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Léa Salelles, Juliane Floury, Steven Le Feunteun. Pepsin activity as a function of pH and digestion time on caseins and egg white proteins in static in vitro conditions. Food and Function, 2021, 10.1039/D1FO02453A . hal-03388703

HAL Id: hal-03388703
https://hal.inrae.fr/hal-03388703v1

Submitted on 20 Oct 2021

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DOI: 10.1039/D1FO02453A

ARTICLE

Pepsin activity as a function of pH and digestion time on caseins and egg white proteins in static *in vitro* conditions

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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The activity of pepsin, the gastric protease, is generally considered to be negligible for pH ≥ 4, based on results obtained with few purified globular proteins. The present study aimed at studying the activity of porcine pepsin on egg white proteins (EWP) and casein micelles (CA) over a broad range of pH (from 1 to 7) at short (3 min) and long (2 h) digestion times. At short time, results confirmed a tendency for a higher rate of hydrolysis with decreasing pH, but with different pH activity profiles for both substrates. More remarkably, the degree of hydrolysis of CA after 2 h of digestion was constant from pH 1 to pH 5, and was only reduced by half at pH 6. This finding demonstrates that pepsin can hydrolyse caseins from the very beginning of gastric digestion. Interestingly, the shape of the reaction kinetics over 2 h appeared to be rather characteristic of the type of substrate and largely independent on pH. Most hydrolysis profiles could be accurately fitted by a power law, an empirical model that was then successfully applied to the static *in vitro* gastric proteolysis of 6 other food matrices. Overall, our results support the idea that pepsin activity in weakly acidic conditions (pH ≥ 4) should not always be neglected, in particular for milk caseins, and that pepsin reaction kinetics during static *in vitro* gastric digestion seem to evolve proportionally to the power of digestion time.

1. Introduction

The behaviour of food matrices in the acidic conditions of the stomach can impact the dynamics of gastric emptying, and hence the postprandial appearance of amino acids in the blood[1–4]. The gastric phase of digestion is, therefore, considered to be of paramount importance in the kinetics of digestion and absorption of dietary proteins[5–7]. At this stage, the enzymatic hydrolysis of proteins is initiated upon the action of pepsin, responsible for the hydrolysis of up to 5-15% of the peptide bonds[8,9]. Pepsin is an aspartic endo-protease showing an activity that is highly dependent on pH. Using serum albumin as a substrate, the activity of human pepsin has been shown to be negligible for pH ≥ 5 and optimal around pH 2[10]. Similar results have been obtained with porcine pepsin, which is extensively used in *in vitro* digestion studies because it is considered as the best substitute of human pepsin[11,12]. For instance, using native haemoglobin as a substrate, the activity of porcine pepsin has been repeatedly found to show a bell-shaped curve rising for pH ≤ 4 with an optimum around pH 2[13–15].

However, it is well known that the pH activity profile of an enzyme can vary from one substrate to another and/or on the exact tri-dimensional conformation of the substrate. In the 1950s, it has for example been shown that the activity of human pepsin on egg albumin[16] and bovine serum albumin[17] was much higher around pH 3 and 4 when the proteins were denatured beforehand. It has even been shown that the apparent pH optimum of pepsin on haemoglobin can be shift from 2.0 to about 3.5, with considerable activity up to pH 5, after urea based denaturation procedures[17]. Considering the great diversity of edible proteins in terms of primary, secondary and tertiary structures, this opens questions on the exact contribution of pepsin hydrolysis during the course of gastric acidification. Indeed, it is well known that the average gastric pH after a meal lowers slowly, typically decreasing from pH ~ 6 down to 2 in about 2 hours[18,19]. Moreover, some values of gastric pH at half-gastric emptying time as high as ~ 4 and 5.5 have been reported in the literature on pig and human digestion[20–22]. Such considerations have been reported to be of key importance to understand the gastric digestion of lipids[22] and starch[23].

With regards to the digestion of proteins, less work have been undertaken since the pioneer works of Christensen (1955)[16] and Schlamowitz and Peterson (1959)[17] quoted above. Yet, of particular note is the recent study of Sams et al. (2018)[24] in which it is shown that the kinetics of hydrolysis of β-casein by pepsin in conditions reproducing the early stage of gastric digestion (pH 5.5, 2.9 µg.mL^{-1} of pepsin) remained in the same order of magnitude as those measured using much more favourable gastric *in vitro* conditions (pH 2.5, 50 µg.mL^{-1} of pepsin)[25]. As stated by the authors, this raises serious concerns about the effects of pH on pepsin activity. Few other hints can be found in the literature suggesting that pepsin activity might not always be much higher at very acidic pH, as for instance in the study of Dekkers et al. (2016)[26] who found similar extents of

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

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gastric proteolysis after 90 min in static pH conditions at pH 1.9 and in dynamic pH conditions from 6 to 2. To improve our understanding of the gastric digestion of food proteins, more data on pepsin activity in weakly acidic conditions are thus clearly needed.

The aim of the present study was to investigate the activity of porcine pepsin on egg white proteins (EWP) and acid-induced micro-aggregates of casein micelles (CA) in static *in vitro* conditions over a broad range of pH: from 1 to 7. Two sets of experiments, with short (3 min) and long (2 h) digestion times, were conducted using the OPA and pH-STAT methods to assess the extent of protein hydrolysis. The substrates were chosen for their content in different proteins, as it is typically the case in a real food or meal, and because these can be considered as good food models of two extreme cases: i) solutions of native globular proteins, and ii) supramolecular assemblies of non-globular proteins. Indeed, the four main proteins of EWP are globular proteins (ovalbumin, ovotransferrin, ovomucoïd and lysozyme)[27], whereas CA consist of agglomerates of casein micelles (average diameter of ~ 150 nm) that are made of a mix of proteins (mainly αs1, αs2, β and κ-caseins) holding together with little individual tertiary structure[28]. The choice for studying acid-induced casein micro-aggregates (prepared in advance) was made to limit the influence of the micellar casein structures on proteolysis, providing that casein micelles remain in suspension for pH > 5 but form uncontrolled clots below[28,29]. For our gastric digestions performed at pH ≥ 5, it should thus be understood that the caseins we studied were not in the form they will normally have in the early stage of *in vivo* milk digestion, these probably better compare to yogurt particles. Sparse data on the effect of pH the hydrolysis of EWP and caseins by pepsin can be found in the literature, as for instance in Ruan, Chi and Zangh (2010)[30] for EW and Tam and Whitaker (1972)[31] for caseins. However, these studies were not undertaken in the objective of studying the gastric digestion of proteins, hence rendering any comparison with realistic gastric digestion conditions very difficult. Our results are, nevertheless, discussed in the light of these previous investigations.

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2. Materials and Methods

2.1. Materials

2.1.1 Food proteins: Eggs were bought at a local supermarket. Egg-whites were separated by hand, homogenised (1 min in 1 L containers using a Ultraturrax Digital Homogenizer IKA-T25 with the axis S25N-/01.814428, 10000 rpm, followed by 5 min of slow spatula homogenization). Concentrated suspensions of native caseins (13 w/w%) were obtained at INRAE (Rennes) from a combination of microfiltration and diafiltration steps of bovine unheated skim milk, as fully described Silva et al. (2015)[32]. The protein content of egg-white was determined to be 10.5 ± 0.1% using the Kjeldhal method with a conversion factor of ×6.25. Both egg-white and caseins were aliquoted in 240 mL flasks and stored at -20°C before use.

2.1.2. Chemicals: Porcine pepsin (P6887-5G), Pepstatin A (P5318-25G) and Ortho-phtalaldehyde (P0657-5G) were all bought from Sigma-Aldrich. The pepsin activity was determined to be 2465 ± 141 U/mg using the pepsin enzymatic assay described in Minekus et al. (2014), Supp data,[12] which relies on dosing hydrolysed TCA-soluble peptides using absorbance at 280 nm after reaction on haemoglobin at pH 2 and 37°C. Pepstatin A, a pepsin inhibitor, was solubilised in ethanol at 0.5 mg/mL and kept a 4°C before use. Water was Milli-Q water and all other chemicals were of classical analytical grade and bought from Sigma-Aldrich, Merck, AnaLR NormaPur, Panreac and VWR chemicals.

