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Effects of Grinding and Thermal Treatments on Hydrolysis Susceptibility of Pea Proteins (*Pisum sativum* L.)

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The effects of three particle sizes with two types of grindings and two thermal treatments on pea protein extraction (PE) and susceptibility to in vitro enzymatic hydrolysis (pepsin plus trypsin) were studied. Degrees of hydrolysis (DH) were calculated. Remaining peptides were detected by SDS-PAGE and identified by immunoblotting and MS/MS spectrometry. The increase in particle size decreased PE and DH due to a restricted access of solvents and enzymes to proteins. The thermal treatment induced a decrease in PE but did not modify DH. Heating improved legumin (αM) and convicilin pepsin hydrolyses but reduced the pea albumin 2 (PA2) hydrolysis. After pepsin and trypsin hydrolysis, only peptides from vicilin and lectin were identified by LC-MS/MS analyses, whatever the treatment.

KEYWORDS: Pea; protein; enzymatic hydrolysis; remaining peptides; technological treatment

INTRODUCTION

Plant seeds represent a major source of dietary proteins. Legume seeds such as soybean, common bean, lentil, or pea are important sources of proteins for human and animal nutrition. However, for feedstuffs, soybean, in the form of soybean meal, is by far the most commonly incorporated in diets. This situation induces the economic dependence of Europe on soybean-producing countries and causes a protein deficit for animal feeds. For these reasons, the European Union is attempting to develop home-grown protein-rich crops, and the production of grain legumes such as pea has been promoted.

Pea is an important source of dietary proteins for monogastric animals, but a high level of incorporation of pea leads to reduced growth performance, especially with young animals (*1*). This could be ascribed to a low protein digestibility and availability of the limiting amino acids, which seem to be multifactorially determined. First, the nutritional quality of pea protein is affected by genotypic and phenotypic variations of pea protein content and composition (*2*, *3*). Second, the presence of antinutritional factors (ANF) and/or fibrous material is partly responsible for the lower susceptibility or accessibility of legume seed proteins to digestive enzymes (*1*, *4*, *5*). However, ANF such as trypsin inhibitors only partly explain the lower digestibility of pea protein because of their rather low activities (*6*, *7*). The globular structure of storage protein also could affect amino acid availability from pea proteins. Two main groups of legume seed proteins are usually considered: albumins, that is, water-soluble proteins; and globulins, that is, salt-soluble proteins. Globulins represent ∼70% of legume seed proteins and are composed of two major groups, legumin-type and vicilin-type families (*8*). Globulins display a very compact structure, rich in β-sheet. The albumin fraction represents ∼20% of legume seed proteins. Pea albumin 2 (PA2) (M_r 26000) is the major component of the pea albumin fraction. Pea albumin 1 (PA1) consists of two post-translational cleaved subunits, PA1a (M_r 6000) and PA1b (M_r 3000), rich in sulfur amino acids. Unlike the 2S albumins the two polypeptides are not linked by interchain disulfide bonds. Lipoxygenases, glycosidases, protease inhibitors, and lectins belong to the albumin family.

Many studies have investigated the susceptibility of pea native proteins hydrolysis in vitro (*9*, *10*), but, in vivo, only total N and amino acid digestibilities were evaluated. Studies reported the impacts of technological treatments on growth performances but not on the susceptibility to hydrolysis or digestibility of processed proteins. Thermal treatment is a physical procedure frequently used in the food industry to modify protein functionality and is one of the widely used methods for feedstuff preservation, sanitization, and pellettization. Feeds are commonly ground at 500 μm and often heated at 85 °C during 3 min for sanitary reasons. However, data on the heating process to increase the storage protein digestibility are conflicting (*9*, *11*, *12*). There is a general consensus on antinutritional factor inactivation by a proper heat treatment (*13*), but, depending on the thermal stability of proteins and heating conditions, proteins may be either partially or completely dissociated and even

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Table 1. Mill Characteristics for Each Grinding Condition

	grinding A			grinding B		
mean particle size ($d50$), μm	200	500	1000	200	500	1000
particle size distribution ($d16$–$d50$–$d84$)	139–253–460	270–559–1157	452–1087–2614	178–293–481	295–521–922	622–1034–1719
mill	hammer 2 mm screen 1 mm screen	hammer 2.5 mm screen	hammer 7 mm screen	roller plus hammer for hulls	roller plus hammer for hulls	roller plus hammer for hulls

denatured and aggregated. Dissociation could increase the enzyme sites accessibility, but unfolded molecules may also aggregate, decreasing their extractability (*14*). Grinding effects on protein digestibility have been less studied than thermal effects and often have been investigated on two very different particle sizes (fine against coarse particles) (*15*). Moreover, the nutritional implications of particle size dispersion are not fully understood (*16*).

The aim of this study was to determine technological treatments that could improve pea protein utilization in animal feeding. We studied three different mean particle sizes (200, 500, and 1000 μm) obtained with two grinding processes (hammer mill or roller mill plus hammer mill) and two temperatures of 3 min steam heat treatment (85 and 95 °C).

