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Application of low-intensity ultrasound to characterise the microstructure of model food systems

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Contents

Symbols and Abbreviation

α	attenuation coefficient [1/m]
α/f^2	ultrasonic attenuation [s^2/m]
α-la	α-lactalbumin
β-lg	β-lactoglobulin
ΔH	enthalpy [J/mol]
κ	compressibility [1/Pa]
κ_s	adiabatic compressibility of the solution [1/Pa]
κ_{s0}	adiabatic compressibility of solvent [1/Pa]
ρ	density [kg/m^3]
ϕ_c	osmotic coefficient for the coil [-]
ϕ_h	osmotic coefficient for the helix [-]
A_n	amplitude of the n-th echo [-]
A_{n-1} [-]	amplitude of the (n-1)-th echo [-]
A_{us}	peak area in the curve of first derivative of the ultrasonic velocity against temperature [m/s]
c	ionic concentration [eq/L]
CMP	caseinomacropeptide
d	distance between the transmitter and receiver [m]
DA	degree of aggregation [%]
DSC	differential scanning calorimetry
f	frequency [1/s]
G’	storage modulus [Pa]
G’’	loss modulus [Pa]
GDL	glucono-δ-lactone

HDL	high density lipoproteins
HPLC	high performance liquid chromatography
K’	bulk modulus [Pa]
LDL	low density lipoproteins
n	numbering [-]
n_h	number of water molecules bound to each molecule solute [-]
n_s	number of mol of solute [-]
n_w	number of mol of water [-]
ϑ_g	gelling temperature [°C]
ϑ_m	melting temperature [°C]
T	absolute temperature [K]
T	periodic time [s]
t_c	coagulation time [min]
T_g	gelling temperature [K]
T_m	melting temperature [K]
v	ultrasonic velocity [m/s]
V_c	coagulation rate [m/s^2]
WPI	whey protein isolate

1 Introduction

Ultrasound is sound with a frequency over 20 kHz, i.e., above the humans' audibility of up to 16-18 kHz (Tietz, 1974). Although studies of inaudible acoustic waves started in the 19th century, modern science of ultrasonics did not occur until about 1917 (Graff, 1981). Ultrasonic technology was first developed as a means of submarine detection in World War I.

Ultrasonic waves are mechanical waves. They propagate as stresses and strains in the physical bonds of the material. The application of ultrasound can be divided in two categories depending on the power level of the applied ultrasound: the low-intensity ultrasound at high frequency (> 1MHz) and the high-intensity ultrasound at low frequency (20-100 kHz) (Povey & Mason, 1998).

Low-intensity ultrasound uses very low power levels (< 1 W/cm^2) so that the physical and chemical properties of the material are not changed by the ultrasound travelling through it. The speed and efficiency of the transmission is sensitive to the nature of the bonds and masses of the molecules present and therefore to composition (Coupland & McClements, 2001). Low-intensity ultrasound can be used as a technique for providing information about the physicochemical properties of the materials. The principle is that the ultrasonic wave can be changed by the molecular interaction of the sample while it travels through the sample. By comparing the incident and resultant ultrasonic wave the structure in the sample can be concluded (McClements, 1995). In the biochemical area the ultrasonic method is a sensitive method for determining the adiabatic compressibility and the hydration state of molecules. Among the applications of low-intensity ultrasound are measurement of gas and liquid flow, measurement of pressure and temperature in elastic materials, quality control of metals and non-metals, measurement of elastic properties, medical diagnosis, and so on.

In contrast, the power levels used in high-intensity ultrasound are large (typically 10-1000 W/cm^2) to cause cavitation and hence to physically and chemically change the material which they are applied to (McClements, 1995). The high-intensity ultrasound can be used to promote many effects, such as heating, stirring, cavitation, diffusion, cleaning, as well as chemical, mechanical, electrolytical and vaccum effects (Martini, 2007). For example, high-intensity ultrasound is used to homogenize or decompose the samples, or to promote certain chemical reactions (e.g., oxidation). The history of high-intensity ultrasound can be traced back to 1927 when it was reported that ultrasound was extremely efficient for the production of an oil and water emulsion (Povey & Mason, 1998).

This work was focused on the application of the low-intensity ultrasound only. The main advantages of the application of low-intensity ultrasound are that it is a rapid, non-destructive and suitable method for concentrated and opaque samples. All these properties make the ultrasonic technique as an interesting method for the monitoring of processes. An important aspect of low-intensity ultrasound is that it may be easily integrated with other sensor modalities. This may be important in enhancing existing process control strategies and in improving understanding the process itself (Povey & Mason, 1998).

Research about the application of low-intensity ultrasound has been conducted in many areas. These include phase transition, emulsion stability, aggregation processes, crystallisation, freezing processes, conformational changes of molecules. Under these many applications, the ultrasonic characterisation of colloids including particle sizing and zeta potential measurement is a well-established area. There are already commercial ultrasonic spectrometers for particle sizing and electroacoustic spectrometers for both particle sizing and zeta potential on the market. A good refrence for fundamentals and applications of ultrasound for characterizing colloids is a book written by Dukhin and Goetz (2002), two of the developers of an electroacoustic spectrometer. In Tab. 1.1 the references about the applications of ultrasonic measurement on different food materials since 1996 are listed. Earlier references were already listed by Povey (1998).

However, due to the complexity of food low-intensity ultrasound response data are often difficult to interpret. In food industries, the applications of the low-intensity ultrasound are restricted to very few areas. Commercial available ultrasonic sensors include sensors for measurement of flow rate and filling level, concentration and density determination. However, as a method for the structure characterisation of the food materials, the ultrasonic method is still not well developed. The non-destructive property of the low-intensity ultrasound makes it especially suitable for the structure characterisation. Due to the applied high frequency, the ultrasonic method can detect changes at the molecular level, which cannot be detected by the oscillatory rheometry. Thus, the ultrasonic method may provide additional information about the microstructure of food systems. To develop the low-intensity ultrasonic method in addition to established analysis methods for more applications in industry or in research, especially for the structure characterisation of food systems, comprehensive information about the dependence of the ultrasonic properties on the structure or structural change in different products is required.

Tab. 1.1: Ultrasonic measurements of food materials.

Overviews	Javanaud, 1998; Povey & Mason, 1998; Coupland & McClements, 2001; Mulet et al., 2002; Prakash & Ramana, 2003; Coupland, 2004
Milk components	Bryant & McClements, 1999; Famelart et al., 1999; Apenten, et al., 2000; Corredig et al., 2004a; Corredig et al., 2004b
Dairy products	Benedito et al., 2000; Buckin & Kudryashov, 2001; Nassa et al., 2001; Smyth et al., 2001; Llull et al., 2002; Chou & Irudayaraj, 2003; Nassar et al., 2004; Dwyer et al., 2005;Dukhin et al., 2005; Gan et al., 2006; Wang et al., 2007
Emulsions, Dispersions	Hibberd et al., 1997; Chanamai et al., 2000; Coupland & McClements, 2001; Bijnen et al., 2002; Dukhin & Goetz, 2002; Saggin & Coupland, 2002a Challis et al., 2005; Gancz et al., 2006; Liu et al., 2008
Frozen products	Sigfusson et al., 2001; Lee et al., 2004; Gülseren & Coupland, 2007
Hydrocolloids	Boulenguer & Langendorff, 2003; Toubal et al., 2003; Aeberhardt et al., 2005
Oils, fats	Saggin & Coupland, 2002b; Benedito et al., 2002; Bijnen et al., 2002; Gan et al., 2006; Martini, 2007
Beverages	Zhao et al., 2003; Becker et al., 2001; Becker et al., 2002; Resa et a., 2004; Resa et al., 2007
Dough	Fox et al., 2004
Starch	Lehmann et al, 2004
Honey	Kulmyrzaev & McClements, 2000
Egg proteins	Bae, 1996; Bae & Kim, 1998; Waris et al., 2001

The desired accuracy of a measurement depends on the changes in the measuring parameter induced by a structure change. The smaller the change in the ultrasonic parameter is induced by a structure change, the higher the measuring accuracy is required to detect this change. Earlier studies showed that the reproducibility of ultrasonic measurement in many cases is very low. Povey and Rosenthal (1984) measured the degradation of starch by α-amylase. In their experiment, the ultrasonic velocity variation between samples was 50 times higher than that due to the action of the enzyme. This large variation of velocity may be caused by the simple construction of the measuring device, which did not consider and compensate the interference from the process, e.g., temperature fluctuation. Nowadays, there are ultrasonic measuring devices with high resolution and high temperature stability for analytical purpose available. This makes it possible to apply the ultrasonic method as a method to track even small changes in food systems. Before the detailed objectives of this study are discussed, the state of knowledge will be presented in order to allow for the full understanding of both motivation and target of this work.

2 State of knowledge

2.1 Physical fundamentals

Ultrasonic waves can be differentiated in two main forms: the longitudinal (compressional) and the transversal (shear) ultrasound. In a longitudinal wave the propagation direction is identical with the oscillation direction, so that the medium is locally compressed and dilated. In a transversal wave the direction of propagation is vertical to that of the oscillation plane, so that the medium is exposed to shear stress. The transversal wave only appears in viscous and solid samples. Low viscous liquid sample does not show rigidity, so that the transversal wave cannot propagate.

2.1.1 Generation of Ultrasound

The often-used method to create ultrasonic waves is the piezoelectric method. It is based on the ability of piezoelectric elements to convert the electric energy to mechanical energy and vice versa. The piezoelectric elements do not have a symmetric centre, so that a mechanical deformation of the element causes a shift of the asymmetrical charge carriers, and therefore, a polarisation of the charges, as shown in Fig. 2.1 in the case of quartz crystal as an example. Conversely, applying an alternating voltage (AC) on the piezoelectric element leads to oscillating (compression and expansion) of the element at very high frequencies producing high frequency mechanical sound waves (Fig. 2.2). The piezoelectric materials include quartz, lithium niobate, lead zirconate ceramic or titanate ceramic.

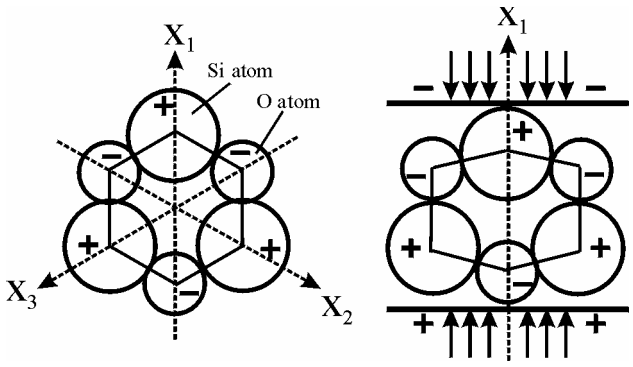


Fig. 2.1: Piezoelectric effect of quartz crystal (Bergmann, 1954).

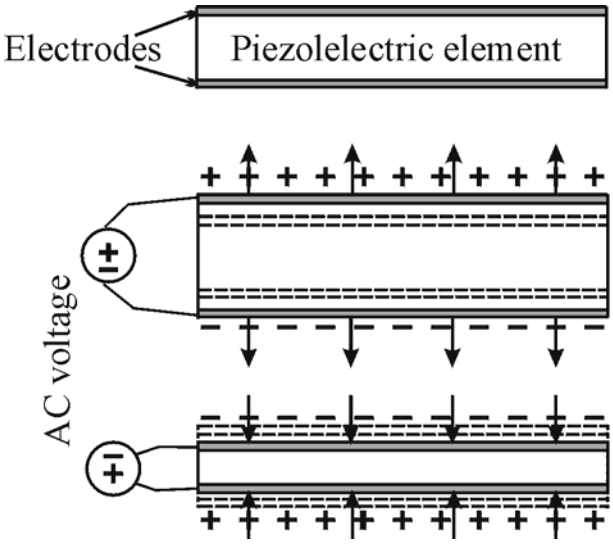


Fig. 2.2: Inverse piezoelectric effect for producing of ultrasound.

2.1.2 Measuring methods for ultrasound

2.1.2.1 Through transmission

Through transmission is the simplest method of ultrasonic measurement. The measuring system consists of a measuring cell, a sound transmitter and a receiver (Fig. 2.3). The transmitter produces a pulse of ultrasound that travels across the sample and is detected by the receiver. The velocity and attenuation of the ultrasound can be determined by measuring the time of flight (t) and amplitude (A) of the ultrasonic pulse. The velocity v is equal to the length of the sample (d) divided by the time (t) taken to travel this distance. The sample length can usually be determined by measuring the time-of-flight through a liquid of known ultrasonic properties. The attenuation coefficient is calculated by comparing the reduction in the amplitude of a pulse that has travelled through the sample.

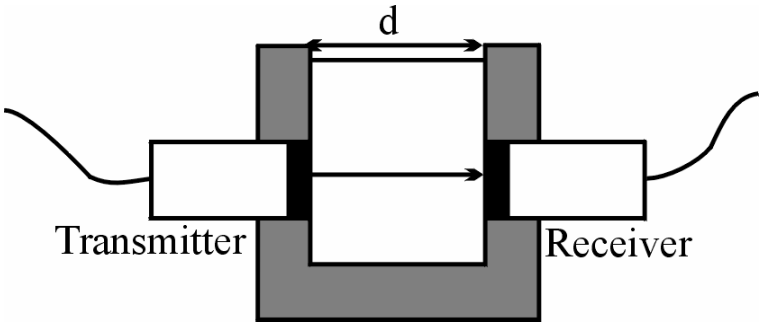


Fig. 2.3: Through transmission (McClements, 1998).

2.1.2.2 Pulse-Echo technique

A typical experimental configuration consists of a measurement cell containing the sample, a pulse generator, an ultrasonic transducer and an oscilloscope. The pulse generator produces an

electrical pulse of appropriate frequency, duration and amplitude. This electrical pulse is converted to mechanical ultrasonic pulse by the transducer. The ultrasonic pulse travels through the sample. It is reflected by the far wall of the measurement cell and comes back to the transducer, which now acts as a receiver and converts the ultrasonic pulse into an electrical pulse. The electrical pulse can be displayed on the oscilloscope. Because each pulse is partially transmitted and partially reflected at the cell walls, a series of echoes is observed on the oscilloscope.

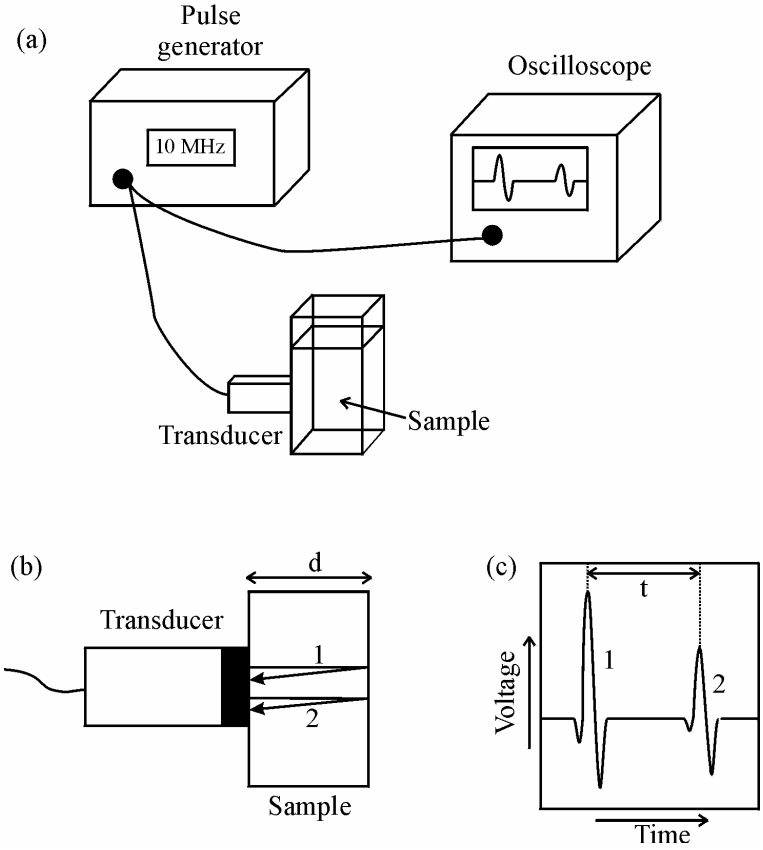


Fig. 2.4: (a) Schematic diagram of the experimental configuration used to carry out a simple ultrasonic experiment. (b) An ultrasonic pulse travels through a sample and is reflected from the far wall. (c) The pulses are partly transmitted on each reflection, thus a series of echoes is detected. The ultrasonic velocity and attenuation coefficient are determined by measuring the time interval (t) between successive echoes and their relative amplitudes. (McClements, 1995).

The velocity and attenuation coefficient can be determined from these echoes. The velocity v can be calculated by measuring the time t, interval between successive echoes and the cell length d:

$$v = \frac{2d}{t} \qquad \textbf{(Eq. 2.1)}$$

The attenuation coefficient α can be determined by measuring the amplitudes of successive echoes:

$$A_n = A_{n-1} \cdot e^{-2 \cdot \alpha \cdot d} \quad \textbf{(Eq. 2.2)}$$

$$\alpha = \frac{1}{2d} \ln\left(\frac{A_{n-1}}{A_n}\right) \quad \textbf{(Eq. 2.3)}$$

with A_{n-1} and A_n for the (n-1)-th and n-th echo, respectively.

2.1.2.3 Interferometric method

In an interferometer the measuring cell containing the sample is located between an ultrasonic transducer (acts as both transmitter and receiver) and a movable reflector plate (Fig. 2.5). A sinusoidal electrical signal of a given frequency is applied to the transducer, where it is converted into a sinusoidal ultrasonic wave that propagates through the sample. This wave is reflected back and forth between the reflector plate and the transducer, which results in the formation of a standing wave in the sample. As the reflector plate is moved vertically through the sample, the amplitude of the signal received by the transducer goes through a series of maxima and minima as destructive and constructive interference occurs. The distance x_{max} between successive maxima is equal to half wavelength of the ultrasonic wave (λ/2) in the sample.

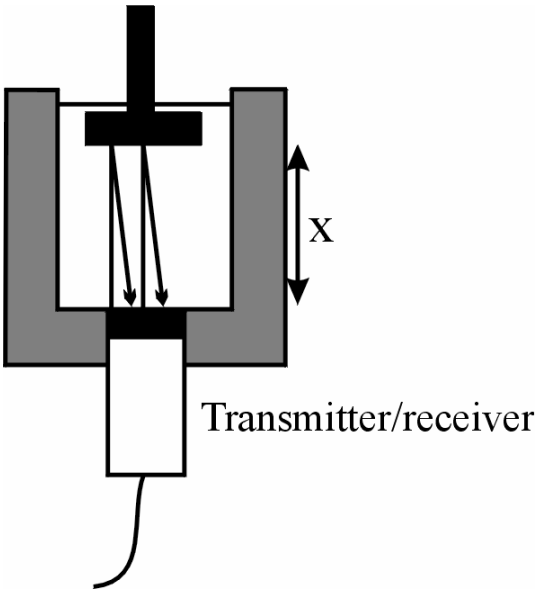


Fig. 2.5: Interferometer (McClements, 1998).

The ultrasonic velocity can be calculated as

$$v = f \cdot \lambda = 2 \cdot f \cdot x_{max} \quad \textbf{(Eq. 2.4)}$$

The amplitude of the maxima decreases as the distance between the reflector and the transducer is increased because of attenuation by the sample, imperfect reflection at the boundary and diffraction. The attenuation coefficient can be determined by measuring the amplitude of the maxima as a function of separation between the transducer and the reflector for both the sample and the calibration material. The frequency of the measurement can be determined by the resonance frequency of the crystal in the transducer.

2.1.2.4 Resonator technique

In the resonance methods, a standing wave is generated in a sample. The sample will resonate, if the path length d is an integer number n of half wavelengths long. Resonance means the wave is positively interfering with itself so that if the wave is being introduced from one side of the sample, a very large response signal will be detected from the second. The conditions for resonance can be related to the ultrasonic velocity v for an ideal system:

$$d = n \cdot \frac{\lambda}{2} = n \cdot \frac{v}{2f} \qquad \textbf{(Eq. 2.5)}$$

where d is the distance between the transducers (Fig. 2.6).

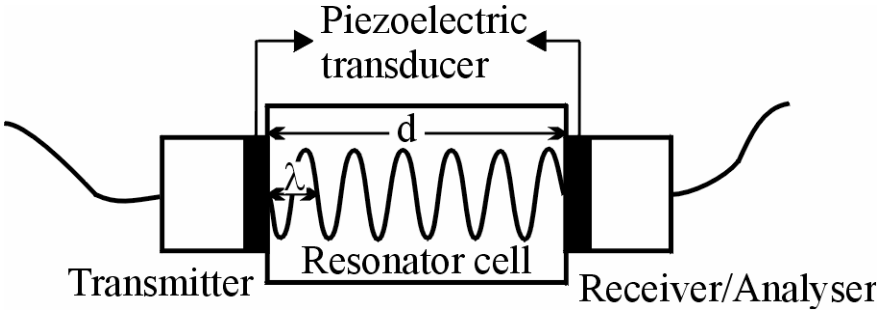


Fig. 2.6: Construction of an ultrasonic measuring system using the resonator technique.

For any sample a series of resonances can be generated by either holding the frequency constant and changing the path length or vice versa. The latter method is the basis for most modern resonance-based ultrasonic devices. Typically, a liquid sample is placed into a precisely-manufactured sample cell and a signal transmitter used to excite one transducer at a precisely known and closely controlled frequency while a second transducer measures the response. The signal transmitter slowly changes the input frequency until a resonance peak is detected. Once located, the position and shape of the peak can be tracked kinetically to a very high precision. The relative change in resonance frequency ($\Delta f_n/f_n$) is equal to relative changes in velocity ($\Delta v/v$). Alternatively, the absolute velocity can be calculated from the

relative positions of two adjacent resonance peaks having measured (f_n-f_{n-1}) by calibration with a reference fluid:

$$v = 2d(f_n - f_{n-1}) \quad \textbf{(Eq. 2.6)}$$

Attenuation causes a broadening of the resonance peaks and can be calculated from the width at half-height of the peak. Some ultrasonic devices also use the peak width at 70% height for the calculation of absorption.

In contrast to light detectors, ultrasonic transducers are phase-sensitive. This can result in a considerable loss in detected signal, which is not due to absorption of ultrasound energy but is due to scattering. Therefore, measurement of ultrasound attenuation tends to be much less reliable than measurement of ultrasonic velocity. The received signal normally requires correction for diffraction, losses at interfaces and relaxation effects (Povey, 1998).

Both the resonance and the pulsed methods are widely used in food characterisation. The major advantage of the resonance techniques is their higher precision. While a pulsed method can struggle to achieve reproducibility within 0.1 $m \cdot s^{-1}$, good-quality resonators can manage five orders of magnitude better. The precision of attenuation measurement is typically somewhat poorer for both techniques. The precision of the resonance devices makes them particularly qualified for measurements of polymer and solution dynamics where the significant changes are very small. Furthermore, resonance cells can easily be constructed to measure less than 1 ml of sample while long path lengths in large samples are needed to cause measurable changes in pulsed measurements (Coupland, 2004).

However, in some cases the phenomenon of interest may cause a massive change in signal (e.g., first order phase transitions, changes in shape or velocity in imaging or Doppler velocimetry applications and concentration determination) and the superior precision of the resonance devices need no longer be a determining factor. Similarly, food materials are often present in abundance and the small cells of resonators may cause more sampling problems than the cost benefits gained. Pulsed methods typically require lower equipment expenditure than resonance methods and are often more robust. Pulsed methods are also extremely rapid and can be a good way to detect fast changes (Coupland, 2004).

2.1.3 Ultrasonic parameters

The most important parameters of ultrasound are ultrasonic velocity and attenuation. If a longitudinal ultrasound wave travels through a viscoelastic medium, the ultrasonic velocity v

is a function of the bulk modulus K' and the storage modulus G' and the density ρ of the medium (Audebrand et al., 1995):

$$v = \sqrt{\left(K' + \frac{4}{3}G'\right) \cdot \frac{1}{\rho}} \qquad \textbf{(Eq. 2.7)}$$

It is to be noted that the values of G' obtained from the rheological measurements at low frequency (normally < 100 Hz) cannot be compared in absolute terms to those from ultrasonic measurements in the MHz range (Audebrand et al., 1995). In fluids and many food systems, such as weak gels, G' is much smaller than K' (Povey & McClements, 1988). Eq. 2.7 can then be simplified to:

$$v = \sqrt{\frac{K'}{\rho}} \qquad \textbf{(Eq. 2.8)}$$

The bulk modulus K' is the reciprocal of the compressibility κ:

$$v = \sqrt{\frac{1}{\rho \cdot \kappa}} \qquad \textbf{(Eq. 2.9)}$$

The compressibility is defined as the relative volume change of a fluid or solid as a response to a pressure change:

$$\kappa = -\frac{1}{V} \cdot \frac{\partial V}{\partial P} \qquad \textbf{(Eq. 2.10)}$$

where V is volume and p is pressure. The propagation of ultrasound in liquid is adiabatic. Therefore, the compressibility determined by the ultrasonic method is the adiabatic compressibility.

An important contribution to the compressibility κ is the hydration of molecules. The hydration usually has a negative contribution to the compressibility since water in the hydration shell is less compressible than that of bulk water (Gekko & Noguchi, 1979; Kharakoz & Sarvazyan, 1993; Nölting et al., 1993). Ultrasonic velocity strongly depends on temperature. In water the ultrasonic velocity varies by approximately 3 $m \cdot s^{-1} \cdot °C^{-1}$ (Povey, 1997). Water has unusual ultrasonic properties. Below 70 °C the temperature coefficient (temperature dependency) of the ultrasonic velocity is positive whilst that of nearly all other liquids is negative (Povey, 1998).

The compressibility of bulk water at 25 °C is about $45 \cdot 10^{-11}$ Pa^{-1} (Gavish et al., 1983). Some authors assumed that the water in hydration shell has ice-like structure with a compressibility

of $18 \cdot 10^{-11}$ Pa^{-1} (Eden et al., 1982; Gekko & Nugguchi, 1979). However, according to the calculation of Kharakoz & Savazyan (1993), the average compressibility in the hydration shell around a protein molecule is $35 \cdot 10^{-11}$ Pa^{-1}. The compressibility of globular proteins in water has been found to be between $1 \cdot 10^{-11}$ Pa^{-1} and $11 \cdot 10^{-11}$ Pa^{-11}, still much less compressible than water (Kharakoz & Savazyan, 1993; Nölting & Sligar, 1993; Gekko & Hasegawa, 1986). These data for the protein compressibility were determined under the conditions < 100 kDa molecular weight, ≤ 25 °C temperature of the aqueous protein solution and < 2% protein concentration (Nölting, 1995).

Attenuation of sound is defined as the loss of energy observed while a sound wave propagates through a sample. In a dispersed system, the attenuation is a sum of the intrinsic loss, the thermal loss, the viscoinertial loss and the scattering (Povey & Mason, 1998). The intrinsic loss is the sound absorption in the continuous and the dispersed phase. When an ultrasonic wave travels through a material, a part of the ultrasonic energy converts into heat due to material viscosity, thermal conduction and molecular relaxation. The reason for the thermal losses is the temperature gradients generated near the particle surface which is a result of the coupling between pressure and temperature. The viscoinertial loss of acoustic energy is induced by the density difference between continuous and dispersed phase. This density difference causes the oscillation of the dispersed phase relative to the surrounding continuous phase because of the different inertia. As a result, scattered waves are generated, which move away from the dispersed phase. Scattering occurs in heterogeneous systems, when an ultrasonic wave is scattered by a discontinuity, so it cannot be detected and is regarded as lost energy (McClements, 1995). Unlike light scattering, the sound scattering may be strongly influenced by mechanical and thermal coupling effects between the continuous and disperse phase. This follows from the physical nature of sound waves, which are nothing less than propagating compressional equilibrium disturbances, coinciding with temperature fluctuations (Babick et al., 2000). The attenuation provides information about the physicochemical properties of materials, e.g. concentration, viscosity, molecular relaxation and microstructure (McClements, 1995).

In literature, attenuation is presented in different forms. The basic form used is the attenuation coefficient α (Eq. 2.3) with the unit neper/m. Neper is a logarithmic unit of measurement that expresses the magnitude of a physical quantity (usually power) relative to a specified or implied reference level. Sometimes, the unit decibel/m (dB/m) is used. Like the decibel, it (dB/m) is a unit in a logarithmic scale, the difference being that where the decibel uses "$\log_{10}$" to compute ratios, the neper uses "ln". Both neper and decibel are dimensionless. Therefore,

the unit for the attenuation coefficient can also be given in 1/m. In order to consider the frequency, the attenuation is also often presented as α/f [s/m] or α/f^2 [s^2/m], where f is frequency. Because the ultrasonic attenuation is influenced by many factors, for the interpretation of the ultrasonic attenuation, a lot of physical chemical parameters are required. For complex systems these parameters are often unknown. Therefore the interpretation for complex system is difficult.

While an ultrasonic wave propagates in a medium, the local temperature in the sample changes, which is accompanied by changes of volume. Therefore, all equilibriums, which are susceptible to temperature and pressure changes (e.g., chemical and conformational) can be influenced by the ultrasound wave. The extent of the influence depends on the frequency of the ultrasound. If the relaxation time τ for changing the equilibriums is much shorter than the periodic time T (T = 1/f) of the ultrasonic wave, the medium is disrupted by the ultrasound; If the relaxation time is much longer than the periodic time of the ultrasonic wave, the ultrasound can not detect the equilibrium change. The equilibrium remains undisturbed; If the relaxation time is in the same range as the periodic time of the ultrasonic wave, the ultrasonic velocity and attenuation will be changed by the medium (Mathson, 1971). By altering the frequency of the ultrasound, the ratio of the wavelength to the equivalent diameter of the particles in the sample varies (Fig. 2.7). This influences the variation of the interaction between the ultrasound and the particle and leads to changes in the ultrasonic properties. Therefore, the particle size can be determined by ultrasonic spectroscopic measurement over a wide frequency range.

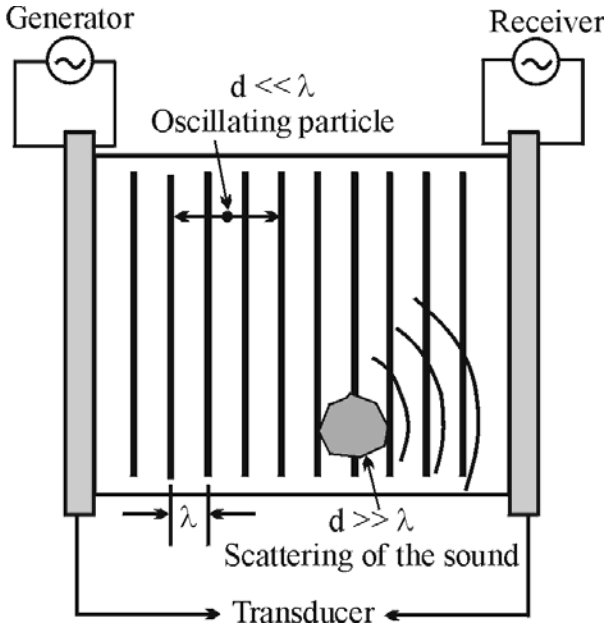


Fig. 2.7: The frequency dependence of ultrasonic propagation (λ=v/f) (Behrens & Pankewitz, 2002).

Dukhin et al. (2005) compared the effect of fat content and fat droplet size distribution in dairy products and type and concentration of salts in solution on the ultrasonic velocity and attenuation. They concluded that the acoustic attenuation/frequency spectrum is a better property for characterizing the dispersed phase and phase composition, whereas sound speed is better for characterizing chemical composition or chemical reactions that occur on a molecular level.

Most food systems are aqueous systems. The change of structure in a sample is always accompanied by the change of hydration of molecules, which causes a change of the compressibility. Thus, it is possible to detect a structural change by ultrasonic measurement in a product during processing or to compare different products.

2.2 High-resolution ultrasonic measurement devices on the market

Although the ultrasonic measurement has been applied for a long time, there was no measuring device with high resolution and temperature scan until a few years ago. Some manufacturers developed ultrasonic spectrometer specific for particle sizing. Commercial measuring devices are provided by Sympatec GmbH (Clausthal-Zellerfeld, Germany), Dispersion Technology, Inc. (Bedford Hills, NY, U.S.A.) and Malvern Instruments Ltd (Worcestershire, UK) for both laboratorial and in-line particle sizing. Compared to light scattering methods, the ultrasonic method can be used for particle sizing of highly concentrated emulsions and dispersions with a volume fraction up to 70%. Besides the function of particle sizing, the electroacoustic spectrometer from Dispersion Technology is able to measure zeta potential. Other Manufactures, such as TF Instruments GmbH (Heidelberg, Germany) and Ultrasonic Scientific (Dublin, Ireland) offer high-resolution ultrasonic devices with the function of a temperature scan for general purpose, which can be used for the measurement of ultrasonic velocity and attenuation in different samples at different temperatures. The latter allows the characterisation of different phase and structure transitions over temperature. The specifications of these devices according to the manufacturers are collected in Tab. 2.1.

Tab. 2.1: Ultrasonic devices on the market. The specifications are given by the manufacturers.

Devices for General Purpose		
Manufacturer	TF Instruments	Ultrasonic Scientific
Products	ResoScan®	HR-US 102 range
Frequency range	7-9 MHz	2 -18 MHz
Volume of the sample cells	0.2 ml	1-1.5 ml
Temperature control	Peltier-elements	Water bath
Temperature range	5-85 °C	-20-120 °C
Resolution	±1 mm/s for velocity 1-3% for attenuation	±0.2 mm/s for velocity 0.2% for attenuation

Devices for Particle Sizing			
Manufacturer	Sympatec GmbH	Malvern Instruments Ltd.	Dispersion Technology, Inc.
Products	Sympatec OPUS (in-line) and NIMBUS (laboratory)	Ultrasizer MSV (Modular Small Volume)	DT-1200 (Electro-Acoustic Spectrometer) DT-100 (Acoustic Spectrometer)
Particle size	0.01 – 3000 µm	0.01-1000 µm	0.005 -1000 µm
Frequency range	0.1-100 MHz	1 - 150 MHz	1-100 MHz
Sample volume	15 – 50 ml (OPUS) 0.5-1 L (NIMBUS)	> 45 ml	20 - 110 ml

In this work, the ResoScan® system was used. Because the frequency range of this device is narrow, spectroscopic measurements were not possible. The measurements were performed at a fixed frequency. Consequently, the application of the ResoScan® is restricted for characterizing structure changes or reactions, which can be detected in this frequency range. However, the ResoScan® system has the advantage that it can automatically chose the best resonance peaks for accurat measurements by evaluating the quality of the resonance peaks considering the symmetry and amplitude of the peaks using the software of the system. This is an advantage especially for unexpierenced users. Furthermore, the temperature of the measuring cells of the ResoScan® system is controlled by Peltier-elements, so that high temperature stability can be achieved.

The HR-US 102 from Ultrasonic Scientific can work at different frequencies, so that it can be used for spectroscopic measurements. However, the manual adjustment of the signal intensity

and selection of the resonance peak requires comprehensive experiences to choose the optimal peak for the measurement. The temperature of the measuring cell of the HR-US 102 is controlled by an external water bath. The heat loss during the circulation process in the connecting tubes makes it difficult to adjust the temperature to a target value at high accuracy.

Both the ResoScan® and the HR-US 102 systems have build-in measuring cells, whose surface is directly in contact with the sample. For the applications in food area, both systems have the disadvantage that it is difficult to be cleaned, especially if the protein containing food systems are heated up to temperatures above the denaturation temperature of the proteins. The denatured proteins can form a layer on the surface of the measuring cell and affect the measuring results. Further improvement is required considering the cleaning.

2.3 Hydration of sugars

As a taste component, a thickening or conservation agent, sugars are important components in many foods. They are also applied as a protective agent during drying of biological systems such as starter culture. Although some sugars have similar chemical structures, their interaction with water differs in strength. This difference may play a role in the protective effect on the biological systems and in their thickening effect. Branca et al. (2001) compared the hydration state of trehalose, maltose and saccharose using ultrasonic and density measurements. They found that trehalose, which has the highest effectiveness in stabilizing biomolecules, cells and tissues during air-drying and freezing-drying, has the highest hydration number per molecule. Therefore, information about the hydration state of sugars helps to understand their effects.

It is known that the hydration of carbohydrates depends on the percentage of axial and equatorial hydroxyl groups. It is more favourable when the hydroxyl group is at the equatorial position (Tait et al., 1972; Franks et al., 1973).

The hydration number of the sugar n_h can be calculated from the adiabatic compressibility κ of the sugar solution and the water (Shiio, 1958; Galema & Høiland, 1991). The hydration number n_h denotes the median number of water molecules bound to each molecule solute.

$$n_h = \frac{n_w}{n_s} \cdot \left(1 - \frac{\kappa_s}{\kappa_{s0}}\right) \qquad \textbf{(Eq. 2.11)}$$

where n_w is mol fraction of water, n_s mole of the solute, κ_s the adiabatic compressibility and κ_{s0} the adiabatic compressibility of water.

The κ_s and κ_{s0} can be determined using Eq. 2.9 by measuring the ultrasonic velocity and density in the sugar solution and water, respectively. This equation is only valid for diluted solutions. Furthermore, it is assumed that n_h is the number of water molecules of the first layer surrounding the solute, and these water molecules are trapped so tightly that they can be considered as incompressible (Junquera et al., 2002). For low molecular weight compounds, the intrinsic compressibility is small, since it is mostly determined by the compressibility of covalent bonds and external electron shells (Chalikian, 1998).

2.4 Gelation of hydrocolloids

The gelation process is a sol/gel phase transition. It causes changes in rheological properties. The rheological method is often used to investigate the gelling process. During the last years, the ultrasonic method has been applied as a new method for the monitoring of the gelation process in different food systems, e.g. egg white (Bae, 1996; Bae et al., 1998), polysaccharide (Gormally et al. 1982; Audebrand et al., 1995; Toubal et al. 2003) and milk gels (Benguigui et al. 1994; Gunasekaran & Ay, 1994; Nassar et al., 2001). Changes in ultrasonic properties were observed due to the formation and aging of a gel. These investigations showed that the gelation process of different systems leads to different changes in the ultrasonic properties. The mechanism of detection is still unclear.

2.4.1 Carrageenans

Carrageenans are hydrocolloids extracted from red seaweeds. They are sulphated D-galactans linked alternately via α (1→3) and β (1→4) bonds. Carrageenans are used widely in the food industry as gelling and stabilizing agents.

The main types of carrageenans are κ-, ι- and λ-carrageenans. κ- and ι-carrageenans form gels at low temperatures. Different models for the gelation mechanism have been proposed by different researchers. The double helix model proposed by Anderson et al. (1969) and later modified to the domain model by Morris et al. (1980) is widely accepted (Fig. 2.8). The domain model assumes that in the sol state at high temperature the carrageenan molecules exist as random coils. A temperature decrease induces the formation of double helices of the polymer chains. The carrageenan molecules contain sections with irregular molecular structures, which cannot form double helices. These sections enable the intermolecular association of double helices and lead to the formation of small independent domains involving a limited number of chains. Aggregation of helices in different domains via cations as counterions enables more long-range cross-linking for the gel formation.

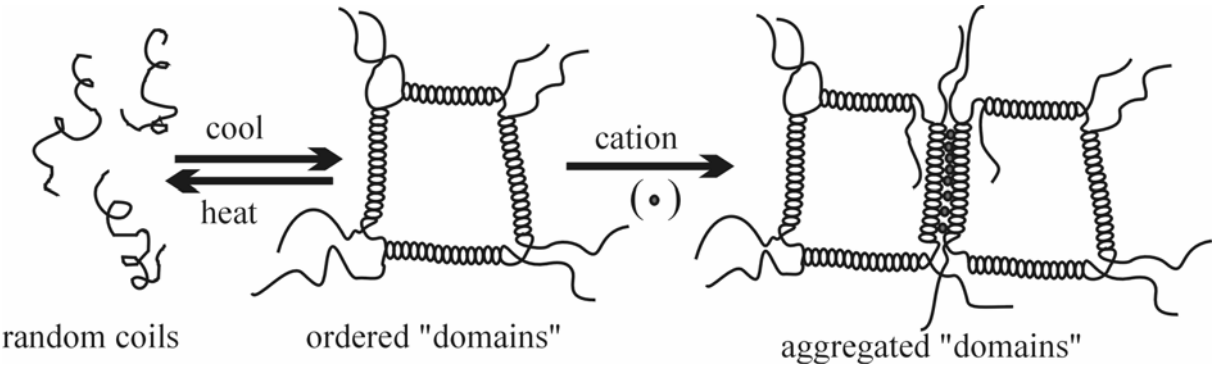


Fig. 2.8: Domain model suggested by Morris et al. (1980).

The gels of κ- and ι-carrageenans have different properties. κ-carrageenan forms a firm and brittle gel, which shows syneresis. In contrast, the ι-carrageenan gel is weaker and cohesive and does not show syneresis. The gelation process depends on the presence of specific couterions, which promote the aggregation process by reducing the repulsion forces of the negative charged carrageenan molecules (Robinson et al., 1980). The firmness of κ-carrageenan sensitively depends on the K^+-concentration, while the gel firmness of the ι-carrageenan is rather sensitive to the Ca^{2+}-concentration. The gelation temperature of carrageenan depends on the carrageenan and gelation-promoting counterions concentration.

Considering their promoting effect on the helix formation and helix aggregation of κ-carrageenan, the counterions can be divided into three main categories, i.e., the non-specific monovalent cations [Li^+, Na^+ and $(CH_3)_4N^+$], the divalent cations [Mg^{2+}, Ca^{2+}, Ba^{2+}, Co^{2+} and Zn^{2+}] and the specific cations [NH_4^+, K^+, Cs^+ and Rb^+]. The former two categories affect the conformational transition primarily by long-range Coulomb interactions. By contrast, the latter binds specifically to the carrageenan chain and stabilizes the helix much more effectively than even the divalent ions (Piculell, 2006).

