



In vitro digestibility of β -casein and β -lactoglobulin under simulated human gastric and duodenal conditions: A multi-laboratory evaluation

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ARTICLE INFO

Article history:

Received 12 March 2009

Available online 31 August 2009

Keywords:

In vitro digestion

β -Casein

β -Lactoglobulin

Physiological protocol

Allergy

ABSTRACT

Initially the resistance to digestion of two cow's milk allergens, β -casein, and β -lactoglobulin (β -Lg), was compared using a "high-protease assay" and a "low-protease assay" in a single laboratory. The low-protease assay represents an alternative standardised protocol mimicking conditions found in the gastrointestinal tract. For the high-protease assay, both proteins were incubated with either pepsin or pancreatin and digestion monitored by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and reverse phase-high performance liquid chromatography. The low-protease assay involved gastroduodenal digestion in the presence or absence of phosphatidylcholine (PC). Both β -casein and β -Lg were susceptible to hydrolysis by pepsin and pancreatin in the high-protease assay. In contrast, the kinetics of β -casein digestion in the low-protease assay were slower, β -Lg being pepsin resistant. During duodenal digestion, β -Lg was gradually degraded and addition of PC slowed digestion. Subsequently, the reproducibility of the low-protease assay was assessed in 12 independent laboratories by visual assessment of the gels and densitometric analysis: the inter- and intra-laboratory variability was affected by sampling and electrophoresis method employed. The low-protease assay was shown to be reproducible. Future studies will extend these findings using a broader panel of proteins.

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1. Introduction

It has become apparent that many food allergens share certain properties, notably stability as defined using denaturants (such as heat) and biochemical measures of stability, such as resistance to pepsinolysis (Breiteneder and Mills, 2005). Indeed, resistance to digestion is a property common to many, although not all, dietary

proteins thought to sensitise by the human gastrointestinal tract (GIT)¹ (Mills et al., 2004). In order to sensitise an individual via the GIT, an allergen must have properties which preserve its structure from degradation (such as resistance to low pH, bile salts, and proteolysis), thus allowing enough allergen to survive in a sufficiently intact form to be taken up by the gut and sensitise the mucosal immune system (Taylor and Hefle, 2001; Mills et al., 2004). The

¹ Abbreviations used: β -Lg, β -lactoglobulin; GIT, gastrointestinal tract; PC, phosphatidylcholine; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; RP–HPLC, reverse phase-high performance liquid chromatography; GMOs, genetically modified organisms; SGF, standard gastric fluid; SIF, standard intestinal fluid; TLC, thin layer chromatography; MALDI–TOF, matrix assisted laser desorption/ionization–time of flight; BAEE, benzoylarginine ethyl ester; BTEE, benzoyltyrosine ethyl ester; BBI, Bowman–Birk inhibitor; T&C, trypsin and chymotrypsin; ABC, ammonium bicarbonate.

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importance of understanding the impact of physiologically-relevant digestion is emphasized by the fact that raised intragastric pH caused by anti-ulcer medication impairs digestion of fish allergens and increases the allergenic activity of fish (Untersmayer et al., 2005, 2007). Such observations are also relevant regarding allergenic potential of foods in infants who have a raised intragastric pH; this may be a contributory factor to allergenicity of food proteins in infants and older individuals who may have reduced gastric functioning (Untersmayer and Jensen-Jarolim, 2008).

Investigations into the role of digestion in allergenicity of proteins have been hampered by a lack of common approaches and protocols for modelling gastrointestinal digestion *in vitro*. For food allergens, this is further complicated by the utilisation of pepsin resistance tests for assessing allergenic potential of novel foods, including target transgenes in genetically modified organisms (GMOs) (FAO-WHO, 2001). Rather than seeking to model physiological processes, pepsinolysis assays can be interpreted as a biological measure of protein stability. This is because aspartyl proteases, such as pepsin, require a certain degree of flexibility in their substrates as they act on six to eight residue sections of a protein substrate, which must lie across their active site in an extended conformation (Hubbard et al., 1998; Dunn and Hung, 2000). Pepsinolysis tests employ conditions which are far from physiological (Astwood et al., 1996) and are based on a standard gastric fluid (SGF) employed for pre-clinical testing of pharmaceuticals, such as described by the United States Pharmacopeia (2000). The ability of this test to distinguish between allergenic and non-allergenic proteins was initially described by Astwood and co-workers (1996) and whilst not completely confirmed by subsequent studies (Asero et al., 2001; Fu, 2002; Fu et al., 2002; Tanaka et al., 2002), digestion assays are still considered to have some utility when used in integrative risk assessment (EFSA, 2007). For example, the pepsinolysis assay has been applied to studying the stability of transgenes such as the Cry proteins from *Bacillus thuringiensis* (Herman et al., 2003) and novel food proteins such as the ice structuring protein (Baderschneider et al., 2002).

Irrespective of whether the stability of a protein is being assessed using the pepsin resistance test or more physiologically relevant models of digestion, the lack of a standardised protocol is a cause of significant variability (Thomas et al., 2004). For example, factors such as pH will affect both the kinetics and specificity of enzymes such as pepsin (Cornish-Bowden and Knowles, 1969) as will the ratio of proteases to test proteins, with proteolysis proceeding more rapidly at higher protease: protein ratios. In addition, inclusion of phosphatidylcholine (PC) at physiologically-relevant molar ratios of 60:1 PC/protein protected some proteins, such as β -Lg, from proteolysis (Moreno et al., 2005; Mandalari et al., 2009; Macierzanka et al., 2009).

In this paper a “high-protease assay” was initially performed in a single laboratory using the pepsin resistance test in standard gastric fluid (SGF) coupled with pancreatin digestion in simulated intestinal fluid (SIF) as reported by Fu et al. (2002) to compare the resistance to digestion of two model allergenic proteins from cow's milk. The first model allergen is the globular whey protein β -lactoglobulin (β -Lg) (Mantylarvi et al., 2000) which is highly resistant to pepsinolysis but is digested by duodenal enzymes (Mandalari et al., 2009). The second is β -casein which, despite being rapidly digested to low molecular weight (<3.5 kDa) peptides, is generally considered to be a potent allergen (Wal, 2001). Subsequently, a more physiologically-relevant digestion protocol with (low-protease + PC assay) and without (low-protease assay) addition of surfactants was performed (Moreno et al., 2005; Mandalari et al., 2009).

The comparison of these methods was then followed by an inter-laboratory comparison of the physiologically-relevant digestion system using both model proteins. This was undertaken in

two stages, initially by 12 laboratories without addition of PC, a surfactant present in milk and also secreted by the gastric mucosa, and then in 5 laboratories able to include physiological levels of PC in the form of single-shelled liposomes.

