Peptide bonds cleaved by pepsin are affected by the morphology of heat-induced ovalbumin aggregates

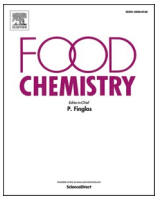
Ousmane Suwareh, David Causeur, Steven Le Feunteun, Julien Jardin, Valérie Briard-Bion, Stéphane Pezennec, Francoise Nau

Food Chemistry 458 (2024) 140260

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



Peptide bonds cleaved by pepsin are affected by the morphology of heat-induced ovalbumin aggregates

Ousmane Suwareh [a], David Causeur [b], Steven Le Feunteun [a], Julien Jardin [a], Valérie Briard-Bion [a], Stéphane Pezennec [a], Françoise Nau [a,*]

[a] STLO, INRAE, Institut Agro, 65 rue de Saint-Brieuc, 35042 Rennes, France
[b] IRMAR UMR6625, CNRS, Institut Agro, 65 rue de Saint-Brieuc, 35042 Rennes, France

ARTICLE INFO

Keywords:
Cleavage site
Statistical model comparison
Aggregation
Denaturation
Statistical analysis
In vitro digestion

ABSTRACT

The study aimed to assess the extent to which protein aggregation, and even the modality of aggregation, can affect gastric digestion, down to the nature of the hydrolyzed peptide bonds. By controlling pH and ionic strength during heating, linear or spherical ovalbumin (OVA) aggregates were prepared, then digested with pepsin. Statistical analysis characterized the peptide bonds specifically hydrolyzed *versus* those not hydrolyzed for a given condition, based on a detailed description of all these bonds. Aggregation limits pepsin access to buried regions of native OVA, but some cleavage sites specific to aggregates reflect specific hydrolysis pathways due to the denaturation-aggregation process. Cleavage sites specific to linear aggregates indicate greater denaturation compared to spherical aggregates, consistent with theoretical models of heat-induced aggregation of OVA. Thus, the peptides released during the gastric phase may vary depending on the aggregation modality. Precisely tuned aggregation may therefore allow subtle control of the digestion process.

1. Introduction

Proteins are essential in the human diet as they provide the nitrogen and amino acids necessary to maintain protein metabolism. It is well known that the action of pepsin, the first digestive protease, is influenced by the presence of certain amino acid residues (AAR) close to a given peptide bond, which would facilitate or, on the contrary, disfavor its hydrolysis (Hamuro et al., 2008; Palashoff, 2008; Powers et al., 1977; Vreeke et al., 2023). In particular, Leu and Phe strongly favor the hydrolysis of peptide bonds by pepsin, regardless of whether they are in the N- or C-terminal position of the bond, in contrast to Gly, which is always unfavorable. Six other AARs also influence the action of pepsin, but only when they are at the N-terminal side of the peptide bond, either favorably (Glu and Met) or unfavorably (Pro, Lys, His, Ser). Beyond the nature of the AARs along the primary protein sequence, the physicochemical environment of the peptide bonds, namely hydrophobicity, charge and structural constraints within native proteins, also has a significant influence on their susceptibility to hydrolysis by pepsin (Suwareh et al., 2021).

However, proteins are rarely in their native state in food matrices. Most foods undergo technological treatments that change protein structure, from the molecular and supramolecular scale to the macroscopic scale (Della Valle et al., 2013). In particular, beyond a certain threshold, heat treatments can cause the denaturation of proteins, especially globular proteins. Indeed, the interactions responsible for the secondary and tertiary structures of proteins, such as hydrogen and disulfide bonds can break down, leading to the disruption of their spatial structure without breaking their peptide bonds (Doi, 1993). As a result, hydrophobic areas and/or reactive groups initially buried in the core of protein molecules may be exposed, altering the balance between attractive and repulsive forces, and leading to interactions between these more or less unfolded proteins, culminating in the formation of high molecular weight complexes called aggregates (Bryant & McClements, 1998; Croguennec et al., 2004).

Abbreviations: OVA, ovalbumin; AA, amino acid; AAR, amino acid residue; SASA, solvent-accessible surface area; PTM, Posttranslational modification; GRAVY, grand average of hydropathy; AIC, Akaike information criterion; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; VMD, visual molecular dynamics..

* Corresponding author.
E-mail addresses: ousmane.suwareh@agrocampus-ouest.fr (O. Suwareh), david.causeur@institut-agro.fr (D. Causeur), steven.le-feunteun@inrae.fr (S. Le Feunteun), julien.jardin@inrae.fr (J. Jardin), valerie.briard-bion@inrae.fr (V. Briard-Bion), stephane.pezennec@inrae.fr (S. Pezennec), francoise.nau@institut-agro.fr (F. Nau).

https://doi.org/10.1016/j.foodchem.2024.140260
Received 25 January 2024; Received in revised form 24 June 2024; Accepted 26 June 2024
Available online 27 June 2024

Protein aggregation is a phenomenon that often occurs during food processing and can affect the properties of foods, but also the digestion process (Barbé et al., 2013). It can also affect the peptide composition of digests, some of which may be bioactive peptides (Barbé et al., 2014). This is due to the changes in protein structure that accompany their aggregation, affecting digestion processes. However, the consequences for proteolysis, which could be favored or disfavored, depend on the structure of the aggregates (Loveday, 2023). For example, in the case of ß-lactoglobulin, a direct relationship has been shown between the extent of gastric digestion and the degree of surface hydrophobicity of the aggregates (Ma et al., 2021). Similarly, the greater resistance to gastric digestion of soluble aggregates of myofibrillar proteins was explained by a reduction in surface hydrophobicity (X. Chen et al., 2022).

The structure and morphology of aggregates can be modulated depending on the physicochemical conditions applied during the heat-induced aggregation process of globular proteins. In the presence of strong repulsive electrostatic forces, the possibilities of interaction between proteins are limited, resulting in the formation of linear aggregates of highly denatured proteins. In contrast, when repulsive electrostatic forces are limited, aggregation occurs more rapidly, on less denatured proteins, and several interaction possibilities appear, leading to the formation of spherical aggregates (Doi, 1993). In the case of ovalbumin (OVA), the major globular protein in egg white, it has previously been shown how the shape and size of the aggregates created by heating can be modulated by varying the pH and ionic strength of the environment (Doi, 1993; Nyemb, Guérin-Dubiard, et al., 2014). It was further shown that the susceptibility of these aggregates to proteolysis depends on their supramolecular structures and was always enhanced after aggregation in comparison with the native OVA (Nyemb, Guérin-Dubiard, et al., 2014). Furthermore, the nature of the peptides released at the end of gastrointestinal digestion varied depending on whether the protein is included in an aggregate or not, and on the type of aggregates created. This suggests that the accessibility of the different regions of the protein would differ depending on the aggregation modality (Nyemb, Jardin, et al., 2014). This raises the question of how the way in which a globular protein such as OVA is aggregated may affect digestion from the gastric phase onwards. Indirectly, this also raises the question of whether it would be possible, by comparing the digested peptide bonds of different types of OVA aggregates, to hypothesize about the structural changes associated with heat-induced aggregation. However, this requires to consider the kinetics of the digestion events, particularly the very first moments of digestion, *i.e.*, when the aggregates are still close to their initial structure. It is only under these conditions, when digestion occurs on "undamaged" aggregates, that digestion can provide an insight into their characteristics and therefore into what happens during the denaturation-aggregation process of a globular protein such as OVA.

