

The bile salt content of human bile impacts on simulated intestinal proteolysis of β -lactoglobulin

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Abbreviations:

α La, α -lactalbumin; β Lg, β -lactoglobulin; BS, bile salt(s); ERCP, endoscopic retrograde cholangio-pancreatography; HB, human bile; NaGDC, sodium glycodeoxycholate; NaTC, sodium taurocholate; PC, phosphatidylcholine; SDS, sodium dodecyl sulfate; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; WPI, whey protein isolate.

Abstract

The gastrointestinal hydrolysis of food proteins has been portrayed in scientific literature to predominantly depend on the activity/specificity of proteolytic enzymes. Human bile has not been considered to facilitate proteolysis in the small intestine, but rather to assist in intestinal lipolysis. However, human bile can potentially influence proteins that are largely resistant to gastric digestion, and which are mainly hydrolysed after they have been transferred to the small intestine. We used purified and food-grade bovine milk β -lactoglobulin (β Lg) to assess the impact of bile salts (BS) on the *in vitro* gastrointestinal digestion of this protein. Quantitative analysis showed that the proteolysis rate increased significantly with increasing BS concentration. The effect was consistent regardless of whether individual BS or real human bile samples, varying in BS concentrations, were used. The total BS content of bile was more important than its BS composition in facilitating the proteolysis of β Lg. We also show that the impact of human bile observed during the digestion of purified β Lg and β Lg-rich whey protein isolate can be closely replicated by the use of individual BS mixed with phosphatidylcholine. This could validate simple BS/phosphatidylcholine mixtures as human-relevant substitutes of difficult-to-obtain human bile for *in vitro* proteolysis studies.

Keywords

Bile salts; Human bile; *In vitro* proteolysis; β -lactoglobulin; Whey proteins.

1. Introduction

Bile salts (BS) are physiological biosurfactants, most commonly known for their crucial role in facilitating intestinal digestion and transport of dietary lipids (Wilde & Chu, 2011). They are a major organic component of bile, which is actively secreted into the small intestine in response to food ingestion (Boyer, 2013). Because of their high surface activity, the BS aid in emulsifying ingested triglycerides to small lipid droplets and facilitate interfacial lipolysis, as well as solubilise lipolysis products (monoglycerides and fatty acids) into mixed micelles, which allows for their transport towards the intestinal epithelium (Maldonado-Valderrama, Wilde, Macierzanka, & Mackie, 2011). The negative charge imparted by adsorption of BS to the surface of partially-digested lipid droplets and other colloidal particles has also been shown to facilitate their transport through the intestinal mucus layer that coats the epithelial surface (Macierzanka et al., 2011).

Up until now, there has only been very limited data available in the scientific literature regarding the role of BS in protein digestion. An early report by Christensen (Christensen, 1951) briefly suggested the BS might play a physiological role in accelerating intestinal proteolysis. Much more recently, Robic and co-workers (Robic, Linscott, Aseem, Humphreys, & McCartha, 2011) found that the catalytic efficiencies of native pancreatic proteases, trypsin and chymotrypsin, was not affected by bile acids (cholic and glycocholic acids) in their study on *in vitro* digestibility of chromogenic substrates. This is despite the fact that BS are capable of altering structural conformation of proteases, as reported recently for trypsin (Najar et al., 2021). The effect depended on the hydrophobicity and concentration of BS (conjugated and unconjugated BS were tested). Gass and co-workers (Gass, Vora, Hofmann, Gray, & Khosla, 2007) showed that it is actually possible to enhance the *in vitro* trypsin-chymotrypsin proteolysis of several dietary proteins by increasing the total concentration of conjugated BS in a simulated intestinal digestion mix. However, it is not clear

whether a similar dose dependent effect of BS can be expected after exposing proteins to real human bile under small intestinal digestion conditions. This is especially important as the composition of bile can vary significantly between individuals, in both the bile acid profile and the total BS concentration (Rossi, Converse, & Hofmann, 1987). Additionally, the secretion of bile into the small intestine, and therefore the concentration of BS in the intestinal lumen, changes between fasted and fed states and is also dependent on age, health status, etc. For example, the luminal BS concentration in healthy adults varies from 0.57 mM to 6.0 mM in the fasting state, and can increase to 16 mM in the postprandial state, whereas in the full-term infants the postprandial concentration ranges only from 0.4 mM to 1.5 mM (Bourlieu et al., 2014). Moreover, infant bile is dominated by tauro-conjugated BS, whereas glyco-conjugated BS usually dominate in adult bile. The bile composition as well as its transport rate through the bile duct to the small intestine can also be significantly affected by numerous ill-health conditions, including cholestasis, choledocholithiasis, malignant biliary obstructions, etc. (Li & Apte, 2015), (Costi, Gnocchi, Di Mario, & Sarli, 2014).

Since the concentration and composition of the BS available to assist in the intestinal digestion processes can depend on a number of physiological and pathological mechanisms, it is important to evaluate if and how variations in the BS concentration and composition of human bile can impact digestion. In this study, we collected and analysed bile samples from a number of adults. Selected samples were then used in experiments simulating human intestinal and/or gastrointestinal proteolysis of bovine milk β -lactoglobulin (β Lg). We choose β Lg as it is a major globular protein in the milk of ruminant species (Kontopidis, Holt, & Sawyer, 2004), and examined the digestibility of purified, intact β Lg as well as β Lg in a food-grade whey protein isolate. Apart from the high nutritional value, β Lg is widely used in food industry because of its excellent emulsifying and foaming properties as well as the ability to form gels upon heating (De Wit, 1998). It is also an example of a dietary protein that is largely resistant to gastric digestion by pepsin (Takagi, Teshima,

Okunuki, & Sawada, 2003). However, the level of resistance can be affected to some extent by food processing methods, such as emulsification or heat-induced gelation, which are used for formation of various β Lg-based food microstructures (Macierzanka, Sancho, Mills, Rigby, & Mackie, 2009), (Sarkar, Goh, Singh, & Singh, 2009), (Macierzanka et al., 2012). The resistance to pepsin has been suggested to be one of the factors contributing to the allergenicity of β Lg in humans (Wal, 2004). β Lg is also considered a so-called 'fast protein', meaning it does not coagulate under the low-pH conditions of the stomach and is rapidly transferred to the small intestine after consumption of a meal containing milk proteins (Boirie et al., 1997). This characteristic is important, for example, in sport nutrition, where the focus on fast intestinal proteolysis and amino acid absorption has led to a common use of the β Lg-rich whey protein fraction of milk. Because of all the above aspects, it is crucial to consider the impact that physiological surfactants, such as bile salts delivered with bile into the small intestine, may have on the intestinal proteolysis of β Lg.

Our work had two goals, the major one being a quantitative evaluation of the influence of BS concentration in real human bile samples on the extent of β Lg digestion *in vitro*. The second goal was to verify whether any effect the human bile might exert on the proteolysis could be reproduced by using simple mixtures of individual BS and other organic components that make up bile. We hypothesise that human bile can have an impact on the intestinal proteolysis of β Lg, and that this impact can be simulated, in terms of producing the same/similar extent of the protein breakdown, by using selected, individual components of bile. If proven, it would potentially validate the use of individual BS as human-relevant substitutes of difficult-to-obtain human bile for *in vitro* proteolysis studies.

2. Materials and methods

2.1. Human bile collection

The collection and study of human bile (HB) were approved by the Ethics Committee of the Regional Medical Chamber in Rzeszów, Poland (certificate 15/B/2016). All methods were planned and conducted in accordance with the ethical principles outlined in the Declaration of Helsinki. Samples of HB were collected at the Department of Gastroenterology and Hepatology (Teaching Hospital No 1, Rzeszów, Poland) during endoscopic retrograde cholangio-pancreatography (ERCP) examination of 76 patients, who had a medical indication for this therapeutic procedure (Supplementary Table S1). All patients were fasted for a minimum of 8h before the ERCP procedure. Informed consent was obtained from all participants to collect and use their bile samples in the study. This HB collection method was chosen in order to reduce the risk of contaminating bile with digestive enzymes present in the intestinal lumen and the pancreatic juice. ERCP is an endoscopic procedure which involves assessment and therapy of the bile ducts and/or pancreatic ducts (Kozarek, 2017). A duodenoscope is inserted into the second part of the duodenum and ampulla of Vater has to be identified (Fig. 1a). The ampulla of Vater, also known as the hepatopancreatic ampulla or the hepatopancreatic duct, is formed by the union of the pancreatic duct and the common bile duct. The ampulla is located at the major duodenal papilla. As a routine part of ERCP, the bile duct was cannulated with a sterile catheter, which was inserted selectively over the guide wire once its position had been confirmed under fluoroscopy (X-ray guidance, Fig. 1b). The position of the catheter was confirmed for all of the patients (n = 76). A syringe was attached to one end of the catheter. The assisting endoscopy nurse performed aspiration of the bile by applying a gentle suction with a syringe. The catheter was moved back and forward from the extrahepatic bile ducts to the intrahepatic bile ducts (Fig. 1c). Approximately 2-3 mL of bile was aspirated from one subject.

Immediately after aspiration, the samples were transferred into 0.5-mL plastic test tubes. The tubes were sealed and instantly immersed in liquid nitrogen for snap freezing. Samples were stored at -80 °C prior to further examination.

