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Characterization of pea (*Pisum sativum*) seed protein fractions

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Abstract

BACKGROUND: Legume seed proteins have to be chemically characterized in order to properly link their nutritional effects with their chemical structure.

RESULTS: Vicilin and albumin fractions devoid of cross-contamination, as assessed by mass peptide fingerprinting analysis, were obtained from defatted pea (*Pisum sativum* cv. Bilbo) meal. The extracted protein fractions contained 56.7–67.7 g non-starch polysaccharides kg^{-1}. The vicilin fraction was higher than legumins in arginine, isoleucine, leucine, phenylalanine and lysine. The most abundant amino acids in the albumin fraction were aspartic acid, glutamic acid, lysine and arginine, and the amounts of methionine were more than double than those in legumins and vicilins. The pea albumin fraction showed a clear enrichment of protease inhibitory activity when compared with the seed meal. *In vitro* digestibility values for pea proteins were 0.63 ± 0.04, 0.88 ± 0.04 and 0.41 ± 0.23 for legumins, vicilins and albumins respectively.

CONCLUSION: Vicilin and albumin fractions devoid of cross-contamination with other proteins were obtained from pea seed meal. The vicilin fraction also contained low amounts of soluble non-starch polysaccharides and was enriched in isoleucine, leucine, phenylalanine and lysine. *In vitro* digestibility values for pea proteins were similar or even numerically higher than those for control proteins.

Keywords: albumins; legumins; *Pisum sativum*; protease inhibitors; protein digestibility; vicilins

INTRODUCTION

It is at present acknowledged that food proteins are not only a source of constructive and energetic compounds such as amino acids (AA) but may also play a bioactive role and/or can be precursors of biologically active peptides with various physiological functions. The best-known examples of this are probably casein-derived peptides, which have been proved to possess immunomodulating, antihypertensive, antithrombotic and opioid activities.[1] In this context, plant proteins and their derived protein hydrolysates are increasingly being used as an alternative to proteins from animal sources in human nutrition.[2] Among plants, legume seeds such as soybean (*Glycine max*), beans (*Phaseolus* spp.), peas (*Pisum sativum*), lupins (*Lupinus* spp.) and lentils (*Lens culinaris*) represent rich sources of proteins, carbohydrates, several water-soluble vitamins and minerals.[3] The dietary importance of legume seeds is expected to grow in the coming years owing to the protein (and other nutrients) demand of the increasing world population and to the need to reduce the risks related to consumption of animal food sources, especially in developed countries.[4] Moreover, there is at present great interest in the use of home-grown protein sources such as legume seeds because of the high market price of soybean meal, which is entirely imported from non-European markets. Moreover, the forbidden use of genetically modified organism (GMO) feed ingredients and solvent-extracted oil seeds in organic farming increases the interest in alternative protein sources.[5] In this context, field pea constitutes a significant sector of agricultural grain production, as approximately 25 million hectares are grown annually worldwide.[6]

It is generally recognized that grain legumes contribute effectively to a balanced diet and can prevent non-communicable diseases, including type II diabetes and cardiovascular diseases.[7] However, which chemical components are responsible for the observed protective effects, their mechanism(s) of action and even the nutritional properties of seed constituents (proteins, carbohydrates, ether extract, fiber) *in vivo* are still unclear. Proteins in legume seeds represent from about 200 g kg^{-1} (dry weight) in pea and beans up to 380–400 g kg^{-1} in soybean and lupin. Traditionally, the classification of legume proteins is based on their solubility properties: albumins are soluble in water, globulins are soluble in salt water solutions and prolamins are soluble in ethanol/water solutions.[8] Most of them are storage proteins, with the most abundant in grain legumes being globulins. These are generally classified as 7S and 11S globulins according to their sedimentation coefficients (*S*). The 7S and 11S globulins of pea are named vicilin and legumin respectively, so that the corresponding proteins of

