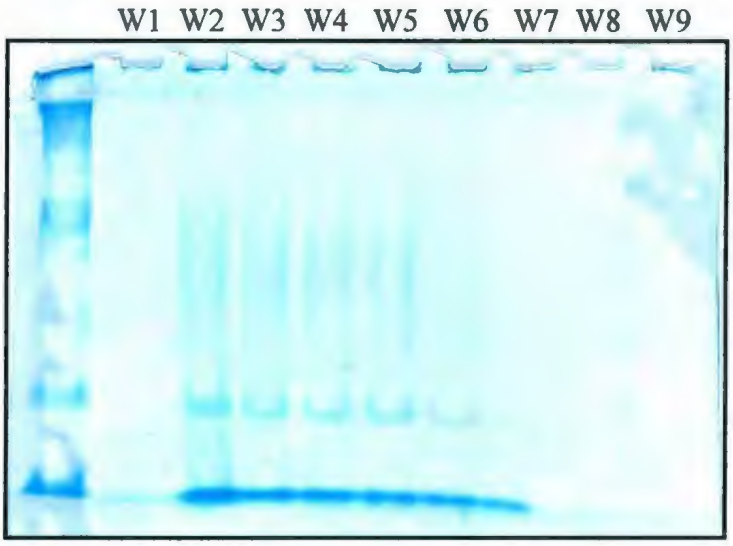

Figure 2.1. SDS-PAGE analysis of washes 1 to 9 eluted from the Sephacryl S-300 HR column after 2 hours of Flavourzyme protease (10 mg) digestion. Fractions (20μL/lane) were loaded on a 4 % stacking/7.5% separating gel. The gel was stained with PAS. Contaminating protein bands were present in wash 2 – wash 6. Molecular Weight Marker 30,000-200,000 kDa (Sigma).

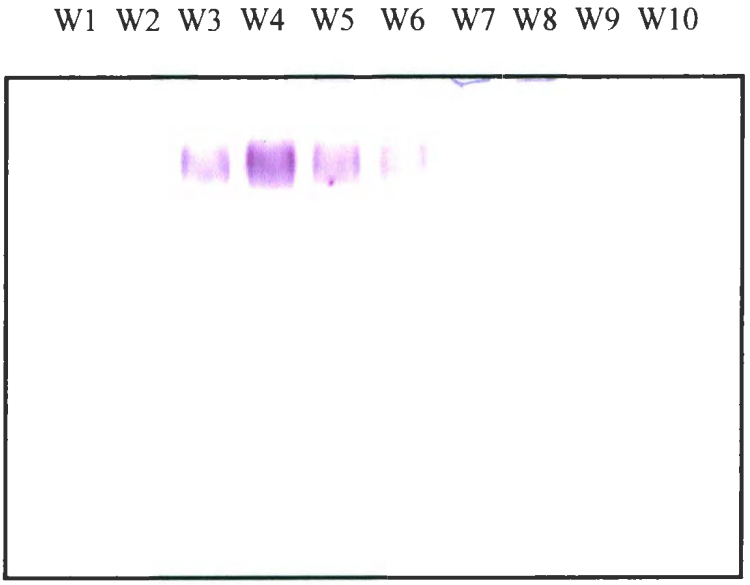

Figure 2.2. SDS-PAGE analysis of washes 1 to 10 eluted from the Sephacryl S-300 HR column after increasing the Flavourzyme to 20 mg and incubation time to 17 hours. Fractions (20µL/lane) were loaded 4 % stacking/7.5% separating gel. The gel was stained with PAS.

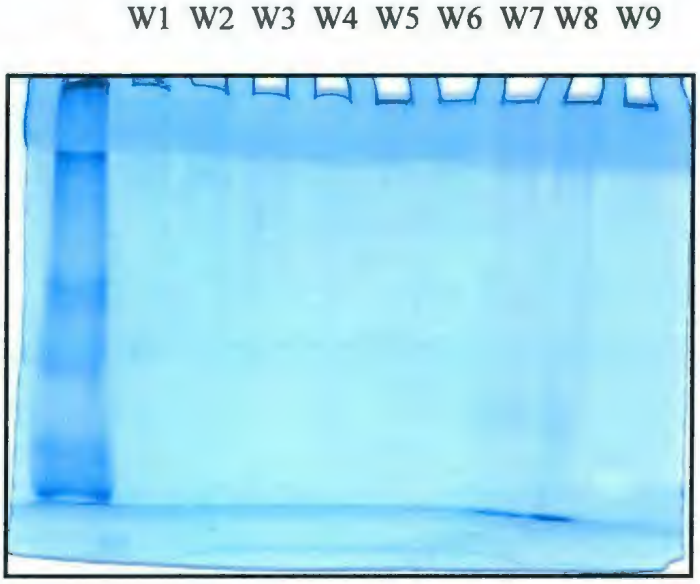

Figure 2.3. SDS-PAGE analysis of washes 1 to 9 eluted from the Sephacryl S-300 HR column after increasing the Flavourzyme to 20 mg and incubation time to 17 hours. Fractions (20μL/lane) were loaded 4 % stacking/7.5% separating gel. The gel was stained with Coomassie Brilliant Blue G-250. No contaminating protein bands are present in any of the washes. Molecular Weight Marker 30,000-200,000 kDa (Sigma).

3.3 Mucosal and Mucin Protein Synthesis Rates

The protein synthesis rates of mucosal proteins and mucin are presented in **Table 1.4.** There were different numbers of samples within the mucosal protein and mucin groups since there was not enough mucosal tissue available for mucin isolation in one of the treated pigs. Because the PBS group was not a direct control of varying threonine levels, we analyzed the data for the three threonine treatments only. The PBS group was included to confirm results obtained by Adegoke et al and validate our model system. Within the three groups for mucosal protein, the loops perfused with the solution containing threonine at 200% and 0% of the estimated requirement had the highest and lowest rate of protein synthesis, respectively **(Figure 2.4 and Figure 2.5)** ($P < 0.05$). The same pattern was observed for the mucin synthesis rates **(Figure 2.6 and Figure 2.7)** ($P < 0.05$). Threonine levels 0%, 75% and 200% were significantly different from each other ($P < 0.05$).

The PBS infused loops had protein synthesis rates of 170%/day and mucin synthesis rates of 661%/day.

