

CLINICAL–ALIMENTARY TRACT

Enhancement of Dietary Protein Digestion by Conjugated Bile Acids

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See Corazza GR et al on page 838 and Wahnschaffe U et al on page 844 in the July 2007 issue of *CGH*.

Background & Aims: Conjugated bile acids promote absorption of dietary lipids by solubilizing them in mixed micelles. Bile acids are not considered to facilitate the digestion of other nutrients. **Methods:** The effect of conjugated bile acids on the rate of protein hydrolysis by trypsin and chymotrypsin was examined in vitro. Common dietary proteins and 2 bacterial glutenases (proposed oral therapies for celiac sprue) were proteolyzed in the absence or presence of a 10 mmol/L conjugated bile acid mixture, simulating human bile composition. Lipolysis products (monoolein) and fatty acid were also evaluated to simulate postprandial intestinal contents. **Results:** Conjugated bile acids dramatically enhanced the proteolysis of several dietary proteins, including β -lactoglobulin, bovine serum albumin, myoglobin, and a commercially available dietary protein supplement. For β -lactoglobulin, a cow's milk allergen that is resistant to pepsin cleavage, bile acids enhanced its proteolysis by pancreatic proteases even after incubation under gastric conditions. Exposure of prolyl endopeptidases to bile acids made them more susceptible to pancreatic proteases under simulated intestinal conditions. The conjugated bile acid effect was most pronounced in the presence of dihydroxy bile acids and was observable at bile concentrations below the critical micellar concentration but to a much greater extent at concentrations above the critical micellar concentration. **Conclusions:** We propose that, in addition to promoting lipid absorption, conjugated bile acids affect the digestion and assimilation of dietary proteins by accelerating hydrolysis by pancreatic proteases. These findings have implications for intraluminal protein breakdown and assimilation in the upper small intestine.

A typical Western diet contains approximately 100 g protein/day.¹ Protein digestion begins in the stomach, where pepsin hydrolyzes proteins into oligopeptides of widely varying sizes. Fluctuations in gastric emptying

times, gastric pH, pepsin activity levels, and the extent of emulsification result in variable and often incomplete hydrolysis of protein as it exits the stomach.^{2–5} As a result, the primary site for protein digestion in humans is the small intestine.

When gastric content enters the small intestine, the pH rises rapidly to near neutrality due to pancreatic and duodenal bicarbonate secretion. At the same time, under the influence of cholecystokinin, bile and pancreatic enzymes are secreted into duodenal content. There are 5 recognized pancreatic proteolytic enzymes (trypsin, chymotrypsin, elastase, carboxypeptidase A, carboxypeptidase B), and these have broad substrate specificity. The pancreatic enzymes digest incoming proteins and polypeptides into short peptides (typically 2–6 residues in length), which are further processed by brush border oligopeptidases and ultimately absorbed as amino acids, dipeptides, and tripeptides through the intestinal epithelium.^{6,7} A few dietary proteins, such as gluteins, are relatively resistant to small intestinal proteolysis. Gluten proteins have uniquely high proline (~15%) and glutamine (~30%) contents, 2 residues that are not preferred by endogenous endoproteases. As a result, many high-molecular-weight oligopeptides (10 to >30 amino acid residues) persist in the small intestinal lumen, some of which are capable of triggering the inflammatory process associated with celiac sprue.⁸ Additionally, several allergenic proteins are also known to be resistant to luminal pancreatic protease digestion. For example, soybean trypsin inhibitor (STI) is stable under simulated intestinal conditions for 2 hours.⁹

In this study, we investigated a possible role for conjugated bile acids in protein digestion. Conjugated bile acids are amphipathic molecules whose identified physiologic role is to solubilize the lipolysis products of dietary triglyceride as well as fat-soluble vitamins.¹⁰ Bile acids also have antimicrobial effects.¹¹ An older German study reported a modest enhancement of fibrin digestion

Abbreviations used in this paper: BLG, β -lactoglobulin; BSA, bovine serum albumin; C, chymotrypsin; FM, *Flavobacterium meningosepticum*; MX, *Myxococcus xanthus*; Na-GCDC, sodium glycochenodeoxycholate; Na-TCDC, sodium taurochenodeoxycholate; PEP, prolyl endopeptidase; SDS-PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; STI, soybean trypsin inhibitor; T, trypsin.

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in the presence of bile acids,¹² although, to our knowledge, no follow-up studies have been reported in the past 70 years. In the course of our studies on the role of bacterial prolyl endopeptidases (PEPs) as potential therapeutic agents for celiac sprue, we observed marked differences between enzyme stability in standard simulated intestinal fluids (devoid of conjugated bile acids) and actual intestinal fluids obtained from experimental animals. These differences could not be reconciled even after carefully adjusting the pancreatic protease and brush border membrane composition of simulated intestinal fluids to more closely reflect conditions in vivo. We therefore evaluated the effect of conjugated bile acids on protein digestion. Our findings have potential implications for understanding the mechanisms of dietary protein assimilation in human nutrition and diseases of the upper gastrointestinal tract.

