JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2006,** *54,* 425−433 **425**

Genetic Variation in Pea Seed Globulin Composition

EMMANOUIL N. TZITZIKAS,[†,§] JEAN-PAUL VINCKEN,[*,†] JOLAN DE GROOT,[#,⊥] HARRY GRUPPEN,[#] AND RICHARD G. F. VISSER[†]

The Graduate School Experimental Plant Sciences, Laboratory of Plant Breeding, Wageningen University, P.O. Box 386, 6700 AJ Wageningen, The Netherlands; Center for Protein Technology WU/Laboratory of Food Chemistry, Wageningen University, Bomenweg 2, 6703 HD Wageningen, The Netherlands; and Wageningen Centre for Food Sciences, Diedenweg 20, 6700 AN Wageningen, The Netherlands

A quantitative characterization of seeds from 59 pea (*Pisum sativum* L.) lines and relative taxa with various external characteristics and wide geographical origin was performed to explore the genetic variation of pea concerning its starch and protein contents and globulin composition. Pea lines, which produce round, wrinkled, flat, and round-dimpled seeds, have starch as the major reserve, with an average content of 46%. Protein content varied from 13.7 to 30.7% of the seed dry matter, with an overall average of 22.3%. Densitometric quantification of the individual globulins (legumin, vicilin, convicilin, and globulin-related proteins) based on SDS-PAGE gels showed no lines lacking any particular globulin. Among the lines tested, variation was shown in both their total globulins content and their globulin composition. The total globulin content ranged from 49.2 to 81.8% of the total pea protein extract (TPPE). Legumin content varied between 5.9 and 24.5% of the TPPE. Vicilin was the most abundant protein of pea, and its content varied between 26.3 and 52.0% of the TPPE. Both processed and nonprocessed vicilins occurred. The processed vicilin was the predominant one, with values between 17.8 and 40.8%, whereas the nonprocessed ones constituted between 3.1 and 13.5% of the TPPE. Convicilin was the least abundant globulin, and its content ranged from 3.9 to 8.3%. Finally, the globulin-related proteins were present in amounts ranging from 2.8 to 17.3%. They were less abundant in comparison with legumin and vicilin, but they showed the largest relative variation of the four globulin classes. Correlations between the different external characteristics and globulin composition were determined. Comparison with soybean showed that pea lines show more variety in the abundance of globulin proteins, enabling a wider range of food application.

KEYWORDS: Peas; *Pisum sativum* L.; globulins; vicilin; convicilin; legumin; seed proteins; SDS−polyacrylamide gel electrophoresis; in vivo processing

INTRODUCTION

Peas (*Pisum sativum* L.) have a high nutritional value and are used as animal feed or as food ingredient. They are a relatively high-quality source of proteins and starch. However, peas and pea proteins are not widely used in food application due to the competitiveness of soybean. Soybeans are readily available in bulk quantities, have better agronomic traits, and have been studied in greater detail with respect to protein quality parameters (*1*). A number of drawbacks are associated with the use of soybeans in foods, such as their beany or green flavor and the presence of antinutritional factors (see ref *2* and references cited therein). This, together with the concern about the introduction of genetically modified organism soybeans, has roused the interest in alternative vegetable protein sources, including peas.

The major component of pea is starch, which accounts for up to 50% of the seed dry matter (DM) (*3*, *4*). Protein and total dietary fiber account for about 24 and 20% of the DM, respectively. Lipids are present in lower amounts (2.5% DM) (*5*). High variations in starch and protein contents are often observed, whereas the variations in the contents of other components are usually lower (*3*).

According to the Osborne fractionation (*6*) pea proteins can be classified into two major classes: the salt-soluble globulins and the water-soluble albumins. Both fractions account in total for ∼80% of the total seed protein content. All globulins and some albumins are storage proteins, which are used as nitrogen sources for the new embryos after seed germination. The globulins have been subdivided into two major groups on the

* Corresponding author (e-mail jean-paul.vincken@wur.nl).
† The Graduate School Experimental Plant Sciences, Wageningen University.
§ Present address: Department of Horticultural Genetics and Biotechnology, Mediterranean Agronomic Institute of Chania, Chania 73100, Greece.
# Center for Protein Technology, Wageningen University.
⊥ Wageningen Centre for Food Sciences.

10.1021/jf0519008 CCC: $33.50
Published on Web 12/20/2005

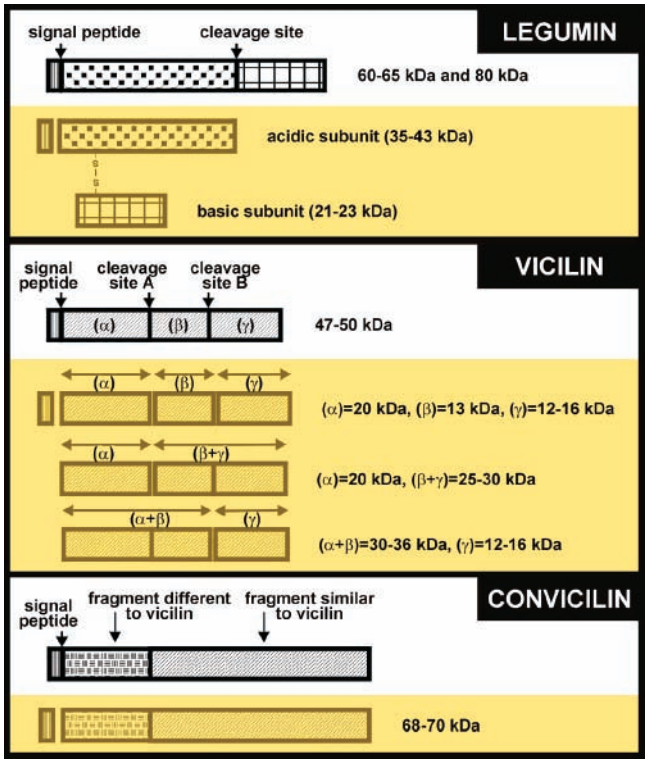

Figure 1. Schematic representation of globulin processing in pea. All globulins have a signal peptide for import into the endoplasmic reticulum (ER), which is removed co-translationally during ER entry. Legumins are processed by a vacuolar processing enzyme into two subunits of about 40 and 20 kDa (the acidic and basic subunit, respectively), which remain linked by a disulfide bridge. Vicilins have two potential cleavage sites (A and B) depending on the isoform. Cleavage at both sites yields fragments of about 20 kDa (α), 13 kDa (β), and 12–16 kDa (γ); cleavage at site A yields fragments of about 20 kDa (α) and 25–30 kDa ($\beta + \gamma$); cleavage at site B gives 30–36 kDa ($\alpha + \beta$) and 12–16 kDa fragments (γ). Convicilins are not known to undergo post-/co-translational modifications other than removal of the signal peptide.

basis of their sedimentation coefficients: the 11S fraction (legumin) and the 7S fraction (vicilin, convicilin). These two groups differ considerably in molecular weight and structure.