Intestinal tissue in between the experimental loops and not directly perfused with any solution was also extracted and analyzed (in the same manner as the experimental loops) to determine the extent of arterial extraction of the luminal flooding dose. The protein-bound and free phenylalanine specific radioactivity in these untreated loops was 3-11%

and 6-8%, respectively, of that found in the luminally flooded loops; these data were similar to those found by Adegoke et al. (1999a). .

Table 1.4. Protein synthesis rates (%/day) of mucosal protein and mucin at varying threonine levels (n=7 for mucosal protein analysis, P<0.05; n = 4 mucin protein synthesis analysis, P<0.05)

Threonine Level (% Requirement)	Mucosal PSR (%/day)	Standard Deviation	Mucin PSR (%/day)	Standard Deviation
0	42	*23*	323	*145*
75	53	*16*	347	*99*
200	66	*10*	414	*63*

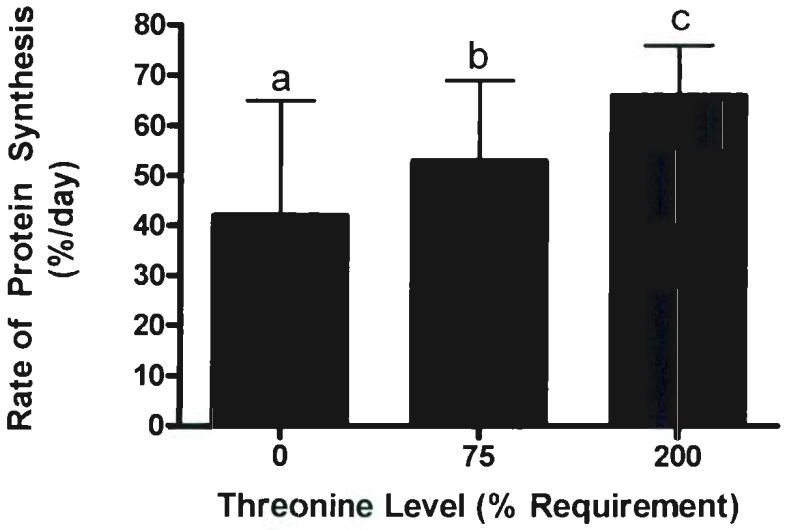

Figure 2.4. Mean (n = 7) mucosal protein synthesis rates (%/day) at varying threonine levels which were set as a percentage of the estimated threonine requirement of the small intestine (Bertolo et al., 1998). Bars with different letters are significantly different from one another ($P < 0.05$) by ANOVA with repeated measure (to account for blocking within pigs) and Student Newman Keuls multiple comparisons.

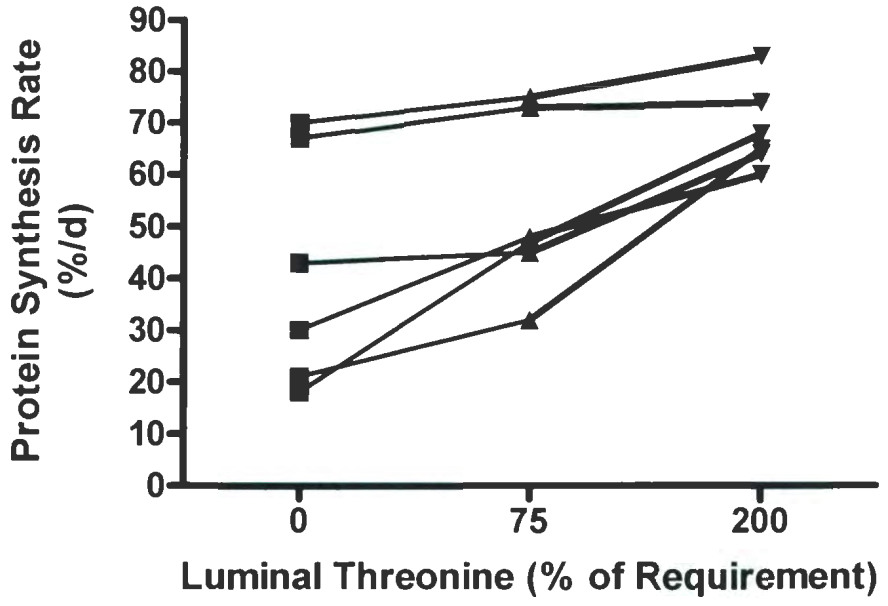

Figure 2.5. Scatter plot of mucosal protein synthesis rates (%/day) at varying threonine levels which were set as a percentage of the estimated threonine requirement of the small intestine (Bertolo et al., 1998). Each point represents a loop; loops within the same pig are connected by lines to demonstrate blocking effect of the experimental design. Analyzed by ANOVA with repeated measures (to account for blocking within pigs) and Student Newman Keuls multiple comparisons.

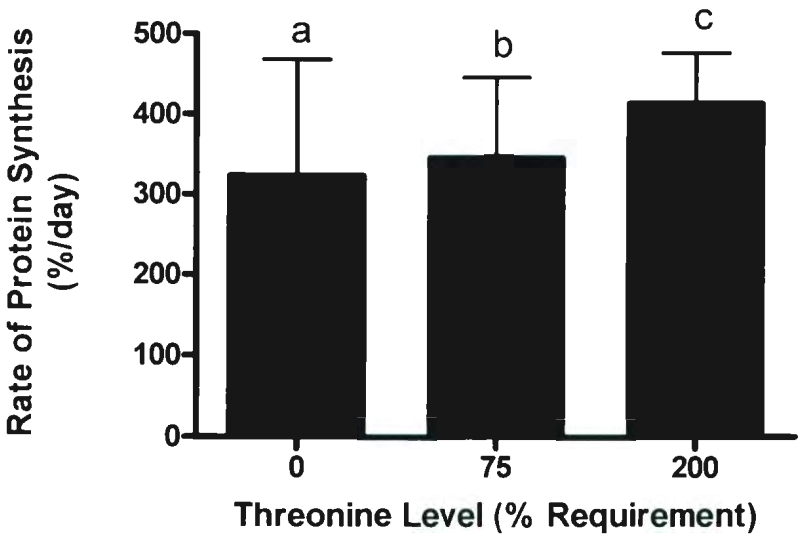

Figure 2.6. Mean (n = 4) mucin synthesis rates (%/day) at varying threonine levels which were set as a percentage of the estimated threonine requirement of the small intestine (Bertolo et al., 1998). Bars with different letters are significantly different from one another ($P < 0.05$) by ANOVA with repeated measure (to account for blocking within pigs) and Student Newman Keuls multiple comparisons.

