Prediction of heat-induced polymerization of different globular food proteins in mixtures with wheat gluten

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Abstract

Egg, soy or whey protein co-exists with wheat gluten in different food products. Different protein types impact each other during heat treatment. A positive co-protein effect occurs when heat-induced polymerization of a mixture of proteins is more intense than that of the isolated proteins. The intrinsic protein characteristics of globular proteins which enhance polymerization in mixtures with gluten are unknown. Here, a model was developed to predict potential co-protein effects in mixtures of gluten and globular proteins during heating at 100 °C. A negative co-protein effect with addition of lysozyme, no co-protein effect with soy glycinin or egg yolk and positive co-protein effects

with bovine serum albumin, (S-)ovalbumin, egg white, whole egg, defatted egg yolk, wheat albumins and wheat globulins were detected. The level of accessible free sulfhydryl groups and the surface hydrophobicity of unfolded globular proteins were the main characteristics determining co-protein effects in gluten mixtures.

Key words

Network formation, cross-links, co-protein effect, egg, soy, whey, wheat gluten

1. Introduction

Food proteins are of great nutritional, sensorial and technological importance. Examples of the latter are that egg, soy, surimi, meat and milk proteins denature and form gels upon heating (Belitz, Grosch & Schieberle, 2009) and that the texture and structure of wheat-based food products such as pasta and bread are mainly determined by network formation of the storage proteins gliadin and glutenin (Delcour, Joye, Pareyt, Wilderjans, Brijs & Lagrain, 2012). Upon mixing with water, these proteins form gluten and give wheat dough unique viscoelastic properties (Veraverbeke & Delcour, 2002). Network formation of proteins through formation and reshuffling of covalent cross-links and non-covalent interactions impacts the structure of food products (Delcour et al., 2012; Mine, 1995). Food processing unit operations such as heating and exposure to different media often induce changes in conformation of proteins with exposure of reactive groups. For instance, sulfhydryl (SH) groups and disulfide (SS) bonds become accessible for intermolecular SH oxidation and SH-SS exchange reactions (Visschers & de Jongh, 2005). At moderate temperatures, free SH groups can initiate cross-linking. Replacing the only cysteine (and thus also free SH group) of β-lactoglobulin by a serine residue obstructs heat-induced covalent network formation (Jayat, Gaudin, Chobert, Burova, Holt, McNae et al., 2004). At elevated temperatures and/or under alkaline conditions β-elimination reactions of SS bonds form free SH groups and dehydroalanine which can then react with cysteine and lysine to form lanthionine (LAN) and lysinoalanine (LAL), respectively (Friedman, 1999).

Even within one protein source, different proteins are present which can impact each other's network formation during heating. Wheat flour proteins are classified as albumin, globulin, gliadin and glutenin by sequential extraction with water, salt solution, aqueous ethanol and dilute acid or alkali (Osborne, 1907). In bread baking, fresh pasta drying and cooking, glutenin polymerizes first while α- and γ-type gliadins are incorporated in the protein network at higher temperatures (Bruneel, Lagrain, Brijs & Delcour, 2011; Lagrain, Thewissen, Brijs & Delcour, 2008). As far as whey proteins are concerned, a mixture of β-lactoglobulin and α-lactalbumin gels at low protein concentration while the separate proteins do not. Both proteins interact through SH-SS exchange reactions which form and stabilize the gel network (Matsudomi, Oshita, Sasaki & Kobayashi, 1992). Also, a mixture of bovine serum albumin (BSA, containing one free SH group) and α-lactalbumin (containing no free SH groups) forms heat-induced SS cross-linked polymers made up by the separate proteins and polymers of both (Havea, Singh & Creamer, 2000). The higher gel strength of a β-lactoglobulin/BSA mixture than that of the isolated proteins has been attributed to the increased extent of SH oxidation reactions during heating (Matsudomi, Oshita & Kobayashi, 1994). According to Gezimati *et al.* (1997) heat-induced gelation between whey proteins depends on the thermal transition temperatures of proteins, the availability of free SH groups and the ability to form non-covalent protein aggregates prior to SS bond formation. Also, at neutral pH, mixtures of the egg white proteins ovalbumin with lysozyme form stronger gels than do the isolated proteins. The ionic interactions between these oppositely charged proteins impact gel strength (Arntfield & Bernatsky, 1993). Furthermore, different types of proteins in one food source can show synergistic effects. Heat-induced gels made with whole egg have higher strength than gels made from egg white or egg yolk. The molecular weight (MW) of aggregates formed by heating (70 °C, 2 min) egg white or yolk also differ from those when heating whole egg (Raikos, Campbell & Euston, 2007). However, the precise impact of different egg proteins on each other's polymerization remains to be investigated.

Literature also provides evidence that interactions and reactions between proteins of different sources impact the characteristics of food systems (Erickson, Campanella & Hamaker, 2012). Addition

of whey to egg albumen proteins enhances the strength of the resultant heat-induced gels. More hydrophobic groups are exposed and β-sheet structures formed in gels from mixtures of proteins than in those from isolated proteins (Ngarize, Adams & Howell, 2005; Ngarize, Adams & Howell, 2004). Also, inclusion of casein increases the mechanical strength of zein-based resins and the amount of intermolecular β-sheets structures (Erickson, Renzetti, Jurgens, Campanella & Hamaker, 2014). At pH 8.0, heat-induced polymerization in a BSA/gluten mixture is similar to that in gluten alone. Gluten decelerates cross-linking of BSA and increases its denaturation temperature (Rombouts, Lagrain & Delcour, 2012). Wouters *et al.* (2016) found a synergistic effect in gel firmness and heat-induced polymerization with gluten/egg white but not with gluten/casein blends.

It is important to mention that thermodynamic incompatibility of proteins in various media makes protein blends difficult to analyze (Polyakov, Grinberg & Tolstoguzov, 1997). However, differences in solubility do not prevent the occurrence of a positive co-protein effect in a BSA/gliadin mixture in water. A positive co-protein effect is here referred to as more intense heat-induced polymerization than expected based on the isolated proteins (Lambrecht, Rombouts & Delcour, 2016). In many cereal-based food products, wheat proteins co-exist with milk, soy and egg proteins. This is the case in some cake, cookie, pancake, milk bread and egg noodle systems. While the above illustrate the importance of co-protein effects in mixtures of gluten and globular proteins, the underlying mechanisms are poorly understood. Basic knowledge on this topic would be helpful for predicting co-protein effects and can form a basis for developing new food recipes and products. It is especially important to investigate how proteins influence each other's functionality and which protein characteristics favor heat-induced polymerization.

