This is the final draft post-refereeing.

The publisher's version can be found at https://pubs.acs.org/doi/10.1021/acs.jafc.0c05868

Please cite this article as: Lambrecht, M.A., Monge-Morera, Godefroidt, T., Vlumanys, N., Deleu, L.J., Goos, P., Schymkowitz, J., Rousseau, F. and Delcour, J.A. 2021. Hydrothermal treatments cause wheat gluten derived peptides to form amyloid-like fibrils, Journal of Agriculture and Food Chemistry, 69, 1963-1974.

Hydrothermal treatments cause wheat gluten derived peptides to form amyloid-like fibrils

Marlies A. Lambrecht[1], Margarita Monge-Morera[1*], Thibault Godefroidt[1], Nele Vluymans[1], Lomme J. Deleu[1], Peter Goos[2], Joost Schymkowitz[3], Frederic Rousseau[3], Jan A. Delcour[1]

[1] Laboratory of Food Chemistry and Biochemistry and Leuven Food Science and Nutrition Research Centre (LFoRCe), KU Leuven, Kasteelpark Arenberg 20, B-3001 Leuven, Belgium

[2] Division of Mechatronics, Biostatistics and Sensors (MeBioS), KU Leuven, Kasteelpark Arenberg 30, B-3001 Leuven, Belgium

[3] Switch Laboratory, VIB, B-3000 Leuven, Belgium, and KU Leuven, Department of Cellular and Molecular Medicine, Herestraat 49, B-3001 Leuven, Belgium

* Corresponding author: margarita.mongemorera@kuleuven.be, Tel.: +32 16329082; fax: +32 16321997

ABSTRACT

Formation of amyloid fibrils (*i.e.* protein structures containing a compact core of ordered β-sheet structures) from food proteins can improve their techno-functional properties. Wheat gluten is the most consumed cereal protein by humans and extensively present in food and feed systems. Hydrolysis of wheat gluten increases the solubility of its proteins and brings new opportunities for value creation. In this study, the formation of amyloid-like fibrils (ALFs) from wheat gluten peptides (WGPs) under food relevant processing conditions was investigated. Different hydrothermal treatments were tested to maximize the formation of straight ALFs from WGPs. Thioflavin T (ThT) fluorescence measurements and transmission electron microscopy (TEM) were used to study the extent of fibrillation and the morphology of the fibrils, respectively. First, the formation of fibrils by heating solutions of tryptic WGPs [degrees of hydrolysis 2.0% (DH 2) or 6.0% (DH 6)] was optimized using a response surface design. WGP solutions were incubated at different pH, times and temperatures. DH 6 WGPs had a higher propensity for fibrillation than did DH 2 WGPs. Heating DH 6 WGPs at 2.0% (w/v) for 38 hours at 85 °C and pH 7.0 resulted in optimal fibrillation. Secondly, trypsin, chymotrypsin, thermolysin, papain and proteinase K were used to produce different DH 6 WGPs. After enzyme inactivation and subsequent heating at optimal fibrillation conditions, chymotrypsin and proteinase K DH 6 WGPs produced small worm-like fibrils whereas fibrils prepared from trypsin DH 6 WGPs were long and straight. The surface hydrophobicity of the peptides was key for fibrillation. Thirdly, peptides from the wheat gluten components gliadin and glutenin fractions formed smaller and worm-like fibrils than did WGPs. Thus, peptides of both gluten protein fractions jointly contribute in gluten fibrillation.

KEYWORDS: glutenin, gliadin, thioflavin T, amyloid, trypsin

INTRODUCTION

The ability of proteins to aggregate and react with each other impacts their functionality in the production of food products such as egg noodles, cake, bread, and cookies[1]. Amyloid fibrils (AFs) from food proteins have gained interest because they can have better foaming[2], gelling[3] and emulsifying properties than other protein aggregates[4]. Characteristic for AFs is their compact core of ordered β-sheet structures which are stacked perpendicularly to the fibril axis, and which mainly are stabilized by hydrogen bonds[5]. Food proteins such as egg, cereal, milk and legume protein or peptides thereof can form amyloid (-like) fibrillar protein structures[6,7] with functionalities that, in food systems, may allow to reduce the level of animal-based protein while maintaining product quality. To lower the carbon footprint, it would even be more interesting to fully replace animal by plant-based proteins in food products.

Formation of fibrils from food proteins has mainly been induced under conditions uncommon to food processing such as extended (several hours to days) heating at moderate temperatures (*ca*. 60 to 85 °C) and low pH (< 2)[6,7]. Of interest is that 15 min boiling induces the formation of about 1% to 3% AFs in fresh hen egg white[8] and of around 0.5% of amyloid-like fibrils (ALFs) in boiled wheat gluten dispersions[9]. Unfortunately, the concentrations of ALFs in boiled wheat gluten dispersion remain low.

While to benefit from the techno-functional properties of AFs in food processing, it may be beneficial to increase their levels, the analysis of AF formation in food systems is challenging. Indeed, methodologies for studying the extent of fibrillation require that aggregates and even precipitates[6,7] are discerned from fibrils. Fortunately, targeted use of peptidases allows concentrating AFs. This has clearly been shown in the case of egg white proteins[8].

Legume [*e.g.* soy and pea][3,10,11] and cereal protein[6] fibrillation has been studied. Wheat gluten is the most important cereal protein consumed [16 g/capita/day][12]. It is the co-product of the industrial isolation of wheat starch[13] and while extensively used in food as well as in animal feed systems[14], there is an ongoing search for additional applications of this plant-based protein. Wheat gluten contains two fractions, *i.e.* monomeric gliadin and polymeric disulfide-linked glutenin[15]. Aqueous dispersions of wheat gluten can form ALFs by exposing them to conditions similar to those in slow cooking[9]. In addition, hydrolysis of gluten enhances its solubility in aqueous media which creates opportunities for a whole array of additional applications[16]. One route to creation of novel applications is the formation of fibrils from gluten hydrolysates. Such hydrolysates have been prepared by incubating gluten with trypsin for two weeks at 37 °C and pH 8.0 under continuous gentle stirring[17]. As a result of subsequent drying at room temperature,

fibrillar structures were formed which were composed of cross-β sheet structures as indicated by ThT fluorescence, Fourier transform infrared spectrometric and X-ray diffraction measurements[17].

In particular, some glutamine-rich peptides formed by tryptic hydrolysis self-assembled into fibrils of nanometer to micrometer size which were enclosed by unassembled peptides[17]. In contrast to those of glutenin, tryptic hydrolysates of gliadin formed cross-β sheet structures. Fibrils from gliadin hydrolysates were not larger than those obtained with wheat gluten peptides (WGPs)[18]. Finally, changes in pH (pH 4 to 10 range), temperature (22 to 80 °C range) and ionic strength (0 to 100 mM NaCl range) affect the extent, morphology and size of fibrils formed from tryptic WGPs[19].

In spite of the above, conditions enhancing the level of fibrillation from WGPs have not been optimized under food processing relevant conditions and prior to the present work it has been unclear which features of the peptides make them prone to fibrillation. Against this background, the AF formation from differently prepared WGPs was studied using different hydrothermal treatments. The impact of hydrothermal treatments on tryptic WGPs [degrees of hydrolysis of 2.0% (DH 2) or 6.0% (DH 6)] fibrillation was examined by varying the temperature and time, pH and WGP concentration. The extent of fibrillation was evaluated by measuring ThT fluorescence. Next, different enzymes were used to hydrolyze gluten to similar degrees, thereby producing peptide populations with different amino acid sequences and properties. The water-soluble peptides were then heat-treated using optimized conditions, and the extent of fibrillation as well as the morphology of fibrils was investigated. Lastly, the contribution of gliadin and glutenin derived peptides to heat-induced fibrillation was studied. For this, the formation and morphology of fibrils from both gliadin and glutenin extensively hydrolyzed [degrees of hydrolysis *ca.* 9.0% (DH 9) and *ca.* 4.0% (DH 4) respectively] were studied. We here report on the outcome of this work.

MATERIALS AND METHODS

Materials

Wheat gluten containing 75% protein (N x 5.7) [determined in triplicate with an automated Dumas protein analysis system (EAS Variomax N/CN, Elt, Gouda, The Netherlands) using an adaptation of the AOAC Official Method 990.03] was obtained from Tereos Starch & Sweeteners (Aalst, Belgium). Trypsin (EC 3.4.21.4, T0303) from porcine pancreas, chymotrypsin (EC 3.4.21.1, 44 U/mg protein, 76220) from bovine pancreas, thermolysin (EC 3.4.24.27, 30-175 U/mg, P1512) from *Geobacillus stearothermophilus*, papain (EC 3.4.22.2; 3 U/mg, 76220) from *Carica papaya* and proteinase K (EC 3.4.21.64, P4850) from *Tritirachium album* were from Sigma-Aldrich (Bornem, Belgium). All enzyme units (U) are as defined by the supplier.

Sodium azide was from Acros Organic (Geel, Belgium). Sodium dodecyl sulfate (SDS), sodium dihydrogen phosphate dihydrate and sodium chloride were from VWR International (Leuven, Belgium). Ovalbumin, albumin chicken egg grade III*, ca.* 94% protein on dry matter basis, A5370) and all other chemicals, reagents and solvents were of at least analytical grade and from Sigma-Aldrich. The highly amylogenic peptide (residues: 103-112, *i.e*. NFNYNNNLQG) derived from sup35 yeast was produced in-house[20].

Separation of wheat gluten into gliadin and glutenin

Gliadin was extracted (60 min, room temperature, 150 rpm) from wheat gluten (25.0 g) with 250.0 ml 60% (v/v) ethanol. After two extractions and intermediate and final centrifugation (10,000 g, 10 min), the combined gliadin containing supernatants were filtered and the ethanol was evaporated by rotary evaporation at 35 °C (Rotavapor R3000, Büchi, Flawil, Switzerland). Starch was washed away from the obtained glutenin enriched pellets with deionized water. The thus obtained glutenin and gliadin fractions were freeze-dried following flash-freezing with liquid nitrogen, ground with a laboratory mill (IKA, Staufen, Germany) and sieved (250 µm). The resultant materials contained 79.2 and 91.8% protein (N x 5.7) on dry matter basis.

Hydrolysis of wheat gluten

First, a suspension of wheat gluten in deionized water containing 2.0% protein (w/v) was hydrolyzed with trypsin to a degree of hydrolysis of 2.0% (DH 2) or 6.0% (DH 6) (see Figure 1, left). Enzyme preparation to substrate protein ratios (EP:SP) were 1:320 (for DH 2) or 1:13 (for DH 6). Incubation was at 37 °C and the pH was kept constant at 8.0 by gradually adding 0.05 M sodium hydroxide using a Titrino 718 device (Metrohm, Herisau, Switzerland). When the desired DH was reached, the enzymes were inactivated by heating at 95 °C for 4 min. Afterwards, the samples were cooled and centrifuged (15 min, 18,600 *g*), and the resultant supernatants were freeze-dried as above and used in the response surface design experiment described below.

