# Heat denaturation of soy glycinin

## Structural characteristics in relation to aggregation and gel formation

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# Stellingen

1. Voor de koppeling van moleculair werk aan bestaande theorieën voor de vorming van deeltjesgelen is het noodzakelijk eerst tot een duidelijke definitie van het begrip "deeltje" te komen.

2. Het hittedenaturatie mechanisme van glycinine is niet tot in detail opgehelderd omdat in hittedenaturatie studies meestal geen rekening gehouden is met het feit dat glycinine bestaat uit een heterogene groep eiwitten (*dit proefschrift*).

3. Indeling van soja-eiwitten op basis van Svedberg sedimentatie coëfficiënten leidt eerder tot verwarring dan tot begrip.

4. Gezien de gebruikte experimentele condities (pH, I) is 80% van de artikelen over soja-eiwitten die gepubliceerd zijn in op levensmiddelen georiënteerde tijdschriften niet direct toepasbaar op levensmiddelen (*dit proefschrift*).

5. De veelal gebruikte aanduiding "moeilijke eiwitten" voor sojaeiwitten gaat wellicht voorbij aan hun werkelijke functie in de plant.

6. De classificatie van een eiwit als opslageiwit is vaak gerelateerd aan onbekendheid met de functie van het eiwit.

7. De wijze waarop het woord "stelling" is gedefinieerd in de van Dale, nl. "thesis waarop men, na verdediging aan een universiteit promoveert" is tekenend voor de kloof tussen universiteit en de rest van de maatschappij.

8. Het hebben van een chronische bronchitis kan adembenemend zijn.

9. De onderzoeker die met beide benen op de grond blijft staan komt niet ver.

10. Net als veel analyse apparatuur functioneren AIO's vaak ook beter in een passende temperatuurgecontroleerde ruimte.

Stellingen behorende bij het proefschrift

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Wageningen, 30 augustus 2001

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Catriona M. M. Lakemond

# Heat denaturation of soy glycinin

## Structural characteristics in relation to aggregation and gel formation

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit
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# Abstract

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key words soy protein; glycinin; thermal stability; pH; ionic strength; genetic variant; solubility; gelation

The main aim of this thesis was to study structural changes of soy glycinin at different conditions (pH and ionic strength) during thermal denaturation and their effect on aggregation and gel formation.

The results show that, generally, glycinin is predominantly present in the 7S form at pH 3.8, while at pH 7.6 the major component is the 11S form. When, at ambient temperatures, the ionic strength at pH 7.6 is lowered from 0.5 to 0.2 or 0.03 the basic polypeptides within the glycinin molecule shift more to the exterior of the glycinin complex. This structural reorganisation caused the pH of minimal solubility to shift to higher values. The 7S form, which is more unfolded than the 11S form, denatures at a lower temperature than the 11S form. Changes in secondary structure take place simultaneously with denaturation.

While at I = 0.03 the gels were found to be fine stranded, gel coarseness increased when the ionic strength was higher. At I = 0.03 finer gel network structures were formed at pH 3.8 than at 7.6, whereas for I = 0.2 and 0.5 the reverse was found. The observed differences in gel stiffness did not correspond to coarseness. The gel structure was clarified into more detail by the use of physico-chemical and spectroscopic techniques. The nature of the primary network particles was different at pH 7.6 (basic polypeptides) than at pH 3.8 (acidic and basic polypeptides).

The heat denaturation process develops at 1% protein similarly as the heat denaturation process at 10% protein. However, the final gel structure and strength cannot be predicted from measurements performed at 1% protein. The heat denaturation mechanisms were not solved into more detail due to the fact that the genetic variants of glycinin differ in thermostability. It was found that the denaturation temperatures of these variants increase in the order G1/G2/G3/< A4<G5<G4.

# Abbreviations

| | |
|---|---|
| A | acidic polypeptides |
| B | basic polypeptides |
| BBI | bowman birk trypsin inhibitor |
| BSA | bovine serum albumin |
| CD | circular dichroism |
| CE | capillary electrophoresis |
| CSLM | confocal scanning laser microscopy |
| DSC | differential scanning calorimetry |
| DTT | dithiothreitol |
| FITC | fluoresceine isothiocyanate |
| GPC | gel permeation chromatography |
| I | ionic strength |
| IR | infrared |
| KSTI | kunitz trypsin inhibitor |
| MALDI-TOF MS | matrix assisted laser desorption/ionization time of flight mass spectrometry |
| PDCAAS | protein digestibility correceted amino acid score |
| PDI | protein dispersibility index |
| SDS-PAGE | sodiumdodecylsulphate polyacrylamide gelelectrophoresis |
| SPI | soy protein isolate |
| UV CD | ultraviolet circular dichroism |
| UV | ultraviolet |

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Chapter 1

# Introduction

## 1 Soy

Soybeans are an important agricultural crop. In the year 2000 the global soy production was estimated at 150 million tons (Liu, 2000). The major producers are the USA (50%), Brazil (18%), China (12%) and Argentina (9%) (Liu, 1997).
It is widely believed that the soybean originated in China around 4000-5000 years ago (Liu, 1997). Soybean production and utilisation as food arose in China no later than the 11$^{th}$ century B.C. (Hymowitz, 1970). Soybeans were first introduced to Europe in 1712. Soybeans have favourable agronomic characteristics, including a good capability to grow on a wide range of soil and an adaptability to fix nitrogen, which makes the soybean a good rotational crop for use together with high nitrogen consuming crops, such as corn and rice (Liu, 1997). Furthermore, soybeans are an economically interesting agricultural product because of their chemical composition. On an average dry matter base, soybeans contain about 40% protein and 20% oil. With this composition, soybeans have the highest protein content among all the food crops and second highest oil content of all food legumes. Soy oil contains a high proportion of unsaturated fatty acids (e.g. linoleic and linolenic acids) which are generally considered to be healthier than saturated fatty acids. Soy proteins contain all the essential amino acids, which are present in amounts that closely match those required for humans, except for methionine and cysteine. When evaluated by the method known as the Protein Digestibility Corrected Amino Acid Score (PDCAAS), that is based on human amino-acid needs and the digestibility of the protein, the nutritional quality of soy protein isolate is the same as that of beef (PDCAAS = 0,92) (Liu, 1997).

## 2 Soy applications in food

For thousands of years the inhabitants of oriental countries have consumed soybeans in various forms of traditional foods, such as tofu, soy sauce, tempe, miso etc. In contrast, in the Western world most of the soybeans are converted into oil and defatted meal. Although soybean oil is produced almost entirely for human consumption, soy meal is mainly used as animal feed. Only a small portion of the defatted meal is processed into soy protein products, such as soy protein isolate and soy protein concentrate. They are not directly consumed as

food but are applied as an ingredient in numerous food systems, including bakery, dairy, meat, breakfast, cereal, beverages, infant formula, and dairy and meat analogues (Liu, 1997). Based on their nutritional properties soy proteins are often used to replace (expensive) animal protein in food products. They can also be used for obtaining certain functional properties, including gelling, emulsifying, water holding and fat absorbing properties. Depending on the specific food product different functional properties are needed. A key constraint limiting soy use as food is the characteristic beany or greeny flavour that is associated with soybean products. This flavour is particularly problematic to Western consumers, who are unaccustomed to it. A key factor in the development of beany flavour in soy products is the presence of lipoxygenases. Other constraints include the oxidative instability of soy oil, lack of certain functional properties of soy protein products, and the presence of several anti-nutritional factors. However, the proteineceous anti-nutritional factors can be inactivated mostly by giving a proper heat treatment (Liu, 2000).
Among the many soy components examined, soy protein (isolate) and isoflavones are the most promising compounds with regard to their health benefits (Anderson et al., 1995; Messina, 1999). Epidemiological studies have indicated that populations that consume soyfoods generally have lower incidences of breast, colon and prostate cancers, osteoporosis and menopausal symptoms (Anderson et al., 1995; Barnes, 1998; Messina, 1999) than populations in which soyfoods are not common. The US Food and Drug Administration approved a health claim in 1999 for soy protein. The approval allows food products which contain 6.25 g of soy protein per serving to carry the claim that soy based protein, combined with a diet that is low in saturated fat and dietary cholesterol, may reduce the risk of heart disease. These findings about the health benefits of soy have improved the image of soy as a food, and increased consumer interest in soyfoods and soy enriched foods. Nowadays, the soyfood market is one of the fastest growing categories in the food industry (Liu, 2000).

## 3 Soy proteins

The soybean consists of about 40% of protein. The protein fraction can be divided into three groups: 1) the proteins involved in metabolism 2) the structural proteins (ribosomal, chromosomal, and membrane proteins) and 3) storage proteins, which include approximately 80-90% of the total soybean protein content (Liu, 1997). When a protein is present in amount of 5% or more of the total protein fraction it is generally defined as a storage protein (Derbyshire et al., 1976). However, we would define storage proteins as proteins that do not have any significant enzymatic activity or other biological function except as being source of

nutrition during germination. These soy storage proteins are globulins (salt soluble at neutral pH), and they precipitate at pH 4.5 to 4.8, for which they are often called acid-precipitable proteins (Liu, 1997). The storage proteins were first characterised by Osborne and Campbell (in 1889), who believed that the soybean contained one major globulin component, which they named glycinin. Later it was demonstrated that this globular component was heterogeneous and consisted of four components, which were denoted 2S, 7S, 11S, and 15 S, as derived from ultracentrifugal analysis (Naismith, 1955; Wolf and Briggs, 1956). This nomenclature, based on sedimentation constants, is still being used throughout literature. It is, however, insufficient since, in fact, these sedimentation constants as well as the separation patterns of the different fractions depend largely on the type of buffer used, the pH, etc. Another system frequently used in literature is one that is based on immunological methods to differ between proteins. Immunologically soybean globulins can be separated into glycinin, β-conglycinin, γ-conglycinin and α-conglycinin. Under conditions used often in literature (I = 0.5 pH 7.6) the 2S fraction consists of the Bowman Birk- (BBI) and Kunitz trypsin inhibitor (KSTI), cytochrome C and α-conglycinin (Catsimpoolas and Ekenstam, 1969; Wolf, 1970). The 7S component consists mainly of β-conglycinin, but also γ-conglycinin and basic 7S globulin are part of the 7S fraction under standard conditions (Wolf, 1970). The 11S fraction consists at I = 0.5 and pH 7.6 of glycinin (Wolf, 1970) and the 15 S fraction of polymers of glycinin (Wolf and Nelsen, 1996).

Several studies have been performed to examine the distribution of protein over the different fractions (e.g. Naismith, 1955; Wolf and Briggs, 1956; Wolf et al., 1962; Saio et al., 1969; Thanh and Shibasaki, 1976; Hughes and Murphy, 1983; Murphy and Resurreccion, 1984; Sato et al., 1986; Iwabuchi and Yamauchi, 1987). The literature data shows considerable variation in the 11S and 7S content of soybeans. It has been found that this variation can be (partly) explained by genetic differences between the soybean varieties (Saio et al., 1969; Hughes and Murphy, 1983; Murphy and Resurreccion, 1984) and the method of determination (Saio et al., 1969). It was also found that the environmental conditions influence the 11S content within one variety (Murphy and Resurreccion, 1984). Table 1 gives an overview about the distribution of protein over the different fractions.

**Table 1** Storage protein composition of soybeans obtained by ultracentrifugational (U), densitometrical (D) or immunological (I) methods.

| Reference | method | 2S (%) | 7S (%) | 11S (%) | 15S (%) |
|---|---|---|---|---|---|
| Wolf et al. (1962) | U | 22 | 37 | 31 | 11 |
| Saio et al. (1969) | U | 13-18[1] | 30-46[1] | 36-53[1] | 0-4[1] |
| Thanh and Shibasaki (1976) | U | 16 | 52 | 31 | 1 |
| Saio et al. (1969) | D | | 38-50[1] | 50-62[1] | |
| Hughes and Murphy (1983) | D | | n.d. | 31-38[1] | |
| Sato et al. (1986) | D | 3[2] | 37 | 37 | |
| Murphy and Resurreccion (1984) | I | | 17-21[1] | 47- 57[1] | |
| Iwabuchi and Yamauchi (1987) | I | | 23 | 32 | |

[1]Values depend on soybean variety; [2] Value for KSTI only

For densitometrical and immunological determination: 7S is equivalent to β-conglycinin and 11S to glycinin

The minor proteins in soybeans include monophosphatase, phophodiesterase, calmodulin, α-galactosidase, lactate dehydrogenase, lipoxygenase and β-amylase. Also lectins or agglutinins are present in soybeans. Furthermore a polygalacturonase-inhibiting protein, metallothionein, urease and β-glucosides are detected in soybean (Garcia et al., 1997).

## 4 Soy glycinin

Fischer and Goldberg (1982) described three glycinin genes (G1 - G3) and Scallon et al. (1985) reported the existence of two additional genes (G4 and G5). The five genes have diverged into two subfamilies that are designated as Group I and Group II glycinin genes (Nielsen, 1984). The different genetic variants of glycinin are also referred to as "subunits". The sequence identity in each group is about 80% and that between groups is about 45% (Adachi et al., 2001). The molecular weight is lower for group I subunits than for group II subunits (Utsumi et al., 1997). The different glycinin subunits were identified on the basis of their amino acid sequences (Moreira et al., 1979; Moreira et al., 1981; Staswick et al., 1981; Staswick et al., 1984a). Each subunit consists of an acidic and a basic polypeptide (Staswick et al., 1981), linked by a single disulfide bridge (Staswick et al., 1984b), except for the acidic polypeptide $A_4$ that is present in G4 (Staswick et al., 1981). The two polypeptide chains result from posttranslational cleavage of proglycinin precursors (Tumer et al., 1981). An overview of the constituents of glycinin is given in Figure 1. In literature also the labels $A_{1a}B_{1b}$ (e.g. Utsumi et al. 1997) or $A_{1a}B_x$ (e.g. Utsumi et al., 1987a) are used for G1 and $A_{1b}B_2$ for G3 (e.g. Utsumi et al., 1997). The molecular weights given in Figure 1 are calculated from the DNA

sequence of the different glycinin genes. The calculated molecular weights vary for the acidic polypeptides from 31.5 to 36.4 kDa (except for $A_5$). However, using electrophoretic methods the molecular weight of the acidic polypeptides is around 38 kDa (e.g. Staswick et al, 1981). For the basic polypeptides similar molecular weights are found when using both techniques. Figure 2 gives an overview of the proposed positions of disulfide bonds and free cysteine residues in glycinin subunits.

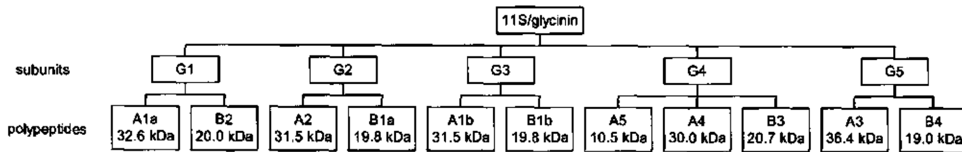


**Figure 1** The constituents of the soy protein glycinin. Also the labels $A_{1a}B_{1b}$ or $A_{1a}B_x$ are used for G1 and $A_{1b}B_2$ for G3. The molecular weights (exclusive signal and propeptides) are calculated from the amino acid sequences as derived from the DNA sequence of the five different glycinin genes (based on Scallon et al. (1987); Nielsen et al. (1989); Sims and Goldberg (1989); Thanh et al. (1989); Cho and Nielsen (1989)).

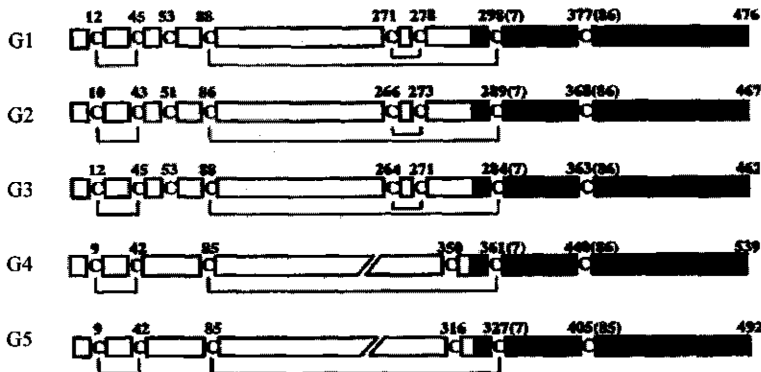


**Figure 2** Schematic representation of the proposed positions of disulfide bonds and free cysteine residues in glycinin subunits. Cysteine residues are designated by C. The number of residues from the N-terminus is indicated (in parentheses the number of residues from the N-terminus is indicated). Open and black areas are the acidic and basic polypeptides, respectively (Utsumi et al., 1997).

For glycinin it is known that pH and ionic strength influence glycinin quaternary structure. At neutral pH and ionic strengths above 0.35 glycinin sediments as a single, well-defined peak of approximately 11S in the ultracentrifuge (Wolf and Briggs, 1958). The molecular weight of

glycinin has been investigated by a variety of techniques and is most often reported to be 320.000-360.000 Da (Derbyshire et al., 1976; Iyengar and Ravestein, 1981), corresponding to the 11S or hexameric form). At ionic strengths below 0.01 and neutral pH a small amount of the 11S dissociates into a 7S (trimer) and a 3S form (monomer). Readjustment to higher ionic strength results in the conversion of the 7S form into the 11S form, but 3S form is not converted into the 11S form. Dissociation into a 7S and a 3S form has also been found at pH<3.8 (Wolf and Briggs, 1958). The 7S form has a more non-structured conformation at a secondary level than the 11S form (Utsumi et al., 1987b).

Badley et al. (1975) proposed a model for the 11S form of glycinin, in which 11S glycinin consists of six acidic polypeptides and six basic polypeptides. These twelve polypeptides are packed into identical hexagons placed one on the other, yielding a hollow oblate cylinder (Figure 3). Badley et al. (1975) could not postulate anything directly about the relative arrangements of the acidic and basic polypeptides, since the resolution of the electron micrographs was too low.

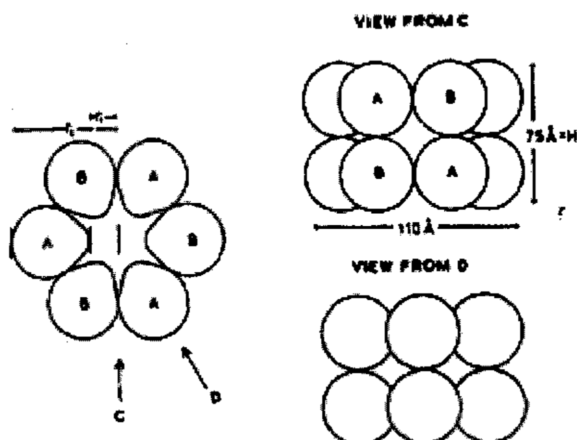


**Figure 3** Schematic representation of 11S glycinin (Badley et al., 1975).

The overall structure of proteins can be solved by crystallography if it is possible to crystallise the protein. The crystal structure of a protein gives information about its (primary), secondary, tertiary and quaternary structure of a protein. The primary structure is the covalent structure, which is defined by the amino-acid sequence and any post-transitional covalent modifications. The secondary structure refers to regular local structures of linear segments of polypeptide chains. Generally, three defined types of secondary folding are present in proteins, which are

α-helix, β-sheet, and β-turn. The tertiary structure is the overall topology of the folded polypeptide chain. The quaternary structure of a protein refers to polypeptide-polypeptide interactions. With regard to quaternary structure, however, there is often uncertainty about whether interactions between protein molecules present in a crystal lattice are relevant to the protein structure and/or to the crystallisation process (Creighton, 1993). No crystallographic information on glycinin is available since the crystallisation is hampered by the heterogeneity of subunits (Lawrence et al., 1994; Adachi et al., 2001). On the basis of amino acid sequence alignment it is predicted that the 11S family is related evolutionary to the 7S family. Until now the crystal structures of two 7S globulins, canavalin from Jack Beans (Ko et al., 1993; Ko et al., 2000) and phaseolin from Kidney Beans (Lawrence et al., 1990; Lawrence et al., 1994) have been reported. Recently, Adachi et al. (2001) determined the crystal structure of proglycinin G1, which is a precursor of the glycinin G1 subunit. This subunit was expressed in *Escherichia coli* to obtain a homogeneous glycinin. A trimeric molecule was found in an asymmetric unit of crystals. The constituent protomers are arranged around a 3-fold symmetry axis with dimensions of 95Å x 95Å x 40Å. The core of the protomer consists of two jellyroll β-barrels and two extended helix domains. This structure of proglycinin is similar to those of canavalin and phaseolin belonging to the 7S globulin family, strongly supporting the hypothesis that both 7S and 11S globulins are derived from a common ancestor. The crystal structure of 7S globulin is given in Figure 4 (Lawrence et al., 1994).

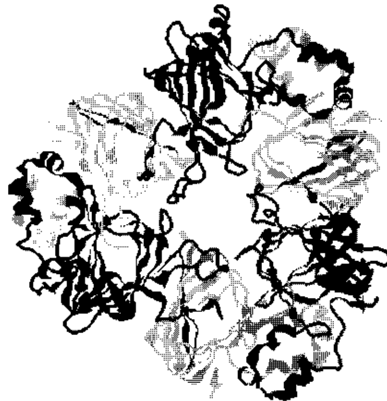

**Figure 4** The crystal structure of the 7S globulin (Lawrence et al., 1994). Based on sequence alignment with glycinin the position of the acidic (black) and basic polypeptides (grey) were estimated.

Crystallography is a highly time consuming technique. Alternative techniques are applied widely to study structural properties of proteins. For examining structural properties under a variety of conditions these methods are better suitable than crystallography. In this thesis, for determining secondary structure we used IR spectroscopy and far-UV Circular Dichroism (CD). Tertiary structure was studied by fluorescence spectroscopy (excitation of tryptophan residues) and near-UV CD, whereas changes in quaternary structure were followed by gel permeation chromatography (GPC) and ultracentrifugational analysis in sucrose gradients.

## 5 Heat denaturation; Aggregation and gel formation

As stated above soy proteins are used in many food products. Foods that contain soy proteins are like most foods subjected to some form of heat treatment (e.g. pasteurisation, sterilisation) during processing to obtain (1) a certain shelf life, (2) an inactivation of proteineceous anti-nutritional properties, and (3) functional properties. In industrial processes the effects of heating on functional properties are not well understood. The method of "Trial and Error" is often used to obtain the desired functional property. This means that the effect of each change in processing conditions needs to be evaluated in view of the functional properties. The term "functionality" as applied to food ingredients has been defined as any property besides nutritional attributes that influences an ingredient's usefulness in foods (Boye et al., 1997). Functional properties of a protein are related to the physical, chemical and conformational properties, which include e.g. size, shape, amino-acid composition and sequence, and charge distribution (Boye et al., 1997). A better understanding of the effects of heating on protein structure and how these structural changes are related to functional properties will enable the development of new products with predictable functional properties.

Heat treatment is the most important food-processing operation that contributes to protein structural changes, but also variations in pH can induce these changes. Heat treatment of globular proteins in solvent increases their thermal motion, leading to rupture of various intermolecular and intramolecular bonds that stabilise the native protein structure. This results in hydrophobic amino acid residues becoming exposed to the solvent and a reorganisation of the protein structure (Boye et al., 1997). Therefore, denaturation of food proteins has been defined as a process in which the spatial arrangement of polypeptide chains within the molecule has been changed from that typical of the native protein to a more disordered arrangement (Kauzmann, 1959). Protein denaturation can be defined, more specifically, as any modification in conformation (secondary, tertiary or quaternary) not accompanied by the

rupture of peptide bonds involved in the primary structure (Tanford, 1968). Various levels of denaturation can be distinguished according to whether the secondary, tertiary, or quaternary structure of the protein is involved in the process. Depending on the protein and conditions, denaturation may be confined to a region of the protein or may involve the complete molecule (Tanford, 1968). The amount of ordered structure within the protein molecule generally decreases after thermal denaturation (Boye et al., 1997). Unfolded molecules associate to form aggregates of irreversibly denatured molecules, which may lead to extended aggregation, precipitation and gelatine (Utsumi et al., 1997). The formation of the network or aggregate/precipitate arises from a combination of covalent bonds and non-covalent bonds. The main types of covalent bonds that can be formed are the disulfide bonds of cysteine residues. These bonds stabilise the structure of many proteins and are stronger than non-covalent interactions. The main types of non-covalent bonds include hydrogen bonds, electrostatic bonds and hydrophobic interactions. Electrostatic bonds may be stronger than other non-covalent bonds, but their existence is determined by the pH and the salt concentration (I = ionic strength) (Oakenfull et al., 1997). The formation of a thermally induced gel matrix or coagulum from proteins involves the following events (Figure 5):

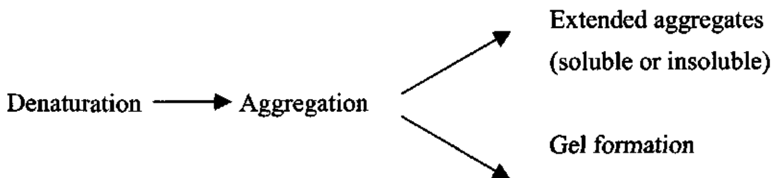


**Figure 5** Formation of thermally induced protein aggregates or gels (modified from Utsumi et al., 1997).

Denaturation is often a prerequisite for the formation of protein aggregates or gels. Thermal aggregation (coagulation) is defined as the random interaction of protein molecules by heat treatment, leading to formation of aggregates that could be either soluble or insoluble (Hermansson, 1978). Thermal gelation on the other hand refers to the formation of a three dimensional network exhibiting a certain degree of order (Kilara and Sharkasi, 1986). A gel is semi-solid; it has a rigidity but it deforms under stress. Being visoelastic, a gel has both solid like and liquid like rheological properties (Oakenfull et al., 1997). For globular proteins generally two different types of gel networks can be distinguished, fine stranded and coarse networks, but intermediate structures have also been reported (Tombs, 1974). In fine stranded networks the proteins are attached to each other like a string of beads. This type of gel is

usually transparent, indicating that the protein aggregates within the gel are smaller than about 50 nm. Coarse networks are non-transparent and are thought to be formed by random aggregation of proteins into clusters, which aggregate to thick strands. When the network structure becomes more coarse, the ability of the gels to retain water decreases (Hermansson, 1986).

## 6 Aggregation and gel formation of soy proteins

In recent decades, considerable information about the structural properties of soy proteins at the primary, secondary and tertiary and quaternary structural levels has been accumulated. The basic mechanisms involved in aggregation and gelation properties are becoming well-understood (Utsumi et al., 1997).

Denaturation of glycinin is affected when pH, ionic strength or protein concentration are modulated. For analysing thermal denaturation of glycinin the heating temperature should be chosen carefully, such that it is the same as the thermal denaturation temperature under the conditions used. Mori et al. (1981) proposed an overall scheme of heat induced changes in glycinin at I = 0.5 and pH 7.6 as shown in Figure 6. In step 1 glycinin aggregates (8 x $10^6$ Da) are formed when glycinin solution is heated both at low and high protein concentrations. On subsequent heating at low protein concentrations, the soluble aggregate disaggregates to acidic and basic polypeptides (step 2'), while at high protein concentrations it undergoes association resulting in gel formation (step 2). Although the formation of network structure is completed by step 2, the stabilisation of network structure through further formation of non-covalent bonding and disulfide cross-links by subsequent heating (step 3) proceeds.

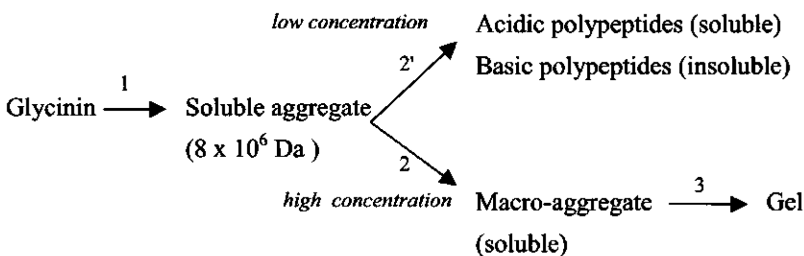


**Figure 6** Overall scheme of thermal change of glycinin at pH 7.6 and 0.5 ionic strength (Mori et al., 1981).

The soluble aggregates and gel network have been visualised by transmission electron microscopy to understand the mechanism of network formation in the thermal gelation process of glycinin (Nakamura et al., 1984b). Based on these results they proposed a string of beads model for glycinin at I = 0.5 and pH 7.6 in which the 11S molecules associate to form a linear string (Figure 7). In this model a "bead" corresponds to the 11S form of glycinin. A brief description of the proposed scheme is as follows: (1) Within a short time of heating (about 15 seconds) short strands consisting of about six beads are formed (Strand I); (2) Strand I associates with itself to form straight strands (strand II); (3) Strand II associates with itself or with strand I to form both branched and unbranched strands (strand III). The gel network could then form from these strand III units. Hermansson (1985) also proposed a string of beads model for glycinin gelation by a process similar to that described above, with exception of the mechanism that triggers association. Hermansson suggested that the glycinin 11S form dissociates into constituent subunits and reassembles into a glycinin form with the formation of gels.