Legumin is expressed as a protein of 60−80 kDa, which is usually present in a hexameric form. Three families of legumin polypeptides can be distinguished on the basis of sequence similarities: *Leg*A (consisting of *leg*A, A2, B, C, and E), *Leg*J (consisting of *leg*J, K, L, and M), and *Leg*S (the only member) (*7*, *8*). The representatives of the first two families have apparent subunit molecular masses of ∼60−65 kDa, whereas *Leg*S has an apparent subunit molecular mass of ∼80 kDa. Each legumin polypeptide (like all globulins) has a signal peptide for import into the endoplasmic reticulum (ER), which is removed co-translationally during ER entry. Subsequently, legumin polypeptides form trimers, which are transported into a prevacuolar compartment (*9*). Here, they are processed by a vacuolar processing enzyme into two polypeptides of about 40 and 20 kDa (referred to as the acidic and basic polypeptides, respectively), which remain linked by a disulfide bridge (**Figure 1**). After processing, the trimers assemble into hexamers, forming the mature protein.

Vicilin is a protein of 47−50 kDa, which can form trimers of a molecular mass of ∼150 kDa (*10*). Some, but not all, vicilins can undergo post-translational cleavage (*11*). Two potential cleavage sites (A and B) are present in vicilin polypeptides, which can be processed separately (**Figure 1**). Cleavage at both sites yields fragments of about 20 kDa (α), 13 kDa (β), and 12−16 kDa (γ). Cleavage at site A alone yields fragments of about 20 kDa (α) and 25−30 kDa ($\beta + \gamma$); cleavage at site B alone gives 30−36 kDa ($\alpha + \beta$) and 12−16 kDa fragments (γ) (*11*, *12*). Processed polypeptides remain associated through non-covalent interactions (*8*, *11*). Some of the vicilins are glycosylated, and glycosylation occurs close to the C terminus of the γ fragment (*13*).

Convicilin is a protein of ∼70 kDa, which can form trimers with a molecular mass of ∼210 kDa. The occurrence of heteromeric trimers consisting of vicilin and convicilin polypeptides has been reported as well (*1*, *14*). Convicilin is not known to undergo any post-/co-translational modifications other than removal of the signal peptide (**Figure 1**), and it is not glycosylated (*15*). Convicilin has extensive homology with vicilin from its amino acid residues 122−166 (depending on the isoform) to its C terminus. It differs from vicilin by an N-terminal extension (*15*, *16*), which is highly charged in contrast to the rest of convicilin and to vicilin.

Due to their differences in structure and molecular weight, globulins can vary considerably in their physicochemical properties. Vicilin has a significantly higher foaming capacity than legumin and a slightly lower emulsifying capacity (*1*, *17*). Various concentrations of legumin and vicilin, alone or in mixtures, can form good gels, depending on the conditions used. On the contrary, convicilin can hinder the gel formation of pea isolates (*18*). Therefore, peas with a specific globulin composition, for instance, enriched in legumin and vicilin or lacking convicilin, would be desirable as a raw material for the food industry (*8*). In addition, the ability of an isolate to form good gels is not only a matter of the ratio of globulins but also depends on the specific isoforms of the globulin present in the isolate (*19*, *20*). The existing genetic variation for pea globulin composition might provide a useful resource for obtaining more appropriate seed material for food applications. It has been shown that there is considerable natural variation in pea protein content and composition (*21*−*23*). These variations can be affected by genetic factors such as the *r* locus, which influences starch biosynthesis and has pleiotropic effects in protein content and composition (*24*, *25*). Very little information on in planta processing of proteins is available, although this may be an important criterion for application of pea proteins as ingredients in the food industry.

This study explores the genetic variation in the starch and protein content/composition of pea. In addition, the level of post-translational processing in the different lines was investigated. To include a wide range of natural genetic variation in the tested material, 59 genotypes were selected on the basis of differences in leaf and seed characteristics and different geographic distribution. Results were correlated with external characteristics of seeds and plants to obtain phenotypical markers for initial screening in breeding programs.

MATERIALS AND METHODS

Seeds. A collection of seeds from 54 pea (*Pisum sativum* L.) lines, as well as from 5 pea wild relatives (taxa) (*Pisum sativum abyssinicum*, *Pisum arvense*, *Pisum elatius* Marbre, *P. elatius 1140175*, and *P. elatius* 1140176), were obtained from Cebeco Zaden B.V. (Lelystad, The Netherlands) and the Center for Genetic Resources (CGN) (Wageningen, The Netherlands), respectively. The lines originate from different parts of the world. They were grown by the provider at the same place and time to eliminate compositional differences due to growth in different environments (except for the wild relatives). The collection includes wild-type (wt; normal leaves) and *afila* (af; semileafless) leaf

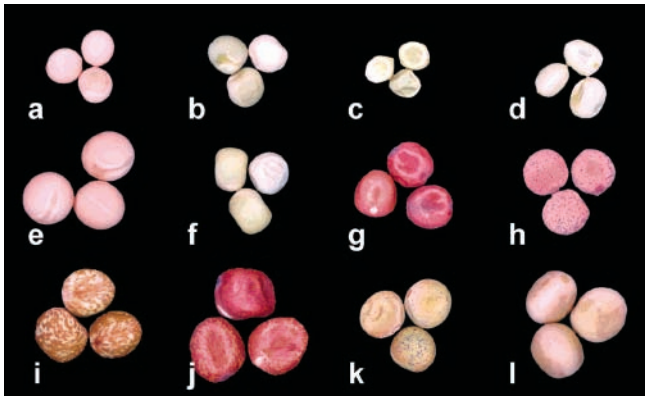

Figure 2. Different morphology (shape and color) of representative pea seeds used in this study: (**a**) [Vreta] (round); (**b**) [NGB 102920 Iran] (round-dimpled); (**c**) [Cisca] (wrinkled); (**d**) [6 S 41.4] (flat); (**e**) [American yellow] (yellow); (**f**) [PV 13] (green); (**g**) [Timo] (brown); (**h**) [*P. elatius* 1145176] (brown-gray-spotted); (**i**) [NGB 102149 Iran] (brown-green-spotted); (**j**) [Courier] (brown-spotted); (**k**) [NGB 101293 Jordan] (green-spotted); (**l**) [NSA 93-0030-2] (green-yellow).

shape varieties. The seeds have diverse external characteristics such as weight, shape, and seed coat color. Some representative examples are shown in **Figure 2**. The morphological characteristics of the different lines are summarized in **Table 1**.