MATERIALS AND METHODS

Plant Material. A spring pea cultivar (cv. Baccara) with yellow seeds and low trypsin inhibitor content [(<0.5 TUI mg $^{-1}$ of DM) determined according to the method described by Valdebouze et al. (*17*)] was used in this study. The cultivar was chosen with a very low trypsin inhibitor content in order to avoid a potential effect of this ANF. Pea was harvested at INRA, Rennes, France. The protein (nitrogen × 5.5) content was 18% of dry matter (DM).

Technological Treatments. *Grinding.* The seeds were ground to obtain particles with three median particle diameters ($d50$): 200, 500, and 1000 μm. In each case, two different distributions of particle size were obtained, A and B: for the first (A, higher dispersion), grinding was performed in a single step with a hammer mill Forplex, and for the second (B, lower dispersion), grinding was performed in two steps, the first with a roller mill Socam, followed by sieving, and the second on the coarser particles with a hammer mill. Particle size dispersion was characterized by dx, where x was the percentage of particles with diameter lower than dx. The experimental flours were characterized by the $d16-d84$ diameter range. The characteristics of the milling process to obtain the selected median diameters are described in **Table 1**. The particle size distributions of the flours were determined using 12 sieves with different openings according to the method described in TECALIMAN (*18*).

Thermal Treatments. The flours were heated in a vertical mixer in batches of 5 kg for 3 min at 85 or 95 °C.

Extraction of Protein. The experimental flour samples (250 mg) were extracted with a diluted hydrochloric acid solution (HCl, pH 2). Suspensions were stirred at 37 °C for 1 h and then centrifuged (10000*g*, 10 min) (T_0). The amount of extracted proteins was determined on supernatants according to the bicinchoninic acid (BCA) micro method (Pierce).

Enzymes. Porcine pepsin (EC 3.4.2.3.1; 3.46 units/mg of protein; P-6887) and porcine trypsin (EC 3.4.2.1.4; 16 units/mg of protein; T-0303) were purchased from Sigma.

Hydrolysis Procedure. Hydrolysis was performed using a constant enzyme-to-protein ratio of 1:51. For each sample, an amount of flour corresponding to 51 mg of protein was introduced into 100 mL vessels. Thirty milliliters of HCl (10 mM) was then added. Suspensions were stirred at 37 °C for 1 h. At zero time (T_0) 1 mg of enzyme solubilized in HCl (1 mM) was added. Aliquots (0.4 mL) were taken after 15 min (T_1) and 3 h (T_2) of hydrolysis by pepsin. Then, pH was adjusted at 8.0 using NaOH (1 N), and 1 mg of trypsin was added. Aliquots (0.4 mL) were taken 15 min (T_3), 1 h and 30 min (T_4), and 3 h (T_5) after trypsin addition.

Hydrolysis Characterization. For each aliquot time sample, the enzymatic reaction was stopped by trichloroacetic acid (TCA) precipitation (10% w/v, final concentration). Samples were then centrifuged (10000*g*, 10 min), and supernatants were recovered for analysis of the TCA-soluble fraction. For each aliquot, the concentration of NH_2 groups was determined according to the *o*-phthaldialdehyde method (*19*). For each flour, the total of NH_2 groups ($[NH_2]_{(total)}$) was determined after hydrolysis in 6 M HCl for 24 h at 105 °C. The degree of hydrolysis (DH) was calculated according to the equation

$$DH = ([NH_2]_{(T_x)} - [NH_2]_{(T_0)})/([NH_2]_{(total)} - [NH_2]_{(T_0)}) \times 100$$

with $[NH_2]_{(T_0)}$ the NH_2 concentration at zero time and $[NH_2]_{(T_x)}$ the NH_2 concentration of the TCA soluble fraction after different times of hydrolysis.

Electrophoresis. For each hydrolysis time, aliquots (0.4 mL) were immediately mixed with 0.1 mL of SDS (200 g/L), heated at 100 °C for 3 min, and centrifuged (10000*g*, 10 min), and supernatants were stored at −20 °C. The samples were diluted 2-fold with Tris-HCl buffer (0.16 M, pH 8.8) containing 300 g/L sucrose, 40 g/L SDS, and 0.02 g/L bromophenol blue. The disulfide bridges were reduced with β-mercaptoethanol for 2 min at 100 °C. Samples of 20 μL were submitted to an electrophoresis in polyacrylamide gel (15%) according to the method of Laemmli (*20*). Proteins were fixed using TCA (125 g/L) and stained with Coomassie blue G 250 (2 mg/L).

Gels with blue-stained proteins were scanned (Amount One, version 4.1; Bio-Rad). Each protein band was quantified by densitometry.