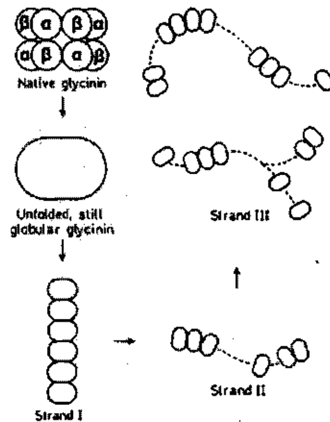


**Figure 7** Schematic representation of formation of soluble aggregates in the course of gelation of glycinin. The α and β indicate acidic and basic polypeptides, respectively (Nakamura et al., 1984b).

Although some models considering glycinin aggregation and gel formation have been postulated, no attention has been given to the heterogeneity in glycinin subunits (genetic variants). It is known that each glycinin subunit exhibits different behaviour on heating (Mori et al., 1982; Nakamura et al., 1985). This means that glycinins having different subunit compositions exhibit distinguishable functional properties. In fact, also glycinins from different cultivars differ in their functional properties (Nakamura et al., 1984a).

## 7 Aim

Despite all research performed on soy proteins in the last decades, much is still unknown about the relationship between the molecular structure of soy proteins and their functional properties. In this work we studied to which extent differences in glycinin structure lead to variations in functional properties (solubility and gel formation). Furthermore, in this study the effect of pH and ionic strength was examined over a range covering conditions often studied in literature (I = 0.5 pH 7.6) and those which are more representative for food (I = 0.02-0.2, pH 3-7).

## 8 Outline

Chapter 2 describes the relationship between the solubility of glycinin and its structural properties at a quaternary, tertiary and secondary folding level under conditions representative for food products at ambient temperatures. Chapter 3 describes heat denaturation of glycinin (1%). It was studied how the structural properties of glycinin at ambient temperatures relate to denaturation temperatures, structural characteristics and solubility. In Chapter 4 glycinin was heat denatured at a protein concentration of 10%. In this chapter we report on glycinin gel formation, the resulting gel structure and its molecular basis at different pH values and ionic strengths. Rheological, microscopic, physico-chemical and spectroscopic techniques were used. The latter two are non-traditional techniques for studying gel structure. In Chapter 5 it is studied if the different genetic variants of glycinin display a different thermostability, since it is known from literature that the proportions of genetic variants of glycinin could determine the functional properties. Chapter 6 deals with the effect of pH on the gel forming properties of both a soy protein isolate and purified glycinin in relation to denaturation and aggregation. In Chapter 7 the results obtained in the previous chapters are discussed.

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Chapter 2

# Soy glycinin; influence of pH and ionic strength on solubility and molecular structure at ambient temperatures [1]

**Abstract**

This study describes the relationship between the solubility of glycinin, a major soy protein, and its structural properties at a quaternary, tertiary and secondary folding level under conditions representative for food products. When the ionic strength is lowered from 0.5 to 0.2 or 0.03, the basic polypeptides shift more to the exterior of the glycinin complex, as determined at pH 7.6 by labelling solvent-exposed lysines, supported by the study of the proteolytic action of clostripain on glycinin. This structural reorganisation caused the pH of minimal solubility to shift to higher values. Ultracentrifugational analysis shows that at pH 7.6 and an ionic strength of 0.5 glycinin forms hexameric complexes (11S), whereas at pH 3.8 and at an ionic strength of 0.03 glycinin exists as trimers (7S). Intermediate situations are obtained by modulation of pH and ionic strength. The observed quaternary dissociation correlates with an increased amount of non-structured protein at a secondary level and with changes in tertiary folding as determined using circular dichroism. Tryptophan fluorescence shows no significant structural changes for different ionic strengths but demonstrates a more tightly packed fluorophore environment when the pH is lowered from 7.6 to 3.8.

key words: soy; glycinin; solubility; pH; ionic strength; protein structure

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## 1 Introduction

Soy proteins are applied in a wide range of food products. Despite all of the research performed in the past decades much is still unknown about the behaviour of soy proteins at a molecular level under conditions present in food. Generally, the pH of food products ranges from pH 3 to 7, and the ionic strength varies from 0.02 to 0.2, whereas the majority of soy protein studies have been carried out at pH 7.6 at an ionic strength of 0.5, as it is known that soy proteins are soluble under these conditions. Furthermore, it is known that lowering the ionic strength and pH affects soy protein structure, which in some cases seems to be related to changes in the functional behaviour in food, such as solubility (Peng et al., 1984). Good protein solubility generally correlates with "optimum" gelation, emulsifying, and foaming activity (see, for example, Kinsella (1979)).

This research focuses on glycinin, a major storage protein in soybeans. Glycinin represents ca. 30% of total protein in soybeans. It is composed of an acidic (ca. 38 kDa) and a basic polypeptide (ca. 20 kDa) (Staswick et al., 1981) linked by a single disulfide bridge, except for the acidic polypeptide $A_4$ (Staswick et al., 1984). Each pair of acidic and basic polypeptides is encoded by a single gene and cleaved post-translationally (Tumer et al., 1981). The solubility of glycinin in dilute Tris buffer is minimal around pH 5.5 according to Thanh and Shibasaki (1976) and from pH 4.3 to 6.0 according to Yagasaki et al. (1997). Furthermore, it is known that the solubility of glycinin depends strongly on ionic strength (Eldridge and Wolf, 1967; Thanh and Shibasaki, 1976; Yagasaki et al., 1997). It has been shown that pH and ionic strength can also influence glycinin molecular structure. At pH 7.6 and at an ionic strength of 0.5 glycinin is mainly present in a hexameric form of 360 kDa with a sedimentation coefficient of 11S (Badley et al., 1975). Lowering the ionic strength to 0.01 at pH 7.6 causes glycinin to dissociate from the 11S form mainly into the 7S form (Wolf and Brigs, 1958; Utsumi et al., 1987), believed to be the trimeric form, which has a more non-structured conformation (Utsumi et al., 1987). Wolf et al. (1958) found that at pH 3.8-2.2 the glycinin complex is present in the 7S and/or 3S form. Tertiary unfolding (Catsimpoolas et al., 1969; Koshiyama, 1972) and secondary unfolding (Koshiyama, 1972) at low pH's (<3) also have been reported.

This work presents a detailed description of the influence of both the ionic strength and the pH on glycinin in a detailed way at all different protein structural levels under conditions relevant for food products.

## 2 Materials and methods

### 2.1 Isolation of glycinin

Glycinin was purified from Williams 82 soybeans (harvest 1994). Broken soybeans were milled into particles with a diameter of 0.5 mm. The milling was performed in the presence of solid $CO_2$ (volume ratio soybean : $CO_2$ = 2 : 1) to prevent heat denaturation of soy protein. After defatting with hexane at 20 °C glycinin was extracted and purified according to the method of Thanh and Shibasaki (1976), except that the purification procedure was performed at 20 °C. The purified glycinin (in 35 mM potassium phosphate buffer pH 7.6 with 0.4 M NaCl and 10 mM 2-mercaptoethanol) was stored at − 20°C at 12 mg/ml. The purity of glycinin was determined by SDS-PAGE under reducing and non-reducing conditions using 10-15% gradient-gels in a Pharmacia Phast System according to the instructions of the manufacturer. The protein bands were stained using Coomassie Brilliant Blue. Glycinin purity was estimated to be above 95% by densitometric analysis of the gel.

Prior to each experiment, the purified glycinin was dialysed (Visking 8/32 tubings, Medicell, London, U.K.) at 20 °C against pH 7.6 buffers of the desired ionic strength. The $I = 0.5$ buffer consisted of 35 mM potassium phosphate and 0.4 M NaCl, the $I = 0.2$ buffer of 35 mM potassium phosphate and 0.1 M NaCl, and the $I = 0.03$ buffer of 10 mM potassium phosphate. For experiments carried out at pH 3.8 and 5.2, the pH of the sample was subsequently adjusted using HCl. Consequently, for pH 3.8 the ionic strength was increased by about 0.015 M for $I = 0.5$ and 0.2 and by 0.008 M for $I = 0.03$, on the basis of the amount of HCl needed to lower the pH to 3.8.

### 2.2 Determination of solubility

The pH of glycinin solutions (0.6 mg/ml) in pH 7.6 buffer at $I = 0.5$, 0.2, or 0.03 was lowered by adding different amounts of HCl stock solutions (0.05-5 M) to obtain final pH values ranging from 7.6 to 2.5 with approximately 0.2 pH unit intervals. After incubation of the glycinin samples for 16 hours at 20 °C the samples were centrifuged for 5 min at 15,800 g and 20 °C (precipitate consists of particles larger than approximately 0.5 μm as was determined using the Stokes equation). The protein concentration of the supernatants was determined in triplicate using the Bradford assay (Bradford, 1976) using BSA as a standard. Prolonged incubation of the samples for 16 hours at 20 °C did not result in any proteolytic digestion of the material at any of the conditions studied as analysed by SDS-PAGE (results not shown).

## 2.3 Fluorescein Isothiocyanate (FITC)-labelling

Labelling experiments of glycinin were performed at pH 7.6 only, because at acidic pH (FITC) is not reactive to ε-amino groups (Stark, 1970). It has been reported that the surface SH content of glycinin depends on the method of glycinin preparation (Wolf, 1993). For our material no free SH groups could be detected at pH 7.6 (unpublished results) using the Ellman reagent (Ellman, 1959).

FITC (Fluka, 46950) was dissolved in the appropriate buffer (I = 0.5, 0.2, and 0.03 at pH 7.6) containing 10% ethanol. The FITC solution was incubated with the glycinin solutions (12 mg/ml) in a molar ratio of 1:4.2 (volume ratio = 1:10) during 16 hours at 20 °C. Next, cysteine was added to the mixture (molar ratio of glycinin/cysteine = 1:12.5) to quench the reaction. After 2 hours the solution was dialysed (Visking 8/32 tubings) against the appropriate buffer to isolate the labelled glycinin. Next, the labelled glycinin samples were denatured in a 10 mM potassium phosphate buffer (pH 6.6) containing 6 M urea and 20 mM 2-mercaptoethanol to dissociate the acidic and basic polypeptides. The denatured samples were applied onto an HPLC system (Spectra Physics, San Jose, USA) using a mono Q 5/5 column (Pharmacia Biotech, Uppsala, Sweden) and eluted at 1 ml/min. The basic polypeptides (no contamination with acidic polypeptides) were eluted with the buffer mentioned above. The fraction bound to the column (the acidic polypeptides; containing < 5% basic polypeptides) was eluted by adding 1 M NaCl to the elution buffer. The absorbance at 280 nm ($A_{280}$) (SpectraSYSTEM UV 3000) and fluorescence (Spectra SYSTEM FL3000; excitation at 493 nm and emission at 522 nm) were detected simultaneously. Measurements were carried out in a range in which fluorescence intensity depended linearly on protein concentration (data not shown). The absorbance at 280 nm ($A_{280}$) was corrected for the expected difference in molar extinction coefficient between the acidic and basic polypeptides (factor = 1.8). This factor was based on the average amount of aromatic amino acids of the basic and acidic polypeptides as was calculated from the amino acid composition of the different glycinin isoforms (Scallon et al., 1987; Nielsen et al., 1989; Sims and Goldberg, 1989; Thanh et al., 1989; Cho and Nielsen, 1989). The fluorescence signal of the basic polypeptides was multiplied by a factor of 2.5 because the basic polypeptides possess less reactive groups than the acidic ones. The factor of 2.5 was based on the amount of lysine present in the acidic and basic polypeptides (Scallon et al., 1987; Nielsen et al., 1989; Sims and Goldberg, 1989; Thanh et al., 1989; Cho and Nielsen, 1989), as arginine is not expected to be labelled significantly at pH 7.6 because of its $pK_a$ of about 11. The ratio between the corrected fluorescence integrated peak area of acidic and basic polypeptides and the corresponding corrected integrated absorbance at 280 nm was used as a measure for the

relative exposure of the acidic and basic polypeptides under the different conditions. This ratio will be referred to as "the relative exposure". All experiments were carried out in duplicate.

### 2.4 Proteolytic digestion

Clostripain (Sigma, C7403) was dissolved in the appropriate buffer (I = 0.5, 0.2, or 0.03 at pH 7.6). Ten microliters of enzyme solution (0.01 mg/ml) was reacted with 100 μl glycinin samples (4 mg/ml) in buffers with 0.5, 0.2, and 0.03 ionic strengths at pH 7.6 in the presence of 1 mM $CaCl_2$ for 30 min at 30 °C. After the addition of EDTA (molar ratio EDTA/$Ca^{2+}$ = 10 :1), the samples were analysed by reduced SDS-PAGE (12% polyacrylamide gels) using the Mini-PROTEAN II system (Bio-Rad, Hercules, California, USA) according to the instructions of the manufacturer using Coomassie Brilliant Blue for staining. The amount of digested acidic and basic polypeptides was calculated by quantification of the protein bands by densitometric analysis.

### 2.5 Ultracentrifugation experiments

To determine sedimentation coefficients of the glycinin samples, 5% - 20% sucrose step gradients (four steps; 12 ml total volume) were prepared in buffers with I = 0.5, 0.2, and 0.03 at both pH 7.6 and 3.8. Prior to the experiments the gradients were allowed to diffuse to linearity during 24 hours at 4 °C. Glycinin samples (0.3 ml, 4 mg/ml) in I = 0.5, 0.2, and 0.03 buffers at pH 7.6 and 3.8 were loaded on top of the gradient. Next, the tubes were centrifuged in a Beckman L60 centrifuge at 186,000 g (at $r_{max}$) for 16 hours at 20 °C. After ultracentrifugation, the gradient was fractionated in 0.5 ml aliquots of which the absorbance at 280 nm was measured. The experiments were performed in duplicate. Svedberg (S)-values were estimated after calibration of the gradient using proteins with known S values (11.2 S for γ-globulin, 7S for katalase, 4.4S for BSA, 2.5S for trypsin, and 1.78S for ribonuclease).

### 2.6 Fluorescence spectroscopy

Fluorescence spectra of 0.2 mg/ml glycinin samples in pH 7.6 and 3.8 buffers at different ionic strengths (0.5, 0.2, and 0.03) were recorded on a Perkin-Elmer luminescence spectrometer LS 50 B at 20 °C. Excitation was at 295 nm, and the emission spectrum was recorded as the average of three spectra from 300 to 450 nm using a scan speed of 50 nm/min and a resolution of 0.5 nm. Both the excitation and emission slit were set at 2.5 nm.

### 2.7 Circular dichroism spectroscopy

Far- and near-UV CD spectra of 0.6 and 1.2 mg/ml glycinin samples, respectively, were recorded at a Jasco J-715 spectropolarimeter (Jasco Corp., Japan) in 0.5, 0.2, and 0.03 ionic strengths at pH 7.6 and 3.8 at 20 °C. For near-UV CD measurements quartz cells with a path length of 10 mm were used, whereas for far-UV CD measurements quartz cells with a path length of 0.2 mm were used. The scan interval for near-UV CD was 350-250 nm and for far-UV CD measurements, 260-190 nm. Spectra were recorded as averages of 25 spectra using a scan speed of 100 nm/min, a bandwidth of 1 nm, a response time of 64 ms, and a step resolution of 0.5 nm.

## 3 Results

### 3.1 Solubility of glycinin

Although many studies on soy protein have been performed at pH 7.6 and I = 0.5, this investigation was aimed at measuring changes in the solubility of glycinin when the ionic strength and pH were lowered to conditions more representative for food products. From Figure 1 it can be seen that glycinin solubility at 20 °C depends strongly on ionic strength and pH. At I = 0.5 and pH 7.6 all glycinin is soluble (100%), but the solubility gradually decreases from 100 to 30% when the pH is lowered from 6.5 to 2.5. The precipitation of glycinin below pH 3.8 is not instantaneous but requires several hours of incubation. At ionic strengths of 0.2 and 0.03 the solubility profiles show one minimum; at I = 0.2 about 95% of the protein precipitates in the pH range from 5.8 to 4.7, whereas at I = 0.03 complete precipitation occurs between pH 6.2 and 4.7. In the latter case the precipitation occurs almost immediately after adjustment of the pH. Glycinin is completely soluble below pH 3.8 at both I = 0.2 and I = 0.03.

To investigate whether the solubility behaviour observed relates to differences in molecular organisation of glycinin, experiments described in the following sections were performed at the three ionic strengths at both pH 7.6 and 3.8. These particular pH values were chosen because they represent the solubility maxima of glycinin. No pH values at solubility minima were chosen because the spectroscopical techniques used require the material to be soluble.

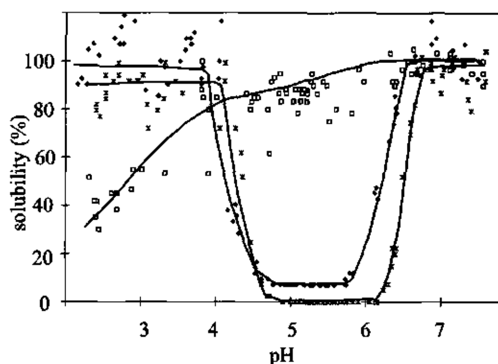


**Figure 1** pH-dependent solubility profiles of glycinin (I = 0.5 (□); I = 0.2 (♦); I = 0.03 (✱) ) determined after incubation of 0.6 mg/ml samples for 16 hours at 20 °C.

### 3.2 Structural arrangement of acidic and basic polypeptides within glycinin

To investigate whether the exposure of acidic and basic polypeptides in the glycinin molecule could explain the different solubility curves, FITC labelling experiments were performed at pH 7.6. As explained in the methods section this probe is expected to label under these conditions merely lysines present at the surface of the molecule, which makes it possible to detect conformational rearrangements of acidic and basic polypeptides in the glycinin complex. Figure 2 shows that the relative exposure of both basic and acidic polypeptides depends on the ionic strength. The acidic polypeptides are exposed about 12 times more than the basic ones at I = 0.5. The relative exposure at I = 0.2 and I = 0.03 of the acidic polypeptides is about half of that at I = 0.5, whereas the relative exposure of the basic polypeptides increases by a factor of about 1.5. When the ionic strength is lowered to 0.2 and 0.03, the acidic polypeptides are still 4 times more exposed than the basic ones. These results clearly indicate a rearrangement of the polypeptides within the glycinin molecule, modulated by ionic strength.

The arrangement of the acidic and basic polypeptides in the complex was alternatively studied by testing their accessibility to proteolytic action by clostripain, resulting in degradation products of approximately 15 and 25 kDa using SDS-PAGE under reducing conditions (results not shown). At an ionic strength of 0.5 and 0.2, 85 ± 1% (n = 5) of the acidic polypeptides are degraded, whereas at an ionic strength of 0.03 only 62 ± 4% (n = 5) of the acidic polypeptides are degraded. This means that the relative exposure of the acidic polypeptides has decreased by a factor of 1.4. The basic polypeptides are not affected significantly by the presence of clostripain at any of the tested ionic strengths. This could be either due to the fact that the overall relative exposure of the acidic polypeptides is higher

than that of the basic polypeptides at all ionic strengths or due to the interference of electrostatic repulsions between the basic polypeptides and the $Ca^{2+}$ required for clostripain action. Qualitatively, these data support the FITC labelling results.

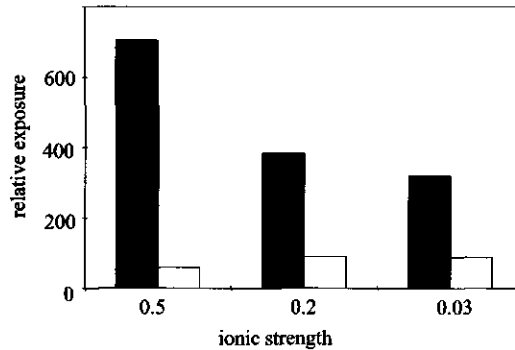


**Figure 2** Relative exposure (for definition, see materials and methods section) at pH 7.6 of acidic (black bars) and basic polypeptides (white bars) after labelling of glycinin with FITC at different ionic strengths at pH 7.6.

### 3.3 Quaternary folding of glycinin

To study possible differences in the quaternary folding of glycinin, ultracentrifugation experiments were performed (Figure 3). At pH 7.6 and an ionic strength of 0.5 or 0.2 glycinin has a sedimentation coefficient of 11S, as estimated by calibration with proteins with a known Svedberg coefficient. A fraction with a higher Svedberg coefficient, probably the 15S fraction as described by Wolf and Nelsen (1996), also seems to be present at I = 0.5. At I = 0.03, next to an 11S fraction, a 7S fraction, representing 15-25% of all protein, could be observed. At pH 3.8 and I = 0.5 about half of the glycinin molecules are present in the 7S form, whereas at I = 0.2 and 0.03 glycinin is predominantly present in the 7S form.

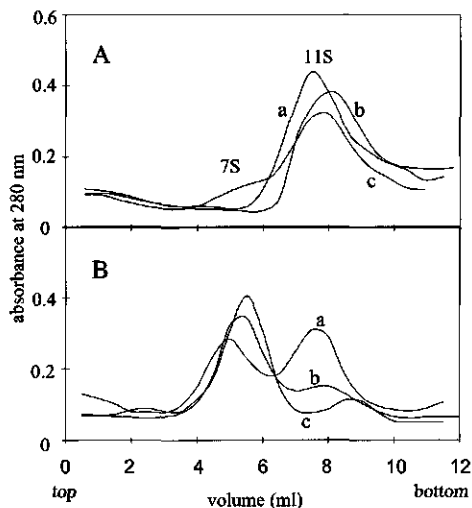


**Figure 3** Protein elution profiles using 5-20% sucrose density gradients after ultracentrifugation at pH 7.6 (A) and 3.8 (B) (I = 0.5 (a); I = 0.2 (b); I = 0.03 (c)).

### 3.4 Tertiary folding of glycinin

To determine differences in the tertiary interactions within the glycinin molecule, tryptophan fluorescence spectra were recorded at pH 7.6 and 3.8 at I = 0.5, 0.2, and 0.03. Only the spectra recorded at I = 0.5 (normalised at 345 nm) are shown (Figure 4A), to give typical examples. Fluorescence spectroscopy shows at I = 0.5 a shift of ca. 2 nm of $\lambda_{max}$ to lower wavelengths when the pH is lowered from 7.6 to 3.8, demonstrating that the local environment of tryptophan is changed into a more apolar one. A similar effect, although smaller (ca. 1 nm), can also be observed at I = 0.2 and 0.03. No significant changes in $\lambda_{max}$ can be observed when the ionic strength is varied either at pH 7.6 or at pH 3.8 (results not shown).

All recorded near-UV CD spectra (Figure 4B) show positive ellipticity between 250 and 300 nm, with a maximum at 285 nm and a resolved shoulder at 291 nm. The near-UV CD spectra of samples of different ionic strength all show a comparable shape at each particular pH. When the ionic strength is lowered at pH 7.6 a decrease of 20% in intensity can be observed when the spectrum at I = 0.5 is compared with that recorded at I = 0.03. At pH 3.8 a similar trend is observed when the ionic strength is lowered, although it is less pronounced than at pH 7.6 (about 10% decrease of intensity). Such a decrease in intensity of the near-UV CD bands generally points at a destabilisation of the protein tertiary structure (Vuilleumier et al., 1993).

When the pH is lowered from 7.6 to 3.8 at all three ionic strengths, the total intensity of the CD spectra decreases significantly, about 35-45%. Furthermore, the ratio between the intensity at 285 and 291 nm decreases slightly. Because tryptophan generally absorbs at higher wavelengths than tyrosine in the 280-295 nm region (Vuilleumier et al., 1993), this could suggest that the local environment of the tyrosines is more destabilised than that of the tryptophans when the pH is lowered. These results confirm those found with fluorescence spectroscopy. It is concluded that the tertiary folding of glycinin depends more strongly on pH than on ionic strength.

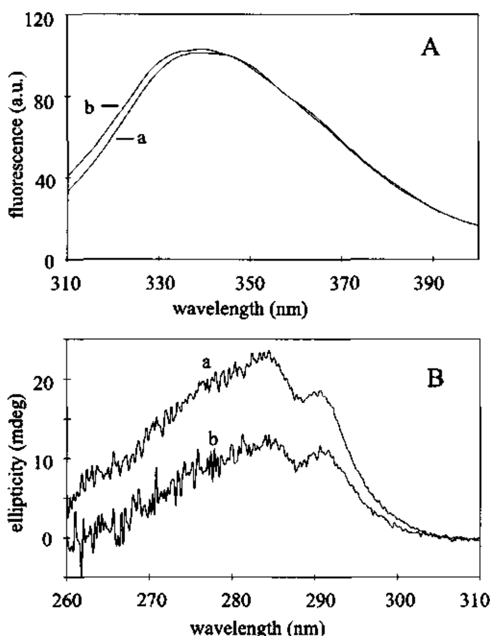


**Figure 4** Tertiary folding of glycinin at I = 0.5 M at pH 7.6 (a) and 3.8 (b) studied with tryptophan fluorescence spectroscopy (A) and near-UV CD spectroscopy (B).

### 3.5 Secondary folding of glycinin

The secondary structure of glycinin was studied by far-UV CD (Fig. 5). Only the results at I = 0.5 are presented in Figure 5A in order to give typical examples. All far-UV CD spectra exhibit a negative extreme around 208 nm and a zero crossing around 200 nm. Spectra could not be recorded below 195 nm due to high concentrations of chloride ions in the I = 0.2 and 0.5 buffers. On the basis of comparison with reference spectra (Johnson, 1990), glycinin predominantly consists of $\alpha$-helical structures. Using curve-fitting procedures with reference spectra (de Jongh and de Kruiff, 1990), the secondary structure content was estimated. Figure

5B presents the amount of non-structured protein for the various conditions. The amount of non-structured protein increases significantly when the pH is lowered from 7.6 to 3.8. Furthermore, at pH 3.8 the amount of non-structured protein also increases when the ionic strength is lowered, whereas at pH 7.6 such a correlation is not observed.

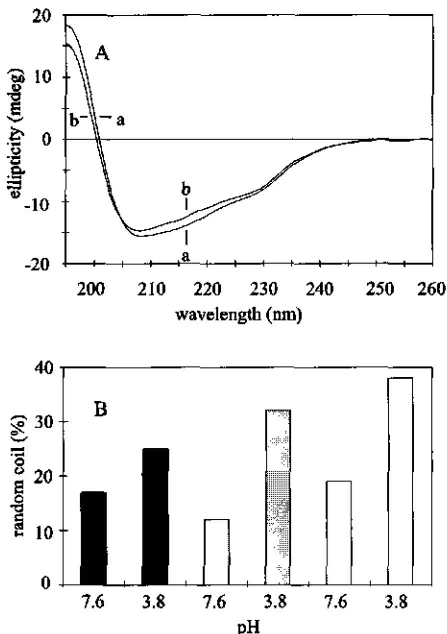


**Figure 5** Far-UV CD spectra of glycinin at I = 0.5 at pH 7.6 (a) and 3.8 (b) (A) and the estimated amount of non-structured glycinin as a function of pH and ionic strength (B) (I = 0.5 (black bars); I = 0.2 (grey bars); I = 0.03 (white bars)).

## 4 Discussion

This study was undertaken to determine glycinin structure under conditions (pH, ionic strength) used frequently in the literature in comparison to conditions more representative for food systems. Its structural properties at different folding levels are related to the solubility.

Our investigations show that the quaternary structure of glycinin is modulated by both the ionic strength and, more effectively, pH. Whereas at I = 0.5 and pH 7.6 glycinin is present in the 11S form, at I = 0.03 and pH 3.8 glycinin has dissociated into the 7S form (Figure 3). The dissociation of 11S into 7S seems to be correlated with significant changes at the secondary (Figure 5) and, to a lesser extent, the tertiary folding level (Fig. 4). Apparently there is no correlation between the 11S/7S ratio and the solubility at a concentration of 0.6 mg/ml, because at both conditions the solubility is 100% (Figure 1). Intermediate situations of the

11S/7S ratio can be obtained by varying pH and ionic strength (Figure 3). Indications for such an 11S/7S dissociation/association have been reported previously (Wolf and Briggs, 1958; Wolf et al., 1958; Utsumi et al., 1987).

Although the relative exposure of the basic polypeptides increased significantly at I = 0.03 compared to at I = 0.5, the relative exposure of the acidic polypeptides at all ionic strengths was still higher than that of the basic polypeptides (Figure 2). Such a structural reorganisation with changing ionic strength is qualitatively confirmed by probing the accessibility of the acidic and basic polypeptides by proteolytic digestion, as could explain the results of Kamata et al. (1982), who observed ionic strength dependent digestion of glycinin. This structural rearrangement does have a strong influence on the solubility profile (Figure 1). At I = 0.2 precipitation occurs between pH 5.8 and 4.7, which is in about the same range of the pI values of the acidic polypeptides (pH 4.8 to 5.4) (Catsimpoolas, 1969). At I = 0.03 this region of precipitation occurs at slightly higher pH (6.2-4.7) (Figure 1), suggesting that it shifts toward the isoelectric points of the basic polypeptides, which vary from pH 8 to 8.5 (Catsimpoolas, 1969). This correlates well with the observation that at all ionic strengths the acidic polypeptides are predominantly facing the outside of the glycinin complex (Figure 2), whereas when the ionic strength is lowered, the basic polypeptides partly displace the acidic ones from the exterior of the complex. The obtained data on the relative exposure of the acidic and basic polypeptides are in line with the model proposed by Marcone et al. (1998), which is based on the model of Plietz et al. (1983), who suggested that the basic polypeptides are present in the interior of the glycinin molecule.