In the present study, native OVA and the four types of OVA aggregates previously described and studied by Nyemb, Guérin-Dubiard, et al. (2014), namely linear, linear-branched, spherical and spherical-agglomerated aggregates, have been digested by pepsin in acidic conditions (pH 3.0). Pepsin has been used here because it is the first protease that foods encounter during digestion, and it is therefore the digestive enzyme that protein aggregates would be subjected as such *in vivo*. In addition, its specificity has been previously studied in relation to the nature of the AARs along the protein sequence, and to physicochemical and structural characteristics of several globular proteins (Suwareh et al., 2021). The first aim of the study was therefore to determine the extent to which aggregation, or even the modalities of aggregation, can modify the course of gastric digestion, right down to the nature of the hydrolyzed peptide bonds. If this were the case, we could then consider using pepsin as a tool to investigate the modifications undergone by OVA during heat-induced aggregation, depending on the modalities of this aggregation. To do this, the peptide bonds hydrolyzed during pepsin digestion were first identified for each of the five conditions tested (native OVA and the four types of aggregates) throughout the 60 min of gastric digestion, with a focus on the early stages (30 s). Each of the hydrolyzed peptide bonds was then described using 24 variables, including the nature of the 14 AARs flanking them, their accessibility to pepsin, their physicochemical and structural environment, and their probability of cleavage in native OVA. Finally, the comparison of cleavage sites between the different conditions, using innovative statistical approaches, enabled us to understand the effects of heat-induced aggregation on structural changes in OVA, and the consequences of these changes on its hydrolysis by pepsin.

2. Materials and methods

2.1. Origin of peptidomic data

Four types of ovalbumin (OVA) aggregates were produced according to the method proposed by Nyemb, Guérin-Dubiard, et al. (2014). Briefly, OVA (P01012; 10VA) was purified from egg white (purity ≥85%) according to the procedure proposed by Croguennec et al. (2000). To create each type of aggregates, 1 g of purified OVA was dissolved in 50 mL of ultrapure water with 0.05% NaN_3 to prevent bacterial growth. The OVA solution, previously poured into a hermetically sealed glass vessel, was heated in a water bath at 80 °C for 6 h while maintaining a specific combination of pH and ionic strength (IS) through NaOH, HCl or NaCl addition. The four types of aggregates obtained had linear (pH 9 / IS 0.03 M), linear-branched (pH 7 / IS 0.03 M), spherical (pH 7 / IS 0.3 M) or spherical-agglomerated (pH 5 / IS 0.8 M) morphologies (Supplementary material 1). Subsequently, these four types of aggregates and native OVA were digested by porcine pepsin (2,000 U/mL in the final volume) at pH 3.0 after dilution of the solutions in simulated gastric fluid (50:50). Digestions were performed in triplicate for each of the five conditions, and 200 µL samples were collected at seven different time points (0.5, 2, 5, 10, 20, 30, and 60 min) throughout the digestion, in which 5 µL of 0.73 mM Pepstatin A were immediately added to stop pepsinolysis. Samples were then analyzed by LC-MS/MS to identify the generated peptides as described by Torcello-Gómez et al. (2020). After separation on a C18 PepMap RSLC column (Dionex, Fisher Scientific, Illkirch, France) using an acetonitrile gradient (2% to 95%) with 0.08% formic acid and 0.01% trifluoroacetic acid, the peptides were analyzed using a nano-LC Dionex U3000 system fitted to a Q-Exactive mass spectrometer (Thermo Scientific, San Jose, CA) equipped with a nano-electrospray ion source operating in positive ion mode (m/z range 250–2,000; resolution set to 70,000 for the mass analyzer and to 17,500 for MS/MS). The X!Tandem pipeline software (Langella et al., 2017) was used to identify the peptides from the MS/MS spectra.

2.2. Deduction of cleaved peptide bonds from peptidomic data

To determine the cleaved peptide bonds from the peptidomic data, *i.e.*, the list of peptides released, the AAR sequence of each identified peptide was aligned with the complete AAR sequence of OVA. This enabled the identification, for each of these peptides, of the two peptide bonds (N- and C-terminal ends) the hydrolysis of which was responsible for their release. A list of hydrolyzed peptide bonds (or "cleavage sites") was thus compiled for each condition and digestion time (Supplementary material 2). We assumed that if a peptide bond is cleaved for a certain condition at a given digestion time, it will remain cleaved at subsequent digestion times.

2.3. Generation of variables describing cleavage sites

Data processing was performed using the R software (R Core Development Team, 2020). A total of 24 explanatory variables were used to describe the cleavage sites when OVA is in its native form.

2.3.1. Variables for univariate analyses

2.3.1.1. Description of the peptide bonds (16 variables). The first 14 variables were the nature of the AARs flanking each peptide bond from the P7 (N-terminal side) to P7' position (C-terminal side) (Supplementary material 3). The fifteenth and sixteenth variables were the class of the AAR at positions P1 ("P1 classes") and P1' ("P1' classes"), respectively, based on their propensity scores proposed by Suwareh et al. (2021). AARs with propensity scores below −2 are considered unfavorable for pepsinolysis; these AARs are Cys, Gly, His, Ile, Lys, Pro, Arg, Ser and Val for P1, and Pro, Ser, Gly and Thr for P1'. AARs with propensity scores between −2 and 2 are considered neutral; these AARs are Glu, Asp, Asn, Gln and Thr for P1, and Ala, Cys, Asp, Glu, His, Lys, Met, Asn, Gln, Arg and Trp for P1'. AARs with propensity scores above 2 are considered to promote pepsinolysis; these AARs are Phe, Leu, Met, Tyr, Trp and Ala for P1, and Phe, Leu, Tyr, Ile and Val for P1'.

2.3.1.2. Accessibility of the peptide bonds to pepsin (2 variables). The accessibility of peptide bonds to pepsin was illustrated by two different variables. The first was the Solvent Accessible Surface Area ratio (hereafter referred to as "SASA") calculated as described by Suwareh et al. (2021). The second variable was the distance of a peptide bond to the pepsin accessible periphery (hereafter referred to as "distance from periphery"). To calculate it, the triangulated convex hull of OVA was inferred using the "cxhull" function of the cxhull package (Barber et al., 2023). The three-dimensional coordinates of each AAR were obtained from the Protein Data Base (PDB) file of OVA (10VA). For each facet of the convex hull, one thousand random points were generated on the plane formed by the three vertices of each facet. Only the points located inside the triangle formed by the three vertices of a facet were considered as peripheral points of the OVA convex hull. Finally, the distance from periphery of a given peptide bond was determined as the minimum distance measured between that bond and one of the peripheral points of the OVA convex hull. A peptide bond is assumed to be located at the barycenter of two AARs it links.

2.3.1.3. Physicochemical and structural environment of the peptide bonds (5 variables). Five variables were considered to describe the physicochemical and structural environment of peptide bonds. The distance between each peptide bond and the nearest unstructured zone (coil) along the primary sequence, environmental hydrophobicity (GRAVY) and net charge considering a radius of 9 Å as detailed by Suwareh et al. (2021) were considered. The "secondary structure" variable indicated the conformation of the protein segment containing the two AARs on both sides of the considered peptide bond as detailed by Suwareh et al. (2021). Briefly, the different α-helix types specified in the PDB file (H, G, I) were grouped into the single modality "helix" (H), the different β-sheet types (E and B) into the single modality "sheet" (E), and finally the less ordered structures (S, T and C) into the single modality "coil" (C) (Reeb & Rost, 2019). The "secondary structure" variable was then simplified, considering the "EC" modality as "EE" while the "HC" modality was considered "HH". The number of post-translational modifications (PTMs) within 9 Å of the peptide bond was also considered.