2.2. Human bile composition and bile salt profile

The concentration of total bile salts (BS) in HB was analysed using the bile acids (enzymatic cycling) test kit (Alpha laboratories, Hampshire, UK) as described in detail previously (Mackie, Rigby, Harvey, & Bajka, 2016). The concentration of phosphatidylcholine (PC) in HB was determined by HPLC (Shimadzu x1 HPLC 4FLC XR, Shimadzu, Japan) equipped with an evaporative light scattering detector (Shimadzu ELSD LT II) using a method modified from the procedure described by Persson et al. (Persson et al., 2007). Details of the method have been given in Supplementary Section S1.2. The total BS and PC concentrations of all HB samples have been shown in Fig. 1d. Results are presented as means \pm SD (n = 3).

The bile salt composition of selected HB samples was analysed using an Agilent 1260 HPLC system coupled to an AB Sciex 4000 QTrap triple quadrupole mass spectrometer (Sciex, Cheshire UK). HB (10 μ L) was diluted with 990 μ L of 0.9% NaCl. Next, 50 μ L of diluted HB was mixed with 50 μ L of methanol in a HPLC vial. The analysis was carried out according to the method described in detail previously (Mackie et al., 2016). The following BS reference standards were used: sodium chenodeoxycholate (C8621, Sigma-Aldrich, Dorset, UK), sodium glycodeoxycholate (G9910, Sigma-Aldrich), sodium glycochenodeoxycholate (G0759, Sigma-Aldrich), sodium glycocholate (G7132, Sigma-Aldrich), sodium taurodeoxycholate (T0875, Sigma-Aldrich), sodium taurochenodeoxycholate (T6260, Sigma-Aldrich), sodium cholate (C6445, Sigma-Aldrich) and sodium taurocholate (86339, Sigma-Aldrich). Representative chromatograms of the BS standards as well as of individual BS species in HB have been shown in Supplementary Fig. S2.1.

2.3. Selection of human bile samples for *in vitro* digestion

Prior to using HB samples in digestion experiments, they were examined for any residual proteolytic activity that might have appeared due to potential contamination with human pancreatic enzymes during the bile aspiration. For this purpose, bovine milk β -lactoglobulin was put through the simulated intestinal proteolysis procedure (according to section 2.4.1) carried out for 120 min under control conditions, i.e., with no proteolytic enzymes added but in the presence of HB. A HB sample was considered free of proteolytic enzymes if the concentration of the protein remained unchanged over the time course of experiment, meaning the protein was not digested. Samples collected during the experiment were analysed for β -lactoglobulin contents by SDS-PAGE and HPLC, using the methods described below (sections 2.5.1 and 2.5.2).

2.4. *In vitro* static digestion models

For the initial evaluation of the effect of individual BS concentration and the BS content of HB on the proteolysis progress, a simple intestinal model of *in vitro* proteolysis was used. The model was adapted from the method described previously (Gass et al., 2007). The *in vitro* intestinal proteolysis procedure was later replaced with a gastrointestinal proteolysis model in order to include both the gastric and the intestinal phases of proteolysis in *in vitro* simulation. The simulated gastrointestinal digestion procedure was used for evaluating the effect of individual BS and/or phosphatidylcholine (PC) as well as the BS and PC in HB. The gastrointestinal model was based on the procedures described previously (Mandalari, Adel-Patient, et al., 2009), (Moreno, Mackie, & Mills, 2005), (Böttger et al., 2019), with some modifications. All buffers and simulated digestive fluids were prepared with ultra-pure water (HLP5s system, Hydrolab, Poland).

2.4.1. Simulated intestinal proteolysis

Protein stock solutions (4 mg/mL) of either β -lactoglobulin (β Lg) purified from bovine milk (90%, L3908, Sigma-Aldrich, Germany), or food-grade whey protein isolate (WPI, 93% protein; BiPRO[®], Davisco Foods International Inc., Eden Prairie, MN) were prepared in 20 mM sodium phosphate buffer (pH 7.0) containing 0.01% (w/v) sodium azide. In the next step, 0.5-mL aliquots were transferred into 1.5-mL screw-cap Eppendorf tubes and one of the following surfactant stock solutions was added: (i) bile salt (BS) stock (50 mM or 100 mM), comprising equimolar quantities of two individual BS, sodium taurocholate (NaTC; $\geq 95\%$, T4009, Sigma-Aldrich, Germany) and sodium glycodeoxycholate (NaGDC; $\geq 97\%$, G9910, Sigma-Aldrich, Germany), dissolved together in the sodium phosphate buffer. These BS compounds were selected to mimic the properties of BS in human bile (Mandalari, Adel-Patient, et al., 2009), (Dupont et al., 2009), (Chu et al., 2009), (Krupa et al., 2020). The tubes were treated during the digestion as individual time-point samples (Brodkorb et al., 2019). The final concentration of individual BS in the digestion mixture (i.e., after addition of intestinal enzymes to the tubes, etc., as described below) was 0, 1, 2, 3, 3.64, 4, 6, 8 or 10 mM. Alternatively, the tubes were completed with 50 μ L of selected human bile samples (HB sample no. 46(B), 65(B) or 51 (A)), which produced 0.65 mM, 3.64 mM or 6.51 mM BS, respectively, and 0.18–0.49 mM PC concentrations in the final digestion mix.

The digestion was carried out with intestinal enzymes, trypsin from porcine pancreas (T0303, Sigma-Aldrich, Germany; activity of 13,800 U/mg of protein calculated using BAEE as substrate) and α -chymotrypsin from bovine pancreas (C7762, Sigma-Aldrich, Germany; activity of 40 U/mg of protein calculated using BTEE as substrate), at 37 °C and 170 rpm (Heratherm IMH60 incubator, Thermo Scientific, Waltham, MA). Prior to use, the enzymes were dissolved separately in a cold (4 °C) sodium phosphate buffer. The enzyme stocks (5 mg/mL each) were prepared directly before the proteolysis experiments to limit autolysis of the enzymes, and stored on ice until needed.



Just before adding the enzyme stock solutions, sample tubes were topped up to 980 μL with the sodium phosphate buffer and incubated for 10 min at 37 °C (170 rpm). A 10- μL aliquot of each enzyme stock was added to the digestion mixtures to obtain the final, total concentration 0.1 mg/mL of trypsin and chymotrypsin (1:1 w/w). The final volume of digestion mixture in each sample tube was 1 mL, and the final concentration of protein (βLg or WPI) at the start of digestion was 2 mg/mL. The intestinal proteolysis was carried out at pH 7.0 (37 °C, 170 rpm) for 0.25, 1, 2, 5, 10, 15, 30, 60 or 120 min. The proteolysis in individual time-point samples was stopped by mixing with PMFS (0.1 M stock in EtOH, 93482, Sigma-Aldrich, Germany) to the final 3 mM concentration of the inhibitor. Control experiment without enzymes, BS or HB were carried out by replacing these components of digestion mixture with the sodium phosphate buffer. All post-digestion samples were stored at -80 °C before further analysis. All *in vitro* intestinal digestion experiments of purified βLg with individual BS were done in triplicated ($n = 3$) for each conditions used (e.g., presence/absence of BS, change in BS concentration, etc.). For any other intestinal digestion experiments (i.e., βLg or WPI with HB samples, WPI with individual BS), $n = 5$ for each conditions used.

2.4.2. Simulated gastrointestinal proteolysis

Stock solutions (pH 3.0) of purified βLg or WPI (protein conc., 2 mg/mL) were prepared with a simulated gastric fluid (SGF; 150 mM NaCl, pH 3.0) containing 0.01% (w/v) sodium azide. After 10 min pre-incubation of protein solution (10 mL) at 37 °C (170 rpm), a stock of porcine pepsin (P6887, Sigma-Aldrich, Germany; activity: 3,300 U/mg of protein calculated using haemoglobin as substrate), prepared in SGF (20 mg/mL), was added to give the final concentration of 100 $\mu\text{g}/\text{mL}$ (pepsin:protein, 1:20, w/w). In order to limit the autolysis of pepsin, the enzyme stock solution was prepared just before the experiment using a cold (4 °C) SGF and stored on ice until needed. Protein samples were digested under gastric simulated conditions for 60 min (pH 3.0, 170 rpm, 37 °C).

Aliquots (0.5 mL) of digestion mixture were taken from the digestion vial to individual 1.5-mL Eppendorf tubes at different time points, between 0.25 min and 60 min of gastric digestion, and mixed with 100 μ L of 0.5 M ammonium bicarbonate (09830, Sigma-Aldrich) aqueous solution. This inactivated pepsin in the time-point samples by rapidly increasing the pH to approx. 7.5. After 60 min, the pepsinolysis in the digestion vial was terminated by increasing the pH to 7.5 with 0.1 M NaOH (kept for 10 min at 37 °C). In order to produce a control sample (i.e., undigested sample), a 0.5-mL aliquot of the original protein stock solution was mixed with 100 μ L of 0.5 M ammonium bicarbonate solution.

