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Food Research International 134 (2020) 109204

Contents lists available at ScienceDirect

Food Research International

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

Hydrolysis of plant proteins at the molecular and supra-molecular scales during *in vitro* digestion

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ARTICLE INFO

Keywords:
INFOGEST protocol
Gluten
Soybean
Tofu
Seitan
Soya juice
Pea
Emulsion

ABSTRACT

The digestion of plant protein is highly dependent on multiple factors, with two of the most important being the protein source and the food matrix. The present study investigated the effects of these two factors on the digestion of seitan (a wheat-based food), tofu, soya juice, and a homemade emulsion of soy oil and water that was stabilised with pea protein. The four plant matrices and their respective protein isolates/concentrates (wheat gluten, soya protein, pea protein) were subjected to *in vitro* static digestion following the INFOGEST consensus protocol. We monitored the release of α-amino groups during digestion. We found that food matrix had a strong influence on protein digestion: soya juice was more hydrolysed than fresh tofu (51.1% versus 33.1%; $P = 0.0087$), but fresh tofu was more hydrolysed than soya protein isolate (33.1% versus 17.9%; $P < 0.0001$). Likewise, the pea-protein emulsion was better hydrolysed than the pea-protein isolate ($P = 0.0033$). Differences were also detected between the two solid foods investigated here: a higher degree of hydrolysis was found for tofu compared to seitan (33.1% versus 11.8%), which was perhaps a function of the presence of numerous dense protein aggregates in the latter but not the former. Furthermore, freeze-drying more than doubled the final degree of hydrolysis of seitan ($P < 0.0001$), but had no effect on tofu ($P = 1.0000$). Confocal microscopy revealed that protein networks in freeze-dried seitan were strongly altered with respect to the fresh product; instead, protein networks in freeze-dried and fresh tofu were largely similar. Finally, we found that the protease:protein ratio had a strong effect on the kinetics of proteolysis: a 3.7-fold increase in the concentration of the soya protein isolate with respect to that of the soya juice decreased the final degree of hydrolysis from 50.3 to 17.9% ($P = 0.0988$).

1. Introduction

Compared to animal protein, proteins derived from plants are known for their reduced environmental and economic impacts (González, Frostell, & Carlsson-Kanyama, 2011). However, their two principal drawbacks are the lack of certain essential amino acids to fulfil human nutritional needs and the presence of anti-nutritional factors, i.e. proteins or associated compounds (enzyme inhibitors, fibre, saponins, and tannins) that hinder the digestive process. Moreover, plant albumins are generally thought to be resistant to proteolysis due to their compact structure, a high number of disulfide bonds, and their capacity for self-association (Carbonaro, Maselli, & Nucara, 2015). To improve the nutritional properties of plant-based protein sources, proteins from legumes and cereals are often mixed to create an amino-acid profile that is more nutritionally complete.

To estimate protein quality, the most commonly used reference indicators are the Protein Digestibility-Corrected AA Score (PDCAAS) and the more recently proposed Digestible Indispensable Amino Acid Score (DIAAS), which takes into account the digestibility of the limiting amino acid. These values are typically determined through *in vivo* experiments, which are not always desirable due to the ethical and economic considerations involved. As an alternative, efforts have been made to develop *in vitro* systems that could help to investigate questions associated with digestion. For example, one recent study investigated the relationship between *in vitro*-based measures of digestibility and PDCAAS values of chickpea flour and its protein fractions. The authors found that measurements of peptide-bond hydrolysis gave the highest correlation with *in vivo*-generated results (Tavano, Neves, & da Silva

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https://doi.org/10.1016/j.foodres.2020.109204
Received 3 September 2019; Received in revised form 20 March 2020; Accepted 27 March 2020
Available online 02 April 2020

Júnior, 2016). Admittedly, *in vitro* protocols do not perfectly reproduce all aspects of digestive physiology—for instance, endopeptidases, neurohormonal control, and intestinal absorption are lacking, and the geometry of the gastrointestinal tract and gastric mixing are poorly mimicked. They do, however, have the advantage of being rapid and economical ways to estimate protein degradation under digestive conditions, particularly compared to *in vivo* experiments on animals or clinical studies, which require more resources and are accompanied by ethical concerns (Bohn et al., 2018). Moreover, in a well-controlled *in vitro* system, the volume of bolus and the quantity of endogenous secretions are known, which enables the precise determination of their contributions to the monitored parameters. Sampling can be performed frequently without extensive disruptions to the system, and inter-trial variability is usually lower than the inter-individual variation of *in vivo* trials. Finally, potential structural changes during digestion can be easily detected.

One of the most important factors in determining the digestibility, bioaccessibility, and ultimate bioavailability of food components for an organism is the food matrix. For example, protein digestion varies considerably among cheese matrices (Lorieau et al., 2018), but to a lesser extent among plant-based food matrices (Rozan et al., 1997). Food processing, such as heat treatment, has also been shown to improve plant protein digestibility, as a consequence of heat-induced protein dissociation or unfolding and/or the elimination or deactivation of thermolabile antinutritional factors, such as trypsin inhibitors (Liu, Song, Maison, & Stein, 2014). In the present study, we aimed to further investigate the matrix effect on protein digestion of plant-based foods. Specifically, we used the *in vitro* INFOGEST digestion protocol (Minekus et al., 2014) to perform comparisons of food products that differed in many key aspects: structure (liquid versus solid), protein source (wheat, soya, pea), and the presence/absence of a food matrix (whole food preparation versus protein isolate/concentrate). We analysed four manufactured foods—seitan, tofu, soya juice, and pea emulsion—that represented three of the most widely produced crops worldwide (among legumes, cereals, and pulses, in tonnes): wheat (8–12%$_{Prot}$ (w/w); 2rd), soybean (40–45%$_{Prot}$ (w/w); 4th), and green pea (22–24%$_{Prot}$ (w/w); 30th) (FAOSTAT, 2017). Seitan is an east Asian wheat-based food, mostly constituted of gluten and starch that has undergone mild thermal treatment. Soya juice and tofu share the same base ingredient, but we included both here to investigate the effects of their highly different macrostructures: soya juice is obtained simply from soaking soya beans in water, while tofu, also called soya bean curd, undergoes one more step of chemical and thermal coagulation (induced by the addition of glucono-δ-lactone, magnesium chloride, or calcium sulfate; Fukushima, 1981). The green pea emulsion was created via ultra-pressure homogenisation of a commercial pea protein isolate (also included in the study) and soya oil in water, and was designed to mimic as closely as possible the nutrient composition and structure of soya juice. With these four foods our study was able to examine differences in the digestion of solids (seitan, tofu) and liquids (soya juice, pea emulsion). All of these results were then compared to those obtained from digestion of the corresponding protein isolate/concentrate for each food—wheat gluten, soya protein (two concentrations, representing soya juice and tofu), and commercial pea protein isolate. To our knowledge, this is one of the first time that digestion of a protein isolate (soya) has been compared to the digestion of the same protein in two different food matrices made with the same batch i.e. a liquid soya juice and a solid tofu. Also, this is the first time that seitan has been studied regarding to the digestive degradation of its proteins.