2. Materials and methods

2.1. Enzymes, proteins and other reagents

Samples and enzymes from a single lot were provided to participating laboratories as freeze-dried powders: pepsin from porcine gastric mucosa (Sigma, activity: 3300 U/mg of protein calculated using haemoglobin as a substrate), porcine pancreatic trypsin (Sigma, activity: 13,800 U/mg of protein using benzoylarginine ethyl ester, BAEE, as substrate), bovine pancreatic α -chymotrypsin (Sigma, activity 40 U/mg of protein using benzoyltyrosine ethyl ester, BTEE, as substrate), bovine β -lactoglobulin (β -Lg mixed variants A and B; Sigma, ~90% PAGE), bovine β -casein (Sigma, $\geq$ 90% as β -casein by electrophoresis), Bowman-Birk trypsin-chymotrypsin inhibitor (Sigma), sodium taurocholate (Sigma, minimum 95% by TLC), and sodium glycodeoxycholate (Sigma, minimum 97% by TLC). The other reagents required were the following: HPLC water, AnalaR sodium chloride, 0.5 M ammonium carbonate solution, Bis-Tris hydrochloride (Sigma). Proteins were adjudged >95% pure by SDS-PAGE analysis prior to commencing the study.

2.2. Preparation of phospholipid vesicles

Phospholipid vesicles were prepared using a previously described method (Mandalari et al., 2009). Egg $\text{l-}\alpha$ -phosphatidylcholine (PC, lecithin grade 1, 99% purity) was obtained from Lipid Products (South Nutfield, Surrey, UK) and provided to each participating laboratory in glass vials kept at -20°C . Solvent was removed from a 0.94 ml aliquot of PC stock solution (63.5 mM in chloroform), under rotary evaporation with vacuum at 5°C . Any residual solvent was removed at room temperature under vacuum overnight after purging three times with nitrogen. The resulting film of phospholipids was then suspended in 12.2 ml of warmed simulated gastric buffer (0.15 M NaCl, pH 2.5), and sonicated to produce single-shelled liposomes. Liposomes were then filtered through a Nalgene 0.22 μm nylon syringe filter to remove any titanium deposited by the sonicator. The sonication systems used in the five laboratories participating in stage 2 were as follows: Lab 1 used a Sonics Vibracell™ (Beun de Ronde, The Netherlands) for 7 min with amplitude of 80 and pulsar at 8 s whereas the output watts were 20; Lab 9 used a sonicator (Schröder, Rødovre, Denmark) medium power amplitude 7 for 7 min; Lab 10 used a sonication probe (Status US 200, Avestin, UK) at 5°C in a coolant-jacketed vessel for 7 min with a pulsed cycle of 30% full power on for 0.9 s and 0.1 s off; Lab 11 used a digital sonifier 250 from Branson Ultrasonics Corporation (Danbury, USA) for a total of 7 min (probe on for 50 s and off for 10 s, power 40%); Lab 13 used a Sonics & Materials Inc., VibraCell, VC 40 (Branson Ultrasonics B.V., The Netherlands), and performed sonication for 40 min on ice. The liposomes were then put back in the shaking incubator for 20 min to equilibrate at 37°C .

2.3. High-protease assay

The protocol described by Fu et al. (2002) was used as follows. Briefly, for the high-protease assay, 10 μl of the test protein (5 mg/ml) was added to 6 vials containing SGF (200 μl), which consisted of pepsin (3.2 mg/ml, 12.8:1 pepsin to protein ratio, w/w) in 0.03 M NaCl (adjusted to pH 1.2 with HCl). Samples were incubated in a water bath at 37°C and 1 M NaOH (75 μl) added to stop the

reaction at intervals of 0.1, 0.5, 5, 15, 60, and 120 min. For the standard simulated intestinal fluid (SIF) assay, 10 μ l of the test protein (5 mg/ml) was added to 6 vials containing standard intestinal fluid (64 μ l), which consisted of pancreatin (10 mg/ml, Sigma, 12.8:1 pancreatin to protein ratio, w/w) in 0.05 M K_2HPO_4 (pH 7.5). Samples were incubated in a water bath at 37 °C and at intervals of 0.1, 0.5, 5, 15, 60, and 120 min the reaction was stopped by placing the tube in a boiling water bath for 10 min.

2.4. Low-protease assay

As previously reported, a simulated human gastroduodenal protocol of digestion (Moreno et al., 2005; Mandalari et al., 2009) was used to assess the digestibility of β -casein and β -Lg under gastric followed by duodenal conditions.

2.4.1. Simulated gastric digestion

β -Lg or β -casein were dissolved in simulated gastric buffer (0.15 M NaCl, pH 2.5, 1 mg/ml) and the pH adjusted to 2.5 with 0.5 M HCl. After incubation at 37 °C for 10 min, pepsin (gastric buffer pH 2.5, 5 mg/ml) was added (100 μ l), to give 165 U of pepsin per mg of protein in the final digestion mix containing 10 mg of protein and 0.5 mg of pepsin (pepsin: protein ratio of 1:20, w:w). Samples were placed in an orbital shaking incubator (170 rpm, 37 °C) and aliquots (100 μ l) withdrawn after 0.3, 1, 2, 5, 10, 20, 40, and 60 min. Digestions were stopped by raising the pH to 7.5 by addition of 0.5 M ammonium bicarbonate (20 μ l), to irreversibly inactivate pepsin. All assays were performed in triplicate.

2.4.2. Simulated duodenal digestion

Digests resulting from 60 min of *in vitro* gastric digestion (5 ml) were used as the starting material. The pH was adjusted to 6.5 with 0.1 M NaOH and solutions of bile salts, Bis-Tris, and enzymes added to give the following: 4 mM sodium taurocholate, 4 mM sodium glycodeoxycholate, 26.1 mM Bis-Tris buffer pH 6.5, 0.4 U per mg of test protein bovine α -chymotrypsin, 34.5 U per mg of test protein porcine trypsin. This gave enzyme:protein ratios of 1:400 (w:w) for trypsin and 1:100 (w:w) for chymotrypsin (5 mg of protein incubated with 2.5 μ g of trypsin and 0.05 mg of chymotrypsin). Digestions were performed in a shaking incubator (170 rpm, 37 °C) for 30 min and aliquots (100 μ l) taken at 0.3, 1, 2, 5, 10, 20, and 30 min for further analysis. Reactions were stopped by adding a solution of Bowman-Birk trypsin-chymotrypsin inhibitor dissolved in 150 mM NaCl pH 6.5 from soybean (Sigma) at a concentration calculated to inhibit twice the amount of trypsin and chymotrypsin present in the digestion mix.

2.4.3. Ring trial study

The low-protease assay was used for the ring trial study: the two test proteins were subjected to *in vitro* digestion under gastric followed by duodenal conditions in the absence (stage 1) or in the presence (stage 2) of PC. Batches from a single lot of proteins, enzymes, inhibitor and surfactants were sent to all participating laboratories from the study coordinator (IFR).

2.4.4. Participating laboratories

Stage 1: Institute of Food Research (Norwich, UK), Food Science Australia (Werribee, Australia), 2 laboratories from Department of Pathophysiology Medical University of Vienna (Vienna, Austria), Department of Systems Biology (Lyngby, Denmark), CNR-Institute of Science of Food Production (Turin, Italy), Allergy Research Centre of 2nd Paediatric Clinic, University of Athens (Athens, Greece), Laboratoire INRA d'Immuno-Allergie Alimentaire, CEA de Saclay (Gif-sur-Yvette, France), Department of Experimental Immunology, Academic Medical Center (Amsterdam, the Netherlands), Division

of Food Science Polish Academy of Science (Olsztyn, Poland), Syn-genta Biotechnology Inc. (Research Triangle Park, North Carolina, US), Dupont (Wilmington, Delaware, US).