2.2. Preparation of food proteins

Casein micelles curdle at pH ≤ 5. To limit the differences between the initial casein substrate's macro- and microstructure in our experiments, we have chosen to always produce casein micro-aggregates with the same procedure (same beaker, pH, temperature, and speed of acidification). The pH of diluted caseins (2% w/w) at 20°C was slowly decreased down to 4.0 using 900 µL of 300 mM HCl with a 20 µL step-wise procedure. In all that follows, it should thus be understood that caseins were initially in the form of a suspension of micro-aggregates (CA: casein aggregates). Egg-white proteins (EWP) do not precipitate at acidic pH, and could be studied as a native protein solution at the same protein concentration (2% w/w).

2.3. Static *in vitro* gastric digestions at different pH

Two sets of experiments were conducted at different pH with both CA suspensions and EWP solutions. The first set was used to monitor the kinetics of protein hydrolysis during 2 h of digestion with a high temporal resolution using the pH-STAT method. The degree of hydrolysis (DH) of end samples were determined with the ortho-phtaladehyde (OPA) method. The second set of experiments was used to assess the initial reaction rate of hydrolysis (after 3 min) with the OPA method.

2.3.1. Gastric digestions lasting 2 h: Static *in vitro* gastric digestions monitored by pH-STAT were carried out for 2 h at various pH (from 1 to 7) on both EWP and CA. Gastric pH excepted, all experimental conditions complied with the recommendations of the INFOGEST protocol for the gastric phase of static digestion[12], where details can be found on the composition of digestive fluids and enzyme activities. We specifically followed the recommendation on the replacement of $NaHCO_3$ by NaCl at the same molar ratio in the electrolyte solutions to avoid unwanted pH drifts during pH-STAT measurements[11,33]. All experiments were performed in a Metrohm 20-90 mL jacketed beaker maintained at 37°C by water circulation, with a magnetic stirring at 350 rpm. An

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automatic titration unit (Titrando 842 titration unit from Omega Metrohm with dosing unit Dosino 800/807, France) was mounted on this set-up. For each experiment, 7.5 g of EWP solution or CA suspension (2% w/w of proteins) were mixed with 7.5 mL of simulated salivary fluid (SSF) with no salivary α-amylase (freshly added with 37.5 µL of a 0.3 M $CaCl_2$ solution) . 13 mL of simulated gastric fluid (SGF) electrolyte solution were subsequently added, and this solution was warmed up until stabilisation at 37 °C. 7.5 µL of a 0.3 M $CaCl_2$ solution was added and the pH of the solution was adjusted to the desired pH (from 1.0 to 7.0) using an appropriate HCl (range: 0.05-2 M) or NaOH (range: 0.05-0.5 M) solution. Water was added to bring the volume to 29.25 mL and the titration was started in pH-STAT mode. After a waiting time of 10 min for pH stabilisation, 0.75 mL of a 80,000 U/mL pepsin solution (prepared on ice and adjusted at pH = 4.0) was finally added to reach 2,000 U/mL in the reaction mixture. The pH-STAT device was programmed to maintain the pH at the desired value, with a data acquisition frequency of 1 Hz, using an appropriate titrant depending on the substrate and pH (HCl 0.05 M; HCl 0.15 M or NaOH 0.05 M). After 2 h, 300 µL of pepstatin A (0.5 mg/mL in ethanol) was added to stop the reaction. The pH was then neutralized to 7.5 using low volume (< 2.5 mL) of appropriate NaOH solutions to ensure a suitable analysis of all the end samples by the OPA method (as it relies on a chemical reaction at alkaline pH). The final reaction mixture was then collected and stored at -20°C until further analysis with the OPA method. For each couple pH/substrate, the experiment was run in triplicate, and two blank experiments were conducted with a heat-inactivated pepsin solution (85°C, 5 min in 2 mL Eppendorf tubes) adjusted to pH 4.0. The mean curve of these blank titrations was subtracted to each titration curve obtained in the presence of pepsin to correct the pH-stat data from the contribution induced by the unbalanced pH of the pepsin solution.

2.3.2. Gastric digestions lasting 3 min: Substrates were prepared as previously described (same beaker and conditions). Three samples of 3.9 mL were collected, adjusted at the desired pH, and warmed at 37°C in a water bath as verified with a thermometer. 100 µL of a 80,000 U/mL pepsin solution was then added and the reaction was stopped exactly 3 min after by adding 40 µL of pepstatin A (0.5 mg/mL in ethanol) and rising the pH up to 7.5 with pre-calibrated volume of NaOH solutions. For each couple pH/substrate, the experiment was run in triplicate, and two blank experiments were conducted with addition of pepstatin A at pH 7.5 before the pepsin addition.

2.4. Degree of hydrolysis (DH) of proteins

2.4.1. DH measured with the OPA method: The DH of proteins was measured with the o-phthalaldehyde (OPA) method on the end samples of the gastric digestions lasting 3 min and 2 h. The protocol we used has been previously described [34], and relies on the method of Church et al. (1983),[35] adapted to microplate. Briefly, 100 mL of reagent were prepared with 2.5 mL of OPA (10 mg/mL in ethanol), 2.5 mL of SDS 20%, 50 µL of β-mercaptoethanol, and 95 mL of sodium tetraborate 20 mM. The reagent was protected from light with an aluminium foil, and stored at 4°C for maximum 30 h if not used immediately. UV transparent 96-well plates and a Multiskan™ GO microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA USA) were used to measure the absorbance at 340 nm after 10 min of contact between the OPA reagent (100 µL) and diluted samples (50 µL) using on/off shaking cycles of 5/40 s. Each sample was measured 3 times using 3 different wells on the same plate. The quantity of α-amino groups released by hydrolysis, which corresponds to the quantity of hydrolysed peptide bonds (*h*), was estimated from the difference in mean absorbance at 340 nm between hydrolysed and unhydrolyzed samples, using L-methionine for the calibration curve (0 - 2 mM). The DH of proteins (%) was then calculated using:

$$DH = \frac{h}{h_{tot}} \times 100 \qquad (1)$$

where h_{tot} is the total number of peptide bonds per gram of protein, taken as 8.2 meqv/g of caseins, and 8.0 meqv/g of EWP[36].

2.4.2. DH measured with the pH-STAT method: pH-STAT can be used to determine the DH of proteins using Eq. 2 at neutral or alkaline pH with a basic titrant[37], and Eq. 3 at acidic pH with an acidic titrant[38] :

$$DH\ (\%) = \frac{V \times N}{m \times h_{tot}} \times \frac{1}{\alpha_{NH_3^+/NH_2}} \times 100 \qquad (2)$$

$$DH\ (\%) = \frac{V \times N}{m \times h_{tot}} \times \frac{1}{1 - \alpha_{COOH/COO^-}} \times 100 \qquad (3)$$

where *V* is the volume of added titrant (mL), *N* is the normality (meq/mL) of the titrant, *m* is the mass of protein (g), h_{tot} as the same meaning and values as in Eq. 1, α_{COOH/COO^-} is the mean dissociation degree of peptides' C-terminus carboxylic groups, and $\alpha_{NH_3^+/NH_2}$ is the mean dissociation degree of peptides' N-terminus amine groups.