2. Materials and methods

2.1. Preparation of products

2.1.1. Protein isolates & concentrates

As sources of isolated/concentrated protein, we used readymade commercial pea isolate (Pisane M9, Lot: N16231O04, Cosucra, Belgium) and wheat gluten; concentrated soya proteins were prepared in our laboratory. Wheat gluten and two batches of husked soya beans were graciously provided by Nutrition & Santé (Revel, France). Proteins were extracted from soya beans using a pH precipitation protocol that combined steps from two previous studies (Li et al., 2007; Sorgentini & Wagner, 2002). Briefly, husked soya beans were milled with an ultra-centrifugal mill (ZM2000, Retsch GmbH, Germany) using a 0.5-mm sieve and an airstream to avoid heating. Then, the remaining flour was dehulled with hexane (52750, Sigma-Aldrich, USA; 1:4 hexane:flour ratio (w/w)) three times using an overhead shaker at 48 tr.min^{-1} (Reax 2, Heidolph, Germany) and vacuum filtration (WhatMan Phase Separator 70 mm, GE Healthcare, USA). Finally, residual hexane was allowed to evaporate under a fume hood overnight. The defatted soya flour was extracted for 2 h at room temperature with distilled water (10:1 water:flour ratio) that had been adjusted to pH 8.0 with 2 N $(NH_4)_2CO_3$ (T122523, Brenntag AG, Germany). The suspension was then centrifuged at 10,400 g for 15 min at 20 °C (Sorvall, Lynx-4000 superspeed centrifuge, Thermo Fisher Scientific, USA); the supernatant was adjusted to pH 4.5 with 1 N HCl before being stored at 4 °C for 2 h. The mixture was again centrifuged at 10,400 g for 20 min at 4 °C and the pellet was washed with distilled water, resolubilised by neutralisation to pH 8.0 with 2N $(NH_4)_2CO_3$, and freeze-dried. Final tofu-related and soya juice-related protein isolates contained, respectively, 83.3% and 80.2% protein (conversion factor: 5.50).

2.1.2. Food matrices

In addition to the protein isolates described above, this study was conducted using two solid food matrices and two liquid food matrices that we prepared ourselves. The wheat gluten was used to make seitan; the two soya bean batches were used to prepare tofu and soya juice. Soya juice was UHT (Ultra-High Temperature) packed. A portion of the tofu and seitan was minced, freeze-dried (Eurolyo, France), and then rehydrated (with the same quantity of distilled water previously lost) under agitation for 30 min. These were labelled freeze-dried tofu and freeze-dried seitan, respectively. The pea emulsion was prepared using the commercial pea isolate and commercial soya oil (Emile Noël, France). After assessing the protein and fat content of the pea isolate, both ingredients were added to water in proportions designed to replicate the protein and fat content of soya juice. The pre-emulsion was created using a disperser (T-50 Homogeniser, Ultra-Turrax, IKA, Germany) and a 15 G Dispersing Element (IKA, Germany) for 1 min at 10000 rpm. The solution was then homogenised two times using a table-top homogeniser (PandaPLUS 2000, GEA, USA) at 1000 bar. Temperature was checked at the end of processing and never exceeded 46 °C.

2.2. Food characterisation

2.2.1. Nutrient content

Dry matter (DM) content was determined by oven-drying at 105 °C overnight. Similarly, ash content was measured according to AOAC 923.03 (AOAC, 1990). Crude fat content was determined by the Randall method after 12 h incubation in 1 M HCl (ISO, 2008). Total nitrogen content was measured using the Kjeldahl method according to Thiex, Manson, Anderson, and Persson (2002). To calculate crude protein content, conversion factors of 5.36 for pea proteins, 5.50 for soya, and 5.50 for wheat proteins were used according to (Mariotti, Tomé, & Mirand, 2008). Starch content was measured using a polarimeter (European Commission, 2009). Non-starch polysaccharide content was checked (Englyst & Cummings, 1984), but as seed coats were removed from food during processing, only traces were found.

2.2.2. Structure of solid foods

2.2.2.1. Confocal laser scanning microscopy of solid food before and after freeze-drying. Sections of 0.1-mm thickness were cut with a sharp razor

blade and placed on microscopy slides between two gene frames with 10 μL of fluorescent probes for protein (Alexa Fluor 546, Thermo Fisher Scientific, Inc., USA) and fat (Nil Red, Thermo Fisher Scientific, Inc., USA). Slides were examined using a confocal microscope (green laser) (Eclipse A1+, Nikon, Japan) in spectral mode. Pictures taken (presented here) were representative of the whole slide.

2.2.2.2. Scanning electron microscopy (SEM) of freeze-dried food. Freeze-dried products were ground into small particles 0.1–1 mm in diameter. Observation was made with an Environmental Quanta 200 FEG (FEI Company, USA) accelerated at 5 kV and under vacuum pressure of 40 Pa. Pictures shown here were considered representative of the sample.

2.3. In vitro digestion protocol

2.3.1. Set-up

This study used the INFOGEST *in vitro* digestion protocol (Minekus et al., 2014), with a specific focus on proteolysis (Lotti Egger et al., 2015). The enzymes used were protease, *i.e.* pepsin (P6887, Sigma-Aldrich, USA) during the gastric phase and porcine pancreatin (P7545, Sigma-Aldrich, USA) during the intestinal phase. Enzyme activity was determined according to EC 3.4.23.1 for pepsin and EC 3.4.21.4 for trypsin of porcine pancreatin, following the protocol described in the supplementary data of (Minekus et al., 2014). Biliary acid content in porcine bile (B8631, Sigma-Aldrich, USA) was checked using a colorimetric reaction-based kit (ref. 1 2212 99 90 313, DiaSys Diagnostic System GmbH). Digestions were conducted in an orbital shaking water bath (1092, GFL, Germany) at 37 °C and 150 rpm. Five mL of a commercial skim milk powder (Régilait, France) (1/10 dissolution in H_2O, w/v) was also digested two times as a control.

2.3.2. Oral phase

Solid foods were ground using a manual mincer until they passed through a 2-mm sieve (Edington Mincer Pro, Product Code 86002, Berkshire, UK), and were then incubated for digestion. In order to simulate the oral phase, **5 g** (of solids) or **5 mL** (of liquids) were mixed with 4 mL of 1.25x concentrated simulated saliva fluid (1.25x SSF), 975 μL of distilled water, and 25 μL of 0.3 M $CaCl_2$ in a 50-mL Schott bottle for two minutes. Each digestion was performed with the same initial amount of food. Incubations of isolates or concentrates contained the same amount of protein as found in those of the related food. Digestions were performed at least in triplicate, in addition to a digestion without enzyme for each group.

