THE ROLE OF MUCIN IN ESTABLISHMENT OF *ESCHERICHIA COLI* IN PORCINE SMALL INTESTINE

by

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ADP	Adenosine diphosphate
cAMP	Cyclic adenosine monophosphate
CAYE	Casamino acids-yeast extract medium
CFU	Colony-forming units
cGMP	Cyclic guanosine monophosphate
DNA	Deoxyribonucleic acid
EDTA	Disodium (ethylenedinitrilo) tetracetate
ETEC	Enterotoxigenic *Escherichia coli*
Fuc	Fucose
Gal	Galactose
GalNAc	N-acetylgalactosamine
GLC	Gas liquid chromatography
GluNAc	N-acetylglucosamine
GTP	Guanosine triphosphate
HexNAc	N-acetylhexosamines
HGM	Hog gastric mucin
LT	Heat-labile enterotoxin
MS	Mucosal scrapings medium
NAD	Nicotinamide adenine dinucleotide
NANA	N-acetylneuraminic acid
NETEC	Non-enterotoxigenic *Escherichia coli*

PBS	Phosphate buffered saline
PSIM	Porcine small intestinal mucin
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
ST	Heat-stable enterotoxin
ST_a	Heat-stable enterotoxin a
ST_b	Heat-stable enterotoxin b
Tris	Tris(hydroxymethyl)aminomethane

CHAPTER ONE

INTRODUCTION

Everyday man ingests literally billions of bacteria from his food chain. Many, if not most, of these bacteria will pass through the intestinal tract and exit via feces virtually unnoticed. However, a small number will become established in one of the sections of the digestive tract (ie. mouth, pharynx, stomach, small intestine, or colon). The intestinal flora is extremely complex and may be populated by over 500 species of bacteria at any time (117). Escherichia coli are isolated frequently as non-pathogenic members of this population (4,159). However, the intestine can also be colonized by enterotoxigenic E. coli (ETEC) which inflict the host with a sometimes deadly diarrheal disease. ETEC enteritis is a major cause of economic loss to the livestock industry. Piglets, calves, and lambs are susceptible to ETEC. Different serotypes of ETEC also cause diarrheal disease in human infants.

The vertebrate gastrointestinal tract is coated with a renewable layer of complex carbohydrates in structural macromolecules collectively termed mucous gel. Mucous gel

is composed of glycoproteins (mucin) and glycolipids containing carbohydrate moieties in the form of oligosaccharide chains. Mucous gel functions to protect the epithelial cell membranes from the harsh intestinal environment.

Several studies have shown that gastric and intestinal mucins are extensively degraded and used for growth by fecal bacteria (81,84-87) while others have reported little or no utilization (12,140,146,147). Enzymes produced by pathogenic and non-pathogenic E. coli are able to hydrolyze ovomucin (11). However, E. coli are unable to extensively degrade (> 25%) the carbohydrate available on hog gastric mucin (HGM) (84).

Enteropathogens, such as E. coli, are able to resist the effects of intestinal motility by adherence to mucosal sufaces (157). Furthermore, enteropathogens can form microcolonies immediately adjacent to apical portions of absorptive cells along the villi of pig small intestine. Nonenteropathogens tend to occur primarily in the central lumen of the small intestine (11,84). Lipopolysaccharides from non-enterotoxigenic E. coli (NETEC) F-18 bind to a 26,000 molecular weight protein from mouse colonic mucous gel (28). Once bound to mucosal gel receptors, E. coli

should be able to metabolize mucous gel proteins. Thus, complex carbohydrates in mucous gel appear to have a role in host microbial association in the gut (83).

Prior to colonization, E. coli present in the intestinal mileau must somehow associate with the mucosal surface. One possible method is chemotaxis which would allow the organism to reach the mucin layer by directed motility rather than by random movement. Bacteria capable of chemotaxis are at a selective advantage in mixed microbial populations (133). The chemical composition of mucin provides an ideal source of taxins to attract E. coli. Strain AW405 was reported in a preliminary study to be chemoattracted to a pepsin digest of rabbit intestinal mucosa (8). Non-chemotactic E. coli mutants were not attracted to pepsin-digested mucosa. Chemotaxis to intestinal mucosa surfaces has been reported for Vibrio cholerae (61) and Salmonella typhimurium (8).

The objectives of this study are 1) to determine chemical and physical properties of mucin prepared for incorporation into bacteriological media by reduction and proteolysis of porcine small intestinal mucous gel, 2) to determine the ability of E. coli to grow and produce enterotoxins in vitro when mucin provides the sole-source

of carbon and nitrogen, 3) to identify the enzymes responsible for hydrolysis of mucin, and 4) to determine whether mucin is a positive *in vitro* chemoattractant for *E. coli*.

CHAPTER TWO

LITERATURE REVIEW

A. HOST MICROFLORA

Many different bacterial species have been isolated from the intestinal tract. However, which factors control the microflora composition of the intestine are not well-understood. Savage (148) has described two types of intestinal microbial habitants. Autochothonous microorganisms, such as the Lactobacillus and Bacteroides species, have developed a symbiotic relationship with their hosts. The allochthonous microorganisms are potential pathogenic microbes which, under proper conditions, may predominate (bacterial overgrowth) and impair normal gastrointestinal function (172). Examples are some species of E. coli and clostridia. Together these groups are termed the indigenous flora. Non-indigenous microorganisms are those acquired from the environment of the host.

Indigenous microorganisms have been shown to influence host characteristics such as rate of growth, resistance to infection, nutritional status, response to toxins, efficiency in digestion of nutrients, and response

to other stressful situations (19,24). In contrast, the host can also influence the enteric flora through the composition of salivary, gastric, and intestinal secretions; intestinal motility; intraluminal pH; redox potential; and fluid and electrolyte compostion of intestinal contents (148,172).

Distribution of bacteria varies along the length of the swine intestinal tract (159,176). In general, pig small intestines are occupied by 10^6 to 10^9 bacteria per gram of contents (4,159) while colon and cecum contain 10^9 to 10^{10} bacteria per gram of contents (159). Bacterial counts in contents of a particular intestinal segment will vary with movement of contents down the intestinal lumen. In general, lower numbers of organisms are present in the anterior portion (stomach and duodenum) of the intestinal tract and higher numbers in the posterior (rectum). The stomach of adult pigs is not sterile as once thought despite its low pH (4.5). When full, the stomach contains 10^4 viable, anaerobic bacteria per gram of contents while an empty stomach contains only 10^2/g (117).

The fetal gastrointestinal tract is sterile until birth (70). During the birth process, infants and baby animals aquire microorganisms from the vagina and

genitalia of the mother and other environmental sources to which they are exposed (38). Many of these microorganisms are unable to colonize the gastrointestinal tract and disappear shortly after birth because of inability to colonize. Others will become established in the intestine and become part of the autochothonous intestinal flora.

During the first day of life, the piglet alimentary tract is colonized by large numbers of E. coli, streptococci, lactobacilli, and Clostridium perfringens (109,159). All of these species except lactobacilli decrease in number after the first day. Lactobacilli are a principal component of the indigenous flora throughout the animal's life.

E. coli also remains as part of the indigenous flora (4,109,159). E. coli is unique because it can exist as a member of the indigenous (allochthonous) intestinal flora with no pathogenic effect to the host as well as a non-indigenous microorganism capable of causing diarrhea. The factors which differentiate the two types (non-pathogenic vs. pathogenic) are beginning to be understood in more detail.

B. ESCHERICHIA COLI PATHOGENICITY

Jensen (89) first identified E. coli as a cause of piglet diarrhea in 1899. Colibacillosis is colonization of the small intestine with an enterotoxigenic strain of E. coli (ETEC). Enterotoxins cause release of fluids and electrolytes from intestinal epithelial cells resulting in diarrhea, dehydration, and sometimes death (95).

Immunological response to surface components on E. coli is used to serologically group and identify ETEC and NETEC. The most common antigens are the O (cell wall lipopolysaccharide), K (acidic polysaccharides of the capsule), and H groups (flagellar proteins). The K antigen has been connected with ability of E. coli to colonize small intestine mucosa. Bacteria which possess specific plasmid mediated K antigens produce adherence structures on their surface which enable binding to the brush border of intestinal mucosa (139). K antigens responsible for adherence occur as filamentous, protein appendages similar to fimbriae or pili (34,169). Four serologically distinct pili (K88, K99, F41 and 987P) have been isolated from E. coli that are enterotoxigenic for neonatal pigs (95). ETEC strains isolated from humans possess similar plasmid-mediated adhesins called

colonization factor antigens (CFA). Two types have been identified; CFA/I (43) and CFA/II (45).

Once ETEC adhere to intestinal cell surfaces they can multiply and produce symptoms in the host by secreting enterotoxin. Enterotoxin production is controlled by plasmids which can be transmitted between strains of E. coli during conjugation (154,158). Therefore, the opportunity exists for a NETEC strain to become ETEC through mating. Smith and Linggood (160) demonstrated that K88 antigen and enterotoxin production are controlled by separate plasmids. Two different enterotoxins have been identified among the extracellular products of E. coli isolated from humans and other mammals with diarrheal disease; one is heat-stable (ST; 144) and the other heat-labile (LT; 145).

Strains of LT-producing E. coli have been implicated in cases of human traveler's diarrhea and animal colibacillosis. LT protein has a molecular weight of approximately 91,000 (27) and is composed of an A protomer of 25,500 to 29,000 M_r (26,32) and a B protomer of 59,000 M_r (65). The A protomer is synthesized as a polypeptide chain (26) that can be converted to a nicked form by trypsin treatment. The result is a toxic A_1 polypeptide

chain (21,000 M_r) linked by a disulfide bond to an A_2 chain (26). The B protomer is composed of five noncovalently linked identical polypeptides (B chains) of 11,800 M_r (31). LT is immunologically similar to cholera toxin and has common antigenic determinants with the A and B subunits of choleragen (25). The toxins also possess a large degree (80%) of sequence homology in the B chain (31) and amino terminal region of the A_1 chains (164). LT and cholergen bind to ganglioside GM_1 (36) of small intestine epithelial cells and possess the same biological activity in several different toxin assays.

The B chains are responsible for binding to intestinal epilthelium by interacting with ganglioside GM_1 or glycoprotein receptors (79,80). After binding, a conformational change occurs in the toxin molecule which allows the A subunit to cross the cell membrane and gain access to the adenylate cyclase system (49,65). Nicotinamide adenine dinucleotide (NAD) and guanosine triphosphate (GTP) are required by the A subunit to activate adenylate cyclase. NAD serves as substrate for an A-catalyzed adenoside diphosphate (ADP)-ribosylation of a 42,000 molecular weight GTP-binding protein that is involved in regulation of adenylate cyclase activity (66).

The role of A_1 and A_2 chains in binding is unknown. The reaction also requires a cytoplasmic macromolecule whose role is undetermined. ADP-ribosylation causes a greater stability of the catalytically active complex composed of GTP, modified GTP-binding protein, and catalytic subunit that actually synthesizes cyclic adenosine monophosphate (cAMP) (66). Increased levels of cAMP in epithelial cells result in secretion of chloride ions into the intestinal lumen and subsequent secretion of fluid characteristic of LT-induced diarrhea.

No unusual factors are necessary for in vitro production of LT (67). Culture pH must be maintained between 7.5 and 8.0 to obtain complete release of toxin from the cells. A basal minimal medium containing methionine, lysine, and either aspartic acid or glutamic acid will yield maximum production of toxin. Addition of glucose to the medium supports maximal LT synthesis despite catabolite repression (67).

ST has been identified as the toxin implicated in certain cases of traveler's and childhood diarrhea, as well as animal colibacillosis. ST is a small, non-antigenic peptide with a molecular weight of 1972 to 5100 depending on strain of ETEC (97,102,166). All

peptides have similar amino acid compositions, are devoid of basic amino acids and contain six half-cysteine residues per molecule. No antigenic cross-reactivity has been observed between ST and LT or cholera toxin (57).

ETEC strains may produce more than one type of ST. These are differentiated by methanol solubility and animal species specificity. ST_a is methanol-soluble, produced in human, bovine, and porcine strains of ETEC, and is detected in suckling mouse bioassay and in unweaned piglets. Methanol-insoluble ST_b is found only in porcine ETEC strains and has activity in weaned piglets but not suckling mice (20). Both forms are immunologically (57) and genetically (163) distinct. ST_a is the most extensively studied. This enterotoxin is very resistant to proteases, lipases, nucleases, phospholipase C, and amylase (6,97). ST_a maintains biological activity between pH 2 and 10 and after heating at 100 C for 15 min, but is inactivated at 121 C. In addition, biological activity is lost in presence of reducing agents (27).

ST_a causes an increase in cGMP levels in intestinal mucosal cells by activation of guanylate cyclase (47,63). The mechanism by which guanylate cyclase is stimulated is unknown. The mode by which ST_b causes diarrhea is even

more obscure, but apparently does not involve cGMP (138). The effect of ST_a appears to be tissue specific since guanylate cyclase activity is not stimulated in a variety of other tissues (73).

The intestinal membrane receptor for ST_a differs from that for LT and cholera toxin. LT and mixed gangliosides do not alter the binding of ST_a to rat intestine epithelial cells and brush-border membranes (56). Binding is time and temperature dependent and saturable. Furthermore, ST_a binds on brush-border membranes to a single-class of high affinity receptors of 100,000 molecular weight (39). Evidence suggests ST_a may be linked to this protein via a disulfide bridge (39).

Defined medium supplemented with vitamins, lactate, or oleate caused slightly higher levels of <u>in vitro</u> ST production (128). However, maximum toxin production can be acheived in a defined medium containing Mg^{2+}, Mn^{2+}, and Fe^{3+} salts, proline, aspartic acid, alanine, serine, and the chelator tricine (128). Increased growth was obtained after addition of glucose (0.25%) but ST production was repressed (5).

C. BACTERIAL CHEMOTAXIS

Enteropathogens, such as *E. coli*, are able to resist the effects of intestinal motility by adherence to mucosal surfaces (157). Furthermore, enteropathogens can form microcolonies immediately adjacent to apical portions of absorptive cells along the villi of pig small intestine. Nonenteropathogens tend to occur primarily in the central lumen of the small intestine (11,92). Whether chemotactic attraction is involved in association of *E. coli* with intestinal mucosa has not been determined.

Chemotaxis is movement toward or away from chemicals or compounds by motile bacteria and was first reported late in the 19th century (41). Chemotaxis is important to survival of the bacterial cell in natural environments. Once a stimulus or repellant is detected, a signal is sent to the flagella to migrate toward (positive chemotaxis) or away (negative chemotaxis) from the stimulus (2, 3). Adler (2) reviewed in detail the mechanisms involved in bacterial chemotaxis. Important points will be discussed below.

"Chemosensors" located in the bacterial membrane measure and respond to changes in chemical concentrations (17,98,99). A "Chemoreceptor" is a component of the

chemosensor that recognizes or binds the chemicals detected. An additional component is the "signaller" that communicates to the flagella the fraction of chemoreceptor occupied by the chemical. Often proteins of the chemosensor also function in a transport system (ie. sucrose binding protein) in which the positive stimuli are substrates for metabolism (99). However, the chemotaxis and transport systems have other independent components and transport is not required for chemotaxis.

The chemosensors transmit information (via unknown pathways) to the chemoreceptor and signaller, which are methylated by a methyltransferase (165). Methyl-accepting chemotaxis proteins require S-adenosyl-methionine for chemotaxis and for the change in membrane potential caused by chemotaxis. No chemotactic response is observed if mutants lack the methyltransferase essential for methylating the chemoreceptor and signaller.

A role for bacterial motility and chemotaxis in pathogenesis of infection has been suggested (15,58,72). Freter et al. (58) have shown chemotactic attraction is an important factor in the association of Vibrio cholerae with mucus gel. Subsequent adherence to animal intestinal epithelial cell receptors occurs after a series of

interactions. In the first phase, motile vibrios detect gradients of chemoattractants and move from the intestinal lumen to the mucosal surface (8,58). Next, vibrios penetrate into and through mucus gel for considerable distances in short periods of time (90). Lastly, vibrios adhere to the intestinal brush borders (60).

Non-motile mutants of V. cholerae associate less frequently with the mucosal surface of mouse and rabbit intestine than their motile parent strains (90). Furthermore, motile vibrios are more widely distributed and penetrate deeper between villi (60,91). Although motility is important for vibrios to enter the mucus gel, movement is random unless directed by chemotaxis (58,61). Experiments with motile, non-chemotactic vibrios demonstrated that these mutants could penetrate mucus gel better than non-motile vibrios, but were less effective than motile, chemotactic parent strains (58).

D. STRUCTURE AND FUNCTION OF GASTROINTESTINAL MUCUS

Gastrointestinal mucus (synonomous with mucous gel) is a general term used to describe the viscoelastic gel which adheres to mucosal cell surfaces and resists aqueous solubilization. Mucus consists of two principal

organic components: the glycocalyx and mucin. Glycocalyx is composed of glycoproteins and glycolipids intimately associated with microvilli membranes and is not affected by intestinal peristalisis. Glycocalyx cell surface oligosaccharides provide a variety of potential binding sites for bacterial toxins (37,78), plant lectins (42), and microorganisms (94).

The major organic component of mucus is a group of macromolecular glycoproteins (mucins). Mucins coat the glycocalyx and are secreted from goblet cells of the intestinal epithelium. The balance of mucus is composed of water (up to 95% by weight), electrolytes, sloughed epithelial cells, plasma proteins, pancreatic enzymes, bacteria and bacterial products, digested food, and bile salts. In this review I will concentrate on the major organic component (mucin) of mucous gel from the small intestine, although much of the information presented here can be applied to virtually all other systems of the body covered with epithelial mucosa despite minute differences in composition. Several excellent reviews on mucin (13,18,22,23,48,53) have appeared recently in the literature.

Mucin has a variety of important functions including

protection, lubrication, entrappment, and locomotion (53). The major function is to protect the underlying epithelial cells from mechanical damage caused by movement of food or feces and other stresses of the digestive process.

Heatley (75) first suggested digestive acids were neutralized by a mucosal alkaline secretion contained within the mucous gel. Williams and Turnberg (178,179) reported pH gradients across the gastric mucous gel from a luminal pH of 2 to a value between pH 6 and 7 at the mucosal surface. Therefore, mucus (which is readily permeable to H^+) provides a barrier at the mucosal surface preventing the relatively small amounts of HCO_3^- from mixing with bulk H^+ in the lumen and confining neutralization to the mucosal surface. Acids released to the duodenum during digestion are neutralized in a similar manner. Flemstrom and Garner (50) demonstrated secretion of mucosal HCO_3^- and protection of the small intestine epithelia from acid.

Intestinal mucin probably does not protect the mucosa from proteolytic enzymes. Shora et al. (155) could not demonstrate protection against trypsin or chymotrypsin. To the contrary, mucin stimulated proteolytic digestion of large substrates (155). Therefore, mucin's role may be to

aid in the luminal degradation of dietary proteins and exfoliated epithelial cells (55).

Mucins lubricate food and fecal material to ease intestinal transit (51,52). The process begins early in the digestive process when saliva coats foodstuffs with mucus to ease passage through the throat (71). Particles of India ink injected into the stomach of a cat become coated with mucin during transit through the intestinal tract (52). In the lower portion of the intestinal tract, the mucin-coated droplets coalesce and appear in feces as small masses firmly held together by mucin.

The role of oral mucin in entrappment of microorganisms is well established. Pathogenic streptococci are aggregated by salivary mucin and washed away from the buccal mucosa to prevent infection (93). Gastric mucin competes with cholera toxin in binding to small intestinal receptor sites (170). However, if mucin blocked potential adhesin sites on the microorganism this activity could not be demonstrated. More research is needed to determine the role of entrapment by mucin in prevention of pathogenesis.

Locomotion of foodstuffs by mucin through the intestinal tract works in tandem with lubrication. Food

is mixed with saliva and salivary mucin and swallowed. Peristalisis by cilia and mucin lining the entire length of the digestive tract carries the lubricated foodstuffs to their final metabolic end-products (53).

Mucins are glycoproteins with a molecular weight of about 2×10^6 and have viscoelastic properties which account for the gelling characteristics of whole mucus. Mucins are characterized by a predominance of carbohydrate which generally accounts for 80% of the macromolecule's weight. Mucin oligosaccharides vary in composition, sequence, and chain length (53). Oligosaccharides consist of N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc), galactose (Gal), fucose (Fuc), and N-acetyl- (NANA) or N-glycollyl- neuraminic acids. Uronic acids and glucose are not present, nor are significant amounts of mannose. Sequence of the oligosaccarides is controlled by specificity of glycosyltransferases. Carbohydrate chain lengths can vary from 2 to 22 sugars and in some cases are branched (125). Oligosaccharide chains are joined to the central protein core by O-linkage of GalNAc to the hydroxyl of either threonine or serine. The protein core is composed mainly (over 70%) of serine, threonine, and proline. Concentration of aromatic amino acids is low.

All human epithelial mucin oligosaccharides have α-linked glycosides on the outer, nonreducing terminal ends (Table 2.1) (68). Carbohydrates are added in a specific order and linkage-type during biosynthesis by glycosyltransferases whose presence is dictated by the individual's ABO(H), Lewis, and secretor blood type genes (68). Hogs appear to have only blood groups A and H in common with humans (105).