For the digestion experiments performed at pH 5 and 6, that is between purely acidic and neutral conditions, preliminary experiments were conducted to determine whether pH-STAT remained a suitable method to monitor protein hydrolysis by pepsin, and if yes, with which kind of titrant (*i.e.* basic or acidic). Although the sensitivity of the method was far from optimal in these mildly acidic conditions, results showed that the kinetics of protein hydrolysis could be assessed using an acidic titration (HCl 0.05 M) for both EWP and CA at pH 5, and a basic titration (NaOH 0.05 M) for CA at pH 6. No pH variations could be detected during *in vitro* digestions of EWP at pH 6 because of a lack of protein hydrolysis by pepsin, as confirmed by lack of EWP hydrolysis detected by OPA at this pH. Moreover, no pH variation could be detected either at pH = 1 for both EWP and CA, most probably because of the very high buffering capacity of such strongly acidic solution[39].

As depicted in Eq. 2 and 3, the underlying reason for the use of an acidic or basic titrant is related to the exact values taken by $\alpha_{NH_3^+/NH_2}$ and α_{COOH/COO^-}. These are needed to convert pH-STAT results into

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DH but depend on both the operating pH and the protein substrate. For each couple substrate/pH, these values could be estimated *a posteriori* from the OPA measurements of DH at 2 h of digestion (§2.4.1), as previously proposed[33]. The values of α_{COOH/COO^-} at pH 2, 3, 4, 5 for EWP and CA were estimated to be 0.02, 0.19, 0.70, 0.96 and 0.02, 0.16, 0.66, 0.95, respectively. The values of $\alpha_{NH_3^+/NH_2}$ at pH ≤ 5 were all negligible (< 0.03) for both EWP and CA. The pH-STAT results obtained in conditions where pH ≤ 5 were therefore converted into DH using corresponding α_{COOH/COO^-} values for EWP and CA in Eq. 3. For CA at pH 6, the value of α_{COOH/COO^-} was estimated to be 1.00, *i.e.* fully dissociated peptides' C-terminus carboxylic groups that have no effect on pH (Eq. 3 becomes invalid), and the value of $\alpha_{NH_3^+/NH_2}$ was estimated to be 0.23. The pH-STAT results obtained for CA at pH 6 were therefore converted into DH using a value of 0.23 for $\alpha_{NH_3^+/NH_2}$ in Eq. 2. Only the gastric digestions lasting 2 h were monitored by pH-STAT. The DH values obtained at 3 min with pH-STAT therefore correspond to the DH values measured at 3 min of the gastric digestions lasting 2 h.

2.4.3. Empirical modelling of the DH measured by pH-STAT: A power law model was used to fit the DH recovered from pH-STAT experiments using the relation:

$$DH = a \times t^b \qquad (4)$$

where a is a pre-factor, t is the time (min) and b is a power exponent. This relation has been previously used to model the hydrolysis kinetics of bovine lactoferrin by pepsin[40], and was found to accurately simulate most of our experimental data. With this model, the value estimated for the power exponent (b) remained very stable for a given substrate (EWP or CA) at all pH. In a second step, the power law model was therefore adjusted to the hydrolysis kinetics using a common power exponent for each substrate at all pH. All fittings were performed using Excel 2016 and its evolutionary solving method by minimizing the sum of the squared distance between model predictions and experimental data within the range from 3 min to 2 h.

2.5. Characterisation of protein substrates

2.5.1. Morpho-granulometry: CA suspensions at different pH (from 1 to 6) were prepared using the exact same procedure as for the hydrolysis experiments (§2.3.1) with no addition of pepsin. The 30 mL of CA suspension were thereafter diluted to 300 mL in SGF electrolyte solution at the appropriate pH. The surface weighted mean diameter ($d_{3,2}$) of the particles were measured on the 300 mL CA suspension using the morpho-granulometry setup QCIPIC (QP0205) with LIXELL dispersion system and M6 cuvette (5-1, 705 µm) from SYMPATEC. All experiments were performed in triplicate.

2.5.2. Confocal microscopy: Confocal laser scanning microscopy (CLSM) observations of the CA suspensions at different pH were performed using a ZEISS LSM 880 inverted confocal microscope (Carl Zeiss AG, Oberkochen, Germany) set at magnification 20× (dry objective lens, NA = 0.5). The CA samples were prepared the same way as for hydrolysis measurements (§2.3.1) at pH ranging from 1 to 6. Then, they were mixed with a 24 µM solution of SYTO™ 9 green fluorescent nucleic acid stain (Invitrogen, Thermo Fisher Scientific) at a sample/probe volume ratio of 10:1 in order to mark the proteins. A 50 µL drop of suspension was put between a glass slide and a cover slip sealed with an adhesive frame (Geneframe, ABgene House, UK).

Images were acquired using an argon laser with an excitation wavelength of 488 nm, and acquired using a PMT detector with a 500-530 nm emission wavelength, and a pixel dwell time of 0.66 µs. For each pH condition, a 90 µm depth Z-stack was acquired on a typical casein particle chosen on the tile scan image, using 1 µm steps (91 images per stack).

2.6. Statistical analyses :

Statistical analyses were all performed using the R software. The datasets analysed were: the mean surface weighted diameters ($d_{3,2}$) of CA as a function of pH, the DH (%) measured at 3 min for EWP and CA as a function of pH, and the DH (%) measured at 2 h for EWP and CA as a function of pH.

A one-way ANOVA was performed first for each data set to determine the impact of pH and test the normality and homogeneity of the residuals using a Shapiro-Wilk test and a Bartlett test, respectively. When both hypotheses were validated ($d_{3,2}$, DH_{3min} of EWP and DH_{2h}), a pair-wise Tukey's HSD test was performed to show which groups were significantly different from the others. When one of the two hypotheses was not validated (DH_{3min} of CA), a Kruskal-Wallis test followed by a pairwise Wilcoxon test were used instead. Normality of the data itself was also tested, and was validated except for the DH_{3min} and DH_{2h} of CA. Statistically significant effects were accepted at the 95% level.

3. Results

3.1. pH-induced modifications of the casein aggregates

Table 1 shows the evolution of the mean surface weighted diameters ($d_{3,2}$) measured by morpho-granulometry of the CA suspended in SGF (without pepsin) at different pH, as well as a typical image of an aggregate obtained using 3D confocal microscopy. At pH 6, no particle could be observed in confocal microscopy and almost no particle was detected in morpho-granulometry (on average: < 1% of the particles detected in the pH range from 1 to 5), indicating that the CA produced at pH 4 were almost fully dissociated upon an increase of pH up to 6. For pH ≤ 5, $d_{3,2}$ was significantly impacted by the pH of the SGF solution (p = 0.010), with statistically smaller aggregates at pH 2 than at pH 4 (p = 0.012) and pH 5 (p = 0.032). The morphology of the CA also appeared dependent on pH in the 3D confocal images. The surface of the aggregates looked both much smoother and denser at acidic pH in comparison to the highly granular structures were observed at pH 4 and 5. Altogether, these results therefore suggest that CA essentially consisted of agglomerates of individual casein particles that have retained a high level of integrity in weakly acidic conditions (pH range: 4-5). In more

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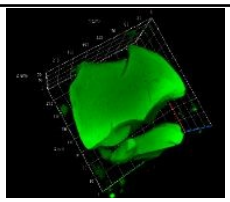
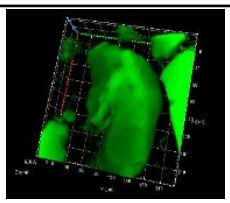
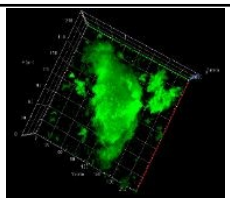
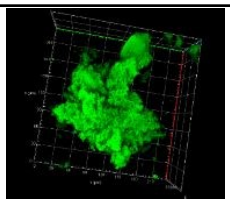
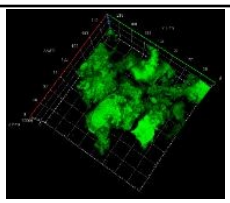
acidic conditions (pH < 3), however, casein particles seem to have interpenetrated and fused together to a large extent, hence leading to a more compact and homogeneous casein network. This can be explained by the establishment of tighter and stronger junctions induced by the decrease of electrostatic repulsions as the pH is moved away from the isoelectric point of caseins[28].

