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Characterization of Pea Vicilin. 2. Consequences of Compositional Heterogeneity on Heat-Induced Gelation Behavior

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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 hypothesized that the highly charged N-terminal extension region on these 70 kDa subunits hinders gelation of this vicilin fraction at pH 7.6 and $I = 0.2$ 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, which offers the possibility to control or modify the gelation behavior of vicilin on the basis of 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 texturizing technique. Because 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, which 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. This paper will address the heat-induced gelation of vicilin.

Vicilin is composed from different combinations of heterogeneous subunits of ∼50 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*), although the subunits remain intact under nondissociating 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 ∼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.

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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 varieties: Solara and Supra (Cebeco Seeds, Lelystad, The Netherlands; grown and harvested in 1998).

Gel Electrophoresis. Samples were prepared by mixing the protein sample 1:1 with sample buffer [1.4 mL of distilled water, 2.0 mL of 0.5 M Tris-HCl at pH 6.8, 2.0 mL of 10% (w/v) SDS, 2.0 mL of glycerol, and 0.4 mL of 0.05% (w/v) bromophenol blue]. A 10−20% linear gradient, Tris-HCl Ready Gels (Bio-Rad), was 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 6−16% (w/v) concentration, at pH 7.6 in 75 mM potassium phosphate buffer. All samples were heated (in sealed glass tubes to avoid evaporation) in a boiling water bath for 30 min. Samples were cooled to room temperature for 1 h and then stored at 4 °C overnight. The next day the tubes were inverted, and the samples that did not flow were considered to have gelled; thus, the minimum gelling concentration was determined.

Gel Sample Preparation. Samples were prepared with 75 mM potassium phosphate buffer, pH 7.6, in 2.0 mL Eppendorf tubes. After the protein had been dissolved, the pH was adjusted with 0.5 M NaOH or HCl, and then samples were left mixing in a test tube rotator for 1 h at room temperature. The Eppendorfs were locked into a heating block (to prevent the lids from opening), and samples were heated in a boiling water bath for 20 min and subsequently cooled at 4 °C overnight before being further analyzed. 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 concentrations were 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 of 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 with 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 (CD). Loss of secondary structure upon heating was monitored using a Jasco J-715 spectropolarimeter (Jasco Corp.) 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) prior to analysis. Heating rate was 1.0 °C/min, scan speed was 100 nm/min, and cell path length 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 nonlinear regression procedure as previously described in detail (*15*); the results are 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 h and filtered through a 0.2 μm sterile filter (Schleicher & Schuell) prior to loading onto the column. The Mono P column (Mono P HR 5/20, Amersham Biosciences) was treated as instructed in the manual. First, it was run with the starting buffer until the pH was stabilized at pH 7.1. Second, Polybuffer 74, pH 4.0 (prepared according to the instructions), was run through the column until the pH reached 4.0. Last, rerunning 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. Three milliliters 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 to guarantee the reproducibility of the elution to within 0.02 pH unit. 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, in which the method is described in detail (*9*), there is also a detailed characterization 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 denaturations at pH 7.6 were not different. This, however, was not the case with their gelation behavior. 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.

Heat-Induced Unfolding. 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 that despite some differences in their native states, 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 the α-subunit (*9*)] was hypothesized to hinder vicilin 2° gelation at pH 7.6 and $I = 0.2$ 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 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.