Native mucin molecules are negatively charged under physiological conditions because: NANA residues are in terminal positions on the oligosaccharide chains, ester sulfate residues (156), and there is a net excess of negatively charged amino acids in the protein core. However, the amount of negative charge will vary from one type of mucin to another since there is heterogeneity in amounts of sialic acids, ester sulfates, and caboxyl groups in the amide form at physiological pH.

HGM secretions are polymeric structures composed of glycoprotein subunits joined by disulfide bridges between their protein cores (161). Reduction of native glycoprotein (2×10^6 M_r) from HGM with mercaptoethanol produces an average of four glycoprotein subunits (5×10^5 M_r) and release of a link protein (131,161). The link

Table 2.1. Structures responsible for A, B, and H(O) blood-group specificities.

Specificity	Structure
A	GalNAc α1-3Gal β 1-3GlcNAc.. 2 | Fuc α 1
B	Gal α1-3Gal β 1-3GlcNAc.. 2 | Fucα 1
H(O)	Gal β 1-3GlcNAc.. 2 | Fuc α1

protein has a molecular weight of 70,000 and is important in holding the subunits together in the native molecule through disulfide bridges.

Glycoproteins from human gastric mucus are similar in structure to HGM (131,132). Both have a polymeric structure of glycoprotein subunits joined by disulfide bridges with link proteins. The link protein from human gastric mucin has a molecular weight of 118,000 (107).

The structure of PSIM (1.8×10^6 M_r) is more complex than HGM (106). Reduction of pig native small intestinal glycoprotein produces a single polydisperse glycoprotein component with a molecular weight of 2.4×10^5 (106) and releases a link protein of 90,000 molecular weight which comprises approximately 5% (by weight) of the native glycoprotein. One link protein is joined to each glycoprotein molecule by disulphide bridges.

Human small intestinal mucin differs structurally from PSIM. Glycoproteins from human small intestinal mucus do not dissociate when incubated in 10 mM dithiothreitol and 6 M guandinium chloride for four hours (54). Additionally, human small intestinal mucin does not possess a link protein (54). The macromolecule appears to be stabilized by non-covalent interactions between a

large, highly glycosylated polypeptide and a small, poorly glycosylated polypeptide (54).

Mucous glycoproteins form weak gels through noncovalent interactions strong enough to resist osmotic pressure and solubilzation but not strong enough to resist gel mobility or mechanical disruption (14). If penetrated, mucus gel will flow and anneal itself. Drastic shear forces (e.g. homogenization) will break the noncovalent bonds between the glycoprotein molecules and solubilize the gel. The polymeric structure, described above, is required for gel formation since treatment with reducing agents or proteases will disrupt the covalent structure and rapidly solubilize mucus.

Native glycoproteins isolated from PSIM are expanded molecules with intrinsic viscosities of 500 ml/g (105). Viscosity of purified small intestinal mucin slowly rises with increasing concentration until about 10 to 12 mg/ml when gelation occurs. This is approximately the <u>in vivo</u> concentration of glycoprotein in PSIM (105). The ability to form a gel does not depend on structure and length of carbohydrate chains as evidenced by gel formation of mucins from different sources [ie. gastric (7), bronchial (141), and submaxillary (77)].

Mucins are synthesized in and secreted from specialized epithelial cells called goblet cells (Fig. 2.1). Goblet cells differentiate in the lower portion of the crypts and migrate to the villi tips. Although mucin is synthesized and secreted as cells migrate, synthesis and secretion proceed more rapidly after goblet cells have reached the villi tips (123,124). Mucin synthesis, with regard to antigenicity and amino acid content, is controlled at the transcriptional and translational levels (69). Once mucins are assembled within Golgi apparatus, they are released at the intestinal cell surface by exocytosis (161). Goblet cells have a unique characteristic in that intracellular mucous granules are stored in tightly packed vesicles at the apical end of the cell (161). These vesicles are enclosed by a filament-rich, cup-shaped sheath of peripheral cytoplasm. Factors which control synthesis and secretion of mucin are unknown. More details on mucin biosynthesis can be found in recent reviews (55,122).

E. DIGESTION OF MUCIN BY ENTERIC MICROFLORA

The use of mucin glycoproteins as metabolizable substrates by indigenous enteric microflora has been the

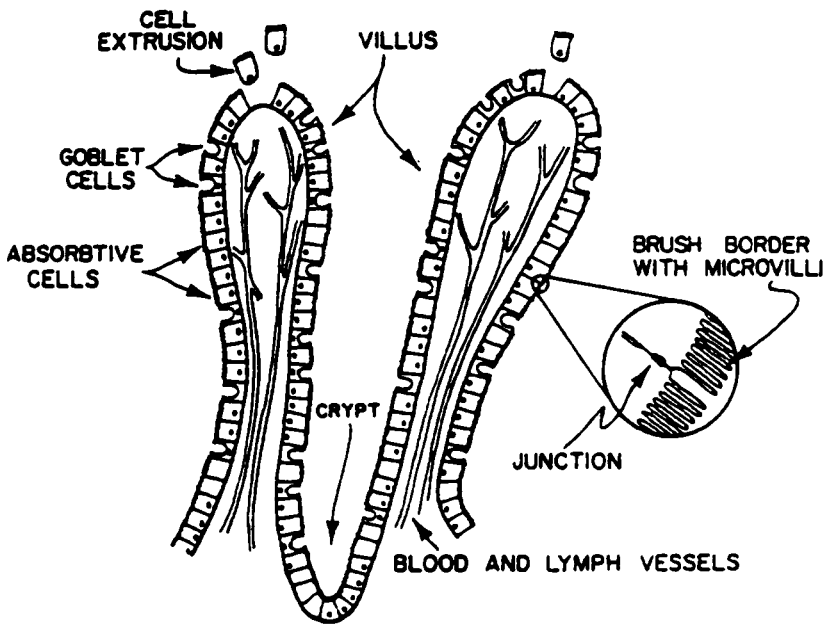

Fig. 2.1. Diagram of porcine small intestine mucosal epithelia (29).

subject of a recent review (83). Studies with germ-free rats have shown mucin glycoproteins are major constituents of water soluble, non-dialyzable fractions obtained from feces and intestinal contents (181). Germ-free rats fed a diet of glucose in Ringer's solution excreted greater quantities of mucin than conventional rats fed the same diet (87). Radiolabeled goblet cell mucin instilled into conventional rat small intestine was partially degraded into smaller deglycosylated fragments (127). Human A, B, and H blood group immunoactivities are present in the water soluble, non-dialyzable fractions of feces from germ-free animals but not from conventional rats (87). High titers of blood group A immunoreactivity were obtained in lumenal contents of the human proximal small intestine while activity diminished near the ileocecal valve and was absent in colon contents (180). As would be expected, distribution of human blood group A-degrading enzymatic activity was highest in the colon and nearly absent in proximal small intestine. For many years this enzymatic activity was thought to be host produced, however, Hoskins (81) demonstrated that indigenous bacteria produce enzymes capable of degrading blood group A-, B-, and H- antigenicities.

HGM has been used to study degradation *in vitro* because it is structurally similar to human intestinal mucin (167) and commercially available. Human cell-free fecal extracts or cell-free supernatants from anaerobic fecal cultures caused a reduction in the total hexose (55 to 90%) and protein (8 to 50%) content of HGM. Degradation rates of mucin carbohydrate by fecal extracts and fecal culture supernatants were comparable.

Mucin oligosaccharide degradation requires cleavage of individual linkages by glycosidases from enteric bacteria. Blood group degrading enzymes from enteric bacteria act as exoglycosidases by specifically cleaving glycosides from the nonreducing terminal end of oligosaccharide chains. Several enzymes which act on glycosidic linkages of mucin have been detected in intestinal contents, fecal extracts or anaerobic fecal cultures of man and rats. These include β-D-galactosidase, β-N-acetyl-D-glucosaminidase, neuraminidase, β-N-acetyl-D-galactosaminidase (A blood group degrading), α-D-galactosidase (B blood group degrading), and α-L-fucosidase (H blood group degrading) (81,82,85,86,121,136,175). Bacteria indigenous to man also produce endoglycosidases (16,62,120), but their

involvement in mucin glycoprotein degradation is unknown.

Although glycosidase production is known to occur in mixed anaerobic fecal cultures, little is known about subpopulations which produce extracellular glycosidase(s) for *in vitro* HGM degradation. All healthy, human subjects possess mucin-degrading bacteria in their feces (115). However, Miller and Hoskins (115) reported that only approximately 1% of the total fecal flora population is capable of degrading greater than 25% of the hexose in HGM. Later, Hoskins and Boulding (86) reported production of both cell-bound and extracellular glycosidase activity in all inocula levels of serially diluted fecal cultures. However, the majority of glycosidase activity required for mucin degradation are exclusively cell bound in a large fraction (10^{10} to 10^{11} /g dry wt) of the bacterial population from feces (81). The role of cell-bound glycosidases in utilization of mucin by intestinal microflora is not currently known.

Bacteroides species comprise approximately 20% of the normal human colonic flora (76,118). Despite this, Salyers et al. (146) found that none of the 188 strains studied from 10 *Bacteroides* species fermented HGM in a defined medium (174). Further work by this group (147)

with 22 additional species (total of 154 strains) present in human colon detected only eight strains from two species, Ruminococcus torques and Bifidobacterium bifidum, capable of fermenting HGM. However, monitoring HGM utilization by detecting decreases in media pH may not be sufficiently sensitive. Roberton and Stanley (140) demonstrated that Bacteroides fragilis is able to utilize 15% of the carbohydrate in pig colon mucin for growth without a significant decrease in medium pH. Other species of anaerobic, fecal bacteria capable of mucin utilization have been isolated after enrichment on HGM (12). Four isolates were characterized but could not be assigned to a specific genus. One isolate was judged to be an undescribed species of Bifidobacterium.

Mucin degradation by pathogenic or potentially pathogenic bacteria has also been observed. V. cholerae produce proteases and an ill-defined enzyme called mucinase which can hydrolyze mouse intestinal mucin (150). Mucin glycoproteins obtained from the cecum of germ-free mice could sustain growth of S. flexneri (135). Furthermore, high titers of α-galactosidase activity were detected in fecal supernatants. Thus, blood group B glycoproteins, such as those on murine mucin, were readily

attacked by α -galactosidase (135). Indirect evidence has been obtained for growth of *E. coli* F-18 on murine colon mucin (28).

The purpose of this study is 1) to determine chemical and physical properties of mucin prepared for incorporation into bacteriological medium by reduction and proteolysis of porcine small intestinal mucous gel, 2) to determine the ability of *E. coli* to grow and produce enterotoxins when mucin provides the sole-source of carbon and nitrogen, 3) to identify the glycosidases responsible for hydrolysis of mucin carbohydrates, and 4) to determine whether mucin is a positive chemoattractant for *E. coli*.

CHAPTER THREE

MATERIALS AND METHODS

A. MATERIALS

Molecular weight markers (Blue Dextran, myosin, β-amylase, alcohol dehydrogenase, β -galactosidase, phosphorylase B, bovine serum albumin, egg albumin, pepsin, carbonic anhydrase, trypsinogen, lysozyme, and α -lactalbumin), nitrophenyl glycosides (p-nitrophenyl- α -L-fucopyranoside, o-nitrophenyl- β-D-galactopyranoside, p-nitrophenyl- α-D-galactopyranoside, and p-nitrophenyl- α -D-mannopyranoside), DNase (Type III), trypsin (Type IX), DNA (Type III from salmon testes), gentamicin, Sepharose CL-4B (manufactured by Pharmacia Fine Chemicals, Piscataway, NJ), sodium dodecyl sulfate (SDS), galactose, mannose, N-acetylglucosamine (GluNAc), N-acetylgalactosamine (GalNAc), N-acetylneuraminic acid, 2-mercaptoethanol, disodium (ethylenedinitrilo) tetracetate (EDTA), tris(hydroxymethyl)aminomethane (Tris), Coomassie Brilliant blue, bovine serum albumin, pentanedione, and sodium 2,2' bicinchonintate were purchased from Sigma Chemical Co., St. Louis, MO. Ham's F-10 nutrient mixture and fetal bovine serum were from

GIBCO Laboratories, Grand Island, NY. Silver stain was purchased from Bio-Rad Laboratories, Richmond, CA. Yeast extract, brain heart infusion agar, trypticase soy broth, and trypticase soy agar were from Baltimore Biological Laboratories, Cockeyesville, MD. Tryptone was from Difco Laboratories, Detroit, MI. Swiss Webster mice were obtained from Laboratory Animal Resources at Virginia Tech. Adrenal cells, strain CCL 79, were from American Type Culture Collection, Rockville, MD. All other chemicals were reagent grade quality.

B. PIG SMALL INTESTINAL MUCIN PREPARATION

Small intestinal mucin was prepared by slight modification of the procedure of Roberton and Stanley (140). Mucin was collected from 20 healthy, freshly slaughtered adult hogs of either sex. The jejuno-ileum was excised from the small intestine and used for mucin preparation. Digestive material was removed by gently flushing intestines with ice-cold physiological saline (0.15 M NaCl). Mucous gel was collected by scraping the mucosal surface with a glass slide and was dispersed in 2 volumes (v/v) of 10 mM sodium phosphate, pH 7.0, containing 0.1 M NaCl (PBS) with a Polytron homogenizer

(Brinkmann Instruments, Westbury, NY). Mucin gel (supernatant) was obtained by centrifugation at 2,000 x g for 10 min at 4 C and redispersed in an equal volume (v/v) of PBS. Homogenization and centrifugation were repeated once. Insoluble mucin was dispersed in 4 volumes (v/v) of 0.1 M sodium phosphate, pH 7.5, containing 0.1 M NaCl and 0.4 M 2-mercaptoethanol and was extracted with stirring under nitrogen for 12 hr at 4 C. Debris was removed by centrifugation at 10,000 x g for 10 min at 4 C and the supernatant was dialyzed in 6000 MW cut-off tubing against several changes of distilled water at 4 C. The final 12 hr of dialysis was against 1 mM sodium phosphate, pH 7.0, containing 0.2% NaN_3 (w/v), 10 mM $MgCl_2$ and 5 mM $CaCl_2$. Debris was removed by centrifugation at 10,000 x g for 10 min at 4 C. The supernatant was then digested with DNase (0.025 mg/g wet weight of original mucus) for 6 hr and subsequently treated with trypsin (0.025 mg/g wet weight) at 37 C. After 12 hr, an equivalent amount of trypsin was added and digestion continued for another 12 hr. The solution was dialyzed as before, insoluble debris was removed by centrifugation at 10,000 x g for 10 min and the supernatant was lyophilized. All mucin preparations were pooled.

C. CHROMATOGRAPHIC PROCEDURES

Carbohydrate composition of mucin was determined by gas-liquid chromatography (GLC). Mucin samples were hydrolyzed with 1 N H_2SO_4 in sealed-tubes for 5 hr at 100 C. Subsequently, the hydrolyzate was derivatized to form aldononitrile acetate derivatives of the neutral sugars using N-methylimidazole as catalyst and solvent (111). Samples were analyzed with a Gow-Mac Series 750 gas chromatograph (Gow-Mac Instument Co., Bridgewater, NJ) containing a 3.05 m nickel alloy column (3.22 mm, i.d.) packed with 1% diethylene glycol adipate on chromosorb WHP (100-120 mesh). Aldononitrile acetates were eluted isothermally at 195 C with nitrogen as carrier gas at 25 ml/min. Relative response factors were determined from peak areas of standard solutions for each sugar.

Descending gel filtration chromatography was performed on a column (35 x 1.0 cm) packed with Sepharose CL-4B and equilibrated with distilled water. Samples (1 ml) were eluted with distilled water and fractions (1 ml) collected were monitored by absorbance at 280 nm. Carbohydrate content was ascertained by the method of Mantle and Allen (104). Void volume of the column was determined using Blue Dextran. Molecular weights were

determined from the column using β -amylase (200,000), alcohol dehydrogenase (150,000), bovine serum albumin (66,000), and lysozyme (14,500).

D. SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

SDS Polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Fairbanks et al. (46). Samples (0.5 mg/ml) were dispersed in Fairbanks' buffer (0.1 M Tris-HCl, pH 8.0, with 1 mM EDTA and 1% (w/v) SDS) and dialyzed (3500 MW cut-off) at room temperature for 16 hr against the same buffer. Samples were heated at 100 C for 2 min and applied to either a 7.5% (w/v) polyacrylamide, 0.1% (w/v) SDS gel or a 5% (w/v) polyacrylamide, 0.1% SDS gel. After electrophoresis (100 v constant current), gels were stained with Coomassie Brilliant blue, silver (112), or periodic acid-Schiff reagent (PAS) (152). The following molecular weight markers were used: myosin, 205,000; β -galactosidase, 116,000; phosphorylase B, 97,400; bovine serum albumin, 66,000; egg albumin, 45,000; pepsin, 34,700; carbonic anhydrase, 29,000; trypsinogen, 24,000, and α -lactalbumin, 14,200.

E. DETERMINATION OF INTRINSIC VISCOSITY

Measurements were made using a Brookfield Syncro-Lectric Model LVT viscometer (Stoughton, MA) at 25 C between the shear rates of 22.5 and 450 sec^{-1}. Viscosity was measured at glycoprotein concentrations of 2, 4, 8, and 10 mg dry wt/ml equilibrated in 0.05 M sodium phosphate, pH 6.2, containing 0.2 M NaCl and 0.02% NaN_3.

F. ANALYTICAL PROCEDURES

Total hexose was determined by the anthrone method (116). Galactose (1 mg/ml) was used as a standard. Total sialic acids were measured after hydrolysis for 1 hr at 80 C with 0.2 M sulfuric acid by the Warren method (177). Hexosamine content was quantified by the method of Rondle and Morgan (143) as modified by Kraan and Muir (96). Reagent A was made by adding 1 ml of pentanedione to 98 ml of 1 N $NaCO_3$. Reagent B was prepared by adding 678 mg p-dimethylaminobenaldehyde in 25 ml of an equal mixture of 95% ethanol and concentrated HCl. Hydrolyzed material (1 ml) was mixed with 1 ml of reagent A. After heating at 90 C for 45 min and cooling, 4 ml of 95% ethanol was added to the sample mixture. Reagent B (1 ml) was added and the samples were incubated at room temperature. After 1 hr,

absorbancies were read at 540 nm. Equal concentrations of GluNAc and GalNAc (20-100 μg) were used as standard.

McFeeters' (110) method was used to measure the increase in reducing ends (calculated as galactose) after E. coli was grown in mucin medium. Reagent A was 4 mM $CuSO_4 \cdot 5 H_2O$ - 28 mM aspartic acid. Reagent B was 0.36 M Na_2CO_3 - 5 mM sodium 2,2'bicinchoninate. Reagent C was reagents A and B mixed 1:1 just before use. An aliquot of sample (final volume 2 ml) was mixed with 2 ml of reagent C. Samples were boiled for 2.5 min, cooled for 10 min, and absorbancies were read at 560 nm. The number of reducing ends was quantified from a standard of galactose.

Free hexosamine end groups were measured by the method of Reissig, Strominger, and LeLoir (138). An aliquot (0.1 ml) of sample was mixed with 0.1 ml of 0.8 M potassium borate, pH 9.2 and heated at 100 C for 5 min. After cooling, 1 ml of p-dimethylaminobenzaldehyde reagent (138) was added and the mixture was incubated at 37 C for 15 min. Absorbance of the color produced was measured at 585 nm.

Contamination of mucin by DNA was quantitated by a diphenylamine method (64). Protein concentrations were determined by the method of Lowry et al. (101) using

bovine serum albumin (5 mg/ml) as standard. Sulphate content of mucin was determined on triplicate samples using the benzidine method of Antonopoulos (10). Mucin was hydrolyzed in sealed tubes with 25% formic acid for 24 hr at 105 C.

A modified micro-Kjeldahl procedure (142) was used to determine total nitrogen in mucin. Mucin (10 mg) was digested with 2 ml of concentrated sulfuric acid. Sodium sulphate (0.16 g) and copper sulphate (0.01 g) were added as catalysts. Flasks were allowed to cool (~30 min) after digestion and 25 ml of distilled water was added. Flasks were attached to a micro-distillation apparatus and 5 ml of 50% NaOH added slowly to the digestion mixture. Ammonia was steam distilled for 8 min into 20 ml of 4% boric acid with 0.08 ml Tashino indicator. Ammonia was titrated with hydrochloric acid (0.1 N) using a syringe microburet (Micro-Metric Instrument Co., Cleveland, OH). Protein was calculated as N x 6.25. Glycine was analyzed as a control. All samples, including blanks, were run in duplicate.

Amino acid content of mucin was determined by the method of Reddy and Salunkhe (137). Samples (75-100 mg) of mucin were hydrolyzed in evacuated Teflon screw-capped

test tubes with 20 ml of 6.0 N HCl at 100 C. After 24 hr, 10 ml of 0.2 M citrate buffer, pH 2.2, were added to the hydrolyzate. The pH was adjusted to 2.2 with 0.1 N NaOH or 0.1 N HCl and the hydrolyzate made up to a final volume of 50 ml with distilled water. Analyses were done by injecting 0.1 ml samples into a Beckman model 122 Amino Acid Analyzer. Norleucine was used as an internal standard.