Table 1: Typical 3D (box l-green×L-red×h-blue = 210×210×90 µm³) confocal images and surface weighted mean diameters ($d_{3,2}$), determined using morpho-granulometry, of the CA prepared at pH 4.0 and thereafter suspended in SGF at different operating pH.

pH	Confocal microscopy	$d_{3,2}$ (µm) Mean ± Std
1		148 ± 35
2		106 ± 16
3		199 ± 63
4		236 ± 33
5		217 ± 25

3.2. The pH-dependence of pepsin activity at short digestion time depends on the protein substrate

The pH-dependence of the initial reaction rates (DH_{3min}) for EWP and CA are presented in Figure 1A and 1B, respectively, according to i) the pH-STAT results at 3 min of the gastric digestions lasting 2h (open symbols), and ii) the OPA results on the end samples of the gastric digestions lasting 3 min (filled symbols). Figure 2 presents the pH-dependence of final degree of hydrolysis (DH_{2h}) for both EWP and CA measured by OPA at the end of the gastric digestions lasting 2h. A significant influence of pH ($p \leq 0.001$) was observed on DH_{3min} and DH_{2h} for both EWP and CA substrates, and the pH-activity profiles of pepsin at short and long times showed comparable overall trends as a function of pH for a given substrate.

For EWP, peptic hydrolysis was negligible at pH 6 after both 3 min and 2 h of digestion, and steadily increased as the pH was lowered down to pH 1. Slight modifications can, nonetheless, be noticed in the pH activity profiles at short and long digestion times, with a straighter relationship observed for DH_{2h} (all values being statistically different from one another, $p < 0.005$).

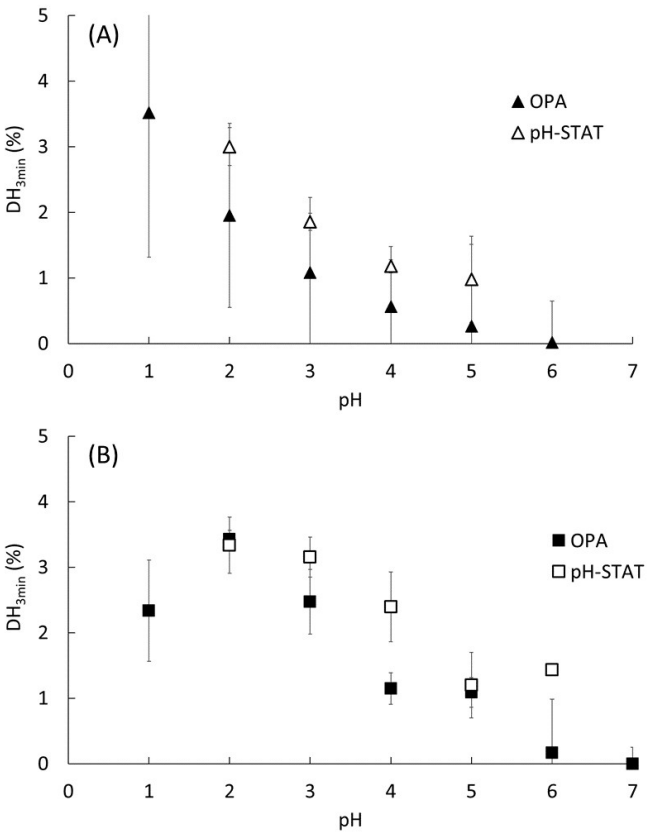


Figure 1: Degree of hydrolysis measured after 3 min of gastric digestion (DH_{3min}) at different pH for EWP (A) and CA (B), as estimated from (i) the OPA method on the end samples of the gastric digestion lasting 3 min (filled symbols) and (ii) the pH-STAT method at 3 min of the gastric digestion lasting 2 h (open symbols). Data represent mean ± std over 3 replicates.

For CA, some pepsin activity could be measured at pH 6 at both 3 min and 2 h, followed by a less pronounced increase with decreasing pH. Remarkably, the extent of CA hydrolysis by pepsin after 2 h was about constant between pH 1 and 5, and was still substantial at pH 6 (40% of the value measured at pH 3), and noticeable at pH 7 (15% of the value measured at pH 3).

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Three different groups could indeed be distinguished from the statistical analysis of these results: the value from pH 1 to 5 ($1.00 > p > 0.05$ between them) were higher than the value measured at pH 6 ($p < 0.001$), the latter being also higher than the value measured at pH 7 ($p < 0.03$). This apparent plateau from pH 1 to 5 contrasts somewhat with the trends observed after 3 min. It is tenting to attribute these changes to the impact of the initial structures of CA. However, it is not straightforward to relate the initial rate of hydrolysis (DH_{3min}) to the size and microstructure of CA particles (Table 1). On the one hand, the mean particle size was slightly smaller at low pH, which could suggest a higher surface accessibility for pepsin action. On the other hand, however, the granular structures observed for pH ≥ 3 could also be put forward to postulate a higher surface accessibility and/or a higher sensitivity of the building blocks to pepsinolysis. Another possible explanation for this apparent plateau is that the substrate concentration (2% w/w) was the rate limiting factor. However, this hypothesis seems hardly compatible with the observations that 1) the DH values in the pH range from 5 to 2 estimated by pH-stat are very similar after only 15 min of digestion, and 2) no similar plateau appeared in the EWP results despite an identical protein concentration. As further discussed, a third option is to assume that the modification of the pH-activity profile at 2 h of digestion predominantly reflects the long-term evolution of the reaction kinetics for these substrates.

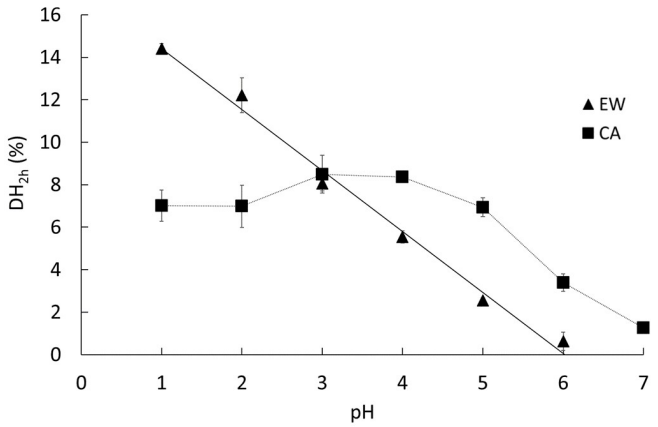

Figure 2: Degree of hydrolysis measured after 2 h of gastric digestion (DH_{2h}) at different pH for EWP (triangles) and CA (squares), as estimated from the OPA method on the end samples of the gastric digestion lasting 2 h. Data represent mean ± std over 3 replicates (some error-bars are smaller than symbol size). The dotted line is a guide for eyes, whereas the solid line is a linear regression on EWP data (DH_{2h} = -2.87 × pH + 17.27, R^2 = 0.99)