Second, suspensions of wheat gluten in deionized water (2.0% protein, w/v) were hydrolysed to DH 6 in triplicate with trypsin (EP:SP 1:22.5), chymotrypsin (EP:SP 1:22.5), thermolysin (EP:SP 1:150), papain (EP:SP 1:10) or proteinase K (62.5 µl EP containing *ca.* 5 U/2.0 g protein) at 40 °C and pH 8.0 (see Figure 1, middle left). The enzymes were then inactivated by heating at 100 °C for 4 min. After cooling to room temperature and centrifugation (10 min, 12,000 *g*), the pH of the resultant supernatants was adjusted to 7.0 using 0.05 M hydrochloric acid. All thus obtained WGPs were freeze-dried as above and further used for evaluating their fibrillation by different hydrothermal treatments as described below.

Third, hydrolysis of wheat gluten to DH 6 was performed with trypsin (EP:SP 1:22.5) at 40 °C and pH 8.0 as above but without subsequent enzyme inactivation (see Figure 1, middle right). After centrifugation (10 min, 12,000 *g*), the pH of the resultant supernatants was adjusted to 7.0 using 0.05 M hydrochloric acid as above, the samples were freeze-dried as above and further used for evaluating their fibrillation by different hydrothermal treatments as described below.

DH was defined as the percentage of the number of peptide bonds hydrolyzed (*h*) to the total number of peptide bonds per unit weight present in wheat gluten protein (h_{tot}) and calculated as follows:

$$DH\ (\%) = \frac{h}{h_{tot}} = \frac{B\ .\ M_b\ .100}{\alpha\ .\ m_p\ .h_{tot}} \qquad \text{(eq 1)}$$

with *B* the total amount of base added, M_b the molarity of the base added, α the measure of the degree of dissociation of the $\alpha\text{-}NH_3^+$, m_p the mass of protein, *h* the hydrolysis equivalents [milli-equivalents (meqv)/g protein] and h_{tot} the theoretical number of peptide bonds per unit weight present in protein. The latter is 8.3 meqv/g protein for wheat gluten as calculated before[21]. At pH 8.0, α is 0.790 at 37 °C and 0.834 at 40 °C[22].

Hydrolysis of gliadin and glutenin

Gliadin and glutenin (2.0% protein, w/v, in 80 ml deionized water) were extensively hydrolyzed with trypsin (EP:SP 1:22.5) at 40 °C and pH 8.0 using the Titrino 718 device (9.4 and 19.8 ml 0.05 M sodium hydroxide were added, respectively) (see Figure 1, right). The calculated h_{tot} of wheat gliadin and glutenin resembled that of wheat gluten and the experimental h_{tot} value (8.3 meqv/g protein) of wheat gluten was used to determine the DH of its fractions. After hydrolysis, the samples were centrifuged (10 min, 12,000 *g*), the pH of the resultant supernatants was adjusted to 7.0 as indicated above, the samples were freeze-dried as above and further used for evaluating their fibrillation by different hydrothermal treatments as described below.

Experimental design for optimizing tryptic wheat gluten peptides fibrillation

The optimal hydrothermal treatments to induce protein fibrillation - judged by the intensity of ThT fluorescence - of tryptic DH 2 and DH 6 WGPs were identified with a response surface design selected using the I-optimality criterion[23]. The main effects of the factors incubation time (0 to 60 h), temperature (65 to 85 °C), pH (5.0 to 7.0) and protein concentration [0.50 to 2.0% in 0.02% (w/v) sodium azide] as well as their interactions and quadratic effects were studied using JMP® Pro 14.0 (SAS Institute, Cary, NC,

USA)[23]. Forty different experimental conditions were selected to study the four factors by heating samples (1.50 ml) while shaking at 75 rpm. Each hydrothermal treatment was performed in triplicate (see Figure 1, left). Factors levels are shown in Table 1S. After heating, samples were cooled on ice-water and centrifuged (9,300 *g*, 10 min, room temperature). The protein contents and ThT fluorescence intensities of supernatants 1 (see Figure 1, left) were analysed both as described below. Also, the pellets formed after heating and centrifugation were extracted as indicated below, and supernatants 2 (see Figure 1, left) were analyzed both for protein content and ThT fluorescence as described below. For the ThT fluorescence response variable, the natural logarithm (Ln) was modeled to obtain strictly positive predictions for the processing conditions under study and thus increase the predictive validity of the model. The resultant ThT fluorescence values (Table 1S) were used to fit a surface model using ordinary least squares regression to identify significant effects ($P < 0.05$). The initial model fitted in our backward model selection procedure was the full second-order response surface model, after which insignificant effects were removed. The resulting model was evaluated based on the coefficient of determination (R^2) and the actual-by-predicted plot, which compares observed and predicted response values. Both supernatants 1 and 2 were used to identify optimal fibrillation conditions of DH 2 and DH 6 WGPs using the prediction profile tool in JMP® Pro 14.0 (SAS Institute).

Hydrothermal treatment for inducing fibril formation

First, tryptic DH 2 and DH 6 WGPs (0.50, 1.25 or 2.00% protein, w/v) were heated at 65, 75 or 85 °C and pH 5.0, 6.0 or 7.0 (adapted by adding 0.05 M hydrochloric acid) for 0, 30 or 60 h according to the response surface design as described above while shaking [75 rpm) (see Figure 1, left). *Second*, WGPs (2.0% protein, w/v) prepared with the different peptidases as described above were heated for 40 h at 85 °C in deionized water (see Figure 1, middle left). *Third*, tryptic DH 6 WGPs (2.0% protein, w/v) prepared without the enzyme inactivation step as described above were heated for 40 h at 85 °C in deionized water (see Figure 1, middle right). *Fourth*, the impact of boiling (100 °C, 15 min) on fibrillation of tryptic WGPs (2.0% protein, w/v) was further investigated by hydrothermal treatment (see Figure 1, middle left and right). *Fifth*, wheat gliadin and glutenin hydrolysates (2.0% protein, w/v) were also heated for 40 h at 85 °C in deionized water (see Figure 1, right). Afterwards, in all five cases, samples were cooled on ice water and centrifuged (10 min, 10,000 *g*) and while the resultant supernatants 1 and 3 (see Figure 1) were analyzed for their protein content, ThT fluorescence and protein morphology with transmission electron microscopy (TEM) as described below, the obtained pellets were treated as described in the following section.

Protein extraction from pellets

First, protein in the pellets of tryptic DH 2 and DH 6 WGPs used in the response surface design experiments was extracted with 0.05 M Tris/HCl/1-propanol (50:50, v/v) buffer (pH 7.5, room temperature, 16 h, 150 rpm, see Figure 1, left). After centrifugation (10 min, 10,000 *g*), supernatants 2 (see Figure 1) were further analyzed for their protein content and ThT fluorescence as described below. *Second*, proteins in the pellets of all the other WGPs were treated with proteinase K [10 µl enzyme solution in 1.5 ml 0.02% (w/v) sodium azide, 37 °C, 48 h, 70 rpm] and centrifuged [10 min, 10,000 *g*] (see Figure 1). Proteinase K was added to extract AFs from the pellets as described by before[8]. Supernatants 4 (see Figure 1) obtained by proteinase K treatment were further analyzed for their protein content, ThT fluorescence and/or protein morphology with TEM.

Protein content determination

The protein content of (un)heated samples were determined by measuring ultraviolet (UV) extinction at 280 nm in triplicate. Samples (200 µL) were analyzed with UV-star plates (Greiner Bio-One, Vilvoorde, Belgium) in a Synergy Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA). Absorbance values were converted to protein concentrations using a calibration curve constructed with their respective unheated WGPs as prepared above of which the protein concentration had been determined with the Dumas method mentioned before.

Thioflavin T measurements

ALF formation was monitored with thioflavin T (ThT) fluorescence measurements in (un)heated samples in triplicate as described before[8]. An increase in ThT fluorescence suggested the presence of β-sheet structures such as the ones present in AFs[24]. Within one well plate, samples and standards [2.0% (w/v) ovalbumin heated at 78 °C for 22 h] were diluted to the same protein concentration with 0.05 M sodium phosphate buffer (pH 7.0). To 190 µl sample, 10 µl 200 µM ThT in the same buffer was added in a black 96-well plate (Greiner Bio-One). Analyses were performed with a Synergy Multi-Mode Microplate Reader (BioTek) using excitation and emission wavelengths of 440 nm and 480 nm, respectively. The ThT fluorescence of samples was expressed relative to the fluorescence intensity of the above mentioned sup35 yeast peptide[8].

Analysis of surface hydrophobicity

The surface hydrophobicity of the proteins in *unheated* supernatants 3 (see Figure 1) was determined in triplicate with 1-anilino-8-naphthalene sulfonate (ANS) and calculated as described previously[25]. Samples were diluted with 0.01 M sodium phosphate buffer (pH 7.0) to obtain protein concentrations ranging from 0.05 to 0.50 mg/ml. Aliquots (200 µl) of diluted samples were then transferred to a black 96-well plate (Greiner Bio-One) and 10 µl ANS solution [8.0 mM in 0.01 M sodium phosphate buffer (pH 7.0)] was added. Fluorescence was analysed in a Synergy Multi-Mode Microplate Reader (BioTek) using excitation and emission wavelengths of 390 nm and 480 nm, respectively. The relative fluorescence intensity was calculated as the intensity of the fluorescence of the protein-ANS mixture minus the control sample (ANS with buffer) fluorescence, which then was divided by the reading of the control sample. The slope of the plot of relative fluorescence intensity as a function of protein concentration for each sample represents the surface hydrophobicity.

Zeta potential measurements

The zeta potential of supernatants 3 (see Figure 7.1) of *unheated* DH 6 WGPs obtained by hydrolysis with different enzymes as described above was measured in a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) instrument at 20 °C based on laser Doppler micro-electrophoresis. To this end, duplicates of aqueous solutions (2.0 protein/ml) were transferred to a disposable capillary zeta cell (Malvern Instruments) in which an electric potential was applied. In this type of measurement, the charge of the peptides results in specific electrophoretic mobility, which then is converted to a zeta potential with the Henry equation. In addition, the isoelectric point of supernatants 1 (see Figure 7.1) of *unheated* tryptic DH 2 and DH 6 WGPs was determined as the pH at which their zeta potential was zero. For this, the zeta potential of aqueous solution (1.5 mg protein/ml) contained in a similar cell was monitored as a function of pH changes resulting from adding 0.25 M or 0.50 M hydrochloric acid (HCl) using the autotritrator device of the Zetasizer Nano ZS.

