Formation, structure and rheological properties of soy protein gels

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This study was performed to understand the factors determining heat-induced formation and properties of soy protein gels the relations between gel properties and network structure in order to support application of soy proteins in food products. Three soy protein preparations were used: soy protein isolate, which is a mixture of soy proteins, purified glycinin and a β-conglycinin rich fraction. Glycinin and β-conglycinin are the main proteins in soybeans. Protein denaturation was studied by differential scanning calorimetry. Rheological properties of the soy protein gels were investigated in small and large deformation tests. Information on coarseness of the network structure was obtained by permeability measurements and confocal scanning laser microscopy.

Heat denaturation proved to be a prerequisite for gel formation at all conditions of pH and ionic strength studied. β-Conglycinin gels were formed at temperatures of about 55-70°C and glycinin gels at about 70-95°C. Soy protein isolate gels were formed on heat denaturation of β-conglycinin at pH lower than 6 and on heat denaturation of glycinin at pH higher than 6. On further heating at 90 or 95°C, gels became stiffer, which was explained by further incorporation of protein in the network and, at pH 7 and 7.6, by the occurrence of rearrangements in the network structure. Gel stiffening on cooling was thermoreversibel and did not involve covalent bond formation and rearrangements.

Gel properties like stiffness, fracture behaviour and water holding capacity strongly depend on conditions during gel formation, such as pH, salt concentration, protein concentration, heating conditions and addition of oil droplets. Also the type of protein, glycinin or β-conglycinin, and their mixing ratio affect gel properties. The differences in gel properties could, for a large part, be related to differences in the network structure of the gels. The most important structural characteristics are pore size, thickness and curvature of the strands. Another factor determining gel properties is the amount of protein incorporated in the network. At pH > 5, less protein (mainly acidic polypeptides) participated in network formation than at lower pH values.

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Chapter 1

Introduction

In comparison with other legumes, soybean has a high protein content (~ 40%) and a relatively high oil content (~ 20%) on a dry weight basis (Piper & Morse, 1923). Its high protein level and well-balanced amino-acid composition makes the soybean an important source of proteins, with potential to replace meat and dairy products, if necessary. At the moment less than 1% of the world soybean production is being used for human food, the rest is used for animal feed. In the future, a need for vegetable proteins is expected, especially in areas where population growth is high. Also, the recently accepted health claim that soy protein helps to reduce the blood cholesterol level will increase market growth of soy foods in western countries. On the other hand, some recent studies also show negative health aspects of soy proteins.

To facilitate use of soy proteins in food products, basic knowledge on their functional properties under food conditions is required.

1.1 Soybean

1.1.1 Origin and production

It is widely believed that the soybean originated in China, 4000-5000 years ago. The soybean (*Glycine max* (L.) Merrill) was introduced in Europe in about 1700. However, due to poor climate and soil conditions, soybean production has been limited in Europe (Liu, 1997). In North America, the soybean was first introduced in the 18th century, but a large-scale official introduction did not occur until the early 1900s. By 1920, thousands of new varieties were collected, combines were first used to harvest soybeans, and the first soybean processing plant was opened. As a result, large-scale production had begun (Liu, 1997).

At the moment, the United States of America has become the world leader in soybean production. In 1999, 71 milliard kg of soybeans were produced in the USA which is 46% of the world market. Other countries with high soybean production are Brazil (20%), Argentina (14%), and China (9%). In 1999, largest importers of whole soybeans were Europe and Japan (Anonymous, 2000).

1.1.2 Use and processing

Soybean is mainly cultivated for its seeds. In the Far East, soybeans are used to make traditional soy foods like tofu, tempeh and soymilk, whereas in the West soybeans are crushed into oil and defatted meal. The meal is mainly used as animal feed. A small portion is further processed into food ingredients including, soy flour, concentrates, isolates and textured

protein. These ingredients can be used as emulsifiers, foaming agents, and texture-enhancers in foods or have nutritional value in new soy foods like soy burgers and soy cheese.

The process commonly used for preparation of oil and soybean meal (Procter, 1997) is schematically shown in Figure 1.1. First the beans are cleaned, cracked and dehulled. After a conditioning step at about 70°C, the beans are flaked to improve the subsequent oil extraction by hexane. The remaining hexane is removed in a desolventiser toaster (DT) or in a flash desolventiser system (FDS) (Hettiarachchy & Kasapathy, 1997; De Meester et al., 2000). The heat treatment in a DT is quite harsh and results in darker meals, due to Maillard reactions, with a low solubility in water as indicated by a low Protein Dispersability Index (PDI). Heat treatment in FDS systems is milder and easier to control and results in whiter meals with higher PDI values. The latter meals are preferred for food and fermentation applications (De Meester et al., 2000).

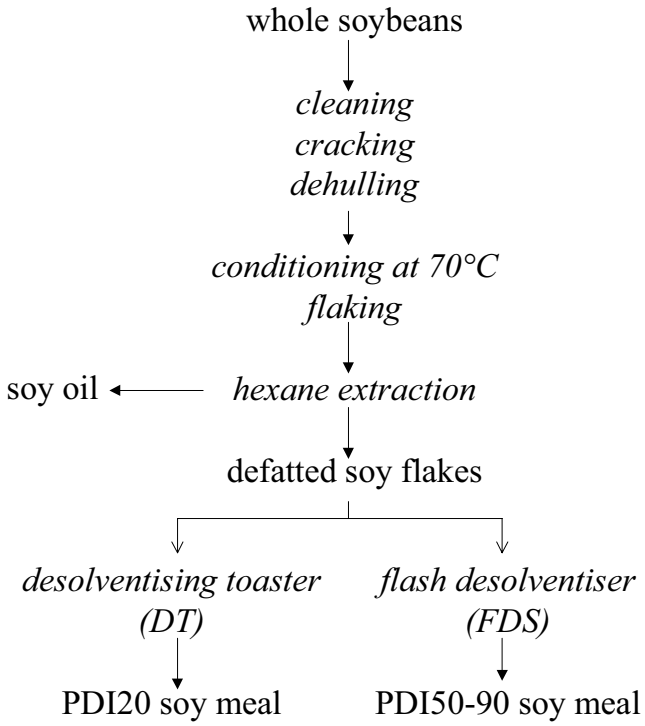

Figure 1.1 Extraction of soy oil and production of defatted soymeal.

1.1.3 Protein composition

Soybean proteins can be divided in albumins (10%), extracted by water, and globulins (90%), extracted by dilute salt solutions (Fukushima, 1991). Soybean globulins consist of four fractions, 2S (15%), 7S (34%), 11S (41.9%) and 15S (9.1%), according to their sedimentation rates when dissolved in a pH 7.6, 0.5 M ionic strength buffer (Koshiyama, 1969). The 11S

and 15S fractions are pure proteins: glycinin and polymers of glycinin, respectively (Wolf, 1970). The 7S fraction is more heterogeneous, the majority is β-conglycinin, but also γ-conglycinin, lipoxygenases, α-amylases and hemagglutenins (or lectins) are found (Nielsen, 1985). The 2S fraction consists of Bowman-Birk and Kunitz trypsin inhibitors, cytochrome C, and α-conglycinin (Catsimpoolas & Ekenstam, 1969; Wolf, 1970).

Glycinin and β-conglycinin are the most important soy proteins. Together with α- and γ-conglycinin, they form the storage proteins, which function as a protein source for the growing seedling. The other proteins have a metabolic function and have to be inactivated by heating to eliminate unwanted and anti-nutritional effects when they are used in human or animal foods. Unfortunately, this heating may also result in a lower solubility of the storage proteins and loss of their functionality.

Glycinin

Glycinin (Figure 1.2) consists of one basic and one acidic polypeptide, which are linked by a single disulphide bond, except for the acidic polypeptide A_4 (Staswick et al., 1984). At least six acidic polypeptides ($A_{1,a}$, $A_{1,b}$, A_2-A_4, and A_5) and five basic polypeptides (B_{1a}, $B_{1,b}$, B_2-B_4) have been isolated (Nielsen, 1984). At ambient temperatures and pH 7.6, glycinin forms hexameric complexes (11S) with a molecular mass of about 360 kDa, while at pH 3.8 it is mainly present as trimeric complexes (7S) with a molecular mass of about 180 kDa. Lowering the ionic strength from 0.5 to < 0.1 also induces dissociation of 11S glycinin into 7S glycinin (Wolf et al., 1958; Lakemond et al., 2000). The isoelectric point of glycinin is 4.90 (Koshiyama, 1983).

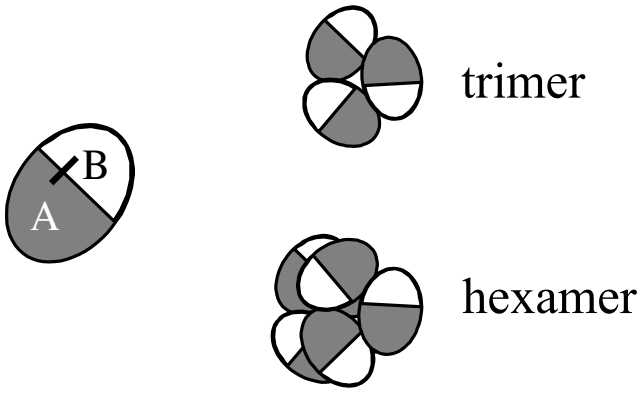

Figure 1.2 Schematic presentation of a glycinin molecule and its trimeric and hexameric complexes. A and B denote the acidic and basic polypeptides, respectively. The small bar connecting A and B represents the disulphide bond.

β-Conglycinin

β-Conglycinin (Figure 1.3) is a trimeric glycoprotein (7S) consisting of three types of subunits, α' (57-72 kDa), α (57-68 kDa), and β (45-52 kDa) (Yamauchi et al., 1991), in seven different combinations (βββ, ββα', βββα, βαα', βααα, ααα', and αααα) (Sykes & Gayler, 1981; Yamauchi et al., 1981). The subunits are associated via hydrophobic and hydrogen bonding without any disulphide bonds (Thanh & Shibasaki, 1978). At pH 5 and higher, β-conglycinin is a trimer (7S) at an ionic strength of 0.5 M, whereas it predominantly exists as a hexamer (9S) at an ionic strength less than 0.1 M. In the intermediate ionic strength region, varying amounts of 7S and 9S β-conglycinin are present (Koshiyama, 1968). At pH 2-5, β-conglycinin reversibly dissociates into a 2-3S and 5-6S fraction at ionic strength less than 0.1 (Wolf, 1972). The isoelectric point of β-conglycinin is 4.64 (Koshiyama, 1983).

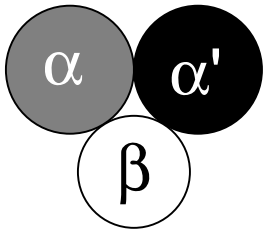

Figure 1.3 Schematic presentation of a β-conglycinin molecule (type βαα').

1.1.4 Protein isolation

In this thesis, three different protein preparations were used: soy protein isolate (SPI), purified glycinin and a β-conglycinin rich protein fraction. SPI is a mixture of soy proteins, which consists mainly of glycinin and β-conglycinin. In Figure 1.4, the isolation procedure for SPI is schematically shown (Hettiarachchy & Kasapathy, 1997). First, defatted soy meal is dispersed at pH 8 to extract the soluble protein and carbohydrates. Then, the protein is precipitated by lowering the pH to 4.8. Consecutively, the protein isolate is dried, or first neutralised before drying, depending on the user's needs.

Glycinin and β-conglycinin were isolated according to a method described by Thanh & Shibasaki (1976), in which both fractions were prepared simultaneously. Again, extraction of the soluble protein and carbohydrates takes place at pH 8. Then, the pH is lowered to pH 6.4. At this pH, most of the glycinin precipitates. Consecutively, the pH of the supernatant, containing β-conglycinin and whey proteins, is lowered to pH 4.8, where β-conglycinin precipitates. The glycinin fraction is quite pure (~95%), whereas the β-conglycinin fraction, used in this study, consists of β-conglycinin (~60%), glycinin (~15-20%) and other proteins (~20-25%) (Renkema et al., 2001).

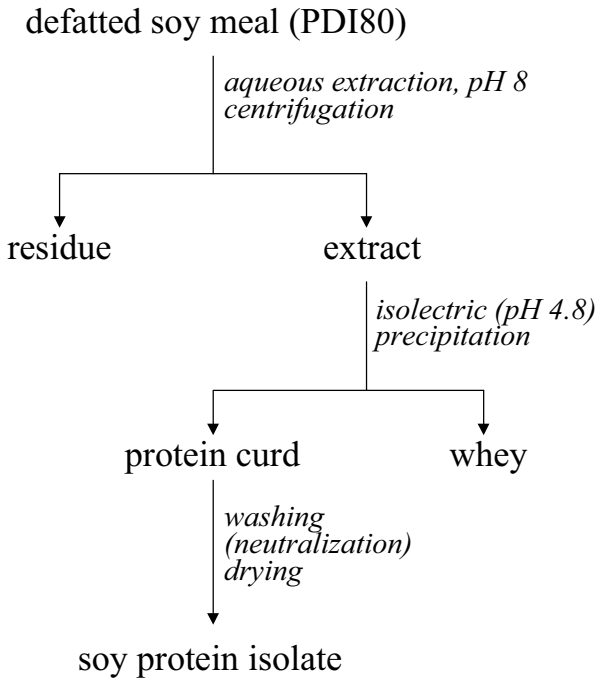

Figure 1.4 Preparation of soy protein isolate from defatted soymeal.

1.2 Gel formation by soy proteins

Soy protein products (flour, concentrates, isolates, and textured soy protein) are applied in virtually every type of food, including bakery, dairy, meat, breakfast cereal, beverages, infant formula, and dairy and meat analogues. They are used in these food systems to increase protein content and to provide desired functional properties such as gelling, emulsifying, water-holding, and fat-absorbing properties (Liu, 2000). Since this thesis deals with the gel forming properties of soy proteins, some general information on gel formation by proteins and methods to describe gel formation and gel properties will be given in this section.

1.2.1 Food gels

A gel is a three-dimensional network in which water is entrapped. In food products, gels provide structure and stability. Moreover, food ingredients such as flavours, sugar, and oil may be hold in the gel network. Food gels are often visco-elastic, which means that they behave like a solid at short time-scales, but more like a fluid at long time-scales. Examples of food gels are cheese, (hard) boiled egg, jelly pudding, and tofu (the most famous soy protein gel). These are so-called stand-up gels. Also, chocolate milk can be regarded as a gel. It

consists of a very weak network, which slows down the sedimentation of cocoa particles during storage, but which is not observed during pouring and drinking. Food gels can be made from polysaccharides (starch, carrageenans, pectin, alginate) and proteins.

Depending on the type of protein that forms the gel, protein gels can be divided in gels of cross-linked, flexible proteins (*e.g.* gelatine, keratin) and gels of protein aggregates, which are formed from low-structured proteins (*e.g.* casein) or globular proteins (*e.g.* ovalbumin, whey proteins, soy proteins). Here, I will focus on gel formation by globular proteins.

1.2.2 Gel formation by globular proteins

Gel formation by globular proteins is a complex process which often involves several reactions such as denaturation, dissociation-association, and aggregation (Hermansson, 1986). Denaturation is the change of a native protein conformation to another, more unfolded conformation, in which functional groups (such as sulfhydryl groups or hydrophobic groups) become exposed. Subsequently, these exposed groups can interact with each other to form aggregates (Wang & Damodaran, 1991). When the protein concentration is high enough, aggregation leads to formation of a gel. At lower concentrations, aggregation leads to precipitation of the protein.

For soy protein isolate, rheological methods showed that the minimal protein concentration, c_o, for gelation is ~6.6% (Bikbov et al., 1979). For globular proteins, this is a normal value, *e.g.* bovine serum albumin at pH 6.3 (c_o = ~6.8%) (Clark & Lee-Tuffnell, 1986), whereas compared to other biopolymers, this value is quite high, *e.g.* agar (c_o = ~0.2%) and gelatine (c_o = ~1.2%) (Clark et al., 1983). For glycinin, the lowest protein concentration required for gel formation is 2.5% at pH 7.6 and ionic strength of 0.5, for β-conglycinin it is 7.5% (Nakamura et al., 1986). However, the method to determine the minimal concentration for gel formation was less accurate in the latter case.

1.2.3 Network structure

The network that is formed upon gel formation by globular proteins can have different structures. Hermansson (1994) divided gel structure roughly into fine-stranded and coarse-aggregated networks (Figure 1.5) based on microscopic observations. Fine-stranded gels may be completely transparent and are composed of strands with a thickness up to a few times the size of a single protein molecule. Coarse gels are non-transparent and are composed of particles with diameters in the range of 100-1000 times a single protein molecule. Intermediate structures containing fine-stranded and coarse structures simultaneously do also exist. The type of gel that is formed depends on conditions during gel formation. In general,

gels become coarser as the pH approaches the isoelectric point or when the ionic strength is increased (Doi, 1993).

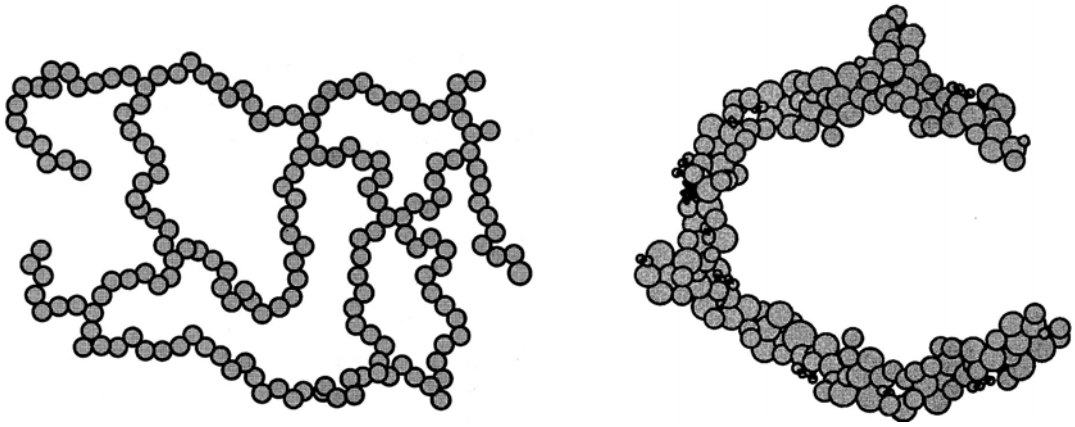

Figure 1.5 Schematic presentation of a fine-stranded (left) and part of a coarse-aggregated network (right).

Mellema et al. (2001) categorised gels based on their mechanical behaviour. They used two parameters: the number of deformable links in a strand and the dominant type of microscopic deformation, *i.e.* bending or stretching. Five categories of gels with specific strand types were distinguished: 1. Random; 2. Curved; 3. Hinged; 4. Straight; and 5. Rigid. The dominant type of deformation of category 1-3 is bending and that of category 4-5 is stretching.

Network structure affects gel properties, including permeability, the ability to retain water, and rheological properties, such as stiffness, fracture strain and fracture stress. The permeability of the gels and the ability to retain water decreases when the network structure coarsens. However, no direct relationship between the coarseness of gels and their rheological properties is observed (Stading & Hermansson, 1991). As a function of strand type, one expects that gel stiffness increases going from category 1 to 5, whereas fracture strain is expected to decrease.

1.2.4 Rearrangements

During and after aggregation of protein particles, rearrangements of the aggregate structure may take place. Protein particles may change their position with respect to each other, *e.g.* by rolling around each other until (most) particles have acquired bonds with two or more particles. This process may lead to the formation of dense aggregates, which are clearly larger than the original particles. These aggregates may aggregate further and form a gel. Extensive rearrangements during aggregation may result in protein precipitation instead of gel

formation (Bremer et al., 1995). Otherwise, it gives a coarser network structure (van Vliet et al., 1997).

After the gel is formed, further rearrangements of the network structure may take place depending on factors such as (heating) temperature, pH, and the presence of salts and specific ions. Particles can fuse resulting in denser and stiffer strands and an increase of protein-protein interactions per cross section. Another type of rearrangements happens at the strand level and involves fracture or yielding of strands leading to regions with high and low densities of protein. This fracture is likely to happen in thin strands or when the average life-time of the protein-protein bonds is short (van Vliet et al., 1991; van Vliet et al., 1997; van Vliet, 2000). These rearrangements will also result in a coarser gel with larger pores and poorer water-holding properties. The permeability of the gel increases and the gel is more sensitive to exhibit syneresis (van Vliet et al., 1997).

1.2.5 Measuring techniques to study gels

Microscopy

The microstructure of gels is mostly studied by electron microscopy, because this technique has a high resolution. Structural elements of the size of about 1 nm can be distinguished. A disadvantage of this technique is the elaborate sample preparation, which can change the original gel structure. Combination of techniques is therefore necessary to detect possible artefacts (Hermansson, 1994).

In this study, confocal scanning laser microscopy (CSLM) was used, which is based on fluorescent light microscopy. This method does not need extensive sample preparation, but only requires addition of a fluorescent dye that binds to the protein fraction. A laser beam is focussed via a pinhole on a spot in the gel. The transmitted fluorescent light is focussed via a pinhole on a detector in which the different grey values are transformed into a microscopic picture. The use of two pinholes improves the resolution in the horizontal plane and in the depth. It makes it possible to view the gel at different depths and to obtain a three-dimensional picture of the gel by combination of the pictures. The resolution of this technique is > 0.2 μm.

Permeability measurements

Information on the microstructure of gels can also be obtained from permeability measurements (van Dijk & Walstra, 1986). In this method a permeability coefficient is determined from the liquid flow through a protein gel. The permeability coefficient is proportional to the square of the pore radius (Bremer et al., 1989), so by this technique a measure of the pore size is obtained. High permeability coefficients indicate that the water holding ability of the gels is low and that the gels are prone to syneresis.

Rheological methods

Rheological measurements determine the relation between the deformation (strain) of a material and the stress applied to achieve such a deformation as a function of time. They can be performed at small and large deformations. At small deformation, the measurement is performed in such a way that the microstructure of the gel is not damaged and that the obtained modulus is independent of the applied strain (*i.e.* in the linear regime). At large deformation measurements, the gels are mostly deformed until macroscopic fracture takes place.

Small deformation measurements are often performed using dynamic mechanical spectroscopy, in which the applied strain (or stress) varies sinusoidally. Parameters obtained from dynamic measurements are the storage or elastic modulus G', which is a measure of the amount of energy that is stored during a periodic application of stress or strain, the loss or viscous modulus G'', which is a measure of the energy loss, and their ratio $tan\ \delta = G''/G'$, which is called the loss tangent. With dynamic measurements the onset of gel formation can be determined and the stiffening of the gel can be followed.

Parameters obtained in large deformation or fracture measurements are stiffness (Young's modulus), fracture stress, fracture strain, and fracture energy (Walstra & van Vliet, 1992). These mechanical properties of gels are the most important ones for practice, *i.e.* during handling, slicing and eating of the gels.

1.3 Aim and outline of the thesis

The aim of this study is to understand the factors determining formation and properties of soy protein gels and to understand the relations between gel properties and network structure in order to support application of soy proteins in food products. Changes in the molecular conformation of soy proteins during gel formation were investigated in a parallel project (Lakemond, 2001). Former work on soy protein gelation was mostly performed with highly purified soy protein fractions at pH 7.6 and ionic strength 0.5, where soy proteins are highly soluble, but which is not a very relevant condition for food products (normally pH 3-7 and ionic strength 0.02-0.2), or it was applied research on ill-defined systems. There is also a lack in knowledge of fracture properties of soy protein gels, whereas these properties are important for food applications.

In chapter 2, heat-induced gelation of laboratory-prepared soy protein isolate (SPI) at neutral pH was studied by small deformation rheology in relation to denaturation. Using different heating temperatures, heating times and heating rates, several stages in the gel formation process were studied, including the onset of gel formation, gel stiffening during

heating by protein incorporation in the network and the occurrence of rearrangements, and the thermoreversibel stiffening of the gel during cooling.

Chapter 3 describes the influence of pH and NaCl concentration on gel formation and gel properties of SPI in relation to denaturation and protein aggregation/precipitation.

Chapter 4 focuses on the relations between rheological properties and network structure of soy protein gels as a function of pH and NaCl concentration. Permeability measurements, CSLM, and large deformation rheology were used to elucidate network structure.

In chapter 5, the role of the two main proteins, glycinin and β-conglycinin, was studied using small and large deformation rheology.

Chapter 6 presents results on the concentration dependence of the dynamic moduli of soy protein gels for different soy protein preparations. Experimental data were compared with existing theoretical models.

In chapter 7 the presence of emulsified oil droplets on gel formation and gel properties was investigated using small and large deformation rheology.

Finally, in the summary the results obtained are outlined and general conclusions are presented.

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Chapter 2

Heat-Induced Gel Formation by Soy Proteins at neutral pH

Abstract

Heat-induced gel formation by soy protein isolate at pH 7 is discussed. Different heating and cooling rates, heating times, and heating temperatures were used to elucidate the various processes that occur and to study the relative role of covalent and non-covalent protein interactions therein. Gel formation was followed by dynamic rheological measurements. Heat denaturation was a prerequisite for gel formation. The gelation temperature (84°C) was just above the onset denaturation temperature of glycinin. The stiffness of the gels, measured as the elastic modulus, G', increased with the proportion of denatured protein. An increase in G' was also observed during prolonged heating at 90°C. This increase is explained by the occurrence of rearrangements of the network structure and probably also by further incorporation of protein in the network. The increase in G' upon cooling was thermoreversibel indicating that disulphide bond formation and rearrangements do not occur upon cooling.

Renkema, J. M. S., & van Vliet, T. *submitted for publication.*

2.1 Introduction

Soy proteins are often used in food products to improve texture. In this respect the most important property is their ability to form a gel with a good water holding capacity upon heating. It is expected that in future soy proteins will play a major role as meat replacer, because of their high nutritional value. However, despite their importance and all research performed, gel formation by soy proteins is still not clearly understood.

Soy protein isolate consists of two major components, β-conglycinin and glycinin, which are also called 7S and 11S globulin. β-Conglycinin is less heat-stable than glycinin; the denaturation temperature of β-conglycinin is about 70°C and that of glycinin about 90°C at neutral pH (Hermansson, 1978). Denaturation is believed to be a prerequisite for gel formation (Kinsella, 1976), so purified β-conglycinin will form a gel at lower temperatures than purified glycinin, which is indeed observed by Nagano et al. (1994a).

Heat-induced gel formation by soy proteins and the molecular interactions involved were studied by various researchers (Babajimopoulos et al., 1983; Utsumi & Kinsella, 1985; Mori et al., 1986; van Kleef, 1986; Wang & Damodaran, 1991; Nagano et al., 1994b; Puppo et al., 1995). Hereto, protein structure destabilizers and stabilizers, such as urea, SDS, β-mercaptoethanol, NaSCN, and NaCl, were used. For soy protein isolate and purified glycinin, it was concluded that disulphide bridges are involved in the gelation process, whereas in β-conglycinin gels they do not play a role. For all three protein systems (glycinin, β-conglycinin and soy protein isolate), non-covalent interactions such as hydrogen bonding and hydrophobic interactions play a role in gelation. It is not clear yet in what stages of gel formation these covalent and non-covalent bonds are important.

In this article, we used a somewhat other approach to study heat-induced gel formation. To elucidate the different processes during gel formation, heating conditions were varied. Dynamic rheological measurements were used to follow gel formation by soy protein isolate at pH 7 and to study if the protein-protein interactions were covalent or non-covalent.

2.2 Material and methods

2.2.1 Preparation of soy protein isolate

Soy protein isolate (SPI) was prepared from mildly treated, defatted PDI80 soy flakes (Cargill BV, Amsterdam, The Netherlands). The flakes were milled in a Fritsch Pulverisette 14702 using a 0.5 mm sieve. Milling was performed in the presence of solid CO_2 (volume ratio flakes:CO_2 is 4:1) to prevent heat denaturation of the proteins. The flour was suspended

in a 100 mM Tris-HCl buffer of pH 8 in a 1:10 ratio (w/v), and stirred at room temperature for one hour. After removal of the insoluble parts by centrifugation (30 minutes, 12,000 *g*, 10°C), the supernatant was brought to pH 4.8 with 2 M HCl to induce precipitation of the soy proteins. After 2 hours at 4°C the dispersion was centrifuged (30 minutes, 12,000 *g*, 10°C). The precipitate was washed twice with a 10 mM sodium acetate buffer of pH 4.8 in a 1:8 ratio (w/v) and freeze-dried afterwards. This material will be referred to as SPI. The protein content was 97% using N × 6.25.

2.2.2 Preparation of protein dispersions

Protein dispersions were prepared by suspending SPI in double-distilled water in higher concentrations than required for the experiments. After stirring for one hour at 4°C the suspension was brought to pH 7 with 0.5 M NaOH, after which the volume of the dispersion was adjusted by adding double-distilled water to obtain the desired protein concentration. The protein dispersions were stirred overnight, at 4°C, to get the protein better dissolved. The preparation of the dispersions was performed at 4°C to prevent proteolysis by endogenous enzymes.

2.2.3 Small deformation experiments

Gel formation was followed by dynamic measurements using a Bohlin CVO rheometer with a serrated concentric cylinder geometry (C25). The measurements were performed at a constant strain of 0.01, which was within the linear region, and at an angular frequency of 0.63 rad/s. To prevent solvent evaporation, a thin layer of soy oil was put on top of the samples. To induce gel formation protein dispersions were consecutively heated from 20 to 90°C at a heating rate of 1 K/min, kept for one hour at 90°C, and cooled to 20°C at a cooling rate of 1 K/min, unless stated otherwise. Applied variations in the above-mentioned temperature profile were heating/cooling rate (1 and 5 K/min), heating time at 90°C (0, 1, and 5 hours), and maximum heating temperature (80-95°C). After gel formation, frequency dependence was studied from 0.0063 to 63 rad/s at several temperatures.