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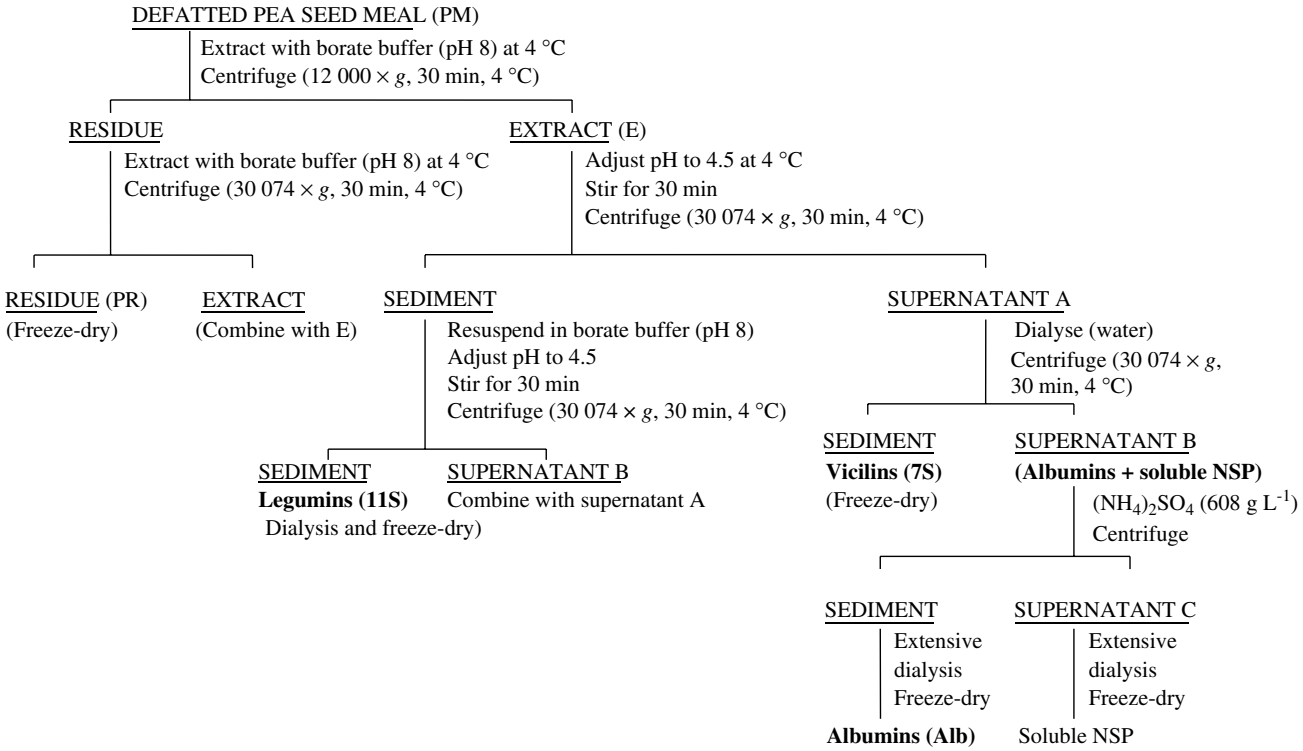

Figure 1. Procedure for extraction of pea (*Pisum sativum* cv. Bilbo) seed meal protein fractions.

other seeds are often indicated as vicilin- and legumin-like globulins.[9] Both albumins and globulins have been claimed to induce a number of health beneficial effects (anticarcinogenic, antihypertensive, hypoglycemic, hypocholesterolemic, etc.) upon dietary consumption.[4] Efforts towards large-scale procedures for protein fractionation from pea[10] or chickpea[11,12] seeds have been mainly focused on globulins and albumins. Albumin separation from globulins has not always been successfully achieved owing to the intermediate solubility behaviors of some proteins.[13] Several physicochemical methods have been developed for the fractionation of 7S and 11S proteins, including ultracentrifugation, fractionation and reverse phase high-performance liquid chromatography (HPLC); however, the yields and purities of isolated fractions were usually low, with cross-contamination among fractions being a main drawback.[11,14] When the nutritional or functional properties of purified legume globulins have been evaluated, complex mixtures of 11S and 7S proteins have generally been used, and only a few nutritional studies with protein fractions have been reported so far. Given that 7S and 11S protein fractions differ in both protein composition and potential nutritional applications, it is clear that the establishment of a reliable protocol for fractionation of sufficient amounts of these proteins would open up novel opportunities to investigate the use of these fractions in preventive and/or therapeutic medicine.

In order to be tested *in vivo* and for their nutritional or physiological effects to be properly addressed, proteins from legume seeds have to be (1) extracted in sufficient amounts for their inclusion in diets for experimental animals and (2) chemically characterized so that their putative effects *in vivo* can be related to their chemical structure. Accordingly, pea (*P. sativum* cv. Bilbo) seed meal was subjected to a chemical procedure to isolate and characterize its constituent albumins and globulins before these fractions are eventually utilized in *in vivo* trials.

MATERIALS AND METHODS

Materials

Trypsin (type III) and α-chymotrypsin (type VII) from bovine pancreas, *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) and *N*-benzoyl-L-tyrosine ethyl ester (BTEE) were obtained from Sigma-Aldrich (Alcobendas, Spain).

