MOLECULAR CHARACTERISATION AND HEAT-INDUCED GELATION OF PEA VICILIN AND LEGUMIN

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ABSTRACT

The most important globular pea proteins are legumin and vicilin, and a minor protein is convicilin. The first two have extensive molecular heterogeneity that is well documented in literature, and the latter possesses a distinctive highly charged N-terminal extension region. Characterisation of two vicilin fractions (one contaminated by convicilin) via column chromatography, gel electrophoresis, differential scanning calorimetry (DSC), circular dichroism and solubility experiments lead to the conclusion that convicilin is not a separate protein. It was denoted as the α-subunit of vicilin, and is another heterogeneous factor of this protein. Further experiments showed that when present in large amounts these α-subunits increase the minimum gelling concentration of purified pea proteins at near-neutral pH, and cause transparent heat-induced gels to be formed. This behaviour was attributed to the repulsive forces on the N-terminal extension region at near-neutral pH, and was supported by the fact that no difference in the gelation behaviour of the two vicilin fractions was observed a low pH values where the repulsive charges would have been neutralised. These α-subunits also appeared to have an impact of the gelation of the pea protein isolates when present in sufficient quantity. Heat-induced gelation of legumin was compared with its analogous protein in soybean, namely glycinin. Overall the results of DSC and small deformation rheology showed that both the proteins have the same physical and chemical driving forces acting during gelation, but soybean glycinin, unlike legumin, was consistently able to form reheatable gels. Comparison of the amino acid profiles of the two proteins gave no indication as to why these homologous proteins form gels with different gel network stabilities. When comparing protein isolates and legumin from different pea cultivars it was shown that the contribution of legumin to pea protein isolate gelation was cultivar specific and that disulphide bonds played a role in gelation, but they did not demonstrate the gel strengthening ability that they are often reported to posses.

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INTRODUCTION

Chapter 1

BACKGROUND

In the early 1990's a programme entitled Sustainable Technological Development (STD) was carried out in The Netherlands with the aim of identifying technologies that could aid the population to meet its social needs more efficiently. To this end, the consumption of energy, space and raw materials, and the environmental burden of current production systems were all identified as needing to be reduced. To achieve such reductions in food production systems the idea was to develop novel protein foods based on plant proteins that could ultimately be consumed as a partial alternative to meat, because meat production systems were considered to place a heavy strain on the environment. Space and raw materials are consumed first to grow the crops that are then used to produce the animal feed, secondly to keep the livestock, and thirdly to process the meat into food products. Furthermore, space is needed to dispose of the manure created by the animals, and this manure emits gases into the environment that add to the total environmental burden of the production system. By contrast, novel protein food production using plant proteins requires less space because the crop itself would be processed into food products, and manure and its emissions would no longer be a relevant factor.

To be successful however, novel protein foods must be acceptable to consumers. Nutritionally they should provide the same amount and quality of protein as meat products, and thus be able to be used instead of meat. Only in this way will meat consumption reduce, and can a consequential reduction in meat production be achieved. At the time when the STD programme was active, the presence of novel protein foods on the market was predominantly in the form of "vegetarian meat" products made from soybean proteins. The products were considered by many as having poor textures and flavours however, meaning that they were unpleasing to the consumer. It was therefore suggested that future studies on novel protein food development should target texture and flavour, rendering them desirable to the consumer. Furthermore, future studies should use alternative protein sources to soybean because this crop is not suited to the temperate climate of Northern Europe, and soybean import is not in line with the ideals of a more environmentally efficient production system.

To tackle the issues brought forward by the STD programme the interdisciplinary project Profetas - PROtein Foods Environment Technology And Society - was established. The project contained all the elements needed to answer the question: Is the partial replacement of meat by novel protein foods technologically, environmentally and socially acceptable? The two production systems confronted within the framework of Profetas were pork meat products and novel protein foods based on peas. Peas were chosen as the protein source because it was believed that the analogous globular proteins in pea and soybean would have similar functional properties - existing knowledge of the strengths and weaknesses of soybean protein texturisation in novel protein foods could thus be taken advantage of.

PISUM

Dry pea (*Pisum sativum* L.) is a pulse crop of the family *Leguminacea* (*1*) that is well suited to the temperate conditions of Northern Europe. Dry pea seeds contain approximately 20-25% protein (*2*) of which 65-70% are the salt extractable globular storage proteins (*3*), legumin, vicilin and convicilin. Globular proteins are recognised as contributing to the texture of food products, and so their relative content tends to be increased by processing pea flours (obtained from the milled seeds) into concentrates and isolates. Commonly protein concentrates are produced by air-classification of the pea flour, which is a dry processing method that blows away the lighter starch granules, thus removing them from the protein. Concentrates have ~50% protein content. Protein isolates instead undergo a wet processing in which low molecular weight water-soluble components and the salt soluble proteins are extracted from the flour and the globular proteins are subsequently isolated by a selective precipitation step at the isoelectric point. Final protein content is ~85%. Full details of the average composition of pea seeds/flour, concentrate and isolate are given in table 1.

Composition of the globular proteins is a varying factor among pea genotypes and the ratio legumin: vicilin has been shown to vary between the extremes of 0.2 - 1.5 (*4*) though for most cultivars vicilin is the predominant protein. Variability has also been shown to exist in the subunit composition of the globular proteins within a given cultivar, as will be discussed in more detail in the successive sections on legumin and vicilin.

Table 1: Approximate composition (protein, starch and fat) of different pea protein preparations.

	Whole dry peas	Concentrate	Isolate
% protein	25	50	85
% starch	50	17	0
% fat	5-6	4	<3

Legumin

Pea legumin is a protein of ~60 kDa that is more commonly referred to as being a subunit of the hexamer into which it assembles at pH 7-9 (*5*). Proteolytic processing of legumin precursors splits the protein into two polypeptides, though they remain covalently linked via a disulphide bond. Consequently, each subunit can be reduced into a characteristic acidic and basic polypeptide of ~ 40 and ~20 kDa, respectively. The production of legumin precursors from a number of gene families results in heterogeneity of these legumin polypeptides: four/five acidic and five/six basic polypeptides have been identified. The exact sizes and isoelectric points reported in the literature have differed among authors, but the generally accepted ranges are as follows: Acidic polypeptides - 38-40 kDa with pIs 4.5-5.8; Basic polypeptides - 19-22 kDa with pIs 6.2-8.8 (*6-9*).

As a food protein, legumin is recognised for its sulphur containing amino acid residues. It has been reported to contain approximately two cysteine and three methionine residues per ~60 kDa protein by Casey (*10*), but seven and four, respectively by Croy (*11*). Regardless, it contrasts strongly with pea vicilin that is reported to contain few methionine residues and no cysteine residues (2*, 11*). Increasing the legumin content via plant breeding could thus be nutritionally beneficial (*10*). Changes in protein composition must also be beneficial for the texturising properties of the protein preparation.

Vicilin

Pea vicilin is produced as a precursor of ~50 kDa which is more commonly referred to as a subunit of the trimer into which it assembles *in vivo* (*12*). Vicilin can be cleaved at one or two sites (called the α:β and β:γ processing sites) as specified by the coding sequence of the vicilin genes. Specifically, proteolysis is believed to occur when the

potential cleavage site is located in a polar region, at an acidic or amide residue (*13*). Cleavage at the α:β site produces fragments of 19 and 30 kDa. Cleavage at the β:γ site produces fragments of 33 and 16 or 12.5 kDa. The smaller fragment is believed to be 16 kDa when it is glycosylated. Cleavage at both sites produces fragments of 19, 13.5 and 16 or 12.5 kDa (*14*). It should be noted however that these small fragments of vicilin are only apparent under dissociating conditions (*12*), such as SDS.

The small fragments of vicilin is one of the factors that contributes to the extensive heterogeneity that vicilin is reported to exhibit (*15*). Other contributing factors are differential glycosylation (*16*) and surface charge heterogeneity (around the potential site of cleavage) (*15*).

Convicilin

Pea convicilin is reported as a third globulin protein of ~70 kDa. Its most commonly recognised oligomeric form is as a trimer. Convicilin has an extensive homology with vicilin along the core of its protein, yet is distinguished by the presence of a highly charged, hydrophilic N-terminal extension region consisting of 122 or 166 residues (see refs *17-18*, respectively). Convicilin and vicilin are shown schematically in figure 1.

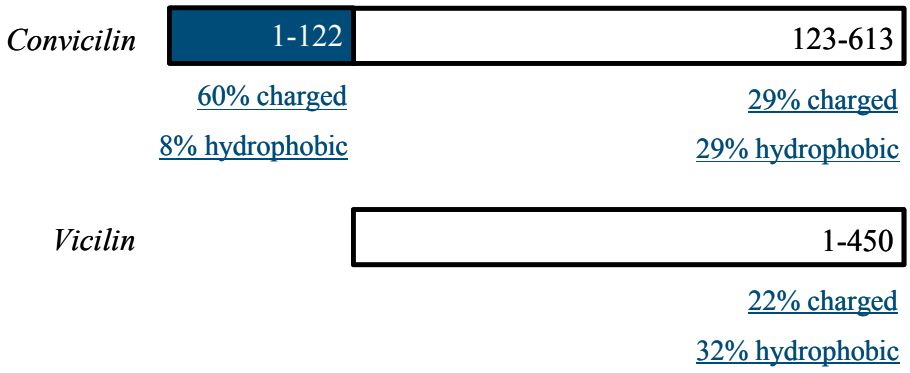

Figure 1: Schematic diagram of the highly charged N-terminal extension region (residues 1-122) present in convicilin molecules. The core of convicilin (residues 123-542) is highly homologous to vicilin, as shown by the percentages of charged and hydrophobic residues. Percentages were calculated based upon published amino acid sequences (*17-20*).

Despite the high level of homology, convicilin differs from vicilin along the core region by lacking the polar regions that code for vicilin's post-translational processing sites (*17*, 2*1*). Convicilin is also reported to contain one cysteine residue, which is another property that distinguishes it from vicilin (*11*, *17*).

Early studies on pea vicilin considered convicilin as a subunit of vicilin (*22*) due to the frequency with which convicilin was present in purified fractions of vicilin. However, after its purification by Croy *et al.* (*11*) it became accepted as a third separate protein; one that is a contaminant of vicilin purified on a large-scale (*23*).

HEAT-INDUCED GELATION

The ability of proteins to form heat-induced gels has been considered of great importance for the structure and properties of many food products (*24*). The structure comes from the protein network that constitutes the gel, though the water, lipids and flavour components that can become trapped with the network all contribute to the overall sensory properties of foods with a gel base.

Heat-induced gelation of globular proteins is a process of three successive steps: (i) unfolding (denaturation) of the protein exposing residues previously buried in the core, (ii) interaction of the exposed residues to form aggregates, and (iii) arrangement of the aggregates into a continuous network. Heat-induced gel networks thus result from a balance of both protein-protein (positive and negative) and protein-solvent interactions (*25*). Factors such as pH and ionic strength influence these interactions, alter their balance and thus the type of network formed (*26*). Renkema (*27*) demonstrated this by comparing the storage modulus, fracture strain, and permeability of soy protein isolates gels formed at 0, 0.2 and 0.5 M NaCl at pH 3.8, 5.2 and 7.6. Gels formed at pH 3.8 had a consistently higher storage modulus and lower fracture strain than those formed at higher pH values, while the permeability of the gels (which reflects the pore size of the protein networks) was influenced more by ionic conditions than by pH. Renkema concluded however that to relate the rheological properties to the network structures formed under different conditions of pH and ionic strength required additional information on the curvature of the strands. Since the strands that make up the gel network are composed of protein

aggregates, studying how the successive steps of the gelation process (unfolding, aggregation and arrangement into a gel network) are influenced by pH and ionic strength could aid the understanding of the underlying factors that determine the type of gel network formed. However, methods that are able to provide information on protein unfolding (circular dichroism), or the size of the aggregates formed (light scattering or size exclusion chromatography) are all methods that require clear solutions of protein aggregates. Practically this means that the protein concentration must be kept well below that of the minimum gelling concentration. However, since gelation is a macroscopic state transition from a liquid to a solid-like material, understanding only the molecular events that occur at these low concentrations is not sufficient (*28*).

To complement information on the aggregation process non-destructive rheological techniques can be used. Measurements of this type can be performed on concentrated, turbid systems. By constant oscillation of one part of the apparatus a fixed strain (deformation) is applied to the sample throughout the gelation process, and the stress developed is constantly measured. The stress developed is dependent upon the nature of the sample and its intrinsic material properties (*29*). Though a valid technique when comparing gel formation under a given condition, the possibility that two structurally different gels could express the same stress response can not be ruled out. Full understanding of gel networks, their formation, and control there of requires also the employment of microscopic techniques. Being able to visualise the gel network structures offers the possibility to confirm the presence of structural features that have been hypothesised to be present, based on the results of rheological testing.

Returning to the three steps that constitute the process of gel network formation the first is often considered as the initiation step, since without heat-induced unfolding of the protein and exposure of residues there is no strong driving force for protein-protein interactions to occur. This initiation step, as well as the rest of the process, is under some extent of kinetic control (*30*), and is thus influenced by the time and temperature profile used to heat and cool the sample. The kinetics of unfolding and aggregation tend towards an orderly assembly of aggregates when aggregation is occurring slowly by comparison to unfolding (e. g. when using a slower heating rate). The opposite is assumed true for samples heated more quickly, that is that aggregation proceeds quickly by comparison to

unfolding and is thus not orderly (*25*). A similar process can be envisaged during the cooling phase, which for leguminous plant proteins is the phase in which the gel network develops. Therefore, a slow cooling rate gives more time at each temperature for aggregates to interact and assemble themselves, thus they can do so in an orderly manner. Orderly arrangement of aggregates into a network creates a 'fine-stranded network'. Gels with such networks are transparent (*31-37*). By contrast, a more random arrangement of aggregates into a network when the successive processes occur too quickly creates larger aggregate clusters, and the resultant gels are turbid (*31-34*).

Whether it be the heating rate or the chemical composition of the environment that changes the kinetics of the process, the essential element for heat-induced gelation to occur is to have a favourable balance of protein-protein and protein-solvent interactions. Following from this comes the reasoning that the intermolecular interactions determine the structure of heat-induced gel networks (as proposed by Zheng *et al.*, (*38*) and Ikeda and Nishinari (*39*)). Good understanding of these interactions should thus be important when there is a desire to control and/or modify the texture of foods created by such protein gels.

PEA PROTEIN GELATION

The current position of understanding

Pea proteins were identified in the 60's and 70's as a potential alternative to soybean proteins, and so some studies on their functionality have been reported in the literature. Specifically with a focus on gelation there has been little progress however. Bora *et al.* (*40*) compared the heat-induced gelation of crude and purified preparations of the major pea globulins, legumin and vicilin, to that of a globulin mixture. They concluded that legumin did not gel, and the amount of legumin in a legumin/vicilin mixture was inversely proportional to the gel hardness. Bacon *et al.* (*41*) compared the gelation characteristics of two protein preparations, each prepared via a different method, but both containing legumin and vicilin (in undefined proportions). Formed in acid conditions using food-grade procedures the gels were described as having clarities that made them a suitable replacement for gelatin in vegetarian foods. Bacon *et al.* (*24*) also showed that

pea vicilin could form transparent gels at low ionic strength with a pH far from the isoelectric point. They reasoned that under such conditions, electrostatic repulsive forces could be maintained and so the formation of large aggregates could be minimised. No other studies on the gelation of pea proteins are known to exist.

Bringing pea proteins forward as a potential alternative to soybean in novel protein foods needs much more knowledge about their gelation behaviour to be generated. As a starting point there needs to be more understanding of the molecular basis of the gelation mechanisms of vicilin and legumin, with particular focus on those mechanisms that determine the structural properties of the gels. Furthermore, it should also be important to determine how genetic variation in the globular protein composition effects the gelation behaviour of crude protein preparations.

Since gelation of globular proteins gives texture to foodstuffs, research should eventually aim to link the molecular understanding of their gelation mechanisms to the textural properties of the gel networks formed.

AIM

The work carried out in this thesis aimed to extend the molecularly based understanding of pea protein gelation that is missing in the literature. Moreover, having selected the purified globulins as the protein source it was also important to determine if such an approach was suitable to understanding the gelation behaviour of the pea protein isolate, which will eventually be a much more likely food ingredient than the purified proteins themselves.

THESIS OUTLINE

Chapter 2 of this thesis describes the isolation and purification procedure used throughout this thesis to obtain purified proteins for functionality testing. Thereafter, a series of experiments are described that used various analytical methods to characterise two fractions of the vicilin protein that were obtained during purification. In chapter 3 the gelation behaviour of the two vicilin fractions were compared under various conditions of

pH and ionic strength in order to determine how the subunit composition effected the gelation process.

Chapter 4 describes a series of experiments designed to deduce the intermolecular forces that produce pea legumin gels with different gel strengths (measured using small deformation rheology). Comparison of legumin with the molecularly homologous soybean glycinin gelled under the same conditions enabled the hypothesis to be tested that a common model for legumin-like protein gelation can be built with a molecular basis.

Chapter 5 presents a comparison of the gelation behaviour of the protein isolates from five different pea cultivars, and explores to what extent knowledge on the gelation of the individual globular proteins can be used to predict the gelation behaviour of the isolate.

The thesis is concluded in chapter 6 with a critical evaluation of the experimental approach taken in this thesis. It is discussed in relation to the aim of testing if it was possible to relate the gelation behaviour of the protein isolates to the molecular and gelling characteristics of its constituent proteins. Recommendations for future work are given.

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Chapter 2

CHARACTERISATION OF PEA VICILIN PART I: DENOTING CONVICILIN AS THE α-SUBUNIT OF THE *PISUM* VICILIN FAMILY

ABSTRACT

Vicilin, a major globulin protein of pea that has been described as extremely heterogeneous in terms of its polypeptide composition, was extracted from pea flour under alkaline conditions, and subsequently fractionated by salt under acid conditions. This procedure induced the separation of vicilin into two fractions, which, after purification, were called vicilin 1° and vicilin 2°. Vicilin 2° was seen on SDS-PAGE to contain the third globulin protein of pea, convicilin (a band at ~70 kDa). Vicilin fractions were thus characterised using gel electrophoresis, differential scanning calorimetry, circular dichroism and pH-dependent solubility in order to determine whether or not the convicilin should in fact be considered as a third separate globulin protein of pea. Based on the results obtained it was concluded that this distinct polypeptide of the *Pisum* vicilin gene family should be further denoted as a subunit of the salt extractable protein vicilin. The definition of vicilin heterogeneity should therefore be extended to acknowledge the possible oligomeric inclusion of the 70 kDa polypeptide that we here denote as the α-subunit.

KEYWORDS: *Pisum*; storage proteins; purification; vicilin; convicilin; subunit composition; heterogeneity.

INTRODUCTION

When aiming to develop plant proteins as food ingredients it is important to study in detail the structural features and structure-function relationships of proteins so that they are understood, and strategies for rational modification of functional properties can be developed (*1*). Pea protein globulins as food ingredients have not received much attention within the literature, especially by comparison with soybean. They have however been studied quite extensively at a genetic level. An observation made by all the early researchers was that heterogeneity was exhibited, (i) in the protein composition of different pea cultivars, with a legumin: vicilin ratio varying from 0.2-1.5 (*2*), and (ii) in the polypeptide composition of individual proteins from a single cultivar (*3-9*).

Pea legumin heterogeneity is exhibited in the size of the acidic and basic polypeptides that the subunits can be separated into (*5*, *7*, *10-12*). Pea vicilin heterogeneity is more complex however. Its heterogeneity derives from a combination of factors, including production of vicilin polypeptides from several small gene families encoding different primary sequences, differential proteolytic processing and differential glycosylation (*13*). Different gene encoding is believed to produce the group of polypeptides ~ 50 kDa (*14*) that are denoted as the subunits that assemble into higher molecular weight oligomers. Cleavage at one or both of two potential processing sites (the α:β site and/or β:γ site) on the subunits accounts for the presence of the small fragments seen on SDS-PAGE. The resulting fragments are as follows: 33 kDa (αβ), 30 kDa (βγ), 19 kDa (α), 13.5 kDa (β) and 16 or 12.5 kDa (γ) (*15*).

A third globulin protein of pea that has received little attention is convicilin. In the early genetic studies (pre-1980) referred to above the 70 kDa polypeptide of convicilin was considered to belong to vicilin. Yet, Croy *et al.* (*16*) showed it was a separate protein, able to be purified. Convicilin was shown to be highly homologous with vicilin along the core of its amino acid sequence, yet possess an extended N-terminus. This extended region was highly charged with acidic residues and contained few hydrophobic residues (*17-18*).

Despite its identification as a separate protein, convicilin has not been considered in functionality studies of pea proteins. Instead, as for other plants, authors have focused on the functionality of the two main proteins legumin and vicilin. Convicilin is often present however as a contaminating protein, visible on SDS-PAGE. Kyoro and Powers (*19*) mentioned convicilin contamination of their protein preparations, but made no attempt to remove it, and did not refer to its presence during their conclusions on the effect of the legumin: vicilin ratio on emulsification and foaming of the pea globulin proteins. A reason for these authors to work with convicilin contaminated protein preparations could have been the difficulty encountered in trying to remove it, while still obtaining sufficient quantities of protein for functionality studies. As demonstrated by Gueguen *et al*. (*20*), and Larré and Gueguen (*21*) in two papers on the large-scale purification of pea globulins, convicilin contamination is difficult to avoid when isolating a vicilin-rich fraction. The legumin fraction can also be contaminated with convicilin.

This paper presents the purification of pea globulin proteins for use in functionality studies. The purification procedure used caused the fractionation of vicilin into two fractions, one of which stained intensively for a band at 70 kDa on SDS-PAGE, indicating it was heavily contaminated with convicilin. Here we report on the chromatographic techniques selected to remove the contaminant protein. Further, we present results on the physico-chemical characterisation of the two vicilin fractions that was carried out in order to determine whether or not convicilin should be considered as a separate contaminating protein.

MATERIALS AND METHODS

Preparation of enriched protein fractions. Vicilin and legumin were purified from peas (*Pisum sativum* L.), cv. Solara (Cebeco Seeds, Lelystad, NL, grown and harvested in 1998), by a non-denaturing fractionation procedure adapted from the method of Kyoro and Powers (*19*) and Bora *et al.* (*22*). Peas were milled in a Waring commercial blender (New Hartford, Connecticut, USA) 2:1 (w/w) with dry ice to avoid any heat denaturation of the proteins. Salt-soluble proteins were then extracted into a 100 mM Tris-HCl buffer, pH 8.0, with a flour to buffer ratio 1:10. Extraction time was 1 hour at room temperature and extract was collected by centrifugation (11 900 x g, 10°C, 25 min). Isoelectric precipitation, pH 4.8, was used to isolate the globulin proteins; the pH was adjusted with 1 M HCl. Precipitated protein was left for 2 hr, 4°C before it was collected by centrifugation (11 900 x g, 10°C, 25 min). Washing the protein pellet with water (pellet to water ratio 1:10) removed unwanted albumin proteins. Again the pellet was collected by centrifugation (11 900 x g, 4°C, 25 min). The crude pellet was suspended in the extraction buffer, pH 8.0 (10 mg/ml) and dialysed at 4°C against McIlvaine's buffer (0.2 M Na_2HPO_4 + 0.1 M citric acid, containing 0.2M NaCl), pH 4.8. Sample to buffer ratio was 1:20, and the dialysis buffer was changed three times over a 24h period. Centrifugation of the sample (18 900 x g, 4°C, 25 min) collected a precipitated fraction (referred to as *legumin enriched*) and a clear supernatant. This supernatant was desalted by further dialysis at 4°C against McIlvaine's buffer (0.2 M Na_2HPO_4 + 0.1 M citric acid), pH 4.8, with no salt. Centrifugation of the sample (18 900 x g, 4°C, 25 min) obtained a second precipitated fraction (referred to as *vicilin enriched*). These fractions were freeze-dried before purification.

Purification of legumin, vicilin2° and vicilin1°. Freeze-dried protein enriched fractions were dissolved in buffer A (35 mM potassium phosphate, containing 0.075 M NaCl), pH 7.6, at protein concentration of 25 mg/ml (which gives a suitably low final sample viscosity for loading onto the column). Legumin enriched isolate was only a suspension, and it was centrifuged (11 900 x g, 4°C, 25 min) before further use. The solutions of both legumin and vicilin enriched fractions were then filtered through sterile membrane filters, 0.2 μm (Schleicher & Schuell, Keene, NH, USA).

Legumin enriched fraction (1200 ml) was loaded onto a DEAE Sepharose Fast Flow column (5 cm diameter, 343 ml volume; Amersham Biosciences, Uppsala, Sweden), previously equilibrated with buffer A. Elution was performed with a linear salt gradient (0.075 - 0.5 M NaCl over 6 column volumes) in the same potassium phosphate buffer. The eluate was monitored at 280 nm, and 15 ml fractions were collected and analysed for purity on an SDS-PAGE gel (Bio-Rad Ready Gel Tris-HCl Gels, 10-12% linear gradient). Fractions containing only bands belonging to vicilin/convicilin (70, 50, 33-14 kDa) or legumin (40, 20 kDa) were pooled together. Pooled fractions were desalted and freeze-dried. This procedure resulted in two pure proteins: *legumin* and *vicilin2°*.

Vicilin enriched fraction (800 ml) was loaded onto the same DEAE Sepharose Fast Flow column, previously equilibrated with buffer A, and eluted by the same linear salt gradient referred to above. The eluate was monitored at 280 nm, and 15 ml fractions were collected and analysed for purity on an SDS-PAGE gel (Bio-Rad Ready Gel Tris-HCl Gels, 10-12% linear gradient). Fractions containing only bands belonging to vicilin/convicilin (70, 50, 33-14 kDa) were pooled together, desalted, and freeze-dried. The yielded protein was called *vicilin1°*.

Gel electrophoresis. Samples were prepared by mixing the protein sample 1:1 with sample buffer (1.4 ml distilled water, 2.0 ml 0.5 M Tris-HCl at pH 6.8, 2.0 ml 10% SDS, 2.0 ml glycerol and 0.4 ml 0.05% bromophenol blue). 10-20% linear gradient, Tris-HCl Ready Gels (Bio-Rad) were used and volume containing 2-10 μg of protein were applied to each well. Low molecular weight protein standards, ranging from 94 to 14 kDa (Amersham Biosciences, Uppsala, Sweden) were made according to the instructions, and 10 μl was applied to each well. Gels were run at a constant 200 V. Staining was done using Coomassie Blue R-250 Bio-safe stain (Bio-Rad).

Differential scanning calorimetry. Thermal denaturation measurements were done in a VP-DSC Microcalorimeter (MicroCal Inc., Northampton (MA), USA). Pure protein fractions were dissolved in potassium phosphate buffer (I = 0.03, 0.2 and 0.5), pH 7.6, at 0.3% (w/v) concentration. All samples were degassed prior to loading into the cell, and were run against a reference sample of buffer (as used for making the protein sample).

Samples were preheated at 45°C for 15 minutes, then heated from 45-115°C at 60°C/hour, and cooled to 20°C. Each sample was reheated one time to verify that there was no reversibility of denaturation.

Determination of minimum solubility. Vicilin fractions were dissolved in 75 mM potassium phosphate buffer, pH 7.6, at 4% concentration (w/v). 1 ml aliquots were put into eppendorf tubes, and the pH was adjusted with a known volume of 1 M HCl or NaOH as necessary. When the pH values were stable the samples were left to settle for 2 hours at 4°C. Subsequently they were centrifuged (15 min, room temperature, 15 000 rpm, MicroCen13 table top centrifuge, Herolab) and the supernatant was carefully removed with a Pasteur pipette. The amount of dissolved protein present in the supernatant was determined with the Bradford method. The percentage of dissolved protein at a given pH value was subsequently calculated from a BSA calibration line.

Estimated composition of dissolved protein at different pH values. Equal volumes of the supernatant (from above) of vicilin 1° and vicilin 2° were prepared for SDS-PAGE, (as described above). After running and staining, the subunit composition of the dissolved protein was determined by densitometry (G-710 Imaging Densitometer, Bio-Rad), and the results were expressed as the relative percentage of vicilin and convicilin. Vicilin was assumed to be composed of all the subunits 50 kDa and smaller, and convicilin of the subunits ~70 kDa.