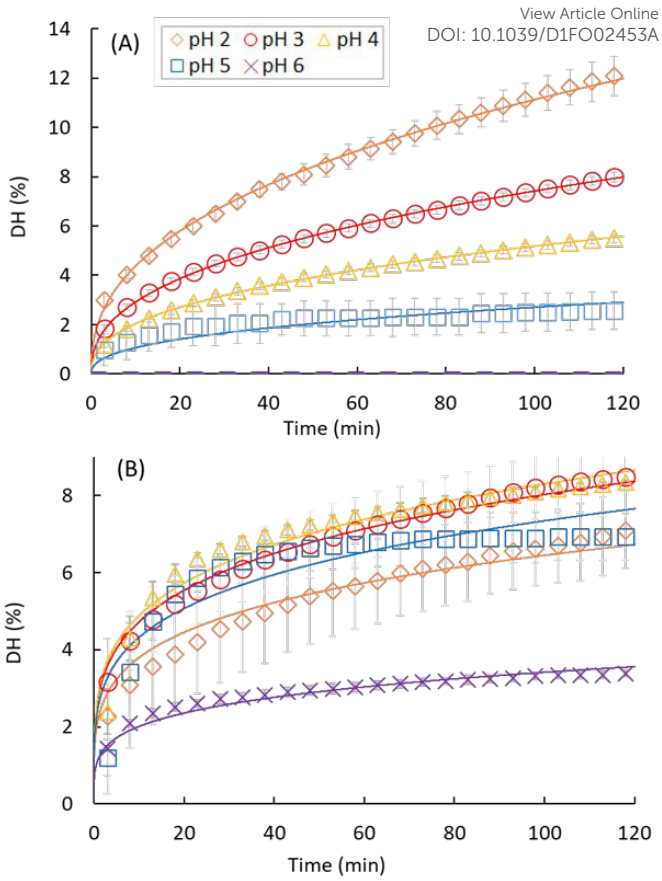

Figure 3: Degree of hydrolysis monitored by pH-STAT during the course of the gastric digestions lasting 2 h for EWP (A) and CA (B) at different pH: pH 2 (diamonds), pH 3 (circles), pH 4 (triangles), pH 5 (squares), pH 6 (crosses). Data represent mean ± std over 3 replicates (some error-bars are smaller than symbol size). Full lines represent the fittings of the power law model: $DH = a \times t^b$.

3.3. The dynamics of proteolysis depends on the type of substrate

Figure *3*A and 3B present the DH evolution of EWP and CA, respectively, during the course of the gastric digestions lasting 2 h in the pH range from 2 to 6. We may indeed recall that pH-STAT measurements did not work at pH 1, most probably because of the very high buffering capacity of this strongly acidic solution[39]. The kinetics of proteolysis follow the same general trend for both EWP and CA, with a high initial reaction rate followed by a progressive slowdown over the duration of the experiments. As shown in Figure *3* and Table 2, most of the experimental curves could be fairly reproduced using the power law model with a common value of the exponent (*b*) for each substrate at all pH ($R^2 > 0.96$), with a notable exception for CA at pH 5 ($R^2 = 0.74$), and to a lesser extent for CA at pH 4 ($R^2 = 0.92$). The fact that the power

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law exponent largely appeared depend on the protein substrate but not on the pH is noteworthy. Providing that this parameter solely governs the shape of the curve, it can be concluded that the pepsin activity profiles measured for a given substrate at different pH were directly proportional (scale invariance). For a given *b* value, and hence substrate, the value taken by the pre-factor of the power law model (*a*) therefore reflects the evolution of the mean reaction rate as a function of pH. Consistently with the trend previously described for DH_{2h}, the value taken by the pre-factor *a* increased linearly with decreasing pH for EWP (Table 2), leading to an extrapolated DH_{2h} value of 14.6% at pH 1, in excellent agreement with the measured 14.4% (Figure 2). The same holds for CA, with a comparable evolution of the pre-factor (Table 2) and DH_{2h} (Figure 2) values as a function of pH.

Table 2: Coefficient of determination (R^2) and estimated parameters of the power law model $DH = a \times t^b$ as a function of both pH and substrate.

	EWP			CA		
pH	***a* ***	***b***	**R^2**	***a***	***b***	**R^2**
2	1.72	0.41	> 0.99	2.23	0.23	> 0.99
3	1.15		> 0.99	2.78		> 0.99
4	0.80		> 0.99	2.88		0.92
5	0.42		0.96	2.55		0.74
6	-		-	1.18		0.98

**For EWP: a = - 0.43 × pH + 2.5 (R^2 = 0.99), with b = 0.41*

4. Discussion

4.1. At short digestion time, the pH activity profile of pepsin depends on the protein substrate and can be substantial in weakly acidic conditions (pH ≥ 4)

The DH measured after 3 min of pepsin hydrolysis (Figure 1), obtained with two sets of *in vitro* digestion experiments performed in compliance with the INFOGEST protocol, show that the pH dependence of the initial reaction rate of porcine pepsin is different on EWP and CA. Despite different experimental conditions, notably in regards of the enzyme to substrate ratio and of the ionic environment, our results are in good agreement with the available literature on the pH dependence of pepsin. For EWP, they are in line with previous findings showing that the initial velocity of porcine pepsin on this substrate increases rapidly with decreasing pH from 3 to 1.5[30]. They can also be compared to the reported activity of human pepsin on native egg albumin, the main protein of egg white, showing an optimal pH close to 1 and a low activity above pH 2.5[16]. This clear tendency for an increased pepsin activity as the pH is reduced fits well the common assumption that an acid denaturation of proteins favour pepsin activity[17,24,25]. For CA, some pepsin activity could be measured at pH 6, and even at pH 7 after 2 h (Figure 2). This also appears consistent with the literature on cheese making, in which the proteolytic activity of porcine pepsin has been studied at pH ≥ 6 in the objective of using it as a substitute of rennet[31,41,42]. Using the same theoretical framework, this higher sensitivity of casein aggregates to pepsin in weakly acidic conditions can probably be attributed to their very particular structural properties, which consist of a highly porous network of individual caseins interacting together with little tertiary structures[28].

Because the casein substrate was initially in the form of casein micro-aggregates in all our experiments, it is noteworthy that we cannot ascertain that the results we obtained for pH ≥ 5 can be transposed to native casein micelles. Still, it is noteworthy that the literature on cheese making leaves no doubt on a noticeable pepsin activity on native casein micelles at pH close to neutrality, and that the mean diameter of casein micelles (of about 150 nm) appears very favourable to pepsin action when considering that it is 3 orders of magnitude smaller than the diameter we measured for our micro-aggregates (Table 1). Therefore, a substantial initial pepsin activity at high pH (i.e. ≥ 5) is very likely to exist as well for native milk casein micelles. This would also convincingly explain why milk can curdle in less than 15 min of digestion at pH ranging from 5.5 to 6.0 during semi-dynamic *in vitro* gastric digestion, whereas it curdles in about 75 min at pH 5.0 in the absence of pepsin[29]. A key contribution of pepsin action in the aggregation of casein micelles may, in fact, appear critical to fully understand the digestion of milk proteins by neonates. Despite their limited capacity to acidify their gastric content[43], it seems critical that both i) an early formation of casein aggregates take place in their stomach, and ii) pepsin can thereafter hydrolyse these neo-formed particles to ensure a controlled transit of protein through the stomach[44]. We may therefore assume there is a true biological advantage for the capability of pepsin to favour both the early curdling of milk (through its non-negligible hydrolytic activity on casein micelles at almost neutral pH), and a subsequent hydrolysis at a relatively pH-independent rate. Alongside pH-induced effects on caseins, both of these latter mechanisms can contribute to a well-controlled gastric hydrolysis of milk, and hence to explain why caseins are categorized as particularly slow dietary proteins[4].

To broaden the discussion on the influence of the protein substrate on the pH dependence of porcine pepsin activity, Figure 4 compares our DH_{3min} values (OPA results) with other initial rates of pepsinolysis extracted from the literature, arbitrary setting an activity value of 100% at pH = 2. As commonly stated, this graph clearly shows that pepsin activity increases with increasing acidity for pH ≥ 2, whatever the substrate considered. However, it also illustrates that pepsin activity in extremely acidic (pH < 2) and weakly acidic (pH ≥ 4) conditions is highly dependent on the protein substrate. Among the considered data, caseins clearly appear to be the most sensitive substrate to peptic hydrolysis in the pH range from 6

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to 3. Conversely, wheat gluten seems particularly resistant to pepsin action in this pH range. The same probably holds for native bovine β-lactoglobulin (not represented in Figure 4 because of a lack of data in the literature), which has been shown to remain intact after 2 h of *in vitro* gastric digestion at pH 2.5[25]. Depending on the considered substrate, porcine pepsin may thus start to show an appreciable activity for pH ≥ 4-5 or only below pH ~ 3. This view is somewhat different from what is too frequently assumed to be a general rule from the widespread data obtained with haemoglobin as a substrate, *i.e.* a bell-shaped curve with a rise around pH 3.5 and maximum around pH 2 (Figure 4).