2.3.1.4. Cleavage probability of peptide bonds in native OVA. The probability of peptide bond cleavage when included in the native OVA was calculated using the logistic regression model proposed by Suwareh et al. (2021).

2.3.2. Variables for multivariate analyses

Among the variables presented in Section 2.3.1, only the variables "P1 classes", "P1' classes", those related to pepsin accessibility and the physicochemical and structural environment of the peptide bonds were considered for the multivariate analyses (9 variables). The variables "P1 classes" and "P1' classes" were considered a good compromise to summarize the categorical variables P1 and P1', for which the high number of modalities would have made the multivariate analysis too difficult. This helps to maintain the robustness and interpretability of the multivariate analysis, ensuring that the results are both meaningful and generalizable.

2.4. Statistical and descriptive analyses

All the analyses were performed using the R software (R Core Development Team, 2020). Venn diagrams were constructed using the "venn.diagram" function of the R package VennDiagram (H. Chen, 2022). Univariate analyses were performed using the "wilcox.test" function, for continuous variables, or the chisq.test function, for categorical variables, of the stats package. For multivariate analyses, maximum-likelihood estimation of logistic regression models was implemented using the "glm" function of the stats package. A stepwise search of the best subset of explanatory variables in the logistic regression model was implemented, aiming at a minimal Akaike Information Criterion (AIC). The forward search was preferred, with a possibility at each step to remove an explanatory variable previously selected, using function stepwise in the R package RcmdrMisc. Type II Analyses of deviance tables for the minimum AIC submodel were finally produced using the "Anova" function of the car package (Fox et al., 2019) and non-significant explanatory variables at level 0.05 were removed one at a time starting from the largest *p*-value.

3. Results and discussion

3.1. The rules of pepsinolysis relating to the nature of AARs, as established for native OVA, remain relatively unchanged for OVA aggregates

Pearson's chi-square tests were first implemented to identify significant associations between each of the categorical explanatory variables and cleavage or not of a peptide bond in at least one of the conditions (native OVA or one of the four types of aggregates). For numerical variables, non-parametric mean comparison tests were performed between the cleaved peptide bonds and those never cleaved. Table 1A and Table 1B indicate the *p*-values for the significant associations and effects, respectively. Note that 232 OVA peptide bonds were hydrolyzed throughout the 60 min digestion in at least one of the conditions, whereas 152 peptide bonds were not. The latter have globally lower

Table 1
Statistically significant variables discriminating peptide bonds hydrolyzed by pepsin under at least one of the conditions (native OVA or one of the aggregate types) from those never hydrolyzed. Univariate analysis was performed using (A) chi-square tests for categorical variables and (B) non-parametric tests of the mean differences for numerical variables. Significance code: *** < 0.001; ** < 0.01; * < 0.05.

A

Variable	p-value	significance
P1 classes	3.9e-15	***
P1	1.6e-12	***
P2	5.4e-03	**
P3	3.6e-04	***
P4	1.2e-02	*
P6	5.4e-03	**
P7	4.2e-02	*
PTM 9A	2.3e-02	*

B.

Variable	p-value	significance
Cleavage probability	< 2.2e-16	***
SASA	4.3e-02	*

cleavage probabilities (p-value <2.2e-16, Table 1B), likely due to the presence of AARs, especially at the P1 position (p-value = 1.6e-12, Table 1A), which Suwareh et al. (2021) found to be unfavorable for pepsinolysis (Supplementary material 4). Accordingly, the variable "P1 classes" also has a highly significant effect (p-value = 3.9e-15). Moreover, the importance of the AARs at the N-terminal side on pepsinolysis (Hamuro et al., 2008; Suwareh et al., 2021), whether OVA is native or aggregated, is demonstrated by the significant effect of the nature of the AARs at positions P1, P2, P3, P4, P6 and P7 (Table 1A). Taken together, these results led us to conclude that the structural changes induced by aggregation do not seem to alter the favorable or unfavorable character of the different AARs.

The proportion of peptide bonds with a post-translational modification (PTM) <9 Å apart is twice as high among those that have never been cleaved (23.6%) as among cleaved peptide bonds (11.5%) (Supplementary material 5). In fact, the presence of PTM significantly disfavors pepsinolysis (p-value = 2.3e-02, Table 1A). OVA glycosylation, in addition to an intramolecular disulfide bridge and two phosphorylation sites, has been reported to be responsible for its relative resistance to pepsinolysis (Dupont et al., 2010). Glycosylation and disulfide bonds would increase pepsin resistance by increasing the stability of the protein structure and decreasing the accessibility to the cleavage sites (steric hindrance) (Y. Liu et al., 2023; Ma et al., 2021; Niu et al., 2016). As for phosphorylation, it would be unfavorable to pepsinolysis by locally decreasing the surface hydrophobicity (J. Liu et al., 2021).

With regard to accessibility variables, the two groups of peptide bonds ("cleaved" *vs.* "never cleaved") differ significantly on the "SASA" criterion in univariate analysis (p-value = 4.3e-02, Table 1B), with cleaved peptide bonds surprisingly appearing less accessible (in the native structure) than never cleaved peptide bonds. However, the present analysis applies to all peptide bonds, whether or not they are cleaved over the 60 min of digestion, by which time proteolysis is relatively extensive. Yet, pepsin shows a preference for hydrophobic AARs, particularly aromatic AARs (Vreeke et al., 2023), and the proportion of hydrophobic AARs at the core of the native OVA structure is logically much higher than at the molecule surface. Thus, 185 of the 204 peptide bonds with a SASA value below 0.2 (*i.e.*, 90.7%) have a hydrophobic AAR in position P1 and/or P1', compared with only 91 of the 180 peptide bonds with a SASA value above 0.2 (*i.e.*, 50.6%). A higher proportion of cleaved bonds in the core of native OVA was therefore expected in the case of advanced proteolysis, as observed after 60 min of digestion. Moreover, it should be noted that the significance of this variable is low (Table 1B).

In the end, the determinants of pepsin activity related to the nature of AARs do not seem to be significantly affected by OVA aggregation. However, differences were observed in the course of gastric digestion, depending on whether OVA is native or aggregated, and on the type of aggregates. These differences are presented below.

3.2. The nature of the peptide bonds hydrolyzed by pepsin varies depending on whether OVA is native or aggregated

To go further and try to highlight the structural changes induced by heat aggregation through their potential impact on the other rules of pepsinolysis, a visualization of the number of common *vs* specific cleavage sites of the native OVA and different aggregates, throughout digestion, is proposed in Fig. 1. Although most cleavage sites that are initially specific to one condition or set of conditions finally become common to all conditions (Fig. 1A-G), some of the cleavage sites that are specific to one condition or set of conditions after 30 s show another trend during the course of the digestion. In particular, among the 49 cleavage sites observed only in aggregates at 30 s (Fig. 1A), 14 are still missing in native OVA at 60 min, including 11 common to all aggregates (Fig. 1G).

