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Thermal aggregation of whey proteins under shear stress and the effects on microparticulation in a high moisture extrusion process

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Contents

Abbreviations

Latin symbols

Asp	Asparagin	
B	β-Lg monomers	
B_2	β-Lg dimers	
BSA	Bovine serum albumin	
B^{SH}	Unfolded β-Lg with exposed SH-group	
B_2^{SH}	Reactive β-Lg dimer with exposed SH-group	
B_i^{SH}	Reactive aggregate with exposed SH-group	
c_0	Native protein concentration at t = 0	%
c_t	Native protein concentration at t	%
Ca	Calcium	
CFD	Computational Fluid Dynamics	
$c_{Protein}$	Protein concentration	%
Cys	Cysteine	
d	Particle diameter	µm
$d_{10,3}$	10th-percentile of the particle size distribution	µm
$d_{50,3}$	Volume based median of the particle size distribution	µm
$d_{90,3}$	90th-percentile of the particle size distribution	µm
D	Extruder diameter	mm
DD	Degree of denaturation	%
E_a	Activation energy	kJ mol^{-1}
J	Number of collisions	
k_0	Rate constant	s^{-1}
k_{agg}	Aggregation rate	
k_B	Boltzmann constant	J K^{-1}

K_G	Equilibrium constant	
$\dot{m}$	Mass flow rate	$kg\ h^{-1}$
$\dot{m}_{Powder}$	Mass flow rate of the powder	$kg\ h^{-1}$
MWP	Microparticulated whey protein	
n	Reaction order	
n	Screw speed	rpm
N	Number of particles	
n_{act}	Actual screw speed	rpm
n_{max}	Maximum screw speed	rpm
P	Maximum engine power	kW
pI	Isoelectric point	
Q_3 (x)	Volume based particle size distribution	%
RP-HPLC	Reverse Phase High Performance Liquid Chromatography	
SH-group	Thiol group	
SME	Specific mechanical energy input	$Wh\ kg^{-1}$
SSHE	Scraped surface heat exchanger	
T	Temperature	°C
t	Time	s
$T_{P,max}$	Maximum product temperature	°C
$T_{P,out}$	Product outlet temperature	°C
WPC	Whey protein concentrate	
WPI	Whey protein isolate	
x	Particle size	µm

Greek symbols

α	Degree of unfolding	
α-La	α-Lactalbumin	
β-Lg	β-Lactoglobulin	
$\dot{\gamma}$	Shear rate	s^{-1}
δ	Clearance	mm
η_k	Viscosity of the continuous phase	Pa s
ϑ	Temperature	°C
ϑ_{Barrel}	Barrel temperature	°C
τ	Motor torque	%

1 General Introduction

The controlled, thermally induced aggregation process, during which heat and shear are applied simultaneously or sequentially to limit aggregate size, is referred to as microparticulation. Whey proteins processed accordingly represent a possibility to impart specific structural and physical properties to food. These aggregates can be applied as a fat replacer or functional ingredient to e.g. modify viscosity or increase heat stability of various food products. This targeted formation of particles with limited particle sizes can be achieved by combination of thermal and mechanical treatment. Large particles must be avoided due to their sensorially negative impact, such as e.g. sandiness or mealiness. To realize a combined heating and shearing process, different options are possible at an industrial scale. Microparticulation of whey proteins can be accomplished in a scraped surface heat exchanger or in a tubular heat exchanger followed by high pressure treatment. An alternative technique is extrusion. The shearing by the screws and the heating of the extruder barrel induce simoultanious mechanical and thermal stresses on the whey proteins. Due to this thermo-mechanical treatment, the native protein structure can be modified by denaturation and aggregation. Using extrusion for protein modification allows the variation of different process parameters in a wide range, compared to other available techniques. Another advantage is the possibility of coping with higher viscosities and therefore higher protein concentrations. Such high concentrations also allow shorter residence times due to accelerated denaturation kinetics and long run times as a result of negligible product caking, which is due to the self-cleaning effect of the screws.

The following sections will give a more detailed description of the theoretical background of the properties, and the thermal denaturation and aggregation behavior of whey proteins in relation to their influence on microparticulation, as well as the fundamentals of extrusion processes.

1.1 Bovine whey proteins

Milk proteins can be subdivided into two major fractions. Caseins are, by definition, insoluble at pH 4.6, whereas whey proteins are soluble under the ionic conditions of milk and stable against degradation by rennet. Whey proteins have typically a globular structure (rather susceptible to denaturation by heat), with high levels of secondary and tertiary structures, in which acidic/basic and hydrophobic/hydrophilic amino acids are distributed in a balanced way along their polypeptide chains. Whey proteins consist of different subfractions. The major whey protein is β-lactoglobulin (β-Lg), while minor whey proteins are mainly α-lactalbumin (α-La) and bovine serum albumin (BSA). Immunoglobulins, lactoferrin, and lactoperoxidase also belong to the whey proteins (Walstra et al., 1984; Edwards et al., 2009). It can be assumed that β-Lg dominate the reaction kinetics during processing, although it cannot be excluded that the minor whey proteins might play a certain role with regard to the denaturation and aggregation behavior. In order to understand protein interaction during denaturation and aggregation, the molecular properties of the individual whey protein fractions, especially of the major ones, would have to be known.

However, whey proteins are mainly used in their natural mixture in form of whey protein concentrate (WPC) and whey protein isolate (WPI). To produce these, the liquid native whey has to be concentrated (Foegeding et al., 2011; Huffman & James Harper, 1999). The main components of sweet whey are lactose (4.8% w/w), protein (0.6% w/w) and minerals (0.6% w/w). Using ultrafiltration allows to increase or modify the composition of the dry mass in whey and to increase the percentage of whey proteins up to 65% (w/w) of the dry mass. To further increase protein concentration, a subsequent diafiltration can follow. To this end, water is added to the retentate of the ultrafiltration process with simultaneous removal of the permeate. As a result, salt and lactose concentrations decrease, while the protein concentration reaches up to 80% (w/w) in dry matter (Bake, 2012). Liquid WPC is usually further evaporated and spray dried. To reach even higher protein concentrations (>90% (w/w)), WPI can be produced using additionally ion exchange chromatography (Foegeding et al., 2011).

β-Lactoglobulin

β-Lg being the major whey protein in bovine milk represents about 50% of the total whey proteins. Sweet whey contains ~3.3 g/L of β-Lg. The molecule has a very compact globular structure composed of 162 amino acids, each monomer having a molecular weight of 18.3 kDa (Creamer et al., 2011; Fox, 2009; Kinsella & Whitehead, 1989; Wit & Klarenbeek, 1984). The diameter of β-Lg is about 4.5 nm (Gebhardt et al., 2012) and the isoelectric point (pI) ~5.2 (McKenzie, 1971). Ten genetic variants of bovine β-Lg (A to J) could be identified. The most common variants are A and B, which differ by two amino acids, Asp_{64} and Val_{118}, and Gly_{64} and Ala_{118} in variant A and B, respectively (Edwards et al., 2009; Farrell, Jr. et al., 2004). Studies on the secondary structure of β-Lg revealed that it comprise 6 - 15% α-helix, 43 - 52% β-sheets, 8 - 10% turn and 32 - 35% random coil (Qi et al., 1997; Fox, 2009; Mulvihill & Donovan, 1987; Sawyer & Kontopidis, 2000). β-Lg belongs to the family of the lipocalins (Sawyer & Kontopidis, 2000; Brownlow et al., 1997; Kontopidis et al., 2004). The tertiary structure consists of a cup-shaped eight-stranded β-barrel (calyx) flanked by a three-turn α–helix. In this hydrophobic pocket, small apolar molecules can be bound (Brownlow et al., 1997; Edwards et al., 2009; Jameson et al., 2002). β-Lg comprises of five cysteine residues in the amino acid sequence, which form two stabilizing intramolecular disulfide bonds in the native protein. A first one between Cys_{66} and Cys_{160} as well as a second one between Cys_{106} and Cys_{119}. In the native state, the free thiol group, mainly at Cys_{121}, is buried inside the hydrophobic pocket. For this reason, it is not accessible for further interactions. Yet, during thermal denaturation the free thiol group interacts with intermolecular disulfide bonds of other protein molecules (Papiz et al., 1986; Walstra et al., 1984; Fox, 2009). Values for the denaturation temperature found in literature vary, ranging from 65 - 70 °C (Iametti et al., 1996; Qi et al., 1997; Dissanayake et al., 2013; Griffin et al., 1993). The quaternary structure varies between monomers, dimers and oligomers depending on pH, temperature and ionic strength. At the physiological conditions of milk, dimers are predominant (McKenzie & Sawyer, 1967; Gottschalk et al., 2003; Kinsella & Whitehead, 1989).

α-Lactalbumin

α-La represents about 20% of the total whey protein (~1.2 g/L) and is a compact, highly structured globular protein. It consists of 123 amino acids with a molecular weight of 14.2 kDa (Fox, 2009; Brew, 2011; Wit & Klarenbeek, 1984). A Stokes radius of 1.9 nm is reported for α-La monomers (Gast et al., 1998). The pI lies between pH 4.2 and 4.5 (Farrell, Jr. et al., 2004; Belitz et al., 2001). Two predominant genetic variants are presently known, which differ in one amino acid, Glu_{10} in variant A and Arg_{10} in variant B (Gordon, 1971). The secondary structure is divided into two domains (α- and β- domain). The larger α-domain is composed of three major α-helices and two short helices. The small β-domain has barely any regular secondary structure. It is composed of a series of loops, a small three stranded antiparallel β-sheet and a short 3_{10} helix (Pike et al., 1996). α-La is genetically and structurally very similar to c-type lysozyme (Kuwajima, 1996; Gordon, 1971; Farrell, Jr. et al., 2004). The polypeptide chain contains eight cysteine residues that are connected by four intramolecular disulfide bonds: Cys_{6}-Cys_{120}, Cys_{28}-Cys_{111}, Cys_{60}-Cys_{77} and Cys_{73}-Cys_{90} (Edwards et al., 2009). In contrast to β-Lg, α-La does not contain a free thiol group (Brew, 2011; Considine et al., 2007). It is a metalloprotein, being able to bind one Ca^{2+} per molecule in a pocket at the physiological conditions of milk (holo-form). When the pH is reduced to pH < 4, the four Asp residues forming the pocket become protonated and lose their ability to bind Ca^{2+} (apo-form). The Ca^{2+}-containing protein is characterized by an increased heat stability (Patocka & Jelen, 1991; Fox, 2009). The denaturation temperature of holo α-La is ∼62 °C (Wit & Klarenbeek, 1984; Yutani et al., 1992), while for the apo α-La this temperature is reduced by ∼20 °C (Bernal & Jelen, 1984; Zhong et al., 1999).

Bovine serum albumin

BSA being present in whey with a concentration of ∼0.3 g/L represents about 8% of the total whey protein (Farrell, Jr. et al., 2004; Wit & Klarenbeek, 1984). It consists of 583 amino acids with a molecular weight of 66.4 kDa (Hirayama et al., 1990; Belitz et al., 2001). The pI of BSA lies between pH 4.7 and 4.9 (Farrell, Jr. et al., 2004). The secondary structure of BSA is comprised of 44% α-helices, 14% β-strands and 42% random coil at pH 7 (Saikia et al., 2014). However, the secondary as well as the tertiary structure strongly depend on the pH (Navarra et al., 2016). The tertiary structure usually reveals three equal sized globular domains. It has one free thiol group (Cys_{34}) and 17 intramolecular disulfide bridges, which hold the protein in a multi-loop structure (Walstra et al., 1984; Giancola et al., 1997). The free thiol group can be involved in covalent protein cross-linking, which is based on thiol-disulfide reactions during heating. The unfolding temperature is reported at around 64 °C (Giancola et al., 1997; Wit & Klarenbeek, 1984; Hendrix et al., 2000).

1.2 Thermal denaturation of whey proteins

The denaturation of whey proteins can be described in a simplified way by two successive steps, the unfolding of the protein and the subsequent aggregation. The thermal denaturation mechanism of whey proteins and particularly β-Lg is the subject of various studies resulting in a number of different models (Roefs & Kruif, 1994; Tolkach & Kulozik, 2007; Mulvihill & Donovan, 1987; Aymard et al., 1996; Elofsson et al., 1996; Iametti et al., 1996; Nicolai et al., 2011). The majority of the studies on thermally induced whey protein denaturation and aggregation are conducted at physiological conditions (pH 6.7, ionic strength and concentration similar to that of whey).

1.2.1 Mechanism and kinetics of unfolding and aggregation

Thermal denaturation mechanism

The thermally induced denaturation of β-Lg is a multistep reaction (Fig. 1-1). At 25 °C and under physiological conditions of milk and whey, β-Lg exists as a non-covalently linked dimer stabilized by hydrogen bonds. The initiation step of heat-induced β-Lg denaturation involves a reversible dissociation of native dimers to native monomers at a temperature above 40 °C, shifting the reversible equilibrium between dimers and monomers [$(\beta\text{-Lg})_2$ ↔ 2 β-Lg] to the right (Wit, 2009; Iametti et al., 1996; Qi et al., 1997). Upon further heating, the native monomers undergo a reversible intramolecular transition into the so-called R-state that differs from the native state only by minor conformational changes of some side chains. This Tanford transition can be detected by a change in optical rotation (Tanford et al., 1959). These small conformational changes in the tertiary structure of the β-Lg result in better accessibility of the free thiol group, which is masked by the α-helix in the native state (Tolkach & Kulozik, 2007; Qi et al., 1997; Oldfield et al., 1998; Wit, 2009; Sakurai & Goto, 2006). In this state, monomers can in principle form aggregates. However, the aggregation rate is extremely low and is not considered in the aggregation processes discussed in the present work. When the temperature increases above ∼65 - 70 °C, the β-Lg monomers undergo further conformational changes and partially unfold, which is called “molten globule state”. As a consequence, the free thiol group is fully exposed onto the outer surface of the molecule (Roefs & Kruif, 1994; Qi et al., 1997; Schokker et al., 1999; Prabakaran & Damodaran, 1997). Furthermore, the hydrophobic amino acids become exposed, thereby increasing the hydrophobic attraction between them (Relkin & Mulvihill, 1996; Considine et al., 2007; Havea et al., 2004). These unfolding related changes in structure are for a big part reversible.

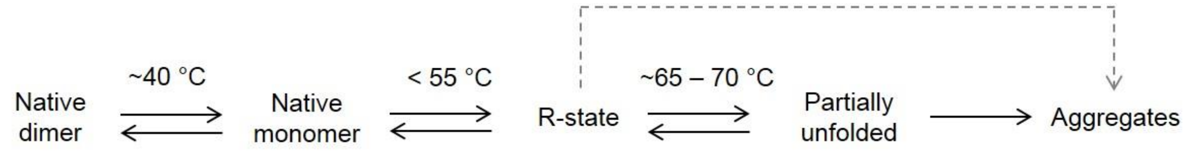

Fig. 1-1: Thermal denaturation mechanism of β –Lg.

The aggregation of the partially unfolded β-Lg monomers follows in a next step. In contrast to unfolding, aggregation is an irreversible reaction, taking place mainly by intermolecular linkage via disulfide bonds and hydrophobic interactions. The aggregation rate strongly depends on milieu and conditions during heat treatment, which are discussed in section 1.2.2. At temperatures above ∼130 °C, complete unfolding of the remaining protein structure is observed, probably induced by chemical breakdown of disulphide bonds (Wit & Klarenbeek, 1981; Shimada & Cheftel, 1988; Paulsson et al., 1985).

The focus of the present work is on aggregation of whey proteins, which is therefore looked upon in more detail. At temperatures above ~60 °C, heat induced denaturation of β-Lg can simply be characterized by a three-state/two-step model (Fig. 1-2) as stated by Tolkach & Kulozik, (2007).

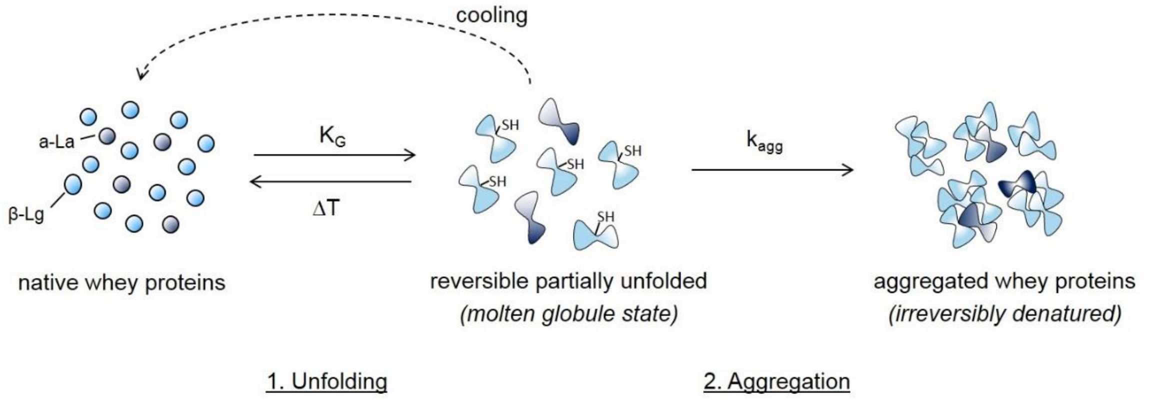

Fig. 1-2: Reaction schema for the two-step process for the heat denaturation of whey proteins (adapted from Tolkach & Kulozik (2007)).

It involves native molecules, reversible partially unfolded molecules and irreversibly denatured, aggregated molecules. In the first step, the native molecules partially unfold until a dynamic equilibrium (with the equilibrium constant K_G) between these two states is reached. In the subsequent second step, the partially unfolded molecules irreversibly aggregate with the aggregation rate k_{agg}. If the temperature of the protein solution is cooled under the denaturation temperature, the protein molecules in their partially unfolded state can refold into the native state. In contrast, the aggregated molecules are irreversibly denatured and cannot fold back. Due to the equilibrium between the native and the partially unfolded form, an increased k_{agg} causes an increased unfolding rate. Since molecules are withdrawn from the unfolded state during aggregation, unfolding increases to maintain the equilibrium between the native and the partially unfolded proteins. Consequently, the total denaturation reaction accelerates with increasing k_{agg} (Tolkach & Kulozik, 2007).

The kinetics and mathematical equations of β-Lg depletion are described in detail in section 3 in Wolz & Kulozik (2015).

Mechanism of whey protein aggregation and relevant types of molecular interactions

Different models try to describe the denaturation process and in particular the aggregation mechanism. On a molecular scale, the model of Roefs & Kruif (1994) is widely recognized and applied (Considine et al., 2007; Hoffmann et al., 1996; Hoffmann et al., 1997b; Verheul, 1998; La Fuente et al., 2002; Schokker et al., 1999).

It postulates a thiol-disulfide exchange by analogy with polymer radical chemistry. The model proposes an initiation step, a propagation step and a termination step (Roefs & Kruif, 1994):

$$B_2 \leftrightarrows B \rightarrow B^{SH} \qquad (1.1)$$

$$B + B_i^{SH} \rightarrow B_{i+1}^{SH} \qquad i \geq 1 \qquad (1.2)$$

$$B_i^{SH} + B_j^{SH} \rightarrow B_{i+j} \qquad i, j \geq 1 \qquad (1.3)$$

The initiation step (eq. 1.1) is the dissociation of the β-Lg dimers (B_2) into monomers (B) and the reversible partial protein unfolding associated with exposure of the free thiol group (B^{SH}). This expose of the free SH-group activates the β-Lg monomer. The propagation step (eq. 1.2) corresponds to the aggregation via thiol-disulphide exchange reactions. This step is characterized by formation of an intermolecular disulphide bond between an exposed SH-group of an activated β-Lg intermediate (B^{SH}) and one of the disulfide bonds of a native β-Lg (B). The resulting reactive dimer (B_2^{SH}) results in reactive aggregates ($B_{i/j}^{SH}$), upon repeated reactions. This propagation step is very fast and can be happen multiple times resulting in a chain reaction. The reaction terminates when an aggregate without a reactive SH-group is formed. In the termination step (eq. 1.3), two activated intermediates ($B_{i/j}^{SH}$) react with each other via oxidation and form larger aggregates linked by disulphide bonds. Molecular oxygen or other oxidizing agents can induce oxidation of the free thiol group to a disulphide bond. This reaction is only possible if the thiol group is sterically free to do so. This holds particularly true if the protein molecule, to which the thiol group is attached, is comparatively large (Wit, 2009; Livney et al., 2003). If other whey proteins, such as α-La or BSA, are present in the solution, the reaction scheme can be extended by an extra propagation step to accommodate the reaction between β-Lg and α-La or BSA (Roefs & Kruif, 1994).

Roefs & Kruif (1994) derived this model from kinetic experiments showing an overall reaction order of 1.5. The model can explain the aggregation of β-Lg in solution at a relatively low temperature of 65 °C over serval hours, at low ionic strength and at neutral pH. Wit (2009) questions the free radical induced mechanism and proposes a reaction based on the presence of thiolate ions (B^{S^-}) instead of an activated thiol group (B^{SH}). Thiolate ions are specific catalysts for the exchange of disulphide groups in proteins and explain the pH dependency of the aggregation reaction (Cecil, 1963).

The aggregation via disulfide bonds can describe the findings of many studies (especially at low temperatures) and is commonly accepted as the major mechanism during β-Lg denaturation. However, it cannot describe the complete aggregation mechanism. Multiple studies showed that, next to disulphide-linked aggregates, aggregates linked by non-covalent interactions are also formed (Verheul et al., 1998; Oldfield et al., 1998; Havea et al., 2004). The non-covalent interactions between the aggregates are mainly of hydrophobic nature or mediated by hydrogen bonds (La Fuente et al., 2002; Nicolai et al., 2011; Havea et al., 2004). Just as the free thiol group, the hydrophobic groups buried in the inner structure of the native protein are also exposed during heating and thus, accessible for interactions (Hoffmann & Mil, 1997). In fact, formation of larger aggregates occurs via a complex mechanism involving both physical aggregation (by non-covalent interactions) and chemical aggregation (by disulfide exchange reactions) (Mulvihill & Donovan, 1987; Verheul, 1998). The contribution of the different types of bonds to the aggregation process is not fully understood and also depends on process and environmental conditions (La Fuente et al., 2002; Mounsey & O'Kennedy, 2007). For example, chemical aggregation is favored at high heating temperatures, high pH values, and low ionic strength, whereas physical reactions are enhanced at low heating temperatures, low pH values and high ionic strength (Wijayanti et al., 2014; Guyomarc'h et al., 2015).

Another way to examine the mechanism of whey protein aggregation, besides the one at molecular sacle (described above), is the one on particular level. For reactions at pH > 5.7, Nicolai et al. (2011) proposed a mechanism to describe the thermally induced aggregation process of β-Lg (Fig. 1-3) at particular level. The mechanism strongly depends on the prevailing conditions, in particular the pH.

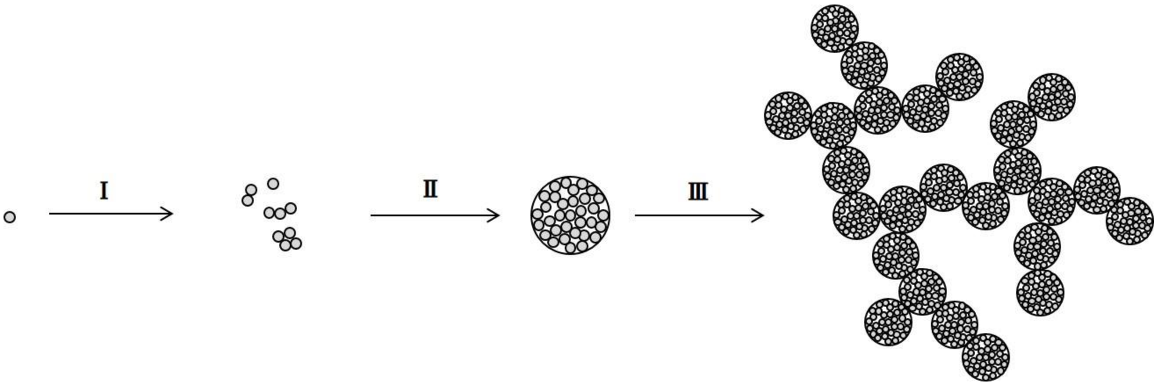

Fig. 1-3: Schematic representation of the mechanism of the β–Lg aggregation process (not in scale) (adapted from Nicolai et al. (2011) and Aymard et al. (1996)).

During heating, native β-Lg molecules undergo conformational changes as described in Fig. 1-1. When the molecules reach the partially unfolded state, first small oligomers are formed (I). Above a critical association concentration, the oligomers associate into relatively monodisperse, so-called primary aggregates (Ⅱ). Size and shape of the primary aggregates as well as the critical association concentration depend on the environmental conditions. At higher protein concentrations, the primary aggregates form larger self-similar aggregates (Ⅲ). In mixtures with other whey proteins e.g. α-La or in WPI, aggregates with similar structures are formed by co-aggregation of the different proteins present in the mixture

(Nicolai et al., 2011; Aymard et al., 1996). A similar mechanism is already described by Verheul et al. (1998). They observed that the concentration of these primary particles grows as the amount of non-aggregated protein decreases in time. The native β-Lg acts as a source that produces primary particles. Above a certain concentration of primary particles, a secondary aggregation mechanism (Schmoluchowski-type) becomes predominant (Verheul et al., 1998). At high concentrations, secondary aggregation of the primary products leads to larger, randomly branched clusters with a self-similar structure (Phan-Xuan et al., 2013; Aymard et al., 1996; Mehalebi et al., 2008). Above a critical gel concentration, a system spanning gel network is formed (Durand et al., 2002; Nicolai et al., 2011). Furthermore, Zhang et al. (2016) observed larger aggregates, whose form suggest a self-similar fractal structure. These structures are formed via a cluster-cluster type aggregation mechanism (Zhang et al., 2016; Kolb et al., 1983).

Aggregation of mixtures of different whey proteins

Although it is accepted that the characteristics of β-Lg dominate the behavior of the whey protein aggregation, other whey proteins can also play an important role in the aggregation process. Molecular complexes can be formed between the different whey proteins during heat-induced aggregation. However, the mechanism of these interactions are still not completely elucidated (La Fuente et al., 2002). Petit et al., (2016) observed that the aggregation rate of β-Lg increases in the presence of other whey proteins, such as α-La and BSA, which are able to form disulphide bonds with β-Lg. Similar results were also reported by Kehoe et al., (2007). Replacing β-Lg with increasing amounts of BSA, while keeping the total protein concentration constant, significantly increased the denaturation rate of β-Lg (Kehoe et al., 2007).

In contrast to β-Lg, α-La does not aggregate by itself, if heated up to 75 °C. However, at high temperatures pure α-La aggregates via hydrohobic interactions (McGuffey et al., 2005). The protein is characterized by a strong reversibility against unfolding. α-La has a high apparent heat stability (holo- α-La), due to the absence of a free thiol group, which prohibits the formation of intermolecular covalent bonds (Mulvihill & Donovan, 1987; Schokker et al., 2000; Gezimati et al., 1997). If proteins containing a free thiol group (such as β-Lg or BSA) are present, the intramolecular disulphide bonds of α-La also react with partially unfolded β-Lg molecules, catalyzing new intra- and intermolecular bonds between the proteins (Calvo et al., 1993; Schokker et al., 2000; Hong & Creamer, 2002). Furthermore, BSA can also accelerate the denaturation of β-Lg (Gezimati et al., 1996) and α-La (Havea et al., 2000). In a mixture of β-Lg, α-La and BSA each fraction can aggregate with itself as well as with each other via disulfide bonds and hydrophobic interactions (Havea et al., 2001; Dalgleish et al., 1997). The latter gain in importance when the protein concentration increases (Havea et al., 1998). The aggregation mechanism of a whey protein mixture is illustrated in Fig. 1-4 .

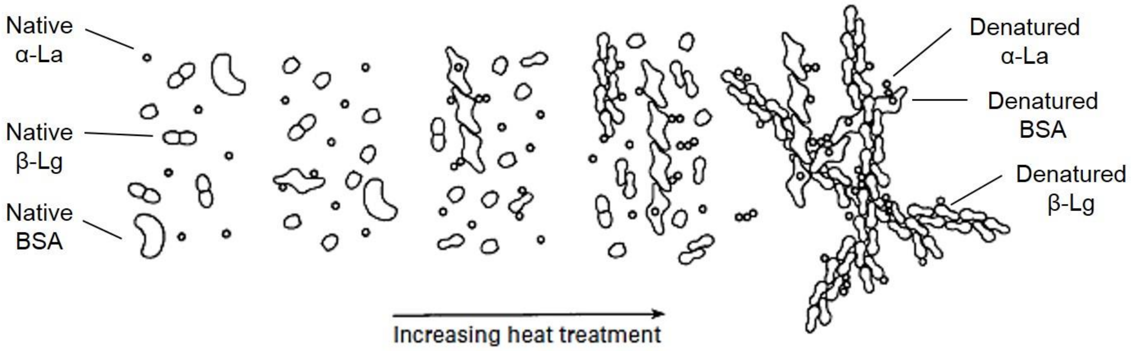

Fig. 1-4: Schematic representation of the aggregation of mixtures of β-Lg, α-La and BSA in heated solutions (adapted from Havea et al. (2001)).

The aggregation mechanism of single whey proteins as well as of whey protein mixtures, like in commercial WPC and WPI, strongly depends on environmental conditions. The proportion of different whey proteins in the aggregates, the extent of aggregation as well as the contribution of the stabilizing forces are studied to a limited extent only and strongly depend on concentration and ionic environment.

1.2.2 Factors affecting thermally induced denaturation and the resulting aggregate size

From literature, it is clear that the denaturation of β-Lg (as well as of all whey proteins) depends on temperature, heating time, shear stress, concentration, other additives (like lactose), pH and ionic strength. These numerous factors make this process very complex. Depending on the values of the other parameters, the dependence of the rate of denaturation on any of these parameters may be different. The complexity can be explained, in part, by the fact that unfolding and aggregation are not influenced in the same way when conditions are varied (Nicolai et al., 2011). Environmental factors are only briefly addressed since their variation was not part of this study (except protein concentration), but may account for diverging findings of other studies. The main focus of this work was the influence of the processing conditions.

1.2.2.1 Processing conditions

Temperature and heating time

Increasing temperature can negatively impact the thermodynamic stability of proteins. Destabilizing effects include reduction of activation energy, increased protein diffusion and increased frequency of molecular collisions as well as enhancement of hydrophobic interactions. Consequently, higher temperatures are a common parameter to accelerate protein denaturation. The influence of temperature already becomes apparent in Fig. 1-1. Heating time and temperature are two of the main process variables influencing the temperature-dependent denaturation reaction. With increasing temperature or heating time, the degree of denaturation increases (Hillier & Lyster, 1979; McSwiney et al., 1994; Dannenberg & Kessler, 1988; Kerche et al., 2016).The depletion rate constant of β-Lg ($k_{T/n}$) strongly depends on the temperature and can be described by the Arrhenius equation (eq. 3.3). The temperature dependence of the depletion rate can be shown in the Arrhenius representation. Above a critical temperature (for β-Lg at 90 °C and for α-La at 80 °C in milk), Dannenberg & Kessler (1988) already observed a significant change in slope and a strong reduction of activation energy. This bend temperature divides the Arrhenius plot into two parts. The low temperature range (below the bend temperature) is attributed to limitation of the unfolding reaction, while at the high temperature range (above the bend temperature) the rate limiting step is considered to be aggregation (Dannenberg, 1986; Sava et al., 2005; Tolkach, 2008). To give a mathematical interpretation to the sharp edge of the denaturation rate constant in the Arrhenius plot, Tolkach & Kulozik (2007) developed a model in which they distinguish explicitly between the unfolding and the subsequent aggregation step (section 1.2.1).

Temperature influences both unfolding and aggregation behavior, and thus, size and the structure of the aggregates. Kennel (1994) investigated whey protein isolate solutions (8% (w/w)) at temperatures between 90 and 150 °C (DD>90%) without shear stress (perikinetic aggregation) and observed an increasing aggregate size with increasing temperature. At temperatures <100 °C, porous and loosely structured aggregates with a high amount of combined water were formed. At temperatures >120 °C, aggregates were larger and denser. Furthermore, the heating up time can influence the aggregate structure (Kennel, 1994). Elofsson et al. (1996) and Ndoye et al. (2013) also found increasing aggregate sizes with rising temperature (59 - 63 °C and 75 - 90 °C, respectively) and heating time. In contrast, Bon et al. (1999) showed that, at a lower temperature level (55 to 87.8°C), the size and structure of β-Lg aggregates is independent of temperature.

At conditions with applied shear stress (orthokinetic aggregation), larger aggregates are formed with increasing heating temperature (<90 °C) (Erabit et al., 2014; Steventon, 1992; Ndoye et al., 2013). Plock (1994) related the increase of aggregate size to the increase in degree of denaturation (e.g. with increasing heating time).

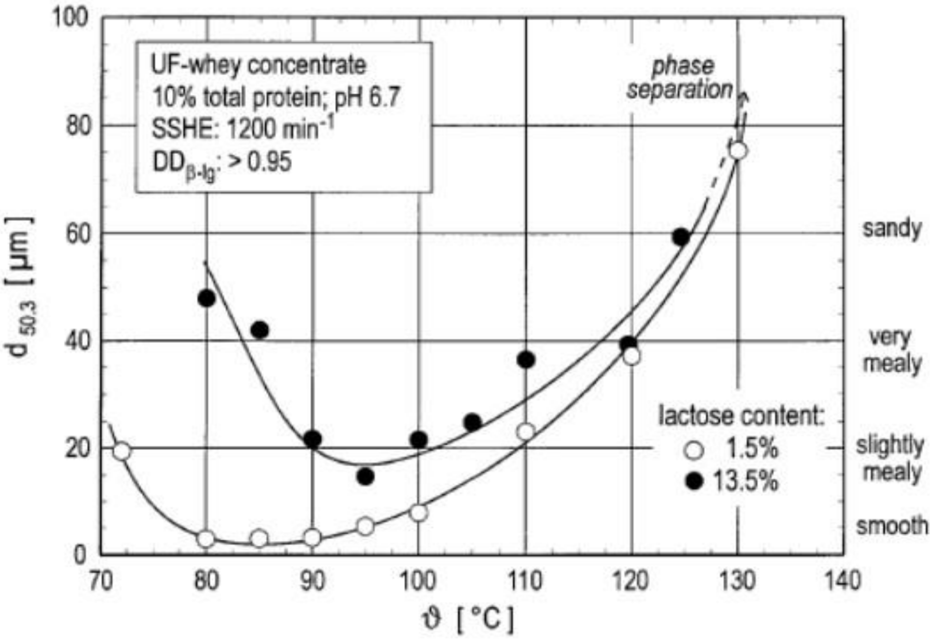

Fig. 1-5: Effect of the heating temperature on the aggregate size of heat- and shear-treated whey protein concentrate (Spiegel, 1999b).

Simmons et al. (2007) and Spiegel (1999a) found smaller, more weakly bonded aggregates at lower temperatures (unfolded limited area) and denser, more rigid aggregates at higher temperatures (and higher shear rates). According to Spiegel (1999a), the smallest particles are formed when the proteins are heated at temperatures near the bend temperature, where unfolding and aggregation occur at almost the same rate (Fig. 1-5). For extrusion, Quéguiner et al. (1992b) observed an increased proportion of large WPI aggregates coagulated at pH 3.9 at high barrel temperatures (120 °C).

Furthermore, the heating rate also plays an important role. Moakes et al. (2015) heated WPI solutions ($c_{Protein}$ = 10%) at 80 °C for 10 min with varying heating rates (1 - 10 °C min^{-1}), different shear rates (200 - 800 s^{-1}) and with quiescent cooling. In the aforementioned study, they presented that fast heating rates promote particles that interact quickly consequently leading to formation of large aggregates. On the other hand, low heating rates or increasing shear rates lower the aggregation rate causing particles to be more affected by the flow. Therefore, smaller aggregates are produced (Moakes et al., 2015).

Shear stress

At quiescent conditions, a thermal treatment of whey protein solutions above a critical concentration causes the formation of a gel network. During concurrent heat and hydrodynamic shear treatment, formation of a gel network can be inhibited and single aggregates are built. Shear stress can have three different effects on the size and structure of proteins and protein aggregates (Taylor & Fryer, 1994): Mechanical forces/shear stress can denature proteins, break-up particles or induce aggregation.

To achieve denaturation of small globular proteins in water, Jaspe & Hagen (2006) calculated that extreme shear rates of ~10^7 s^{-1} would be required. For whey proteins, Steventon (1992) found no indication of denaturation at a shear rate of up to 3000 s^{-1} at temperatures below 60 °C. Thomas & Geer (2011) came to the conclusion that hydrodynamic forces alone are not responsible for structural changes in proteins, but that interfacial phenomena are also critical. Denaturing during shearing is influenced by synergetic temperature effects or by additional turbulences in fluid flow (Walkenström et al., 1998).

A more detailed explanation regarding the effect of shear stress, including the equation for perikinetic and orthokinetic aggregation as well as the mechanism of fragmentation, is given in section 4 in Wolz et al. (2016b).

Decreasing aggregate size with increasing shear stress could be observed during concurrent heating and shearing of whey protein suspensions at neutral pH in a scraped surface heat exchanger. Studies on this topic were conducted by Plock (1994) for re-suspended sweet whey powder at low shear rates from 16.1 to 80.5 s^{-1} and by Spiegel (1999a) for whey concentrate at shear rates of 105 to 628 s^{-1}. Spiegel (1999a) also observed that decreasing particle sizes are particularly pronounced for shear rates <500 s^{-1}. His findings were confirmed by measurements with a rheometer. Steventon (1992) also studied the thermal aggregation behavior in a rheometer using a protein suspension ($c_{Protein}$=7% (w/w)) of WPC35 at 80 °C and shear rates from 288 to 1476 s^{-1}. He ascertained a decrease of particle size with increasing shear rate. This observation was explained by lower collision effectiveness and not by break-up of aggregates, which conflicts with his later publication in which he attributed the result to shear-induced fragmentation (Steventon et al., 1994). Furthermore, Steventon (1992) observed an increase in particle size with increasing shear rate at short periods of heating, but a decrease in size at longer heating times (Fig. 1-6).

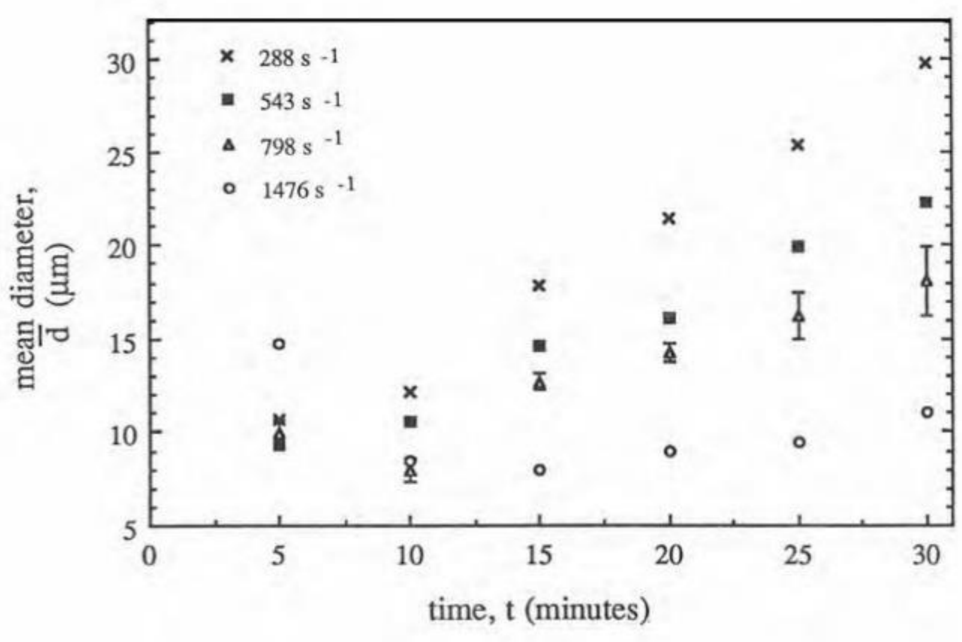

Fig. 1-6: Effect of shear rate and heating time on the aggregate mean diameter at ϑ = 80 °C after t = 30 min (Steventon, 1992).