Size exclusion high performance liquid chromatography

The apparent molecular weight distribution of the constituents (*i.e.* supernatants 3 in Figure 1) in the WGPs prepared with different peptidases as described above was evaluated in triplicate with size exclusion high performance liquid chromatography (SE-HPLC) as described before[26]. Samples [1.0 mg protein/ml 0.050 M sodium phosphate buffer (pH 6.8) containing 2.0% (w/v) SDS] were shaken (60 min, room temperature). After centrifugation (10 000 *g*, 10 min) and filtration (Millex-HP, 0.45 µm,

polyethersulfone, Millipore, Carrigtwohill, Ireland), solutions of the peptides (20 µl) were separated at 0.5 ml/min on an BioSep-SEC-S2000 column (Phenomenex, Torrance, CA, USA) and detected at 214 nm on a Shimadzu (Kyoto, Japan) Prominence modular system with automated injection. Bovine serum albumin (66 kDa), chicken egg ovalbumin (44 kDa), aprotinin (6.5 kDa), angiotensin III (931 Da) and $(Ala)_5$ (373 Da) were used as protein markers.

Transmission electron microscopy

The morphologies of peptides in supernatants 3 and 4 (see Figure 1) were visualized with TEM using negative staining with uranyl acetate. Samples [10 µL containing 0.1% protein (w/v)] were prepared as described previously[8] and examined on a JEM-1400 TEM (Jeol, Toyko, Japan) instrument at 80 keV. The average length of the protein aggregates/fibrils was determined with Image J 1.52a software (National Institutes of Health, Bethesda, MD, USA) using at least three TEM images and measuring at least ten different aggregates in the images.

RESULTS AND DISCUSSION

The impact of hydrothermal treatments on the fibrillation of tryptic wheat gluten peptides

A response surface design and modeling approach was used to optimize protein fibrillation in tryptic DH 2 and DH 6 WGPs. Various concentrations of both WGP samples (0.5 to 2.0% protein, w/v) were incubated at temperatures from 65 to 85 °C for 0 to 60 h in a pH range (5.0 to 7.0) relevant for food processing. The upper temperature limit was set at 85 °C. Heating for several hours at temperatures exceeding 85 °C fosters polymerization and gelling of protein[1] which can negatively impact fibrillation[6,7]. Protein fibrillation in supernatants 1 and 2 (see Figure 1) was monitored with ThT fluorescence measurements. The proteins extracted in supernatants 2 and those present in supernatants 1 both originated from the same starting material and hydrothermal treatment. Thus, both were considered for optimizing the fibrillation conditions. The resultant Ln (ThT fluorescence) prediction profiles and the estimated effects of the terms included in those models are shown in Figure 2 and Table 1, respectively. The final response surface models obtained had a good fit, as demonstrated by the large coefficient of determination values and the correspondence between the predicted and experimentally observed ThT fluorescence values shown in the actual-by-predicted plots in Supplementary Figure S1.

Fibrillation of tryptic wheat gluten peptides with a degree of hydrolysis of 2.0%

Temperature had a significant impact on the ThT fluorescence of supernatants 1 through its interaction with time (P = 0.0306), but it did not have any significant effect on the ThT fluorescence of supernatants 2 of DH 2 WGPs (see Table 1). In the case of supernatants 1, the ThT fluorescence remained constant over time for samples treated at 65 °C whereas it increased over time for samples treated at 85 °C (see Figures 2.A and S2.A). That heating at 85 °C led to higher ThT fluorescence than at 65 °C logically indicated that protein unfolding occurred to a greater extent at the higher temperature, which corresponded well with the fact that (partial) unfolding of proteins is required for fibril formation[6]. When proteins (partially) unfold, stacked β-sheet structures may be formed in which hydrogen bonding is key for their increase stability[27]. In addition, an increased level of β-sheet structures as a result of heating at 85 °C suggests that ordered aggregation was more important than random polymerization.

The pH had a large significant positive or negative effect on ThT fluorescence in the case of supernatants 1 and 2 of DH 2 WGPs, respectively (see Figure 2.A and Table 1). Peptides in supernatants 1 of DH 2 WGPs randomly aggregated at pH conditions close (*e.g*. 5.0) to their isoelectric point, which was 4.7 (± 0.1), in line with the earlier reported value (*ca.* 5.0[28]). In contrast, at pH 7.0, DH 2 WGPs are not prone to aggregate randomly which favours protein fibrillation as denoted by the high levels of ThT fluorescence in supernatants 1 at pH 7.0 (see Figure 2.A). As most β-sheet structures were present in supernatants 1, mainly amorphous structures were contained in supernatants 2 of DH 2 WGPs at pH 7.0, as suggested by the lowest levels of ThT fluorescence in the latter (Figure 2.A). As a result, the model considers pH 5.0 as optimal for protein fibrillation in DH 2 WGPs. As pH had the largest effect (Table 1) on ThT fluorescence of DH 2 WGPs, it seems that rapid aggregation promoted by pH conditions close to the isoelectric point of DH 2 WGPs are key for determining the type of protein aggregates formed.

While time was a highly significant factor for predicting the ThT fluorescence of supernatants 1, it was not significant in the case of supernatants 2 (see Table 1). In supernatants 1 of DH 2 WGPs, provided the temperature was high enough, longer incubation times resulted in higher ThT fluorescence values than shorter incubation times (see Figure 2.A), which is in line with protein fibril formation usually being a slow process[7] and with earlier reports of extended times required to produce protein fibrils in tryptic WGPs[18].

Protein concentration did not have a significant effect on the ThT fluorescence of supernatants 1 of DH 2 WGPs. Ovalbumin fibrillation was also less dependent on a similar tested protein concentration range than on temperature (between 40 °C to 80 °C)[29]. Nonetheless, β-lactoglobulin fibrils prepared by heating

at 80 °C for 16 h (pH 2) showed a different β-sheet content and morphology when prepared at different protein concentrations (*e.g.* 3.0% or 7.5%)[30]. Therefore, the effect of protein concentration seems to be protein dependent and related to the concentration range studied. Indeed, protein concentration had a significant effect on ThT fluorescence of supernatants 2 of DH 2 WGPs ($P < 0.00001$ for the main effect and $P = 0.0154$ for the interaction with pH; see Table 1). In supernatants 2, the ThT fluorescence was higher at 2.0% than at 0.5% protein (see Figure 2.A). Due to the interaction with pH, ThT fluorescence remained similar over the pH range studied at 0.5% protein, whereas at 2.0% protein ThT fluorescence was higher at pH 5.0 than at pH 7.0 (see Figure S2.B).

Based on above, the ThT fluorescence obtained when 2.0% (w/v) DH 2 WGPs were heated at 85 °C for 60 h at pH 5.0 (see Figure 2.A) was considered to be optimal even if it was affected in an opposite way by pH in both supernatants 1 and 2.

Fibrillation of tryptic wheat gluten peptides with a degree of hydrolysis of 6.0%

For both supernatants 1 ($P = 0.0097$) and 2 ($P = 0.0025$), temperature had a significant quadratic effect on ThT fluorescence of DH 6 WGPs (see Table 1), with an optimal fibrillation temperature of 85 °C (see Figure 2.B). So, as with DH 2 WGPs, heating at 85 °C favoured DH 6 WGP fibrillation probably by promoting their (partial) unfolding and the formation of ordered β-sheet structures stabilized by hydrogen bonds. The pH had a significant effect on ThT fluorescence of DH 6 WGPs for both supernatants 1 and 2 (see Table 1). As observed for DH 2 WGPs, the pH effect on ThT fluorescence differed for supernatants 1 and 2 (see Figure 2.B). While for supernatants 1 pH had a large and significant positive effect on ThT fluorescence ($P < 0.0001$ for the main effect and $P = 0.0224$ for the quadratic effect of pH), the pH effect was smaller and negative ($P < 0.0001$ for the main effect) for supernatants 2 (see Table 1). The isoelectric point in supernatants 1 of tryptic DH 6 WGPs was about 4.8 (± 0.1) and slightly higher (*ca.* 4.0) than that described before[28], which indicated that pH conditions far from the isoelectric point (*e.g.* neutral pH) enhanced protein fibrillation in supernatants 1 of DH 6 WGPs (see Figure 2.B). In contrast to DH 2 WGPs, the level of β-sheet structures were similar in both supernatants 1 and 2 of DH 6 WGPs and thus neutral pH was considered optimal for their fibrillation (see Figure 2.B).

Time had a small significant effect ($P = 0.0134$) on the ThT fluorescence of supernatants 1, but no significant effect for supernatants 2 of DH 6 WGPs (see Table 1). Longer incubation times resulted in better fibrillation conditions, with an optimum at 38 h (see Figure 2.B), and mainly determined by the effect of time on supernatants 1. The optimal incubation time of DH 6 WGPs is therefore slightly shorter than for

DH 2 WGPs. Nevertheless, a more extensive hydrolysis thus facilitated protein fibrillation. In addition, time exhibited a significant interaction effect (P = 0.0003) with pH (see Table 1) on the ThT fluorescence of supernatants 1 of DH 6 WGPs. ThT fluorescence was very low at pH 5.0 without incubation, which corresponded well with the fact that pH conditions close to isoelectric point promoted random aggregation even without heating (see Figure S2.C). In contrast, ThT fluorescence was higher at pH 7.0 when samples were not heated suggesting that this condition promoted the formation of β-sheet structures (see Figure S2.C). While at pH 7.0 the high ThT fluorescence values only increased slightly with incubation time, at pH 5.0 they increased more pronouncedly but still to a lower extent than noted for pH 7.0 (see Figure S2.C). As with DH 2 WGPs, protein concentration had a positive effect (P < 0.00001) on ThT fluorescence for supernatants 2 of DH 6 WGPs (see Table 1), but no significant effect for supernatants 1. Therefore, for supernatants 2, protein fibrillation was more intense at 2.0% than at 0.5% protein (see Figure 2.B). Much as in supernatants 2 for DH 2 WGPs, not all protein was extracted in the corresponding supernatants of DH 6 WGPs (see Table 1S).

Optimal ThT fluorescence was obtained when heating 2.0% (w/v) DH 6 WGPs at 85 °C for 38 h and neutral pH (see Figure 2.B). Comparing the optimal settings in Figures 2.A and 2.B shows that the ThT fluorescence under optimal conditions was higher for DH 6 than for DH 2 WGPs. As DH 2 WGPs were evidently average wise larger than DH 6 WGPs (Supplementary Figure S3), it seems that the smaller peptides aggregated more easily in ordered β-sheet structures than the larger ones.

