Food Research International 169 (2023) 112825

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Combination of three null mutations affecting seed protein accumulation in pea (*Pisum sativum L.*) impacts positively on digestibility

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ARTICLE INFO

Keywords:
Digestibility
Lectin
Mutants
Pea
Pea albumin 2
Seed proteins
Trypsin inhibitors

ABSTRACT

The presence of so-called anti-nutritional factors can reduce the bioavailability of nutrients following consumption of seeds which are otherwise an excellent source of proteins, carbohydrates and micronutrients. Among the proteins associated with negative effects on quality in pea (*Pisum sativum* L.) seeds are lectin, pea albumin 2 (PA2) and trypsin inhibitors (TI). Here we have investigated the impact of these proteins on protein digestibility and amino acid availability, using naturally occurring and derived mutant lines of pea lacking these proteins. The mutations were stacked to generate a triple mutant which was compared with a wild-type progenitor and a line lacking the major seed trypsin inhibitors alone. In vitro digestions following the INFOGEST protocol revealed significant differences in the degree of hydrolysis, protein profile and apparent amino acid availability among the pea variants. Proteins resistant to digestion were identified by MALDI-TOF mass spectrometry and amino acid profiles of digested samples determined. The results indicate that pea seeds lacking certain proteins can be used in the development of novel foods which have improved protein digestibility, and without negative impact on seed protein concentration or yield.

1. Introduction

The increasing demand on food production to meet the needs of a growing population, coupled to the need to protect the environment, makes it necessary to change the food production system. Legume crops are a great choice as food crops to fulfil many needs, including nurturing the human population with plant-derived foods while also reducing the impact of agriculture on the planet, chiefly through biological nitrogen fixation in roots as a consequence of bacterial symbiosis.

Although much emphasis has been placed on the concentration of protein in seeds from many food crops, it has become clear that quality in terms of digestibility and amino acid availability in human and farm animal diets is of greater importance (FAO, 2013). Besides the amino acids that are indispensable (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine), others (arginine, cysteine, glycine, glutamine, proline, tyrosine) are considered as conditionally indispensable during illness and stress.

The seeds of many pulse crops, including pea, are rich sources of protein (typically 20–30%, depending on genotype and environment) (Robinson & Domoney, 2021). However, seed proteins typically include proteins which have been documented as being poorly digested by farm animals or which interfere directly with digestion through inhibition of digestive enzymes. Where proteins are poorly digested, they can then contribute positively both to the development of sarcopenia and frailty in older age within the human population (Lonnie et al., 2018; Tournadre, Vial, Capel, Soubrier, & Boirie, 2019), and to the eutrophication of lands and water courses as excreted nitrogen (Leip et al., 2015). In pea seeds, three groups of water-soluble proteins or albumins fall into this group: pea albumin 2 (PA2) and lectin, both of which are associated with poor digestion (Crevieu et al., 1997; Le Gall, Quillien, Seve, Gueguen, & Lalles, 2007; Salgado et al., 2003), and the trypsin inhibitors (TI) (Clemente et al., 2005; Rubio et al., 2006). While lectin and TI have been described as anti-nutritional proteins, pea albumin 2, shown to be poorly or hardly digested, can be predicted to contribute little to the value of dietary seed proteins (Vigeolas et al., 2008).

Earlier screens of pea germplasm collections led to the identification

Abbreviations: CIA, chymotrypsin inhibitory activity; DH, degree of hydrolysis; IU, inhibitor units; TI, trypsin inhibitors; TIA, trypsin inhibitory activity; PA2, pea albumin 2; SEC, size exclusion chromatography.

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https://doi.org/10.1016/j.foodres.2023.112825
Received 16 November 2022; Received in revised form 6 March 2023; Accepted 11 April 2023
Available online 14 April 2023

of naturally occurring mutations which abolished the synthesis of pea albumin 2 (Vigeolas et al., 2008) and double-headed trypsin-chymotrypsin inhibitors (Clemente et al., 2015) in seeds of the wild accessions JI1345 and JI0262, respectively. Screening a mutant population of pea, generated by fast neutron mutagenesis using the accession JI2822, led to the identification of a mutant lacking seed lectin (Domoney et al., 2013). Here we have used high-throughput genetic screens to generate lines lacking all three proteins to investigate whether their removal from seeds could lead to an improvement in protein digestibility and, secondly, whether an overall reduction in protein concentration occurs in such mutants.

2. Materials and methods

2.1. Plant material

Three mutant pea lines were used in a crossing programme to generate single, double and triple null mutants lacking PA2, lectin and TI proteins. Briefly, the PA2 null mutant is a natural variant identified in a pea germplasm screen (JI1345, Vigeolas et al., 2008). A lectin A (LecA) null line (FN1063/1_1_1/19) was identified by screening a fast neutron mutagenized population of pea as previously described (Domoney et al., 2013). A null mutant (JI0262) for the two genetically linked major trypsin-chymotrypsin inhibitor genes, *TI1* and *TI2*, was identified by screening diverse germplasm (Clemente et al., 2015). The derivation of single, double and triple mutants is described in full in the Supplementary file of material and methods. The details of all the primers and the assay conditions used for the detection of mutants are provided in Supplementary Table S1.

Seeds from homozygous segregants were multiplied for all combinations, along with control parental lines (cv. Birte, JI2822, cv. Caméor). Seeds from the TI mutation, back-crossed six times into cv. Caméor, were multiplied, alongside a control wild-type segregant. Seeds from the TI mutant, triple mutant and control lines were used to investigate the impact of the mutations on seed protein and digestibility traits.

In order to study the impact that desirable mutations might have on agronomic traits, the mutations were introgressed into a common cultivated genetic background (cv. Prophet), using TaqMan assays to identify mutants and their combinations (Supplementary Table S1). Ten plants of every combination at the backcross (BC) 6 generation were grown alongside wild-type segregants and the parental cultivar under greenhouse conditions. Bulking early-generation triple mutant (non-backcrossed) lines allowed a preliminary assessment under field conditions in comparison to the cv. Caméor. Microplots (1 m^2) were sown with 100 seeds per plot in spring 2018 (F_4) and 2021 (F_6) using standard agronomic practices.

2.2. Total protein determination

Cotyledonary meal samples were prepared by drilling five seeds per sample and using three biological replicates for every genotype with three technical replicates for every sample. Protein was extracted with Tris buffer (50 mM, pH 8) containing NaCl (500 mM) buffer, using 125 μL per mg meal, for 2 h at 4 °C and then centrifuged (16,000*g*, 5 min, 4 °C). Extracts (2 μL of supernatants) were spotted onto Direct Detect™ cards (Millipore Corporation) and read in a Direct Detect™ spectrophotometer against a buffer blank and using the NIST BSA AM2q3 programme. Percentage protein content was calculated for every sample.