LC-MS/MS Analyses. Bands of interest were excised from Coomassie blue-stained gel, reduced, alkylated using dithiothreitol and iodoacetamide, respectively, and then subjected to digestion with trypsin (Promega). Extracted peptides were dried and stored at −80 °C until use. For LC-MS/MS analyses, peptides were solubilized in buffer A (H_2O/acetonitrile/FA, 96:4:0.1, v/v). Nanoscale capillary liquid chromatography−tandem mass spectrometry (LC-MS/MS**)** analyses of the digested proteins were performed using an UltiMate capillary LC system (LC packings/Dionex) coupled to a hybrid quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer (Q-TOF Global, Waters). Chromatographic separations were conducted on a reversed-phase (RP) capillary column (Pepmap C18, 75 μm i.d., 15 cm length, LC Packings) with a 200 nL/min flow. The gradient profile used consisted of a linear decrease from 100 to 45% B (H_2O/acetonitrile/FA, 10:90:0.085, v/v) in 50 min, followed by a linear increase to 100% B in 10 min. Mass data acquisitions were performed by MassLynx software (Waters) using automatic switching between MS and MS/MS modes ("survey scan" mode). Peptides eluted from the chromatographic column were detected for 1 s; when their signal reached a user-defined threshold (8 counts/s), they were selected for fragmentation in MS/MS. Acquisitions were performed with the dynamic exclusion of m/z ratios of already fragmented ions (exclusion during 60 s of a ±0.5 Da mass window around the m/z ratio of previously selected precursors). Mass data collected were processed with Protein Lynx Global Server v. 2.0 (Waters). Protein identifications were obtained by comparison of experimental data to the NCBInr or Swissprot databases. Searches were done with a tolerance on mass measurement of 0.15 Da in MS mode and 0.25 Da in MS/MS mode.

Immunoblotting. Rabbit polyclonal sera against legumin, vicilin, lectins, and PA2 were obtained by subcutaneous immunization with the purified protein. Each injection contained 1 mg of protein in

Table 2. Effects of Grinding and Thermal Treatments on Protein Extraction (PE) at pH 2 (Extracted Protein as Percentage of Total Protein)

	grinding A			grinding B			statistical analysis
mean particle size (d50), μm	200	500	1000	200	500	1000	probability of no effect of
nonheated[a] PE (%)	73.6a	58.1c	37.4f	63.8b	52.3d	36.4f	(1) grinding A vs B: <0.0001[b] (2) mean particle size: <0.0001[b,c] (3) heating: <0.0001[b,c]
							interactions
heated at 85 °C[a] PE (%)	62.6bc	50.4d	31.5g	53.2d	45.1e	31.9g	(1) × (2): 0.0013[b] (1) × (3): 0.6743[b] (2) × (3): 0.0549,[b] 0.0319[c]
heated at 95 °C[a] PE (%)	52.3d	37.4f	25.4h	NR[d]	NR	NR	(1) × (2) × (3): 0.9732[b] SEM = 1.57

[a] Values with different letters are significantly different ($P < 0.05$) according to the Duncan test for 15 group means. [b] Variance analysis without heat treatment at 95 °C. [c] Variance analysis within grinding A, with heat treatment at 95 °C. [d] Nonrealized treatment.

Table 3. Effects of Grinding and Thermal Treatments on Hydrolysis Susceptibility Pea Proteins (DH)

	grinding A			grinding B			statistical analysis
mean particle size (d50), μm	200	500	1000	200	500	1000	probability of no effect of
nonheated[a] DH (%)	24.0ab	21.9bc	15.9e	25.4a	26.3a	16.7de	(1) grinding A vs B: 0.0016[b] (2) mean particle size: 0.0001[b,c] (3) heating: 0.6313,[b] 0.0787[c]
							interactions
heated at 85 °C[a] DH (%)	25.2a	23.5ab	12.2f	26.6a	23.8ab	16.0e	(1) × (2): 0.5552[b] (1) × (3): 0.7110[b] (2) × (3): 0.0801,[b] 0.0234[c]
heated at 95 °C[a] DH (%)	25.7a	19.9cd	9.8f	NR[d]	NR	NR	(1) × (2) × (3): 0.0853[b] SEM = 1.10

[a] Values with different letters are significantly different ($P < 0.05$) according to the Duncan test for 15 group means. [b] Variance analysis without heat treatment at 95 °C. [c] Variance analysis within grinding A, with heat treatment at 95 °C. [d] Nonrealized treatment.

phosphate-buffered saline (10 mM Na_2HPO_4, 150 mM NaCl, pH 7.2) (PBS), emulsified with complete (first injection) or incomplete (boost injections) Freund's adjuvant. Boosting was repeated every 15 days, and serum was collected after the fourth injection.

Proteins separated by electrophoresis were electroblotted on nitrocellulose sheets (pore diameter = 0.2 μm) according to the method of Towbin et al. (*21*). After quenching of the free sites by skimmed milk (50 g/L), sera were used to reveal specific bands. The final revelation was obtained after incubation with goat anti-rabbit IgG conjugated with horseradish peroxidase and addition of the peroxidase staining mixture [0.06% 4-chloronaphthol, 0.02% hydrogen peroxide in methanol/PBS (1:10, v/v)].

Statistical Analysis. Variance analyses were performed for testing the effects of particle size distribution (A and B dispersion), particle median diameter, heating, and distribution × median diameter, distribution × heating, median diameter × heating, and distribution × median diameter by heating interaction effects. Group means corresponding to the latter second-order interaction were compared by Duncan's multiple-range test. The effect of particle size was partitioned into linear and quadratic contrasts. The effects of heating and distribution on the linear and quadratic components of median particle size effects were compared as components of the median diameter × heating and distribution × median diameter first-order interaction effects, respectively. All statistical analyses were performed using the General Linear Model procedure of SAS.