Similar results were obtained by Simmons et al. (2007) for protein concentrations of ~0.5% and shear rates of 111 to 624 s^{-1} in a rheometer (Fig. 1-7). In this study, the shear rate was step-changed (from 111 s^{-1} to the targeted shear rate) after 20 min of the experiment, in order to separate the effects of denaturation and aggregation. It was postulated that aggregation is a function of the applied shear field, whilst temperature mainly affects the unfolding step.

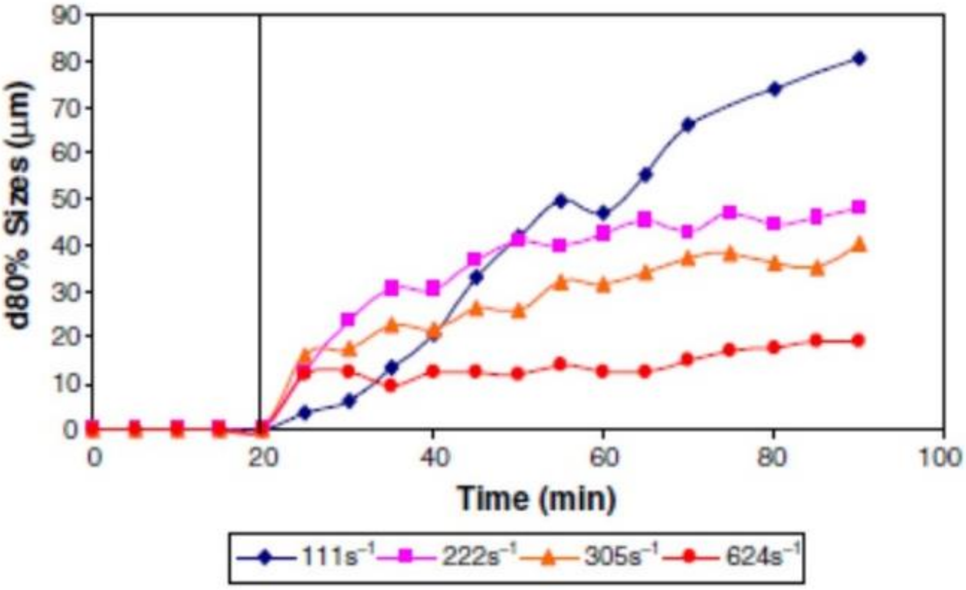

Fig. 1-7: Effect of shear rate and heating time on the aggregate size d_{80} during step change experiments from 111 s^{-1} after t = 20 min at ϑ = 70 °C (Simmons et al., 2007).

Erabit et al. (2014), in contrast, measured a decreasing concentration of small aggregates (1-10 µm) and an increasing concentration of large aggregates (20 - 200 µm) while applying shear stress ($\dot{\gamma}$ = 0 - 400 s^{-1}) during the heat treatment (ϑ = 67 - 95 °C, t = 0 - 240 s). Upon shear treatment, larger and more stable aggregates were observed, which was attributed to an increased number of collisions of molecules (Erabit et al., 2014). It was, thus, concluded that an increasing shear rate up to a critical value increases the probability of collisions and the aggregation rate, resulting in larger particles. At shear rates above the critical value, the lower effectiveness of collisions or the break-up of aggregates is responsible for the formation of smaller particles. The results obtained by Erabit et al. (2014) are in line with the one described above, due to the short heating times. During extrusion of whey proteins ($c_{Protein}$ = 20%) at acidic pH, Quéguiner et al. (1992b) observed smaller particles at higher screw speed, thus higher shear forces (75 to 200 rpm) in spite of shorter residence times. In the aforementioned study, much higher protein concentrations were used.

1.2.2.2 Environmental conditions

Protein concentration

The influence of protein concentration on denaturation of whey proteins and in particular on β-Lg at quiescent conditions (perikinetic conditions) was shown in various studies e.g. in Kessler & Beyer (1991), Verheul et al. (1998), Hoffmann & Mil (1997), Anema et al. (2006) and Nielsen et al. (1996). The higher the protein concentration, the faster the denaturation reaction is. A reaction order n = 1.5 could be determined for the denaturation of β-Lg in UF whey concentrate (Spiegel, 1999a) of skim milk (Dannenberg, 1986; Beyer, 1990), reconstituted whole milk (Anema & McKenna, 1996) and whey protein concentrate (Plock et al., 1997; Tolkach & Kulozik, 2007). The reaction order n reflects the dependence of the reaction on the initial concentration. n>1 indicates that an increase in denaturation is expected with increasing concentration. This can be explained by the mechanism of denaturation (section 1.2.1). Thermal denaturation behavior was looked at in previous studies at protein concentrations of up to 5% (w/w) by Donovan & Mulvihill (1987), Kessler & Beyer (1991), Bon et al. (1999), Plock et al. (1997) and Zuniga et al. (2010) and up to 10% (w/w) by Fitzsimons et al. (2007) and Roefs & Kruif (1994). Only a few studies targeted protein contents of up to 20% (w/w) (Dissanayake et al., 2013; Nielsen et al., 1996).

Aggregate size is also affected by the protein concentration. At conditions without shear, Hoffmann et al. (1997a) showed that the molecular weight of the aggregates increases with increasing β-Lg concentration (10 - 100 g/L), demonstrating that the average size of the aggregates increases. At a temperature of 65 °C, this shift towards higher molecular masses was much stronger than the shift that was observed as a function of heating time. The study of Iametti et al. (1995) and Aymard et al. (1996) suggested that the protein unfolding is independent of protein concentration, whereas aggregation is highly dependent on concentration. The aggregation rate is proportional to the β-Lg concentration (Elofsson et al., 1996; Bon et al., 1999; Mehalebi et al., 2008). A measureable increase of the size occurred after a lag period, which length is dependent on heating temperature and protein concentration (Elofsson et al., 1996; Steventon, 1992; Bon et al., 1999; Verheul, 1998).

Findings obtained at conditions without shear cannot simply be transferred to aggregation processes under shear stress. Thermal aggregation at conditions with applied shear stress must be considered separately. Under concurrent heating and shearing, Spiegel (1999a) noted a decrease in aggregate size with increasing protein concentration (5 to 20% (w/w)). Steventon (1992) also confirmed that aggregate size decreases with increasing protein concentration (from 3.5 to 10% (w/w)) during heating at 86 °C for 30 min and a constant shear rate of 543 s^{-1} (see Fig. 1-8).

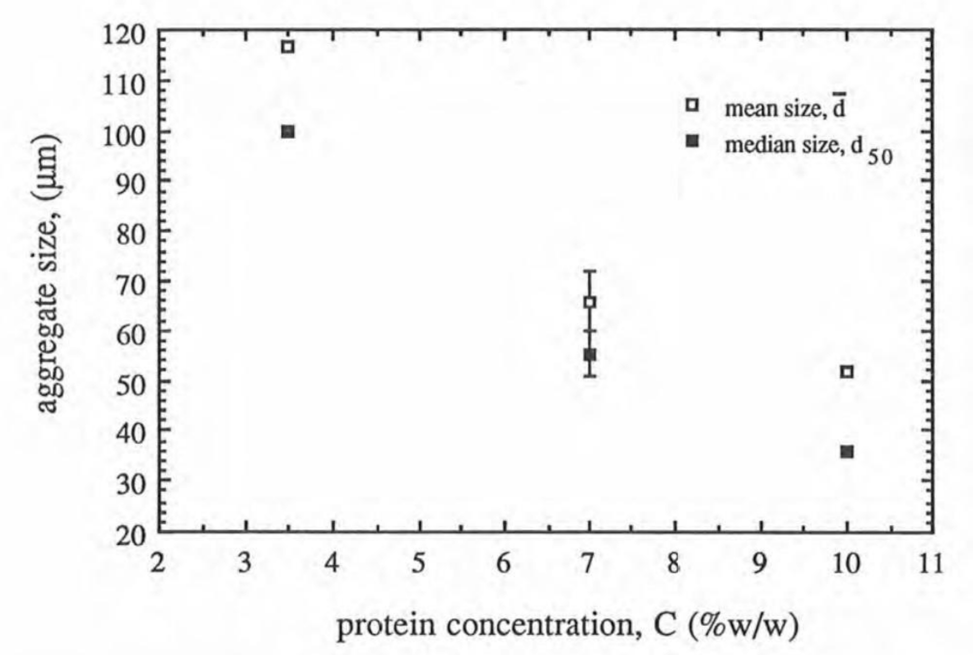

Fig. 1-8: Effect of protein concentration on the aggregate size formed at $\vartheta = 86$ °C, $\dot{\gamma} = 543\ s^{-1}$ and t = 30 min (Steventon, 1992).

The increase in viscosity can be considered as a possible reason for these results. Due to the higher viscosity, diffusion and motion of whey protein molecules and aggregates is limited, subsequently the collision rate is reduced. Additionally, shear forces and shear stress are increased, which reduces the effectiveness of collisions (Steventon, 1992; Spiegel, 1999a; Kennel, 1994).

Lactose concentration

Due to the protecting effect of sugars on the protein unfolding, low lactose concentrations result in an increase in denaturation temperature of β-Lg (Wit & Klarenbeek, 1984; Plock et al., 1998b). A high lactose concentration decelerates the denaturation rate of whey proteins (Anema et al., 2006; Hillier et al., 1979). Furthermore, an increase in the kinetic bend temperature has been reported for increasing lactose concentrations or decreasing pH values (Dannenberg & Kessler, 1988; Spiegel & Huss, 2002; Tolkach & Kulozik, 2005). The effect of the lactose concentration on β-Lg denaturation can most likely be explained using the 'preferential hydration' theory of Arakawa & Timasheff (1982). For globular proteins such as α-La and β-Lg, increasing concentrations of sugars increase the ordering of the water structure around the protein molecules. Addition of lactose results in an unfavorable free energy change of the system. This effect increases with increasing surface area of the proteins since unfolded proteins have more unfavorable protein-water interactions than native proteins. It also explains the stabilizing effect of lactose on the native protein structure and its enhancing effect of protein associations (Arakawa & Timasheff, 1982). However, lactose seems to stabilize the proteins mainly at lower temperatures. At higher temperatures (> 90 °C), almost no protective effect of lactose can be observed (Plock et al., 1998b; Anema & McKenna, 1996).

During concurrent heating and shearing in a scraped surface heat exchanger (SSHE), lactose concentration also influences size and structure of whey protein aggregates. Plock (1994) reported that the measured aggregate growth started at a lower level and increased already at lower degrees of denaturation with increasing lactose concentration. However, for high lactose concentrations, he reported, only results for low degrees of denaturation. For low lactose concentrations, only high degrees of denaturation are given. Spiegel (1999b) observed that smaller aggregates are formed at lower lactose levels. As the lactose concentration increases, whey protein aggregates become bigger and softer. This fact is probably due to the ability of lactose to steer particulation of loosely structured aggregates (Spiegel, 1999b). Contrary results were obtained by Toro-Sierra (2016), who noted a decreasing particle size of β-Lg and WPI aggregates with increasing lactose concentration and attributed this to the unfolding limiting effect of lactose on the whey proteins.

pH value

The pH value directly affects the charge of the proteins and thereby influences their conformation stability. For decreasing pH values, an increase in denaturation temperature, i.e. an increasing stabilization of the native conformation of β-Lg, was shown. In the pH range from 4.0 to 3.0, maximum stability was detected (Verheul, 1998; Wit & Klarenbeek, 1981; Relkin & Mulvihill, 1996; Hegg, 1980; Harwalkar &

Ma, 1992; Liu et al., 1994). Furthermore, a decrease in the rate of denaturation with decreasing pH is observed at a temperature up to 90 °C (Farrag et al., 1997; Tolkach & Kulozik, 2005; Guy et al., 1967).

The effect of pH on aggregation, and thus on aggregate size, is complex because pH affects protein charge, conformation, and sulfhydryl reactivity. A change in pH value affects intra- and intermolecular interactions between β-Lg molecules. The thermal stability maximum of globular proteins lies around the isoelectric point. In this pH range, most of the amino acids a protonated. Therefore, the intramolecular electrostatic repulsive forces are at their minimum. As a result, the conformational stability of the proteins is high (Donovan & Mulvihill, 1987; Kella & Kinsella, 1988). The high stability of β-Lg against unfolding in the pH range between pH 3.5 and 5.0 is associated with the highly ordered structure of octamers (Spiegel, 1999a; Kella & Kinsella, 1988). At low pH values, proteins are positively charged and electrostatic interactions between monomers are purely repulsive. Hence, aggregation is prohibited (Verheul, 1998; La Fuente et al., 2002). Moreover, a decrease in denaturation rate at acidic pH is attributed to a lower accessibility of the free thiol group. The mechanism of aggregation under these conditions is considerably different from that at neutral pH. The disulphide bond interchange, which is involved in the aggregation process at neutral pH, is highly unlikely to play a role, because the thiol groups are very stable at low pH (La Fuente et al., 2002; Shimada & Cheftel, 1988; Hoffmann & Mil, 1997).

At heating conditions without shear stress, different structures are formed at varying pH values. For example, Jung et al. (2008), who studied aggregation at pH 2.0, 5.8 and 7.0, observed, long rigid strands (fibrils), spheres and small curved strands, respectively. Loveday et al. (2017) reported that β-Lg assembles into fibrils when heated at low pH and low inonic strength. Individual fibrils have a diameter of 5 – 10 nm and the length can exceed 10 µm (Fig. 1-9).

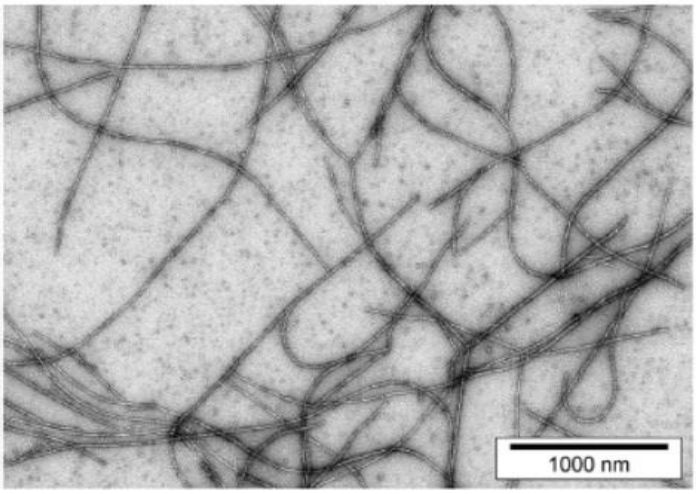

Fig. 1-9: Transmission electron micrograph of bovine β-Lg fibrils formed by heating at pH 2 and 80 °C (Loveday et al., 2017).

Partially unfolding of the tertiary structure allows β-sheets to come into contact and form hydrogen-bonded stacks. Low inonic strength and low pH ensure that electostatic repulsion among positively-charged monomers and peptides inhibits random aggregation (Loveday et al., 2017).

The size of random aggregates increases with increasing pH values (pH 4 to pH 7) at conditions without (Donato et al., 2009) and with shear stress (Spiegel, 1999a; Quéguiner et al., 1992b; Steventon, 1992). However, these studies compare particle sizes for aggregates formed at different pH values, yet at identical heating conditions. Thus, different degrees of denaturation as a consequence of unequal denaturation kinetics are compared. At pH values > 5.5, denser and more compact aggregate structures were observed. This phenomenon was attributed to a more ordered aggregation mechanism (Steventon, 1992).

Ionic environment

Electrolytes can affect the colloidal stability of proteins by four related mechanisms. First, electrolytes impact solubility of proteins, observed as either salting-in or salting-out of native proteins (Hofmeister effects). Second, electrolytes impact heat-induced protein unfolding. Third, an increased ionic strength weakens electrostatic repulsion. Fourth, salts may have protein-specific effects such as ligand binding or as part of their inherent structure. For example, α-La with bound calcium (holo form) is more heat stable than without calcium (apo form) (Ryan et al., 2013). Thus, depending on the conditions during heating (e.g. pH value, lactose concentration and temperature) salts and especially calcium can have different effects on whey protein unfolding and aggregation. Due to changes in intramolecular interactions, binding of calcium on the protein can increase its stability against thermally induced unfolding (Plock, 1994; Harwalkar & Ma, 1992; Petit et al., 2016). In contrast, at high salt concentrations, hydrophobic attraction is strengthened and electrostatic attraction is weakened. As a result, the aggregation rate increases (Verheul, 1998; Petit et al., 2011; O'Kennedy & Mounsey, 2009). Furthermore, calcium-induced aggregation occurs via ion binding between calcium and the negatively charged carboxyl group of the protein molecule (Rham & Chanton, 1984; Petit et al., 2011; Simons et al., 2002). In the presence of salts, and in particular calcium, larger and denser aggregates are induced (Schmitt et al., 2007; Donovan & Mulvihill, 1987; Pouzot et al., 2005; Phan-Xuan et al., 2014; Croguennec et al., 2004; Sağlam et al., 2012). For aggregates produced under shearing conditions, different results are reported. Erabit et al. (2013) observed an increasing particle size with increasing calcium concentration. In contrast, Simmons et al. (2007) saw that the addition of minerals to a WPC solution resulted in the formation of smaller aggregates, but also increased deposition onto the surface of the heating equipment used. However, it should be taken into account that very high protein concentrations were used in the latter study, which might lead to crystallization of calcium.

1.3 Microparticulation of whey proteins

Principle and Methods

Microparticulation is a controlled, thermally induced aggregation process, during which shear forces are applied simultaneously or sequentially to limit aggregate growth or to reduce resulting aggregate size Fig. 1-10.

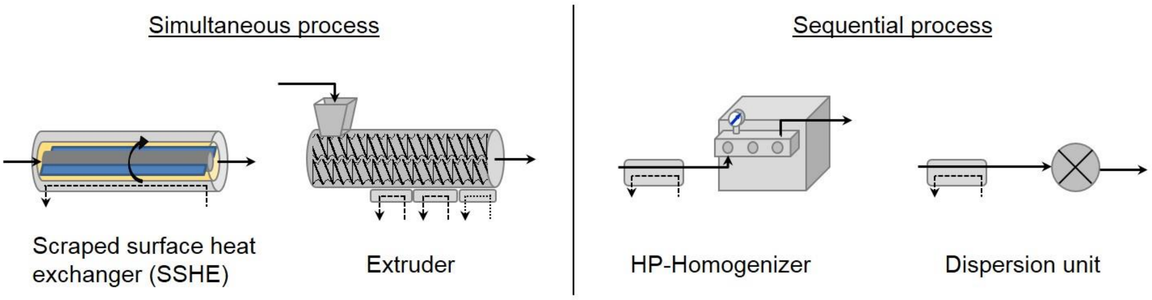

Fig. 1-10: Overview of different methods of microparticulation.

Both the process and the use of microparticulated whey proteins (MWP) was originally patented by Singer et al. (1988). They heated a WPC50 dispersion at acidic pH (pH 3.5 - 5.0) together with an emulsifier in a specially designed SSHE. High soluble spherical whey protein particles in a size range from 0.1 – 3 µm were reported (Singer et al., 1988; Singer & Dunn, 1990). Based on this work, a commercial fat replacer (Simpless®) was launched by NutraSweet (today CPKelco). Plock (1994) also used a SSHE to particulate whey proteins. Solutions with 10% protein and at pH of 6.5 were heated at 80 °C until different degrees of denaturation (max. 70%) were archieved. A strong increase in particle size was observed with increasing degree of denaturation, decreasing both shear rate and lactose concentration. First systematic investigations on the influence of different variables were conducted by Spiegel (1999a). He particulated concentrated whey corresponding to WPC35 and WPC80 in a SSHE at different process and milieu conditions to achieve a high degree of denaturation. He found that the particle properties depend on the different reaction mechanisms in the unfolding and aggregation limited temperature area. The smallest aggregates were observed at the bend temperature (Spiegel, 1999b). At low pH values (pH 4 - pH 5), small particles (< 5 µm) were induced independently of the heating temperature and the lactose concentration. This was attributed to the low reactivity of the free thiol group at this pH-range (Spiegel & Huss, 2002). Based on these results, a commercial process with a specifically designed SSHE was brought to the market (APV LeanCreme™, SPX Flow

Technology). Lactose can partially be replaced by inulin to reduce calorie content (Tobin et al., 2010).

An alternative technique is extrusion, as used by Quéguiner et al. (1992b) for microparticulation. In this study, WPI was coagulated by thermo-mechanical processing in a twin-screw extruder. However, their goal was to produce semi-solid spreads, which were only obtained at acidic pH between 3.5 - 3.9 and at an extruder barrel temperature of 90 - 100°C and a screw speed of 100 - 200 rpm. At these conditions, mean particle diameters of 11.5 µm were obtained. In contrast, higher pH values produced a grainy texture. However, the conditions during processing were only varied to a limited extent. Experiments were conducted at a fixed protein concentration of 20% and a total mass flow rate of 20 kg/h. The influence of the barrel temperature was investigated in the range of 85 - 120 °C and the screw speed from 75 - 200 rpm.

Micro-particles can also be produced by a sequential process (commercial process: CreamoProt, LTH Dresden). In this case, the whey protein concentrate or solution is heated in a first step e.g. in a tubular heat exchanger and then high-pressure treated in a second step e.g. by a microfluidizer (Paquin et al., 1992; Dissanayake & Vasiljevic, 2009). Compared to the previously described process, heating and shearing are not applied simultaneously. Solutions with a protein concentration of 10% (w/w) were heated until complete denaturation. The obtained aggregates were subsequently microfluidized at 75 MPa (Paquin et al., 1992) or 140 MPa (Dissanayake & Vasiljevic, 2009). This resulted in particle sizes ranging from 0.26 to 71.9 µm (average diameter of 4.77 µm) and particles around 10 µm, respectively.

Another process was developed by Asher et al. (1992), applying no additional mechanical treatment. The fat substitute Dairy-Lo^{TM} is produced by heating whey concentrate (9% total solids, pH 6.1) in a plate heat exchanger at ~80 °C for ~17 s. In this process, the degree of denaturation must be between 60% and 80% (Asher et al., 1992).

On laboratory scale, whey protein behavior during concurrent thermal and mechanical treatment was studied by Steventon, Donald, & Gladden (1994), Erabit et al. (2014), Simmons et al. (2007) and Walkenström et al. (1998) using a couette apparatus.

Application

MWP can be used to partially mimic the effect of fat and to generally design structure in food products in terms of texture and mouthfeel. The use of MWP as a fat substitute provides an opportunity to develop low-fat products, which have comparably sensory properties of full-fat alternatives (Civille, 1990). The application of MWP in various dairy products has already been extensively investigated. This includes ice cream (Yilsay et al., 2006; Koxholt et al., 1999; Prindiville et al., 2000), yoghurt (Torres et al., 2011; Tamime et al., 1995; Sandoval-Castilla et al., 2004; Barrantes et al., 1994; Janhøj et al., 2006), emulsions (Sun et al., 2015; Çakır-Fuller, 2015; Chung et al., 2014), and cheese (Sturaro et al., 2014; Steffl, 1999; Lee et al., 2013; Hinrichs, 2001; Sturaro et al., 2015; Schenkel et al., 2013). Furthermore, MWP can be used to increase the heat stability of high protein food (Sağlam et al., 2014) and beverages (Ryan & Foegeding, 2014). Purwanti et al. (2012) added MWP to protein gels to reduce their stiffness. Renard et al. (2002) and Torres et al. (2016) showed that the effect of MWP on protein network properties is also influenced by aggregate size.

However, enhancement of creaminess in low-fat products is the main reason for application of MWP. The creamy and smooth texture can be explained by the so-called ‘ball bearing mechanism’. MWP particles are expected to roll freely over one another in response to applied shear (Cheftel & Dumay, 1993). Liu et al. (2016) showed that MWP poses good lubrication properties by reducing the friction coefficient effectively. They attributed this to the ‘ball bearing mechanism’. Frøst & Janhøj (2007) reviewed the understanding of creaminess. They concluded that texture properties play an important role for the creaminess of dairy products. Sensory properties correlating most with creaminess irrespective of product type are: smoothness, fatty after-mouth-feel and cream flavor (Frøst & Janhøj, 2007). However, MWP contributes to fat-related sensations in a different way than oil does. The perception of MWP particles is related to the size of the particles, as well as the properties of the surrounding matrix (Liu et al., 2016).

However, depending on the application, the function of added MWP is not only to ensure the desirable creaminess, but also to ensure gel strength and avoid graininess and syneresis of yoghurt. The functionality of MWP in such systems depends on the ability of the particles to interact with the rest of the protein matrix during processing (i.e., heat treatment and acidification) (Ipsen, 2017). In other systems, notably cheese, MWP should, however, not interact with the rest of the matrix, but rather act as an inert filler within the system (Ipsen, 2017).

1.4 Extrusion

Screw extruders are used in a wide variety of applications. They are the major processing machines in the thermoplastics industry (White & Kim, 2010; Kohlgrüber & Bierdel, 2008; Rauwendaal, 2010). Screw extruders are also employed in large numbers in both the food and feed industry (Moscicki & Zuilichem, 2011; Kohlgrüber & Bierdel, 2008).

1.4.1 Basics of extrusion

Extrusion is a continuous high temperature short time process that transforms a variety of raw materials into modified intermediate or finished products. It combines several unit operations within one machine including heating, conveying, mixing, cooking, kneading, shearing and shaping (Riaz, 2000; Guy, 2001). Extrusion processing is widely used to restructure starch- and protein based raw materials to manufacture a variety of different textured foods (Harper, 1981; Guy, 2001; O'Connor, 1987), ranging from the simplest expanded snacks to highly processed meat analogues. The most popular extrusion-cooked products include: direct extruded snacks, ready-to-eat cereal flakes, baby food, pet food, texturized vegetable protein, crispbread and confectionery (Moscicki, 2011; Frame, 1994; Mercier et al., 1990; Riaz & Rokey, 2012). Upon heating and shearing during the extrusion process, the macromolecules in food ingredients lose their native, organized tertiary structure (Harper, 1986). Cooking is accomplished by application of heat, either directly via steam injection or indirectly via jackets, as well as by dissipation of mechanical energy during shearing. Cooking of food ingredients during the extrusion process results in gelatinization of starch, denaturation of protein, inactivation of enzymes, destruction of naturally occurring toxic substances and reduction in microbial counts of the final product (Harper, 1981; Maskan & Altan, 2011).

The principal advantages of the modern food extruder are (Kumar et al., 2010; Harper, 1981; Guy, 2001; Moscicki, 2011): (1) *Versatility* – The extruder can produce a wide variety of products of different shape, texture and appearance by changing ingredients and processing conditions. (2) *Automated production* – The extruder can provide continuous, high throughput and can be fully automated which can increase productivity. (3) *High product quality* – As cooking is done at high temperatures for a very short time, less destruction of heat sensitive ingredients occurs. At the same time, major undesired components of food, such as enzymes and microorganisms are destroyed. (4) *Reduced cost* – Extrusion has a lower processing cost compared to other cooking and forming processes. Extrusion processing also requires less space per unit of operation than traditional cooking systems. (5) *Absence of process effluents* – Extrusion produces little or no waste streams and thus, spares processors the effort to install effluent treatment systems.

Extrusion refers to a process during which a soft material is forced through a die. Several designs are possible for extruders. Screw extruders contain one or more rotating screws to either mechanically and/or thermally process and form a material. This work focuses on co-rotating, intermeshing twin-screw extruders, as these are the most relevant for processing food products and biopolymers (Lengerich et al., 2007; Kumar et al., 2010; Harper, 1981; Frame, 1994). In these extruders, the screws are in constant interaction with each other, creating natural wiping action. Hence, the screws create a positive movement of the material although the machine is not fully filled. The intermeshing, co-rotating twin screw extruder is a positive displacement pump which allows handling of a large range of materials (Bouvier & Campanella, 2014; Fellows, 2009). Theses extruders provide a high degree of heat transfer, improved conveying and superior mixing capabilities, controlled narrow residence time distribution (RTD), self-cleaning mechanisms and a uniform, yet flexible process (Frame, 1994; Riaz & Rokey, 2012; Zuilichem & Stolp, 1984).

1.4.2 The extrusion process

An extrusion-cooker is a process reactor, including screws with a certain lay-out, an installed main drive as well as a barrel with a heating and cooling jacket to control the reactions (Fig. 1-11). Adequate use of these components allow the manufacture of processed materials due to heating and shearing. For example, denaturation of proteins in the presence of water and rupture of starches are both affected by the combined effects of heat and shear (Moscicki & Zuilichem, 2011).

1.4.2.1 Extruder setup

A typical twin-screw extrusion system is shown in Fig. 1-11. Dry solid ingredients from storage bins are conveyed to the solids **feeders** and are metered into the extruder at the feed hopper. A variable speed metering and feeding screw is used to discharge continuous and uniform materials to the extruder. Typically, gravimetric feeders are used to maintain control of mass flow rates into the extruder barrel (Mercier et al., 1990; Kumar et al., 2010; Riaz & Rokey, 2012). Liquid feeds can be pumped into the extruder at desired locations. The **extruder barrel** is the cylindrical casing, which fits tightly around the screw of the extruder. A barrel is made up of several segments. The barrel is constructed of special hard alloys to withstand the pressure developing inside the barrel and to resist wear. The interior surface of the barrel is grooved to prevent slippage of materials on the walls. The extruder barrel is equipped with a heating jacket to regulate the barrel temperature either electrically or by fluid or steam heating. The outside of the barrel is covered with cooling channels for circulation of a heating or cooling medium e.g. water (Lengerich et al., 2007; Guy, 2001; Riaz, 2000).

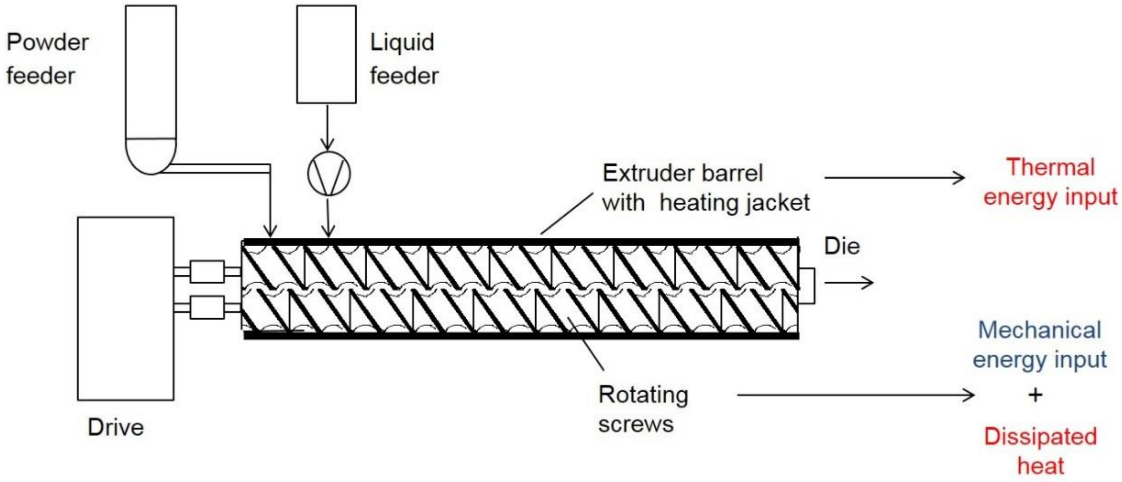

Fig. 1-11: Schematic illustration of a twin-screw extruder.

The **electrical drive** needs to provide sufficient torque to process and convey the material along the screws and to force it through the extruder die (Lengerich et al., 2007; Riaz, 2000). The **screws** are the central part of an extruder. The major change in molecular conformation of the raw or preconditioned food ingredients, occurs within the extrusion screws. The screws rotate inside the barrel and convey the material form the feed end to the discharge end. The screw elements are assembled on splined shafts and can be removed from the extruder barrel. The number and design characteristics of screw elements depend on process requirements. The screws of an extruder can be composed of different types of screw elements with different geometries tailored to specific process and product requirements. They can be configured to enhance conveying, kneading, shearing, pressure development, and filling of the screw. Conveying sections are usually only partially filled with product and therefore impart relatively little energy and shear (Harper, 1986; Kumar et al., 2010; Lengerich et al., 2007). Kneading elements create a significant shearing/mixing action and dissipate large amounts of mechanical energy. Reverse screw elements or kneading elements can be used to increase screw fill. The amount of shear imparted is affected by the kneading-block design. Shear increases with increasing numbers of lobes on the kneading blocks (Riaz, 2000; Harper, 1986). The extruder barrel is equipped with a **die** having one or more openings through which the extrudate flows. The openings shape the final product and provide a resistance against the flow of extrudate (Harper, 1986; Kumar et al., 2010; Riaz, 2000). In fact, there are applications where no die is required (Bouvier & Campanella, 2014).

Heat is applied by viscous dissipation of mechanical energy, by heat transfer via the barrel jacket, and/or by incorporation of steam injected through the barrel wall and mixed into the product. Through hydration, denaturation, and melting processes, these energy additions transform the food ingredients into a continuous viscous, plasticized material (Harper, 1986; Moscicki & Zuilichem, 2011).

1.4.2.2 Process parameters

Independent process parameters include variables associated with the extruder equipment (e.g. screw configuration, barrel temperature), as well as parameters associated with the product formulation (e.g. moisture content, raw material characteristics) and operational variables (e.g. screw speed, mass flow rate). These parameters can be controlled directly. Dependent parameters (also called system parameters) are those variables, which are dependent on the magnitude of the independent variables. Independent process parameters and raw material characteristics affect the properties of the final product by influencing the extruder response inside the barrel, and thus, the system parameters. Such parameters include the specific mechanical energy input (SME), specific thermal energy input and the residence time distribution (Kumar et al., 2010; Guy, 1998).

The properties of an extruded product are dependent on the composition and properties of **feed ingredients**. Additional water can be added directly to the feed, injected into the barrel or added in form of steam to a preconditioner or into the barrel. The actual moisture in an extrusion process strongly affects viscosity and flow behavior of the product (Mercier et al., 1990; Maskan & Altan, 2011). Changing the **mass flow rate** (at constant product composition) also changes the degree of filling in the extruder. This directly affects the area of heat exchange and thus the amount of heat exchanged (Bouvier & Campanella, 2014). In contrast to this positive effect, increasing mass flow results in decreasing energy input per mass unit delivered by the screws (at otherwise constant process parameters), while the torque increases. Furthermore, studies showed that mass flow also affects the flow conditions and the residence time distribution in the extruder. An increasing mass flow changes the flow conditions towards plug flow behavior (Gogoi & Yam, 1994; Yeh et al., 1992). Extruders have a variable speed drive which enables the **screw speed** to be changed. Screw speed directly affects the degree of barrel fill and hence residence time distribution as well as shear stress on the material being extruded. Screw speed also affects the mechanical energy input. Increasing screw speed elevates the friction between product and screw and thus more mechanical energy is produced (Kumar et al., 2010; Frame, 1994; Maskan & Altan, 2011). The **configuration of the screw** can be changed by using different conveying and mixing elements such as forward screws, kneading screw, and reverse screw elements. These elements can be combined in different ways to achieve variation in the geometric configuration of the extruder. Using flow restricting elements such as reverse elements will increase the degree of filling and the residence time (Yacu, 2011; Harper, 1981; Riaz, 2000). Set point **temperature of the barrel** can be changed to achieve a particular temperature profile within the barrel. Barrel heating causes conductive and convective heat transfer into the product. As with all heat transfer equipment, the rate of heat transfer is a function of the surface area, the temperature difference between material boundary layer and metal barrel and heat transfer coefficients. Moreover, increasing the barrel temperature increases product temperature resulting in a decrease in product viscosity. Most extruders run with temperature control while the degree of indirect heating or cooling depends on how the extruder is operated (Yacu, 2011; Frame, 1994; Kumar et al., 2010).

1.4.3 Approaches to analyze the effects of extrusion processes

A major challenge is to identify decisive variables of the extrusion process to predict process behavior and structural changes, which determine product characteristics. Extrusion is a continuous process operating in a dynamic steady state equilibrium, where the input variables are balanced with the outputs. Extruders are often considered as black boxes. There is a lack of knowledge on the effect of process parameters on resulting conditions in the extruder and the, thereby, induced chemical and biological changes in the product. For this reason, process control, optimization and design of new extrusion processes and products are still mostly based on empirical knowledge and trial and error experiments (Guy, 2001; Frame, 1994; Emin & Schuchmann, 2017). The **empirical approach** links independent process variables (e.g. screw speed, barrel temperature, feed composition) to final product characteristics. These parameters determine the extent of macromolecular changes taking place during extrusion, which in turn influence the rheological properties of the product inside the extruder and, consequently the characteristics of the extrudates (Meng et al., 2010). This empirical approach was successfully used for various conventional food products. However, this approach cannot account for the mechanisms causing product changes neither can it provide fundamental understanding of the process. Furthermore, process scale-up remains challenging, as gained information is highly material and machine dependent (Emin & Schuchmann, 2017).

The development of new processes or new materials is often assisted by the use of additional approaches, which are able to contribute information on the process conditions in the extruder and consequently the product characteristics (Della Valle et al., 2011). Hence, Meuser et al. (1982) developed the **system analytical approach** for extruded starch (Fig. 1-12).

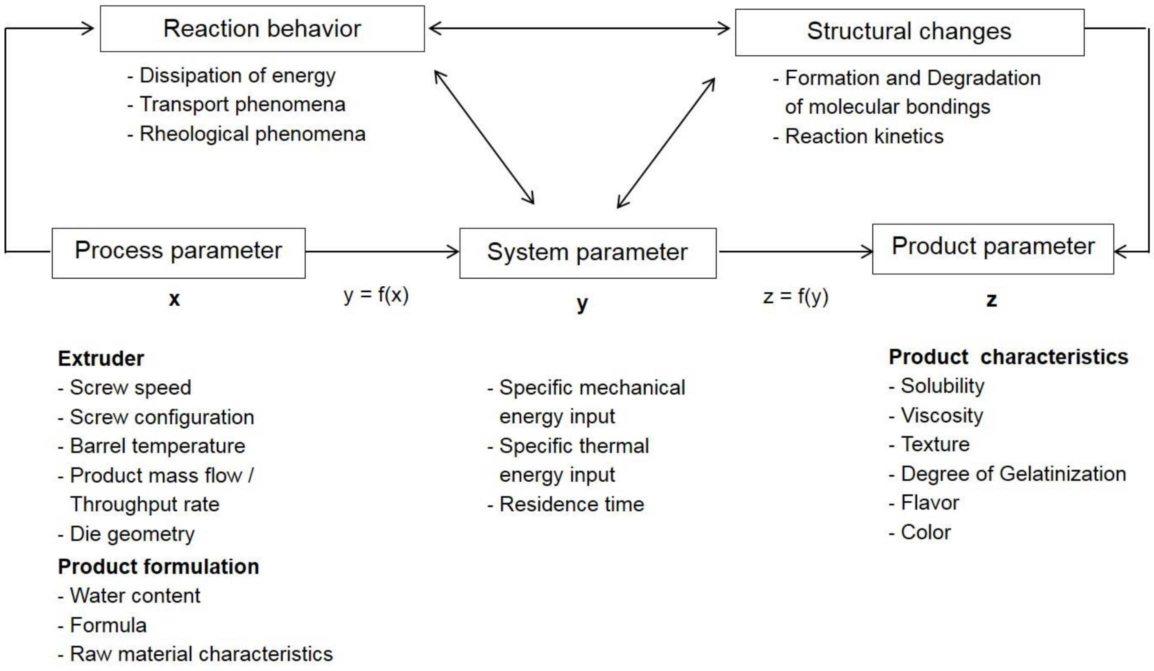

Fig. 1-12: System analytical model for the extrusion of starch containing food materials (Meuser & Lengerich, 1984).

