CONDITIONS GOVERNING FOOD PROTEIN AMYLOID FIBRIL FORMATION. PART I: EGG AND CEREAL PROTEINS

Koen J.A. Jansens[a,c], Marlies A. Lambrecht[a,*], Ine Rombouts[a,d], Margarita Monge Morera[a], Kristof Brijs[a], Frederic Rousseau[b], Joost Schymkowitz[b] and Jan A. Delcour[a]

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

[b] Switch Laboratory, VIB, B-3000 Leuven, Belgium, and KU Leuven, Department of Cellular and Molecular Medicine, B-3000 Leuven, Belgium

Current working addresses:

[c] Nutrex NV, Achterstenhoek 5, B-2275 Lille, Belgium

[d] KU Leuven, ECOVO, Kasteelpark Arenberg 21, B-3001 Leuven, Belgium

[*]**Corresponding author**: Tel.: +32 16 376781; fax: +32 16 321997.

E-mail address: marlies.lambrecht@kuleuven.be

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ABSTRACT

Conditions including heating mode, time, temperature, pH, moisture and protein concentration, shear, and the presence of alcohols, chaotropic/reducing agents, enzymes and/or salt influence amyloid fibril (AF) formation as they can affect the accessibility of amino acid sequences prone to aggregate. As some conditions applied on model protein resemble conditions in food processing unit operations, we here hypothesize that food processing can lead to formation of protein AFs with a compact cross β-sheet structure. This paper reviews conditions and food constituents that affect amyloid fibrillation of egg and cereal proteins. While egg and cereal proteins often coexist in food products, their impact on each other's fibrillation remains unknown. Hen egg ovalbumin and lysozyme form AFs when subjected to moderate heating at acidic pH separately. AFs can also be formed at higher pH, especially in presence of alcohols or chaotropic/reducing agents. Tryptic wheat gluten digests can form fibrillar structures at neutral pH and maize and rice proteins do so in aqueous ethanol or at acidic pH, respectively.

Keywords: amyloids, fibers, ovalbumin, lysozyme, gluten

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61 **1 INTRODUCTION**

The German physician scientist Rudolph Virchow introduced the term amyloid in 1854. Based on a positive iodine staining reaction he falsely identified a macroscopic tissue abnormality as starch and named it amyloid, with the term being derived from the Latin *amylum* or the Greek *amylon*. In reality, the tissue consisted of fibrillary protein deposits ranging in size from nanometers to micrometers, as evidenced by electron microscopy (Sipe & Cohen, 2000). Amyloid fibrils (AFs) have a rigid structure with superior mechanical strength (Schleeger et al., 2013). Their characteristic molecular structure is the cross-β motif (Figure 1). It consists of β-strands stacked perpendicularly to the long axis of the fibrils and linked by inter-strand hydrogen bonds (Sunde et al., 1997). As already reported in 1935 for β-keratin (Astbury, Dickinson, & Bailey, 1935), this structure results in a fiber X-ray diffraction pattern with reflections at 4.7 Å and around 10 Å (Sunde et al., 1997). Furthermore, that amyloid-like aggregates occur is often suggested based on photometric evidence. Indeed, the cross-β structures have high affinity for Congo red and thioflavin T (ThT) with concomitant green birefringence and fluorescence, respectively (Harrison, Sharpe, Singh, & Fairlie, 2007).

Amyloid formation has attracted much attention because of its association with over 30 diseases. These include Alzheimer, Huntington, Parkinson, Creutzfeldt-Jacob, prion disorders, Lou Gehrig's disease and type II diabetes (Harrison et al., 2007). The folding pathways of disease-related amylogenic peptides and proteins and key factors affecting the occurrence and structure of AFs have been recurrently reviewed (Chiti & Dobson, 2006; Eisenberg & Jucker, 2012; Harrison et al., 2007; Rousseau, Schymkowitz, & Serrano, 2006; Sipe & Cohen, 2000). However, many non-disease related globular proteins also

form fibrils under specific experimental conditions, of which some are relevant for food systems and in biotechnology (Lassé, Gerrard, & Pearce, 2012). Amyloid formation by ovalbumin (OVA) (Lara, Gourdin-Bertin, Adamcik, Bolisetty, & Mezzenga, 2012; Tanaka et al., 2011), lysozyme (LYS) (Hill, Robinson, Matthews, & Muschol, 2009; Mishra et al., 2007) and β-lactoglobulin (Hamada et al., 2009; Loveday, Wang, Rao, Anema, & Singh, 2012) has been reported. Furthermore, wheat gluten (Athamneh and Barone, 2009a; Mackintosh et al., 2009; Ridgley and Barone, 2013; Ridgley et al., 2012; Ridgley et al., 2014; Ridgley et al., 2011), maize zein (Erickson et al., 2012) and soy glycinin (Akkermans et al., 2007; Phoon et al., 2013; Tang and Wang, 2010; Wang et al., 2011) can form fibrous β-sheet rich protein networks. Also, functional amyloid structures are ubiquitous in nature (Gebbink, Claessen, Bouma, Dijkhuizen, & Wosten, 2005; Schwartz & Boles, 2013).

AFs have unique techno-functional properties (Jansens et al., 2019). The presence and morphology of fibrillar structures have been related with foaming and emulsifying properties (Blijdenstein, Veerman, & van der Linden, 2004; Kroes-Nijboer, Venema, & van der Linden, 2012). Furthermore, macroscopic gel properties (*e.g.* transparent versus opaque) have been linked to the dominant gel type (fine-stranded versus particulate) and even aggregate morphology (spherical particles, flexible strands, semi-flexible fibrils, and fractal clusters) (Nicolai & Durand, 2013).

AF formation, which seems to be a generic property of proteins and many peptides, holds promise for various (non-)food applications (Dobson, 2003; Eichner & Radford). The mechanism of self-assembly seems protein specific (Dobson, 2003; Humblet-Hua, Sagis, & van der Linden, 2008; Sagis, Veerman, & van der Linden, 2004). Indeed, at least three different mechanisms have been defined for amyloid fibrillation: (i) nucleated

polymerization, (ii) nucleated conformational conversion, and (iii) downhill polymerization (Figure 2) (Eisele et al., 2015).

Amyloid formation under acidic conditions is usually described to be by **nucleated polymerization** (Hill et al., 2009). Typically, an oligomeric nucleus rich in β-sheet structures is formed to which monomers are non-covalently added. For example, hen egg LYS first without nucleation barrier aggregates into small oligomers of uniform size. After a lag period in which a critical threshold concentration of oligomers is reached, protofibril nucleation starts from oligomers. The protofibrils then grow by adding oligomers to their ends. Finally, the protofibrils self-assemble into much longer and stiffer mature fibrils (Hill et al., 2009).

The **nucleated conformational conversion** process is characterized by an equilibrium between monomers and heterogeneously structured oligomers. The rate-limiting step is the conversion of the oligomers into nuclei which further grow into AFs.

The two above processes can be accelerated by **seeding** with nuclei which may or may not contain cross-β-sheet structures (Eisele et al., 2015). These nuclei, *i.e.* seeds, contain amino acid sequences that promote ordered protein aggregation and end up in the fibril core (such sequences are hence referred to as amyloid core peptides). Seeding is particularly interesting since it holds promise for tailoring aggregation and, hence, protein techno-functionality (Jansens et al., 2019).

Seeding apparently does not affect the rate of **downhill aggregation**. In this mode of aggregation, the change in conformation of the native protein is rate-limiting. Once it has happened, aggregation proceeds rapidly and both AFs and amorphous aggregates are formed (Eisele et al., 2015). Fibrillation and amorphous aggregation are mainly determined

by hydrogen bonding and hydrophobic interactions, respectively (Fitzpatrick, Knowles, Waudby, Vendruscolo, & Dobson, 2011). The balance between both interaction types determines the dominance of each type of aggregates (Fitzpatrick et al., 2011; Saha & Deep, 2014).

Chaotropic agents [*e.g.* sodium dodecyl sulfate (SDS)] or alcohols can also induce fibril formation. While native and denatured proteins do not aggregate easily in aqueous environments due to their buried hydrophobic regions, partially unfolded proteins with notable secondary structure are prone to aggregate (Chi, Krishnan, Randolph, & Carpenter, 2003). Alcohols weaken hydrophobic interactions and enhance polar interactions thereby facilitating protein denaturation (Thomas & Dill, 1993). Aso et al. (2007) showed that 25 out of 38 commercially available non-disease-related proteins form fibrils in 5.0% ethanol at pH 2.0 and 57 °C. In 5.0% trifluoroethanol instead of 5.0% ethanol, fibril formation has been observed for 28 proteins. Nicolai and Durand (2013) and Raynes et al. (2014) reviewed the most important parameters that control protein aggregate morphology, including changes in temperature, pressure, shear stress, sonication and hydrolysis. Numerous recent studies have thus increased the understanding of amyloidosis of one or more food proteins. However, while such studies focused on the impact of different conditions and the consequences for protein techno-functionality, an overview of the current knowledge on the topic for specific food proteins is missing.

Many publications describe the conversion of food proteins into fibrillary structures by relatively simple and straightforward manipulations such as altering the pH or salt

concentration or applying heat or shear. In addition, the impact of various food constituents and/or chemicals on food protein fibrillation is often investigated in model systems. Only in particular cases, the formation of AFs from isolated food protein in specific conditions has undisputedly been proven. Current questions are whether AFs are already present in food, whether they can establish interesting techno-functionalities in complex food systems and to what extent they are formed during food processing unit operations. In addition, the resistance and stability of AFs during unit operations and digestion is questioned. To bridge the gap between model systems and food, the conditions and mechanisms governing AF formation of egg and cereal proteins are reviewed. Both protein sources often coexist in cereal-based food products. Examples include cake, cookie, waffle, pasta and noodle systems. During heating, both protein sources impact each other's protein network formation and product quality (Lambrecht, Deleu, Rombouts, & Delcour, 2018). An overview of the current knowledge on amyloid formation of egg and cereal proteins will help further research of food proteins as will an accompanying paper dealing with AF formation from dairy and legume proteins (Lambrecht et al., 2019). Knowledge on the exact conditions and mechanisms of AF formation, their techno-functionality and impact on human health is necessary. The significance of AF formation in food systems for techno-functional properties and nutritional quality has recently been discussed by Jansens et al. (2019). In this review, all conditions that are known to induce fibril formation with hen egg, wheat, maize and rice proteins are listed and the mechanisms and resulting fibril morphology are described. Similar trends in mechanism of fibril formation have been observed in measurements at similar pH values. Therefore, information on various

conditions that impact each protein type is subdivided in fibril formation under strongly acidic conditions (pH ≤ 3.5) or at higher pH values (pH ≥ 4.0).

