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Characterization of Pea Vicilin. 1. Denoting Convicilin as the α-Subunit of the *Pisum* Vicilin Family

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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 characterized using gel electrophoresis, differential scanning calorimetry, circular dichroism, and pH-dependent solubility in order to determine whether the convicilin should in fact be considered as a third separate globulin protein of pea. On the basis of 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 is here denoted as the α-subunit.

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

INTRODUCTION

When one is 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 of the early researchers was that heterogeneity was exhibited (i) in the protein composition of different pea varieties, with a legumin/vicilin ratio varying from 0.2 to 1.5 (*2*) and (ii) in the polypeptide composition of individual proteins from a single variety (*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 of ∼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 the *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 (α*β*), 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 possessing 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

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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 inevitable when a vicilin-rich fraction is isolated. 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 intensely 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. Furthermore, we present results on the physicochemical characterization of the two vicilin fractions that was carried out in order to determine whether 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, The Netherlands; 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, CT) 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 of 1:10. Extraction time was 1 h at room temperature, and extract was collected by centrifugation (11900*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 h at 4 °C before it was collected by centrifugation (11900*g*, 10 °C, 25 min). Washing the protein pellet with water (pellet-to-water ratio of 1:10) removed unwanted albumin proteins. Again the pellet was collected by centrifugation (11900*g*, 4 °C, 25 min). The crude pellet was suspended in the extraction buffer, pH 8.0 (10 mg/mL), and dialyzed at 4 °C against McIlvaine's buffer (0.2 M Na_2HPO_4 + 0.1 M citric acid, containing 0.2 M NaCl), pH 4.8. Sample-to-buffer ratio was 1:20, and the dialysis buffer was changed three times over a 24 h period. Centrifugation of the sample (18900*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 (18900*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, Vicilin 2°, and Vicilin 1°. Freeze-dried protein-enriched fractions were dissolved in buffer A (35 mM potassium phosphate, containing 0.075 M NaCl), pH 7.6, at a 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 (11900*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).

The 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 analyzed 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 *vicilin 2°*.

The 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 analyzed 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 *vicilin 1°*.

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% SDS, 2.0 mL of glycerol, and 0.4 mL of 0.05% bromophenol blue). A 10−20% linear gradient and Tris-HCl Ready gels (Bio-Rad) were used, and a volume containing 2−10 μg of protein was applied to each well. Low molecular weight protein standards, ranging from 94 to 14 kDa (Amersham Biosciences) 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 (DSC). Thermal denaturation measurements were done in a VP-DSC microcalorimeter (MicroCal Inc., Northampton (MA). 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 min, then heated from 45 to 115 °C at 60 °C/h, 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). One milliliter 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 h at 4 °C. Subsequently, they were centrifuged (15 min, room temperature, 15000 rpm, MicroCen13 tabletop centrifuge, Herolab), and the supernatant was carefully removed with a Pasteur pipet. 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. Samples were made and analyzed in duplicate, and results are presented as an average.

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 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. Densitometry was performed with duplicate gels, and the average percentage of vicilin and convicilin is presented.

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) and stained with the Shiffs-PAS staining method. Ovalbumin with 2% glycosylation was used as a positive control. A low molecular weight gel electrophoresis calibration kit (Amersham Biosciences) was used as a negative control.

Rechromatography of the Purified Vicilin Fractions. Vicilin 1° and vicilin 2° were loaded onto a Source 15 Q PE 4.6/100 column (Amersham Biosciences) (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. On the basis of initial gel electrophoresis results, the eluted protein was pooled into four fractions, 1−4. These fractions 1−4 were dialyzed 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 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 of protein load), and the eluted protein was detected at 280 nm and collected in 300 μL aliquots. Samples were run in triplicate to guarantee the reproducibility of the elution to within 0.02 pH unit. One sample is presented.

Circular Dichroism (CD). The secondary structure of the native proteins was determined at 20 °C using a Jasco J-715 spectropolarimeter (Jasco Corp.) 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 path length 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) prior to analysis. The relative percentages of secondary structure were calculated using a nonlinear 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 a constant wavelength of 203 nm. This wavelength was selected on the basis of 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 JASCO J-715 Spectra Analysis software.

RESULTS

Extraction, fractionation, and purification of legumin and vicilin were 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 dialyzed against McIlvaine's buffer, pH 4.8, 0.2 M NaCl, a fraction of vicilin coprecipitated with the legumin. As described already, this fraction was later called vicilin 2°. The vicilin fraction precipitating after desalting was called vicilin 1°.

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**). 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 1c** (although the four lanes on the gel represent only the beginning and the end of the overlap region).