2.2.4 Differential scanning calorimetry (DSC)

The degree of protein denaturation in soy protein isolate as a function of maximum heating temperature was determined by differential scanning calorimetry and calculated by dividing the change in enthalpy, ΔH, associated to the denaturation peak of glycinin of a preheated

suspension by that of an unheated suspension. Thereto protein dispersions were first heated in 0.9 ml stainless-steel vessels in a micro-DSC (Setaram, France) from 20 to a maximum temperature ranging from 76 to 94°C at a heating rate of 1 K/min, maintained at that temperature for one hour, and cooled to 20°C at a cooling rate of 1 K/min. Consecutively, the sample was scanned from 20 to 115°C at a scanning rate of 1 K/min. A sample that was not preheated was scanned in the same way.

The temperature at which denaturation starts, the onset denaturation temperature T_o, was calculated by taking the intercept of the baseline and the extrapolated slope of the peak. For the peak denaturation temperature T_p, the temperature at maximum heat flow was taken.

2.3 Results

2.3.1 DSC

A DSC-thermogram of a 10% SPI dispersion that was not preheated is shown in Figure 2.1. Two endothermic transitions were observed caused by heat denaturation of β-conglycinin at the lowest temperature and of glycinin at the highest temperature (Hermansson, 1978). The onset (T_o) and peak (T_p) denaturation temperatures were 63 and 68°C for β-conglycinin and 80 and 88°C for glycinin. Heating the dispersion for a second time showed a DSC-thermogram without endotherms indicating that heat-induced denaturation of the proteins was followed by irreversible processes such as aggregation.

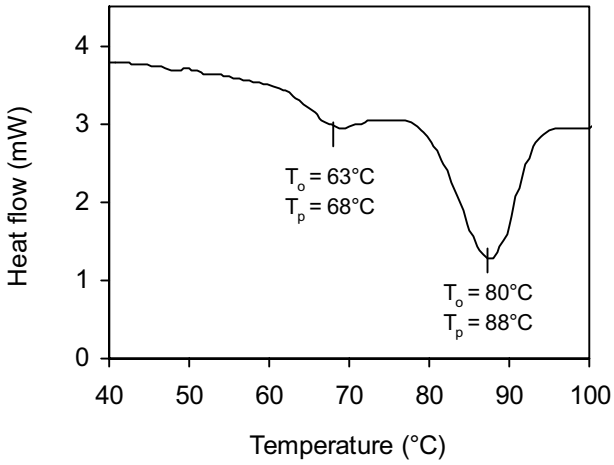


Figure 2.1 DSC-thermogram of a 10% soy protein isolate dispersions in double-distilled water (pH 7). The onset (T_o) and peak (T_p) denaturation temperatures of both endotherms are given.

Figure 2.2 shows the degree of denaturation of SPI dispersions that were heated for one hour at temperatures ranging from 76 to 94°C. These maximum heating temperatures corresponded with the temperatures covered by the endotherm of glycinin, which means that for all maximum heating temperatures β-conglycinin was already denatured. As the maximum heating temperature increased, the degree of denaturation increased. At 88°C, SPI was completely denatured.

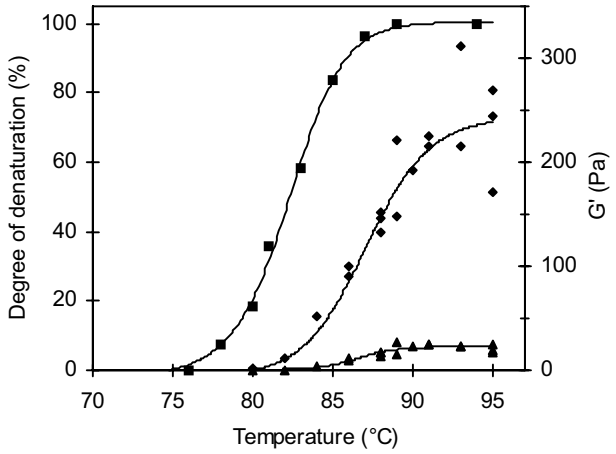


Figure 2.2 Degree of denaturation (■) and storage modulus *G′* after heating for one hour at maximum temperature (◆) and after cooling (▲), of 10% soy protein isolate dispersions in double-distilled water (pH 7) as a function of maximum heating temperature.

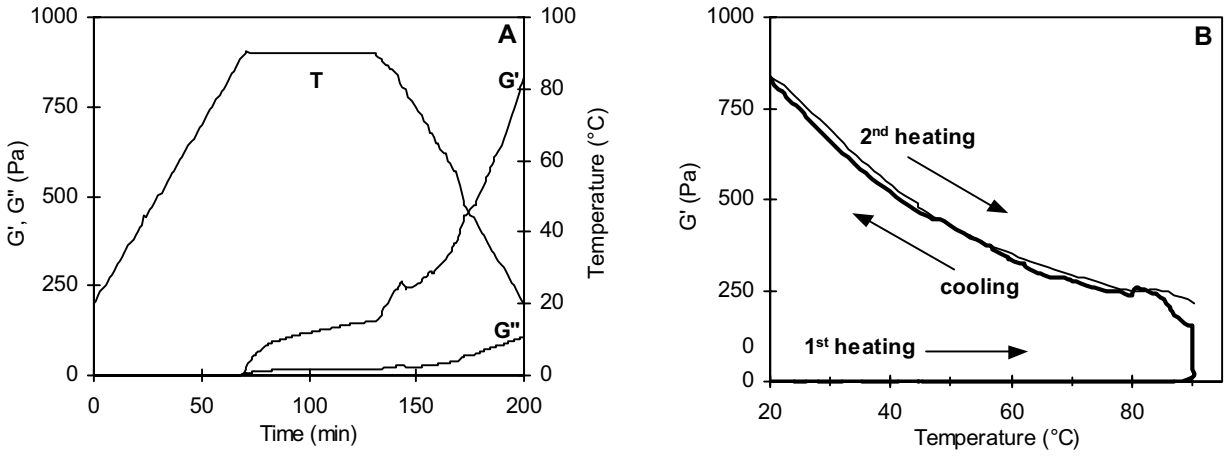
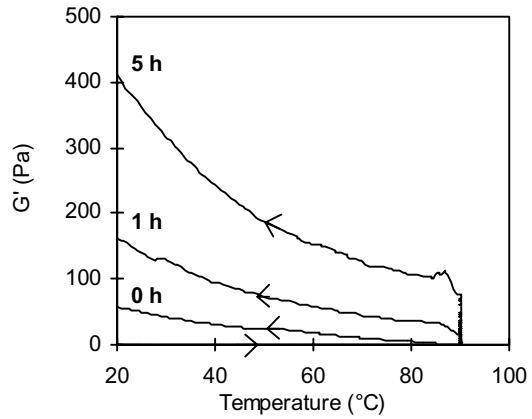


Figure 2.3 Dynamic moduli, *G′* and *G″*, of an 11% soy protein isolate dispersion in double-distilled water at pH 7 as a function of time during a heating and cooling cycle (T) (A) and the storage modulus *G′* as a function of temperature (B). In B, thick line represents first heating and cooling curve; thin line represents second heating curve.

2.3.2 Gel formation

In Figure 2.3A a typical example of a gelation curve of soy protein isolate at pH 7 as a function of time is presented. At 84°C (t = 64 min), the storage modulus *G′*, which is a measure of the stiffness of the gel, started to increase; this temperature is defined as the gelation temperature. *G′* kept increasing upon further heating, but a much stronger increase in *G′* was observed upon cooling. In Figure 2.3B the same gelation curve is presented (solid line), but in this case as a function of temperature. A second curve (dotted line) is plotted which resembles reheating of the gel from 20 to 90°C at 1 K/min. The second heating curve overlapped the cooling curve for the largest part indicating that gel stiffening during cooling was almost completely thermoreversibel.

The effect of heating time at 90°C on the gelation curves is shown in Figure 2.4. *G′* increased gradually on prolonged heating at 90°C, resulting in higher *G′* values just after the heating step, and even higher values after cooling. The effect of heating and cooling rate was studied for 1 and 5 K/min (Figure 2.5). The actual maximum cooling rate was in the latter case 3 K/min. At a heating rate of 1 K/min gelation started at a lower temperature than at 5 K/min, which resulted in a stiffer gel after cooling. Cooling rates did not seem to affect *G′* during and after cooling, when heating was performed at a heating rate of 1 K/min (data not shown).



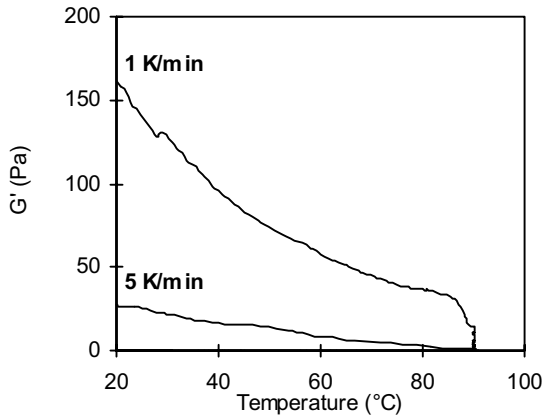


Figure 2.4 (left) Storage modulus *G′* during heating and subsequent cooling of 10% soy protein isolate dispersions in water (pH 7) for heating times at 90°C of 0, 1, and 5 hours respectively. Heating and cooling rates were 1 K/min. >, heating; <, cooling.

Figure 2.5 (right) Storage modulus *G′* during heating and subsequent cooling of 10% soy protein isolate dispersions in water (pH 7) as a function of heating rate. Heating time at 90°C was one hour.

Like for the degree of denaturation, the effect of maximum heating temperature on gel formation was studied. Figure 2.2 shows *G′*, immediately after heating for one hour, and *G′*, after cooling down to 20°C, as a function of maximum heating temperature. Gels were formed at maximum heating temperatures higher than 80°C. Up to 90°C, the stiffness of the gels increased with increasing heating temperatures. At temperatures higher than 90°C, *G′* seems to reach a plateau value. It is not clear if a plateau value or a maximum in the curve is obtained, because from this point the scatter in the data became large.

Combination of the y-data from Figure 2.2 gives *G′* as a function of the degree of denaturation (Figure 2.6). At least 20% of the glycinin fraction had to be denatured to achieve notable gel formation. As more protein became denatured, higher *G′* values were observed.

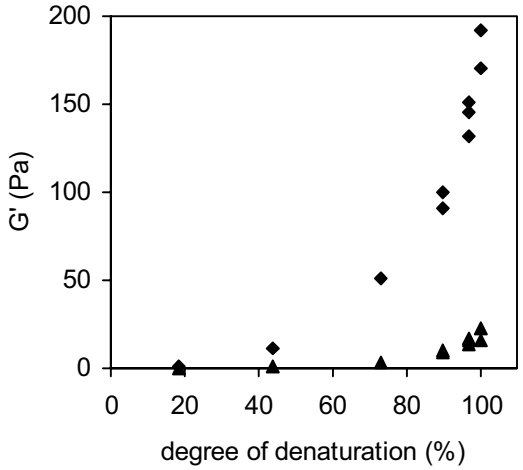


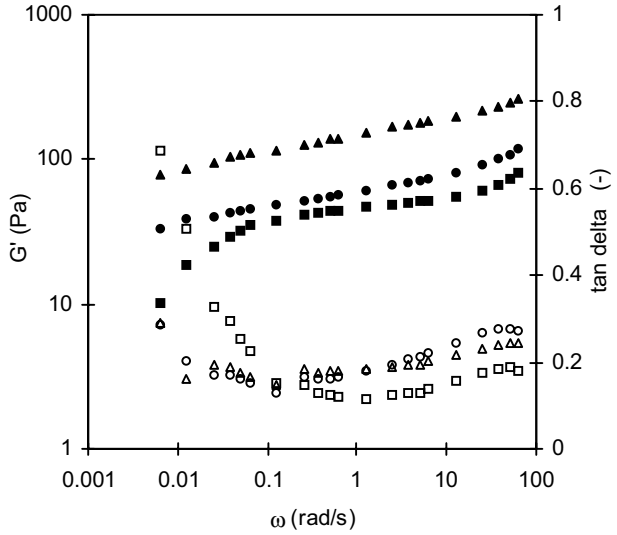
Figure 2.6 Storage modulus *G′* after heating for one hour at maximum temperature (◆) and after cooling (▲) of 10% soy protein isolate dispersions in water (pH 7) as a function of the degree of protein denaturation.

2.3.3 Frequency dependence

Figure 2.7 shows the frequency dependence of the storage modulus, *G′*, and the loss tangent, *tan* δ, at 20, 50 and 90°C. At 20°C, *G′* increased linearly with increasing frequency and *tan* δ was only slightly dependent on frequency. At temperatures of 50°C and higher, *G′* and *tan* δ became more frequency dependent (Figure 2.7 and 2.8).

2.4 Discussion

Below, we will discuss consecutively different stages in the gelation process: the onset of gel formation and the role of protein denaturation therein, the development of the storage



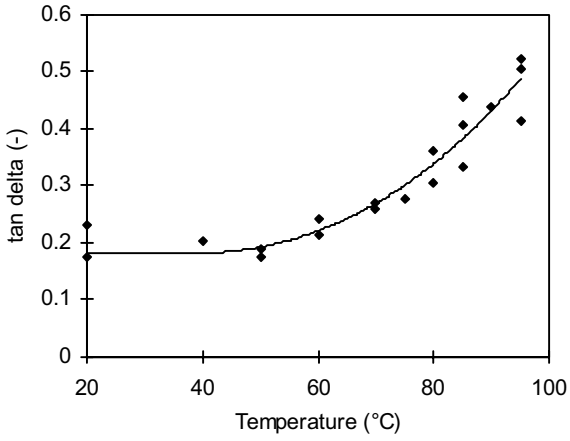


Figure 2.7 (left) Storage modulus *G′* (closed symbols) and loss tangent *tan* δ (open symbols) of a 10% soy protein isolate gel (pH 7) as a function of angular frequency, ω, at 20 (▲), 50 (●) and 90°C (■). Gel was prepared according to the standard procedure.

Figure 2.8 (right) Loss tangent, *tan* δ, of a 10% soy protein isolate gel (pH 7) as a function of temperature at angular frequency, ω = 0.0063 rad/s. Gel was prepared according to the standard procedure.

modulus *G′* and the occurrence of rearrangements in the network structure during (prolonged) heating, and the reversible stiffening of the gel on cooling.

The temperature at which *G′* started to increase (84°C), the onset of gel formation, is in between the onset and peak denaturation temperature of glycinin (Figure 2.1). At a heating rate of 5 K/min, *G′* started to increase later than at 1 K/min, *i.e.* 3 min after the program had reached 90°C. At higher heating rates denaturation temperatures are higher (*e.g.* Nagano et al., 1992), which would explain the higher gelation temperature at 5 K/min compared to 1 K/min. The results confirm the idea that heat denaturation is a prerequisite for gel formation. It is remarkable that gelation did not start at lower temperatures, namely after heat denaturation of β-conglycinin, which has a peak denaturation temperature of 68°C at these conditions. Likely, the explanation is that the β-conglycinin concentration was too low (< 4%) to result in a notable increase of *G′* (Renkema, 2001b).

The conclusion that protein denaturation is a prerequisite for gel formation is confirmed by the data in Figure 2.6. This figure shows that a certain amount of protein had to denature before a gel is formed and that the storage modulus increased with the amount of denatured protein.

In principle, heat denaturation of proteins is a reversible process. At each temperature, a specific equilibrium exists between proteins in the native state and proteins in the denatured state. However, on (partial) unfolding of soy proteins, functional groups such as sulfhydryl groups and hydrophobic groups become exposed and immediately interact with each other leading to irreversible protein aggregation and network formation (gelation). The equilibrium of native and denatured protein is restored continuously after aggregation of the denatured protein, until the point where no native protein is left. In practice, this means that heat denaturation of proteins is an irreversible process.

The data show (*a*) broad endothermic transitions in the DSC thermogram (Figure 2.1) and (*b*) that different amounts of denatured glycinin could be obtained by using a range of heating temperatures (Figure 2.2). This is explained by the fact that both the β-conglycinin and the glycinin fraction are composed of several genetic variants, which have a different thermal stability (Maruyama et al., 1998; Maruyama et al., 1999; Lakemond et al., 2001). For the data in Figure 2.2 this means that at a certain degree of denaturation some of the glycinin variants are denatured and some are still native. An additional reason for obtaining different amounts of denatured protein is that the heating conditions might have been such that denaturation was not yet completely irreversible.

After the onset of gelation, an increase in G' is observed on further heating. G' increased because more protein becomes incorporated into the network leading to a further built-up of the network structure. G' might also have increased by rearrangements in the network. A type of rearrangements that might occur is fusion of the protein aggregates in the strands, which results in an increase of protein-protein interactions per cross-section and so in denser and stiffer strands. Another type of rearrangements involves fracture or yielding of strands leading to regions with high and low densities of protein (van Vliet et al., 1991; van Vliet et al., 1997; van Vliet, 2000). This fracture is likely to happen in thin strands and/or when the average lifetime of the protein-protein bonds is short. Breaking of the strands due to relaxation of the intermolecular protein bonds is induced by thermal motion.

An indication for the possible occurrence of rearrangements in the network structure during heating was obtained from the frequency dependence data (Figure 2.7 and 2.8). High *tan* δ values at low frequencies (ω) show that gels have a more viscous behavior at longer time scales ($t = 1/\omega$). A high *tan* δ means that the average lifetime of the protein-protein bonds is rather small. Since such bonds are more likely to break and reform due to thermal motion, it indicates that rearrangements can occur (van Vliet et al., 1991; van Vliet, 1999). The high *tan* δ at 90°C (Figure 2.7 and 2.8) shows that rearrangements in soy protein gels possibly occur during prolonged heating at high temperatures in contrast to temperatures lower than 50°C.

Another indication that rearrangements take place at high temperatures was obtained by confocal scanning laser microscopy (CSLM). Gels that were heated for 4 hours at 95°C showed somewhat larger aggregates and significantly more contrast between protein and background than gels that were heated for 1 hour (data not shown). It is likely that rearrangements induced the changes on prolonged heating as observed in the micrographs and that these changes affected the stiffness of the gels.

No distinction could be made between the contribution to G' by continuous protein incorporation into the network and that by rearrangements. It is obvious that the steep increase of G' at the onset of gelation is caused by further incorporation of protein after the gel is formed. Yet, we do not know if the slow increase in G' on prolonged heating at 90°C (Figure 2.3) is also caused by additional protein built into the network or by rearrangements. The former is the case for whey protein gels (Verheul et al., 1998) while the latter occurs in casein gels (Mellema, 2000).

It is not clear why gels prepared at a heating rate of 5 K/min had a lower G' than gels at 1 K/min. The same phenomenon has been observed for other globular proteins such as β-lactoglobulin (Stading et al., 1993), vicilin and ovalbumin (Arntfield & Murray, 1992). The higher heating rate might have affected the aggregation kinetics resulting in gels with a different network structure. It is known that differences in network structure affect G' (Bremer et al., 1990; Mellema et al., 2001; Renkema, 2001a).

The last stage of the gelation curve, the gel stiffening during cooling, was almost completely thermoreversibel (Figure 2.3B) and independent of cooling rate. The reversibility is an indication that no covalent bonds are formed during this stage and that no rearrangements take place involving fracture of strands, because these processes are irreversible. Also the absence of an effect of cooling rate implies that no chemical reactions between protein molecules take place, because the reaction kinetics would be affected by the cooling rate. The increase in G' upon cooling is probably caused by a decreasing mobility of the proteins with decreasing temperature, which allows enhanced bond formation in and between the protein molecules.

The bump in the cooling part of the gelation curve (Figure 2.3A: t = 130-150 min; Figure 2.3B: T = 80-90°C) is partly explained by malfunctioning of the cooling program; G' reacted strongly on temperature fluctuations. However, also rearrangements in the network structure, resulting in an increase of G', might partly cause the bump in the cooling stage, because at 85°C these may still take place as indicated by the high *tan* δ at low ω (Figure 2.8). The occurrence of rearrangements also explains why G' was not fully thermoreversibel at temperatures above 85°C.

It is known that formation of intermolecular disulphide bonds via thiol-disulphide interchange and oxidation reactions plays a role in aggregation of soy protein (Yamauchi et al., 1991). We doubt, however, the importance of disulphide bridges for the stiffening of the initially formed network during heating. Disulphide bridges will essentially not be broken over time scales of minutes, which is in contrast with the results obtained for *G'* and *tan* δ at low ω and 90°C (time scale = $1/\omega$) (Figure 2.8). This implies that soy protein isolate gels do not consist of a so-called covalent network. The nature of the non-covalent protein-protein bonds that exist in the network was not studied by us.

Summarising, heat-induced gel formation by soy proteins involves several processes like denaturation, aggregation (in which disulphide bridges play a role), network formation and gel stiffening. Gel stiffening during prolonged heating is caused by rearrangements in the network structure and probably to some extent by further incorporation of protein into the network. Gel stiffening during cooling is a thermoreversibel process and does, therefore, not involve disulphide bond formation or rearrangements in the network structure.

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Chapter 3

The influence of pH and ionic strength on heat-induced formation and rheological properties of soy protein gels in relation to denaturation and their protein compositions

Abstract

The influence of pH and ionic strength on gel formation and gel properties of soy protein isolate (SPI) in relation to denaturation and protein aggregation/precipitation was studied. Denaturation proved to be a prerequisite for gel formation under all conditions of pH and ionic strength studied. Gels exhibited a low stiffness at pH > 6 and a high stiffness at pH < 6. This might be caused by variations in the association/dissociation behaviour of the soy proteins on heating as a function of pH as indicated by the different protein composition of the dissolved protein after heating. At pH 3-5 all protein seems to participate in the network, whereas at pH > 5 less protein and especially less acidic polypeptides take part in the network, coinciding with less stiff gels. At pH 7.6, extensive rearrangements in the network structure took place during prolonged heating, whereas at pH 3.8 it did not happen.

Renkema, J. M. S., Gruppen, H., & van Vliet, T. *submitted for publication.*

3.1 Introduction

The ability of soy proteins to form a gel on heating makes them well suited to improve texture of food products. Nevertheless, soy proteins are not frequently used as texture-enhancers. Partly, this is due to the difficulties to predict gel properties and to control food texture for all the different conditions (*e.g.* pH, ionic strength and heating temperature) during manufacturing of food products.

The main soy proteins are glycinin and β-conglycinin. Their quaternary structure is determined by pH and ionic strength. Glycinin is composed of acidic (*ca.* 38 kDa) and basic polypeptides (*ca.* 20 kDa) linked by a single disulphide bridge, except for the acidic polypeptide A_4 (Staswick et al., 1984). At ambient temperatures and pH 7.6 glycinin forms hexameric complexes (11S), while at pH 3.8 it is mainly present as trimeric complexes (7S) (Wolf et al., 1958; Lakemond et al., 2000a). At pH 2-10 and ionic strength higher than 0.1, β-conglycinin is a trimeric glycoprotein (a 7S globulin) (Koshiyama, 1983) consisting of three different subunits (α', α, and β with molecular masses of 57-72, 57-68 and 45-52 kDa, respectively (Yamauchi et al., 1991)) in at least six different combinations (Thanh & Shibasaki, 1978). At an ionic strength less than 0.1, β-conglycinin exists as a hexamer (9S) at pH 5 and higher, whereas at pH 2-5 β-conglycinin dissociates into a 2-3S and 5-6S fraction (Koshiyama, 1983).

Heat denaturation is often a prerequisite for gel formation of globular proteins. Denaturation temperatures depend strongly on pH and ionic strength (Hermansson, 1986; Damodaran, 1988). The onset denaturation temperature of glycinin is around 80-90°C for the 11S form and 60-70°C for the 7S form (Danilenko et al., 1987; Lakemond et al., 2000b). β-Conglycinin denatures at 60-75°C (Maruyama et al., 1998; Maruyama et al., 1999; Puppo & Añón, 1999; Renkema, 2001b). The relation between denaturation and the onset of gelation has not extensively been studied for soy proteins as a function of pH and ionic strength.

Ionic strength and pH also affect the characteristics of soy protein gels (Catsimpoolas & Meyer, 1970; Bau et al., 1985; Utsumi & Kinsella, 1985; van Kleef, 1986; Nagano et al., 1994a; Nagano et al., 1994b, c; Puppo et al., 1995; Renkema et al., 2000; Renkema, 2001b). The influence of pH on gel formation by soy proteins has been studied by rheology (van Kleef, 1986; Nagano et al., 1994c), microscopy (Hermansson, 1994), gel swelling or dissolving experiments (van Kleef, 1986; Puppo et al., 1995), and FTIR spectroscopy (Nagano et al., 1994c). Beside different methods, different materials (glycinin, β-conglycinin or soy protein isolate (SPI)) were used for each of these studies, which makes comparison and an integration of the results into an overall picture of gel formation difficult.

This study is focussed on the influence of pH on gel formation by SPI in relation to

denaturation at three salt concentrations. Furthermore, gel properties are determined by rheological measurements at small deformation and related to the effect of heating on protein aggregation/precipitation and to the extent of participation of polypeptides/subunits in the network formation. The relations between network structure and rheological properties of SPI gels as a function of pH will be studied in future work.

3.2 Material and methods

3.2.1 Sample preparation

For the experiments, a soy protein isolate (SPI) was used with a calculated protein content of 97% using N × 6.25. The SPI (pH 4.8) was prepared from mildly treated, defatted PDI 80 soy flakes (Cargill, Amsterdam, The Netherlands) according to a method described previously (Renkema, 2001b). Protein dispersions (120 mg/g) for gel formation and DSC experiments were prepared by suspending 6 g SPI in 35 g double-distilled water, 0.2 or 0.5 M NaCl solution. After stirring for one hour at 4°C the suspension was brought to pH 7.6 with 0.5 M NaOH. After two hours the pH was adjusted with 0.5-1 M HCl to the desired value. If necessary, the mass of the dispersion was adjusted to 50 g by adding water or salt solution to obtain the desired protein concentration. The protein dispersions were stirred overnight to enhance protein dissolution. Protein dispersions were prepared at 4°C to prevent proteolysis by endogenous enzymes.

As a reference for DSC experiments, protein dispersions (0.1 g/g) of purified glycinin and a β-conglycinin rich fraction in double-distilled water were used. Isolation of these proteins was described in a previous paper (Renkema, 2001b).

3.2.2 Determination of protein solubility and protein composition

SPI dispersions (10 mg/ml) were prepared by stirring 1 g SPI in 90 ml double-distilled water or salt-solution for 1 hour at 4°C. In all cases, the protein dispersions were first brought to pH 7.6 with a defined amount of 0.5 M NaOH. After one hour, the pH was adjusted to the desired pH with 0.5 M HCl or NaOH. After pH adjustment, the volume of the dispersion was adjusted to 100 ml. The dispersions were stirred for 16 hours at 4°C. Preparation of the dispersions was performed at 4°C to prevent proteolysis by endogenous enzymes. Part of the protein dispersion was heated from 20 to 95°C at a rate of 1 K/min, kept at 95°C for one hour, and cooled down at a rate of 1 K/min to 20°C. The unheated and heated dispersions were centrifuged at 32000 *g* for 30 minutes at 4°C. The protein content of the dispersions (total

protein) and of the supernatants (dissolved protein) was determined in duplicate by the micro-Kjeldahl method (AOAC, 1980) using a Kjeldahl factor of 6.25. Solubility was defined as (dissolved protein/total protein) × 100%.

The protein composition of the supernatants of the heated and unheated dispersions was determined by SDS-PAGE under both reducing (with β-mercapto-ethanol) and non-reducing conditions on a Phast System (Pharmacia, Sweden) according to the instructions of the manufacturer. Equal volumes of each supernatant were diluted in sample buffer. The samples were left overnight at 20°C. Gradient gels (10-15%) were used, which were stained with Coomassie Brilliant Blue. The gel was calibrated with low molecular mass markers ranging from 14-94 kDa (Pharmacia, Sweden).

3.2.3 Differential scanning calorimetry (DSC)

Denaturation temperatures of SPI dispersions (120 mg/g) were determined by differential scanning calorimetry at a scanning rate of 1 K/min. The measurements were performed in a micro-DSC (Setaram, France) equipped with 0.9 ml stainless steel sample vessels. The temperature at which denaturation starts, the onset denaturation temperature (T_o), was estimated by taking the intercept of the baseline and the extrapolated slope of the peak. For the peak denaturation temperature (T_{max}), the temperature at maximum heat flow was taken.

3.2.4 Gelation

Gel formation was followed in duplicate by dynamic measurements in a Bohlin CVO rheometer using a serrated concentric cylinder geometry (C25). The storage (G') and loss (G'') modulus were measured in the linear region at a constant maximum strain of 0.01 and an angular frequency of 0.63 rad/s. To induce gel formation, protein dispersions were heated from 20 to 95°C at a rate of 1 K/min, kept for one hour at 95°C, and cooled down to 20°C at a rate of 1 K/min. To prevent solvent evaporation, a thin layer of soy oil was put on top of the samples. Selected gels were reheated from 20 to 95°C at a rate of 1 K/min. At 95°C, the time-dependent behavior of the gels was studied by a frequency sweep up and down from 0.063 to 63 rad/s in 16 logarithmic steps, followed by a frequency sweep down and up from 0.063 to 0.0063 rad/s in 6 logarithmic steps. The temperature at which G' started to increase over 0.5 Pa/K was defined as the gelation temperature. At most conditions, a slow increase in G' (totally about 1 Pa) was observed before the steep increase. We chose to neglect this slow increase.

3.3 Results

3.3.1 Solubility

Figure 3.1 shows the protein fraction that remains dissolved after centrifuging of unheated and heated SPI dispersions (10 mg/ml) in water, 0.2 M and 0.5 M NaCl. This protein fraction consists of proteins, polypeptides, subunits and aggregates smaller than approximately 0.2 μm, as calculated by using Stokes equation, and is further denoted as solubility.