The decreased solubility of glycinin below pH 3.8 (Figure 1) can be attributed to pH denaturation of the protein caused by protonation of the carboxyl groups. It is only observed at I = 0.5, where apparently the screening of positive charges of the salt is as efficient to overcome electrostatic repulsions. For I = 0.5 an additional complexity is observed. No minimum in solubility could be observed between pH 7.6 and 3.8 (Figure 1) at 20 °C, probably due to the salting-in effect. At 4°C, however, precipitation does occur at pH 4.6 (instantaneous; 40% solubility), whereas the solubility is 100% at pH 7.6 and 80% at pH 3.8 (unpublished results). At 20 °C at I = 0.5 and pH 7.6 the solvent exposure of the acidic polypeptides is maximal. Generally, the preference of the basic polypeptides to reside at the inner part of the glycinin complex could be attributed to the higher content of hydrophobic amino acids in the basic polypeptides compared to that of the acidic ones (Catsimpoolas et al., 1971). When the temperature is lowered, the role of electrostatic interactions increases as the role of hydrophobic interactions decreases. This could favour the shift of the basic polypeptides to the exterior of the molecule, similar to the rearrangements induced by

lowering the ionic strength at pH 7.6. To what extent this hypothesis is valid needs to be examined. Conclusively, the solubility behaviour of glycinin at low pH is related to the relative arrangement of acidic and basic polypeptides at pH 7.6. The solubility profile of glycinin described in this work is in line with the results of Thanh and Shibasaki (1976), although in the latter work the ionic strength dependent shift in solubility was found at a lower ionic strength than presented here.

Because it can be expected that the observed modulation of quaternary structure and reorganisation of polypeptides within the glycinin complex, as described in this work, has a strong influence on the functional properties of the protein, it is of great importance to study this protein under "food conditions" (pH 3 to 7 and I = 0.02 to 0.2).

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Chapter 3

# Heat denaturation of soy glycinin; influence of pH and ionic strength on molecular structure[1]

**Abstract**

The 7S/11S glycinin equilibrium as found in Chapter 2 at ambient temperatures influences heat denaturation. It is found that the 7S form of glycinin denatures at a lower temperature than the 11S form, as demonstrated by a combination of calorimetric (DSC) and circular dichroism (CD) experiments. At pH 7.6, at which glycinin is mainly present in the 11S form, the disulfide bridge linking the acidic and the basic polypeptide is broken during heat denaturation. At pH 3.8 (at which glycinin has dissociated partly into the 7S form) and at pH 5.2 this disruption does not seem to take place, as demonstrated by solubility and gel electrophoretic experiments. A larger exposure of the acidic polypeptides (Lakemond et al., 2000) possibly correlates with a higher endothermic transition temperature and with the appearance of an exothermic transition as observed with DSC. Denaturation/aggregation (studied by DSC) and changes in secondary structure (studied by far-UV CD) take place simultaneously. Generally, changes in tertiary structure (studied by near-UV CD) occur at lower temperatures than changes in secondary structure.

key words: soy; glycinin; pH; ionic strength; heat denaturation; protein structure

[1] This Chapter has been published as Lakemond, C. M. M.; de Jongh, H. H. J.; Hessing, M.; Gruppen, H.; Voragen, A. G. J. Heat denaturation of soy glycinin: Influence of pH and ionic strength on molecular structure. *J. Agric. Food Chem.* **2000**, *6*, 1991-1995.

## 1 Introduction

Soy proteins are widely applied in food products. Generally, food products containing soy are heated to inactivate antinutritional factors, for food preservation reasons, and to obtain desired functional properties, such as solubility or textural properties. Glycinin is one of the major soy proteins, representing ca. 30% of total protein in soybeans. It is composed of an acidic (about 38 kDa) and a basic polypeptide (about 20 kDa) (Staswick et al., 1981), linked by a single disulfide bridge, except for the acidic polypeptide $A_4$ (Staswick et al., 1984). Each pair of acidic and basic polypeptides is encoded by a single gene and cleaved post-translationally. Generally, multiple genes encoding for glycinin are present in each soy species (Tumer et al., 1981).
Several studies have been reported on glycinin heat denaturation. Koshiyama et al. (1980/81) and Hashizume et al. (1975) found that at pH 7.6 the heat denaturation temperature of glycinin was influenced by ionic strength. Koshiyama et al. (1980/81) found no indications for a correlation between the denaturation temperature and the protein structure at ambient temperatures. Furthermore, according to Yamagishi et al. (1987) and Hashizume and Watanabe (1979), at I = 0.5 and pH 7.6 the acidic polypeptides were present in the soluble fraction after heat treatment, whereas the basic polypeptides were found in the precipitate. However, these studies do not describe the effect of heat treatment of soy glycinin at low pH, whereas as demonstrated in the preceding paper, pH has a dominant influence on the structural properties of glycinin. At ambient temperatures at pH 7.6 glycinin forms hexameric complexes (11S), whereas at pH 3.8 glycinin is mainly present in trimeric complexes (7S) and more pronounced at lower ionic strength. The dissociation into the 7S form seems to correlate with changes in secondary and tertiary structure as described in Lakemond et al. (2000). Furthermore, at pH 7.6 when the ionic strength is lowered from 0.5 to 0.03 the basic polypeptides shift more to the exterior of the glycinin complex. This altered arrangement of acidic and basic polypeptides seems to influence solubility. In this work the relationship is studied between the structural properties of glycinin at ambient temperatures modulated by pH and ionic strength and its heat denaturation.

## 2 Materials and methods

### 2.1 Sample preparation

Glycinin was purified from Williams 82 soybeans (harvest 1994) as described in Lakemond et al. (2000). Prior to an experiment, the purified glycinin was dialysed at 20 °C against pH 7.6 phosphate buffers of ionic strengths of 0.5, 0.2, and 0.03. The pH and ionic strength conditions studied are similar to those used in the preceding paper to allow direct comparison. For experiments carried out at pH 3.8 and 5.2, the pH of the sample was subsequently adjusted using HCl.

Enriched 7S and 11S glycinin fractions were obtained from a glycinin sample at I = 0.5 and pH 3.8, where about equal amounts of 7S and 11S were present (Lakemond et al., 2000). The fractions were separated on a 5-20% sucrose gradient as described in Lakemond et al. (2000) and subsequently dialysed against potassium phosphate buffer (I = 0.5, pH 3.8) at 20 °C to remove sucrose. To investigate the purity and stability of the sample, the enriched 7S and 11S glycinin fractions were again analysed by ultracentrifugation on a sucrose gradient after 8 hours. The results demonstrated that the obtained 7S and 11S glycinin fractions had a cross-contamination of maximally 10-20%. The protein concentration used in this work was below 1.5 %, at which concentration no gelation of the protein solution occurs (Yamauchi et al., 1991).

### 2.2 Differential scanning calorimetry (DSC)

DSC thermograms were recorded on a micro DSC III (Setaram, Caluire, France) using 0.9 ml vessels. Glycinin (0.9 ml; 3 mg/ml) at different ionic strengths and pH's was heated from 20 to 115 °C at a heating rate of 1 K/min and subsequently cooled to 20 °C at the same rate. To investigate the reversibility of denaturation a subsequent heating/cooling cycle was performed. The transition temperature ($T_t$) is defined as the temperature at the minimum/maximum heat flow of the endothermic/exothermic transition. Enthalpies were calculated using the Setaram software, based on integration of the area of the transitions. The detection limit for transitions was 84 $\mu J\ g^{-1}K^{-1}$.

### 2.3 Circular dichroism (CD) spectroscopy

Far- and near-ultraviolet (UV) CD temperature scans of glycinin samples at different pH values and ionic strengths were recorded on a Jasco J-715 spectropolarimeter (Jasco Corporation, Japan) at temperatures ranging from 20 to 98 °C with a heating rate of 1 K/min. The protein concentration used for near-UV CD measurements was 1.2 mg/ml and for far-UV

CD measurements 0.2 mg/ml, using cells with pathlengths of 10 and 1 mm, respectively. Temperature scans were recorded using a step resolution of 0.2 °C and a response time of 8 s. Detection wavelengths were 285 and 215 nm for near-UV and far-UV CD. Spectra recorded at I = 0.5 and pH 7.6 at different temperatures showed that at these wavelengths the ellipticity was most sensitive to temperature (results not shown).
In addition, far-UV CD spectra of 0.6 mg/ml of glycinin were recorded at 20°C as described previously (Lakemond et al., 2000).

### 2.4 Determination of glycinin solubility

Glycinin samples (12 mg/ml) at various ionic strengths and pH conditions were heated from 20 to 98 °C at a rate of 1 K/min, kept at 98 °C for 30 min, and subsequently cooled to 20 °C. Next, the glycinin samples were centrifuged for 5 min at 20 °C at 15800g. The protein content of the supernatants was determined in triplicate using the Bradford assay (Bradford, 1976) using BSA (A-4503, Sigma, St. Louis, USA) as standard.

### 2.5 Gel electrophoresis

The protein composition of the supernatants and precipitates obtained after heat treatment of glycinin at the various ionic strength and pH conditions was determined using reduced and non-reduced SDS-PAGE on a Phast System (Pharmacia, Uppsala, Sweden) according to the instructions of the manufacturer. Gradient gels (10-15%) were used, which were stained with Coomassie Brilliant Blue and calibrated with low molecular weight markers ranging from 14 to 94 kDa (Pharmacia).

### 2.6 Ultracentrifugation experiments

To determine the sedimentation coefficients of the glycinin fraction that remained soluble after heat treatment, ultracentrifugation of sucrose gradients was performed as described in the preceding paper. Samples (0.3 ml) were loaded on top of the sucrose gradient [I = 0.5, 0.2, and 0.03 at pH 7.6 (3 mg/ml) and I = 0.03 at pH 3.8 (4.5 mg/ml)].

### 2.7 Fluorescence spectroscopy

Fluorescence spectra of 0.2 mg/ml glycinin samples were recorded on a Perkin-Elmer Luminescence Spectrometer LS 50 B as described previously (Lakemond et al., 2000).

## 3 Results

In this work we investigated how pH and ionic strength influence solubility and molecular structure during heating under conditions relevant to food systems. The conditions studied are I = 0.5, 0.2 and 0.03 at pH 7.6, 5.2, and 3.8; the condition I = 0.5 and pH 7.6 is included for comparison to the literature.

### 3.1 Denaturation temperatures of glycinin

To determine if pH and ionic strength influence the denaturation temperature of glycinin, DSC experiments were performed. Figure 1 shows DSC thermograms of glycinin for two typical examples. From these thermograms the transition temperatures of glycinin were determined as the temperature at which the heat flow is maximal (Table 1). The highest transition temperatures at a particular ionic strength are found at pH 5.2. The transition temperatures at each pH decrease generally when the ionic strength is lowered. For I = 0.2 and I = 0.03 endothermic transitions are observed, and remarkably, at pH 3.8 and at pH 5.2 and I = 0.03 two endothermic transitions are present. The enthalpy of the transitions is also presented in Table 1. For I = 0.5 and 0.2 the highest enthalpies are observed at pH 7.6, whereas for I = 0.03 the highest enthalpy is observed at pH 5.2.

At I = 0.5 both exothermic and endothermic transitions are observed. Interestingly, at pH 7.6 the exothermic transition is observed at a higher temperature than the endothermic transition, whereas at pH 5.2 and 3.8 the opposite is found. This exothermic transition was not observed when 10 mM 2-mercaptoethanol was present in the sample (results not shown). The position of this exothermic transition depends strongly on the heating rate (results not shown). All transitions found are irreversible as in the second heating scan no transitions could be observed at all conditions studied (results not shown).

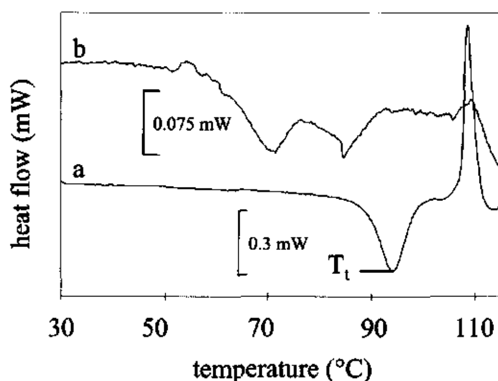


**Figure 1** DSC thermograms of glycinin at I = 0.5 pH 7.6 (a), and I = 0.03 pH 3.8 (b), recorded at a rate of 1 K/min.

**Table 1** Transition temperatures of glycinin at I = 0.5, 0.2, and 0.03 at pH 7.6, 5.2, and 3.8 as measured by DSC and far-UV CD temperature scanning at 215 nm and the enthalpy of the transitions as determined by DSC.

| | | $T_t$ (°C) | | | | Enthalpy (J/g) | |
|---|---|---|---|---|---|---|---|
| I | pH | DSC endo. | DSC exo. | far-UV CD | near-UV-CD | Endotherm | Exotherm |
| 0.5 | 7.6 | 94 | 109 | >98 | >98 | 41 | -42 |
| | 5.2 | 96 | 92 | n.d. | n.d. | 18* | -16 |
| | 3.8 | 85 | 73 | 70/83 | 61/89 | 10* | -19 |
| 0.2 | 7.6 | 87 | - | 89 | 54/80 | 37 | - |
| | 5.2 | 92 | - | n.d. | n.d. | 22 | - |
| | 3.8 | 67/82 | - | 68-81 | 67/79 | 30* | - |
| 0.03 | 7.6 | 78 | - | 78 | 73 | 39 | - |
| | 5.2 | 58/88 | - | n.d. | n.d. | 53* | - |
| | 3.8 | 71/85 | - | 72-82 | 52/66 | 25* | - |

* different transitions are not well separated; n.d. = not determined

### 3.2 Secondary and tertiary folding of glycinin as a function of temperature

CD temperature scans were recorded to determine if changes in secondary and tertiary structure play a role in glycinin denaturation. The pH 5.2 conditions could not be studied because of precipitation of the protein at ambient temperatures, whereas the CD technique requires the protein to be soluble. Figure 2A shows the recorded far-UV CD temperature scans at I = 0.5 at pH 7.6 and 3.8 to give typical examples. The transition temperatures are determined by taking the first derivative of the temperature scans (Figure 2B) and are presented in Table 1. At pH 3.8 and I = 0.5 two subsequent transitions are observed, whereas at I = 0.2, and 0.03 transitions over a broad temperature range are observed. At I = 0.5 and pH 7.6 the first derivative does not show a clear maximum because it was not possible to heat above 98 °C. Generally, the transitions observed with far-UV CD are found at temperatures comparable to those at which the transitions are observed with DSC.

In all samples, except at I = 0.03 and pH 3.8, precipitation of the protein is observed after heating, as indicated by an increase of the turbidity of the sample. A decrease in ellipticity at higher temperatures can therefore be attributed to changes in secondary structure as well as to precipitation. The temperatures at which aggregation/precipitation appear are detected by the applied voltage for the CD photomultiplier signal, which is related to the optical density of the sample. Figure 2C displays the first derivative of these turbidity signals. Because the turbidity curves do not entirely coincide with changes in the ellipticity, these CD curves display to some extent changes in the secondary structure of the material.

Similar experiments have been performed in the near-UV region by monitoring a spectral region that was shown previously to be sensitive to tertiary interactions (results not shown). The observed transition temperatures are presented in Table 1. Apart from the condition I = 0.2 and pH 7.6, no changes in tertiary structure could be observed prior to aggregation/ precipitation.

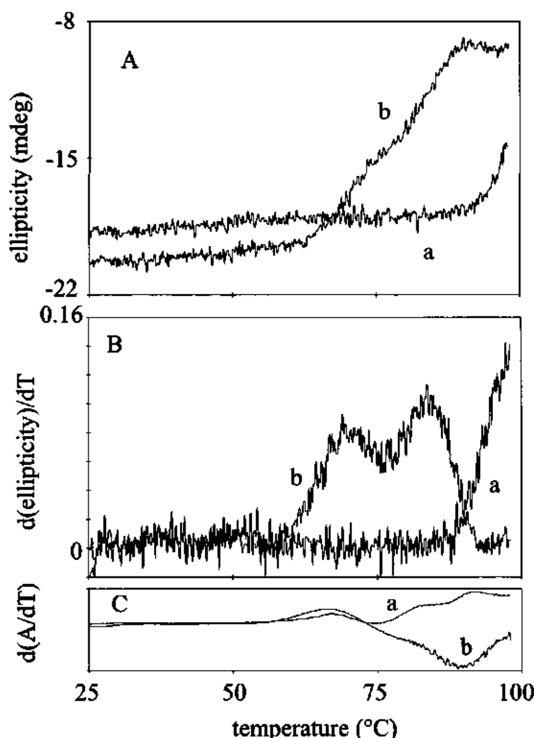


**Figure 2** Far-UV CD temperature scans (A) of glycinin at I = 0.5 pH 7.6 (a), and I = 0.5 pH 3.8 (b), recorded at 215 nm at a heating rate of 1 K/min, the first derivative of the heating curves (B) and the first derivatives of the signal as measured by the applied voltage of the CD photomultiplier (C).

### 3.3 Thermal denaturation of 11S versus 7S glycinin

To explain the nature of the double-endothermic transition observed for glycinin at particular conditions (Figure 1; Table 1), glycinin was heated at I = 0.2 and pH 3.8 up to 72 °C, to obtain partly denatured and precipitated glycinin (only the transition at the lowest denaturation temperature occurred). Subsequent ultracentrifugational analysis of this sample no longer showed a 7S peak, whereas the 11S peak was mainly intact (results not shown). This demonstrates that 7S glycinin denatures at a lower temperature than 11S glycinin.

Because at I = 0.5 and pH 3.8 a comparable amount of 7S and 11S glycinin is present (Lakemond et al., 2000), this condition was used to obtain enriched 7S and 11S fractions as described in the methods section. Far-UV CD temperature scans of these enriched fractions were recorded (Figure 3). The enriched 7S and 11S fractions underwent a single transition at 73 and 80 °C, respectively. This demonstrates that the denaturation profile of the total glycinin fraction is composed of a separate 7S and 11S contribution (compare Figure 2A). Because the protein concentrations of the enriched 7S and 11S fractions were too low for

DSC analysis and protein concentration techniques could not be used because of the danger of modifying protein structure, DSC experiments were omitted.

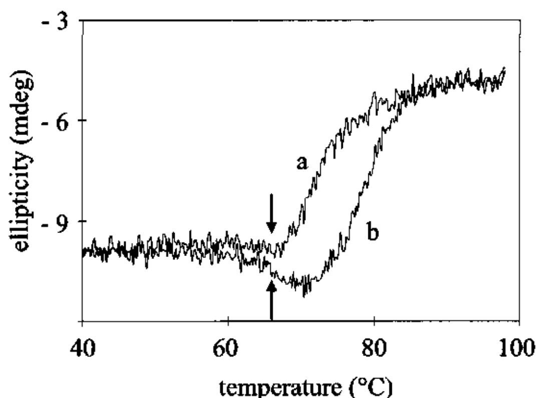


**Figure 3** Far-UV CD temperature scans of 7S glycinin (a) and 11S glycinin (b) at I = 0.5, pH 3.8, recorded at 215 nm at a heating rate of 1 K/min.

### 3.4 Solubility and aggregate formation of acidic and basic polypeptides after heat treatment

To study to which extent glycinin precipitates at different conditions after heat treatment, the solubility of the supernatant, obtained by centrifugation, was determined (Table 2). SDS-PAGE analysis under reduced and non-reduced conditions was performed to determine whether acidic and/or basic polypeptides are present in the supernatants and precipitates obtained after heat treatment and to determine the involvement of disulfide bridges (results not shown). The results of the non-reduced SDS-PAGE are qualitatively presented in Table 2. Whereas the solubility of glycinin is 100% at pH 7.6 before heating, about 50% of the protein remains soluble after heating at all ionic strengths. Only acidic polypeptides are present in the supernatants at pH 7.6, partly in aggregates that could be dissociated upon reduction in SDS. The precipitates at pH 7.6 contain mainly basic polypeptides, although also acidic polypeptides are present (corresponding to approximately 10-20% of the amount present before heat treatment). These polypeptides are all present in SDS as large aggregates containing intermolecular S-S bridges.

After heating at pH 5.2, at which pH solubility was very low before heating at I = 0.2 and 0.03, almost all material appears in the precipitate, which consists of large aggregates that are linked by S-S bridges, as in SDS no individual acidic and basic polypeptides could be observed.

At pH 3.8 and I = 0.5 and 0.2 all protein precipitates, whereas at I = 0.03 about 75% of the protein remains soluble after heat treatment. At I = 0.5 and 0.2 the precipitate consists predominantly of large aggregates in SDS. In contrast to the precipitate at I = 0.5, at I = 0.2 no covalently linked acidic and basic polypeptides are found. In the supernatant I = 0.03 acidic as well as basic polypeptides were present, both as aggregates and as individual acidic (A) or basic (B) polypeptides or as AB subunits in the presence of SDS.

**Table 2** Composition of precipitates and supernatants of glycinin after heat treatment at I = 0.5, 0.2, and 0.03 at pH 7.6, 5.2, and 3.8 as determined by non reduced SDS-PAGE (S = supernatant, P = precipitate).

| I (M) | pH | Solubility (%) | S/P | A polypeptides | B polypeptides | AB subunits | Aggregates (>100 kDa) |
|---|---|---|---|---|---|---|---|
| 0.5 | 7.6 | 53 ± 4 | S | + | - | - | + |
| | | | P | - | - | - | + |
| | 5.2 | 9 ± 8 | P | - | - | - | + |
| | 3.8 | 4 ± 1 | P | + | + | + | + |
| 0.2 | 7.6 | 52 ± 3 | S | + | - | - | + |
| | | | P | - | - | - | + |
| | 5.2 | 2 ±8 | P | - | - | - | + |
| | 3.8 | 5 ± 6 | P | - | - | - | + |
| 0.03 | 7.6 | 54 ± 1 | S | + | - | - | + |
| | | | P | - | - | - | + |
| | 5.2 | 1 ± 1 | P | - | - | - | + |
| | 3.8 | 72 ± 6 | S | + | + | + | + |

## 3.5 Quaternary, tertiary, and secondary structure of soluble acidic polypeptides obtained after heat treatment

To examine the molecular structure of the non-precipitated polypeptides in the supernatants obtained after heat treatment, ultracentrifugational analysis (Figure 4A), tryptophan fluorescence spectroscopy, and circular dichroism spectroscopy (Fig. 4B) were performed. Only the proteins in the supernatants obtained after heating at pH 7.6 at all three ionic strengths and at pH 3.8 at I = 0.03 were studied, because at the other pH/ I combinations over 90% of the protein has precipitated after heat treatment.

Figure 4A shows that all ultracentrifugational profiles demonstrate two major populations. At pH 7.6 the acidic polypeptides present in the supernatants are present partly as large (soluble)

aggregates and partly as oligomers and monomers (4S or smaller). The AB complexes found in the supernatant at pH 3.8 and I = 0.03 (Table 2) are apparently present as large aggregates or as (oligomers of) the individual AB subunit and not as an 11S/7S form.

Tryptophan fluorescence spectra of the supernatants of glycinin demonstrated that the difference in $\lambda_{max}$ after heating compared to the $\lambda_{max}$ of the spectra recorded before heating differs significantly for the various conditions (pH 3.8 and I = 0.03: about 1 nm; pH 7.6 and I = 0.5: about 0.5 nm; pH 7.6 and I = 0.2: about 2 nm; pH 7.6 and I = 0.03: about 1.5 nm). In all cases the local environment of the tryptophans is more polar after heating than before.

The CD spectrum of the supernatant of glycinin obtained after heat denaturation at I = 0.03 and pH 3.8 (Figure 4B) has a different shape (zero-crossing around 196 nm) from the spectra of the soluble glycinin fraction obtained at pH 7.6 at I = 0.5, 0.2, and 0.03 (zero crossing below 190 nm). From comparison with reference spectra (Johnson, 1990) it can be concluded that the amount of random coil at I = 0.03 at pH 3.8 is the lowest of all conditions studied. For comparison also the spectrum of non-heated glycinin at I = 0.03 at pH 3.8 is shown in Figure 4B. This spectrum resembles most the spectrum at I = 0.03 at pH 3.8 after heat treatment, indicating that at this ionic strength and pH secondary structure has changed less than for the soluble polypeptides at pH 7.6 at all ionic strengths.

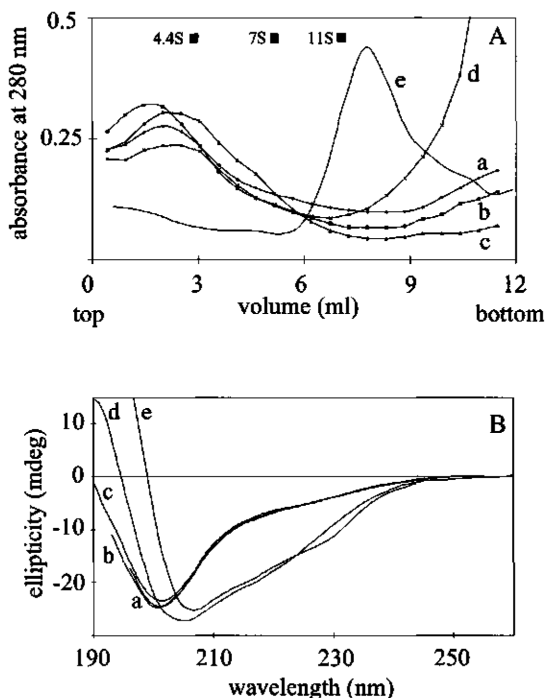


**Figure 4** Protein elution profiles of supernatants of heat-denatured glycinin after ultracentrifugation in 5-20% sucrose density gradients (A) and far-UV CD spectra (B) at pH 7.6 at I = 0.5 (a), I = 0.2 (b), and 0.03 (c), at I = 0.03 M at pH 3.8 (d), and as non heated glycinin at I = 0.5 and pH 7.6 (e) (■ = standard proteins).

## 4 Discussion

This study was performed to investigate the influence of pH and ionic strength on glycinin solubility and structure during and after heat treatment. It was determined whether glycinin behaves differently under conditions used frequently in the literature (I = 0.5 and pH 7.6) in comparison to conditions more representative for food systems (I = 0.02-0.2 and pH 3-7).

### 4.1 Heat denaturation of glycinin

It has been shown that both the denaturation temperature and the enthalpy vary strongly with pH and ionic strength (Table 1). The highest denaturation temperatures of glycinin at a particular ionic strength are found at pH 5.2. This is in accordance with the general opinion that globular proteins are most stable close to their apparent pI (Privalov and Khechinashvili,

1974). The enthalpies of the transitions found in this study are generally higher than those reported in the literature. At pH 7.6 enthalpies are reported ranging from 7-17 J/g for different ionic strengths (Koshiyama et al., 1980/81; Marshall and Zarins, 1989; Bogracheva et al., 1996). Exothermic transitions, probably due to aggregation (Marshall and Zarins, 1989), are observed only at I = 0.5.

Denaturation/aggregation (as studied by DSC) and changes in secondary structure (far-UV CD) take place simultaneously (Table 1). Upon heating, changes in tertiary structure, as determined by near-UV CD, occur at lower temperatures than changes in secondary structure, except for I = 0.2 at pH 3.8 (Table 1). This is more remarkable because it is generally believed that the energy involved in these transitions is required to compensate for the increased exposure of hydrophobic sites of the protein to the solvent, as expected to occur in tertiary unfolding.

### 4.2 Glycinin heat denaturation in relation to the 7S/11S ratio

This work shows that the 7S/11S glycinin ratio influences heat denaturation of glycinin. At all conditions studied glycinin 7S denatures at a lower temperature than 11S glycinin. This is complementary to the results of Utsumi et al. (1987), who reported this phenomenon at I = 0.01 and pH 7.6 (ratio 7S/11S = 1:2). Although at pH 3.8 and I = 0.5 about 50% of the protein is present in the 7S form, its endothermic transition could not be detected (Table 1). This could be a combination of the fact that (1) due to its lower denaturation temperature the enthalpy of 7S glycinin is by definition lower than that of 11S, assuming that the folding state of 7S and 11S glycinin is equivalent at a tertiary and secondary folding level (Privalov and Khechinashvili, 1974) and (2) the 7S glycinin endotherm could be compensated by the exothermic transition.