G. CULTURE MEDIA

Galactose minimal medium and tryptone broth were as described by Adler (1). Casamino acids-yeast extract (CAYE) medium was used as described by Evans et al. (44). Minimal medium was identical to Minca medium (74) except the nitrogen- and carbon-sources were omitted. Ammonium sulphate (1 g/l) and carbohydrates (3 mM) were added to minimal medium for experiments utilizing carbohydrate broths.

Mucin medium contained (per liter distilled water): K_2HPO_4, 1.38 g; NaH_2PO_4, 10.1 g; 1.0 ml trace salt solution; and, mucin, 2.0 g. Trace salt solution was composed of (w/v): $MgSO_4$, 1%; $MnCl_2$, 0.1%; $FeCl_3$, 0.135%; and, $CaCl_2$, 0.04%. The pH was adjusted to 7.0.

Mucin broth medium was filter-sterilized (Millipore, 0.30 μm). Mucin semi-solid medium was made by mixing equivalent amounts of double-strength mucin broth and 1% tempered agar. Mucosal scrape (MS) medium was prepared by mixing 5 g of fresh mucosal scrapings from a hog small intestine with 10 ml of minimal medium. Sterilization of the medium was not possible, therefore a control had to be used in experiments utilizing MS medium. Trypticase soy broth and agar and brain heart infusion agar were prepared according to package directions.

H. BACTERIA

The bacterial strains used are listed in Table 3.1. Cultures were maintained on trypticase soy agar slants at room temperature.

I. ENTEROTOXIN ASSAYS

ST enterotoxin was measured by the suckling mouse assay (33). Mice (3 days of age) were separated from their dams immediately before use and randomly divided into groups of three. Mice were injected percutaneously into the stomach with 0.1 ml of sterile culture filtrate to which had been added 0.01 ml of 0.002% Evan's blue dye

Table 3.1. Strains of *E. coli* used in this study.

Strain[1]	Serotype	Source[2]	Toxin[3]
G-9[a]	O8:K87:88ab:H19	PF	LT/ST
P-155[a]	O149:K91,88ac:H10	PF	LT/ST
431[a]	O101:K30:K99:NM	PF	ST
B-47[a]	O9:K35:K99:NM	PF	ST
1592[a]	O9:K103:987P:NM	PF	ST
1413[a]	O20:K101:987P:NM	PF	ST
FS-8001[b]	O43:K87:H?	PF	ST
V-517[b]	NT[4]	HF	LT/ST
3-7[b]	NT	M	-
B-42[b]	NT	PSI	-
FS-8201[b]	NT	HF	-
FS-8202[b]	NT	HF	-
FS-8204[b]	NT	HF	-
FS-8216[b]	NT	FF	-
FS-8211[b]	NT	MSI	-
123[a]	O43:K-:H28	PF	-
1466-56[a]	O8:K-:H16	PF	-
FS-8301[b]	NT	CF	-
FS-8401[b]	NT	DF	-
FS-8402[b]	NT	OF	-
FS-8404[b]	NT	OSI	-
ATCC 23721[c]	NT	-	-
ATCC 23722[c]	NT	-	-
ATCC 23723[c]	NT	-	-

[1] Obtained from:
a: Richard Wilson, Penn State University
b: Department of Food Science & Technology Stock Collection; Virginia Tech
c: American Type Culture Collection; Rockville, MD

[2] PF, porcine feces; HF, human feces; M, milk; PSI, porcine small intestine; FF, feline feces; MSI, mouse small intestine; CF, canine feces; DF, duck feces; OF, ovine feces; and OSI, ovine small intestine

[3] LT, heat-labile; ST, heat-stable; -, negative

[4] NT, not typed

(2% w/v in distilled water). After incubation at room temperature (23-25 C) for 3 hr, each animal was euthanized with chloroform vapors and the entire intestinal tract was removed. The mouse was discarded if dye was not present in the stomach at sacrifice. The intestines and carcasses from three mice were pooled and the intestinal weights compared with remaining body weight to determine the gut:body ration. A ratio of 0.083 or greater was considered positive for ST production.

LT enterotoxin was measured by the Y-1 adrenal tumor cell assay of Donta et al. (35). Adrenal cells were maintained on Ham's F-10 medium supplemented with 15% fetal bovine serum and 0.05 mg of gentamicin per ml. Assays for entertoxin activity were done in 24-well cluster dishes (Costar, Cambridge, MA). Each well contained 1 ml of medium seeded with approximately 10^5 cells. Dishes were incubated at 37 C in a humidified atmosphere of 95% air and 5% CO_2. After cells formed a confluent monolayer (usually 1 day) sterile culture filtrate (0.05 ml) was added to each well. Toxin activity was determined using light microscopy by counting the number of rounded and unrounded cells in 20 fields. Rounding of greater than 50% of the cells was considered

LT positive. Wells with uninoculated cells had < 20% rounding. All samples were tested in triplicate.

J. CULTURE PROCEDURES

Prior to growth studies, bacteria were inoculated (1%) into 5 ml of minimal medium, glucose medium, or mucin medium in 125 ml screw-cap Erlenmeyer flasks. Cultures were incubated with shaking (150 rpm; Lab-Line Environ-Shaker, Melrose Park, IL) for 6 hr at 37 C. A 1% inoculum was introduced into 50 ml of each respective medium in 500 ml Erlenmeyer flasks and incubated under identical conditions. Samples (1 ml) were withdrawn at 0, 2, 4, 6, and 8 hr for enumeration in brain heart infusion agar. Aliquots (7 ml) were also centrifuged at 12,000 x g for 30 min at 4 C to separate culture supernatant from bacterial pellet. The bacterial pellet was discarded and the culture supernatant was heated at 100 C for 5 min to inactivate enzymes. Supernatant was stored at -20 C for later analyses.

Mucin medium was neutralized to pH 7.0 with 1 N NaOH after growth (8 hr) of E. coli P-155 or 123, filter-sterilized, inoculated with a fresh culture of E. coli P-155 or 123, and incubated as described above. After an

additional 8 hr of growth, cultures were enumerated to determine if the "spent" mucin could still support growth. Additionally, aliquots (7 ml) were centrifuged and heated as in growth studies described above. A portion (3 ml) of the aqueous supernatant was frozen at -20 C for later analyses and a portion (4 ml) was dialyzed (3500 MW cut-off tubing) against distilled water at 4 C. After several water changes, the mucin was lyophilized for use in capillary chemotaxis assays described below.

For glycosidase assays, mucin broth (15 ml in a 125 ml screw-cap Erlenmeyer flask) was inoculated with 0.15 ml of a 4 hr-old culture of E. coli growing in minimal broth and incubated at 37 C with shaking (60 oscillations/min; Precision Water Bath, Chicago, IL). After 4 hr, cells were enumerated in brain heart infusion agar and assayed for cell-bound and extracellular glycosidase activity as described below. For extracellular assays, 5 ml of culture was centrifuged at 8,000 x g for 10 min at 20 C and the supernatant passed through a 0.45 μ m Gelman Acrodisc filter into a sterile test tube. Culture conditions were the same as with mucin broth when inducer carbohydrates were added to minimal medium. Lactose and melibiose (3 mM) were used to induce β-galactosidase and

α-galactosidase, respectively.

K. GLYCOSIDASE ASSAYS

Activities of α-galactosidase (E.C. 2.3.1.22), α-fucosidase, (E.C. 3.2.1.51) and α-mannosidase (E.C. 3.2.1.24) were determined using a modification of the *in vivo* assay of Burstein and Kepes (21). Briefly, 5 ml of culture fluid (with or without bacterial cells) was incubated at 35 C with the appropriate nitrophenyl glycoside (3 mM final concentration) and the change in absorbance was measured at 420 nm ($E = 4.7 \times 10^3$). One unit of glycosidase activity is the amount of enzyme which releases one micromole of nitrophenol/min/mg dry cell wt.

The procedure of Miller (114) as modified by Huber et al. (88) was used to assay β-galactosidase (E.C. 3.2.1.23). Briefly, cells in culture (1 ml) were solubilized with 0.075 ml each of 0.1% SDS and chloroform. The tube was vortexed for 10 sec and the chloroform layer allowed to separate. Aliquots (0.05 ml) of the aqueous layer were added to 0.75 ml of 0.1 M sodium phosphate, pH 7.6, with 1 mM $MgSO_4$ in a cuvette. The reaction was initiated by adding 0.25 ml of 12 mM ONPG (in the above buffer. The change in absorbance was monitored at 420 nm

and units expressed as above.

Enumeration of viable cells was related to dry weight. Dry weight was determined as follows: a culture of P-155 grown on glucose medium was sampled periodically. Enumerations were done in brain heart infusion agar pour plates and a portion of the sample was filtered through a pre-weighed nitrocellulose filter (0.45 μm size). Cells were washed with sterile, distilled water and dried overnight (14 to 18 hr) at 80 C before weighing. This procedure was repeated twice and a standard curve was constructed of viable counts vs. dry wt.

L. AGAR MOTILITY ASSAY

Tubes of mucin semi-solid agar were stab-inoculated with *E. coli* actively growing in tryptone broth. Cultures were grown at 32 C for 24 hr. Positive motility was determined to be growth away from the stab line and on top of the mucin agar; negative motility was determined as growth only along the stab line.

M. CAPILLARY CHEMOTAXIS ASSAY

Bacteria from a mother culture were grown for 15 hr in tryptone broth (10 ml in a 125 ml flask) at 32 C with shaking (60 oscillations/min). Bacteria from this culture were used to inoculate (1%) 10 ml of galactose minimal medium (1) in a 125 ml flask. The culture was incubated as above. After 4 hr, the culture was centrifuged for 10 min at 8000 x g at 20 C. An aliquot (5 ml) of chemotaxis buffer (see below) was used to gently resuspend the bacterial pellet and centrifugation was repeated. The final pellet was gently resuspended with 10 ml of chemotaxis buffer or buffers of different pH. Chemotaxis buffer was 10 mM K_2HPO_4, pH 7.0. The optimum pH for chemotactic activity was determined in 3 mM citrate-potassium phosphate (pH 4 to 6), 10 mM potassium phosphate (pH 6 to 8), and 9 mM Tris-hydrochloride (pH 9 and 10). All buffers were supplemented with 0.1 mM EDTA and had an ionic strength of approximately 0.03 M.

The capillary chemotaxis assay of Adler (1) was modified as by Freter and O'Brien (59). Briefly, the ends of sealed 20 µl capillaries filled with approximately 5 µl of chemoattractant were placed open-end down into small test tubes (6 x 50 mm) containing washed bacteria in

chemotaxis buffer. After incubation for 30 min at 32 C, contents of the capillaries were cultured in brain heart infusion agar pour plates. Counts were done in duplicate and each measurement represented the mean count of two capillaries. Random migration was monitored by using unsupplemented chemotaxis buffer. Tryptone (1%, w/v) was used as a positive chemoattractant control.

N. STATISTICS

The 95% confidence intervals were calculated as described by Snecdor and Cochran (162). Standard methods were used for t-test evaluations (162). Molecular weight and concentration values were determined from linear regression plots of values obtained in standards.

CHAPTER FOUR

RESULTS AND DISCUSSION

A. CHEMICAL AND PHYSICAL PROPERTIES OF MUCIN

Mucin was isolated in this study by reduction and proteolysis of small intestinal mucous gel. This method was chosen over isolation by CsCl gradient (105) because large quantities were needed for incorporation into bacteriological media. Commercially available HGM was not used since *E. coli* enterotoxins originate in the small intestine (95) and because HGM contains slightly less carbohydrate than PSIM (23). Chemical analysis of PSIM showed the carbohydrate, protein, and sulfate concentrations (Table 4.1) to be higher than reported for PSIM isolated by preparative ultracentrifugation (105). Mucin prepared in this study contained (% by weight) hexosamines, 20.5%; galactose, 13.5%; fucose, 4.6%; mannose, 9.4%; and, sialic acid, 10.7%. Uronic acid and xylose were not detected indicating the preparation was not contaminated with glycosylaminoglycans. Trace amounts of DNA (< 1 μg/ml) were detected in mucin used in this study.

Interestingly, this preparation of PSIM had a

Table 4.1. Comparison of human and porcine small intestinal mucin.

Constituent	Composition % by wt. glycoprotein		
	Human[1]	Porcine[2]	Porcine[3]
Carbohydrate	77.6	77.5	58.7
Protein	16.0	19.6	37.2
Sulfate	1.0	2.8	3.7
	Molar Ratio		
Hexosamine	1	1	1
Gal	1.8	1.1	0.7
Fuc	1.2	0.5	0.2
NANA	0.4	0.8	0.5
Man	0.01	0	0.5

1 Data from (54)

2 Data from (105)

3 Data from the current study

carbohydrate composition similar to mucin prepared in a CsCl gradient (105) except that mannose concentration was considerably higher (9.4%). Mannose has been detected in trace amounts in human (126), rat (103), and monkey (100) salivary mucins, human bronchial mucin (30), and human small intestinal mucin from patients with cystic fibrosis (108). The high mannose content of this preparation may be indicative of contamination by blood proteins or mucosa epithelial membranes. However, sialic acid concentration (11%) was lower than would be expected if contamination were a major contributor (68). Therefore, presence of high levels of mannose may indicate mucins (or components of them) are "mixed" glycoproteins as proposed by Mantle et al. (108). Thus, mucin biosynthesis would involve, at least partially, the same processing reactions used in formation of N-linked oligosaccharides (149).

Mucin (2 mg dry wt/ml) was excluded completely by Sepharose CL-4B (Fig. 4.1) as detected by absorbance at 280 nm (protein) and by PAS colorimetric assay (glycoprotein). However, after filter-sterilization of the mucin suspension, two peaks were observed on Sepharose CL-4B: one eluted at the void volume and the other at a molecular weight of 14,500 (Fig. 4.2). In both fractionations, the

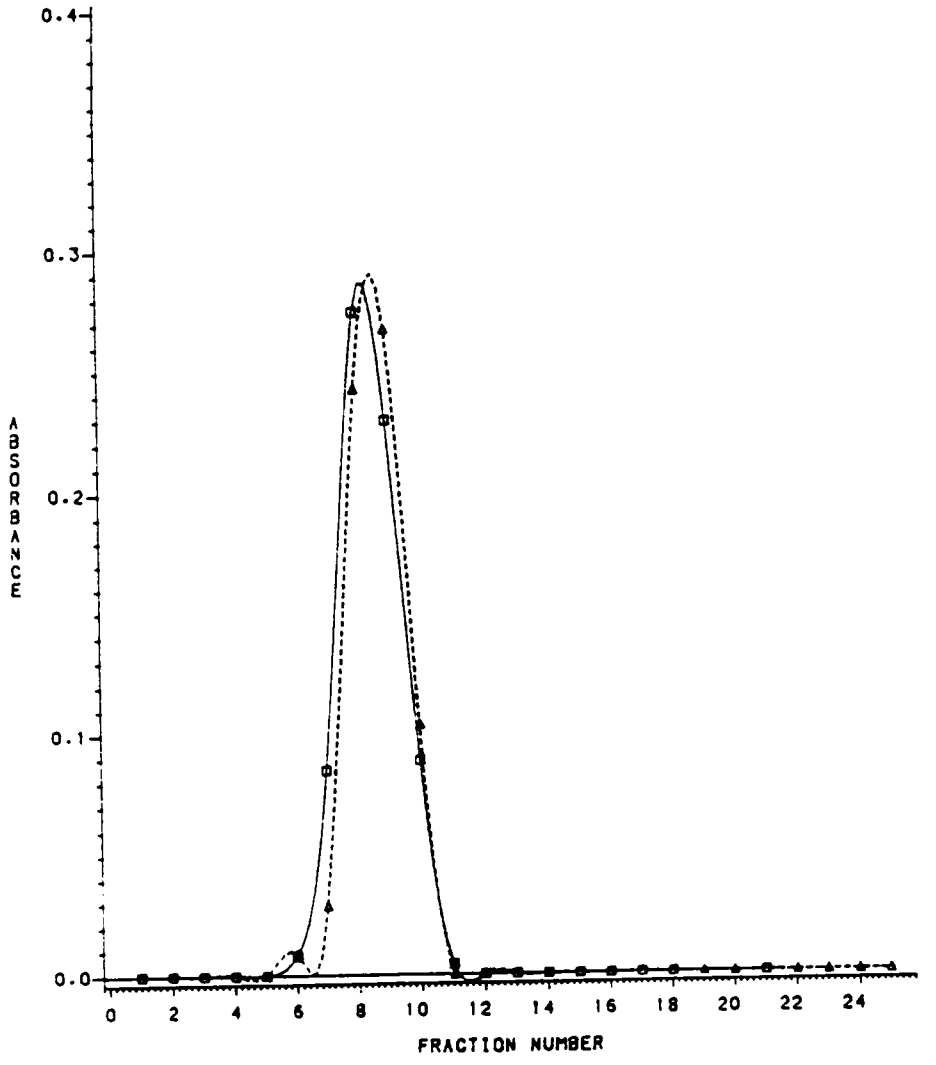

Fig. 4.1. Gel filtration profile on Sepharose CL-4B of PSIM (1 mg dry wt/ml) before filter sterilization. Fractions were monitored by absorbance at 280 nm (□) or 555 nm (△). The void volume (V_o) for this column was 8 ml (fraction 8).

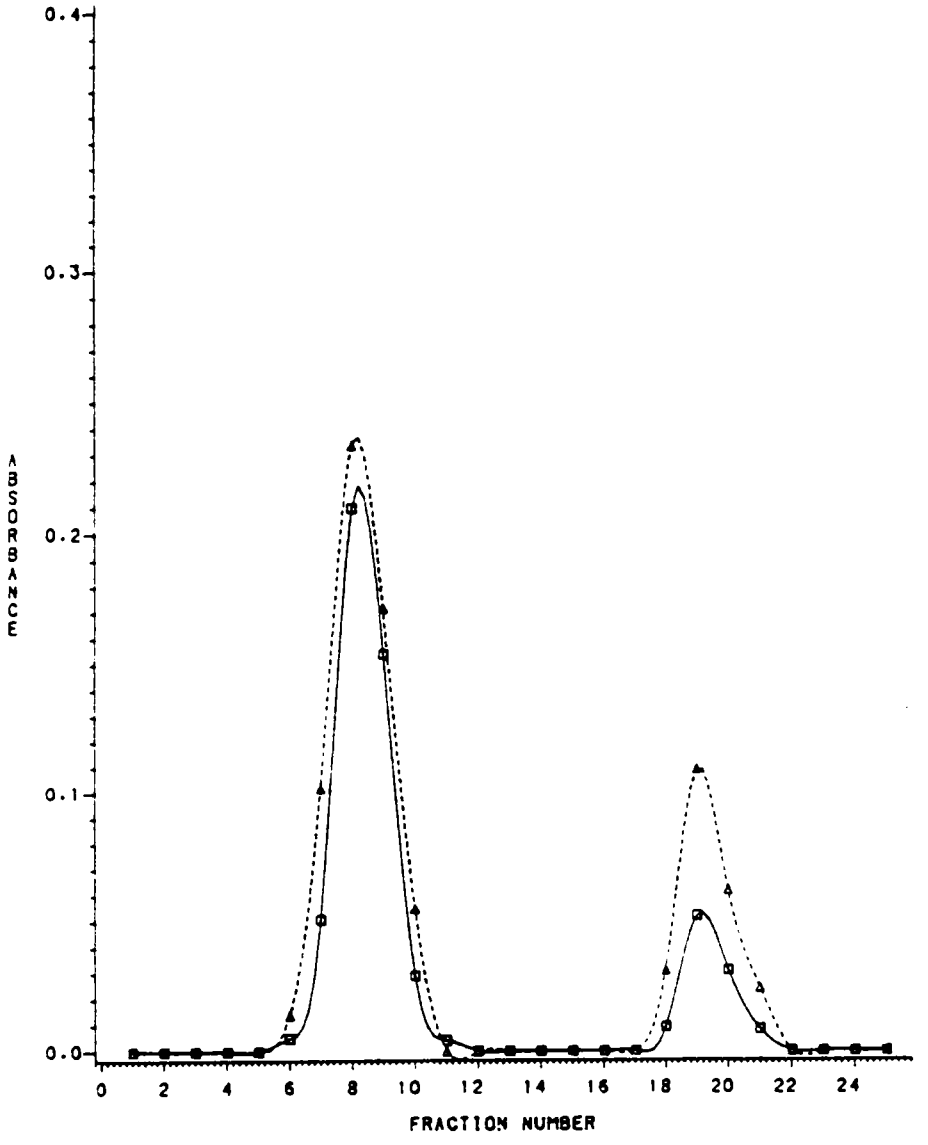

Fig. 4.2. Gel filtration profile on Sepharose CL-4B of PSIM (1 mg dry wt/ml) after filter sterilization. Symbols are the same as in Fig. 4.1.

peaks detected by PAS overlapped the protein peak. The small molecular weight fraction may have been dissociated from the excluded macromolecule by physical forces during filter-sterilization (77). Insoluble material was also deposited on the filter membrane.