The so-called “system analytical model” uses the specific mechanical energy input (SME) and the specific thermal energy input (STE) calculated from the motor torque and the product temperature at the die, respectively. With this method, they linked process parameters with the resulting product characteristics. This approach has shown that these dependent system parameters, SME and STE, are important variables of the extrusion process (Meuser et al., 1982; Meuser & Lengerich, 1984; Meuser et al., 1992). In addition, the residence time distribution can also be defined as a system parameter. As a result, system parameters can be used to describe or compare extrusion processes under different operating conditions. Furthermore, it helps to simplify process control, optimization and scale-up processes. This approach can be assisted by applying response surface methodology (Meuser et al., 1992; Pansawat et al., 2008; Xu et al., 2016) or neural networks (Eerikäinen et al., 1994; Fan et al., 2013).

In contrast, the basic **modeling approach** includes deterministic models based on equations of continuum mechanics. These equations are typically mass, energy and momentum transfer, completed by constitutive equations describing the rheological behavior and the physicochemical transformation of the studied material. The main advantage of models based on continuum mechanics is that they do not require any adjustable parameter and thus, are predictive. However, the parameters involved in the various equations have to be known. In fact, determining all these values is very difficult if not impossible to perform (Della Valle et al., 2011). Indeed, many studies could already show that, predictive models for some issues of the extrusion process could be developed with in- and offline-measurements of different material parameters, as well as reasonable assumptions. These models are used, for example, for 3D numerical CFD simulations, which analyze the flow and the heat transfer in the extruder (Dhanasekharan & Kokini, 2003) or to calculate the mixing efficiency (Valette et al., 2009; Avalosse & Rubin, 2000; Emin & Schuchmann, 2013; Ficarella et al., 2006).

A more recent idea is a **mechanistic approach** published by Emin & Schuchmann (2017). The idea is to use different methods (numerical, rheological etc.) to gain more detailed information about the processes in the extruder. The methods therefore can be of different nature, i.a. direct measurements during the extrusion process (e.g. product temperature), readjustment of the extrusion process in a rheometer, as well as numerical simulations of the process.

1.4.4 High moisture extrusion of proteins

The most common and most thoroughly investigated extrusion type is the one at low moisture levels (< 40%) being widely used in food industry for starch based products (Harper, 1981; Guy, 2001). In contrast, extrusion at higher moisture levels (also called wet extrusion) (> 40%) has been studied less extensively. Yet, it enables the production of innovative food products (Akdogan, 1999; Camire, 1991; Cheftel et al., 1992; Chen et al., 2011). High moisture extrusion applications utilize twin screw extruders due to their efficient conveying capabilities. In twin screw extruders, the product is transferred as a bulk from one screw to the other. This makes the forward conveying more efficient compared to single screw extruders, where conveying is caused by the friction between the barrel and the product, as well as the screw and the product (Akdogan, 1999; Noguchi, 1990). The rheological properties, torque, pressure build-up and energy requirements of high moisture extrusion systems are different from those in low moisture (Akdogan, 1999). Wet extrusion is characterized by lower friction and low viscous dissipation. Water content is usually an effective extruder parameter influencing the rheological behavior of the product in the extruder during low moisture extrusion. Since viscosity of a high moisture extruding system is considerably lower, viscous dissipation in such systems is of less importance. Therefore, the energy required to work the product into the screw channel mostly relies on thermal input through the barrel walls rather than the friction between molecules (Akdogan, 1999). Thus, high moisture extrusion enables proteins to be texturized. For example, fibrous meat-like structures from plant proteins can be obtained. Therefore, proteins form different sources, such as soy (Liu & Hsieh, 2008; Lin et al., 2000; Chen et al., 2011; Fang et al., 2014; Hayakawa, 1992), pea (Osen et al., 2014) or wheat gluten (Pietsch et al., 2017) can be used. Due to the thermo-mechanical treatment in the extruder, the proteins become denatured. By attaching a long cooling die to the end of the extruder, the proteins realign in flow direction and form fibrous structures (Cheftel et al., 1992; Noguchi, 1990; Areas, 1992). The conditions during the process induce molecular transformations and chemical reactions of the protein molecules which contribute to stabilization of the three-dimensional network formed after the extrusion step in the cooling die (Chen et al., 2011; Liu & Hsieh, 2007, 2007; Osen et al., 2014). Furthermore, proteins can be restructured to resemble chicken meat (Alvarez et al., 1990; Cheftel et al., 1992) or fish surimi (Thiébaud et al., 1995). Moreover, conjugates composed of soy protein with lactose and sucrose (Guerrero et al., 2012) or whey proteins with citrus pectin (Koch et al., 2017) can be formed in an efficient way using an extrusion process. Conjugates are covalently linked molecules composed of polysaccharides and proteins. Conjugation is one of the first reaction steps of the Maillard reaction. Such conjugates possess improved functional properties such as emulsifying activity. In dairy applications, extrusion cooking can also be applied to manufacture processed cheese (Zuber et al., 1987) or sodium caseinate (Cheftel et al., 1992; Fichtali et al., 1995).

Extrusion can also be used to produce protein based additives for different food applications. For example, whey proteins can be texturized by high moisture extrusion (Onwulata et al., 2010; Qi & Onwulata, 2011). These extrudates can be added to starch based snacks in order to improve the textural and nutritional properties (Onwulata et al., 2010; Day & Swanson, 2013; Onwulata et al., 1998). Yet in these studies, the addition of these extruded whey protein led to less expanded products with higher breaking strength (Onwulata et al., 1998; Purwanti et al., 2010; Allen et al., 2007). However, the aim of these aforementioned studies was not aggregation of whey proteins. Thus, the particle size was not measured. Furthermore, whey proteins can be used to produce aggregates with specific functional properties using a microparticulation process at acidic pH (Quéguiner et al., 1992b; Quéguiner et al., 1992a; Cheftel et al., 1992). This process is already described in more detail in the section above (section 1.3).

2 Objective and Outline

Controlled thermal unfolding and subsequent aggregation can give whey proteins new functional properties. The last decades have seen the development of a broad knowledge base concerning the thermally induced denaturation and aggregation behavior of whey proteins. Furthermore, different processes are available to achieve aggregated micro-particles. A comprehensive summary about the thermal denaturation behavior and the methods of microparticulation was presented in the theoretical background. However, the focus was so far on aggregation mechanisms without shear. Furthermore, low protein concentrations were mainly considered to gain insights into the underlying mechanisms as well as to explore processes for microparticulation. Indeed, shear and protein concentration are fundamental factors affecting the frequency of collisions between molecules and particles as well as the effective shear stress acting on aggregates.

Hence, the aim of this thesis was to investigate the thermal aggregation mechanism of whey proteins under shear stress with a particular interest on high protein concentrations. Based upon these findings, the goals were to develop a novel high moisture extrusion process for microparticulation and to gain fundamental understanding of the processes in the extruder. Thereby, identification of the major influencing factors on the aggregation process and thus product properties was crucial. Thus, this thesis sought to describe a way to control and monitor the microparticulation process.

The structure of this thesis is outlined in Fig. 2-1. A stepwise approach was used to develop a novel high moisture extrusion process for microparticulation of whey proteins and to understand the underlying aggregation processes on a mechanistic level. This typically enables optimization and control of both the process and product properties.

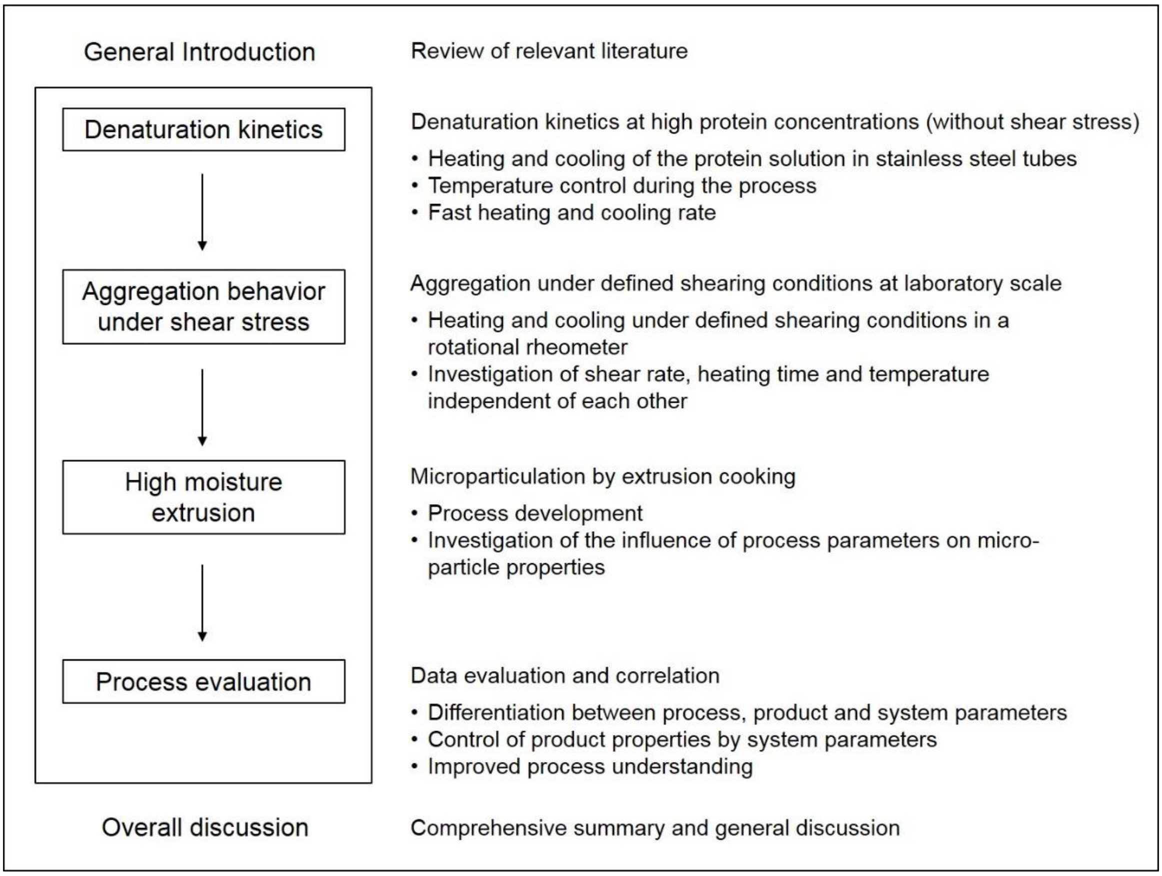

Fig. 2-1: Overview of the structure and the general approach applied in this thesis.

Heat treatments that whey proteins undergo during extrusion or during processing in general affect the native state and the stability of these proteins. Consequently, a better understanding of their behavior during heating is essential to control their functional properties and characteristics. For the development a new microparticulation process and its subsequent optimization, detailed information about the kinetics and the mechanism of whey protein unfolding and aggregation was needed, in particular at high protein concentrations (section 3). Furthermore, aggregation behavior under defined shearing conditions needed to be studied alongside with the independent effect of shear rate, heating time and temperature (section 4). Based on these results, a new extrusion process for microparticulation of whey proteins was developed and the influence of different parameters was examined (section 5). Subsequently, the obtained data were evaluated and novel correlations became apparent by differentiating between process, product and system parameters. These correlations gave insight in the field of process control, understanding and optimization (section 6). Besides, the results provide a deeper understanding of the aggregation processes in the extruder. With all sections taken together, the wider aim of this thesis was to not only describe the effects of high moisture extrusion, but also to explore and explain the underlying mechanisms.

3 Thermal denaturation kinetics of whey proteins at high protein concentrations[1]

Abstract

A detailed kinetic study of the thermal reaction kinetics of whey protein concentrate was conducted at high protein concentrations. Whey protein solutions with protein concentrations of up to 40% (w/w) were heated at different temperatures for varying periods of times. The denaturation of β-lactoglobulin followed a reaction order of 1.5 and depended strongly on temperature and protein concentration. The rate of denaturation was shown to increase with increasing temperature. This could be explained by the strong influence of the temperature on the unfolding reaction. Furthermore, the protein concentration induced a faster thermal denaturation, most likely due to the increased probability of collision between whey protein molecules with increasing protein concentration which promotes protein aggregation. The results of this study are of industrial relevance for extrusion processes and the production of protein concentrates in evaporators where high protein concentrations are frequently used.

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[1] Original publication: Wolz, M.; Kulozik, U. (2015). Thermal denaturation kinetics of whey proteins at high protein concentrations. International Dairy Journal, 49, 95–101. doi: 10.1016/j.idairyj.2015.05.008.
Adapted original manuscript. Adaptions of the manuscript refer to numbering of sections, figures, tables and equations, abbreviations, format and style of citation. Permission for the reuse of the article is granted by Elsevier Limited.

3.1 Introduction

Knowledge about the thermal reaction kinetics of whey protein denaturation is important for many processes in dairy technology, such as fouling in heat exchangers or evaporators (Davies et al., 1997; Simmons et al., 2007). Furthermore, functional properties of whey proteins can be specifically modified by controlled denaturation and aggregation (Dissanayake & Vasiljevic, 2009; Nicolai & Durand, 2013).

The thermal denaturation behaviour under conditions without shear stress was investigated in previous studies at protein concentrations of up to 5% (w/w) by Donovan & Mulvihill (1987), Kessler & Beyer (1991), Bon et al. (1999), Plock et al. (1997) and Zuniga et al. (2010), and up to 10% (w/w) by Fitzsimons et al. (2007) and Roefs & Kruif (1994). Various results have been reported depending on milieu, material and process conditions. Details regarding these studies at low protein concentrations can be found in, e.g., Boye et al. (1997), Nicolai et al. (2011), and Wit (2009). Only few studies investigated protein contents of up to 20% (w/w) at maximum (Dissanayake et al., 2013; Nielsen et al., 1996).

To our knowledge, the reaction kinetics of whey proteins at higher protein concentrations have not been studied so far. Information about the thermal behaviour at higher protein levels is interesting to obtain insights into the effects taking place at more elevated protein concentrations. Evaporation of whey to produce concentrates or the functionalisation of whey proteins by microparticulation in a cooking extrusion are both dependent on thermal impact in a wanted or undesired manner. For both of these examples it would be desirable to be able to know the reaction kinetics of whey protein denaturation to better control heat induced fouling or to steer the microparticulation reaction, for instance.

A deeper understanding of the structural and physical changes taking place during denaturation is essential to be able to characterize the related reaction kinetics. The major whey proteins affecting denaturation kinetics are β-lactoglobulin (β-Lg) and α-lactalbumin (α-La). β-Lg is a globular protein with two intramolecular disulphide bonds and one free thiol group, which is hidden in the native state. α-La has four intramolecular disulphide bonds and no free thiol group. The denaturation takes place in two successive steps. First, the native globular proteins unfold during hydrophobic groups. In the second step, the unfolded proteins aggregate via intermolecular disulphide bonds and hydrophobic interactions (Havea et al., 2001; Hong & Creamer, 2002; Wijayanti et al., 2014; Zuniga et al., 2010).

The rate of the denaturation reaction is determined by environmental factors as well as processing conditions. The reaction kinetics of whey proteins strongly depend on the protein concentration: the higher the concentration, the faster the reaction (Verheul et al., 1998). Protein concentration mainly affects the aggregation step rather than the unfolding of whey proteins. Thus, higher concentrations result in high molecular weight aggregates (Dissanayake et al., 2013; Fitzsimons et al., 2007). During heating, the native whey proteins unfold and the free thiol group of the β-Lg becomes accessible. A dynamic equilibrium with the equilibrium constant (K_G) between the protein in its native conformation and the partially unfolded protein is reached. Unfolding is reversible and the protein molecules can refold in its native conformation if the temperature is reduced by rapid cooling below the unfolding temperature before aggregation has taken place.

However, if two unfolded molecules collide before they can refold, they aggregate. This second step of the reaction is irreversible. Due to the equilibrium between the native and the partially unfolded form of the molecules an increased aggregation also causes an increased unfolding of the proteins. This is necessary to maintain the equilibrium between the native and the partially unfolded proteins, as molecules are withdrawn from the unfolded state during aggregation. As a consequence, the total denaturation reaction becomes accelerated with increasing aggregation rate (k_{agg}) (Tolkach & Kulozik, 2007).

The main factors affecting the unfolding reaction are temperature, the composition of the protein solution as well as the concentration of unfolded, but not yet aggregated protein molecules. Factors affecting the aggregation rate k_{agg} include temperature, the probability of collision of the unfolded proteins, which is the prerequisite for an irreversible aggregation and determines the degree of unfolding of the proteins.

Furthermore, the lactose concentration is known to affect the denaturation kinetics. An increase in denaturation temperature of β-Lg with increasing lactose concentration has been reported (Plock et al., 1998a; Wit & Klarenbeek, 1984). The pH value directly affects the charge of the proteins and thereby influences the stability of the protein conformation. This effect was already studied by, e.g., Nicolai et al. (2011) and Zuniga et al. (2010). Salts, in particular calcium, influence electrostatic interactions and often have a promoting effect on aggregation depending on the prevailing pH value of the solution (Erabit et al., 2013; Giroux et al., 2010; Spiegel & Huss, 2002). Another key factor for the denaturation of whey proteins is the temperature dependent reaction rate. With increasing temperature, the degree of denaturation increases. At low temperatures, the unfolding step is the rate determining step. At higher temperatures, the aggregation reaction is the dominating reaction. The degree of denaturation of whey proteins depends, next to temperature and substrate composition, also on the heating time. Longer heating times result in a higher degree of denaturation due to progressive denaturation (Schokker et al., 2000; Verheul et al., 1998; Zuniga et al., 2010).

As briefly mentioned above, concentrates with high protein contents are generated or applied in various processes. However, the thermal denaturation behaviour has not been investigated so far at protein concentrations as high as up to 40%, which is of relevance for evaporation processes at temperatures up to 70 °C, extrusion cooking and membrane processes at elevated temperatures. Therefore, the objective of the current study was to determine the denaturation kinetics of whey proteins as a function of temperature, heating time and protein concentration with a particular interest in high protein concentrations. The kinetics were studied by assessing the degree of denaturation during heating as a function of temperature/time and protein concentration.

3.2 Material and Methods

3.2.1 Sample preparation

Whey protein concentrate (WPC80, Germanprot Sachsenmilch, Leppersdorf, Germany) was dissolved in demineralized water to the desired protein concentration (10, 20, 30 and 40%, w/w). The composition of the whey proteins in the WPC80 is shown in Tab. 3-1. All experiments were carried out at pH 6.7, unvaried salt concentration and salt composition. The obtained whey protein solutions were stored for at least 12 h at 4 °C prior to experimental use to ensure complete dissolution.

Tab. 3-1: Whey protein composition of the used whey protein concentrate (WPC80).

Protein	percentage [%]
β-Lactoglobulin (β-Lg)	50.2
α-Lactalbumin (α-La)	12.0
Bovine serum albumin (BSA)	2.4
Lactoferrin (LF)	1.2
Caseinomakropeptide (CMP)	18.2
Denatured whey protein (with pH 4.6 criteria)	16.0

3.2.2 Heat treatment

For thermal treatment and evaluation of kinetic data, temperature controlled oil and water baths were used. Samples (9.4 mL) were filled in stainless steel tubes with a length of 120 mm and an inner diameter of 5 mm. A temperature sensor was integrated in the screw cap of the tubes to track the temperature during heating and cooling by a data logger Almemo (Ahlborn, Holzkirchen, Germany). An oil bath with a temperature of 170 °C was used to ensure a fast heating up (heating rate > 3.5 C s^{-1}). After the whey protein solutions reached the desired temperature, tubes were transferred into a water bath of constant temperature of 70, 75, 80, 85, 90 and 95 °C for varying holding times of 5 - 5000 s. After heating the samples were cooled down immediately in an ice bath (cooling rate > 5.5 °C s^{-1}). As an example, a heating and cooling profile is shown in Fig. 3-1. Product fouling at the tube surfaces did not influence the results, which was controlled by visual inspection of all tubes upon removal of the samples. In any case the whole sample contents were collected by a mechanical piston, which was necessary due to the high viscosity the samples partially had, especially when the protein concentration was high. It was considered to use an available continuous capillary system for heating, but the unit could not be used because of the high viscosity of the concentrated protein solutions and the gel formation during heating. Therefore, the study was conducted using a batch heating system with as short as possible heating-up and cooling-down ramps, as depicted in Fig. 3-1.

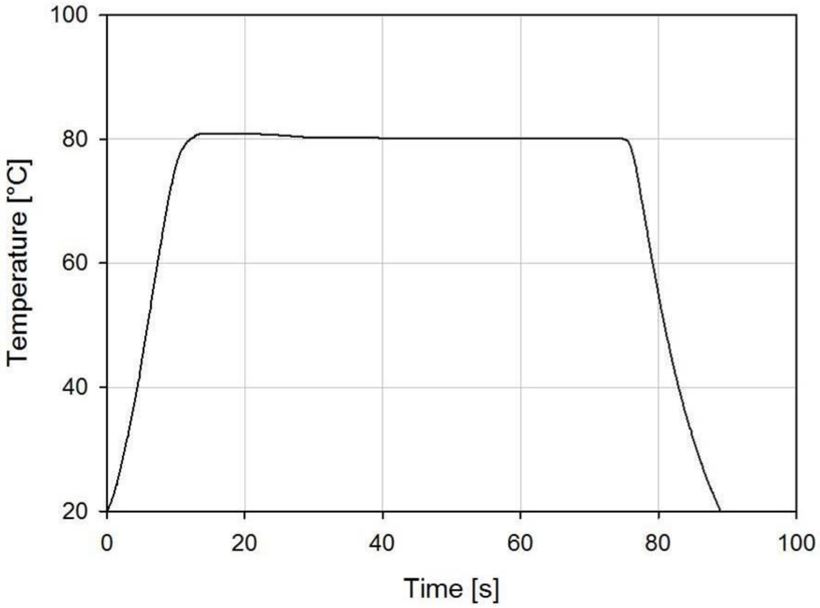

Fig. 3-1: Exemplary heating and cooling profile during heat treatment.

3.2.3 Analysis of degree of denaturation

To determine the degree of denaturation, the native and the residual native amount of β-Lg after heating were measured using reversed phase-high performance liquid chromatography (RP-HPLC). The criterion therefore was the loss of solubility of the denatured whey protein at a pH of 4.6 (Dannenberg & Kessler, 1988; Kessler & Beyer, 1991; Roefs & Kruif, 1994). The content of native β-Lg was determined after pH 4.6 precipitation by RP-HPLC by using the sample preparation and method described by (Toro-Sierra et al., 2013). Using this method, the amount of native β-Lg and α-La could be quantified. The degree of denaturation (DD) was calculated as the ratio of the amount of native protein before (c_0) and after (c_t) heating.

3.2.4 Data analysis

The thermal changes occurring during heating can be mathematically described. The reaction rate v of the denaturation reaction is determined by the reaction order n, the native protein concentration c and the temperature dependent rate constant $k_{T/n}$.

$$v = -\frac{dc}{dt} = k_{T/n} \cdot c^n \qquad (3.1)$$

The integration of Eq. (3.1) results in:

$$\left(\frac{c_t}{c_0}\right)^{1-n} = k_{T/n} \cdot t \cdot (n-1) \cdot c_0^{(n-1)} + 1 \qquad \text{for } n \neq 1 \qquad (3.2)$$

The temperature dependence of the rate constant $k_{T/n}$, which is independent of protein concentration, can be described by the Arrhenius equation:

$$k_{T/n} = k_{0/n} \cdot e^{\left(\frac{E_a}{R \cdot T}\right)} \qquad (3.3)$$

Hence, the concentration independent rate constant $k_{T/n}$ depends on the activation energy E_a, the universal gas constant R, the temperature T as well as the pre-exponential factor $k_{0/n}$. In this case, the stoichiometric coefficient n can be seen as formal reaction order of the irreversible denaturation.

The analysis of the degree of unfolding and the aggregation rate was conducted by applying the model of Tolkach & Kulozik (2007). The thermally induced unfolding of the whey proteins can be seen as an activation of the proteins and is the starting point for further reactions. The building up and degradation of the activated protein can be described by the equilibrium between the native protein and the partially unfolded protein. Therefore, an important requirement is a fast protein unfolding. This correlates with a fast equilibrium between native and partially unfolded state. In this case, the equilibrium constant K_G can be defined as the ratio between the concentrations of unfolded protein c_{unfold} and the concentration of native protein c_{native}. The degree of unfolding describes the ratio between the concentration of protein existing in its unfolded conformation c_{unfold} and the concentration of molecules detected using RP-HPLC c_{HPLC}.

$$\alpha = \frac{c_{unfold}}{c_{unfold} + c_{native}} = \frac{c_{unfold}}{c_{HPLC}} = \frac{K_G}{K_G + 1} \qquad (3.4)$$

The degree of unfolding α can assume values in the range of $0 < \alpha < 1$. If the total whey protein is present in its native state $\alpha = 0$. When all molecules are unfolded $\alpha = 1$.

Using RP-HPLC, native proteins as well as renatured proteins are detected. Renatured proteins are whey proteins that did not aggregate (and thereby were not irreversibly denatured) and thus can fold back to their native conformation during cooling. For this reason, the changes over time of the concentrations measured by RP-HPLC are equal to the temporal changes of the irreversibly aggregated and denatured proteins ($\frac{dc_{agg}}{dt}$).

$$-\frac{dc_{agg}}{dt} = \frac{dc_{HPLC}}{dt} = k_{agg} \cdot (\alpha \cdot c_{HPLC})^n \qquad (3.5)$$

The rate of the decline of native protein does not only depend on the rate of aggregation k_{agg}, but also on the degree of unfolding of the proteins (α). Combining Eq. (3.5) with Eq. (3.1) gives:

$$k_{T/n} = \alpha^{n} \cdot k_{agg} \qquad (3.6)$$

Thus, Eq. (3.3) can be linearised by logarithmic conversion:

$$\ln\left(k_{T/n}\right) = \ln\left(\alpha^{n} \cdot k_{agg}\right) = n \cdot \ln \alpha + \ln k_{agg} \qquad (3.7)$$

By this equation, the degree of unfolding α and the aggregation rate k_{agg} can be determined from the data.

3.2.5 Statistical analysis

All experiments were performed in triplicate. Curves were fitted using SigmaPlot for Windows Version 11.0 Build 11.0.0.75 (Systat Software Inc., Chicago, USA). Mean values ± 95% confidence levels are reported. The Student t-test was applied to estimate differences between mean values at a confidence level of 95%.

3.3 Results and Discussion

3.3.1 Influence of protein concentration on denaturation rate

Since β-Lg represents over 50% of the total whey protein in bovine milk, thereby dominating whey protein denaturation, this protein was selected as the lead component for the present study. A similar approach was already chosen by other authors (Dissanayake et al., 2013; La Fuente et al., 2002). To identify the reaction order, the logarithmic change of the native β-Lg concentration over time was plotted. The slope of this function characterizes the reaction order n. A reaction order of n =1.5 for β-Lg could be identified from the results obtained, which is in accordance with the findings of previous studies for lower protein concentrations at neutral pH (Dannenberg & Kessler, 1988; Erabit et al., 2013; Bon et al., 1999; Roefs & Kruif, 1994; Tolkach & Kulozik, 2007). Other authors partially reported different reaction orders between 1.5 and 2 (Hillier & Lyster, 1979; Kessler & Beyer, 1991), but the majority of published works report n = 1.5. The reasons for the differences are probably the different evaluation methods used for the analysis and the different conditions during the heat treatment and extent of denaturation. If the conversion of native β-Lg to aggregated β-Lg is in the low range, it is difficult to determine the reaction order n precisely. Fig. 3-2 shows the thermal denaturation behaviour of β-Lg at different whey protein concentrations at a heating temperature of 80 °C and a reaction order of n = 1.5. An increase in protein concentration accelerates the denaturation reaction. The explanation of this accelerated thermally induced denaturation reaction is the fundamental reaction mechanism. The number of protein molecules increases with increasing protein concentration, thereby raising the probability of collision between the molecules. This results in an increased aggregation rate and leads to an accelerated overall thermal denaturation reaction.

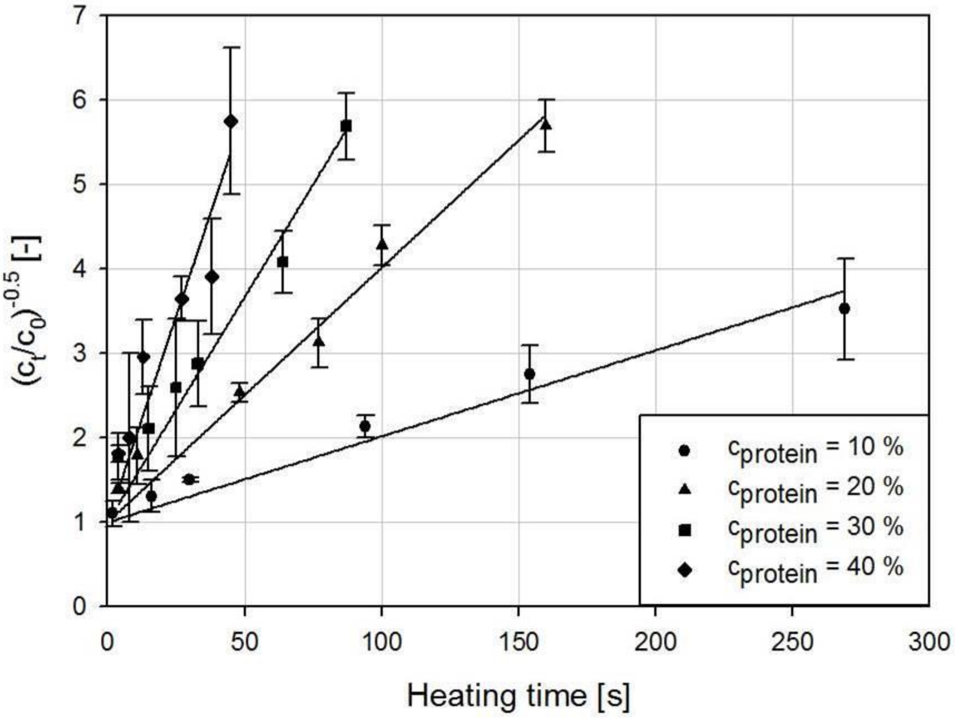

Fig. 3-2: Denaturation behaviour for different protein concentrations at 80 °C and a reaction order of 1.5.

3.3.2 Influence of temperature on denaturation rate

Due to the dependency of the denaturation reaction on the heating temperature (Eq. (3.3)), temperature is an essential process variable. Its influence was investigated for high protein concentration between 10 and 40%. Fig. 3-3 shows the temperature dependency of the denaturation reaction. With increasing temperature, the slope increases considerably illustrating the strong increase of the reaction rate with increasing heating temperature. Similar reaction patterns were observed for all investigated protein concentrations (results not shown). The results are in agreement with other authors, such as Dannenberg & Kessler (1988) and Wit (2009) for lower protein concentrations.

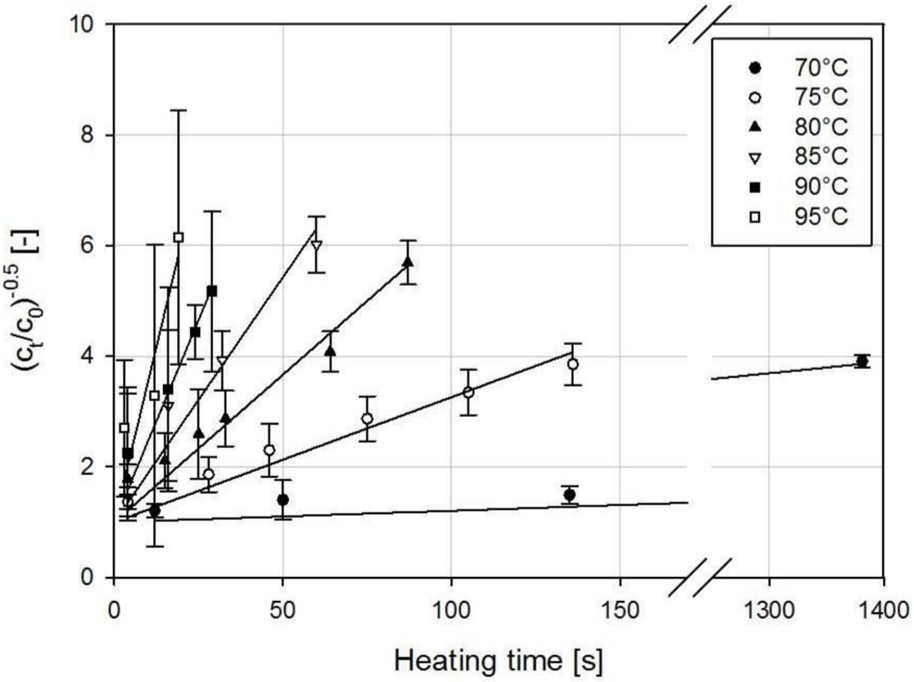

Fig. 3-3: Denaturation behaviour of whey protein at different temperatures for n = 1.5 and a protein concentration of 30%.

Using the Arrhenius approach (Fig. 3-4) for the concentration independent rate constant $k_{T/1,5}$ of the β-Lg denaturation, a two-step reaction could be identified. At low temperatures, the reaction rate is limited by unfolding of the proteins while at high temperatures, the reaction rate is limited by aggregation. This explains the sharp change of slope for the reaction rate in the Arrhenius plot, see Fig. 3-4. This bend temperature separates the Arrhenius plot into two parts. At temperatures above the bend temperature, unfolding of the molecules takes place faster than aggregation. The total reaction is limited by the irreversible aggregation step. In case the temperature lies below the bend temperature, partial unfolding of molecules is the limiting step of the total reaction.

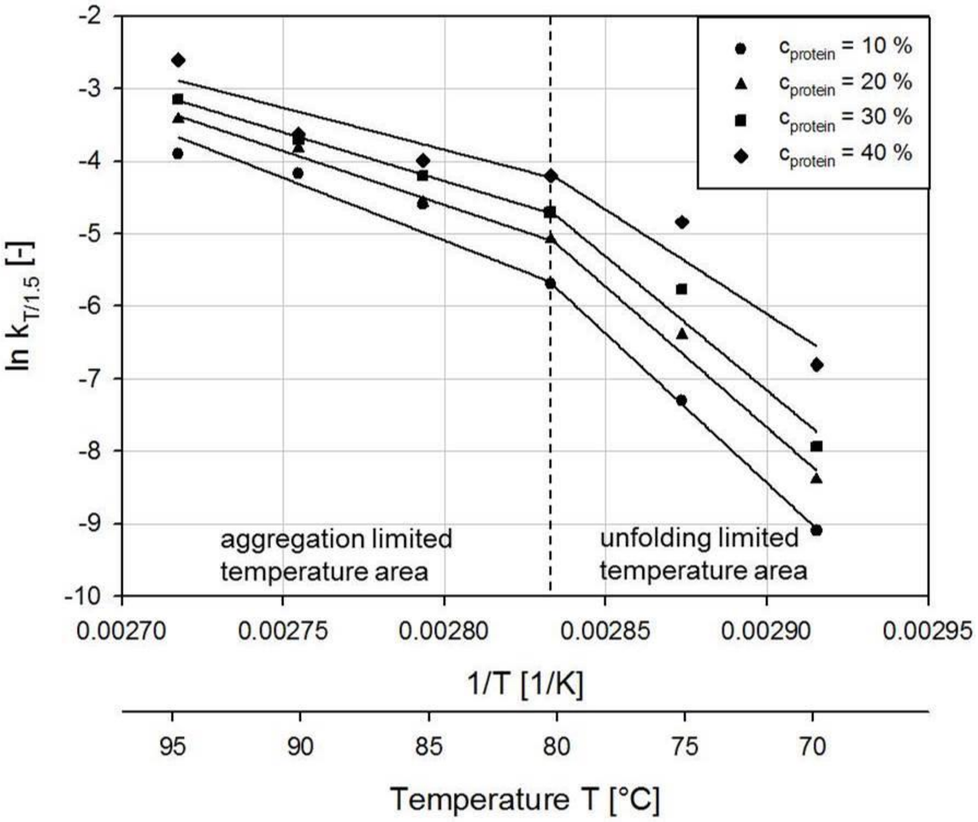

Fig. 3-4: Temperature dependence of the denaturation velocity rate constant at different protein concentrations.

The temperature dependent denaturation rate constant at different protein concentrations is represented in Fig. 3-4. Independent of the protein concentration, the bend temperature is 80 °C. With decreasing protein concentration, the rate decreases and the reaction slows down. The bend temperature of β-Lg in milk and whey as well as in UF-milk and whey concentrate is reported to be 90 °C (Dannenberg & Kessler, 1988; Oldfield et al., 1998; Tolkach & Kulozik, 2007). Milieu conditions, especially the lactose concentration and the pH value of the protein solution have a significant influence on the value of the bend temperature. A reduction of lactose concentration or an increase of the pH value causes a decrease of the kinetic bend temperature (Dannenberg & Kessler, 1988; Spiegel & Huss, 2002; Tolkach & Kulozik, 2005). This suggests that, due to the lower lactose concentration used in the present work, the bend temperature was shifted to lower temperatures (80 °C) compared with pure milk or whey.

On basis of Eq. (3.3), the activation energy of the reaction E_a could be determined as the slope of the curves in Fig. 3-4. The activation energy is a criterion for the temperature dependency of the reaction. For the unfolding limited reaction, an increase in activation energy with decreasing protein concentration was observed (Tab. 3-2).

Tab. 3-2: Activation energy (E_a) and rate constant (k_0) of the reaction for different protein concentrations ($c_{protein}$).

Limiting reaction	$c_{protein}$ [%]	E_a [kJ mol^{-1}]	k_0 [s^{-1}]
unfolding	10	342	$1.7 \cdot 10^{48}$
	20	334	$2.2 \cdot 10^{47}$
	30	326	$2.0 \cdot 10^{46}$
	40	263	$1.5 \cdot 10^{37}$
aggregation	10	126	$2.0 \cdot 10^{16}$
	20	123	$1.2 \cdot 10^{16}$
	30	111	$3.3 \cdot 10^{14}$
	40	110	$3.1 \cdot 10^{14}$

This points at an increasing impact of temperature with decreasing protein concentration on the unfolding step of the temperature increase has a lower effect. This also shows that the effect of concentration dominates the reaction with increasing protein concentration, while unfolding is progressively less dependent on the heating temperature. For the aggregation limited step of the denaturation almost no effect of the protein concentration on the activation energy could be observed. Therefore, it can be assumed that the temperature mainly influences the unfolding step rather than the aggregation step.