2.3. Albumin extraction

The albumin fraction from pea seed meal was extracted following a method previously described (Rubio et al., 2014). Briefly, seeds were ground and 20 mg meal was extracted with 0.2 M borate buffer pH 8 containing 0.5 M NaCl and centrifuged (30,000*g*, 30 min, 4 °C). The supernatant was adjusted to pH 4.5 with glacial acetic acid at 4 °C, stirred for 60 min and centrifuged (30,000*g*, 30 min, 4 °C). The supernatant was dialysed extensively against distilled water and centrifuged (30,000*g*, 30 min, 4 °C), to give a soluble fraction containing the albumins. Trypsin-chymotrypsin inhibitors were isolated from albumin fractions using trypsin-agarose chromatography. Briefly, 2 mg of albumins were loaded on a trypsin-agarose affinity column (T1763 Sigma); TI, if present, were eluted using 10 mM acetic acid.

2.4. Measurement of protease inhibitory activities

Seeds were screened for their relative trypsin (TIA) and chymotrypsin inhibitory activity (CIA), as described previously (Clemente et al., 2005). Finely ground meal from pooled seeds of each pea line was used to measure TIA and CIA with N-α-benzoyl-DL-arginine-p-nitroanilide (BAPNA) and N-α-benzoyl-L-tyrosine-p-nitroanilide (BTpNA) as specific substrates, respectively (Clemente et al., 2005). TIA and CIA, expressed as inhibitor units (IU) per mg of meal, were calculated.

2.5. In vitro protein digestion

The *in vitro* digestion assays followed the harmonized INFOGEST protocol (Brodkorb et al., 2019; Minekus et al., 2014) and, for every pea line tested, was performed at least in triplicate. Enzyme activities and bile concentrations were measured according to the assays described previously (Minekus et al., 2014). The digestion involved oral, gastric and intestinal phases within a final volume of 8 mL. For the oral phase, 1 g of pea meal was mixed with pre-warmed simulated saliva fluid and α-amylase (A1031, Sigma; 96 U/mg) and shaken gently in a water bath at 37 °C for 2 min. For the gastric phase, the oral bolus was mixed with simulated gastric fluid, the pH was adjusted to 3.0, porcine pepsin (P6887, Sigma; 3359U/mg) was added to achieve 40,000 U in the final mixture and this was immediately incubated in a water bath at 37 °C, while shaking gently for 2 h. After gastric digestion, the pH was adjusted to pH 7.0. For the intestinal phase, the mixed simulated intestinal fluid and 4000 U of pancreatin (P7545, Sigma; 5.18 IU trypsin/mg) was added to the mixture together with 160 mM bile solution (B8756, Sigma; 2.40 mmol of bile salts/g). The digests were incubated for 2 h in a water bath at 37 °C while mixing at 80 rpm. In order to stop the intestinal digestion, samples were immediately frozen in liquid nitrogen. Samples were defrosted afterwards on ice and centrifuged at 10,000*g* at 4 °C. Supernatants were used for the following analyses. A blank digestion was performed by using the same concentration of enzymes, where the pea sample was replaced with Milli-Q water.

2.6. Degree of hydrolysis (DH)

All reagents were purchased from Merck/Sigma-Aldrich. The DH of the digested pea samples was evaluated spectrophotometrically at 340 nm by reaction of primary amino groups using the OPA (o-phthaldialdehyde) methodology of Nielsen, Petersen, and Dambmann (2001), with slight modifications. The OPA reagent was prepared by dissolving 160 mg OPA in 4 mL ethanol, adding a borate/SDS solution (7.62 g disodium tetraborate decahydrate and 200 mg Na-dodecyl-sulfate (SDS) in 150 mL deionized water), followed by 176 mg dithiothreitol (DTT) and adjusting the final volume of the solution to 200 mL with distilled water. The free amino group concentrations were determined with reference to a calibration curve using L-serine (12.5–100 mg L^{-1}), which shows a response close to the average response of amino acids in OPA reactions. DH (%) was estimated according to the following equation (Zahir, Fogliano, & Capuano, 2018):

$$DH\,(\%) = [[NH_2(\text{final}) - NH_2(\text{initial})]/[NH_2(\text{acid}) - NH_2(\text{initial})]] \times 100$$

where NH_2 (final) is the concentration of free amino groups in the

digested sample after each phase, NH_2 (initial) is the concentration of free amino groups before digestion, and NH_2 (acid) is the total content of completely hydrolysed sample in 6 N HCl at 110 °C for 24 h. All measurements were carried out at least in triplicate for each digestion.

2.7. SDS-PAGE analysis of protein profiles

Denaturing gel analyses of pea proteins were carried out using either gradient 4–12 % or 12% Bis-Tris pre-cast gels (Invitrogen), according to the manufacturer's instructions, with 2-N-morpholine-ethane sulphonic acid (NuPAGE MES, Invitrogen) or 3-N-morpholino-propanesulfonic acid (NuPAGE MOPS, Invitrogen) as running buffer. Immediately before loading, samples (20 μL) were denatured with 7.5 μL of sample buffer (NuPAGE lithium dodecyl sulphate (LDS), Invitrogen NP0007), reduced with 3 μL DTT (Sigma-Aldrich D9163, 0.5 M), and heated at 90 °C for 10 min. LDS sample buffer is supplied as a 4× concentrate to which protein, water and DTT are added to give 1× LDS buffer. To prevent re-oxidation of reduced proteins during electrophoresis, 0.5 mL of NuPAGE antioxidant (Invitrogen NP0005) was added to the upper buffer chamber. Gels were stained using either Colloidal Blue or InstantBlue. Protein standards used in gel analyses included Mark12TM (LC5677, Invitrogen) with proteins in the range of 2.5–200 kDa or SeeBlue Plus2 pre-stained standards ranging from 3 to 198 kDa (LC5925, Invitrogen).

2.8. Size exclusion chromatography (SEC)

The molecular weights of the soluble proteins after each digestion phase were monitored by SEC. Samples were filtered through a 0.22 μm filter and equal amounts of sample were loaded onto a HiPrep 26/60 Sephacryl S-100 HR column (flow rate of 0.3 mL per min) in 50 mM phosphate buffer containing 150 mM NaCl, pH 8.0. Three replicates per sample were analysed. Fully digested samples (after the intestinal phase) were first filtered through 30 KDa Amicon® filters in order to eliminate the majority of the intestinal enzyme background. The column was calibrated using the following molecular weight standards: bovine serum albumin (63.5 kDa), ovalbumin (48.1 kDa), chymotrypsinogen A (20.4 kDa), ribonuclease A (15.6 kDa), aprotinin (6.5 kDa), and vitamin B12 (1.3 KDa).