Detection of glycoproteins. Glycosylation was determined in samples of both vicilin 1° and vicilin 2°. Samples were prepared according to the protocol for gel electrophoresis. The proteins were separated on a PhastGel gradient 10-15 on the PhastSystem (Amersham Biosciences, Uppsala, Sweden) and stained with the Shiffs-PAS staining method. Ovalbumin with 2% glycosylation was used as a positive control. Low molecular weight gel electrophoresis calibration kit (Amersham Biosciences) was used as a negative control.

Re-chromatography of the purified vicilin fractions. Vicilin 1° and vicilin 2° were loaded onto a Source 15 Q PE 4.6/100 column (Amersham Biosciences, Uppsala, Sweden) (100 ml, 25 mg/ml concentration in buffer A as referred to above). They were

eluted at 10 ml/min with a linear salt gradient from 0.075 to 0.5 M NaCl in running buffer A over 6 column volumes. The eluate was monitored at 280 nm and 5 ml fractions were collected. Based on initial gel electrophoresis results the eluted protein was pooled into four fractions, 1 to 4. These fractions 1-4 were dialysed against nanopure water and freeze-dried. Each fraction (1-4) was subsequently loaded (5 mg/ml concentration in buffer A in successive applications of 1 ml) onto a Mono Q HR 5/5 column (Pharmacia Biotech, Uppsala, Sweden). Each fraction was eluted at 1 ml/min with a linear salt gradient from 0.075 to 0.5 M NaCl (in the same potassium phosphate running buffer) over 10 column volumes, and the eluate was monitored at 280 nm.

Chromatofocusing. Samples were prepared by dissolving the purified proteins vicilin 1° and vicilin 2° in starting buffer (0.025 M Tris-HCl with saturated imidazole) at pH 7.1 at 2 mg/ml concentration. Samples (5 ml) were gently stirred for 2 hours and filtered through a 0.2 μm sterile filter (Schleicher & Schuell, Keene, NH, USA) prior to loading onto the column. The Mono P column (Mono P HR 5/20, Amersham Biosciences, Uppsala, Sweden) was treated as instructed in the manual. Firstly, it was run with the starting buffer until the pH was stabilised at pH 7.1. Secondly, polybuffer 74, pH 4.0 (prepared according to the instructions) was run through the column until the pH reached 4.0. Lastly, re-running in starting buffer took the pH to 7.0, and the column was then ready for sample application. Flow rate was 0.5 ml/min at all times. 3 ml of sample was applied (6 mg protein load) and the eluted protein was detected at 280 nm, and collected in 300 μl aliquots.

Circular Dichroism. Secondary structure of the native proteins was determined at 20°C using the Jasco J-715 spectropolarimeter (Jasco corporation, Japan) in the far-UV range 260-190 nm, at a scan speed of 50 nm/min. Each spectrum was recorded as the average of 30 accumulations. Cell pathlength was 0.1 mm. Sample concentration was 0.2 mg/ml in 10 mM potassium phosphate, pH 7.6, and all samples were filtered through a 0.2 μm sterile filter (Schleicher & Schuell, Keene, NH, USA) prior to analysis. The relative percentages of secondary structure were calculated using a non-linear regression procedure as previously described in detail (*23*), and the results were presented as an average of three replicates. Subsequently, the loss of secondary structure upon heating

was monitored at constant wavelength of 203 nm. This wavelength was selected based on previous experiments as the wavelength at which there was the biggest change of signal upon protein denaturation. Heating rate was 1.0°C/min, and measurements were made at 0.1°C intervals. Data were baseline corrected and the peak of the denaturation was determined using the JASCO J-715 Spectra Analysis software.

RESULTS

Extraction, fractionation and purification of legumin and vicilin was done according to a method (*19*) in which a salt fractionation of the extracted protein was intended to separate these two proteins - legumin precipitating in salt, and vicilin remaining soluble. This method was adapted from that originally used by Thomson *et al.* (*4*) who indeed induced an 11S / 7S fractionation with salt. In our work however, the separation was not clear-cut. When the protein isolate was suspended in extraction buffer and dialysed against McIlvaine's buffer, pH 4.8, 0.2 M NaCl, a fraction of vicilin co-precipitated with the legumin. As described already, this fraction was later called vicilin 2°. The vicilin fraction precipitating after desalting was called vicilin 1°.

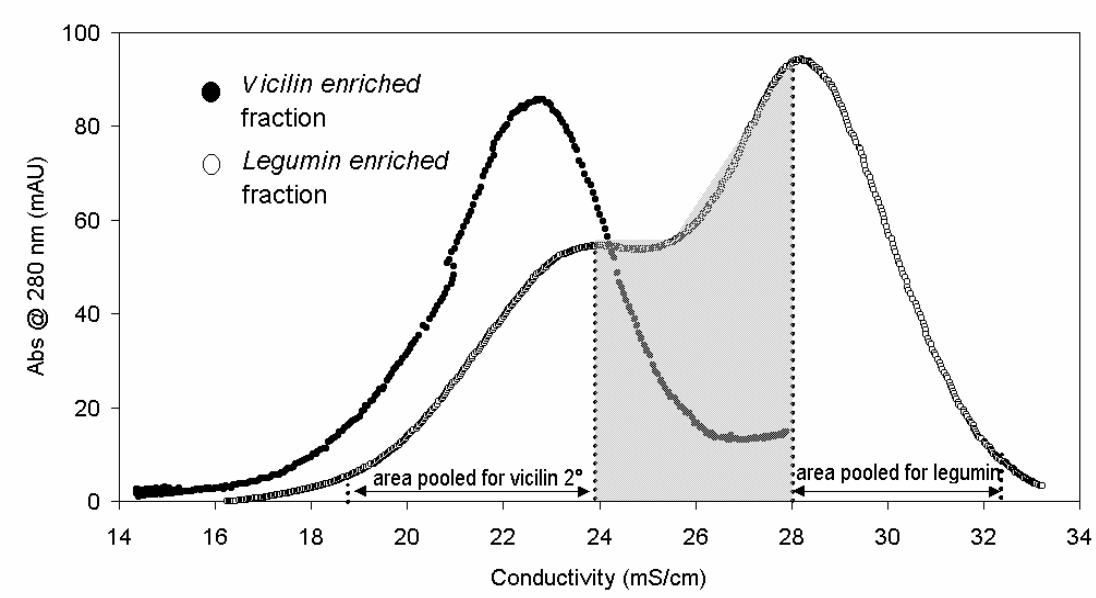

Figure 1a: Elution profile of *vicilin* and *legumin enriched protein fractions* from the DEAE Sepharose Fast Flow column under a linear salt gradient. NB: Absorbance value of legumin enriched fractions was reduced by a factor of 10 to make the profiles more comparable.

Purification of these two fractions (*legumin* and *vicilin enriched*) using DEAE Sepharose Fast Flow showed immediately that the two vicilin fractions (1° and 2°) contained proteins that had different surface charges. Figure 1a shows that the *vicilin enriched* fraction (from which comes vicilin 1°) started eluting at a lower salt

concentration than the *legumin enriched* fraction. SDS-PAGE of the eluting proteins showed that the *vicilin enriched* fraction contained only vicilin (50-14 kDa) and convicilin (70 kDa) polypeptides (figure 1b, left hand side). The entire fraction was thus pooled. The double peaked *legumin enriched* fraction caused some problems for the purification of large amounts of representative samples. The first peak was vicilin 2°, the latter peak legumin. However, as highlighted in figure 1a (shaded area), there was a considerable overlap between the two proteins that had to be discarded. Consequently, the pooled protein (indicated in figure 1a) was not a completely representative sample of the entire protein. The region of overlap is also indicated on the SDS-PAGE in figure 1b (right hand side), (though the four lanes on the gel only represent the beginning and the end of the overlap region).

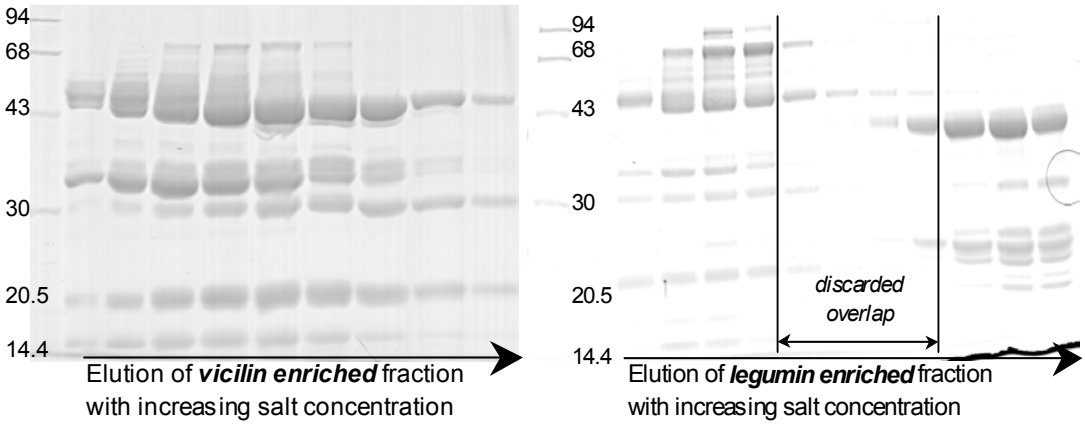

Figure 1b: SDS-page profile of protein fractions as they eluted from the DEAE column (according to figure 1a). Arrow indicates the order of elution. Standard markers are indicated (in kDa) on the left hand side of each picture.

Gel electrophoresis was done as a first step in characterising the vicilins obtained, and determining the difference between them. In figure 2 vicilin 2° is distinguishable from vicilin 1° in its content of the 70 kDa convicilin polypeptide. Using a densitometer, with an average of 8 samples, vicilin 2° and vicilin 1° were estimated to have a convicilin content of 55% and 5%, respectively. Differences in their small fragment composition (bands < 50 kDa) were not detectable.

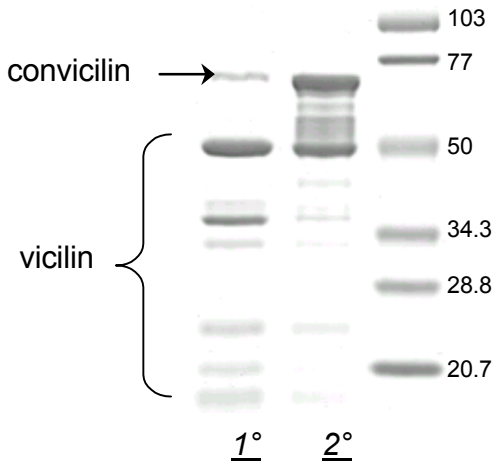

Figure 2: SDS-PAGE of the purified vicilins - *1° and 2°*. The bands belonging to vicilin and convicilin are indicated and the standard markers are indicated (in kDa) on the right hand side.

It should be noted that the dark area between the bands at 70 and 50 kDa was not considered in the densitometric analysis of the purified vicilin fractions. We acknowledge that such a spread of bands could well be due to proteolysis of the 70 kDa polypeptide, but it is not an issue that we have investigated. Qi *et al.* (*24*) reported the action of a soybean protease that is involved the in mobilisation of β-conglycinin. This protease cleaves 1 or 2 kDa fragments from the α and α' subunits, producing a succession of intermediates, until it finally produces two polypeptides of 50 and 48 kDa. Presence of a similar protease in our protein preparations would explain our observations but no references to such a protease from pea could be found in the literature.

Separation of vicilin and convicilin was decided to be done to be able to better determine if, in the absence of convicilin, the two vicilins obtained were identical. It was seen on SDS-PAGE (figure 1b) that when the *legumin enriched* fraction eluted from the DEAE column, the leading edge of the peak contained no 70 kDa convicilin polypeptide, and the relative amount of this polypeptide increased with the increased salt in the gradient. With this result, it shows that at pH 7.6 the 70 kDa polypeptide of convicilin is more highly charged than the polypeptides of vicilin. Re-chromatography of the purified vicilin fractions, 1° and 2°, was thus done on analytical anion-exchange columns (Source

Q and Mono Q). Firstly, the vicilin 1° eluting from the Source Q column was collected as four sub-fractions, numbered 1 to 4 as indicated below figure 3a.

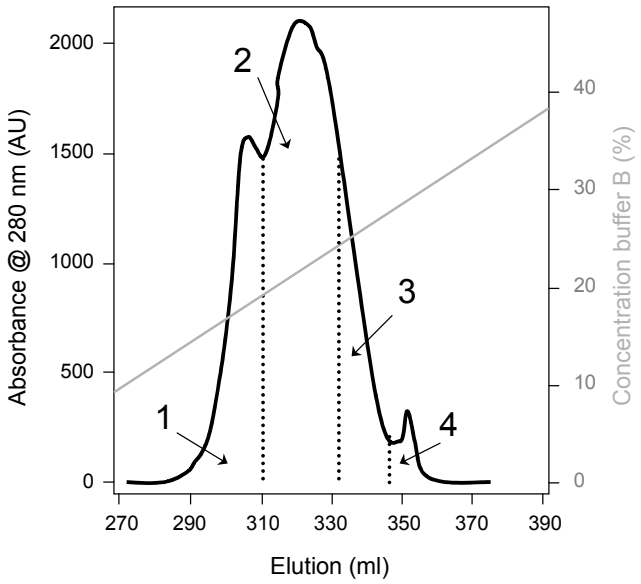

Figure 3a: Elution of vicilin 1° from the Source 15Q column under a linear salt gradient. Each section was applied to an analytical Mono Q column and their respective elutions can be seen in figure 3b.

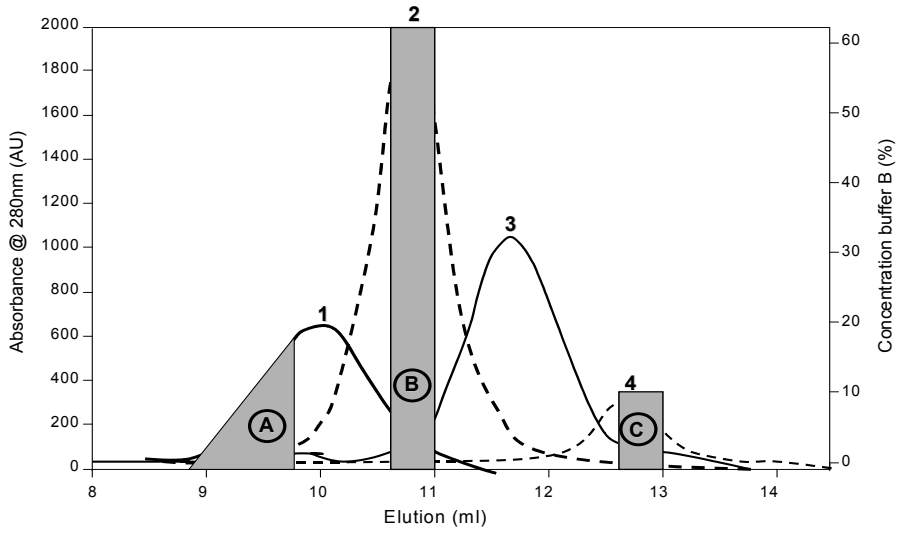

Figure 3b: Elution of vicilin 1° from the Mono Q column under a linear salt gradient. Numbers 1 to 4 above each peak refer to division of the protein (as explained for figure 3a). Shaded areas, labelled A, B and C, indicate the fractions kept for further analysis.

Each sub-fraction was reapplied to the Mono Q column and after overlaying the chromatograms of each sub-fraction (figure 3b) and visualising their composition on SDS-PAGE, fractions A, B and C were selected. Note that no fraction was taken from number 3 because its composition on SDS-PAGE was no different from taken from number 2.

Figure 3c shows that although this experiment did not achieve its objective of separating the 70 and 50 kDa polypeptides from each other, it did (for vicilin 1°) separate the protein into three fractions (A, B and C), each with a different predominance of small fragments. Fraction A: 50, 33, and 16 kDa polypeptides. Fraction B: 50, 33, 30, 19, 16 and 14 kDa polypeptides. Fraction C: 50, 30, and 19 kDa polypeptides. Repeating this procedure with vicilin 2° achieved neither convicilin/vicilin separation, nor separation of fractions with a different small fragment composition (no results shown).

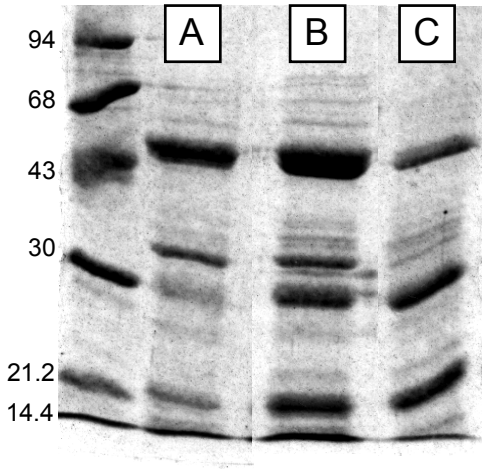

Figure 3c: SDS-page of fractions A, B and C kept from the protein eluting from the analytical Mono Q column (labelled according to figure 3b). Standard markers are indicated (in kDa) on the left hand side.

Because the small fragments of vicilin were the focus of early research on the *heterogeneity of pea vicilin* (*4*, *6*, *8*, *25-26*) it was considered worthwhile to keep the sub-fractions of vicilin 1° (A, B and C) and determine if the small fragment composition effected the thermal denaturation temperature (T_d). There was a maximum 2°C shift in the T_d (see table 1) that was not considered to be important.

Table 1: Thermal denaturation temperatures (T_d) at pH 7.6, and ionic strengths I = 0.03, 0.2 and 0.5, of fractions A, B and C of *vicilin 1°* that were separated by analytical chromatography with the Mono Q column (figure 3b). The polypeptides present in each fraction are listed in the table (and are visible on the SDS-PAGE in figure 3c).

Fraction	Polypeptides present in sample (kDa)	T_d (°C)		
		I = 0.03	I = 0.2	I = 0.5
A	50, 33, 16	70	73.7	82.7
B	55, 33, 30, 19, 16	69.4	72.6	82.1
C	50, 30, 19	71.8	74.6	84.1

Returning now to the separation of vicilin and convicilin, an alternative method for separating proteins according to their charge is chromatofocusing. Using a Mono P column with Polybuffer 74, vicilin 1° and 2° were applied to the column, and eluted in a linear pH gradient from 7 to 4. The elution profiles (figure 4a) showed immediately that vicilin 2° was more acidic than vicilin 1°. Furthermore, SDS-PAGE visualisation of the eluting fractions (figure 4b) showed that the relative amount of convicilin (the band at ~70 kDa) increased as the pH of elution decreased, in both vicilin 1° and vicilin 2°. Overall these results indicated that convicilin is more acidic than vicilin. Its acidity is conferred by its extension region, as is also true for the similarly extended α and α' subunits (*27-29*) of soybean β-conglycinin.

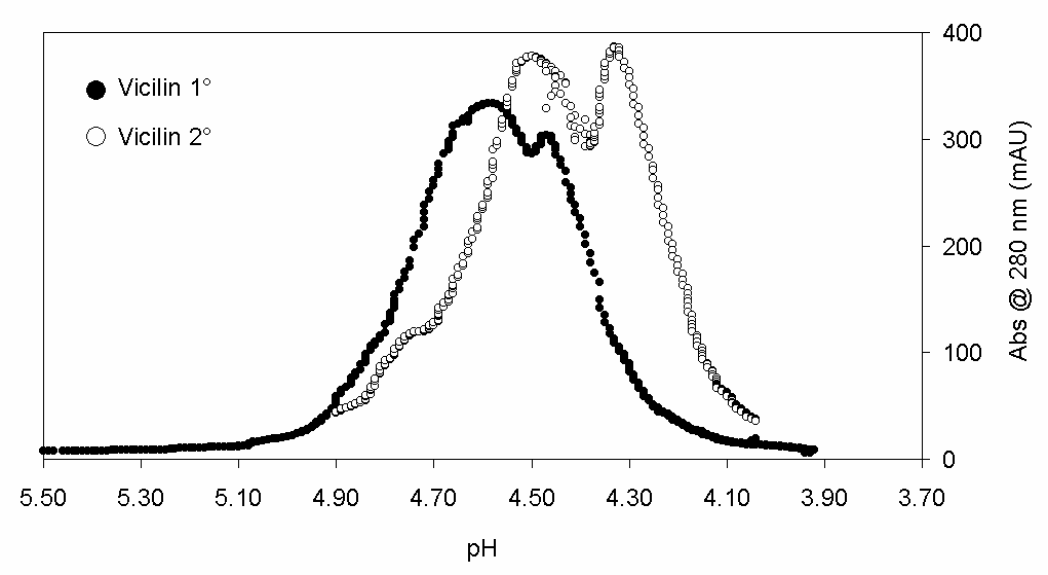

Figure 4a: Elution profiles of *vicilin 1° and vicilin 2°* from the Mono P column under a linear pH gradient from pH 7 to 4.

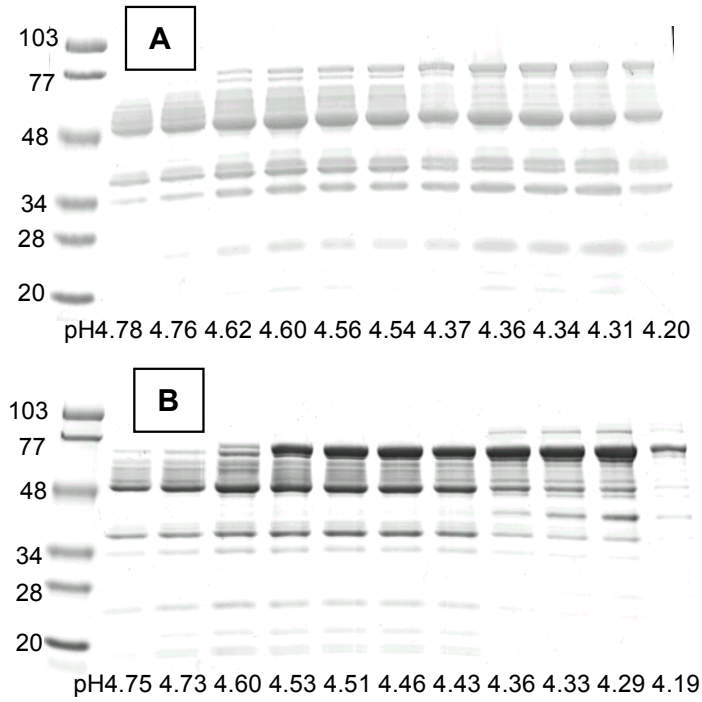

Figure 4b: SDS-PAGE of samples taken over the entire range of elution from the Mono P column. The pH of elution is indicated under each lane. Standard markers are indicated (in kDa) on the left hand side. (A) *vicilin 1°* and (B) *vicilin 2°*.

Looking in more detail at figures 4a and 4b vicilin 1° can be described as extremely heterogeneous in its composition, with no dominant species; it eluted as a wide peak, and the composition of each successive lane on the SDS-PAGE differed slightly from the previous one. Vicilin 2° eluted as two resolvable peaks, but still the composition of the eluting peaks seemed to be a mixture of both convicilin and vicilin polypeptides.

pH of minimum solubility of the two vicilin proteins was calculated from data on the amount of dissolved protein in the supernatant at each pH value. Immediately it was seen that the profiles did not differ: both vicilin 1° and vicilin 2° had a minimum amount of dissolved protein at pH 4.8-5.0 (figure 5). However, since a difference in solubility was the apparent cause of their fractionation it was decided to determine the composition of the dissolved protein (in the supernatant). The supernatant was therefore analysed by SDS-PAGE. A clear difference in composition of the soluble protein could not be seen at first sight. This is to say that the polypeptides of both convicilin (70 kDa) and vicilin (50-14 kDa) could be seen across the entire pH range of both vicilin fractions.

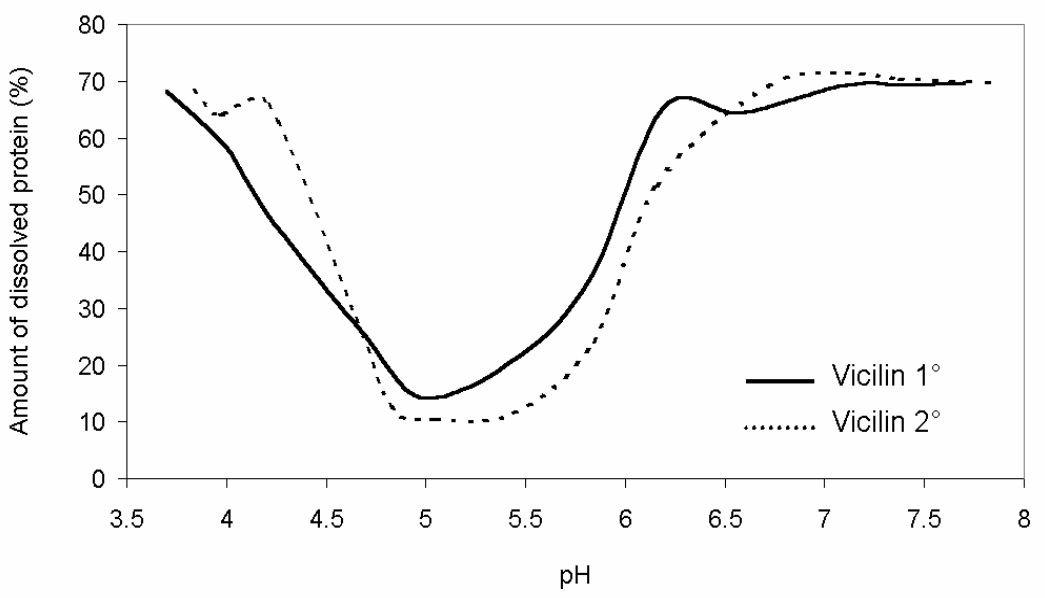

Figure 5: Plot of the percentage of dissolved protein versus pH. The percentage of dissolved protein was determined by the amount of nitrogen in the supernatant (see methods for further details). Solid line: vicilin 1°. Dashed line: vicilin 2°.

Further analysis by densitometry highlighted one feature however, that the relative amount of convicilin in the dissolved protein was reduced at the pH of minimum solubility. It reduced to approximately 30% (from ~55%) in vicilin 2°, and to <1% (from ~5%) in vicilin 1°. This means that under the conditions used for legumin/vicilin fractionation the convicilin is less soluble than vicilin. Considering that convicilin has more acidic residues than vicilin it had not been expected to be less soluble than vicilin. In fact, Casey and Sanger (*10*) commented on an unusually low solubility in acid/salt of a similar vicilin fraction that they obtained while purifying legumin. Despite the lack of apparent understanding of its behaviour, it does seem to explain why the vicilin 2° fraction that precipitated with legumin was heavily contaminated by convicilin.

Glycosylation of the two preparations, vicilin 1° and vicilin 2°, did not show any apparent difference (no results shown). For both samples the band at ~14 kDa stained positively, with a similar intensity. No other bands were visible. This was in agreement with other authors (*6*). The possibility that differential glycosylation was an additional factor contributing to the fractionation of vicilin1° from vicilin 2° was thus ruled out.

Table 2: Relative percentage of secondary structural features in native *vicilin 1°* and *vicilin 2°* at pH 7.6. Values were determined using the CD-Fit modelling program as described in materials and methods.

Secondary structure component	Relative amount (%)	
	Vicilin 1°	*Vicilin 2°*
α-helix	36	35
β-sheet	49	40
beta turns	0	0
random coil	15	7

Secondary structure of the native proteins was determined (at 20°C), with far-UV circular dichroism. Results showed that the native proteins were not dissimilar. α-helix contents were 36 and 35% for vicilin 1° and vicilin 2°, respectively. β-sheet contents 49%

and 40 % in vicilin 1° and vicilin 2°, respectively. The CD fit model used for the analysis gave zero random coil for the vicilin 1° fraction, and 15% beta turns. Vicilin 2° however was predicted to contain 18% random coil and 7% beta turns. These similarities and differences between the two vicilins were consistent for each replicate sample. Since the aim was to directly compare the two vicilins, and the model was therefore considered to have performed sufficiently. This said however, zero random coil in vicilin 1° was considered an unlikely result, and it is more likely an artefact of the sample. These results are presented in table 2.