In addition, at conditions at which the 11S form dominates over the 7S form (at pH 7.6), 80-90% of the acidic polypeptides remain in solution after heat treatment (Table 2). This implies a disruption of the S-S bridge and the non-covalent bonds between the acidic and basic polypeptide. This is in line with the results of Wolf and Tamura (1969), Hashizume et al. (1975), Mori et al. (1981), and Yamagishi et al. (1987). It is likely that the disruption observed at pH 7.6 does not take place at pH 5.2 and 3.8, on the basis of the presence of both acidic and basic polypeptides in the precipitate or the supernatant at these conditions (Table 2).

### 4.3 Glycinin heat denaturation in relation to the A/B polypeptide arrangement

A larger exposure of acidic polypeptides (Lakemond et al., 2000), as observed in the preceding paper, could possibly correlate with a higher endothermic transition temperature. It is also remarkable that the exothermic transition could be observed only at high ionic strength at which the acidic polypeptides are exposed the most. Although the previous work (Lakemond et al., 2000) shows a correlation between the relative exposure of the polypeptides and the solubility at ambient temperature, this correlation could not be observed for the heated material.

In conclusion, the temperature at which and the mechanism by which glycinin denatures depends on pH and ionic strength and seems to be correlated to glycinin quaternary, tertiary, and secondary structure before heating. Because ionic strength and pH affect the solubility of glycinin after heating, it is important to take these parameters into account when soy proteins are applied in food. It can be expected that pH and ionic strength also affect gel formation, another important functional property in food. This will be the subject of our next studies.

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Chapter 4

# Gel formation of soy glycinin: influence of pH and ionic strength on network structure in relation to protein conformation[1]

**Abstract**

Formation and structure of glycinin gels were studied in relation to protein conformation for two pH values and three ionic strengths. While at I = 0.03 the gels were found to be fine stranded, gel coarseness increased when the ionic strength was higher. At I = 0.03 finer gel network structures were formed at pH 3.8 than at pH 7.6, whereas for I = 0.2 and 0.5 the reverse was found. The observed differences in gel stiffness (rheological dynamical measurements) did not correspond to coarseness.

To clarify gel structure into more detail also the molecular basis of gel formation was studied using physico-chemical and spectroscopic techniques. It was found that the nature of the primary network particles was different at pH 7.6 compared to pH 3.8, since at pH 7.6 only 51 - 69% of total protein was incorporated in the gel network (predominantly basic polypeptides), while at pH 3.8 all protein was present in the network. The higher water holding capacities observed at pH 7.6 compared to pH 3.8 support the idea that at pH 7.6 the non-network protein resides in the pores.

It was found that the observed differences in gel structure are related to differences during the gel formation process (e.g. disruption of the disulfide bond linking the acidic and basic polypeptide at pH 7.6 which is likely not to occur at pH 3.8). At all conditions studied denaturation coincides with the induction of β-sheet at a secondary level (IR measurements), and with gel formation (except for I = 0.03). The largest increase in gel stiffness did not take place directly after denaturation but during the cooling part of the temperature cycle used. This increase in gel stiffness could not be related to changes in secondary structure.

key words: soy; glycinin; pH; ionic strength; heat denaturation; gel; network

[1] This Chapter has been submitted to *Food Hydrocolloids* as Lakemond, C. M. M.; de Jongh, H. H. J.; Paques, M.; Vliet, T.; Gruppen, H.; Voragen, A.G.J. Gel formation of soy glycinin: Influence of pH and ionic strength on network structure in relation to protein conformation.

## 1 Introduction

Soy proteins are used as an ingredient in many different food products. The ability of soy proteins to form gels on heating is an important functional property. This research focuses on glycinin, one of the major soy proteins. It represents about 30% of total protein in soybeans. It is composed of an acidic (~38 kDa) and a basic polypeptide (~20 kDa) (Staswick et al., 1981), linked by a single disulfide bridge. Each pair of acidic and basic polypeptides is encoded by a single gene and cleaved posttranslationally. Generally multiple genes encoding for glycinin are present in each soy species (Tumer et al., 1981).

Heat denaturation, gel formation and resulting gel structures of soy glycinin have been studied extensively at pH 7.6, but not at pH's below 7 which is more common for food products. For globular proteins generally two different types of gel networks can be distinguished, fine stranded and coarse networks, but intermediate structures have also been reported (Tombs, 1974). In fine stranded networks the proteins are attached to each other like a string of beads. This type of gel is usually transparent, indicating that the protein aggregates within the gel are smaller than about 50 nm. Coarse networks are non-transparent and are thought to be formed by random aggregation of proteins into clusters, which aggregate to thick strands. When the network structure becomes more coarse, the ability of the gels to retain water decreases (Hermansson, 1986). For glycinin fine stranded network structures are found at pH 7.0-7.6 (Nakamura et al., 1984; Hermansson, 1985; Mori et al., 1986). Increasing ionic strength at pH 7.0 leads to the formation of more coarse glycinin gels (Hermansson, 1985). The microstructure of glycinin gels at acidic pH 's has not been studied.

Although the type of gel formed strongly determines its rheological properties (Doi and Kitabatake, 1989; Stading and Hermansson, 1991; Langton and Hermansson, 1992; Stading et al., 1993; Verheul and Roefs, 1998), there is no general relationship between the coarseness of a gel and its rheological properties. Different models exist that describe the relation between rheological properties and network structure (e.g. Mellema, 2000).

Most of the numerous studies performed on glycinin gel formation from a physico-chemical point of view are focussed on the size of the aggregates formed at pH values above 7 (Mori et al., 1981; Nakamura et al., 1984; Nakamura et al., 1985; Mori et al., 1986) and the type of bonds involved in aggregation (Mori et al., 1981; Nakamura et al., 1984; Utsumi and Kinsella, 1985a; Mori et al., 1986; van Kleef, 1986; Mori et al., 1989; Utsumi et al., 1993). Few studies have been published on the molecular basis of gel formation by glycinin and the properties of the resulting gel network. These studies have exclusively been performed at pH

values above 7. Ker et al. (1993) found at pH 7.0 that a higher α-helix content in the denatured glycinin correlated with the formation of a more coarse network. Furthermore, it is known that at high pH the acidic polypeptides in glycinin gels are water soluble both at high (I = 0.5, pH 7.6; Nakamura et al., 1985; Yamagishi et al., 1987) and low ionic strength (I = 0.012; Utsumi and Kinsella (1985b). It can be hypothesised that the acidic and basic polypeptides of glycinin contribute to a different extent to the network at pH > 7, a subject that is studied in this work.

In this paper we report on glycinin gel formation, the resulting gel structure and its molecular basis at different pH values and ionic strengths using rheological, microscopic, physico-chemical, and spectroscopic techniques.

## 2 Materials and methods

### 2.1 Glycinin purification

Glycinin was purified from William's 82 soybeans (harvest 1994). Milling and defatting of the soybeans was performed according to Lakemond et al. (2000a). Glycinin was extracted and purified according to a modified method of Thanh and Shibasaki (1976). First a soy protein isolate was obtained from defatted soy by alkaline extraction at pH 8.0 followed by acidic precipitation at pH 4.8. The precipitate was resolubilised at pH 7.8. Subsequently, a crude glycinin fraction was obtained by acidic precipitation at pH 6.4 and the pelleted glycinin was resolubilised in 10 mM potassium phosphate buffer at pH 7.8 containing 10 mM 2-mercaptoethanol. For further purification ammoniumsulphate was added to the resolubilised crude glycinin fraction up to 50% saturation. After centrifugation (30 min at 12000 g; 4°C) additional ammoniumsulphate was added to the supernatant to a concentration of 70% saturation. The precipitate obtained after centrifugation (30 min at 12000 g; 4°C) was resolubilised in a 10 mM potassium phosphatebuffer at pH 7.8 in the presence of 10 mM 2-mercaptoethanol and 20% glycerol. This fraction, purified glycinin, was stored at -40°C. After defrosting part of the purified glycinin, it was extensively dialysed against Millipore water and freeze-dried afterwards (storage at -20°C). The purity of glycinin, as determined by SDS-PAGE under reducing and non- reducing conditions, was estimated to be > 95% by densitometric analysis of the gel. SDS-PAGE analysis was performed on a Phast System (Pharmacia, Uppsala, Sweden) according to the instructions of the manufacturer. Gradient gels (10-15%) were used, which were stained with Coomassie Brilliant Blue. The gel was calibrated with low molecular weight markers ranging from 14-94 kDa (Pharmacia).

## 2.2 Preparation of glycinin dispersions and gels

Prior to an experiment, glycinin (5-15% w/v) was suspended in pH 7.6 buffers at 20°C with ionic strengths of 0.5, 0.2 and 0.03. The I = 0.5 buffer consists of 35 mM potassium phosphate and 0.4 M NaCl, the I = 0.2 buffer of 35 mM potassiumphosphate and 0.1 M NaCl and the I = 0.03 buffer of 10 mM potassium phosphate. For experiments performed at pH 7.6 the ionic strength was increased by ~ 0.02 and for experiments performed at pH 3.8 by ~0.07 based on the amount of NaOH and HCl, respectively, needed to adjust the pH. We will refer in the article to the ionic strength of the buffers used since ionic strength is also increased slightly due to relatively high protein concentrations.

Gel formation of glycinin suspensions (10% (w/v)) was induced consecutively by heating from 20 to 95°C at a rate of 1 K/min, keeping temperature constant at 95°C for 30 min and cooling down to 20°C at a rate of 1 K/min. For the experiments to isolate network protein (section 2.4) and for confocal scanning laser microscopy (CSLM) at I = 0.03 and pH 3.8 gels were made with 15% (w/v) protein, because 10% gels were too weak to handle.

## 2.3 Isolation of non-network protein from glycinin gels

Glycinin gels were centrifuged at 15800g for 30 minutes at 20°C. As estimated from the Stokes equation protein aggregates in the supernatant must be smaller than ~0.2 μm. The protein in the supernatant obtained was defined as non-network protein. The volume of the supernatant was determined as a measure of water holding capacities of the gels. The protein concentration of the supernatant was determined using the Dumas combustion method on a NA 2100 protein/nitrogen analyser (CE Instruments, Thermoquest Italia, Milano, Italy) according to the instructions of the manufacturer. Atropine and aceetanilide were used for calibration.

## 2.4 Isolation of network protein from glycinin gels

Slices of about 40 μm thickness were cut from glycinin gels in a microtome cryostat Biomed HM500 OM (Micron Laborgeräte GmbH, Heidelberg, Germany) and transferred to the appropriate buffer (250 times excess w/v). Sodium azide (0.01%) was added to prevent microbial growth. The soluble protein in the samples was allowed to diffuse out of the slices for 24 hours, which is sufficiently long for reaching equilibrium based on the slice thickness. This was also illustrated by a diffusion experiment for 48 hours yielding similar results. Next, the slices were gathered by centrifugation (870g for 15 min) and the precipitate was washed twice with the appropriate buffer. The final residue was defined as "network protein".

### 2.5 Composition of network and non-network protein

The ratio of acidic to basic polypeptides in the non-network and the network protein was studied by SDS-PAGE analysis under reducing conditions performed on a Phast System (Pharmacia, Uppsala, Sweden) followed by densitometric analysis (n>4) of the gel. Gradient gels (10-15%) were used, which were stained with Coomassie Brilliant Blue. The gel was calibrated with low molecular weight markers ranging from 14-94 kDa (Pharmacia). The obtained values were corrected for the difference in intensity on the gels between the acidic and basic polypeptides based on data from native glycinin. This difference is caused by the different composition and the different molecular weights. From the protein content of the non-network protein and the polypeptide ratio, the amount of the acidic and basic polypeptides present in the non-network and the network fraction was calculated. For the calculation it was assumed that 10% of the total volume was not available for non-network protein due to part of the volume being occupied by network protein and the occurrence of steric exclusion effects. The magnitude of the steric exclusion effects is determined by the type of "non-network" protein (van Boekel and Walstra, 1989), who studied steric exclusion effects of serum proteins with respect to (para) casein micelles.

### 2.6 Confocal Scanning Laser Microscopy

Confocal scanning laser microscopy (CSLM) measurements of glycinin gels were performed on a Leica TCS SP Confocal Scanning Light Microscope (Leica, Heidelberg, Germany), in single photon mode, configured with an inverted microscope (model Leica DM IRBE), and using an Ar/Kr laser. Slices of approximately 0.2-0.5 cm thick were cut of the cylindrical shaped gels and the protein was labeled with Fluorescein Isothiocyanate (FITC). Two drops of a 0.1% aqueous solution of FITC were added to each sample slice and incubated at ambient temperature for 30 min. During this incubation the dye will diffuse equally over the sample volume, as was followed by direct observation using the microscope. The excitation wavelength was 488 nm, and the emission maximum was at 518 nm. The following Leica objective lenses were used: 20x/0.7NA/dry/HC PL APO, 63x/UV/1.25NA/water immersion/PL APO. In addition to 2D images, 3D image stacks were recorded and presented in 2D projections of the sample volume.

Optical imaging techniques introduce a distortion in the image, in particular in the 3$^{rd}$ dimension due to the point-spread function which is a characteristic of the imaging instrument. Therefore, a selection of the data files from the samples I = 0.5 at pH 7.6 and pH 3.8 was processed by application of Huygens-2 software (Scientific Volume Imaging, Hilversum). The "estimate background" tool was applied to a spherical area (radius 0.5

micron) to determine the average background pixel value, which was set as threshold value. Next, a deconvolution and subsequent apodisation of the data was performed to correct for the distortion.

### 2.7 Rheological measurement at small deformation

Gel formation of 10-15% (w/v) glycinin suspensions was studied by dynamic measurements in a Bohlin CVO rheometer (Bohlin Instruments LTD, Cirencester, United Kingdom) using a smooth concentric cylinder geometry C14 unit (cell content 3 ml). To prevent evaporation of the solvent, a thin layer of soy oil was put on top of the samples. Measurements were performed at a constant strain of 0.01 and an angular frequency of 0.63 rad/s. To induce gel formation, samples were heated according to the same temperature profile as described in section 2.2. Gelation temperature, is defined as the temperature at which the storage modulus, $G'$, started to increase over 0.5 Pa/min. In this work we define that a gel has been formed when $G'$ is larger than 1 Pa. All experiments were performed (at least) in duplicate.

### 2.8 Circular dichroism spectroscopy

Non-network protein was analyzed by far- and near-UV CD. Spectra were recorded at 20 °C using a Jasco J-715 spectropolarimeter (Jasco corporation, Japan). For near-UV CD measurements quartz cells with a pathlength of 10 mm were used, while for far-UV CD measurements quartz cells with a pathlength of 0.2 mm were used. The scan interval for near-UV CD was 350-250 nm, for far-UV CD measurements 260-190 nm. Spectra were recorded as averages of 25 spectra using a scan speed of 100 nm/min, a bandwidth of 1 nm, a response time of 64 msec, and a step resolution of 0.5 nm. The far-UV CD spectra at $I = 0.5$ and $I = 0.2$ could not be recorded below 194 nm and 197 nm respectively, due to strong absorption of the chloride ions in these buffers.

### 2.9 Gel permeation chromatography

The aggregation state of non-network protein was analyzed at 20°C by gel permeation chromatography using a Superdex 200 column 10/30 (Pharmacia, Uppsala, Sweden) attached to an FPLC system (Pharmacia, Uppsala, Sweden). The elution buffer was equal to the sample buffer. The flow rate was 1 ml/min and the eluate was monitored at 280 nm. The column was calibrated with a high molecular weight gel filtration calibration kit (Pharmacia).

**2.10 Differential scanning calorimetry (DSC)**

DSC thermograms were recorded using a micro DSC III (Setaram, Caluire, France) with 0.9 ml vessels. Glycinin suspensions (10% w/v) at different pH values and ionic strengths were heated from 20 to 115 °C at a rate of 1 K/min and subsequently cooled to 20°C at the same rate. The denaturation temperature $T_d$ was defined as the temperature at maximum heatflow of the endothermic transition. The onset denaturation temperature ($T_o$) was defined as the temperature at the crosspoint of the tangent of the endotherm and the baseline.

**2.11 Infrared spectroscopy**

Lyophilized glycinin was suspended in potassium phosphate buffers as described in section 2.2 prepared in $D_2O$ instead of $H_2O$ at different pD values and ionic strengths. To obtain H-D exchange of labile protons in the protein, the suspensions (10% (w/v)) were stirred for about 16 hours at 20°C in the buffer at different ionic strengths at pD 7.6 and adjusted to the appropriate pD afterwards. Infrared (IR) spectra were recorded using a Perkin Elmer 1725 Fourier transform IR spectrometer equipped with a liquid nitrogen-cooled mercury/cadmium/telluride detector with a temperature controlled cell interfaced to a computer. The spectral resolution of aqcuisition was 4 $cm^{-1}$, but was enhanced to 1 $cm^{-1}$ by zero-refilling prior to Fourier Transformation.

To prevent evaporation during heating, the protein sample was placed between two $CaF_2$ windows inside a ring of stretched parafilm (< 0.1 mm). The temperature profile used was identical to the one described in section 2.2. To study the reversibility of the conformational changes a second scan from 20 °C to 95°C was recorded after cooling.

In this work we use pD = pH - 0.1 in accordance to Bundi and Wütrich (1979), who suggest that in protein studies pD = pH -0.1 should be used, instead of the generally used pD = pH + 0.4, because of the isotope effect on the acidity constants of the ionizable groups of proteins. During the experiment we noticed that the protein in $D_2O$ buffers tends to form a layer with high viscosity on the bottom of the solution when stirring was stopped. This was not observed in $H_2O$ buffers.

Spectral analysis and display were carried out using Infrared Data Manager Analytical Software (Perkin-Elmer, Beaconsfield, UK). Second derivative spectra were calculated over the region between 1800 and 1500 $cm^{-1}$. Changes in protein secondary structure were monitored from discrete shifts in position of these bands with temperature. The temperature at which changes in secondary structure start to take place ($T_{ir}$) is defined as the crosspoint of the tangent of the baseline observed with increasing temperature and the transition.

## 3 Results

In this work formation and structure of glycinin gels were studied in relation to protein conformation for two pH values and three ionic strengths. We compared conditions often studied in literature (I = 0.5 and pH 7.6) to conditions more relevant for food systems (I = 0.02-0.2 and pH 3-7). The specific conditions studied are I = 0.03, 0.2 and 0.5 at pH 3.8 and 7.6. Intermediate pH conditions (4-6.5) could not be studied, since poor solubility of the material (Lakemond et al., 2000a) prohibited the formation of homogeneous gels, especially at lower ionic strengths (results not shown).

### 3.1 Gelation and denaturation temperatures of glycinin gels

The relation between gelation and denaturation was determined using dynamical rheologic measurements and differential scanning calorimetry (DSC), respectively (Table 1). At pH 3.8 two endothermic transitions were observed at all ionic strengths studied. The endothermic transition at the lower temperature originates from denaturation of 7S glycinin and the one at higher temperature of 11S glycinin (Lakemond et al., 2000b). At pH 7.6 only the 11S glycinin form is present resulting in a single endothermic transition (Lakemond et al., 2000b). Table 1 shows that at I = 0.2 and 0.5 gelation temperatures ($T_{gel}$) were 3-7°C higher than the onset denaturation temperatures ($T_o$) of 11S glycinin. Remarkably, at I = 0.03 noticeable gelation started only at a temperature about 20°C higher than the onset denaturation temperature of 11S glycinin. When at I = 0.03 the protein concentration was increased to 15%, the measured gelation temperatures decreased to 82 °C and 83 °C for pH 3.8 and 7.6, respectively (results not shown). Concentration dependency was also found for the other ionic strengths, but $T_{gel}$ was always higher than $T_o$.

The temperatures at which changes in secondary structure start to take place ($T_{ir}$) are also given in Table 1 (see also Figure 6). The results show that $T_{ir}$ is between the onset denaturation temperature and the denaturation temperature as measured in $D_2O$ (results not shown), indicating that denaturation takes place simultaneously with changes in secondary structure. The denaturation temperatures in $D_2O$ are 3-5°C higher than those in $H_2O$ (results not shown).

**Table 1** Denaturation temperatures ($T_o$, $T_d$), gelation temperatures ($T_{gel}$), and temperatures at which changes in secondary structure start to take place ($T_{ir}$) in °C of 10% (w/v) glycinin solutions at different pH values and ionic strengths as determined by DSC, dynamic rheological measurements, and IR spectroscopy, respectively.

| I | pH | $T_o$ ($H_2O$) | $T_d$ ($H_2O$) | $T_{gel}$ ($H_2O$) | $T_{ir}$ ($D_2O$) |
|---|---|---|---|---|---|
| 0.5 | 7.6 | 85 | 91 | 91 | 95 |
| | 3.8 | 63/72 | 71/86 | 75 | 77 |
| 0.2 | 7.6 | 85 | 91 | 92 | 92 |
| | 3.8 | 60/73 | 69/84 | 77 | 74 |
| 0.03 | 7.6 | 74 | 84 | 95 | 85 |
| | 3.8 | 59/74 | 68/84 | 95 | 71 |

## 3.2 Gel characteristics

### *3.2.1 Visual and microscopic characterization of glycinin gels*

The structure of the glycinin gels was characterized both by visual appearance and CSLM. Table 2 summarizes visual appearance for the various gels. At I = 0.03 the gels were (semi) transparent, while at I = 0.2 and 0.5 they appeared turbid. At the latter ionic strengths they are white and granular at pH 3.8 and yellowish and smooth at pH 7.6. Since granularity and gel transparency are related to aggregate size (Doi, 1993) these results demonstrate that large differences exist in the aggregation process when the pH and ionic strength are varied.

**Table 2** Visual appearance of 10% (w/v) glycinin gels.

| | pH 3.8 | pH 7.6 |
|---|---|---|
| I = 0.03 | transparent, smooth | semi transparent, smooth |
| I = 0.2 | turbid, white, granular | turbid, yellowish, smooth |
| I = 0.5 | turbid, white, granular | turbid, yellowish, smooth |

Aggregate size is related to the thickness of the strands and, therefore, to gel network structure. CSLM pictures (Figure 1) confirm the conclusions based on the visual appearance of the gels. Both at pH 7.6 and 3.8 a finer gel structure was observed when the ionic strength was lower. At I = 0.03 the gel structure was less coarse at pH 3.8 than at pH 7.6. At I = 0.2 and 0.5 no clear differences were seen before deconvolution of the micrographs. However, after deconvolution for I = 0.5 (Figure 1B) the gels at pH 3.8 were seen to consist of significantly thicker strands, smaller pores, and likely with more protein present in the strands than at pH 7.6.

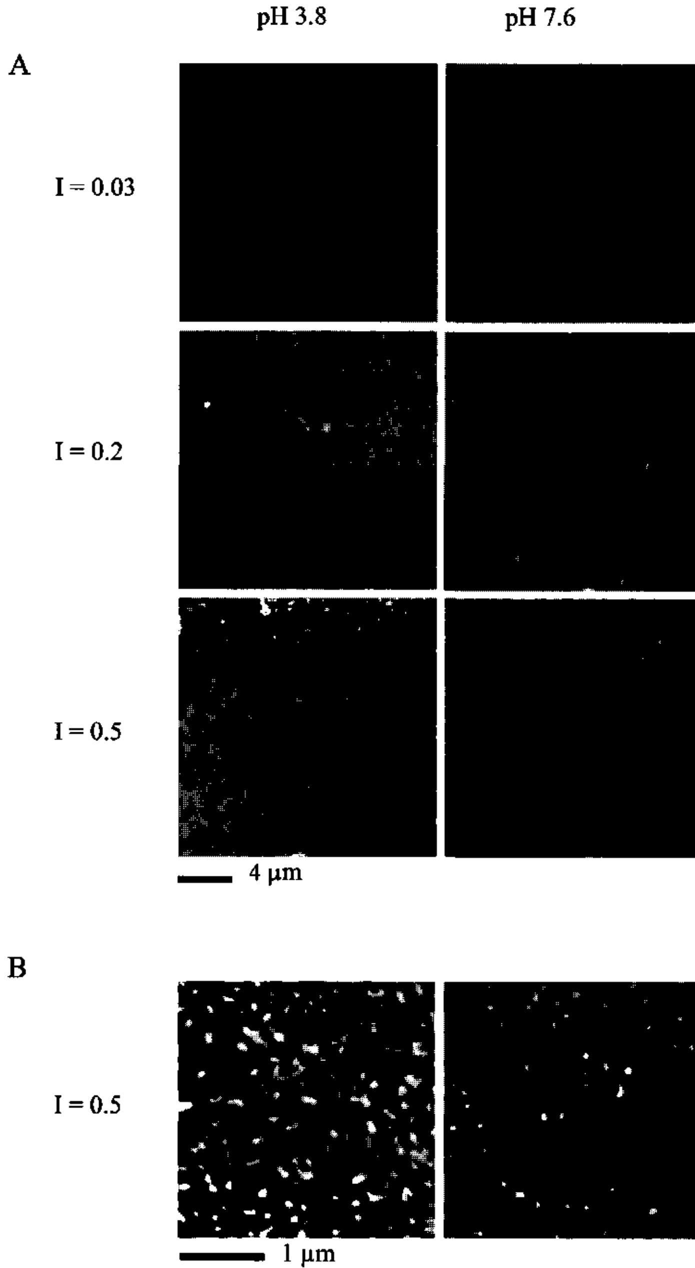


**Figure 1** CSLM pictures of 10% (w/v) glycinin gels (A) (I = 0.03 and pH 3.8 (15% gel); I = 0.03 and pH 7.6; I = 0.2 and pH 3.8; I = 0.2 and pH 7.6; I = 0.5 and pH 3.8; I = 0.5 and pH 7.6) and (B) after deconvolution at I = 0.5 and pH 3.8, and at I = 0.5 and pH 7.6.

*3.2.2 Water holding capacity of glycinin gels*

The water holding capacity of the glycinin gels was determined by centrifugation (Figure 2). At I = 0.03 and pH 3.8 it was not possible to centrifuge liquid out of the gels, even when centrifugal force was increased to 186000 g (for 1 hour). If there is no exudation of water after centrifugation we define water holding capacity to be 100%. The results show that both at pH 3.8 and 7.6 the water holding capacity increased when ionic strength was decreased. This dependency is larger at pH 3.8 that at pH 7.6. At I = 0.03 the gels retained more water at pH 3.8 than at pH 7.6. On the contrary, at I = 0.2 and 0.5 the gels retained more water at pH 7.6 than at 3.8.

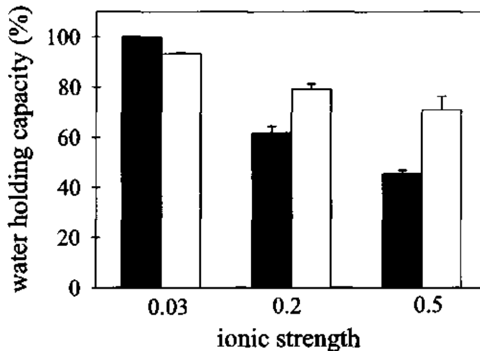


**Figure 2** Water holding capacity of 10% (w/v) glycinin gels pH 3.8 (black) and pH 7.6 (white) at ionic strengths of 0.03, 0.2 and 0.5 (standard deviation on top of bars, n = 3).
(At I = 0.03 pH 3.8 also a 15% gel was used)

*3.2.3 Stiffness of glycinin gels*

Stiffness of glycinin gels (5-15% (w/v)) was determined by rheological dynamic measurements. Figure 3A shows the elastic modulus G' at 20°C of 10% (w/v) glycinin gels after completion of a temperature cycle as described in section 2.2. Generally G' was higher at pH 3.8 than at pH 7.6, although not significantly at I = 0.03. At both pH values G' decreases in the order I = 0.2 > 0.5 > 0.03. At I = 0.2 and 0.5 gel formation was observed at 5% protein concentration at both pH 3.8 and 7.6, while at I = 0.03 at both pH values no noticeable gel formation was observed at this concentration.

Figure 3B gives typical examples of the effect of protein concentration on the elastic modulus G' after completion of a temperature cycle. While at low ionic strength at least 7% protein is required to obtain gel formation (modulus above 1 $Nm^{-2}$) at high ionic strength (I = 0.2 and 0.5) such a modulus or higher was already obtained at 5% (no further results shown). Straight lines are observed in log-log plots implying that G' is proportional to $[protein]^x$. As shown in

Table 3, the values for the slope are clearly higher at I = 0.03 than at I = 0.2 and 0.5 for both pH values. The results show furthermore that at I = 0.03 the value for the slope was higher at pH 3.8 than at 7.6, while at I = 0.2 and 0.5 the reverse is observed.