In the next step, 5 mL of the post-gastric digestion mixture was transferred to a new vial for intestinal digestion. The mixture was made up with 275 μ L of 0.5 M Bis-Tris HCL (B6032, Sigma-Aldrich, Germany) buffer prepared in simulated intestinal fluid (SIF; 150 mM NaCl, pH 7.0). For experiments with HB, 283 μ L of the HB sample no. 65(B) was added to the digestion vial to make the final (i.e., after addition of intestinal enzymes, etc., as described below) 20-fold dilution of the HB, which produced 3.64 mM BS and 0.18 mM PC concentrations in the final digestion mix. Alternatively, for experiments with individual BS with/without PC, the stock solution of individual BS (i.e., NaTC and NaGDC; prepared as in section 2.4.1, but with SIF) and/or the vesicular PC dispersion were added to obtain the same, final BS and/or PC concentrations as in the digestion experiments with HB, i.e., BS 3.64 mM and PC 0.18 mM. The mixture was topped up with SIF in order to replicate the volume brought about with the HB sample. If the digestion was carried out in the presence of PC, the vesicular dispersion of the lipid was prepared first, according to the method adapted from previous studies (Moreno et al., 2005), (Mandalari et al., 2008), (Macierzanka et al., 2009). Namely, egg yolk L- α -phosphatidylcholine (61755, Sigma-Aldrich, Germany) was dissolved in a chloroform/methanol mixture (1:1 v/v) to the PC concentration of 4.62 mg/mL. After transferring 5 mL of the mixture to a 50 mL round bottom flask, the solvents were evaporated using a rotary

evaporator in order to form a thin film of PC. Any residual solvent was removed at RT under vacuum after three purges with nitrogen. The lipid film was then suspended in 5 mL of SIF (170 rpm, 10 min at 37 °C), and finally the dispersion (PC conc. 4.62 mg/mL) was sonicated in an ice bath for 5 min using a sonication probe (80% intensity, 2 s impulses with 5 s breaks between them; Vibra-Cell Ultrasonic Liquid Processor, Sonics, CT). The size of PC vesicles was determined by a dynamic light scattering method using a Nano-ZS Zetasizer (Malvern Instruments Ltd., Malvern, UK) operating in size-measure mode. The mean particle size (Z-average) was 146.4 ± 2.4 nm ($n = 4$). The vesicular PC dispersion was added to the digestion mix in quantities required to obtain 0.18 mM PC concentration.

Subsequently, the pH of the digestion vial content was adjusted to 7.0, if required. After 10 min pre-incubation at 37 °C, the intestinal proteolysis was carried out at pH 7.0 for up to 120 min (37 °C, 170 rpm) using trypsin from porcine pancreas (T0303, Sigma-Aldrich, Germany) and α -chymotrypsin from bovine pancreas (C7762, Sigma-Aldrich, Germany). Stock solutions of both enzymes were prepared as in section 2.4.1, but with chilled SIF. The intestinal digestion was initiated by a simultaneous addition of 50 μ L of the trypsin stock and 50 μ L of the chymotrypsin stock, which produced a comparable ratio of the protein being digested to the intestinal enzymes as in the procedure described in section 2.4.1. Time-point samples (500 μ L) were withdrawn after 0.25, 1, 2, 5, 10, 15, 30, 60 and 120 min, and digestion in the samples stopped by mixing with 0.1 M PMSF inhibitor solution to its final concentration of 3 mM. Control experiment without enzymes, BS/PC or HB were carried out by replacing those components of digestion mixture with SGF or SIF. All post-digestion samples were stored at -80°C before further analysis. All *in vitro* gastrointestinal digestion experiments of purified β Lg and WPI were done five times ($n = 5$) for each conditions used.

Comparative gastrointestinal digestion experiments have also been done for bovine milk caseins from various sources: β -casein purified from bovine milk (98%, C6905, Sigma-Aldrich,



Germany), food-grade sodium caseinate (90% protein, DMV International, The Netherlands) as well as Micellar Casein (Reflex Nutrition, UK) – a food supplement containing 80% protein (casein/whey protein, 80/20 w/w), 4% carbohydrate and 1.5% fat. Protein stock solutions (protein conc., 10 mg/mL) were prepared individually for all three casein sources by gentle mixing of protein powders in 150 mM NaCl (pH 7.0) containing 0.01% (w/v) sodium azide. Finally, stock solutions were diluted with SIF to give the protein conc. of 2 mg/mL, and pH adjusted to 3.0, if required. All *in vitro* gastrointestinal digestions of caseins were carried out in the same way as described above in this section for the purified β Lg or WPI. They were done in triplicate (n = 3) for each conditions used.

2.5. Characterisation of digestion samples

2.5.1. Qualitative analysis of proteolysis progress

Progress of the proteolysis was initially evaluated qualitatively by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of the collected time-point samples (Böttger et al., 2019). A detailed procedure has been given in the Supplementary Section S1.3.

2.5.2. Quantitative analysis of proteolysis progress

A quantitative analysis of the proteolysis progress and extent was carried out by HPLC using a method described by Hernandez-Ledesma et al. (Hernández-Ledesma, Dávalos, Bartolomé, & Amigo, 2005) with some modifications. Analysis of the digestion time-point samples was performed with the Agilent 1100 Series LC (Agilent Technologies Inc., Santa Clara, CA) system consisting of a vacuum degassing unit, a binary pump, an autosampler injector, a column thermostat, and a diode array detector (DAD). For the chromatographic separation, Zorbax 300SB-C8 column (4.6 × 250 mm, 5 μ m; Agilent Technologies Inc., Santa Clara, CA) was used. The injection volume was 5 μ L. The

column was maintained at 30 °C. The binary gradient elution system consisted of solvents: A (water acidified with 0.1% v/v of trifluoroacetic acid) and B (0.1% v/v trifluoroacetic acid solution in acetonitrile). The samples were eluted at a flow rate of 0.8 mL/min. The gradient for solvent B was as follows: 5% (0 min), 20% (20 min), 60% (50 min), 60% (53 min). Detection was carried out at 214 nm and 280 nm. The concentration of β Lg (total of the variants A and B of β Lg) was determined in digestion time-point samples and normalised against the value recorded for the undigested control (Meyer, 1995), (Vincent, Elkins, Condina, Ezernieks, & Rochfort, 2016).

HPLC analysis was performed for samples obtained from either five ($n = 5$) or three ($n = 3$) individual digestion experiments carried out for every individual set of conditions. These have been specified in sections 2.4.1 and 2.4.2. Results are presented as the mean \pm SD. Statistical comparisons between two groups were made using a Student's t -test, and three (or more) groups were evaluated using 1-way ANOVA (significance level, $\alpha = 0.05$). The statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA).

2.6. Circular dichroism (CD) spectroscopy

Far-UV CD spectra were recorded using a Jasco J-710 Spectropolarimeter (Jasco, Tokyo, Japan) and a 0.1 mm path length quartz cell (Hellma UK Ltd, Essex, UK). β Lg (L3908, Sigma-Aldrich, Germany; final concentration of 2 mg/mL) was dissolved in 20 mM sodium phosphate buffer (pH 7.0), and analysed in the absence or presence of individual BS, i.e., NaTC and NaGDC (1:1, mol/mol; total 3.64 mM) and/or PC (0.18 mM and 0.5 mM). The BS stock solution and the PC dispersion were prepared as in sections 2.4.1 and 2.4.2, respectively, but with the 20 mM sodium phosphate buffer, and added in quantities required to obtain the above concentrations of BS and/or PC. The protein was incubated with the BS and/or PC for 5 min at 37°C (170 rpm) before analysis. CD spectra represent the average of four accumulations collected at 20 nm/min in the range between 185 and 260 nm,

with a 4 s time constant and a 0.5 nm resolution. They are presented as molar CD, after the buffer blank has been subtracted.

3. Results and discussion

3.1. Effect of bile salt concentration on *in vitro* intestinal proteolysis of β Lg

Before we looked at the influence of BS content in real human bile (HB) on the extent of β Lg digestion, we first quantitatively analysed the effect of the concentration of individual BS on the extent of protein hydrolysis. In this initial part of the study, we used a simple model of *in vitro* intestinal digestion with pancreatic enzymes, trypsin and chymotrypsin. A similar, but only qualitative, study was conducted before with a mixture of six tauro- and glyco-conjugated BS (Gass et al., 2007), and the SDS-PAGE results obtained by the researchers showed a gradual increase in protein digestibility upon increasing BS concentration. Our goal was to conduct a quantitative analysis of the proteolysis progress for purified native β Lg as well as β Lg in a food-grade WPI. Importantly, we only used a mixture of two individual BS (NaTC and NaGDC) to mimic the presence of HB in the digestion environment. The two BS were used before in numerous *in vitro* studies simulating the human small intestinal environment, as summarised recently (Macierzanka, Torcello-Gómez, Jungnickel, & Maldonado-Valderrama, 2019). However, these individual BS have never been shown to directly impact the intestinal digestion of milk proteins. Neither have they been validated against real HB.