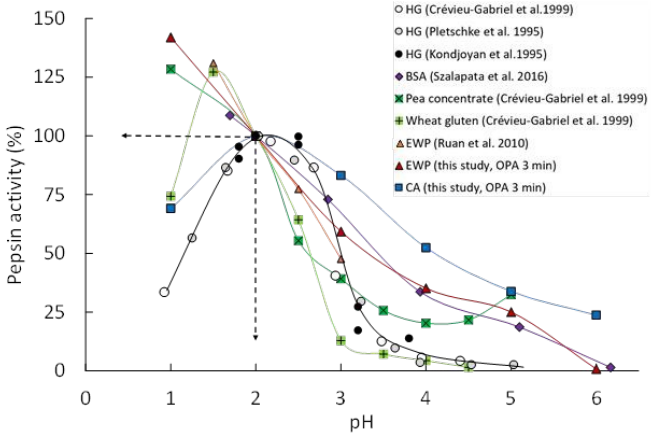

Figure 4: pH dependence of porcine pepsin activity at short time on different protein substrates: Haemoglobin (HG; ○, ● & ○), Bovine serum albumin (BSA, ◆), Pea concentrate (▣), Wheat gluten (▣), Egg white proteins (EWP; ▲ & ▲) and Casein aggregates (CA; ■). Lines are guides for the eyes and activity values were set arbitrary at 100% at pH = 2.0. Experimental data arise from the present study (means of OPA and pH-STAT data at 3 min) and the literature [13–15,30,45]

The contrasted behaviours of substrate here reported might be of particular interest for the research community working in the field of dietary protein digestion, notably because the structure of the protein substrate and their extent of denaturation have been suggested to be the rate-determining factor for peptic digestion in weakly acidic conditions[16,17]. One should, indeed, not forget that the gastric pH after a meal decreases very slowly from about 6 to 2 before it remains about stable. This decrease can take about 2 h for a meal according to Malagelada et al. (1979)[19] and Dressman et al. (1986)[18] and pH values ranging from ~ 4 to 5.5 have been reported at half-gastric emptying time for egg white gels in pigs[21], as well as for liquid and solid meals in humans[20,22]. Important pH gradients can also take place over the entire stomach[20,21], hence possibly leading to an even longer persistence of weakly acidic conditions in the proximal stomach. It is thus defendable that foods are exposed to pH ≥ 4 during a considerable amount of time in the stomach. Similarly to the conclusion reached by Sams et al. (2018)[24] with their results on the hydrolysis of β-casein in early gastric conditions (pH 5.5), we might therefore wonder if the contribution of gastric hydrolysis of dietary proteins during this time window has been too much neglected so far, and whether this may influence the gastric emptying kinetics and/or the downstream intestinal proteolysis kinetics to a substantial extent.

4.2. At long digestion time, the shape of the hydrolysis kinetics by pepsin is independent of pH but seems characteristic of the substrate

The degrees of hydrolysis measured after 2 h of gastric digestion (Figure 2), all between 0 and 15%, are consistent with the values reported in the literature that typically fall within this range[30,38,46]. The pH dependence of DH_{2h}, similar in trend to the one observed for DH_{3min} (Figure 1), also confirms that pH is one of the key factors governing the extent of protein hydrolysis by pepsin at long digestion times. The gaps between the smallest and highest DH values as a function of pH tended to be reduced after 2 h of digestion, nevertheless, leading to a straighter tendency for EWP and a more flattened curve for CA. For instance, the DH_{3min} of CA at pH 2 (~ 3.4%) was 3 times higher on average (p = 0.09) than the one measured at pH 5 (~ 1.1%), whereas similar DH_{2h} were measured for CA at both pH (6.98% at pH 2 *vs* 6.93% at pH 5). Overall, the final extents of casein hydrolysis by pepsin were about the same in a remarkable broad range of pH, from 1 to 5, with a value only 40-50% smaller at pH 6 on average. Such a finding clearly reinforces the idea that the *in vivo* contribution of peptic hydrolysis in weakly acid conditions should not always be neglected, at least for caseins.

Slight changes could also be noticed when comparing the DH of EWP and CA at short and long digestion times. For instance, the DH_{3min} at pH 3 for CA (~ 3%) was about twice (p = 0.001) the one measured for EWP (~ 1.5%), whereas similar DH_{2h} were obtained for both kinds of substrate at this pH (8.05% for EWP *vs* 8.50% for CA). The time evolution of DH monitored by pH-STAT (

Time (min)

Figure *3*) enables a better view on how these gaps were progressively filled. The excellent temporal resolution of these data, and their fair fittings by a power law, show that the shape of the reaction kinetics observed for EWP and CA was largely pH independent. Instead, it appeared to be rather specific to the considered substrate, with a more pronounced transition from a high to a slow reaction rate at all pH for CA (*b* << 1, Table 2) than for EWP (*b* closer to 1). Therefore, the gap between EWP and CA was progressively filled as the digestion time increased because of a lesser reduction of the instantaneous reaction rate with EWP. This also shows that caution should be taken when willing to extrapolate at long times (*e.g.* 2 h) the pH-dependence of pepsin activity measured at short time (*e.g.* 3 min). The only noticeable deviation from a profile shape that is substrate specific was observed for CA at pH 5 (and to a lesser extent at pH 4). This pH is close to the isoelectric point of caseins at 4.6, and corresponds to the pH at which casein micelles of milk naturally clot when slowly acidified[47,48]. Therefore, this

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singular behaviour was most probably induced by the very peculiar physicochemical properties of caseins around pH 5.

Because very few studies have investigated the gastric hydrolysis of proteins at various pH over long digestion times, it is difficult to determine how general can be the observed trend for a substrate specific peptic hydrolysis profile between pH ~ 6 to 2, *i.e.* the typical range of gastric pH *in vivo*. It is also difficult to explain why such a trend would exist. During gastric proteolysis, the substrate continuously evolves from large polypeptides to smaller peptides, meaning that the substrate is not the same as a function of time. The shape of the hydrolysis curve could thus reflect the progression of the substrate transformation. However, the overall advancement of the reaction, and hence the state of the substrate at a given time, is largely influenced by pH (*e.g*. Fig. 3A) while the shape of the hydrolysis profile is largely conserved. If confirmed, this trend would thus call for a better understanding on the exact phenomena governing the progressive loss of pepsin activity as a function of reaction time and pH. It would also prove useful to extrapolate the extent of protein hydrolysis at long times for different pH, and possibly to predict the evolution of the pepsin activity during the course of a physiologically relevant gastric acidifying kinetics (*i.e.* with continuous HCl secretions). More experimental studies would thus be very valuable to address these questions.

With regards to the power law used in our study, it is noteworthy that more elaborated models of peptic hydrolysis have been proposed in the literature. Some are based on the Michaelis-Menten equation, as for instance in Ruan et al. (2010)[30]. Some others are based on the first order reaction model, sometimes considering a two stages reaction scheme to improve the fittings under the hypothesis that globular proteins need to unfold before the hydrolysis of peptide bonds can take place[49]. Although more mechanistic, a common issue with these models is that they are often specific of the protein substrate considered, and/or that the model parameters, sometimes numerous, might be difficult to interpret and discuss. This is probably the reason why there is no unique widespread model of pepsin reaction kinetics. In the present study, the power law model was found to provide the best fittings on our data sets among five different models, including those described in Kondjoyan et al. (2015)[13], Väljamäe et al. (2003)[50], Deng et al. (2018)[51] and Ruan et al. (2010)[30]. Although empirical, this model relies on only two model parameters, each related to a strict interpretation: the pre-factor (a) is a proportionality constant, and the scaling factor (b) solely governs the shape of the curve.