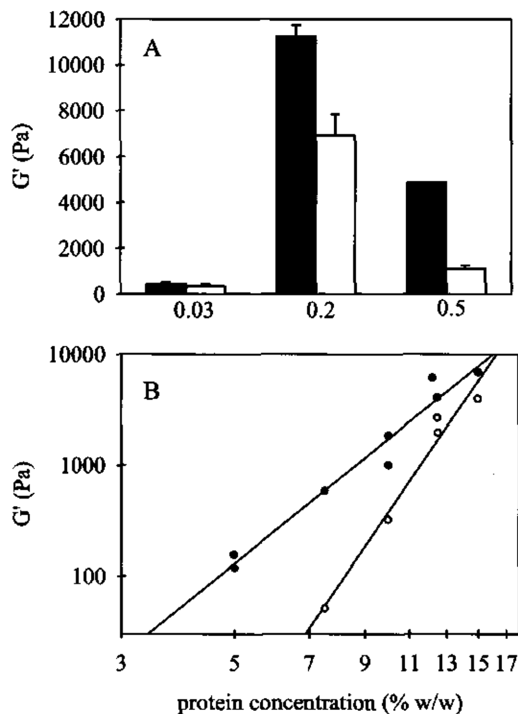


**Figure 3** (A) Elastic modulus (G') of 10% (w/v) glycinin gels after a complete temperature cycle at pH 3.8 (black) and pH 7.6 (white) at ionic strengths of 0.03, 0.2 and 0.5 (standard deviation on top of the bars, n = 2) (B) Elastic modulus (G') of glycinin gels after a complete temperature cycle as a function of protein concentrations (w/v) plotted on a log-log scale at pH 7.6 at I = 0.03 (open circle) and I = 0.5 (closed circle).

**Table 3** Slope of log-log plot of G' versus protein concentration after completion of a temperature cycle for pH 3.8 and 7.6 at I = 0.03, 0.2 and 0.5.

| Ionic strength | slope at pH 3.8 | slope at pH 7.6 |
|---|---|---|
| 0.03 | 7.5 | 6.7 |
| 0.2 | 2.9 | 3.5 |
| 0.5 | 3.0 | 3.7 |

*3.2.4 Amount and composition of network and non-network protein*

The proportion of protein in the gels that is present as non-network protein at the different conditions is presented in Table 4. At pH 3.8 the amount of non-network protein is over a factor of 8 lower than at pH 7.6. At pH 7.6 the amount of non-network protein at I = 0.2 is about one third lower than at I = 0.5 and 0.03. It is unclear if at pH 3.8 and I = 0.03 any non-network protein is present, because it was not possible to centrifuge moisture out of the gel.

The total amounts of acidic and basic polypeptides in the non-network and network protein at pH 7.6 are presented in Figure 4. The results are calculated assuming that the: (1) molar amount (acidic + basic) non-network protein + molar amount (acidic + basic) network protein = 100% and (2) the molar amount of acidic polypeptides = molar amount of basic polypeptides at the beginning of each experiment (before heating). The results show that the major component of the non-network protein at pH 7.6 are acidic polypeptides. The ratio acidic/basic polypeptides did not depend significantly on ionic strength for the network protein. However, it was found for the non-network protein that the ratio acidic/basic polypeptides is lower at I = 0.5 than at I = 0.2 and 0.03.

At pH 3.8 the total amount of non-network protein is not significant compared to the amount of network protein (Table 4), although the results indicate that consequently about 10% more basic polypeptides than acidic polypeptides were present in the network (results not shown). No significant differences were observed between different ionic strength at this low pH.

**Table 4** Amounts of non-network protein in 10% (w/w) glycinin gels based on protein concentration measured in the supernatants extracted from gels at pH 3.8 and 7.6 at I = 0.03, 0.2 and 0.5 (values $\bar{x} \pm$ SD, n = 2).

| | Non-network protein (%) | |
|---|---|---|
| Ionic strength | pH 3.8 | pH 7.6 |
| 0.03 | * | 49 ± 0.5 |
| 0.2 | 4 ± 1 | 31 ± 2 |
| 0.5 | 3 ± 1 | 48 ± 0.1 |

* It was not possible to centrifuge moisture out of the glycinin gel at I = 0.03 at pH 3.8.

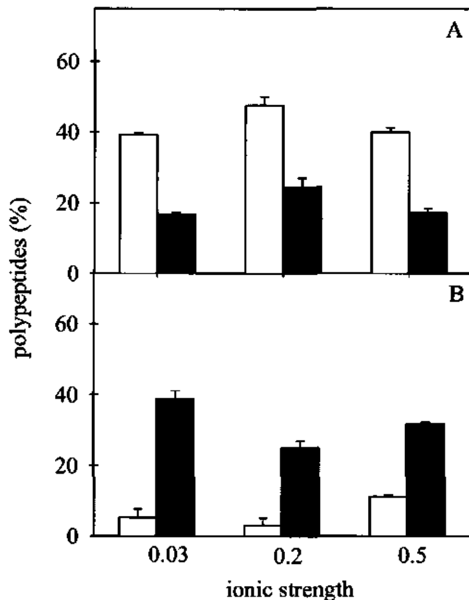


**Figure 4** Distribution of acidic (black) and basic polypeptides (white) over glycinin gels at I = 0.03, 0.2 and 0.5 at pH 7.6 ((A) network protein; (B) non-network protein). Values are obtained by protein determination and measuring the ratio acidic/basic polypeptides by densitometric analysis of SDS-PAGE gels (standard deviation on top of the bars, n ≥ 4). For further details see text.

### 3.3 Changes in secondary structure during gel formation

Gel formation was followed by rheological dynamic measurements. Figure 5 shows the increase of G' during the applied temperature profile of glycinin at I = 0.2 at pH 7.6 as a typical example. During cooling the largest increase in stiffness is observed. For the other conditions studied similar trends are found.

To determine how gel formation is related to changes in secondary structure, IR measurements were performed. The use of IR spectroscopy for the study of protein secondary structure is based primarily on the examination of C=O streching bands in the region 1700-1600 $cm^{-1}$ (amide I). Both theoretical and experimental studies with model peptides and proteins have shown that the shape of the amide I band shape is determined by the secondary structure content (Susi et al., 1967; Surewicz and Mantsch, 1988; Haris et al., 1989; Bandekar, 1992; Goormaghtigh et al., 1994). Figure 6A shows IR spectra in the amide I region before heating, at 95°C and after cooling of glycinin at I = 0.2 and pH 7.6 as typical examples. Upon heating the frequency of the top of the amide I band shifts to higher

wavenumbers. This effect is not only caused by changes in structure but also by the temperature increase itself. After cooling the position of the top of the amide I band has shifted from 1641 $cm^{-1}$ (before heating) to 1640 $cm^{-1}$. This irreversible shift is larger (up to about 3 $cm^{-1}$) for the other conditions studied. It is not expected that this shift is due to additional H-D exchange caused by unfolding of the protein since the intensity of the amide II band (predominantly N-H vibrations) relative to the amide I band is not significantly affected as could have been expected when additional NH-ND vibrations had taken place (results not shown). Furthermore, a shoulder (top at 1618-1614 $cm^{-1}$) in the amide I band occurs around 91°C and is still present after cooling (visible in Figure 6A). This shoulder also occurs at the other conditions studied. Both the shift to lower wavenumber of the top of the amide I band and the occurrence of the shoulder indicate an increased β-sheet content (Goormaghtigh et al., 1994; Boye et al., 1996).

Figure 6B shows a typical example of a wavenumber versus temperature plot of glycinin at I = 0.2 and pD 7.5 of the intensity maximum of the amide I band. It is observed that the wavenumber shifts gradually to higher values with increasing temperature. This is considered to be an intrinsic effect. Above 92 °C a sigmoidal shaped increase is observed that is interpreted as a conformational change. The gradual decrease is again an effect caused by the temperature decrease itself. Furthermore, this gradual decrease is completely reversible for all conditions studied (results not shown) as was investigated by performing a reheating scan. For each condition the wavenumber versus temperature plot shows similar trends for the shoulder that occurs upon heating (a typical example for I = 0.2 and pD = 7.5 is shown in Figure 6C).

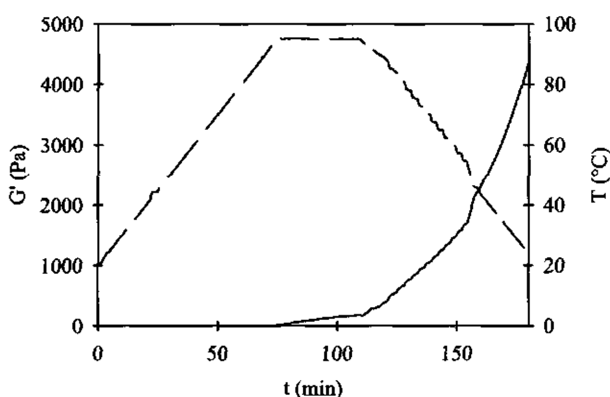


**Figure 5** The increase in gel stiffness as a function of time and temperature of 10% (w/v) glycinin at I = 0.2 and pH 7.6 as a typical example. The temperature profile is indicated with a dashed line.

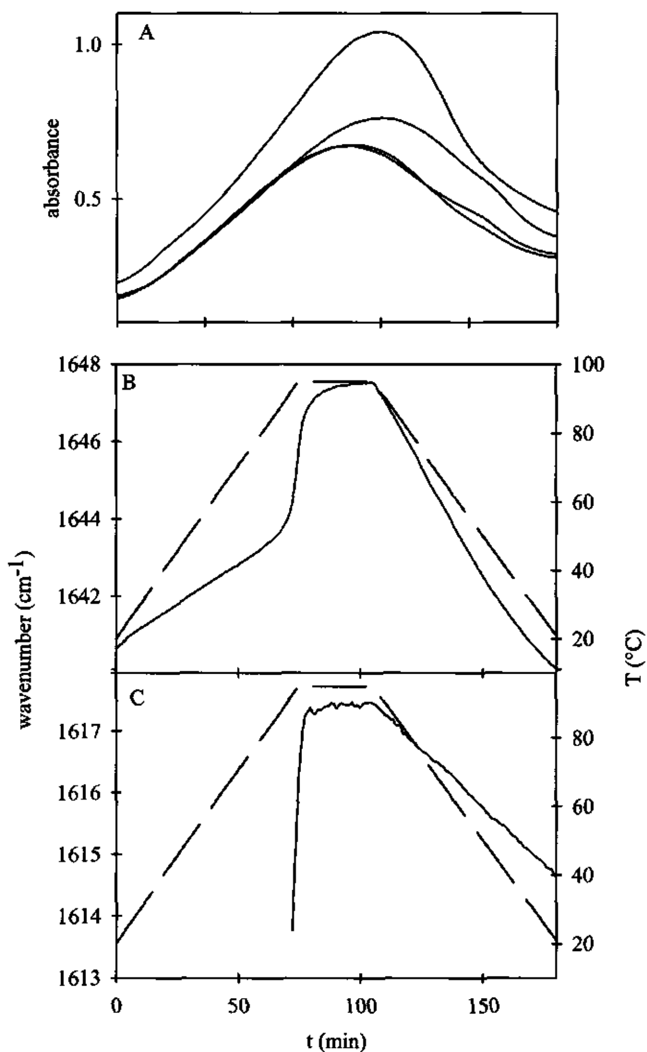


**Figure 6** Typical example of 10% (w/v) glycinin at I = 0.2 and pD 7.5 (A) deconvoluted IR spectra of the 1700 -1600 cm$^{-1}$ region at different stages of the temperature profile recorded in $D_2O$ ((a) 20°C before heating; (b) 0 min at 95°C; (c) 30 min at 95°C; (d) 20°C after cooling) (B) wavenumber versus temperature plot of the top of the amide I band (C) wavenumber versus temperature plot of the shoulder occurring on the amide I band. The temperature profile is indicated with a dashed line.

### 3.4 Characterization of the non-network protein at a quaternary, tertiary and secondary level

The size of the non-network protein was studied by GPC (Figure 7A) in order to obtain information about the extent of protein aggregation. The elution pattern of non-network protein at pH 7.6 (a typical example at I = 0.5 has been given) changed significantly compared to the elution pattern of the non-heated glycinin. The elution patterns of the non-network fractions obtained at pH 7.6 at I = 0.5, 0.2 and 0.03 showed one major peak around the void volume and a minor peak at 12.6, 13.8 and 10.7 ml for I = 0.03, 0.2 and 0.5, respectively. This means that in *all* cases the majority of the *soluble* aggregated proteins had apparent molecular weights higher than approximately 700 kDa.

The non-network protein was also studied by near-UV CD to obtain information about changes occurring at a tertiary folding level (Figure 7B) The total intensity of the near-UV CD spectra of the non-network protein obtained after heating had decreased by about 90% compared to unheated glycinin indicating a large tertiary destabilization of the protein (Vuilleumier et al., 1993). The near-UV CD spectrum at I = 0.5 had a comparable shape as the spectra at I = 0.2 (results not shown) and 0.03.

Far-UV CD was used to obtain information about the changes occurring at secondary folding level (Figure 7C). The figure shows that upon heating the negative extreme in the far-UV CD spectrum shifts to a lower wavelength (about 5 nm). Furthermore, at I = 0.5 the non-network glycinin had a negative extreme around 203 nm, while the spectra at I = 0.03 and 0.2 (results not shown) exhibited a negative extreme around 201 nm. The secondary structure content was estimated using curve fitting procedures with reference spectra (de Jongh and de Kruijff, 1990). The amount of non-structured protein in heated glycinin is clearly higher at I = 0.03 and 0.2 (61%) compared to I = 0.5 (53%). This amount of non-structured protein was found to be increased by a factor 2-3 compared to unheated glycinin for all ionic strength studied (Lakemond et al., 2000a).

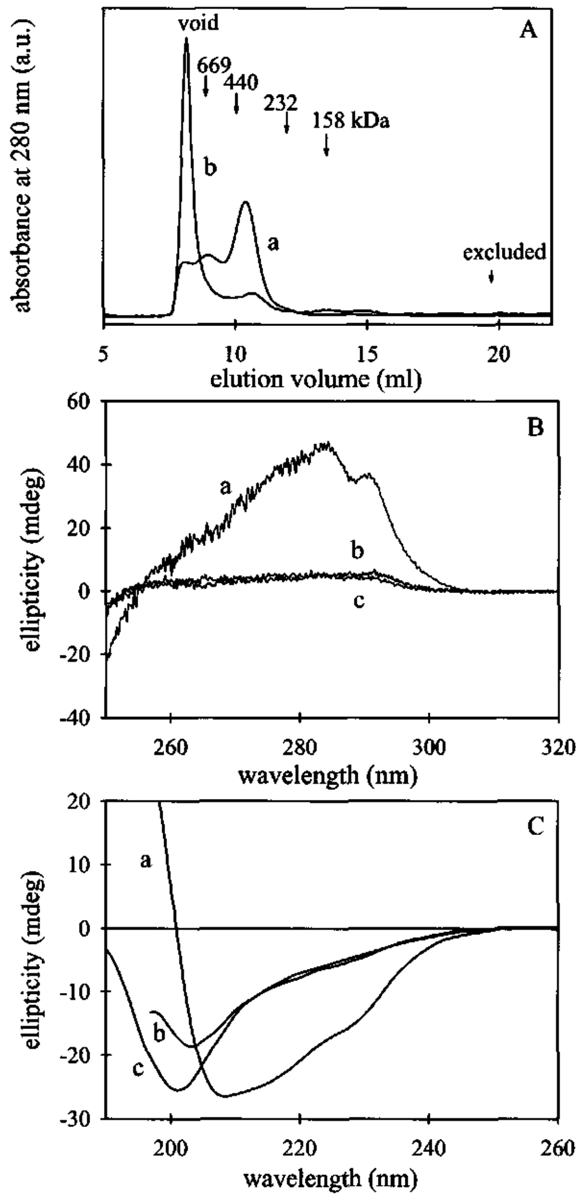


**Figure 7** Characterization of the non-network protein at pH 7.6 at a quaternary, tertiary and secondary level as measured by (A) gel permeation chromatography on a Superdex 200 column (B) near-UV CD, and (C) far-UV-CD. ((a) unheated glycinin at I = 0.5; (b) non-network protein at I = 0.5; (c) non-network protein at I = 0.03).

## 4 Discussion

This study was performed to investigate the relation between protein conformation, gel formation and gel structure for soy glycinin. A comparison was made between conditions used frequently in literature (I = 0.5; pH 7.6) and conditions more representative for food systems (I = 0.02- 0.2; pH 3-7).

### 4.1 Glycinin network structure; Low (0.03) versus high ionic strength (0.2 and 0.5)

At I = 0.03 for both pH's the gels are (relatively) transparent (Table 2), have a high water holding capacity (Figure 2) and a fine structure (Figure 1), characteristics reported to be associated with fine stranded network structures (Hermansson, 1986). At I = 0.2 and 0.5 the gels have a more turbid appearance (Table 2), a relatively low water holding capacity (Figure 2) and a clear microstructure (coarse) (Figure 1), that generally point at the presence of a coarse network (Hermansson, 1986). A more coarse gel at a higher ionic strength was also found at pH 3.5 for soy protein isolate gels (Puppo and Añón, 1998) and at pH 7.0 for glycinin gels (Hermansson, 1985). In contrast with our observations even at high ionic strength (I = 0.5) fine stranded network structures have been reported for glycinin at pH 7.6 (Nakamura et al., 1984; Mori et al., 1986).

For both pH values the gels seem to be more coarse when the ionic strength is higher, based on the water holding capacity (Figure 2) and the CSLM pictures (Figure 1). However, the highest G' values (Figure 3) are observed at I = 0.2 and the lowest at I = 0.03. This shows that G' does not correlate (solely) to gel coarseness or thickness of the strands. This is not surprising since G' also depends on the curvature of the strands (Bremer et al., 1989; Mellema, 2000), the type and number of bonds in the strands, and the size of the primary particles (van Vliet, 2000).

The values for the slope "x" (derived from the log-log plot of G' versus protein concentration) (Table 3) are lower as the gel coarseness increases (Figure 1; Table 2), except for I = 0.2 and 0.5, where for the slope no significant differences are present between pH 3.8 and 7.6. The slopes at I = 0.03 are high (Table 3) compared to literature. At I = 0.2 and 0.5 these values are in the range of what is generally found for proteins (van Vliet, 2000). Assuming that the clusters forming the gels can be described as fractal clusters, differences observed in slope values "x" relate to differences in gel structure, since $x = \alpha/(3 - D_f)$, where $D_f$ is the fractal dimensionality and $\alpha$ relates to the nature of the strands (Bremer et al., 1989). The value of $\alpha$ is around 3 for hinged strands and 2 for straight strands (Mellema, 2000). In our laboratory

(unpublished results) it is found for glycinin at I = 0.2, that at pH 7.6 the strands within the gel network are curved or hinged, while they are straight at pH 3.8. In analogy, we use $\alpha = 2$ for pH 3.8 and $\alpha = 3$ for pH 7.6 for estimating $D_f$, which then turns out to be lower at pH 3.8 ( $D_f$ = 2.1) than at pH 7.6 ($D_f$ = 2.3).

### 4.2 Glycinin network structure; High pH (7.6) versus low pH (3.8)

The results show that at I = 0.03 finer gel network structures were formed at pH 3.8 than at pH 7.6, since at pH 3.8 the gels are fully transparent (Table 2), have the highest water holding capacity (Figure 2) and show the finest structure when analyzed by CSLM (Figure 1). On the other hand, at I = 0.2 and 0.5, coarser network structures were formed at pH 3.8 than at pH 7.6, as can be concluded from the low water holding capacities at pH 3.8 (Figure 2) and the differences in visual appearance (Table 2) of the glycinin gels. Although gels at I = 0.5 and 0.2 were turbid at both pH values, at pH 3.8 the gels were also granulated which indicates the existence of even larger aggregates than 1 μm and thus more coarse networks. The yellowish color observed at pH 7.6 could in principle be related to the size of the aggregates, but is more likely caused by the presence of traces of phenolic compounds that color yellowish at pH 7.6, since this color difference was already observed prior to heating.

The fact that the CSLM pictures obtained after deconvolution (Figure 1B) of 10% glycinin gels show that at pH 7.6 less network protein was present than at pH 3.8 indicates also that it is insufficient to describe gel network structure only in terms of coarseness.

The fact that lower G' values are found at pH 7.6 compared to pH 3.8 (Figure 3) is probably due to the fact that at pH 7.6 less protein is incorporated in the network (Table 4), and the curved nature of the strands at pH 7.6 versus straight at pH 3.8 as found in our laboratory (unpublished results). Since curved strands are easier to bend than straight strands, this implies a lower stiffness (G') for gels build of curved strands (Bremer et al., 1989; Mellema, 2000).

### 4.3 Glycinin network structure; Distribution of acidic and basic polypeptides

Since at pH 3.8 less than 5% of total protein was found to be non-network protein (Table 4), 95% of the protein was present in the network. At pH 7.6 the non-network protein (Table 4), which is assumed to be spread homogeneously over the network, is not visible any more in the deconvoluted CSLM pictures (Figure 1B) due to the threshold value. This explains why more network protein is present at pH 3.8 than at pH 7.6. The relatively high water holding capacity at pH 7.6 compared to pH 3.8 (Figure 2) supports the idea that at pH 7.6 non-network protein is present in the pores. Solely based on the size of the pores, which are

relatively large at pH 7.6 compared to pH 3.8 (Figure 1B) one would expect the reverse. It implies that non-network protein is important for the final gel characteristics, as was also suggested by Yamagishi et al. (1987), although it likely does not attribute to gel stiffness. Characterization of the non-network protein showed that it was mainly present as soluble aggregates with molecular sizes above 720 kDa or 15S (Figure 7A), as is also in line with Yamagishi et al. (1987). The non-network protein was denatured to a large extent after heating for all ionic strengths, especially at the tertiary level (Figure 7B and C). The secondary structure of the non-network protein at I = 0.5 was slightly different from I = 0.03 and I = 0.2, while for tertiary structure no differences were found (Figure 7B and C). No correlation could be found between these observations and gel structure.

It was found that the nature of the primary particles in the strands at pH 7.6 is different from those at pH 3.8 since, at pH 7.6 the network protein consists predominantly of basic polypeptides (Figure 4) (only 51 to 69 %, depending on ionic strength, of all protein takes part in the network (Table 4)). The results show that at pH 3.8 the network protein consists of both polypeptides (all protein takes part in the network). It is not clear why consequently about 10% more basic than acidic polypeptides are found in the network protein at this pH.

These results imply that at pH 7.6 disruption of the disulfide bridges and the non-covalent bonds between the acidic and basic polypeptides occurred during heating, as is in agreement with previous findings (Nakamura et al., 1985; Utsumi and Kinsella, 1985b; Yamagishi et al., 1987). The presence of a minor amount of basic polypeptides at pH 7.6 in the non-network protein (Figure 4B) corresponds to the results of Yamagishi et al. (1987), implies that not all disulfide bridges and/or non-covalent bonds between the acidic and basic polypeptides were broken. It is not expected that at pH 3.8 the disulfide bridge between the acidic and basic polypeptides breaks during gel formation since the disulfide bridge was likely intact after heat denaturation of protein at a concentration of 1% (Lakemond et al. (2000b).

### 4.4 Denaturation and changes in secondary structure during gel formation

The observed differences in gel structure can be related to differences in the structural changes of the subunits during the gel formation process. The results show that denaturation is in all cases a prerequisite for gel formation. At I = 0.03, 0.2 and 0.5 denaturation coincides with the induction of β-strand at a secondary level (Table 1), as is in line with results for β-conglycinin (Nagano et al., 1995) and for β-lactoglobulin (Boye et al., 1996). However, denaturation and β-sheet induction only coincides with gel formation at I = 0.5 and 0.2 (Table 1). An explanation is that the minimum amount of protein necessary to form a gel is at I = 0.03 (≥ 7%) much higher than at I = 0.2 and 0.5 (≤ 5%). At I = 0.03 at both pH values the

endothermic transitions ends around 95°C and one could assume that only then the concentration of denatured protein is high enough to form a gel. This is confirmed by the fact that when protein concentration is increased the gelation temperature decreases drastically. The gelation temperature never dropped below the onset denaturation temperature of 11S glycinin also for protein concentrations of 12.5 and 15%.
The largest increase in gel stiffness takes place in the cooling part of the temperature cycle (Figure 5). The IR measurements show that this increase in stiffness is not caused by a change in secondary structure during cooling (Figure 6). This process does not seem to be related to changes in molecular structure at a secondary level.

## 5 Conclusion

From the results presented in this work it can be concluded that large differences exists in the structure and formation of glycinin networks when pH and ionic strength are varied. The results show that denaturation, coinciding with the induction of β-strand at a secondary folding level, is a prerequisite for gel formation at all conditions studied, but occurs at different temperatures for the various conditions studied. The increase in gel stiffness, especially during cooling cannot be explained by changes in secondary structure.
Gel coarseness (results obtained from CSLM and visual appearance) differs for the various conditions studied. By examining additional parameters (as gel stiffness, the presence of non-network protein, its structure and waterholding capacities) gel structure could be clarified in more detail. It can be derived from the results that gel coarseness is not linked to these parameters.
It is emphasized that the use of non-traditional techniques (physico-chemical and spectroscopic) in gelation studies provides additional information on gel formation and gel structure.

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Chapter 5

# Differences in denaturation of genetic variants of soy glycinin[1]


**Abstract**

In heat denaturation studies, so far, the genetic variants of glycinin have been considered as a homogeneous group of proteins. In this work the validity of this assumption was tested. It was found by calorimetric studies that glycinin denatures heterogeneously at pH 7.6. When the temperature of isothermal treatment is increased from 70°C to 82°C the proportion of glycinin remaining native gradually decreases from 95% to 5% while the denaturation temperature of the glycinin remaining native increases from 88.5 to 95°C. Similar trends were found for pH 3.8. Fractionation and subsequent analysis (MALDI-TOF and CE) of isothermally treated samples demonstrated that at pH 7.6 the heterogeneous denaturation is caused by differences in thermal stability of the genetic variants of glycinin. The stability increases in the order G2/G3/G1< $A_4$< G5 < G4.


key words: soy; glycinin; pH; genetic variant; subunit; heat denaturation

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# 1 Introduction

Soy proteins have found applications in many different food products. Generally, such food products are heated to inactivate antinutritional factors, for food preservation reasons, and to obtain desired functional properties. The major seed storage protein in soybeans is glycinin. It represents about 30% of total protein in soybean (Yamauchi et al., 1991). Fischer and Goldberg (1982) described three glycinin genes (G1 - G3) and Scallon et al. (1985) reported the existence of two additional genes (G4 and G5). The five genes have diverged into two subfamilies that are designated as Group I and Group II glycinin genes (Nielsen, 1984). The different genetic variants of glycinin are also referred to as "subunits" throughout literature and this work. The different glycinin subunits were identified on the basis of their amino acid sequences (Moreira et al., 1979; Moreira et al., 1981; Staswick et al., 1981; Staswick et al., 1984a). Each subunit consists of an acidic and a basic polypeptide (Staswick et al., 1981), linked by a single disulfide bridge (Staswick et al., 1984b), except for the acidic polypeptide $A_4$ present in G4 (Staswick et al., 1981). The two polypeptide chains result from posttranslational cleavage of proglycinin precursors (Turner et al., 1981). Table 1 shows the molecular weights of different glycinin subunits as calculated from the reported DNA sequences of the different glycinin genes.

**Table 1** Molecular weights (exclusive signal and propeptides) of the five different glycinin subunits and their polypeptides (A = acidic polypeptide; B = basic polypeptide) as calculated from their amino acid sequences as derived from the DNA sequence of the five different glycinin genes (based on Scallon et al. (1987); Nielsen et al. (1989); Sims and Goldberg (1989); Thanh et al. (1989); Cho and Nielsen (1989)).

| Subunit | MW (kDa) of Subunits | Polypeptides | Mw (kDa) of Polypeptides |
|---|---|---|---|
| G1 | 52.6 | $A_{1A}B_2$ | 32.6; 20.0 |
| G2 | 51.3 | $A_2B_{1A}$ | 31.5; 19.8 |
| G3 | 51.3 | $A_{1b}B_{1b}$ | 31.5; 19.8 |
| G4 | 61.2 | $A_5A_4B_3$ | 10.5; 30.0; 20.7 |
| G5 | 54.4 | $A_3B_4$ | 36.4; 19.0 |

Indications exist that more than five different variants (subunits) of glycinin are present (Mori et al., 1981; Lei et al., 1983; Nielsen et al., 1989). Nielsen et al. (Nielsen et al., 1989) suggest the presence of more than five genes, but it was also suggested that glycinin undergoes, like other 11S storage proteins, a complex series of posttranslational events (Dickinson et al., 1989). Some studies report that a small portion of glycinin is glycosylated (Wolf and Sly, 1966; Fukushima, 1968; Lei and Reeck, 1987), which is contradicted by others (Koshiyama and Fukushima, 1976). It is, however, important to realise that the precise subunit composition depends on the soybean variety used (Mori et al., 1981).

It was found that glycinin associated into 7S or 11S aggregates depending on the type of glycinin subunits present (Utsumi et al., 1987; Yagasaki et al., 1997). Since 7S glycinin denatures at lower temperatures than 11S glycinin (Danilenko et al., 1987; Lakemond et al., 2000a), it could be speculated that there is a relation between denaturation temperatures and subunit composition. However, in articles in which models for glycinin heat denaturation are proposed (for example in Peng et al. (1984) and Yamauchi et al. (1991)) glycinin has never been considered to be heterogeneous. This complication may have contributed to the fact that the glycinin heat denaturation mechanism has not been elucidated fully.

