Validation of an In Vitro Digestive System for Studying Macronutrient Decomposition in Humans1–3

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Abstract

The digestive process transforms nutrients and bioactive compounds contained in food to physiologically active compounds. In vitro digestion systems have proven to be powerful tools for understanding and monitoring the complex transformation processes that take place during digestion. Moreover, the investigation of the physiological effects of certain nutrients demands an in vitro digestive process that is close to human physiology. In this study, human digestion was simulated with a 3-step in vitro process that was validated in depth by choosing pasteurized milk as an example of a complex food matrix. The evolution and decomposition of the macronutrients was followed over the entire digestive process to the level of intestinal enterocyte action, using protein and peptide analysis by SDS-PAGE, reversed-phase HPLC, size exclusion HPLC, and liquid chromatography-MS. The mean peptide size after in vitro digestion of pasteurized milk was 5–6 amino acids (AA). Interestingly, mostly essential AA (93.6%) were released during in vitro milk digestion, a significantly different relative distribution compared to the total essential AA concentration of bovine milk (44.5%). All TG were degraded to FFA and monoacylglycerols. Herein, we present a human in vitro digestion model validated for its ability to degrade the macronutrients of dairy products comparable to physiological ranges. It is suited to be used in combination with a human intestinal cell culture system, allowing ex vivo bioavailability measurements and assessment of the bioactive properties of food components. J. Nutr. doi: 10.3945/jn.111.148635.

Introduction

To study the physiological effects of in vitro digested food, the composition of an in vitro digestion system needs to be chosen carefully. Knowing the molecular players is a prerequisite for the investigation of digestive downstream events induced by released nutrients. The specific digestive enzymatic action on the ingested macromolecules might have a great influence on the production and action of bioactive compounds. More importantly, the testing of in vitro digested products in human intestinal cell culture models needs in-depth validation of the digestive processes. A reliable interpretation of the results is only possible by knowing the molecular composition of the original products, their fate, and the outgoing metabolites.

So far, most in vitro digestion systems that included an absorption cell culture step simply focused on compound bioavailability and were not designed to determine physiological effects (1–3). Very few studies have concentrated on the functional properties of food components after digestion and absorption of nutrients (4). Digestion models used in such studies either contained nonmammalian enzymes or applied ion and enzyme concentrations far different from physiological ranges.

Milk is a large repository of different nutrients and bioactive compounds and, for a long time, bovine milk has been one of the most important nutrient sources in the human diet. Therefore, milk was chosen as a complex food matrix for validation of the in vitro digestion system. According to the USDA nutrient database (no. 01211), bovine milk contains ~3.15 g protein, 3.27 g fat, and 5.05 g carbohydrates (lactose)/100 g milk (100 mL milk corresponds to 103 g). The major protein fractions in milk are caseins and whey proteins, accounting for 80 and 17% of total milk proteins, respectively. The remaining 3% consist of hundreds of minor proteins. The casein fraction is dispersed in the form of calcium-containing casein micelles (5). In the course
of digestion, the proteins in milk are denatured by gastric acidification and subsequently degraded by pepsin and pancreatic proteases into peptides and AA. The mean peptide size in the jejunum, including the amino- and di-peptidase activity of the enterocytes, is 3–6 AA (6).

Native milk fat is dispersed in the form of milk fat globules, consisting mainly of TG (95%) enclosed by the milk fat globule membrane with embedded minor milk proteins, enzymes, diacylglycerols (2%), phospholipids (1%), cholesterol (<0.5%), and FFA (<0.5%) (5,7). TG are digested primarily by pancreatic lipase in the upper jejunum after emulsification by bile acids, leading to a liquid-crystalline interface at the surface of the emulsion particles (8). The main activity of pancreatic lipase on the sn1 and sn3 positions of the TG results in the release of 2-monoacylglycerol and FFA (9,10). Hydrolysis and absorption across enterocytes is facilitated by their inclusion into mixed micelles (11).

Lactose, the major carbohydrate present in milk, besides a small number of oligosaccharides, is cleaved by lactase secreted by intestinal enterocytes and therefore requires an additional digestive step not included in this system.