Thermally induced unfolding of the two vicilin proteins (1° and 2°) at the secondary and tertiary level was measured with circular dichroism (CD) and differential scanning calorimetry (DSC), respectively. Figure 6 shows spectra measured at 203 nm while heating the vicilin proteins from 45 to 95°C. It can be seen that there was no obvious difference between the unfolding behaviour of these two proteins.

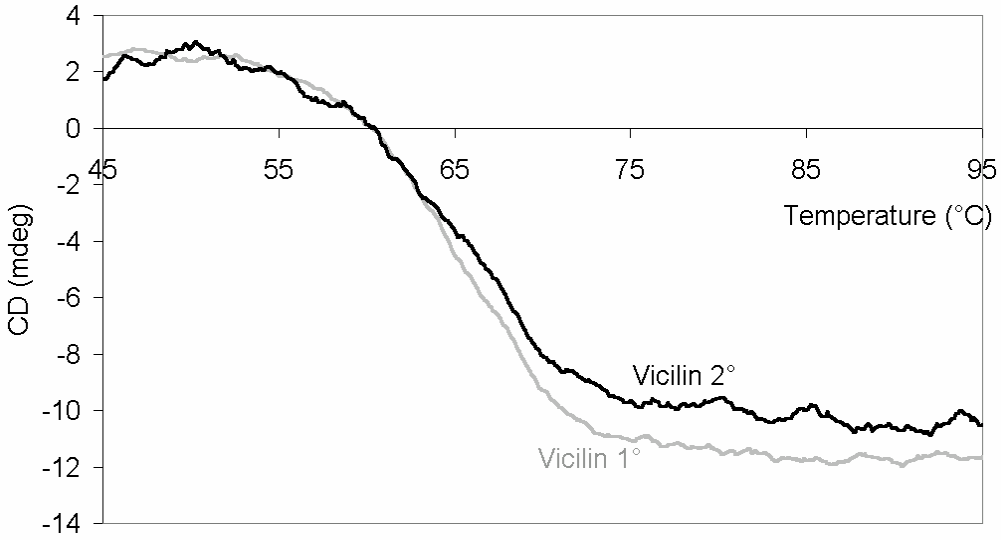

Figure 6: Thermally induced unfolding of secondary structure of *vicilin 1°* (grey line) and *vicilin 2°* (black line) as determined by far-UV scans at a constant wavelength of 203 nm, when heating from 45 to 95°C at a rate of 1.0°C/min.

Fitting these data using the JASCO Spectra Analysis software also gave no real difference between the samples. The thermal denaturation temperature (T_d) of the

secondary structure at ionic strength (I) 0.03 was ~66°C, compared to ~ 70°C at the tertiary level (see table 3).

Table 3: Thermal denaturation temperatures (T_d) of native *vicilin 1°* and *vicilin 2°* at pH 7.6, as determined by circular dichroism and differential scanning calorimetry.

Sample	T_d (°C) I = 0.03	I = 0.2	I = 0.5
Vicilin 1°	66[a] / 71.8[b]	76.4[b]	84.3[b]
Vicilin 2°	66[a] / 69.9[b]	75.6[b]	84.7[b]

[a] measured by CD at 203 nm

[b] measured by DSC

Actual differences in T_d between vicilin 1° and vicilin 2° at the tertiary level (see again table 3) were not considered important. Further, it can be said that vicilin 1° and vicilin 2° both had an increased T_d from ~70°C to ~84°C in response to an increase in ionic strength from 0.03 to 0.5.

DISCUSSION

This paper presented a purification scheme for pea globulins that resulted in the separation of the vicilin protein into two fractions, namely vicilin 1° and vicilin 2°. Initial work aimed at removing the contaminating 70 kDa polypeptide of convicilin from the vicilin proteins, and then determining if the resulting vicilins were the same or not. However, removal of the contaminating convicilin was not possible. The two vicilin fractions were thus characterised as they were.

Structurally, vicilin 1° and vicilin 2° were determined to be similar. Newbigin *et al.* (*18*) used the model of Garnier *et al.* (*30*) to predict the secondary structure of convicilin versus vicilin, and the model predicted that the N-terminal extension region would contribute an additional 15% α-helix. Our experimental determination, as well as that of Newbigin (using convicilin from transgenic tobacco), showed no such difference however. Similar determinations using soybean β-conglycinin showed the α_3 form (which is analogous to convicilin) to contain only 3% more α-helix than the β_3 form (*31*). Though the authors considered this a large difference, it was much smaller than the 15% predicted by the model (*30*). With an extensive sequence homology along the core regions of convicilin and vicilin (*17*), these two proteins can well be folded in a similar way, hence why so little structural difference was detected between the two vicilin preparations. The presence of convicilin as a heavy contaminant in the vicilin 2° preparation was not detected to influence the solubility profiles, or the thermal denaturation behaviour of the protein preparations either.

As a polypeptide, convicilin is genetically distinct from vicilin (*18*, *32*), in the sense that it has its own encoding genes. After 1980 when Croy *et al.* (*16*) purified convicilin it also became considered as a distinct, separate, third globulin protein of pea. To compare pea with soybean proteins, the two polypeptides that are similar to convicilin are those that are denoted as the α and α' subunits of β-conglycinin. Though their gene families are related to each other (*33*), these two polypeptides are genetically distinct from the β-subunit of β-conglycinin (*34*). The β-subunit is that which is similar to the 50 kDa vicilin polypeptide of pea, yet it does not undergo post-translational proteolysis (*35*). When

defining the polypeptides under these terms it becomes apparent that genetic distinctness has been considered differently for pea, than for soybean. Because, although genetically distinct from each other, the α and α' and β-polypeptides of soybean have always been denoted as subunits of β-conglycinin. Conversely, convicilin has become defined as a separate third protein in pea.

If convicilin is indeed a separate, third globulin of pea, distinct from vicilin, we would have expected two peaks of denaturation to have been apparent. This, however, was certainly not the case at the secondary or tertiary level. Only when performing chromatofocusing was more than one species of protein apparent by the resolution of vicilin 2° into two peaks, though still both peaks contained a mixture of convicilin and vicilin polypeptides.

Considering also how vicilin 1° and vicilin 2° were obtained, we have commented already on the unusual solubility behaviour of convicilin in acid/salt conditions. Though this explained why so much convicilin was in the *legumin enriched* precipitate, it did not give reason to the concomitant vicilin polypeptides. If we consider for a moment however, that convicilin and vicilin polypeptides form heterogeneous oligomers, the concomitant vicilin would be explained.

In view of the points presented in this discussion we propose that the consideration of convicilin as a separate, third globulin of pea has been wrongly interpreted within the literature. Convicilin is a distinct polypeptide of the *Pisum* vicilin gene family, but should be further denoted as the α-subunit of the salt extractable pea protein vicilin. Its possible oligomeric inclusion in pea vicilin should now be taken into consideration in structure-function studies aimed at developing pea proteins as a food ingredient.

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Chapter 3

CHARACTERISATION OF PEA VICILIN PART II:

CONSEQUENCES OF COMPOSITIONAL HETEROGENEITY

ON HEAT-INDUCED GELATION BEHAVIOUR

ABSTRACT

The gelling characteristics of two vicilin fractions from pea (*Pisum sativum* L.) were compared over a range of pH and salt conditions after preliminary results showed that despite having equal opportunity to unfold, and expose hydrophobic residues, they had different minimum gelling concentrations (at pH 7.6). Furthermore, at this pH one fraction formed turbid gels and the other formed transparent gels. The fraction that formed transparent gels contained a substantial amount of the 70 kDa α-subunits of vicilin, and thus it was hypothesised that the highly charged N-terminal extension region on these 70 kDa subunits hinders gelation of this vicilin fraction at pH 7.6, I 0.2 due to repulsion of the net negative charge. The experiments designed to test this hypothesis are presented and discussed in this paper and prove that the hypothesis was true, and offers the possibility to control or modify the gelation behaviour of vicilin based on information of its subunit composition.

KEYWORDS: *Pisum*; storage proteins; heterogeneity; N-terminal extension region; aggregation; gelation; turbid and transparent gels

INTRODUCTION

Plant proteins are an important functional ingredient in many processed food products. Beyond improving the nutritional value of the food, protein isolates also impart texture to food (*1*). The formation of a protein gel network upon heating is an important texturising technique. Since these networks essentially result from a balance of both attractive and repulsive protein-protein and protein-solvent interactions, their formation can be influenced by changes in the protein, and/or the environment (*2*). With this in mind, it is reasonable to consider that the molecular heterogeneity of globular proteins, that Utsumi *et al.* (*3*) referred to as seeming to be an inherent property of the major storage proteins of legume seeds, may cause them to exhibit functional heterogeneity. It was seen in the literature that functional heterogeneity of leguminous proteins has only really been addressed for the major soybean globulins, glycinin and β-conglycinin (*4-8*). Most likely this is because soybean is used so extensively as a food ingredient and such research enables its further development for new applications. Development of alternative leguminous plant proteins, meanwhile, is left lagging behind. A potential alternative to soybean is pea (*Pisum sativum* L.). Its two major globulin proteins are legumin and vicilin. Heat-induced gelation of pea legumin has been dealt with in another paper. The current paper will address the heat-induced gelation of vicilin.

Vicilin is composed from different combinations of heterogeneous subunits of ~50 kDa and ~ 70 kDa (*9*). The polypeptides that are denoted the ~50 kDa subunits can be split at one or both of two potential cleavage sites (*10-11*), though the subunits remain intact under non-dissociating conditions (*12*). The larger subunits (~70 kDa) have a core region that is highly homologous with the uncleaved 50 kDa subunit, yet is distinguished by the presence of a highly charged, acidic, N-terminal extension region. Being a 166-amino acid sequence (*13*) this extension region constitutes approximately 20% of the total subunit. This distinguishing feature makes these subunits very similar to the α and α' subunits of soybean's vicilin-like protein, β-conglycinin.

When associated into trimeric combinations, the vicilin subunits cause a considerable compositional heterogeneity. Considering only the charge heterogeneity at the potential

cleavage sites of vicilin polypeptides, Gatehouse *et al.* (*12*) already came to the idea that molecules with different physical properties could well be expected. The impact of the hydrophilic extension region has not been considered however. Its effect on functional properties was referred to, by Casey (*14*), as being unclear, because the N-terminally extended subunits have never been purified in sufficient amounts for detailed investigation. Now, in this paper, we present results that show that a high amount of these subunits in the vicilin preparation has a distinct effect on the heat-induced gelation of this pea protein.

MATERIALS AND METHODS

Preparation of enriched protein fractions, and purification of two vicilin fractions. The preparation of two vicilin fractions, later named vicilin 1° and vicilin 2° was previously described in detail (*9*). In this paper the vicilin fractions were prepared in the same way, but from two pea cultivars: Solara and Supra (Cebeco Seeds, Lelystad, NL, grown and harvested in 1998).

Gel electrophoresis. Samples were prepared by mixing the protein sample 1:1 with sample buffer (1.4 ml distilled water, 2.0 ml 0.5 M Tris-HCl at pH 6.8, 2.0 ml 10% (w/v) SDS, 2.0 ml glycerol and 0.4 ml 0.05% (w/v) bromophenol blue). 10-20% linear gradient, Tris-HCl Ready Gels (Bio-Rad) were used and 10-20 µl of sample was applied to each well (the amount judged according to the value of absorbance at 280 nm as the protein eluted from the chromatography column). Low molecular weight protein standards, ranging from 94 to 14 kDa (Amersham Biosciences, Uppsala, Sweden) were made according to the instructions, and 10 µl was applied to each well. Gels were run at a constant 200 V. Staining was done using Coomassie Blue R-250 Bio-safe stain (Bio-Rad).

Minimum gelling concentration was determined by making 3 ml protein solutions of 8-16% (w/v) concentration, at pH 7.6, in 75 mM potassium phosphate buffer. All samples were heated, (in sealed tubes to avoid evaporation), in a boiling water bath for 30 minutes. Samples were cooled to room temperature for 1 hour, then stored at 4°C overnight. Next day the tubes were inverted and the samples that did not flow were considered to have gelled, hence was determined the minimum gelling concentration.

Gel sample preparation. Samples were prepared with 75 mM potassium phosphate buffer, pH 7.6, in 2.0 ml eppendorfs. After dissolving the protein the pH was adjusted with 0.5M NaOH or HCl, and then samples were left mixing in a test-tube rotator for 1 hour at room temperature. The eppendorfs were locked into a heating block (to prevent the lids opening), and samples were heated in a boiling water bath for 20 minutes, and

subsequently cooled at 4°C overnight before being further analysed. Further details about the specific sample concentration and pH are as follows:

- At pH 6.1 sample concentration was 10% (w/v)
- At pH 3.8 sample concentration was 10, 8, 6, 4 and 2 % (w/v)
- At pH 7.6 in the presence of salt (from 0 to 1 M NaCl) sample concentration was 10% (w/v)
- At pH 7.6 mixed samples were made with legumin: vicilin ratios 1: 0.22, 1: 0.57 and 1: 1.2. The final sample concentration was always 11% w/v.
- At pH 7.6 samples made with the protein from the overlap region were 14% (w/v) (which was predetermined as the minimum gelling concentration)
- At pH 7.6 samples made with the legumin were 10.5% (w/v) (which was predetermined as the minimum gelling concentration)

Texture Analyzer (TXA). Samples were prepared as described above, using a 16% (w/v) concentration at pH 7.6, no added salt. The gel force was determined with a probe that was moving at only 0.01 mm/min, for a total depth of 6 cm from the trigger point. The trigger point (to start the measurement) was 0.01 N. All TXA tests were performed at room temperature, with triplicate samples, and the results are presented as the average of all three measurements.

Circular Dichroism. Loss of secondary structure upon heating was monitored using the Jasco J-715 spectropolarimeter (Jasco corporation, Japan) by heating the protein from 40 to 90°C at 10°C intervals and measuring in the far-UV range (260-190 nm) at each interval. Each spectrum was recorded as the average of 30 accumulations. Sample concentration was 0.2 mg/ml, sample buffer was 10 mM potassium phosphate, pH 7.6, and all samples were filtered with a 0.2 μm sterile filter (Schleicher & Schuell, Keene, NH, USA) prior to analysis. Heating rate was 1.0°C/min, scan speed was 100 nm/minute, cell pathlength was 0.1 mm. Data were baseline corrected (from the data of heating buffer alone), and the relative percentages of secondary structure were calculated using a non-linear regression procedure as previously described in detail (*15*), and the results were presented as an average of three replicates.

Chromatofocusing. Samples were prepared by dissolving the purified proteins, vicilin 1° and vicilin 2°, in starting buffer (0.025 M Tris-HCl with saturated imidazole) at pH 7.1 at 2 mg/ml concentration. Samples (5 ml) were gently stirred for 2 hours and filtered through a 0.2 μm sterile filter (Schleicher & Schuell, Keene, NH, USA) prior to loading onto the column. The Mono P column (Mono P HR 5/20, Amersham Biosciences, Uppsala, Sweden) was treated as instructed in the manual. Firstly, it was run with the starting buffer until the pH was stabilised at pH 7.1. Secondly, polybuffer 74, pH 4.0 (prepared according to the instructions) was run through the column until the pH reached 4.0. Lastly, re-running in starting buffer took the pH to 7.0, and the column was then ready for sample application. Flow rate was 0.5 ml/min at all times. 3 ml of sample was applied (6 mg protein load) and the eluted protein was detected at 280 nm, and collected in 300 μl aliquots. All samples were run in triplicate and the elution profile and further analysis of one sample is presented

RESULTS

The two vicilin fractions, vicilin 1° and vicilin 2°, were previously obtained in a salt fractionation procedure of the salt-extracted globular proteins of pea. In the previous paper where the method is described in detail (*9*), there is also a detailed characterisation of the two vicilin fractions, vicilin 1° and vicilin 2°. We showed that despite differences in the subunit composition (specifically the ~70 kDa subunit) of the native proteins, their thermal denaturation at pH 7.6 was not different. This however was not the case with their gelation behaviour. Initial experiments showed that vicilin 1° had a minimum gelling concentration of 10% (w/v) concentration, yet vicilin 2° needed a minimum 14% (w/v) concentration to gel. Furthermore, vicilin 1° formed turbid gels, yet those of vicilin 2° were transparent.

Having noted the different minimum gelling concentrations of the two vicilin proteins, the loss of secondary structure upon heating was looked at in more detail because if vicilin 2° unfolded less than vicilin 1°, thus exposing fewer hydrophobic residues, its higher minimum gelling concentration could be understood. Figure 1 shows though that despite some differences in their native state the thermal unfolding of the two vicilins proceeded in the same way. Moreover, at 90°C, the unfolded proteins had negligible differences in the relative amount of residual structure. Thus, with equal opportunity to unfold, and expose hydrophobic residues, the highly charged N-terminal extension region on the 70 kDa subunit, (previously denoted as the α-subunit (*9*)), was hypothesised to hinder vicilin 2° gelation at pH 7.6, I 0.2 due to repulsion of the net negative charge. Further experiments were designed to test this hypothesis.

Gel strength comparisons. The first tests with the Texture Analyzer (TXA) were done with gels made from the vicilin fractions from two different pea cultivars, Solara and Supra, using 16% (w/v) sample concentration, pH 7.6. All samples were slightly brown/orange coloured solutions before heating, and after heating the vicilin 2° gels were transparent, and vicilin 1° gels were turbid. The overall result after probing these gels with the TXA (figure 2) showed that the turbid gels were stronger than the transparent gels. Furthermore, the gels made with vicilin 1° from cv. Supra were stronger than those

from cv. Solara. No varietal difference was observed for vicilin 2° gels. Visually, there was a notable difference between the gels as they were probed. Vicilin 2° gels appeared to break into small pieces, yet vicilin 1° gels were in effect squashed by the probe.

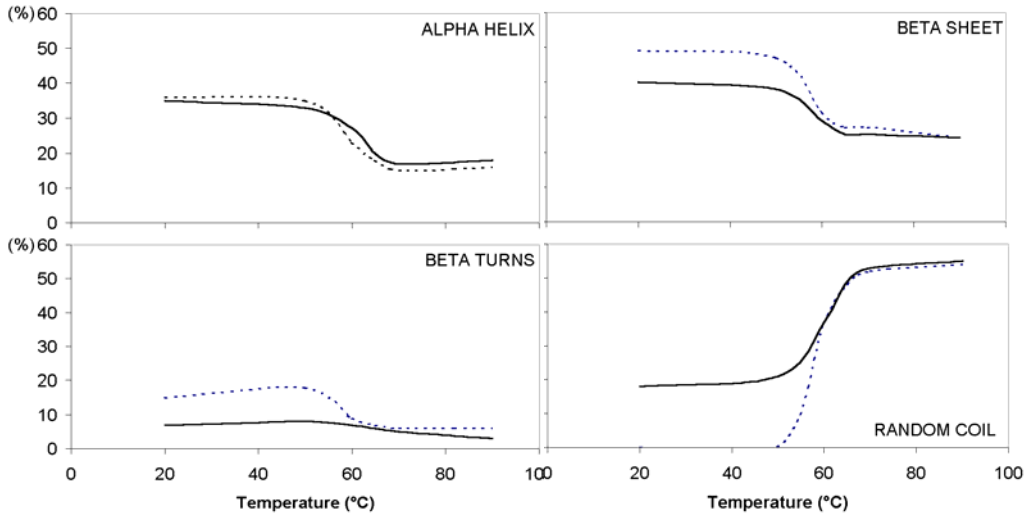

Figure 1: Plot of the relative percentage of secondary structure against temperature, for *vicilin 1°* and *vicilin 2°* from the pea cv. Solara, showing the pattern of structural loss upon heating for *vicilin 1°* (solid line) and *vicilin 2°* (dashed line).

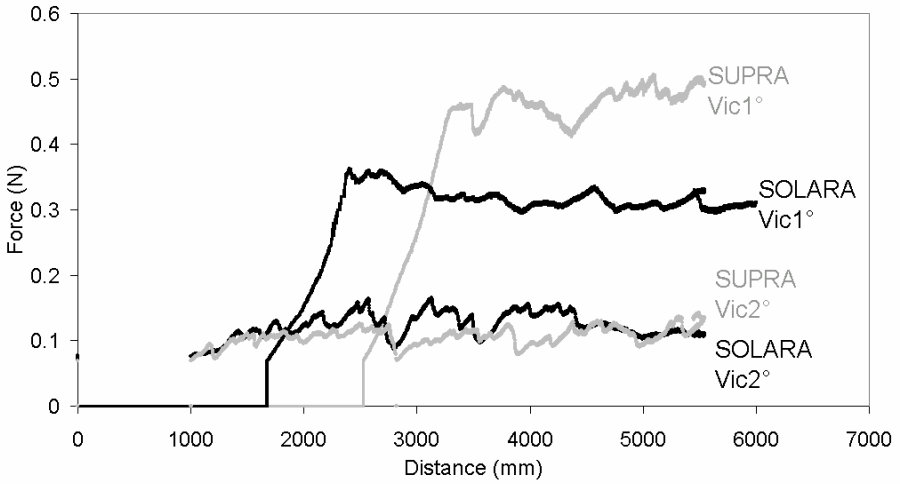

Figure 2: Plot of force against distance for a probe going into 16% vicilin gels at a speed of 0.01 mm/minute. Results are shown for vicilin 1° and vicilin 2° for two pea cv. - Solara (black) and Supra (grey).

Specific features of the plots of force against distance in figure 2 can be explained as follows:

Vicilin 2° gel measurements appear to start sooner than the other gels.

Data recording started the moment that the trigger force (0.01 N) was asserted on the probe. With such a low trigger force the measurement started as soon as the sides of the probe touched the most upper/outer part of the meniscus. Vicilin 2° gels had a flatter meniscus than vicilin1° gels, so the distance to pass through the meniscus and into the bulk of the gel was shorter for vicilin 2° than for vicilin 1°.

Vicilin 1° gel measurements have a large initial increase in force.

The large increase in force exerted on the probe at 1500 and 2500 mm is again an effect of the probe passing through the meniscus into the gel, but this time into the stronger gels of vicilin 1°. The difference between the distances at which the bulk gel is reached is an artifact of the samples, and is not a characteristic difference between the two vicilin 1° samples.

Varietal differences of the protein preparations. Due to the observed varietal difference in the TXA measurements (for vicilin 1°) we characterised the vicilin proteins using chromatofocusing. This technique was selected, based on previous experience, as being effective at highlighting compositional heterogeneity of the vicilin proteins. The vicilin 2° preparations eluted from the Mono P column over the same pH range (figure 3, upper panel), but vicilin 1° from cv. Supra was seen to be less acidic than that from cv. Solara (figure 3, lower panel). Being less acidic was attributed to this protein containing fewer α-subunits. SDS-PAGE of the Mono P fractions as they eluted from the column are shown as inserts within figure 3. It can be seen that vicilin 1° cv. Solara had a greater relative amount of the α-subunit throughout its profile. Being more acidic it carried a larger net negative charge at pH 7.6, thus was not as well able to form protein-protein interactions as its equivalent fraction from cv. Supra.

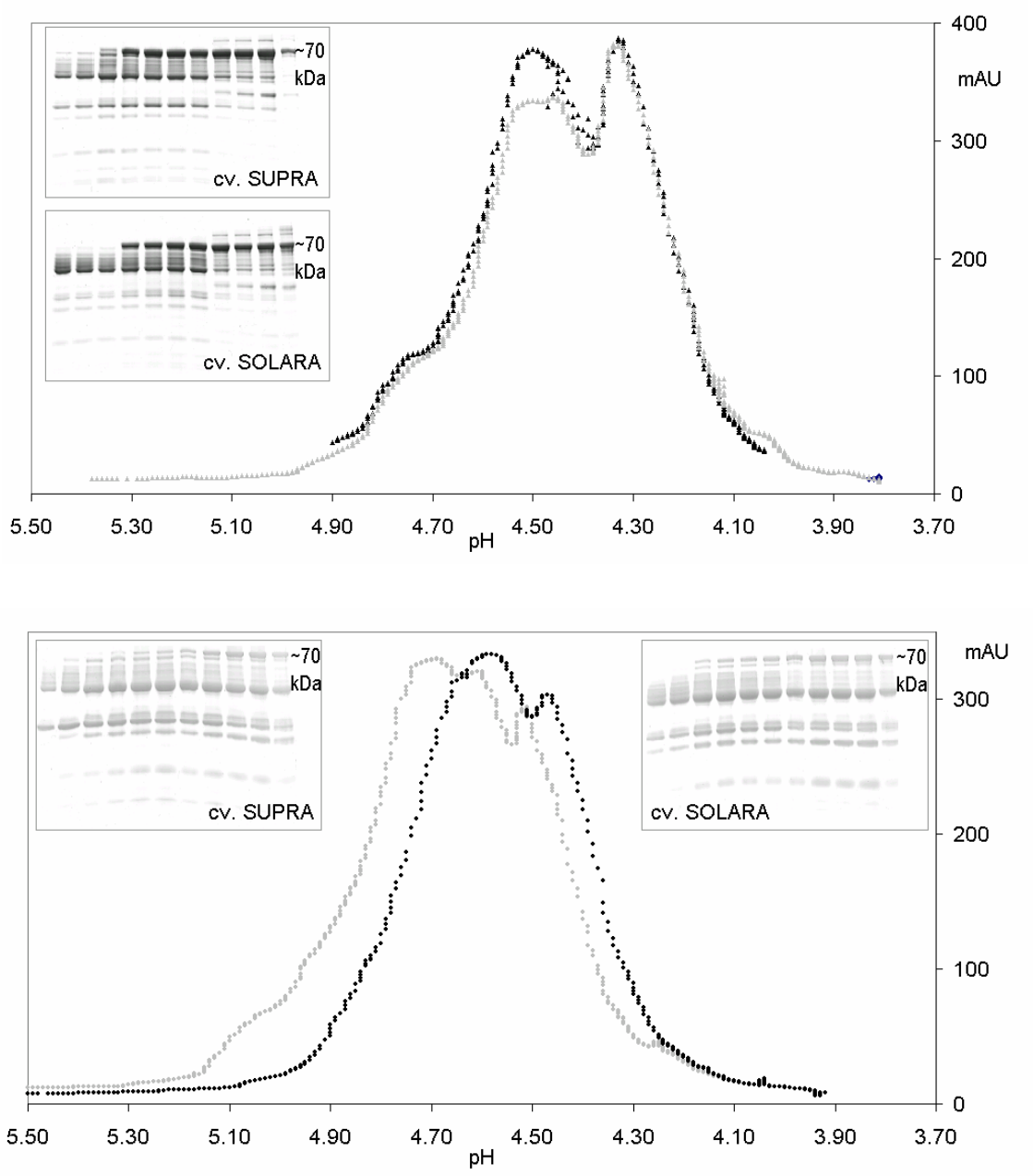

Figure 3: Elution profiles of *vicilin 2°* (upper panel, triangles) and *vicilin 1°* (lower panel, circles) from the Mono P column under a linear pH gradient (pH 7 to 4), for the two cv. Solara (black) and Supra (grey). SDS-PAGE profile of the eluting protein is inserted aside the chromatograms.

pH induced gelation. As described earlier, the α-subunit of vicilin has a highly charged acidic N-terminal extension region that carries a net-negative charge at pH 7.6. In order to remove the excessive repulsive forces the pH of the sample was reduced to pH

6.1, which is the theoretical pI of the α-subunit (as calculated based on its published amino acid sequence (*16*)). Samples (10% w/v) of vicilin 1° and vicilin 2° at pH 6.1 were visually different before heating, as described in table 1. The vicilin 2° sample was slightly turbid, but the sample did not settle, even after 30 minutes, whereas vicilin 1° had visible aggregates within a clear solution, and these aggregates settled within a few minutes. To be consistent both samples were vortexed immediately before heating. After heating and cooling both samples had formed brilliant white gels. For vicilin 2° the control sample (pH 7.6) was a slightly orange/brown coloured clear solution, and for vicilin 1° it was grey/white opaque gel. An additional comment on the gels formed at pH 6.1 is that when they were squashed the vicilin 1° gel had a homogeneous, paste-like consistency, but the vicilin 2° gel broke immediately into small pieces of gel ~2 mm^3, (as had been observed for the vicilin 2° samples probed with the TXA). These small pieces had a homogenous appearance when squashed.