Mucin (PSIM) used in the present study differs from mucin prepared by ultracentrifugation in percent by weight protein (Table 4.1) and amino acid composition (Table 4.2). PSIM contained 37% protein (by weight) compared to only 18% protein (by weight) in a more purified preparation (105). However, PSIM did contain less protein than reported for crude small intestinal mucin (70%, by weight) (105) and HGM (60%, by weight) (167). Presence of intestinal secretions and lysed cells from the intestinal microflora may contribute to protein content. Higher levels of protein in this preparation probably account for differences observed in amino acid content (Table 4.2). Mucin protein isolated on a CsCl gradient is composed of over 50% (by weight) proline, serine, and threonine, and 3% (by weight) half-cysteine residues. Mucin prepared in the present study contained lower proportions of these amino acids (Table 4.2). Proline, serine, and threonine accounted for 26% (by weight) of the amino acids while half-cysteine

Table 4.2. Amino acid composition of porcine small intestinal mucin.

Amino acid	nmol/mg dry wt
Asp	259
Thr	363
Ser	254
Glu	287
Pro	242
Gly	251
Ala	264
1/2 Cys	33
Val	274
Met	78
Ilu	160
Leu	152
Tyr	117
Phe	195
His	90
Lys	147
Arg	146
Total	3312

residues accounted for another 1%. PSIM was low in aromatic and high in polar amino acids.

Complex electrophoretic patterns were obtained for PSIM on SDS-PAGE (Fig. 4.3). Two acrylamide concentrations were used to estimate molecular weights. Higher molecular weights (> 62,000) were determined from a 5% polyacrylamide gel (Fig. 4.3a) and lower (< 62,000) from a 7.5% polyacrylamide gel (Fig. 4.3b). Polypeptide molecular weights were determined with standards of known molecular weight (Table 4.3). The pattern for PSIM contained two major (II and V) and seven minor polypeptide bands. Five bands (I, III, V, VII, and IX) stained positively with PAS. All bands stained positively with silver and Coomasssie blue stains. Coomassie blue- and PAS-positive material could be observed at the interface between the stacking and running gel in the 7.5% polyacrylamide gel. Despite all efforts, smearing in the gels could not be eliminated.

Viscosity analyses revealed pronounced shear-dependence in this mucin preparation (Fig. 4.4). Reduced viscosities were determined by manual extrapolation. Intrinsic viscosity of PSIM was low (135 ml/g) and did not demonstrate the intermolecular properties of a gel-forming substance (Fig. 4.5).

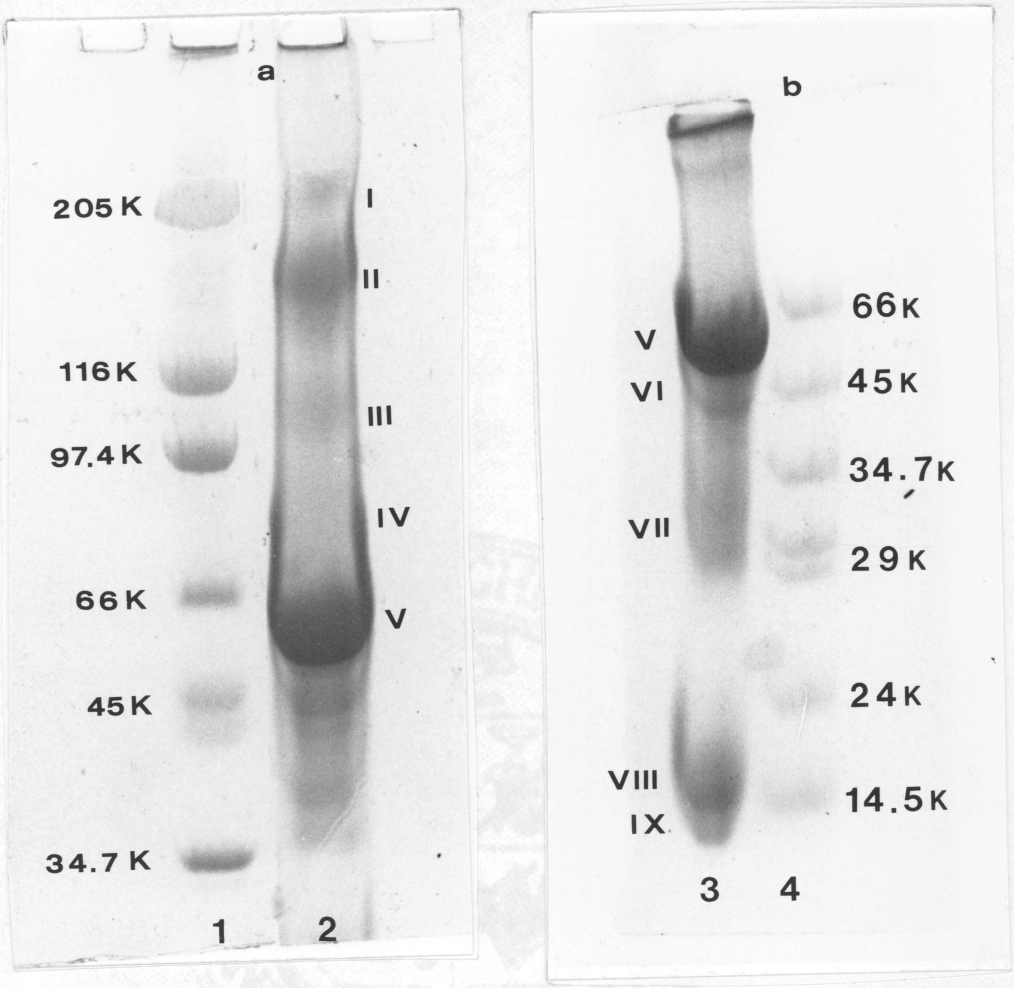

Fig. 4.3. SDS-PAGE patterns of PSIM. (a) 5% polyacrylamide gel. Lane 1, high molecular weight markers - myosin, β-galactosidase, phosphorylase B, bovine serum albumin, egg albumin, and carbonic anhydrase ; lane 2, PSIM. (b) 7.5% polyacrylamide gel. Lane 3, PSIM; lane 4, low molecular weight markers - bovine serum albumin, egg albumin, pepsin, carbonic anhydrase, trypsinogen, and α-lactalbumin. Gels were stained with Coomassie blue. Polypeptide bands are numbered in order of increasing electrophoretic mobility.

Table 4.3. Molecular weights and staining patterns obtained for components of porcine small intestinal mucin separated by SDS-PAGE (Fig. 4.3).

Polypeptide band[1]	Calculated molecular weights	Ag[2]	CB[3]	PAS[4]
I	>205,000[5]	+	+	+
II[6]	170,000	+	+	+
III	112,000	+	+	-
IV	81,000	+	+	-
V[6]	52,500	+	+	+
VI	45,000	+	+	-
VII	40,000	+	+	+
VIII	16,000	+	+	-
IX	14,500	+	+	+

1 Roman numerals I to IV correspond to mucin polypeptide bands in Fig. 4.3a. Numerals V to IX correspond to mucin polypeptide bands in Fig. 4.3b.

2 Silver stain positive (+).

3 Coomassie blue positive (+).

4 Periodic acid-Schiff reagent positive (+) or negative (-).

5 Molecular weight of band I could not be accurately determined because highest molecular weight standard was 205,000.

6 Polypeptide bands which stained more intensely with Coomassie blue and were designated as major polypeptides.

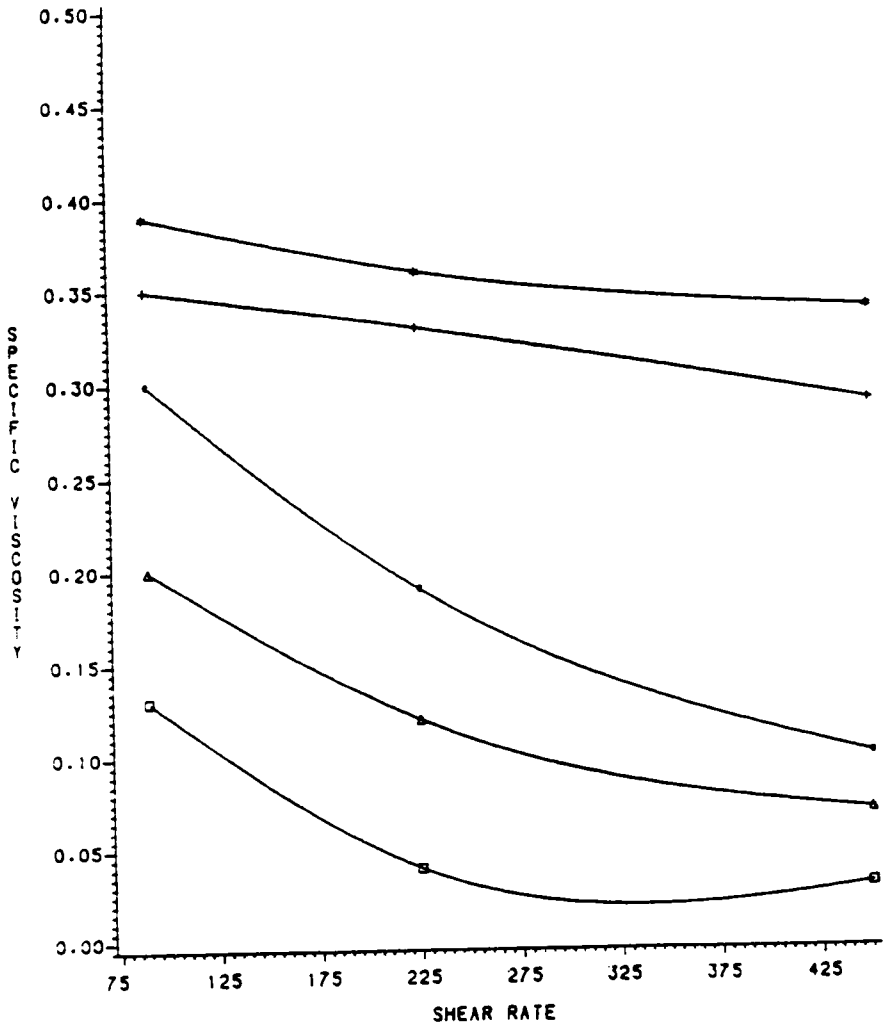

Fig. 4.4. Effect of different concentrations of PSIM on viscosity. Mucins were studied at the following concentrations: □, 1 mg/ml; Δ ; 2 mg/ml; O , 4 mg/ml; +, 8 mg/ml; and, *, 10 mg/ml. Extrapolation to zero shear was done manually.

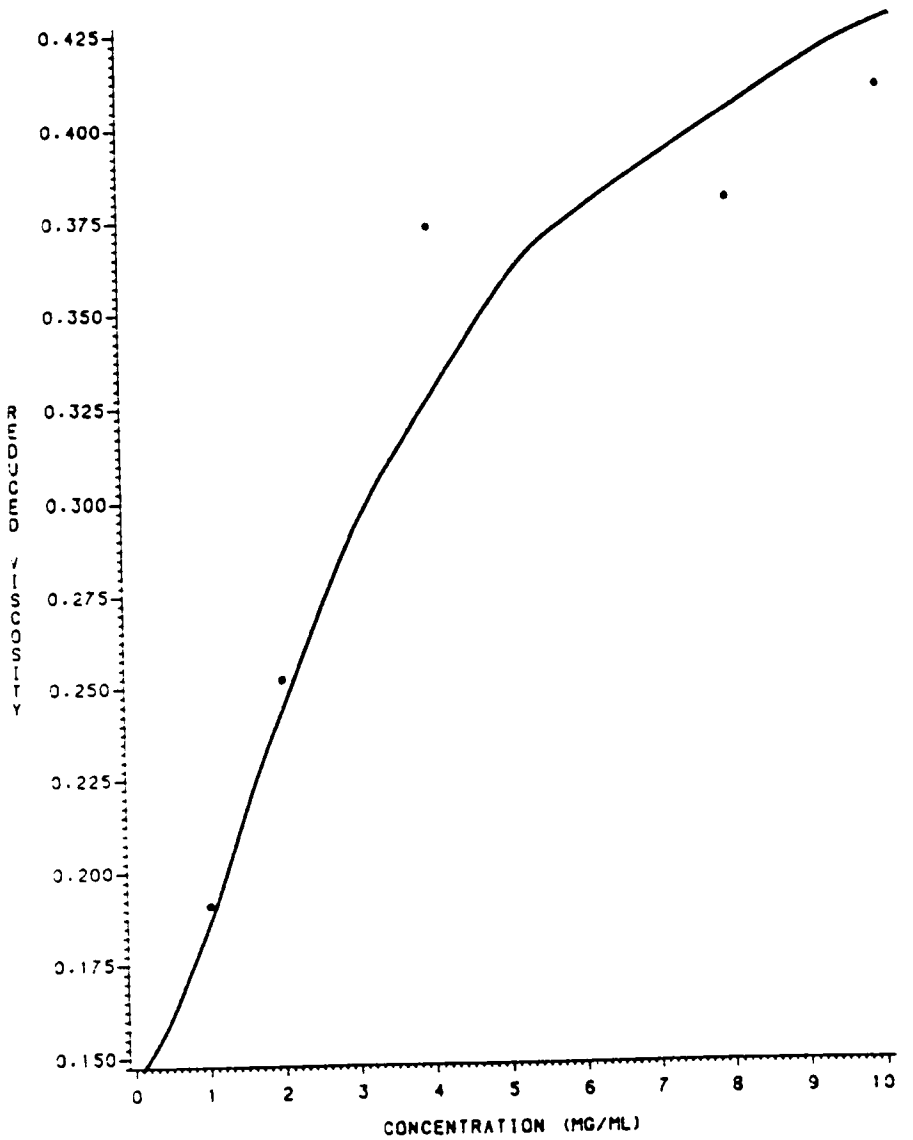

Fig. 4.5. Reduced viscosity vs. concentration of PSIM. Intrinsic viscosity (135 ml/g) was determined by extrapolation to zero concentration.

Mucin isolated on a CsCl gradient has an intrinsic viscosity of 500 ml/g (105). Lipids and other proteins associated with mucosal gel have a role in viscosity and gel-formation of mucin. Extraction of lipids associated with mucin results in an 80 to 85% decrease in viscosity (119). Although little is known about the effect of contaminating proteins, non-covalently bound protein and nucleic acids appear to adversely effect rheological properties of mucin (105). However, incubation of delipidated mucin with albumin or IgA prior to viscometric analyzes results in an increase in viscosity (119). Lowered viscosity in PSIM may be attributed to protein contamination. From results presented in this study, preparation of mucin by reduction of disulfide bonds and proteolysis of the protein core should be avoided for rheological studies. However, this method is suitable for preparation of large quantities of enriched mucin for use in bacteriological media.

B. MUCIN UTILIZATION BY *E. COLI*

1. Bacterial growth on mucin

E. coli strains P-155, B-42, 1466-56, and 123 (Table

3.1) grew very well with mucin as the sole-source of carbon and nitrogen (Figs. 4.6a to 4.9a). Pre-enrichment growth in glucose and mucin media was diluted (1:10) prior to inoculation to assure that all three media received a comparable inoculum. All strains were in exponential growth phase from 2 to 6 hr after inoculation in mucin medium. For each strain, the rates of growth and final numbers of colony-forming units (CFU) were comparable to growth in 3 mM glucose broth medium. Poor growth was obtained for all strains in minimal broth medium. Bacterial cells grown on mucin maintained their typical rod shape and vigorous motility (if a motile strain). Cells in minimal medium appeared as short rods which were almost spherical shaped and were non-motile. Mucin medium had a final pH of 6.8 after growth of *E. coli*.

2. Carbohydrate and protein utilization

All four strains began utilizing protein and hexose from mucin medium by 2 hr after inoculation (Figs. 4.6b to 4.9b). Most strains used no more than 6% of the peptides available, but strain P-155 used nearly 10% probably due to better growth in mucin medium than the other strains. Between 2 and 10% of the total hexose available in mucin

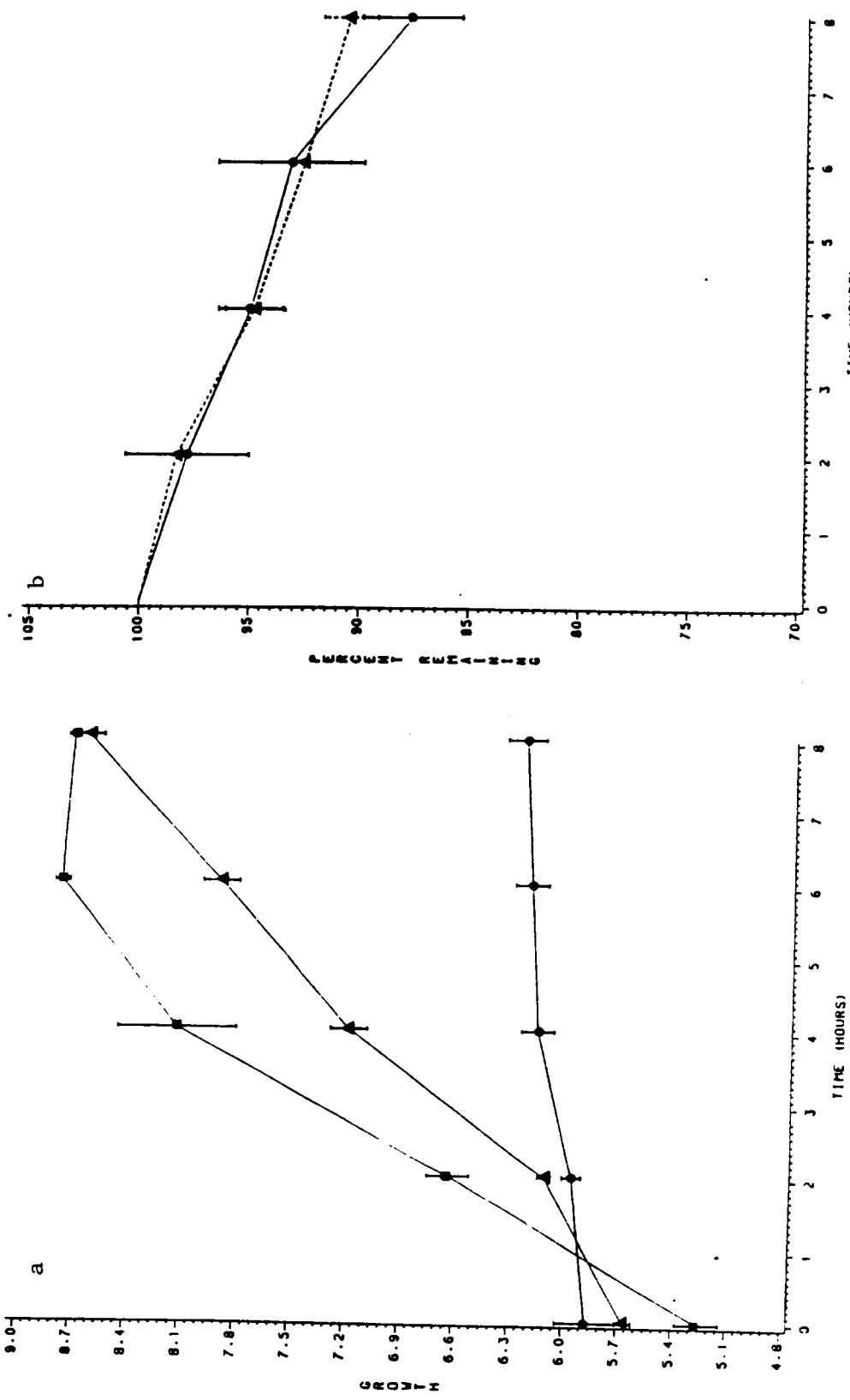

Fig. 4.6. (a) Growth ($\log_{10}$ CFU/ml) of E. coli P-155 in minimal medium (●), glucose medium (▲), and mucin medium (■).
(b) Utilization of protein (▲) and total hexose (●) from mucin medium.