Preparation of Samples. For all of the biochemical analyses performed in this study, samples were prepared as follows: seeds (~1 g) were peeled and ground with a mortar and pestle at room temperature until a fine powder was obtained. This powder was dried at 40 °C in a vacuum desiccator in the presence of P_2O_5 until the weight remained constant (~2 days).

Determination of Starch Content. Ground pea seed (~40 mg) was suspended in 2.5 mL of 8 M HCl and 10 mL of DMSO and incubated for 1 h at 60 °C. After incubation, 15 mL of H_2O, 4 mL of 5 M NaOH, and 18.5 mL of 0.1 M sodium citrate buffer (pH 4.6) were added to obtain appropriate conditions for the enzymes used in the starch determination assay. After vigorous mixing, 1 mL of this suspension was centrifuged at 13600*g* for 10 min, and 20 μL of the supernatant was used for the enzymatic determination of starch. For that, a starch assay kit obtained from Boehringer (Mannheim, Germany) was used with a procedure slightly modified from the one described by the manufacturer. The modification consisted of a 5× reduction of the reaction volume of the enzymatic hydrolysis of the starch to adapt the procedure for determining the total amount of glucose with a microplate reader (3550-HV, Bio-Rad Labs, Hercules, CA). For each line, at least three independent samples were prepared and analyzed.

Protein Content Determination. Protein content was measured using the combustion (Dumas) method on a nitrogen and protein analyzer (model ThermoQuest NA 2100, CE Instruments, Milan, Italy). The sample (20−30 mg of ground pea seed) was weighed into a sample cup and analyzed using D-methionine as an external standard. For each line, at least three independent samples were prepared and analyzed. The protein content was calculated using 5.25 as the nitrogen-to-protein conversion factor, which was determined for field peas (*26*). This factor was based on the amino acid composition of field peas (*27*) and was corrected for non-protein nitrogen, which is ~5% in pea (*26*, *28*, *29*).

Determination of the Composition of Extractable Proteins. Total pea protein extract was prepared by stirring 100 mg of ground pea seed in 1.5 mL of 0.1 M Tris-HCl buffer (pH 8) for 1 h at 30 rpm at room temperature. Subsequently, the samples were centrifuged at 1500*g* for 7 min at 20 °C to precipitate insoluble material. The protein composition of the supernatant, referred to as total pea protein extract (TPPE), was determined in triplicate by SDS-PAGE, without and with 2% (v/v) β-mercaptoethanol, on a Mini-Protean II electrophoresis system from Bio-Rad Labs, according to the instructions of the manufacturer. Five hundred microliters of TPPE was diluted 1+1 (v/v) in sample buffer, consisting of 0.02 M Tris-HCl buffer (pH 8), 2 mM EDTA, 20% glycerol, 2% SDS, and 0.002% Bromophenol Blue. The samples, sealed in 1.5 mL tubes, were heated in boiling water for 5 min, and proteins were separated using 12% Tris-HCl polyacrylamide

Table 1. Summary of the Various Genotypes Used in This Study with Their Morphological Seed and Leaf Characteristics

line	name	color[a]	weight[b]	shape[c]	leaf[d]	line	name	color	weight	shape	leaf
1	93125-07 Canada	Gr	0.238	R-D	*af*	31	Classic	Yel	0.312	F	*af*
2	M 98 Canada	Yel	0.358	R-D	*af*	32	Baccara	Yel	0.361	R-D	*af*
3	Chilean grano de Oro	Yel	0.309	F	wt	33	Solara	Gr	0.282	R	*af*
4	KPMR 146 India	Yel	0.260	F	wt	34	NSA 93-0030-2	Gr-Yel	0.393	R	*af*
5	MP 790 Canada	Yel	0.263	R	wt	35	CEB 1475.1	Yel	0.345	R	*af*
6	Russian yellow	Yel	0.427	R	wt	36	CEB 1466	Yel	0.231	R	*af*
7	Tanganyka	Yel	0.458	R	wt	37	FDP 9023/16	Yel	0.257	R	*af*
8	Russian fodder pea green	Gr	0.267	R	wt	38	484	Gr	0.335	R	*af*
9	Mexican yellow	Yel	0.260	R	wt	39	6 S 41.4	Gr	0.233	F	*af*
10	American yellow	Yel	0.284	R	wt	40	A 5052/4	Gr	0.323	F	*af*
11	FAL 49110 Mongolia	Yel	0.216	R	wt	41	DS 4-9309	Gr-Yel	0.270	R	*af*
12	FAL 48919 Ethiopia	Br-sp	0.262	F	wt	42	LPKE 8020	Gr	0.236	F	*af*
13	Fal 49142 China	Yel	0.224	R	wt	43	SCHW 67.89.35	Gr	0.360	R	*af*
14	NGB 102349 China	Yel	0.224	R	wt	44	SWS 97-112-13	Gr-Yel	0.363	R	*af*
15	NGB 102920 Iran	Gr	0.337	R-D	wt	45	UN 407	Gr-Yel	0.334	R	*af*
16	NGB 102149 Iran	Br-Gr-sp	0.179	F	wt	46	CEB 1475.2	Yel	0.233	R	*af*
17	NGB 101293 Jordan	Gr-sp	0.163	F	wt	47	CEB 1488	Gr-Yel	0.280	R	*af*
18	NGB 100180 Hungary	Br-Gr-sp	0.123	R	wt	48	CEB 1222	Gr	0.518	W	*af*
19	PV 13	Gr	0.202	F	wt	49	93126–610	Gr	0.486	W	*af*
20	Timo	Br	0.218	F	wt	50	CEB 1162	Gr	0.232	R	*af*
21	Vreta	Yel	0.223	R	wt	51	Finale	Gr	0.320	R	wt
22	Capella	Yel	0.237	R	*af*	52	Supra	Gr	0.473	W	*af*
23	Courier	Br-sp	0.272	F	*af*	53	Celica	Gr	0.346	F	*af*
24	Fallon	Yel	0.254	R	*af*	54	Espace	Gr	0.276	F	*af*
25	Tremont Scotch	Gr	0.180	F	wt	55	*P. sativum abyssinicum* (CGN 16636)[e]	Br	0.165	F	wt
26	Solido	Br	0.396	W	*af*	56	*P. elatius*, Marbre (CGN 03351)	Br-Gr-sp	0.213	F	wt
27	CEB 1312 (Racer)	Br-Gr-sp	0.224	F	*af*	57	*P. arvense* (CGN 10193)	Br-Gr-sp	0.062	F	wt
28	Mylosa (CEB 1811)	Gr	0.235	W	*af*	58	*P. elatius*, 1140175 (CGN 10205)	Br-Gray-sp	0.149	R	wt
29	Tango	Gr	0.164	R	wt	59	*P. elatius*, 1145176 (CGN 10206)	Br-Gray-sp	0.145	R	wt
30	Cisca	Gr	0.119	W	wt						

[a] Color abbreviations: Yel, yellow; Gr, green; Br, brown; sp, spotted. [b] Weight: average weight of 10 mature seeds (g). [c] Shape abbreviations: F, flat; W, wrinkled; R, round; R-D, round-dimpled. [d] Leaf shape abbreviations: wt, wild type; *af*, semileafless (*afila* mutant). [e] CGN accession numbers.

