

Hydrostatic high pressure treatment of casein to generate defined particle and gel structures

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Erklärung

Hiermit versichere ich, diese Arbeit selbständig verfasst und nur die angegebenen Quellen benutzt zu haben.

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Merel, E., Budde, T. & Hinrichs, J. (2005). Formation of new casein structures by high pressure, Poster presentation - *Joint 20th AIRAPT - 43rd EHPRG Conference on Science and Technology of High Pressure*, Karlsruhe.

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Symbols and abbreviations

Latin symbols

c	concentration	[g ml ⁻¹], [g 100ml ⁻¹]
d	diameter	[m]
D	diffusion coefficient	[m ² s ⁻¹]
D _f	fractal dimensionality	[-]
d _H	mean hydrodynamic diameter	[nm]
D _m	mutual diffusion coefficient	[m ² s ⁻¹]
G′	storage modulus	[Pa]
G′′	loss modulus	[Pa]
h	mean head of capillary	[m]
i	number of independent experiments	[-]
J	encounters per unit time	[s ⁻¹]
K	calibration constant for a falling-ball viscometer	[-]
K	capillary viscometer conversion factor	[-]
k _{ow}	Ostwald factor	[-]
K _{vector}	norm of the scattered light vector	[-]
l	length of capillary	[m]
M	torque	[Nm]
N	number of particles per unit volume	[m ⁻³]
n	flow index	[-]
r	radius	[m]
RI	refraction index	[-]
T	absolute temperature	[K]
t	time	[s]
V	volume	[m ³]
V _a	voluminosity	[ml g ⁻¹]

Greek symbols

$\dot{\gamma}$	shear rate	[s ⁻¹]
ϑ	temperature	[°C]
α	angle of cone	[rad]
δ	loss angle	[°]
η	dynamic viscosity of the solution	[Pa·s]
Θ	scattered light angle	[°]
λ	wavelength of the light	[nm]
ν	kinematic viscosity	[m ² s ⁻¹]
ρ	density of the solution	[kg m ⁻³]
τ	shear stress	[Pa]
φ	volume fraction	[-]
Ω	angular velocity	[rad s ⁻¹]

Indices

0	time t = 0
A	aggregate
app	apparent
B	ball
d	delay
f	fractal
H	hydrodynamic
max	maximum
ortho	orthokinetic
P	particle
peri	perikinetic
S	liquid sample
S	sphere
t	time

Abbreviations

AFM	atomic force microscopy
CaB	calcium bridge
CCP	colloidal calcium phosphate
CI	confidence interval
EB	electrostatic bonds
EDTA	ethylenediaminetetraacetic acid
G	gel
HDPE	high density polyethylene
Hy	hydrophobic bonds
IEP	isoelectric point
mC	micellar casein
MF	microfiltration
n.a.	non analyzed
nb	non-bond proteins
PCS	photon correlation spectroscopy
S	sol
s.d.	standard deviation
SS	disulfide bridges
T	transition
UFP	ultrafiltration permeate
WB	hydrogen bonds

Constants

g	acceleration of gravity on earth	$g = 9.81 \text{ m s}^{-2}$
k_B	Boltzmann constant	$k_B = 1.38066 \cdot 10^{-23} \text{ J K}^{-1}$

If nothing else is noted, concentrations in percent are given in weight per volume.

Chapter I

Scope and outline

Scope

In high pressure treatment, the structure of casein micelles can be modified by the process parameters and milieu conditions resulting in new functional properties. Several studies have shown that during pressure build-up phase casein micelles dissociate into submicelles (Schmidt & Buchheim, 1970; Needs et al., 2000; Regnault et al., 2004). This pressure-induced dissociation was explained by the weakening of hydrophobic and electrostatic interactions between submicelles (Mozhaev et al., 1996), and the solubilisation of colloidal calcium phosphate out of the micellar framework (Shibauchi et al., 1992; Lee et al., 1996; Schrader et al., 1997). During pressure release, the binding forces are regained and new calcium bridges are built up (Shibauchi et al., 1992). Instead of the original casein micelles new hyper-structures may be built up. Most of the studies on casein micelles under high pressure are focused on the influence of pressure level and temperature but knowledge about the influence of pressure release rate is still lacking.

The focus of the work was to study the influence of different high pressure process parameters especially pressure release rate but also pressure build-up, pressure level and holding time and milieu conditions on pressure-induced casein structures. The experiments were carried out with an enriched micellar casein powder gained by diafiltration of skim milk at the Institute for Food Process Engineering in Freising, Germany (Kersten, 2001). Pressure release and pressure build-up rates were varied from 20 to 600 MPa min⁻¹, pressure level from 200 to 600 MPa and holding time from 0 to 30 min. A better understanding of the pressure-induced structure formation of the casein micelles on ultra-high pressure treatment may offer opportunities for the creation of novel dairy products.

Outline of the thesis

Chapter II provides basic information about casein, casein micelles but also aggregation and gelation. Hypotheses about the action of high pressure on casein structures are presented. Furthermore, theories about the used analysis methods are given. All these aspects will allow the discussion and interpretation of the results in the following chapter and therefore give a better understanding of the behavior of casein under high pressure.

Chapter III describes the influence of pressure release rate and protein concentration on the formation of pressure-induced casein structures.

This Chapter has been submitted to Journal of Dairy Research:

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Chapter IV is focussed on the influence of pressure build-up rate, pressure holding time and pressure release rate on casein. *Ex situ* and *in situ* results are presented and compared.

Chapter V describes the influence of casein concentration and calcium content, combined with pressure release rate on the formation of new casein structures.

This Chapter was published in the proceedings CD of the AIRAPT-EHPRG conference in Karlsruhe:

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Chapter VI describes the influence of pressure level, pressure release rate and temperature on the formation of pressure-induced casein structures

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Coauthors

My work was supervised by Prof. Jörg Hinrichs. The research was done in parallel with researchers of the Chair for Food Process Engineering and Dairy Technology of the Technical University of Munich, headed by Prof. Ulrich Kulozik. Their research was mainly based on the formation of high pressure induced casein/hydrocolloids and whey proteins/hydrocolloids structures.

This dissertation comprises studies that were carried out in cooperation with several researchers. Dr. Gebhardt of the research group of Dr. Doster of the Physics Department of the Technische Universität München worked with our casein powder and investigated the effect of high pressure (*in situ* PCS) on the particle size distribution. A part of the work presented in Chapter III was supervised by Prof. Ulrich Kulozik. Chapter IV presents research supervised by Prof. Antonio Delgado and Dr. Albert Baars of the Chair for Fluidmechanics and Processautomation of the Technische Universität München. Natalie Pereyra-Grünhagen of the working group of Prof. Antonio Delgado provided the results of the *in situ* viscosity. Results presented in Chapter V to VI were obtained with the help of Tanja Budde and Ioana Paula Duma who did their master theses in our institute in Hohenheim.

The project was financially supported by the DFG (Deutsche Forschungsgemeinschaft) as part of the high pressure project FOR 358/2.

Chapter II

General introduction

The work was focused on the pressure-induced transformation of the structure of casein micelles. In this chapter basic information about casein, casein micelles but also aggregation and gelation are provided. Hypotheses about the action of high pressure on casein structures are presented. Furthermore, theories about the used analysis methods are given. All these aspects will allow the discussion and interpretation of the results in the following chapter and therefore give a better understanding of the behavior of casein under high pressure.

The theories presented in this chapter are mostly assembled from the following books: Walstra & Jenness, 1984; Walstra et al., 1999; Belitz et al., 2001 and Walstra, 2003.

The caseins

Milk is a three phase system composed of a solution (milk serum), an emulsion and a dispersion. In the serum, the major parts of the milk are whey proteins, salts, vitamins, urea and other diluted milk components. The emulsion is composed of the milk fat in form of dispersed fat drops. Caseins represent the dispersion phase. The milk proteins (about 3.4 %) are composed of two major protein fractions: the caseins and the whey proteins at the rate of 4:1.

About 80 % of the milk proteins consist of casein, phosphoproteins that precipitate at about pH 4.6 and 20 °C. Due to their electrophoretic mobility, caseins are divided in four major fractions: α_{s1} -casein (39 %), α_{s2} -casein (10 %), β -casein (36 %) and κ -casein (13 %) (Table II.1).

Table II.1: Characteristics of the casein fractions (Walstra et al., 1999)

Fraction	α_{s1}	α_{s2}	β	κ
Molecular weight	23.6	25.2	24.0	19.0
Phosphoserine (res./mol)	8	11	5	1
Cysteine (res./mol)	0	2	0	2
Hydrophobicity (kJ/res.)*	4.9	4.7	5.6	5.1
Net charge / residues at pH 6.6	-0.10	-0.07	-0.06	-0.02
Isoelectric pH	4.1-4.8	5.1	5	5.5-5.8
Calcium sensitivity	++	+++	+	-

*Tanford-bigelow hydrophobicity scale

The casein fractions differ from each other in their amino acid composition (Table II.2), their charge distribution and in their tendency to aggregate in the absence and presence of calcium.

Table II.2: Amino acid composition of the cow casein fractions *

Amino acid	α_{s1} -Cas		α_{s2} -Cas		β -Cas		κ -Cas	
	%	F	%	F	%	F	%	F
Ala	4.5	0.6	3.9	0.5	2.4	0.3	8.3	1.1
Arg	3.0	0.6	2.9	0.5	1.9	0.4	3.0	0.6
Asn	4.0	1.0	6.8	1.6	2.4	0.6	4.7	1.1
Asp	3.5	0.7	1.9	0.4	1.9	0.4	2.4	0.4
Cys	0.0	0.0	1.0	0.6	0.0	0.0	1.2	0.8
Gln	7.0	1.8	7.7	2.0	9.6	2.4	8.9	2.2
Glu	12.6	1.9	11.6	1.7	9.1	1.4	7.1	1.1
Gly	4.5	0.7	1.0	0.1	2.4	0.3	1.2	0.2
His	2.5	1.1	1.4	0.6	2.4	1.0	1.8	0.8
Ile	5.5	0.9	5.3	0.9	4.8	0.8	7.1	1.2
Leu	8.5	0.9	6.3	0.7	10.5	1.1	4.7	0.5
Lys	7.0	1.2	11.6	2.0	5.3	0.9	5.3	0.9
Met	2.5	1.1	1.9	0.8	2.9	1.2	1.2	0.5
Phe	4.0	1.0	2.9	0.7	4.3	1.1	2.4	0.6
Pro	8.5	1.8	4.8	1.0	16.7	3.5	11.8	2.5
Ser	8.0	1.2	8.2	1.2	7.7	1.1	7.7	1.1
Thr	2.5	0.5	7.2	1.3	4.3	0.8	8.9	1.6
Trp	1.0	0.9	1.0	0.8	0.5	0.4	0.6	0.5
Tyr	5.0	1.6	5.8	1.9	1.9	0.6	5.3	1.7
Val	5.5	0.8	6.8	1.0	9.1	1.4	6.5	1.0

* The amino acid sequences of the mature chains of the different casein fractions of *Bos taurus* (cow) were obtained from the protein database SWISS-PROT (<http://www.expasy.org/sprot>; Boeckmann et al., 2003; SWISS-PROT-IDs: CAS1_BOVIN, CAS2_BOVIN, CASB_BOVIN, CASK_BOVIN)

%. occurrence of given amino acid per 100 amino acids in protein, F: factor describing the over or under representation of this amino acid in the casein fractions (occurrence of amino acid in given protein divided by the average composition of this amino acid in the complete data base of proteins (SWISS PROT))

The amino acid composition (% and F) were calculated using the program PEPSTATS of the EMBOSS program package (Rice et al., 2000)

The casein fractions contain a large amount of proline inhibiting helix formation because of its ring form (helixbraker). The formation of a secondary and tertiary structure is therefore obstructed and caseins can not be denatured (Holt & Sawyer, 1993; Walstra et al. 1999). Caseins contain a large amount of phosphor (0.9 %), mostly as phosphoserine. These phosphoserine residuals are able to build stable bonds with bivalent ions like Ca^{2+} . All caseins tend to self-associate in solution, also self association of α_{s1} -casein, α_{s2} -casein, β -casein and κ -casein is possible (Walstra et al., 1999; Belitz et al., 2001; Rollema & de Kruif, 2003).

α_{s1} -casein

The α_{s1} -casein fraction consists of five genetic variants (A, B, C, D, E). The most frequently found variant B has a peptide chain with 199 amino acids and a molecular weight of 23,600. The polypeptide chain of α_{s1} -casein consists of two predominantly hydrophobic regions (residues 1-44 and 90-199) and a highly charged polar zone (45-89). The amino acid proline is equally dispersed along the peptide chain and inhibits the formation of larger regular secondary structure elements. α_{s1} -casein builds an insoluble calcium salt in the presence of calcium concentrations typically found in milk.

α_{s2} -casein

The α_{s2} -casein fraction has a peptide chain with 207 amino acids and a molecular weight of 25,200. α_{s2} -casein contains two cysteine residues wherefore disulfide bonds can be formed. α_{s2} -casein is more sensitive to precipitation by Ca^{2+} than α_{s1} -casein. α_{s2} -casein has a dipolar structure with a concentration of negative charges near the N-terminus and positive charges near the C-terminus.

β -casein

From the seven genetic variants (A^1 , A^2 , A^3 , B, C, D, E) the variant A^2 is the most frequent one in bovine milk. This variant has a peptide chain of 209 amino acids and a molecular weight of 24,000. β -casein is the most hydrophobic casein. Due to its uncharged essentially hydrophobic tail and its negatively charged head, this casein molecule is acting as an anionic detergent. It contains, like α_{s1} -casein, no cysteine and precipitates in the presence of calcium in milk common concentrations. The calcium salt can be resolubilized at a temperature below 1 °C.

κ -casein

Four genetic variants (A, B, C, D) are known. The variant B is the most frequent variant in bovine milk. It has a peptide chain of 169 amino acids and a molecular weight of 18,000. κ -casein consists of a mixture of trimers or higher polymers presumably held together by intermolecular disulfide bonds. The protein contains a carbohydrate-free major component and six other components differing in the amount of carbohydrates. κ -casein is the only casein fraction being soluble in the presence of calcium in milk concentration and is able to protect the other calcium insoluble fractions by building complexes. This property of κ -casein is very important for the building of casein complexes and casein micelles. κ -casein is hydrolyzed at the Phe(105)-Met(106) bond by the enzyme chymosin. Chymosin cleaves the κ -casein into the hydrophobic para- κ -casein and the hydrophilic caseinomacropptide. This reaction is the initial step of curdling the milk (by rennet) and thus essential for cheese-making.

Interactions between the casein fractions

An important functional property of the caseins is their ability to self associate and to associate with the other casein fractions. For the formation of intra- and inter-molecular interactions between the casein fractions, the amino acid composition of these fractions is important (Table II.2). Disulfide bridges can be formed by thiols and disulfide interchange or by oxidation of thiol groups. This formation can only occur if cysteine is present in the protein structure. Two cysteine groups are located in α_{s2} - and κ -casein. So, these proteins are able to form disulfide bridges. α_{s1} - and β -casein do not contain cysteine.

Electrostatic interactions result from attraction of ionized groups of opposite charge. The terminal carboxyl groups are negatively charged at the physiological pH of milk (≈ 6.7), the terminal amino groups are positively charged. Electrostatic bonds are either influenced by pH or the milieu (e.g. ionic strength). Ionized groups of equal charge repel each other. At the physiological pH of milk, the carboxyl groups of aspartic acid (IEP = 2.8) and glutamic acid (IEP = 3.2) are negatively charged. In contrary, the amino group of lysine (IEP = 9.6), the imidazol ring of histidine (IEP = 7.5) and the guanidine group of arginine (IEP = 10.8) are positively charged. All casein fractions in table II.2 possess these amino acids, and therefore all casein fractions are able to form electrostatic bonds.

For the formation of calcium bridges, proteins must contain carboxyl groups (aspartic acid, glutamic acid) or phosphorylated amino acids (phosphoserine, phosphothreonine, phosphotyrosine), and calcium has to be in the system. All casein fractions contain carboxyl groups and different amounts of phosphoserine. Especially α_{s2} -casein has a high amount of

phosphoserine and is known as the most calcium sensitive fraction of the caseins. In summary, caseins are able to build calcium ion salt bridges.

The association of non polar groups in water induces hydrophobic interactions. When two hydrocarbon groups associate, some of the water molecules which were more ordered around a hydrocarbon group than elsewhere in the solution, are released and become less ordered (entropy increase). This increase of entropy of water induces the formation of hydrophobic bonds. These hydrophobic interactions are especially important for the protein folding. Non-polar groups are ordered inside the structure, polar groups outside of it. Amino acids with non-polar side groups (alanine, valine, leucine, isoleucine, phenylalanine and methionine) are found in high quantity in all casein fractions. Thus hydrophobic interactions can become active in and between all casein fractions.

Hydrogen donors and acceptors can be found in many amino acids; hydrogen donors in serine, threonine und tyrosine, acceptors in methionine, asparagine, glutamine, aspartic acid und glutamic acid. The acid amide group of the main chain (donor: -NH, Acceptor: =O) can contribute to the formation of hydrogen bonds. Thus all caseins contain hydrogen donors and acceptors and hydrogen bonds can be built.

Casein micelles

The major part of casein (90 %) is structured in casein micelles. Casein micelles in skim milk generally have a diameter between 40 and 300 nm. The variation of the casein micelle size depends principally on the protein composition and the proportion of κ -casein. Casein micelles contain inorganic substances, mainly calcium phosphate (8 %). Many models exist about the structure of the casein micelle. The most commonly used one is the submicelles model: casein micelles are aggregates composed of smaller units, the submicelles (10-20 nm) which are bound together by calcium phosphate bonds (Schmidt & Buchheim, 1970; Walstra et al., 1999). Two submicelle types exist: one type essentially contains α_s -casein and β -casein, the other one α_s -casein and κ -casein. The hydrophobic and calcium sensitive fractions α_s -casein and β -casein are situated in the inner of the micelle, the calcium insensitive κ -casein at the surface, protecting the micelles against calcium precipitation. The hydrophilic caseinomacropeptide of the κ -casein is oriented outside the micelles into the surrounding medium as a flexible “hair” and builds a hydrate envelope stabilizing the micelle because of steric and electrostatic repulsions. Aggregation of submicelles would go on until the surface of the micelle was more or less covered with κ -casein. Figure II.1 shows the model of a casein micelle as described by Walstra et al. (1999).

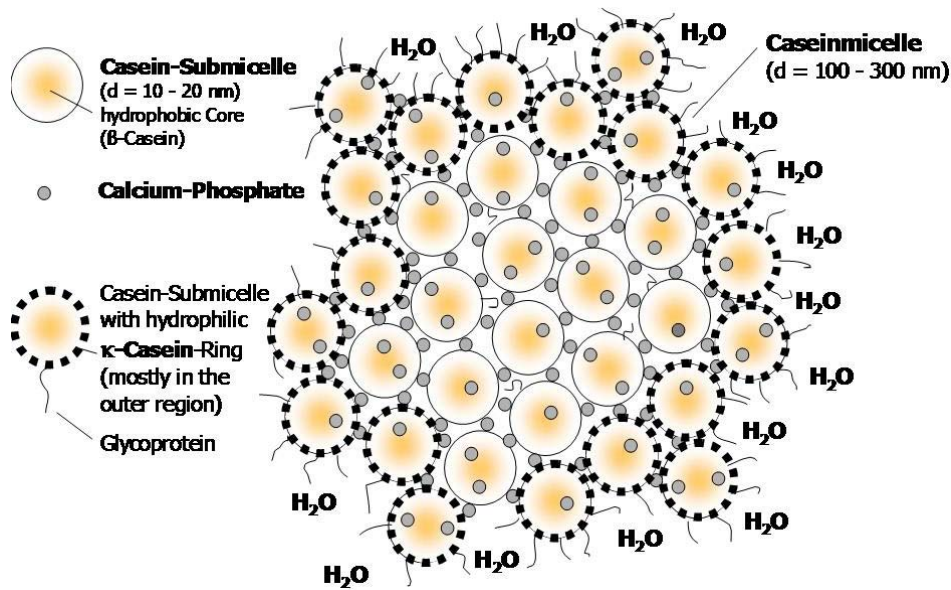


Figure II.1: Schematic model of a cross-section through a casein micelle (according to Walstra et al. 1999; Schmidt & Payens, 1976 and Walstra, 1979)

Intermolecular interactions stabilizing the casein micelles are illustrated in Table II.3. Some authors proposed that the micelles are stabilized essentially by calcium bridges and hydrophobic interactions. Others mention that electrostatic interactions and/or hydrogen bonds are also involved. According to Law et al. (1998), casein monomers aggregate to submicelles due to electrostatic and hydrophobic interactions and the casein submicelles are bound together by calcium bridges to form the casein micelle structure.

Table II.3: Proposed stabilizing interactions in casein micelles

Stabilizing interactions	Sources
CaB, Hy	Schmidt and Buchheim, 1970; Horne, 1998; Haque et al., 2001; Johnston et al., 2002
CaB, Hy, WB	Lucey and Singh, 1998; Horne, 1999
CaB, Hy, EB	Law et al., 1998
CaB, Hy, EB, WB	Walstra and Jenness, 1984; Walstra et al., 1999
CaB, EB, WB	Keim, 2005

EB: electrostatic bonds, CaB: calcium bridge, Hy: hydrophobic bonds, WB: hydrogen bonds

By applying different buffer systems, Keim (2005) measured that calcium bonds and the sum of electrostatic interactions, hydrogen bonds and non bound proteins dominated in a casein concentrate with 15% casein content (Table II.4).

Table II.4: Stabilizing bonds in casein concentrate with 15 % casein content (Keim, 2005)

	Stabilizing bonds [%] Mean \pm CI 99
SS	0.9 \pm 4.8
Hy	1.3 \pm 8.0
CaB	36.6 \pm 4.8
EB+WB+nb	60.3 \pm 7.4
EB+WB	n.a.
nb	n.a.
Total	99.1 \pm 4.8
i	8

SS: disulfide bridges, EB: electrostatic bonds, CaB: calcium bridge, Hy: hydrophobic bonds, WB: hydrogen bonds, nb: non bond proteins; n.a.: non analyzed because liquid sample; i: number of independent experiments; CI: confidence interval

A casein micelle and its surroundings keep exchanging components. The principal exchanges occur between:

- casein molecules and submicelles
- submicelles and colloidal calcium phosphate
- submicelles and micelles

The stability of casein micelles is affected by modifications of external conditions like temperature, pH and proportion of calcium, but also high pressure, modifications which can lead to aggregation of the micelles. The aggregation theory is discussed later in this chapter. The various causes for the aggregation of casein micelles are reported in Table II.5.