In the present work it is studied if the different genetic variants of glycinin display a different thermal stability. We compare heat denaturation at pH 7.6, a pH often used in soy protein literature, with pH 3.8 which is more representative for conditions present in food (pH 3-7). Because glycinin is insoluble between pH 3.8 and 7.0 at the ionic strength (0.2) used, which gives experimental complications, pH values between 3.8 and 7.0 were not studied.

## 2 Materials and methods

### 2.1 Glycinin purification and preparation of glycinin dispersions

Glycinin was purified from William's 82 soybeans (harvest 1994). Milling and defatting of the soybeans was performed according to Lakemond et al. (2000b). Glycinin was extracted and purified according to a modified method of Thanh and Shibasaki (1976).

A soy protein isolate was obtained after extraction of protein at pH 8.0 from the defatted soy meal and subsequent acidic precipitation at pH 4.8. The precipitate obtained was resolubilised at pH 7.8 and subsequently a crude glycinin fraction was obtained by acidic precipitation at pH 6.4. The pelleted glycinin was resolubilised in 10 mM potassium phosphate buffer at pH 7.8 containing 10 mM 2-mercaptoethanol. For further purification ammoniumsulphate was

added to the resolubilised crude glycinin fraction up to 50% saturation. After centrifugation (30 min 12000 g; 4°C) more ammoniumsulphate (70% saturation) was added to the supernatant. The precipitate obtained after centrifugation (30 min 12000g; 4 °C) was resolubilised in a 10 mM potassium phosphatebuffer at pH 7.8 in the presence of 10 mM 2-mercatoethanol and 20% glycerol. Batches of this fraction, purified glycinin, were stored at -40°C. After defrosting part of the purified glycinin, it was extensively dialysed against Millipore water and freeze-dried afterwards (storage at -20°C). The purity of glycinin was determined by SDS-PAGE under reducing and non-reducing conditions on a Phast System (Pharmacia, Uppsala, Sweden) according to the instructions of the manufacturer. Gradient gels (10-15%) were used, which were stained with Coomassie Brilliant Blue. The gels were calibrated with low molecular weight markers ranging from 14-94 kDa (Pharmacia). The purity of glycinin was estimated to be over 95% by densitometric analysis of the gels.
Prior to an experiment 1% (w/v) glycinin was suspended in 35 mM potassium phosphate buffer at pH 7.6 containing 0.1 M NaCl. For experiments performed at pH 3.8 the pH was adjusted by adding HCl. The ionic strength was increased by ~ 0.015 by the amount of HCl needed.

### 2.2 Separation and identification of the genetic variants of glycinin

At pH 7.6 anion exchange chromatography (section 2.6) was used to separate unheated glycinin into several fractions. The different fractions pooled were analysed for subunit composition by MALDI-TOF MS spectroscopy (section 2.7) and by capillary electrophoresis (CE) (section 2.8). Prior to MALDI-TOF MS analysis the acidic polypeptides were separated from the basic polypeptides with anion exchange chromatography as described previously (Lakemond et al., 2000b). These fractions were also analysed with CE.

### 2.3 Differential scanning calorimetry (DSC)

For experiments carried out at pH 7.6 DSC thermograms were recorded on a micro DSC III (Setaram, Caluire, France) using 0.9 ml vessels. For the experiments carried out at pH 3.8 a VP-DSC MicroCalorimeter (MicroCal Incorporated, Northhampton MA, USA) with 0.5 ml cells was used, because the micro DSC III was not sensitive enough to carry out the experiments at pH 3.8. Glycinin suspensions (1% w/v) at pH 7.6 and 3.8 were (partly) heat denatured consecutively by heating from 20 to 53-83 °C at a rate of 1 K/min, keeping temperature constant at set temperatures between 53-83°C for 16 hours and cooling down to

20°C at a rate of 1 K/min. This sequence of heating conditions is further denoted as isothermal treatment. The total time for the isothermal treatment step was set at 16 hours to ensure the denaturation process being in an equilibrium state. Next, the samples were reheated from 20 to 115 °C at a rate of 1 K/min and subsequently cooled to 20°C to determine the proportion of denaturation and the denaturation temperature of the protein fraction unaffected by the isothermal treatment (referred to as glycinin "remaining native"). The denaturation temperature $T_d$ is defined as the temperature at maximum heatflow of the endothermic transition. The proportion of denaturation was calculated from the enthalpy remaining after isothermal treatment divided by the total enthalpy of unheated glycinin. At pH 3.8 the inaccuracy of the data obtained was about 10% ($\sigma_{n-1}$) and at pH 7.6 about 3% ($\sigma_{n-1}$), as was derived from duplicate experiments.

### 2.4 Isolation of the glycinin remaining native after isothermal treatment

At pH 7.6 glycinin samples were (partly) heat denatured heated according to the temperature profile described in section 2.3 (isothermal treatment). To isolate the glycinin fractions remaining native (see section 2.3) first a supernatant was obtained by centrifugation (30 min; 15800 g) at 20°C. The native protein fraction was separated from the soluble fraction by gel permeation chromatography (section 2.5). Protein that did not dissociate or aggregate (corresponding to 11S and 15S glycinin) after heat treatment was considered to be native and will be referred to as "purified native glycinin". These fractions were analysed for their subunit composition by anion exchange chromatography (section 2.6).

### 2.5 Gel Permeation Chromatography

Gel permeation chromatography was performed at 20°C on a Superdex 200 column 10/30 (Pharmacia, Uppsala, Sweden) attached to an FPLC system (Pharmacia, Uppsala, Sweden). The elution buffer consisted of 35 mM potassium phosphate and 0.1 M NaCl at pH 7.6. The flow rate was 1 ml/min and the eluate was monitored at 280 nm. The column was calibrated with a high molecular weight gel filtration calibration kit (Pharmacia; 152-669 kDa).

### 2.6 Anion Exchange Chromatography

Anion exchange chromatography was performed at 20°C on a mono Q HR 5/5 column (Pharmacia, Uppsala, Sweden) attached to an FPLC system (Pharmacia, Uppsala, Sweden). First, the protein was brought onto the column with 10 mM potassium phosphate buffer

containing 6 M urea at pH 6.6 and eluted afterwards in the same buffer. The protein bound to the column was eluted using a linear gradient from 0 to 0.5 M NaCl in the same buffer over 17.5 column volumes. The flow rate was 1 ml/min and the eluate was monitored at 280 nm.

### 2.7 MALDI-TOF MS spectroscopy

MALDI-TOF MS spectroscopy was performed using a Voyager DE RP instrument (PerSeptive Biosystems, Framingham, USA). In order to prepare the matrix 10 mg/ml 3,5 dimethoxy-4-hydroxycinnamic acid (sinapinic acid, Sigma D-7927, St Louis, USA) in 0.6% (v/v) aqueous trifluoroacetic acid was mixed with acetonitril in a ratio of 7:3 (by volume). The samples were diluted 10 times with matrix, and allowed to crystallise for 30 minutes on a gold plated welled plate. External calibration was performed according to the description of the manufacturer using insulin (5734 Da), cytochrome C (12361 Da), myoglobulin (6952 Da) and BSA (66431 Da) to obtain an inaccuracy of < 50Da.

### 2.8 Capillary Electrophoresis

Capillary electrophoresis was performed on a Beckman P/Ace system 5500 equipped with a Diode array detector (Beckman Instruments, Fullerton, USA). Separation was obtained using a 50 μm hydrophilic coated capillary of 57 cm (Celect P-150, Supelco, Bellefonte, USA). The fractions were analysed at a concentration of 5 mg/ml in a 10 mm potassium phosphate buffer containing 6 M urea, 4.5 mM DTT at pH 6.6. The running buffer contained 8 M urea, 0.38 M citric acid, and 0.05% methyl-hydroxy-ethyl-cellulose at pH 2.7. The separation voltage was 25 kV, the temperature 45 °C, detection was at 214 nm (data collection rate 1 Hz) and injections were carried out by pressure (injection time 10s). Replicate experiments were performed.

# 3 Results

## 3.1 Gradual denaturation of glycinin

DSC measurements were performed to establish glycinin fractions denature to different extents at pH 7.6 and pH 3.8. In Figure 1 the influence of the isothermal treatment on the proportion of glycinin remaining native after isothermal treatment at pH 7.6 and 3.8 is presented using the enthalpies of the endothermic transitions of unheated glycinin as a reference value. At pH 7.6 it is found that when the heating temperature increases from 70 °C to 82 °C the proportion of glycinin remaining native gradually decreases from about 95 to 5%. At pH 3.8 it is found that when the isothermal heating temperature is increased from 54 to 75 °C the proportion of native glycinin decreases gradually from 81 to 9%. From 54 °C to about 66 °C only 7S glycinin denatures and at higher temperatures also the 11S form starts to denature, since it is known from earlier work (Lakemond et al., 2000a) that at pH 3.8 two endothermic transitions (at 68 °C and 82 °C) are observed that are related to the presence of both a 7S and an 11S form. In addition, at pH 7.6 only one endothermic transition is observed (at 87°C) since at this pH glycinin is present in the 11S form only (Lakemond et al., 2000a).
To investigate whether the denaturation process is measured in an equilibrium state we compared heating for 16 hours to heating for 24 hours at 77.5°C at pH 7.6. The prolonged incubation shows that the nativity of the glycinin decreases with only additional 4%, which is within the experimental error of the enthalpy determination. Thus, the samples can be assumed to be measured in an equilibrium state. The fact that it is possible to obtain glycinin fractions that are denatured to different extents points at a heterogeneous heat denaturation process at both pH 7.6 and 3.8.

Furthermore, it was observed that at pH 7.6 the denaturation temperature of the protein remaining native increases from 88.5 to 95 °C as the temperature of isothermal treatment increases from 70 to 82 °C (Figure 2), suggesting differences in the thermostability. For pH 3.8 a similar trend is observed. Only the denaturation temperature of the 11S glycinin transition at low pH is shown in Figure 2, since the denaturation temperatures of the 7S form could not be determined accurately (the relatively smaller 7S endotherm is not separated well from the 11S endotherm).

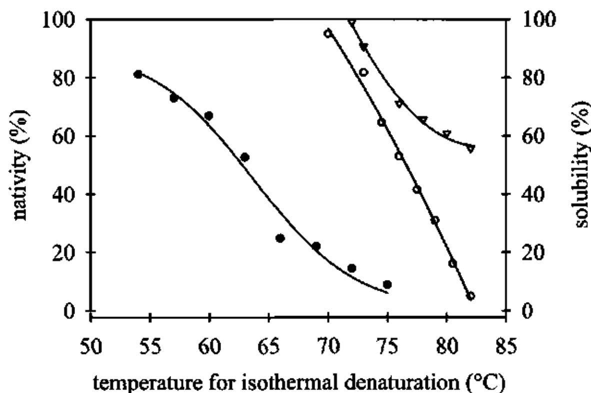


**Figure 1** The influence of the isothermal temperature (heating during 16 hours) on the proportion of glycinin remaining native at pH 3.8 (•) and pH 7.6 (o) (DSC measurements) and the solubility at pH 7.6 (Δ).

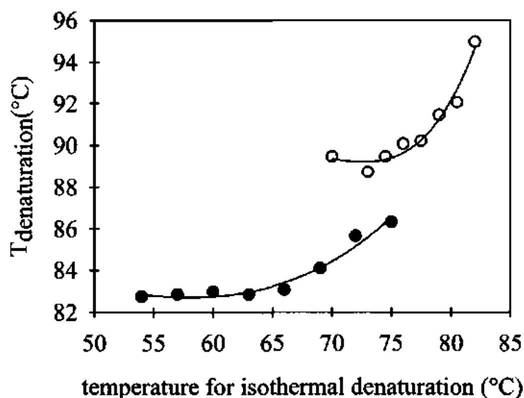


**Figure 2** The denaturation temperatures of the protein remaining native after isothermal treatment at different temperatures at pH 3.8 (•) and pH 7.6 (o) as obtained with DSC.

### 3.2 Aggregation state of glycinin after isothermal treatment

To investigate the aggregation state of the glycinin fractions after isothermal treatment the amount of protein remaining soluble was determined at pH 7.6 (Figure 1). The results indicate that upon increasing the temperature of isothermal treatment to 82°C the solubility decreases gradually to about 50 % (solubility of unheated glycinin was set at 100%), while the protein

was denatured over 95%. The soluble protein fraction consists of protein aggregates smaller than approximately 0.2 μm, as calculated using the Stokes equation from the g-force applied. Gel permeation chromatography was used to separate aggregated or dissociated protein from native protein (11S and 15S glycinin). Figure 3 shows the elution patterns of the different soluble fractions obtained after isothermal treatment at different temperatures. The elution patterns in Figure 3 show that the unheated glycinin is predominantly present in the 11S form, which is in agreement with observations throughout literature. Also a 15 S fraction was present and a fraction consisting of even larger aggregates (together ~ 20-30%). Upon increasing the temperature the intensity of the 11S and 15S peaks decreases gradually. At 73 and 74 °C a large void peak is observed, containing aggregates with apparent molecular weights larger than 700 kDa. At higher temperatures these large aggregates have disappeared and in the elution patterns also a protein peak with a molecular weight below 160 kDa occurs. This dissociation is probably linked to the disruption of the disulfide bridge between the acidic and basic polypeptides, after which the basic polypeptides precipitate and the acidic polypeptides stay soluble (Lakemond et al., 2000a). The fractions containing native protein in an 11S or 15S structural organisation are pooled for further analysis as indicated in Figure 3.

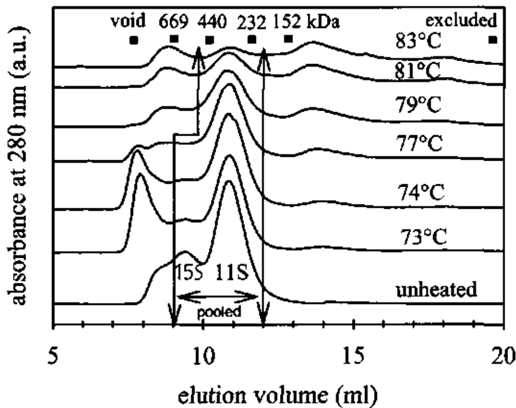


**Figure 3** The elution patterns of the soluble fractions obtained after isothermal treatment at pH 7.6 at different temperatures on a Superdex 200 10/30 column. Fractions were pooled to obtain the purified native protein as indicated in the figure.

### 3.3 Analysis of the purified native glycinin

The purified native protein fractions obtained by GPC after isothermal treatment at different temperatures were analysed with anion exchange chromatography to study if the different subunits of glycinin denature at different temperatures (at pH 7.6). In Figure 4 the elution patterns of the different purified native glycinin fractions are presented. In the elution pattern of the unheated sample five major fractions are observed, one in the non-bound material (fraction a) and four in the bound material (fraction b-e). Fraction a is still present at a temperature at which fraction b-e cannot be observed any more. When the temperature is increased the intensity of fraction c decreases somewhat faster than the intensity of fraction b. The intensity of fraction e decreases relatively faster with increasing temperature than that of fraction d. The intensity of fraction b and c decreases at lower temperatures compared to fraction d and e.

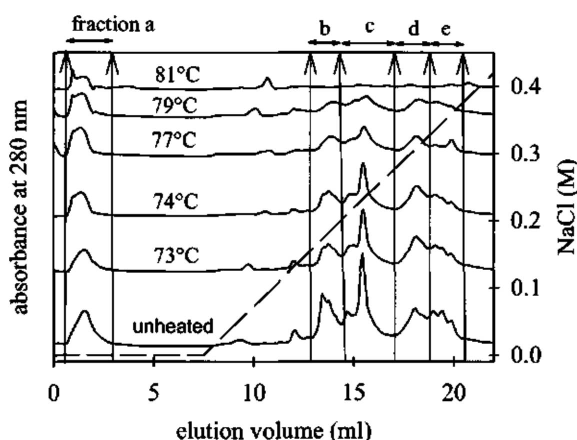


**Figure 4** The elution pattern of purified native glycinin fractions obtained after isothermal treatment at different temperatures on a mono Q column. The arrows indicate the different protein fractions. The NaCl gradient is indicated with a dashed line.

### 3.4 Separation and identification of the different glycinin subunits

In order to analyse the protein composition in the various anion exchange chromatography fractions obtained of unheated glycinin (Figure 4) both CE and MALDI-TOF MS were used. The CE electropherograms of the different fractions of unheated glycinin show that the different polypeptides in the fractions have retention times varying from about 17 to 28 minutes (Figure 5A). The number of peaks in the electropherogram (over 25 in the total unheated reference sample) exceeds the number of polypeptides identified from literature

(Table 1). Significant differences between CE electropherograms are observed when a comparison is made between the electropherograms of fractions (a) to (e), indicating that the different fractions of the unheated glycinin contain multiple (genetic) variants of glycinin. It is possible to identify which peaks originate from basic or acidic polypeptides by separating the acidic from the basic polypeptides prior to capillary electrophoresis, as is shown for fraction (d) (Figure 5B). It can be concluded that the peaks present around 18 minutes originate from acidic polypeptides. Between 20 and 22 minutes about five peaks from acidic polypeptides, and one from basic polypeptides are visible. Furthermore, the electropherogram of the basic polypeptides shows over seven peaks with retention times from 22-27 minutes.

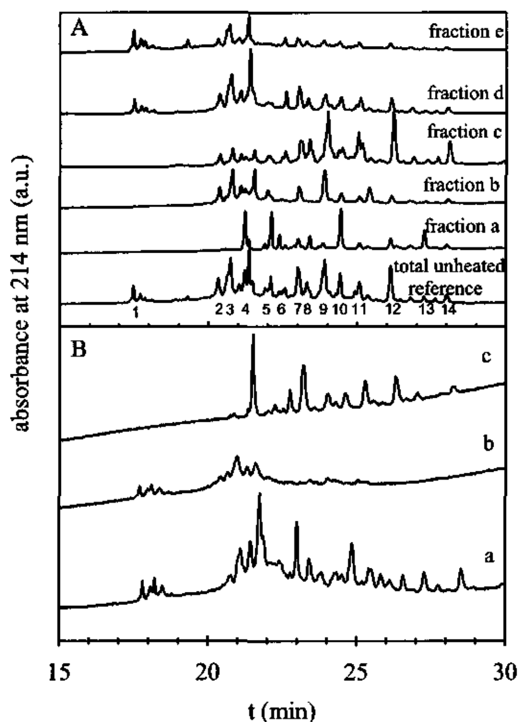


**Figure 5** (A) Electropherograms of the different peaks that were obtained after separation of unheated glycinin on a mono Q column as shown in Figure 4, elution pattern a. (The different fractions analysed are indicated in the Figure); (B) Electropherograms of all polypeptides (a), the acidic polypeptides (b) and the basic polypeptides (c) present in fraction (d) derived from unheated glycinin.

MALDI-TOF MS spectrometry was used to analyse the subunit composition of the fractions obtained after anion exchange chromatography of unheated glycinin (Figure 4). The MALDI-

TOF MS spectra (Figure 6) of the acidic polypeptides present in the total (non fractionated) unheated glycinin sample show five major peaks at ~10.6, 30.2, 31.6, 32.6 and 36.2 kDa. The spectrum of the basic polypeptides shows three major peaks at 19.3, 20.2 and 20.4 kDa. In the spectra of different fractions obtained after anion exchange chromatography of unheated glycinin, however, also a peak at 20.3 kDa was clearly visible (spectra not shown).

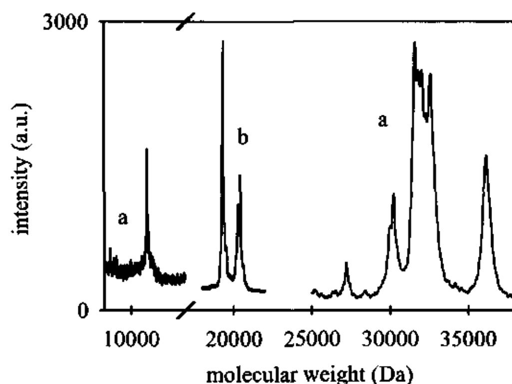


**Figure 6** MALDI-TOF MS spectra of the acidic (a) and basic polypeptides (b) of total (non-fractionated) unheated glycinin.

The peaks could be assigned to specific acidic and basic polypeptides (Table 2) based on their theoretical molecular weights (Table 1). It was not possible to distinguish between the polypeptides in G2 and G3 since they have comparable molecular weights (Table 1). The peaks observed at 20.2 and 20.3 kDa could not be assigned unambiguously, since the observed molecular weights do not match exactly the theoretical molecular weights (Table 1). From the MALDI-TOF MS spectra of the different fractions indicated in Figure 4 it was derived which subunits or polypeptides were present as a major or as a minor component (Table 2). It was assumed that the different acidic and basic polypeptides ionise similarly. Fraction (a) (Figure 4) contained mainly subunit G4 (without polypeptide $A_4$). In fraction (b) subunit G1 was present, while fraction (c) contained predominantly subunit G2 and/or G3. The main components of fraction (d) were subunit G5 and the acidic polypeptide $A_4$. Fraction (e) contained predominantly the acidic polypeptide $A_4$. This acidic polypeptide, which is part of glycinin G4 is not covalently bound to a basic polypeptide (Staswick et al., 1981) and elutes, therefore, in a different fraction than the polypeptides $A_5$ and $B_3$, the other constituents of G4. Each fraction contained also minor components as indicated in Table 2.

**Table 2** The subunit composition of the different purified native glycinin fractions as measured with MALDI-TOF MS spectrometry.

| | $M_{w\ acidic\ polypeptides}$ (kDa) | | | | | $M_{w\ basic\ polypeptides}$ (kDa) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | $A_5$ | $A_4$ | $A_2/A_{1B}$ | $A_{1A}$ | $A_3$ | $B_4$ | $B_{1A}/B_{1B}$ | $B_{1B}/B_2$ | $B_3$ | subunit/polypeptide | |
| Ref. | 10.6 | 30.2 | 31.6 | 32.6 | 36.2 | 19.3 | 20.2 | 20.3 | 20.4 | major | minor |
| Fr. a | ++ | | | | | | | + | ++ | G4* | $B/B_2$ |
| Fr. b | | | | ++ | | | | ++ | + | G1 | $B_3$ |
| Fr. c | | | ++ | + | | | ++ | + | | G2/G3 | G1 |
| Fr. d | + | ++ | + | + | ++ | ++ | + | + | + | G5, $A_4$ | G1,G2/G3,G4 |
| Fr. e | + | ++ | + | + | + | + | + | + | + | $A_4$ | G1,G2/G3,G4,G5 |

The peaks (Figure 6) present in the unheated glycinin were assigned to specific basic and acidic polypeptides. Based on the composition of the different fractions obtained it was assigned which subunits were present in which purified native glycinin fraction.

* the subunit G4 did not contain the acidic polypeptide $A_4$

++ = major component; + = minor component; A = acidic polypeptide; B = basic polypeptide

We attempted to designate the different peaks in the CE electropherogram of the unheated sample (14 major peaks were identified) to specific basic or acidic polypeptides using the data from Table 2 (based on MALDI-TOF MS spectra). The results are given in Table 3. Since the different fractions all contained minor constituents we assumed that a major component of a specific fraction gives rise to major contributions in the electropherograms.

**Table 3** Identification the different peaks present in the capillary electrophoresis electropherogram of the total unheated reference sample (Figure 5A).

| peak | Polypeptide | peak | Polypeptide | peak | polypeptide | Pea k | polypeptide | peak | polypeptide |
|---|---|---|---|---|---|---|---|---|---|
| 1 | $A_4$ | 4b | $B_3$ | 6 | $B_4$* | 9b | $A_2/A_{1B}$ | 13 | $A_5$ |
| 2 | $A_{1A}/A_5$* | 4c | $A_3$ | 7 | $B_{1A}/B_{1B}$ | 10 | $A_5$ | 14 | $B_{1A}/B_{1B}$ |
| 3 | $A_{1A}$ | 4d | $A_{1A}$ | 8 | $A_2/A_{1B}$ | 11 | $A_2/A_{1B}$ | | |
| 4a | $B_4$ | 5 | $B_3$ | 9a | $B_2$ | 12 | $B_{1A}/B_{1B}$ | | |

* peak constituent uncertain

## 4 Discussion

### 4.1 Separation and analysis of the different glycinin subunits

In order to investigate the heterogeneous denaturation of glycinin a procedure was developed to separate the different genetic variants of glycinin. It was proven that it is possible to separate the genetic variants of glycinin subjected to isothermal treatment with anion exchange chromatography by analysing the different fractions obtained with Capillary Electrophoresis (Figure 5) and MALDI-TOF MS analysis (Table 2). The results are in line with the work of Staswick et al. (1981), who identified the different genetic variants based on their amino acid composition.

The number of polypeptides identified by MALDI-TOF (5 acidic and 4 basic polypeptides; Table 2) is in line with the number of polypeptides identified in literature (Fischer and Goldberg, 1982; Scallon et al., 1985; Table 1). The number of peaks in the CE electropherogram (over 25 in the unheated glycinin sample) exceeds the number of polypeptides identified from MALDI-TOF MS spectrometry. The number of five (genetic) variants consisting of 11 different polypeptides in total (Fischer and Goldberg, 1982; Scallon et al., 1985; Table 1) seems to be insufficient to explain the CE electropherograms, supposing that one specific polypeptide leads to one peak in the electropherogram. The large number of peaks in the CE electropherogram could be explained by the presence of more than five (genetic) variants as is also suggested by others (Mori et al., 1981; Nielsen et al., 1989). These glycinin variants possibly do not differ much in molecular weight, which could explain the wide peaks in the MALDI-TOF data. CE is a very sensitive method and could be of future interest to analyse glycinin fractions for subunit composition on the condition that the peak composition in the electropherograms is studied more thoroughly. Conclusively, the above described analyses are sufficient to investigate the possible relation between the genetic variants and the glycinin structural stability upon isothermal treatment.

### 4.2 Heterogeneous denaturation of glycinin subunits

At pH 7.6, at which glycinin is present in the 11S form (Lakemond et al., 2000b), the different genetic variants of glycinin display a different thermostability (Figure 4; Table 2). Although the intensity of genetic variant G2 and/or G3 (fraction (c)) decreases somewhat more progressively than the intensity of genetic variant G1 (fraction (b)) (Figure 4), this difference is not designated as significant. The free acidic polypeptide $A_4$ denatures at lower

temperatures than G5, since the intensity of fraction (e) decreases relatively quicker than that of fraction (d). The fact that the intensity of G1 and G2/G3 (fractions (b) and (c)) decreases less progressively compared to G5 and $A_4$ (fraction (d) and (e)) means that G1 and G2/G3 denature at lower temperatures than G5 and $A_4$. The intensity of fraction (a), in which G4 (without the polypeptide $A_4$) is present, is still present at a temperature at which fractions (b-e) cannot be observed any more. This means that the denaturation temperature of the glycinin subunits increases in the order G2/G3/G1 $<$ $A_4$ $<$ G5 $<$ G4 at pH 7.6. The fact that it is possible to obtain glycinin fractions that are denatured to different extents (Figure 1) and the increasing denaturation temperatures of the native glycinin fractions that were observed after increasing the temperature for isothermal treatment (Figure 2) also point at a heterogeneous heat denaturation of glycinin at pH 7.6.

At pH 3.8, which is a pH more representative for conditions occurring in foods than pH 7.6, it is found that glycinin also denatures heterogeneously because 1) it is possible to obtain glycinin fractions that are denatured to different extents (Figure 1) and 2) the heat denaturation temperatures of the purified native glycinin fraction increases as the temperature of isothermal denaturation increases (Figure 2). It is likely that, in analogy to pH 7.6, the heterogeneity in heat denaturation at pH 3.8 is linked to different thermal stabilities of the genetic variants of glycinin. However, the situation at pH 3.8 is more complicated than at pH 7.6, because this fraction does not only contain glycinin in the 11S form but also in the 7S form, which denatures at lower temperatures than the 11S form (Lakemond et al., 2000a). Gel electrophoresis showed that at pH 3.8 the 7S form contained the acidic polypeptide $A_3$ and the 11S form not (results not shown), which is in line with the results of others (Utsumi et al., 1987; Yagasaki et al., 1997). This means that a relation exists between aggregate size and subunit type at pH 3.8. It was not studied if the order of denaturation is equal to pH 7.6.

Since heating took place for 16 hours it is expected that in this study glycinin is deamidated during isothermal treatment. For example, significant deamidation levels are reached after heating for only 1 hour at 95 °C (22%) (Matsudomi et al., 1985) and for 9 hours at 70°C (25%) (Wagner and Guéguen, 1995). For glycinin, unlike for many other globular proteins, the denaturation temperatures are that high (Danilenko et al., 1987; Lakemond et al., 2000a) that denaturation always coincides with deamidation. Therefore, in this article deamidation is considered to be part of the denaturation process and has not been studied separately. Figure 2 shows that heating of glycinin for 16 hours at 52-68°C did not influence the denaturation temperature of 11S glycinin. In literature also no evidence was found for a relation between

increasing denaturation temperatures and increasing deamidation levels (Catanzano et al., 1997; Lupano, 1994).