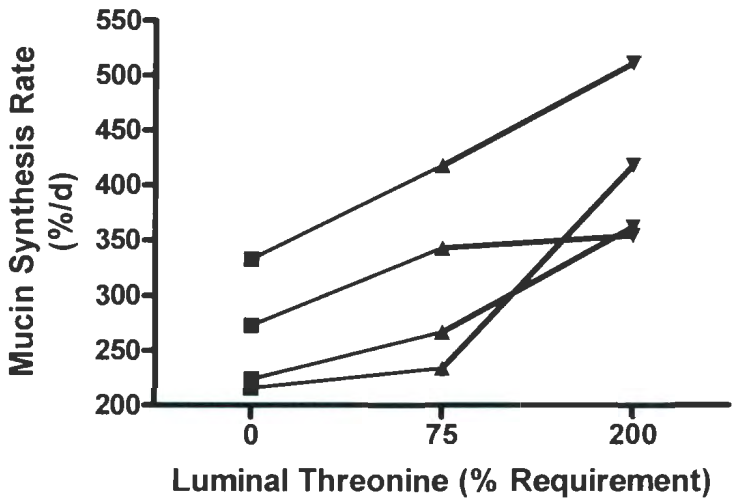

Figure 2.7. Scatter plot of mucin synthesis rates (%/day) at varying threonine levels which were set as a percentage of the estimated threonine requirement of the small intestine (Bertolo et al., 1998). Each point represents a loop; loops within the same pig are connected by lines to demonstrate blocking effect of the experimental design. Analyzed by ANOVA with repeated measures (to account for blocking within pigs) and Student Newman Keuls multiple comparisons.

4.0 Discussion

The protein synthesis rate of the small intestine is of particular interest due to the fact that it accounts for 9-12% of the total body protein synthesis even though it constitutes only 4-6% of the total body mass. In addition, it has a unique amino acid requirement when compared to the rest of the body. In particular, the amino acid threonine is of interest since it is the single most utilized essential amino acid by the portal drained viscera (Schaart et al., 2005). It has been found that the threonine requirement of the body is reduced by 60% when the gut is bypassed through the use of TPN feeding (Bertolo et al., 1998) indicating the importance of threonine in normal gut function. Therefore, the aim of the present study was to develop the gut loop model and flooding dose technique as an initial approach to the determination and understanding of the threonine requirement of the small intestine.

Over the years, the structure of mucin has been studied extensively and it has been found that this glycoprotein's protein backbone is rich in the amino acids threonine, proline and serine. Since threonine is an essential amino acid, the requirement of the body and the small intestine for this amino acid is of particular interest. We used the concept of the rate limiting amino acid as a control mechanism of the protein synthesis rates in our loops. This concept allows us to control the rate of protein synthesis by limiting an essential amino acid which is crucial for protein synthesis to occur. When the amino acid is not present, protein synthesis should be at a minimum. As the amino acid level is

increased, so does the rate of protein synthesis. However, once the amino acid is present at an amount that meets or surpasses the requirement, the rate of protein synthesis will reach a maximum and therefore, will not change with further additions of the amino acid. With this in mind, it would be expected that the mixture which contains no threonine (0%) would result in the lowest rate of protein synthesis for both the mucosal protein and for mucin alone. Indeed, this was observed in our study. As the threonine level was increased, the protein synthesis rates increased from 0 to 75% and from 75 to 200% threonine levels. This correlation between luminal threonine and protein synthesis was observed for both total mucosal protein and for mucin protein. These results demonstrated the novel finding that protein synthesis was acutely dependent on luminal supply of amino acids even in the presence of adequate arterial supply to the loops. These results are novel and are an important step in eventually determining the threonine requirement of the small intestine.

Another study was carried out by Faure et al. (2005), in which rats were fed for 14 days with a diet that containing varying levels of threonine. They examined the effect of chronic dietary threonine supply on mucin synthesis rates, using the whole body flooding dose method and the mucin purification technique previously developed in their lab (Faure et al., 2002). In our study, we made use of the gut loop model established by Adegoke et al. (1998) to study the acute effects of varying dietary threonine levels on mucosal and mucin protein synthesis rates as opposed to the long term feeding of the threonine diets examined by Faure's group (2005). Our results differed from those found

by Faure et al. (2005) in that their data indicated no difference in protein synthesis rates in mucosal tissues in response to the diets containing varying threonine levels, whereas our results showed a significant difference between the loops ($P < 0.05$) that contained varying threonine levels **(Table 2.4 and Figure 2.5)**. This difference between our study and the Faure et al. (2005) study may be a result of the different designs used. We employed the young, rapidly growing piglet whereas Faure and colleagues used adult rats. Moreover, the sensitivity of the small intestine to varying threonine levels may be an acute alteration. The 14 day feeding period used by Faure et al. may have allowed for an adaptation period by the mucosa, during which time, the body may have upregulated threonine extraction from systemic circulation to the small intestinal tissue to compensate for the chronic dietary deficit. In our study, an immediate response of the small intestine was observed which probably did not allow for such compensation.

Faure and colleagues' results did, however, indicate a difference in the mucin synthesis rates, similar to results in our study. However, our mucin synthesis rates were double those observed by Faure and coworkers which may be a result of the acute effect mentioned above, species differences or age differences (young weaned pigs vs adult rats). The fact that the mucin synthesis rates are higher than those observed in the mucosal tissue was not surprising. Faure et al. (2002) observed that the protein synthesis rates of mucin in the small intestine of the rat was always higher than 100% per day which indicates that the mucin glycoprotein pool is being renewed at least every 24 hours. Overall, the mucosa turns over total protein (primarily in enterocytes) at a slower

rate than the secreted glycoproteins, indicating the importance of the mucus layer in the small intestine.