In water and 0.2 M NaCl, SPI had a low solubility between pH 4 and 5 (Fig. 3.1A). In 0.5 M NaCl, SPI also had a low solubility in this pH range, but the actual minimum was observed at pH values below 3. An increase of salt concentration caused an increase in the amount of dissolved protein between pH 4 and 5 and a decrease at pH values lower than 3 and higher than 7. This agrees with results by others (Hermansson, 1973; Shen, 1976). Heating did not affect solubility in the absence of NaCl (Fig. 3.1B). At 0.2 M NaCl solubility decreased with 20-30% at pH values higher than 5, and at 0.5 M NaCl it decreased with 10-30% at pH 3-7.

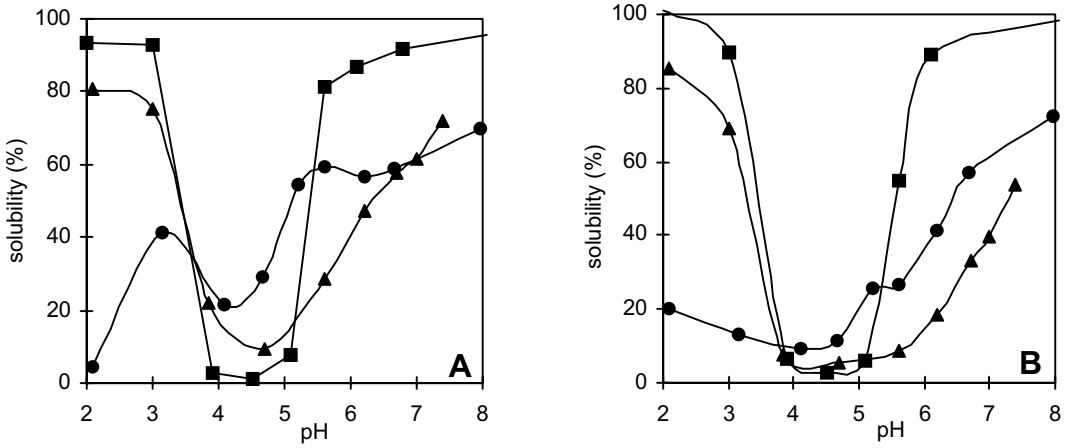

Figure 3.1 Solubility of unheated (A) and heated (B) 1% soy protein dispersions as a function of pH at added salt concentrations of 0 (■), 0.2 (▲), and 0.5 M NaCl (●).

3.3.2 Protein composition of dissolved fractions

Protein compositions of the dissolved fractions of the unheated and heated dispersions (10 mg/ml) were determined by SDS-PAGE analysis under reduced and non-reduced conditions and are presented in Table 3.1.

Table 3.1 Protein composition of supernatant of heated 1% soy protein isolate dispersions as determined by SDS-PAGE.

NaCl	pH	**Unheated** *reduced conditions*				**Heated** *reduced conditions*				**Heated** *non-reduced conditions*					
		A	B	α, α'	β	A	B	α, α'	β	A	B	AB	α, α'	β	Agg
0 M	3	+++	+++	+++	+++	++	++	+	+++	?	?	++	+	+	++
	3.9	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	4.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	5.1	-	-	+	-	-	-	-	-	-	-	-	-	-	-
	5.6	+++	+++	+++	+++	+++	+++	+++	+++	+	-	-	+	+	+++
	6.1	+++	+++	+++	+++	+++	+++	+++	+++	+	-	-	+	+	+++
	6.8	+++	+++	+++	+++	+++	+++	+++	+++	+	-	-	+	+	++
	8.1	+++	+++	+++	+++	+++	+++	+++	+++	++	-	-	++	++	++
0.2	2.1	+++	+++	+++	+++	++	++	-	++	-	-	+	-	++	-
	3	+++	+++	+++	+++	++	++	-	++	-	-	+	-	++	+
	3.9	+	+	++	++	-	-	-	-	-	-	-	-	-	-
	4.7	-	-	+	-	-	-	-	-	-	-	-	-	-	-
	5.7	+	+	++	++	+	-	-	-	-	-	-	-	-	-
	6.2	++	++	+++	+++	++	-	+	-	+	-	-	-	-	++
	6.7	+++	+++	+++	+++	+++	+	++	++	+	-	-	+	+	++
	7	+++	+++	+++	+++	+++	+	++	++	+	-	-	+	+	+++
	7.4	+++	+++	+++	+++	+++	+	++	++	+	-	-	+	+	+++
0.5	2.1	-	-	+++	+++	-	-	-	-	-	-	-	-	-	-
	3.2	+++	+++	++	++	-	-	-	-	-	-	-	-	-	-
	4.1	+	+	++	++	-	-	-	-	-	-	-	-	-	-
	4.7	++	++	+++	+++	-	-	-	-	-	-	-	-	-	-
	5.2	++	++	+++	+++	+	+	-	-	-	-	-	-	-	-
	5.6	+++	+++	+++	+++	++	+	-	-	-	-	+	-	-	-
	6.2	+++	+++	+++	+++	++	+	+	-	+	-	+	-	-	+
	6.7	+++	+++	+++	+++	+++	++	++	++	++	-	++	++	+	++
	8	+++	+++	+++	+++	+++	++	+++	++	++	-	++	++	+	++

A, B acidic and basic polypeptides of glycinin

α, α', β subunits of β-conglycinin

Quantification is based on visual evaluation of the SDS-PAGE profiles and with the condition at pH 8.1 and 0 M NaCl as a reference:

- no polypeptides/subunits present in the dissolved fraction

+++ all polypeptides/subunits present in the dissolved fraction

++, + part of the polypeptides/subunits present in the dissolved fraction

? no clear profile

Unheated The SDS-PAGE profiles of the supernatants of the unheated dispersions at pH 2-3 and pH 5.6 and higher showed a normal pattern of glycinin polypeptides and β-conglycinin subunits, except for pH 2-0.5 M. At this condition, only β-conglycinin was present in the supernatant. At the intermediate pH values, dissolved protein was only observed at 0.2 and 0.5 M NaCl and consisted of more β-conglycinin than glycinin.

Heated At pH > 5 (further denoted as *high pH*) and 0 M NaCl, as much acidic (A) as basic (B) polypeptides of glycinin were found under reduced conditions, whereas in the presence of salt more acidic than basic polypeptides remained dissolved. Non-reducing SDS-PAGE revealed that the original AB subunit was not present anymore at 0 and 0.2 M NaCl in contrast to 0.5 M NaCl. At all salt concentrations, free acidic polypeptides and aggregates larger than 100 kDa were observed in the non-reduced SDS-PAGE gels, whereas free basic polypeptides were not noticed. The basic and the remaining acidic polypeptides must be present as (part of the) dissolved aggregates and, at 0.5 M NaCl, also as part of the AB subunits. Reduced SDS-PAGE showed that on heating the amount of dissolved β-conglycinin subunits decreased with increasing salt concentration, whereas the composition of β-conglycinin remained largely unchanged. Under non-reduced conditions, however, less β than α and α' subunits were observed at 0.5 M NaCl. This means that also part of the β subunits is present as (part of the) dissolved aggregates.

At pH 3-5, no protein could be detected on the SDS-PAGE gels because of the low amount of dissolved protein.

At pH 2 and 3 (further denoted as *low pH*), the A and B polypeptides of glycinin were present in the same amounts at 0 and 0.2 M NaCl when analysed at reduced conditions. At 0.5 M NaCl, no dissolved protein was detected. Under non-reduced conditions, the original AB subunit and aggregates larger than 100 kDa were observed and no free polypeptides were present at 0 and 0.2 M NaCl. This means that the acidic and basic polypeptides were only present in the AB subunit. At 0 M NaCl, the composition of the β-conglycinin subunits differed between reduced and non-reduced conditions. This indicates that part of the β subunits was present as (part of the) soluble aggregates.

The aggregates larger than 100 kDa formed at low and high pH could only be seen on SDS-PAGE gels under non-reduced conditions. The results indicate that at least part of every polypeptide and subunit is present in an aggregated form. Whether aggregates consist of only acidic or basic polypeptides of glycinin or subunits of β-conglycinin or of complexes formed by specific polypeptides or subunits has not been studied.

3.3.3 Denaturation

The influence of pH and ionic strength on denaturation was studied by differential scanning calorimetry (DSC). Figure 3.2 shows typical examples of DSC-thermograms of soy protein isolate (a-d) at several pH values. All thermograms showed two endothermic transitions, except for pH 3. Separate analysis of purified glycinin (e) and β-conglycinin (f) at this pH showed that heat denaturation took place for both proteins. The enthalpy, ΔH, however, was very low at pH 3. β-Conglycinin had an onset and peak denaturation temperature of 55°C and 61°C, respectively. The thermogram of glycinin showed a broad endothermic transition with an onset denaturation temperature of 45°C and two peak denaturation temperatures at 55°C and 63°C.

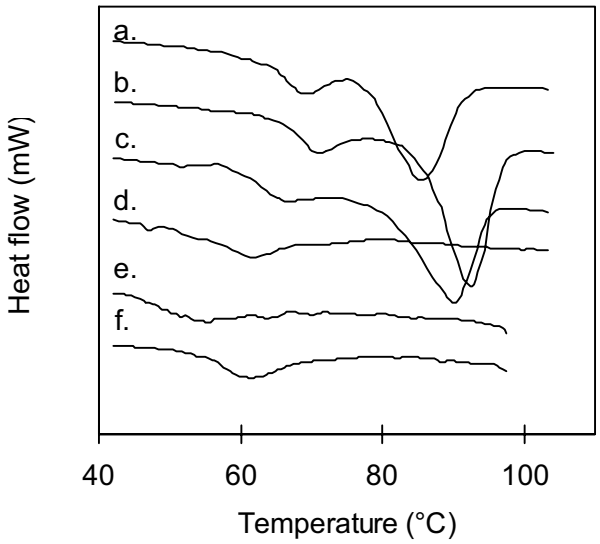

Figure 3.2 Examples of DSC thermograms of 12% soy protein isolate dispersions (a-d) and 10% dispersions of purified glycinin (e) and β-conglycinin (f) at different pH values and 0 M NaCl. Scanning rate was 1 K/min. a, pH 7.8; b, pH 5.2; c, pH 3.8; d-f, pH 3.

In Figure 3.3, the onset and peak denaturation temperatures of SPI were maximal close to the iso-electric point and minimal at pH values smaller than 4. Similar results were obtained for SPI (Hermansson, 1978), β-conglycinin (Nagano et al., 1994c; Renkema, 2001b) and glycinin (Lakemond et al., 2000b; Wongprecha et al., 2000). Figure 3.3 also shows that heat denaturation temperatures were higher at higher salt concentrations than at low salt concentrations. Furthermore, with increasing salt concentration the pH dependence became less pronounced at pH 5-8.

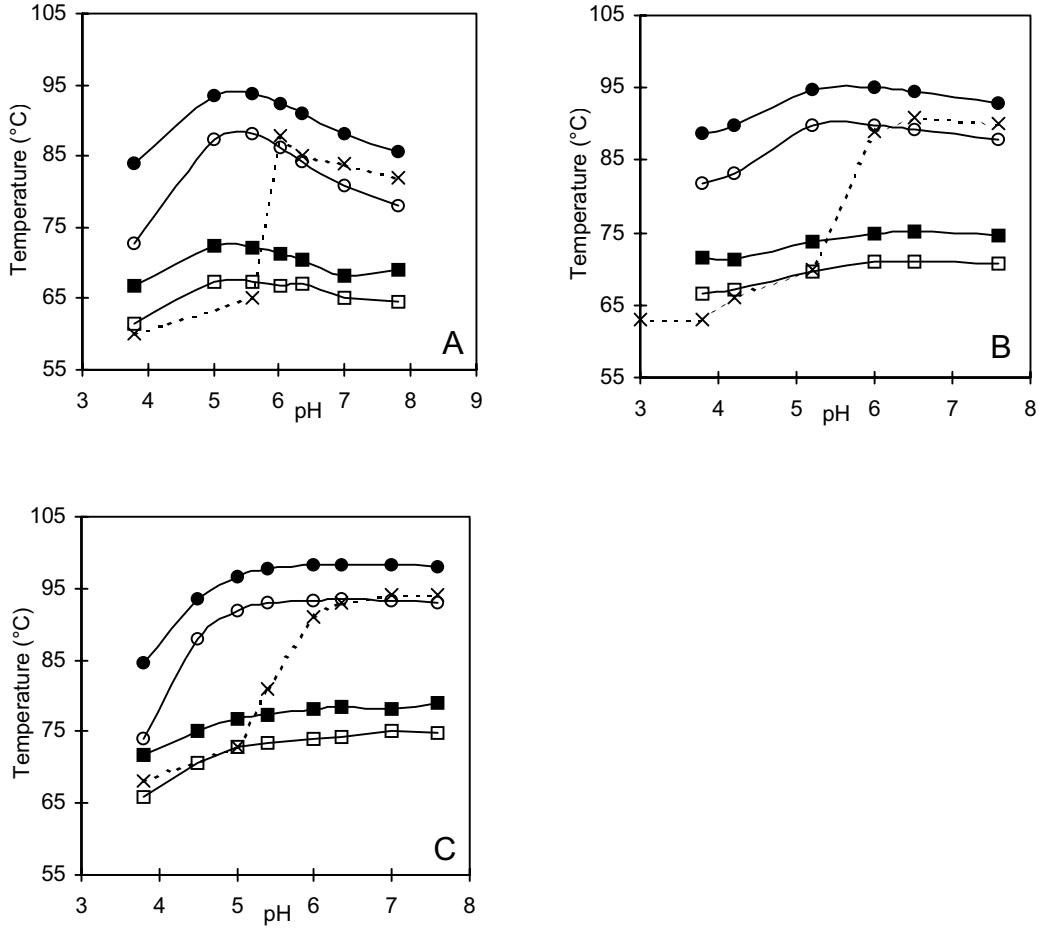

Figure 3.3 Onset (□,○) and peak (■,●) denaturation and gelation (×) temperatures of 12% soy protein isolate dispersions as a function of pH at 0 (A), 0.2 (B), and 0.5 M (C) added NaCl. ■,□ denaturation temperatures of the first endothermic transition; ●,○ those of the second endothermic transition.

3.3.4 Gelation

Figure 3.4 shows typical gelation curves of SPI gels in 0.2 M added NaCl at pH 3.8, 5.2 and 7.6 as observed by dynamic rheological measurements. At a certain temperature, the gelation temperature, G' starts to increase. During subsequent heating at 95°C G' kept increasing, but a much stronger increase was observed on cooling.

Gelation temperatures are plotted in Figure 3.3 together with the denaturation temperatures. At a pH lower than 6, gelation temperatures were lowest and coincided with the first denaturation peak for SPI. At pH 6 and higher, gelation temperatures were higher and coincided with the second denaturation peak.

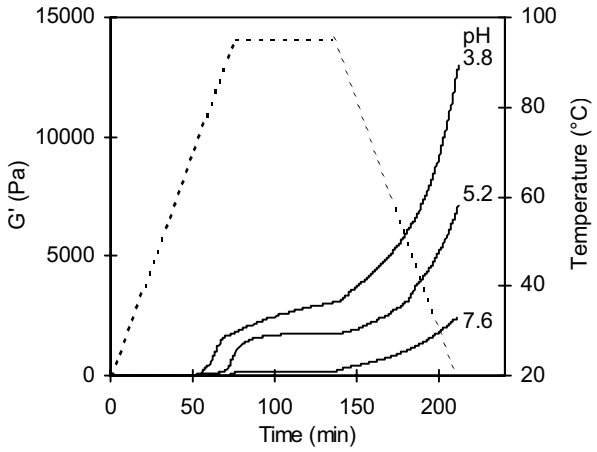

Figure 3.4 Typical gelation curves of 12% soy protein isolate at 0.2 M NaCl at three different pH values. ── storage modulus *G'*; ----- temperature.

Figure 3.5 shows the storage or elastic modulus, *G'*, of soy protein isolate gels after completion of the heating and cooling cycle as a function of pH at the three different salt concentrations. *G'* is a measure of the stiffness of gels or in other words of the resistance to deformation. At every salt concentration studied, gels were stiffer at low pH than at high pH. In the absence of NaCl, a maximum in *G'* was found at pH 6.5, but an even higher stiffness of the gels was measured at pH values lower than 6. At pH values 4.5-5.5 and in the absence of NaCl, no measurements could be performed owing to protein precipitation before heating. At

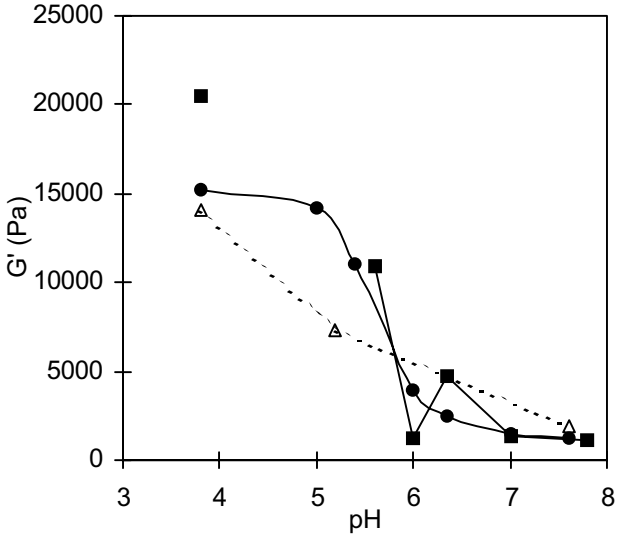

Figure 3.5 Storage modulus *G'* after a complete temperature cycle of 12% soy protein dispersions as a function of pH at added salt concentrations of 0 (■), 0.2 (▲), and 0.5 M NaCl (●).

0.2 M NaCl, only three data points are given. However, another series of measurements, performed with SPI from a different batch, showing a gradual increase in *G′* with decreasing pH (not shown) allowed drawing of the dotted line. Also, at 0.5 M NaCl, *G′* increased gradually with decreasing pH. Similar results were obtained by van Kleef (1986).

3.3.5 Thermoreversibility and frequency dependence

Thermoreversibility and the occurrence of rearrangements at high temperatures, were investigated by reheating gels to 95°C, where the frequency dependence was tested, and consecutively cooling gels down to 20°C. Figure 3.6 shows the increase in storage modulus, *G′*, of a 12% SPI dispersion in 0.2 M NaCl of pH 3.8, 5.2 and 7.6 during a normal heating and cooling cycle (line with one arrow). At pH 7.6, *G′* is thermoreversibel on reheating up to 40°C. At higher temperatures, the reheating curve started to deviate from the first cooling curve. The second cooling curve started at a lower stiffness and differed completely from the previous curves. Immediate cooling down after reheating of the gel did not result in such a large difference at 20°C (data not shown). At pH 3.8, the reheating curve follows the first cooling curve completely, but *G′* of the second cooling curve is about 1 kPa higher at each temperature. At pH 5.2, the reheating and second cooling curves were only slightly deviating, which means that this gel was almost completely thermoreversibel. Keeping the gels for a long time at 95°C apparently caused changes in (the properties of) the gels at pH 7.6 and to a small extent at pH 3.8 and pH 5.2.

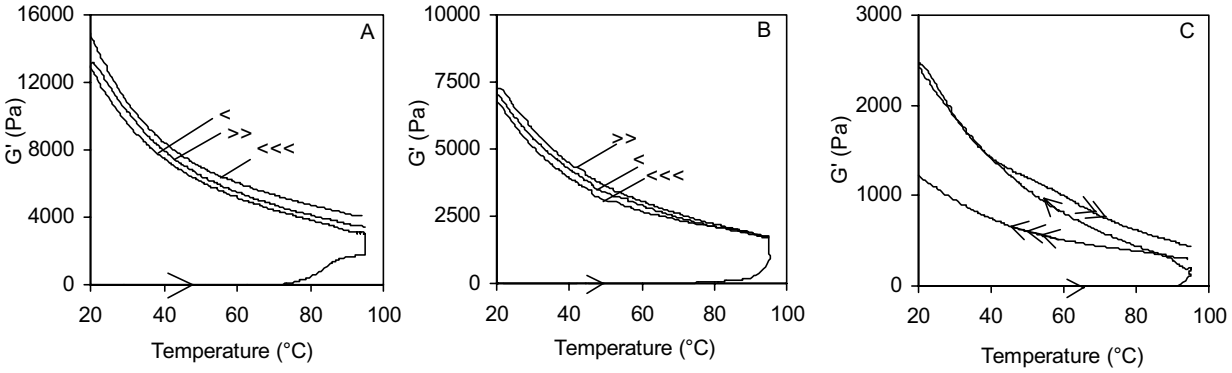

Figure 3.6 Thermoreversibility of the storage modulus *G'* of a 12% soy protein isolate gel at pH 3.8 (A), 5.2 (B) and 7.6 (C) and 0.2 M NaCl. Line with one arrow represents first heating and cooling curve, line with two arrows the second heating curve and line with three arrows the second cooling curve (after performance of the frequency sweeps at 95°C (duration: ~10 hours)).

Figure 3.7 shows the frequency dependence of the storage modulus G' (A) and the loss tangent $tan\ \delta$ (B) at 95°C of SPI gels at 0.2 M NaCl and pH 3.8, 5.2, and 7.6. At pH 3.8, G' values were slightly higher for a higher angular frequency, ω, whereas at pH 5.2 and, especially, pH 7.6, G' was more frequency dependent. At low frequencies lower G' values were measured than at high frequencies, because at larger experimental time scales (= 1/ω) more protein-protein bonds have the opportunity to become stress-free during the periodic

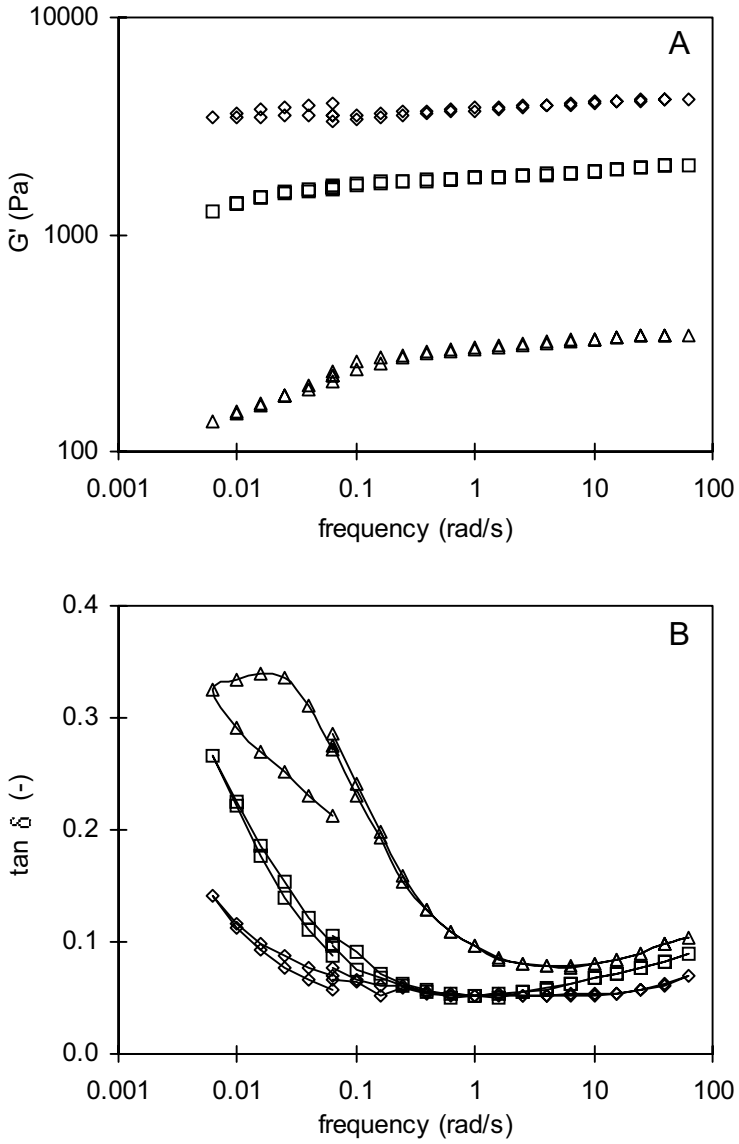

Figure 3.7 Frequency dependence of the storage modulus (A) and loss tangent (B) at 95°C of 12% soy protein isolate gels at pH 3.8 (◇), 5.2 (□) and pH 7.6 (Δ) and 0.2 M NaCl.

deformation. Figure 3.7B shows that *tan δ* is also more frequency dependent at pH 5.2 and 7.6 than at pH 3.8. At pH 7.6 the curves of the frequency sweeps down and up from 0.063 to 0.0063 rad/s were not the same, which showed that long incubation times at 95°C induced large changes in the pH 7.6 gel leading to a decrease in *tan δ*. A control experiment in a Bohlin VOR rheometer, in which the dynamic moduli of a pH 7.6 gel were recorded during heating at 90°C for 10 hours at a constant strain of 0.01 and an angular frequency of 0.0063 rad/s, confirmed that *tan δ* decreased on prolonged heating (data not shown).

3.4 Discussion

3.4.1 Heat denaturation as a prerequisite for gelation

Heat denaturation temperatures vary as a function of pH and ionic strength (Figure 3.3). Highest denaturation temperatures were found close to the iso-electric point (pH ~ 5.5) and at higher salt concentrations. This is common for most globular proteins (Privalov, 1979), for proteins tend to be most stable against denaturation when they have no net charge or when their charge is screened.

Gel formation started at the onset of denaturation, which shows that heat denaturation is a prerequisite for gel formation by soy proteins. This is valid for all the studied conditions of pH and ionic strength. Unexpectedly, gelation of SPI coincided with the first denaturation peak at pH < 6, whereas at pH > 6 gelation started at the second denaturation peak (Figure 3.3). At the studied pH range, the endothermic transition observed at the lowest temperature is caused by heat denaturation of β-conglycinin and the one at the highest temperature by glycinin (Hermansson, 1978; German et al., 1982). At pH 3.8, the endothermic transition at the lowest temperature might be partly caused by heat denaturation of the 7S form of glycinin which is present predominantly at this pH (Lakemond et al., 2000a) and has a lower denaturation temperature than the 11S form (Danilenko et al., 1987; Utsumi et al., 1987; Lakemond et al., 2000b). Although this might suggest that at pH < 6 gelation is also caused by denaturation of 7S glycinin, results from our laboratory showed that, for the present conditions, gel formation by glycinin did only start at heat denaturation of the 11S form (Renkema et al., 2000).

The results suggest that β-conglycinin cannot form a gel at pH > 6, but other studies (Nagano et al., 1994c; Renkema, 2001b) showed that gel formation does take place at pH > 6. However, at the SPI concentration (120 mg/g) in this study, the initial increase in G' as a result of denaturation of β-conglycinin was too low (<<0.5 Pa/K) to be considered as gel formation. This is due to the lower efficiency of β-conglycinin to form a gel with a certain G' at pH 7.6

compared to pH 3.8 (Renkema, 2001b). The critical protein concentration of β-conglycinin required for gel formation did not differ much at low or high pH values (Renkema, 2001b).

At pH 3, only one broad endothermic transition is observed (Figure 3.2). Further analysis showed that this is a result of denaturation of both glycinin and β-conglycinin. Exceptionally, the denaturation temperatures of glycinin were lower than those of β-conglycinin. According to Wolf et al. (1958) most of the glycinin is in a 3S (1AB) and part of the glycinin in a 7S form at pH 3 and low ionic strength. The 3S form does not give a cooperative transition (Danilenko et al., 1987), which implies that at this condition 7S glycinin is not very heat stable.

3.4.2 Rheological properties of soy protein gels

The stiffness of soy protein gels varies as a function of pH and ionic strength (Figure 3.5). In general, higher values for G' were obtained at pH < 6 than at pH > 6. Irrespective of pH, the stiffness of the gels decreased when the temperature of the gels was increased. At pH 3.8 and 5.2, reheating of the gels completely undid the stiffening of the gels that was induced by cooling in contrast to pH 7.6 (Figure 3.6). The fact that the gels were not thermoreversibel on reheating at pH 7.6 indicates that at high temperatures changes in the network occur. We believe that these changes are most likely induced by rearrangements. Indications for the occurrence of these rearrangements are obtained from the frequency dependence of G' and $tan\delta$ at 95°C. Higher $tan\delta$ values at lower frequencies (Figure 3.7), as found at pH 7.6 and to some extent 5.2, mean that these gels had a stronger viscous-like behavior than at pH 3.8. It implies that, at 95°C, bonds between protein molecules can be broken and reformed more easily at pH 7.6 and 5.2 (van Vliet et al., 1991).

Low G' values, as found at higher pH values, correlate with a high amount of dissolved protein in heated 1% dispersions (Figure 3.1B and 3.5). It is expected that the protein that remained dissolved after heating is not incorporated in the network in contrast to the precipitated polypeptides and subunits. These lower concentrations of aggregated protein result in a lower stiffness of the gels (Verheul et al., 1998). However, despite the higher amounts of dissolved protein, gels at pH 5.2-0.5 M NaCl have higher G' values than at 0.2 M NaCl. This means that the amount of protein incorporated in the network cannot fully explain the differences in G'. Indeed from other work, we know that variations in network structure of the soy protein gels also contribute to variations in gel stiffness (Renkema, 2001a).

The dip in the G' curve at pH 6 and 0 M NaCl is not an experimental artifact (Figure 3.5). Measurements of G' at pH 6 as a function of NaCl concentration (data not shown) also showed that G' is very low at NaCl concentrations of 0-0.03 M. Up to 0.1-0.2 M NaCl, G'

increased owing to a salting-in effect, which was followed by a decrease in G' at higher salt concentrations. The dip in the G'-pH curve has also been observed by van Kleef (1986) for soy protein isolate (at pH 4.5) and glycinin (at pH 6) under conditions without salt. It might indicate the presence of a (small) pH zone in which the network had a different structure than at both sides of the zone, as was observed for heat-induced ovalbumin gels (Doi & Kitabatake, 1989).