3.3.3 Iso-effect-lines for different temperature time combinations

Fig. 3-5 depicts the lines of constant degree of denaturation for whey protein denaturation at different concentration and degrees of denaturation of 10 and 90%. The strong dependency of the denaturation rate on the temperature and the two-step reaction become again obvious. With increasing temperature, the native whey proteins unfold faster. The free thiol group, which is masked in the inner structure of the native conformation, is exposed. Disulphide bonds and hydrophobic interaction lead to the formation of aggregates (La Fuente et al., 2002; Wit, 2009). Proteins in their native conformation cannot participate in this aggregation. During cooling, partially unfolded molecules, which have not been aggregated, fold back into the native conformation and are detected as native proteins by RP-HPLC.

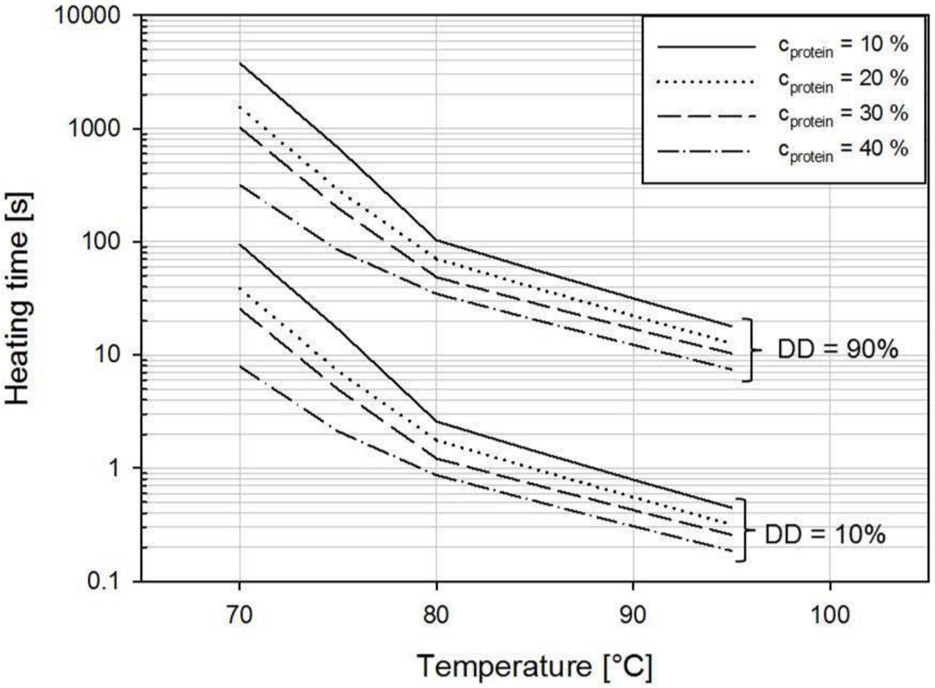

Fig. 3-5: Lines of constant degree of denaturation with DD = 10% and DD = 90% for different whey protein concentrations from 10 to 40%.

Fig. 3-5 shows iso-effect-lines for a degree of denaturation of 10 and 90%. The total reaction is very fast due to the high protein concentration and the low lactose concentration. The reaction is strongly dependent on the protein concentration (as discussed in Section 3.3.1). The higher the protein concentration, the shorter is the heating time required to induce a certain degree of denaturation (at constant temperature). These findings can, for instance, be used for the design of heating units for the treatment of concentrated whey. For example, at a temperature of 70 °C and highly concentrated protein solutions ($c_{Protein}$ = 40%) 8 s are already sufficient to cause a degree of denaturation of 10%, and thereby a remarkable change in protein structure. The findings can also be useful to optimise processing conditions for protein functionalising (e.g., microparticulation) where protein concentrates could be applied and where a high protein degree of denaturation should be achieved.

3.3.4 Determination of the degree of unfolding and the aggregation rate

To determine the degree of unfolding and the aggregation rate the model of Tolkach & Kulozik (2007) was used. Experiments to measure directly the content of unfolded b β-Lg and thereby the degree of unfolding is difficult or even impossible to perform. For this reason the model is based on formal kinetic data generated by ex-situ measurements with a novel reaction kinetic approach described in Section 3.2.5. This allows deeper insights regarding explanations for the sharp edge on the Arrhenius plot observed by different kinetic studies. The rate constant $k_{T/n}$, which describes the denaturation of the proteins without differentiating between unfolding and aggregation, consists of two terms (Eq. (3.7)). Each term characterises one part of the reaction. Due to the fact that α varies between 0 and 1, ln α is always negative. If the heating temperature is high enough and all proteins are unfolded a tends towards 1 and ln α is about 0. In this case, the total denaturation rate of the whey proteins is determined by the aggregation rate constant k_{agg} and the unfolding step is completed. Hence, the degree of unfolding a can be determined from the slope of the lines in Fig. 3-4.

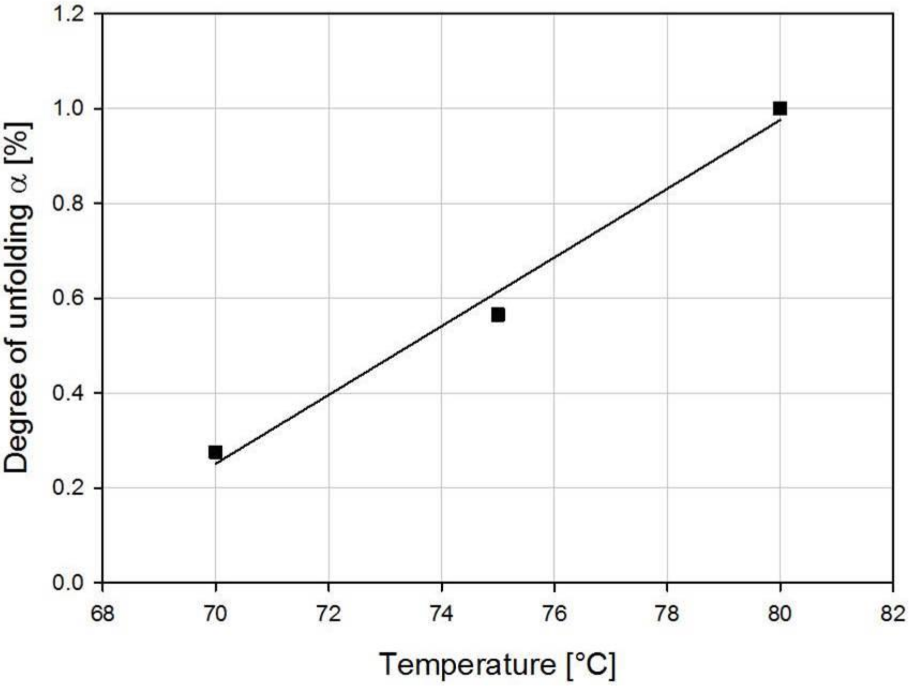

Fig. 3-6: Degree of unfolding as a function of temperature at a protein concentration of 30%.

Fig. 3-6 shows the degree of unfolding of β-Lg at a concentration of 30% as a function of temperature. A linear increase in the degree of unfolding is observed with increasing temperature. Complete unfolding of the proteins is reached (α = 1) at the bend temperature (ϑ = 80 °C). The degree of unfolding α decreases rapidly with decreasing temperature, whereby the negative term n ln α gains in importance. The lower the heating temperature, the smaller α and the higher is the absolute value of n ln α (Eq. (3.6)). This induces the kink in the Arrhenius plot of the kinetic rate constant for the thermal denaturation of β-Lg.

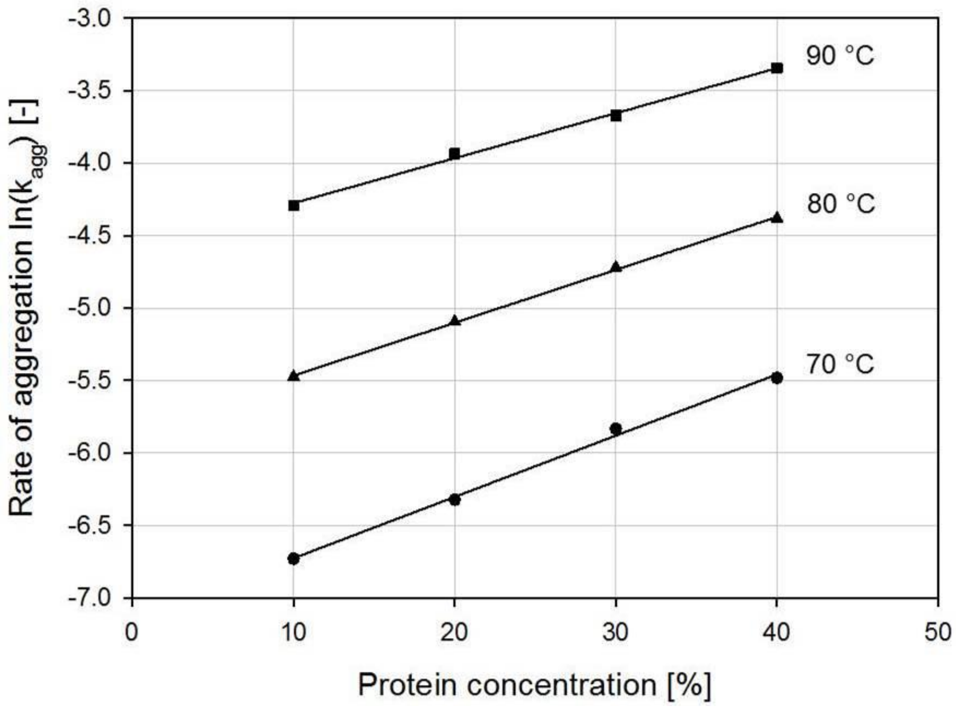

Fig. 3-7: Rate of aggregation as a function of protein concentration for temperatures of 70 and 90 °C.

The aggregation rates in Fig. 3-7 were determined from the slope of the lines of Fig. 3-4. On the one hand, Fig. 3-7 demonstrates the influence of the temperature. On the other hand, it also shows the strong impact of the protein concentration on the aggregation rate. The rate of aggregation exponentially increases with the protein concentration, which is due to the interaction between the protein molecules.

Since aggregation rate and unfolding rate directly affect each other, each change in the unfolding process also causes changes in the aggregation process and vice versa. Factors supporting the irreversible aggregation step of the reaction cause an accelerated unfolding at the same time. The level of acceleration depends on the degree of unfolding. The higher α, the stronger is the effect of changes of the aggregation rate on the unfolding rate. Such a behaviour occurs at temperatures below the bend temperature. At higher heating temperatures α equals 1, which implies that the unfolding is completed and the unfolding rate is zero.

3.4 Conclusion

The knowledge of the formal reaction kinetics with regard to thermal treatment of whey proteins could be widened to higher protein concentrations (up to 40%). Elevated whey protein concentrations result in an enhanced denaturation rate. From the calculated activation energy, it can be derived that the temperature mainly affects the unfolding step of the reaction. For all investigated protein concentrations, a shift of the bend temperature from 90 °C for diluted whey proteins to 80 °C could be observed due to the low lactose concentration applied. This study forms the basis for an improved understanding of the mechanism of the denaturation of highly concentrated protein solutions. The obtained results are helpful for choosing process conditions during the production of whey protein concentrates and whey protein powders to ensure a highly native protein product. The new insights allow for the extension of the known reaction kinetics to higher protein concentrations as they occur as a result of thermal concentration in evaporators or as they are applied in a targeted manner in different industrial processes such as extrusion or membrane concentration at higher temperatures. An extension of this study to include variables such as lactose content, pH and ionic composition would be useful.

Summary and Contribution of the Doctoral Candidate

Thermal denaturation kinetics of whey protein play an important role in many dairy processes (e.g. fouling in heat exchangers or evaporators). Furthermore, the denaturation reaction can be targeted and utilized to produce micro-particles with specific functional properties. Thermal denaturation behavior was investigated in previous studies for low protein concentrations of up to 5% (w/w). Until now, there is little known about the reaction rate and influencing factors at higher protein concentrations, especially at concentrations >20% (w/w). However, such knowledge would deliver insight into effects taking place during production of whey protein concentrates or during microparticulation, and thus, help to control and steer the occurring reactions. Hence, this study focused on denaturation kinetics of whey proteins at high protein concentrations. The influence of temperature, heating time and protein concentration on the denaturation progress was also studied. For this purpose, whey protein solutions with protein concentrations of up to 40% (w/w) were heated at different temperatures for varying periods of time.

A reaction order of 1.5 for the denaturation of β-Lg was identified, which is in line with literature data. The observed shift of the bend temperature from 90 °C to 80 °C was attributed to the low lactose concentration used in this study. The reaction rate strongly depended on heating temperature and protein concentration. The rate of denaturation increased with rising temperature. The temperature mainly affected the unfolding step of the reaction rather than the aggregation step. Furthermore, it was found that increasing protein concentration induced a faster reaction. This was explained by the increased probability of collisions between unfolded protein molecules. Thus, increasing protein concentration promotes aggregation and, thereby, the total denaturation reaction. These findings are of relevance when choosing process conditions for different industrial processes such as extrusion for microparticulation of whey proteins.

The substantial contributions of the doctoral candidate include conception and design of the experiments based on preceding critical literature review, as well as the writing and the revision of the majority of the manuscript. The doctoral candidate carried out major parts of the data analysis, interpreted the data set, and discussed it.

4 Thermal aggregation of whey proteins under shear stress[2]

Abstract

Processed food protein such as whey protein, used as hydrocolloid, is a possibility to impart specific structural and physical properties of food. The specific protein properties can be achieved by controlled denaturation or rather aggregation. The resulting aggregate structure and thereby the functional properties at the macroscopic scale depend strongly on the process conditions. Heating conditions, shear stress and material composition, determine the reaction kinetics as well as the resulting particle size distribution and the structure of the aggregates. These conditions often cannot be investigated separately in industrial processes. Therefore, the impact of shear rate, heating time and protein concentration on the particle characteristics was investigated by a rotational rheometer at 80 °C. By increasing the protein concentration (from 5 to 30% w/w), smaller more compact and stable aggregates were produced. This is due to the higher viscosity and the higher shear stress. The aggregates appearance changes from long and crystalline like structure to a spherical shape. The influence of shear rate is dependent on protein concentration. In suspension with 5% protein the aggregate size initially increases with increasing shear rates because of the predominant effect of increasing number of collisions; and decreases subsequently due to limitation of the particle growth. At high concentrations the size of the aggregates decreases with the shear rate, because of increasing shear stress. The size of whey protein aggregates can thus be regulated by the applied shear rate during processing.

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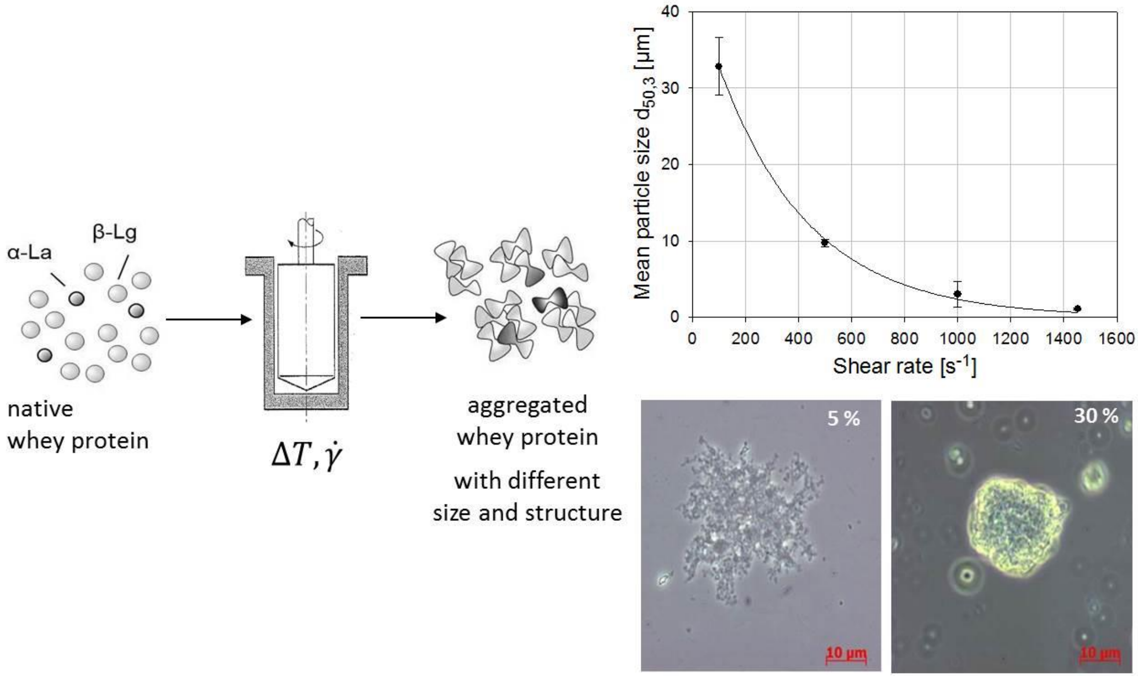
[2] Original publication: Wolz, M.; Mersch, E.; Kulozik, U. (2016b). Thermal aggregation of whey proteins under shear stress. Food Hydrocolloids, 56, 396–404. doi: 10.1016/j.foodhyd.2015.12.036. Adapted original manuscript. Adaptions of the manuscript refer to numbering of sections, figures, tables and equations, abbreviations, format and style of citation. Permission for the reuse of the article is granted by Elsevier Limited.

Graphical Abstract



Highlights

- Investigation of whey protein aggregation under shear stress.
- By increasing the protein concentration smaller, more compact and stable aggregates were formed.
- The influence of shear rate is dependent on protein concentration.
- At low concentration, the aggregate size initially increases with the shear rate.
- At high concentration, the shear rate is the critical factor to limit particle size.

4.1 Introduction

Whey proteins are often heat treated to change their physical and functional characteristics in food industry. The variation whey protein properties can be achieved by controlled denaturation. The principle mechanism of the irreversible denaturation reaction is a two-step reaction. In the first step the whey proteins unfold during heating and in the second step the unfolded protein molecules aggregate mainly by disulfide bonds and hydrophobic interactions. Processing conditions, such as temperature, heating time and shear stress determine the reaction kinetics as well as the size and the structure of the aggregates (Havea et al., 2001; Tolkach & Kulozik, 2007; Zuniga et al., 2010). Depending on the properties of the aggregates they can be used as food hydrocolloids for different applications: as fat replacer in food products (Sandoval-Castilla et al., 2004), as stabilizer in foams, as functional ingredient for viscosity and structure modification in many food products (Çakır-Fuller, 2015; Damodaran & Paraf, 1997) or to enhance the yield of cheese (Hinrichs, 2001). The knowledge about the unfolding and aggregation of whey proteins during concurrent heating and shearing is also fundamental for the fouling mechanism in heat exchanger during the manufacturing of whey protein concentrates and powders (Davies et al., 1997; Simmons et al., 2007). Whey proteins behavior during concurrent thermal and mechanical treatment was studied by Steventon (1992), Erabit et al. (2014) and Simmons et al. (2007) using a coquette apparatus. By Spiegel & Huss (2002) with a scraped surface heat exchanger. Paquin et al. (1992) and Dissanayake & Vasiljevic (2009) used a high pressure system, but in this case heating and shearing was not applied at the same time. Various factors such as heating conditions, shear stress, protein concentration, pH, Calcium etc. affecting the aggregation of whey proteins have been already identified (Donovan & Mulvihill, 1987; Onwulata et al., 2010; Plock et al., 1998a; Quéguiner et al., 1992b; Steventon, 1992). This study focused on protein concentration, heating time and shear rate.

Protein concentration: The reaction kinetics strongly depend on the protein concentration. The higher the concentration the faster is the total reaction (Kessler & Beyer, 1991; Spiegel & Huss, 2002; Verheul et al., 1998; Wolz & Kulozik, 2015). Aggregate size is also influenced by the protein concentration. At low protein concentrations (<5 - 10%) an increase of concentration results in an increase of aggregate size under conditions without shear stress (Boulet et al., 2000; Elofsson et al., 1996; Hoffmann & Mil, 1997). At higher protein concentrations (>5 - 10%) and under shear stress a decrease of the aggregate size can be measured (Steventon, 1992). However, molecular crowding was reported in cell biology works as an effect in contrast to our experience in whey protein technology. Molecular crowding was reported to even stabilize proteins against external stress factors (Ellis, 2001; Mittal et al., 2015). These contrary observations will be discussed in conjunction with the experimental results.

Heating: Heating time and temperature are one of the main process parameters influencing the temperature depending denaturation reaction. With increasing temperature and constant heating time the degree of denaturation increases. Using the Arrhenius approach for the concentration independent rate constant of the β-Lg denaturation, a two-step reaction could be identified. The bend temperature separates the Arrhenius plot into two parts. At temperatures below the bend temperature unfolding is the rate dominating step. At temperatures above the bend temperature the aggregation reaction is rate limiting (Wit, 2009; Wolz & Kulozik, 2015). Additionally, structure and size of aggregates built during heating are influenced by the temperature (Giroux et al., 2010; Zuniga et al., 2010). In the unfolding limited temperature area, smaller and more porous aggregates are achieved. In the aggregation limited area, bigger and more rigid aggregates are formed (Spiegel, 1999b).

Shear rate: Studies on the aggregation of whey proteins during concurrent heating and shearing show a decrease of the aggregate diameter with increasing shear stress (Cheftel et al., 1992; Quéguiner et al., 1992a). But Steventon (1992) also ascertained an opposite tendency at short periods of heating. A possible reason for this is a reduced efficiency of the protein collision at high shear rates. In principle shearing can have different effects on the protein structure. 1. It is possible to denature proteins by mechanical forces, but for that to occur extremely high shear rates have to be applied (Thomas & Geer, 2011). 2. The aggregates can break up due to increasing shear stress. This can be attributed to three mechanisms: (i) Deformation and fragmentation caused by pressure fluctuation in fluid flows. (ii) Erosion, i.e. abrasion of primary aggregates from the particle surface (iii) Fragmentation of the aggregates in large fragments caused by the mechanisms (i) and (ii) (Steventon, 1992; Taylor & Fryer, 1994). 3. Due to the enforced rate of collision during shearing, an increased aggregation can be achieved. The growth of the aggregates is first induced by the Brownian motion (thermal motion) and is independent of shear stress. This collision of particles causes the formation of primary aggregates. If the primary aggregates are large enough (0.25 - 1 µm), the particle motion is influenced by fluid flow and is hydrodynamically shear controlled. In consequence, the number of collisions between primary aggregates increase. The probability of particle interaction increase and the aggregation is intensified (Ker & Toledo, 1992; Simmons et al., 2007; Taylor & Fryer, 1994). The frequency of collisions and thereby the probability of aggregation can be mathematically described by the coagulation theory of Smoluchowski (1916). As a simplification all particles are assumed to be spherical. The theory is only valid for laminar shear flow. The number of collisions J_1 per volume and time is limited by the Brownian motion and thereby by diffusion in the first step (perikinetic aggregation).

$$J_1 = \frac{4 \cdot k_B \cdot T \cdot N^2}{3 \cdot \eta_k} \tag{4.1}$$

With the constant of Boltzmann k_B, the absolute temperature T, the number of particles per volume unit N and the viscosity of the continuous phase η_k. Thus, in this case the aggregation is independent of the particle size and only depends on the particle number. In the second step of the aggregation process, the number of collisions strongly depends on the particle size. Shear stress promotes the probability of collision and thereby the aggregation of the whey proteins (orthokinetic aggregation). The number of collisions in this second step J_2 can be described by eq. (4.2).

$$J_2 = \frac{2 \cdot d^3 \cdot N^2 \cdot \dot{\gamma}}{3} \tag{4.2}$$

The number of collisions J_2 depends on the particle diameter d, the number of particles N and the shear rate $\dot{\gamma}$ (Walstra et al., 1984). If Brownian motion or hydrodynamic shear forces are dominant they can be described by the ratio between the numbers of collisions of both steps (J_2/J_1). J_2/J_1 means number of collisions induced by shear proportional to the number of collisions induced by thermal diffusion (Fischer et al., 2009). Next to the frequency of collision also the intermolecular bonds can only be created, if the energy of the collision is high enough to overcome the shear stress of the fluid. Additionally, the particles must face each other correctly and the kinetic energy must be sufficient. At higher shear rates the encounter time between particles is also reduced. Hence, under these conditions there is insufficient contact time for successful primary particle attachment and thus these particles are more likely to remain as individual particles in the submicron range (Steventon, 1992; Zumaeta et al., 2006). The mentioned studies already gave deeper insights to control aggregate size and structure during the shear process. While most research has focused on low protein concentration (<10% (w/w)), rather little attention has been paid to higher protein concentration. Concentrates with high protein contents are generated or applied in various processes. The influence of process conditions during shear treatment has not been investigated so far at protein concentrations as high as up to 30%, which is of relevance for evaporation processes and extrusion cooking. Therefore, the objective of the current study was to investigate the structure formation during thermal aggregation of whey proteins under shear stress as a function of protein concentration, heating time and shear stress with a particular interest at high protein concentrations.

4.2 Material and methods

4.2.1 Preparation of protein solutions

Whey protein concentrate (WPC80, GermanProt, Sachsenmilch, Leppersdorf, Germany) with a protein concentration of 80% was diluted in deionized water to obtain solutions with protein concentrations of 5%, 10%, 20% and 30% (w/w). Thus, the relation between protein and ions was constant. For this reason the ionic strength and the pH was not adjusted. The pH changes slightly from pH 7.0 for 5% protein content up to pH 6.7 for 30% protein concentration. Analysis of the main the components in the WPC80 yielded the contents as: protein 80.0%, lactose 4.4%, ash 3.2%. The whey protein solution was stored for at least 12 h at 4 °C to ensure complete hydration.

4.2.2 Thermo-mechanical treatment

Heat treatments were performed using a rotational rheometer AR1000 (TA Instruments, Eschborn, Germany) equipped with a customer designed Mooney/Ewart geometry (cup diameter: 30 mm, bob diameter: 28 mm, angle: 4°), The Mooney/Ewart design combines cylindrical and cone/plate geometry. The same shear rate in the annular flow gap and in the conical bottom can thus be achieved. This device makes the application of high heating and cooling rates possible: up to 15 °C min^{-1} during heating and 9 °C min^{-1} during cooling, with two water baths, one for heating and one for cooling. The effect of the heating and cooling ramps cannot be fully avoided, but this method of being able to combine heat and defined shear rates simultaneously is the only and best way to assess the impact of shear on the denaturation and aggregation of the proteins. The temperature profile for a heat holding time of 10 s is depicted in Fig. 4-1. The product sample was placed in the gap between two cylinders constituting the Mooney/Ewart geometry of the rheometer where a shear rate can be applied. Protein solutions were heated at a temperature of 80 °C for varying periods of time (10 s - 30 min) under controlled shear conditions (100 s^{-1} to 1452 s^{-1}). Shear stress was applied during heating as well as during cooling. The samples were covered to avoid evaporation of water during the measurement.

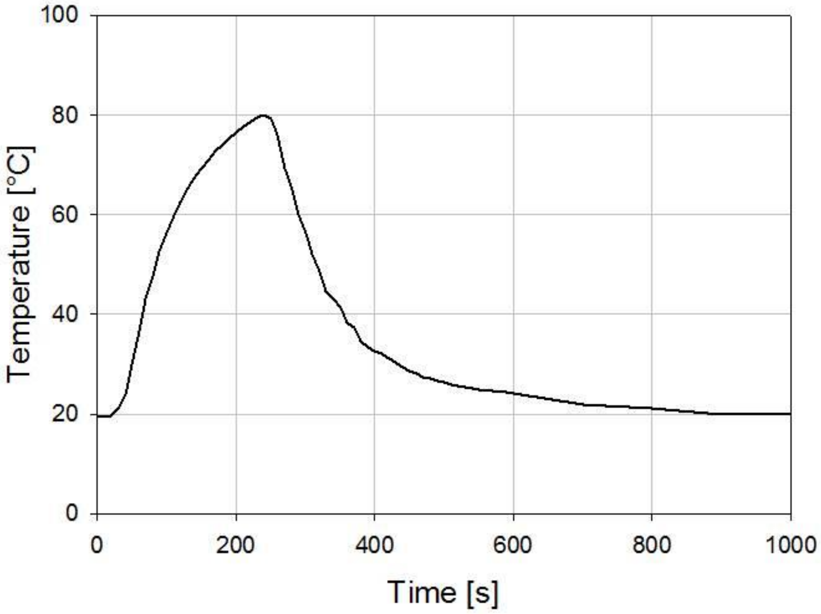


Fig. 4-1: Temperature profile for a heat holding time of 10 s at 80 °C.

4.2.3 Viscosity measurement

Rheological measurements were performed with a rotational rheometer AR1000 (TA Instruments, Eschborn, Germany) at a controlled temperature of 20 °C. In contrast to the thermomechanical treatment the viscosities of the samples were measured using a standard DIN rotor (cup diameter: 30 mm, bob diameter: 28 mm). The shear viscosity of samples was measured over the shear rate range 1-1452 s^{-1}. All measurements were performed at constant temperature of 20 °C. First measurements were taken 2 min after the geometry was immersed in each sample, to allow thermal equilibrium in the sample, and to eliminate the effect of immediate time dependence on the measurement data. The shear rate versus shear stress was interpreted according to the power law expression. Viscosities of the protein solutions were compared at 100 s^{-1}. The critical shear rate for the occurrence of Taylor vortices were calculated by the method of (Mezger, 2006). For protein solutions with protein concentrations of 5% and 10% Taylor vortices could not be excluded for shear rates over 300 s^{-1} and 600 s^{-1}, respectively. For higher concentration the critical shear rate is far above the studied shear rates. The calculated critical shear rates were also controlled by the analysis of the flow curves.

4.2.4 Particle size measurement

Particle size distributions of the samples were measured using Malvern Mastersizer Hydro 2000S (Malvern Instruments Ltd, Malvern, Worcestershire, UK). Particle size distributions were calculated by analyzing the sample's scattering pattern with an optical model (Mie theory). The refractive indices of the dispersed phase (1.45), the dispersant medium (1.33) and the absorbance of the protein solution (0.001) were selected (Dybowska, 2011; Erabit et al., 2013; Gordon & Pilosof, Ana M. R., 2010). The size measurements were reported as $d_{10,3}$, $d_{50,3}$ and $d_{90,3}$, diameters at 10, 50 and 90% of cumulative volume, respectively.

4.2.5 Analysis of the degree of denaturation

After the thermal treatment, the irreversible denaturation degree was determined using reversed phase high pressure liquid chromatography (RP-HPLC), as described by (Toro-Sierra et al., 2013). The method analyzes the amount of native whey proteins after precipitation and separation of the denatured and aggregated whey proteins at pH 4.6, and thereby the protein solubility (Dannenberg & Kessler, 1988; Kessler & Beyer, 1991; Roefs & Kruif, 1994). The solubility is related to colloidal stability, and does not measure denaturation directly, but rather the aggregation that follows extensive thermal denaturation (Loveday, 2016). The denaturation degree represents the difference between native protein concentration before and after the thermo-mechanical treatment in relation to the native protein concentration before the treatment. The major whey protein affecting denaturation kinetics is β-lactoglobulin (β-Lg) since it represents over 50% of the total whey protein in bovine milk. For this reason β-Lg was selected as the lead component for the present study. In this study RP-HPLC for the assessment of β-Lg content was used, since this technique provides information about protein molecules remaining native or refolded to the native state after heat treatment.

4.2.6 Optical microscopy

The morphology of the aggregates was visually assessed using an optical microscope (Axioskop, Carl Zeiss AG, Jena, Germany). The aqueous aggregate suspensions were diluted to 0.05% (w/w) and photographed at 100-fold magnification.

4.2.7 Statistical analysis

All experiments were performed in triplicate. Mean values with 95% confidence levels are reported. t-Test was applied to estimate differences between mean values at a confidence level of 95%. Curves were fitted using SigmaPlot for Windows Version 11.0 Build 11.0.0.75 (Systat Software Inc., Chicago, USA).

4.3 Results and discussion

4.3.1 Influence of protein concentration

Increasing whey protein concentration accelerates the thermal denaturation rate not only under quiescent conditions (Verheul et al., 1998; Wolz & Kulozik, 2015) but also when shear stress is applied. Fig. 4-2 shows this increase of the degree of denaturation (DD) with protein concentration.

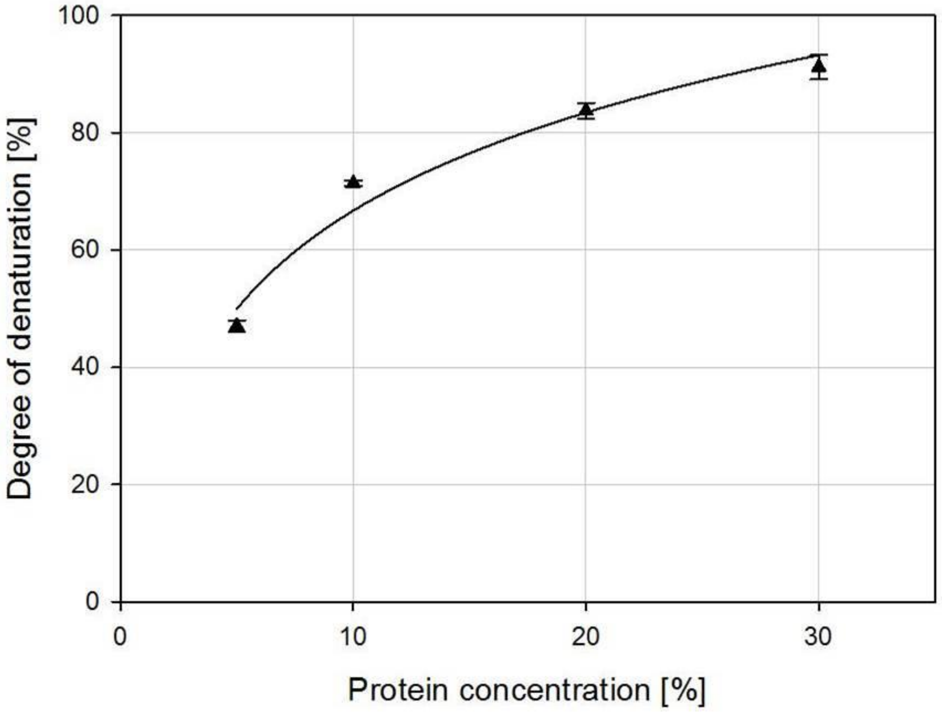


Fig. 4-2: Influence of protein concentration on the degree of denaturation for solutions heated at 80 °C for 10 s and a shear rate of 1452 s^{-1}.

After heating the protein solutions with a concentration of 30% at 80 °C for 10 s the degree of protein denaturation is already DD > 90%. However, solutions with 5%

only reach a DD < 50% under the same conditions. These findings are consistent with the previous experience in whey protein technology. Findings reported in studies in cell biology (Ellis, 2001; Mittal et al., 2015) on the stabilizing effect of molecular crowding, i.e. at higher protein concentrations, could not be confirmed for whey proteins, probably because of the different type of reaction and extreme conditions in terms of protein concentration and temperature in comparison to physiological conditions. With increasing protein concentration the number of protein molecules increases. Hence, the probability of collision between the protein molecules rises. This results in a higher aggregation rate and an accelerated denaturation reaction. However, although the mass ratio of protein to mineral was not changed, the ionic strength and the slight pH changes could have an additional effect on protein properties (e.g. zeta potential) and aggregation behavior (Engelhardt et al., 2013; Schmitt et al., 2007). Furthermore, the particle size is also affected by the protein concentration.

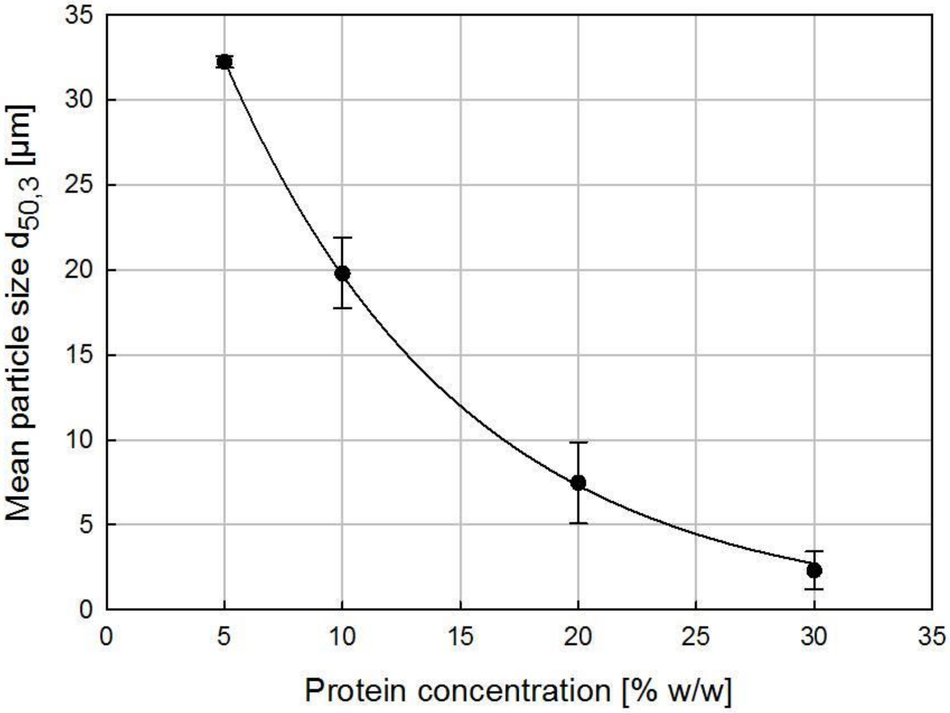


Fig. 4-3: Mean particle size $d_{50,3}$ as a function of whey protein concentration for heating at 80 °C for 30 min with a shear rate of 1452 s^{-1}.

After a heating time of 30 min all whey proteins are denatured and a DD of at least 98% for all protein concentrations is reached. We ensure the particle size was mainly influenced by protein concentration. Increasing whey protein concentration results in an exponential decay of the mean particle size ($d_{50,3}$) as shown in Fig. 4-3. The obtained results are in line with the results of Steventon (1992) for lower protein concentrations. Opposite findings from Purwanti et al. (2011) and Hoare et al. (1982) resulted from experiments performed under conditions without shear stress or at low pH, respectively. The results of the present study can be attributed on the one hand to the accelerated denaturation rate and, on the other hand, to the increasing viscosity. Hence, the shear stress affecting the proteins increases.

