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CONDITIONS GOVERNING FOOD PROTEIN AMYLOID FIBRIL FORMATION.

PART II: MILK AND LEGUME PROTEINS

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ABSTRACT

Both intrinsic and extrinsic factors impact amyloid formation of food proteins. We here review the impact of various conditions and food constituents on amyloid fibrillation of milk and legume proteins. Much less is known about casein and legume protein amyloid-like fibril (AF) formation than about that of whey proteins such as β -lactoglobulin, α -lactalbumin and bovine serum albumin. Proteins of both sources are often studied after heating under strong acidic ($\text{pH} < 3$) conditions. The latter induces changes in protein conformation and often peptide hydrolysis. At higher pH values, alcohols, chaotropic and/or reducing agents induce the conformational changes required to enhance fibrillation. Different types of food proteins can impact each other's fibrillation. Also, the presence of other food constituents can enhance or reduce it. No general conclusions on the mechanisms or impact of different food constituents on food proteins can be made. Optimal conditions for AF formation, *i.e.* heating for several days at low pH, are rare in food processing. However, this does not exclude the possibility of AF formation in food products. For example, slow cooking of hydrolyzed proteins may enhance it. Future research should focus on the prevalence of AFs in complex food systems or model systems relevant for food processing.

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41 **Keywords:** amyloids, fibers, β -lactoglobulin, soy, bean

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75 **1 INTRODUCTION**

76 A major challenge for the food industry with respect to proteins is to replace animal by
77 more sustainable plant-based proteins and/or to reduce the level of the former in food
78 products. A route hereto is to improve the functionality of such animal and plant-based
79 proteins, most preferably by applying ‘clean label’ approaches. Aggregation of food
80 proteins into fibrillar structures holds much promise as they can increase viscosity, act as
81 gelling agent and/or stabilize foams or emulsions. Based on a review of the literature, it has
82 most recently been hypothesized that food-derived amyloid fibrils (AFs) likely have
83 minimal impact on human health (Jansens, Rombouts, et al., 2019).

84

As summarized for egg proteins by Jansens et al. (2019), AFs are typically formed by nucleated aggregation process under acidic conditions. Monomeric proteins or peptides thereof associate and form amylogenic oligomers (Figure 1). These oligomers and further monomers or peptides associate and elongate into protofibrils (Hill, Robinson, Matthews, & Muschol, 2009) or protofilaments (Adamcik & Mezzenga, 2012). After gaining some length, protofibrils further self-assemble into mature AFs (Hill et al., 2009). They laterally associate and align prior to twisting into mature AFs (Adamcik & Mezzenga, 2012). In some cases, the growth of protofibrils ends when loops are formed (Kalapothakis et al., 2015). The amino acid sequences which are frequently involved in fibrillation and which actually promote the formation of β -sheet structures are called core or amylogenic regions (Jansens, Rombouts, et al., 2019). Such core regions are usually short sequences (5 to 15 amino acids) with a high hydrophobicity and low net charge (De Baets, Schymkowitz, & Rousseau, 2014). Identified and predicted core regions in food proteins and their importance in aggregation have been reviewed by Jansens et al. (2019). Both intrinsic (amino acid composition, hydrophobicity, etc.) and extrinsic (temperature, time, pH, ionic strength, shear flow, etc.) factors impact the level and morphology of specific food protein aggregates as well as the exact aggregation mechanism (Jansens, Lambrecht, et al., 2019). Knowledge on core regions of food proteins and the impact of processing thereupon is of key importance to create new functionalities for proteins in food (Jansens, Rombouts, et al., 2019).

In industrial practice, legume derived products such as soymilk undergo processing steps such as homogenization, heat-treatments, or fermentation to make them resemble cow's

milk or fermented dairy-type products. Over the last years, the interest in and market of plant-based milk substitutes has increased. However, many of these products are still not accepted by mainstream Western consumers (Mäkinen, Wanhalinna, Zannini, & Arendt, 2016). Controlled protein aggregation and, more specifically, fibrillation holds much promise to increase protein techno-functionality (*e.g.* gelling, emulsifying and foaming properties) in food systems. The latter may help developing legume-based alternatives which mimic the structure and texture of popular products. Furthermore, new knowledge on protein fibrillation may increase the functionality of milk proteins in order to reduce their levels in food products or to use them in new applications. In this manuscript, the formation of milk and legume protein AFs under a variety of processing conditions are reviewed.

2 BOVINE MILK PROTEINS

Milk proteins are divided into two groups: caseins and whey proteins. Whey proteins are co-products of cheese production (Smithers, 2008). The major whey proteins are β -lactoglobulin (LG, 51%), α -lactalbumin (LA, 19%), immunoglobulins (11%) and bovine serum albumin (BSA) (6%) (Raikos, 2010; Wijayanti, Bansal, & Deeth, 2014).

2.1 β -Lactoglobulin

Monomers of β -LG (*ca.* 18 kDa, 162 amino acids) are stable at pH values below 3.5 and exceeding pH 7.5 (Belitz, Grosch, & Schieberle, 2009). β -LG usually appears as dimers at pH values exceeding its isoelectric point (pI) 5.4 (de la Fuente, Singh, & Hemar, 2002). Reduction of the ionic strength or heating induces the dissociation of dimers into monomers (Aymard, Durand, & Nicolai, 1996). Heating aqueous dispersions of β -LG at different pH values can lead to spherical particles, flexible strands or semi-flexible fibrils (Figure 2)

(Amagliani & Schmitt, 2017). We here discuss the impact of pH and processing conditions on the level and morphology of fibrils is discussed.

2.1.1 Fibril formation under strongly acidic conditions (pH < 2.5)

Heating β -LG at low pH increases its level of intermolecular β -sheets (Kavanagh, Clark, & Ross-Murphy, 2000; Lefevre & Subirade, 2000; Oboroceanu, Wang, Brodkorb, Magner, & Auty, 2010). Fibrillar aggregates with lengths of several micrometers have been detected after heating at low pH in the absence of salts (Akkermans et al., 2006; Aymard, Nicolai, Durand, & Clark, 1999; Veerman, Ruis, Sagis, & van der Linden, 2002). Such aggregates bind Congo red and thioflavin T (ThT) and the fibrils show the specific amyloid X-ray pattern (with reflections at 4.6 Å and 10.6 Å) (Bromley, Krebs, & Donald, 2005). β -LG fibrils typically consist of protofilaments that are laterally aligned and twisted along the long axis resulting in a twisted ribbon structure (Adamcik et al., 2010; Adamcik & Mezzenga, 2011; Bolisetty, Adamcik, & Mezzenga, 2011). The development of fluorescence is typically preceded by a lag phase, after which its intensity rapidly increases in a growth phase. It ends with a stationary phase (Bromley et al., 2005; Loveday et al., 2010). Increasing the heating temperature shortens the lag phase. The drop of ThT fluorescence during prolonged heating at 110 °C (> 3 h 18 min) and 120 °C (> 5 h) has been attributed to fibril breakdown and/or local gelation (Loveday, Wang, Rao, Anema, & Singh, 2012). Fibrils formed by heating at 80°C for up to a few hours disintegrate during slow cooling, whereas those formed by heating for longer periods remain intact during cooling (Arnaudov, de Vries, Ippel, & van Mierlo, 2003). Heating at pH values below 2.0 accelerates fibril formation during the growth phase, but has little effect on the lag phase (Loveday et al., 2010). Genetic variability in β -LG (A, B and C) reflected by differences in

some amino acid residues affects neither fibril formation kinetics nor their morphology (Dave, Loveday, Anema, Jameson, & Singh, 2015).