Table 1: Description of the visual appearance of 10% (w/v) *vicilin 2°* and *vicilin 1°* samples before and after heating at pH 7.6 and pH 6.1, in the absence of added salt. All samples were cv. Solara.

Sample	pH 7.6, no added salt		pH 6.1, no added salt	
	Before heating	*After* heating	*Before* heating	*After* heating
Vicilin 2°	Transparent solution	Transparent liquid	Slightly turbid suspension	Brilliant white particle gel
Vicilin 1°	Transparent solution	White/grey opaque gel	White aggregates[a]	Brilliant white smooth gel

[a] sample was vortexed immediately prior to heating to re-suspend the aggregates. These aggregates dissolved upon heating.

Subsequent samples were made at pH 3.8 so that the highly ionisable N-terminus would be neutralised. At this pH samples were also made at 10, 8, 6, 4 and 2% (w/v) concentration. Before heating both the vicilin proteins were very well suspended: the samples were turbid and off-white, with no apparent settling after 20 minutes. Regardless, all samples were vortexed immediately before heating. After cooling the samples were slightly different for the two vicilin preparations. Vicilin 2° gels all had an off-white,

opaque appearance, while vicilin 1° gels were noticeably more white, but for both proteins the samples of 10, 8 and 6% (w/v) concentration had gelled. No further results are presented.

Salt induced gelation. Adding NaCl was chosen as a means of shielding the net negative charges on the extension region. For this test, samples were made at pH 7.6, and only at 10% (w/v) concentration. Before heating there was no observable difference in any of the samples, with respect to both the protein and the added salt. After heating, the different effects of added salt were apparent though (see table 2 for full details). The most noticeable results were with vicilin 2°. Addition of 0.2 M salt was the lowest concentration to have any effect on the sample - the heated sample was turbid, though liquid. Addition of 0.5M NaCl had caused phase separation, and 1.0 M NaCl had induced gel formation. As described in table 2, this latter gel had a brown/cream turbid appearance, and was firm and smooth.

Table 2: Description of the visual appearance of 10% (w/v) *vicilin 2°* and *vicilin 1°* samples before and after heating at pH 7.6, in the presence of added salt (from 0 to 1 M NaCl). All samples were cv. Solara.

Added NaCl	Vicilin 2° pH 7.6		Vicilin 1° pH 7.6	
	Before heating	*After* heating	*Before* heating	*After* heating
0 M	Transparent solution	Transparent liquid	Transparent solution	White/grey opaque gel
0.1 M	Transparent solution	Transparent liquid	Transparent solution	White/grey opaque gel
0.2 M	Transparent solution	Turbid liquid	Transparent solution	White/grey opaque gel
0.5 M	Transparent solution	Phase separated	Transparent solution	White/grey opaque gel[a]
1.0 M	Transparent solution	Brown/cream smooth gel	Transparent solution	White/grey opaque gel[a]

[a] the gels had a white tip, assumed to be due to protein that precipitated out during heating and settled to the bottom of the eppendorf.

By contrast, all the vicilin 1° samples had gelled, and all had a white/grey opaque appearance. The only noticeable effect of salt on vicilin 1° gelation was the appearance of

a white tip on the bottom of the gels formed in the presence of 0.5 and 1.0 M NaCl. As stated underneath table 2 these white tips were believed to be due to the settling of protein that aggregated upon heating.

Mixed vicilin/legumin gels. Pea legumin at pH 7.6 formed opaque gels, so vicilin 2° and 1° were added to this protein to determine if the presence of the α-subunits would effect the opacity. Addition of vicilin 1° had no apparent effect: the samples remained opaque, and all the samples gelled (no results shown). By contrast though in the samples with a legumin: vicilin 2° ratio of 1: 0.57 and 1.2, vicilin 2° reduced the opacity after heating and prevented gel formation. The sample with a legumin: vicilin 2° ratio of 1: 0.22 gelled however, and the gel was opaque like that of legumin alone (which is shown in figure 4).

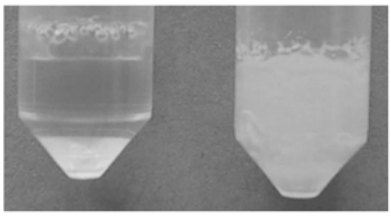

Figure 4: Picture of gels made from "overlap" region (left) and legumin (right). All protein was from cv. Supra.

The gel made with the "overlap" region was completely transparent, and resembled the gels formed by vicilin 2° alone. The protein from the overlap region also had the same minimum gelling concentration (14% w/v) as vicilin 2°. The overlap region was obtained during purification of the pea globulins, and it is a mix of the last eluting part of vicilin and the first eluting part of legumin (for full details refer to (*9*)). SDS-PAGE profile of the overlap region (under non-reducing conditions) showed bands at ~70 kDa (α-subunit vicilin), 60 kDa (legumin subunit) and 50 kDa (intact subunit vicilin), constituting 45, 34 and 21% respectively of this protein fraction. The small vicilin fragments (<50 kDa) were too weakly stained for inclusion in this analysis.

DISCUSSION

The results presented in this paper strongly indicate that the hypothesis that the highly charged N-terminal extension region on the α-subunit of vicilin hinders vicilin 2° gelation at pH 7.6, I 0.2 due to repulsion of the net negative charges, is true.

First of all, vicilin 2° samples at pH 7.6, I 0.2 were all transparent after heating, while those of vicilin 1° were turbid. For the gelled samples (at 16% w/v concentration) transparency versus turbidity is believed to indicate different gel networks. Generally, transparent globular protein gels are considered to reflect a fine network structure, composed of linear aggregates of heat denatured proteins (*17-24*) and turbid gels tend to have randomly agglomerated heat-denatured molecules (*17-18*, *20-21*). Though our observations do not allow us to define the type of networks in our gels, it is undeniable that vicilin 1° and vicilin 2° form distinct gel types at pH 7.6, I 0.2. Having a large repulsive area on the α-subunits of vicilin 2° there could well be a reduced aggregate agglomeration relative to vicilin 1°, hence the transparent gel. In a series of studies Maruyama *et al.* (*6*) came to this result with respect to the α-subunits of soybean β-conglycinin. These authors used a normal recombinant protein system that expressed subunits that were not glycosylated, and mutant recombinants that also lacked the extension region. Comparing the subunits enabled the functionality of the N-terminus to be studied without any interference from glycosylated residues. Overall, the extension regions on the α/α'-subunits of β-conglycinin were shown to hinder heat-induced association at pH 7.6 (*6*). The highly charged extension region was explained to favour protein-solute interactions, thus keeping the protein in solution at low ionic strength, and hindering protein-protein interaction after heat-denaturation of soybean's β-conglycinin. Such a hindrance of protein-protein interaction was evident to a small extent in the results from the Texture Analyzer (TXA) presented in this paper. Not only was there the difference between vicilin 1° and vicilin 2° (which differ greatly in the amount of α-subunits), but there was also a difference between the vicilin 1° gels from the two cultivars. Again, the difference appeared to be related directly to the amount of α-subunits present.

Secondly, lowering the pH to 6.1, the theoretical pI of the α-subunit of pea vicilin, gave a significant result of gel formation of vicilin 2° at 10% (w/v) concentration. Logically, gels were formed with both vicilin 1° and vicilin 2°, but the gelation of vicilin 2° at only 10% (w/v) concentration speaks volumes for the effect of removing the net negative charge from the N-terminal extension region. Acidifying the samples (pH 3.8) enabled gels to be formed from sample concentrations as low as 6% (w/v) concentration. At this pH no significant difference was observed between the gels of the two proteins. This can be explained because the N-terminus was neutralised, thus not acting repulsively, and the remainder of the 70 and 50 kDa polypeptides are highly homologous, so with a similar net charge. Thus, at this pH there was no great difference between the two vicilins with respect to the driving forces of protein-protein interaction and gel network formation. Again, to compare these results with the equivalent protein in soybean, β-conglycinin, Maruyama *et al.* (*6*) concluded that at pH 3.8 protein-protein interactions occurred after heat denaturation because the carboxyl groups were neutralised, and the repulsive force substantially reduced.

Lastly, returning to pH 7.6 where there was a dominant net negative charge on the vicilin 2°, distinct differences between the samples were highlighted when salt was added. Though qualitative, the results in table 2 gave further evidence that when the repulsive negative charges from vicilin 2° were "removed", the protein was able to gel at lower concentrations. Similarly, when heating solutions of only 0.5% (w/v) β-conglycinin at pH 7.5 no aggregate formation was detected unless salt was added (*25*), even though (as we showed here for vicilin) the protein denatured and exposed its hydrophobic residues. The authors, as we are doing here, suggested that mutual repulsion of the hydrophilic domains was superior to the hydrophobic interaction, and thus inhibited aggregation.

A final observation where the distinctive behaviour of the N-terminal extension region was apparent, was when vicilin was mixed with legumin. When vicilin 2° constituted 50% the sample had a reduced opacity after heating, and it was prevented from gelling, and no such changes were observed in the presence of vicilin 1°. Moreover, gels made from the "overlap" region, (a sample with an approximate legumin: vicilin

ratio of 1: 2), were transparent. Overall, these results again indicated that when present in sufficiently high amounts the α-subunits of vicilin cause a transparent gel network to form. In soybean (*26*) it was shown (at pH 8.0) that when added to samples of soybean glycinin (in high enough amounts), β-conglycinin formed an electrostatic complex with the glycinin. Then, when heating, the highly charged character of the β-conglycinin suppressed aggregation and a clear solution remained. Adding salt above 0.4 M shielded this net negative charge on the β-conglycinin and the basic subunits of glycinin under went heat aggregation, as they did in the absence of the β-conglycinin. In their experiment an electrostatic complex was formed between the two soybean proteins. Whether or not such a complex forms between pea vicilin and legumin has not been studied in this paper. Regardless, the results for both pea and soybean again exemplified very well the ability of the N-terminal extension region to overpower hydrophobic interactions and inhibit or hinder heat-induced aggregation.

To summarise, the results presented in this paper show that the large hydrophilic N-terminal extension that is present on the α-subunits of vicilin has a distinct effect on the protein-protein interactions of the heat-denatured protein, when present in large amounts. In near-neutral conditions its negative charge reduces the gelling ability of the protein (with respect to the concentration needed), yet it enables a transparent gel to be formed. In acidic conditions it has no effect, and so compositional heterogeneity does not effect vicilin functionality. The similarity between the effect of the respective α-subunits on pea vicilin gelation and soybean β-conglycinin gelation was remarkable. Overall, it lead us to the conclusion that the distinct behaviour of the N-terminally extended α-subunits of pea vicilin, (and other similarly composed proteins), can be exploited when trying to modify or control the gelation behaviour of this protein at near-neutral conditions.

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Chapter 4

HEAT-INDUCED GELATION OF PEA LEGUMIN: A COMPARISON WITH SOYBEAN GLYCININ

ABSTRACT

Gel network formation of pea legumin was monitored via dynamic rheological measurements in the absence and presence of the thiol-blocking reagent *N*-ethylmaleimide, at different rates of heating and cooling. Overall, it was shown that pea legumin gel formation was not effected by changes in the heating rate, and the two differently heated samples were unaffected by the addition of 20 mM NEM, which indicated that disulphide bonds were not essential within the network strands of these legumin gels. However, slowly cooling the legumin samples caused disulphide bonds to become involved within the network: this was observed by a large increase in gel strength that was then substantially reduced when repeating the sample in the presence of NEM. These experiments were repeated with soybean glycinin in order to determine whether a common model for gel formation of legumin-like proteins could be built, based upon molecular reasoning. The two proteins responded in the same way to changes in the conditions used, but when applying a procedure of reheating and recooling the gel networks responded differently. Pea legumin gel networks were susceptible to rearrangements that caused gel strengthening, yet glycinin gel networks not. It was concluded that the same physical and chemical forces drove the processes of denaturation, aggregation and network formation, but that pea legumin and soybean glycinin gel networks had structurally different building blocks however. A model of gelation aimed at texture control therefore requires additional information.

KEYWORDS: *Pisum*; legumin; glycinin; gelation; small deformation rheology; texture control

INTRODUCTION

Globular proteins from various sources (in the form of isolates) play an important role in many foodstuffs, both because of their nutritional value, and their contribution to food texture (*1*). These texture contributions come from the network structures created by the proteins. Since gelation is one of the most important functional properties of the globular proteins used to modify food texture (*2*), it should be important to understand which factors determine the gel network are how they are effected by processing parameters. Such understanding would enable better control of food textures.

Protein isolates from soybean predominate in the market, though presently there is a trend for alternative protein isolates having similar functional and nutritional properties as soya (*3*). A potential alternative plant protein in Europe is pea (*Pisum sativum* L.). Just as for soybean it contains two major globulin proteins, namely legumin and vicilin. Pea vicilin functionality has been dealt with in a previous paper (*4*), so only legumin will be given further consideration in this paper. Legumin is a polypeptide of ~60 kDa, though this polypeptide is commonly denoted as a legumin subunit that assembles into higher molecular weight oligomers. A feature of legumin subunits is that they split into acidic (40 kDa) and basic (20 kDa) polypeptides via disulphide bond reduction. Similar subunits compose the legumin-like proteins of *Glycine max*. (*5*), and *Vicia faba* (*6*). In all cases the disulphide-bonded acidic and basic polypeptides are formed when the protein precursor is proteolytically processed in the plant (*7*). As a protein type legumins, in contrast to vicilins, are recognised for their cysteine content: pea and fababean legumin contain approximately 5 residues per 60 kDa subunit, and soybean glycinin approximately 8.

Studies on the emulsification and foaming (*8-12*) and gelation (*12-14*) of pea legumin have been reported, but none of these studies compared pea legumin functionality to that of its related protein in other leguminous plants. Even though the literature exploring the functional properties of alternative leguminous protein sources is quite extensive (*15-21*) it is difficult to compare and contrast the functional properties as often the experimental conditions used are different. Such differences make it difficult to identify the basis

needed to build a common model for gelation of legumes, and in turn hampers the introduction of alternative plant protein sources as a direct replacement for soybean.

This paper presents results on gelation of the legumin protein from peas, and demonstrates how gel formation was affected by the heating process, in both the absence and presence of the thiol-blocking reagent *N*-ethylmaleimide. The results are compared with soybean glycinin gels formed under the same conditions in order to determine whether a common model for gel formation can be built, based upon molecular reasoning.

MATERIALS AND METHODS

Preparation of enriched protein fractions. Legumin was purified from peas (*Pisum sativum* L.), cv. Solara (Cebeco Seeds, Lelystad, NL, grown and harvested in 1998), by a non-denaturing fractionation procedure adapted from the method of Kyoro and Powers (*11*) and Bora *et al.* (*14*). Peas were milled in a Waring commercial blender (New Hartford, Connecticut, USA) 2:1 (w/w) with dry ice to avoid any heat denaturation of the proteins. Salt-soluble proteins were then extracted into a 100 mM Tris-HCl buffer, pH 8.0, with a flour to buffer ratio 1:10 (w/v). Extraction time was 1 hour at room temperature and extract was collected by centrifugation (11 900 x g, 10°C, 25 min). Isoelectric precipitation, pH 4.8, was used to isolate the globulin proteins from the extract; the pH was adjusted with 1 M HCl. Precipitated protein was left for 2 hr, 4°C before it was collected by centrifugation (11 900 x g, 10°C, 25 min). Washing the protein pellet with water (pellet to water ratio 1:10 w/v) removed unwanted albumin proteins. Again the pellet was collected by centrifugation (11 900 x g, 4°C, 25 min). The crude pellet was suspended in the extraction buffer, pH 8.0 (10 mg/ml) and dialysed at 4°C against McIlvaine's buffer (0.2 M Na_2HPO_4 + 0.1 M citric acid, containing 0.2M NaCl), pH 4.8. Sample to buffer ratio was 1:20, and the dialysis buffer was changed three times over a 24h period. Centrifugation of the sample (18 900 x g, 4°C, 25 min) collected a precipitated fraction (referred to as *legumin enriched*).

Purification of legumin. Freeze-dried *legumin enriched* fraction was suspended in buffer A (35 mM potassium phosphate, containing 0.075 M NaCl), pH 7.6, at a concentration of 25 mg/ml (which gave a suitably low final sample viscosity for loading onto the column). Because the legumin enriched isolate was only a suspension, and it was centrifuged (11 900 X g, 4°C, 25 min) before further use. Clear l*egumin enriched fraction* (1200 ml) was loaded onto a DEAE Sepharose Fast Flow column (5 cm diameter, 343 ml volume; Amersham Biosciences, Uppsala, Sweden), previously equilibrated with buffer A. Elution was performed with a linear salt gradient (0.075 - 0.5 M NaCl) in the same potassium phosphate buffer, over 6 column volumes. The eluate was monitored at 280 nm, and 15 ml fractions were collected and analysed for purity on an SDS-PAGE gel (Bio-Rad Ready Gel Tris-HCl Gels, 10-12% linear gradient) under non-reducing

conditions. Fractions containing only the band belonging to legumin (~60 kDa) were pooled together. Pooled fractions were desalted by extensive dialysis against distilled water, and freeze-dried. This procedure resulted in pure *legumin*.

Purification of glycinin from soybean. The glycinin used in this paper was purified as previously described (*22*).

Nitrogen content determination. The percentage nitrogen content in the purified proteins was determined using the dynamic flash combustion method (NA 2100 nitrogen and protein analyser, CE Instruments, Milan, Italy). Triplicate samples of 5, 10 and 15 mg were used for the determination, methionine was used for the calibration and the protein conversion factor used was 6.25.

Gel electrophoresis. Samples were prepared in the absence and presence of the reducing agent β-mercaptoethanol. For both, 10 µl of the protein (either as it eluted from the column solution, or a 1 mg/ml solution in 10 mM potassium phosphate buffer, pH 7.6) was mixed at a ratio of 1:1 with sample buffer (1.4 ml distilled water, 2.0 ml 0.5 M Tris-HCl at pH 6.8, 2.0 ml 10% SDS, 2.0 ml glycerol and 0.4 ml 0.05% bromophenol blue) and heated for 10 minutes in a sealed eppendorf tube in a boiling water bath. When used, 20 µl of β-mercaptoethanol was added to 0.78 ml of sample buffer. 10-20% linear gradient, Tris-HCl Ready Gels (Bio-Rad) were used and between 7 and 20 µl of sample was applied to each well (according to the protein concentration of the sample). Low molecular weight protein standards, ranging from 94 to 14 kDa (Amersham Biosciences, Uppsala, Sweden) were made according to the instructions, and 10 µl was applied to each well. Gels were run at a constant 200 V. Staining was done using Coomassie Blue R-250 Bio-safe stain (Bio-Rad).

Thermal denaturation. Legumin and glycinin were dissolved in 75 mM potassium phosphate buffer pH 7.6, at 0.3% (w/v) concentration. All samples were centrifuged and degassed prior to use. Measurements were made in a VP DSC MicroCalorimeter (MicroCal Inc., Northampton (MA), USA) using the sample buffer in the reference cell. Samples were preheated to 45°C for 15 minutes, and subsequently heated to 115°C at a rate of 1°C/min or 0.5°C/min. One replicate of each sample was reheated after cooling to

check if any of the denaturation was reversible. One sample each protein was also heated at 1°C/min in the presence of 20 mM *N*-ethylmaleimide (NEM) to check if its presence effected the temperature of denaturation. For samples in buffer triplicates were run, and the results are presented as an average.

Small deformation rheology. Samples of purified proteins, 9.9% for pea legumin and 7.5% for soybean glycinin on a protein basis, were prepared in 75 mM potassium phosphate buffer, pH 7.6. Where used, the thiol-blocking agent *N*-ethylmaleimide (NEM) was added at a concentration of 20 mM. Gelling was done by heating samples in a Bohlin CVO rheometer concentric cylinder (C-14) (Bohlin Instruments Ltd., Gloucestshire, UK). Heating profile was 45°C to 98°C, holding at 98°C for 30 minutes, cooling to 25°C, and holding at 25°C for 30 minutes. Sample volume was 2.8 ml, and a few drops of vegetable oil were put on the top of the sample to prevent evaporation during heating. Heating and cooling rate was 1°C/min for control samples. One sample was heated slowly at 0.5°C/min (yet cooled at 1.0°C/min), and the other sample was cooled slowly at 0.2°C/min (after having been heated at 1°C/min). Dynamic measurements were taken at 60 second intervals, under a constant strain of 0.015 for legumin and 0.01 for glycinin (values within the linear viscoelastic strain region of the gels under the given conditions), and 0.1 Hz frequency. When analysing the results, the temperature at which the elastic modulus (G') became greater than viscous modulus (G") was determined as the initiation of gelation. When performing dynamic rheological measurements this is a measure of the gel point (*2*, *23-25*), that is most commonly referred to in the literature as the G'-G" crossover. Samples were run in duplicate, but the gel formation and development of only one of the samples is presented.

Transmission electron microscopy. Legumin samples were prepared at 10.4% concentration on a protein basis, pH 7.6, I=0.2, in sealed eppendorf tubes (1.5 ml) and were heated in the water bath connected to the Bohlin rheometer. Heating and cooling rates for the three samples were; 1.0°C/min heating & cooling; 0.5°C/min heating & 1.0°C/min cooling; 1.0°C/min heating & 0.2°C/min cooling. All samples were heated from 45°C to 98°C, held for 30 minutes at 98°C, cooled to 25°C, and held at 25°C for 30 minutes. Gel samples were then prepared as follows. They were cut into approximately 1

mm^3 cubes and fixed using 2.5% glutaraldehyde in distilled water. After washing with distilled water the samples were dehydrated using a graded series of ethanol followed by infiltration with LX112 epoxy resin. These plastic embedded blocks were polymerised at 60°C for 24 hours and sectioned using Leica Ultracut S. The ultrathin sections obtained (60-80 nm) were collected in 100 mesh Collodion coated grids and stained using Reynolds lead-citrate and uranyl acetate. The grids were examined in a Philips CM12 transmission electron microscope operated at 80 kV accelerating voltage.

Solubility of gels. Samples of pea legumin (9.9% concentration on a protein basis) were prepared in the same buffer as used for the Bohlin experiments. 1ml of sample was put into each test-tube (5 ml with screw-cap) and heated in a water bath at 95°C for 30 minutes. After heating, the samples were cooled at 4°C for 2 hours. Subsequently, 5 ml of each of the following solutions was added to one of the test-tubes: 8M urea; 8M urea with 2% (w/v) β-mercaptoethanol; 1.5% (w/v) sodium dodecyl sulfate (SDS). Each tube was then re-sealed and continuously rotated in a test-tube rotor in order to keep the gel mobile and allow for good diffusion of the solution into the gel network. Samples were rotated for 24 hours at room temperature. The amount of gel that had dissolved in each reagent was then judged visually according to the clarity/turbidity of the sample as it rotated, and the presence/absence of sediment after leaving samples to stand for 1 hour at room temperature.

RESULTS

Gel formation using different heating and cooling rates. Samples concentrations of 9.9 and 7.5% (on a protein basis) were used because they were determined as the minimum gelling concentrations of pea legumin and soybean glycinin, respectively. Figure 1b shows legumin and glycinin gel formation at these concentrations using different heating and cooling rates, as measured by the storage modulus (G'). In these figures G' is plotted as a function of temperature, rather than against time as is more commonly done, but the direction in which to follow the data points is indicated by successive numbering from 1 to 4. (The numbers 1 to 4 in figure 1b correspond with those in figure 1a where the storage and loss modulii are plotted more traditionally as a function of time.) A first comment on the gel formation of legumin and glycinin as a function of the heating/cooling rate is that the two proteins appeared to respond in the same way to changes in the rates. For both proteins, using a slower heating rate (0.5°C/min) instead of the control rate (1.0°C/min) did not effect the gel formation of the legumin or the glycinin gels as detected by the rheometer. A slower cooling rate however caused a stronger gel to be formed, for both proteins. Looking in more detail at the plots of G' it can be seen that it was during the beginning of cooling (phase 3 in the figures, 98-87°C) that the slowly cooled gel attained much of its "additional" strength. Thereafter, the relative increase in G'/1°C was similar to that of the two other gel samples (both cooled at 1°C/min), as indicated by the slopes of the plots being so similar.

Though it is not plotted, the loss modulus (G") was monitored during all the measurements presented in figure 1b because the temperature at which G' became larger that G" (the G'-G"crossover) was used as a measure of the initiation of gel formation. The temperature of the G'-G" crossovers for pea legumin and soybean glycinin are presented in table 1. It can be seen that the samples heated slowly (0.5°C/min) initiated gel formation at a lower temperature than the control samples (heated at 1°C/min).

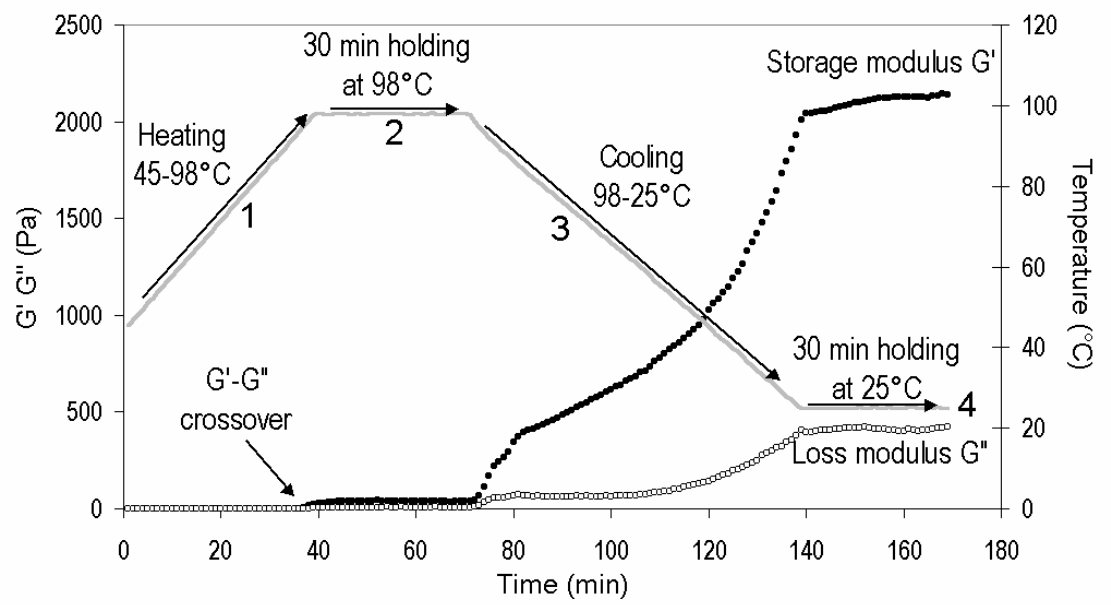


Figure 1a: Presentation of storage (G') and loss (G") modulii as a function of time. Heating and cooling phases are plotted on a secondary axis. Nos.1-4 correlate with Figure 1b where the time axis has been removed, and the storage modulus is plotted as a function of temperature. (NB: Loss modulus is not plotted in figures 1-3).

Table 1: Table showing the temperature of the G'-G" crossover point and the thermal denaturation temperature (T_d) for pea legumin and soybean glycinin solutions heated at 0.5°C/min and 1.0°C/min.

Heating rate (°C/min)	G'-G" crossover (°C)		Td (°C/min), I = 0.2	
	legumin	glycinin	legumin	glycinin
0.5	88	86	87	86
1.0	94	95	88	87

Despite this, the gel networks that developed with continued heating and cooling had the same strength. Table 1 also shows that for the slowly heated samples (0.5°C/min) gel initiation corresponded with the peak thermal denaturation temperature (T_d), as measured by differential scanning calorimetry. However, when heating at 1°C/min gel initiation was not detected until the end point of the peak of denaturation (~94°C).