In this study, protein characteristics are linked to covalent network formation of protein model systems during heating at 100 °C based on assessing of the formation of SS bonds, LAN and LAL, changes in MW and changes in extractability in sodium dodecyl sulfate (SDS) containing medium. Gradually increasing the complexity of the systems allowed developing a model for predicting

potential co-protein effects between gluten and globular proteins. First, the polymerization of isolated food proteins was studied using five well-documented food proteins varying in amino acid composition and structure. BSA [66.3 kDa (Belitz et al., 2009), pI 4.7] is present in blood, milk and whey. It has 17 intramolecular SS bonds and one free SH group (Anand & Mukherjee, 2013). Ovalbumin (44.5 kDa, pI 4.5) is the most abundant egg white protein (*ca.* 54%) (Belitz et al., 2009). S-Ovalbumin results from L-to-D isomerization of Ser-164, Ser-236 and Ser-320 (Yamasaki, Takahashi & Hirose, 2003). It is more thermostable than ovalbumin itself due to the isomerization of Ser-164 and Ser-320 (Takahashi, Maeda, Yamasaki & Mikami, 2010). Ovalbumin has four SH groups and only one SS bond. Egg lysozyme (14.3 kDa, pI 10.7) is used in some food systems as food preservative. It hydrolyses β-1-4 glycosidic bonds in the cell walls of Gram-negative bacteria. Lysozyme contains four SS bonds and no free SH groups (Huopalathi, López-Fandiño, Anton & Schade, 2007). Glycinin [*a.* 360 kDa (Belitz et al., 2009), pI 4.5-6.0 (Liu, Lee & Damodaran, 1999)] is one of the most abundant proteins in soy flour. It contains *ca.* 20 SS bonds and no free SH groups (Draper & Catsimpoolas, 1978). Second, heat-induced changes in mixtures of proteins derived from the same source were investigated, with a focus on egg and wheat proteins. Third, co-protein effects between the above mentioned proteins and gluten were analyzed and linked to specific protein characteristics.

2. Materials and methods

2.1 Materials, their preparation and characterization

Gluten [83.2% protein, on dry matter (dm) basis] from wheat flour (cultivar Paragon, RAGT, Ickleton, United Kingdom) and soy glycinin (98.1% protein on dm) from soy flour (L.I. Frank, Twello, The Netherlands) were isolated as described by Lambrecht *et al.* (2015). Albumins (42.9% protein on dm) and globulins (69.1% protein on dm) were extracted (60 min, room temperature) from wheat flour (50.0 g) with respectively 100.0 ml water or 100.0 ml 0.050 M sodium phosphate buffer (pH 7.6) containing 0.4 M sodium chloride. After three extractions with each medium and intermediate and final centrifugation (10 000 g, 10 min) steps, the combined water or buffered supernatants were

dialyzed for 24 h against 0.01% acetic acid. Gliadin (87.7% protein on dm) was obtained by extraction with 70% (v/v) ethanol as in Lambrecht (2016). The residue obtained after gliadin extraction was further extracted twice with 60% (v/v) ethanol. These extracts were discarded. Starch was then washed from the pellet with deionized water and sieving and the resultant residue was called glutenin (82.4% protein on dm). Commercial eggs (55.6% protein on dm) were whipped with a whisk or separated in egg white (90.1% protein on dm) and yolk (33.6% protein on dm) with removal of the vitelline membrane. All protein fractions were freeze-dried and gently ground in a mortar. Lipids were removed from freeze-dried egg yolk (50.0 g) with hexane. After five extractions (each with 250.0 ml) and filtration, the defatted egg yolk (67.2% protein on dm) was air dried. Lysozyme (chicken egg white, 100.0% protein on dm) and ovalbumin (albumin chicken egg grade III, 94.1% protein on dm) were from Sigma-Aldrich (Bornem, Belgium). Ovalbumin (6.67 mg/ml) was shaken for 24 h at 55 °C in a 0.10 M glycine-sodium hydroxide buffer (pH 9.9) for converting it into S-ovalbumin. After dialyzing for 24 h against deionized water, S-ovalbumin was freeze-dried and ground (91.6% protein on dm). BSA (fraction V for biochemistry, 98.2% protein on dm) was from Acros Organics (Geel, Belgium). Whey protein isolate (97.7% protein on dm) was from Fonterra (Amsterdam, The Netherlands). Moisture contents were determined in triplicate according to AACC-I Approved Method 44-15.02 (AACC, 1999). Protein contents were determined in triplicate, with an automated Dumas protein analysis system (EAS Variomax N/CN, Elt, Gouda, The Netherlands) using an adaptation of AOAC Official Method 990.03 (AOAC, 1995). Conversion factors (5.7 for gluten; 6.25 for all other proteins) were used to calculate protein from nitrogen contents. All chemicals were of analytical grade and from Sigma-Aldrich (Steinheim, Germany) unless specified otherwise. Dithiothreitol (DTT), disodium hydrogen phosphate and sodium dihydrogen phosphate were from VWR International (Leuven, Belgium).

2.2 Heat treatment

Solutions or dispersions of the isolated proteins or mixtures thereof with gluten in deionized water (ratio 2:1 w/w, 100.0 mg protein/ml) were heated in hermetically sealed tubes (glass, inner and outer diameter 27 and 34 mm respectively, height = 100 mm) at 100 °C for 0, 6, 60 and 120 min. Heat-treated sample tubes were immediately cooled by putting them in water. The pH of unheated samples was determined in duplicate after shaking for 60 min at room temperature. All samples were freeze-dried and ground.

2.3 Differential scanning calorimetry

Denaturation properties were determined with differential scanning calorimetry (DSC) using a Q2000 DSC (TA instruments, New Castle, DE, USA) calibrated with indium. Samples (2.00-4.00 mg) were accurately weighed in an aluminum pan (Perkin-Elmer, Waltham, MA, USA) and deionized water [1/3 (w/w) dm protein/water] was added. Pans were hermetically sealed and heated from 0 °C to 120 °C at 4 °C/min. An empty pan was used as reference. Analyses were performed at least in triplicate. The denaturation onset, peak, conclusion temperatures, temperature ranges and enthalpies were determined using Universal Analysis 2000 software (TA Instruments).

2.4 Surface hydrophobicity

The protein surface hydrophobicity was determined in duplicate with 1-anilino-8-naphthalene sulfonate (ANS). Unheated and heated (60 sec; 100 °C) proteins (10.0 mg protein/ml) 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. Samples (200 µl) were transferred to a 96-well plate and 10 µl ANS solution [8.0 mM in 0.01 M sodium phosphate buffer (pH 7.0)] was added. The fluorescence of the protein samples was measured with a Synergy MX Multi-Mode Reader (BioTek, Winooski, VT, USA). Wavelengths of excitation and emission were 390 nm and 480 nm, respectively. The protein surface hydrophobicity was calculated as in Chaudhuri *et al.* (1993). Hereto, the relative fluorescence was calculated as the difference in fluorescence intensity of the protein-ANS mixture and that of solution in buffer without protein (control ANS) and divided by the fluorescence of control ANS. The slope of the plot of relative

158 fluorescence intensity as a function of protein concentration represents the protein surface
159 hydrophobicity.

160 **2.5 Monomeric particle size and ζ-potential measurements**

161 Duplicate samples (10.0 mg protein/ml) were extracted (60 min, room temperature) with 0.10 M
162 NaCl. After centrifugation (5 000 *g*, 5 min) the particle size distribution of protein in the supernatant
163 was determined using dynamic light scattering (Zetasizer Nano ZS series, Malvern Instruments,
164 Worcestershire, UK). In this method, the Brownian motion of particles is related to their size based
165 on the Stokes-Einstein equation. Monomeric globular albumins and globulins are here represented as
166 spheres. The ζ-potential was determined on unheated and heated (1 min; 100 °C) samples with
167 particle electrophoresis (Zetasizer Nano ZS series). A short heating step was performed to unfold the
168 globular proteins while minimizing polymerization. All measurements were carried out at 20 °C.