In contrast to the previous reports of wheat gluten hydrolysates fibrillation mainly as a result of drying at room temperature for extended incubation times (*i.e*. 2 weeks)[17], the above-optimized settings showed the potential of food relevant processing conditions to induce WGP fibrillation. As the overall ThT fluorescence values were low for WGPs in both supernatants (Figure 2), it seems that the amount of protein fibrils was limited, which was in line with the earlier finding that approximately only 0.1 to 0.5% of wheat gluten proteins assembled into ALF during boiling[9]. Nevertheless, WGPs fibrillation under food relevant conditions may be improved by *seeding*, that is, adding preformed protein fibrils to accelerate fibril formation[4].

The impact of the use of different enzymes for producing wheat gluten peptides on their heat-induced fibrillation

Structurally different WGPs were prepared by hydrolyzing wheat gluten with various enzymes to DH 6. Indeed, the resultant supernatants 3 of WGPs (see Figure 1) showed some differences in properties such

as surface charge, hydrophobicity and molecular weight distribution (see Table 2 and Figure 3). All WGPs had a negative zeta potential, which indicated an overall negative charge. WGP prepared with papain had the least negative zeta potential value, while the other WGPs had similar and much more negative zeta potential values (Table 2).

The surface hydrophobicity of WGPs varied more than their surface charge (Table 2). The highest surface hydrophobicity was noted for tryptic WGPs (see Table 2). Trypsin selectively cleaves only next to the positively charged amino acids lysine and arginine, which results in conserved patches of hydrophobic amino acids in the released peptides[31]. As these positively charge amino acids are not abundant in wheat gluten proteins[32], large peptides with increased hydrophobicity can be obtained by treating wheat gluten with trypsin[33]. Indeed, supernatants 3 of tryptic WGPs (see Figure 1) contained the largest peptides (elution time *ca.* 12 min to 14 min, see Figure 3). WGPs prepared by thermolysin treatment showed a more or less uniform molecular weight distribution in the 15 min to 21 min elution time range (see Figure 3). Thermolysin preferentially cleaves before hydrophobic amino acids (*e.g.* alanine, leucine, isoleucine, phenylalanine, valine and methionine) and results in smaller WG peptides than those prepared with trypsin or chymotrypsin[34]. Indeed, WGPs prepared with trypsin, chymotrypsin and proteinase K showed higher levels of a relatively large peptide (elution time *ca.* 15 min, see Figure 3) than those prepared with thermolysin and papain. Moreover, WGPs by thermolysin had the lowest hydrophobicity (see Table 2) which corroborates that this enzyme disrupted patches of hydrophobic amino acids in wheat gluten. In contrast, WGPs prepared by chymotrypsin or proteinase K treatments had a higher surface hydrophobicity than those prepared with thermolysin or papain (see Table 2). Chymotrypsin preferentially cleaves next to large hydrophobic amino acids (*e.g.* tyrosine, tryptophan and phenylalanine) but also to methionine, histidine and leucine[35], while proteinase K is a non-specific peptidase[36]. Thus, chymotrypsin and proteinase K disrupted hydrophobic regions in wheat gluten proteins but to a lower extent than thermolysin. As papain has a strong proteolytic activity[37], it is not surprising that the smallest peptides (present in the large peak eluting at *ca.* 22 min) were produced by this enzyme (see Figure 3).

Heating the different WGPs to inactivate the enzymes (4 min at 100 °C) prior to inducing fibrillation (40 h at 85 °C, pH 7.0, 70 rpm) and subsequently executing centrifugation barely affected the level of ThT fluorescence (see Table 2). in their corresponding supernatants 3 (see Figure 1). While the ThT fluorescence of supernatants 3 (see Figure 1) of WGPs prepared with trypsin and papain was little affected by the above heating treatments, that of those prepared with proteinase K and chymotrypsin slightly increased. However, as a result of such heating, the ThT fluorescence of supernatants 3 (see Figure 1) of

WGPs made with thermolysin was slightly lowered (see Table 2). TEM images of supernatants 3 (see Figure 1) of tryptic WGPs after heating as before contained long (*ca.* 500 nm to 1.3 µm) and straight fibrils in combination with some amorphous structures (see Figure 4 and Supplementary Figure S4). In contrast, supernatants 3 (see Figure 1) of WGPs obtained by chymotrypsin or proteinase K treatments after similar heating showed smaller and worm-like fibrils with an approximate length of 183 nm and 163 nm respectively (see Figure 4 and Supplementary Figure S4). Supernatants 3 (see Figure 1) of WGPs by papain and thermolysin treatments did not did not contain heat-derived fibrillar structures (see Figure 4 and Supplementary Figure S4). In these samples, after heating almost all protein (see Table 2) was present in the supernatants 3 (see Figure 1) and amorphous. However, the formation of large and straight fibrils by heating of tryptic WGPs did not result in enhanced ThT fluorescence in supernatants 3 (see Figure 1). It should be noted here that about one quarter of the protein precipitated during heating and that thus additional fibrils may have been present in the pellet. Nevertheless, applying enzymes other than trypsin to produce DH 6 WGPs did not improve fibrillation.

The WGPs containing more of the smallest peptides with the lowest surface hydrophobicity (*i.e*. thermolysin and papain WGPs) did not form fibrils during heating. The largest and most straight fibrils were formed with the tryptic WGPs which contained larger and more hydrophobic peptides. The main differences in characteristics of tryptic WGPs on the one hand, and WGPs resulting from chymotrypsin or proteinase K treatments on the other hand, were their average peptide size and corresponding surface hydrophobicity. Therefore, it is suggested that WGP size and the associated surface hydrophobicity are key for the morphology of ALFs formed during heating. No general trend in the zeta potential measurements could be related to the fibrillation of different WGPs.

When tryptic WGPs were not boiled prior to heating under optimal fibrillation conditions, exceeding the hydrolysis time beyond that needed to obtain DH 6 did not further increase the DH (results not shown). It is of interest that *boiling* tryptic DH 6 WGPs enhanced the formation of amorphous aggregates (see Figure 5 and Supplementary Figure S5). Indeed, after boiling for 15 min, amorphous protein structures were present both in supernatants 3 (see Figure 1) and in the material recovered by proteinase K treatment in supernatants 4 (see Figure 7.1) from the pellet formed after boiling (see Figure 5 and Supplementary Figure S5). It is worth noting that when tryptic WGPs were not boiled for trypsin inactivation prior to heating under optimal fibrillation conditions, the resultant supernatants 3 (see Figure 1) contained clusters of large (*ca*. 400 nm to 1 µm) and straight fibrils, while the corresponding

supernatants 4 (see Figure 1) mainly contained straight ALFs of variable length (*ca*. 200 nm to 1 µm) (see Figure 5 and Supplementary Figure S5).

The above results indicated that specific heating conditions affected tryptic WGPs fibrillation in a different way by probably influencing their protein aggregation kinetics[38]. Boiling mainly induced the formation of amorphous structures in tryptic WGPs as these conditions may result in fast and disordered aggregation, while heating at moderately high temperatures for extended time (*i.e.* 85 °C, 40 h) resulted in fibrillar structures by probably allowing the formation of more ordered β-sheet structures. In this sense, the structural characteristics (*e.g*. size, surface charge and hydrophobicity) of WGPs also determined their fibrillation propensity as boiling conditions had also induced AF formation in egg white[8]. Thus, the identification of specific protein sequences with a high tendency to form β-sheet structures (*i.e*. aggregation prone regions) in wheat gluten proteins will increase our understanding of their fibrillation propensity and even induce fibril formation in a more effective way.

The impact of glutenin and gliadin on heat-induced fibrillation of wheat gluten peptides

To study the contribution of glutenin and gliadin peptides on the hydrothermally induced formation of wheat gluten fibrils, both protein fractions were also extensively hydrolyzed with trypsin. Under the experimental conditions employed, glutenin and gliadin reached a DH of about 9 and 4, respectively.

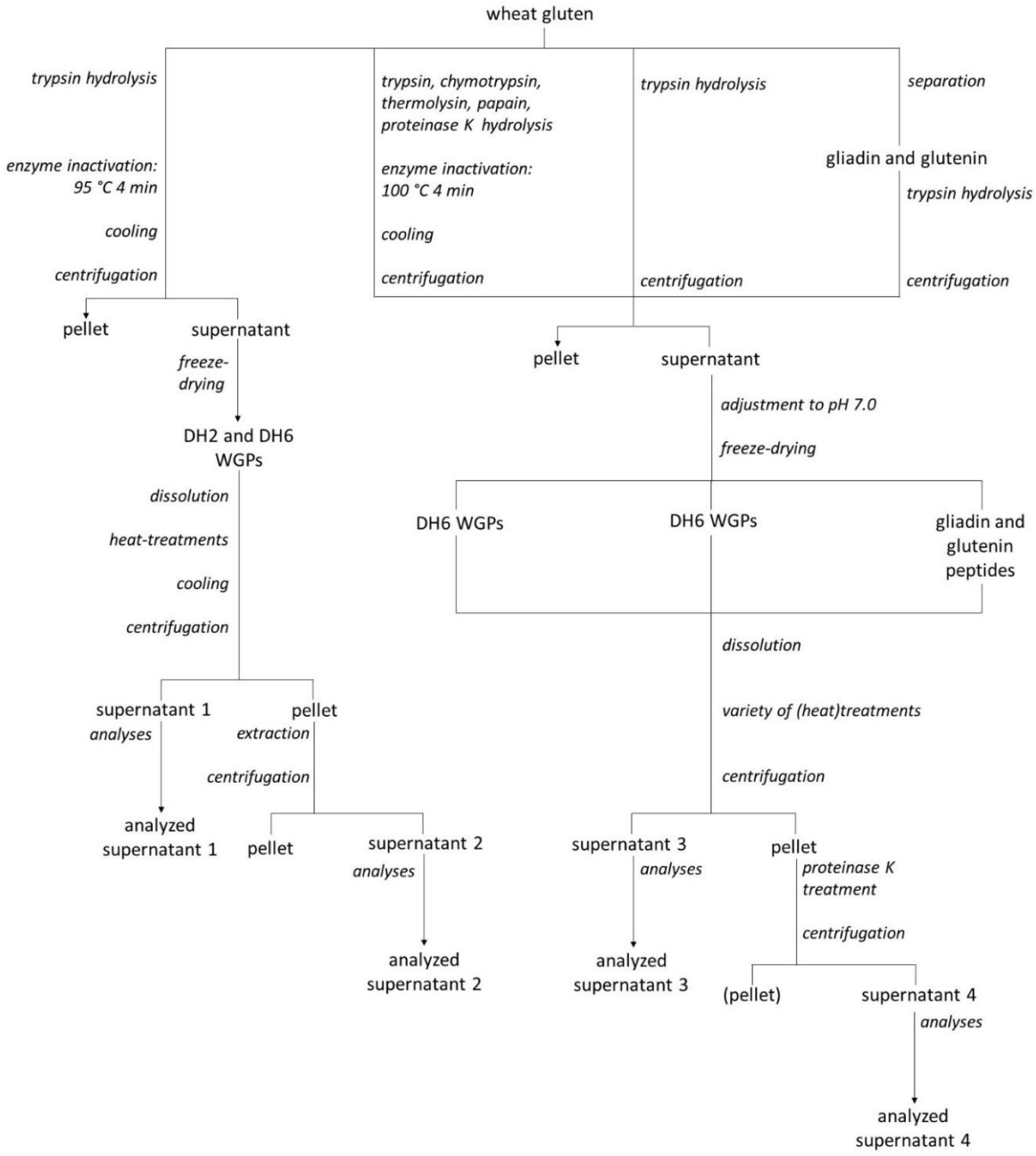
After heating (85 °C, 40 h, pH 7.0) 2.0% (w/v) glutenin and gliadin hydrolysates in water, no differences in ThT levels were noted (6.3% ± 1.6% and 5.7% ± 1.0%, respectively) for their corresponding supernatants 3 (see Figure 1). For both glutenin and gliadin, TEM pictures showed curly worm-like fibrils in their supernatants 3 (see Figure 1) both of which were present as separate entities or as large clusters (see Figure 6). However, the fibrils recovered in supernatants 3 of heated glutenin (see Figure 7.1) seemed to be shorter (average length *ca.* 230 nm) than those in supernatants 3 of heated gliadin (*ca.* 200 nm to 2 µm) (see Figure 6 and Supplementary Figure S6).