Materials and Methods

Materials

All bile acids were from Sigma (St Louis, MO) except for glycochenodeoxycholate (Calbiochem, a division of Merck KGaA, Darmstadt, Germany). All dietary proteins except for gluten (Bob's Red Mill, Milwaukie, OR), β -lactoglobulin (BLG; MP Biomedicals, Inc, Solon, OH), and Premium Pro-Rated dietary protein supplement (chocolate flavored; Wellements, Phoenix, AZ) were from Sigma. Pancreatic enzymes were from Sigma (Trypsin from Bovine Pancreas, T4665; α -Chymotrypsin Type II from Bovine Pancreas, C4129), while pepsin was obtained from American Laboratories (Omaha, NE). Additional materials included cholestyramine resin (Sigma), monoolein (TCI America, Portland, OR), and sodium oleate (JT Baker, Phillipsburg, NJ). Substrates for all chromogenic enzyme assays, except for PEP, were from Sigma; the PEP substrate (Z-Gly-Pro-p-Nitroanilide) was from Bachem (Torrance, CA). Recombinant PEPs from *Flavobacterium meningosepticum* (FM) and *Myxococcus xanthus* (MX) and recombinant cysteine endoprotease EP-B2 from barley were purified in-house based on existing protocols.^{13–15} All other materials were food or reagent grade.

Enzyme Activity Assays

All enzyme activity assays (PEP, trypsin, chymotrypsin, and EP-B2) were performed as described previously.^{15,16}

Sodium Dodecyl Sulfate/Polyacrylamide Gel Electrophoresis Analysis of Dietary Proteins

The sensitivity of dietary proteins to conjugated bile acid-induced proteolysis was evaluated with respect to the following conditions: (1) physiologic bile acid mixture alone, (2) mixed micelles (conjugated bile acid/oleic acid/monoolein mixed micelles), and (3) pretreatment under gastric conditions, with and without pepsin. The impact of the physiologic human bile acid mixture

and bile acid/oleic acid/monoolein mixed micelles was measured on BLG, bovine serum albumin (BSA), STI, chicken ovalbumin, myoglobin, and a commercially available dietary protein supplement. The impact of the gastric and acidic pretreatments was performed on select dietary proteins (BLG, BSA, myoglobin, and the dietary protein supplement).

Physiologic Conjugated Bile Acid Mixture

Solutions of dietary protein (4 mg/mL) and physiologic bile acid mixture (40 mmol/L total bile acid) were prepared in 20 mmol/L sodium phosphate, pH 7, buffer. The 40 mmol/L physiologic bile acid mixture contained 12.8 mmol/L sodium glycocholate, 12.8 mmol/L sodium glycochenodeoxycholate (Na-GCDC), 6.4 mmol/L sodium glycodeoxycholate, 3.2 mmol/L sodium taurocholate, 3.2 mmol/L sodium taurochenodeoxycholate (Na-TCDC), and 1.6 mmol/L sodium taurodeoxycholate because these are representative of the major bile acid species in the human gut.^{17,18} Stock solutions of trypsin (10 mg/mL) and chymotrypsin (10 mg/mL) were prepared. The dietary protein solution (50 μ L), the physiologic human bile acid mix (25 μ L), additional 20 mmol/L sodium phosphate, pH 7, buffer (23 μ L), trypsin (1 μ L), and chymotrypsin (1 μ L) were added together to obtain the following final composition: ~2 mg/mL dietary protein, 10 mmol/L physiologic bile acid mixture, and 0.1 mg/mL trypsin and chymotrypsin. A conjugated bile acid-free control was performed by replacing the physiologic human bile acid mixture with 20 mmol/L sodium phosphate, pH 7, buffer. In addition, for BLG, protein stability was measured as a function of conjugated bile acid concentration in the 0–14 mmol/L range. To visualize the initial proteolytic process, trypsin and chymotrypsin levels were reduced in these experiments to a final concentration of 0.01 mg/mL.

Conjugated Bile Acid/Oleic Acid/Monoolein Mixed Micelles

These experiments used a concentrated bile acid/oleic acid/monoolein mixed micelle mixture in 50 mmol/L sodium phosphate, pH 6.5. Na-TCDC (14.4 mmol/L), sodium taurocholate (9.6 mmol/L), sodium oleate (24 mmol/L), and monoolein (12 mmol/L) were added to 50 mmol/L sodium phosphate, pH 6.5, buffer. The mixture was sonicated and heated until it became uniformly turbid. A 4 mg/mL dietary protein solution was prepared in 50 mmol/L sodium phosphate, pH 6.5. The dietary protein solution (48 μ L) was mixed with the concentrated bile/oleic acid/monoolein mixed micelle mixture (50 μ L), 10 mg/mL trypsin (1 μ L), and 10 mg/mL chymotrypsin (1 μ L). The final concentrations were 12 mmol/L bile acids, 12 mmol/L sodium oleate, 6 mmol/L monoolein, and 0.1 mg/mL trypsin and chymotrypsin. For these experiments, both a conjugated bile acid- and a fatty acid/monoglyceride-free control were

performed by replacing the concentrated bile/oleic acid/monoolein mixed micelle system with 50 mmol/L sodium phosphate, pH 6.5, and sodium taurodeoxycholate (14.4 mmol/L) and sodium taurocholate (9.6 mmol/L) in 50 mmol/L sodium phosphate, pH 6.5, respectively.