The amino acid profile used was an adaptation of that found by Davis et al. (1994) with a few modifications. Firstly, we added glutamine at the expense of glutamate to our profile. Since during hydrolysis, glutamate is converted to glutamine, the analysis by Davis et al (1994) gave an overestimation of glutamate and no data for glutamine. Tryptophan is also destroyed during acid hydrolysis and therefore was not measured by Davis et al. (1994). We used the tryptophan levels known to be adequate in a piglet from a previous study (Bertolo et al., 1998). Finally, taurine was added because of its importance as a required nutrient in neonates (Heird 2004).. Lysine was also slightly lowered to ensure that the amino acid profile met NRC requirements for essential amino acids when expressed as a ratio to lysine for piglets of this age group. The total amino acid concentration in the perfusion solution was taken from the experiments of Adegoke and colleagues but where we used this milk-based amino acid profile, they used estimates from hydrolyzed intestinal digesta.

An estimation of the threonine requirement for the orally fed piglet was made by Bertolo et al. (1998). They observed, with the use of the indicator amino acid oxidation technique, that the mean requirement level for threonine was 28 mg/g of amino acids. With respect to the total amino acid concentration in the perfusion solutions, the amount of threonine found in this solution was set at 2.8% of the total concentration and

considered to be the 100% test level. The 0% group contained no threonine with the other two test levels containing 75% and 200% of this 28 mg/g level.

It is unknown at this point whether or not the threonine levels used in this experiment encompass the breakpoint for intestinal threonine requirement. There may have been an overestimation or underestimation of the requirement. It is possible that protein synthesis plateaus below or above the 200% values. This is, however, why we used 75% and 200% of requirement levels; we wanted to establish whether protein synthesis is sensitive to luminal threonine supply at all. The next step is to expand the range with more threonine levels in order to determine if the requirement falls above or below the 200% level. This will then indicate the actual threonine requirement of the small intestine.

Another interesting concept that may be occurring is that threonine may be directly stimulating protein synthesis independent of its role as a limiting amino acid. It has been shown that essential amino acids, in particular leucine, can act as a nutrient signal for the translation of proteins and therefore induce protein synthesis through an mTOR-regulated pathway (Kimball, 2002). It has been shown that if an amino acid is missing, the enzyme p70 S6 kinase, is inhibited. This enzyme is used to facilitate the translation of capped mRNA (Anthony et al. 2001, Hara et al, 1998). Specifically, the deletion of threonine has been shown to diminish p70 S6 kinase activity moderately (Hura et al. 1998). Using our model, it is unlikely that intracellular threonine concentrations were negligible given the treatment protocol and the fact that arterial threonine supply was available to the

intestinal mucosa. It may be possible that the higher than normal luminal threonine concentrations may have resulted in an intracellular activation of protein synthesis and this may be a contributing factor in our results.

The flooding dose phenylalanine concentration of 2 mM was determined by Adegoke et al. (1999b) through a study using an intravenous flood. In that study, they found that the plasma phenylalanine concentration did not rise above 2 mM after an oral flood. In addition, when they circulated a 2 mM phenylalanine solution through a 10 cm jejunal loop of a pig or rat, there was no change in the plasma phenylalanine concentration. In past studies it has also been determined that after a protein meal has been administered, the phenylalanine concentration was found to be 2 mM (Adibi et al., 1973). Therefore, this evidence was used to set the flooding dose phenylalanine concentration to 2 mM.

Adegoke et al. (1999a) carried out an initial study to validate the flooding dose method in the gut loop model. They observed that the small intestine was sensitive to the presence of a 30 mmol/L amino acid solution when compared to a PBS control (Adegoke et al., 1999b). An interesting finding observed by both our group and Adegoke et al. (1999a) was that when the lumen of the small intestine was exposed to PBS only, without any nutrients, very high rates of protein synthesis were observed when compared to those observed with amino acids present. In the present study, we employed this PBS infused loop to see if our results verified these surprising findings. Because this loop did not contain any nutrients, it was not deemed a direct control treatment for the varying

threonine treatments. Adegoke et al. (1999b) developed a hypothesis for the suppression of protein synthesis in the small intestine when exposed to a luminal solution of amino acids. Since protein synthesis utilizes high amounts of ATP to assemble a single peptide bond, it could be possible that energy preservation may be occurring to ensure that adequate amounts of energy are available for nutrient absorption into systemic circulation. Therefore, protein synthesis is suppressed when exposed to luminal nutrients.

We observed the effect of varying threonine concentrations on protein synthesis rates acutely. The gut loop model allowed for these observations and was another important development of our study. This model will allow us to not only observe the threonine requirement of the gut, but will also assist us in understanding the effect that secretagogues have on this threonine requirement. As reviewed above, some known mucin secretagogues are cholera toxin, fibers and dietary protein sources such as casein. When the lumen of the intestine is exposed to these factors, an increase in mucin production and secretion is observed. Therefore, it is of interest nutritionally, to investigate how the small intestinal threonine requirement is altered upon exposure to these substances.

In conclusion, the present study demonstrates that during acute threonine restriction, the small intestinal mucosal and mucin protein synthesis rates are limited. This has important implications in situations where gut barrier functions are impaired, such as in

various small intestinal diseases. If gut barrier function is impaired, then more mucin is required to ensure adequate protection. In turn, more threonine is utilized for mucin production which relies on dietary threonine, since threonine is an essential amino acid. This process could possibly result in an increased threonine requirement. The dietary threonine supply may therefore need to be increased to maintain adequate function.