The in vitro digestion model presented in this work is designed for the investigation of physiological effects when used in combination with cellular absorption. Therefore, ionic compositions, enzyme concentrations, and pH profiles of all digestive juices were chosen to be close to human physiology, compositions, enzyme concentrations, and pH profiles of all in combination with cellular absorption. Therefore, ionic compositions, enzyme concentrations, and pH profiles of all digestive juices were chosen to be close to human physiology (12,13) to ensure a physiological degree of macronutrient degradation. System validation was performed by analyzing macronutrient degradation and comparing the results with physiological data from human studies.

**Materials and Methods**

**Chemicals and reagents.** Pasteurized and homogenized whole milk was obtained commercially (Migros). The macronutrient content was determined by standard analytical methods (proteins: International Organization for Standardization 8968, fat: International Dairy Federation 001D-1996, lactose: pH differential method using an EC Microlab EFA instrument and the kit provided by the manufacturer). The composition was: protein, 30.5 ± 0.7 g/L; fat, 34 ± 0.1 g/L; and lactose, 46.9 ± 0.4 g/L. The chemicals for the determination of AA and L-norleucine were from Merck (Merck Nr. 24560). All other chemicals and enzymes used were from Sigma Aldrich.

**In vitro digestion model.** Milk was digested in vitro according to a modified protocol developed by Versantvoort et al. (14). Briefly, the ion concentrations in the digestive juices were taken from the literature (12,13), including physiological concentrations of calcium and magnesium (Supplemental Table 1). Human lysozyme was added to the saliva (18.7 mg/L). Mucin type II and type III (Sigma Aldrich) were added to the digestive juices. Pasteurized homogenized whole milk (2.25 mL) was incubated with 3 mL of saliva for 5 min, then with 6 mL of gastric juice for 120 min and, subsequently, with 6 mL of pancreatic juice plus 3 mL of bile juice for 120 min (Supplemental Fig. 1). All incubations were performed at 37°C on a rotating wheel. After digestion, all samples were immediately placed on ice and frozen at −80°C for further analysis. Details of the composition of the digestive juices are listed in Supplemental Table 2.

**Gel electrophoresis.** Equal amounts of proteins were separated by SDS-PAGE after quantification with the BCA protein determination kit (PIERCE). A molecular weight marker (Benchmark, Invitrogen) was included on each gel. Gels were stained with colloidal Coomassie (15). Samples from pasteurized whole milk and controls (water) were collected at each step and at different time points (30, 60, 90, and 120 min) of the gastric and pancreatic phases. The samples were analyzed by SDS-PAGE, separating proteins >5 kDa in size.

**In-gel tryptic digestion.** Polyacrylamide gel pieces were manually excised from the protein bands of interest. Gel plugs were washed three times, alternating between 100 µL ABC (25 mmol/L) in acetonitrile (50%) and 100 µL ABC (25 mmol/L). They were subsequently digested with trypsin (4 mg/L) in 20 µL of ABC (10 mmol/L) in acetonitrile (5%) at 25°C overnight.

**LC-MS analysis after tryptic digestion.** Peptides were separated on a Rheso 2200 HPLC (Flux Instruments) equipped with an X Terra MS C18 column (3.5 µm, 1.0 mm × 150 mm, Waters). The HPLC was directly coupled to a linear ion trap mass spectrometer (LTQ, Thermo Scientific) using an electron spray ionization interface.

**Detection of low-molecular peptides, AA, and amines (OPA method).** Proteins and longer peptides were precipitated with perchloric acid (0.5 mol/L). AA and peptides in the supernatant were measured after reaction with OPA in the presence of mercaptoethanesulfonic acid at 335 nm (16). In detail, 25 µL of the samples and 25 µL of perchorlic acid (5 mol/L) were mixed, diluted with 75 µL water, and incubated at 4°C for 15 min. After centrifugation, 30 µL of supernatant were mixed with a reagent solution (0.05 mol/L borate, 10 g/L laurylsulfate, 0.8 g/L OPA, 5 g/L Na-MES, and 5 g/L Triton X-100) at a ratio of 1:30, incubated at room temperature in the dark for 40 min, and measured at 335 nm. Absorbance readings from the samples and the water controls were compared to the values of a glutamate standard curve.

**AA analysis (HPLC).** Proteins from samples (25 µL) were precipitated with acetonitrile (200 µL) together with 3.125 nmol of norleucine as an internal standard. After centrifugation (4500 × g, 1 min), the supernant was dried and derivatized with 6 µL phenylisocyanate in methanol: water: triethylamine (420 µL:60 µL:60 µL) and then dried. AA were analyzed by HPLC on a Nova-Pak column (C18, 4 µm, 3.9 mm × 150 mm, Waters) using a gradient method (5–60% acetonitrile, 50°C) and detected with a diode array detector (Summit Dionex) at 247 nm.