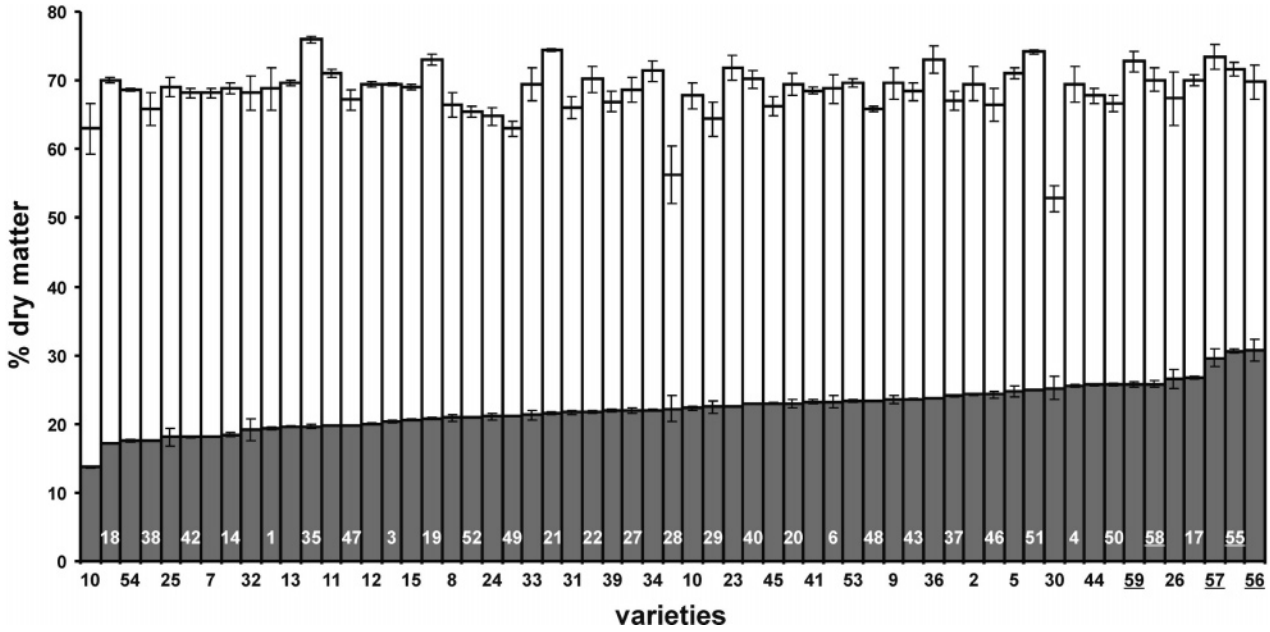

Figure 3. Protein (gray bars) and total reserve size (starch + protein; open bars) of the tested pea seed material. The various pea lines are ranked from low to high protein content. Values are the result of at least three replications (with standard deviation of <1). The pea wild relatives are underlined.

Ready Gels from Bio-Rad Labs. Gels were run at a constant voltage of 200 V and were calibrated with low molecular mass markers ranging from 14 to 94 kDa from Amersham Biosciences (Uppsala, Sweden). Ten microliters of sample was loaded into each well. All samples contained an amount of protein that was well within the loading range for sample application. The gels were run in a buffer solution of 0.025 M Tris, 0.19 M glycine, and 0.1% SDS (pH 8.3). The gels were stained in a solution of 0.1% Coomassie Brilliant Blue R250, 40% methanol, and 10% acetic acid and destained in a solution of 30% methanol and 10% acetic acid. The different bands were quantified by scanning densitometry, using a Molecular Dynamics laser scanner and the ImageQuant software package, both from Amersham International Plc (Buckinghamshire, U.K.). The proportions of legumin, vicilin, convicilin, and globulin-related proteins were calculated according to the area underneath the staining density peak. The bands were assigned to legumin, vicilin, and convicilin according to their molecular weights reported in the literature (*7*, *8*, *10*−*12*, *15*, *21*) and on the basis of relative motilities on SDS-PAGE gels of purified proteins from pea isolates (*14*, *18*) in a similar way as was done for soybean globulins (*30*). The bands of a molecular mass of 60−65 kDa, which disappear after β-mercaptoethanol treatment, are referred to as legumin. The bands of a molecular mass of 47−50 kDa were assigned as nonprocessed vicilins; processed vicilins were related to the bands of 36, 35, 33, 30, 25, 20, 16, and 14−12 kDa. The bands with a molecular mass of 68−70 kDa were quantified as convicilins. The bands present on the SDS gel, which were not described in the literature as legumins, vicilins, and convicilins, but which belong to the soluble fraction because they are coprecipitating with legumin and vicilin under acidic conditions (*14*), were denoted globulin-related proteins. They had a molecular mass between 52−66 and 45 kDa, which did not disappear under reducing conditions. The bands present in the gels not meeting the criteria for legumin, vicilin, convicilin, or globulin-related proteins were assigned as non-globulins.

Statistical Analysis. To verify the statistical significance of the results, the values of mean and standard deviation (mean ± SD) were calculated with a 95% confidence interval (CI). When necessary, a *T* test (Bonferroni; $P < 0.05$) and a correlation coefficient test (Pearson two-tailed; $P < 0.01$) were performed with the statistical software package SPSS 10.0 for Windows (SPSS Inc., Chicago, IL).