Fractionation procedures

Peas (*P. sativum* cv. Bilbo) were a gift from Limagrain UK Ltd (Rothwell, UK). Seeds were ground in a Retsch hammer mill (Biometa SA, Llanera, Spain) fitted with a 1 mm mesh screen. The meal was treated twice with chloroform/methanol (2:1 v/v) for lipid extraction and then air dried. The protein extraction procedure (Fig. 1) was as in Rubio *et al.*[15] with some modifications. Defatted meal was extracted (1:10 w/v) with 0.2 mol L^{-1} borate buffer (pH 8) containing 0.5 mol L^{-1} NaCl and centrifuged (30 074 × *g*, 30 min, 4 °C). The supernatant was retained (supernatant A) and the borate-insoluble sediment was re-extracted as above and centrifuged. The new sediment (pea residue, PR) was recovered by freeze-drying. The extract (supernatant B) was combined with supernatant A, adjusted to pH 4.5 with glacial acetic acid in the cold, stirred for 30 min and centrifuged (30 074 × *g*, 30 min, 4 °C). The sediment was redissolved in borate buffer, dialysed extensively against distilled water and freeze-dried (legumins 11S). The supernatant was also dialysed extensively against distilled water and centrifuged (30 074 × *g*, 30 min, 4 °C). The new sediment was freeze-dried (vicilins 7S). The supernatant was treated with 608 g L^{-1} $(NH_4)_2SO_4$, stirred for 2 h in the cold and centrifuged (30 074 × *g*, 30 min, 4 °C). The sediment (albumins, Alb) was dialysed extensively against distilled water and freeze-dried. The supernatant containing mainly soluble non-starch polysaccharides (NSP) was also dialysed extensively against distilled water and freeze-dried.

Chemical analysis

All analyses were performed in duplicate except for carbohydrates, which were run in triplicate. Nitrogen content was determined according to the Dumas procedure using a Truspec CN analyser (LECO Corporation, St Joseph, MI, USA). AA were determined, after protein hydrolysis in 6 mol L^{-1} HCl plus 10 g L^{-1} phenol in sealed tubes at 110 °C for 24 h, by HPLC according to the Waters Pico Tag method,[16] using pre-column derivatization with phenylisothiocyanate and a Waters 2695 separation module (Waters Cromatografía SA, Madrid, Spain). A Millenium 32 chromatography manager system (Waters Cromatografía) was used for gradient control and data processing. Cysteine and methionine were determined as cysteic acid and methionine sulfone respectively, obtained by oxidation with performic acid before 6 mol L^{-1} HCl hydrolysis.[17] Tryptophan was not analysed. Starch (amyloglucosidase/α-amylase method, AOAC 996.11) was determined using a Megazyme K-TSTA analysis kit (Bray, Ireland). NSP analyses in feedstuffs and freeze-dried biological samples were carried out by gas/liquid chromatography.[18] Concentrations of individual derivatized sugars were determined in a Hewlett-Packard (Avondale, PA, USA) gas chromatograph equipped with a flame ionization detector and fitted with a 30 m × 0.25 mm × 0.2 µm Supelco SP 2380 capillary column (Bellefonte, PA, USA).[19]

Protease inhibitory activity

Pea seed meal and the albumin fraction were assessed for trypsin inhibitory activity (TIA) and chymotrypsin inhibitory activity (CIA). Finely ground samples (50 mg) were extracted with 1.5 mL of 50 mmol L^{-1} HCl at 4 °C for 2 h and centrifuged at 12 000 × *g* for 10 min at room temperature. TIA was measured by means of a modified small-scale quantitative assay with BAPNA as specific substrate, using 50 mmol L^{-1} Tris (pH 7.5) instead of 0.01 mol L^{-1} NaOH. One trypsin inhibitor unit (TIU) was defined as that which gives a reduction in absorbance at 410 nm of 0.01, relative to trypsin control reactions, in a defined assay volume of 10 mL.[20] CIA was measured using BTEE as specific substrate. One chymotrypsin inhibitor unit (CIU) was defined as that which gives a reduction in absorbance at 256 nm of 0.01, relative to chymotrypsin control reactions, in a defined assay volume of 10 mL.[21]

In vitro protein digestibility (IVPD)