There have been several studies looking at BS–protein molecular interactions in aqueous solutions. Far-UV circular dichroism (CD) spectroscopy revealed that an incubation of malate dehydrogenase and luciferase with cholate and/or deoxycholate caused a significant unfolding and aggregation of the proteins (Cremers, Knoefler, Vitvitsky, Banerjee, & Jakob, 2014). The observed



changes in the proteins' secondary structures were indicative of an increase in random coil formation, which occurred at the expense of α -helical fragments. BS have also been claimed to have the ability to denature glycoproteins and render them susceptible to enzymatic deglycosylation (Kimzey & Haxo, 2016). Martos and co-workers studied the interaction of egg white ovalbumin (OVA) with NaTC and NaGDC (Martos, Contreras, Molina, & López-Fandiño, 2010). Although, the BS did not appear to destabilise the molecular structure of OVA, the IgG binding to the protein was significantly decreased in the presence of BS. This led the authors to the conclusion that OVA could associate with the BS, which also increased the protein's exposure to pancreatic proteases. Since the proteolytic activity of trypsin and chymotrypsin is not affected by BS (Gass et al., 2007), the considerably enhanced digestibility of OVA in the presence of BS, observed by Martos et al. under simulated intestinal conditions (Martos et al., 2010), was likely to be the results of non-specific interactions of the protein with the BS. Gass and co-workers (Gass et al., 2007) found that conjugated BS can significantly enhance the digestibility of several globular dietary proteins, including myoglobin, bovine serum albumin and β Lg, under simulated duodenal conditions with trypsin and chymotrypsin. In the case of β Lg, the researchers suggested that some BS could bind into the hydrophobic, interior domains of the protein, which would destabilise the protein structure and make the domains susceptible to the action of the intestinal proteases. However, the effect of BS on the structure of β Lg was not confirmed in that study. The incubation of NaTC and NaGDC with bovine milk β Lg was studied by a far-UV CD and results presented in our previous report (Maldonado-Valderrama et al., 2011). The BS were found to interact with the protein and partially destabilise its secondary structure when used at 7.4 mM concentration. A similar experiment has been conducted in the present study (Supplementary Fig. S2.2.). The incubation of β Lg with an equimolar mixture of the same, individual BS (i.e., NaTC and NaGDC) caused a similar change in the secondary structure of the protein, despite the fact that the total BS concentration was more than

two times lower than in the previous work (Maldonado-Valderrama et al., 2011) and the BS/protein ratio reduced over four-fold. Regardless of its limited extent, the BS-mediated structural changes might be responsible for the significantly enhanced intestinal digestibility of β Lg observed in the present proteolysis study (Fig. 2). In the absence of NaTC and NaGDC, the relative amount of native β Lg that remained undigested after 5 min incubation with trypsin and chymotrypsin was reduced to 70.5 ± 3.7 % of its original amount before digestion (Fig. 2a). However, the extent of protein hydrolysis was twice as high when the proteolysis was repeated in the presence of 1 mM BS. Increasing the concentration to 2 mM resulted in only 16.9 ± 8.3 % β Lg left undigested after the 5 min exposure to intestinal proteases, and the HPLC analysis revealed that no intact β Lg could be observed in digestions carried out at higher concentrations of BS. The SDS-PAGE showed that at these elevated BS concentrations ($c \geq 3$ mM) the proteolysis progress was still BS-dependent, as evident from the gradually increasing intensity of the bands corresponding to peptides ($M_r \leq 6$ kDa) produced during the digestions at increasing concentrations of BS (Fig. 2a).

The extent of β Lg digestion in WPI was, in general, higher than for the purified β Lg. For example, there was 15.1 ± 5.8 % intact β Lg remaining after digestion of WPI at 1 mM BS (Fig. 2b), compared to 35.2 ± 17.4 % in the experiment on purified β Lg at the same BS concentration (Fig. 2a). However, a higher digestibility of β Lg in WPI was also observed for the control proteolysis experiments in the absence of BS: 42.7 ± 8.2 % (Fig. 2b) vs 70.5 ± 3.7 % remaining intact (Fig. 2a). The explanation for this increased susceptibility of β Lg to enzymatic hydrolysis may lie in the way food-grade WPI is produced. The conventional manufacturing of WPI involves moderate heat-treatment, which can cause partial denaturation of β Lg. Heat-treatment has been shown to facilitate gastrointestinal proteolysis of purified β Lg and β Lg in WPI (Kitabatake & Kinekawa, 1998). However, the heat-induced enhancement of protein digestibility depends very much on the conditions of heating and the resulting interplay between the extent of protein unfolding and aggregation

(Macierzanka et al., 2012). The type and degree of food processing is often a major determinant of protein digestibility (Asensio-Grau, Peinado, Heredia, & Andrés, 2019), (Asensio-Grau, Calvo-Lerma, Heredia, & Andrés, 2021). For example, a recent *in vitro* study of cheese digestion showed that the gastrointestinal degradation of protein fraction was only dependent on the characteristics of cheese matrix produced from cow, sheep and goat milks (Asensio-Grau, Peinado, et al., 2019). Moreover, the protein digestibility, determined in that study by measuring the soluble protein fraction at different times of digestion, was found not to be affected by intestinal BS concentration (1–10 mM). However, the authors did not present detailed results for digestibility of individual proteins in cheese samples, majority of which must have been caseins.

3.2. *In vitro* intestinal proteolysis of β Lg in the presence of human bile

After the effect of the concentration of individual BS on protein digestion had been analysed, we performed *in vitro* intestinal proteolysis experiments in the presence of real HB. This aimed to investigate whether the BS-mediated effect can also be observed for HB samples that (i) vary in total BS contents and (ii) differ considerably from the mixture of individual BS in their complexity of BS composition.

We collected bile from patients who had been diagnosed with various health conditions that can affect bile composition (Supplementary Table S1). Determination of BS and PC contents showed that the collected HB samples varied substantially in the concentrations of these two major biliary surfactants (Fig. 1d). This allowed selection of several HB samples that would meet the following criteria: (i) contrasting BS contents, (ii) similar and low PC contents, and (iii) not contaminated with pancreatic proteases. Based on the BS and PC concentration results (Fig. 1d), three HB samples (i.e., 46(B), 65(B) and 51(A)) were chosen for *in vitro* intestinal proteolysis experiments. Fig. 3a shows the BS and PC concentrations obtained in digestion mixtures after the selected HB samples had been

used, which involved their 20-fold dilution in a digestion mix (see section 2.4.1.). By selecting these samples, we intended to focus predominantly on investigating a potential impact of the BS content of HB on proteolysis. Therefore, the HB samples were required to contain low concentrations of PC (<0.5 mM was obtained in the digestion mix with the HB samples used) but, at the same time, it was essential they differed considerably in total BS concentrations (from 0.65 mM to 6.51 mM BS was produced in the digestion mix with the HB samples used), Fig. 3a. All three HB samples showed no proteolytic activity (Supplementary Fig. S2.3.), which confirmed they had not been contaminated with pancreatic proteases during aspiration from the bile duct.

As with the individual BS (Fig. 2a,b), the HB samples were used in the *in vitro* intestinal digestion of β Lg. After 2 min incubation with trypsin and chymotrypsin, and in the presence of only 0.65 mM BS derived from HB, the amount of purified β Lg was reduced to 56.7 ± 8.1 % of its original amount (i.e., before digestion), which was significantly less than nearly 80% of intact β Lg remaining in the control digestion without HB (Fig. 4a). Using the HB samples with higher BS contents resulted in a progressively more profound hydrolysis of the protein; for 3.64 mM BS there was 40.2 ± 9.1 %, and for 6.51 mM BS only 3.5 ± 2.0 % β Lg left undigested after the 2 min proteolysis. Increasing the digestion time to 5 min enhanced the degree of hydrolysis in all analysed digestion samples (Fig. 4a). Experiments on WPI showed a very similar trend of significantly more enhanced digestibility of β Lg when using HB samples with increasing concentrations of BS (Fig. 4b). Although, the degree of hydrolysis was, in general, more substantial than for the purified β Lg (Fig. 4a). In experiments on WPI, the incubation with trypsin and chymotrypsin seemed to affect α -lactalbumin to a lesser extent than β Lg, as suggested by SDS-PAGE (Fig. 4b).

The trend of a significantly increased degree of β Lg hydrolysis with an increasing content of BS in HB (Fig. 4) is consistent with what was observed for the proteolysis experiment in the presence of increasing concentrations of individual BS (Fig. 2). This is despite the fact that the individual BS

used comprised an equimolar mixture of only two BS (i.e., NaTC and NaGDC), whereas the three HB samples differed considerably between each other not only in the total BS concentration (Figs. 1d and 3a) but also in BS profiles (Fig. 3b). This suggests that the total BS content of HB might be more important than the BS composition in facilitating protein digestion. However, there was a noticeable difference in the extent of β Lg digestion between experiments with individual BS and HB. Namely, a smaller concentration of individual BS, relative to the BS concentration delivered with HB, was required to exert a similar effect on protein digestibility. For example, the amount of intact β Lg after 5 min digestion of WPI in the presence of 1 mM individual BS was 15.1 ± 5.8 % (Fig. 2b), whereas a roughly comparable 12.6 ± 1.8 % of undigested β Lg was only observed after digesting WPI for 5 min in the presence of 3.64 mM BS from HB (Fig. 4b). In experiments with individual BS, no intact β Lg was left after increasing the BS concentration over 3 mM (Fig. 2b). Similar differences in digestion extent were also observed for proteolysis of purified β Lg (Fig. 2a vs Fig. 4a; 5 min digestions). In order to look more closely at this effect, we repeated the intestinal proteolysis of β Lg with HB sample 65(B) (i.e., at 3.64 mM BS concentration produced by the HB) as well as with individual BS used at the total concentration of 3.64 mM in the digestion mix. The digestion experiments were carried out for up to 120 min (Fig. 5). The blank experiments (without enzymes but in the presence of individual BS or HB) showed no hydrolysis of the protein, which confirmed the lack of proteolytic activity in the digestion environments (Fig. 5a,b). Using trypsin and chymotrypsin in the absence of BS, resulted in progressive degradation of β Lg, and after 60 min of digestion only a faint band of the protein was observed in SDS-PAGE gel (Fig. 5c). Addition of the HB that delivered 3.64 mM BS concentration to the digestion mix reduced this time to 15 min (Fig. 5d). However, replacing the HB with 3.64 mM individual BS accelerated the proteolysis even more substantially; the SDS-PAGE showed no intact β Lg remaining in the samples taken after 5 min of intestinal digestion (Fig. 5e).