Gastric Pretreatment (With and Without Pepsin)

A stock solution of dietary protein (4 mg/mL) was prepared in 0.01N HCl (pH 2). Pepsin NF powder was added to a final concentration of 0.6 mg/mL, and the mixture was incubated at 37°C for 30 minutes. After this 30-minute incubation, the gastric-treated dietary protein solution (48 μ L) was mixed with the 40 mmol/L physiologic bile acid mixture (25 μ L), 500 mmol/L sodium phosphate, pH 7, buffer (25 μ L), 10 mg/mL trypsin (1 μ L), and 10 mg/mL chymotrypsin (1 μ L). The final concentrations were as follows: ~2 mg/mL dietary protein, 10 mmol/L physiologic bile acid mixture, and 0.1 mg/mL trypsin and chymotrypsin. A conjugated bile acid-free control was performed using 20 mmol/L sodium phosphate, pH 7, buffer. The effect of incubating the dietary proteins under acidic conditions (ie, no pepsin) was also assessed. This was performed in an identical fashion as above, except that no pepsin was used and the 4 mg/mL dietary protein solution was prepared in 0.001N HCl (pH 3).

For sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) experiments, the final mixtures at pH 6.5 or 7 were incubated at 37°C for approximately 15 minutes. Samples (20 μ L) were withdrawn at 1, 5, and 15 minutes. Each sample was quenched by adding 20 μ L Laemmli solution (composition 3% [wt/vol] SDS, 32.5 mmol/L Tris, pH 6.8, 50% [vol/vol] glycerol, 10% [vol/vol] 2-mercaptoethanol, and 10 mg/mL bromophenol blue) and heating the mixture at 95°C for >5 minutes. Samples were analyzed using NuPAGE 4/12% Bis-Tris gels (Invitrogen, Carlsbad, CA). For studies on the STI, the above protocols were modified slightly. No trypsin enzyme was used in the digestion and additional chymotrypsin (0.5 mg/mL final concentration) was used. For gastric stability evaluations, samples (10 μ L) were withdrawn at various time points throughout the 30-minute incubation. Each sample was neutralized with 10 μ L 0.1 mol/L NaHCO₃ before adding 20 μ L Laemmli solution.

High-Performance Liquid Chromatography Analysis of Gluten and Conjugated Bile Acids

In vitro gluten digestion assays developed previously were modified to evaluate the sensitivity of gluten to conjugated bile acid-induced pancreatic digestion.^{14,16} To simulate gastric digestion, gluten flour (45 mg) was added to 1.485 mL 0.01N HCl, pH 2, and incubated at 37°C for 20 minutes. Pepsin NF powder was added to obtain a 0.6 mg/mL final concentration. The mixture was incubated at 37°C for 30 minutes, and then a 250- μ L aliquot was mixed with 125 μ L 40 mmol/L physiologic bile acid mixture (in 20 mmol/L sodium phosphate, pH

7), 121 μ L 500 mmol/L sodium phosphate, pH 7, buffer, 1.9 μ L 50 mg/mL trypsin, and 1.9 μ L 50 mg/mL chymotrypsin to obtain 15 mg/mL gluten, 10 mmol/L physiologic bile acid mixture, and 0.375 mg/mL trypsin and chymotrypsin at pH 7. This material was incubated at 37°C for 30 minutes. Samples (200 μ L) were withdrawn at 10 and 30 minutes during both the gastric and intestinal phase of the digestion. The gastric samples were quenched by the addition of 60 μ L 0.1N NaOH, and the intestinal samples were quenched by adding 60 μ L of 2 mg/mL aprotinin. High-performance liquid chromatography analysis of the samples was performed as detailed previously.¹⁴

PEP Activity in the Presence of Conjugated Bile Acids

The stability of 2 bacterial PEP enzymes was evaluated in a solution containing 10 mmol/L physiologic conjugated bile acid mixture and 15 mg/mL pepsin-digested gluten. A stock solution of pepsin-digested gluten (30 mg/mL) was prepared by incubating gluten with 0.6 mg/mL pepsin for 60 minutes at 37°C at pH 2. After 60 minutes, sodium phosphate was added to adjust the pH to 7. Stock solutions of the physiologic conjugated bile acid mixture (40 mmol/L) and pancreatic enzymes (10 mg/mL trypsin or 10 mg/mL chymotrypsin) were also prepared in a buffer containing 42 mmol/L HCl, 50 mmol/L KCl, 50 mmol/L NaHCO₃, 140 mmol/L NaCl, 0.8 mmol/L MgCl₂, and 2 mmol/L CaCl₂. Before stability testing, all stock solutions were adjusted to pH 7 by addition of 1 mol/L HCl or 1 mol/L NaHCO₃. Stability testing was performed on a 100- μ L scale by mixing specified amounts of the stock solutions, equilibrating to 37°C for 5 minutes, adding the concentrated PEP stock solution (3–6 mg/mL), incubating at 37°C, and measuring residual activity. Samples were collected at 0, 2.5, 5, 10, 15, and 30 minutes. Analogous experiments were performed to evaluate PEP stability in the presence of individual conjugated bile acids without pancreatic enzymes or a gluten buffer.

Effect of Cholestyramine Resin on Conjugated Bile Acid-Induced Enzyme Inactivation

The impact of cholestyramine resin, a bile acid sequestrant, on bile-induced inactivation of PEP enzymes was evaluated. Na-GCDC (5 mmol/L concentration) was mixed with varying concentrations of cholestyramine resin (1 mg/mL or 10 mg/mL) in 20 mmol/L sodium phosphate, pH 7, buffer. The pH of this mixture was readjusted to 7 after the resin addition. This mixture was incubated at 37°C for approximately 15 minutes with manual agitation every 5 minutes to suspend the resin. PEP (5 μ L of 1–10 mg/mL stock) was then added to this mixture. The final mixture was incubated at 37°C with periodic agitation. PEP activity was measured as a function of incubation time.