RESULTS

Protein Extraction (PE) at pH 2. *Grinding Effects.* The effect of particle size on PE was highly significant ($P < 0.0001$). When the highest temperature, which was applied with the first grinding process (A) only, was not included in the analysis, the level of PE linearly ($P < 0.0001$) and quadratically ($P < 0.01$) decreased when the particle size increased (**Table 2**), regardless of heating and dispersion (grinding process). The quadratic effect meant a lower difference between 200 and 500 μm than between 500 and 1000 μm of median diameter. However, the median diameter × distribution interaction effect was significant ($P < 0.001$), and this interaction primarily affected the linear component of the particle size effect ($P < 0.001$): from a higher value at 200 μm with grinding A (higher dispersion) than with grinding B (lower dispersion) PE decreased to similar values at 1000 μm of particle size, regardless of heating.

Effects of Thermal Treatments. The level of PE at pH 2 decreased significantly ($P < 0.05$) when a thermal treatment was applied (**Table 2**). However, the median diameter × heating interaction effect was significant ($P < 0.01$), and this interaction also primarily affected the linear component of the particle size effect ($P < 0.02$): the negative effects of heating on protein extraction were lower at 1000 μm than at 200 μm of particle size, regardless of distribution. Separate analysis within grinding A (high dispersion) data also confirmed a significant median diameter × heating interaction effect ($P < 0.05$) primarily affecting the linear component of the particle size effect: heating at 95 °C further decreased the protein extraction to a lower extent at 1000 μm than at 200 μm of particle size (linear component contrast between the nonheated and the 95 °C heated pea, $P < 0.05$).

Hydrolysis. *Degrees of Hydrolysis.* The effects of technological treatments on susceptibility of pea proteins to enzymatic hydrolysis were studied through the DH criterion. Only results obtained for 3 h of pepsin hydrolysis plus 3 h of trypsin hydrolysis (T_5) are presented (**Table 3**). The general effect of the increase in particle size was to reduce significantly the DH ($P < 0.0001$). When the highest temperature, which was applied with the first grinding process (A) only, was not included in analysis, the level of DH linearly ($P < 0.0001$) and quadratically ($P < 0.0002$) decreased when particle size increased, regardless of heating and dispersion (grinding process). The quadratic effect reflected the lower difference between 200 and 500 μm than