To check whether the power law model could be satisfactory applied to other substrates during gastric *in vitro* digestions, we tested it on other pH-STAT data of ours (Supp. Mat.), all obtained at pH 3 using the INFOGEST protocol[12]. Results showed that the power law very accurately modelled (all $R^2 > 0.99$) the gastric proteolysis of: native and denatured dairy whey proteins[52], gluten based and pea protein based gels, wheat based and pea-based cakes also containing eggs, oil and sugars (unpublished data). These results (Supp. Mat.), together with the ones presented as part of the present study, therefore suggest that a power law might be suitable to model the gastric proteolysis of various edible proteins, and even some complex foods. The extensive use of the INFOGEST static *in vitro* protocol now calls for standardized ways of analysing and presenting digestion data to enable comparisons across studies. In this regard, the use of a power law might prove useful for its capability to summarize the gastric proteolysis kinetics in only two well-defined parameters.

Conclusions

This study shows that the pH dependence of pepsin activity is highly dependent on the protein substrate at both short and long digestion times, and does not always show a bell shaped curve as frequently assumed. Most remarkably, after 2 h of static *in vitro* digestion, the extent of hydrolysis of casein micellar aggregates was almost the same from pH 1 to pH 5, and remained noticeable up to pH 7. This can be viewed as a biological advantage for neonates, and may explain why casein micelles can aggregate only few minutes after ingestion. More generally, our results suggest that gastric proteolysis in weakly acidic conditions, as typically observed during the first hour of gastric digestion (and possibly later on in the proximal part of the stomach), should not always be neglected. They also show that the shape of the hydrolysis profiles of EWP and CA by pepsin was largely independent on pH and seemed characteristic of the substrate. These proteolysis profiles could be fairly fitted by a power law model, which was found very useful to interpret and summarize our experimental data.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

We would like to thank Yann Le Gouar and Olivia Ménard for their technical help in the laboratory, and Françoise Nau for helpful discussions on the properties of egg white proteins. Alicia Dérand and Isabelle Souchon are also acknowledged for their agreement to publish the wheat and pea based results as part of the Supplementary Material file.

Abbreviations

DH: Degree of hydrolysis; EWP: Egg white proteins; CA: Casein aggregates

References

1 F. Barbé, O. Ménard, Y. Le Gouar, C. Buffière, M.-H. Famelart, B. Laroche, S. L. Feunteun, D. Rémond and D. Dupont, Acid and rennet gels exhibit strong differences in the kinetics of milk protein digestion and amino acid bioavailability, *Food Chemistry*, 2014, **143**, 1–8.

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Food & Function Accepted Manuscript

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2 T. T. Lambers, W. G. van den Bosch and S. de Jong, Fast and slow proteins: modulation of the gastric behavior of whey and casein in vitro, *Food Digestion*, 2013, **4**, 1–6.

3 S. Le Feunteun, F. Barbé, D. Rémond, O. Ménard, Y. Le Gouar, D. Dupont and B. Laroche, Impact of the dairy matrix structure on milk protein digestion kinetics: mechanistic modelling based on mini-pig in vivo data, *Food and Bioprocess Technology*, 2014, **7**, 1099–1113.

4 Y. Boirie, M. Dangin, P. Gachon, M.-P. Vasson, J.-L. Maubois and B. Beaufrère, Slow and fast dietary proteins differently modulate postprandial protein accretion, *PNAS*, 1997, **94**, 14930–14935.

5 J. Sicard, P.-S. Mirade, S. Portanguen, S. Clerjon and A. Kondjoyan, Simulation of the gastric digestion of proteins of meat bolus using a reaction–diffusion model, *Food Funct.*, 2018, **9**, 6455–6469.

6 R. G. M. van der Sman, S. Houlder, S. Cornet and A. Janssen, Physical chemistry of gastric digestion of proteins gels, *Current Research in Food Science*, 2020, **2**, 45–60.

7 Q. Luo, W. Zhan, R. M. Boom and A. E. M. Janssen, Interactions between acid and proteins under in vitro gastric condition – a theoretical and experimental quantification, *Food Funct.*, 2018, **9**, 5283–5289.

8 M. Griffiths, *Crash Course: Gastrointestinal System E-Book*, Elsevier Health Sciences, 2012.

9 J. E. Norton, G. A. Wallis, F. Spyropoulos, P. J. Lillford and I. T. Norton, Designing food structures for nutrition and health benefits, *Annu. Rev. Food Sci. Technol.*, 2014, **5**, 177–195.

10 D. W. Piper and B. H. Fenton, pH stability and activity curves of pepsin with special reference to their clinical importance, *Gut*, 1965, **6**, 506–508.

11 A. Brodkorb, L. Egger, M. Alminger, P. Alvito, R. Assunção, S. Ballance, T. Bohn, C. Bourlieu-Lacanal, R. Boutrou, F. Carrière, A. Clemente, M. Corredig, D. Dupont, C. Dufour, C. Edwards, M. Golding, S. Karakaya, B. Kirkhus, S. Le Feunteun, U. Lesmes, A. Macierzanka, A. R. Mackie, C. Martins, S. Marze, D. J. McClements, O. Ménard, M. Minekus, R. Portmann, C. N. Santos, I. Souchon, R. P. Singh, G. E. Vegarud, M. S. J. Wickham, W. Weitschies and I. Recio, INFOGEST static in vitro simulation of gastrointestinal food digestion, *Nature Protocols*, 2019, **14**, 991–1014.

12 M. Minekus, M. Alminger, P. Alvito, S. Ballance, T. Bohn, C. Bourlieu, F. Carrière, R. Boutrou, M. Corredig, D. Dupont, C. Dufour, L. Egger, M. Golding, S. Karakaya, B. Kirkhus, S. Le Feunteun, U. Lesmes, A. Macierzanka, A. Mackie, S. Marze, D. J. McClements, O. Ménard, I. Recio, C. N. Santos, R. P. Singh, G. E. Vegarud, M. S. J. Wickham, W. Weitschies and A. Brodkorb, A standardised static in vitro digestion method suitable for food – an international consensus, *Food Funct.*, 2014, **5**, 1113–1124.

13 A. Kondjoyan, J.-D. Daudin and V. Santé-Lhoutellier, Modelling of pepsin digestibility of myofibrillar proteins and of variations due to heating, *Food Chemistry*, 2015, **172**, 265–271.

14 B. I. Pletschke, R. J. Naudé and W. Oelofsen, Ostrich pepsins I and II: A kinetic and thermodynamic investigation, *The International Journal of Biochemistry & Cell Biology*, 1995, **27**, 1293–1302.

15 I. Crévieu-Gabriel, J. Gomez, J. P. Caffin and B. Carré, Comparison of pig and chicken pepsins for protein hydrolysis, *Reprod. Nutr. Dev.*, 1999, **39**, 443–454.

16 L. K. Christensen, Concerning the pH optimum of peptic hydrolysis, *Archives of Biochemistry and Biophysics*, 1955, **57**, 163–173.

17 M. Schlamowitz and L. U. Peterson, Studies on the optimum pH for the action of pepsin on "native" and denatured bovine serum albumin and bovine hemoglobin., *The Journal of biological chemistry*, 1959, **234**, 3137–45.

View Article Online DOI: 10.1039/D1FO02453A

18 J. B. Dressman, Comparison of canine and human gastrointestinal physiology, *Pharmaceutical Research*, 1986, **3**, 123–131.

19 J.-R. Malagelada, V. L. W. Go and W. H. J. Summerskill, Different gastric, pancreatic, and biliary responses to solid-liquid or homogenized meals, *Digestive Diseases and Sciences*, 1979, **24**, 101–110.

20 F. Carrière, C. Renou, E. Ville, P. Grandval and R. Laugier, Advantage of expressing the variations in some digestive parameters as a function of gastric emptying instead of time, *Digestion*, 2001, **64**, 46–53.