The difference in the way a mixture of individual BS and an *ex vivo* HB can contribute to intestinal proteolysis was further explored in the *in vitro* gastrointestinal digestion model.

3.3. *In vitro* gastrointestinal proteolysis: Simulating the impact of human bile by a mixture of individual bile salts and phospholipids

Having analysed the impact of HB on the extent of proteolysis, we applied a simulated gastric followed by intestinal digestion to investigate whether the effect of HB can be reproduced with individual BS. This aimed to validate the use of BS as a replacement for difficult-to-obtain HB in *in vitro* simulations of gastrointestinal digestion of β Lg. We used the gastrointestinal model of digestion in order to check whether applying this more complex *in vitro* digestion procedure would yield a similar pattern of β Lg hydrolysis as compared to the one observed in the solely intestinal model (sections 3.1. and 3.2.). If so, it would confirm the robustness of our findings. This is especially important in view of the fact that applying various static *in vitro* gastrointestinal scenarios for the evaluation of proteolysis can produce different extents of the hydrolysis, despite using *in vitro* models that are considered physiologically relevant. This has recently been shown for digestion of several dietary proteins (Torcello-Gómez et al., 2020).

Both, the food-grade WPI and purified β Lg were first subjected to the gastric phase of digestion with pepsin, after which the intestinal phase was carried out under various conditions, according to section 2.4.2. HPLC analysis of the time-point samples collected during the digestion of purified β Lg showed that approx. 90% of the protein remained intact after 60 min pepsinolysis (Fig. 6a). This high resistance of native β Lg to pepsin is consistent with previous studies (Kitabatake & Kinekawa, 1998), (Mandalari, Adel-Patient, et al., 2009) and most likely attributed to the burial of potential cleavage sites between particular amino acids of the protein in the folded calyx structure of β -strands (Reddy, Kella, & Kinsella, 1988). When the digestion mix was transferred to the control

intestinal conditions, i.e., with individual BS but in the absence of intestinal proteolytic enzymes (Fig. 6a; '-E+BS'), the amount of intact, post-gastric β Lg remained the same for the entire duration of the intestinal phase (120 min). Using the opposite conditions, i.e., exposing the protein to trypsin and chymotrypsin in the absence of BS (Fig. 6a; '+E'), caused a progressive breakdown of β Lg, and after 60 min of the intestinal hydrolysis there was only 6.5 ± 2.9 % undigested protein in the system. However, when the proteolysis was repeated in the presence of BS, β Lg was hydrolysed rapidly and completely within the first 10-15 min of the intestinal phase of digestion (Fig. 6a; '+E+BS'). HPLC chromatograms obtained for the digestion time-point samples suggest that the presence of BS might have also altered the profile of peptides produced from β Lg during the digestion with trypsin and chymotrypsin (Fig. 7), apart from increasing the extent of proteolysis. This overall trend of enhanced proteolysis in the presence of BS is consistent with the SDS-PAGE results of the simpler, intestinal model of digestion (Fig. 5), and likely attributed to the partial denaturation of β Lg by the BS (Supplementary Fig. S2.2.), which might have made the protein more susceptible to the intestinal proteases. Same as in this initial evaluation of the intestinal proteolysis progress (section 3.2.), 3.64 mM concentration of individual BS was used in the gastrointestinal digestion in order to compare directly the results obtained with individual BS with results observed in the presence of HB sample 65(B). The HPLC and SDS-PAGE analyses showed that replacing the individual BS with HB made β Lg less susceptible to the intestinal enzymes (Fig. 6a-c; '+E+BS' vs '+E+HB'). The observed difference might have been due to the presence of other components of HB that could affect the protein digestibility, such as phospholipids. Using the HB sample 65(B) in the digestion mixture meant that a 0.18 mM concentration of phosphatidylcholine (PC) was introduced with the bile, apart from 3.64 mM BS (Fig. 3a). In this case, the molar concentration of PC was higher than the one of β Lg, which was ca. 0.05 mM. When these exact concentrations of the biliary PC and BS were replicated in the digestion mixture with individual BS and egg yolk PC (Fig. 6a, '+E+BS+PC'), the extent of β Lg



hydrolysis was reduced relative to the digestion carried out only in the presence of individual BS (Fig. 6a, '+E+BS'). Importantly, the statistical analysis of the results revealed that the addition of PC was crucial in tuning the protein digestibility in such a way that it did not differ significantly ($P>.05$) from the one observed in the presence of HB (Fig. 6b). This might have been due to the PC interacting with β Lg and/or because of a possible complexation of PC with BS, which would make some BS molecules less available to interact with the protein, as discussed below.

Apart from bile salts, phospholipids are another substantial class of highly surface-active compounds in the human bile, with PC being the major phospholipid; whereas cholesterol is the predominant sterol, and very little diglycerides, triglycerides or fatty acids are found in bile (Boyer, 2013). The HPLC-ELSD analysis showed that the PC concentrations in individual HB samples collected for this study was substantially lower than the relevant total BS concentrations (Fig. 1d). In general, we did not detect phosphatidylethanolamine in HB samples, as evident from chromatograms of representative samples (Supplementary Fig. S2.4.). This is in agreement with a previous report that concluded the PC accounts for approx. 95% of the biliary phospholipids (Gilat & Sömjen, 1996).

The effect of PC on the resistance of bovine β Lg to *in vitro* gastrointestinal proteolysis has been studied previously (Mandalari, Mackie, Rigby, Wickham, & Mills, 2009), (Bossios et al., 2011). The PC was found to reduce the extent of intestinal digestion of the protein by trypsin and chymotrypsin. It was suggested the effect was attributed to the steric hindrance caused by monomeric PC binding to a secondary fatty acid binding site along the single strand of α -helix in β Lg, which blocked the action of intestinal proteases. Several scientific studies have shown that PC–protein interaction can significantly reduce the *in vitro* gastrointestinal digestibility of a number of other globular proteins (Berecz et al., 2013), (Moreno et al., 2005), (Vassilopoulou et al., 2006). The decreased rate of proteolysis has been believed to relate to an increased stability and rigidity of the protein scaffold offered by the formation of additional hydrogen bonds between the PC ligand and

protein. A previous study using far-UV CD spectroscopy revealed that incubation of β Lg (1 mg/mL) with PC (2.32 mg/mL) had no direct effect on the protein secondary structure (Maldonado-Valderrama et al., 2011). However, when β Lg was incubated with PC and BS (7.4 mM) under simulated gastrointestinal conditions, the lipid seemed to prevent the protein from structural changes that could otherwise be observed after subjecting β Lg to BS alone. We have done a similar CD experiment for the present study (Supplementary Fig. S2.2.) but with 3.64 mM total concentration of individual BS (NaTC and NaGDC) and 0.18 mM PC in order to reflect the BS and PC concentrations that were produced in the digestion mix with the HB sample 65(B) (Fig. 3a). The CD spectra of β Lg incubated only with PC showed that the protein structure has not been affected; even if the lipid concentration was increased to 0.5 mM (Fig. S2.2.). However, in the experiment with BS and PC, 0.18 mM PC was sufficient to avoid structural changes of β Lg caused by BS. Partial denaturation of the protein was only observed after it had been incubated with the BS (Fig. S2.2.). As with the previous study (Maldonado-Valderrama et al., 2011), the underlying mechanism might involve either (i) non-specific interactions of PC with β Lg, which attenuated the denaturing effect of BS, or (ii) reduction in the concentration of monomeric BS, and thus in interaction of BS molecules with β Lg, by binding the BS and PC into mixed micelles formed by these two surfactants. The protective effect of PC has also been confirmed under the *in vitro* conditions of intestinal proteolysis used in this study. The concentrations of undigested β Lg in the time-point samples withdrawn during the proteolysis in the presence of PC (Fig. 6a, '+E+PC') were consistently higher than in digestion without PC (Fig. 6a, '+E').

Interaction between emulsified triglycerides and BS/PC can also impact on protein digestibility. Previous *in vitro* proteolysis study of β Lg-stabilised emulsions have shown that interfacial adsorption seems to give rise to a pepsin-susceptible form of the protein (Macierzanka et al., 2009), (Sarkar et al., 2009). After 60 min pepsinolysis, only about 40% β Lg was found to remain



intact in emulsion relative to >90% intact protein in a control experiment, where β Lg was exposed to pepsin in solution (Macierzanka et al., 2009). In a subsequent, duodenal stage of digestion presented in that report, the remaining, adsorbed β Lg was rapidly displaced from the interface by the BS present in the digestion mix, and the protein hydrolysed completely in the aqueous phase of emulsion by trypsin and chymotrypsin within 30 min. However, in the presence of BS and PC, the duodenal proteolysis was limited, and after 30 min there was still over 30% undigested β Lg in the system. The protection was suggested to be due to the ability of β Lg to interact with PC in aqueous solution (Mandalari, Mackie, et al., 2009), after the protein was displaced from the interface.