169 **2.6 Amino acid analysis**

170 Amino acids, including cross-links as cystine, LAN and LAL, were analyzed with high-performance
171 anion-exchange chromatography with pulsed amperometric detection. To that end, (iso)peptide
172 bonds of samples (10.0 mg protein/ml) were hydrolyzed by heating at 110 °C for 24 h with 6.0 M HCl
173 containing 0.1 % (w/v) phenol and 3.0 mM norleucine (as internal standard). Then, mixtures were
174 diluted (800-fold) in deionized water, filtered (Millex-GP, 0.22 µm, polyethersulfone, Merck Millipore,
175 Carrigtwohill, Ireland) and separated using a Dionex ICS3000 system (Sunnyvale, CA, USA) as in
176 Rombouts *et al.* (2009) with some modifications. To avoid underestimation of the total amount of
177 cysteine due to acid-induced degradation, cysteine and cystine were oxidized to cysteic acid prior to
178 acid hydrolysis as in Moore (1962). Performic acid was formed by adding 1.0 ml 35% hydrogen
179 peroxide to 9.0 ml formic acid and resting for 60 min at room temperature. The resulting performic
180 acid containing solution was then cooled to 0 °C on ice. An aliquot (4.0 ml) was then added to the
181 samples which were then left overnight (16 h, 0 °C). To remove the excess performic acid, 0.6 ml 48%
182 hydrogen bromide was added. Bromine and formic acid were evaporated from the samples with a

183 Rotational Vacuum Concentrator (Q-Lab, Vilvoorde, Belgium) at 60 °C and 100 Pa, followed by amino
184 acid analysis as described in Rombouts *et al.* (2009) with 0.15 mM norleucine as internal standard.
185 Mixtures were diluted (200-fold) in deionized water before filtration and quantification of cysteic
186 acid. All analyses were performed in triplicate. The expected levels of LAN and LAL were calculated as
187 the weight average of the experimental values of the corresponding isolated proteins.

188 **2.7 Quantification of free SH groups**

189 Free SH groups were determined colorimetrically with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB).
190 Samples (1.0-1.5 mg protein) were shaken for 60 min at room temperature in 1.0 ml sample medium
191 [0.10 mol/l tris(hydroxymethyl)aminomethane-HCl (pH 7.0) containing 2.0% SDS, 3.0 M urea and 1.0
192 mM tetrasodium ethylenediaminetetraacetate]. Then, 100 µl of DTNB reagent [0.1% (w/v) in sample
193 buffer] was added and the samples were shaken for 10 min. After filtration (Millex-HP, 0.45 µm,
194 Merck Millipore), the absorbance at 412 nm was read exactly 55 min after addition of DTNB reagent.
195 Extinction values were converted to concentrations of free SH groups using a calibration curve with
196 glutathione. Controls without DTNB or sample were used to correct for background absorbance of
197 DTNB and sample. All analyses were performed in triplicate. Based on the weight average of the
198 experimental values of the corresponding isolated proteins an expected free SH content was
199 calculated for mixtures of globular proteins with gluten (ratio 1:2 w/w).

200 **2.8 Size exclusion high performance liquid chromatography**

201 The impact of heating on the MW distribution and extractability in SDS-containing media was
202 evaluated in triplicate with size exclusion high performance liquid chromatography (SE-HPLC) and
203 conducted as in Lambrecht *et al.* (2015). Samples (1.0 mg protein/ml) were extracted (60 min, room
204 temperature) with sodium phosphate buffer (0.050 M; pH 6.8) containing 2.0% (w/v) SDS. Extraction
205 under reducing conditions was performed under nitrogen atmosphere with inclusion of 1.0% (w/v)
206 DTT in the SDS-containing buffer. The total extractable protein in SDS-containing media (SDS-EP) was
207 calculated from the corresponding peak areas and expressed as a percentage of assumed total area,

that is, the area recorded under reducing conditions. The expected SDS-EP values were calculated as the weight average of the experimental SDS-EP values of the corresponding isolated proteins.

2.9 Kinetics of extractability loss in sodium dodecyl sulfate containing medium

Proteins lose extractability in SDS-containing medium following first-order kinetics during heat treatment (Rombouts et al., 2012). Protein extractability in SDS-containing medium (y, expressed as SDS-EP) decreases during heating towards a minimum and can be presented as follows:

$$-\frac{\mathrm{dy}}{\mathrm{dt}} = \mathrm{k[y]} \qquad \text{(Equation 1)}$$

with k the first-order reaction rate constant of polymerization (min^{-1}). Hence, with $[y]_0$ and $[y]_t$ the SDS-EP at time zero and time t respectively is given by

$$[\mathrm{y}]_t = [\mathrm{y}]_0\, e^{-kt} \qquad \text{(Equation 2)}$$

The protein extractability decreases towards a minimum during heating time according to the equation

$$[\mathrm{y}]_t = [\mathrm{y}]_0\ \mathrm{e}^{-\mathrm{kt}} + [\mathrm{y}]_{\mathrm{minimal}} \qquad \text{(Equation 3)}$$

with $[y]_{minimal}$ the extractability of protein resisting polymerization under the experimental conditions. Trend lines of protein polymerization according to first order kinetics were estimated using JMP® Pro 11.2.0 (SAS Institute, Cary, NC, USA). Due to the limited heating time points, first order kinetics were used to support the experimental data but no k, [y] or R^2 values are reported in the text.

2.10 Prediction model

To predict whether a food protein would experience a co-protein effect in the presence of gluten, a multiple linear regression model was developed which takes into account a number of experimentally determined protein characteristics [i.e. monomeric particle size, free SH content, cysteine level, denaturation temperature of the largest fraction, ζ-potential and surface

hydrophobicity from the unheated and heated (1 min; 100 °C) samples]. For each heating time, the co-protein effect (in %) was calculated as the ratio of experimental to expected SDS-EP. A ratio higher, similar or lower than 100% reflects a positive, no or a negative co-protein effect respectively. The experimental protein characteristics of ten isolated protein (mixtures) were related to their corresponding co-protein effect ratio when mixed with gluten. Because only a limited amount of samples was available, main-effects-only regression models were considered. An "all possible subsets" model selection procedure was executed using the Akaike Information Criterion (AIC) (Akaike 1973) and Adjusted R^2 as statistics to define the best model (Kutner et al. 2005). In this final model all terms significantly contributed to the prediction capability. These steps were performed using the JMP® Pro 11.2.0 software.

3. Results and discussion

3.1 Cross-linking of different isolated food proteins

Solutions of five isolated proteins were heated for various times at 100 °C in water. Their DSC peak denaturation temperatures were lower than 100 °C (Table 1).

BSA is the least heat-stable protein in this study (denaturation temperature *ca.* 62 °C). It has a larger portion of its hydrophobic residues at the surface than the other model proteins. Heating at 100 °C unfolds BSA exposing more negatively charged and less hydrophobic amino groups at the protein surface. BSA polymerizes rapidly during heating at 100 °C in water through SH oxidation and SH-SS exchange reactions (Lambrecht et al., 2016), decreasing the SDS-EP of BSA to 3% ± 1% already after 6 min of heating (Figure 1.A). The level of its accessible free SH groups also rapidly decreased (6 min of heating) due to SH oxidation and slightly increased during extended heating (≥ 120 min) due to β-elimination reactions (Table 2).

Ovalbumin contains less cysteine (112 µmol/g protein) than BSA (528 µmol/g protein). However, one molecule of ovalbumin contains four free SH groups while a molecule of BSA contains only one such functional group (Tables 1 and 2). When ovalbumin unfolds, more of its negatively charged and

hydrophobic amino acids become accessible at the protein surface (Table 1). During heating, the portion of free SH groups decreased (Table 2). The level of monomeric (eluting at *ca.* 9 min 5 sec) and dimeric (eluting at *ca.* 8 min 5 sec) ovalbumin decreased during heating with formation of polymers which remained extractable in SDS-containing medium (eluting between 5 min 20 sec and 7 min 50 sec) and a low fraction of polymers not extractable in such medium (Figures 1.A and 2.1B). Only half of the accessible free SH groups of ovalbumin were consumed after 120 min heating (Table 2).