Pea seed proteins (legumins, vicilins and albumins) were digested *in vitro* as described previously[22] by following the procedure described in Glahn *et al*.[23] with some modifications. To verify the suitability of the method, casein and lactalbumin were included as control proteins in the assay. All analyses were performed in quadruplicate. Purified or control proteins (30 mg) were suspended in 24 mL of 120 mmol L^{-1} NaCl. The pH was then adjusted to 10 with 0.1 mol L^{-1} NaOH, and samples were allowed to stand at room temperature for 15 min. Aliquots (50 µL) were taken from each tube to determine protein at time 0. The pH was then adjusted to 2 with 5 mol L^{-1} HCl and the volume was made up to 30 mL with 120 mmol L^{-1} NaCl solution. For the gastric digestion step, 0.3 mL of pepsin solution (5 mg in 2.5 mL of 0.1 mol L^{-1} HCl) was added to each sample, and tubes were placed in a shaker (100 oscillations min^{-1}) at 37 °C for 1 h. For the intestinal digestion step, the pH was raised to 6 with 1 mol L^{-1} $NaHCO_3$, and 7.5 mL of pancreatin/bile salt mixture (2.5 mg of pancreatin and 15 mg of bile extract in 25 mL of 100 mmol L^{-1} $NaHCO_3$) was added. The pH was adjusted to 7.5 with 1 mol L^{-1} NaOH and the volume was made up to 45 mL with 120 mmol L^{-1} NaCl. Intestinal digestion of proteins was carried out at 37 °C for 2 h. Controls containing only the digestive enzymes in buffered solution were included in the assay. After protein digestion, enzymes were inactivated by heating at 85 °C for 5 min in a water bath. Tubes were allowed to stand for 5 min on ice and centrifuged at 12 000 × *g* for 10 min at 4 °C. Aliquots (5 mL) of the supernatant were concentrated in a CentriVap concentrator (Labconco Corporation, Kansas City, MO, USA), and total N was determined according to the Dumas procedure using a Truspec CN analyser (LECO Corporation). Digested protein was considered as that soluble in 100 g L^{-1} trichloroacetic acid (TCA). IVPD was calculated as follows: N (mg) soluble in 100 g L^{-1} TCA after digestion/total amount (mg) of N initially added.

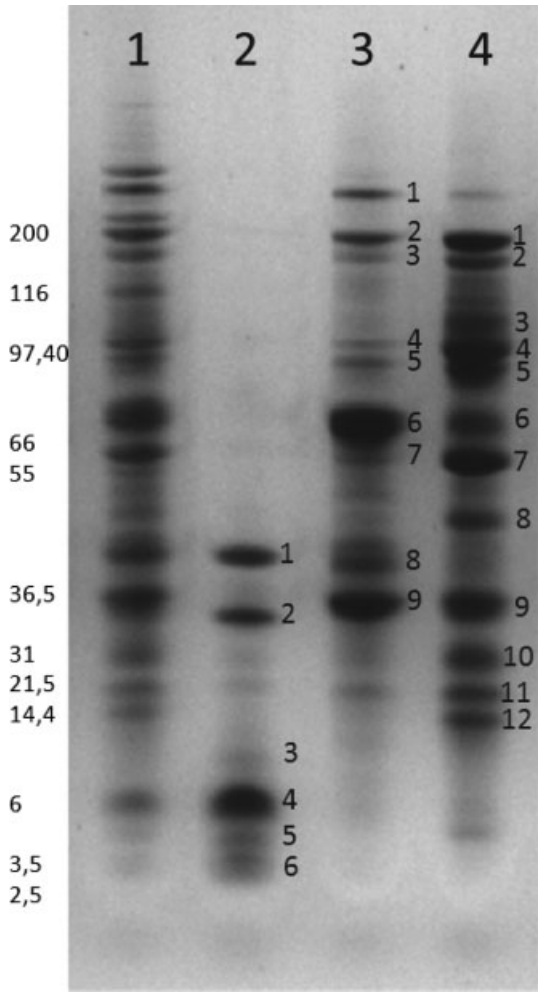

Figure 2. SDS-PAGE of extracted pea (*Pisum sativum* cv. Bilbo) protein fractions: lane 1, defatted pea seed meal; lane 2, albumin fraction; lane 3, legumin (11S) fraction; lane 4, vicilin (7S) fraction. For band identification and relative amounts, see Table 1.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

SDS-PAGE analysis was carried out to monitor the electrophoretic pattern of the different protein extracts. Samples containing 1 mg protein mL^{-1} (32.5 µL) were mixed with 5 µL of 0.5 mol L^{-1} dithiothreitol (DTT) and 12.5 µL of NuPAGE® LDS sample buffer (4×) (Invitrogen, Barcelona, Spain). Aliquots of 20 µL were loaded onto the gel. Separation was performed on 4–12% linear gradient polyacrylamide NuPAGE® Novex Bis-Tris precast gels using a

Table 1. Putative identification by mass peptide fingerprinting and amount (%) of different proteins in each protein lane (lane 2, albumins; lane 3, legumins 11S; lane 4, vicilins 7S) extracted from defatted pea seed meal