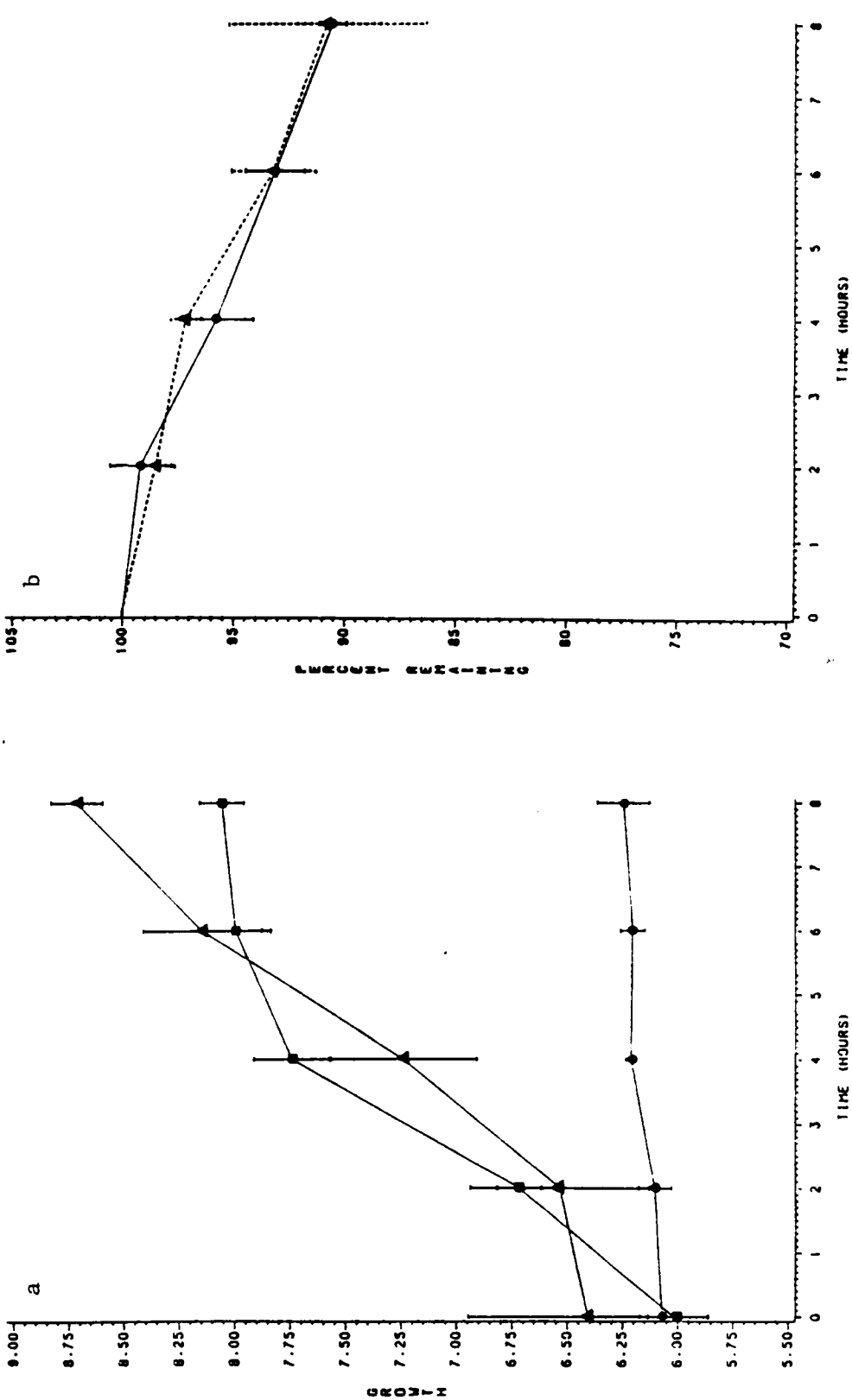

Fig. 4.7. (a) Growth ($\log_{10}$ CFU/ml) of E. coli B-47 in minimal medium (●), glucose medium (▲), and mucin medium (■).
(b) Utilization of protein (▲) and total hexose (●) from mucin medium.

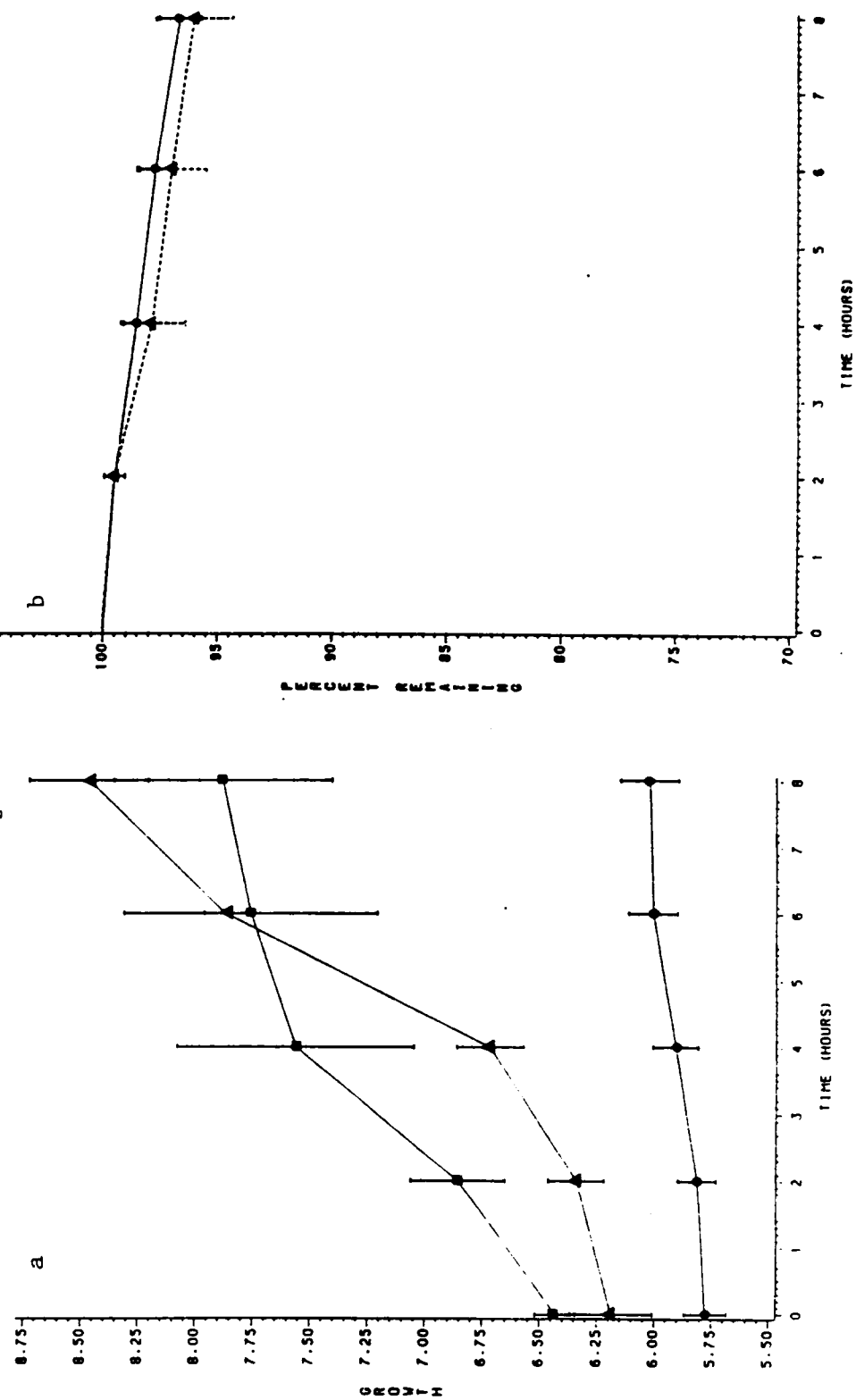

Fig. 4.8. (a) Growth ($\log_{10}$ CFU/ml) of E. coli 1466-56 in minimal medium (●), glucose medium (▲), and mucin medium (■).
(b) Utilization of protein (▲) and total hexose (●) from mucin medium.

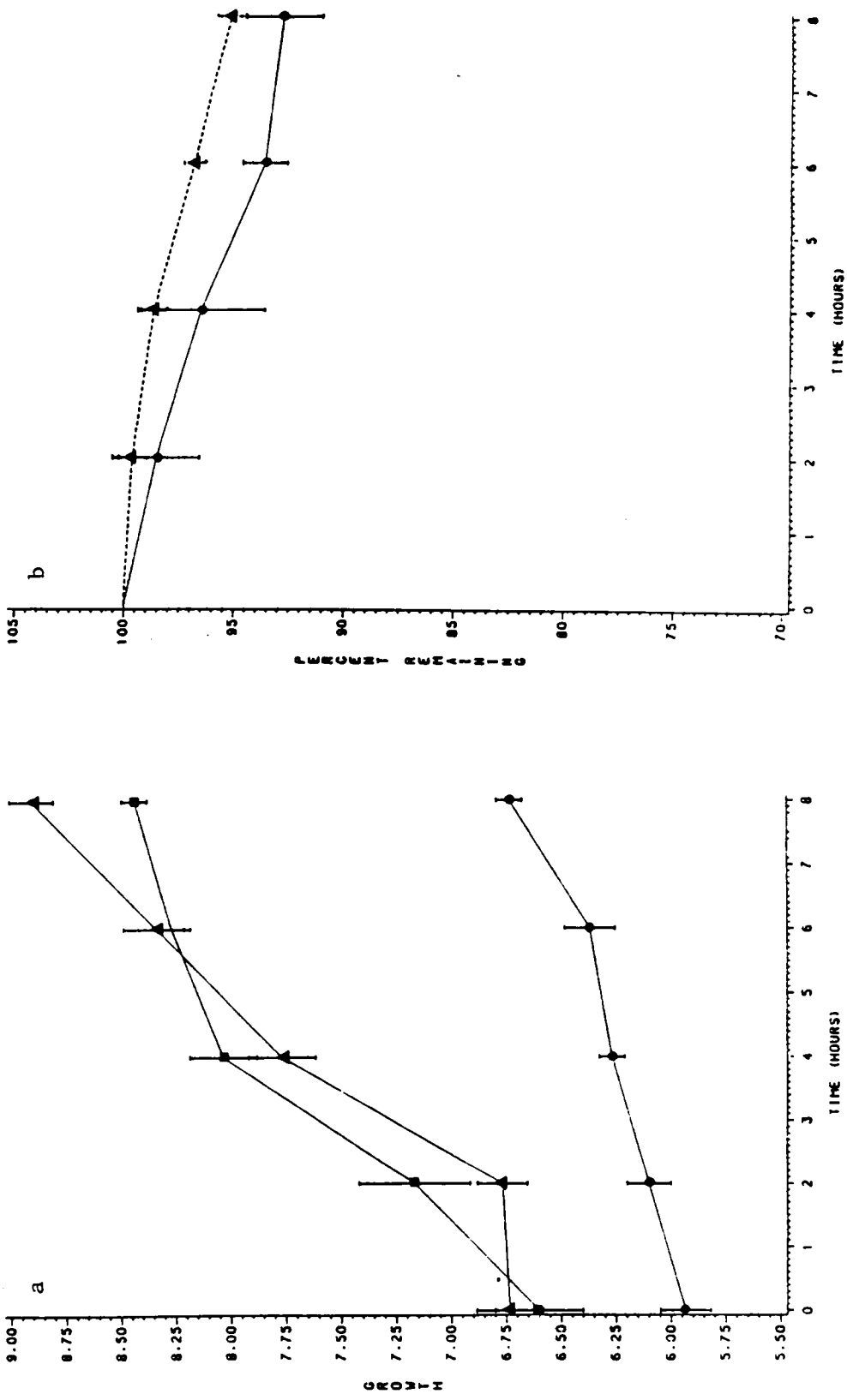

Fig. 4.9. (a) Growth ($\log_{10}$ CFU/ml) of E. coli 123 in minimal medium (●), glucose medium (▲), and mucin medium (■).
(b) Utilization of protein (▲) and total hexose (●) from mucin medium.

medium was used during growth by E. coli. Strain P-155 utilized glucose more extensively (Fig. 4.10) than the complex carbohydrate substrate available in mucin (Fig. 4.6b).

Carbohydrate and protein concentrations in uninoculated mucin medium did not change during incubation. Therefore, residual trypsin, which may have been present from mucin preparation, was not responsible for hydrolysis and subsequent utilization of mucin protein detected during E. coli growth. However, peptidases and proteases associated with the outer membrane and periplasmic space of E. coli (129,171) may have been involved.

Mucin medium was fractionated on Sepharose CL-4B before and after growth of E. coli. Elution patterns obtained prior to inoculation (Figs. 4.11 to 4.14) were similar to the pattern previously shown for filter-sterilized mucin (Fig. 4.2). E. coli growth in mucin medium did not generate any additional peaks, but a decrease in the amount of protein (280 nm) and glycoprotein (555 nm) absorbing material was observed in both the excluded and included peaks (Figs. 4.11 to 4.14). These results suggest that both large and small molecular weight components of PSIM are degraded by proteolysis during

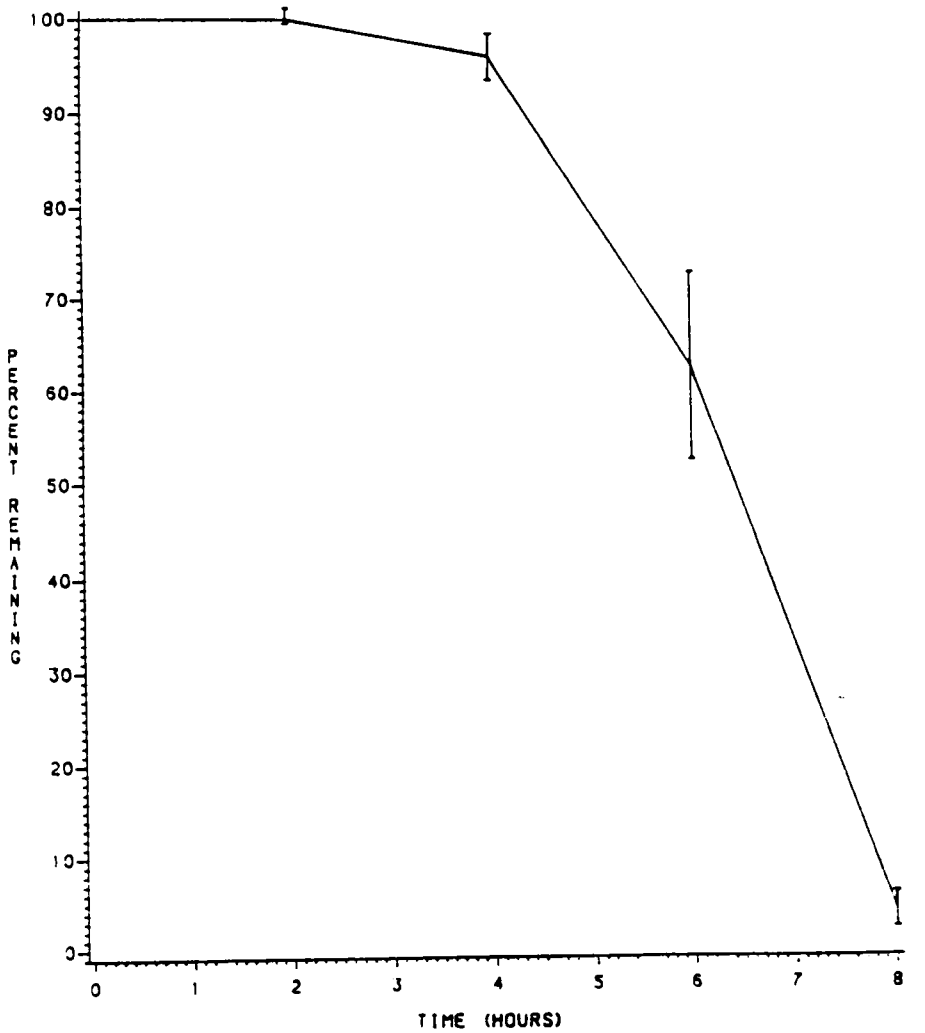

Fig. 4.10. Pattern of total hexose depletion by growth of E. coli P-155 in glucose medium.

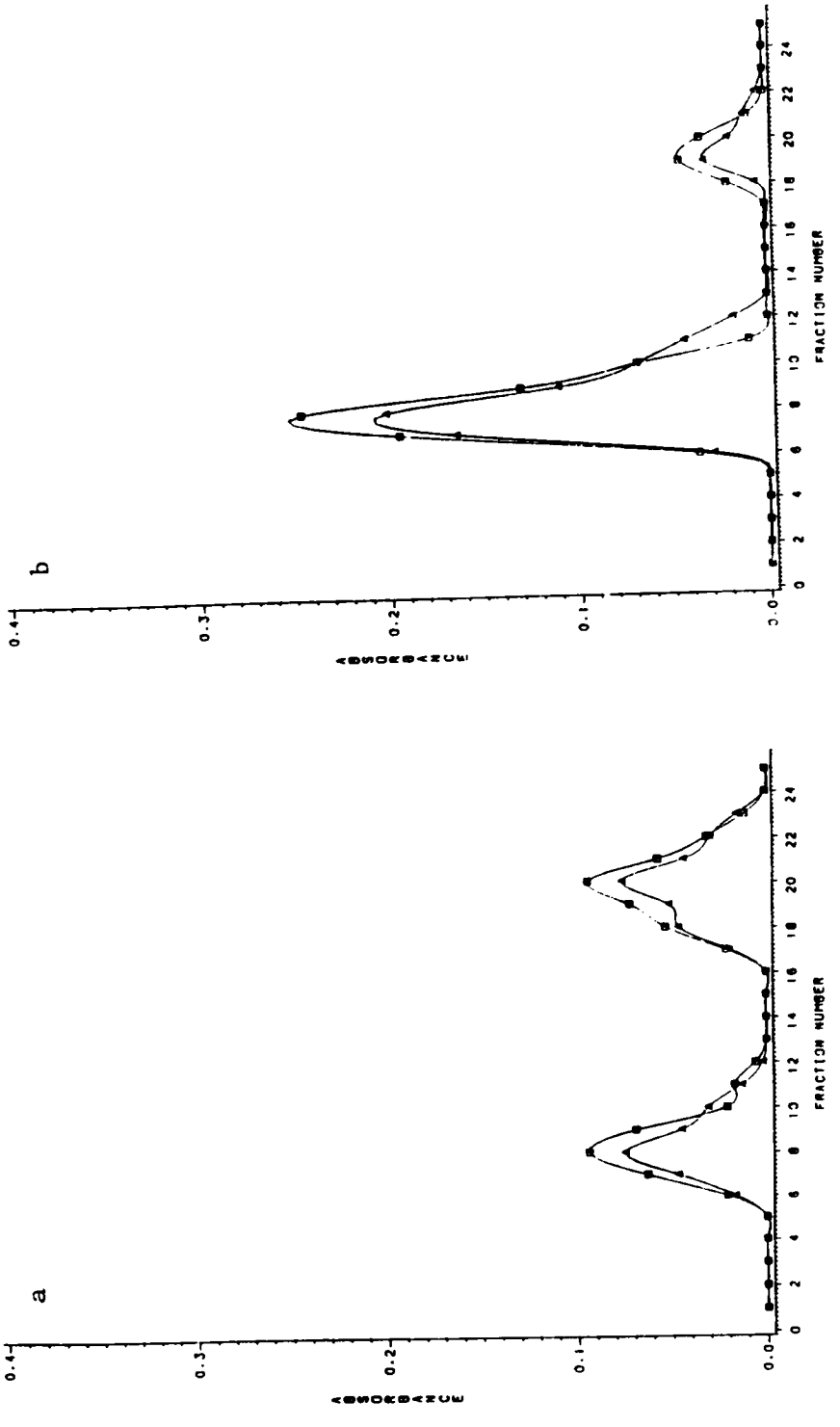

Fig. 4.11. Gel filtration profile on Sepharose CL-4B of mucin medium before (□) and after (Δ) growth of E. coli P-155. (a) Absorbance at 280 nm. (b) Absorbance at 555 nm.

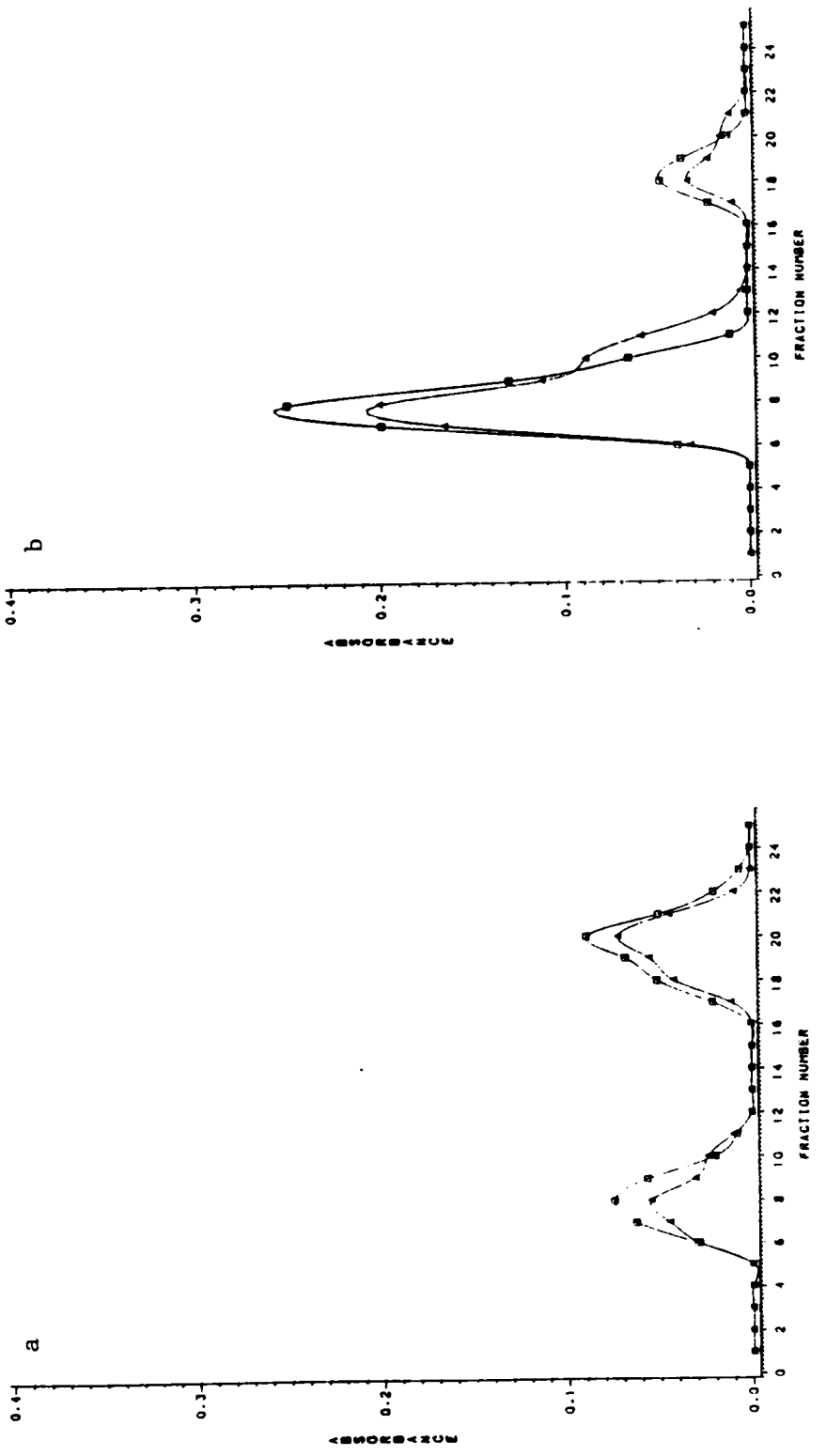

Fig. 4.12. Gel filtration profile on Sepharose CL-4B of mucin medium before (□) and after (△) growth of E. coli B-47. (a) Absorbance at 280 nm. (b) Absorbance at 555 nm.