The ability of other luminal lipids to alter the intestinal proteolysis has also been presented in the scientific literature. A combined effect of a mixture of conjugated BS (12 mM) with monoglycerides (monoolein; 6 mM) and fatty acids (oleic acid; 12 mM) on simulated duodenal proteolysis of β Lg was studied by Gass and co-workers (Gass et al., 2007). This lipid mixture was used in order to simulate the simultaneous lipolysis of triglycerides and formation of mixed micelles of BS and lipolysis products. The SDS-PAGE analysis of a control experiment showed that trypsin and chymotrypsin were able to completely hydrolyse β Lg within the first 5 min under the *in vitro* duodenal conditions used in that study, providing the proteolysis was carried out solely in the presence of 12 mM BS. That enhancement effect of BS was found to be largely attenuated after introducing monoglycerides and fatty acids to the digestion mixture, although the protein breakdown was still accelerated relative to the proteolysis carried out in the presence of trypsin and chymotrypsin alone. As suggested by the authors, a competition for BS incorporation into the mixed micelles could have reduced the concentration of free BS that were available for interacting with β Lg molecules, and hence explain the attenuated effect.

The gastrointestinal digestion experiments have also been conducted for WPI in our study (Fig. 6d–f). In general, all the conclusions drawn from the results obtained for purified β Lg (Fig. 6a,b)

also hold for the digestion of β Lg in WPI. However, the relative concentrations of undigested β Lg in the WPI digestion samples (Fig. 6d,e), assessed at various stages of gastrointestinal proteolysis, were up to 15–20% lower than observed in corresponding experiments on purified β Lg (Fig. 6a,b). This effect was consistent regardless of the presence or absence of BS, PC or HB. The most plausible explanation for this is the aforementioned higher susceptibility of β Lg to enzymatic action of proteases caused by a conventional manufacturing of food-grade WPI that may involve limited denaturation of some β Lg molecules. Another noticeable effect was a fast hydrolysis of α La during the gastric proteolysis with pepsin (Fig. 6f). In contrast to β Lg, α La is known to be highly susceptible to pepsin digestion (Sah, McAinch, & Vasiljevic, 2016).

We have also examined the digestibility of other milk proteins under the simulated gastrointestinal conditions applied in this study. Purified β -casein as well as food-grade casein concentrates, sodium caseinate and a casein food supplement (Micellar Casein), were put through the gastric followed by intestinal proteolysis, according to the procedure described in section 2.4.2. Unsurprisingly, in all three cases, the caseins were fully hydrolysed by pepsin within the first 10–20 min of gastric digestion (Supplementary Fig. S2.5.), and only low molecular weight peptides were transferred to the intestinal phase of digestion (data not shown). Their interactions with BS and/or PC were not analysed in this study. It is well-documented in the scientific literature that an overall resistance of caseins to pepsin is relatively low (Mandalari, Adel-Patient, et al., 2009), (Dupont et al., 2009), (Böttger et al., 2019), which is because of the enzyme preference for mobile, loosely structured polypeptides; typical of those offered by caseins (Dupont & Tomé, 2020). The results of the present study are in good agreement with the previous reports.

Together, our results and the results of previous studies described above imply that the presence of BS and other polar lipids, including phospholipids, in the aqueous environment of intestinal digestion can impact on the extent of β Lg proteolysis. However, to our knowledge, this is

the first time the importance of considering a combined effect of two major surface-active components of human bile – BS and PC – on the progress of gastrointestinal proteolysis has been shown. This is also the first time that analogous effects have been reported for real HB and a BS/PC mixture, the latter being prepared and used in order to mimic bile. The results obtained in this study can validate the use of a mixture of individual BS and PC as a human-relevant substitute of HB for *in vitro* proteolysis studies.

The data presented in this report also suggest that the use of mixtures of individual BS and PC might be a more convenient solution for simulating the impact of HB on gastrointestinal proteolysis than the use of animal bile (e.g., fresh bovine or porcine bile, or commercially available bile extracts). Animal bile extracts have widely been used in *in vitro* models of digestion, as they are considered to reflect the complexity of the BS profile in HB, and recommended to use in quantities required to achieve predefined, total BS concentrations (Brodkorb et al., 2019). However, less attention has been paid to the concentrations of other components (e.g., phospholipids) that are brought with such animal bile preparations to the digestion environment. Our results imply that, at least for the proteolysis of β Lg, it is not only the total BS concentration but also the BS/PC ratio that might define the digestion progress and be crucial for simulating the impact exerted by HB. The ratio can most easily and precisely be controlled by applying individual BS and PC, especially as the necessity of replicating the complexity of the BS profile of HB seems to be of less importance, as suggested by the results presented in this report.

4. Conclusions



Quantitative analysis showed that the extent of the proteolysis of bovine milk β Lg by trypsin and chymotrypsin is strongly depended on the concentration of BS under simulated small intestinal conditions, with a significant enhancement of proteolysis already seen for 1 mM BS in the digestion environment. The effect was increasing progressively upon increasing the total concentration of BS, and observed for simple mixtures of two individual BS as well as for real HB samples with complex BS compositions. According to the results of the simulated intestinal digestion experiments carried out in the presence of various HB samples, the total BS content of HB seemed to be more important in defining the extent of β Lg proteolysis than the BS profile of HB. This suggests that under physiological conditions of the small intestine, it is the overall luminal BS concentration – which depends on either the BS content of bile secreted into the intestinal lumen or the bile secretion rate – that defines the kinetics of protein digestion. Our study has also shown that the magnitude of the HB impact on the gastrointestinal proteolysis of β Lg (purified and in a food-grade whey protein isolate) can be closely replicated *in vitro* by the use of individual BS mixed with PC of animal origin. Although, this needs to be confirmed in a follow-up study that would involve a larger number of HB samples varying in BS and PC contents.

The results presented in this report might aid in defining the full physiological role of HB. They also shed more light on what the consequences of BS deficiency in the gut might mean for the effectiveness of food digestion. In addition, this study shows how a difficult-to-obtain HB could be reliably replicated in *in vitro* human-relevant proteolysis models simulating the small intestinal conditions. However, it is important to notice that any results presented in this work were obtained with static *in vitro* digestion models. Further studies are required to fully define the role of BS in protein digestion. These should be conducted with the use of dynamic *in vitro* digestion procedures as well as by applying *in vivo* animal and human models.



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Supplementary Material

Additional characterisation of methods and supplementary data.