Stage 2: Institute of Food Research (Norwich, UK), Department of Systems Biology (Lyngby, Denmark), Laboratoire INRA d'Immuno-Allergie Alimentaire, CEA de Saclay (Gif-sur-Yvette, France), Department of Experimental Immunology, Academic Medical Center (Amsterdam, the Netherlands), Laboratory of Food Chemistry (Wageningen, the Netherlands).

Laboratories were coded by a random number and are referred to in this manner in the remainder of the manuscript.

2.4.5. Ring trial study stage 2

In stage 2 of the study gastric digestions were performed in the presence of PC. The protein was firstly dissolved as above and then mixed with PC vesicles (3 mM, which corresponds to the human concentration found *in vivo*, Carey and Hernell, 1992) in a molar ratio phospholipid/test protein of 60:1.

2.5. SDS-PAGE analysis

Precast gels were used by 12 out of 13 laboratories to ensure the reproducibility of the method. One laboratory used 17.5% SDS-PAGE gels following the Laemmli method (1970). Samples taken at different time-points during *in vitro* gastric and duodenal digestion were kept at –20 °C until SDS-PAGE analysis. Samples (32.5 μ l) were added to 5 μ l of 0.5 M DL-dithiothreitol solution (Sigma) in deionised water and 12.5 μ l of 4 $\times$ NuPAGE lithium dodecyl sulphate sample buffer (Invitrogen, Carlsbad, CA) and incubated in a water bath at 70 °C for 10 min. Samples were then loaded (15 μ l/lane) on a 10% polyacrylamide NuPAGE Novex Bis-Tris precast gels (Invitrogen, Carlsbad, CA, USA). A continuous buffer system was used consisting of 50 ml of 20 $\times$ NuPAGE MES SDS running buffer with 950 ml of ultrapure water. NuPAGE antioxidant (NP 0005, 0.5 ml) was added to 200 ml buffer and used to fill the inner tank. Gels were run for 35 min at 120 mA/gel and 200 V and then fixed for 30 min with a solution of 50% (v:v) methanol and 10% (v:v) acetic acid in ultrapure water. After washing 2 $\times$ for 5 min with deionised water, gels were stained using a Colloidal Blue Staining Kit (Simply Blue Safe Stain, Invitrogen). Molecular weights markers comprised the following mix of proteins (Invitrogen, Mark 12 MW standard 1 $\times$): insulin α -chain (*Mr* 2500 Da), insulin β -chain (*Mr* 3500 Da), apro-tinin (*Mr* 6000 Da), lactalbumin (*Mr* 14,200 Da), lysozyme (*Mr* 14,400 Da), trypsin inhibitor (*Mr* 20,000 Da), carbonic anhydrase (*Mr* 31,000 Da), lactate dehydrogenase (*Mr* 36,500 Da), ovalbumin (*Mr* 45,000 Da), glutamic dehydrogenase (*Mr* 55,400 Da), bovine serum albumin (*Mr* 66,300 Da), phosphorylase (*Mr* 97,400 Da), β -galactosidase, (*Mr* 116,300 Da), myosin (*Mr* 200,000 Da). Laboratories were asked to run markers in the first and last lane on gels to facilitate data analysis.

2.6. In-gel-tryptic digestion

Polyacrylamide gel pieces were manually picked from the protein bands of interest and in-gel trypsin digested using a ProGest™ Protein Digester (Genomic Solutions, Huntingdon, UK). Gel plugs were conditioned with two 20 min incubations in 200 mM ammonium bicarbonate (ABC) in 50% (v:v) acetonitrile (50 μ l) followed by 10 min incubations with acetonitrile (50 μ l). Any cysteine thiol side chains were then reduced by incubation with 10 mM dithiothreitol in 50 mM ABC (40 μ l) for 10 min at 60 °C before being alkylated with 100 mM iodoacetamide in 50 mM ABC (30 μ l) for 45 min at room temperature. The gel plugs were then conditioned with two 15 min incubations in 25 mM ABC (50 μ l) followed by 10 min in acetonitrile (50 μ l). A final 5 min incubation with

acetonitrile (50 μ l) preceded the addition of sequencing grade porcine trypsin (Promega UK, Southampton, UK) using 155 ng trypsin in 25 mM ABC (10 μ l). After 10 min 25 mM ABC (15 μ l) was added and digestion performed at 37 °C for 3 h. Digestion was stopped and peptides extracted using 10% (v:v) formic acid (7 μ l). Acidified digests (0.4 μ l of each sample) were automatically spotted onto pre-spotted anchor chip plastic MALDI target plates (PAC384 target plates, Bruker Daltonics Ltd., Coventry, UK) using a MALDI-Auto-Prep (MAP II) spotting robot (Bruker Daltonics Ltd.). When the samples had dried the whole plate was dipped into 1.5 l of 10 mM ammonium phosphate, 0.1% trifluoroacetic acid for 3–5 s, removed and air-dried.

Analysis was carried out on an Ultraflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonics Ltd. at the Institute of Food Research-John Innes Centre joint proteomic centre). A 200 Hz nitrogen laser was used to desorb/ionise the matrix/analyte material, and ions were detected in positive ion reflection mode. All spectra were acquired automatically using the Bruker fuzzy logic algorithm. Successful spectral packets for each sample were summed, calibrated, annotated and background subtracted using FlexAnalysis 3.0, the results of which were then automatically set up for searching against the relevant sequence database using the pre-assigned Biotoools method which submits searches to the Mascot 2.1 search engine (Matrix Science Ltd., UK).

2.7. Reverse phase-high performance liquid chromatography

β -Casein and β -Lg samples after high-protease assay (70–80 μ l) were applied to a Phenomenex Jupiter (Macclesfield Cheshire, UK) C4 300 Å pore size, 5 μ m particle size (250 $\times$ 4.6 mm i.d.) column attached to a Dionex HPLC system with a diode array detector following a method as previously described (Mandalari et al., 2009).

2.8. Data evaluation

Images of the gels annotated with the lanes corresponding to a given time-course experiment were identified by numbers, together with the band corresponding to the parent protein, and printed out using a colour Laserjet 3600 dn printer (Hewlett Packard, UK). A panel of six individuals were selected by the study coordinator (IFR) and asked to report the highest lane number where the band could still be seen for each gel image (i.e. the last time-point). The results were tabulated and analyses of variance (ANOVAs) carried out to examine the differences across the panel and between laboratories (results reported at 1% significance level). The mode (consensus value by majority vote) of the six responses was calculated for each gel. This was carried out for 34 gels for β -casein (laboratories 1–12, with three replicates for all laboratories except 5 and 10 which had two), 35 gels for β -Lg (laboratories 1–12, with three replicates for all laboratories except laboratory 5 which had two), 10 gels for β -casein with added PC (laboratories 1, 9, 10, 11, with three replicates for all laboratories except 9 and 10 which had two), and 12 gels for β -Lg with added PC (laboratories 1, 9, 10, 11, with three replicates for all laboratories). Lab 12 was excluded from the gel analysis as the quality of the images was poor with a number of time-points not properly distributed across the gels. The gels were also analysed by densitometry with Totallab TL120 software (Nonlinear Dynamics Ltd., UK) as previously described (Mandalari et al., 2009), and a first-order decay model fitted to the data corresponding to each gel. The time at which the intensity had decreased by 90%, DT_{90} , (Herman et al., 2003) was calculated in Matlab (The MathWorks Inc., Natick, USA) and analysis of variance carried out to examine differences between laboratories (results reported at 1% significance level).