Table II.5: Various causes for the aggregation of casein micelles (Walstra et al., 1999)

Cause	Micelles changed?	Aggregation reversible?	Aggregation at low temperatures?
Long storage (age gelation)	Yes	No	No
At air-water interface	Spreading	No	No
High temperature (heat coagulation)	Chemically	No	-
Acid to pH \approx 4.6	No CCP left	(Yes) ¹	No
Ethanol	Presumably	No	?
Renneting	κ -casein split	No	No
Excess Ca ²⁺	More CCP	Yes	?
Freezing plus thawing	Presumably	(Yes) ²	-
Addition of some polymers	No	Mostly	Yes

¹ At neutral pH, the aggregates dissolve again but the natural micelles do not reappear

² Partly, depending on conditions

CCP: colloidal calcium phosphate

Not reported by Walstra et al. (1999) is the sensitivity of the casein micelles under high pressure.

Influence of high pressure on casein

High pressure treatment can be applied in food processing for different objectives: to destroy microorganisms, to inactivate enzymes or to modify proteins. Pressure effects are governed by Le Chatelier's principle, which states that at equilibrium a system tends to minimize the effect of any external factor by which it is perturbed. Thus, reactions that result in reduced volume will be promoted under high pressure whereas those associated with a volume increase are retarded (Masson, 1992; Mozhaev et al., 1996). Covalent bonds are largely insensitive to pressure treatment up to 1000 MPa. The main targets of pressure treatment are electrostatic and hydrophobic interactions which are accompanied by a positive reaction volume (+10 to +20 ml mol⁻¹) and therefore weakened by high pressure. Hydrogen bond formation is almost pressure insensitive (-4 to 1 ml mol⁻¹) (Masson, 1992).

Pressure treatment can cause substantial modification of the casein micelles. High pressure parameters (pressure level, holding time, release rate, temperature) and milieu conditions (casein and calcium content, pH) influence dissociation and re-association of casein and induce the formation of new casein structures.

Pressure level

Casein micelles dissociate into smaller particles called submicelles during the pressure build-up phase of a pressure treatment up to 400 MPa (Schmidt & Buchheim, 1970; Schrader & Buchheim, 1998; Needs et al., 2000; Regnault et al., 2004). This pressure-induced casein micelle dissociation was explained by the weakening of the hydrophobic and electrostatic interactions between the submicelles (Mozhaev et al., 1996) due to their positive reaction volume (+10 to +20 ml mol⁻¹), and by the solubilisation of colloidal calcium phosphate (CCP) out of the micellar framework (Shibauchi et al., 1992; Lee et al., 1996; Schrader et al., 1997). An increase of the casein micelles size at pressures of 200 to 250 MPa (Gaucheron et al., 1997; Huppertz et al., 2004) followed by a decrease of the micelles size up to 400 MPa (Desobry-Banon et al., 1994; Gaucheron et al., 1997; Needs et al., 2000; Huppertz et al., 2004; Regnault et al., 2004) was observed by photon correlation spectroscopy (PCS), laser granulometer studies and transmission electron microscopy.

Pressure release rate

Keenan et al. noted in 2003 that the gelling process in concentrated milk occurred during decompression. Fertsch et al. (2003) demonstrated that the pressure release rate significantly influences the structure formation of pressure-induced 15 % casein gels. The higher the pressure release rate, the firmer and more homogeneous the structures after high pressure treatment (Table II.6 and Figure II.2).

Table II.6: Median of the gel firmness of the pressure induced micellar casein (mC) gels with a protein content of 15 % (number of independent experiments n=3) (Fertsch et al, 2003)

Build-up rate / holding time / release rate in min	mC gel \pm s.d. in N
3/0/1	0.14 \pm 0.14
3/15/1	0.70 ^a \pm 0.11
3/30/1	0.73 ^a \pm 0.20
3/0/3	0.08 \pm 0.05
3/15/3	0.41 ^b \pm 0.07
3/30/3	0.54 ^b \pm 0.23
3/0/30	0.12 ^c \pm 0.03
3/15/30	0.31 ^c \pm 0.10
3/30/30	0.21 ^c \pm 0.12

s.d.: standard deviation; a, b, c: values with the same index do not differ significantly with $p < 0.05$

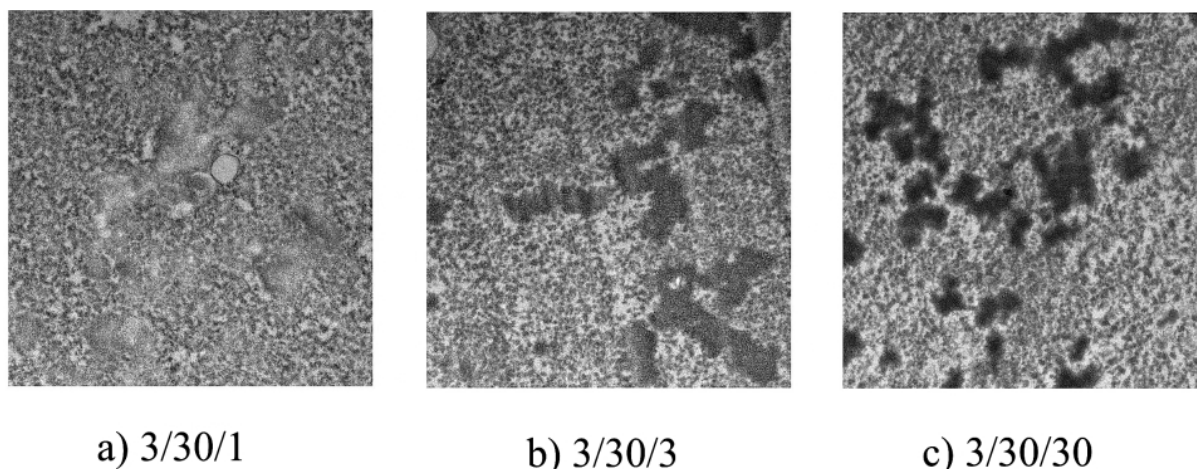


Figure II.2: Electron micrographs (image 3030 X 3030 nm) of pressure-induced 15 % casein gels (600 MPa) as a function of the pressure release rate. The name of the samples consists of the time in min for pressure increase / holding time / pressure release (Fertsch et al., 2003)

During pressure release, the non covalent interactions weakened by high pressure are reactivated (Suzuki & Taniguchi, 1972; Hinrichs, 2000) and calcium bonds are rebuilt (Shibauchi et al., 1992; López-Fandiño et al. 1998). The casein submicelles aggregate to form new microstructures (Ohmiya et al., 1989; Johnston et al. 1992a; Johnston et al. 1992b; Masson, 1992; Hinrichs, 2000; Johnston et al., 2002; Fertsch et al., 2003).

Pressure holding time

As essentially non covalent bonds stabilize the structure of casein micelles it can be assumed that the high pressure-induced reformation of these bonds occurs during pressure release and that pressure holding time has no influence. However, Fertsch et al. (2003) observed that the firmness of 15 % casein gels was higher when a holding time of 15 min was applied than without holding time at 600 MPa (Table II.4). They assumed that the dissociation of the micelles may not be completed when pressure is not hold for a certain time. Therefore, association initiated during pressure release leads to inhomogeneous gelling since intact micelles or bigger fragments are present. The texture of the gels appears very soft. Furthermore, an increase of the casein micelles size was observed at 200 MPa (Anema et al., 2005) and 250 MPa (Huppertz et al., 2004) by increasing the treatment time from 5 to 60 min.

Temperature

Gaucheron et al. (1997) observed that increasing the temperature from 4 to 40 °C during a pressure treatment at 250 MPa increased the casein particle size (252 nm at 40 °C compared to 184 nm at 20 °C, 133 nm at 4 °C and 190 nm for control). No influence was observed at treatment at 450 and 600 MPa. Garcia-Risco et al. (2000) and Huppertz et al. (2004) showed the presence of large casein aggregates for milk treated at pressures between 250 and 400 MPa and temperatures from 40 to 60 °C. Anema et al. (2005) noticed that increases in temperature promoted the aggregation reactions of casein micelles. Needs et al. (2000) and Anema et al. (2005) suggested that the hydrophobic interactions, which increase with rising temperature, may be involved in the reassociation of casein micelles.

Casein content

The pressure-induced structure of the casein micelles is affected by protein concentration and firm gels were obtained at a protein concentration of about 10 % (Velez-Ruiz et al., 1998; Hinrichs, 2000; Fertsch et al., 2003). According to Snoeren et al. (1982) the influence of casein content on gel formation is due to the inner friction of the dispersed particles with the outer phase and to the high water binding capacity of the casein.

Calcium content

Calcium phosphate is essential for the stability of casein micelles. Micelles dissociate into smaller units after treatment with calcium binding substances (e.g. sodium citrat, EDTA, Oxalate) or dialyze against calcium-free solution. Shibauchi et al. (1992) and Lee et al. (1996)

showed that the dissociation of casein micelles during high pressure treatment is accompanied by an increase in the levels of soluble calcium and phosphate. Lee et al. (1996) also showed that the resistance to pressure-induced solubilisation of colloidal calcium phosphate out of the micellar framework is increased by soluble calcium.

Aggregation and gelation

Kinetics of Aggregation

When colloidal particles meet each other, aggregation can occur due to Brownian motion (perikinetic aggregation) or to velocity gradient (orthokinetic aggregation). Colloidal interaction forces are responsible for aggregation.

Perikinetic fast aggregation

In his theory, Smoluchowski (1916, cited by Walstra, 2003) assumes that colloidal particles stick and remain aggregated when encountering each other due to the Brownian motion. Smoluchowski defined the number J_{peri} of encounters per unit volume and time (flux or aggregation rate) [s^{-1}] for equal-sized spheres to be:

$$J_{peri} = 4\pi D_m r N \quad (II.1)$$

where N is the number of particles per unit volume (or particle number concentration) [m^{-3}], D_m the mutual diffusion coefficient of two particles (for a sphere equal the sum of the diffusion coefficient of both spheres) [$m^2 s^{-1}$] and r the collision radius (for a sphere equal the sum of the radii of both spheres) [m].

The diffusion coefficient D is given by the Stokes-Einstein equation:

$$D = \frac{k_B \cdot T}{3 \cdot \pi \cdot \eta_0 \cdot d} \quad (II.2)$$

where d is the diameter of the particle [m], k_B the Boltzmann constant [$J K^{-1}$], T the absolute temperature [K], η_0 the viscosity of the continuous phase [$Pa \cdot s$] and D the diffusion coefficient [$m^2 s^{-1}$].

For an equal sphere equation II.1 is transformed:

$$J_{peri} = \frac{8k_B T}{3\eta_0} N \quad (II.3)$$

where η_0 is the viscosity of the continuous phase [$Pa \cdot s$], k_B the Boltzmann constant [$J K^{-1}$] and T the temperature absolute [K].

With the assumption that each collision of two particles reduces the particle number by unity, the change in number of particles per unit volume with time is obtained by:

$$-\frac{dN}{dt} = \frac{1}{2} J_{peri} N = \frac{4k_B T}{3\eta_0} N^2 \quad (\text{II.4})$$

During the aggregation process, N keeps decreasing, the aggregate size increasing and therefore the aggregation rate decreases.

The time needed for halving the number of particles is given by:

$$t_{0.5} = \frac{2}{J_0} = \frac{3\eta_0}{4k_B T N_0} = \frac{\pi d_0^3 \eta_0}{8k_B T \varphi} \quad (\text{II.5})$$

where $J_{0,peri}$, N_0 and d_0 are the initial values of J_{peri} , N and d and φ the volume fraction of the particles defined as:

$$\varphi = \frac{\pi d^3 N}{6} \quad (\text{II.6})$$

The number of particles as a function of aggregation time is described by the following equation:

$$N_t = \frac{N_0}{1 + \frac{t}{t_{0.5}}} \quad (\text{II.7})$$

For the case of aggregation by encountering doublets or triplets particles, equation II.8 will be used:

$$\frac{N_k}{N_0} = \frac{\left(\frac{t}{t_{0.5}}\right)^{k-1}}{\left(1 + \frac{t}{t_{0.5}}\right)^{k+1}} \quad (\text{II.8})$$

Figure II.3 illustrates this time dependent aggregation process.

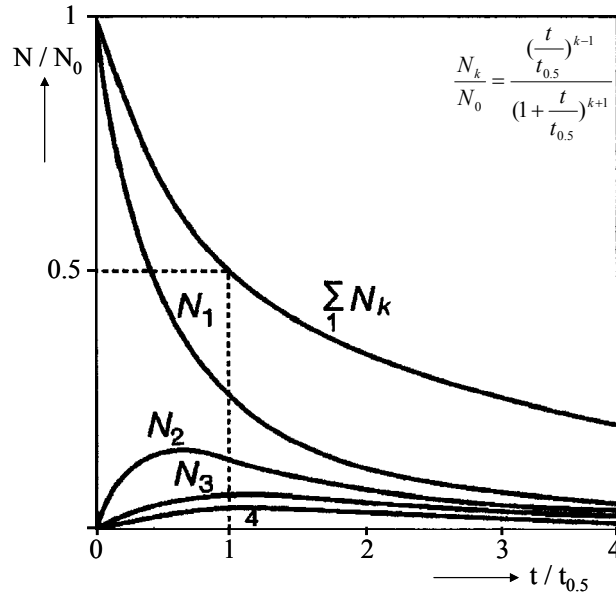


Figure II.3: Modification of the particles composition in a dispersion during a perikinetic aggregation (Walstra, 2003)

The presented theory of Smoluchowski describes the aggregation process of colloidal solution under following conditions:

- The solution contains N_0 particles at t_0 . The particles are spherical, have the same size and have the same aggregation readiness at every time.
- Each collision between two particles implies a fast aggregation and a durable contact.
- The new formed spherical particles from two aggregated particles with the radius r have the radius $2r$.
- The aggregation time is independent of the number of non aggregated particles.

For dispersions not following one or more of these conditions, the theory can be used for the first few aggregation steps, but correction factors have to be considered.

Orthokinetic fast aggregation

During an orthokinetic aggregation, particles aggregate due to velocity gradients in the liquid. A theory of Smoluchowski for the case of simple shear flow assumes that particles will stick and remain aggregated when encountering each other. Assuming spheres of equal size, he defined the particle number concentration N as:

$$-\frac{dN}{dt} = \frac{1}{2} J_{ortho} N = \frac{2}{3} d^3 N^2 \dot{\gamma} = \frac{4}{\pi} \phi N \dot{\gamma} \quad (\text{II.9})$$

where $\dot{\gamma}$ is the velocity gradient (here equal the shear rate) [s^{-1}].

The time needed for halving the number of particles is given by:

$$t_{0.5} = \frac{\pi \ln 2}{4\varphi\dot{\gamma}} \quad (\text{II.10})$$

The ratio of the initial rates of orthokinetic over perikinetic aggregation is given by:

$$\frac{J_{0,ortho}}{J_{0,peri}} = \frac{d^3 \eta_0 \dot{\gamma}}{2k_B T} \quad (\text{II.11})$$

If the particles are large (above 1 μm), orthokinetic aggregation tends to be faster than perikinetic. One reason is the d^3 in the numerator of Equation II.11. A second reason is the small velocity gradients being induced by temperature fluctuations and leading to significant orthokinetic aggregation for particles above 4 μm .

In practice, slow aggregation often prevails mostly due to electrostatic repulsions, hydrodynamic interaction, disaggregation of particle doublets, high volume fractions, sedimentation of the particles or anisometric particles. For all these cases, a capture efficiency (probability that two particles stick upon closely encountering each other) has to be determined.

Fractal aggregation

A fractal is an irregular geometric object that is self-similar to its substructure at any level of refinement. When particles bond together during perikinetic aggregation and stay in the same relative position as during bond formation, fractal aggregates are formed. Fractal aggregates are scale-invariant because of the repetition of the same type of structure in the whole aggregate. A simple equation describes fractal formation:

$$N_p = \left(\frac{r_a}{r_p} \right)^{D_f} \quad (\text{II.12})$$

where N_p is the number of particles in the aggregate, r_a the radius of the aggregate [m], r_p the particle radius [m] and D_f the fractal dimensionality.

The fractal dimensionality is a measure of the irregularity of the boundary of the aggregate.

The number of particles in a sphere of radius R if closely packed is:

$$N_s = \left(\frac{r_a}{r_p} \right)^3 \quad (\text{II.13})$$

and the volume fraction of the particles in this spherical fractal aggregate:

$$\varphi_A = \frac{N_p}{N_s} = \left(\frac{r_a}{r_p} \right)^{D_f-3} \quad (\text{II.14})$$

In a three dimensional space, the fractal dimensionality is always smaller than 3. It means that the volume fraction of particles decreases with increasing size of the aggregate and the amount of water bound to the particles increases. The fractal aggregate thus grows until the volume fraction of particles in the aggregate equals the volume fraction of particles in the liquid. The system is then packed with aggregates inducing the formation of a particle gel.

Casein micelles essentially encounter each other because of Brownian motion. Their aggregation follows the theory of perikinetic aggregation of Smoluchowski and the aggregates have a fractal nature. Bremer et al. (1989) studied the fractal structure of casein gels formed of casein micelles aggregates. They showed that acid casein gels can be described as formed from fractal aggregates with a fractal dimension of 2.3. The aggregation of casein micelles is illustrated by Walstra et al. (1999):

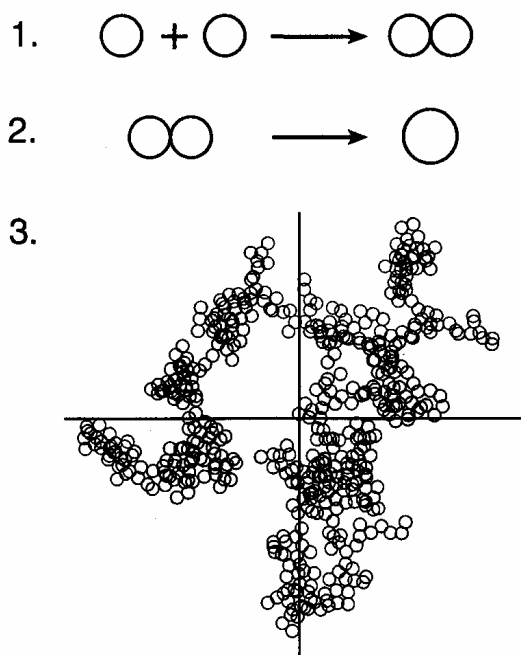


Figure II.4: Aggregation of casein micelles. 1: Aggregation reaction, 2: Fusion of two micelles, 3: Example of an aggregate of micelles formed during ongoing aggregation (Walstra et al., 1999)

The fusion of two casein micelles (reaction 2. in Figure II.4) is the same reaction as the fusion of submicelles to form micelles. It is a low reaction compared to with the one of aggregation but it can be accelerated for example by the cleaving of κ -casein. If the fusion is faster than the aggregation, large and dense particles are formed and no gelation occurs.

High pressure-induced gelation of casein

According to Aguilera & Stanley (1999), a gel is a three-dimensional network formed by the association or cross-linking of long polymeric molecules, which entraps and immobilizes the liquid solvent forming a rigid structure. A classification of gels related to their formation mechanisms was done by Djabourov (1991). Cross-linked or fishing nets are chemical gels built from linear flexible chains linked by covalent bonds (e. g. acrylamide gels). Thermoreversible physical gels are formed by partial crystallization of chains or by conformational coil-to-helix transitions. Depending on the temperature, the structure can switch from sol to gel. Soft and highly deformable gels like gelatin gels but also hard and brittle gels like agarose gels belong to the thermoreversible ones. Junction zones linked by ionic complexation with a divalent cation (like Ca^{2+}) bridging two strands of the polymer form an egg-box structure (e. g. alginate and pectin gels). A cluster arrangement of more or less spherical particles forms a particle or colloidal gel (casein and whey proteins). The junction zones and supramolecular structures of some gels are illustrated by Aguilera & Stanley, 1999 (Figure II.5).

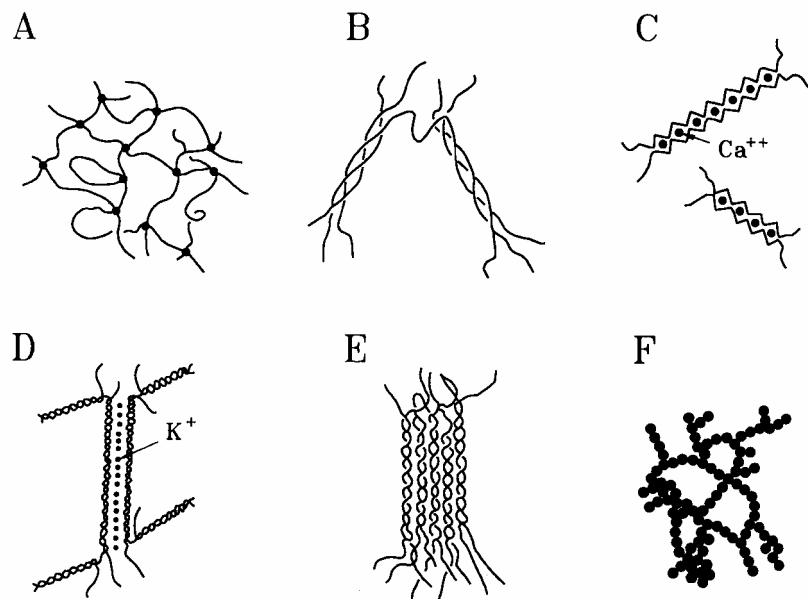


Figure II.5: Schematic representation of some supramolecular structures of pure gels. (A) Cross-linked or fishing net type (chemical gel). (B) Triple-helices of gelatin gels. (C) Egg-box structures of pectin and alginate gels in the presence of calcium ions. (D) Aggregated domains after carrageenan gelation. (E) Bundles of double helices agarose gels. (F) Particulate gels formed by globular proteins (like casein and whey protein). Not at the same scale (Aguilera & Stanley, 1999)

Casein forms particle gels. Casein gelation occurs because of the destabilization of the casein micelles and can be induced by different processes like temperature treatment, high pressure treatment or renneting (cleaving of the κ -casein).

Keim (2005) showed that gels can be induced by high pressure at a casein concentration of about 12 % at a pressure of 600 MPa. She also measured the stabilizing bonds in casein concentrate and pressure-induced casein gels with 15 % casein content (Table II.7).

Table II.7: Stabilizing bonds in casein concentrate and in pressure-induced (600 MPa, 30 °C, 30 min, pH 6.5) casein gels with 15 % casein content (Keim, 2005)

	Bonds in casein concentrate [%] Mean \pm CI 99	Bonds in pressure-induced casein gels [%] Mean \pm CI 99
SS	0.9 \pm 4.8	-3.5 \pm 3.8
Hy	1.3 \pm 8.0	1.5 \pm 8.0
CaB	36.6 \pm 4.8	35.5 \pm 5.3
EB+WB+nb	60.3 \pm 7.4	63.0 \pm 8.8
EB+WB	n.a.	63.0 \pm 8.8
nb	n.a.	0.0 \pm 0.0
Total	99.1 \pm 4.8	96.5 \pm 4.0
i	8	4

SS: disulfide bridges, EB: electrostatic bonds, CaB: calcium bridge, Hy: hydrophobic bonds, WB: hydrogen bonds, nb: non bond proteins; n.a.: non analyzed because liquid sample; i = number of independent experiments; CI: confidence interval.