More of the initial protein was present in the pellet of heat-treated glutenin (13.5% ± 2.0%) than in that of gliadin samples (7.9% ± 2.9%). When treating these pellets with proteinase K, the material recovered in supernatants 4 (see Figure 1) from the glutenin pellet had a higher ThT fluorescence (19.5% ± 0.9%) than that of the corresponding gliadin sample (2.0% ± 0.5%). While TEM of supernatants 4 (see Figure 7.1) of gliadin materials showed only amorphous aggregates, the corresponding glutenin materials showed a combination of straight fibrils as well as clusters of worm-like and amorphous structures (see Figure 6 and Supplementary Figure S6). Our results indicated that both tryptic gliadin and glutenin hydrolysates

455 components were able to form ALFs when applying optimal fibrillation conditions [2.0% (w/v) protein, 85
456 °C, 40 h, pH 7.0]. Previously, tryptic hydrolysates of gliadin formed cross-β sheet structures by drying at
457 room temperature in contrast to those prepared from glutenin[18]. Nonetheless, an insoluble glutenin
458 fraction in SDS containing medium has been reported to form protein fibrils by incubation (25 °C, *ca.* 105
459 days) in the presence of either 2.0 M urea or 30% (v/v) trifluoroethanol at pH 6.0 to 7.0[39].

460 While WGPs formed long straight fibrils by heating at optimal fibrillation conditions, those from gliadin
461 and glutenin components were predominantly worm-like and smaller. As the morphology and size of
462 gliadin and glutenin fibrils did not resemble those from the WGPs, both protein fractions are believed to
463 jointly contribute to the formation of wheat gluten ALFs. As indicated before, the identification of
464 aggregation prone regions in wheat gluten protein fractions will allow understanding their contribution
465 during protein fibrillation and even steering protein fibril morphology.

466 Finally, the here described enzymatic and further hydrothermal treatment delivers long and straight
467 protein fibrils from wheat gluten peptides, the functionality of which should be explored. To the best of
468 our knowledge, this is the first report that WGPs may self-assembled into ALFs by food relevant processing
469 conditions. As the yield of protein fibrils was still low, further research may show whether different
470 heating modes, addition of fibrillating agents and/or mechanical treatments can further increase the rate
471 and level of wheat gluten peptides fibrillation.



472

473 Figure 1. Experimental set-up for optimizing the fibrillation hydrothermal treatment conditions of wheat
474 gluten peptides (WGPs) produced by imposing a degree of hydrolysis of 2.0% (DH 2) or 6.0% (DH 6) by
475 trypsin (left) treatment, as well as for studying protein fibrillation of DH 6 WGPs by trypsin, chymotrypsin,
476 thermolysin, papain or proteinase K treatments (middle left), in DH 6 WGPs produced with trypsin but
477 without enzyme inactivation (middle right) and in tryptic gliadin and glutenin peptides (right).
478 Supernatants 1 and 2 were obtained after incubation of tryptic DH 2 and DH 6 WGPs at different
479 hydrothermal conditions and extraction with 0.05 M Tris/HCl/1-propanol (50:50, v/v) buffer (pH 7.5) of

the resultant pellets formed after the heat treatments, respectively. The variety of (heat) treatments leading to supernatants 3 and 4 were the following: a) dissolving 2.0% protein (w/v) of different samples in water, b) heating solutions containing 2.0% protein (w/v) of the these samples at 85 °C for 40 h in water and c) heating solutions containing 2.0% protein (w/v) of tryptic DH 6 WGPs at 100 °C for 15 min in water.

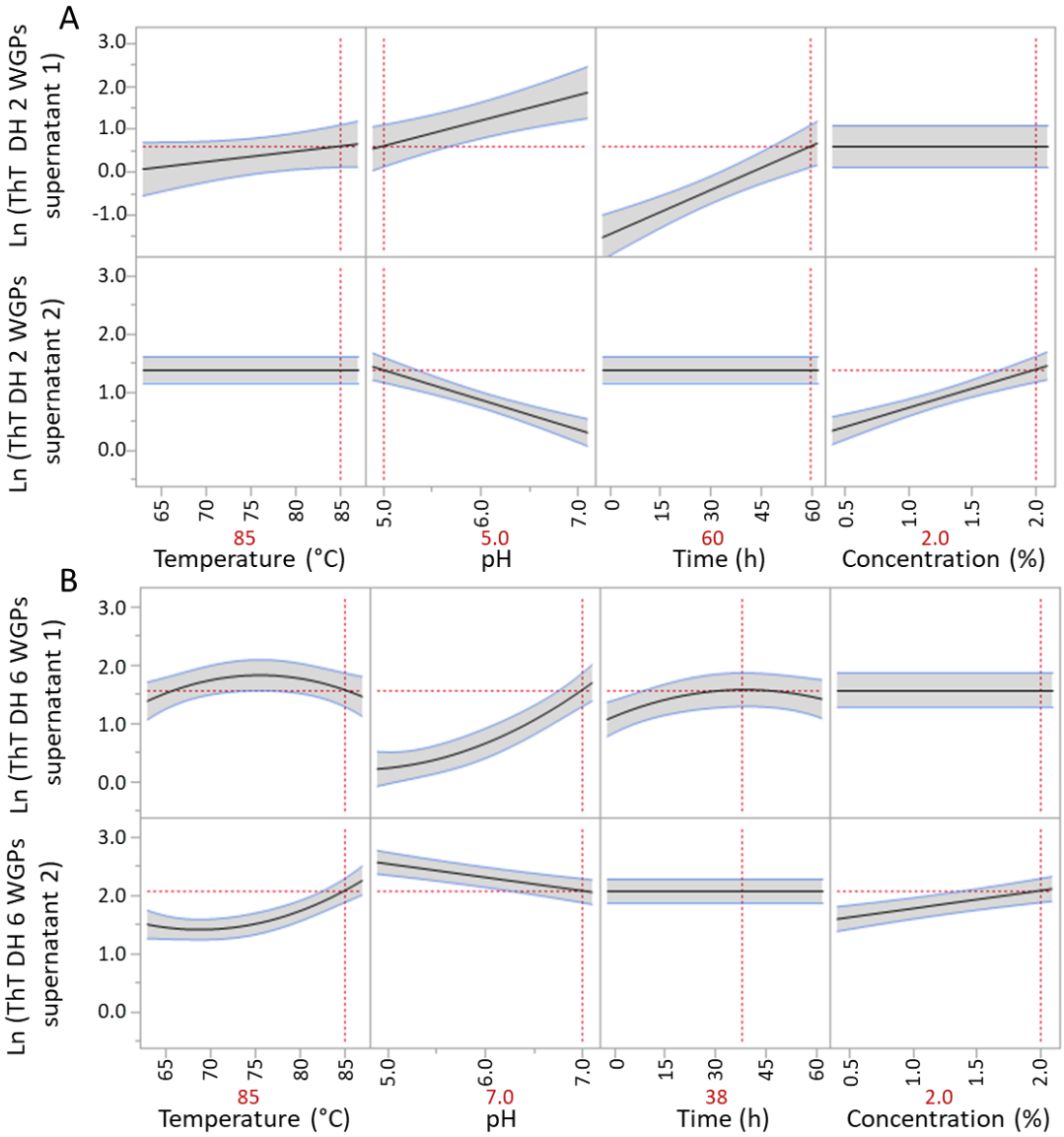


Figure 2. Prediction profiler of wheat gluten peptides (WGPs) produced by tryptic hydrolysis of wheat gluten to a degree of hydrolysis of 2.0% (DH 2; A) or 6.0% (DH 6; B) showing the predicted natural logarithm (Ln) of thioflavin T (ThT) fluorescence (%) as a function of temperature, pH, time and protein concentration. Fibrillation conditions are shown by vertical red dotted lines [DH 2 WGPs: 85°C, pH 5.0, 60 h, 2.0% (w/v) protein; DH 6 WGPs: 85°C, pH 7.0, 38 h, 2.0% (w/v) protein] and were found to be optimal

when both the resultant water-soluble fractions (*i.e.* supernatants 1 in Figure 1) as well as the material extracted from the pellets (*i.e.* supernatants 2 in Figure 1) displayed optimal levels of ThT fluorescence. Peptides were extracted from the pellets with 0.05 M Tris/HCl/1-propanol (50:50, v/v) buffer (pH 7.5). Confidence intervals are colored grey.

Table 1. Estimated effect of the factors included in the response surface models for the natural logarithm (Ln) of thioflavin T (ThT) fluorescence of wheat gluten peptides (WGPs) produced by tryptic hydrolysis to a degree of hydrolysis of 2.0% (DH 2) or 6.0% (DH 6). Non-significant parameters were removed from the models. Effects are shown for both the water-soluble fraction (*i.e.* supernatants 1 in Figure 1) and the peptides extracted from the pellets with 0.05 M Tris/HCl/1-propanol (50:50, v/v) buffer (pH 7.5) [*i.e.* supernatants 2 in Figure 1].