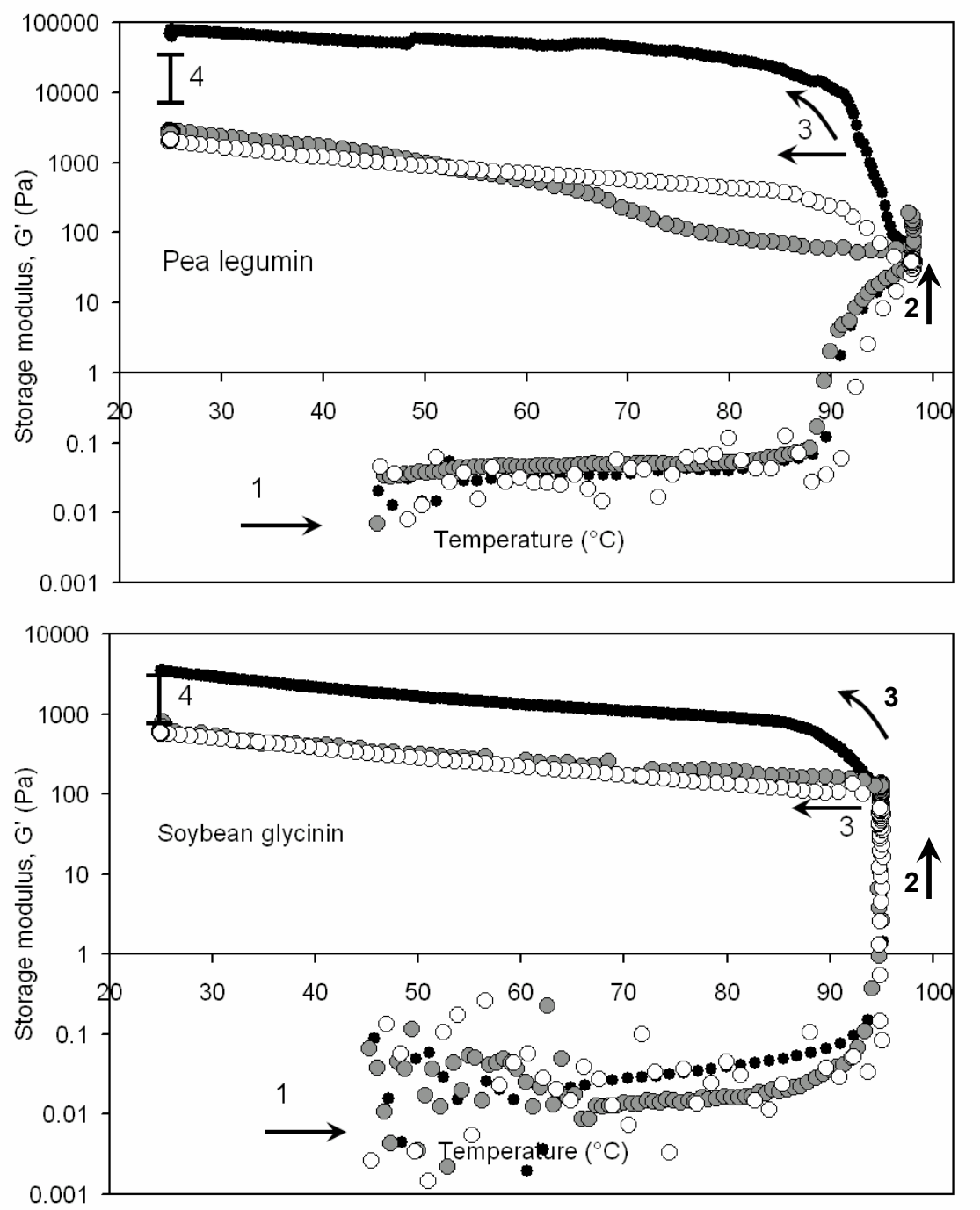


Figure 1b: Effect of heating/cooling rate on development of storage modulus (G') during heat-induced gelation of *pea legumin* (upper panel) and *soybean glycinin* solution (lower panel). 1°C/min heating & cooling (white circles). 0.5°C/min heating & 1°C/min cooling (grey circles). 1°C/min heating & 0.2°C/min cooling (black circles).

Addition of *N*-ethylmaleimide. In the control samples (heated at 1°C/min), and those heated slowly (at 0.5°C/min), the blocking of disulphide bond formation by the action of *N*-ethylmaleimide caused a negligible effect on the gel formation of legumin and glycinin. It was negligible in that gelation proceeded regardless, and the gel strength was unaffected, but a slight destabilization of the network during formation was apparent, as seen by a slight wavering of the data points (see control sample in figure 2; black diamonds). (Data for the samples heated at 0.5°C/min are not plotted in this figure because they were the same as for the control.) However, when the samples were cooled slowly (0.2°C/min) the effect of the added NEM was very noticeable (figure 2, grey circles) because it caused a substantial reduction of the "additional" gel strength that was previously pointed out in figure 1b (between 98-87°C).

For pea legumin (figure 2, upper panel) this reduction was such that the value of G' at ~87°C was of the same order of magnitude at it was in the control sample (also shown in figure 2). Thereafter however during continued cooling, an increase in G' between 85-75°C caused the plot of G' to deviate from the control, and in the end the slowly cooled sample was somewhat stronger than the control. Soybean glycinin cooled slowly in the presence if NEM had no such increases during continued cooling, and the plot of G' (figure 2, lower panel) was of the same order of magnitude as the control sample (cooled at 1°C/min in the presence and absence of NEM). A final observation worth noting is that all samples heated in the presence of NEM formed transparent gels.

Reheating/recooling of the gel. After formation, gel samples were reheated and recooled using a constant rate of 1°C/min. Pea legumin and soybean glycinin behaved strikingly differently to this treatment. Soybean glycinin gels were what we described as being ‘reheatable’. Exactly what this means is that the part of the gel that originally formed between 85 and 25°C of the cooling phase (phase 3) was thermally reversible. Thus when reheated (phase 5) and recooled (phase 7) the plot of G' between 85 and 25°C went backwards and forwards along itself. This ‘reheatability’ of soybean glycinin can be clearly seen in figure 3a.

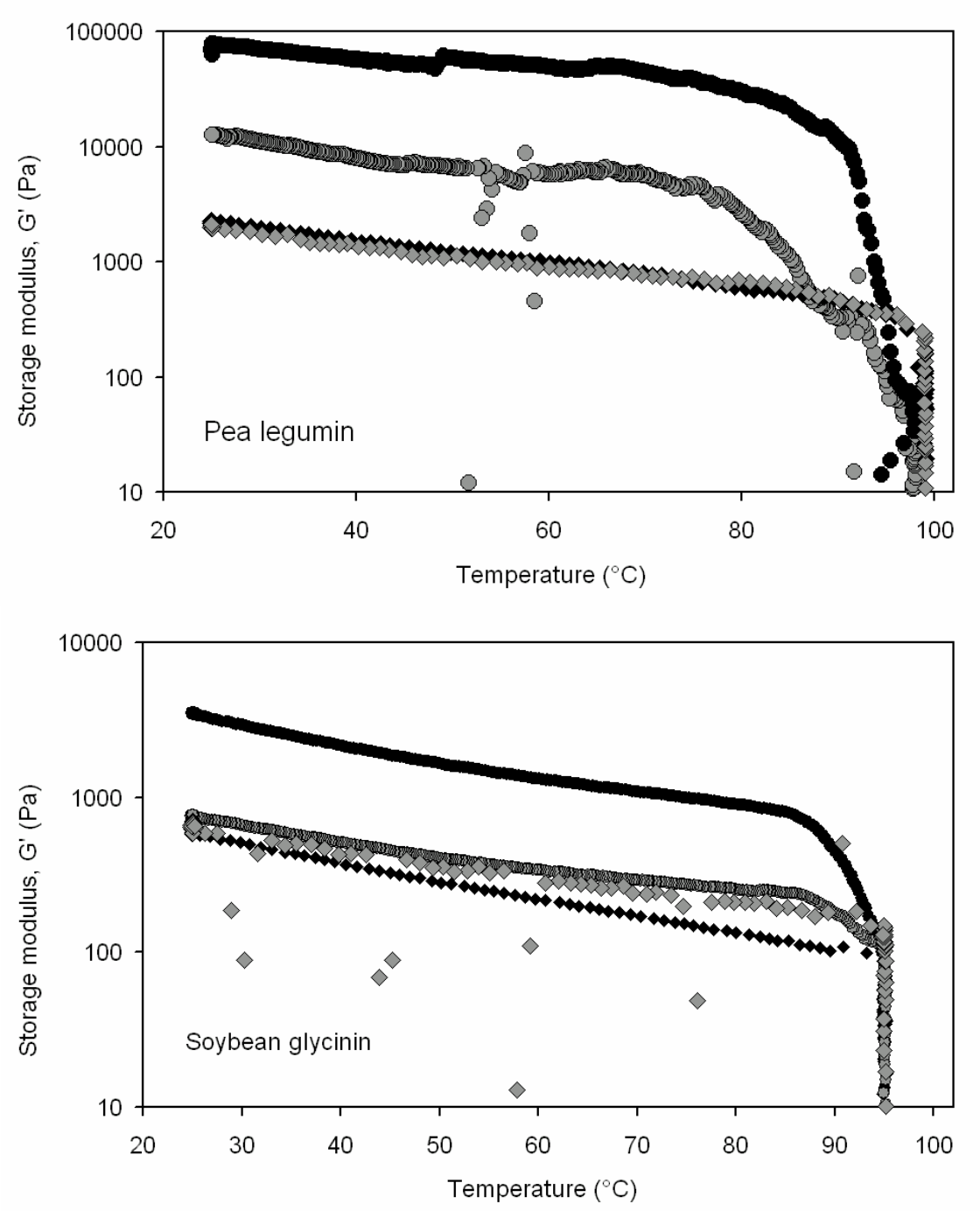


Figure 2: Effect of the addition of 20 mM N-ethylmaleimide (NEM) on the development of the storage modulus during heating and cooling a 9.9% protein concentration *pea legumin* solution (upper panel) and a 7.5% protein concentration *soybean glycinin* solution (lower panel). 1°C/min heating & cooling (black diamonds); + 20 mM NEM (grey diamonds). 1°C/min heating & 0.2°C/min cooling (black circles); +20 mM NEM (grey circles).

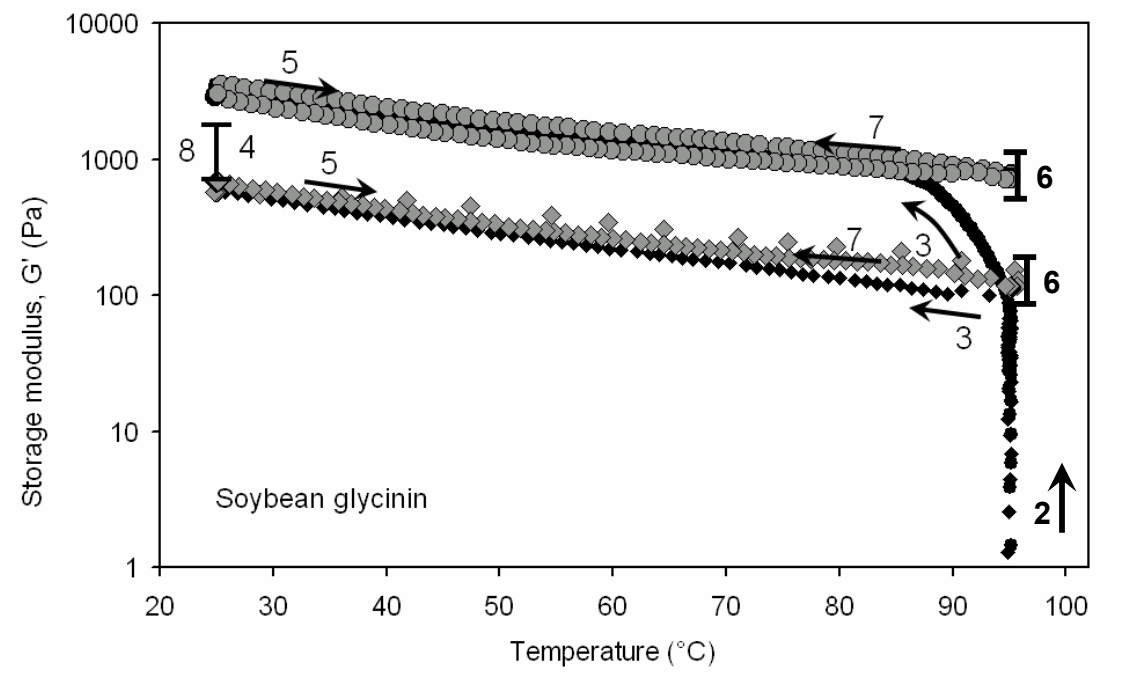


Figure 3a: 'Reheatability' of *soybean glycinin* gels. 1.0°C/min heating & cooling (black diamonds) with reheating & recooling at 1.0°C/min (grey diamonds). 1.0°C/min heating & 0.2°C/min cooling (black circles) with reheating & recooling at 1.0°C/min (grey circles).

The behaviour of pea legumin to the procedure of reheating/recooling differed according to the cooling rate: the slowly cooled sample (figure 3b, upper panel) was seen to be 'reheatable' like soybean glycinin, yet the control sample (figure 3b, lower panel) became stronger by one log scale after reheating/recooling. This same phenomenon of becoming stronger after reheating/recooling was also seen for the pea legumin sample heated at 0.5°C/min.

The procedure of reheating/recooling was also done with samples that were gelled in the presence of NEM. The soybean glycinin gels were again all 'reheatable' (no results shown). The pea legumin gels however were not reheatable because the gel networks became disrupted, either during holding at 98°C (phase 6), or during recooling (phase 7) (no results shown).

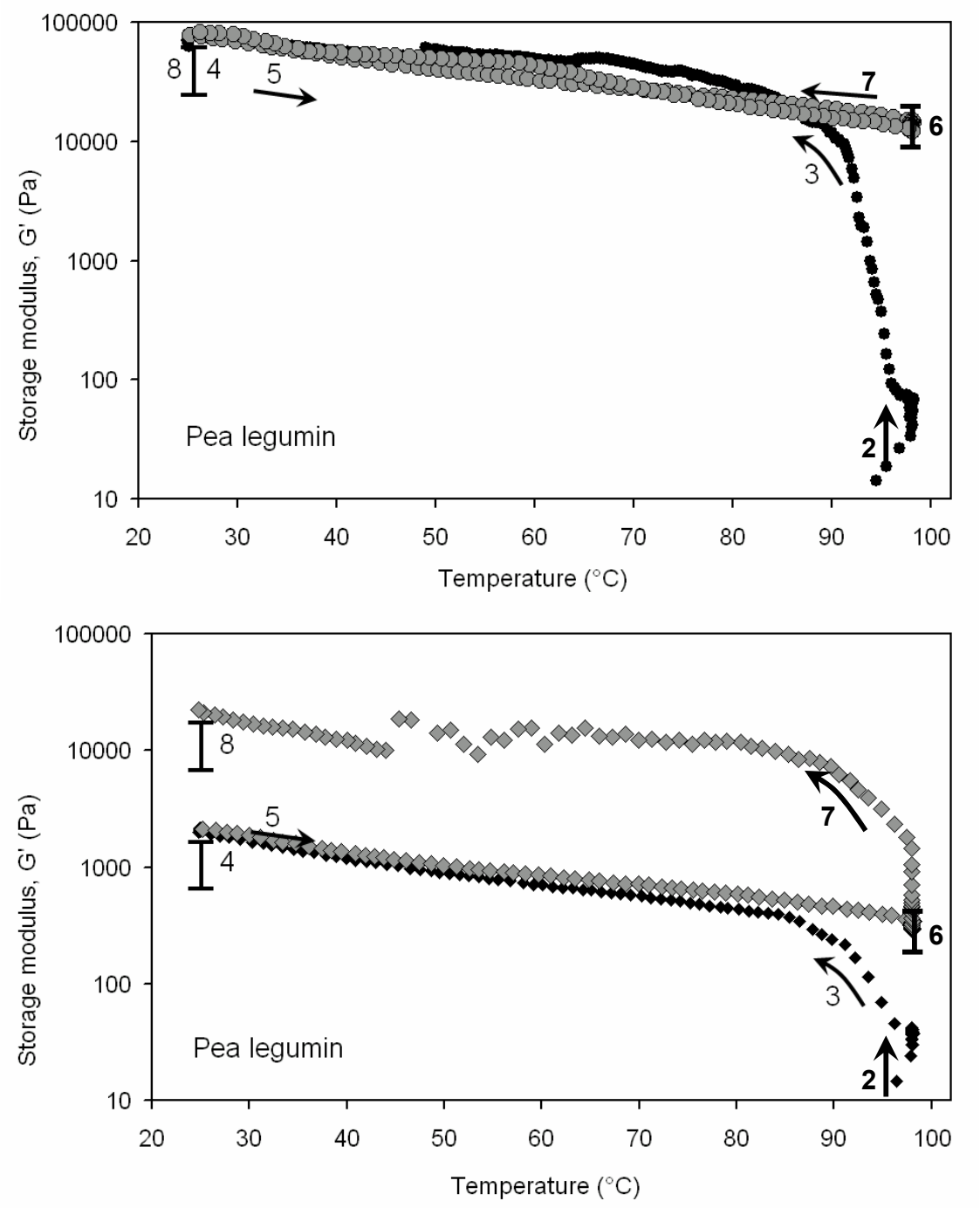
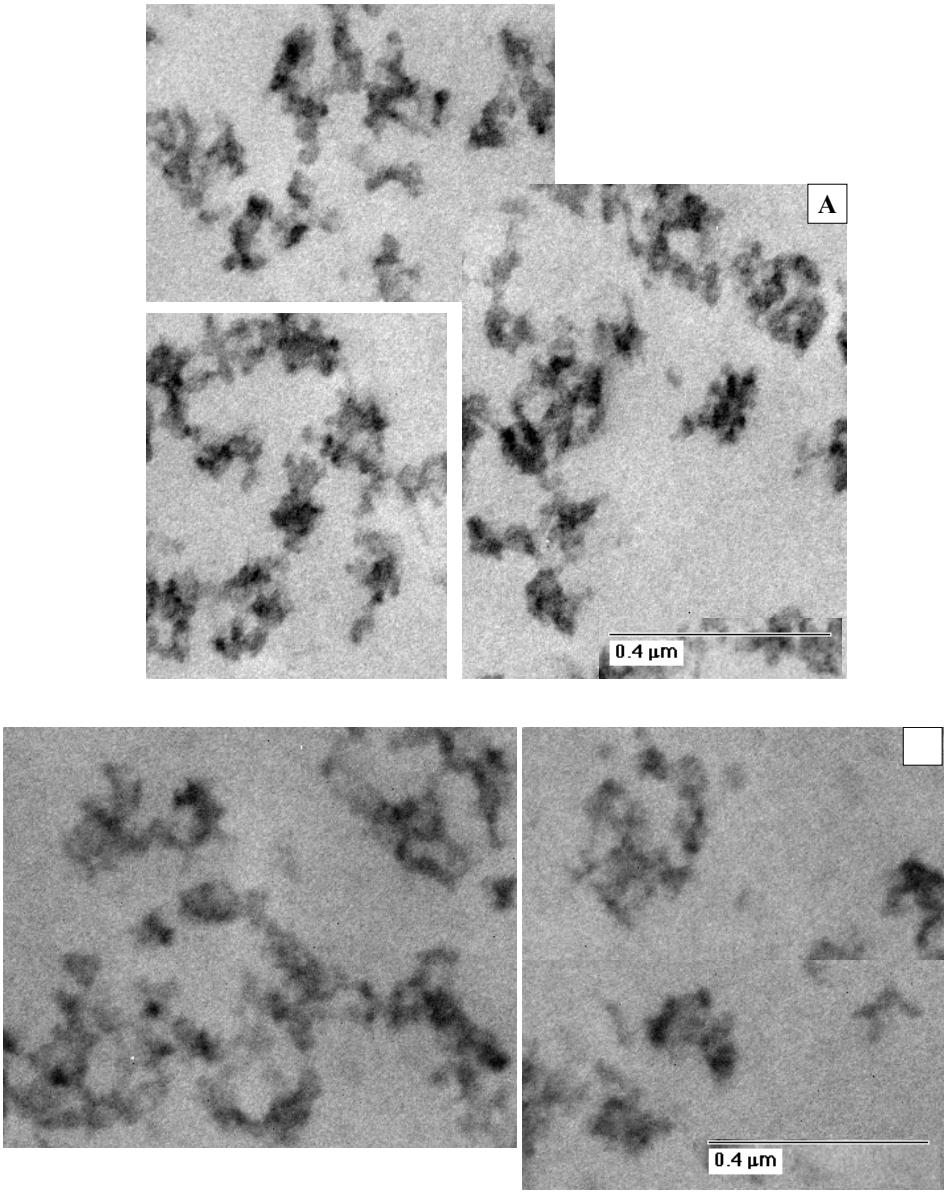


Figure 3b: 'Reheatability' of *pea legumin* gels. Upper panel: 1°C/min heating & 0.2°C/min cooling (black circles), with reheating & recooling at 1°C/min (grey circles). Lower panel: 1°C/min heating & cooling (black diamonds) with reheating & reccoling at 1°C/min (grey diamonds.

Transmission Electron Microscopy (TEM). Figures 4A, 4B, and 4C show TEM pictures of cross sections of the pea legumin gels formed with the different heating and cooling rates, in the absence of NEM. Figures 4A and 4B show similar clustered agglomerate gel networks. These are in fact the two legumin gels (1.0°C and 0.5°C/min heating) that had no detectable differences in their gel strengths (as measured by the rheometer). Moreover, both these systems had the behaviour of strengthening after being reheated/recooled.



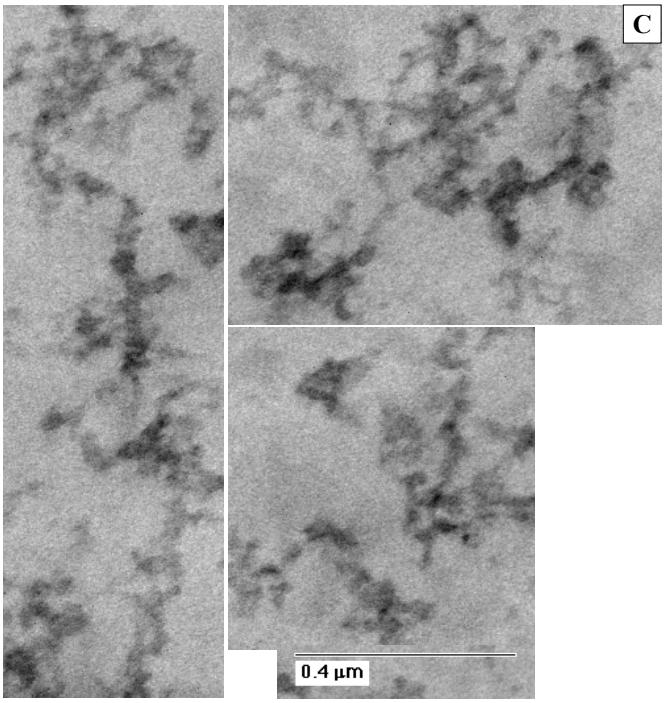


Figure 4: TEM cross-section of pea legumin gels. A: 1.0°C/min heating & cooling. B: 0.5°C/min heating & 1.0°C/min cooling. C: 1.0°C/min heating & 0.2°C/min cooling. The relevant scale is indicated with the bar of 0.4 μm.

The slowly cooled sample is pictured in figure 4C, and is shown to have a more branched and connected network than the other two samples. Also, the darkened patches of agglomerated protein are smaller. A side from the differences, there was one observation relevant to all the legumin gels presented in figures 4A-C: none of them appeared to have a homogeneous network.

Solubility of gels in different reagents. Tests for solubility of gels in a combination of reagents were performed for pea legumin gels as a way of determining the bond types that were structurally important for the gel network. Considering that legumin contains approximately five cysteine residues, it was important to determine the effect of the thiol-reducing reagent β-mercaptoethanol on the gel structure. This reagent was then used alone, and in combination with urea, so making a mixture of reagents capable of disrupting both covalent and non-covalent bonds. Urea was then used alone so that any covalent disulphide bonds would be left intact, thus enabling the structural importance of

the non-covalent bonds to be deduced. Finally, the detergent SDS was used to disrupt hydrophobic residues, while leaving hydrogen bonds intact. The gels used for these tests had been heated in a water bath without a rate control. Due to the relative velocity of the heating and cooling, these gels were very likely to have had a randomly clustered network (similar to that formed with a 1.0°C/min heating/cooling rate). The gels dissolved to a clear solution within 1 hr in the urea/β-me mixture. In β-me alone the gel broke into small lumpy pieces, which settled to leaving a clear solution. In urea alone after 12 hr there was a turbid liquid containing very small pieces of gel. There was some settling of these small gel pieces after 1 hr standing, but the turbidity remained. Finally, submergence in SDS disturbed the gel structure making it swollen and "fluffy", but the gel remained as one piece, and the clarity of the SDS solution was unchanged.

DISCUSSION

As stated earlier in this paper, the purpose of comparing the gelation of pea legumin with soybean glycinin under various conditions was to determine if a common model for gel formation could be built, based upon molecular reasoning. This paper therefore also tested the assumption that is often passed around in the literature that intermolecular forces determine the structure of heat-induced gel networks, and that better understanding of these forces will enable modification and control of the resultant textural properties of the foods (*2*, *26*).

It was seen in this paper that gel formation of pea legumin and soybean glycinin, under the conditions used, was initiated at or after the peak temperature of denaturation, and their gel networks predominantly developed during cooling. These observations indicated that these proteins followed the three-step process of gelation that is generally accepted for heat-induced gelation of such globular proteins. This process, which was documented in detail by Clark *et al*. (*27*), can be summarised as follows: (i) denaturation of the protein with subsequent exposure of hydrophobic residues, (ii) intermolecular hydrophobic interaction of the unfolded proteins (aggregation) and (iii) agglomeration of aggregates into a network structure. The equilibrium that exists between the native and unfolded states of globular proteins causes their process of heat-induced gelation to be under a certain extent of kinetic control (*27*). Moreover, the slower the rate of aggregation relative to denaturation, the more fine stranded and ordered is the resultant gel network (*28*). As shown in this paper however, a reduction in the heating rate from 1 to 0.5°C/min caused no observable changes in either the gel formation of the legumin and glycinin gels (figure 1b), or the network structures of the pea legumin gels (see figure 4A and 4B). However, when cooling the system more slowly the process of agglomeration of the aggregates into a network structure was significantly altered, as observed by the formation of a strong branched network of the pea legumin gel (figure 4C). Slow cooling was believed to maintain the protein in its unfolded state for a longer time, thus enable more optimal interactions to occur, and disulphide bonds were believed to be active at the strand-junctions. This was deduced by the observation that even in the presence of NEM the slowly cooled gel was stronger than the control (figure 2, upper panel), and thus the

more optimal gelation (seen in figure 4C) was enhanced, but not dependent on network disulphide bonds. With soybean glycinin the results were slightly different. Though slow cooling increased gel strength due to the involvement of disulphide bonds in network branching (*29*) it had a negligible effect in the presence of NEM. This would suggest that the interaction of unfolded glycinin molecules is already close to optimal under the conditions of the control sample.

Referring back for a moment to the legumin and glycinin gels heated at 0.5 and 1°C/min, the addition of NEM caused no change in the measured gel strengths (despite the slight wavering of the data points in figure 2). Disulphide bonds should therefore be considered as having been non-essential in these gels. This result agrees with that of Utsumi and Kinsella (*30*) who saw that glycinin gels formed in the presence of NEM were fragile, but of equal strength to the control sample (formed at pH 8 in 30 mM tris-HCl buffer).