2 HEN EGG PROTEINS

Hen eggs contain egg white and yolk in a ratio of 2 to 1 on wet weight base. Egg white is an aqueous solution (about 12% solids on wet base), the solids of which are mainly protein (about 88%) (Belitz, Grosch, & Schieberle, 2009). Its major proteins (based on dry matter weight) are 54.0% OVA, 12.0% ovotransferrin, 11.0% ovomucoid, 3.5% ovomucin, 3.4% LYS and ovoglobulins (Mine, 1995). Most egg yolk proteins appear as low and high density lipoproteins. Other egg yolk proteins are livetins (about 10% of dry matter) and phosvitin (about 4% of dry matter). To the best of our knowledge, literature on amyloid formation of egg proteins is limited to reports on OVA, LYS and egg white as a whole. The conditions used to induce fibrillation of OVA and LYS, which are discussed in the following sections, are listed in Table 1.

2.1 Ovalbumin

OVA (about 45 kDa, 385 amino acids) is a glycophosphoprotein with an isoelectric point (pI) of 4.5. The self-assembly far below the pI differs significantly from that at pH values exceeding the pI (Veerman, de Schiffart, Sagis, & van der Linden, 2003). The protein denatures around 84 °C at neutral pH (Belitz et al., 2009) and around 57 °C at pH 2.2 (Tatsumi, Yoshimatsu, & Hirose, 1999). During storage or heating under basic conditions, the more heat stable S-OVA with a denaturation temperature of 92.5 °C is formed from native OVA (Belitz et al., 2009; Mine, 1995).

2.1.1 Fibril formation under strongly acidic conditions (pH ≤ 3.5)

At low pH, OVA adopts a partially denatured conformation, referred to as the molten globule state (Naeem, Khan, Muzaffar, Ahmad, & Saleemuddin, 2011; Tatsumi & Hirose, 1997). The secondary structure of OVA at pH 2.0 is almost the same as that at pH 7.0, but the native tertiary interactions are strongly disrupted (Koseki, Kitabatake, & Doi, 1988; Naeem et al., 2011). Heating OVA at acidic pH decreases the contents of the α-helical and random coils and increases that of β-sheets. Both the ThT and the 8-anilino-1-naphthalene-sulfonic acid (ANS) fluorescence are clearly higher after heating, indicating that the aggregates display amyloid structures which are more hydrophobic than OVA itself (Bhattacharya & Dogra, 2015; Bhattacharya, Jain, Dogra, Samai, & Mukhopadhyay, 2013; Jansens, Brijs, Stetefeld, Delcour, & Scanlon, 2017). The presence of 20 mM trifluoroacetic acid or 30 mM trichloroacetic acid at room temperature even induces OVA aggregation as evidenced by increased ThT fluorescence and β-sheet contents (Naeem et al., 2011). OVA aggregates with increased ThT fluorescence are also formed at room temperature when OVA solutions (pH 2.5) are subjected to continuous shaking for multiple days (Tufail et al., 2015).

2.1.1.1 Fibril morphology

Several authors have reported the formation of semi-flexible fibrils when OVA solutions (pH 2.0) are kept at 80 °C (Veerman et al., 2003; Weijers, Sagis, Veerman, Sperber, & van der Linden, 2002). However, also annular pore-like aggregates and low levels of worm-like structures can be formed under acidic conditions (4 h, 65 °C, pH 2.2) (Bhattacharya & Dogra, 2015; Bhattacharya, Jain, et al., 2013). The contour length of semi-flexible OVA fibrils obtained by heating (1 h, 80 °C, pH 2.0) increases with OVA concentration in a 2.0

to 7.0% range. While Weijers et al. (2002) noticed increased fibril length with higher ionic strength (0.0 to 0.03 M), under the same conditions no significant differences in contour length were observed when the ionic strength ranged from 0.01 to 0.30 M (Veerman et al., 2003). At a salt concentration of 0.03 M, the aggregates were linear, but they started to form clusters. The degree of clustering increased with pH in a 2.0 to 3.5 range as van der Waals forces and hydrophobic interactions became more important than electrostatic repulsion when approaching the pI. At pH 2.0, electrostatic repulsion dominates and linear aggregates are formed (Weijers et al., 2002).

Lara et al. (2012) reported the simultaneous formation of several types of OVA fibrils during heat treatment for 1 to 265 hours at 90 °C and pH 2.0 irrespective of whether the solution contained salt. In the absence of salt, three types of fibrils were distinguished after heating. The first type are the most rigid ones which grow up to 10 μm after 24 hours. These fibrils are rich in β-sheet and show a diffraction pattern typical for amyloids. The second type are quite flexible and are typically 500 nm long. These fibrils are believed to be intermediate structures that assemble into thicker multi-stranded fibrils upon further incubation. The third type of fibrils are the most flexible ones. During the first few hours of incubation they occur as point-like aggregates. These then grow over time into very flexible fibrils of up to a micrometer long and with a typical worm-like morphology. They do not have the typical amyloid fingerprint (Lara et al., 2012).

Incubation of OVA (pH 2.5, room temperature) under continuous shaking for 10 days results in fibrillar structures whereas aggregates with a suprafibrillar structure are observed after 15 days (Tufail et al., 2015).

2.1.1.2 The importance of (amylogenic) peptides and peptide bond hydrolysis for (amyloid) fibril formation

Removing the N-terminal region (amino acid residues 1 to 22) of OVA by peptic hydrolysis lowers its rate of aggregation during heating for up to 1 h at 65 °C and pH 2.2. In addition, spherical aggregates rather than semi-flexible fibrils are formed. Presumably, the N-terminal region facilitates the conformational transition of OVA α-helices into β-sheet structures. Addition of the enzymatically released N-terminal peptide to native OVA and heating results in straight rather than semi-flexible fibrils. Interestingly, when the first 8 amino acid residues of the peptide were removed, the resulting peptide did not significantly promote heat-induced aggregation of OVA. This suggests that an amphiphilic structure is necessary for promoting protein aggregation (Kawachi, Kameyama, Handa, Takahashi, & Tanaka, 2013).

The sequence MVLVNAIVFK, which can form AFs on its own at neutral pH (Tanaka et al., 2011), was detected in fibrils formed under acidic conditions (Lara et al., 2012).

2.1.2 Fibril formation at higher pH (pH ≥ 4.5)

Heating OVA at 40 to 90 °C and neutral pH decreases the percentage of helical structures, and increases ThT fluorescence (Azakami, Mukai, & Kato, 2005; Pearce, Mackintosh, & Gerrard, 2007). Also, incubation of OVA at room temperature (pH 7.0) under continuous shaking for up to 15 days yields aggregates with lower percentages of helical structures and higher levels of ThT fluorescence than the starting material (Tufail et al., 2015).

2.1.2.1 Fibril morphology

Based on transmission electron microscopy studies, some authors describe the aggregates formed by heating OVA at neutral pH as amorphous (Pearce et al., 2007), whereas others

consider them to be semi-flexible (Tanaka et al., 2011). According to Pouzot et al. (2005), the structure of OVA aggregates formed during heating at 75 to 80 °C for 24 hours at neutral pH is compatible with that of semi-flexible strings of monomers. Fibrillar aggregates have also been reported to result from incubation (pH 7.0) at room temperature during agitation for 15 days (Tufail et al., 2015). At low ionic strength, heat-induced aggregates are weakly branched and with increasing ionic strength, the degree of branching and the flexibility increases (Pouzot et al., 2005). At neutral pH, temperature is a more important factor governing amyloid-like aggregation than salt (0 to 200 mM NaCl) or protein concentration (1.0 to 20 mg/mL) (Pearce et al., 2007). Nevertheless, protein charge clearly affects fibril morphology. Using methylation and succinylation, Broersen et al. (2007) and Weijers et al. (2008) prepared a range of OVA samples with different net charge. Charge modification significantly affected the denaturation temperature, which is an important factor determining OVA aggregation propensity as well as the morphology of the aggregates obtained by heating. The degree of branching and the flexibility decreased with increasing net charge. Furthermore, shielding the introduced charge with salt resulted in aggregates with morphology similar to that of aggregates with a low charge. Not just the charge itself, but also its distribution over the protein molecule may play a crucial role in determining fibril properties (Broersen et al., 2007; Weijers et al., 2008).

2.1.2.2 The impact of peptides on fibril formation

When OVA is incubated (80 °C, pH 7.5) with IAIMSA, a peptide located in the N-terminal region of OVA, long straight fibrils are obtained that are distinct from the semi-flexible fibrils formed under the same conditions in absence of this peptide. Presumably, the N-terminal region acts as the core for fibril formation (Tanaka et al., 2011). Furthermore, with

the algorithms TANGO, AGGRESCAN and PASTA, Tanaka et al. (2011) identified other regions in OVA with high β-aggregation propensity. The OVA peptides LAMVYL, MVLVNAIVFK and FLFCIK can form AFs on their own when heated at 80 °C and pH 7.5. For more information on prediction algorithms as a tool to identify regions of protein sequences with high β-aggregation propensity the interested reader is referred to the review of Jansens et al. (2019).

2.1.2.3 The relation between disulfide bond and fibril formation

OVA contains six cysteine residues of which two are involved in an intramolecular disulfide (SS) bond. OVA is the only egg white protein which contains free sulfhydryl (SH) groups (Belitz et al., 2009). Reduction of the only SS bond has little effect on the overall conformation of unheated OVA (Takahashi, Koseki, Doi, & Hirose, 1991). However, it lowers the temperature at which conformational changes occur prior to amyloid formation (Jansens, Brijs, Delcour, & Scanlon, 2016). Heat treatment (80 °C, 1 h, pH 7.5, 20 mM NaCl) of OVA with reduced SS bonds yields long straight fibrils whereas semi-flexible fibrils are formed from intact OVA treated under the same conditions. No differences in level of β-sheets between intact and SS reduced OVA are observed after heating (Tanaka et al., 2011). A kinetic study of SS reduced OVA fibril formation at neutral pH showed differences with the classic nucleation growth mechanism. Fibril formation, as evidenced by ThT fluorescence, increased sharply from the start of heating with no apparent lag phase. The initial growth phase was followed by (i) a second growth phase in which the ThT fluorescence still increased, but more slowly than in the initial growth phase and, (ii) by a subsequent plateau phase with constant ThT fluorescence. A model for fibril formation which includes seeded linear growth, end-joining and fibril fragmentation shows that

311 especially end-joining (hence the formation of loops) impacts the growth of fibrils at
312 neutral pH (Kalapothakis et al., 2015). Oxidation of SH groups has little – if any – impact
313 on fibril formation as evidenced by ThT fluorescence (Jansens et al., 2016). Furthermore,
314 the rate of heat-induced OVA aggregation (70 °C and pH 7) appears to be unaffected by the
315 introduction of additional SH groups. SS bond formation was preceded by non-covalent
316 interactions and thus not the driving force for OVA aggregation. The morphology of heat-
317 induced OVA aggregates, as investigated by transmission electron microscopy, depends on
318 the number of introduced SH groups. With increasing levels of introduced SH groups the
319 aggregates transform from fibrillar into amorphous (Broersen et al., 2006).