References

1. Adibi, SA, Mercer, DW (1973). Protein digestion in human intestine as reflected in luminal, mucosal and plasma amino acid concentration after meals. J of Clin Invest, 52:1586-1594.
2. Adegoke, OAJ, McBurney, MI, Baracos, VE (1999a). Jejunal mucosal protein synthesis: validation of luminal flooding dose method and effect of luminal osmolarity. Am J Phys. 276 (Gastrointest. Liver Physiolo. 39):G14-G20.
3. Adegoke, OAJ, McBurney, MI, Samuels, SE, Baracos, VE (1999b). Luminal amino acids acutely decrease intestinal mucosal protein synthesis and protease mRNA in piglets. J Nutr. 129:1871-1878.
4. Adegoke, OAJ, McBurney, MI, Samuels, SE, Baracos, VE (2003). Modulation of intestinal protein synthesis and protease mRNA by luminal and systemic nutrients. Am J Physiol Gastrointest Liver Physiol 284:G1017-G1026.
5. Allen, A (1981). The structure and function of gastrointestinal mucus. In: Johnson LR, ed. Physiology of the gastrointestinal tract. Vol. 1. New York: Raven, 617-639.
6. Allen, A, Bell, A, Mantle, M, Pearson, JP (1982). The structure and physiology of gastrointestinal mucus. Adv Exp Med Biol 144:115-133.
7. Allen, A, Carroll, NJ (1985). Adherent and soluble mucus in the stomach and duodenum. Dig Dis Sci 30:55S-62S.
8. Alpers, DH (1972). Protein synthesis in intestinal mucosa: the effect of route of administration of precursor amino acids. J Clin Invest 51:167-173.
9. Anthony TG, Reiter AK, Anthony JC, Kimball SR, Jefferson LS (2001). Deficiency of dietary EAA preferentially inhibits mRNA translation of ribosomal proteins in liver of meal-fed rats. Am J Physiol Endocrinol Metab 281:E430-9.
10. Atuma, C, Strugala V, Allen, A, Holm, L (2001). The adherent gastrointestinal mucus gel layer: thickness and physical state in vivo. Am J Physiol 280:G922-G929.
11. Baracos, VE, Samuels, SE, Adegoke, OAJ (2000). Anabolic and catabolic mediators of intestinal protein turnover: a new experimental approach. Curr Opin Clin Nutr Metab Care 3:183-189.
12. Barcelo, A, Claustre, J, Moro, F, Chayvialle, J, Cuber, JC, Plaisancie, P (2000). Mucin secretion is modulated by luminal factors in the isolated vascularly perfused rat colon. Gut 46: 218-224.
13. Bell, AE, Seller, LA, Allen, A, Cunliffe, WJ, Morris, ER, Ross-Murphy, SB (1985). Properties of gastric and duodenal mucus: effects of proteolysis, disulfide reduction, bile, acid, ethanol and hypertonicity on mucus gel structure. Gastroenterology 88:269-280.
14. Bertolo, RF, Brunton, JA, Pencharz, PB, Ball, RO (2000). Steady state is not achieved for most plasma amino acids during 12 hours of fasting in the neonatal piglet. Pediatr Res 48:701-7.
15. Bertolo RFP , Pencharz PB, Ball RO (2005). Role of Intestinal First-pass Metabolism on Whole Body Amino Acid Requirements. **Book Chapter** in Biology of Growing Animals. Volume 3: Biology of Metabolism in Growing

Animals, (Editors: DG Burrin and HJ Mersmann), Elsevier Science, London pp 127-156.
16. Bertolo, RFP, Chen, CZL, Law, G, Pencharz, PB, Ball, RO (1998). Threonine requirement of neonatal piglets receiving total parenteral nutrition is considerably lower than that of piglets receiving an identical diet intragastrically. J Nutr 128:1752-1759.
17. Bradford, MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254.
18. Buisine, MP, Devisme, L, Savidge, TC, Gespach, C, Gosselin, B, Porchet, N, Aubert, JP (1998). Mucin gene expression in human embryonic and fetal intestine. Gut 43:519-524.
19. Burrin, DG, Davis, TA, Fiorotto, ML, Reeds, PJ (1991). Stage of development and fasting affect protein synthetic activity in the gastrointestinal tissues of suckling rats. J Nutr, 121:1099-1108.
20. Chadee, K, Keller, K, Forstmer, J, Innes, DJ, Ravdin, JI (1991). Mucin and nonmucin secretagogue activity of entamoeba histolytica and cholera toxin in rat colon. Gastroenterology 100:986-997.
21. Clamp, JR, Allen, A, Gibbons, RA, Roberts, GP (1978). Chemical aspects of mucus. British Med Bull 34(1): 25-41.
22. Chesson, A. (1993). Feed enzymes. Anim. Feed Sci. TEchnol., 45, 65-79.
23. Claustre, J, Toumi, F, Trompette, A, Jourdan, G, Guignard, H, Chayvialle, JA, Plaisancie, P (2002). Effects of peptides derived from dietary proteins on mucus secretion in rat jejunum. Am J Physiol 263(Gastrointest. Liver Physiol.):G521-G528.
24. Coconnier, M-H, Dlissi, E, Robard, M, Laboisse, CL, Gaillard, J-L, Servin, AL (1998). *Listeria monocytogenes* stimulates mucus exocytosis in cultured human polarized mucosecreting intestinal cells through action of Listeriolysis O. Infect Imm 66(8):3673-3681.
25. Corfield, AP, Myerscough, N, Longman, R, Sylvester, P, Arul, S, Pignatelli, M (2000). Mucins and mucosal protection in the gastrointestinal tract; new prospects for mucins in the pathology of gastrointestinal disease. Gut 47:589-594.
26. Corfield AP, Carroll, D, Myerscough, N, Probert, CS (2001). Mucins in the gastrointestinal tract in health and disease. Frontiers in Bioscience Oct 1;6:D1321-57.
27. Cross, CE, Halliwell, B, Allen, A (1984). Antioxidant protection: a function of tacheobronchial and gastrointestinal mucus, Lancet 1:1328-1330.
28. Davies JR, Carlstedt, I. Isolation of large gel-forming mucins. In: Glycoprotein methods and protocols: the mucins 2000 (Corfield AP, ed.), pp. 3-14. Humana Press, Totowa, NJ.
29. Davis, TA, Nguyen, HV, Garcia-Bravo, R, Fiorotto, ML, Jackson, EM, Lewis, DS, Lee, DR, Reeds, PJ (1994). Amino acid composition of human milk is not unique. J Nutr 124:1126-1132.
30. Dekker, J, van Beurden-Lamers, WMO, Oprins, A, Strous, GJ (1998). Isolation and structural analysis of rat gastric mucus glycoprotein suggests a homogeneous