According to Record (1975) and Manning (1972) the enthalpy of melting for helix-coil conformational transition can be predicted by considering the counterions and the electrostatic screening due to the ionic strength. The melting enthalpy can be expressed by the Eq. 2.12 (Rochas & Mazet, 1984):

$$\Delta H = -R \cdot (\phi_c - \phi_h) \cdot \frac{d \ln[c]}{d(1/T_m)} \qquad \textbf{(Eq. 2.12)}$$

where ΔH is the enthalpy of melting, ϕ_c the osmotic coefficient for the coil, ϕ_h the osmotic coefficient for the helix, c the total ionic concentration in equivalent per litre, and T_m the melting temperature in Kelvin, respectively. The ΔH value is expressed per unit charge, namely, per disaccharide residue of the κ-carrageenan molecule (Rochas & Rinaudo, 1982).

Many researchers found that the gelation of κ-carrageenan includes different levels of aggregation of κ-carrageenan (Borgström et al., 1996; Hermansson, 1989; Hermansson et al., 1991; Ikeda et al., 2001), as described in the following scheme:

single chain → double helix → superhelical rod → bundle of rods

κ-carrageenan shows thermal hysteresis in temperature related conformational transitions. The transition temperature during cooling is lower than that during heating. This hysteresis effect in polysaccharide order-disorder transition has its origin in aggregation of the helix. Rochas and Rinaudo (1982) investigated the melting enthalpy of the carrageenans and concluded that there are two contributions to the melting enthalpy: the conformational contribution (helix-coil transition) and the contribution of cross-linking in stabilizing the 3-dimentional network structure (melting of the aggregates of helix).

Pure ι-carrageenan, uncontaminated by κ-carrageenan, does not display thermal hysteresis in the conformational transition, regardless of the monovalent ion form (Piculell, 2006). However, in commercial products, caraggenans are normally not pure. Each carrageenan contains trails of other carrageenans. Using small angle x-ray scattering, Yuguchi et al. (2002) compared the K^+-type of κ-carrageenan and ι-carrageenan. They concluded that κ-carrageenan forms two or three associated double helices during gelation, while gelation of ι-carrageenan mostly induced by the transition from two or single chain to double helix without association between helices.

The sol-gel and coil-helix transition of carrageenan can be measured by different methods, e.g., rheology (Hermansson, 1989), optical rotation (McKinnon et al., 1969; Bryce, et al., 1974), dielectric conductivity (Takemasa et al., 2002), calorimetry (Rochas & Rinaudo, 1982; Rochas & Mazet, 1984) and NMR (Nuclear Magnetic Resonance) (Hinrichs et al., 2003).

In literature, there are only very few ultrasonic investigations of carrageenan gelation reported. Each of these investigations had a different focus. Gormally et al. (1982) performed the ultrasonic spectroscopic measurement (0.5-20 MHz) of carrageenan in different states of molecular organisation by varying the polymer composition and the ionic environment. They observed a higher attenuation in the gel state than in the sol state, which is predominantly arising from motion of the solvent within the polymer network. Passage of a sound wave through the sample creates regions of high and low pressure. The system will then respond to this perturbation by transport of solvent to offset the pressure differential. Isolated flexible polymer chains would be expected to offer little resistance to flow, but transport of the solvent through the pores of a cross-linked network of large aggregates will be more restricted, with a

consequent increase in the energy dissipated. Toubal et al. (2003) measured the ultrasonic velocity in a 0.75% ι-carrageenan from 90-20 °C at 0.5 MHz. They observed a maximum in ultrasonic velocity at the gelling temperature without being able to explain this phenomenon, however.

Carrageenans in solution are strongly hydrated, because their polar groups interact with the polar solvent water (Oakenfull, 1987). Hinrichs (2004) investigated the water mobility of carrageenan sols and gels using Low Resolution NMR (LR-NMR). In the NMR measurement, water is differentiated in four different phases according into its mobility: the immobile phase (water chemically bounded to the molecules), the weakly mobile phase (water trapped in small pores and capillaries), the mobile phase (water trapped in middle large pores and capillaries) and the very mobile phase (bulk water trapped in large pores and capillaries). Hinrichs (2004) concluded that the gel formation does not change the proportion of the four water phases of different mobility much. Most water remains in the very mobile phase even in the gel state. However, the mobility of the very mobile phase in the gel state is lower than that in the sol state, because the bulk water is trapped in the small cavities formed by the gel network and its mobility is restricted. Even the addition of potassium, which is known to be an influencing factor for the gel properties and structures, does not induce much variation in the proportion and mobility of different water phases. Probably the LR-NMR used by Hinrichs (2004) is not sensitive enough to detect the changes in the water mobility during sol/gel transition.

The optical rotation and calorimetric methods measure the optical and thermal changes induced by coil-helix transition, while the NMR and ultrasonic methods measure the water mobility. However, in contrast to NMR, ultrasonic meathod is only sensitive to transition between bulk water and chemically bound water because of their different compressibilities. The ultrasonic method cannot differtiate between water trapped in small capillaries and that in big pores, as long as the compactness and compressibility of water is not changed by chemically bonding to a molecule.

2.4.2 Gelatine

Gelatine is obtained by degradation of collagen. The collagen monomer is a triple helix or rod about 300 nm long and 1.5 nm in diameter of molecular weight about 300,000 (Ledward, 2000). At temperatures above 35-40 °C gelatines in solutions behave as random coils, which can take up an infinite number of transient configurations, on cooling the solution aggregation occurs and at concentrations above about 1%, depending on the quality of the gelatine and pH.

The thermal reversibility of gelatine with a melting point at 35-40°C makes gelatine such a useful and unique food ingredient since such gels will melt in the mouth (Ledward, 2000).

During cooling of gelatine solution intermolecular triplex helices are formed. The network formation of these helices leads to gelation. The pyrolinide (proline and hydroxyproline)-rich regions of the gelatine chains act as nucleation sites for the formation of potential junction zones in that, theses regions tend to take up the poly-L-proline II helix (which is relatively open and has no internal hydrogen bonding compared to poly-L-proline I) during cooling. Aggregation of three such helices leads to the formation of a collagen-like triple helix, which acts as the gel junction points or zones. The junction zones are stabilized by inter-chain hydrogen bonds, which break at 35-40 °C and cause gel melting (Ledward, 2000; Johnston-Banks, 1990).

In dilute solutions, at concentrations below 0.5%, gelatine will gel unimolecularly (Fig. 2.9, step A), while at concentrations above this the mechanism tends increasingly towards a bi- or trimolecular one (Fig. 2.9, step B).

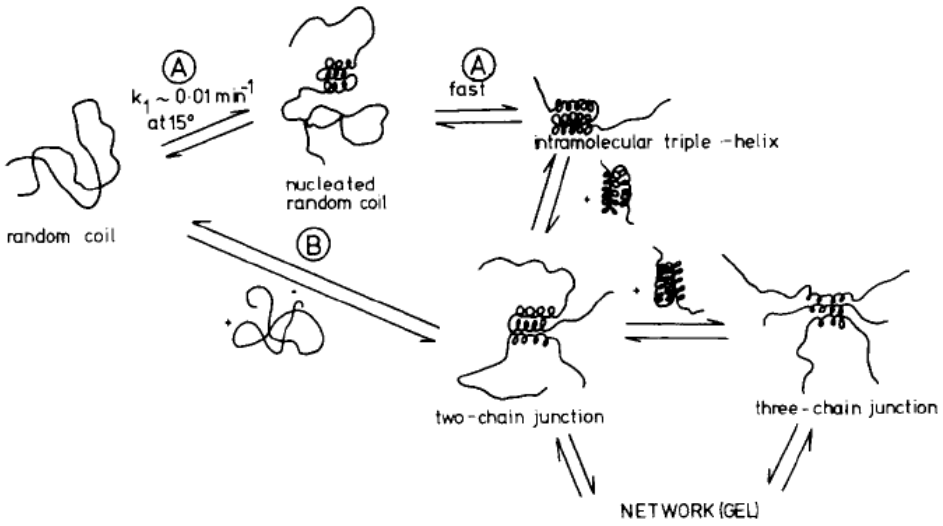


Fig. 2.9: Schematic representation of the possible conformational changes undergone by α-gelatine molecules during the formation of a gel by quenching a solution of random-coil molecules (Finer et al., 1975).

2.5 Milk gelation

Milk is a colloidal dispersion of highly hydrated protein particles, the casein micelles (Walstra & Jenness, 1984). The casein micelle system is an excellent example of a colloidal dispersion. There are different models for the casein structure. The model of the casein micelle as a roughly spherical, fairly small particle of about 100 nm diameter with a hairy outer layer

consisting of κ-casein, is generally accepted (Walstra, 1999). But there are different descriptions for the internal structure of the micelles. In the "sub-micelle model" of Walstra and Jenness (1984), the core of the micelle is built of sub-micelles, which are held together in the micelle by calcium phosphate bridges and hydrophobic interaction. The "hairy casein micelle model" of Holt and Horne (1996) does not agree with the notation of sub-micelles. In this model, the casein micelle is regarded as a colloidal particle, where a fairly open structure of polypeptide chains is cross-linked by calcium phosphate nanoclusters in the core. The external region with low segment density forms the hairy layer of the micelle.

The casein micelles are stabilized by hydration, negative charges and steric repulsion (Mulvihill & Grufferty, 1995). Because the casein micelles are negatively charged, they have a zeta potential of about -20 mV. This charge is reduced by about 50% on rennet treatment (Dalgleish, 1984). The outer layer of casein micelles consists of κ-casein, the C-terminal portion of which extends out into the solution providing a steric stabilizing layer. By enzymatic hydrolysis of the κ-casein renneting or acidification, the casein micelle can be destabilised, resulting in gel formation.

2.5.1 Rennet gelation of milk

The rennet gel formation is described in Fig. 2.10. It is initiated by the proteolysis of κ-casein molecules, which is accompanied by the release of the hydrophilic caseinomacropeptide (CMP) into serum. The hydration state of casein micelles thus changes due to the loss of the hydrophilic part of κ-casein on its surface and the radius of casein micelles shrinks (De Kruif & Holt, 2003). The proteolysis of CMP is called the primary phase of rennet gelling. The loss of CMP is accompanied by a decrease of the zeta potential, which results in destabilization of the micelle. The casein micelles start to aggregate due to increased hydrophobicity of their surfaces. Finally, the aggregates form a three-dimensional gel network. The aggregation and network formation are called the secondary phase of rennet gelling. The rennet gel formation and properties of the formed gel can be influenced by many factors, such as calcium concentration, pH and heat treatment of the milk.

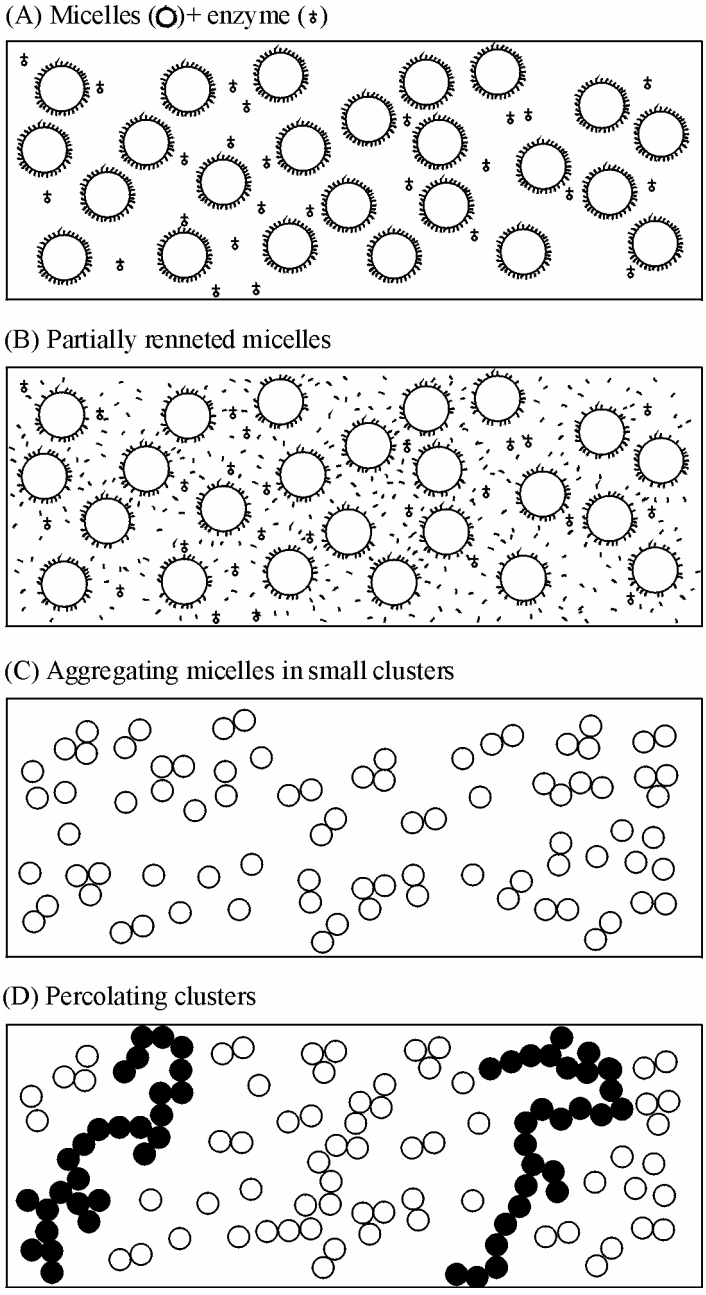


Fig. 2.10: A schematic description of the various stages envisaged in the enzymatic coagulation of milk, starting from the initial mixture of casein micelles and enzyme (A) and proceeding through proteolysis (B), initial aggregation into small clusters (C) and reaching a gel point at percolation (D) (Horne & Banks, 2004).

A variety of different methods exist to investigate milk gel formation: oscillatory rheometry (Bohlin et al., 1984), thermal conductivity (Hori, 1985), refractometry (Korolczuk & Maubois, 1988) and dynamic light scattering (Horne & Davidson, 1990) to name but a few.

Many techniques use a mathematical model based on a mechanistic description of the rennet reaction, which can predict the behaviours of interesting variables such as temperature, pH, milk composition and pre-treatment. However, few of them provide direct relationships

applicable over the whole course of the renneting from the micelle to gel. Indeed, this is perhaps one reason why the rennet gelation process is divided into the primary and secondary phases, since the early aggregation phase can be followed by turbidity or light scattering, whereas the gel formation and development is most easily monitored by rheometry. Each technique has its limitations. Light scattering requires a dilute dispersion of particles. Direct conversion to molecular weight or size is also limited by the ratio of particle size to light wavelength. Studies using light scattering are thus limited to the early stage of aggregation, where growth of the molecular weight is observed. In contrast, the limitation of the rheological method is instrument sensitivity. A detectable signal is realized only if the reaction has progressed to a significant extent (Horne & Banks, 2004).

In the last few years ultrasound was also used to investigate the milk gel formation with inconsistent results. Gunasekaran and Ay (1994) investigated rennet milk coagulation using ultrasound at a frequency of 1 MHz and found that the ultrasonic velocity and attenuation decreased during coagulation of casein. In contrast, Corredig et al. (2004a) observed an increase in ultrasonic velocity and attenuation at higher frequencies (14.667 & 7.835 MHz) during milk coagulation by rennet. At 200 KHz, Nassar et al. (2004) also measured an increase in ultrasonic velocity. Dwyer et al. (2005) could clearly measure a decrease of ultrasonic attenuation during enzymatic cleavage of CMP at 14.5 MHz. Corredig et al. (2004a), however, measured little change in attenuation at both 7.835 MHz and 14.667 MHz during enzymatic hydrolysis.

Thus, low-intensity ultrasound as a novel method seems to offer a new option to obtain more information regarding the rennet action in its primary phase and the secondary aggregation phase. In the studies mentioned above the focus was on the ultrasonic measurement alone. In order to use ultrasonic measurement as a new analysis method, it is important to assess the quantitative correlation between this method and an established method such as rheology.

2.5.2 Acid gelation of milk

Milk can be acidified by bacterial cultures, which ferment lactose to lactic acid, by the addition of inorganic or organic acids such as HCl and citric acid, or by the use of the acid precursor glucono-δ-lactone (GDL). The addition of inorganic and organic acids causes locally strong acidification of milk. This results in local precipitation of casein, if the acidification is performed at temperature higher than 10 °C (Hammelehle, 1994). In contrast, GDL acidifies the milk gradually by hydrolysing to gluconic acid and enable a gel network formation without precipitation even at higher temperature. The rate of acidification is

different between milk acidified with GDL and bacterial cultures. The pH decreases immediately after addition of GDL, whereas the pH in the bacterially inoculated milk does not change much initially after the addition of starter bacteria. The final pH in GDL-induced gels depends on the amount initially added to milk, whereas starter bacteria can continue to produce until a very low pH (e.g., pH < 4.0) (Lucey & Singh, 1998).

The formation of acid gels is a complex process. The mechanism of acid-induced gel is still largely unknown (Holt & Horne, 1996). It is thought that coagulation at pH close to the isoelectric point of casein is simply due to charge neutralisation. As the pH is reduced, colloidal phosphate and small amount of magnesium and citrate are fairly rapidly dissolved and casein monomers are released into the milk serum. According to Dalgleish and Law (1988; 1989), the calcium phosphate dissolved in the soluble phase by around pH 5.1. There is increased solubilization of the individual caseins. β-casein dissociated from the casein micelle into serum upon acidification (Heertje et al., 1985). Therefore, Holt and Horne (1996) put forward the questions how the micelle re-organises itself at low pH, and whether the material which forms the gel network are casein micelles.

Heertje et al. (1985) as well as Parnell-Clunies and Kakuda (1988) suggested, from electron microscopy studies of acidified milk, that the casein micelle dissociates into subparticles in pH interval 5.1-4.8 due to conversion of colloidal Ca^{2+} into ionic Ca^{2+}, before the general aggregation takes place at the isoelectric point.

El-Shobery (1983) proposed a model of the structure change of casein over pH by measuring the relaxation time, i.e., the time necessary to reach a new pH equilibrium after addition of a certain amount of acid, in a titration experiment (Fig. 2.11). At the natural pH of milk the casein exists in a closed micellar structure. With decreasing pH the micelles begin to aggregation. After a maximal aggregation of micelles at pH 6.0, the micellar structure begins to dissolve to open peptide chains, which associate with further pH decrease.

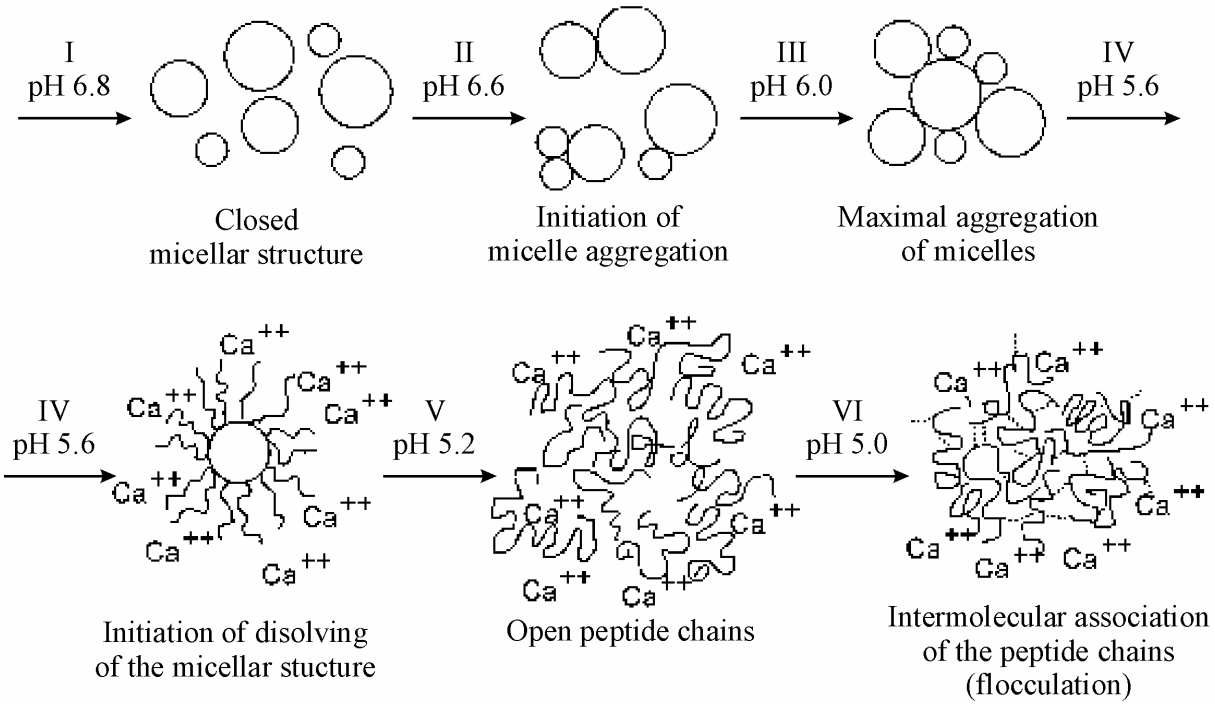


Fig. 2.11: Structure alteration of casein micelles during acidification of milk (El-Shobery, 1983).

Heertje et al. (1985) observed a minimum of zeta-potential of casein in skim milk at pH 5.2. This pH coincides with the isoelectric point (pI) of β-casein and the start of the aggregation phase. At this pH calcium is almost removed from the micelle and a relative maximum in the micelle voluminosity occurs. Heertje et al. (1985) suggested that after the release of β-caseins from the casein micelle at pH higher than pI, leaving a framework of α_s-caseins, the reabsorption of β-caseins starts to occur at its pI. Horne (2003) studied acidified milk gels produced over a wide range of acidification conditions using GDL and concluded that the internal changes in micellar structure with pH decrease play a role in the viscoelastic properties of the resulting gel.

For the investigation of acid gel formation, the same methods such as rheological methods or light scattering are often applied as for the rennet gelation. Recently, Kudryashov (2000) as well as Dalgleish and co-workers (2004; 2005) made the first ultrasonic investigations to characterise acid gelation. They observed an increase in both ultrasonic velocity and attenuation during acidification. This increase occurs immediately after the addition of acidification agent GDL. According to Kudryashov et al. (2000) and Dalgleish et al. (2005) the increase in ultrasonic velocity can be explained by the dissolution of the micellar calcium phosphate, which alters the ultrasonic properties of the milk serum because of the high degree of hydration of the released calcium ions. Kudryashov et al. (2000) suggested that the changes

in attenuation have been suggested to arise from the changes in elasticity and hydration of the casein micelles during acidification. In contrast, Dalgleish et al. (2005) suggested that attenuation is closely correlated with the release of calcium as acidification proceeded.

2.5.3 Caseinomacropeptide

Caseinomacropeptide (CMP) is the C-terminal residue of κ-casein following cleavage by rennet enzyme from casein. CMP can contain glycosylated carbohydrate chains. According to Mollé and Léonil (2005), about 50% of CMP are in glycosylated form. CMP has a high content of sialic acid, which is responsible for the high negative charge over the whole pH area (Saito & Itoh, 1992; Tran et al., 2001). Due to the glycosylation CMP possesses hydrophilic properties.

Kawasaki et al. (1993) determined the molecular weight of CMP using gel filtration and found a molecular weight of 20-50 kDa at pH 7 and 10-30 kDa at pH 3.5. Both Kawasaki et al. (1993) and Minkiewicz (1996) concluded that the apparent molecular weight of CMP decreases with decreasing pH. Kawasaki et al. (1993) suggested that at neutral pH CMP associate to oligomers through non-covalent interactions, which dissociate at acid pH, while Minkiewicz (1996) supposed that the lower apparent molecular weight at low pH is caused by the lower voluminosity due to the depressed electrostatic repulsion.

CMP has received much attention in recent years due to its nutritional, physiological and biological properties, such as its ability to bind *cholera* and *Escherichia coli* enterotoxins, to inhibit bacterial and viral adhesion, to suppress gastric secretions, to promote bifido-bacterial growth and to modulate immune system responses (Brody, 2000). Therefore, CMP has a good potential for application in functional foods, dietary products and infant products.

In view of these benefits, information about the technological functional properties of CMP is of great interest for its application in innovative foods. Past research showed that CMP has emulsifying, foaming and gelling properties (Brody, 2000; Thomä-Worringer et al. 2006b). Compared to other emulsifying and foaming properties, there is only little information about the gelling properties of CMP. Burton and Skudder (1987) found that a solution containing 9.3% CMP form a gel at pH 4.5 at 20 °C, but not when heated to 90 °C. However, Marshall (1991) could not reproduce these findings. The gelation of CMP was described by a few authors. Hiroshi and Kawasaki (2001) patentet the application of CMP as a gelling agent at pH below 5 and CMP concentration between 0.1% and 10%. However, no detailed information is available about this. In the rheological investigation of fermented goats' milk

supplemented with CMP, Martin-Diana et al. (2004) found that the addition of CMP increased the elasticity and resulted in a more ordered and structured gel compared to that made with whey protein concentrate.

2.6 Thermal denaturation of proteins

Protein denaturation is a common process in the heat treatment during food processing. The thermal denaturation of globular proteins often involves the unfolding of the protein molecules and the following irreversible aggregation of the unfolded molecules. The functionality of proteins depends on the degree of their thermal denaturation. Therefore, it is important to understand the denaturation kinetics of proteins.

An established method for determining the degree of irreversible aggregation of proteins is reversed phase high-performance liquid chromatography (RP-HPLC). RP-HPLC measures soluble proteins and separates different proteins on the basis of their different molecular hydrophobicities (Aguilar, 2004). By measuring, for instance, the difference between the native α-la concentration in unheated and heated samples, the degree of the irreversibly aggregated α-la during thermal denaturation can be determined.

Another method for investigating protein denaturation is calorimetry (Manji & Kakuda, 1987). Differential scanning calorimetry (DSC) is a valuable tool for studying thermally induced changes in a protein. In this method the change in thermal energy in a sample during heating or cooling is measured, which results in a heat flux or a change in the thermal energy. Denaturation of a protein causes an endothermic peak in the thermogram (Privalov & Khechinashvili, 1974). According to the area of the endothermic peak, the denaturation of α-la can be determined quantitatively.

The compressibility of proteins in solution can be measured by measuring the ultrasonic velocity. The compressibility of proteins is composed of the intrinsic compressibility, the hydration compressibility and the compressibility related to relaxation processes (Gekko & Hasekawa, 1986). The intrinsic compressibility is the compressibility due to imperfect packing of the molecule. The hydration compressibility is the change in solvent compressibility due to the interactions of solvent molecules with the solvent accessible atomic groups of the protein. The hydration compressibility is usually negative since water in the hydration shell is less compressible than bulk water (Gekko & Noguchi, 1979; Kharakoz & Sarvazyan, 1993). The presence of relaxation increases the compressibility, while an increased level of hydration causes a decrease in compressibility (Nölting & Sligar, 1993).

Depends on conditions, globular proteins can assume different conformational states, including native, compact intermediate, partially unfolded and fully unfolded. The native state of a globular protein is the most compact conformation amongst the possible states (Chalikian & Breslauer, 1996). It exhibits the lowest solvent accessible surface area and the most tightly packed core with the compressibility close to organic solid (Kharakoz & Savarzyan, 1993). The molten globule state is an intermediate state of protein during unfolding. The molten globule state is characterised by a moving tertiary structure but a higher degree of compactness and a large content of secondary than the unfolded state (Ohgushi & Wada, 1983). Kharakoz & Bychkova (1997) characterised the molten globule of human α-lactalbumin and concluded that it has an increased hydration of the interior and an increased compressibility compared to the native state.

Brandts et al. (1970), Zipp and Kauzmann (1973), and Hawley (1971) analysed the pressure-induced denaturation of protein. They concluded that the compressibility of denatured protein is larger than that of the native one. This conclusion is contrary to the expectation that in the denaturation process the hydrophobic groups buried in the interior of the protein are exposed to the solvent accompanying the decrease in void and increase in hydrophobic or hydrogen bound hydration, which should lead to a decrease in the compressibility of the denatured state compared to the native state. Gekko and Noguchi (1979) estimated the contributions of voids in the interior of the molecules and the hydration to the compressibility of proteins. They concluded that a large positive contribution to the compressibility due to the void compensates a negative contribution to the compressibility due to the hydration of the protein, resulting in a small positive value for compressibility. Such a large compressibility of the void supports the proposition that the increase in compressibility of the protein by denaturation is due to the high local concentration of non-polar groups of the denatured protein. According to Bøje and Hvidt (1971), the volume change accompanying the exposure of non-polar groups to water, i.e., the rupture of hydrophobic bonds, is positive, and therefore, results in an increase in the compressibility. Zipp and Kauzmann (1973) concluded that the conformational change of proteins induced by pressure is similar to that observed upon denaturation by acid, heat and urea. Therefore, the same aspects as mentioned above can be applied to explain the compressibility changes of the protein during heat-induced denaturation.

During denaturation, the globular protein molecule unfolds, which leads to changes in the compactness of the interior of the protein molecules and changes in the hydration state of the molecules due to the enlarged surface area that is accessible to the solvent. Furthermore, protein unfolding causes an increased relaxation contribution due to conformational relaxation

or an increased number of proton-exchanges due to a higher degree of exposure of side chains to solvent (Kamiyama & Gekko, 1997). These modifications lead to changes in the ultrasonic velocity in the protein solution. Many researchers have investigated conformational changes in protein molecules due to chemical denaturation using ultrasonic velocimetry (Kamiyama & Gekko, 1997; Chalikian & Breslauer, 1996; Nölting et al., 1993).

However, temperature-dependent changes in ultrasonic properties of proteins were barely investigated. By using modern high-resolution ultrasonic measuring equipment, which is able to perform continuous temperature scanning, it is possible to exactly quantify the denaturation of proteins similar to in the DSC method.

2.6.1 Whey protein α-lactalbumnin

α-lactalbumin (α-la) is a small, Ca^{2+}-binding whey protein with a molecular mass of 14.2 kDa. It is a globular protein with a secondary structure composed of 26% α-helix, 14% β-sheet and 60% irregular structure (Kinsella et al. 1989). α-la contains four disulphide bonds and no free thiol group and has a strong Ca^{2+} binding site. The Ca^{2+} binding stabilizes the conformation of α-la and increases its thermal stability against denaturation. The secondary structure of α-la molecules can be easily changed by heating, but the α-la molecule is capable of refolding to a conformation similar to that of its native state in the presence of Ca^{2+} (Boye et al., 1997; Brew, 2003). An additional reason for the high thermal stability of α-la is the absence of a free thiol group in the molecule (Chaplin & Lyster, 1986).

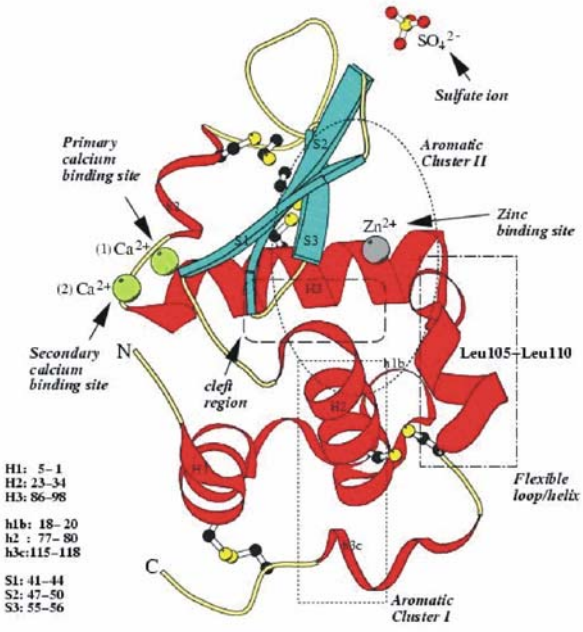


Fig. 2.12: Structure of α-lactalbumin (Chrysina et al., 2000).

If α-la is heated alone, aggregates via disulphide bonds can only be formed at extreme time and temperature combinations (Chaplin & Lyster, 1986). In the presence of β-lactoglobulin (β-lg), the thermal stability of α-la decreases because β-lg, with its free thiol group, acts as a catalyst to open the disulfide group in α-la, thus enabling the formation of intermolecular disulfide bonds (Calvo et al., 1993).

Most investigations focused on the different states of proteins separately by denaturing the proteins with chemical agents (Taulier & Chalikian, 2001; Nölting et al., 1993). Corredig et al. (2004a; 2004b) did the first investigation of the in situ thermal denaturation of whey protein isolate (WPI) and β-lg using a high-resolution ultrasonic instrument. Their investigation focused on a description of the course of ultrasonic properties of WPI and β-lg over a temperature range of 20-80 °C and a comparison of WPI and β-lg with regard to their ultrasonic properties. They found that, during heating, the ultrasonic velocity difference between sample and reference decreased continuously with temperature, which was explained by a rearrangement of the hydration layer the protein and an increase in compressibility of the protein shell. A sharp decrease in ultrasonic velocity difference between sample and reference and an increase in in the attenuation at 70 °C indicate the protein denaturation and formation of gel network. WPI and β-lg showed similar ultrasound properties during heating. No further investigations exist referring to the ultrasonic characterization of the thermal denaturation.

2.6.2 Egg proteins

Due to their technological functionalities such as coagulation ability, foaming ability and emulsifying properties, egg white and egg yolk are applied to many foods (e.g., baked goods, noodles, confectionery, pastry products, mayonnaise and other salad dressings, soup powders, margarine, meat products, ice cream and egg liqueurs).

Egg white contains different protein fractions, the main ones being ovalbumin, conalbumin, ovomucoid, lysozyme and others. The main protein fraction of egg white and their characteristics are shown in Tab. 2.2. All egg white proteins except lysozyme are glycoproteins, at which continuous polypeptide chains oligosaccharides of different numbers and compositions are covalently bound. Ovalbumin is a glycoprotein, carbohydrate being present to the extent of 3.2%. It is a compact, roughly spherical molecule. Conalbumin is an iron binding protein (Painter & Koenig, 1976). The proteins in egg white do not only have different denaturation temperatures, but also a different tendency to coagulate. The aggregation of egg white proteins is faster at higher protein concentrations, leading to formation of more brittle gels (Trziszka, 1994). The pI values of the proteins are also different.

The pH-value of egg white from fresh eggs is between 7.6 and 8.5. During storage the pH increases. The pH influences the stability of egg white proteins by influencing the net charge of the proteins. Ovalbumin has the highest stability at neutral pH. At pH 6 conalbumin has a very low thermal stability, while at pH 9.0 its thermal stability increases (Trziszka, 1994). During the storage of eggs ovalbumin converts to a stable form called S-ovalbumin. S-ovalbumin is highly resistant to the thermal aggregation process (Painter & Koenig, 1976).

Tab. 2.2: Protein composition of egg white (main fractions only) (Belitz & Grosch, 1992).

Protein	% of total egg white proteins	Denaturation temperature	Molecular weight	Isoelectric point
Ovalbumin	54	84.5	44 500	4.5
Conalbumin	12	61.5	76 000	6.1
Ovomucoid	11	70.0	28 000	4.1
Ovomucin	3.5	-	$5.5\text{-}8.3\cdot10^6$	4.5-5.0
Lysozyme	3.4	75	14 300	10.7

When egg white is heated, individual egg white protein fractions have different temperature ranges of heat sensitivity. Donovan et al. (1975) studied the denaturation of egg white proteins using DSC with a heating rate of 10 °C/min. They found two major endothermic peaks for the sample at pH 7. These two peaks are produced by the denaturation of conalbumin and ovalbumnin, respectively. The peaks shift to higher temperatures by raising the pH to 9 or by adding saccharose to the egg white. Rossi and Schiraldi (1992) used a heating rate of 2 °C/min and deconvolted the endotherminc signal of fresh egg white into three main Gaussian components, which are related to the denaturation of conalbumin, lysozyme and ovalbumin.

Painter and König (1976) reported that the formation of intermolecular β-sheet structure during thermal denaturation of various egg white proteins was observed by Raman spectroscopy (Fig. 2.13).

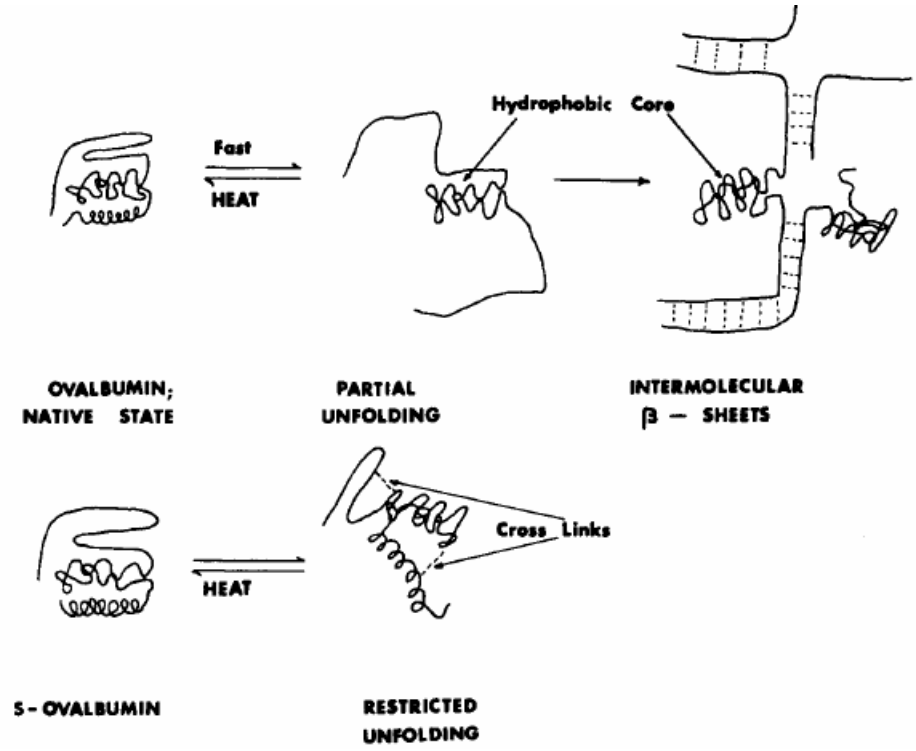


Fig. 2.13: Thermal denaturation mechnism for ovalbumin proposed by Painter and Koenig (1976). S-ovalbumin is a more stable form of ovalbumin, which is occurred in vivo during storage of eggs.

Egg yolk is a natural oil-in-water emulsion containing 52% dry matter, of which fat represents about 65%, proteins about 31%, the remaining 4% being carbohydrates, vitamins and minerals (Li-Chan et al., 1995; Burley & Vadehra, 1989). All lipids of egg yolk are associated with proteins to form lipoproteins, which are commonly classified in low-density lipoproteins (LDL), which apoproteins are called lipovitellenins, and high-density lipoproteins (HDL), which apoproteins are called lipovitellins (Anton, 1998). Egg yolk can be separated into plasma (supernatant) and granules (sediment) after dilution in 1% NaCl followed by centrifugation. The plasma contains lipid-free globular glycoproteins known as α-, β- and γ-livetins, as well as the LDL. Granules contain a lipid-free phosphoprotein known as phosvitin, as well as the HDL (Burley & Vadehra, 1989). Bernardi and Cook (1960) have shown that the HDL fraction of egg yolk consists of two forms called α- and β-lipovitellin. The construction of egg yolk in detail is presented in Fig. 2.14. Granules exist as insoluble complexes in 1% NaCl solution, because negatively charged phosphoserine residues of high density HDL and phosvitin molecules are linked through the divalent cations Ca^{2+} (Chang et al., 1977; Causeret et al., 1991). At high NaCl concentration phosphocalcium bridges are broken due to the substitution of divalent Ca^{2+} by monovalent Na^{+}, while HDL and phosvitin are soluble (Causeret et al., 1991).

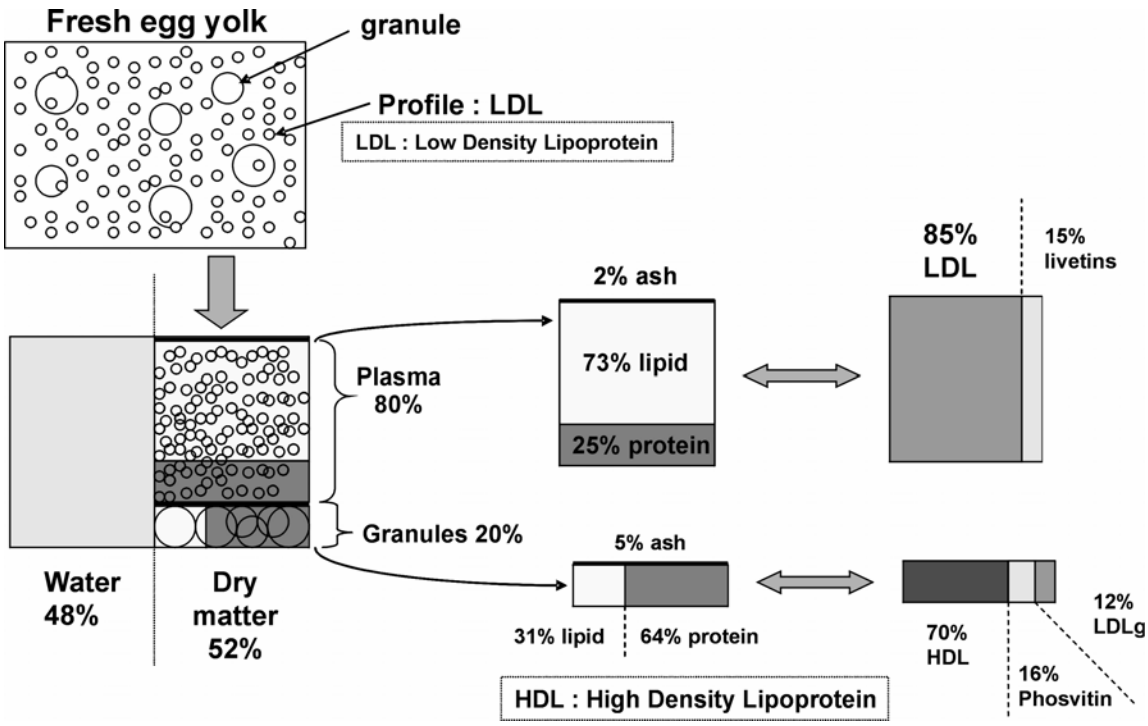


Fig. 2.14: Construction of egg yolk (Saari et al., 1964; McBee & Cotterill, 1979; Guilmineau, 2004).