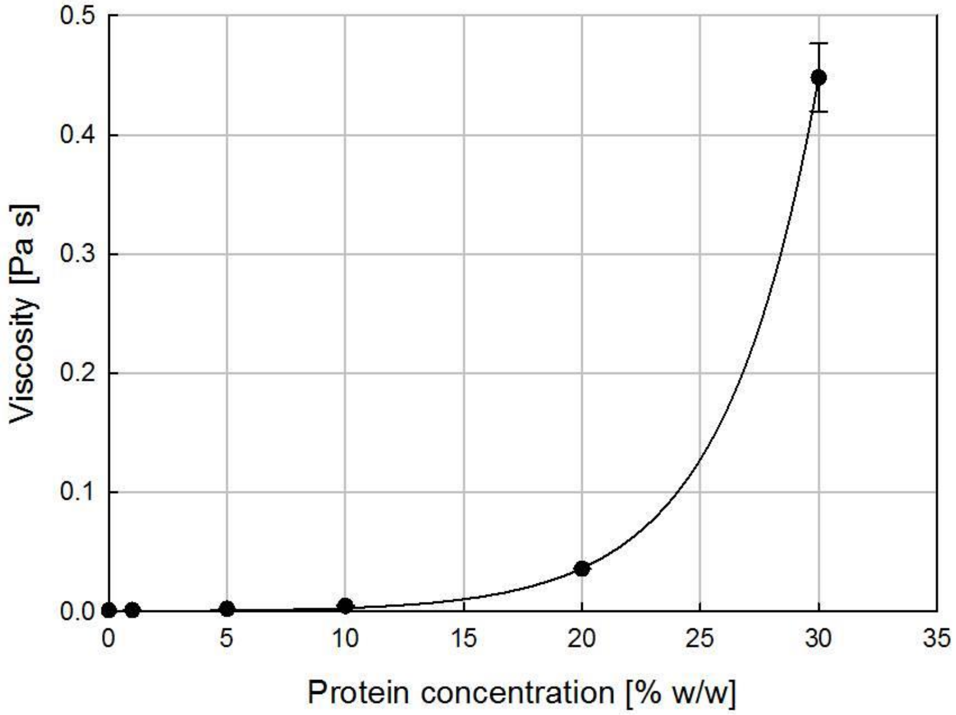


Fig. 4-4: Change of viscosity with whey protein concentration of unheated whey protein solutions at 20 °C and 100 s^{-1}.

Fig. 4-4 shows the exponential increase of viscosity of the native whey protein solutions with increasing protein concentration. This qualitative curve progression can also be estimated for denatured protein solutions, but cannot be measured without the effect of different particle size distributions (Brouwers, 2010). The viscosity increase can be responsible for the limitation of the mean particle size (Fig. 4-3). A higher viscosity restricts the potential of motion of the single particles. Simultaneously, the shear stress acting on the particles rises with the viscosity. The effectiveness of collisions is thus reduced. Both effects leads to a decrease of particle size at higher protein concentration.

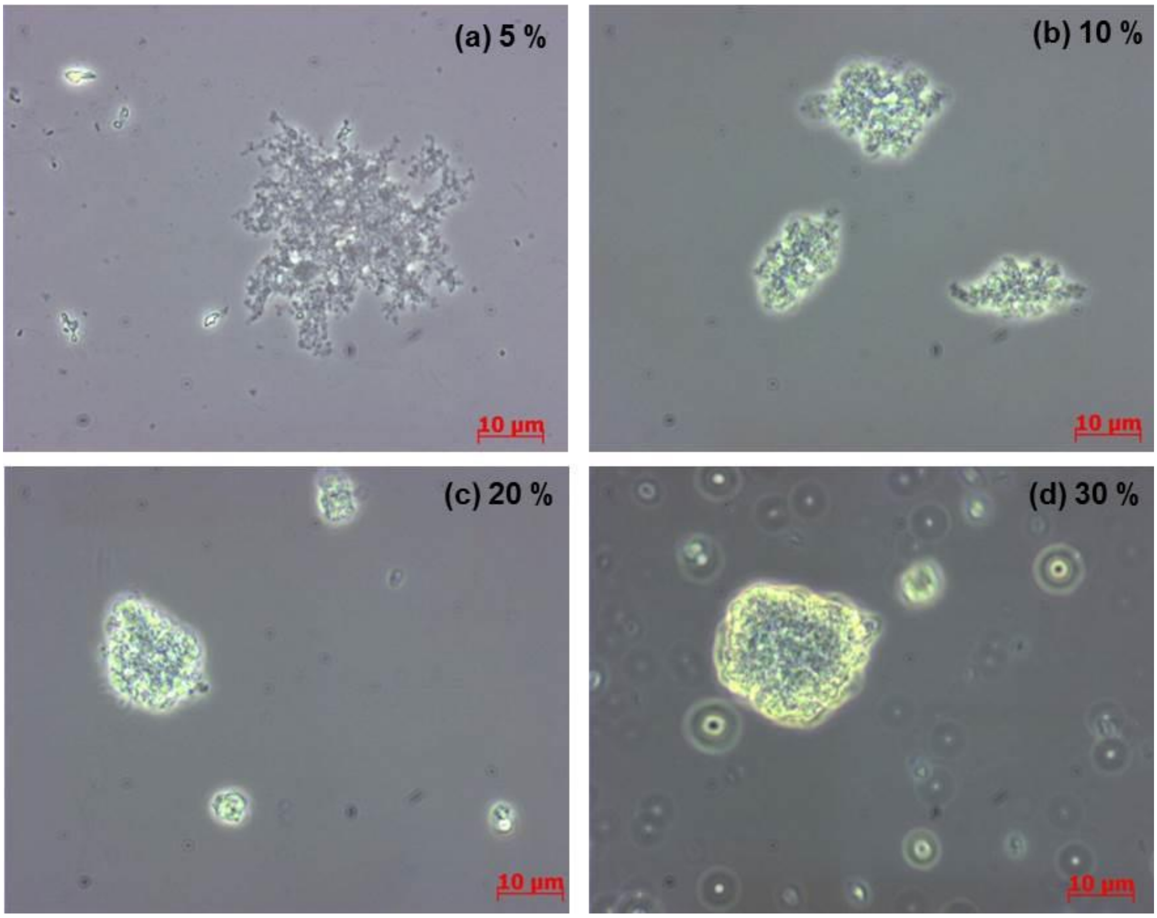


Fig. 4-5: Structure of whey protein aggregates heated and sheared at 80 °C with protein concentrations of (a) 5% (b) 10% (c) 20% and (d) 30% with DD > 95%.

Protein concentration also influences the structure of the formed aggregates, besides the degree of denaturation and the particle size. This influence on the structure can be observed by the light microscopy picture with 100x magnification in Fig. 4-5.

The structure and appearance of the aggregates changes with increasing protein concentration. It changes from branched porous aggregates at low concentration to a round and compact form at high protein concentrations (Fig. 4-5a-d). Large aggregates with many branches and a loosely structure are observed for suspension with 5% of protein (Fig. 4-5a). Furthermore, the parts of the particles at the surface are not firmly associated with the core of the aggregate. With increasing concentration the aggregates become smaller, denser and more compact. The shape of the aggregates changes from irregular formed particles towards spherical ones. Due to the lower viscosity at lower protein concentrations a reduced shear stress acts on the particles, and consequently particles can stick to each other at many contact points. Thereby, due to high shear stress. Accordingly, the aggregates obtain a more spherical shape and a smooth surface. The resulting aggregates become more compact due to the higher force (or rather shear stress) acting on the particles.

4.3.2 Influence of heating time

The denaturation reaction of whey proteins is strongly affected by the temperature-time-conditions during heating. For this reason the changes of particle size over time were studied. The influence of the heating time depends, at the meanwhile, on the protein concentration.

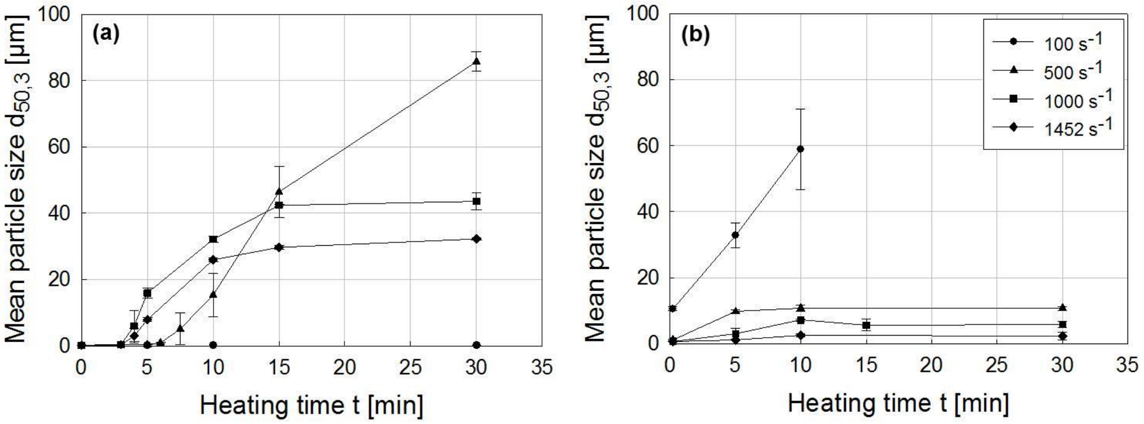


Fig. 4-6: Development of the mean particle size $d_{50,3}$ over heating time at 80 °C at different shear rates and protein concentrations of (a) 5% (w/w) and (b) 30% (w/w).

At low protein concentrations with short periods of heating, nearly no change of aggregate size can be detected (Fig. 4-6a). After this lag-phase the particle sizes increase steeply for the shear rate of 500 s^{-1}, while the curves for the higher shear rates of 1000 and 1452 s^{-1} level off, when the denaturation reaction is completed. It is an interesting observation that for the shear rate of 100 s^{-1} there is hardly any particle growth. This could be explained by the lower collision rate in combination with the low protein concentration. Under these conditions the proteins denature without measurable aggregation. The occurrence of the lag-phase and the abrupt rise of the aggregate size is in accordance with expectations. In the first step primary aggregates are created by contact through Brownian motion. When these aggregates are large enough, the particle movement is influenced by the shear flow, whereby the rate of collision between the aggregates increases (second step). At higher protein concentrations (Fig. 4-6b) no lag-phase can be determined. The reaction rate is very fast. The aggregate size increases with the heating time, as long as the denaturation reaction is not completed and proteins still aggregate (Fig. 4-6). This behavior can be observed for different shear rates, but the mean particle size is lower for higher shear rates (see 4.3.3). For very small shear rates (100 s^{-1}) the aggregate size increases subsequently. The shear force acting on the aggregates is not high enough to cause a disruption of the particles. No single aggregates are created, but rather a gel network.

Tab. 4-1: Quotient of the collision number by shear diffusion and thermal diffusion J_2/J_1 for different shear rates and particle sizes at 80 °C for a protein concentration of 5%.

x [µm]	$\dot{\gamma}$ [s^{-1}]			
	100	500	1000	1452
0.1	$3.6 \cdot 10^{-2}$	$1.8 \cdot 10^{-1}$	$3.6 \cdot 10^{-1}$	$5.3 \cdot 10^{-1}$
0.2	$2.9 \cdot 10^{-1}$	$1.4 \cdot 10^{0}$	$2.9 \cdot 10^{0}$	$4.2 \cdot 10^{0}$
0.5	$4.5 \cdot 10^{0}$	$2.3 \cdot 10^{1}$	$4.5 \cdot 10^{1}$	$6.6 \cdot 10^{1}$
1	$3.6 \cdot 10^{1}$	$1.8 \cdot 10^{2}$	$3.6 \cdot 10^{2}$	$5.3 \cdot 10^{2}$
5	$4.5 \cdot 10^{3}$	$2.3 \cdot 10^{4}$	$4.5 \cdot 10^{4}$	$6.6 \cdot 10^{4}$
10	$3.6 \cdot 10^{4}$	$1.8 \cdot 10^{5}$	$3.6 \cdot 10^{5}$	$5.3 \cdot 10^{5}$

As an explanation of the abrupt rise of the particle size at low protein concentrations (5%), Tab. 4-1 shows the quotient of the collision numbers J_2/J_1 for different shear rates and particle sizes. J_1 is independent from the particle size and the shear rate and is mainly affected by the number of particles. J_2 in contrast is dependent on the shear rate, on the number of particles and strongly also on the particle size (with the power of 3). The higher the shear rate and larger the particle size, the higher is J_2/J_1. The collisions induced by shear forces are more predominant. The lower the shear rate and the smaller the particles, the smaller is J_2/J_1 and the more prevailing are the collisions induced by Brownian motion. For the process with short heating time the particles are small, which leads to low J_2/J_1, in the beginning of the heating and shearing process. This means that collisions are dominated by Brownian motion. Under these conditions no increase of particle size can be observed. The low protein concentration of 5% is subcritical. The number of particles is not sufficient to cause enough effective collisions between the proteins to observe a measurable increase of particle size by thermal motion. For high shear rates the point in time beyond which the particles increase is earlier compared to lower shear rates (Fig. 4-6a). This is also expressed by Tab. 4-1, where the distinctive influence of the particle size on J_2/J_1 is highlighted. When particles become big enough to be affected by the convective flow higher collision rates occur and the particle size starts to increase steeply (Fig. 4-6a). At this point the number of collision caused by shear stress is much higher compared to the number of collisions by thermal diffusion. This is also demonstrated in Fig. 4-7: At the beginning the already bigger primary aggregates grow, in contrast to the smaller particles which are limited by the Brownian motion. The mean particle size is nearly not affected. At longer heating times the smaller particles aggregate as well. When these particles are big enough and the transition between the Brownian motion limited range to the shear induced range of aggregation happens, the particle size increases abruptly. After approx. 15 min the particles are nearly completely aggregated, this results in a monomodal particle size distribution. For the higher protein concentration the effect of shear is not required to induce collision (Fig. 4-6b). Here, particle grow fast due to high molecule density and shear becomes the dominating factor is limiting particle growth.

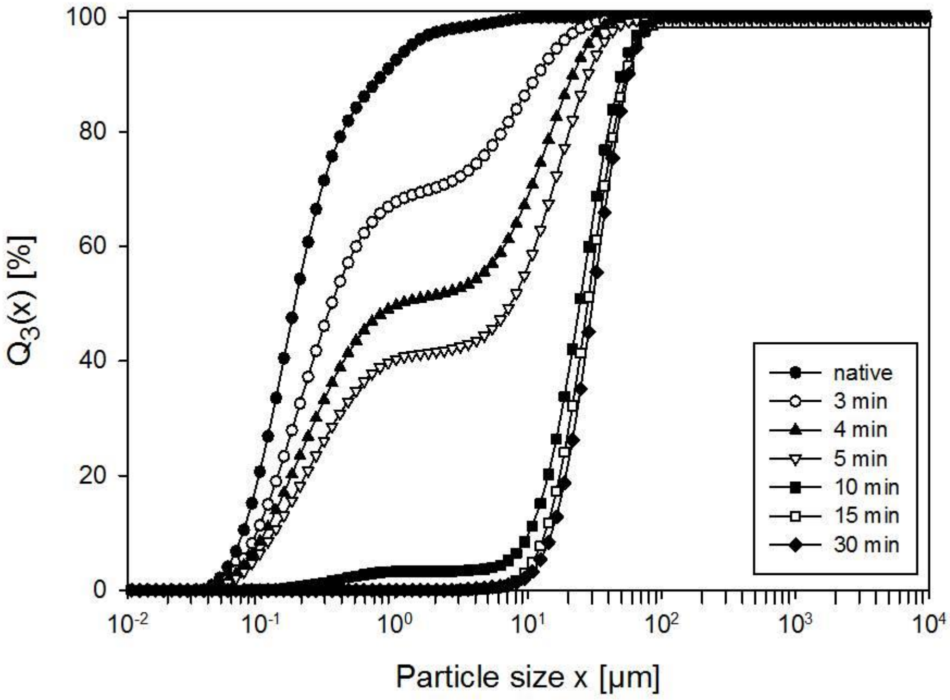


Fig. 4-7: Influence of heating time on the volume based particle size distribution $Q_3(x)$ for a protein concentration of 5% heated at 80 °C and 1452 s^{-1}.

4.3.3 Influence of shear rate

Fig. 4-8 depicts the influence of the shear rate on the degree of denaturation for different protein concentrations.

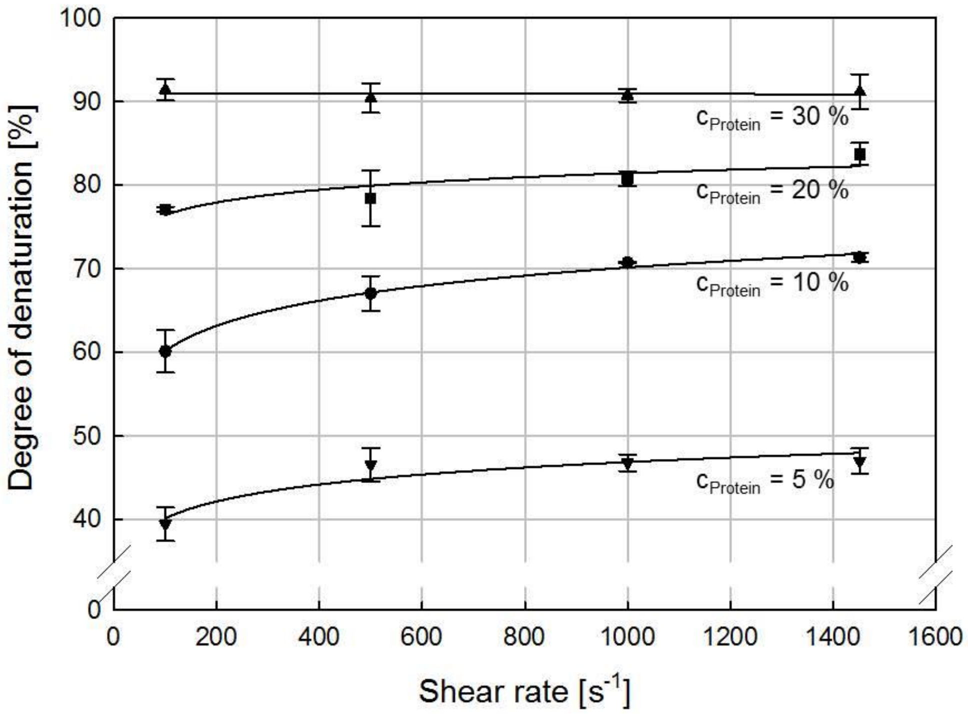


Fig. 4-8: Influence of shear rate on the degree of denaturation for different protein concentrations (5-30%) heating at 80 °C for 10 s.

At high protein concentrations ($c_{Protein}$ = 30%) after a short heating time of 10 s the denaturation reaction is nearly finished and all whey proteins are denatured. For this reason the shear rate has no measurable effect on the DD (Fig. 4-8). At lower protein concentrations, increasing the shear rate causes an increasing DD. These findings can be attributed to the higher probability of collision between the proteins and the accelerated aggregation rate correlated to the rate of collision. To maintain the equilibrium between native and partially unfolded protein molecules the unfolding of the proteins is accelerated. However, if the temperature or the protein concentration is high, the denaturation reaction is already very fast and an increasing shear rate has no additional effect on the aggregation rate. Experiments carried out at temperatures below the unfolding temperature of the proteins did not show any denaturation or rather aggregation effect on the proteins per se (data not shown). In other words, shear has a synergistic effect on the thermal denaturation and aggregation due to the effect of increasing the probability of collision. It should also be noted that the effect of mechanical stress alone on the native molecule is by far not sufficient to induce unfolding or even breaking the molecule in parts. Temperature and protein concentration, however, together have a larger impact on the rate of the denaturation reaction compared to the shear rate. Temperature induces unfolding and shear increases the convective flow and the collision rate.

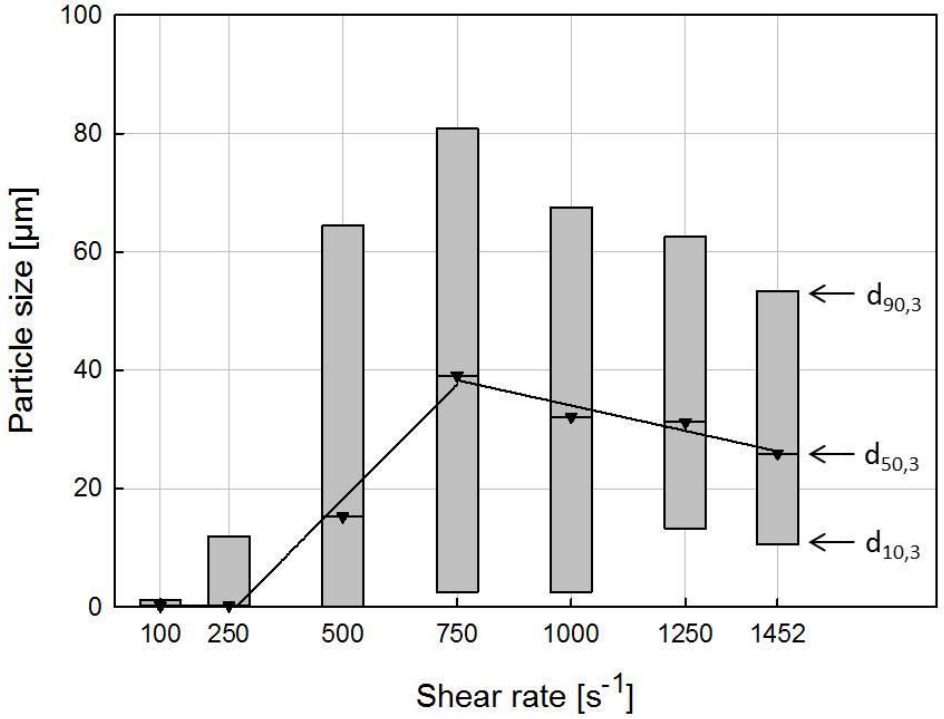


Fig. 4-9: Influence of shear rate on the particle size of whey protein solutions with $c_{Protein}$ = 5% heated at 80 °C for 10 min with a degree of denaturation of >95%.

Moreover, the shear rate also has an effect on the particle size of whey protein aggregates. This effect is furthermore dependent on the protein concentration. Fig. 4-9 depicts the aggregation results, i.e. particle sizes at low protein concentrations.

At low protein concentrations ($c_{Protein}$ = 5%) the particle size initially increases with the shear rate (100 - 750 s^{-1}), as described by the theory of collision by Smoluchowski (1916). At higher shear rates (750 - 1452 s^{-1}), the particle size tends to decrease. This could additionally be a result of Taylor vortices occurring in the annular gap of the rheometer at high shear rates and at low viscosity. Overall, the decrease in size can be attributed to the increasing shear stress acting on the particle. Under these conditions the effects are the same as for the high protein concentration explained in the following.

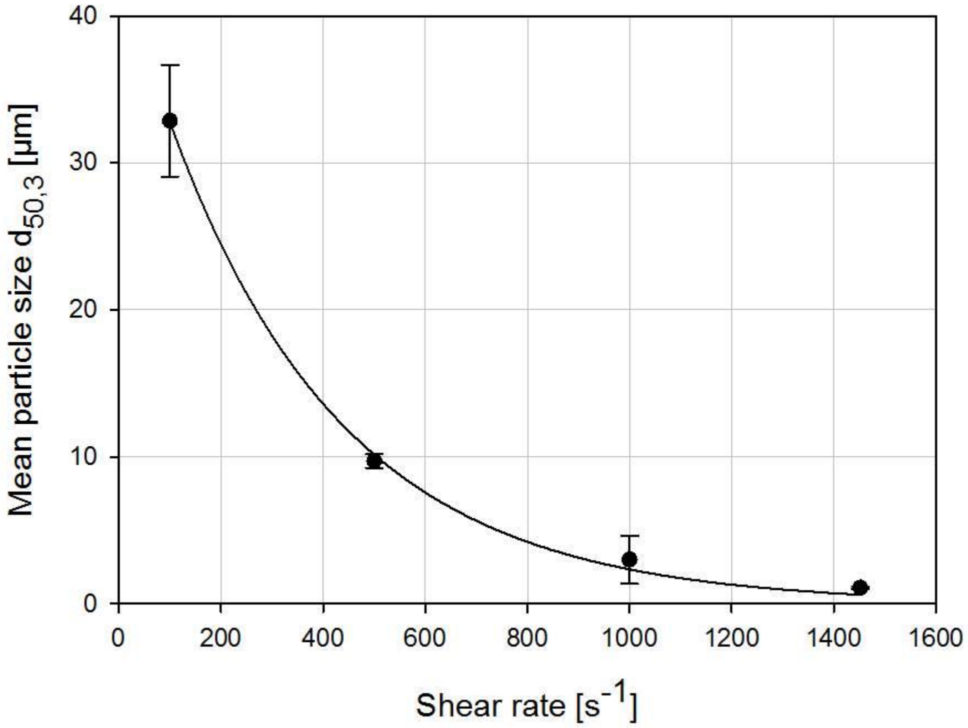


Fig. 4-10: Influence of shear rate on the mean particle size $d_{50,3}$ for a protein concentration of 30% heated at 80 °C for 10 s with a degree of denaturation of >95%.

Fig. 4-10 shows the influence of the shear rate at $c_{Protein}$ = 30%. At this high protein concentration a decrease of particle size with increasing shear rate was observed. The mean particle size $d_{50,3}$ decreases exponentially with the shear rate. The reason for this is the accelerated aggregation rate at high protein concentration. The probability of collision is higher or high enough for aggregation at elevated temperatures simply due to the increased number of protein molecules. This is why the shear rate has a lower relative impact as compared to the lower protein concentration of 5%.

In other words, the high tendency to aggregate at 30% protein concentration is mainly driven by the high density of reactive molecules under elevated thermal conditions. In this environment the shear rate limits the growth of particles, thus limiting their final size. However, particle collisions will only lead to permanent aggregation if cross-links can be formed with sufficient energy to resist the fluid shear stresses. Furthermore, the collision rate increases with shear rate, while at the same time the contact time becomes shorter. Therefore, the proteins and already existing aggregates may not have the time to orientate themselves for effective interactions. In summary, this means that under these conditions (high protein concentration, high shear rate, low contact time) large particles cannot be formed in the first place. This explanation agrees with Steventon (1992) who investigated whey protein aggregation under concurrent thermo-mechanical treatment, however, at low concentrations.

A decrease of particle size with shear rate was also found by other authors for isoelectrical precipitation of whey proteins (Byrne et al., 2002; Zumaeta et al., 2006) and of soy protein (Bell & Dunnill, 1982; Hoare et al., 1982) under stirring, however, without thermal treatment above the denaturation temperature. These researchers attributed the effect of shear rate on aggregate size to the break-up of already existing aggregates. However, in our case the results show that narrow particle size distributions are obtained, when higher the shear stresses are applied. This observation supports the hypothesis that particles reach the final size while being formed instead of being formed and then being destructed. Otherwise, wider particle sizes would be expected, because fragments in different sizes would be formed if larger particles would be destroyed.

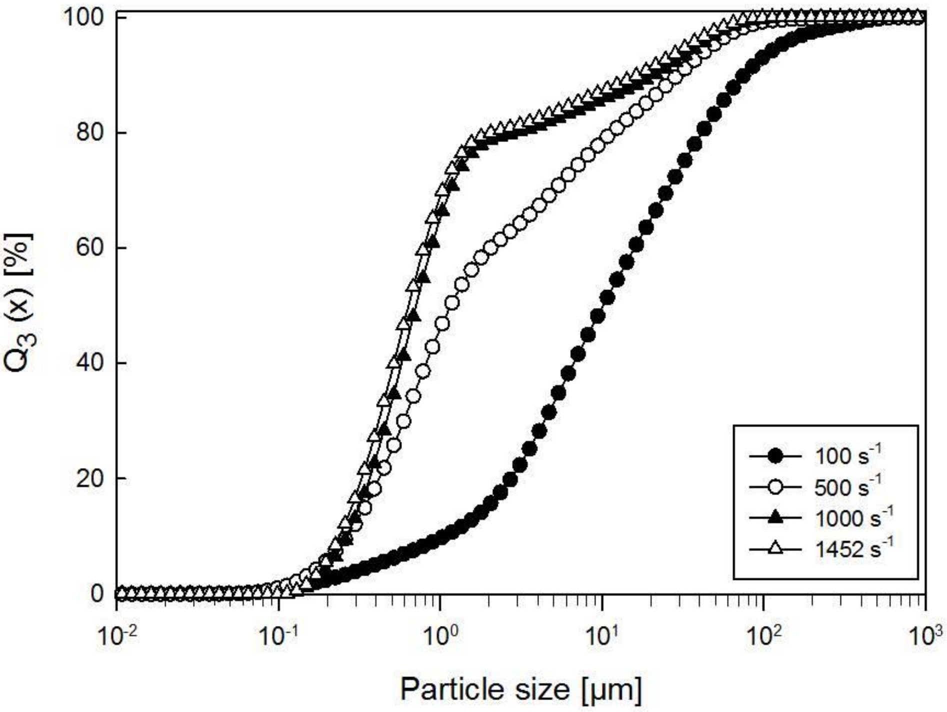


Fig. 4-11: Influence of shear rate on the volume based particle size distribution Q_3(x) for a protein concentration of 30% heated at 80 °C for 10 s with a degree of denaturation of >95%.

The influence of shear rate on the volume based particle size distribution for protein concentrations of 30% is shown in Fig. 4-11. A decrease of the size can be observed as the shear rate increases. At high protein concentrations some bigger aggregates remain also at high shear rates. The influence of the shear rate on the aggregate size decreases with increasing shear rate. This can be attributed to a limited particle size. A hard aggregate core is created upon thermal denaturation, which could not be destructed by shear forces (Zumaeta et al., 2006).

4.4 Conclusion

The used experimental methodology applying a rotational rheometer enables to observe the influence of process conditions on the aggregation of whey proteins with the focus on the behavior of highly concentrated protein solutions. The heating time, shear rate and protein concentration were investigated independently of each other to assess the individual impact. This study formed the basis for a deeper understanding of the mechanism of the aggregation in concentrated protein solutions. By increasing the protein concentration, smaller more compact and stable aggregates are formed. The influence of shear rate is dependent on protein concentration. In suspension of 5% protein the aggregate size initially increases as the shear rate increases due to the predominant effect of increasing number of collisions; and decreases subsequently due to the increasing shear stress. At high concentrations the size of the aggregates decreases with the shear rate, because of increasing shear stress. The shear rate is the critical factor to limit particle size. The results obtained are helpful for selecting process conditions during the production of whey protein micro-particles by simultaneous heating and shearing processes using scraped surface heat exchanger or extrusion cooking. These insights also allow better process designs for the production and preservation of whey protein concentrates or powders. In these cases, aggregation of whey protein is not desired or even has to be avoided completely. A more detailed study including variables such as temperature, lactose content, pH and ionic composition will further extend the knowledge gained from the study presented here. In the future, it is also worth to study the influence of pure solutions of β-lactoglobulin and α-lactalbumin to analyze the role of the single proteins and to investigate also the changes of particle size on molecular scale.

Summary and Contribution of the Doctoral Candidate

The irreversible thermal denaturation of whey proteins can basically be considered as a two-step reaction: the unfolding of the protein during heating and the subsequent aggregation of the unfolded molecules. The size and structure of the resulting protein aggregates strongly depend on the reaction kinetics and the conditions during processing. These conditions include heating temperature, heating time and shear stress. Thermal aggregation of whey proteins during concurrent heating and shearing takes place, for example, in heat exchangers, evaporators or extruders. However, there is a lack of knowledge about the influence of process conditions during shear treatment at elevated protein concentrations. Some studies focusing on low protein concentrations indicate that the protein concentration strongly influences the aggregation step of the denaturation reaction and accordingly the resulting aggregate properties. For this reason, this study carried out a systematic investigation of the impact of protein concentration, heating time and shear stress on aggregate size and degree of protein denaturation. Since processing conditions often cannot be varied separately in industrial processes, experiments were conducted in a rotational rheometer at lab scale. This offered the possibility to determine the single contribution of the process conditions independently of each other and assess their individual impact.

Smaller and more compact aggregates were formed at higher protein concentrations. This was explained by the higher viscosity and the associated higher shear stress acting on the particles. Different effects of the shear rate were obtained, depending on the protein concentration. At low protein concentrations, the aggregates size initially increased with increasing shear rate. Above a critical value, the particle size decreased. These trends were attributed to two different predominant effects: first, the increasing number of collisions that promotes the aggregation and, second, the limitation of the particle growth due to increasing shear stress. At high protein concentrations, only the second effect was observed. A major finding of this study was that the shear rate was the critical factor for the limitation of the aggregate size. Thus, the shear rate can be used to control the particle size during simultaneous heating and shearing processes for microparticulation.

The substantial contributions of the doctoral candidate include conception and design of the experiments based on preceding critical literature review, as well as the writing and the revision of the majority of the manuscript. The doctoral candidate carried out major parts of the data analysis, interpreted the data set, and discussed it.

5 High moisture extrusion for microparticulation of whey proteins - Influence of process parameters[3]

Abstract

Defined functional properties of whey proteins can be achieved by controlling thermal unfolding and subsequent aggregation. To achieve a controlled formation of micro-particles, thermo-mechanical treatment by high moisture twin screw extrusion was used. The aim was to control the degree of denaturation as well as the particle size by variation of the process parameters. The temperature of the extruder has a strong impact on the maximum product temperature and increasing the extruder temperature increases the degree of denaturation. An increasing mass flow in contrast decreases the specific heat transfer to the product and leads to a decrease in denaturation. Increasing the screw speed has almost no effect on the degree of denaturation, but results in an exponential decrease in particle size due to increasing mechanical shear stress. Thus, the degree of irreversible protein denaturation as well as the particle size distribution can be controlled by these process parameters and aggregates with specific properties can be produced.

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[3] Original publication: Wolz, M.; Kastenhuber, S.; Kulozik, U. (2016a). High moisture extrusion for mi-croparticulation of whey proteins –Influence of process parameters. Journal of Food Engineering, 185, 56–61. doi: 10.1016/j.jfoodeng.2016.04.002.
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Highlights

- Novel extrusion process set-up for microparticulation of whey proteins.
- A heating and a cooling zone allows the microparticulation also at neutral pH values.
- Particle properties can be controlled by process parameters.
- Aggregates with individual properties can be produced.

5.1 Introduction

Processing functions of extruders can include conveying, mixing, shearing, separation, heating or cooling, shaping, co-extrusion, venting volatiles and moisture, flavor generation, encapsulation and sterilization of biomaterials (Guy, 2001). Hence, extrusion processes can change the molecular structure of food ingredients, e.g. of proteins. Extrusion processes at low moisture levels are widely used in food industry for production of instant snack foods, cereals, pasta and pet foods (Guy, 2001; Harper, 1981; Kokini et al., 1992). In contrast, extrusion cooking at higher moisture contents (wet extrusion) has been studied much less frequently. Wet extrusion applications utilize twin screw extruders due to their efficient conveying capabilities. The rheological properties, torque, pressure build-up and energy requirements of high moisture extrusion systems are different from those of low moisture systems (Akdogan, 1999). Extrusion at high moisture levels is distinguished by lower friction and low viscous dissipation. Wet extrusion allows the production of innovative food products such as texturized proteins. Examples include extruded crab analog (Thiébaud et al., 1995), texturized soybean foods (Shen & Wang, 1992), fibrous structures for meat analog (Cheftel et al., 1992; Osen et al., 2014; Thiébaud et al., 1996) and sodium caseinate (Cheftel et al., 1992; Fichtali et al., 1995). Onwulata et al. (2010), Quéguiner et al. (1992a) and Qi & Onwulata (2011) processed whey protein by high moisture extrusion and extrude it subsequently a second time together with a starch matrix in order to obtain improved textural properties. Furthermore, whey proteins can be used to produce aggregates with specific functional properties using a microparticulation process at acidic pH (Onwulata et al., 2010; Quéguiner et al., 1992a). For the extrusion process of Quéguiner et al. (1992b), pH values of at least below 3.9 were necessary to achieve small aggregates. However, micro-particles with an acidic pH can limit their application in food products (Cheftel & Dumay, 1993).

Microparticulation is a controlled thermally induced aggregation process, during which shear forces are applied simultaneously or sequentially to limit the aggregate size. Whey proteins processed accordingly, used as additives, represent a possibility to impart specific structural and physical properties to food. These aggregates can be applied as fat replacer (Sandoval-Castilla et al., 2004) or functional ingredient to modify viscosity in various food products (Çakır-Fuller, 2015; Damodaran & Paraf, 1997; Lee et al., 2013). A specific variation of whey protein properties can be achieved by controlled unfolding and subsequent aggregation mainly mediated via disulfide bonds and hydrophobic interactions (Havea et al., 2001; Wijayanti et al., 2014; Zuniga et al., 2010). This targeted formation of particles with limited particle sizes to avoid a sensorially relevant impact, e.g. sandiness or mealiness induced by larger particles can be achieved by combination of thermal and mechanical treatment. The fundamental molecular mechanisms of unfolding and aggregation of whey proteins, which form the basis for particle formation, have been extensively studied (e.g. (Bon et al., 1999; Erabit et al., 2014; Hollar et al., 1995; Nicolai et al., 2011; Roefs & Kruif, 1994; Steventon, 1992; Wolz et al., 2016b). To realize a combined heating and shearing process, different options are possible at industrial scale. Microparticulation of whey proteins can be realized in a scraped surface heat exchanger (Spiegel & Huss, 2002) or in a tubular heat exchanger followed by high pressure treatment (Iordache & Jelen, 2003; Paquin et al., 1992; Singer & Dunn, 1990). An alternative technique is extrusion cooking. Using extrusion for the thermomechanical modification of proteins allows the variation of different process parameters, such as e.g. temperature, screw speed and protein concentration, in a wide range. Another advantage is the possibility of coping with higher viscosities and therefore higher protein concentrations compared to other available techniques like scarped surface heat exchangers. Such high concentrations also allow a short residence time due to accelerated denaturation kinetics with increasing whey protein concentration (Wolz & Kulozik, 2015). Furthermore, a long run time can be expected as a result of negligible product caking which is due to the self-cleaning effect of the screws.

The aim of the present study is to control the degree of denaturation as well as the particle size distribution for microparticulation of whey protein concentrate by high moisture extrusion. In comparison to literature, a neutral instead of an acidic pH is used to enable possible applications in food products with neutral pH. Furthermore, a relatively low lactose concentration is chosen and no other additives are added to increase the reaction kinetics and to avoid a browning reaction during the thermal process. Depending on the future application the aim is to produce aggregates with different particle sizes and various degrees of protein denaturation. However, the most challenging task is to produce small aggregates (between 0.5 and 10 µm) and a narrow particle size distribution with a high degree of denaturation (high yield). Addition of such particles increase creaminess and heat stability of the product during further processing and which can otherwise cause coarse protein aggregation and grittiness. The resulting physical and chemical properties at macroscopic scale depend strongly on the processing conditions. The influence of temperature, mass flow and screw speed on the resulting aggregate properties are extensively investigated in this study.

5.2 Material and methods

5.2.1 Materials

Whey protein concentrate (WPC80, Germanprot Sachsenmilch, Leppersdorf, Germany) with a protein concentration of 80% was used as feed material. By addition of deionized water, the protein concentration was adjusted to 30% (w/w) during the extrusion process. This corresponds to a dry matter concentration of about 38% and a pH of 6.7. Analysis of the major components present in the used WPC80 yielded the following contents: protein 80.0%, lactose 4.4% and ash 3.2%.

5.2.2 Extrusion process

Extrusion experiments were carried out using a co-rotating intermeshing twin-screw extruder (ZSK25, Coperion, Stuttgart, Germany) with a screw diameter of 25 mm, a smooth barrel, and a total length of the screw of 38D. The barrel consisted of nine segments, each segment (except the first one) being equipped with an independent temperature control which is heated by an electric cartridge heating system and cooled with water (Fig. 5-1). The second and third heating segment were constantly set at 30 °C and 50 °C respectively, and the other heating segments at different extrusion temperatures between 90 °C and 120 °C ± 0.5 °C (in the following named extruder barrel temperature). For all process conditions, the maximum product temperature reached at the end of the heating zone as well as the product outlet temperature was measured by a thermocouple temperature sensor touching the product. Several pretests have been conducted to ensure that the actual product temperature was measured and not the barrel temperature. The die end plate has one hole of a 10 mm diameter.