Another way to determine the role of different bond types in the network structure was the solubility of the pea legumin gels in different reagents. Overall, the results showed that unless the samples were cooled very slowly disulphide bonds were involved within the individual aggregates, but it was hydrophobic and hydrogen bonds that supported network formation. The gel solubility tests were not repeated with soybean glycinin due to a lack of protein availability, yet other authors have done such tests. Utsumi and Kinsella (*30*) used 8M urea and 0.2 M β-mercaptoethanol, and found that 90% and 30% of the gel dissolved in each reagent, respectively. This indicated a role of hydrophobic interactions, hydrogen bonds and disulphide bonds in maintaining the gel matrix. While this may be true, it must be repeated that according to the rheological measurements performed in this paper disulphide bonds within the gel network of glycinin are non-essential.

To summarise thus far it can be said that the molecular driving forces of the heat-induced gelation of pea legumin and soybean glycinin are the same, and they can be manipulated by using a slow rate of cooling. The question that then arises is why the gels of these two molecularly similar proteins have a different response to the procedure of

reheating/recooling. If intermolecular forces really do determine the structure of heat-induced gel networks (as proposed by Zheng *et al*. (*26*) and Ikeda and Nishinari (*2*)), then legumin and glycinin, when gelled under the same conditions, should be expected to have the same network structures, and thus the same behaviour of 'reheatability'. This was clearly not the case though. Soybean glycinin was completely reheatable under all conditions (fast or slow heating and cooling, in both the presence and absence of NEM). Results are only shown for selected conditions in figure 3a. Pea legumin on the other hand was only 'reheatable' after having been cooled slowly in the absence of NEM (figure 3b, upper panel). The pea legumin gels formed in the absence of NEM became stronger after the procedure of reheating/recooling (figure 3b, lower panel), while those reheated in the presence of NEM were not 'reheatable' (no results shown). In literature the phenomena of increased gel strength after reheating was found for whey protein isolates gelled at pH 8.0 (*31*) and at neutral pH in distilled water (*32*). Two possible explanations were offered by Rector (*31*): (i) More disulphide bonds form during reheating reducing the flexibility of the network chains, bringing them closer together, consequently enabling more extensive short-range crosslinks to form during recooling. (ii) Aggregates unfold upon reheating making more residues accessible for interaction. Considering that it was the gel with enhanced disulphide bonding that was 'reheatable', the first explanation offered seems most likely to apply to pea legumin gels. Thus, a branched network well stabilised with covalent bonds can be said to be important in making pea legumin gels structurally stable against rearrangements during reheating. By contrast, soybean glycinin appears to be inherently able to form structurally stable gel networks in both the absence and presence of disulphide bonds.

In keeping with the idea that soybean glycinin is inherently better able to form a well structured gel network is the fact that it has a 2.4% lower minimum gelling concentration than pea legumin. For two molecularly similar proteins that gel via the same bonding mechanisms, this difference in concentration is significant. It could reflect two possible characteristics: more organised formation of network strands, and/or inclusion of a higher amount of available protein in the network. References from the literature support the formation of more organised strands. Hermansson (*33*) formed gels of glycinin at pH 7.0 and, using electron microscopy to visualise the structures described the strands of the

glycinin gels as "very regular". Nakamura *et al.* (*29*) studied the aggregation of glycinin at pH 7.6 and identified an ordered mechanism of network formation. Also, Zheng *et al.* (*34*) compared fababean legumin with soybean glycinin and found that under equal conditions a higher amount of legumin than glycinin was needed to form a gel of equal strength due to the more irregular network strands of fababean legumin.

Having compared the processes of heat-induced gelation of pea legumin and soybean glycinin from a molecular basis, and having compared structural elements of the two gels, it seems that the information collected is not sufficient for building a common model of gelation. Based upon molecular reasoning changes in the gelation mechanism may well be achieved. Control over the food texture is more difficult however because the structural quality of the network strands appears to be determined by inherent features of the protein, rather than the molecular interactions that drive the gelation mechanism.

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Chapter 5

GELATION BEHAVIOUR OF PROTEIN ISOLATES EXTRACTED FROM FIVE CULTIVARS OF *PISUM SATIVUM* L.

ABSTRACT

Protein isolates were extracted from five pea (*Pisum*) cultivars and their gelation behaviours were compared at pH 7.6. Gel formation and development was monitored via constant oscillation dynamic measurements. The standard heating and cooling rate was 1.0 °C/min, but samples were also heated at 0.5 °C (and cooled at 1.0 °C/min), and others were heated at 1.0 °C/min and cooled slowly at 0.2 °C/min. When heating more slowly no changes in gel formation were detected for any of the cultivars. When cooling slowly, the cultivar Solara, with the highest legumin content, formed a stronger gel than all the other cultivars. It did the same when the thiol-blocking agent *N*-ethylmaleimide (NEM) was added to the system. This indicated that the strengthened gel system formed independently of any disulphide bonds formed by the legumin. The cultivars Supra and Classic formed stronger gels only when cooled slowly in the presence of NEM, and so disulphide bond formation in their gel systems was apparently a factor that prevented gel network strengthening. The cultivars Finale and Espace were unable to form strong and self-supporting gels. This was believed to be because of the repulsive forces on the α-subunits of vicilin, which were at their highest level in the cultivars Finale and Espace. The contribution of legumin to the pea protein isolate gels was shown to be cultivar specific and related to its disulphide bonding ability rather than the absolute amount of legumin protein present.

KEYWORDS: *Pisum*; protein isolate; globular protein composition; heat-induced gelation; gelation behaviour; legumin; vicilin

INTRODUCTION

Understanding structure-function relationships of food proteins can aid further development of their applications, and enable substitution of one protein by another. The major leguminous plant protein that is established as a food ingredient is soybean protein and its products (protein isolates and concentrates) (*1*). Other legume proteins do not appear to have found a way into the spotlight. The reason why is not clear. It can be that the protein functionality is not sufficiently understood to promote their development, that the quality of their functionality is inferior relative to that of soybean, or that the functionality varies too much from one leguminous plant to another.

Pisum (pea), as a protein source, is genetically variable. Not only does the total protein content vary, so does the ratio of the two major globular proteins. Ratios of legumin: vicilin can be found between the extremes of 0.2 and 1.5 (*2*). Furthermore, the specific amino acid profile of the globulin proteins varies depending on the sequence of the encoding gene. As a consequence the position and number of post-translational cleavage sites can differ from one protein precursor molecule to another. Post-translational proteolysis of legumin is responsible for the formation of the acidic and basic polypeptides (that can be separated under reducing conditions). Four/five acidic polypeptides (38-40 kDa; pI 4.5-5.8) and five/six basic polypeptides (19-22 kDa; pI 6.2-8.8) have been reported (*3-6*). The same type of genetic variability is apparent in the analogous glycinin molecules of soybean. A study on soybean glycinin gelation even reported gel hardness to be directly proportional to the percentage of AS-III polypeptides present (the biggest of the acidic polypeptides) (*7*). Similarly, tofu made from soybeans containing the A5 polypeptide was reported to be harder and more solid-like that that prepared from cultivars that lacked the A5 polypeptide (*8*). In the Vicilin gene family nine genes encode for the smaller protein ~50 kDa, and two for the larger protein ~70 kDa. Depending on the specific gene, the 50 kDa protein can have two, one or no post-translational cleavage sites. Proteolytic processing thus creates a range of small fragments between 36-12 kDa. In a study that separated the protein into subfractions with different small fragment compositions it was concluded that the differences in their thermal denaturation temperatures were too small to be important (*9*). No studies comparing the

functionality of peas lacking one or more of the small fragments are known to exist. The larger protein (~70 kDa) has been shown to be responsible for the heterogeneous gelation behaviour exhibited by pea vicilins at near-neutral pH conditions (*10*), though not because of any compositional variation of this protein itself. Pea cultivars with a different total content of this protein may well exhibit different functional properties.

Understanding which, if any, of the above factors influence the functionality of pea flours and isolates would enable cultivars to be screened and selected for given applications. This paper presents the results on a study of the gelation of pea protein isolates extracted from five different *Pisum* cultivars. Having previously gained knowledge on the heat-induced gelation behaviour of the individual pea proteins, legumin and vicilin (*10-11*), the aim was to determine if this knowledge enabled the gelation behaviour of the pea protein isolates to be predicted.

MATERIALS AND METHODS

Pea protein isolate was obtained by isoelectric precipitation of a total protein extract of pea flour from the cultivars Solara, Supra, Classic, Finale and Espace. All cultivars were grown and harvested under the same conditions by Cebeco Seeds (Lelystad, NL) in 1998. The peas were subsequently air-dried. Peas were milled in a Waring commercial blender (New Hartford, Connecticut, USA) 2:1 (w/w) with dry ice to avoid any heat denaturation of the proteins. Salt-soluble proteins were then extracted into a 100 mM Tris-HCl buffer, pH 8.0, with a flour to buffer ratio 1:10 (w/v). Extraction time was 1 hour at room temperature and the extract was collected by centrifugation (11900 x g, 10°C, 25 min). Isoelectric precipitation, pH 4.8, was used to isolate the globulin proteins from the total protein extract; the pH was adjusted by dropwise addition of 1 M HCl. Precipitated protein was left for 2 hr, 4 °C, before it was collected by centrifugation (11900 x g, 10°C, 25 min). Washing the protein pellet with water (pellet to water ratio 1:10) removed unwanted albumin proteins. Again the pellet was collected by centrifugation (11900 x g, 4°C, 25 min), freeze-dried and stored at -20°C. The dried product was called the pea protein isolate.

Gel electrophoresis samples were prepared by mixing the protein sample 1:1 with sample buffer (1.4 ml distilled water, 2.0 ml 0.5 M Tris-HCl at pH 6.8, 2.0 ml 10% (w/v) SDS, 2.0 ml glycerol and 0.4 ml 0.05% bromophenol blue), and heating in a boiling water bath in sealed eppendorfs for 10 minutes. The protein samples were made by mixing with 75 mM potassium phosphate buffer, pH 7.6, for 2 hours, and then spinning down to avoid insoluble material in the sample being applied on the gel and causing a drag effect that rendered the protein bands indecipherable. Polyacrylamide Tris-HCl (12%) gels were used and a volume containing 2-10 μg of protein was loaded into each well. Low molecular weight protein standards, ranging from 14 to 94 kDa (Amersham Biosciences, Uppsala, Sweden) were made according to the instructions, and 10 μl was applied to each well. Gels were run at a constant 200 V. Staining was done using Coomassie Blue R-250 Bio-safe stain (Bio-Rad).

Estimation of protein composition of the protein isolate was determined by imaging densitometry (Bio-rad Densitometer G-710) using the gels obtained as described above. The percentage of legumin and vicilin were calculated according to the area under the peak of staining density. The protein bands were selected according to those previously assigned to the pure proteins (which are highlighted by a circle around the relevant bands of the pure proteins in figure 1). In addition, the proportion of vicilin that was α-subunits (the largest protein band ~70 kDa) was also calculated. The pure proteins used as a reference were obtained from the legumin enriched isolate of cv. Solara according to the method previously described in detail in O'Kane *et al.* (*9*).

Protein content determination. The nitrogen content (%) in the protein isolates was determined using the dynamic flash combustion method (NA 2100 nitrogen and protein analyser, CE Instruments, Milan, Italy). Triplicate samples of 5, 10 and 15 mg were used for the determination, methionine was the calibration sample and the conversion factor used to calculate the protein content was 6.25.

Minimum gelling concentration was determined by making 5 ml protein isolate suspensions of 10-20% (w/v) concentration, at pH 7.6. All samples were heated, (in sealed tubes to avoid evaporation), in a boiling water bath for 30 minutes. Samples were cooled to room temperature for 1 hour, then stored at 4°C overnight. Next day the tubes were inverted and the samples that did not flow within 24 hrs were considered to have gelled.

Small deformation rheology. Samples of the protein isolates, 18% concentration (w/v), were prepared in 75 mM potassium phosphate buffer, and adjusted to pH 7.6 with dropwise addition of 1 M NaOH, and maintained at this pH throughout. Samples were mixed well for 2 hours at room temperature to get a good dispersion of the isolate in the buffer. Where used, the thiol-blocking agent *N*-ethylmaleimide (NEM) was added at a concentration of 20 mM. Gelling was done by heating samples in a Bohlin CVO rheometer concentric cylinder (C-14) (Bohlin Instruments Ltd., Gloucestshire, UK). Heating profile was 45°C to 98°C, holding at 98°C for 30 minutes, cooling to 25°C, and holding at 25°C for 30 minutes. Sample volume was 3 ml, and a few drops of vegetable

oil were put on the top of the sample to prevent evaporation during heating. The standard conditions were samples heated and cooled at 1.0°C/min. Other sample conditions were heating slowly at 0.5°C/min (yet cooling at 1.0°C/min), and cooling slowly at 0.2°C/min (after having been heated at 1.0°C/min). Dynamic measurements were taken at 60 second intervals, under a constant strain of 0.015 (value within the linear viscoelastic strain region of the gels under the given conditions), and 0.1 Hz frequency. Samples were run in duplicate, and one is presented.

Determination of free thiol groups using Ellman's reagent. The amount of free thiol groups was estimated in samples containing approximately 5 mg of protein per ml dissolved in 0.1 M sodium phosphate buffer (pH 8.0) containing 8 M urea. L-Cysteine hydrochloride (8-60 mg/l final concentration) was used as a standard. To 50 µl of sample or standard 250 µl of buffer (0.1 M sodium phosphate buffer, pH 8.0, containing 8 M urea) was added. To this mixture 20 µl of a 2 mg/ml solution of 5,5'-dithio bis(2-nitrobenzoic acid) in buffer was added and 200 µl of each sample or standard was transferred to a microtiter plate. After 15 min of incubation the absorbance of the samples and standards was measured at 412 nm using a microtiter plate reader.

Total amino acid analysis. Amino acid analysis was performed by Ansynth BV (Roosendaal, The Netherlands). Amino acid analysis was performed after protein hydrolysis using an amino acid analyser equipped with a ninhydrin detection system. Acid hydrolysis was carried out in 6 M HCl for 22 h at 105-110°C. In order to analyse cysteine and methionine samples underwent oxidation with performic acid for 16 h at 0–5°C, followed by acid hydrolysis in 6 M HCl for 22 h at 105-110°C. For tryptophan analysis an alkaline hydrolysis was performed in 4.2 M NaOH for 22 h at 105-110°C.

RESULTS AND DISCUSSION

To start, the protein isolates were characterised in various ways. Firstly, the total protein content. The results were as follows: Solara 93%, Supra 92%, Classic 91%, Finale 94% and Espace 93%. Secondly, the globular protein composition. The relative percentages of vicilin and legumin in the soluble fraction of the protein sample were estimated according to the intensity of staining of bands belonging to each protein. The stained samples are shown in figure 1, and the compositions are summarised in table 1. Solara and Espace had the highest (28%) and lowest (21%) legumin contents, respectively, and Finale had the highest percentage of α-subunits (14%). Finally, the minimum gelling concentration of each isolate was determined. For all isolates the minimum gelling concentration was 16% (w/v). These gels were disrupted by applying a small force (with a glass rod) however, so it was decided to perform all subsequent gelling experiments at 18% (w/v). The 18% (w/v) protein concentration is a higher gelling concentration than needed to make soybean protein isolate gels (*12*) however, so it was investigated if insoluble material was hindering the gel network formation. Samples were spun down and the supernatant (14% protein by weight) was gelled in the Bohlin rheometer under standard conditions. The strength of the gel was unchanged with respect to the uncentrifuged sample (no results shown) and so it was decided to proceed with the unspun sample. It was upon the results of these preliminary tests that it was also decided to use only the soluble fraction for estimating the protein composition of the isolates.

Table 1: Ratios of protein composition of the protein isolate soluble in potassium phophate buffer, as estimated from the staining intensity of the bands on SDS-PAGE. The bands belonging to legumin and vicilin are indicated on the SDS-PAGE gel in figure 1.

Pea cultivar	Legumin (%)	Vicilin bands ≤50 kDa (%)	Vicilin α-subunits (%)
Solara	28	62	10
Supra	25	64	11
Classic	22	67	11
Finale	25	61	14
Espace	20	67	13

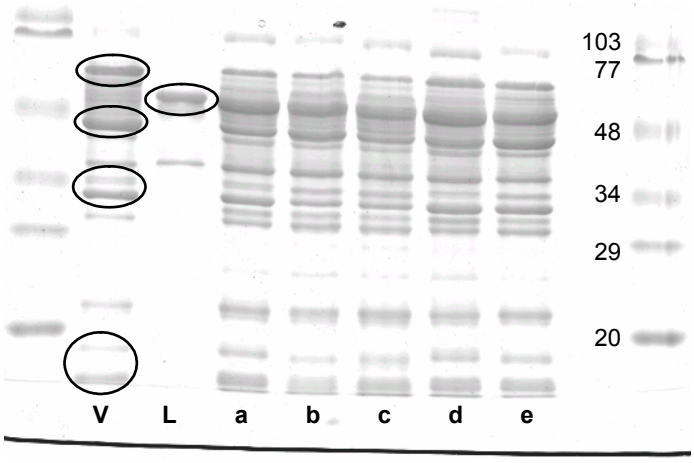


Figure 1: SDS-PAGE of pea protein isolates dissolved in potassium phosphate buffer, run on a 12.5% polyacrylamide Tris-HCl gel. From left to right: (V) purified vicilin fraction; (L) purified legumin; protein isolates from cv. (a) Solara, (b) Supra, (c) Classic, (d) Finale, (e) Espace. External lanes are standard markers (molecular weights indicated in kDa on right hand side). Bands previously assigned to pure proteins are highlighted by circles. The largest band of vicilin (~70 kDa) was taken separately to calculate the percentage of α-subunits.

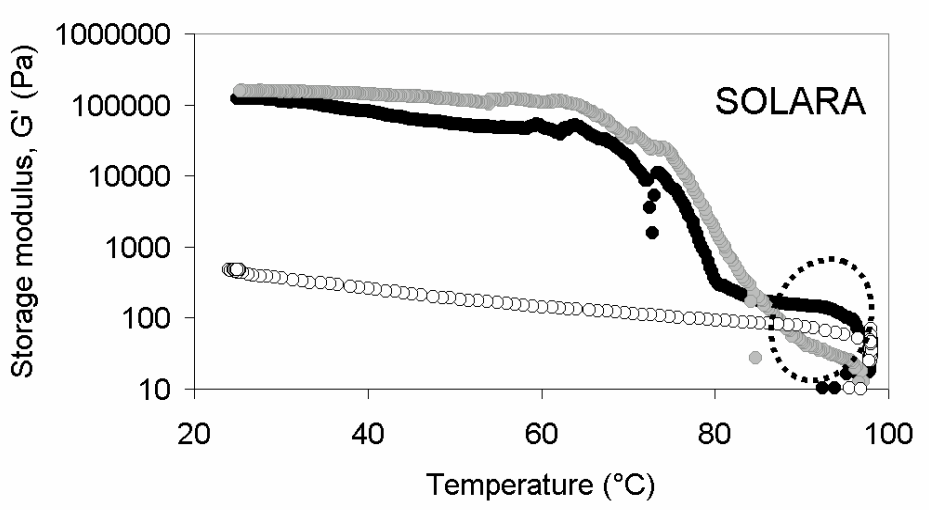
Samples were prepared at pH 7.6, and gelled in a Bohlin rheometer with constant oscillation, taking dynamic measurements throughout the heating and cooling cycles. All the results (in figures 2 and 3) are plotted as storage modulus against temperature. Figure 2b indicates with arrows and numbers phase 1 (heating to 98 °C and holding for 30 minutes) and phase 2 (cooling to 25 °C and holding for 30 minutes). The plot of phase 1 is only visible for values above 10 Pa. It is for this reason that for some samples the plot of storage modulus is only visible at 98°C, while for others there is a visible increase in storage modulus with temperature during the heating ramp up to 98°. For phase 2 the plots must be read from right to left, following an increasing storage modulus as the temperature decreases.

The first comment to make is that when heating/cooling was performed at 1.0°C/min (standard conditions) or heating at 0.5°C/min and cooling 1.0°C/min there was little difference between the cultivars. Figures 2a-e (white circles) show that all the gels formed by heating/cooling at 1.0°C/min had a final gel strength (at 25°C) of the same magnitude (~1000 Pa). Samples heated at 0.5°C/min showed no divergence from the plot

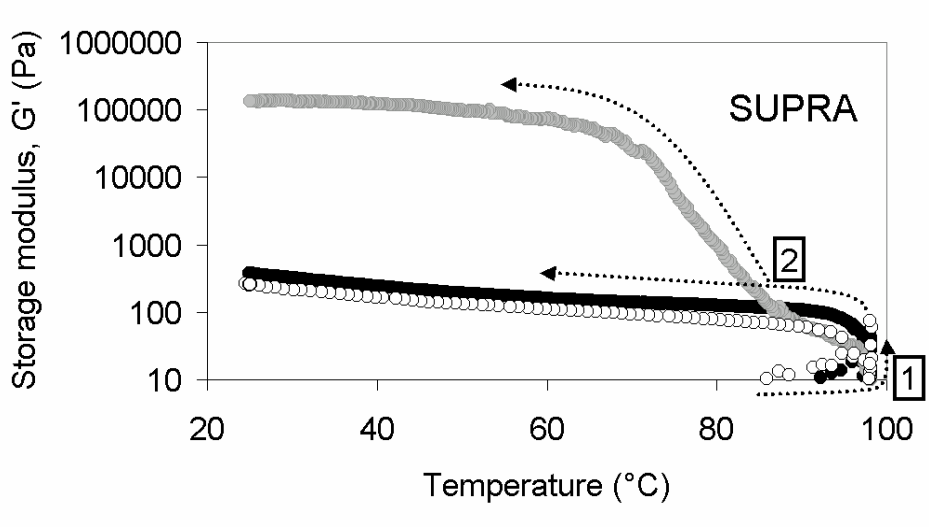
of storage modulus of the standard sample (1.0°C/min heating/cooling) so no results are shown in order to avoid confusion. By contrast, varietal differences in gel formation were seen when the samples were cooled slowly at 0.2°C/min, and for all cultivars there was a difference between the gel formation (at this same rate) in the presence and absence of the thiol-blocking reagent *N*-ethylmaleimide (NEM). Taking first variety Solara (figure 2a), this had the highest proportion of legumin (table 1), and when cooled slowly it had a clear increase in the storage modulus during the initial phase of cooling (98-88°C of phase 2 highlighted by a circle). Gel formation within this temperature range in the presence of NEM was different - the plot of grey circles did not have the same increase in storage modulus at the high temperature (see again the circled area in figure 2a). Since this increased gel formation at high temperatures disappeared in the presence of NEM it was a strong indication that it was generated by the legumin protein forming disulphide bonds that co-operated within the gel network.

Staying with the cultivar Solara, but following the storage modulus at lower temperatures (in phase 2), the development and maturation of the gel network appeared to proceed in the same way in the presence and absence of NEM. This implied that the bonds that strengthened and stabilised the gel networks in this pea cultivar formed independently of any disulphide bonds. The increased number of these bonds (indicated by the increased value of G') when cooling the gel slowly is believed to be because slow cooling gave more time for the proteins to interact. With more time in motion they were able to orientate themselves into more orderly configurations, and maximise their attractive potential (*11, 13*).

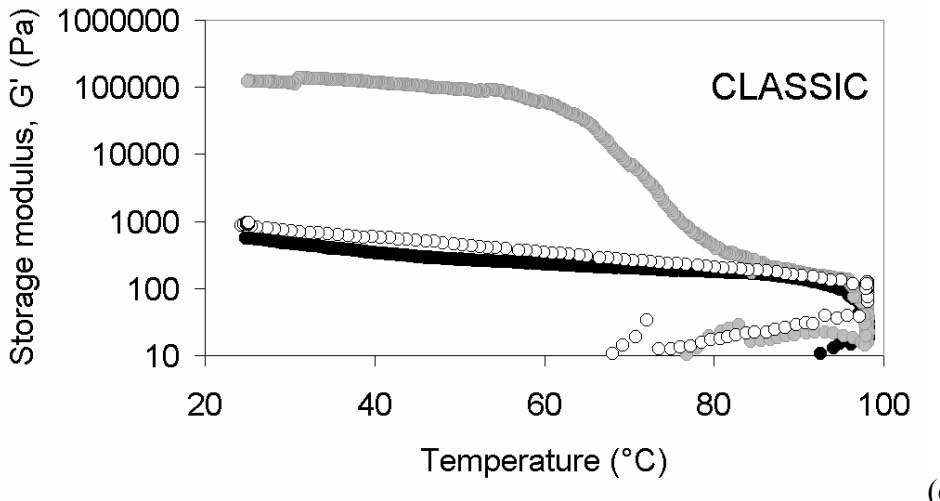
For the cultivar Supra (figure 2b) it can be seen immediately that stronger gels formed only in the presence of NEM. In other words, only when disulphide bonds were absent from the gel network did slow cooling assist a more extensive network interactions to form. From this result, it can be reasoned that in cultivar Supra the formation of disulphide bonds (black circles in figure 2b) somehow restricts extensive non-covalent bond formation and strengthening of the gel network that would otherwise happen when cooling slowly. The cultivar Classic (figure 2c) exhibited the same gelation trends as Supra.


SOLARA
Storage modulus, G' (Pa)
1000000
100000
10000
1000
100
10
20
40
60
80
100
Temperature (°C)

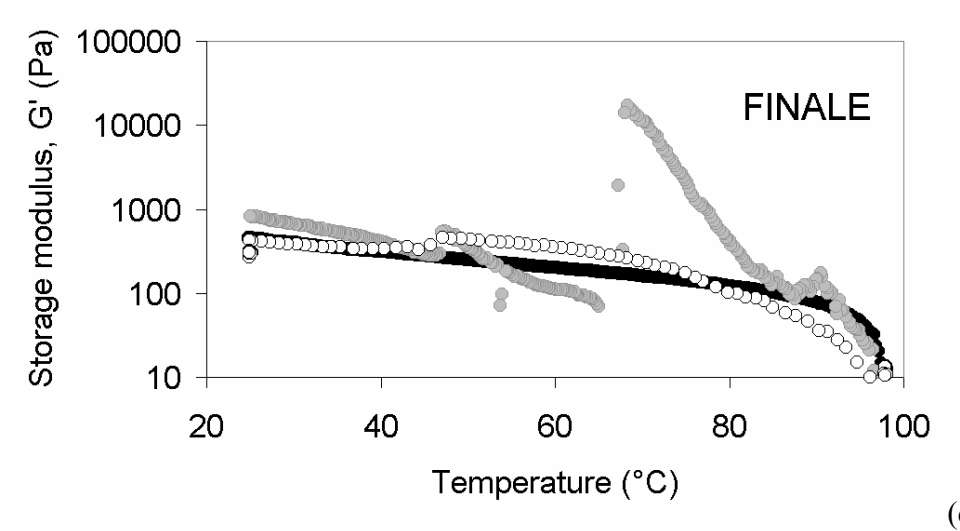
(a)


SUPRA
Storage modulus, G' (Pa)
1000000
100000
10000
1000
100
10
2
1
20
40
60
80
100
Temperature (°C)

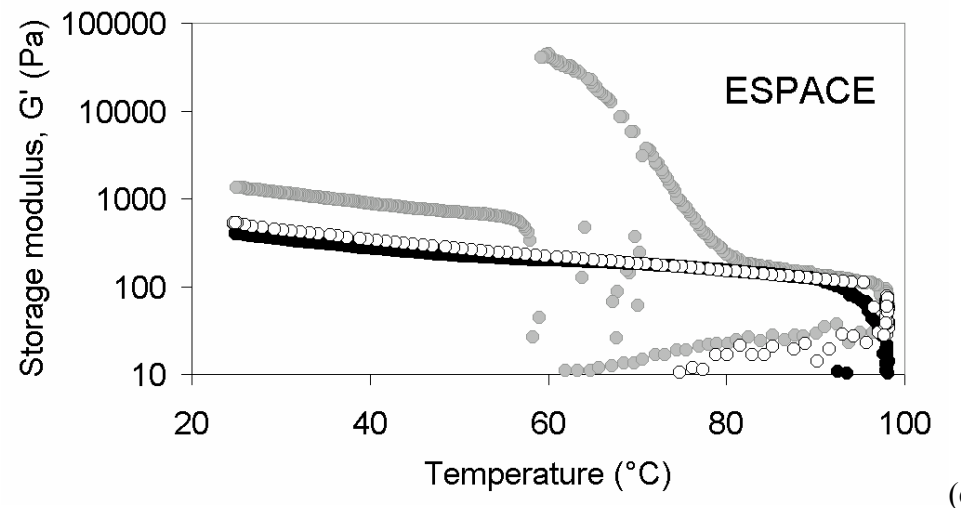
(b)


CLASSIC
Storage modulus, G' (Pa)
1000000
100000
10000
1000
100
10
20
40
60
80
100
Temperature (°C)

(c)



(d)



(e)

Figure 2. Plots of storage modulus (G') against temperature, for protein isolate samples (18% w/v concentration) formed in a Bohlin rheometer with a target strain of 0.015. Symbols: (white circles) heated/cooled 1°C/min; (black circles) heated 1°C/min & cooled 0.2°C/min; (grey circles) heated 1°C/min & cooled 0.2°C/min in the presence of 20 mM *N*-ethylmaleimide (NEM). Pea cultivars used: (a) Solara, (b) Supra, (c) Classic, (d) Finale and (e) Espace.