Calcium bonds and the sum of electrostatic interactions, hydrogen bonds and non-bound proteins dominated in the non treated casein concentrate but also in the pressure-induced casein gel. Although the texture changed from sol (liquid) to gel (solid), no difference in the sum of the stabilizing bonds was determined.

In addition, the firmness of high pressure-induced gels depends on various factors besides protein concentration. For example, Fertsch et al. (2003) found that the higher the pressure release rate, the firmer and more homogeneous the gels structures after high pressure treatment for a casein solution with 15 % casein.

At the beginning of our work, we took the following initial hypothesis: casein micelles dissociate during pressure build-up phase and holding phase because of the weakening of non-covalent bonds. After a certain holding time, all micelles are dissociated in subunits. The longer the holding phase, the smaller the units being present. A certain holding time is required for the complete dissociation of the micelles, but this holding time should not be too

long to avoid the re-association of the submicelles due to hydrogen bonds. During pressure release, the non-covalent bonds are reactivated and depending on the release rate structures with different structures can be induced. This model is illustrated in Figure II.6.

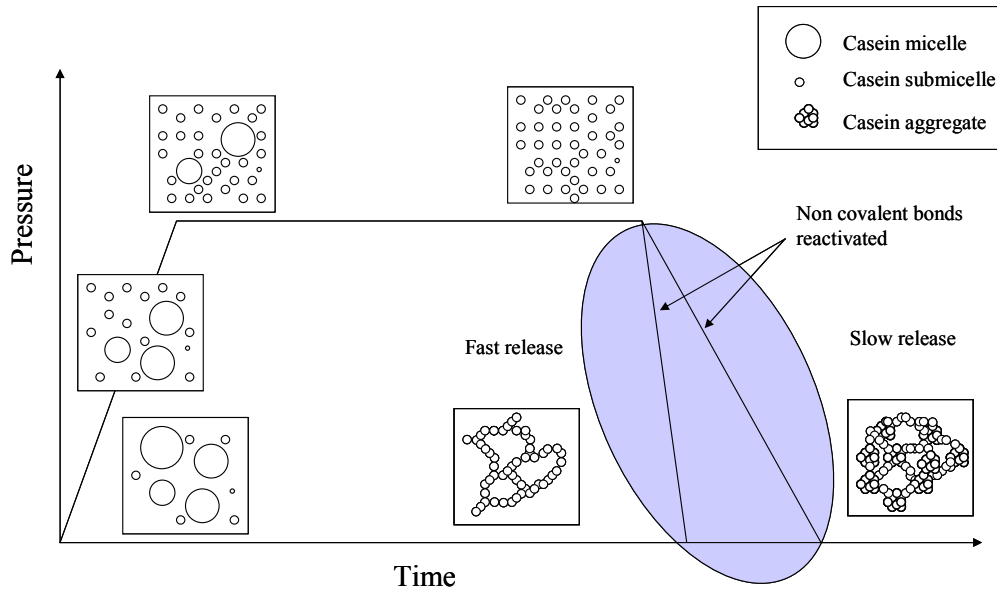


Figure II.6: Model of the dissociation and aggregation of casein micelles during a high pressure treatment of a casein solution

Characterization of structural modifications

Principle of rheology

Rheology is defined as the study of the deformation and flow of matter. Rheology studies the relations between the stress (defined as the force divided by the area over which the stress is acting) acting on a material, its relative deformation also called strain (deformation or change in distance divided by the original distance) and the shear rate involved.

Viscosity

When a stress is applied to a fluid, it will flow. Viscosity is the measure of the internal friction of a liquid or its tendency to resist flow. The viscosity of liquids generally increases with molar mass and decreases with increasing temperature.

The dynamic viscosity η , frequently called “viscosity” or “absolute viscosity”, is defined by equation:

$$\eta = \frac{\tau}{\dot{\gamma}} \quad (\text{II.15})$$

where τ is the shear stress [Pa] and $\dot{\gamma}$ the shear rate [s^{-1}].

Shear stress is the stress component applied tangential to the plane on which the force acts, shear rate the velocity gradient established in a fluid as a result of an applied stress.

For a so-called Newtonian liquid, the shear stress is proportional to the shear rate at constant temperature and so the viscosity does not depend on the stress or shear rate applied within a laminar flow range. The viscosity is given by the slope of the shear stress-shear rate curve, called flow curve. Many liquids are not Newtonian. For these liquids, the ratio of shear stress over shear rate is called the apparent viscosity. The apparent viscosity will change depending on shear rate and temperature.

In a rotational viscometer with cone-plate geometry, the fluid is held by its own surface tension between a cone of small angle that just touches a flat surface. The torque caused by the drag of the fluid on the cone is measured as the cone geometry is rotated while the plate remains stationary. For Newtonian fluids, the following equation applies for viscosity:

$$\eta = \frac{3\alpha M}{2\pi r^3 \Omega} \quad (\text{II.16})$$

where α is the angle of cone [rad], M the torque [Nm], r the radius of the cone [m], and Ω the angular velocity of the rotating cone geometry [rad s^{-1}].

Dynamic viscosity can also be determined using a falling-ball viscometer. The viscosity is calculated by the following equation:

$$\eta = K(\rho_B - \rho_S)\Delta t \quad (\text{II.17})$$

where K represents a calibration constant depending on pressure and viscosity of the investigated medium at ambient pressure, ρ_B the density of the ball [kg m^{-3}] and ρ_S the density of the liquid sample [kg m^{-3}].

Kinematic viscosity ν is defined as the dynamic viscosity η divided by the density of the fluid ρ :

$$\nu = \frac{\eta}{\rho} \quad (\text{II.18})$$

where ρ is the density of the solution [kg m^{-3}].

Kinematic viscosity can be determined using a capillary viscometer where the time for a standard volume of fluid to pass through capillary tube is measured. Kinematic viscosity is obtained by multiplying the measured time by the instrument conversion factor K :

$$\nu = Kt \quad (\text{II.19})$$

where K is the conversion factor and t the time measured [s].

$$K = \frac{\pi h g r^4}{8Vl} \quad (\text{II.20})$$

where h is the mean head [m], g the acceleration of gravity [m s^{-2}], r the radius of capillary [m], V the volume of flow [m^3] and l the length of capillary [m].

Voluminosity

An important characteristic of proteins is their degree of hydration, defined as the number of grams of water bound per gram of protein. A common way to measure the hydration is to measure the voluminosity, defined as the volume of solution occupied by one gram of dry micelle material. The voluminosity can be determined using viscosity measurements and various relations exist to connect viscosity measurements with voluminosity.

For very dilute dispersions of solid spherical particles, Einstein derived the following relation:

$$\eta = \eta_0(1 + 2.5\varphi) \quad (\text{II.21})$$

where η is the dynamic viscosity of the solution [$\text{Pa}\cdot\text{s}$], η_0 the viscosity of the solvent [$\text{Pa}\cdot\text{s}$] and φ the volume fraction of the particles.

For the viscosity of less dilute systems, Eilers' equation (eq. II.22) (Eilers, 1941; Eilers, 1943) and the model by Gleissle & Baloch and Windhab (eq II.23) (cited by Windhab, 1986) are used. Both models assume solid spherical particles and monodispersity.

$$\frac{\eta}{\eta_0} = \left(1 + \frac{1.25\varphi}{1 - \frac{\varphi}{\varphi_{\max}}} \right)^2 \quad (\text{II.22})$$

where φ is the volume concentration occupied by the hydrated particles and φ_{\max} the maximum volume concentration occupied by the hydrated particles in the solution.

The model by Gleissle & Baloch and Windhab describes the viscous behaviour of suspensions based on the concentration of suspended particles and can be applied for volume concentrations up to φ_{\max} .

$$\frac{\eta}{\eta_0} = \left(1 - \frac{\varphi}{\varphi_{\max}} \right)^{-1} \quad (\text{II.23})$$

In a study regarding shear stability of fat globules, Hinrichs & Kessler (1997) found that the particles in laminar shear condition are already coming into contact with each other at volume concentration of $\phi_{\max} \sim 0.4$.

The voluminosity V_a is related to the volume fraction with the equation $\phi = c \cdot V_a$ where c is the concentration of the particles [g ml^{-1}].

Assuming that $\phi_{\max} = 0.4$, eqs (II.22) and (II.23) become:

$$\frac{\eta}{\eta_0} = \left(\frac{1 - 1.25c \cdot V_a}{1 - 2.5c \cdot V_a} \right)^2 \quad (\text{II.24})$$

$$\frac{\eta}{\eta_0} = (1 - 2.5c \cdot V_a)^{-1} \quad (\text{II.25})$$

The voluminosity can be determined by measuring the viscosity of the same sample at different concentrations.

Viscoelasticity and dynamic measurements

A substance is called viscoelastic if after exerting a stress on it, it deforms at first elastically, then starts to flow and upon release of the stress it regains part of the original shape. A substance is defined as ideal elastic if after an external action of a strain the substance comes back in its original state. If the material is purely elastic, the resulting shear stress is always proportional to the strain and the ratio shear stress/strain is called the elastic or storage modulus G' . It is the measure of the mechanical energy stored during the deformation. For viscoelastic materials, the shear deformation is not reversible and the material stays deformed after action of a strain. This deformation energy describing the viscous compartment of a substance is defined as loss modulus G'' . The loss tangent, $\tan \delta$, is a measure of the nature of the material:

$$\tan \delta = \frac{G''}{G'} \quad (\text{II.26})$$

The loss angle is defined as:

$$\delta = \text{Arc tan} \frac{G''}{G'} \quad (\text{II.27})$$

The loss angle δ ($0^\circ \leq \delta \leq 90^\circ$) is a measure of the (visco) elasticity of the sample. The smaller the loss angle, the more elastic is the sample.

Viscoelastic values are resumed by Keim (2005):

Table II.8: Viscoelastic comportment, models and examples (Keim, 2005)

Effect	G', G''	δ	Models	Examples
Ideal elastic deformation	$G'' = 0$	$\delta = 0^\circ$	Hooke (spring)	Strong networked polymer
Viscoelastic solid substance, gel	$G' > G''$	$0^\circ < \delta < 45^\circ$	Kelvin / Voigt (spring + dampers, in parallel)	Polymer, protein gel
Viscous and elastic part equal	$G' = G''$	$\delta = 45^\circ$		Sol-Gel-Transition, Gel point by gelation
Viscoelastic liquid substance	$G' < G''$	$45^\circ < \delta < 90^\circ$	Maxwell (spring + dampers in series)	Protein concentrate, blood
Ideal viscous flowing	$G' = 0$	$\delta = 90^\circ$	Newton (dampers)	water

A gel is a viscoelastic material for which G' is greater than G'' .

Principle of the photon correlation spectroscopy

Photon correlation spectroscopy is a dynamic scattered light method used to measure particles with a size between 5 nm and 5 μm . Due to the Brownian molecular motion, molecules are always in motion bringing time dependent variation of the scattered light. A big particle diffuses at a slower rate than a small one. The measured time dependant intensity differences are therefore more pronounced for small particles than for large one (Tabatt, 2003; Walstra, 2003).

The time dependant variation of the scattered light is determined with a mathematic method, the autocorrelation. The measure autocorrelation function can be adapted to a theoretical correlation function (Müller & Schuhmann, 1996):

$$g(\tau) = e^{-2 \cdot D \cdot K_{\text{vector}}^2 \cdot t_d} \quad (\text{II.28})$$

where D is the diffusion coefficient [$\text{m}^2 \text{s}^{-1}$], K_{vector} the norm of the scattered light vector and t_d the delay time [s].

The norm of the scattered light vector K depends on the refraction index RI , the scattered light angle Θ and the wavelength of the light λ :

$$K = \frac{4 \cdot \pi \cdot RI}{\lambda} \cdot \sin \frac{\Theta}{2} \quad (\text{II.29})$$

The diffusion coefficient D , describing the velocity of the particles, can be calculated using Equation II.14 and II.15.

If the temperature and the viscosity of the solvent are known, the diameter of the particles is determined using the Stokes-Einstein equation (Eq. II.2).

Many theories about the behavior of casein, for example under different temperature conditions, are available but most of them refer to ambient pressure. Pressure represents an additional parameter for experiments with casein. Studies reported so far in the literature about the structure modification of casein under pressure are mostly concentrated on the influence of pressure level. Influence of other important parameters like pressure release rate and *in situ* observations of the modification of the structure are still missing.

As shown in this introduction, high pressure treatment of casein micelles can lead to the formation of casein particles, aggregates or gels. In the following chapters, casein solutions are treated with high pressure under different process and milieu conditions. The obtained structures are analyzed with the different methods presented above for a better understanding of the behavior of casein micelles in the different phases of high pressure treatment.

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Chapter III

Influence of pressure release rate and protein concentration on the formation of pressure-induced casein structures

Summary

The formation of pressure-induced casein structures (600 MPa for 30 min at 30 °C) was investigated for different pressure release rates (20 to 600 MPa min⁻¹) and casein contents (1 to 15 %). Structures from liquid (sol) to solid (gel) were observed. The higher the protein content and the pressure release rate, the more viscous appeared the macrostructures. A firm gel was built up at a casein content of 7 % for a pressure release rate of 600 MPa min⁻¹, while lower release rates resulted in weak gels with a rough microstructure (200 MPa min⁻¹) or liquid structures (20 MPa min⁻¹). In a 5 % casein solution and at a pressure release rate of 600 MPa min⁻¹, casein aggregates built from smaller casein particles with a larger hydrodynamic diameter and higher voluminosity than in the untreated solution are generated. After a slow release rate casein micelles have a smaller hydrodynamic diameter and a lower voluminosity, but are similar in shape and diameter as compared to the micelles in solution before high pressure treatment.

Merel, E., Hinrichs, J. & Kulozik, U. Influence of pressure release rate and protein concentration on the formation of pressure-induced casein structures, *Journal of Dairy Research* (in first revision).

Introduction

High pressure can be applied to modify casein micelles. Parameters like pressure level (Desobry-Banon et al., 1994; Gaucheron et al., 1997; Schrader & Buchheim, 1998; Velez-Ruiz et al., 1998; Needs et al., 2000; García-Risco et al., 2000; Huppertz et al., 2004a; Regnault et al., 2004), holding time (Fertsch et al., 2003; Huppertz et al., 2004a) and temperature (Gaucheron et al., 1997; García-Risco et al., 2000; Huppertz et al., 2004a; Regnault et al., 2004; Huppertz et al., 2004b) as well as the milieu like calcium content (Lee et al., 1996) or pH (Arias et al., 2000; Huppertz et al., 2004a) influence dissociation and reassociation of caseins.

Casein micelles in milk were dissociated in submicelles at a pressure of 400 MPa according to Schmidt & Buchheim (1970), Schrader & Buchheim (1998), Needs et al. (2000) and Regnault et al. (2004). In more detail, several photon correlation spectroscopy (PCS) and laser granulometer studies showed an increase of the casein micelles size at pressures of 200 to 250 MPa (Gaucheron et al., 1997; Huppertz et al., 2004a) followed by a decrease of the micelles size up to 400 MPa (Desobry-Banon et al., 1994; Gaucheron et al., 1997; Needs et al., 2000; Huppertz et al., 2004a; Regnault et al., 2004). The decrease of the micelles size after treatment at 400 MPa and 600 MPa was also confirmed by transmission electron microscopy (Gaucheron et al., 1997; Schrader & Buchheim, 1998; García-Risco et al., 2000; Needs et al., 2000; Keenan et al., 2001). This pressure-induced casein micelle dissociation was explained by the weakening of hydrophobic and electrostatic interactions between the submicelles (Mozhaev et al., 1996) and the solubilisation of colloidal calcium phosphate (CCP) out of the micellar framework (Shibauchi et al., 1992; Lee et al., 1996; Schrader et al., 1997).

The increase in micelle size up to 250 MPa accompanied by an increase of viscosity and hydration was considered to be due to the solubilisation of CCP rendering the casein micelles less compact and due to the formation of casein micelles chains and clusters (Shibauchi et al., 1992; Desobry-Banon et al., 1994; Gaucheron et al., 1997; Walstra, 1990; Huppertz et al., 2004b).

The pressure-induced structure of the casein micelles was also affected by protein concentration and firm gels were obtained after pressure treatment according to the protein content (Velez-Ruiz et al., 1998; Hinrichs, 2000).

Hydrophobic and electrostatic interactions in the casein micelles which are weakened under pressure are reactivated during pressure release (Suzuki & Taniguchi, 1972; Hinrichs, 2000). Keenan et al. (2001) noted that the gelling process in concentrated milk occurred during decompression. Fertsch et al. (2003) showed that a low pressure release rate induced the

formation of a rough and weak casein gel while a homogeneous and firm microstructure was formed at a high release rate. However, only few studies noticed the importance of the pressure release phase on properties of the resulting structures.

The objective of this study was to investigate the influence of casein content and pressure release rate on formation of pressure-induced casein micelles structures in more detail. The sol-gel transition of casein structures were characterised after high pressure treatment at 600 MPa for 30 min at 30°C depending on protein content varied from 1 to 15 % and applying pressure release rates between 20 and 600 MPa min⁻¹. In addition, pressure-induced modifications of the casein structure in the sol phase were described by measuring voluminosity and particle size and by atomic force microscopy.

Material and methods

Sample preparation and high pressure treatment

The experiments were carried out with an enriched micellar casein powder produced by diafiltration of skim milk at the Institute for Food Process Engineering in Freising, Germany (Kersten, 2001). Skim milk was diafiltrated by means of microfiltration (MF) (MF module 7P19-40GL; cut off: 100nm, APV, 8600 Silkeborg, Denmark). The MF-permeate obtained was ultrafiltrated (cut off: 25 kDa, DDS AS, 4900 Nakskov, Denmark) and used for diafiltration. After six washing steps the casein retentate was concentrated by microfiltration (concentration factor 4) and spray dried (Niro Atomizer, 2860 Soeborg, Denmark). The powder contained 6.5 % water, 68.4 % total protein including 68.0 % of casein, 16.6 % lactose and 8.4 % minerals including 2.3 % calcium.

The casein powder was diluted in a reconstituted ultrafiltration permeate obtained from milk (UFP) (Ingredia Dairy Ingredients, 3602 Thun, Switzerland) to adjust the casein content in the sample from 1 to 15 % and mixed for 3h at room temperature. The UFP powder is composed of 4.8 % water, 2.9 % total protein, 84.9 % lactose and 7.4 % minerals including 0.5 % calcium and was rehydrated with distilled water to achieve a total water content of 94.8 %. The protein content was checked with a nitrogen analyzer using Dumas method (LECO FP-528, Leco Instrumente GmbH, 41199 Moenchengladbach, Germany). Prior to high pressure treatment pH was adjusted to pH 6,0 by adding lactic acid 10 % (VWR, 64283 Darmstadt, Germany). The samples were filled into 20 ml HDPE tubes (inner diameter 32 mm, filled height 14 mm) (Nalgene, Novodirect, 77694 Kehl, Germany) and closed with a silicone plug

(VWR, 64283 Darmstadt, Germany). The samples were tempered for more than 5 min at the appropriate temperature of 30 °C before being pressurized.

In the high pressure autoclave (inner volume 125 ml; height 100 mm; diameter 40 mm) (Resato High Pressure Technology, 9301 Roden, The Netherlands) pressure was built up with a rate of 200 MPa min⁻¹ and held constant at 600 MPa for 30 min at 30 °C. A temperature increase of about 2 °C in the autoclave was noticed during the pressure build-up phase. Equilibration took place within about 5 minutes. Pressure release was varied from 20 to 600 MPa min⁻¹. After treatment, the samples were stored overnight at 4 °C before analysis. The following results are the mean of 2 to 3 independent pressure experiments carried out on different days.

Viscosity measurements

Dynamic viscosity

Dynamic viscosity η of the 5 % casein solution was determined before and after high pressure treatment at 10 °C using a rotational rheometer (Advanced Rheometer AR 2000, TA Instruments, 63755 Alzenau, Germany). The shear rate was increased to 500 s⁻¹ in 3 min, then held for 5 min at 500 s⁻¹ and then decreased to 0 s⁻¹ in 3 min. The apparent dynamic viscosity was defined as the viscosity at the end of the 5 min holding time at a shear rate of 500 s⁻¹. It will be called viscosity in this work. 4 to 6 viscosity measurements from the 2 to 3 independent high pressure treatments were carried out.

Kinematic viscosity

Each sample was diluted in five steps in UFP to adjust casein contents from 1 % to 2.5 %. The kinematic viscosity ν of each dilution was determined using a capillary viscometer (Type 53710, VWR, 64283 Darmstadt, Germany). In addition, the density ρ was determined using an oscillating U-tube (DMA 5000, Anton Paar, 73760 Ostfildern, Germany). In both cases, the temperature was set to 10 °C. The dynamic viscosity η was calculated from:

$$\eta = \nu \cdot \rho \tag{III.1}$$

4 to 8 kinematic viscosity measurements from 2 to 3 independent high pressure treatments were carried out for each dilution.

Particle size of the casein micelles in the sol

The mean hydrodynamic diameter d_H of the casein micelles in the untreated and high pressure treated 5 % casein solution was determined by photon correlation spectroscopy (PCS) at 25 °C (HPPS, Malvern Instruments Ltd, Malvern WR14 1XZ Worcestershire, UK). This particle sizer uses back scattering with an angle of 173 ° which also enables to measure in undiluted solutions preventing dissociation of the casein micelles due to dilution effects. Three light scattering measurements of 60 s were carried out for each sample. The results, expressed in nm, are determined from the intensity distribution curves and are the average of 10 to 15 measurements from 2 to 3 independent high pressure treatments.

Atomic force microscopy (AFM)

To visualize the PCS results atomic force microscopy was carried out in analogy to Regnault et al. (2004). A dimension 3100 microscope equipped with the nanoscope IIIa electronic device in the contact mode (Digital Instruments-Veeco, CA 93117 Santa Barbara, USA) was used. The untreated and high pressure treated 5 % casein solutions were diluted 30-fold in milk UFP to avoid dissociation of the casein particles. 8 µl of each dilution were placed on a teflon disk and dried in ambient air for about 30 min. The pictures were analyzed with the Digital Nanoscope Software (version 4.43r2, Digital Instruments-Veeco, CA 93117 Santa Barbara, USA).

Structure diagram

The formation of pressure-induced casein structures depending on pressure release rate and casein content was illustrated in a structure diagram for sol-gel transition. Liquid samples (sol) were characterized means of a flow curve using the same method as for the determination of the dynamic viscosity with the rotational rheometer. The flow index n of the samples was determined from the upward flow curve using Ostwald's equation:

$$\tau = k_{ow} \cdot \dot{\gamma}^n \quad (\text{III.2})$$

τ : shear stress [Pa]; k_{ow} : Ostwald factor; $\dot{\gamma}$: shear rate [s^{-1}]; n : flow index

Samples with a flow index $n \geq 0.7$ were considered to be part of the sol phase. The firmness of gel-like-samples was determined at 4 °C with a texture analyzer (Zwick I, 89079 Ulm, Germany). A cylinder with a 5 mm diameter penetrated the sample with 7 mm depth at a rate of 0.5 mm s^{-1} . The maximum strength was defined as firmness. Samples with a firmness higher than 0.02 N were classified as gel in the structure diagram.