Term	Estimate	Standard error	t ratio	Prob > |t|
Ln ThT of the DH 2 WGP supernatants 1				
Intercept	−5.62966	1.01196	−5.56	<0.0001
pH	1.03225	0.10170	10.15	<0.0001
pH*Time	−0.01427	0.00393	−3.63	0.0009
Time	0.01089	0.00328	3.32	0.0021
Temperature*Time	0.00091	0.00040	2.26	0.0306
Temperature	−0.00365	0.01014	−0.36	0.7211
Ln ThT of the DH 2 WGP supernatants 2				
Intercept	2.08331	0.33770	6.17	<0.0001
pH	−0.34827	0.05382	−6.47	<0.0001
Protein concentration	0.43346	0.07175	6.04	<0.0001
pH* Protein concentration	−0.22344	0.08788	−2.54	0.0154
Ln ThT of the DH 6 WGP supernatants 1				
Intercept	−4.29058	0.62074	-6.91	<0.0001
pH	0.74082	0.06163	12.02	<0.0001
Time	0.01364	0.00202	6.75	<0.0001
pH*Time	−0.00772	0.00240	−3.21	0.003
Temperature*Temperature	−0.00281	0.00102	−2.75	0.0097
Time*Time	−0.00030	0.00012	−2.62	0.0134
pH*pH	0.25241	0.10521	2.40	0.0224
Temperature	0.00319	0.00623	0.51	0.6116
Ln ThT of the DH 6 WGP supernatants 2				
Intercept	0.15327	0.50073	0.31	0.7613
Temperature	0.03149	0.00498	6.33	<0.0001
pH	−0.23212	0.04987	−4.65	<0.0001
Protein concentration	0.30419	0.06630	4.59	<0.0001

Temperature*Temperature	0.00256	0.00079	3.25	0.0025

Table 2. A. The zeta potential, surface hydrophobicity and thioflavin T (ThT) fluorescence of supernatants 3 (see Figure 1) of unheated wheat gluten peptides (WGPs) produced from wheat gluten by imposing a degree of hydrolysis (DH) of 6.0% trypsin, chymotrypsin, thermolysin, papain or proteinase K treatments. B. Protein yields (expressed as percentage of the levels in the unheated samples) and ThT fluorescence of supernatants 3 (see Figure 1) of similar WGPs after heating to inactivate the enzymes (4 min at 100 °C) and subsequent heating under optimal fibrillation conditions (*i.e*. 2.0% protein w/v, pH 7.0, 85 °C, 40 h).

	A. *Unheated* supernatants 3			B. *Heated* supernatants 3	
WGPs produced with	Zeta potential (mV)	Surface hydrophobicity $(mg/ml)^{-1}$	ThT fluorescence (%)	Protein yield (%)	ThT fluorescence (%)
Trypsin	-21.1 (0.5)	8.0 (0.6)	6.9 (0.3)	73.4 (4.3)	6.3 (0.4)
Chymotrypsin	-22.9 (2.8)	4.8 (0.3)	2.1 (0.3)	65.7 (2.0)	3.7 (0.4)
Thermolysin	-20.7 (1.0)	0.6 (0.1)	2.4 (0.4)	98.9 (2.2)	1.5 (0.4)
Papain	-10.5 (3.6)	3.2 (0.2)	1.6 (0.5)	89.9 (4.7)	1.1 (0.2)
Proteinase K	-17.9 (1.2)	5.3 (0.4)	1.1 (0.1)	49.9 (1.7)	2.0 (0.1)

Standard deviations are between brackets.

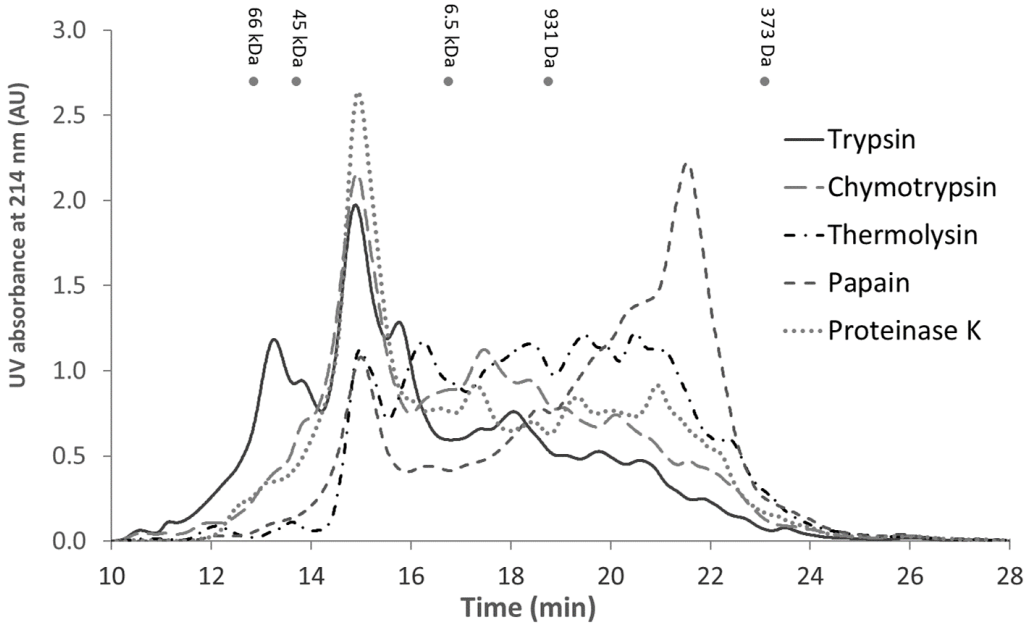


Figure 3. SE-HPLC profiles of supernatants 3 (see Figure 1) of unheated wheat gluten peptides (WGPs) prepared from wheat gluten by imposing a degree of hydrolysis (DH) of 6.0% by trypsin, chymotrypsin, thermolysin, papain or proteinase K treatments. Molecular weight markers (▪) are indicated at the top of

513 the profiles [66 kDa, bovine serum albumin; 44 kDa, ovalbumin; 6.5 kDa, aprotinin; 931 Da, angiotensin
514 III; 373 Da, $(Ala)_5$]. AU, arbitrary units.
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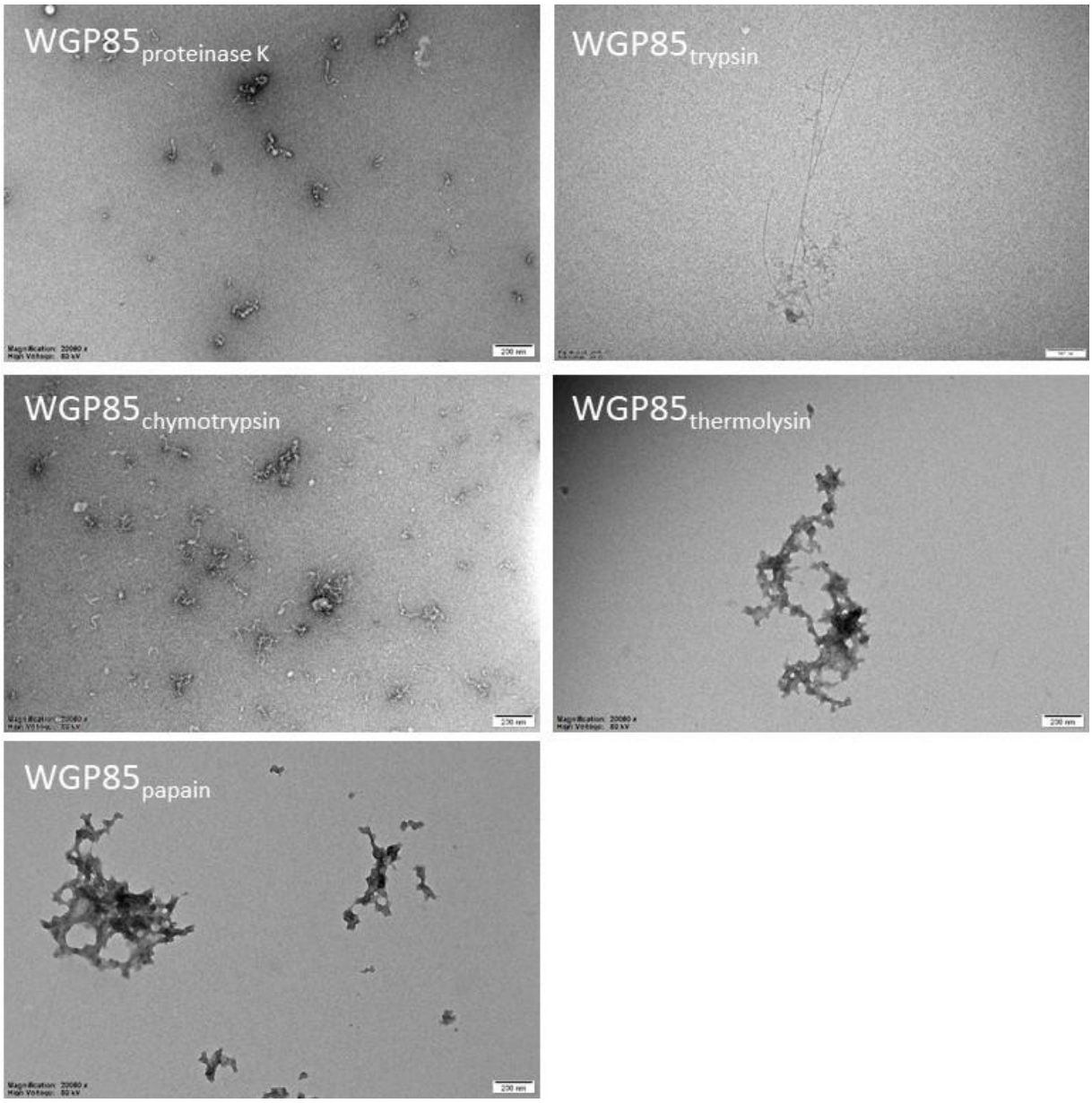