320 ### 2.1.3 The stability of fibrils

321 OVA fibrils formed during heating for one hour at 80 °C and pH 2.0 do not fall apart up to
322 24 hours after dilution, indicating that the aggregation is irreversible (Veerman et al.,
323 2003). However, Tufail et al. (2015) found aggregation of OVA fibrils over several days to
324 be reversible. After fibril formation (room temperature, pH 2.5 or 7.0) and suspension of
325 the fibrils at pH 7.4, monomeric OVA was released from the fibrils in a slow and steady
326 manner. The extent of release was less for fibrils which required more days to aggregate
327 during fibril formation (Tufail et al., 2015).
328 OVA fibrils formed under acidic conditions from reduced OVA were to some extent
329 resistant towards in vitro proteolysis (Lassé, Ulluwishewa, Healy, Thompson, Miller, Roy,
330 Chtcholtan, et al., 2016).

331 ## *2.2 Lysozyme*

332 Hen LYS (about 14 kDa, 129 amino acids, pI 10.7) is one of the most studied proteins in an
333 amyloid context due to its similarity with human LYS. Trexler et al. (2007) and

Swaminathan et al. (2011) reviewed amyloid formation for both human and hen egg LYS. Here, only hen LYS is discussed. Fibrils based on LYS can be formed under different conditions (Krebs et al., 2000; Swaminathan et al., 2011) but most literature reports deal with the formation of LYS fibrils at acidic pH.

2.2.1 Fibril formation under strongly acidic conditions (pH < 3.5)

At low pH, LYS is partially denatured (Babu & Bhakuni, 1997) and heating results in formation of AFs. The initial stages of LYS amyloid formation at acidic pH involve its irreversible unfolding (Xu et al., 2005; Xu, Ermolenkov, Uversky, & Lednev, 2008; Xu et al., 2007). Overall, fibril assembly from LYS under acidic conditions follows the classic nucleation-growth process (Hill et al., 2009). At 57 °C and pH 2.0, fibrillar aggregates are formed after a lag time of 2 days, whereas the lag time is 11 days at pH 3.0. At pH 4.0 aggregates were not even detected after a 42 day period (Arnaudov & de Vries, 2005).

Both the rate of formation and the final amounts of AFs formed at acidic pH strongly depend on the incubation temperature and for LYS are maximal at 65 °C. At higher temperatures, amorphous aggregation competes with AF formation (Ow & Dunstan, 2013). In contrast to Arnaudov and de Vries (2005), Mishra et al. (2007) reported that the lag phase of LYS fibril formation at pH 1.6 is concentration dependent. The lag time decreases with increasing salt concentration. However, at salt concentrations exceeding 350 mM disordered protein aggregates are formed upon incubation at acidic pH. Repulsive charge interactions appear to be a prerequisite for ordered fibril assembly (Hill, Miti, Richmond, & Muschol, 2011). Furthermore, which fibril assembly mechanism occurs depends on the salt concentration. It has been reported that when it increases to levels exceeding 150 mM a switch from a monomeric to an oligomeric assembly pathway occurs (Hill et al., 2011).

Also, the efficiency of monovalent anions at enhancing fibrillation kinetics is inversely related with the Hofmeister series (Ponikova et al., 2015). Acetylation of lysine residues in LYS prior to fibril formation reduces the lag time and the formation of more unbranched and neat fibrils than those formed from intact LYS (Morshedi, Ebrahim-Habibi, Moosavi-Movahedi, & Nemat-Gorgani, 2010). In the presence of low levels of SDS, no obvious lag phase is observed during LYS fibril formation at pH 2.0 and 55 °C (Hung, Lin, Chen, & Wang, 2010). Fibril formation during heating at pH 2.0 does not involve the formation of covalent cross-links (Arnaudov & de Vries, 2005).

Based on a study with LYS mutants, Harada et al. (2008) suggested that mainly the C-helix (residues 88-99) is involved in the α- to β-transition during amyloid formation. Both the β-sheets in the oligomers as well as those in the mature AFs formed at low pH and elevated temperature are in a parallel conformation (Zou et al., 2014; Zou, Li, Hao, Hu, & Ma, 2013). However, oligomers formed at room temperature contain an antiparallel β-sheet conformation (Zou et al., 2013). Stabilization of native α-helices of sequences with high fibrillation propensity in the partially-unfolded state decelerates amyloid fibril formation. The stabilization of α-helices in non-amylogenic sequences can probably accelerate fibril formation of globular proteins (Chaudhary, Vispute, Shukla, & Ahmad, 2017). X-Ray diffraction patterns with reflections at 4.7, 9.7 and 12.6 Å have confirmed the amyloid nature of the fibrillar structures obtained by heating LYS solutions at pH 2.0 (Krebs et al., 2000).

2.2.1.1 Fibril morphology

Fibril morphologies have been reported to vary with the heating conditions. Heating at 80 °C yields a combination of fibrillar and spherical aggregates at pH 2.0, whereas only

spherical aggregates are observed as a result of heating at pH 3.0 and 4.0 (Arnaudov & de Vries, 2005). Yagi et al. (2009) reported that spherulites in which the AFs three dimensionally extend in all directions result from incubation of LYS for 30 to 60 days at 60 °C and pH 1.6. Lara et al. (2011) reported the formation of giant multi-stranded twisted and helical ribbons from LYS as a result of incubation at pH 2.0 and 90°C for 20 to 30 hours.

2.2.1.2 The impact of peptide bond hydrolysis on fibril formation

Heating at pH 2.0 results in aggregates that contain both hydrolysis products as well as full-length protein. SDS-polyacryl amide gel electrophoresis (PAGE) under non-reducing conditions has indicated that hydrolysis is not necessarily a prerequisite for fibril formation (*e.g.* at pH 3) (Arnaudov & de Vries, 2005). However, it is possible that limited protein hydrolysis is not detected with SDS-PAGE when executed under non-reducing conditions (Mishra et al., 2007). According to Mishra et al. (2007), intact full-length LYS does not dominate the composition of AFs during heating at 65 °C and pH 1.6. During heating, LYS unfolds and hydrolysis of one peptide bond results in nicked LYS. Prolonged heating (5-40 hours) sets free other fragments such as the peptide containing the LYS residues 49 to 101, which is considered to be the most amylogenic region of LYS. The 49 to 101 fragment is believed to form the rate-determining nucleus while nicked full-length LYS is efficiently incorporated into AFs. Mature LYS fibrils are mainly composed of nicked LYS, but during prolonged heating, the AFs undergo a fibril shaving process in which the non-amylogenic parts of the nicked LYS (residues 1-28 and 102-129) are removed from the fibrils (Mishra et al., 2007).

AFs formed by incubating LYS solutions at pH 2.0 and 65 °C for 10 days predominantly consist of fragments of LYS corresponding to residues 49 to 100/101 and 53 to 100/101 formed by partial hydrolyses of Asp-X peptide bonds (Frare, de Laureto, Zurdo, Dobson, & Fontana, 2004). Peptides corresponding to part of the amino acid sequence of LYS can also form fibrils on their own. The peptide corresponding to residues 49 to 64 of LYS readily forms fibrils when incubated at 37 °C and pH 4.0 for 24 hours, whereas full-length LYS requires heat at pH 2.0 or addition of organic solvents followed by incubation for prolonged periods to form similar fibrils as that formed by the peptide within 24 hours (Krebs et al., 2000). The peptide corresponding to residues 57 to 107 of LYS forms AFs during incubation at 65 °C and pH 2.0 whereas the fragment 1 to 38/108 to 129 does not aggregate under similar conditions (Frare et al., 2004).

Lara et al. (2011) reported that formation of helical ribbons by incubation of LYS at pH 2.0 (90 °C for 20-30 hours) only took place after hydrolysis of LYS and that small fragments (< 6 kDa) participated in the formation of these structures. Subsequent research showed that the fragment ILQINS (corresponding to residues 55 to 60 of LYS) is present in most of the sequences constituting the ribbons. The fragment itself also forms fibrillar structures when incubated at room temperature and pH 2.0. Interestingly, the ribbons from this fragment show a right-handed twist, whereas ribbons formed from LYS are left-handed (Lara et al., 2014).

Replacing Trp62 with Gly in SS bond reduced LYS yielded a variant that does not yield ThT positive structures after 2 weeks of incubation at pH 2.0 and 25 °C (Ohkuri, Shioi, Imoto, & Ueda, 2005). Interestingly, replacing both Trp62 and Trp111 of reduced LYS by Gly yields a variant that results in fibril formation (Mishima, Ohkuri, Monji, Imoto, &

Ueda, 2007). These results suggest that the structure of reduced LYS at pH 2.0 is important for amyloid formation. Disruption of specific hydrophobic clusters in reduced LYS enhanced or retarded AF formation (Mishima, Ohkuri, Monji, Imoto, & Ueda, 2006; Mishima et al., 2007; Ohkuri et al., 2005).

2.2.1.3 The impact of seeding and cross-seeding on fibril formation

LYS fibril formation at 37 °C and pH 2.0 is significantly accelerated when preformed LYS fibrils are added, a phenomenon known as seeding (Figure 3). That seeding accelerates fibril formation confirms that fibril growth is dominated by a nucleation mechanism. Interestingly, Krebs et al. (2000) noted that seeding can be carried out both with fibrils based on full-length LYS as with fibrils based on the residues 49 to 64 peptide of LYS discussed above. Seeding with nuclei or with mature fibrils shortens the lag phase and accelerates AF formation. Seeding of nicked LYS abolishes the lag phase completely. Adding native LYS at the end of the lag phase delays fibril formation (Mishra et al., 2007). Extensive sonication of mature fibrils (formed by incubation during 11 days at pH 2.2 and 57 °C) yields fibril fragments which act as seeds. In the presence of these seeds, α-helix to β-sheet transition starts immediately at 57 °C and pH 2.2 and thus without any lag time (Sasaki et al., 2008).

Cross-seeding between different types of proteins can also occur. However, when the sequence identity between egg white LYS and preformed fibrils from other protein decreases, the efficiency of seeding also decreases. This may be related to the importance of long-range interactions in stabilizing the core of AFs (Krebs, Morozova-Roche, Daniel, Robinson, & Dobson, 2004).