Egg yolk proteins have also been shown to be sensitive to heat, particularly the LDL (Tsutsui, 1988) and some of the livetins (Ternes & Werlein, 1987). An egg yolk dispersion (5.5 wt% protein) is reported to start getting thicker after a few minutes at temperatures above 64 °C and to form a gel at temperatures above about 69 °C (Anton et al., 2001). Tsutsui (1988) observed that the amount of extractable lipid in the heated LDL decreases compared to that of the unheated sample. He supposed that the aggregates of LDL may contain lipids in its interior and the protein may play an important role in aggregation between LDL particles.

Egg white and yolk sold for industrial use must be pasteurised in order to ensure its microbiological safety. The heat treatment results in denaturation of heat sensible protein fractions and leads to alterations in their technological functionalities. In order to understand these alterations, it is important to assess the thermal-induced changes in the egg proteins.

The methods for investigation of thermal denaturation of egg proteins used in the literature are gel electrophoresis (Chang et al., 1970; Mine et al., 1990; Guilmineau et al., 2005), solubility measurement (Guilmineau & Kulozik, 2006a; Guilmineau & Kulozik, 2006b), DSC (Mine, 1997; Rossi & Shiraldi, 1992), chromatography (Sajdok et al., 1989; McBee & Cotterill, 1979), turbidity measurement (Kitabatake & Kinekawa, 1995), immunochemical assay (Sajdok et al., 1989) and ultrasonic method (Takagi et al., 1986; Bae, 1996; Bae et al., 1998).

Researchers in Japan and Korea have studied a few ultrasonic investigations on thermal-induced changes in egg proteins. Takagi et al. (1986) measured the ultrasonic velocity and attenuation in the thin portion of egg white at 3 MHz at a range of temperature. They observed a two-stage increase in ultrasonic attenuation near and after the gel transition temperature. The first increase was explained by the interaction of constructed network and the solvent, but Takagi et al. (1986) could not explain the mechanism of the second increase. Later, Bae et al. (1998) made a step forward. They investigated the gelation of egg white as well as the pure form of different egg white protein fractions using the ultrasonic method. They also observed the two-stage increase in absorption over temperature in the egg white sample. They assigned these two stages to the aggregation of conalbumin and ovalbumin, respectively. These investigations in the past are restricted to a qualitative description of the gelling or aging process only. It is of interest to acquire the capability of ultrasound for quantitative investigation of egg proteins.

2.6.3 Protective effect of sugars on the protein stability

Sugars have large effects on the structure and properties of the protein. When sugar is added to the protein solution, the OH groups of sugar interact with both protein and water. Sugar interacts more strongly with water molecules than with protein. The protein is preferentially hydrated and the sugar preferentially excluded from protein (Fig. 2.15). The addition of stabilizer such as sugar increases the chemical potential of the protein and, thus, the free energy of the system. This is a thermodynamically unfavourable situation. Since denaturation leads to protein unfolding and an increase in structural asymmetry, the solvent accessible surface of protein increases. The unfolding results in an increase of the zone of co-solvent exclusion which leads to a thermodynamically even more unfavourable state with a higher chemical potential, by the Le Chatelier principle, the reaction is pushed towards the native state (Timasheff & Arakawa, 1989).

Waris et al. (2001) studied the stabilisation of ovalbumin by measuring density, ultrasonic velocity and viscosity. They suggested that the stabilisation of ovalbumin is mainly due to two mechanism: Firstly, the primary interaction between sugar and water molecules leads to the formation of clusters in the vicinity of the protein. This will favour an increase in the degree of water molecule organisation and will thus limit the denaturation of the protein. Secondly, the hydrophobic interactions are strengthened by the unfavourable (or polar) environment produced by sugar molecules preventing the exposure of the hydrophobic groups to the solvent.

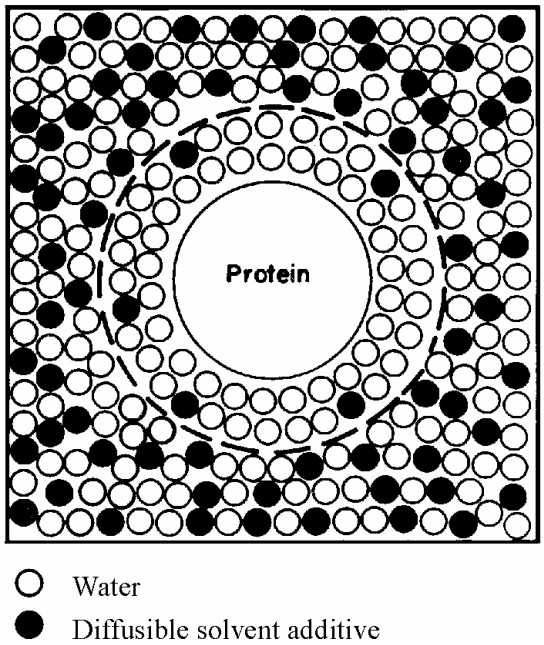


Fig. 2.15: Schematic representation of preferential hydration. (Timasheff & Arakawa, 1989).

2.7 Hydrolysis of lactose

Lactose is the only sugar in milk. The lactose concentration in the milk is about 4-5 % and 37 % of the dry mass. Many people, especially people in Asia and Africa, cannot digest dairy product, because of their lactose intolerance. This problem can be solved by the hydrolysis of lactose to glucose and galactose (Fig. 2.16). Further benefits of hydrolysing lactose are increased solubility, higher osmotic pressure, lower viscosity and an increased level of reducing sugars and sweetness value (Baer et al., 1980). By use of lactose-hydrolysed whey, the sandy structure caused by the formation of lactose crystals can be prevented.

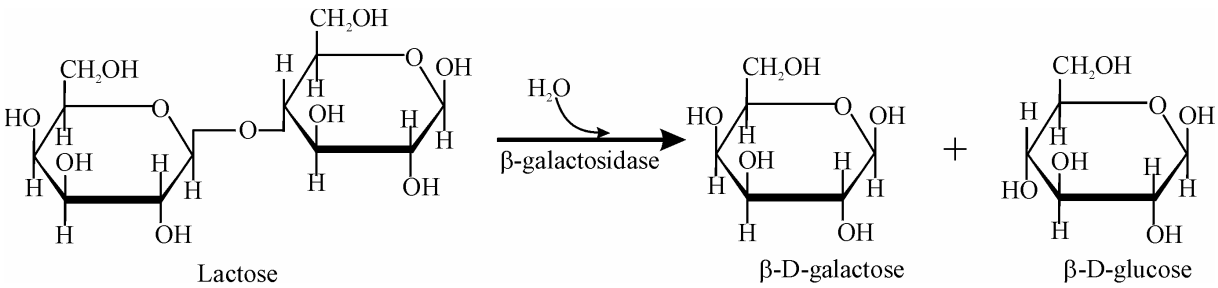


Fig. 2.16: Enzymatic hydrolysis of lactose to galactose and glucose.

Acid hydrolysis of lactose is possible, but colour and bitterness are produced. Also the process has shown to be relatively inflexible (only demineralized, deproteinized streams can be processed). Commercialisation of this process has not, therefore, taken place. Enzymatic hydrolysis with β-galactosidase (lactase) presents a good alternative. As the enzyme is inhibited by galactose (one of the reaction products) it is very difficult to achieve complete

hydrolysis unless high concentrations of enzyme are used. Lactases can be immobilized on a variety of supports for industrial use.

Lactose hydrolysis is applied in liquid milk, milk powders, fermented milk products, concentrated milk products (e.g. ice cream), whey for animal feed, whey, deproteinized whey (permeate of milk ultrafiltration).

There are a few methods to determine the degree of hydrolysis during processing. They include (a) cryoscopic method, which is based on the depression of the freezing point of the solution induced by the increase in the molarity of the soluble compounds due to the splitting of lactose into galactose and glucose (Zarb & Hourigan, 1979), (b) differential pH technique, which uses an additional enzymatic step causing a pH variation proportional to the glucose content in the sample (Luzzana et al. 2001; Luzzana et al. 2003) and (c) chromatographic method (Jelen, 2002; Ferreira et al., 1998). However, these methods normally require sample preparation. The differential pH technique requires an additional enzymatic step — the phosphorylation of the hydrolysis product, glucose, catalysed by hexokinase. For chromatographic methods, fat and proteins have to be removed from the sample. The ultrasonic technique offers a good potential to determine the degree of lactose hydrolysis due to its sensitivity to hydration changes and its on-line or in-line applicability. By breaking down lactose into galactose and glucose, the overall hydration is supposed to increase due to the increasing surface accessible to the solvent. According to Eq. 2.9, the ultrasonic velocity is expected to increase with decreasing hydration or increasing hydrolysis of lactose. So it is possible to determine the degree of hydrolysis by measuring the change of ultrasonic velocity during fermentation.

In the literature, the application of high intensity ultrasound for the enhancement of the lactose hydrolysis during fermentation using lactic acid bacteria was described, which based on the enhanced release of lactic acid bacteria cells to the medium. (Sakakibara et al. 1994; Wang & Sakakibara, 1997). However, there is no information in the literature referring to the application of ultrasound as an analysis method for monitoring the hydrolysis process. For such application, fundamental knowledge about the change in ultrasonic velocity depending on the hydrolysis is required.

3 Target of this work

To understand the structure of complex systems such as food, it is often necessary to apply more than one method of analysis. The known analytical methods for structure characterisation are optical microscopy, electron microscopy, rheology, NMR, laser diffraction, to name a few. In the last few years the low-intensity ultrasound is developed as a new analytical method for characterizing the structure in food systems.

Due to the complexity of food systems, the low-intensity ultrasound technique is, despite of its many benefits, still in its infancy in food science and process. To establish the low-intensity ultrasonic technique as a new method of analysis, the first step is to get fundamental knowledge about the ultrasonic properties in different model food systems.

The aim of this work was to assess the applicability of the low-intensity ultrasound for different systems in the food research on the one hand, and to obtain the fundamental knowledge for the potential application of ultrasound as an online sensor on the other hand.

This work focuses on the application of ultrasound to characterise the gelation of hydrocolloids, the gelation of milk proteins and protein denaturation. Furthermore, the enzymatic hydrolysis of lactose was investigated. To assess the applicability of the ultrasonic method, appropriate reference methods were applied for different investigations.

Firstly, the gelation and melting properties of different carrageenans and gelatine as well as the rennet and acid gelation of milk proteins and the gelation of caseinomacropeptides was investigated using ultrasonic and oscillating rheological method. These systems have different gelation mechanism. The purpose of these experiments was to understand the influence of the gelation mechanism on the ultrasonic responses and to find out if there is a correlation between the rheological and the ultrasonic method.

To acquire the applicability of the ultrasonic method for the characterisation of protein denaturation, the degrees of aggregation of whey protein α-lactalbumin were measured by the ultrasonic method, Differential Scanning Calorimetry (DSC) and High Performance Liquid Chromatography (HPLC), respectively. These three methods were compared regarding to their capability on the determination of the degrees aggregation. Furthermore, the denaturation of the egg white proteins and egg yolk proteins were monitored by the ultrasonic and DSC methods.

Finally, the enzymatic hydrolysis of lactose in UF-permeate was monitored by the ultrasonic method. The degree of hydrolysis was determined over incubation time using RP-HPLC. In this experiment, the relationship between the ultrasonic properties and the degree of hydrolysis of lactose should be investigated.

Most of the earlier investigations are qualitative description of the ultrasonic changes during a process. In this work the main focus was put on the quantitative assessment of changes in sample using ultrasonic method by comparing to reference methods auch as rheology, DSC, or HPLC, in order to acquire new knowledge regarding complex food systems, which cannot simply be characterised by one single analytical technique alone.

4 Material and methods

4.1 Analytical methods

4.1.1 Ultrasonic measurements using the ResoScan® system

All ultrasonic measurements were performed using the ultrasonic device ResoScan® from TF Instruments GmbH (Heidelberg, Germany). The ResoScan® system measures ultrasonic velocity v and attenuation (α/f^2), where α is the attenuation coefficient of the sample and f the frequency of the ultrasonic wave. The ResoScan® system is based on the resonance technique. The sample cells are constructed as ultrasonic resonators in which a standing wave is stimulated. Fig. 4.1 shows a schematic representation to describe the principle for the ultrasonic measurement. The ultrasonic wave is generated by a high frequency alternating voltage using an ultrasonic transducer. The ultrasonic wave travels through the sample and reaches the receiver, which transforms the ultrasonic wave into alternating currency. An analyser compares the AC signal used to generate the ultrasonic wave at the sender transducer with that transformed from the receiver transducer. In this way, the ultrasonic velocity v and the attenuation α/f^2 are determined.

During the initialization, a frequency range of about 7 to 9 MHz is scanned. Series of resonance peaks are obtained within this frequency range (Fig. 4.2). By locating the resonance frequencies within this range, the order of the resonance peaks can be calculated. The system automatically selects an optimal resonance peak (the master peak) for the measurement. According to this peak, the ultrasonic velocity and attenuation are calculated. For the measurements in this work the selected master peak was at 7.8 MHz.

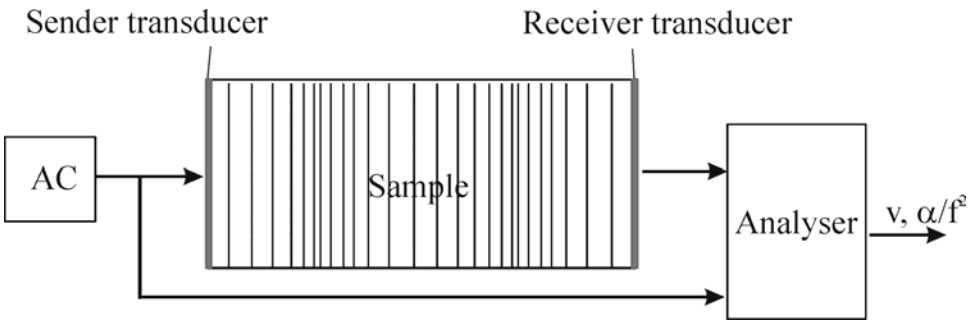


Fig. 4.1: Schematic representation of the principle for the ultrasonic measurement.

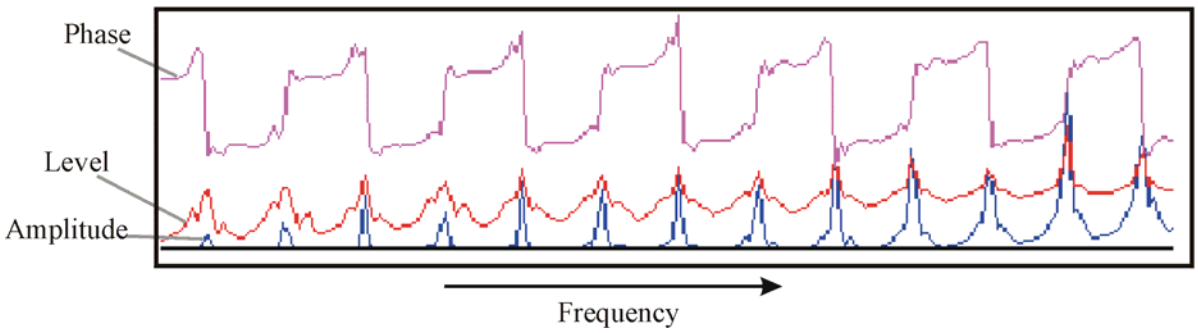


Fig. 4.2: Typical amplitude-frequency and phase-frequency diagrams of a peak scan of ResoScan®.

The ResoScan® system uses special intrinsic procedures to calculate the velocity and attenuation. Ultrasonic velocity v is calculated from fundamental frequency f_1 and the path length of the sample cells d:

$$v = 2df_1 \qquad \textbf{(Eq. 4.1)}$$

Fundamental frequency f_1 is indirectly obtained by measuring the frequency at the maximum of the master resonance peak f_n. In the ideal model of the ultrasonic resonator, the resonances are described by harmonic overtones of the fundamental frequency. Hence, f_n should be an integer multiple of the fundamental frequency f_1. In the real ultrasonic resonator, however, the measured frequencies differ somewhat from the theoretical resonance frequency. These deviations are used to evaluate the series of real resonances by system specific mathematical correction routines integrated in the software of the ResonScan system (Funck & de Maeyer, 2001).

The attenuation α/f^2 is calculated from changes in the width of the chosen master peak caused by the sample. The ResoScan® system uses a special procedure containing a corrective factor for the calculation. The determined attenuation is the total loss of energy by the sample itself including the scattering by large particles. The energy loss that occurs due to other effects, e.g. scattering, refraction and absorption by the resonator, is considered by the corrective factor. The ultrasonic intensity in the sample is in the range 10^{-4} - $5 \cdot 10^{-3}$ W/cm^2.

The ResoScan® system has two closed sample cells with a path length of 7.0 mm. Evaporation of the sample is prevented by the closed lids. In the cells, the samples can be heated or cooled with a rate of 0.1-0.35 °C/min via Peltier elements. The absolute accuracy of the thermostat temperature is 0.01 °C. The resolution of the ultrasonic velocity is 0.001 m/s, i.e., 1 mm/s. The repeatability of absolute velocity after automatic reinitialization is ± 0.01 m/s. The resolution of ultrasonic attenuation is 1-3%, depending on the extent of attenuation.

Data Analysis of ultrasonic velocity and attenuation

For many case of data analysis, differentiated values of the ultrasonic velocity were used, in order to acquire the changes in ultrasonic velocity and attenuation more clearly. The first derivation of Δv and $\Delta(\alpha/f^2)$ versus time, i.e., d(Δv)/dt and d $(\Delta(\alpha/f^2))/dt$, was calculated by using Mathcad 2001i Professional from MathSoft Engineering & Education, Inc. (Cambridge, Massachusetts, U.S.A.). A smoothened curve was created by calculating an average value of several consecutive data points. For the data smoothing, the following equations were applied:

$$\left\{\left[\frac{d(\Delta v)}{dt}\right]_m\right\}_j = \frac{\sum_{i=j}^{j+p-1}\left(\frac{\Delta v_{i+1}-\Delta v_i}{t_{i+1}-t_i}\right)}{p} \qquad \textbf{(Eq. 4.2)}$$

$$\left\{\left[\frac{d(\Delta(\alpha/f^2)}{dt}\right]_m\right\}_j = \frac{\sum_{i=j}^{j+p-1}\left[\frac{\Delta(\alpha/f^2)_{i+1}-\Delta(\alpha/f^2)_i}{t_{i+1}-t_i}\right]}{p} \qquad \textbf{(Eq. 4.3)}$$

$$(\vartheta_m)_j = \frac{\sum_{i=j}^{j+p-1}\left(\frac{\vartheta_{i+1}+\vartheta_i}{2}\right)}{p} \qquad \textbf{(Eq. 4.4)}$$

$$(t_m)_j = \frac{\sum_{i=j}^{j+p-1}\left(\frac{t_{i+1}+t_i}{2}\right)}{p} \qquad \textbf{(Eq. 4.5)}$$

i: numbering of the raw data rows, i = 1, 2, 3,…n, with n for the total number of data rows.

j: numbering of the smoothed data rows

p: number of data points included to calculate a average value

$(d(\Delta v)/dt)_m$: average d(Δv)/dt calculated from p consecutive data points

$\{d[\Delta(\alpha/f^2)]/dt\}_m$: average d(Δv)/dt calculated from p consecutive data points

ϑ_m: average temperature of p consecutive data points

t_m: average time of p consecutive data points

4.1.2 Oscillating rheological measurements

All rheological measurements for this work were non-invasive oscillating measurements. Oscillating rheological method provide information about the vicoelastic properties of samples. It was used in this work to follow the gelation process.

For a controlled stress rheometer, the input stress is applied to the sample in a sinusoidal manner with very small amplitude, so that the structure of the sample is not destroyed. The input stress:

$$\tau(t) = \tau_A \cdot \sin \varpi t \qquad \textbf{(Eq. 4.6)}$$

with τ for stress, τ_A for amplitude of stress, ω for angular frequency and t for time.

The input stress induces a sinusoidal output strain with a phase shift δ (also called loss angle) relative to the strain.

$$\gamma(t) = \gamma_A \cdot \sin(\varpi t + \delta) \qquad \textbf{(Eq. 4.7)}$$

with γ for strain and γ_A for amplitude of strain.

The storage modulus G' and the loss modulus G'' and can be calculated as follows:

$$G' = \left(\frac{\tau_A}{\gamma_A}\right) \cdot \cos\delta \qquad \textbf{(Eq. 4.8)}$$

$$G'' = \left(\frac{\tau_A}{\gamma_A}\right) \cdot \sin\delta \qquad \textbf{(Eq. 4.9)}$$

G' represents the elastic storage of energy. It is also a measure of how well structured a material is. If G' > G'', the sample is predominantly elastic or highly structured. G'' represents the viscous dissipation or loss of energy, and is related to the dynamic viscosity. If G'< G'', the sample is predominantly viscous. The tangent of δ (tan δ) is called loss factor and is equal to the ratio of G'' to G'. If G' = G'' or tan δ = 1, the elastic and viscous properties are equal.

All oscillating rheological measurements in this work were performed by using an AR 1000N controlled stress rheometer (TA Instruments, New Castle, Delaware, USA). Different geometries were chosen for the measurement of different gelling systems. The details regarding the geometry and performance for the individual experiments are described in chapter 4.2. For oscillating measurements at high temperatures or those with a temperature ramp, a protective solvent trap was used, in order to reduce the evaporation of the sample solvent.

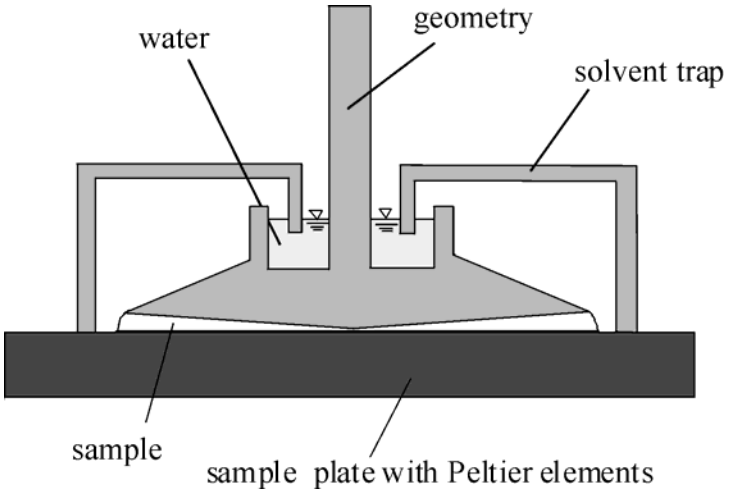


Fig. 4.3: Construction of the rheometer measuring system with solvent trap.

4.1.3 DSC method

DSC is a valuable tool to study thermal induced changes in the sample. In this method the change of thermal energy in a sample during heating or cooling is measured, which result in a heat flux or change of the thermal energy. Denaturation of protein causes an endothermic peak in the thermogram (Privalov & Khechinashvili, 1974). According to the area of the endothermic peak the denaturation of α-la can be acquired quantitatively (Manji & Kakuda, 1987).

The DSC measurements were carried out using the DSC equipment Q1000 from TA Instruments, Alzenau, Germany. The pans used for the DSC measurements were high volume pans made of stainless steel with a sample volume of 75 mg.

4.1.4 HPLC method

High Performance Liquid Chromatography (HPLC) is used to separate components of a mixture by using a variety of chemical interactions between the substance being analysed (analyte) and the chromatography column. There are different types of HPLC. In this work, the reversed phase chromatography is applied for the concentration determination of the CMP and whey proteins, and ion exchange chromatography for the sugar analysis.

The details about reversed phase and ion exchange HPLC analysis for the protein and sugar analysis are stated in chapter 4.2.

4.1.5 Determination of total protein content

When necessary, the total protein content in the sample was determined by a nitrogen analyser FP-528 (Leco Instrumente GmbH, Mönchengladbach, Germany) following the Dumas method and using a transformation factor of 6.38. The sample is flushed with pure oxygen for combustion at 850 °C. Aliquot amounts of gas containing NO_x formed are swept through a CuO/Pt-catalysator to reduce the NO_x into N_2, which is measured by a thermal conductivity detector. The protein content can be calculated from the total nitrogen content.

4.2 Experimental performance

4.2.1 Experiments for the determination of the hydration of sugar

To characterise the hydration of sugars, saccharose and lactose for microbiology (Merck, Darmstadt, Germany) were used. The sugars were weighed with an accuracy of ± 0.1 mg and mixed with deionized water under stirring. The solution was stayed for a few hours prior to use, in order to reach the hydration equilibrium of sugar molecules. For the experiments, the measuring cells of ResoScan® were filled with water and sugar solution, respectively. Then, the ultrasonic velocity and attenuation were measured at different temperatures.

The densities of the sugar solutions and water were measured using the oscillating tube densiometer DMA 45 from Anton Paar K. G. (Graz, Austria) was applied. In the DMA 45 densiometer the measuring cell is made up of a U-shaped tube (occupied by a sample), which is placed inside a double glass-walled cylinder sealed at both ends and filled with a gas of high thermal conductivity. The accuracy of the densiometer is ± 0.1 kg/m^3. The measuring temperature in the tube oscillator was hold in an accuracy of ± 0.1 °C.

4.2.2 Experiments for characterising the gelation behaviours of carrageenanen

Three different carrageenans were used for the experiments: κ-, ι- and κ/ι-hybrid-carrageenan. The carrageenans were provided by Danisco Cultor, Braband, Denmark. All the carrageenans were not standardized respecting their gel strength. The natural κ/ι-hybrid-carrageenan has a κ:ι-ratio about 0.6. This ratio was determined by the supplier using FT-IR calibrated by reprecipitated carrageenans from Sigma, St. Louis, USA. (Hansen & Wichmann, 1999). It was shown by electrophoresis that the κ and ι-units are located in the same macromolecule chain and not a mixture of κ- and ι-macromolecules (De Vries, 2002). All carrageenans contained traces of other carrageenans (< 5%) and salt ions. Tab. 4.1 shows the ion

concentrations in 0.3% carrageenan solutions determined with the flame photometer ELEX 6361 from Eppendorf AG, Hamburg, Germany.

Tab. 4.1: Ion concentrations in 0.3% (w/w) carrageenan solutions.

	Na^+ [mg/l]	K^+ [mg/l]	Ca^{2+} [mg/l]
κ-carrageenan	0.3	14.6	0.3
ι-carrageenan	3.7	12.8	7.2
hybrid-carrageenan	0	24.8	1.5

0.3%, 0.5%, 1% and 2% (w/w) aqueous solutions of different carrageenans were investigated. To investigate the influence of potassium ions K^+ on the gelation of κ-carrageenan, 0.5% κ-carrageenan with 0.04%, 0.08%, 0.16% and 0.32% (w/w) K^+ added was used. K^+ was added as KCl (purity: ≥ 99.5%, Merck KGaA, Darmstadt, Germany).

In order to obtain a homogeneous solution, carrageenan, KCl and deionised water were added by weight to a total amount of 50 g and placed in a preheated water bath of 70 °C under stirring for 20 min. 0.18 ml of the solution was filled in one of the cells of the ResoScan® device at 70 °C. The other cell was filled with 0.18 ml of a reference. References were deionized water for the samples without K^+ addition and KCl solutions for the samples with K^+ addition, respectively. The KCl solutions had the respective concentrations as in the carrageenan solutions. For the ultrasonic measurements the samples and references were cooled from 70 °C to 10 °C by a rate of 0.1 °C/min, held for 15 min at 10 °C and then heated again.

As a reference method to investigate the gelation of carrageenans, the storage modulus G', the loss modulus G" and tangent of the loss angle tan δ were measured using a stress-controlled rheometer AR 1000N (TA Instruments, New Castle, USA). The geometry for the measurement was a plate/cone system with 40 mm cone diameter and 4 ° cone angle. The samples were cooled from 70 °C to 10 °C, held for 10 min at 10 °C and then heated again. A solvent trap cover was used in order to avoid evaporation of solvent during the measurement. The rate of cooling and heating was 1 °C/min. We chose this higher cooling and heating rate as compared to the ultrasonic measurements in order to avoid losses of solvent due to evaporation, which could happen at too long processing times. At low heating rates time for the measurement would be very long, so that the concentration of sample could possibly be changed during the measurement. The oscillation frequency was 1 Hz, and the displacement of the geometry was $5 \cdot 10^{-4}$ rad.

4.2.3 Experiments for characterisation of gelatine gelation

Rosselot™ (L'isle sur la sorgue, France) Gelatine 150 LH 30 was used for characterisation of the gelation of gelatine. It is a limed hide edible gelatine with a gel bloom strength of 140 to 160 g (measured at 6.67%, 10 °C). The powder gelatine was swollen in cold water for 30 min, then heated to 65 °C and stirred for 30 at 65 °C.

The homogenous gelatine solution was added to the measuring cell of ResoScan® at 65 °C and sample plate of the rheometer, respectively. For the ultrasonic measurement, water was used as reference. The solution was cooled to 10 °C, hold for 5 min at 10 °C, then heated again. The cooling/heating rate 0.3 °C/min for the ultrasonic measurement and 1 °C/min for the rheological measurement.

4.2.4 Experiments to investigate the rennet gelation

Production of casein solution and heating experiments

Pasteurised skim milk (72°C, 15 s) was obtained from a local dairy. The whey proteins were removed by using microfiltration (MF) in combination with ultrafiltration (UF) according to Kersten (2001). The resulting "whey protein-free" casein solution with 3% (w/w) casein and ≤ 0.02% (w/w) whey protein had similar lactose and salt contents as the original skim milk. The whey protein content was determined by RP-HPLC (Beyer, 1989). The casein solution was stored at -18°C prior to use.

The MF Module (APV, Silkeborg, Denmark) has 7 multi-channel elements (SCT, Bazet, France) with 19 channels (total area 1.68 m2) each. The membrane material is α-aluminium oxide and an active layer of zirconium oxide. The nominal pore-size of the membrane was 0.1 µm. The MF plant works according to the Uniform Transmembrane Pressure (UTP) principle, which reduces the surface layer formation and therefore allows a higher permeation flux compared to traditional cross-flow microfiltration. The UF module (DSS, Nakskov, Denmark) consists of a polysulphone membrane with a total area of 3 m^2 and the molecular weight cut-off of 25,000 Da.

The pre-treatment by heat under UHT conditions of the casein solution was carried out in small stainless steal tubes with a volume of 20 mL in a lab scale heating plant. The casein solution was pre-treated at 120, 130 and 140°C for up to 300 s.

Sample preparation for the analysis

The casein solution, unheated or heated under variation of the UHT heating temperature and time, was tempered at 30°C. After the temperature equilibrium was reached, 0.02% CaCl2 (Merck KGaA, Darmstadt, Germany) was added to the casein solution. Then the pH value of the solution was adjusted to 6.5 using 9% lactic acid (Merck KGaA, Darmstadt, Germany). This solution was used as starting solution for the renneting experiments. The renneting was performed at 30°C with 0.02% rennet addition. According to the specification from the manufacture, the rennet extract (Standard plus 175, Chr. Hansen, Nienburg, Germany) consisted of 80% chymosin and 20% pepsin. It was applied in the form of a 5% solution to adjust the rennet concentration.

Rheological measurements

Casein gels are viscoelastic (Bohlin et al., 1984) and their rheological properties upon small deformation can be determined using low amplitude dynamic oscillation. An AR 1000 N Rheometer (TA Instruments, New Castle, Delaware, USA) was used to measure the dynamic rheological properties of the samples. The measuring geometry consisted of an acryl cone (diameter 6 cm and 2° angle) and a plate. Two hundred µL of 5% rennet solution was added to 50 g casein solution with adjusted $CaCl_2$ concentration, pH and temperature. After 30 s stirring the sample was transferred to the plate of the rheometer with an adjusted temperature of 30°C. The sample was measured during 60 min at a frequency of 1 Hz. The strain was 0.03. The storage modulus G' was measured.

In this work, the coagulation time was considered as the time necessary for the gel to reach a G' value of 1 Pa according to Walsh-O'Grady et al. (2001) and Srinivasan & Lucey (2002). The gel strength was defined as the storage modulus after 60 min oscillation time.

Ultrasonic measurement

Prior to the addition of rennet, both sample cells of ResoScan® were filled with 3% casein solution with adjusted $CaCl_2$ concentration, pH and temperature by means of a syringe, for the initialisation of the system to select the resonance peak. After completion of this procedure, 200 µL of 5% rennet solution was externally added to 50 g of the starting solution with adjusted $CaCl_2$ concentration, pH and temperature and stirred for 30 s. Subsequently, the casein solution in cell 2 was replaced by rennet containing casein solution. In order to determine the effect of rennet alone and to minimize even small temperature fluctuations, the differences of ultrasonic velocity and attenuation between a sample, casein solution with

rennet, and a reference, casein solution without rennet, were used instead of the absolute values v and α/f^2. The differences are abbreviated as Δv and $\Delta(\alpha/f^2)$ respectively. The ultrasonic velocity Δv and attenuation $\Delta(\alpha/f^2)$ were measured over incubation time. In total, the measurement was started approximately 120 s after adding the rennet in both rheological and ultrasonic measurements.

Determination of the CMP content in serum during renneting

50 ml of the same whey-protein-free casein solution with adjusted pH and Ca^{2+} content as used for the ultrasonic and rheological measurements was equilibrated at 30 °C. After addition of 200 µL 5% rennet and 30 s stirring, the sample was divided and transferred in 10-11 tubes with 4 mL solution in each tube. After different incubation times at 30 °C, the renneting reaction in the tubes was stopped by adding 1 mL 15 % (w/w) perchloric acid (Merck, Darmstadt, Germany). The precipitated casein was separated by centrifugation at 3000 g for 15 min. The CMP content in the supernatant was determined by RP-HPLC according to Thomä et al. (2006a). A sample without rennet addition was prepared in the same way as a control. This sample was considered as the sample with incubation time 0. The relative CMP release was calculated according to following equation:

$$rel.\,CMP\ release = \frac{CMP\ content\ in\ sample}{\max.\,attainable\ CMP\ content} \qquad \textbf{(Eq. 4.10)}$$

4.2.5 Experiments to investigate the acid induced milk gelation

The GDL was obtained from Fluka Chemie AG (Buchs, Switzerland). The culture for yoghurt fermentation was ABT 21 in frozen form from Christian Hansen (Nienburg, Germany). The culture is composed of milk lactic acid bacteria *Streptococcus thermophilus*, *lactobacillus acidophilus* und *Bifidobacterium sp.* (Hansen, 2001). In order to make the dosage easier, a 10% solution of the culture was used. For this purpose, 5 g frozen culture was dispersed in 45 g cold pasteurized skim milk and stored at 4°C prior to use. At 4°C the fermentation does not start.

For the ultrasonic measurements the measuring cells of the ultrasonic device ResoScan® was filled with pasteurised skim milk und equilibrated at 42 °C. The initialising process was started to select the optimal peaks for the measurement. After that, 100µl 10% culture solution or 1.5 g GDL was added externally to 50 ml skim milk, which was equilibrated at 42 °C (for yoghurt fermentation) or 30 °C (for direct acidification). The sample was stirred for 1 minute. Subsequently, the skim milk in the sample cell of ResoScan® was replaced by the sample

containing yoghurt culture or GDL. The measurement was started. The rest samples containing yoghurt culture or GDL were hold in water bath at 42 °C or 30 °C and the pH value in these samples were measured.

4.2.6 Experiments for characterizing CMP gelation

Two different commercial CMP products were used. One was the Lacprodan CGMP-20 from Arla Foods Ingredients amba (Viby J, Denmark), and the other one was BioPure-GMP™ from Davisco Foods International, Inc. (Eden Prairie, MN, USA). The specifications of these two products are described in Tab. 4.2. The glycosylation degree of these CMP samples was determined by HPLC. Lacprodan CGMP-20 and BioPure-GMP™ have a glycosylation degree of about 76% and about 65% respectively.

Tab. 4.2: Specification of the CMP Lacprodan CGMP-20 from Arla Foods Ingredients amba and BioPURE-GMP™ from Davisco Foods international, Inc.

	Lacprodan CGMP-20	BioPURE-GMP™
Protein	80 ± 2%	83.3 ± 3.0
CMP content in protein	> 95 %	90.0 ± 3.0
Lactose	< 1.0%	> 2%
Fat	< 0.2%	< 1.0%
Ash	< 7.0%	< 7.0%
Moisture	< 5.0%	< 7.0%

In order to obtain a 5% CMP solution of different pH, at first a 6% CMP solution was produced by dispersing the CMP powder in bi-distilled water and stored in refrigerator for at least 15 hours prior to use. Then, the pH of the CMP solution was adjusted using a 1 M and a 0.1 M HCl solution. The weight of added HCl solution was measured. After that, the CMP solution was diluted to 5%. Finally, the pH of the 5% CMP solution was measured and adjusted again. In this way, the concentration deviation due to pH adjustment was prevented. Solutions of different pH measured by ultrasonic and DSC methods with a heating rate of 0.3 °C/min. Deionized water was used as the reference sample in both methods.

4.2.7 Experiments to determine the degree aggregation of α-lactalbumin

α-lactalbumin

α-la used was produced from whey protein concentrate by means of micro- and ultrafiltration at adjusted pH, whey protein, calcium and lactose concentrations and with subsequent thermal denaturation. The details have been described by Tolkach et al. (2005). The obtained solution of native α-la was freeze-dried and stored prior to use. The α-la/β-lg ratio in the powder was about 10:1 according to the HPLC analysis.

To acquire the general course of ultrasonic velocity in an α-la solution with a variation in temperature, a 4% (w/w) native α-la was used. To achieve different degrees of irreversible α-la aggregation for the measurements, 6% and 10% native α-la were pre-heated at 90°C for varying times. The high concentrations were chosen to avoid overly low native α-la concentrations for measurements in samples with high degrees of aggregation, which could be under the detection limit of the methods applied.

To obtain the α-la solutions of different concentrations, the α-la powder was dissolved in milk serum (UF-permeate) produced by ultra- and diafiltration of milk using a membrane with a nominal cut-off value of 25,000 Da. The UF-permeate has the same composition of lactose and minerals as milk, but casein and whey proteins were excluded. The exact α-la concentrations in the samples were determined by HPLC.

Pre-heating experiments

The pH value of the solution was adjusted to 6.5 with NaOH and HCl solutions (Sigma, Taufkirchen, Germany). The solutions were added to small stainless steel tubes with an inner diameter of 4 mm, a length of 260 mm and a volume capacity of 3.3 ml. The tubes with samples were heated in a water bath at 90 °C. Different degrees of aggregation of α-la were obtained by varying the heating time.

HPLC method

The concentration of native α-la was determined by reversed phase HPLC (RP-HPLC) according to a method described in details by Kessler and Beyer (1991) as well as Tolkach and Kulozik (2005). According to this method, the pH value of the samples was adjusted to 4.6 using 1 M and 0.1 M HCl solution. The irreversibly aggregated α-la coagulates. The aggregates were separated using a membrane filter (ø 45 µm, Chromafil® RC-45/25, Macherey-Nagel, Düren, Germany). The concentration of native α-la in the filtrate was

determined by RP-HPLC. A PLRP-S 8µm, 300Å column from Latek (Eppelheim, Germany) was used. Elution was performed by using a gradient from a mixture of 57% eluent A [0.1% trifluoroacetic acid (TFA) in water] and 43% eluent B (80% acetonitrile, 19,1445% water and 0.0555% TFA) to 100% eluent B in 23 min. The temperature of the column was kept at 40 °C. The flow rate was 1.0 ml/min. The absorbance was recorded with a UV detector at 226 nm.

Fig. 4.4 shows the HPLC chromatograms of a 6% α-la solution, unheated and pre-heated at 90 °C for 2 min. The difference in the α-la absorption peak areas between the unheated and pre-heated sample correlates with the amount of the irreversibly aggregated α-la.

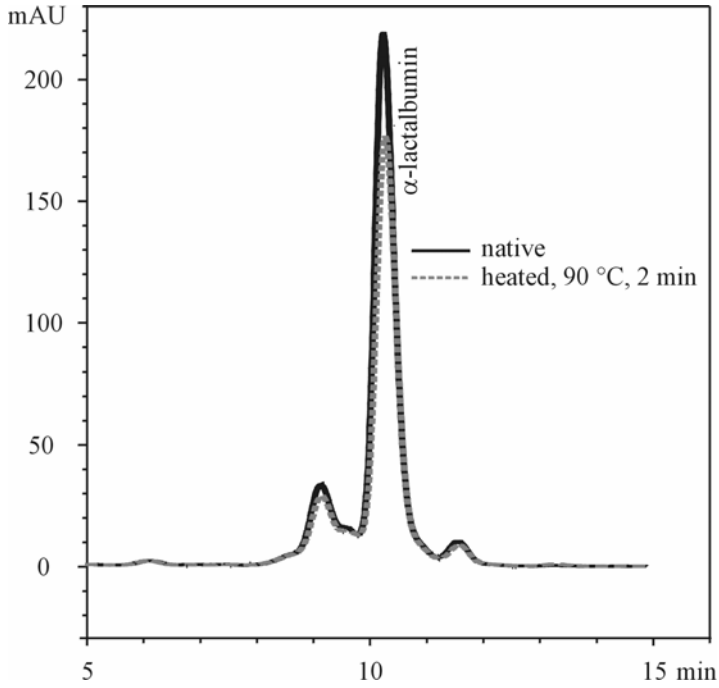


Fig. 4.4: HPLC chromatograms of a 6% α-la solution, native and pre-heated at 90°C for 2 min.

