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Hydrolyzed Casein Influences Intestinal Mucin Gene Expression in the Rat

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The effect of hydrolyzed casein (HC) on the expression of three mucin genes (Muc2, Muc3, and Muc4) in the rat intestine was investigated using quantitative real-time polymerase chain reaction. After a 10 day acclimatization period, rats received for 8 days the test diets containing either HC or a synthetic amino acid (SAA) mixture as the sole source of nitrogen or a protein-free (PF) diet ($n =$ 12 per treatment). The addition of HC or the SAA mixture to the diet significantly improved average daily gain, average daily food intake, and gain:feed ratio as compared with the PF diet. Terminal ileal endogenous N flow was significantly higher for the HC-fed rats in comparison with either the SAA or the PF rats ($p \leq 0.001$). HC supported a significant increase of Muc3 mRNA (277 and 229% of that for diets PF and SAA, respectively; $p \leq 0.05$) in the small intestinal tissue and Muc4 mRNA (325 and 265% of that for diets PF and SAA, respectively; $p \leq 0.05$) in the colon. In conclusion, HC enhances ileal endogenous N flow and up-regulates in vivo the expression of some individual mucin genes.

KEYWORDS: Hydrolyzed casein; rat mucin gene; ileal endogenous N; real-time PCR

INTRODUCTION

The mucus layer is a complex mixture containing cellular material, microorganisms, water, ions, and large amounts of a high molecular weight glycoprotein, mucin, which protects the mucosal cells from noxious substances and pathogens, regulates epithelial hydration, and participates indirectly in the immune response (*1*). Mucins consist of a core protein, containing tandemly repeated amino acid (AA) sequences rich in serine and threonine residues, to which large amounts of carbohydrate (about 50−85%) are linked by *O*-glycosylation (*2*). The oligosaccharide chains vary in length and antigenic structure among the different mucins (*3*).

Gastrointestinal mucus layers are affected, quantitatively and qualitatively, by intestinal stress. Intestinal diseases such as Crohn's disease and ulcerative colitis, for example, may induce alteration in mucin secretion and a reduction in the number of mucin-containing goblet cells, thus causing degradation of the mucus layer (*4*). Interestingly, dietary factors can alter mucin production quantitatively, which ultimately affects the physiological efficiency of the protective barrier (*5, 6*). Dietary compounds like fiber and short-chain fatty acids can modify the dynamics of mucus by changing the expression of mucin or the numbers of goblet cells (*5, 7*). Dietary proteins and their degradation products are also involved in the regulation of mucin secretion (*8, 9*). Recently, β-casomorphin derived from bovine β-casein was reported to induce the release of mucin in rat jejunum (*10*).

Various biological activities of milk protein peptides have been well-documented (*11*), and an effect of dietary peptides on gut endogenous protein flow is apparent (*12*). Although it is clear that milk peptides affect the secretory activity of mucus cells and may play an important role in the protective function of mucin, to date, only a few studies have been conducted (*6, 8, 10*).

In humans, at least 17 different mucin genes encoding secretory or membrane-associated proteins have been identified (*13*). As for the human intestine, the major mucins produced in rodent intestine are Muc2, Muc3, and Muc4 (*14, 15*). Muc2, the main secretory mucin, is confined to the goblet cells, while Muc3 and Muc4, the transmembrane mucins, are detectable in both goblet cells and enterocytes (*16*). The present study was undertaken to investigate the effect of hydrolyzed casein (HC) on the expression of three mucin genes (Muc2, Muc3, and Muc4) at the transcriptional level in the intestinal tract of the rat.