In trying to relate the observations to the protein composition for these first three cultivars (Solara, Supara and Classic), it made it seem that the 28% legumin of cultivar Solara (table 1) should be a critical level of legumin for disulphide bonds not to be detrimental to the gel strength. Maybe below this percentage the network branch points

that are believed to be created by disulphide bonding (*11*, *13-14*) were too sparse to bring the network strands close together. This was therefore tested by adding legumin purified from cultivars Solara and Supra to the protein isolate of cultivar Classic. The level of substitution (40-80 mg) boosted the percentage of legumin in the isolate of Classic to 28-33% (≥ the percentage of legumin in the isolate of cultivar Solara). Gel formation was monitored in the Bohlin rheometer exactly as had been done for the isolates and the results are presented in figures 3a and 3b. It was obvious immediately that rather than the quantity of legumin being an issue, it was a cultivar effect. This was concluded because the legumin purified from cultivar Solara added to the Classic isolate sample strengthened the gel system and shifted the shape of the plot towards that of a gel made from 100% isolate cultivar Solara (figure 3a). The addition of different amounts of legumin purified from cultivar Supra made the plot of storage modulus and temperature equal to that of a gel made from 100% isolate cultivar Supra, which was in fact slightly weaker than that of the gel made from 100% isolate cultivar Classic (figure 3b).

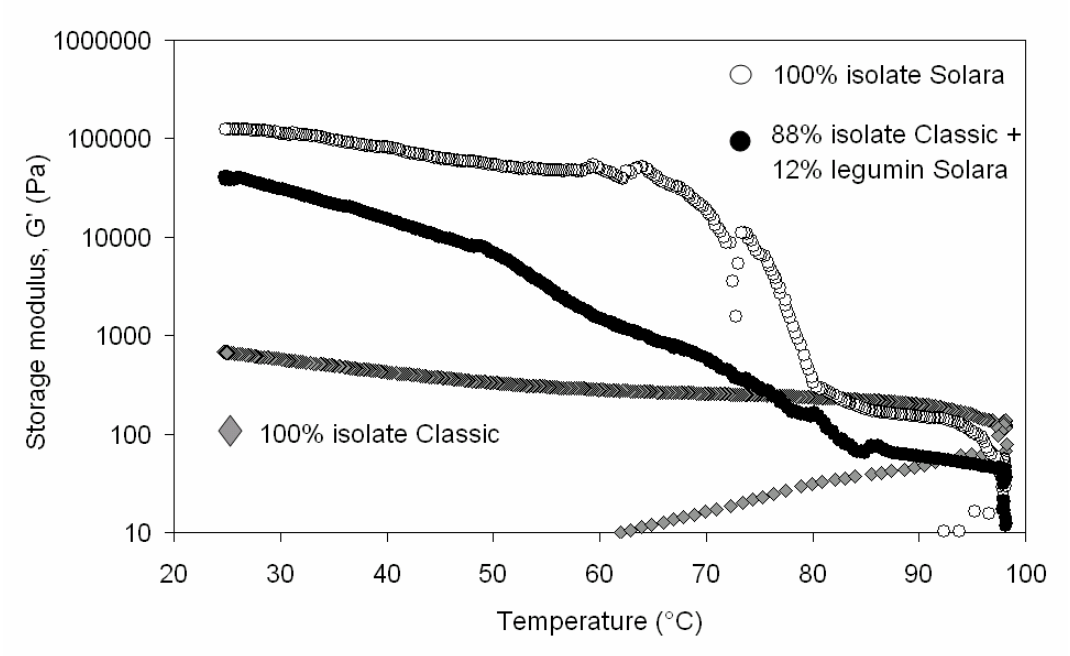


Figure 3a: Plot of storage modulus against temperature for three different gels, all with 18% (w/v) protein concentration, all heated 1°C/min & cooled 0.2°C/min. Symbols: isolate cv. Classic (grey diamonds); isolate cv. Solara (white circles); isolate cv. Classic with 12% (by weight) substituted by purified legumin protein cv. Solara (black circles).

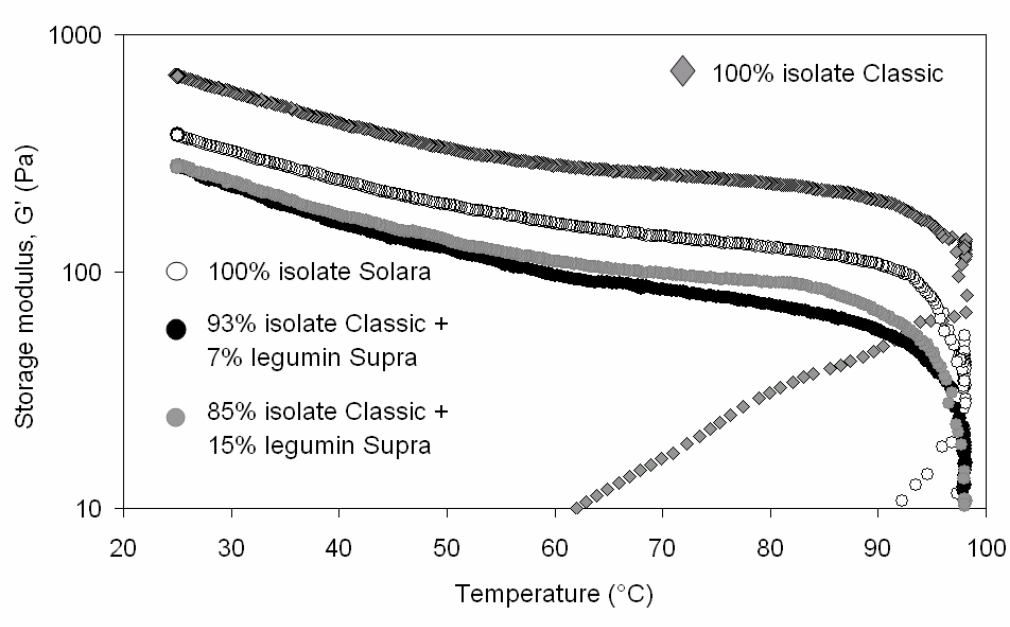


Figure 3b: Plot of storage modulus against temperature for four different gels, 18% (w/v) protein concentration, heated 1°C/min & cooled 0.2°C/min. Symbols: isolate cv. Classic (grey diamonds); isolate cv. Supra (white circles); isolate cv. Classic with 7% (by weight) substituted by purified legumin protein cv. Supra (black circles); isolate cv. Classic with 15% (by weight) substituted by purified legumin protein cv. Supra (grey circles).

Being a cultivar specific difference in gelation behaviour, it may relate to one of the genetically controlled features of legumin, such as the presence of different acidic/basic polypeptides. Under reducing conditions on SDS-PAGE, the size and number of the acidic and basic polypeptides of the purified legumins from cultivars Solara and Supra were the same though (no results shown), and so this was not investigated further. Instead, the amount of free-sulphydryl groups and the total cysteine content of the purified legumin proteins and the protein isolates were determined. This was done because the isolates of Solara, and Supra and Classic, exhibited their differences when disulphide bonds formed within the gel network. The hypothesis put forward at this point was that legumin from cultivar Solara should have less potential for forming disulphide bonds than that from Supra or Classic. Thus, the network branches created by the disulphide bonds in cultivar Solara are so few that they impose very little strength, leaving the aggregate strands free and flexible and able to come close together and form

extensive non-covalent bonds during further cooling. Hence why a strong gel could form in both the presence and absence of NEM (figure 2a). By the same reasoning the higher number of disulphide bonds in Supra and Classic gel networks could restrict the strand flexibility and prevent an optimal gel network formation with respect to the overall gel strength that could develop. This reasoning seems contrary to the gel strengthening ability that disulphide bonds are generally considered to have (*15*-*18*), but it is not. The amount of legumin present in the pea protein isolates is simply proposed to be too little for a sufficient distribution of disulphide bonds throughout the whole gel network. Yet at the same time too much for the effect of the disulphide bonds too many to go unnoticed.

Table 2: Amount of free-sulphydryl groups, and cysteine residues determined to be present in purified legumin of cultivars Solara and Supra, and in the protein isolates of cultivars Solara, Supra and Classic.

Pea cultivar and protein sample	**Free-sulphydryl groups detected (mg /g protein)**	**Total amount of cysteine residues in the sample (g/kg)**
Solara, pure legumin	0.16	6.82
Supra, pure legumin	0.10	7.30
Solara, isolate	1.58	7.38
Supra, isolate	2.29	7.45
Classic, isolate	2.24	8.54

Returning now to the analyses performed, the results obtained are presented in table 2. The purified legumins had a very low free-sulphydryl content. For the protein isolate the levels were higher than for the purified protein, and the isolate from varities Supra and Classic contained slightly more free-sulphydryl groups than the isolate from cultivar Solara. It should be noted that the α-subunits of vicilin are reported to contain one (*19*-*21*) or two (*22*) cysteine residues per ~70 kDa polypeptide, and so these subunits will have contributed to the figures presented in table 2 for the isolates for both the free-sulphydryl and total cysteine content. Even accounting for this, the figures in table 2 indicate the presence of other proteins in the isolate, possibly albumins, that contain significant amounts of cysteine. With respect to the purified proteins, based on an average

molecular weight of the legumin ~ 58 kDa the total cysteine contents of the purified proteins correspond to approximately 4 and 5 cysteine residues per molecule for purified legumin from the cultivars Solara and Supra, respectively, which is an intermediary cysteine content relative those reported by other authors (*19*, *23*). Overall, the results presented in table 2 gave no clear indication that the legumin of Solara has less potential for forming disulphide bonds during heat-induced gelation, and so the hypothesis presented was not proven. The gel strengthening effect of cultivar Solara must therefore be contributed to by other factors. One possibility is that legumin from cultivar Solara contains a large number of hydrophobic residues (that were previously buried in the core of the protein) and that their interaction is what encourages a more extensive gel network formation. A study on the gelation behaviour of the glycinin fraction from four soybean protein isolates (*24*) showed that the storage modulus of the gels correlated positively with the molar percentage of hydrophobic residues, as well as correlating negatively with the molar percentage of cysteine residues. They therefore concluded that gel-forming properties might be related to more than just protein content - also to the amount and type of amino acid in the fraction. Lack of available protein prevented such a comparison being made of the legumin of Solara and Supra. However, a comment to be added is that if the different ability to strengthen gels was related to the number of hydrophobic interactions formed in the gel network, the change observed when NEM was added would not have been expected.

Seeking again an involvement of disulphide bond formation, maybe the difference lies in the aggregate size and shape. Reconsidering the effect that NEM was seen to have on the protein isolates from cultivars Supra and Classic (figures 2b and 2c), it might be speculated that the free-sulphydryl and cysteine residues in these cultivars are spatially very close together when the protein unfolds, and they immediately interact, forming large disulphide bonded aggregates. Larger aggregates would generate aggregate strands with fewer links from one aggregate to the next. With a lesser number of network interactions ultimately the gel would be weaker (have a lower storage modulus). Adding NEM would remove this driving force to form such large aggregates, and enable the benefit of slow cooling to become apparent (as actually observed). In otherwords, without disulphide-driven aggregation the unfolded proteins could have remained in motion for

longer and arranged themselves into more orderly network strands, with smaller aggregates and thus a larger total number of bonds contributing to the network strength (as reflected by the increased storage modulus).

Having discussed the impact of legumin on the gelation behaviour of the pea protein isolates, now the effect of vicilin's oligomeric composition will be discussed. Figures 2d and 2e show that although the two pea cultivars Finale and Espace had the same tendency to form stronger gels when cooled slowly in the presence of NEM, their gel networks were not able to extensively strengthen. According to the staining intensity of the bands of these two cultivars they were calculated to have an α-subunit content of 14 and 13%, respectively in Finale and Espace. Though only 3 and 2% more than present in Supra and Classic, it is proposed to be a critical level. It was shown in a previous paper (*10*) that when a vicilin fraction (50% composed from α-subunits) was added to legumin at a level of 36% (by weight of the total sample), it was able to inhibit gelation at pH 7.6. At this level the percentage of α-subunits in the sample was approximately 18%. Considering that this was able to prevent the gelation of a purified legumin gel containing disulphide bonds it seems reasonable that at 14% in the absence of any disulphide bonds the repulsion of the α-subunits can reduce the gelation ability of the protein isolates Finale and Espace. What was unusual about the gel disruption was that it happened as the temperature dropped. For the cultivars Finale and Espace it can therefore be said that the α-subunits override the attractive forces that would otherwise form at lower temperatures during slow cooling. Why the gels strengthens initially is believed to be because the repulsive α-subunits are in effect too few to override the strong attraction of the hydrophobic residues. They can override the weaker short-range hydrogen bonds however. Without the additional strength and stabilisation offered by hydrogen bonds the continuous movement of the repelled strands is believed to disrupt some of the network that has already formed and cause the drop in storage modulus seen in figures 2d and 2e.

Some studies on globular pea proteins have compared the functionality of crude protein preparations to that of the isolate as a first step in determining what should be the ratio of the proteins for an optimum functionality of the protein isolate (*25-27*). In this paper, evidence for relating the gelation behaviour of the isolate to its protein

composition could only be done for the cultivars Finale and Espace, with respect to the percentage of α-subunits in the vicilin fraction. The contribution of legumin to the pea protein isolate gels was shown to be cultivar specific and possibly more dependent on spatial proximity of the reactive residues when the protein unfolds than the amount of legumin protein present.

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Chapter 6

GENERAL DISCUSSION

RESTATING THE AIM

The work carried out in this thesis aimed to extend the molecularly based understanding of pea protein gelation that is missing in the literature. Moreover, having selected the purified globulins as the protein source it was also important to determine if such an approach was suitable for understanding the gelation behaviour of the pea protein isolate, which will eventually be a much more likely food ingredient than the purified proteins themselves.

A MOLECULARLY BASED UNDERSTANDING

The aim of extending the understanding of the molecular events that leads to gel network formation was chosen for the simple reason that molecular understanding is believed to give scientists the key for controlling and/or modifying the functional behaviour of proteins (via changes in the environmental conditions). For proteins used as food ingredients molecular understanding aids further development of their applications. One of the questions posed in the Profetas project on the design of novel protein foods, was if pea proteins offer a technologically sustainable future for novel protein foods. With this regard, this thesis intended to explore if the pea proteins were a suitable and pliable food texturising ingredient for novel protein foods. The importance of gels in food structure led to the investigation of the heat-induced gelation of the pea proteins presented in this thesis.

Pea vicilin

The first of the globular pea proteins that was investigated was vicilin. The polypeptides produced from the vicilin genes are molecularly heterogeneous: the majority contain different post-translational cleavage sites, and a small number have a highly polar N-terminal extension region (that constitutes ~20% of the total amino acid sequence). Despite this heterogeneity the separation of the extracted vicilin protein into two fractions was unexpected, and not in accordance with the literature (*1*), where legumin and vicilin were reported to separate from one another under the acid/salt conditions used in this thesis. Characterisation of the two vicilin fractions (chapter 2) showed the fractionation to

have been caused by the N-terminally extended polypeptides, which have an unusually low solubility in acid/salt conditions. This was concluded after observing on SDS-PAGE that at the minimum solubility of the vicilin fraction (pH 4.8-5.0) the percentage of ~70 kDa polypeptides present in the soluble fraction decreased from ~55 to ~30%.

It is worth to add at this point that although this shift in the proportion of ~70 kDa polypeptides could be argued to be due to the precipitation of the separate protein convicilin, we argued instead that this shift is due to the precipitation of oligomers containing only, or predominantly ~70 kDa polypeptides. For the sake of explanation let's assume the vicilin to be present as a trimer at pH 4.8-5.0, and that the polypeptides ~50 kDa are known as β-subunits. The precipitated protein should thus be the α_3 or $\alpha_2\beta$ form of the vicilin protein. That which remained dissolved was therefore different $\alpha\beta_2$ and β_3 trimers (different with respect to the numerous genetic forms of the β-subunits). If the α-subunits formed only separate oligomers, thus existed as the separate protein convicilin, the shift in the composition of the dissolved protein would have been expected to have been reduced by more than the 25% observed. Thus, as already concluded in chapter 2, the consideration of convicilin as a separate protein, when referring to oligomeric assemblies, is misleading.

As done by Thanh and Shibasaki for soybean β-conglycinin (*2-3*) it would be interesting to separate the individual oligomers of pea vicilin and build a model of the different combinations that exist, eg. α_3, $\alpha_2\beta$, $\alpha\beta_2$, β_3. It was shown in chapter 3 however when the vicilin fractions were separated into subfractions using chromatofocusing, the differentially processed ~50 kDa subunits introduce a factor of heterogeneity that does not exist in β-conglycinin. Consequently, the separation and classification of different pea vicilin oligomers presents a greater challenge than that tackled by Thanh and Shibasaki.

Transparent gels

When the vicilin preparations were gelled by heating (chapter 3) the N-terminally extended polypeptides, the α-subunits, became evident again. With a net negative charge at near-neutral pH conditions, the extended regions were sufficiently repulsive as to

hinder the aggregation and network formation of the unfolded proteins. Consequently, more protein was needed to form a continuous network. Yet when formed, the gel was transparent. Despite the high protein concentration needed to form a transparent gel, this behaviour of the N-terminally extended polypeptides is undoubtedly a tool for product developers, because transparency is not a property that leguminous plant protein gels tend to offer. It is a gel property that is most often reported in relation to ovalbumin, lysozyme and bovine serum albumin (*4*).

Whatever the source of protein, transparent gels are believed to be created by a linear arrangement of aggregates into fine network strands (*4-6*). It follows from this that transparent gel networks can be created by manipulating the way in which the protein aggregates form and arrange themselves. For example, transparent gels were successfully created by adding fatty acid salts with a sufficiently long unsaturated carbon chain to the 13S globulin protein of sesame seeds (15% w/v protein concentration) (*7*). The authors proposed that the added fatty acid salts interacted with the hydrophobic area of the protein molecules, and that this depressed the protein-protein hydrophobic interaction that would otherwise enable extensive aggregation of the sesame seed protein. The reactive parts of the proteins left exposed were highly hydrophilic, and so the aggregates arranged into fine hydrophilic strands.

Returning now to the discussion of the N-terminally extended polypeptides of the vicilin, these polypeptides are not post-translationally cleaved, and are encoded for by only two genes. They are therefore the least genetically heterogeneous polypeptides of the pea. Moreover, in terms of the amount produced within the plant they are considered as a minor polypeptide of the pea. It seems ironic therefore that their consistent characteristic of being N-terminally extended is what dominated the extraction and purification, and the heat-induced gelation of pea vicilin. What it demonstrates however is the importance of the positive/negative balance of interactions in the formation and maintenance of a gel network.

The soybean analogue

In addition to extending the knowledge of pea protein gelation, comparison of the gelation behaviours of the major pea globulins with the much studied analogous globulins of soybean (as done in table 1) can help to identify common molecular based characteristics of gelation. The soybean analogue of pea vicilin is β-conglycinin. As stated earlier, unlike vicilin it does not undergo post-translational proteolysis, thus on SDS-PAGE only the intact monomers of its oligomers are visible. The smallest (the β-subunit) is ~50 kDa, thus of a similar size to the subunits that can undergo post-translational proteolysis in pea. The other two subunits, α- and α'-, are larger ~67 and ~71 kDa respectively, and both contain N-terminally extended regions. The extension regions have 125 and 141 residues respectively in the α- and α'-subunits and they have the same polar characteristic as that on the α-subunits of vicilin (*8*). In addition, its impact on the heat-induced aggregation of purified β-conglycinin at near-neutral pH conditions appears to be the same (*9-10*), though not all authors recognise the extension region as the cause of their observations. Renkema (*11*) for example noticed that the gels of soybean protein isolate were much weaker than the gels of soybean glycinin. Moreover, the gel formation of the protein isolate was detectable only after the denaturation of the glycinin (which denatures at a higher temperature than β-conglycinin). The conclusion came that β-conglycinin did not contribute much to the gel network formation. Under the conditions used the explanation would seem instead to be that the extension regions on the α/α' subunits were acting against the process of aggregation and network formation.

Concluding remarks

It is believed that all vicilin-like proteins that contain highly polar N-terminally extended polypeptides will have the same repulsive forces acting against extensive aggregation and network formation under near-neutral pH conditions. If considered desirable, this characteristic can be used as a tool to modify the gelation behaviour. Alternatively, it can be neutralised by maintaining the protein at pH values below the isoelectric point, or by adding salt at a suitably high level to shield the charges.

Table 1: Summary of the size and heterogeneous features of polypeptides belonging to the vicilin- and legumin-like proteins of pea and soybean. The molecular characteristic that effects gelation behaviour is indicated for both the purified and isolated protein systems.

	Pea vicilin	**Soybean β-conglycinin**	**Pea legumin**	**Soybean glycinin**
Size of proteins generated	~50 kDa ~70 kDa (N-terminally extended)	~50 kDa (b-subunit) ~67 kDa (a-subunit) ~71 kDa (a'-subunit) (a/a' N-terminally extended)	~60 kDa	~60 kDa
Differential post-translational proteolysis	Two, one or no cleavage sites on ~50 kDa polypeptides, depending on encoding gene	No post-translation cleavage of any polypeptides	Site-specific cleavage generates acidic and basic polypeptides than remain linked via a disulphide bond	Site-specific cleavage generates acidic and basic polypeptides than remain linked via a disulphide bond
Characteristic effecting gel network formation in a pure protein system	Percentage of polypeptides ~70 kDa in protein preparation (chapter 3)	N-terminally extended α/α'-subunits (9-10)	Rate of refolding/aggregation (chapter 4)	Unknown (chapter 6)
Characteristic effecting gel network formation in a protein isolate system	Percentage of polypeptides ~70 kDa in protein preparation (chapter 5)	N-terminally extended α/α'-subunits (speculated in chapter 6)	Spatial proximity of cysteine residues generates large aggregates and weaker gels (chapter 5)	Believed to be the same as for the pure protein system because it is a reheatable gel network.
Role of disulphide bonds as a gel strengthener	n/d	n/d	Only in pure protein samples cooled slowly (chapter 4)	In pure protein samples cooled slowly (chapter 4). For protein isolates n/d.

n/d: not determined

Pea legumin

Pea legumin is no less heterogeneous than vicilin in its genetic variants. Produced from several small families of genes, each encoded protein has a slightly differing amino acid profile. After post-translational proteolysis there are then numerous acidic and basic polypeptides with different sizes and isoelectric points generated, though they remain disulphide bonded until, for example, the protein undergoes heat-induced gelation. At this point the acidic and basic polypeptides may dissociate from each other (depending on the sample conditions) and act independently from one another - the separation of different legumin molecules for functionality testing is not a feasible option though. Added to this that in the purification scheme used in this thesis (chapter 2) all forms of legumin were obtained as a single preparation staining with a single band on SDS-PAGE (~60 kDa), its heterogeneous composition was not investigated.

Instead, the opportunity was taken to study pea legumin and its analogue from soybean, namely glycinin, under the same conditions of experimentation. The work set out to test the hypothesis that a common model for the gelation of legumin-like proteins can be built upon a molecular based understanding of the gelation behaviour. Changes in the heating/cooling rate of the gel samples was done to alter the kinetics of the gelation process. The thiol-blocking agent *N*-ethylmaleimide (NEM) was also added to test the importance of disulphide bonds within the gel network, because pea legumin and soybean glycinin are recognised for their disulphide bonding potential. The results showed the hypothesis to be false: for a given condition the two molecularly homologous proteins had the same physical and chemical forces driving their gelation processes. Despite this soybean glycinin was able to form more orderly gel networks. Reasons for this are not yet understood. The information gained was therefore not sufficient to build a predictive model of gelation. Aside from this conclusion, disulphide bonds were shown to be important for creating pure pea legumin gel networks that were stable to reheating (stable against rearrangements at high temperature), and when present in samples that were cooled slowly they contributed to the additional gel strength. This contribution to gel strength and stability is in agreement with the role that disulphide bonds are often promoted to have in diverse gel systems (*12-15)*.

Later in chapter 5, the role of legumin, and especially its disulphide bonding potential was tested with the heat-induced gelation of five different pea protein isolates. Previously in chapter 4 the gel strengthening ability of disulphide bonds had been clearly visible in samples that had been cooled slowly. In the pea protein isolates only one cultivar, Solara, exhibited the same type of gel strengthening when cooled slowly, though it was shown to occur regardless of any involvement of disulphide bonds. The legumin protein present in other cultivars apparently hindered a more orderly arrangement of the protein aggregates, because only when NEM was added to the samples were the effects of slow cooling apparent.

Transparent gels

In this thesis transparent gels were obtained with both pea legumin and soybean glycinin when NEM was added (chapter 4). A report of transparent gel formation by soybean glycinin has also been made by Lakemond *et al.* (*16*) when the sample conditions were pH 3.8, I = 0.03. In the first cases, the formation of disulphide bonds was suppressed by the NEM covalently bound to sulphydryl groups, and in the second case disulphide bond formation would have been minimised by the acid/low ionic strength conditions used. For all these situations the transparency can then be explained to have derived from a less branched network being formed (*17*), since it is disulphide bonds that contribute to network branching and subsequent strengthening (chapter 4).

The soybean analogue

Soybean glycinin is molecularly homologous to pea legumin, more so than β-conglycinin is to vicilin. Both proteins have a similar size, and undergo the same post-translational proteolysis, generating a range of acidic and basic polypeptides with different sizes and isoelectric points (as detailed in table 1). In chapter 4 it was even shown, by changes in the conditions that influenced the kinetics, that both the proteins have the same molecular forces driving the gelation process. What remains the question therefore is why soybean glycinin aggregates arrange themselves in a more orderly manner. Already it was deduced that the reheatability of the glycinin gels was not

dependent on disulphide bonding in the aggregates or the network. The next thought that arose was if glycinin has a much larger number of hydrophobic residues that once exposed drive an ordered aggregation mechanism. To answer this question a total amino acid analysis was performed, comparing the purified legumin of cultivar Solara with the soybean glycinin from cultivar William's 82 (as used in chapter 4). The results are presented in table 2. Looking specifically at the relative amounts of hydrophobic residues, no direct correlation can be made between the amino acid composition and the gelation behaviour. Moreover, apart from the sulphur containing residues, there is not much difference in the amino acid profiles.

Table 2: Amino acid compositions of pea legumin cv. Solara and soybean glycinin cv. William's 82. Hydrophobic residues are highlighted in bold.

Amino acid	Pea legumin, cv. Solara (mol%)	Soybean glycinin, cv. William's 82 (mol%)
Asp + Asn	11.4	12.0
Thr	3.4	3.9
Ser	6.4	6.9
Glu + Gln	20.7	20.1
Pro	5.3	6.4
Gly	**7.8**	**8.2**
Ala	**6.5**	**5.7**
Val	**4.4**	**4.3**
Cys	0.9	1.4
Met	0.6	1.1
Ile	**3.1**	**3.6**
Leu	**7.5**	**7.2**
Tyr	2.3	2.7
Phe	**3.5**	**3.9**
Lys	4.6	4.2
His	2.4	1.8
Trp	0.5	0.7
Arg	8.6	6.0

Though having similar amino acid profiles is in line with both proteins having the same forces driving the successive processes of heat-induced gelation (chapter 4), it makes it seem that the reheatablility of glycinin gels is determined by inherent features of the glycinin protein, and not by the molecular interactions that occur during gelation.