These results indicate that certain cleavage sites, especially those specific to native OVA, are specific only at the beginning of digestion, probably because they are not yet accessible in the aggregates. Another hypothesis would be that other cleavage sites are preferentially cleaved in the aggregates, which could notably be the sites located in the hydrophobic regions of the protein, exposed after denaturation-aggregation to create surface hydrophobic patches (Bryant & McClements, 1998). In contrast, a set of cleavage sites observed in aggregates are never observed in native OVA, *i.e.*, without the events occurring during heat-induced aggregation. These sites are analyzed in more detail in Section 3.4.

We therefore confirmed a positive impact of OVA aggregation on its proteolysis by pepsin, in the sense that it increases the number of cleavage sites, thus reinforcing the assumption previously put forward by Nyemb, Jardin, et al. (2014). In fact, the effect of protein aggregation on digestion, particularly gastric digestion, has already been reported, but with opposite results depending on the aggregate structure (Loveday, 2023). However, to our knowledge, the effect of protein aggregation on the nature of the peptide bonds hydrolyzed by pepsin had not yet been studied. The present results therefore offer a new perspective from this point of view, and even more so with the comparison between aggregates of different morphology and structure but obtained from the same protein, as described in sections 3.4 and 3.5.

3.3. Native OVA vs aggregates: Mainly a difference in pepsinolysis kinetics

To investigate what changed due to heat-induced aggregation, a distinction is made hereafter between the 11 native OVA-specific cleavage sites and the 49 aggregate-specific cleavage sites after 30 s of digestion (Fig. 2A). No significant association was found following the Pearson's chi-square tests performed between the aggregate or native OVA specificity and the variables related to the nature of the AARs flanking the cleavage sites (Fig. 2B). Thus, it seems that heat-induced aggregation of OVA did not significantly change the pepsin preferences regarding the nature of the AARs flanking the peptide bonds, consistently with the results presented in section 3.1.

Significant mean differences in univariate analyses were only observed for the variables "distance from periphery" (p-value = 6.6e-05), "GRAVY 9A" (p-value = 6.9e-04) and "SASA" (p-value = 1.5e-02) (Fig. 2B, Supplementary material 6). Interestingly, the best model to illustrate the difference between the two groups was only composed by the variable "distance from periphery", although other physicochemical variables were proposed to be added. This suggests that the significant mean differences observed for the variables "distance from periphery", "GRAVY 9A" and "SASA" illustrate the same event here, namely the more or less buried nature of the cleavage sites in the native OVA molecule. Specifically, the cleavage sites that are native OVA-specific at the very beginning of digestion are generally farther from the periphery of the protein molecule than the sites observed only in aggregates (Supplementary material 6A). Thus, these native OVA-specific cleavage sites share the feature of being buried in the protein molecule. This suggests that the structure of the aggregates limits pepsin access to the buried sites normally accessible in native OVA at the start of digestion. In other words, pepsin is able to reach the buried zones of native OVA more quickly than in aggregates, probably because molecules involved in the aggregates are less accessible to pepsin than monomers in solution, and because of the compactness and/or rigidity of the aggregates. Similarly, Zhang et al. (2023) have reported slower *in vitro* gastric digestion of egg white proteins previously pH-treated, likely due to reduced accessibility of the cleavage sites, protein structure changes and rigidity increase.

The assumption of a change in the accessibility of the cleavage sites is supported firstly by the fact that the cleavage sites observed in both OVA and aggregates after 30 s of digestion have mean distances from periphery intermediate between the groups of OVA-specific and aggregate-specific cleavage sites (Supplementary material 7). Secondly, all native OVA-specific cleavage sites after 30 s of digestion become common to all

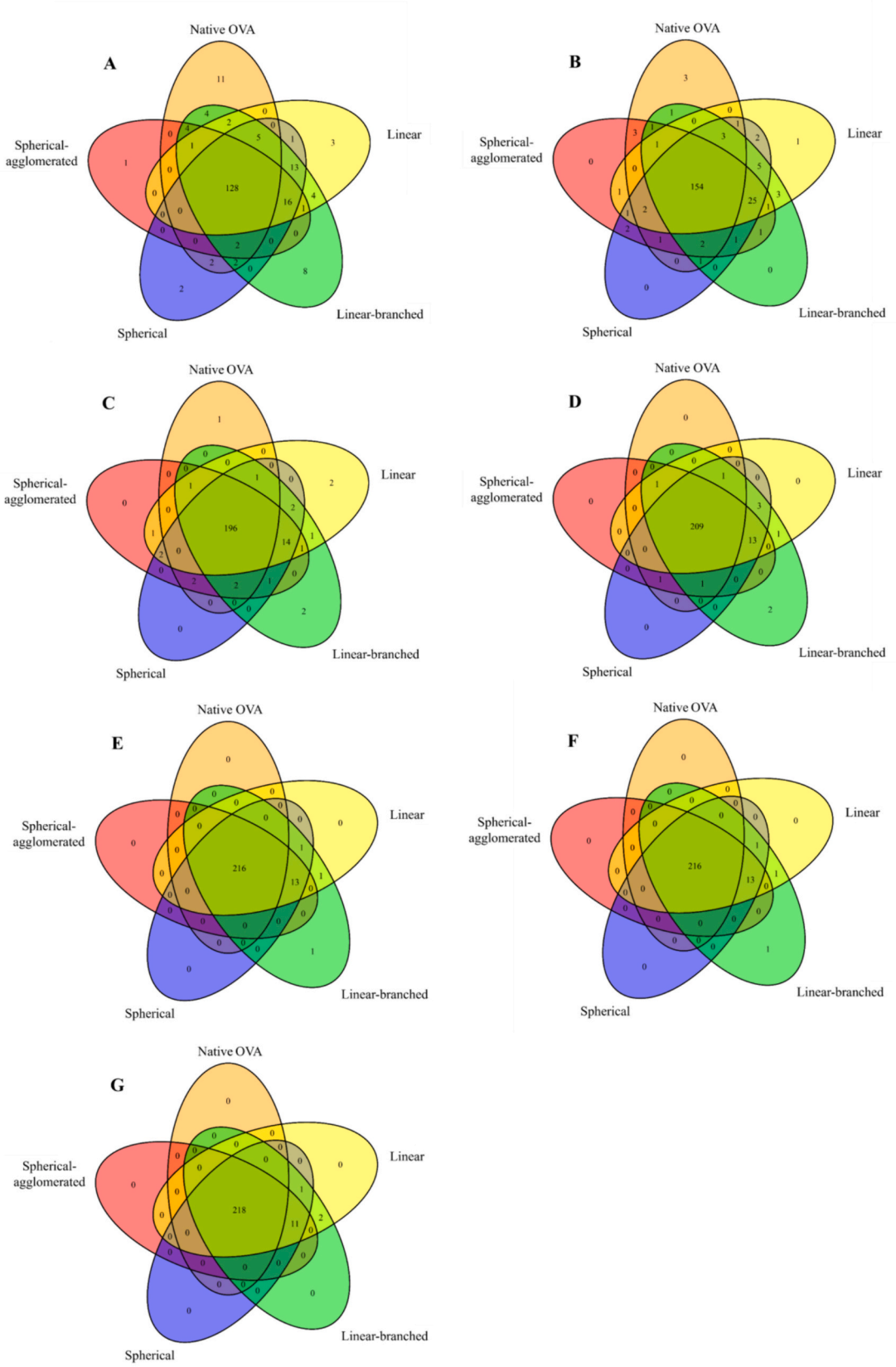
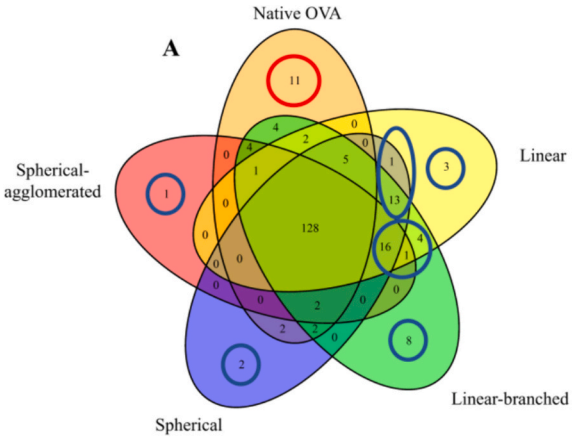