MATERIALS AND METHODS

Animal Experiment. This experiment was approved by the Massey University Animal Ethics Committee (protocol 05/04). Thirty-six Sprague−Dawley male rats (250 ± 2 g of body weight) were housed individually in stainless steel cages in a room maintained at 21 ± 2 °C with a 12 h light/dark cycle. Water was continuously available. For the entire study, rats received eight equally sized meals daily given at hourly intervals and each for a 10 min period. The rats were acclimatized from days 0 to 9 while receiving a casein-based preliminary diet (**Table 1**), and then, the rats were randomly allocated ($n = 12$ per treatment) to one of the three test diets for days 10−17. The diets included a protein-free (PF) diet and two diets containing enzyme-HC or a free L-AA mixture simulating the HC (synthetic amino

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Table 1. Ingredient Compositions of the Diets Given to Growing Rats

ingredient	diet			
	preliminary	PF	SAA	HC
	g/kg air dry weight			
corn starch[a]	634	723	621	628
purified cellulose[b]	50	50	50	50
vitamin mix[c]	50	50	50	50
salt mix[d]	50	50	50	50
$NaHCO_3$		19	23	
sucrose	70	70	70	70
soya oil	35	35	35	35
acid casein[e]	111			
AA mixture[f]			98	
HC[e]				114
titanium dioxide[g]		3	3	3

[a] Golden Harvest, Primary Foods Ltd., Auckland, New Zealand. [b] Avicel PH101, Commercial Minerals Limited, Auckland, New Zealand. [c] Crop & Food Research, Palmerston North, NZ. The mixtures were formulated to meet the vitamin and mineral requirements of the growing rat (*20*). The mixture supplied (mg/kg diet): retinol acetate, 5.0; DL-α-tocopheryl acetate, 200; menadione, 3.0;, thiamin hydrochloride, 5.0; riboflavin, 7.0; pyridoxine hydrochloride, 8.0; D-pantothenic acid, 20; folic acid, 2.0; nicotinic acid, 20; D-biotin, 1.0; myo-inositol, 200; and choline chloride, 1500; (μg/kg diet): ergocalciferol, 25; and cyanocobalamin, 50. [d] Crop & Food Research, Palmerston North, NZ. The mixtures were formulated to meet the vitamin and mineral requirements of the growing rat (*20*). The mixture supplied (g/kg diet): Ca, 6.29; Cl, 7.79; Mg, 1.06; P, 4.86; K, 5.24; and Na, 1.97; (mg/kg diet): Cr, 1.97; Cu, 10.7; Fe, 424; Mn, 78.0; and Zn, 48.2; (μg/kg diet): Co, 29.0; I, 151; Mo, 152; and Se, 151. [e] New Zealand Milk Products, Wellington, New Zealand. [f] SAAs (Ajinomoto Co., Japan). The AA mixture simulated the AA content of the HC and contained (g/kg diet): Thr, 4.0; Val, 6.5; Ile, 5.0; Leu, 9.5; Phe, 5.2; Tyr, 5.6; Lys, 7.9; His, 3.0; Glu, 22.2; Pro, 11.2; Arg, 3.4; Try, 1.3; Asp, 6.9; Ser, 4.8; Gly, 1.9; Ala, 4.1; Cys, 0.3; and Met, 2.7. [g] Indigestible marker included for determination of ileal ENFL.

acid, SAA) as the sole source of nitrogen (**Table 1**). All diets were formulated to meet the nutrient requirements of the growing rat (*17*). Food intake was recorded daily, and the rats were weighed on days 9 and 16. On day 17, 6 h ± 15 min after the first meal, the rats were asphyxiated with carbon dioxide gas and decapitated. The abdomen was opened by an incision along the midventral line. The whole digestive tract was dissected and divided into four parts: proximal intestine, median intestine, terminal ileum (last 20 cm of the small intestine), and cecum/colon. Digesta from the terminal ileum were gently removed using ice-cold saline solution and then immediately frozen at −80 °C, then freeze-dried, and ground for subsequent analysis of AAs and titanium. Because of complications with the ultrafiltration of digesta for the HC-fed rats, digesta were collected from the same number of rats under identical conditions in a separate study. Each intestinal section was weighed, immediately frozen on dry ice, and then stored at −80 °C for subsequent analysis of mucin gene expression.