2.1.1.1 The impact of peptide bond hydrolysis on fibril formation

Heating β -LG at 85 °C and pH 2.0 for 20 h induces peptide bond cleavage, especially before and after aspartic acid residues. Some of the resulting peptides with a molecular weight between 2 kDa and 8 kDa form AFs (Akkermans, Venema, van der Goot, Gruppen, et al., 2008; Oboroceanu et al., 2010). None of these fibrils contain β -LG residues 64 to 96. In contrast, its residues 1 to 11 (LIVTQMKGLD) are consistently present and residues 12 to 33 are frequently present in the fibrils. Whether or not peptides are present in fibrils depends on their hydrophobicity, net charge, charge distribution and β -sheet forming capacity (Akkermans, Venema, van der Goot, Gruppen, et al., 2008). Peptide bond hydrolysis typically precedes fibril formation upon heating under acidic conditions (Kroes-Nijboer, Venema, Bouman, & van der Linden, 2011), but does not seem to be a prerequisite to fibril formation (Loveday, Wang, et al., 2012). While some discuss the presence of monomers in fibrillar aggregates obtained by heating β -LG at low pH for relatively short times (Jung & Mezzenga, 2010; Oboroceanu et al., 2010; Schokker, Singh, Pinder, & Creamer, 2000), peptide hydrolysis seems to occur rapidly with conventional heating (Dave et al., 2013). However, monomers and large peptides have been found in amyloid-like β -LG fibrils after microwave heating at 80 °C and pH 2.0 (Hettiarachchi, Melton, Gerrard, & Loveday, 2012). Fibrils can also be formed at room temperature and pH 2.0 from a peptide mixture obtained by hydrolysis of β -LG with AspN endoproteinase (Akkermans, Venema, van der Goot, Boom, & van der Linden, 2008). Ghadami et al. (2011) suggested that some of the lysine residues in β -LG are of critical importance in the aggregation process.

2.1.1.2 Fibril morphology

The contour and the persistence length of fibrils decreases with increasing β -LG concentration (measured at concentrations between 3.0 and 7.5%). Short, worm-like fibrils formed at high β -LG concentrations have a lower β -sheet content than the long straight fibrils formed at low protein concentrations (vandenAkker, Engel, Velikov, Bonn, & Koenderink, 2011). The critical protein concentration below which no significant fibril formation occurs (*ca.* 2.5% in absence of salt) decreases with increasing ionic strength (Arnaudov & de Vries, 2006; Arnaudov et al., 2003) and does not depend on temperature in a 70 to 100 °C interval (Kroes-Nijboer, Venema, Bouman, & van der Linden, 2009). Kitchen salt accelerates fibril formation at pH 2.0 (Arnaudov & de Vries, 2007; Bromley et al., 2005). It only affects the growth phase whereas calcium chloride affects both the lag and growth phases (Loveday et al., 2010; Loveday, Wang, Rao, Anema, & Singh, 2011). The persistence length of β -LG fibrils formed by heating at 80 °C and pH 2.0 decreases with increasing ionic strength (Aymard et al., 1999; Loveday et al., 2010). At high ionic strength the formed fibrils are short and curvy whereas those formed at low ionic strength are long and straight (Arnaudov & de Vries, 2006; Kavanagh et al., 2000; Veerman et al., 2002). The aggregates are entirely held together by non-covalent bonds (Schokker et al., 2000). Lara et al. (2011) reported the formation of single filament structures in combination with few giant multi-stranded twisted and helical ribbons upon incubation of β -LG at pH 2.0 and 90°C for 30 hours. At pH 2.0 and 90°C, peptides of β -LG start to fibrillate before they reach final fragmentation by hydrolysis. The relative large size of the peptides limit the growth of single filament structures into multi-stranded structures (Lara et al., 2011).

2.1.1.3 The impact of processing on fibril formation

β -LG fibril formation is enhanced by shear flow which generates precursors that act as seed-like initiators. The level of precursors formed increases with shear rate (Hill, Krebs, Goodall, Howlett, & Dunstan, 2006). Both shear pulses and continuous shear enhance fibril formation. Therefore, it seems that mainly the onset of shear flow is important (Akkermans et al., 2006). Not only the rate of fibril formation, also the morphology of the obtained fibrils is affected by shear flow and the flow regime (Dunstan, Hamilton-Brown, Asimakis, Ducker, & Bertolini, 2009; Sharma, Furusawa, Fukui, & Sasaki, 2014).

Microwave heating (80 °C, pH 2.0) results in faster formation of AFs from β -LG than does conventional heating. However, prolonging such treatment causes fibril disintegration. Fibrils induced by microwave and conventional heating have similar secondary structures, but the former consist of larger peptides and show higher surface hydrophobicity. The latter is attributed to the larger peptides which can better extend out of the core structure (Hettiarachchi et al., 2012). Depending on the experimental conditions, microwave heating can result in AFs with different helical pitch, diameter and length. For example, at a given optimal energy influx the length, thickness and uniform characters of the AFs formed depends on the irradiation time interval (λ) (Lee et al., 2015).

2.1.1.4 The impact of food constituents on fibril formation

At β -LG: β -casein ratios exceeding 80:1, β -casein acts as a chaperone and slows down β -LG fibril formation during heating at 80 °C and pH 2.0 in a concentration-independent way (Dave, Loveday, Anema, & Singh, 2016). Prior glycation strongly inhibits β -LG fibril formation with glycation by lactose having a much stronger effect than that with glucose

(Dave, Loveday, Anema, Jameson, & Singh, 2014a). Both glycerol and sorbitol decrease the rate of β -LG self-assembly, but do not affect fibril morphology (Dave, Loveday, Anema, Jameson, & Singh, 2014b). Whereas copper ions (Cu^{2+}) significantly increase the rate of β -LG fibril nucleation without altering fibril morphology (Zappone, De Santo, Labate, Rizzuti, & Guzzi, 2013), ferric ions (Fe^{3+}) inhibit its fibrillation at high temperature (Guzzi, Rizzuti, Labate, Zappone, & De Santo, 2015). Preformed β -LG AFs reduce ferric to ferrous (Fe^{2+}) ions better than native β -LG but less effective than its non-fibril peptides. In addition, preformed β -LG AFs can be used as a carrier for iron nanoparticles which increase the bioavailability of the latter in rats (Shen et al., 2017).

2.1.2 Fibril formation at higher pH (pH $\geq$ 4.0)

The formation of β -LG fibrils at pH values of at least 7.0 has been reported, but always in the presence of denaturing agents (Hamada & Dobson, 2002; Rasmussen et al., 2007).

2.1.2.1 The impact of chaotropic agents on fibril formation

At pH 7.0, fibril formation from β -LG can be induced by its incubation in 3.0 to 5.0 M urea for 10 to 30 days. A number of peptides corresponding to individual β -strands of native β -LG also readily form amyloid-like aggregates in the presence of urea. Addition of these preformed fibrils to solutions of disulfide (SS)-reduced β -LG containing urea accelerates fibril formation by a seeding process which results in the absence of a lag phase (Hamada & Dobson, 2002). Preformed fibrils corresponding to only one of the β -strands of β -LG (β A) even promote fibril formation of intact β -LG in the presence of urea. Based on these results, it has been suggested that most regions of high intrinsic aggregation propensity only give rise to aggregation when at least partial unfolding takes place (Hamada et al., 2009). Rasmussen et al. (2007) reported that prolonged exposure (> 90 days) of β -LG to sub-

denaturing concentrations of either urea (5.0 M) or potassium thiocyanate (2.0 M) results in fibrils which are mainly stabilized by intermolecular SS bonds or hydrophobic interactions, respectively. The pathway for β -LG fibril formation in the presence of 3.0 to 5.0 M urea differs from that in water at pH 2.0 (Loveday, Wang, et al., 2012).