2.3.3. Gastric phase

Then, 8 mL of 1.25x concentrated simulated gastric fluid (1.25x SGF) and 5 μL of 0.3 M $CaCl_2$ were added to the oral bolus, and the pH of the mixture was gently adjusted to 3 using 1 M HCl. Distilled water was added to create a final volume of 19 mL. After allowing the mixture to reach 37 °C, 1 mL of pepsin solution (40 000 $U.mL^{-1}$) was rehydrated and added to the digestion reactor to obtain a final concentration of 2 000 $U.mL^{-1}$.

2.3.4. Intestinal phase

After 2 h of the gastric phase, 11 mL of 1.25x concentrated simulated intestinal fluid (1.25x SIF), 40 μL of 0.3 M $CaCl_2$, and 2.5 mL of bile solution (160 $mmol.L^{-1}$) were added to the chyme. The pH of the mixture was adjusted to 7 using 1 M NaOH and the reactor was filled with distilled water to reach a final volume of 35 mL, without considering the volume of the food. When the temperature reached 37 °C, 5 mL of pancreatin solution (800 $U.mL^{-1}$ diluted in SIF) were added. The incubation lasted 2 h.

2.3.5. Sampling and pH regulation

pH was adjusted between each step and checked every 30 min using a glass electrode (N61, SI Analytics GmbH, Germany). We considered the mixture to be homogenous, and took 500-μL samples before incubation with enzymes and 10, 30, 60, and 120 min after the gastric and intestinal phases started. Two initial samplings were performed during the oral phase. At each step, < 3 mL of content were collected, representing 15% and 7.5% of the chyme volume during the gastric and intestinal period, respectively. Immediately after each sample was taken, enzymatic reactions were blocked with enzymatic inhibitors. For gastric samples, we used 10 μL of 0.5 $mg.mL^{-1}$ Pepstatin A (P5318, Sigma-Aldrich, USA), diluted in 9:1 methanol:acetic acid; for intestinal samples, we added 30 μL of 0.1 M Pefabloc SC (76307, Sigma-Aldrich, USA), diluted in distilled water. Samples were then vortexed for 5 s, put on ice, and stored at –20 °C until further analysis was carried out.

2.4. Release of α-amino groups and determination of degree of hydrolysis (DH)

The concentration of α-amino groups was determined using the ninhydrin reaction described in (Moore & Stein, 1954; Sarin, Kent, Tam, & Merrifield, 1981). Ninhydrin reagent (250 mL) was prepared by mixing 0.75 g of D (-) fructose (F0127, Sigma-Aldrich), 1.5 g of ninhydrin (151173, Sigma-Aldrich), 15 g of monopotassium phosphate (PHR1330, Sigma-Aldrich), and 9.914 g of sodium phosphate, dibasic anhydrous (71639, Sigma-Aldrich). The mixture was adjusted to pH 6.7, completed with milliQ water, and stored in the dark until use. For each digest, the sample was centrifuged (5000 g for 10 min at 20 °C), and the supernatant was diluted with milliQ water and mixed with the prepared ninhydrin reagent at a ratio of 1:2 (v/v ninhydrin reagent:diluted solution). After heat treatment at 95 °C for 16 min, the solution was cooled on ice for 20 min. Dilution solvent (3:2 milliQ water:96% ethanol with 0.2% w/v of potassium iodate (215929, Sigma-Aldrich)) was added at a 5:1 ratio (v/v diluent:solution diluted). Absorbance at 570 nm was quantified with a Spark 20 M microplate reader (TECAN, Switzerland). A calibration curve was constructed using data generated with known concentrations of glycine in distilled water (ranging from 0.04 to 0.21 mM; G8898, Sigma-Aldrich). The analysis was performed in duplicate for each sample.

To determine the total amount of α-amino groups released from food, we used the ninhydrin protocol described above on food samples that had first undergone total acid hydrolysis (2 mg of total protein incubated with 1.5 mL of 6 M HCl solution at 110 °C for 24 h, then cooled and diluted with water). Two measures of degree of hydrolysis (DH) were calculated from these data, using the following formulas:

- DH using the total amount of food nitrogen in the mix, $N_{tot\ food}$: $DH(t) = (N(t)-N(0))/(N_{tot\ food}-N(0)) \times 100$, where N(t) was the concentration of α-amino groups in the mixture at time t, corrected for endogenous input, and N(0) was the concentration of α-amino groups at time 0 (before the addition of pepsin).
- DH using the total amount of nitrogen from α-amino groups released after total acid hydrolysis, $N_{100\%}$, according to (Petitot et al., 2009): $DH_{N100\%}(t) = (N(t)-N(0))/(N_{100\%}-N(0)) \times 100$, where N(t) and N(0) are the same as described above.

2.5. Data & statistical analysis

Data are presented as mean ± SD (standard deviation). Gastric and intestinal steps were analysed independently. DH kinetics were compared using the repeated option of the SAS PROC MIXED procedure (SAS University Edition, v. 3.71; SAS Institute Inc., Cary, NC, USA), with time of digestion, food, and their interactions (written as time × food on figures) as fixed effects. If the effect of the interaction was significant, the LSMEANS procedure was used, followed by Tukey adjustment, to test differences between foods of interest at specific time points. The alpha level for our statistical tests was 0.05.

Table 1
Nutritional composition of studied products (g per 100 g (wet base)).

Protein source (N factor)	Food	DM	Ash	Protein	Fat	Starch	Name and protein content of the related protein isolate/concentrate
Wheat (5.50)	Seitan	38.4	1.3	27.4	2.1	3.0	49.8 (Gluten)
Soya (5.50)	Tofu	25.0	1.3	11.0	8.5	0	83.3 (Tofu-related protein isolate)
	Soya juice	8.0	0.5	3.0	2.1	0	80.2 (Soya juice-related protein isolate)
Pea (5.36)	Pea emulsion	5.22	0.25	3.0	2.1	0	73.6 (Pea protein isolate)