S-Ovalbumin, the most thermo-stable form of ovalbumin (Table 1), already polymerized during its production (SDS-EP of 84% ± 2%). It contained less accessible free SH groups than ovalbumin (Table 2). Also, residual buffer present as a result of the S-ovalbumin production could cause a higher pH reading of the S-ovalbumin solution than that of the ovalbumin solution (Table 1). SH groups (pK_a 8.3) are more reactive under mild alkaline conditions than at lower pH (Visschers & de Jongh, 2005). With a similar isoelectric point and a higher pH, S-ovalbumin was more negatively charged than ovalbumin. More hydrophobic groups were already exposed in unheated S-ovalbumin than in ovalbumin but the increase during heating was lower. After 6 min of heating, monomeric and dimeric S-ovalbumin were converted to polymers which were either extractable or unextractable in SDS-containing medium. SS bond formation (Figure 1.A, profile not shown) was crucial in this context. . S-Ovalbumin formed polymers which were not extractable in SDS-containing medium in water to a larger extent than did ovalbumin.

Lysozyme is positively charged in water and has low surface hydrophobicity (Table 1). This small protein with four SS bonds did not extensively polymerize after 6 min of heating at 100 °C (Figures 1.A and 2.1C). Prolonged heating (≥ 60 min) increased the level of free SH groups through β-elimination reactions (Table 2) which initiated polymerization (Figures 1.A and 2.1C). Eventually, lysozyme formed more polymers which were not extractable in SDS-containing medium than did ovalbumin.

Soy glycinin is a large protein. It has a lower cysteine density than the other used model proteins (Table 2). During heating, the ζ-potential of soy glycinin solutions decreased and some hydrophobic patches were exposed. This protein polymerized faster and to a larger extent than lysozyme (Figure 1.A). A small portion of free SH groups formed during isolation initiated covalent network formation (Table 2, Figure 1.A). In agreement with Lambrecht *et al*. (2016), polymers extractable in SDS-containing medium were formed after 6 min of heating which polymerized further to polymers unextractable in this medium during prolonged heating (Figure 2.1D).

Although ovalbumin rapidly initiated SH oxidation as a result of it containing four free SH groups per molecule, no continuous protein network was formed (Figure 2.1B). Even lysozyme, which needed β-elimination reactions to initiate SH oxidation and SH-SS exchange reactions, formed more polymers which were not extractable in SDS-containing medium than ovalbumin (Figure 1.A). Free SH groups are required to initiate polymerization. However, these data indicate that the accessibility of intramolecular SS bonds also impacts the extent and continuity of the covalent network. For example, BSA (1 SH group, 17 SS bonds) formed larger polymers which were unextractable in SDS-containing medium than did ovalbumin (4 SH groups, 1 SS bond).

3.2 Cross-linking of egg proteins

The intrinsic characteristics and cross-linking of **egg white** were compared to those of its most abundant protein, *i.e*. ovalbumin. The pH of unheated lyophilized egg white solution was higher and its ζ-potential lower than those of a solution of ovalbumin of the same concentration (100.0 mg/ml) (Table 1). Heating for 60 sec at 100 °C increased the surface hydrophobicity in both samples, but slightly more in ovalbumin than in egg white. Egg white proteins formed more rapidly and to a larger extent polymers which were no longer extractable in SDS-containing medium than did isolated ovalbumin (Figures 1.A and 1.B). Indeed, at 100 °C isolated ovalbumin formed more polymers that were still extractable in such medium, while egg white proteins immediately formed a network to an extent that they were no longer extractable in it. Ovalbumin is the only egg white protein which

304 contains free SH groups (Huopalathi et al., 2007). Isolated lysozyme, which does not contain free SH
305 groups, barely formed polymers which were not extractable in SDS-containing medium (Figure 1.A).
306 Even without concrete information on the extractability loss of the egg white proteins other than
307 ovalbumin and lysozyme, it is undisputable that more polymers were formed in egg white which
308 were not extractable in SDS-containing medium than what could be expected based on data for the
309 isolated proteins. The level of free SH groups in egg white decreased more rapidly than expected
310 based on the loss of free SH in ovalbumin (Table 2). The formation of polymers not extractable in
311 SDS-containing medium in heated egg white implies that SH-SS exchange reactions occurred
312 between ovalbumin and other egg white proteins containing more intramolecular SS bonds than
313 ovalbumin. These results indicate that ovalbumin rapidly initiated polymerization in egg white
314 thereby interconnecting other egg white proteins and leading to a continuous protein network. To
315 the best of the authors' knowledge, the role of egg white proteins other than ovalbumin as network
316 formers to date has been overlooked in the relevant food science literature.

317 In contrast to egg white, **egg yolk** solids contain *ca.* 62.5% lipids next to *ca.* 33% protein (Powrie &
318 Nakai, 1985). Prior defatting of egg yolk with hexane increased the extent of polymerization upon
319 subsequent heating (Figure 1.B). Prior removal of lipids increased the opportunity of egg yolk
320 proteins to react and the loss of free SH groups upon heating (Table 2). Egg yolk proteins polymerized
321 to a smaller extent upon heating than egg white proteins even after defatting (Figure 1.B). Defatted
322 egg yolk proteins also contained less cysteine and were less charged and hydrophobic at the protein
323 surface than egg white proteins (Table 1).

324 The loss of SDS-EP in **whole egg**, which contains egg white and egg yolk in a ratio 2:1 (Powrie &
325 Nakai, 1985), equaled the sum of those of its fractions (Figure 1.B). There was hence no indication of
326 a synergistic effect between egg white and yolk proteins.

327 **3.3 Cross-linking of wheat proteins**

328 **Wheat gluten** proteins contain high levels of glutamine and proline and low levels of charged amino
329 acids. Even if cysteine is a minor amino acid in gluten, it largely impacts the structure of wheat-based
330 food products (Wieser, 2007). Before heating, 75% ± 2% of wheat gluten was extractable in SDS-
331 containing medium (Figure 1.C). As a result of heating, the extractability of glutenin (eluting between
332 *ca.* 5 min and 7 min 55 s) in this medium decreased more rapidly than that of gliadin (eluting
333 between *ca.* 7 min 55 sec and 9 min 40 sec) (Figure 2.1A). Glutenin and gliadin proteins polymerized
334 about ten times faster during heating at 100 °C in water in each other's presence than absence
335 (results not shown). Isolated glutenin polymerized fast during the first minutes of heating. At longer
336 heating times (≥ 60 min), increased polymerization in gluten was attributed to increased gliadin
337 incorporation. **Wheat albumin and globulin** were fully extractable before heating (Figure 1.C). The
338 former polymerized more slowly than the latter, but reached a lower plateau value in terms of
339 extractability in SDS-containing medium. All wheat fractions had little free SH groups which rapidly
340 oxidized into SS bonds during heating (Table 3). However, wheat gluten contained less cysteine (200
341 ± 7 µmol cysteine/g) than wheat albumin (322 ± 15 µmol/g) and globulin (353 ± 6 µmol/g) (Table 1).