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Figures

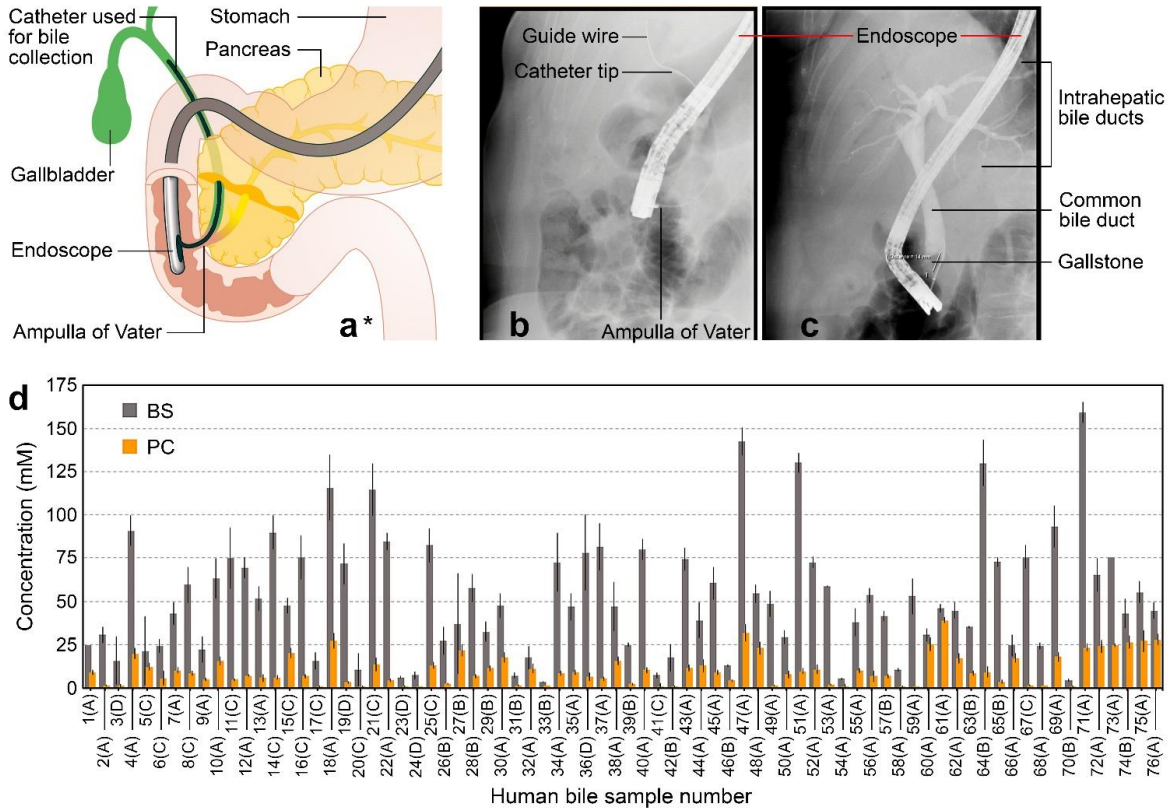


Fig. 1. Human bile (HB) collection and composition. (a) Schematic representation of HB collection during the endoscopic retrograde cholangio-pancreatography, ERCP. (b, c) Fluoroscopy images taken during the ERCP examination of a choledocholithiasis patient. Image (b) shows cannulation of the common bile duct with a catheter and its positioning for bile aspiration. Image (c) shows the post-aspiration application of a contrast in the same patient for visualising the intrahepatic and extrahepatic bile ducts and localising the position of a biliary gallstone. (d) The bile salt (BS) and phosphatidylcholine (PC) concentrations (means \pm SD, $n = 3$) of HB samples collected from 76 individuals. A sample number is followed by an ERCP indication code (see Supplementary Tab. S1). * The diagram was modified from the original material – ©Detailed diagram of an endoscopic retrograde cholangio pancreatography (ERCP) by Cancer Research UK (original email from CRUK, <https://commons.wikimedia.org/w/index.php?curid=34332566> / CC BY-SA 4.0, <https://creativecommons.org/licenses/by-sa/4.0/deed.en>) – and is available to use after modifications under the same license as the original.

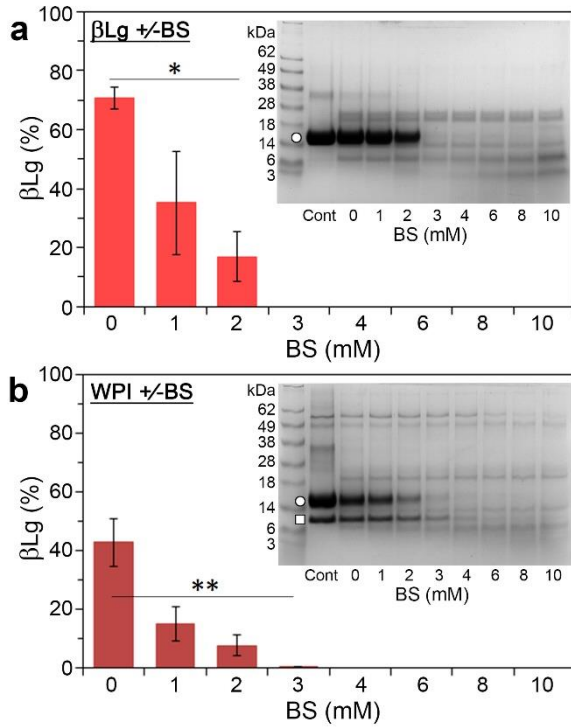


Fig. 2. Effect of the total concentration of individual bile salts (BS; NaTC and NaGDC, 1:1, mol/mol) on the *in vitro* intestinal proteolysis of (a) purified β -lactoglobulin (β Lg) or (b) β Lg in whey protein isolate (WPI). The bar graphs show HPLC data (mean \pm SD; (a) $n = 3$, * $P < .01$; (b) $n = 5$, ** $P < .001$) for the percentage of β Lg remaining intact after 5 min digestion with trypsin and chymotrypsin (1:1 w/w; total concentration of 0.1 mg/mL) at 37 °C. The HPLC data are accompanied by corresponding SDS-PAGE results (reducing conditions; 'Cont' refers to undigested control; \circ , β Lg; \square , α -lactalbumin).

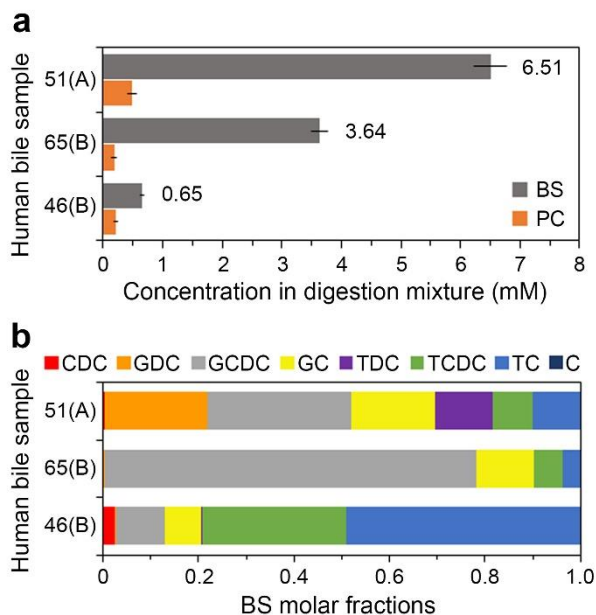


Fig. 3. The human bile (HB) samples used in *in vitro* digestions. (a) Total bile salt (BS) and phosphatidylcholine (PC) concentrations obtained in *in vitro* digestions with selected HB samples (i.e., after their 20-fold dilution in a digestion mix). (b) BS profiles of the HB samples; individual BS molar fractions in total BS tested (chenodeoxycholate, CDC; glycodeoxycholate, GDC; glycochenodeoxycholate, GCDC; glycocholate, GC; taurodeoxycholate, TDC; taurochenodeoxycholate, TCDC; taurocholate, TC; cholate, C).

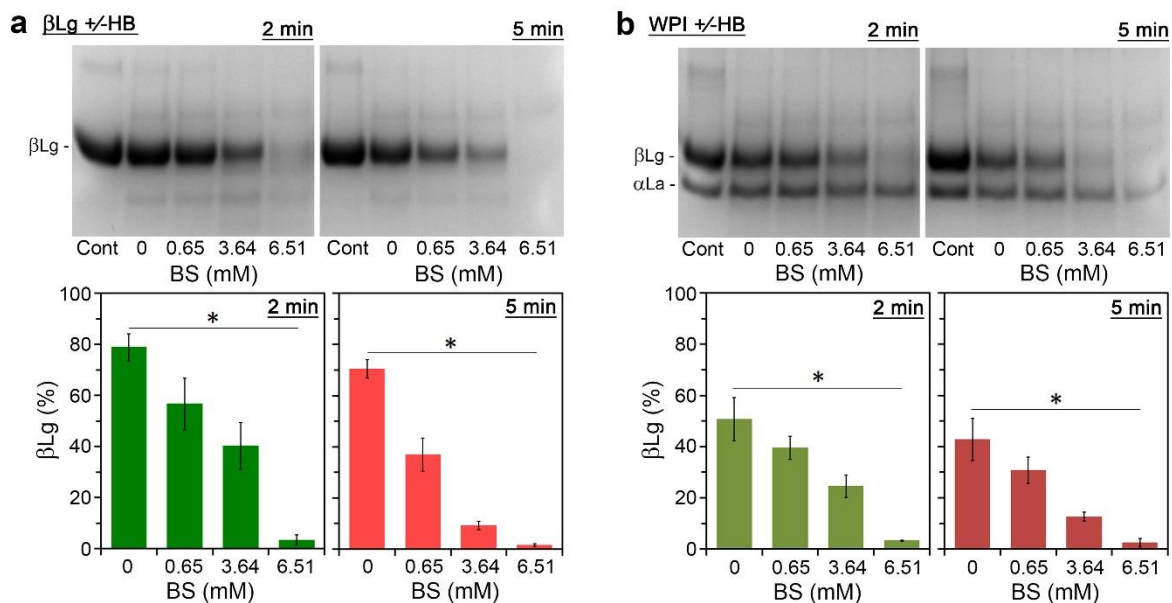


Fig. 4. Effect of the total bile salt (BS) content of human bile (HB) on the *in vitro* intestinal proteolysis of (a) purified β -lactoglobulin (β Lg) or (b) β Lg in whey protein isolate (WPI). The bar graphs show HPLC data (mean \pm SD, n = 5; * $P < .001$) for the percentage of β Lg that remained intact after 2 min or 5 min digestion (37 °C) with trypsin and chymotrypsin (1:1 w/w; total concentration of 0.1 mg/mL) in the absence of HB or in the presence of various concentrations of BS obtained with different HB samples (see Fig. 3a). The HPLC data are accompanied by corresponding SDS-PAGE results (reducing conditions; 'Cont' refers to undigested control; α La, α -lactalbumin).