The degree of irreversible aggregation of α-la molecules (DA) in pre-heated samples was calculated from the native α-la concentration in the pre-heated and unheated sample by the following equation:

$$DA_{HPLC} = (1-C_{pre\text{-}heated} / C_{unheated}) \cdot 100\% \qquad \textbf{(Eq. 4.11)}$$

DSC method

The reference sample was UF-permeate. The heating rate was 0.3 °C/min, the same as that in the ultrasonic measurements. The small heating and cooling rate was chosen so that DSC and ultrasonic methods could be compared on the one side, and so that a better thermal equilibrium within the sample could be achieved on the other side. The heat flow from the DSC device to the sample was measured as watts per gram of sample. The denaturation enthalpy ΔH was calculated by integrating the areas of the endothermic peaks obtained from

heated and unheated samples. Fig. 4.5 shows the DSC thermograms of a solution containing 6% native α-la, unheated and pre-heated at 90 °C for 2 min.

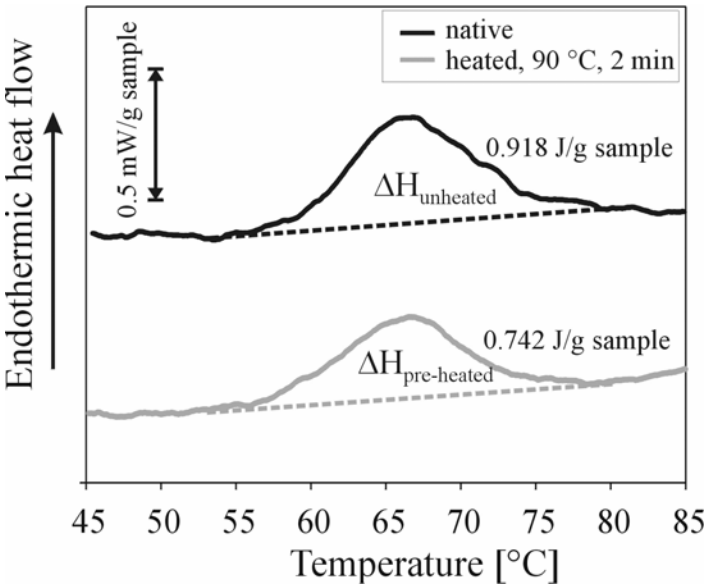


Fig. 4.5: DSC thermograms of a solution containing 6% α-la, native and pre-heated at 90 °C for 2 min.

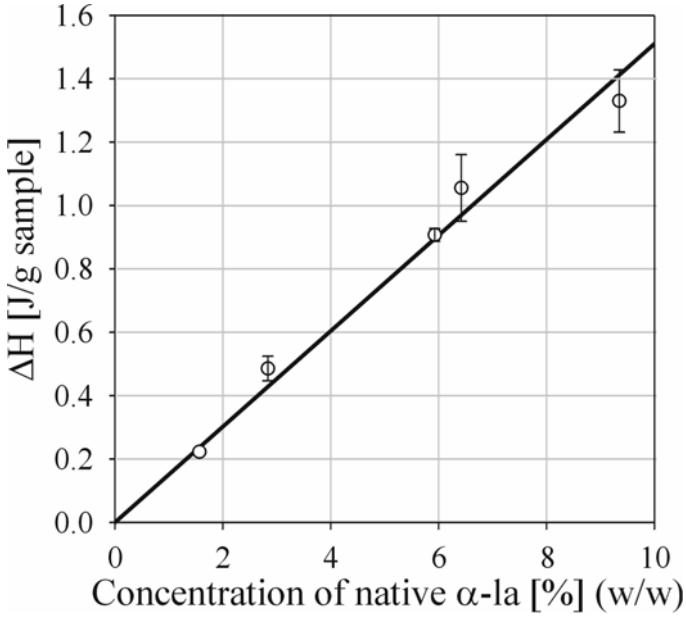


Fig. 4.6: Denaturation enthalpy as a function of the concentration of native α-la in the sample.

To assess the relationship between the denaturation enthalpy, i.e., the area of the endothermic peak and the content of native α-la in the sample, solutions with different concentrations of native α-la were measured. A linear correlation between these two parameters was found (Fig. 4.6). Therefore, the degree of irreversible aggregation in heated samples can be calculated from the denaturation enthalpy (peak area) of the pre-heated and the unheated samples during heating:

$$DA_{DSC} = (1-\Delta H_{DSC,\ pre\text{-}heated} / \Delta H_{DSC,\ unheated}) \cdot 100\% \qquad \textbf{(Eq. 4.12)}$$

Ultrasonic method

All experiments were carried out by measuring the ultrasonic velocity in the α-la solution against that in the reference UF-permeate. The reference UF-permeate and the α-la solutions were added to cells 1 and 2, respectively. The samples were equilibrated at 45 °C before the initialization process of the ResoScan® unit. In the initialization process a resonance peak at 7.8 MHz was chosen for the measurement. The sample was heated from 45 °C to 85 °C at a rate of 0.3 °C/min. The ultrasonic velocity in cells 1 and 2 during heating was measured. To determine the heat effect on α-la alone, the difference between the velocity in an α-la solution and that in the reference UF-permeate Δv was used for data analysis.

Calculation of velocity constant of irreversible α-la aggregation

Many researchers found that the denaturation of α-la follows a reaction order of one (Anema, 2001; Plock et al., 1997). For a first-order reaction the following relationships applies:

$$\ln \frac{C_t}{C_0} = -k \cdot t \qquad \textbf{(Eq. 4.13)}$$

with C_t is the concentration of the reactant (here: the native α-la) at time t, C_0 the concentration at t = 0, and k the velocity (rate) constant of the reaction.

The velocity constant of irreversible α-la denaturation (i.e., aggregation), k was determined according to Eq. 4.13. The decrease in the native α-la concentration was calculated from the degrees of aggregation determined by the respective method:

$$(C_t/C_0) = 1\text{-}DA_t \qquad \textbf{(Eq. 4.14)}$$

where DA_t is the degrees of aggregation at time t.

To compare the velocity constants of α-la aggregation determined by ultrasound, DSC and HPLC, an ANOVA test was performed using Statgraphics Centurion XV (StatPoint, Inc, Herndon, VA, U.S.A.). A P-value α of 0.05 was used as a threshold of statistical significance. The velocity constants k for all 24 measuring points for the samples with 6% α-la (eight points per methods) were calculated using Eq. 4.13 and Eq. 4.14.

4.2.8 Experiments to characterise the thermal denaturation of egg proteins

The eggs were from a local farm. Egg white and yolk were manually separated. The egg white was gently stirred for a few hours, in order to obtain homogeneous egg white. The total protein concentration in undiluted egg white was 10.75% according to the protein analysis.

The protein concentration of the egg white solutions was adjusted using 0.5 % NaCl. To adjust the pH value, 1 M as well as 0.1 M HCl and NaOH solutions were applied. The sugar concentration was adjusted using commercial sugar for household form Südzucker (Mannheim, Germany). The samples were measured in ResoScan® against a reference, 0.5% NaCl or sugar solution with the same concentration as it is in the sample.

The concentration of egg yolk proteins was adjusted by using NaCl solutions of different concentrations. In the experiment, the egg yolk was separated in plasma and granules. For this purpose the complete egg yolk was 1:3 diluted (i.e., 25% egg yolk solution) using 1% NaCl and stirred for at least 1h. The homogeneous diluted dispersion was centrifuged at 10 °C and 10 000 g for 30 min. The supernatant and the sediment were the plasma and the granules fractions for the experiment, respectively. In order to remove the residual plasma in the granules, the sediment from the first centrifugation was dispersed in 1% NaCl, stirred for at least 30 min. Then the suspension was centrifuged at 10 000 g for another 30 min. The protein concentration in the complete egg yolk solution, plasma and granules were determined. The concentration of the granules was adjusted by using 3% NaCl. The complete egg yolk, the plasma and the granules were adjusted to the same protein and NaCl content by using 3% NaCl solution and solid NaCl. In 3% NaCl the granules were soluble. Because the plasma has the lowest protein content, the maximally attainable protein concentration was limited by the protein content of the plasma.

The homogenous samples were measured by ultrasonic and DSC methods using a temperature scan by a rate of 0.3 °C/min. The small heating and cooling rate was chosen in order to get DSC and ultrasonic methods compared on the one side and to achieve a better thermal equilibrium within the sample on the other side. The reference in both methods was the NaCl solution used to dilute the proteins.

4.2.9 Experiments for determination of the degree of lactose hydrolysis

Enzyme

The lactase (β-galactosidase) ((E.C. 3.2.1.23) used for the hydrolysis of lactose was Maxilact® LX2000 from DSM Food Specialities, Delft, Netherlands. Maxilact® is a purified lactase preparation isolated from a selected strain of the dairy yeast *Saccharomyces (Kluyveromyces) marxianus va. lactis*.

Substrate UF-permeat

As a substrate for the enzyme lactase, the milk srum, i.e., permeate from the ultrafiltration of the milk, was used. This UF-permeate has the same lactose and salt concentration as those in the milk. The lactose concentration in the UF-permeate was about 4.6 g/l according to HPLC analysis. The permeate was stored frozen at -30 °C as substrate for the experiments.

Ultrasonic measurement

21 g defrosted UF-permeate in a closed bottle was equilibrated in a water bath at 39 °C. The pH of the substrate was adjusted to 6.5. After the temperature equilibrium in permeate was reached, 1 ml of the pre-equilibrated substrate was sucked out by a syringe, and then filled in the two sample cells of ResoScan®, which was pre-equilibrated at 37 °C. After the temperature equilibrium initialising was started to select an appropriate resonance peak for the measurement. After that the resonance peak was selected and a few measurement points was registered, the measurement was stopped. The substrate in cell 2 was removed by suction. The bottle with substrate was put on a magnet stirrer. The lactase undiluted (5000 U/g) or diluted, depending on the used concentration for the experiment, was immediately added to substrate under stirring. After 30 s the mixture of substrate and enzyme was filled in cell 2 and the measurement was continued. The time from the addition of lactose to the registration of the first measurement value in continued measurement (Δt) was noted. Later in the interpretation of the result, Δt was added to the automatically registered time by the control program of ResoScan®.

There is always a difference of ultrasonic velocity between the two sample cells (Δv) for the same sample, even though the two sample cells are identical. The difference caused by sample cell was eliminated by subtracting the measured Δv of cell 2 and cell 1, while both cells were filled with substrate without enzyme.

HPLC measurement

The substrate with adjusted pH (6.5) was filled in many small tumblers (10-13). All tumblers were equilibrated put in a at 37 °C pre-equilibrated water bath. The temperature stability of the water bath is ± 0.1 °C. Lactase was added to the pre-equilibrated substrate and stirred with a plastic rod for 30 s. After a certain incubation time, one of the tumblers was taken out and put in another water bath pre-equilibrated at 85 °C for 5 minutes to inactivate the lactase. Then the tumbler with sample was cooled down in a water/ice mixture. The incubation time

was counted from the addition of lactase to the substrate. A reference sample without enzyme was also heated at 85 °C for 5 min and then cooled.

The cooled samples were prepared for HPLC measurement as following: 50 µl of 60 % perchloric acid was added to 1 ml sample and then diluted with deionised water. The dilution factor was calculated by weight. The diluted sample was filtrated and filled the concentration of lactose in the samples was determined using HPLC. The column was Aminex HPX-87H (300·7.8 mm). The eluent was a 0.005 M sulphuric acid solution, which was pumped through the column at a flow rate of 0.6 ml/min. The measuring temperature was 50 °C. A refractive index detector was used to determine the lactose concentration.

The degree of lactose hydrolysis was calculated by:

$$\text{Degree of hydrolysis} = (1-c_{Lactose,\,t}/c_{Lactose,\,t0}) \cdot 100\% \qquad \textbf{(Eq. 4.15)}$$

With $c_{Lactose,\,t0}$ as lactose concentration in the sample without enzyme, and $c_{Lactose,\,t}$ as lactose concentration in the sample with incubation time t.

5 Results and discussion

5.1 Hydration state of sugars

As mentioned above, the ultrasonic method is a very sensitive method to measure the compressibility. One of the applications of this method is to investigate the hydration state of different sugars. Due to the low compressibility of sugar molecules compared to that of other substances such as protein, the sugar molecules themselves can be considered as incompressible. This simplifies the interpretation of the ultrasonic data and the calculation of the hydration state. The ultrasonic method has already been used to determine the sugar content of fruit juice and drinks (Contreras et al. 1992).

In this chapter we studied the dependence of the hydration of saccharose and lactose on temperature and concentration. For this purpose, the ultrasonic velocity and attenuation of saccharose and lactose solutions of different concentrations were measured at 20-70 °C in 10 °C-steps. For the saccharose 1%, 10%, 20% und 50% (w/w) were used, and for the lactose 1% und 10% (w/w) solutions.

Contreras et al. (1992) measured the ultrasonic velocity in pure sugar solutions for a range of sugars and concentrations between 10 °C and 30 °C. A two way analysis of variance of the velocity data gave rise to the following equation for velocity:

$$v = A_v + B_v \cdot x + C_v \cdot \vartheta + D_v \cdot x^2 + E_v \cdot \vartheta^2 + F_v \cdot x \cdot \vartheta \qquad \textbf{(Eq. 5.1)}$$

with v for the ultrasonic velocity in m/s, ϑ for the temperature in °C, x for the sugar concentration in g/100ml and A-F for the coefficients determined from fitting the data. The regression coefficients for saccharose are A_v=1405.9; B_v=3.44; C_v=4.46; D_v=0.0176; E_v=-0.0331; F_v=-0.0319 (Contreras et al., 1992).

Using the Eq. 5.1 and the coefficients given above, the ultrasonic velocity was calculated for the saccharose solutions used in our experiments. The calculated values are compared with the measured values. For the calculation the w/w-concentration of the saccharose solutions used in this experiment was converted to w/v-concentration by deviding the density of the respective solution at 20°C. In Fig. 5.1(a) the measured and predicted ultrasonic velocity in deionized water and saccharose solution of different concentrations is plotted against temperature. Up to 50 °C the measured values matches the predicted curve very well. At temperatures higher than 50 °C the given equation for prediction loses more and more its

validity with increasing temperature, especially for the solution with high concentration. A new fitting of the curve is necessary for describe the ultrasonic velocity as a function of temperature up to a higher temperature.

The velocity increases with the temperature and sugar concentration. In our results, water shows a velocity maximum at 74 °C due to its temperature abnormality. This agrees exactly with the temperature given by Del Grosso and Mader (1972). With increasing sugar concentration this velocity maximum shifts to lower temperature, which can be especially clearly seen in the 50% saccharose solution. Furthermore, Fig. 5.1(a) shows that with increasing sugar concentration the effect of temperature on the velocity decreases.

The attenuation α/f^2 decreased with increasing temperature and increased with increasing sugar concentration (Fig. 5.1(b)). The decrease in attenuation with increasing temperature is caused by decreased viscosity of the solution with increasing temperature. In contrast to ultrasonic velocity, the effect of temperature on the attenuation increases with increasing sugar concentration, because the decrease of viscosity with increasing temperature is more distinctive for sugar solution at high concentration.

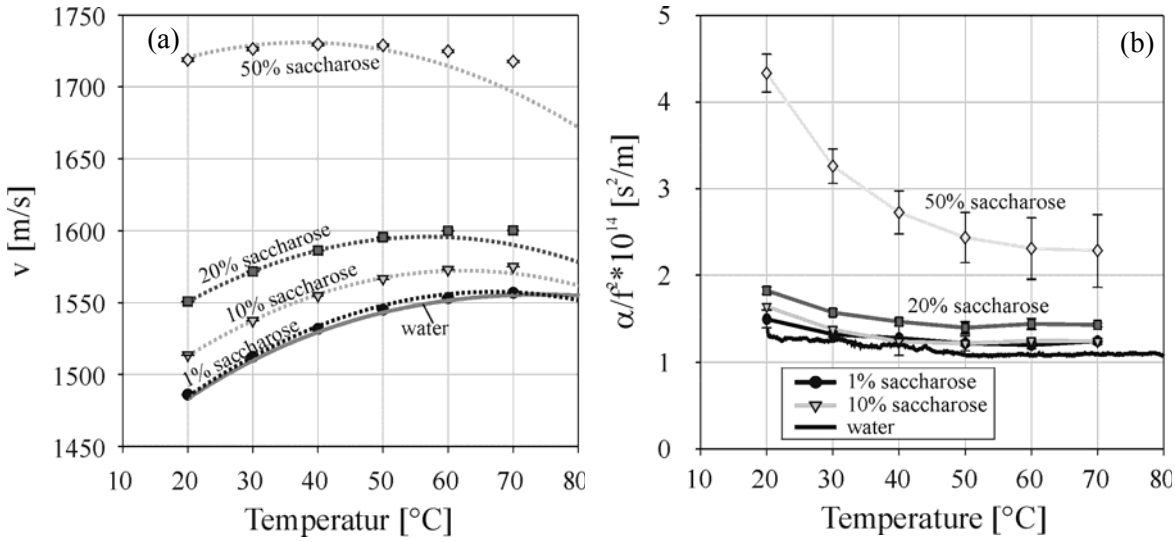


Fig. 5.1: (a) Measured and predicted ultrasonic velocity v in deionized water and saccharose solutions of different concentrations as a function of temperature. The symbols and dotted lines denote the measured and predicted ultrasonic velocity in saccharose solutions, respectively. The solid line denotes the measured ultrasonic velocity in deionized water. (b) Measured ultrasonic attenuation α/f^2 in deionized water and saccharose solutions of different concentrations as a function of temperature.

The hydration numbers of the sugars was calculated using Eq. 2.11. Because this equation is only valid for diluted solutions, the calculated value for the sugar solutions with higher concentrations used in this work probably deviates from the real one. Even so, information about the hydration state of sugar molecules can be obtained, in order to qualitatively compare them considering their dependency on temperature and concentration.

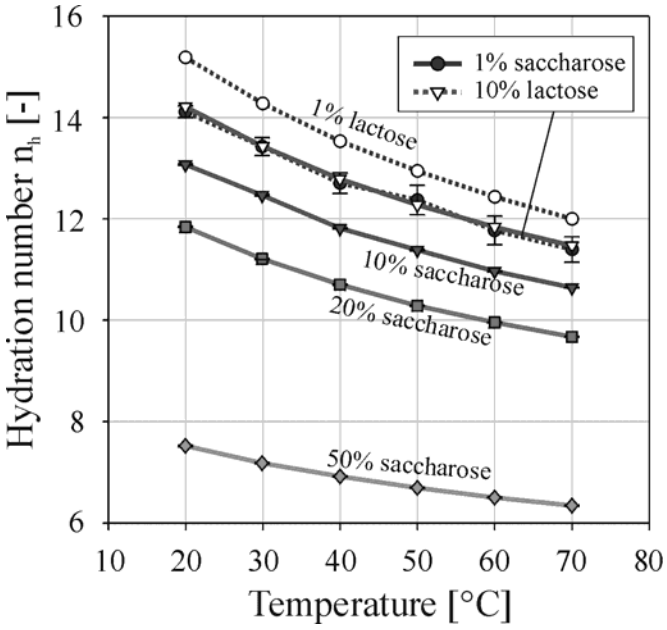


Fig. 5.2: The hydration number of saccharose and lactose depending on temperature und and concentration.

Fig. 5.2 shows the hydration number n_h depending on temperature and concentration. With increasing temperature and increasing concentration the hydration number of saccharose and lactose decreases. The reasons are that the kinetic energy of water molecules is higher at higher temperature on the one side, and the interaction of sugar molecules increases at higher concentration on the other side. With increasing concentration the available water amount decreases. Therefore, the hydration number decreases. Fig. 5.2 reveals that lactose can bind more water than saccharose. This is in agreement with the results in the literature (Galema & Høiland, 1991).

The results show that the ultrasonic method is very sensitive for the measurement of the hydration state of molecules. The measurement of the hydration level of different sugars may help to understand different protective effects of different sugars on proteins or micro organisms.

5.2 Gelation of hydrocolloids

5.2.1 Gelation of Carrageenans[1]

The sol/gel and gel/sol transition in different carrageenan solutions was investigated by measuring the ultrasonic velocity and attenuation during cooling and heating. The results are compared with the rheological data obtained from oscillating rheological measurements. It was the purpose of this study to investigate an important gel and structure forming system, namely carrageenan, and to assess the applicability of low-intensity ultrasound as a suitable technique for the characterisation of mixtures of various types of carragerenans versus the rheological method as an established technique.

5.2.1.1 Influence of the carrageenan type and concentration on the gelation

The concentration of carrageenans is decisive for the gel network formation and therefore the gel properties. For the gel formation a certain minimum concentration of carrageenan is necessary. The difference of ultrasonic velocity (Δv) and attenuation ($\Delta(\alpha/f^2)$) between κ-carrageenan of different concentrations and the reference (water) as a function of temperature is plotted in Fig. 5.3.

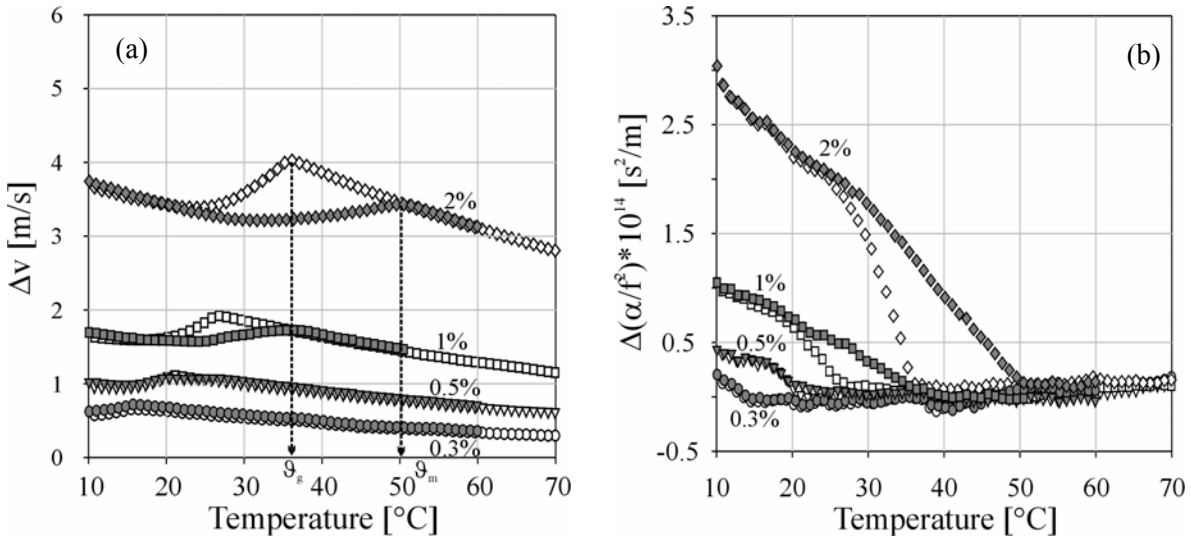


Fig. 5.3: Ultrasonic velocity difference Δv (a) and attenuation difference $\Delta(\alpha/f^2)$ (b) of κ-carrageenan depending on the temperature and carrageenan concentration during cooling (open symbols) and heating (filled symbols). Reference: water; Heating/coolling rate: 0.1 °C/min

During cooling Δv increases with decreasing temperature because of the different temperature dependencies of the ultrasonic velocity in the carrageenan samples and in water. At a certain

[1] Chapter published as Wang et al. (2005): *Innovative Food Science and Emerging Technologies, 6*, 465-472.

temperature Δv starts to decrease (Fig. 5.3(a)). We define this temperature as the gelling temperature ϑ_g. The reason for the decrease in velocity might be the coil/helix transition and the aggregation of carrageenan double helices, a precondition for gel formation. In protein science, it is known that water is extruded from the bound state into the bulk during the transition of DNA from hydrated random coil to double helix (Lewin, 1974). We suggest that the same water extrusion process occurs in the case of coil/helix transition of carrageenan. Due to the cation-mediated aggregation of carrageenan double helices, the overall area for water to access the carrageenan molecules decreases resulting in additional water release from bound into bulk water. According to the fact that the water in the hydration shell is less compressible than the bulk water, the ultrasonic velocity decreases while the carrageenan aggregation increases (Eq. 2.9). When the aggregation is completed, the temperature effect dominates again, and Δv increases.

The melting of a gel is the reverse process to gel formation. However, the melting of κ-carrageenan takes place at higher temperatures than the onset temperature because the aggregates formed during cooling are stable, so that more energy must be supplied to melt them (Liang et al. 1979; Ramakrishnan & Prud'homme, 2001). We define the temperature, at which the value of Δv during the heating phase reaches a local maximum, as the melting temperature ϑ_m (Fig. 5.3(a)).

Fig. 5.3(b) shows that during cooling the attenuation remains constant at a temperature higher than ϑ_g. At ϑ_g the attenuation starts to increase with decreasing temperature. The reason for the increase in attenuation is the energy loss due to friction between the matrix of carrageenan aggregates formed during cooling and the mobile water. The ultrasonic wave causes a local pressure difference in the sample. The system attempts to balance the pressure difference by transporting water to the places with lower pressure. Hence, there is a relative displacement between carrageenan aggregates or networks and water molecules which leads to friction (Gormally et al., 1982). In the melting process during heating the attenuation decreases with increasing temperature until ϑ_m is reached, and then assumes constant values.

In Fig. 5.3, one can see that ϑ_g, ϑ_m and the changes of Δv and $\Delta(\alpha/f^2)$ increase with increasing carrageenan concentration. Because the precision of ultrasonic attenuation measurement is lower than that of ultrasonic velocity measurement, we mainly used the ultrasonic velocity as characteristic of gel formation in this work, in order to exactly determine the gelling and melting points.

The results of the oscillating rheological measurements of 0.5% and 1% κ-carrageenan solutions during gelling and melting are presented in Fig. 5.4. In the rheological measurement, the gelling and melting temperatures are defined as the temperature with tan δ = 1, i.e. G' = G", during cooling and heating, respectively. According to this definition, 0.5% κ-carrageenan does not form a gel within the temperature range investigated (G' < G" and tan δ > 1) (Fig. 5.4(a)), while 1% κ-carrageenan clearly shows a sol/gel transition (Fig. 5.4(b)). This indicates that there are not enough κ-carrageenan molecules to form a continuous gel network in a 0.5% solution. Even though, at about 21 °C a steep increase in G' and G" upon cooling and a kink point of tan δ could be observed in 0.5% κ-carrageenan (Fig. 5.4(a)). The loss factor is a parameter for the ratio of viscous to elastic property. Upon cooling the ratio of viscous to elastic property of 0.5% κ-carrageenan increases at temperatures above and decreases at temperatures below the temperature at the kink point. The ultrasonic parameters also show a kink point at a similar temperature. This kink might be associated with the aggregation of carrageenan double helices. The gelling and melting temperatures increase with increasing carrageenan concentration. (ϑ_m-ϑg) gives information about the stability of the aggregates formed during cooling. The aggregates formed at low concentrations are probably very small. They need less energy to melt than the bigger aggregates formed at higher concentration. Therefore (ϑ_m-ϑg) decreases with decreasing carrageenan concentration.

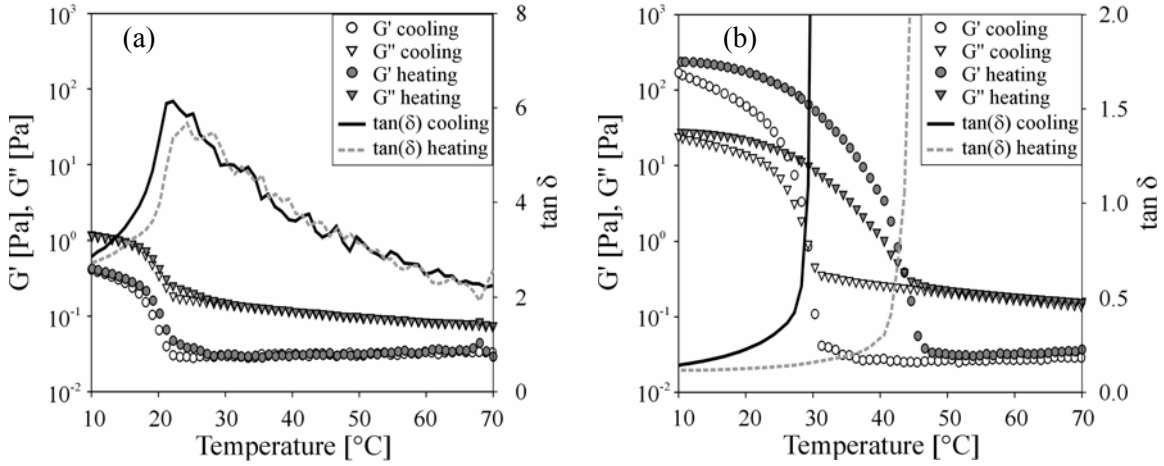


Fig. 5.4: G', G'' and tan δ of 0.5% (a) and 1% (b) κ-carrageenan depending on the temperature during cooling and heating. Heating/coolling rate: 1 °C/min.

Comparing Fig. 5.3 with Fig. 5.4(b) it is clear that the gelling and melting temperatures in the oscillating rheological measurement are higher than those determined by the ultrasonic measurement. In this work the cooling and heating rates of 1 °C/min used in rheological measurements is higher than that in ultrasonic measurements, but chosen as a common

condition in rheological measurements. In the case of higher cooling rates, gel formation is rather expected to be initiated at lower temperature, because there is less time available for the stabilisation of junction zones of the gel network (Fonkwe et al., 2003). Therefore, the higher ϑ_g and ϑ_m in rheological measurements cannot be explained by the higher cooling and heating rate compared to ultrasonic measurements. They are probably caused by the different definitions of gelling and melting temperatures in the two methods. In ultrasonic measurements we mainly measure changes of compressibility κ by measuring changes in ultrasonic velocity. ϑ_g and ϑ_m are defined by the kink point with changed compressibility of the sample, which may correlate to the formation and melting of double helices aggregates respectively. In rheological measurements, however, ϑ_g and ϑ_m are determined by storage modulus G' and loss modulus G", which correlate with the viscoelastic behaviour of a sample.

The hybrid-carrageenan forms weaker gels than κ-carrageenan. Therefore, the change of Δv and $\Delta(\alpha/f^2)$ during gelation is smaller (Fig. 5.5). A sol/gel transition is only distinctive in 2% hybrid-carrageenan. In the rheological measurement a sol/gel transition was observed in 1% hybrid-carrageenan (Fig. 5.6(a)). However, the increase in G’ and G’’ upon cooling is less steep compared to κ-carrageenan due to the lower gel strength.

In order to investigate the influence of the molecular structure on the gelation behaviours, the gelation of 1% κ/ι-hybrid-carrageenan was compared to that of a mixture of 0.4% κ- and 0.6% ι-carrageenan. These two samples had the same content of κ- and ι-units. However, in the hybrid-carrageenan κ- and ι-units are located in the same molecule, whereas in the mixture the κ- and ι-units exist as separate molecules. The results from rheological and ultrasonic measurements are shown in Fig. 5.6 and Fig. 5.7.

A solution of 1% (w/w) κ/ι-hybrid-carrageenan forms a weak gel. It shows a hysteresis in the rheological measurement (Fig. 5.6(a)). In contrast, the hysteresis is not distinctive in the ultrasonic measurement (Fig. 5.7). A mixture of κ- and ι-carrageenan, however, forms a firm gel and shows hysteresis in both methods (Fig. 5.6(b) and Fig. 5.7). Because κ- and ι-carrageenan have different coil/helix transition temperatures (Ridout et al., 1996), the κ- and ι-units in the same molecule of hybrid carrageenan might influence each other during the gelation process, so that the network formation will be affected. However, in the mixture of κ- and ι-carrageenan both carrageenans form a gel in two stages independently of each other as was also found by Piculell et al. (1992), Parker et al. (1993), and Ridout et al. (1996). G' and G" show a 2-stage increase during cooling (Fig. 5.6(b)). This can be explained by the coil/helix transitions of ι- and κ-carrageenan, which take place at different temperatures. In

contrast, the ultrasonic data plotted in Fig. 5.7 show only one transition temperature for the mixture during cooling and heating.

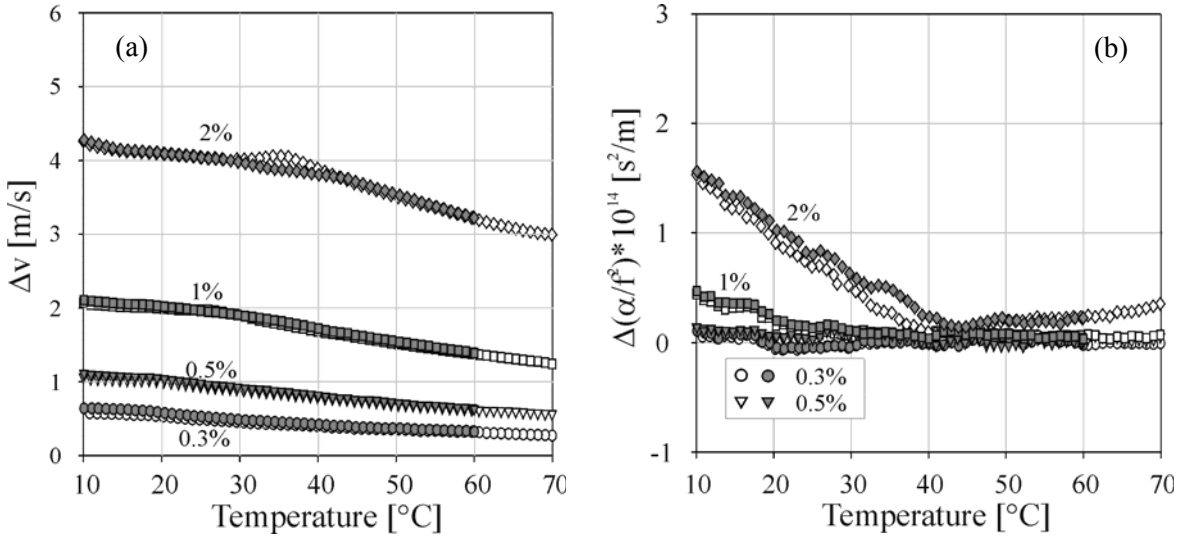


Fig. 5.5: Ultrasonic velocity difference Δv (a) and attenuation difference $\Delta(\alpha/f^2)$ (b) of κ/ι-hybrid-carrageenan depending on the temperature and carrageenan concentration during cooling (open symbols) and heating (filled symbols). Reference: Water; Heating/coolling rate: 0.1 °C/min.

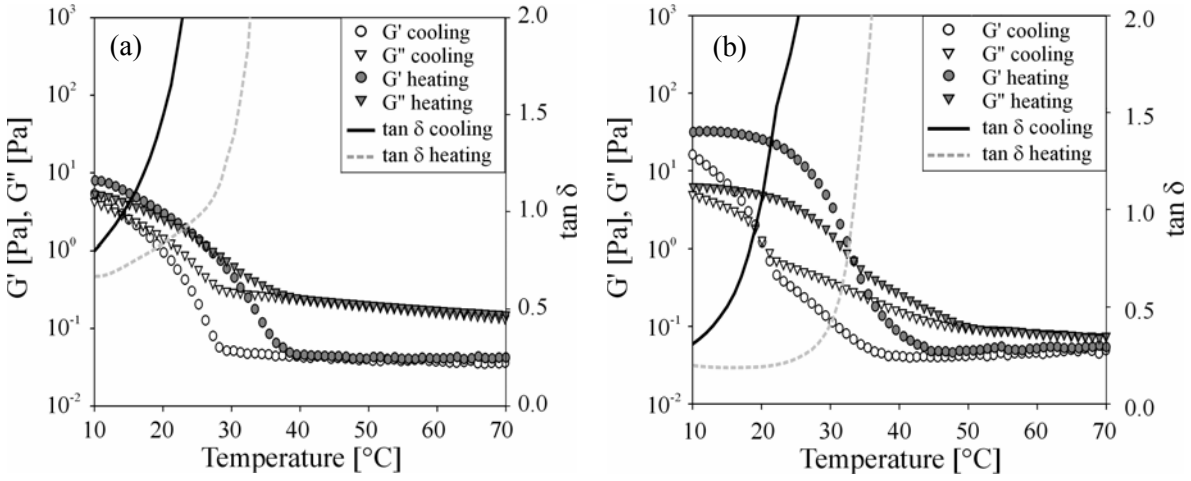


Fig. 5.6: G', G'' and tan δ of 1% κ/ι-hybrid-carrageenan (a) and a mixture of 0.4% κ- and 0.6% ι-carrageenan (b) depending on the temperature during cooling and heating. Heating/coolling rate: 1 °C/min.

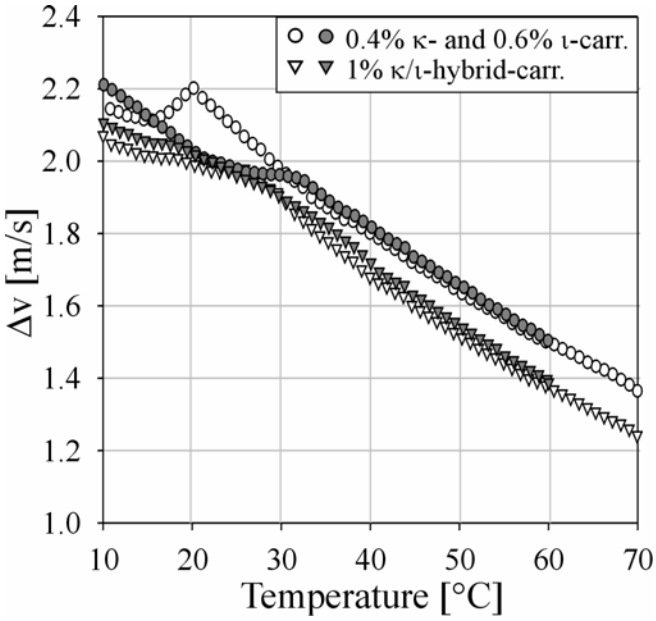


Fig. 5.7: Δv of 1% κ/ι-hybrid-carrageenan and a mixture of 0.4% κ- and 0.6% ι-carrageenan depending on the temperature during cooling (open symbols) and heating (filled symbols). Reference: water; Heating/coolling rate: 0.1 °C/min.

Ultrasonic velocity of 1% and 2% ι-carrageenan solutions do not show any transition in the temperature range between 10 °C and 70 °C (Fig. 5.8(a)). The ultrasonic attenuation shows a small increase at low temperature (Fig. 5.8(b)). But this increase is so small that no clear transition can be recognised. In contrast, the rheological measurement of 1% ι-carrageenan clearly shows a gel formation (Fig. 5.9). The transition of ι-carrageenan is apparently not detected by the ultrasonic method at the frequency used in this study. This explains why the ultrasonic method only detects one transition during the gelation of the mixture of 0.4% κ- and 0.6% ι-carrageenan (Fig. 5.7).

As mentioned above, the decrease in ultrasonic velocity during gelation is a result of the aggregation of double helices. Compared to κ-carrageenan, ι-carrageenan has more sulphate groups per monomer unit, which hinder extensive aggregation of double helices. Therefore, the degree of aggregation in ι-carrageenan gel is lower than that in κ-carrageenan gel (Oakenfull & Morris, 1987). It must be highlighted that the concentration of cations in these solutions is very low. This is an additional factor for the weaker aggregation. It appears that the aggregation of double helices in ι-carrageenan is so weak that the small hydration change can not cause a detectable change in ultrasonic velocity.

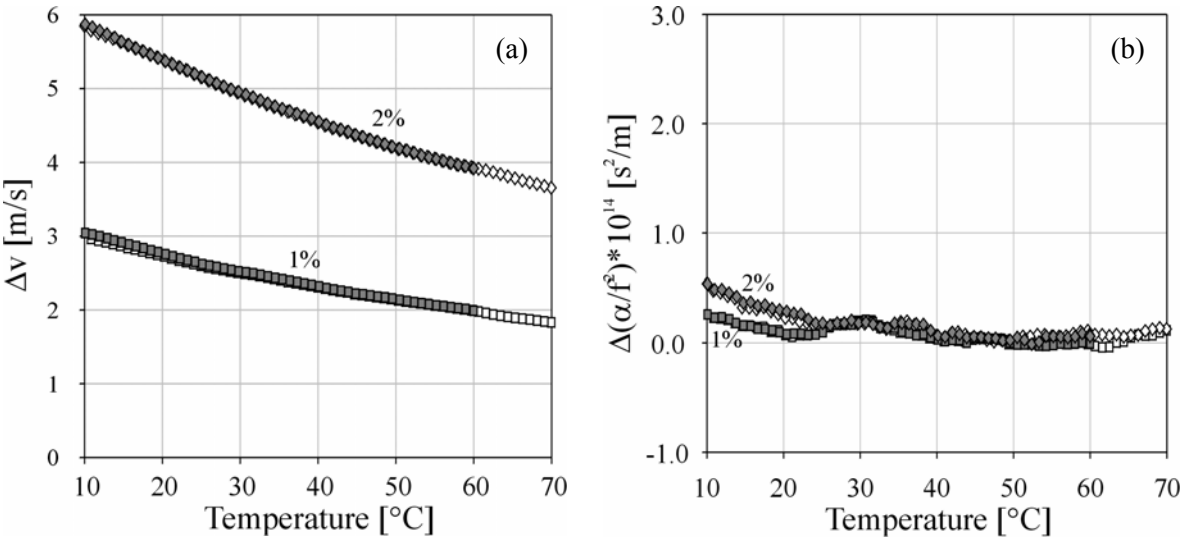


Fig. 5.8: Ultrasonic velocity difference Δv (a) and attenuation difference $\Delta(\alpha/f^2)$ (b) of ι-carrageenan depending on the temperature and carrageenan concentration during cooling (open symbols) and heating (filled symbols). Reference: water; Heating/coolling rate: 0.1 °C/min.