342 **3.4 Cross-linking between wheat gluten and (mixtures of) other food proteins**

343 In some food products wheat gluten and other types of protein co-exist. Here, gluten was mixed with
344 different globular proteins (ratio 2:1 w/w) and potential co-protein effects were monitored. The
345 heat-induced SDS-EP losses of protein mixtures of wheat gluten with either BSA, ovalbumin or S-
346 ovalbumin were larger than expected based on the SDS-EP losses of the isolated proteins (Figures
347 3.A, 3.B and 3.C). Such synergistic network formation is referred to as a positive co-protein effect. In
348 the case of BSA (Figure 3.A), the difference between measured and expected SDS-EP readings was
349 mainly due to increased gliadin incorporation (Figure 2.2A). In addition, gluten increased the
350 denaturation temperature of BSA to 79.3°C ± 0.3°C, in agreement with findings by Rombouts *et al.*
351 (2012). Mixtures of ovalbumin and S-ovalbumin with gluten also polymerized faster and to a larger
352 extent than expected based on observations for the isolated proteins (Figures 3.B and 3.C). After 6

353 min of heating, more gliadin was incorporated in the protein network of mixtures of gluten with (S-
354)ovalbumin than with BSA (Figures 2.2A and 2.2B). The ovalbumin co-protein effect with gluten was
355 more pronounced than that of BSA (Figures 3.A, 3.B and 3.C). Gliadin lacks free SH groups. As a
356 result, the presence of other proteins with free SH groups can initiate SH-SS exchange reactions and
357 thereby substantially enhance protein cross-linking. The free SH content of (S-)ovalbumin-gluten
358 mixtures was lower than expected based on observations for the isolated proteins after 6 min of
359 heating (Table 3) implying increased SH oxidation in the mixture. A negative co-protein effect was
360 noted for the lysozyme/gluten mixture. The extractability loss of lysozyme-gluten mixtures was lower
361 than expected based on the isolated proteins (Figure 3.D). After 6 min of heating, more free SH
362 groups were accessible in this mixture with gluten than expected based on the isolated proteins
363 (Table 3), and gliadin was not yet incorporated into the protein network (Figure 2.2C). As mixtures of
364 equal weight basis were used, more protein molecules were present in lysozyme-gluten than in the
365 other mixtures. The formation of a continuous protein network evidently requires more covalent
366 cross-linking between smaller than between larger proteins. Thus, besides the absence of free SH
367 groups, the small MW (*ca*. 14 kDa) could explain the negative co-protein effect. Soy glycinin showed
368 no co-protein effect with gluten. The SDS-EP loss during heating was as expected based on that of
369 the isolated protein (Figure 3.E). As soy glycinin has a high MW (*ca.* 360 kDa), the low amount of free
370 SH groups seemed to be the factor limiting the triggering of a co-protein effect with gluten. In
371 contrast with the isolated proteins, gluten contains sufficient intramolecular SS bonds to form a
372 continuous network.

373 We also investigated the occurrence of co-protein effects of complex protein mixtures of one source
374 with gluten. The protein mixtures described in section 3.2 were heated at 100 °C with gluten (ratio
375 1:2 w/w). Both egg white and whole egg showed a positive co-protein effect with gluten (Figures 3.F
376 and 3.G). However, the difference between measured and expected SDS-EP was higher for egg white
377 than for whole egg protein. Egg yolk lipids prevented the yolk proteins from exerting a co-protein
378 effect with gluten (Figure 3.H). Indeed, defatted egg yolk proteins induced a co-protein effect with

379 gluten (Figures 3.I). Furthermore, the measured SDS-EP of whole egg-gluten mixtures equaled the
380 sum of the readings for egg white-gluten and egg yolk-gluten mixtures. Wheat albumin and globulin
381 both had a positive effect on protein network formation (Figures 3.J and 3.K). However, after 120
382 min of heating the polymerization of the mixtures was almost as expected based on the isolated
383 proteins, because ω-gliadin and part of the albumin/globulin remained extractable (results not
384 shown). So, even wheat proteins contribute to each other's polymerization. In all protein mixtures,
385 less LAN was formed after 120 min heating than expected based on its formation in the isolated
386 proteins (Table 3). After 120 min heating at 100 °C, LAL was formed neither in the isolated protein
387 (fractions) nor in their mixtures with gluten. In these mixtures, SS bond formation seemed to be
388 favored above other covalent reactions. While gluten increased the denaturation temperature of BSA
389 largely, the impact of gluten on the denaturation temperatures of the other tested food proteins was
390 less pronounced (results not shown). All globular proteins denatured during heating at 100 °C in
391 mixtures with gluten.

392 To reveal which protein characteristics impacted the co-protein effects, the experimentally
393 determined monomeric particle size, free SH content, cysteine level, denaturation temperature of
394 the largest fraction, ζ-potential and surface hydrophobicity of unheated and heated samples of ten
395 proteins (Tables 1 and 2) were linked to co-protein effects after 120 min of heating in the presence of
396 gluten. Egg yolk was excluded as its lipids affected protein network formation. The resulting model
397 describes whether the inclusion of specific globular proteins would induce a positive, no or negative
398 co-protein effect in a mixture with gluten. Equation 4 was found with an R^2 of 0.79:

399 Co-protein effect$_{120\ min}$ (%) = 85.00 + 0.82 x free SH content (µmol/g) + 0.50 x surface hydrophobicity
400 heated sample [$(mg/ml)^{-1}$] (Equation 4)

401 The free SH content of unheated proteins and the surface hydrophobicity of heat-induced unfolded
402 proteins were the main protein characteristics impacting co-protein effects in mixtures with gluten.
403 Gluten contains little free SH groups. The accessible free SH groups of the added proteins play a key

role because they can initiate SH-SS exchange reactions and thereby trigger the rapid formation of a protein network which involves both gluten and globular proteins. Furthermore, the model demonstrates the importance of non-covalent interactions for the formation of a covalent network in protein mixtures. When both protein types contain hydrophobic protein patches, these attractive forces reduce the distance between proteins and increase the opportunity for SH oxidation and SH-SS exchange reactions.

Applying this model for whey protein isolate, which had a free SH content of 25.4 µmol/g ± 0.3 µmol/g and a surface hydrophobicity value after heating of 19.6 $(mg/ml)^{-1}$ ± 1.5 $(mg/ml)^{-1}$, showed a slightly positive co-protein effect after 120 min of heating at 100 °C of 115.6%. Heating experiments with whey protein isolate and gluten showed an experimental co-protein effect of *ca.* 114.1%. These results confirm the validity of the here developed model to predict co-protein effects when heating globular proteins in combination with gluten in water at 100 °C. Indeed, while this model is based on an observational study with limited number of samples, it still seems a valuable tool to predict heat-induced polymerization of mixtures between wheat gluten on the one hand and any water or salt soluble globular protein on the other hand.