2.9. Amino acid analysis

Quantitative amino acid analysis was carried out, using a Biochrom 30 amino acid analyzer based on ion-exchange liquid chromatography and post-column continuous reaction with ninhydrin. The ninhydrin derivatives eluted from columns were monitored at 570 and 440 nm (the latter for proline). The resultant chromatograms gave the identity and amount of the amino acids present in samples. Free amino acid contents in the soluble fraction after digestion were determined, following deproteinization of the samples with TCA precipitation (500 μL of 20% TCA with 2 mM of norleucine as internal standard per 500 μL of the soluble fraction) and centrifugation (2,700g for 1 h at 5 °C). Supernatants were stored at −20 °C until analysis. Free amino acid analysis was carried out once for each digestion phase and sample.

2.10. Peptide mass fingerprinting

For protein identification in the albumin fraction, bands were excised from Colloidal Blue-stained gels and subjected to in-gel trypsin digestion. Peptide fragments from digested proteins were desalted and concentrated using C18 ZipTip columns and then loaded directly onto the matrix-assisted laser desorption/ionisation (MALDI) plate, using α-cyano-4-hydroxycinnamic acid as the matrix for MALDI-mass spectrometry (MS) analysis. MS spectra were obtained automatically in a 4700 Proteomics Analyzer (Applied Biosystems, Cheshire, UK) operating in reflectron mode with delayed extraction. Peptide mass data were used for protein identification against the MS protein sequence database (https://www.matrixscience.com).

Identification of proteins resistant to digestion was carried out by liquid chromatography and mass spectrometry (nanoLC-MS/MS). The analysis was performed on a nanoLC (easy nanoLC II, Proxeon) directly connected to an Ion Trap Mass Spectrometer (Amazon Speed ETD, Bruker) with a CaptiveSpray source. Peptide separation was performed on a C18 column (75 μm × 15 cm, 3 μm, 100A, Ac-claim PepMap100, Thermo Scientific) over a 180 min acetonitrile gradient from 5 to 30% B (solvent A: 0.1% formic acid in water; solvent B: 0.1% formic acid in acetonitrile) with a flow rate of 300 nL/min. The Ion Trap was set to analyse the survey scans in the mass range m/z 250–2500 in Enhanced Resolution MS mode (speed 8100 m/z/sec) and the top 10 most intense ions in each duty cycle selected for MS/MS in UltraScan mode (32,500 m/z/sec). Fragmentation parameters were based on active exclusion after two spectra and active release of 0.4 min, within a scan range of 50 m/z – 2 × precursor. Protein identification was performed using the ProteinScape (Bruker) program and MASCOT (Matrix Science) as search engine. Searches were made in the UniProt Trembl and UniProt SwissProt databases with a filter for *Pisum sativum*. In all cases, cysteine carbamidomethylation was considered as a fixed modification, and methionine oxidation as a variable modification, with one "missed cleavage" for the enzyme.

2.11. Statistical analysis

The data derived from the chemical composition analysis and DH (%) at each phase of digestion (oral, gastric and intestinal) are presented as mean ± standard deviation (SD) of at least three experimental replicates. Statistical significance between samples and at each digestion stage was assessed by repeated-measures analysis of variance (ANOVA) using IBM SPSS statistics 25 link (NY:IBM Corp). Significant differences ($p < 0.05$) between means were determined by Tukey's test.

3. Results

Certain proteins such as lectins and TI in the seeds of pea and other legume crops have been linked with negative impacts on the digestibility of seed proteins in farm animals (Clemente et al., 2015; Vigeolas et al., 2008), while others have structures that are associated with low digestion rates. The use of pea mutants lacking such proteins and their combination provide excellent tools with which to investigate the changes that can be made to seed protein profiles to enhance digestibility-associated amino acid scores. Here we have used an induced mutant line and two natural variants to investigate whether benefits to digestion systems can be demonstrated when the mutations are combined.

Following back-crossing to BC6 in a cultivated genetic background (cv. Prophet), the seed protein profile of the selected mutant and wild-type lines showed very similar patterns apart from the proteins (PA2, lectin and TI in decreasing size order) which are absent from the mutants (Fig. 1A). None of the mutations is associated with a reduction in seed protein concentration, as measured using Direct Detect™ (Fig. 1B). Here the TI and PA2 nulls showed a marginal but significant increase in seed protein concentrations compared with the *LecA* null or the control (wild-type segregant) lines. Seed protein concentrations were higher for the triple and two double null combinations than for either the wild-type segregants or the parental cultivar (Fig. 1C).

As the null mutations all impact on the expression of genes encoding albumin proteins, the albumin fraction was analysed in some detail, using lines carrying a single (TI) or triple mutant combinations. Fig. 2A shows that the albumin profile was almost identical for the control and TI mutant lines, in contrast to the triple null mutant line, where two of the predominant albumins were missing. The polypeptides of relative molecular mass (M_r) ~25,000 and ~17,000 evident in Fig. 2A were confirmed by peptide mass fingerprinting to be PA2 and the large