Gel electrophoresis was done as a first step in characterizing 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 eight samples, vicilin 2° and vicilin 1° were estimated to have convicilin contents of 55 and 5%, respectively. Differences in their small fragment composition (bands < 50 kDa) were not detectable. 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 in the mobilization 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. The presence of a similar protease in our protein preparations would explain our observations, but no references to such protease from pea could be found in the literature.

Separation of vicilin and convicilin was 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. This result shows that at pH 7.6 the 70 kDa polypeptide of convicilin is more highly charged than the polypeptides of vicilin. Rechromatography of the purified vicilin fractions, 1° and 2°, was thus done on analytical anion-exchange columns (Source Q and Mono Q). First, the vicilin 1° eluting from the Source Q column was collected as four subfractions, numbered 1−4 (as indicated in **Figure 3a**). Each subfraction was reapplied to the Mono Q column, and after the chromatogram of each subfraction had been overlaid (Figure 3b) and their compositions visualized on SDS-PAGE, fractions A−C were selected. Note that no fraction was taken from subfraction 3 because its composition on SDS-PAGE was no different from that of fraction B. **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−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).

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 subfractions of vicilin 1° (A−C) and determine if the small fragment composition affected the thermal denaturation temperature (T_d). There was a maximum 2 °C shift in the T_d (see **Table 1**), which was not considered to be important.