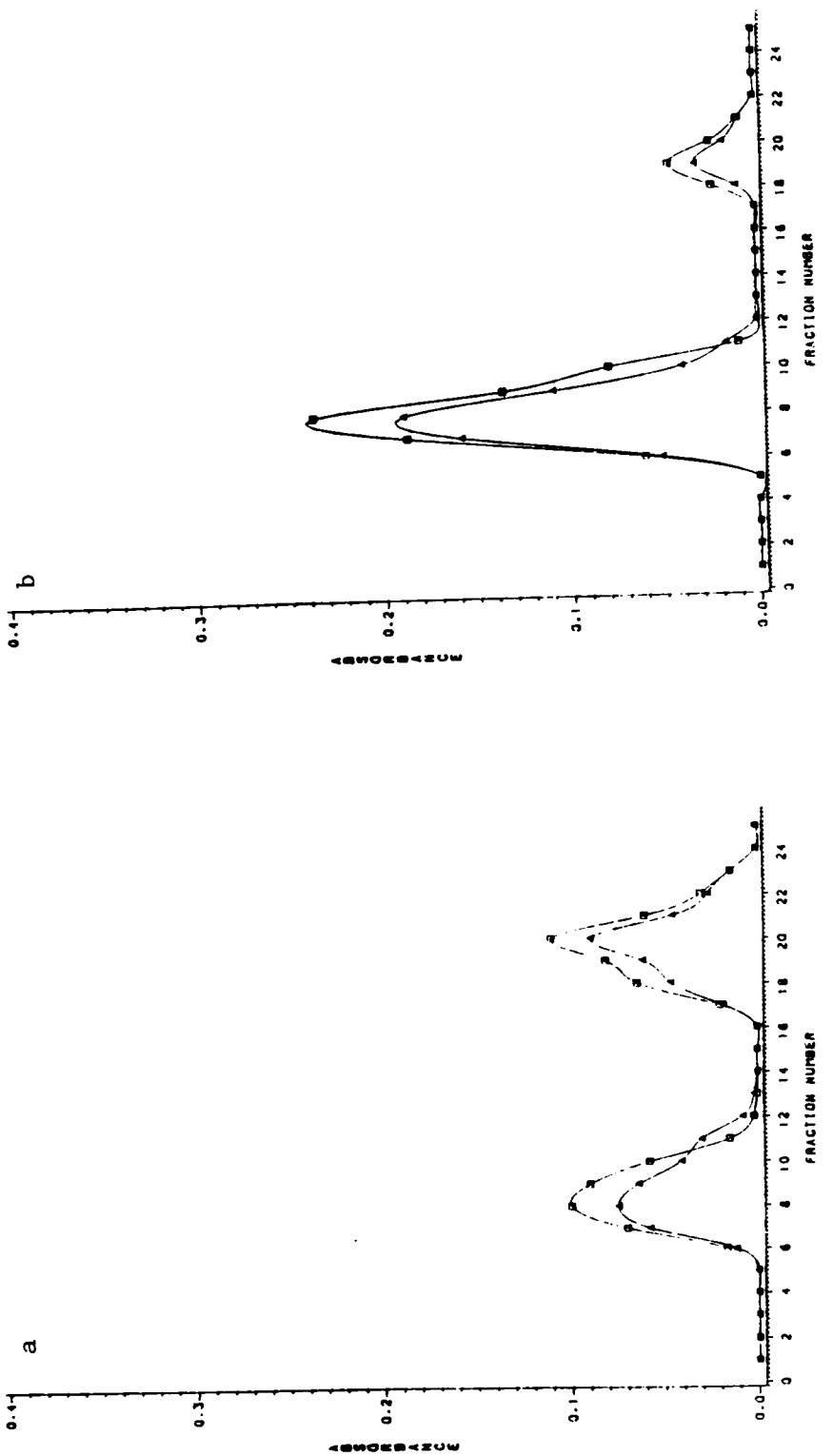

Fig. 4.13. Gel filtration profile on Sepharose CL-4B of mucin medium before (□) and after (△) growth of E. coli 1466-56. (a) Absorbance at 280 nm. (b) Absorbance at 555 nm.

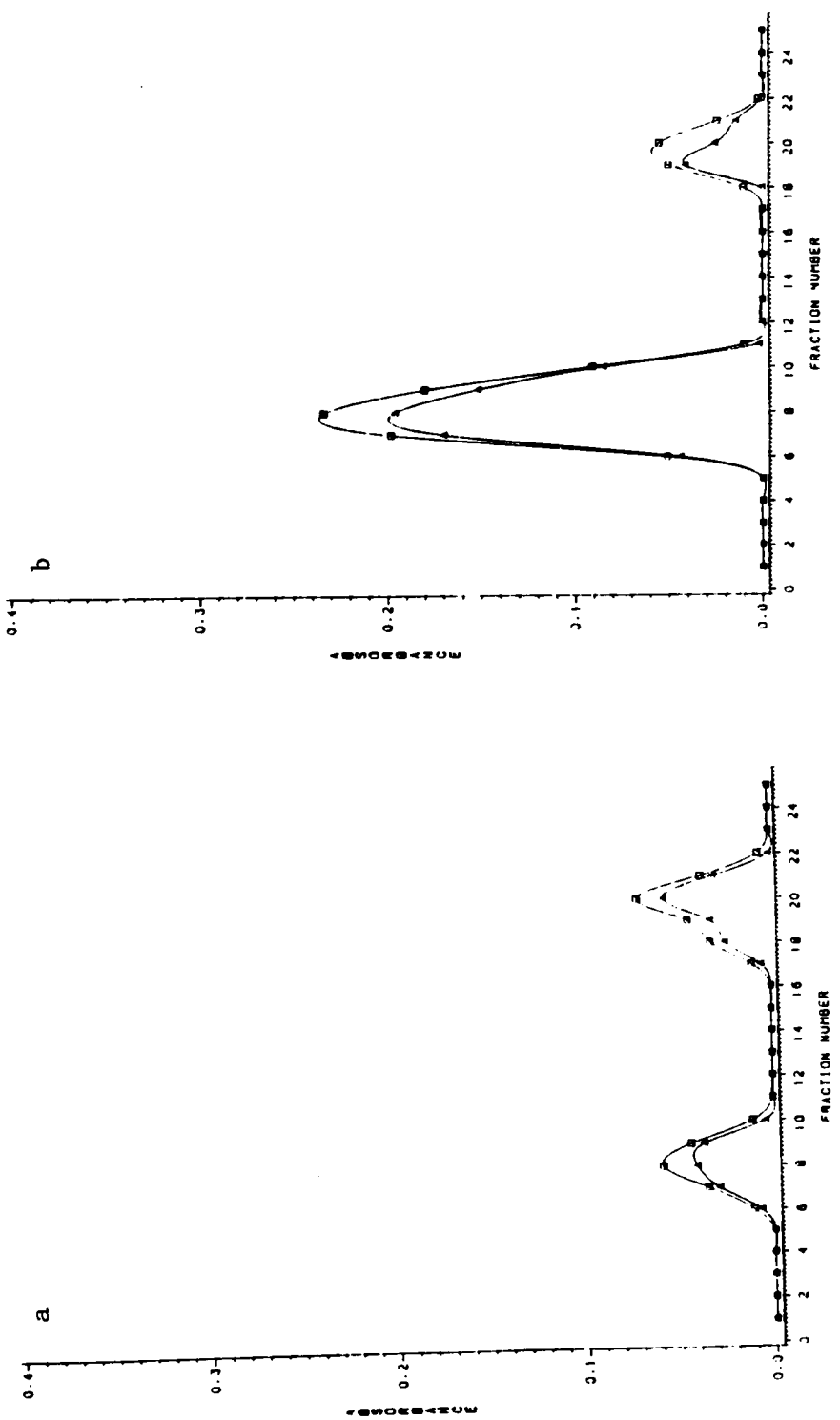

Fig. 4.14. Gel filtration profile on Sepharose CL-4B of mucin medium before (□) and after (△) growth of E. coli 123. (a) Absorbance at 280 nm. (b) Absorbance at 555 nm.

growth of E. coli.

Further evidence indicates that glycosidases produced by E. coli are involved in mucin utilization. Growth of E. coli in mucin medium resulted in an increase in the number of reducing ends on the carbohydrate chains and an increase in the number of free amino sugar end groups (Table 4.4). When concentrations of monosaccharides were determined in mucin medium before and after (8 hr) E. coli growth (Table 4.5), an average of 13 to 16% of the mucin carbohydrate was utilized. Galactose was extensively utilized (33 to 37%), while mannose and fucose were intermediate (24 to 28%). Concentrations of neuraminic acid and hexosamine were not affected by growth.

E. coli growth in mucin medium did not appear to be limited by lack of available substrate. E. coli inoculated into spent mucin broth grew through two log cycles and utilized protein, galactose, mannose, and fucose as when initially inoculated into fresh mucin medium (Table 4.6). Although growth in spent mucin medium was significantly less than growth in fresh mucin medium, sufficient substrate to support growth apparently still remained following the initial 8 hr incubation. Initial growth may have been limited by product inhibition.

Table 4.4. Reactions involved in degradation of small intestinal mucin by *E. coli*.[1]

Strain	Reducing group increase	Amino sugar end group increase
P-155	.34 (±.05)	.04 (±.008)
B-47	.27 (±.07)	.05 (±.004)
123	.29 (±.06)	.04 (±.01)
1466-56	.22 (±.04)	.03 (±.008)

[1] All values are in μ moles/ml

Table 4.5. Utilization of pig small intestinal mucin by *E. coli*.

	Concentration of mucin carbohydrate (mM)[1]					
Strain	Fuc	Man	Gal	NANA	HexNAc	Total
Undigested	.74	.58	1.23	.55	1.67	4.76
P-155	.61	.44	.83	.55	1.68	4.11
B-47	.63	.43	.79	.55	1.60	3.98
123	.67	.42	.78	.55	1.60	4.08
1466-56	.60	.42	.79	.55	1.66	3.97

[1] No glucose or uronic acids were detected by GC analysis. NANA was determined by the Warren procedure (184). No free sialic acid was detected in the medium.

Table 4.6. Growth of *E. coli* on spent mucin medium.

	Growth[1]	Protein[2]	Gal[3]	Man	Fuc
E. coli 123					
1st growth	7.75	6.3	28.4	19.8	8.4
2nd growth	7.0	9.8	49.1	24.0	15.1
E. coli P-155					
1st growth	7.87	9.0	24.2	18.2	15.4
2nd growth	7.17	12.6	46.2	24.8	21.8

[1] Log_{10} of CFU/ml after growth for 8 hr

[2] Percent protein degraded after growth for 8 hr compared to uninoculated medium

[3] Percent of monosaccharide utilized for growth compared to uninoculated medium

3. Enterotoxin production

E. coli P-155 was assayed for toxin production after growth (15 hr) in minimal, glucose, mucin, CAYE, and MS broth media. ST and LT toxins were present in sterile culture supernatants after growth in glucose, CAYE, mucin, and MS broths (Figs. 4.15 and 4.16, respectively). Minimal medium did not support production of either LT or ST. Growth in CAYE produced the highest gut-to-body fluid ratios in infant mouse bioassay for ST and the greatest rounding in adrenal cell assay for LT. Catabolite repression of ST synthesis in glucose medium has been reported (6). However, in the present study, low concentrations of glucose in the growth medium did not repress ST production by E. coli P-155 (Fig. 4.15).

C. ENZYMES PRODUCED BY E. COLI FOR MUCIN UTILIZATION

1. Glycosidase production after growth on mucin

Although further study of mucin protein utilization by E. coli is needed, more attention was given to carbohydrate utilization in this study. Hoskins and Boulding (86) reported blood group degrading enzymes from anaerobic fecal cultures appeared to be glycosidases rather than proteases.

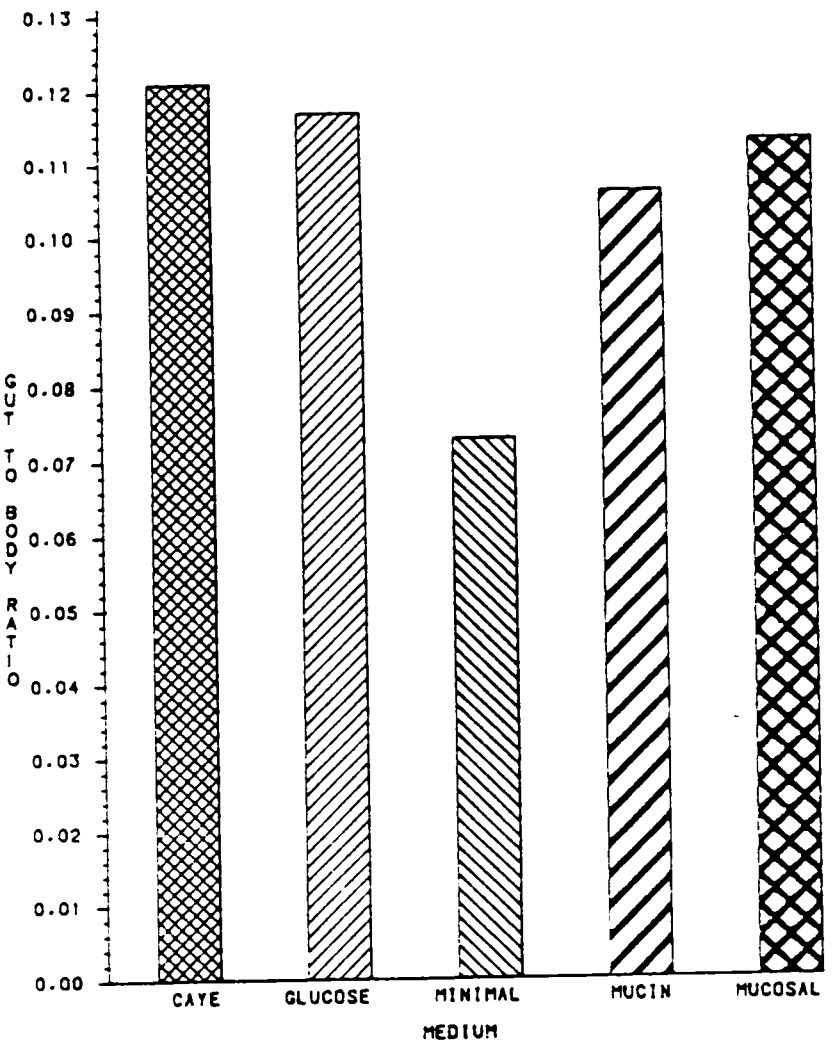

Fig. 4.15. Production of ST-enterotoxin by E. coli P-155 after growth in various media. Positive ST response is a gut:body ratio >0.083.

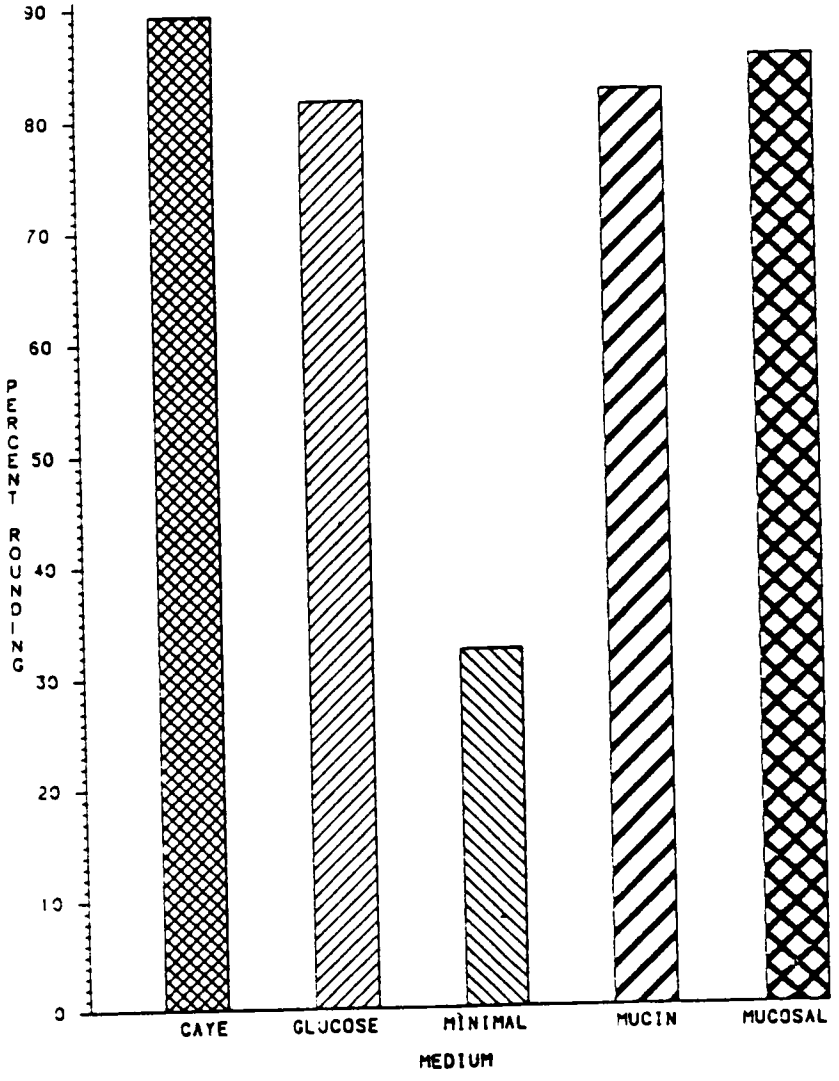

Fig. 4.16. Production of LT-enterotoxin by E. coli P-155 after growth in various media. Positive LT response is >50% rounding of Y1 adrenal cells in cell culture.

The increases in reducing end groups, free amino sugar groups, and utilization of carbohydrates (Tables 4.4 and 4.5) indicates E. coli possess glycosidases necessary for mucin utilization. Mucin glycoproteins offer intestinal bacteria a variety of sugars in different anomeric linkages for growth. Certainly, not all bacteria will have a full complement of glycosidases necessary for complete utilization and will only grow to a limited extent.

All E. coli strains used in growth studies (P-155, B-47, 1466-56, and 123) were examined for α-galactosidase activity (Table 4.7). Eight additional strains were also screened. Strains G-9, 431, and 1413 are porcine ETEC strains, strain B-42 is a porcine NETEC strain, and strain V-517 is a human ETEC strain (Table 3.1). Strains ATCC 23721, ATCC 23722, and ATCC 23723 were screened for α-galactosidase activity because positive chemotaxis of strain ATCC 23723 was not observed in mucin semi-solid agar (see below). Eleven of the strains produced α-galactosidase activity (Table 4.7). Moreover, activity was detected in strain P-155 only when grown in media containing carbohydrates having α-glycosidic linkages (Table 4.8). The average α-galactosidase activity was 0.38 units for the 11 strains which did produce the enzyme.

Table 4.7. Glycosidase activity of a variety of *E. coli* strains after growth in mucin medium.[1]

	α -galactosidase[2]	α -fucosidase	
Strain	Cell-bound	Cell-bound[3]	Extracellular
P-155	.79	1.02	.74
B-47	.21	.70	.32
1466-56	.33	.96	.61
123	.37	1.08	.59
G-9	.51	1.11	.63
431	.57	1.12	.68
1413	.21	.63	.43
V-517	.20	.66	.46
B-42	.21	1.13	.63
ATCC 23721	.68	.67	.58
ATCC 23722	.23	.66	.30
ATCC 23723	0	.56	.30

[1] Activities are expressed in units. 1 unit= 1 μmole nitrophenyl released/min/mg dry cell wt

[2] no extracellular activity was observed

[3] corrected for extracellular activity

Table 4.8. Cell-bound glycosidase activity of *E. coli* P-155 after growth on various carbohydrate substrates.

Substrate	α-galactosidase units[1]	β-galactosidase units[1]	α-fucosidase units[1]
Lactose	0	1.98	ND[2]
Glucose	0	ND	1.05
Melibiose	4.38	ND	ND
Mucin	.79	.021	1.76
None	0	.020	1.51

[1] 1 unit= 1 μmole nitrophenyl released/min/mg dry cell wt

[2] ND, not determined

Extracellular activity was not detected in sterile filtrates of the growth medium. The only strain which did not have α-galactosidase activity was ATCC 23723. This strain lacks the gene for galactoside permease.