RESULTS

Starch and Protein Contents. A qualitative and quantitative characterization of seeds from 59 pea lines was performed to explore the genetic variation of pea concerning its major seed reserves. Tested lines were grown under the same conditions and on randomized blocks (except the wild relatives of pea). Therefore, any variation found can be related to genetic differences in the plant material. **Figure 3** shows the natural variation in starch and protein contents of the 59 genotypes. The average starch content is 46.0% of DM and varied from 27.6 to 56.3%. In all lines tested, the starch content is higher than the protein content. On average, the wrinkled lines showed significantly (*T* test, Bonferroni) lower starch contents than the lines with other seed shapes, with an average of 38.5% compared to 46.7, 48.0, and 47.0% for flat, round-dimpled, and round varieties, respectively. Nevertheless, certain wrinkled lines, such as 48 (CEB 1222) and 52 (Supra), had higher starch contents than lines with other seed shape types. No relationship was found between starch content and leaf shape or seed color. The *Pisum sativum* wild relatives have on average a starch content of ∼43.0%, which is significantly lower than that of the *P. sativum* accessions (46.2%) (*T* test, Bonferroni).

Results on protein content showed a variation from 13.7 to 30.7% of DM with an overall average content of 22.3%. No significant differences were observed between varieties with different seed shapes (*T* test, Bonferroni). Wrinkled varieties scored 23.3% compared to 22.6, 22.8, and 20.8% for flat, round, and round-dimpled varieties, respectively. No significant differences were observed between lines with different leaf shapes, whereas differences were observed between lines with different seed colors (*T* test, Bonferroni). The brown lines had on average significantly higher protein content (26.7%) than the green and yellow lines, which have average contents of 21.5 and 21.8%, respectively. The wild relatives had an average protein content of 28.5%, showing that the wild relatives have on average a significantly (*T* test, Bonferroni) higher protein content than the *P. sativum* lines, which have an average of 21.8%. Nevertheless, there were also *P. sativum* lines scoring high when compared with the overall average protein content. For instance, line 17 (NGB 101293 Jordan) had a protein content of 26.8%, which falls in the range of that of the wild relatives (25.7−30.7%).

Protein content correlated negatively with starch (significance, −0.597; Pearson two-tailed; $P < 0.01$). **Figure 3** shows the

Table 2. Total and Individual Globulin Composition of the Tested Material as a Percentage of the Total Protein Content[a]

line	*n*glob	globulins	legumins	vicilins	*np*vicil	*p*vicil	conv	globRP	line	*n*glob	globulins	legumins	vicilins	*np*vicil	*p*vicil	conv	globRP
1	50.8	49.2	8.3	32.4	9.2	23.2	5.7	2.8	31	35.6	64.4	11.5	32.6	10.7	21.9	5.5	14.8
2	33.5	66.5	15.1	35.1	10.4	24.7	5.7	10.6	32	29.3	70.7	14.3	33.8	11.4	22.4	5.4	17.2
3	34.7	65.3	10.5	37.4	8.5	28.9	6.1	11.2	33	28.8	71.2	21.3	29.7	5.0	24.7	7.6	12.7
4	21.5	78.5	18.4	45.5	11.3	34.2	7.2	7.3	34	33.5	66.5	15.4	30.9	11.3	19.7	5.6	14.7
5	28.2	71.8	14.1	38.6	6.6	31.9	7.4	11.8	35	30.6	69.4	16.8	35.6	8.5	27.1	7.6	9.4
6	30.1	69.9	13.4	39.4	9.0	30.4	6.2	10.9	36	35.2	64.8	16.9	33.5	7.6	25.9	5.2	9.2
7	24.3	75.7	11.2	47.9	10.4	37.4	4.2	12.4	37	34.7	65.3	11.4	33.2	10.0	23.2	7.5	13.1
8	32.8	67.2	13.6	38.9	10.3	28.6	5.9	8.9	38	29.2	70.8	14.2	34.8	13.3	21.4	5.8	16.0
9	25.7	74.3	18.5	41.4	9.2	32.3	5.4	9.0	39	34.3	65.7	10.5	37.8	7.9	29.9	5.8	11.6
10	29.8	70.2	17.8	37.1	7.2	29.9	4.5	10.8	40	34.3	65.7	10.5	36.7	7.7	28.9	6.4	12.2
11	30.7	69.3	14.2	39.5	6.8	32.6	3.9	11.7	41	35.8	64.2	7.3	37.1	7.3	29.8	5.9	13.9
12	27.5	72.5	13.6	37.9	10.8	27.1	7.4	13.6	42	26.0	74.0	13.3	44.1	12.8	31.3	6.1	10.6
13	27.0	73.0	13.4	41.3	9.6	31.6	5.2	13.2	43	30.4	69.6	14.4	34.6	11.8	22.8	5.8	14.7
14	28.6	71.4	13.9	38.3	8.9	29.4	5.2	13.9	44	40.5	59.5	10.2	30.4	7.0	23.4	6.3	12.5
15	31.0	69.0	12.3	34.2	6.3	27.9	5.1	17.3	45	33.0	67.0	16.0	33.6	11.7	22.0	6.5	10.8
16	23.5	76.5	5.9	48.2	12.8	35.4	8.3	14.0	46	29.1	70.9	16.1	37.0	9.6	27.4	7.5	10.4
17	25.6	74.4	16.5	38.1	7.2	31.0	7.8	12.0	47	28.8	71.2	18.5	31.8	11.7	20.1	6.6	14.2
18	19.2	80.8	10.9	52.3	12.0	40.3	6.6	10.9	48	19.3	80.7	13.9	50.8	10.8	40.0	5.4	10.7
19	26.6	73.4	16.2	41.6	8.8	32.8	5.2	10.4	49	34.5	65.5	12.0	33.8	10.6	23.2	5.7	14.0
20	22.3	77.7	16.6	42.8	11.7	31.1	8.3	10.0	50	31.7	68.3	18.1	26.3	8.5	17.8	7.6	16.3
21	38.1	61.9	14.5	29.1	7.1	22.0	6.7	11.6	51	29.5	70.5	13.5	38.8	7.8	31.0	6.3	11.8
22	26.9	73.1	15.4	38.6	7.5	31.1	7.8	11.3	52	32.6	67.4	20.7	30.6	7.3	23.3	4.3	11.8
23	23.2	76.8	14.3	41.9	12.6	29.3	7.0	13.6	53	37.3	62.7	14.8	29.6	8.5	21.0	6.7	11.6
24	27.7	72.3	12.4	39.7	7.3	32.4	7.4	12.8	54	37.2	62.8	13.0	29.1	6.3	22.8	7.0	13.7
25	31.1	68.9	9.3	41.9	7.6	34.3	5.0	12.6	55	23.0	77.0	21.4	38.5	13.5	25.0	6.3	10.8
26	28.2	71.8	21.0	33.7	9.4	24.3	6.1	11.0	56	18.2	81.8	19.4	41.1	8.7	32.3	6.8	14.6
27	29.6	70.4	16.4	37.0	7.6	29.5	5.5	11.5	57	26.8	73.2	24.5	31.2	7.3	23.9	6.9	10.6
28	33.2	66.8	9.4	38.4	7.7	30.8	7.5	11.4	58	36.3	63.7	17.4	26.4	3.1	23.3	5.3	14.7
29	32.4	67.6	14.3	36.3	6.8	29.5	5.6	11.4	59	32.4	67.6	17.1	31.5	5.1	26.4	6.3	12.7
30	22.7	77.3	11.0	45.8	5.1	40.8	4.2	16.2									