3.4.3 Association/dissociation behaviour on heating

The increase in solubility of the unheated SPI at pH 4-5 with increasing ionic strength was in the first place due to an increased solubility of β-conglycinin, but also more glycinin became dissolved (Table 3.1). At other pH values no difference in solubility between both proteins was observed, except at pH 2 and 0.5 M NaCl, where all glycinin had precipitated. This latter observation agrees with results for glycinin (Lakemond et al., 2000a). At pH 4-5, salting-in effects can explain the increase in the amount of dissolved proteins at higher salt concentrations. The solubility decrease at pH < 3 and pH > 7 with increasing ionic strength seems to be a salting-out process. However, salting-out of globular proteins normally happens at NaCl concentrations much higher than 0.5 M (Tanford, 1961). At pH < 3 and pH > 7, the proteins have a strong net charge, which promotes dissolution at low ionic strength. At higher ionic strength, the charge is screened resulting in a lower electrostatic repulsion and lower solubility.

After heating, a different protein composition in the supernatant was found for samples at pH 2 and 3 (further denoted as low pH) than for samples at pH > 5 (further denoted as high pH). At high pH the absence of the AB subunit and the presence of free acidic polypeptides in the supernatants of the heated dispersions imply rupture of the disulphide bond and the non-covalent interactions between the acidic and the basic polypeptide of glycinin. Reshuffling of S-S/SH groups probably broke the disulphide bond. This is in agreement with results by others (Yamagishi et al., 1987; Lakemond et al., 2000b). At 0.5 M NaCl, part of the AB subunit stayed intact on heating. This might be explained by the dissociation/association processes being slower compared to those at 0.1 M NaCl as observed by Wolf and Tamura (1969). At low pH, the disulphide bonds seem to remain intact, because intact AB subunits were observed at non-reduced conditions and as much acidic as basic polypeptides at reduced conditions. This suggests a difference in denaturation mechanism at low and high pH, which might be due to the much higher activity of the S-S/SH interchange reaction around pH 7 than around pH 3.

In SPI, part of the basic polypeptides remained dissolved as (part of the) soluble aggregates on heating a 1% dispersion at high pH. In the case of purified glycinin all the basic polypeptides would have been precipitated (Hashizume et al., 1975; Yamagishi et al., 1987; Lakemond et al., 2000b). The presence of β-conglycinin in SPI prevented complete precipitation of the basic polypeptides as was observed earlier by other researchers (Damodaran & Kinsella, 1982; Yamagishi et al., 1983). They concluded that a heat-induced complex was formed between basic polypeptides of glycinin and β subunits of β-conglycinin. The smaller amount of dissolved β subunits compared to α and α' at 0.5 M in our results might be explained by this complex formation. At low pH, there are no indications of complex formation between polypeptides of glycinin and subunits of β-conglycinin. The lower solubility of α, α' subunits of β-conglycinin with regard to the β-subunits is not understood.

Heat-induced aggregates larger than 100 kDa could only be seen on SDS-PAGE gels under non-reduced conditions. As is discussed before, it is very likely that disulphide bridges play a role in aggregate formation at high pH. The absence of aggregates at low pH under reduced conditions might indicate that disulphide bridges are also important for aggregation at low pH. However, it is more likely that the reduction of the S-S bridge between the acidic and basic polypeptides during the analysis has facilitated the break-up of the aggregates.

3.5 Conclusions

In conclusion, variations in the association/dissociation behaviour of the soy proteins on heating as a function of pH might be a reason for differences in the rheological properties of the soy protein gels. At pH 3-5 all protein seems to participate in the network, whereas at pH > 5 less protein and especially less acidic polypeptides take part in the network resulting in less stiff gels. In this study, the effect of pH on gel formation and gel properties was larger than that of salt concentration. Denaturation proved to be a prerequisite for gel formation under all conditions studied.

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Chapter 4

Relations between rheological properties and network structure of soy protein gels

Abstract

This paper focuses on the relations between network structure and rheological properties of soy protein gels as a function of pH and ionic strength. Network structure has been characterised independently by permeability measurements and confocal scanning laser microscopy in terms of coarseness. Results showed that gels at pH 3.8 and 5.2 were coarser than at pH 7.6, except for pH 3.8-0 M NaCl. Rheological properties determined were dynamic moduli, Young's modulus, fracture stress and fracture strain. Gels at pH 3.8 had lower fracture strains and higher moduli than gels at pH 5.2 and 7.6, while fracture stresses were about the same. To relate the rheological properties to network structure in a self-consistent way, an additional structural parameter was required: curvature of the strands.

Renkema, J. M. S. *submitted for publication.*

4.1 Introduction

Heat-induced soy protein gels are important for the texture of several food products. During manufacturing of food products conditions vary greatly due to variations in pH, salt content, combination of ingredients, etc. Such variations will affect the gel formation process, network structure, rheological and water holding properties of the formed gel.

It has been shown experimentally that the rheological properties of soy protein gels vary with pH and ionic strength (van Kleef, 1986; Nagano et al., 1994; Renkema, 2001a). These differences in rheological properties could partly be explained by the amount of protein incorporated into the network. Gels were less stiff (lower storage modulus G') when more protein remained dissolved after heating instead of being part of the network (Renkema et al., 2000; Renkema, 2001a). However, this factor can not explain the whole difference. From work on other proteins, (*e.g.* van Kleef, 1986; Doi & Kitabatake, 1989; Bremer et al., 1990; Stading & Hermansson, 1990; Verheul & Roefs, 1998a, b; Mellema et al., 2001), it is known that also network structure determines the rheological properties of gels. For soy proteins, gel stiffness in relation to network structure has not been studied yet.

Hermansson (1994) divided network structure roughly into fine-stranded and coarse-aggregated networks. Fine-stranded gels may be completely transparent and are composed of strands with a thickness up to a few times the size of a single protein molecule. Coarse gels are non-transparent and are composed of particles with diameters in the range of 100-1000 times a single protein molecule. Intermediate structures containing fine-stranded and coarse structures simultaneously do also exist. The type of gel that is formed depends on conditions during gel formation. In general, gels become coarser as the pH approaches the isoelectric point or when the ionic strength is increased (Doi, 1993).

The permeability of gels increases and the ability to retain water decreases when the network structure coarsens. However, the relationship between the coarseness of gels and their rheological properties is not clear. Stading and Hermansson (1991), for example, observed that fracture strain and stress of transparent, fine-stranded gels formed at a pH higher than 6 were larger than those of fine-stranded gels formed at a pH lower than 4. For the same gels, microscopic observations showed that the strands at high pH are more curled and have a larger contour length between the junctions than those at low pH (Langton & Hermansson, 1992). Bremer et al. (1990) observed that coarse acid casein gels with straight strands were stiffer (higher storage modulus G') and had a smaller fracture strain than those with curved strands. Both examples indicate that besides coarseness also curvature of the strands affects the rheology of the gels.

The curvature of the strands together with strand connectivity determines in what way strands will be deformed microscopically, *i.e.* by bending or by stretching (Mellema et al.,

2001). The dominant type of deformation of gels with curved strands is bending and that of gels with straight, interconnected strands is stretching. In general, gels with curved strands show higher fracture strains than those with straight strands, because strong deformation of curved strands involves first straightening and then stretching of the strands. Further, one expects that gel stiffness is lower for gels with curved strands than for those with straight strands, because the resistance against bending is lower than against stretching.

In this paper, we investigated the relations between rheological properties of soy protein gels and their network structure, in terms of curvature of the strands and coarseness, as a function of pH and ionic strength. Permeability measurements and confocal scanning laser microscopy were used to study coarseness of the gels. Gel properties, such as stiffness, fracture stress and strain, were determined by small and large deformation rheology.

4.2 Material and methods

4.2.1 Soy protein isolate (SPI)

SPI was prepared from mildly treated, defatted PDI 80 soy flakes (Cargill BV, The Netherlands) according to a method described earlier (Renkema, 2001b). SPI had a protein content (N × 6.25) of 97%.

4.2.2 Preparation of protein dispersions

SPI dispersions were prepared by suspending the freeze-dried SPI in 0, 0.2 and 0.5 M NaCl solutions at higher concentrations than required for the experiments. After stirring for one hour the suspension was brought to pH 7.6 with a defined amount of 0.5 M NaOH. For experiments carried out at pH 3.8 and 5.2, the pH of the sample was adjusted after one hour using 0.5-1 M HCl. The SPI dispersions were stirred overnight to enhance protein dissolution. Finally, they were diluted by adding water or salt solution to obtain the desired protein concentration. None of the dispersions was completely transparent.

4.2.3 Rheological measurements at small deformation

Gel formation of soy protein dispersions (120 mg/g) was induced by heating from 20 to 95°C at a heating rate of 1 K/min, keeping the temperature at 95°C for 60 min, and cooling down to 20°C at a cooling rate of 1 K/min. Dynamic rheological measurements were performed in a Bohlin CVO rheometer using the serrated concentric cylinder geometry C25 (content 13 ml). The storage (G') and loss (G'') modulus were measured at a constant strain of

0.01, which was within the linear region, and at an angular frequency of 0.63 rad/s. A thin layer of soy oil was put on top of the samples to prevent evaporation of water.

4.2.4 Rheological measurements at large deformation

For large deformation measurements, gels were prepared by heating soy protein dispersions (120 mg/g) in cylindrical glass moulds with an inner diameter of 18 mm and a height of 115 mm. The moulds were filled three-quarters full to enable air bubbles to escape, and placed vertically in a waterbath. Heating conditions were the same as described for the small deformation experiments. After preparation the gels were removed from the moulds and cut into test pieces of 20 mm height by means of a stainless steel wire.

Fracture properties of the gels were tested in uniaxial compression in a Zwick material-testing machine, fitted with a 50 N load cell. The test pieces were compressed between two parallel plates at an initial relative deformation rate of 0.05 s^{-1} until fracture occurred. Measurements were performed at 20°C and repeated 4-6 times. Mean values with their standard deviations for fracture stress and strain and Young's modulus were calculated as described before (Renkema, 2001b).

4.2.5 Permeability measurements

Permeability coefficients were determined by measuring the flow rate of water or salt solutions through the soy protein gels. The flow rate Q is related to the permeability coefficient B according to the Darcy relation:

$$Q = \frac{B \cdot A_c}{\eta} \cdot \frac{\Delta P}{l}$$

in which A_c is the cross-sectional area of the gel through which the liquid is permeating, ΔP the applied pressure difference over a distance l and η the viscosity of the water or salt solution.

Measurements were performed according to the method developed by van Dijk and Walstra (1986). Gels (80 mg/g) were prepared in open-end glass tubes with an inner diameter of 4 mm and a height of about 25 cm. The tubes were placed in a glass cylinder that was filled with SPI dispersion. The height of the dispersion in the tubes was about 8 cm. The cylinder was closed airtight and placed in a waterbath. The same heating conditions were applied as described for the small deformation experiments. After heat treatment, 12 tubes together with 4 reference tubes were placed in a rack in a thermostated measuring vessel (20°C) made of Plexiglas. The vessel was filled with the solution (water, 0.2 or 0.5 M NaCl) used for dissolving the SPI. The level of the solution was higher than the top of each gel, so there was

a pressure gradient over the gel (Figure 4.1). The initial pressure gradient was about 10 kPa/m. The level of the liquid on top of the gel h_t was monitored at regular time intervals by means of a travelling microscope. Permeability coefficients were calculated with the following equation:

$$B = -\frac{1}{\Delta t} \cdot \frac{\eta \cdot l}{\rho \cdot g} \cdot \ln\left[\frac{h_\infty - h_t}{h_\infty - h_0}\right]$$

where h_∞ is the level in the reference tubes, h_0 the level at time 0, ρ the density of the permeating liquid and Δt the time difference.

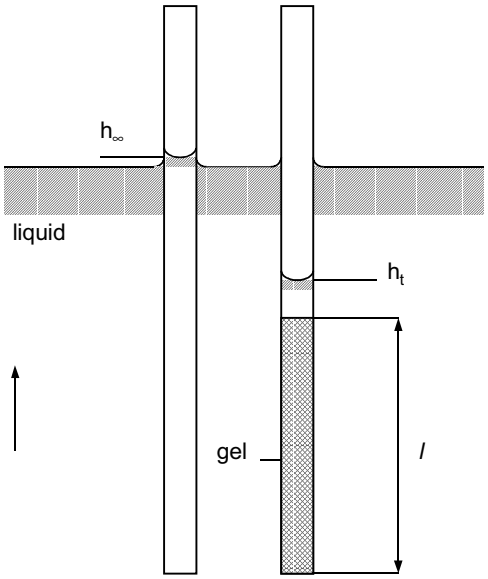

Figure 4.1 Schematic representation of a permeability measurement. For explanation of the symbols see text.

4.2.6 Confocal scanning laser microscopy (CSLM)

SPI dispersions (100 mg/g) were made with the fluorescent dye Rhodamine B (~10 µg/g protein) that stains the protein phase. Gels were prepared in special slides with a shallow hole. After addition of the protein dispersion, the sample was covered with a glass cover slip and sealed with nail polish to prevent evaporation. The slides were transferred to a plastic box, covered with a thin layer of soy oil to enhance heat transfer and heated in a waterbath according to the previously mentioned temperature profile. The gels were studied in single photon mode in a Leica TCS SP Confocal Scanning Laser Microscope, configured with an inverted microscope using a Ar/Kr laser for excitation of the dye (wavelength 543 nm) and spectroscopic filtering of the emitted fluorescence (625 nm). Pictures were taken with a water-immersed 63× objective at a depth of 20 µm below the cover slip surface.

4.3 Results

In this work, we investigated the effect of pH and ionic strength on rheology and network structure of soy protein isolate gels at conditions relevant to food systems: pH 3-7 and salt concentration of 0-0.2 M. To allow comparison with literature, also the conditions 0.5 M salt added and pH 7.6 were studied. Gel formation was induced by heating at 95°C. At every condition studied, this temperature was higher than the onset denaturation temperature (Renkema, 2001a). For practical reasons, protein concentration was not the same in each experiment. Large deformation experiments needed a relatively high protein concentration to obtain a stand-up gel, especially at pH 7.6, whereas for permeability measurements the protein concentration had to be as low as possible. At pH 7.6, permeability measurements could only be performed at protein concentrations of 8-10%. At higher concentrations permeability was too low to measure, whereas at lower concentrations the gels were not firm enough.

4.3.1 Rheological measurements at small deformation

Figure 4.2 shows the storage modulus G', which is a measure of the stiffness of gels, after completion of the temperature program as a function of pH and ionic strength. Highest values of G' were observed at pH 3.8, whereas at pH 7.6 G' was lowest. At pH 5.2 intermediate values were obtained. The influence of the NaCl concentration seemed to depend on pH. At pH 3.8, G' was highest at 0 M NaCl, whereas at pH 5.2 G' was highest at

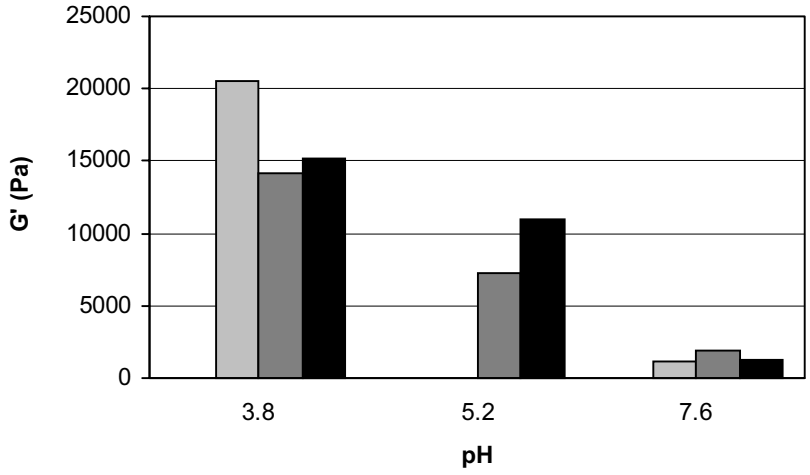

Figure 4.2 Storage modulus, G', of 12% (w/w) SPI gels after a complete temperature cycle at pH 3.8, 5.2 and 7.6. Concentration of added NaCl: 0 M (light gray bars), 0.2 M (dark gray bars) and 0.5 M (black bars).

0.5 M NaCl and at pH 7.6 at 0.2 M NaCl. No data at pH 5.2 and 0 M NaCl could be given owing to protein precipitation before and during heating.

4.3.2 Rheological measurements at large deformation

Fracture properties of soy protein gels were determined by large deformation rheology. Apart from the relevance of these properties for food applications, the fracture properties give information on the curvature of the strands (Bremer et al., 1990; Mellema et al., 2001). Namely, strand curvature determines how far one has to pull the two end points of a strand apart in order to straighten it. Figure 4.3 shows representative stress-strain curves as a function of pH and ionic strength. Fracture took place at the point where stress values were at a maximum, except for pH 5.2 (0 and 0.2 M NaCl). In these cases, an arrow marks fracture. Table 4.1 gives values for fracture strain, fracture stress and Young's modulus.

Gels at pH 5.2 (0 and 0.2 M NaCl) had very poor water holding properties. Water flew out as soon as the gels were pressed. These gels fractured at a high strain and stress and returned to their old shape after removal of the pressure. The gels had a sponge-like character, although their stress-strain curves did not resemble those of sponges' (Gibson & Ashby, 1988). At a higher salt concentration (0.5 M NaCl), gels were slightly humid after compression and broke at a smaller stress and strain than gels at a lower salt concentration.

Gels at pH 3.8 were also slightly humid after compression. They exhibited the lowest fracture strain and broke into several fragments. Fractures stresses at pH 3.8 were comparable to those at pH 7.6. At pH 7.6 gels were jelly-like and somewhat sticky. Young's moduli (Table 4.1) show the same trends as the storage moduli (Figure 4.2).

Table 4.1 Fracture strain and stress and Young's modulus of soy protein isolate gels (120 mg/g). Mean values (4-6 samples) and standard deviations are given.

		Fracture strain (-)	Fracture stress (kPa)	Young's modulus (kPa)
pH 3.8	0 M	0.28 ± 0.01	13.7 ± 0.4	42.1 ± 0.3
	0.2 M	0.38 ± 0.02	12.0 ± 2.4	31.8 ± 5.4
	0.5 M	0.40 ± 0.04	12.9 ± 2.6	34.2 ± 2.6
pH 5.2	0 M	1.1 ± 0.02	30.9 ± 4.8	10.5 ± 0.2
	0.2 M	1.05	25.0	5.9 ± 2.8
	0.5 M	0.68 ± 0.03	20.7 ± 2.9	24.5 ± 2.2
pH 7.6	0 M	0.79 ± 0.03	10.4 ± 1.0	3.9 ± 0.,1
	0.2 M	0.61 ± 0.05	7.8 ± 1.3	4.9 ± 0.3
	0.5 M	0.69 ± 0.04	10.3 ± 1.0	5.5 ± 0.3

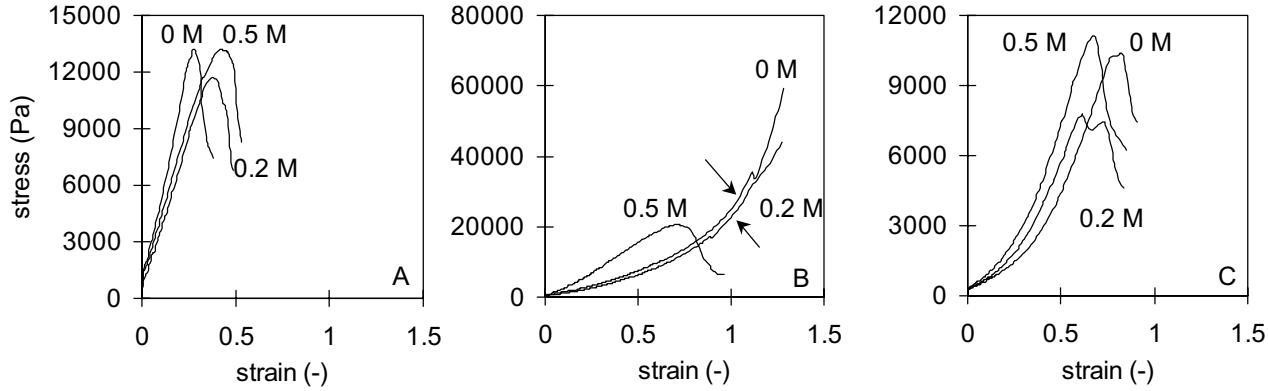

Figure 4.3 Representative examples of stress-strain curves of 12% (w/w) SPI gels at pH 3.8, 5.2 and 7.6 and 0, 0.2 or 0.5 M added NaCl.

4.3.3 CSLM

The microstructure of soy protein gels was studied using confocal scanning laser microscopy. CSLM pictures (Figure 4.4) show in most cases a clear difference in network structure as a function of pH and ionic strength. White and gray areas in the pictures represent protein while black areas represent the pores of the network containing the continuous phase.

At pH 3.8, coarse networks with a clear contrast between strands and pores were found at 0.2 M (Figure 4.4B) and 0.5 M NaCl (data not shown). A finer structure was observed at 0 M NaCl (Figure 4.4A).

At pH 5.2 and 0.2 M NaCl (Figure 4.4C), a very coarse structure was observed, whereas at 0 M the sample was too inhomogeneous to get a representative picture. The inhomogeneity of the samples was either due to intensive protein precipitation or because the gel was so coarse that no proper network could be seen within the geometry of the microscopic slide. At pH 5.2 (0.5 M NaCl) (Figure 4.4D) and pH 7.6 (all NaCl concentrations), no clear network structures could be observed due to large white spots and a bad contrast between strands and pores. Likely, the poor contrast is caused by the presence of soluble protein in the pores. The presence of white spots can not be explained. It could be related to the amount of soluble protein present in the pores. However, at a condition with no soluble protein present (pH 3.8-0 M NaCl), also some large white spots were observed. More research is necessary to establish the identity of the white spots.

4.3.4 Permeability measurements

Figure 4.5 shows the permeability coefficient *B* as a function of time at several pH values at 0.2 M NaCl. Note that a log scale is used for *B*. *B* is constant during the time span of the experiment (~24 hours) and is correlated to the square of the pore radius which in turn is

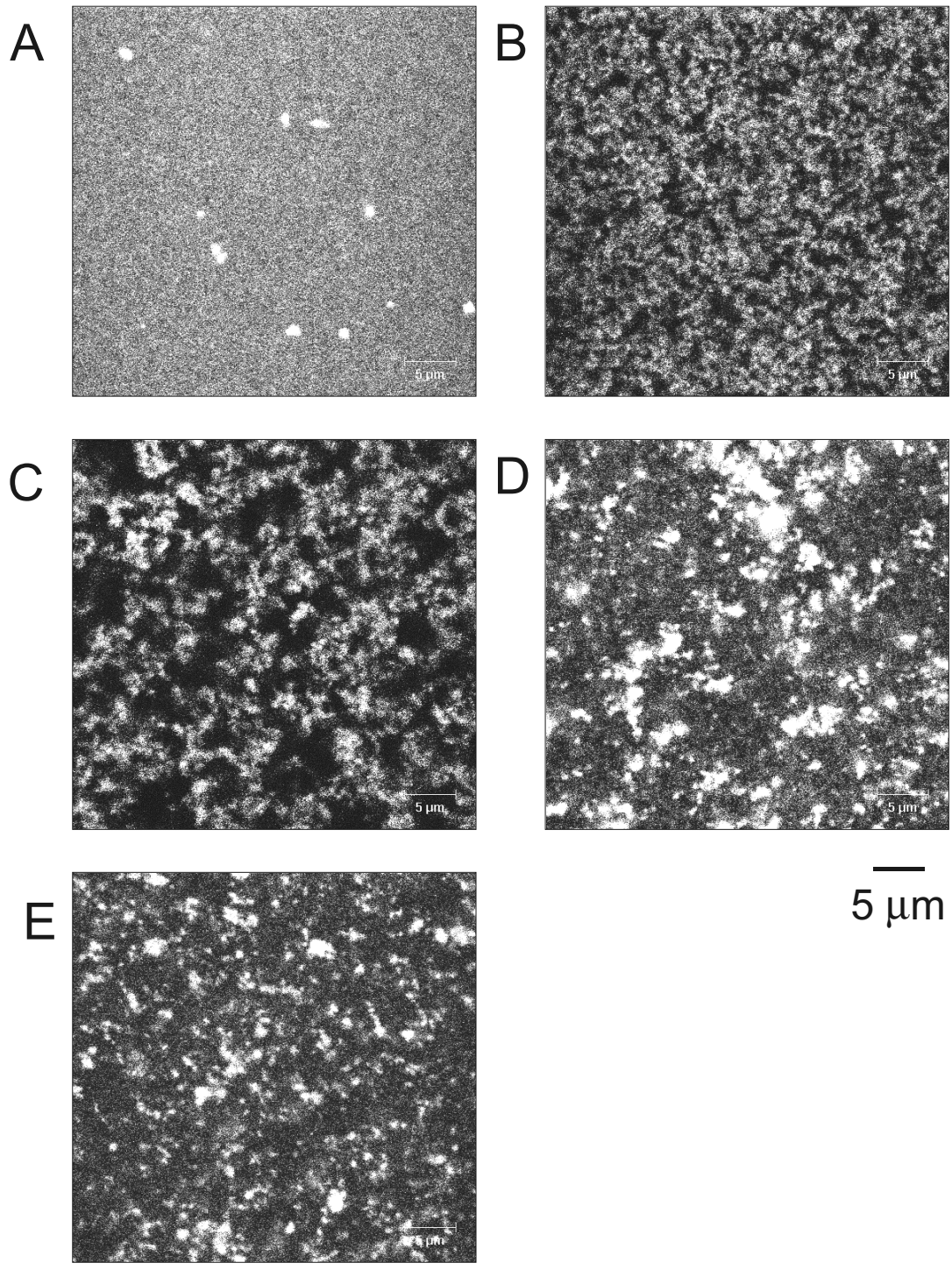

Figure 4.4 CSLM pictures of 10% (w/w) SPI gels. A: pH 3.8, 0 M NaCl; B: pH 3.8, 0.2 M NaCl; C: pH 5.2, 0.2 M NaCl; D: pH 5.2, 0.5 M NaCl; E: pH 7.6, 0.5 M NaCl.

correlated to the square of the aggregate radius (Bremer et al., 1989). At pH 5.2, permeability was much higher than at pH 3.8 and pH 7.6, which means that at this pH the coarsest gels were formed. However, at a higher salt concentration (Table 4.2), *B* of pH 5.2 gels was much lower and in the same order of magnitude as *B* of pH 3.8 gels. At 0 M NaCl, no experiments could be performed at pH 5.2 because of instability of the protein dispersion against precipitation before and during heating. At pH 3.8 (0 M), *B* is lower than at 0.2 and 0.5 M NaCl. At pH 7.6, permeability was low at both salt concentrations. Apart from the finer network structure at this pH, the presence of soluble protein in the pores might have retarded the liquid flow resulting in lower *B* values.

A high permeability of gels means in practice that the water holding capacity of the gels is very low. At pH 5.2 (0 and 0.2 M NaCl) this low water holding capacity was also clear from the water loss when pressing the gels.

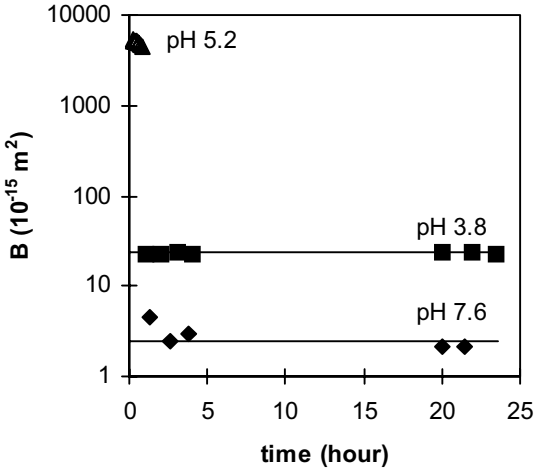

Figure 4.5 Examples of the permeability coefficient *B* of 8% (w/w) SPI gels as a function of time at pH 3.8, 5.2 and 7.6 and 0.2 M added NaCl.

Table 4.2 Permeability coefficient *B* of gels of soy protein isolate (80 mg/g) as a function of pH and ionic strength. Mean values of 4-12 samples are given.

	B (10^{-15} m^2)		
	0 M NaCl	0.2 M NaCl	0.5 M NaCl
pH 3.8	3.4	24	17
pH 5.2	precipitated	5050	12
pH 7.6	too weak	5.1	4.1

4.4 Discussion

Table 4.3 gives a summary of the results obtained in this study on the relation between rheological properties and network structure of SPI gels as a function of pH and ionic strength. A first characterization will be in terms of coarseness, which refers to the inhomogeneity of the gel (*i.e.* pore size and thickness of the strands). Information on coarseness of gels was obtained from CSLM and permeability measurements. For the latter, a high permeability means a large pore size and thus a coarser gel. From the appearance of the gels, one expects a coarser network structure at pH 3.8 and 5.2 (white gels, indicating particles of about 1 μm) than at pH 7.6 (turbid gels). Both CSLM and permeability measurements showed that indeed a coarser network structure was formed at pH 3.8 and 5.2 compared to pH 7.6, except for pH 3.8-0 M NaCl. At this condition, the gels had a more fine-stranded structure with a low permeability. At pH 5.2-0.2 M NaCl, the coarsest gels were observed. At pH 5.2 and higher salt concentration, a less coarse network structure was formed which is especially clear from the permeability data. CSLM pictures of pH 7.6 gels did not show a distinct network structure, but they were clearly different from those of the gels at the other pH values. This observation, in combination with the low permeability of these gels, strongly indicates that pH 7.6 gels are relatively fine-stranded.

From Table 4.3 it follows that there is no unequivocal relation between the modulus and the fracture strain of the gel and its coarseness. For example, at pH 3.8 (0 M), the structure is *not coarse* and yields a *high modulus* and *low fracture strain*, while at pH 7.6 the structure is *not coarse* and yields a *low modulus* and *intermediate fracture strain*.