Samples with a very weak gel structure with phase separation which could not be characterized by a flow curve or by the firmness test were located in the transition phase of the structure diagram. Each point in the structure diagram represents the mean value of three independent high-pressure experiments.

Calculation of the voluminosity

The voluminosity V_a of the solution was calculated using Eilers' equation (Eilers, 1941; Eilers, 1943) and the models by Gleissle and Baloch (cited by Windhab, 1986) and Windhab (1986). Both models assume solid spherical particles and monodispersity. For simplification we also followed this assumption for the calculation of the voluminosity in this study.

Eilers' equation connects viscosity measurements with voluminosity when solutions are not too concentrated.

$$\frac{\eta}{\eta_0} = \left(1 + \frac{1.25 \varphi}{1 - \frac{\varphi}{\varphi_{\max}}} \right)^2 \quad (\text{III.3})$$

η : dynamic viscosity of the solution [Pa·s]; η_0 : dynamic viscosity of the outer phase (here UFP) [Pa·s]; φ_{\max} : maximum volume concentration occupied by the hydrated particles in the solution; φ : volume concentration occupied by the hydrated particles

The model by Gleissle & Baloch and Windhab describes the viscous behaviour of suspensions based on the concentration of suspended particles and can be applied for volume concentration up to φ_{\max} .

$$\frac{\eta}{\eta_0} = \left(1 - \frac{\varphi}{\varphi_{\max}} \right)^{-1} \quad (\text{III.4})$$

In a study regarding shear stability of fat globules, Hinrichs & Kessler (1997) found that the particles in laminar shear condition are already coming into contact with each other at volume concentration of $\varphi_{\max} \sim 0.4$. The voluminosity V_a is related to the volume fraction with the equation $\varphi = c_{\text{casein}} \cdot V_a$ where c_{casein} is the casein concentration of the particles [g/ml].

Assuming that $\varphi_{\max} = 0.4$, Eqs (III.3) and (III.4) become

$$\frac{\eta}{\eta_0} = \left(\frac{1 - 1.25 c_{\text{casein}} \cdot V_a}{1 - 2.5 c_{\text{casein}} \cdot V_a} \right)^2 \quad (\text{III.5})$$

$$\frac{\eta}{\eta_0} = (1 - 2.5 c_{\text{casein}} \cdot V_a)^{-1} \quad (\text{III.6})$$

Both equations were applied to calculate voluminosity from viscosity measurements:

i.: direct determination using viscosity data of the 5 g/100ml casein solution (rotational rheometer).

ii.: regression of viscosity data of the dilutions series (capillary viscometer).

Results given represent the average of four to six viscosity measurements from two to three independent high pressure treatments.

Results

Structure diagram for sol-gel transition

Structures from liquid (sol) to solid (gel) were generated depending on casein concentration and pressure release rate. The observed structures are summarized in the structure diagram in Figure III.1.

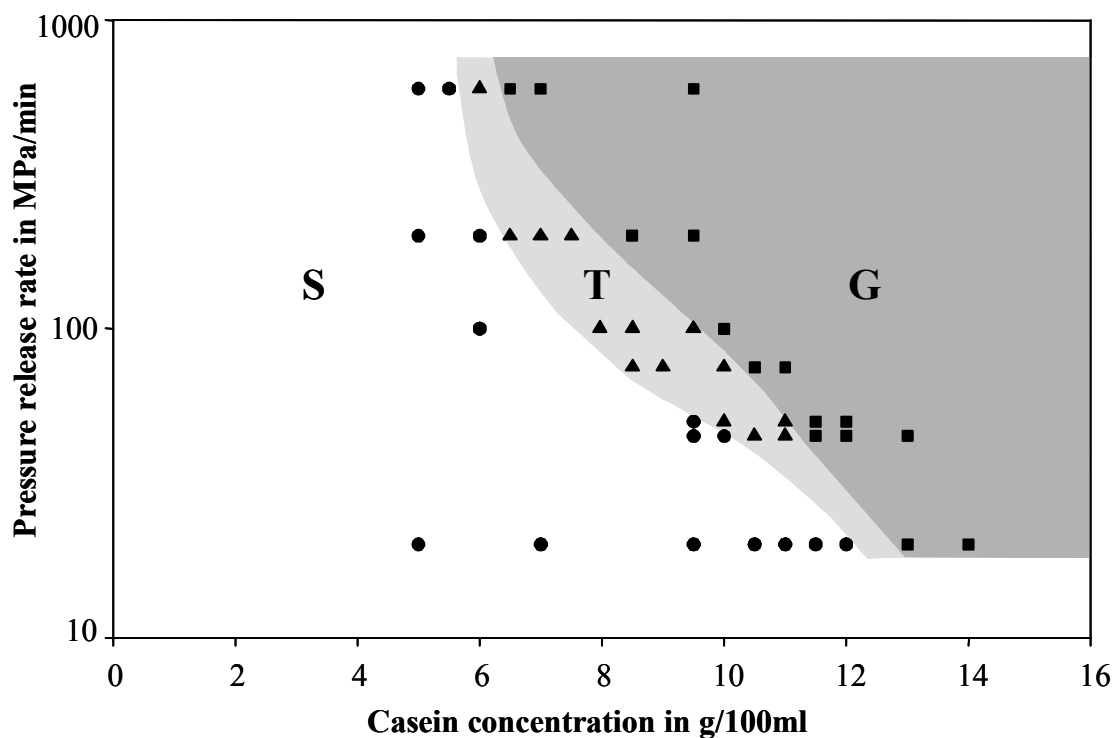


Figure III.1: Structure diagram: Influence of pressure release rate and casein content on the gel-sol-transition of casein structures. S: sol; T: transition; G: gel; ●: indication for sol, ▲: indication for transition, ■: indication for gel

At low casein content ($\leq 5\%$), the structures were still liquid. At increased release rates and higher protein contents the macrostructures appeared to be more viscous. At a slow release rate of 20 MPa min^{-1} a gel was formed at casein content of 13% . Higher pressure release rates with a casein content of 8.5% (200 MPa min^{-1}) and 6.5% (600 MPa min^{-1}) already induced

gel structures. At a casein concentration of 7 % a homogeneous and firm microstructure was built up for a pressure release rate of 600 MPa min⁻¹, while the lower release rates produced weak gels with a rough structure (200 MPa min⁻¹) or liquid structures (20 MPa min⁻¹).

Voluminosity of the casein micelles

Changes in the voluminosity V_a as a function of pressure release rate are shown in Table III.1.

Table III.1: Voluminosity of casein micelles from a 5 % casein solution pressurized at 600 MPa / 30 °C for 30 min

A. Calculated from apparent viscosity data of the rotational viscometer*

Pressure release rate [MPa min ⁻¹]	Voluminosity V_a [ml g ⁻¹]	
	by Eilers' (Eq. III.5)	by Gleissle and Baloch (Eq. III.6)
untreated	4.78 ± 0.09 ^a	5.37 ± 0.10 ^a
600	5.19 ± 0.01 ^b	5.84 ± 0.01 ^b
200	4.42 ± 0.13 ^c	4.95 ± 0.15 ^c
20	3.35 ± 0.31 ^d	3.69 ± 0.36 ^d

* Data within a column followed by different letters are significantly different at $p < 0.05$

B. Calculated from viscosity data of the capillary viscometer

Pressure release rate [MPa min ⁻¹]	Voluminosity V_a [ml g ⁻¹]	
	by Eilers' (Eq. III.5)	by Gleissle and Baloch (Eq. III.6)
untreated	6.06 ± 0.25	6.55 ± 0.29
600	7.32 ± 0.23	8.01 ± 0.22
200	6.09 ± 0.24	6.61 ± 0.26
20	4.82 ± 0.21	5.15 ± 0.22

The influence of the pressure release rate on voluminosity is clearly shown for both calculations based on Eilers (Eq. III.5) and Gleissle & Baloch (Eq. III.6): the higher the pressure release rate, the higher the voluminosity of the micelles. Table III.1A illustrates that the voluminosity (calculated from viscosity data of the rotational viscometer) after a pressure release rate of 600 MPa min⁻¹ was significantly ($p < 0.05$) higher than the one of the untreated solution. After treatment with a release rate of 200 MPa min⁻¹ and 20 MPa min⁻¹ the voluminosity was significantly lower than the one of the untreated solution. Voluminosity values obtained from viscosity values of the capillary viscometer (Table III.1B) were higher

than the one calculated from viscosity data of the rotational viscometer but the influence of pressure release rate is still evident: the high pressure release (600 MPa min^{-1}) induced a higher voluminosity than the low pressure release (20 MPa min^{-1}). The voluminosity of the sample after pressure release rate of 200 MPa min^{-1} was nearly unchanged compared to the untreated sample.

Hydrodynamic diameter of the casein micelles

Changes in the hydrodynamic diameter d_H of the casein micelle as a function of pressure release rate are collected in Table III.2.

Table III.2: Average hydrodynamic diameter of casein micelles in untreated and pressurized 5 % casein solution

Pressure release rate [MPa min ⁻¹]	Average hydrodynamic diameter d_H [nm]
untreated	377 ± 46^a
600	477 ± 65^b
200	$395 \pm 93^{a,b,c}$
20	231 ± 7^d

* Data within a column followed by different letters are significantly different at $p < 0.05$

The largest hydrodynamic diameter was obtained with the highest pressure release rate. The hydrodynamic diameter at a pressure release rate of 600 MPa min^{-1} was significantly higher (about 30 % more) than the one of the untreated solution. At a pressure release rate of 200 MPa min^{-1} the hydrodynamic diameter was not significantly different neither to the untreated solution nor to the 600 MPa min^{-1} treated one while the pressure release rate of 20 MPa min^{-1} induced the smallest diameter (about 30 % less than in the untreated solution).

Atomic force microscopy

Parallel to particle size measurements, atomic force microscopy (AFM) observations were used to characterize pressure-induced changes of the structure of the casein micelles. Figure III.2 shows AFM images for the untreated solution and the treated solution after pressure release of 600 , 200 and 20 MPa min^{-1} .

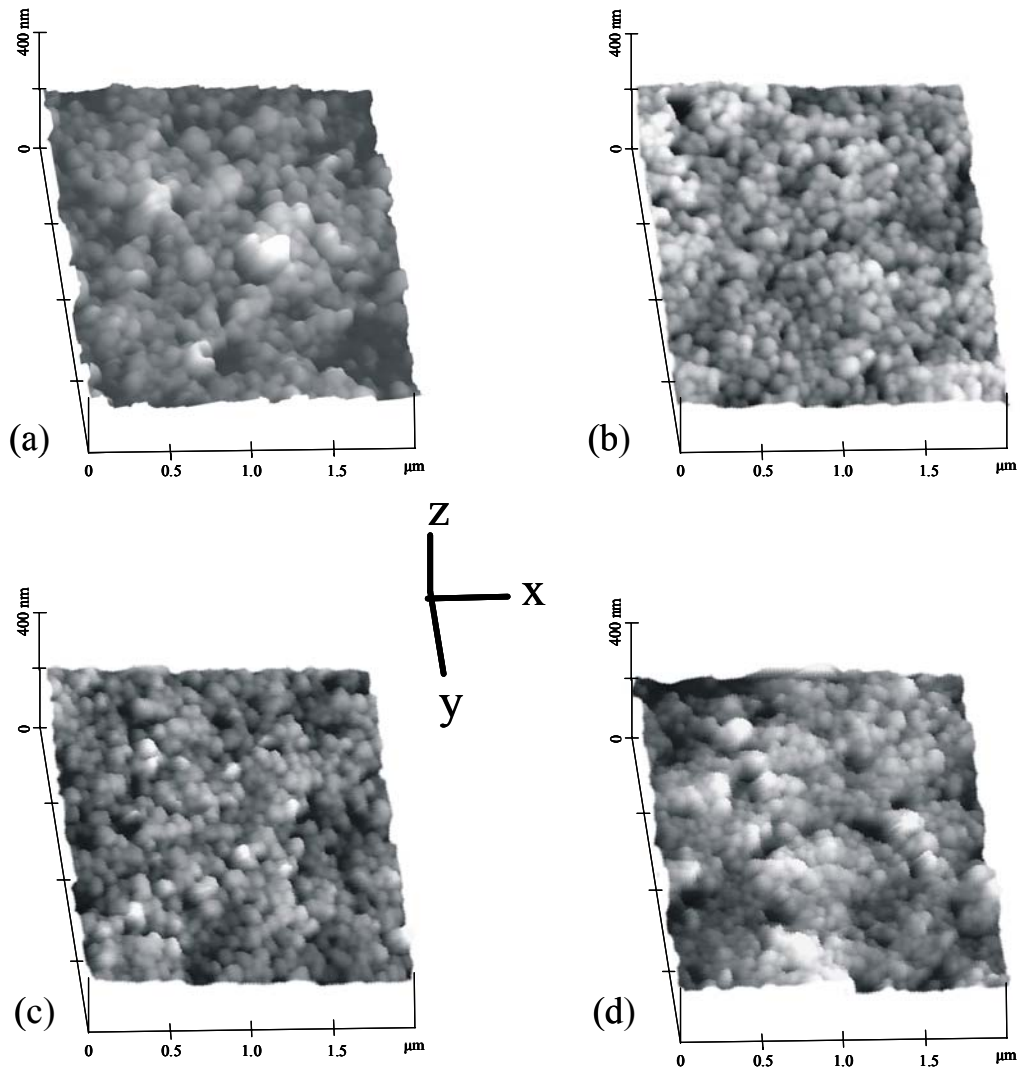


Figure III.2: Atomic force microscopic images of casein solutions with 5 g casein/100ml UFP after high pressure treatment with variation of the pressure release rate, (a) untreated casein solution, (b) pressure release rate 600 MPa min^{-1} , (c) pressure release rate 200 MPa min^{-1} and (d) pressure release rate 20 MPa min^{-1}

All pictures show spherical casein micelles. Smaller casein particles were detected in casein solutions treated at a 600 MPa with a pressure release of 600 and 200 MPa min^{-1} (Figure III.2b and III.2c) than in the untreated solution (Figure III.2a). After a slow pressure release of 20 MPa min^{-1} , casein micelles appeared comparable to the untreated solution.

Discussion

Protein content and pressure release rate influence the properties of high pressure induced casein micelles. At a casein content of about 7 %, depending on the pressure release rate, sol as well as gel structures were observed. The higher the casein content and the faster the

pressure release, the firmer were the structures built up and the finer were the microstructures. The influence of the casein content is due to the inner friction of the dispersed particles with the outer phase, and to the high water binding of the casein (Snoeren et al., 1982). The difference observed between voluminosity data from the rheometer and the capillary viscometer may be due to disintegration of the casein aggregates due to the shearing. Nevertheless, in both cases, the influence of pressure release rate was illustrated. This influence is in good agreement with the results of Fertsch et al. (2003) which showed that the firmness of pressure-induced casein gels with a 15 % casein content was mainly affected by the pressure release rate. The hydrodynamic diameter of casein particles treated at 600 MPa with a release rate of 600 MPa min⁻¹ was significantly larger than before the treatment. The voluminosity of the particles was also higher. However, the AFM pictures showed smaller casein micelles after treatment with a fast pressure release (600 MPa min⁻¹) than in the untreated solution. Casein aggregates from smaller casein micelles are created by high pressure. The non covalent interactions in the casein micelles are weakened during pressure build-up and pressure holding phase inducing the dissociation of casein micelles into submicelles. Due to the reactivation of these interactions during pressure release phase (Suzuki & Taniguchi, 1972; Hinrichs, 2000) and the reformation of calcium bridges from free calcium in the serum phase (Shibauchi et al., 1992; López-Fandiño et al., 1998; Abbasi et al., 2002), casein micelle aggregates are generated (Ohmiya et al., 1989; Johnston et al., 1992a; Johnston et al., 1992b; Masson, 1992; Hinrichs, 2000; Johnston et al., 2002; Fertsch et al., 2003). According to Needs et al. (2000), the extensive reaggregation of the casein particles during pressure release is due to the high hydrophobicity of the submicellar particles at pressure above 400 MPa (Johnston et al., 1992). The presence of large casein aggregates has already been shown by Gaucheron et al. (1997), Law et al. (1998), Garcia-Risco et al. (2000) and Huppertz et al (2004a) for milk treated at pressures between 250 and 400 MPa and temperatures from 40 to 60 °C. These modifications are accompanied by hydration changes. The high pressure induced casein aggregates of smaller casein micelles have a larger hydrodynamic diameter and higher voluminosity. The results confirmed the observations of Anema & Creamer (1993) and Gaucheron et al. (1997) who noticed that smaller particles had greater degrees of hydration. According to Masson (1992), hydration changes are mainly caused by pressure induced ionization, solvent exposure change of amino acid side chains and of peptide bonds (Carter et al., 1978), and diffusion of water into cavities located in the hydrophobic core of proteins.

As for a release rate of 600 MPa min⁻¹, a release rate of 200 MPa min⁻¹ induces a structure with smaller casein particles. However, voluminosity and hydrodynamic diameter of these particles are not higher but almost similar to the particles in the untreated solution. Aggregates of small casein micelles are still induced but these aggregates are smaller than those after the faster pressure release rate of 600 MPa min⁻¹. They have the size of the initial casein micelles before high pressure treatment.

After a slow release rate of 20 MPa min⁻¹ casein micelles have a similar shape and diameter than those of the solution before high pressure treatment. Nevertheless, the initial structure is not rebuilt, the micelles show a smaller hydrodynamic diameter and a lower voluminosity than in the solution before treatment.

Conclusion

The formation of pressure-induced casein structures (600 MPa for 30 min at 30 °C) has been investigated for different pressure release rates (20 to 600 MPa min⁻¹) and casein contents (1 to 15 %). The present study shows that the choice of a pressure release rate is as quite important factor depending on which structure has to be built up. A better understanding of the pressure-induced structure formation of the casein micelles on ultra-high pressure treatment may offer opportunities for the creation of novel dairy products.

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Chapter IV

The high pressure induced modification of casein as influenced by pressure release rate and holding time

Summary

The properties of bovine casein (5 % casein content), treated at 600 MPa and 30 °C, as influenced by pressure build-up rate (20 to 600 MPa min⁻¹), pressure holding time (0 to 30 min) and pressure release rate (20 to 600 MPa min⁻¹) was studied. By *ex situ* experiments, the samples were analysed regarding viscosity and hydrodynamic diameter before and after pressure treatment. Pressure build-up rate had no influence on the resulting pressure-induced casein structures. The modification of casein was more influenced by either the pressure holding time or the pressure release rate. The higher the pressure release rate, the more viscous the pressure treated casein solution and the larger the hydrodynamic diameter of the colloid particles (390 nm for a release of 600 MPa min⁻¹ compared to 230 nm for a release of 20 MPa min⁻¹). Further experiments were established to follow the viscosity *in situ* while pressure treatment. During pressure increase to 600 MPa the viscosity of the casein solution increased by more than twofold due to hydration of the dissociated casein fragments. In comparison, no significant changes were observed in the holding phase of 30 min. The pressure release rate of 600 MPa min⁻¹ induced a dramatic increase in viscosity resulting in gel-like properties, but release rates of 200 and 20 MPa min⁻¹ resulted in lower viscosity than in the untreated solution.

Introduction

High pressure treatment can be applied in food processing for several advantages, like the inactivation of microorganisms, modification of proteins, or the creation of new food structures. In this context the effect of high pressure treatment on casein micelles has been studied. Herein the studies mainly focussed on the influence of pressure level (Desobry-Banon, Richard & Hardy, 1994; Gaucheron, Famelart, Mariette, Raulot, Michel & Le Graet, 1997; Schrader & Buchheim, 1998; Velez-Ruiz, Swanson & Barbosa-Canovas, 1998; Needs, Stenning, Gill, Ferragut & Rich, 2000; García-Risco, Olano, Ramos & López-Fandiño, 2000; Huppertz, Fox & Kelly, 2004a; Regnault, Thiebaud, Dumay & Cheftel, 2004) and temperature applied during pressurization (Gaucheron et al., 1997; García-Risco et al., 2000; Huppertz et al., 2004a; Regnault et al., 2004; Huppertz, Fox & Kelly, 2004b) on the stability of the micelles.

As observed by photon correlation spectroscopy (PCS), laser granulometer studies (Desobry-Banon et al., 1994; Gaucheron et al., 1997; Needs et al., 2000; Huppertz et al., 2004a; Regnault et al., 2004) and transmission electron microscopy (Gaucheron et al., 1997; Schrader et al., 1998; García-Risco et al., 2000; Needs et al., 2000; Keenan, Young, Tier, Jones & Underdown, 2001) after a treatment with pressures up to 400 MPa, casein micelles size decreases. The reduction of the micelle size was explained by two phenomena, the solubilisation of colloidal calcium phosphate (CCP) out of the micelle (Shibauchi, Yamamoto & Sagara, 1992; Lee, Anema, Schrader & Buchheim, 1996; Gaucheron et al., 1997; Schrader, Buchheim & Morr, 1997; López-Fandiño, De la Fuente, Ramos & Olano, 1998; Keenan et al. 2001; Abbasi & Dickinson, 2002) and the weakening of hydrophobic and electrostatic interactions between the submicelles (Mozhaev, Heremans, Frank, Masson & Balny, 1996; Needs et al., 2000).

However, not only the pressure level but also pressure holding time and especially the pressure release rate may influence dissociation and association of caseins. During pressure release the binding forces take effect again (Suzuki & Taniguchi, 1972; Hinrichs, 2000), calcium bonds are rebuilt (Shibauchi et al., 1992; López-Fandiño et al. 1998) and casein submicelles or fragments associate and form new microstructures (Ohmiya, Kajino, Shimizu & Gekko, 1989; Johnston, Austin & Murphy, 1992a; Johnston, Austin & Murphy, 1992b; Masson, 1992; Hinrichs, 2000; Johnston, Rutherford & McCreedy, 2002; Fertsch, Müller & Hinrichs, 2003). Fertsch et al. (2003) demonstrated that the pressure release rate significantly influences the structure formation of pressure-induced 15 % casein gels. The higher the pressure release rate, the firmer the gels after high pressure treatment. Furthermore, the

firmness of gels was higher after fast pressure release when the solution was kept at a pressure of 600 MPa for 15 to 30 minutes compared to pressure treatment without holding time. Fertsch et al. (2003) assumed dissociation of the micelles may not be finally completed when the holding time is too short. Therefore, self-association of the caseins initiated during pressure release lead to inhomogeneous gelling since intact micelles or bigger fragments are present. The texture of the gels appears soft.

The aim of this work was to determine which phase of the treatment has an influence on the formation of high-pressure-induced casein structures, the pressure build-up, the holding time or the pressure release. Pressure-induced modification of the casein solution (*ex situ*) was analysed by measuring apparent viscosity and particle size before and after treatment. In order to observe in more detail the change in casein solution during holding phase and pressure release, the viscosity was also measured *in situ*.