2.1.2.2 The impact of aqueous alcohol on fibril formation

Fibrillar β -LG structures are also formed during heating at 80 °C in aqueous alcohols such as those of methanol, ethanol and propanol at pH 2.0 or trifluoroethanol at pH 7.0 (Gosal, Clark, Pudney, & Ross-Murphy, 2002; Gosal, Clark, & Ross-Murphy, 2004). Fibrils formed during heating in water at 90 °C and pH 2.0 disassemble and then reassemble into a different type of fibril when incubated in aqueous ethanol (Jordens, Adamcik, Amar-Yuli, & Mezzenga, 2011). The presence of acetonitrile and benzyl alcohol at neutral pH can also induce amyloid-like structures (Pal, Maity, Sardar, Chakraborty, & Halder, 2016).

2.1.2.3 The impact of (food) constituents on fibril formation and stability

Increasing concentrations of the macromolecular crowding agents dextran 70 and polyethylene glycol accelerate AF formation of β -LG at neutral pH in the presence of guanidine hydrochloride. However, they can reduce fibril yield and alter fibril morphology (Ma, Xie, Wei, & Li, 2013). Whereas aluminum (Al^{3+}) and zinc (Zn^{2+}) ions catalyze fibrillation of β -LG at pH 7.0 and 70 °C, complexation of curcumin with these ions inhibits its fibrillation (Pal, Maity, Sardar, Parvej, et al., 2016). The aroma compound β -ionone inhibits β -LG fibril formation in the presence of urea in a concentration-dependent fashion (Ma, You, & Lu, 2014). Under certain conditions, addition of chitosan or polyethylenimine

265 to dispersions of β -LG fibrils increases their stability upon pH increases from 2.0 to up to
266 7.0 (Gilbert, Campanella, & Jones, 2014).

267 **2.2 α -Lactalbumin**

268 At acidic pH, α -LA (*ca.* 14 kDa, 123 amino acids) is present in a molten globule state
269 (Ku wajima, 1996). Its denaturation temperature is *ca.* 66 °C (Wijayanti et al., 2014) and
270 removal of the calcium ion from its structure reduces its thermal stability (Veprintsev et al.,
271 1997; Wijayanti et al., 2014).

272 **2.2.1 Fibril formation under strongly acidic conditions (pH < 2.5)**

273 Incubation of α -LA solutions in the presence of kitchen salt at pH 2.0 at 37 °C yields
274 fibrillar structures with high β -sheet content. These structures display ThT fluorescence.
275 The lag phase during fibril formation at low pH can be drastically reduced by increasing
276 salt concentration (Goers, Permyakov, Permyakov, Uversky, & Fink, 2002).

277 **2.2.1.1 The impact of peptide bond hydrolysis on fibril formation**

278 α -LA of which either (i) the peptide bond between residues 40 and 41 has been hydrolyzed
279 (nicked α -LA) or (ii) a chain segment of 12 amino acid residues (residues 41-52) located at
280 the level of the β -subdomain of the native protein has been hydrolytically removed (gapped
281 α -LA) aggregates much faster at pH 2.0 than the intact protein and forms long and well-
282 ordered fibrils. Furthermore, these α -LA derivatives can also form regular fibrils at neutral
283 pH whereas native α -LA does not. Thus, protein hydrolysis can enhance AF formation and
284 cause protein aggregation and fibrillation (de Laureto et al., 2005).

2.2.1.2 The impact of food constituents on fibril formation

Glutathione, crocin and safranal can inhibit amyloid formation of calcium ion depleted α -LA at acidic pH (Ebrahim-Habibi, Amininasab, Ebrahim-Habibi, Sabbaghian, & Nemat-Gorgani, 2010; Wang, Liu, Wen, & Wang, 2011).

2.2.2 Fibril formation at higher pH (pH $\geq$ 4.0)

Yang et al. (2006) reported that AFs of calcium-ion-depleted α -LA are formed when decreasing the pH of their solutions to pH 4.5. AFs of the latter were not observed in a pH 5.0 to 7.0 interval (Yang et al., 2006). Incubating such α -LA at neutral pH and 60 °C for 15 h yields fibrillar aggregates (Kumar, Kumar, & Prabhu, 2015). As further discussed, fibril formation of α -LA at neutral pH is usually studied in the presence of denaturing agents, SS reducing agents or enzymes.

2.2.2.1 The impact of disulfide reducing and denaturing agents on fibril formation

Incubation at 37 °C and pH 7.4 (without denaturing agent) of S-(carboxymethyl)- α -LA (a disordered form of the protein with three out of four SS bridges reduced) (Goers et al., 2002) yields fibrillar structures. So does that of α -LA subjected to the SS reducing agent dithiothreitol (Ghahghaei, Divsalar, & Faridi, 2010).

Addition of low concentrations (< 0.2 mM) of sodium dodecyl sulfate (SDS) nearly doubles the lag time upon incubation at neutral pH and 60 °C whereas the fibril elongation rate remains unaltered. At this concentration, SDS can neutralize the charge of α -LA without exposure of hydrophobic groups. At SDS concentrations exceeding 0.2 mM but below micellar concentrations, the lag time is up to *ca.* 60 times higher and fibril elongation is accelerated 3- to 6-fold. Here, hydrophobic groups of α -LA are exposed which interact

with SDS. Probably, semi protein-SDS micellar aggregates are formed which delay nucleation. At concentrations exceeding 2 mM SDS, α -LA fibril formation is inhibited and amorphous aggregates are formed (Kumar et al., 2015).

2.2.2.2 The impact of peptide hydrolysis on fibril formation

Partial enzymatic hydrolysis of α -LA at neutral pH and 50 °C can result in formation of AFs (Ipsen, Otte, & Qvist, 2001; Otte, Ipsen, Bauer, Bjerrum, & Waninge, 2005). The predominant fragments in such fibrils have a molecular mass of 8.8 or 9.8 kDa and are respectively identified as the sequences 26-37 or 26-47 of α -LA linked to sequence 50-113 via a SS bond (Otte et al., 2005; Otte, Ipsen, Ladefoged, & Sorensen, 2004). Self-assembly of peptidase-induced α -LA nanotubes is possible over a broad pH range (pH 4-7.5). These nanotubes consist of ten fibrils which align in a right-handed twisted tubular structure with an outer and inner diameter of *ca.* 20 and *ca.* 8 nm, respectively. A decrease in pH dramatically decreases the hydrolysis rate, whereas the self-assembly process is stimulated (Geng, Bjerrum, Arleth, Otte, & Ipsen, 2016). The concentration of α -LA has a major influence on the morphology of the obtained aggregates (Ipsen & Otte, 2007; Otte et al., 2005). At protein concentrations lower than 30 g/L fibrillar aggregates are formed, whereas nanotubes are formed at higher concentrations. These nanotubes are formed by nucleated polymerization and are stabilized by intermolecular calcium ion bridges (Ipsen & Otte, 2007). The presence of calcium ions accelerates self-assembly of hydrolyzed α -LA in nanotubes. However, higher concentrations thereof favor the formation of random aggregates (Graveland-Bikker, Ipsen, Otte, & de Kruif, 2004). Further information on α -LA nanotubes can be found in the review of Graveland-Bikker and de Kruif (2006).

2.2.2.3 The impact of food constituents on fibril formation

ThT fluorescence measurements have shown that the rate and extent of amyloid formation of α -LA at neutral pH in the presence of dithiothreitol can be increased with the crowding agent dextran. In contrast, α -casein, α -crystallin and arginine can reduce the extent of its fibril formation (Ghahghaei et al., 2010; Ghahghaei & Mohammadian, 2014). Oleic acid completely inhibits fibril formation at pH 4.5, but does not have such effect at pH 2.0 or 3.0 (Yang et al., 2006).