Fig. 1. Visualization of the number of common and specific cleavage sites of the native OVA and the different aggregates throughout *in vitro* digestion by pepsin, (A) after 30 s; (B) 2 min; (C) 5 min; (D) 10 min; (E) 20 min; (F) 30 min; (G) 60 min.



B

Variable	p-value	significance
Log (distance from periphery)	6.6e-05	***
GRAVY 9Å	6.9e-04	***
SASA	1.5e-02	*

Fig. 2. Characterization of the cleavage sites specific to native OVA *vs* those specific to aggregates. (A) Identification of the different sets of cleavage sites to be compared to understand the impact of heat-induced aggregation. The 11 native OVA-specific cleavage sites observed after 30 s of digestion are inside the red circle, and the 49 aggregate-specific cleavage sites are inside the blue circles. (B) Significant effects determined by univariate analyses using non-parametric mean comparison for numerical variables and chi-square tests for categorical variables. Significance code: *** < 0.001; * < 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

conditions as digestion continues (Fig. 1B–E), presumably due to the progressive disintegration of the aggregates, which makes these cleavage sites progressively accessible to pepsin. In fact, while 128 cleavage sites are common to all conditions after 30 s of digestion (Fig. 1A), this number increases progressively until reaching 218 after 60 min (Fig. 1G). This significant increase in the number of cleavage sites common to all conditions suggests that the differences observed between the OVA states mainly reflect differences in the kinetics of pepsinolysis.

This result is nonetheless interesting, as differences in pepsinolysis kinetics can have metabolic effects, by modulating amino acid (AA) delivery in the bloodstream, which controls the balance between catabolic and anabolic utilization of AAs (Pinckaers et al., 2021). This has been extensively studied in the case of milk proteins, whose fate during digestion primarily depends on the rate of gastric emptying, which in turn depends on the nature of the milk proteins and their ability to coagulate or not under gastric conditions (Horstman & Huppertz, 2023). But beyond this "nature of the proteins" effect, the present study suggests that heat-induced aggregation of OVA can be used with the aim of modulating and/or directing gastric proteolysis, and likely by cascade intestinal proteolysis. In this sense, this study supports the prospects proposed by Loveday (2023) of using food processing as an effective tool to optimize protein digestion and absorption for desired metabolic outcomes. However, such a perspective means that we need to understand what changes, induced by heat treatment, are involved.

3.4. *Denaturation prior to aggregation enables hydrolysis of peptide bonds never cleaved in native OVA*

Looking at the digestion kinetics as a whole, a total of 63 peptide bonds were hydrolyzed either more rapidly in the aggregates than in native OVA (49 peptide bonds), or specifically in the aggregates, *i.e.,* they were never cleaved in native OVA until the end of digestion (14 peptide bonds). All of these cleavage sites indicate by their very existence that the denaturation-aggregation process allows the "unmasking" of certain areas of OVA that were initially poorly accessible or even inaccessible to pepsin. Interestingly, the 14 aggregate-specific cleavage sites have a mean cleavage probability, based on the model proposed by Suwareh et al. (2021), significantly lower (*p*-value = 3.7e-03) than that associated with the 49 cleavage sites finally observed in native OVA, and similar (p-value = 0.73) to that of the 152 peptide bonds that were never cleaved in any condition (Supplementary material 8). It can then be inferred that these 14 cleavage sites would have never been observed without the denaturation-aggregation process. Since 11 out of these 14 cleavage sites are common to all aggregates (Fig. 1G), regardless of the aggregate morphology, it is more likely that the changes involved are related to the denaturation step rather than the aggregation step. In contrast, the 49 cleavage sites initially observed in the aggregates and that ended up being observed in native OVA as well, could correspond to cleavage sites expected in the native OVA, but which would be hydrolyzed as a priority in the aggregates due to the structural changes caused by the denaturation-aggregation process. These changes would either facilitate their hydrolysis by pepsin, or slow down the hydrolysis of other peptide bonds cleaved first in the native OVA. Actually, the corresponding 49 peptide bonds have cleavage probabilities indicating that their cleavage was expected in native OVA.

Table 2 shows the variables that compose the best model to distinguish the 49 cleavage sites initially observed in aggregates only and finally observed also in native OVA, from the 14 aggregate-specific cleavage sites never observed in native OVA. It is noticeable that these 14 aggregate-specific cleavage sites are located in a significantly more hydrophobic environment in the native form of OVA (within which the GRAVY index has been calculated), in comparison to the 49 peptide bonds that were finally hydrolyzed in native OVA as well. The preferential hydrolysis of the 14 cleavage sites in question, in aggregates composed of unfolded OVA is thus consistent with the positive effect of a higher surface hydrophobicity on gastric digestion (Ma et al., 2021). More generally, it is assumed that the folding state of the protein substrate can affect hydrolysis by pepsin, because of more or less accessible peptide bonds (Vreeke et al., 2023). As for the fact that these bonds are never cleaved in native OVA, one hypothesis would be that the hydrolysis cascades lead to configurations such that these 14 peptide bonds can no longer be hydrolyzed (*e.g.*, because they are too close to one end

Table 2
Characterization of the 49 aggregate-specific cleavage sites finally observed also in native OVA at the end of the digestion *vs* the 14 aggregate-specific cleavage sites never observed in native OVA until the end of digestion. Analysis of deviance (type II) was performed for the best model obtained by forward stepwise selection in order to illustrate the difference between the two groups following forward stepwise model selection. The p-value for each effect reflects its importance with respect to all other effects. Significance code: * < 0.05; · < 0.1.

Variable	p-value	significance
GRAVY 9A	2.0e-03	*
P1' classes	1.5e-02	*
PTM 9A	5.2e-02	.

of a released peptide, given the endoprotease character of pepsin). Vreeke et al. (2023) have referred to this type of peptide bonds as "masked cleavage sites".