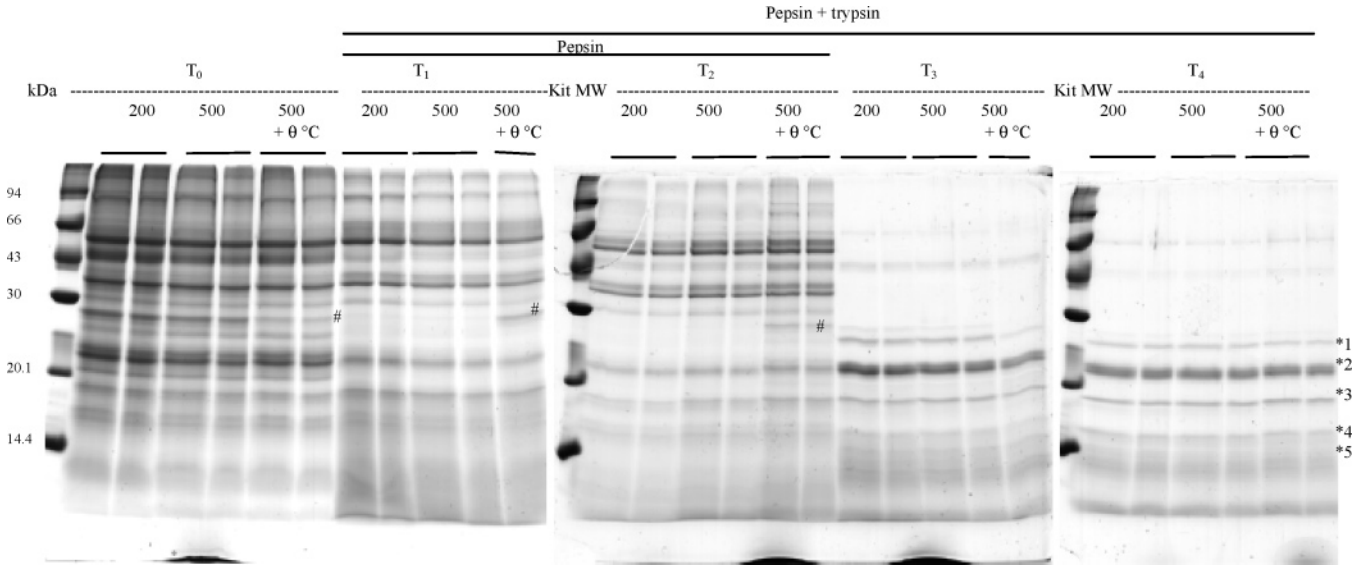

Figure 1. SDS-PAGE and Coomassie blue staining in reducing conditions of total protein extracted before (T_0) and after hydrolysis with pepsin (T_1 = 15 min and T_2 = 3 h) after hydrolysis with pepsin + trypsin (T_3 = 3 h + 15 min and T_4 = 3 h + 1 h and 30 min). (E/S = 1/51 for each enzyme.) **200**, flour ground at 200 μm and not heated; **500**, flour ground at 500 μm and not heated; **500** + θ °**C**, flour ground at 500 μm and heated at 85 °C for 3 min. Molecular weight markers are on the left-hand side of the gels. Bands denoted by an asterisk (*) were analyzed by MS/MS spectrometry. #, PA_2, 26 000Da.

between 500 and 1000 μm of median diameter for both grinding processes. The main effect of the distribution was highly significant ($P < 0.0016$) with higher DH values for grinding B (lower dispersion) than for grinding A (higher dispersion). There was no significant median diameter × distribution interaction effect.

There was no significant effect of the heat treatment at 85 °C whatever the mean particle size and the type of grinding. In the separate analysis within grinding A (high dispersion) data a trend to an effect of heating was noticeable ($P < 0.10$). This trend mainly reflected a lower DH with heating at 95 °C compared to no heating or heating at 85 °C ($P \approx 0.05$). Furthermore, there was a significant median diameter × heating interaction effect ($P < 0.05$) primarily affecting the linear component of the particle size effect: from a similar value at 200 μm, regardless of heating, increasing the particle size to 1000 μm reduced more drastically the DH with heating at 95 °C than without heating ($P < 0.005$).

Analysis of Remaining Peptides. To analyze by electrophoresis and immunoblotting all of the remaining (nonextracted, nonhydrolyzed, partly hydrolyzed) peptides, a SDS extraction was realized. The flours ground with the hammer mill (grinding A) were studied only for the 200 and 500 μm flours. In the 1000 μm flour, the particle size was too coarse to extract protein in a reproducible manner. The effect of heating was assessed through comparison of the 85 °C heated and nonheated 500 μm flours.

The electrophoretic patterns of these three samples (**Figure 1**) were compared at each time of hydrolysis by densitometry analyses (data not shown). At the initial time (T_0), protein extraction rates by SDS were affected by particle size and the thermal treatment. The band intensities of the various polypeptides slightly decreased when particles size increased (500 vs 200 μm). The band of 26 kDa (#) was specifically and drastically decreased after the thermal treatment. This protein was no more extracted by the SDS buffer after heat action. At T_1 (15 min of hydrolysis by pepsin), pepsin hydrolysis induced new protein bands in the range of 45−50 and 35 kDa. At the same time, protein bands of 25 and 20 kDa totally disappeared. The quantitative differences of band intensities due to the particle size remained, but no qualitative difference in the electrophoretic patterns was observed between the 200 and 500 μm flours. After heat treatment, most of the polypeptides, extracted by the SDS buffer, were qualitatively and quantitatively similar to those in the nonheated 500 μm flour, except for the band at 26 kDa (#), which showed qualitative differences. This polypeptide, which disappeared in the nonheated flours at T_1, was almost not hydrolyzed when the 500 μm flour was heated at 85 °C. There were few differences in the electrophoresis patterns between T_1 and T_2 because in our hydrolysis conditions (enzyme/substrate), the enzyme action was very fast. At T_3 (15 min after addition of trypsin), only a few traces of high molecular mass polypeptides were detected. However, several polypeptides with molecular mass lower than 25 kDa including a large band of 19 kDa (*2) were clearly observed. Like after the short time of pepsin hydrolysis (T_1), mainly quantitative differences between the electrophoretic patterns of the nonheated 200 μm, the nonheated 500 μm, and the 85 °C heated 500 μm flours were observed. The band intensities were weaker when particle size increased and when the thermal treatment was applied. These differences disappeared at T_4 (3 h after trypsin addition).

Identification of Remaining Peptides. The protein origin of the polypeptides, still detected after hydrolysis, was studied by immunoblotting identification. Antibodies against legumin, vicilin, albumin PA2, and lectin were used (**Figure 2**).

The legumin antibodies detected the major acidic (αM) and the basic (β) polypeptides, with the same intensity for the three (the nonheated 200 μm, the nonheated 500 μm, and the 85 °C heated 500 μm) flours at T_0 (**Figure 2A**).The β polypeptides, no longer detected at T_1, appeared to be more sensitive to hydrolysis than the αM polypeptides, which totally disappeared only at T_2. The detection of the αM polypeptides from the heated flour slightly decreased at T_1. These polypeptides were consequently more sensitive to pepsin for the heated flour.