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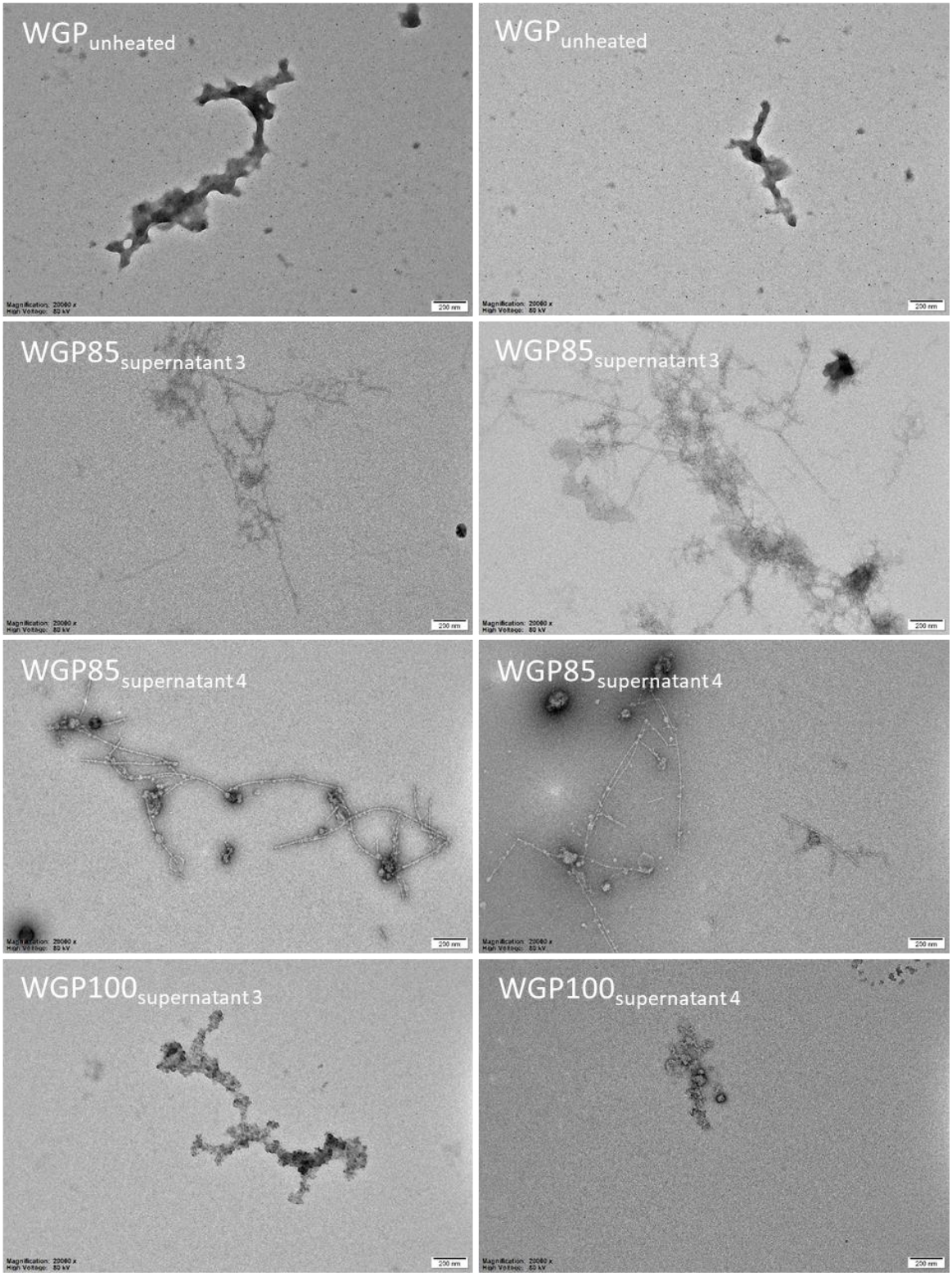
517 Figure 4. Transmission electron microscopy (TEM) images of supernatants 3 (see Figure 1) of wheat gluten
518 peptides (WGPs) prepared from wheat gluten by imposing a degree of hydrolysis (DH) of 6.0% by
519 proteinase K, trypsin, chymotrypsin, thermolysin or papain treatments. After treatments, the samples
520 were heated to inactivate the enzymes (4 min at 100 °C) and subsequently heated under optimal
521 fibrillation conditions (*i.e*. 2.0% protein w/v, pH 7.0, 85 °C, 40 h). Scale bar: 200 nm.



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523 Figure 5. Transmission electron microscopy (TEM) images of (i) supernatants 3 (see Figure 7.1) of
524 unheated (in subscript) tryptic wheat gluten peptides (WGPs) with a degree of hydrolysis (DH) of 6.0%, (ii)

supernatants 3 (see Figure 7.1) of similar WGPs heated for 40 h at 85 °C (WGP85) or for 15 min at 100 °C (WGP100), and (iii) supernatants 4 (see Figure 7.1) containing peptide material recovered by proteinase K treatment (37 °C, 48 h, 70 rpm) from the pellets formed after previous heat treatments. Scale bar: 200 nm.

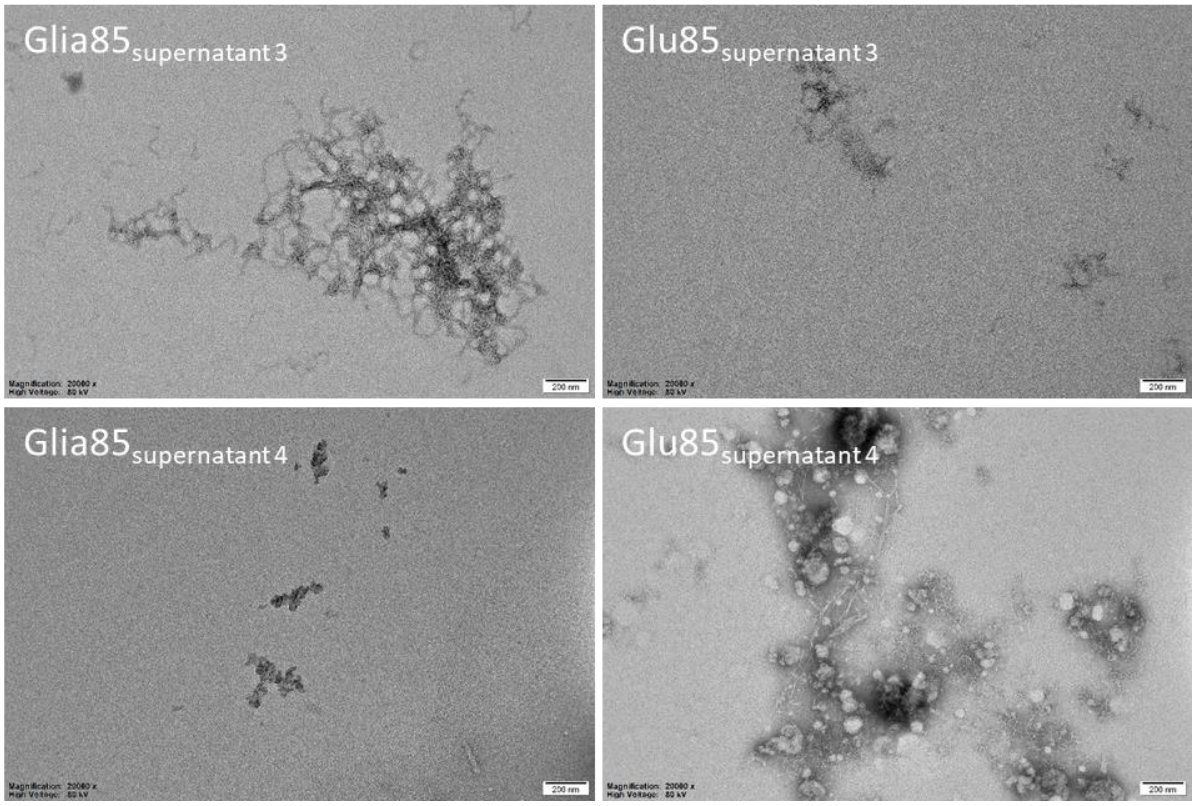

Figure 6. Transmission electron microscopy (TEM) images of (i) supernatants 3 (see Figure 7.1) of tryptic wheat gliadin (Glia85) and glutenin (Glu85) peptides heated at optimal fibrillation conditions [*i.e.* 2.0% protein (w/v), pH 7.0, 85 °C, 40 h], and (ii) supernatants 4 (see Figure 7.1) containing peptide material recovered by proteinase K treatment (37 °C, 48 h, 70 rpm) from the pellets formed after previous heat treatment. Scale bar: 200 nm.

SUPPORTING INFORMATION DESCRIPTION

Plots of predicted and observed thioflavin T (ThT) fluorescence of wheat gluten tryptic peptides. Surface plots of the thioflavin T (ThT) fluorescence model of wheat gluten peptides (WGPs) by trypsin. Molecular weight distribution of wheat gluten peptides by trypsin. Protein morphology of wheat gluten peptides produced by different enzymes and further heating. Protein morphology of wheat gluten peptides by

trypsin and further heating. Protein morphology of gliadin and glutenin peptides by trypsin and further heating.

ACKNOWLEDGMENTS

This work is part of the Methusalem programme “Food for the future” at the KU Leuven. M. A. Lambrecht acknowledges the Research Foundation-Flanders (FWO, Brussels, Belgium) for a position as postdoctoral researcher (grant 12V6718N). The authors acknowledge FWO for the SBO grant Profibfun (S003918N). Switch Laboratory was supported by grants under the EU's Horizon 2020 Framework Programme ERC Grant agreement 647458 (MANGO) to J. Schymkowitz, , the Flanders Institute for Biotechnology (VIB), the KU Leuven, the FWO, the IWT (SBO grant 60839) and Belspo (IUAP, grant P7/16). J. A. Delcour is W. K. Kellogg Chair in Cereal Science and Nutrition at the KU Leuven. V. Depoortere and O. Chen are thanked for technical assistance and S. Vercruysse for fruitful discussions. M. Monge-Morera thanks the University of Costa Rica for the permission to perform research abroad.

ABBREVIATIONS USED

WGPs, wheat gluten peptides; ThT, thioflavin T; DH X, degree of hydrolysis X.0%; AFs, amyloid fibrils; ALFs, amyloid-like fibrils; U, enzyme units; SDS, sodium dodecyl sulfate; EP:SP, enzyme preparation to substrate protein ratios; Ln, natural logarithm; ANS, 1-anilino-8-naphthalene sulfonate; SE-HPLC, size exclusion high performance liquid chromatography; TEM, transmission electron microscopy; WGP85, wheat gluten peptide treated 40 h at 85 °C; WGP100, wheat gluten peptides treated 15 min at 100 °C; Glia85, tryptic wheat gliadin peptides treated 40 h at 85 °C; Glu85 tryptic wheat glutenin peptides treated 40 h at 85 °C.