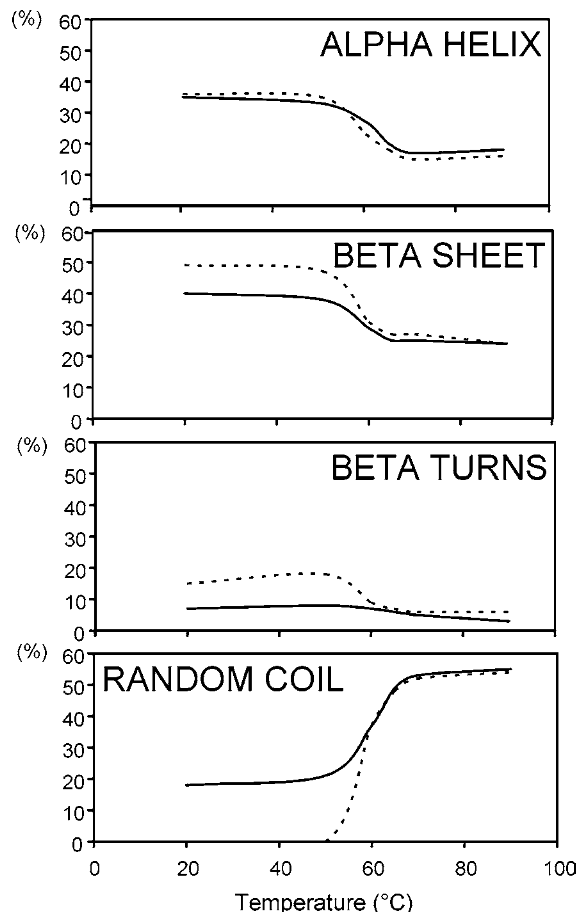

Figure 1. Plot of the relative percentage of secondary structure against temperature, for vicilin 1° and vicilin 2° from the pea cv. Solara, showing the pattern of structural loss upon heating for vicilin 1° (dotted line) and vicilin 2° (solid line).

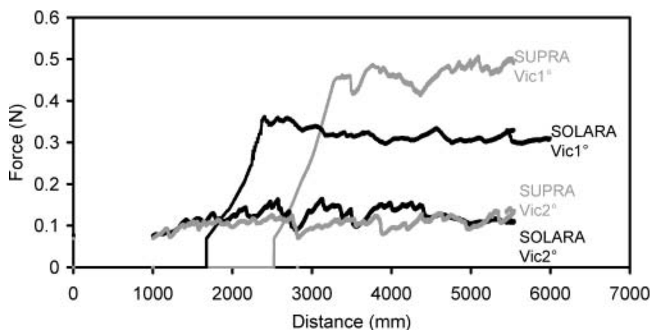

Figure 2. Plot of force against distance for a probe going into 16% vicilin gels at a speed of 0.01 mm/min. Results are shown for vicilin 1° and vicilin 2° for two pea cultivars, Solara (black) and Supra (gray).

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 upper- or outermost part of the meniscus. Vicilin 2° gels had a flatter meniscus than vicilin 1° 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.

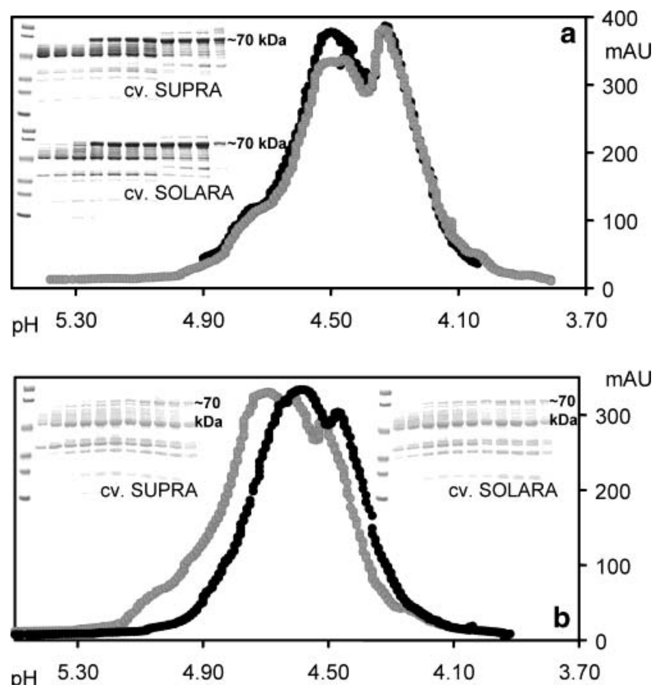

Figure 3. Elution profiles of vicilin 2° (**a**) and vicilin 1° (**b**) from the Mono P column under a linear pH gradient (from pH 7 to 4) for the two cultivars Solara (black circles) and Supra (gray circles). The SDS-PAGE profiles of the eluting proteins are beside the chromatograms.

Varietal Differences of the Protein Preparations. Due to the observed varietal difference in the TXA measurements (for vicilin 1°) we characterized the vicilin proteins using chromatofocusing. This technique was selected, on the basis of 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 3a**), but vicilin 1° from cv. Supra was seen to be less acidic than that from cv. Solara (**Figure 3b**). Being less acidic was attributed to this protein containing less of the extended α-subunits. SDS-PAGE of the Mono P fractions as they eluted from the column are shown as insets within **Figure 3**. It can be seen that vicilin 1° from 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 and thus was not as well able to form protein−protein interactions as its equivalent fraction from cv. Supra.