4. Conclusion

This work highlights the importance of some intrinsic protein characteristics for protein network formation, either in absence of other proteins, in the presence of proteins derived from the same source, or in the presence of proteins from a different source. For isolated globular proteins, key roles reside in the presence of free SH groups and SS bonds during heat-induced protein cross-linking. Accessible free SH groups initiate covalent network formation through SH oxidation and SH-SS exchange reactions. The level of monomeric BSA (one free SH group) and ovalbumin (four free SH groups) decreased more rapidly than that of soy glycinin and lysozyme (no free SH groups). However, the polymers formed by (S-)ovalbumin remained largely extractable in SDS-containing medium. It was hypothesized that the small amount of intramolecular SS bonds in ovalbumin hinders the

formation of a continuous covalent protein network. Different proteins from one source can impact each other's polymerization. More polymers which are not extractable in SDS-containing medium are formed in egg white than expected based on ovalbumin alone. Egg white proteins interconnect, which is referred to as a synergistic polymerization behavior. Nevertheless, the extractability in SDS-containing medium of whole egg proteins equaled the sum of that of egg white and egg yolk proteins. In mixtures with gluten, a negative co-protein effect when adding lysozyme, no co-protein effect when adding of soy glycinin and positive co-protein effects with BSA, ovalbumin and S-ovalbumin were noted. The level of accessible free SH groups and the surface hydrophobicity of unfolded proteins were the main protein characteristics determining co-protein effects in mixtures with gluten. These novel insights into protein cross-linking of complex protein mixtures are important in light of the growing demand for sustainable alternatives for globular animal proteins. Predicting the effect of food proteins on each other's covalent network formation will be helpful to develop new food formulations.

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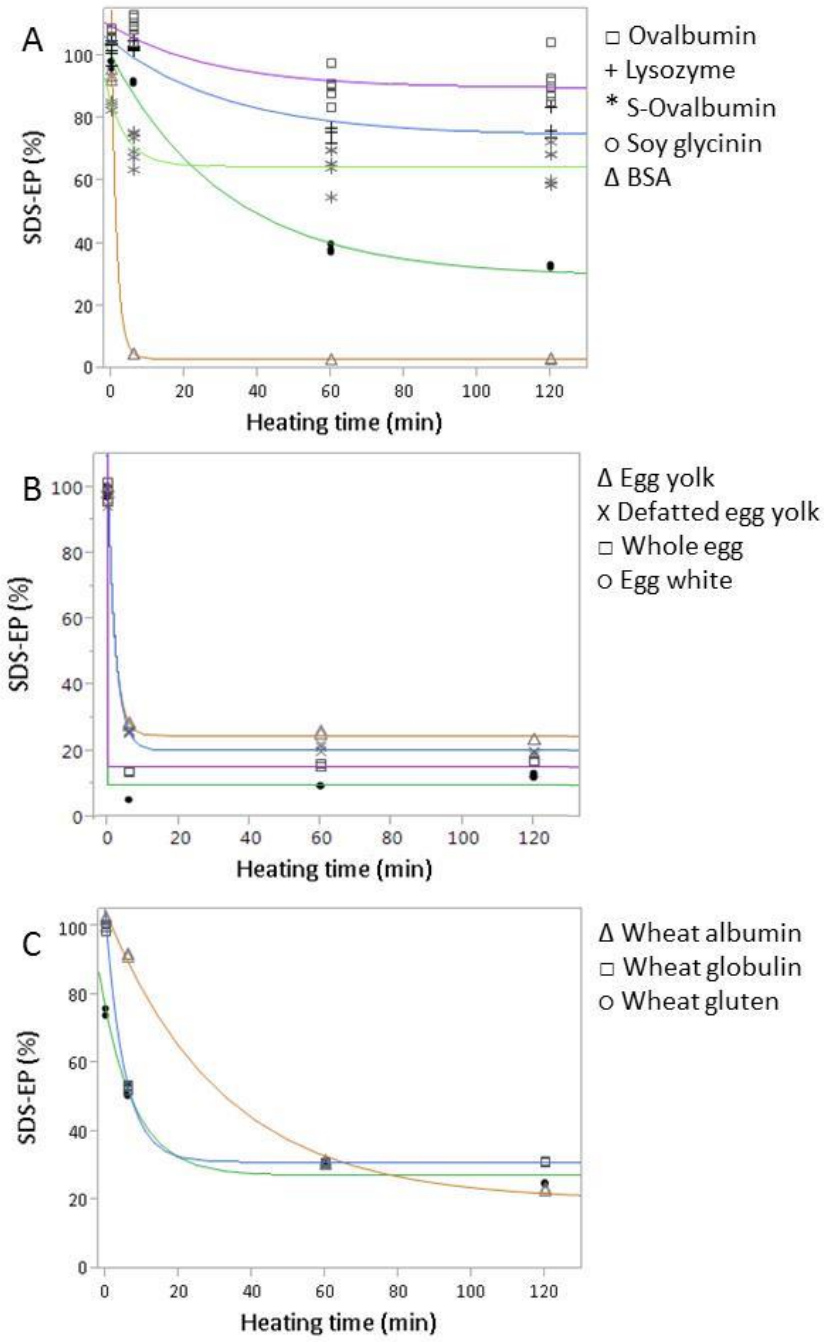
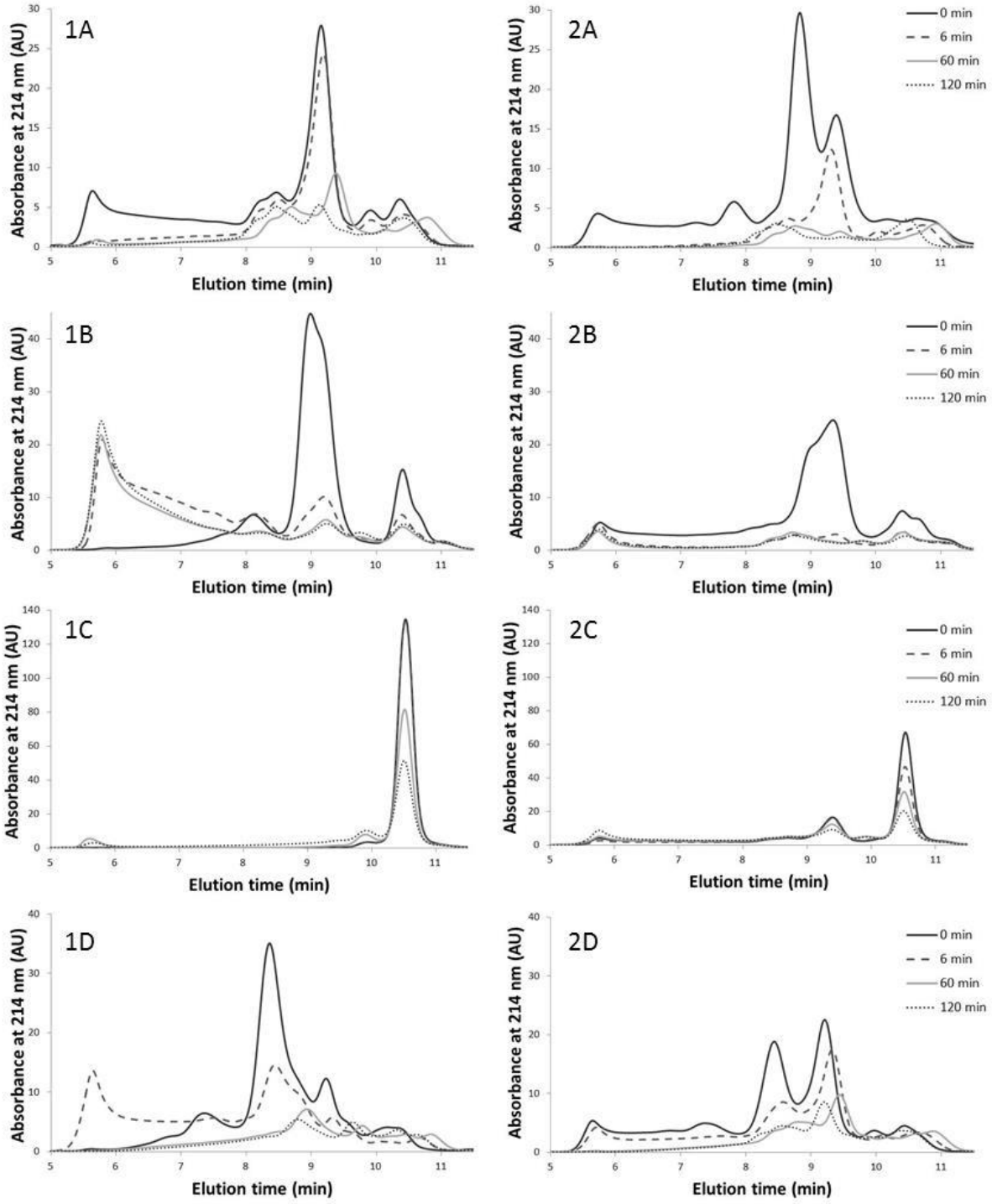
551