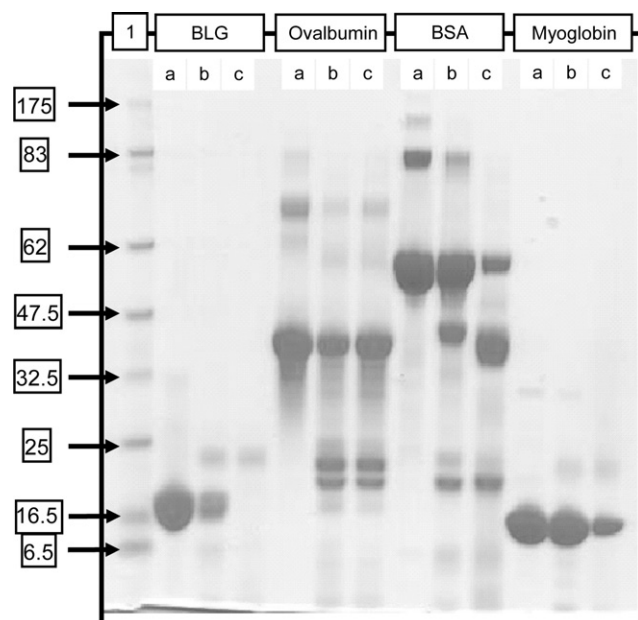


Figure 1. SDS-PAGE analysis of dietary proteins that were exposed to trypsin and chymotrypsin in the presence or absence of conjugated bile acids. Four dietary proteins (BLG, chicken ovalbumin, BSA, and myoglobin) are presented. For each protein, the following treatments were performed: (a) protein control in 20 mmol/L sodium phosphate, pH 7 (no incubation); (b) protein treated with 0.1 mg/mL trypsin and chymotrypsin at 37°C for 15 minutes; (c) protein treated with 0.1 mg/mL trypsin and chymotrypsin and 10 mmol/L physiologic bile acid mixture at 37°C for 15 minutes. All mixtures were heat deactivated and applied onto SDS-PAGE. Protein marker is presented in lane 1.

Effect of Bile Acids on Pancreatic Enzymes

Whether conjugated bile acids accelerate autolysis of trypsin and chymotrypsin was examined. This analysis was performed in an analogous fashion as with the PEP enzymes. Trypsin and chymotrypsin (1 mg/mL) were incubated with individual bile acids (Na-GCDC, sodium glycocholate, and sodium taurodeoxycholate) at 37°C at concentrations between 0 and 20 mmol/L in 20 mmol/L sodium phosphate buffer. Enzyme activity was measured as a function of incubation time and compared with a control lacking bile acids.

Results

Impact of Conjugated Bile Acids on Dietary Protein Hydrolysis

We investigated the effect of conjugated bile acids on the proteolysis of several dietary proteins, including BLG, BSA, STI, chicken ovalbumin, myoglobin, and gluten. The digestion of BLG, BSA, and myoglobin was enhanced in the presence of 10 mmol/L physiologic bile acid mixture (Figure 1). For these proteins, some digestion was observed over 15 minutes without bile, whereas proteolysis was appreciably accelerated in the presence of the conjugated bile acid mixture. In contrast, the digestion rates of chicken ovalbumin (Figure 1) and STI (data

Table 1. The Effect of Conjugated Bile Acids or Mixed Micelles of Conjugated Bile Acids With Lipolytic Products (Fatty Acids and Monoolein) on the Proteolysis of Selected Dietary Proteins by Trypsin and Chymotrypsin

Substrate	Effect of conjugated bile acids	Effect of mixed micelles
Dietary protein		
BLG	Increased	Smaller increase
Myoglobin	Increased	Further increase
BSA	Increased	No effect
Chicken ovalbumin	No effect	No effect
STI	No effect	No effect
Pepsin-treated gluten	No effect	Not tested
Dietary protein supplement	Increased	Not tested

NOTE. All results are relative to digestion of the particular dietary protein by trypsin and chymotrypsin in the absence of conjugated bile acids or mixed micelles.

not shown) remained unaffected in the presence of conjugated bile acids. The bile acids also had no effect on further proteolysis of pepsin-treated gluten as shown by high-performance liquid chromatography analysis (data not shown). Results are summarized in Table 1.