3. Results

3.1. Comparison of different digestion protocols

The kinetics of digestion of β -casein and β -Lg and the resulting patterns of digestion products were compared using the “high-protease assay” and the “low-protease assay”, with and without inclusion of PC (Figs. 1 and 2). β -Casein was rapidly degraded by pepsin in the low-protease assay (Fu et al., 2002), and after 0.1 min only a faint band of M_r 26,000 Da corresponding to the intact protein could be observed by SDS-PAGE (Fig. 1A). Due to its high concentration pepsin appears together with other proteinaceous material which may be contaminants and/or pepsin autolysis products (SGF only lane, Fig. 1A), whose presence may interfere with data evaluation. In-gel-tryptic digestion of bands running at the same molecular weight as β -casein after 0.1, 0.5, and 5 min digestion in SGF did not produce any significant data for band identification (Table 1). HPLC analysis of β -casein samples after the low-protease assay showed a similar degradation pattern of that observed in the SDS-PAGE gels, with no intact protein remaining after 0.1 min incubation with SGF, and qualitative changes in the abundance of certain peptides observed during the time-course of digestion. Peptides eluting at 23, 34, 41, 44, 46 min increased in abundance during the time-course (Fig. 3A). Given the high level of pepsin included, it is possible that some of these peaks are pepsin autolysis products and impurities. In contrast, digestion of β -casein using a simulated physiological gastric digestion protocol was much slower and even after 10 min a M_r 26,000 Da polypeptide corresponding to the intact protein could still be observed (Fig. 1B). β -Casein degradation was accompanied by the appearance of lower molecular weight fragments (M_r between 2500 and 21,500 Da), some of which persisted after 60 min incubation. Reducing the pH of the physiological system to 1.2 did not alter the degradation pattern for β -casein (results not shown). As previously reported (Macierzanka et al., 2009), the inclusion of physiological levels of PC did not affect the breakdown of β -casein during simulated gastric digestion (Fig. 1C). The differences in protein loading between Fig. 2B and C indicated by the molecular weight marker and sample lanes are a result of data coming from two laboratories participating in the ring trial. They are presented to show inter-laboratory variation in primary data gathered in the ring trial (see below).

In the assay with simulated intestinal fluid, β -casein was incubated directly (without prior pepsin digestion) with pancreatin and it was immediately degraded by this crude mix of pancreatic enzymes. β -Casein digestion products could not be distinguished from pancreatic components by SDS-PAGE (Fig. 1D). In contrast, for the low-protease assay the pH of 60 min post gastric digest of β -casein was raised to 7.0, irreversibly inactivating pepsin and mimicking the transfer of gastric contents into the duodenal compartment. With the addition of trypsin and chymotrypsin the low molecular weight fragments of β -casein remaining after gastric digestion were rapidly broken down so that after 5 min duodenal digestion no trace of β -casein-derived peptides was visible by SDS-PAGE (Fig. 1E). The inclusion of PC during gastric digestion did not alter the pattern of β -casein digestion products generated under duodenal conditions (Fig. 1F), apart from differences in protein loading between two laboratories.

Although β -Lg is generally accepted to be resistant to pepsinolysis in SGF (Herman et al., 2007), it was degraded in the presence of a gross excess of pepsin (Fig. 2A) in the high-protease assay, with only a faint trace of the M_r 18,300 Da intact protein remaining after 120 min. In-gel-tryptic digestion and MALDI-TOF analysis confirmed this polypeptide was β -Lg (Table 1). Both variants A and B

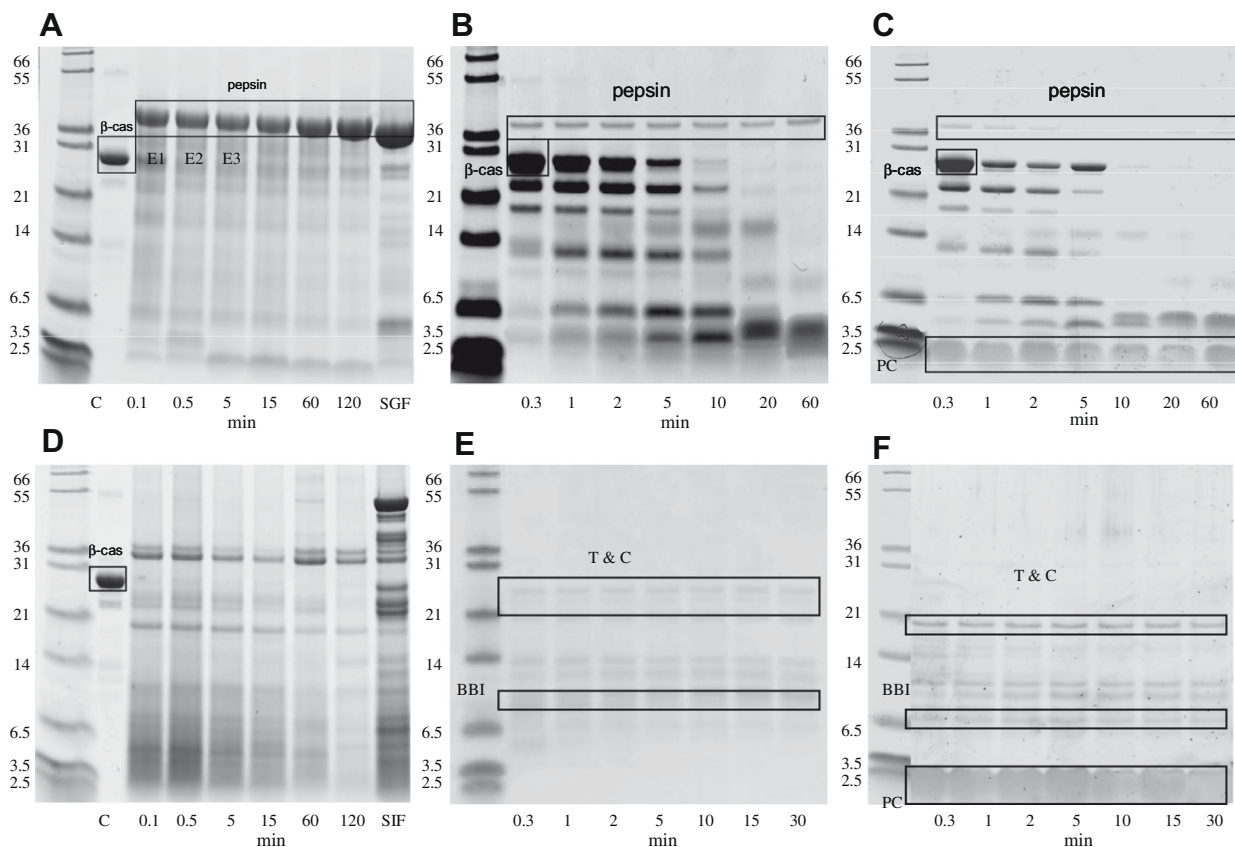


Fig. 1. SDS-PAGE analysis of β -casein subjected to gastric stability assay (A, laboratory 10), physiological gastric digestion (B, laboratory 3), physiological gastric digestion in the presence of PC (C, laboratory 9), intestinal stability assay (D, laboratory 10), physiological gastric plus duodenal digestion (E, laboratory 4), and physiological gastric plus duodenal digestion in the presence of PC (F, laboratory 10). SDS-PAGE was performed under reducing conditions. C, no enzyme added; β -cas, β -casein; SGF, standard gastric fluid; SIF, standard intestinal fluid; BBI, Bowman-Birk inhibitor; T&C, trypsin and chymotrypsin. Bands with code (E1, E2, and E3) subjected to in-gel-tryptic digestion as reported in Table 1.