To reflect on the relationship between gel network formation and the protein's bonding potential, it was seen that the amino acid profile of the cultivar William's 82 (as presented in table 2) was very similar to the cultivar IL2 used by Riblett *et al.* (*18*). Cultivar IL2 was that which had the lowest gel strengths of the four cultivars studied, supposedly because it had the lowest percentage of hydrophobic residues. In another study that compared four soybean cultivars (*19*), the amino acid profile of the glycinin fraction again showed a positive correlation between hydrophobicity and gel strength. Despite these correlations between glycinin hydrophobicity and gel strength, in this thesis there was a lack of correlation between glycinin reheatability and its amino acid profile when compared with that of pea legumin. Though a speculative comment, it may be that each legumin-like protein has its own gelation behaviour, thus if comparing different cultivars a correlation between gel strength and the amino acid profile may well be found. But if comparing one legumin-like protein with another the correlation will be lacking, because there is no common process of gelation. After comparing physical characteristics of a milk protein, a blood protein and two egg white proteins and finding it hard to identify a protein characteristic that was crucial for gel formation, Hegg (*20*) similarly concluded that the answer to good gelling behaviour might well be finding the right conditions for the protein in question. A comparison of pea legumins from different cultivars would be worthwhile to test this idea. Comparing table 2 with results reported by Casey and Short (*21*) for six different pea legumins shows differences in the amino acid profiles, so maybe a correlation with pea legumin gelation behaviour could be found.

Concluding remarks

The gelation behaviour of the legumin-like proteins has proven to be difficult to systematically describe with a molecularly-based explanation. No generic importance of disulphide bonds in stabilising and strengthening gel networks could be acknowledged.

Finding the differences among the similarities of legumin-like proteins

Before proceeding to a critical view on the experimental approach taken in this thesis as a whole, it is worth to reflect a little longer on the lack of molecular understanding of the different gelling behaviours of legumin-like proteins (as summarised in table 1). In this thesis the comparison was made between pea legumin and soybean glycinin. To expand the view, published results on comparisons between broad bean legumin and soybean will also be introduced.

As already stated, legumin-like proteins are the most molecularly similar proteins among different leguminous plants. Generated as molecules of ~60 kDa they undergo post-translational processing that splits the molecules into an acidic and a basic polypeptide. However, these polypeptides remain linked via a disulphide bond until the protein is denatured by heating, or the disulphide bond is chemically reduced. For pea, soybean and broad bean numerous acidic and basic polypeptides with different sizes and isoelectric points are acknowledged to exist: the cause is the generation of the legumin-like molecules from many small gene families. Now we come back to the title, because as explained above, the molecular similarity of the legumin-like proteins can not be disputed. And it is this that makes it difficult to deduce why these proteins have different gelation behaviours.

The covalent disulphide bonding capacity of legumin-like proteins has long been recognised, but like Alting showed by comparing whey protein isolate with ovalbumin (*22*), no generic role of disulphide bonds in network formation could be shown. In the absence of any clear correlation between gel strength and the number of thiol groups, a statement often made is that the gelation behaviour of the protein depends not only the number of thiol groups, but on their topology and reactivity. While this may be a relevant statement, this thesis was able to go a little further and show that it was when the involvement of disulphide bonds within the gel network was not maximised (eg. by slow cooling) that the number of thiol groups lost its relevance. Under control conditions (1°C/min heating and cooling) the spatial proximity of reactive residues may well drive the aggregation process and determine the characteristics of the final gel network. In this

thesis at least no direct correlation between the bonding capacity and gel network characteristics could be found for the legumin-like proteins studied.

This said however, other authors have taken a different approach to legumin-like gelation. Using mutant molecules Utsumi *et al.* (*23*) compared mixed soybean glycinin-broad bean legumin mutants to the native proteins, and correlated the presence of certain acidic subunits with the gel hardness. Of particular interest in their study was the fact that the broad bean legumin formed increasingly transparent gels at higher protein concentrations. This again was correlated to the presence of a specific acidic subunit, and its possible ability to remain associated with the basic subunit. Insolubility of the dissociated basic subunits is what was believed to cause glycinin gels to be turbid. Remaining associated also implies that the sulphydryl group involved in that association is unavailable for creating network branches. This fits with the explanation put forward for the transparent pea legumin and soybean glycinin gels formed when heated in the presence of the thiol-blocking reagent, which ultimately suppresses the acidic-basic polypeptide dissociation.

Another study that compared soybean glycinin with broad bean legumin (*24*) showed with TEM that despite their transparency legumin formed less regular network strands than glycinin. This irregularity was believed to be the cause of the higher minimum gelling concentration of legumin and the weaker gels of legumin at a given concentration compared to glycinin. Although the pea legumin gels in this thesis were not transparent, the similarity of their results with those presented in chapter 4 make it worthwhile to use both broad bean and pea in studies aimed at further elucidating why soybean glycinin has a superior gelation behaviour relative to other legumin-like proteins.

To summarise, the experiments performed in all the above examples drew conclusions about the factors determining the gelation behaviour of the legumin-like behaviours that respected what was observed. Thus it becomes evident the difficulty there is to synthesise the observations of different legumin-like proteins, and find a single determinant that can explain the gelation behaviours of all these molecularly similar proteins.

THE APPROACH

As explained already, this thesis was approached from the bottom up, first working to extend the molecular based understanding of the gelation behaviour of the purified pea globulins, and secondly testing if the understanding helps to explain the gelation behaviour of pea protein isolates. Since pea seeds have a highly variable globular protein composition (*26*) it was assumed that the protein isolates from different cultivars would also have different protein compositions and functionality - it was in this way that the applicability of the molecular understanding of the pure proteins was expected to be tested. The first comments should therefore be on the similar protein compositions of all the protein isolates used in chapter 5, even though the total protein extracts of these five cultivars had been shown to be different. A summary of the protein composition before and after isoelectric precipitation is given in table 3. The only apparent explanation seems to be that the isoelectric precipitation step with hydrochloric acid caused a high level of irreversible precipitation of particularly the legumin. Such loss due to irreversible precipitation has been shown to occur also with soybean glycinin (*25*). The equalisation of the protein composition can not be explained by this phenomena however, and is something that is undoubtedly worth investigating in more detail because it is the variability in pea protein composition that is most often referred to as a potential tool for controlling the functional behaviour of protein isolates used as food ingredients.

Table 3: Relative composition of the globular proteins in a total extract of pea flour (pH 8), and in protein isolates (isolated from the total extract by precipitation at pH 4.8) resuspended in 75 mM potassium phosphate buffer at pH 7.6. Percentages were calculated according to the area under the peak of the staining intensity.

Pea cultivar	Legumin (%)		Vicilin subunits ≤ 50 kDa (%)		Vicilin α-subunits (%)	
	extract	isolate	extract	isolate	extract	isolate
Solara	54	*28*	27	*62*	19	*10*
Supra	54	*25*	35	*64*	11	*11*
Classic	34	*22*	50	*67*	16	*11*
Finale	37	*25*	43	*61*	20	*14*
Espace	35	*20*	49	*67*	16	*13*

To discuss now to what extent the pure protein gel behaviour was apparent within the pea protein isolates, the discussion will start again with vicilin. In chapter 3 when vicilin was added to legumin, the repulsive force was not sufficient to disrupt the gel network when α-subunits constituted 10% of the total protein present. They were disruptive at a level of 18%. Intermediary α-subunit contents were not tested, but a content of 13/14% α-subunits in the protein isolate is considered sufficient to be the cause of network destabilisation in the cultivars Finale and Espace. The remainders of gel samples were not evaluated for turbidity/opacity when removed from the Bohlin rheometer so it can not be commented whether or not the α-subunits are effective at reducing the turbidity of the protein isolate gels. Regardless, it can be concluded that the repulsive force of N-terminally extended polypeptides of pea vicilin at near-neutral pH act in the same way in all types of gel samples, hindering the gel network formation when present in sufficient quantity.

The behaviour of pea legumin in a purified gel sample or in a protein isolate exhibited differences, the understanding of which was complicated by legumin also exhibiting cultivar specific behaviours. Without re-summarising the results in words, they are presented in table 4. In view of everything, it can be stated that for the cultivar Solara the gelation behaviour of purified legumin (chapter 4) was not reflected in the gelation behaviour of the protein isolate (chapter 5). Both systems formed more orderly, strengthened networks with slow cooling, but while that of the pure protein was promoted by the involvement of disulphide bonds, that of the protein isolate formed regardless of disulphide bonds being present or not.

For the cultivar Supra, despite the legumin having a higher disulphide bonding potential than the cultivar Solara, it did not exhibit any gel strengthening ability. This makes it seem that in this cultivar disulphide bond formation inhibited the gel strengthening that it would otherwise be expected to promote.

Table 4: Summary of the gel strengths obtained in gel samples containing the protein legumin when they were heated 1.0°C/min & cooled 0.2°C/min, in the presence and absence of the thiol-blocking agent *N*-ethylmaleimide (20 mM). The amount of free-sulphydryl groups and total cysteine residues for the different samples is also listed (expressed in g/kg).

Protein sample	**Final gel strength, G' (Pa) at 25°C**		**Free-SH content (g/kg)**	**Cysteine content (g/kg)**
	in buffer	**+NEM**		
Purified legumin cv. Solara	$\sim 10^5$	$\sim 10^4$	0.16	6.82
cv. Supra	n/d	n/d	0.10	7.30
Protein isolate cv. Solara	$\sim 10^5$	$\sim 10^5$	1.58	7.38
cv. Supra	$\sim 10^3$	$\sim 10^5$	2.29	7.45
cv. Classic	$\sim 10^3$	$\sim 10^5$	2.24	8.54
Protein isolate cv. Classic with added purified legumin				
cv. Solara (60 mg)	$\sim 10^5$	n/d	n/d	n/d
cv. Supra (40 and 80 mg)	$\sim 10^3$	n/d	n/d	n/d

(n/d: not determined)

Concluding remarks

The ultimate goal of the Profetas project was to offer product developers the necessary tools for establishing a consumer-orientated product development process for novel protein foods. The results presented in this thesis have shown that there is not always one answer to protein functionality, and the globular pea proteins can be said to have exhibited a functional complexity that well reflects their heterogeneous composition. The approach to first expand the molecular understanding of the behaviour of purified proteins was useful because differences in gelation behaviour of purified proteins could be satisfactorily explained. A clear example is the dominant character of the α-subunits when gelling vicilin-like proteins at near-neutral pH conditions. Admittedly though, the results are not yet sufficient to predict or control the complex gelation behaviour of the globular pea proteins within a food matrix. This requires more research on the interaction between proteins as well as between proteins and other

components, such as the starch in pea flour. Nevertheless, it is believed that the results of this thesis form a basis and give guidance for such further research.

FUTURE POSSIBILITIES

The potential for modifying the gelation behaviour of pea globulins to suit the creation of a desirably textured product is in the hands of both the product developers and plant breeders. This thesis has demonstrated that under conditions where they carry a net negative charge the α-subunits of vicilin offer the opportunity to make transparent gels. Moreover, this possibility can be seized using a vicilin-like protein preparation from any other legume that contains extended polypeptides with the same characteristic of being highly polar.

For pea legumin the future is in research aimed at fully understanding the difference in the aggregation and network forming processes between pea (and faba bean) legumin and soybean glycinin, using the protein from many different cultivars of each plant source. Until the difference can be related to a characteristic of the protein, it is difficult to target legumin for improved or modified functionality. This thesis yielded what appeared to be conflicting results because while for one pea cultivar the gel strengthening ability of disulphide bonds was appreciated in the pure legumin gels, it was an irrelevant factor in the protein isolate gel. For another cultivar the disulphide bonds were an apparent hindrance against gelation of the protein isolate. Future studies should focus on the important factors of legumin-like protein gelation mechanisms as blank loose leaf pages, and not as a book already bound by disulphide bonds.

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SUMMARY

This thesis was completed within the framework of the Profetas project, an acronym for PROtein Foods, Environment, Technology And Society. Profetas is a multi-disciplinary project containing all the elements needed to address the question: Is the partial replacement of meat by novel protein foods technologically, environmentally and socially acceptable? As part of the technological team, this thesis intended to explore if the pea proteins were a suitable and pliable food texturising ingredient for novel protein foods. The importance of gels in food structure led to the investigation of the heat-induced gelation, with the aim to extend the molecular-based understanding of globular pea protein gelation. For proteins used as food ingredients molecular understanding aids further development of their applications.

Chapter 1 introduces the subject with a literature review concerning pea protein composition and characteristics, heat-induced gelation of proteins in general and pea proteins in particular. The most important globular pea proteins are legumin and vicilin, and a minor protein is convicilin. Their extensive heterogeneity is described. The problem statement, goal and outline of the present work is stated at the end of Chapter 1.

Chapter 2 describes the isolation and purification procedure used throughout this thesis to obtain purified proteins for functionality testing. An unexpected separation of vicilin into two fractions triggered the additional work in this chapter on the characterisation of pea vicilin. Before that however, a series a chromatographic techniques were used to remove the convicilin that heavily contaminated one of the vicilin fractions. This separation was not possible, and instead the results obtained indicated that convicilin is not a contaminant, but a subunit of the extracted vicilin protein. This was further supported by gel electrophoresis, differential scanning calorimetry (DSC), circular dichroism (CD) and solubility experiments. Convicilin was denoted as the α-subunit, in an analogous manner to soybean. The definition of vicilin heterogeneity should consequently be extended to acknowledge the possible inclusion of α-subunits in the oligomeric assembly of vicilin.

The implications on gelation of such a subunit heterogeneity was explored in Chapter 3, where the two vicilin fractions (with differing subunit compositions) were compared under the same conditions with regard to gelling properties upon heating and cooling. As is usual for heat-induced protein gelation, a three-step process was observed, first denaturation (unfolding), then aggregation of denatured proteins, followed by further aggregation into network structures upon cooling. It was clearly demonstrated that at near-neutral pH conditions the highly charged N-terminal extension region on the α-subunits contributed a net negative charge that ultimately hindered extensive gel network formation, i.e. increased the minimum gelling concentration. What was interesting however was that the α-subunits caused transparent gels to form. Transparency is a characteristic of gels that indicates that the aggregates that make up the gel network are small in size. This observation is in keeping with proteins that carry a large net negative charge. The finding was further substantiated by the observation that the inhibiting effect of the α-subunits disappeared at low pH as well as at high ionic strength. Another observation was that mixing of the vicilin fraction containing α-subunits with legumin had an effect on the transparency of the resulting gel. This chapter concluded that the subunit composition of vicilin can be targeted as a tool to modify the gelation of these pea proteins.

Having explored vicilin, Chapter 4 describes a series of experiments designed to compare the intermolecular forces that create and support different pea legumin gels. The techniques used were DSC, rheology, and transmission electron microscopy. In view of the importance of sulphydryl groups, use was made in some experiments of *N*-ethylmaleimide, a compound that blocks sulphydryl groups. Overall, it was shown that pea legumin gel formation was not effected by changes in the heating rate, and two differently heated samples were unaffected by the addition of NEM, which indicated that disulphide bonds were not essential within the network strands of these legumin gels. Comparison of legumin with the molecularly homologous soybean glycinin gelled under the same conditions enabled the hypothesis to be tested that a common model for legumin-like protein gelation can be built with a molecular basis. Overall the results showed that both the proteins have the same physical and chemical driving forces acting during gelation, but soybean glycinin, unlike legumin, was consistently able to form

reheatable gels. Legumin formed stronger gels upon reheating and cooling. The hypothesis tested was thus proven to be false. What enables the soybean gels to be reheatable was not identified in this chapter, but was returned to in chapter 6 after having performed total amino acid analysis of both pea legumin and soybean glycinin.

Chapter 5 presents a comparison of the gelation behaviour of the protein isolates from five different pea cultivars, and explored to what extent it could be predicted based on knowledge of how the individual globular proteins gelled. One cultivar (Solara) stood out for its ability to form strengthened gels both in the presence and absence of NEM, while other varieties formed stronger gels only when NEM was added. The purified legumin from cv. Solara also exhibited gel strengthening ability when added to the protein isolate from a cultivar (Classic) that formed weaker gels than did the protein isolate from cv. Solara. Determination of the free-thiol and total cysteine contents of purified legumin and protein isolates highlighted no direct relationship between the disulphide bonding potential of the cultivars and their gel behaviours, so an indirect explanation for why NEM affected the gel strength for all pea cultivars except Solara was put forward. This was that the spatial arrangement of SH-groups has an effect on the types of aggregates formed and consequently on the resulting network formation. Isolates of two cultivars (Finale and Espace) gave only weak gels and this could be related to the content of α-subunits of vicilin in these isolates, in line with the findings reported in Chapter 3.

The thesis is concluded in chapter 6 with a critical evaluation of the experimental approach taken in this thesis. A comparison is made between the analogous pea and soy proteins. The aim of relating the gelation behaviour of the pea protein isolates to the molecular and gelling characteristics of its constituent proteins is critically discussed, and suggestions for future work are put forward. Overall, it is concluded that the influence of vicilin α-subunits on gelation behaviour is a quantity dependent behaviour. That of legumin however is a variety specific behaviour, and depends on protein characteristics as yet unidentified. In view of the observed differences between pea legumin and soy glycinin an additional amino acid analysis was performed, but this did not reveal significant differences: there was no relation between amino acid composition and gelation behaviour. Spatial proximity of reactive residues when the legumin and glycinin

proteins unfold has been speculated to be a possible factor that can effect the aggregation process, and in turn influence the strength of the resultant gel network. All in all, no unique answer could be given as to what determines protein functionality, though heterogeneity of pea proteins is one apparent cause of heterogeneous functionality. Differences in functionality, once completely understood, will aid the creation of desirably textured food products.

SAMENVATTING

Het onderzoek beschreven in dit proefschrift werd verricht in het kader van het zogenaamde Profetas project, een Engels acroniem voor Protein Foods, Environment, Technology and Society. Profetas is een multi-disciplinair project waarin onderzocht wordt of de gedeeeltelijke vervanging van vlees door zogeheten *novel protein foods*, levensmiddelen gebaseerd op niet-dierlijke eiwitten, technologisch mogelijk is, milieuwinst oplevert t.a.v. vleesproductie, en maatschappelijk gewenst is. In het Profetas project is gekozen voor de erwt als plantaardige eiwitbron. Dit proefschrift beschrijft een onderdeel van het technologische onderzoek, namelijk de vraag of en hoe eiwitten uit de erwt geschikt zijn om een gewenste textuur te geven aan *novel protein foods*. Aangezien gelvorming een belangrijk aspect is van textuur, is er voor gekozen om hitte-geïnduceerde gelering te bestuderen. De filosofie was dat moleculaire kennis van de belangrijkste globulaire erwten-eiwitten en hun gedrag bij verhitten nuttige informatie zou opleveren om gelering van erwteneiwit isolaat te begrijpen en dat dit zou helpen bij verdere toepassingen van eiwitten als ingredient om een bepaalde textuur te geven in levensmiddelen, en *novel protein foods* in het bijzonder.

Hoofdstuk 1 leidt het onderwerp in met een literatuuroverzicht van wat er bekend is t.a.v. erwteneiwit, hitte-geïnduceerde gelering van eiwitten in het algemeen en erwteneiwit in het bijzonder. De belangrijkste globulaire erwten-eiwitten zijn viciline en legumine, en in mindere mate conviciline. De eiwitten zijn erg heterogeen. Uit het literatuuroverzicht volgde de probleem- en doelstelling van het proefschrift. Aan het eind van het hoofdstuk is de gevolgde aanpak uiteengezet.

Hoofdstuk 2 beschrijft de isolatie en de zuivering van de eiwitten. De uiteindelijk gevolgde procedure vormde de basis voor het verdere onderzoek naar eiwit functionaliteit. Bij de isolatie en zuivering kwam onverwachts naar voren dat de viciline fractie eigenlijk uit twee fracties bestaat, met een verschillende verhouding aan viciline en conviciline, en dit bleek grote consequenties te hebben voor de karakterisering van viciline uit de erwt. Er zijn verschillende chromatografische technieken gebruikt om te pogen conviciline te scheiden van viciline, maar dit bleek niet goed mogelijk. Andere

gebruikte technieken ter karakterisering van de eiwitfracties waren gelelectroforese, DSC (differential scanning calorimetry), CD (circulair dichroisme) en oplosbaarheidsproeven. De conclusie uit dit alles was dat conviciline geen contaminant is van de viciline fractie zoals oorspronkelijk gedacht maar een onderdeel van de viciline fractie. Naar analogie van de nomenclatuur van soja-eiwitten werd conviciline daarom geduid als de α-subunit van de viciline fractie.

Hoofdstuk 3 beschrijft de implicaties van de heterogeniteit op subunit niveau, zoals vastgesteld in Hoofdstuk 2, op gelerende eigenschappen na verhitten en koelen. Hiervoor werden met name reologische technieken gebruikt. Zoals te doen gebruikelijk bij eiwitgelering is gelering een drie-staps proces: eerst moeten de eiwitten denatureren (ontvouwen), daarna kunnen ze gaan aggregeren bij afkoeling, en tenslotte vormen de aggregaten een netwerk dat zich uit als een gel. De twee viciline fracties, die verschilden in subunit samenstelling, werden vergeleken op geleergedrag onder dezelfde condities. Bij neutrale pH bleek het N-terminale gedeelte van de α-subunit een dusdanig hoge netto negatieve lading te hebben dat de vorming van een uitgebreid gelnetwerk werd gehinderd. Dit uitte zich in een toename van de minimum concentratie van de viciline fractie waarbij gelvorming optrad. Een interessant fenomeen was dat de aanwezigheid van α-subunits tot een transparant gel leidde. Dit is een aanwijzing dat de aggregaten die gelvorming veroorzaken klein zijn, en dit is in lijn met de gedachte dat de betrokken eiwitten negatief geladen zijn. De remmende effecten van de α-subunits konden worden weggenomen door de pH te verlagen of door de ionsterkte te verhogen. Ook na menging van de viciline fractie met α-subunits met legumine bleken de α-subunits een invloed te hebben op gelering en transparantheid. Een conclusie die in dit hoofdstuk werd getrokken is dat de samenstelling t.a.v. de α-subunit gebruikt kan worden om de gelering van erwten-eiwit te beïnvloeden.

Hoofdstuk 4 beschrijft een aantal experimenten met het erwten-eiwit legumine, met als doel om de intermoleculaire krachten te bestuderen die leiden tot hitte-geïnduceerde gelvorming. De gebruikte technieken waren electroforese, DSC, reologie en transmissie electronenmicroscopie. Er werd een vergelijking gemaakt met het moleculair homologe soja-eiwit glycinine, om de hypothese te testen dat op basis van moleculaire kennis een

algemeen model kan worden opgesteld om gelering van legumine-achtige eiwitten te voorspellen. Gezien het belang van zwavelbrugvorming bij gelering van legumine en glycinine werd in sommige experimenten N-ethylmaleïmide (NEM) toegevoegd om sulfhydryl-groepen uit te schakelen. Dit gaf als resultaat dat de gelvorming niet beïnvloed werd door verschil in verhittingssnelheid en de verschillend verhitte monsters niet beinvloed werden door de toevoeging van NEM, hetgeen aangeeft dat disulfide brugvorming niet essentieel is in de vorming van het legumine netwerk De resultaten lieten zien dat dezelfde fysisch-chemische factoren van invloed zijn op de gelering van legumine en glycinine. Een langzamere koelsnelheid na verhitten gaf aanleiding tot steviger gelen voor beide eiwitten, Een kenmerkend verschil was echter dat glycinine-gelen bij opnieuw verhitten op een reversibele manier weer dezelfde gelen vormden na afkoeling terwijl dat bij legumine gelen niet het geval was: deze werden steviger bij opnieuw verhitten en afkoelen. De bovengenoemde hypothese bleek dus niet juist te zijn. Wat het verschil veroorzaakte tussen de beide eiwitgelen is niet duidelijk geworden uit het werk beschreven in hoofdstuk 4, maar leidde wel tot de conclusie dat er naast gemeenschappelijke moleculaire interacties ook specifieke eiwit eigenschappen zijn die van invloed zijn op gelering.

Hoofdstuk 5 beschrijft een vergelijking tussen het geleergedrag van eiwitisolaten verkregen uit vijf verschillende erwten cultivars. Deze cultivars verschilden in de onderlinge verhouding aan legumine en viciline en de hoeveelheid α-subunit in de viciline. Het doel was om te onderzoeken in hoeverre het geleergedrag voorspeld kon worden op basis van de verkregen kennis beschreven in de vorige hoofdstukken t.a.v. het geleergedrag van de individuele eiwitten. De cultivar Solara sprong eruit vanwege het vermogen om sterke gelen te vormen in zowel de aan- als afwezigheid van NEM (een stof die sulfhydryl groepen blokkeert, en daarmee de vorming van zwavelbruggen). De overige cultivars vormden alleen stevige gelen in de aanwezigheid van NEM. Gezuiverd legumine verkregen uit de cultivar Solara zorgde ook voor een sterker gel wanneer het werd toegevoegd aan een eiwitisolaat van een cultivar dat van zichzelf geen sterk gel vormde. Om te zien of het verschil te wijten was aan een verschil in sulfhydryl en totaal cysteine gehalte werd het gehalte hieraan bepaald. De resultaten gaven geen directe aanwijzing voor een verband tussen het vermogen om zwavelbruggen te vormen en het

waargenomen geleergedrag. Een conclusie was dan ook dat er nog onbegrepen cultivar-specifieke effecten zijn. Een hypothese werd voorgesteld om te verklaren waarom NEM de gelsterkte beïnvloedde van de bestudeerde ertwen cultivars (behalve die van Solara), namelijk dat de ruimtelijke rangschikking van sulfhydrylgroepen een invloed heeft op de vorm van aggregaten en de daaropvolgende netwerkvorming. Isolaten van twee cultivars (Finale en Espace) gaven zwakke gelen hetgeen gerelateerd kon worden aan de hoeveelheid α-subunits van viciline in deze isolaten, in lijn met de bevindingen beschreven in Hoofdstuk 3.

In hoofdstuk 6 tenslotte zijn de resultaten in perspectief gezet en worden onderlinge verbanden besproken. De verschillen en overeenkomsten tussen de analoge erwten- en sojaeiwitten zijn geanalyseerd. De doelstelling om geleergedrag van eiwitten te kunnen voorspellen op basis van moleculaire kennis is kritisch geëvalueerd. Niet alles kon verklaard worden, maar de resultaten hebben wel duidelijk gemaakt in welke richting verder onderzoek nuttig is en in het hoofdstuk worden daartoe een aantal aanbevelingen gegeven. Een van de belangrijkste conclusies is dat de hoeveelheid α-subunit van de viciline fractie een bepalende factor is voor het geleergedrag van erwtenisolaat. Naar aanleiding van de geconstateerde verschillen tussen het erwteneiwit legumine en het sojaeiwit glycinine, is van beide eiwitten nog een aminozuuranalyse gedaan, maar er was geen significant verschil te constateren. Er kon geen verband worden gelegd tussen geleergedrag en aminozuursamenstelling. Als hypothese voor verder onderzoek is gesteld dat een bepaalde ruimtelijke orientatie van reactieve aminozuur-residuen een invloed heeft op de aggregatie die volgt na eiwit-ontvouwing, en dat heeft weer invloed op de sterkte van het resulterende gel netwerk. Al met al kan gesteld worden dat er nog geen eenduidig antwoord gegeven kan worden op de vraag wat eiwitfunctionaliteit bepaalt. De heterogene samenstelling van erwteneiwit leidt blijkbaar ook tot een heterogeniteit in functionaliteit. Dit verschil in functionaliteit, eenmaal begrepen, biedt juist mogelijkheden om verschillende gewenste getextureerde producten te maken.

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O'Kane FE, Happe RP, Vereijken JM, Gruppen H, van Boekel MAJS. Characterization of pea vicilin Part I: Denoting convicilin as the α-subunit of the *Pisum* vicilin family. *J. Agric. Food Chem.* In print.

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