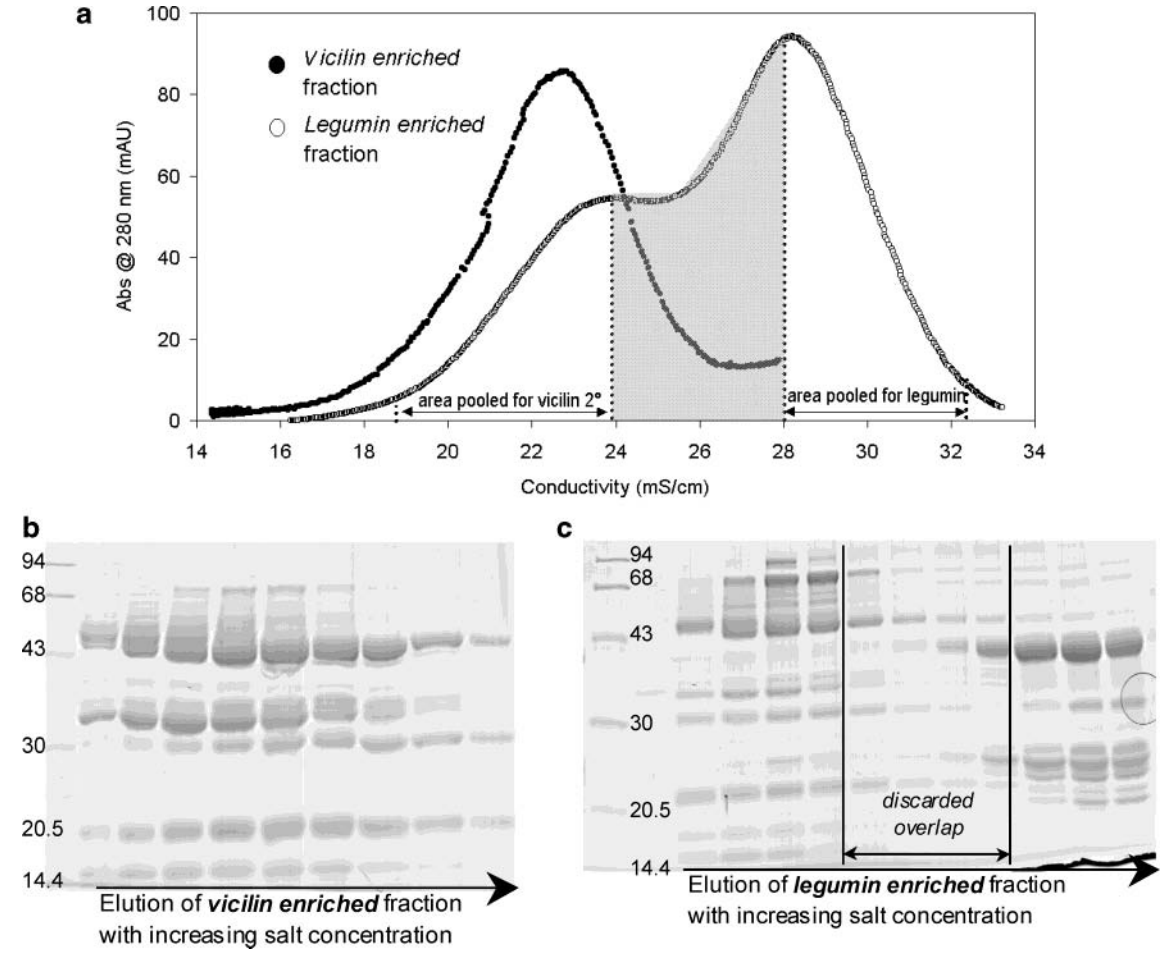

Figure 1. (**a**) Elution profile of vicilin- and legumin-enriched protein fractions from the DEAE Sepharose Fast Flow column under a linear salt gradient. Absorbance value of legumin-enriched fractions was reduced by a factor of 10 to make the profiles more comparable. (**b**) SDS-PAGE profile of vicilin-enriched protein fraction as it 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 the picture. (**c**) SDS-PAGE profile of legumin-enriched protein fraction as it 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 the picture.

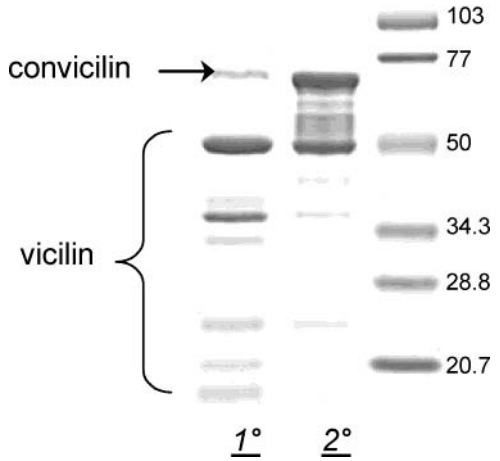

Figure 2. SDS-PAGE of purified vicilins 1° and 2°. The bands belonging to vicilin and convicilin are indicated (left) and the standard markers are indicated (in kDa) on the right-hand side.

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, vicilins 1° and 2° were applied to the column and eluted in a linear pH gradient from 7 to 4. The elution profiles (**Figure 4**) showed immediately that vicilin 2° was more acidic than vicilin 1°. Furthermore, SDS-PAGE visualization of the eluting fractions (**Figure 5**) 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.

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–C of Vicilin 1° That Were Separated by Analytical Chromatography with the Mono Q Column (**Figure 3b**)

	polypeptides presen in sample[a] (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

[a] The polypeptides present in each fraction are listed in the table (and are visible on the SDS-PAGE in **Figure 3c**).

Looking in more detail at **Figures 4** and **5**, 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