of β -Lg showed identical behaviour (results not shown), although others have reported that only β -Lg A was degraded under these conditions (Fu et al., 2002). HPLC analysis of β -Lg samples from the high-protease assay showed intact β -Lg eluting on a 300 Å pore size column at 62 min as a double peak, reflecting the presence of both variants A and B (Fig. 3B). No intact β -Lg was observed after 0.1 min incubation with SGF, with qualitative changes in the abundance of certain peptides observed during the time-course. As for β -casein these may represent pepsin autolysis products and impurities.

In contrast β -Lg was resistant to physiological gastric digestion (Fig. 2B) and was unaffected by inclusion of physiological levels of PC (Fig. 2C). Incubation of β -Lg with pancreatin in the assay with simulated intestinal fluid (i.e. without prior digestion with pepsin) resulted in rapid degradation of the intact protein, which seemed to co-run with a polypeptide present in the standard intestinal fluid mixture on SDS-PAGE (Fig. 2D). Pancreatin, a major component of SIF, is a mixture of many enzymes, including amylase, trypsin, lipase, ribonuclease and protease. Although Fu et al. (2002) reported β -Lg was stable in SIF for 5 min, in-gel-tryptic digestion only confirmed the presence of β -Lg after 0.1 min incubation, the bands running with the same molecular weight as β -Lg after 0.5, 5, 15, 60, and 120 min being identified as carboxypeptidase A1 precursor (Table 1). The higher calculated M_r shows this is likely to be a peptide originating from the pancreatin mixture used in the assay.

In the low-protease assay, β -Lg was gradually degraded by trypsin and chymotrypsin during simulated gastrointestinal digestion, and after 30 min only a faint band corresponding to intact β -Lg was

observed by SDS-PAGE (Fig. 2E) with a range of lower molecular weight polypeptides (M_r between 2500 and 14,400 Da) being evident. The presence of PC during gastric digestion slowed or prevented digestion of β -Lg by duodenal enzymes (Fig. 2F) as previously reported (Mandalari et al., 2009).

These results showed that the kinetics of digestion of β -casein and β -Lg were much slower using more physiological digestion assays (enzymes:protein ratio of 1:20 w/w in the low-protease assay) compared to the unphysiological high-protease assays (enzymes:protein ratio of 12.8:1 w/w). Given the need for harmonisation of test protocols, we undertook a ring trial for the low-enzyme assay using β -casein and β -Lg with 12 laboratories. The multi-laboratory evaluation approach of Thomas et al. (2004) was used to assess the final time-point at which the intact protein was detectable on the SDS-PAGE gel. The first trial was undertaken in 12 laboratories without addition of gastric PC and was followed by a second trial with five laboratories when gastric PC was included.

3.2. Ring trial stage 1

The analyses of variance (ANOVAs) of the panel assessment showed that there were no statistically significant differences between panel members (1% level), hence consensus values are discussed.

For β -casein gastric digestions performed in the absence of PC, the consensus value for the time at which the β -casein band was last observed on SDS-PAGE gels was 10 min in 62% of cases, 20 min in another 26% of cases, and either 5 or 40 min in 6% of cases (Fig. 4A). For half the laboratories there was no

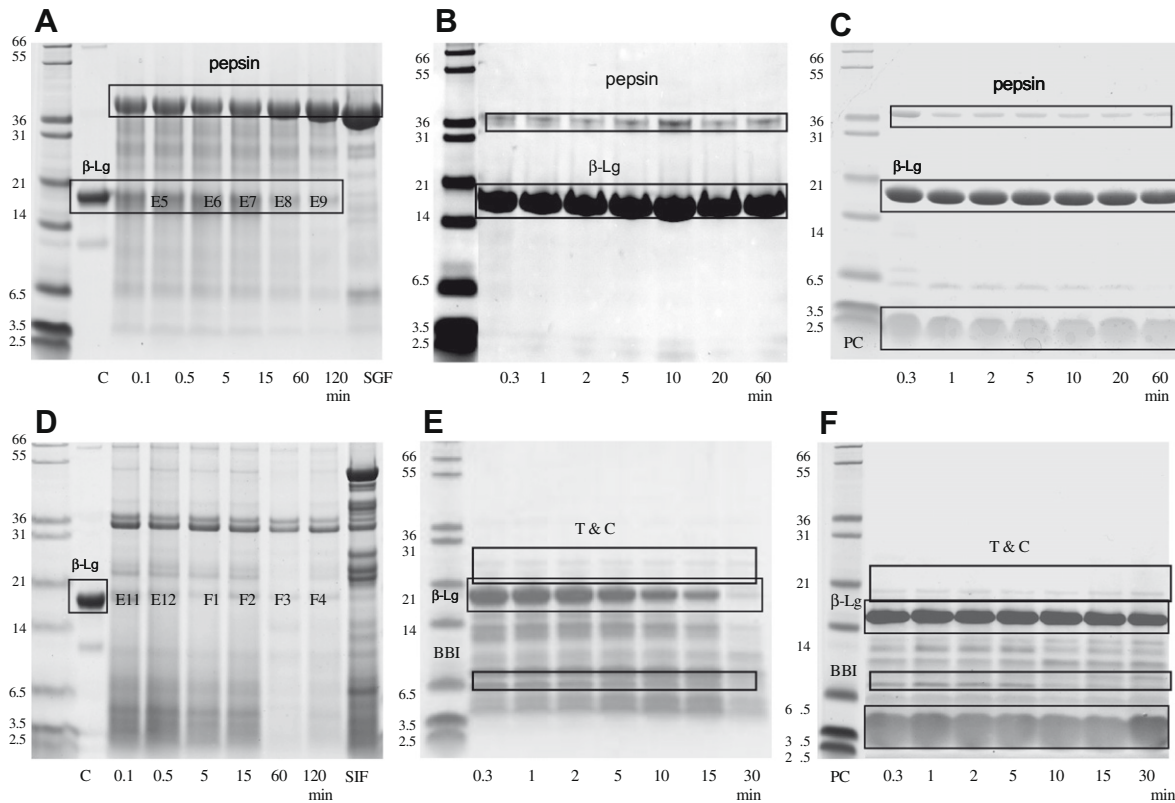


Fig. 2. SDS-PAGE analysis of β -lactoglobulin subjected to gastric stability assay (A, laboratory 10), physiological gastric digestion (B, laboratory 10), physiological gastric digestion in the presence of PC (C, laboratory 9), intestinal stability assay (D, laboratory 10), physiological gastric plus duodenal digestion (E, laboratory 3), and physiological gastric plus duodenal digestion in the presence of PC (F, laboratory 10). SDS-PAGE was performed under reducing conditions. C, no enzyme added; β -Lg, β -lactoglobulin; SGF, standard gastric fluid; SIF, standard intestinal fluid. Bands with code (E5–E9, E11, E12, and F1–F4) subjected to in-gel-tryptic digestion as reported in Table 1.