protein backbone. Biochem J 260:717-723.
31. Deplancke, B, Gaskins, HR (2001). Microbial modulation of innate defense: goblet cells and the intestinal mucus layer. Am J Clin Nutr 73(suppl):1131S-1141S.
32. Dunsford, BR, Haensly, WE, Knabe, DA (1990). Neutral and acidic goblet cell concentration in the small intestine of the unweaned pig. Biol Neonate 57:194-199.
33. Dunsford, BR, Haensly, WE, Knabe, DA (1991). Effects of diet on acidic and neutral goblet cell populations in the small intestine of early weaned pigs. Am J Vet Res 52(10):1743-1746.
34. Ece, F, Hatabay, NF, Erdal, N, Gedik, C, Guney, C, Aksoy, F (2004). Does infection play a role in lung cancer? Respiratory Medicine 99(10): 1258-1262.
35. Elliott, HL, Carpenter, CCJ, Sack, BR, Yardley, JH (1970). Small bowel morphology in experimental canine cholera: a light and electron microscope study. Laboratory Investigation 22(2):112-120.
36. Epple, HJ, Kreusel, KM, Hanski, C, Schulzke, JD, Riecken EO, Fromm, M (1997). Differential stimulation of intestinal mucin secretion by cholera toxin and carbachol. Pflugers Arch 433(5):638-647.
37. Faure, M, Moennoz, D, Montigon, F, Fay, LB, Breuille, D, Finot, PA, Ballevre, O, Boza, J (2002). Development of a rapid and convenient method to purify mucins and determine their in vivo synthesis rate in rats. Analytical Biochemistry 307:244-251.
38. Faure, M, Moënnoz, D, Montigon, F, Mettraux, C, Breuillé, D, Ballèvre, O (2005). Dietary threonine restriction specifically reduces intestinal mucin synthesis in rats. J Nutr 135(3):486-91.
39. Flach, CF, Lange, S, Jennische, E, Lonnroth, I (2004). Cholera toxin induces expression of ion channels and carriers in rat small intestinal mucosa. FEBS Letters 561:122-126.
40. Fogg, GJJ, Hutton, DA, Jumel, K, Pearson, JP, Harding, SE, Allen, A (1996). Characterization of pig colonic mucins. Biochem J 316:937-942.
41. Forstner, GG (1995). Signal transduction, packaging and secretion of mucins. Annu Rev Physiol 57:585-605.
42. Forstner, G, Wesley, A, Forstner, J (1982). Clinical aspects of gastrointestinal mucus. Adv Exp Med Biol 144:199-224.
43. Forstner, JF, Jabbal, I, Forstner, GG (1973). Goblet cell mucin of rat small intestine. Chemical and physical characterization. Can J Biochem 51:1154-1166.
44. Forstner, JF, Roomi, NW, Fahim, REF, Forstner, GG (1981). Cholera toxin stimulates secretion of immunoreactive intestinal mucin. Am J Physiol 204 (Gastrointest. Liver Physiol. 3):G10-G16.
45. Francois, C, Marshall, RD, Neuberger, A (1962). The determination of mannose in hen's-egg albumin by radioisotope dilution. Biochem J 83(2):335-341.
46. Fuller, MF, Milne, A, Harris, CE, Reid, TM, Keenan, R (1994). Amino acid losses in ileostomy fluid on a protein-free diet. Am J Clin Nutr 59:70-73.
47. Garlick, PJ, McNurlan, MA, Preedy, VR (1980). A rapid and convenient technique for measuring the rate of protein synthesis in tissues by injection of ^{3}H-

phenylalanine. Biochem J 192:719-723.
48. Gendler, SJ, Spicer, AP (1995). Epithelial mucin genes. Ann Rev Physiol 57:607-634.
49. George, MH, Leitch, GJ (1983). Separation of rabbit ileum mucus from electrolyte and water secretion by cholera enterotoxin, verapamil and A23187. Life Sci 32:839-846.
50. Gerard C. Purification of glycoproteins. Purification of glycoproteins. In: Guide to protein purification 1990 (Deutscher MP, ed.), Methods in enzymology vol 182, pp. 529-538. Academic Press Inc., San Diego, CA.
51. Gum, JR, Hicks, JW, Lagace, RE, Byrd, JC, Toribara, NW, Siddiki, B, Fearney, FJ, Lamport DTA, Kim, YS (1991). Molecular cloning of rat intestinal mucin: lack of conservation between mammalian species. J Biol Chem 266(33):22733-22738.
52. Haas H, Falcone FH, Schramm G (1999). Dietary lectins can induce in vitro release of IL-4 and IL-13 from human basophils. Eur J Immunol. 29:918-927.
53. Hara K, Yonezawa K, Weng QP, Kozlowski MT, Belham C, Avruch J (1998). Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism. J Biol Chem 273:14484-94.
54. Heird WC (2004). Taurine in neonatal nutrition--revisited. Arch Dis Child Fetal Neonatal Ed 89:F473-4.
55. Karam SM (1999). Lineage commitment and maturation of epithelial cells in the gut. Frontiers in Bioscience 4:286-298.
56. Kennedy JF, Pagliuca G. Oligosaccharides. In: Carbohydrate analysis: a practical approach 1994. (Chaplin MF & Kennedy JF, ed.) 2nd ed, pp. 43-72. IRL Press, New York, NY.
57. Kimball SR, Farrell PA, Jefferson LS (2002). Invited Review: Role of insulin in translational control of protein synthesis in skeletal muscle by amino acids or exercise. J Appl Physiol 93(3):1168-80.
58. King, TP, Pusztai, A, Clarke, EM (1980). Kidney bean (Phaseolus vulgaris) lectin induced lesions in the small intestine: 1. Light microscope slide. J Comp Pathol 90(4):585-95.
59. Kleessen, B, Hartmann, L, Blaut, M (2003). Fructans in the diet cause alterations of intestinal mmucosal architecture, released mucins and mucosa-associated bifidobacteria in gnotobiotic rats. British J Nutr 89:597-606.
60. Laemelli, UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.
61. Lamont, JT (1992). Mucus: The front line of intestinal mucosal defense. An NY Acd Sci 664:190-201.
62. Leitch, GJ (1988). Cholera enterotoxin-induced mucus secretion and increase in the mucus blanket of the rabbit ileum in vivo. Infect Imm 56(11):2871-2875.
63. Lien, KA, McBurney, MI, Beyde, BI, Thomas, AB, Sauer,WC (1996). Ileal recovery of nutrients and mucin in humans fed total enteral formulas supplemented with soy fiber. Am J Clin Nutr 63:584-595.
64. Lien KA, Sauer WC, Fenton M. (1997). Mucin output in ileal digesta of pigs fed a protein-free diet. Z Ernähurng-swiss. 36:182-190.