Porter et al. (134) first reported the presence of α-galactosidase in E. coli. Growth in a melibiose medium requires inducement (130) of a galactoside permease and α-galactosidase. A variety of α-galactosides induce synthesis of α-galactosidase (153), but activity has not been reported after growth on glycoprotein substrates.

The only blood group containing α-linked galactosides is type B. However, pigs possess only A and H blood groups in common with humans. Therefore, results obtained in this study indicate PSIM contains terminal α-linked galactosides. PSIM oligosaccharides have not been sequenced, but terminal α-galactosides have been reported on HGM (156). A significant portion of the galactosides in PSIM must be in the α-linkage as evidenced by low E. coli β-galactosidase activity (Table 4.8) and utilization of 36% of the total galactose during initial growth in mucin medium (Table 4.5) and an additional 21% utilization during growth on spent mucin medium (Table 4.6).

Cell-bound and extracellular α-fucosidase activity

was detected in all strains of *E. coli* examined (Table 4.7). Extracellular fucosidase activity probably is not an artifact due to bacterial cell lysis since α-galactosidase activity was always cell-bound. In addition, α-fucosidase could not be detected in PSIM prepared for this study (not shown) as has been reported for cervical mucin (151). Moreover, fucose could not be detected by GLC in the other media used to assay fucosidase activity. Fucosidase activity was observed at time of inoculation and thus appears to be a constitutive enzyme. Enzyme activity was highest after growth on mucin medium (Table 4.8). The average cell-bound and extracellular activities were 0.86 and 0.52 units, respectively in the strains examined. The rate of nitrophenyl liberation by strain P-155 was linear for at least 10 min (Fig. 4.17) with both α-galactosidase (cell-bound) and α-fucosidase (cell-bound and extracellular).

Minimal β-galactosidase activity was observed in strain P-155 after growth on mucin medium (Table 4.8). Since this enzyme did not appear to be involved in galactose utilization by strain P-155, no other strains were examined for β-galactosidase activity. Cell-bound and extracellular α-mannosidase activity could not be

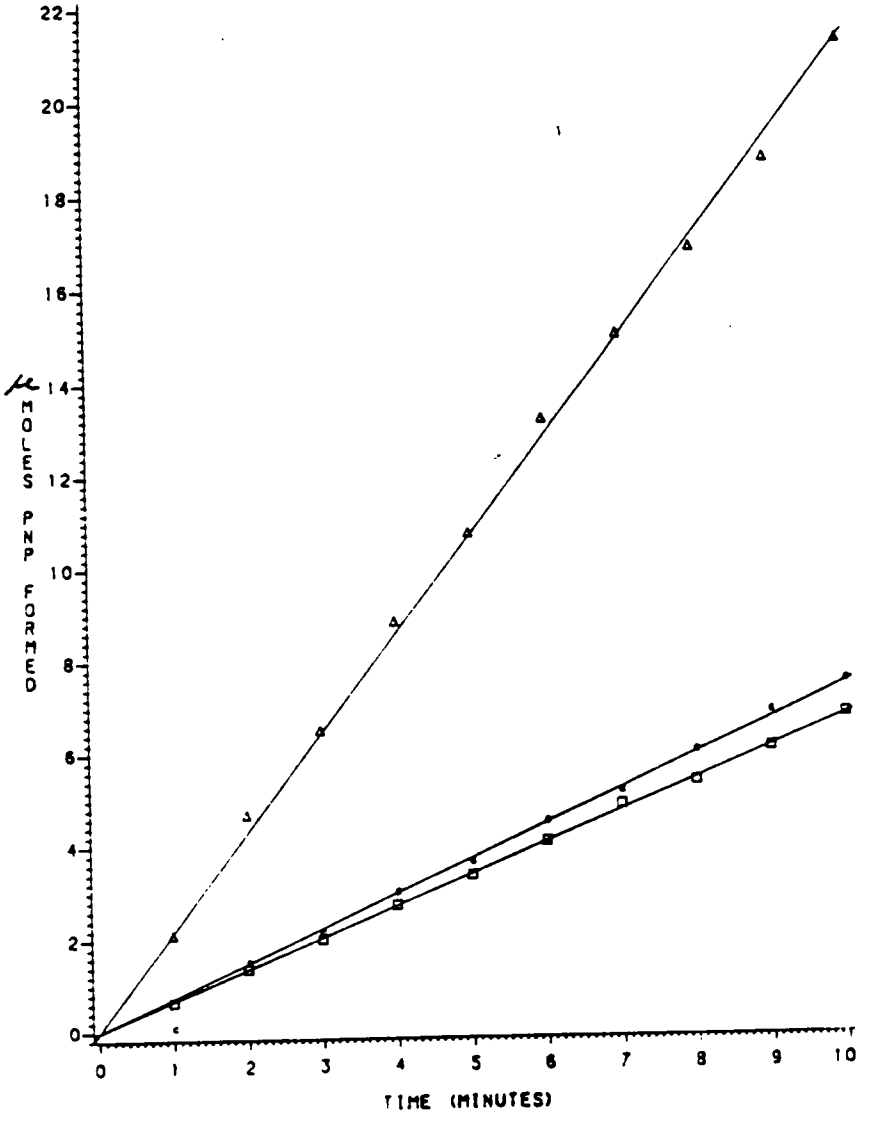

Fig. 4.17. Liberation of nitrophenyl by extracellular α-fucosidase (○), cell-bound α-fucosidase (△), and cell-bound α-galactosidase (□) at 35 C. Substrate concentration was 3 mM.

detected in any of the strains examined. If E. coli possess an α-mannosidase, this enzyme may not be active against nitrophenyl glycosides as has been reported for other glycosidases (9). Alternatively, mannose may have been utilized by E. coli through the action of endoglycosidases (182) which would not be detected in the present study.

Hoskins et al. (84) obtained results which conflict with the present study. Cell-bound and extracellular α-galactosidase and α-fucosidase activity were not detected in human, fecal E. coli strains grown in HGM medium. However, they did observe that approximately 19% of the total hexoses in HGM medium were used for E. coli growth. Cell-bound glycosidase activity was measured using cell lysates. Burstein and Kepes (21) reported α-galactosidase activity was absent in cell lysates, but present when intact cells were used as in the present study. Absence of α-fucosidase activity in Hoskin's study can not be explained as readily.

2. Activity of cell-bound α-galactosidase and α-fucosidase during growth on small intestinal mucin

Results from GLC and enzyme analyses during growth were used to correlate degradation of mucin by E. coli P-155 glycosidases. Induction of α-galactosidase (Fig. 4.18) had occurred by 2 hr after inoculation into mucin medium, while α-fucosidase activity (Fig. 4.19) (1.02 units) could be detected at inoculation. Organisms which possess important constitutive enzymes are at a selective advantage in their natural environment over nonconstitutive strains where substrate concentration may be limiting growth (40). Constitutive fucosidase activity would enable E. coli to begin growth and metabolism on fucose linked to mucin after a short lag time. Maximum activities for both enzymes were obtained during exponential growth of the organism (Figs. 4.18 and 4.19).

E. coli P-155 utilized 27% of the galactose and 17% of the fucose in mucin medium. Galactose was utilized at a rate of 30 nmoles/hr for the first 2 hr after inoculation and at a faster rate (45 nmoles/hr) during the last 6 hr. Once α-galactosidase was induced and synthesized by the cell, α-linked galactosides were hydrolyzed by the

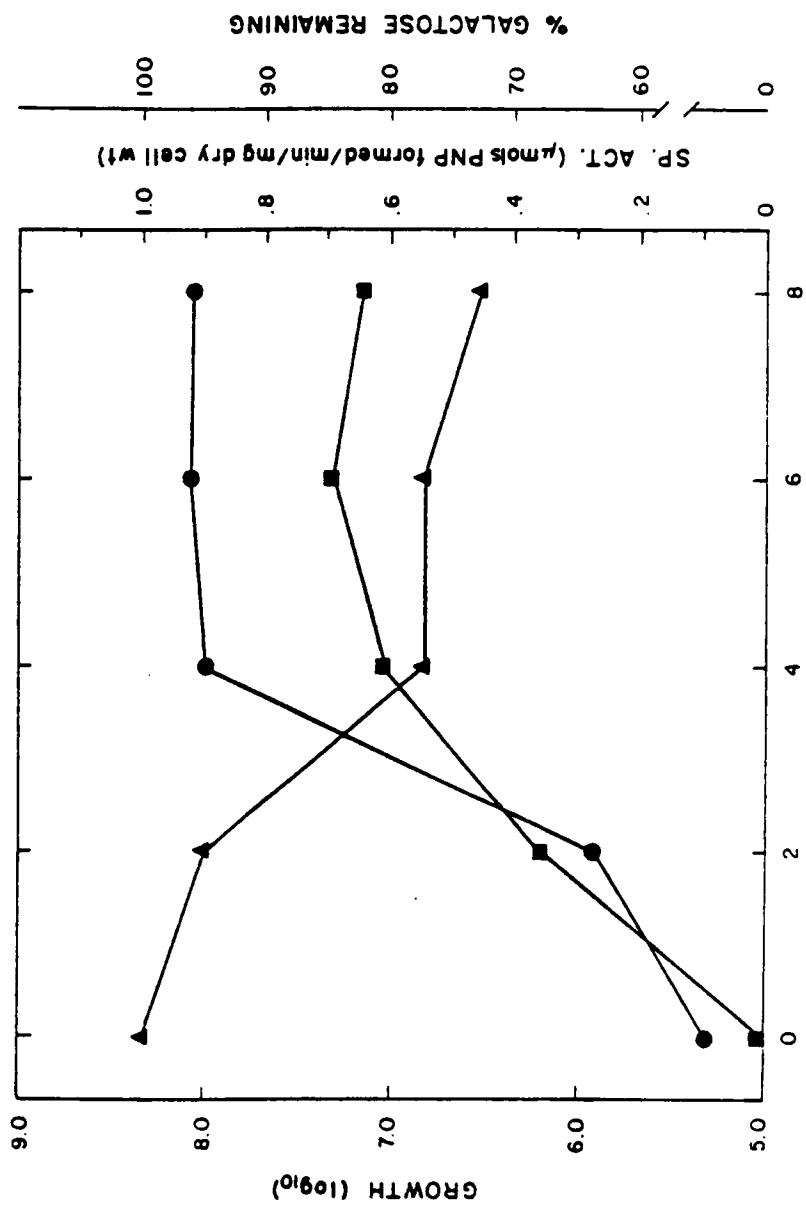

Fig. 4.18. Induction of α-galactosidase during growth of E. coli P-155 in mucin medium. Symbols: growth, ● ; α-galactosidase specific activity, ■ ; utilization of galactose, ▲ .

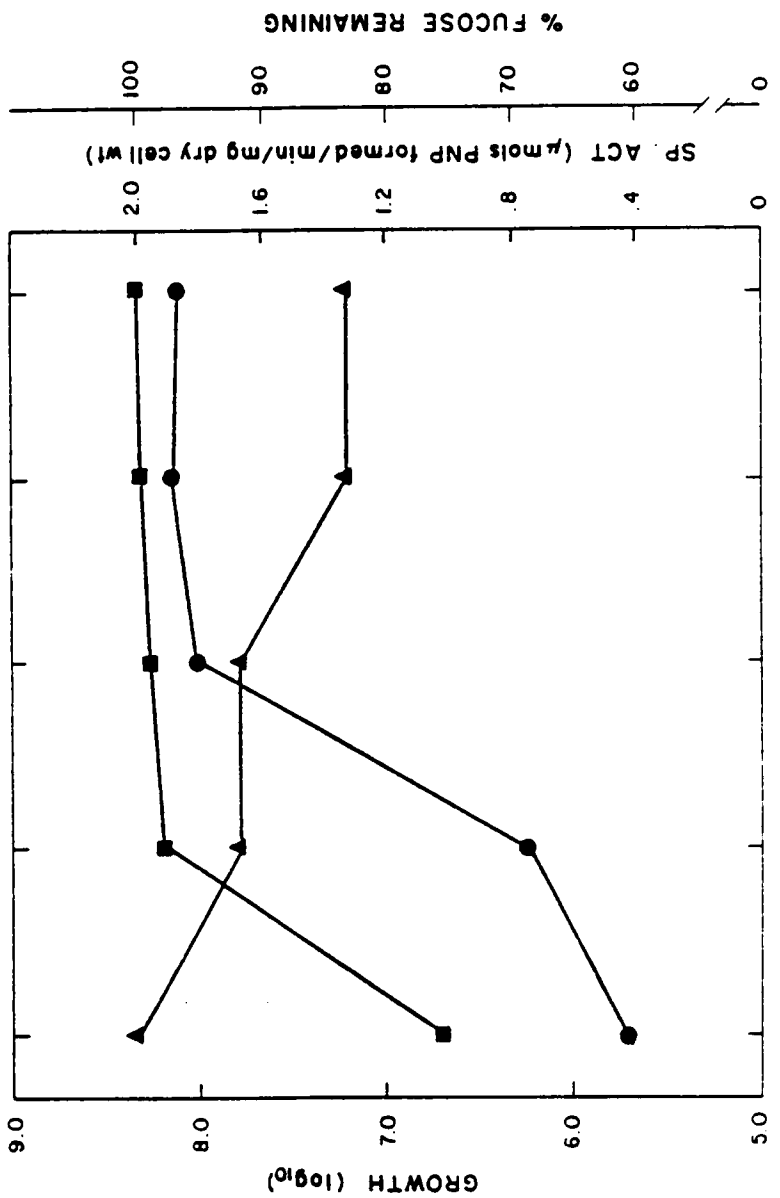

Fig. 4.19. α-Fucosidase activity during growth of E. coli P-155 in mucin medium. Symbols: growth, ●; α-fucosidase specific activity, ■; and, utilization of fucose, ▲.

bacterium as a source of additional growth nutrients. In contrast, fucose was utilized at a steady rate of 10 nmoles/hr during the entire 8 hrs of growth.

Exoglycosidase or endoglycosidase activity was not determined in this study. Exoglycosidases cleave monosaccharide units from the non-reducing terminal end of oligosaccharides, whereas endoglycosidases randomly attack the oligosaccharide chain. Fucose and galactose utilization (Table 4.5) and increases in reducing ends (Table 4.4) implies these sugars are in terminal positions accessible to exoglycosidases. However, mannose is usually found in the core of oligosaccharides. Thus, utilization of this monosaccharide by E. coli (Table 4.5) as well as the small increase observed in free amino sugar groups (Table 4.4) suggests the possibility that endoglycosidase activity may be produced by E. coli. Escherichia fruendii, a soil bacterium, produces an endo- β-galactosidase which is induced by growth on HGM (62). Although not assayed in this study, β-D-N-acetylglucosaminidase is a cell-bound enzyme of E. coli responsible for hydrolysis of outer cell membranes (182). If bound to the exterior of the cell, this enzyme may be responsible for endoglycosidase activity with subsequent loss of mannose.

D. ROLE OF MUCIN IN *E. COLI* CHEMOTAXIS

All 24 strains of *E. coli* from various animal and environmental sources described in Table 3.1 were examined for motility in mucin semi-solid agar. Fifteen strains showed positive motility by growing away from the stab line and along the air-agar interface (Table 4.9). Positive strains grew as rings and gradually progressed down the agar as oxidizable substrates were utilized. All positive strains were also motile when observed under high-power in a light microscope (Table 4.9). Two strains (FS-8216 and FS-8301) did not grow in mucin semi-solid agar. One motile strain of *E. coli* (ATCC 23723) did not exhibit positive motility in mucin semi-solid agar even though the parent strain ATCC 23721 and strain ATCC 23722 did. As mentioned above, this strain (ATCC 23723) is a mutant of *E. coli* K12 (ATCC 23721) and lacks the gene coding for galactoside permease (114). Studies comparing establishment of this strain and its parent (ATCC 23721) in hog small intestine would be of considerable interest.

E. coli strain P-155 (Table 3.1) was attracted to PSIM in capillary chemotaxis assays in numbers comparable to the positive chemoattractant (tryptone; Fig. 4.20). Motile

Table 4.9. Results of motility in mucin semi-solid agar.

Strain	Agar[1]	Microscopy
G-9	+	+
P-155	+	+
431	-	-
B-47	-	-
1592	-	-
1413	-	-
FS-8001	+	+
V-517	-	-
3-7	+	+
B-42	+	+
FS-8201	+	+
FS-8202	+	+
FS-8204	-	-
FS-8216	NG[2]	-
FS-8211	+	+
123	+	+
1466-56	+	+
FS-8301	NG	+
FS-8401	+	+
FS-8402	+	+
FS-8404	+	+
ATCC 23721	+	+
ATCC 23722	+	+
ATCC 23723	-	+

1 +, positive; -, negative

2 NG, no growth

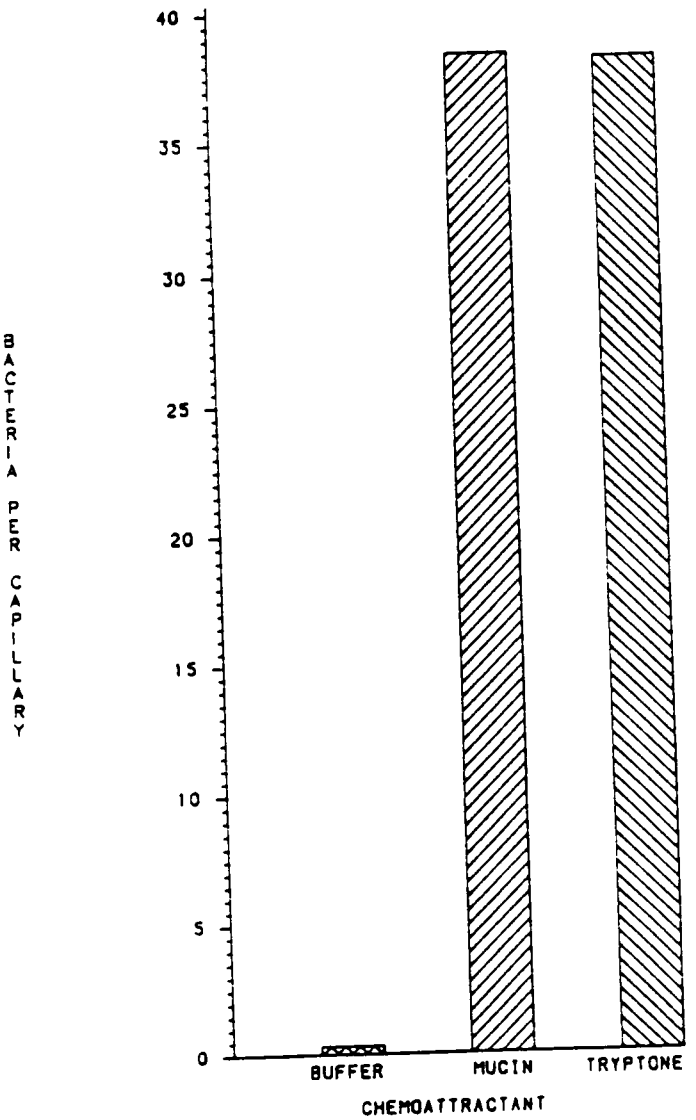

Fig. 4.20. Chemotactic effect (x 10^5) of mucin (1 mg/ml), chemotaxis buffer (pH 7), and tryptone (1%, w/v). Capillaries were incubated with E. coli P-155 cells (~1 x 10^8) for 30 min at 32 C.

bacteria accumulated in the capillaries with an optimal incubation time of 30 min (Fig. 4.21). The attracting power of mucin and tryptone was 85-fold greater than random migration. Similar results were observed in capillary experiments using the NETEC strain 123 (Fig. 4.22).

The components of mucin responsible for positive chemotaxis are difficult to surmise. The complexity and heterogeneity of mucin make this difficult to clarify. Optimal concentrations of monosaccharides (3) and amino acids (113) involved in positive chemotaxis of E. coli have been obtained. All of the sugars linked on mucin (105) are chemoattractants to E. coli in their monosaccharide form (3). Sulfates associated with mucin may also be involved since some metallic sulfates are positive chemoattractants for E. coli (173).

Mucin pH did affect chemotactic attraction. Maximum mucin chemotaxis occurred at pH 7.0 (Fig. 4.23). Mucin remains a positive chemoattractant over a broad pH range. Digestion causes sudden and drastic pH changes in the upper small intestine; small intestinal pH will drop as low as 5.0 when the pylorus valve opens to empty digesta to the duodenum (178). A positive chemotactic response was noted even at a low pH of 5.0 in this study (Fig. 4.23).