2.3 Bovine serum albumin

BSA (*ca.* 66 kDa, 582 amino acids, pI 4.7) contains one free sulfhydryl group and 17 SS bonds. Its denaturation temperature in water is *ca.* 56 °C (Michnik, 2003). The single polypeptide of BSA is composed of three similar domains each of which is formed by six helices (Borzova et al., 2016; Hirayama, Akashi, Furuya, & Fukuhara, 1990). The intramolecular SS bonds provide rigidity to each domain but do not prevent the structure from changing under different environmental conditions (*e.g.* pH, ionic strength and heat). Seven pH-dependent BSA isomeric forms with differences in α -helical content exist. For example, at pH < 4.0, BSA reversibly loses its α -helical structure and adopts an ‘extended’ conformation (Curvale, Masuelli, & Padilla, 2008) of much higher length (26.7 nm) than that of the ‘normal’ compact state (8.3 nm) at pH 4 to 9 (Jachimska & Pajor, 2012). Studies of the conformational changes and aggregation of BSA in the presence of D₂O have revealed that repulsion between proteins (at pD far from pI) enhances the formation of small ordered aggregates containing β -sheet structures. More disordered and larger aggregates of BSA containing more random coil structures are formed at pD values close to the pI (Militello et al., 2004).

2.3.1 Fibril formation under strongly acidic conditions (pH ≤ 3.0)

Amyloid formation during incubation of BSA solutions *inter alia* depends on its concentration, pH, temperature and exposure time. A high level of Congo red absorbance, which indicates the presence AFs, is found when heating 5.0 mg/ml BSA for 48 h at 72 °C and pH 3.0 (Arasteh, Habibi-Rezaei, Ebrahim-Habibi, & Moosavi-Movahedi, 2012). Also the rate of amyloid formation, measured by ThT fluorescence, during heating at 65 °C and pH 3.0 is higher than that with similar heating at other pH values (pH 1.6, 7, 9 and 10.5) (Bhattacharya, Jain, & Mukhopadhyay, 2011). The critical protein concentration required for BSA self-assembly decreases with increasing ionic strength (between 0.20 and 0.30 M) (Veerman, Sagis, Heck, & van der Linden, 2003). When heating BSA solutions of different pH, independently of protein concentration, fibril formation proceeds without a lag phase. Thus, its fibril formation cannot be described solely by the classical nucleation mechanism. Presumably, at room temperature, BSA already partially unfolds into a molten globule state with exposure of hydrophobic regions at low pH and protein concentrations. Increasing the protein concentration or ionic strength results in formation of molten oligomers. In these oligomers, β -sheet structures are formed at 65°C. The latter can associate and form AFs (Bhattacharya et al., 2011).

Heating at 90 °C and pH 2.0 both denatures and hydrolyzes BSA. After 40 h under these conditions, fragments smaller than 6.5 kDa are formed. Already after 6 h, relatively short and flexible fibrils occur. Until up to 40 h of incubation, their lengths and numbers grow. From that point in time onwards, they coexist with rigid fibrils. Two subtypes of left-handed twisted flexible fibrils (thin and thick) and four subtypes of rigid fibrils (thin and thick both with or without right-handed helical ribbons) can be distinguished. Probably, one

flexible fibril transforms in a rigid fibril without helical ribbons while two flexible fibrils wind together and form a rigid right-handed twisted ribbon (Usov, Adamcik, & Mezzenga, 2013a). Dilution and storage for 7 days at 4 °C does not change the morphologies of these fibrils, suggesting irreversible fibrillation of BSA. In contrast, Veerman et al. (2003) reported that BSA fibrils formed after heating for 1 h at 60 °C and pH 2.0 are converted back to monomers upon dilution. Whether or not BSA fibril formation is irreversible upon subsequent dilution very likely depends on the incubation conditions. Prolonged heating (100 and 145 h) at pH 2.0 and 90 °C further increases the length of the fibrils. Stirring the resultant large fibrils causes them to entangle and aggregate (Usov, Adamcik, & Mezzenga, 2013b).

2.3.2 Fibril formation at higher pH (pH ≥ 4.0)

Amyloid-like aggregates are formed when BSA is heated (60-80 °C) at neutral pH. The obtained worm-like aggregates bind ThT and display the typical amyloid X-ray fiber diffraction pattern (Holm et al., 2007; Pearce, Mackintosh, & Gerrard, 2007). Depending on the temperature, fibril formation occurs over time frame of up to several hours without a lag phase and is accompanied by an increase in β -sheet structures (Holm et al., 2007). Amyloid formation during heating at 80 °C at pH values close to 7.0 is slower for bovine than for human serum albumin. Not only the secondary structure of the final aggregates but also the size and the quantity of AFs differ between these two proteins even if they have 88% sequence identity (de la Arada, Seiler, & Mantele, 2012). Even at room temperature, oligomerization of BSA takes place at pH 7.4 at a concentration of 150 μ M. This oligomerization process is concentration dependent and involves the formation of β -sheet structures which bind ThT. The oligomers formed under such conditions dissociate into

their native monomeric state upon dilution (Bhattacharya, Prajapati, Chatterjee, & Mukherjee, 2014).

At pH values close to the pI (4.7) of BSA, disordered amorphous aggregates are formed during heating at 60 °C. Under these conditions, non-specific (*e.g.* hydrophobic) interactions are favored and no changes in secondary structure occur (Vetri, Librizzi, Leone, & Militello, 2007). At pH values exceeding its pI, ionic repulsive interactions can cause BSA aggregation to proceed in a more ordered way. Under such conditions, small aggregates are formed which contain intermolecular β -sheet structures (Militello et al., 2004). At pH 8.9, repulsion between proteins slows down their aggregation and favors formation of cross- β sheet structures. The formation of protofibrils starts with the assembly of destabilized monomers through transition of α -helical to β -sheet structures. At low BSA concentrations, the aggregation mechanism resembles diffusion limited aggregation of polymers without nucleation occurring. At intermediate pH values, multiple mechanisms impact protein aggregation leading to a mixture of amorphous and fibrillary aggregates (Vetri et al., 2011).

Sonication of BSA solutions (pH 7.8) yields amyloid-like aggregates which appear very stable and do not re-dissolve within a timeframe of a few weeks. Furthermore, the sonication-induced aggregates seem to act as seeds when native proteins are incubated with them (Stathopoulos et al., 2004). However, seeding with preformed amyloid-like BSA aggregates subjected to sonication in order to disperse them and to expose growing ends appears unsuccessful (Holm et al., 2007).

2.3.2.1 The impact of disulfide reducing and denaturing agents on fibril formation

BSA at a concentration of 40 μ M does not aggregate when incubated at 37 °C and pH 7.2. However, when its intramolecular SS bonds are first reduced, it forms non-fibrillar or amorphous aggregates which display ThT fluorescence lower than that of mature AFs. Indeed, in some instances, amorphous aggregates bind ThT and display fluorescence. ThT can probably bind to the hydrophobic pockets of amorphous BSA aggregates and thereby give rise to some fluorescence (Yang, Dutta, & Tiwari, 2015).

Fibers can also be formed by drying BSA solutions under a laminar flow, especially at pH values close to the protein's pI and in the presence of sodium sulfate and dithiothreitol. Removing water surrounding proteins makes them unfold and subsequently aggregate at the water/air interface. Cross-linking of the formed fibers by formaldehyde or glutaraldehyde improves their mechanical properties. The cross-linked fibers can be twisted into yarns with mechanical properties similar to those of silk (Wu, Wang, Buschle-Diller, & Liles, 2013).

Eluting BSA on a Superdex-200 column with 25 mM Tris-HCl buffer pH 8.0 containing 1.0 mM EDTA, 0.1 M salt and 0.05% SDS induces the formation of fibrillar structures (Huang, Liang, Chu, & Liang, 2009). In the presence of guanidinium hydrochloride, the aggregation of BSA is delayed and the mechanism changes from downhill to nucleated polymerization. Electrostatic repulsion between guanidinium-bound BSA molecules increases the activation energy to induce aggregation (Saha & Deep, 2014).