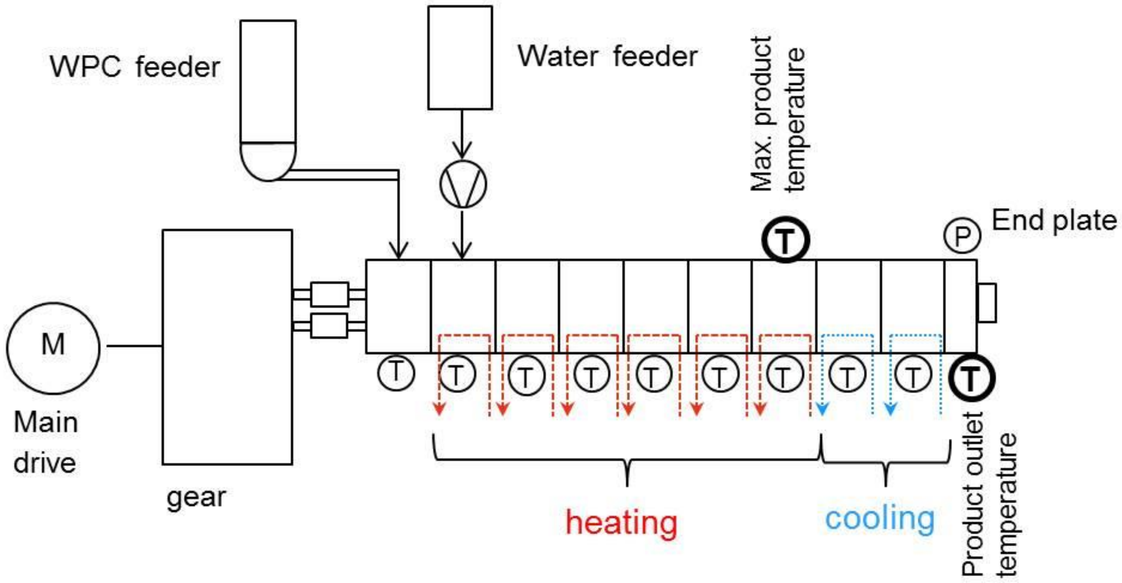

Fig. 5-1: Schematic illustration of the applied extrusion process.

The screw speed was set between 100 and 800 rpm. Whey protein powder was fed using a distinct weight feeder (K-Tron Soder, Niederlenz, Switzerland) and water was fed using a membrane pump (Grundfos, Erkrath, Germany). The total feed rate ranged from 5.3 to 21.1 kg/h. The screw profile was consisted of different screw elements that can be assembled on shafts. The screw profile is depicted in Fig. 5-2.

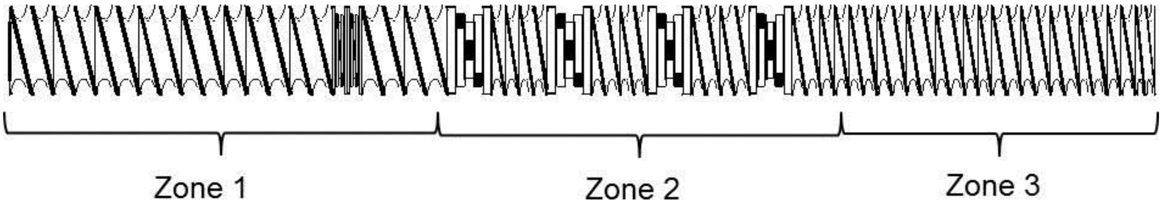

Fig. 5-2: Screw configuration used for microparticulation.

The screw profile comprised 3 zones (from feed to exit). Zone 1 (feeding zone) had a length of 366 mm and consisted of forwarding screws with a pitch of 36 mm intercepted by 2 kneading blocks (5 disks with an offset of 45° and a length of 12 mm). Zone 2 had a length of 336 mm and consisted of 4 alternating kneading blocks (5 disks with an offset of 45° and a length of 36 mm) and of 48 mm forwarding screw with a pitch of 24 mm. Zone 3 with a length of 256 mm was the cooling zone, which was comprised of a pure forwarding screw to avoid unnecessary energy input.

5.2.3 Sample collection and determination of process response

Samples were collected after the extruder system parameters (product temperature and motor torque) reached a steady state condition. Samples were filled in glass containers and immediately cooled in an ice bath. Maximum product temperature, product outlet temperature and motor torque were recorded. Samples were stored at 4 °C until analysis.

Specific mechanical energy (SME) was calculated from the maximum screw speed (n_{max} = 1200 rpm), the maximum engine power (P = 20 kW), the actual screw speed n_{act} [rpm], the torque τ [%] and the total mass flow rate $\dot{m}$ [kg/h] according to equation (5.1).

$$SME = \frac{n_{act}}{n_{max}} \cdot \frac{\tau}{\dot{m}} \cdot P \quad (5.1)$$

5.2.4 Particle size measurement

To determine the particle size distribution of extruded samples, a laser diffractometer Malvern Mastersizer Hydro 2000S (Malvern Instruments Ltd, Malvern, Worcestershire, UK) was used. Particle size was calculated using the Fraunhofer approximation. The light sources were a red He-Ne laser (632.8 nm) and a blue

LED laser (470 nm). The size measurements were reported as volume based particle size distribution or as $d_{10,3}$, $d_{50,3}$ and $d_{90,3}$, representing the diameters at 10, 50 and 90% cumulative volume, respectively.

5.2.5 Analysis of the degree of denaturation using RP-HPLC

Analysis of native whey proteins and the degree of irreversible denaturation was done by RP-HPLC. The samples were dissolved in deionized water to obtain whey protein concentrations of 1% (w/ w). Subsequently, the pH of the solutions was adjusted to 4.6 (whey protein isoelectric point) by addition of HCl. At this pH value, remaining whey proteins do not precipitate unless they are irreversibly denatured (Dannenberg & Kessler, 1988; Kessler & Beyer, 1991; Roefs & Kruif, 1994). Solutions were held for 30 min prior to HPLC analysis. The supernatant was collected and filtered using a 0.45 µm syringe filter (Chromafil RC-45/25; Macherey-Nagel GmbH & Co. KG, Düren, Germany). Analysis was performed using an Agilent 1100 series chromatograph (Agilent Technologies, Santa Clara, CA, USA) with a PLRP-S 300-Å 8-µm column (Latek, Eppelheim, Germany) using the method described by (Toro-Sierra et al., 2013). The degree of denaturation represents the difference in native protein concentration before and after the thermo-mechanical treatment in relation to the native protein concentration before the treatment. β-Lactoglobulin (β-Lg) representing over 50% of the total whey protein in bovine milk and thus being the major protein affecting denaturation kinetics in whey, was selected as the lead component for the present study.

5.2.6 Statistical analysis

All experiments and analyses were carried out in triplicate. Mean values with 95% confidence levels are reported. Curves were fitted using SigmaPlot for Windows Version 11.0 Build 11.0.0.753 (Systat Software Inc., Chicago, USA). Correlation coefficients are given in the diagrams.

5.3 Results and discussion

5.3.1 Influence of extruder temperature

Temperature mainly affects protein denaturation kinetics. The degree of irreversible protein denaturation increases with increasing temperature due to reaction kinetics taking place at higher speed. This behavior has already been extensively studied applying conditions without shear stress (Dannenberg & Kessler, 1988; Donovan & Mulvihill, 1987; Tolkach & Kulozik, 2007; Verheul et al., 1998; Wolz & Kulozik, 2015). During extrusion, next to thermal stress mechanical stress is applied to the product as a consequence of screw rotation. During extrusion of whey proteins, the corresponding reaction kinetics also increases with increasing extruder temperature. However, it must be pointed out that during the extrusion processes, the extruder barrel temperature is not equivalent to the effective product temperature.

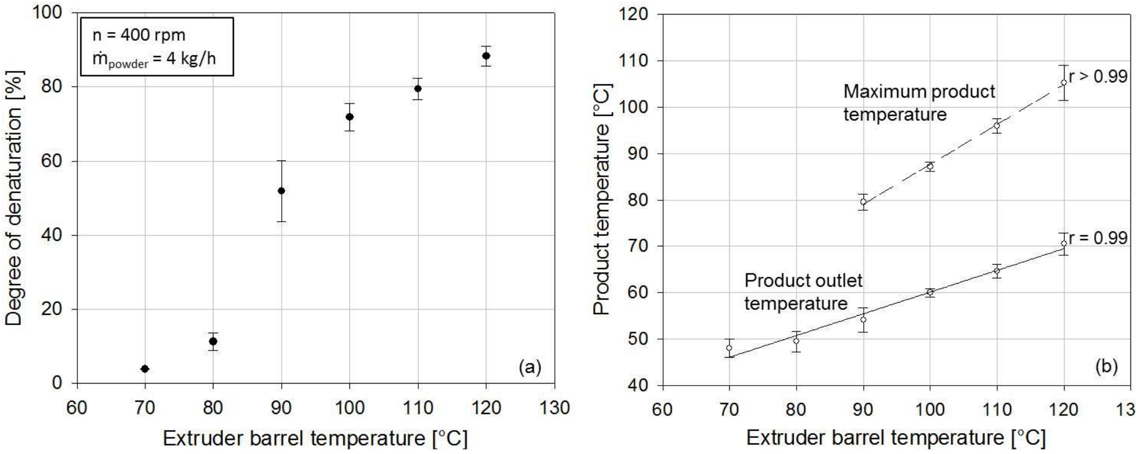

Fig. 5-3: Influence of the extruder barrel temperature on the degree of denaturation (a) and the product temperature (b) for a screw speed of 400 rpm.

Fig. 5-3a shows that the degree of denaturation increases with increasing maximal temperature of the extruder barrel in the heating zone. These results are in-line with our expectation of accelerated denaturation kinetics with increasing temperature as well as with literature data concerning the extrusion of whey proteins using a different process set-up (Quéguiner et al., 1992b). The maximum product temperature also increases with increasing temperature of the extruder barrel (Fig. 5-3b). It can therefore be concluded that the increased maximum product temperature represents the main reason for the elevated degree of irreversible denaturation. Nevertheless, the maximum product temperature always stays 10 - 15 °C below the extruder barrel temperature in the investigated temperature range (Fig. 5-3b). This suggests that the whey protein concentrate was only submitted to a heating up phase and not to a heat holding phase during the extrusion process. The product outlet temperature is considerably lower than the maximum product temperature due to the novel extruder set-up including a cooling zone at the end of the barrel. If the extruder barrel temperature becomes too high (above 110 °C) or rather the maximum product temperature is too high, the product outlet temperature exceeds the denaturation temperature of whey proteins ~68 °C) due to insufficient cooling. This causes an uncontrollable protein aggregation at the product outlet, which should therefore be avoided. Thus, the highest extruder temperature that can be selected applying the aforementioned temperature profile is 110 °C. This highest possible extruder barrel temperature could vary depending on the length of the cooling zone on the one hand. On the other hand also the protein concentration and milieu conditions used and subsequently the different denaturation kinetics can have an influence. The rate of denaturation highly depends on the temperature, the protein concentration and the milieu conditions. If the denaturation reaction become too fast, undesired large particles are formed. For this reason the highest possible extruder barrel temperature can also be limited.

5.3.2 Influence of screw speed

Fig. 5-4 shows the influence of screw speed on the degree of irreversible protein denaturation and the particle size.

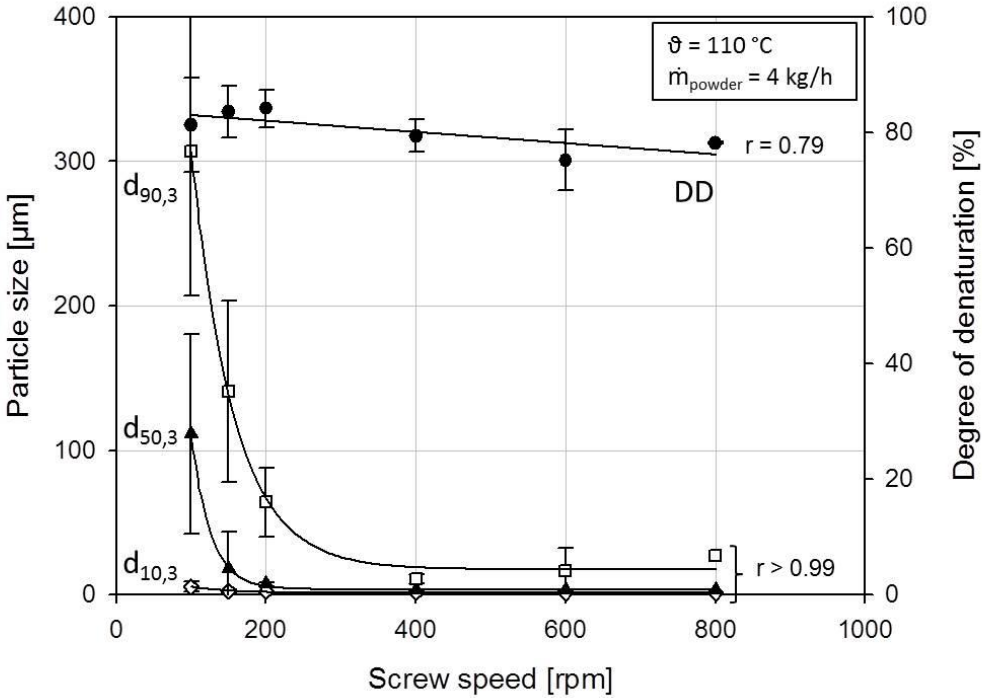

Fig. 5-4: Degree of denaturation and particle size distribution ($d_{10,3}$, $d_{50,3}$ and $d_{90,3}$) as a function of screw speed for an extruder barrel temperature of 110 °C and a powder mass flow of 4 kg/h.

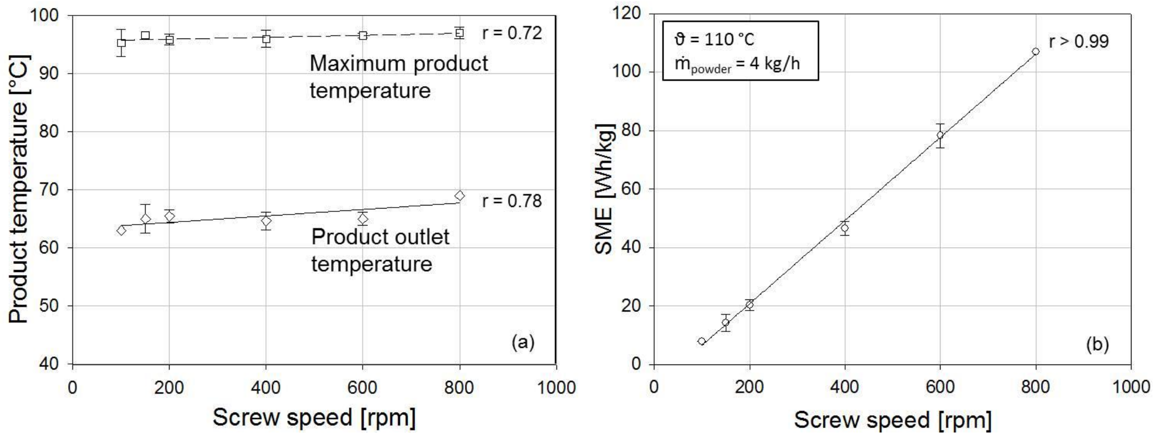

Fig. 5-5: Influence of screw speed on product temperature and degree of denaturation (a) as well as on the SME (b) at an extruder barrel temperature of 110 °C and a powder mass flow of 4 kg/h.

Contrary to the assumed first hypothesis, there is hardly any influence of the screw speed on the degree of denaturation, although the residence time decreases with increasing screw speed (data not shown). This finding regarding residence time also correspond with literature data (Altomare & Ghossi, 1986; Kumar et al., 2010; Ruyck, 1997). The effect screw speed on the degree of denaturation can possibly be associated with the resulting product temperature. Fig. 5-5 depicts the product temperature as function of screw speed.

There are hardly any changes in the degree of denaturation with increasing screw speed. At the same time, there are hardly any changes in the maximum product temperature at the end of the heating zone (Fig. 5-5a). The improved heat transfer by the accelerated screw rotation seems to compensate the reduction in residence time. The same maximum temperature can be measured for varying screw speeds. This indicates that even if the residence time decreases the maximum product temperature is mainly responsible for the resulting degree of denaturation. Examination of the product outlet temperature at the end of the cooling zone only shows a small increase. However, the denaturation temperature should again not be surpassed.

The size of the particles decreases exponentially with increasing screw speed (Fig. 5-4). Particle sizes are shown as $d_{50,3}$, $d_{10,3}$ and $d_{90,3}$ representing, respectively, the median, the smallest and the biggest particles of the distribution. At an elevated screw speed small aggregates can easily be produced in the desired size range. In contrast to the minor effect of screw speed on product temperature, the influence of screw speed on the SME input is a major one (Fig. 5-5b). With increasing screw speed the SME increases linear. SME combine the impact of interacting material, machine and process parameters.

A decrease in aggregate size with increasing shear stress can also be observed for other simultaneous heating and shearing systems at industrial scale such as extrusion at acid pH (Quéguiner et al., 1992b) or analytical scale rheometry (Simmons et al., 2007; Steventon, 1992). As the results show, small whey protein microparticles at neutral pH can be formed applying the described combination of a heating and cooling zone.

5.3.3 Influence of mass flow

In contrast to the screw speed, the mass flow has a strong impact on the degree of denaturation. The degree of denaturation decreases linearly with increasing mass flow (Fig. 5-6).

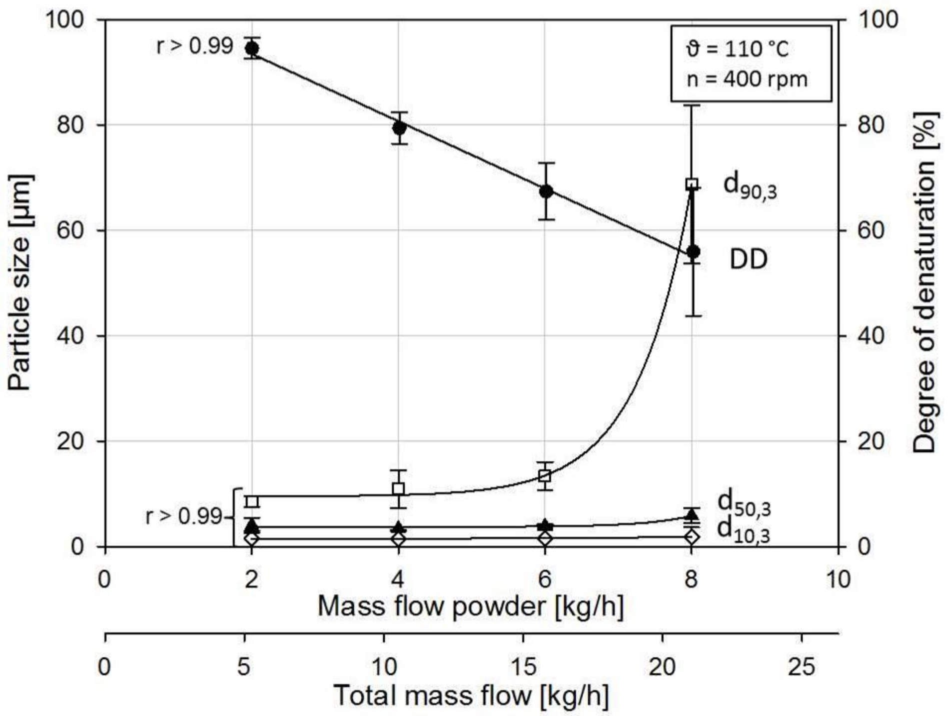

Fig. 5-6: Particle size and degree of denaturation as a function of mass flow for an extruder barrel temperature of 110 °C and a screw speed of 400 rpm.

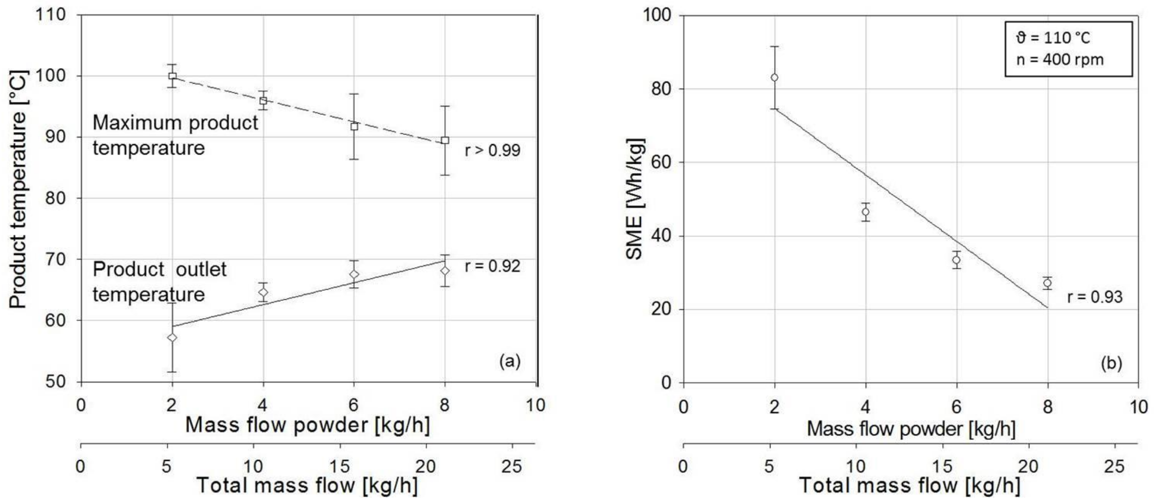

Fig. 5-7: Influence of mass flow on product temperatures (a) and SME (b) for an extruder barrel temperature of 110 °C and a screw speed of 400 rpm.

At constant screw speed, an increase in mass flow also leads to an increase in the screw filling level. This can cause a decrease in residence time and thereby result in a reduced reaction time for the proteins to unfold and aggregate. As a consequence, the degree of denaturation decreases. However, as already shown for the influence of screw speed, the residence time plays an insignificant role in this particular extrusion process. However, axial mixing is also reduced. The lower the level of screw filling, the better is the contact between the product on the heated wall and in the middle of the screw (Yeh et al., 1992). The specific thermal energy, which is transferred from the heated extruder wall to the product, is also reduced with increasing mass flow. As a consequence, the maximum product temperature and thereby also the degree of protein denaturation decrease. With decreasing mass flow, the degree of denaturation as well as the maximum product temperature increases (Fig. 5-7a). This suggests again that the maximum product temperature is the major responsible factor for protein denaturation.

Looking at the particle size distribution parameters in Fig. 5-6, there is hardly any influence of the mass flow on the median particle size $d_{50,3}$ and the small particles $d_{10,3}$. For the $d_{90,3}$, the biggest particles of the distribution, the value increases at elevated mass flows. With increasing mass flow, the resulting mechanical energy input per mass unit and thereby the shear stress acting on the aggregates also decreases (Fig. 5-7b). The SME input seems to be the main factor influencing particle size. Furthermore, the product outlet temperature increases with increasing mass flow (Fig. 5-7a) and reaches values above the denaturation temperature of whey proteins, which is most likely due to insufficient cooling at the end of the barrel due to a high product load. For high mass flows, this could result in an undesired and uncontrolled protein aggregation after the product outlet.

5.4 Conclusion

In the current study, high moisture extrusion was applied for the microparticulation of whey proteins. The results obtained show that variation of extruder barrel temperature, screw speed and mass flow strongly affects product temperature and SME and thereby the degree of denaturation and the particle size. As our study demonstrates, high moisture extrusion is a suitable technique to produce microparticles in the desired size range of 0.5 - 10 µm at neutral pH. Applying an extruder set-up with a heating and cooling zone, the process is no longer limited to low pH values. The degree of irreversible protein denaturation as well as the particle size distribution can be controlled by the process parameters, extruder barrel temperature, screw speed and mass flow. The targeted control of the resulting particle sizes in the low µm-range as achieved in this study with extrusion technology cannot be realized by any other technical device with simultaneous application of heat and shear such as scraped surface heat exchangers. Future studies should further investigate the functional properties of extruded compared to conventionally produced micro-particles. Furthermore, it would be worthwhile studying the structure of such particles in order to understand their formation and properties.

Summary and Contribution of the Doctoral Candidate

The properties of whey proteins are particularly influenced by thermally controlled unfolding and subsequent aggregation. Whey protein aggregates can be used to partially mimic the effect of fat and to generally support structures in food products. Aggregate size has to be limited to avoid a negative sensory impact, e.g. sandiness induced by larger particles. Therefore, a thermo-mechanical treatment by high moisture twin screw extrusion was used, to achieve a controlled formation of these micro-particles. As part of this process, the degree of protein denaturation as well as the particle size distribution had to be controlled. Depending on the targeted application, the aim was to produce aggregates with different particle sizes and various degrees of protein denaturation. However, the most challenging task was to achieve a high degree of denaturation and small aggregates with a narrow particle size distribution at the same time. Due to the extruder set-up with a heating and a cooling zone, the process was no longer limited to acidic pH values, as was the case for other reported processes. This enables these micro-particles to be also applied in food products of neutral pH. The prevailing conditions during the extrusion process strongly affect the aggregate properties. Hence, the influence of temperature, mass flow rate and screw speed on the resulting aggregate properties were extensively investigated in this study.

A major finding of this study was that high moisture extrusion was a suitable technique to produce micro-particles in the desired size range of 0.5 - 10 µm at neutral pH. In addition, the targeted control of the resulting particle sizes and the degree of denaturation by the process parameters distinguished this process from others (e.g. the SSHE process).

The obtained results showed that the degree of denaturation strongly increased with increasing extruder barrel temperature. At the same time, an increasing mass flow reduced the specific heat transfer to the product and, consequently, the degree of denaturation decreased. Screw speed was found to have almost no effect on the degree of denaturation. In contrast, screw speed had a strong effect on aggregate size. The size was exponentially reduced with increasing screw speed due to the rising mechanical shear stress.

The substantial contributions of the doctoral candidate include conception and design of the experiments based on preceding critical literature review, as well as the writing and the revision of the majority of the manuscript. The doctoral candidate carried out major parts of the data analysis, interpreted the data set, and discussed it.

6 System parameters in a high moisture extrusion process for microparticulation of whey proteins[4]

Abstract

Whey proteins can be texturized by thermo-mechanical treatment during high moisture extrusion. Thereby, protein aggregates with specific functional properties can be obtained. In this study, a heated co-rotating twin screw extruder was used to particulate whey protein concentrate. For data evaluation, process, product and system parameters affecting the extrusion process were distinguished. To characterize the process by system parameters is an important step towards an improved process understanding. The maximum product temperature ($T_{P,max}$) correlated linearly with the extruder barrel temperature and the mass flow. The specific mechanical energy input (SME) was found to be mainly affected by screw speed and mass flow. Independent of the respective adjusted process parameters, aggregate size was only affected by the resulting SME value. Equally, the degree of denaturation was only affected by the $T_{P,max}$. Thus, manipulation of system parameters (SME and $T_{P,max}$) can be applied for an indirect control of micro-particle properties.

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[4] Original publication: Wolz, M.; Kulozik, U. (2017). System parameters in a high moisture extrusion process for microparticulation of whey proteins. Journal of Food Engineering, 209, 12–17. doi: 10.1016/j.jfoodeng.2017.04.010
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Highlights

- Correlations between process, system and product parameters during high moisture extrusion.
- Only the SME determines the size of the micro-particles.
- Only the maximum product temperature determines the degree of denaturation.
- Product outlet temperature should be controlled during the extrusion process.

6.1 Introduction

Microparticulation is a controlled, thermally induced aggregation process, during which shear forces are applied simultaneously or sequentially to limit the aggregate growth or to reduce the resulting aggregate sizes. Microparticulation of whey proteins can be realized in a scraped surface heat exchanger (SSHE) (Spiegel & Huss, 2002) or in a tubular heat exchanger followed by high pressure treatment (Iordache & Jelen, 2003; Paquin et al., 1992). The downside of the established approaches is that only diluted solutions ($c_{\text{Protein}} \leq 10\%$ (w/w)) can be handled. Furthermore, shear stresses do not reach higher levels compared to SSHE. However, shear stress is a key factor in terms of aggregate size. This is of significant importance, as particles exceeding a certain size would create a mealy or even sandy mouthfeel. This would affect consumer acceptance negatively. We therefore applied the concept of extrusion for the microparticulation of whey proteins as an alternative to SSHE. In a previous publication, we reported that high moisture extrusion is a suitable technique to produce micro-particles in a desired size range of 0.5 - 10 µm at neutral pH. The targeted control of the resulting particle sizes in the low µm-range as achieved with extrusion technology, cannot be realized by any other technical devices with simultaneous application of heat and shear (Wolz et al., 2016a).

What remained to be studied is the functional relationship between the prevalent processing and technical conditions and the resulting product properties. The aforementioned study, as well as most of the other food extrusion studies focused on the direct influence of process parameters and raw material characteristics on product properties. However, due to the interdependence of influencing factors, e.g., screw speed, volume flow, and residence time, this is of limited success. The extrusion process is a complicated, empirically controlled multi-input-output system for which it is hardly possible to establish process-function-property correlations (Emin & Schuchmann, 2017). Therefore, the concept of system parameters will be applied, which analyzes the process and the outcome at a higher level above individual processing or technical factors. Thus, the number of variables can be reduced by defining system parameters, which describe the effects an extruder has on the material processed by it. As direct and in-line measurements of product properties is challenging, other approaches are required. In this context, system parameters offer the potential to control the product quality during processing (Chen et al., 2010; Onwulata et al., 1994). Moreover, manipulation of system parameters can be applied for an indirect control of product properties. Furthermore, this can simplify process control and optimization, transfer to other extruder systems and scale-up processes. The systems can be compared in the case of comparable values for the system parameters (Meuser et al., 1992), similar to the concept of dimensionless numbers in engineering in general.

Shearing by the screws and heating of the extruder barrel induce mechanical and thermal stress on proteins during high moisture extrusion. This thermo-mechanical treatment can modify the native structure of proteins from different sources in terms of denaturation and aggregation (Chen et al., 2010; Fang et al., 2014; Koch et al., 2017; Liu & Hsieh, 2007; Osen et al., 2014; Pietsch et al., 2017). The fundamental mechanisms of thermally induced denaturation and aggregation of whey protein in absence of shear (Havea et al., 2001; Roefs & Kruif, 1994; Tolkach & Kulozik, 2007; Zuniga et al., 2010) or presence of shear (Byrne et al., 2002; Cheftel et al., 1992; Simmons et al., 2007; Steventon, 1992) have already been extensively studied. The heating temperature was identified as the rate-determining factor of protein denaturation. Aggregates are built during denaturation where disulfide bonds and hydrophobic interactions are formed between the protein molecules (Havea et al., 2001; La Fuente et al., 2002; Nicolai et al., 2011; Wit, 2009). The aggregate size can be limited by shear stress acting on the particles. Quéguiner et al. (1992b) also used extrusion for the microparticulation of whey proteins. With the process conditions used in the aforementioned study, low pH values (<3.9) were necessary to achieve small micro-particles.

The approach of this study was to distinguish between independent processing and technical parameters, to combine these into system parameters and to correlate these with the resulting product properties. The fundamental approach is similar to the concept first proposed by Meuser et al. (1992) for the extrusion of cereals. In this study we extend this concept for the microparticulation of whey proteins. Fig. 6-1 depicts the influencing factors for the microparticulation of whey proteins by extrusion. Independent process parameters and raw material characteristics affect the properties of the final product by influencing the extruder response inside the barrel, and thus, the system parameters. Such parameters include the specific mechanical energy input (SME), specific thermal energy input and the residence time distribution. System parameters combine the effects of the different process parameters and raw material characteristics on the processed product. As a result of different combinations of extrusion conditions, the system parameters can be used to describe or to compare extrusion processes under different operating conditions or machine sites.

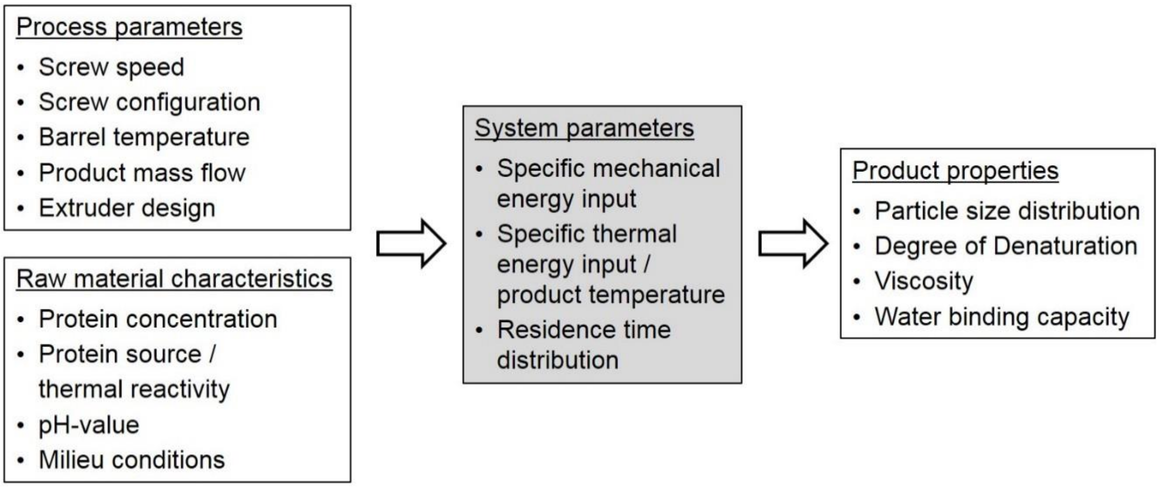


Fig. 6-1: Factors influencing the high moisture extrusion process for microparticulation of whey proteins.

The aim of this study was to correlate process and system parameters as well as system and product parameters by evaluating extrusion data. Thus, a tool for the control of the process and the product properties by inline-measurement of system parameters should be proposed. This is still a major gap in empirically controlled practical applications. The processing parameters barrel temperature, screw speed and mass flow were varied to assess their effects on the system parameters SME, maximum product temperature ($T_{P,max}$) as well as on the product properties particle size and degree of denaturation.

6.2 Material and methods

6.2.1 Materials

Whey protein concentrate WPC80 was kindly supplied by Germanprot Sachsenmilch (Leppersdorf, Germany). Its composition was: protein 80.0%; lactose 4.4%; ash 3.2%. By addition of deionized water during the extrusion process, the protein concentration was adjusted to 30% (w/w). This corresponds to a dry matter concentration of 38%, the pH was 6.7.

6.2.2 Extrusion process

A co-rotating intermeshing twin-screw extruder (ZSK25, Coperion, Stuttgart, Germany) equipped with two screws with a diameter of 25 mm and a die end plate with a hole of 10 mm in diameter was used. Due to the process design and the high moisture content, there was no pressure build-up towards the extruder exit. The barrel length was 38D. The barrel was divided in a heating and a cooling zone consisting of nine single segments, each with the option of individual temperature set points (except segment 1). Barrel temperature (ϑ_{Barrel}) was varied as shown in

Tab. 6-1. The extrusion process and the screw profile applied are described in more detail by Wolz et al. (2016a). The screw speed was set to 100 - 800 rpm. A screw feeder (K-Tron Soder, Niederlenz, Switzerland) was used to feed the whey protein powder based on weight. The powder feed rate ranged from 2 to 8 kg h^{-1}. A membrane pump (Grundfos, Erkrath, Germany) was used to dose the deionized water. To obtain a protein concentration of 30% (w/w) in the final product, the total water feed rate ranged from 5.3 to 21.1 kg h^{-1}, depending on the WPC80 feed rate.

Tab. 6-1: Barrel temperature settings (ϑ_{Barrel}) for segment 1 to 9.

1	2	3	4	5	6	7	8	9
-	30 °C	50 °C	90 °C	90 °C	90 °C	90 °C	50 °C	30 °C
-	30 °C	50 °C	100 °C	100 °C	100 °C	100 °C	50 °C	30 °C
-	30 °C	50 °C	110 °C	110 °C	110 °C	110 °C	50 °C	30 °C
-	30 °C	50 °C	120 °C	120 °C	120 °C	120 °C	50 °C	30 °C

After steady state conditions were reached and extruder system parameters (product temperature and motor torque) were constant, samples were collected and immediately cooled in an ice bath. The $T_{P,max}$ is a measure of the thermal energy input. For all process conditions, the $T_{P,max}$ reached at the end of the heating zone (7th segment) as well as the product outlet temperature ($T_{P,out}$) (in front of the die) was measured by a thermocouple temperature sensor reaching into the product stream. Pre-tests were conducted to ensure that the actual product temperature was measured, not the barrel temperature. For all samples, $T_{P,max}$, $T_{P,out}$ and the motor torque during processing were recorded. Samples were stored at 4 °C until analysis.

According to Lengerich (1984) the SME [Wh/kg] was calculated by equation (6.1).

$$SME = \frac{n_{act}}{n_{max}} \cdot \frac{\tau}{\dot{m}} \cdot P \tag{6.1}$$

with the maximum screw speed (n_{max} = 1200 rpm), the actual screw speed n_{act} [rpm], the motor torque τ [%], the maximum engine power (P = 20 kW) and the total mass flow rate $\dot{m}$ [kg/h].

6.2.3 Degree of irreversible whey protein denaturation

For the present study, β-lactoglobulin (β-Lg) was selected as the lead component since it represents over 50% of the total whey protein in bovine milk and thus, majorly affects denaturation kinetics. RP-HPLC was used to determine the degree of irreversible denaturation. After precipitation of aggregated whey proteins at pH 4.6, the content of native β-Lg before and after extrusion was measured by RP-HPLC applying the sample preparation and analytical method described by Toro-

Sierra et al. (2013). The denaturation degree represents the difference between the native β-Lg concentration before and after the extrusion process in relation to the native β-Lg concentration before processing.

6.2.4 Particle size

The particle size distribution of extruded samples was analyzed using a Malvern Mastersizer with a Malvern Mastersizer Hydro 2000S measurement unit (Malvern Instruments Ltd, Malvern, Worcestershire, UK). The light sources were a red He-Ne laser and a blue LED laser with a wavelength of 632.8 nm and 470 nm, respectively. Particle size was calculated using the Fraunhofer approximation. All samples were measured in triplicate. Size measurements were reported on a volume basis as $d_{10,3}$, $d_{50,3}$ and $d_{90,3}$, representing the diameters at 10, 50 and 90% cumulative volume, respectively.

6.2.5 Statistical analyses

All experiments were carried out in triplicate. Mean values ± 95% confidence intervals are given. The statistical processing and curve fitting was done using SigmaPlot for Windows Version 11.0 Build 11.0.0.75 (Systat Software Inc., Chicago, IL).

6.3 Results and discussion

6.3.1 Relationship between process parameters and system parameters

The effect of the extruder on the material can be described by system parameters in terms of energy input affecting structural properties. The energy input can either be of thermal or mechanical origin. The product temperature reached is a measure for the thermal energy input and can therefore be expressed by $T_{P,max}$, the mechanical energy input by the SME (Bouvier & Campanella, 2014; Brümmer et al., 2002).

6.3.1.1 Impact of processing parameters on the SME

Fig. 6-2 shows the influence of the screw speed and total mass flow on the system parameter SME during whey protein extrusion. Each combination of these process parameters resulted in a specific SME.