HPLC Gel Filtration. Enzyme-HC, an acid-precipitated casein that had been hydrolyzed by pancreatin, was obtained from New Zealand Pharmaceuticals Ltd. (Palmerston North, NZ). The molecular weight distribution of the HC was determined using a high-performance liquid chromatography (HPLC) gel filtration column (TSKGel G2000SWXL, 30 cm, Phenomenex, CA). The effluents were eluted using 36% acetonitrile containing 0.1% trifluoroacetic acid (TFA) with detection at a wavelength of 205 nm.

Chemical Analysis. Digesta were pooled across randomly selected pairs of rats within each treatment to give six ileal digesta samples. Ileal digesta from rats (six pooled samples each of two rats) fed diet HC collected in a separate related study were rehydrated overnight and then centrifuged (1400*g*, 30 min, and 3 ± 1 °C). The supernatant was ultrafiltered (Centriprep-10 devices, 10 kDa MW cutoff; Amicon Inc., Beverly, MA) as described previously (*18*). The resulting retentate (MW > 10 kDa) was added to the precipitate from the centrifugation step, freeze-dried, finally ground, and thus provided a measure of endogenous AA flow for the HC diet. Diets and digesta samples were analyzed for TiO_2 and AAs. AAs were determined after acid hydrolysis using an ion exchange HPLC system (Waters Co., Milford, MA) (*19*). Cys, Met, and Trp destroyed during acid hydrolysis were not determined. TiO_2, which was included in the diets as an indigestible marker compound, was determined by a colorimetric assay after ashing of the sample and digestion of the minerals (*20*).

Endogenous AAs were measured directly in digesta from rats fed diet PF and for the SAA, assuming a virtual complete absorption of synthetic AA (*21*) or after centrifugation and ultrafiltration of digesta for the rats fed diet HC. Ileal endogenous N (EN) was determined from the sum of the endogenous AA residues divided by 6.25. Ileal endogenous nitrogen flow (ENFL, μg/g DMI) was determined with reference to the dietary marker as follows: ENFL = EN × (TiO_2 in diet/TiO_2 in digesta).

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (PCR). Four rats were randomly selected from each diet group to determine the expression of mucin genes. Total RNA was extracted from the colon or small intestinal tissues (duodenum, jejunum, and ileum combined) with Trizol reagent (Invitrogen Co., Carlsbad, CA) according to the manufacturer's guidelines. For the small intestinal tissues, equal amounts of tissue were collected from the middle section of each of the three parts and then combined. To be sure of the absence of contamination by genomic DNA, total RNA was digested by RNA-free DNase I (Promega Co., Madison, WI). The first strand of cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Basel, Switzerland). One microgram of DNase-treated RNA and anchored-oligo$(dT)_{18}$ primer (2.5 μM) was heated at 65 °C for 10 min to denature the RNA template. Transcriptor reverse transcriptase (10 U), deoxynucleotide mix (1 mM each), RNase inhibitor (20 U), and reaction buffer (8 mM $MgCl_2$) were added to yield a final volume of 20 μL, and the reaction mixture was incubated at 50 °C for 60 min and then at 85 °C for 10 min.

For real-time PCR, the synthesized cDNA was diluted (1:5) with RNA-free water. The real-time PCR measures were performed using the LightCycler 480 system with the LightCycler 480 probes master kit (Roche Diagnostics). Primers and probes for mucin genes and an internal reference β-actin gene were designed with the assistance of Roche Universal ProbeLibrary Assay Design Center (https://www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp) (**Table 2**). The reaction mixture contained 2 μL of diluted cDNA (1:5), forward and reverse primers (0.4 μM each), probe (0.1 μM), and 10 μL of LightCycler 480 probes master in a final volume of 20 μL. The cycling conditions of PCR were as follows: preincubation at 95 °C for 10 min, followed by 50 amplification cycles (95 °C, 10 s, and 55 °C, 45 s), and cooling (40 °C, 10 s). Real-time monitoring was achieved by measuring the fluorescence at the end of the elongation phase.