2.2.1.4 The impact of processing on fibril formation

Agitation can facilitate formation of LYS fibrils during heating at acidic pH (Lieu, Wu, Wang, & Wu, 2007; Ow & Dunstan, 2013; Sasahara, Yagi, Naiki, & Goto, 2007). Furthermore, the morphology of LYS fibrils varies considerably depending on the applied flow. With increasing shearing or stirring rates more rod-like and shorter fibrils are obtained whereas longer semi-flexible fibrils are formed at rest or at low shear rates (Humblet-Hua et al., 2008). Incubation of LYS for 15 days at pH 2.0 and 37 °C without agitating results in curve-linear and relatively thick (about 2.5 to 3.0 mm) fibrils while incubation under those conditions for 7 days while agitating leads to straight and thin (about 2.0 to 2.5 mm) fibrils. These aggregates are more efficient in self-seeding than in cross-seeding under swapped incubation conditions (Sivalingam, Prasanna, Sharma, Prasad, & Patel, 2016). Of further note is that extensive sonication of preformed LYS fibrils can result in their degradation into much smaller round-shaped particles, which may act as seeds (see above) (Sasaki et al., 2008).

LYS fibrils exist in a high-volume and high-compressibility state and are sensitive to pressure. Exposure to pressure can accelerate the dissociation of AFs into monomeric LYS (Akasaka et al., 2007; Latif, Kono, Tachibana, & Akasaka, 2007; Shah, Maeno, Matsuo, Tachibana, & Akasaka, 2012). Furthermore, the interface area between hydrophilic (e.g. aqueous salt solutions) and hydrophobic (e.g. air) surfaces impacts fibrillation kinetics due to protein unfolding and subsequent fibrillation at the interface. With decreasing vial diameter, the rate and level of fibrillation decreases (Jayamani & Shanmugam, 2017).

2.2.1.5 The impact of (food) constituents on fibril formation

Numerous researchers have investigated the inhibition of LYS amyloid formation. This has resulted in a list of compounds that either partially or completely inhibit fibril formation at acidic pH: food constituents like short-chain phospholipids (pH 2.0 and 55 °C) (Wang, Hung, Wen, Lin, & Chen, 2011), curcumin (Liu et al., 2012; Wang, Liu, & Lee, 2009) or its water soluble derivatives (Wang et al., 2018), myricetin (He et al., 2014), the osmolytes proline, hydroxyproline, sarcosine and trimethylamine N-oxide (Choudhary & Kishore, 2014), cysteine (Takai et al., 2014) (Wang, Liu, Wu, & Lai, 2009), glutathione (Wang, Chou, Liu, & Wu, 2009), carnosine (Wu et al., 2013), trehalose, magnesium chloride (Chatterjee, Kolli, & Sarkar, 2017), safranal, crocin (Joloudar et al., 2017), zinc ions (Ma, Zhang, Wang, & Zhu, 2017), aroma components (*e.g.* phenyl ethyl alcohol, N,N,N,N'-tetramethylethylenediamine or cinnamaldehyde) (Seraj et al., 2018), rosmarinic acid, resveratrol (Shariatizi, Meratan, Ghasemi, & Nemat-Gorgani, 2015), chemicals such as p-benzoquinone (Lieu et al., 2007; Wang, Chen, & Hung, 2006), 4-aminophenol and 2-amino-4-chlorophenol (Vieira, Figueroa-Villar, Meirelles, Ferreira, & De Felice, 2006), tris(2-carboxyethyl)phosphine (Wang, Liu, & Lu, 2009), SDS concentrations of at least 0.25 mM (Hung et al., 2010), nonionic detergents like triton X-100 and n-dodecyl-β-D-maltoside (Siposova, Kozar, & Musatov, 2017), the ionic liquid tetramethyl guanidinium acetate (Kalhor, Kamizi, Akbari, & Heydari, 2009), clotrimazole (Sarkar, Kumar, & Dubey, 2011b), sodium tetrathionate (Sarkar, Kumar, & Dubey, 2011a), β-mercaptoethanol (Sarkar et al., 2011a), glycol-acridines (Vuong et al., 2013), 2-acetyl amin-3-[4-(2-amintgo-5-sulfo-phenyl]-propionic acid (Maity et al., 2013), indole, indole 3-acetic acid, indole 3-carbinol, indole 3-propionic acid and tryptophol (Morshedi, Rezaei-Ghaleh, Ebrahim-

Habibi, Ahmadian, & Nemat-Gorgani, 2007) and other components such as melatonin (Wang et al., 2006), glutathione-covered gold nanoparticles (Antosova et al., 2012), manganese-salen derivatives (Bahramikia & Yazdanparast, 2012; Bahramikia, Yazdanparast, & Gheysarzadeh, 2012), the crowding agents Ficoll 70 and dextran 70 (Ma et al., 2012), bovine serum albumin and its combination with Ficoll 70 (Zhou, Zhou, Hu, Chen, & Liang, 2008) and type I collagen (Dubey & Mar, 2014).

Some additives cannot only inhibit fibril formation, they can also lead to dispersion of preformed aggregates. This is the case for the manganese-salen derivatives (Bahramikia & Yazdanparast, 2012; Bahramikia et al., 2012). The phospholipids 1,2-dimyristoyl-*sn*-glycero-3-phophocholine and 1,2-dihexanoyl-*sn*-glycerol-3-phosphocholine inhibit fibril formation through the binding of LYS monomers but they do not depolymerize LYS AFs (Ponikova et al., 2017). Addition of gum Arabic or pectin to LYS enhances the association of AFs into clumps and higher order fibrillary aggregates (Ow, Bekard, & Dunstan, 2018).

Ma et al. (2013) reported a result which is interesting from a practical point of view. LYS AFs are typically formed under acidic conditions and at elevated temperature, whereas their characterization is often performed at room temperature. Interestingly, they observed the formation of non-fibrillar β-sheet aggregates during storage at room temperatures of LYS solutions after prior incubation at elevated temperatures. Hence, it is important to reduce the time between the heat treatment and the analysis of the fibrils.

2.2.2 Fibril formation at higher pH (pH ≥ 4.0)

Heating LYS under agitation at 65 °C at pH 6.0 or 2.7 yields fibrillar aggregates. The lag phase is longer at pH 6.0 than at pH 2.7 and the morphology of the fibrils is somewhat different. At both pH values linear non-branched fibrils are formed, but those formed at pH

6.0 are thicker and shorter than those formed at pH 2.0 (Mocanu et al., 2014). Based on density and ultrasonic velocity measurements, Akasaka et al. (2007) reported that the fibrils formed at pH 4.0 are highly voluminous and compressible, indicating cavity-rich structures. Incubation of LYS at alkaline pH (12.2) and 25 °C also yields structures displaying ThT fluorescence (Homchaudhuri, Kumar, & Swaminathan, 2006; Swaminathan et al., 2011). Prolonged incubation leads to a mixture of amorphous aggregates and amyloid-like fibrils (Kumar, Ravi, & Swaminathan, 2008; Ravi, Swain, Chandra, & Swaminathan, 2014). The fibril formation at alkaline pH is accelerated by heating the samples to 50 °C (Li et al., 2014). The initial step of fibril formation at alkaline pH is the formation of small β-sheet rich oligomers. Next, the oligomers assemble into nuclei, followed by formation of mature fibrils (Li et al., 2014).

2.2.2.1 Amylogenic peptides and the relation between disulfide bond reduction and fibril formation

Sugimoto et al. (2011) reported that the peptide corresponding to residues 54-62 (GILQINSRW) aggregates at pH 7.5 and 25 °C into fibrils with positive ThT fluorescence. Although fibrils are also formed at neutral pH, fibrils from this peptide are formed faster at pH 4.0. Tryptophan appears critical in this sequence since no fibrils are formed after deletion or substitution of this amino acid. Furthermore, the peptide also needs a certain sequence of hydrophobic amino acids for fibril formation to occur (Tokunaga, Sakakibara, Kamada, Watanabe, & Sugimoto, 2013).

Fibrillar structures are also formed at room temperature when incubating LYS of which one of the four SS bonds is reduced at pH 7.5 (Takase, Higashi, & Omura, 2002). However, incubation of SS-reduced LYS at pH 7.2 and 37 °C for several days results in amorphous

aggregates (Yang, Dutta, & Tiwari, 2015). Niraula et al. (2004) reported the formation of amyloid-like fibrils when incubating a genetically engineered SS-deficient variant of LYS for weeks to months at 25 °C and pH 7.5, 4.0 and 2.0 to 2.7. Fibrils from wild-type hen LYS are fairly thick (~5 nm) and straight, whereas those from SS-deficient LYS are thin (~2 mm) and curvy (Latif et al., 2007; Shah et al., 2012).

Based on the effect of pressure on AFs from SS-deficient LYS, Kamatari et al. (2005) concluded that protofibrils grow by multiple mechanisms such as successive addition of monomers to the growing end of protofibrils in the early phase and end-to-end association of shorter protofibrils to form long fibrils in the later phase of growth. In subsequent research, the authors reported that formation of AFs from SS-deficient LYS fibrils proceeds largely by attaching monomers to the end of the protofibrils (Latif et al., 2007).

2.2.2.2 The impact of denaturing agents on fibril formation

That it is also possible to form amyloid-like structures using denaturing agents supports the view that partial unfolding is a prerequisite for fibril formation. Fibrils are rapidly formed in LYS solutions at pH 6.3 and 50 °C in the presence of 2.0 to 5.0 M guanidine hydrochloride but not at very low or very high concentrations thereof (Vernaglia, Huang, & Clark, 2004). Wang et al. (2007) reported the formation of AFs when LYS at pH 7.4 was heated (37 to 55 °C) in 4.0 to 8.0 M urea. However, the AFs disappeared during prolonged incubation. Fibril formation also occurs upon incubation of LYS at pH 9.2 in the presence of SDS (Khan et al., 2012; Moosavi-Movahedi et al., 2007). Based on these findings, it was concluded that both electrostatic and hydrophobic forces are prerequisites for fibril formation, with the former playing a leading role (Khan et al., 2012). Fibril formation in the presence of SDS at pH 9.2 is prevented by β-cyclodextrin (Moosavi-Movahedi et al., 2007).

In the presence of SDS (0.0 to 0.6 mM) fibril formation occurs over a broad pH range (1.0 to 10.0), but most pronounced at pH 1.0 (Khan et al., 2014). Emadi et al. (2014) reported that incubation of LYS at pH 7.0 in 4.0 M guanidine hydrochloride or 1.0 to 2.0 M guanidine thiocyanate yields structures which exhibit ThT fluorescence, although no clear fibril formation is detected.