Electrophoretic band	% of protein in lane[a]	Homologous protein	Mascot score[b]	Protein sequence coverage (%)	UniProtKB/Swiss-Prot Accession No.
2.1	17.4	Pea albumin 2 (PA2)	214	67	P08688
2.2	17.3	Pea albumin 2 (PA2)	89	42	P08688
2.3	12.6	Bowman–Birk inhibitors[c]			
2.4	24.5	Pea albumin 2 (PA2)	58	26	P08688
2.5	13.4	Defensins 1 and 2	57 and 54	80 and 72	P81929
2.6	14.8	Pea albumin 2 (PA2)	47	8	P08688
3.1	4.3	Convicilin	28	18	P13915
3.2	5.3	Convicilin	113	25	P13915
3.3	4.2	Convicilin	131	32	P13915
3.4	3.9	Vicilin	123	30	P13918
3.5	6.6	Provicilin	38	28	P02855
3.6	24.9	Legumin	119	26	P15838
3.7	6.9	Legumin	44	16	P15838
3.8	17.7	Pea albumin 2	111	51	P08688
3.9	26.2	Legumin	88	28	P15838
4.1	7.0	Convicilin	165	29	P13915
4.2	5.1	Convicilin	225	39	P13915
4.3	5.7	Convicilin	201	37	P13915
4.4	7.4	Vicilin	291	60	P13918
4.5	7.7	Vicilin	119	31	P13918
4.6	8.7	Mixture of vicilin and convicilin	68 and 62	25 and 22	P13918, P13915
4.7	12.5	Provicilin	130	34	P02854
4.8	7.2	Vicilin	122	33	P13918
4.9	14.2	Vicilin	82	24	P13918
4.10	10	Provicilin	70	23	P02854
4.11	7.6	Provicilin	78	21	P02854
4.12	6.9	Provicilin	90	25	P02854

[a] The relative polypeptide composition of each fraction was analysed using Quantity One software (Bio-Rad).
[b] Protein scores greater than 57 were significant ($P < 0.05$).
[c] The in-gel digestion of this electrophoretic band showed peptides with molecular masses of 1693.6920 and 2256.8777 Da, which matched those derived from *in silico* trypsin digestion of TI1, a major pea isoinhibitor.

continuous buffer system (NuPAGE® MES SDS running buffer, Invitrogen) for 50 min at a constant voltage of 200 V. Unstained protein molecular markers (Mark 12™, Invitrogen) were applied to the gel. Electrophoretic bands were stained with a Coomassie Blue staining kit (Invitrogen), and the electrophoretic pattern was analysed using Quantity One software (Bio-Rad, Madrid, Spain).

Protein identification by mass peptide fingerprinting

After staining, visualized electrophoretic bands with proteins of interest were excised using an EXQuest spot cutter (Bio-Rad) and digested using a DigestPro MS (Intavis AG, Valencia, Spain). Proteins were reduced with 10 mmol L^{-1} DTT (Sigma-Aldrich) for 45 min at 56 °C, alkylated with 55 mmol L^{-1} iodoacetamide (Sigma-Aldrich) for 30 min in the dark at laboratory temperature and digested with 15 µL of 10 ng μL^{-1} trypsin (Promega, Alcobendas, Spain) for 4 h at 37 °C. Peptides were extracted from the gel with 40 µL of 2 g L^{-1} trifluoroacetic acid (TFA), 20 µL of 50 mmol L^{-1} ammonium bicarbonate and 20 µL of acetonitrile and concentrated under vacuum centrifugation to a final volume of 10–15 µL. Samples were passed through a ZipTip µ-C18 column (Millipore, Madrid, Spain) using a DigestPro MS (Intavis AG). The ZipTip was previously treated with acetonitrile and 2 g L^{-1} TFA, and peptides were eluted with 600 mL L^{-1} acetonitrile/2 g L^{-1} TFA. Samples were crystallized in the analysis plaques with a CHCA matrix (LaserBio Labs, Sophia-Antipolis, France). Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) analysis of the tryptic digests was carried out with a Voyager DE-PRO mass spectrometer (Applied Biosystems, Alcobendas, Spain). Spectra were calibrated internally using the peptidic molecular weights of the trypsin digestion or with external standards. The identification of proteins from the peptidic molecular weights was carried out through the NCBI and Swiss-Prot databases using the Mascot search engine (Matrix Science, London, UK).