2.3.2.2 The impact of food constituents on fibril formation

In heated mixed protein systems, proteins from different sources can impact each other's polymerization (Lambrecht, Deleu, Rombouts, & Delcour, 2018). During heating at 70°C and pH 7.4, mixtures of BSA, insulin and/or lysozyme aggregate and form amyloid-like fibrils. The aggregation of the protein mixtures is faster than that of the individual proteins and no lag phase is observed. Adding preformed AFs of BSA, insulin or lysozyme to solutions of the different proteins prior to heating enhances aggregation (as evidenced by ThT fluorescence measurements) during heating. Amyloid-like aggregation in the tested protein mixtures is faster than that when adding seeds to monomers of a same protein. The driving force for co-aggregation and cross-seeding seems to depend on the reactivity of the partially denatured proteins rather than on sequence similarities (Dubey, Anand, Temgire, & Kar, 2014). The amino acid sequences of the core regions of fibrils formed in mixed protein systems were not further characterized. Whether different protein types participated in the formation of a single fibril remains unknown. Also, A β ₂₅₋₃₅, a fragment of the amyloid β -peptide which is present in the form of senile plaques in the brain of patients suffering from Alzheimer's disease, can act as seed and accelerate BSA fibril formation at 37 °C (Ferdousi et al., 2016).

Sodium salicylate and sodium deoxycholate reduce the rate of BSA fibrillation when added prior to heating. They impact the secondary but not the tertiary structure of preformed BSA fibrils (Dey et al., 2015). Cuprous (Cu²⁺) and zinc (Zn²⁺) ions impact the conformation of BSA but not its denaturation temperature. Zinc in contrast to cuprous ions enhance BSA aggregate growth (Navarra, Tinti, Leone, Militello, & Torreggiani, 2009). Glycation of BSA with D-ribose but not with D-xylose or D-glucose enhances the formation of globular

amyloid-like fibrils (Wei, Chen, Chen, Ge, & He, 2009). Ionic liquid surfactants impact the self-assembly of BSA in aqueous media. The ester group of 1-dodecyl-3-methylimidazolium ester chloride easily forms hydrogen bonds and enhances the formation of right handed twisted BSA AFs (Singh & Kang, 2015). While glycine betaine promotes fibrillation of BSA, other osmolytes suppress its aggregation. Hydroxyproline is more efficient at suppressing BSA fibrillation than sarcosine and proline (Dasgupta & Kishore, 2017). Eugenol suppresses both spontaneous and seed-induced fibrillation of BSA (Dubey, Anand, Shekhawat, & Kar, 2017).

2.4 Casein

Bovine casein is a mixture α_{s1} -casein (*ca.* 24 kDa), α_{s2} -casein (*ca.* 25 kDa), κ -casein (*ca.* 19 kDa) and β -casein (*ca.* 24 kDa). The latter can be degraded by endogenous milk peptidases into γ -casein (*ca.* 12-21 kDa). Each type of casein has various genetic variants. Casein does not denature as it lacks tertiary structure (Belitz et al., 2009). Holt et al. (2013) and Thorn et al. (2015) reviewed the structure of casein and its AFs. At physiological pH and temperature, α_{s2} - (Thorn, Ecroyd, Sunde, Poon, & Carver, 2008) and κ -casein (Farrell, Cooke, Wickham, Piotrowski, & Hoagland, 2003; Leonil et al., 2008; Thorn et al., 2005) do fibrillate. Lencki (2007) suggested that short fibril-like structures similar in length (7 to 10 nm) to protofibrils occur in casein micelles. However, there is no clear evidence that milk contains AFs. Even though all four casein types exhibit propensity to self-associate (Holt et al., 2013), no mention has been made of α_{s1} - and β -casein fibrillation at neutral pH. Furthermore, none of the predicted amyloid sequences of bovine casein are fully conserved in sequences of casein from other species (Holt, 2016).

2.4.1 Fibril formation under strongly acidic conditions (pH ≤ 3.0)

While β- and κ-casein form amyloid-like fibrils when heated at 90 °C and pH 2.0 for 48 h, α-casein does not. During the first 12 h already, hydrolysis of proteins into peptides smaller than 10 kDa occurs. Acid hydrolysis of β- and κ-casein limits the rate at which they form fibrils (Pan & Zhong, 2015).

2.4.2 Fibril formation at higher pH (pH ≥ 4.0)

Thorn *et al.* (2005) suggested that monomeric κ-casein aggregates into AFs after several hours of incubation at pH 7.0 and 37 °C. Oxidation of its two methionine residues into methionine sulfoxide also increases the rate of its fibrillation (Koudelka, Dehle, Musgrave, Hoffmann, & Carver, 2012). Amyloid formation from α_{s2}-casein at 37°C is optimal at pH 6.5-6.7. In a 25 to 50 °C temperature interval, the rate of fibril formation increases with temperature. Under physiological conditions, α_{s2}-casein forms twisted, ribbon-like fibrils (*ca.* 12 nm in diameter) with, occasionally, loop structures (Thorn et al., 2008). The SS-linked dimer of α_{s2}-casein is more susceptible to amyloid formation than α_{s2}-casein itself (Thorn et al., 2015).

2.4.2.1 The impact of disulfide reducing and carboxymethylating agents on fibril formation

κ-Casein normally occurs as trimers or larger aggregates (Belitz et al., 2009). However, reduction of its SS bonds causes it to be mainly present as monomers (Thorn et al., 2005). Monomeric κ-casein shows higher propensity to form fibrils than its dimeric or trimeric forms. Multimeric κ-casein even inhibits its fibril formation (Ecroyd, Thorn, Liu, & Carver, 2010).

Reduced and carboxymethylated κ -casein (hereafter referred to as κ -casein derivative) at 37 °C easily forms fibrillar structures and spherical particles (Farrell et al., 2003). κ -Casein derivative seems to be in equilibrium with its micellar aggregate at pH 8.0 and 37 °C (Leonil et al., 2008). According to Leonil *et al.* (2008), under such conditions conformational rearrangement of the protein in these micelles limits the rate of κ -casein derivative fibrillation. Nevertheless, when present in concentrations lower than its critical micelle concentration it also forms fibrillary structures (Ecroyd et al., 2010). Probably, the dissociation of oligomeric micelles into monomeric κ -casein derivative limits the rate of amyloid fibrillation (Ecroyd et al., 2010; Liu et al., 2016). The rate of fibril formation of κ -casein derivative does not change by seeding with preformed fibrils. Its residues 25-86 are incorporated in the AF cores formed at 37 °C and pH 7.2 (Ecroyd et al., 2008).

2.4.2.2 The impact of food constituents on fibril formation

AF formation of reduced and native κ -casein is hindered by adding either 8-anilinonaphtalene-1-sulfonic acid (ANS) or α_s - and β -casein. They all reduce the degree to which hydrophobic interactions occur (Thorn et al., 2005). The presence of α_{s1} - and β -casein induces a lag phase and decreases the rate of κ -casein derivative fibrillation at pH 8.0 and 37 °C (Leonil et al., 2008). In the presence of β -casein, reduced κ -casein forms fibrils faster than does native κ -casein. In such mixtures, fibrils from native κ -casein are shorter and more flexible than those from reduced κ -casein (Portnaya et al., 2016). Much as is the case for κ -casein, fibrillation of α_{s2} -casein is inhibited by α_{s1} -casein and partially by β -casein (Thorn et al., 2008). Casein proteins can also inhibit amyloid formation of other proteins. This for example is the case with ovalbumin, carbonic anhydrase (Khodarahmi, Beyrami, & Soori, 2008) or β -peptide involved in Alzheimer's disease (Carrotta et al.,

2012; Ghahghaei & Shahraki, 2016). Casein proteins can act as chaperones which prevent partially unfolded globular proteins from aggregating and precipitating (*e.g.* α -LA and β -LG). Casein proteins have extensive regions of little order which can facilitate their interaction with a broad range of destabilized proteins (Morgan, Treweek, Lindner, Price, & Carver, 2005). The chaperone action of casein has been reviewed by Holt *et al.* (2013). While calcium ions, lactose or heparin sulfate have little – if any – impact on the kinetics of κ -casein derivative fibrillation under physiological conditions, some lipids and crowding agents do. Longer-chain phosphatidylcholine lipids enhance its fibrillation whereas various crowding agents reduce the extent of its fibrillation (Liu et al., 2016). Also flavonoids with a high degree of hydroxylation and molecular planarity inhibit its fibrillation (Carver et al., 2010). Furthermore, ginsenosides from *Panax ginseng* seem to inhibit fibrillation of reduced κ -casein (Liu et al., 2015).