To simulate physiologic conditions during digestion, a mixed micelle system containing bile acids, monoglycerides (monoolein), and fatty acids (oleic acid) was prepared. In the presence of mixed micelles, trypsin and chymotrypsin accelerated the breakdown of BLG and myoglobin relative to bile acid-free controls (see Figure 2

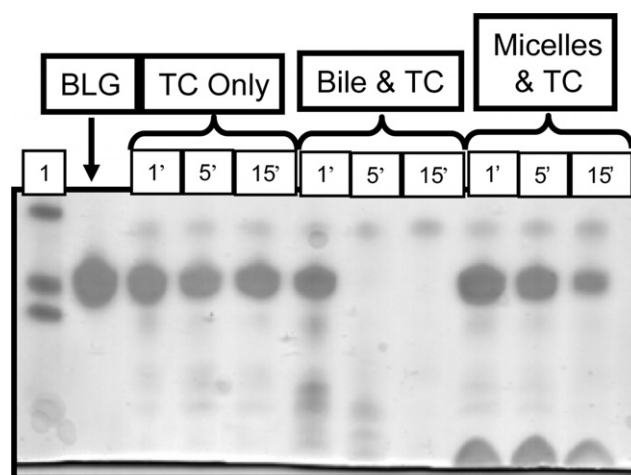


Figure 2. SDS-PAGE analysis of BLG that was exposed to trypsin and chymotrypsin in the presence or absence of conjugated bile acids or mixed micelles containing conjugated bile acids, monoolein, and oleic acid. The stability of BLG under 3 conditions is presented: lanes 3–5, BLG with 0.1 mg/mL trypsin and chymotrypsin, and no bile acids; lanes 6–8, BLG with 0.1 mg/mL trypsin and chymotrypsin, and 12 mmol/L conjugated bile acid mixture; lanes 9–11, BLG with 0.1 mg/mL trypsin and chymotrypsin, and mixed micelles. All mixtures were incubated at 37°C for specified times, followed by heat deactivation and application onto SDS-PAGE. Protein marker is presented in lane 1, and BLG in 50 mmol/L sodium phosphate buffer is presented in lane 2.

for BLG). The enhancement effect of the mixed micelles on BLG was not as dramatic as that of conjugated bile acids alone (Figure 2). In contrast, the proteolysis of myoglobin was accelerated further in the presence of mixed micelles as compared with bile acids alone (data not shown). For BSA, STI, and chicken ovalbumin, no difference was observed between the mixed micelle and bile acid-free control conditions (data not shown).

The effect of preincubating dietary proteins under gastric conditions was evaluated for the 3 proteins whose digestion under simulated duodenal conditions was accelerated by the presence of conjugated bile acids. For each protein, the impact of acidic conditions and pepsin was evaluated separately. Protein substrates (4 mg/mL) were incubated at low pH (2–3) with or without pepsin (0.6 mg/mL) for 30 minutes. Samples were then neutralized, and the effect of conjugated bile acids on further hydrolysis by pancreatic proteases was assessed. For all 3 proteins, acidic pretreatment alone did not influence the sensitivity of the proteins to bile acid-induced proteolysis by pancreatic proteases (data not shown). For BLG, there was also no impact of pepsin on bile-induced destabilization (Figure 3), whereas both myoglobin and BSA were extensively digested by pepsin (data not shown).

The effect of conjugated bile acid concentration was also evaluated. BLG (2 mg/mL) was incubated with 0–14 mmol/L of physiologic bile acid mixture for 15 minutes at 37°C, pH 7, in the presence of 0.01 mg/mL trypsin and chymotrypsin. As little as 2 mmol/L bile accelerated protein digestion (Figure 4), although the effect was more

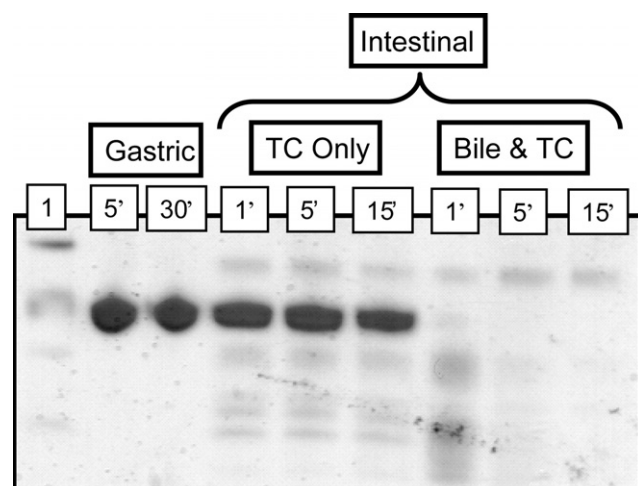


Figure 3. SDS-PAGE analysis of acid- and pepsin-pretreated BLG that has been exposed to trypsin and chymotrypsin in the presence or absence of conjugated bile acids. Lane assignments are as follows: lane 1, protein marker; lanes 2 and 3, BLG with 0.6 mg/mL pepsin, pH 2; lanes 4–6, acid- and pepsin-pretreated BLG with 0.1 mg/mL trypsin and chymotrypsin; lanes 7–9, acid- and pepsin-pretreated BLG with 0.1 mg/mL trypsin and chymotrypsin, and 10 mmol/L physiologic conjugated bile acid mixture. All mixtures were incubated at 37°C for the specified times, followed by heat deactivation and application onto SDS-PAGE.