RESULTS AND DISCUSSION

Fractionation procedures

The rationale behind the isolation of the different fractions by this procedure was the differences in solubility (water for albumins and salt solutions for globulins) and in isoelectric points between legumins and vicilins.[24] About 70.7 g kg^{-1} was extracted as globulin material from defatted pea (*P. sativum* cv. Bilbo) meal, of which 40.6 g kg^{-1} corresponded to 7S vicilins and 30.1 g kg^{-1} to 11S legumins. In previous studies,[15,25] where we used a similar protein extraction procedure on a small scale, the yield was higher (about 160 g kg^{-1} as globulin material from lupin and faba

bean seed meals). In the present work we have used a large-scale extraction procedure starting with 9 kg of defatted seed meal. Crévieu *et al*.,[26] using a different large-scale procedure, obtained about 1000 g of globulin fraction from 15 kg of pea flour, which represents 66.7 g kg^{-1}, a value closer to that obtained here. For the albumin fraction we obtained 21.3 g kg^{-1}, while Crévieu *et al*.[26] reported a yield of 25.3 g kg^{-1} flour. Protein content in different varieties is also known to be variable, and a positive relationship between protein content and legumin/vicilin ratio in pea seeds has recently been reported.[27]

Electrophoretic profiling of the three major protein fractions (11S, 7S and albumins) (Fig. 2) and putative identification of their major components by mass peptide fingerprinting (Table 1) were carried out. Data analysis demonstrated a lack of cross-contamination in the vicilin and albumin fractions, but not in the legumin fractionm, where vicilins and albumins were also detected. As expected, the 7S fraction showed major electrophoretic bands corresponding to vicilin and convicilin polypeptides, the latter being of higher relative mobility (Mr) ($\geq$70 kDa). Polypeptides of Mr lower than 50 kDa have been reported to be derived from vicilin precursors by limited post-translational processing.[28] In the albumin fraction the major identified proteins were pea albumin 2 (PA2), defensins 1 and 2 and Bowman–Birk inhibitors (BBI). In the case of BBI, the search for peptide mass data for protein identification against databases was unsuccessful. This could be due to the presence of several BBI isoforms with sequence variation, which can make putative identification more difficult.[29] Further attempts to identify pea BBI isoforms were carried out. After trypsin digestion, electrophoretic band 3 of the albumin fraction (Fig. 2) showed peptides with molecular masses of 1693.6920 and 2256.8777 Da, which matched those derived from *in silico* trypsin digestion of TI1 (accession number Q9M3X3P from Uni-Prot KB/TrEMBL), a major pea isoinhibitor described previously; an additional peptide mass, 3805 Da, could be associated with TI2 (accession number Q41066), another major pea isoinhibitor.[21] The 11S fraction consisted of a heterogeneous mixture of relatively impure polypeptides with the presence of vicilin, convicilin and PA2, representing 104, 95 and 170 g kg^{-1} total protein respectively. A major electrophoretic band corresponding to acidic polypeptides of legumins ($\sim$40 kDa; 26.2% of total protein) was detected.

Chemical analysis

Despite the present interest in pea seed as a protein source, particularly in animal nutrition, not much information can be found in the literature concerning its carbohydrate composition. In addition, where found, information from animal studies usually refers to neutral and acid detergent fibre analysis.[30,31] Total free sugars, including oligosaccharides, were not analysed here, because they are soluble in water and lost through dialysis and therefore not likely to be found in the protein fractions, which are the main interest of the designed procedure. The values determined here (Table 2) for NSP content and composition of pea seed meal were similar to those reported previously.[32–34] Uronic acids, glucose (mainly as cellulose) and arabinose were the most abundant constituent sugars. The extracted protein fractions (legumins, vicilins and albumins) contained between 56.7 and 67.7 g NSP kg^{-1}, probably deriving mostly from the soluble NSP fraction, which constitutes about 52 g kg^{-1} in pea seed meal,[32] and also from the carbohydrate fraction of the proteins themselves. A small amount of starch (0.3–35.7 g kg^{-1}) was also determined in the protein fractions. The carbohydrate fraction therefore amounted to between 68.0 and 98.3 g kg^{-1} extracted proteins, with vicilins containing the lowest amount.