2.5 Mixtures of milk proteins

2.5.1 Whey proteins

Fibrils can also be produced by heating whey protein concentrate (WPC) and whey protein isolate (WPI) at low pH (Akkermans, van der Goot, Venema, van der Linden, & Boom, 2008; Bolder, Hendrickx, Sagis, & van der Linden, 2006; Gao, Xu, Ju, & Zhao, 2013; Loveday, Su, Rao, Anema, & Singht, 2012; Munialo, de Jongh, Broersen, van der Linden, & Martin, 2013). WPI (between 1.0 and 6.0% protein) forms fibrils and spherulites when heated at 80 °C and pH 2.0. Since pure α -LA and pure BSA do not form fibrils when heating for 10 h under such conditions, fibrils made from WPI presumably consist of β -LG (Bolder et al., 2006). Whey fibrils formed by heating at 90 °C and pH 2.0 for 20 h are not

stable when changing the pH. When increasing it from 2.0 to 8.0, the fibrils become shorter and at pH 5.0 to 7.0 clusters are observed (Akkermans, van der Goot, et al., 2008).

In contrast to what is the case with unstirred samples, AF formation in stirred samples does not benefit from seeding. More fibrils of a similar length are formed when a WPI solution is stirred while heating than when it is not. Stirring likely breaks up immature fibrils thereby producing more fibrils which then accelerate the rate of fibrillation (Bolder, Sagis, Venema, & van der Linden, 2007a, 2007b). Fibril formation by heating WPI solutions of pH 2.0 at 80 °C is accelerated by sodium or calcium chloride addition. Straight fibrils are obtained in absence of calcium chloride, whereas highly flexible, curly fibrils are obtained in the presence of 80 mM calcium chloride (Loveday, Su, Rao, Anema, & Singh, 2011). The type of salt has no marked effects on fibril morphology (Loveday, Su, Rao, Anema, & Singh, 2012).

Freeze-drying breaks straight WPI fibrils into short rods whereas curly fibrils encounter little damage. Both freeze-dried straight and curly fibrils can be used to seed fresh WPI solutions (Loveday, Su, Rao, Anema, & Singht, 2012). Pretreatment of WPC with trypsin, protease A, pepsin or protease M to bring about a low degree of hydrolysis (0.1-0.3%) affects fibril formation during heating at 90 °C and pH 2.0. Fibril formation of trypsin treated WPC is faster than that of native WPC and yields higher levels of long fibrils. Pretreatment with protease A, pepsin and protease M slows down the aggregation and yields low levels of curved fibrils (Gao et al., 2013).

2.5.2 Mixtures of casein and other milk proteins

In raw milk already 18% α_{s2} -casein, 25% β -LG and 46% κ -casein are present in SS linked aggregates. These levels further increase during heating (Chevalier & Kelly, 2010).

Noteworthy is that isolated β -LG and κ -casein co-aggregate into AFs during heating at 94 °C and pH 6.7 for 1 h. Even though these two proteins are structurally unrelated, they have sufficient sequence similarity which allows them to co-fibrillate (Raynes, Day, Crepin, Horrocks, & Carver, 2017). While β -casein inhibits fibril formation of β -LG during heating at temperatures exceeding 75 °C at neutral pH, it does not prevent fibrillation during heating at 80 °C and pH 2.0. When β -casein is heated with β -LG, fibril assembly is retarded and twisted irregular fibrils coexist with long semi-flexible fibrils (Dave et al., 2016).

3 LEGUME PROTEINS

Most literature on amyloid-like aggregation of legume proteins deal with those of soy. Few papers also report fibril formation by pea and kidney bean proteins. Recently, Amagliani and Schmitt (2017) reviewed the aggregation and fibrillation of soy and pea proteins and their relation with foaming and emulsifying properties.

3.1 Soy proteins

The main storage proteins of soy are β -conglycinin (*ca.* 200 kDa) and glycinin (*ca.* 360 kDa). They occur in ratios ranging from 0.5 to 1.3 (Nishinari, Fang, Guo, & Phillips, 2014). Each glycinin subunit consists of an acidic (pI *ca.* 5) and a basic (pI *ca.* 8.2) polypeptide linked by a single SS bond (Belitz et al., 2009; Staswick, Hermodson, & Nielsen, 1984). Under physiological conditions, glycinin subunits form a compact hexameric complex (Draper & Catsimpoolas, 1978) which at pH 3.8 and/or low ionic strength dissociates in two trimeric complexes (Lakemond, de Jongh, Hessing, Gruppen, & Voragen, 2000). The three major subunits of conglycinin (α , α' , and β) are held together mainly by hydrogen bonds and hydrophobic interactions (Singh, Meena, Kumar, Dubey, & Hassan, 2015).

Amyloid-like aggregation has been investigated for soy glycinin, β -conglycinin and soy protein isolate (SPI). The latter is a highly refined form of soy protein with a minimum protein content of 90% and is produced from defatted soy flour (Singh, Kumar, Sabapathy, & Bawa, 2008).

3.1.1 Fibril formation under strongly acidic conditions (pH < 2.5)

Heating soy glycinin or SPI (10, 20 or 40 g protein/L) at 85 °C and pH 2.0 induces the formation of long fibrillar semi-flexible aggregates with β -sheet structures (Akkermans et al., 2007; Wan, Yang, & Sagis, 2016). Higher protein concentrations (*e.g.* 40 g protein/L) as well as longer heating times (*e.g.* 20 h) seem to yield more fibrils. Exposing soy glycinin solutions to shear flow while heating for 20 h at 85 °C and pH 2.0 does not significantly impact fibril formation (Akkermans et al., 2007). However, it does seem to enhance fibril formation when short heating times (2 h) are applied. The amyloid-like fibrils formed by heating at 85 °C and pH 2.0 have contour lengths of *ca.* 1 μ m, persistence lengths of 2.3 μ m, and are a few nanometers thick.

Comparison of fibrillar aggregation at 80 °C and pH 2.0 of soy β -conglycinin, glycinin, and a 1:1 mixture thereof suggests that the former has a much higher potential to form heat-induced fibrillar aggregates than the latter (Tang & Wang, 2010). This has been ascribed to differences between the two proteins in terms of conformational changes and the extent of polypeptide hydrolysis upon heating. The different fibrillation kinetics and morphologies of α , α' , and β subunits of β -conglycinin are ascribed to differences in their primary structures (Wang, Yang, et al., 2011). Increasing the salt concentration (0-300 mM NaCl) increases the level and length of β -conglycinin fibrils obtained by heating at 80 °C and pH 2.0 for 2 h to 16 h. These higher salt concentrations favor the extent of β -sheet formation and

disruption of tertiary and quaternary structures (Tang, Wang, & Huang, 2012). When β -conglycinin is selectively hydrolyzed and removed from SPI by dialysis, the fibrils formed by heating the resulting SPI preparation for 60 min at 95 °C and pH 2.0 are long and semi-flexible instead of short and worm-like (Xia et al., 2017). The acidic subunits of glycinin form thin, long and semi-flexible fibrils when heated for 20 h at 95 °C and pH 2.0 in contrast to the unordered amorphous aggregates of the basic subunits of glycinin formed under similar conditions. β -Conglycinin enhances and soy glycinin and its basic subunits hinder fibrillation of the acidic subunits of glycinin. The glycinin basic subunits probably inhibit the transition of α -helices into β -strands of the acidic subunits (Dong, Xu, Li, Cheng, & Zhang, 2016).