Table 1
Proteins identified by in-gel-tryptic digestion.

Sample	Accession ^a and organism	Protein ID	% Coverage	PMF MOWSE score ^b	Calc. Mr	Obs. Mr	Band ID
β -Casein SGF 0.1 min	NI	NI	NI	ND	NI	27.0	E1
β -Casein SGF 0.5 min	NI	NI	NI	41	NI	27.0	E2
β -Casein SGF 5 min	NI	NI	NI	20	NI	27.0	E3
β -Lg SGF 0.5 min	P02754 <i>Bos taurus</i> (Bovine)	β -Lactoglobulin	51	124	20.3	18.0	E5
β -Lg SGF 5 min	P02754 <i>Bos taurus</i> (Bovine)	β -Lactoglobulin	64	109	20.3	18.0	E6
β -Lg SGF 15 min	P02754 <i>Bos taurus</i> (Bovine)	β -Lactoglobulin	51	95	20.3	18.0	E7
β -Lg SGF 60 min	P02754 <i>Bos taurus</i> (Bovine)	β -Lactoglobulin	44	65	20.3	18.0	E8
β -Lg SGF 120 min	P02754 <i>Bos taurus</i> (Bovine)	β -Lactoglobulin	61	99	20.3	18.0	E9
β -Lg SIF 0.1 min	P02754 <i>Bos taurus</i> (Bovine)	β -Lactoglobulin	61	91	20.3	18.0	E11
β -Lg SIF 0.5 min	P02754 <i>Bos taurus</i> (Bovine)	β -Lactoglobulin	39	42	20.3	18.0	E12
β -Lg SIF 5 min	P09954 <i>Sus scrofa</i> (Porcine, pancreatic)	Carboxypeptidase A1 precursor	35	76	47.3	18.0	E12
β -Lg SIF 5 min	NI	NI	NI	ND	NI	18.0	F1
β -Lg SIF 15 min	P09954 <i>Sus scrofa</i> (Porcine, pancreatic)	Carboxypeptidase A1 precursor	33	101	47.3	18.0	F2
β -Lg SIF 60 min	P09954 <i>Sus scrofa</i> (Porcine, pancreatic)	Carboxypeptidase A1 precursor	33	107	47.3	18.0	F3
β -Lg SIF 120 min	P09954 <i>Sus scrofa</i> (Porcine, pancreatic)	Carboxypeptidase A1 precursor	36	99	47.3	18.0	F4

ND, no data; NI, not identified.

^a Swiss-Prot/GenBank Accession code.

^b Protein scores greater than 67 are significant ($p < 0.05$).

intra-laboratory variability at all, whilst the other half showed widespread differences with replicate digestions end-times of between 5 and 40 min. In addition ANOVA suggested there were significant differences between laboratories; notably differences were highlighted for laboratories 3, 11, and 12. However, the ANOVA on the DT₉₀ obtained by fitting a first-order decay model showed no significant differences between laboratories (1% level); these are not inconsistent results as for DT₉₀ only the data for laboratories 1–9 were included, due to quality issues for the images from laboratories 11 and 12. The DT₉₀ was 8 min on average, ranging between 4 and 12.4 min for averages of replicates for each laboratory

(Table 2). This was consistent with the panel's observations, given the fact they could only identify discrete time-points corresponding to sampling times. Importantly, both qualitative and quantitative approaches consistently indicated the digestion of the protein being complete by 10–20 min in 10 out of 12 centres.

The ANOVA of the panel data for the β -Lg gastric digestion also only highlighted differences between laboratories (i.e. not between panel members). This was due to the estimated time at which β -Lg was last observed, being 15 min for some of the assessments of gels from lab 10 and of one gel from lab 8, whereas it was 30 min for all other gels and all panel members. Thus, in 91% cases

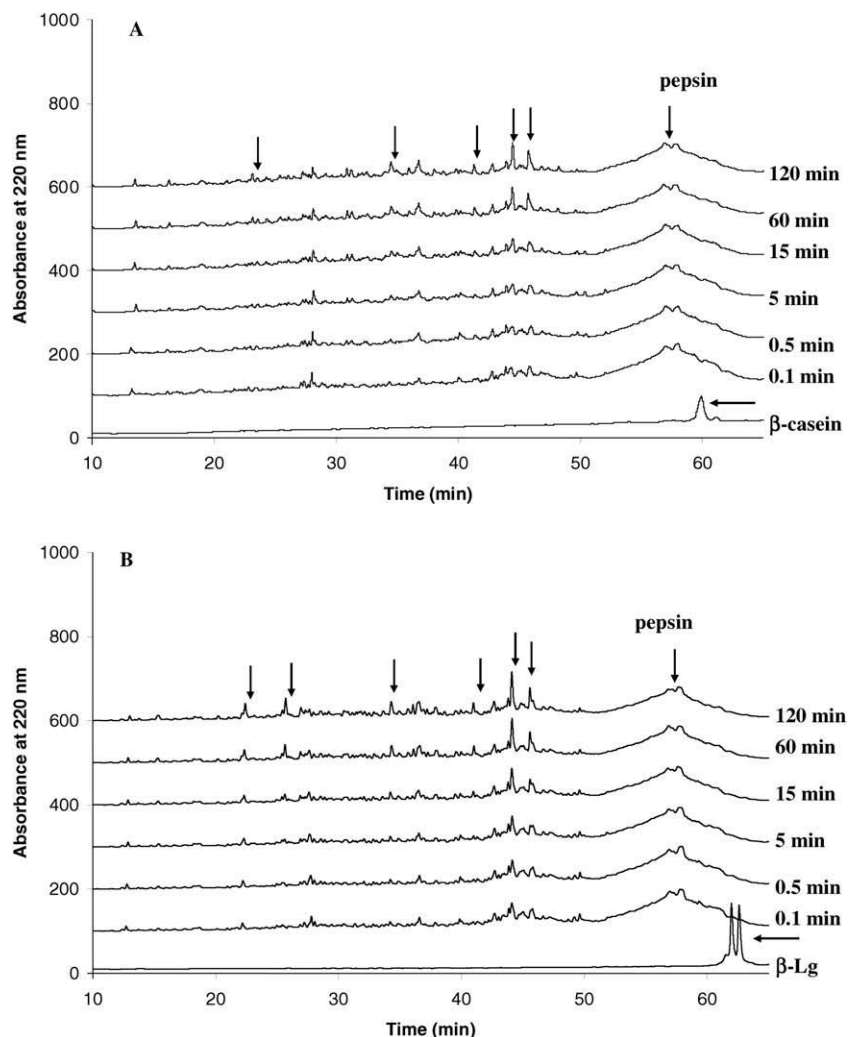


Fig. 3. RP-HPLC analysis of β -casein (A) and β -Lg (B) time-point samples subjected to digestion stability assay using 300 Å pore size column. Digestions were performed in standard gastric fluid for 0.1, 0.5, 5, 15, 60, and 120 min. Peaks corresponding to β -casein, β -Lg and pepsin are indicated. Closed arrows indicate peptide peaks increasing in size during the time-course.

the mode value (consensus) for the estimated time at which β -Lg was last observed by the panel during duodenal digestion was 30 min, being 15 min only for 3 gels (Fig. 5A). One of those (lab 8, rep 3) was from a time-course where the sample analysis was split between 3 gels, 15 and 30 min time-points being analysed on different gels. The fitting of a first-order decay model gave a DT_{90} of 45 min on average with all values except one in the range 33–57 min (Table 3). The DT_{90} was extrapolated beyond the last sampling point, which explains why the panel detected the protein at 30 min in 90% cases. The ANOVA for DT_{90} suggested that there were no significant differences between labs (1% level): lab 10 had a lower average DT_{90} value but, unlike for the panel data, was not highlighted. Again the later quantitative approach afforded higher precision in parameter estimates of kinetics. However, regardless of the approach, these data consistently confirmed that incubation of β -Lg with duodenal enzymes, trypsin and chymotrypsin, resulted in gradual digestion of the protein.