REFERENCES

(1) Lambrecht, M. A.; Deleu, L. J.; Rombouts, I.; Delcour, J. A. Heat-Induced Network Formation between Proteins of Different Sources in Model Systems, Wheat-Based Noodles and Pound Cakes. *Food Hydrocoll.* **2018**, *79*, 352–370.

(2) Peng, D.; Yang, J.; Li, J.; Tang, C.; Li, B. Foams Stabilized by β-Lactoglobulin Amyloid Fibrils: Effect of PH. *J. Agric. Food Chem.* **2017**, *65*, 10658–10665.

(3) Munialo, C. D.; Martin, A. H.; van der Linden, E.; De Jongh, H. H. J. Fibril Formation from Pea Protein and Subsequent Gel Formation. *J. Agric. Food Chem.* **2014**, *62*, 2418–2427.

(4) Jansens, K. J. A.; Rombouts, I.; Grootaert, C.; Brijs, K.; Van Camp, J.; Van der Meeren, P.; Rousseau, F.; Schymkowitz, J.; Delcour, J. A. Rational Design of Amyloid-like Fibrillary Structures for Tailoring Food Protein Techno-Functionality and Their Potential Health Implications. *Compr. Rev. Food Sci. Food Saf.* **2019**, *18*, 84–105.

(5) Sunde, M.; Serpell, L. C.; Bartlam, M.; Fraser, P. E.; Pepys, M. B.; Blake, C. C. F. Common Core Structure of Amyloid Fbrils by Synchrotron X-Ray Diffraction. *J. Mol. Biol.* **1997**, *273*, 729–739.

(6) Jansens, K. J. A.; Lambrecht, M. A.; Rombouts, I.; Monge-Morera, M.; Brijs, K.; Rousseau, F.; Schymkowitz, J.; Delcour, J. A. Conditions Governing Food Protein Amyloid Fibril Formation—Part I: Egg and Cereal Proteins. *Compr. Rev. Food Sci. Food Saf.* **2019**, *18*, 1256–1276.

(7) Lambrecht, M. A.; Jansens, K. J. A.; Rombouts, I.; Brijs, K.; Rousseau, F.; Schymkowitz, J.; Delcour, J. A. Conditions Governing Food Protein Amyloid Fibril Formation—Part II: Milk and Legume Proteins. *Compr. Rev. Food Sci. Food Saf.* **2019**, *18*, 1277–1291.

(8) Monge-Morera, M.; Lambrecht, M. A.; Deleu, L. J.; Gallardo, R.; Louros, N. N.; De Vleeschouwer, M.; Rousseau, F.; Schymkowitz, J.; Delcour, J. A. Processing Induced Changes in Food Proteins: Amyloid Formation during Boiling of Hen Egg White. *Biomacromolecules* **2020**, *21*, 2218–2228.

(9) Monge-Morera, M.; Lambrecht, M. A.; Deleu, L. J.; Louros, N. N.; Rousseau, F.; Schymkowitz, J.; Delcour, J. A. Heating Wheat Gluten Promotes the Formation of Amyloid-like Fibrils. *ACS Omega* **2021**, *6*, 1823–1833.

(10) Tang, C. H.; Wang, C. S. Formation and Characterization of Amyloid-like Fibrils from Soy β-Conglycinin and Glycinin. *J. Agric. Food Chem.* **2010**, *58*, 11058–11066.

(11) Akkermans, C.; van der Goot, A. J.; Venema, P.; Gruppen, H.; Vereijken, J. M.; van der Linden, E.; Boom, R. M. Micrometer-Sized Fibrillar Protein Aggregates from Soy Glicinin and Soy Protein Isolates. *J. Agric. Food Chem.* **2007**, *55*, 9877–9882.

(12) FAOSTAT. Production statistics of the Food and Agriculture Organisation of the United Nations http://faostat3.fao.org/compare/E (accessed Aug 7, 2020).

(13) Van Der Borght, A.; Goesaert, H.; Veraverbeke, W. S.; Delcour, J. A. Fractionation of Wheat and Wheat Flour into Starch and Gluten: Overview of the Main Processes and the Factors Involved. *J. Cereal Sci.* **2005**, *41*, 221–237.

(14) Day, L.; Augustin, M. A.; Batey, I. L.; Wrigley, C. W. Wheat-Gluten Uses and Industry Needs. *Trends Food Sci. Technol.* **2006**, *17*, 82–90.

(15) Wieser, H. Chemistry of Gluten Proteins. *Food Microbiol.* **2007**, *24*, 115–119.

(16) Wouters, A. G. B.; Rombouts, I.; Fierens, E.; Brijs, K.; Delcour, J. A. Enzymatically Hydrolyzed Wheat Gluten as a Foaming Agent in Food: Incorporation in a Meringue Recipe as a Proof-of-Concept. *J. Food Sci.* **2018**, *83*, 2119–2126.

(17) Athamneh, A.; Barone, J. Hierarchical Self-Assembly of Tryptic Peptides from Wheat Gluten. *ASME* **2009**, *2*, 693–699.

(18) Athamneh, A.; Barone, J. Enzyme-Mediated Self-Assembly of Highly Ordered Structures from Disordered Proteins. *Smart Mater. Struct.* **2009**, *18*, 1–8.

(19) Ridgley, D.; Claunch, E.; Barone, J. The Effect of Processing on Large, Self-Assembled Amyloid Fibers. *Soft Matter* **2012**, *8*, 10298–10306.

(20) Maurer-Stroh, S.; Debulpaep, M.; Kuemmerer, N.; Lopez de la Paz, M.; Martins, I. C.; Reumers, J.;

Morris, K. L.; Copland, A.; Serpell, L. C.; Serrano, L.; Schymkowitz, J. W. H.; Rousseau, F. Exploring the Sequence Determinants of Amyloid Structure Using Position-Specific Scoring Matrices. *Nat. Methods* **2010**, *7*, 237–242.

(21) Nielsen, P. M.; Petersen, D.; Dambmann, C. Improved Method for Determining Food Protein Degree of Hydrolysis. *Food Chem. Toxicol.* **2001**, *66*, 642–646.

(22) Adler-Nissen, J. *Enzymatic Hydrolysis of Food Proteins*; New York, NY, USA: Elsevier Applied Science Publishers, 1985.

(23) Goos, P.; Jones, B. *Optimal Design of Experiments: A Case Study Approach*, 1st ed.; Hoboken, NJ, USA: Wiley, 2011.

(24) Vassar, P. S.; Culling, C. F. Fluorescent Stains, with Special Reference to Amyloid and Connective Tissues. *Arch. Pathol.* **1959**, *68*, 487–498.

(25) Lambrecht, M. A.; Rombouts, I.; De Ketelaere, B.; Delcour, J. A. Prediction of Heat-Induced Polymerization of Different Globular Food Proteins in Mixtures with Wheat Gluten. *Food Chem.* **2017**, *221*, 1158–1167.

(26) Lambrecht, M. A.; Rombouts, I.; Van Kelst, L.; Delcour, J. A. Impact of Extraction and Elution Media on Non-Size Effects in Size Exclusion Chromatography of Proteins. *J. Chromatogr. A* **2015**, *1415*, 100–107.

(27) Serpell, L. Amyloid Structure. *Essays Biochem.* **2014**, *56*, 1–10.

(28) Wouters, A. G. B.; Fierens, E.; Rombouts, I.; Brijs, K.; Joye, I. J.; Delcour, J. A. Exploring the Relationship between Structural and Air-Water Interfacial Properties of Wheat (Triticum Aestivum L.) Gluten Hydrolysates in a Food System Relevant PH Range. *J. Agric. Food Chem.* **2017**, *65*, 1263–1271.

(29) Pearce, F. G.; Mackintosh, S. H.; Gerrard, J. A. Formation of Amyloid-like Fibrils by Ovalbumin and Related Proteins under Conditions Relevant to Food Processing. *J. Agric. Food Chem.* **2007**, *55*, 318–322.

(30) Vandenakker, C. C.; Engel, M. F. M.; Velikov, K. P.; Bonn, M.; Koenderink, G. H. Morphology and Persistence Length of Amyloid Fibrils Are Correlated to Peptide Molecular Structure. *J. Am. Chem. Soc.* **2011**, *133*, 18030–18033.

(31) Wouters, A. G. B.; Rombouts, I.; Fierens, E.; Brijs, K.; Delcour, J. A. Relevance of the Functional Properties of Enzymatic Plant Protein Hydrolysates in Food Systems. *Compr. Rev. Food Sci. Food Saf.* **2016**, *0*, 1–15.

(32) Delcour, J. A.; Hoseney, R. C. *Principles of Cereal Science and Technology*, 3rd ed.; St. Paul, MN, USA: AACC International Inc.: Minnesota, 2010.

(33) Wouters, A. G. B.; Rombouts, I.; Legein, M.; Fierens, E.; Brijs, K.; Blecker, C.; Delcour, J. A. Air-Water Interfacial Properties of Enzymatic Wheat Gluten Hydrolyzates Determine Their Foaming Behavior. *Food Hydrocoll.* **2016**, *55*, 155–162.

(34) Lutz, E.; Wieser, H.; Koehler, P. Identification of Disulfide Bonds in Wheat Gluten Proteins by

Means of Mass Spectrometry/Electron Transfer Dissociation. *J. Agric. Food Chem.* **2012**, *60*, 3708–3716.

(35) Colgrave, M. L.; Byrne, K.; Howitt, C. A. Food for Thought: Selecting the Right Enzyme for the Digestion of Gluten. *Food Chem.* **2017**, *234*, 389–397.

(36) Petrotchenko, E. V.; Serpa, J. J.; Hardie, D. B.; Berjanskii, M.; Suriyamongkol, B. P.; Wishart, D. S.; Borchers, C. H. Use of Proteinase K Nonspecific Digestion for Selective and Comprehensive Identification of Interpeptide Cross-Links: Application to Prion Proteins. *Mol. Cell. Proteomics* **2012**, *11*, 1–13.

(37) Fernández-Lucas, J.; Castañeda, D.; Hormigo, D. New Trends for a Classical Enzyme: Papain, a Biotechnological Success Story in the Food Industry. *Trends Food Sci. Technol.* **2017**, *68*, 91–101.

(38) Rousseau, F.; Schymkowitz, J.; Serrano, L. Protein Aggregation and Amyloidosis: Confusion of the Kinds? *Curr. Opin. Struct. Biol.* **2006**, *16*, 118–126.

(39) Mackintosh, S. H.; Meade, S. J.; Healy, J. P.; Sutton, K. H.; Larsen, N. G.; Squires, A. M.; Gerrard, J. A. Wheat Glutenin Proteins Assemble into a Nanostructure with Unusual Structural Features. *J. Cereal Sci.* **2009**, *49*, 157–162.

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