Heating soy proteins at 80 °C and pH 2.0 for up to 16 h causes peptide bond hydrolysis (Tang & Wang, 2010). However, under such conditions, the extent hydrolysis considerably varies among various soy globulins. Polypeptides of β -conglycinin are generally more readily hydrolyzed than those of glycinin. Moreover, the α and α' subunits of β -conglycinin are more easily hydrolyzed than the β subunit, whereas in glycinin, the acidic subunits are more susceptible to hydrolysis than the basic subunits. Next to hydrolysis, heat-induced conformational changes largely determine the potential of soy proteins to assemble into highly ordered fibrils (Tang & Wang, 2010). Wang *et al.* (2011) have come to similar conclusions. They related the distinct hydrolysis rates and cleavage sites of three subunits of β -conglycinin with the kinetics of fibril formation and the resultant morphology of amyloid structures.

3.2 Pea proteins

Pea protein (pI *ca.* 4.5) consists mainly of albumins and globulins. Much as what is the case in soy, the main storage proteins are 7S (vicilin and convicilin) and 11S (legumin) globulins. The latter occur as a hexameric complex in which each subunit contains a basic and an acidic polypeptide linked by an SS bond. Vicilin (*ca.* 150 kDa) and convicilin (*ca.* 210 kDa) form trimeric homomeric complexes. Heteromeric trimers composed of vicilin and convicilin also occur (Tzitzikas, Vincken, de Groot, Gruppen, & Visser, 2006).

3.2.1 Fibril formation under strongly acidic conditions (pH < 2.5)

Micrometer-long fibrillar aggregates are observed after heating pea protein solutions at pH 2.0 for 20 h (Munialo, Martin, van der Linden, & de Jongh, 2014). Such heat treatment releases peptides from all pea proteins. About 50% of the released peptides assemble into fibrils. Structural changes observed by circular dichroism, transmission electron microscopy and particle size analysis and concomitant increases in ThT fluorescence suggest the formation of amyloid-like structures. The pea protein fibrils are curly with a worm-like appearance which is comparable to that of the soy β -conglycinin fibrils prepared at pH 2.0 and 80 °C, but distinct from that of the β -LG fibrils. Whereas similarities between pea protein and soy β -conglycinin fibrils are related to similarities in the way they thermally aggregate, differences in morphology of pea protein and β -LG fibrils are attributed to inherent differences between plant storage and animal proteins as well as to differences in their quaternary structure and solubility. Gels prepared from pea and soy protein fibrils are weaker than those derived from whey protein fibrils (Munialo et al., 2014).

3.3 Kidney, mung and red bean proteins

As in pea, the 7S and 8S globulins (vicilins) and 11S globulins (legumins) are the major bean storage proteins (Liu & Tang, 2013).

3.3.1 Fibril formation under strongly acidic conditions (pH < 2.5)

Heating a 1.0% (w/v) kidney bean vicilin solution of pH 1.6-2.0 at 80-85 °C for 15 min to 22 h at an ionic strength not exceeding 20 mM induces AF formation (Lassé et al., 2016; Tang, Zhang, Wen, & Huang, 2010). The fibrils formed by incubation at pH 2.0 and 85 °C for 15 min to 12 h are similar, but their ThT fluorescence and mean contour lengths increase with heating time (Tang et al., 2010). Heating for 15 min decreases the β -strand content from 39 to 23%, while further heating increases it. Gel electrophoresis has indicated that heating at low pH induces AF formation but also disrupts the kidney bean vicilin polypeptides as it releases fragments smaller than 10 kDa. A clear link between peptide bond hydrolysis and AF formation of vicilin has to the best of our knowledge not been demonstrated. However, both the disruption of polypeptides and conformational changes at secondary, tertiary, and quaternary structural levels have been suggested to play a key role in AF formation (Tang et al., 2010). Vicilin from mung bean has respectively a higher or lower ability than that from red or kidney bean to form fibrils during heating at 85 °C and pH 2.0 for 1 to 24 h. The yield of fibrils from all these vicilin types increases with protein concentrations in a 0.25-1.00% (w/v) range but their size remains unaffected. While the diameter of kidney bean vicilin fibrils increases with the salt concentrations in a 0-200 mM range, this is not the case for such fibrils from red or mung beans (Liu & Tang, 2013).

4 DISCUSSION AND PERSPECTIVES

Taken together, the myriad of examples discussed here and elsewhere clearly indicate that food proteins are no exception to the observation that 98% of the proteome harbors at least one amyloid nucleating region (Rousseau, Serrano, & Schymkowitz, 2006). Formulated differently, given the right conditions that will expose said aggregation prone sequences, almost any protein will undergo amyloid-like aggregation (Dobson, 2003), whether they are food-borne or not. Conditions that favor aggregation thus usually require the destabilization of the folded native state, but if any general rule can be extracted harsher conditions tend to favor less ordered systems than milder denaturing conditions, which tend to favor the highly ordered amyloid fibrils, whose techno-functional properties are thought to be attractive for food applications. Hence, as summarized by Jansens *et al.* (2019), different factors such as heating mode, time, temperature, pH, moisture content, protein concentration, shear or the presence of alcohols, chaotropic/reducing agents, enzymes, salt and/or other food constituents govern fibrillation of food proteins in model systems. Especially amyloid(-like) fibrillation of egg and milk proteins has been extensively studied by heating these proteins at high temperatures (e.g. 80-95 °C) and acidic pH (≤ 2.5) for several hours to days. By applying such conditions on ovalbumin and β -LG, Lara et al. (2011) showed next to protofibrils of different length scales even the formation of multi-stranded mature AF. What remains unclear is whether food processing induces amyloid formation in real food systems: These systems are typically more complex and more concentrated (lower water content) than the dilute solutions under which ‘optimal fibrils’ are usually obtained of purified proteins. On the one hand this molecular crowding effect, in conjunction with the application of strongly denaturing conditions such as cooking

temperatures, would seem to favor a lower degree of order, and the net effect of all the non-protein constituents is impossible to predict. On the other hand the presence of amyloid fibrils has convincingly been demonstrated in foie gras (Solomon et al., 2007) and X-reflection patterns compatible with the cross-beta structure of amyloid were observed in stretched films of poached egg white already in the 1930s (Astbury, Dickinson, & Bailey, 1935). These cases suggest that in fact, during common processing steps of widespread food products, amyloid fibrils could be formed to a significant degree.

Food processing involves a combination of unit operations (*e.g.* pasteurization, sterilization, fermentation, filtration etc.) to achieve intended changes in food products. During food processing changes in temperature, acidity, shear and/or moisture content are often applied and constituents such as enzymes, salt, sugars and/or preservatives are added. Processing can, depending on the applied conditions, impact the structure of food proteins and their interactions and/or reactions (Belitz et al., 2009). Heating at higher pH values (≥ 4.0) for several minutes is relevant for food processing (*e.g.* cooking of rice, peas, beans or pasta, milk pasteurization/sterilization, baking of cake or bread). Heating (ca. 60-80 °C) ovalbumin (Tufail et al., 2015) or BSA (Holm et al., 2007) at neutral pH for several hours to days yields amyloid-like aggregates. At higher pH (≥ 4.0) values, fibrillation of these and other proteins such as β -LG, lysozyme and wheat proteins generally have been studied in the presence of denaturing or reducing agents. Again, what this implies for common food products in terms of the relative content of oligomers, protofibrils and mature amyloid fibrils remains unclear, but a complex mixture of all three would seem to be the most likely.