65. Lien KA, Sauer WC, He JM (2001). Dietary influences on the secretion into and degradation of mucin in the digestive tract of monogastric animals and humans. J Anim Feed Sci 10:223-245.
66. Lobley, GE, Harris, PM, Skene, PA, Brown, DS, Milne, E, Calder, AG, Anderson, SE, Garlick, PJ, Nevison, I, Connell, A (1992). Response in tissue protein synthesis to sub- and supra-maintenance intake in ruminant lambs: comparison of large dose and continuous infusion techniques. Br J Nutr 68:373-388.
67. Lundin, E, Zhang, JX, Haung, CB, Reuterving, CO, Hallmans, G (1993). Oat bran, rye bran, and soybean hull increase goblet cell volume density in the small intestine of the golden hamster. Scand J Gastroenterol, 28:15-22.
68. Majamaa, H, Isolauri, E (1997). Probiotics: a novel approach in the management of good allergy. J Allergy Clin Immunol 99:179-185.
69. Mantle, M, Allen, A (1981a). Isolation and characterization of the native glycoprotein from pig small-intestinal mucus. Biochem J 195:267-275.
70. Mantle, M, Mantle, D, Allen, A (1981b). Polymeric structure of pig small-intestinal mucus glycoprotein. Biochem J 195:277-285.
71. Mantle, M, Thakore, E, Hardin, J, Gall, DG (1989). Effect of Yersinia enterocolitica on intestinal mucin secretion. Am J Physiol 256(Gastrointest. Liver Physiol 19):G319-G327.
72. Mariscal-Landin G, Sève B, Colléaux Y, Lebreton Y (1995). Endogenous amino nitrogen collected from pigs with end-to-end ileorectal anastomosis is affected by the method of estimation and altered by dietary fiber. J Nutr 125:136-146.
73. McCool, DJ, Forstner, JF, Forstner, GG (1994). Synthesis and secretion of mucin by the human colonic tumour cell line LS180. Biochem J 302:111-118.
74. McCormick, DA, Horton, LWL, Mee, AS (1990). Mucin depletion in inflammatory bowel disease. J. Clin. Pathol. 43:143-146.
75. McGuckin MA, Thronton DJ. Detection and quantitation of mucins using chemical lectin, and antibody methods. In:Glycoprotein methods and protocols: the mucins 2000 (Corfield AP, ed.), pp. 99-109. Humana Press, Totowa, NJ.
76. McNurlan, MA, Tomkins, AM, Garlick, PJ (1979). The effect of starvation on the rate of protein synthesis in rat liver and small intestine. Biochem J 178:373-379.
77. Montagne, L, Toullec, R, Lalles, JP (2000). Calf intestinal mucin: isolation, partial characterization, and measurement in ileal digesta with an enzyme-linked immunosorbent assay. J Dairy Sci, 83:507-517.
78. Montagne, L, Piel, C, Lalles, JP (2004). Effect of diet on mucin kinetics and composition: nutrition and health implications. Nutr Reviews 62(3):105-114.
79. Moore, BA, Sharkey, KA, Mantle, M (1993). Neural mediation of cholera toxin-induced mucin secretion in the rat small intestine. Am J Physiol 265(Gastrointest. Liver Physiol. 28):G1050-G1056.
80. Neutra, MR, Forstner, JF, Gastrointestinal mucus: synthesis, secretion, and function, in: L.R. Johnson (Ed.), Physiology of the gastrointestinal Tract, Raven Press, New York, 1987, pp. 975-1009.
81. Njoku, OO, Leitch, GJ (1983). Separation of cholera enterotoxin-induced mucus secretion from electrolyte secretion in rabbit ileum by acetazolamide, colchicines, cycloheximide, cytochalasin B and indomethacin. Digestion 27:174-184.

82. Oliver, MG, Specia, RD (1990). Cytoskeleton of intestinal goblet cells: role of actin filaments in baseline secretion. Am J Physiol. 259 (Gastrointest. Liver Physiol. 22):G991-G997.
83. Ota H, Nakayama, J, Momose M (1998). Helicobacter pylori infection produces reversible glycosylation changes to gastric mucins. Virchows Arch 433:419-426.
84. Pearson JP, Allen A, Hutton DA. Rheology of mucin. In: Glycoprotein methods and protocols: the mucins 2000 (Corfield AP, ed.), pp. 99-109. Humana Press, Totowa, NJ.
85. Phillips, TE (1992). Both crypt and villus intestinal goblet cells secrete mucin in response to cholinergic stimulation. Am J Physiol 262(Gastrointest. Liver Physiol. 25):G327-G331.
86. Piel, C, Montagne, L, Salgado, P, Lalles, J-P (2004). Estimation of ileal output of gastro-intestinal glycoprotein in weaned piglets using three different methods. Reprod Nutr Dev 44:419-435.
87. Piel, C, Montagne, L, Seve, B, Lalles, J-P (2005). Increasing digesta viscosity using carboxymethylcellulose in weaned piglets stimulates ileal goblet cell numbers and maturation. J Nutr 135:86-91.
88. Plaisancié, P, Barcelo, A, Moro, F, Claustre, J, Chayvialle, JA, Cuber, JC (1998). Effects of neurotransmitters, gut hormones, and inflammatory mediators on mucus discharge in rat colon. Am J Physiol 275(Gastrointest. Liver Physiol 38):G1073-G1084.
89. Pullan RD, Thomas, GAO, Rhodes, M (1994). Thickness of adherent mucus on colonic mucosa in humans and its relevance to colitis. Gut 35:353-359.
90. Pusztai, A, Greer, F (1985). Toxicity of kidney beans (Phaseolus vulgaris) in rats: changes in intestinal permeability. Digestion 32(1): 42-6.
91. Reeds, P, Burrin, D, Stoll, B, Vangoudoever, J (1999). Consequences and regulation of gut metabolism. In: Lobley, GE, White, A. and MacRae, JC, eds. Proceedings of the VIIIth international symposium on protein metabolism and Nutrition. Wageningen, The Netherlands: EAAP Publication no 96;127-153.
92. Roomi, N, Laburthe, M, Fleming, N, Crowther, R, Forstner, J (1984). Cholera-induced mucin secretion from rat intestine: lack of effect of cAMP, cycloheximide, VIP, and colchicines. Am J Physiol 247(Gastrointest. Liver Physiol. 10):G140-G148.
93. Satchithanandam, S, Vargofcak-Apker, M, Calvert, RJ, Leeds, AR, Cassidy, MM (1990). Alteration of gastrointestinal mucin by fiber feeding in rats. J Nutr 120:1179-1184.
94. Satchithanandam, S, Klurfeld, DM, Calvert, RJ, Cassidy, MM (1996). Effects of dietary fibers on gastrointestinal mucin in rats. Nutr Res 16:1163-1177.
95. Scawen, M, Allen, A (1976). The action of proteolytic enzymes on the glycoprotein from pig gastric mucus. Biochem J 63(2):363-8.
96. Schaart, MW, Schierbeek, H, van der Schoor, SRD, Stoll, B, Burrin, DG, Reeds, PJ, van Goudoever, JB (2005). Threonine utilization is high in the intestine of piglets. J Nutr 135:765-770.
97. Schmidt-Wittig U, Enss ML, Coenen M, Gartner K, Hedrich HJ (1996). Response of rat colonic mucosa to a high fiber diet. Ann Nutr Metab 40:343-350.