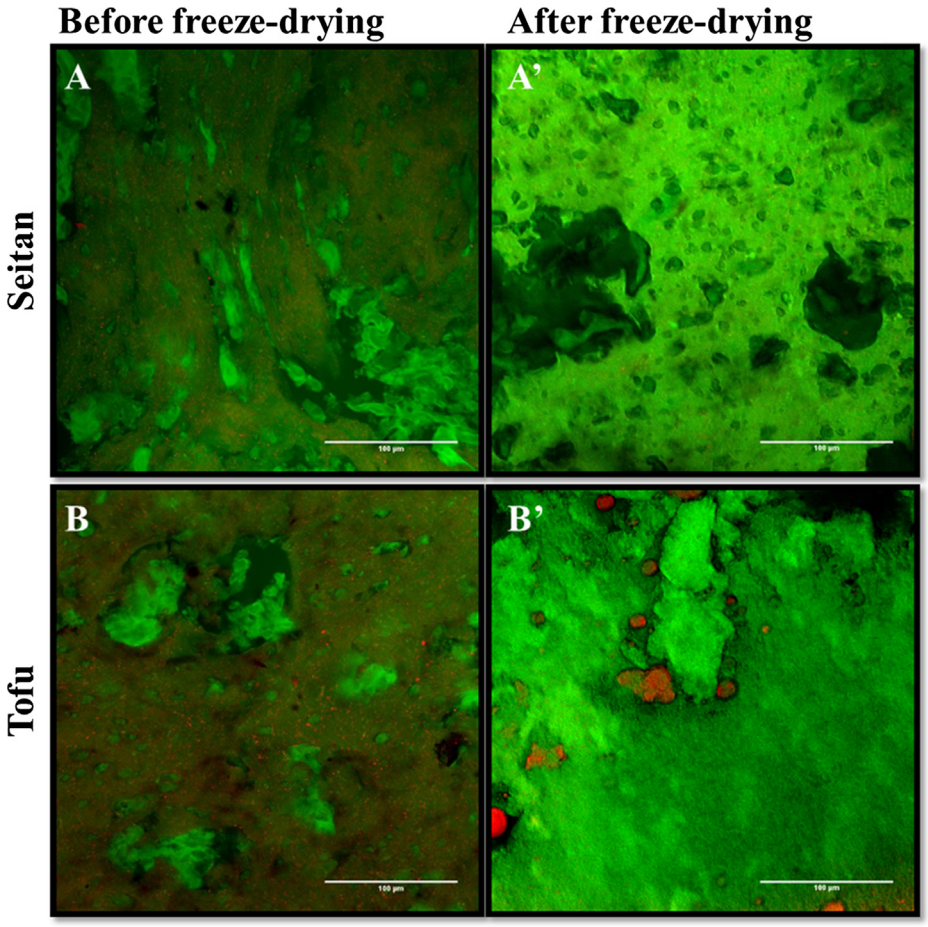

Fig. 1. Representative images from confocal laser scanning microscopy of seitan and tofu, before and after freeze-drying. Proteins were labelled in green and lipids in red (scale bar is 100 μm).

3. Results and discussion

3.1. Nutritional and structural characterisation

Table 1 shows the composition of the different foods investigated here. Liquids were intentionally prepared to have a fat and protein content that was comparable to their solid counterparts Seitan was characterised by a high starch content (3.0 g.100 g^{-1}), whereas tofu contained more fat (8.5 g.100 g^{-1} versus 2.1 g.100 g^{-1} for seitan).

Qualitatively, three distinct structures were identified in seitan and tofu (Fig. 1): (i) a hydrated gel with proteins that were more or less homogeneous, with droplets in the micrometer range, (ii) highly dense nuclei of proteins (diameter ~10 mm), (iii) and shadow areas containing water, perhaps created by shearing. Globally, seitan was more heterogeneous in composition than tofu.

As the seitan and the tofu were produced from wheat gluten or soya flour respectively, intact cells were still present in the final food. After the material had been freeze-dried, both matrices appeared more homogeneous. This might have been due to the fact that the freezing process degraded cell walls and ducts, thus facilitating the diffusion of fluorescent markers through the cell networks (Harnkarnsujarit, Kawai, Watanabe, & Suzuki, 2016). Likewise, the cell damage caused by freeze-drying also gave other components, such as enzymes, easier access to macronutrients. Since tofu had appeared more homogeneous than seitan prior to freeze-drying, we considered the resulting structural changes in seitan to be more consequential than those in tofu. Instead, in freeze-dried tofu, the lipids seemed to have coalesced. These qualitative observations were consistent with images taken with SEM (Fig. 2), in which porosity seemed to be higher in the freeze-dried seitan than in freeze-dried tofu.

3.2. Degree of hydrolysis (DH)

The degree of hydrolysis was calculated two different ways: using the total amount of food nitrogen ($N_{tot\ food}$) or the total amount of nitrogen in α-amino groups released after the total acid hydrolysis of food ($N_{100\%}$). These two evaluations of the degree of hydrolysis were referred to as DH and $DH_{N100\%}$, respectively.

All $DH_{N100\%}$ values were higher than DH values, which could be explained by the difference between the total amount of food nitrogen

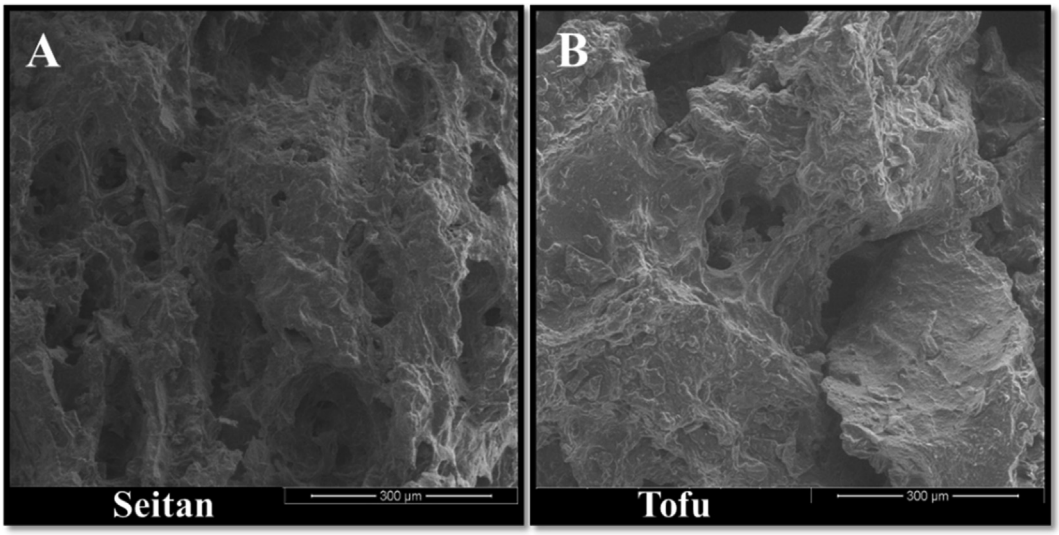

Fig. 2. Representative images from scanning electron microscopy of seitan and tofu (scale bar is 100 µm).