Material and methods

Sample preparation

The experiments were carried out with highly enriched micellar casein powder produced by diafiltration of skim milk (Kersten, 2001). The powder contained 6.5 % water, 68.4 % total protein including 68.0 % casein, 16.6 % lactose and 8.4 % minerals including 2.3 % calcium. The powder was diluted in ultra filtration permeate (ultra filtration permeate powder, containing 4.8 % water, 2.9 % total protein, 84.9 % lactose and 7.4 % minerals including 0.5 % calcium was reconstituted with distilled water to a total water content of 94.8 %) (Ingredia Dairy Ingredients, Switzerland) to achieve a total protein content of 5 %, mixed for 3h and then stored at 4 °C for one day. The protein content of the samples was checked with a nitrogen analyzer using Dumas method (LECO FP-528, Leco Instrumente GmbH, Germany). The pH of the solutions was adjusted to pH 6,0 by adding lactic acid 10 % (Merck, Germany).

High pressure treatment, ex situ viscosity and particle size measurement

Samples were filled into 20 ml HDPE tubes (inner diameter 32 mm, filled height 14 mm) (Nalgene, Novodirect, Germany) leaving no headspace, closed with a silicone plug (VWR, Germany). Prior to the pressure application, the samples were tempered for more than 5 min at the appropriate temperature of 30°C. In a high pressure apparatus (Resato High Pressure Technology, The Netherlands) pressure was built up with 200 MPa min⁻¹ and held constant at

600 MPa and 30 °C. Holding time was varied from 0 to 30 min and pressure release by applying 20, 200 and 600 MPa min⁻¹. In further experiments, the pressure built up rate was varied (20, 200 and 600 MPa min⁻¹) and pressure was kept constant at 600 MPa for 30 min at 30 °C. Pressure was released with 200 MPa min⁻¹. After the treatment, the samples were stored overnight at 4 °C before analysis.

The apparent dynamic *ex situ* viscosity of the solution was determined using a rotational rheometer (Advanced Rheometer AR 2000, TA Instruments, Germany) tempered at 10 °C. The shear rate was linearly increased to 500 s⁻¹ in 3 min, held for 5 min at 500 s⁻¹ and then decreased to 0 s⁻¹ in 3 min. The apparent dynamic viscosity was defined as the viscosity at the end of the 5 min holding time at a shear rate of 500 s⁻¹. The results are the average of 4 to 6 viscosity measurements from 2 to 3 independent high pressure experiments.

The mean hydrodynamic diameter d_H of particles was determined by photon correlation spectroscopy (PCS) at 25 °C using a high performance particle sizer (HPPS, Malvern Instruments Ltd., UK). The particle sizer uses back scattering at an angle of 173° that enables to measure undiluted samples preventing the dissociation of the casein micelles due to dilution. For each sample, three light scattering measurements of 60 s were carried out. The results, expressed in nm, are determined from the intensity distribution curves and are the average of 10 to 15 measurements from 2 to 3 replicate high pressure experiments carried out at different days.

High pressure treatment and *in situ* viscosity measurement

In situ viscosity measurement was carried out with a rolling ball viscometer. Detailed information about the measurement system is given by Först, Werner and Delgado (2000, 2002). It consists of a high pressure tube with an inner diameter of 1.6 mm. During measurements a sphere of steel (diameter 1.39 mm) is rolling in the inclined tube, which is filled with the pressurized sample. Two coils, situated around the tube in an axial distance L , detect by change of inductance the time Δt the sphere needs to traverse the length L . The temperature of the sample is set by a cooling jacket around the pressure tube. A capillary connects the high pressure tube to the pressure generation unit, a manual piston pump (Sitec Sieber Engineering AG, Switzerland) and to a pressure transducer (Wika GmbH, Germany). The viscosity η is calculated by

$$\eta = K \cdot (\rho_B - \rho_S) \cdot \Delta t \quad (1)$$

where K represents a calibration constant depending on pressure as well as on viscosity of the investigated sample at ambient pressure. The values ρ_B and ρ_S denote the density of the sphere

respectively of the sample. Within the measurement the pressure was build-up with a rate of 200 MPa min^{-1} and held constant at 600 MPa and $30 \text{ }^\circ\text{C}$ for 30 min. Different pressure release rates were applied: 20, 200 and 600 MPa min^{-1} . The viscosity of the sample was measured prior to high pressure treatment, at 600 MPa with an interval of 10 min and after pressure release. During the pressure release experiment with 20 MPa min^{-1} , the viscosity was followed at 500, 300 and 100 MPa. The results are the average of 3 *in situ* viscosity measurements.

Due to small dimensions of the measurement tube and of the disperse phase, an approximately homogeneous treatment (Delgado & Hartmann 2003) can be expected in the pressure holding phase. In contrast, this assumption can not hold in the pressurization and depressurization phase, as pressure and temperature differences as well as shear gradients apply. However, these affects are considered to be not the subject of the present contribution.

Statistical analysis

The statistical analysis was carried out by means of a t-test (Sigma Plot 8.0, SPSS, Inc.) with a significance of $p < 0.05$.

Results

Ex situ

The apparent viscosity and the hydrodynamic diameter of the differently pressurized casein solutions are shown in Table IV.1. For the pressure release rate of 600 MPa min^{-1} , the viscosity (*ex situ*) increased with extended holding time (0 to 30 min). Hereby, a significant difference to the control sample (untreated solution) appeared at holding times of 20 and 30 min. At a pressure release rate of 200 MPa min^{-1} , a small, but not significant influence of the pressure holding time on viscosity was found. The pressure release rate of 20 MPa min^{-1} resulted in the lowest viscosity, being lower than control, and no influence of holding time was observed. At constant holding time, viscosity increased significantly with increased pressure release rate (about 7 mPa s for a release of 600 MPa min^{-1} compared to 3.5 mPa s for a release of 20 MPa min^{-1} , at a holding time of 30 min).

Table IV.1: Influence of the pressure holding time and pressure release rate on apparent dynamic viscosity (*ex situ*, measured at 10 °C) and mean hydrodynamic diameter of a 5 % casein solution treated at 600 MPa, 30 °C, pressure build-up 200 MPa min⁻¹ *

Holding time in min	Pressure Release rate in MPa min ⁻¹	Apparent dynamic <i>ex situ</i> viscosity in mPa s	Mean hydrodynamic diameter in nm
Control		4.4 ± 0.3	(3.8 ± 0.6)·10 ²
0	600	4.7 ± 0.5 ^{control}	(3.4 ± 0.4)·10 ^{2 control}
	200	3.6 ± 0.1	(2.7 ± 0.3)·10 ²
	20	3.2 ± 0.2	(2.6 ± 0.2)·10 ^{2 0(200)}
10	600	4.9 ± 0.7 ^{control, 0(600)}	(3.5 ± 0.6)·10 ^{2 control, 0(600)}
	200	4.3 ± 0.7 ^{control,10(600), 0(200)}	(3.5 ± 0.6)·10 ^{2 control, 10(600)}
	20	3.2 ± 0.2 ⁰⁽²⁰⁾	(2.4 ± 0.3)·10 ²
20	600	5.0 ± 0.4 ⁰⁽⁶⁰⁰⁾	(4.2 ± 0.8)·10 ^{2 control}
	200	4.4 ± 0.5 ^{control,20(600), 10(200)}	(3.3 ± 0.4)·10 ^{2 10(200)}
	20	3.1 ± 0.1 ^{0(20), 10(20)}	(2.3 ± 0.2)·10 ^{2 10(20)}
30	600	6.9 ± 1.9 ²⁰⁽⁶⁰⁰⁾	(3.9 ± 0.6)·10 ^{2 control, 10(600), 20(600)}
	200	4.8 ± 0.9 ^{control, 10(200), 20(200)}	(3.3 ± 0.6)·10 ^{2 10(200), 20(200)}
	20	3.5 ± 0.4 ^{0(20), 10(20)}	(2.3 ± 0.1)·10 ^{2 10(20), 20(20)}

* Each value was compared with control, and values with the same release rate are also compared. A value followed by ^{control} is not significantly different to control. A value followed by ⁰⁽⁶⁰⁰⁾ is not significantly different to the value of the solution treated with holding time of 0 min and pressure release of 600 MPa min⁻¹ at p < 0.05.

In parallel to viscosity, the values of the mean hydrodynamic diameter were higher for the pressure release rate of 600 MPa min⁻¹ compared to 200 MPa min⁻¹ and 20 MPa min⁻¹. The hydrodynamic diameter of the solution treated with 600 MPa min⁻¹ was not significantly different to control, and no significant influence of holding time was observed. In addition, after a pressure release of 200 MPa min⁻¹ particles with the same size as control or smaller were detected. The pressure release rate of 20 MPa min⁻¹ resulted in the smallest mean hydrodynamic diameter independent from applied pressure holding time.

In order to illuminate the influence of pressure build-up rate, different values were applied (20, 200 and 600 MPa min⁻¹) at constant holding time and pressure release rate (Table IV.2).

Table IV.2: Influence of the pressure build-up rate on apparent dynamic viscosity (*ex situ*, measured at 10 °C) and mean hydrodynamic diameter of a 5 % casein solution treated at 600 MPa, 30 min, 30 °C, pressure release rate 200 MPa min⁻¹ *

Pressure build-up rate in MPa min ⁻¹	Pressure release rate in MPa min ⁻¹	Apparent dynamic <i>ex situ</i> viscosity in mPa s	Mean hydrodynamic diameter in nm
600	200	4.7 ± 0.4	(2.9 ± 0.3)·10 ²
200	200	4.8 ± 0.9 ⁶⁰⁰	(3.3 ± 0.6)·10 ² ⁶⁰⁰
20	200	4.4 ± 0.5 ^{600,200}	(3.0 ± 0.5)·10 ² ^{600,200}

* A value followed by ⁶⁰⁰ is not significantly different to the value of the solution treated with the pressure build-up rate of 600 MPa min⁻¹ at p < 0.05.

Hereby, this parameter seems not to affect significantly viscosity and hydrodynamic diameter (p < 0.05).

In situ

The measured *in situ* viscosity data are given in Table IV.3. The viscosity of the casein solution increased more than twofold from 2.0 (control) to 4.7 mPa s while pressure build-up phase (Table IV.3). During holding time at 600 MPa, the viscosity of the casein solution remained nearly constant: after 30 minutes only a little, but not significant decrease in viscosity became visible compared to the value at the beginning of the holding phase. After pressure release to 0.1 MPa with a rate of 600 MPa min⁻¹ the ball didn't roll anymore in the tube of the viscometer indicating the formation of a gel like structure of the sample. Hence, viscosity was not measurable with this system. The pressure release rates of both, 200 and 20 MPa min⁻¹, resulted in almost the same viscosity range than control. Only minor changes were observed being significant for 200 MPa but not for 20 MPa. Finally, the course of viscosity was followed during the slowest pressure release rate of 20 MPa min⁻¹. The most important change of viscosity occurred between 300 MPa and 0.1 MPa from about 4.4 to 1.9 mPas.

Table IV.3: *In situ* viscosity measured at 30 °C during pressure treatment at 600 MPa of a 5 % casein solution and ultra filtration permeate *

	Apparent dynamic <i>in situ</i> viscosity of the casein solution in mPa s	Apparent dynamic <i>in situ</i> viscosity of the ultra filtration permeate in mPa s
Control	2.0 ± 0.1 ^a	0.9 ± 0.0
After pressure build-up at 600 MPa (after 0 min)	4.7 ± 0.5 ^b	1.2 ± 0.1 ^a
During holding time at 600 MPa:		
after 10 min	4.6 ± 0.4 ^b	1.1 ± 0.1 ^a
after 20 min	4.6 ± 0.3 ^b	1.1 ± 0.1 ^a
after 30 min	4.5 ± 0.4 ^b	1.1 ± 0.1 ^a
After pressure release of 600 MPa min ⁻¹	not measurable	
After pressure release of 200 MPa min ⁻¹	1.7 ± 0.1	
During pressure release of 20 MPa min ⁻¹ :		
at 500 MPa	4.8 ± 0.6 ^b	
at 300 MPa	4.4 ± 0.6 ^b	
at 100 MPa	2.3 ± 0.2	
at 0.1 MPa	1.9 ± 0.2 ^a	

* Values within a column followed by the same letter are not significantly different at $p < 0.05$.

In addition, the *in situ* viscosity of the outer phase, the ultra filtration permeate, was measured at a pressure of 600 MPa after 0, 10, 20 and 30 min holding time (Table IV.3). During the build-up phase the viscosity of the permeate increased more than 20 % compared to control. During holding time viscosity remained constant.

Discussion

The experimental data of *in situ* and *ex situ* measurements provide the basis for discussion of the effects of pressure build-up phase, holding phase and pressure release phase on casein.

Pressure build-up phase: *In situ* experiments showed that the viscosity of the casein solution increased more than twofold due to pressure rise from 0.1 to 600 MPa (Table IV.3). In comparison, the viscosity of the solvent (ultra filtration permeate) increased only 20 %, which

has the same order like water, see also Först et al. (2002). The latter effect results from more dense packed molecules and more intense intermolecular interaction. Hence, pressure induced changes in structure of the casein may be responsible for the dramatic increase in viscosity under pressure of the solution compared to the permeate. Several researchers explained the reduction of the micelle size after pressure treatment by two phenomena, the solubilisation of colloidal calcium phosphate (CCP) out of the micelle and the weakening of hydrophobic and electrostatic interactions between the submicelles (Shibauchi et al., 1992; Lee et al., 1996; Mozhaev et al., 1996; Gaucheron et al., 1997; Schrader et al., 1997; López-Fandiño et al., 1998; Needs et al., 2000, Keenan et al., 2001; Abbasi et al., 2002). Gebhardt, Doster and Kulozik (2005) demonstrated by means of *in situ* photon correlation spectroscopy at 300 MPa that casein micelles (they used the same micellar casein for the experiments as in this work) dissociate into smaller fragments and casein monomers.

The pressure induced change in viscosity of the casein solution results from compound counteracting effects. Considering the casein solution as a suspension of spherical particles, the viscosity increases with the volume fraction of the solute (Barnes, Hutton & Walters, 1993). On the one hand side the dissociation of the casein micelles leads to a reduction in volume fraction of the solute, and therefore to a decrease in solvent viscosity if molecular interactions between solvent and solute are neglected. On the other hand, the surface of the solute for interactions with water increases due to disintegration of the casein micelle. This results in a higher hydration (Anema & Creamer, 1993; Walstra, 2003) and in a higher effective volume fraction of the solute. The latter phenomenon and the increased solvent viscosity at 600 MPa cause a raise in solution viscosity in comparison to 0.1 MPa. A further effect could originate from different behaviour of solvent and solute density with pressure. This also may influence the volume fraction of the solute but can be assumed to be small. The twofold increase in viscosity of the casein solution compared to 20 % of permeate indicate the hydration of the casein fragments as the dominant impact on the solvent viscosity. Finally, it has to be pointed out that the rate of pressure build-up has no significant influence on the *ex situ* viscosity and hydrodynamic diameter of the casein solution after pressure treatment (Table IV.2).

Pressure holding phase: Only minor but not significant changes of the *in situ* viscosity were observed during holding time (Table IV.3). It can be assumed that during pressure build-up phase (200 MPa min⁻¹) the disintegration of the casein micelle took mainly place so that equilibrium was nearly achieved when the holding time starts. Nevertheless, *ex situ* experiments demonstrated an influence of the pressure holding time especially when the

pressure release rate was high (Table IV.1). For 20 and 30 min holding time at 600 MPa combined with a pressure release of 600 MPa min⁻¹, viscosity and mean hydrodynamic diameter were higher than control. Fertsch et al. (2003) also observed an effect of the holding time on gelation for a 15 % micellar casein solution. They proposed that the dissociation of the micelles may not be finally completed when the holding time is too short resulting in an inhomogeneous gelation during pressure release phase. The texture of the gels appeared soft compared to gels formed after a holding time of 30 min.

Pressure release: Both *ex situ* and *in situ* experiments showed an influence of the pressure release rate on properties of the casein solution (Table IV.1 and IV.3). The higher the release rate was, the more viscous the solution and the bigger the mean hydrodynamic diameter appeared. First of all, this observation is in contrast to other researchers (Gaucheron et al., 1997; Needs et al., 2000; Huppertz et al., 2004a; Regnault et al., 2004) who found smaller micelles after pressure treatment. But one has to bear in mind that i) pressure of 400 MPa was often not exceeded and ii) the pressure release rate was not investigated explicitly.

The observations of this work are in good agreement with the results of Fertsch et al. (2003) who found that the firmness of pressure-induced casein gels with a 15 % casein content was mainly affected by the pressure release rate. This observation can be explained by the fact, that during pressure build-up casein micelles disintegrate into smaller fragments by the weakening of non-covalent bonds and solubilisation of colloidal calcium phosphate (CCP) out of the micelle. During pressure release phase, the binding forces take effect again (Suzuki et al., 1972; Shibauchi et al., 1992; Masson, 1992; Hinrichs, 2000; Johnston et al., 2002; Fertsch et al., 2003). Thus, protein-protein interactions are reestablished, the self-association of the caseins (Rollema & de Kruif, 2003) starts and calcium bridges from free calcium in the serum phase are reformed. For the high release rate, the reassociation of casein micelles leads to an increase of the hydrodynamic diameter and therefore of the volume fraction of the solute, explaining the observed increase in solution viscosity. Considering once more these interactions and our experimental data, it can be assumed that a slow release rate may lead more or less to the original casein micelles structure due to the ability of the caseins to self-association. But high pressure release rates, far from equilibrium, disturb the self-association mechanisms of the caseins and new structures or aggregates made of casein fragments build up.

Conclusion

The influence of the different phases during pressure treatment on the properties of micellar casein solution containing 5 % protein was studied in detail. Hinrichs (2000) proposed a model for the disintegration and association of casein micelles during high pressure treatment. This model can now be completed with the results of the present study and the results of Gebhardt et al. (2005). High pressure build-up induces a dissociation of casein micelles in casein fragments and casein monomers resulting in the *in situ* measured increase of viscosity and decrease of mean hydrodynamic diameter. Hereby, the rise in viscosity of the solution comes predominantly from an increase of volume fraction of the solute due to hydration. During pressure holding time the casein fragments and monomers will come very fast to an equilibrium. Finally, the pressure release, the last phase of a high pressure process, seems to affect significantly the rearrangement of the casein fragments and monomers. The self-association process of the caseins is initiated during pressure release because protein-protein interactions and protein-environment interactions are reestablished. Consequently, association rate of the casein fragments and monomers depends on the release rate and the formed casein particles may differ from native casein micelles.

Further studies must be conducted to examine the casein particle structure in more detail as well as the stability of the pressure-induced structures. Besides pressure level, holding time and temperature, the release rate should be taken into account in future when working with milk or casein systems.

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Chapter V

Formation of new casein structures by high pressure

Summary

The effect of pressure release rate (20 to 600 MPa min⁻¹), protein content (1 to 15 %) and calcium content (ionic strength 0.1 to 2 mol l⁻¹) on the formation of pressure-induced casein structures was investigated. The experiments were carried out with highly enriched micellar casein concentrate, which was treated at 600 MPa and 30 °C. The formed structures were analyzed regarding viscosity and texture. Depending on the different parameters, liquid (sol) to solid (gel) structures were generated. The higher the casein content and the release rate, the firmer the textures. An ionic strength of 0.3 mol l⁻¹ induced firmer textures than lower ionic strengths (0 and 0.1 mol l⁻¹) but the addition of more calcium (ionic strength of 2 mol l⁻¹) induced liquid structures with low viscosity.

Merel, E., Budde, T. & Hinrichs, J. (2005). Formation of new casein structures by high pressure, *Proceedings CD, joint 20th AIRAPT - 43rd EHPRG Conference on Science and Technology of High Pressure*, Karlsruhe.

Introduction

In high pressure treatment, the structure of casein micelles can be modified by the process parameter and milieu conditions resulting in new functional properties. Several studies showed that during pressure build-up phase casein micelles dissociate into submicelles (Schmidt & Buchheim, 1970; Needs et al., 2000; Regnault et al., 2004). This pressure-induced dissociation was explained by the weakening of hydrophobic and electrostatic interactions between submicelles (Mozhaev et al., 1996), and the solubilisation of colloidal calcium phosphate out of the micellar framework (Shibauchi et al., 1992; Lee et al., 1996; Schrader et al., 1997). During pressure release, the binding forces are regained and new calcium bridges are built up (Shibauchi et al., 1992). Instead of the original casein micelles new hyper-structures may be built up. In addition, Fertsch et al. (2003) observed that pressure release rate influences significantly the structure formation of pressure-induced casein gels. The faster the pressure is released, the firmer are the gels that are formed. Furthermore, the pressure-induced structure formation of casein particles and casein gels are not only influenced by processing but also by the composition. Due to the high water binding capacity of casein micelles, highly viscous structures and firm gels are built up depending on the casein content (Snoeren et al., 1982, Velez-Ruiz et al., 1998, Hinrichs, 2000). According to Lee et al (1996), the solubilisation of colloidal calcium phosphate out of the micellar framework is decreased with increasing level of soluble calcium in the solution before pressure treatment.

The aim of this work was to study the influence of casein and calcium content combined with the influence of pressure release rate on the formation of pressure-induced casein structures.

Experimental Method

The experiments were carried out with highly enriched micellar casein powder (containing 68.0 % of casein and 2.3 % of calcium) produced by diafiltration of skim milk (Kersten, 2001). The powder was diluted in ultrafiltration permeate (Ingredia Dairy Ingredients, Thun, Switzerland) to adjust the protein content from 1 to 15 %, stirred for 3h and then stored at 4 °C for 1 day. The pH of the solutions was adjusted to pH 6,0 by adding 10 % lactic acid (Merck, Darmstadt, Germany). Samples were filled into 20 ml HDPE tubes (inner diameter 32 mm, filled height 14 mm) (Nalgene, Novodirect, Kehl, Germany) leaving no headspace and closed with a silicone plug (VWR, Darmstadt, Germany). Prior to the pressure application

the samples were tempered for more than 5 min at the appropriate temperature. In the high pressure pilot apparatus (Resato High Pressure Technology, Roden, The Netherlands), pressure was built up with a rate of 200 MPa min⁻¹ and held constant at 600 MPa for 30 min at a temperature of 30 °C. Pressure release was varied from 20 to 600 MPa min⁻¹. After pressure treatment, the samples were stored 1 day at 4 °C until analysis.

Variation of the ionic strength

To investigate the influence of calcium content at a casein content of 5%, three different amounts of calcium chloride (CaCl₂ • 2H₂O, crystalline, Merck, Darmstadt, Germany) were added to get an additional ionic strength of calcium chloride of 0.1, 0.3 and 2 mol l⁻¹ before pH adjustment.