The genetic variant composition of glycinin is of industrial relevance since for example Mori et al. (1982) and Tezuka et al. (2000) found links between gelation properties and subunit composition. The fact that it is known that 7S glycinin denatures at lower temperatures (over 10°C difference) than 11S glycinin combined with the fact that glycinin associates into 7S or 11S aggregates depending on the types of genetic variants present (Utsumi et al., 1987; Yagasaki et al., 1997) possibly explains the relation that is found between subunit composition and gelation.

### 4.3 Conclusion

It is concluded that the thermal stability of the different genetic variants of glycinin increases at pH 7.6 in the order G2/G3/G1 $< A_4 <$ G5 $<$ G4 (without $A_4$). At pH 3.8 the genetic variants also display a different thermostability. Therefore, glycinin cannot be considered as a homogeneous group of proteins as has been the case in many heat denaturation studies.

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Chapter 6

# The effect of pH on heat denaturation and gel forming properties of soy proteins[1]

**Abstract**

This study is focussed on the influence of pH on the gel forming properties of soy protein isolate and purified glycinin in relation to denaturation and aggregation. At pH 7.6 more fine-stranded gels were formed characterised by low G' values, and a smooth, slightly turbid appearance, whereas at pH 3.8 more coarse gels were obtained with a high stiffness and a granulated, white appearance. Low G' values, as found at pH 7.6, correlate with a high solubility of glycinin and soy protein isolate (ca. 50%) after heating at low protein concentration. At pH 3.8 all protein precipitated upon heating, which correlates with relatively high G' values. The role of β-conglycinin during gelation of SPI seems to be minor at pH 7.6, which is indicated by the fact that, in contrast to pH 3.8, notable gel formation did not start upon heat denaturation of β-conglycinin. Furthermore, the mechanism of gel formation seems to be affected by pH, because at pH 7.6, in contrast to pH 3.8, the disulphide bridge between the acidic and the basic polypeptide of glycinin seems to be broken upon heating.

keywords: soy protein isolate; glycinin; rheology; network

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## 1 Introduction

Soy proteins are applied in a wide range of food products. The ability of soy proteins to form a gel upon heating is generally considered as an important functional property. Conditions during gel formation in food products vary greatly due to variations in pH, salt content, the combination of ingredients, etc. This will certainly affect the properties of the formed gel.
The water extractable soy proteins (about 90% of total protein present in soybeans) consist for 25-35% of glycinin and for 20-35 % of β-conglycinin (Yamauchi et al., 1991). Glycinin is composed of acidic (ca. 38 kDa) and basic polypeptides (ca. 20 kDa) (Staswick et al., 1981). Each acidic and basic polypeptide is linked by a single disulphide bridge, except for the acidic polypeptide $A_4$ (Staswick et al., 1984). At ambient temperatures and at pH 7.6 glycinin forms hexameric complexes (11S), while at pH 3.8 it is mainly present in trimeric complexes (7S) (Wolf et al., 1958). Three different β-conglycinin subunits are known (α', α, and β with molecular masses of ca. 65 kDa, 62 kDa and 47 kDa, respectively). Generally, β-conglycinin forms a trimer (7 possible combinations), which corresponds to the 7S form, however, also a hexameric form (9S) has been reported (Koshiyama, 1983).
Heat denaturation and gel formation of soy proteins have been extensively studied. Heat denaturation is often a prerequisite for gel formation. Glycinin has a denaturation temperature of 90°C at neutral pH and an ionic strength of 0.25 M, whereas β-conglycinin already unfolds at 74°C. Reducing the ionic strength lowers the denaturation temperatures (Hermansson, 1986; Damodaran, 1988). Gel characteristics of pure glycinin and β-conglycinin are affected by ionic strength (Utsumi and Kinsella, 1985; van Kleef, 1986), heating temperature (Nagano et al., 1994a) and pH during heating (van Kleef, 1986; Nagano et al., 1994b). For soy protein isolate it is also known that pH, temperature, and ionic strength affect gel formation (Catsimpoolas and Meyer, 1970; Bau et al., 1985; Utsumi and Kinsella, 1985; Puppo et al., 1995; van Kleef, 1986).
Generally, two different types of gel networks can be distinguished, fine-stranded and coarse networks (Tombs, 1974). In fine-stranded gels the proteins are moderately unfolded and attached to each other as a "string of beads". Coarse gels are formed by random aggregation of the proteins to clusters, which aggregate to thick strands (Hermansson, 1986). The type of gel that is formed depends on the conditions during gel formation.
Most studies on gel formation by soy proteins are limited to pH 7.6. The pH value of 7.6 is less relevant for the pH values generally occurring in food products (pH 3-7). This study is, therefore, focussed on the influence of pH on the gel forming properties of soy proteins in relation to denaturation. Furthermore, the effect of heating on protein aggregation/precipitation

is studied in order to clarify which polypeptides/subunits participate in network formation. Both glycinin and soy protein isolate are studied in order to obtain information about the role of glycinin in the soy protein isolate network.

## 2 Materials and methods

### 2.1 Isolation of soy proteins

Two different protein preparations were used for the experiments: purified glycinin and soy protein isolate, which consists for about 60% of glycinin and 30% of β-conglycinin. Glycinin was isolated from Williams'82 soybeans (harvest 1994) by isoelectric precipitation at pH 6.4 as described by Lakemond et al. (2000a). The purified glycinin was resuspended (12 mg $ml^{-1}$) in a 35 mM potassium phosphate buffer with 0.4 M NaCl and stored at −20°C. Soy protein isolate (SPI) was prepared from mildly treated, defatted PDI 80 soy flakes (Cargill BV, 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 $CO_2$/soy flakes was 1:4) to prevent heat denaturation of the proteins. The flour was suspended in a 100 mM Tris-HCl buffer of pH 8.0 in a 1:10 ratio (w/v) and stirred for one hour at room temperature. After removal of the insoluble parts by centrifugation (30 min; 12000*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 min; 12000*g*; 10°C). The precipitate obtained was washed twice with a 10 mM sodium acetate buffer at pH 4.8 in a 1:8 ratio (w/v) and was freeze-dried afterwards. This material had a protein content (N x 6.25) of 97% and will be referred to as SPI.

### 2.2 Preparation of protein dispersions

Prior to DSC (section 2.3) and solubility experiments (section 2.5), purified glycinin was dialysed at 20°C against a 35 mM potassium phosphate buffer containing 0.1 M NaCl (pH 7.6, ionic strength 0.2). For experiments carried out at pH 3.8 or 5.2, the pH was subsequently adjusted with 0.1-5 M HCl. For gelation experiments (section 2.4), the purified glycinin was dialysed at 20°C against double-distilled water, freeze-dried (97% of protein) and subsequently dispersed in 35 mM potassium phosphate buffers with 0.1 M NaCl of pH 7.6 or 3.8 (ionic strength is 0.2). At pH 5.2, the gelation experiment could not be performed due to sedimentation of the protein prior to heating. After adjusting the pH the glycinin dispersions were stirred for 1.5 hours to enhance protein dissolution.

SPI dispersions were prepared by suspending the freeze-dried SPI in 0.2 M NaCl solutions at higher concentrations than required for the experiments. After stirring for one hour at 4°C 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, at 4°C, to enhance protein dissolution. Finally, the SPI dispersions were diluted by adding salt solution to obtain the desired protein concentration. Preparation of the SPI dispersions was performed at 4°C to prevent possible proteolysis by endogenous enzymes. Prolonged incubation of glycinin samples at 20°C did not result in any detectable proteolytic digestion as was tested by SDS-PAGE.
The use of different combinations of salts to reach the same ionic strength may have an effect on functional properties, but we expected a negligible effect compared to the effects of pH at the ionic strength studied.

### 2.3 Differential scanning calorimetry (DSC)

Protein heat denaturation was monitored by differential scanning calorimetry in a micro-DSC (Setaram, France). The protein concentration of glycinin solutions was 0.3% (w/v), for SPI dispersions the concentration was 12% (w/w). The stainless steel vessels contained 0.9 ml of these samples. The samples were scanned from 20 to 115°C at a scanning rate of 1 K $min^{-1}$ 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 $T_{max}$, the peak denaturation temperature, the temperature of maximum heat flow was taken.

### 2.4 Gelation

Gel formation of glycinin (10% (w/w)) and SPI (12% (w/w)) dispersions was followed by dynamic measurements in a Bohlin CVO rheometer using the smooth concentric cylinder geometry C14 (content 3 ml) for glycinin and the serrated C25 (content 13 ml) for SPI. 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 $s^{-1}$.
To prevent evaporation of water, a thin layer of soy oil was put on top of the samples. To induce gel formation, samples were consecutively heated from 20 to 95°C at a heating rate of 1 K $min^{-1}$, kept at 95°C for 30 min (glycinin) or 60 min (SPI), and cooled to 20°C at a cooling rate of 1 K $min^{-1}$. The temperature at which gelation starts, the gelation temperature, is defined as the temperature at which the storage modulus, G', started to increase over 0.5 Pa $min^{-1}$.

In order to study macroscopic appearance of the protein gels, glycinin (10% (w/w)) and SPI (12% (w/w)) dispersions were heated in closed glass tubes (Kimax culture tube, Kimble glass Inc., USA) with the same temperature profile as for the dynamic measurements.

### 2.5 Determination of "solubility"

Glycinin samples (12 mg $ml^{-1}$) were heated from 20 to 98°C at a rate of 1 K $min^{-1}$, kept at 98°C for 30 min and subsequently cooled to 20°C. Next, the glycinin samples were centrifuged for 5 min at 20°C at 15800*g*. The protein content of the supernatants was determined in triplicate with the Bradford assay (Bradford, 1976) using BSA (A-4503, Sigma, USA) as standard. SPI dispersions (10 mg $ml^{-1}$) were heated from 20 to 95°C at a rate of 1 K $min^{-1}$, kept at 95°C for one hour, and cooled at a rate of 1 K $min^{-1}$ to 20°C. The heated dispersions were centrifuged for 30 min at 5°C at 32000*g*. The protein content of the dispersion (total protein) and of the supernatant (dissolved protein) was determined in duplicate by a micro-Kjeldahl method using a Kjeldahl factor of 6.25. Solubility was defined as (dissolved protein/total protein) x 100%.

The protein composition of the supernatants of the heated protein dispersions, was determined by SDS-PAGE at both reducing and non-reducing conditions on a Phast System (Pharmacia, Sweden) according to the instructions of the manufacturer. 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 Results and Discussion

Below we will discuss successively the relation between heat denaturation and gel formation, the stiffness and appearance of the gels formed and protein solubility after heating. Finally the possible relation between these phenomena is discussed.

### 3.1 Heat denaturation and gelation

Figure 1 shows DSC-thermograms of glycinin and SPI at pH 3.8 and 7.6. The thermogram of glycinin at pH 7.6 shows one endothermic transition around 88°C due to denaturation. At pH 3.8, two endothermic transitions were observed at 68°C and 82°C. At this pH, glycinin was both present in the 11S or hexameric form (360 kDa), and in the 7S or trimeric form (180 kDa) (Lakemond et al., 2000a). The 7S form unfolds at the lowest temperature, the 11S form at the highest temperature (Utsumi et al., 1987; Lakemond et al., 2000b). The thermograms of SPI show two endothermic transitions at both pH values caused by heat denaturation of β-

conglycinin at the lowest temperature and by glycinin at the highest temperature (Hermansson, 1978). In SPI, the 7S form of glycinin is also expected to be present at pH 3.8. This might be indicated by the asymmetrical wide shape of the transition at the highest temperature, but it is also possible that denaturation of the 7S form of glycinin coincides with denaturation of β-conglycinin. Lowering pH from 7.6 to 3.8 causes the denaturation temperature to shift to lower values. This was also observed by Hermansson (1978) for SPI, and by Nagano et al. (1994b) for β-conglycinin. At pH 3.8, the charge distribution differs from that at pH 7.6, and apparently leads to differences in quaternary structure and protein stability.

Figure 1 also shows that heat denaturation temperatures of purified glycinin were lower than heat denaturation temperatures of glycinin in the SPI. Additional DSC experiments (results not shown) suggested that β-conglycinin or other components in SPI have a stabilising effect on glycinin, and that also protein concentration partly influenced denaturation temperatures. The difference in soybean variety was not responsible for the observed difference in denaturation temperatures (results not shown).

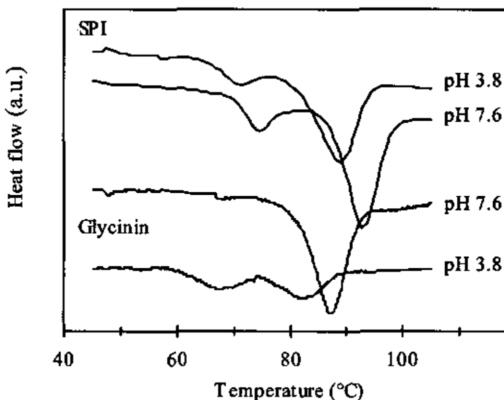


**Figure 1** DSC-thermograms of 0.3% (w/v) glycinin and 12% (w/w) soy protein isolate (SPI) dispersions at pH 7.6 and 3.8 and ionic strength of 0.2 M. Scanning rate was 1 K $min^{-1}$.

To study if heat denaturation is a prerequisite for gel formation at all pH conditions, the onset denaturation temperatures, $T_o$, were compared with gelation temperatures as a function of pH for both glycinin and SPI (Figure 2). For SPI (Figure 2A), gel formation coincided with heat denaturation of β-conglycinin at pH 3.8 and 5.2, whereas at pH 7.6 gel formation started simultaneously with denaturation of glycinin. Figure 2B shows that gelation of glycinin

always started at a temperature above that for heat denaturation of the 11S form of glycinin, indicating that the 7S form did not seem to give gel formation at the applied concentration. Apparently, β-conglycinin plays a more important role in gel formation of SPI at low pH than at high pH. The minimal protein concentration for gel formation by β-conglycinin might be higher at pH 7.6 than at lower pH possibly due to a different aggregation mechanism or a different type of gel. Another possibility is that glycinin has to denature before β-conglycinin can aggregate with glycinin polypeptides and form a gel, but results from Nagano (Nagano et al., 1992; Nagano et al., 1994a; Nagano et al., 1994b) contradicted this hypothesis.

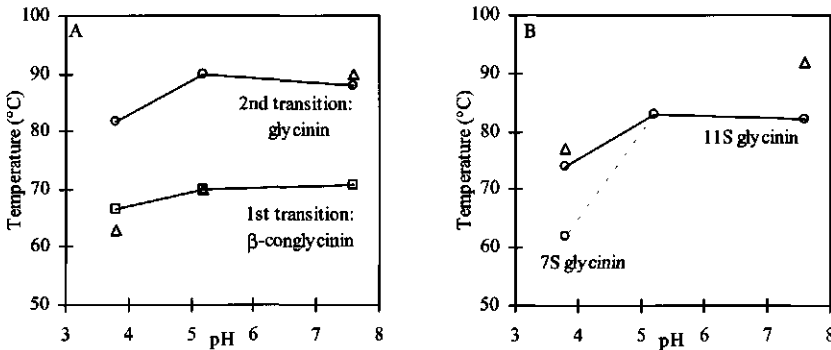


**Figure 2** Gelation (△) and onset denaturation (O, □) temperatures of SPI (A) and glycinin (B) as a function of pH at ionic strength of 0.2. Protein concentration of glycinin samples was 10% (w/w) and 0.3% (w/v) respectively, protein concentration of SPI was 12% (w/w).

### 3.2 Stiffness and appearance of soy protein gels

An indication of the type of network that is formed is given by the appearance of the gels. At pH 7.6 the gels were slightly turbid and smooth, whereas the gels at pH 3.8 were white and granular. The white colour of the gels indicates that they consisted of particles of 1 μm or larger. Considering the granular appearance, the particles were probably even larger. This implies that at pH 3.8, coarse gels with large aggregates, thick strands, and large pores were formed. The pH 7.6 gels consisted of much smaller aggregates and probably had a more fine-stranded network structure with small pores. This agrees with results of Hermansson (1985) and Puppo and Añón (1998) who visualised soy protein gel structure by electron microscopy.

Figure 3 shows the elastic or storage modulus, G', after completion of the temperature cycle for pH 3.8 and 7.6 for glycinin and SPI. G' is a measure of the stiffness or, in other words, the

resistance to deformation of the gels. Gels at pH 3.8 had higher G' values than gels at pH 7.6 for both glycinin and SPI. Similar results were obtained by van Kleef (1986) for SPI and glycinin and by Nagano et al. (1994b) for β-conglycinin. The difference in stiffness might be related to the earlier onset of gelation at pH 3.8 or to the formation of different types of network.

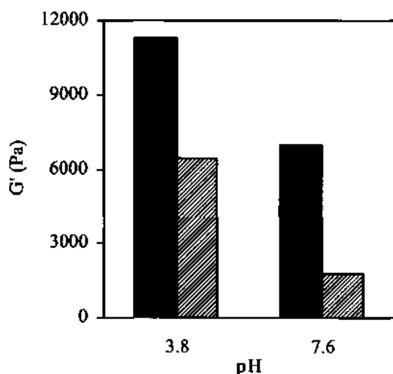


**Figure 3** Storage modulus, G', of 10% (w/w) glycinin gels (black bars) and 12% (w/w) SPI gels (hatched bars) after a complete temperature cycle at pH 7.6 and 3.8 ionic strength of 0.2.

### 3.3 Protein "solubility" after heating

In order to identify which polypeptides or subunits participate in network formation, the effect of heating on solubility and protein composition of 1% protein dispersions was studied. Table 1 shows the amount of protein that remains dissolved after heating together with the composition of the dissolved fractions as determined by gel-electrophoresis. Solubility in these experiments refers to proteins that were not aggregated or were present in aggregates too small to sediment upon centrifugation. In the case of glycinin, the sediment would have consisted of particles larger than approximately 0.5 μm as calculated by using the Stokes equation. For SPI, the precipitated particles were approximately 0.2 μm or larger.

For glycinin, the "solubility" at pH 7.6 was reduced from 100% to about 50% after heating. The majority of the acidic polypeptides remained in solution, whereas all the basic polypeptides precipitated on heating. This implies disruption of the S-S bridge and of the non-covalent interactions between the acidic and basic polypeptide, probably due to reshuffling of the S-S/SH groups. This is in agreement with earlier observations (Hashizume et al., 1975; Yamagishi et al., 1987). In SPI, however, part of the basic polypeptides remained in solution at pH 7.6. Damodaran and Kinsella (1982) claimed that complete precipitation of the basic

polypeptides is prevented by the formation of a heat-induced complex between basic polypeptides of glycinin and β subunits of β-conglycinin. At pH 3.8 solubility was very low; hence, any subunits or polypeptides could not be detected in the supernatants for both glycinin and SPI. Since protein solubility of SPI at pH 3.8 was already low prior to heating, results obtained at pH 3 were included in the table. At this pH, solubility was high (76%) prior to heating. Heating caused a small decrease in protein solubility mainly due to precipitation of the α', α and β subunits of β-conglycinin. At these low pH values, it is likely that the disulphide bridge between acidic and basic polypeptides was not broken, which is confirmed by the fact that at non-reduced conditions the AB subunit was found to be intact (no further results given). This implies that the denaturation mechanism at pH 3.8 is different from that at pH 7.6. This may be due to the S-S/SH interchange reaction occurring more readily around pH 7 than around pH 3-4.

**Table 1** Protein "solubility" of 1.2% (w/v) glycinin and 1% (w/v) SPI dispersions after heating as a function of pH, and the composition of the dissolved fraction as determined by reduced SDS-PAGE.

| pH | Glycinin | | | SPI | | | | |
|---|---|---|---|---|---|---|---|---|
| | Solubility (%) | Composition[a] | | Solubility (%) | Composition[a] | | | |
| | | A | B | | A | B | α, α' | β |
| 7.6 | 52 | ++ | - | 54 | ++ | + | ++ | ++ |
| 3.8 | 5 | - | - | 7 | - | - | - | - |
| 3.0 | n.d. | n.d. | n.d. | 69 | ++ | +++ | - | + |

[a] Quantification is based on visual evaluation and comparison of the SDS-PAGE profiles; A, B acidic and basic polypeptides of glycinin; α, α', β, subunits of β-conglycinin; -, 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; n.d. = not determined.

### 3.4 General discussion

The results obtained indicate that gel structure and gelation mechanism at pH 7.6 are different from those at pH 3.8, for both glycinin and SPI. At pH 7.6, more fine-stranded gels were formed with low G' values, and a smooth and slightly turbid appearance, whereas at pH 3.8 more coarse gels were obtained with a high stiffness, and a granulated and white appearance. At pH 3.8 the proteins tend to aggregate to a larger extent, resulting in more coarse gels, whereas at pH 7.6 the proteins were less aggregated, resulting in more fine-stranded gels. Since a high G' value correlates with a low solubility after heating at low protein

concentrations, our hypothesis is that only subunits or polypeptides that precipitate upon heating participate in the network that is formed at higher protein concentrations. It is expected that all protein that remains in solution upon heating is not incorporated in the network. That would mean that in the more coarse gel at pH 3.8 all of the protein is incorporated in the network, and in the more fine-stranded gel at pH 7.6 only a part.

There are no indications for basic differences in aggregation and gelation mechanism between glycinin and soy protein isolate. The role of β-conglycinin during gelation seems to be minor at pH 7.6, which is supported by the fact that G' of the SPI suspension is significantly lower than G' of glycinin suspensions (glycinin concentration in SPI gels is 6%), and secondly that no gel formation was observed as long as heat denaturation was restricted to β-conglycinin. However, the presence of β-conglycinin in SPI prevents precipitation of the basic subunits in SPI after heat treatment at pH 7.6, which could also lead to lower G' values according to our hypothesis. At pH 3.8 the role of β-conglycinin is more pronounced since notable gel formation started immediately after heat denaturation of β-conglycinin in SPI.

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Chapter 7

# Discussion

## 1 Introduction

Many studies have been performed on glycinin heat denaturation, aggregation and gel formation (e.g. Hashizume et al., 1975; Hashizume and Watanabe, 1979; Mori et al., 1981a; Koshiyama et al., 1981; Mori et al., 1982; Nakamura et al., 1984a; Nakamura et al., 1984b; Nakamura et al., 1985; Hermansson, 1985; Hermansson, 1986; Mori et al., 1986; Yamagishi et al., 1987; Mori et al., 1989). However, relationships between molecular structure and functional properties of this soy protein are not resolved into detail. The goal of this work was to study solubility and gel formation of the major soy protein glycinin in relation to its structural characteristics both at ambient temperatures and after heat denaturation. First, the pH and ionic strength conditions were varied in order to modify the structure of glycinin at ambient temperatures after which the structural aspects were studied in detail (Chapter 2). Most studies reported in literature have been performed at one "standard" condition, which is pH 7.6 and ionic strength I = 0.5. The knowledge of the influence of glycinin structure on functional properties is, therefore, limited to this condition. Furthermore, this standard condition is less relevant for the conditions present in food products (pH 3-7 and I = 0.02-0.2). Therefore, a further part of this work is to study the influence of pH and ionic strength on glycinin structure and its functional properties, in order to be able to compare heat denaturation of glycinin under standard literature conditions with conditions generally occurring in food products.
In this chapter the main findings obtained during these PhD studies are discussed.

In this work three ionic strengths and three pH conditions were studied. I = 0.03 and 0.2 were chosen because of their relevance for food products. The ionic strength of 0.5 was included for comparison with literature data. The pH values 3.8 and 5.2 were studied since these pH's are relevant for food products and pH 7.6 was studied for comparison to literature. One complication that was encountered while studying glycinin at ambient temperatures in Chapter 2 was the (expected) decrease in solubility in the pH range of about 4-7 for I = 0.03 and 0.2. The number of techniques that is suitable for studying insoluble protein is limited, as no spectroscopic techniques are possible. At pH 5.2 it was not only impossible to use spectroscopic techniques, but it was also not possible to investigate gel formation, since we found that at this pH gels were not homogeneous. Two different layers could be distinguished

in the glycinin gels; at I = 0.03 and 0.2 a turbid gel layer was observed with the solvent on top; at I = 0.5 two gel layers could be distinguished of which the upper was less turbid than the lower one (results not shown).

## 2 Glycinin nativity

The results presented in Chapter 2 show that pH and ionic strength modulate glycinin structure at all three structural levels (secondary, tertiary and quaternary). Denaturation of protein is defined as any modification in conformation (secondary, tertiary and quaternary structure) leaving primary structure intact (Tanford, 1968; Chapter 1). Since glycinin is a storage protein that is present in an aggregated non-soluble form in the protein bodies of the soybean seeds, it can be stated that solubilisation at any pH and ionic strength denatures glycinin even at ambient temperatures. In theory, the structure of glycinin within the protein body should be referred to as the native form. However, only after extraction the structure can be studied. It is, therefore, an arbitrary assumption to define the 11S form of glycinin at pH 7.6 and I = 0.5 as a native like structure as has been assumed in many literature studies (e.g. reviewed in Brooks and Morr (1985)). This statement is rather based on historically used extraction conditions than on generally accepted criteria, as the conditions used for extraction were also the (first) conditions used for analyses of the quaternary structure.

## 3 Glycinin quaternary model

The widely used model of Badley et al. (1975) (Figure 1a) that is also presented in Chapter 1 proposes one glycinin molecule (11S) to consist of six subunits which each consist of an individual acidic and basic polypeptide. Throughout literature the 11S structure of glycinin is referred to as the glycinin molecule, probably because it is mainly present in the 11S form at pH and ionic strengths conditions that are used in all isolation methods. However, depending on pH and ionic strength it may also occur in the 7S and 3S form. This definition is, therefore, arbitrary. In this thesis we have adapted our terminology to terminology generally used in literature for uniformity reasons. However, we do realise that the glycinin subunit should be referred to as the glycinin molecule and the 7S or 11S form as its trimer or hexamer, since each subunit is encoded by a single gene (Staswick et al., 1984; Fischer and Goldberg, 1982; Scallon et al., 1985).

The model for 11S glycinin of Badley et al. (1975) (Figure 1a) can be extended based on the work that is described in Chapter 2. First, the basic and acidic polypeptides have different

sizes (Staswick et al., 1981) resulting in the radius of the acidic polypeptide being about 1.25 times larger than the radius of the basic polypeptide. This is not clear from Badley's model. Secondly, it was found that the basic polypeptides of glycinin are predominantly present at the interior of the glycinin molecule at pH 7.6. Based on these findings it is suggested that the model that is proposed by Plietz et al. (1983) for sunflower and rapeseed 11S globulins is also valid for soy glycinin (Figure 1b). This model is to our opinion more truthful than the model of Badley et al. (1975) and can lead to a better understanding of glycinin structural rearrangements, as has been described in Chapter 2; At pH 7.6 the basic polypeptides shift more to the exterior of the glycinin complex when the ionic strength is lowered from 0.5 to 0.2 and 0.03. This is schematically represented in Figure 1c. The relative arrangement is at pH 3.8 expected to be different from that at pH 7.6. However, this was not studied since the method used was not suitable for low pH values.

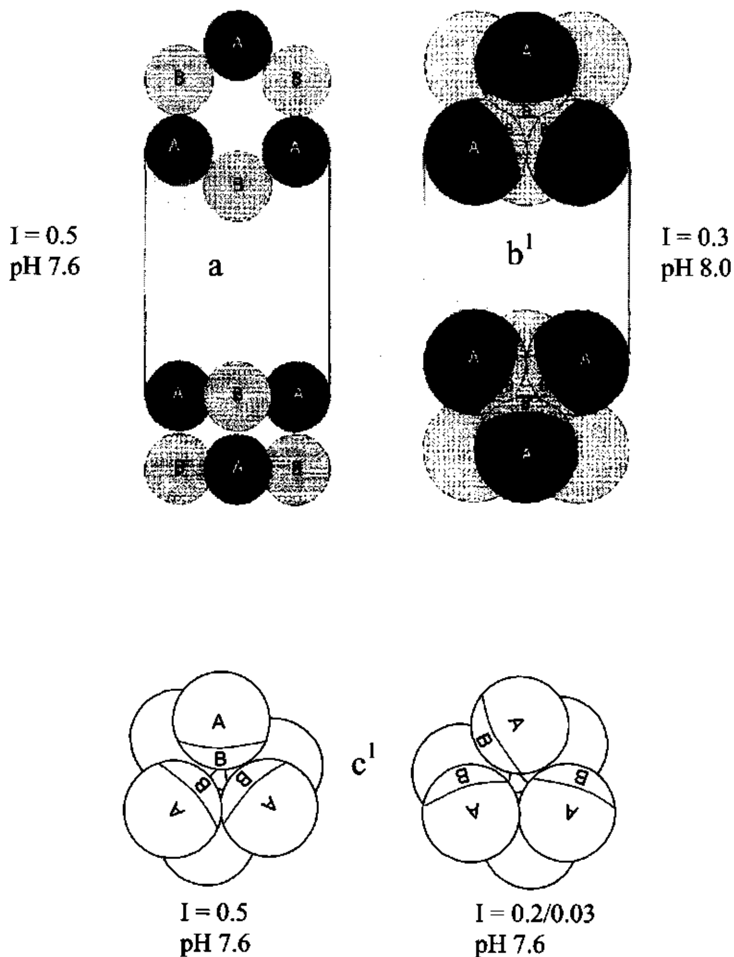


**Figure 1** Schematic representation of 11S glycinin (Modified model of Badley et al. (1975) (a); modified model of Plietz et al. (1983) (derived from Marcone et al. (1998) (b) extended model of Plietz et al. (1983) based on results from Chapter 2 (c).