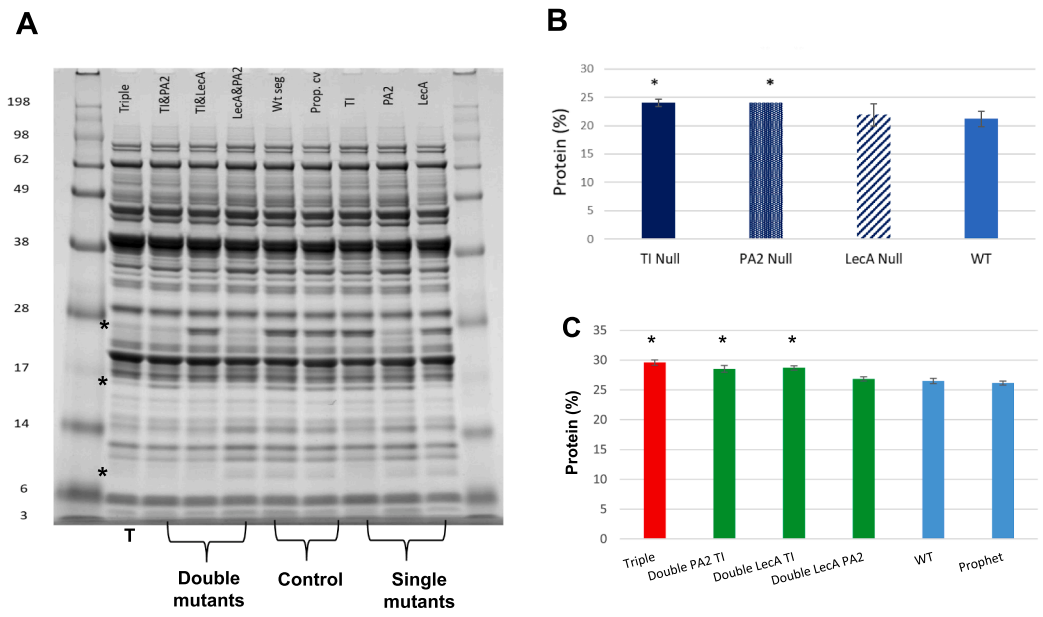

Fig. 1. Analysis of the impact of mutations introgressed into a cultivated background in pea (BC6). **A.** The protein profile of triple (T), double and single null segregant lines, analysed by gel electrophoresis (12% gel). The positions of the three proteins eliminated from the mutants, in comparison with control lines, are highlighted with asterisks (PA2, lectin and TI in decreasing size order). The extreme left and right-hand tracks show protein standards (molecular masses indicated in the range 3–198 kDa). **B.** Mean protein concentration (%) determined for seeds from single null and wild-type segregants ($BC6F_3$). **C.** Mean protein concentration (%) determined for seeds from double and triple null mutants compared with wild-type segregants ($BC6F_4$). Protein assays were performed in triplicate, each with three technical replicates; asterisks denote significant differences from the wild type segregant. WT, wild-type segregant lines; Prophet, the parental cultivar.

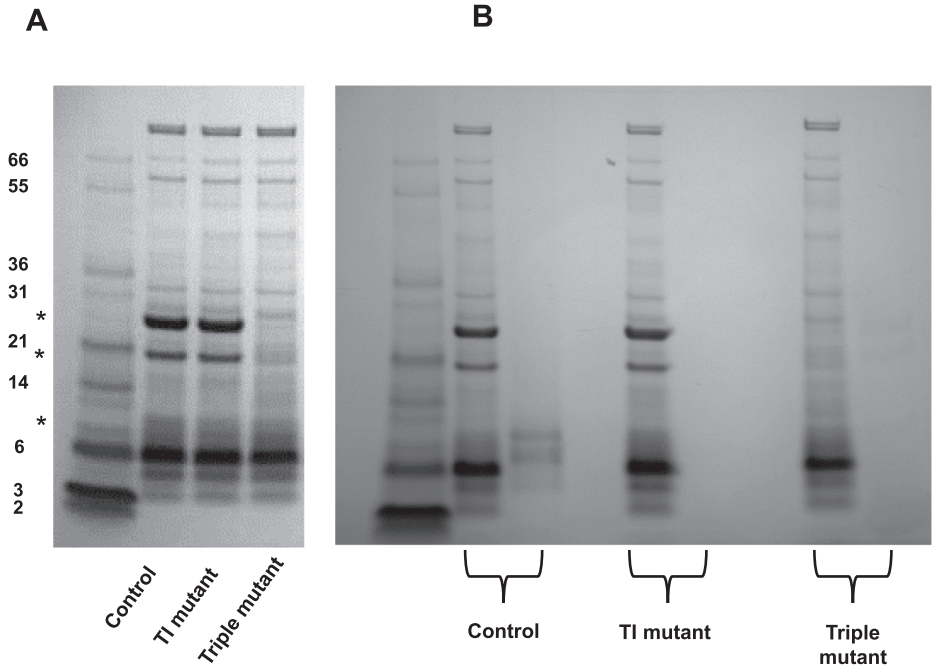

Fig. 2. Analysis of the albumin fraction from control and mutant pea seeds by gel electrophoresis (4–12% gels). **A.** Albumin fraction from control (BC6 TI wild-type segregant), TI mutant (BC6 TI mutant) and F6 triple null mutant line (lacking PA2, lectin and trypsin/chymotrypsin inhibitors, in decreasing order, asterisked). **B.** Analysis of fractions following purification of trypsin-chymotrypsin inhibitors by trypsin-agarose affinity chromatography. For control (BC6 TI wild-type segregant), TI mutant (BC6 TI mutant) and triple null line (lacking PA2, lectin and trypsin/chymotrypsin inhibitors), the flow-through proteins are shown in the left lane, whereas proteins eluted from the column are shown in the right-hand lane in every case. The extreme left-hand track of both A and B shows protein standards (molecular masses indicated in the range 2–66 kDa).

subunit of lectin, respectively (Supplementary Table S2). The smaller subunit of pea lectin (~6000) (Higgins, Chandler, Zurawski, Button, & Spencer, 1983; Trowbridge, 1974) is not visible in either Figs. 1 or 2 due to the relative abundance of a predominant albumin, pea albumin 1, composed of two peptides PA1a and b with molecular masses of ~6 and 4 kDa, respectively (Higgins et al., 1986). This protein makes analysis of other low molecular weight albumins difficult without further purification steps.

Although the position of the trypsin-chymotrypsin inhibitors can be inferred from analysis of mutant and control lines in Fig. 1A, these proteins resolve poorly on such gels, unless alkylation or pyridylethylation of disulphide bonds is carried out prior to gel analyses, and hence do not run according to their true molecular mass (7–8 kDa) (Domoney, Welham, & Sidebottom, 1993, Domoney, Welham, Sidebottom, and Firmin, 1995). Measurement of overall trypsin and chymotrypsin inhibitory activities of control and mutant seed protein extracts revealed that inhibitory activity could not be detected in the single TI and triple null mutants; control segregants had 4.09 and 6.36 trypsin and chymotrypsin inhibitory units per mg meal, respectively (Table 1). The absence of inhibitors in the back-crossed single TI and

Table 1
Comparison of trypsin (TIA) and chymotrypsin inhibitory activities (CIA) in seeds of control, TI and triple mutants.

	Control (BC6 wild-type segregant)	TI mutant (BC6)	Triple null (PA2,LecA, TI)
TIA	4.09 ± 0.01	n.d.	n.d.
CIA	6.36 ± 0.12	n.d.	n.d.

Trypsin (TIA) and chymotrypsin (CIA) inhibitory activities are presented as inhibitory units per mg of meal. Data are means ± SD (n = 3). n.d. not detected.

triple mutant lines was confirmed when the albumin fractions of mutant and control lines were fractionated by chromatography on an immobilised trypsin affinity column. Trypsin inhibitors were eluted from control lines, where a group of low molecular weight peptides was evident, but not from mutant lines (Fig. 2B).

In order to investigate whether removal of certain proteins from pea seeds would impact favourably on digestibility or not, seed meals were subjected to *in vitro* digestion following the INFOGEST protocol (Brodkorb et al., 2019; Minekus et al., 2014) and a range of parameters associated with the digestion process analyzed. The *in vitro* digestion protocol developed as INFOGEST is the internationally accepted standard alternative to *in vivo* assays, available as a standardised protocol format that includes recommended standard enzyme assays and videos pertaining to oral, gastric and intestinal phases (Brodkorb et al., 2019).