[a] Results are means of the determinations of three independent samples. Protein separation was performed by SDS–gel electrophoresis using 12% polyacrylamide gels. The quantification of the individual bands was performed by scanning densitometry. Values are given as percentage of total pea protein extract. *n*Glob, non-globulins, *np*vicil, nonprocessed vicilins; *p*vicil, processed vicilins; conv, convicilins; GlobRP, globulin-related proteins.

sum of the protein and starch contents of the different pea lines. The average sum corresponds to a value of 68.5%. Only 13 of 59 lines differed significantly from this average (standard error, ±0.05), which is much lower than the 36 and 39 lines of 59 found for individual starch and protein contents, respectively. This indicates that the pea's total reserve size remains more or less constant but that differences in the starch-to-protein ratio are tolerated.

Protein Composition. Globulins were quantified on the basis of SDS-PAGE analysis of the samples without β-mercaptoethanol, because in these samples, no overlap of legumin with processed vicilin subunits occurred. When β-mercaptoethanol was added, the bands of processed vicilins and those of the basic subunits of legumin overlapped. It should be noted that without β-mercaptoethanol some overlap of legumin and globulin-related protein bands (see Materials and Methods) was observed, which may overestimate the quantity of legumin. However, the analysis of the protein samples treated with β-mercaptoethanol showed that this overestimation was negligible for all lines and was not further accounted for. A representative SDS-PAGE gel is shown in **Figure 4**. Prior to analysis of the various pea lines, we have investigated the reproducibility of the extraction of the pea material. For sample 1, six independent extractions were carried out, and the TPPE was analyzed by SDS-PAGE/densitometry. The extraction procedure was highly reproducible with standard deviations of <2% for the amount of protein in the various globulin classes. The amounts of protein (as percentage of TPPE) in the various classes were 8.27 ± 0.09 for legumin, 9.17 ± 0.14 for nonprocessed vicilin, 23.23 ± 0.42 for processed vicilin, 5.72 ± 0.06 for convicilin, and 2.82 ± 0.03 for globulin-related proteins.

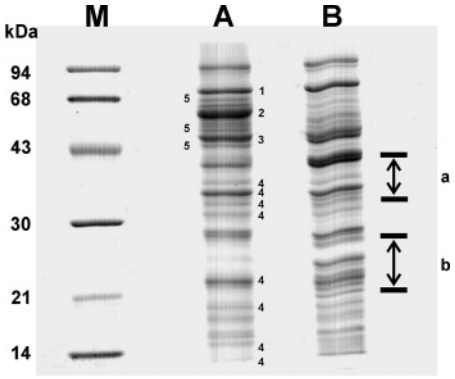

Figure 4. Representative 12% polyacrylamide gel electrophoretic separation of pea proteins contained in TPPE: lane M, marker; lane A, TPPE without β-mercaptoethanol; lane B, TPPE with the addition of 2% β-mercaptoethanol. Bands indicated by 1, 2, 3, 4, and 5 correspond to convicilin, legumin, nonprocessed vicilin, processed vicilin, and globulin-related proteins, respectively. The extra bands appearing in the region indicated by a and b upon β-mercaptoethanol treatment represent the acidic and basic subunits of legumin, respectively.

Table 2 shows the results of TPPE compositional analysis by densitometric measurement. Two main protein classes can be distinguished in TPPE, globulins and non-globulins (together equaling 100% of the proteins). The non-globulins were not subdivided further, whereas the quantities of the globulins are presented both as total and for the individual classes. Globulins were the major category of seed proteins in all lines tested, except line 1. Their contents varied between 49.2 and 81.8%, with an average content of 69.8%. No significant difference in

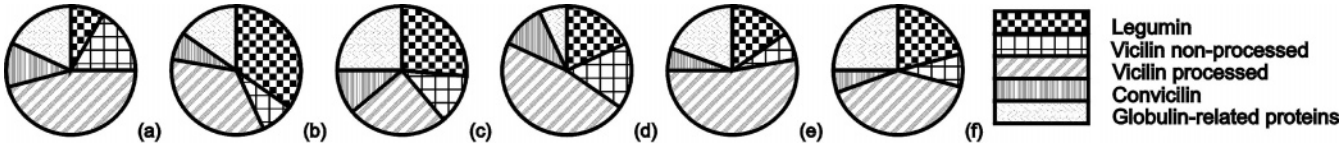

Figure 5. Examples of extremes in globulin composition in various pea lines. Diagrams **a** (line 16 [NGB 102149 Iran]) and **b** (line 57 [*P. arvense*, CGN 10193]) show the extremes in legumin composition, diagrams **c** (line 50 [CEB 1162]) and **d** (line 1 [93125-07 Canada]) the extremes in vicilin composition, diagrams **e** (line 30 [Cisca]) and **d** the extremes in convicilin composition, and diagrams **d** and **f** (line 15 [NGB 102920 Iran]) the extremes in globulin-related proteins.

globulin content was observed between the *P. sativum* species (69.7%) and the wild relatives (72.6%) (*T* test, Bonferroni).

Densitometric quantification of the individual globulins showed that the legumin content varied between 5.9 and 24.5% of the proteins of TPPE. The wild relatives contained on average 20.0% of legumin, which is significantly higher than the average of 14.0% found for the *P. sativum* lines (*T* test, Bonferroni). Interestingly, legumin content correlated significantly (0.548) with that of total seed protein (Pearson two-tailed); a similar relationship was found for processed vicilin (Pearson two-tailed). A separate quantification of the *Leg*A and *Leg*J families was not performed, because their similarities in molecular weight made it impossible to distinguish between members of these two families with SDS-PAGE. Vicilin was the most abundant protein of pea, and its content varied between 26.3 and 52.0% of the TPPE. There was no correlation between the amount of vicilin and legumin or that of vicilin and convicilin (Pearson two-tailed). In all of the materials tested, both processed and nonprocessed vicilins were found. The processed vicilin was the predominant of the two, with values between 17.8 and 40.8%, whereas the nonprocessed ones constituted between 3.1 and 13.5% of the TPPE. The amounts of processed and nonprocessed vicilin did not correlate with each other (Pearson two-tailed). Convicilin was the least abundant globulin, having an average content of 6.1%. Its content ranged from 3.9 to 8.3%. Finally, the globulin-related proteins were present in amounts ranging from 2.8 to 17.3% of the TPPE; they showed the largest relative variation of the four globulin classes.