The two groups of cleavage sites also differ significantly on the "P1' classes" variable (Table 2). In particular, the 14 cleavage sites that are aggregate-specific until the end of gastric digestion, are characterized by a high proportion (57%) of AARs not favorable to hydrolysis by pepsin at the P1' position (Supplementary material 9). Hence, the modifications induced by the OVA denaturation-aggregation process allow pepsin to hydrolyze peptide bonds that, based on the AAR sequence, should not have been hydrolyzed. This suggests that, if the 3D-structure of the protein is favorable, pepsin is able to hydrolyze peptide bonds that are supposed to be resistant. The 3D-structure of the protein would therefore have a major effect on the action of pepsin, which predominates over that of the sequence of AARs. This could explain why this protease has often been described as "unpredictable" when authors have tried to determine its specificity based on AARs alone (Ahn et al., 2013). Moreover, this suggests that simple technological processes such as heat treatment, which induce sufficient structural modifications in proteins known to be pepsin-resistant, could be effective ways of improving their gastric digestibility. This prospect is especially worthy of investigation given that the link between pepsin resistance and allergenicity is generally recognized (Foster et al., 2013; Wang et al., 2022).

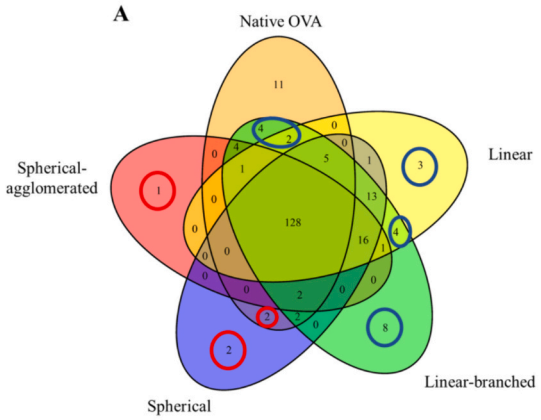
In addition to the comparisons of native and aggregated OVA reported above, which manly reflect the overall consequences of denaturation-aggregation, it then seemed appropriate to take a closer look at the peptide bonds specifically cleaved according to the type of OVA aggregates. In fact, with OVA, we have a protein model whose heat denaturation-aggregation mechanisms, as a function of pH and ionic strength, have been widely described (Doi, 1993). We therefore hoped to be able to link the differences in supramolecular organization between the aggregates and their hydrolysis by pepsin, if hydrolysis varied at all.

3.5. The characteristics of the cleavage sites specific to each type of OVA aggregates support the theoretical mechanisms of heat-aggregation

Consistently with the theoretical models of heat-induced aggregation of globular proteins (Bryant & McClements, 1998; Nicolai & Durand, 2013), and similarly to what was described by Nyemb, Guérin-Dubiard, et al. (2014), the spherical and spherical-agglomerated aggregates of OVA were produced under conditions (weak electrostatic repulsive forces between protein molecules) that favor protein aggregation over denaturation, suggesting weak or moderate denaturation of OVA before aggregation, and therefore subsequent little or no exposure of areas initially deeply buried within the native protein. In contrast, the low IS (0.03 M) associated with a high pH level (pH 7 or pH 9, *i.e.,* much higher than the isoelectric point of OVA, which is 4.5), applied to produce the linear and linear-branched aggregates, would result in a great denaturation before aggregation, and thus potentially an important exposure of initially deeply buried peptide bonds. As a result, it can be assumed that the conformation of OVA in spherical and spherical-agglomerated aggregates is much more similar to that of the native protein, especially with regard to the nature of buried areas, than in linear and linear-branched aggregates. Indeed, this hypothesis is supported by the fact that a large proportion of the native OVA-specific cleavage sites first become common to the spherical and spherical-agglomerated aggregates, before becoming common to all aggregates (Supplementary material 10).

To understand what distinguishes linear and linear-branched aggregates from spherical and spherical-agglomerated aggregates (Fig. 3A), different three-variable models have been constructed. Specifically, these models aimed to identify what distinguishes the cleavage sites observed in spherical and/or spherical-agglomerated aggregates but missing in linear and linear-branched aggregates, from the cleavage sites observed in linear and/or linear-branched aggregates but missing in spherical and spherical-agglomerated aggregates. By construction, the first two variables in each model were "P1 classes" and "distance from periphery", chosen because they are the most influential variables, as highlighted in the previous sections. Including them in the models therefore makes it possible to focus on the real differences between the groups currently being compared. Then, the third variable in each model was one of the remaining variables available for multivariate analyses. Fig. 3B and Fig. 3C show the tables of analysis of deviance (type II) of the only two models for which significant effects were observed.

The "GRAVY 9A" variable has a significant effect (p-value = 1.9e-02, Fig. 3B), with a negative coefficient (−1.8) indicating lower hydrophobicity, as measured in the native protein, around the cleavage sites observed only in spherical and/or spherical-agglomerated aggregates, in comparison with those specific to linear and/or linear-branched aggregates. This means that the cleavage sites observed specifically in linear and/or linear-branched aggregates are located, in native OVA, in more hydrophobic environments. Thus, it appears that the most hydrophobic areas of native OVA, which can reasonably be assumed to be buried deeply in the core of the molecule, are more exposed in linear and/or linear-branched aggregates, since they are more accessible to cleavage by pepsin in the early stages of digestion. This result is therefore



B

Variable	p-value	significance
P1 classes	4.7e-01	
Log (distance from periphery)	1.2e-02	*
GRAVY 9Å	1.9e-02	*

C

Variable	p-value	significance
P1 classes	9.2e-01	
Log (distance from periphery)	3.5e-01	
Charge 9Å	3.7e-02	*

Fig. 3. Characterization of the 5 cleavage sites observed in spherical or spherical-agglomerated aggregates at 30 s digestion but missing in linear and linear-branched aggregates (red circles) *vs* the 21 cleavage sites observed in linear or linear-branched aggregates but missing in spherical or spherical-agglomerated aggregates (blue circles). (A) Identification of the two sets of cleavage sites to be compared. (B, C) Analysis of deviance (type II) of the two three-variable models showing significant effect in the distinction of the two groups of cleavage sites. The p-value for each effect reflects its importance with respect to all other effects. Significance code: * < 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

consistent with the theoretical mechanism of denaturation-aggregation of globular proteins whereby linear and linear-branched aggregates are produced under conditions in which proteins are assumed to undergo an extensive denaturation prior to aggregation (Bryant & McClements, 1998; Doi, 1993; Nicolai & Durand, 2013). Interestingly, the variable "distance from periphery" also has a significant effect in the three-variable model, *i.e.*, when the variables "GRAVY 9A" and "P1 classes" are taken into account, whereas no significant difference was observed for this variable in the univariate analysis. This may reflect the fact that, due to conformational constraints, not all hydrophobic regions of globular proteins such as OVA are buried in the core of native molecules (Supplementary material 11). In other words, the hydrophobicity score alone does not ensure that a given peptide bond is located in the core of the protein molecule.

The "Charge 9A" variable also has a significant effect (p-value = 3.7e-02, Fig. 3C), with a coefficient (1.86) indicating higher charge, as measured in the native protein, around the cleavage sites observed only in spherical and/or spherical-agglomerated aggregates, in comparison with those specific to linear and/or linear-branched aggregates. This indicates that the most charged areas of OVA, which are preferentially present on the surface of the native protein, are proportionally more exposed in spherical and spherical-agglomerated aggregates than in linear and linear-branched aggregates, suggesting less denaturation of the protein in the former category of aggregates. Once again, this result is therefore consistent with the theoretical mechanisms used to describe the heat denaturation-aggregation process of globular proteins (Bryant & McClements, 1998).