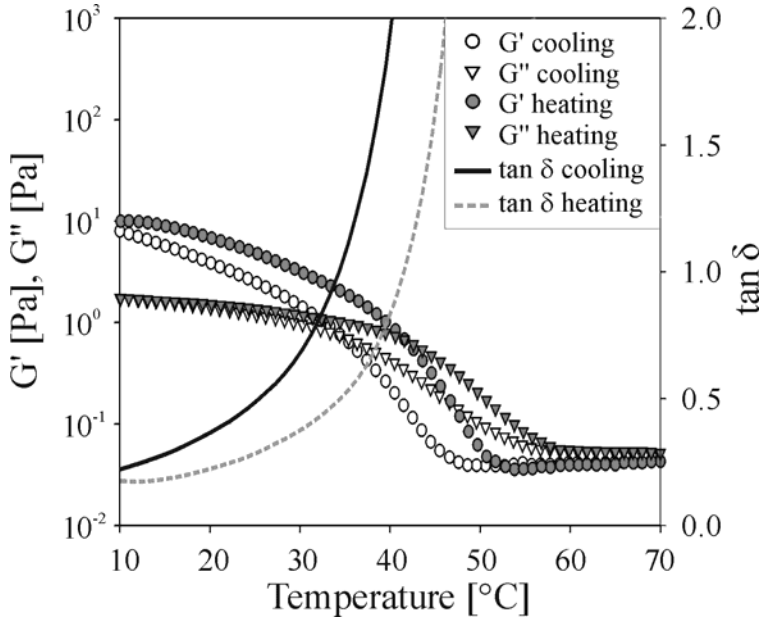


Fig. 5.9: G', G'' and tan δ of 1% ι-carrageenan depending on the temperature during cooling and heating. Heating/coolling rate: 1 °C/min.

In contrast to the results in this work, Toubal et al. (2003) observed an increase in ultrasonic velocity corresponding to the sol/gel transition of a ι-carrageenan solution. However, the frequency used in their experiments was 0.5 MHz. The wave length at 0.5 MHz is almost 15 times higher than that used in our work. This wave length is more sensitive to the macro property of the sample such as the elastic property (G'), whereas the wave length at 7.8 MHz used in this work is more sensitive to the micro property of the sample such as molecular hydration. The increase in G' during the sol/gel transition can be measured much more

sensitively at 0.5 HMz than at 7.8 MHz. According to Eq. 2.7, the ultrasonic velocity increases with increasing increasing G'. This may be the explaination of the increase in ultrasonic velocity observed by Toubal et al. (2003).

5.2.1.2 Influence of K^+ on the gelation of κ-carrageenan

Fig. 5.10(a) depicts the Δv between 0.5% κ-carrageenan with added K^+ at different concentrations and KCl solution of the same K^+-concentration as in the respective sample as a function of temperature. It can be seen that the melting temperature ϑ_m, the geling temperature ϑ_g and their difference (ϑ_m-ϑ_g) increase with increasing K^+-concentration (Fig. 5.10(a) and Fig. 5.10(b)), because K^+ compensates the negative charges of the carrageenan molecules and promotes their ability to form double helices and respective aggregates. The effect of K^+ on the gelation of κ-carrageenan was confirmed in many investigations (Morris et al., 1980; Lai et al., 2000; Takemasa et al., 2001). Similar to the observation above (chapter 5.2.1.1), the gelation temperature ϑ_g, the melting temperature ϑ_m and their difference (ϑ_m-ϑ_g) determined using the ultrasonic measurement are again lower than those determined by the rheological measurement (Fig. 5.10(b)).

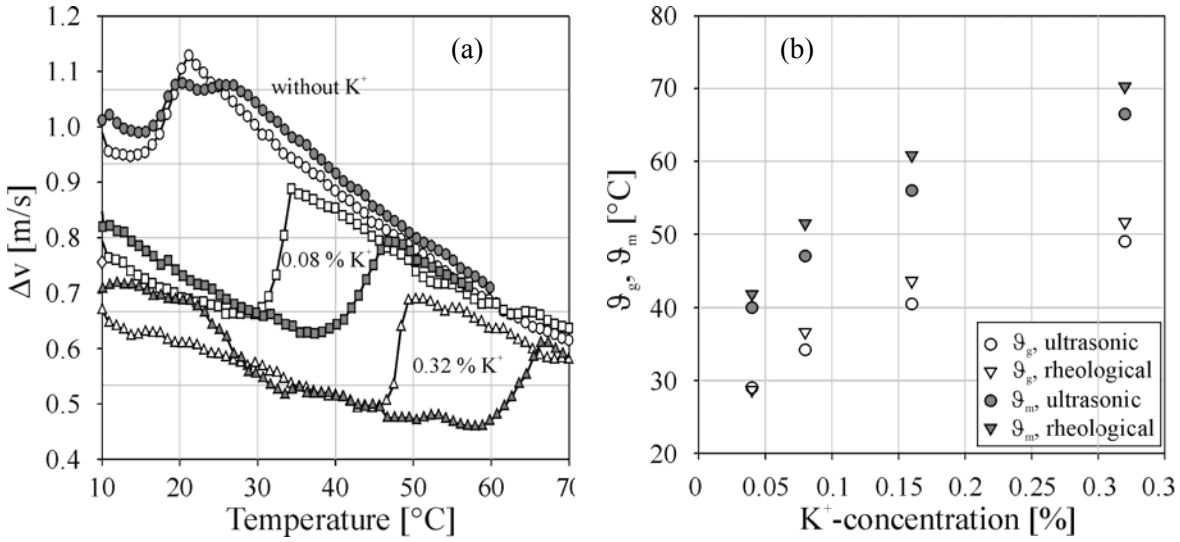


Fig. 5.10: (a) Δv ($v_{(sample)}$- $v_{(KCl\ solution)}$) of 0.5% κ-carrageenan with addition of different K^+-concentrations depending on the temperature during cooling (open symbols) and heating (filled symbols) by a rate of 0.1 °C/min. References: KCl solutions; (b) Ultrasonic and rheological measurements of gelling and melting temperatures ϑ_g and ϑ_m depending on K^+-concentration in 0.5% κ-carrageenan.

In Fig. 5.11 the ϑ_m and ϑ_g determined by the ultrasonic and rheological measurements are plotted against each other. There is a linear correlation between the transition temperatures ϑ_m and ϑ_g determined by these two methods.

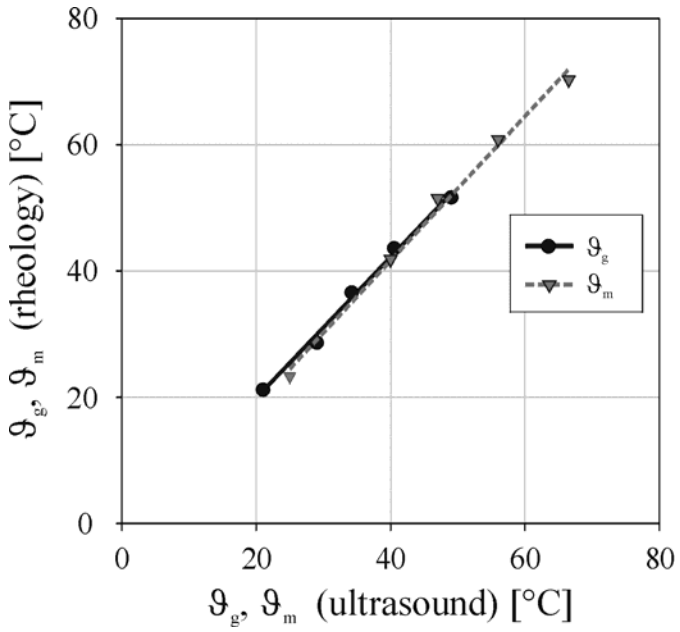


Fig. 5.11: Correlation of gelling temperature ϑ_g and melting temperature ϑ_m from the ultrasonic and the rheological methods.

Using the ionic concentrations in the samples and the respective transition temperatures, it is possible to determine the transition enthalpy. Investigations of the conformational transition of carrageenans by Rochas and Rinaudo (1980; 1982) as well as Rochas and Mazet (1984) using optical rotation and microcalorimetry show that the logarithms of the total ionic concentration ln[c] in [eq/l] is a linear function of the reciprocal of the conformational transition temperature $1/T_m$ or $1/T_g$ in [K^{-1}] (Eq. 2.12). Using this relationship, the melting enthalpy of carrageenan can be calculated. In Fig. 5.12 the $1/T_m$ and $1/T_g$ from rheological and ultrasonic measurement are plotted against the the logarithm of total ionic concentration. The ionic concentration c was calculated from the added K^+-concentration and the ionic concentration contained in the carrageenan used (Tab. 4.1). Similar to the results from the optical rotation measurements by Rochas and Rinaudo (1980), Fig. 5.12 shows a linear relationship between $1/T_m$ or $1/T_g$ and ln[c] for both rheological and ultrasonic measurement. The slopes of the regression lines (ln[c] versus $1/T_m$) obtained in Fig. 5.12 correspond to the term $d\ln[c]/d(1/T_m)$ in Eq. 2.12. Using the slopes, the melting enthalpies calculated from the ultrasonic and rheological data are presented in Tab. 5.1.

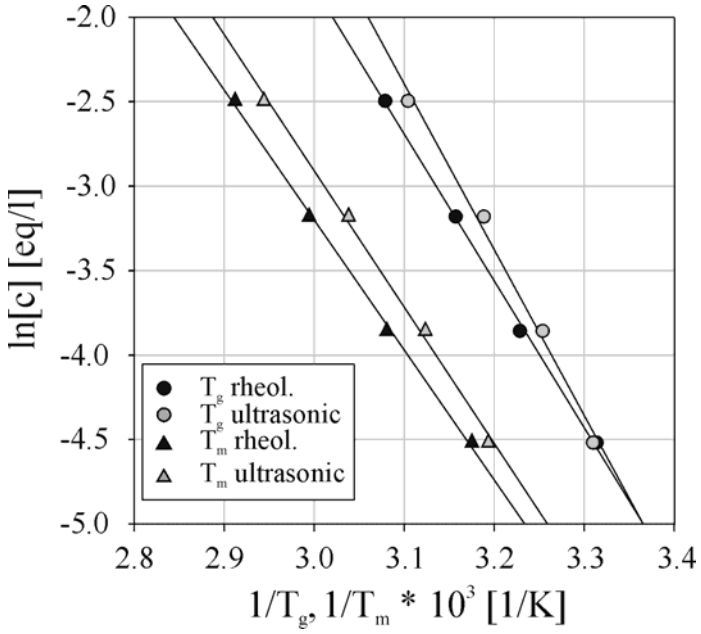


Fig. 5.12: Dependence of the logarithm of the total ionic concentration (ln[c]) and the reciprocal of the gelling temperature ($1/T_g$) and melting temperature ($1/T_m$) in 0.5% κ-carrageenan determined by rheological and ultrasonic method.

Tab. 5.1 presents the enthalpy of κ-carrageenans in the presence of K^+. According to the results, the ultrasonic and rheological methods provide similar values of melting enthalpy. Both enthalpy values agree quite well with values obtained by Rochas and Rinaudo (1982). This supports the view that the characteristic kink point in the ultrasonic measurement, as defined for ϑ_m and ϑ_g, indeed correlates to the conformational transition of κ-carrageenan molecules and the consequent sol/gel transition.

Tab. 5.1: Melting enthalpy of κ-carrageenans in 0.5% carrageenan solution with added K^+. The osmotic coefficients $\phi_c = 0.601$ and $\phi_h = 0.359$ are obtained by interpolating the measuring values of Rochas and Rinaudo (1982) for κ-carrageenan in water**.**

	dln[c]/d(1/T_m) [1/K]	ΔH [kJ/mol]
Rheological measurement	-7701.30	15.49
Ultrasonic measurement	-8074.18	16.25

The results show that gelation of different carrageenans induces different changes in ultrasonic parameters despite their similar molecular structures. Gelation of κ-carrageenan clearly caused a decrease in ultrasonic velocity and an increase in attenuation, while gelation of ι-carrageenan did not cause any change in ultrasonic velocity at 7.8 MHz. This suggests that the molecular change or the extent of the molecular change during gel formation of ι-

carrageenan may be different from that of κ-carrageenan. A mixture of κ- and ι-carrageenans showed very different ultrasonic characteristics compared to a natural κ/ι-hybrid-carrageenan. This confirms that the molecular structure of carrageenans has an enormous influence on their gelation properties. In particular, it allows to sensitively differentiate between carrageenan types (κ/ι-hybrid-carrageenan versus mixture of κ- and ι-carrageenan) as an alternative to rheological measurements. The results obtained indicate that ultrasound appears to be able to differentiate different carrageenans. It could be used for the control of carrageenan in its molecular properties in quality control. The fact that the ultrasonic method does not detect sol/gel transition of ι-carrageenan, while the rheological method does detect the sol/gel transition, provides additional information regarding the gel formation mechanism, i.e., the different extent of aggregation of the helices during gelation.

5.2.2 Gelation of Gelatine

To investigate gelling properties of gelatine, which has another gelling mechanism unlike carrageenan, temperature scans using ultrasonic and rheological method, similar to those for the carrageenan, were performed.

The ultrasonic velocity and attenuation difference in a 4% gelatine solution as a function of temperature are depicted in Fig. 5.13. The ultrasonic velocity difference Δv increases with decreasing temperature during cooling from 50 °C to 10 °C. The curve for the heating process is almost the reversed curve of the cooling process. At temperature above 22 °C, Δv increases linearly with the temperature. This linear change is only a temperature effect induced by the different temperature dependencies of the ultrasonic velocity in water and gelatine solution. In the temperature range below 22 °C, the cooling curve is slightly above the heating curve which indicates a slight hysteresis. No characteristic changes associated with the sol/gel transition were observed. The attenuation difference $\Delta(\alpha/f^2)$ also increases with decreasing temperature till about 28 °C. Between 28 °C and 22 °C $\Delta(\alpha/f^2)$ remains almost constant, which may be caused by the gelation process. If the temperature effect (The ultrasonic velocity increases with decreasing temperature.) is excluded, the constant $\Delta(\alpha/f^2)$ over temperature between 28 °C and 22 °C would mean that the structure changes (conformation transition and network formation) in this temperature range alone leads to a decrease in the attenuation. In contrast to that, the gelation of κ-carrageenan induces an increase in ultrasonic attenuation, as described in chapter 5.2.1. The decrease in attenuation due to sol/gel transition may be a result of the increased homogenity/continuity of the microstrucutre, which results in a more effective propagation of the ultrasonic energy. During the transition from sol to gel,

the microstructure of gelatine may become more homogenous/continuous, whilst the microstructure of κ-carrageenan becomes more inhomogenous due to the high extent of aggregation of double helices. From 22 °C $\Delta(\alpha/f^2)$ increases again with decreasing temperature. In parallel to the ultrasonic velocity, the attenuation also shows a slight hysteresis in the temperature range below 22 °C.

Fig. 5.14 shows the rheological measurement of a 4% gelatine solution during cooling and heating. During cooling G' and G'' remain almost constant down to 30 °C, then increase with decreasing temperature. The intercept point of G' and G'' at 18 °C corresponds to the gelling point of the gelation solution. In contrast to the ultrasonic measurement, the rheological measurement shows a marked hysteresis between the heating and cooling curves. The melting point is at 28 °C, i.e., 10 °C higher than the gelling point.

The negligible change in the ultrasonic velocity during the gelation process let us suppose that the sol-gel transition of gelatine does not result in a marked change the compressibility of the whole system. Either the hydration state of the molecules does not change much due to gelation process, or the effect of the decrease of the hydration due to aggregation is compensated by the effect of the increasing elasticity due to the network formation.

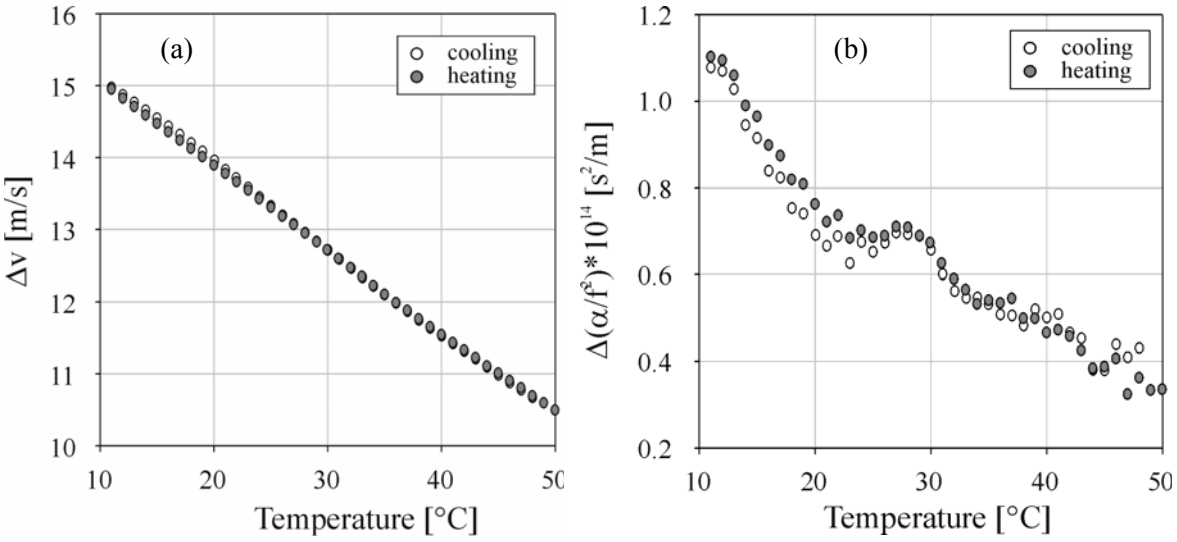


Fig. 5.13: Ultrasonic velocity difference Δv (a) and attenuation difference $\Delta(\alpha/f^2)$ (b) of 4% gelatine depending on the temperature and gelatine concentration during cooling and heating. Reference: water; Heating/coolling rate: 0.3 °C/min.

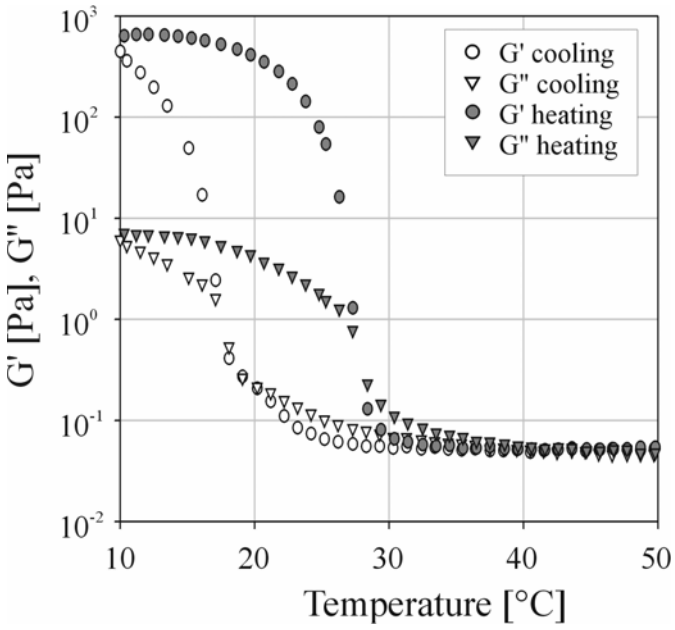


Fig. 5.14: G', G'' of 4% gelatine depending on the temperature during cooling and heating. Heating/coolling rate: 1 °C/min.

The ultrasonic measurement of gelation of carrageenans and gelatine shows that the change in ultrasonic parameters, especially in ultrasonic velocity, depends on the mechanism of the gelation. Gelation of κ- and κ/ι-hybrid-carrageenan induces a decrease in ultrasonic velocity, while gelation of ι-carrageenan and gelatine does not. Audebrand et al. (1995) could not observe any variation in the velocity during gelation of amylase and alginate, either. However, an increase in the attenuation is induced by the gelation of both amylase and alginate.

Conclusion of these results is that the ultrasonic parameters are not specific for the detection of sol/gel phase transition. The ultrasonic parameters react differently to different gelling systems. According to description in the literature, carrageenans and gelatine have similar gelation mechanism. They all go through following steps: the conformational transition from random coil to helix, aggregation of the helices, and network formation to form a gel. Why ultrasonic parameters differently respond to gelling process of different carrageenans and gelatine with similar gelation mechanism, is still not clear. However, this difference in the ultrasonic properties indicates that there is a difference between the gelation mechanism or gel structures. Ultrasonic velocity is sensitive to hydration of molecules. The difference in the behaviour of ultrasonic velocity in different gelation systems may provide information about changes the hydration state of the molecules during gelation. κ-carrageenan molecules become less hydrated due to the high aggregation extent, while the hydration of ι-carrageenan and gelatine does not change noticeably during the sol/gel transition.

5.3 Investigation of the gelation of milk proteins

In order to evaluate the applicability of the ultrasonic method for the characterisation of gelation of milk or its components and the impact of different milk gelation mechanism on the ultrasonic properties, the rennet and acid gelation of milk as well as the gelation of CMP were investigated.

5.3.1 Rennet gelation of casein solutions: Influence of the UHT treatment and rennet concentration[2]

The purpose of our study was to investigate the ultrasonic method in comparison to rheometry as an established method to describe the enzymatic renneting process, i.e., the action of the rennet enzyme on the casein fraction of milk protein. The casein solution used in this study was milk, depleted of whey proteins by means of membrane filtration. This solution was preheated at temperatures of 120, 130, or 140°C for various heating times to induce heat related changes in the casein fraction. Preheating is known to affect the renneting process (Bulca & Kulozik, 2003; Bulca et al., 2004; Bulca & Kulozik, 2004), and therefore, differences in the ultrasonic assessment were expected.

5.3.1.1 Ultrasonic velocity and attenuation during rennet gel formation

The ultrasonic velocity and attenuation over time during rennet gel formation was measured in an unheated 3% casein solution with 0.02% rennet addition. The typical course of these two dependent variables over time is shown in Fig. 5.15. In order to eliminate slight sample to sample variations between measurements, the Δv and $\Delta(\alpha/f^2)$ values are normalized (index n) as $(\Delta v)_n$ and $(\Delta(\alpha/f^2))_n$ by subtracting the respective starting value. A two-stage increase of the ultrasonic velocity in the gel formation process is observed. The first increase can be correlated to the enzymatic hydrolysis. In this stage, the rennet enzyme cuts off the hydrophilic hair, caseinomacropeptid (CMP), from the surface of the casein micelle. This causes changes in the hydration state of casein micelles und leads to a more hydrophobic surface. On the other hand, the CMP released into the solution has a larger surface area accessible to water (Fig. 5.16). The overall hydration degree of the casein solution increases. This leads to an increase in ultrasonic velocity, as shown in Fig. 5.15 during the first increase of Δv. After a certain amount of the total CMP is cut off, the casein micelles begin to coagulate. Green et al. (1978) found that the viscosity of rennet treated milk begins to rise

[2] Chapter published as Wang et al. (2007): *International Dairy Journal, 17*, 50-58.

when the enzymatic reaction is about 86% complete. In the ultrasonic measurement a second increase in ultrasonic velocity in parallel with the coagulation phase can be observed. Corredig et al. (2004a) and Nassar et al. (2004) considered this increase in ultrasonic velocity connected with the increased elasticity of the sample. Another possible reason for the increase in the ultrasonic velocity is the scattering effect due to the increased particle size. When a sound wave is incident on a spherical particle, a proportion of the energy is scattered from the forward direction of the wave. The phase changes in the forward component of the wave manifest themselves as a change in the apparent velocity of the sound wave. Thus, the velocity becomes a function of particle size (Pinfield et al. 1995).

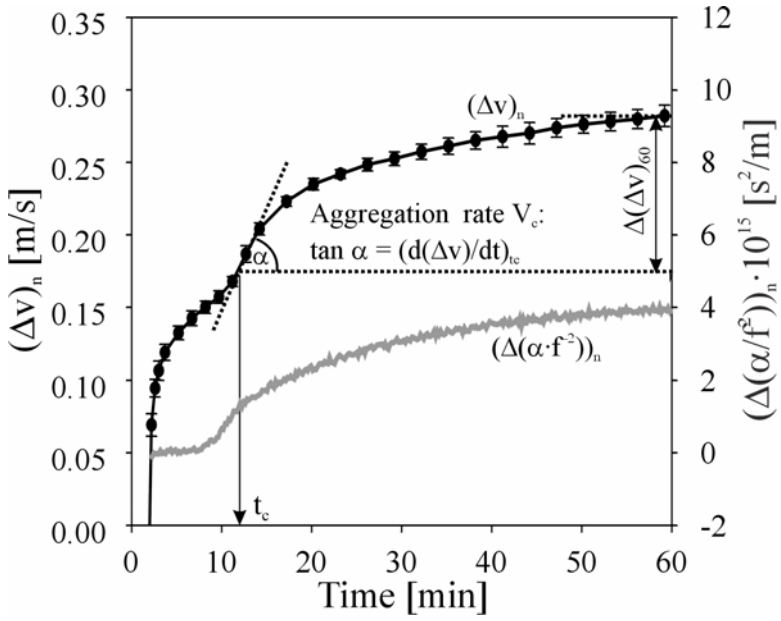


Fig. 5.15: Course of normalized ultrasonic velocity and attenuation, $(\Delta v)_n$ and $(\Delta(\alpha/f^2))_n$, over time during rennet gel formation and definitions of different parameters for the characterisation gel formation. The error bars are the confidence intervals ($p \leq 0.05$) calculated from 5 measurements of the ultrasonic velocity in 3% unheated casein solution with 0.02% rennet. Reference: 3% casein solution without rennet.

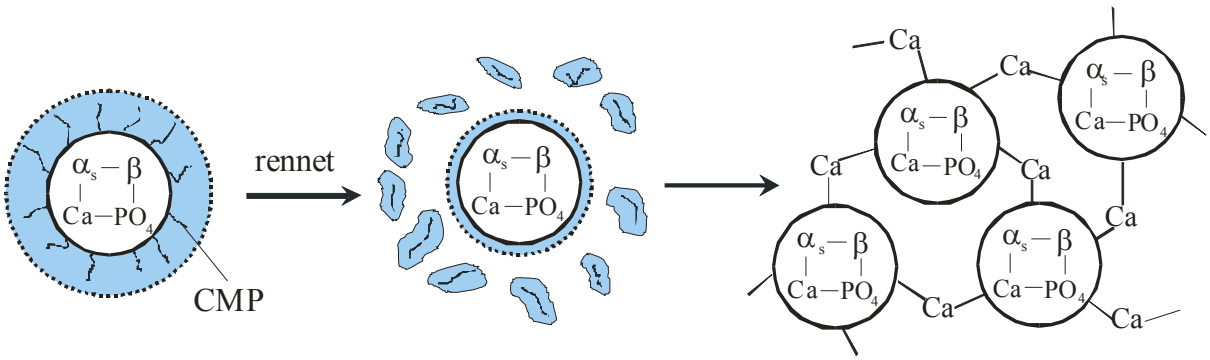


Fig. 5.16: Schematic description of the gel formation process.

In order to assess the reproducibility of the ultrasonic measurement, five measurements of the ultrasonic velocity in rennet treated unheated casein solution were carried out over time. In Fig. 5.15 the error bars are the confidence intervals ($p \leq 0.05$). As can be seen there is a good reproducibility of the ultrasonic velocity measurement and the renneting process. The coagulation time t_c determined from these five measurements was 12.67 ± 0.39 min.

According to the course of Δv over time (Fig. 5.15) we defined the parameters for the characterisation of coagulation process as following:

- The coagulation time t_c as the time at the inflexion point. If Δv is differentiated against time, this point corresponds to the peak maximum of the curve $d(\Delta v)/dt = f(t)$.
- The aggregation rate V_c as the slope of the curve $\Delta v = f(t)$ at time t_c which corresponds to $d(\Delta v)/dt$ at the point of t_c.
- The $\Delta(\Delta v)_{60}$ as Δv at 60 min after rennet addition (Δv_{60min}) subtracted by Δv at t_c (Δv_{tc}), because the starting point of the curve immediately after adding the rennet enzyme could not be detected, as described above.

During the enzymatic hydrolysis the ultrasonic attenuation changes only slightly at the frequency used. This agrees with the results of Corredig et al. (2004a) who measured at 7.835 MHz. According to the scattering theory, particle size influences the attenuation. The hydrodynamic length of CMP is 7 nm (De Kruif, 1999). Compared to the wave length at the frequency used (about 0.2 mm) the decrease in diameter of the casein micelles due to enzymatic cleavage is relatively small, so that this change could not be detected by measuring ultrasonic attenuation. This change could probably be detected by ultrasound at higher frequencies which has a smaller wave length and is therefore more sensitive to smaller diameter changes of particles. Dwyer et al. (2005) measured a decrease of attenuation in the enzymatic phase at different frequencies between 2.5 MHz and 14.5 MHz. However, Corredig et al. (2004a) could not measure this decrease of attenuation at 7.835 MHz or 14.665 MHz. Dwyer et al. (2005) used a skim milk prepared from a skim milk powder. In contrast, Corredig et al. (2004a) used fresh skimmed milk. Casein micelles have a sensible structure. Any pre-treatment may cause changes in the hydration state of the casein micelles and its renneting properties. With the beginning of the coagulation process the attenuation $\Delta(\alpha/f^2)$ increases more steeply, which correlates with the increase of particle size due to aggregation. This increase was also measured by both Dwyer et al. (2005) and Corredig et al. (2004a).

5.3.1.2 Influence of heating temperature and time

In order to assess the effects of the heat-treatment on the renneting action and the capability of the ultrasonic technique to detect the difference in rennetability in comparison to rheology, the renneting process of the samples preheated at 120, 130, or 140°C for up to 300s was tracked by the ultrasonic and rheological measurements. The renneting properties of the unheated casein solution were used for comparison.

Rheological measurements

Fig. 5.17 shows the change of G' in casein solutions unheated and pre-treated by heat at 120 °C, 130 °C, or 140 °C for 100 s during renneting. The storage modulus G' remains constant at first. This correlates with the primary enzymatic phase. After a certain time, which depends on the extent of the heat pre-treatment, G' starts to increase. This increase indicates the beginning of the coagulation of casein micelles. The higher the temperature of the heat treatment, however, the slower the increase of G'. This clearly indicates an effect of the heat treatment on the casein micelles, in spite of the negligible low concentration of the whey proteins. In the past, whey proteins were made responsible for the negative effect of heat treatment at higher temperature on the renneting action. Bulca et al. (2004a) observed that with decreasing whey protein concentration in skim milk the influence of a UHT-pre-heating on the coagulation time and gel strength becomes smaller but is still detectable. Bulca et al. (2003; 2004b) measured an increased dissociation of α_s-, β- and κ-casein from micelles into the serum and an increased polymerisation degree of the casein fractions with increasing extent of UHT treatment. These changes indicate the sensitivity of the casein micelle to heat, which has an effect on the rennet gel formation.

We defined the storage modulus G' at 60 min after start of the measurement (G'_{60}) as a criteria for the strength of gel formed. The G'_{60} values are plotted against the heating time in Fig. 5.18. It can be seen that G'_{60} decreases with increasing heating time and temperature. This indicates that, the higher the temperature and the longer the heating time, the weaker the gel formed.

The coagulation time t_c, defined as the time at G'=1 Pa, is also plotted against heating time at different temperatures (Fig. 5.19). A linear increase of the coagulation time t_c with increasing heating time of the pre-heating step was found for all pre-treatment temperatures.

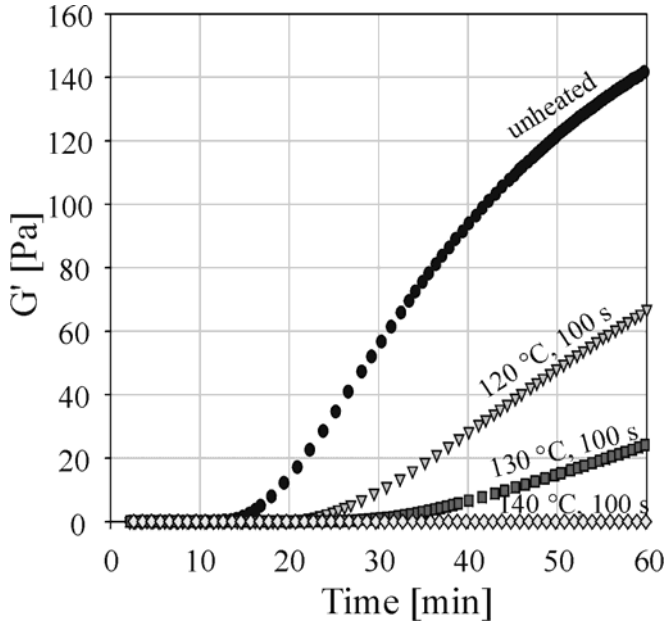


Fig. 5.17: Influence of the pre-heating conditions of the casein solution on the storage modulus G' during gel formation.

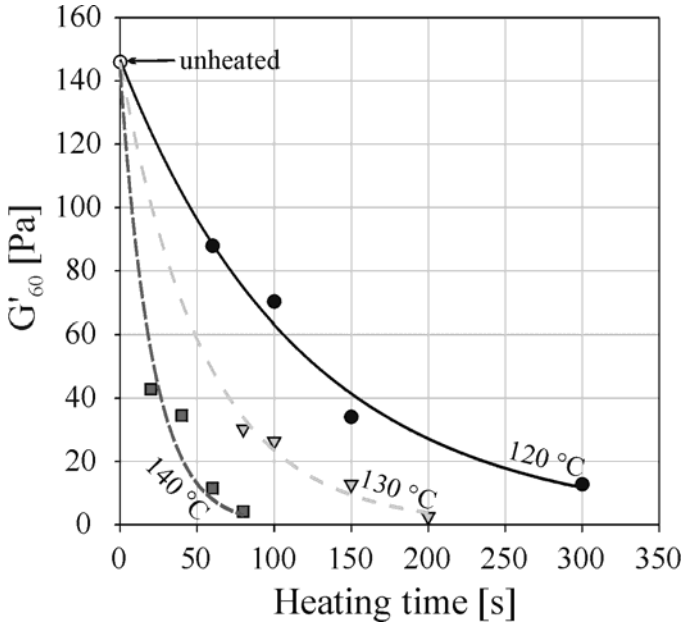


Fig. 5.18: G' value of the rennet gel at 60 min after rennet addition (G'_{60}) depending on the pre-heating condition of the casein solution.

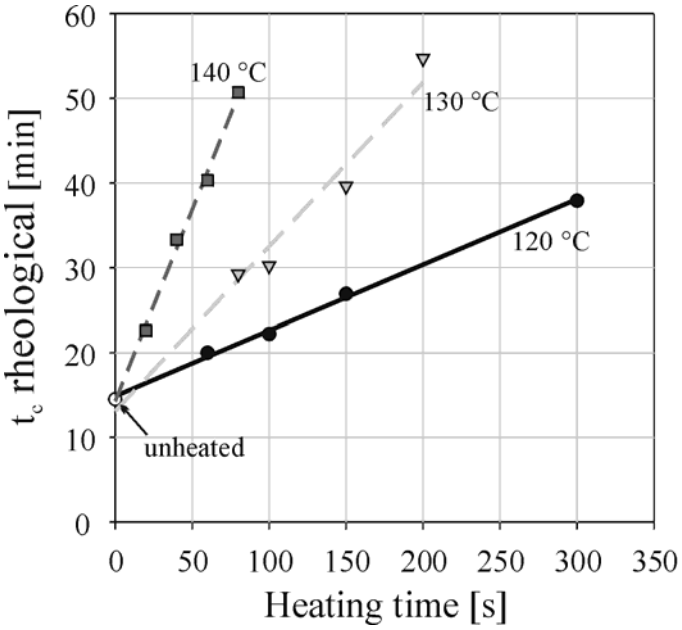


Fig. 5.19: Influence of the pre-heating condition of the casein solution on the coagulation time t_c determined by the oscillating rheological method.

Ultrasonic measurement

Fig. 5.20 shows the change of the normalized attenuation $(\Delta(\alpha/f^2))_n$ over time after rennet addition in samples unheated and pre-treated at 120 °C, 130 °C or 140 °C for 100 s during rennet gelation. The increase of the attenuation becomes weaker with increasing intensity of preheating. This confirms the poor coagulation capability of preheated samples.

The characteristic shape of the curve $(\Delta(\alpha/f^2))_n$ as a function of time is similar to that of G' as plotted in Fig. 5.17. But the increase of the ultrasonic attenuation appears much earlier than that of the G'. The ultrasonic method seems to be more sensitive to detect the starting of the aggregation process. The rheological measurement, however, can not detect a change in G' until a certain extent of the aggregation is reached. Similar to G' the increase of the ultrasonic attenuation decreases with increasing intensity of the pre-heating. This indicates that the higher the intensity of pre-heating is, the slower the coagulation process is.

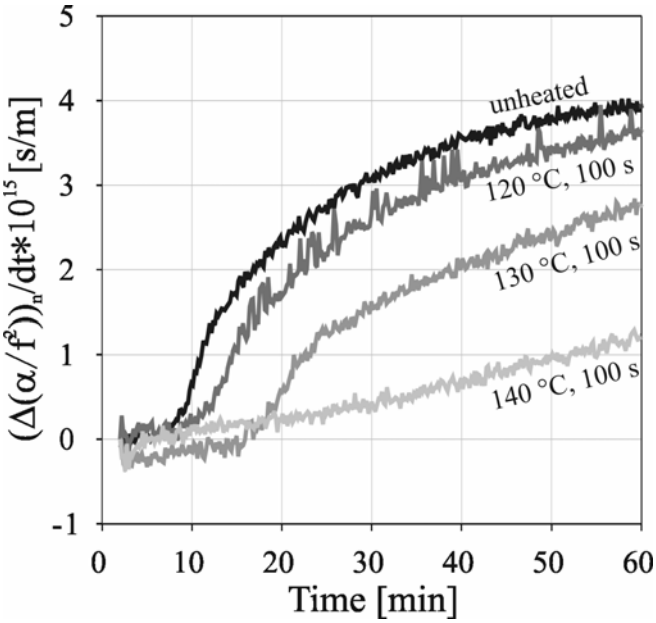


Fig. 5.20: Change of normalized attenuation difference between the sample and the reference $(\Delta\alpha/f^2))_n$ over time during renneting in casein solutions unheated and pre-treated at 120 °C, 130 °C or 140 °C for 100 s.

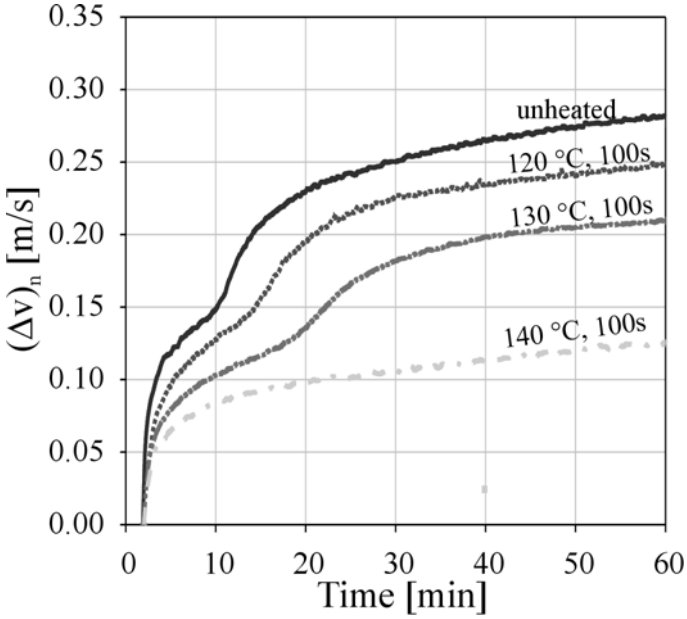


Fig. 5.21: Change of normalized velocity difference between the sample and the reference $(\Delta v)_n$ over time during renneting in casein solutions unheated and pre-heated at 120 °C, 130 °C or 140 °C for 100 s.

In Fig. 5.21 $(\Delta v)_n$ is plotted against the time after rennet addition. The different slopes of the first increase in $(\Delta v)_n$ indicate that pre-heating affects the enzymatic phase of renneting, too. To assess the effect of preheating on the enzymatic cleavage of CMP, the CMP (glycosylated and non-glycosylated) content released into the serum was determined during renneting. The relative CMP release is plotted against the incubation time in Fig. 5.22. With increasing

heating intensity, the CMP cleavage was decelerated. This explains the different slopes of the first increase in $(\Delta v)_n$ and confirms the hypothesis that the first increase correlates with the CMP cleavage. Heating at high temperature causes modifications of casein micelles even in absence of whey proteins, e.g., dissociation of casein micelles (Aoki et al., 1974) and cross-linking reactions on the surface or within the micelle (Bulca et al., 2004b). These modifications of casein micelles may impair the access of the rennet enzyme to the binding sites at the κ-casein.

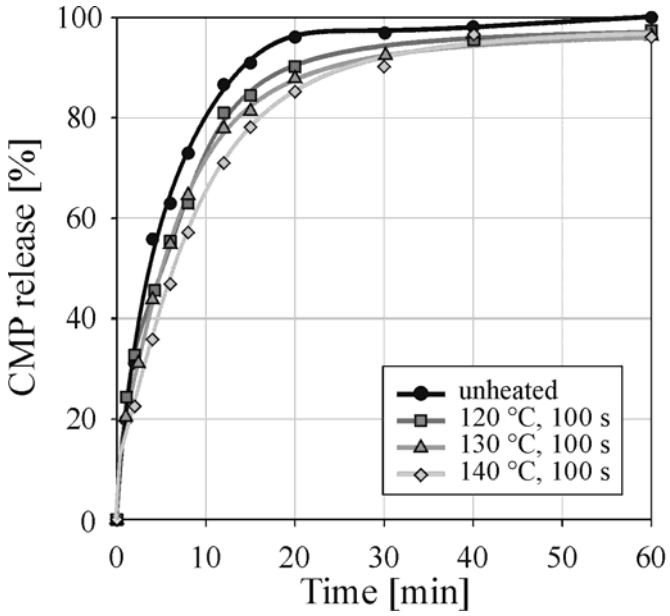


Fig. 5.22: Relative CMP release depending on the incubation time of rennet in 3% casein with 0.02% rennet addition at 30 °C.