552 **Figures**

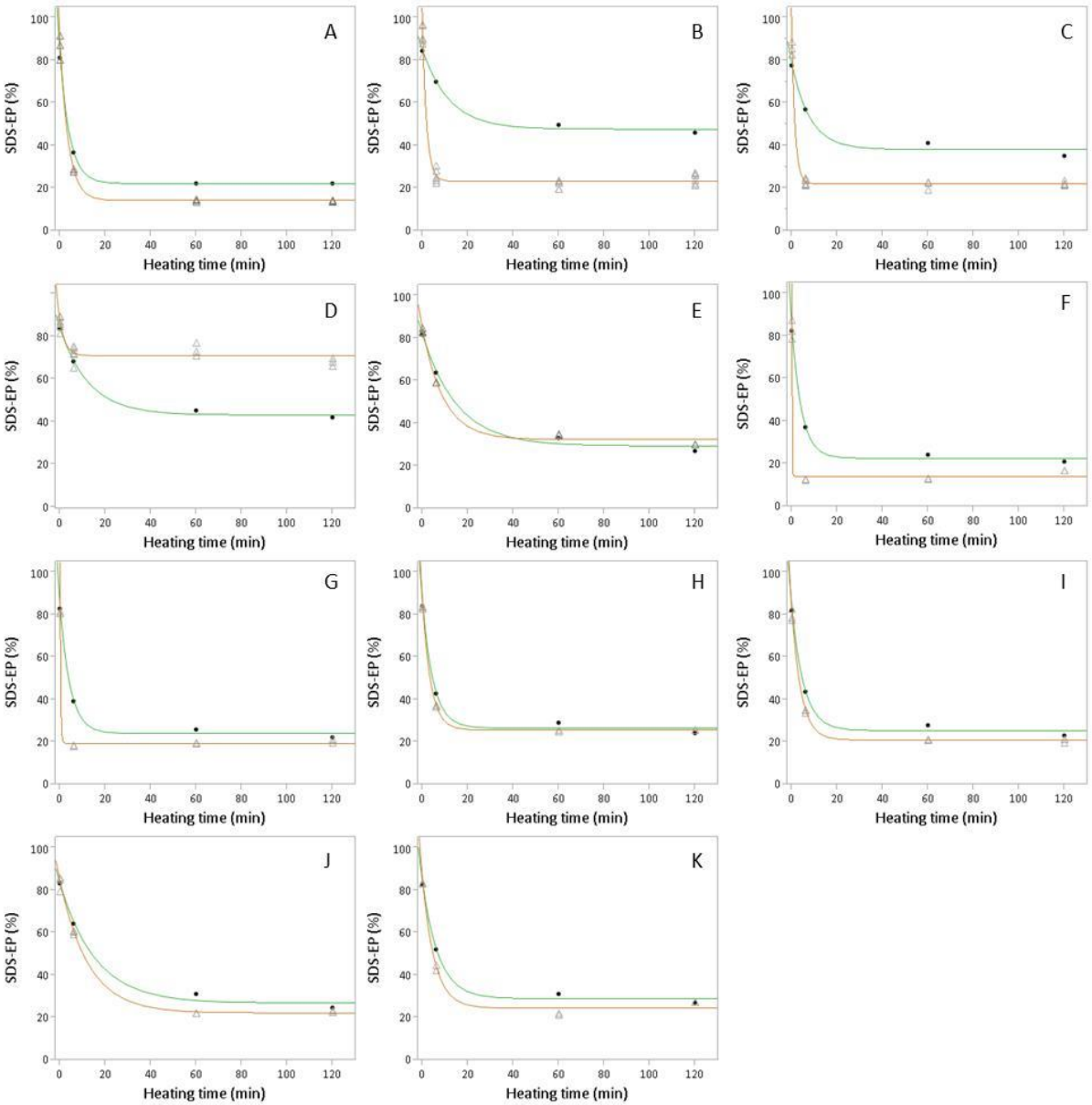


553

554 Figure 1. Protein extractability in sodium dodecyl sulfate containing medium (SDS-EP) of different
555 proteins [A, bovine serum albumin (BSA, Δ), ovalbumin (□), S-ovalbumin (∗), lysozyme (+) and soy
556 glycinin (○)], egg [B, whole egg (□), egg white (○), egg yolk (Δ) and defatted egg yolk (x)] and wheat
557 [C, gluten (○), albumin (Δ) and globulin (□)] fractions heated for various times at 100 °C in water.
558 Trend lines were constructed assuming first-order kinetics.



559
560 Figure 2. SE-HPLC profiles of protein extracted in sodium dodecyl sulfate (SDS) containing medium of
561 different proteins and mixtures thereof with gluten (ratio 1:2 w/w). Gluten (1A), ovalbumin (1B),
562 lysozyme (1C), soy glycinin (1D) and mixtures of gluten with bovine serum albumin (2A), ovalbumin
563 (2B), lysozyme (2C) and soy glycinin (2D) before and after heat treatment at 100 °C for 6, 60 and 120
564 min in water. AU, arbitrary units.



565

566 Figure 3. Protein extractability in sodium dodecyl sulfate containing medium (SDS-EP) of mixtures of
567 gluten with bovine serum albumin (A), ovalbumin (B), S-ovalbumin (C), lysozyme (D), soy glycinin (E),
568 egg white (F), whole egg (G), egg yolk (H), defatted egg yolk (I), wheat albumin (J) and wheat globulin
569 (K). Results (Δ) are compared with values expected based on the isolated proteins (○). Trend lines
570 were constructed assuming first-order kinetics.

571

572 **Tables**

573

574 Table 1. The pH of solutions/dispersions of unheated globular proteins (100.0 mg protein/ml), monomeric particle size, total cysteine contents, peak
575 denaturation temperatures (T_d), ζ-potential and surface hydrophobicity of different food proteins and protein mixtures. Samples were also heated for 60 sec
576 at 100 °C to determine the ζ-potential and surface hydrophobicity.