3.3. Ring trial study stage 2

When β -casein was incubated in the presence of PC under gastric conditions, the estimated time at which the intact β -casein band was last observed was again 10 or 20 min in the majority of cases (Fig. 4B). For two of the laboratories there was little intra-

or inter-variability in the estimated time at which the β -casein band was last seen, with all reported values corresponding to either 10 or 20 min. However, laboratories 9 and 10, for which there were only two replicates, gave more disparate results, being 5 or 10 min for one replicate and 60 min for the other. Addition of physiological relevant levels of PC to the gastric digestion mix did not alter stability to gastric digestion but did protect β -Lg from proteolysis by duodenal enzymes, all the reported values being 30 min with no intra- or inter-laboratory variability, or even disagreement between volunteers (Fig. 5B).

4. Discussion

The digestibility of two allergenic milk proteins, β -casein and β -Lg, was assessed using a high-protease assay and a low-protease assay. An inter-laboratory comparison of the low-enzyme assay was then performed with and without addition of surfactants. In the high-enzyme assay, the protocol reported by Fu et al. (2002) was used to investigate the stability of β -casein and β -Lg in SGF and SIF. The ratio of pepsin and pancreatin to test protein was 12.8:1, whilst in the “low-enzyme assay” much lower enzyme to test protein ratios were used, being 1:20, 1:100, and 1:400 for pepsin, chymotrypsin, and trypsin, respectively (Rodbro et al., 1967; Hardorn, 1974; Clemente et al., 1972; Allan and White,

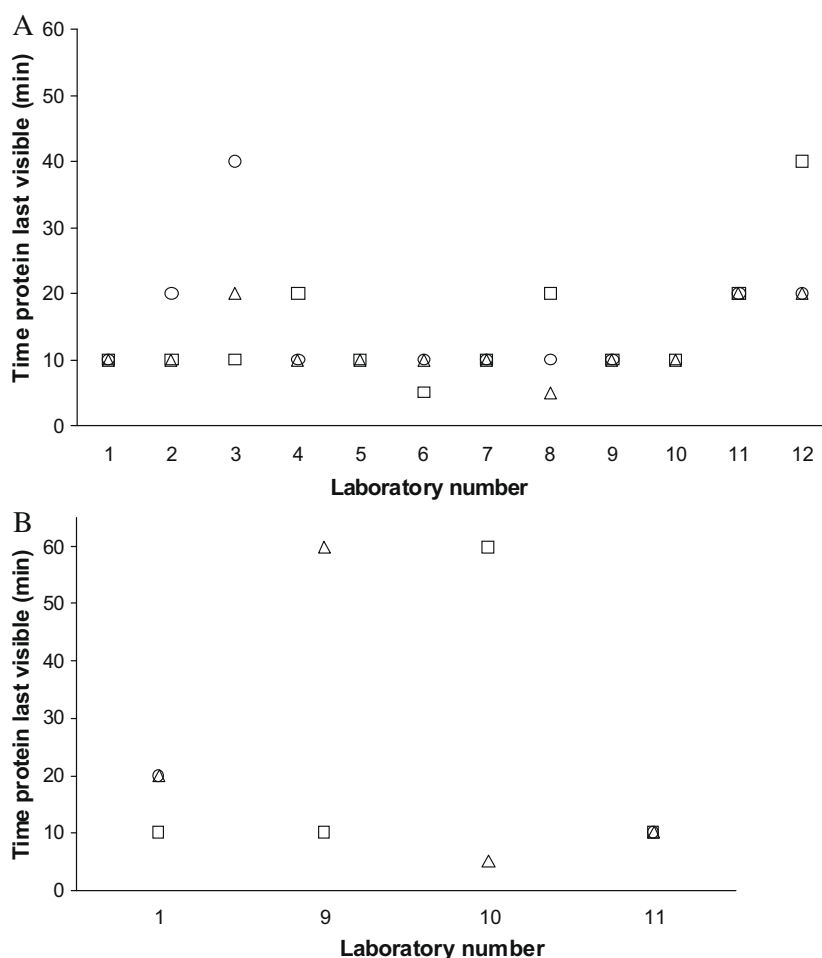


Fig. 4. Mode of times estimated to be the last time at which the parent protein (β -casein) band is visible for laboratories 1–12 and replicates 1–3 (square, triangle, and round) in the absence of PC (A) and for laboratories 1, 9, 10, and 11 and replicates 1–3 (square, triangle, and round) in the presence of PC (B). Digestions were performed under physiological gastric conditions.

Table 2

Average DT_{90} for all gels, and for gels corresponding to each laboratory, for β -casein gastric digestions performed in the absence of PC.

	Overall	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9
Mean	8	11.3	8.4	10.0	12.4	5.6	6.9	4.0	6.1	6.6
Std	3.6	1.8	2.8	0.7	0.2	2.1	2.1	1.5	2.2	4.8
CV	45.0	48.1	47.8	51.7	54.7	38.2	47.4	49.1	48.8	67.3

1975). This factor alone accounts for the differences in protein digestion kinetics.

A second difference was pH, with the high-protease assay using a pH of 1.2, whilst the low-protease assay used a higher pH of 2.5. When the pH of the low-protease assay was decreased to 1.2 it did not affect the kinetics of digestion. However, it is possible such a pH would contribute to changes in the patterns of proteolysis products generated by the two assays since pepsin exhibits different specificities at those different pH values (Cornish-Bowden and Knowles, 1969).

Although β -Lg is generally resistant to pepsinolysis (Schmidt et al., 1995), the protein (as variants A and B) was degraded by the high pepsin concentration in SGF. This effect results from the fact that, like all hydrolysis reactions, the cleavage of the amide bonds in a protein substrate is an equilibrium reaction. Thus, inclusion of sufficient pepsin will allow this degradation reaction to take place. Our results are in contrast to those reported by Fu et al. (2002), who found β -Lg variant B was resistant in SGF for

120 min, whereas variant A was degraded within 0.5 min. However, others (Kitabatake et al., 2001) also found variant A was resistant to pepsin hydrolysis at pH 2.0.