21 F. Nau, K. Nyemb-Diop, V. Lechevalier, J. Floury, C. Serrière, N. Stroebinger, T. Boucher, C. Guérin-Dubiard, M. J. Ferrua, D. Dupont and S. M. Rutherfurd, Spatial-temporal changes in pH, structure and rheology of the gastric chyme in pigs as influenced by egg white gel properties, *Food Chemistry*, 2019, **280**, 210–220.

22 L. Sams, J. Paume, J. Giallo and F. Carrière, Relevant pH and lipase for in vitro models of gastric digestion, *Food Funct.*, 2016, **7**, 30–45.

23 D. Freitas, S. Le Feunteun, M. Panouillé and I. Souchon, The important role of salivary α-amylase in the gastric digestion of wheat bread starch, *Food Funct.*, 2018, **9**, 200–208.

24 L. Sams, S. Amara, P. Mansuelle, R. Puppo, R. Lebrun, J. Paume, J. Giallo and F. Carrière, Characterization of pepsin from rabbit gastric extract, its action on β-casein and the effects of lipids on proteolysis, *Food Funct.*, 2018, **9**, 5975–5988.

25 A. Macierzanka, A. I. Sancho, E. N. C. Mills, N. M. Rigby and A. R. Mackie, Emulsification alters simulated gastrointestinal proteolysis of β-casein and β-lactoglobulin, *Soft Matter*, 2009, **5**, 538–550.

26 B. L. Dekkers, E. Kolodziejczyk, S. Acquistapace, J. Engmann and T. J. Wooster, Impact of gastric pH profiles on the proteolytic digestion of mixed βlg-Xanthan biopolymer gels, *Food Funct.*, 2016, **7**, 58–68.

27 T. Strixner and U. Kulozik, Egg proteins, in *Handbook of Food Proteins*, eds. G. O. Phillips and P. A. Williams, Woodhead Publishing, 2011, pp. 150–209.

28 J. A. Lucey and H. Singh, Formation and physical properties of acid milk gels: a review, *Food Research International*, 1997, **30**, 529–542.

29 A.-I. Mulet-Cabero, A. R. Mackie, P. J. Wilde, M. A. Fenelon and A. Brodkorb, Structural mechanism and kinetics of in vitro gastric digestion are affected by process-induced changes in bovine milk, *Food Hydrocolloids*, 2019, **86**, 172–183.

30 C. Q. Ruan, Y.-J. Chi and R.-D. Zhang, Kinetics of hydrolysis of egg white protein by pepsin, *Czech Journal of Food Sciences*, 2010, **28**, 355–363.

31 J. J. Tam and J. R. Whitaker, Rates and extents of hydrolysis of several caseins by pepsin, rennin, endothia parasitica protease and mucor pusillus protease1, *Journal of Dairy Science*, 1972, **55**, 1523–1531.

32 J. V. C. Silva, D. Legland, C. Cauty, I. Kolotuev and J. Floury, Characterization of the microstructure of dairy systems using automated image analysis, *Food Hydrocolloids*, 2015, **44**, 360–371.

33 D. J. L. Mat, S. Le Feunteun, C. Michon and I. Souchon, In vitro digestion of foods using pH-stat and the INFOGEST protocol: Impact of matrix structure on digestion kinetics of macronutrients, proteins and lipids, *Food Research International*, 2016, **88**, 226–233.

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34 L. Lorieau, A. Halabi, A. Ligneul, E. Hazart, D. Dupont and J. Floury, Impact of the dairy product structure and protein nature on the proteolysis and amino acid bioaccessiblity during in vitro digestion, *Food Hydrocolloids*, 2018, **82**, 399–411.
35 F. C. Church, H. E. Swaisgood, D. H. Porter and G. L. Catignani, Spectrophotometric assay using o-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins1, *Journal of Dairy Science*, 1983, **66**, 1219–1227.
36 P. M. Nielsen and H. S. Olsen, Enzymic modification of food protein., in *Enzymes in food technology*, Sheffield Academic Press ; CRC Press, Sheffield; Boca Raton, FL, 2002.
37 J. Adler-Nissen, S. Eriksen and H. S. Olsen, Improvement of the functionality of vegetable proteins by controlled enzymatic hydrolysis, *Plant Foods for Human Nutrition*, 1983, **32**, 411–423.
38 D. J. L. Mat, T. Cattenoz, I. Souchon, C. Michon and S. Le Feunteun, Monitoring protein hydrolysis by pepsin using pH-stat: In vitro gastric digestions in static and dynamic pH conditions, *Food Chemistry*, 2018, **239**, 268–275.
39 D. D. Van Slyke, On the measurement of buffer values and on the relationship of buffer value to the dissociation constant of the buffer and the concentration and reaction of the buffer solution, *Journal of Biological Chemistry*, 1922, **52**, 525–570.
40 A. Tonda, A. Grosvenor, S. Clerens and S. Le Feunteun, In silico modeling of protein hydrolysis by endoproteases: a case study on pepsin digestion of bovine lactoferrin, *Food Funct.*, 2017, **8**, 4404–4413.
41 P. F. Fox, Milk-clotting and proteolytic activities of rennet, and of bovine pepsin and porcine pepsin, *Journal of Dairy Research*, 1969, **36**, 427–433.
42 J. Winwood, Rennet and rennet substitutes, *International Journal of Dairy Technology*, 1989, **42**, 1–2.
43 C. Bourlieu, O. Ménard, K. Bouzerzour, G. Mandalari, A. Macierzanka, A. R. Mackie and D. Dupont, Specificity of infant digestive conditions: some clues for developing relevant in vitro models, *Critical Reviews in Food Science and Nutrition*, 2014, **54**, 1427–1457.
44 T. Huppertz and L. W. Chia, Milk protein coagulation under gastric conditions: A review, *International Dairy Journal*, 2021, **113**, 104882.
45 K. Szałapata, M. Osińska-Jaroszuk, J. Bryjak, M. Jaszek and A. Jarosz-Wilkołazka, Novel application of porous and cellular materials for covalent immobilization of pepsin, *Braz. J. Chem. Eng.*, 2016, **33**, 251–260.
46 N. Ahmad, M. Imran, M. K. Khan and M. un Nisa, Degree of hydrolysis and antigenicity of buffalo alpha S1 casein and its hydrolysates in children with cow milk allergy, *Food and Agricultural Immunology*, 2016, **27**, 87–98.
47 M. H. Famelart, F. Lepesant, F. Gaucheron, Y. Le Graet and P. Schuck, pH-Induced physicochemical modifications of native phosphocaseinate suspensions: Influence of aqueous phase, *Lait*, 1996, **76**, 445–460.
48 S. Banon and J. Hardy, Study of acid milk coagulation by an optical method using light reflection, *Journal of Dairy Research*, 1991, **58**, 75–84.
49 R. Herman, Y. Gao and N. Storer, Acid-induced unfolding kinetics in simulated gastric digestion of proteins, *Regulatory Toxicology and Pharmacology*, 2006, **46**, 93–99.
50 P. Väljamäe, K. Kipper, G. Pettersson and G. Johansson, Synergistic cellulose hydrolysis can be described in terms of fractal-like kinetics, *Biotechnology and Bioengineering*, 2003, **84**, 254–257.
51 Y. Deng, F. van der Veer, S. Sforza, H. Gruppen and P. A. Wierenga, Towards predicting protein hydrolysis by bovine trypsin, *Process Biochemistry*, 2018, **65**, 81–92.
52 D. J. L. Mat, I. Souchon, C. Michon and S. Le Feunteun, Gastro-intestinal in vitro digestions of protein emulsions monitored by pH-stat: Influence of structural properties and interplay between proteolysis and lipolysis, *Food Chemistry*, 2020, **311**, 125946.

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DOI: 10.1039/D1FO02577F

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