Table 2. Primers and Probes for Quantitative Real-Time PCR[a]

gene	primers	probes	Genbank accession no.
Muc2	5′-TGAGGTAGACAGAGCGACCA 5′-GGAGTCCAAGCAGGGAGAG	5′-GCCTGAAG	U68172
Muc3	5′-CTTGAGGAGGTGTGCAAGAAA 5′-CCCCAGGGTGACATACTTTG	5′-GGAGAGGA	U76551
Muc4	5′-GCTTGGACATTTGGTGATCC 5′-GCCCGTTGAAGGTGTATTTG	5′-CATCACCA	AH003319
β-actin	5′-CCCGCGAGTACAACCTTCT 5′-CGTCATCCATGGCGAACT	5′-CAGCTCCT	NM_031144

[a] Muc, mucin.

Statistical Analysis. Data are given as mean ± SEM and were analyzed using ANOVA (GLM procedures of SAS, SAS Institute, Cary, NC). Means were compared using Duncan's multiple range test.

RESULTS AND DISCUSSION

The effect of dietary protein source on growth performance and gut weight is shown in **Table 3**. Rats fed the SAA and HC diets grew faster and consumed more food than rats receiving the PF diet ($p \leq 0.0001$). Small intestine and colon weights were not influenced by dietary treatment.

Ileal EN losses were determined for rats fed the PF and SAA diets, assuming a virtual complete absorption of synthetic AA,

Table 3. Mean ($n = 12$) Weight Gain, Food Intake, Gain:Feed Ratio, and Small Intestine and Colon Weights for Rats at the End of the Experimental Period[a]

	diet		
	PF	SAA	HC
ADG (g/day)	−0.91 ± 0.20 b	2.93 ± 0.43 a	3.73 ± 0.30 a
ADFI (g/day)	10.44 ± 0.52 b	14.74 ±0.26 a	15.56 ± 0.49 a
gain:feed	−0.53 ± 0.37 b	1.16 ± 0.46 a	1.43 ±0.27 a
small intestine weight (g/100 g BW)	2.13 ± 0.13	2.24 ± 0.09	2.42 ± 0.04
colon weight (g/100 g BW)	1.21 ± 0.06	1.31 ± 0.06	1.34 ± 0.05

[a] Values are means ± SEM. Means in a row without a common letter differ, $p \leq 0.0001$. ADG, average daily gain; ADFI, average daily food intake; and BW, body weight.

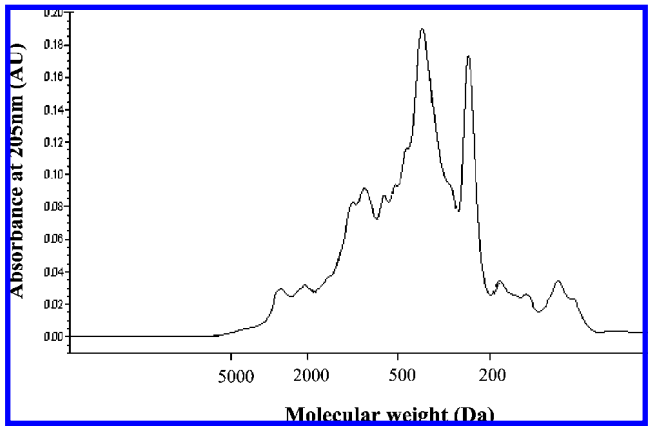

Figure 1. HPLC gel elution profile for the freeze-dried HC. The effluents were eluted using 36% acetonitrile containing 0.1% TFA and monitored at 205 nm.

and for rats fed the HC diet after centrifugation and ultrafiltration (MW > 10 kDa) of digesta. Ileal EN loss was significantly higher ($p \leq 0.001$) for rats fed the HC diet [1127 ± 24 μg/g dry matter intake (DMI)] as compared with that for rats fed diets PF and SAA (578 ± 56 and 613 ± 29 μg/g DMI, respectively). The latter finding is in accord with earlier studies (*12*) and a recent tightly controlled study with the growing rat (*22*). It appears that the higher ileal EN flow is a response to the HC peptides rather than to a higher food intake or body N retention, as food intake and growth rate were the same for rats for the HC and SAA diet, but EN flow was much higher for the rats fed HC.