**Determination of total AA.** Samples were hydrolyzed in the gas phase with hydrochloric acid (6 mol/L) containing 0.1% (by volume) phenol at 115°C for 24 h under vacuum (17). The liberated AA were treated as described above. Tryptophan could not be detected after hydrolysis due to oxidative degradation during acid hydrolysis (18).

**Peptide length determination (size-exclusion HPLC).** The digested samples were filtered through Microcon columns (Ultracel YM-30, Millipore) and the samples were separated on a size-exclusion column (BioBasic SEC-300, 7.8 mm × 150 mm, Thermo Scientific) using Buffer A (50% water:50% acetonitrile:0.1% formic acid). The flow rate was 0.25 mL/min at 40°C and the detection was performed at 200 nm. A standard mix of α-casein, α-lactalbumin, a 20-Å fragment of miraculin (DSAPVPVLIDDIQQKLRTGTN, Bachem), and the tripeptide VPP were used for size comparison.

**Peptide length analysis (LC-MS).** After sample filtration through Microcon columns (Ultracel YM-30, Millipore), peptides (>5 AA) were separated and analyzed according to the LC-MS method described above. The samples were measured in multiple overlapping, narrow-mass windows (100 m/z) and all raw files were merged for an identification search with Mascot (Matrix Science) by using a database containing the major milk proteins. The results were validated manually and the identified peptides were summed up according to their length.

**FFA determination (GC).** The digestion of fat was monitored through the quantification of FFA before and after digestion. After addition of 10 mL ethanol to the internal standards (C5, C7, and C13) and samples, the FFA were extracted 3 times with a mix of diethyl ether:heptane (1:1; v/v). The combined extracts were dried with sodium sulfate and subsequently separated over an aninopropyl column (DB-FFAP 15 m, 530 µm, PH, Stelheld & Cie). The extracted fat was excluded with a mix of 2-propanol:hexane 2:3 (v/v). FFA were eluted with diethylster containing 2% formic acid. The eluate was analyzed by GC (19).
**Statistics.** A water (control) digestion was performed with each digestion and values were subtracted from the milk digestions. All the experiments were repeated independently at least three times. Values in the text are mean ± SD. The data were analyzed with SYSTAT 13 (Systat Software). Repeated measurements (free AA and OPA) were analyzed as linear mixed models with the restricted maximum likelihood algorithm with correlated errors (first order auto-regression) (20). Technical replications (OPA) were averaged before analysis to keep the covariance structure simple. Multiple comparisons among least squares means were performed using the Tukey-Kramer procedure and paired observations (FFA) were analyzed by the paired t test (after log-transformation) testing the null hypothesis 

\[ \log(H_0: \mu_{\log(ST0)} = \mu_{\log(ST3)}) \]

Differences of \( P < 0.01 \) were considered significant.

**Results**

**Protein digestion.** A comparison of the protein pattern of the digested samples revealed that all caseins and \( \alpha \)-lactalbumin were completely digested within 30 min by the gastric juice (Fig. 1A, lane 2). Surprisingly, \( \beta \)-lactoglobulin was digested only after incubation with pancreatic juice (Fig. 1A, lanes 6 and 10), indicating that pepsin alone was not digesting \( \beta \)-lactoglobulin but that the presence of pancreatic enzymes and bile is needed. Protein bands were cut and identified from milk samples and controls at different sampling times (Fig. 1). After complete digestion (ST 3), only digestive enzymes and traces of milk-derived peptides were identified (Supplemental Table 3). These results showed that the system degraded most of the proteins into fragments smaller than 5 kDa, which is the lower protein separation limit of SDS-PAGE.