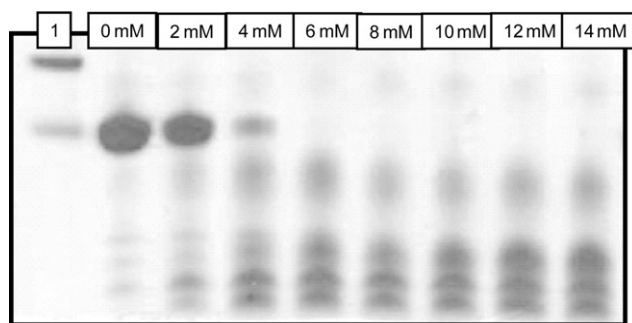


Figure 4. Effect of conjugated bile acid concentration on proteolysis of BLG. BLG was incubated with 0.01 mg/mL trypsin and chymotrypsin and increasing levels of physiologic bile acid mixture (0–14 mmol/L). All mixtures were incubated at 37°C for 15 minutes before heat deactivation and application onto SDS-PAGE. Protein marker is presented in lane 1.

pronounced above the critical micelle concentration of the physiologic bile acid mixture (3.5 mmol/L).¹⁹

Impact of Conjugated Bile Acids on the Digestion of a Complex Dietary Protein Supplement

All studies detailed previously were performed on dietary proteins purified from the original food source. To verify that our observations were relevant to complex dietary protein mixtures, a commercially available dietary protein supplement (Premium Pro-Rated, chocolate; 75%–80% protein content; ingredients include whey proteins, casein, and egg albumin) was studied. SDS-PAGE analysis showed that digestion of this protein supplement was significantly enhanced by bile. As seen in Figure 5, the protein supplement contains 2 major protein bands at <10 and 18 kilodaltons, both of which rapidly disappear in the presence of 10 mmol/L physiologic bile acid and 0.1 mg/mL trypsin and chymotrypsin. Preexposure of the protein supplement to simulated gastric conditions (pH 2, 0.6 mg/mL pepsin) did not alter the observed bile effect on the 18-kilodalton protein. In contrast, the <10-kilodalton protein was extensively hydrolyzed under gastric conditions (Figure 5).

Effect of Conjugated Bile Acids on Pancreatic Enzymes

In view of the above results, we wished to investigate whether conjugated bile acids could also accelerate the autolysis of pancreatic proteases such as trypsin and chymotrypsin. The activity of both proteases was measured in the presence of high concentrations of individual bile acids. Bile acids were found to have negligible effect on the activity profile of either enzyme (results not shown).

Effect of Conjugated Bile Acids on PEP Stability

PEPs are under consideration as potential oral therapeutic agents for in vivo gluten detoxification in

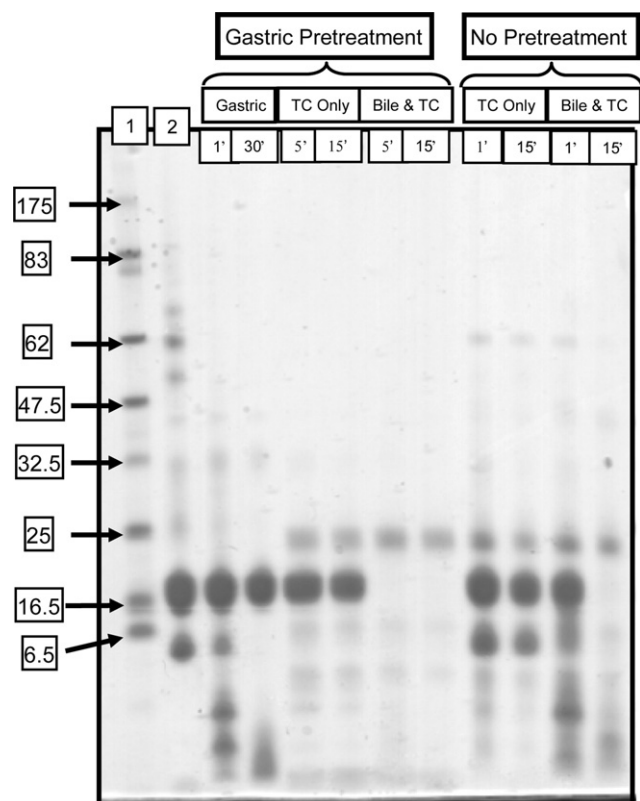


Figure 5. Trypsin and chymotrypsin catalyzed proteolysis of a complex dietary protein supplement with and without conjugated bile acids. Both gastrically treated protein supplement (lanes 3–8) and untreated protein supplement (lanes 9–12) are presented. For the gastrically treated protein supplement, the protein supplement was initially digested with 0.6 mg/mL pepsin, pH 2, for 30 minutes (lanes 3 and 4). No bile was added to these simulated gastric digests. The resulting mixture was then digested with 0.1 mg/mL trypsin and chymotrypsin without (lanes 5 and 6) and with (lanes 7 and 8) 10 mmol/L physiologic bile acid mixture. Untreated protein supplement was also digested in the absence (lanes 9 and 10) or presence (lanes 11 and 12) of 10 mmol/L physiologic bile acid mixture and 0.1 mg/mL trypsin and chymotrypsin. All mixtures were incubated at 37°C for specified times, followed by heat deactivation and application onto SDS-PAGE. Protein marker is presented in lane 1, and the protein supplement in 20 mmol/L sodium phosphate buffer is presented in lane 2.