The study thus demonstrates that the nature of the peptide bonds cleaved by pepsin depends not only on the aggregation of OVA, but also on the modalities of aggregation, which determine the structure of the aggregates at the molecular and supramolecular scales. The example of OVA thus illustrates what Loveday (2023) considered a likely hypothesis for all proteins, considering that the type and degree of alteration to the protein structure determine the effect of processes on their digestion. However, very few studies demonstrating the importance of the microstructural characteristics of protein aggregates for their fate during digestion have been reported to date. We can cite the study by Ma et al. (2021) who demonstrated variable kinetics and levels of pepsin hydrolysis between three types of heat-induced ß-lactoglobulin aggregates (fibrils, nanoparticles, and worm-like aggregates), but without going as far as to compare the nature of the hydrolyzed peptide bonds between the three conditions. Nevertheless, beyond the opportunities offered by protein aggregation as a technological lever to guide their fate during digestion, the differences highlighted here between the different types of OVA aggregates suggest that precisely tuned processes of aggregation could even allow subtle control of peptide bonds cleaved at the start of digestion, and therefore probably of peptides released at the end of digestion.

4. Conclusions

The present study aimed to elucidate the impact of protein aggregation on the gastric digestion process. To this end, an original approach was developed for analyzing hydrolyzed peptide bonds. This approach involved applying statistical analyses to peptidomics data, with a detailed characterization of each protein's peptide bonds and their environment. It enabled us to confirm, for the major globular egg white protein, *i.e.*, OVA, the impact of heat-induced aggregation on the course of digestion by pepsin, with an effect not only on digestion kinetics (faster for the native protein than for aggregated forms), but also on the nature of the hydrolyzed peptide bonds. Specific cleavage sites for the aggregated forms were observed, made possible by the denaturation of the proteins during the aggregation process. OVA aggregation therefore modifies the peptide profile during the gastric phase and it is reasonable to assume that this also has an effect on the peptide profile at the end of the intestinal phase, in line with a previous study (Nyemb, Guérin-Dubiard, et al., 2014). We also wanted to find out whether the morphological characteristics of protein aggregates could also have an impact on their digestion by pepsin. To this end, we took advantage of the possibility of modulating the denaturation-aggregation process of OVA, like globular proteins in general (Nicolai & Durand, 2013), to prepare linear (fibrillar) aggregates on the one hand and spherical (particulate) aggregates on the other, and subject them to digestion by pepsin. To our knowledge, the impact of the morphology of protein aggregates on the nature of the peptide bonds hydrolyzed by pepsin had never been studied, particularly given the attention we paid to the very first moments of gastric digestion and to the detailed characterization of the cleaved peptide bonds. Thus, we could show that certain cleavage sites are specific to one or other of these types of aggregates, particularly in the early stages of gastric digestion. We can therefore assume that by finely controlling the aggregation of a globular protein such as OVA, we can influence the course of gastric digestion, and consequently the nature of the peptides released. So, it is not just a question of looking at aggregation as a lever for controlling the digestive fate of proteins, but of controlling the modalities of aggregation even more precisely. However, this approach needs to be confirmed by further studies on a wide range of proteins, and by validating its relevance through precise monitoring of peptide bonds cleavage throughout the digestion process, *in vitro* and ideally *in vivo*. Therefore, there is probably still a long way to go before such strategies can be applied to the development of specific food products.

Furthermore, one of the interesting outcomes of this study is that the original approach we adopted to explore the question of the impact of aggregation seems to be validated. Applied to the specific cleavage sites at the first moment of gastric digestion, to either linear aggregates or spherical aggregates, the dual approach that combined peptidomics and statistical analyses testified to the differences between these two aggregation modalities, consistently with theoretical models of heat-induced denaturation-aggregation of OVA, and of globular proteins in general. Thus, pepsin enabled to gain access to heat-induced structural modifications in OVA, at a molecular and supramolecular scale. It would be interesting, in order to validate the approach developed here, to apply it to other enzymes which, like pepsin, are characterized by a relatively fluctuating specificity, as this is strongly affected by the environment of the peptide bonds. It should be noted, however, that this implies having sufficient data concerning the specificity rules of the enzyme studied on the one hand, and structural data for the substrate proteins on the other, which could constitute limiting points.

Funding

This work was supported by INRAE and Conseil Régional de Bretagne.

CRediT authorship contribution statement

Ousmane Suwareh: Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation. **David Causeur:** Writing – review & editing, Validation, Supervision, Software, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Steven Le Feunteun:** Writing – review & editing. **Julien Jardin:** Investigation, Data curation. **Valérie Briard-Bion:** Investigation, Data curation. **Stéphane Pezennec:** Writing – review & editing, Software, Methodology. **Françoise Nau:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.140260.

References

Ahn, J., Cao, M.-J., Yu, Y. Q., & Engen, J. R. (2013). Accessing the reproducibility and specificity of pepsin and other aspartic proteases. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics, 1834*(6), 1222–1229. https://doi.org/10.1016/j.bbapap.2012.10.003

Barbé, F., Le Feunteun, S., Rémond, D., Ménard, O., Jardin, J., Henry, G., Laroche, B., & Dupont, D. (2014). Tracking the in vivo release of bioactive peptides in the gut during digestion : Mass spectrometry peptidomic characterization of effluents collected in the gut of dairy matrix fed mini-pigs. *Food Research International, 63*, 147–156. https://doi.org/10.1016/j.foodres.2014.02.015

Barbé, F., Ménard, O., Le Gouar, Y., Buffière, C., Famelart, M.-H., Laroche, B., Le Feunteun, S., Dupont, D., & Rémond, D. (2013). The heat treatment and the gelation are strong determinants of the kinetics of milk proteins digestion and of the peripheral availability of amino acids. *Food Chemistry, 136*(3), 1203–1212. https://doi.org/10.1016/j.foodchem.2012.09.022

Barber, C. B., Center, T. G., & Laurent, S. (2023). Cxhull: Convex Hull (0.7.2) [Logiciel]. https://CRAN.R-project.org/package=cxhull.

Bryant, C. M., & McClements, D. J. (1998). Molecular basis of protein functionality with special consideration of cold-set gels derived from heat-denatured whey. *Trends in Food Science & Technology, 9*(4), 143–151. https://doi.org/10.1016/S0924-2244(98)00031-4

Chen, H. (2022). VennDiagram: Generate high-resolution Venn and Euler plots (1.7.3) [Logiciel]. https://CRAN.R-project.org/package=VennDiagram.

Chen, X., Chen, K., Cheng, H., & Liang, L. (2022). Soluble aggregates of Myofibrillar proteins engineered by Gallic acid : Colloidal structure and resistance to *in vitro* gastric digestion. *Journal of Agricultural and Food Chemistry, 70*(13), 4066–4075. https://doi.org/10.1021/acs.jafc.1c05840

Croguennec, T., Nau, F., Pezennec, S., & Brule, G. (2000). Simple rapid procedure for preparation of large quantities of ovalbumin. *Journal of Agricultural and Food Chemistry, 48*(10), 4883–4889. https://doi.org/10.1021/jf991198d

Croguennec, T., O'Kennedy, B. T., & Mehra, R. (2004). Heat-induced denaturation/aggregation of β-lactoglobulin a and B : Kinetics of the first intermediates formed. *International Dairy Journal, 14*(5), 399–409. https://doi.org/10.1016/j.idairyj.2003.09.005

Della Valle, G. G., Souchon, I. I., & Anton, M. (2013). La matrice alimentaire : Définition, classification et caractérisation. In *Structure des aliments et effets nutritionnels* (p. 470 p.). Editions Quae https://hal.inrae.fr/hal-02803058.