The multi-laboratory evaluation approach previously reported by Thomas et al. (2004) was used to investigate the reproducibility of β -casein and β -Lg during gastrointestinal digestion, with or without addition of surfactants. As every laboratory used shared reagents it was not possible to evaluate the variability resulting from each laboratory conducting the assay with their own supplies of reagents. β -Casein was last detected under physiological gastric conditions consistently by 10–20 min in 10 out of 12 laboratories. We hypothesize that the variation in rate of digestion across the laboratories could be due, in part, to errors during sampling. As previously reported by Thomas et al. (2004), the gel electrophoresis system affected the detection of intact protein and digestion fragments: one laboratory participating in the ring trial used gels based on the traditional Laemmli buffer system and it was not possible to analyze SDS-PAGE results due to the poor quality of the gel

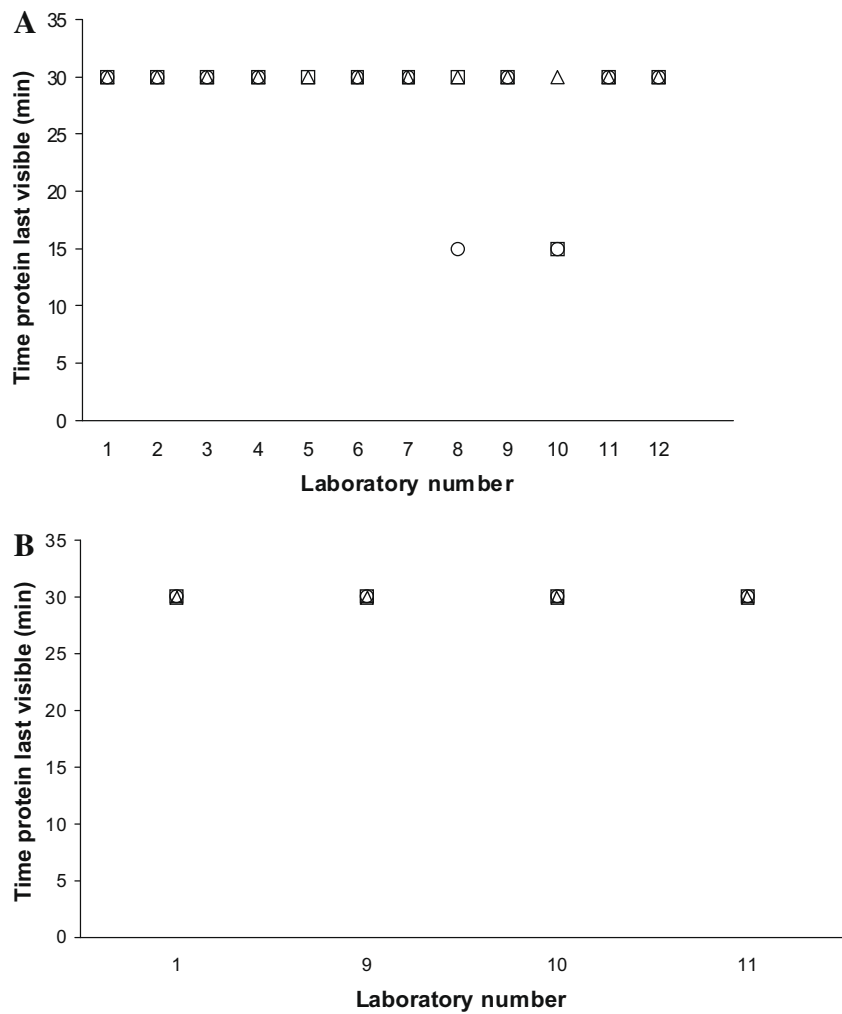


Fig. 5. Mode of times estimated to be the last time at which the parent protein (β -Lg) band is visible for laboratories 1–12 and replicates 1–3 (square, triangle, and round) in the absence of PC (A) and for laboratories 1, 9, 10, and 11 and replicates 1–3 (square, triangle, and round) in the presence of PC (B). Digestions were performed under physiological duodenal conditions.

Table 3
Average DT₉₀ for all gels, and for gels corresponding to each laboratory, for β -Lg duodenal digestions performed in the absence of PC.

	Overall	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10
Mean	45.1	85.2	37.8	33.6	47.1	57.1	33.0	44.8	42.2	41.6	33.0
Std	19.6	18.6	7.1	2.5	5.2	41.7	9.7	2.0	13.8	7.1	6.8
CV	43.1	47.1	47.5	51.3	49.8	73.1	47.2	53.1	47.7	47.9	47.3

images. The fitting of a first-order decay model to densitometry data was also applied, as per [Herman et al. \(2003\)](#) to determine the time at which the parent protein had decayed by 90%. This approach has much more scope for an in-depth analysis of intra- and inter-laboratory variability, given the higher precision of the parameters describing the kinetics of digestion (see CVs).

We have recently shown that inclusion of physiologically relevant concentrations of vesicular PC in simulated gastroduodenal digestion has a protective effect against proteolysis of cows' milk β -Lg ([Mandalari et al., 2009](#)). Therefore it is important to investigate the interactions between allergens and other food components, especially lipids, which may affect the allergenicity of proteins sensitising via the GIT and proteolytic fragments generated during digestion. In this study we have demonstrated that presence of PC consistently did not affect β -casein degradation under physiological gastric conditions, whereas it altered the kinetics of β -Lg digestion in the duodenal environment, protecting the pro-

tein from degradation. Therefore, the inclusion of surfactants into the digestion mix affects certain proteins but not others. Given the harmonisation need for a standardised protocol to assess protein digestibility, the results obtained in this study showed consistency between laboratories for two allergenic milk proteins, lending support for the use of a gastroduodenal digestion system simulating physiological *in vivo* conditions including lower levels of proteases and surfactants. Much of the interest in simulated digestion studies for assessment of allergenic potential of novel proteins stems from the original study of [Astwood et al. \(1996\)](#). Subsequently efforts have focussed on refining and amending this assay which is primarily simulating gastric pepsinolysis ([Thomas et al., 2004](#); [Fu et al., 2002](#); [Herman et al., 2007](#)) and it has become enshrined in the allergenic risk assessment process for novel proteins ([EFSA, 2007](#)) since resistance of a protein to digestion is generally considered to be an indicator for enhanced likelihood of allergenicity. However, this relationship is not proven ([Bannon](#)

et al., 2003) and several food proteins not known to be allergens show resistance to degradation whilst other allergens are susceptible (Fu et al., 2002; Herman et al., 2007). In general few studies have assessed the utility of simulated intestinal digestion (Fu et al., 2002) and this has not generally been applied in allergenic risk assessment. However, given the role of gastroduodenal digestion in presenting allergenic proteins to the immune system either in sensitisation or elicitation of allergic reactions (Untersmayer et al., 2005; Eiwegger et al., 2006), assessing resistance to gastroduodenal digestion may add value. Although the current study does demonstrate the low-protease assay is robust and reproducible, further validation should be undertaken using a more extensive panel of proteins.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

This research was funded by the Biotechnology and Biological Research Sciences Council (BBSRC, UK) and the EuroPrevall Integrated Project FOOD-CT-514000. We thank Drs. Mike Naldrett and Gerhard Saalbach of the JIC/IFR Joint Proteomic Facility for performing the MALDI-TOF analysis, and Jack Dainty for help with the panels' data analyses of variance.

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