2.2.2.3 The impact of aqueous alcohol on fibril formation

Alcohols (*e.g.* ethanol, trifluoro-ethanol, hexafluoro-propanol) promote AF formation from hen egg white LYS (Aso et al., 2007; Bhattacharya, Ghosh, Dasgupta, & Roy, 2013; Goda et al., 2000; Hameed, Ahmad, Khan, Andrabi, & Fazili, 2009; Holley, Eginton, Schaefer, & Brown, 2008; Lin, Lee, Yoshimura, Yagi, & Goto, 2014).

Because of their hydrophobicity alcohols destabilize the native structure of LYS, often yielding α-helical conformations (Cammers-Goodwin et al., 1996; Hoshino, Hagihara, Hamada, Kataoka, & Goto, 1997). Lin et al. (2014) reported that solubility of LYS in water-alcohol mixtures limits its fibrillation. Alcohol-denatured LYS retains meta-stability in water-alcohol mixtures and super-saturation prevents conformational transitions. Nevertheless, under these conditions fibril formation can be triggered by ultra-sonication. Based on their results, Yonezawa et al. (2002) proposed a three stage pathway for LYS fibril formation in alcohol solutions. In a first stage dimers are formed, then protofilaments and finally AFs, the latter via lateral association of protofilaments. That the structure of the fibrils depends on salt concentration suggests that electrostatic interactions play an important role in their formation (Fujiwara, Matsumoto, & Yonezawa, 2003). Also fully reduced LYS can form AFs by using ethanol (Cao, Hu, & Lai, 2004).

2.2.2.4 The impact of processing on fibril formation

Xie et al. (2012) reported that UV illumination for 4 days (about 20 μw/cm³) brings LYS to an energetically higher conformational state which triggers its fibrillation at pH 7.0 after 3 to 10 days. The fibrillar aggregates retained native-like conformation, which is different from the enrichment in β-sheets typically observed during formation of amyloid-like fibrils from LYS under acidic conditions. Furthermore, in contrast to what is generally observed for amyloid-like fibrils, the formation of intermolecular SS bonds was critical for the growth of the fibrils (Xie et al., 2012).

Fibrils from SS-deficient LYS formed in an early-stage dissociate faster when exposed to high pressure than their mature counterparts. The pressure induced dissociation is reversible (Kamatari et al., 2005; Niraula et al., 2004). For AFs in general, high pressure typically leads to dissociation of aggregates formed early in the assembly process, whereas mature fibrils are often more stable at high pressure (Meersman & Dobson, 2006).

Sonication of LYS solutions (pH 7.8) also results in amyloid-like aggregates. It may destabilize LYS through various chemical and physical processes (Stathopulos et al., 2004). Membranes containing negatively charged phospholipids can trigger rapid formation of amyloid-like fibrils from LYS at room temperature and neutral pH (Melo, Ricardo, Fedorov, Prieto, & Coutinho, 2013; Relini, Marano, & Gliozzi, 2014; Zhao, Tuominen, & Kinnunen, 2004). The charge density of the phospholipid membrane substantially influences the extent of LYS fibril formation (Al Kayal et al., 2012). Accardo et al. (2011) reported the formation of LYS fibrils having cross-β structural features under weakly acidic conditions (pH 3.8 to 4.5) in the presence of calcium ions when evaporating a drop of a solution of LYS on a super-hydrophobic surface.

2.2.2.5 The impact of (food) constituents on fibril formation

Amyloid-like fibrils are formed upon long incubation (20 days) of LYS at neutral pH in 50% glyoxal (Fazili, Bhat, & Naeem, 2014). For some proteins, glycation can lead to amyloid formation. However, for LYS, glycation with glucose, fructose or ribose promotes the formation of cross-linked oligomers rather than fibrillary species during incubation at neutral pH and 37 °C. Although they are amorphous, ribose mediated oligomers have ThT fluorescence (Ghosh, Pandey, Roy, et al., 2013). Both anionic and cationic surfactants can promote amyloid fibrillation at low molar ratio (1:10) at 25 °C and pH 9.0 or 13.0, respectively (Chaturvedi, Khan, Siddiqi, Alam, & Khan, 2016).

The inhibitory effect on amyloid formation which some compounds display seems to depend on pH. For instance, rottlerin promotes LYS amyloid formation under acidic conditions, but inhibits it at alkaline pH (Sarkar et al., 2011b). Other compounds that inhibit LYS amyloid formation under alkaline conditions include dithiothreitol (Kumar et al., 2008), cetyltrimethylammonium bromide (Kumar et al., 2008), iodoacetamide (Ravi, Goel, Kotamarthi, Ainavarapu, & Swaminathan, 2014), high molecular weight (HMW) polyethylene glycols (PEG 20,000 and PEG 35,000) (Ghosh, Pandey, & Dasgupta, 2014), green tea polyphenols (Ghosh, Pandey, & Dasgupta, 2013) and triacetylchitotriose (Kumar, Ravi, & Swaminathan, 2009).

Fibril formation in the presence of guanidine hydrochloride is inhibited by curcumin (Borana, Mishra, Pissurlenkar, Hosur, & Ahmad, 2014), kaempferol (Borana et al., 2014) and acridine derivatives. Both the structure of the acridine side chain and molecule planarity influence their anti-amyloidogenic activity (Gazova et al., 2008).

2.3 Mixtures of egg proteins

Heating egg white protein (20 mg/ml) at pH 7.5 in the presence of 200 mM salt at 60 °C to 80 °C increases its ThT fluorescence. The ThT fluorescence depends more on temperature than on protein concentration or ionic strength of the medium (Pearce et al., 2007). Transparent cold-set gels rich in fibrillar structures are formed with egg white powder in absence of ovotransferrin (Weijers, van de Velde, Stijnman, van de Pijpekamp, & Visschers, 2006). Sugimoto et al. (2011) investigated the interaction of LYS with OVA. The interactions appeared most pronounced when mixtures thereof were heated at 72 °C, a temperature slightly lower than that at which OVA denatures. Furthermore, the interaction also took place when unheated LYS at pH 7.5 and 25 °C was mixed with OVA preheated at 72 °C. The temperature of preheating appeared to be critical. Whereas OVA preheated at 72 °C inhibits the enzymatic activity of LYS, OVA preheated at higher temperatures tends to lose its inhibitory effect on LYS. Interaction at pH 7.5 and 25 °C between unheated LYS and OVA preheated at 72 °C results in fibrous aggregates with ThT fluorescence. Sugimoto et al. (2011) applied proteolysis in combination with affinity and reversed phase chromatography to identify regions within the proteins responsible for the interaction. For OVA the region with amino acid residues 229 to 263 and for LYS both the region with residues 112 to 123 and the region with residues 54 to 62 are important for the interaction. The peptide corresponding to the latter residues forms fibrous aggregates with OVA preheated at 72 °C or with the unheated peptide corresponding to residues 229 to 263 of OVA. Also the peptide corresponding to residues 112 to 123 results in aggregation (however not fibrous) with OVA preheated at 72 °C, but not with the peptide corresponding to residues 229 to 263 of OVA (Sugimoto et al., 2011).

3 CEREAL PROTEINS

Cereals are the most important source of protein in the human diet. Cereal production for human consumption is dominated by wheat (65.4 kg/capita), rice (53.9 kg/capita) and maize (17.9 kg/capita) in 2013 (Faostat, 2016).

3.1 Wheat proteins

Wheat gluten contains particularly high levels of glutamine and proline (Delcour et al., 2012). Although wheat gluten is rich in glutamine residues in the repetitive domain which theoretically increases its likelihood to form AFs (Chen, Berthelier, Hamilton, O'Nuallain, & Wetzel, 2002), no data indicates that native wheat gluten spontaneously forms protein fibrils upon temperature or pH changes.

Peptides with low levels of proline and glycine are associated with amyloid formation while higher levels of these amino acids are associated with the formation of elastomers. The latter are proteins which provide elastic recoil which itself is necessary for reversible deformation. The transition between amylogenic or elastomeric peptides is not abrupt. The composition of wheat gluten entails both elastomeric and amylogenic properties (Rauscher, Baud, Miao, Keeley, & Pomes, 2006). It has been suggested that viscoelastic gluten proteins interact through aligned β-sheets corresponding to their repetitive domains (Pézolet, Bonenfant, Dousseau, & Popineau, 1992). In this regard, a shift from α-helix to β-sheet as a result of gluten heat (Bruun, Søndergaard, & Jacobsen, 2007) or transglutaminase (Bagagli, Jazaeri, Bock, Seetharaman, & Sato, 2014) treatments has been noted. Apparently, wheat gluten proteins have intrinsic properties (*e.g*. high contents of glutamine and β-sheets in their native domains) which favor protein fibril formation. However, the

complex structure and lack of solubility of wheat gluten in aqueous media may well inhibit protein fibril formation and impede their analysis to a great extent.

3.1.1 Fibril formation at higher pH (pH ≥ 4.0)

Wheat gluten consists of monomeric gliadins and polymeric glutenins. Glutenins are made up by HMW and low molecular weight (LMW) glutenin subunits (GS) (Delcour et al., 2012). At pH 6.0–7.0 and in the presence of either 2.0 M urea or 30% (v/v) trifluoroethanol, a nanostructure can be derived from HMW-GS which has some characteristic amyloid features, namely significant ThT fluorescence, a fibrillar morphology as observed by transmission electron microscopy and an X-ray fiber diffraction pattern resembling that of typical amyloids (Mackintosh et al., 2009). Their 4.6 Å reflection is consistent with that predicted for the amyloid inter-β-strand, and the absence of the inter-β-sheet distance at 10 to 11 Å is not unprecedented in amyloid-like structures. Attempts to improve the rate and yield of fibril formation by performing tryptic hydrolysis prior to fibril formation or by seeding with preformed fibrils were not successful under the above cited conditions.

3.1.1.1 The impact of peptide bond hydrolysis on fibril formation

Explicit literature reports on gluten AF formation mostly mention some peptide bond hydrolysis (Athamneh & Barone, 2009a; Athamneh & Barone, 2009b; Claunch, Ridgley, & Barone, 2015; Ridgley & Barone, 2013; Ridgley, Claunch, & Barone, 2012; Ridgley, Claunch, Lee, & Barone, 2014; Ridgley, Ebanks, & Barone, 2011; Ridgley, Rippner, & Barone, 2015). Wheat gluten fibers can be prepared from tryptic gluten hydrolysates [2.5% (w/v) protein, 1:1,000 (w/w) enzyme-to-substrate ratio, 37 °C, pH 8.0] over a two week time with continuous stirring. X-ray diffraction patterns, Fourier transform infrared (FTIR)

spectra and ThT binding assays of the resulting dried fibers indicated that they are composed of cross-β structures. It was suggested that tryptic proteolysis of wheat gluten produces glutamine rich peptides with self-assembling propensity (Athamneh & Barone, 2009b).