patients with celiac sprue. Given the variable residence time of food in the stomach, the ability of a PEP to thoroughly detoxify dietary gluten therefore depends on its continued activity after it has mixed with pancreatic proteases and bile in the duodenum. We evaluated the stability of 2 PEP enzymes (FM and MX) toward trypsin and chymotrypsin in the presence and absence of the physiologic conjugated bile acid mixture (10 mmol/L). To simulate actual duodenal conditions, pepsin-digested gluten (15 mg/mL) was added to these assay mixtures. The presence of conjugated bile acids influenced the enzymatic activity of the 2 enzymes differently. Whereas FM PEP activity was stable in the presence of conjugated bile acids alone, a combination of pancreatic enzymes and conjugated bile acids induced loss of enzymatic ac-

tivity (Figure 6A). In contrast, MX PEP activity was lost in the presence of conjugated bile acids alone (ie, when no trypsin or chymotrypsin was added) (Figure 6B). In the presence of a mixed micellar system containing oleic acid and monoolein, FM PEP displayed improved proteolytic stability, whereas MX PEP rapidly lost all activity (data not shown). In addition, SDS-PAGE analysis (analogous to Figure 4) showed a similar relationship between bile concentration and breakdown of the PEP enzymes in the presence of pancreatic enzymes (data not shown). Pancreatic protease-catalyzed deactivation of yet another oral enzyme therapy candidate (cysteine endoprotease EP-B2 from barley)¹⁵ was unaffected by conjugated bile acids (data not shown). Thus, the range of effects of bile on oral protease stability mirrors the range of effects observed on dietary proteins.

The sensitivity and quantitative accuracy of the assay for FM PEP enzyme activity provided a convenient method to examine structure activity relationships for individual conjugated bile acids in the acceleration of

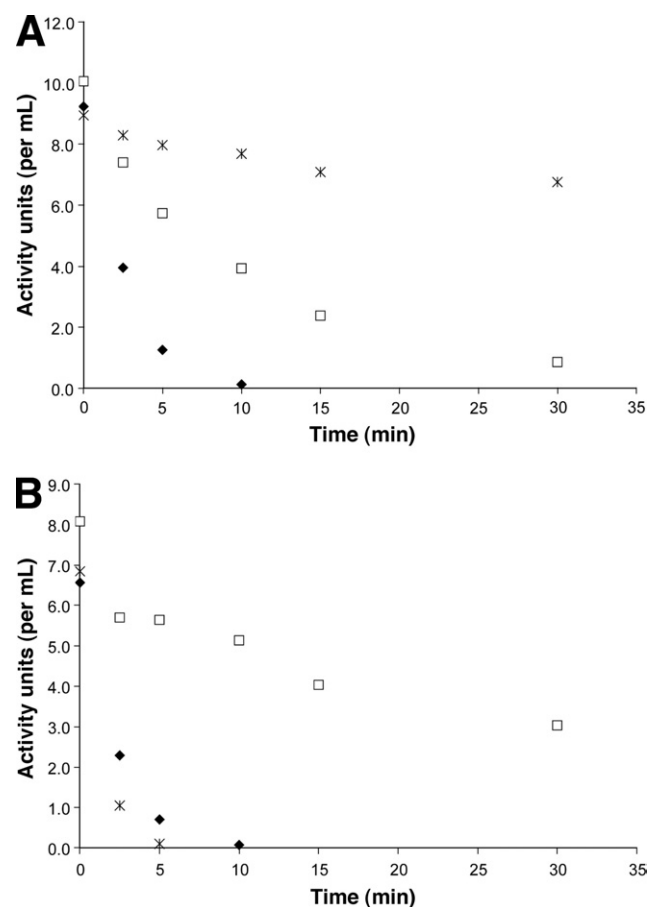


Figure 6. Effect of conjugated bile acids on proteolysis of (A) FM PEP and (B) MX PEP by trypsin and chymotrypsin. Each figure presents that stability of 3 experimental conditions: (1) 10 mmol/L physiologic conjugated bile acid mixture, no trypsin or chymotrypsin (*); (2) no bile, 1 mg/mL trypsin and chymotrypsin (□); and (3) 10 mmol/L physiologic bile acid mixture, 1 mg/mL trypsin, and chymotrypsin (◆). All experiments were performed in a 15 mg/mL gluten buffer at 37°C.

hydrolysis by pancreatic proteases. With Na-GCDC or sodium taurodeoxycholate, 2 dihydroxy conjugated bile acids, a sharp loss of activity was observed, whereas sodium glycocholate, a trihydroxy conjugated bile acid, had no impact on enzyme activity (data not shown). These results indicate that destabilization is more pronounced with the dihydroxy conjugated bile acid, at least for this substrate. The greater destabilizing effect of conjugated dihydroxy bile acids was also observed for glycooursodeoxycholate, another dihydroxy conjugated bile acid (data not shown).

Impact of Cholestyramine Resin on Bile-Induced Enzyme Inactivation

Because of the widespread use of bile acid sequestrants, we evaluated the effect of cholestyramine on conjugated bile acid-induced inactivation of PEP enzymes. Addition of as little as 1 mg/mL cholestyramine resin to 5 mmol/L Na-GCDC significantly attenuated the loss of PEP activity, whereas addition of 10 mg/mL cholestyramine completely protected FM PEP from bile-induced activity loss (Figure 7).

Discussion

Our data indicate that conjugated bile acids markedly accelerate the rate of trypsin- and chymotrypsin-catalyzed cleavage of some, but not all, of a spectrum of dietary proteins. Of the proteins studied, BLG, BSA, myoglobin, and a protein dietary supplement showed much more rapid hydrolysis by trypsin and chymotrypsin when in the presence of conjugated bile acids (Figures 1 and 5 and Table 1). In contrast, the hydrolysis of STI, chicken ovalbumin (Figure 1), and whole gluten was not influenced by the presence of bile acids (Table 1).