Proteins	pH	Monomeric particle size (nm)	Cysteine (µmol/g)	T_d (°C)		ζ-potential (mV)		Surface hydrophobicity $(mg/ml)^{-1}$	
				Peak 1	Peak 2	Unheated	Heated	Unheated	Heated
Bovine serum albumin	6.9 (0.1)	7.4 (0.4)	528 (27)	61.9 (0.3)	n.a.	-8.6 (0.1)	-13.1 (2.0)	128.4 (9.8)	109.8 (2.6)
Glycinin	6.3 (0.1)	11.6 (0.2)	112 (8)	96.8 (0.4)	n.a.	-5.6 (1.1)	-10.2 (1.9)	0.2 (0.1)	0.7 (0.2)
Ovalbumin	7.3 (0.1)	5.7 (0.2)	252 (13)	80.2 (0.1)	n.a.	-9.6 (1.7)	-15.8 (1.8)	7.7 (0.7)	88.8 (5.6)
S-ovalbumin	9.5 (0.1)	5.7 (0.4)	150 (24)	91.1 (0.6)	n.a.	-11.5 (0.3)	-13.2 (0.5)	16.9 (2.6)	45.9 (3.8)
Lysozyme	3.5 (0.0)	3.3 (0.0)	724 (14)	73.0 (0.1)	n.a.	9.8 (0.2)	9.8 (2.8)	0.8 (0.1)	1.2 (0.1)
Whole egg	9.1 (0.1)	7.9 (1.7)	265 (52)	67.6 (0.3)	81.6 (0.2)	-12.9 (0.1)	-14.5 (0.8)	6.8 (0.1)	45.9 (0.1)
Egg white	10.0 (0.1)	6.5 (0.4)	238 (4)	66.9 (0.1)	80.5 (0.1)	-13.4 (0.2)	-13.5 (1.9)	6.7 (0.8)	76.1 (1.9)
Defatted egg yolk	5.9 (0.1)	6.9 (1.2)	174 (11)	74.1 (0.7)	n.a.	-6.9 (1.3)	-8.2 (0.3)	1.6 (0.4)	4.6 (0.2)
Wheat albumin	6.0 (0.1)	6.6 (1.6)	322 (15)	67.3 (0.1)	89.1 (0.8)	-1.8 (0.1)	-1.8 (0.3)	5.9 (0.1)	11.1 (0.7)
Wheat globulin	6.1 (0.1)	10.8 (2.1)	353 (6)	64.0 (0.1)	84.3 (0.3)	-2.9 (0.2)	-3.3 (0.7)	5.6 (0.5)	18.3 (0.8)

577 Standard deviations are between brackets.

578 n.a., not applicable

579 Table 2. Free sulfhydryl (SH) contents of proteins in sodium dodecyl sulfate (SDS) and urea containing
580 medium (pH 7.0) heated at 100 °C in water for various times.

Proteins	Free SH content (μmol/g)			
	0 min	6 min	60 min	120 min
Bovine serum albumin	7.7 (0.3)	1.0 (0.2)	1.3 (0.1)	2.1 (0.1)
Ovalbumin	62.0 (1.0)	50.6 (0.5)	40.5 (1.4)	30.5 (1.3)
S-ovalbumin	29.9 (0.6)	15.3 (0.8)	5.3 (0.2)	7.1 (0.3)
Lysozyme	0.0 (0.3)	0.0 (0.2)	0.3 (0.1)	1.1 (0.1)
Soy glycinin	1.4 (0.3)	0.3 (0.1)	0.1 (0.2)	0.0 (0.1)
Whole egg	27.3 (0.8)	1.2 (0.2)	0.6 (0.1)	0.4 (0.1)
Egg white	32.6 (1.3)	0.9 (0.1)	2.0 (0.1)	2.2 (0.1)
Egg yolk	9.4 (0.6)	3.6 (0.3)	1.4 (0.5)	1.5 (0.2)
Defatted egg yolk	11.5 (0.5)	1.0 (0.5)	0.1 (0.4)	0.1 (0.4)
Wheat gluten	2.4 (0.1)	0.0 (0.3)	0.0 (0.2)	0.3 (0.2)
Wheat albumin	6.3 (0.1)	0.0 (0.1)	0.1 (0.1)	0.8 (0.1)
Wheat globulin	2.7 (0.2)	0.0 (0.5)	0.5 (0.1)	0.0 (0.1)

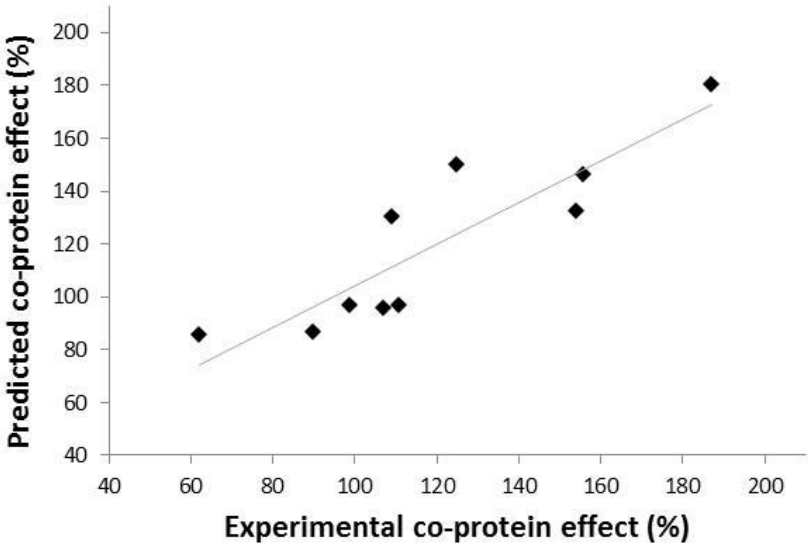
581 Standard deviations are between brackets.

582 Table 3. The pH of solutions/dispersions of unheated globular proteins and gluten (100.0 mg
583 protein/ml). Free sulfhydryl (SH) and lanthionine (LAN) contents of proteins with wheat gluten (ratio
584 1:2 w/w) heated at 100 °C for respectively 6 and 120 min. The expected values are the weight
585 averages of the experimental SH and LAN contents of the corresponding isolated proteins.

Proteins with gluten	pH	Free SH groups (μmol/g)		LAN (μmol/g)	
		Experimental	Expected	Experimental	Expected
Bovine serum albumin	6.9 (0.1)	0.5 (0.0)	0.1 (0.2)	0.0 (0.0)	5.0 (1.1)
Soy glycinin	6.1 (0.1)	0.6 (0.2)	0.0 (0.2)	0.0 (0.0)	0.0 (0.0)
Ovalbumin	6.4 (0.1)	11.8 (0.7)	16.8 (0.3)	4.5 (1.3)	7.7 (0.5)
s-Ovalbumin	8.9 (0.1)	2.7 (0.3)	9.8 (0.3)	8.9 (3.2)	9.4 (5.0)
Lysozyme	4.9 (0.1)	1.7 (1.1)	0.0 (0.2)	0.0 (0.0)	0.0 (0.0)
Whole egg	7.1 (0.1)	4.7 (0.6)	0.2 (0.2)	4.9 (1.2)	12.2 (0.3)
Egg white	8.2 (0.1)	1.7 (0.3)	0.1 (0.2)	26.9 (0.9)	35.0 (2.2)
Egg yolk	6.1 (0.1)	4.1 (1.2)	1.0 (0.2)	0.6 (0.1)	0.8 (0.1)
Defatted egg yolk	5.5 (0.1)	1.0 (0.2)	0.1 (0.3)	1.3 (1.2)	0.7 (0.1)
Wheat albumin	6.0 (0.1)	1.4 (0.4)	0.0 (0.2)	0.0 (0.0)	0.0 (0.0)
Wheat globulin	6.1 (0.1)	0.8 (0.2)	0.0 (0.3)	0.0 (0.0)	0.0 (0.0)

586 Standard deviations are between brackets.

Additional information



Additional information: Goodness of fit between the experimentally determined co-protein effects of globular proteins with gluten after 120 min of heating in water at 100 °C and the predicted co-protein effects based on Equation 4. Linear regression (in grey) revealed an R^2 of 0.79.