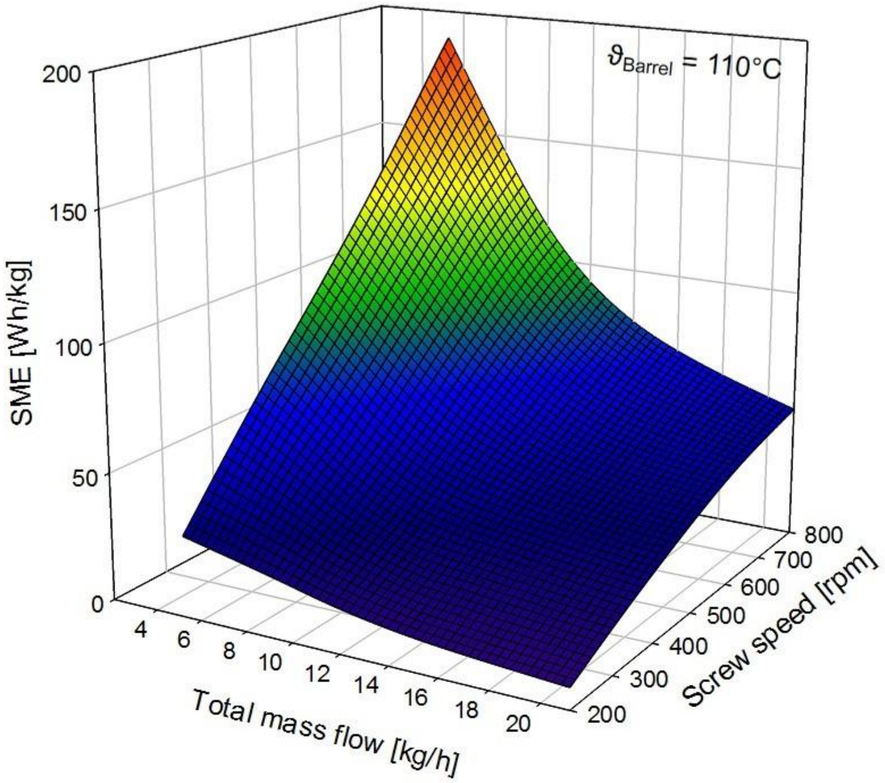


Fig. 6-2: Influence of screw speed and total mass flow on the SME at an extruder barrel temperature of 110 °C.

As can be seen in Fig. 6-2, lowering the screw speed or heightening the mass flow resulted in a lower SME. The SME strongly increased at high screw speed and low mass flow. The results obtained in the present study are in line with the few data available on high moisture extrusion, so far (Akdogan, 1996; Meng et al., 2010). The influence of the screw speed on the SME decreased with increasing mass flow. In comparison, the effect of mass flow on the SME increased with increasing screw speed. Both process parameters showed a strong correlation with the SME within the investigated parameter range. In contrast, the extruder barrel temperature in the heating zone only had a limited effect on the SME. The SME slightly decreased with increasing barrel temperature (Fig. 6-3), most likely due to a reduction in viscosity at higher temperatures. According to Meuser et al. (1992), mechanical energy input along the screw can be indirectly measured by the torque of the screws. For high moisture extrusion of whey proteins, no pressure build-up was measured at the extruder die. Hence, shear forces created by the rotating screws were responsible for the SME in this study rather than pressure build up in front of the die. The higher the SME, the higher the shear forces act on the aggregates.

6.3.1.2 Impact of processing parameters on $T_{P,max}$

Obviously, the $T_{P,max}$ increased with increasing extruder barrel temperature (Fig. 6-3). In contrast to extrusion at low moisture levels, the thermal energy input (and thus the $T_{P,max}$) during extrusion at high moisture levels barely depends on the mechanical energy input. As mentioned above, dissipated heat is therefore less relevant in a high moisture process due to the much lower viscosity (resulting from the high water content) (Akdogan, 1996; Noguchi, 1990). Hence, the product temperature is primarily influenced by the extruder barrel temperature. This particular characteristic of wet extrusion was found to be more pronounced in the present study than in high moisture extrusion processes conducted by Meng et al. (2010) and Chen et al. (2010), who extruded chickpea flour and soybean protein, respectively.

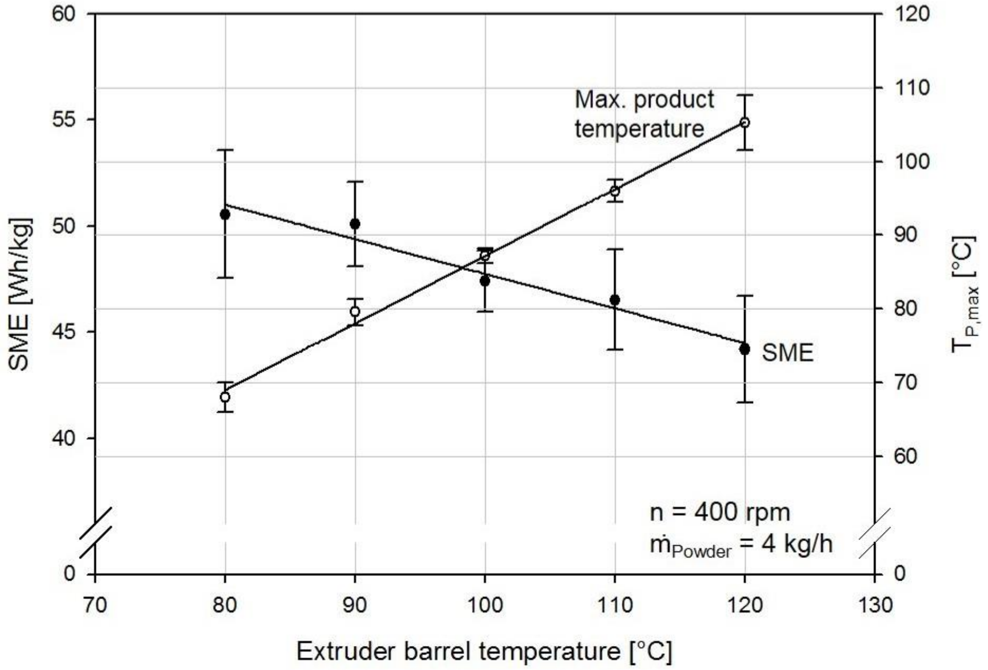


Fig. 6-3: Correlation between extruder barrel temperature and SME as well as between extruder barrel temperature and $T_{P,max}$ at a screw speed of 400 rpm and a mass flow rate of 10.5 kg/h.

Nevertheless, the $T_{P,max}$ always remained below the extruder barrel temperature (Fig. 6-3). This observation distinguishes the process studied here from other extrusion processes, in which the $T_{P,max}$ was reported to be higher than the extruder barrel temperature due to dissipated heat (Bouvier & Campanella, 2014; Guy, 2001). The extruded whey protein was only exposed to a heating-up phase and not to a heat holding phase during the extrusion process. The product temperature increased linearly along the screw until it reached its maximum in the segment 7 of the extruder barrel ($T_{P,max}$) and decreased linearly in the following cooling zone (segment 8 and 9). This was verified by additional measurements of the product temperature along the screw during selected experiments (data not shown).

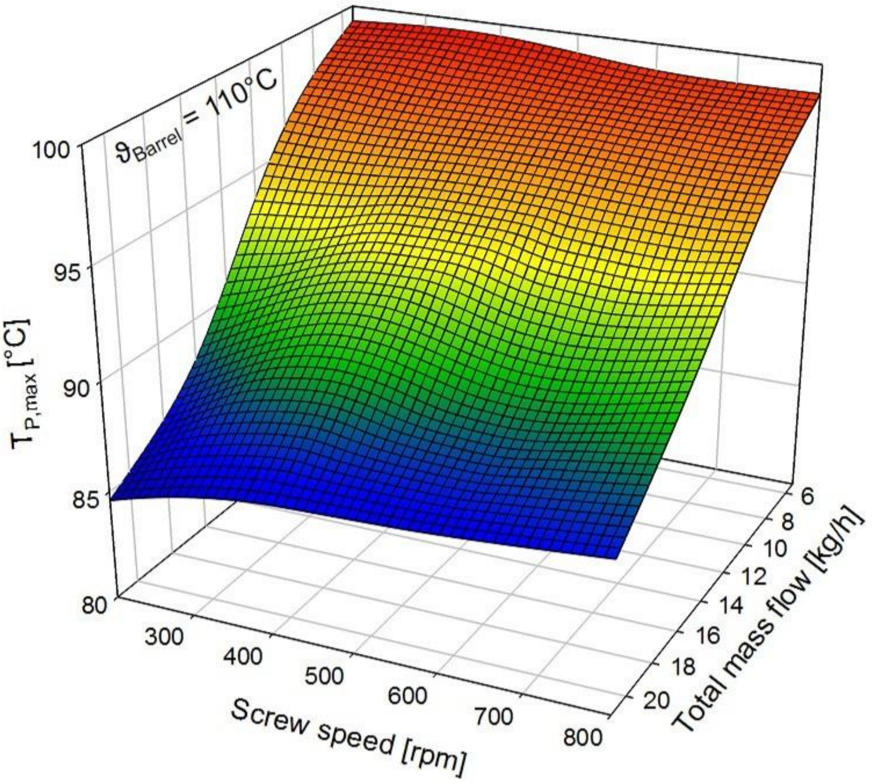


Fig. 6-4: Influence of screw speed and total mass flow on the $T_{P,max}$ at an extruder barrel temperature of 110 °C.

Furthermore, the $T_{P,max}$ correlated almost linearly with the mass flow (Fig. 6-4). The higher the mass flow, the lower the resulting $T_{P,max}$. With increasing mass flow, less specific thermal energy could be transferred to the product (per unit mass) by the barrel heating system. In consequence, $T_{P,max}$ decreased. Due to the low influence of dissipated heat, the screw speed, in contrast, only had a small effect on $T_{P,max}$. $T_{P,max}$ remained almost constant across the investigated screw speed range at otherwise constant process conditions (Fig. 6-4).

6.3.2 Relationship between system parameters and product properties

The correlation between the mechanical and thermal energy (system parameters) and product properties is described in the following. Critical product properties for microparticulation include particle size and degree of protein denaturation.

6.3.2.1 Impact of the SME on particle size

As in previous studies with different raw materials, the system parameters, especially the SME, had a direct impact on the final product (Chen et al., 2010; Fang et al., 2014). Fig. 6-5 (a) depicts the dependency of the median particle size $d_{50,3}$ on the SME. The results shown represent multiple experiments at different process parameter combinations such as barrel temperature, screw speed and mass flow, resulting in the single curve.

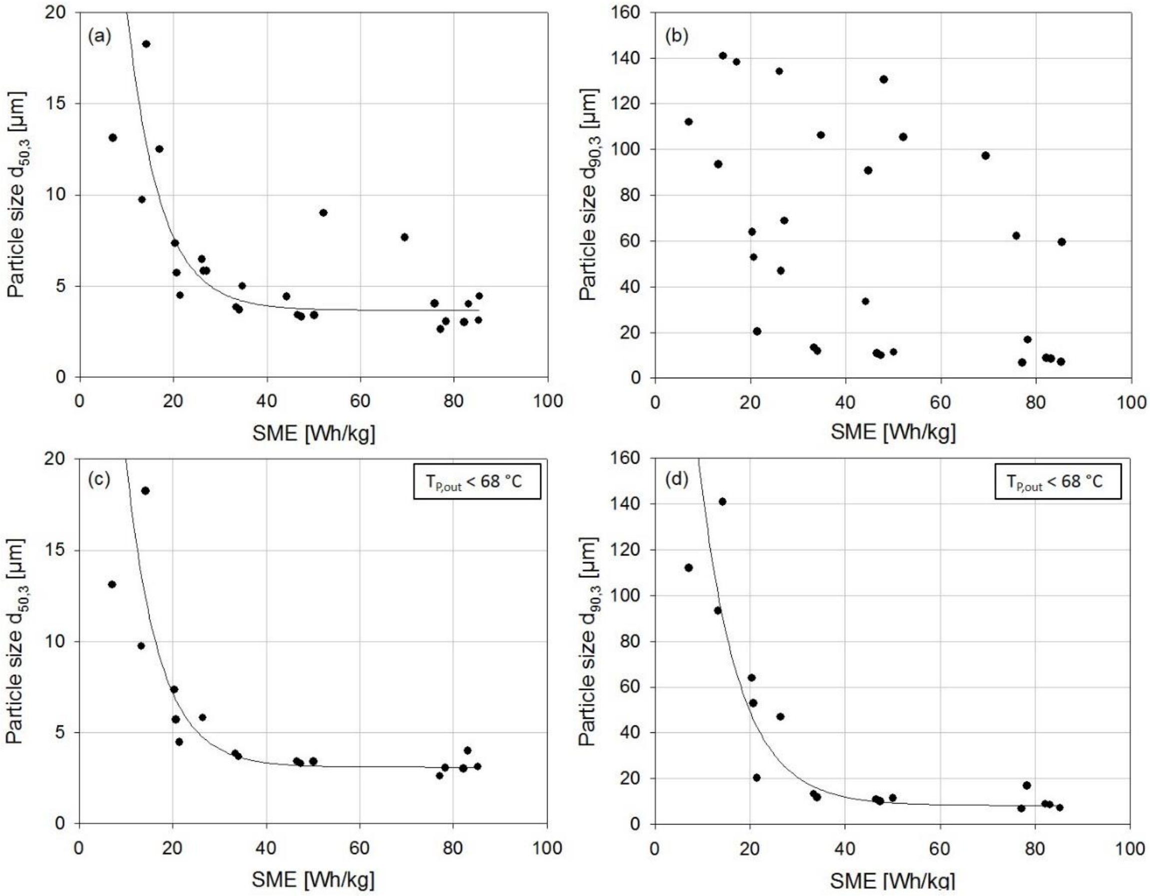


Fig. 6-5: Influence of SME on the particle size $d_{50,3}$ (a and c) and the $d_{90,3}$ (b and d) for all measured $T_{P,out}$ (a and b) and for $T_{P,out}$ below 68 °C (c and d).

An exponential decrease in $d_{50,3}$ was detected with increasing SME. This suggests that, independently of the prevalent processing parameters, the SME was the major factor limiting $d_{50,3}$. With increasing SME, the shear stress acting on the particles increased. Therefore, the growth of the aggregates was limited and their final size was reached directly. This could be explained by observations made by Steventon (1992) and Wolz et al. (2016b) for whey protein aggregation in a rheometer. At this high protein concentration level, thermal aggregation of whey proteins is mainly driven by the high collision rate. However, particle collision only leads to permanent aggregation, if cross-links can be formed resisting the fluid shear stresses. At the same time, the contact time between the molecules is reduced. Therefore, the proteins and already existing aggregates may not have the time to orientate themselves for sustainable interactions. Thus, a high SME limits the growth rate and large particles cannot be formed in the first place.

The influence of the SME on $d_{90,3}$ is depicted in Fig. 6-5 (b). In contrast to $d_{50,3}$ data, scattered data points were observed without a trend related to SME input. To explain this, the product outlet temperature ($T_{P,out}$) had to be considered. In particular, $T_{P,out}$ became critical if the denaturation was incomplete at the extruder outlet. In case $T_{P,out}$ exceeded the denaturation temperature of 68 °C for whey proteins, further aggregation could take place after the product left the extruder. Remaining native whey proteins denatured at the surfaces of larger particles resulting in an uncontrolled growth even though the product had left the extruder processing unit. These changes of the particle size occured after the extrusion process, i.e., they were not an effect of the conditions in the extrusion process itself. By only considering experiments with $T_{P,out} < 68$ °C (Fig. 6-5c), the SME now correlated with both, $d_{90,3}$ and $d_{50,3}$ (Fig. 6-5 (d)). In addition, the curve fitting for the $d_{50,3}$ was also optimized (Fig. 6-5 (c)).

In contrast, $T_{P,max}$, had no influence on the resulting particle size. The same size of the aggregates was obtained for different $T_{P,max}$. It can therefore be concluded that the SME-increase is the major factor responsible for the control of particle size or limitation of particle size growth. This effect was independent of the processing in the SME were based on screw speed or mass flow. An increase in the resulting mechanical energy input per mass unit of protein increased the shear stress acting on the aggregates and thereby, limited their size. This clearly shows that the particle size of the whey protein aggregates can easily be controlled by the system parameter SME. This is not possible in a SSHE, which was used in previous studies to create whey protein micro-particles.

6.3.2.2 Impact of $T_{P,max}$ on the degree of denaturation

As shown in Fig. 6-6, the degree of denaturation was mainly influenced by $T_{P,max}$. The higher the $T_{P,max}$ in the extruder barrel, the higher was the degree of denaturation of the whey proteins. These results correspond to earlier findings, which showed an acceleration of whey protein denaturation kinetics with increasing temperature (Dannenberg & Kessler, 1988; Donovan & Mulvihill, 1987; Tolkach & Kulozik, 2005; Wolz & Kulozik, 2015).

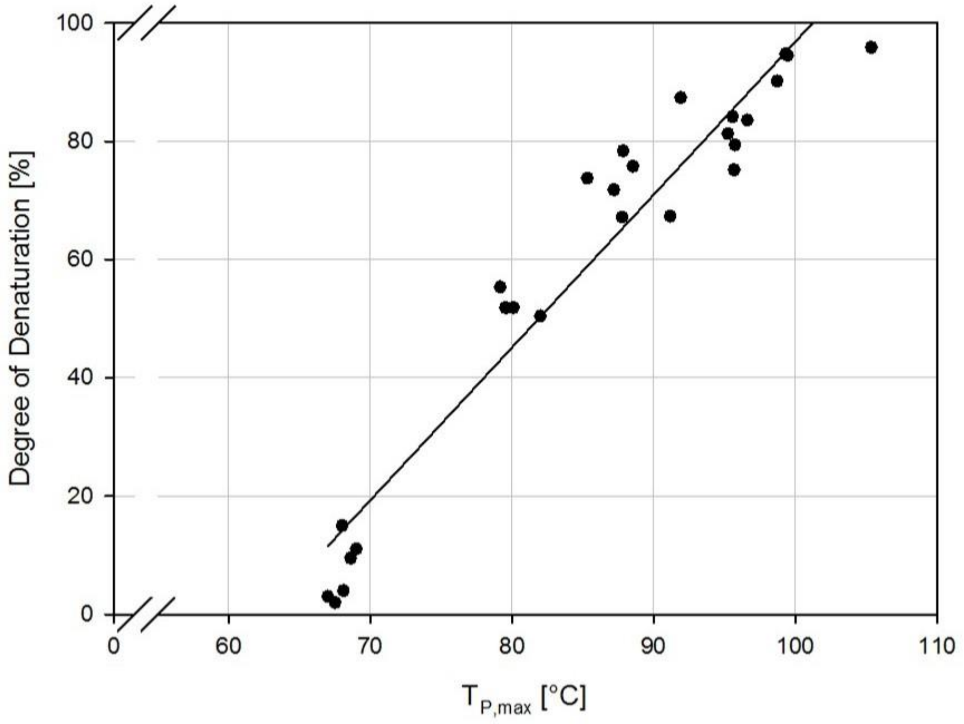


Fig. 6-6: Influence of the $T_{P,max}$ on the degree of denaturation at $T_{P,out}$ below 68 °C.

Fig. 6-6 also shows that a complete denaturation of the whey proteins could be achieved provided that $T_{P,max}$ is above a certain limit, i.e., above approximately 100 °C in this case even if the mean residence time is very short (30 - 90 s). In this case, no reactive native proteins would remain and $T_{P,out}$ would become less important. However, if the surface of the barrel was too hot (>130 °C) fouling could occur. No influence of the SME on the degree of protein denaturation was observed. The degree of denaturation only depended on the temperature with $T_{P,max}$ as the critical measure, no matter which combination of processing parameters was responsible for each particular $T_{P,max}$. Similar findings were also described by Afizah & Rizvi (2014), who used reactive supercritical fluid extrusion of the whey protein concentrate at different extruder temperatures and acidic pH regions to influence their emulsifying properties. It is self-evident that it would be the best to know the temperature-heating time combinations in the extruder. However, in industrial processes the residence time distribution is often difficult to measure. Besides, contrary to our expectation, the residence time seems to have no influence on the degree of protein denaturation. To measure $T_{P,max}$ is sufficient to predict the resulting degree of denaturation. This is very advantageous for the process design of extruders for microparticulation. The residence time changed with process parameters such as screw speed, whereas in contrast, the degree of denaturation remained constant (Fig. 6-4). Subsequently, the degree of denaturation measured at the end of the process, only increases with increasing product temperature, due to a higher denaturation rate. Hence, the heating time are not required to be known to predict the resulting product properties. It is not necessary to know the temperature-time combination and thus, to make elaborate measurements for the residence time distribution.

6.4 Conclusion

SME and the $T_{P,max}$ are sufficient to control particle size and degree of denaturation, respectively. In contrast, no influence of the residence time could be determined. The reason for this is most probably that the product only passes through a fast heating-up and cooling-down phase and not through a heat-holding phase. Different combinations of processing parameters are practically available in order to obtain a specific SME or a specific $T_{P,max}$. The processing parameters can be combined in such a way that the required system parameters and the desired aggregate properties can be achieved. Due to the clear and simple correlations between process and system parameter and between system and product parameters, SME and $T_{P,max}$, can be used for process control and optimization. During scale-up the cooling zone has to be designed in such a way to be long enough to reach a $T_{P,out}$ below 68 °C, i.e. below the critical denaturation temperature of the whey proteins. Future studies are required to assess the influences of moisture content, protein concentration and extruder screw design. Furthermore, it would be worthwhile to study the functional properties of extruded compared to conventionally produced micro- particles.

Summary and Contribution of the Doctoral Candidate

High moisture extrusion, as a thermo-mechanical process, can be used for the microparticulation of whey proteins. This process yields protein aggregates in a low µm range with specific functional properties. However, the functional relationship between the prevalent processing and technical conditions and the resulting product properties is not well understood. In an extrusion process, it is hardly possible to establish process-function-property correlations. Due to the interdependence of influencing factors, e.g. screw speed, volume flow and residence time, investigating the influence of process parameters and raw material characteristics on product properties has so far been of limited success. Thus, the system analytical approach could be used. The independent processing and technical parameters were combined as in the system parameters, describing the effects an extruder has on the material processed by it. In this manner, the number of variables could be reduced. Hence, the objective of the present study was to examine the correlation between processing parameters and system parameters as well as between system parameters and the resulting product properties.

For this purpose, the processing parameters (barrel temperature, screw speed and mass flow rate) were varied, while their effect on system parameters (specific mechanical energy input (SME), maximum product temperature ($T_{P,max}$)) and product properties (particle size and degree of denaturation) was analyzed.

The $T_{P,max}$ was shown to be a linear function of the extruder barrel temperature and the mass flow. The SME was found to be mainly affected by screw speed and mass flow. Independ from which process parameters were changed, the resulting SME value only affected particle size while the $T_{P,max}$ only affected the degree of denaturation. Thus, the effect of the extrusion process on aggregate properties could be described solely by the system parameters. The new insight allows indirect control of product properties by in-line measurement of the system parameters. Furthermore, the desired aggregate properties can be modified adjusting the required system parameters by an adequate combination of process parameters.

The substantial contributions of the doctoral candidate include conception and design of the experiments based on preceding critical literature review, as well as the writing and the revision of the majority of the manuscript. The doctoral candidate carried out major parts of the data analysis, interpreted the data set, and discussed it.

7 Overall Discussion and Conclusions

Whey proteins are characterized by their excellent technological and nutritional properties and are thus used in various different food products. Whey proteins represent about 20% of the total milk protein and remain in the whey during cheese production. Thus, they accumulate in large amounts. One option for their functionalization is microparticulation. This means formation of particles by protein aggregation. Next to incorporation in milk products, micro-particles find possible applications in meat, fish, confectionary and bakery products. Advantages include potential reduction in fat content, improved sensorial quality and increased yield.

The physico-chemical properties of whey protein micro-particles are controlled by thermal treatment and shear. These properties include particle size, degree of denaturation and aggregate structure. They determine the behavior of the protein aggregates in the final product. Until now, scraped surface heat exchangers have been used for concurrent thermal-mechanical treatment (Spiegel, 1999b; Plock, 1994; Singer et al., 1988). This process represents one possibility to particulate whey proteins. However, only low dry matter raw materials with low protein concentrations and high lactose-to-protein-ratios can be processed. In addition, process conditions that can be varied, such as mechanical shear stress and process temperature, are limited. Furthermore, the run time is short due to difficulties controlling product caking. Thus, it was the aim of this work to develop a novel extrusion process, to deal with far higher protein concentrations and lower lactose concentration at once, as well as to be able to vary the process conditions over a much broader range. Using high protein concentrations reduces the mass flow, which has to be heated per unit of micro-particles. Therefore, taken together with the extended run time, extrusion is considered a more energy efficient process.

Quéguiner et al. (1992b) already used extrusion to microparticulate whey proteins. However, their aim was to produce a semi-solid spread. Therefore, the process variables were only varied in a narrow range and the process set-up did not ensure a complete stop of the aggregation process in the extruder. For the extrusion process of Quéguiner et al. (1992b), pH values below 3.9 were necessary to achieve small aggregates. However, micro-particles with an acidic pH can limit their application in food products (Cheftel & Dumay, 1993).

Hence, the aim of the present work was to develop a microparticulation process at neutral pH using extrusion. Beyond this, the underlying aggregation mechanisms and influencing factors under shear stress were to be investigated. For that purpose, processes at high protein concentration were of particular interest.

7.1 Denaturation characteristics in concentrated whey protein systems

High whey protein concentrations have to be handled in various dairy processes. During the production of WPC and WPI, the protein concentration is successively increased by filtration and evaporation. In this case, denaturation during filtration at elevated temperatures or fouling inside the evaporator´s heat exchanger equipment should be avoided as far as possible. Furthermore, high protein concentrations are processed for functionalization of whey proteins by microparticulation in an extruder. In this regard, changes due to denaturation are explicitly desired. In both cases, it is essential to have consolidated knowledge about the thermal reaction kinetics at high protein concentrations.

As already shown for lower protein concentrations, the kinetics of whey protein denaturation are strongly affected by concentration (Anema et al., 2006; Hoffmann & Mil, 1997; Nielsen et al., 1996; Kessler & Beyer, 1991). The depletion rate of β-Lg increases with increasing protein concentration. This could also be confirmed for far higher protein concentrations up to 40%. Particularly at the upper end of the tested protein concentrations, a high degree of denaturation was already reached after heating times of only a few seconds (section 3). Furthermore, also during a concurrent thermo-mechanical treatment it was shown that the protein concentration had a strong effect on the degree of denaturation, independent of the technology used, although side effects of varying heating and cooling rates could not be fully prevented. For this reason, the absolute values were hardly comparable. However, all technologies had in common that the higher the concentration, the faster were the denaturation kinetics. Furthermore, no additional effect of shear stress on the degree of denaturation at high protein concentrations could be detected (Fig. 4-8 → rheometer, Fig. 5-4→ extruder). This is in line with the study of Erabit et al. (2014), who also arrived at the conclusion that shear exerts no impact on the residual native fraction.

What distinguishes high protein from low protein systems, are on the one hand, the higher amount of available molecules and thereby possible reactants, and on the other hand, the higher viscosity. High protein concentrations induce a high number of protein molecules. According to eq. 4.1 and 4.2, this causes a higher number of collisions during perikinetic and orthokinetic aggregation. The aggregation process of colloidal particles in general requires that particles are brought in direct proximity of each other by a transport mechanism, referred to as collision. Then, an aggregate will be formed, if the net inter-particle forces are attractive and strong enough to overcome Brownian motion and hydrodynamic drag forces (assuming shear stress is applied) (Vanni & Baldi, 2002). Accordingly, the probability of collision between protein molecules rises with increasing protein concentration, since the number of protein molecules increases. This results in a higher aggregation rate and an accelerated denaturation reaction.

A higher viscosity could restrict the potential of motion of individual particles in the solution. However, according to the coagulation theory of Smoluchowski (1916) the frequency of collisions is limited by Brownian motion and thus by diffusion in the first step of aggregation (perikinetic aggregation). Therefore, as described in eq. 4.1, the viscosity of the continuous phase η_k is decisive. If the main dissolved component in the solution is whey protein, η_k hardly changes with increasing protein concentration. Thus, viscosity does not restrict the frequency of collision, which was the case with the WPC80 used in this work.

Next to denaturation kinetics, the high protein concentration also affected the resulting aggregate size and structure. The size decreased exponentially with increasing protein concentration (section 4). This was explained by the fact that viscosity increases as protein concentration rises. The viscosity is a measure for a fluid´s internal flow resistance, thus its resistance to being deformed (Mezger, 2006). Hence, the shear stress acting on the aggregates also increases with increasing viscosity. In the case of native whey protein solutions, viscosity increased exponentially with protein concentration (Fig. 4-4). This qualitative curve progression could also be estimated for denatured protein solutions, but could not be measured without including the effect of different particle size distributions (Brouwers, 2010). Furthermore, the structure of the aggregates and thus, their rheology depends on the shear stresses applied (Barthelmes et al., 2003).

Increasing viscosity intensifies the shear stress acting on the particles. In contrast to frequency of collisions, the effective shear stress is a function of the viscosity of the total suspension and not only of the viscosity of the continuous phase. Hence, with increasing protein concentration, viscosity increases and enhances the shear stress acting on the particles. This leads to a decreasing particle size.

7.2 Aggregation in shearing devices

Aggregation refers to a formation of assemblages in a suspension. Aggregation of whey proteins without shear is a matter of course for most scientific research projects in this area (e.g. (Bon et al., 1999; Kennel, 1994; Aymard et al., 1996; Tolkach, 2008). The prevailing underlying mechanisms are described in section 1.2. However, aggregation under shearing conditions is much less investigated, despite the fact that shear is always present in continuous industrial processes. This hinders the transfer of scientific findings to industry and makes any scale-up difficult (Erabit et al., 2014). Studies during concurrent heating and shearing as conducted by Steventon (1992), Simmons et al. (2007), Erabit et al. (2014) and Spiegel (1999a) showed that shear is an important factor affecting aggregation. Furthermore, conflicting results at conditions with and without shear have been observed. As an example, an increasing protein concentration leads to decreased aggregate sizes with shear (Steventon, 1992; Spiegel, 1999a), but increased aggregate sizes without shear (Elofsson et al., 1996; Hoffmann & Mil, 1997; Hoffmann et al., 1997a). Consequently, this contradiction has been investigated in more detail in this thesis.

For this purpose, experiments in two shearing devices were conducted. First, whey proteins were concurrently heated and sheared in a rheometer (section 4). revealing a strong influence of the protein concentration on the denaturation rate as well as on the aggregate size. This also caused differences in the aggregation behavior and the effect of shear rate. The effect of the shear rate was dependent on the protein concentration. At low shear rates and a low whey protein concentration of 5%, an increasing shear rate (from 100 - 750 s^{-1}) resulted in larger particles (Fig. 4-9). This observation conforms with Smoluchowski´s (1916) theory of collision. Mechanisms, which can induce relative movements among particles hence leading to collisions, are, on the one hand, Brownian motion (for submicron particles, <~0.25 - 1 µm), and on the other hand, motion by fluid flow (for particles >~0.25 - 1 µm). The fluid moving alongside a solid particle or boundary will generate shear stress on that particle or boundary (Vanni & Baldi, 2002). Similar observations were made by Erabit et al. (2014). They reported differences in aggregation rate due to the aggregate size. They stated that collisions of β-Lg monomers were not significantly affected by shear, whereas collisions between aggregates >1 µm were. In contrast, the particle size decreased at higher shear rates (750 - 1452 s^{-1}) (Fig. 4-9). This observation was attributed to the increasing shear stress acting on the particles. Particle collisions can only lead to permanent aggregation if cross-links between the molecules are formed. Cross-linking is hindered if the shear stress, acting on the particle in the moving fluid, is high. Furthermore, the collision rate increases with shear rate, while contact time, and thereby the time particles have to cross-link becomes shorter. Therefore, proteins and already existing aggregates may not have sufficient time to orient themselves for effective interactions.

At a protein concentration of 30%, the particle size decreased exponentially, while the shear rate increased over the whole range (Fig. 4-10). At such a high protein concentration, the probability of collisions between molecules was high and, therefore, the aggregation rate accelerated. In other words, the elevated tendency of proteins to aggregate at a concentration of 30% was mainly driven by the high density of reactive particles under elevated thermal conditions. Compared to lower protein concentrations, the shear rate had a lower relative impact, as the frequency of collisions was already very high due to the high number of protein molecules. The prevalent effects limiting particle growth and thus, final particle size were the same as at low protein concentrations and high shear rates. These results were obtained from the experiments carried out at the rheometer under defined shearing conditions (section 4).

The extruder exhibited comparable effects for protein concentrations of 30% (section 5). Due to the short residence time, samples with low protein concentration (<10%) could not be extruded with a noticeable degree of denaturation. Similar to the results in the rheometer, particle size decreased with rising screw speed at 30% protein (Fig. 5-4). This also led to an increasing shear rate. In general, the average shear rate or even the distribution of the shear rate along the screw is nearly impossible to obtain. Although the shear rate varies depending on the location in the extruder barrel, for low-pressure processes, its average value can be approximated by the shear rate in a shearing gap (Moscicki & Zuilichem, 2011). Hence, the average shear rate could be estimated from the different values of the screw speed and the dimensions of the extruder ($D_{Extruder}$ = 25.5 mm, δ = 0.5 mm).

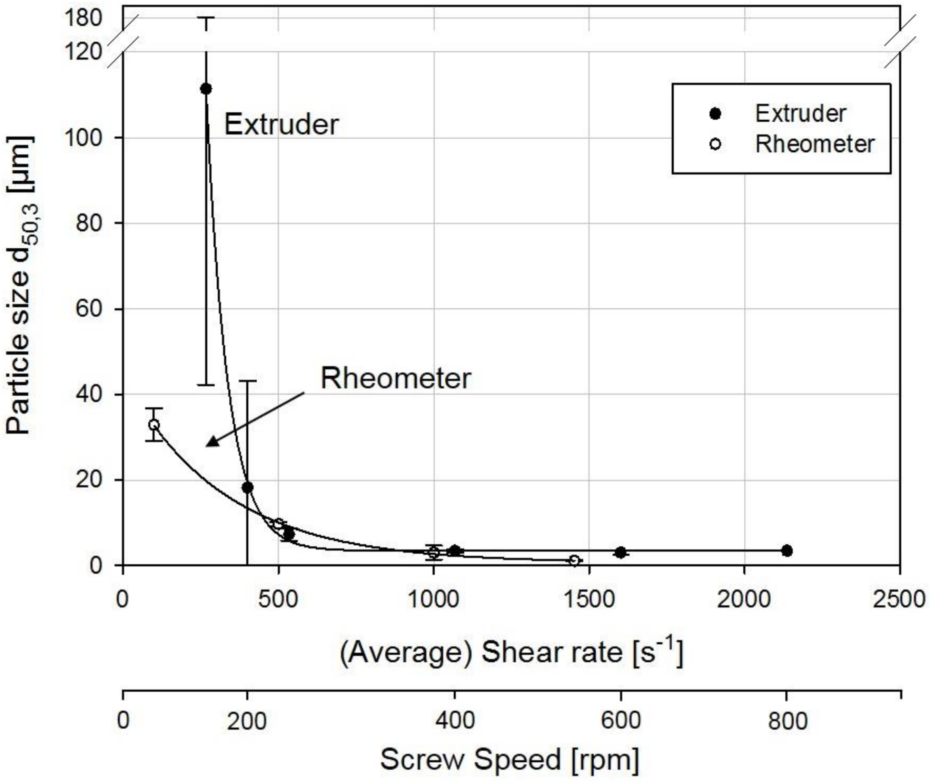


Fig. 7-1: Effect of the shear rate in the rheometer on the particle size $d_{50,3}$ (data from Wolz et al. (2016b)) compared to the effect of the estimated average shear rate in the extruder on the particle size $d_{50,3}$ (data for particle size from Wolz et al. (2016a)).

Fig. 7-1 shows the effect of shear rate on the $d_{50,3}$. Shear rate values in the rheometer were precisely regulated, whereas the values for the shear rate in the extruder represent estimated average shear rates at different screw speeds. The axis of the screw speed served as a reference for comparison purposes.

Results obtained from the extruder experiments versus those from the rheometer showed good agreement. For both shearing devices, the particle size decreased exponentially with increasing shear rate (Fig. 7-1). From the comparison of the shearing devices, it was concluded that a minimum shear rate of 500 s^{-1} or even 1000 s^{-1} was necessary to achieve small particles. This corresponds to screw speeds of 200 – 400 rpm.

Fig. 7-2 sums up the different effects of whey protein aggregation under shear stress. The representation is based on the findings of Aymard et al. (1996) and Nicolai et al. (2011) at quiescent conditions, but was adapted and expanded for aggregation during thermo-mechanical treatment.

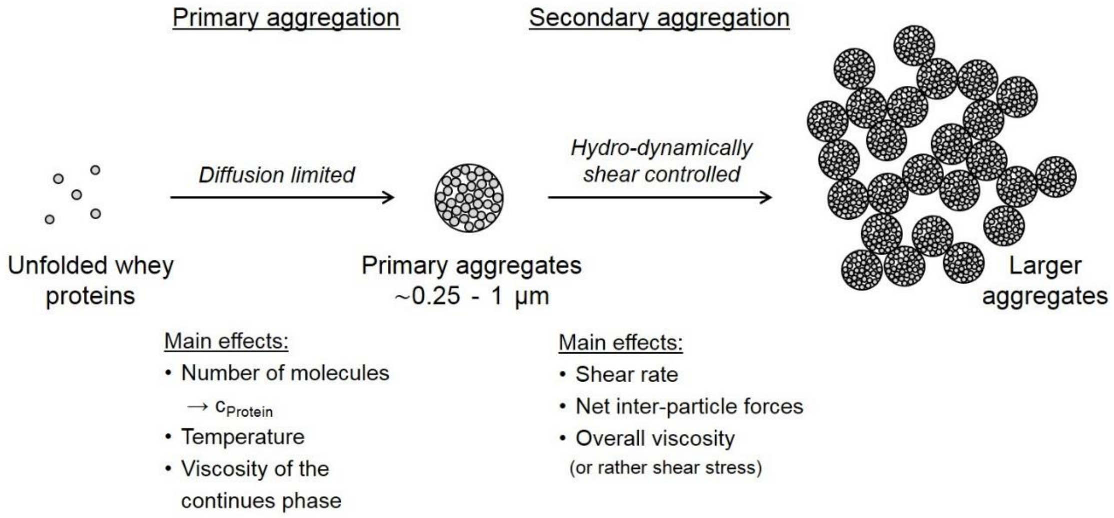


Fig. 7-2: Schematic representation of the aggregation mechanism of β–Lg under shear stress (not in scale).

Under concurrent heating and shearing as occurring in an extruder or other shearing device, the aggregation process of whey protein could be divided into primary and secondary aggregation. First, unfolded whey proteins assembled to so-called primary aggregates. These had a size of ~0.25 – 1 µm. Second, the primary aggregates formed larger aggregates. However, primary and secondary aggregation also took place simultaneously. Primary aggregation was limited by diffusion of unfolded proteins in the surrounding fluid. Thus, it was mainly affected by the number of available protein molecules, the viscosity of the continuous phase, as well as by the prevailing temperature (eq. 4.1). The latter accelerated the diffusion rate, due to faster motion of molecules at elevated temperatures. In contrast, secondary aggregation was hydro-dynamically shear controlled. At high protein concentrations, the resulting aggregate size was not limited by the aggregation rate. At a high density of reactive molecules, aggregation was not substantially enhanced by applied shear stress. Indeed, the shear stress acting on the aggregates limited the particle growth and thus final aggregate size. Shear stress rose with increasing overall viscosity and shear rate. Furthermore, the net inter-particle forces had to be attractive and strong enough to overcome the fluid shear stress. Hence, environmental conditions also played an important role.