The total protein content and AA composition of pea seed meal and its fractions are shown in Table 3. Bibliographic information on AA composition is greater than it is on carbohydrates, and it is generally known that a low content of essential AA such as sulfur-containing AA limits the nutritive value of many food proteins of plant origin, such as soybean and other legumes, including pea.[3] In pea meal, lysine, arginine and leucine were the most abundant among the essential AA, while aspartic acid and glutamic acid were the most abundant among the non-essential AA, which is in agreement with a previous report.[35] However, much less information exists on the AA composition of legume seed proteins and protein fractions. The composition of AA in pea proteins ultimately depends on the proportion of the three major soluble protein fractions present in pea seeds: legumins, vicilins and albumins. According to Owusu-Ansah and McCurdy (1991), as cited by Urbaityte *et al*.,[36] legumin contains more sulfur-containing AA and arginine, whereas vicilin is enriched in isoleucine, leucine, phenylalanine and lysine. This is relevant, as these are essential AA. Also, in another of the few reports on this issue, Croy *et al*.[37] reported that the most abundant AA in vicilins are glutamate, aspartate, leucine and lysine. These previous reports are in line with values obtained here. In the case of the AA composition of pea albumins, there are even fewer reports. Croy *et al*.[38] reported that total albumin proteins obtained by extraction with sodium acetate buffer (pH 5) and chromatography through Sephadex G-150 and DE-cellulose columns were high in aspartic acid, glutamic acid, glycine, alanine, valine and lysine. In the present study the most abundant AA in the albumin fraction were aspartic acid, glutamic acid, lysine and arginine, and the amounts of methionine were more than double than those in legumins and vicilins.

Protease inhibitor activity

The cv. Bilbo showed relatively low TIA and CIA, with 1.8 $\pm$ 0.1 TIU and 1.4 $\pm$ 0.2 CIU mg^{-1} dry weight. In a previous study, several pea genotypes were screened for their relative TIA and CIA.[39] Significant differences in both TIU and CIU mg^{-1} seed meal were found among pea genotypes, ranging from 4.1 to 10.7 TIU mg^{-1} and from 5.3 to 11.1 CIU mg^{-1}. The pea albumin fraction showed a clear enrichment of protease inhibitory activity compared with the seed meal, with values of 56.7 $\pm$ 0.5 TIU and 60.9 CIU mg^{-1}. The major components responsible for these increased inhibitory activities are the water-soluble BBI. Emerging evidence suggests that BBI exert their potential chemopreventive and therapeutic properties via protease inhibition.[40] In previous studies a significant concentration- and time-dependent decrease in the growth of an array of colon cancer cells (HT29, Caco2 and LoVo) has been demonstrated *in vitro* following treatment with BBI variants from several legume sources, including pea,[40] lentil[41] and soybean.[42] The cytotoxic effect of BBI isoforms from soybean on HT29 human colorectal cancer cells is related to their intrinsic ability to inhibit serine proteases. In contrast, the growth of non-malignant colonic fibroblastic CCD18-Co cells was unaffected by BBI. Recently, the antiproliferative effect of rTI1B, a major pea isoinhibitor expressed heterologously in *Pichia pastoris*, has been evaluated using colon cancer cells grown *in vitro*.[40] Comparisons of the effects of rTI1B with those observed using a related synthetic mutant derivative showed that the proliferation of HT29 colon cancer cells was inhibited significantly by rTI1B in a dose-dependent manner, whereas the mutant that lacked trypsin

Table 2. Non-starch polysaccharide (NSP) composition and starch content (g kg^{-1} dry matter) of pea (*Pisum sativum* cv. Bilbo) seed meal and its protein fractions

Component	Meal	Defatted meal	Legumins	Vicilins	Albumins	Residue[a]
Rhamnose	4.9	4.8	4.5	4.4	0.0	7.0
Arabinose	17.1	17.8	3.7	11.7	4.4	18.9
Xylose	8.6	10.2	0.0	7.3	3.1	8.7
Mannose	10.2	9.4	9.4	9.2	10.4	9.2
Galactose	10.8	13.1	15.0	9.3	16.0	9.6
Glucose	48.6	69.3	19.4	21.9	19.4	70.6
Uronic acids	46.7	45.3	10.6	4.1	3.5	47.1
Total NSP	146.9	169.9	62.6	67.7	56.7	171.0
Starch	373.2	397.9	35.7	0.3	32.1	602.3

[a] See Fig. 1.

Table 3. Nitrogen (N) content and amino acid composition (g kg^{-1} dry matter) of pea (*Pisum sativum* cv. Bilbo) seed meal and its protein fractions