98. Schneeman BO, Richter BD, Jacobs LR (1982). Response to dietary wheat bran in the exocrine pancreas and intestine of rats. J Nutr 112:283-286.
99. Sellers, LA, Allen, A, Morris, ER, Ross-Murphy, SB (1991). The rheology of pig small intestinal and colonic mucus: weakening of gel structure by non-mucin components. Biochimica et Biophysica Acta 1115:174-179.
100. Sherr, HP, Mertens, RB, Brook, R (1979). Cholera toxin-induced glycoprotein secretion in rabbit small intestine. Gastroenterology 77:18-25.
101. Shiau, SY, Chang, GW (1983). Effects of dietary fiber on fecal mucinase and β-glucuronidase activity in rats. J Nutr 113:138-144.
102. Shimotoyodome, A, Meguro, S, Hase, T, Tokimitsu, I, Sakata, T (2000). Short chain fatty acids but not lactate or succinate stimulate mucus release in the rat colon. Comparative Biochemistry and Physiology Part A, 125:525-531.
103. Smith AC and Podolsky DK (1987). Biosynthesis and secretion of human colonic mucin glycoproteins. J Clin Invest 80:300-307.
104. Southorn, BG, Kelly, JM, McBride, BW (1992). Phenylalanine flooding dose procedure is effective in measuring intestinal and liver protein synthesis in sheep. J Nutr 122:2398-2407.
105. Specian, RD, Oliver, MG (1991). Functional biology of intestinal goblet cells. Am J Physiol 260(Cell Physiol. 29):C183-C193.
106. Starkey, BJ, Snary, D, Allen, A (1974). Characterization of gastric mucoproteins isolated by equilibrium density-gradient centrifugation in caesium chloride. Biochem J 141:633-639.
107. Steel, DM, Hanrahan, JW (1997). Muscarinic-induced mucin secretion and intracellular signlaing by hamster tracheal goblet cells. Am J Physiol 272:L230-L237.
108. Steinberg, SE, Banwell, JG, Yardley, JH, Keusch, GT, Hendrix, TR (1975). Comparison of secretory and histological effects of shigella and cholera enterotoxins in rabbit jejunum. Gastroenterology 68:309-317.
109. Stoll, B, Henry, J, Reeds, PJ, Yu, H, Jahoor, F, Burrin, DG (1998). Catabolism dominates the first-pass intestinal metabolism of dietary essential amino acids in milk protein-fed piglets. J Nutr 128:606-614.
110. Stoll B, Burrin, DG, Henry, JF, Jahoor, F, Reeds, PJ (1999). Dietary and systemic phenylalanine utilization for mucosal and hepatic constitutive protein synthesis in pigs. Am J Physiol 276(1 Pt 1):G49-57.
111. Strugala, V, Allen, A, Dettmar, PW, Pearson, JP (2003). Colonic mucin: methods of measuring mucus thickness. Proceedings of the Nutrition Society 62:237-243.
112. Tani, S, Okuda, M, Morishige, R, Tanaka, T (1997). Gastric mucin secretion from cultured rat epithelial cells. Biol Pharm Bull 20:103-116.
113. Trompette A, Claustre J, Caillon F, Jourdan G, Chayvialle JA, Plaisancié P (2003). Milk bioactive peptides and beta-casomorphins induce mucus release in rat jejunum. J Nutr 133(11):3499-503.
114. Tytgat, KMAJ, Bovelander F-J, Opdam, FJM, Einerhand AWC, Buller, HA, Dekker, J (1995). Biosythesis of rat muc2 in colon and its analogy with human muc2. Biochem J 309:221-229.

115.Tytgat, KMAJ, Buller, HA, Opdam, FJM, Kim, YS, Einerhand, AWC, Dekker, J (1994). Biosynthesis of human colonic mucin: muc2 is the prominent secretory mucin. Gastroenter 107:1352-1363.
116.Umbach, M, Teschemacher, H, Praetorius, K, Hirschhauser, R, Bostedt, H (1985). Demonstration of a beta-casomoprphin immunoreactive material in the plasma of newborn claves after milk intake. Regul Pept 12:223-230.
117.van Goudoever JB, Sulkers EJ, Lafeber HN, Sauer PJ (2000). Short-term growth and substrate use in very-low-birth-weight infants fed formulas with different energy contents. Am J Clin Nutr 71(3):816-21.
118.Van Der Schoor, SRD, Reeds, PJ, Stoll, B, Henry, JF, Rosenberger, JR, Burrin, DG, Van Goudoever, JB (2002). The high metabolic cost of a functional gut. Gastroenterology 123:1931-1940.
119.Van Klinken, BJ-W, Einerhand AWCE, Buller, HA, Dekker, J (1998). Strategic biochemical analysis of mucins. Analytical Biochemistry 265:103-116.
120.Walsh MD, Jass JR. Histologically based methods for detection of mucin. In: Glycoprotein methods and protocols: the mucins 2000 (Corfield AP, ed.), pp. 29-44. Humana Press, Totowa, NJ.
121.Yardley, JH, Bayless, TM, Leubbers, EH, Halsted, CW, Hendrix, TR (1972). Goblet cell mucus in the small intestine: findings after net fluid production due to cholera toxin and hypertonic solutions. Johns Hopkins Med. J 130:1-10.
122.Zhao XH, Wen ZM, Meredith CN, Matthews DE, Bier DM, Young VR (1986). Threonine kinetics at graded threonine intakes in young men. Am J Clin Nutr 43(5):795-802.