Single AA and oligopeptides released after different digestive steps were determined with the OPA method. Starting with 32 g protein/L of milk, containing 3.37 ± 1.52 mmol glutamate equivalents/L milk of free AA and di- and tripeptides, 112 ± 5.46 mmol glutamate equivalents/L milk (\( P < 0.0001 \), ST 0 compared to ST 3) were liberated by the digestive process, corresponding to 53.9 ± 2.63% of the total amount of milk AA. Samples incubated in the absence of bile were less efficiently digested, namely 90.0 ± 5.24 mmol glutamate equivalents/L milk, corresponding to 43.4 ± 2.63% (\( P < 0.0001 \), ST 3 compared to ST 3 without bile) of total milk AA, showing the importance of bile action for digestion efficiency. To confirm and extend the findings obtained with the OPA method, the individual AA released after milk digestion were quantified by HPLC (Fig. 2). The total amount of free AA after complete digestion (ST 3) of milk was 20.7 mmol/L milk, corresponding to 9.97 ± 1.2% of the inserted milk proteins, whereas undigested milk contained 1.16 ± 0.01 mmol/L milk (Fig. 2A). Interestingly, 93.6 ± 6.0% of the released AA was essential for humans, representing an increase in the relative distribution of essential AA compared to the total AA profile of undigested milk, which contains 44.5 ± 0.9% of essential AA (Fig. 2B; Supplemental Fig. 2A,B). The control digest contained 47.6 ± 6.5% essential AA that originated exclusively from the digestive juices.

Considering the results from the OPA (53.9%) and HPLC (9.97%) analysis, we deduced that ~50% of the proteins were degraded into di- and tripeptides and that 10% of those proteins were degraded to free AA. However, the size distribution of the remaining proteins and peptides in the range between 5 kDa and tripeptides was still unclear. After size-exclusion HPLC, the peptides in the digested milk eluted in 3 major peaks (Fig. 3A). Comparing the chromatograms with those from known proteins and peptides, the first peak of the digested milk eluted between the 20-mer miraculin (Fig. 3A, peak 4, 2.11 kDa) and the tripeptide VPP; the major peak coeluted with VPP (Fig. 3A, peak 5, 313 Da); and the 3rd peak eluted after VPP. These results indicate that none of the peptides present after digestion (ST 3) was longer than 20 AA. Samples taken after each digestive step (ST 1–3 and 3 without bile) showed that the protein and peptide degradation was indeed progressing to smaller peptides (Fig. 3B).

![FIGURE 1](image) Representative Coomassie-stained polyacrylamide gels of pasteurized milk, undigested and at different digestive stages (A); water control at different digestive stages (experiment was repeated 3 times) (B). ST 1–3 and 3 without bile refer to the sample collection times used for all other experiments. Indicated spots were identified by MS. Spots 1–11 are milk proteins, and spots 12–21 are digestion enzymes. Identifications for each spot are listed in Supplemental Table 3. B, bile at different time points (5, 30, 60, 90, and 120 min); GJ, gastric juice; PJ, pancreatic juice; S, saliva; ST, sampling time.
These results could be confirmed with LC-MS/MS by multiple data-dependent identification runs, using overlapping small mass windows (Fig. 3C). Fully digested milk contained a majority of hexapeptides. This result was confirmed by quantitative Edman degradation (Supplemental Fig. 3). Fat digestion. Undigested milk contained 1.04 ± 0.03 mmol FFA/L milk and after complete digestion, 62.8 ± 15.4 mmol FFA/L milk (P < 0.005, ST 0 compared to ST 3) was found, corresponding to 40.8 ± 12.2% of the total milk fat (34 g/L). However, assuming that all TG (molecular weight = 800) present in whole milk were hydrolyzed into two FFA (both Mr = 300) and one monoacylglycerol (21), this value corresponded to 100% of the TG. The relative FFA distribution (Supplemental Fig. 4A) showed that more SCFA (10.9 ± 0.64%) were released after digestion compared to the relative total fatty acid distribution of the inserted milk containing 5.82 ± 0.17% of SCFA (Supplemental Fig. 4B; fatty acid profile: Supplemental Table 4).

Discussion
An in-depth analysis of food digestion is essential for understanding the physiological effects of food consumption. Therefore, a human in vitro digestion system was designed that digests food, especially dairy products, simulating all steps upstream of intestinal enterocyte absorption. Enzyme concentrations were calculated according to averaged physiological values and the system was validated by comparing the digested products with reported human values (6, 9, 22). An increasing number of reports are focusing on the bioactive effects of peptides derived from milk proteins, either present in undigested milk or generated during digestion, probably influencing their physiological function. Although most of the published studies have concentrated on protein-derived compounds, bioactive...
compounds derived from lipids such as CLA and carbohydrates, should also be kept in mind (23–26).