Trypsin-hydrolyzed gliadin peptides [2.5% (w/v) protein, 1:1,000 (w/w) enzyme-to-substrate ratio, 37 °C, pH 8.0] under continuous stirring over 48 h form cross-β structures whereas trypsin-hydrolyzed glutenin does not. Particularly in this case, trypsin thermal inactivation after peptide hydrolysis impacts fibril formation by initiating a self-assembly process through aggregation of shorter extended peptides which results in gluten fibrils of up to about 10 μm in diameter and of about 100 μm length (Athamneh & Barone, 2009a). Processing variables (*e.g.* temperature, pH and ionic strength) affect the size, morphology and modulus of fibrils made from wheat gluten hydrolysates. Wheat gluten fibril formation is enhanced at 37 °C and pH 8.0. However, under these conditions, addition of 100 mM salt decreases the level of fibril formation. On a microscopic level, wheat gluten fibers exhibit two main morphologies, *i.e.* flat ribbons (tapes) and twisted cylinders. The latter contain more β-sheet structures and are more robust than the former (Ridgley et al., 2012).

3.1.1.2 The importance of (amylogenic) peptides for (amyloid) fibril formation

Large fibers (micrometer dimensions) from wheat gluten hydrolysates are formed through hydrophobic packing between short hydrophobic peptides with a high cross-β potential and longer more hydrophilic α-helical peptides. The peptide corresponding to residues 3 to 22 of gliadin (Gd20) which is obtained by tryptic gliadin hydrolysis at 37 °C and pH 8.0 can act as template for wheat gluten fibril formation due to its high hydrophobicity. It has been proposed that Gd20 forms a stable cross-β template that interacts with hydrophilic α-helical

peptides of other proteins, which subsequently undergo α-helix to β-sheet transition, and are added to the amyloid structure. Hydrolyzed gliadin forms short elliptical fibers [diameter (D) = about 12.1 μm] while hydrolyzed wheat gluten forms round fibers of similar size (D = about 10.7 μm). Mixtures of Gd20 and myoglobin or amylases yield longer and wider fibers (D = about 16.2 μm and D = about 19.1 μm, respectively) (Ridgley et al., 2011). Additionally, mixtures of Gd20 and α-helical "adder" proteins (*e.g.* α-lactalbumin, amylase, hemoglobin and insulin) produce large fibrils with a variety of morphologies (Ridgley et al., 2014). Varying the ratio of hydrophobic amino acid groups to polyglutamine sequences impacts the fibril morphology and modulus (Ridgley et al., 2015). Even though considerable advances have been made into understanding wheat gluten fibril formation, it is still necessary to comprehend the process at a molecular level. In addition, the impact of reported processing conditions (*e.g.* tryptic hydrolysis and drying) on wheat gluten fibril formation has not been estimated.

3.2 Maize proteins

The prolamins of maize are called zeins. They consist of one major (α-zein) and several minor (β-, γ-, δ-zeins) groups (Shewry & Halford, 2002). Zeins do not form a viscoelastic matrix when mixed with water. However, they can form β-sheet structures (Mejia, Mauer, & Hamaker, 2007).

The inclusion of small levels of wheat gluten HMW-GS or casein in a maize zein dough recipe can improve its rheological properties. This has been ascribed to an increase in the level and stabilization of β-sheet structures without a link to AF formation (Mejia, Gonzalez, Mauer, Campanella, & Hamaker, 2012). It has been confirmed in subsequent studies that plasticizers and co-proteins influence zein secondary structure in resin systems

742 by decreasing and increasing the levels of β-sheet structures (around 1640-1615 cm^{-1}),
743 respectively (Erickson, Renzetti, Jurgens, Campanella, & Hamaker, 2014). These zein
744 resins were formed via precipitation in aqueous-ethanol environments. Zein's transition
745 from aqueous-ethanol soluble globular aggregates to insoluble β-sheet-rich fibrils shows
746 similarities with that of AFs (Erickson, Campanella, & Hamaker, 2012).
747
748 Furthermore, α-zein forms amyloid-like nanofibrils during heating in aqueous ethanol
749 solutions [50% to 70% (v/v/)]. Evidence that β-sheet formation in these fibrils is enhanced
750 resides in the increased ThT fluorescence, an intensified FTIR peak at 1630 cm^{-1} (which is
751 characteristic for β-sheet structures), and X-ray diffraction peaks at about 0.6 $Å^{-1}$ and about
752 1.5 $Å^{-1}$ which are distinctive for β-strand and β-sheet stacking distances. Nevertheless, the
753 characteristic X-ray diffraction pattern (of about 4.6 $Å^{-1}$ and 10 $Å^{-1}$) of AFs were not
754 observed for these zein fibrils (An et al., 2016).
755 Recombinant maize transglutaminase produced in *E. coli* and unfolded by guanidine
756 hydrochloride exhibits the intrinsic propensity of forming aggregates displaying amyloid-
757 like features when refolded *in vitro* (25 °C, 20 μM protein, one week). The C-terminal
758 sequence comprising residues 465 to 477 has been predicted to be highly amylogenic
759 (Villar-Piqué et al., 2010).

760 ## *3.3 Rice proteins*

761 To the best of our knowledge, only few reports deal with amyloid-like aggregation of rice
762 protein. This is probably due to its insolubility and difficult isolation (Fabian & Ju, 2011).
763 Rice bran protein forms amyloid-like fibrils during heating at 90 °C and pH 2.0 for 2 h
764 (Zhang & Huang, 2014; Zhang, Huang, & Wei, 2014). The mean contour length and

765 particle size of these fibrils increase with heating time (up to 360 min) and protein
766 concentration (2, 10, and 50 mg/mL). The maximum ThT fluorescence intensity after 360
767 min heating is about 2.5 times that after 10 min heating (Zhang et al., 2014). Rice bran
768 protein fibrils formed by heating (90 °C, 2 h) at pH 2.0 are linear strands while adjusting
769 the pH to 7.0 induces fibril clustering (Zhang & Huang, 2014). Increasing the ionic strength
770 (0 to 500 mM salt) promotes fibril assembly of rice bran globulin when heated at 90 °C and
771 pH 2.0 for 2 h. The contour length of these fibrils increases with ionic strength (Huang,
772 Zhang, & Li, 2014). Rice bran albumin AF formation is enhanced by seeding. Adding
773 fibrils to rice bran protein solutions accelerates the rate of gel formation at 90 °C and pH
774 2.0 (Zhang & Huang, 2014; Zhang et al., 2014).

775 **CONCLUDING REMARKS**

776 Different conditions govern food protein amyloid fibrillation (Figure 4). These include the
777 heating mode, time, temperature, pH, moisture content, protein concentration, shear or the
778 presence of alcohols, chaotropic/reducing agents, enzymes, salt and/or other food
779 constituents (Figure 4). All these can impact the mobility, the probability of collision and/or
780 the conformation of food proteins (*e.g*. their secondary or tertiary structures) and therefore
781 the accessibility of their reactive groups or their hydrophobic/hydrophilic balance at the
782 protein surface. Sequences prone to form amyloids, *i.e*. amylogenic core regions, need to be
783 accessible to allow alignment of β-sheet structures in successively oligomers, protofibrils
784 and mature amyloids. Challenges and perspectives regarding protein fibrillation during
785 food processing are discussed in the accompanying paper dealing with formation of AF
786 from dairy and legume proteins (Lambrecht et al., 2019).

OVA can easily form fibrillar structures at low (pH 2.0) or neutral pH and moderate heating (*e.g.* 65-80 °C). Specific peptides are amylogenic and can act as a core in AF formation. The morphology of the formed fibrils can *e.g.* be adapted by changing the salt concentration or reducing its SS bonds.

LYS fibrillation has mostly been studied under acidic conditions. Heating at low pH hydrolyses peptide bonds leading to nicked LYS and/or or a peptide mixture. The latter aggregates through nucleation, a process which can be enhanced by seeding, *i.e.* the addition of amylogenic peptides or preformed fibrils.

Mixtures of (peptides of) LYS and OVA can form amyloids during heating at neutral pH. Heating egg white at neutral pH increases its level of ThT fluorescence but that AFs are formed has not been proven.

In contrast to what is the case for hen egg proteins, knowledge on amyloid fibrillation of cereal proteins is limited. Enzymatic hydrolysis of wheat proteins exposes peptides with high propensity to form β-sheets under alkaline conditions. Maize and rice proteins have the potential to form fibrils in the presence of aqueous ethanol and acidic pH, respectively. Even though egg and cereal proteins often coexist in food products, the impact on each other's fibrillation has not been investigated.

ABBREVIATIONS

Amyloid fibril (AF), Thioflavin T (ThT), isolectric point (pI), 8-anilinonaphtalene-1-sulfonic acid (ANS), ovalbumin (OVA), lysozyme (LYS), disulfide (SS), sulfhydryl (SH), sodium dodecyl sulfate (SDS), polyacryl amide gel electrophoresis (PAGE), high molecular