Doi, E. (1993). Gels and gelling of globular proteins. *Trends in Food Science & Technology, 4*(1), 1–5. https://doi.org/10.1016/S0924-2244(05)80003-2

Dupont, D., Mandalari, G., Molle, D., Jardin, J., Léonil, J., Faulks, R. M., … Mackie, A. R. (2010). Comparative resistance of food proteins to adult and infant *in vitro* digestion models. *Molecular Nutrition & Food Research, 54*(6), 767–780. https://doi.org/10.1002/mnfr.200900142

Foster, E. S., Kimber, I., & Dearman, R. J. (2013). Relationship between protein digestibility and allergenicity : Comparisons of pepsin and cathepsin. *Toxicology, 309*, 30–38. https://doi.org/10.1016/j.tox.2013.04.011

Fox, J., Weisberg, S., Fox, J., & Weisberg, S. (2019). *An R companion to applied regression* (3rd ed.). thousand oaks CA: Sage https://socialsciences.mcmaster.ca/jfox/Books/Companion/. Logiciel.

Hamuro, Y., Coales, S. J., Molnar, K. S., Tuske, S. J., & Morrow, J. A. (2008). Specificity of immobilized porcine pepsin in H/D exchange compatible conditions. *Rapid communications in mass spectrometry : RCM, 22*(7), 1041–1046. https://doi.org/10.1002/rcm.3467

Horstman, A. M. H., & Huppertz, T. (2023). Milk proteins : Processing, gastric coagulation, amino acid availability and muscle protein synthesis. *Critical Reviews in Food Science and Nutrition, 63*(30), 10267–10282. https://doi.org/10.1080/10408398.2022.2078782

Langella, O., Valot, B., Balliau, T., Blein-Nicolas, M., Bonhomme, L., & Zivy, M. (2017). X!TandemPipeline : A tool to manage sequence redundancy for protein inference and Phosphosite identification. *Journal of Proteome Research, 16*(2), 494–503. https://doi.org/10.1021/acs.jproteome.6b00632

Liu, J., Wan, Y., Ren, L., Li, M., Lv, Y., Guo, S., & Waqar, K. (2021). Physical-chemical properties and in vitro digestibility of phosphorylated and glycosylated soy protein isolate. *LWT, 152*, Article 112380. https://doi.org/10.1016/j.lwt.2021.112380

Liu, Y., Hoppenbrouwers, T., Wang, Y., Xie, Y., Wei, X., Zhang, H., … Bastiaan-Net, S. (2023). Glycosylation contributes to Thermostability and proteolytic resistance of rFIP-nha (Nectria haematococca). *Molecules, 28*(17), 6386. https://doi.org/10.3390/molecules28176386

Loveday, S. M. (2023). Protein digestion and absorption : The influence of food processing. *Nutrition Research Reviews, 36*(2), 544–559. https://doi.org/10.1017/S0954422422000245

Ma, R., Lin, Z., Wu, Y., Gao, Z., Hu, B., Xu, L., Fang, Y., & Nishinari, K. (2021). Modulating the in vitro gastric digestion of heat-induced beta-lactoglobulin aggregates : Incorporation with polysaccharide. *Food Chemistry, 354*, Article 129506. https://doi.org/10.1016/j.foodchem.2021.129506

Nicolai, T., & Durand, D. (2013). Controlled food protein aggregation for new functionality. *Current Opinion in Colloid & Interface Science, 18*(4), 249–256. https://doi.org/10.1016/j.cocis.2013.03.001

Niu, C., Luo, H., Shi, P., Huang, H., Wang, Y., Yang, P., & Yao, B. (2016). N-glycosylation improves the pepsin resistance of histidine acid phosphatase Phytases by enhancing their stability at acidic pHs and reducing Pepsin's accessibility to its cleavage sites. *Applied and Environmental Microbiology, 82*(4), 1004–1014. https://doi.org/10.1128/AEM.02881-15

Nyemb, K., Guérin-Dubiard, C., Dupont, D., Jardin, J., Rutherfurd, S. M., & Nau, F. (2014). The extent of ovalbumin in vitro digestion and the nature of generated peptides are modulated by the morphology of protein aggregates. *Food Chemistry, 157*, 429–438. https://doi.org/10.1016/j.foodchem.2014.02.048

Nyemb, K., Jardin, J., Causeur, D., Guérin-Dubiard, C., Dupont, D., Rutherfurd, S. M., & Nau, F. (2014). Investigating the impact of ovalbumin aggregate morphology on in vitro ovalbumin digestion using label-free quantitative peptidomics and multivariate data analysis. *Food Research International, 63*, 192–202. https://doi.org/10.1016/j.foodres.2014.03.041

Palashoff, M. H. (2008). Determining the specificity of pepsin for proteolytic digestion. https://repository.library.northeastern.edu/files/neu:767.

Pinckaers, P. J. M., Trommelen, J., Snijders, T., & Van Loon, L. J. C. (2021). The anabolic response to plant-based protein ingestion. *Sports Medicine, 51*(S1), 59–74. https://doi.org/10.1007/s40279-021-01540-8

Powers, J. C., Harley, A. D., & Myers, D. V. (1977). *Subsite specificity of porcine pepsin* (pp. 141–157). Boston, MA: Springer. https://doi.org/10.1007/978-1-4757-0719-9_9

R Core Development Team. (2020). R Core Team (2020). R: A language and environment for statistical computing. In *R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/* (4.0.0) [R] https://www.R-project.org/.

Reeb, J., & Rost, B. (2019). Secondary structure prediction. In *Encyclopedia of bioinformatics and computational biology* (pp. 488–496). Elsevier. https://doi.org/10.1016/B978-0-12-809633-8.20267-7.

Suwareh, O., Causeur, D., Jardin, J., Briard-Bion, V., Le Feunteun, S., Pezennec, S., & Nau, F. (2021). Statistical modeling of in vitro pepsin specificity. *Food Chemistry, 362*, Article 130098. https://doi.org/10.1016/j.foodchem.2021.130098

Torcello-Gómez, A., Dupont, D., Jardin, J., Briard-Bion, V., Deglaire, A., Risse, K., Mechoulan, E., & Mackie, A. (2020). The pattern of peptides released from dairy and egg proteins is highly dependent on the simulated digestion scenario. *Food & Function, 11*(6), 5240–5256. https://doi.org/10.1039/D0FO00744G

Vreeke, G. J. C., Vincken, J.-P., & Wierenga, P. A. (2023). Quantitative peptide release kinetics to describe the effect of pH on pepsin preference. *Process Biochemistry, 134*, 351–362. https://doi.org/10.1016/j.procbio.2023.10.021

Wang, K., Crevel, R. W. R., & Mills, E. N. C. (2022). Assessing protein digestibility in allergenicity risk assessment : A comparison of in silico and high throughput in vitro gastric digestion assays. *Food and Chemical Toxicology, 167*, Article 113273. https://doi.org/10.1016/j.fct.2022.113273

Zhang, T., Chen, T., Jiang, H., Zhang, M., Gong, P., Liu, J., & Liu, X. (2023). Effect of pH treatment on egg white protein digestion and the peptidomics of their in vitro digests. *Food Research International, 173*, Article 113327. https://doi.org/10.1016/j.foodres.2023.113327