The biological effects underline the importance of an in vitro digestion system that closely mimics the digestive process in humans. To this end, dynamic models have been developed that are closer to human physiology than static models (27). In addition, the mechanical actions caused by muscle contraction of the stomach and small intestine influence the digestive rate (28,29). However, the strength of our model relies on the detailed characterization of macronutrient decomposition along all levels of the in vitro digestive process, which was consistent with results of human digestion studies.

**Protein digestion.** Analysis and identification of proteins showed that after incubation with saliva and gastric juice, all proteins, with the exception of β-lactoglobulin, were degraded to smaller peptides. The peptides were further degraded at the end of the digestive process; besides some minor milk-derived peptides, only peptides from the digestive enzymes could be identified. Earlier reports have shown β-lactoglobulin resistance to gastric pepsin (30) and the necessity of bile for its digestion (31). It was proposed that lipophilic molecules, including fatty acids and TG, bind to β-lactoglobulin and therefore prevent access of proteases (31).

After complete digestion, 54% of the total milk proteins were degraded to free AA, dipeptides, and tripeptides. This rate was significantly reduced without bile (43%), further underlining its importance.

HPLC analysis revealed that 10% of the total milk proteins were degraded to free AA, 94% of which are essential for humans, including arginine and tyrosine, which are essential for children (32). Thus, essential AA are preferentially released from milk containing 45% of essential AA. Negative values for some AA can be explained by autoproteolysis of enzymes and the enhanced digestion of BSA contained in the digestive juices.

To determine mean peptide length after digestion, 3 different approaches were selected. Analysis by size-exclusion HPLC showed that the highest peak eluted with the same retention time as the tripeptide VPP. LC-MS/MS, with a lower identification limit of 5 AA, had the highest number of peptides, with 6 AA in length, a finding that was further confirmed by Edman degradation. The results corresponded to values from human studies that reported a mean peptide length of 3–6 AA after digestion (6).

Therefore, it is apparent that the digestive system is suited to releasing physiological levels of AA and small peptides from food, particularly dairy products.

**Fat digestion.** Lipid digestion in humans starts with lingual and gastric lipases. Emulsification takes place via peristalsis in the stomach and mostly in the duodenum, where the crude emulsions are mixed with bile and pancreatic juice (33). TG hydrolysis occurs through the action of pancreatic lipase in the duodenum with variable importance of preduodenal lipases (11). Lingual and gastric lipases were not included in the system; therefore, pancreatic lipase, present in pancreatin, was the sole enzyme responsible for fat digestion.

The fat in homogenized milk is organized in small droplets and contains only very small amounts of FFA. After full digestion, the result of 40% of liberated FFA was higher than previous reports of 15.5–25% (34–36). Thus, pancreatic lipase efficiently digested the milk fat.

After digestion, the number of SCFA was higher than the theoretical fat composition of milk. This finding can be explained by the preferred location of long-chain fatty acids at the sn2-position in bovine milk (11,35) and the preference of lipase to hydrolyze the sn3 and sn1 positions, therefore leading to the formation of sn2-monoacylglycerol and FFA (36).

Milk fat normally consists of up to 95% TG (5) and its absorption efficiency is >95% (11). This rate is strongly dependent on the initial fat droplet size being increased for smaller fat droplets, such as for homogenized and pasteurized milk (34). With a calculated fat digestion efficiency of ~100% of total TG, this in vitro digestive system can be used to analyze products with different fat droplet sizes or different fat compositions.

**Digestion of pasteurized and homogenized whole milk.** Products from in vitro digestion of all macronutrients present in pasteurized and homogenized whole milk were in line with human data (6,9,22). Moreover, a preferential release of essential AA was observed, and it illustrated the benefit of in vitro systems in identifying important nutritional properties of food whose relevance needs to be confirmed in vivo.

In conclusion, in this study, an in vitro digestion system was designed, modeling the digestion of food macronutrients upstream of enterocyte action. The digestive system was validated by monitoring the degradation of macronutrients in pasteurized and homogenized whole milk. Results for all macronutrients were consistent with human physiological values found in the literature. The digested products can be applied to an intestinal cell culture model to study the last step of digestion and the final absorption of nutrients, both steps being mediated by intestinal enterocytes.

Our in vitro digestion system can be applied as an efficient screening tool for identifying specific properties of food and nutrients and it presents an important prerequisite for downstream absorption and functionality studies.

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