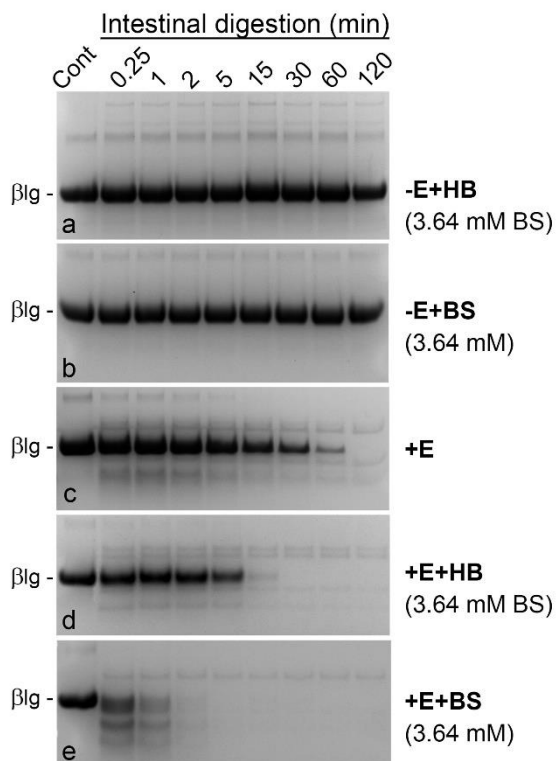


Fig. 5. Representative SDS-PAGE analysis (reducing conditions) of the *in vitro* intestinal proteolysis of purified β -lactoglobulin (β Lg) in the presence or absence of 3.64 mM individual bile salts (BS; NaTC and NaGDC, 1:1, mol/mol), or human bile (HB) providing 3.64 mM BS (HB sample no. 65(B), see Fig. 3). (a,b) Incubation of the protein under the intestinal digestion conditions in the absence of enzymes (-E) and in the presence of HB (a) or individual BS (b). (c–d) Intestinal proteolysis with trypsin and chymotrypsin (+E), and in the absence of BS (c), or in the presence of either HB (d) or individual BS (e). ‘Cont’ refers to undigested control.

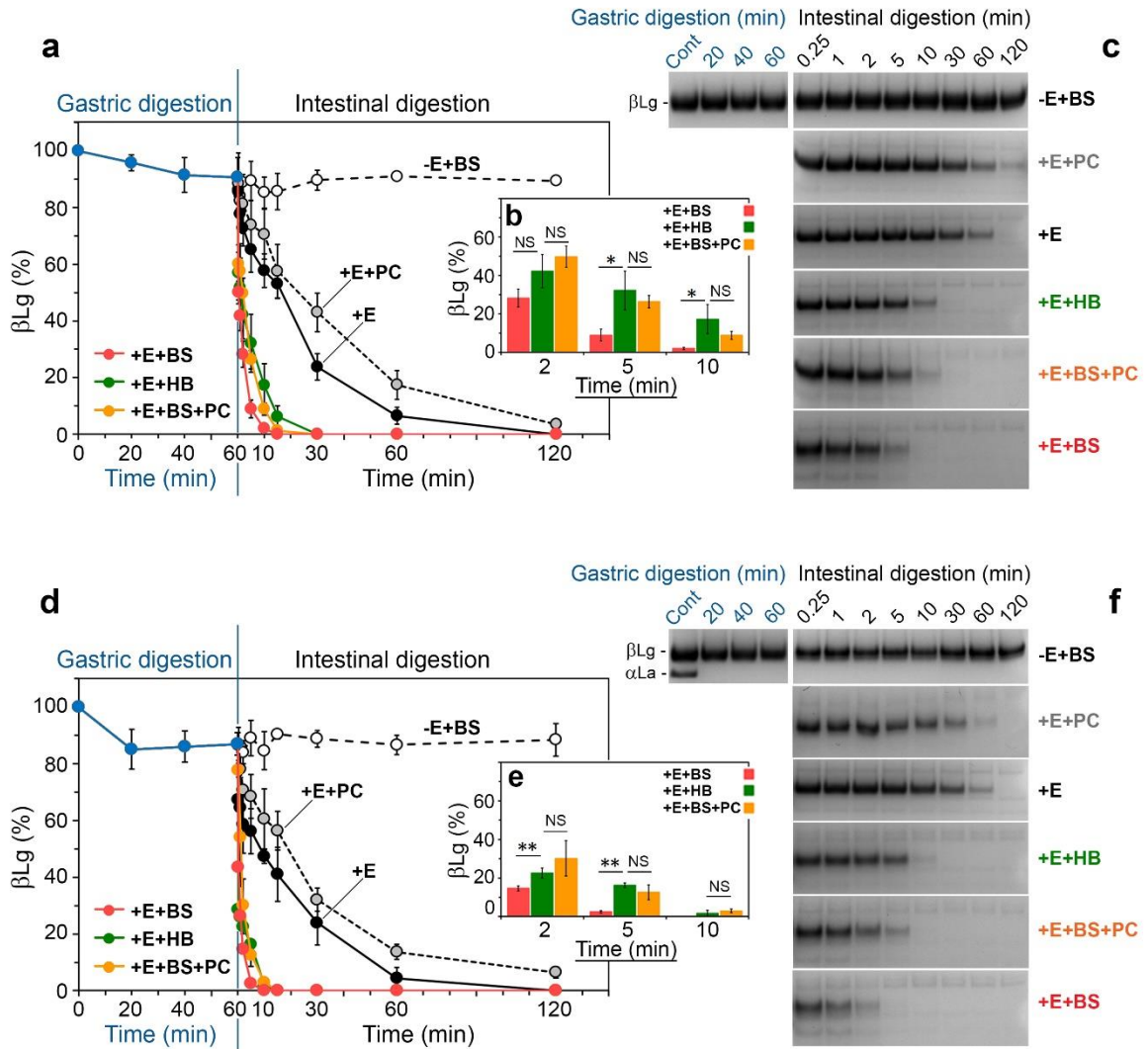


Fig. 6. Effect of individual bile salts (BS), phosphatidylcholine (PC) or human bile (HB) on *in vitro* gastrointestinal proteolysis of (a–c) purified β -lactoglobulin (β Lg) or (d–f) β Lg in whey protein isolate (WPI). HPLC (a, b, d, e) and SDS-PAGE (c, f; reducing conditions) analyses of the digestion time-point samples. The HPLC data (mean \pm SD; $n = 5$) show percentage of intact β Lg remained in the digestion mix. The gastric phase of digestion was performed with pepsin, whereas the intestinal phase was carried out with trypsin and chymotrypsin, and in the presence (+) or absence (-) of individual BS (NaTC and NaGDC, 1:1, mol/mol; total concentration of 3.64 mM), PC (0.18 mM), or HB (sample no. 65(B) diluted to give 3.64 mM BS and 0.18 mM PC in the digestion mix; see Fig. 3a). Some experiments were also done in the absence of the intestinal enzymes (-E). (b,e) * $P < 0.05$; ** $P < 0.01$; NS, not significant ($P > 0.05$). (c,f) 'Cont' refers to undigested control; α La, α -lactalbumin.

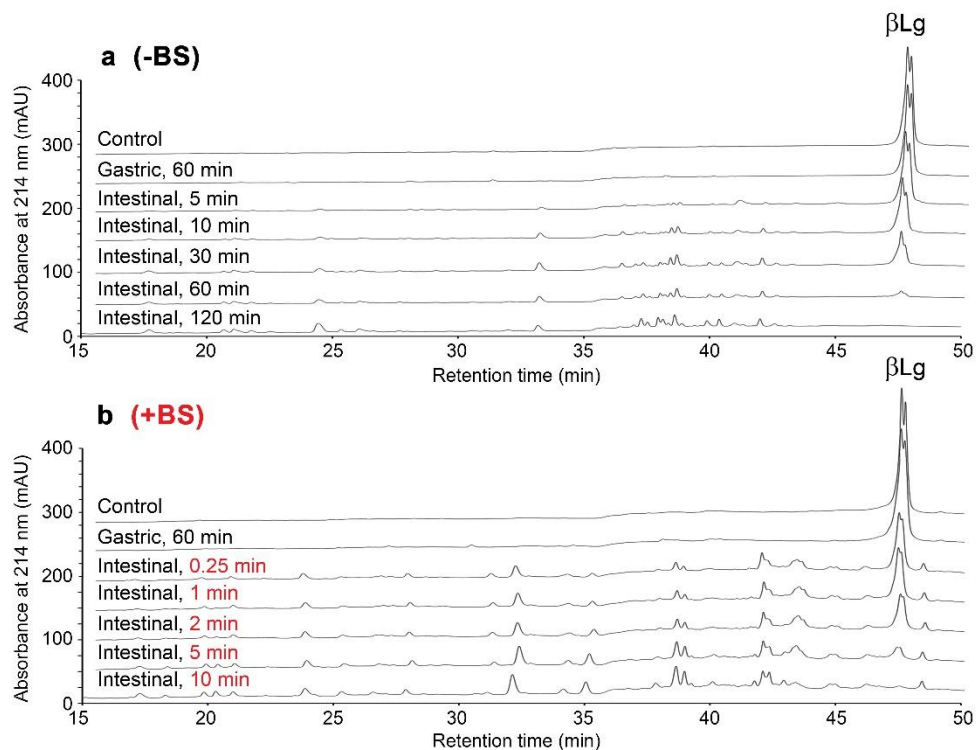


Fig. 7. Representative HPLC chromatograms obtained for undigested protein (purified β -lactoglobulin (β Lg); 'Control') and the *in vitro* gastrointestinal digestion time-point samples. The *in vitro* gastric pepsinolysis of β Lg (60 min) was followed by intestinal proteolysis with trypsin and chymotrypsin (up to 120 min), which was carried out in the absence (a) or presence (b) of individual bile salts (BS; NaTC and NaGDC, 1:1, mol/mol; total concentration of 3.64 mM). Formation of a range of proteolysis products can be observed for retention times ≤ 45 min.