[1] In only three out of the six glycinin subunits the acidic and basic polypeptides are drawn.

## 4 Structure of glycinin in relation to denaturation aggregation and gel formation

The results presented in this thesis are summarised in Table 1. It shows that at pH 7.6 and I = 0.5 glycinin forms hexameric complexes (11S), whereas at pH 3.8 and I = 0.03 glycinin exists as trimers (7S). Intermediate situations are obtained by modulation of the pH and the ionic strength. The dissociation at quaternary level correlates with an increase of non-structured protein at a secondary and tertiary level (Chapter 2). Since glycinin structure can be modified at ambient temperature by changing pH and ionic strength conditions it is likely that also the mechanisms proposed for aggregation, precipitation and gelation at standard conditions (I = 0.5 an pH 7.6) (Chapter 1) are not valid for food conditions. This can indeed be derived from the results presented in Chapter 3 and 4.
The changes in glycinin structure at ambient temperature correlate with changes as a result of heat denaturation; At pH 3.8, at which pH glycinin is mainly present in the 7S form, the protein is more unfolded at a tertiary and secondary level, the denaturation temperatures are lower than at pH 7.6, as are also the gelation temperatures (except for I = 0.03). Furthermore, the acidic polypeptides remain soluble at low protein concentration and form the non-network protein at high protein concentration. The basic polypeptides are insoluble at low protein concentration and form the network protein at high protein concentration (as is schematically shown in Figure 2). This difference between pH 7.6 and 3.8 can be explained by a disruption of the disulfide bonds at pH 7.6, as is in line with the proposed mechanism presented by Mori et al. (1981a), while at pH 3.8 acidic and basic polypeptides seem to stay linked. For I = 0.2 and 0.5 the water holding capacity and the gel stiffness at high protein concentration differ clearly between pH 7.6 and 3.8. From Chapter 4 it is clear that water holding capacity and gel stiffness are not directly correlated to gel coarseness. Also, no clear correlations exist between glycinin structure at ambient temperatures (Chapter 2) and water holding capacity, stiffness and gel coarseness.

**Table 1** Summary of the selected results obtained in Chapter 2 ,3 and 4

| Ch. | property studied | conditions under which glycinin was studied | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | native/ denatured | glycinin concentration[1] | | pH 3.8 I = 0.03 | pH 3.8 I = 0.2 | pH 3.8 I = 0.5 | pH 7.6 I = 0.03 | pH 7.6 I = 0.2 | pH 7.6 I = 0.5 |
| 2 | quaternary structure | native | low | major[3] | 7S | 7S | 7S and 11S | 11S | 11S | 11S |
| | | | | minor[3] | -[5] | 11S | -[5] | 7S | -[5] | -[5] |
| 2 | secondary structure (% non-structured) | native | low | | 38 | 32 | 25 | 19 | 12 | 17 |
| 2 | solubility (%) | native | low | | 90 | 100 | 100 | 100 | 100 | 100 |
| 3 | solubility (%) | denatured | low | | 72 | 2 | 4 | 54 | 52 | 53 |
| 4 | non-network protein (%) | denatured | high | | ?[6] | 4 | 3 | 49 | 31 | 48 |
| 4 | gel coarseness | denatured | high | | -- | + | ++ | - | + | ++ |
| 4 | gel water holding capacity (%) | denatured | high | | 100 | 62 | 45 | 94 | 79 | 70 |
| 4 | gel stiffness (kPa) | denatured | high | | 0.4 | 11.2 | 4.9 | 0.3 | 6.9 | 1.1 |
| 4 | gel appearance | denatured | high | | transparent | turbid, granular | turbid, granular | semi-transparent | turbid, smooth | turbid, smooth |
| 3 | denaturation temperature DSC (°C) | | low | | 71/85 | 67/82 | 85[2] | 78 | 87 | 94[2] |
| 4 | denaturation temperature DSC (°C) | | high | | 68/84 | 69/84 | 71/86 | 84 | 91 | 91 |
| 4 | gelation temperature (°C) | | high | | 95 | 77 | 75 | 95 | 92 | 91 |
| 3 | enthalpy (J/g) of the endothermic transition | | low | | 25[4] | 30[4] | 10[4] | 39 | 37 | 41 |

[1] low protein concentration generally refers to a concentration ≤ 1.2 %; high protein concentration refers to a concentration of 10% or 15%, a concentration at which gelation takes place; [2] also an exothermic transition was observed; [3] Major and minor components are represented; [4] Different transitions are not well separated; [5] not present; [6] could not be determined since it was not possible to centrifuge moist out of the gel

Based on the results obtained from Chapter 4 we present a tentative schematic representation of glycinin gel structure at pH 3.8 compared to pH 7.6 (Figure 2). This scheme does only represent the gel structure at I = 0.2 and 0.5 and is not representative for I = 0.03, since gel structure is fine stranded at both pH values at I = 0.03. Based on the visual appearance and the water holding capacities the strands are likely more fine stranded at pH 7.6 than at pH 3.8, as is visualised in Figure 2. Furthermore, the above-described difference in network and non-network protein for pH 3.8 and 7.6 is visualised in Figure 2. In Chapter 1 the gelation model of Nakamura et al. (1984b) was introduced, which suggested the formation of a string of beads model for glycinin at I = 0.5 and pH 7.6. Our schematic representation is different from that of Nakamura and co-workers.

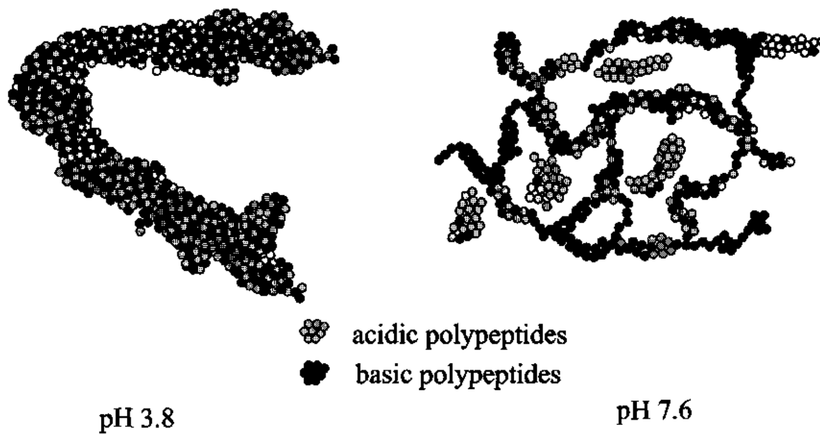


**Figure 2** Schematic presentation of a possible gel structure for glycinin for I = 0.2 and 0.5.

With respect to Table 1, it is found that the denaturation temperatures obtained at low protein concentration (Chapter 3) compared to those obtained at high protein concentration (Chapter 4) are not equal. This change in denaturation temperatures with varying protein concentration is only observed when association or dissociation reactions accompany the process of denaturation (Sturtevant, 1987). The enthalpies observed for unfolding of the glycinin at different conditions differ clearly. It is noted that the enthalpy values are lower at pH 3.8 than at pH 7.6 for every condition studied. Since it is clear from Chapter 3 and 4 that the protein structure after heat denaturation differs between the various conditions, it is not possible to interpret these differences in enthalpy in terms of structural differences before heating.

Another striking difference observed when comparing the DSC results obtained at low with those obtained at high protein concentration is that at low protein concentration exothermic transitions are observed (at I = 0.5 at all three ionic strengths) while these are absent at high protein concentration. These results are in line with those obtained by Marshall and Zarins (1989). They attribute the occurrence of the exothermic transition to aggregation of the basic polypeptides. However, it was found in this work that the basic polypeptides aggregate both at low (Chapter 3) and at high protein concentration (Chapter 4). Furthermore the position of the exothermic transition at low protein concentration depends strongly on the heating rate (Chapter 2). At low scanning rates the exothermic transition even disappears completely, while it is likely that the basic polypeptides still aggregate. Since Marshall and Zarins (1989) observe that at I = 0.5 the exothermic transition occurs before the endothermic transition, they suggest that aggregation of the basic polypeptides occurs before denaturation takes place. Based on the results presented in this thesis their assumption is likely not valid, since the presence of the exothermic transition was found to occur after and not before the endothermic transition at an ionic strength of 0.5 (Chapter 3).

## 5 Genetic variants of glycinin

In Chapters 2-4 glycinin is studied as a homogeneous group of proteins, as is also assumed in many other studies. This approach has provided us with information about aggregation and gelation. However, we did not succeed in studying the heat denaturation and aggregation mechanism in more detail. We found that it was not possible to find a temperature at which the complete denaturation process took place within a measurable timespan (the denaturation process went on slowly; unpublished results). This led to the hypothesis that glycinin, which actually consists of at least five genetic variants/subunits, does not denature homogeneously. It is indeed clear from the work presented in Chapter 6 that these genetic variants denature heterogeneously. It was found in literature (Yagasaki et al., 1997) and also in this thesis that glycinin associates into the 7S or 11S form depending on the type of glycinin subunits present (Chapter 5). The precise subunit composition depends on the soybean variety used (Mori et al., 1981b). Thus, by using different soybean varieties it should be possible to change the 7S/11S equilibrium and thereby the functional properties of glycinin, as was found by Nakamura et al. (1984a). All glycinin subunits dissociate from the 11S form into the 7S form as pH and ionic strength are lowered (Chapter 2). It is our hypothesis that this dissociation process is also dependent on the genetic variant, since it was found by gel electrophoresis that

at pH 3.8 and I = 0.5 the 7S form of glycinin contained the acidic polypeptide $A_3$ and the 11S form did not (Chapter 5).

It can be expected that the heat denaturation and aggregation mechanism of glycinin and the types of bonds involved can be resolved more extensive when heat denaturation of only one genetic variant is studied. To obtain the genetic variants separately the purification procedure, anion exchange chromatography, used in Chapter 4 is, however, not sufficient since we found that enriched fractions are obtained rather than pure fractions. The anion exchange method could possibly be optimised by modulating the elution conditions. Another method, which was studied by us but not presented in the previous chapters, is chromatofocussing. This method was also not successful since the shape of the chromatogram changed inexplicable with time (1, 4, 16 hours of incubation), even though the sample was dissolved in 6M urea. Another option to obtain pure genetic variants of glycinin is to purify it from soybean varieties that express only one genetic variant of glycinin or to express one variant in a suitable expression system.

It should be reminded that the precise number of glycinin variants is not exactly known. Indications exist that more than five different variants (subunits) of glycinin are present (Mori et al., 1981b; Lei et al., 1983; Nielsen et al., 1989; Chapter 5). Nielsen et al. (1989) suggest the presence of more than five genes, but it was also suggested that glycinin undergoes, like other 11S storage proteins, a complex series of posttranslational events (Dickinson et al., 1989). Some studies report that a small portion of glycinin is glycosylated (Wolf and Sly, 1966; Fukushima, 1968; Lei and Reeck, 1987), which is contradicted by others (Koshiyama and Fukushima, 1976).

The discussion about the number of glycinin variants complicates the determination of glycinin purity throughout this thesis and literature. The exact estimation of glycinin purity from SDS-PAGE gels remains arbitrary.

## 6 Industrial implications

Arrese et al. (1991) studied several commercial soy protein isolates, which were found to differ largely in their functional properties such as solubility, gelation and water binding capacity. From these results they concluded that the variation in functional properties was related to the degree of denaturation (also completely denatured commercial isolates were found). An example of the effects of processing on denaturation is given in Figure 3, where we varied heating time of a native protein isolate from 1 to 15 min at 80 °C. The nativity decreases upon increasing heating time. β-Conglycinin is denatured more progressively than

glycinin since the denaturation temperature chosen is closer to that of β-conglycinin than that of glycinin. Thus, for industrial purposes it is also possible to denature the different proteins (glycinin and β-conglycinin) to different extents to modify the functional properties. It should be reminded that the denaturation temperature of glycinin (Chapters 3 and 4) differs strongly on the pH and ionic strength conditions applied (differences in denaturation temperatures of over 20°C are observed).
The work presented in this thesis shows that, apart form varying the denaturation degrees, pH and ionic strength can also clearly modify glycinin structure and its functional properties (solubility and gel formation) (Chapter 2-6); Modifying pH and ionic strength alone results in different gel strengths (varying from 0.3 to 11.2 kPa; Chapter 4), different appearances (varying from turbid and granular to transparent; Chapter 4), different water holding capacities (varying from 45 - 100%; Chapter 4), and different solubilities (varying from 0 to 74 %; Chapter 3). Furthermore, the minimum protein concentration to obtain gel formation can also be varied by changing pH and ionic strength conditions (Chapter 4).
From the work of different authors (Mori et al., 1982; Nakamura et al., 1984a; Nakamura et al., 1985) it is clear that also the protein composition (the ratios of the different variants of glycinin present) influence its functional behaviour. Also, glycinins from different cultivars differ in their functional properties (Nakamura et al., 1984a). In Chapter 5 the heat denaturation behaviour of the genetic variants of glycinin is studied into detail, which gives a basic understanding of their behaviour during heat treatment and is, therefore, relevant for industrial processing.

Denaturation, aggregation and precipitation characteristics of purified fractions of individual soy proteins as a result of heating can be different from those of the individual soy proteins when heated within a soy protein isolate or a food matrix. Therefore, the research in Chapter 6 deals with the effect of pH on heat denaturation and gel forming properties of soy protein isolate compared to those of purified glycinin. This research is a combined work with Marianne Renkema, who performs a PhD project that was entitled "Functional properties of gels formed by soy protein and fractions thereof". The main aim of the latter project is to understand the factors determining the main properties of soy protein isolate gels. In Chapter 6 no indications were found for basic differences in aggregation and gelation mechanisms between glycinin and soy protein isolate, which means that the work performed on purified glycinin (Chapters 2-5) is a good base for predicting the effect of pH and ionic strength on the functional properties of soy protein isolates.

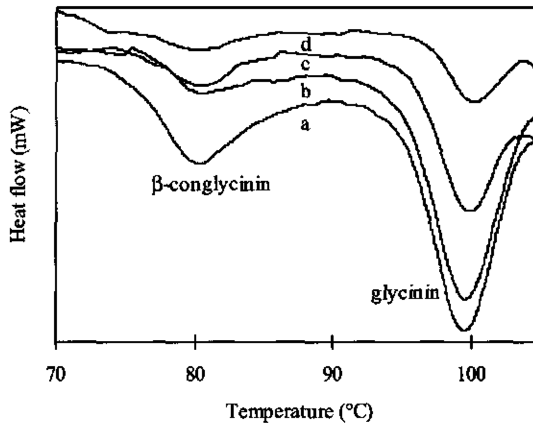


**Figure 3** DSC thermograms of soy protein isolate (SPI) heated at 80°C during different times (t =0 min (a); t = 1 min (b); t = 5 min (c); t = 15 min (d).

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# Summary


Despite all research performed on soy proteins in the last decades, much is still unknown about the relationship between the molecular structure of soy proteins and their functional properties. In this work we studied to which extent differences in glycinin structure lead to variations in functional properties (solubility and gel formation). Furthermore, we examined the effect of pH and ionic strength over a range covering conditions often studied in literature (I = 0.5 pH 7.6) and in addition conditions which are more representative for food (I = 0.02-0.2, pH 3-7).

Chapter 2 describes the relationships between structural properties of glycinin at a quaternary, tertiary and secondary folding level and its solubility under conditions representative for food products. When the ionic strength is lowered from 0.5 to 0.2 or 0.03, the basic polypeptides shift more to the exterior of the glycinin complex, as determined at pH 7.6 by labelling solvent-exposed lysines, supported by the study of the proteolytic action of clostripain on glycinin. This structural reorganisation caused the pH of minimal solubility to shift to higher values. Ultracentrifugational analysis shows that, at pH 7.6 and an ionic strength of 0.5, glycinin forms hexameric complexes (11S), whereas at pH 3.8 and at an ionic strength of 0.03 glycinin exists as trimers (7S). Intermediate situations are obtained by modulation of pH and ionic strength. The observed quaternary dissociation correlates with an increased amount of non-structured protein at a secondary level and with changes in tertiary folding as determined using circular dichroism. Tryptophan fluorescence shows no significant structural changes for different ionic strengths but demonstrates a more tightly packed fluorophore environment when the pH is lowered from 7.6 to 3.8.

The 7S/11S glycinin equilibrium at ambient temperatures influences heat denaturation (Chapter 2). The 7S form of glycinin denatures at a lower temperature than the 11S form, as demonstrated by a combination of calorimetric (DSC) and circular dichroism (CD) experiments (Chapter 3). At pH 7.6, at which glycinin is mainly present in the 11S form, the disulfide bridge linking the acidic and the basic polypeptide is supposed to be broken during heat denaturation. At pH 3.8 (glycinin has dissociated partly into the 7S form) and at pH 5.2 this disruption does not seem to take place, as demonstrated by solubility and gel electrophoretic experiments. A larger exposure of the acidic polypeptides possibly correlates with a higher endothermic transition temperature and with the appearance of an exothermic transition as observed with DSC. Denaturation/aggregation (studied by DSC) and changes in secondary structure (studied by far-UV CD) take place simultaneously. Generally, changes in

tertiary structure (studied by near-UV CD) occur at lower temperatures than changes in secondary structure.

In Chapter 4 formation and structure of glycinin gels were studied in relation to protein conformation. While at I = 0.03 the gels were found to be fine stranded, gel coarseness increased when the ionic strength was higher. At I = 0.03 finer gel network structures were formed at pH 3.8 than at pH 7.6, whereas for I = 0.2 and 0.5 the reverse was found. The observed differences in gel stiffness (rheological dynamical measurements) did not correspond to coarseness.

To clarify gel structure into more detail also the molecular basis of gel formation was studied using physico-chemical and spectroscopic techniques. It was found that the nature of the primary network particles was different at pH 7.6 compared to pH 3.8, since at pH 7.6 only 51 - 69% of total protein was incorporated in the gel network (predominantly basic polypeptides), while at pH 3.8 all protein was present in the network. The higher water holding capacities observed at pH 7.6 compared to pH 3.8 support the idea that at pH 7.6 the non-network protein resides in the pores.

It was found that the observed differences in gel structure are related to differences during the gel formation process (e.g. disruption of the disulfide bond linking the acidic and basic polypeptide at pH 7.6 which is likely not to occur at pH 3.8). At all conditions studied denaturation coincides with the induction of β-sheet at a secondary level (IR measurements), and with gel formation (except for I = 0.03). The largest increase in gel stiffness did not take place directly after denaturation but during the cooling part of the temperature cycle used. This increase in gel stiffness could not be related to changes in secondary structure.

In heat denaturation studies, so far, the genetic variants of glycinin have been considered as a homogeneous group of proteins. The validity of this assumption was tested (Chapter 5). It was found by calorimetric studies that glycinin denatures heterogeneously at pH 7.6. When the temperature of isothermal treatment is increased from 70°C to 82°C the proportion of glycinin remaining native gradually decreases from 95% to 5% while the denaturation temperature of the glycinin remaining native increases from 88.5 to 95°C. Similar trends were found for pH 3.8. Fractionation and subsequent analysis (MALDI-TOF and CE) of isothermally treated samples demonstrated that at pH 7.6 the heterogeneous denaturation is caused by differences in thermal stability of the genetic variants of glycinin. The stability increases in the order G2/G3/G1< $A_4$< G5 < G4.

The work presented in Chapter 6 deals with the effect of pH on heat denaturation and gel forming properties of soy protein isolate compared to purified glycinin. No indications were found for basic differences in aggregation and gelation mechanisms between glycinin and soy

protein isolate, which means that the work performed on purified glycinin (Chapters 2-5) is a good base for predicting the effect of pH and ionic strength on the functional properties of soy protein isolates.

In Chapter 7 the main findings obtained in these PhD studies are discussed.

# Samenvatting

Ondanks al het onderzoek dat in de laatste decennia is verricht naar soja-eiwitten, is er nog veel onbekend over de relatie tussen de moleculaire structuur van soja-eiwitten en hun functionele eigenschappen. In deze studie is onderzocht in hoeverre verschillen in de structuur van het qua hoeveelheid belangrijkste sojaeiwit glycinine leiden tot verschillen in functionele eigenschappen (oplosbaarheid en gelvorming). Er zijn zowel condities bestudeerd die vaak in de literatuur worden onderzocht (I = 0.5 en pH 7.6) als condities die meer representatief zijn voor voedselsystemen (I = 0.02 - 0.2 en pH 3-7).

Hoofdstuk 2 beschrijft de relatie tussen de structuur eigenschappen van glycinine op quaternair, tertiair, secundair vouwingsniveau en oplosbaarheid. De resultaten laten zien dat wanneer de ionsterkte wordt verlaagd de basische polypeptides van glycinine meer naar de buitenkant van het glycinine complex verschuiven. Deze structurele reorganisatie zorgt ervoor dat de pH waarbij de oplosbaarheid van het eiwit minimaal is naar hogere waardes verschuift. Uit analyses met ultracentrifugatie in sucrose gradiënten is geconcludeerd dat glycinine bij pH 7.6 en ionsterkte 0.5 voorkomt als een hexameer (11S) terwijl bij pH 3.8 en een ionsterkte van 0.03 glycinine voorkomt als trimeer (7S). Tussenliggende situaties (verschillende verhoudingen 11S en 7S) kunnen verkregen worden bij andere condities. De dissociatie op quaternair niveau correleert met een toenemende hoeveelheid ongestructureerd eiwit op secundair niveau en met veranderingen in tertiaire vouwing (waargenomen met circulair dichroïsme). Met behulp van tryptofaan fluorescentie zijn op tertiair niveau geen verschillen gevonden tussen de verschillende ionsterktes, maar bij pH 7.6 is omgeving van de fluorofoor dichter gepakt dan bij pH 3.8.

Het 7S/11S evenwicht dat bij kamertemperatuur wordt gevonden beïnvloedt hitte-denaturatie. De 7S vorm van glycinine denatureert bij lagere temperatuur dan de 11S vorm van glycinine, zoals is aangetoond door middel van een combinatie van calorimetrische (DSC) en circulair dichroïsme (CD) experimenten (Hoofdstuk 3). Bij pH 7.6, waarbij glycinine voornamelijk in de 11S vorm voorkomt, wordt de zwavelbrug die de zure met de basische polypeptides verbindt, verbroken tijdens hitte-denaturatie. Bij pH 3.8 en bij pH 5.2 vindt deze zwavelbrug verbreking waarschijnlijk niet plaats, zoals is aangetoond met behulp van oplosbaarheids en gel-electroforese experimenten. Een grotere oplosmiddel expositie van de zure polypeptides correleert mogelijk met een hogere endotherme transitie temperatuur en met het verschijnen van een exotherme transitie, zoals is waargenomen met DSC. Denaturatie/aggregatie (bestudeerd met DSC) en veranderingen in secundaire structuur (bestudeerd met verre UV-

CD) vinden tegelijk plaats. In het algemeen worden veranderingen in tertiaire structuur bij lagere temperaturen waargenomen dan veranderingen in secundaire structuur.

In hoofdstuk 4 werd de vorming en de structuur van glycinine gelen bestudeerd in relatie tot eiwitvouwing. De gelstructuur bij een ionsterkte van 0.03 was "fine stranded". Bij toenemende ionsterkte nam de grofheid van de gel toe. Bij I = 0.03 was de gelstructuur fijner bij pH 3.8 dan bij pH 7.6, terwijl bij I = 0.2 en 0.5 het omgekeerde gevonden werd. De verschillen in gel stijfheid (dynamische reologische metingen) correspondeerden niet met de grofheid van de gelen.

Om de gel structuur in meer detail op te helderen is de moleculaire basis van gel vorming bestudeerd met behulp van fysisch-chemische en spectroscopische technieken. Het blijkt dat de deeltjes die het netwerk vormden bij pH 7.6 verschilden van die bij pH 3.8. Dit werd geconcludeerd uit het feit dat bij pH 7.6 slechts 51-69% van het eiwit deel uitmaakte van het netwerk (voornamelijk basische polypeptides), terwijl bij pH 3.8 al het eiwit in het netwerk aanwezig was. De grotere waterbindende capaciteit bij pH 7.6 (in vergelijking met pH 3.8) ondersteunt het idee dat bij pH 7.6 het niet-netwerk eiwit in de poriën aanwezig was.

De verschillen in gelstructuur zijn gerelateerd aan de verschillen die tijdens het gel vormingsproces optreden (bijvoorbeeld het verbreken van de zwavelbrug die de zure met de basische polypeptides verbindt bij pH 7.6, wat niet bij pH 3.8 optreedt). Bij alle condities die bestudeerd zijn vindt denaturatie gelijktijdig plaats met de inductie van β-sheet op secundair vouwingsniveau (IR metingen) en met gel vorming (behalve voor I = 0.03). De grootste toename in gel stijfheid vindt plaats gedurende het afkoeltraject van de gebruikte temperatuurcyclus. De toename in gel stijfheid kan niet worden gecorreleerd met veranderingen in secundaire structuur.

Tot nu toe worden de genetische varianten van glycinine in hittedenaturatie studies beschouwd als een homogene groep eiwitten. Of dit echt zo is, is getest in hoofdstuk 5. Het bleek dat bij pH 7.6 glycinine heterogeen denatureert (calorimetrische studies): Wanneer de temperatuur voor isotherme behandeling verhoogd werd van 70 tot 82°C daalde de hoeveelheid glycinine die natief bleef geleidelijk van 95 tot 5%, terwijl de denaturatie temperatuur van 88.5 tot 95°C steeg. Vergelijkbare trends zijn voor pH 3.8 gevonden. Fractionering en aansluitende analyse (MALDI-TOF en CE) van de isotherm behandelde monsters bewees dat de heterogene denaturatie, zoals die bij pH 7.6 gevonden werd, veroorzaakt wordt door verschillen in thermische stabiliteit van de genetische varianten van glycinine. De stabiliteit neemt toe in de volgorde G2/G3/G1 $<$ $A_4$$<$ G5$<$ G4.

Het werk dat in hoofdstuk 6 beschreven wordt behandelt het effect van de pH op hittedenaturatie en gelvormende eigenschappen van soja-eiwit isolaat vergeleken met

gezuiverd glycinine. Er zijn geen indicaties gevonden voor basale verschillen in aggregatie- en geleringsmechanismen tussen glycinine en soja-eiwit isolaat. Dit betekent dat het werk dat werd uitgevoerd aan gezuiverd glycinine (hoofdstuk 2-5) een goede basis vormt voor het voorspellen van het effect van pH en ionsterkte op de functionele eigenschappen van soja-eiwit isolaten.

In hoofdstuk 7 worden de belangrijkste bevindingen uit dit AIO onderzoek bediscussieerd, waarbij modellen zijn geformuleerd met betrekking tot gelstructuur en de structurele organisatie van zure en basische polypeptides binnen het glycinine molecuul.

# Nawoord

Ziezo, het werk is gedaan. Ik zie mijn promotietijd vooral als een leerzame periode en dan bedoel ik niet alleen leerzaam op wetenschappelijk gebied. Allereerst wil ik iedereen bedanken die mij in moeilijke tijden heeft gesteund en bijgestaan.

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Lieve Adrie, normaal gesproken schrijft de promovendus nu in het nawoord dat deze de laatste jaren te weing tijd aan de partner heeft besteed maar dat nu wil inhalen. Ook ik wil dat, maar jij hebt het altijd drukker dan ik. Eén ding weet ik zeker, namelijk dat we ook nu geen rustige periode tegemoet gaan, maar naar ik hoop wel een heel bijzondere tijd. Ik weet heus wel dat ik het je de laatste jaren niet altijd makkelijk gemaakt heb met promotieperikelen en andere onzinnigheden. Maar weet dat je relativerende woorden en je onvoorwaardelijke liefde voor mij heel belangrijk zijn.

# Curriculum vitae

Catriona Lakemond werd geboren op 21 september 1970 te Zaandam. Na het behalen van haar VWO diploma in 1988 aan het reformatorisch college Blaise Pascal te Zaandam startte zij in datzelfde jaar met haar studie Levensmiddelentechnologie aan de Landbouwuniversiteit te Wageningen. In het kader van haar studie deed zij afstudeervakken bij de leerstoelgroepen levensmiddelenchemie en toxicologie. Haar derde afstudeervak, met als onderwerp kwaliteisborging, werd uitgevoerd bij Mona te Woerden. Zij heeft haar studie in 1994 cum laude afgerond met een stage op het Department of Applied Biochemistry and Food Science aan de University of Nottingham, UK. Van 1995 tot 2000 werkte zij als Assistent in Opleiding bij het Centrum voor Eiwittechnologie, een samenwerkingsverband tussen TNO Voeding en de Wageningen Universiteit. Vanaf 1997 maakte het onderzoek ook deel uit van het Wageningen Centre for Food Sciences. Het onderzoek dat in deze periode werd uitgevoerd staat in dit proefschrift beschreven.

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