Structure diagram

The formation of pressure-induced casein structures depending on the pressure release rate and the casein content was illustrated in a structure-diagram for sol-gel transition. Casein solutions with a casein content of 1 to 15 % were pressure treated and pressure release was varied. Structures that appeared still liquid after pressure treatment were characterized as sol, and firm structures were characterized as gel. In further experiments, casein content was kept constant at 5 % and the influence of calcium and pressure release rate was investigated in detail regarding viscosity and firmness.

Viscosity measurements

Viscosity of the 5 % casein solution was determined before and after high pressure treatment at 10 °C by means of a rotational rheometer (Advanced Rheometer AR 2000, TA Instruments, Alzenau, Germany). The shear rate was increased to 500 s⁻¹ in 3 min, then held for 5 min at 500 s⁻¹ and decreased to 0 s⁻¹ in 3 min again. The apparent dynamic viscosity, η_{app} was defined as the viscosity at the end of the 5 min holding time at a shear rate of 500 s⁻¹. 4 to 6 viscosity measurements from the 2 to 3 independent high pressure treatments were carried out.

Texture properties

Dynamic rheology measurements (non-destructive measurements) were carried out to characterize the texture of the gels. The storage modulus, G' was determined at 10 °C (Advanced Rheometer AR 2000, TA Instruments, Alzenau, Germany) in the oscillation mode

with the plate geometry (diameter 20mm, stainless steel). The gap was adjusted by applying a normal force of 0.2 N. A stress sweep (1 Hz, 0.1-100 Pa) was first performed with each sample in order to figure out the linear viscoelastic regime. Then a frequency sweep was performed (2 Pa, linear viscoelastic regime, 0.01-100Hz) and the data of the storage modulus, G' were read at 1 Hz. The data present the mean of 4 to 6 measurements from the 2 to 3 independent high pressure experiments.

Results

Structure diagram

The following structure diagram (Figure V.1) illustrates the effect of pressure release after a pressure treatment at 600 MPa for 30 min at a temperature of 30 °C. Depending on the casein concentration and the pressure release rate, liquid (sol) to solid (gel) structures were generated. Samples having a very weak gel structure with phase separation neither to be characterized with a flow curve nor a firmness test were called “Transition”.

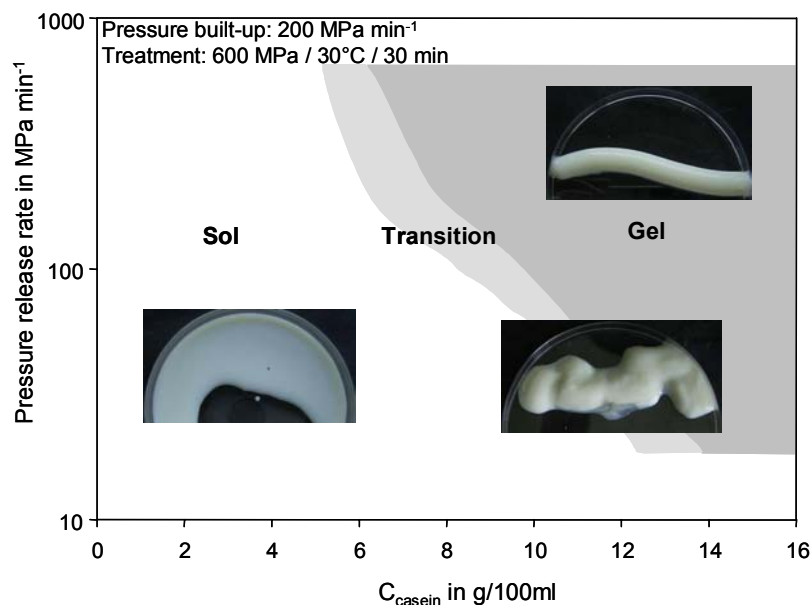


Figure V.1: Structure diagram: Influence of pressure release rate and casein content on gel-sol-transition of casein after high pressure treatment at 600 MPa, 30 min, 30 °C.

In general, sol-gel transition was shift from low casein content for high pressure release rate to high casein content for low pressure release rate. At a low casein content the solutions were still liquid (sol) independent from the release rate. With increased casein content and

increased release rate the samples appeared more viscous, and finally gels were formed. Below 5 % casein content, all samples were still liquid (sol) after high pressure treatment. However, above 7 % casein content, a gel was built up when applying 600 MPa min⁻¹ pressure release rate. The lower the release rate (20 and 200 MPa min⁻¹) the higher the casein content necessary to induce gelation (7.5 % for 200 MPa min⁻¹ and 14 % for 20 MPa min⁻¹). Interestingly, structures with different texture (sol, gel or transition) may be induced at casein content from 7 to 12 % casein only by varying the pressure release rate.

Influence of calcium content

Casein solutions with different ionic strengths (0, 0.1, 0.3 and 2 mol l⁻¹) were pressurized with 600 MPa for 30 min at 30 °C and release rate was varied. Sol viscosity and the storage modulus of the gels were determined and the results are presented in Table V.1.

Table V.1: Influence of the ionic strength and the pressure release rate on structure, apparent viscosity and storage modulus of 5 % casein solutions pressurized with 600 MPa at 30 °C for 30 min depending on pressure release rate

Ionic strength in mol l ⁻¹	Pressure release rate in MPa min ⁻¹	Structure	$\eta_{app} \pm$ s.d. in mPa.s	Storage modulus, $G' \pm$ s.d. in Pa
Without addition of calcium	Control	Sol	5.9 ± 0.7	-
	600	Sol	6.6 ± 1.2	-
	200	Sol	5.4 ± 0.6	-
	20	Sol	4.5 ± 0.4	-
0.1	Control	Sol	12.2 ± 1.2	-
	600	Gel	-	(7.0 ± 1.7)·10 ²
	200	Gel	-	(5.3 ± 1.6)·10 ²
	20	Sol	5.9 ± 1.7	-
0.3	Control	Sol	13.0 ± 2.4	-
	600	Gel	-	(1.33 ± 0.33)·10 ³
	200	Gel	-	(1.17 ± 0.15)·10 ³
	20	Gel	-	(8.4 ± 2.6)·10 ²
2	Control	Sol	20.0 ± 1.2	-
	600	Sol	8.0 ± 0.3	-
	200	Sol	6.7 ± 0.6	-
	20	Sol	4.2 ± 0.2	-

Control: no pressure treatment; Sol: liquid structure; Gel: structure with a firm continuous network; s.d.: standard deviation

The viscosity of the untreated control solutions increased with the amount of calcium added. It was about 3 times higher in the solution with an ionic strength of 2 mol l⁻¹ than in the one without calcium addition. After pressure release rates of 600 and 200 MPa min⁻¹ gelation was

observed for an ionic strength of 0.1 mol l^{-1} , while for an ionic strength of 0.3 mol l^{-1} gels were induced independent from the applied release rate. At 2 mol l^{-1} all samples remained liquid after pressure treatment. Furthermore, it is important to notice the influence of pressure release rate: the higher the pressure release rate the more viscous the sol (higher apparent viscosity) or the firmer the formed gel (higher storage modulus).

Discussion

Influence of the protein content

The influence of casein content is illustrated in the structure diagram (Figure V.1). The higher the casein content the firmer the texture. According to Snoeren et al. (1982) the influence of casein content is due to the inner friction of the dispersed particles with the outer phase, and to the high water binding of the casein. Furthermore it is well-known, that at a certain protein content of about 10 to 14 % in milk concentrates gel structures were built up when high pressure was applied, but the influence of the pressure release rate on structure formation was not studied in detail. This study demonstrated that the casein content necessary for pressure-induced transition from sol to gel is significantly lower when a pressure release of 600 MPa min^{-1} compared to 20 MPa min^{-1} is applied. The sol-gel transition at low casein and high pressure release rate may be due to spontaneous aggregation of caseins into a homogeneous network besides during slow relaxation casein hyper-structures are developed and network formation is retarded.

Influence of the ionic strength

The viscosity of the untreated solution increased with increasing amount of calcium. Shibauchi et al. (1992) and Lee et al. (1996) showed that the dissociation of casein micelles during high pressure treatment is accompanied by an increase in the levels of soluble calcium and phosphate. Lee et al. (1996) also showed that the resistance to the pressure-induced solubilisation of colloidal calcium phosphate out of the micellar framework is increased by soluble calcium. Our results are in good agreement with these observations, higher ionic strength decreases the solubilisation of colloidal phosphate and firmer gels are formed. The latter may be due to the fact, that the higher the calcium content the more electrostatic interactions are formed stabilizing the network structure.

However, at 2 mol l^{-1} the structures remained still liquid after high pressure treatment and viscosities were lower than the untreated solution. Water molecules are needed for the hydration of the ions, and at high ionic strength the solubility of proteins is decreasing (salting out effect, Berlitz et al., 2004). The caseins become more compact, the water binding is reduced resulting in a low viscosity and gel formation is retarded. Finally, it should be pointed out, that for all ionic strengths and independently whether gels or sols were formed, an influence of pressure release rate was observed.

Influence of the pressure release rate

The structure diagram (Figure V.1) and also the experiments regarding calcium ionic strengths (Table V.1) demonstrated the influence of the pressure release rate: the faster the pressure release, the firmer the textures that were induced. Fertsch et al. (2003) also showed that the firmness of pressure-induced casein gels with 15 % casein content was mainly affected by the pressure release rate. During pressure build-up, casein micelles dissociate into submicelles by the weakening of non-covalent bonds, while during pressure release, the binding forces take effect again (Suzuki & Taniguchi, 1972; Hinrichs, 2000). In addition, calcium bridges from free calcium in the serum phase are reformed (Shibauchi et al., 1992) and instead of the original casein micelles, new hyper-structures are built up (Masson, 1992; Johnston et al., 2002; Fertsch et al., 2003). The formation of these new structures depends on the pressure release rate. It is assumed that the slower the pressure is released, the higher is the level of aggregates already formed during release. At low casein content the solution remains liquid but at certain casein content aggregates also conjoin and form a network.

Conclusion

Not only the casein content and the milieu conditions but also the process parameters like pressure build up phase, pressure level and pressure release rate influence the properties of casein structures. The higher the casein and calcium content, and the faster the decompression the firmer are the gels that are built up. However, too high calcium may induce a salting out effect and may inhibit the formation of firm gels. Further research was initiated for better understanding of the mechanism and in order to characterize the different structures. The results will be published later on. To conclude, it is important to note for experimental design as well as for practical application that besides the composition of the solution the pressure

release rate is an important process parameter for casein based structures in pressure treatment.

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Chapter VI

Pressure-induced modification of casein micelles - Influence of pressure build-up rate, pressure level, release rate and temperature on viscosity and particle size

Summary

The objective of the experiments was to study the influence of pressure build-up (20 to 600 MPa min⁻¹), pressure level (200 to 600 MPa) and release rate (20 to 600 MPa min⁻¹) as well as the applied temperature (20 to 50 °C) on casein micelles. The samples (5 % micellar casein solution) were analyzed regarding viscosity and mean hydrodynamic diameter. An increase of viscosity and decrease of mean hydrodynamic diameter was observed in the casein solution when pressure was increased. When pressure was released from 200 MPa, no significant influence of the release rate on viscosity and hydrodynamic diameter was found, but when the pressure was released from 400 or 600 MPa changes were significant. The faster the pressure was released, the higher were viscosity and hydrodynamic diameter. At pressure treatment temperatures of 20, 30 and 40 °C the viscosity increased with increasing release rate while no influence of the release was observed at 50 °C. The highest effect of the pressure release on the viscosity was observed at 30 °C. At 20 and 30 °C the hydrodynamic diameter increased with increasing release rate. Pressure build-up rate had no significant influence on pressure-induced modification of casein.

Merel-Rausch, E., Duma, I.P. & Hinrichs, J. (2006). Pressure-induced modification of casein micelles - Influence of pressure build-up rate, pressure level, release rate and temperature on viscosity and particle size, *Milk Science International* 61(3), 255-259.

Introduction

High pressure treatment can modify the structure of casein micelles depending on the applied parameter resulting in different functional properties of the solution. During pressure build-up phase casein micelles dissociate into submicelles (Needs et al., 2000; Regnault et al., 2004; Schmidt & Buchheim, 1970) due to weakening of hydrophobic and electrostatic interactions between submicelles (Mozhaev et al., 1996) and colloidal calcium phosphate solubilizes out of the micellar framework (Lee et al., 1996; Schrader & Buchheim, 1997; Shibauchi et al., 1992). In photon correlation spectroscopy (PCS) and laser granulometer studies an increase of the casein micelle size at pressures of 200 to 250 MPa was observed (Gaucheron et al., 1997; Huppertz et al., 2004a), followed by a decrease of the micelle size up to 400 MPa (Desobry-Banon et al., 1994; Gaucheron et al., 1997; Huppertz et al., 2004a; Needs et al., 2000; Regnault et al., 2004; Schmidt & Buchheim, 1970; Schrader & Buchheim, 1998). Transmission electron microscopy studies confirmed the measured decrease of the micelle size after treatment at 400 MPa and 600 MPa (García-Risco et al., 2000; Gaucheron et al., 1997; Keenan et al., 2001; Needs et al., 2000; Schrader & Buchheim, 1998).

During pressure release, the binding forces are regained and calcium bridges are rebuilt (Shibauchi et al., 1992). Instead of the original casein micelles new hyper-structures may be formed. Fertsch et al. (2003) demonstrated that the pressure release rate influences significantly the firmness and microstructure of pressure-induced casein gels with 15 % casein. The faster the pressure was released after pressure treatment at 600 MPa, the firmer the gels that were formed. We showed in one of our recent studies (Merel et al., 2005) that depending on the casein concentration and the pressure release rate after pressure treatment at 600 MPa, liquid (sol) to solid (gel) structures can be generated. At low casein content ($\leq 5\%$), the structures were still liquid independent from the release rate. At about 7 % casein the solution remained liquid when a low pressure release of 20 MPa min^{-1} was applied whereas a high release rate of 600 MPa min^{-1} induced gelation. Finally, at a casein content of 15 % a gel was already formed when the slow release rate of 20 MPa min^{-1} was performed.

The aim of this work was to study the influence of pressure build-up rate, pressure level, pressure release rate and temperature on the formation of pressure-induced casein particles in a solution with 5 % casein.

Materials and methods

In order to avoid interaction with whey proteins in the pressure experiment, micellar casein was used. Micellar casein powder (6.5 % water, 68.4 % total protein including 68.0 % casein, 16 % lactose and 8.4 % minerals including 2.3 % of calcium) was produced by diafiltration of skim milk (Kersten, 2001). The powder was diluted in ultrafiltration permeate to achieve a protein content of 5 %, mixed for 3h and stored at 4 °C for one day. The permeate was prepared by reconstitution of powder (Ingredia Dairy Ingredients, Switzerland; composition: 4.8 % water, 2.9 % total protein, 84.9 % lactose and 7.4 % minerals including 0.5 % calcium). The pH of the samples was adjusted to pH 6,0 by adding lactic acid 10 % (Nr.100366, VWR, Germany).

Experiments were carried out in a high pressure apparatus (Resato, The Netherlands). Samples were filled into 20 ml HDPE tubes (Nalgene, Novodirect, Germany) leaving no headspace and closed with a silicone plug (VWR, Germany). Prior to the pressure application the samples were tempered at the appropriate temperature. Pressure level was varied from 200 to 600 MPa and temperature held constant at 30 °C. Then pressure treatment conditions were kept constant (600 MPa, 30 min) and temperature was varied from 20 and 50 °C. In both cases, pressure was built up with a rate of 200 MPa min⁻¹ and release was varied from 20 to 600 MPa min⁻¹. At least, build-up rate was varied from 20 to 600 MPa min⁻¹. After treatment, the samples were stored 1 day at 4 °C until analysis.

Apparent dynamic viscosity η of the solution was determined before and after pressure treatment at 10 °C by means of a rotational rheometer (AR 2000, TA Instruments, Germany). The shear rate was increased to 500 s⁻¹ in 3 min, held for 5 min at 500 s⁻¹ and decreased to 0 s⁻¹ in 3 min. η was defined as the viscosity at the end of the 5 min holding time at a shear rate of 500 s⁻¹. The η values given in the tables represent the average of 6 to 12 measurements from 3 to 6 independent pressure experiments.

The mean hydrodynamic diameter d_H of the casein particles was determined by photon correlation spectroscopy (PCS) at 25 °C (HPPS, Malvern Instruments Ltd., Malvern, UK). For each sample, three light scattering measurements of 30 s were carried out. The results are calculated from the intensity distribution curve and are the average of 30 to 60 measurements from 3 to 6 independent pressure experiments.

Results and discussion

Influence of pressure level and release rate

In Table VI.1 is shown, that the viscosity η increased with increasing pressure level for the release rate of 600 and 200 MPa min⁻¹. A pressure level of 400 and 600 MPa combined with 600 MPa min⁻¹ release rate resulted in viscosities being higher than control. In contrast, a release rate of 20 MPa min⁻¹ showed no significant difference in η on the various pressure levels and was lower than control. At a pressure of 200 MPa η reached similar values and seemed to be independent from the release rate. In addition, all η values were significantly lower than control. The influence of release rate became visible for the pressure level of 400 MPa and even more at 600 MPa.

Table VI.1: Influence of pressure level and release rate on apparent viscosity and mean hydrodynamic diameter of a 5 % casein solution pressurized for 30 min at 30 °C; build-up rate 200 MPa min⁻¹ *

Pressure level in MPa	Pressure release rate in MPa min ⁻¹	Apparent dynamic viscosity $\eta \pm$ s.d. in mPa s	Mean hydrodynamic diameter $d_H \pm$ s.d. in nm
Control		4.8 ± 0.9	$(3.6 \pm 0.6) \cdot 10^2$
200	600	3.7 ± 0.4	$(5.0 \pm 0.6) \cdot 10^2$
	200	3.7 ± 0.4 ²⁰⁰⁽⁶⁰⁰⁾	$(5.0 \pm 0.7) \cdot 10^2$ ²⁰⁰⁽⁶⁰⁰⁾
	20	3.6 ± 0.4 ^{200(600),200(200)}	$(5.4 \pm 0.7) \cdot 10^2$
400	600	6.3 ± 1.6	$(4.8 \pm 1.1) \cdot 10^2$ ²⁰⁰⁽⁶⁰⁰⁾
	200	4.3 ± 0.5 ^{control}	$(2.9 \pm 0.3) \cdot 10^2$
	20	3.7 ± 0.4 ²⁰⁰⁽²⁰⁾	$(2.5 \pm 0.3) \cdot 10^2$
600	600	6.9 ± 1.9 ⁴⁰⁰⁽⁶⁰⁰⁾	$(3.9 \pm 0.6) \cdot 10^2$ ^{control}
	200	4.8 ± 0.9 ^{control,400(200)}	$(3.3 \pm 0.6) \cdot 10^2$ ^{control}
	20	3.5 ± 0.4 ^{200(20),400(20)}	$(2.3 \pm 0.2) \cdot 10^2$

*Each value is compared with control and values with the same pressure release rate. Example: a value followed by ^{control} is not significantly different ($p < 0.05$) to control; a value followed by ²⁰⁰⁽⁶⁰⁰⁾ is not significantly different to the value of the solution treated at the pressure level of 200 MPa with the pressure release rate of 600 MPa min⁻¹. s.d.: standard deviation

As shown in Table VI.1 the mean hydrodynamic diameter d_H of the casein particles decreased with increasing pressure level. Smallest particles were observed at 600 MPa. Higher d_H than control were determined at 200 MPa for all pressure release rates and at 400 MPa for a release

rate of 600 MPa min⁻¹. The d_H measured after treatment at 600 MPa followed by a pressure release rate of 600 and 200 MPa min⁻¹ appeared in the same size as control. In contrast, the d_H was smaller than control after treatment either at 400 MPa followed by 200 and 20 MPa min⁻¹ pressure release or after 600 MPa and 20 MPa min⁻¹ release. In parallel to viscosity data, the influence of release rate became visible at a pressure of 400 and 600 MPa but not at 200 MPa. The slower the pressure was released, the smaller d_H .

An influence of release rate on the structure of casein gels was observed by Fertsch et al. (2003) for casein solution with 15 % casein. They showed that the higher the pressure release rate was, the higher the firmness of pressure-induced casein gels. During pressure build-up, casein micelles dissociate into submicelles due to the weakening of non-covalent bonds (Mozhaev et al., 1996). During pressure release, these interactions are reactivated (Hinrichs, 2000; Suzuki & Taniguchi, 1972) and calcium bridges from free calcium in the serum phase are reformed (Shibauchi et al., 1992). Instead of the original casein micelles new hyperstructures are built up and casein micelle aggregates are generated (Fertsch et al., 2003; Hinrichs, 2000; Johnston et al., 1992a; Johnston et al., 1992b; Johnston et al., 2002; Masson, 1992; Ohmiya et al., 1989). From the results shown in Table VI.1 it can be assumed that during fast pressure release (600 MPa min⁻¹) the submicelles re-associate to loose and big aggregates having a high water binding which is indicated by η which was even higher than control. Not only the pressure release, but also the pressure level is important.

At 400 MPa the pressure release phase still had an influence on d_H and η , but no influence was noted at 200 MPa. In addition, it was observed that the lower the pressure level, the lower was η . The dissociation of casein micelles is probably just starting at 200 MPa. This implies that the water binding of the formed aggregates at 200 MPa level is lower than that of particles built up at higher pressures.

Huppertz et al. (2004a) also reported on casein aggregate formation in pressure treated (250 MPa, 20 °C) raw skim milk. A decrease of 50 % of the casein particle size compared with the untreated milk was observed after pressure treatment in the range of 300 to 800 MPa (Desobry-Banon et al., 1994; García-Risco et al., 2000; Gaucheron et al., 1997; Needs et al., 2000). In our experiments, a decrease in the hydrodynamic diameter was only observed for the low release rate; at the high release rate of 600 MPa min⁻¹, particles were still larger than control. This difference may be explained by the composition and the particle size measurement. The PCS measurement system allows particle measurement without dilution of the casein solution which may help to avoid changes in the particle size due to dissociation effects.

Influence of pressure build-up

In order to verify that the effect observed in Table VI.1 is mainly due to the pressure release, the effect of pressure build-up rate on η and d_H is shown in Table VI.2.

Table VI.2: Influence of pressure build-up rate on apparent viscosity and mean hydrodynamic diameter of a 5 % casein solution pressurized at 600 MPa and 30 °C for 30 min; pressure release rate 200 MPa min⁻¹ *

Pressure build-up rate in MPa min ⁻¹	Apparent dynamic viscosity $\eta \pm$ s.d. in mPa s	Mean hydrodynamic diameter $d_H \pm$ s.d. in nm
600	4.7 \pm 0.4	(2.9 \pm 0.3)·10 ²
200	4.8 \pm 0.9 ⁶⁰⁰	(3.3 \pm 0.6)·10 ²
20	4.4 \pm 0.5 ^{600,200}	(3.0 \pm 0.5)·10 ² ^{600,200}

*A value followed by ⁶⁰⁰ is not significantly different ($p < 0.05$) to the value of the solution treated with the pressure release rate of 600 MPa min⁻¹. s.d.: standard deviation

The viscosity η of the treated solution was independent of the pressure build-up rate. Pressure build-up had no influence on d_H of the casein particles except at 200 MPa min⁻¹ where a bit larger particles than at 600 MPa min⁻¹ and 20 MPa min⁻¹ were observed.