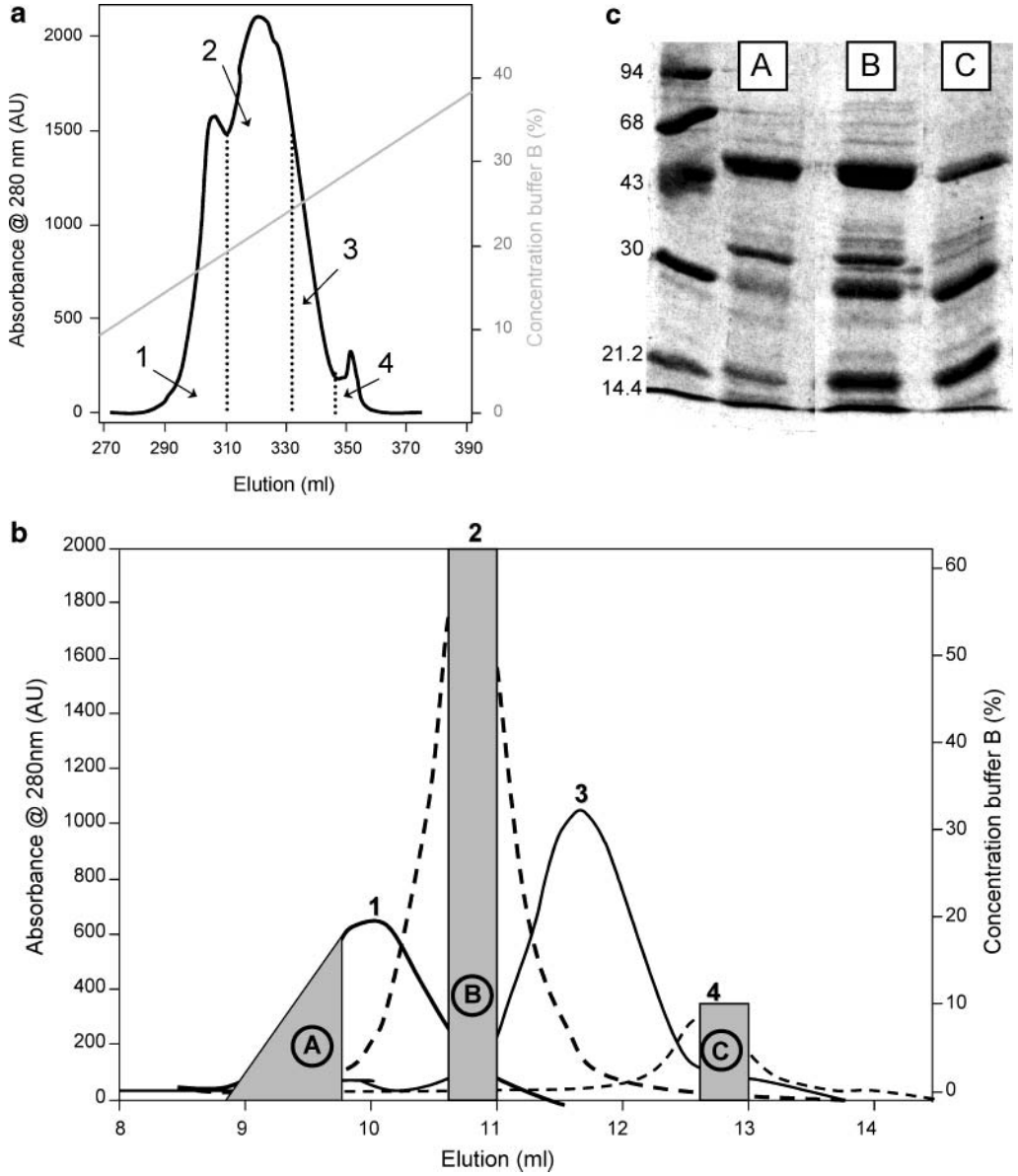

Figure 3. (**a**) Elution of vicilin 1° from the Source 15Q column under a linear salt gradient. The peak is divided into four sections (with broken lines), and successive sections are numbered 1–4 as indicated by the arrows. These fractions were applied to an analytical Mono Q column, and their respective elutions can be seen in **Figure 3b**. (**b**) Elution of vicilin 1° from the Mono Q column under a linear salt gradient. Numbers 1–4 above each peak refer to division of the protein (as explained for **Figure 3a**). Shaded areas, labeled A–C, indicate the fractions kept for further analysis. (**c**) SDS-PAGE of fractions A–C kept from the protein eluting from the analytical Mono Q column (labeled according to **Figure 3b**). Standard markers are indicated (in kDa) on the left-hand side.

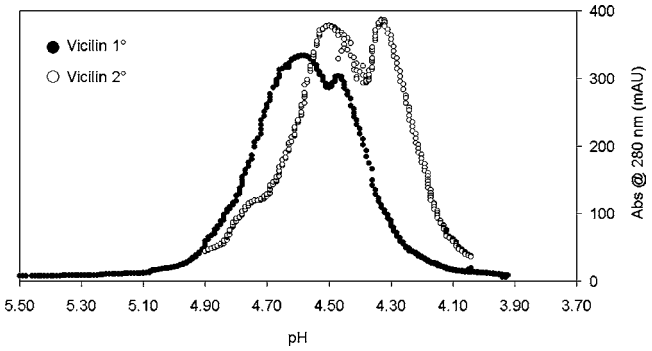

Figure 4. Elution profiles of vicilin 1° and vicilin 2° from the Mono P column under a linear pH gradient from pH 7 to 4.

resolvable peaks, but still the composition of the eluting peaks seemed to be a mixture of both convicilin and vicilin polypeptides.

The 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 6**). However, because 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 analyzed by SDS-PAGE. A clear difference in composition of the soluble protein could not be seen at first. 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. Further analysis by densitometry highlighted one feature, however; 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%

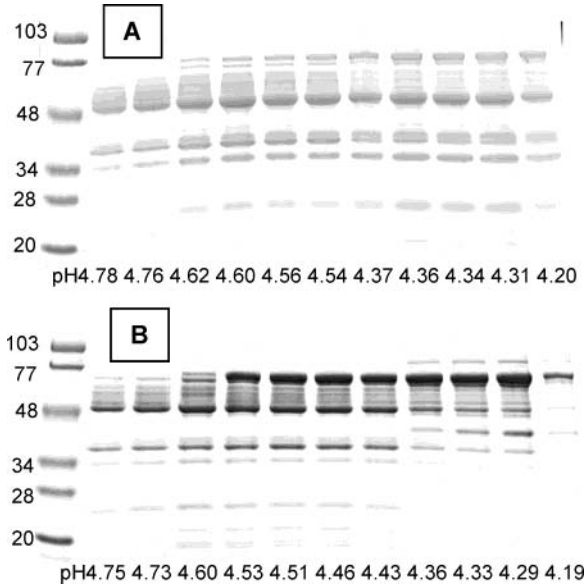

Figure 5. SDS-PAGE of samples taken over the entire range of elution from the Mono P column. The pH of elution is indicated under each lane. Standard markers are indicated (in kDa) on the left-hand side: (**A**) vicilin 1°; (**B**) vicilin 2°.