3.1. Degree of hydrolysis in digested pea samples

The degree of protein hydrolysis after the three digestion stages (oral, gastric and intestinal) was estimated using the OPA method, which measures the free amino groups in the supernatant of the digested samples (Nielsen et al., 2001; Zahir et al., 2018). The DH of the different pea seeds was not significantly different after either the oral or the following gastric phase (Fig. 3). A steady increase in the DH from oral to gastric digestion was observed for all samples with slight but not statistically significant differences between the triple null mutant and the control and single TI mutant samples in the gastric phase. In contrast, the DH showed significant differences when the three genotypes are compared, following the action of the pancreatin in the intestinal phase (Fig. 3). The triple null mutant showed the highest DH at over 75%, whereas the single TI mutant showed around 60% of protein hydrolysis and the control around 50% (Fig. 3). Pancreatin used in the *in vitro* digestions is a mixture of digestive enzymes with proteolytic activity, including trypsin and chymotrypsin. The differences in DH observed after the intestinal phase can be at least partly attributed to the interaction between the trypsin-chymotrypsin inhibitors present in the control seeds and pancreatin, lowering the proteolysis of other proteins. Earlier *in vivo* studies with broilers, fed with near-isogenic lines of peas differing quantitatively in seed TI activity, showed a negative effect of these protease inhibitors on apparent digestibility (Wiseman, Al-Mazooqi, Welham, & Domoney, 2003). The lack of trypsin-chymotrypsin inhibitory activity in the mutant lines (Table 1) would enable the action of both proteases with an overall positive effect over the digestion process. The higher DH of the triple null mutant when compared to the TI mutant can be attributed to the additional absence of lectin and PA2, in agreement with their respective negative digestibility attributes, without compromising total seed protein concentration (Fig. 1B, C).

3.2. Size exclusion chromatography (SEC) analysis of digestion products

Analysis of the hydrolysed proteins, small peptides and amino acids produced during digestion provides a qualitative measure of the molecular size distribution of products. The molecular weight of a protein product is a key factor that determines whether or not it would be absorbed. Small (di- and tripeptides) and large (10–51 amino acids) peptides generated in the diet can be absorbed intact through the intestine and produce biological effects at the tissue level (Roberts, Burney, Black, & Zaloga, 1999). SEC has been described as a technique that enables a qualitative evaluation of differences in protein digestion (Rieder et al., 2021). SEC analysis of the molecular distribution of the soluble fraction of the three digestion phases revealed a broadly similar profile for the control and two mutant pea samples analysed, but with quantitative differences in peak heights (Fig. 4). The three samples showed the same trend with a marked decrease in the peaks containing the higher molecular weight proteins which eluted early in the chromatogram and a corresponding increase in peaks containing lower molecular weight peptides from gastric to intestinal phases (Fig. 4A–C).

After the oral phase, minor differences were observed among samples, mainly in the approximate molecular range of 50–30 kDa (116–140 mL elution volume, Ve) for the triple null mutant; this approximate size range is likely to include PA2 and lectin proteins (Rubio et al., 2014), which are missing from the triple null. After the gastric phase, the height of the peak corresponding to proteins higher than 40 kDa (up to 140 mL Ve) was reduced for all the pea lines compared to the oral phase (Fig. 4A, B), with an increase in the peak areas corresponding to smaller peptides/proteins. Following the intestinal phase, most of the proteins with an apparent molecular weight ≥10 kDa (100–200 mL Ve) are hydrolysed into smaller oligopeptides or free amino acids. The peaks obtained for the triple null mutant showed a marked decrease in height/intensity, compared with the other two

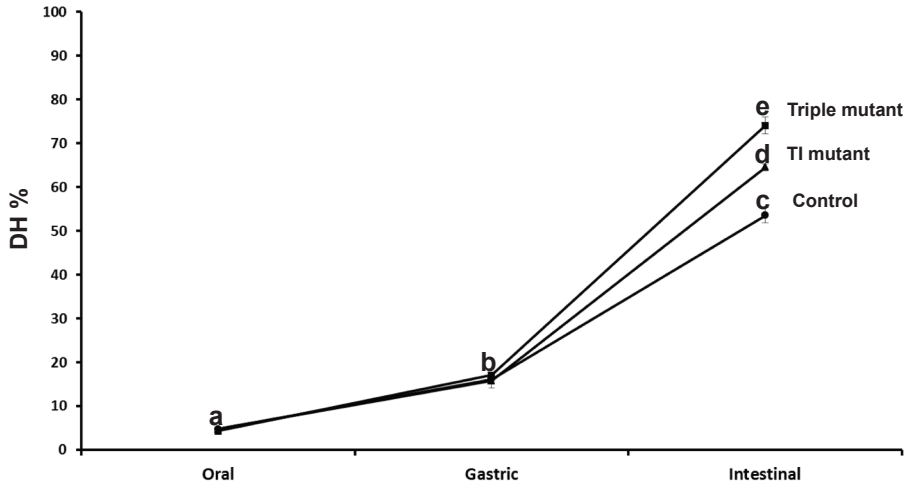

Fig. 3. Degree of hydrolysis of pea proteins (DH%), determined using the OPA method, following use of the *in vitro* digestion model system with three phases (oral, gastric and intestinal). The samples analysed are: TI mutant and control wild-type segregant lines (BC6) and triple mutant (lacking PA2, lectin and TI proteins, F_6). Values are means of at least three independent *in vitro* digestion experiments; standard deviations are indicated by bars. Significant differences between and within phases are indicated (a–e).

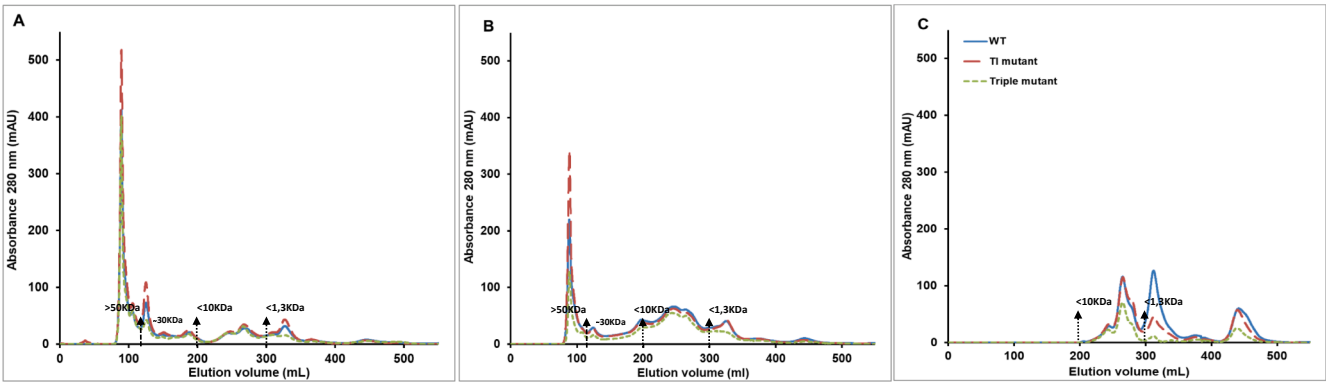

Fig. 4. Qualitative analysis by Size Exclusion Chromatography (SEC) profile of peptides after the three phases of *in vitro* digestion, using TI mutant and control wild-type segregant lines (BC6) and triple mutant (lacking PA2, lectin and TI proteins, F_6). The profiles following oral (A), gastric (B) and intestinal (C) phases are shown.

samples, indicating a greater degradation of peptides (Fig. 4C). It is worth noting that free amino acids are eluted from the column after 350 mL but their detection at 280 nm does not allow quantitative comparisons among samples to be made.