In summary, the pressure build-up rate seems to be not so important for casein micelle modification in pressure treatment. More important are pressure level and holding phase (dissociation), and finally the pressure release phase in which re-association and aggregation take place being mainly influenced by the rate of pressure release (Table VI.1).

Influence of temperature and pressure release

For pressure treatment at 20 °C, 30 °C and 40 °C, a decrease of η was observed with decreasing pressure release rate (Figure VI.1).

The release rate of 600 MPa min⁻¹ resulted in the highest η , which was significantly higher than control, except at 40 °C where η was similar to control. After 200 MPa min⁻¹ release rate η appeared in the same range than control. The slowest release rate (20 MPa min⁻¹) induced the lowest η . It has to be mentioned, that at a temperature of 30 °C the effect of pressure release rate on η change was highest. With increasing temperature (40 °C, 50 °C) the effect of the pressure release became lower, and at 50 °C no significant influence of pressure release was observed. All η values were approximately 70 % of control at 50 °C.

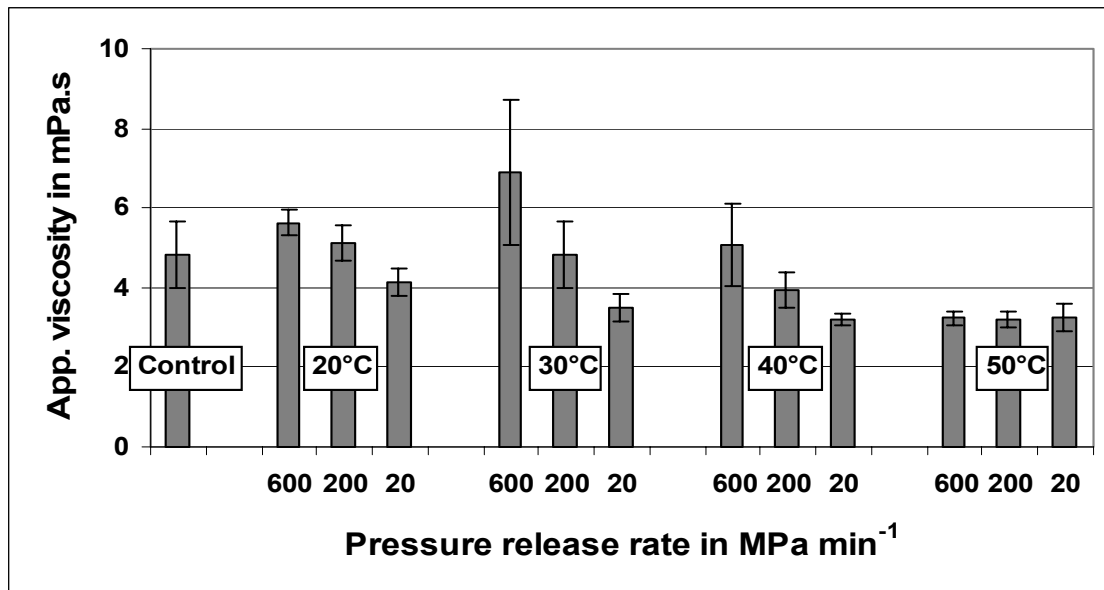


Figure VI.1: Influence of pressure release rate and temperature on apparent viscosity η of a 5 % casein solution treated at 600 MPa for 30 min; build-up rate 200 MPa min⁻¹

At 20 and 30 °C the diameter d_H increased in the treated solution with increasing release rate (Figure VI.2).

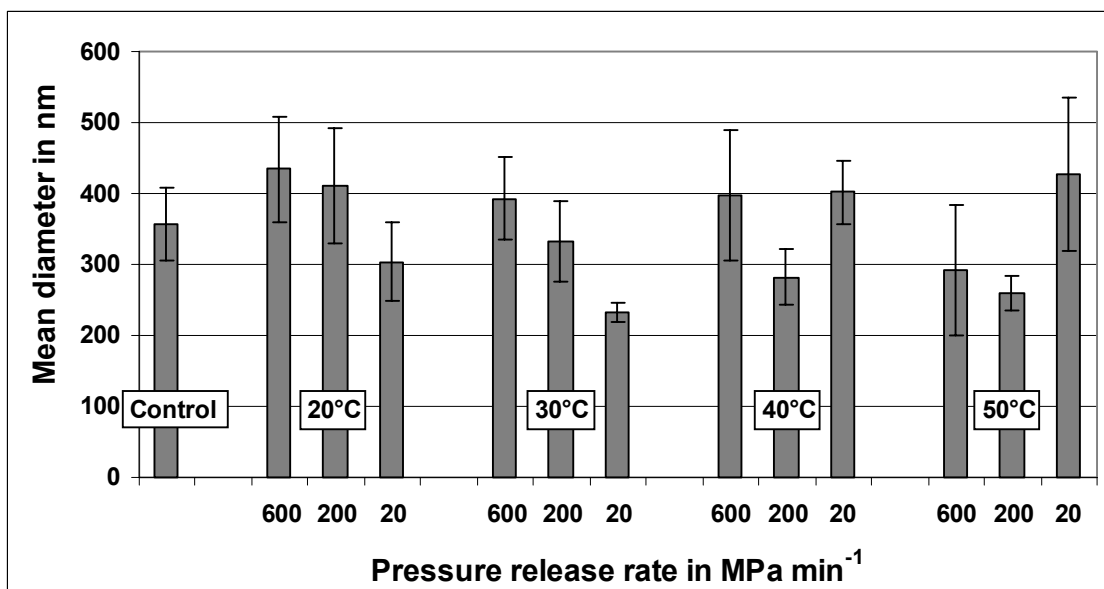


Figure VI.2: Influence of pressure release rate and temperature on mean diameter d_H of a 5 % casein solution treated at 600 MPa for 30 min; build-up rate 200 MPa min⁻¹

The highest d_H of about 420 nm was observed at 20°C after the pressure release of 600 and 200 MPa min⁻¹ and at 30 °C after the release of 600 MPa min⁻¹. At 40 and 50 °C, no clear influence of the release rate on d_H could be observed. At 40 °C combined with release rates of

600 and 20 MPa min⁻¹ and at 50 °C with a rate of 20 MPa min⁻¹, higher diameter than control with a d_H of about 400 nm were detected. In contrast, at 40 °C with a release rate of 200 MPa min⁻¹ and at 50 °C with release rates of 600 MPa min⁻¹ and 200 MPa min⁻¹, particles with a d_H below control (about 300 nm compared to 360 nm for control) were found.

Needs et al. (2000) suggested that during pressure release the association of submicellar particles is induced by the reformation of hydrophobic bonds, which are known to be enhanced with increasing temperature. Their hypothesis may explain some of our results regarding the viscosity η at 40 and 50 °C: the casein particles may be packed denser, water binding is reduced resulting in the observed decrease in viscosity being lower than control (Figure VI.1). Huppertz et al. (2004a) reported a high increase of the micelle size compared to control by increasing the temperature from 5 to 40 °C at 250 MPa. They explained this increase with the extensive formation of hydrophobic bonds between the submicellar particles inducing the formation of casein aggregates. Others (Gaucheron et al., 1997; García-Risco et al., 2000; Huppertz et al., 2004a) as well detected large casein aggregates in milk treated at pressures between 250 and 400 MPa and temperatures from 40 to 60 °C. But none of the authors mentioned the pressure release rate applied which might give an additional effect as demonstrated in Figure VI.2.

In summary, the experimental results demonstrated that casein micelles react very sensitive to the different phases of pressure treatment and also to the temperature applied during treatment. Especially, the effect of pressure release rate should be mentioned when working with casein containing solutions.

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Chapter VII

Concluding remarks

The problematic of the photon correlation spectroscopy measurement is shortly introduced at the beginning of this chapter. Furthermore, the influence of the different phases of pressure treatment on the properties of casein was studied in detail and a model is proposed.

Photon correlation spectroscopy

The size of the casein micelles was measured by means of photon correlation spectroscopy (PCS) using back scattering at an angle of 173° . This method enables to measure undiluted samples preventing the dissociation of the casein micelles because of dilution. The hydrodynamic diameter of the particles was determined and not the real diameter of the casein micelles. If particles aggregate, the diameter of the whole aggregate was determined. With PCS measurements, it is impossible to know whether larger casein particles were induced by a fusion of casein micelles or casein aggregates were built up. Therefore it is important to make microscopic images of the structures for a better understanding.

PCS measurements of a 5 % casein solution treated at 600 MPa at 30 °C for 30 min (Table VII.1, results from chapter III) showed bigger particles after a fast pressure release of 600 MPa min⁻¹. In contradiction, AFM images (Figure VII.1, results from Chapter III) showed smaller casein particles. Furthermore, the viscosity of these new formed structures was higher than before pressure treatment.

Table VII.1: Average hydrodynamic diameter of casein micelles in untreated and pressurized casein solution [5 g casein/100ml UFP].	
Pressure release rate [MPa min ⁻¹]	Average hydrodynamic diameter d_H [nm]
untreated	377 ± 46^a
600	477 ± 65^b
200	$395 \pm 93^{a,b,c}$
20	231 ± 7^d

* Data within a column followed by different letters are significantly different at $p < 0.05$

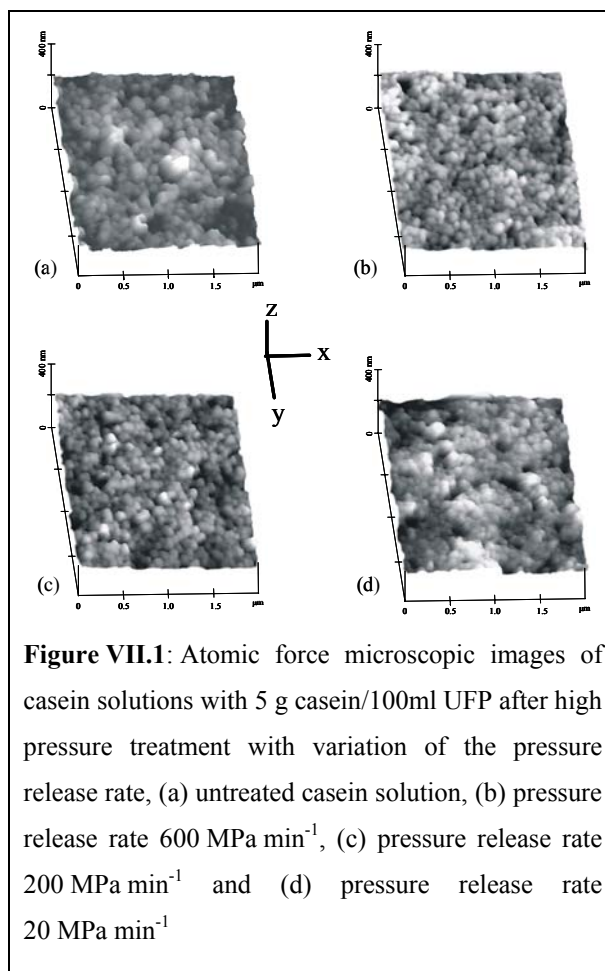


Figure VII.1: Atomic force microscopic images of casein solutions with 5 g casein/100ml UFP after high pressure treatment with variation of the pressure release rate, (a) untreated casein solution, (b) pressure release rate 600 MPa min⁻¹, (c) pressure release rate 200 MPa min⁻¹ and (d) pressure release rate 20 MPa min⁻¹

Combining these observations, it can be assumed that the casein particles with large hydrodynamic diameter measured by means of PCS are built up of small casein micelles. These aggregates of small particles incorporate some serum resulting in the high water binding and in an increased viscosity of the solution compared to the untreated solution. In the case of particle size measurement of casein micelles, it is important to verify the PCS results with microscopic observations of the structures.

Model for the high pressure-induced casein modification

The model presented as initial hypothesis of this work in chapter II (Figure II.6) should be completed with the results presented in chapter III through chapter VI. The influence of the different high pressure process parameters especially pressure release rate but also pressure build-up, pressure level and holding time and milieu conditions on pressure-induced casein structure is summarized in the model illustrated in Figure VII.2 (formation of particles and aggregates) and Figure VII.3 (formation of gels).

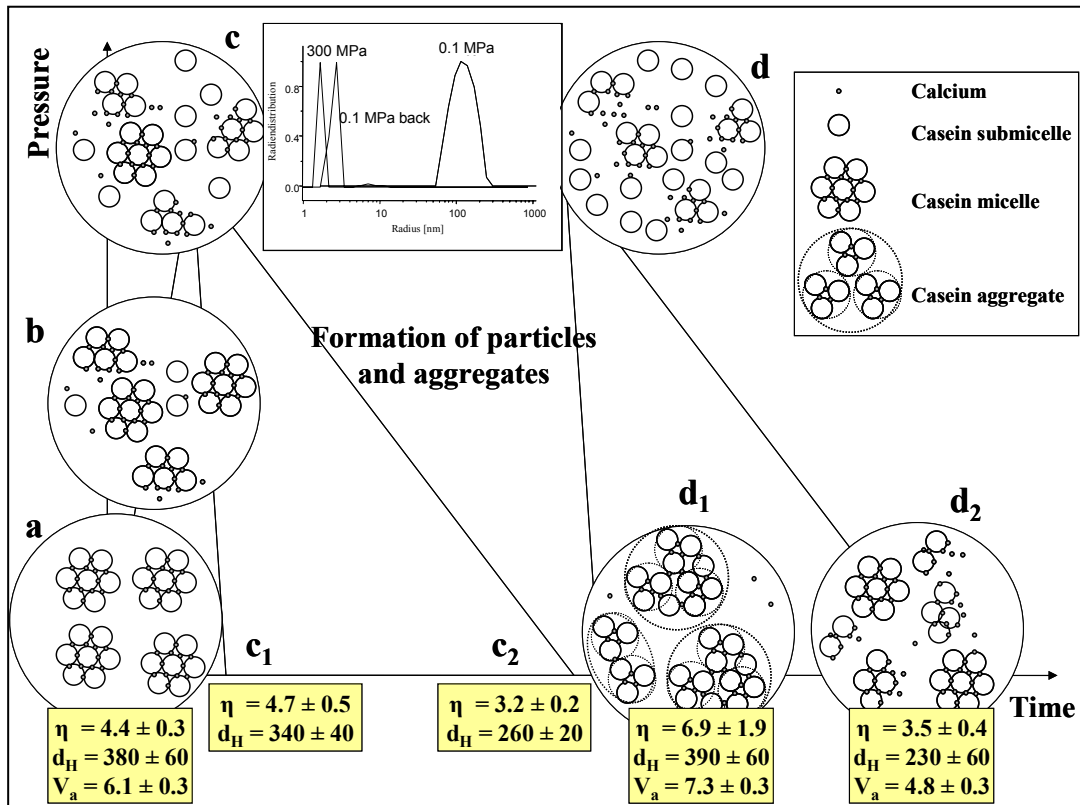


Figure VII.2: Model of the dissociation and aggregation of casein micelles during high pressure treatment of a casein solution with low casein content ($c_{\text{casein}} \leq 5\%$). η : viscosity of the solution [mPa s]; d_H : mean hydrodynamic diameter of the casein micelles [nm]; V_a : voluminosity of the solution [ml g^{-1}] (Distribution function from Gebhardt, 2005)

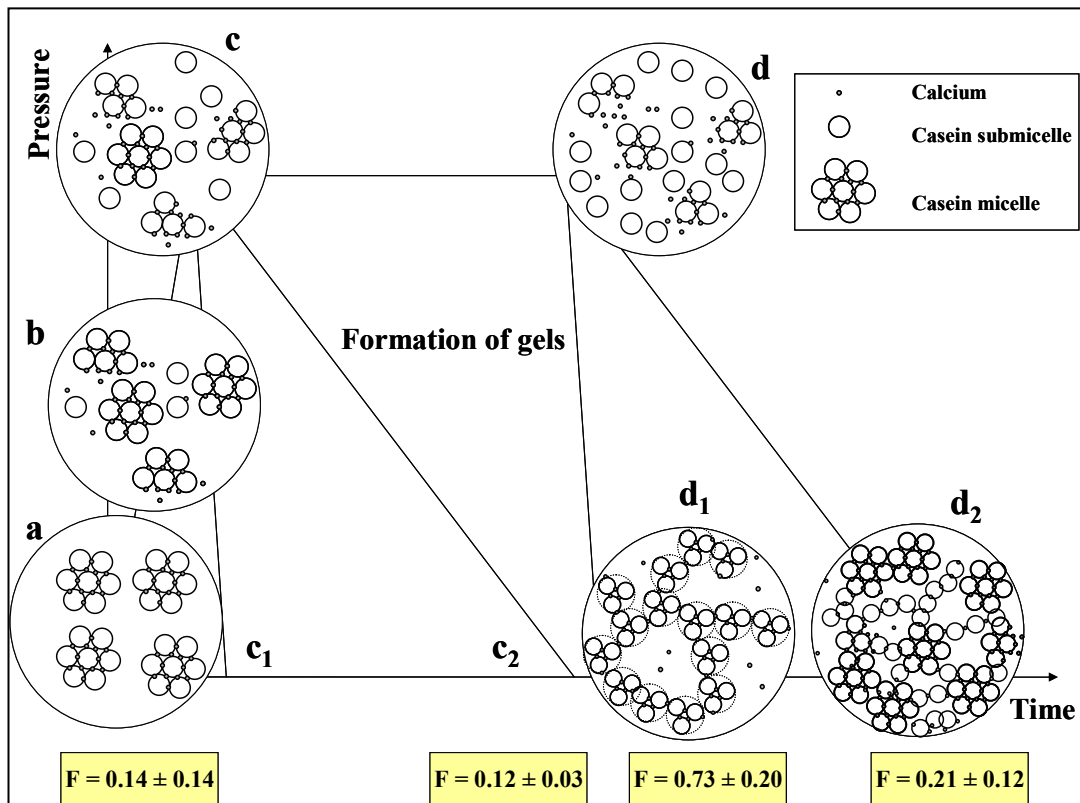


Figure VII.3: Model of the dissociation and gelation of a casein solution (high casein content $c_{\text{casein}} > 7\%$) during a high pressure treatment. F : Firmness of the gel [N].

Pressure build-up phase

Pressures up to 200 MPa induce the dissociation of casein micelles in submicelles and monomers (Figure VII.2 and 3, a→b→c) due to the solubilisation of colloidal calcium phosphate out of the micellar framework and the weakening of the non-covalent bonds stabilizing the casein micelle. At a pressure of 300 MPa, casein micelles are already dissociated into smaller units and casein monomers (Gebhardt et al., 2005), distribution function between c and d on Figure VII.2). This dissociation is accompanied by a decrease of the particle size and an increase of the viscosity of the casein solution after pressure build-up (Table VII.2).

Table VII.2: *In situ* viscosity measured at 30 °C during pressure treatment at 600 MPa of a 5 % casein solution and ultrafiltration permeate

	5 % casein solution	ultrafiltration permeate
	η in mPa s	η_0 in mPa s
Control	2.0 ± 0.1	0.9 ± 0.0
After pressure build-up at 600 MPa	4.7 ± 0.5	1.2 ± 0.1

η : dynamic *in situ* viscosity of the casein solution, η_0 : dynamic *in situ* viscosity of the ultrafiltration permeate

The volume fraction of the hydrated casein can be estimated from the *in situ* viscosity using the model by Gleissle & Baloch and Windhab presented in chapter II and III (Eq. II.25 and III.6, here Eq. VII.1).

$$\frac{\eta}{\eta_0} = (1 - 2.5 \varphi)^{-1} \quad (\text{VII.1})$$

where η is dynamic viscosity of the solution [Pa s], η_0 the viscosity of the solvent [Pa s] and φ the volume fraction of the particles.

Equation VII.1 can be transformed in:

$$\varphi = \frac{\eta - \eta_0}{2.5 \eta} \quad (\text{VII.2})$$

According to the values of Table VII.2, the volume fraction of casein at ambient high pressure is

$$\varphi_{0.1 \text{ MPa}} = 0.22$$

and after pressure build-up at 600 MPa:

$$\varphi_{600 \text{ MPa}} = 0.37$$

The volume fraction of casein increases during pressure build-up. The increase of viscosity must be due to an increase of surface of casein being in interaction with water resulting in high hydration.

The rate of pressure build-up has no significant influence on casein micelles modification (viscosity and hydrodynamic diameter) after pressure treatment (Chapters IV and VI).

Pressure holding phase

During pressure holding time the dissociation into casein fragments and monomers comes to an equilibrium (Figure VII.2 and VII.3, c→d). At 600 MPa, the longer holding time (30 min) induces firmer structures after fast pressure release (Figure VII.2 and VII.3, d→d₁) than no holding time or a shorter one (Figure VII.2 and VII.3, c→c₁) (Chapter IV). Dissociation of the micelles may not be finally completed when the holding time is too short resulting in an inhomogeneous gelation during pressure release phase.

Pressure release phase (Chapter III to Chapter VI)

The last phase of a pressure treatment, the pressure release phase, plays a very important role in the rearrangement of the casein fragments and monomers. The small fragments and monomers aggregate during pressure release phase because the binding forces take effect again. Protein-protein interactions are reestablished and the self association process of the casein submicelles and monomers is reinitiated with the formation of calcium bridges. The formed casein structures differ from the native structure because the association rate of the casein depends on the release rate. To explain the differences, equation VII.3 (Eq II.3 from chapter II) has to be considered:

$$J_{peri} = \frac{8k_B T}{3\eta_0} N \quad (\text{VII.3})$$

where J is the number of encounters per unit time [s^{-1}] for equal-sized spheres, η_0 the viscosity of the continuous phase [Pa s], k_B the Boltzmann constant [$J K^{-1}$], T the absolute temperature [K] and N is the number of particles per unit volume (or particle number concentration) [m^{-3}].

In the case of a fast release, the high number of particles N before pressure release and the fast decrease of the viscosity η_0 during the release induce an aggregation of the particles. All bonds are reactivated and each encounter between particles induces aggregation.

In the case of a slow release, the number of particles before pressure release is high and the viscosity stays first high during release. Bonds are not reactivated immediately and particles

do not move so quickly so that aggregation doesn't occur for each encounter between particles.

Thus, different structures can be obtained by varying the pressure release rate. At 600 MPa, structures from sol to gel could be induced by increasing the release rate and the protein concentration (Figure VII.4, from Chapter V).