To obtain a more direct relation between the elasticity modulus and network structure, one thus should use additional characteristics of the network. One such characteristic is the curvature of the strands. If the strands are curved, the modulus will be low, since the relevant deformation energy will be bending energy. If the strands are straight and interconnected, the modulus is high, since the relevant deformation energy is stretching energy, which is always much higher than bending energy (Mellema et al., 2001). The curvature of the strands can be inferred from the fracture strain, as obtained by large deformation rheology. During large deformations the strands will have to be straightened (involving a bending deformation) before they are stretched. So, the larger the strain at which fracture occurs, the more the strands had to be straightened before fracture. Therefore, high fracture strains denote that the strands are more curved for further identical systems.

Taking into account both the coarseness and strand curvature one *can* explain the difference in modulus between pH 3.8-0 M NaCl and pH 7, as mentioned above. Namely, from the fracture strains in Table 4.3 it follows that at pH 3.8 (0 M NaCl) a large number of

Table 4.3 Summary of rheological properties, permeability and structure of soy protein gels.

	[NaCl]	Modulus G', E	Fracture strain ε_f	Permeability B	CSLM *structure*	Appearance
pH 3.8:	0 M	high	low	low	fine	white
	0.2 M	high	low	intermediate	coarse	white
	0.5 M	high	low	intermediate	coarse	white
pH 5.2:	0 M	intermediate	high	-	-	white, loss of water on compression, elastic
	0.2 M	intermediate	high	high	coarse	white, loss of water on compression, elastic
	0.5 M	high	intermediate	intermediate	?*	white
pH 7.6:	0 M	low	intermediate	-	fine?*	light brown, sticky, jelly-like
	0.2 M	low	intermediate	low	fine?*	light brown, sticky, jelly-like
	0.5 M	low	intermediate	low	fine?*	light brown, sticky, jelly-like

G' = Storage modulus; E = Young's modulus; ε_f = fracture strain; B = permeability coefficient.

* No clear picture could be obtained. The presence of dissolved protein probably prevented a good contrast between strands and pores.

strands is straight, leading to a high modulus, while at pH 7.6 the strands are curved, leading to a lower modulus.

Other features of Table 4.3 can be explained as well using both curvature and coarseness. One can *e.g.* explain the difference in modulus between coarse gels at pH 3.8 and 5.2. Namely, from the fracture strains in Table 4.3 it follows that at pH 5.2, the strands are curved, leading to a lower modulus. Also one can explain that for different salt concentrations at pH 3.8, one did not observe large differences in modulus and fracture strain, although the gels ranged from fine-stranded to coarse. This is because the curvature of the strands did not change much as a function of salt concentration and because it is as hard to stretch ten small thin strands as one thick one which is ten times thicker.

At pH 5.2 and 0.5 M NaCl, a lower fracture strain, a somewhat higher modulus and a less coarse network structure are observed than at 0 and 0.2 M NaCl. The lower fracture strain at 0.5 M can be explained by a lower curvature of the strands, but it is not allowed to explain the higher modulus with a lower curvature of the strands. In all cases, the fracture strain is so high that strands are curved and deformed by bending. A possible explanation for the higher modulus is a change in protein-protein interactions due to a change in ionic strength. Also, the moisture losses during compression at 0 and 0.2 M NaCl might have affected the measurements of the modulus.

The fracture strain at pH 5.2 (0.5 M NaCl) is about the same as at pH 7.6, whereas the modulus is much higher than at pH 7.6. This can not be explained by only considering the effect of curvature, since then the same modulus would be expected. In addition, one has to incorporate the coarseness of the structure, in terms of the thickness of the strands. Namely thin strands are easier to bend than thick strands, since the bending load of a cylinder scales with the diameter^4 (Young, 1989), which implies a lower G' with thinner strands. This will partly explain why the coarse pH 5.2 gels with curved strands have a higher modulus than the fine-stranded pH 7.6 gels with curved strands. Another factor is the attractive interaction energy between the protein molecules, which is expected to be higher closer to the isoelectric point (near pH 5.2) than at pH 3.8 or 7.6.

The above suggests that a direct relation between network structure and elastic modulus can be obtained if both coarseness and curvature of the strands are taken into account, assuming that other factors as interaction forces do not change largely.

Network structure also affects the fracture stress of gels. No clear effect of curvature of the strands on the fracture stress is expected since during large deformations all stressed curved strands will be straightened implying that all strands will break in tension. However, coarseness of the gels is expected to affect the fracture stress. The fracture stress will be lower when the defects in the gel are larger, *i.e.* for a coarser gel (van Vliet & Luyten, 1995). In addition, the fracture stress is determined by the number of protein-protein bonds per cross-

section. This may be exemplified at pH 7.6, where probably less protein is incorporated in the strands (Renkema, 2001a), which might lead to smaller fracture stresses. The combined effects of the three parameters (curvature, coarseness and number of protein-protein bonds) explain that the difference in fracture stress between the coarse gels at pH 3.8 and the relatively fine-stranded gels at pH 7.6 is only small.

The reason why the fracture stress at pH 5.2 is highest could be due to the fact that this pH is close to the isoelectric point, possibly implying that the protein-protein interactions are strongest. The somewhat lower fracture stresses when the ionic strength is higher (pH 5.2) might be due to screening of the net charge of the protein molecules, leading to a decreased strength of the protein-protein interactions. Additionally, at 0.5 M added NaCl less protein participates in the network (Renkema, 2001a).

Summarizing, SPI gels exhibited different network depending on pH and ionic strength (Table 4.4). In order to relate the rheological properties of these gels to their network structure two structural parameters were found to be necessary to arrive at a self-consistent picture: coarseness of the gel and curvature of the strands in the gel. A rheological categorization as a function of these two parameters is given in Figure 4.6.

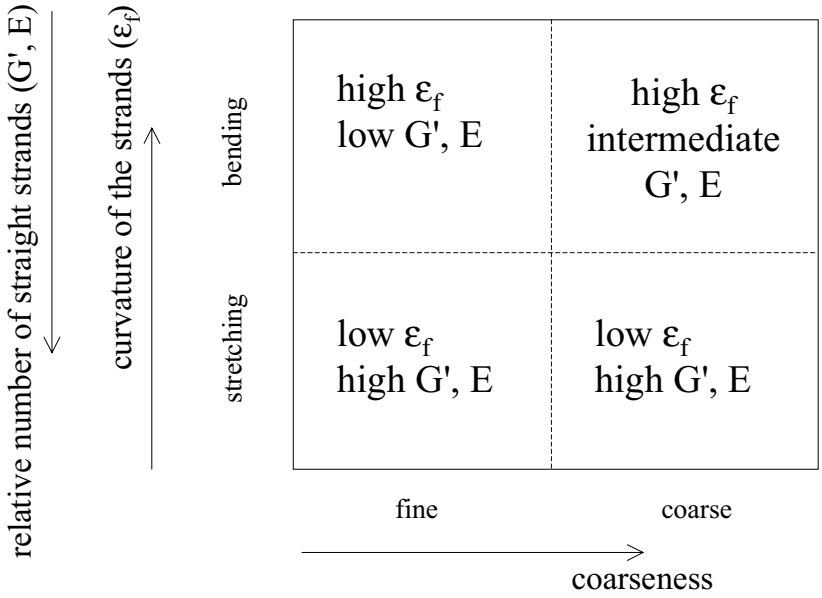

Figure 4.6 Rheological categorization of protein gels as a function of structural parameters, *i.e.* curvature of the strands, number of strands deformed in stretching deformation, and coarseness of the gel (thickness of the strands). The dotted lines between the categories indicate a gradual transition. Only main trends are indicated. ε_f = fracture strain; G' = storage modulus.

Table 4.4 Network structure of SPI gels, in terms of coarseness and curvature of the strands, as a function of pH and ionic strength.

		coarseness[a]	strand curvature[b]
pH 3.8:	0 M NaCl	+	0
	0.2, 0.5 M NaCl	++	0
pH 5.2:	0, 0.2 M NaCl	+++	++
	0.5 M NaCl	++	+
pH 7.6:	0, 0.2, 0.5 M NaCl	+	+

[a] +, fine-stranded; ++, coarse; +++, very coarse

[b] 0, +, ++: increasing degree of curvature of the strands

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Chapter 5

Gel formation by β-conglycinin and glycinin and their mixtures

Abstract

Gel formation and gel properties of β-conglycinin, glycinin and their mixtures were studied as a function of pH using small and large deformation rheology and differential scanning calorimetry. We conclude that heat denaturation is a prerequisite for gel formation. Gelation temperatures of β-conglycinin were lower than those of glycinin and more dependent on protein concentration. At pH 7.6, protein solutions gelled at a higher temperature than at pH 3.8.

Glycinin gels were stiffer than β-conglycinin gels at the same pH and protein concentration, and fractured at a higher strain and stress. At pH 7.6, G' is lower than at pH 3.8 for both proteins and the gels could be deformed to a larger extent. Based on the appearance of the gels (turbid at pH 7.6, white at pH 3.8) and the fracture properties, we conclude that different network structures are formed as a function of pH. The reason why glycinin gives a better gel than β-conglycinin is believed to be due to a difference in network structure as well as in strength of interaction between the protein molecules.

Mixing of both soy proteins resulted in improved gelling properties at pH 3.8. The elastic modulus of the mixture was larger than the weighed sum of the separate contributions. Furthermore, mixing reduced the protein dispersability at pH 7.6. This strongly indicates the presence of an interaction between the proteins. Gels of the 1:1 mixture (pH 3.8) had a fracture stress and strain in between those of the gels of the separate proteins.

Renkema, J. M. S., Knabben, J. H. M., & van Vliet, T. *Food Hydrocolloids*, in press.

5.1 Introduction

Soy proteins are used in several food products because of their highly nutritive value and ability to improve texture. They consist of two major components, β-conglycinin and glycinin. β-conglycinin, a 7S globulin, is a trimeric glycoprotein consisting of three types of subunits (α', α, and β) in seven different combinations (Thanh & Shibasaki, 1976) with a molecular weight of about 180 kDa (Koshiyama, 1983). At pH 7.6 and an ionic strength less than 0.1, β-conglycinin forms dimers (a 9S globulin) (Koshiyama, 1968). Glycinin consists of an acidic and a basic polypeptide which are linked by a disulphide bridge. At pH 7.6 and high ionic strength (0.5 M), it exists as a hexamer (an 11S globulin) with a molecular weight of about 360 kDa. At pH 3.8 and low ionic strength (0.03 M), glycinin is predominantly in a trimeric form (a 7S globulin) with a molecular weight of about 180 kDa (Danilenko et al., 1987; Utsumi et al., 1987; Lakemond et al., 2000a).

At the same pH and ionic strength, β-conglycinin is less heat-stable than glycinin (Puppo & Añón, 1999a). Denaturation temperatures of both proteins are higher at pH 7.6 than at pH 3.8, and they are higher at a higher ionic strength (Maruyama et al., 1998; Maruyama et al., 1999; Lakemond et al., 2000b). Since denaturation is believed to be a prerequisite for gel formation (Kinsella, 1976), one expects β-conglycinin to form a gel at lower temperatures than glycinin, which has indeed been observed by Nagano et al. (1994a).

Gel properties of (pure) β-conglycinin and glycinin are affected by protein concentration, heating temperature, ionic strength, and pH (Babajimopoulos et al., 1983; Hermansson, 1985; Utsumi & Kinsella, 1985; van Kleef, 1986; Nagano et al., 1992; Nagano et al., 1994a; Nagano et al., 1994b, c; Puppo & Añón, 1999b; Wongprecha et al., 2000). In mixed systems like in soy protein isolate also the ratio β-conglycinin:glycinin influences gel formation and gel properties (Nakamura et al., 1986a, b; Kang et al., 1991; Nagano et al., 1996).

Most of the studies on gel formation by soy proteins have been performed at pH 7.6. In a previous study with soy protein isolate, consisting mainly of β-conglycinin and glycinin, we observed that the gelation coincided with heat denaturation of β-conglycinin at pH 3.8 and 5.2, whereas at pH 7.6 gel formation started at the heat denaturation temperature of glycinin (Renkema et al., 2000). This difference in gelation temperature of soy protein isolate as a function of pH suggests a different gel network and different gel properties. In this study we examined gel formation and gel properties of systems containing β-conglycinin, glycinin, and mixtures thereof, as a function of pH, using small and large deformation rheology and DSC.

5.2 Material and methods

5.2.1 Material

Two different protein preparations were used for the experiments: purified glycinin (ca. 95% pure) and a β-conglycinin rich fraction, which consists for about 60% of β-conglycinin, 15-20% of glycinin, and for the rest of other soy proteins (like Kunitz trypsin inhibitor and Bowman Birk inhibitor) as determined by SDS-PAGE analysis. Glycinin was isolated from Williams'82 soybeans (harvest 1994) by isoelectric precipitation at pH 6.4 as described by Lakemond et al. (2000a). The β-conglycinin rich fraction was obtained from the retentate of the glycinin isolation by isoelectric precipitation at pH 4.8. The purified glycinin and the β-conglycinin rich fraction were resuspended (135 and 69 mg/ml, respectively) in a 35 mM potassium phosphate buffer with 0.4 M NaCl containing 20% glycerol and stored at –40°C.

Prior to gelation experiments the protein preparations were dialysed against double-distilled water, freeze-dried, and subsequently dispersed in 0.2 M NaCl solutions at higher concentrations than required. The suspensions were brought to pH 7.6 with a defined amount of 0.5 M NaOH. For experiments carried out at pH 3.8, the pH of the dispersion was adjusted after one hour using 1 M HCl. For the experiments in which the concentration or the ratio of glycinin/β-conglycinin was varied, stock dispersions were prepared of 120 mg/g and 80-90 mg/g, respectively. Protein concentration in the dispersions was checked by micro-Kjeldahl analysis (AOAC, 1980) using a Kjeldahl factor of 6.25.

As a reference, protein dispersions (120 mg/g) of soy protein isolate (SPI) in 0.2 M NaCl were used. Preparation of the isolate and dispersions of it was described earlier (Renkema et al., 2000).

5.2.2 Differential scanning calorimetry (DSC)

Protein heat denaturation was monitored by differential scanning calorimetry in a micro-DSC (Setaram, France). Protein concentration of the glycinin and the β-conglycinin-rich suspensions was 90 mg/g. Stainless steel vessels were used containing 0.9 ml of the samples. The samples were scanned from 20 to 115°C at a scanning rate of 1 K/min and subsequently cooled to 20°C at the same rate. The temperature at which denaturation starts, the onset denaturation temperature (T_o), was calculated by taking the intercept of the baseline and the extrapolated slope of the peak. For T_p, the peak denaturation temperature, the temperature of maximum heat flow was taken.

5.2.3 Rheological measurements at small deformation

Gel formation of soy protein dispersions (20-120 mg/g) was followed in dynamic measurements in a Bohlin CVO rheometer using the serrated concentric cylinder geometry C25 (content 13 ml). Measurements were performed at a constant strain of 0.01, which was within the linear region, and at an angular frequency of 0.63 rad/s. A thin layer of soy oil was put on top of the samples to prevent evaporation of water. To induce gel formation, samples were heated from 20 to 95°C at a heating rate of 1 K/min, kept at 95°C for 60 min, and cooled down to 20°C at a cooling rate of 1 K/min.

5.2.4 Rheological measurements at large deformation

Gels were prepared by heating soy protein dispersions (80 mg/g) in cylindrical glass moulds with an inner diameter of 18 mm and a height of 115 mm. The moulds were filled three-quarters full, to enable air bubbles to escape, and placed vertically in a waterbath. Heating conditions were the same as described in section 2.3. Immediately after preparation, fracture properties of the gels were tested in uniaxial compression in a Zwick material-testing machine, fitted with a 50 N load cell. The gels were removed from the moulds and cut into test pieces of a height of 20 mm by means of a stainless steel wire. The test pieces were compressed between two parallel plates at an initial relative deformation rate of 0.05 s^{-1} until fracture occurred. The measurement temperature was 20°C. Measurements were repeated 3-8 times and mean values with their standard deviations for fracture stress and strain were calculated.

The relative deformation at a certain stage is expressed as a Hencky strain, ε_h (-), defined as:

$$\varepsilon_h = \ln \frac{h(t)}{h_0}$$

where h_0 is the original height of the test piece, and $h(t)$ the height after a certain deformation time t. For compression the Hencky strain is negative, but it will be expressed as a positive figure.

The average stress in the test piece during deformation at time t, $\sigma(t)$ (Pa), is given by:

$$\sigma(t) = \frac{F(t)}{A(t)}$$

where $F(t)$ is the force per unit of area $A(t)$. Assuming that the volume of the test piece does not change during compression and that its shape remains cylindrical, the cross section area of the test piece at time t is:

$$A(t) = \frac{h_0}{h(t)} \cdot A_0$$

From the stress-strain curves Young's modulus E (Pa) was calculated according to:

$$E = \left(\frac{d\sigma}{d\varepsilon} \right)_{\varepsilon \to 0}$$

5.3 Results and discussion

Gelation experiments were carried out on systems containing either glycinin, a β-conglycinin-rich fraction (further denoted as β-conglycinin), or mixtures thereof, at pH 3.8 and 7.6. Because at pH 7.6 the mixing of glycinin and β-conglycinin did not result in homogenous suspensions, especially at increased proportions of β-conglycinin, no data on gel characteristics of these mixtures are reported at this pH. We add that SDS-PAGE analysis demonstrated that both proteins precipitated from the mixtures (data not shown). It was not determined whether the phase separation is accompanied by complexation between the two types of protein.

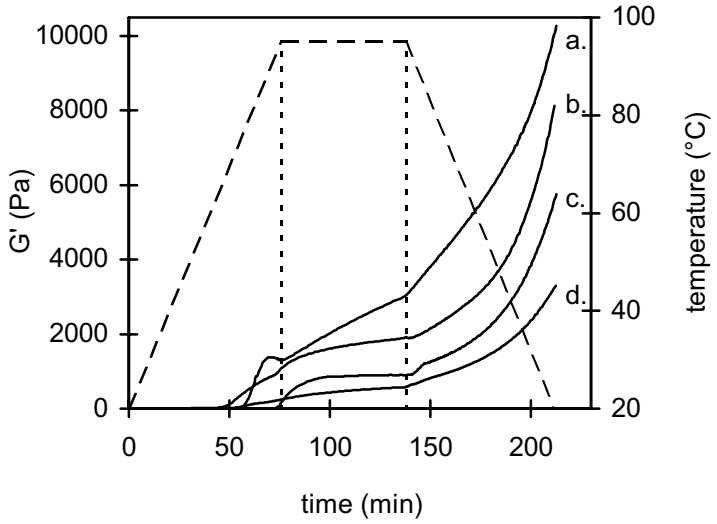

Figure 5.1 Typical gelation curves of soy protein gels at 0.2 M NaCl. a, glycinin (78 mg/g), pH 3.8; b, β-conglycinin (86 mg/g), pH 3.8; c, glycinin (93 mg/g), pH 7.6; d, β-conglycinin (89 mg/g), pH 7.6. The dashed line shows temperature against time.

5.3.1 Gelation temperature

Gel formation was induced by a gradual increase of temperature (1 K/min) and observed by dynamic measurements. Figure 5.1 shows examples of the behaviour of the storage modulus, G', through the temperature stages. At a certain temperature, G' starts to increase. During subsequent heating at 95°C G' kept increasing, but a much stronger increase was observed on cooling. The increase in G' on cooling could almost completely be reversed on heating, which means that no irreversible processes like formation of covalent bonds or rearrangements of the network structure took place at this stage (data not shown). During heating at 95°C, formation of more cross-links between the protein particles and/or ongoing incorporation of protein and, at pH 7.6, rearrangements in the network structure took place, all resulting in an increasing G' (Renkema, 2001). At pH 3.8, the bump in the gelation curve of glycinin at t = 73 min was found for all protein concentrations.

The onset of gelation, T_{gel}, was defined in two ways, namely as the crossover temperature of the storage modulus, G', and the loss modulus, G'', and as the temperature at which G' started to increase over 0.5 Pa/K. Table 5.1 shows gelation temperatures of suspensions of β-conglycinin, glycinin and mixtures of both as a function of pH and protein concentration. For glycinin, the two gelation temperatures were comparable. At pH 7.6, the gelation temperature was not clearly affected by glycinin concentration, whereas at pH 3.8 a gradual increase in T_{gel} was observed with decreasing concentration. For β-conglycinin, it was generally not possible to determine T_{gel} in both ways. At pH 3.8, no crossover point was observed; G' was and remained larger than G'' before and during heat treatment. At pH 7.6, the initial increase in G' was lower than 0.5 Pa/K. At a concentration of 60 mg/g, for example, the total increase of G' at the end of the heating stage from 20 to 95°C was only 4.5 Pa. When comparison was possible, T_{gel} at the crossover point was much smaller than T_{gel} at an increase of G' over 0.5 Pa/K. Moreover, T_{gel} was highly dependent on β-conglycinin concentration. The gelation temperature of mixtures depended on the proportion of β-conglycinin to glycinin. At a 3:1 ratio gelation occurred at the gelation temperature of β-conglycinin, whereas at lower ratios no gelation was observed at temperatures lower than T_{gel} of glycinin.

Table 5.2 shows gelation temperatures of suspensions of β-conglycinin (90 mg/g), glycinin (90 mg/g) and soy protein isolate (120 mg/g) in relation to onset (T_o) and peak (T_p) denaturation temperatures as determined by DSC. DSC-thermograms of glycinin showed one endothermic transition at pH 7.6 and two endothermic transitions at pH 3.8; The transition at the lowest temperature corresponds to the trimeric or 7S form and the transition at the highest temperature to the hexameric or 11S form (Danilenko et al., 1987; Lakemond et al., 2000b). Soy protein isolate and the β-conglycinin rich fraction showed two endothermic transitions at both pH values, corresponding to denaturation of β-conglycinin (lowest temperature) and

Table 5.1 Gelation temperatures of glycinin, β-conglycinin and mixtures as a function of concentration. T_{gel} (c.o.) is the temperature at the crossover point of G' and G". T_{gel} (0.5 Pa/K) is the temperature where G' starts to increase with 0.5 Pa/K.

		pH 7.6		pH 3.8	
	Conc (mg/g)	T_{gel} (c.o.) (°C)	T_{gel} (0.5 Pa/K) (°C)	T_{gel} (c.o.) (°C)	T_{gel} (0.5 Pa/K) (°C)
glycinin	*120*	84.4	91	69	69
	90	90	90	72	71
	60	-[1]	-[1]	76	74
	50	-[1]	-[1]	-[2]	80
	40	88	90	76	77
	30	88	91	75	79
	20	89	93	77	81
β-conglycinin	*120*	38	55	-[2]	55
	90	56	73	-[2]	61
	60	72	-[3]	-[2]	64
	40	72	-[3]	-[2]	65
	30	73	-[3]	68	93
	20	74	-[3]	84	-[3]
Mixture glyc.+ β-congl.	*75 + 25*	-[1]	-[1]	75	76
	50 + 50	-[1]	-[1]	72	72
	25 + 75	-[1]	-[1]	-[2]	64

-[1] not determined
-[2] no crossover point
-[3] increase was less than 0.5 Pa/K

Table 5.2 Gelation temperatures (c.o.; 0.5 Pa/K) of glycinin and β-conglycinin (90 mg/g) and soy protein isolate suspensions (120 mg/g) in relation to onset (T_o) and peak (T_p) denaturation temperatures.

	pH	T_{gel} (°C)	T_o (°C)	T_p (°C)
glycinin	7.6	90; 90	90	97
	3.8	72; 71	61 + 69	72+ 84
β-conglycinin	7.6	56; 73	71 + 93	76 + 99
	3.8	- ; 61	62 + 81	69 + 86
soy protein isolate	7.6	68; 90	71 + 88	75 + 93
	3.8	- ; 63	67 + 82	71 + 89

glycinin (highest temperature), respectively. In SPI, the transition band of glycinin was more intense compared to the band of β-conglycinin, whereas in the β-conglycinin rich fraction the glycinin band was less intense than the β-conglycinin band. At pH 3.8, the endothermic transition of 7S glycinin was not visible, which was likely due to overlap with the endotherm of β-conglycinin. In general, gelation temperatures were higher at pH 7.6 than at pH 3.8. Furthermore, β-conglycinin gelled at a lower temperature than glycinin. For β-conglycinin and glycinin, gelation started at temperatures in between the onset of denaturation, T_o, and the peak denaturation temperature, T_p, from which can be concluded that heat denaturation is a prerequisite for gel formation. Gelation of glycinin at pH 3.8 seems to start at the onset of denaturation of the 11S form and not of the 7S form. At pH 7.6, gelation temperatures of soy protein isolate were dependent on their definition. When looked at the crossover point of G' and G'', gelation coincided with denaturation of β-conglycinin, whereas using the criterion that G' has to increase more than 0.5 Pa/K, gelation concurred with denaturation of glycinin. At pH 3.8, gelation of soy protein isolate started a few degrees lower than the onset denaturation temperature of β-conglycinin, but at about the same gelation temperature as the β-conglycinin-rich fraction.

Determination of the gel point is discussed by several authors (*e.g.* Stading & Hermansson, 1990; Horne, 1999) and the discussion will not be repeated here. In our studies on soy protein gelation, we needed a definition that was appropriate at every condition of pH and ionic strength and for every soy protein preparation. A general definition of the gel point is the point where G' starts growing or where G' becomes greater than the background noise. In our studies these points could not be determined precisely enough over the whole temperature range, especially at conditions where the system already exhibited a gel-like behaviour. The multiple protein character of soy protein isolate also provided difficulties. In soy protein isolate (120 mg/g), heat denaturation of β-conglycinin only resulted in a total initial increase of G' of about 1 Pa at pH > 6, whereas that of glycinin resulted in a steep increase of G'. We chose to neglect this contribution of β-conglycinin by defining the gel point as the temperature at which G' started to increase over 0.5 Pa/K. The value 0.5 Pa/K was a somewhat arbitrary choice. In this study, we needed a second definition for the gel point to investigate whether β-conglycinin formed a gel. We chose the crossover point of G' and G''. This definition of the gel point turned out to work well over the whole concentration range at pH 7.6 only.

5.3.2 Rheological behaviour at small deformation

Figure 5.2 shows the storage modulus, G', after completion of the temperature cycle as a function of protein concentration on a double logarithmic scale. G' is lower at pH 7.6 than at

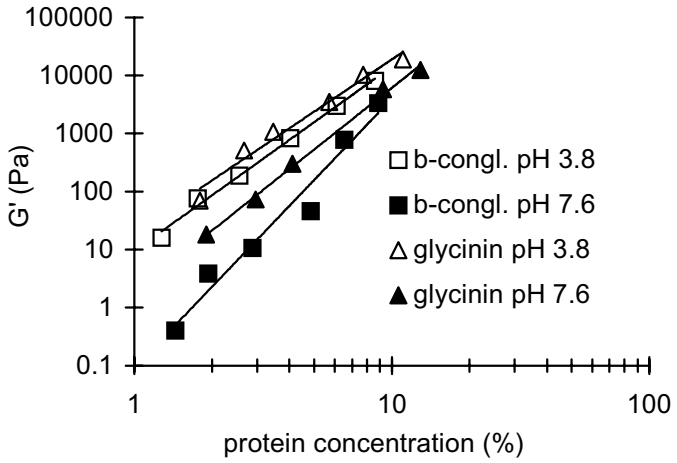

Figure 5.2 Storage moduli, *G'*, after completion of the temperature cycle of gels formed by glycinin and β-conglycinin at pH 3.8 and 7.6 in 0.2 M NaCl.

pH 3.8 for both glycinin and β-conglycinin. At the same pH, G' values of glycinin were higher than those of β-conglycinin.

The difference in stiffness of the gels as a function of pH is mainly determined by the amount of protein that is incorporated into the gel network and by the network structure. For glycinin and soy protein isolate, we observed that if more protein remained in solution during heating, a lower G' was obtained (Renkema et al., 2000). To our opinion, the dissolved protein is located in the pores of the network and will therefore not contribute to the stiffness of the gel. For glycinin, part of the protein remains dissolved after heating at pH 7.6 and an ionic strength of 0.2 M, whereas at pH 3.8 all protein precipitated (Renkema et al., 2000). For β-conglycinin, only solubility data at higher and lower ionic strength are available, which makes it impossible to relate solubility and gel stiffness. At 0.5 M, soluble aggregates were formed at pH 7.6 and insoluble aggregates at pH 3.8; at 0.08 M, soluble aggregates were formed at both pH values (Maruyama et al., 1999). The effect of network structure will be discussed in section 5.3.3.