At T_0, the vicilin antibodies revealed the four main constitutive polypeptides of vicilin characterized by molecular masses of 50, 33, 30, and 19 kDa, but did not detect the lowest polypeptides of 16 and 13 kDa. Convicilin (70 kDa), serologi-

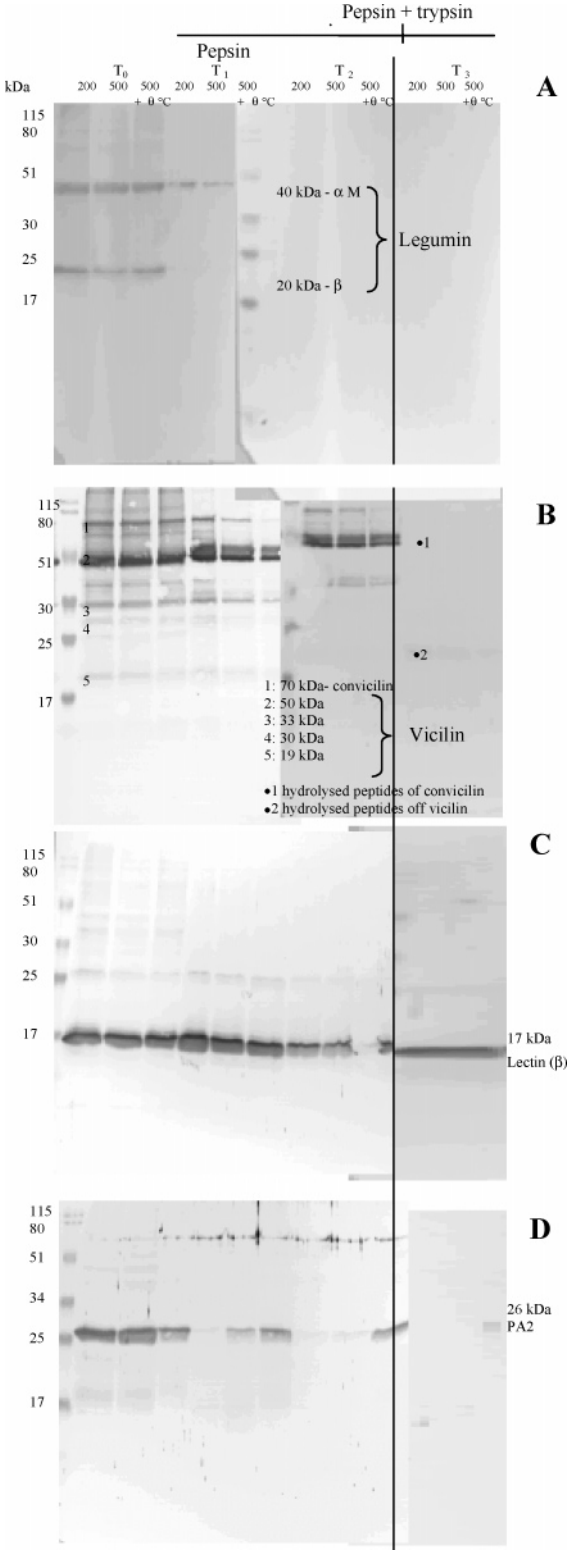

Figure 2. Immunoblotting of total protein extracts before (T_0) and after hydrolysis with pepsin (T_1 = 15 min and T_2 = 3 h) after hydrolysis with pepsin plus trypsin (T_3 = 3 h + 15 min) (E/S = 1/51 for each enzyme) using plasmas raised against legumin (**A**), vicilin (**B**), lectins (**C**), and albumin PA_2 (**D**). **200**, flour ground at 200 μm and not heated; **500**, flour ground at 500 μm and not heated; **500** + θ °**C**, flour ground at 500 μm and heated at 85 °C for 3 min.

cally cross-reacting with vicilin antibodies, was also detected (**Figure 2B**). Polypeptides of vicilin were slightly affected by pepsin hydrolysis whatever the time of hydrolysis and technological treatment applied to the flour. Convicilin susceptibility to pepsin hydrolysis was enhanced by the thermal treatment (85 °C, 3 min). At T_1, only 15 min after addition of the pepsin, the convicilin peptide of 70 kDa was completely degraded in the heated flour and only partly in the unheated one. A new polypeptide appeared at 55 kDa (•1), probably corresponding to a pepsin-cleaved convicilin polypeptide because its molecular mass was higher than those of vicilin subunits. At T_3 (15 min after addition of trypsin), all previously detected protein bands disappeared and a new large band of 19 kDa (•2) was detected with a very weak intensity. This band persisted until the end of hydrolysis.

Lectin (β polypeptides) was detected by immunoblot at 17 kDa with the same intensity for all hydrolysis times (**Figure 2C**). Therefore, lectin was completely resistant to the hydrolysis, and the technological treatments did not modify this behavior.

At T_0, the PA2 antibodies detected PA2 (26 kDa) in all of the different samples but with quantitative variations (**Figure 2D**). PA2 was less extractible by the SDS buffer from the 500 μm flour than from the 200 μm flour and less from the 85 °C heated flour than from the nonheated flour. At T_1, the susceptibility to pepsin hydrolysis increased with the decrease of particle size. The detection of PA2 by immunoblotting, only in 500 μm, showed that this technique is more sensitive than the Coomassie blue staining. After the thermal treatment, PA2 became resistant to pepsin but was rapidly hydrolyzed by trypsin.