The HPLC gel filtration profile of HC indicated that about 12% of the peptides were between 1 and 5 kDa in size and about 88% were less than 1 kDa (**Figure 1**). Pancreatin, used to prepare the hydrolysate, is produced by the exocrine cells of the pancreas and is a mixture of several digestive enzymes and contains trypsin, which hydrolyzes proteins to oligopeptides (*23*). The HC, therefore, would have contained numerous small peptides. Caseins and whey proteins, moreover, are known to be a source of biologically active peptides such as opioid agonists, antihypertensive peptides, and immunostimulants (*24, 25*). In particular, milk protein fragments have been shown to behave like opioid receptor ligands (ORL) (*24*). ORL originating from milk casein are relatively small peptides such as the α-casein exorphins or casoxin D (α-casein), β-casomorphins or β-casorphin (β-casein), and casoxins (κ-casein) (*11, 24*). Because bioactive peptides like β-casomorphin-7 derived from bovine milk protein are found in the intestinal lumen of adult humans during milk digestion (*26*), the biological activity of milk protein

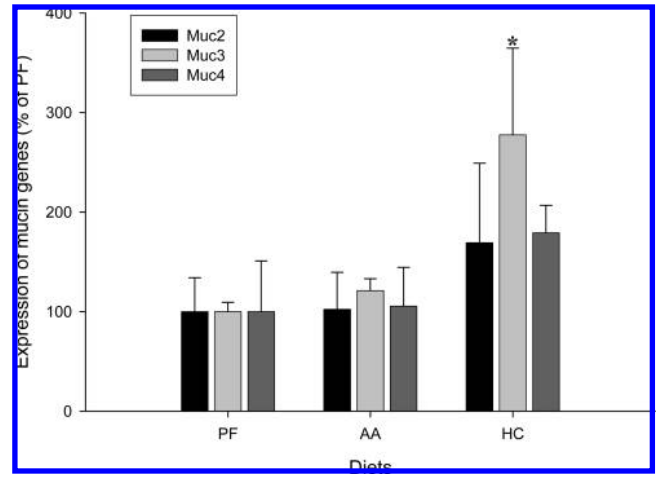

Figure 2. Effect of diet protein source on the mRNA levels of three mucin genes (Muc2, Muc3, and Muc4) in the rat small intestine. The mRNA expression of mucin genes was normalized to the β-actin mRNA level in each sample. Real-time PCR was performed with four rats in each group. The results are expressed as percentage increases as compared with the PF diet. Values are means ± SEM; *$p \leq 0.05$ vs PF diet.

hydrolysates merits investigation. The objective of the present study therefore was to investigate the influence of a casein hydrolysate on mucin gene expression.

So far, there have been only a few reports regarding the effect of casein hydrolysates or bioactive milk peptides on mucin gene expression, and importantly, these studies have been conducted in vitro with cell culture systems or a vascularly perfused rat intestine (*6, 8, 10*). We initiated this study to evaluate the effect in vivo of HC on the mRNA expression of three mucin genes (Muc2, Muc3, and Muc4) in the intestinal tract of the rat using quantitative real-time PCR. As shown in **Table 2**, we designed novel primers and probes for the rat Muc2, Muc3, and Muc4 gene based on the mucin gene sequences recorded in Genbank (NCBI), which were found to be appropriate for quantitative real-time PCR.