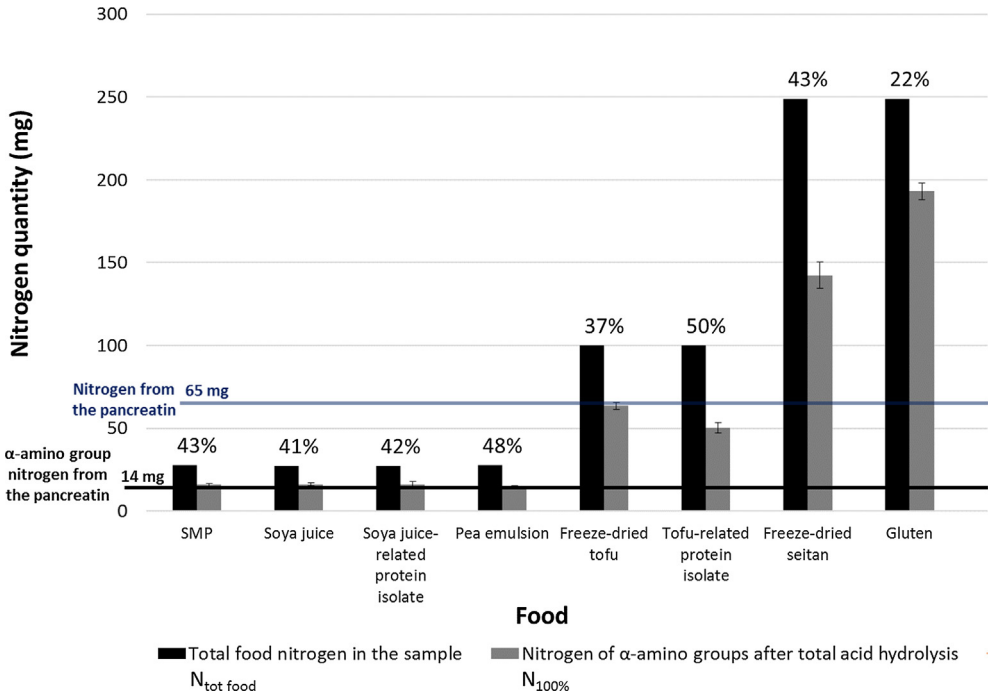

Fig. 3. Amount of total food nitrogen in the reactor ($N_{tot\ food}$) compared to the total amount of nitrogen in α-amino groups released after total acid hydrolysis ($N_{100\%}$) (bars are shown with standard deviations, n = 2 total hydrolyses). The black line indicates the amount of α-amino nitrogen contributed by pancreatin in the intestinal phase. Percentages above bars are relative differences between $N_{tot\ food}$ and $N_{100\%}$: $(N_{tot\ food} - N_{100\%})/(N_{tot\ food})$. SMP: Skim milk powder.

put in the reactor ($N_{tot\ food}$) and the total amount of α-amino-group nitrogen released after the total acid hydrolysis of food ($N_{100\%}$). $N_{100\%}$ was always lower than $N_{tot\ food}$, with the magnitude of the difference ranging from 22 to 50% depending on the food in question (Fig. 3). One contributing factor could have been that the total acid hydrolysis led to the destruction of some amino acids (Trp, Cys, Thr, Ser), which were therefore not taken into account in the determination of $N_{100\%}$ (FAO & WHO, 1991). Moreover, some nitrogen atoms that are not involved in α-amino groups in some amino acids (Gln, Asn, Lys, His) may been included in the measurement of $N_{tot\ food}$ and thus increased this value compared to the real amount of complete hydrolysis. Finally, some differences likely arose from the fact that we used different foods, with differences in amino acid profiles and food matrices that could affect the kinetics of acid hydrolysis.

Non-negligible differences were observed between different foodstuffs regarding the amounts of α-amino groups released after total hydrolysis. For this reason, hydrolysis kinetics were presented and analysed using the total amount of food nitrogen ($N_{tot\ food}$).

In the case of liquid foods, around 27–28 mg of $N_{tot\ food}$ were initially incubated, corresponding to 14–16 mg of α-amino-group nitrogen of totally hydrolysed food (Fig. 3). During the intestinal step, around 65 mg of nitrogen and 14 mg of α-amino group nitrogen were contributed by the addition of pancreatin. In terms of α-amino-group nitrogen, pancreatin therefore represented 31–33% of the total nitrogen in the intestinal mixture and 46–50% of the theoretical maximum amount of α-amino-group nitrogen that could be observed, excluding the contributions of pepsin and bile.

Overall, the amounts of α-amino-group nitrogen detected here were lower than those previously reported from other studies. Most of those studies used the O-Phthalaldehyde (OPA) method, which has become popular in recent years for the evaluation of digestive proteolysis. However, this method has some limits: the risk of false positives caused by ε-amino groups is higher than for the ninhydrin method (Church, Swaisgood, Porter, & Catignani, 1983; Turgeon et al., 1991) and OPA is