3.3. SDS-PAGE analysis of protein profile during the digestion process

Following the three phases of digestion, proteins present in the soluble fraction were analysed by SDS-PAGE gel electrophoresis. Samples from the oral phase were generally identical for control and mutant samples, apart from the absence of proteins with apparent molecular weights corresponding to PA2 and lectin in the triple mutant line (not shown). The presence or absence of TI proteins could not be determined from these analyses (see earlier). Following the gastric phase, an increase in the peptides ≤10 kDa was apparent on gel analyses, in agreement with the results from SEC analysis (Fig. 4B). After the intestinal phase, most of the proteins with a molecular mass greater than 21.5 kDa correspond to pancreatic enzymes and pancreatin, as seen in control digestions without pea meal (Fig. 5, lane b). A significant protein (~20 kDa) persisted in the control and TI mutant samples following all the phases of digestion, but was absent from the triple null (Fig. 5, lanes c–e). Proteins which appeared to survive digestion in the gastric and intestinal phases were selected for identification by MALDI-TOF mass spectrometry. Among those detected after the gastric phase were convicilin, several vicilin polypeptides and legumin in the molecular weight range 14–50 kDa (Supplementary Fig. S1). Proteins detected in the wild-type control sample following the intestinal phase included PA2, lectin, vicilin and legumin (Fig. 5; Table 2). Lectin, notably absent from the triple null mutant, appears to resist digestion totally, based on apparent size (Fig. 5). However, the fact that PA2 was identified among peptides of ~6 kDa following the three phases of digestion suggest that at least a partial hydrolysis of this protein has occurred (Fig. 5, Table 2). Trypsin-chymotrypsin inhibitors were not identified among the digested proteins analysed, presumably because these proteins form very stable complexes with the digestive enzymes trypsin and chymotrypsin.

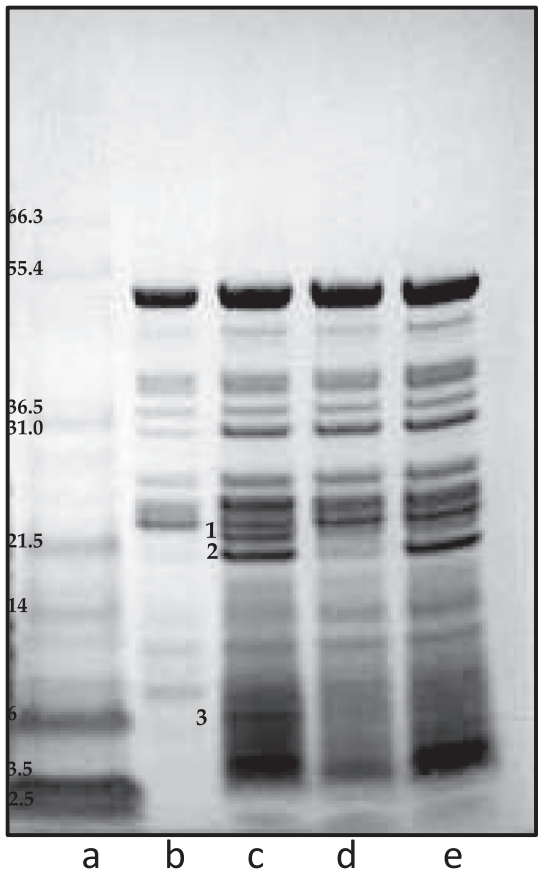

Fig. 5. SDS-PAGE of proteins after complete *in vitro* digestion. a, molecular weight markers (protein standards of molecular masses indicated in the range 3–56 kDa); b, intestinal control without added pea sample; c, wild-type control segregant; d, triple null mutant; e, TI null mutant. Proteins were excised from the regions labelled 1–3 from the control sample and identified by peptide mass fingerprinting.

3.4. Free amino acid profile after in vitro digestion

Since protein quality is ultimately defined in terms of the bioavailability of oligopeptides and amino acids, the total free amino acids which were released after complete *in vitro* digestion were quantified. When expressed per unit of protein digested, the amounts of almost all individual free amino acids were significantly higher for the mutant lines, indicative of a more effective digestion of proteins (Fig. 6). The triple null mutant showed the highest concentrations of most amino acids and the two mutant lines showed higher concentrations of almost all individual amino acids, with the exception of asparagine/ aspartic acid and proline, which were present at the same concentration in all three samples (Fig. 6). Moreover, when indispensable or essential amino acids released after complete digestion were evaluated, the amounts observed in both mutants were significantly higher than in the control wild type samples per unit of digested protein (Fig. 6; Supplementary Table S3).

3.5. Evaluation of seed yield in variant lines

The impact that desirable mutations might have on agronomic traits

Table 2
Identities of pea proteins resistant to digestion following all three phases (oral, gastric and intestinal) using peptide mass fingerprinting.

Gel band	Protein identity	Mascot score	Sequence coverage (%)	Number of peptides	Swiss-Prot/ Trembl Accession No.
1	Vicilin	67	30	13	P13918
	Legumin A2	52	23	11	P02857
2	Lectin	363	23	5	P02867
	Vicilin	47	18	8	D3VNE2
3	Legumin	58	20	11	P15838
	Pea Albumin 2	46	36	8	P08688
	Provicilin	43	21	9	P02854
	Vicilin	88	33	13	Q702P0

After digestion of pea meal, proteins were excised from gels (Fig. 5) and subjected to cleavage and mass fingerprinting.

such as seed yield was assessed under greenhouse conditions, using a cultivated genetic background (cv. Prophet), into which combinations of the PA2, lectin and TI mutations had been introgressed. There was no discernible difference in plant habit among the lines (not shown). The yield of seeds was comparable among mutant lines with one double null yielding a marginally higher mean seed yield than the control lines (Fig. 7), reflecting an average of 20 seeds per plant for the line lacking both PA2 and lectin, compared with 16–17 seeds per plant for the other lines. When the mutant lines were tested under field conditions (F_4, F_6), their yields were comparable to that of cv. Caméor (Supplementary Fig. S2).