Fig. 5.21 also shows that the higher the extent of the heat pre-treatment is, the later the second increase of $(\Delta v)_n$ occurs. In the sample heated at 140 °C for 100 s the second increase of $(\Delta v)_n$ disappeared. This confirms that the casein micelles were unable to form a coagulum as already shown in Fig. 5.17.

In order to detect the time t_c more clearly, the slopes of the curve in Fig. 5.21 were assessed as $d(\Delta v)/dt$. The unheated sample and the heated samples show different patterns (Fig. 5.23). Both the peak maximum (the aggregation rate V_c) and the time at peak maximum (the coagulation time t_c), depend on the extent of heat treatment.

According to the definitions given above, the coagulation time t_c, the aggregation rate V_c and the $\Delta(\Delta v)_{60}$ value of the heated samples at different temperatures and heating time were obtained. The influence of heat treatment on these parameters is demonstrated below.

As can be seen in Fig. 5.24 the coagulation time increases linearly with increasing temperature and increasing heating times. The aggregation rate decreases linearly with increasing extent of heat pre-treatment (Fig. 5.25).

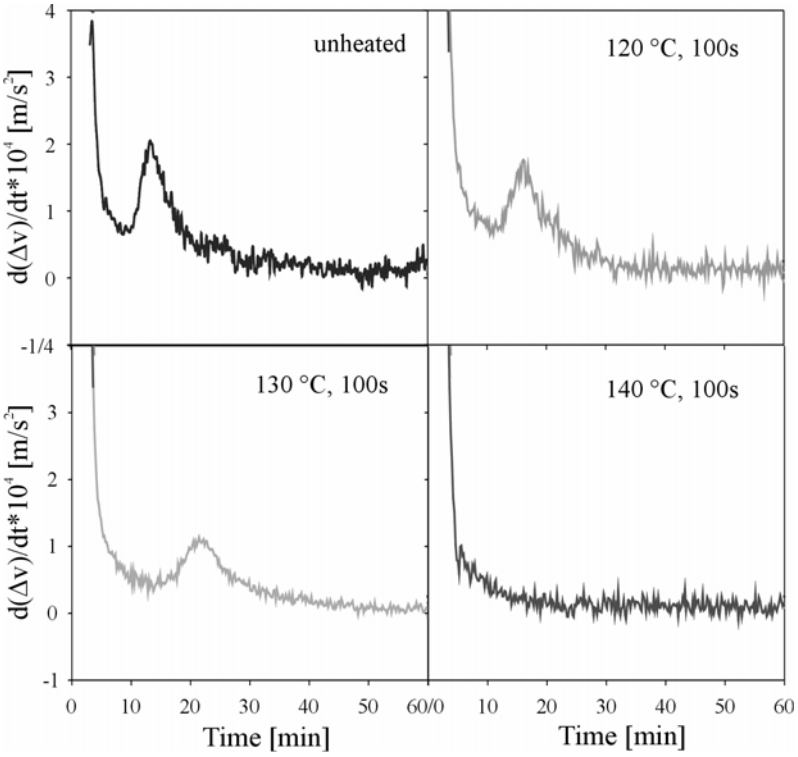


Fig. 5.23: Differentiated velocity difference between the sample and the reference d(Δv)/dt in unheated and pre-heated casein solutions over time during renneting process.

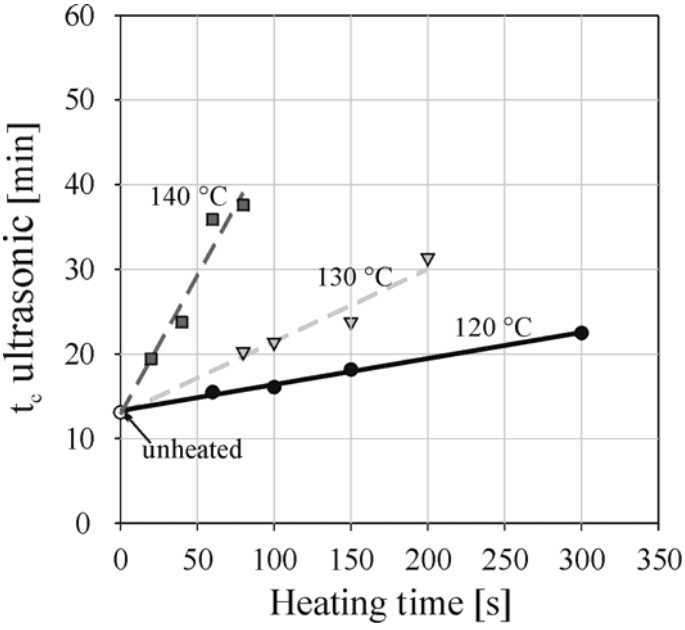


Fig. 5.24: Coagulation time t_c determined by ultrasonic measurements depending on the pre-heating condition of the casein solution.

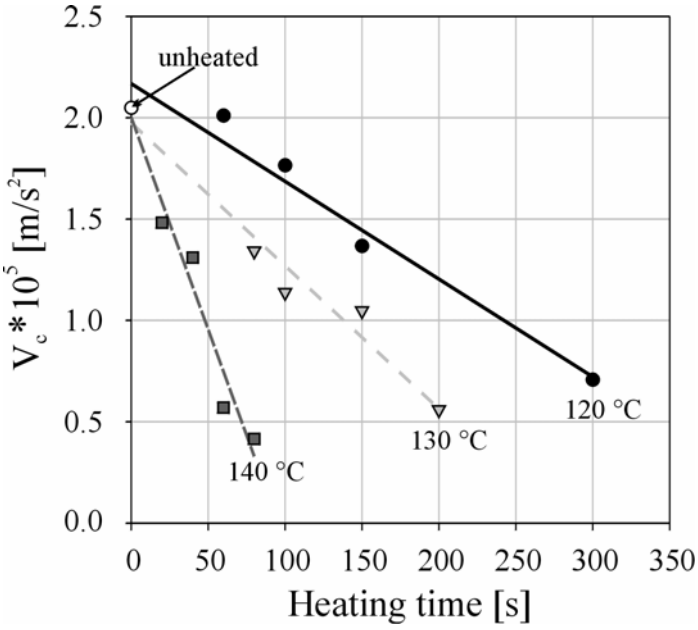


Fig. 5.25: Aggregation rate of the casein V_c determined by the ultrasonic method depending on the pre-heating condition of the casein solution.

In Fig. 5.26 the $\Delta(\Delta v)_{60}$ values derived from ultrasonic measurements depending on the heat treatment are plotted. The $\Delta(\Delta v)_{60}$ values show a decrease with increasing heating intensity. Although the decreasing trend of $\Delta(\Delta v)_{60}$ was represented by a line in Fig. 5.26, the measurement points are scattered relatively broadly around this trend, compared to what was observed with rheological measurements.

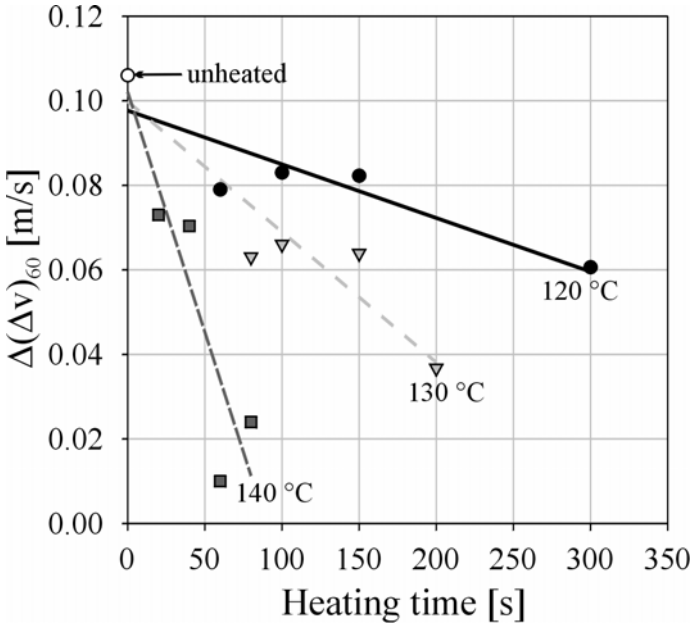


Fig. 5.26: Influence of the pre-heating condition of the casein solution on the $\Delta(\Delta v)_{60}$ value (difference of ultrasonic velocity at 60 min after rennet addition and that at t_c) of the rennet gel in ultrasonic measurements.

As is shown in Fig. 5.26 the $\Delta(\Delta v)_{60}$ values are generally very low. This indicates that the gel formation causes only a small change in the ultrasonic velocity. According to Eq. 2.7, the ultrasonic velocity of compressional ultrasound is influenced by the storage modulus G' and the bulk modulus K'. In many food gels the contribution of G' is much smaller than that of the K' (Povey, 1997). The gelling process mainly causes a change of the elastic properties (G') of the sample. Therefore, the formation of the casein network is more difficult but still possible to be measured by compressional ultrasound.

5.3.1.3 Correlation of ultrasonic and rheological measurements

In order to compare the t_c determined by the two methods used, the t_c in samples heated at 120°C obtained from the rheological measurements is plotted against that from the ultrasonic measurements (Fig. 5.27). As can be seen, t_c from the ultrasonic and the rheological measurements are linearly correlated. The values for t_c from the rheological measurements are higher than those from the ultrasonic measurements. This means that the ultrasonic technique detects changes related to aggregation earlier than the rheological method. Because the aggregation process precedes the network formation, t_c from the rheological measurements correlates with a certain extent of network formation. It can only be measured at a later stage compared to the aggregation related changes in ultrasonic measurements.

The slope of the regression line in Fig. 5.27 is larger than 1. This means that the difference between t_c from ultrasonic and rheological measurements increases with increasing heating time. This indicates that, the more the aggregation process is affected, the longer it takes from aggregation to gelling. The results of ultrasonic and rheological measurements show that UHT heat treatment retards the coagulation process, and therefore, prolongs the gel firming process.

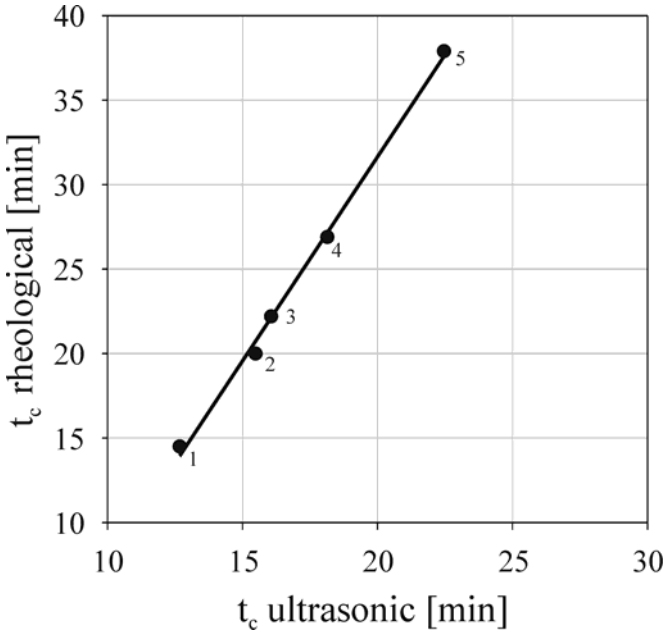


Fig. 5.27: Correlation of the coagulation times determined by ultrasonic and rheological methods in samples heated at 120 °C for varying time. The numbers 1 to 5 are for unheated, 60 s, 100 s, 150 s and 300 s, respectively. $R^2 = 0.9967$.

In contrast to the rheological method, which is not sensitive enough to detect changes during the enzymatic hydrolysis, both the enzymatic phase and the aggregation phase of the casein micelles can be sensitively detected by ultrasonic measurement. A linear correlation exists between the coagulation time determined by rheological and ultrasonic measurements. However, the coagulation times measured by these two methods are different. The growth of the aggregates to a three dimensional casein network is more difficult to be detected by compressional ultrasound. For the characterisation of the formed gel the rheological measurement is more sensitive. The results of this study show that the ultrasonic measurement is a capable method to complement the established rheological measurement for the characterisation of the rennet gel formation and a new option to track the cleavage phase of the rennet enzyme acting on the casein micelle surface. Due to its online applicability the ultrasonic method has good potential for use in monitoring the rennet process in cheese making and quality control of rennet enzyme. However, in this case high resolution and accuracy of the measurement are required, because the changes in the ultrasonic parameters induced by renneting gelation are very small. The online application can be made viable by reconstructing a high resolution measuring device with high temperature stability such as ResoScan®, which may be connected as a bypass of the renneting process line.

5.3.2 Monitoring of the acid gelation of skimmed milk

Fig. 5.28(a) depicts the ultrasonic velocity as a function of the time during acidification using GDL in pasteurized skim milk, UF-permeate and water. The velocity difference Δv is the difference between the velocity in the sample with GDL and that in the sample without GDL. It can be seen that the addition of 3% GDL caused an increase of over 8 m/s in ultrasonic velocity in all samples. Not only in the skim milk, but also in the UF-permeate and water, an increase of Δv was observed. The change of the Δv is caused not only by the changes of the milk components during acidification but also by the hydrolysis of GDL to gluconic acid. This hydrolysis leads to a higher hydration degree of solutes, and therefore, to a higher ultrasonic velocity. The time dependent changes in the slope of the curves in Fig. 5.28(a) are different for pasteurized milk, permeate and water, indicating that the rate of the hydrolysis depends on the milieu condition.

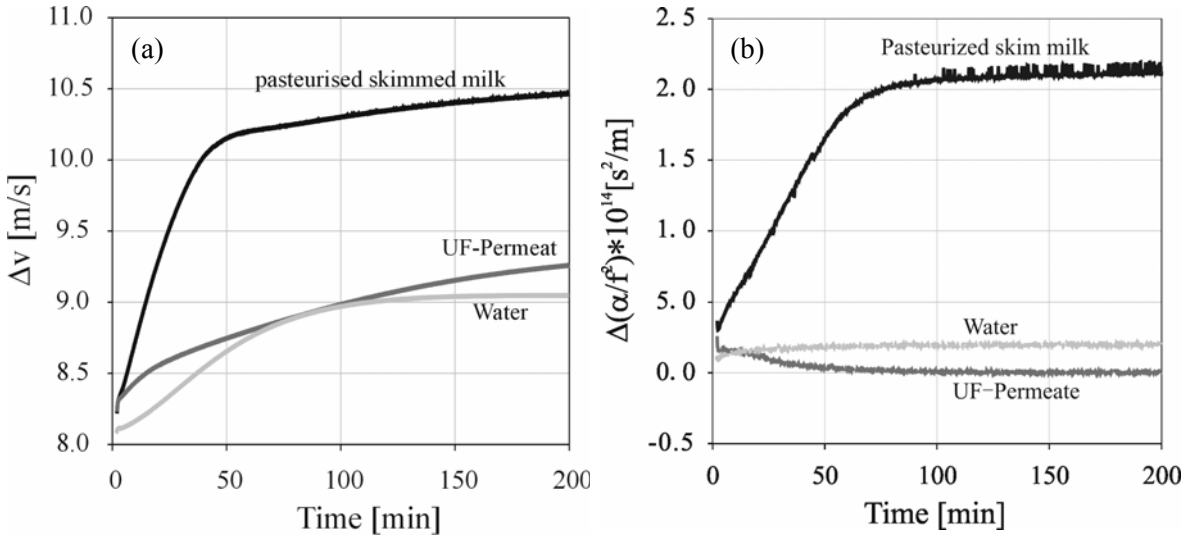


Fig. 5.28: Ultrasonic velocity difference Δv (a) and attenuation difference $\Delta(\alpha/f^2)$ as a function of time during acidification in pasteurized skim milk, milk serum (UF-permeate) and water containing 3% GDL at 30 °C. Reference: pasteurized skim milk, milk serum (UF-permeate) and water without GDL, respectively.

An increase of the ultrasonic attenuation difference could only be observed in the skim milk containing GDL (Fig. 5.28(b)). In water, the attenuation remained constant during the GDL hydrolysis, which indicates that the GDL hydrolysis process itself does not affect the attenuation. In UF-permeate, the ultrasonic attenuation even slightly decreases with the GDL hydrolysis. Compared to water, the UF-permeate contains different solutes, such as lactose and minerals and traces (about 0.02%) of whey proteins. The decrease in the attenuation must be a result of the pH-induced changes of the solutes, e.g., decrease in apparent volume due to

decrease in hydration. The hydration of small molecules like salts and lactose is not sensitive to pH variation. It is more possible that the traces of whey proteins are responsible, because their surface charge is sensitive to pH variation. A pH decrease leads to a compensation of the negative charge on the surface and a decrease in the apparent volume of the solutes.

The rheological measurements show that G' and G'' remained constant down to pH 5. Then an increase in both G' and G'' was observed (Fig. 5.29). The increase of Δv and $\Delta(\alpha/f^2)$ in milk immediately after the GDL addition demonstrates that this increase could not be induced by the gelation process, which does not occur until pH 5 according to the rheological measurement.

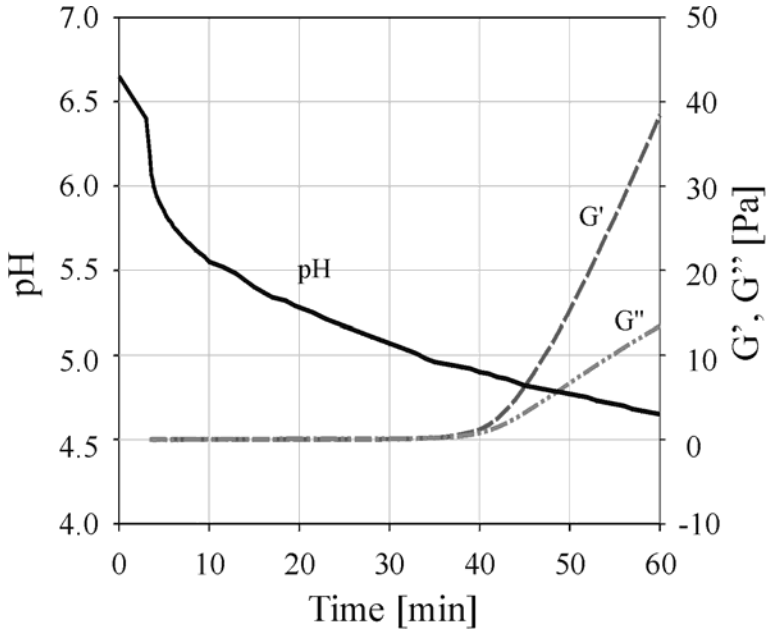


Fig. 5.29: G', G'' and pH as a function of pH during acidification in pasteurized skim milk containing 3% GDL at 30 °C. G'=1 Pa at pH 4.89.

Both Dalgleish et al. (2005) and Kudryashov et al. (2000) explained the increase in ultrasonic velocity by the dissociation of calcium from casein micelles. However, all the past investigations did not consider the contribution of the GDL hydrolysis to the increase in ultrasonic velocity. The results shown here prove that the contribution of GDL hydrolysis is not negligible. It makes up for up to 30% of the overall increase in ultrasonic velocity. The increase in the ultrasonic velocity is a result of both the calcium dissociation and the GDL hydrolysis. Dalgleish et al. (2005) observed that both the ultrasonic velocity and the attenuation correlate to the Ca^{2+} concentration in serum. According to Dalgleish et al. (2005), the changes of the ultrasonic velocity and attenuation during the milk acidification using GDL is rather caused by the changes in milk serum, i.e., dissociation of the calcium from casein micelles, than changes of the casein micelles themselves. The gelation does not have a major

effect on the ultrasonic parameter. According to our measurements, an increase of $CaCl_2$ concentration in milk serum (UF-permeate) by 20 mM induces an increase in ultrasonic velocity by 1.5 m/s, which confirms that the ion concentration in milk serum has a large effect on ultrasonic velocity. However, the addition of $CaCl_2$ by up to 20 mM did not noticeabelly affect the attenuation. Dukhin et al. (2005) also concluded that the impact of chemical variation on attenuation is negligible if concentration varies is less than 0.1 M. This indicates that the increase in ultrasonic attenuation during acidification cannot be explained by the Ca^{2+} dissociation.

Fig. 5.30 shows the ultrasonic velocity and attenuation as a function of pH. The curves of Δv and $\Delta(\alpha/f^2)$ over pH are similar. The attenuation curve is shifted on the pH (also time) axis, which provides again that the ultrasonic velocity and attenuation detect different processes which take place consecutively in the sample. This indicates that the ultrasonic velocity characterises a process which takes place earlier than that characterised by the ultrasonic absorption. The ultrasonic velocity measures changes in hydration, which is associated with the molecular structure of the sample. In contrast, the ultrasonic attenuation measures the interactions between the molecules (e.g., aggregation), which are a result of the forgoing changes in the molecular structure.

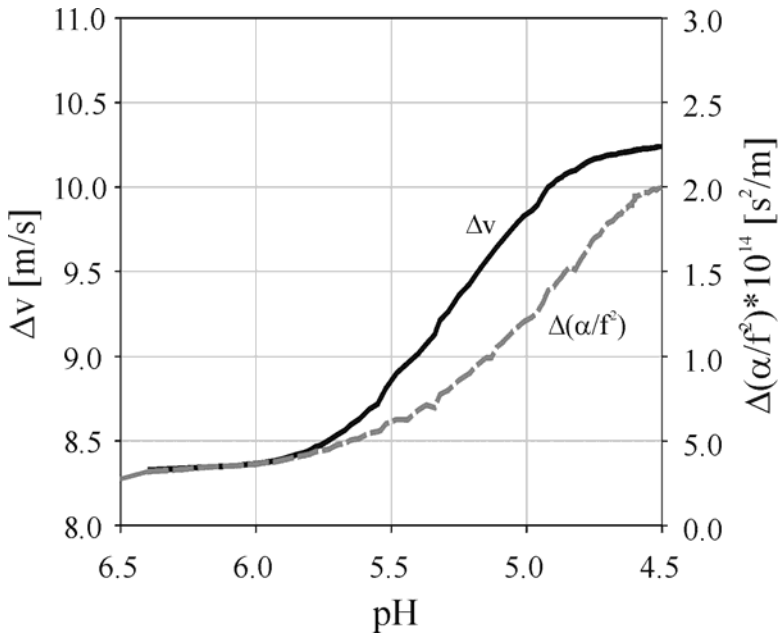


Fig. 5.30: Δv and $\Delta(\alpha/f^2)$ as a function of pH during acidification in pasteurized skim milk containing 3% GDL at 30 °C.

In Fig. 5.31 the ultrasonic velocity and attenuation difference between the skim milk containing yoghurt culture and that without yoghurt culture are plotted against the pH value during fermentation. Both ultrasonic velocity and attenuation increase with decreasing pH

over the whole pH area. The casein micelles rather lose their charges with decreasing pH which leads to a lower hydration degree and smaller volume. Therefore, the increase of the ultrasonic velocity and attenuation cannot be a result of the changes of the charge at the casein surface. It may be caused by the dissociation of Ca^{2+} or by the formation of lactic acid or other metabolic products. In contrast, G' and G'' in the rheological measurements remain constant down to pH 5.4 and then increase steeply. This indicates the start of the aggregation process from pH 5.4 onwards (Fig. 5.32). A steep increase in the ultrasonic attenuation was observed at about the same pH. This indicates that the gelation process influences the $\Delta(\alpha/f^2)$ additional to the effects mentioned above. Similar to acidification using GDL, the steep increase of the ultrasonic velocity takes place later than that of the attenuation.

Between pH 5 and pH 4.8 a shoulder area was found both in ultrasonic velocity and in attenuation (Fig. 5.31). According to El-Shobery (1987), at pH 5 the peptide chains which occur due to the loss of the micellar structure of casein begin to associate. The shoulder areas in Fig. 5.31 are probably caused by the initial stage of the network formation of casein, which initially occurs slowly and is accelerated at decreasing pH. From pH 4.6 the change in the ultrasonic parameter becomes smaller which probably indicates the completion of network formation.

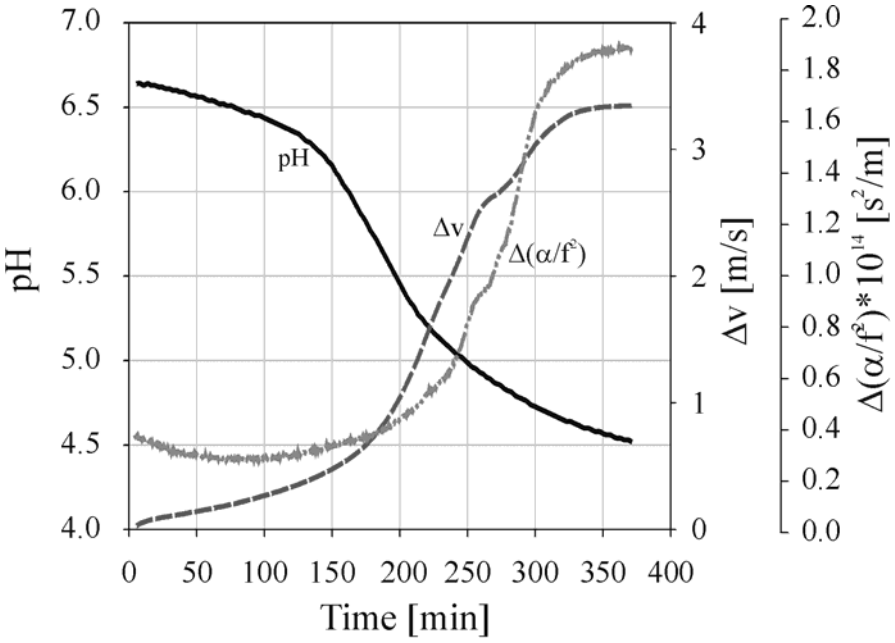


Fig. 5.31: pH, Δv and $\Delta(\alpha/f^2)$ as a function of time during the yoghurt fermentation in pasteurized skim milk at 42 °C. Reference: pasteurized skim milk.

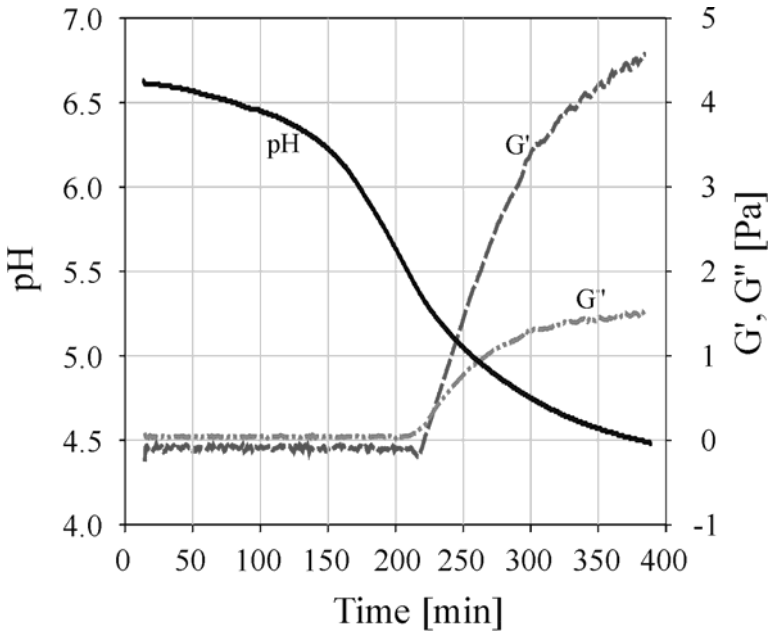


Fig. 5.32: pH, G' and G'' as a function of time during the yoghurt fermentation in pasteurized skim milk at 42 °C.

The results shows that the ultrasonic method rather detects the phenomenon accompanied by the pH decrease during acidification of milk using GDL, e.g., dissociation of Ca^{2+} from casein micelles, than the aggregation and network formation of casein micelles. During yoghurt fermentation the ultrasonic method detects the overlaid effects of the Ca^{2+} dissociation as well as the aggregation and gelation. The mechanism can still not be completely explained. However, it can be seen that the ultrasonic method is sensitive to the pH change during the acidification of milk. It can be used as a non-invasive method to follow the acidification process in milk.

5.3.3 Investigation of the thermal-induced gelation of caseinomacropeptides

In order to assess gel formation with another different mechanism, the gelation of CMPs at different acid pH was investigated. The work of Thomä-Worringer and Kulozik (2006) shows that CMPs are able to form a gel in acid environment. However, the exact mechanism of the gelation is still not clear. We investigated the thermally induced changes in 5% solution of CMP from two different companies (Lacprodan CGMP-20 from Arla Foods and BioPURE-GMP™ from Davisco Foods International) using ultrasound and expected to get information to explain the gelation mechanism of the gelation process of CMP.

Fig. 5.33 and Fig. 5.34 show the ultrasonic velocity difference Δv and attenuation difference $\Delta(\alpha/f^2)$ over temperature in 5% solutions of CMP from Arla and Davisco at different pH. The CMP concentration and pH were chosen in the area, where gelation is expected according to

the results of Thomä-Worringer and Kulozik (2006). At low temperature the Δv of CMP shows a linear decrease at first, which is a temperature effect. From a certain temperature onwards, however, this decrease becomes steeper, which indicates a change of structure additional to the temperature effect (Fig. 5.33(a) and Fig. 5.34(a)). A decrease in the ultrasonic velocity means an increase in the compressibility. CMPs are peptide chains which do not have a secondary structure. Its intrinsic compressibility is supposed to be negligibly low. The increase of the compressibility is mainly contributed by the decrease in the hydration degree. It may be caused by an aggregation process due to hydrophobic interaction, where water is extruded from bound state into bulk resulting in a lower hydration degree. The $\Delta(\alpha/f^2)$ in all CMP solutions decreases with increasing temperature at low temperatures due to the decreasing viscosity, then increases steeply with temperature up to a levelling off (Fig. 5.33(b) and Fig. 5.34(b)). In 5% Lacprodan CGMP-20 sample at pH 3.5 and 5% BioPURE-GMPTM sample at pH 4, no increase in ultrasonic attenuation was observed indicating the absence of gel formation. The increase in ultrasonic attenuation is due to the aggregation of CMP molecules and the gel network formation, which leads to dissipation of ultrasonic energy due to growing molecular size and friction loss due to the relative motion between gel network and water. The levelling off indicates the completing of the gelation process. The higher the pH, the higher the levelling off value of $\Delta(\alpha/f^2)$. This indicates that the gel microstructure formed is depending on the pH. The lower the pH, the more negative charges at the CMP molecule are compensated, the more junction zones under different CMP molecules can be formed. Therefore, the gel network formed at lower pH may be more homogenous and induces less dissipation of ultrasonic energy than that formed at higher pH.

Depicting the same results as the first derivative of Δv over temperature, a peak is a clear demonstration of changes going on in the corresponding temperature area, as shown in Fig. 5.35 and Fig. 5.36 for the Arla and the Davisco samples respectively.

A DSC measurement of a 5% CMP (Davisco BioPURE-GMP^{TM}) at pH 3.5 (Fig. 5.37) shows an exothermic peak which occurs at the similar temperature as the peak in the ultrasonic data. The exothermic peak in the DSC measurement supports the assumption that the additional decrease of Δv (i.e., peaks in Fig. 5.35 and Fig. 5.36) was caused by an aggregation process, which is known to be exothermic.

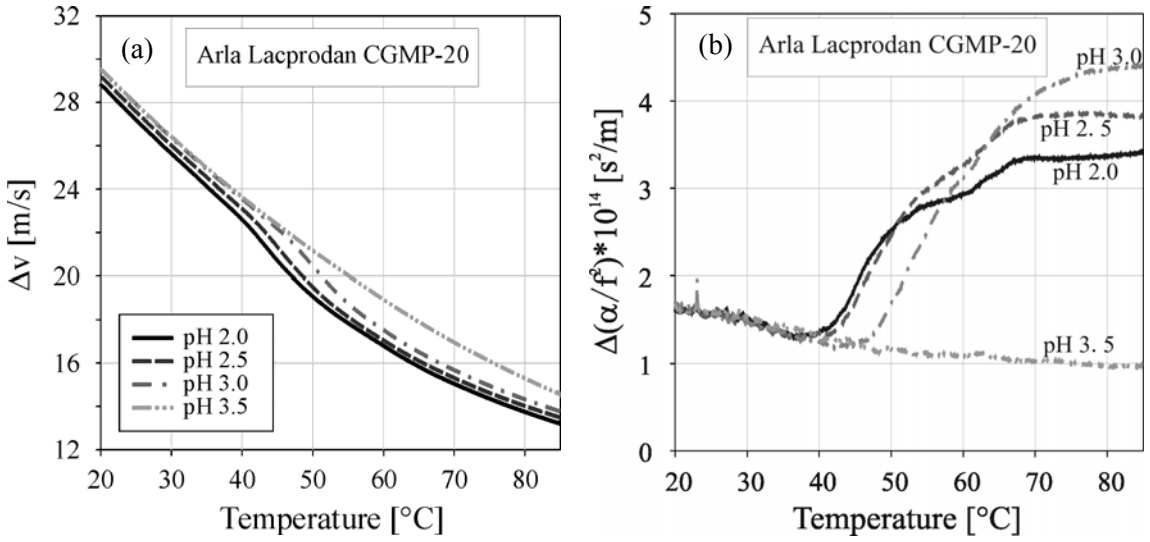


Fig. 5.33: Ultrasonic velocity difference Δv (a) and attenuation difference $\Delta(\alpha/f^2)$ (b) between 5% CMP solution (Arla Lacprodan CGMP-20) at different pH and water depending on the temperature. Reference: water; Heating rate: 0.3 °C/min.

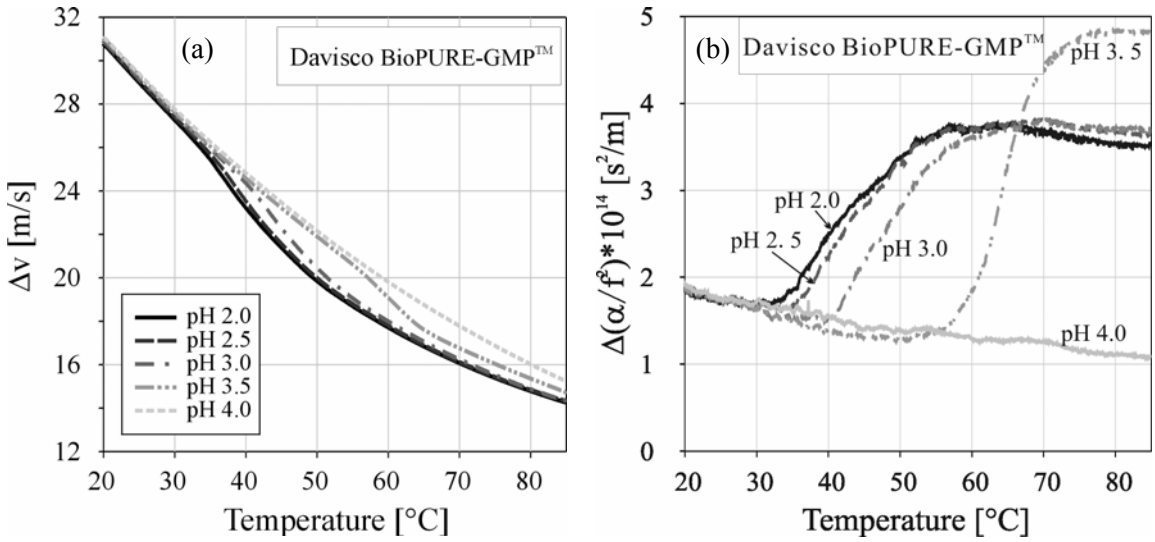


Fig. 5.34: Ultrasonic velocity difference Δv (a) and attenuation difference $\Delta(\alpha/f^2)$ (b) between 5% CMP (Davisco BioPURE-GMP™) at different pH and water depending on the temperature. Reference: water; Heating rate: 0.3 °C/min.

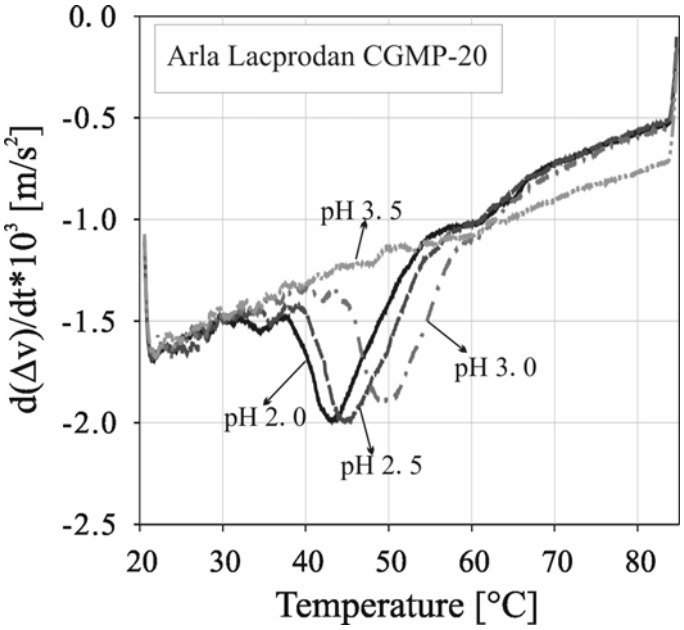


Fig. 5.35: The first derivative of ultrasonic velocity difference d(Δv)/dt in 5% CMP solution (Arla Lacprodan CGMP-20) at different pH depending on temperature. Heating rate: 0.3 °C/min.

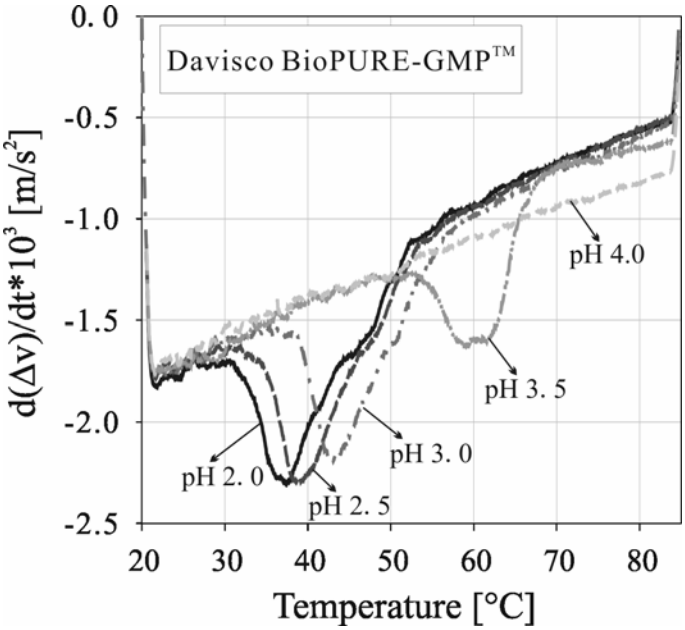


Fig. 5.36: The first derivative of ultrasonic velocity difference d(Δv)/dt in 5% CMP solution (Davisco BioPURE-GMP™) at different pH depending on temperature. Heating rate: 0.3 °C/min.

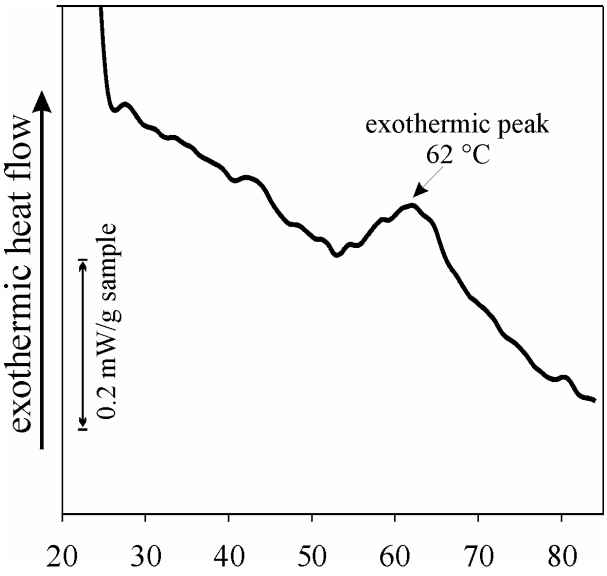


Fig. 5.37: DSC thermogramm of 5% CMP (Davisco BioPURE-GMP™) at pH 3.5. Heating rate: 0.3 °C/min.

The peaks of the first derivative of Δv in Fig. 5.35 and Fig. 5.36 as well as the increase of $\Delta(\alpha/f^2)$ in Fig. 5.33(b) and Fig. 5.34(b) occur only at an acid pH range of about 2-3.5. This pH range corresponds to the pH area for the minimal solubility of CMP and is suggested to be the isoelectric point of the non-glycosylated CMPs (Thomä-Worringer & Kulozik, 2006). At the isoelectric point, the positive and the negative charges of the CMP chains are balanced, so that aggregation is favoured.

Comparing the starting values of CMP solutions of different pH at 20 °C, both CMP samples show that the ultrasonic velocity decreases with decreasing pH within the pH range applied. This means that the hydration degree of CMP decreases with decreasing pH. It is known that the apparent molecular weight of CMP at acid pH is lower than that at neutral pH (Kawasaki et al., 1993; Minkiewicz et al., 1996). The apparent molecular weight consists of the molecular weight of the CMP molecule itself plus that of the hydration shell. This agrees with the ultrasonic data shown here. The hydration shell can provide a protective coating against aggregation via hydrophobic interaction. This explains why the higher the pH, the higher the necessary temperature for the aggregation as shown in Fig. 5.33 and Fig. 5.34, because the higher the temperature, the higher the hydrophobic interaction between the CMP molecules.