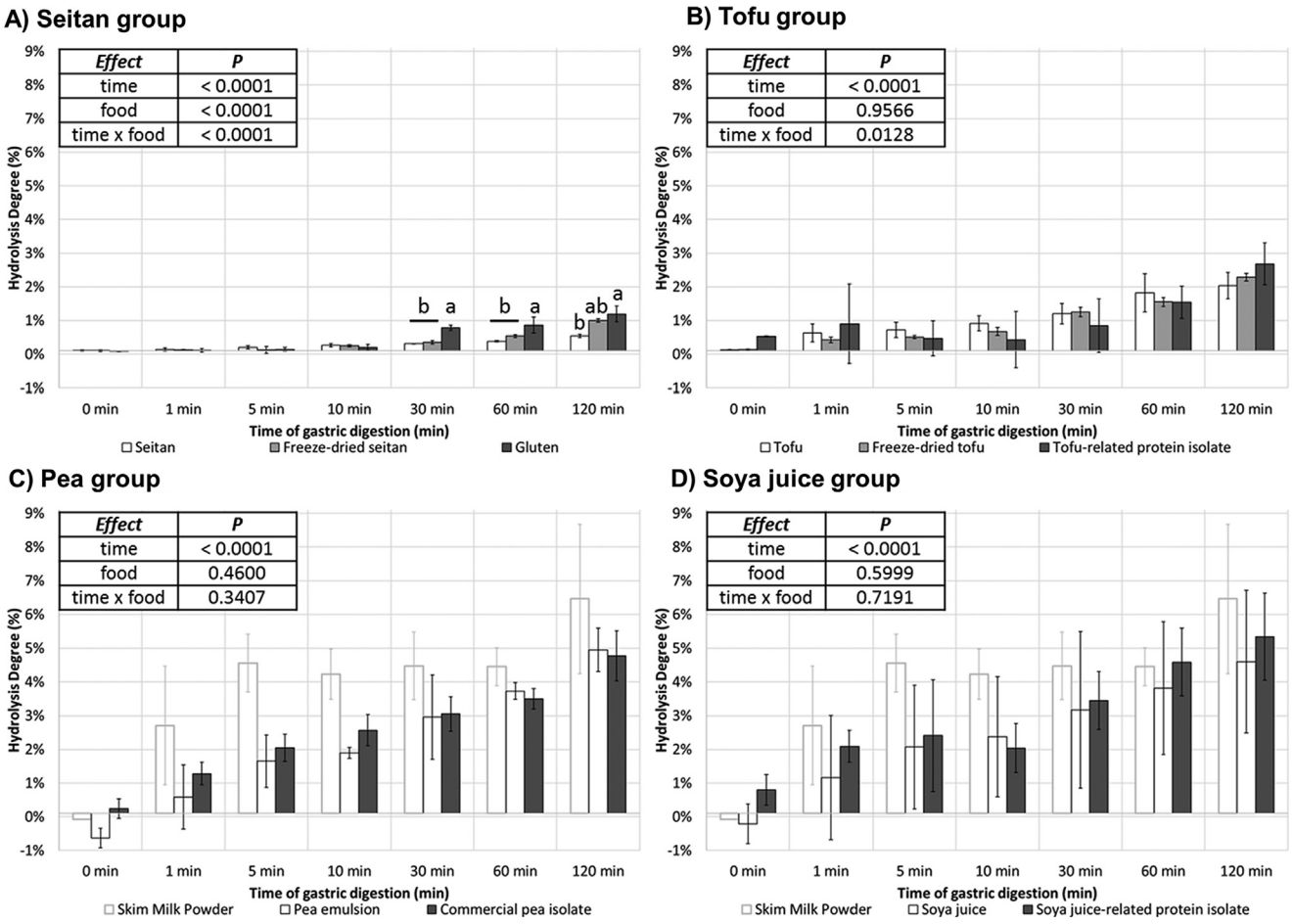

Fig. 4. Profiles of degree of hydrolysis, calculated with $N_{tot\ food}$ (DH), of protein during *in vitro* gastric digestion. Bars represent mean ± SD; n = 3–7 digestions. Panels show the DH of foods before and 1, 5, 10, 30, 60, and 120 min after incubation with pepsin. A) Seitan group; B) Tofu group; C) Pea group; D) Soya juice group. Letters (a – b) above bars indicate results of statistical comparison with post-hoc Tukey tests.

more dangerous to handle than ninhydrin. Our use of ninhydrin could explain the lower content of α-amino groups measured here compared to other studies that used OPA or 2,4,6-trinitrobenzenesulfonic acid (TNBS), as was previously shown in the digestion of pea protein (Panasiuk, Amarowicz, Kostyra, & Sijtsma, 1998).

3.3. Effect of the food structure on proteolysis

3.3.1. Degree of hydrolysis (DH) of food matrices

This section describes only the degree of hydrolysis (DH) of food items; protein isolates/concentrates are analysed in section 3.3.4 in the context of their corresponding foods.

The gastric proteolysis of fresh seitan (Fig. 4A) began late, after 30 min (DH = 0.3 ± 0.0%) of incubation, and reached a final DH of 0.4 ± 0.1% in this stage. Just after the addition of pancreatin, DH increased directly and progressively until it reached 11.8 ± 1.2% at 60 min, at which point it remained stable (120 min: 11.8 ± 2.6%) (Fig. 5A). The DH kinetics of freeze-dried seitan were largely similar to those of fresh seitan; the main exception was found in the intestinal step, when after 30 min the DH of freeze-dried seitan surpassed that of fresh seitan (see section 3.3.3 for more details).

With fresh tofu, gastric hydrolysis began just after the addition of pepsin (Fig. 4B); the DH was 0.5 ± 0.3% after 1 min. At 120 min, the DH arrived at 1.9 ± 0.4%. During the intestinal step (Fig. 5B), the addition of pancreatin induced a direct increase in DH (1 min: DH = 13.4 ± 5.9%), but with a large degree of variation among replicates. DH remained stable around this value for the next 10 min (DH = 12.0 ± 3.5%). Finally, from 30 min to 120 min after the addition of pancreatin, DH continued to rise linearly to a final value of 33.1 ± 4.0%. DH kinetics of freeze-dried tofu were similar to those of fresh tofu (see section 3.3.3 for more details).

Gastric hydrolysis of the pea emulsion (Fig. 4C) increased linearly, from 0.5 ± 1.0% at 1 min to a maximum of 4.9 ± 0.6%. One minute after the addition of pancreatin, the DH increased by more than a factor of seven (35.7 ± 19.4%), but again with extensive variation between tests. As we observed for tofu, during the 10 first minutes of the intestinal phase (Fig. 5C), the DH of the pea emulsion remained relatively stable around 35%. From that point until the end of the phase, the DH doubled linearly, increasing from 35.0 ± 20.6% to 73.6 ± 12.1%.

The DH of soya juice evolved similarly to that of the pea emulsion. In the simulated stomach (Fig. 4D), hydrolysis began immediately, and after 120 min of incubation, arrived at a maximum value of 4.5 ± 2.1%. In the intestinal environment (Fig. 5D), in the first minute following the addition of pancreatin the DH increased to 34.7 ± 14.0% and remained constant (about 30%) for 30 min, at which point the DH increased to reach a final value of 51.1 ± 15.5%.

Overall, the two solid matrices, seitan and tofu, exhibited a lower degree of proteolysis than the two liquid matrices, pea emulsion and soya juice. This might be due to the relative inhibition of pepsin diffusion within a solid matrix compared to a liquid one, as previously demonstrated between water and whey protein isolate gels (Luo, Borst, Westphal, Boom, & Janssen, 2017). Indeed, natural proteolysis typically occurs in heterogeneous phases (solid–liquid) rather than in solution. Among the four foods, seitan demonstrated the least amount of