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. To remove the excessive repulsive forces, the pH of the sample was reduced to pH 6.1, which is the theoretical p*I* of the α-subunit [as calculated on the basis of 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 min, 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 was a slightly orange/brown clear solution, and

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 6.1 in the Absence of Salt[a]

	pH 7.6, no added salt		pH 6.1, no added salt	
sample	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/gray opaque gel	white aggregates[b]	brilliant white smooth gel

[a] All samples were cv. Solara. [b] Sample was mixed with a vortex immediately prior to heating to resuspend the aggregates. These aggregates dissolved upon heating.

Table 2. 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)[a]

	vicilin 2°, pH 7.6		vicilin 1°, pH 7.6	
added NaCl (M)	before heating	after heating	before heating	after heating
0	transparent solution	transparent liquid	transparent solution	white/gray opaque gel
0.1	transparent solution	transparent liquid	transparent solution	white/gray opaque gel
0.2	transparent solution	turbid liquid	transparent solution	white/gray opaque gel
0.5	transparent solution	phase separated	transparent solution	white/gray opaque gel[b]
1.0	transparent solution	brown/cream smooth gel	transparent solution	white/gray opaque gel[b]

[a] All samples were cv. Solara. [b] White tip visible after heating assumed to be due to protein that precipitated out during heating and settled to the bottom of the Eppendorf tube.

for vicilin 1° it was a gray/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 homogeneous appearance when squashed.

Subsequent samples were made at pH 3.8 so that the highly ionizable N terminus would be neutralized. At this pH samples were also made at 10, 8, 6, 4, and 2% (w/v) concentration. Before heating, both of the vicilin proteins were very well suspended: the samples were turbid and off-white, with no apparent settling after 20 min. 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, whereas vicilin 1° gels were noticeably whiter, but for both proteins the samples of 10, 8, and 6% (w/v) concentration had gelled. No further results are presented.

Salt-Induced Gelation. The addition of 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 (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, although liquid. Addition of 0.5 M NaCl had caused phase separation, and 1.0 M NaCl had induced gel formation. This gel had a brown/cream turbid appearance and was firm and smooth. By contrast, all of the vicilin 1° samples had gelled, and all had a white/gray 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 in footnote *b* of **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 vicilins 2° and 1° were added to this protein to determine if the presence of the α-subunits would affect the opacity. Addition of vicilin 1° had no apparent effect: the samples remained opaque, and all of the samples gelled (no results shown). In contrast, though, vicilin 2° reduced the opacity after heating and prevented gel formation, in the samples with a legumin/vicilin 2° ratio of 1:0.57 and 1.2. 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. Gels made from "overlap" region (left) and legumin mixed with vicilin 2° (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 (see **Figure 4**). 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 ref *9*). The SDS-PAGE profile of the overlap region (under nonreducing conditions) showed bands at ~70 (α-subunit vicilin), 60 (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 and $I = 0.2$ due to repulsion of the net negative charges is true.

First of all, vicilin 2° samples at pH 7.6 and $I = 0.2$ were all transparent after heating, whereas 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*). Although 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 and $I = 0.2$. Having a large repulsive area on the α-subunits of vicilin 2°, there could well be a reduced aggregate agglomeration relative to vicilin 1° and 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 favor 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 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 varieties. Again, the difference appeared to be related directly to the amount of α-subunits present.

Second, lowering the pH to 6.1, the theoretical p*I* 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 neutralized, thus not acting repulsively, and the remainder of the 70 and 50 kDa polypeptides are highly homologous and so have similar net charges. 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 neutralized and the repulsive force was substantially reduced.

Last, returning to pH 7.6, at which there was a dominant net negative charge on vicilin 2°, distinct differences between the samples were highlighted when salt was added. Although 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 solutions of only 0.5% (w/v) β-conglycinin were heated 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 behavior 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 underwent 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 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 summarize, 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 affect 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 led us to the conclusion that the distinct behavior of the N-terminally extended α-subunits of pea vicilin (and other similarly composed proteins) can be exploited when one is trying to modify or control the gelation behavior of this protein at near-neutral conditions.

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