809 weight (HMW), polyethylene glycols (PEG), low molecular weight (LMW), glutenin
810 subunits (GS), Fourier transform infrared (FTIR), diameter (D)
811
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819
820 **AUTHORS CONTRIBUTIONS**
821 Jansens K.A., Lambrecht M.A., Rombouts I. and Morera Monge M. collected the
822 references and wrote the manuscript. Brijs K., Rousseau F., Schymkowitz J. and Delcour
823 J.A. corrected and reviewed the manuscript. Lambrecht M.A. made the figures and table.
824
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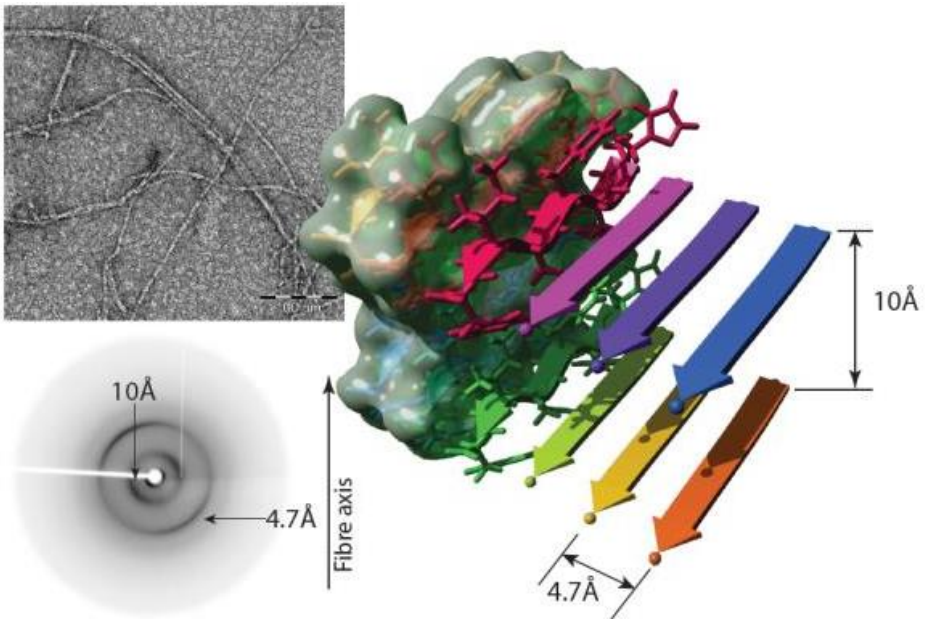
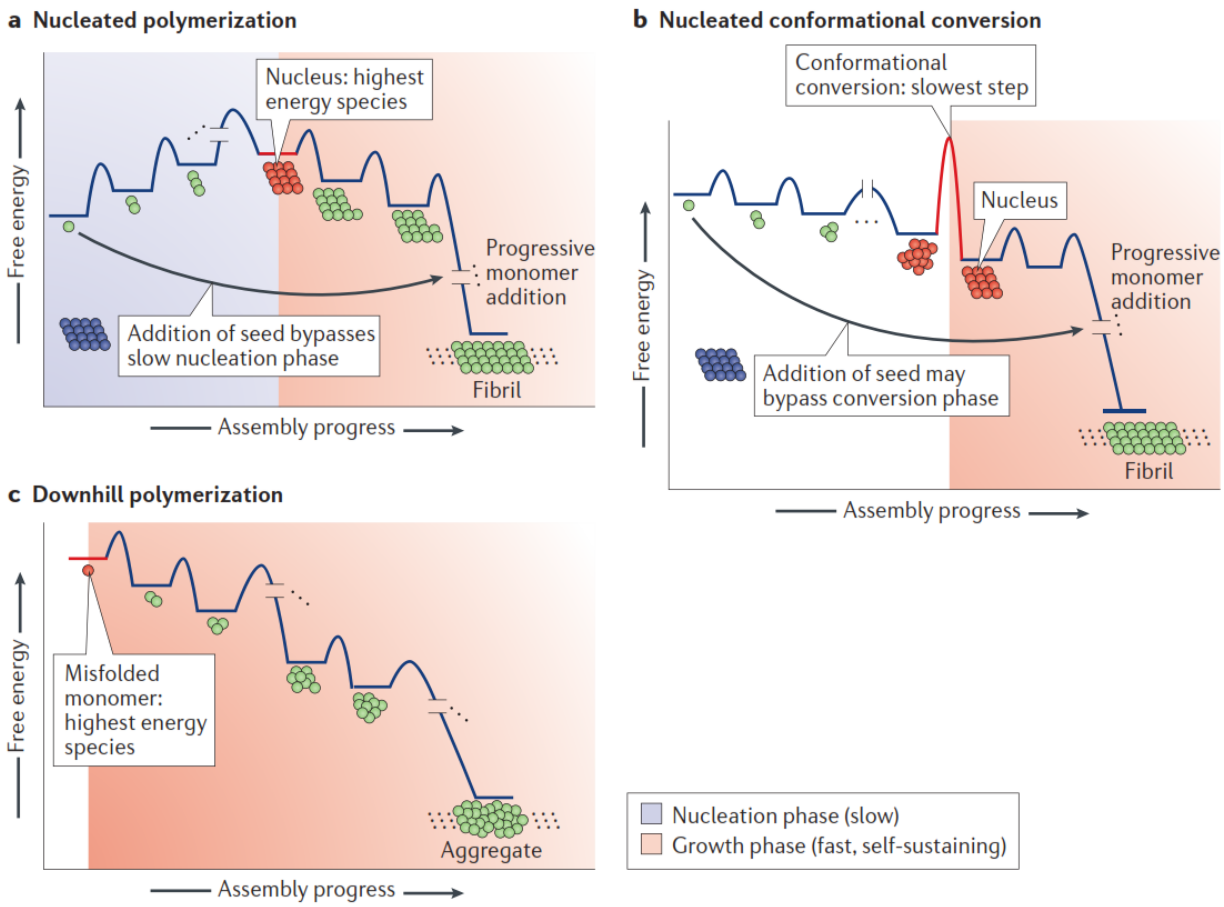


Figure 1. Transmission electron microscopy image, X-ray diffraction pattern and visualization of an amylogenic fibril of the peptide vascin. Reprinted from Jansens et al. (2019).



1505

1506 Figure 2. Thermodynamic energy requirement of various steps in protein aggregation. In
1507 **nucleated polymerization**, presumably an oligomeric nucleus rich in β-sheet structures is
1508 formed to which other oligomers or monomers can bind. In **nucleated conformational**
1509 **conversion**, the monomers are in equilibrium with heterogeneously structured oligomers
1510 which have to undergo conformational changes before initiating fibrillation. Both processes
1511 can be accelerated by adding preformed seeds. In **downhill polymerization**, misfolded
1512 monomer initiates aggregation rapidly which then can result in both amorphous or fibrillary
1513 aggregates. Reprinted from Eisele et al. (2015).

1514

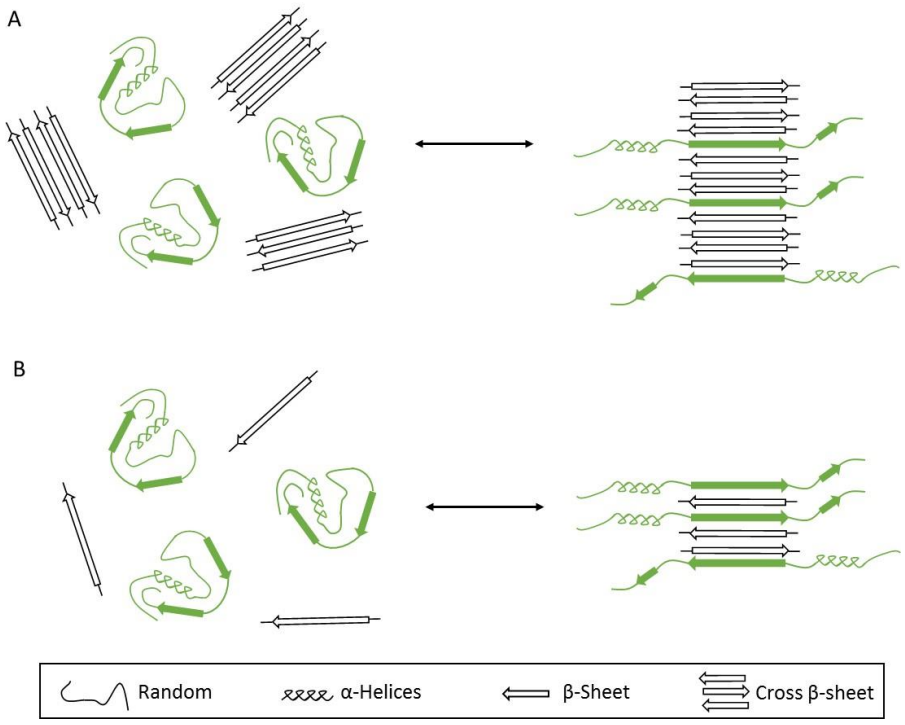
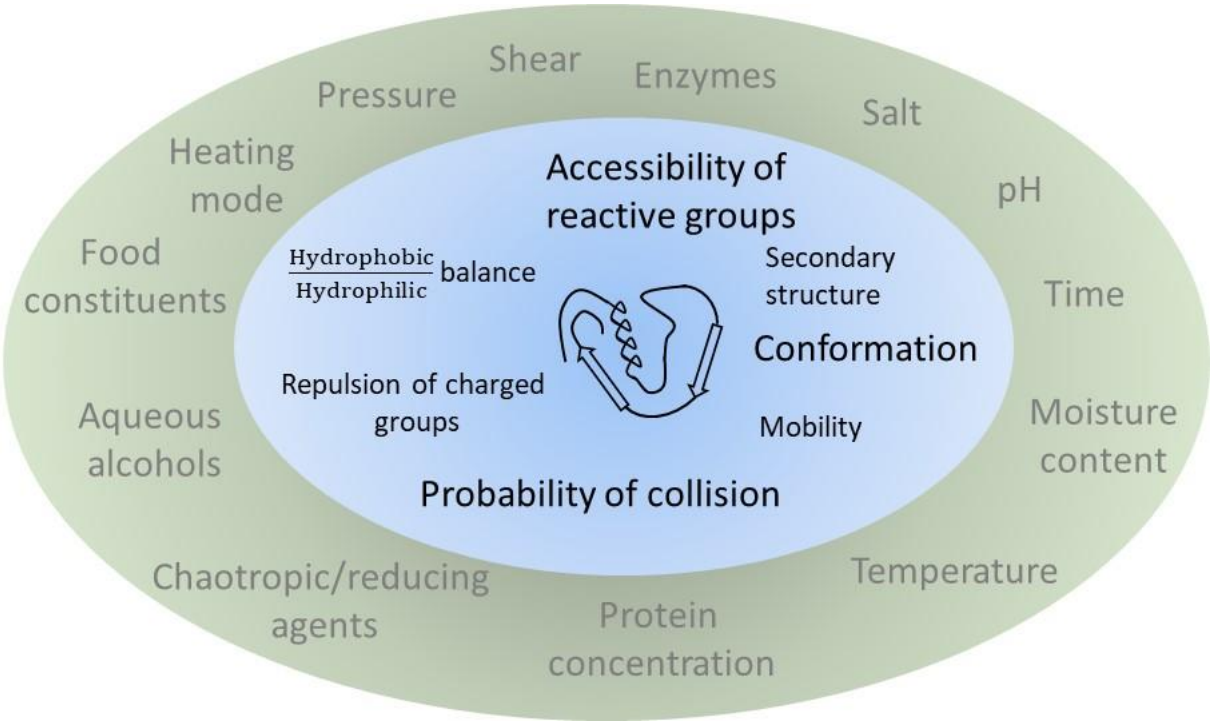


Figure 3. The concept of seeding or cross-seeding of globular proteins (green) with amylogenic oligomers (A) or peptides (B) of the same or different origin (black), respectively.



1521

1522 Figure 4. Food processing factors (grey, peripheral) impacting the properties of proteins

1523 (black, central) and their fibrillation capacity.