Muc3 mRNA expression in the rat small intestine significantly increased for the HC diet as compared with the PF or SAA diets (277 and 229% increase, respectively; $p < 0.05$) (**Figure 2**). Muc2 and Muc4 mRNA expressions also tended to increase with the HC diet, but differences were not statistically significantly different. In the colon, Muc4 mRNA expression was significantly increased for rats fed the HC diet (325 and 265% increase over the PF and SAA diets, $p \leq 0.05$), but expression of the Muc2 and Muc3 mRNA was not affected by diet (**Figure 3**). The higher EN loss found in the present study may have resulted from a higher secretion of mucins into the intestine with diet HC, as suggested by the present results for mRNA expression. It has been previously reported that mucins are an important component of gut endogenous protein losses (*27*).

Our findings are consistent with those of Claustre et al. (*8*) showing that peptides from a casein hydrolysate markedly stimulate the expression of mucin genes in the rat jejunum. Claustre et al. (*8*) measured mucin discharge in the isolated vascularly perfused rat jejunum using enzyme-linked immunosorbent assay and reported that casein hydrolysate-induced mucin secretion is triggered by a neutral pathway and mediated through opioid receptor activation. Trompette et al. (*10*) also showed that luminal administration of β-casomorphin-7, a fragment of β-casein, reproduced mucin release in rat jejunum. Zoghbi et al. (*6*) reported that β-casomorphin-7 increased the expression of Muc2 and Muc3, but not Muc1, Muc4, and

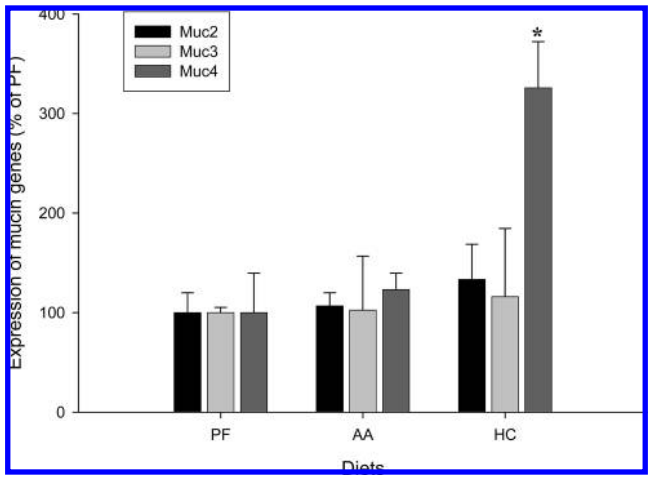

Figure 3. Effect of diet protein source on the mRNA levels of three mucin genes (Muc2, Muc3, and Muc4) in the rat colon. The mRNA expression of mucin genes was normalized to the β-actin mRNA level in each sample. Real-time PCR was performed with four rats in each group. The results are expressed as percentage increases as compared with the PF diet. Values are means ± SEM; $^*p \leq 0.05$ vs PF diet.

Muc5AC, via a direct effect on intestinal goblet cells and the activation of μ-opioid receptors in rat DHE cells.

In humans, at least 17 mucin genes have been described. Among them, 13 mucin genes are expressed in the gastrointestinal tract, coding for gel-forming mucins (Muc2, 5AC, 5B, and 6) expressed at the same chromosome locus, and membrane-bound mucins (Muc1, 3A, 3B, 4, 11, 12, 13, 15, and 17) (*13, 28*).

In contrast, rat mucins and their encoding genes have not been studied as extensively, but so far, at least five distinct mucin genes (Muc1, Muc2, Muc3, Muc4, and Muc5AC) have been identified (*29, 30*). Although the major mucin in the rat intestine is Muc2 produced from goblet cells, in this study, Muc2 mRNA expression was not influenced by HC. However, the expression of Muc3, which is also a predominant intestinal mucin and colonic Muc4, normally expressed in the rat intestine, were significantly enhanced in response to the HC diet (*16, 30*).

In conclusion, HC affects mucin expression in the rat intestine in vivo. Further experiments are required to investigate which peptides from the HC are implicated in mucin expression, whether such peptides are present in other protein hydrolysates, and to describe the underlying mechanisms.

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