This mechanism of primary and secondary aggregation also had implications on the resulting aggregate appearance (Fig. 7-3). The appearance of the aggregates in the present work could be explained by the fast aggregation rate at the conditions applied. The rate of protein aggregation was faster than the rate of protein unfolding so that protein-protein attractive and repulsive interactions were not in balance (Havea et al., 2004; Tang et al., 1995). Under conditions without shear, particulate gels would be formed, whereas shear let to individual randomly aggregated particles.

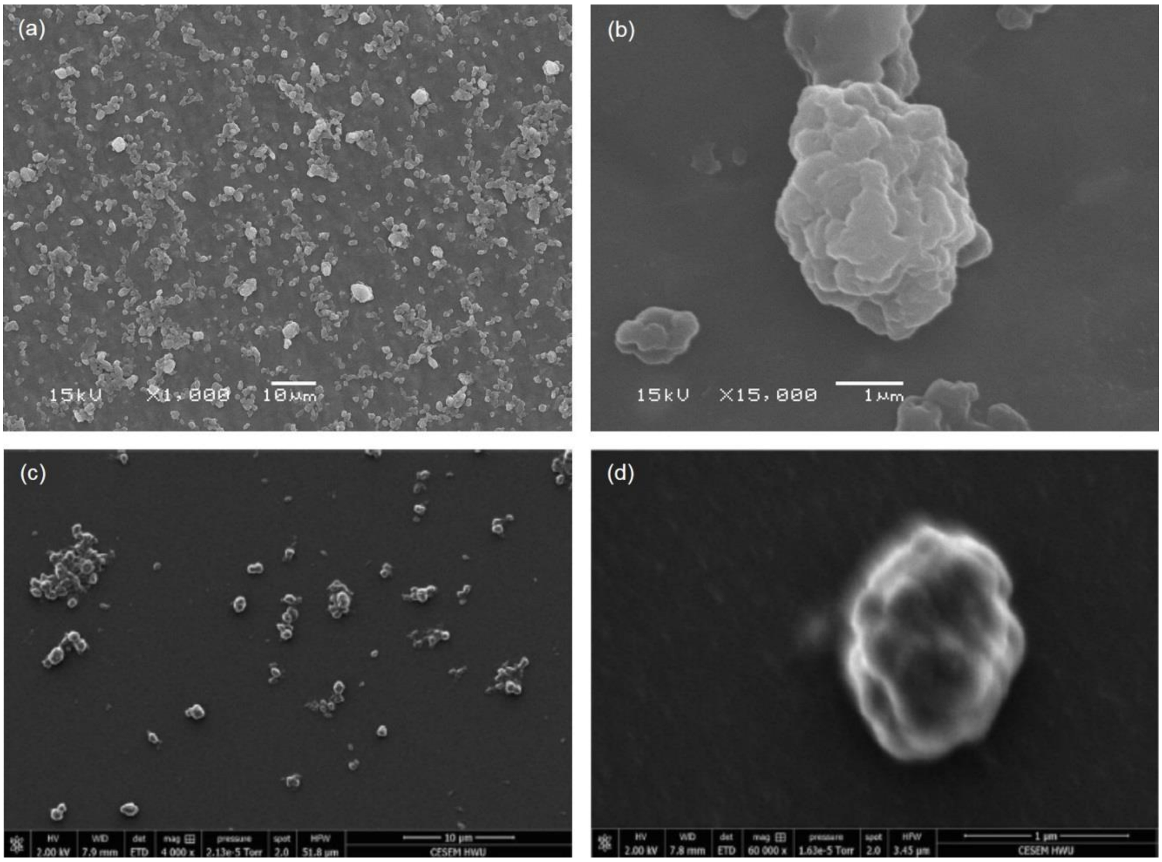


Fig. 7-3: Scanning electron micrographs of whey protein micro-particles (a) 1000x, produced by extrusion, (b) 15000x, produced by extrusion, (c) 4000x, Simpless® 100 (Zhang et al., 2016) (d) 60000x, Simpless® 100 (Zhang et al., 2016).

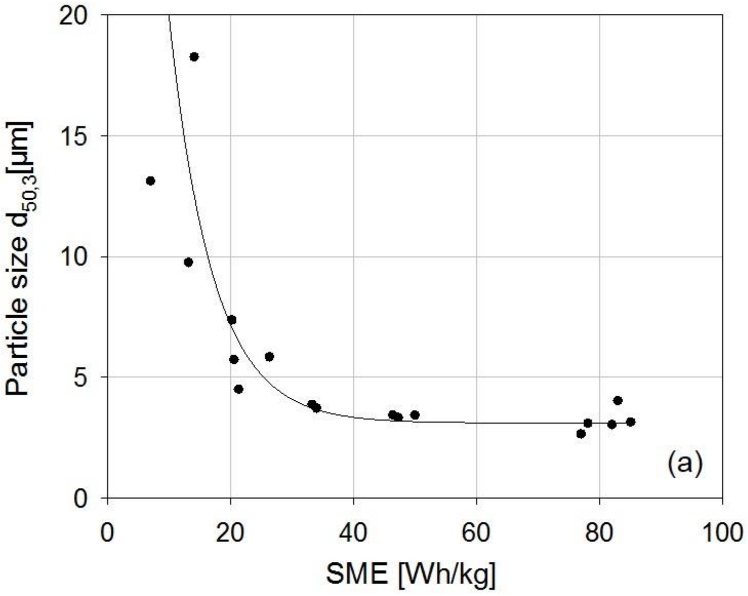
Fig. 7-3 presents micrographs of micro-particles at different magnifications. Micro-particles, produced by high moisture extrusion (Fig. 7-3 (a) and (b)), were compared to a commercial fat replacer (Fig. 7-3 (c) and (d)). A very similar aggregate form and structure is visible. As shown in Fig. 7-3 (b) and (d), larger aggregates were composed of smaller sub-units, the primary aggregates. This structure was also observed for aggregates formed during thermo-mechanical treatment in the rheometer (Fig. 4-5).

Consequently, as far as possible, secondary aggregation should be minimized for microparticulation to avoid formation of very large aggregates.

7.3 Effects on the extrusion process

Using high moisture extrusion, micro-particles comparable to the ones produced by the SSHE could be obtained. However, extrusion offers the advantages mentioned above (section 1.4.1 and 7). For extrusion processes, the concept of system parameters can be applied to obviate the interdependence of different influencing factors (section 1.4.3). The system parameters can be used to describe or compare extrusion processes under different operating conditions. In addition, they offer the potential to control the product quality during processing, as direct and in-line measurements of product properties are challenging (Chen et al., 2010; Onwulata et al., 1994).

Derived from the results shown in section 5, system parameters were identified that combine the effects of different process parameters and raw material characteristics on the processed product (section 6). The SME and the $T_{P,max}$ emerged as relevant system parameters for high moisture extrusion of whey proteins (Wolz & Kulozik, 2017). An exponential correlation between the SME and the resulting particle size was identified (Fig. 7-4 (a)). Furthermore, a linear correlation between the $T_{P,max}$ and the degree of denaturation was detected (Fig. 7-4 (b)), under the condition that the denaturation reaction was stopped in the extruder and the product outlet temperature did not exceed 68 °C.



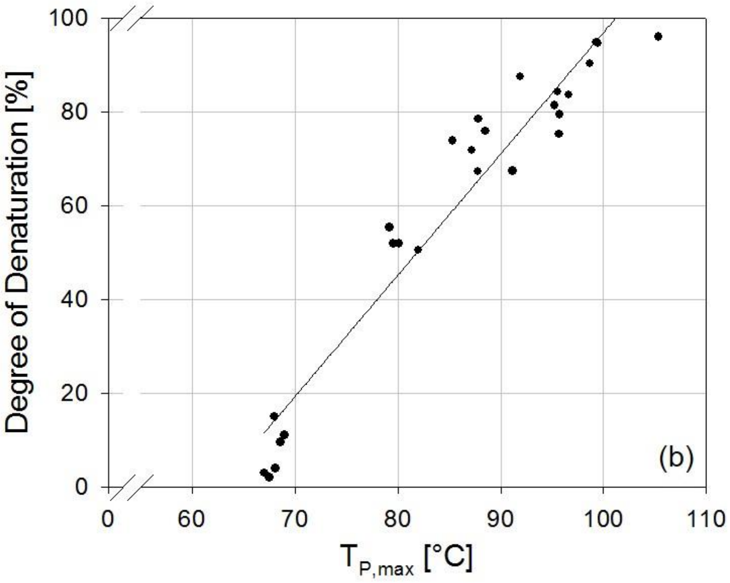


Fig. 7-4: Effect of the system parameters on the product properties of the micro-particles using high moisture extrusion. (a) Effect of SME on the particle size $d_{50,3}$ (b) Effect of $T_{P,max}$ on the degree of denaturation (Wolz & Kulozik, 2017).

The SME as well as the main drive torque were found to be directly proportional to the resulting shear stress. Hence, the SME is a measure for the overall mechanical energy input and, thereby, the applied shear stress. In the present study, it was found that the higher the SME (and thus the acting shear stress), the smaller was the size of the aggregates formed (Fig. 7-4 (a)). Additionally, the higher $T_{P,max}$, the higher was the degree of denaturation of the processed whey proteins (Fig. 7-4 (b)). Since the product only ran through a heating up phase in the extruder without a heat holding phase, the maximum temperature of the product at the end of the heating zone ($T_{P,max}$) was a measure for the received thermal stress. Different combinations of processing parameters are possible in order to obtain a specific SME or a specific $T_{P,max}$. The two curves in Fig. 7-4 (a) and Fig. 7-4 (b) were the result of multiple experiments at different process parameter combinations, such as barrel temperature, screw speed and mass flow. Thereby, the number of variables could be reduced to the system parameters SME and $T_{P,max}$. Independent of the adjusted process parameters, aggregate size was only affected by the resulting SME value. Equally, the degree of denaturation was only affected by the $T_{P,max}$. Indeed, this reduction of variables is only valid for this high moisture extrusion process for microparticulation. This is likely due to the short residence time and the low viscosity compared to other extrusion processes. Overall, the results indicate that the most important micro-particle properties, namely size and degree of denaturation, could be partially uncoupled.

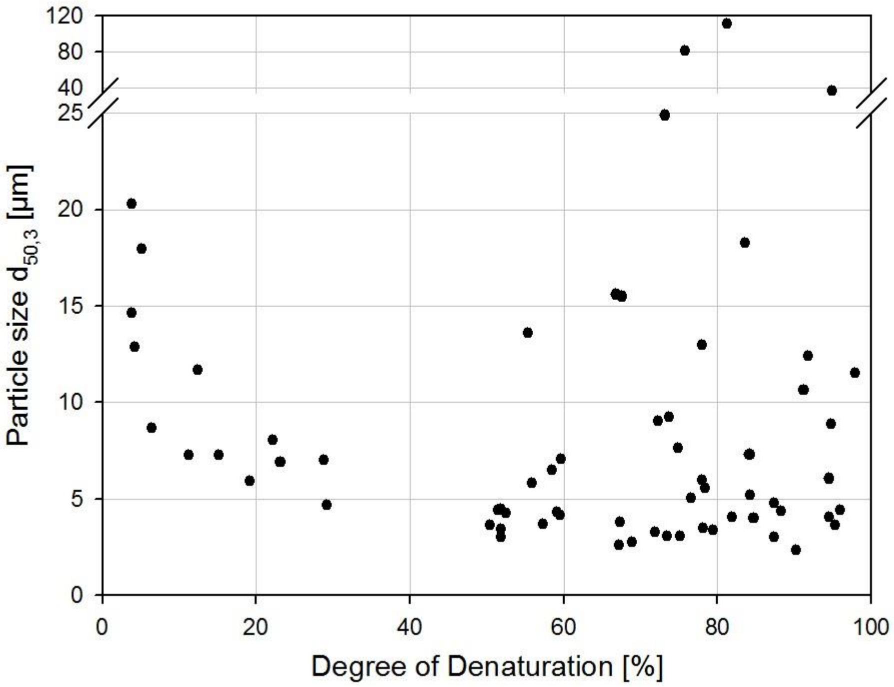


Fig. 7-5: Correlation between degree of denaturation and particle size $d_{50,3}$ (data from (Wolz et al., 2016a)).

A correlation between the degree of denaturation and the $d_{50,3}$ can be derived from Fig. 7-5. The presented extrusion process enables production of aggregates with identical size (i.e. ~2.5 - 5 µm), but different degrees of denaturation (i.e. ~50 – 95%). Thus, small micro-particles can be produced for different applications. On the one side, high degrees of denaturation are required for high heat stability. On the other side, a lower degree of denaturation is preferred to allow building-up of new structures. Furthermore, aggregates with a comparable degree of denaturation (e.g. ~80%), but different sizes (e.g. ~3 – 100 µm) are possible. Consequently, tailored micro-particles can be produced. Particle properties could be individually controlled to produce aggregates for different applications.

7.4 General Conclusion and Outlook

The overall aim of this work was to develop a high moisture extrusion process for microparticulation of whey proteins, as well as to explain the underlying thermal aggregation mechanism. In this study, extrusion has been found to be a suitable technique to produce micro-particles in the desired size range of 0.5 – 10 µm at neutral pH. Using an extruder set-up with a heating and cooling zone, the process is no longer limited to low pH values. During scale-up, the cooling zone has to be designed in such a way that it is long enough to reach a $T_{P,out}$ below 68 °C, i.e. below the critical denaturation temperature of whey proteins. Control of particle sizes in the low µm-range, as achieved in this study with extrusion technology, cannot be realized by comparable technical devices (e.g. SSHE) which simultaneously apply heat and shear.

Using extrusion, SME and $T_{P,max}$ are sufficient to control particle size and degree of denaturation, respectively. In contrast, no influence of the residence time could be determined. The reason for this is most likely that the product only passes through a fast heating-up and cooling-down phase and does not retain heat. Specific SME or $T_{P,max}$ values can be obtained by different combinations of process parameters. Thus, process parameters can be combined in such a way that system parameters required to achieve desired product properties are reached. Due to the simple correlation between process and system parameters on the one side and system and product parameters on the other, both SME and $T_{P,max}$ can be used for process control and optimization. Even if aggregate size and structure in the scanning electron micrographs suggest comparable functional properties, comparative application of micro-particulates produced by different techniques, such as extrusion and SSHE, is still lacking.

Furthermore, it was found that shear stress is the critical factor to limit particle size. Shear stress can be influenced by viscosity and shear rate, while the latter is again dependent on screw speed and extruder dimensions. The results obtained in this work can be put into practice when selecting process conditions during production of whey protein micro-particles.

This thesis presents a high moisture extrusion as an alternative process to the state of the art techniques for microparticulation, such as SSHE. Due to the higher mechanical shear stresses, which can be applied, this high moisture extrusion process makes it considerably easier to limit the resulting particle size to the desired degree. In addition, this process, using an intermeshing co-rotationg twin-scew extruder, largely excludes the formation of product caking. Furthermore, highly concentrated protein solutions can be processed resulting in lower volumetric flows, which is advantageous in regards to energy consumption.

This study forms the basis for an improved understanding of the denaturation and aggregation mechanism of highly concentrated protein solutions. The new insight gained in this work allows for an extension of the known reaction kinetics to higher protein concentrations, as they are applied in a targeted manner in the extrusion process. What is not covered in the present study is the influence of environmental factors (e.g. pH, calcium) on the extrusion process at elevated protein concentrations. It cannot be excluded that, interrelations between process and environmental conditions might arise. Bearing this in mind, it would be worthwhile to extend this study to variables such as lactose content, pH and ionic composition.

8 Summary & Zusammenfassung

8.1 Summary

Thermal aggregation of whey proteins is the basis for microparticulation. Depending on the quantity, size distribution and structure, micro-particles can modify and improve the textural properties of various food products. The denaturation mechanisms during heating have to be known to enable a tailored formation of whey protein aggregates with specific functional properties. β-Lg is commonly used as the lead component when investigating this reaction. During heating β-Lg unfolds und exposes the free thiol group. Thus, it reacts with other unfolded whey proteins via covalent and non-covalent bonds to larger aggregates. The rate of denaturation and aggregation strongly depends on the conditions concerning the process (temperature, heating time, shear) and the environment (concentration, lactose, pH, ionic milieu). Although the unfolding and aggregation behavior of whey proteins have been intensively investigated in the past, investigation at high protein concentrations are still lacking. Thus, the first aim was to extend the established reaction kinetics of β-Lg.

Despite, shear being present in most industrial processes, it is commonly neglected in literature. Whey proteins are, for example, exposed to shear at elevated temperatures in a targeted manner to produce micro-particles. A favorable and energy efficient technique to deal with this issue and offer various advantages is extrusion. This technique allows the variation of many process parameters, e.g. temperature, screw speed and feed rate, in a wide range. Extruders also enable the implementation of much higher protein concentrations as compared to the state of the art technology, the scraped surface heat exchanger. Hence, the aim of this study was to develop a high moisture extrusion process for microparticulation, which allows the targeted control of particle properties. Furthermore, the study sought to explain the aggregation behavior at high protein concentrations, and in particular to point out the effect of shear. The mechanistic understanding of the aggregation mechanism during thermo-mechanical treatment will provide a substantial basis for the future design of more efficient microparticulation processes.

The investigation of the denaturation kinetics at high protein concentrations was performed using thin tubes filled with protein solution and heated rapidly to different temperatures for varying heating times. The depletion of native β-Lg followed a reaction order of 1.5. The denaturation rate increased due to the strong influence of temperature on the unfolding reaction. In addition, the rate progressively increased with protein concentration due to promoted aggregation caused by the increased probability of collisions between the whey proteins. High protein systems are characterized by a high amount of available molecules, which cause a high number of collisions and a high viscosity. The latter could restrict the potential of motion of the single particles in the solution. However, in the first step of aggregation, Brownian motion limits the frequency of collisions; therefore, the viscosity of the continuous phase is decisive and doesn´t restricted the collision frequency of the used high protein concentrate.

Investigations at a rotational rheometer were carried out to further measure the impact of shear rate, temperature and heating time, independent of each other under controlled conditions. Solutions with different concentrations of whey proteins were heated and cooled while applying a constant shear rate. At high protein concentrations, the form of the aggregates was small, compact and almost spherical. This was explained by the increasing viscosity, leading to rises in the shear stress acting on the aggregates. In contrast to the frequency of collisions, the effective shear stress is a function of the total suspension viscosity and not only of the viscosity of the continuous phase. Hence, with increasing protein concentration, the viscosity increases and enhances the shear stress acting on the particles. This leads to a decreasing aggregate size. Another deciding factor to influence the aggregate size was the applied shear rate. Indeed, the effect of the shear rate was depend on the protein concentration. At a protein concentration of 5% and low shear rates, the aggregate size increased as the shear rate rose. This was related to the predominate effect of the growing number of collisions. On the other hand, at higher shear rates, the aggregate size decreased with further increasing shear rate. This relationship was also observed for suspensions with a higher protein concentration over the whole shear rate range. The increasing shear stress with rising shear rate was found to limit the particle growth and thus the aggregates' size. Compared to lower protein concentrations, the frequency of collisions and the aggregation rate at high protein concentrations was already very high due to the large number of protein molecules. The prevalent effects for the limitation of the particle growth and thus the limitation of the final particle size are the same as at low protein concentrations and high shear rates. Hence, the size of whey protein aggregates can be regulated by the applied shear stress during processing. However, the shear stress cannot be directly set in the majority of processes, such as extrusion.

A high moisture extrusion process was developed for microparticulation. For this purpose, a co-rotating twin screw extruder was used to particulate whey protein concentrate. The processing parameters barrel temperature, screw speed and mass flow were varied to assess their effects on the particle size distribution and the degree of denaturation. Furthermore, their effects on the system parameters, specific mechanical energy input (SME) and maximum product temperature ($T_{P,max}$), were obtained and correlation to the product properties were provided. Increasing the extruder barrel temperature elevated the degree of denaturation. In contrast, a higher mass flow led to a decrease in denaturation due to a reduced specific heat transfer to the product. Increasing the screw speed had almost no effect on the degree of denaturation, but resulted in an exponential reduction in particle size due to increasing mechanical shear stress.

To improve the understanding of the process, the system analytical approach was used. This characterized the process by system parameters. The $T_{P,max}$ correlated linearly with the extruder barrel temperature and the mass flow. The degree of denaturation only depended on the temperature with $T_{P,max}$ as the critical measure, no matter which combination of processing parameters was responsible for each particular $T_{P,max}$. The degree of denaturation measured at the end of the process, only increased with rising product temperature, due to a higher denaturation rate. The SME was the major factor limiting $d_{50,3}$. This effect was independent whether or not the change of the SME was based on screw speed or mass flow. An increase in the resulting mechanical energy input per mass unit of protein intensified the shear stress acting on the aggregates and, thereby, limited their size. This clearly shows that the particle size of the whey protein aggregates can easily be controlled by the system parameter SME and the degree of denaturation by the $T_{P,max}$. This offer the opportunity to control the process and the product properties by inline measurement of the system parameters. In addition, this enables to formation micro-particles with a specific size and certain degree of denaturation.

As the SME and the $T_{P,max}$ were sufficient to control particle size and degree of denaturation, respectively, the obtained results indicate that the important micro-particle properties, size and degree of denaturation, could be partially uncoupled. Consequently, this led to aggregates with the same size, but different degrees of denaturation. Furthermore, aggregates with the same degree of denaturation, but with different sizes are shown to be possible. This permits the production of tailored micro-particles. Particle properties could be individually controlled to produce aggregates for different applications.

In conclusion, in this study a high moisture extrusion process for the microparticulation of whey proteins was successfully developed and evaluated. This allows for a deeper understanding of the aggregation processes in the extruder. The effects that occur during microparticulation in a thermo-mechanical process were clearly shown to be related to the underlying aggregation mechanisms.

8.2 Zusammenfassung

Die Grundlage der Mikropartikulierung von Molkenproteinen ist deren Disposition zur thermischen Aggregation. Abhängig von der Anzahl, der Größenverteilung und der Struktur der Mikropartikulate können diese in unterschiedlichen Lebensmitteln, zur Veränderung und Verbesserung der strukturellen Eigenschaften, eingesetzt werden. Um bedarfsgerechte Molkenproteinaggregate mit bestimmten funktionellen Eigenschaften herzustellen, muss der dahinterstehende Denaturierungsmechanismus während des Erhitzens bekannt sein. β-Lg als Leitsubstanz wird allgemein verwendet, um diese Reaktion zu untersuchen. Während der Erhitzung falten die β-Lg-Moleküle auf und die freie Thiolgruppe wird exponiert. Auf diese Weise reagiert β-Lg mit anderen aufgefalteten Molkenproteinen über kovalente und nicht-kovalente Bindungen zu größeren Aggregaten. Die Denaturierungs- und Aggregationsgeschwindigkeit hängt dabei stark von den Bedingungen während des Erhitzens ab. Dabei haben sowohl die Prozessbedingungen (Temperatur, Heißhaltezeit, Scherung) als auch die Milieubedingungen (Konzentration, Lactosegehalt, pH, Ionenmilieu) einen großen Einfluss. Obwohl das Auffaltungs- und Aggregationsverhalten der Molkenproteine in vielen Studien bereits umfangreich untersucht wurde, ist das Verhalten bei hohen Proteinkonzentrationen nahezu unbekannt. Folglich war es das Ziel, die Reaktionskinetiken von β-Lg dahingehend zu erweitern.

Ungeachtet dessen, dass Scherung in den meisten industriellen Prozessen auftritt, wird diese in der Literatur oft nicht berücksichtigt. Zum Beispiel wird eine gleichzeitige Erhitzung und Scherung gezielt eingesetzt, um Mikropartikulate zu erzeugen. Dafür stellt die Extrusion ein geeignetes und energieeffizientes Verfahren dar, welches diverse Vorteile bietet. Bei der Extrusion können die Prozessparameter, wie beispielsweise die Temperatur, die Schneckendrehzahl und der Massenstrom in einem weiten Bereich variiert werden. Auch können dadurch deutlich höhere Proteinkonzentrationen, als bei der aktuellen Technik, den Schabewärmetauschern, gehandhabt werden. Das Ziel war es deshalb, einen Nassextrusions-Prozess für die Mikropartikulierung zu entwickeln, bei welchem die Partikeleigenschaften gezielt kontrolliert werden können. Zudem sollte das Aggregationsverhalten und im Speziellen der Einfluss der Scherung und der Proteinkonzentration darauf aufgeklärt werden. Mithilfe eines mechanistischen Verständnisses des Aggregationsmechanismus während der thermo-mechanischen Behandlung sollen Mikropartikulierungsprozesse künftig effizienter ausgelegt werden können.

Für die Untersuchungen der Denaturierungskinetiken bei hohen Proteinkonzentration wurden dünnwandige Röhrchen mit Proteinlösung gefüllt und schnell auf unterschiedliche Temperaturen und für verschiedene Zeiten erhitzt. Für den Abbau von nativem β-Lg wurde eine Reaktionsordnung von 1.5 ermittelt. Die Denaturierungsgeschwindigkeit stieg, aufgrund des starken Einflusses auf die Auffaltungsreaktion mit der Temperatur. Ferner nahm die Geschwindigkeit mit der Proteinkonzentration stetig zu, da die Aggregation durch die steigende Kollisionswahrscheinlichkeit zwischen den Molkenproteinen verstärkt wurde. Systeme mit hoher Proteinkonzentration zeichnen sich durch eine hohe Anzahl an vorhandenen Molekülen aus, welche eine hohe Kollisionszahl und eine hohe Viskosität verursachen. Die hohe Viskosität kann dabei die Bewegungsmöglichkeiten der einzelnen Partikel in der Lösung einschränken. Allerdings wird die Häufigkeit der Kollisionen, im ersten Schritt der Aggregation durch die Brownsche Molekularbewegung limitiert. Dafür ist die Viskosität der kontinuierlichen Phase entscheidend. Damit begrenzte die Viskosität, bei den hier verwendeten hochkonzentrierten Proteinkonzentraten, nicht die Kollisionshäufigkeit.

Um weiterhin den Einfluss der Scherrate, der Temperatur und der Heißhaltezeit unabhängig voneinander unter kontrollierten Bedingungen zu erfassen, wurden Untersuchungen an einem Rotationsrheometer durchgeführt. Lösungen mit unterschiedlichen Konzentrationen an Molkenproteinen wurden bei einer konstanten Scherrate erhitzt und wieder abgekühlt. Bei hohen Proteinkonzentrationen wurden dabei kleine, kompakte und runde Aggregate beobachtet. Dies konnte mit der zunehmenden Viskosität und der damit einhergehenden, zunehmend auf die Partikel wirkenden Schubspannung erklärt werden. Im Gegensatz zur Kollisionshäufigkeit ist die Schubspannung abhängig von der Viskosität der gesamten Suspension und nicht nur von der Viskosität der kontinuierlichen Phase. Folglich steigt die auf die Partikel wirkende Schubspannung mit zunehmender Proteinkonzentration. Dies führte zu einer Abnahme der Aggregatgröße mit zunehmender Konzentration. Der Effekt der eingestellten Scherrate am Rheometer änderte sich mit der Proteinkonzentration. Bei einer Proteinkonzentration von 5 % und geringer Scherrate erhöhte sich die Aggregatgröße mit steigender Scherrate. Dies konnte mit dem vorwiegenden Effekt der zunehmenden Anzahl an Kollisionen in Verbindung gebracht werden. Bei höheren Scherraten nahm die Aggregatgröße mit zunehmender Scherrate wieder ab. Eine Abnahme der Aggregatgröße mit zunehmender Scherrate wurde ebenfalls für hohe Proteinkonzentrationen (30 %) über den gesamten untersuchten Scherratenbereich festgestellt. Die steigende Schubspannung mit steigender Scherrate ist die Ursache für die Begrenzung des Aggregatwachstums und damit der Größe. Im Vergleich zu niedrigen Proteinkonzentrationen ist die Kollisionswahrscheinlichkeit bei hohen Proteinkonzentrationen aufgrund der hohen Anzahl an Molekülen bereits sehr hoch; auf diese Weise ist auch die Aggregationsrate beschleunigt. Die Ursache für das limitierte Partikelwachstum ist damit in erster Linie die gleiche wie bei geringen Konzentrationen und hohen Scherraten. Die Größe der Molkenproteinaggregate kann also durch die Scherrate im Prozess gesteuert werden. Allerdings kann die Scherrate im Prozess in den seltensten Fällen direkt eingestellt werden, so auch bei der Extrusion.

Für die Mikropartikulierung eines Molkenproteinkonzentrats im Pilotmaßstab wurde ein gleichläufig drehender Doppelschneckenextruder eingesetzt und ein geeigneter Nassextrusionsprozess entwickelt. Die Prozessparameter Gehäusetemperatur, Schneckendrehzahl und Produktmassenstrom wurden variiert, um ihren Einfluss zum einen auf die Partikelgrößenverteilung und den Denaturierungsgrad festzustellen. Zum anderen um den Einfluss auf die Systemparameter, den spezifisch mechanischen Energieeintrag (SME) und die maximale Produkttemperatur ($T_{P,max}$), zu erhalten und um Zusammenhänge zu den Produkteigenschaften herstellen zu können. Bei steigender Gehäusetemperatur des Extruders nahm auch der Denaturierungsgrad zu. Im Gegensatz dazu führt ein steigender Massenstrom zu einer Abnahme des Denaturierungsgrades aufgrund der verringerten spezifischen Wärmeübertragung auf das Produkt. Eine Zunahme der Drehzahl hatte kaum einen Effekt auf den Denaturierungsgrad, resultierte aber in einem exponentiellen Abfall der Partikelgröße durch die zunehmende Schubspannung.

Um das Prozessverständnis entscheidend zu verbessern, wurde der systemanalytische Ansatz verwendet. Dafür wurde der Prozess mittels Systemparametern beschrieben. $T_{P,max}$ korrelierte linear mit der Gehäusetemperatur und dem Massenstrom. Der Denaturierungsgrad hing nur von $T_{P,max}$ ab, unabhängig davon durch welche Kombination an Prozessparametern diese erreicht wurde. Der Denaturierungsgrad nahm nur mit steigender Produkttemperatur und damit höher Denaturierungsgeschwindigkeit zu. Der SME war die Hauptursache für die Beschränkung des $d_{50,3}$. Auch dieser Effekt war unabhängig davon, ob die Veränderung des SME durch die Drehzahl oder den Massenstrom bedingt wurde. Eine Zunahme des mechanischen Energieeintrags pro Masseneinheit an Protein erhöht die Schubspannung, welche auf die Aggregate wirkt und dadurch die Größe limitiert.

Dies macht deutlich, dass die Partikelgröße der Molkenproteinaggregate über den Systemparameter SME und der Denaturierungsgrad über $T_{P,max}$ einfach kontrolliert und gesteuert werden kann. Dadurch eröffnet sich die Möglichkeit, die Prozess- und Produkteigenschaften durch inline Messung der Systemparameter zu kontrollieren. Des Weiteren ist dadurch die Herstellung von Mikropartikulaten mit bestimmter Größe und bestimmten Denaturierungsgrad realisierbar.

Die erzielten Ergebnisse zeigen, dass wichtige Eigenschaften der Mikropartikulate, wie die Größe und der Denaturierungsgrad, teilweise entkoppelt werden können, da mittels des SME die Größe und mittels $T_{P,max}$ der Denaturierungsgrad eingestellt werden kann. Dies erlaubt sowohl die Bildung von Aggregaten mit gleicher Größer, aber unterschiedlichen Denaturierungsgraden als auch die Bildung von Aggregaten mit dem gleichen Denaturierungsgrad, aber unterschiedlichen Partikelgrößen. Dies ermöglicht es, zielgerichtet Mikropartikulate herzustellen und die Partikeleigenschaften individuell je nach Anwendungsgebiet einzustellen.

Im Rahmen dieser Arbeit konnte ein Prozess für die Nassextrusion von Molkenproteinen zur Mikropartikulierung erfolgreich entwickelt und beurteilt werden. Mit dem fundamentalen Verständnis der Aggregationsvorgänge können die Effekte im Extruder bei der Mikropartikulierung erklärt und mit den grundlegenden Aggregationsmechanismen in Beziehung gebracht werden.

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10 Appendix

The following publications and presentations have emerged from this work. They are listed in chronological order. Publications being part of this thesis are marked in bold.

10.1 Peer reviewed publications

1. **Wolz, M., Kulozik, U. (2015).** Thermal denaturation kinetics of whey proteins at high protein concentrations. International Dairy Journal, 49, 95–101.
2. **Wolz, M., Mersch, E., Kulozik, U. (2016).** Thermal aggregation of whey proteins under shear stress. Food Hydrocolloids, 56, 396–404.
3. **Wolz, M., Kastenhuber, S., Kulozik, U. (2016).** High moisture extrusion for micro-particulation of whey proteins –Influence of process parameters. Journal of Food Engineering, 185, 56–61.
4. **Wolz, M., Kulozik, U. (2017).** System parameters in a high moisture extrusion process for microparticulation of whey proteins. Journal of Food Engineering, 209, 12-17.

10.2 Non reviewed publications

1. Merkl, M., Hajdu, T., Berlehner, R., Kulozik, U. (2012). Mikropartikulierung von Molkenproteinen mittels Heißextrusion – Einfluss der Drehzahl. Jahresbericht 2011 der milchwissenschaftlichen Forschungseinheiten am ZIEL, 54, 133-135, ISBN 978-939182436.
2. Merkl, M., Kulozik, U. (2013). Aggregation von Molkenproteinen bei Erhitzung und Scherung – Einfluss der Protein-konzentration. Jahresbericht 2012 der milchwissenschaftlichen Forschungseinheiten am ZIEL, 55, 92-94, ISBN 978-3939182528.

3. Merkl, M., Kulozik, U. (2014). Einfluss der Proteinkonzentration auf das thermisch induzierte Denaturierungsverhalten von Molkenproteinen. Jahresbericht 2013 der milchwissenschaftlichen Forschungseinheiten am ZIEL, 56, 110-112, ISBN 978-3939182634.
4. Wolz, M., Kulozik, U. (2015). Einfluss von Prozessbedingungen bei der Extrusion auf die Mikropartikulation von Molkenproteinen. Jahresbericht 2014 der milchwissenschaftlichen Forschungseinheiten am ZIEL, 57, 86-87, ISBN 978-3939182757.
5. Wolz, M., Kulozik, U. (2015). Mikropartikulierung von Molkenproteinen mittels Heißextrusion. Abschlussbericht zum AiF-FV 17140 N. Forschungskreis der Ernährungsindustrie e.V. (FEI).
6. Wolz, M., Kulozik, U. (2016). Einsatz von extrudierten Mikropartikulaten in Speiseeis. Jahresbericht 2015 der milchwissenschaftlichen Forschungseinheiten am ZIEL, 58, 87-90, ISBN 978-3939182894.
7. Wolz, M., Kuhne, F., Kulozik, U. (2017). Charakterisierung von Bindungsmechanismen bei extrudierten Mikropartikulaten. Jahresbericht 2016 der milchwissenschaftlichen Forschungseinheiten am ZIEL, 59, 61-63, ISBN 978-3-939182-93-1.

10.3 Oral presentations

1. Merkl, M., Kulozik, U.: Heißextrusion von Molkenproteinen - Gezielte Strukturbeeinflussung und Aggregatbildung durch Extrusion. DECHEMA-VDI-GVC ProcessNet, Jahrestreffen der Fachausschüsse Lebensmittelverfahrenstechnik und Trocknungstechnik, Stuttgart-Hohenheim, 20.03.2012.
2. Merkl, M., Kulozik, U.: Particle formation of whey protein by extrusion cooking. International Symposium on Food Rheology and Structure (ISFRS), Zürich, Schweiz. 12.04.2012.
3. Merkl, M., Kulozik, U.: Strukturierung von Proteinen mittels Mikropartikulierung im Extruder. Technologieseminar Weihenstephan „Verfahrens- und Strukturoptimierung in der Lebensmittelherstellung“, Freising, 25.10.2012.
4. Merkl, M., Kulozik, U.: Thermische Aggregation von Molkenproteinen unter Scherung. Jahrestreffen der ProcessNet-Fachgruppe Lebensmittelverfahrenstechnik, Quakenbrück, 25. - 27.02.2013.
5. Merkl, M., Kulozik, U.: Microparticulation of whey proteins by extrusion cooking. Euro-FoodChem XVII, Istanbul, Türkei, 07. - 10.05.2013.
6. Merkl, M., Kulozik, U.: Erzeugung von mikropartikulierten Molkenproteinen mittels Extrusionsverfahren. Weihenstephaner Milchwirtschaftliche Herbsttagung, Freising-Weihenstephan, 10. - 11.10.2013.

7. Merkl, M., Kulozik, U.: Mikropartikulierung von Molkenproteinen mittels Heißextrusion. Projekttreffen zum AiF/FEI-Forschungsvorhaben 17140 N, 05.11.2013.
8. Wolz, M., Kulozik, U.: Extrusion cooking as a new process for the production of whey protein aggregates as functional food colloid. 15th Food Colloid Conference, Karlsruhe, 13. – 16.04.2014.
9. Wolz M., Kulozik U.: Extrusion cooking as a new process for the microparticulation of whey proteins using high protein concentrations, 7th International Whey Conference, Rotterdam, 07. – 09.09.2014.
10. Wolz M., Kulozik U.: Mikropartikulierung von Molkenproteinen mittels Heißextrusion, 2. AiF-Projektausschuss-Sitzung AiF 17140 N, Freising, 04.11.2014.
11. Wolz, M., Kulozik, U.: Extrusion als neues Verfahren zur Mikropartikulierung von Molkenproteinen. Jahrestreffen der ProcessNet-Fachgruppe Lebensmittelverfahrenstechnik, Magdeburg, Deutschland, 16. - 18.03.2015.
12. Wolz, M., Kulozik, U.: Mikropartikulierung von Molkenproteinen mittels Heißextrusion, 3. AiF-Projektausschuss-Sitzung AiF 17140 N, Freising, Deutschland, 23.06.2015.
13. Wolz, M.; Kulozik, U.: New ways of whey protein microparticulation by extrusion cooking for enhanced technological functionalities. Technology Seminar, Freising-Weihenstephan, Deutschland, 16. - 18.09.2015.
14. Wolz, M., Kulozik, U.: Whey protein functionalization by extrusion cooking. 9th NIZO Dairy Conference: Milk Protein Functionality, Papendal, Niederlande, 30.09. - 02.10.2015.

10.4 Poster presentations

1. Merkl, M., Mersch, M. Kulozik, U.: Thermal aggregation of whey protein under defined shearing conditions. Annual General Meeting des International Fine Particle Research Insti-tutes (IFPRI), Ludwigsburg. 27. Juni 2012.
2. Merkl, M., Kulozik, U.: Whey protein functionalization by extrusion cooking. IDF World Dairy Summit, Yokohama, Japan, 28.10.-01.11.2013.
3. Wolz, M., Haler, N.; Kulozik, U.: Extrusion cooking as new process for protein structuring – Microparticulation of whey protein using high protein concentrations. 12th International Congress on Engineering and Food, Québec City, Kanada, 4.-18. Juni 2015.