Taking into account potential heat-induced interactions and reactions between food proteins (Lambrecht et al., 2018) and the conditions for their fibrillation, the initiation phase of protein aggregation can be linked to their preferential aggregation structure (Figure 3). While heating at moderate pH often induces (partial) denaturation, the use of peptidases or heating at low pH also (partially) hydrolyzes food proteins into peptides. Processing can seize the energy barriers needed to partially unfold and aggregate proteins (Figure 4). The resulting protein structures can aggregate further at different rates and into different morphologies. Both non-covalent interactions and covalent cross-links can initiate rapid protein aggregation. Full unfolding of proteins exposes various functional groups which seems to result mostly in amorphous aggregates. Protein AF formation is a slow process which typically involves nucleated polymerization. The exposure of protein amylogenic regions is crucial for it to occur. Indeed, such protein segments assemble into the characteristic cross- β structure and promote amyloid formation. In this regard, we expect more mature amyloid fibrils starting from peptides rather than from nicked proteins (Figure 3). We are thus in line with Lara et al. (2011). They discussed that more mature AFs are formed with ovalbumin than β -LG after heating for 30 h at 90 °C and pH 2 as the larger size of the β -LG peptides limits its growth. Non-amylogenic sequences in nicked or partially denatured proteins may also initiate other types of aggregation thereby hindering amyloid fibrillation (Figure 3). In such cases, the formation of a combination of amorphous aggregates with amylogenic oligomers and/or protofibrils can be expected. In typical food processing, extensive hydrolysis of proteins by enzymes or heating in acidic ($\text{pH} \leq 2.5$) is rare. Therefore, it is likely that in common food products amylogenic oligomers and/or protofibrils co-occur with amorphous protein structures.

759

760 To investigate whether AFs are present in complex food matrices, researchers need a
761 strategy to analyze ‘branched’ fibrils entrapped in a protein network.
762 Insoluble/unextractable AFs hamper fibril analysis. We see high potential in chemical or
763 enzymatic ‘shaving’ of these ‘branched’ fibrils for studying the presence of AFs in complex
764 structures and food products. The core regions of AFs are thermodynamically very stable,
765 often more than native proteins or amorphous aggregates (Figure 4) (Hartl, Bracher, &
766 Hayer-Hartl, 2011). The compact and stable cross β -sheet structures of AFs are more
767 resistant to proteolytic cleavage than other protein structures (Harrison, Sharpe, Singh, &
768 Fairlie, 2007). Adding limited amounts of enzymes or chaotropic/reducing agents may
769 fragment amorphous protein aggregates prior to impacting protofibrils or mature AFs.
770 Amylogenic oligomers are less ordered and less stable than AFs (Figure 4) and are
771 correlated with the exposure of sticky, hydrophobic surfaces and accessible sequences
772 (Hartl et al., 2011). These reactive oligomers are probably affected by applying this
773 strategy. Knowledge on the actual presence or absence of AFs and their reactive species in
774 common food products will help estimating long-term health implications.

775

776 AFs have interesting gelling, emulsifying, foaming or interfacial properties (Jansens,
777 Rombouts, et al., 2019). However, also in this field most research has been limited to
778 fibrillation of food proteins under extreme processing conditions (low pH, long heating
779 times, use of reducing and denaturing agents). The work performed has shown the potential
780 of AFs as techno-functional quality improver but the used conditions are not easily
781 applicable in or relevant for common food processing. As discussed above, a wide range of

strategies can be applied to induce amyloid formation in food. In essence, these involve exposing the amylogenic regions such that amyloid formation is privileged over amorphous aggregation. A better understanding of this process is key to tailoring the fibril formation process. While free amylogenic peptides seem to associate easily into unbranched AFs, nicked or partially denatured proteins usually result in a mixture of branched fibrils and amorphous aggregates (Figure 3). Strategies to enhance AF formation involve seeding, the application of shear and the optimal selection of incubation conditions. The latter can include slow cooking, *i.e.* heating for several hours at 80-95°C, of peptidase-treated food proteins. As reviewed by Jansens et al. (2019), especially the concept of seeding seems promising if one wishes to accelerate the fibrillation process. Nevertheless, successful seeding has only been realized for a handful of food proteins and none have been reported in complex food systems. Another strategy to exploit the techno-functionality of AFs in food is to include AFs prepared under harsh conditions in recipes. Nevertheless, the question remains whether preformed AFs can resist different unit operations in food production.

CONCLUSIONS

Food protein AF formation is most frequently induced by heating under acidic conditions. Such conditions typically result in peptide bond hydrolysis and the release of amylogenic peptides which can form cross β -sheet structures.

Fibril formation from β -LG can take place at higher pH values when alcohols or chaotropic agents induce conformational changes. Fibrils of α -LA or BSA can be formed under acidic conditions or in the presence of denaturing agents. In addition, moderate heating of BSA at

neutral pH also results in fibril formation. Whey proteins (isolated or in a mixture) seem to be more apt than caseins to form AFs under various conditions. While κ -casein readily forms amyloids in a broad pH range, the other types of caseins have limited – if any – tendency to form such structures.

Different milk proteins can impact each other's fibril formation. Fibril formation of legume proteins has only been tested by heating under acidic conditions. Such conditions are optimal for fibril formation but seldom used in food processing.

ABBREVIATIONS

Amyloid fibril (AF), α -lactalbumin (α -LA), β -lactoglobulin (β -LG), bovine serum albumin (BSA), isoelectric point (pI), thioflavin t (ThT), disulfide (SS), sodium dodecyl sulfate (SDS), 8-anilinonaphtalene-1-sulfonic acid (ANS), whey protein isolate (WPI), whey protein concentrate (WPC), soy protein isolate (SPI)

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AUTHORS CONTRIBUTIONS

M.A. Lambrecht, K.A. Jansens and I. Rombouts collected the references and wrote the manuscript. M.A. Lambrecht made the figures. K.Brijs K, F. Rousseau F, J. Schymkowitz and J.A. Delcour corrected and reviewed the manuscript.

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Figure 1. Schematic overview of protein amyloid fibril formation. Monomeric proteins or peptides thereof associate and form amylogenic oligomers. These oligomers and further monomers associate and elongate into protofibrils. The latter can laterally associate and thereby form mature fibrils. The growth of protofibrils ends when loops are formed.

Figure 2. Transmission electron microscopy images of 10 g/L β-LG dispersions heated at 85 °C for 15 min (2 h for pH 2.0) at different pH values. Scale bars are 0.5 μm except for pH 2.0 (0.1 μm) and pH 7.0 (0.2 μm). Reproduced from Amagliani and Schmitt et al. (2017).

Random α-Helices β-Sheet Cross β-sheet

Figure 3. Schematic representation of the link between initiation and the preferential type of aggregation of food proteins during processing. The (partial) denaturation and/or hydrolysis of globular proteins initiate(s) the aggregation of proteins. Fully denatured proteins mainly aggregate in amorphous clusters while peptides fibrillate more easily. Both fibrillar and amorphous aggregates can be formed from nicked or partially denatured proteins.

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1417 Figure 4. Funnel-shaped free-energy surface of proteins and their aggregates. By changes in
 1418 intramolecular contacts native proteins are converted in partially (un)folded states. Proteins
 1419 have to overcome various energy barriers to unfold or aggregate. Chaperones help other
 1420 protein to overcome these barriers to acquire a specific conformation. Amyloid fibrils are
 1421 more stable than amorphous aggregates or even native proteins. Reprinted from Hartl et al.
 1422 (2011).