Fig. 5.33 and Fig. 5.34 show that BioPURE-GMP™ aggregates at a lower temperature than Lacprodan CGMP. According to the HPLC analysis, the Lacprodan CGMP-20 contains about 76% glycosylated CMP, while in the BioPURE-GMP™ only about 66% of the CMP are glycosylated. The aggregation process is initiated by the hydrophobic interaction, which increases with decreasing glycosylysation degree of the CMP and increasing temperature.

Lacprodan CGMP is less hydrophobic than BioPURE-GMP^{TM}. Therefore, it requires higher temperature to reach the critical hydrophobic strength to initiate the aggregation process.

The ultrasonic method can be used to detect the thermally induced aggregation process of CMP at low pH environment. Furthermore, the results suggest that the hydration state of the CMP depending pH can be derived from the ultrasonic measurements.

5.4 Assessment of the heat-induced protein denaturation

Protein denaturation is a molecular reaction that often occurs in the food manufacturing. During denaturation the protein molecule unfolds. Subsequently, the unfolded molecules aggregate with each other. The protein structure and the hydration state of protein molecules change during unfolding, so that the compressibility of the molecules changes, which is expected to be measurable by ultrasound.

5.4.1 Denaturation of whey protein α-lactalbumin[1]

When a new analytical method is introduced, it is important to compare it with established methods. In the literature there is no information about the application of ultrasound for quantitative investigation of protein denaturation. In this work, the focus was put on the quantitative assessment of the thermal denaturation of α-la. α-la was chosen, because it is often used as a model protein of globular proteins. It is a relatively stable protein, so that a wide range of degrees of aggregation can be easily achieved.

The purpose of this work was to acquire the ultrasonic velocity in α-la solutions during the thermally induced protein denaturation process, and to investigate whether the degree of aggregation of this protein can be determined via ultrasonic measurements, and how the results correlate with those of established methods, namely, HPLC and DSC. Next to that, it was of interest, whether the ultrasonic method can provide additional information about the denaturation compared to the established methods was of interest.

5.4.1.1 Changes in ultrasonic attenuation and velocity depending on temperature in α-la

The ultrasonic attenuation difference ($\Delta(\alpha/f^2)$) between the 4% (w/w) native α-la and milk serum (UF-permeate) as a function of temperature is presented in Fig. 6.1. During heating, the $\Delta(\alpha/f^2)$ decreases up to about 50-55 °C at first, which may be caused by the decreased viscosity with increasing temperature in the sample. Then $\Delta(\alpha/f^2)$ increases due to the heat-

[1] Chapter published as Wang et al. (2006): *Journal of Agricultural and Food Chemistry, 54,* 6501-6506.

induced conformational changes of protein molecules. A steeper increase occurs between 60 °C and 75 °C, before the curve levels off. The levelling off indicates the completion of the unfolding process. During the cooling, the course of $\Delta(\alpha/f^2)$ over temperature is a reversed course of the heating curve, but on a higher level and less changeable, however. The sample at 85 °C is a mixture of unfolded and aggregated α-la, and therefore attenuates more ultrasonic energy compared to the native and partly unfolded sample. From 85 °C to about 70 °C, the attenuation increases slightly, because the viscosity of the sample increases with decreasing temperature. In the temperature range from 70 °C to 53 °C, the attenuation decreases due to the refolding process of the not aggregated α-la molecules. Below 53 °C, the attenuation increases again with decreasing temperature due to increasing viscosity.

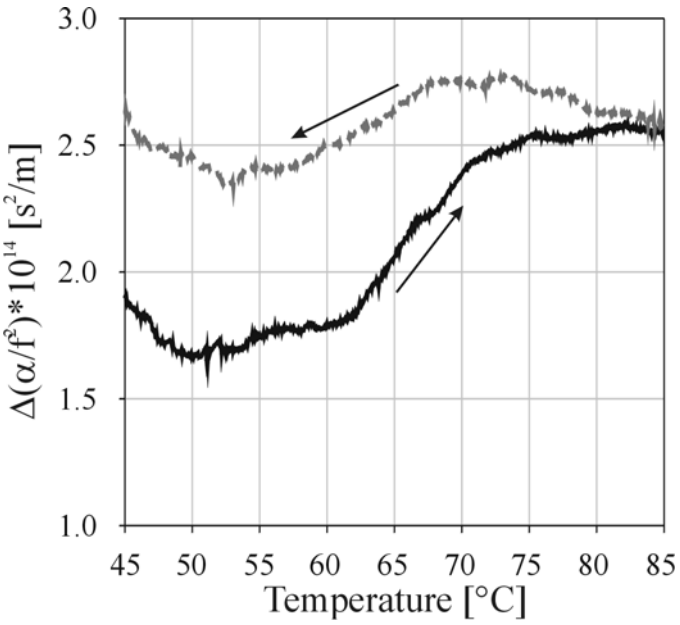


Fig. 6.1: Effect of the temperature on the ultrasonic attenuation difference $\Delta(\alpha/f^2)$ in a 4% native α-la solution at pH 6.5 upon heating and cooling by a rate of 0.3 °C/min.

Fig. 6.2 shows Δv in the 4% native α-la solution depending on temperature during heating and subsequent cooling. It can be seen that upon heating at first a linear decrease in Δv at temperatures between 45 and 54 °C can be observed. The ultrasonic velocity is related to the compressibility of the medium. The decrease in ultrasonic velocity indicates an increase in protein compressibility with an increase in temperature. This increase in compressibility may have been caused by an increasing conformational change and hydrophobic interaction of α-la molecules with an increase in temperature (Corredig et al., 2004b). Furthermore, the hydration degree of molecules decreases generally with an increase in temperature due to the higher kinetic energy of water molecules at higher temperatures which induces an increase in the compressibility of the α-la solution. Above a temperature of about 54 °C, the curve is no

more linear. It becomes steeper. This indicates changes in α-la molecules during denaturation. Gast et al. (1986) found that the hydrodynamic effective molecular dimensions of the molten globule state and the unfolded state of α-la are larger than those of the native state. This rather leads to a decreased compressibility and increased ultrasonic velocity due to an enlarged hydration shell. However, the decrease in ultrasonic velocity correlates with an increase in the overall compressibility.

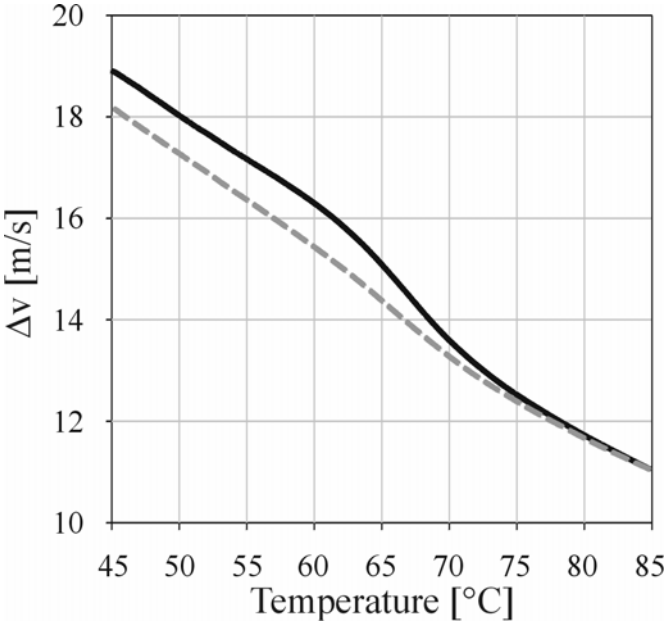
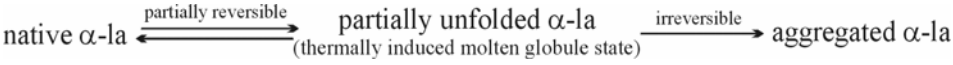


Fig. 6.2: Effect of the temperature on the ultrasonic velocity difference Δv in a 4% native α-la solution at pH 6.5 upon heating and cooling by a rate of 0.3 °C/min.

Many researchers found that compressibility of denatured protein is larger than that of the native one (Brandts et al., 1970, Zipp & Kauzmann, 1973 and Hawley, 1971). This may be caused by the decreased compactness of the unfolded protein, which leads to increased intrinsic compressibility, and by the conformational and chemical relaxation processes (Nölting et al., 1993). During unfolding, the atomic groups of the side chains are exposed to the solvent. The relaxation contribution increases due to an increase in the conformational flexibility of the protein molecules and an accelerated proton exchange process (Nölting & Sligar, 1993). The presence of relaxation terms increases the compressibility and decreases the ultrasonic velocity (Nölting & Sligar, 1993). The negative contribution of intrinsic compressibility and relaxation to the ultrasonic velocity may be greater than the positive contribution of the hydration. This may be the reason for the decrease in the ultrasonic velocity during the thermal denaturation of α-la. After an inflection point, the curve becomes flatter again. Above approximately 80 °C, the decrease in Δv with an increase in temperature is linear again, which indicates the completion of the conformational change.

To follow the changes in ultrasonic velocity as a function of temperature increase more clearly, the first derivative of Δv [i.e., d(Δv)/dt] was calculated and plotted against temperature. Fig. 6.3 shows d(Δv)/dt versus temperature in a 4% native α-la solution. The bottom curve depicts d(Δv)/dt as a function of increasing temperature and the top curve as a function of decreasing temperature following the upward ramp. Both curves show peaks in the temperature region of 54-80 °C. However, the peak area during cooling is smaller than that during heating. This indicates that the molecular changes detected by ultrasound were partially reversible. This complies with the description of α-la denaturation in the literature (Boye et al., 1997). The partial reversibility of α-la denaturation can be described by the following reaction scheme:



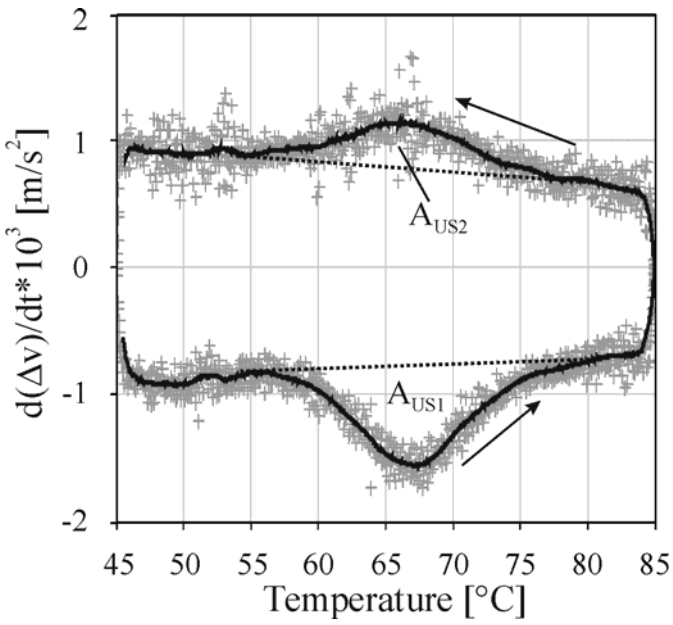


Fig. 6.3: Effect of the temperature on the d(Δv)/dt in a 4% native α-la solution at pH 6.5 on heating and cooling by a rate of 0.3 °C/min. The cross symbols are all measure points. The solid line is a smoothed curve derived from the data points.

The temperature increase beyond 60 °C leads to a partial unfolding and formation of the thermally induced molten globule state of α-la. This partially unfolded state exhibits an increased molecular dimension and hydrophobicity as well as better accessibility of the disulphide bonds to a thiol exchange reaction (Calvo et al., 1993). Thus, it can participate in the following intermolecular aggregation. This aggregation is considerably enhanced especially in the presence of traces of β-lg. Once the partially unfolded α-la molecules are

aggregated, they are not able to refold during cooling. In contrast, the unaggregated molecules refold during cooling. The downward peak in Fig. 6.3 describes the denaturation (unfolding and aggregation) process of α-la, while the upward peak describes the refolding of the unaggregated α-la. The areas (A_{US}) of the downward and upward peak correspond to the overall change in Δv during denaturation and refolding, respectively. The peak maxima correlate with the maximal change rates in ultrasonic velocity. The temperatures at the peak maxima were about 66 °C for both the heating and cooling curves. This agrees with the DSC measurement, where that the temperature at the maxima of the DSC endothermic peak is 65.8 ± 0.2 °C.

According to the results given above, it can be suspected that the peak area A_{US} may be proportional to the native α-la concentration in the sample. To verify this, α-la solutions at different concentrations were investigated by the ultrasonic method. The peak areas A_{US} are plotted against the native α-la concentrations in the samples in Fig. 6.4. It shows a linear correlation between the peak area A_{US} and the native α-la concentration in the sample. The coefficient of determination R^2 was 0.9946. This confirms that the overall change in Δv during thermal denaturation of α-la linearly correlates with the native α-la concentration in the sample.

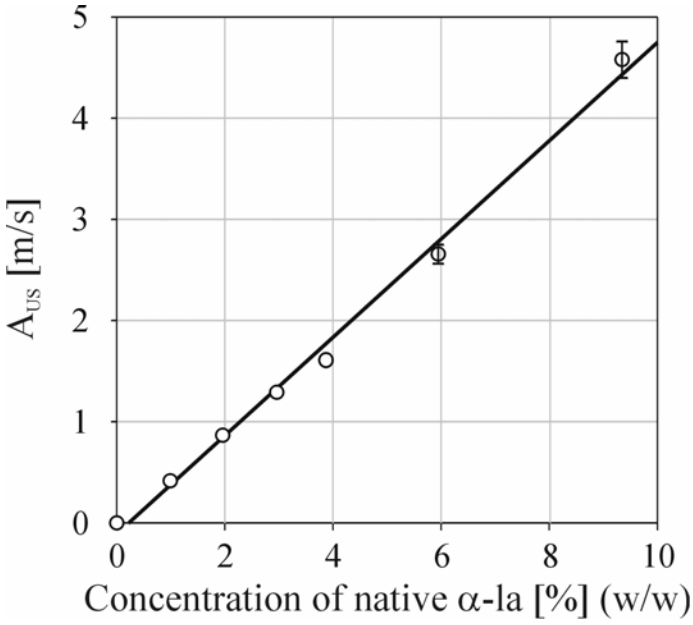


Fig. 6.4: Dependence of peak area A_{US} on the native α-la concentration in ultrasonic measurements. Error bars refer to standard deviation. $R^2 = 0.9946$

Fig. 6.5 shows the dependency of d(Δv)/dt on the temperature in an unheated and a preheated (90 °C for 2 min) 6% α-la solution. According to the linear correlation in Fig. 6.4, the difference between the peak areas of the unheated and pre-heated samples is proportional to

the amount of irreversibly aggregated α-la. Therefore, the degree of aggregation DA in the pre-heated sample can be calculated from the A_{US} of the pre-heated and unheated samples as follows:

$$DA_{US} = (1 - A_{US,\ pre\text{-}heated} / A_{US,\ unheated}) \cdot 100\% \qquad \textbf{(Eq. 6.1)}$$

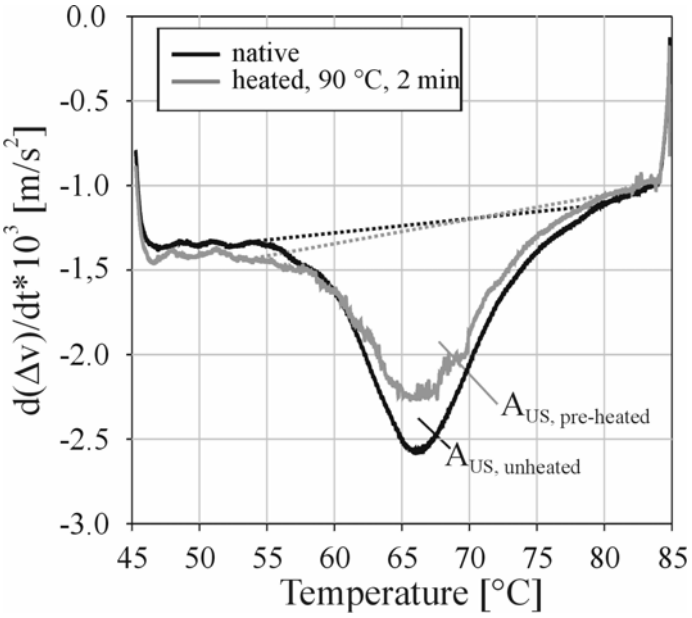


Fig. 6.5: Effect of heat treatment on the temperature dependence of the ultrasonic velocity in the samples: d(Δv)/dt depending on the temperature in a 6% α-la solution, native and pre-heated at 90 °C 2 min. Heating rate: 0.3 °C/min.

5.4.1.2 Kinetics of the thermal aggregation of α-la determined by HPLC, DSC and ultrasound

To compare the ultrasonic method with HPLC and DSC methods for quantitative determination of α-la aggregation over a wide range of degrees of aggregation, we assessed pre-heated α-la solutions with different degrees of aggregation by HPLC, DSC and ultrasound. The degrees of aggregation of α-la determined by these three methods in 6% α-la solutions with different heating times are listed in Tab. 6.1. The degree of aggregation of α-la increases with increasing heating time.

In Fig. 6.6 the DA of the pre-heated 6% α-la solutions (presented in Tab. 6.1) and those of the pre-heated 10% α-la solutions determined by HPLC, ultrasonic and DSC methods are plotted against each other. 10% α-la solution was additionally used to show the influence of the concentration on the determination the degree of α-la aggregation. To determine the reproducibility of the methods two of the 6% α-la solutions were measured three times each. The standard deviations are plotted as error bars.

Tab. 6.1: Increase of degree of aggregation DA with increasing heating time at 90 °C in 6% α-la solution.

Heating time [min]	DA_{HPLC} [%]	DA_{DSC} [%]	DA_{US} [%]
2	15.12	19.15	17.35
3	14.08	22.01	17.37
6	19.89	31.02	23.47
10	28.9	34.53	34.95
20	37.35	38.02	37.47
25	47.88	43.47	51.15
44	73.84	77.78	78.88
55	87.07	91.11	89.57

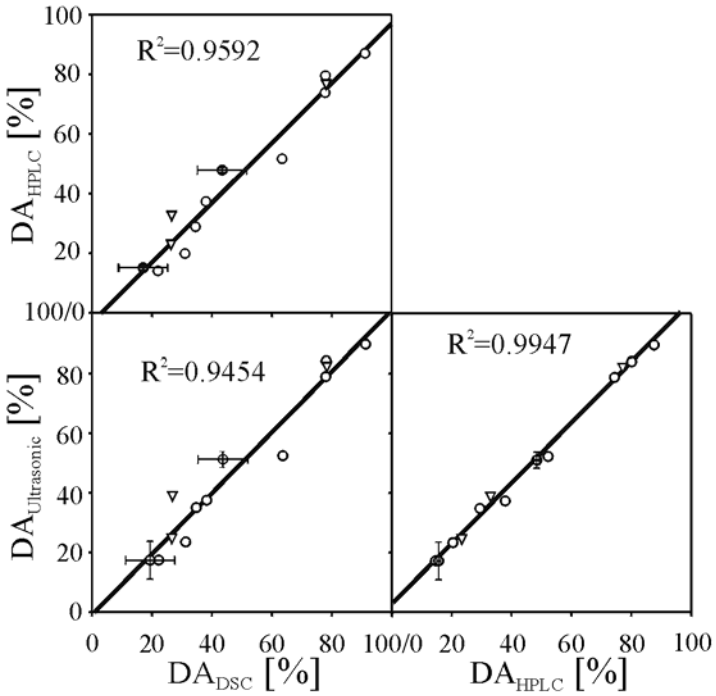


Fig. 6.6: Correlation between degrees of irreversible aggregation of α-la in 6% (circle) and 10% (triangles) α-la solution after heating at 90 °C determined by three different methods. Error bars refer to standard deviation.

Independently of the α-la concentration in the samples, linear correlations are shown between the degrees of α-la aggregation determined by the three methods studied (Fig. 6.6). The DSC method shows the highest standard deviation of these three methods. Manji and Kakuda (1987) also observed a poor reproducibility of DSC measurements for whey protein denaturation in 2-7% protein solutions. At low protein concentrations, the endothermic heat flow is too small

to be accurately detected. Compared to the HPLC method which determines the degree of irreversible aggregation after the heating process, ultrasonic and DSC measurement can be used to follow the conformational change in protein molecules during the heating process.

The velocity constants of α-la aggregation k calculated from the linear regression of the degree of aggregation depending on heating time were $(5.59 \pm 0.49)\cdot10^{-4}$, $(6.19 \pm 0.58)\cdot10^{-4}$ and $(6.24 \pm 0.82)\cdot10^{-4}$ s^{-1} for the HPLC, ultrasonic and DSC methods, respectively. The P-value α of the F-test in the ANOVA analysis to compare k values from the three methods was greater than 0.05 ($\alpha = 0.61$), i.e., no significant difference exists between the means of the aggregation velocity constants k determined by the three methods at a 95% confidence level.

The results show that the thermal denaturation of α-la causes a decrease in the ultrasonic velocity. This decrease linearly correlates with the native α-la concentration in the sample and therefore can be used to quantify the degree of aggregation of α-la. The degrees of aggregation determined by HPLC, DSC and ultrasound correlate linearly and they provide similar velocity constants of α-la aggregation without a significant difference, despite the fact that these methods are based on different principles. Both DSC and ultrasonic methods follow heat-induced changes in protein molecules during the heating process, but the ultrasonic method shows a lower standard deviation and a better correlation with the established HPLC method than with the DSC method. Compared to the HPLC method, the DSC and ultrasonic methods are less sensitive for samples with low native protein concentrations, because at low protein concentrations the overall change in thermal energy and compressibility comes close to the detection limit of the methods.

The ability of ultrasonic method to characterise of the denaturation process of α-la allows this method to be applied for most globular proteins, which undergo a change in compressibility due to conformational changes during heat treatment. This method appears to be a useful additional method for investigating the thermal denaturation of globular proteins.

In summary, the HPLC method measures the amount of remaining native α-la, which is not irreversibly aggregated during the preheating of the samples, while the DSC and ultrasonic methods measure the thermal and compressional changes in the sample induced by the thermal denaturation process, respectively. Therefore, in contrast to the HPLC methods, the DSC and ultrasonic methods assess the degree of irreversible aggregation indirectly. In the ultrasonic measurements, the structure and size of the protein aggregates, affected by different milieu conditions, may influence the ultrasonic velocity. The larger the aggregates formed during the heating process, the more ultrasound is scattered, which leads to changes in the

ultrasonic velocity. Furthermore, the extensive aggregation leads to inhomogeneity of the sample. These changes may affect the determination of the degree of aggregation. The application of the ultrasonic method may be restricted, if large aggregates form in the sample during heat treatment. Further work is required to assess the ultrasonic method in more detail with regard to compositional and processing variables in heating experiments which may lead to different α-la aggregate structures.

5.4.2 Denaturation of egg proteins

In this chapter, the applicability of ultrasound on a complex protein mixture should be evaluated by measuring the denaturation behaviour of egg white and yolk proteins. Egg white contains different proteins. Most of them are globular proteins. Proteins in egg yolk are mostly lipoproteins.

5.4.2.1 Denaturation of egg white proteins

Fig. 6.7 shows the ultrasonic velocity difference (Δv) and attenuation difference ($\Delta(\alpha/f^2)$) between 4% egg white protein solution (diluted using 0.5% NaCl) and a reference (0.5% NaCl) as a function of temperature in an egg white solution with 4% total protein. Up to about 58 °C Δv decreases linearly, which is a temperature effect. After that Δv shows a two-stage steeper decrease. This indicates two successive heat-induced processes in the proteins. Beginning from 78°C the slope of the curve becomes lower again, which indicates the completion of the second change.

The ultrasonic attenuation difference $\Delta(\alpha/f^2)$ decreases slightly up to about 58 °C. This decrease may be caused by the decreased viscosity with increasing temperature. After 58 °C $\Delta(\alpha/f^2)$ shows a two-stage increase. As in the case of Δv, this indicates again two heat-induced changes of the proteins in the temperature range investigated.

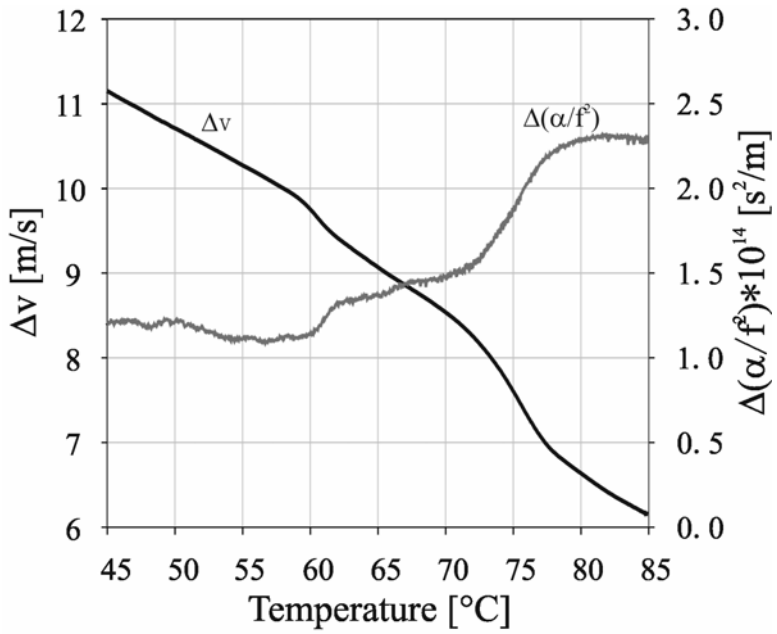


Fig. 6.7: Ultrasonic velocity and attenuation difference between a 4% egg white protein solution in 0.5% and a reference (0.5% NaCl) depending on the temperature during heating by a rate of 0.3 °C/min.

Bae et al. (1998) found that the isothermal denaturation of egg white at temperatures above the gelation temperature of conalbumin, which means to heat the egg white to a certain temperature and hold at this temperature until no changes in ultrasonic parameters are observed, leads to a decrease in ultrasonic velocity and an increase in attenuation. The denaturation reaction of protein is a function of both time and temperature. Both the isothermal process at a constant temperature and the heating process with a constant heating rate lead to a progressive denaturation of proteins. Therefore, our results actually agree well with those of Bae et al. (1998) that the protein denaturation results in a decrease in ultrasonic velocity and an increase in ultrasonic attenuation.

In order to show the changes more clearly, the Δv was differentiated. The course of $\Delta(\alpha/f^2)$ over temperature was less smooth, as it shows in Fig. 6.7. Therefore, we focus on the ultrasonic velocity, in order to achieve a more accurate quantitative analysis.

Fig. 6.8 shows the differentiated Δv, i.e., d(Δv)/dt as a function of the temperature in egg white solutions with different protein concentrations. All samples show two peaks with peak maxima at about 60 °C and 75 °C respectively. Donovan et al. (1975) investigated the heat denaturation of egg white proteins using DSC. They found two endothermic peaks between 50°C and 100 °C using a heating rate of 2-10 °C/min. The first is due to denaturation of conalbumin, the second to denaturation of ovalbumin. The two peaks in the ultrasonic measurement (Fig. 6.8) seem to be correlating with the denaturation of these two egg white

proteins, too. The temperatures at the peak maxima of their measurement were higher than that found in our ultrasonic measurement. This is caused by the much lower heating rate used in our measurement.

By plotting the total decrease of the ultrasonic velocity and increase of attenuation derived from individual isothermal experiments at different temperatures against the temperature, Bae (1996) found two inflexion points for both ultrasonic velocity and attenuation change over temperature at 55 °C and 75 °C relating to the denaturation of conalbumin and ovalbumin, respectively. These inflexion points correspond to the peak maxima in the ultrasonic measurements (Fig. 6.8). The deviation of the denaturation temperature of 60 °C for conalbumin from our measurements may be caused by the origin of the egg white proteins. Bae (1996) used the crystallized and lyophilized samples of ovalbumin and conalbumin, which were dissolved in distilled water, while diluted fresh egg white was used in our investigation.

The peak areas were calculated using Mathcad Professional 2001i and plotted against the respective egg white protein concentration (Fig. 6.9). The areas of both peaks linearly increase with increasing protein concentration. Therefore, the ultrasonic measurement can be used to quantify the heat-induced change in protein solution.

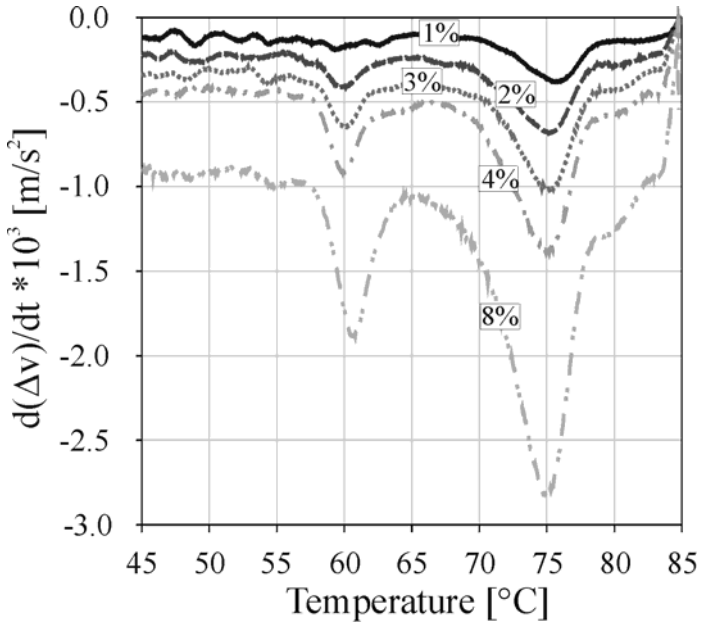


Fig. 6.8: differentiated ultrasonic velocity difference of egg white protein solution of different concentrations during heating by a rate of 0.3 °C/min.

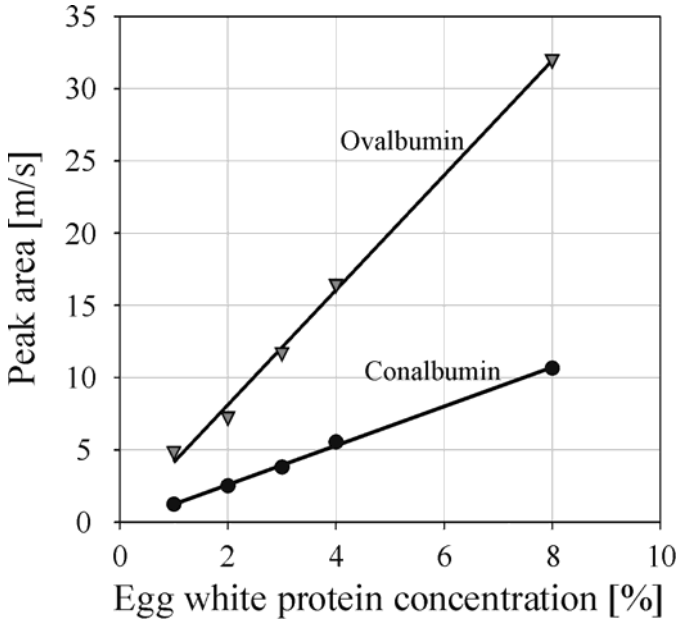


Fig. 6.9: The peak area of the conalbumin and ovalbumin in Fig. 3 depending on the egg white concentration. $R^2 = 0.9992$ for conalbumin and $R^2 = 0.9984$ for ovalbumin.

Protective effect of suagr on the protein denaturation

Fig. 6.10 shows the differentiated ultrasonic velocity difference of a 2% egg white protein solution with different sugar concentrations during heating. The conalbumin and ovalbumin peaks shift to higher temperature with increasing sugar concentration, which indicates that the sugar stabilized the egg white proteins against denaturation.

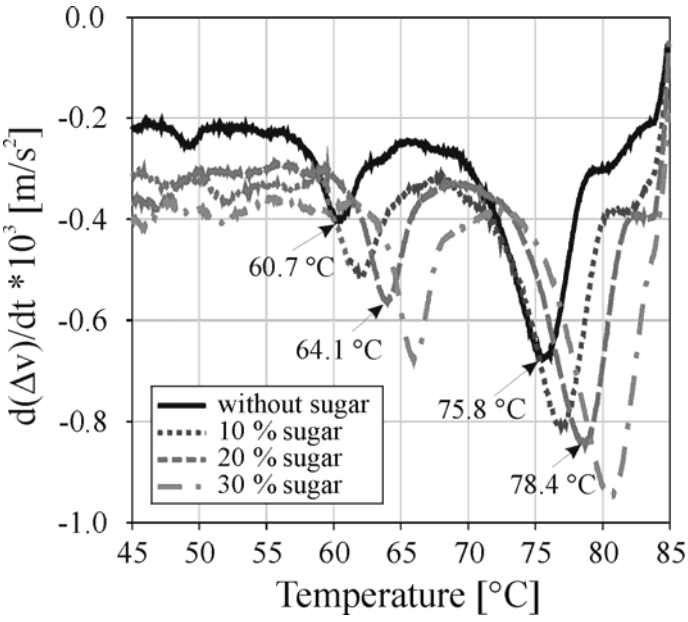


Fig. 6.10: Differentiated ultrasonic velocity of 2% egg white protein solution with different sugar concentrations during heating by a rate of 0.3 °C/min.

Sugar is considered as a stabilizer for the globular proteins. Simpson and Kauzmann (1953) observed that in presence of saccharose the denaturation of ovalbumin in urea decreased. Back et al. (1979) found that the temperature, at which the denaturation rate of ovalbumin is the highest, increases with increasing saccharose concentration. As described in chapter 2.6.3, Arakawa and Timasheff (1982) explained the stabilising effect of sugar on proteins using the preferential hydration theory. Addition of these sugars to an aqueous solution of the protein causes preferential hydration of protein, which results in an increase in the free-energy, and therefore, a thermodynamically unfavourable state. This effect increases with an increase in protein surface area. Therefore, the denatured form of proteins is unfavourable due to their larger surface area compared to the native form. This explains stabilizing effects of sugars on proteins, which retard the unfolding of protein. On the other side, the aggregation of the unfolded protein molecules can be enhanced by the addition of sugar, since the formation of contacts between the protein molecules decreases the total surface area of the protein and hence their chemical potential change per monomeric unit.

Fig. 6.11 shows a DSC thermogram of a 2% egg white protein solution without and with 20% sugar. The DSC thermograms also show two peaks. However, the peaks are less pronounced than those in the ultrasonic measurement. This suggests that the ultrasonic method appears to be more sensitive for the measurement of unfolding of egg white proteins. The maxima of the endothermic peaks are at similar temperatures as those in the ultrasonic measurement. This indicates that the thermal changes during the egg white protein denaturation were accompanied with the compressibility changes of the proteins. It can be seen in Fig. 6.11 that the peaks in the sample with 20% sugar are smaller than that without sugar. This can also be explained by the increased aggregation of unfolded protein molecules caused by addition of sugar. In contrast to the endothermic unfolding process, the aggregation process is an exothermic process. The addition of sugar enhances the aggregation of unfolded protein molecules. Therefore, the area of the endothermic peak of the sample with 20% sugar is smaller than that without sugar (Fig. 6.11).

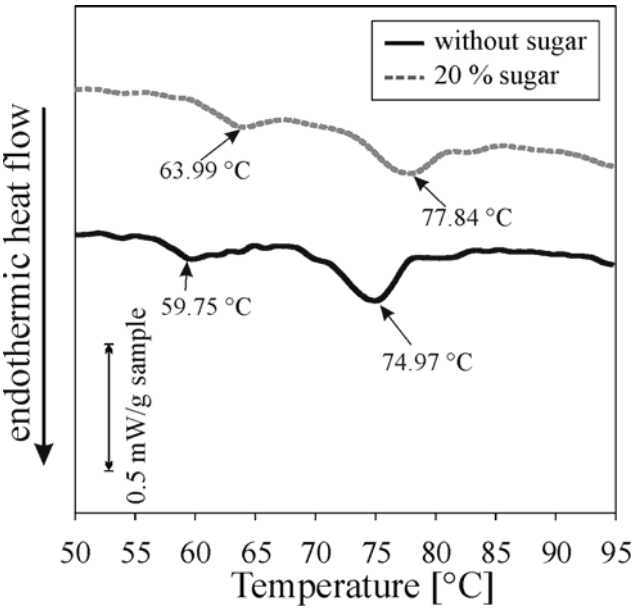


Fig. 6.11: DSC thermogram of 2% egg white protein solution with and without sugar. The heating rate was 0.03 °C/min.

For the ultrasonic measurements, the areas of the conalbumin and ovalbumin peaks in 2% egg white protein solution with different sugar concentrations were calculated and plotted against sugar concentration (Fig. 6.12). Both the conalbumin and the ovalbumin peak areas linearly increase with increasing sugar concentration. The addition of sugar seemed to increase the compressibility change of egg white proteins during denaturation. The larger the peaks, the more the ultrasonic velocity decreases during the denaturation processes. The increase in the d(Δv)/dt peak area with increasing sugar concentration may be caused by the enhanced aggregation due to the sugar addition resulting in a decrease in hydration level. Back et al. (1979) supposed that the sugar enforces the hydrophobic interaction under the hydrophobic groups of the proteins. In this way, the hydration degree decreases and results in a decrease of the ultrasonic velocity. This may be the reason for the increased peak area of conalbumin and ovalbumin.

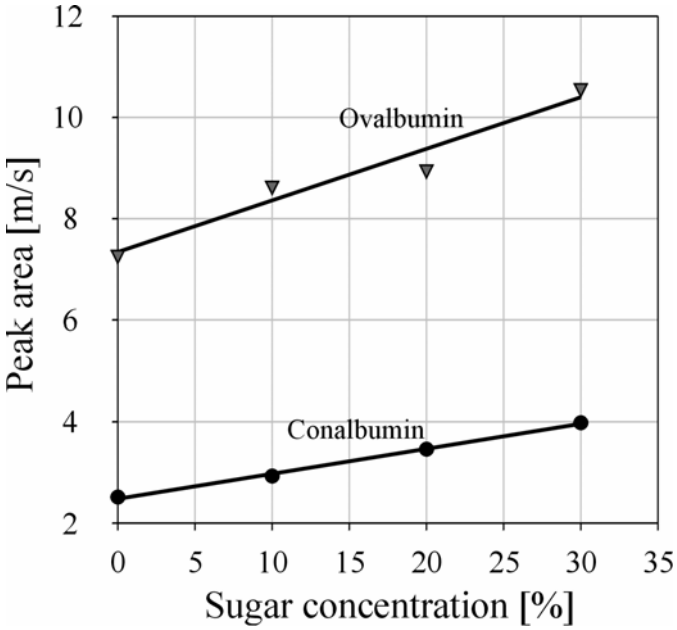


Fig. 6.12: The peak area of conalbumin und ovalbumin in the ultrasonic measurements depending on the sugar concentration in 2% egg white protein solution. $R^2 = 0.9985$ for conalbumin and $R^2 = 0.9736$ for ovalbumin.

5.4.2.2 Denaturation of egg yolk proteins

Fig. 6.13(a) shows the temperature dependence of the ultrasonic velocity difference Δv between egg yolk plasma, granules and complete egg yolk dispersions with the same total protein content (2.4%) in 3% NaCl and the reference (3% NaCl). In 3% NaCl, both plasma and granules are soluble. In contrast to all other measurements in this work, the Δv in dispersions of egg yolk and its fractions is negative. This means, despite of the higher solid content in the sample than in the reference (which was also used to dilute the sample) the ultrasonic velocity in the samples is lower than that in the reference. This may be caused by the high lipid content in the egg yolk. It is known that, in the temperature range investigated, the ultrasonic velocity in most oils is lower than that in Water. The protein/lipid ratios in plasma, granule and complete egg yolk are 25:73, 64:31 and 31:65, respectively. As the samples are adjusted to the same protein concentration, the lipid as well as the dry matter content in the samples is in the order: plasma > complete egg yolk > granules. The plasma dispersion has the highest lipid content and therefore the lowest ultrasonic velocity.

The granule dispersion shows the highest and the plasma the lowest Δv over temperature. For the same protein concentration, the temperature dependency of Δv increases in the order: plasma < complete egg yolk < granules. The different temperature dependencies of the samples are caused by their different compositions. The higher the lipid content in the sample, the stronger Δv depends on the temperature. It is known that the ultrasonic velocity has a

positive temperature coefficient in water and a negative one in almost all other liquid inclusive oil (Povey, 1998). The more lipids are in the dispersion, the more the ultrasonic velocity decreases with increasing temperature.

The attenuation of egg yolk plasma, granules and complete egg yolk dispersions with 2.4% total protein during heating is presented in Fig. 6.13(b). The plasma dispersion shows the highest and the granule dispersion the lowest attenuation over the whole temperature range. This agrees with the order of the dry matter content in the samples. The more solute is in the sample, the more the ultrasonic energy attenuated. In all samples the ultrasonic attenuation decreases with increasing temperature up to 70°C, and then increases in all samples (Fig. 6.13(b)), which is a result of aggregation. The increase in the plasma is the largest, followed by the complete egg yolk and granule dispersion. This indicates that the extent of aggregation in the plasma is the highest and that in the granule dispersion is the lowest. This agrees with the state of knowledge in that granule proteins are more resistant to heat than plasma proteins (Le Denmat et al., 1999).