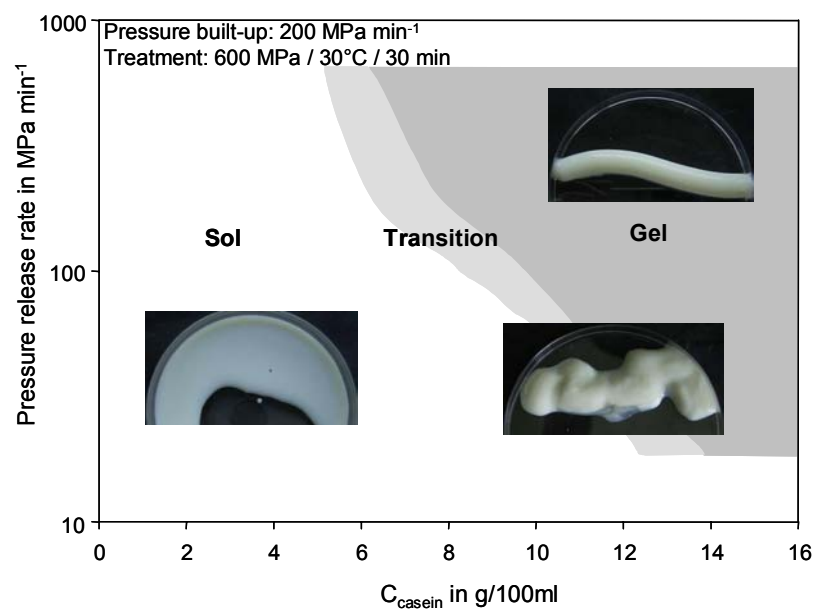


Figure VII.4: Structure diagram: Influence of pressure release rate and casein content on gel-sol-transition of casein after high pressure treatment at 600 MPa, 30 min, 30 °C.

At a casein content of about 7 %, depending on the pressure release rate, sol as well as gel structures were observed. The higher the casein content and the faster the pressure release, the firmer were the structures built up and the finer were the microstructures. The influence of the casein content is due to the inner friction of the dispersed particles with the outer phase, and to the high water binding of the casein.

Casein and calcium concentration

Increasing the casein concentration and calcium content leads to the formation of gel structures after pressure release (Figure VII.4 and results of chapters III and V). The higher the casein and calcium content, and the faster the decompression the firmer are the gels that are built up. However, too high calcium may induce a salting out effect: water molecules are needed for the hydration of the ions and at high ionic strength the solubility of proteins is decreasing. The caseins become more compact, the water binding is reduced resulting in a low viscosity and gel formation is retarded.

This model is supported by the bonds analysis of Keim (2005) demonstrating that despite the texture being changed from sol to gel no difference in the stabilizing bonds between non treated casein and pressure-induced 15 % casein gel was determined. The weakening of the protein bonds could be seen as reversible; however, after pressure release a new network structure is built up.

The model presented in chapter II has been completed with new results. In future besides pressure level, holding time and temperature, the release rate should be taken into account when working with milk or casein systems.

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Summary

Besides the inactivation of microorganisms, high pressure can also be applied to modify proteins. Owing to their molecular composition, their conformation and their quaternary structure, proteins react differently to pressure and temperature, whereby their functional properties are influenced in many cases, depending on the intensity of the treatment. The so far accomplished research about structure formation of milk proteins deals essentially with the effect of a static pressure treatment or the effect of different pressure levels on the structure of the molecules observed *in situ*. A few studies exist about the influence of pressure release, but systematic researches has not been undertaken.

The main component of the milk proteins, the casein, is structured in micelles. Casein monomers aggregate to submicelles due to electrostatic and hydrophobic interactions. The submicelles are bound together by calcium bridges to form the casein micelles. Two submicelle types exist: one type essentially contains α_s -casein and β -casein, the other one α_s -casein and κ -casein. The hydrophobic and calcium sensitive fractions α_s -casein and β -casein are situated in the inner of the micelle, the calcium insensitive κ -casein at the surface. The hydrophilic caseinomacropeptide of the κ -casein is situated outside the micelles reaching into the surrounding medium and builds a hydrate envelope stabilizing the micelle because of steric and electrostatic repulsions. For this reason the hydrophilic caseinomacropeptide is responsible for the stability of casein micelles and inhibits the aggregation of casein in milk.

As shown in preliminary tests, structures stabilized by non-covalent interactions, like casein, are mostly influenced by the pressure release phase. Accordingly, the following work hypothesis was formulated: the pressure release rate plays an important role for the structure formation of caseins, which are mostly stabilized by non-covalent interactions.

The focus of the work was to study the influence of pressure treatment conditions on pressure-induced casein structures in detail. The influence of process parameters like pressure build-up, pressure level, holding time and release rate but also temperature, ionic strength and casein concentration were determined.

The used enriched micellar casein powder was gained by diafiltration of skim milk with ultrafiltration permeate. Casein concentrates (1 to 15 % casein, pH 6,0) were treated with pressures of 200 MPa to 600 MPa. Pressure build-up rate (20 to 600 MPa min⁻¹), holding time (0 to 30 min), release rate (20 to 600 MPa min⁻¹), temperature (20 to 50 °C) and calcium

concentration (ionic strength 0.1 to 2 mol l⁻¹) were varied and the obtained structures characterized regarding *ex situ* and *in situ* viscosity, serum binding, texture and particle size. Depending on the casein concentration and the pressure release rate, liquid (sol) to solid (gel) structures were generated. Structures with a near to Newtonian flow behavior were called “sol”. Structures with a continuous and stable network were called “gel”. Samples having a very weak gel structure with phase separation were classified into the “transition phase” (between “sol” and “gel”). The hypothesis could be confirmed: pressure release rate influences structures stabilized by non-covalent bonds like casein micelles. Another important result is that the higher the release rate, the firmer the structures obtained. The formation of firm pressure-induced casein gels is not only influenced by the protein concentration but particularly by the pressure release rate.

Furthermore the effects of pressure build-up, pressure level, holding time and release rate but also of temperature and ionic strength on particle size, composition of the particle, voluminosity and viscosity were analysed in detail and the mechanisms were examined. Thereby the importance of the above mentioned parameters on the formation of different structures was shown and a model about the pressure-induced modification of casein depending on process parameters and milieu conditions was presented.

This work showed that the structure formation of casein under high pressure treatment depends on numerous factors. Sols but also gels can be formed and could be used for different applications particularly with the choice of the release rate and the milieu conditions, even if pressure conditions and casein concentration are kept constant.

Zusammenfassung

Das Verfahren der Ultrahochdruckbehandlung kann neben der Inaktivierung von Mikroorganismen auch zur Modifikation von Proteinen eingesetzt werden. Proteine reagieren aufgrund ihres molekularen Aufbaus, ihrer Konformation und ihrer quartären Struktur unterschiedlich auf Druck und Temperatur, wodurch sich in vielen Fällen, abhängig von der Intensität der Behandlung, die funktionellen Eigenschaften beeinflussen lassen. Die bisher durchgeführten Untersuchungen zur Strukturbildung von Milchproteinen befassen sich im Wesentlichen mit dem Effekt einer statischen Druckbeaufschlagung oder der Wirkung unterschiedlicher Druckniveaus auf die Struktur der Moleküle, die *in situ* beobachtet werden. Ferner existieren einige wenige Arbeiten zum Einfluss des Druckabbaus; systematische Untersuchungen wurden jedoch bisher nicht durchgeführt.

Betrachtet man die Hauptfraktion der Milchproteine, die Caseine, so sind diese zu Micellen assoziiert. Caseinmonomere aggregieren zu Submicellen aufgrund elektrostatischer und hydrophober Wechselwirkungen. Diese Submicellen werden durch Calciumbrücken gebunden und bilden Micellen. Zwei Arten von Submicellen sind zu unterscheiden: Die eine enthält hauptsächlich α_s -Casein und β -Casein, die andere α_s -Casein und κ -Casein. Die hydrophoben und calciumempfindlichen Fraktionen, α_s -Casein und β -Casein, befinden sich im Inneren der Micelle, die calciumunempfindliche Fraktion, κ -Casein, an der Oberfläche. Das hydrophile Caseinomaclopeptid des κ -Caseins ist in Milchserum gerichtet und bildet aufgrund sterischer und elektrostatischer Abstoßungen eine stabilisierende Hülle. Damit ist das hydrophile Caseinomaclopeptid wichtig für die Stabilität der Caseinmicellen und verhindert zudem die Aggregation der Caseine in der Milch.

Wie sich in Vorversuchen zeigte, werden Strukturen wie Caseine, die über nicht-kovalente Bindungen stabilisiert sind, maßgeblich durch die Druckentspannungsphase beeinflusst. Davon ausgehend wurde die Arbeitshypothese formuliert: Für Caseine, die hauptsächlich durch nicht-kovalente Bindungen stabilisiert sind, besitzt die Entspannungsphase einen maßgeblichen Einfluss auf die sich ausbildenden Strukturen.

Ziel der Arbeit war es, die Druckbehandlungsbedingungen in ihrer Auswirkung auf die Strukturausbildung in Caseinsystemen im Detail zu untersuchen. Neben den Prozessparametern Druckaufbaurate, Druckhöhe, Haltezeit und Entspannungsrate wurde der Einfluss der Temperatur, der Konzentration an Casein aber auch des Ionenmilieus bei der Druckbehandlung untersucht.

Das verwendete Caseinpulver wurde mittels Magermilch-Diafiltration mit einem Ultrafiltrationspermeat hergestellt. Caseinkonzentrate (1 bis 15 % Casein, pH 6) wurden mit Drücken von 200 MPa bis 600 MPa behandelt. Die Druckaufbaurrate (20 bis 600 MPa min⁻¹), Druckhaltezeit (0 bis 30 min), Druckabbaurate (20 bis 600 MPa min⁻¹), Temperatur (20 bis 50 °C) und Calciumkonzentration (Ionenstärke 0.1 bis 2 mol l⁻¹) wurden variiert, und die gewonnenen Strukturen hinsichtlich *ex situ* und *in situ* Viskosität, Serumbindung, Textur und Partikelgröße charakterisiert.

In Abhängigkeit von der Proteinkonzentration und der Entspannungsrate wurden Strukturen von Sol über einen Übergangsbereich bis hin zu festen Gelen ausgebildet. Als „Sol“ wurden Strukturen mit nahezu newtonschem Fließverhalten charakterisiert. Als „Gel“ wurden Strukturen definiert, die ein kontinuierliches und stabiles Netzwerk ausbilden. Zwischen diesen zwei Bereichen, dem Übergangsbereich, bilden sich Strukturen aus, die eine Joghurt-ähnliche Textur und teilweise Phasentrennung aufweisen. Damit wurde die Hypothese bestätigt, dass Strukturen, die über nicht-kovalente Bindungen stabilisiert sind, wie es bei Caseinmicellen der Fall ist, in ihren Eigenschaften insbesondere durch die Entspannungsphase zu modulieren sind. Festzuhalten ist ferner als wesentliches Ergebnis, dass je höher die Entspannungsrate gewählt wird, desto fester die erzeugten Strukturen werden. Das Ausbilden fester druckinduzierter Caseingele hängt somit nicht nur von der Proteinkonzentration, sondern insbesondere von der Entspannungsrate ab.

In weiteren Versuchen wurden die Einflüsse von Druckniveau, Druckaufbaurrate, Druckhaltezeit, Druckabbaurate, Temperatur und Ionenstärke und Caseinomacropetid-Menge hinsichtlich der Partikelgröße, Aufbau der Partikel, Voluminosität und Viskosität im Detail studiert und die Mechanismen untersucht. Dabei wurde die Bedeutung der oben genannten Parameter auf die Ausbildung unterschiedlicher Strukturen und deren Mikro- und Makrostruktur aufgezeigt, und daraus wurde eine Modellvorstellung der druckinduzierten Modifikationen der Caseine in Abhängigkeit von den Prozessparametern und Milieubedingungen erarbeitet.

Mit der Arbeit wurde aufgezeigt, dass die Strukturierung von Casein als Resultat einer Hochdruckbehandlung von zahlreichen Faktoren abhängt. Insbesondere durch die Wahl der Entspannungsrate und der Milieubedingungen lassen sich bei ansonsten gleicher Druckbehandlungsbedingungen und Caseinkonzentration sowohl Sole als auch Gele für die unterschiedlichsten Applikationen erzeugen.

Appendix

Appendix I: Pilot apparatus for high pressure treatment

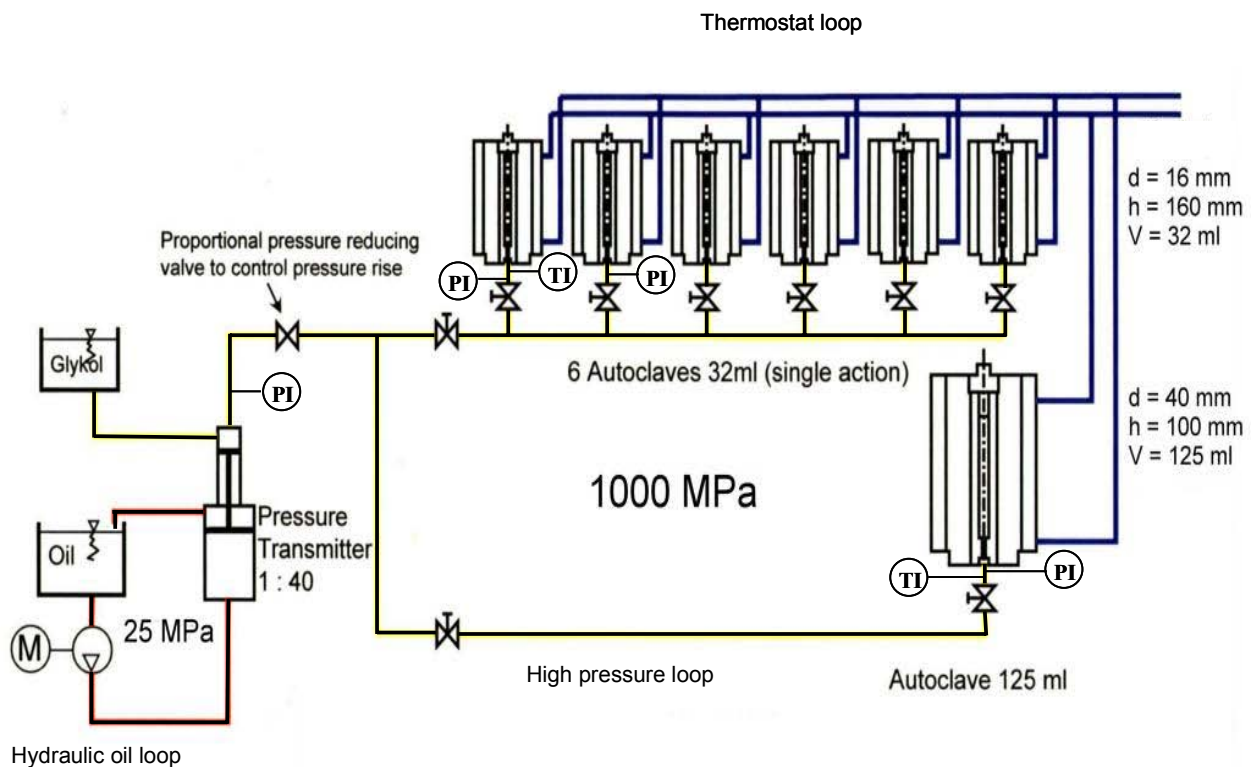


Figure IX.1: High pressure pilot apparatus (Resato High Pressure Technology, Roden, The Netherlands and Knam Schneidertechnik, Langenargen, Germany); d: diameter pressure chamber for the sample, h: height of the chamber, V: volume of the pressure chamber, PI: pressure indicator, TI: temperature indicator.

In the used high pressure pilot apparatus, products can be treated up to temperatures of 100 °C and pressures of 1000 MPa (Figure IX.1). The pilot plant has seven autoclaves, 6 small ones with a volume of 32 ml and a big one with a volume of 125 ml. The 125 ml autoclave was used for all experiments.

Appendix II: Production of the micellar casein powder

The casein powder used in the experiments was an enriched micellar casein powder produced by diafiltration of skim milk at the Institute for Food Process Engineering in Freising, Germany (Kersten, 2001). Skim milk was diafiltrated by means of microfiltration (MF) (MF module 7P19-40GL; cut off: 100nm, APV, Silkeborg, Denmark). The MF-permeate obtained was ultrafiltrated (UF module GR 60 PP; cut off: 25 kDa, DDS AS, Nakskov, Denmark) and used for diafiltration. After six washing steps the casein retentate was concentrated by microfiltration (concentration factor 4) and spray dried (Niro Atomizer, Soeborg, Denmark) (Figure IX.2).

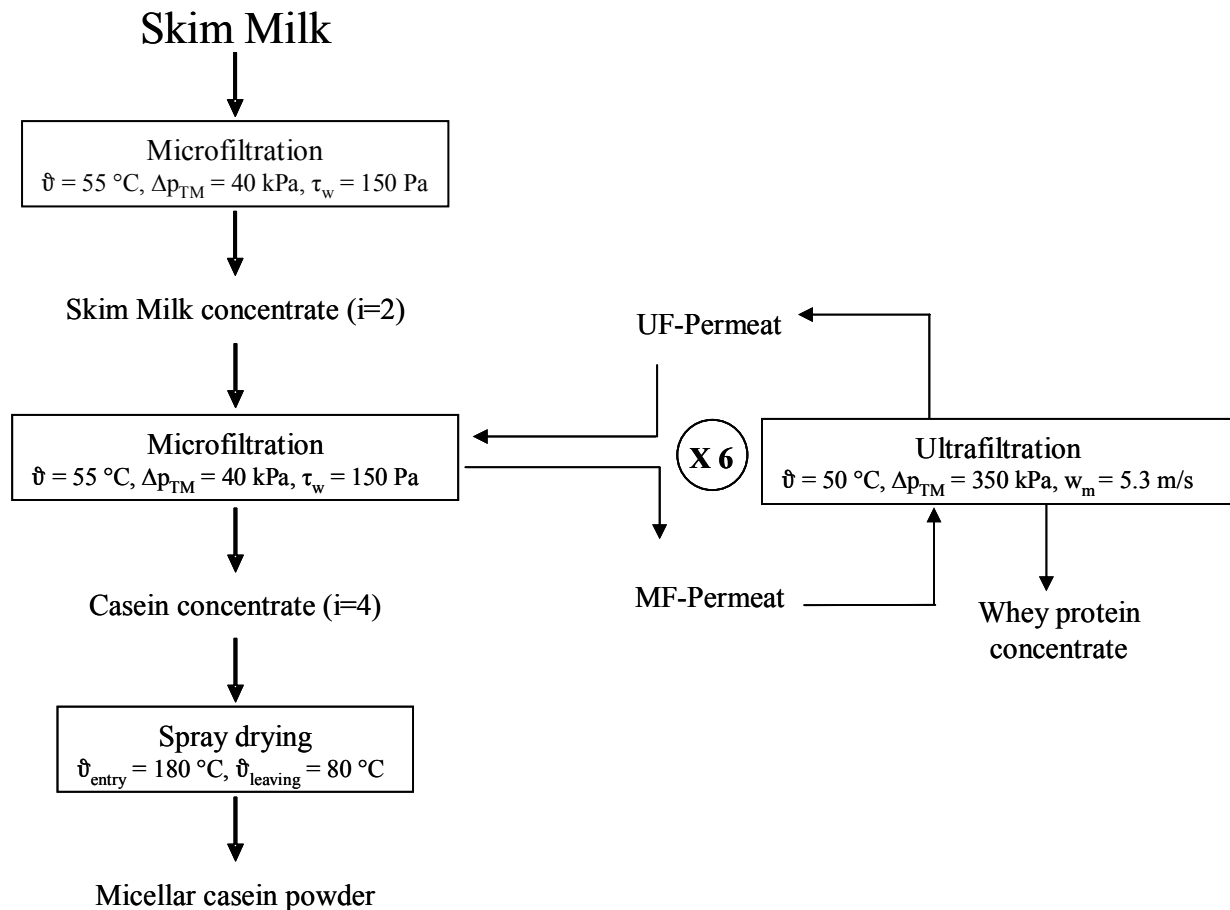


Figure IX.2: Process steps of the production of the micellar casein powder; i : concentration factor, ϑ : temperature during the process, Δp_{TM} : trans membrane pressure, τ_w : wall shear stress, w_m : mean velocity, ϑ_{entry} : temperature at the beginning of the spray drying, $\vartheta_{leaving}$: temperature at the end of the spray drying.

The final micellar casein powder contained 6.5 % water, 68.4 % total protein including 68.0 % of casein, 16.6 % lactose and 8.4 % minerals including 2.3 % calcium.

Appendix III: Results of chapter VI about the influence of temperature and pressure release on apparent viscosity and mean diameter

Table IX.1: Influence of pressure release rate and temperature on apparent viscosity η and mean diameter d_H of a 5 % casein solution treated at 600 MPa for 30 min; build-up rate 200 MPa min⁻¹

Temperature in °C	Pressure release rate in MPa min ⁻¹	Apparent dynamic viscosity $\eta \pm$ s.d. in mPa s	Mean hydrodynamic diameter $d_H \pm$ s.d. in nm
Control		4.8 ± 0.9	$(3.6 \pm 0.6) \cdot 10^2$
20	600	5.6 ± 0.3	$(4.4 \pm 0.8) \cdot 10^2$
	200	5.1 ± 0.5	$(4.1 \pm 0.9) \cdot 10^2$
	20	4.1 ± 0.4	$(3.0 \pm 0.6) \cdot 10^2$
30	600	6.9 ± 1.9	$(3.9 \pm 0.6) \cdot 10^2$
	200	4.8 ± 0.9	$(3.4 \pm 0.6) \cdot 10^2$
	20	3.5 ± 0.4	$(2.3 \pm 0.2) \cdot 10^2$
40	600	5.1 ± 1.1	$(4.0 \pm 1.0) \cdot 10^2$
	200	4.0 ± 0.5	$(2.8 \pm 0.4) \cdot 10^2$
	20	3.2 ± 0.2	$(4.0 \pm 0.5) \cdot 10^2$
50	600	3.2 ± 0.2	$(2.9 \pm 1.0) \cdot 10^2$
	200	3.2 ± 0.3	$(2.6 \pm 0.3) \cdot 10^2$
	20	3.3 ± 0.4	$(4.2 \pm 1.1) \cdot 10^2$

Control: no pressure treatment; s.d.: standard deviation

Appendix IV: Analysis methods

Calcium:

The calcium amount of the casein powder was determined using the EDTA-method (C10.6.8, Methodenbuch (1985), Handbuch der landwirtschaftlichen Versuchs- und Untersuchungsmethodik, Band VI, vierte Auflage, VDLUFA-Verlag, Darmstadt.).

Water content:

The water content of the casein powder was determined with the method for dry milk products (C35.6, Methodenbuch VI, 1985).

Ashes content

The gravimetric measure of the ashes content of the casein powder was determined after the method C35.6 (Methodenbuch VI, 1985).

Protein content

The total protein content was calculated from nitrogen assayed by a nitrogen analyzer using Dumas method (LECO FP-528, Leco Instrumente GmbH, Moenchengladbach, Germany) and using a transformation factor of 6.38.

pH-value:

The pH-value of the samples was determined with a pH-Electrode (Blue line, Schott, Mainz).