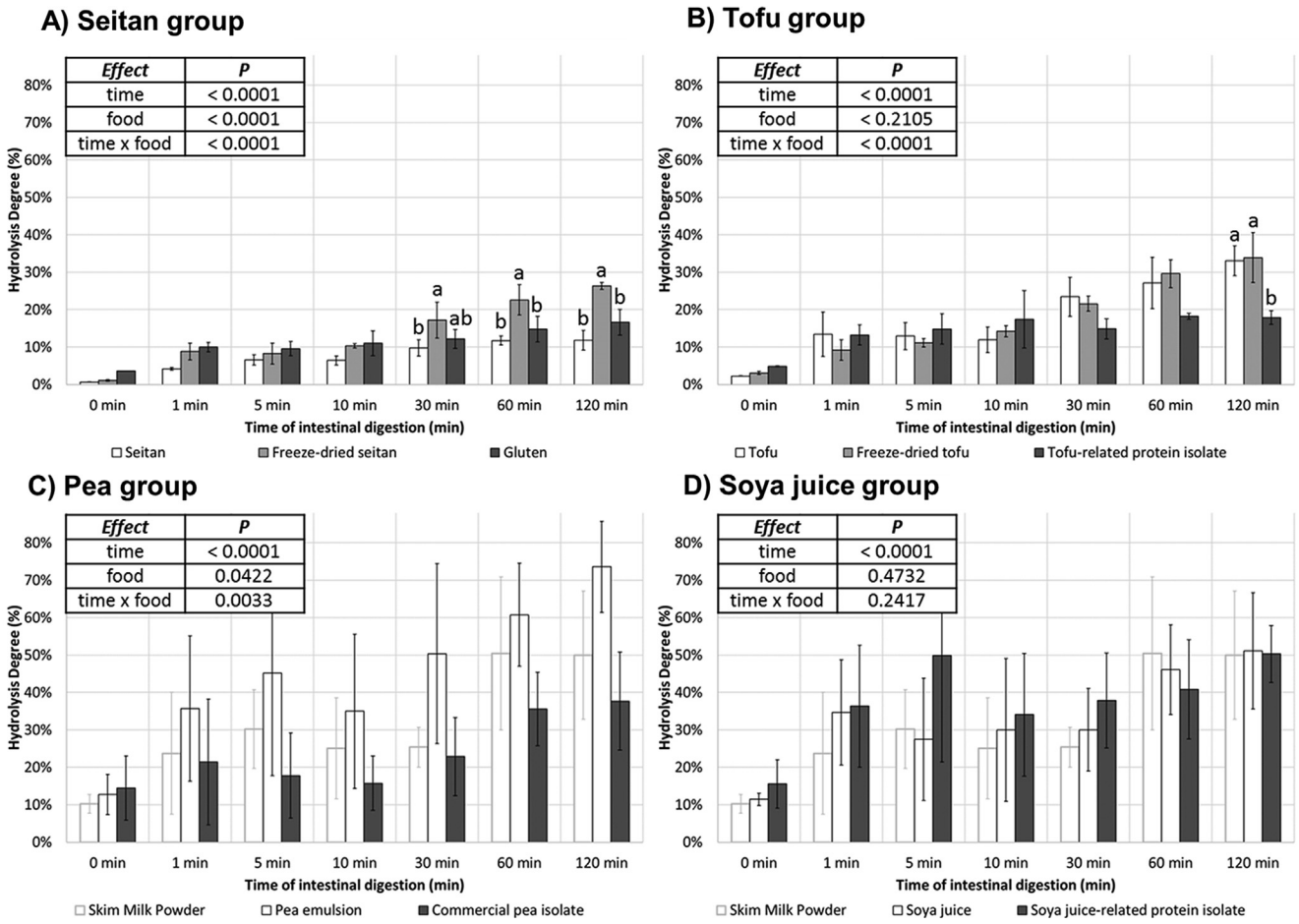

Fig. 5. Profiles of the degree of hydrolysis, calculated with $N_{tot\ food}$ (DH), of protein during *in vitro* intestinal digestion. Bars represent mean ± SD; n = 3–6 digestions. Panels show the DH of foods before and 1, 5, 10, 30, 60, and 120 min after incubation with pancreatin. A) Seitan group; B) Tofu group; C) Pea group; D) Soya juice group. Letters (a – b) above bars indicate results of statistical comparison with post-hoc Tukey tests.

hydrolysis during the gastric phase.

Values of DH in the gastric phase were low compared to final values in the intestinal phase. The differences we observed here between the DH in gastric and intestinal phases of *in vitro* digestion, which were based on similar quantities of protein, were consistent with those obtained from the gastric digestion of pasta (DH 8% after 180 min of pepsin incubation; Petitot et al., 2009) or whey protein (DH around 3–4%; Mat, Cattenoz, Souchon, Michon, & Le Feunteun, 2018). Following standard practice, we also digested a skim milk powder (diluted 1:10 in water) in order to compare our results to previous studies. As was reported by (Mat, Le Feunteun, Michon, & Souchon, 2016), we found that about 60% of dairy proteins were digested using the *in vitro* INFOGEST protocol (L. Egger et al., 2017; Picariello et al., 2015).

3.3.2. Solid and liquid soya-based foods

The effect of the food × time interaction was significant during gastric digestion (P = 0.0429), with the DH of soya juice increasing more quickly (-0.3 ± 0.0% to 4.5 ± 2.1%) (Fig. 4D) than that of tofu (0.0 ± 0.0% to 1.9 ± 0.4%) (Fig. 4B). This result is consistent with the observation that β-conglycinin persists in tofu but not in soya juice after pepsin digestion (Adachi et al., 2009). One possible explanation could be linked to the lower proportion of soluble nitrogen, which is accessible to enzymes, in tofu compared to in soya juice (Rui et al., 2016). Gastric conditions have also been reported to cause more significant structural alterations to soya juice than to tofu (Liu et al., 2019); this could also facilitate further intestinal digestion and lead to higher rates of hydrolysis.

During intestinal digestion, the effect of food was significant (P = 0.0087), but the food × time interaction was not (P = 0.6894); this meant that even if DH values were significantly different between tofu and soya juice, the kinetics of hydrolysis were similar. The final DH values highlighted the cumulative effect of the differences between the two products: soya juice was 51.1 ± 15.5% hydrolysed (Fig. 5D), whereas tofu was only 33.1 ± 4.0% hydrolysed (Fig. 5B).

3.3.3. Effect of freeze-drying

Significant differences were found between fresh and freeze-dried seitan at the end of the intestinal step, with fresh seitan demonstrating a lower DH (11.8 ± 2.6%) than freeze-dried (26.3 ± 0.9%) (P < 0.0001). Instead, this was not the case for tofu (fresh: 33.1 ± 4.0% versus freeze-dried: 33.9 ± 6.7%; P = 1.0000).

Freeze-drying seemed to increase the substrates' accessibility to enzymes by modifying the food structure, but not necessarily altering pepsin proteolysis sites. Although a previous study highlighted pore deformation and wall-thickness changes in tofu during freeze-drying, and leaching during rehydration, no effect was reported on the protein digestion of tofu (Harnkarnsujarit et al., 2016). Freeze-drying could also affect the bioaccessibility of other compounds, as was reported for polyphenol in apples, by altering the microstructure (Dalmaua, Bornhorst, Eima, Rosselló, & Simal, 2017).

3.3.4. Comparison between protein concentrates/isolates and food matrices

With soya-based foods and protein isolates, we found that the food matrix had a significant effect on protein digestion: the gel matrix of tofu seemed to protect peptide bonds from hydrolysis by trypsin (and, to a lesser extent, chymotrypsin), whereas emulsification during the

process of making soya juice appeared to expose them.

The effect of the food × time interaction was significant for tofu and its related preparations during both the gastric and intestinal steps, with the final DH of the tofu-associated protein isolate (17.9 ± 1.8%) markedly lower than that of fresh or freeze-dried tofu (33.1 ± 4.0% and 33.9 ± 6.7%, respectively; $P < 0.0001$ for both) (Fig. 5B). The likely explanation for this is that, compared to the unprocessed protein of the isolate, denaturation occurring during processing probably made the protein in the food matrix more sensitive to digestive proteases by opening protein structures (Desphande & Damodaran, 1989). However, for soya juice and its related isolate, there was no significant food × time interaction (gastric phase: $P = 0.7191$; intestinal phase: $P = 0.2417$) (Fig. 4D and Fig. 5D), which suggests that the protein in soya juice and in the soya juice-related isolate was digested in the same way. This is consistent with previous work performed on humans in which researchers found that, although the gastro-jejunal absorption of nitrogen appeared to be lower for soya juice (49%) than for soya protein isolate (63%), the difference was not statistically significant (Baglieri et al., 1994)).