Assuming a power-law relation between G' and protein concentration, c, according to $G' \propto c^x$, one may determine x at different times during heating and cooling. In Figure 5.3 the exponent x is given at three moments in the temperature programme, namely at the end of the heating stage to 95°C (t_1), of the isothermal stage (t_2), and of the cooling stage to 20°C (t_3). The values for x are within the normal range for protein gels (van Vliet, 2000). At pH 3.8, x remained the same during the heating and cooling cycle, whereas at pH 7.6 an increase was found. At the end of the temperature programme, x was higher at pH 7.6 than at pH 3.8, which

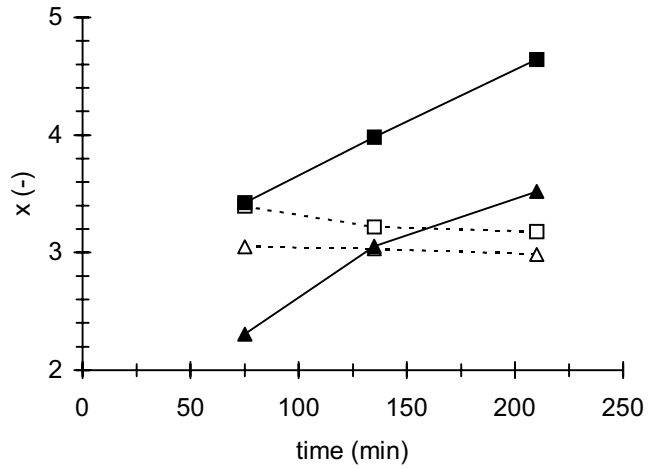

Figure 5.3 The exponent x in the power-law relation $G' \propto c^x$ at three moments in the temperature programme: at the end of the heating stage (t_1), the isothermal stage (t_2), and the cooling stage (t_3). β-Conglycinin (squares) and glycinin (triangles) gels at pH 3.8 (open symbols) and 7.6 (solid symbols) in 0.2 M NaCl.

might indicate that the protein in pH 7.6 gels was used less efficient and was distributed more heterogeneously. The exponent x yields information on the gel structure, in particular on the nature of the strands, α, and the fractal dimensionality of the clusters forming the gel, D_f, assuming that a fractal description holds. The relation between these two parameters is given by $x = \alpha/(3-D_f)$, and α ranges from 2 for straight, deformable strands to 4.3 for curved, very bendable strands (Bremer et al., 1989). Using the relation $x = \alpha/(3-D_f)$, a higher value for x at pH 7.6 implies a higher value for α and/or D_f. Since an increase in α is unlikely (*i.e.* the strands becoming more flexible during ongoing heating) (van Vliet & Mellema, 2000), a rise in D_f is expected. The rise in D_f could be caused by rearrangements. A high loss tangent, $tan\delta$, at high temperatures indicates the occurrence of rearrangements during ongoing heating at pH 7.6 (Renkema, 2001). However, D_f could also be increased because of more protein being incorporated in the gel network on prolonged heating (as observed by Verheul et al., 1998), which implies that a fractal description would not be valid anymore. Clearly, more research is necessary to give a satisfactory explanation of the increase in x with time.

Figure 5.4 shows the stiffness of mixtures of β-conglycinin and glycinin (about 80-90 mg/g) at different ratios at pH 3.8. The results were compared with a theoretical curve, which is the sum of the separate contributions to G' of β-conglycinin and glycinin at the specific protein concentrations assuming that two independent, not interfering networks were formed. The contributions of both proteins were calculated using the concentration dependence

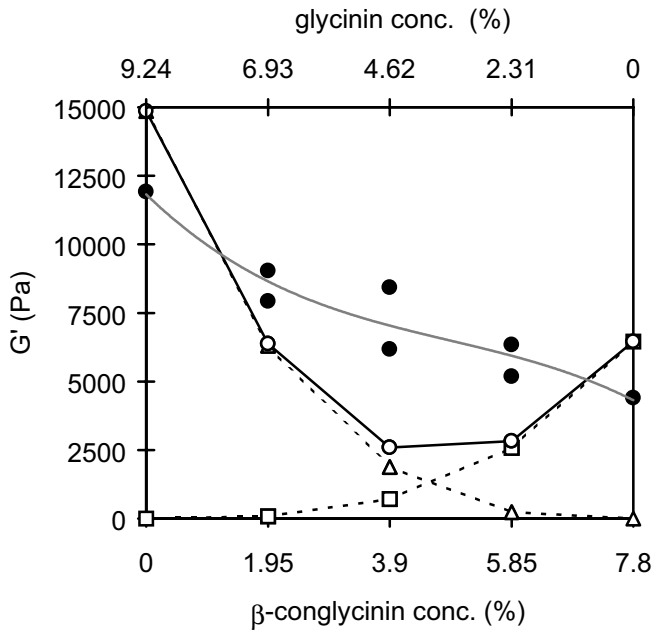

Figure 5.4 Storage moduli, *G′*, after completion of the temperature cycle of gels formed by a mixture of glycinin and β-conglycinin in different ratios at pH 3.8 in 0.2 M NaCl. Measured values (●) are compared with a theoretical curve (○), which is the sum of the separate contributions to *G′* of β-conglycinin (■) and glycinin (▲).

curves. The measured moduli of the mixtures were clearly larger than the "theoretical" moduli. This strongly indicates that there is interaction between the proteins. Indeed, at pH 7.6 the interaction between basic polypeptides of glycinin and β subunits of β-conglycinin has been demonstrated by twodimensional SDS-PAGE (German et al., 1982). Unfortunately, at pH 3.8 no data on interactions between β-conglycinin and glycinin are reported in the literature. This is probably due to the poor solubility of the proteins after heating at this pH, which hinders such a study.

5.3.3 Rheological behaviour at large deformation

The large deformation properties of soy protein gels are important for application in food products, because these are the properties that consumers observe during handling, slicing and eating of the product. Furthermore, mechanical properties give information about network structure. Figure 5.5 shows stress-strain curves for glycinin, β-conglycinin and a 1:1 mixture of both proteins as a function of pH. Table 5.3 presents mean values for fracture stress and strain, Young's modulus and storage modulus. Glycinin gels fractured at a much higher stress and strain than β-conglycinin gels. Gels of both proteins could be deformed to a

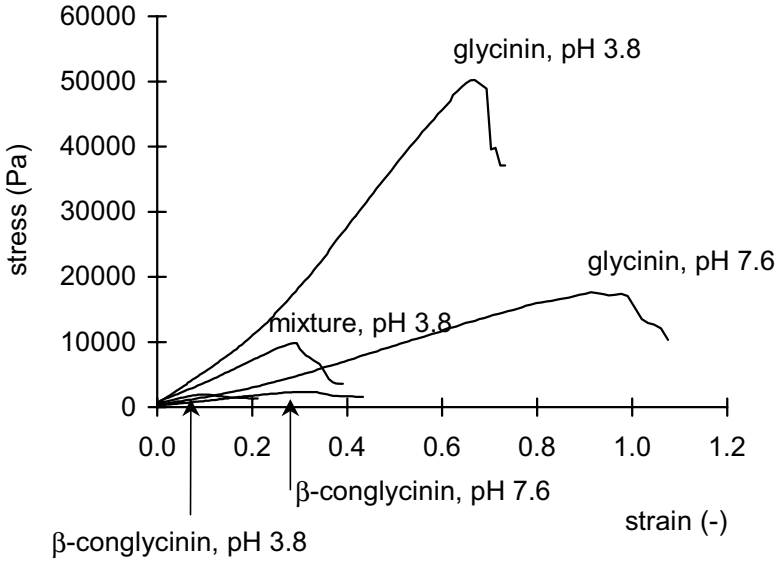

Figure 5.5 Representative stress-strain curves of gels formed by glycinin, β-conglycinin and a 1:1 mixture thereof as a function of pH.

Table 5.3 Mean values and standard deviations of fracture strain, ε_f, and stress, σ_f, and moduli of gels of glycinin, β-conglycinin and 1:1 mixtures.

		Conc. (mg/g)	ε_f (-)	σ_f (kPa)	Young's modulus (kPa)	Storage modulus (kPa)
pH 3.8	glycinin	74	0.65 ± 0.03	46.2 ± 9.3	48.2 ± 7.0	7.7
	β-conglycinin	78	0.10 ± 0.004	2.1 ± 0.18	18.4 ± 0.39	6.5
	1:1 mixture	76	0.30 ± 0.02	10.3 ± 1.0	54.4 ± 4.2	
pH 7.6	glycinin	98	0.88 ± 0.05	18.1 ± 1.9	12.3 ± 0.4	5.7
	β-conglycinin	84	0.31 ± 0.02	2.2 ± 0.15	7.0 ± 0.17	1.8
	1:1 mixture	91	n.r.	n.r.	n.r.	

n.r.: not reproducible due to phase separation

larger extent at pH 7.6 compared to pH 3.8. The 1:1 mixture (pH 3.8) had a fracture stress and strain in between the two proteins.

The differences in fracture strain between the two proteins and as a function of pH imply differences in network structure. Electron microscopy measurements showed that β-conglycinin gels are denser and consist of thicker and more irregular strands than glycinin

gels (Hermansson, 1985). Probably, this is why they break more easily on deformation. Also, the presence of other soy proteins in the β-conglycinin rich fraction might cause irregularities in the gel network. In mixed gels, β-conglycinin probably introduces weak spots in the gel network. The stress-strain curves of mixed gels further imply that interaction takes place between the two proteins (since a gel with two independent networks would generate two fracture points contrary to the observed one fracture point). For both proteins, the appearance of the gels indicates that at pH 7.6 (turbid gels) smaller aggregates and thinner strands were formed than at pH 3.8 (white gels). This is in accordance with the larger fracture strain of pH 7.6 gels compared to pH 3.8 gels.

Young's moduli, E, which were determined from the initial slope of the stress-strain curve, show the same trends as the elastic moduli, G' (Table 5.3). At pH 3.8, higher Young's moduli were obtained than at pH 7.6. The moduli for the glycinin gels were higher than for β-conglycinin gels. In theory, $E=3G'$. In practice, this relation was not obtained exactly, which is probably due to a combination of reasons. Firstly, Young's and the elastic modulus are highly dependent on protein concentration, so a small deviation in the desired protein concentration leads to a large difference in G' and/or E. Secondly, the surfaces of the cylindrical samples in the compression tests were not always smooth or completely parallel to the compression plate, what makes it difficult to determine E. Therefore, E was determined at $\varepsilon = 0.1$ or 0.05, whereas G' was measured at $\varepsilon = 0.01$.

5.4 General discussion and conclusions

Glycinin gives a firmer gel with a higher stiffness and a larger deformability before fracture than β-conglycinin. The protein concentration at which glycinin and β-conglycinin can form a gel is about the same, but the efficiency of β-conglycinin to form a gel with a certain strength is much lower, especially at pH 7.6. This is probably the reason why gel formation in mixed systems like in soy protein isolate (≤ 15 wt% dispersions) has not been observed at denaturation of β-conglycinin at pH 7.6. The initial increase in G' as a result of it was too low (<< 0.5 Pa/K) to be regarded as gel formation.

The reason why glycinin gives a firmer gel than β-conglycinin is not clear. Both soy proteins form gels with a clearly different network structure as is deduced from our large deformation experiments and as demonstrated by microscopic observations by Hermansson (1985). In both cases, the mechanism of gel formation is probably cluster-cluster aggregation of protein particles whereby the size of the clusters depends on the size and shape of the protein molecules and/or heat-induced aggregates (Nakamura et al., 1984; Huang et al., 1999), on the pH (larger clusters at pH 3.8) and on the extent of rearrangements after gel formation (more intensive at pH 7.6). The strength of the bonds between the clusters and possibly the

strength of the bonds between the protein molecules within a cluster seem to be higher for glycinin, as concluded from the much larger differences in fracture stress compared to the differences in moduli for the two types of protein systems.

Summarising, glycinin is a more efficient and better gelling agent than β-conglycinin under the conditions studied. Mixing of both proteins resulted in improved gelling properties at pH 3.8 and a reduced dispersability at pH 7.6. There are strong indications for the presence of an interaction between the two proteins. Further research is necessary to elucidate why gel formation by glycinin is more efficient than that by β-conglycinin.

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Chapter 6

Concentration dependence of dynamic moduli of heat-induced soy protein gels

Abstract

The concentration dependence of dynamic moduli of soy protein gels was studied for different protein preparations (soy protein isolate (SPI), purified glycinin and a β-conglycinin rich fraction) at various pHs and salt concentrations. The concentration dependence of the storage modulus of glycinin and β-conglycinin gels was similar to that of SPI gels. For SPI, the critical protein concentration for gelation was estimated to be 3% at pH 7.6 (0.2 M NaCl), 6.5 % at pH 7 (0 M NaCl), and 0% at pH 5.2 and pH 3.8 (0.2 M NaCl). Relating the experimental data to a fractal model, both rheological and permeability measurements resulted in a consistent value for the fractal dimensionality D_f (= 2.3) for SPI gels at pH 3.8 and 0.2 M NaCl. At pH 5.2 and 7.6 (0.2 M NaCl), and pH 7 (0 M NaCl), the concentration dependence of the modulus could not be analysed satisfactorily using a fractal model.

Renkema, J. M. S., van Vliet, T., & van der Linden, E. *submitted for publication*.

6.1 Introduction

Heat-induced gel formation is an important property of soy proteins for use as (texture-enhancing) ingredient in food products. Soy proteins have a high nutritional value and, therefore, it is expected that in the future they will become important as meat-replacers. In recent work, the effect of several (processing) conditions (heating, pH and ionic strength) on gel formation by soy proteins and the relation between gel properties and network structure were studied (Renkema et al., 2000; Renkema, 2001b, c, d). This paper addresses the concentration dependence of the dynamic moduli of soy protein gels, which gives information on the gelation efficiency and on the structure of the particle network.

Bikbov et al. (1979b) were the first to study the modulus (G) of soy protein gels as a function of protein concentration (c). In the concentration range 10-20% they found the relation: $G \propto c^{4.67}$. In the concentration range 7.5 - 58.4% the data fitted well with a model according to Hermans (1965) taking into account a minimum concentration for gelation of ~6.6% (Bikbov et al., 1979a). The pH was not specified in these studies.

In addition to the model of Hermans, other models describing concentration dependence have been proposed. These are, among others, a model by (Oakenfull, 1984) and a branching (cascade) model (Clark & Ross-Murphy, 1985) (which are both modifications of Hermans' model), percolation models (*e.g.* De Gennes, 1979) and fractal models (*e.g.* Bremer et al., 1989; Mellema et al., 2001). All models, except for the fractal model, lead to a power law relation $G \propto (c\text{-}c_p)^n$, in which is accounted for a critical protein concentration, c_p, for gelation. The power law relation of the fractal model, $G \propto c^n$, does not contain such a critical protein concentration. Nevertheless, a critical protein concentration is defined in the fractal model and may come in due to a finite size of the gelation vessel or due to sedimentation of the aggregating clusters (Bremer et al., 1989; Bremer et al., 1995).

The fractal model assumes that so-called primary particles aggregate into finite clusters with a fractal structure. The number of particles in such clusters then scales with R as $(R/a)^{D_f}$, where R denotes the mean radius of the fractal cluster, a the mean radius of the primary particle and D_f the dimensionality of the fractal cluster ($D_f < 3$). The volume fraction of primary particles in the cluster, $\phi_{cluster,}$ is given by $(R/a)^{D_f - 3}$ and decreases with R. A gel is presumed to have formed when $\phi_{cluster}$ becomes equal to the particle volume fraction in the system, ϕ. The clusters then fill the whole space. Following this description, the dependence of the storage modulus, G', on the volume fraction of primary particles in the gel can be written as (Mellema et al., 2001)

$$G' \propto \phi^{\frac{\alpha}{3-D_f}} \qquad (1)$$

where α is dependent on the nature of the strands (for particle gels, ranging from 1 for rigid, straight strands to 4 for curved, flexible strands (Mellema et al., 2001)). Similarly, the concentration dependence of the permeability, *B*, can be written as (Bremer et al., 1989)

$$B \propto \phi^{\frac{2}{D_f - 3}} \tag{2}$$

In this paper, results are presented of concentration dependence of the modulus for various pH and salt concentrations. Moreover, some data are given on the concentration dependence of the permeability. Fitting of the experimental data with existing power law relations will be discussed.

6.2 Material and methods

6.2.1 Material

For the experiments, a soy protein isolate (SPI) was used with a calculated protein content of 97% using N × 6.25. The SPI (pH 4.8) was prepared from mildly treated, defatted PDI 80 soy flakes (Cargill, Amsterdam, The Netherlands) according to a method described previously (Renkema et al., 2000). Stock dispersions were prepared by suspending SPI in double-distilled water or 0.2 M NaCl solution. After stirring for one hour at 4°C the suspensions were brought to pH 7.6 with 0.5 M NaOH. For experiments carried out at pH 3.8, 5.2 and 7, the pH of the dispersion was adjusted after one hour using 1 M HCl. Protein dispersions (60-150 mg/g) were prepared by diluting portions of the stock dispersions with double-distilled water or salt solution. The protein dispersions were stirred overnight to enhance protein dissolution. Protein dispersions were prepared at 4°C to prevent proteolysis by endogenous enzymes. The protein content of the dispersions was checked in duplicate by micro-Kjeldahl method (AOAC, 1980) using a Kjeldahl factor of 6.25.

As a reference, protein dispersions (20-120 mg/g) of purified glycinin and a β-conglycinin rich fraction (about 60%) in 0.2 M NaCl were used. Preparation of the protein fractions and their dispersions was described earlier (Renkema, 2001d).

6.2.2 Rheological measurements at small deformation

Gel formation of soy protein dispersions (60-150 mg/g) was followed by dynamic measurements in a Bohlin CVO rheometer using the serrated concentric cylinder geometry C25 (content 13 ml). Measurements were performed at a constant strain of 0.01, which was within the linear region, and at an angular frequency of 0.63 rad/s. A thin layer of soy oil was put on top of the samples to prevent evaporation of water. To induce gel formation, samples

were heated from 20 to 95°C at a heating rate of 1 K/min, kept at 95°C for 60 min, and cooled down to 20°C at a cooling rate of 1 K/min. As an exception, pH 7 gels were prepared using a maximum heating temperature of 89°C instead of 95°C.

6.2.3 Permeability measurements

Permeability coefficients of pH 3.8 gels (60-100 mg/g) were determined by measuring the flow rate of 0.2 M NaCl through the protein gel according to the method described earlier (Renkema, 2001c).

6.3 Results and discussion

Figure 6.1 shows the storage modulus G' at the end of a complete temperature cycle as a function of concentration at various pH and ionic strength. At pH 7 and 7.6, G' values are much lower at the same concentration than at pH 3.8 and 5.2. This difference in G' is related to a difference in network structure (Renkema, 2001c) and to the fact that part of the protein is not incorporated in the protein network at pH 7 and 7.6 (Renkema, 2001b).

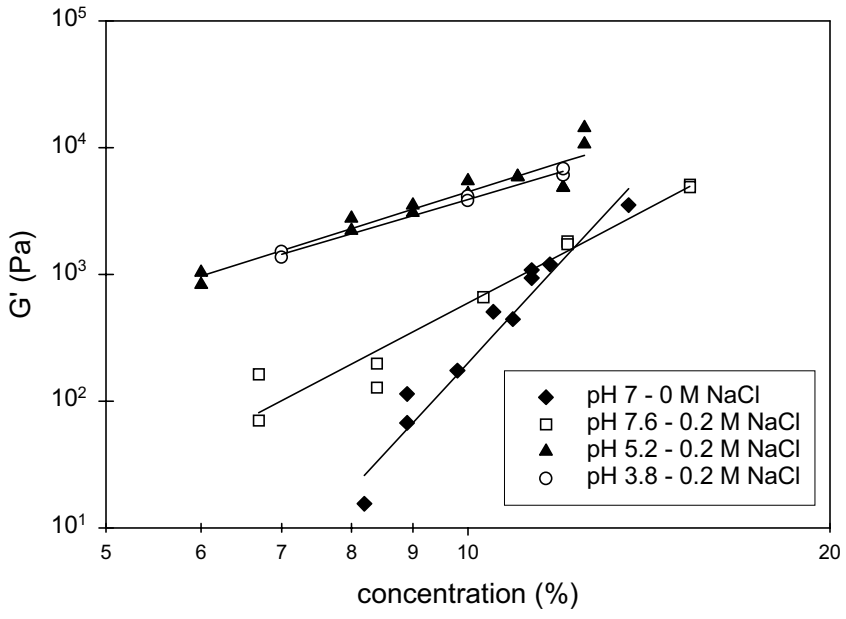

Figure 6.1 The storage modulus G' after completion of the temperature programme as a function of protein concentration of gels formed by soy protein isolate at various pH and salt concentrations.

Assuming a power-law relation between G' and protein concentration, c, according to $G' \propto c^x$, one may determine x at different times during heating and cooling. The correlation

coefficients R^2 of the fitted lines in Figure 6.1 were higher than 0.89. In Figure 6.2 the exponent x is given at the end of the heating stage at 95°C (or 89°C for pH 7) (t_1), at the end of the isothermal stage (t_2), and at the end of the cooling stage at 20°C (t_3). At pH 3.8 and 5.2, x remained constant during the heating and cooling cycle, whereas at pH 7.6 and 7 a decrease in x was found. At all stages of the temperature cycle, x was higher at pH 7.6 and 7 than at pH 3.8 and 5.2.

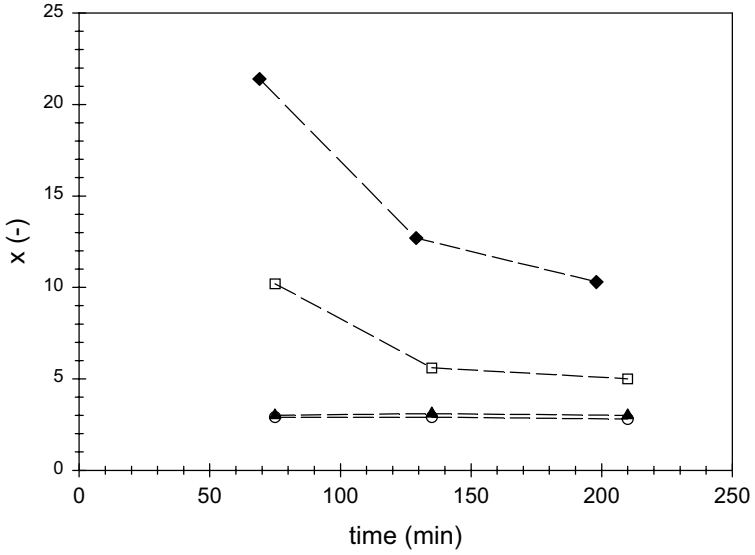

Figure 6.2 The exponent x in the power-law relation $G' \propto c^x$ at three moments in the temperature cycle: at the end of the heating stage (t_1), the isothermal stage (t_2), and the cooling stage (t_3). For explanation of symbols: see Figure 6.1.

For comparison, x values for SPI at t_3 are given together with the x values for glycinin and β-conglycinin (Table 6.1). The 'purified' protein fractions also have higher x values at pH 7.6 than at pH 3.8, but the difference in x for the two pHs is smaller than for SPI. The development of x during the temperature cycle for the 'purified' fractions also differs from SPI (Renkema, 2001d). Firstly, for glycinin and β-conglycinin x increases during heating and cooling at pH 7.6 from 2.3 to 3.5 and from 3.4 to 4.6, respectively. Secondly, at pH 7.6, x for glycinin was lower at t_1, equal at t_2 and higher at t_3 compared to x at pH 3.8, whereas for β-conglycinin and SPI, x was always higher at pH 7.6 than at pH 3.8 during the temperature cycle.

The loss or viscous modulus G'' showed the same concentration dependence, except for β-conglycinin gels at pH 7.6. Consequently, the loss tangent $tan\delta$ was independent of protein concentration. At t_3, $tan\delta$ was around 0.15. For β-conglycinin gels at pH 7.6, $tan\delta$ increased with decreasing concentration at concentrations lower than 4%.

Table 6.1 The exponent x in the power-law relation $G' \propto c^x$ at the end of the temperature cycle (t_3).

	x		
	SPI	glycinin*	β-conglycinin*
pH 7 – 0 M NaCl	10.3		
pH 7.6 – 0.2 M NaCl	5.0	3.5	4.6
pH 5.2 – 0.2 M NaCl	3.0		
pH 3.8 – 0.2 M NaCl	2.8	3.0	3.2

* derived from Renkema (2001d)

Figure 6.3 shows the permeability coefficient B of SPI gels at pH 3.8 and 0.2 M NaCl as a function of protein concentration. The permeability of the gels decreased with increasing protein concentration. A power-law relation was obtained between B and the protein concentration, c, according to $B \propto c^{-2.7}$ (Figure 6.3). At pH 7.6 and 5.2, it was not possible to measure B over a significant concentration range, because at low concentrations (60-70 mg/g) the gels were too weak (pH 7.6) or too inhomogeneous due to protein precipitation (pH 5.2), whereas at concentrations higher than 100 mg/g the flow rate through the gels was too low to measure (pH 7.6).

Assuming that the fractal model can be applied and that c is proportional to ϕ, the exponent x in the rheological measurements is equal to $\alpha/(3-D_f)$, according to equation (1), and the exponent x in the permeability measurements equals $2/(D_f-3)$, according to equation (2). Thus, both exponents give information on the gel structure in terms of the nature of the strands, α, and/or the fractal dimensionality, D_f, of the clusters forming the gel.

At pH 3.8, rheological measurements of SPI gels yield $x = 2.8$. Since these gels exhibit a small fracture strain ($\varepsilon = 0.4$) implying that they probably consist of straight strands (Renkema, 2001c) with $\alpha \approx 2$ (Mellema et al., 2001). Solving D_f using equation (1) gives $D_f \approx 2.3$. Permeability measurements yield $x = -2.7$, which, using equation (2), also leads to $D_f = 2.3$. Thus, assuming a fractal model, both methods give a consistent value for D_f at pH 3.8.

At pH 5.2, large deformation experiments indicated that SPI gels consist of hinged or curved strands ($\alpha = 3$ or $\alpha = 4$) (Mellema et al., 2001; Renkema, 2001c). Using $x = 3$ and equation (1), $D_f = 2.0$ or 1.7, respectively. These values are lower than usually found for protein gels ($D_f = 2.2$-2.4) (van Vliet, 2000). In contrast with pH 3.8 gels, these values could not be checked by concentration dependent permeability measurements. Hence, more research

is necessary for a better structural interpretation of the concentration dependence of G' at this pH.

At pH 7.6 and 7, a fractal model has not been applied for several reasons. Firstly, at these pHs part of the protein is not incorporated in the network at the gel point. This part becomes partly incorporated on further heating due to ongoing denaturation and aggregation of protein molecules and partly remains dispersed (Renkema, 2001a, b). Probably, the additionally incorporated protein is equally divided over the strands in the fractal clusters resulting in denser clusters and loss of their fractal character. It is unknown to what extent the protein fraction remaining dispersed will affect the concentration dependence of G'. It will certainly affect the permeability coefficient B and its concentration dependence.

Secondly, a critical protein concentration, c_p, for gelation was observed at pH 7.6 and 7 in contrast to pH 3.8 and 5.2. For SPI, c_p is estimated to be 3% for pH 7.6 and 6.5% for pH 7 (using $G^{1/n}$ (at t_3) versus c plots with n ranging from 1-5; $n \approx 3.5$ giving the best linear fit). The existence of such a high critical protein concentration is not expected according to the fractal model for particle aggregation for the given conditions.

Thirdly, values for x were very high at pH 7.6 and 7 in view of the fractal model (especially at t_1) and x changed during the temperature cycle (Figure 6.2). The change in x implies changes in the network structure, for which we have indications that they are induced by rearrangements (Renkema, 2001b). These rearrangements undoubtedly affected the fractal character of the gels.

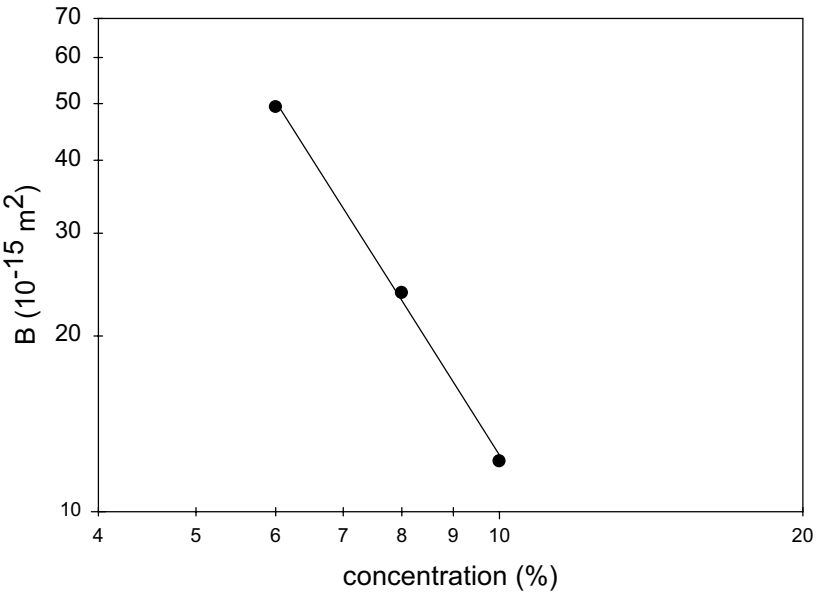

Figure 6.3 The permeability coefficient B as a function of protein concentration of a soy protein isolate gel at pH 3.8 and 0.2 M added NaCl.

The use of models leading to a power law relation $G \sim (c\text{-}c_p)^n$ could not be tested for SPI gels at either pH. At pH 3.8 and 5.2, c_p was estimated zero. At pH 7.6 and 7, c_p was not zero, but differed at the three separate stages in the temperature cycle (t_1, t_2 or t_3). The occurrence of rearrangements at pH 7.6 and 7 will also limit an analysis by these models.

Application of a fractal model on G' versus concentration (eq. 1) of pH 3.8 gels of purified glycinin and the β-conglycinin rich fraction, and assuming that $\alpha = 2$, yields $D_f = 2.3$ and 2.4, respectively. The assumption that these gels have straight strands ($\alpha = 2$) stems from the fact that the fracture strain at pH 3.8 was smaller than at pH 7.6 (Renkema, 2001d) as was observed for SPI gels. At pH 7.6, we again refrain from calculating D_f, for the same reasons as for SPI. Firstly, for glycinin it is known that part of the protein is not involved in network formation at pH 7.6 (Renkema et al., 2000). Secondly, for both proteins the critical protein concentration is estimated to be larger than 0% at pH 7.6. Thirdly, the values for x of glycinin and β-conglycinin gels changed during the temperature cycle at pH 7.6 (Renkema, 2001d). Since x increased, which is in contrast to the observations for SPI, an analysis of the concentration dependence of G' at this pH is even more questionable.

The value for D_f of both the glycinin and the β-conglycinin gels at pH 3.8 is consistent with that of SPI gels. Again, as for SPI gels, calculation of D_f at pH 7.6 was dubious for both glycinin and β-conglycinin gels. This suggests that the effect of pH on gel formation (and network structure) could be similar for the more or less purified proteins and SPI. The interactions and synergistic behaviour of glycinin and β-conglycinin in mixed systems (Renkema, 2001d), like in SPI, does not seem to influence this pH effect.