The TPPE compositional analysis showed that none of the lines lacked any particular globulin. All four classes of globulins were present in all lines investigated, including the wild relatives. However, significant differences were observed between the different lines, not only in their globulin content of TPPE but also in their globulin composition. **Figure 5** shows a number of examples in which the content of one class of proteins is particularly high or low. It can be seen that the differences within the globulin fractions were not correlated with the content of the individual globulins; there were no apparent links between a high content of one globulin and a low content of another one. Line 1 (93125-07 Canada) had the highest proportion of vicilin and convicilin and the lowest proportion of globulin-related proteins (**Figure 5d**).

Table 3 shows that the ratio of vicilin/legumin varied from 1.3 to 8.2 and that on average it was significantly higher in *P. sativum* than in the wild relatives, with averages of 2.8 and 1.7, respectively (*T* test, Bonferroni). No significant differences were found in this ratio between lines with different external characteristics (such as leaf shape, seed shape, and seed color). The vicilin/convicilin ratio varied from 3.5 to 11.4 (**Table 3**). It appeared that this ratio was significantly lower in the *af* lines than in wt, with averages of 5.6 and 6.8, respectively (*T* test, Bonferroni). No differences between the wild relatives and *P. sativum* lines were observed. The ratio of vicilin to globulin-related proteins varied from 1.6 to 11.6 (**Table 3**), and significant differences in this ratio were found neither between lines with different external characteristics nor between wild relatives and *P. sativum* lines (*T* test, Bonferroni).

Table 3. Ratios between Vicilin and the Other Globulins and between the Two Vicilin Fractions of the Tested Lines[a]

line	vicilins/ legumins	vicilin/ conv	vicil/ globRP	*p*vicil/ *np*vicil	line	vicilins/ legumins	vicilins/ conv	vicilins/ globRP	*p*vicil/ *np*vicil
1	3.9	5.7	11.6	2.5	31	2.8	5.9	2.2	2.0
2	2.3	6.2	3.3	2.4	32	2.4	6.3	2.0	2.0
3	3.6	6.1	3.3	3.4	33	1.4	3.9	2.3	4.9
4	2.5	6.3	6.2	3.0	34	2.0	5.5	2.1	1.7
5	2.7	5.2	3.3	4.8	35	2.1	4.7	3.8	3.2
6	2.9	6.4	3.6	3.4	36	2.0	6.4	3.6	3.4
7	4.3	11.4	3.9	3.6	37	2.9	4.4	2.5	2.3
8	2.9	6.6	4.4	2.8	38	2.5	6.0	2.2	1.6
9	2.2	7.7	4.6	3.5	39	3.6	6.5	3.3	3.8
10	2.1	8.2	3.4	4.2	40	3.5	5.7	3.0	3.8
11	2.8	10.1	3.4	4.8	41	5.1	6.3	2.7	4.1
12	2.8	5.1	2.8	2.5	42	3.3	7.2	4.2	2.4
13	3.1	7.9	3.1	3.3	43	2.4	6.0	2.4	1.9
14	2.8	7.4	2.8	3.3	44	3.0	4.8	2.4	3.3
15	2.8	6.7	2.0	4.4	45	2.1	5.2	3.1	1.9
16	8.2	5.8	3.4	2.8	46	2.3	4.9	3.6	2.9
17	2.3	4.9	3.2	4.3	47	1.7	4.8	2.2	1.7
18	4.8	7.9	4.8	3.4	48	3.7	9.4	4.7	3.7
19	2.6	8.0	4.0	3.7	49	2.8	5.9	2.4	2.2
20	2.6	5.2	4.3	2.7	50	1.5	3.5	1.6	2.1
21	2.0	4.3	2.5	3.1	51	2.9	6.2	3.3	4.0
22	2.5	4.9	3.4	4.1	52	1.5	7.1	2.6	3.2
23	2.9	6.0	3.1	2.3	53	2.0	4.4	2.6	2.5
24	3.2	5.4	3.1	4.4	54	2.2	4.2	2.1	3.6
25	4.5	8.4	3.3	4.5	55	1.8	6.1	3.6	1.9
26	1.6	5.5	3.1	2.6	56	2.1	6.0	2.8	3.7
27	2.3	6.7	3.2	3.9	57	1.3	4.5	2.9	3.3
28	4.1	5.1	3.4	4.0	58	1.5	5.0	1.8	7.5
29	2.5	6.5	3.2	4.3	59	1.8	5.0	2.5	5.2
30	4.2	10.9	2.8	8.0					

[a] Results are based on the content of the individual globulins as percentage of the TPPE. For abbreviations see **Table 2**.

Tables 2 and **3** and **Figure 5** clearly demonstrate that the extent of vicilin processing can differ considerably between lines; the ratio of processed to nonprocessed vicilins varied from 1.6 to 8, showing that the processed fraction is always higher than the nonprocessed one. In **Figure 6**, this ratio has been categorized in four groups: <2, $2-4$, $4-6$, and >6. The majority of lines belongs to the group $2-4$ (38 lines), whereas the least number of lines belongs to the group >6 (2 lines). The ratio between the two vicilin fractions does not vary significantly between lines with different seed shape (*T* test, Bonferroni). In contrast, significant differences with respect to the *p*vicil/*np*vicil ratio were found between lines with different leaf shape and seed coat color (*T* test, Bonferroni). The average ratios of *af* and wt lines were 2.9 and 3.9, respectively, whereas the ratio of 6.3 found for brown-gray-spotted seed coat lines (*Pisum elatius* accessions) was significantly higher than that of 3.5, 3.4, 2.4, 2.4, and 2.6 for green, yellow, brown-spotted,

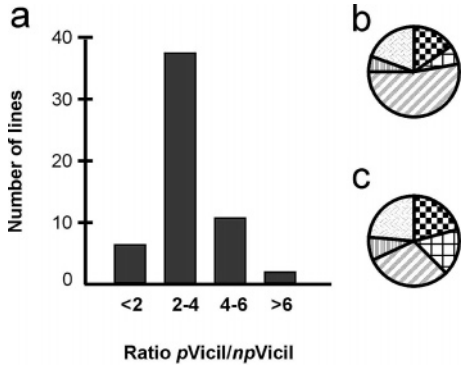

Figure 6. Extent of vicilin processing in the tested pea lines: (**a**) ratio of processed to nonprocessed vicilin categorized in four groups, number of lines belonging to each category indicated; (**b**, **c**) globulin composition of two extreme varieties with respect to processing, *p*vicil/*np*vicil <2 (line 38 [484]) and >6 (line 30 [Cisca]), respectively. For pie diagrams legends, see **Figure 5**.

brown, and green-yellow lines, respectively. No differences with respect to the *p*vicil/*np*vicil ratio between wild relatives and *P. sativum* lines were observed.