The origin of the remaining polypeptides after pepsin plus trypsin hydrolyses was tentatively determined by MS/MS spectrometry analyses. Proteins corresponding to the bands denoted by an asterisk (*) on the Coomassie-stained PAGE (**Figure 1**) were identified. Their sequences are reported on **Table 4**. These analyses allowed the identification of all remaining polypeptides and their localization in the protein sequence. We found lectin as previously detected by antibodies, in the band denoted 3. Bands denoted 1−5 were attributed to vicilin. Bands 1, 5, and 4 were peptides from the COOH-terminal part of the vicilin A and convicilin, respectively. Bands 2 and 3 were peptides from the NH_2-terminal part of the vicilin A or vicilin C. The fragment of 19 kDa (α) (*2) was totally resistant to pepsin and trypsin hydrolysis and should correspond to an accumulation of this peptide due to the hydrolysis of the 50 kDa polypeptide.

DISCUSSION

The technological treatments applied on pea seeds were shown to affect the pea protein accessibility to solvents and enzymes and protein structure. Protein extraction at pH 2 was affected by particle size probably due to an increase in the relative surface of the ground material. Moreover, depending on particle size obtained, grinding could also, more or less, destroy the physical barrier constituted by the cell walls, inducing the release of cellular contents (*22*). The one-step grinding process (hammer mill) led to a higher dispersion of particle size and higher PE than the two-step grinding (roller mill plus hammer mill). The first explanation may be that the use of a roller mill rather than a hammer mill, for the first grinding step, decreased the amount of smaller particles. Second, because narrowing the distribution of particles was more difficult to obtain for the lowest mean size (200 μm), the coarsest particles passed up to six times through the grinding screen of the hammer mill. Therefore, the second step (complementary hammer mill grinding) may have induced significant additional heating of the product as shown by Kaysi and Melcion (*23*). Accordingly, pea protein extraction at pH 2 appeared to be very

Table 4. Results of Protein Identification Obtained from the LC-MS/MS Analyses of the Digestion Products of Five Bands

no. of band on SDS-PAGE	approx molecular mass (kDa)	accession no.[a]	sequences of identified peptides [b]	localization in protein
1	25	(sp | P02855 | VCLA_PEA)	EGSLLLPHYNSR	extremity C-terminal
			GDFELVGQR	
			LTPGDVFVIPAGHPVAVR	
			ASSNLNLLGFGINAENNQR	
			DLTFPGSAQEVDR	
2	19	(sp | P13918 | VCLC_PEA)	SDPQNPFIFK	extremity N-terminal
			FQTLFENENGHIR	
			IFENLQNYR	
			SKPHTIFLPQHTDADYILVVLSGKA	
			AILTVLKPDDR	
			NSFNLER	
			NILEASFNTDYEEIEK	
3	17	(sp | P02867 | Lec Pea)	TETTSFLITK	on all sequence
			FSPDQKNLIFQGDGYTTK	
			TTQTVAVEFDTFYNAAWDPSNR	
		(sp | P13918 | VCLC_PEA)	NSFNLER	extremity N-terminal
			LPAGTIAYLVNR	
			NILEASFNTDYEEIEK	
4	15	(CAB82855.1) CVC PEA	ELTFPGSVQEINR	extremity C-terminal
5	14	(sp | P02855 | VCLA_PEA)	LTPGDVFVIPAGHPVAVR	extremity C-terminal
			DLTFPGSAQEVDR	

[a] The accession number code refers to the Swissprot database. [b] Characteristics of best matching polypeptides from database.

sensitive to temperature as shown by the decrease in protein extraction from the heated flours, whatever the mean particle size. The thermal denaturation can involve a reassociation of only partially unfolded molecules with formation of new complexes (*24*).

The susceptibility to enzymatic digestion, measured by DH, was decreased only for the coarsest particle size. Our in vitro data are in agreement with the in vivo observations (*16*), suggesting that a decrease in particle size distribution induces an increase in digestibility. A reduced accessibility of proteins is the major factor that may decrease pea protein digestibility. The intact cell walls could be important barriers to protein enzymes sites for added enzymes (*25*). When the particle size increased from 200 to 500 μm, the DH values were not significantly different; instead, PE values decreased. Consequently, this would mean that flour proteins may be accessible to enzymes, and the hydrolyzed polypeptides can be released in the supernatant, independently of flour protein ability to be extracted in acidic conditions. These results emphasize the importance of the in vitro technique used to predict protein availability for animal nutrition or to evaluate the technological treatments to improve it. Due to a number of possible component interactions, the control of protein extractibility and accessibility is a challenging problem. The classical protein solubility measurement (PE) discriminated the different processes used in this study. However, determination of the degree of hydrolysis in vitro (DH), reflecting the accessibility to both the solvents and the enzymes, seemed to be better adapted. We also observed that for lower mean particle size (200 and 500 μm), the DH values were rather high, within the range of 22−25%, the average size of the resulting peptides was about four to five amino acids. Whether these peptides could be quickly hydrolyzed in the gastrointestinal tract by carboxypeptidases and aminopeptidases in vivo should be investigated further. In any case, the lower DH value obtained for the coarser flour (1000 μm) clearly indicates that the use of such flour should be avoided.