For pea protein, the only differences between the protein isolate and the emulsion were found during the intestinal step: when analysed over the entire stage, the DH of the emulsion was significantly higher than the DH of the protein isolate ($P = 0.0033$) (Fig. 5C). However, no significant differences were detected at any of the individual sampling times. It should be noted that the coefficients of variation (ratio of the mean to the standard deviation) were particularly high for the pea emulsion, likely influenced by the high relative proportion of pancreatin in the intestinal phase, as discussed in section 3.2. A 1-liter batch of emulsion was prepared before each assay. We considered each batch to be homogenous, but since only small quantities were used in each assay (5 mL), it is possible that there were variations among the samples used, which then introduced variation into our results. Regardless, for pea protein, a simple solubilisation of the isolate appeared to be less conducive to protein hydrolysis than an emulsification. The high-pressure processing used for emulsification could explain the higher rate of protein digestion in this preparation, as previously described for pea isolates (Laguna, Picouet, Guàrdia, Renard, & Sarkar, 2017). This type of processing may cause an increase in protein solubilisation and in the number of lipid droplets, thus increasing the exposure of protein to enzymes.

With respect to wheat protein, isolated gluten was more accessible to enzymes than gluten in a food matrix, as indicated by the higher DH in the simulated stomach for wheat gluten compared to fresh seitan starting from 30 min after the addition of pepsin (0.7–1.1% versus 0.2–0.4%; $P < 0.0086$) (Fig. 4A). A similar difference was observed between gluten and freeze-dried seitan at 30 and 60 min, but not at 120 min. Then, during the intestinal step, the DH kinetics of gluten were similar to those of fresh seitan, but freeze-dried seitan was significantly more hydrolysed. The reduction in gastric DH observed for seitan compared to gluten could be due to the heat treatment used in the seitan-making process, which has been previously reported to lead to lower protein hydrolysis during *in vitro* digestion of gluten products (Rahaman, Vasiljevic, & Ramchandran, 2016; Wu, Taylor, Nebl, Ng, & Bennett, 2017).

3.4. Protein source and effect on proteolysis

3.4.1. Effect of protein source on the proteolysis of solids

When we compared fresh tofu and seitan, the food × time interaction was significant ($P < 0.0001$) in both the simulated stomach and small intestine. From 10 min after gastric output until the end of intestinal digestion, fresh tofu was more hydrolysed than fresh seitan ($P < 0.0276$), with the exception of just after the addition of bile, just before the addition of pancreatin, and 10 min into the intestinal digestion. The final values for DH in the intestinal phase illustrate the difference between these two solid foods: 33.1 ± 4.0% for fresh tofu, 11.8 ± 2.6% for seitan.

These observations may be explained by the higher solubility of soya protein compared to gluten. The supposed higher porosity (Fig. 2) of seitan compared to tofu does not seem to have resulted in higher hydrolysis. The protein aggregates that we observed in confocal images of seitan appeared to be denser than those of tofu (Fig. 1), which could explain this limited proteolysis. Other factors, such as the presence of starch in seitan and its absence in tofu, could also contribute to a higher degree of resistance to hydrolysis (Nawrocka, Szymańska-Chargot, Miś, Kowalski, & Gruszecki, 2016).

3.4.2. Effect of protein source on the proteolysis of liquids

The proteolysis of pea and soya emulsions proceeded similarly: there was no significant interaction observed between protein source and time either during the gastric ($P = 0.9509$) or the intestinal step ($P = 0.1850$). Furthermore, during the gastric phase, no differences were detected between pea and soya isolates ($P = 0.1200$). However, the two isolates did exhibit significant differences during the intestinal phase ($P = 0.0419$): soya isolate appeared to be hydrolysed more quickly than pea isolate, even if none of the differences at individual sampling times were considered significant. This result was surprising given that a recent study, using the same conditions of *in vitro* digestion, found that around 25% fewer α-amino groups were released from a soya isolate compared to a pea isolate (Corgneau, Gaiani, Thanh, & Le, 2019).

3.4.3. Importance of the protease:protein ratio

When we compared the two types of soya protein isolates (representative of tofu and soya juice, respectively), we found a significant effect of the food × time interaction on the kinetics of gastric DH ($P = 0.0186$). Starting from 30 min after the addition of pepsin ($P < 0.0684$), the protein isolate that was representative of tofu seemed to be less hydrolysed (0.7–2.6%) than the one that represented soya juice (3.3–5.2%).

The DH of the soya protein isolates also differed during the intestinal phase ($P = 0.0039$), but not globally (overall interaction effect was not significant: $P = 0.2523$). At the end of the intestinal step, the DH of the tofu-related isolate appeared to be lower (17.9 ± 1.8%) than the soya juice-related isolate (50.3 ± 7.6%), but this difference did not reach the level of statistical significance ($P = 0.0988$).

The slower and more limited hydrolysis that we observed for the tofu-associated isolate could be explained by the fact that it was 3.7-times more concentrated with respect to food protein content than the isolate that represented soya juice. It is also possible that some of these differences arose from inconsistencies between the batches of soya beans used to make the tofu and soya juice.

4. Conclusions

The present study on *in vitro* protein digestion of different food products enabled us to examine multiple factors that affect the digestion of proteins, and, through various comparisons, to untangle their relative effects. Here, the food matrix played an important role in the digestion of proteins, as demonstrated for both pea emulsion and tofu. By comparing the digestion of the proteins alone, only solubilised in water, to the same proteins inside a food matrix, we were able to observe clear differences in the progression of the degree of hydrolysis. In both cases, fresh tofu and pea emulsion were better hydrolysed than their respective protein isolates. However, this effect was dependent on the food matrix and protein of origin, because it was not found for seitan and soya juice. The food matrix also affected how proteases digested food proteins. Globally, we observed that solid foods had a lower degree of hydrolysis than liquid ones. More specifically, after gastric and intestinal digestion, soya juice was better hydrolysed (51%) than tofu (33%). We were also able to document how a treatment that affects the food matrix—freeze-drying, which in seitan appeared to increase

the flexibility of the protein network—also increased the final degree of hydrolysis. With respect to protein source, it was not possible to make generalisations; protein digestion of the soya juice-related isolate was significantly different from that of the pea isolate, but emulsions of both isolates were digested similarly. Another of our comparisons enabled us to evaluate the effect of different concentrations of the same protein: when incubated with the same amount of digestive enzyme, a larger quantity of soya protein substrate (representative of tofu) was hydrolysed more slowly than a smaller amount of the same protein (representative of soya juice). To conclude, efforts to characterise proteolysis-related data during *in vitro* digestion represent a useful starting point in screening and comparing the responses of different kinds of plant-based food protein to digestive processes.

Acknowledgements

We thank Dominique Chevalier from Nutrition & Santé for kindly providing food products and advice. We are grateful to Lindsay Higgins for correcting the English of the manuscript. We also gratefully acknowledge Sophie Legall for quantifying non-starch polysaccharide dosages and Thomas Auxenfans for taking pictures on the scanning electron microscope.

Funding information

The present article is part of a CIFRE thesis (CIFRE n°2016/0719) funded by IMPROVE SAS and INRA.

Disclosure

The authors have no competing interests.

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