1524 Table 1. Overview of conditions used to induce fibrillation of ovalbumin or lysozyme proteins and/or peptides. RT, room temperature.

reference	solution properties					incubation temperature (°C)	protein concentration	incubation time	shear
	pH	pH adjusted with	salt	alcohol	chaotropic/ reducing agent				
Ovalbumin									
Bhattacharya et al. (2013)	2.2	50 mM glycine-HCl buffer	50 mM NaCl			65	100 µM	15 min - 4 h	
Bhattacharya & Dogra (2015)	2.2	50 mM glycine-HCl buffer	20 mM NaCl			65	10 µM	15 min - 4 h	
Jansens et al. (2017)	2.0	1.0 or 0.1 M HCl	0 or 0.15% (w/w) NaCl			60 - 80	2.0% (w/w)	5 - 1200 min	
	7.0	1.0 or 0.1 M HCl	0 or 0.15% (w/w) NaCl			60 - 80	2.0% (w/w)	5 - 1200 min	
Naeem et al. (2011)	2.0	10-35 mM trichloroacetic acid or 10-25 mM trifluoroacetic acid				RT	0.5% (w/v)	3 h	
Tufail et al. (2015)	2.5	glycine-HCl buffer				RT	0.1% (w/v)	2 - 15 days	90 rpm
	7.0	saline phosphate buffer				RT	0.1% (w/v)	2 - 15 days	90 rpm
Veerman et al. (2003)	2.0		10 - 35 mM			80	2.0 - 7.0% (w/v)	1 h	
Weijers et al. (2002)	2.0 - 4.0	0.1 - 5 M HCl	0 - 60 mM NaCl			80	5.0 - 10% (w/v)	60 - 80 min	
Lara et al. (2012)	2.0	1.0 M HCl	0 or 50 mM NaCl			90	2.0% (w/w)	1 - 265 h	stirred
Pearce et al. (2007)	7.0	50 mM sodium phosphate buffer	0 - 200 mM NaCl			60 - 80	0.05 - 2.0% (w/v)	5 - 30 min	
Pouzot et al. (2005)	7.0		3 - 100 mM NaCl			75 - 80	0.4 - 6.0% (w/v)	22 - 24 h	
Ovalbumin peptides									
Kawachi et al. (2013)	2.2	100 mM potassium phosphate buffer				65	0.026 - 2.0% (w/v)	1 h	
Tanaka et al. (2011)	7.5	20 mM Tris-HCl buffer	20 mM NaCl			80	0.05% (w/v)	1 h	
Modified ovalbumin									
Lassé et al. (2016)	1.6	HCl	100 mM NaCl		15 mM β-mercapthoethanol	80	1.0% (w/v)	22 h	

Tanaka et al. (2011)	7.5	20 mM Tris-HCl buffer	20 mM NaCl		15 mM dithiothreitol	80	0.05% (w/v)	1 h	
Kalapothakis et al. (2015)	6.8	10 mM ammonium acetate buffer			10 mM dithiothreitol	60 - 80	0.01 - 1.28% (w/v)	3 - 5 days	
Jansens et al. (2016)	7.0	1.0 or 0.1 M HCl			0.6% (w/w) dithiothreitol and 0.3 or 0.6% potassium iodate	60 - 80	2.0% (w/w)	60 or 1200 min	
Broersen et al. (2006)	7.0	50 mM phosphate buffer	0 or 150 mM NaCl			64 - 76	0.3% (w/v)	5 - 380 min	
Lysozyme									
Xu et al. (2005; 2008; 2007)	2.0	HCl				65	1.4% (w/v)	1 - 8 days	
Hill et al. (2009)	2.0	1.0 M HCl	175 mM NaCl			50	1.7% (w/v)	4 - 5 days	
Arnaudov & de Vries (2005)	2.0 - 4.0	1.0 M HCl	13 mM			57 - 80	1.0 - 3.0% (w/w)	5 min - 42 days	
Ow & Dunstan (2013)	1.6	0.1% HCl				55 - 80	0.02 - 0.25% (w/v)	1 - 16 h	stirred 840 rpm
Mishra (2007)	1.6	25 mM HCl				65	50 - 500 µM	5 - 140 h	
Hill et al. (2011)	2.0	25 mM potassium phosphate buffer	50 - 400 mM NaCl or 50 - 70mM $MgCl_2$ or 100 - 150 mM NaBr			20 -65	0.5 - 2.0% (w/v)	3 h - 8 days	
Ponikova et al. (2015)	2.7	70 mM glycine-HCl	80 - 200 mM NaCl, Na_2SO_4, $NaClO_4$, NaBr, NaSCN or $NaNO_3$			65	5 µM	1 - 40 min	stirring 1200 rpm
Morshedi et al. (2010)	2.5	1.0 M HCl after adding 50 mM phosphate buffer				57	0.10 - 0.20% (w/v)	1 h - 5 days	stirred 150 rpm
	3.0 - 7.0	1.0 M HCl after adding 50 mM phosphate buffer				57	0.20% (w/v)	2 days	stirred 150 rpm

Hung et al. (2010)	2.0	HCl	136.7 mM NaCl and 2.68 mM KCl		0.1 - 20.0 mM sodium dodecyl sulfate	55	0.2% (w/v)	1 - 400 h	30 rpm
Harada et al. (2008)	2.0	10 mM glycine-HCl buffer				65	0.1 mM	48 - 192 h	
Zou et al. (2013)	1.6 or pD 2.0	DCl	140 mM NaCl			62	1.5 % (w/v)	5 - 124 h	
Chaudhary et al. (2017)	1.5					65	40 µM	1 - 150 h	230 rpm
Krebs et al. (2000)	2.0	HCl				37 or 65 or 1 min heating at 100 °C followed by incubation at 37 °C	1 mM	1 - 56 days	
	7.4	NaOH		0 or 30% 2,2,2-trifluoroethanol		37 or 65	1 mM	1 - 8 days	
Yagi et al. (2009)	1.6	HCl				60	1.0% (w/v)	30 - 60 days	
Lara et al. (2011)	2.0					90	2.0% (w/v)	3 - 100 h	stirred 300 rpm
Frare et al. (2004)	2.0	10 mM HCl				65	1 mM	15 h - 10 days	
Sasaki et al. (2008)	2.2	HCl	80 mM NaCl			57	0.4% (w/v)	1 h -11 days	
Lieu et al. (2007)	2.0	HCl	136.7 mM NaCl and 2.68 mM KCl			45 - 55	0.2% (w/v)	12 h - 48 days	30 rpm
Sasahara et al. (2007)	2.0 - 6.0	0.1 M HCl	1.0 M NaCl			37 or heating from 10 to 100 °C at 60°C/h	30 µM	2 - 24 h	310 rpm
Humblet-Hua et al. (2008)	2.0	10 mM HCl				57	2.0% (w/v)	1 - 160 h	steady shear and turbulent flow (290 - 550 rpm)
Sivalingam et al. (2016)	2.0	50 mM glycine-HCl buffer				37 or 65	1 mM	2 - 15 days	static and agitated

Shah et al. (2012)	2.2	HCl	80 mM NaCl			57	0.8% (w/v)	4.5 h - 11 days	
Jayamani et al. (2017)	2.0	HCl	100 mM NaCl	10% (v/v) ethanol		65	0.125% (w/v)	240 min	150 rpm
Vernaglia et al. (2004)	6.3	20 mM potassium phosphate			0 - 8 M guanidine hydrochloride	50	0.2% (w/v)	10 min - 19 h	stirred
Wang et al. (2007)	7.4	10 mM phoshate buffer	136.7 mM NaCl and 2.68 mM KCl		0 - 8 M urea	37 - 55	0.2% (w/v)	1 - 27 days	
Khan et al. (2012)	9.2 or 13.2	20 mM glycine-NaOH (pH 9.2) or KCl-NaOH (pH 13.2)			500 µM sodium dodecyl sulfate	25	5 µM	1 h	
Aso et al. (2007)	2.0 - 12.0	20 mM HCl (pH 2), sodium phosphate (pH 7.0) and 20 mM NaOH (pH 12)	0 and 100 mM NaCl	0, 5 or 50% ethanol		37 or 57	0.3% (w/v)	2 months	
Bhattacharya et al. (2013)	7.0 or 11.0	20 mM phosphate buffer	20 mM NaCl	80% (v/v) ethanol		60	150 µM	6 h	
Goda et al. (2000)			10 mM NaCl	90% (v/v) ethanol		25	1.0% (w/v)	1 week	
Hameed et al. (2009)	12.7			20% butanol		25	1 µM	1 - 8 h	
Holley et al. (2008)				80% ethanol		22	0.3% (w/v)	1 - 60 days	with and without agitation
Lin et al. (2014)	4.8	25 mM sodium acetate buffer		0 - 90% ethanol, 2,2,2-trifluoroethanol and 1,1,1,3,3,3-hexafluoro-2-propanol		25	1.5 - 10% (w/v)	5 min - 48 h	stirred 0 or 600 rpm
Yonezawa et al. (2002)				0 - 90% (v/v) ethanol		20	0.21 - 0.96% (w/v)	24 h	
Fujiwara et al. (2003)			0.3 - 2.0 mM NaCl	90% (v/v) ethanol		10 or 20	1% (w/v)	10 min - 9 h	

Lysozyme peptides									
Frare et al. (2004)	2.0	10 mM HCl				37	0.55 mM	15 - 140 h	
Sugimoto et al. (2011)	7.5	50 mM phosphate buffer				25	50 mM	1 - 10 days	
Tokunaga et al. (2013)	2.0 - 9.0	20 mM glycine-HCl (pH 2), sodium acetate (pH 4), potassium phosphate (pH 7) or Tris-HCl (pH 9) buffer				37	0.2% (w/v)	1 - 14 days	
Modified lysozyme									
Ohkuri et al. (2005)	2.0	50 mM sodium maleate				25	0.8% (w/v)	1 - 14 days	
Mishima et al. (2007)	2.0	50 mM sodium maleate				30	0.8% (w/v)	1 day - 10 weeks	
Latif et al. (2007)	4.0	20 mM sodium acetate buffer	30 mM NaCl			25	0.8% (w/v)	6 - 8 months	
Niraula et al. (2004)	2.0 - 7.5	50 mM sodium maleate (pH 2.0 - 2.7), sodium acetate (pH 4.0) or Tris-HCl (pH 7.5) buffer				25	0.25 - 1.00% (w/v)	1 day - 9 months	
Kamatari et al. (2005)	4.0	20 mM sodium acetate buffer	30 mM NaCl			25	0.8% (w/v)	6 - 14 months	
Takase et al. (2002)	7.5	20 mM Tris-HCl buffer	0 or 0.15 M NaCl		4 mM dithiothreitol	RT	0.1% (w/v)	1 week	
Yang et al. (2015)	7.2	20 mM sodium phosphate buffer	150 mM NaCl		0 or 10 mM dithiothreitol	37	40 µM	4 - 350 h	
Cao et al. (2004)	4.0 - 5.0	20 mM acetic acid		90% (v/v) ethanol	0 or 2 - 6 mM dithiothreitol	RT	0.2% (w/v)	3 min - 1 week	