Identification by antibodies of the four major pea proteins (legumin, vicilin, lectin, and albumin PA2) or their remaining nonhydrolyzed peptides revealed differences between the technological treatments (nonheated 200 μm, nonheated 500 μm, and 85 °C heated), but only after pepsin hydrolysis. Pepsin hydrolysis takes place at pH 2 when the 11S legumin is dissociated into unfolded 3S monomeric form (*26*). Under these conditions, legumin is extracted and hydrolyzed by pepsin. In contrast, the 7S globulins are more stable at acidic pH (*26*). Keeping their quaternary structure, these globular proteins were less extractible when the particle size increased and were not hydrolyzed by pepsin. This result and those obtained on PA2 for the nonheated flour were in agreement with previous in vitro studies (*10*, *12*). The PA2 was sensitive to pepsin hydrolysis, but it became resistant to pepsin after the thermal treatment. In fact, it was the SDS protein extractability that was decreased at T_0 for the heated flour. This albumin is constituted of two subunits of 26 kDa each. Each subunit has three cysteine residues; one is free, and the others are involved in a disulfide bond (*27*). The thermal treatment could induce the access to the two free cysteines and the formation of a new disulfide bond. The S−S formation induces the cross-linking of the protein and decreases its extractability even with SDS, which disrupts the hydrophobic interactions only. Whereas in vitro and in vivo studies had shown PA2 to be sensitive to pepsin and resistant to trypsin (*10*, *15*, *27*), we clearly show that although PA2 became resistant to pepsin hydrolysis after heating, it was rapidly hydrolyzed by trypsin. The PA2 compact structure, rich in β sheet, could have been modified by the thermal treatment, inducing the susceptibility to trypsin attack. The lectin was resistant to hydrolysis by pepsin and trypsin, as shown earlier by many authors (*10*, *28*). Its resistance was demonstrated in vivo (*29*) and could be due to a very compact structure rich in β-sheets (*30*). The technological treatments applied in this study had no effect on lectin resistance to the enzymatic hydrolysis. After pepsin and trypsin hydrolysis, whereas only one remaining peptide having a molecular mass of 19 kDa was identified by immunoblotting, five peptides ranging from 15 to 25 kDa could be identified by MS/MS spectrometry. Thus, although likely belonging to vicilin and convicilin, those remaining peptides could not be recognized by antibodies that were produced

against native protein and not against hydrolyzed peptides. Immunoblotting is more adapted to identify nonhydrolyzed native protein than to detect all of the resistant peptides. The MS/MS spectrometry allowed the identification of five peptides of different molecular masses, ranging from 15 to 25 kDa, as N-terminal or C-terminal peptides of hydrolyzed vicilin. The 7S vicilin is made of different combinations of heterogeneous subunits of ~50 kDa. The corresponding polypeptides of ~50 kDa can be post-translationally modified at one or at both of the two potential cleavage sites (the α:β site and/or the β:γ site). The resulting fragments are as follows: 33 kDa (αβ), 30 kDa (βγ), 19 kDa (α), 13.5 kDa (β), and 16 or 12.5 kDa (γ) (*31*). The C-terminal part of vicilin subunits is resistant to trypsin hydrolysis only when it is not, initially, post-translationally cleaved (vicilin A, 50 kDa). The fragment α (N-terminal part of vicilin) is totally (19 kDa) or partially (17 kDa) resistant whatever its origin (αβ or α). These results are in agreement with in vitro trypsin hydrolysis (*10*) or in vivo studies. Indeed, some 7S proteins are known to be resistant to stomach and intestinal digestion (*32*), and these proteins have been shown to present immunogenic properties in the young animal (*33*). We suppose that the structure of these polypeptides prevents the accessibility to enzymes as was shown for phaseolin (*34*, *35*). Indeed, phaseolin, constituted by three 50 kDa subunits without post-translational cleavage and glycosylation, was shown to be highly resistant to trypsin (*31*).

In conclusion, the results of the present study showed that the grinding parameters and the thermal treatments had an effect only on protein extractability, which is lower for the coarsest grinding and when the moderate thermal treatment is applied. The impact of technological treatments must be evaluated by an in vitro hydrolysis test and not only by a protein solubility test. The thermal treatment induced a decrease in pea protein susceptibility to pepsin hydrolysis, but seemed to enhance trypsin hydrolysis. After pepsin and trypsin hydrolysis, peptides of vicilin and lectin were identified, regardless of technological treatment. In vivo studies are now necessary to confirm (I) if the observed changes induced in proteins by thermal treatments will affect digestibility and (II) if a median particle size of 500 μm, as generally obtained under industrial grinding conditions, is low enough to ensure a good bioavailability of pea amino acids.

ABBREVIATIONS USED

DH, hydrolysis degree; PE, protein extraction; ANF, antinutritional factors; DM, dry matter; BCA, bicinchoninic acid; TCA, trichloroacetic acid; PBS, phosphate-buffered saline; FA, formic acid.

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