6.4 Conclusions

Using a fractal model, the concentration dependence of the storage modulus and the permeability yields a consistent value of 2.3 for the fractal dimensionality of SPI gels at pH 3.8. At pH 7.6, gel formation is more complex and application of existing theoretical models is questionable. The effect of pH on the concentration dependence of the storage modulus seems to be independent of the soy protein preparation (glycinin, β-conglycinin rich fraction or SPI).

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Chapter 7

Rheological properties of soybean protein isolate gels containing emulsion droplets

Abstract

Rheological properties of soybean protein gels containing various volume fractions oil droplets have been studied at small and large deformations. Dynamic viscoelastic properties of soybean protein isolate gels were determined as a function of the volume fraction of oil droplets stabilised by the same protein, both in absence and presence of 0.2 M sodium chloride (NaCl). The storage and loss moduli were higher if NaCl was added. For both conditions, they increased with increasing oil volume fraction during the heating as well as the cooling stage. Furthermore, gel formation started at a lower temperature with increasing oil volume fraction. The increase in the moduli with increasing volume fraction of oil droplets was stronger than predicted by Van der Poels theory for a simple filled gel containing single interacting stiff emulsion droplets. This effect was attributed to aggregation of the emulsion droplets. Fracture properties of gels with different oil volume fraction and oil droplet size were determined at pH 7.0 in the presence of 0.2 M NaCl by a uniaxial compression test. Compressive stresses of the gels containing oil droplets of 1.17 μm were higher than those containing oil droplets of 2.70 μm, especially at higher volume fractions of oil droplets. The fracture strain did not depend on the volume fraction of oil droplets.

Kim, K.-H., Renkema, J. M. S., & van Vliet, T. *Food Hydrocolloids* 15 (2001), 295-302

7.1 Introduction

Soybean proteins are extensively used for processed foods because of their high nutritional value and their contribution to food texture due to their ability to form a gel on heating (Nagano et al., 1994). Gel formation by soybean protein is a complex process, which often involves several reactions such as denaturation, dissociation-association and aggregation (Hermansson, 1985). Due to the industrial importance of gel formation by soy proteins, much research has been done that was focused on the mechanisms involved (Utsumi & Kinsella, 1985a, b; Arrese et al., 1991; Kang et al., 1991). A complication is that soy proteins consist of various proteins (mainly glycinin and β-conglycinin), which have different gel formation properties (Petruccelli & Añón, 1994).

An important aspect for food industry is the effect of the presence of emulsified fat on gel properties. Fat plays an important role in the texture of many foods, *e.g.*, by imparting desirable mouthfeel to milk, cheese, ice cream, cakes and processed meats (Xiong et al., 1991). Gels containing oil droplets can be considered as a protein gel matrix, in which oil droplets are embedded. The rheological properties of such composite gels depend strongly on the volume fraction of dispersed oil droplets and on the interaction between the gel matrix and the filler particles (van Vliet & Dentener-Kikkert, 1982; Bargeau & Kinsella, 1987; van Vliet, 1988; Aguilera & Kessler, 1989; Langley & Green, 1989; Luyten & van Vliet, 1990; Xiong & Kinsella, 1991; Yost & Kinsella, 1992; McClements et al., 1993; Chen & Dickinson, 1998). Filler particles that strongly interact with the gel matrix, so-called active fillers, reinforce the gel strength (Ring & Stainsby, 1982; van Vliet, 1988). For filled protein gels containing protein-covered oil droplets, gel stiffness may become enhanced if the adsorbed protein layer interacts with the protein gel matrix (van Vliet, 1988; Xiong et al., 1991; Matsumura et al., 1993). The size of the dispersed particles may also affect rheological and especially fracture properties of the gels (Ross-Murphy & Todd, 1983; Brownsey et al., 1987; van Vliet, 1988; Langley & Green, 1989; Matsumura et al., 1993; Kim et al., 1996).

As part of a large research project on gel formation by soy proteins (Renkema et al., 2000; Renkema, 2001) this work focuses on the effect of soy protein stabilised oil droplets on the rheological properties of heat-set soy protein gels at small and large deformations. pH 7 and an added salt concentration of 0.2 M NaCl was chosen because under these conditions the used soy protein isolate could be dissolved well while they were not abnormal for food products.

7.2 Materials and methods

7.2.1 Materials

Soybean protein isolate (SPI) was prepared from defatted soybean meal (PDI 80) produced by Cargill (The Netherlands). The soybean meal was dispersed in 0.1 M Tris HCl-buffer solution at pH 8.0 (1:10 w/v). The dispersion was stirred at room temperature for 1 h, and then centrifuged at 10,000 *g* for 30 min at 10°C. Supernatant was adjusted to pH 4.8 with 2 M HCl and kept at 4°C overnight and next centrifuged again at 10,000 *g* for 30 min. After being washed with 5 mM Na-acetate buffer (pH 4.8) for 2 times (1:8 w/v), the pellet was stored in a freezer overnight and next freeze-dried at –40°C for 3 days. Finally, the dry pellet was ground in a mortar. The protein content (N × 6.25) of the soy protein isolate was 95 wt% as determined by the micro-Kjeldahl method (AOAC, 1980).

Commercial soybean oil of food grade quality was purchased from Reddy (The Netherlands). The specific gravity of the soybean oil was 961 kg m^{-3}.

7.2.2 O/W emulsion preparation

Fine SPI powder was dispersed in distilled water or in 0.2 M NaCl solution and stirred for 30 min at room temperature. SPI dispersions were adjusted to pH 7.0 with 0.2 M NaOH and stirred overnight at 4°C. SPI concentration was always 13% total mass (on w/v basis). Finally soybean oil was added directly to the protein dispersions to an oil volume fraction of 0.1, 0.2, 0.25 and 0.3, respectively.

Standard emulsions were prepared at 22 ± 0.5°C by homogenisation (homogenizer 12705, A/S N Foss Electric, Denmark) at a constant pressure of 75 atm. The emulsion was circulated through the homogenizer 8-11 times. Emulsions with larger oil droplet diameters were prepared at an oil volume fraction of 0.1, 0.2 and 0.3 by emulsifying with a dispenser (type T20b, Janke & Kunkel GmbH & Co. KG, Germany) for 1, 1 and 2 minutes, respectively.

The emulsion droplet size distribution and the volume surface average droplet diameter d_{32} ($\Sigma_i n_i d_i^3 / \Sigma_i n_i d_i^2$) were determined by spectroturbidimetry (type M4 GII, Carl Zeiss Inc., Germany) after dilution with a surfactant solution to disaggregate possible aggregates present after emulsification (Walstra, 1965). The emulsions were used for gel formation within one day.

7.2.3 Small deformation experiments

Small deformation properties were determined in dynamic oscillation using a Bohlin CVO rheometer with a serrated concentric cylinder geometry (C25), diameter of bob 24 mm and of cup 27 mm. Samples were covered with a thin layer of paraffin oil to prevent evaporation. Gelation was induced by heating the samples from 20 to 95°C at 1 K/min, maintaining temperature at 95°C for 60 min and cooling to 20°C at a rate of 1 K/min. The development of the storage (G') and loss modulus (G'') was followed at a frequency of 0.1 Hz and at a maximum strain of 0.01 which is in the linear region. Gelation experiments were performed in triplicate.

7.2.4 Large deformation experiments

Gels for large deformation measurements were prepared by heating the protein dispersion or emulsion in a cylindrical glass container (inside diameter 18 mm, height 115 mm). The glass containers were only filled half so that emerging gas bubbles could escape. Samples were covered with a thin layer of paraffin oil to prevent evaporation during heating. Heating conditions were the same as in the small deformation experiments. Gels were cooled by keeping them for 30 min at room temperature and next for 30 min in a water bath set at 20 ± 0.1°C.

Large deformation and fracture properties were determined in uniaxial compression within a few hours after gel preparation by using a material-testing instrument (Zwick GmbH & Co., Germany) equipped with a 50 N load cell. Gels were cut into sections of 20 mm in height. Test pieces were compressed between two parallel plates to a deformation ($\Delta h/h_0$) of 80% at a constant displacement speed of 20 mm/s at 20 ± 0.5°C. Measurements were repeated 3-7 times and mean values for fracture stress and strain were calculated.

The relative deformation at a certain stage is expressed as a true or Hencky strain ε_H (-) which is defined as:

$$\varepsilon_H = \ln\frac{h(t)}{h_0} \tag{1}$$

where h_0 is the original height of the test piece, and $h(t)$ the height after a certain deformation time t. For compression, the Hencky strain is negative, but it will be expressed as a positive figure. The average stress $\sigma(t)$ in the test piece at a certain deformation at time t is given by:

$$\sigma(t) = \frac{F(t)}{A(t)} \tag{2}$$

where $F(t)$ is the measured force after a deformation time t and $A(t)$ the area of the test piece.

Assuming that the volume of the test piece does not change during compression and the shape stays cylindrical, $A(t)$ is equal to $(h_0/h(t)) \times A_0$.

7.2.5 Light microscopy

Light microscopy was performed using a Zeiss Axiomat microscope. Gel samples were made by placing a small amount of the emulsion soy protein dispersion in a hollow slide. Next it was covered with a cover slide and placed in the same dispersion. Gel formation was induced by applying the standard temperature cycle.

7.3 Results and discussion

7.3.1 Characterisation emulsions

Emulsions with different droplet sizes were prepared for the experiments. The average volume-surface diameter d_{32} of the emulsion droplets formed by homogenisation at 75 atm was 1.17 ± 0.19 μm, independent of the oil volume fraction (data not shown). The relative standard deviation C_2 of the droplet size distribution was always about 0.32. For the emulsions with salt d_{32} was 1.16 μm and C_2 0.29. The emulsions made with the dispenser had an average d_{32} of 2.70 ± 0.27 μm and a C_2 of 0.7.

Due to adsorption of soy protein on the emulsion droplets during emulsification, the protein concentration in the aqueous continuous phase decreased somewhat. We assume that the adsorbed amount is 2.5 mg m^{-2}, as being a typical value for globular proteins (Smulders, 2000). Then the total adsorbed amount of protein in the emulsions with the smaller droplets was about 1.25, 2.50 and 3.75 kg m^{-3} for volume fractions of oil of 0.1, 0.2 and 0.3, respectively. This resulted in small (0.1-0.5%) corrections for the bulk protein concentration. For the emulsion with a droplet diameter of 2.70 ± 0.26 μm the total adsorbed amount was 0.55, 1.11 and 1.68 kg m^{-3} for a volume fraction of oil of 0.1, 0.2 and 0.3, respectively. It gave a similar small correction (0.1–0.3%) for the bulk protein concentration of the emulsions with the large droplets.

7.3.2 Small deformation properties

Figures 7.1 and 7.2 show the effect of the presence of oil droplets on the storage presence of 0.2 M NaCl during a temperature cycle. The curves for the loss modulus G'' were

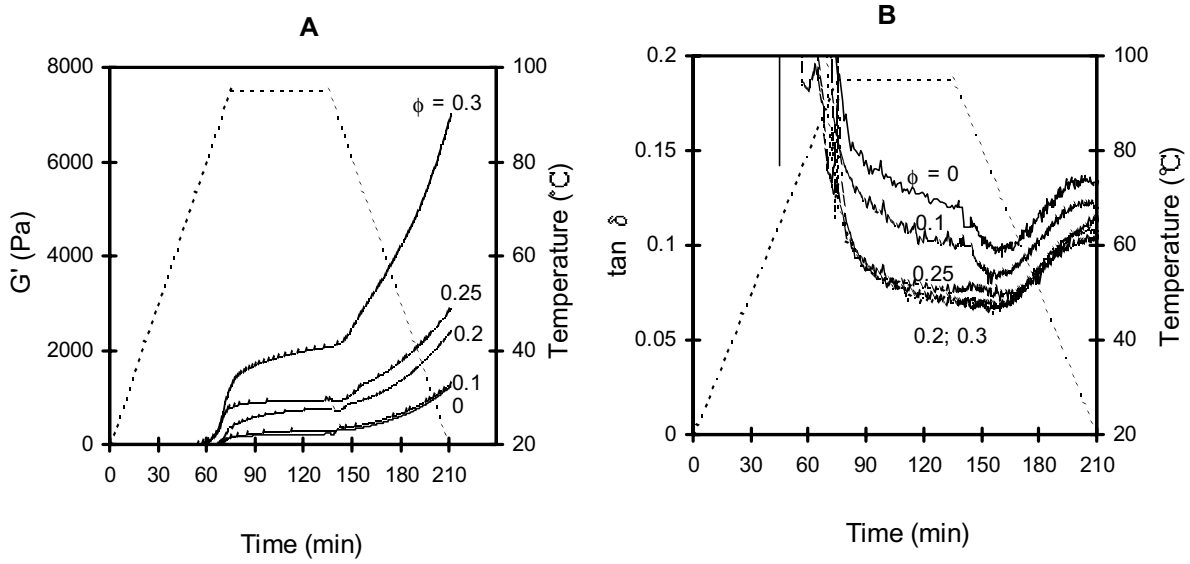

Figure 7.1 Storage modulus G' (A) and loss tangent tan δ (B) of soybean isolate gels at pH 7.0 as a function of time during a heating and cooling cycle for various volume fractions of oil droplets (indicated). The dashed lines show temperature against time.

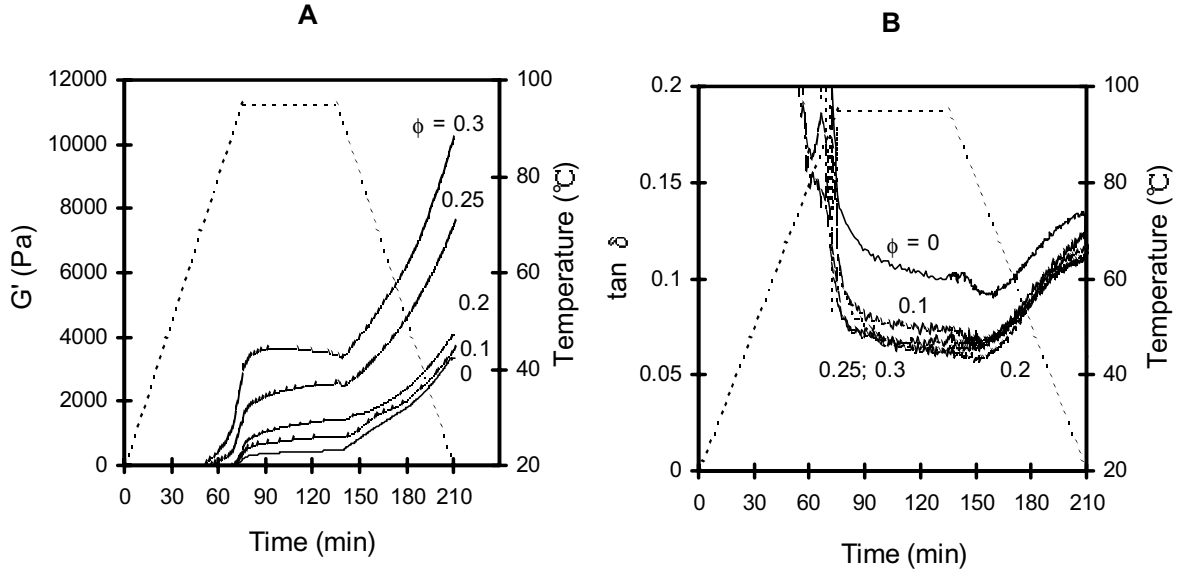

Figure 7.2 Storage modulus G' (A) and loss tangent tan δ (B) of soybean isolate gels at pH 7.0 with 0.2 M NaCl added as a function of time during a heating and cooling cycle for various volume fractions of oil droplets (indicated). The dashed lines show temperature against time.

rather similar to those for G' (not shown). During gel formation and ageing, the storage modulus G' and loss modulus G'' increased, and tan δ decreased. As the temperature increased above 80 °C, both G' and G'' increased almost linearly with temperature up to 95°C while tan δ decreased. It indicates the transition from a liquid-like dispersion into a more solid-like gel structure. During the constant temperature regime at 95°C, G' tended to increase somewhat while G'' was about constant, and tan δ decreased somewhat. During cooling both G' and G'' increased strongly. They were higher for a higher oil volume fraction ϕ, while tan δ tended to be lower. The dynamic moduli for gels with 0.2 M NaCl were remarkably higher compared with the gels without NaCl, but the effect of the incorporation of oil droplets was similar.

In Figure 7.3, the effect of oil volume fraction and NaCl is shown on the onset temperature for gel formation, T_{gel}, by soy protein isolate. T_{gel} was defined as the temperature at which the dynamic moduli started to increase more than 0.5 Pa/K. Both in the absence and presence of NaCl, T_{gel} decreased in the presence of oil droplets, especially at oil volume fractions higher than 0.2. Salt concentration did not affect the gelation temperature, except for ϕ is 0.3. At that ϕ, T_{gel} was much lower in the presence of salt.

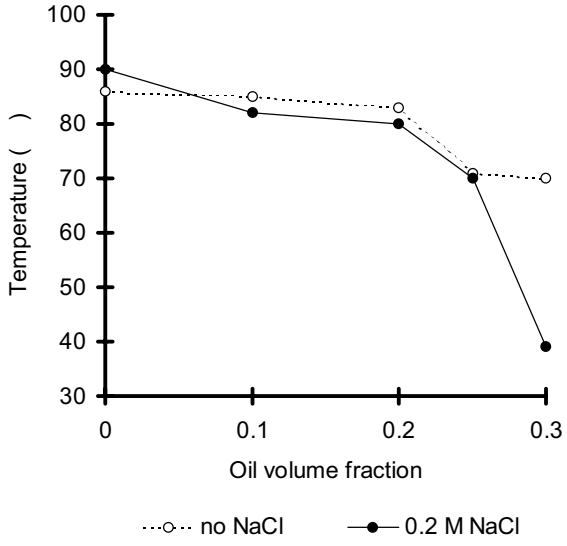

Figure 7.3 Effect of oil volume fraction on the onset temperature for gel formation of soy protein isolate dispersions. Dashed curve represents gels without added NaCl, full curve 0.2 M NaCl containing systems.

As was observed before (*e.g.* Renkema et al., 2000), heat denaturation of soy protein is a prerequisite for gel formation. At pH 7 and 13% soy protein isolate, T_{gel} coincides with the denaturation temperature of glycinin (Renkema et al., 2000), which was higher than that of β-conglycinin (*e.g.* Puppo & Añón, 1999). Under these conditions the concentration of β-

conglycinin is too low (~4.5%) to give a clear increase of G' (>0.5 Pa/K) due to denaturation of β-conglycinin (Renkema, 2001). However, in the presence of soy protein stabilised emulsion droplets notable gel formation was already observed at temperatures below the denaturation temperature of glycinin, which likely can be attributed to denaturation of β-conglycinin. At ϕ is 0.1 and 0.2 the observed increase in G' due to denaturation of β-conglycinin was very small compared to that of glycinin (5 and 20 Pa, respectively, before G' started to increase as a result of glycinin denaturation). It resulted only in a small decrease in T_{gel}. At ϕ is 0.25 and 0.3, the increase in G' due to denaturation of β-conglycinin was much stronger (>100 Pa) than at low ϕ. The presence of emulsion droplets causes the apparent soy protein concentration in the system to be higher, because the emulsion droplets act as huge soy protein particles. Since T_{gel}, as defined above, depends strongly on the concentration of β-conglycinin and therewith on soy protein concentration (Renkema, 2001), the apparent higher soy protein concentration in systems with emulsion droplets likely explains the greatest part of the observed decrease in T_{gel} in Figure 7.3.

At ϕ is 0.3 and 0.2 M NaCl, gel formation took place at a temperature below the denaturation temperature of β-conglycinin. Moreover, if at low ϕ the part in the decrease of T_{gel} due to denaturation of β-conglycinin was neglected still a small decrease was observed. Besides the effect discussed above aggregation of soy protein stabilised emulsion droplets might have decreased the measured T_{gel}. Emulsion droplets are more inclined to aggregation during heating than protein molecules are for the following reasons. Firstly, the Hamaker constant between oil droplets will be higher than between the protein particles (van Vliet, 1988). Secondly, oil droplets are larger than protein molecules. More than a hundred protein molecules have to aggregate to form a protein strand equal in size to the average free distance between the emulsion droplets, so the latter have time to aggregate before they are trapped in the gel matrix. It causes that oil droplets may aggregate in a secondary minimum before and during gel formation. Finally, adsorption of protein goes likely together with some changes in their conformation and this may affect their denaturation behaviour. This may also lead to a lower denaturation temperature and, with that, to a lower aggregation temperature of the emulsion droplets.

The experimentally observed effect of the volume fraction of oil droplets on the dynamic moduli was compared with theoretical predictions according to the Van der Poel theory (van der Poel, 1958; Smith, 1975). For that, the ratio of the storage moduli of the filled gels (G'_c) divided by the storage modulus of oil-free gel (G'_m) was calculated as a function of ϕ. Results obtained for the gels at the end of the heating period at 95°C and after cooling until

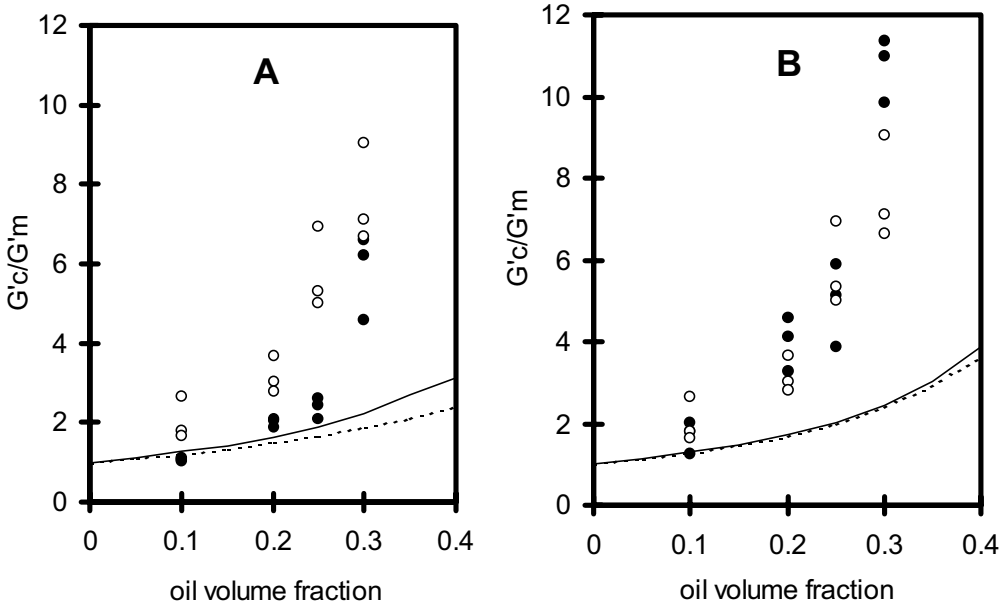

Figure 7.4 Storage moduli of filled gels G'_c divided by those of the gels without emulsion droplets G'_m as a function of the volume fraction of emulsion droplets. (o) 0.2 M NaCl, (•) no NaCl added. Dashed curves represents the theoretical relation calculated according to the Van der Poel theory for the 0.2 M NaCl containing systems and the full curves the no salt systems, repectively. (A) at 20°C and (B) at 95°C.

20°C are shown in Figure 7.4. At both temperatures and salt concentrations G'_c/G'_m increased with increasing oil volume fraction. However, the increase in G'_c/G'_m with ϕ is about the same at 20 and 95°C in the presence of 0.2 M NaCl while in the absence of NaCl the increase at 95° C was sharper than at 20°C.

The theoretical curves in Figure 7.4 were calculated using a simplification of the Van der Poel formula as given by Smith (1975). The modulus G_c can be obtained from the following equation:

$$\alpha X^2 + \beta X + \delta = 0 \qquad (1)$$

which has to be solved for X. The positive root is equal to $(G'_c/G'_m)-1$. Taking the Poisson ratio to be equal to 0.5, α, β and δ are given by:

$$\alpha = [8P - \phi^{7/3}S][Q - 3(M-1)\phi] - 126P(M-1)\phi(1-\phi^{2/3})^2 \qquad (2a)$$

$$\beta = 17.5P[(3M+4.5) - 3(M-1)\phi] - 7.5[8P - S\phi^{7/3}](M-1)\phi \qquad (2b)$$

$$\delta = -131.25P(M-1)\phi \qquad (2c)$$

where ϕ is the volume fraction emulsion droplets, $P = 9.5M + 8$ and $S = 166.25\ M - 9.5P$. M is the ratio of the moduli of the filler material (the dispersed emulsion droplets) and of the matrix material (the oil free soy protein gel) ($M = G'_f / G'_m$) and is given in Table 7.1 for the various gels. The storage modulus G'_f of the dispersed oil droplets may be taken to be equal to $2\gamma/R$, where γ is the interfacial tension and R is the droplet radius (van Vliet, 1988). The theoretical curves depend somewhat on the salt concentration due to the lower relative stiffness of the oil droplets (G'_f/G'_m) for the gels containing 0.2 M NaCl (Table 7.1).

Table 7.1 Characteristics of the soybean oil emulsion droplets and the ratio of the shear modulus of the droplets, G_f and the storage modulus of the soybean isolate gel matrix, G_m. R is droplet radius and γ is interfacial tension. $G_f = 2\gamma/R$ (van Vliet, 1988). A, without NaCl at 20°C; B, without NaCl at 95°C; C, with 0.2 M NaCl at 20°C; D, with 0.2 M NaCl at 95°C.

	R (μm)	γ (mN m^{-1})	G_f (Pa)	G_f/G_m			
				A	B	C	D
Oil droplets	0.6	10	3.3×10^4	27	170	9.8	72

For both salt concentrations, G'_c/G'_m increased much stronger with ϕ than predicted by the Van der Poel theory. The discrepancy between experimental results and theory may be caused by several factors. Firstly, the interaction between the filler particles and the gel matrix is not perfect. However, this would lead to an even larger discrepancy (van Vliet, 1988). Secondly, the presence of a very stiff adsorbed protein layer around the dispersed droplets would lead to an effective increase of ϕ. However, even in the unlikely case that such a layer, with a thickness of 20 nm, would be formed around the droplets it would only lead to an increase of the effective ϕ by at most 10%. This can never explain the observed discrepancy.

A similar discrepancy, as found in this study, has been observed for acid milk gels, containing recombined milk fat globules (van Vliet & Dentener-Kikkert, 1982; van Vliet, 1988) and for heat-set whey protein gels, containing whey protein stabilised emulsion droplets (Chen & Dickinson, 1998). They suggest that the discrepancy is due to the formation of aggregates by the emulsion droplets during the gel formation process. The Van der Poel theory is based on a uniform distribution of the dispersed particles. If the emulsion droplets aggregate to stiff aggregates during gel formation, these aggregates should be considered as the dispersed particles in the gel. Because the volume of such an aggregate is greater than the sum of the volumes of the contributing emulsion droplets, the effective volume fraction of particles responsible for the increase in G'_c is higher than the volume fraction of added emulsion

droplets. A good fit of the experimental data is obtained for $\phi_{effective}/\phi \approx 2$.

The possibility that the emulsion droplets aggregate during gel formation has already been discussed above. Moreover, there is some experimental evidence that at least some aggregation occurs. The increase in the viscosity of the soy protein dispersions with ϕ before heating was stronger than can be expected for separate droplets. Moreover, microscopic evaluation of the systems with a high ϕ indicated aggregation of the emulsion droplets both in the unheated systems as in the gels (Figure 7.5). No difference was observed if salt conditions were changed (not shown). Chen and Dickinson (1998) found a large increase in apparent particle size due to droplet flocculation. The extent was depending on protein concentration. The latter was standardised in this research. They could also not provide a definitive explanation for droplet aggregation.

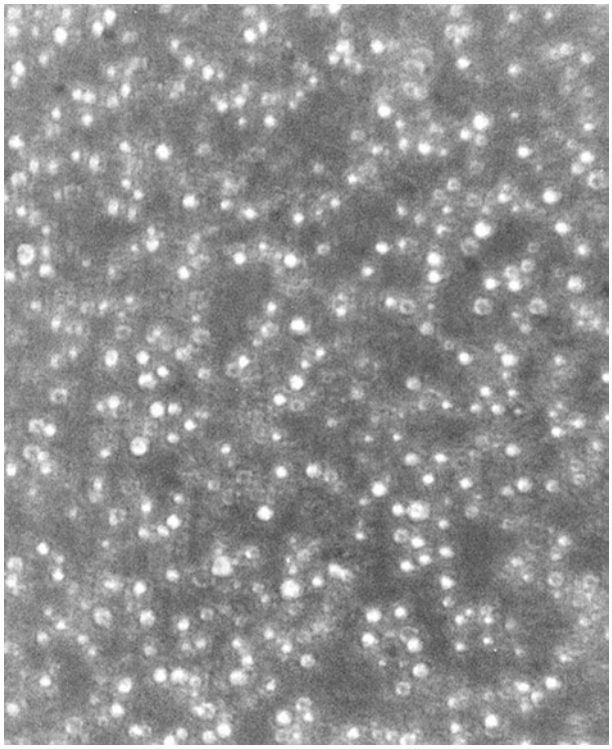

Figure 7.5 Microphotograph of a soy protein isolate gel with a volume fraction of oil droplets of 0.3. Width of photograph 120 μm.