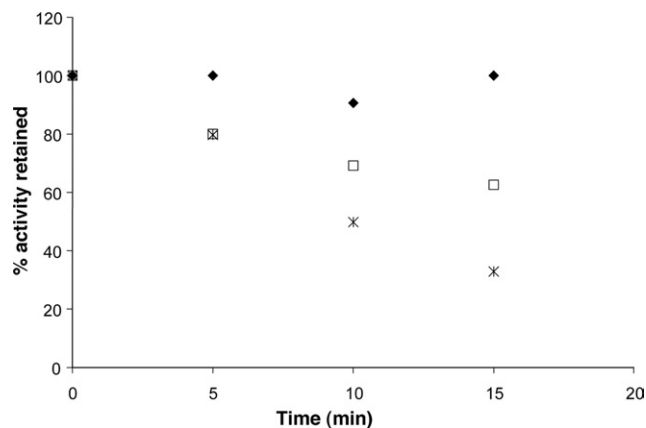


Figure 7. Impact of cholestyramine on FM PEP susceptibility to bile-induced inactivation. Three experimental conditions were evaluated: (1) 5 mmol/L Na-GCDC, no cholestyramine control (*); (2) 5 mmol/L Na-GCDC, 1 mg/mL cholestyramine (□); and (3) 5 mmol/L Na-GCDC, 10 mg/mL cholestyramine resin (◆). All experiments were performed in a 20 mmol/L sodium phosphate buffer, pH 7, at 37°C. Cholestyramine was added to the bile acid solution and incubated at 37°C for 15 minutes before addition of the PEP enzyme.

The mixture of conjugated bile acids was also evaluated with the addition of lipolysis products consisting of oleic acid and monoolein. The fatty acid/monoglyceride mixture simulates small intestinal content during triglyceride digestion. The impact of the mixed micelle mixture on the conjugated bile acid-enhanced proteolysis was protein dependent (Figure 2 and Table 1). Extensive acid and pepsin pretreatment of a subset of dietary proteins, most notably BLG and the pepsin-resistant ingredient of the dietary supplement, had no effect on their intrinsic hydrolytic resistance toward simulated duodenal conditions (Figures 3 and 5). Our study was limited to the effects of trypsin and chymotrypsin, and we have not investigated the effect of conjugated bile acids on peptide bond cleavage by elastase or carboxypeptidases.

Additional experiments explored what concentration of conjugated bile acids was required for promotion of proteolysis, as well as whether the structure of the steroid nucleus was important. We observed that BLG hydrolysis was moderately enhanced in the presence of submicellar (2 mmol/L) concentrations of conjugated bile acids with marked further enhancement at concentrations above the critical micelle concentration (3.5 mmol/L) (Figure 4). Similar results were also observed with a PEP (data not shown). These results suggest that conjugated bile acids exert their effects on protein digestion as individual molecules and also as micelles.

Different bile acids also showed remarkable variability in their effect on trypsin- and chymotrypsin-catalyzed breakdown of PEPs, for which accurate quantitative assays have been developed. PEPs are potential oral therapeutic agents for the treatment of celiac sprue. For maximal efficacy, they must be reasonably resistant to the duodenal environment. For these enzymes, conjugated dihydroxy bile acids were more potent destabilizers than trihydroxy acids, presumably because they are more surface active and have a greater hydrophobic surface area.²⁰ Together, these results suggest that certain bile acids accelerate protein digestion in the duodenum most likely by destabilizing the tertiary structures of dietary proteins, thereby making them more susceptible to attack by pancreatic endoproteases such as trypsin and chymotrypsin.

Bile acids appear to possess characteristics that enable them to facilitate the proteolysis of several digestion-resistant dietary proteins. For example, BLG is known to bind lipophilic molecules, including fatty acids and triglycerides, in a hydrophobic cavity.²¹ If certain bile acids bind to hydrophobic pocket(s) in the protein substrates, they may destabilize its structure, making additional interior domains of the dietary protein available for luminal endoprotease action. Competition for bile acid incorporation into the polar mixed micelle lowers the free bile acid concentrations; this would explain our observation that, in the presence of fatty acids and monoglycerides, the bile effect is attenuated (Figure 2).

To the extent our *in vitro* findings can be verified *in vivo*, one must consider the potential consequences of bile-enhanced protein digestion for human nutrition and disease. There are 4 implications of our findings. First, for *in vitro* studies concerned with proteolysis, the effect of conjugated bile acids with and without the addition of lipolysis products should be scrutinized. Current US Pharmacopeia recommendations are that dodecyl sulfate be present in simulated small intestinal content; it is not known whether the effects observed in our study will be mimicked by dodecyl sulfate. Dodecyl sulfate is a polar surfactant with a flexible alkane hydrophobic body. Bile acids are rigid, planar amphipaths with a hydrophobic side and a hydrophilic side. Second, bile acid sequestrants may influence protein digestion by removing bile acids from solution. Bile acid sequestrants are known to cause dyspepsia, and our findings raise the possibility that impaired protein digestion might contribute. Third, in light of our observations reported here, the stability of oral protein therapeutics in the presence of pancreatic proteases and conjugated bile acids must be considered during product development. Lastly, dietary proteins such as bovine BLG are well-known allergens, especially among children. Our results suggest that bile acids may reduce the intestinal persistence of intact BLG, possibly decreasing its antigenic activity.

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