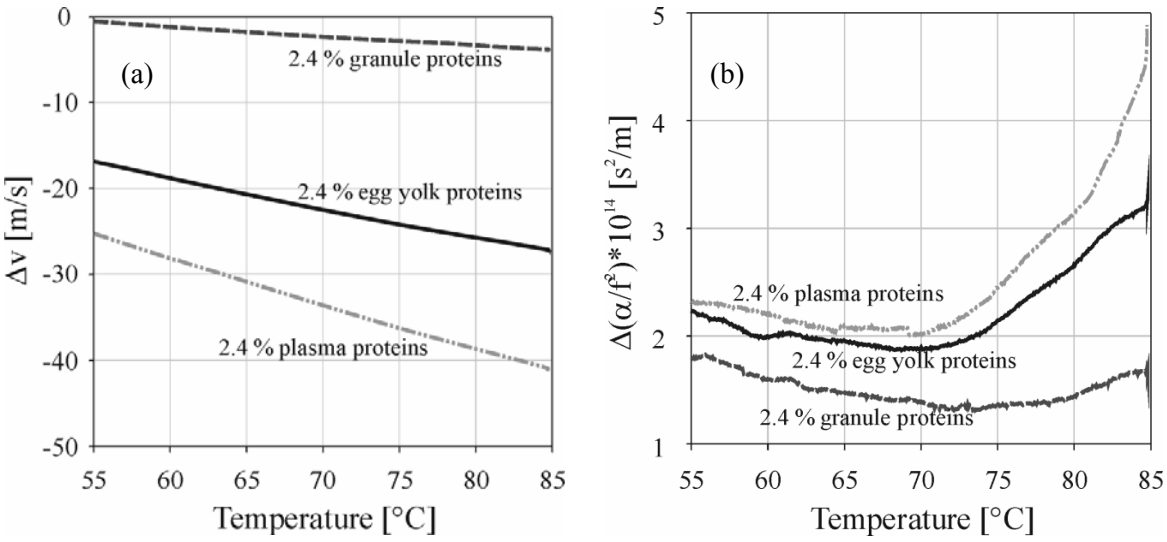


Fig. 6.13: Ultrasonic velocity difference Δv (a) and attenuation difference $\Delta(\alpha/f^2)$ (b) in 2.4% plasma proteins, granule proteins and complete egg yolk proteins dispersed in 3% NaCl depending on the temperature by a heating rate of 0.3 °C/min. Reference: 3% NaCl.

Fig. 6.14 shows the first derivative of the ultrasonic velocity (d(Δv)/dt) and attenuation ($d(\Delta(\alpha/f^2)/dt$). The d(Δv)/dt in plasma and complete egg yolk dispersion shows two upward peaks upon temperature increase, while the d(Δv)/dt in granule dispersion shows one downward peak between 70 °C and 85 °C. The $d(\Delta(\alpha/f^2)/dt$, however, shows upward peaks in all three samples. This indicates that the heat-induced change in granule dispersion causes a decrease in ultrasonic velocity and an increase in ultrasonic attenuation. In contrast, the

denaturation of plasma proteins induced an increase in both ultrasonic velocity and attenuation. The results for the denaturation of α-la and egg white proteins show that the protein denaturation normally shows a downside peak in the first derivative of the ultrasonic velocity over temperature. It seems that in the granule dispersion the effect of protein denaturation dominates, while in the plasma and complete egg yolk dispersions the impact of the lipids overcomes that of the protein denaturation. Furthermore, the result in Fig. 6.14 also shows that there were two main heat-induced changes, probably denaturation of two different fractions of proteins, in the plasma and complete egg yolk dispersion, while only one in the granule dispersion. These changes can be more sensitively followed by ultrasonic attenuation than ultrasonic velocity. However, there is no information about the denaturation temperature of egg yolk proteins. Therefore, it is difficult to assign the proteins to the denaturation peaks. Ternes and Werlein (1987) measured the viscosity of egg yolk during heating. They found that the viscosity of egg yolk increases above 65 °C and reaches a maximum at about 74 °C before decreasing slightly and increasing again at temperatures above 78 °C. This course of viscosity over temperature is similar to the ultrasonic data presented in Fig. 6.14. Ternes and Werlein (1987) assumed that the first maximum in the viscosity corresponds to the denaturation of livetins, whereas the second one would be due to the aggregation of LDL and HDL apaproteins. Based on the assumption of Ternes and Werlein (1987), the two peaks in plasma and complete egg yolk dispersions would be the denaturation of livetins and the aggregation of LDL apoproteins respectively, while the one peak in the granule dispersion would be the aggregation of LDL and HDL apoproteins.

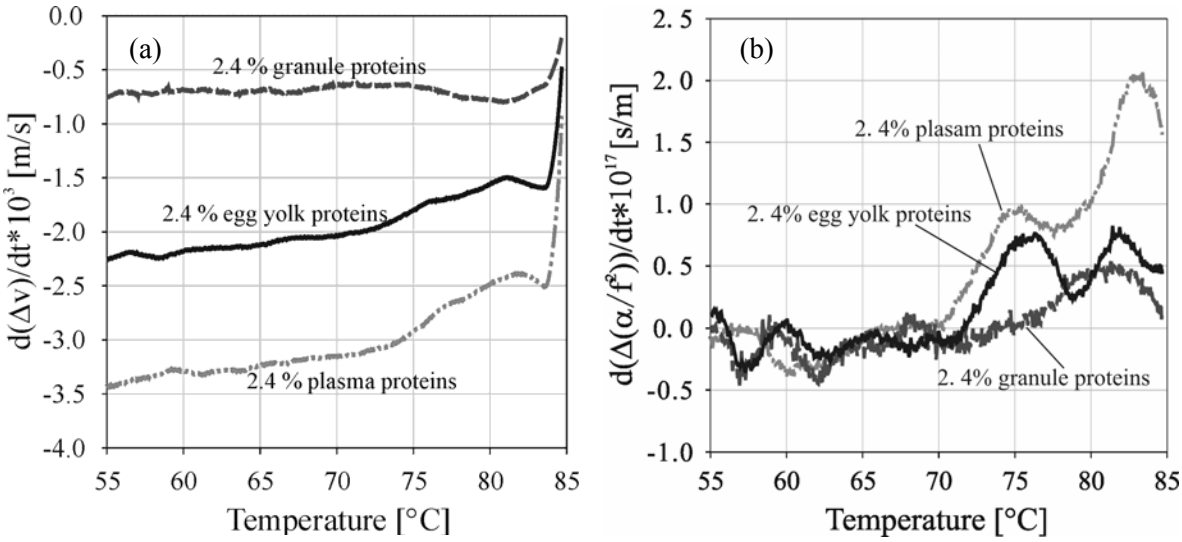


Fig. 6.14: Differentiated ultrasonic velocity difference (a) and attenuation difference (b) in dispersions of plasma, granule and complete egg yolk with 2.4% total protein content depending on the temperature by a heating rate of 0.3 °C/min. Reference: 3% NaCl.

Anton et al. (2000) found that granules disrupted by dispersing in a 0.67 M (4% w/w) NaCl dispersion (24% ± 0.5% dry matter) show a large increase in viscosity from about 70 °C. This is consistent with our ultrasonic measurements, which show an increase in ultrasonic attenuation from this temperature. Anton et al. (2000) suggested that the increase of the viscosity is a consequence of aggregation of soluble proteins of the disrupted granules during heating, which causes a gelation at around 80 °C. The aggregation involves mainly LDL and α-HDL. Two hypotheses were suggested for the mechanism of the aggregation. One of them is that the aggregation of native granules could be caused by unbound calcium that binds to protein during heating. This calcium could be involved in phosphor-calcium bridges between proteins belonging to different granules and cause aggregation. The other hypothesis is that soluble proteins are partly denatured, exposing their hydrophobic sites and causing aggregation of granules through hydrophobic interactions.

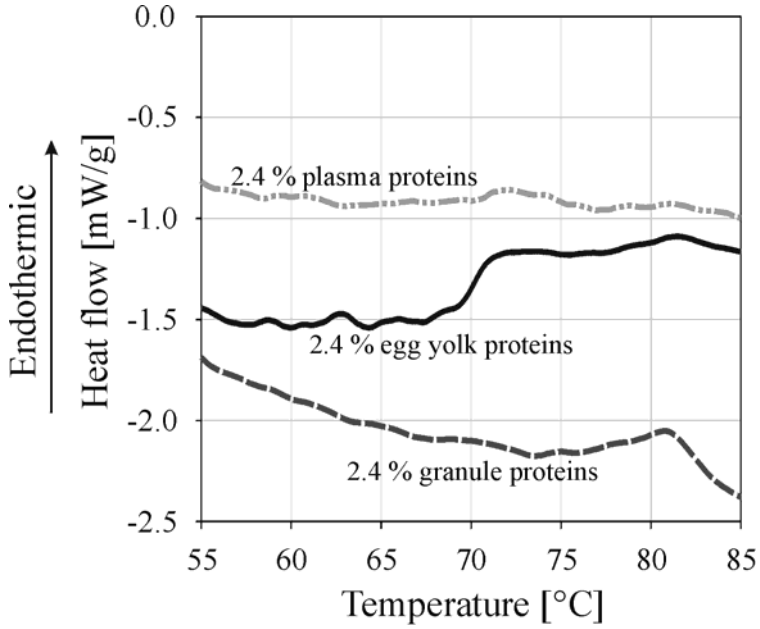


Fig. 6.15: DSC-thermograms of dispersions of plasma, granule and complete egg yolk with 2.4% total protein content depending on the temperature by a heating rate of 0.3 °C/min. Reference: 3% NaCl.

The DSC-thermograms of plasma proteins, granule proteins and complete egg yolk proteins depending on the temperature in Fig. 6.15 show that heating of plasma proteins, of the granule proteins and of the complete egg yolk proteins cause in all samples endothermic peaks relating to the denaturation process of proteins. The peaks in the DSC measurement agree well with those in the ultrasonic measurement and occurred at the similar temperatures as those in the ultrasonic measurement. In their DSC measurement of undiluted egg yolk (heating rate 2 °C/min), Rossi and Shiraldi (1992) observed a broad peak with a maximum at

84 °C and a shoulder between 70 °C and 80 °C. These temperatures agree with the peak temperatures in plasma and complete egg yolk dispersions in our ultrasonic measurement.

The results show that the ultrasonic method is suitable to measure the heat-induced denaturation of protein not only in pure form, but also in a protein mixture. It is even more sensitive than the DSC method.

5.5 Determination of the degree of lactose hydrolysis

In order to assess the applicability of ultrasound in monitoring of enzymatic lactose hydrolysis, the ultrasonic velocity and attenuation were measured during lactose hydrolysis in a milk serum (UF-permeate, $c_{Lactose}$ = 4.6 g/l) using different concentrations of lactase. The degree of hydrolysis was determined by HPLC. The correlation between the ultrasonic velocity and the degree of hydrolysis was investigated.

The ultrasonic velocity difference Δv is plotted against the incubation time in Fig. 6.16. It can be seen that the ultrasonic velocity increases with increasing incubation time, which is caused by the hydrolysis of lactose. Due to the hydrolysis process, lactose is separated to glucose and galactose, which leads to a larger area accessible to the solvent water. The amount of hydrated water in the sample increases causing an increase in ultrasonic velocity. With increasing lactase concentration the ultrasonic velocity difference Δv changes faster. This indicates a faster hydrolysis rate at higher lactase concentration.

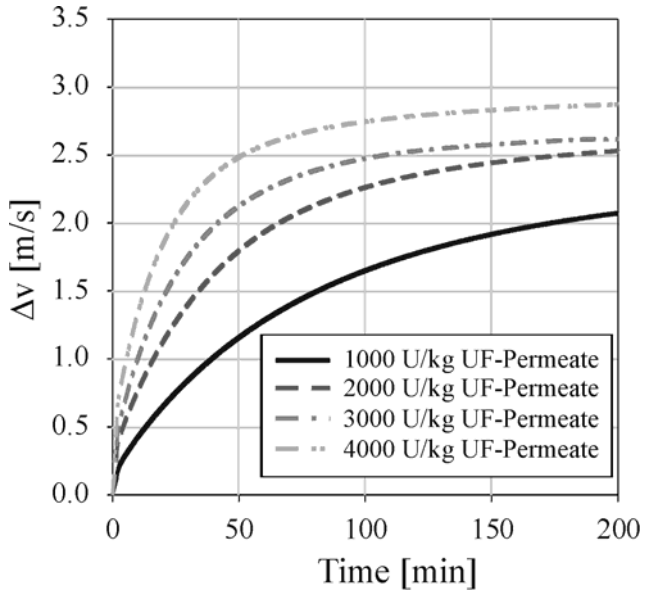


Fig. 6.16: The change of ultrasonic velocity as a function of the incubation time during lactose hydrolysis in milk serum $c_{Lactose}$ = 4.6 g/l at 37 °C. The enzyme concentration is referred to 1 kg milk serum (UF-permeate).

In contrast to ultrasonic velocity, the attenuation remains almost constant over the whole hydrolysis time (Fig. 6.17), because both changes in the sample viscosity and in the volume of the solute (particle size) due to the hydrolysis are negligibly low.

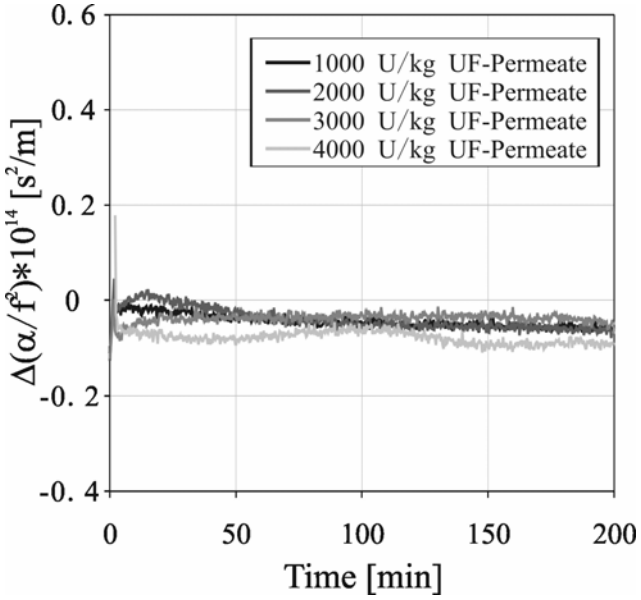


Fig. 6.17: The change of ultrasonic attenuation as a function of the incubation time during lactose hydrolysis in milk serum ($c_{Lactose}$ = 4.6 g/l) at 37 °C. The enzyme concentration is referred to 1 kg milk serum (UF-Permeate),

To compare the change of ultrasonic velocity with the degree of hydrolysis of lactose, a hydrolysis experiment parallel to the ultrasonic measurement was performed in a water bath using the same condition as that for the ultrasonic measurement. The enzymatic reaction was stopped by heating at 85°C for 5 min. The degrees of hydrolysis in the samples were determined by HPLC.

The degree of hydrolysis determined by HPLC was plotted against the Δv at the same incubation time (Fig. 6.18). There is a linear correlation between the degree of hydrolysis and the ultrasonic velocity difference for measuring points of samples for all lactase concentrations. The relationship of the degree of hydrolysis and the ultrasonic diffenrence Δv can be described by the linear equation: Degree of hydrolysis = -0.0176 + 0.3659 · Δv, with R^2 = 0.9843. The prediction limits in Fig. 6.18 presents the limits for the forecast. It is estimated that 95% of additional measuring points would fall within these limits. The confidence limits provide 95% confidence intervals for the mean value of the degree of hydrolysis at any selected ultrasonic velocity defference Δv.

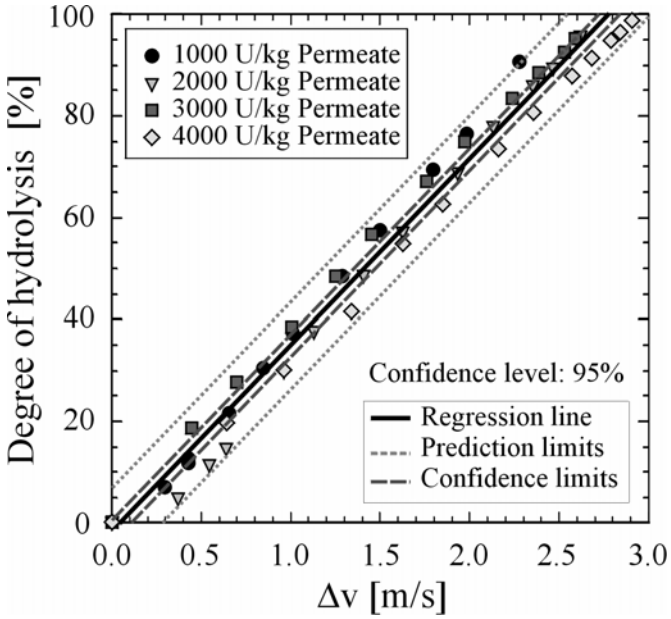


Fig. 6.18: Correlation between the degree of lactose hydrolysis in milk serum ($c_{Lactose}$ =4.6 g/l) and the change in ultrasonic velocity for all measuring points in samples with different lactase concentrations.

The change of ultrasonic velocity by up to 3 m/s during the lactose hydrolysis is relatively high compared to that during the gelation process. This makes it more viable to apply the ultrasonic method as a technique for process control during lactose hydrolysis with the help of a calibration curve. However, it is to be noted that ultrasonic velocity strongly depends on temperature. Therefore, calibration is necessary for each process temperature applied. Furthermore, variations in the process conditions may become a potential source of error. An exact measurement is only viable either by using a well thermostatically controlled measuring cell in the bypass or by compensating the effect of temperature fluctuation, if a priori information is available for the temperature dependency of ultrasonic velocity.

6 Conclusions

The following key conclusions can be drawn from the results obtained:

- **Ultrasonic velocity is sensitive for the detection of changes on molecules, while ultrasonic attenuation is more sensitive to changes in particle size and molecular interaction.** This can be seen from a comparison of the measurements of lactose hydrolysis and of rennet gelation of casein. During lactose hydrolysis, the enhanced hydration due to splitting of one molecule into two led to an increase in the ultrasonic velocity up to 3 m/s, while the ultrasonic attenuation remained almost constant. During rennet gelation, the variation in ultrasonic velocity caused by the rennet gelation was only less than 10% of that caused by the lactose hydrolysis. However, the aggregation process of casein after CMP hydrolysis induced an increase in ultrasonic attenuation as a result of increasing particle size. Because of the high sensitivity of the attenuation on particle size, the aggregation process could be measured earlier by the ultrasonic method than by the rheological method. The rheological method could not measure the aggregation, until the extent of aggregation resulted in a noticeable change in the viscosity (G'') or elasticity (G').

- **In the gelation process, the ultrasonic method is rather sensitive to the gelling mechanism than the gel properties.** It detects the gelation process by measuring the compressibility or particle size changes during the gelation process. Therefore, its capability on the characterisation of the gelation processes depends on the gelation mechanism of investigated the system. In contrast to the rheological method, which generally measures the firming process of the sample structure during a gelation process (i.e., the increase of G' and G''), the ultrasound responds differently on samples with different gelling mechanism. The gelation of κ-carrageenan and CMP causes a decrease in ultrasonic velocity, the gelation of ι-carrageenan does not cause any change in ultrasonic velocity, while the rennet and acid gelation of milk lead to an increase in ultrasonic velocity. The high frequency low-intensity ultrasound measures the mechanical response of the sample at molecular level, e.g., hydration change on molecules or the association of molecules to a small extent. As soon as the aggregation progresses to a continuous network, the behaviour of the gel network as a whole system will be too large to be detected by the high frequency ultrasound. Therefore, the ultrasonic method cannot be used to characterise the growth of the gel

network and gel firming. It is not sensitive to the gel strength, which is determined by the gel network as a result of interaction and arrangement of many molecules. The rheological method is performed at much lower frequency at a higher wavelength and can detect the behaviours of gel networks much better than the ultrasonic method. Therefore, the rheological method is more suitable for the characterisation of the gel macroscopic structure.

- **The ultrasonic method is a sensitive method to follow the denaturation process of globular proteins both in their pure form and in a mixture.** The denaturation of globular protein induces an increase in the compressibility and a decrease in the ultrasonic velocity. It is expressed as a peak in the first derivative of the ultrasonic velocity change over temperature. The peak area can be used quantitatively to characterise the denaturation process. The peaks obtained from ultrasonic measurements are more distinctive than those in DSC measurements, which indicate the high sensitivity of the ultrasonic method.

7 Summary

To assess the structure of complex systems such as food, different measuring methods are required. Low-intensity ultrasound as a non-invasive method has been attracting the attention of food scientists since the last several years. It has many advantages, such as a simple construction of the measuring equipment, capability to measure opaque samples, and ease of integration with other sensor modalities. However, due to the complexity of food matrix systems much information about the ultrasonic properties depending on the food structure or structure changes is required for the capability of ultrasonic method.

The target of this work was to investigate the responses of ultrasound on different structure changes and to evaluate the capability of the ultrasonic method for characterisation of structure and structure changes in food systems. For this purpose, different model systems were measured using the ultrasonic system ResoScan® at 7.8 MHz in comparison to an appropriate reference method.

First, the ultrasonic properties in a very simple system, i.e., 1-50% (w/w) aqueous solutions of sugars (saccharose and lactose), were investigated. The ultrasonic velocity and attenuation increased with increasing sugar concentration. The ultrasonic velocity showed a maximum in the temperature range investigated (20 to 70 °C). The higher the sugar concentration, the lower was the temperature at the velocity maximum. The attenuation decreased with increasing temperature due to the viscosity decrease. The hydration numbers calculated from the ultrasonic velocities and densities of both the solvent water and the sugar solutions showed that lactose can bind more water compared to saccharose. The investigation of hydration level of sugars may help to understand the protective effect of sugars on microorganisms.

In order to evaluate the responses of ultrasound on the gelling mechanism, different gelling systems were investigated using ultrasonic method in comparison with the established oscillating rheological method: (a) Gelation of different carrageenans and gelatine, which is based on a cold-induced coil/helix transition of the macromolecules followed by aggregation of the helices; (b) Gelation of milk and its components, which is based on the hydrophobic interaction as a result of modification of the molecule surface properties by enzymatic hydrolysis, pH or temperature.

The gelation of κ- and κ/ι-carrageenans induced a decrease in ultrasonic velocity and an increase in attenuation. The decrease in the velocity is supposed to be caused by the coil/helix

transition and the aggregation of the double helix strands, which results in extrusion of water from bound to bulk. However, the gelation of ι-carrageenan could not be detected by the ultrasonic method, which indicates that the gelation mechanism of ι-carrageenan differentiates from that of κ-carrageenan. The extent of aggregation in κ-carrageenan is suggested to be much smaller than in the case of ι-carrageenan. There is a linear correlation between the gelling temperature determined by the ultrasonic and the rheological method. The gelation of gelatine did not cause any change in the ultrasonic velocity, but a decrease in the ultrasonic attenuation. Comparing the ultrasonic and oscillatory rheological method for the measurement of gelation of hydrocolloids, we can conclude that the rheological method generally measures the firming process of the sample structure during a gelation process (the increase of G' and G''), while changes in ultrasonic properties depends on the gelling mechanism of the respective sample.

During the rennet gelation of a 3% casein solution, both the enzymatic and the aggregation phases could be measured by the ultrasonic method, while the oscillatory rheology could not detect the enzymatic phase. The ultrasonic velocity shows a two-stage increase during the renneting process. These two stages correlate with the enzymatic and the aggregation processes, respectively. The first increase is induced by the release of caseinomacropeptide (CMP) into serum inducing an increased total water accessible area and hence an increased hydration level. The second increase is a result of the aggregation induced scattering effect. The ultrasonic attenuation remained almost constant in the enzymatic phase of the renneting. In the aggregation phase, the ultrasonic attenuation increases due to the increase of the particle size. There was a linear correlation between the coagulation times determined by the ultrasonic and rheological method for the casein solutions treated at UHT conditions with different time/temperature combinations. However, the ultrasonic method was able to detect the aggregation phase earlier than the rheological method.

Additionally, the acid gelation of pasteurised milk induced by the addition of glucono-δ-lactone (GDL) and by fermentation using yoghurt culture was investigated. Immediately after the GDL addition, both the ultrasonic velocity and attenuation increase upon time due to the calcium dissociation from the casein micelle into serum. The hydrolysis of GDL alone was found to contribute to the increase of the ultrasonic velocity, too. However, it did not affect the ultrasonic attenuation. No characteristic changes in the ultrasonic parameters were found at the gel point determined by the rheological method. The ultrasonic parameters seemed to be rather sensitive to the changes in the ionic balance in casein micelles and serum than to the

gelation process. Similar to GDL induced milk gelation, the ultrasonic parameters during yoghurt fermentation also change immediately after the addition of the yoghurt culture.

The gelation of CMP occurs only at lower pH and higher temperature, where the hydrophobic interaction is strong. Due to the aggregation and gelation of CMP molecules, the ultrasonic velocity decreases and the ultrasonic attenuation increases. The lower the pH and the lower the glycosylation degree of the CMP, the lower the necessary temperature for gelation. This is because the hydrophobic interaction between CMP molecules becomes stronger with decreasing pH and glycosylation degree of CMP. The decrease of ultrasonic velocity in CMP solutions with decreasing pH indicates that the hydration degree of CMP molecule decreases with decreasing pH.

The different responses of ultrasound on different gelling systems show that the ultrasonic method is not as specific for the firming process of gelation as the rheological method. It can differentiate gelling mechanism on molecular level, probably the change of molecular hydration during sol/gel transition and the extent of aggregation.

Furthermore, the applicability of the ultrasonic method for the characterisation of protein denaturation was evaluated. Hence, α-lactalbumin (α-la) is chosen as an example of protein in pure form and egg proteins as an example for protein mixture. The heat-induced denaturation of these proteins was investigated by the ultrasonic method using a temperature scan up to 85 °C by a heating rate of 0.3 °C/min in comparison to DSC and HPLC. Generally, denaturation of globular proteins induces a decrease in the ultrasonic velocity and an increase in attenuation, which is a result of the unfolding and aggregation of protein molecules. The denaturation process of a protein exhibits as a peak in the first derivative of the ultrasonic velocity ($d(\Delta v)/dt$) and attenuation ($d(\Delta(\alpha/f^2))/dt$) over temperature.

In case of α-La, a subsequent cooling process after heating shows a peak with a smaller peak area than that during heating, which corresponds to the refolding of α-la and indicates the partly reversible denaturation of α-la. The peak area during heating process is a linear function of the concentration of native α-la. Based on this relationship, a quantitative determination of the native α-la concentration is possible. By comparing the degrees of aggregation in α-la solutions heated at 90 °C for various times using ultrasonic, DSC and HPLC methods, a linear correlation was found between the degrees of aggregation determined by all these three methods.

Heating of egg white solution results in two peaks in the first derivative of ultrasonic velocity between 55 °C and 65 °C as well as 65 °C and 80 °C, which correspond to the denaturation of

ovalbumin and conalbumin, respectively. The peak maxima temperatures agree with those of the endothermic peaks from the DSC measurements. The peak area correlates linearly with the protein concentration. Addition of sugar leads to a shift of the peak to higher temperature as a result of the stabilizing effect of sugar and an increase of the peak area as a result of enhanced aggregation of the unfolded protein molecules.

In contrast to all the other systems investigated in this work, ultrasonic velocity in egg yolk dispersions is lower than that in the buffer, which was used to dilute the egg yolk. This is a result of the high lipid content in egg yolk, because ultrasonic velocity in lipids is lower than in water. In complete egg yolk and plasma dispersions denaturation of two main fractions of proteins was detected between 70 °C and 85 °C, which is shown as two peaks in the first derivative of ultrasonic velocity and attenuation, while the heating of granule dispersion induces only one peak within the same temperature range. Due to the complexity of the egg yolk protein composition and lack of information about their denaturation behaviours, it is difficult to assign the protein fractions to the peaks. The results show that ultrasonic method can be used to measure the denaturation of proteins both in pure solution and in mixture of proteins.

Finally, the enzymatic hydrolysis of lactose in milk serum at 37 °C was investigated. The splitting of lactose into galactose and glucose induces an increase in ultrasonic velocity up to 3 m/s due to the enhanced hydration, but no changes in attenuation. There is a linear correlation between the degree of hydrolysis and the increase in ultrasonic velocity induced lactose hydrolysis. The large change in ultrasonic velocity during lactose hydrolysis compared to that detected for example in the gelation processes makes it possible, to apply the ultrasonic method as online sensor for monitoring the hydrolysis process.

In summary, a linear correlation between ultrasonic and established methods was found for the determination of gelation point, degrees of aggregation of globular protein and degree of lactose hydrolysis. Different behaviours of ultrasonic parameters during gelation of different systems point out the different gelation mechanism and microstructure formed, such as the change in the hydration state of molecules during gelation. Although not all measured responses in ultrasonic parameters can be explained in detail at this stage, there is no doubt that the ultrasonic method is very sensitive to the hydration of molecules, which is difficult to be detected by any other method, as proven during the hydrolysis of lactose and GDL as well as the cleavage of CMP from casein micelle. The information about molecular hydration can help to understand and differentiate the mechanism of the microstructure formation.

8 Kurzfassung

Um die Strukturen komplexer Systeme, wie sie in Lebensmitteln vorkommen, zu erfassen, sind oft unterschiedliche Methoden notwendig. Der niederenergetische Ultraschall hat als eine nichtzerstörende Methode in den letzten Jahren immer häufig das Interesse von Lebensmittelwissenschaftlern auf sich gezogen. Diese Methode hat viele Vorteile, z.B. ein einfacher Aufbau der Messinstrumente, eine Anwendbarkeit für opake Proben und leichte Integrierbarkeit in andere Sensorsysteme. Aufgrund der Komplexität vieler Lebensmittel sind für die Anwendung des Ultraschalls jedoch umfangreiche Informationen bezüglich der Abhängigkeit der Schalleigenschaften von den Lebensmittelstrukturen bzw. Strukturänderungen erforderlich.

Ziel dieser Arbeit war es, die Reaktion des Ultraschalls auf unterschiedliche Strukturänderungen zu untersuchen und die Anwendbarkeit der Ultraschallmethode zu evaluieren. Dafür wurden unterschiedliche Model-Lebensmittelsysteme mittels des Ultraschallmesssystems ResoScan® bei 7,8 MHz gemessen und mit einer geeigneten Referenzmethode verglichen.

Zunächst wurden die Ultraschalleigenschaften in einem sehr einfachen System, nämlich 1-50 %ige Zuckerlösungen (Saccharose und Lactose) untersucht. Sowohl die Schallgeschwindigkeit als auch die Schalldämpfung steigen mit zunehmender Zuckerkonzentration an. Die Schallgeschwindigkeit erreicht im untersuchten Temperaturbereich (20-70 °C) ein Maximum. Dabei ist die Maximumstemperatur umso niederiger, je höher die Zuckerkonzentration ist. Die Schalldämpfung sinkt aufgrund der abnehmenden Viskosität mit steigender Temperatur. Die aus der Ultraschallmessung ermittelten Hydratationszahlen zeigen, dass Lactose mehr Wasser binden kann als Saccharose. Die Untersuchung der Hydratationslevel von Zucker könnte helfen, den schützenden Effekt der Zucker auf Mikroorganismen besser zu verstehen.

Um die Reaktion des Ultraschalls auf die Gelbildungsmechanismen zu evaluieren, wurden verschiedene gelbildende Systeme mittels Ultraschall untersucht und mit der bereits etablierten Oszillationsmethode verglichen: (a) Gelbildung von unterschiedlichen Carrageenanen und einer Gelatine, die auf einem Kälte-induzierten Coil/Helix-Übergang der Makromoleküle und einer anschließenden Aggregation der Helices basiert; (b) Gelbildung von Milch und seiner Komponenten, die auf hydrophoben Wechselwirkungen als eine Folge

der Modifikation der Moleküloberfläche durch enzymatische Hydrolyse, Temperatur oder pH basiert.

Die Gelbildung von κ- and κ/ι-hybrid-carrageenan führt zu einer Abnahme der Schallgeschwindigkeit und einer Zunahme der Schalldämpfung. Die Abnahme der Schallgeschwindigkeit wird vermutlich durch den Coil/Helix-Übergang und die Aggregation der Doppelhelices verursacht, die in einer Verdrängung des an den Molekülen gebundenen Wassers resultieren, wohingegen die Gelbildung von ι-Carrageenan nicht gemessen werden konnte. Das deutet drauf hin, dass sich der Gelbildungsmechanismus des κ-Carrageenans von dem des ι-Carrageenans unterscheidet. Das Ausmaß der Aggregation ist bei κ-Carrageenan vermutlich viel kleiner als bei ι-Carrageenan. Zwischen den Gelbildungstemperaturen aus den Ultraschallmessungen und den rheologischen Messungen besteht eine lineare Korrelation. Die Gelbildung von Gelatine verursacht keine Änderung in der Schallgeschwindigkeit, jedoch eine Abnahme der Schalldämpfung. Der Vergleich der Ultraschallmethode mit der oszillationsrheologischen Methode in Bezug auf ihre Anwendbarkeit zur Charakterisierung der Gelbildung von Hydrokolloiden zeigt, dass die rheologische Methode generell den Verfestigungsprozess der Probenstruktur während der Gelbildung misst (die Zunahme von G' und G''). Dagegen hängen die Änderungen der Ultraschallparameter vom Gelbildungs-mechanismus der jeweiligen Probe ab.

Bei der Labgelbildung einer 3 %igen Caseinlösung konnte sowohl die enzymatische Phase als auch die Aggregationsphase mittels Ultraschall detektiert werden. Hingegen konnte die Oszillationsmethode die enzymatische Phase nicht detektieren. Die Schallgeschwindigkeit zeigt einen 2-stufigen Anstieg während der Labgelbildung, die jeweils mit der enzymatischen Phase und der Aggregationsphase korrelieren. Der erste Anstieg ist eine Folge der Freisetzung von Caseinomakropeptid (CMP), die zu einer Vergrößerung der gesamten Moleküloberfläche führt, die sich aus der Summe der einzelnen Moleküle von Para-κ-Casein und CMP ergibt. Dadurch kann sich mehr Wasser an die Moleküle binden, was zu einer stärkeren Hydratation führt. Der zweite Anstieg wird durch den Streuungseffekt der gebildeten Aggregate verursacht. Die Schalldämpfung bleibt während der enzymatischen Phase fast konstant. Erst in der Aggregationsphase steigt sie aufgrund der Zunahme der Partikelgrößen. Zwischen den Koagulationszeiten aus den Ultraschallmessungen und rheologischen Messungen der Labgelbildung von Caseinlösungen, die bei UHT-Bedingungen unter unterschiedlichen Zeit/Temperatur-Kombinationen erhitz wurden, besteht eine lineare Korrelation. Die

Ultraschallmethode konnte die Aggregationsphase früher detektieren als die rheologische Methode.

Des weiteren wurde die durch Zugabe von Glucono-δ-lacton (GDL) oder durch eine Joghurtfermentation induzierte Säuregelbildung pasteurisierter Milch untersucht. Unmittelbar nach der Zugabe von GDL steigt sowohl die Schallgeschwindigkeit als auch die Schalldämpfung aufgrund der Calciumdissotiation aus den Caseinmicellen in das Serum mit zunehmender Reaktionszeit an. Die Hydrolyse von GDL verursacht alleine auch eine Zunahme der Schallgeschwindigkeit, aber keine Änderung in der Schalldämpfung. Beim anhand der rheologischen Messung bestimmten Gelpunkt wurde keine charakteristische Änderung der Ultraschallparameter festgestellt. Die Ultraschallparameter sind anscheinend eher sensitiver bezüglich einer Änderung in der Ionenbilanz von Caseinmicellen und Serum als gegenüber der Gelbildung. Ähnlich wie bei der GDL-induzierten Milchgelbildung ändern sich die Ultraschallparameter bei der Joghurtfermentation auch sofort nach Zugabe der Joghurtkultur.

Die Gelbildung von einer 5%igen CMP-Lösung tritt nur bei niedrigem pH und hoher Temperatur, bei der die hydrophoben Wechselwirkungen stark sind, auf. Aufgrund der Aggregation und Gelbildung der CMP-Moleküle nimmt die Schallgeschwindigkeit ab und die Schalldämpfung zu. Je niedriger der pH und der Glykosylierungsgrad von CMP sind, desto niedriger ist die für die Gelbildung benötigte Temperatur. Der Grund dafür ist, dass die hydrophoben Wechselwirkungen zwischen den CMP-Molekülen mit sinkendem pH-Wert und Glykosylierungsgrad stärker werden. Die Schallgeschwindigkeit in der CMP-Lösung nimmt mit sinkendem pH-Wert ab. Das deutet darauf hin, dass der Hydratationsgrad von CMP-Molekülen mit sinkendem pH-Wert abnimmt.

Weiterhin wurde die Anwendbarkeit des Ultraschalls für die Charakterisierung der Proteindenaturierung evaluiert. Dabei wurde α-Lactalbumin (α-La) als ein Beispiel für Protein in reiner Form und Eiproteine als Beispiele für Proteine in einer Mischung gewählt. Die Denaturierung dieser Proteine während einer Erhitzung wurde bei einer Temperaturrampe von 0,3 °C/min bis zu 85 °C mittels Ultraschallmethode untersucht. Die Ergebnisse wurden mit denen aus der DSC- und HPLC-Methode verglichen. Im Allgemeinen führt eine Denaturierung von globulären Proteinen zu einer Abnahme der Schallgeschwindigkeit und einer Zunahme der Schalldämpfung aufgrund der Auffaltung und Aggregation der Proteinmoleküle. Der Denaturierungsprozess stellt sich als ein Peak in der ersten Ableitung der Schallgeschwindigkeit ($d(\Delta v)/dt$) und –dämpfung ($d(\Delta(\alpha/f^2))/dt$) über die Temperatur dar.

Bei α-La wurde bei einer anschließenden Abkühlung nach der Erhitzung ein Peak mit kleinerer Fläche im Vergleich zu dem während des Erhitzens festgestellt. Dieser Peak entspricht der Rückfaltung von α-La. Die Peakfläche beim Erhitzen ist eine lineare Funktion von der Konzentration von nativem α-La. Diese lineare Abhängigkeit ermöglicht eine quantitative Bestimmung der Konzentration von nativem α-La. Die Denaturierungsgrade in den α-La-Lösungen erhitzt bei 90 °C für unterschiedliche Heißhaltezeiten wurden mittels Ultraschall-, DSC- und HPLC-Methode ermittelt. Zwischen den Denaturierungsgraden aus allen drei Methoden wurde eine lineare Korrelation festgestellt.

Bei der Erhitzung der Eiweißlösung zeigen sich zwischen 55 °C und 65 °C sowie zwischen 65 °C und 80 °C zwei Peaks in der ersten Ableitung der Schallgeschwindigkeit, die jeweils mit der Denaturierung von Ovalbumin und Conalbumin korrelieren. Die Peakmaximums-temperaturen stimmen mit denen der endothermischen Peaks aus der DSC-Messung überein. Die Peakfläche korreliert linear mit der Proteinkonzentration. Eine Zugabe von Zucker führt zu einer Verschiebung des Peaks zu einer höheren Temperatur als eine Folge dessen stabilisierender Wirkung. Darüber hinaus nimmt die Peakfläche mit der Zuckerkonzentration zu, weil die Aggregation durch Zucker begünstigt wird.

Im Gegensatz zu allen anderen in dieser Arbeit untersuchten Systemen ist die Schallgeschwindigkeit in Eigelbdispersion niedriger als im Puffer, der zum Verdünnen des Eigelbs verwendet wurde. Das ist auf den hohen Lipidgehalt im Eigelb zurückzuführen, da die Schallgeschwindigkeit in Lipiden niedriger ist als die im Wasser. In der Dispersion des kompletten Eigelbs und in der Plasmadispersion wurde die Denaturierung von zwei Hauptproteinfraktionen zwischen 70 °C und 85 °C detektiert, die als zwei Peaks in der ersten Ableitung der Schallgeschwindigkeit und –dämpfung darstellt sind. Hingegen zeigt die Granuladispersion nur einen Peak in dem selben Temperaturbereich. Aufgrund der Komplexität der Eigelbzusammensetzung und dem Mangel an Informationen über ihr Denaturierungsverhalten ist es schwierig, die Proteinfraktionen den jeweiligen Peaks zuzuordnen. Die Ergebnisse zeigen, dass die Ultraschallmethode zur Charakterisierung der Denaturierung von Proteinen, sowohl in reiner Form als auch in einer Mischung, eingesetzt werden kann.

Abschließend wurde die enzymatische Hydrolyse von Lactose in Milchserum (UF-Permeate) bei 37 °C untersucht. Die Spaltung der Lactose in Galactose und Glucose führt aufgrund der verstärkten Hydratation zu einer Zunahme der Schallgeschwindigkeit bis zu 3 m/s, aber zu keiner Änderung in der Schalldämpfung. Die durch Lactosehydrolyse verursachte Änderung

der Schallgeschwindigkeit hängt linear von dem Hydrolysegrad ab. Die relative große Änderung der Schallgeschwindigkeit im Vergleich zu der, die verursacht wird durch z.B. die Gelbildungsprozesse, ermöglicht es, die Ultraschallmethode als Online-Sensor zum Monitoring der Hydrolyseprozesse einzusetzen.

Eine lineare Korrelation zwischen der Ultraschallmethode und den anderen Methoden wurde in Bezug auf die Bestimmung des Gelpunktes, des Denaturierungsgrades von α-La und des Hydrolysegrades von Lactose festgestellt. Das unterschiedliche Verhalten der Ultraschallparameter während der Gelbildung verschiedener gelbildender Systeme deutet auf Unterschiede bezüglich Gelbildungsmechanismen sowie gebildeter Mikrostruktur hin, z.B., die Änderung im Hydratationszustand der Moleküle während der Gelbildung. Obwohl noch nicht alle Änderungen der Ultraschallparameter im Detail erklärt werden konnten, ist es offensichtlich, dass die Ultraschallmethode eine sehr sensitive Methode zur Messung der Hydratation von Molekülen ist, die mit allen anderen Methoden schwierig zu erfassen ist, wie es bei der Hydrolyse von Lactose und GDL sowie bei der CMP-Abspaltung nachgewiesen wurde. Informationen über molekulare Hydratation können dabei helfen, die Mechanismen der Mikrostrukturbildung besser zu verstehen und zu unterscheiden.

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