7.3.3 Large deformation properties

Fracture properties, like fracture stress and strain, are important quality characteristics

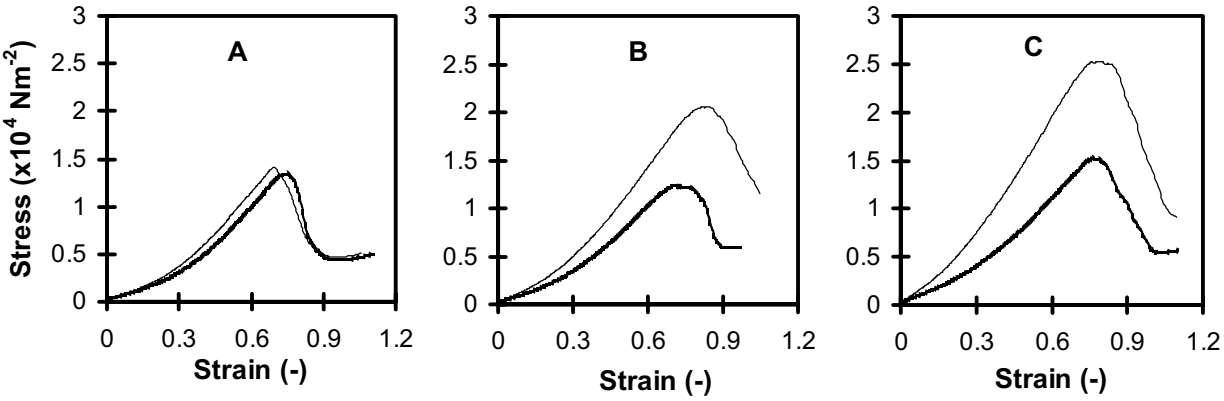

Figure 7.6 Stress-strain curves for soy protein isolate gels containing emulsion droplets with a mean diameter d_{32} of 1.17 μm (thin curves) and 2.70 μm (thick curves). Volume fraction of emulsion droplets (A) 0.1; (B) 0.2 and (C) 0.3.

of solid and solid-like food products (van Vliet & Luyten, 1995). Fracture stress and strain are a measure of the strength of the material and of its deformability before fracture occurs, respectively. In this study, fracture properties of 13 w/v% soy protein isolate gels with 0.2 M NaCl added have been investigated as a function of the volume fraction of emulsion droplets (0 – 0.3) by uniaxial compression. The sizes of the added emulsion droplets were 1.17 and 2.70 μm.

In Figure 7.6A-C average stress-strain curves of the gels are shown for ϕ is 0.1, 0.2 and 0.3, respectively. For all three volume fractions of emulsion droplets, stress increases faster with strain for the gels containing the small oil droplets, especially at a high volume fraction. Figure 7.7 shows the fracture stress and strain of the filled and unfilled gels as a function of the volume fraction of emulsion droplets. The fracture stress of the gels containing emulsion droplets with a diameter of 1.17 μm was higher than that of the gels containing large emulsion droplets (diameter of 2.70 μm). In both cases the increase in fracture stress with ϕ was much smaller than was found for G', especially at high ϕ (compare Figures 7.4 and 7.7). For explaining this difference, one has to consider two phenomena. Firstly, as already discussed above, it is likely that the emulsion droplets aggregate before and during the gel formation process. Secondly, for all gel systems studied the stress increased more than proportional with the strain (Figure 7.6). The gels exhibited strain hardening. The total stress is due to the resistance against deformation of the continuous soy protein network and due to the contribution of the dispersed particles. The resistance of the continuous soy protein network increases more than proportional with the strain while the resistance of the dispersed aggregates of emulsion droplets likely will not or much less. This would imply that the ratio

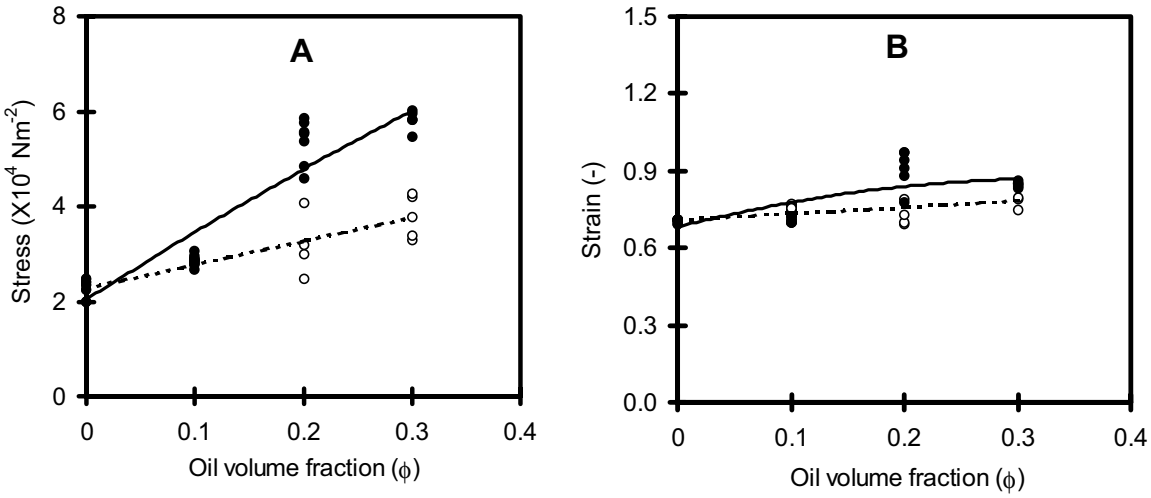

Figure 7.7 Fracture stress (A) and fracture strain (B) of soy protein isolate gels as a function of the volume fraction of emulsion droplets. The volume surface diameter of the emulsion droplets d_{32} was 1.17 μm (•) and 2.70 μm (o), respectively.

of the resistance against deformation of the aggregates of the emulsion droplets, $G'_{\text{aggregates of emulsion droplets}}$, over the resistance of the protein matrix, G'_m, decreased with ongoing deformation. It will cause a diminution of the effect of the dispersed emulsion droplet on the resistance against deformation with increasing deformation of the filled gel. However, this does not offer an explanation for the observed effect of the droplet size. Another explanation for the smaller increase of the stress with the volume fraction of emulsion droplets at large deformations than observed for small deformations may be the occurrence of slip between the droplets and the gel matrix at the higher stresses involved (Brownsey et al., 1987). The precise reason for the much stronger resistance (larger stresses involved) against large deformations for the gels containing small emulsion droplets is not clear. The storage modulus of the small droplets was about 2.3 times higher than that of the large ones. The observed effect is in line with experimental results of others (Matsumura et al., 1993; Kim et al., 1996) for whey protein and agar gels with dispersed emulsions droplets, respectively, and qualitatively in line with the Van der Poel theory. However, a rough calculation according the Van der Poel theory shows that the observed effect is much larger than may be expected in view of the difference in droplet modulus.

In contrast to fracture stress, there was no significant effect of the volume fraction of emulsion droplets and of droplet size on fracture strain. The fracture strain of a gel depends strongly on the inherent defect length, *i.e.* the size (length) of the largest inhomogeneities (weak spots), present in the system (van Vliet & Luyten, 1995; van Vliet & Walstra, 1995). These results indicate that the inherent defect length present in soy protein isolate gels is probably clearly more than 10 μm, the size of the largest emulsion droplets. For starch gels and for Dutch type of cheese, which is in essence a casein gel, inherent defect lengths were

observed of about 0.1 mm (van Vliet et al., 1991), so a defect length of more than 10 μm is not unlikely. In view of these data, the absence of a clear effect of ϕ and droplet diameter on the fracture strain is not unexpected.

7.4 Conclusions

The incorporation of soy protein stabilised emulsion droplets clearly affects the rheological properties of soy protein isolate gels. The increase in the dynamic moduli with the volume fraction of dispersed emulsion droplets was much stronger than predicted theoretically. This is likely caused by aggregation of the emulsion droplets before and/orf during the gel formation process. The effect of the emulsion droplets on the fracture stress was less than for the dynamic moduli and nearly absent for the fracture strain. A clear effect of droplet size on the fracture stress was observed. No definitive explanation for the effects on fracture parameters could be given.

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Summary
Samenvatting

Summary

Soy proteins can improve texture of food products, because they can form a gel on heating. Moreover, soy proteins are very suitable to replace meat and dairy products, because of their well-balanced amino-acid composition. However, soy proteins are not widely applied in food products. One of the reasons for this is that the varying conditions in food products make it difficult to predict gel properties and to control food texture. Most of the published work on soy protein gelation was performed with highly purified soy protein fractions (β-conglycinin or glycinin) at pH 7.6 and ionic strength 0.5, where soy proteins are highly soluble, but which is not a very relevant condition for food products (normally pH 3-7 and ionic strength 0.02-0.2), or concerns applied work with ill-defined soy protein preparations. There is also a lack in knowledge of fracture properties of soy protein gels, whereas these properties are important for food applications. Therefore, the aim of this study is to understand the factors determining formation and properties of soy protein gels and to understand the relations between gel properties and network structure in order to support application of soy proteins in food products.

In chapter 2, heat-induced gelation of laboratory-prepared soy protein isolate (SPI) at neutral pH was studied at various heating conditions by small deformation rheology. Heat-induced gel formation by soy proteins involves several processes like denaturation, aggregation (in which disulphide bridges play a role), network formation and gel stiffening. Heat denaturation was found to be a prerequisite for gel formation. This followed from the fact that: 1. The gelation temperature (84°C) was just above the onset denaturation temperature of glycinin, and 2. The stiffness of the gels, measured as the elastic modulus, G', increased with the proportion of denatured protein. Gel stiffening took place during prolonged heating at 90°C (increase in G') and was explained by the occurrence of rearrangements in the network structure and, to some extent, by further incorporation of protein in the network. Gel stiffening upon cooling was thermoreversibel and does, therefore, not involve disulphide bond formation and rearrangements in the network structure.

Chapter 3 describes the influence of pH and NaCl concentration on formation and rheological properties of SPI gels in relation to denaturation and protein aggregation/precipitation. It was found that at pH < 6 gel formation coincided with heat denaturation of β-conglycinin (~60-70°C), whereas at pH > 6 gelation concurred with heat denaturation of glycinin (~80-95°C). Gels exhibited a high stiffness at pH < 6 and a low stiffness at pH > 6. This might be caused by variations in the association/dissociation behavior of the soy proteins on heating as a function of pH as indicated by the different protein composition of the protein that remained dissolved after heating. At pH 3-5 all protein seems to participate in the network, whereas at pH > 5 less protein and especially less acidic

polypeptides take part in the network resulting in less stiff gels. Another explanation for the difference in stiffness of the gels is a difference in network structure as a function of pH, which was discussed in chapter 4. At pH 7.6, extensive rearrangements in the network structure took place during prolonged heating, whereas at pH 3.8 it did not happen and at pH 5.2 only to a small extent.

Chapter 4 focuses on the relations between rheological properties of SPI gels and their network structure, in terms of curvature of the strands and coarseness, as a function of pH and NaCl concentration. Coarseness of the network structure has been characterized independently by permeability measurements and confocal scanning laser microscopy. Results showed that gels at pH 3.8 and 5.2 were coarser than at pH 7.6, except for pH 3.8 and no extra NaCl added. Rheological properties determined were dynamic moduli, Young's modulus, fracture stress and fracture strain. In particular the fracture strain, *i.e.* to what extent the strands can be deformed before they break, gives information on the curvature of the strands and with that on the type of deformation energy, *i.e.* bending or stretching energy. Stretching energy will always be much higher than bending energy, so gels with mainly straight strands will have higher moduli than gels with mainly curved strands. Results showed that gels at pH 3.8 had lower fracture strains and higher moduli than gels at pH 5.2 and 7.6, while fracture stresses were about the same. This indicates that pH 3.8 gels consist mainly of straight strands and pH 5.2 and 7.6 gels of curved strands. Based on the results, relations between network structure and rheological properties were established, which can be generally applied to (protein) gels.

In chapter 5, gel formation and gel properties of β-conglycinin, glycinin and their mixtures were studied at pH 3.8 and 7.6 using small and large deformation rheology and differential scanning calorimetry. One of the aims of this study was to understand the difference in gelation temperatures of SPI between pH < 6 and pH > 6 as observed in Chapter 3. Glycinin exhibited higher gelation temperatures than β-conglycinin agreeing with their difference in denaturation temperature. Glycinin gels were stiffer than β-conglycinin gels at the same pH and protein concentration, and fractured at a higher strain and stress. The effect of pH on rheological properties of glycinin and β-conglycinin gels is similar as for SPI gels (see Chapter 3 and 4). The reason why glycinin is a more efficient and better gelling agent than β-conglycinin is believed to be due to a difference in network structure as well as in strength of the interactions between the protein molecules. Mixing of both soy proteins resulted in improved gelling properties (higher modulus) at pH 3.8 and reduced protein dispersability at pH 7.6. This strongly indicates the presence of an interaction between the proteins. Gels of the 1:1 mixture (pH 3.8) had a fracture stress and strain in between those of the gels of the separate proteins. The gelation temperature of SPI was higher at pH > 6 than at pH < 6, because at pH > 6 the concentration of β-conglycinin present in the studied systems

resulted in a too low increase of G' on denaturation of β-conglycinin to be regarded as gelation.

Chapter 6 presents results on the concentration dependence of the dynamic moduli of soy protein gels for different soy protein preparations (soy protein isolate (SPI), purified glycinin and a β-conglycinin rich fraction) at various pHs and salt concentrations. The concentration dependence of the storage modulus of glycinin and β-conglycinin gels was similar to that of SPI gels. For SPI, the critical protein concentration for gelation was estimated to be 3% at pH 7.6 (0.2 M NaCl), 6.5 % at pH 7 (0 M NaCl), and 0% at pH 5.2 and pH 3.8 (0.2 M NaCl). Relating the experimental data to a fractal model, both rheological and permeability measurements resulted in a consistent value for the fractal dimensionality D_f (= 2.3) for SPI gels at pH 3.8 and 0.2 M NaCl. At pH 5.2 and 7.6 (0.2 M NaCl), and pH 7 (0 M NaCl), the concentration dependence of the modulus could not be analysed satisfactorily using a fractal model.

In chapter 7 the presence of emulsified oil droplets on formation and properties of SPI gels was investigated at neutral pH using small and large deformation rheology. The storage and loss moduli of SPI gels were higher if NaCl was added. Both in absence and presence of 0.2 M NaCl, higher moduli were obtained at higher volume fractions of oil droplets. Furthermore, gel formation started at a lower temperature with increasing oil volume fraction. In the presence of 0.2 M NaCl, fracture stresses increased with increasing oil volume fraction. Also the size of the oil droplets affect fracture stress. Fracture stresses of gels containing oil droplets of 1.17 μm were higher than those containing oil droplets of 2.70 μm. The fracture strain did not depend on the volume fraction of oil droplets or the size of the oil droplets. The increase in the moduli with increasing volume fraction of oil droplets was stronger than predicted theoretically. This effect was attributed to aggregation of the emulsion droplets before and/or during the gel formation process.

In conclusion, this thesis shows that functional properties of soy protein gels strongly depend on conditions during gel formation, such as pH, salt concentration, protein concentration, heating conditions and addition of oil droplets. Gel properties like stiffness, fracture behavior and water holding ability are largely determined by gel network structure, which in turn depends on gelation conditions. The most important structural characteristics are pore size, thickness of the strands, and curvature of the strands. Also, the ratio β-conglycinin/glycinin affects gel properties.

Samenvatting: Vorming, structuur en reologische eigenschappen van soja-eiwitgelen

Soja-eiwitten kunnen de textuur van levensmiddelen verbeteren, omdat ze een gel kunnen vormen bij verhitting. Verder zijn soja-eiwitten erg geschikt om vlees en zuivelproducten te vervangen vanwege hun uitgebalanceerde aminozuursamenstelling. Soja-eiwitten worden echter nog niet veel toegepast in levensmiddelen. Een van de redenen hiervoor is dat de omstandigheden in de producten zo variëren dat het moeilijk is om de geleigenschappen te voorspellen of in de hand te houden. Veel van het onderzoek dat al gepubliceerd is op het gebied van de gelering van soja-eiwitten, is ofwel uitgevoerd met zuivere soja-eiwitfracties (β-conglycinine en glycinine) bij pH 7,6 en ionsterkte 0,5 -een conditie waarbij soja-eiwitten goed oplossen, maar die irrelevant is voor levensmiddelen (meestal pH 3-7 en ionsterkte 0,02-0,2)-, ofwel betreft toegepast onderzoek met slecht-gedefinieerde soja-eiwitproducten. Verder is er een gebrek aan kennis van de breukeigenschappen van soja-eiwitgelen, terwijl deze eigenschappen erg belangrijk zijn voor toepassing van soja-eiwit in levensmiddelen. Om de toepassing van soja-eiwitten in levensmiddelen te vergemakkelijken heeft dit onderzoek tot doel zowel de factoren die bepalend zijn voor de vorming en eigenschappen van soja-eiwitgelen als de relaties tussen geleigenschappen en netwerkstructuur te begrijpen.

In hoofdstuk 2 werd de hitte-geïnduceerde gelering van een eigengemaakt soja-eiwitisolaat (SPI) bij neutrale pH bestudeerd door middel van reologische metingen bij kleine vervorming onder verscheidene verhittingsomstandigheden. De hitte-geïnduceerde gelering van soja-eiwitten betreft verschillende processen zoals denaturatie (ontvouwing van het eiwit), aggregatie (waarin disulfidebruggen een rol spelen), netwerkvorming en het stijver worden van het gel. De resultaten toonden aan dat hitte-denaturatie een voorvereiste was voor gelering: 1. De geleringstemperatuur (84°C) was iets hoger dan de temperatuur waarop denaturatie van glycinine begon, en 2. De stijfheid van de gelen, gemeten als de elasticiteitsmodulus G', nam toe met de hoeveelheid gedenatureerd eiwit. Het stijver worden van gelen gedurende langdurig verhitten bij 90°C (toename in G') werd verklaard door het optreden van herrangschikkingen in de netwerkstructuur en, in mindere mate, door verdere inbouw van eiwit in het netwerk. Het stijver worden van gelen tijdens afkoelen was thermoreversibel, wat betekent dat er in dit traject geen disulfidebindingen gevormd werden en dat herrangschikkingen in het netwerk niet optraden.

Hoofdstuk 3 beschrijft de invloed van de zuurtegraad (pH) en zout op de vorming en reologische eigenschappen van gelen van soja-eiwitisolaat (SPI) met betrekking tot denaturatie en eiwitaggregatie en -precipitatie. Er werd gevonden dat gelvorming bij pH-waarden lager dan 6 tegelijk optrad met hittedenaturatie van β-conglycinine (~60-70°C), terwijl gelvorming bij pH-waarden hoger dan 6 samenviel met hittedenaturatie van glycinine

(~80-95°C). Gelen hadden een hogere stijfheid bij pH lager dan 6 en een lagere stijfheid bij pH hoger dan 6. Dit wordt waarschijnlijk veroorzaakt door het verschil in associatie/dissociatiegedrag van soja-eiwitten als gevolg van verhitten als functie van pH, wat duidelijk werd uit de verschillende eiwitsamenstelling van de oplosbare fractie na verhitten. Bij pH 3-5 lijkt al het eiwit deel te nemen aan de netwerkvorming, terwijl bij pH-waarden hoger dan 5 minder eiwit, vooral minder zure polypeptiden van glycinine, in het netwerk zit, wat resulteert in minder stijve gelen. Een andere uitleg voor het verschil in gelstijfheid is een verschil in netwerkstructuur als functie van pH. Dit wordt besproken in hoofdstuk 4. Bij pH 7,6 vinden herrangschikkingen in de netwerkstructuur in hoge mate plaats, terwijl ze bij pH 5,2 nauwelijks en bij pH 3,8 niet plaatsvinden.

Hoofdstuk 4 gaat over het verband tussen reologische eigenschappen van SPI-gelen en hun netwerkstructuur, in termen van gekromdheid van de strengen en grofheid van de gelen, als functie van pH en zoutgehalte. De grofheid van de netwerkstructuur werd bepaald door twee onafhankelijke metingen: permeabiliteitsmetingen en confocal scanning laser microscopie. Resultaten hiervan lieten zien dat gelen bij pH 3,8 en 5,2 grover waren dan bij pH 7,6, met uitzondering van pH 3,8 gelen waaraan geen zout toegevoegd was. Reologische eigenschappen die bepaald werden, waren de elasticiteits- en viscositeitsmodulus, Young's modulus, spanning bij breuk en vervorming bij breuk. Met name de breukvervorming, oftewel de mate waarin strengen uitgerekt kunnen worden voordat breuk optreedt, levert informatie over de gekromdheid van de strengen en daarmee over het soort vervormingsenergie, buig- of rekenergie. Rekenergie zal altijd veel hoger zijn dan buigenergie, waardoor gelen met voornamelijk rechte strengen stijver zijn (hogere moduli) dan gelen met voornamelijk gekromde strengen. Resultaten van de reologische metingen lieten zien dat gelen bij pH 3,8 een kleinere breukvervorming en hogere moduli hadden dan bij pH 5,2 en 7,6, terwijl hun breukspanningen vrijwel hetzelfde waren. Dit wijst erop dat pH 3,8 gelen voornamelijk uit rechte strengen en pH 5,2 en 7,6 gelen hoofdzakelijk uit gekromde strengen bestaan. Op grond van deze resultaten werden verbanden tussen netwerkstructuur en reologische eigenschappen vastgesteld, die algemeen toepasbaar zijn voor (eiwit)gelen.

In hoofdstuk 5 werden de vorming en de eigenschappen van gelen, die gemaakt werden met β-conglycinine, glycinine en mengsels daarvan, onderzocht met reologische metingen bij kleine en grote vervorming en differential scanning calorimetrie. Een van de doelen van dit onderzoek was het begrijpen van het verschil in geleringstemperatuur van soja-eiwitisolaat tussen pH < 6 en pH > 6, zoals werd waargenomen in hoofdstuk 3. Glycinine heeft een hogere geleringstemperatuur dan β-conglycinine, wat overeenkomt met het verschil in denaturatietemperatuur. Glycininegelen zijn stijver dan β-conglycininegelen bij dezelfde pH en eiwitconcentratie en breken bij een grotere vervorming en spanning. De invloed van pH op de reologische eigenschappen van glycinine- en β-conglycininegelen is vergelijkbaar met

die van SPI-gelen (zie hoofdstuk 3 en 4). Het feit dat glycinine een efficiënter en beter geleermiddel is dan β-conglycinine, komt waarschijnlijk door een verschil in netwerkstructuur en in wisselwerkingskrachten tussen de eiwitmoleculen. Mengen van beide eiwitten resulteerde in verbeterde geleigenschappen (hogere modulus) bij pH 3,8 en verslechterde dispergeerbaarheid bij pH 7,6. Dit wijst in sterke mate op de aanwezigheid van een wisselwerking tussen beide eiwitten. Gelen, gemaakt met een 1:1 mengsel van glycinine en β-conglycinine (pH 3,8), hadden een breukspanning en –vervorming, die tussen die van gelen van de afzonderlijke eiwitten in lag. De geleringstemperatuur van SPI was hoger bij pH > 6 dan bij pH < 6, omdat bij pH > 6 de concentratie van het aanwezige β-conglycinine in de bestudeerde systemen een te lage toename in G' veroorzaakte bij denaturatie om beschouwd te worden als gelering.

Hoofdstuk 6 geeft resultaten van de concentratieafhankelijkheid van de dynamische moduli van soja-eiwitgelen voor verschillende soja-eiwitproducten (soja-eiwitisolaat (SPI), gezuiverd glycinine en een β-conglycinine-rijke fractie) bij verschillende pH waarden en zoutgehaltes. De concentratieafhankelijkheid van de elasticiteitsmodulus van glycinine- en β-conglycininegelen was vergelijkbaar met die van SPI-gelen. De minimale eiwitconcentratie die nodig was voor gelering van SPI werd geschat op 3% bij pH 7,6 (0,2 M NaCl), 6,5% bij pH 7 (zonder zout) en 0% bij pH 5,2 en 3,8 (0,2 M NaCl). Wanneer de experimentele data gerelateerd worden aan een theoretisch, fractaal model, komen zowel de reologische als de permeabiliteitsmetingen uit op een consistente waarde voor de fractale dimensionaliteit D_f (=2,3) voor SPI-gelen bij pH 3,8 en 0,2 M NaCl. Bij pH 5,2 en 7,6 (0.2 M NaCl) en pH 7 (zonder zout) kon de concentratieafhankelijkheid van de modulus niet goed gerelateerd worden aan een fractaal model.

In hoofdstuk 7 werd de aanwezigheid van geëmulgeerde oliedruppels op de vorming en eigenschappen van SPI-gelen bij neutrale pH onderzocht met reologische metingen bij kleine en grote vervorming. De opslag- en verliesmoduli van SPI-gelen waren hoger wanneer zout toegevoegd was. Zowel in aan- als afwezigheid van 0.2 M NaCl werden hogere moduli verkregen bij hogere volumefracties van de oliedruppels. Bovendien begon de gelering bij een lagere temperatuur met toenemende volumefractie aan olie. In de aanwezigheid van 0.2 M NaCl namen breukspanningen toe met toenemende volumefractie. Ook de grootte van de oliedruppels heeft invloed op de breukspanning. Breukspanningen van gelen met oliedruppels van 1,17 μm waren hoger dan die van gelen met oliedruppels van 2,70 μm. De vervorming bij breuk was onafhankelijk van de volumefractie olie of de druppelgrootte. De toename van de moduli met de volumefractie was groter dan voorspellingen aan de hand van theoretische modellen. Dit effect werd toegeschreven aan de aggregatie van oliedruppels voor en gedurende het geleerproces.

Dit proefschrift laat zien dat functionele eigenschappen van soja-eiwitgelen sterk afhangen van de omstandigheden tijdens de gelvorming, zoals pH, zoutgehalte, verhittingscondities en aanwezigheid van oliedruppels. Geleigenschappen als stijfheid, breukgedrag en watervasthoudend vermogen worden in grote mate bepaald door de netwerkstructuur, die weer afhangt van de geleringscondities. De meest belangrijke structuurkenmerken zijn poriegrootte, dikte van de strengen en gekromdheid van de strengen. Verder heeft de verhouding glycinine/β-conglycinine invloed op de geleigenschappen.

Nawoord

Heerlijk, het proefschrift is af! Na al die jaren mag ik nu eindelijk het nawoord van mijn proefschrift schrijven. In tegenstelling tot de meeste AIO's ben ik niet meteen na mijn afstuderen begonnen met mijn promotie-onderzoek, maar heb ik eerst ruim anderhalf jaar bij het ATO gewerkt. Werken op dit onderzoeksinstituut in Wageningen was echt een eye-opener voor mij, omdat er zoveel niet-Wageningers werkten. Ik heb er veel geleerd en ook erg veel lol gehad. Toch wilde ik nog steeds graag een AIO-baan en ik hoefde dan ook niet lang na te denken, toen Ton van Vliet mij vertelde dat hij een onderzoeksproject had over soja-eiwitgelen. Dit project combineerde mijn specialisatie en interesse in de levensmiddelennatuurkunde op een perfecte manier met de kennis over eiwitten die ik had opgedaan op het ATO.

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Marianne

Curriculum vitae

Jacoba Maria Sophie (Marianne) Renkema werd op 21 december 1968 geboren te Vlaardingen. In 1987 behaalde zij het gymnasium-β-diploma aan het Revius Lyceum te Doorn. Aansluitend begon zij aan de studie Levensmiddelentechnologie aan de toenmalige Landbouwuniversiteit te Wageningen (LUW). Onderdelen van deze studie waren afstudeervakken Levensmiddelennatuurkunde en Proceskunde, uitgevoerd op de LUW, en stages bij Unilever Research Laboratory Colworth House in Engeland en bij MONA te Woerden. In 1993 studeerde zij af.

Begin 1994 was zij gedurende enkele maanden toegevoegd onderzoeker bij de toenmalige sectie Zuivel en Levensmiddelennatuurkunde (LUW). Vervolgens werkte ze van mei 1994 tot en met december 1995 als onderzoeker bij het Instituut voor Agrotechnologisch Onderzoek (ATO-DLO) in de groep Industriële Eiwitten. Van januari 1996 tot mei 2000 was zij assistant in opleiding (AIO) bij het Centrum voor Eiwittechnologie TNO-WU en de leerstoelgroep Levensmiddelennatuurkunde van de Wageningen Universiteit. Het onderzoek tijdens deze laatste periode staat beschreven in dit proefschrift. Van januari tot en met maart 2001 had zij een aanstelling als toegevoegd docent bij de leerstoelgroep Productontwerpen en kwaliteitskunde.

Marianne woont samen met Wiet Jenniskens. Zij hebben een zoon, Maurits, geboren op 15 augustus 1999 te Arnhem. Hun tweede kind verwachten zij in januari 2002.

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Stellingen

1. Het verschil in netwerkstructuur en reologische eigenschappen van soja-eiwitgelen als functie van pH heeft zijn oorsprong in het verschil in invloed van de thiol/disulfide-uitwisselingsreactie op aggregatieprocessen als functie van pH.

   dit proefschrift

2. Tijdens koelen zijn zwavelbrugvorming en herrangschikkingen in de netwerkstructuur niet van belang voor het stijver worden van soja-eiwitgelen.

   dit proefschrift

3. Voor de stijfheid van gelen is gekromdheid van de strengen een belangrijker structuurkenmerk dan poriegrootte.

   dit proefschrift

4. Ondanks de grote verscheidenheid aan theoretische modellen met betrekking tot aggregatie en gelering van eiwitten zijn deze processen nog steeds een slecht begrepen fenomeen.

5. De door Chen en Dickinson gebruikte term emulsiegelen voor verhitte emulsies bereid met 10% wei-eiwitoplossingen en 11-45 volume% olie suggereert ten onrechte dat emulsiedruppels de enige netwerkvormer zijn.

   J. Chen and E. Dickinson. Journal of Texture Studies 29 (1998) 285-304

6. Genetische modificatie kan beter ingezet worden om planten geschikt te maken voor droge of zoute gebieden, dan om het gebruik van bestrijdingsmiddelen te reduceren.

7. De Engelse uitdrukking “in labour” is een betere weergave van de werkelijkheid dan het Nederlandse equivalent “bevallen”.

8. Het is verstandig als ouders hun werk en zorgtaken onder elkaar gelijk verdelen.

9. De term “functional foods” suggereert dat er levensmiddelen zijn die geen functie hebben.

J. M. S. Renkema
Formation, structure and rheological properties of soy protein gels
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