4. Discussion

Until technologies such as genetic engineering and particularly gene editing approaches are available more widely to allow for the precise fine tuning of plant traits such as seed composition, genetic variants offer huge potential to investigate the biological role and value of nutritionally important seed constituents, and to modulate the content of so-called anti-nutrients. In this work, we have availed of naturally occurring mutations which abolish the synthesis of PA2 and trypsin-chymotrypsin inhibitors in seeds (Clemente et al., 2015; Vigeolas et al., 2008) and combined these with an induced deletion of the *LecA* gene (Domoney et al., 2013), which encodes seed lectin, to investigate the benefits that such losses might confer to seed protein quality. All these proteins, which are albumins, have been reported to be associated with negative effects on digestibility, in addition to the potential allergenicity of PA2 (Vigeolas et al., 2008). In the studies of Park, Kim, and Baik (2010) all three were identified as proteolytically resistant proteins, even after thermal treatment which often improves digestibility by denaturation. In general, albumins from different pulses have been shown to be less digestible than globulins (Clemente et al., 2000; Crevieu et al., 1997; Rubio et al., 2014). Other studies have described lectins as resistant to the action of digestive enzymes and studies with animals have shown that these proteins can be found at the end of the intestinal tract (Le Gall et al., 2005, 2007; Salgado et al., 2003). Trypsin inhibitors from peas have been identified as proteins resistant to proteolytic enzymes in many *in vitro* and *in vivo* studies (Crevieu et al., 1997; Jayathilake et al., 2018; Le Gall et al., 2007; Recoules et al., 2017;

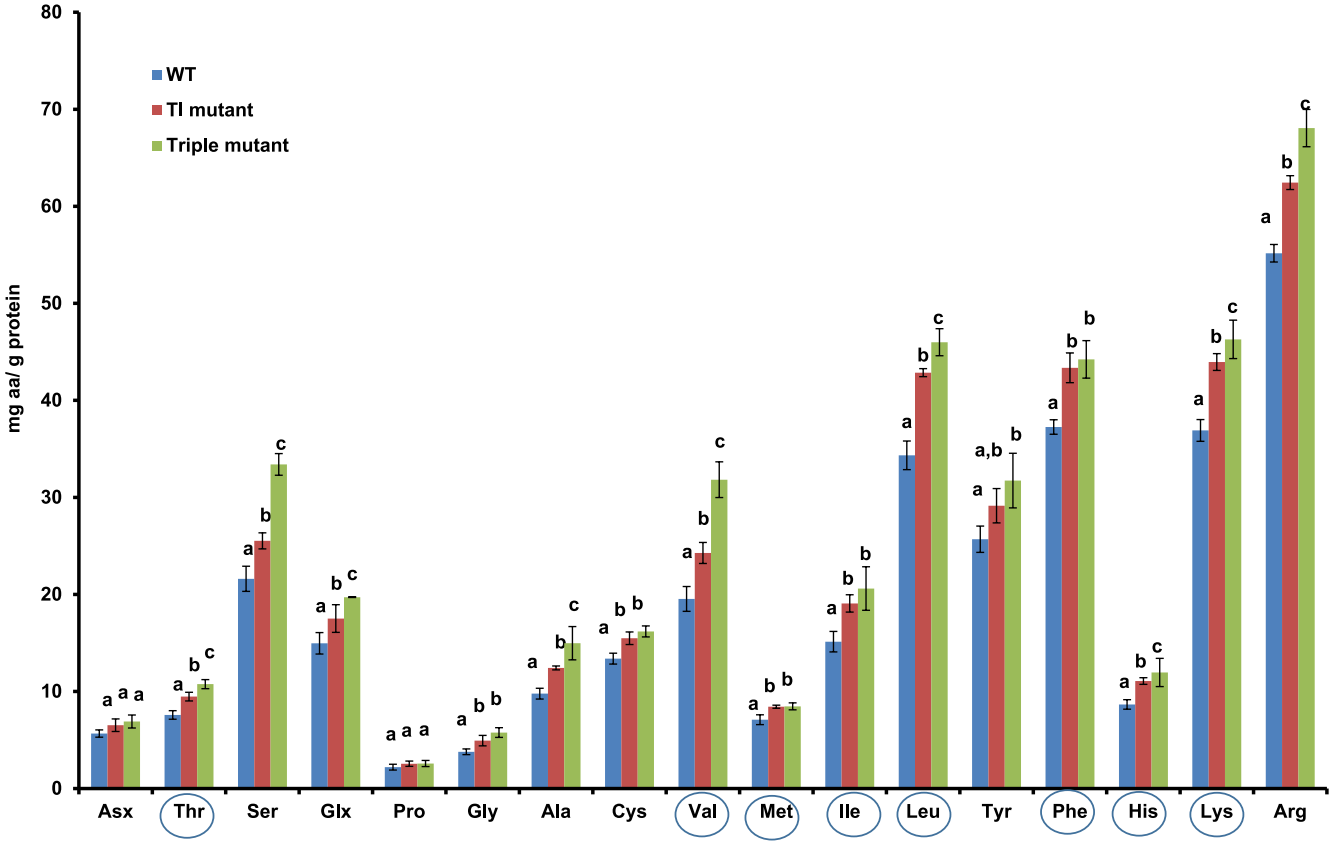

Fig. 6. Free amino acid profile following *in vitro* digestion of pea samples (control wild type, TI and triple null mutant). The content of each amino acid is expressed as mg/g of protein digested. Data are means of at least three independent reactions, each carried out in duplicate. Asx: aspartic acid (Asp) and asparagine (Asn); Glx: glutamic acid (Glu) and glutamine (Gln). Cysteine is measured as cysteic acid and methionine as methyl sulfone. Essential amino acids are highlighted (eight within ovals); amounts of tryptophan were not determined.