Component	Meal	Defatted meal	Legumins	Vicilins	Albumins	Residue[a]
Total N	40.0	41.4	138.9	160.8	145.3	14.5
Protein (N × 5.6)[b]	224.0	231.7	778.0	900.0	813.7	81.0
Essential amino acids						
Methionine	5.8	6.0	9.7	6.9	17.9	1.06
Cystine	0.0	0.0	0.1	0.1	0.1	0.01
Lysine	19.1	20.2	50.1	111.3	101.4	6.68
Histidine	8.6	9.6	23.6	32.1	36.1	3.03
Arginine	13.4	16.5	30.2	41.4	84.5	4.17
Tyrosine	8.8	9.5	26.4	35.0	39.0	4.53
Leucine	13.4	15.8	49.1	86.9	34.6	5.64
Isoleucine	7.8	9.5	25.4	41.8	28.3	3.43
Threonine	14.2	16.0	50.4	63.6	44.2	7.24
Valine	8.8	10.4	29.7	39.2	36.4	4.56
Phenylalanine	9.7	10.7	30.4	54.7	30.6	3.79
Non-essential amino acids						
Aspartate	25.8	34.3	87.4	151.4	130.1	8.20
Glutamate	34.1	40.9	126.3	204.7	154.0	9.70
Serine	10.8	12.5	34.7	55.4	44.2	4.16
Glycine	9.8	11.0	27.4	33.3	56.7	4.06
Alanine	8.4	10.2	25.7	31.3	51.3	3.39
Proline	13.5	13.7	39.4	49.2	45.0	8.32

[a] See Fig. 1.
[b] Mossé.[50]

and chymotrypsin inhibitory activity did not show any significant effect on colon cancer cell growth.

In vitro protein digestibility

As shown in Table 4, digestibility values for pea proteins (0.63 ± 0.04, 0.88 ± 0.04 and 0.41 ± 0.23 for legumins, vicilins and albumins respectively) were similar or even numerically higher than those for control proteins (0.48 ± 0.05 and 0.73 ± 0.23 for lactalbumin and casein respectively). This is in agreement with previous *in vitro*[43] and *in vivo*[25] studies. It is generally thought that the presence of antinutritional factors (ANF) together with the slower digestion rate of legume proteins compared with animal proteins could explain the lower nutritional efficiency of legume proteins *in vivo*. Nevertheless, this conclusion is mostly based on experiments in which the whole seed meal was used in the diet. Where purified proteins were tested, only *in vitro* values were usually reported. One of the few reports on *in vivo* digestibility studies with purified proteins is that by Aubry and Boucrot,[44] who showed that, after 2 h of gastric emptying, the intestinal absorption of pea vicilin and legumin in rats was as high as that of casein. However, the nutritional value (measured as protein efficiency ratio and biological value) of diets based on purified legume proteins or even seed meals containing low amounts of or no ANF is below that of control diets, even though both fecal and ileal digestibilities of globulins purified from legume seeds such as soya bean (*G. max*), faba bean (*Vicia faba*) and narrow-leafed lupin (*Lupinus angustifolius*) were not different from control values in the rat.[15,45] Furthermore, ileal and fecal N digestibilities of whole legume seed meals low in ANF are usually similar or close to control values.[25,46,47] These results suggest that undenatured legume globulins are highly digestible in the small intestine, so the lower digestibility of legume proteins when the whole meal is used

Table 4. *In vitro* protein digestibility of lactalbumin, casein and purified pea (*Pisum sativum* cv. Bilbo) seed protein fractions

Protein or seed protein fraction	*In vitro* protein digestibility[a]
Lactalbumin	0.48 ± 0.05
Casein	0.73 ± 0.23
Legumins	0.63 ± 0.04
Vicilins	0.88 ± 0.04
Albumins	0.41 ± 0.23

[a] Values are mean ± standard deviation.

in the diet is likely to be due to other factors such as lectins, tannins and/or trypsin inhibitors. However, feeding growing animals with diets based on legume seed meals as the main source of protein results in performance values that are lower than expected based on the chemical composition of the diets. Previous results[48,49] suggest that AA from legume protein isolates are absorbed at slower rates than those from animal proteins, which might explain the lower nutritional utilization of legume storage proteins as compared with lactalbumin or casein.

CONCLUSIONS

Very little information is at present available in the literature on the chemical characterization of legumes, particularly pea seed fractions. Even less information exists on the composition of constituent protein fractions, particularly vicilins. This is very relevant in order to properly link the nutritional effects of a given component with its chemical properties. By the procedure here described, vicilin and albumin fractions devoid of cross-contamination with other proteins were obtained from pea (*P. sativum* cv. Bilbo) seed meal. The legumin fraction consisted of a heterogeneous mixture of relatively impure polypeptides. The extracted protein fractions (legumins, vicilins and albumins) contained between 56.7 and 67.7 g NSP kg^{-1}, probably deriving mostly from the soluble NSP fraction, with vicilins containing the lowest amount. Legumins contained more sulfur-containing AA and arginine, whereas vicilins were enriched in isoleucine, leucine, phenylalanine and lysine. The pea albumin fraction showed a clear enrichment of protease inhibitory activity when compared with the seed meal. *In vitro* digestibility values for pea proteins were similar to or even numerically higher than those for control proteins.

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