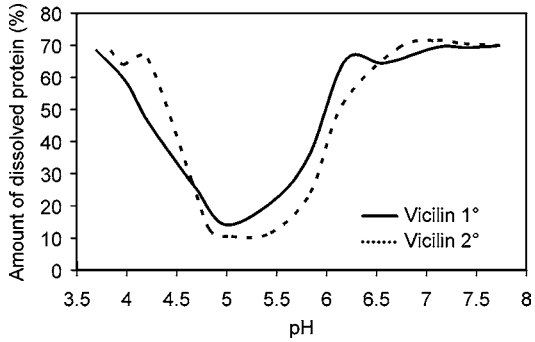

Figure 6. Plot of the percentage of dissolved protein versus pH. The percentage of dissolved protein was determined by the amount of nitrogen in the supernatant (see Materials and Methods for further details).

(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 behavior, 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 vicilin 1° from vicilin 2° was thus ruled out.

The secondary structure of the native proteins was determined (at 20 °C) with far-UV CD. 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 were 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% β-turns. Vicilin 2°, however, was predicted to contain 18% random coil and 7% β-turns. These similarities and differences between the two vicilins were consistent for each replicate sample. 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 to be an unlikely result, and it is more likely an artifact of the sample. These results are presented in **Table 2**.

Table 2. Relative Percentage of Secondary Structural Features in Native Vicilin 1° and Vicilin 2° at pH 7.6[a]

secondary structure component	relative amount (%)	
	vicilin 1°	vicilin 2°
α-helix	36	35
β-sheet	49	40
β-turns	15	7
random coil	0	18

[a] Values were determined using the CD-Fit modeling program as described under Materials and Methods.

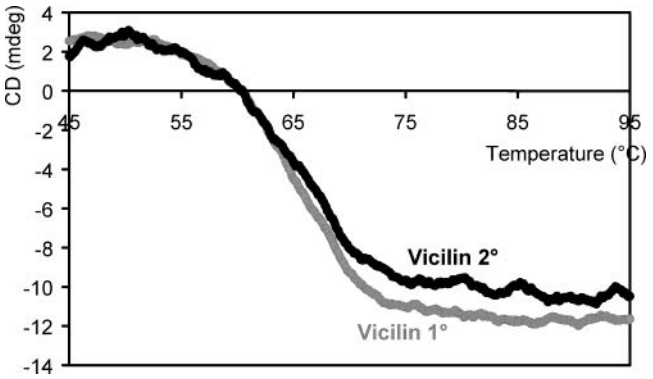

Figure 7. Thermally induced unfolding of secondary structure of vicilin 1° (gray line) and vicilin 2° (black line) as determined by far-UV scans at a constant wavelength of 203 nm, when heating from 45 to 95 °C at a rate of 1.0 °C/min.

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.

Thermally induced unfolding of the two vicilin proteins (1° and 2°) at the secondary and tertiary levels was measured with CD and DSC, respectively. **Figure 7** shows spectra measured at 203 nm during heating of the vicilin proteins from 45 to 95 °C. It can be seen that there was no obvious difference between the unfolding behaviors of these two proteins. Fitting these data using 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**). Actual differences in T_d between vicilin 1° and vicilin 2° at the tertiary level (see again **Table 3**) were not considered to be important. Furthermore, it can be said that vicilin 1° and vicilin 2° both had an increased T_d from ~70 to ~84 °C in response to an increase in ionic strength from 0.03 to 0.5.

DISCUSSION

This paper presents a purification scheme for pea globulins that results 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 characterized 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*). Although 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, 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 behavior 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 the α- and α′-subunits of β-conglycinin. Although 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 the polypeptides are defined under these terms, it becomes apparent that genetic distinctness has been considered differently for pea than for soybean. Although genetically distinct from each other, the α-, 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 chromatofocusing was performed was more than one species of protein apparent by the resolution of vicilin 2° into two peaks, although 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 behavior of convicilin in acid/salt conditions. Although 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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