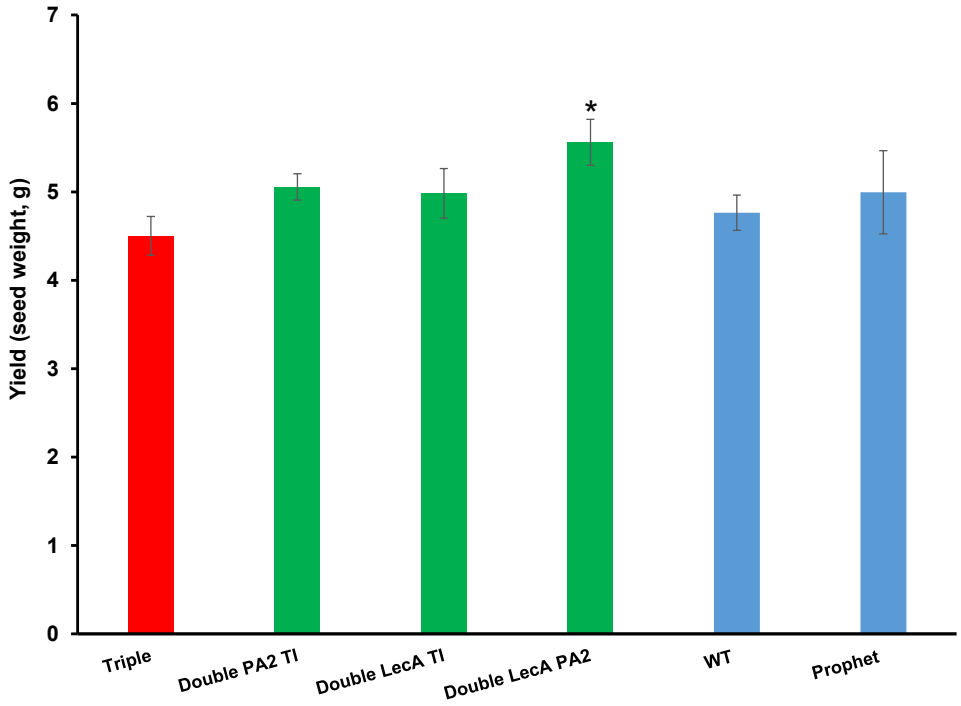

Fig. 7. Mean yield (weight of seeds per plant) obtained from double and triple null and control lines ($BC6F_4$). An asterisk denotes a significant difference from the wild-type segregant. WT, wild-type segregant lines; Prophet, the parental cultivar.

Ribeiro et al., 2017; Santos-Hernández et al., 2020; Szczurek & Świątkiewicz, 2020). Taken together, these studies support the investigations of mutations for a range of seed proteins with negative properties in order to improve protein bioavailability.

In the present work, when the mutations for the three albumins were introgressed into a commercial genetic background, two single mutants, two double combinations and the triple mutant showed significant increases in overall protein concentration (Fig. 1B, C), in agreement with earlier observations of a negative correlation between total seed protein and albumin concentrations and a positive correlation of the former with globulin concentration (Park et al., 2010). Earlier studies of the PA2 null mutation had also suggested that backcrossed lines carrying the mutation had an elevated seed protein concentration (Vigeolas et al., 2008). Previous studies of pea lines with quantitative variation in seed trypsin and chymotrypsin inhibitory activity showed that this was independent of the concentration of total seed protein (Griffiths, 1984). Lectin mutants have not been analysed previously for their seed protein concentration, but it was established that such mutants did not appear to be compromised in their capacity to form symbiotic root nodules, despite the fact that lectin is also expressed in pea roots and implicated in bacterial recognition (Rayner, Moreau, Isaac, & Domoney, 2018).

The digestibility assays employed in the present work are based on a protocol now accepted internationally as an alternative to *in vivo* models (Brodkorb et al., 2019) which, although providing direct information from a digestion process, are often difficult to perform, expensive or unjustifiable on ethical grounds. The *in vitro* digestion protocol developed as the INFOGEST model makes use of a sequential mixture of enzyme preparations, resulting in protein hydrolysis products that are a heterogeneous mixture of oligopeptides and free amino acids. The results from the assays reported here provide strong evidence for the improvement in protein hydrolysis in mutant lines carrying either the TI or all three mutations, as measured by both the degree of hydrolysis, SEC profiles and free amino acid measurements (Figs. 3-6); as expected, the majority of the hydrolysis occurred during the intestinal phase of digestion. The identification of peptides by MALDI-TOF mass spectrometry supported the resistance of lectin to digestion in wild-type control samples. A smaller peptide derivative of PA2 was detected, although the original protein could not be discerned among the pancreatic enzymes which co-migrated on gels. Although PA2 has been described as a protein resistant to digestion in experiments with broilers (Crevieu et al., 1997), other studies with piglets found that part of PA2 was susceptible to digestion in the gut of mammals, in contrast to poultry (Le Gall et al., 2007).

Since protein quality refers not only to amino acid profile but also its bioavailability and digestibility, allowing absorption of the amino acids, the free amino acid profile at the end of digestion is an extremely important parameter to assist in determining the capacity of a food protein to satisfy metabolic demands for amino acids and nitrogen. As noted earlier, oligopeptides up to 50 amino acids may also be absorbed (Roberts et al., 1999). The free amino acids quantified from digestion of the three pea seed samples supported the greater digestibility of the mutant samples, with the mutants providing higher amounts of most amino acids, including the sulphur-containing amino acids, methionine and cysteine, and the triple mutant showing the highest values for many other amino acids, notably many of which are defined as essential (Fig. 6). The elevated amounts of essential amino acids which are released following digestion of protein from the mutant lines (Supplementary Table S3) will have relevance to feed and food manufacturers, particularly in cases where formulations for poultry and farm animal rations require supplementation to overcome limitations.

The data presented for protein and digestibility traits suggested that the loss of three albumins from pea seeds could confer advantages to amino acid bioavailability. However, such mutations should not be associated with negative agronomic traits in order for them to be used economically in breeding programmes. The analysis of seed yield from the variant lines grown under greenhouse conditions suggests that there is no penalty associated with the mutations (Fig. 7). Assessment of bulked early generation mutant lines under field conditions supported the conclusion that there were no negative pleiotropic consequences associated with the mutations (Supplementary Fig. S2). Thus the combination of an elevated seed protein concentration, loss of trypsin/chymotrypsin inhibitors and a potential allergen, together with

improved digestibility and amino acid bioavailability makes these mutants an attractive proposition to improve protein bioavailability in pulse crops. Moreover, since albumins are associated with a range of functional properties (e.g. foaming, emulsification, gelling, water-binding capacity) which are of interest to the industry seeking to modify plant proteins for a variety of end-uses, these variant lines should now be tested in a range of industrial applications.

Funding

We gratefully acknowledge support for this work from the Department for Environment, Food and Rural Affairs (Defra) (CH0103, CH0111, Pulse Crop Genetic Improvement Network), the Biotechnology and Biological Sciences Research Council (BBSRC) (BB/P012523/1, BBS/E/J/000PR9799) and the John Innes Foundation. We acknowledge the financial support of AGL2017-83772-R funded by MCIN/AEI/ https://doi.org/10.13039/501100011033 and the European Union, and also the support from grant P20_00242 (PAIDI 2020) funded by Junta de Andalucía.

Informed Consent Statement: Not applicable.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data is contained within the article or supplementary material.

Acknowledgments

We are grateful to Dr Pete Isaac (IDna Genetics Ltd, Norwich, UK) for designing the genomic screening methods, and the proteomics services of Instituto Lopez Neyra (CSIC-Granada, Spain) for carrying out the peptide analysis. We are very grateful to Catherine Taylor and Lionel Perkins for their assistance with plant husbandry during the greenhouse experiments, and Cathy Mumford and Darryl Playford for their assistance with field trials.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2023.112825.

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