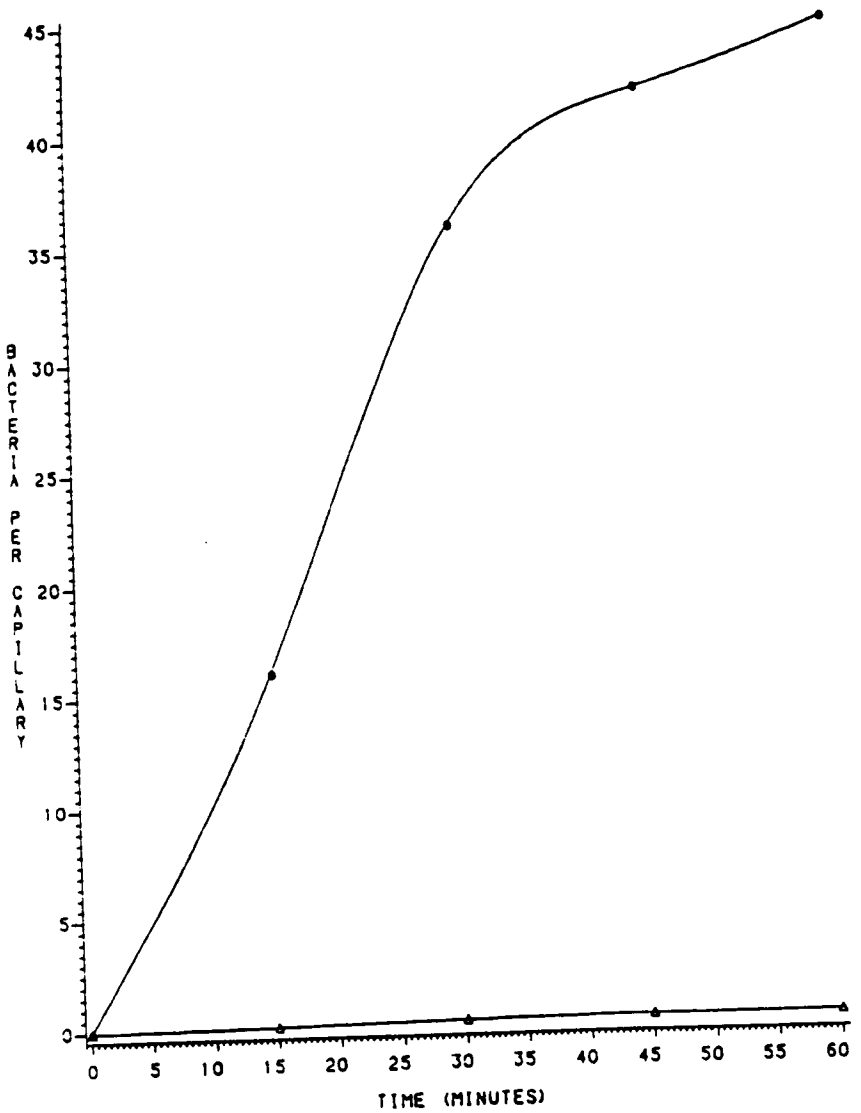

Fig. 4.21. Accumulation (x 10^5) of E. coli P-155 in capillaries containing mucin (●; 1 mg/ml) or unsupplemented chemotaxis buffer (△; pH 7).

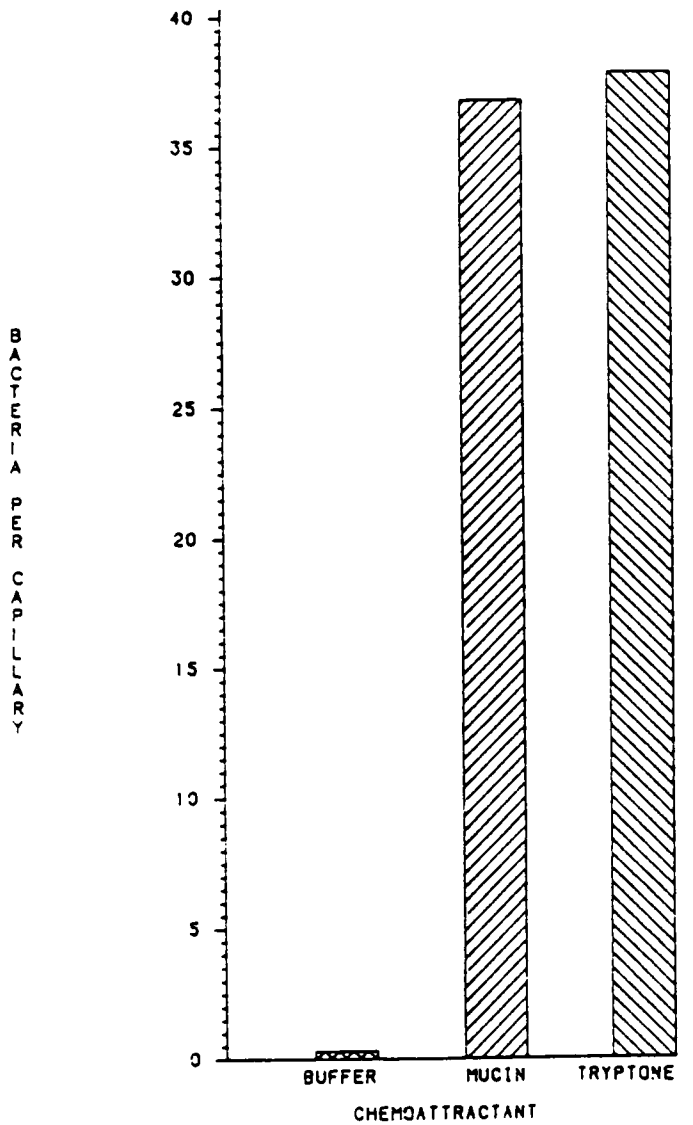

Fig. 4.22. Chemotactic effect ($x10^5$) of mucin (1 mg/ml), chemotaxis buffer (pH 7), and tryptone (1%, w/v). Capillaries were incubated with E. coli 123 cells (~1 x 10^8) for 30 min at 32 C.

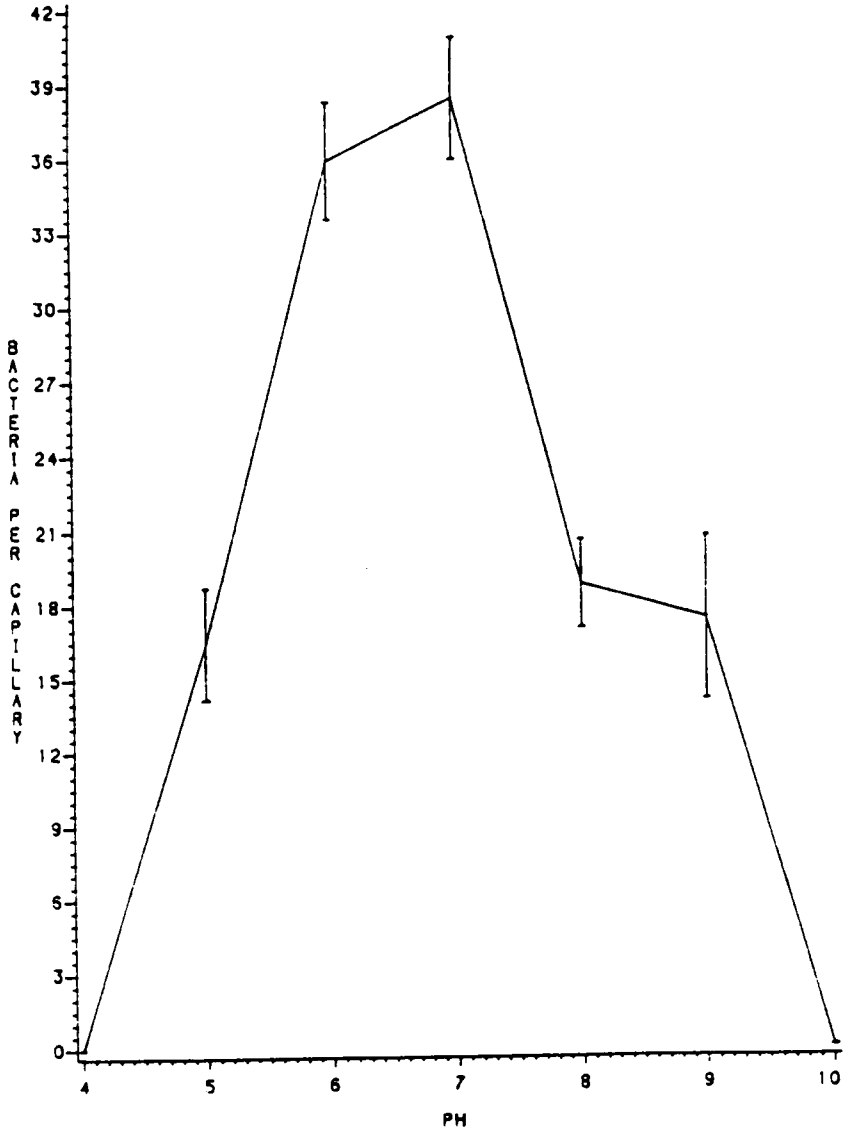

Fig. 4.23. Dependence of pH on chemotaxis. Capillaries of mucin (1 mg/ml) at different pH were incubated with E. coli P-155 cells for 30 min. Accumulation of bacteria in capillaries is $x10^5$.

Furthermore, mucin remained a positive chemoattractant to a pH of 9.0. This is well within the physiological pH range in porcine small intestine.

In vitro bacterial attraction occurred over a broad mucin concentration range (Fig. 4.24). However, an optimal concentration of 1 mg/ml was obtained. A 1.5-fold decrease was observed at 2 mg/ml and, as concentration of mucin increased, a greater decrease was observed (2.5-fold at 10 mg/ml). Higher accumulations observed in diluted mucin could be due to differences in viscosity that would allow greater bacterial movement. Presence of inhibitory substances (eg. lysozyme, lactoferrin, or secretory immunoglobulin) is unlikely because this strain grew fairly well on mucin prepared for this study (Fig. 4.5a).

No significant difference was observed in using spent mucin as chemoattractant to both strains of E. coli (Table 4.10), despite a loss of 15% of the carbohydrate present (Table 4.5). Thus, removal of carbohydrate moieties from mucin by E. coli growth does not appear to destroy the positive taxins.

Motility and chemotaxis appear to be important virulence factors for V. cholerae (61). The rate of penetration into mucous gel by V. cholerae is relatively

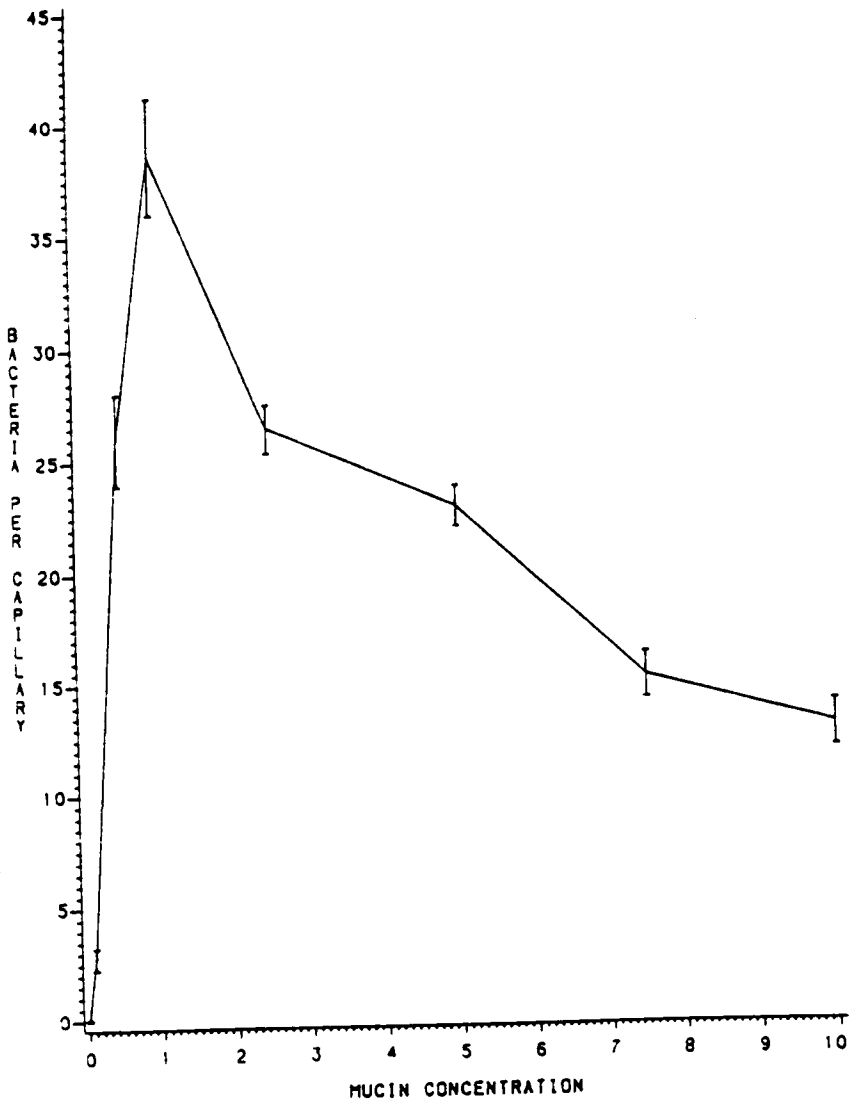

Fig. 4.24. Accumulation (x 10^5) of E. coli P-155 in capillaries containing different concentrations (mg/ml) of mucin in chemotaxis buffer (pH 7). Capillaries were incubated for 30 min at 32 C.

Table 4.10. Effect of spent mucin on *E. coli* chemotaxis[1].

	# Bacteria/Capillary[2]	
Strain	Native Mucin	Spent Mucin
123	38.8	29.1
P-155	38.3	31.5

[1] Capillaries were filled with chemoattractant (native mucin or "spent" mucin; concentration of 1 mg dry wt/ml chemotaxis buffer, pH 7.0) and immersed in washed bacterial cells (~1 x 10^8 organisms) for 30 min at 32 C

[2] x 10^5

rapid when the organism possess genes for motility and chemotaxis (61). However, both properties are necessary since motile bacteria without chemotactic guidance are unable to enter the gel. In the present study, motility was observed to be an important property for chemotaxis. However, NETEC were chemoattracted as well as ETEC. Therefore, chemotaxis may not be as important to *E. coli* pathogenesis as suggested for *V. cholerae*. A combination of motility, chemotaxis, and adhesin factors may be needed for increased virulence of ETEC. Certainly this would need further *in vivo* study.

CHAPTER FIVE

SUMMARY AND CONCLUSIONS

The presence of *E. coli* as an indigenous intestinal organism in man and animals is well documented. Many organisms, including *E. coli*, may utilize glycoproteins and glycolipids from the intestinal mucosa for survival in the digestive tract. In this study, *in vitro* methods were used to examine the role of mucosal glycoproteins (mucin) in establishment of *E. coli* in pig small intestine.

Porcine small intestinal mucin isolated by solubilization of mucous gel with 2-mercaptoethanol, was composed of (% by wt): 58.7% carbohydrate; 37.2% protein; and 3.7% sulfate ester. Electrophoretic patterns of mucin were more complex than the gel filtration profile. Mucin eluted as a single peak at the void volume after fractionation on Sepharose CL-4B, whereas 2 major and 7 minor polypeptide bands were detected by SDS-PAGE. Thus, mucin peptides appear to aggregate in the absence of dissociating agents probably through strong non-covalent interactions. Filter-sterilization of mucin released a small amount of material which had an approximate molecular weight of

14,500 by gel filtration. This protein is probably dissociated from the macromolecule by shear forces involved in filter-sterilization. Mucin prepared by reduction and proteolysis of mucus gel had a much lower viscosity (intrinsic viscosity= 135 ml/g) and should not be used in rheological studies. However, this procedure does produce sufficient quantities of enriched mucin for use in bacteriological media.

Results reported herein support the hypothesis that mucin, as the sole source of carbon and energy, can support growth of E. coli. ETEC do not have an advantage over NETEC in either growth on or utilization of mucin. However, mucin does support production of both LT and ST enterotoxins by E. coli P-155.

E. coli are capable of degrading both carbohydrate and protein available in mucin medium. Gel filtration (Sepharose CL-4B) of mucin medium before and after growth demonstrated no major degradation patterns. Decreases in peak areas suggest that both large and small molecular weight components are degraded. However, increases in the number of reducing end groups and free amino groups indicate oligosaccharide chains are also hydrolyzed and sugars removed. Growth of E. coli resulted in utilization

of galactose (35%), mannose (26%), and fucose (15%) in mucin medium.

Utilization of carbohydrate in PSIM by E. coli was selected for further examination in these studies. A constitutive cell-bound and extracellular α-L-fucosidase and an induced cell-bound α-D-galactosidase were present when E. coli was grown on PSIM. Activities for α-fucosidase and α-galactosidase were 1.02 units and 0.79 units, respectively. β-Galatosidase activity was minimal in E. coli P-155 after growth on PSIM and did not appear responsible for cleavage of galactose residues from PSIM oligosaccharides. α-Galactosidase and α-fucosidase appear to be responsible for cleavage of sugars from mucin during E. coli growth. Utilization of mannose from mucin by E. coli needs further investigation. Mannosidase activity could not be detected using nitrophenyl substrates. However, other glycosidases do not have activity toward nitrophenyl substrates (9). Mannose may also have been removed by the activity of endoglycosidases not assayed in this study.

Motile strains of E. coli used in this study were chemoattracted to PSIM. Positive strains grew on the surface of mucin semi-solid agar and moved down into the

agar as oxidizable substrates were used. Negative and non-motile strains only grew along the stab line. Capillary chemotaxis assays were used with two strains to monitor the numbers of bacteria which would be chemoattracted to mucin. Approximately 3.7×10^6 organisms were attracted into capillaries containing mucin. Mucin was a positive chemoattractant over a wide pH (5.0 to 9.0) and concentration range (0.25 to 10.0 mg/ml). Therefore, motile *E. coli* appear to have chemosensors which can detect and initiate movement toward the mucosal surface from the lumen.

This research has established that mucin plays an important role in establishment of *E. coli* in hog small intestine. *E. coli* can be attracted from the small intestinal lumen to the mucosal surface by chemotaxins on mucin. Once at the mucosal surface, *E. coli* binds to and utilizes mucin for growth. Cleavage of sugars from mucin oligosaccharide chains is accomplished, at least partially, by an induced α-galactosidase and a constitutive α-fucosidase. Constitutive fucosidase activity would enable *E. coli* to begin growth and metabolism after a short lag time. Furthermore, fucose is utilized at a steady rate (10 nmoles/hr) throughout

growth. Once α-galactosidase is induced and synthesized by the cell, α-linked galactosides are hydrolyzed by the organism to be used as additional nutrients for growth. E. coli α-galactosidase is induced within two hours after inoculation. Endo- or exo-glycosidase activity by these glycosidases could not be determined from this study. Results observed in this study suggest α-fucosidase and α-galactosidase are acting as exoglycosidases. Further study is needed to better understand mucin utilization by E. coli.

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THE ROLE OF MUCIN IN ESTABLISHMENT OF *ESCHERICHIA COLI* IN PORCINE SMALL INTESTINE

by

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(ABSTRACT)

Mucin was isolated for incorporation in bacteriological media by reduction and proteolysis of mucous gel from porcine small intestine. Mucin prepared in this study contained (by weight) 37.2% protein, 58.7% carbohydrate (4.6% fucose, 9.4% mannose, 10.7% sialic acid, 13.5% galactose, and 20.5% hexosamine), and 3.7% ester sulfate. Fractionation of mucin on Sepharose CL-4B yielded one peak which eluted at the void volume. However, sodium dodecyl sulfate gel electrophoretic patterns contained 9 polypeptide bands of which 6 stained with periodic acid-Schiff reagent. Proline, serine, and threonine residues accounted for 26% (by weight) of the total protein in the preparation. Half-cysteine residues made up another 1%. Intrinsic viscosity of mucin prepared by reduction and proteolysis was 135 ml/g.

Mucin was incorporated into a minimal bacteriological medium as the sole-source of carbon and nitrogen. Enterotoxigenic and non-enterotoxigenic Escherichia coli grew equally well in mucin medium at levels comparable to growth in 3 mM glucose medium. Growth did not appear to be limited by availability of metabolizable substrates. Spent mucin medium supported growth in comparable numbers. E. coli P-155 produced heat-stable and heat-labile enterotoxins during growth in both mucin medium and fresh mucosal scrapings medium. E. coli utilized total hexose and protein in mucin medium at comparable levels (6 to 10%). Increases in reducing end groups (0.28 μ moles/ml) and free amino sugar end groups (0.04 μmoles/ml) during growth were detected. E. coli used approximately 15% of the total carbohydrate in mucin medium including 36% of the galactose, 15% of the fucose, and 27% of the mannose. Utilization of mucin by E. coli produced minor changes in gel filtration patterns on Sepharose CL-4B.

Twelve strains of E. coli were examined for glycosidase activity during growth on mucin. All twelve produced a cell-bound and an extracellular α-fucosidase although the majority of activity was cell-bound.

Although α-fucosidase was a constitutive enzyme of E. coli P-155, maximum activity was observed during exponential growth in mucin medium. Eleven strains produced cell-bound α-galactosidase. No extracellular activity of this enzyme was detected. Maximum levels of induced α-galactosidase activity were obtained in late exponential to early stationary growth of E. coli. E. coli ATCC 23723, a mutant of E. coli K12 lacking the galactoside permease gene, did not produce α-galactosidase activity during growth on mucin. No α-mannosidase activity was detected using nitrophenylmannoside as substrate.

Porcine small intestinal mucin was a positive chemoattractant for E. coli in capillary assays. Optimal chemotactic response by E. coli P-155 in capillary experiments was obtained at a mucin concentration of 1 mg dry wt/ml at a pH of 7.0. Spent mucin was still a positive chemoattractant for E. coli P-155 and 123 despite losing 15% of the total mucin carbohydrate.