DISCUSSION

In this study, the genetic variation of pea (*P. sativum* L.) in its starch and protein contents and protein composition has been explored, with the aim of identifying lines with extreme variation. Such could be of importance for industrial applications of pea or its isolated proteins, because pea globulins have different physical properties (*8*). Additionally, it is known that starch and different mixtures of globulins give different extrusion products (*25*). Our results with the 59 tested lines are discussed with respect to the quality of starch, pea meal, pea protein isolates, and purified globulins. The biodiversity identified has been analyzed for correlations with external seed and plant characteristics that might be used as an initial screen to assist in breeding programs.

External Characteristics. The pea lines investigated, producing round, wrinkled, flat, and round-dimpled pea seeds, have starch as the major pea seed reserve (average content of 46%). It is known that the starch content and quality are affected by the presence of the *r* locus, which results in the production of wrinkled instead of round peas, when near-isogenic lines are compared (*4*, *31*). The wrinkled varieties appeared to have on average lower starch content than the varieties with other seed shapes. Nevertheless, there are wrinkled varieties scoring higher on starch content than some nonwrinkled ones (such as lines 48 and 52). This indicates that the genetic background of a line is crucial in overcoming the effects of the *r/r* locus on starch content.

The protein contents of the material tested varied considerably. Seed shape and leaf type did not seem to be related to protein content, but seed color was. The three brown pea lines tested had significantly higher protein contents than the other lines. Differences between green and yellow peas, which are the cultivated varieties for human consumption, were not observed, indicating that color cannot be a selection criterion for pea protein content in breeding programs for human consumption. We have not observed with our lines that wrinkled peas have more protein than round ones, as was reported by other researchers (*22*, *32*). The wild relatives of pea have a protein content of 25−30%, which is above average, but below that of soybean (42−45%), the most important protein source for food applications (*33*).

We found a negative correlation between the starch and protein content of the lines tested, but the sum of these appeared to be remarkably constant. This demonstrates that the amounts of these two reserves are balanced. However, there are some exceptions to this (**Figure 4**), such as the lines 28 [Mylosa (CEB 1811)] and 30 (Cisca), which are both wrinkled peas; the other wrinkled lines do not deviate with respect to the sum of protein and starch content.

Protein Composition. Pea lines lacking a specific globulin such as convicilin would be desirable because convicilin's N-terminal extension reduces the gelation ability of pea globulin isolates (*18*). Interestingly, none of the tested lines lacked any particular globulin class. The absence of such lines can be explained by the fact that the globulin classes legumin, vicilin, and convicilin are encoded by multigene families (*34*, *35*).

The lines tested showed variation in both total globulin content and globulin composition. Vicilin is the most abundant globulin; it can be present in 8-fold higher amounts than legumin and in up to 11-fold larger amounts than convicilin and globulin-related proteins. The ratio vicilin/legumin has been studied previously. Our results showed a higher vicilin/legumin ratio compared to that reported by others (*21*, *22*, *32*, *36*). We did not find significant differences in the vicilin/legumin ratio between round and wrinkled peas. Others (*22*, *32*), using rocket immunoelectrophoresis and ultracentrifugation, have reported that the ratio vicilin/legumin is slightly higher for wrinkled peas than for round peas. Probably, the differences in results between these studies and our study are due to different determination methods and plant material used.

All lines tested contained processed vicilin. This showed that the vicilin-processing enzyme, which has not been identified yet, is present and active in all lines. This gene could be a potential genetic marker for pea genetic studies. In all lines, the amount of processed vicilin was higher than that of nonprocessed vicilins. However, the amounts of processed vicilin and nonprocessed vicilin did not correlate with each other. This was expected, because not all vicilins are processed. Thus, the extent of processing is related to the availability of the isoform that can be cleaved, and not to the total amount of vicilin. It has been shown that fragmentation of a protein can alter its physicochemical properties (*37*). This indicates that the varieties high in processed vicilin might have different properties compared to those low in processed vicilin. It would be worthwhile to further explore the effect of the extent of in planta processing of vicilin in relation to the properties of protein mixtures.

Globulins have different physicochemical properties, and this is reflected in their protein isolates. Gelation studies with globulin isolates derived from the varieties Classic, Solara, Finale, Supra, Espace (**Table 1**, lines 31, 33, 51, 52, and 54, respectively), which vary in globulin composition, also showed different gelation behaviors (*20*). Similarly, it has been shown that genetic soybean variants in glycinin have different denaturation behaviors (*38*). Our results showed significant biodiversity in globulin composition, which may have potential for applications in the food industry. In soybean, the globulins of which are widely used for various applications, the genetic variation for protein composition is much less than that observed for pea. For instance, the ratio of 7S/11S globulins in soybean ranged from 0.47 to 0.79 (*39*, *40*), whereas we have shown that the same ratio (excluding convicilin) varied between 1.2 and 8 in pea. Moreover, the content of glycinin, which is the soybean analogue to pea legumin, is always higher than that of β-conglycinin (the soybean analogue of pea vicilin + convicilin).

On the contrary, in peas, with some exceptions (*21*, *32*), vicilin is always higher than legumin. This is another major difference in the globulin composition of pea and soybean, which could make the use of pea proteins, complementary to those from soybean, attractive to the food industry.

It is known that a low vicilin/convicilin ratio in pea or a low ratio of its soybean equivalents (the β subunit of β-conglycinin corresponds to vicilin; the α and α' subunits of β-conglycinin correspond to convicilin) hinders gelation in both pea (*18*) and soy isolates (*41*). On the basis of data provided by Yaklich (*30*) and Fehr et al. (*39*), it can be calculated that the $\beta/(\alpha + \alpha')$ ratio of the subunits of β-conglycinin in soybean varies between 0.7 and 1.5 (*30*, *39*), whereas in pea the ratio vicilin/convicilin varies between 3.5 and 11.4. This suggests that pea isolates might have a more favorable protein composition for gelling applications compared to those from soybean. Moreover, the genetic variation for this trait appears to be larger in pea than in soybean, which might offer opportunities to reduce the convicilin content further.

ABBREVIATIONS USED

DM, dry matter; ER, endoplasmic reticulum; wt, wild type; *af*, *afila*; TPPE, total pea protein extract; SD, standard deviation; CI, confidence interval.

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Received for review August 4, 2005. Revised manuscript received November 2, 2005. Accepted November 18, 2005. We thank STW for financing this project as part of the PROFETAS program.

JF0519008